DEPARTMENT OF ELECTRONIC & ELECTRICAL ENGINEERING

University of Strathclyde Engineering

ANTIMICROBIAL 405 NM LIGHT FOR CLINICAL DECONTAMINATION: INVESTIGATION OF THE ANTIVIRAL EFFICACY AND POTENTIAL FOR BACTERIAL TOLERANCE

A thesis presented in fulfilment of the requirement for the degree of

Doctor of Philosophy

Rachael Margaret Tomb (BSc Hons)

2017

Department of Electronic and Electrical Engineering

University of Strathclyde

Glasgow, UK

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

Signed:

Date:

This study was funded by the Scottish Infection Research Network and Scottish Government Health and Social Care Directorates, through a Doctoral Fellowship in Healthcare Associated Infections 2013-2017.

Reference: SIRN/DTF/13/02

Acknowledgments

I would firstly like to thank my supervisors Dr Michelle Maclean and Professor John Coia. I am so grateful for the opportunity to undertake this PhD and their support and guidance throughout. I cannot thank them enough for their time, ideas and motivation. I also wish to thank Professor John Anderson, Professor Scott MacGregor, Dr Libby Graham and Professor Helen Grant, who have all been generous with their time and help throughout the project.

I also wish to thank David Currie, Katie Henderson, Mike McDonald and Tracy White for their technical expertise and support. Additionally, thank you to Maureen Cooper for all her administrative assistance and the technicians in the workshops for their help.

My PhD experience was made all the more special by sharing it with other students, who have become great friends. A special mention to Karen, Sian, Jonah, Michael, Dan, Caron, Athanasios, Laura and Justine.

I would also like to thank my family and friends, your continued support throughout my PhD has meant so much to me. I also hope that my Dad and Grandparents would be proud - you are in my thoughts every day.

Finally, I cannot thank Chris and my Mum enough for their love, support, patience and cups of tea! Thank you for believing in me. I lovingly dedicate this thesis to you both.

Abstract

Hospital associated infections result in longer patient stays, increased treatment costs and morbidity and mortality. Novel technologies utilising UV light, hydrogen peroxide vapour and ozone have been designed to provide high-level environmental decontamination, in an attempt to prevent patient acquisition of nosocomial pathogens. However due to safety concerns, these technologies are only suitable for intermittent terminal cleaning, and surfaces can become quickly re-contaminated. Recently developed systems which utilise antimicrobial 405 nm violet-blue visible light have been successfully used for continuous decontamination of surfaces and air, in the presence of patients. This study investigated some fundamental questions surrounding the use of antimicrobial 405 nm light for clinical decontamination.

Initial investigations studied the antiviral efficacy of 405 nm light on a model for the nosocomial pathogen, Norovirus. Studies showed positive antiviral effects in suspension and on clinically-relevant surfaces when virions were exposed in minimal media (DPBS), however this inactivation efficacy was significantly improved (85% lower dose) when the virus was suspended in organically-rich, biologically-relevant media (such as saliva and blood plasma). This enhanced inactivation is likely due to photoexcitation of the suspending media, and was demonstrated using fluorescence spectrophotometry, with excitation peaks seen for all suspending media except minimal media. A systematised review also compared 405 nm light inactivation of viruses with other clinical pathogens (bacteria, fungi), and found that viruses (exposed in minimal media) are more resilient structures, requiring higher doses for equivalent reductions, likely due to differing inactivation mechanisms.

Further studies also investigated the potential for proliferating and non-proliferating bacteria, *Staphylococcus aureus*, to become tolerant to 405 nm light. Results demonstrated that exposure to 405 nm light during cultivation resulted in higher dose requirements for complete inactivation and increased stress tolerance, however the process was unlikely to be selective. Results also demonstrated that repeated sub-lethal exposure of antibiotic sensitive and resistant vegetative cells did not give rise to tolerance or alter antibiotic susceptibility.

This study has provided significant fundamental information about antimicrobial violet-blue light. The results demonstrate proof-of-concept evidence of the virucidal efficacy of 405 nm light, as well as demonstrating that bacterial tolerance is unlikely. These results further support the clinical use of antimicrobial 405 nm light for continuous environmental decontamination, with implementation likely to aid infection control and reduce hospital associated infections.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

aHP – Aerosolised hydrogen peroxide

ALA - Aminolaevulinic acid

CA-MRSA – Community associated methicillin-resistant *Staphylococcus aureus*

CAPP – Cold atmospheric pressure plasma

CCV – Canine calicivirus

CFU – Colony forming units

cl – Chlorine

CO_2 – Carbon dioxide

DMEM – Dulbecco's Modified Eagle medium

DMSO – Dimethyl sulfoxide

DPBS – Dulbecco's phosphate buffered saline

EDS – Environmental Decontamination System

EDTA – Ethylenediaminetetraacetic acid

EUCAST – European Committee on Antimicrobial Susceptibility Testing

FBS – Fetal Bovine Serum

FCV – Feline calicivirus

FEA – Feline embryonic cells

FWHM – Full-width-half maximum

GGC – Greater Glasgow and Clyde

HAI – Hospital associated infection

HA-MRSA – Hospital associated methicillin-resistant *Staphylococcus aureus*

HAV – Hepatitis A virus

HCW – Health care worker

HINS – High intensity narrow spectrum

HPLC – High performance liquid chromatography

HPS – Health Protection Scotland

HPV – Hydrogen peroxide vapour

H_2O_2 – Hydrogen peroxide

ICU – Intensive care unit

LED – Light emitting diode

MALDI-TOF – Matrix assisted laser desorption/ionization time-of-flight mass spectrometry

MDR – Multidrug-resistant

MIC – Minimum inhibitory concentration

MNV – Murine Norovirus

MPIX – Mesoporphyrin IX

MSSA – Methicillin-sensitive *Staphylococcus aureus*

MRSA – Methicillin-resistant *Staphylococcus aureus*

NA – Nutrient agar

NaOH – Sodium hydroxide

NB – Nutrient broth

NCTC – National Collection of Type Cultures

NIPCM – National Infection Prevention and Control Manual

NO – Nitrogen oxide

NO_3 – Nitrate

NoV – Norovirus

$^1O_2$ – Singlet oxygen

O_2^- – Superoxide

O_3 – Ozone

$^{\cdot}OH$ – Hydroxyl radical

PBP – Penicillin binding protein

PBS – Phosphate buffered saline

PDI – Photodynamic inactivation

PEC – Porcine enteric calicivirus

PFU – Plaque forming units

PVC – Polyvinyl chloride

PUV – Pulsed UV

ROLEST – The Robertson Trust Laboratory for Electronic Sterilisation Technologies

ROS – Reactive oxygen species

RT-PCR – Reverse transcription polymerase chain reaction

SCVs – Small colony variants

SDS-PAGE – Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SNBTS – Scottish National Blood Transfusion Service

SSI – Surgical site infection

TEM – Transmission electron microscopy

TFTC – Too few to count

TiO_2 – Titanium dioxide

TNTC – Too numerous to count

TuV – Tulane virus

UPI – Uroporphyrin I

UTI – Urinary tract infection

UV – Ultraviolet

VBNC –Viable but non culturable

VRE – Vancomycin resistant Enterococci

WHO – World Health Organisation

5PI – 5-Carboxylporphyrin

6PI – 6-Carboxylporphyrin

7PI – 7-Carboxylporphyrin

Chapter One

Thesis Introduction

1.0 Overview

According to the World Health Organisation (WHO 2011), hospital associated infections (HAIs) affect on average 7% of patients in developed countries and 10% in developing countries at any given time. Acquisition of HAIs can lead to increased morbidity and mortality, as well as increased durations of patient stays and treatment costs (Cosgrove 2006). It is now accepted that contaminated surfaces play a major role in the transmission of HAI-associated pathogens, which are becoming increasingly resistant to antibiotics. It is therefore of upmost importance that new technologies are developed to help address the problem of environmental contamination within hospitals. The High Intensity Narrow Spectrum-light Environmental Decontamination System (HINS-light EDS) is one such technology, which emits visible violet-blue light to continuously reduce microbial bioburden in occupied hospital environments. As part of the ongoing research surrounding the clinical use of the EDS, there are several areas which require further investigation to ensure optimisation of this system and prevention of adverse effects. These study areas include:

- Investigating the potential antiviral efficacy of high-intensity 405 nm light
- The potential for viral inactivation using the EDS in hospital related scenarios
- Evaluation of the inactivation of microorganisms using 380-480 nm violet-blue light
- Investigating the potential for bacterial tolerance to 405 nm light in proliferating and non-proliferating clinically relevant organisms

Investigating these four objectives will provide crucial, novel information on this emerging healthcare technology. The results will aid the improved application of antimicrobial violet-blue light for decontamination, both with regards to expanding its use for viral inactivation and understanding the potential for bacterial resistance. This will ensure that the technology can be used more effectively in the hospital environment to reduce contamination, and subsequently the transmission of hospital associated pathogens.

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

Chapter 2 *(Background and Literature Review)*: Discusses the current problems associated with infectious pathogens in the hospital environment, and issues which arise due to inadequate environmental cleaning. This chapter looks at important nosocomial pathogens including Norovirus and methicillin-resistant *Staphylococcus aureus*; standard hospital decontamination practices; and provides information on novel decontamination technologies, with a focus on violet-blue light technology.

Chapter 3 *(Methodology)*: Details the microorganisms, cell culture techniques, media, chemical preparations, enumeration techniques, biochemical tests, and light arrays used throughout this study.

Chapter 4 *(Proof-of-Concept of the Antiviral Efficacy of 405 nm Light)*: Investigates the antiviral efficacy of high-irradiance 405 nm light using feline calicivirus as a surrogate for Norovirus. This chapter compares the inactivation of viral particles when suspended in organically-rich and biologically-relevant media including human blood plasma, artificial saliva and artificial faeces.

Chapter 5 *(Viral Inactivation using Low-Irradiance 405 nm Light)*: Investigates the potential for using the HINS-light EDS for viral decontamination. Studies focus on the virucidal efficacy of low-irradiance 405 nm light (emitted by the HINS-light EDS) against bacteriophage and viruses when suspended in organically-rich media. Additionally, inactivation of viral contamination on clinically-relevant surfaces was also evaluated.

Chapter 6 *(Comparison of the Virucidal Efficacy of Violet-Blue Light with the Wider Antimicrobial Efficacy)*: Compares the average dose requirements of violet-blue light for inactivation of viruses with a range of microorganisms including vegetative bacteria, bacterial endospores, yeasts and fungi. Firstly, data from the present research study and the wider research carried out at the ROLEST laboratory was analysed, followed by a systematised review of published literature on microbial inactivation using violet-blue light between 380-480 nm. Comparisons are also made between the efficacy of different wavelengths of violet-blue light, the irradiance used and starting population on the antimicrobial efficacy of this technology.

Chapter 7 (*Potential for Bacterial Tolerance to 405 nm Light*): Discusses the potential for bacterial tolerance to 405 nm light, using *S. aureus* as a model organism. The potential for tolerance was investigated in proliferating and non-proliferating bacteria, with inactivation kinetics and antibiotic susceptibility investigated before and after repeated sub-lethal exposure. Additionally, HPLC was performed to analyse the porphyrin content within *S. aureus*, to provide further evidence on the antimicrobial mechanism of violet-blue light.

Chapter 8 *(Conclusions and Recommendations for Future Work)*: Concludes the scientific findings from each chapter, and highlights the beneficial use of 405 nm light for decontamination in the hospital environment with regards to the key findings within this study. Recommendations for future work, to expand on the novel results found in this study, are also discussed.

Chapter Two

Background & Literature Review

2.0 Overview

Modern medicine is becoming increasingly complex with more invasive procedures and complicated equipment than ever before (Dancer 2008). To add to the challenge, antibiotic resistant organisms are a common occurrence in the hospital environment and cause increased morbidity and mortality of patients (Cosgrove 2006). Hospital surfaces have been linked to transmission of these antibiotic resistant pathogens, with up to 50% of surfaces untouched or uncleaned following terminal cleaning with disinfectants (Carling *et al*., 2008; Dancer *et al*., 2008). As hospital cleaning strategies require improvement, there is an increased demand for novel technologies which can improve environmental cleanliness and reduce spread of nosocomial pathogens. One such technology is antimicrobial violet-blue light, which is the focus of the present study.

This chapter discusses common nosocomial pathogens, routes of transmission, hospital cleaning methods and novel decontamination technologies in order to clarify the current situation within hospitals. Additionally, this chapter will focus on antimicrobial violet-blue light technology to fully understand the mechanism of action and its potential benefit for many clinical applications, as well as discussing current research on this technology.

2.1 Hospital Associated Infections

HAIs are localised or systemic infections in patients who did not present any evidence as to the presence or incubation of pathogenic organisms, before admission to hospital (Horan *et al*., 2008). HAIs can be caused by infectious agents from endogenous sources such as skin, nose, mouth or exogenous sources such as health care workers (HCWs), visitors, the patient environment or medical devices (Horan *et al*., 2008). Old, young and immunocompromised patients are at particular risk of HAIs, which can arise due to: surgery, invasive medical devices, immunosuppressive treatment, previous antibiotic treatment, blood transfusion, underlying illness, sepsis and long hospital stays (Inweregbu *et al*., 2005). HAIs include surgical site infections (SSIs), blood stream infections, urinary tract infections (UTIs),

respiratory tract infections, gastric infections or ears, eyes, nose and throat infections, as well as device-related infections.

HAIs can be associated with many pathogenic organisms, as detailed in Table 2.1. Infection with antibiotic resistant organisms such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* spp. (VRE), *Escherichia coli* and *Klebsiella pneumoniae* (β-lactamase and carbapenemase producing), can result in increased morbidity and mortality as well as increased length of patients stays and increased treatment costs (Cosgrove 2006). Filice *et al.* (2010) noted that infections caused by MRSA are twice as expensive to treat as those caused by methicillin-sensitive *S. aureus* (MSSA) in the USA, with average costs of $35,000 and 24% mortality for MRSA and costs of $16,000 and 11.5% mortality for MSSA. Delays in treatment, or inappropriate treatment, may also increase morbidity, with some antimicrobial treatments thought to be toxic. It is estimated that in long-term care facilities in Europe there are > 100,000 HAIs on any given day, with an estimated total of 4.2 million HAIs for the entire year, which results in 37,000 deaths and 16 million extra days of hospital stay (European Centre for Disease Prevention and Control 2008, 2015).

With regards to HAIs in Scotland, it is estimated that there is 4.9% prevalence in acute care and 2.5% in non-acute hospitals (Health Protection Scotland (HPS) 2012). UTIs and SSIs are the most common, estimated to account for 22.6% and 18.6% of inpatient HAIs, respectively within NHS Scotland, followed by infections of the blood stream (10.8%), gastrointestinal tract (6.8%), and skin and soft tissue (4%) (HPS 2012). Additionally, the highest prevalence of HAIs in Scotland have been linked to intensive care units (ICU) (16.1%) and surgery (6.3%) (HPS 2012). The latest data on HAIs in Scotland, during 2015, shows increasing trends in resistance observed among *E. coli* urinary isolates, with significant increases in resistance to the third generation cephalosporins, carbapenems and nitrofurantoin (HPS 2016). During 2015 there were 62 outbreaks and incidents reported, with respiratory and gastrointestinal infections accounting for 47% of all outbreaks and incidents reported (HPS 2016). Table 2.2 summarises the key pathogenic organisms within Scottish hospitals and the latest data on incidence during 2015.

Table 2.1. Common nosocomial bacteria and viruses and their associated clinical illnesses.

Adapted from Murray *et al*. (2008a).

Nosocomial Bacteria	**Associated Clinical Illness**
Acinetobacter baumannii	Wound infections; Urinary tract infections; Respiratory tract infections; Septicaemia
Clostridium difficile	Antibiotic associated diarrhoea; Pseudomembranous colitis
Enterococcus spp.	Bacteraemia; Endocarditis; Urinary tract infections; Wound infections
Escherichia coli	Gastroenteritis; Urinary tract infections; Haemorrhagic colitis; Haemolytic uremic syndrome; Bacteraemia
Klebsiella pneumoniae	Wound infections; Soft tissue infections; Urinary tract infections; Pneumonia
Pseudomonas aeruginosa	Burn wound infections; Skin, ear and eye infections Bacteraemia; Endocarditis; Pulmonary infections
Stenotrophomonas maltophilia	Bacteraemia; Pneumonia
Staphylococcus aureus	Wound infections; Impetigo; Pneumonia; Empyema; Bacteraemia; Endocarditis; Septic shock
Streptococcus pyogenes	Pyoderma; Cellulitis; Necrotizing fasciitis; Scarlet fever; Pharyngitis
Nosocomial Viruses	**Associated Clinical Illness**
Adenovirus	Conjunctivitis; Gastroenteritis; Respiratory tract infections; Pneumonia; Haemorrhagic cystitis; Myocarditis
Hepatitis A/B/E	Liver disease
Influenza A	Respiratory tract infections; Diarrhoea; Vomiting; Pneumonia; Respiratory system failure
Norovirus	Diarrhoea; Nausea; Vomiting; Abdominal cramps; Fever
Respiratory syncytial virus	Respiratory tract infections; Pneumonia
Rotavirus	Diarrhoea; Nausea; Vomiting; Abdominal Cramps; Fever

Table 2.2 Annual cases of hospital associated infection within Scotland in 2015. Adapted from Health Protection Scotland Annual Report 2015.

Pathogen	Annual cases in Scotland 2015
Staphylococcus aureus (including MRSA)	1600 cases of bacteraemia: 110 (6.9%) were MRSA, 2.2 per 100,000 acute bed days 1,490 (93.1%) were MSSA, 29.5 per 100,000 acute bed days
Escherichia coli	4596 cases of *E. coli* bacteraemia, 90.9 per 100,000 acute bed days
Carbapenemase Producing Organisms (including *Enterobacteriaceae* spp.)	66 Cases of Carbapenemase producing organisms in Scottish hospitals
Clostridium difficile	543 cases in 15-64 year olds, 38.3 per 100,000 bed days 1133 cases in ≥ 65 year olds, 31.2 per 100,000 bed days
Norovirus	1,390 confirmed cases*

*This data remains provisional (Norovirus Scotland Annual Totals on 28 July 2016, Health Protection Scotland)

As this study focusses particularly on application of 405 nm light with regards to the potential antiviral efficacy against Norovirus (NoV) and bacterial tolerance in *Staphylococcus aureus*, the following sections provide a general overview of these two HAI-associated pathogens with regards to prevalence within hospitals and clinical symptoms:

2.1.1 Norovirus

Viruses are obligate intracellular parasites that have the ability to invade human and bacterial cells, replicate using host machinery and ultimately cause cell death (Murray *et al.*, 2008a). NoV, a common nosocomial pathogen, is a member of the *Caliciviridae* family, has a non-enveloped icosahedral structure with a (+ve) single stranded RNA genome, and measures 27-40 nm in diameter (Siebenga *et al*., 2010; Cromeans *et al*., 2014).

This virus is of particular importance in the hospital environment as it causes 19-21 million cases of associated illness in the USA annually, and has become the most common cause of epidemic acute gastroenteritis (Patel 2009; Hall *et al*., 2013). It is estimated that globally over 200,000 people die annually due to NoV with > 100,000 of these occurring in children up to 14 years and > 77,000 in adults over 55 years (Bartsch *et al*., 2016).

NoV is highly transmissible through several routes: faecal-oral pathway; via contaminated food and water; aerosolised particles; and contaminated surfaces, and can occur via multiple routes during an outbreak (Glass *et al*., 2009). The virus is highly contagious with as little as 18-100 viral particles needed for infection, and ~30 million viral particles released during a vomiting episode and > 10^9 particles/g in faeces. (Caul 1994; Lee *et al*., 2007; Tenuis *et al*., 2008; Karst 2010; Bentley *et al*., 2012; Robilotti *et al*., 2015). NoV shedding in stools can continue for four to eight weeks in those who have recovered, and will last even longer in those with persistent infections (Atmar *et al*., 2008, 2103; Schorn *et al*., 2010).

NoV has a short incubation period of 24-48 hours after which the main symptoms include vomiting, diarrhoea and abdominal cramps for one to three days (Murray *et al*., 2008a). The illness is usually mild and self-limiting however can be more serious for infants, the elderly, the immunocompromised and transplant patients, the latter two of which can suffer persistent infections with symptoms lasting over 2 years (Karst 2010; Lopman *et al*., 2012; Schorn *et al*., 2010). Due to the features outlined above and the lack of any licensed vaccines to prevent NoV, transmission can be a major issue amongst hospitalised patients, and those affected can only be offered nursing support care and rehydration solutions, as there are no available specific treatment options (Gonzalez *et al*., 2016).

At least 6 genogroups have been recognised (GI-GVI), with GI, GII and GIV generally responsible for human infections (Glass *et al*., 2009; Morter *et al*., 2011; Knipe and Howley 2013; Eden *et al*., 2014). The virus is highly flexible and diverse, and genetic variability between genotypes can differ by 14-44% (Zheng *et al*., 2006). Such variability is due to the replication of RNA which is carried out by virus-encoded RNA-dependant RNA polymerase which does not 'proof-read' resulting in errors occurring during transcription (Bull *et al*., 2010). The virus evolves in an epochal manner as there are periods of stability followed by a quick accumulation of mutations and emergence of new variants (Siebenga *et al*., 2007). Due to the nature of NoV replication, antigenic variants arise every three to four years, with GII.4_Sydney_2012 thought to be currently responsible for outbreaks within Scottish hospitals (Bull *et al*., 2010; Bennet *et al*., 2013).

Recombination also contributes to the genetic variation seen in NoV isolates. A recombinant is a NoV isolate which contains genomic information regarding the viral capsid and polymerase from two distinct groups of NoV (Bull *et al*., 2007). At present, new recombinant variants may be emerging, with GII.P16-GII.2. identified in German hospitals (Neindorf *et al*., 2017). This variant was found to be responsible for 48% of sporadic cases and 42% outbreaks in Germany in the winter of 2016 (Neindorf *et al*., 2017). The genetic and antigenic variability, as well as lack of long term-immunity, means that one person can have repeated infections. Therefore, there is the potential for repeated outbreaks which enables NoV to be an enduring threat to the healthcare environment (Wyatt *et al*., 1974; Glass *et al*., 2009).

If a NoV outbreak does occur within the clinical environment, this can result in substantial operational and financial losses for healthboards. Hospital bays and wards may be closed following confirmation of an outbreak which will prevent admissions and cause procedures and operations to be postponed (Barclay *et al*., 2014). Additionally, if staff become infected they cannot return to work for 48 hours. As a result, NoV outbreaks can have a severe financial impact. For example, a study by Danial *et al*. (2011) investigated the effect of NoV gastroenteritis within NHS Lothian between 2007-2009, during which period there was a loss of £1.2 million. Losses were incurred as a result of lost bed days, due to restricted admissions and therefore treatments; as well as due to staff absences and agency staffing costs (Danial *et al*., 2011). The global economic impact is even greater, with NoV infections thought to result in additional costs of approximately £3.2 billion ($4.2 billion US Dollars) per year to global health systems (Bartsch *et al*., 2016).

2.1.2 *Staphylococcus aureus*

S. aureus bacteria are Gram positive cocci, 0.5 to 1.5 μm in diameter, non-motile and facultatively anaerobic (Murray *et al*., 2008a). According to the WHO (2014), one of the most commonly identified antibiotic resistant bacteria to cause nosocomial infections worldwide is *S. aureus*. Antibiotic resistant isolates of *S. aureus* were first identified 75 years ago, shortly after the introduction of the antibiotic penicillin (Rammelkamp and Maxon 1942). Resistance to penicillin occurred as a result of bacterial production of penicillinase, a β-lactamase enzyme, encoded by the *blaZ* gene residing on a large plasmid transposon (Peacock and Paterson 2015). This enzyme is able to inactivate penicillin via hydrolysation of the β-lactam ring, and is now thought to be present in > 90% of human *S. aureus* isolates (Kirby 1944; Peacock and Paterson 2015).

A semisynthetic antibiotic, methicillin, was subsequently developed to help overcome penicillin-resistant *S. aureus*. This β-lactamase stable β-lactam antibiotic was designed to target cell wall synthesis, however resistance also occurred rapidly following clinical implementation, and these resistant bacteria were termed methicillin-resistant *S. aureus* (MRSA) (Jevons *et al*., 1963). The term MRSA is now used to describe isolates resistant to other β-lactam antibiotics, such as flucloxacillin and amoxicillin. Methicillin-resistance has emerged due to alterations in the penicillin-binding-protein, PBP. The altered PBP, known as PBP2a, has reduced affinity for β-lactam antibiotics allowing cell wall biosynthesis to continue (Peacock and Paterson 2015). PBP2a is encoded by the *mecA* gene carried on a distinct mobile genetic element, staphylococcal chromosomal cassette (SCC*mec*) (Peacock and Paterson 2015).

Recently, isolates of MRSA have also been identified with resistance to vancomycin, which is a last resort drug for treating MRSA associated infections (Gardete and Tomasz 2014). This resistance mechanism is due to plasmid-borne copies of the transposon *Tn1546*, which was acquired from vancomycin-resistant *Enterococcus faecalis*, which lowers affinity of vancomycin to the precursor units of the bacterial cell wall (Sivert *et al*., 2008).

Although attention has recently shifted to Gram negative multi-drug resistant (MDR) organisms, such as *E. coli, K. pneumoniae* and *P. aeruginosa* (Table 2.1), *S. aureus* continues to be a persistent problem within healthcare settings and a major cause of nosocomial infections, with Epidemic MRSA-15 and EMRSA-16 regularly identified (Coia 2016). *S. aureus* can be transmitted by direct contact with colonised/infected individuals or via environmental surfaces and air. This organism is both a commensal and a pathogen, with around 20% of people persistently nasally colonised and a further 30% intermittently colonised (Wertheim *et al*., 2005). Screening and decolonisation of *S. aureus* carriers in hospitals is an important strategy to help control MRSA, as carriers are more likely to develop nosocomial infections (Wertheim *et al*., 2005; Coia *et al*., 2006). This includes: skin and soft tissue infections, device-related infections, pulmonary infections, endocarditis, bacteraemia and sepsis, which can become systemic and life-threatening (Tong *et al*., 2015).

Recently there has also been widespread emergence of community associated MRSA (CA-MRSA). CA-MRSA is genetically distinct from hospital associated MRSA (HA-MRSA) and can be rapidly spread in the community, affecting younger, healthier people, compared to HA-MRSA, which usually affects hospitalised, elderly patients with a history of surgery or indwelling devices (Naimi *et al*., 2003; Chambers and DeLeo 2009;

Otter and French 2011). CA-MRSA strains such as USA300 are beginning to replace/overtake traditional HA-MRSA lineages as a common cause of hospital infection (Otter and French, 2011; Thurlow *et al*., 2012).

The emergence of CA-MRSA is a further burden for hospitals. These pathogens can cause infections in previously healthy individuals and therefore can infect wider groups of patients and HCWs, increasing hospitals costs and resource utilisation. Reservoirs of MRSA in the community (as well as reservoirs within livestock) provide opportunities in which new antimicrobial resistance and virulence traits can be selected (Coia 2016). These can be passed on to HA-MRSA, and in turn CA-MRSA may also develop additional resistance mechanisms due to the antibiotic selective pressure in hospitals, making infections harder to treat (Otter and French 2011). Finally, and perhaps most importantly, as CA-MRSA can be constantly re-introduced into the hospital environment, this will reduce current hospital control of MRSA, with nasal screening only detecting 67% of CA-MRSA carriers (Yang *et al*., 2009; Otter and French 2011).

2.2 Transmission of Nosocomial Pathogens

Due to the relentless prevalence of infectious pathogens within the hospital environment it is important to understand the general routes in which they are released into the environment and are transferred amongst patients. The main routes of potential transmission in the clinical setting include transmission via aerosols, droplets, contact, food, water and common environmental sources such as surfaces, equipment and water outlets (and in warmer climates also vectors such as mosquitos and ticks).

Microorganisms can be dispersed into the air via talking, coughing, laughing and sneezing, in both small droplets (< 5 μm) and large droplets (> 5 μm), which are involved in airborne transmission or droplet transmission, respectively (Fernstrom and Goldblatt 2013). The small droplets form aerosols which can spread over wide areas and can also evaporate to form droplet nuclei. Droplet nuclei can remain infectious for long periods of time depending on size, velocity, density, humidity and light (Memarzadeh *et al*., 2010). Droplet nuclei can travel via air currents, which can be enhanced by ventilation systems, and cover wide areas within hospitals. These infectious particles can be inhaled by susceptible patients and HCWs and as a result cause infection (Memarzadeh *et al*., 2010). However, this is dependent on close proximity, high levels of dispersal, high susceptibility or a combination of these factors (Coia *et al*., 2013). Large respiratory droplets can travel directly from the respiratory tract to

HCWs or other patients by depositing on mucosal surfaces such as eyes, nose, or mouth. When in close contact, this can be an efficient means of transmission as droplets contain high numbers of microorganisms (Coia *et al*., 2013). Droplet transmission was only thought to be effective over short distances, with sedimentation occurring within 1-2 m, due to the large droplet size (Memarzadeh *et al*., 2010). However recent studies observing sneezing and coughing indicated that droplets can travel up to 6-8 m, and stay suspended in clouds for up to 10 min, before landing on surfaces to act as a secondary source of transmission (Bourouiba *et al*., 2014). Although the range of pathogens transmitted by airborne transmission is fairly small, this can include respiratory and enteric viruses. A prime example being NoV, which can be inhaled directly following aerosolisation of vomit, as well as droplets of vomit and faeces being able to settle on near-by surfaces and act as a secondary source of transmission if touched.

Additionally, aerial dispersal involves the distribution of pathogenic organisms on particles such as skin squames. This occurs in the case of MRSA, which is commonly found on the skin of colonised patients. MRSA can be shed into the air directly from patients or following changing of wound dressings or bed sheets, and remain in the air for up to an hour (Sergent *et al*., 2012; Bache *et al*., 2015). These skin particles travel via air and land on different surfaces, with bigger scales landing nearby, and smaller travelling the length of wards and often settling on hard to reach surfaces and ledges (Eames *et al*., 2009). These MRSA particles can then survive for long periods of time, with MRSA capable of surviving in dust for up to a year (Wagenvoort *et al*., 2000).

Contact transmission, either through direct or indirect contact, is the most common route of pathogen transmission, and is illustrated in Figure 2.1. Direct contact occurs when susceptible patients are touched by a HCW or visitor who is colonised or infected with a pathogenic organism. Indirect transmission can also occur when HCWs transmit pathogens from an infected/colonised patient to a susceptible patient, usually via their hands. Indirect transmission can also occur if an infected/colonised patient touches and contaminates surfaces or inanimate objects which can also be referred to as fomites. Fomites can become contaminated through contact with droplets or bodily fluids such as faeces, vomit, blood and nasal discharge (Boone and Gerba 2007). Subsequent contact with these fomites by susceptible patients may result in colonisation and infection, especially if they come into contact with hands.

Common sources such as food, water, medications, solutions, devices, or equipment can also act as vehicles of transmission of nosocomial pathogens, with many people exposed and

becoming ill. Sinks and taps have acted as common sources of *P. aeruginosa* within the clinical environment, with biofilms contaminating water, resulting in outbreaks and many unnecessary deaths (Hota *et al*., 2009; Walker *et al*., 2014; Garvey *et al*., 2016). Additionally, NoV is not only spread via the air and fomites but also via the faecal-oral route. Infected individuals can contaminate food and water, and in turn these can act as common sources of transmission (Maunula 2005; Raj *et al*., 2017; Smith *et al*., 2017). Contaminated fomites such as inanimate objects and equipment can also become common sources of outbreaks, with incidences of *K. pneumoniae* linked to ultrasound gel (Galliout 1998) and outbreaks of *P. aeruginosa* and *E. coli* linked to surgical probes (Bancroft *et al*. 2013; Seki *et al*. 2013). Even unexpected items such as 'non-slip' socks have recently become implicated as vectors in the transmission of MDR organisms around hospitals (Mahida and Boswell 2016).

2.2.1 Transmission via Healthcare Workers Hands

It is apparent that transmission of nosocomial pathogens can be via multiple routes and is interlinked (Fig 2.1). HCWs play a pivotal role in transmission, with hands thought to be the main way in which nosocomial pathogens are spread (Allegranzi and Pittet 2009). It is therefore imperative that HCWs wash their hands (i) before touching patients; (ii) before clean/aseptic care techniques; (iii) after contact with body fluid; (iv) after touching patients and (v) after touching patient surroundings (Pittet *et al*., 2006; WHO 2009).

Numerous studies have demonstrated that improvement of hand hygiene compliance helps to reduce HAIs (Pittet *et al*., 2000; Lam *et al*., 2004; Pessoa-Silva *et al*., 2007; Grayson *et al*., 2008; Salama *et al*., 2013; Sickbert-Bennet *et al*., 2016). The long-term effect of hand hygiene improvement was demonstrated almost twenty years ago, by Pittet *et al*. (2000), who investigated hand hygiene compliance over a 3 year period. Following an awareness campaign with posters and implementation of alcohol rubs, hand hygiene compliance improved from 47.6% pre-intervention to 66.2% post intervention. Increased hand hygiene then lead to a reduction of nosocomial infections from 16.9% pre-intervention to 9.9% over the 3 year period (Pittet *et al*., 2000). Increased hand hygiene has also recently been shown to reduce infections with MDR organisms. Salama *et al*. (2013) demonstrated hospital hand hygiene compliance rose from 43 % to 65% following a multifaceted intervention using lectures, posters, leaflets and installations of alcohol based hand sanitizers. Following intervention, the total number of nosocomial infections fell from 37.2 to 15.1 per 1,000 patient-days, with 35.5 %, 50 %, 74.4% and 74.4% reductions of infections due to MDR *Acinetobacter baumannii*, *E. coli*,

K. pneumoniae and MRSA (Salama *et al*., 2013). Additionally, no infections occurred due to *Clostridium difficile* and *P. aeruginosa*, following improved hand hygiene practices post-intervention (Salama *et al*., 2013).

Transmission via hands is likely due to hand contamination following direct contact with an infected/colonised patient or contact with surfaces and equipment surrounding patients' bed spaces. An interesting study by Barker *et al*. (2004) demonstrated that clean fingertips inoculated with NoV could contaminate common hand touch surfaces and up to 7 clean surfaces, demonstrating the importance of hand hygiene to prevent spread. Several studies have also demonstrated the contamination of HCW's hands or gloves after contact with environmental surfaces surrounding patients (Bhalla *et al*., 2004; Stiefel *et al*. 2011; Guerrero *et al*., 2012; Morgan *et al*., 2012). For example, Bhalla *et al*. (2004) demonstrated that 53% of hands were contaminated with one or more pathogens following contact with environmental surfaces near patients. Furthermore, hands are just as likely to become contaminated following exposure to surfaces as exposure to patients. This was demonstrated by Stiefel *et al.* (2011) who found a 40% chance of gloved hand contamination following contact with colonised skin and 45% chance of contamination following surface contact.

Hand hygiene compliance is therefore equally as important when touching patients as it is when touching surfaces surrounding patients. However, Randle *et al*. (2010) demonstrated compliance differs between these two clinical contact scenarios. A 24 hour observational study of hand hygiene in two hospital wards, found that hand hygiene compliance is reduced when HCWs touch surfaces compared to patients, with only 50% compliance when touching surfaces compared to 80% compliance after patient contact (Randle *et al*., 2010).

2.2.2 Transmission via Environmental Surfaces

It is now widely accepted that environmental surfaces may facilitate the transmission of nosocomial pathogens (Boyce *et al*., 2007; Dancer *et al*., 2008; Weber *et al*., 2010). There is likely to be cross-infection between patients and their environment, with those infected or colonised shedding pathogens into their surrounding environment. Not only can MRSA be shed into the environment on skin and respiratory secretions but it can also be shed via stools. Boyce *et al*. (2007) demonstrated that patients with gastrointestinal tract colonization with MRSA caused increased environmental contamination, with > 50% of the surfaces sampled contaminated with identical or similar strains of MRSA. NoV is similarly shed into the environment via stools: asymptomatically, before symptom onset, during infection, and following infection resolution, as well as via aerosolised vomitus which can settle onto

surfaces (Atmar *et al*., 2008; Lopman *et al*., 2012; Sabrià *et al*., 2016). Additionally, MDR organisms such as *K. pneumoniae* cause environmental contamination, with the same strain infecting a patient found on environmental sites including handrails, shower faucets, light switches and mattress covers (Judge *et al*., 2013).

As well as environmental surfaces, modern technological equipment such as computers, keyboards, mobile phones and up to 95% of bed control handsets can become contaminated, so these items may also play a role in nosocomial pathogen transmission (Neely *et al*. 1999; Bures *et al.* 2000; Hartmann *et al.* 2004; Brady *et al.* 2007; Ulger *et al*., 2009; Messina *et al.* 2013). Portable equipment such as blood pressure monitors and radiograph machines have also been found to be inadequately cleaned by nursing and technical staff, with *A. baumannii, K. pneumoniae* and *P. aeruginosa* isolated (Levin *et al*. 2009; Havil *et al*. 2011).

Upon release, nosocomial bacteria can thrive in the warm hospital environment and subsequently survive on these hospital environmental surfaces and in the air (Dancer 2014). Surfaces can act as long term reservoirs of both bacterial and viral pathogens, and as can be seen in Table 2.3, these pathogens can survive from hours to years. The long-term survival of vegetative organisms may be attributed to biofilms on dry surfaces, with some species shown to survive even after terminal decontamination (Vickery *et al*., 2012). A key example of ineffective terminal cleaning against nosocomial pathogens was demonstrated by Manin *et al*. (2011), who found MRSA and *A. baumannii* on surfaces after 4 rounds of terminal cleaning and disinfection with bleach. Another example of substandard cleaning was demonstrated by Morter *et al*. (2011) who identified NoV on 31% of environmental swabs post cleaning on keyboards, computers, soap dispensers, equipment as well as surfaces around bedside furniture and fixtures.

This failure to fully decontaminate surfaces has been shown to increase the risk of acquisition of nosocomial pathogens, with an increased risk of infection if a room was previously occupied by a patient infected with *A. baumannii, C. difficile*, MRSA, MDR *P. aeruginosa* and VRE (Huang *et al*., 2006; Drees *et al*., 2008; Shaughnessy *et al*., 2011; Nseir *et al.*, 2011; Mitchell *et al*., 2014). However, if cleaning is improved or increased as part of an outbreak control bundle, this can lead to the resolution of outbreaks (Mayfield *et al*., 2000; Denton *et al*., 2004; Hayden *et al*., 2006; Palmore and Henderson 2013).

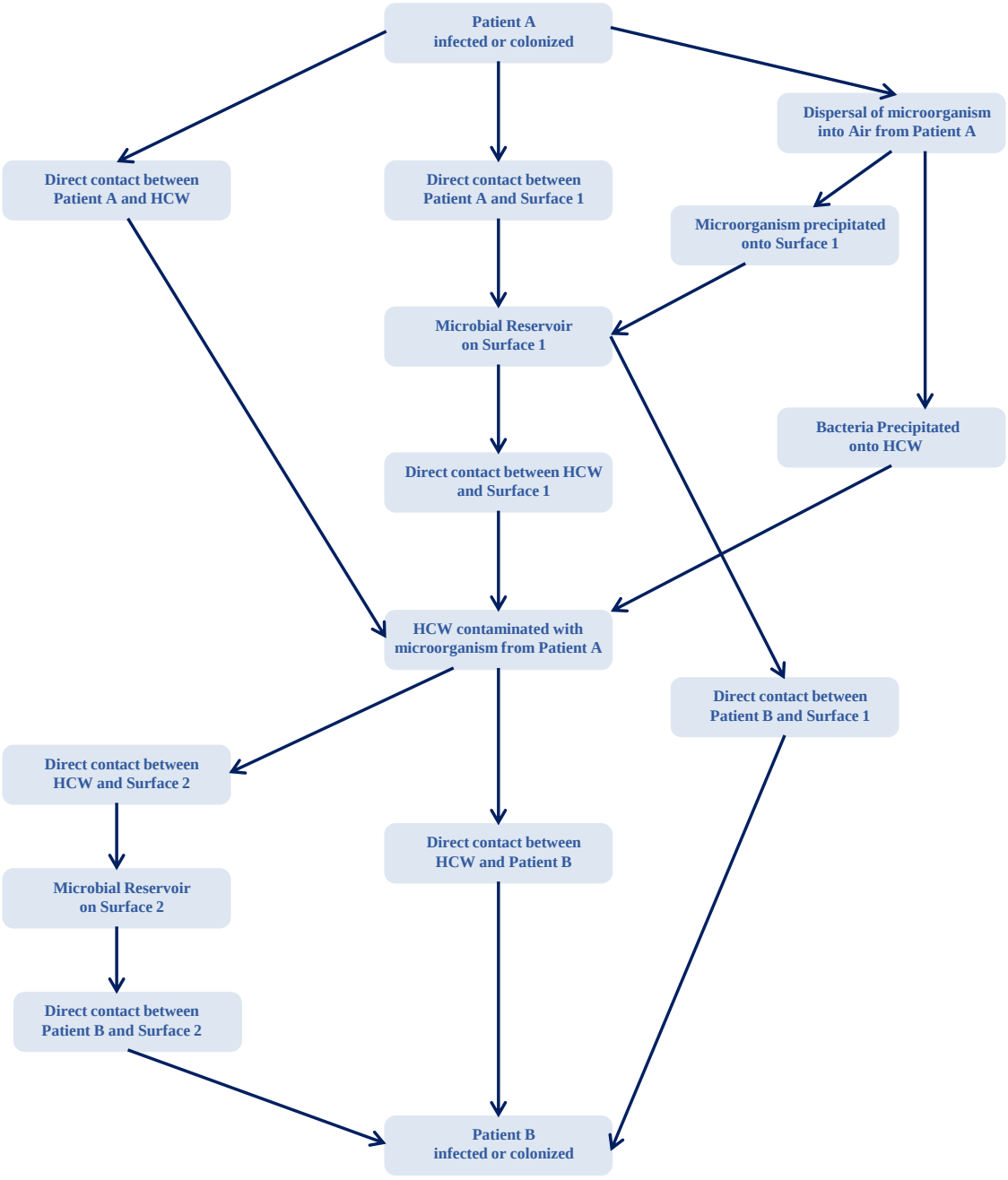


Figure 2.1 Routes of direct and indirect transmission between colonised/infected patients and susceptible patients. Adapted from Bache (2013).

Table 2.3 Survival rates of common hospital associated pathogens on surfaces. Adapted from Kramer *et al*. (2006), Otter *et al*. (2013) and Kotwal and Cannon (2014).

Nosocomial Bacteria	**Survival on Environmental Surfaces**
Acinetobacter spp.	3 days – 11 months
Clostridium difficile (spores)	> 5 months
Escherichia coli	1.5 hours – 16 months
Enterococcus spp. (incl VRE)	5 days – > 46 months
Klebsiella spp.	2 hours – > 30 months
Pseudomonas aeruginosa	6 hours – 16 months
Staphylococcus aureus (incl MRSA)	7 days – 12 months
Nosocomial Viruses	**Survival on Environmental Surfaces**
Adenovirus	7 days – 3 months
Hepatitis B	> 1 week
Influenza	1– 2 days
Norovirus	8 hrs – > 1 month
Respiratory syncytial virus	6 hours
Rotavirus	6 – 60 days

2.3 Environmental Decontamination in Hospitals

As contaminated hands and environmental surfaces have an interlinked and integral role in transmission of nosocomial pathogens (Fig 2.1), environmental decontamination is an important method for reducing the threat of nosocomial pathogens. Decontamination includes (i) cleaning and disinfection of non-critical and semi-critical items, or (ii) cleaning and sterilisation of critical items, both in an effort to reduce the risk of patients acquiring HAIs (Veerabadran and Parkinson 2010). Decontamination of non-critical environmental surfaces and non-invasive equipment, like blood pressure cuffs, first requires cleaning with detergents for the physical removal of pathogens and organic matter (Veerabadran and Parkinson 2010). Subsequently, the surfaces are treated for > 1min using low-level chemical disinfectants (detailed in Table 2.4) which physically or chemically destroy any pathogens present (Veerabadran and Parkinson 2010; Rutala and Weber 2013). Inadequate cleaning can, however, interfere with disinfection if organic matter is not completely removed from surfaces and equipment.

Isolation of patients and increased decontamination measures are also required to control environmental contamination when patients are infected or colonised with pathogens such as *C. difficile*, MDR Gram negative bacilli, MRSA, NoV and VRE (Veerabadran and Parkinson 2010; Dancer 2014). In the case that transmission occurs between several patients and results in an outbreak, additional measures are required to help to reduce the spread of infection. These include closure of wards and restriction of patient movement (Danial *et al*., 2011). After an isolated patient is discharged or an outbreak is resolved, wards and isolation rooms must undergo a terminal clean whereby the area is stripped of rubbish, curtains and bed linen, and all equipment and surfaces are decontaminated before rendering safe for new patients (Danial *et al*., 2011).

Table 2.4 Comparison of commonly used disinfectants within hospitals. Adapted from Rutala and Weber (2013).

Disinfectant	Advantages	Disadvantages
Alcohol	• Effective against bacteria, fungi & viruses • Rapid • Easy to use • No toxic residue	• Not effective against spores • Affected by organic matter • May cause material damage (e.g. rubber, glue) • Cannot be used for large surface areas • Flammable
Chlorine	• Broad antimicrobial efficacy against bacteria, fungi, viruses & spores • No toxic residue • Inexpensive • Not affected by water hardness • Stable (30 day half-life)	• Exposure can cause eye irritation, oropharyngeal, oesophageal and gastric irritation and burns • Affected by organic matter • May corrode metal surfaces and discolour fabrics • Can release toxic fumes if mixed with acids or ammonia
Idophors	• Effective against bacteria, mycobacteria & viruses	• Not effective against spores • Requires long exposures for fungal inactivation • More commonly used as an antiseptic than disinfectant • Can cause material damage (e.g. silicon)
Improved Hydrogen Peroxide*	• Effective against bacteria, mycobacteria & viruses • Safe for users • Not affected by organic matter • Non-corrosive and non-staining • Compatible with common surface materials • 12 month shelf life	• Expensive • Limited proof of antimicrobial efficacy
Phenolics	• Effective against bacteria, fungi & viruses • Inexpensive	• Not effective against spores • Absorbed by porous materials • Irritates tissues and causes depigmentation of skin • If not prepared correctly may cause hyperbilirubinemia in infants
Quaternary Ammonium	• Effective against bacteria, fungi & enveloped viruses • Compatible with common surface materials • Undisturbed solutions can have persistent antimicrobial activity	• Not effective against spores • Not effective against non-enveloped viruses • Less microbicidal when used with hard water and cotton • Exposure may potentially cause asthma • Affected by organic matter

* Improved hydrogen peroxide contains very low levels of anionic and/or non-ionic surfactants, which help to increase the speed of antimicrobial activity (Rutala *et al*., 2012).

2.3.1 Routine and Terminal Decontamination

For effective decontamination, it is important that staff are aware of their specific responsibilities and schedules, including frequency and method of environmental decontamination, to prevent confusion and inadequate cleaning (Anderson *et al*., 2011). In general, domestic staff should clean surfaces, tables, floors and under bed areas, with nursing staff cleaning all patient care equipment and the base of the bed, mattress and rails (NHS GGC 2016). The National Infection Prevention and Control Manual (NIPCM 2012) has been developed in Scotland to help provide guidance on standard procedures to help reduce the risk of HAIs. Procedures within the manual are mandatory for all NHS Scotland staff, and therefore ensure a constant, effective infection control practice across Scottish hospitals, allowing better-quality auditing and improvements.

The NIPCM (2012) states that routine cleaning of the patient environment should be carried out using a fresh solution of neutral pH detergent, however detergent wipes are commonly used for this purpose for convenience (NHS GGC 2016). Additionally, chlorine based disinfectants should be used routinely on sanitary fittings including toilets, sinks, basins, baths, taps and fixtures. Cleaning of isolation or cohort rooms housing patients suspected, colonised or infected with a contagious pathogen, must be carried out using either detergent followed by disinfection solution of 1000 ppm chlorine (cl), or a combined detergent/disinfectant solution (1000 ppm cl). Cleaning in these cases is required at least daily and even more frequently in the case of high-touch surfaces and toilet/commodes (NIPCM 2012).

When patients have been discharged, transferred, or are no longer an infection risk, terminal decontamination is required. The NIPCM (2012) states that firstly all rubbish, screens, curtains and laundry should be bagged and removed from the patient area. Using the previously described chlorine based disinfectants, all surfaces, basins, showers, toilets, patient equipment, computing equipment, beds, mattresses, rails, buzzers, lockers and tables in each patient space must then be cleaned, with cleaning starting at the top and finishing on the lower surfaces (NIPCM 2012). Supplies should then be replenished and surfaces left to dry before the area can be re-opened (NHS GGC 2016).

2.3.2 Detergent Wipes

Detergents wipes are becoming increasingly popular for environmental cleaning in hospitals due to their convenience, ease of use and disposal. Detergents within these wipes are water

soluble chemical compounds such as surfactants (Ramm *et al*., 2015). These disposable wipes have been demonstrated to reduce bacterial burden on high risk sites such as bed rails and over-bed tables, reducing levels to below 5 CFU/cm^2 for up to 24 hours (Boguz *et al*., 2013).

However, the effectiveness of these wipes has been challenged by Ramm *et al*. (2015), who found that bacterial reduction could not only vary between microorganisms, but also brands of wipes tested. Following 10 seconds of wiping, the average removal of bacteria from stainless steel surfaces ranged from 0.96 log_{10}, 1.5 log_{10} and 3.5 log_{10} reduction for *C. difficile, S. aureus and A. baumannii,* respectively (Ramm *et al*., 2015). Furthermore, vegetative bacteria and spores could also be transferred over 3 surfaces via detergent wipes (Ramm *et al*., 2015). Therefore, these wipes may be useful for removing bacterial bioburden, but do not have a universal efficacy against the various organisms found in the environment. Wipes can spread pathogens if users do not change wipes in between surfaces, and may necessitate the need for additional disinfectants following use.

2.3.3 Chlorine Based Disinfectants

Chlorine based disinfectants such as bleach made of 0.1% sodium hypochlorite (1000 ppm cl) are commonly used for disinfection in hospitals as they can inactivate a broad range of organisms (Tanner 2009). However, these disinfectants can be toxic to humans, may be corrosive to surfaces, and also ineffective if diluted or if contact time is not long enough (Tanner 2009; Veerabadran and Parkinson 2010).

Actichlor is one such chlorine based disinfectant commonly used in Scottish hospitals (NHS GGC 2016). With sodium dichloroisocyanurate as the active ingredient, Actichlor has been demonstrated to inactivate vegetative bacteria as well as spores (Vohra and Poxton 2011). Actichlor also has greater efficacy than other disinfectants, with > 3 log_{10} inactivation of *C. difficile* spores in organic soiling compared to only 0.7 log_{10}, 0.5 log_{10} and 0.2 log_{10} reductions achieved using Mirasol 3+, TriGene Advance and Virkon, respectively (Vohra and Poxton 2011).

However, Actichlor has been shown to be less effective during NoV outbreaks. Morter *et al*. (2011) studied environmental cleanliness during NoV outbreaks for 4 months and demonstrated that many surfaces were contaminated with the virus even after cleaning with Actichlor. After an initial clean, NoV was detected in 31.4% of the 239 environmental swabs tested, with ~50% of swabs from soap dispensers and patient equipment testing

positive. Furthermore, in two of the wards studied, surfaces were re-cleaned but NoV was still detected on 13.2% and 19.4% of the surfaces swabbed (Morter *et al*., 2011).

2.4 Novel Decontamination Technologies for Hospitals

Although there is greater awareness of the importance of environmental cleaning for reducing HAIs, cleaning can be inefficient, particularly in busy, understaffed wards (Harbarth *et al*., 1999). This has resulted in the development of technologies which are designed for 'whole-room' decontamination. These novel technologies are designed to provide more thorough environmental decontamination over and above normal procedures used in hospitals. The following section will discuss the mechanisms and antimicrobial efficacy of terminal decontamination technologies such as hydrogen peroxide vapour, ozone, cold atmospheric pressure plasma and UV light, as well as the HINS-light Environmental Decontamination System which is alternatively used for continuous environmental decontamination.

2.4.1 Hydrogen Peroxide Vapour

Hydrogen peroxide vapour technologies are becoming increasingly popular for terminal decontamination of surfaces and objects within hospital rooms and wards. These technologies release hydrogen peroxide (H_2O_2) that forms oxidizing hydroxide and ferryl free radicals, which inactivate microorganisms by reacting with nucleic acids, proteins and membrane lipids, preventing them from functioning correctly and halting replication and infection (Pottage *et al.*, 2010; Linley *et al*., 2012). Hydrogen peroxide is commonly used in one of two forms for hospital disinfection: aerosolised hydrogen peroxide (aHP) or hydrogen peroxide vapour (HPV) (Otter *et al*., 2013)

Aerosolised HP systems, such as ASP Glosair and Oxypharm Nocospray, deliver a pressure generated fine mist aerosol of H_2O_2. The chemical composition is usually 5%-6% hydrogen peroxide, 50 ppm silver and 95% de-ionised water (Barbut *et al*., 2009). The recommended dose for hospitals is 6 ml/m^3 per cycle, during which electrically charged particles circulate in the air, adhere to microbial particles in the air and on surfaces and inactivate them (Barbut *et al*., 2009; Boyce *et al.*, 2009). As the H_2O_2 used in aHP systems naturally decomposes, no aeration systems are required. Alternatively, HPV works by generating a 30-35% w/w vapour of hydrogen peroxide through a high-velocity air stream, with a dose of 10 g/m^3 recommended per cycle (Boyce *et al*., 2009; Fu *et al*., 2012). HPV systems differ. The Steris

VHP system produces non-condensing vaporised hydrogen peroxide, at a constant concentration, which does not condense onto surfaces as the air is continually dried (Otter *et al*., 2011). In contrast, the Bioquell BQ-50 system fills the air with hydrogen peroxide vapour, which is not controlled, and the vapour subsequently condenses on surfaces (Boyce *et al*., 2009). Unlike aHP systems, HPV systems require active catalytic conversion to aid decomposition of the hydrogen peroxide into non-toxic by-products (Otter *et al*., 2009).

Both aHP and HPV have broad antimicrobial efficacy. aHP has efficacy against nosocomial pathogens including *A. baumannii*, *C. difficile*, MRSA and VRE (Barbut *et al*., 2009; Boyce *et al*., 2008; Chan *et al.*, 2011). Decontamination of various surfaces in hospital rooms previously occupied by *C. difficile* infected patients, using aHP, resulted in 91% reduction of contamination compared to only 50% using conventional cleaning with 0.5% sodium hypochlorite (Barbut *et al.*, 2009). Although aHP is easier to use, has lower costs, and can naturally decompose, HPV is thought to have superior efficacy against nosocomial pathogens (Barbut *et al*., 2009; Herruzo *et al*., 2014). Fu *et al*. (2012) confirmed this, by demonstrating $\geq 2 \log_{10}$ inactivation of *A. baumannii, C. difficile* and MRSA using HPV, over and above the level of inactivation achieved using aHP. Tuladhar *et al.* (2012) also demonstrated that HPV can be used for viral decontamination on surfaces, with $> 4 \log_{10}$ reduction of several respiratory and enteric viruses including avian Influenza virus, swine Influenza virus, human Adenovirus type 1 and surrogates of NoV and severe acute respiratory syndrome coronavirus (Goyal *et al*., 2014). However, Fu *et al*. (2012) demonstrated that organic soiling reduced the efficacy of both aHP and HPV inactivation of MSSA, indicating the need for pre-clean before use.

2.4.2 Ozone

Ozone is a very strong oxidizing agent which has also been utilised for terminal decontamination. It is thought that free radicals produced cause oxidative damage to the bacterial cell wall and cytoplasmic membrane, and lipid peroxidation of viruses and subsequent lipid envelope and protein shell damage (Li and Wang 2003; de Boer *et al*., 2006; Murray *et al*., 2008b). Systems used for ozone decontamination include the Meditrox 100, which takes approximately one hour for a full cycle, including conversion of ozone to safe levels using a catalytic convertor or quench gas at the end of the cycle (Sharma and Hudson 2008; Moat *et al*., 2009).

Ozone can inactivate a wide range of microorganisms including *A. baumannii, C. difficile*, MRSA and NoV (Hudson *et al.*, 2007; Sharma and Hudson 2008). Hudson *et al*. (2007) demonstrated that a 3 $\log_{10}$ reduction of the NoV surrogate, feline calicivirus, could be achieved within one hour when the virus was dried onto hard surfaces and fabrics in conditions relative to the healthcare setting. However, this technology is less efficacious against spores and fungi, with only a 1.3 $\log_{10}$ reduction of *C. difficile* spores and *Bacillus cereus* achieved after exposure (Moat *et al*., 2009; Doan *et al*., 2012).

Interestingly, recent studies have also indicated that a high level of decontamination can be achieved when ozone is used in conjunction with HPV. In a study by Zoutman *et al*. (2011) of room decontamination using 80 ppm ozone and 1% hydrogen peroxide, $\geq 6 \log_{10}$ reduction of *E. coli*, MRSA and VRE was achieved after 60 mins and ~ 6 $\log_{10}$ reduction of *B. subtilis* and *C. difficile* was achieved after 90 minutes.

2.4.3 Cold Atmospheric Pressure Plasma

The use of cold atmospheric pressure plasma (CAPP) is also gaining attention for potential environmental decontamination applications. CAPP systems release reactive oxygen and nitrogen species such as $^1O_2$, O_3, NO_2, peroxynitrous acid and UV radiation, with combinations released depending on the plasma-generating mechanism e.g. plasma jet, di-electric barrier charge (O'Connor *et al*., 2014; Aboubakr *et al*., 2015). A mixture of the above properties, in different proportions can be used to inactivate microorganisms, through disruption of bacterial cell walls, destruction of viral capsids, and damage to microbial DNA/RNA (Cahill *et al*., 2014; Aboubakr *et al*., 2015, 2016).

CAPP has a wide antimicrobial efficacy and has demonstrated potential for many different clinical applications including skin decontamination, wound healing and dermatitis treatment (Daeschlein *et al*., 2012; Klämpfl *et al*., 2012; Isbary *et al*., 2012, 2013a, b; Emmert *et al*., 2013; Ahlfeld *et al*., 2015). Recent laboratory studies have focused on using this technology for decontamination of clinical surfaces. A 'CAPP Single Jet' system generally achieved $\geq 3 \log_{10}$ reductions of MDR *A. baumannii*, MDR *E. coli*, VRE and MRSA on clinically relevant surfaces including mattresses, marmoleum, polypropylene and stainless steel, when exposed for 90s, 1 cm from the surfaces (Cahill *et al*., 2014). Additionally, Aboukhar *et al*. (2015) demonstrated an approximate 6 $\log_{10}$ reduction of a NoV surrogate in distilled water when exposed to Argon plus 1% O_2 plasma for 15 seconds. As exposure only requires a matter of minutes, this may point to using CAPP for direct

applications on specific surfaces such a spot cleaning of areas which have been in contact with biological fluids from infected patients.

2.4.4 UV Light Decontamination Technologies

Ultra-violet (UV) light (100-400 nm) is electromagnetic radiation, which is divided into four distinct spectral areas (Fig 2.2): UVA (315-400 nm), UVB (280-315 nm), UVC (200-280 nm) and vacuum UV (100-200 nm) (Kowalski 2010). UV exposure of DNA causes the formation of pyrimidine (cytosine & thymine) dimers in the same strand of nucleic acid by covalent bonding. The bonding forms cyclobutane dimers and pyrimidine-pyrimidone 6-4 photoproducts (Maclean *et al*., 2008c). These photoproducts prevent the transcription and replication processes of the microorganism so it can no longer function (Maclean *et al*., 2008c; Ben Said 2009). Less is known about UV damage to RNA, although damage is thought to occur due to photochemical modification, crosslinking, and oxidative damage (Wurtmann and Wolin 2009). UV in the region of 250-270 nm is considered most effective for microbial inactivation, due to the nucleotide base components of DNA having peak absorbencies in this region (Maclean *et al*., 2008c; Kowalski 2010).

UV light is particularly popular for terminal decontamination in hospitals, with mobile devices designed for room decontamination utilising either continuous or pulsed UV light (Rutala *et al*., 2010; Otter *et al*., 2013; Maclean *et al*., 2015). It is popular due to the fact it has reliable biocidal activity against a range of hospital associated pathogens; does not produce by-products; does not require storage, handling or disposal of corrosive chemicals; does not require sealing of rooms (unlike HPV and Ozone); and the cycle times are relatively short (15-45 mins) (Rutala and Weber 2013b). There are two main type of UV device which emit either continuous or pulsed UV light, as discussed below.

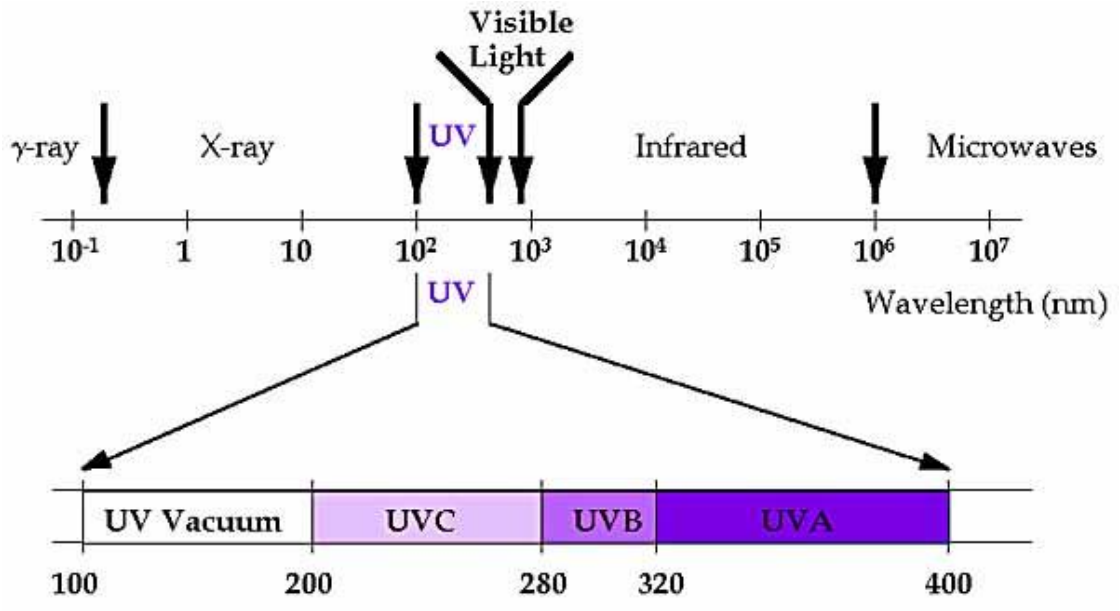


Figure 2.2 Electromagnetic spectrum. Adapted from Soehnge *et al*. (1997).

2.4.4.1 Continuous UV Light

There are several continuous UV light systems available for hospital decontamination including: Clinell UV-360; Ultra-V (Hygiene solutions); Helios Tripe UV system (Surfacide) and Tru-D Smart UVC (Lumalier). These continuous UV systems generate UVC light using low pressure mercury vapour lamps, which emit UVC at a wavelength of 254 nm (Boyce 2016).

The majority of studies to date have investigated inactivation using the Tru-D device using doses of 12 mJ/cm^2 for vegetative bacteria and 22 mJ/cm^2 for spores (Rutala *et al*., 2010). Studies have shown that these devices can decontaminate rooms spiked with important hospital pathogens including *C. difficile* spores, MDR *A. baumannii*, MRSA and VRE, with 2-4 $\log_{10}$ reduction achieved when in direct line of UVC irradiation (Nerandzic *et al*., 2010; Havil *et al.*, 2012; Mahida *et al*., 2013; Rutala *et al*., 2010, 2013a, 2014). Inactivation of vegetative cells is relatively quick at around 15-45 mins, however spores require double that normally between 45-90 mins (Nerandzic *et al*., 2010; Rutala *et al*., 2010, 2013a, b, 2014; Havil *et al*., 2012; Mahida *et al*., 2013).

However in all these aforementioned studies there was significantly less inactivation when organisms were out of direct line of sight. This was apparent in a study by

Mahida *et al*. (2013), with ≥ 4 $\log_{10}$ reduction of *A. baumannii*, *Aspergillus* spp. and VRE, in direct site after a dose of 12 mJ/cm^2, but only 1.7 $\log_{10}$, 2 $\log_{10}$ and 2.3 $\log_{10}$ reduction, respectively, when in shaded areas. Continuous UV has also been demonstrated to reduce bacterial contamination in patient rooms of infected or colonised patients (Boyce *et al*., 2011; Anderson *et al*., 2013). Anderson *et al*. (2013) demonstrated an overall 1.71 $\log_{10}$, 1.16 $\log_{10}$ and 1.68 $\log_{10}$ reduction of *A. baumannii*, *C. difficile* and VRE on common surfaces such as bed rails, following UVC exposure.

2.4.4.2 Pulsed UV Light

Pulsed UV (PUV) light technology is an alternative UV technology, which uses xenon flash lamps. These flash lamps can be operated to give a high-energy pulsed-output (milliseconds/pulse) and produce an intense light pulse of polychromatic light with output ranging from UV to infrared wavelengths (Lamont *et al.,* 2007). Currently the first and only commercially available system making use of PUV for hospital decontamination, is the Light Strike Germ Zapping Robot™ (Xenex). It is recommended these UV-robots undergo 2 to 3 5-min cycles per room, with each cycle emitting ~ 450 flashes (Jindatha *et al*., 2014).

Following a 10 min exposure in a sluice room, 5 $\log_{10}$ reduction of *A. baumannii*, MDR enterococci, MRSA and VRE could be achieved on spiked plates when 1.2 m away from a pulsed UV device (Hosein *et al*., 2016). Even greater inactivation efficacy can be achieved, with 8.7-9.3 $\log_{10}$ reduction of *A. baumannii, E. coli, K. pneumoniae, P. aeruginosa* and *S. aureus* when exposed for 5 mins, 1 m away from the sources (Stibich and Stachowiak 2016). PUV also has efficacy against bacterial spores and to a lesser extent fungi, with 3.3-3.8 $\log_{10}$ reduction of *Bacillus atrophaeus* and *Geobacillus stearothermophilis* and 0.33 $\log_{10}$ reduction of *Aspergillus* spp. following 5 mins of exposure. (Stibich and Stachowiak 2016). The virucidal efficacy has also been demonstrated with 0.86-1.54 $\log_{10}$ reduction of avian infectious bronchitis virus, Middle East respiratory syndrome coronavirus and vaccinia virus, when exposed in liquid and, > 5 $\log_{10}$ reduction of vesicular stomatitis virus on a petri dish after 5 min exposure (Stibich and Stachowiak 2016). Additionally a > 7 $\log_{10}$ reduction of Ebola virus was achieved after 1 min exposure (Stibich and Stachowiak 2016).

This technology has also been shown to reduce up to 99% of contamination on surfaces within hospitals, with 15 min exposures thought to improve general hospital hygiene (Jinadatha *et al*., 2014; Hosein *et al*., 2016). Stibich *et al*. (2011) demonstrated enhancement of hospital decontamination of VRE, with 23.3% VRE positive plates before cleaning,

8.2% positive after terminal clean and 0% present after PUV treatment. Unlike continuous UV, PUV is also effective if there is no pre-cleaning carried out, however increasing distance from the device does reduce killing efficiency (Jinadatha *et al*., 2015; Nerandzic *et al*., 2015)

2.4.5 HINS-Light Environmental Decontamination System

The HINS-light EDS is a new technology developed at The Robertson Trust Laboratory for Electronic Sterilisation Technologies (ROLEST), at the University of Strathclyde. Unlike the previously discussed technologies the EDS is designed for continuous decontamination of the hospital environment rather than for terminal cleaning purposes (Maclean *et al.*, 2010, 2013a; Bache *et al*., 2012). This technology can be installed into hospital ceilings and contains light emitting diodes (LEDs) which emit 405 nm violet-blue light at an irradiance of 0.1-0.5 mW/cm^2 (Fig 2.3) (Maclean *et al.*, 2008a, 2014). This wavelength of violet-blue light has been proven to inactivate many common bacterial pathogens (as will be discussed in Section 2.5.3) including *C. difficile, E. coli* and MRSA (Guffey and Wilborn 2006; Enwemeka *et al.*, 2008; Maclean *et al*., 2008a, 2009, 2013b; Murdoch *et al*., 2012, 2013).

Figure 2.3 HINS-light EDS installed into a hospital isolation room.

2.4.5.1 Light Emitting Diodes

LEDs, such as those used in the EDS, are semiconductors, which emit light when an electrical current is passed through. In the centre of the LED is the semiconductor chip made from nitride based materials, n-type and p-type, with the n-type material acting as a source of electrons and the p-type material providing mobile vacancy called 'holes' (Stevenson 2009). When a voltage is applied across the semiconductor, the electrons recombine with the positive electron holes, and in the active region between the n-type and p-type layers, the energy is released as photons of light (Stevenson 2009). The wavelength of light emitted is determined by the materials used for the band gap, with blue LEDs normally composed of gallium nitride, with the n-type doped with silicon and the p-type doped with magnesium and with the quantum well of the active region made of indium gallium nitride (Stevenson 2009).

The use of LEDs within the HINS-light EDS has many advantages and allows the colour output of this technology to be specifically violet-blue, peaking at 405 nm. LEDs are compact, long lasting, and can generate a bright level of light with less heat and energy consumed than normal fluorescent lighting (Ghate *et al*., 2013; Yeh *et al*., 2015). Additionally, several LEDs can be used to increase brightness rather than needing a higher current (Yeh *et al*., 2015). With LEDs increasingly being used in medical treatments, phototherapy, aquaculture, agriculture, biomass production and for photocatalyst activation, they are a smart choice for the EDS, as they are likely to become even more cost effective and efficient as their uses grow (Yeh *et al*., 2015).

2.4.5.2 Clinical Efficacy Testing

The HINS-light EDS has undergone numerous clinical evaluations within in-patient and out-patient settings. An initial study by Maclean *et al*. (2010) demonstrated the reduction of *Staphylococcus* contamination in burns isolation rooms using two HINS-light EDS units. Environmental surfaces were sampled using Baird parker agar contact plates, before, during and after EDS use. When the EDS was used in an unoccupied room there was a 91% reduction after 24 hours use, with a 40% increase in environmental contamination 24 hours after the lights were no longer in use. Similar patterns were seen when the EDS was used in an occupied burns patient room with 62-86% reduction in contamination over five days use, and sampling six days post use revealed an 126% increase in environmental contamination (Maclean *et al*., 2010).

Further evidence of the efficacy of the HINS-light EDS has been demonstrated through use in burns unit outpatient rooms as well as an ICU isolation room (Bache *et al*., 2012; Maclean *et al*., 2013a). The normal increase in contamination of environmental surfaces, throughout the duration of a burns outpatient clinic, decreased by 61.3% when the EDS was utilised (Bache *et al*., 2012). The investigations in an ICU isolation room also demonstrated 37.9% and 66.8% reduction in *Staphylococcus* counts after one and five days use (Maclean *et al*., 2013a). Reductions in total viable bacterial counts (through sampling with tryptone soya agar plates) were also demonstrated, with a 53.3% reduction of CFU when the EDS was in use for two days, and a 75% increase in CFU following sampling carried out two days after the lights had been switched off (Maclean *et al*., 2013a).

More recently this technology has been licensed in the USA, with 'Indigo-Clean' devices (Kenall, USA) available for use in American hospitals. These units emit low levels of 405 nm light at an irradiance of 0.1-0.2 mW/cm^2. Studies on the clinical efficacy of these units were presented at the ID Week Conference 2016. Sandhu *et al*. (2016) sampled 20 sites in an ICU suite for *Staphylococcus* species. Sampling was carried out at 7 am three times a week for four weeks. Sampling during the first week of the study, when the lights were not utilised, indicated 2456 CFU/plate. However, when indigo-clean was used during the second and third weeks of the study, there was an 88.8% reduction in week two (275 CFU/plate) and 99.4% reduction in week three (14 CFU/plate). Sampling in week four, when the lights were no longer in use demonstrated an 8714% increase in contamination (1234 CFU/plate) compared to week three. Additionally, Sutton *et al*. (2016) measured total CFU on 5 sampling sites in a trauma room, using Replicate Organism Detection and Counting (RODAC) plates, before and after two and fifteen weeks use of Indigo-Clean. Before use, there was a mean 25 CFU/plate when there was an average 254 patient min/day, after 2 weeks there was similar 27 CFU/plate however room utilisation was increased, with 368 patient min/day . The benefit of Indigo-Clean was evident after 15 weeks use, with total CFU reduced to 5 CFU/plate and increased room use with 490 patient min/day.

These studies all indicate that the long-term use of 405 nm light in the clinical setting may be beneficial to reduce microbial contamination on surfaces and air, with up to 86% reductions in bacterial contamination over and above regular cleaning (Maclean *et al*., 2010). Discontinuing use resulted in environmental contamination levels rising to similar levels of that before use, further demonstrating the ability of this technology to maintain low levels of pathogenic bioburden with regular use (Maclean *et al*., 2014).

2.4.6 Operational Considerations of Novel Decontamination Technologies

The aforementioned technologies have an established antimicrobial efficacy however there are several limitations which generally make UV light, ozone, HPV and CAPP more appropriate for terminal decontamination. Conversely the HINS-light EDS can be used continuously to maintain low levels of environmental contamination within hospitals. The benefits and limitations of each system are discussed below and summarised in Table 2.5.

2.4.6.1 Safety Considerations

The primary concern about many of the novel technologies discussed is their potentially harmful effects to both hospital staff and patients. UV light has detrimental effects on human health, can damage the skin and components of the eye, as well as having mutagenic effects on cells, so must not be used in the presence of patients and staff (Matsumura and Ananthaswany 2004; Young 2006). HPV and ozone are also potentially toxic at high concentrations, meaning that rooms and wards must be also be vacated, as well as being sealed to prevent any gas leakage (Fu *et al*., 2012; Otter *et al*., 2013; Herruzo *et al*., 2014; Maclean *et al*., 2015). Less is known about the safety implications of CAPP however there could be potential for harm as toxic gases such as O_3, NO, and NO_2 are released as well as UV light (Isbary *et al*., 2013a, b). Although these CAPP systems have been used for wound healing and dermatological purposes, there is large variation in system design and composition of generated plasma so further safety testing of systems designed for environmental decontamination is required (Isbary *et al*., 2013a, b).

As these technologies can be harmful, it is essential that a high level of staff training is carried out before use. Training is also required to ensure correct placement of devices and to reduce the level of human error. UV light systems need to be carefully placed, with the Ultra-V system requiring 6 ‘spectromes’ to be placed around the room, so that the system can respond depending on the amount of UV light the spectromes receive. Additionally, as multiple cycles of PUV are required due to objects being out of the line of sight, these devices must be moved at least 2-3 times in a single room. Staff are also required to correctly seal rooms and put up hazard signage when using HPV and ozone and must be able to operate equipment to check levels of H_2O_2 are below health and safety limits within rooms afterwards (Otter *et al*., 2013; Dancer 2014).

Material damage and corrosion are also important to consider. Ozone can be very corrosive to metals such as stainless steel and if used at high humidity (> 80%) will degrade rubber,

therefore may be impractical if surfaces in the hospitals are not able to tolerate exposure (Brown and Duquette 1993; Davies *et al*., 2011). UV light may also have a deleterious effect on the hospital environment and can damage plastics and polymers upon repeat exposures (Yin *et al*., 2013). As further studies are required to discover how CAPP would be applied in the environment, these will confirm if CAPP would have any detrimental effects on surfaces and equipment (O'Connor *et al*., 2014).

However, the EDS is able to overcome many of the aforementioned limitations. The EDS can firstly be used in the presence of patients and staff, with the optical output carefully evaluated to ensure its safe use. The violet-blue wavelengths utilised (in the region of 405 nm) are out-with the UVB region, which is associated with photokeratitis (burn of the cornea) and erythema (sunburn), and are also out-with the higher wavelength blue light regions which are associated with photoretinitis (~435 nm; damage to the retina), and circadian rhythm (~480 nm). In addition to the wavelengths used, the irradiance levels used have also been selected so that they are well below the accepted international safety threshold levels for continuous use, set by the International Commission on Non-Ionizing Radiation Protection (ICNIRP) and the American Conference of Governmental Industrial Hygienists (ACGIH) (Bache *et al*., 2012). Only basic staff training is required with the EDS, and once installed, staff only need to 'flick a switch'. Therefore this technology can be used continuously to enhance current hospital cleaning regimes inactivating a wide range of nosocomial pathogens, without the need for rooms to be vacated (Maclean *et al*., 2015).

Additionally, 405 nm light does not cause photo-degradation of plastics/polymers that is associated with 254-260 nm wavelengths of UV light (Mitsuoka *et al*., 1993; Maclean *et al*., 2009). Irving *et al*. (2016) demonstrated this by exposing endoscope tubing to 2.6 mW/cm^2 405 nm, UVC and broadband light for 400 hours. No visible cracking or increases in the surface roughness were seen in the endoscope material after storage in 405 nm light and broadband light, however following storage in UVC there was visible cracking and a significant increase in surface roughness from 2.3 mm to 68.7 mm. Additionally, there was no increase in bacterial adhesion to the endoscope material following 405 nm or broadband light exposure whereas there was a significant increase (~87%) in adhesion following UV light exposure for 400 hours.

2.4.6.2 Time Considerations

As patient rooms generally have to be pre-cleaned before use of these terminal whole-room decontamination technologies (UV light, HPV, ozone), turnaround times can be long.

Pre-cleaning is required not only for aesthetic reasons but also to reduce organic soil which may reduce inactivation effect (Piskin *et al*., 2011; Tuladhar *et al.*, 2012). Moving furniture and positioning aHP and UV devices can also be time consuming, and this has to be done before decontamination cycles have even began.

Cycle times vary between decontamination systems, with UV light requiring at least 15 mins, ozone 1 hour, and HPV 1.5-3 hours (Sharma and Hudson 2008; Moat *et al*., 2009; Passaretti *et al*., 2013; Rutala and Weber 2013b). In the case of CAPP, treatments of 5 cm^2 areas require up to 90 seconds, when this is extrapolated to every surface in an isolation room, decontamination could take a considerable amount of time (Cahill *et al*., 2014). Therefore these technologies may be impractical in busy ward settings which have high bed occupancy and are unlikely to be able to be utilised daily for decontamination (Dancer 2009; Otter *et al*., 2009).

However, as the HINS-light EDSs are installed in the ceiling, this technology can be continuously used throughout the day and also removes issues regarding portability, room preparation and re-positioning, as well as the requirement to put up warning signs/barriers which are required with the other technologies discussed.

2.4.6.3 Reduction of Nosocomial Infections

It is also important to consider the effects of these technologies on overall hygiene levels of hospitals and their potential to reduce acquisition of HAIs. Studies have demonstrated that following HPV decontamination rooms can become quickly re-contaminated with pathogens, which is detrimental to hygiene levels (Hardy *et al*., 2007). Furthermore, Haas *et al*. (2014) reported a reduction in rigorous manual cleaning due to reliance on the technology. These novel technologies could therefore present additional problems if staff consider them a replacement rather than a supplementary treatment for environmental decontamination. As the HINS-light EDS is designed to be integrated into existing lighting systems, it is less likely that staff will be aware of the technology and therefore it is less likely to cause a reduction in manual cleaning.

As with any new decontamination technology is it essential to carry out clinical evaluations to investigate if implementation leads to reduction in HAIs. A recent review by Weber *et al*. (2016), discussed the reduction in HAIs following the implementation of HPV or UV light into hospital terminal decontamination procedures. The review highlighted a 30 month prospective-cohort study which demonstrated that patients admitted to rooms which had been decontaminated using HPV were 64% less likely to acquire a MDR pathogen and

80% less likely to acquire VRE, however there was no significant reduction in acquisition of *C. difficile,* MDR Gram negative bacilli or MRSA (Passaretti *et al*., 2013 Weber *et al*., 2016). The review also highlighted a randomised clinical trial which used UVC light in 9 hospitals and showed that decontamination using UV light reduced acquisition of *C. difficile,* MRSA and VRE by 10-30% (Anderson *et al*., 2015; Weber *et al*., 2016). However to the best of my knowledge there have been no studies conducted to investigate the reduction in HAIs following implementation of ozone for terminal decontamination, following long-term use of the HINS-light EDS, or implementation of CAPP decontamination systems (however CAPP systems are in an earlier stage of development). It is therefore essential for clinical evaluations to be carried out to ensure that the technologies discussed do indeed reduce the acquisition of nosocomial pathogens and prove they are a worthwhile investment for health boards.

2.4.6.4 Running Costs

It is inevitable that the initial capital costs of these decontamination technologies would be substantial compared to traditional cleaning using disinfectants. However, it is likely the running costs of the EDS will be lower than those of HPV, Ozone and UV light. Use of the HINS-light EDS (Section 3.5.3) for 12 hours per day, has a monthly cost of approx. £3.65 per EDS per bed-space (based on a laboratory prototype, and current electricity costs), which would be around £365 if 100 EDS units were installed in high-risk areas. The monthly costs of using HPV and ozone for staff, equipment and resources are estimated to be much higher at £1154.98 and £1232.67, respectively (Doan *et al*., 2012). Additionally, the estimated monthly cost of UV-robots, before staff costs are included, is £2438.58 (Ghantoji *et al*., 2015).

The EDS may also be better value for money as it can be used daily, whereas HPV, ozone, UV light and CAPP can only be used sporadically depending on how busy the hospital is, and the availability of trained staff. Furthermore, UV devices will require more maintenance and bulb replacements, with bulbs lasting between 400-1600 hours (Veenhuis 2017) but EDS LEDs being able to be utilised for 125,000 hours (Indigo-Clean 2016).

The EDS could also be considered to be more environmentally friendly than the other decontamination technologies discussed. There is no requirement for disposal of harmful chemicals (unlike HPV and chlorine based disinfectants) and LEDs are more compact and easier to dispose of than mercury-containing UV bulbs (Hamamoto *et al*., 2007; Mori *et al*., 2007).

2.4.7 Summary

The novel decontamination technologies discussed all have benefits and disadvantages. UV light, HPV, and ozone are targeted towards terminal cleaning, and have rapid and broad antimicrobial efficacy but with this comes quick room recontamination once the systems are no longer in use. The HINS-light EDS overcomes this by providing continual decontamination however prolonged use is required for the benefits to be seen. A best-case scenario might be to develop a decontamination protocol whereby technologies such as UV or HPV are utilised for terminal decontamination, immediately followed by use of the EDS, and spot cleaning of high-risk areas using CAPP. This combination may result in lower levels of microbial bioburden during patients stays, and reduce the risk of acquisition of nosocomial pathogens.

Table 2.5 Comparison of traditional and novel hospital decontamination technologies. Adapted from Maclean *et al*. (2015).

Technology	Operational Advantages	Operational Disadvantages
Cleaning (Chlorine Based Disinfectants)	• Broad antimicrobial efficacy • Simple to operate • Suitable for hard & soft surfaces • Standard protocols in place (e.g. NIPCM)	• Episodic use • Exposure to chemicals can be harmful • Labour intensive • Relies on user to ensure all surfaces decontaminated • Air not treated • May corrode surfaces • Surfaces quickly re-contaminated
Ozone	• Broad antimicrobial efficacy • Suitable for hard & soft surfaces & air • Effective for terminal decontamination	• Episodic use • Exposure can be toxic • Requires staff training • Rooms require to be vacated and sealed • May cause corrosion of materials • Surfaces quickly re-contaminated
UV-Robots	• Broad antimicrobial efficacy • Suitable for hard & soft surfaces & air • Effective for terminal decontamination	• Episodic use • Exposure can be harmful and mutagenic • Requires staff training • Rooms require to be vacated • Causes material degradation • Surfaces quickly re-contaminated
HPV	• Broad antimicrobial efficacy • Suitable for hard & soft surfaces & air • Effective for terminal decontamination	• Episodic use • Exposure can be toxic • Requires staff training • Rooms require to be vacated and sealed • Surfaces quickly re-contaminated
CAPP	• Broad antimicrobial efficacy • Suitable for hard & soft surfaces • Effective for high level decontamination of surfaces	• Episodic use • Safety implications unknown • Likely to require staff training • No established system in place
HINS-light EDS	• Broad antimicrobial efficacy • Can be used continuously • Suitable for hard & soft surfaces & air • Simple to operate • Safe for exposure • No compliance issues • Maintains low levels of surface contamination	• Slower efficacy against microorganisms than UV/HPV/Ozone/CAPP • Bacterial and fungal spores require long exposure • Virucidal efficacy unknown • Not suitable for terminal decontamination

2.5 Violet-Blue Light Technology

As the HINS-light EDS is a promising addition to infection control procedures within hospitals, this next section of the literature review includes an in-depth review of violet-blue light technology. This will allow a greater understanding of the mechanism of inactivation and spectrum of antimicrobial activity.

2.5.1 Photoexcitation Mechanism of 405 nm Light

The narrow band of violet-blue visible light, peaking at 405 nm, utilised in the HINS-light EDS causes the photoexcitation of endogenous porphyrin molecules within microbial cells, causing them to act as photosensitizers (Fig 2.4). (Maclean *et al*., 2009). Upon absorption of a photon of light, the porphyrins are excited to the first excited singlet state. This excited state is short lived and undergoes intersystem crossing to a third excitation state which is longer lived or returns to the ground state by releasing heat and fluorescence (Maisch 2009). In the triplet state, the porphyrin can interact with ground state molecular oxygen, which can then follow two pathways (Yin *et al*., 2013). The type I pathway involves electron or hydrogen atom transfer to molecular oxygen resulting in the formation of reactive oxygen species (ROS) such as peroxides, superoxide ions and hydroxyl radicals (Luksienė and Zukauskas 2009; Maisch 2009). The type II pathway involves energy transfer from the triplet state porphyrin to molecular oxygen to produce singlet oxygen ($^1O_2$) (Maisch 2009; Vatansever *et al*., 2013).

It is widely accepted that the ROS produced by photo-excited porphyrins can target a range of biomolecules within microbial cells, and cause widespread oxidative damage (Hamblin and Hassan 2004; Maclean *et al*., 2008b, 2009; Lubart *et al*., 2011). Maclean *et al*. (2008b) demonstrated that oxygen was integral for photo-inactivation and showed significantly reduced inactivation of *S. aureus* in the presence of reactive oxygen scavengers; ascorbic acid, catalase, and dimethylthiourea. The production of intracellular ROS in *Staphylococcus epidermidis* was demonstrated using non-fluorescent carboxyl-H_2DCFDA dye, which became fluorescent following 405 nm light exposure, indicating oxidative stress (Ramakrishnan *et al*., 2016). Ramakrishnan *et al*. (2016) also demonstrated through the use of scavengers, that H_2O_2 may play a predominant role in the cytotoxic mechanism of 405 nm light within bacteria (Ramakrishnan *et al*., 2016).

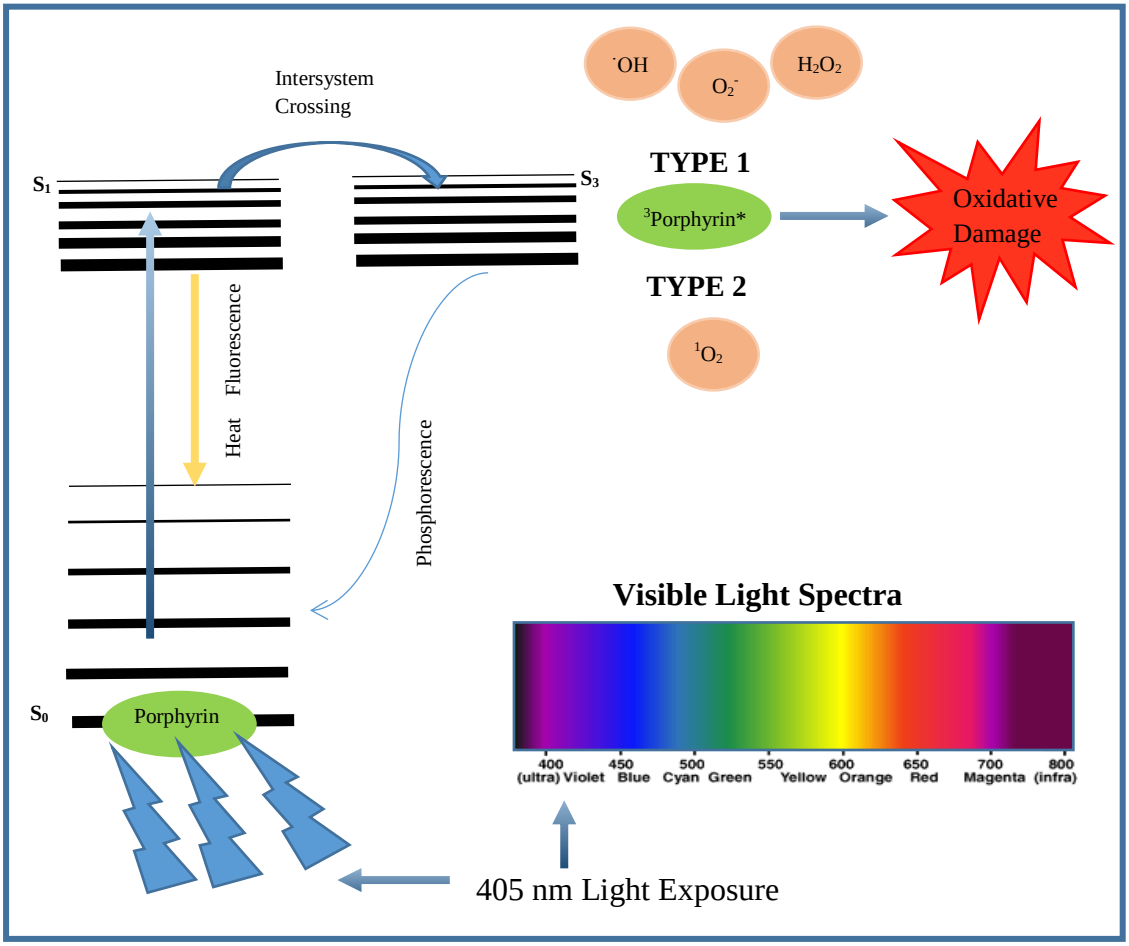


Figure 2.4 Diagram illustrating the photoexcitation mechanism of porphyrins following 405 nm light exposure. Demonstrating the potential photoexcitation of porphyrins within microbial cells following exposure to high intensity narrow spectrum visible light. Adapted from Luksienė and Zukauskas (2009), Maisch (2009), and Vatansver *et al*. (2013).

2.5.1.1 Porphyrins

The endogenous photosensitizers within microorganisms, porphyrins, are naturally occurring molecules which are involved in a wide range of biological processes including photosynthesis, oxygen transport and catalysis (Goldoni 2001). This ubiquitous class of molecules have a basic porphine macrocycle structure with 16 atom rings containing 4 nitrogen atoms, obtained by linking 4 tetrapyrrolic subunits with 4 methane bridges (Fig 2.5a) (Goldoni 2001). Porphyrins strongly absorb light around 400 nm (known as the soret band), and also absorb light between 450-700 nm (q-bands) (Goldoni 2001). Violet-blue light excitation of porphyrins results in characteristic fluorescence emission peaks around 612 nm (Ashkenzai *et al*., 2003).

Porphyrins have been identified in violet-blue light sensitive organisms by using fluorescence spectrophotometry or high performance liquid chromatography (HPLC) analysis. Several studies have indicated the presence of coproporphyrin (Fig 2.5b) within microorganisms including *B. cereus, E. coli, Listeria monocytogenes, Propionibacterium acnes, P. aeruginosa Salmonella* spp. and *S. aureus* (Ashkenzai *et al*., 2003; Hamblin *et al*., 2005; Kumar *et al*., 2015, Amin *et al*., 2016, Kim and Yuk 2017). Additionally, other porphyrins such as protoporphyrin and uroporphyrin have been identified within light sensitive organisms such as *A. baumannii, Helicobacter pylori*, and *P. aeruginosa* (Hamblin *et al*., 2005; Soukos *et al*., 2005; Dai *et al*., 2013a; Wang *et al*., 2016). It has been suggested that coproporphyrin may be the most important porphyrin involved in violet-blue light inactivation, and has been shown to be the porphyrin that produced the majority of free radicals (Nitzan *et al*., 2004). Additionally, light sensitive strains of *S. aureus* have been found to contain 10 fold more porphyrins than the more resistant strains (Liposky *et al*., 2009).

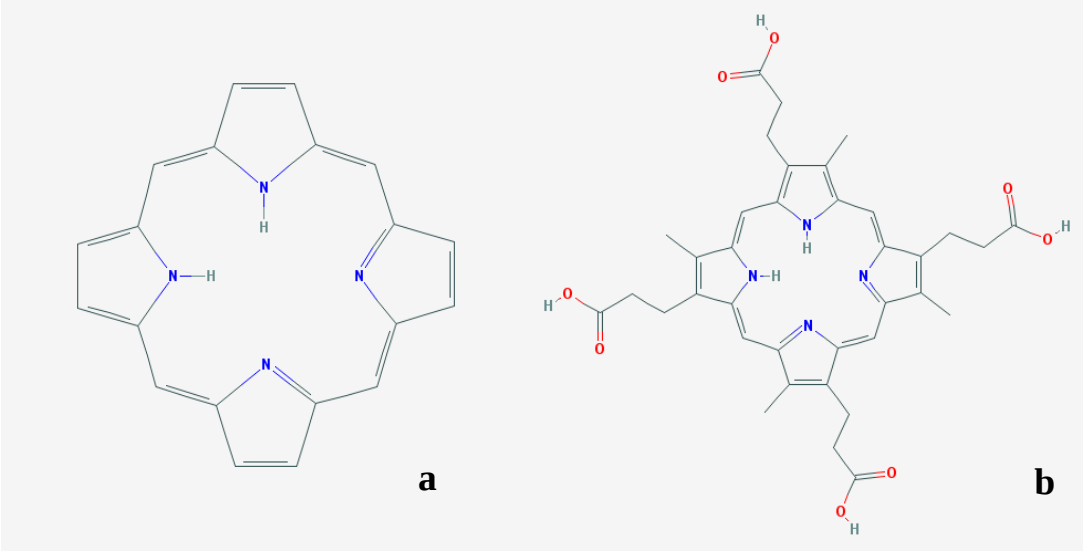


Figure 2.5 Basic structure of (a) porphine and (b) coproporphyrin molecules. Adapted from the Open Chemistry Database (www.pubchem.com).

2.5.2 Inactivation Mechanism

There are differing opinions over the inactivation mechanism of violet-blue light. Enwemeka *et al*. (2008) hypothesised that damage may occur in the double bond between

pyrimidine bases of DNA, causing new bonds to form between incorrect base pairs. As the dose delivered would cause the rate of damage to exceed the rate of repair, cells are likely to die after exposure to violet-blue light and not photo repair (Enwemeka *et al*., 2008). A recent study by Kim and Yuk (2017) supports this hypothesis, with TEM revealing disorganisation of chromosomes and ribosomes following violet-blue light exposure as well as DNA oxidation. The authors hypothesised that violet-blue light inactivation was due to DNA damage and loss of efflux pump activity rather than membrane peroxidation, as no noticeable changes to the cell envelope were witnessed using microscopy (Kim and Yuk 2017).

However, a greater amount of evidence has been produced which indicates that inactivation is not due to DNA damage but rather membrane damage. Kim *et al*. (2015, 2016), exposed *B. cereus, E. coli, L. monocytogenes, Salmonella sonnei; Salmonella typhimurium; and S. aureus* to 405 nm light and found no DNA damage. There was no evidence of DNA fragmentation or changes in the DNA ladder profile after performing a comet assay and DNA ladder analysis respectively (Kim *et al*., 2015, 2016). Additionally, TEM has been used to demonstrate structural damage following 415 nm light exposure of *P. aeruginosa* and MRSA. Membrane degradation, large vacuole formation, release of cytoplasmic material and complete cell disruption was witnessed in *P. aeruginosa,* whilst there was disruption of cytoplasmic contents, breakage of bacterial cell walls and cell debris seen in MRSA samples (Dai *et al*., 2013a, b). TEM of *Candida albicans* also revealed decomposition of inner organelles, deformed cell walls and unusual vacuole growth following a dose of 35.1 J/cm^2, and complete loss of cytoplasmic contents due to disrupted cell walls after a dose of 70.2 J/cm^2 415 nm light (Zhang *et al*., 2016).

Loss of membrane integrity has also been investigated in two recent studies by Kim *et al*. (2016) and McKenzie *et al*. (2016). In both studies there was a significant decrease in salt and bile tolerance in a range of organisms including *E. coli, S. sonnei, S. typhimurium* and *S. aureus* following violet-blue light exposure, which implies a loss of outer membrane function as a permeability barrier. Both studies also used staining to visualise membrane damage. McKenzie *et al*. (2016) demonstrated membrane damage in *E. coli* using SYTOX analysis, with > 500% increase in fluorescence, demonstrating permeated membranes. Furthermore the use of LIVE/DEAD® Backlight™ staining by Kim *et al*. (2016) indicated light exposed samples had a loss of membrane integrity with the increase in red fluorescence compared to green fluorescing control cells, with cells only able to stain red after a loss of membrane function as a permeability barrier.

2.5.3 Scope of Antimicrobial Efficacy

Violet-blue light has been proven to inactivate many organisms in both liquid suspensions and on surfaces including HAI-associated pathogens such as *A. baumannii, Aspergillus niger, B. cereus, C. albicans, C. difficile, E. coli, K. pneumoniae, P. aeruginosa, S. aureus, S. epidermidis* and *Mycobacteria* (Guffey and Wilborn 2006; Enwemeka *et al.*, 2008; Maclean *et al*., 2008a, 2009, 2013b; Murdoch *et al*., 2012, 2013).

The fact that ROS produced by endogenous porphyrins are not likely to be site specific, unlike antibiotics, explains why these antimicrobial light wavelengths have such broad-spectrum action, and can also inactivate antibiotic resistant pathogens (Ramakrishnan *et al*., 2014). Successful inactivation of antibiotic resistant organisms, such as β-lactam resistant *E. coli*, MDR Enterobacteriaceae, and MRSA has been demonstrated, with little difference in lethal doses required (Maclean *et al*., 2009: Rhodes *et al*., 2016; Barneck *et al*., 2016; Halstead *et al*., 2016).

The ability of 405 nm light to inactivate MRSA and *E. coli* as well as *C. difficile* spores is also particularly important considering their prevalence in Scottish Hospitals (Table 2.2) and the ability of *C. difficile* spores in particular to survive in the environment and resist disinfectants (Wilcox 2003). It should be noted however that vegetative *C. difficile* cells are far more susceptible to violet-blue light inactivation, with a dose of 250 J/cm^2 required for > 3 $\log_{10}$ inactivation whereas endospores required almost 10× that for a similar level of inactivation (Moorhead *et al*., 2016a). Additionally, the virucidal efficacy of 405 nm light has not yet been established, however initial studies have indicated that in certain circumstances bacteriophage can be inactivated by violet-blue light (Tomb *et al*., 2014).

2.6 Violet-Blue Light Research

In addition to the use of violet-blue light for environmental decontamination, there are a number of other areas of research currently investigating the potential of this antimicrobial light for clinically-relevant applications. This section will focus on the current laboratory, animal and clinical studies involving violet-blue light between 400-420 nm.

2.6.1 Wound Decontamination Studies

As SSIs are one of the most common HAIs, and wound infections are the principle cause of death in burns patients, there is potential for using antimicrobial violet-blue light to

decontaminate these lesions (McDonald *et al*., 2011; HPS 2016). Decontamination of artificially spiked skin abrasions and burn wounds has been demonstrated in mice models. Skin abrasions were created by scraping mouse skin using scalpels, and burns created using heated blocks pressed on skin for ~ 3 seconds, prior to adding a bacterial suspension to the affected area (Dai *et al*., 2013 a, b; Zhang *et al*., 2014). Inactivation of pathogens was measured via bioluminescence intensity, which is linearly proportional to viable bacterial population (Dai *et al*., 2013 a, b; Zhang *et al*., 2014).

Initial studies by Dai *et al.,* (2013b) used 415 nm light at an irradiance of 15 mW/cm^2 for treatment of early and established MRSA infections in mouse skin abrasions. Exposing wounds 30 mins after inoculation (as a prophylactic measure) resulted in near complete inactivation following a dose of 41.4 J/cm^2 (46 mins) (Dai *et al.,* 2013b). Near complete inactivation was also achieved in 24 hour old wounds, requiring higher doses of 108 J/cm^2 (120 mins) (Dai *et al.,* 2013b). However as complete inactivation was not achieved in the established wounds, this allowed bacterial re-growth after 24 hours following exposure (Dai *et al.,* 2013b). It is likely that complete bacterial inactivation did not occur due to biofilm formation, bacterial penetration into deeper tissues, and the issue of light transmission through blood and other tissue and wound fluids, which indicated higher doses, and different application measures were required in future studies.

Several subsequent studies demonstrated successful inactivation in wound models. Following a dose of 55.8 J/cm^2 30 mins after inoculation, > 4 $\log_{10}$ reduction of *A. baumannii* in a mouse burn model was achieved with no subsequent recurrence of infection (Zhang *et al*., 2014). Exposure of *P. aeruginosa* resulted in 3 $\log_{10}$ and 5 $\log_{10}$ reductions in burn and wound models following doses of 55.8 J/cm^2 and 48 J/cm^2, respectively (Dai *et al*., 2013a; Amin *et al*., 2016). Fungal inactivation has also been demonstrated, with up to 99.3% reduction of *C. albicans* in a burn model 12 hours after inoculation, using a dose of 43.2 J/cm^2 (Zhang *et al*., 2016) The results in the case of *Pseudomonas* inactivation highlight the benefit of violet-blue light for wound decontamination as the control mice died of sepsis, whereas all the violet-blue light treated mice survived (Dai *et al*., 2013a).

A. baumannii biofilms have also been reduced in mice burn wounds following 24 and 48 hours of growth. Biofilms were harder to eradicate, with high doses of 360-540 J/cm^2 required for 3 $\log_{10}$ reduction (Wang *et al*., 2016). However assessment of mouse skin biopsies following exposure to these high doses (540 J/cm^2), demonstrated very few apoptotic cells and almost no blue light induced DNA damage, up to 48 hours after exposure

(Zhang *et al*., 2014; Wang *et al*., 2016). Although further research is required to investigate the effect on human wounds, these results indicate potential for violet-blue light decontamination of a multitude of lesions including surgical sites and burn wounds, either as a prophylactic treatment or as an adjunct treatment alongside antibiotics. This could be particularly useful when medical assistance is required in situations with limited resources, as light units can be portable and battery powered (Zhang *et al*., 2014).

2.6.2 Mammalian Cell and Tissue Studies

Investigating the potential cytotoxic effect of violet-blue light on mammalian cells is additionally important. This will help to establish its safety for wound decontamination as well other application areas where human tissue is to be exposed.

As mammalian cells also contain intracellular porphyrins, there may be the potential for these to become photosensitised and produce ROS (Lavi 2004, Ramakrishnan *et al*., 2016). The overproduction of ROS could have serious consequences in cells. Normally, free radicals play a role in cell signalling and homeostasis however over production would alter redox balance and oxidation of cellular components which could lead to damaged cell structures (Devasagayam 2004: Ramakrishnan *et al*., 2016). Exposure of fibroblasts, kidney epithelial cells and human keratinocytes to 400-410 nm light induced the production of H_2O_2 in peroxisomes and mitochondria, which was thought to be associated with cell pathology (Hockberger *et al*., 1999). Additionally, cytotoxic effects have been seen in cells which were exposed to 412 nm and 419 nm light using doses of 66-100 J/cm^2, every 24 hours for 3 days. Results indicated that high irradiance exposure was cytotoxic for the skin derived endothelial cells and keratinocytes, with non-toxic levels of light also reducing proliferation (Liebmann *et al*., 2010).

Bacterial cells however have greater sensitivity to 405 nm light than mammalian cells, which may be due to greater antioxidant defence mechanisms in mammalian cells compared to bacteria (Dai *et al*., 2013a, b; MacDonald *et al*., 2013; Ramakrishnan *et al*., 2014). Therefore, there is potential for a 'critical window' whereby cells can be exposed to violet-blue light without detrimental effects occurring, whilst microbial numbers are reduced (Dai *et al*., 2013a, b; Zhang *et al.*, 2014). Several studies have investigated the effect of 415 nm light on human keratinocytes (HaCaT). Doses of 70.2 J/cm^2, 109.9 J/cm^2 and 168 J/cm^2 have been shown to cause 4 $\log_{10}$, 4.6 $\log_{10}$ and 7.5 $\log_{10}$ reductions of *A. baumannii*, *P. aeruginosa* and MRSA respectively whilst only 0.1 $\log_{10}$, 0.16 $\log_{10}$ and 0.29 $\log_{10}$ reductions of HaCaT cells occurred (Dai *et al*., 2013a, b: Zhang *et al*., 2014). In

the case of *Pseudomonas* inactivation there was up to 21-fold faster inactivation of bacteria compared to cells, demonstrating the therapeutic window in which violet-blue light can be used without causing damage to host cells (Zhang *et al*., 2014). Zhang *et al*. (2016) also demonstrated a 42-fold increase in susceptibility of *C. albicans* compared to keratinocytes, with 5.4 $\log_{10}$ reduction of *C. albicans* and only 0.11 $\log_{10}$ reduction of HaCaT cells, following exposure to a dose of 70.2 J/cm^2 violet-blue light.

Additionally, McDonald *et al*. (2011) investigated if violet-blue light would affect wound healing by using a fibroblast-populated collagen matrix of 3T3 mouse cells, as an *in vitro* model. Exposure for 1 hour using irradiances up to 1.8 mW/cm^2 did not affect cell contraction, morphology, expression of α-smooth muscle actin (a marker of fibroblast contractile activity) or cause a decrease in cell numbers. The maximum intensity that could be used, before contraction was affected was 5 mW/cm^2, however at this irradiance a 3 $\log_{10}$ reduction of *S. epidermidis*, could be achieved; therefore, supporting the use of violet-blue light for wound decontamination purposes.

The use of 405 nm light for environmental decontamination could be extended from use in patient isolation rooms and wards to other high-risk areas such as operating theatres. To investigate the safety implications of this, the sensitivity of (rat) osteoblasts to 405 nm light has been investigated (McDonald *et al*., 2013; Ramakrishnan *et al*., 2014, 2016). In a study by Ramakrishnan *et al*. (2014) exposure of osteoblasts to doses of up to 36 J/cm^2 405 nm light resulted in no significant effects on cell viability, function, proliferation rate or morphology, but resulted in complete inactivation (2 $\log_{10}$ reduction) of common surgical pathogens such as *P. aeruginosa, S. aureus* and *S. epidermidis*, thus indicating the potential of utilising 405 nm light during orthopaedic surgery to prevent contamination (Ramakrishnan *et al*., 2014).

Interestingly, intracellular inactivation of bacteria has also been demonstrated. A study by Wasson *et al*. (2012) demonstrated the inactivation of *Chlamydia trachomatis* in endothelial epithelial cells (HeLa) during active and persist infections. Significant inactivation of active infections was achieved using doses of between 5-20 J/cm^2, and higher doses of 20 J/cm^2 were required for persistent infections (Wasson *et al*., 2012).

2.6.3 *Helicobacter* Infections

Alongside the potential for microbial inactivation in wounds and cells, there is also potential to use this technology to eradicate bacteria within the human body. *Helicobacter pylori* inactivation is of particular interest as this pathogen can cause peptic ulcers and cancer of the

stomach, with current treatment options having significant side effects and/or little therapeutic effect (Chey and Wong 2007). A preliminary study by Ganz *et al*. (2005) delivered 405 nm light to 1 cm spots in the gastric antrum in 10 patients and found a 91% reduction of *H. pylori* in tissue samples after treatment. This treatment method was further investigated by Lembo *et al*. (2009) using a device which delivered 408 nm light to the whole stomach of 18 patients for between 15-60 minutes. Following exposure there was up to 99% inactivation, however urease breath tests 5 weeks post treatment, indicated that *H. pylori* had regrown. These studies indicate potential for violet-blue light eradication of *H. pylori* however further work is required to improve therapy by using different methods of delivery, using adjuvants alongside violet-blue light or using 405 nm light as an initial treatment followed by additional exposures using lower doses or antibiotics (Lembo *et al*., 2009).

2.6.4 Violet-Blue Light for Blood Product Storage

The use of 405 nm light during storage of *ex vivo* blood plasma is another novel clinical application of this technology, which could help to reduce the occurrence of transfusion transmitted infections. As normal pathogen reduction technologies for treatment of blood components require the use of additional chemicals, photosensitizers and/or exposure to UV light, use of 405 nm light would reduce risk of residual toxicity due to detergents or UV damage (Aubuchon *et al*., 2011; Tsen *et al*., 2014). A study by Maclean *et al*. (2016) demonstrated the potential for 405 nm light for decontamination of human blood plasma, with proof-of-concept results showing a 99.9% reduction in *S. aureus* contamination using a dose of 144 J/cm^2. Significantly, due to the higher penetrability of 405 nm light compared to UV light, the treatment effect was able to be achieved within sealed transfusion bags, meaning that decontamination can be applied after blood products have been bagged and sealed, reducing processing stages which may introduce further contamination to the products (Maclean *et al*., 2016). Further work is required to investigate if there would be any detrimental effects seen in the violet-blue light irradiated products, such as the effect of irradiation on plasma proteins such as immunoglobulins. However, this study clearly indicates a potential for using 405 nm light to prevent contamination in injectable blood products.

2.6.5 Violet-Blue Light for Ophthalmological Purposes

There is also potential to use violet-blue light for inactivation of pathogens which cause ocular infection and disease such as infectious keratitis. 415 nm light has been shown to

significantly reduce bioluminescent bacteria within infected corneas, using light at an irradiance of 100 mW/cm^2 on the surface of the cornea (Zhu *et al*., 2017). Exposure of *P. aeruginosa* to 415 nm light 6 hours after inoculation caused a 3 $\log_{10}$ reduction in *ex vivo* rabbit corneas following a dose of 84 J/cm^2 and 2 $\log_{10}$ reduction in *in vivo* mice corneas following exposure to a dose of 36 J/cm^2 (Zhu *et al*., 2017). More established infections of keratitis required higher doses, with 304 J/cm^2 (50 min) for a similar 3 $\log_{10}$ reduction in rabbit corneas, 24 hours after inoculation (Zhu *et al*., 2017). Inactivation of 24 hour infections in mice corneas required a dose of 144 J/cm^2 for > 2.5 $\log_{10}$ reduction (Zhu *et al*., 2017). However bacterial infection reoccurred in mice corneas after treatment of both 6 and 24 hour infections (Zhu *et al*., 2017). Similar to the studies investigating *H. pylori* infection reduction, repeated violet-blue light treatment would be required for complete bacterial eradication in corneas or a combination treatment with antibiotics. Additionally, as blue light in the region of 435 nm can be toxic to the retina, it will be critical to select a light source which has light emission < 420 nm. Also, exposures should be kept to a minimum as the current doses used on the *ex vivo* rabbit corneas would cause retinal damage if there is 100% transmission thorough the cornea, however if the cornea is cloudy due to infection, this will reduce the risk of damage (Zhu *et al*., 2017).

A more practical option may be to implement violet-blue light during the storage of ocular products such as contact lenses. This would be particularly beneficial as there are approx. 2.44 per 10,000 cases of microbial keratitis per year in contact lens wearers (Seal *et al*., 1999). Additionally, the chemicals used for contact lens decontamination can cause ocular cell damage and some bacterial strains are even becoming resistant (Cheung *et al*., 2016; Matoba *et al*., 2016). A novel study by Hoenes *et al*. (2016) demonstrated that 405 nm light could be effective against *B. subtilis* and *E. coli* when exposed in contact lens cases, with a dose of 75.6 J/cm^2 delivered over 60 min resulting in 1.5-2 $\log_{10}$ reductions. Future studies should investigate if there are any interactions between 405 nm light and contact lens cleaning solutions, materials within the contact lenses, as well as the effect ocular bioburden may have on the antimicrobial efficacy, allowing optimisation of the contact lens decontamination process.

2.6.6 Biofilm Inactivation

A microbial biofilm can be defined as a '*microbially derived sessile community characterised by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene*

transcription' (Donlon and Costerton 2002). Biofilms protect microorganisms against environmental stresses and allow greater resistance to antibiotics, disinfectants and germicides, with multispecies biofilms having greater resistance than single species biofilms (Ceri *et al*., 1999; Harriot and Noverr 2009; Smith and Hunter 2008; Wang *et al*., 2013a; Lee *et al*., 2014). As discussed earlier, biofilms are commonly found in hospitals on indwelling devices, sinks and showers and can exist for long periods of time on clinical surfaces, even after cleaning (Lindsay and von Holy 2006; Espinal *et al*., 2012; Vickery *et al*., 2012; McLean *et al*., 2013; Soto-Giron *et al*., 2016). It is important to establish the efficacy of violet-blue light for inactivation of biofilms as the majority of studies have focussed on microbial inactivation in liquid suspension or on agar surfaces, but when in the environment bacteria are more likely to be found in these complex communities (Hall-Stoodley *et al*., 2004; Halstead *et al*., 2016).

McKenzie *et al*. (2013) demonstrated inactivation of *E. coli* biofilms on acrylic and glass. After 4 hours growth 3-4 $\log_{10}$ reductions could be achieved on glass and acrylic following 15 min exposure to 405 nm light at an irradiance of 141 mW/cm^2. Additionally, 5 $\log_{10}$, 7 $\log_{10}$ and 8 $\log_{10}$ reductions of mature biofilms were achieved following 24, 48 and 72 hours growth on glass, and ~5 $\log_{10}$ reductions were achieved on acrylic following 24 and 48 hour growth. A dose of 168 J/cm^2 also achieved 2.48-3.72 $\log_{10}$ inactivation of *L. monocytogenes*, *P. aeruginosa* and *S. aureus* after 4 hours development on glass (McKenzie *et al*., 2013). Mixed biofilms which had evolved over 24 hours on glass were also susceptible, with 2.19 $\log_{10}$ reduction of the total bacterial counts following 30 min exposure (McKenzie *et al*., 2013).

Inactivation of mature biofilms has also been demonstrated by Wang *et al*. (2016). Doses of 432 J/cm^2 415 nm light achieved > 3 $\log_{10}$ reductions of 24 hour old biofilms of *A. baumannii* and *P. aeruginosa* (Wang *et al*., 2016). Reduced inactivation was seen in more mature 72 hour biofilms, with 3.12 $\log_{10}$ and 3.18 $\log_{10}$ reductions respectively, following exposure to the same dose. Halstead *et al*. (2016) also demonstrated differing levels of biofilm inactivation dependent on strain susceptibility to 400 nm light, by measuring optical density of samples following exposure. Of the 34 bacterial isolates studied, the most susceptible was *A. baumannii*, with a 93.5% reduction in biofilm following a dose of 54 J/cm^2 whereas *Enterobacter cloacae* was least susceptible following the same dose, with an increase in biofilm development seen (Halstead *et al*., 2016).

2.6.7 Enhancing 405 nm Light Inactivation

There is also potential to enhance 405 nm light inactivation of microorganisms, if they are exposed to violet-blue light whilst subjected to sub-lethal stresses. Enhanced inactivation of *E. coli* and *L. monocytogenes* was evidenced by McKenzie *et al*. (2014) with inactivation increased by up to 50% when exposed to violet-blue light during temperature stress (4 & 45 °C), osmotic stress (10% sodium chloride) and acid pH stress (pH 3 & 3.5). These results indicate the potential for enhanced bacterial inactivation in hospitals, where these organisms have been sub-lethally stressed by desiccation or disinfectants during normal cleaning procedures (Maclean *et al*., 2014).

An example of enhanced inactivation was recently demonstrated by Moorhead *et al*. (2016a). Inactivation of *C. difficile* spores was enhanced when exposed to 405 nm light alongside diluted chlorinated disinfectants. Following a dose of 1.6 kJ/cm^2, there was ~1.3 $\log_{10}$, 0.9 $\log_{10}$ and 1.1 $\log_{10}$ greater reductions of spores compared to 405 nm light inactivation alone, when exposed with sodium hypochlorite, Tristel and Actichlor respectively (Moorhead *et al*., 2016a). Results not only demonstrated the sporicidal efficacy of 405 nm light, but also indicated that 33% less dose is required in presence of diluted disinfectants, with reduced concentration beneficial to domestic cleaning staff: reducing risk of respiratory irritation, corrosion of surfaces and equipment (Table 2.4).

2.6.8 Research on 405 nm Light Research yet to be Addressed

There are several areas of violet-blue light research which require further investigation to ensure safe and effective use of this technology within the hospital environment. This includes investigating the potential for antiviral efficacy using high and low-intensity 405 nm light, an evaluation of published data on the antimicrobial efficacy of violet-blue light and also the potential for bacterial tolerance to 405 nm light in clinically relevant organisms.

Little is known with regards to the potential antiviral efficacy of 405 nm light. It is hypothesised that viral inactivation is unlikely. This is due to the lack of endogenous porphyrins within the viral structure (Hamblin *et al*., 2005; Guffey and Wilborn 2006; Enwemeka *et al*., 2008; Maclean *et al*., 2008b, 2009, 2013b). However, a recent study investigating bacteriophage exposure to 405 nm light demonstrated successful inactivation when the viral surrogate was suspended in nutritious photosensitive media (Tomb *et al*., 2014). Therefore, it is not unlikely that the same effect could occur when mammalian viruses are exposed in similar photosensitive media. This could prove

particularly beneficial if the HINS-light EDS was shown to have virucidal efficacy against nosocomial viral pathogens such as NoV (Section 2.1.1). This may reduce viral transmission which would not only benefit patient and staff well-being, but may help to reduce costs associated with NoV outbreaks.

Another important aspect of violet-blue light research which requires attention is the comparison of inactivation profiles of microorganisms via review. Although many proof-of-concept studies have been carried out within this rapidly expanding research area, there are very few reviews published analysing the data generated to date (Dai *et al*., 2012; Maclean *et al*., 2014; Hessling *et al*., 2017). The preparation of a comprehensive review would allow in-depth analysis and evaluation of the efficacy of violet-blue light. This would help to draw conclusions about the inactivation kinetics when different irradiances of 405 nm light are used and the effect of bacterial population on inactivation kinetics. Additionally, it would be important to compare the efficacy of 405 nm light to a broader range of violet-blue wavelengths used for microbial inactivation (380-480 nm). A review of the published data would also help to identify areas of research on violet-blue light which require further evaluation and allow conclusions to be drawn about the scope of this technology for clinical applications.

To ensure safety of use of 405 nm light for clinical applications the potential for microbial resistance to 405 nm light must also be investigated. As bacteria are rapidly evolving, with some strains now found resistant to last resort drugs (McGann *et al.*, 2016), it is important to ensure that bacteria will not also evolve to become resistant to antimicrobial 405 nm light treatments. The potential for tolerance development is considered to be unlikely due to the non-specific mechanism of action and multiple targets of ROS generated by 405 nm light (Dai *et al.*, 2013a; Maclean *et al.*, 2014; Amin *et al*., 2016). However, there has been little evidence published to demonstrate this with only three studies published to date specifically investigating potential for tolerance following violet-blue light exposure in bacteria. An initial study by Guffey *et al*. (2013a) demonstrated potential for *S. aureus* to become tolerant to 405 nm light, but conversely, two studies using 415 nm light demonstrated no evidence of tolerance formation in *A. baumannii and P. aeruginosa* (Zhang *et al*., 2014; Amin *et al*., 2016). Therefore, further work is required to clarify whether there is potential for tolerance in proliferating and non-proliferating organisms.

Research must be carried out to investigate these key areas, and will provide novel and important information regarding this decontamination technology. This will enable improved

application of antimicrobial violet-blue light within and out with the clinical environment, and may influence future research surrounding antimicrobial violet-blue light.

2.7 Overall Summary

This literature review has highlighted the issue of environmental contamination within hospitals and the role that surfaces play in infection transmission. Recent developments in whole-room decontamination technologies have been discussed, with each system displaying both practical advantages and limitations. As discussed, the HINS-light EDS, developed to provide continuous environmental decontamination, may be a more practical solution than the terminal decontamination systems. This thesis addresses some important questions surrounding the antimicrobial efficacy of 405 nm violet-blue light utilised by the HINS-light EDS, including the potential for (i) viral inactivation, (ii) anti-viral efficacy compared to other microorganisms, and (iii) bacterial tolerance development.

Chapter Three

Methodology

3.0 Overview

This chapter provides details of the cultivation and sample preparation of the microbial samples (viruses, bacteriophage and bacteria) used throughout this study. Details of the 405 nm light arrays used throughout the study are also included.

3.1 Viral Methodology

Viral studies utilised feline calicivirus and feline embryonic host cells. This section details the procedures for cell passage, maintenance, enumeration and seeding; and viral preparation, co-incubation and enumeration.

3.1.1 Virus and Cell Strains

The virus and cell strains used were as follows:

- Mammalian virus, feline calicivirus (FCV) vaccine strain F9; University of Glasgow Culture Collection (Glasgow, UK)
- Mammalian cell line of feline embryonic cells, strain FEA (Jarrett *et al*., 1973); University of Glasgow Culture Collection (Glasgow, UK).

3.1.1.1 Passage and Maintenance of Mammalian Cells

Initial cell stocks were grown as monolayers in 75 cm^2 and 150 cm^2 tissue culture flasks (0.2 µm vent cap treated flasks, Corning, UK) in supplemented 10% fetal bovine serum-Dulbecco's Modified Eagle medium (FBS-DMEM) (Table 3.2) in a humidified atmosphere of 5% CO_2 in air at 37 °C. When the cell monolayer was confluent, usually after 24 hours growth in 150 cm^2 flasks, the medium was removed and 10 ml 10% Trypsin-EDTA in phosphate buffered saline (PBS) (Section 3.4.4) was used to wash cells in the flask twice. Following the second wash, 2 ml of the wash was left in the flask and used to roll over the cell layer, using a tapping action to dislodge cells from the surface. Following this, 10% FBS-DMEM was added to the flask and washed over the cell monolayer several times

to inhibit the action of trypsin and break any cell clumps. The cells were normally suspended in 18 ml 10% FBS-DMEM and transferred to a sterile universal tube. When using 75 cm^2 flasks the described volumes were halved.

A small volume of cell suspension was removed from the universal tube to perform a cell count (Section 3.1.3) and then the cells were split into new tissue culture flasks according to requirements. A 1:3 split was normally carried out to ensure 80-90% confluency of cells the following day, with the flasks kept in the incubator set at 37 °C and 5% CO_2 for the appropriate time required. Large 150 cm^2 flasks were generally used to culture cells, to ensure there was a sufficient number of viable cells available to seed 6-well plates for plaque assays (Section 3.1.5).

To ensure adequate stocks of FEA cells (replacing those in use after 25 passages and in case of contamination) stock populations of FEA cells were produced. Cells were gathered after growing to a confluence of 90%, and if the population was > 1×10^6 cells/ml, they were centrifuged at 960 ×*g* for ten minutes and then re-suspended in 10% DMSO in DMEM (Section 3.4.4). Vials of cell stock were slowly frozen at a rate of -1°C/minute using a Mr Frosty™ freezing container (Thermo Fisher Scientific, UK) and then placed in liquid nitrogen at -190°C for long term storage. When new cells were required, vials were quickly defrosted in a 37°C water bath for 3 minutes, re-suspended in 10% FBS-DMEM and incubated for several days in 5% CO_2 in air at 37 °C.

3.1.2 Enumeration and Seeding of Mammalian Cells

As described in Section 3.1.2, confluent cells were gathered in 20 ml universal tubes. The cell suspensions were shaken to allow equal distribution of cells throughout the liquid before removing 10 µl and adding to the counting chamber of an Improved Neubauer Haemocytometer, via capillary action. The layout of the counting chamber of the haemocytometer is shown in Figure 3.1a, with the typical appearance of FEA cells in the counting chamber seen in Figure 3.1b.

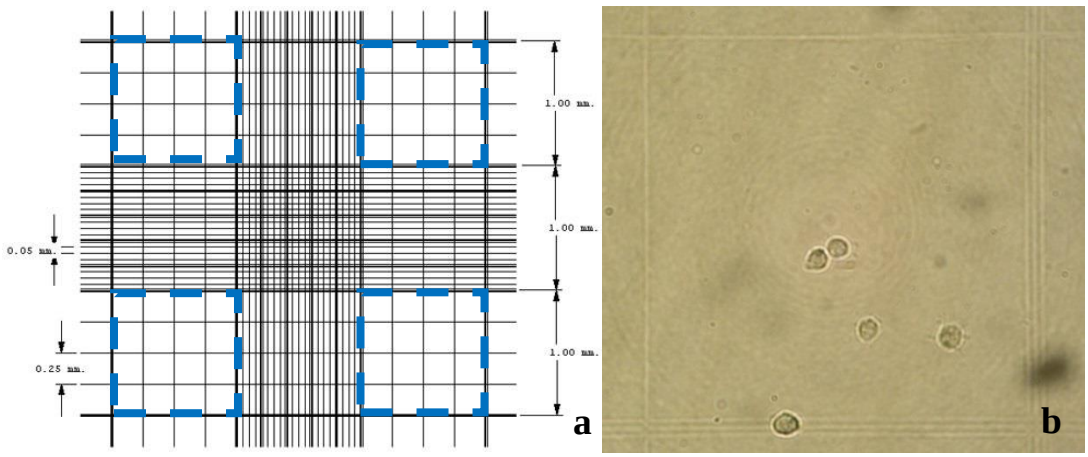


Figure 3.1 Appearance of (a) counting chamber within the Improved Neubauer haemocytometer and (b) FEA cells in a single square of the counting chamber at ×400 magnification. Blue squares highlight the 4 quartiles in which cells were counted in the Improved Neubauer haemocytometer, adapted from ww.laboratoryinfo.com. Cells were pictured using a Nikon Coolpix 4500 microscope camera.

To count the cells, the haemocytometer was viewed under a microscope (×400 magnification). All cells were counted in each of the 4 quartiles of the counting chamber (Fig 3.1a, highlighted in blue). To ensure consistency, the following counting rule was used: when cells were touching the 4 perimeter sides of a corner square, cells were only counted on 2 sides, either the 2 outer sides or 2 inner sides. The number of cells was calculated using equation (3.1):

$$\textit{Total cells/ml} = \textit{Total Cells Counted} \times \frac{\textit{Dilution Factor}}{\textit{Number of Squares}} \times \textit{10,000 cells/ml} \qquad (3.1)$$

Once enumerated, FEA cells were diluted in 10% FBS-DMEM to allow seeding of 6-well plates at a density of 7×10^5 cells/well or 6×10^5 cells/well, depending on experimental requirements. Seeded 6-well plates were then incubated overnight in a humidified atmosphere of 5% CO_2 in air at 37 °C, ready for use for viral plaque assay (Section 3.1.5).

3.1.3 Propagation and Maintenance of Viral Stocks

Viral stocks of FCV were used to prepare a viral pool. To prepare, firstly the growth medium from confluent FEA monolayers in 850 cm^2 cell culture roller flasks (Corning, USA) was removed and 6 ml of the virus inoculum was added to each flask, along with 6 ml 10% FBS-DMEM to ensure coverage of the cells. The flasks were filled with CO_2 gas and were then incubated at 37 °C on a roller stand for 90 minutes to allow for virus absorption. Each flask then received 12 ml 10% FBS-DMEM and were again filled with CO_2 gas. The flasks were then incubated at 37 °C for 24 hours on the roller which resulted in virus-induced destruction of nearly 90% of the monolayer. The medium from each roller bottle was collected and each bottle was washed with 7 ml 10% FBS-DMEM and collected into the same universal tube. The universal tube was then frozen and thawed twice and the contents centrifuged at 3300 ×*g* for 10 minutes to remove cell debris. The virus-containing supernatant was dispensed in 300 µl volumes and stored at −80 °C until required.

3.1.4 Co-Incubation and Enumeration of Viruses

To enumerate FCV, samples were co-incubated with FEA cells, in pre-seeded 6-well plates (Detailed in Section 3.1.3). To co-incubate, the growth medium was aspirated from the FEA cells and replaced with 1 ml of FCV sample, with different dilutions in each well. Plates were co-incubated at 37 °C in a humidified 5% CO_2 incubator for 90 minutes, with the plates gently rocked every 15 minutes to ensure even distribution of the inoculum over each monolayer. The inoculum was then aspirated and the well washed with medium (10% FBS-DMEM or Dulbecco's phosphate buffered saline (DPBS)) before adding 4 ml overlay mixture consisting of 2× supplemented DMEM 1:1 with 2× agarose (Table 3.2/Section 3.4.4). The overlay was left to set before the plates were incubated at 37 °C in 5% CO_2 for 44-48 hours.

Post-incubation, the monolayers were fixed and stained overnight with 0.5% crystal violet in 10% buffered neutral formalin (Sigma Aldrich, UK). The agarose plugs and stain were then removed, the plates left to dry, plaques counted, and the virus infectivity titre calculated (Table 3.1).

To calculate the reduction in viral population densities during inactivation studies, the population before and after light treatment where enumerated by counting the number of plaques formed per cell monolayer respectively. This allowed the level of inactivation to be calculated in plaque forming units per millilitre (PFU/ml).

3.2 Bacteriophage Methodology

Bacteriophage studies utilised bacteriophage ϕC31, and host bacterium *Streptomyces coelicolor*. This section details the procedure for cultivation of both organisms as well as sample preparation and enumeration of ϕC31.

3.2.1 Bacteriophage and Host Bacteria Strains

The strains used were as follows:

- *Streptomyces* bacteriophage ϕC31, strain c*Δ25*; University of Strathclyde Culture Collection (Glasgow, UK)
- *Streptomyces coelicolor*, A3(2) ΔpglW; University of Strathclyde Culture Collection (Glasgow, UK).

3.2.2 Cultivation and Maintenance of Bacterial Stocks

For culture of *Streptomyces coelicolor* spores, 50 µl of stock culture was spread onto soya flour mannitol agar plates and incubated at 30 °C. Following a 7-day incubation period, 10 ml sterile water was added to each plate. A pooled spore suspension was obtained after scraping the spores from the plates and centrifuging at 3939 ×*g* for 10 minutes. The resultant pellet was re-suspended in 20% (W/V) glycerol (Thermo Fisher Scientific, UK). The suspension was stored at −20 °C, and small aliquots were defrosted when spores were required.

3.2.3 Cultivation and Maintenance of Bacteriophage Stocks

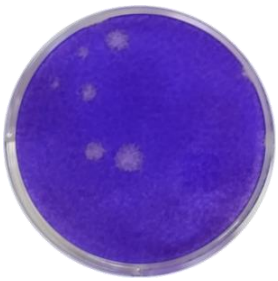
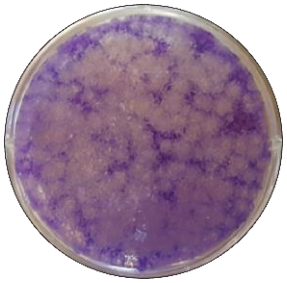
The bacteriophage, ϕC31, was cultivated to create a stock population. ϕC31 was diluted in nutrient broth, and 100 µl of each dilution was pipetted onto enriched nutrient agar plates (Table 3.3). A thin layer of molten soft agar (Table 3.3) containing 0.1% *S. coelicolor* spores was poured onto the plates. The plates were swirled to ensure even distribution of ϕC31 and incubated at 28 °C overnight. The following day, 10 ml nutrient broth was added to the plates which had complete bacterial clearance, and these were left for 3 hours. Following this, the 10 ml liquid was removed and filtered using a Minisart 0.25 µm filter (Sartorious, UK). The filter only allowed passage of the bacteriophage and NB, which was then stored at 4 °C for experimental use.

3.2.4 Co-Incubation and Enumeration of Bacteriophage

With regards to enumerating bacteriophage populations, this was carried out via co-incubation with *S. coelicolor* spores through a double-agar layer method. Several dilutions of bacteriophage samples were pipetted in various volumes (100, 200 and 500 µl volumes) onto enriched nutrient agar plates before a soft agar overlay containing 0.1% *S. coelicolor* spores thinly poured on top. The plates were swirled, left to set and then co-incubated at 28 °C overnight. Post-incubation, the surviving ϕC31 were enumerated and the bacteriophage infectivity titre calculated (Table 3.1).

To calculate the reduction in bacteriophage densities during inactivation studies, the populations before and after light treatment were enumerated by counting the number of plaques formed in bacterial lawns. This allowed the level of inactivation to be calculated in plaque forming units per millilitre (PFU/ml).

Table 3.1 Appearance of plaques formed by feline calicivirus in FEA cells, and ϕC31 in Streptomyces coelicolor lawns. Plaques were formed following: (a) incubation at 37 °C for 48 hours and (b) incubation at 28 °C overnight.

Apperance of Viral & Bacteriophage Plaques			
a			
FCV			
b			
ϕC31			
	No Plaques Formed (TFTC)	**Individual Plaques (Countable)**	**Lysis of Host Cells (TNTC)**

*TFTC: too few to count; TNTC: too numerous to count

3.3 Bacterial Culture and Methodology

Bacterial studies utilised methicillin-sensitive and methicillin-resistant *S. aureus*. This section details the procedures for cultivation, sample preparation and enumeration.

3.3.1 Bacterial Strains

The bacterial strains used were as follows:

- Methicillin-sensitive *Staphylococcus aureus* NCTC 4135; National Collection of Type Cultures (Colindale, UK)
- Methicillin-resistant *Staphylococcus aureus* EMRSA-15; Scottish Microbiology Reference Laboratories (SMiRL; Glasgow, UK).

3.3.2 Culture and Maintenance of Bacterial Stocks

For culture of *Staphylococcus aureus*, a loop of *S. aureus* was transferred from a stock culture (stored on a nutrient agar (NA) slope/plate) and inoculated into 100 ml nutrient broth (NB). The broth was incubated under rotary conditions (120 rpm) at 37 °C for 18-24 hours. For short term storage, and as a source of inoculum for daily experimental work, *S. aureus* was cultured on a NA slope and refrigerated at 4 °C. All bacterial strains stored on slopes were re-streaked onto a new agar slope every four weeks, and Gram stained (Section 3.3.5.1) to ensure purity. For long term storage, *S. aureus* was kept on Microbank™ beads (Pro-Lab Diagnostics Inc., UK) at −70 °C.

3.3.3 Re-Suspension and Serial Dilutions

Following bacterial cultivation, broths were centrifuged at 3939 ×*g* for 10 minutes. The supernatant was discarded and the resultant pellet was re-suspended in 100 ml phosphate buffered saline (PBS). In order to achieve the desired starting population of bacteria for experimental use, the stock suspension was serially diluted (10-fold dilutions; 1 ml bacterial suspension into 9 ml PBS) until the desired starting population was achieved. At each stage of dilution, the suspension was mixed using a vortex (Thermo Fisher Scientific, UK) to ensure the microorganism was equally dispersed throughout the suspension.

3.3.4 Plating and Enumeration

To calculate the reduction in bacterial population densities following exposure to antimicrobial 405 nm light, the population before and after light treatment was enumerated,

allowing the level of inactivation in colony forming units per millilitre (CFU/ml), to be calculated.

In most cases to enumerate *S. aureus*, samples in suspension were plated onto agar and incubated at 37 °C for 18-24 hours. When bacterial populations where expected to be high, suspensions were serially diluted prior to plating, to ensure quantifiable results. Additionally, depending on the expected population, the method of plating differed. The two main techniques used for plating samples were spiral plating and spread plating:

Spiral plating, using a WASP2 spiral plater (Don Whitley Scientific, UK), was used to plate samples that were expected to be between 10^3 and 10^5 CFU/ml. Spiral plating deposited 50 µl sample volumes in a logarithmic spiral across the agar surface

Manual spread plating was used when the CFU count was expected to be less than 10^3 CFU/ml. The spiral plater was used to plate 'spread plates' by linear deposition of 100 µl samples. Spread plates were also prepared manually by pipetting 100, 200 or 500 µl sample volumes onto agar plates and spreading using L-shaped spreaders.

Following plating, agar plates were incubated at 37 °C for 18-24 hours and then enumerated. Logarithmic spiral plates were enumerated using a counting grid and reference table (provided by the manufacturer), with results reported as colony-forming units per millilitre (CFU/ml). For enumeration of spread plates, all bacterial colonies grown on the plate were counted, and results calculated to give the CFU/ml.

3.3.5 Bacterial Identification Tests

Several identification tests were used to check that bacterial stocks were not contaminated. These tests were also used to ensure bacterial samples were not contaminated during repeated culture (discussed in Chapter 7).

3.3.5.1 Gram Staining

This staining technique is used to aid bacterial identification by confirming the Gram status as well as providing information on the cell morphology. The procedure used was as follows:

Preparation of a heat-fixed sample: A single bacterial colony was mixed into a drop of water on a microscope slide, or a drop of bacterial suspension, was smeared over the slide forming a thin film. The slide was then air-dried at room temperature before passing through a Bunsen flame several times to fix the bacterial film.

Staining procedure: the slide was flooded with crystal violet and left for 30 seconds. The slide was then briefly rinsed with Gram's Iodine before being flooded with the Iodine for 60 seconds. The Iodine was rinsed off with tap water and the sample decolourised using ethanol until all violet colour had drained from the slide. The ethanol was then rinsed off using tap water and the sample counterstained with safranin for 60 seconds. The slide underwent a final rinse using tap water and was blotted dry. All reagents were from Sigma Aldrich, UK.

The slide was then viewed under the microscope, using the ×100 oil-immersion objective lens giving a magnification of ×1000. Gram-positive bacteria retained the crystal violet and appeared as purple, while Gram-negative bacteria were decolourised and stained by safranin and appeared pink/red.

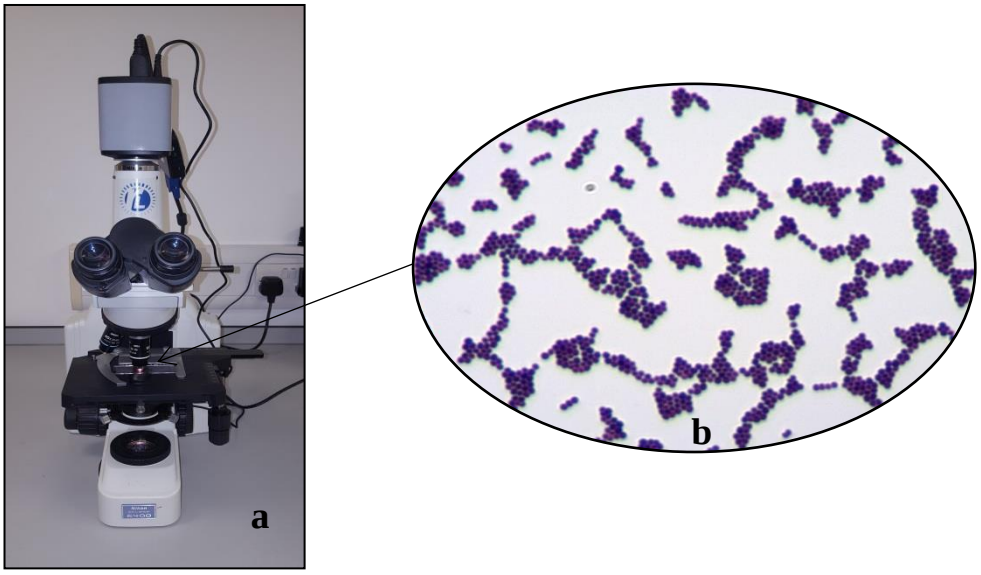


Figure 3.2 Viewing a Gram stain of Staphylococcus aureus under a microscope. Demonstrating: (a) Nikon Eclipse E400 Microscope used to view Gram stain under ×100 oil immersion lens (b) Photograph of Gram stain taken with a Euromex Image 4.0 Microscope Camera.

3.3.5.2 Latex Agglutination Tests

Latex agglutination tests were used to confirm the identity of *S. aureus* and MRSA.

Staphaurex Plus

Staphaurex® Plus was used to confirm samples were *S. aureus* (Remel, Thermo Fisher Scientific, UK). This test used yellow latex particles coated with human fibrinogen to detect clumping factor, and were also coated with rabbit IgG for detection of protein A and cell surface antigens characteristic of MRSA and MSSA strains.

The test was conducted following the manufacturer's instructions. In brief, single colonies of the isolate were mixed with a 'test' latex and a 'control', the samples were rocked for 30 seconds, and observed for agglutination (Fig 3.3). Agglutination indicated the presence of either coagulase, protein A or surface antigens commonly found on *S. aureus*. A known strain of *S. aureus* was used as a positive control.

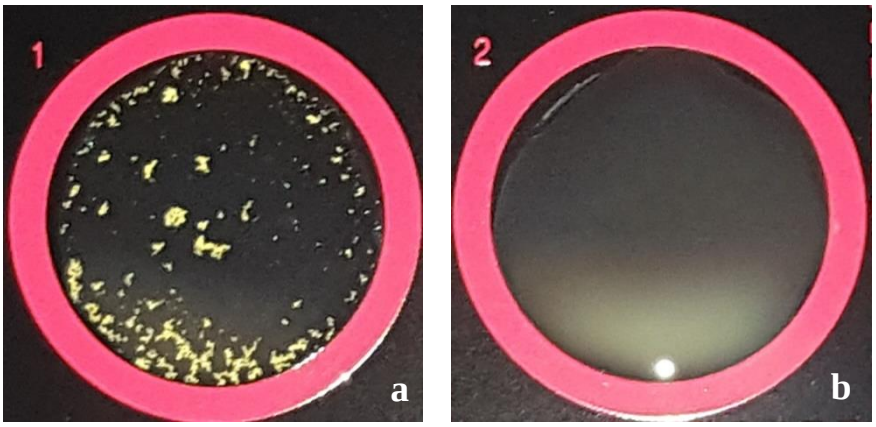


Figure 3.3 Appearance of latex agglutination test, Staphaurex® Plus. (a) agglutination of test latex and (b) no agglutination of control latex.

Penicillin-Binding Protein 2a Test

The Penicillin-Binding Protein 2a (PBP2a) test (Oxoid, UK) was used to confirm methicillin resistance. This test used latex particles sensitised with a monoclonal antibody against PBP2a, an altered penicillin-binding protein with reduced affinity for β-lactam antibiotics.

The test was conducted following the manufacturer's instructions. In brief, four drops of 'extraction reagent 1' and a loopful of bacterial cells were vortexed in a microcentrifuge tube to produce a turbid suspension. The tube was heated in a water bath at 98 °C for 3 minutes before cooling at room temperature. A drop of 'extraction reagent 2' was then added, mixed and the centrifuge tube was centrifuged at 1500 ×*g* for 5 minutes. The resultant supernatant was then used for the latex agglutination test. 50 µl of sample supernatant was added to 'test latex' or 'control latex', and samples rocked for up to three minutes to identify agglutination. Agglutination confirmed the presence of methicillin-resistance. Known strains of methicillin-resistant and methicillin-sensitive *S. aureus* were used as positive and negative controls, respectively.

3.4 Media

The following section details the cell culture media used for mammalian cell culture and the agar and broths used for microbial culture.

3.4.1 Culture Media for Mammalian Cells

Supplemented, 2× and 10× DMEM were prepared as detailed in Table 3.2. All media was stored at 4 °C, with small volumes of supplemented 10% FBS-DMEM held in a 37 °C incubator for 48 hours to ensure no contaminants were present. Once prepared, 10× DMEM was also sterile filtered to remove any contaminants.

3.4.2 Culture Media for Microorganisms

Agar and broths used for microbial cultivation were prepared according to manufacturer guidelines (Table 3.3). Media was sterilised by autoclaving at 121°C for 15 minutes before use.

3.4.3 Biologically-Relevant Suspending Media

The biologically-relevant media used in this study include human blood plasma, artificial saliva and artificial faeces as can be seen in Figure 3.4.

Human blood plasma was obtained from the Scottish National Blood Transfusion Service (SNBTS, UK). The blood was stored at −20°C until required and was slowly defrosted and decanted into a sterile container before use. Artificial saliva was a modified version of that used by Margomenou *et al*. (2000) and artificial faeces was a modified version of that by

Colón *et al.* (2015). The constituents of both artificial media can be seen in Table 3.6 and after preparation (and sterilisation of artificial faeces at 121 °C) both media were adjusted to pH 7 (pH210 microprocessor pH meter; Hannah Instruments, UK). The modifications made to the original formulations, to ensure compatibility with the FEA cells, are provided in further detail in Chapter 4.

Additionally, samples were also exposed in minimal media (DPBS) with the addition of 5% or 10% FBS, which acted as organic soiling.

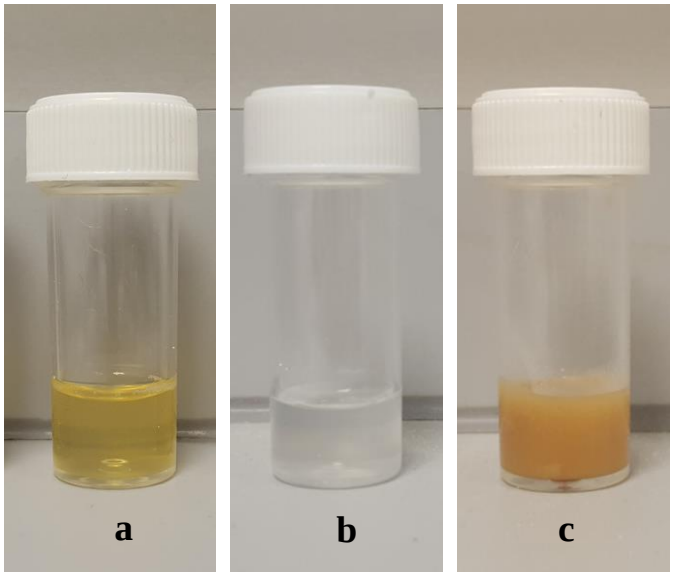


Figure 3.4 Appearance of biologically-relevant media used within this study. Appearance of: (a) human blood plasma, (b) artificial saliva and (c) artificial faeces.

3.4.4 Diluents and Other Media

- **Phosphate Buffer Saline** [BR0014G] (Oxoid, UK) – 1 tablet in 100 ml distilled water, sterilised at 121 °C for 15 minutes, and used for bacterial and viral suspension.
- **HyClone™ Dulbecco's phosphate-buffered saline** [SH3026401] (GE Healthcare Life Science, USA) – premade filter sterilised salt solution containing magnesium and calcium used for viral suspension.
- **10% Trypsin-EDTA in PBS** – 20 ml of 0.5% Trypsin-EDTA (10×) [15400054] (Gibco, UK) added to 180 ml phosphate buffered saline, used to dissociate mammalian cells from cell culture flasks.
- **Low Melting Point Agarose** [A9045 and 16520] (Sigma Aldrich and Invitrogen, UK, respectively) – 2× agarose was prepared by dissolving 20 g agarose in 1 l deionised distilled water and was sterilised at 121 °C. Used 1:1 with 2× supplemented DMEM to form an overlay for viral experiments.
- **Dimethyl sulfoxide** (DMSO) [D8418] (Sigma Aldrich, UK) – used in 10% concentration during cell storage to prevent ice crystal formation.

Table 3.2 Supplemented media for mammalian cell culture.

Media	Additives	Quantity Used	Manufacturer	Product Code
10% FBS-DMEM	Dulbecco's Modified Eagle Medium	1 l	Gibco, UK	42430025
	Fetal Bovine Serum, Heat Inactivated	100 ml/l	Gibco, UK	10500064
	200 mM L-Glutamine (×100)	10 ml/l	Gibco, UK	25030081
	100 mM Sodium Pyruvate (×100)	10 ml/l	Gibco, UK	11360039
	Penicillin-Streptomycin (10000 U/mL)	24 ml/l	Gibco, UK	15140122
10× DMEM	Dulbecco's Modified Eagle Medium Powder	140 g/l	Gibco, UK	52100021
	Sterile H_2O	1 l	-	-
2× DMEM	10× DMEM	200 ml/l	-	-
	Fetal Bovine Serum, Heat Inactivated	200 ml/l	Gibco, UK	10500064
	200 mM L-Glutamine (×100)	20 ml/l	Gibco, UK	25030081
	100 mM Sodium Pyruvate (×100)	20 ml/l	Gibco, UK	11360039
	Penicillin-Streptomycin (10000 U/mL)	40 ml/l	Gibco, UK	15140122
	Sodium Bicarbonate (7.5%)	98.6 ml/l	Gibco, UK	25080094
	Sterile H_2O	421.4 ml/l	-	-

Table 3.3 Broth and agar media for microbial cultivation, with required additives.

Media	Additives	Quantity Used	Manufacturer	Product Code
Nutrient Broth	N/A	13 g/l	Oxoid, UK	CM0001
Nutrient Agar	N/A	28 g/l	Oxoid, UK	CM0003
Mueller-Hinton Agar	N/A	38 g/l	Oxoid, UK	CM0337
Soya Flour Mannitol Agar	Soya Flour	20 g/l	Holland & Barrett, UK	-
	Mannitol	20 g/l	ACROS Organics, UK	125341000
	Agar Bacteriological	20 g/l	Oxoid, UK	LP0011
Enriched Nutrient Agar	Nutrient Agar	28 g/l	Oxoid, UK	CM0003
	Glucose	5 g/l	Thermo Fisher Scientific, UK	G/0500/53
	10 mM Magnesium Sulfate ($MgSO_4$)	10 ml/l	ACROS Organics, UK	413485000
	8 mM Calcium Nitrate ($Ca(NO_3)_2$)	10 ml/l	Thermo Fisher Scientific, UK	C/1882/53
Enriched Soft Agar Overlay	Nutrient Broth	13 g/l	Oxoid, UK	CM0001
	Agar Bacteriological	3 g/l	Oxoid, UK	LP0011
	Glucose	5 g/l	Thermo Fisher Scientific, UK	G/0500/53
	10 mM Magnesium Sulfate ($MgSO_4$)	10 ml/l	ACROS Organics, UK	413485000
	8 mM Calcium Nitrate ($Ca(NO_3)_2$)	10 ml/l	Fisher Scientific, UK	C/1882/53

Table 3.4 Constituents of biologically-relevant media; artificial saliva and faeces.

Media	Additives	Quantity Used	Manufacturer	Product Code
Artificial Saliva	Sterile H_2O	1 l	-	-
	Sodium Bicarbonate ($NaHCO_3$)	5.2 g/l	ARCOS Organics, USA	217125000
	Sodium Chloride (NaCl)	0.88 g/l	Thermo Fisher Scientific, UK	10428420
	Dipotassium Phosphate (K_2HPO_4)	1.36 g/l	BDH Chemicals Ltd, UK	9266
	Potassium Chloride (KCl)	0.48 g/l	Sigma Aldrich, UK	208000
	α-Amylase	2000 I.U	Sigma Aldrich, UK	A3176
	Mucin from Porcine Stomach	2 g/l	Sigma Aldrich, UK	M1778
Artificial Faeces	Distilled H_2O	920 g/l	-	-
	Inactivated Yeast *(S. cerevisiae)*	30 g/l	Marigold, UK	-
	Psyllium Powder	7 g/l	Buy Whole Foods Online, UK	-
	Miso Paste	11 g/l	Yutaka, UK	-
	Cellulose	8 g/l	Sigma Aldrich, UK	435236
	Oleic Acid	N/A	Sigma Aldrich, UK	75096
	Sodium Chloride (NaCl)	1.6 g/l	Thermo Fisher Scientific, UK	10428420
	Potassium Chloride (KCl)	1.6 g/l	Sigma Aldrich, UK	208000
	Calcium Chloride ($CaCl_2$)	0.8 g/l	Sigma Aldrich, UK	746495

3.5 405 nm Light Exposure Systems

For this study, LED arrays were used as the light sources for exposure of microorganisms to 405 nm light. Several different arrays were used throughout the study: an ENFIS PhotonStar 24-LED array, a 9-LED array, and a HINS-light EDS. The optical emission spectra of the systems were measured using a spectrometer (model HR4000, Ocean Optics). The peaks of violet-blue light from these light sources are in the region of 405 nm, and their bandwidth 18-21 nm at full-width-half maximum (FWHM), but these light sources will all be referred to as 405 nm LEDs throughout the text.

The different arrays used in this study were selected to allow a range of irradiances from 0.5-155 mW/cm^2, with the irradiance of the light sources measured at the sample surface, using a radiant power meter and photodiode detector (LOT Oriel, USA). Details of how each array was utilised are described in subsequent experimental chapters, however an overview of each system is provided in the following subsections.

3.5.1 ENFIS PhotonStar 24-LED Array

The ENFIS PhotonStar, a 24-LED array (PhotonStar Technologies, UK) powered by a 40 V Xitanium LED Driver (Phillips, Netherlands) was used for high-intensity exposure of viral and bacterial samples (Chapter 4 & 7, respectively). The array had a peak wavelength of 407 nm and a FWHM of 19 nm (Fig 3.5). The system was mounted onto a polyvinyl chloride (PVC) stand, which consisted of a PVC base and holding plate with a connecting metal pole, allowing the distance between the light source and surface sample to be changed depending on experimental requirements (Fig 3.6a). The 405 nm LED array (Fig 3.6b) was connected to two variable resistors, to allow the irradiance of the array to be altered when at a fixed height. The array was also connected to a heat sink and a cooling fan (Fig 3.6c) to dissipate heat and prevent transfer to the test samples. For viral exposure experiments the distance between the array and sample surface was 4 cm and for bacterial tolerance experiments 6 cm, with the output irradiance measured as 155.8 mW/cm^2 and 60 mW/cm^2 respectively.

3.5.2 9-LED Array

The 9-LED array system was used for experiments investigating bacterial cultivation in low-intensity 405 nm light (Chapter 7). The system was composed of a 9-LED array (GE Illumination, USA) with LEDs arranged in a 3 × 3 grid pattern (Fig 3.7) powered by a variable DC power supply (Velleman, Belgium). The array was also connected to a heat sink to dissipate heat. The LEDs had a peak wavelength of 406 nm and a FWHM of 18 nm

(Fig 3.5). It should be noted that the LEDs were purchased as warm white LEDs, however the hemispherical phosphor coated caps were removed, to expose the 405 nm diodes. The voltage and current supplied to the LEDs differed during experiments, depending on the irradiance required, which was between 0.15-1 mW/cm^2.

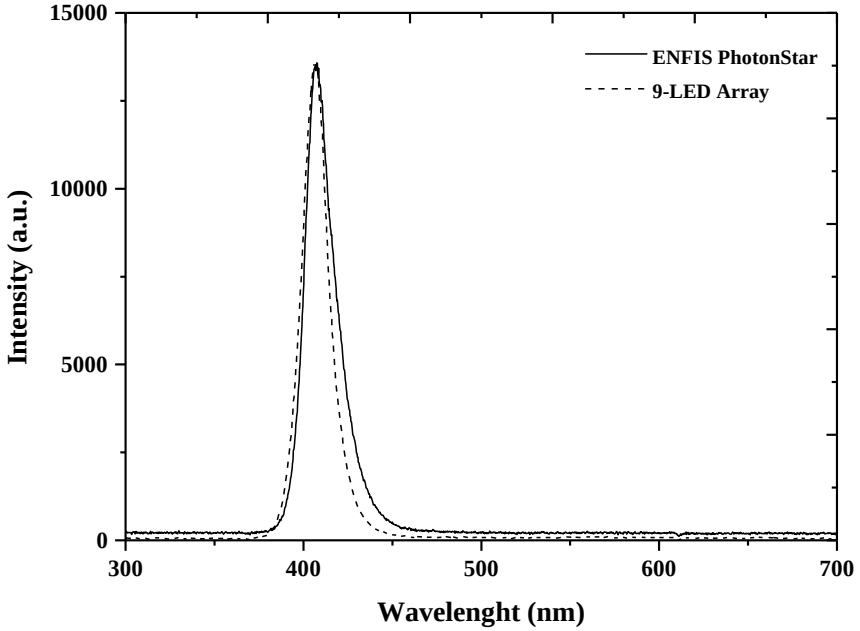


Figure 3.5 Optical emission spectrum of the ENFIS PhotonStar 24-LED and 9-LED array used during this study. Measured using an HR4000 spectrometer (Ocean Optics, Germany) and Spectra Suite software version 2.0.151.

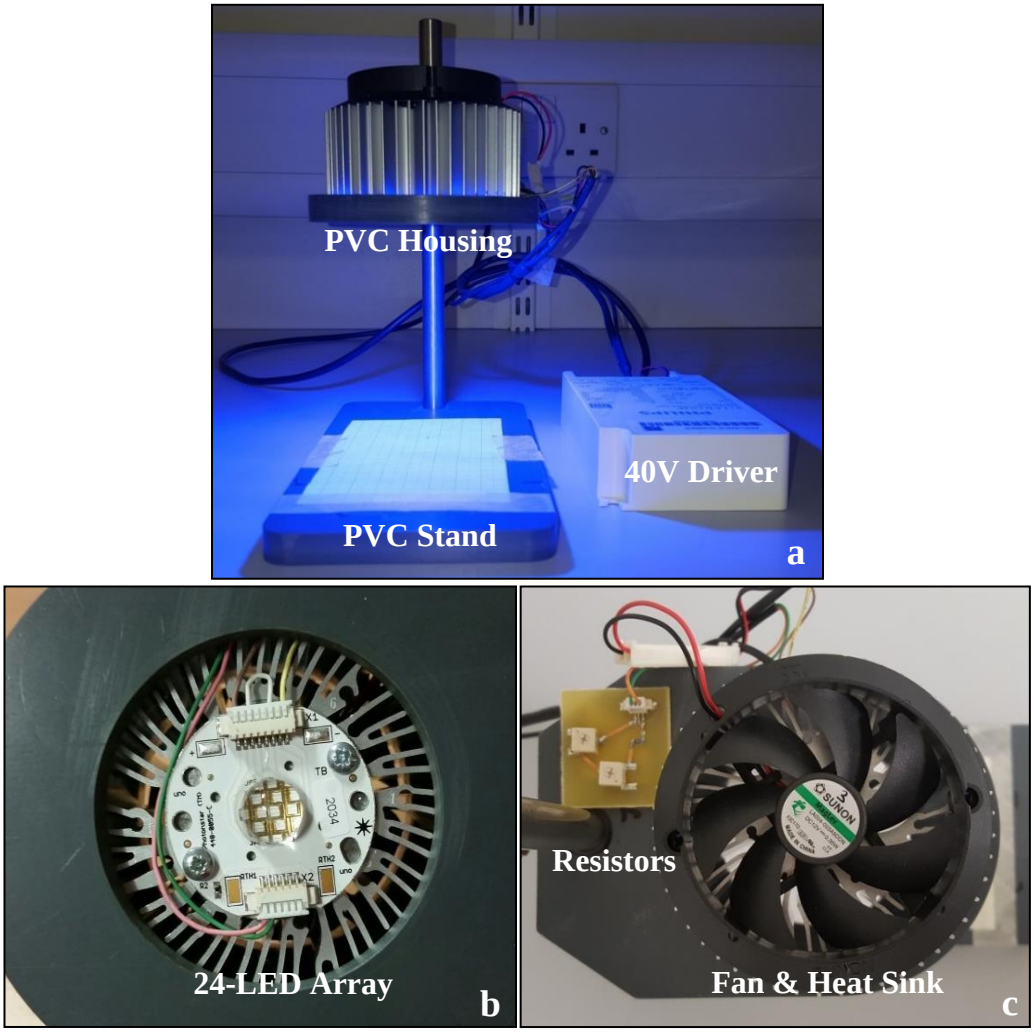


Figure 3.6 ENFIS PhotonStar 405 nm Lighting System. Appearance of: (a) system placed on movable PVC housing, (b) 24-LED Array and (c) heat sink, fan and control circuit.

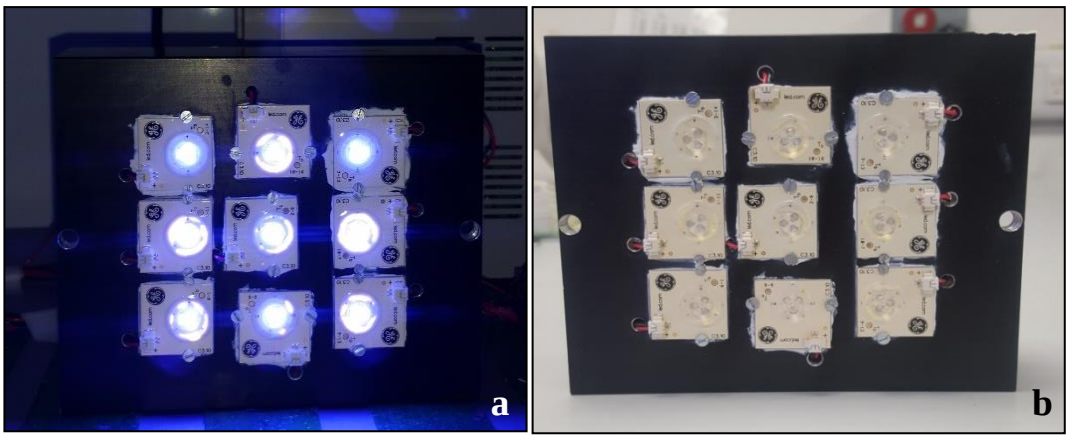


Figure 3.7 Appearance of 9-LED array arranged in a 6 × 6 cm grid pattern. (a) lights on and (b) lights off.

3.5.3 HINS-Light EDS Prototype

The HINS-light EDS is a prototype lighting system developed at the University of Strathclyde, device patent numbers: 2211914 (Europe) and 8,398,264 (USA) (Fig 3.8). The system has been designed to be retrofitted in place of conventional ceiling tiles, to continually emit low levels of blue 405 nm light (0.1-0.5 mW/cm^2) to aid environmental decontamination. In addition to the violet-blue 405 nm light output, the system incorporates a white light output to ensure that the colour output is more similar to commonly used fluorescent lighting (Fig 3.8b). This ensures that the system is aesthetically acceptable when utilised in occupied environments.

The prototype EDS used in this study, used only violet-blue LEDs to investigate virucidal efficacy of 405 nm light, with the white LEDs disconnected (Fig 3.9). The system was a more recent design, based on 4-LED arrays rather than a matrix of 16 405 nm 99-LED arrays used in older systems (Bache *et al.*, 2012). The violet-blue LEDs had a peak wavelength of 408 nm and a full-width-half maximum of 21 nm (Fig 3.9) and when white LEDs were connected the system had an additional output of white light between 500-700 nm (Fig 3.8; Fig 3.9).

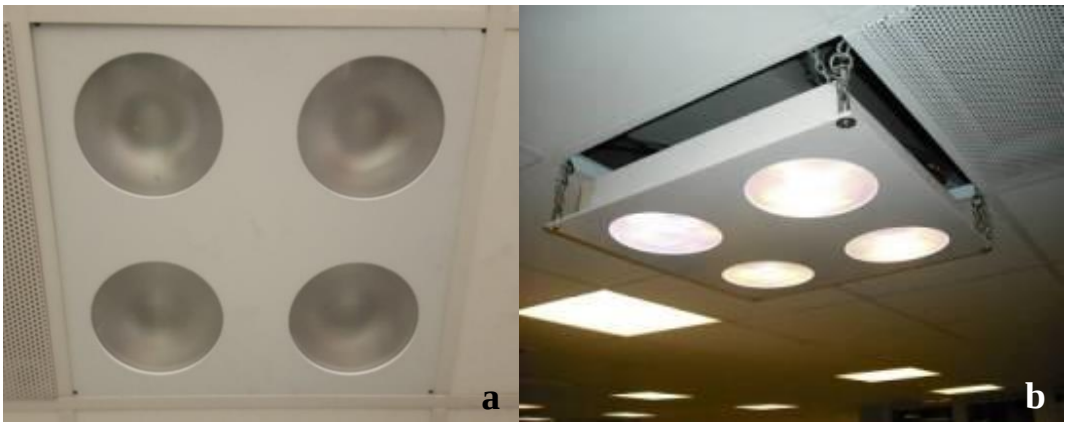


Figure 3.8 High Intensity Narrow Spectrum-light Environmental Decontamination System (HINS-light EDS). Appearance of: (a) the view of the EDS unit in place of a ceiling tile, and (b) EDS switched on utilising both white and 405 nm LEDs.

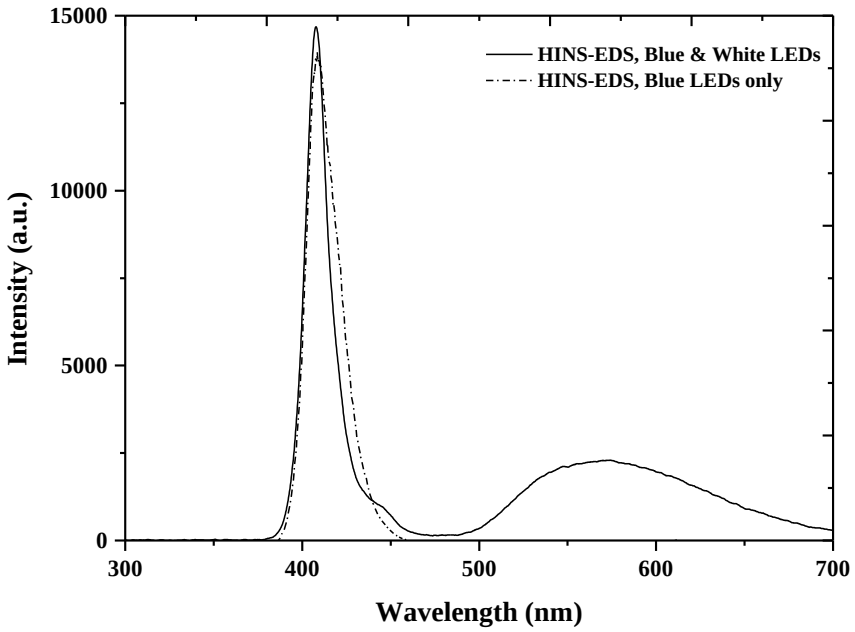


Figure 3.9 Optical emission spectrum of the HINS-light EDS used during this study. Spectrum of the EDS was measured when both the blue and white LEDs were utilised, and when only the blue LEDs were utilised. Measured using an HR4000 spectrometer (Ocean Optics, Germany) and Spectra Suite software version 2.0.151.

3.6 Data Analysis

The following section details how dose was calculated for each of the light sources used in the study, as well as methods of statistical analysis.

3.6.1 Calculating Dose

For antimicrobial tests, microbial samples were subjected to 405 nm light for different exposure periods. Using the measured irradiance and exposure times, the applied dose of 405 nm light could be calculated using equation (3.2):

$$E = Pt \tag{3.2}$$

Where E = dose in J/cm^2, P = irradiance in W/cm^2 and t = exposure time in seconds.

3.6.2 Statistical Analysis

Data points on graphs represent the mean results (± standard deviation) of triplicate independent experiments, with a minimum of two replicates for bacterial sample experiments ($n \geq 6$) and at least one replicate for viral experiments ($n \geq 3$). Significant differences were calculated at the 95% confidence interval using either T-tests (paired or 2-sample) or ANOVA (one way), depending on the data, using Minitab software release 17. Results were found to be significant when $P < 0.05$. Significant differences are highlighted in figures using an asterisk '*'.

Chapter Four

Proof-of-Concept of the Virucidal Efficacy of 405 nm Light

4.0 Overview

As NoV regularly causes outbreaks within the hospital setting, studies in this Chapter focussed on establishing the antiviral efficacy of high-intensity 405 nm light, using feline calicivirus (FCV) as a NoV surrogate. This involved investigating the virucidal efficacy of 405 nm light when viral particles were suspended in different liquid media, including minimal, organically-rich and biologically-relevant media. Additionally, investigations were carried out to discover the photometric properties of the suspending media. The results provide the first proof-of-concept evidence of the virucidal efficacy of 405 nm light, and the enhancement that can be achieved when viral particles are surrounded by biologically-relevant media.

4.1 Introduction

NoV causes acute vomiting and diarrhoea, which can result in major outbreaks within a short period of time. NoV outbreaks are a global issue not only in hospitals but also places where people are in close contact such as nursing homes, schools and cruise ships (Vivancos *et al*., 2010; Lai *et al*., 2013; Xu *et al*., 2013). It is estimated that there are up to 700 million cases of NoV each year globally (Bartsch *et al*., 2016). Most NoV outbreaks are thought to occur in health facilities with 79% of outbreaks in England and Wales occurring in hospitals and residential care facilities (Lopman *et al*., 2003).

Due to the low infectious dose, high infectivity, persistent shedding in stools and environmental survival of NoV, (as discussed in Section 2.1.1 and 2.2.2) cleaning during an outbreak is of particular importance in the hospital setting, as otherwise surfaces can play a role in transmission (Wu *et al*., 2005). However, if hospitals are inadequately cleaned transmission can be rapid among staff and patients. Upon confirmation of a NoV outbreak the bay or ward will be closed to new patients and occasionally outbreaks can result in

complete hospital closure. This can lead to a huge loss for health boards as was demonstrated by Danial *et al*. (2011) who calculated a loss of £1.2 million between 2007 and 2009 for NHS Lothian, Scotland, indicating the increasing need for novel decontamination systems to help prevent NoV outbreaks.

As described in Section 2.4.5, 405 nm light is one such technology designed to aid environmental decontamination, and can inactivate a wide range of bacteria and fungi including nosocomial pathogens. However, to date there has been little work carried out to investigate whether this technology can be used for the inactivation of viruses. A brief report which exposed a viral surrogate, *Streptomyces* bacteriophage ϕC31, to 405 nm light, indicated that when in nutritious media the bacteriophage was inactivated and no longer able to infect *Streptomyces coelicolor* (Tomb *et al*., 2014). The results indicated that 405 nm light was able to inactivate viruses in photosensitive media and suggested that inactivation may also be possible in non-nutritious media (e.g. PBS) with higher doses of 405 nm light (Tomb *et al*., 2014).

Further evidence of the virucidal effect of 405 nm light needs to be established, and confirming the inactivation of viruses using light alone would strengthen the case for use of 405 nm light for environmental decontamination. Currently, inactivation studies of NoV are limited: as there is no established cell-culture system, it is not possible to quantify viral inactivation (Duzier *et al*., 2004a; Richards 2012; Cromeans *et al*., 2014). To overcome this, this study used FCV as a model to study the antiviral effects of 405 nm light on NoV.

This chapter focusses on establishing the first proof-of-concept evidence of the antiviral effects of narrowband 405 nm light, using FCV as a model for NoV. The study investigates the influence of various types of suspending media on viral inactivation and is divided into three main subsections as follows:

- Inactivation of FCV when suspended in minimal and organically-rich media, using high-irradiance 405 nm light (~150 mW/cm^2) in order to generate proof-of-principle inactivation kinetics.
- Inactivation of FCV when suspended in biologically-relevant media including artificial saliva and faeces using high-irradiance 405 nm light (~150 mW/cm^2), in order to generate inactivation kinetics in clinically-relevant scenarios.
- Assessment of the photometric properties of the range of minimal, organically-rich and biologically-relevant media in which viruses were suspended and exposed to high-intensity 405 nm light.

4.2 Inactivation of FCV when Suspended in Minimal and Organically-Rich Media

Previous studies have shown successful inactivation of a viral surrogate, bacteriophage ϕC31, using 405 nm light when suspended in nutrient-rich media and, to a lesser extent, when suspended in minimal media. The aim of this study was to determine whether similar results could be achieved using FCV as a NoV surrogate.

4.2.1 Viral Preparation

The viral stock of FCV was previously prepared as described in Section 3.1.4. Small aliquots of viral stocks were defrosted at room temperature when required and then serially diluted in a particular suspending medium to achieve the desired starting population of 1-2 × 10^5 PFU/ml.

4.2.2 Cell Plate Preparation

Mammalian FEA Cells were maintained as described in Section 3.1.2. Prior to experiments, 6-well cell culture plates (Thermo Fisher Scientific, UK) were seeded with 7.5 × 10^5 FEA cells per well by adding 3 ml of the cell suspension to each well and incubating at 37 °C in 5% CO_2 in air for approximately 20 hours, resulting in confluent monolayers.

4.2.1 Suspending Media Preparation

For suspension in minimal media, phosphate buffered saline or Dulbecco's phosphate buffered saline supplemented with Ca^{2+} and Mg^{2+}, were used. Dulbecco's Modified Eagle medium supplemented with 240 U/ml penicillin streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate (Table 3.2), with or without a 10% FBS addition, was used as an organically-rich media for viral suspension.

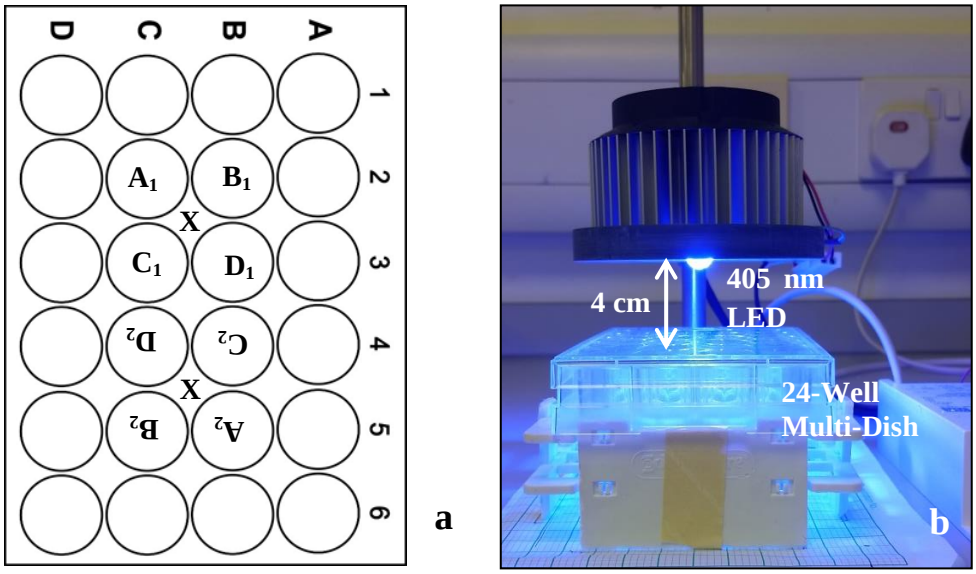
4.2.2 405 nm Light Source

To investigate viral inactivation using 405 nm light, experiments were conducted using the ENFIS PhotonStar 24-LED array, as described in Section 3.5.1. The light source was held on a PVC stand at a distance of 4 cm from viral samples giving an irradiance of 155.8 mW/cm^2 at the sample surface, measured using a radiant power meter and photodiode detector (LOT Oriel, USA).

4.2.3 High-Irradiance Exposure of Viral Suspensions

FCV stock was defrosted at room temperature and diluted to 2×10^5 PFU/ml in the selected minimal media or organically-rich media. 1.5 ml volumes of viral suspension were transferred into the central wells of a 24-well plate (Techno Plastic Products, Switzerland) with only wells A, B & D used (Fig 4.1) (scoping experiments revealed reduced transmission of 405 nm light through well C compared to wells A, B & D). The plate was positioned on a raised rack stand, with the sample wells 4 cm directly below the light source. The plates were held on the rack stand, rather than a solid base in order to permit airflow around the sample dish and prevent a build-up of heat. Furthermore, the plate lid was kept on to prevent evaporation.

Test samples were exposed to increasing doses of 405 nm light at room temperature, with the dose calculated as described in Section 3.6. Control samples were set up under identical environmental conditions but without 405 nm light illumination. Post-exposure, FCV samples were serially diluted in minimal media for enumeration by plaque assay.



24–Well Multi-Dish **405 nm Light System**

Figure 4.1 Experimental arrangement for exposure of viral suspensions to 405 nm light. Illustrating: (a) 24-well multi-dish used to hold viral suspensions, with central wells A_1, B_1 & D_1 used initially and then the dish was rotated and re-used, utilising wells A_2, B_2 & D_2, and (b) ENFIS PhotonStar 24-LED array held on a PVC housing 4 cm above viral samples held in the 24-well multi-dish on a PVC stand with ventilation.

4.2.4 Plaque Assay

Post-exposure, the viral suspension and control were diluted in DPBS. The growth medium was then aspirated from the FEA cells in the pre-prepared 6-well plates and replaced with 1 ml of viral sample. As described in Section 3.1.5, the plates were co-incubated at 37 °C in a humidified 5% CO_2 incubator for 90 minutes, with plates rocked every 15 minutes to ensure even distribution of the inoculum over each monolayer.

Following the viral incubation period, the inoculum was aspirated and the well washed with medium (10% FBS-DMEM or DPBS) before adding 4 ml overlay mixture consisting of 2× supplemented DMEM 1:1 with 2× agarose (Section 3.4.4). The overlay was left to set before the plates were incubated at 37 °C for 44-48 hours in 5% CO_2.

Post-incubation, the monolayers were fixed and stained overnight with 0.5% crystal violet in 10% buffered neutral formalin. The agarose plugs and stain were then removed, the plates left to dry, plaques counted, and the virus infectivity titre expressed as PFU/ml.

4.2.5 Results: FCV Inactivation in Minimal and Organically-Rich Media

Viral suspensions were firstly exposed to high-irradiance light whilst suspended in PBS. Figure 4.2 highlights the inactivation kinetics when the virus was suspended in this minimal medium, with a significant 4.2 $\log_{10}$ reduction of FCV found after a final dose of 2.8 kJ/cm^2. However, upon analysis of the results, it was firstly noted that the initial starting population (6×10^4 PFU/ml) was significantly lower (P = 0.029) than the expected population ($1\text{-}2 \times 10^5$ PFU/ml) established during viral titre assays (in DMEM) after initial viral preparation. There was also a decrease in the non-exposed control population from 6×10^4 PFU/ml to 2.5×10^4 PFU/ml after the equivalent exposure time. This indicated that there was less viral attachment and infection of FEA cells than expected. To overcome this, the experiment was repeated with FCV suspended in modified PBS, Dulbecco's PBS (DPBS) which contained calcium and magnesium cations (Ca^{2+} & Mg^{2+} respectively), used to aid viral attachment and infection of cells.

Using DPBS as a suspending medium ensured that the initial population was the expected 10^5 PFU/ml population (Fig 4.2). There was also no significant difference in control populations over the duration of the experiments (P = 0.656). Therefore, the 3.9 $\log_{10}$ inactivation occurring after a dose of 2.8 kJ/cm^2, when FCV is suspended in DPBS, is more likely the true level of inactivation due to exposure to 155.8 mW/cm^2 405 nm light. These initial results indicate that high doses of 405 nm light are required for FCV inactivation when

suspended in minimal media, with relatively linear inactivation occurring after doses of 561 J/cm^2, 1683 J/cm^2 and 2804 J/cm^2, all of which were found to be significantly different to the final control population (P = 0.003, 0.002, 0.001 respectively).

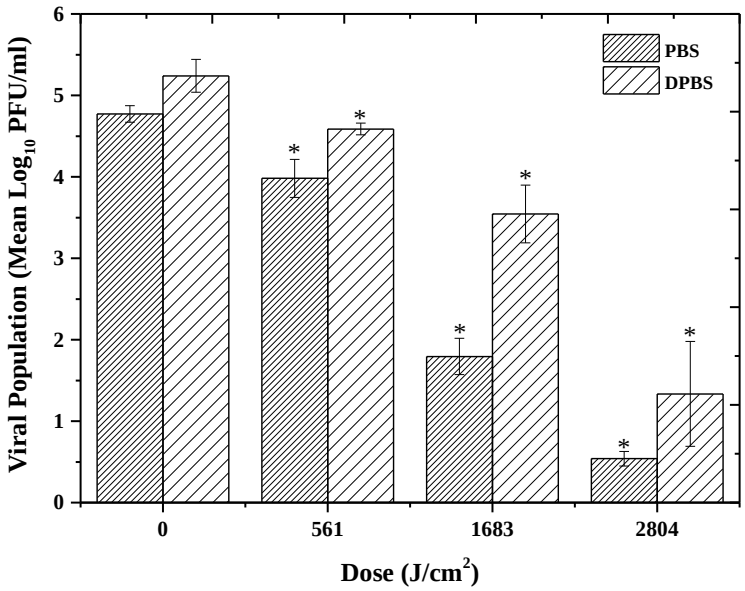


Figure 4.2 Inactivation of feline calicivirus suspended in minimal media (phosphate buffered saline and Dulbecco's phosphate buffered saline), exposed to high-irradiance 405 nm light. Samples were exposed to 405 nm light at an irradiance of 155.8 mW/cm^2. Data points show the mean counts (n = 2 for PBS and n = 3 for DPBS respectively) ± SD. Asterisks '*' indicate light-exposed samples that were significantly different to the non-exposed final control samples ($P \leq 0.05$), using one-way ANOVA. No significant decrease was observed in the final control populations ($P \geq 0.05$).

To investigate if the virucidal efficacy of 405 nm light could be enhanced, FCV was suspended in organically-rich media: supplemented DMEM (Table 3.2), normally used as a cell-culture medium. As can be seen in Figure 4.3, when exposed in this nutritious medium, a significantly lower dose was required for viral inactivation compared to when in the minimal medium, with a 4.8 $\log_{10}$ reduction achieved after a dose of 421 J/cm^2 in 10% FBS-DMEM compared to 3.9 $\log_{10}$ reduction achieved after a dose of 2.8 kJ/cm^2 in minimal medium.

As the presence of FBS (10%) in DMEM is thought to reduce the level of oxidation upon exposure to normal laboratory lighting (Grzelak *et al*., 2001), the exposure was repeated with FCV suspended in DMEM without FBS to observe any differences in inactivation kinetics. Results (Fig 4.3) demonstrate that slightly greater inactivation was observed, when the serum additive was omitted from the medium, however statistical analysis showed this difference was not significant ($P > 0.05$). Control samples also showed no significant decrease ($P > 0.05$).

As components of DMEM have been shown to be photosensitive (Grzelak *et al*., 2001), exposures were repeated with riboflavin added to DPBS with and without tyrosine, tryptophan, pyridoxine and folic acid, in the same concentrations as found in DMEM (Table 4.1). Results demonstrated that exposure of FCV suspended in DPBS with riboflavin only resulted in a 1.3 $\log_{10}$ reduction after a dose of 421 J/cm^2 however when all components were present, enhanced inactivation occurred and a 5.1 $\log_{10}$ inactivation was achieved.

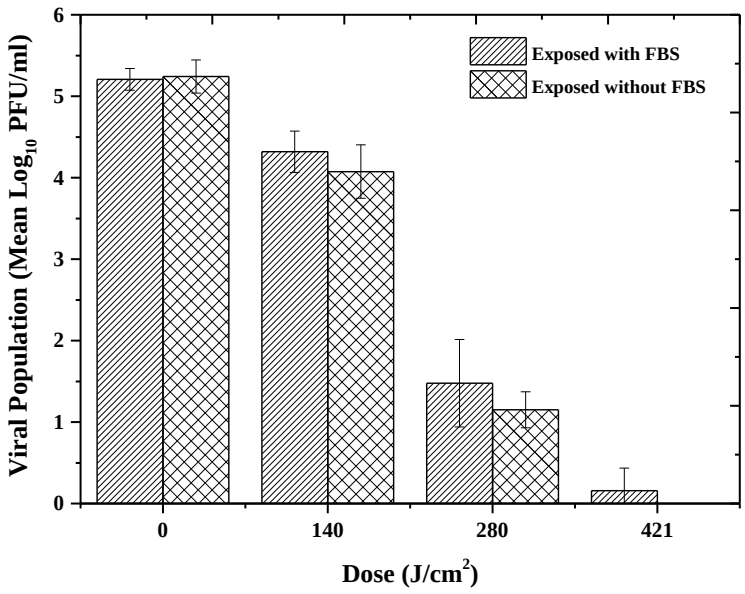


Figure 4.3 Comparison of the inactivation of feline calicivirus suspended in organically-rich media (Dulbecco's Modified Eagle medium (DMEM), without and without 10% fetal bovine serum (FBS)), exposed to high-irradiance 405 nm light. Samples were exposed to 405 nm light at an irradiance of 155.8 mW/cm^2. Data points show the mean counts (n = 3) ± SD. Statistical analysis showed no significant difference between inactivation in the two media ($P > 0.05$), using a paired T-test. No significant decrease was observed in the final control populations ($P \geq 0.05$).

Table 4.1 ***Comparison of the inactivation of feline calicivirus when suspended in Dulbecco's phosphate buffered saline supplemented with riboflavin alone or alongside tyrosine, tryptophan, pyridoxine and folic acid, upon exposure to high-irradiance 405 nm light.*** Samples were exposed to 405 nm light at an irradiance of 155.8 mW/cm^2. Table entries represent the mean count (n = 3) ± SD. Asterisks '*' indicate light-exposed samples that were significantly different to the non-exposed final control samples ($P \leq 0.05$).

	DPBS Supplements	
Photosensitive Components	**Riboflavin (0.4 mg/l)**	**Riboflavin (0.4 mg/l) Tyrosine (104 mg/l) Tryptophan (16 mg/l) Pyridoxine (4 mg/l) Folic Acid (4 mg/l)**
Starting Population Log_{10} PFU/ml (± SD)	5.01 ± 0.02	5.15 ± 0.03
Exposed Viral Population Log_{10} PFU/ml (± SD)	3.77 ± 0.61	0.00 ± 0.00
Non-Exposed Control Population Log_{10} PFU/ml (± SD)	5.05 ± 0.06	5.12 ± 0.07
Log_{10} Reduction PFU/ml (± SD)	1.28* ± 0.60	5.12* ± 0.00

4.3 Inactivation Kinetics of FCV when suspended in Biologically-Relevant Media

Once the baseline inactivation kinetics for FCV in minimal and organically-rich media were established, experiments were conducted to determine if FCV could be inactivated when in biologically-relevant media. This was carried out to investigate whether there was potential for enhanced virucidal activity when using 405 nm light for clinical decontamination applications. The three different biologically-relevant media used were: artificial saliva,

human blood plasma and artificial faeces, representing biological substances which commonly contaminate the environment and can potentially carry viral particles.

4.3.1 Artificial Saliva Preparation

Artificial saliva was based on that used by Margomenou *et al.* (2000), modified to contain a minimal number of ingredients which would represent the protein (mucin) and enzyme (amylase) content of human saliva. The formula was prepared as detailed in Table 3.4. The amount of α-amylase was reduced following initial tests in which higher concentrations were found to be not compatible with FEA cells. NaN_3 was also excluded as it has oxygen scavenger properties and therefore may protect against oxidative damage occurring (Egyeki *et al*., 2003). Once dissolved the artificial saliva was adjusted to a pH of between 7-7.5 to replicate the pH of saliva (Edgar *et al*., 2004). Artificial saliva was prepared immediately before it was required, and stored at 4 °C between use on the same day.

4.3.2 Human Blood Plasma Preparation

The human blood plasma used in viral inactivation studies was acquired from the Scottish National Blood Transfusion Service (SNBTS, Glasgow). The SNBTS have very stringent donation and screening procedures to ensure that blood products are not contaminated and safe for use (NHS National Services Scotland, 2016), therefore it was not necessary to filter the plasma before use to remove contaminants. The plasma was stored at −20 °C and slowly defrosted at 4 °C overnight, it was then decanted into a sterile container and mixed thoroughly before use.

4.3.3 Artificial Faeces Preparation

The artificial faeces was based upon a formulation detailed by Colón *et al*. (2015) (Section 3.4.3). Inactivated *Saccharomyces cerevisiae*, oleic acid, psyllium powder, miso paste and cellulose were used to represent microbial debris, fats, fibre, nitrogen and carbohydrates, respectively. The original formulation (Table 4.2; Column 1), was tested with the FEA cells but was found to be extremely thick, causing the FEA cell layer to dislodge from 6-well plates, and also left a fatty residue on the remaining cells. This occurred even if the formulation was diluted by a factor of 10^4.

To ensure compatibility with FEA cells, work was conducted to adapt the formulation to ensure it was suitable for use. As the original formula was extremely viscous, the formulation was adapted to have an increased water content of 900 g (Formula 1), which is also more indicative of the water level of diarrhoea (Wenzl *et al*., 1995). Upon incubation of

the FEA cells with Formula 1, the solution was still viscous and cells still appeared stressed with oily deposits on the surface. Subsequently several different formulations were produced to reduce fatty residue and cell stress, firstly by further increasing the water content and gradual reduction in the levels of constituents, with reductions in psyllium and miso found to be most favourable for reducing visible cell stress and removal of the faeces from cell wells (Formula 2). However, although the increased water content reduced viscosity to a favourable level and cell stress appeared somewhat reduced, the fatty residue was still present. Several attempts were made to remove the fatty residue from cells with various washing techniques and formulations were tested with reduced oleic acid, but it was found that complete removal of oleic acid was ultimately the best way to resolve this and remove any additional stress incurred (Formula 3). As Formula 3 was seen to cause little stress and there were no oily deposits, it was selected for use as the artificial faeces simulant, and the pH was adjusted to pH 7-7.5 to ensure that there would not be any negative effects on FCV.

Although the chosen formula had an increased total percentage of water (~94% vs 80%), and an increased percentage dry weight of all components, except psyllium powder and oleic acid, compared to the original formulation (Colón *et al*., 2015), it was found to be the most suitable formulation that could be used during virucidal efficacy testing and least likely to cause additional inactivation of FCV and stress to FEA cells. Results when FCV was suspended in these artificial faeces are preliminary indicators of the likelihood of viral inactivation in real human faecal matter but cannot completely replicate the true inactivation kinetics.

4.3.4 Viral and Cell Plate Preparation

FCV was prepared and suspended in the different biologically-relevant media, in a manner similar to that described in Section 4.2.1. Cell plates were also prepared as described in 4.2.2.

4.3.5 Experimental Arrangement

Experimental arrangement, exposure to 405 nm light at an irradiance of 155.8 mW/cm^2 and resultant plaque assays were carried out in the same manner as detailed in Sections 4.2.3-4.2.4.

Table 4.2 Combinations of the different components of artificial faeces, allowing compatibility with FEA cells.

Component	Formula by Colon *et al.* (g)	Solid Dry Weight (%)	Formula 1 (g)	Solid Dry Weight (%)	Formula 2 (g)	Solid Dry Weight (%)	Formula 3 (g)	Solid Dry Weight (%)
Distilled Water	800	-	900	-	920	-	920	-
Inactivated Yeast	60	30	30	30	30	37.5	30	50
Microcrystalline Cellulose	20	10	10	10	8	10	8	13.3
Psyllium powder	35	17.5	17.5	17.5	7	5.6	7	11.7
Miso Paste	35	17.5	17.5	17.5	11	8.8	11	18.3
Oleic Acid	40	20	20	20	20	16	/	/
NaCl	4	2	2	2	1.6	1.3	1.6	2.7
KCl	4	2	2	2	1.6	1.3	1.6	2.7
$CaCl_2$	2	1	1	1	0.8	1	0.8	1.3

4.3.6 Results: FCV Inactivation when in Biologically-Relevant Media

Artificial saliva, human blood plasma and artificial faeces were selected as organically-rich media, which are biologically-relevant in terms of media in which viral particles may be found in the environment, with NoV regularly identified in faeces. Exposure of FCV when suspended in artificial saliva yielded results similar to those achieved when suspended in DMEM, with a 5.1 $\log_{10}$ reduction of infectivity achieved after a dose of 421 J/cm^2 (Fig 4.4a). (In this case, inactivation was measured to a sensitivity of ten PFU/ml, as the artificial saliva in the undiluted samples adversely reacted with the FEA cells causing them to dislodge from the plate). Control samples showed no significant change when FCV was suspended in artificial saliva (P = 0.618).

The dose required for inactivation when FCV was suspended in blood plasma was slightly greater than that of artificial saliva, with 561 J/cm^2 required for 4.8 $\log_{10}$ inactivation of the NoV surrogate (Fig 4.4b). Control samples showed no significant change when FCV was suspended in plasma (P = 0.101).

Exposure of FCV in artificial faeces required far greater doses of 405 nm light for inactivation, with 4.5 $\log_{10}$ inactivation achieved after a dose of 1.4 kJ/cm^2 (Fig 4.4c). Control samples showed no significant change when FCV was suspended in artificial faeces (P = 0.747).

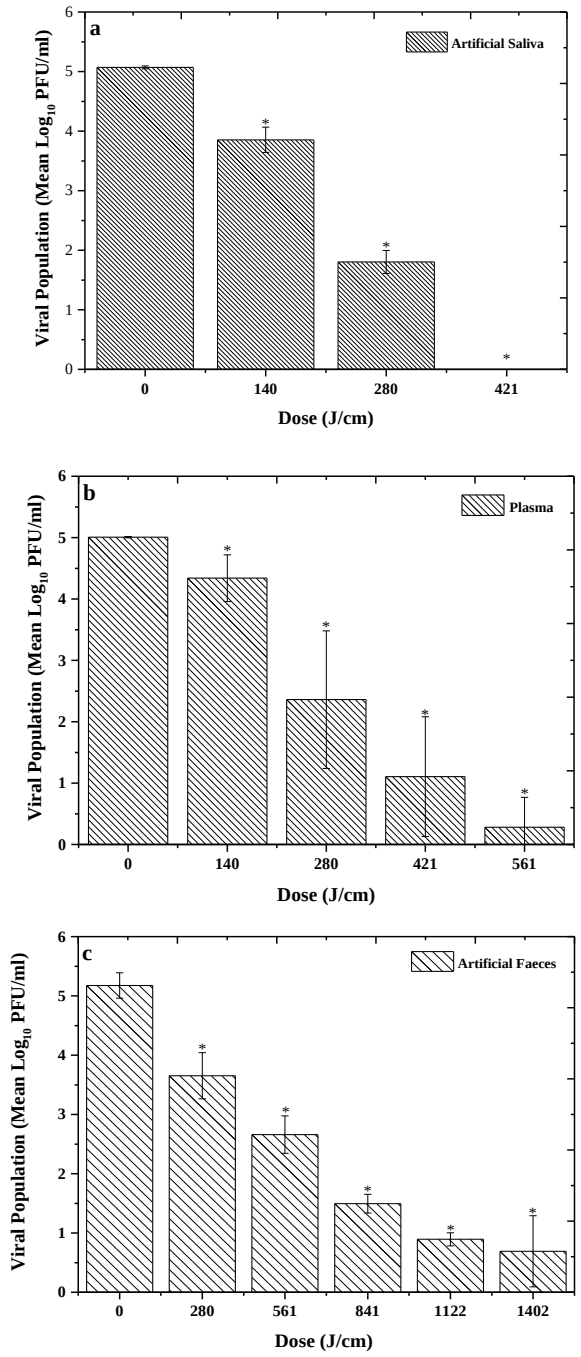


Figure 4.4 Inactivation of feline calicivirus suspended in biologically-relevant media upon exposure to high irradiance 405 nm light. Samples were exposed to 405 nm light at an irradiance of 155.8 mW/cm^2. Feline calicivirus was suspended in (a) artificial saliva, (b) blood plasma and (c) artificial faeces. Data points show the mean counts (n = 3) ± SD. Asterisks '*' indicate light exposed samples that were significantly different to non-exposed final control samples ($P \leq 0.05$), using one-way ANOVA. No significant decrease was observed in the final control populations ($P \geq 0.05$).

Comparison of the inactivation kinetics of FCV when suspended in the different selected media can be seen in Figure 4.5. Results demonstrate three distinct clusters within the inactivation profile of FCV in the differing media. The first cluster of inactivation occurs when FCV was suspended in organically-rich and biologically-relevant media including DMEM, artificial saliva and blood plasma, with doses of 420-560 J/cm^2 required for 5 $\log_{10}$ reduction. This is followed by > 3× increase in dose requirements for a similar level of inactivation when FCV is exposed in artificial faeces. These differences in dose requirements when FCV is suspended in biologically-relevant media could be due to reduced levels of transmission through the media, as detailed in Section 4.4.2.

The final inactivation cluster requires the greatest doses of 405 nm light for viral inactivation. When suspended in minimal media (DPBS) a dose of 2.8 kJ/cm^2 was required for 3.9 $\log_{10}$ reduction of FCV. This is likely due to the differing inactivation mechanism when the virus is suspended in minimal media compared to organically-rich media as discussed in section 4.5.

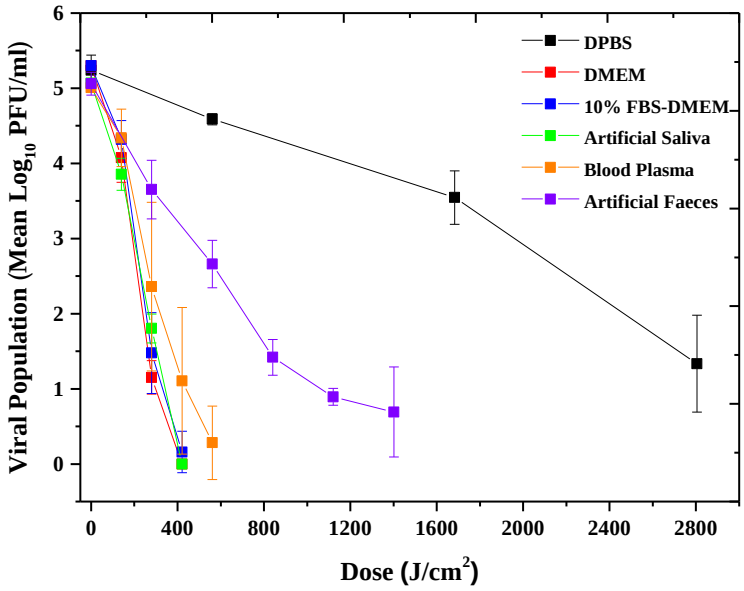


Figure 4.5 Comparison of inactivation kinetics of feline calicivirus when suspended in a range of media. FCV was suspended in minimal medium (Dulbecco's phosphate buffered saline (DPBS)), organically-rich media (Dulbecco's Modified Eagle medium (DMEM), 10% fetal bovine serum supplemented DMEM (10% FBS-DMEM)) and biologically-relevant media (artificial saliva, human blood plasma and artificial faeces), upon exposure to increasing doses of 405 nm light at an irradiance of 155.8 mW/cm^2. Data points show the mean counts (n = 3) ± SD.

4.4 Assessment of the Photosensitivity of the Suspending Media

This section provides analysis of the suspending media used for viral exposures in this study. The aim was to assess the potential photosensitivity of the different suspending media, and discuss the potential affect that these may have exerted on FCV during high-intensity 405 nm light exposure.

4.4.1 Spectrophotometry Techniques

The transmission and absorbance of 405 nm light through the suspending media was measured using a Biomate 5-UV-Visible Spectrophotometer (Thermo Fisher Scientific, UK).

The presence of porphyrins, or other components with the ability to absorb 405 nm light and emit fluorescence, within the suspending media was determined using fluorescence spectrophotometry. Media were freshly prepared and fluorescence measurements were carried out using a RF-5301 PC spectrofluorophotometer (Shimadzu, USA). Excitation was carried out at 405 nm and emission spectra were recorded between 450 and 700 nm.

4.4.2 Results

The transmission of light through the suspending media can be seen in Figure 4.6a. The greatest transmission of 405 nm light occurred through minimal media (DPBS) with 99.9% transmission. Least transmission occurred in blood plasma (2.1%) and artificial faeces (0.05%). Transmission of 405 nm light through DMEM and artificial saliva was similar, between 30-41%, with transmission through DMEM reduced from 40.9% to 30.6% when the FBS additive was present.

The absorbance measurements of the suspending media were also recorded (Fig 4.6b). Maximum absorbance of 405 nm light was by blood plasma and artificial faeces (1.8 and 3.3 a.u, respectively). Again there were differences in the measurements of DMEM with and without FBS, with absorbance of 405 nm light greater in 10% FBS-DMEM (0.5 a.u) compared to DMEM (0.4 a.u). Minimal absorbance of light occurred in DPBS (0.0003 a.u).

The fluorescence emission spectra of all the suspending media can be seen in Figure 4.7. When excited at 405 nm, emission peaks for organically-rich media were seen between 518-520 nm. Additionally, the excited biologically-relevant media had emission peaks at 461, 510 and 519 nm for artificial saliva, blood plasma and artificial faeces, respectively.

Selected components of artificial saliva and faeces were then individually excited at 405 nm. Constituents of artificial saliva and artificial faeces were suspended in distilled water in the concentrations described in Table 3.4, with the exception of the yeast which underwent a 10-fold dilution as the neat solution was very opaque. As can be seen in Figure 4.8 small peaks appear around 465-470 nm for both pig gastric mucin and α-amylase, both of which are components of artificial saliva. With regards to artificial faeces, small peaks were found at 671 nm for psyllium (Fig 4.9a) and 478 nm and 520 nm for miso and yeast, respectively (Fig 4.9b).

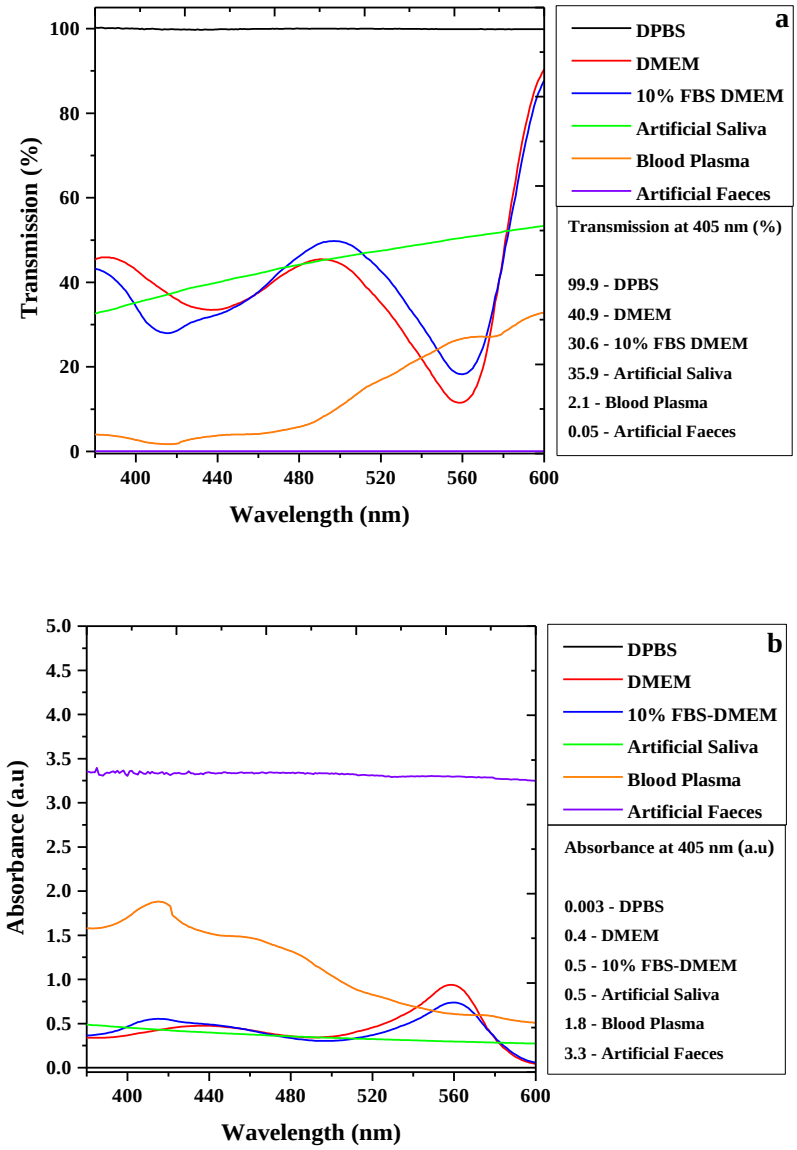


Figure 4.6 Light transmission and absorbance through different suspending media. Illustrating: (a) light transmission and (b) light absorbance, through minimal medium (Dulbecco's phosphate buffered saline (DPBS)) organically-rich media (Dulbecco's Modified Eagle medium (DMEM), 10% fetal bovine serum supplemented DMEM (10% FBS-DMEM)) and biologically-relevant media (artificial saliva, artificial faeces and blood plasma).

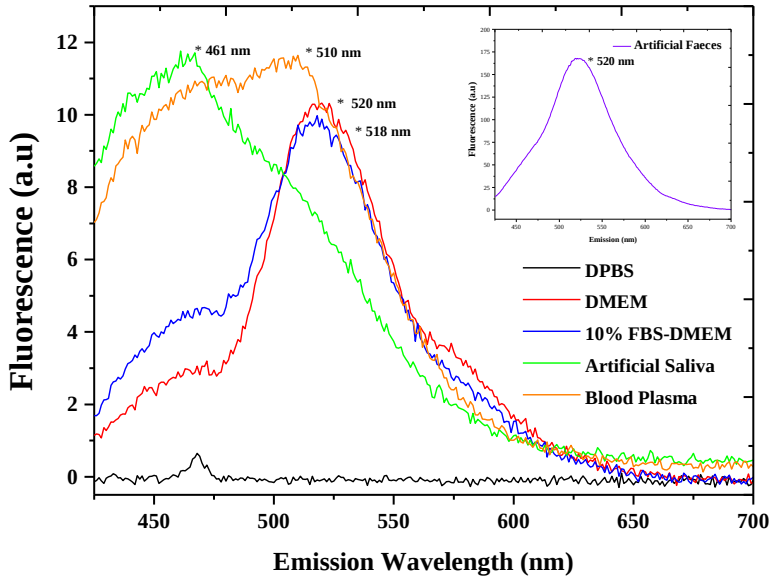


Figure 4.7 Fluorescence emission spectra of suspending media used for feline calicivirus exposure, using an excitation wavelength of 405 nm. Media included minimal medium (Dulbecco's phosphate buffered saline (DPBS)), organically-rich media (Dulbecco's Modified Eagle medium (DMEM), 10% fetal bovine serum supplemented DMEM (10% FBS-DMEM)) and biologically-relevant media (artificial saliva, artificial faeces and plasma). Asterisks '*' indicate peak emission wavelengths.

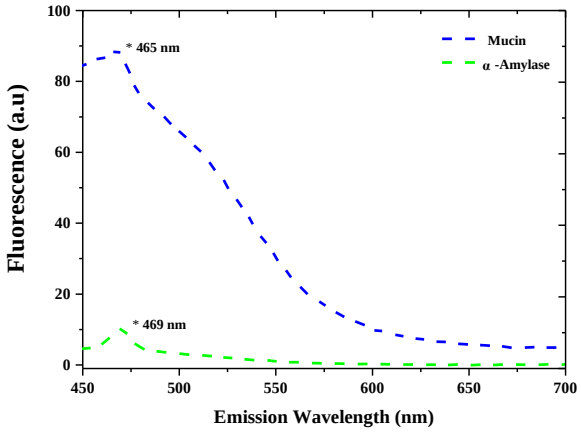


Figure 4.8 Fluorescence spectra of the main components of artificial saliva (pig gastric mucin and α-amylase), using an excitation wavelength of 405 nm. Asterisks '*' indicate peak emission wavelengths.

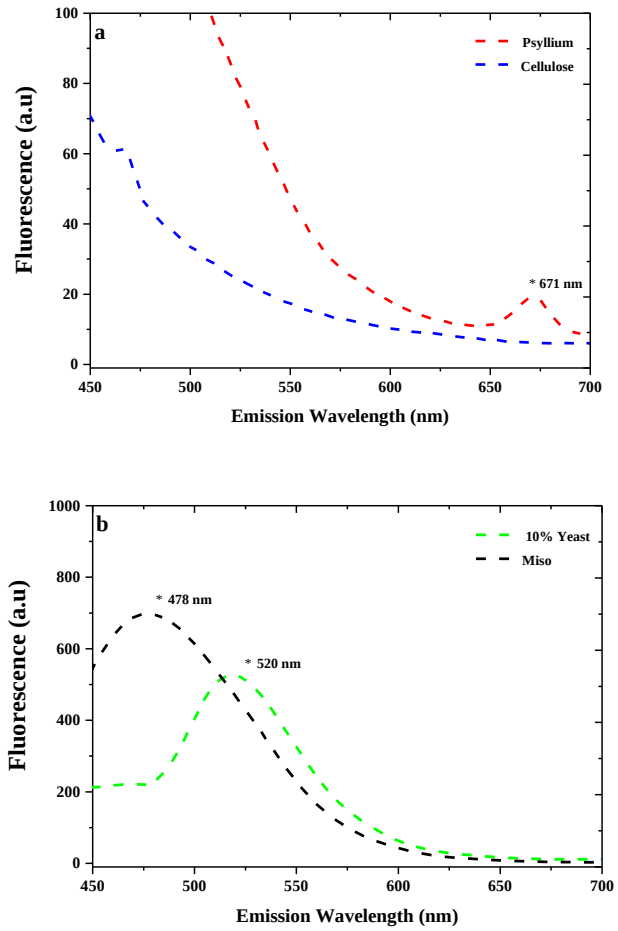


Figure 4.9 Fluorescence spectra of the main components of artificial faeces, using an excitation wavelength of 405 nm. Components included (a) psyllium and cellulose and (b) 10% yeast and miso. Asterisks '*' indicate peak emission wavelengths.

4.5 Discussion

This study was designed to investigate the effect of 405 nm light on the clinically important virus, NoV, which is highly contagious and one of the leading causes of acute gastroenteritis (Atmar and Estes 2006; Cromeans *et al*., 2014). Currently, the efficacy of decontamination technologies cannot be tested against NoV. This is because there is no established cell culture method, and recent attempts using 3D organoid models of intestinal epithelial cells have had mixed success (Duzier *et al*., 2004a; Straub *et al*., 2011; Papafragkou *et al*., 2013; Takanishi *et al*., 2014). Detection of NoV using reverse transcription-polymerase chain reaction (RT-PCR) can also be unreliable. As only a small region of the RNA genome is amplified from the virus, this method cannot distinguish the infectivity of viruses and may

underestimate the efficacy of decontamination treatments (Duizer *et al*., 2004a; Diez-Valcarce *et al*., 2011; Park *et al.*, 2011; Cromeans *et al*., 2014**)**. Current studies are underway to tackle these problems, including the study of *in-situ* capture quantitative RT-PCR developed to differentiate between active and inactive viruses; and investigating B-cells and stem cell–derived human enteroids for NoV replication (Jones *et al*., 2014; Wang and Tian 2014; Ettayebi *et al*., 2016). However, these methods are yet to be standardised.

To overcome this lack of a NoV cell culture model, this study used FCV as a surrogate mammalian virus, which similarly belongs to the *Caliciviridae* family (Siebenga *et al.*, 2010). FCV and NoV share physiochemical and genomic similarities with similar genomic size and organisation (7.5 kb genome, 3 Open Reading Frames) and similar non-enveloped structures containing 180 capsid proteins arranged into 90 dimers (Jiang *et al*., 1993; Slomka *et al*., 1998; Vasikova *et al*., 2010; Hoelzer *et al*., 2013; Kneil *et al*., 2014). The use of FCV as a surrogate for NoV is well-established and has a standardised cell-culture protocol (Doultree *et al*., 1999; Bidawid *et al*., 2003; Duizer *et al*., 2004b; Kampf e*t al*., 2005; Lee *et al*., 2007; Chander *et al*., 2012). In this study, FCV was used to infect feline embryonic cells (strain FEA) to form countable plaques indicating virus inactivation which allowed for quantification (Jarrett *et al*., 1973; Omerod and Jarrett 1978).

Similar studies investigating the virucidal effects of other decontamination technologies such as UV light, ozone, HPV and CAPP have also used FCV as a NoV surrogate (Nuswalen *et al*., 2002; Hudson *et al*., 2007; Bentley *et al*., 2012; Aboubakr *et al*., 2015; Holmdahl *et al*., 2016). However, there has been a recent move towards using Murine Norovirus (MNV) and Tulane Virus (TuV), alongside FCV, as NoV surrogates (Cromeans *et al*., 2014; Kniel 2014; Chiu *et al*., 2015; Esseili *et al*., 2015; Zonta *et al*., 2016). Other viruses such as Porcine Enteric Calicivirus (PEC), Canine Calicivirus (CCV), Hepatitis A Virus (HAV) and MS2 coliphage have also been used in some studies as alternative human enteric viruses and NoV surrogates (Duizer *et al*., 2004b; D'Souza and Su 2010: Wang *et al.*, 2013b: Cromeans *et al*., 2014). In addition, some studies have used Virus Like Particles (VLPs) and RT-PCR to investigate NoV inactivation, however this can be inaccurate as previously discussed (Park *et al*., 2010; Sato *et al*., 2016).

When comparisons are made between FCV and these alternative NoV surrogates there are differences in susceptibility to varying physiochemical and environmental factors such as alcohol, pH level and temperature. FCV is thought to be more sensitive to low pH levels than MNV, TuV and PEC but more resistant than CCV (Duizer *et al*., 2004; Cannon *et al*., 2006; Cromeans *et al*., 2014). With regards to alcohol resistance, FCV and CCV are more resistant

to 70% and 90% ethanol than MNV and PEC, however, TuV has been shown to be more resistant than FCV (Duzier *et al*., 2004; Cannon *et al*., 2006; Sattar *et al*., 2011; Cromeans *et al*., 2014). Cannon *et al*. (2006) demonstrated FCV was more stable than MNV when at an elevated temperature of 56 °C but displayed similar inactivation levels at higher temperatures of 63 °C and 72 °C. Conversely, in a more recent study by Cromeans *et al.* (2014) raising the temperature to 56 °C for 20 minutes resulted in similar 5 $\log_{10}$ inactivation of FCV, MNV and TuV with only a 2 $\log_{10}$ reduction of PEC.

However, when comparing FCV's susceptibility to decontamination technologies such as UV light, HPV, ozone and photodynamic inactivation (PDI) against other surrogates, little difference is seen (Nims and Plasvic 2013). In the case of UVC irradiation, similar doses of 254 nm light, 29 mJ/cm^2 and 25 mJ/cm^2, were required for 4 $\log_{10}$ inactivation of MNV and FCV respectively (Park *et al*., 2011). Additionally, there is also very little difference in irradiation inactivation between FCV and CCV with a 99.9% reduction after a dose of 34 mJ/cm^2 UVB radiation (280-320 nm) (Duizer *et al*., 2004; Nims and Plasvic 2013). When exposed on surface coupons, there was a > 4 $\log_{10}$ reduction of MNV and FCV after exposure to 12% hydrogen peroxide gas for one hour or 30% hydrogen peroxide vapour for 20 minutes, respectively. (Tulhadar *et al*., 2012; Bentley *et al*., 2012). Additionally, although difficult to compare due to different experimental arrangements, using ozone there was ≥ 3 $\log_{10}$ reduction of FCV after 20 minutes using ~20-25 mg/l and a 2-3 $\log_{10}$ reduction of MNV following treatment using 1 mg/l ozone for 2 minutes (Hudson *et al*., 2007; Lim *et al*., 2010). Furthermore, oxidative damage caused by a 60 min exposure to fluorinated titanium dioxide and 10 μW/cm^2 fluorescent light resulted in 1.7 $\log_{10}$, 2.6 $\log_{10}$ and 2.6 $\log_{10}$ reduction of MS2, FCV and MNV respectively (Cho *et al*., 2010).

Therefore, it is not unreasonable to hypothesise that the antiviral efficacy of 405 nm light against FCV may be commensurate to other NoV surrogates and small non-enveloped viruses. As there is no scientific agreement on the use of a single surrogate for NoV, regardless of the surrogate used, decontamination studies may under or overestimate the antiviral efficacy. Therefore, using FCV as a NoV surrogate was thought to be the best choice due to ease of use; reproducibility of results; and similarity in inactivation to other surrogates with regards to novel decontamination technologies. Using FCV would provide the initial proof-of-concept of the virucidal efficacy of 405 nm light, with ≥ 4 $\log_{10}$ reduction in the number of viable viruses, demonstrating virucidal efficacy (Steinmann 2004; Tulhdar *et al*., 2012).

Violet-blue light between 400-420 nm has been used for microbial inactivation, with 405 nm thought to have the optimal antimicrobial efficacy. Due to the lack of endogenous porphyrins within virions, however, inactivation by 405 nm light has been thought unlikely (Hamblin *et al*., 2005; Guffey and Wilborn 2006; Enwemeka *et al*., 2008; Maclean *et al*., 2008b, 2009, 2013b). However, a recent study by Tomb *et al*. (2014) indicated that a bacteriophage, used as a surrogate for mammalian viruses, could be inactivated by 405 nm light. The study demonstrated that, following a dose of 306.2 J/cm^2, a 2.7 $\log_{10}$ reduction of the bacteriophage could be achieved when in nutrient-rich media. However, upon exposure to 405 nm light when in minimal media, the bacteriophage was still able to infect the bacterial host, but a small yet significant 0.3 $\log_{10}$ inactivation was observed after the same dose of 306.2 J/cm^2 (Tomb *et al*., 2014).

To ascertain whether the same effect could occur in a virus, the virucidal efficacy of 405 nm light was determined using FCV suspended in both a minimal medium and organically-rich media. Exposure in a minimal medium would provide a better indication of the interaction of 405 nm light with the virus alone, whilst suspension in organically-rich media, which is likely to contain photosensitive components, would assess how viral susceptibility to the light can potentially be influenced by the surrounding media.

Successful FCV inactivation was achieved when suspended in a minimal medium, although the dose required was substantial, with 2.8 kJ/cm^2 giving a 3.9 $\log_{10}$ reduction (Fig 4.2). In the case of bacteria and fungi in minimal medium, doses in the range of 18-576 J/cm^2 are typically required for 5 $\log_{10}$ inactivation (Maclean *et al.*, 2009; Murdoch *et al*., 2012, 2013). The increased susceptibility of bacteria and fungi compared to viruses is accredited to the presence of endogenous photosensitive porphyrins within these cells (Hamblin and Hasan 2004; Maclean *et al*., 2008b; Murdoch *et al.*, 2013). Low sensitivity of FCV in minimal medium was anticipated due to the absence of porphyrins in the viral structure, together with the fact that minimal medium does not contain any photosensitive substances which absorb light at 405 nm (Fig 4.7), suggesting that viral inactivation, in this case, is due to a differing mechanism.

An alternative mechanism of inactivation when FCV is suspended in minimal medium may be associated with the LED emission spectrum extending slightly into the UVA region (Fig 3.5), meaning the virus is exposed to very low-level UVA photons (~390 nm). Over an extended period, this could cause oxidative damage to proteins (Girard *et al*., 2011; Pattison *et al*., 2012), for example, to the viral capsid, and therefore contribute to the observed inactivation. Another possibility is that the small amount of 420-430 nm light

emitted from the source may contribute to viral inactivation. Antiviral effects of 420-430 nm have been demonstrated against murine leukaemia virus, with long exposures thought to cause photo-damage to the virion-associated reverse transcription complex (Richardson and Porter 2005). Although this virus differs in structure to FCV, these findings suggest that prolonged exposure to wavelengths at the tail ends of the 405 nm LED emission spectrum such as 390 and 420 nm, as well as 405 nm, may affect the viruses' ability to infect and replicate in host cells, and have a role in the inactivation of FCV by the LEDs used in this study.

To investigate whether exposure in organically-rich media had any effect on viral susceptibility, FCV was first suspended in DMEM with and without 10% FBS, thought to aid protection against ROS (Grzelak *et al*., 2001). Results (Fig 4.3) demonstrated near complete reduction in infectivity of a 10^5 PFU/ml population after a dose of 421 J/cm^2. As can be seen in Figure 4.3 slightly greater inactivation occurred when FCV was suspended in DMEM without the FBS serum additive, however, no significant difference was seen between the inactivation kinetics. As the inactivation dose of 421 J/cm^2 is 85% less than that required for a similar level of inactivation in the minimal medium, it is likely that components of the organically-rich media are influencing FCV inactivation. The aforementioned study by Tomb *et al*. (2014), which investigated the susceptibility of bacteriophage ϕC31, demonstrated similar results to those of the current study. Little inactivation was observed when exposed in a simple salt solution, however susceptibility was significantly enhanced when suspended in a nutrient-rich medium, with a 5.4 $\log_{10}$ reduction of ϕC31 achieved after exposure to 510 J/cm^2. This was hypothesised to be due to the complex protein and amino acid rich composition of the nutrient-rich medium, suggesting some components could be photosensitive and when exposed to 405 nm light in the presence of oxygen would produce ROS and other toxic photoproducts which damage the bacteriophage (Tomb *et al*., 2014). This same phenomenon is likely to account for the enhanced inactivation of FCV when in DMEM and 10% FBS-DMEM, as these contain a complex mixture of amino acids, vitamins and sugar, which have the potential to absorb 405 nm light (Fig 4.7) and act as photosensitizers.

The photosensitisation of components of DMEM upon exposure to light is known, with riboflavin shown to produce ROS which is further enhanced by tryptophan, tyrosine, pyridoxine and folic acid (Grzelak *et al*., 2001). Riboflavin is known to be photosensitive in the presence of visible light and oxygen, and can produce singlet oxygen and superoxide anions by an energy transfer from the photo-excited riboflavin to molecular oxygen

(Joshi 1985). Furthermore, violet-blue light wavelengths are thought to be the most efficient for the photo-decomposition of riboflavin and the generation of ROS (Cheng *et al.*, 2015). To investigate this, riboflavin was added to DPBS with and without tyrosine, tryptophan, pyridoxine and folic acid in the same concentrations found in DMEM (Table 4.1). Results support this, with only 1.3 $\log_{10}$ reduction when only riboflavin was present. However, when all vitamins and amino acids were present, enhanced inactivation of FCV was achieved with complete inactivation of a 10^5 PFU/ml population.

It is possible that ROS such as hydrogen peroxide, superoxide, hydroxyl radicals and/or singlet oxygen are generated from the organically-rich media upon 405 nm light illumination. These ROS could cause damage to several different sections of the viral structure such as the capsid, the core proteins, as well as the nucleic acid, which could lead to viral inactivation. Attempts were made during this study to identify the oxidative damage which had occurred in exposed FCV virions, using transmission electron microscopy (TEM). The objective was to observe and identify any capsid damage which had occurred and investigate if there were differences in structural damage when FCV was exposed to 405 nm light suspended in minimal or organically-rich media. Unfortunately attempts to visualize the virus were unsuccessful, but future work to identify damage, will be discussed in Section 8.2.2.

Upon review of studies using decontamination technologies to inactivate NoV surrogates, it is possible to hypothesise that damage is likely to have occurred to the viral capsid, when FCV was exposed to 405 nm light suspended in ROS-generating media. Studies investigating oxidative damage to NoV surrogates have used several techniques to visualize viral damage including TEM, matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and OxyELISAs^{TM}. (Sigstam *et al*., 2013; Park *et al*., 2014; Shionoiri *et al*., 2015; Predmore *et al*., 2015; Bekele *et al*., 2016).

Several of the aforementioned techniques were utilised in a recent study by Shionoiri *et al.* (2015), investigating damage to FCV caused by electrochemical treatment. Firstly, SDS-PAGE performed to identify protein modification, demonstrated a smeared band representing the major viral capsid protein VP1 (62 kDa), indicating damaged capsid proteins (Shionoiri *et al*., 2015). However, as it was not fragmented or degraded, it indicated oxidation was attributable to the electrochemical viral inactivation. MALDI-TOF analysis was also carried out and identified capsid peptides in the terminal arm and shell of the major protein complex (VP1) which had been altered due to oxidisation (Shionoiri *et al*., 2015).

Additionally, TEM used to visualise viral particles and identify morphological changes demonstrated that the viral numbers did not change after electrochemical treatment but the viral particles were damaged and the 32 cup-like hollows of the of VP1 capsid were no longer as visibly clear (Shionoiri *et al*., 2015). Therefore, this study demonstrated several techniques and several potential alterations of the viral capsid which are altered due to oxidative damage.

Further, in a study by Park *et al*. (2014) an OxyELISA^{TM} was carried out to identify the oxidative damage occurring to viral capsid proteins after exposure to light-activated fluorinated titanium. This technique demonstrated an increase in oxidised protein concentration of MS2 and G1.1 NoV capsid proteins, after treatment, increasing from 5% to 61% and 69% for MS2 and G1.1 NoV respectively. The results in this study indicated that ROS such as singlet oxygen and hydroxyl radicals generated from the light-activated fluorinated titanium had targeted the viral capsid proteins and oxidised them. Additionally, RT-PCR assays were also carried out before and after treatment, which demonstrated that there was no reduction in viral RNA, suggesting inactivation was not due to RNA damage (Park *et al*., 2014).

The aforementioned methods, TEM, SDS-PAGE and RT-PCR, were also used to identify damage to MNV and TuV after ozone treatment. The results demonstrated by Predmore *et al*. (2015) indicated that inactivated viruses had damaged structures and capsid proteins whilst the RNA remained intact, further supporting the hypothesis that oxidative damage alters the viral capsid and does not affect the viral RNA.

It is therefore likely that any damage occurring to FCV during exposure to 405 nm light in organically-rich media is oxidative damage to the viral capsid proteins. When FCV is illuminated with 405 nm light it is likely that the photo-sensitive chromophores within the suspending medium become excited and release harmful ROS. These ROS may cause oxidative damage to the fundamental structures of the viral capsid complex, namely the major and minor capsid proteins (VP1 and VP2 respectively), with the VP1 protein responsible for viral attachment and host cell entry (Bhella *et al*., 2008). Therefore, as FCV is no longer able to infect FEA cells after 405 nm light exposure, the VP1 protein may be damaged or altered, preventing FCV from recognising host cellular receptors and initiating their viral replication cycle. In terms of NoV inactivation, if 405 nm light were to have the same efficacy and mechanism as that against FCV, then perhaps the capsid proteins would also be damaged. This would prevent the protruding (P) domain of the VP1 protein from

recognising receptors on host cell surfaces such as the histo blood group antigens and prevent NoV infection from occurring (Glass *et al.,* 2009; Thorne and Goodfellow 2014).

As this study is focussed on the applied use of 405 nm light for decontamination, it was important to consider how light-induced inactivation would be influenced when viral particles were suspended in more biologically-relevant, naturally occurring matrices such as body fluids or secretions. The three different biologically-relevant media used were artificial saliva, artificial faeces and human blood plasma. There were several benefits of exposing FCV in these biologically relevant media. Firstly, artificial saliva and artificial faeces could be readily prepared and, once the correct formulations were established, these media were much easier to collect and safer to work with than the equivalent human samples. Secondly, if the inactivation of FCV in artificial faeces was successful this would be an indicator of the potential efficacy of 405 nm light against NoV. Finally, establishing viral inactivation kinetics, using FCV as a model virus, in artificial saliva and blood plasma would indicate the potential for inactivation of other viruses which are likely to be found suspended in, and surrounded by, these biological fluids in the acute setting (Aitken and Jeffries 2001).

Results demonstrated that, similarly to inactivation in organically-rich media (DMEM and 10% FBS-DMEM), viral susceptibility was significantly increased when suspended in these biologically-relevant fluids. Of the three model fluids used, sensitivity was highest when suspended in artificial saliva (Fig 4.4a), with a 5.1 $\log_{10}$ reduction of FCV infectivity achieved after a dose of 421 J/cm^2 – the same as that observed when in organically-rich media. Susceptibility was slightly reduced when suspended in blood plasma (4.8 $\log_{10}$ inactivation with 561 J/cm^2), and further reduced when in artificial faeces, with more than three times the dose required to achieve a 4.5 $\log_{10}$ reduction (Fig 4.4b & 4.4c).

When inactivation profiles were compared in Figure 4.5, there is a clear clustering effect between the different suspending media. To achieve a 4 $\log_{10}$ reduction of FCV, there are three distinct inactivation clusters: (i) inactivation in nutrient rich media, artificial saliva and blood plasma requiring doses of ~400-500 J/cm^2; (ii) inactivation in artificial faeces requiring doses of ~1 kJ/cm^2; and (iii) inactivation in minimal media requiring doses of ~3 kJ/cm^2. It is likely that inactivation of FCV in blood plasma, in cluster 1, requires slightly higher doses of ~500 J/cm^2 due to an average 2.12% transmission of 405 nm light through this media. Additionally, the reduced levels of 405 nm light transmission through artificial faeces contributes to the slower inactivation rate, seen in cluster 2, with 0.05% transmission of 405 nm light recorded, compared to 30-40% transmission in all other organically rich media used (Fig 4.6). Interestingly, similar levels of transmission (30-40%) and absorbance

(0.4-0.5 a.u.) of 405 nm light were evidenced between artificial saliva and organically-rich media (Fig 4.6) which may have influenced the similar dose requirement for FCV inactivation, when in these particular media. Inactivation requires much higher doses in cluster 3 when FCV is suspended in minimal media, likely due to differing inactivation mechanism as discussed previously.

The linear inactivation of FCV in blood plasma is a particularly important and novel result in this study. This result indicates the potential for the use of 405 nm light for viral decontamination of blood and blood products, with common viral contaminants including Hepatitis Viruses (HBV, HCV), Human Immunodeficiency Viruses (HIV-1 & HIV-2), Human T-cell Lymphotrophic Virus and Cytomegaloviruses (Bihl *et al.,* 2007). Caliciviruses (unspecified strains) have been shown to be inactivated in blood plasma previously, with a 4 $\log_{10}$ reduction achieved using methylene blue as a photosensitizer and visible light (Mohr *et al.,* 1995). More recent advances in plasma decontamination generally use a riboflavin additive, which is illuminated with 313 nm UV light to photo-dynamically inactivate viral pathogens (Goodrich *et al*., 2006; Marschner *et al*., 2011). However, 405 nm light has been shown to have potential for blood decontamination without the need for photosensitizers. This has been established by Maclean *et al*. (2016), who demonstrated the inactivation of key bacterial pathogens: *S. aureus* and *S. epidermidis* in human blood plasma. Using 405 nm light at an irradiance of between 5-100 mW/cm^2, results demonstrated successful inactivation of these pathogens in 3 ml plasma samples, with doses between 270-360 mW/cm^2 required for 5 $\log_{10}$ reduction (Maclean *et al,* 2016). Not only was inactivation achieved in directly exposed plasma but also in plasma contained within sealed transfusion bags. The small-scale tests, similar to those used in the present study, demonstrated similar inactivation kinetics for bacteria as found with FCV (360 vs 421 J/cm^2 respectively). Therefore, with the bactericidal and viricidal efficacy of 405 nm light on blood plasma, this technology could be developed for blood decontamination and would overcome many of the current problems using PDI. This would eliminate the requirement for additional photosensitizers and detergents for treatment, which can be toxic if not completely removed from blood products (AuBuchon *et al*., 2011; Tsen *et al*., 2014).

The successful inactivation of FCV in artificial saliva and artificial faeces is also an exciting result in terms of the potential virucidal efficacy of 405 nm light. Inactivation of viruses in saliva is of particular importance with regards to viruses which are transmitted in the hospital environment via droplets such as respiratory syncytial virus and Influenza

(Weedon *et al*., 2013). The ability of 405 nm light to inactivate FCV in artificial faeces is also particularly significant in terms of NoV, which is transmitted via the faecal-oral route.

The artificial faeces used in these experiments contained inactivated *Saccharomyces cerevisiae*, oleic acid, psyllium powder, miso paste and cellulose, chosen to represent microbial debris, fats, fibre, nitrogen and carbohydrates, respectively. The proportions in which these components are present in human faeces will differ as there will be an array of fats, carbohydrates and proteins dependent on food intake. The water content will also be influenced by food intake and the state of hydration of the relevant individual (Arnaud 2003), as well as the presence of minerals such as sodium and potassium (Nishimuta *et al*., 2006). Additionally, the composition of the gut microbiome will influence the consistency of human faecal matter (Vandeputte *et al*., 2016). Therefore, the adapted artificial faeces used in this study can never truly replicate naturally produced faeces, however are still a good indicator of the likelihood of NoV inactivation in faeces.

It is interesting to note that human faecal matter will also contain bilirubin, a photosensitive breakdown product of blood plasma (McDonagh 2008). This may act as a photosensitizer when in contact with 405 nm light, enhancing inactivation levels seen over and above that in this study. Additionally, as the artificial faeces formula used in this study was very turbid (0.05% transmission) it may be that lower doses would be required in the environment if NoV particles are released in diluted faecal matter, for example if faecal aerosols are formed after flushing the toilet. Other hospital acquired viruses such as Hepatitis A, Hepatitis E, Adenovirus and Rotavirus can also be transmitted via the faecal-oral route (Lemon 1997; Nuanualsuwan *et al*., 2002; Gleizes *et al.,* 2006; Rutala *et al*., 2006; Kumar *et al*., 2013). Therefore, there may be potential for 405 nm light to have virucidal efficacy against these other infectious viruses which are transmitted in respiratory secretions and faecal matter.

To further investigate the reaction of the minimal, organically-rich and biologically-relevant media after exposure to narrow-spectrum 405 nm light, fluorescence spectrophotometry was used to record any fluorescent output from the media (Fig 4.7). Excitation of the minimal medium displayed no emission peaks, which ratifies the previously discussed findings that large doses of 405 nm light are required for FCV in DPBS. The lack of peaks in the emission spectrum indicates that there are no components in DPBS acting as exogenous photosensitizers, and the inactivation seen is potentially due to low-levels of near UVA or 420-430 nm light.

Emission peaks were found between 517-520 nm for the organically-rich media, and 460 nm, 510 nm, 519 nm for artificial saliva, blood plasma and artificial faeces respectively. As viruses do not contain porphyrins, and the fact that the range of organically-rich and biologically-relevant suspending media used contained components that were excited by 405 nm light, the results indicate that FCV inactivation using 405 nm light was enhanced by the photosensitisation of suspending media, with 50-85% lower doses required for similar levels of viral inactivation.

These results indicate the potential for NoV susceptibility to 405 nm light to be enhanced when in organically-rich media, or host secretions in which they are released such as faeces, blood and vomit. Although the consistency and transparency/opacity may differ to those used in this study, these fluids are likely to be rich in molecules which could potentially be sensitive to 405 nm light, thereby aiding in NoV inactivation.

In all scenarios when FCV was exposed to 405 nm light in minimal, organically-rich or biologically-relevant media, there was ≥ 4 $\log_{10}$ inactivation achieved (Fig 4.5), using doses of 421-2.8 kJ/cm^2, demonstrating the virucidal efficacy of violet-blue light (Steinmann 2004; Tulhdar *et al*., 2012). Therefore, this study provides the first proof-of-concept evidence demonstrating that the antimicrobial efficacy of 405 nm light can be extended to medically important viruses, with susceptibility significantly enhanced when the viral particles are contained within biologically-relevant media, representing how viruses are found in the environment. The high-irradiances used are indicative of 405 nm light applications in contained areas not in direct contact with patients and staff. This could include decontamination of clinical equipment and long-term storage of high-risk medical supplies and devices.

Chapter Five

Viral Inactivation using Low-Irradiance 405 nm Light

5.0 Overview

This chapter expands on the proof-of-concept findings of the previous chapter, and looks to establish the antiviral efficacy of low-irradiance 405 nm light, using the HINS-light EDS. This novel decontamination system emits low-irradiance 405 nm light and can be integrated into hospitals alongside normal lighting, to help reduce the level of environmental contamination (Maclean *et al*., 2010, 2013a; Bache *et al*., 2012). This chapter investigates the virucidal efficacy of the EDS, firstly using bacteriophage and viruses suspended in a range of media, including biologically-relevant liquids (blood, plasma, faeces). Subsequently, the antiviral efficacy of the EDS for decontamination of materials representative of typical contact surfaces within the clinical environment, was assessed.

5.1 Introduction

As discussed in Chapter 2, NoV is easily transmissible and can be spread via multiple routes including food and water, person to person contact and environmental surfaces (Glass *et al*., 2009). Surfaces and fomites play a particularly important role in NoV transmission, with surfaces linked to repeat outbreaks as a result of inadequate cleaning (Jones *et al*., 2007). As NoV outbreaks have become regular occurrences in health-care settings, health boards require more advanced cleaning techniques to operate alongside normal cleaning regimes, to prevent further transmission (Nerandzic *et al*., 2010; Otter *et al*., 2013).

New technologies which have been developed to aid environmental decontamination include UV light, HPV, ozone and CAPP. These technologies have faster bactericidal activity than the HINS-light EDS, however due to their operating requirements, they are more suited to terminal clean activities. The HINS-light EDS on the other hand can be employed for continuous environmental decontamination in the presence of patients and staff. Although there is published evidence of the antibacterial efficacy of the HINS-light EDS within

laboratory and clinical environments (Maclean *et al*., 2009, 2010, 2013 a, b; Murdoch *et al*., 2012, 2013), there is currently no available data quantifying antiviral efficacy.

As can be seen in Chapter 4, high-intensity 405 nm light is able to inactivate the NoV surrogate, FCV, with an increased level of inactivation seen when the viral particles are suspended in photosensitive media. This chapter was designed to investigate the virucidal efficacy of low-intensity 405 nm light from the EDS, with exposure times far longer than with high-irradiance sources. Exposures were carried out over a number of days rather than hours, which is more similar to practical application of the EDS being used continuously to support decontamination. The study is divided into three main sections:

- Inactivation of a viral surrogate, bacteriophage ϕC31.
- Inactivation of FCV, when suspended in minimal, organically-rich and biologically-relevant media.
- Inactivation of FCV contamination on surfaces relevant to the hospital environment.

5.2 Inactivation of a Viral Surrogate using Low-Irradiance 405 nm Light

The first stage of experiments used a viral surrogate, bacteriophage ϕC31, to investigate the antiviral efficacy of low-irradiance light. In a previous study by Tomb *et al*. (2014) the bacteriophage was shown to be sensitive to high-intensity 405 nm light, therefore this organism was selected to allow initial confirmation of the virucidal effect of low-irradiance light, and to allow calculation of doses required for subsequent FCV exposure experiments.

5.2.1 Experimental Methodology

The light source used for exposure of bacteriophage samples was the prototype HINS-light EDS unit, as detailed in Section 3.5.3. For sample exposure, the EDS was mounted in the ceiling, and the sample plates were positioned directly under the EDS, at a distance of 1.6 m (Fig 5.1a).

For exposure, 3 ml volumes of bacteriophage suspension with a density of 10^5 PFU/ml (prepared as described in Section 3.2.3) were held in 12-well plates on the worktop surface (Fig 5.1b). Bacteriophage were suspended in either PBS (minimal media) or nutrient broth (nutritious growth media) for exposure for 1-14 days. The sample dishes were placed in the

centre of the worktop with their lids kept on to prevent any sample evaporation. The average irradiance at the surface of the samples was approximately 0.5 mW/cm^2 (taking into account a sample depth of 6 mm, and a 4% loss of irradiance through the sample plate lid). The non-exposed control samples were prepared in the same way but exposed to fluorescent laboratory lighting, which measured at 0.02 mW/cm^2 and 60 Lux.

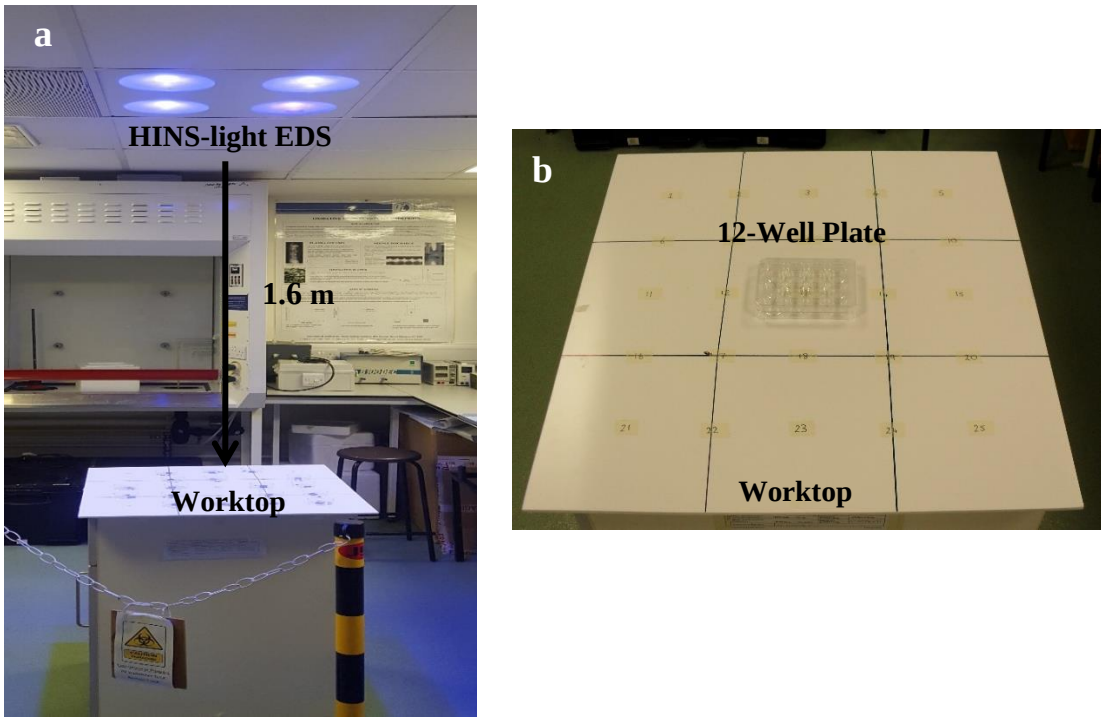


Figure 5.1 Experimental arrangement for inactivation of bacteriophage and virus suspensions using low-irradiance 405 nm light. The light source used was the HINS-light EDS. Illustrating: (a) the HINS-light EDS at a distance of 1.6 m from the sample dishes and (b) location of the 12-well plate on the worktop giving an average irradiance of 0.5 mW/cm^2 at the surface of the 3 ml samples.

5.2.2 Assessment of Surviving Bacteriophage Populations

Post exposure, samples (100, 200 and 500µl volumes) were pipetted onto nutrient agar plates and a soft agar containing 0.1% *S. coelicolor* spores was thinly poured on top. The plates were left to set and then co-incubated overnight at 28°C. Following incubation, the surviving ϕC31 were enumerated by counting the plaque-forming units on the plate (PFU) and counts were converted to PFU/ml.

5.2.3 Results: Bacteriophage Inactivation using Low-Irradiance 405 nm Light

Inactivation of ϕC31 when suspended in PBS is demonstrated in Figure 5.2. Significant inactivation (0.3 $\log_{10}$ reduction; $P = 0.001$) was not observed until exposure to a dose of 173 J/cm^2 (4 days). 1 $\log_{10}$ reduction of ϕC31 was achieved after 14 days of exposure (605 J/cm^2; $P = 0.000$).

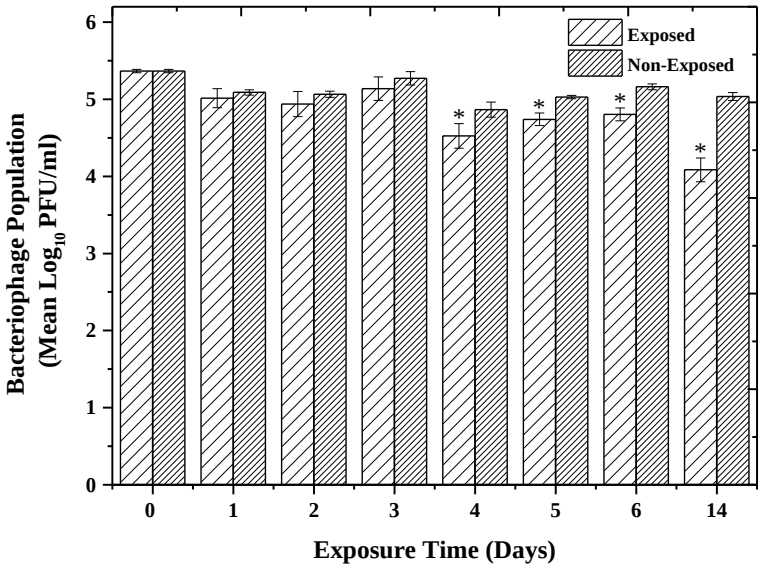


Figure 5.2 Inactivation of bacteriophage ϕC31 suspended in phosphate buffer saline upon exposure to low-irradiance 405 nm light. Samples were exposed to 405 nm light at an irradiance of 0.5 mW/cm^2 using the EDS. Data points show the mean counts (n = 3) ± SD. Asterisks '*' indicate light exposed samples that were significantly different to non-exposed final control samples ($P \leq 0.05$), using one-way ANOVA.

Inactivation of ϕC31 using the EDS was enhanced when the bacteriophage was suspended in NB. There was a significant 0.4 $\log_{10}$ inactivation after 1 day ($P = 0.00$), and following 6 days exposure, 4.8 $\log_{10}$ inactivation was achieved (Fig 5.3)

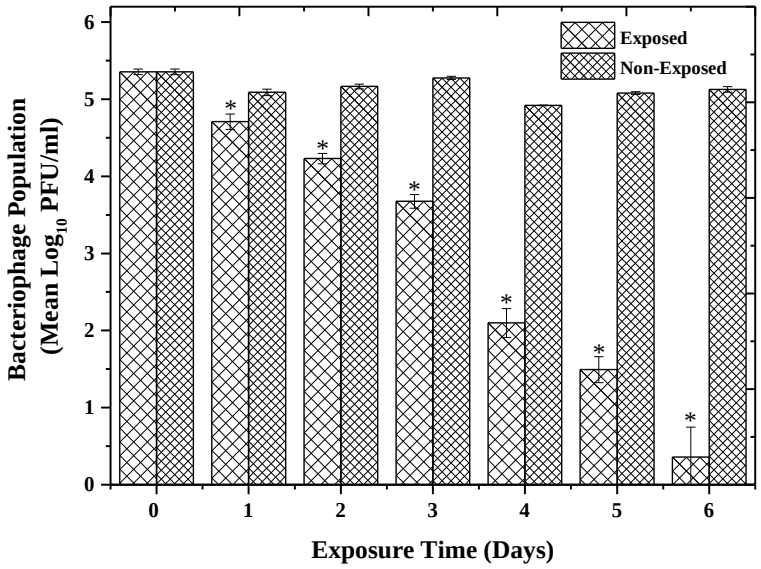


Figure 5.3 Inactivation of bacteriophage ϕC31 when suspended in nutrient broth upon exposure to low-irradiance 405 nm light. Samples were exposed to 405 nm light at an irradiance of 0.5 mW/cm^2 using the EDS. Data points show the mean counts (n = 3) ± SD. Asterisks '*' indicate light exposed samples that were significantly different to non-exposed final control samples ($P \leq 0.05$), using one-way ANOVA.

5.3 Inactivation of FCV in Liquid Suspension using Low-Irradiance 405 nm Light

Following the positive results exposing ϕC31 in liquid suspension to low irradiance 405 nm light from the HINS-light EDS, the NoV surrogate, FCV, was exposed to the EDS in a range of minimal, organically-rich and biologically-relevant media, to investigate if a similar virucidal effect could occur.

5.3.1 Viral Preparation

The viral stock of FCV was prepared as described in Section 3.1.4. When required, aliquots of the viral stocks were defrosted at room temperature and then serially diluted in the suspending media of choice, to achieve the desired starting population of 1-2 × 10^5 PFU/ml.

5.3.2 Cell Plate Preparation

The FEA cells were cultured as described in Section 3.1.2. As the cell culture equipment used in this chapter differed from that of the experiments in Chapter 4, it was found that it was more favourable to decrease the density of cells used to seed the 6-well plates, from 7.5×10^5 cells/well to 6×10^5 cells/well, as the previous seeding population was too high and caused the cells to detach from the 6-well plates.

5.3.3 Suspending Media Preparation

Several types of media (Section 3.4) were used for FCV suspension during exposure to the EDS: minimal media (Dulbecco's phosphate buffered saline), minimal media with organic soiling (DPBS supplemented 5% or 10% fetal bovine serum), organically-rich media (Dulbecco's Modified Eagle medium supplemented with 10% FBS, 240 U/ml penicillin streptomycin, 2mM L-glutamine and 1 mM sodium pyruvate) and biologically-relevant media (human blood plasma, artificial saliva and artificial faeces), as detailed in Section 3.4.3.

5.3.4 Experimental Arrangement

The experimental arrangement for FCV exposure in liquid suspension is as described in Section 5.2.3 and Figure 5.1. 3 ml samples were exposed to 0.5 mW/cm^2 for 7 day (1 week) and 14 day (2 week) periods, and control samples were held under fluorescent laboratory lighting throughout the experiment.

5.3.5 Plaque Assay

Prior to experiments, 6-well cell culture plates were seeded with 6×10^5 FEA cells per well. Plaques assays, cell fixation and staining were then carried out as described in Section 4.2.4. Following which, viral plaques were counted, and the virus infectivity titre expressed as PFU/ml.

5.3.6 Results: Inactivation of FCV in Liquid Suspension using Low-Irradiance 405 nm Light

Results in Figure 5.4 demonstrate FCV inactivation when suspended in DPBS, with and without 5% or 10% FBS, acting as organic soiling. As can be seen in Figure 5.4a there was 2.0 $\log_{10}$ and 3.4 $\log_{10}$ reduction of FCV in DPBS, compared to the starting population, following 7 and 14 day exposures, respectively. However, there was also a natural decrease

in the non-exposed FCV controls, with 0.9 $\log_{10}$ and 2.1 $\log_{10}$ reductions seen after these same exposure periods. Therefore, to take into account this natural loss of viral infectivity, the difference between the exposed sample and non-exposed controls was calculated and used as the value of $\log_{10}$ reduction due to the EDS. The difference in infectivity of FCV is significantly different between exposed and non-exposed suspensions in DPBS, with a 1.1 $\log_{10}$ reduction following a dose of 302 J/cm^2 (7 days; $P = 0.006$) and a 1.3 $\log_{10}$ PFU/ml reduction following a dose of 605 J/cm^2 (14 days; $P = 0.000$).

Similarly, when FCV was suspended in 5% FBS-DPBS there was a significant decrease in viable viruses after 7 and 14 days of exposure (Fig 5.4b), with 1.2 $\log_{10}$ ($P = 0.000$) and 1 $\log_{10}$ ($P = 0.001$) reductions, respectively.

FCV was also suspended in 10% FBS-DPBS to represent a 2-fold increase in organic soiling. The results in Figure 5.4c follow the same pattern as earlier results with 0.5 $\log_{10}$ and 0.8 $\log_{10}$ inactivation achieved after doses of 302 J/cm^2 and 605 J/cm^2, respectively ($P = 0.005$ & 0.001).

A comparison of inactivation of FCV after 14 days of exposure showed that there was a significant reduction in the level of inactivation with each addition of FBS ($P < 0.001$). Only a 1.0 $\log_{10}$ and 0.8 $\log_{10}$ reduction of FCV was achieved in 5% and 10% FBS-DPBS respectively, compared to 1.3 $\log_{10}$ reduction when in DPBS alone. These results indicate that the addition of FBS, acting as an organic soil, has reduced the 405 nm light inactivation of FCV, and prevented some damage to the viral particles.

To investigate if the addition of FBS had reduced light penetration through DPBS, the transmission was measured using a Biomate 5-UV-Visible Spectrophotometer (Thermo Fisher Scientific, UK). As can be seen in Figure 5.5, there was 99.9% transmission in DPBS alone, whereas when supplemented with 5% FBS, transmission was reduced to 84.3% and additionally reduced to 72.1% through 10% FBS-DPBS.

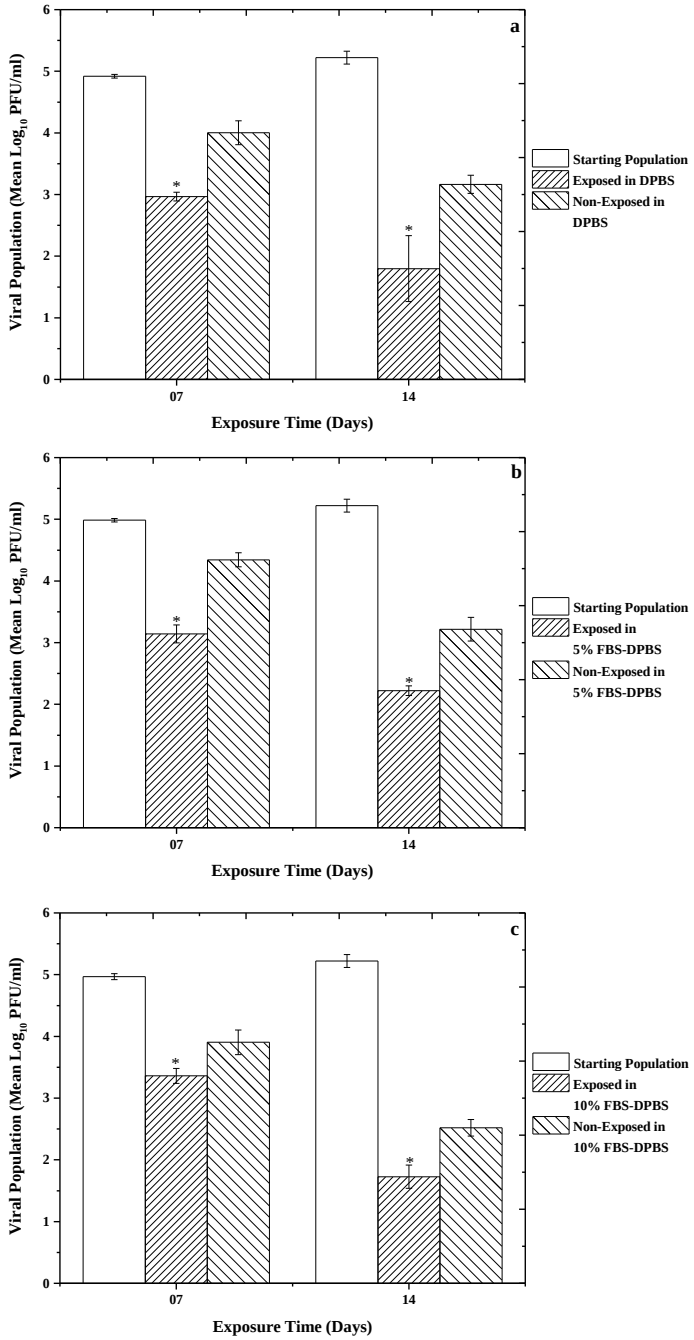


Figure 5.4 Inactivation of feline calicivirus when suspended in Dulbecco's phosphate buffered saline supplemented with 0, 5 or 10% fetal bovine serum (FBS) and exposed to low-irradiance 405 nm light. Samples were exposed to 405 nm light at an irradiance of 0.5 mW/cm^2 using the EDS. Data points show the mean counts (n = 3) ± SD. Asterisks '*' indicate light exposed samples that were significantly different to non-exposed final control samples ($P \leq 0.05$), using one-way ANOVA.

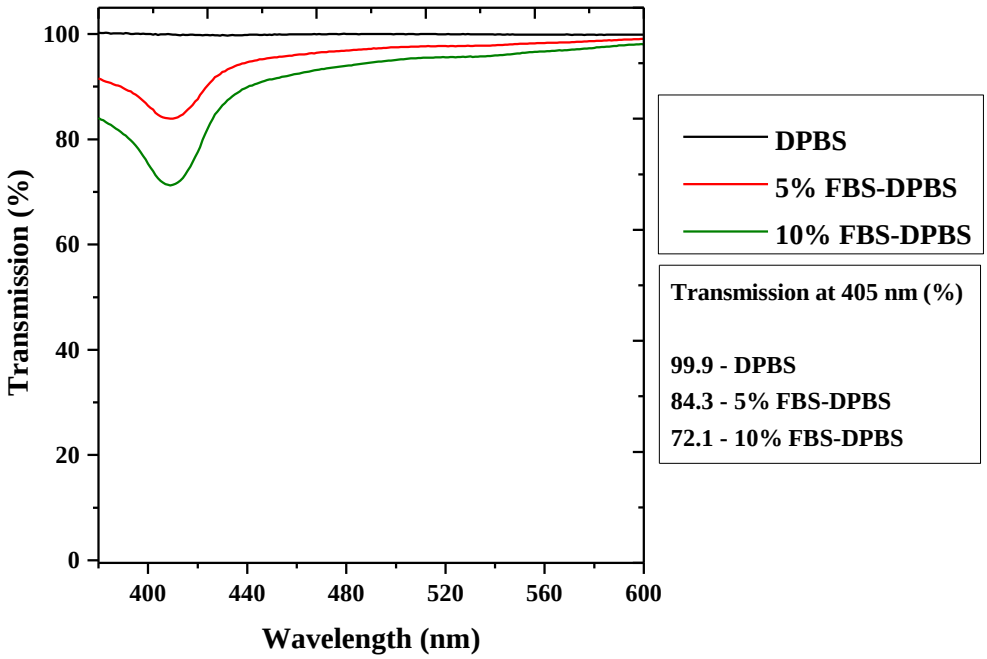


Figure 5.5 Light transmission through minimal medium (Dulbecco's phosphate buffered saline (DPBS)) with and without 5% or 10% fetal bovine serum (FBS) acting as organic soiling.

Following exposure in minimal media, FCV was light-exposed whilst suspended in 10% FBS-DMEM, which has previously been shown to be photosensitive and enhance inactivation (Section 4.2.5). Inactivation of FCV occurs very quickly when exposed to the EDS in 10% FBS-DMEM with a 2.4 $\log_{10}$ reduction after 1 day and complete inactivation after 2 days compared to the starting population (Fig 5.6). Again the controls also decreased during this time and there was a 1.0 $\log_{10}$ and 4.7 $\log_{10}$ reduction in the non-exposed control after 1 and 2 days respectively. Therefore, this equates to a significant 1.4 $\log_{10}$ reduction of FCV after 1 day of exposure and a subsequent 0.2 $\log_{10}$ reduction after 2 days compared to non-exposed controls (P = 0.007 & 0.116, respectively).

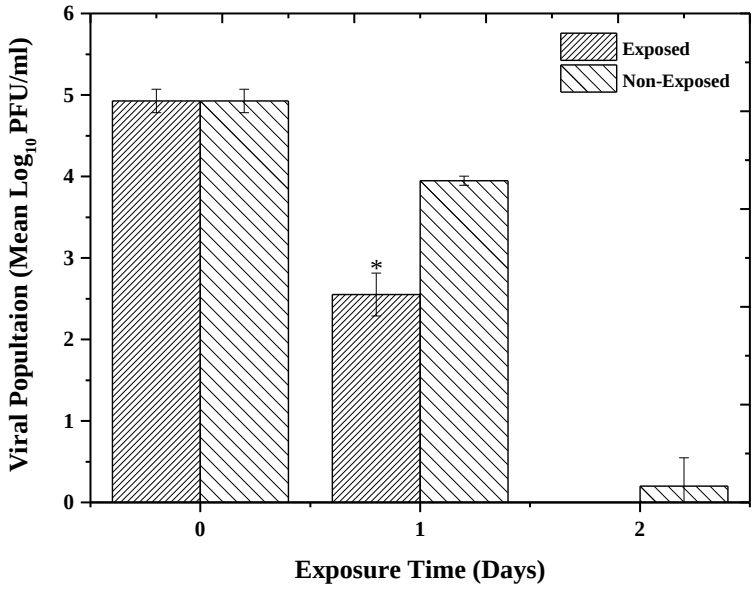


Figure 5.6 Inactivation of feline calicivirus when suspended in 10% fetal bovine serum supplemented Dulbecco's Modified Eagle medium (10% FBS-DMEM), and exposed to low-irradiance 405 nm light. Samples were exposed to 405 nm light at an irradiance of 0.5 mW/cm^2 using the EDS. Data points show the mean counts (n = 3) ± SD. Asterisks '*' indicate light exposed samples that were significantly different to non-exposed final control samples ($P \leq 0.05$), using one-way ANOVA.

Although viral inactivation in DPBS is slow using low-irradiance light from the HINS light EDS, results show that this can be accelerated when the virus is in photosensitive media (10% FBS-DMEM), similar to that found in Chapter 4. When FCV was suspended in human blood plasma a 1.8 $\log_{10}$ reduction ($P = 0.038$) occurred after 2 days, compared to the non-exposed control. A further 1.5 $\log_{10}$ inactivation was seen following 3 days of exposure (130 J/cm^2), however due to the large standard deviation in the test samples this was only deemed significant to a confidence level of 90% ($P = 0.075$). It should also be noted that the value for the final time point was measured to a sensitivity of 10 as the neat samples reacted badly with the cells, possibly due to the length of time held at room temperature.

Inactivation of FCV using low-irradiance light was also successful when the virus was suspended in artificial faeces. A 1 $\log_{10}$ reduction was achieved after a dose of 43 J/cm^2 (1 day; $P = 0.024$). Following 5 days of exposure (216 J/cm^2) there was complete

inactivation of FCV compared to the starting population, which equated to a significant 3.9 $\log_{10}$ reduction in viral infectivity compared to the equivalent non-exposed control (P = 0.00). This was measured to a sensitivity of 2, as the neat samples of artificial faeces caused some cell dislodgement from the outer edges of the cell layer in the 6-well plates.

When the surrogate virus was exposed to low-irradiance light in artificial saliva it was not possible to elucidate viral inactivation. The experimental procedure was to take samples after every day of exposure, however complete inactivation of the exposed and control samples occurred after 1 day of exposure when in artificial saliva, indicating that a component of the saliva (such as the salivary enzyme α-amylase) may be toxic to FCV during long term exposure in the environment. Due to time constraints, further studies using artificial saliva could not be carried out, however it would be beneficial to carry out future experiments using viruses suspended in human saliva to replicate a more-realistic scenario.

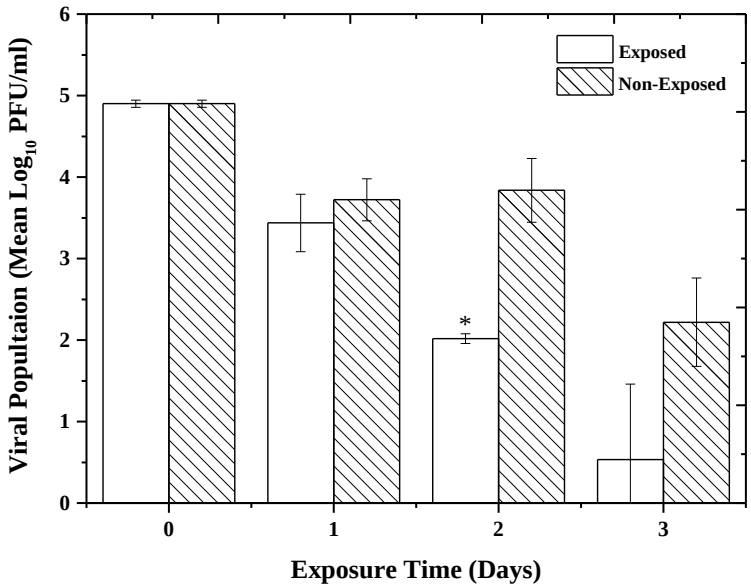


Figure 5.7 Inactivation of feline calicivirus when suspended in human blood plasma and exposed to low-irradiance 405 nm light. Samples were exposed to 405 nm light at an irradiance of 0.5 mW/cm^2 using the EDS. Data points show the mean counts (n = 3) ± SD. Asterisks '*' indicate light exposed samples that were significantly different to non-exposed final control samples ($P \leq 0.05$), using one-way ANOVA.

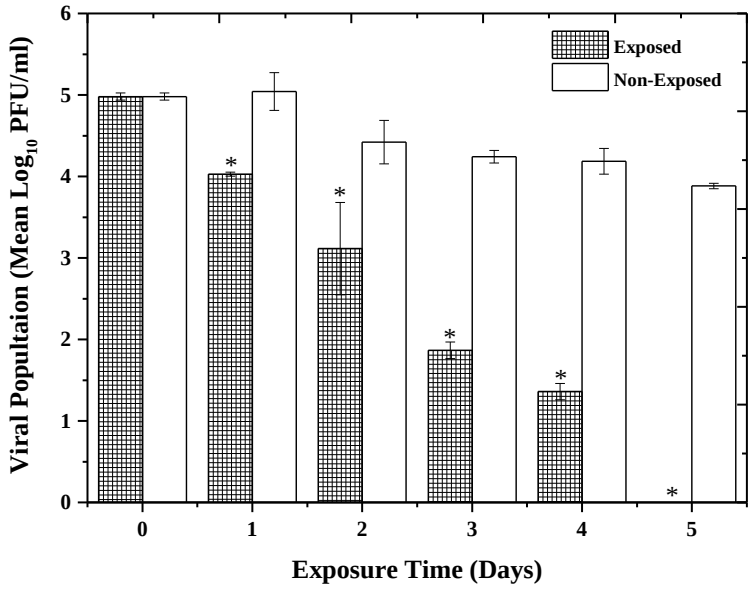


Figure 5.8 Inactivation of feline calicivirus when suspended in artificial faeces and exposed to low-irradiance 405 nm light. Samples were exposed to 405 nm light at an irradiance of 0.5 mW/cm^2 using the EDS. Data points show the mean counts (n = 3) ± SD. Asterisks '*' indicate light exposed samples that were significantly different to non-exposed final control samples ($P \leq 0.05$), using one-way ANOVA.

5.4 Inactivation of FCV on Clinically Relevant Surfaces using Low-Irradiance 405 nm Light

This section of the study investigated antiviral efficacy of low-irradiance 405 nm light for inactivation of viral particles dried on surface coupons, representing a scenario of how viruses are likely to be found in the hospital environment.

5.4.1 Surface Coupon Preparation

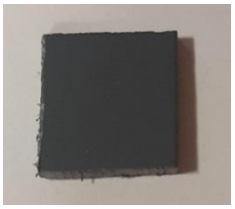
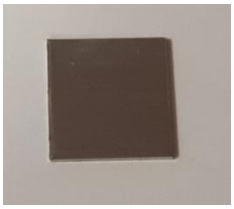
Coupons were made of polyvinyl chloride (PVC), stainless steel and vinyl flooring (2000 Pur, Polyflor Ltd, UK), and can be seen in Table 5.1. Coupons were 15 × 15 mm in size and had depths of 3, 1 and 2 mm, respectively. When arranging coupons on the experimental surface, as discussed in Section 5.4.3, an average depth of 2 mm was used to calculate irradiance at the surface of the coupons, as there was likely to be minimal difference in irradiance between the different coupon heights.

To clean the coupons before use, the coupons were soaked in 5% Decon 90 for 5 minutes, rinsed using 500 ml sterile water, soaked in 70% ethanol for 1 min and rinsed with 500 ml sterile water. The coupons were then left to dry in a microbiological safety cabinet before being individually wrapped in aluminium foil. The vinyl coupons were left to dry in a heated cabinet, whilst the PVC and stainless steel coupons were autoclaved. All coupons were then allowed to dry for a further 18 hours overnight in the heated cabinet before use.

5.4.2 Seeding Surface Coupons

Once the coupons had been decontaminated and dried, they were placed in individual 50 mm dishes in the microbiological safety cabinet using sterile callipers. FCV was diluted to 10^6 PFU/ml in the required suspending medium (DPBS with and without 5% FBS, and artificial faeces) before seeding onto coupons. 100 µl of the viral suspension was placed on the centre of each coupon, and spread across the coupon using a sterile pipette tip. FCV was seeded onto 3 coupons of each material: one to calculate the initial starting population, one to be light-exposed, and one as a non-exposed control sample. These coupons were then left to dry for 1.5 hours in the dark inside the microbiological safety cabinet.

Table 5.1 Coupons of common hospital surface materials. FCV was dried onto surface coupons to investigate the efficacy of low irradiance 405 nm light. Adapted from Warnes *et al*. (2015).

Surface	Polyvinyl Chloride (PVC)	Stainless Steel	Vinyl (2000 Pur)
Surface Appearance			
Properties	Low cost; Hard but can be made flexible with plasticizers; Wipe clean	Resistant to corrosion; Easy to clean	Easy to clean; Quick drying
Use(s)	Window sills, flooring, pipes	Trolleys, sinks, surfaces, bowls	Bathroom and kitchen flooring

5.4.3 Experimental Arrangement

The seeded coupons were placed under the EDS at 100 mm intervals, over the worktop surface, in individual 50 mm petri dishes with the lids removed (Fig 5.9). The irradiance across the whole 60 × 60 cm worktop surface was measured and mapped, and can be seen in Figure 5.10. The irradiance was measured at the average height (2 mm) the coupons would sit in the petri dishes: 158 cm from the EDS unit. The maximum irradiance of 0.501 mW/cm^2 was measured at the centre of the worktop surface (Fig 5.10), however this varied over the surface of the experimental worktop. To ensure that inactivation could be compared between the different surface coupons, the petri dishes containing the coupons were arranged so that the average irradiance across the exposed surfaces, in triplicate, averaged 0.48 mW/cm^2 (Fig 5.9). Control plates were held in identical conditions but exposed to normal laboratory fluorescent lighting (0.02 mW/cm^2; 60 Lux).

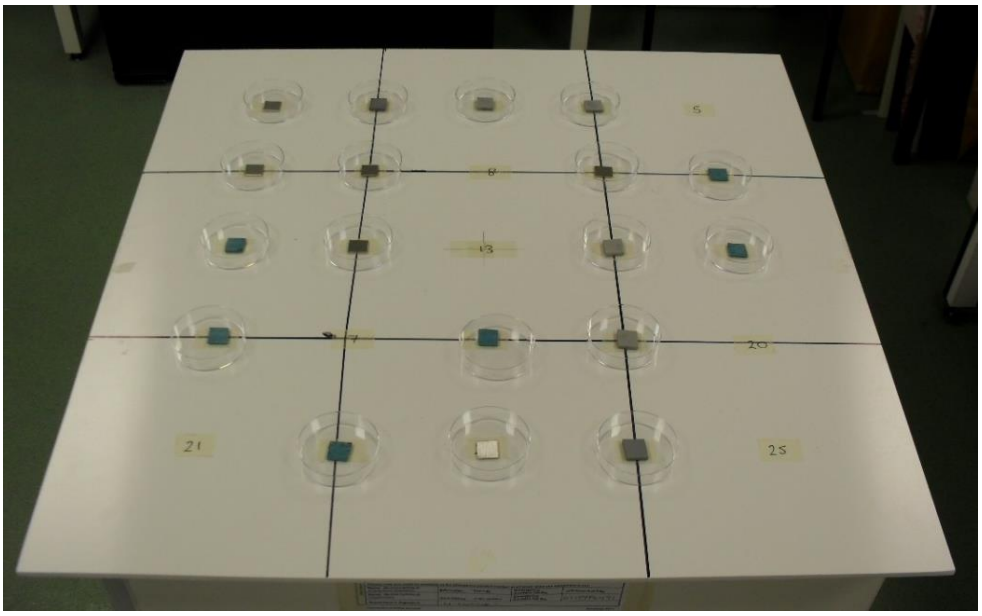


Figure 5.9 Arrangement of surface coupons on the experimental worktop surface below the HINS-light EDS. Coupons were arranged so that the average irradiance of 405 nm light over coupons exposed in triplicate was equivalent to 0.48 mW/cm^2.

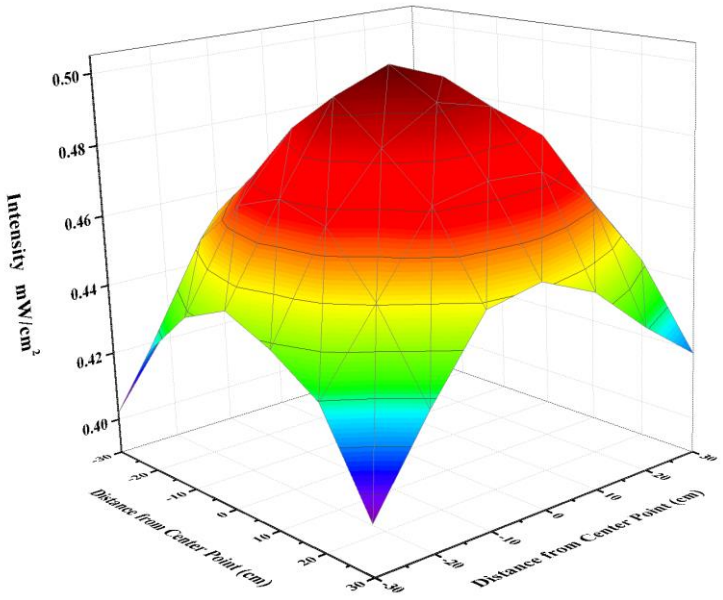


Figure 5.10 3D model of the 405 nm light irradiance across the 60 × 60 cm experimental surface, measured at the average distance of 158 cm below the HINS-light EDS. The maximum irradiance measured at the centre point of the exposure surface was 0.501 mW/cm^2.

5.4.4 Viral Elution from Surface Coupons

To elute the virus from the surface coupons, the coupons were placed in sterile 50 ml tubes containing 2 ml of supplemented DMEM. The supplemented DMEM acted as an elution buffer, and the coupons were vortexed for 30-60 seconds until the virus containing medium had been visually removed from the coupon surface. The elution medium was then pipetted over the surface of the coupons several times to ensure all viral particles were removed. It was found most challenging to remove viral samples from stainless steel coupons and easiest from vinyl coupons.

5.4.5 Viral Plaque Assay and Enumeration

As described previously (Section 5.3.1) 6-well plates were seeded with 6×10^5 FEA cells for 18-24 hours prior to elution, to allow viral enumeration via the plaque assay technique, which was carried out as described in Section 4.2.4.

Following incubation, fixation, washing and drying of the cell layers in 6-well plates, the number of plaque forming units were counted in each well which represented a PFU/ml. However, to calculate the viral concentration on the coupons, the number of plaques counted per well was multiplied by 20, to account for dilution in the 2 ml elution buffer. The final value calculated represents the population of viruses present on the surface of the coupons in the 100 µl sample.

5.4.6 Results: Inactivation of FCV using Low-Irradiance 405 nm Light when Dried on Clinically Relevant Surface Coupons

The level of FCV inactivation when dried on surfaces in DPBS is similar for PVC, stainless steel and vinyl (Fig 5.11). When FCV was exposed to the EDS for 7 days, equivalent to a dose of 290 J/cm^2, there was approximately 0.9 $\log_{10}$, 0.7 $\log_{10}$ and 0.6 $\log_{10}$ reduction compared to the equivalent non-exposed controls on PVC, stainless steel and vinyl, respectively. Although the level of inactivation seen was greater than that of non-exposed controls, the inactivation was not deemed significant. This is likely due to large standard deviation as a result of the number of surviving viral samples which ranged from 0-100 PFU/coupon after exposure.

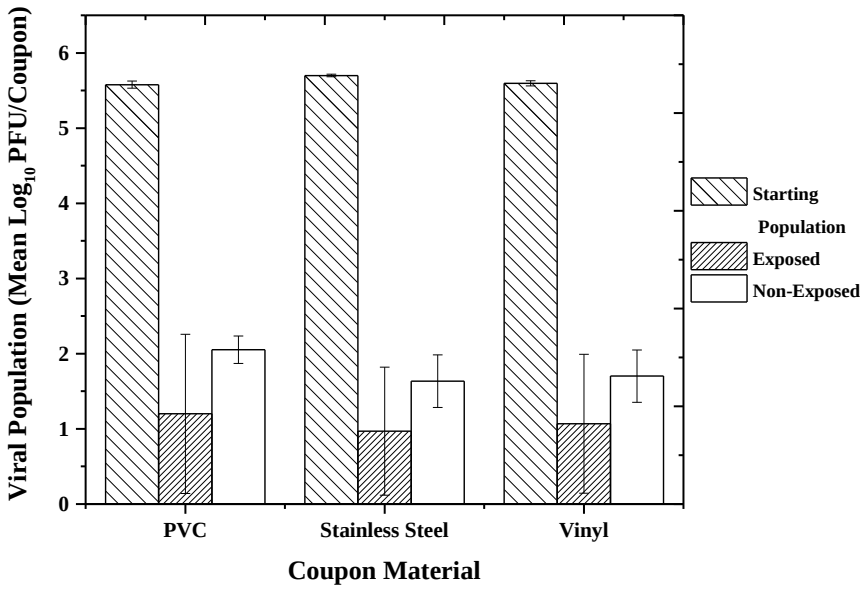


Figure 5.11 Inactivation of feline calicivirus in Dulbecco's phosphate buffered saline (DPBS) when dried onto surfaces, and exposed to low-irradiance 405 nm light. Samples were exposed to 405 nm light at an irradiance of 0.48 mW/cm^2 for 7 days using the EDS. Data points show the mean counts (n = 3) ± SD.

Similarly, when FCV was dried on surfaces in 5% FBS-DPBS, little difference in inactivation was seen between exposed and non-exposed samples after 7 days. As can be seen in Figure 5.12, when the virus was dried on PVC and stainless steel with organic soiling there was a 0.2 $\log_{10}$ reduction of FCV compared to controls. A significant 0.4 $\log_{10}$ reduction of FCV on vinyl ($P = 0.003$) was achieved for the same exposure.

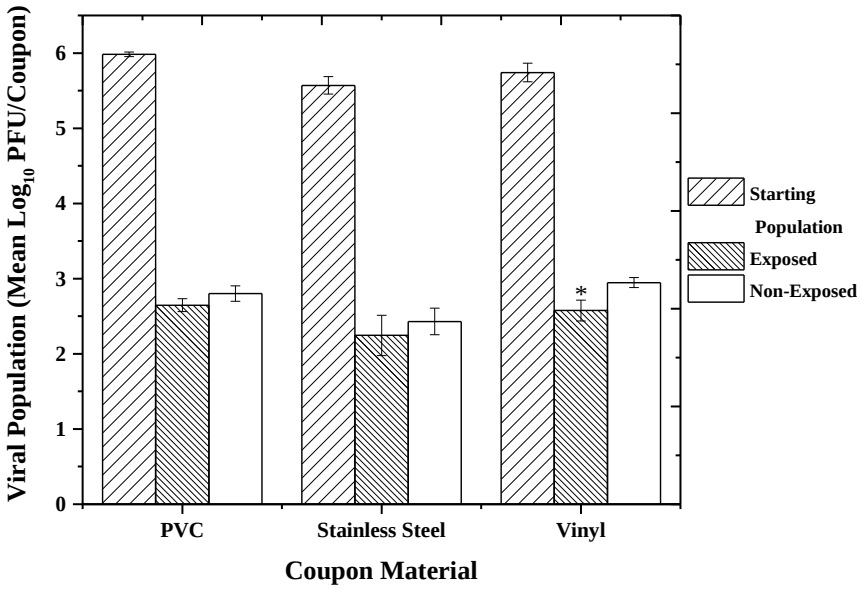


Figure 5.12 Inactivation of feline calicivirus in Dulbecco's phosphate buffered saline supplemented with 5% fetal bovine serum (5% FBS-DPBS) when dried onto surfaces, and exposed to low-irradiance 405 nm light. Samples were exposed to an irradiance of 0.48 mW/cm^2 for 7 days using the EDS. Data points show the mean counts (n = 3) ± SD. Asterisks '*' indicate light exposed samples that were significantly different to non-exposed final control samples ($P \leq 0.05$), using one-way ANOVA.

When FCV was dried onto coupons in artificial faeces, there was significant inactivation on all surfaces after 7 days of exposure (Fig 5.13). A 1.4 $\log_{10}$, 0.9 $\log_{10}$ and 1.6 $\log_{10}$ reduction of FCV occurred on PVC, stainless steel and vinyl coupons respectively compared to the equivalent non-exposed control. Furthermore, significant inactivation also occurred after a 14 day exposure, equivalent to a dose of 581 J/cm^2 405 nm light. After this exposure period, there was a 1.1 $\log_{10}$, 0.9 $\log_{10}$ and 1.3 $\log_{10}$ reduction of FCV on PVC, stainless steel and vinyl respectively, compared to the equivalent non-exposed controls ($P < 0.05$ for all).

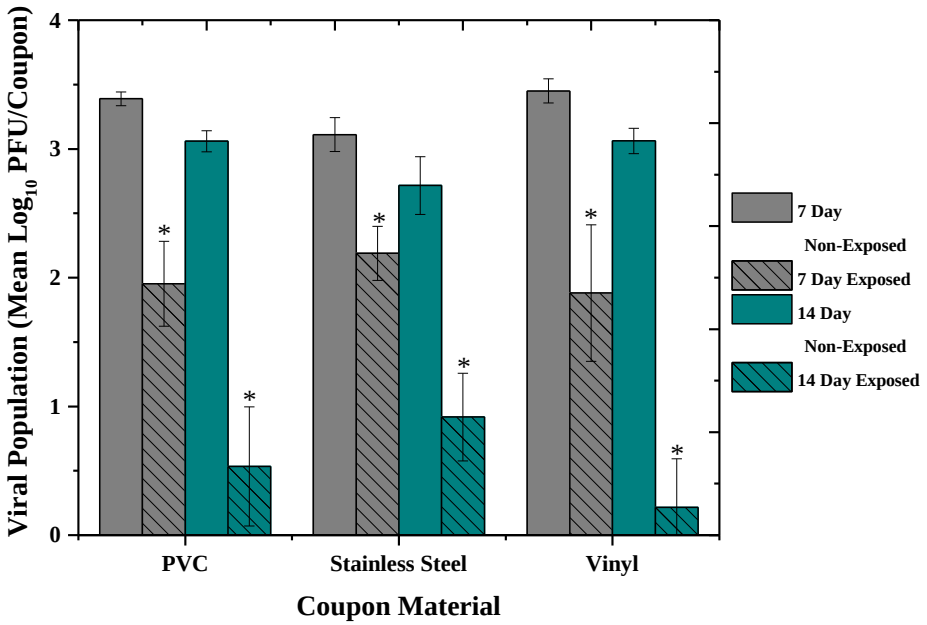


Figure 5.13 Inactivation of feline calicivirus in artificial faeces when dried onto surfaces, and exposed to low-irradiance 405 nm light. Samples were exposed to an irradiance of 0.48 mW/cm^2 for 7 and 14 days using the EDS. Data points show the mean counts (n = 3) ± SD. Asterisks '*' indicate light exposed samples that were significantly different to non-exposed final control samples ($P \leq 0.05$), using one-way ANOVA.

Results in Table 5.2, compare the dose required for FCV inactivation in minimal and biologically-relevant media, when exposed to low-irradiance light in liquid suspension or dried onto surfaces. Similar doses of 405 nm light, resulted in greater inactivation of FCV when suspended in liquid rather than dried on surfaces, for both DPBS and artificial faces. In the case of DPBS, there was no significant inactivation when dried on surfaces after a dose of 290 J/cm^2, however following a slightly higher dose of 302 J/cm^2, a significant 1.4 $\log_{10}$ inactivation could be achieved when in liquid suspension. When FCV was dried on surfaces in artificial faeces there was significant 0.9-1.6 $\log_{10}$ inactivation, however there was at least 2 × greater $\log_{10}$ inactivation when suspended in liquid, following a lower dose of 216 J/cm^2 405 nm light.

Additionally, far greater inactivation of FCV was achieved when the virus was suspended in artificial faces compared to minimal media. 2.5 × greater $\log_{10}$ inactivation was achieved following doses of 1683 J/cm^2 and 1402 J/cm^2 when using high-intensity 405 nm light, or doses of 216 J/cm^2 and 302 J/cm^2 using low-irradiance light. Greater inactivation in artificial faeces was also achieved when the virus was dried on surfaces, with no significant inactivation when dried in DPBS but between 1.3–2.6 × greater $\log_{10}$ inactivation when dried in artificial faeces, following a dose of 290 J/cm^2.

Table 5.2 Comparison of the 405 nm light inactivation of feline calicivirus when suspended in minimal media (DPBS) or biologically-relevant media (artificial faeces). Comparisons are made between the different low irradiance and high irradiance experimental arrangements, as described in Chapter 4 and Chapter 5. Asterisks '*' indicate significant inactivation compared to the equivalent non-exposed control population ($P < 0.05$).

Experimental Arrangement			**Exposed in DPBS**		**Exposed in Artificial Faeces**	
Light Source	**Average Irradiance (mW/cm^2)**	**Exposure Conditions**	**Dose (J/cm^2)**	**Log_{10} Reduction**	**Dose (J/cm^2)**	**Log_{10} Reduction**
High-intensity 405 nm light from single array	155.8	1.5 ml in 24-well plate	1683	1.7*	1402	4.5*
Low-intensity 405 nm light from EDS	0.50	3 ml in 12-well plate	302	1.4*	216	3.9*
	0.48	100µl dried on PVC	290	0.8	290	1.4*
	0.48	100µl dried on steel	290	0.7	290	0.9*
	0.48	100µl dried on vinyl	290	0.6	290	1.6*

5.5 Discussion

Despite extensive cleaning of the hospital environment, outbreaks of NoV regularly occur, due to NoV's ability to survive on environmental surfaces for long periods of time (Wu *et al*., 2005; Lopman *et al*., 2012; Kambhampati *et al*., 2015; Robollotti *et al*., 2015). The results in this chapter demonstrate for the first time the antiviral efficacy of low-irradiance 405 nm light, with inactivation enhanced when viruses are exposed in biologically-relevant media. These findings suggest the potential for use of the HINS-light EDS for environmental decontamination of NoV.

Results in the first stage of this study demonstrate inactivation of the viral surrogate, bacteriophage ϕC31, using low-irradiance 405 nm light, when suspended in minimal and organically-rich media. ϕC31 was initially investigated for several reasons: (i) working with bacteriophage was must much faster and cheaper than working with mammalian virus FCV, (ii) results following exposure of ϕC31 could be generated overnight whereas a minimum of 5 days was required before results could be recorded when working with the FCV-FEA cell infectivity based assays and (iii) the bacteriophage should have similar resistance to environmental factors as enteric mammalian viruses (Leclerc *et al*., 2000; Costa *et al*., 2012) and therefore the approximate doses that would be required for FCV in future experiments could be estimated.

When ϕC31 was suspended in minimal media and exposed to low-irradiance light for 4 days using a dose of 173 J/cm^2, a significant 0.3 $\log_{10}$ reduction compared to the equivalent control was achieved (Fig 5.2). Further exposure increased the level of bacteriophage inactivation seen, with a maximum dose of 605 J/cm^2 causing a 1 $\log_{10}$ reduction. However, when the phage was suspended in an organically-rich media (NB), inactivation was much faster (Fig 5.3). An initial exposure for 1 day and equivalent dose of 43 J/cm^2, resulted in a 0.4 $\log_{10}$ reduction of the bacteriophage and after 6 days of exposure and an equivalent dose of 259 J/cm^2, a 5 $\log_{10}$ reduction of ϕC31 was achieved.

These results are similar to those in the previous study by Tomb *et al.* (2014) using high-irradiance 405 nm light to inactivate ϕC31. As can be seen in both studies, far greater inactivation of the bacteriophage occurs when suspended in organically-rich media compared to minimal media. Upon exposure to the high-irradiance source, at an irradiance of 56.7 mW/cm^2, a 0.3 $\log_{10}$ reduction of ϕC31 suspended in PBS occurred after exposure to a dose of 306.2 J/cm^2, whereas when suspended in NB a 5.4 $\log_{10}$ could be achieved following a dose of 510 J/cm^2 (Tomb *et al.*, 2014).

As the viral surrogate was successfully inactivated using the EDS, the study then went onto investigate if the NoV surrogate, FCV, could also be inactivated using similar low-irradiance light. Firstly, FCV was exposed using the EDS whilst suspended in minimal media (DPBS). As can be seen in Figure 5.4a there is reduction of the viral infectivity of the non-exposed control populations with a 0.9 $\log_{10}$ and 2.1 $\log_{10}$ reduction after 7 and 14 days, respectively. This loss of viral integrity could be due to environmental factors such as temperature, light and humidity (Vasickova *et al*., 2010). As the non-exposed controls were held in fluorescent laboratory lighting, this could also have had an effect on the viral infectivity titre. Standard fluorescent lighting has emission in the range 350-750 nm (Webb 2006), so UV and near-UV emissions (350-400 nm) may have damaged the viral particles. Decreased environmental survival of FCV in liquid has also been demonstrated by Allwood *et al.* (2005), with > 6 $\log_{10}$ reduction of FCV when held in tap water for 21 days. Therefore, the loss of viral integrity in the non-exposed control populations was to be expected.

Although there was a decrease in the non-exposed controls, there was still significant inactivation of FCV in DPBS after 7 days of exposure (302 J/cm^2) resulting in a 1 $\log_{10}$ reduction compared to the equivalent non-exposed control. Extending exposure to 14 days also resulted in a 1.3 $\log_{10}$ reduction of FCV compared to the equivalent control. These results indicate that although a proportion of the inactivation occurs due to environmental factors, there is increased inactivation of FCV in minimal media, over and above natural reduction, as a result of exposure to 405 nm light emitted from the EDS.

The HINS-light EDS was additionally able to reduce viral infectivity of FCV in the presence of organic soiling, over and above natural environmental reduction. FCV was exposed in DPBS containing 5% or 10% FBS, to represent organic matter surrounding the viral particles (Jean *et al*., 2011; Goyal *et al*., 2014). Although there was a reduction in the non-exposed controls for both suspending media, there was a 1.2 $\log_{10}$ and 0.5 $\log_{10}$ reduction of FCV infectivity after 7 days when in 5% and 10% FBS-DPBS respectively (Fig 5.4 b & c). Additionally, following 14 days (602 J/cm^2), there was a 1 $\log_{10}$ and 0.7 $\log_{10}$ reduction of FCV in 5% and 10% FBS-DPBS, respectively.

When the inactivation kinetics of FCV in DPBS with and without the FBS additives were compared, it was found that there was significantly less inactivation of FCV after a final dose of 602 J/cm^2 when in DPBS containing FBS than without ($P < 0.05$ for all). Furthermore, there was significantly less inactivation when in DPBS containing 10% FBS compared to 5% FBS ($P = 0.00$). The reduced inactivation of FCV in the presence of organic matter was also demonstrated by Jean *et al*. (2011). FCV was subject to pulsed UV light with

and without FBS, and a reduction in inactivation by 3 orders of magnitude was seen when FBS was present (Jean *et al*., 2011). It was hypothesised that this reduction in inactivation was a result of aggregates of FBS in the suspension, which may have reduced the penetration of the pulsed UV light. This may also be the case during 405 nm light exposure with reduced transmission of 405 nm light in 5% FBS-DPBS (~16% less) and 10% FBS-DPBS (~28% less) compared to DPBS, which will contribute to the decreased level of inactivation seen.

Significant inactivation of FCV in minimal media, using low irradiance-light, required long exposure periods (7-14 days). In an effort to reduce the length of time of exposure, FCV was suspended in an organically-rich medium, 10% FBS-DMEM, which was found to aid viral inactivation when using high-irradiance 405 nm light (Section 4.2.5). As can be seen in Figure 5.6 there was enhanced inactivation of the virus, with a significant 1.4 $\log_{10}$ reduction following exposure for 1 day and an additional 0.2 $\log_{10}$ reduction after an additional day, compared to the equivalent control. A significant level of inactivation also occurred in the non-exposed controls which may have been due to the fluorescent laboratory lighting activating photosensitive molecules within DMEM, causing them to produce ROS and in turn damage FCV (Grzelak *et al*., 2001), albeit at a slower rate than the EDS. It would be interesting to repeat this experiment with the control held in darkness, as it is likely there would be less excitation of the DMEM, resulting in a more stable control population, which will help to calculate the true level of inactivation of FCV when light-exposed in photosensitive media. However, the fact that there was 95% reduction compared to the equivalent control after 1 day, supports results in Chapter 4, which demonstrated that constituents of DMEM such as riboflavin, tryptophan, tyrosine, pyridoxine and folic acid are photosensitive and aid the inactivation of FCV using 405 nm light.

As FCV displayed sensitivity to 405 nm light inactivation when suspended in photosensitive media this indicates a potential for using photosensitive products alongside the low-irradiance EDS for enhanced environmental decontamination. These products could include using photocatalytic surface coatings, photosensitive wipes and photosensitive surface sprays (McDonald *et al*., 2000; Page *et al*., 2009; Verhaelen *et al*., 2014). There has been much interest in the use of photocatalytic surfaces such as those coated in titanium dioxide (TiO_2) which cause bacterial and viral inactivation once illuminated with UV or broadband light (Kühn *et al*., 2003; Vohra *et al*., 2005; Zan *et al*., 2007; Nakano *et al*., 2012). For example, a recent study by Park *et al*. (2014) demonstrated that fluorinated TiO_2 on surfaces caused a 2-3 $\log_{10}$ reduction of bacteriophage MS2, FCV and MNV using 10 $\mu W/cm^2$ UVA light for

60 minutes. Furthermore, studies have also demonstrated the use of 405 nm light to activate these photosensitive surfaces (McKenzie 2014; Sadowski *et al*., 2015). McKenzie (2014) demonstrated increased inactivation of *E. coli* when on surface samples which had been coated in TiO_2 nanoparticles. After exposure to 18 J/cm^2 405 nm light, there were 3.8 log_{10}, 4.4 log_{10} and 5 log_{10} reductions of *E. coli* on TiO_2 coated wet room tiles, glass and vinyl flooring, respectively whereas only 0.8 log_{10}, 0.9 log_{10} and 3 log_{10} reductions could be achieved using violet-blue light alone (McKenzie 2014).

However, it is unlikely that when the EDS is used in hospitals, all surfaces will have the benefit of photocatalytic coatings to allow for improved viral inactivation. Artificial enhancement of antiviral activity by addition of exogenous photosensitizers may not be required though due to the enhanced inactivation seen when the NoV surrogate was suspended in biologically-relevant media.

Increased antiviral efficacy of the EDS was seen when FCV was exposed in human plasma and artificial faeces. When FCV was suspended in human blood plasma a 1.8 log_{10} reduction occurred after 2 days of exposure compared to the non-exposed control (Fig 5.7). Further enhancement of inactivation occurred after 3 days (130 J/cm^2), which resulted in an additional 1.5 log_{10} inactivation of FCV. The inactivation level compared to the final control point was only found significant to a confidence interval of 90%. This was likely due to the deviation in the levels of inactivation seen after 3 days, which may have been affected by the suspending plasma, with transmission ranging between 0.2-14.5% (n = 3), therefore varying the level of inactivation seen.

When suspended in artificial faeces, near complete inactivation of FCV occurred after 5 days with only a 1 log_{10} reduction seen in the control samples (Fig 5.8). Although successful, the rate of inactivation in faeces was reduced compared to that in plasma, and this is likely due to the reduced transmission of 405 nm light through the media (0.01%), and because viruses surrounded by colloidal matter, organic matter or human faeces are stabilised and protected (Vasickova *et al*., 2010). As components of the artificial faeces were seen to be very photosensitive (Fig 4.9) they may have enhanced FCV inactivation upon exposure to 405 nm light from the EDS. The survival of the control samples was far greater than that observed by Cannon *et al*. (2006) who demonstrated a 3.5 log_{10} reduction of FCV, strain F9, when held at room temperature in an artificial faeces solution for 7 days, however this could be due to differing artificial faeces matrixes used. As NoV is shed in diarrhoeal stools, these results are a positive indicator for the use of the EDS to aid inactivation of viruses in contaminated

bodily elutions that have been missed by conventional cleaning (Atmar *et al*., 2008; Morter *et al*., 2011).

Comparison of the dose requirements for FCV inactivation in photosensitive and biologically-relevant media using high-irradiance and low-irradiance 405 nm light, found the doses required when using low-irradiance light are far lower. When in photosensitive 10% FBS-DMEM only 43 J/cm^2 405 nm light is required for a 1.4 $\log_{10}$ reduction of FCV using the EDS whereas for a similar 1.2 $\log_{10}$ reduction using the high-irradiance source a far higher dose of 140 J/cm^2 was required. Additionally, as can be seen in Figure 4.4b & c, doses of 560 J/cm^2 and 1.4 kJ/cm^2 were required for 4.5-5 $\log_{10}$ reduction of FCV in plasma and artificial faeces using high-irradiance light, respectively. However, using the EDS far lower doses of 130 J/cm^2 and 216 J/cm^2 were required for similar levels of inactivation in plasma and artificial faeces. This phenomenon was also demonstrated when the bacteriophage ϕC31 was exposed to the EDS with approximately 40-50% less dose required than when exposed in minimal and organically-rich media using the high-intensity light source (Tomb *et al*., 2014).

The lower requirements could be a result of the doses from the high-intensity source being greater than the maximum dose which can be absorbed by photosensitive molecules within the viral structure and suspending media i.e. there is an excess of photons which cannot be absorbed by photosensitive molecules over and above a critical level, so will not contribute to the inactivation effect seen (Maclean *et al*., 2016). This would also result in inefficiencies, as using systems delivering a dose higher than that of the threshold, would result in energy which cannot be absorbed and is therefore wasted.

Lower dose requirements for microbial inactivation have also been demonstrated when exposing bacteria to the EDS compared to single array high-irradiance light sources. Maclean *et al*. (2009) demonstrated a dose requirement of between 30-110 J/cm^2 for inactivation of *A. baumannii*, *P. aeruginosa*, *S. aureus* and *Streptococcus pyogenes* when in liquid suspension and exposed to 405 nm light at an irradiance of 10 mW/cm^2. Whereas when these aforementioned bacteria where seeded onto agar plates and exposed to the HINS-light EDS at an irradiance of 0.5 mW/cm^2, far lower doses of between 7.2-12.6 J/cm^2 were required for a similar 2 $\log_{10}$ reduction (Bache 2013).

As bacterial inactivation using the EDS only requires 4-7 hours exposure for 2 $\log_{10}$ reductions (Bache 2013), compared to 24-336 hours (1-14 days) required for viral inactivation, this further highlights that the mechanism of bacterial inactivation differs to that

of viral inactivation. As hypothesised in Chapter 4, it is probable that an accumulation of effects causes the inactivation seen in viruses. It is likely that damage to the viral particles will occur as a result of the small amount of UVA and 420-430 nm light being released by the light source (Fig 3.5), which may cause oxidation of proteins such as those in the viral capsid (Girard *et al*., 2011; Pattison *et al*., 2012). Following exposure in liquids it was important to investigate if low doses would also be required for inactivation when FCV was suspended on surface coupons.

It is thought that environmental transmission plays an important role in the spread of NoV. Surfaces can become contaminated after direct contact with bodily fluids or indirect contact with aerosols or fomites, and viral particles can subsequently be transferred by hands, food and other inanimate objects, and remain viable for > 28 days (Barker *et al*., 2004; Vasickova *et al*., 2010; Lopman *et al*., 2012). Studies have demonstrated recovery of the virus from many different surfaces including keyboards, toilet seats, hand rails, taps, soap dispensers and medical equipment even after clinical cleaning (Wu *et al*., 2005; Morter *et al*., 2011). Therefore, to replicate how NoV would be found in the hospital environment, the next stage of this study used the EDS to expose FCV dried onto PVC, stainless steel and vinyl flooring, in minimal and biologically-relevant media.

When dried onto surfaces in minimal media (DPBS), with and without 5% FBS acting as organic matter, there was a 2-3 $\log_{10}$ reduction in control populations after 7 days, as seen earlier with controls in suspension. A reduction in infective viral particles on surfaces has also been demonstrated by D'Souza *et al*. (2006) who described a 6-7 $\log_{10}$ reduction in infectious viral particles recovered from Formica, stainless steel and ceramic surfaces after 7 days. The inactivation of FCV exposed to low-irradiance light when dried on all three surfaces in DPBS with and without 5% FBS, was not significantly different to the reduction in infectivity of the non-exposed controls, with the exception of a significant 0.4 $\log_{10}$ inactivation when FCV was dried on vinyl in 5% FBS-DPBS (Fig 5.11 & 5.12). Although inactivation when dried on coupons in DPBS was not deemed to be significant, there is a greater level of FCV inactivation when exposed on coupons compared to equivalent controls (Fig 5.11) with an average 0.9 $\log_{10}$, 0.7 $\log_{10}$ and 0.6 $\log_{10}$ reduction compared to the equivalent non-exposed controls on PVC, stainless steel and vinyl, respectively. The fact these were not found to be significantly different is likely due to the variation in PFU/coupon counted, with low recoverability of the virus (38-50%) potentially affecting this. FCV inactivation on vinyl was seen to be significant when in 5% FBS-DPBS and could be due to the higher recoverability of the virus from vinyl when dried in this medium (57.0 ± 17.0%).

However, results were more encouraging when surfaces seeded with FCV in artificial faeces were exposed to low-irradiance 405 nm light. As can be seen in Figure 5.13 there was a significant 1.4 log_{10}, 0.9 log_{10} and 1.6 log_{10} inactivation of FCV after 7 days, and a further 1.1 log_{10}, 0.9 log_{10} and 1.3 log_{10} reduction after 14 days, when exposed on PVC, stainless steel and vinyl, respectively. Although inactivation was significant, far greater inactivation of FCV occurs when suspended in artificial faeces rather than dried on surfaces (Table 5.2). A 3.9 log_{10} reduction of FCV was achieved after a dose of 216 J/cm^2 when suspended in liquid whereas only 0.9-1.6 log_{10} reduction was achieved when on surfaces. This could be a result of the ROS produced by photosensitive molecules, having a short life span (10^{-6} s) and reduced distance of diffusion due to the viruses being immobilised on the surface coupons (Murdoch *et al*., 2013).

Differences in behaviour of viral inactivation, when dried on surfaces compared to those suspended in media, have also been documented when viruses have been exposed to disinfectants. In a study by Terpstra *et al*. (2007), canine parvovirus and HAV were dried on surfaces in blood plasma or culture media and it was found that in a dried state the viruses were less susceptible to disinfectants, especially when dried in blood plasma. Furthermore, Park *et al*. (2007), demonstrated a 3 log_{10} reduction of MS2 phage and NoV using hypochlorous acid solution, with a 20 second exposure to 18-188 ppm required when in liquid, whereas when on ceramic tile or stainless steel, a significantly higher exposure time of 1-10 min was required for the equivalent level of inactivation. The reasons why inactivation is reduced on surfaces are unclear but it is assumed that the reduced sensitivity of FCV to the HINS-light EDS when in a dried, static state, may be due to reduced ability of 405 nm to light to irradiate the entire viral particle (Park *et al*., 2007). However, if future studies were able to sample the level of viral inactivation on a daily basis, there may be a lesser level of control decline evidenced per day, which could in turn reveal greater virucidal efficacy of 405 nm light on surface deposited viruses.

Additionally, it is also important to compare the virucidal efficacy of low-irradiance 405 nm light to other decontamination technologies such as UV light, ozone, CAPP and HPV. Several studies have investigated UV inactivation of FCV and other NoV surrogates when in liquid and on surfaces (Nuanualswan *et al*., 2002; Thurston-Enriquez *et al*., 2003; de Roda Husman *et al*., 2004; Duizer *et al*., 2004b). A study by Park *et al*. (2011) using a 253.7 nm mercury vapour lamp, demonstrated inactivation of three NoV surrogates (MNV, FCV & Echovirus-12) in liquid, using a maximum dose of 40 mJ/cm^2. Furthermore, pulsed UV light, ranging from 200-1100 nm, has also been demonstrated to inactivate MNV on

surfaces and in liquid (Jean *et al*., 2011). The results by Jean *et al*. (2011) demonstrated a dose of 7.62 J/cm^2 was required for a 5 $\log_{10}$ reduction of MNV on PVC and stainless steel coupons as well as when exposed in liquid suspension. The equal level of inactivation of MNV on surfaces and in liquid, conflict with those in this study, however differences could be due to different inactivation mechanisms of UV light compared to 405 nm light, with UV light causing inactivation due to modifications within genetic material of an organism (Maclean *et al*., 2008c).

Although viral inactivation using UV light requires significantly less dose than the EDS, (mJ vs J), there are several features of UV light which make the 405 nm light EDS a more favourable option including material degradation and safety concerns (Section 2.4.6). Additionally, photo-reactivation can occur in bacteria following exposure to UV light if not fully inactivated (Maclean *et al*., 2008c). Photoreaction allows the cell to survive as nucleic acid damage is repaired by photolyase enzymes, which capture energy from photons of light, and split the dimers formed (Maclean *et al*., 2008c; Ben Said *et al*., 2009). This reactivation is also possible if a virus is able to infect multiple host cells which can provide repair enzymes or machinery, or if the virus has repair genes, therefore allowing enhanced survival of DNA and RNA viruses (Yamamoto and Shimojo 1971; Ben Said *et al*., 2009). This reactivation is unlikely to occur using 405 nm light as the ROS produced will target a number of sites within viral structures (Dai *et al.*, 2013a; Maclean *et al.*, 2014).

HPV is also increasingly being used for decontamination within hospitals, and several studies have demonstrated inactivation of FCV and other NoV surrogates using this technology (Tulhadar *et al*., 2012; Goyal *et al*., 2014; Holmdahl *et al*., 2016). Notably Bentley *et al*. (2012) demonstrated that HPV could cause greater than 4 $\log_{10}$ reduction of FCV on different surfaces related to the hospital environment. Furthermore, results by Bentley *et al*. (2012) also demonstrated greatest inactivation of FCV when the virus was dried onto vinyl and least when dried onto stainless steel, which concurred with the results of this study when FCV in artificial faeces was dried onto these coupons (Fig 5.13). The difference in inactivation on these surfaces may be due to the different affinities of FCV to vinyl and stainless steel, with electrostatic and hydrophobic interactions as well as ionic strength affecting interaction between surfaces and viral particles (Gerba 1984; Vasickova *et al*., 2010). However, inactivation using HPV is far quicker than low irradiance 405 nm light. Zonta *et al*. (2016) used 7% H_2O_2 to cause 4.84 $\log_{10}$ and 4.85 $\log_{10}$ reduction of MNV and FCV on glass, and $\geq$ 3.90 $\log_{10}$ and 5.30 $\log_{10}$ reduction on stainless steel discs respectively, after 90 seconds.

Additionally, ozone and CAPP are also gaining attention for environmental decontamination purposes. Hudson *et al*. (2007) demonstrated that within one hour, a 3 $\log_{10}$ reduction of NoV RNA in faecal matter could be achieved when dried onto plastic, stainless steel, glass, cotton and carpet using ozone. Furthermore, a study by Aboukhar *et al*. (2016) demonstrated ~6 $\log_{10}$ reduction of FCV when suspended in distilled water and exposed to a CAPP treatment using Argon + 1% O_2 plasma after 15 seconds. However, if the virus was suspended in Modified Eagle Medium with a FBS additive, only a 1 $\log_{10}$ reduction occurred after 120 seconds (Aboukhar *et al*., 2016). This later result, additionally supports those in this study whereby the presence of FBS inhibits viral inactivation. This was also demonstrated in Chapter 4. When DMEM was supplemented with 10% FBS there was less inactivation of FCV seen than when in DMEM alone, likely due to FBS quenching some of the ROS produced.

It is clear to see that the aforementioned technologies have a greater virucidal efficacy than low-irradiance 405 nm light from the HINS-light EDS, and these are ideal for terminal clean regimes. The EDS, however, has many benefits (section 2.4.6) which make it ideally suited to continuous disinfection of the hospital environment. Patient rooms and wards do not have to be vacated during operation, and it is safe for use around patients, unlike some of the other technologies discussed (Maclean *et al*., 2015). Furthermore, biological soiling also reduces the efficacy of UV light and HPV whereas results in this study indicate an enhanced efficacy of 405 nm light when viruses are exposed in biologically-relevant media (Piskin *et al*., 2011; Fu *et al*., 2012; Haas *et al*., 2014). Additionally, with all the technologies discussed there is opportunity for malfunction especially if operators are not correctly trained. This could result in potential harm to the operators and any others in close proximity whereas the HINS-light EDS can be used with no requirement for staff training, no catastrophic effects upon malfunction and therefore no compliance problems (Maclean *et al*., 2014).

As NoV can survive and remain infectious in the environment for several weeks, the HINS-light EDS could help target NoV contaminated areas which are in hard to reach places (Doultree *et al*., 1999; Wu *et al*., 2005; D'Souza *et al*., 2006; Ha *et al*., 2015). Furthermore, it could provide effective viral inactivation on areas which are not regularly cleaned such as walls, window sills, curtains, rails, and complex equipment (Maclean *et al*., 2013a). The use of the EDS may be particularly effective as part of a comprehensive approach against NoV and other nosocomial pathogens, supplementing regular cleaning and terminal decontamination (Maclean *et al*., 2014).

Further work is required to investigate if the EDS can inactivate other NoV surrogates, such as MNV and TuV, and to establish the exact mechanism of viral inactivation (as discussed in Chapter 4). However, similar susceptibility to UVB and UVC light treatments has been demonstrated between FCV and other NoV surrogates, suggesting the inactivation results using the EDS may be extrapolated to these other NoV surrogates as well as NoV itself (Duizer *et al*., 2004b; Park *et al*., 2011). It is also important to investigate the inactivation effect of the EDS on additional pathogenic viruses such as Influenza and Rotavirus which also cause outbreaks within the hospital environment (Aitken and Jeffries 2001). Although out-with the scope of this project, the results also indicate that the EDS could be integrated into other locations vulnerable to NoV outbreaks such as schools, cruise ships, nursing homes, etc. (Viviancos *et al*., 2010; Lai *et al*., 2013: Xu *et al*., 2013). Additionally, as the NoV surrogate used, FCV, is a veterinary pathogen, this could increase the scope of HINS-light EDS for use within agriculture, aquaculture and veterinary care (Roh *et al*., 2016).

Chapter Six

Comparison of the Virucidal Efficacy of Violet-Blue Light with the Wider Antimicrobial Efficacy

6.0 Overview

As demonstrated in the previous chapters, viral inactivation can be included within the antimicrobial scope of 405 nm light. This review chapter provides a comparison of the efficacy of violet-blue light against viruses, with currently available data on the efficacy against a range of microorganisms including Gram positive and Gram negative bacteria, endospore forming bacteria, yeasts and fungi. Analysis has been carried out through a systematised review of peer-reviewed published studies focussing on violet-blue light between 380-480 nm.

6.1 Introduction

Traditionally, light inactivation of microorganisms has been associated with PDI. This inactivation method utilises exogenous photosensitizers such as methylene blue, rose bengal or cationic porphyrins, which in the presence of oxygen, become excited when exposed to different wavelengths of light, including violet-blue (380-500 nm) and red (625-740 nm) (Schagen *et al*., 1999; Hamblin and Hassan 2004; Lambrechts *et al*., 2005; Almeida *et al*., 2011; Manoil *et al*., 2016). These excited photosensitizers produce ROS, such as singlet oxygen and hydroxyl radicals, which can damage many structures within microorganisms, including proteins, lipids and nucleic acids. This leads to cellular damage and inactivation of bacteria, yeasts, fungi, viruses and parasites (Hamblin 2016).

Research carried out in the last decade has indicated that exogenous porphyrins are not always required for visible light inactivation of microorganisms. Early studies by Hamblin *et al*. (2005) indicated *H. pylori* could be inactivated by at least 99% following a dose of 20 J/cm^2 of 405 nm light. The authors believed that this was due to the presence of

high levels of the intracellular porphyrins coproporphyrin and protoporphyrin IX, which produce ROS upon illumination. These findings were further supported in 2006 by Guffey and Wilborn, who demonstrated a successful 88% and 91% reduction of *S. aureus* and *P. aeruginosa* following exposure to 405 nm light. Additionally, 470 nm light also appeared to have antimicrobial efficacy against these organisms with a 62% reduction of *S. aureus* and 97% reduction of *P. aeruginosa* after doses of 15 J/cm^2 and 5 J/cm^2, respectively (Guffey and Wilborn 2006). Shortly after this, studies by Maclean *et al*. (2008a, b) demonstrated that oxygen enhanced visible light inactivation of *S. aureus* and violet-blue light peaking at 405 nm was most effective for microbial inactivation.

Subsequently, many studies have investigated the antimicrobial efficacy of 405 nm light, with recent investigations using violet-blue light with central wavelengths other than 405 nm. For example, Haughton *et al*. (2012) inactivated *Campylobacter jejuni* using 395 nm light whilst Bumah *et al.* (2015a, b) demonstrated the antimicrobial efficacy of 470 nm light against *Salmonella enterica* and *S. aureus*. Additionally, a small number of bacterial endospores, fungi and yeasts have been inactivated using violet-blue light (De Lucca *et al*., 2012; Imada *et al*., 2013; Maclean *et al*., 2013; Murdoch *et al*., 2013; Gupta *et al*., 2015; Moorhead *et al*., 2016 a, b). To date, the data in Chapter 4 is the first evidence of violet-blue 405 nm light inactivation of a mammalian virus without using additional photosensitizers.

This chapter provides a systematised review comparing the inactivation efficacy of visible violet-blue light on a range of microorganisms compared to viruses. Inactivation kinetics generated during this study, and data from peer-reviewed research studies using wavelengths between 380-480 nm, was analysed to compare the scope and efficacy of microbial inactivation.

The analysis of the antimicrobial efficacy of violet-blue light included the following parameters:

- The effect of population density.
- The effect of irradiance.
- Comparison of the dose required for 1 $\log_{10}$ reduction using light between 380-480 nm.

The chapter focuses on two main areas of analysis. The first compares inactivation kinetics from the present study with other previously published data from the ROLEST research group, specifically focusing on 405 nm. The second part expands the review to include a comparison of the antimicrobial scope and efficacy of violet-blue light between 380-480 nm.

6.2 Determining the Antimicrobial Efficacy of 405 nm Light

This section of the study compares the inactivation kinetics of a range of microorganisms exposed to 405 nm light. The data for comparison was either generated during this study (Chapter 4, Chapter 7) or retrieved from studies published by the ROLEST research group (Murdoch *et al*. (2012, 2013), Tomb *et al*. (2014) and Moorhead *et al*. (2016a, b)). This allowed direct comparison of the antimicrobial efficacy of 405 nm light on representative organisms from across the different microbial categories (Table 6.1).

Table 6.1 Representative organisms used to compare the germicidal efficacy of 405 nm light, carried out by the ROLEST research group.

Microbial Classification	Representative Organism
Gram negative vegetative bacteria	*Escherichia coli*
Gram positive vegetative bacteria	*Staphylococcus aureus*
Gram positive bacterial endospores	*Clostridium difficile*
Yeast	*Candida albicans*
Fungal hyphae (vegetative)	*Aspergillus niger*
Fungal conidia (spores)	*Aspergillus niger*
Bacteriophage	ϕC31
Mammalian Virus	Feline calicivirus

6.2.1 Comparison of Inactivation Kinetics

The inactivation kinetics of Gram positive and Gram negative vegetative bacterial cells, fungal conidia and yeast cells with a population density of 10^5 CFU/ml, compared to mammalian viruses can be seen in Figure 6.1a. Gram positive bacteria are the most susceptible to 405 nm light, with doses of 160 J/cm^2 required for a 3 $\log_{10}$ reduction, compared to approximately 1.8, 3, 11 and 16 × greater dose required for an equivalent level of inactivation of Gram negative bacteria, yeasts, fungal conidia and viruses respectively.

Additionally, the inactivation kinetics of 10^3 CFU/ml population densities of fungal hyphae, bacteriophage and bacterial endospores are shown in Figure 6.1b. Both the fungal hyphae and bacterial endospores require very high doses of 405 nm light for a 3 $\log_{10}$ reduction, with doses of 1.4 kJ/cm^2 and 2.4 kJ/cm^2 required for complete inactivation, respectively. Although complete inactivation of the bacteriophage was not achieved, it is likely that very high doses would also be required with only a 0.3 $\log_{10}$ reduction following a dose of 306 J/cm^2, suggesting doses in the region of 1 kJ/cm^2 would be required for complete inactivation.

As discussed in Chapter 4, virucidal activity is enhanced when viruses are exposed whilst suspended in organically-rich media such as NB and DMEM. Results in Figure 6.2 reiterate this finding, demonstrating that > 5 $\log_{10}$ reductions of FCV and ϕC31 can be achieved following doses of 421 J/cm^2 and 510 J/cm^2 respectively; increasing viral susceptibility to levels more comparable with those evidenced with vegetative bacterial cells.

In order to compare the susceptibility of these microbial groups to 405 nm light, germicidal efficiency ($\log_{10}$ reduction per unit dose in joules per square centimetre) was compared. Results in Table 6.2 demonstrate that 405 nm light has the greatest germicidal efficacy against Gram positive vegetative cells (0.0231 $\log_{10}$/J/cm^2), closely followed by Gram negative vegetative cells (0.0174 $\log_{10}$/J/cm^2). The germicidal efficacy of 405 nm light was decreased for yeasts, fungal hyphae and fungal conidia, with the lowest germicidal efficacy seen for bacteriophage (0.0008 $\log_{10}$/J/cm^2). Interestingly the germicidal efficacy for Gram positive spores and mammalian viruses was the same (0.0014 $\log_{10}$/J/cm^2) indicating that using the same dose on these organisms may result in similar levels of inactivation.

Table 6.2 Comparison of the germicidal efficacy of 405 nm light on microorganisms investigated by the ROLEST research group.

Organism	Dose (J/cm^2)	$Log_{10}(N/N_0)$ Reduction	Germicidal Efficiency ($Log_{10}(N/N_0)/J/cm^2$)
Gram negative vegetative bacteria	288	5	0.0174
Gram positive vegetative bacteria	216	5	0.0231
Gram positive bacterial endospores	2430	3.3	0.0014
Yeast	576	5	0.0086
Fungal hyphae (vegetative)	1440	3	0.0021
Fungal conidia (spores)	2300	5	0.0022
Bacteriophage	306	0.3	0.0008
Mammalian Virus	2800	3.9	0.0014

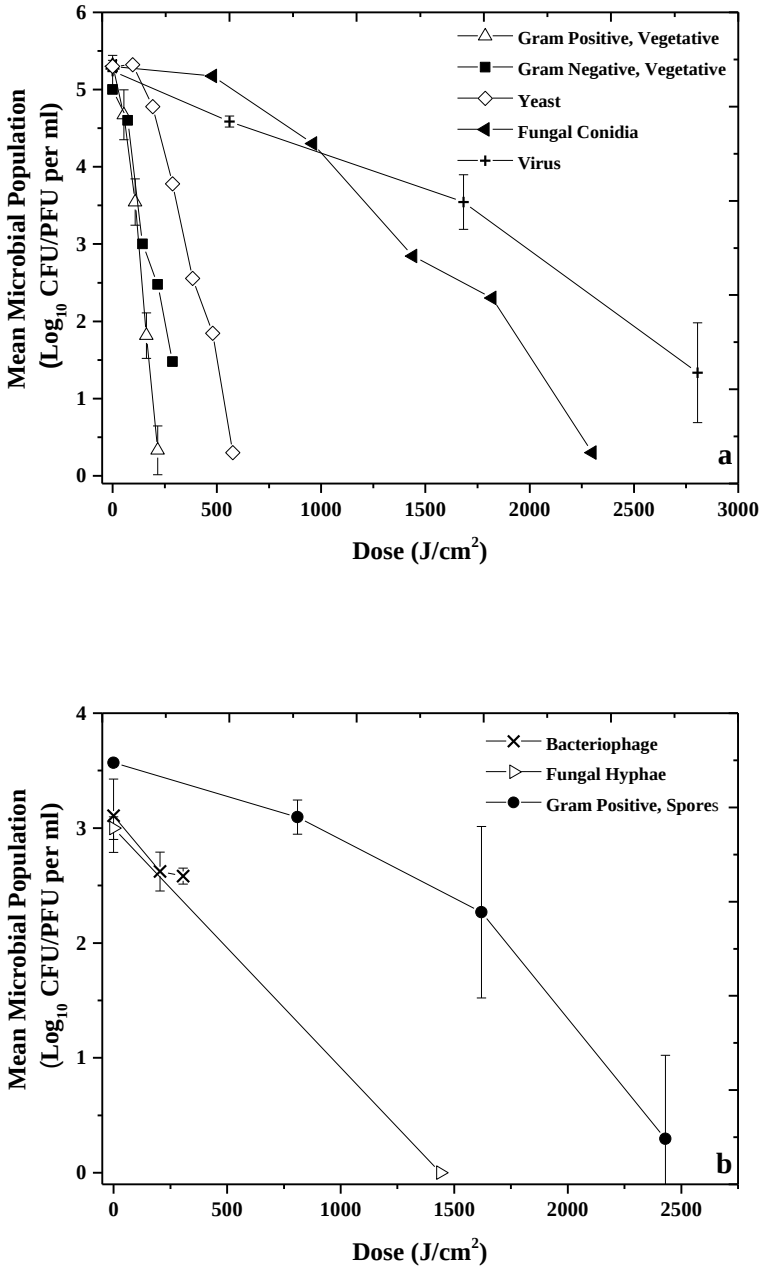


Figure 6.1 Comparison of the efficacy of 405 nm light against a range of microorganisms, using data generated by the ROLEST research group. Inactivation of (a) 10^5 CFU/PFU per ml populations of Gram positive bacteria (*Staphylococcus aureus*), Gram negative bacteria (*Escherichia coli*), yeast (*Candida albicans*), fungal conidia (*Aspergillus niger*), and virus (feline calicivirus) and (b) 10^3 CFU/PFU per ml populations of bacteriophage (ϕC31), fungal hyphae (*Aspergillus niger*), and Gram positive endospores (*Clostridium difficile*), upon exposure to 405 nm light. Adapted from Murdoch *et al*. (2012, 2013); Tomb *et al.* (2014); Moorhead *et al.* (2016a, b).

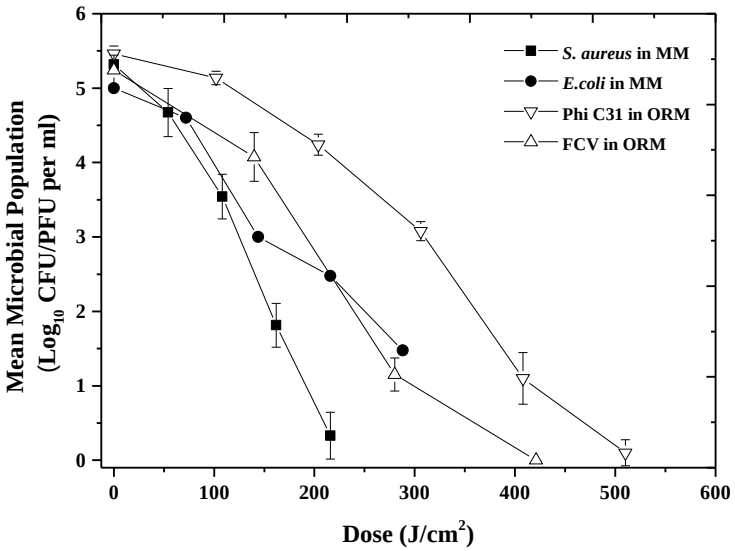


Figure 6.2 Comparison of 405 nm light inactivation of bacteria and viruses, when viruses are exposed in organically-rich media. Inactivation of Gram positive bacteria (*Staphylococcus aureus*) and Gram negative bacteria (*Escherichia coli*) when suspended in minimal media (MM) compared to bacteriophage (ϕC31) and virus (feline calicivirus) when suspended in organically-rich media (ORM). Adapted from Murdoch *et al*. (2012) and Tomb *et al*. (2014).

6.3 Systematised Review of the Antimicrobial Scope and Efficacy of Violet-Blue Light between 380-480 nm

The next stage of analysis was the systematised review of peer-reviewed published studies using violet-blue light between 380-480 nm. The methodology for this review is based on that of a systematic review, however cannot be classed as such due to several factors including lack of a second reviewer, time constraints and level of unavoidable bias, and is therefore classed as a systematised review (Grant and Booth 2009).

6.3.1 Database Search and Study Inclusion

Two Databases, PubMed and Science Direct, were searched for articles from the past thirty years (1987-2017). Search terms used key words associated with violet-blue light technology (i.e. individual wavelengths between 380-480 nm, blue light, visible light) and decontamination (inactivation, kill, antimicrobial, decontamination, disinfection, antibacterial, antiviral, antifungal, antimicrobial, photoinactivation, photosensitizers). The first 20 pages of each web search were screened (equivalent to 400 papers), or all pages if

the search generated less than 20 pages. Additional references added to the screening list included research papers (found in the references section of review papers) as well as a PhD thesis by Bache (2013). Once combined and duplicates excluded, the search yielded 9055 articles (Fig 6.3). The titles and abstracts were then screened to identify relevant peer-reviewed papers, and those which were not relevant to this review were excluded, e.g. studies on PDI, studies inducing porphyrin production and those which used wavelengths of light out-with 380-480 nm. Following the initial screening process, 150 articles underwent a full-literature review. An additional 74 papers were subsequently removed, including reviews on violet-blue light or research studies which performed experiments out-with the scope of this review, e.g. violet-blue light inactivation of: microorganisms exposed on food; bacterial biofilms; pathogens on environmental surfaces.

6.3.2 Data Extraction and Analysis

The final 76 research articles reviewed contained inactivation data on clinically-relevant and food associated microorganisms exposed to violet-blue light between 380-480 nm. Data from each article was extracted, summarised and tabulated (Appendix A). This included information on: microorganism (categorised by species and strain number); wavelength of light; irradiance of light source; applied dose and population exposed.

Subsequent analysis of inactivation kinetics was based upon that of Hessling *et al.* (2017). Log_{10} reduction values were extracted from text and tables or if unavailable, extracted from figures. The highest dose reported, for the greatest significant inactivation of each exposed population was recorded. However if prominent tailing of results occurred, the previous dose which caused a similar level of inactivation, was used in the analysis. Recording data in this way allowed the calculation of the average dose required for 1 log_{10} reduction and therefore allowed comparison between studies investigating different: organisms; irradiances of light; wavelengths of violet-blue light and exposed populations.

The data extracted includes inactivation at low (< 10 °C) or high (≥ 37 °C) temperatures, which has been indicated in the appendices (Appendix A). However as violet-blue light inactivation of bacteria has been shown to be enhanced in low or elevated temperatures (McKenzie e*t al*., 2014) the results of these studies were excluded from any figures on the dose analysis. Similarly, information on inactivation in anaerobic conditions is included in Appendix A, but similarly excluded from any additional figures on the dose analysis.

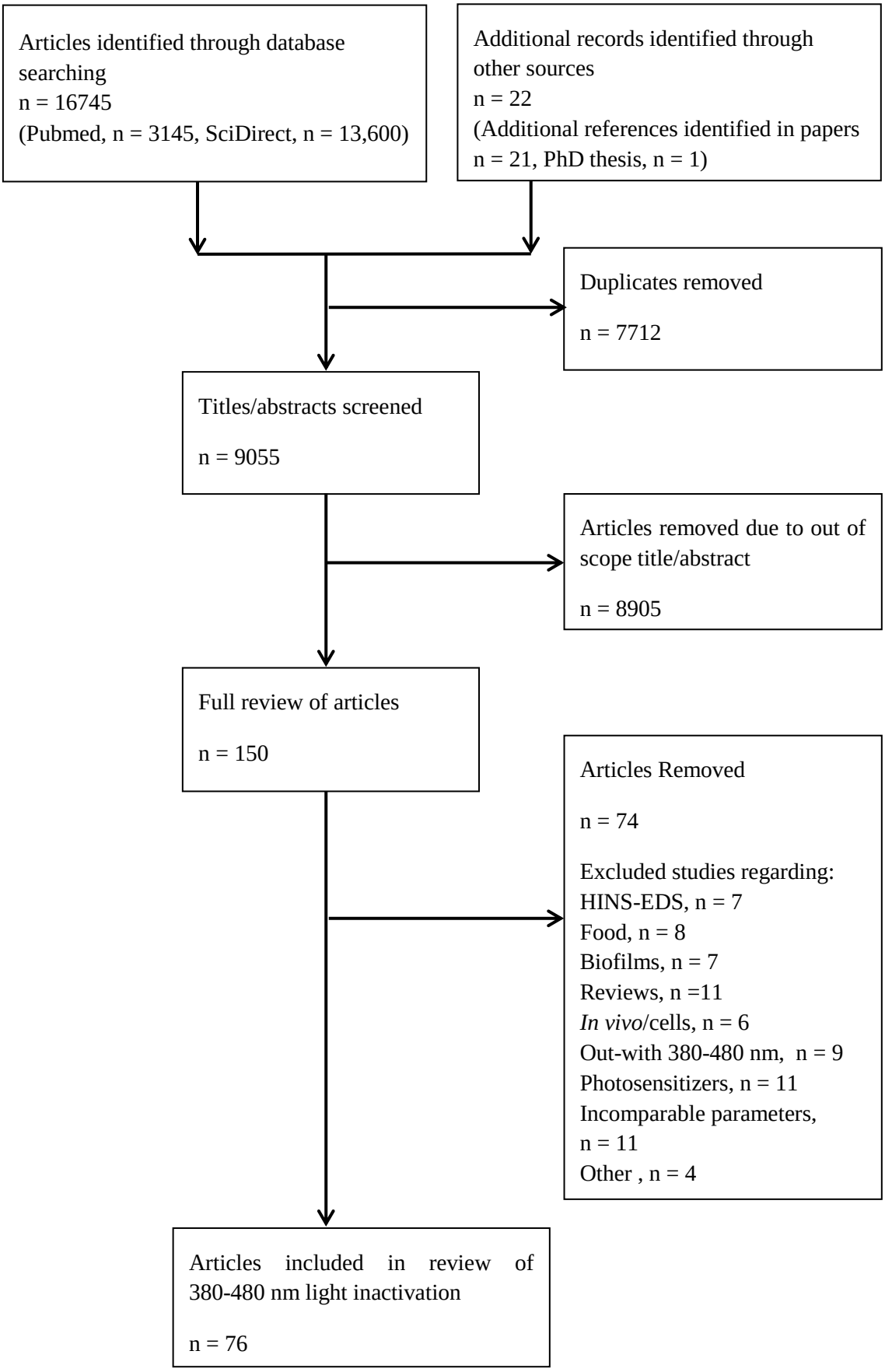


Figure 6.3 Flow chart of the study selection process during the systematised review.

6.3.3 Microbial Inactivation Using Violet-Blue Light

Data was retrieved from 76 sources providing information on the inactivation kinetics of a range of microorganisms using violet-blue light between 380-480 nm. There were 365 individual entries accumulated, including 56 bacterial strains, 8 yeasts and fungi, 1 bacteriophage and 2 mammalian viruses. The full data set gathered during the systematised review can be seen in Appendix A.

As an overall comparison of the data gathered, Figure 6.4 represents the dose required for 1 $\log_{10}$ inactivation of the different microbial species investigated. The majority of light inactivation studies have been carried out using violet-blue light peaking at 405 nm. Additionally, there is interest in inactivation at longer wavelengths of blue light including 415 nm, 450 nm and 470 nm. The majority of Gram positive and Gram negative vegetative cells required doses < 200 J/cm^2. However, Gram positive endospores, fungal spores, yeast, viruses and bacteriophage generally required > 400 J/cm^2 for a 1 $\log_{10}$ reduction.

It was possible to perform further analysis on the data collected with regards to individual Gram positive and Gram negative bacteria. The most common Gram positive organisms that have been investigated are *L. monocytogenes* and *S. aureus*, and the most common Gram negative bacteria are *E. coli* and *P. aeruginosa*. Comparisons were made between the dose for 1 $\log_{10}$ reduction using 405 nm light and the starting population or irradiance used. Additionally, the dose for 1 $\log_{10}$ reduction of these organisms was compared between the differing wavelengths (380-480 nm) of violet-blue light.

Upon comparison of the starting population with the dose of 405 nm light required for 1 $\log_{10}$ reduction, the linear fit indicates that with increasing populations of *S. aureus*, *E. coli* and *L. monocytogenes* higher doses of 405 nm light are required, whereas in the case of *P. aeruginosa* it appears that as concentration increases the dose required decreases (Fig 6.5). However, as the Pearson's r values for the linear correlation analysis of all the bacteria investigated are between −0.12 and 0.27, these indicate that there is no linear relationship, so no true conclusions can be drawn.

Upon comparison of the irradiance of 405 nm light used, the linear fit suggests that for *S.aureus* and *P. aeruginosa* there is little change in dose required for 1 $\log_{10}$ reduction as irradiance increases (Fig 6.6). However, regarding inactivation of *E. coli* and *L. monocytogenes* this is not the case, with higher irradiances resulting in the dose increasing for *E. coli* inactivation and the dose decreasing for *L. monocytogenes* inactivation. Upon

analysis of the Pearson's r values for the linear correlation, these indicate no linear relationship for *E. coli* (0.22), and a weak negative linear relationship (−0.3) for *L. monocytogenes*, therefore no true significant conclusions can be drawn.

As a final comparison, the wavelength of violet-blue light used was compared with the dose for 1 $\log_{10}$ reduction of the 4 vegetative bacteria. As can be seen in Figure 6.7, there is very little difference in dose required when exposing *S. aureus* and *P. aeruginosa* to different wavelengths of violet-blue light however this is not the case with *L. monocytogenes* and *E. coli*. As the wavelength increases the dose for 1 $\log_{10}$ reduction increases, with Pearson's r values of 0.39 and 0.73, indicating that there is a moderately strong positive correlation between the wavelength used and dose required for these organisms.

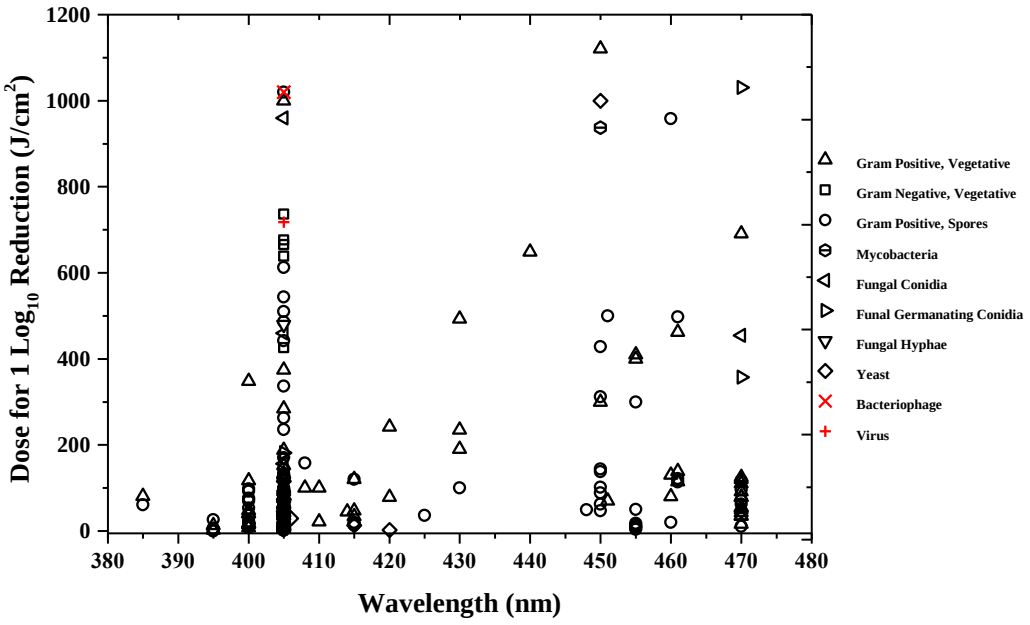


Figure 6.4 Comparison of the dose for a 1 $\log_{10}$ reduction of a range of microorganisms, compared to the wavelength of violet-blue light used for irradiation.

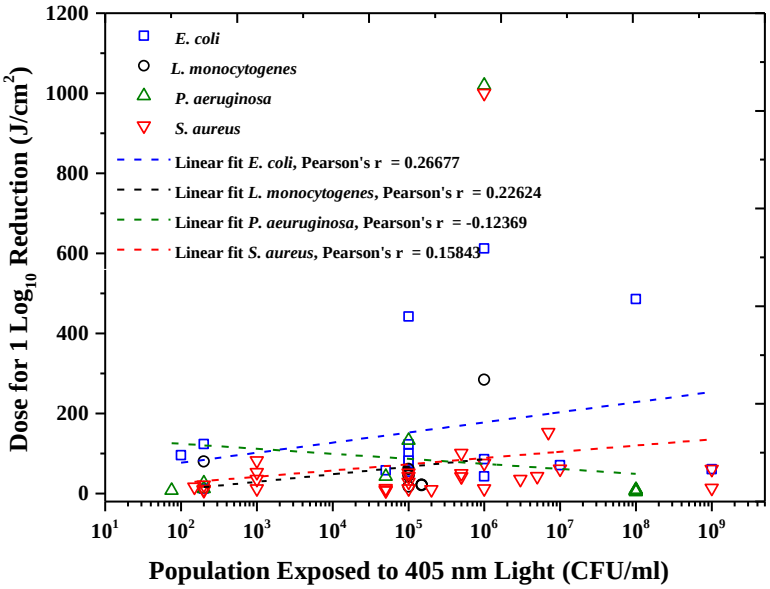


Figure 6.5 Comparison of the dose for 1 log_{10} reduction of Gram positive and Gram negative bacteria, using 405 nm light, when there are differing population densities.

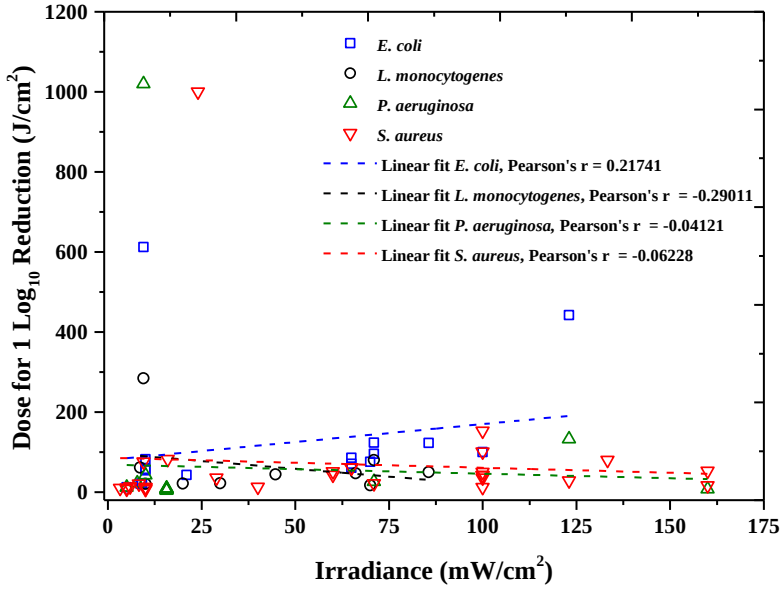


Figure 6.6 Comparisons of the dose for 1 log_{10} reduction of Gram positive and Gram negative bacteria, when exposed to differing irradiances of 405 nm light.

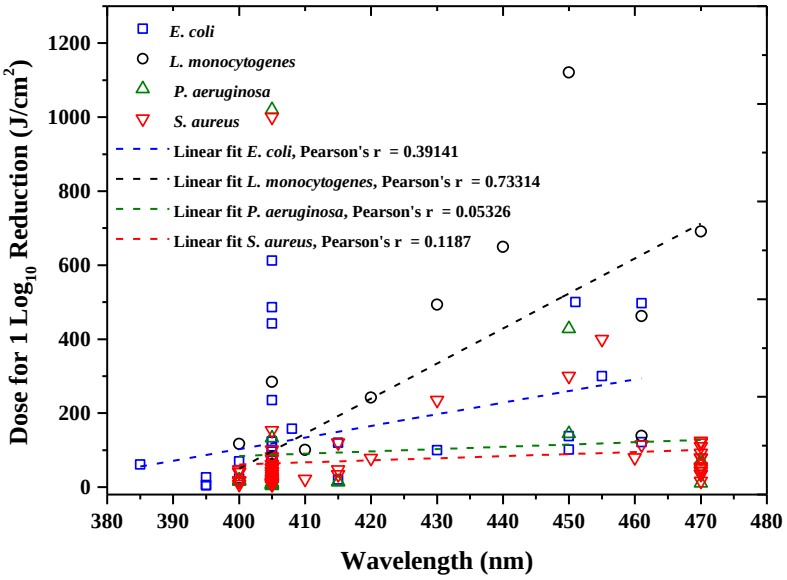


Figure 6.7 Comparisons of the dose for 1 log_{10} reduction of Gram positive and Gram negative bacteria, when exposed to differing wavelengths of violet-blue light.

6.4 Discussion

Comparison of the susceptibility of microorganisms using data generated by research conducted at ROLEST (Fig 6.1) indicates that Gram positive bacteria appear to be the most susceptible to 405 nm light inactivation, and viral particles least susceptible, requiring doses of 200 J/cm^2 and 2.8 kJ/cm^2, respectively. Results also indicate that higher doses of 405 nm light are required for bacterial and fungal spores, with > 1000 J/cm^2 needed for complete inactivation. The varying dose requirements are likely due to the specific properties of each microorganism, which could include structural differences, differences in types and levels of intracellular porphyrins, and porphyrin absorption maxima (Maclean *et al*., 2009; Murdoch *et al*., 2012). Additionally, spores are likely to require higher doses of violet-blue light for inactivation due to their innate resistance to stressful environmental conditions, and may have additional resistance features such as pigments within the walls of fungal spores, which would protect against photochemical damage (Moorhead *et al*., 2016b). The high doses required by the bacteriophage and mammalian virus are likely due to the lack of porphyrins in the viral structures and their reliance on small amounts of near-UV light and

light > 410 nm emitted from the LED array, which may induce inactivation (as discussed in Chapter 4).

As shown in Chapter 4 (Fig 4.3), viral susceptibility is enhanced when exposed whilst suspended in organically-rich media. Comparison of this enhanced viral inactivation with that of bacterial susceptibility (Fig 6.2), indicates that exposure in organically-rich media reduces the required virucidal doses to levels that are more comparable with bacterial inactivation: the lethal dose required for viral inactivation decreased from 14 × to 2 × that of *S. aureus*, when in organically-rich media. This indicates that 405 nm inactivation of viruses in photosensitive fluids, such as bodily fluids, may be similar to that of bacteria, and therefore there is potential to use 405 nm light for viral inactivation in clinical applications, as demonstrated in Chapters 4 & 5.

To further investigate the antimicrobial scope and efficacy of violet-blue light a systematised review (Section 6.3) was carried out to compare the dose required for 1 $\log_{10}$ reduction of a range of microorganisms. This:

- allowed comparison of the inactivation data generated at ROLEST (including this study) to that generated by other research groups;
- indicated how the efficacy of violet-blue compares between organisms; and
- confirmed areas of antimicrobial violet-blue light research which require further investigation.

The systematised review indicated many differences in the light sources and experimental arrangements used between research groups. Major differences in the experimental arrangement included how microorganisms were exposed, with some studies exposing organisms on agar plates whilst others exposed organisms in suspension (1 µl-40 ml). Light sources varied with different groups using single LEDs and arrays (1-144 LEDs), lasers, or broadband sources with filters, which emitted violet-blue light at irradiances ranging from 1.2-520 mW/cm^2. Exposure times also ranged from 20 seconds-24 hours, with exposed population densities of between 10^1-10^9 CFU. Furthermore, as complete data sets were not always available and at times the experimental arrangement was unclear, it was not possible to use mathematical models of inactivation kinetics such as the Kamau; Gompertz; Weibull; or Hom models (Kumar *et al*., 2015). Therefore, dose for 1 $\log_{10}$ reduction, as demonstrated by Hessling *et al*. (2017), was the most appropriate means of comparison.

Despite these methodological variations, several comparisons were able to be made using the data gathered in the systematised review, including comparing the overall susceptibility of microorganisms to violet-blue light between dose for 1 $\log_{10}$ reduction. The data in Figure 6.3 compares the dose of 380-480 nm light required between different organisms, with further analysis of this data provided in Figure 6.8, comparing the average dose requirements between microbial groups. As can be seen in Figure 6.8, Gram positive and Gram negative vegetative bacteria appear to require similar doses of violet-blue light for 1 $\log_{10}$ inactivation, with mean doses of 129 J/cm^2 (6-747.5 J/cm^2) and 100 J/cm^2 (0.3-552.9 J/cm^2) required, respectively. This differs from the ROLEST data presented in Section 6.2, which indicated that Gram positive bacteria are more sensitive to violet-blue light inactivation then Gram negative (Maclean *et al*., 2009; Murdoch *et al*., 2012; MacDonald *et al.*, 2013). However, the results in this review, do mirror those of Hessling *et al*. (2017) who also demonstrated no evidence of increased susceptibility of Gram positive bacteria when compared to Gram negative.

Additionally, results in Figure 6.8 indicate that yeast cells have similar dose requirements to vegetative bacteria; with the average dose of 125.6 J/cm^2 required for 1 $\log_{10}$ reduction. Mycobacteria, germinating fungal conidia, fungal conidia, fungal hyphae and bacterial endospores all required increasingly greater doses of violet-blue light compared to vegetative bacteria. The mean average doses being: 354 J/cm^2 for mycobacteria; 440.3 J/cm^2 for fungal conidia; 480 J/cm^2 for fungal hyphae; 523.2 J/cm^2 for germinating fungal conidia; and 640.5 J/cm^2 for bacterial endospores. Viruses appeared to be least susceptible with the highest mean doses of 718 J/cm^2 and 1020 J/cm^2 required for 1 $\log_{10}$ reduction for viruses and bacteriophage respectively.

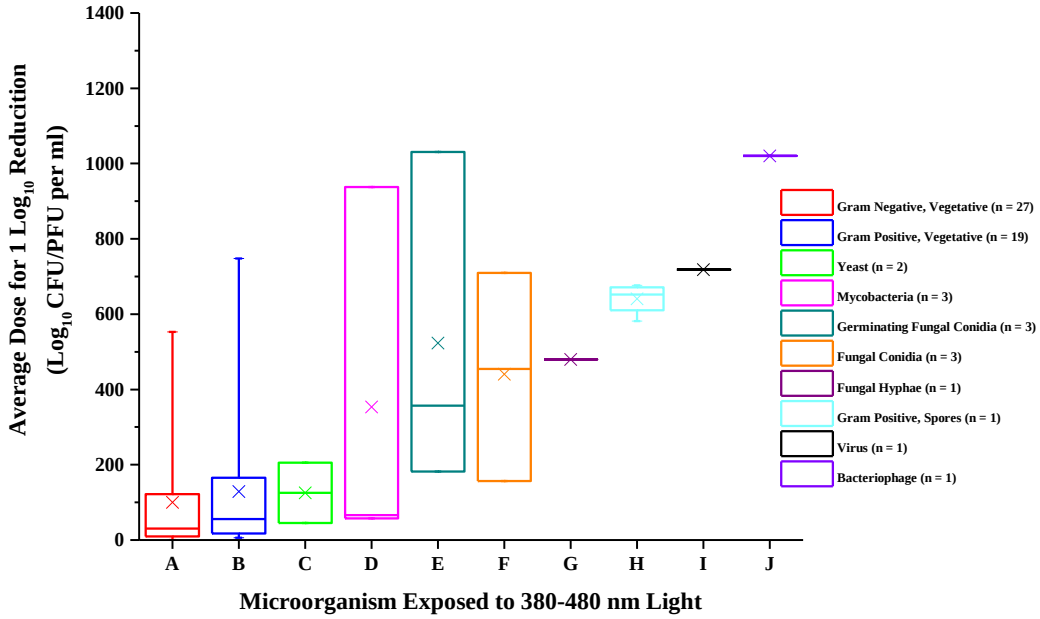


Figure 6.8 Box plot analysis of the average dose for 1 log_{10} reduction between different microbial groups. Crosses '×' indicate the mean dose for a 1 log_{10} reduction.

Comparisons were also made between several species of bacteria due to the large number of results collected during the systematised review. The effect of population density and irradiance of 405 nm light on the average dose for 1 log_{10} reduction was compared between *E. coli*, *L. monocytogenes*, *P. aeruginosa* and *S. aureus*. With regards to the population density used, the linear correlation trends indicated a slight increase in the dose requirements upon increasing population, with the exception of *P. aeruginosa*. This is reflected in a study by Maclean *et al*. (2009) who demonstrated that a dose of 36 J/cm^2 was required for a 3 log_{10} reduction of 10^3 and 10^7 CFU/ml populations of *S. aureus* compared to a slightly increased dose of 41 J/cm^2 for equivalent reduction of a 10^9 CFU/ml population. The increase in dose was attributed to attenuation of light passing through a 10^9 CFU/ml population of *S. aureus*. Light irradiance reduced from 10 mW/cm^2 at the sample surface to 5.6 mW/cm^2 after passing through the sample, which was not seen in samples with a lower population density (Maclean *et al*., 2009). Additionally, Bumah *et al*. (2013) demonstrated that bacterial density does not affect the bactericidal effect of 405 nm and 470 nm light, but the reduced light penetration of suspending liquids, due to increased bacterial concentration, is likely to limit bactericidal effect. These results are similar to that of Hessling *et al*. (2017) who also found no significant correlation between the starting population and dose requirements.

Trends in Figure 6.6 indicate little change in the dose for 1 $\log_{10}$ reduction with increasing irradiance of 405 nm light (with the exception of *L. monocytogenes*), with no significant correlation seen. However in the case of *L. monocytogenes*, as the irradiance increased there was a slight decrease in the dose required. This opposes results demonstrated by Murdoch *et al*. (2012) who exposed *L. monocytogenes* to different irradiances (10, 20, 30 mW/cm^2) of 405 nm light. Following a dose of 108 J/cm^2 there was a slight decrease in inactivation from 5.18 $\log_{10}$ to 4.9 $\log_{10}$ reduction when irradiance was increased from 10-30 mW/cm^2. However, as the linear relationship was deemed weak in Figure 6.6, it is still likely that there is an absorption maxima regardless of the irradiance of light used.

Stronger correlation was observed with the relationship between dose and violet-blue light wavelength. In the case of *S. aureus* and *P. aeruginosa* there was little change in dose with increasing wavelength, however stronger positive linear correlations were seen for *E. coli* and *L. monocytogenes* (Fig 6.7). This indicates that some bacterial strains may be more sensitive to shorter wavelengths closer to 405 nm rather than those towards 470 nm (Fig 6.7). 405 nm light was also demonstrated to be the most effective wavelength for microbial inactivation by Endarko *et al*. (2012). Exposure of *L. monocytogenes* to wavelengths between 400-450 nm, achieved maximum inactivation (1.45 $\log_{10}$ reduction) following exposure to 405 nm light and least inactivation, (0.04 $\log_{10}$ reduction) using 450 nm light following a dose of 123.3 J/cm^2 (Endarko *et al*., 2012).

As the methodology to calculate dose for 1 $\log_{10}$ reduction was adapted from Hessling *et al*. (2017), it is interesting to compare results between reviews. There were several differences with regards to the calculation for 1 $\log_{10}$ reduction, this is likely due to differing interpretation of inactivation kinetics from figures. For example, several studies stated that they exposed 100-300 CFU/plate and Hessling *et al*. (2017) recorded this as 1×10^3 CFU/ml so this may also have affected dose calculation with regards to $\log_{10}$ reduction. Additionally, there were several instances during comparisons that major differences were noted. The first case of this was regarding the inactivation of *B. subtilis* vegetative cells. Hessling *et al*. (2017) stated that using 408 nm and 451 nm light resulted in an average dose of 69.8 J/cm^2 and 100 J/cm^2, respectively, for 1 $\log_{10}$ reduction (adapted from Hoenes *et al*., 2015). However upon review of work by Hoenes *et al*. (2015) it appears the dose requirements had been mixed-up between wavelengths. Another difference was noted for the dose requirements for *C. difficile*. Hessling *et al*. (2017) stated that the dose for 1 $\log_{10}$ reduction of spores was 76.4 J/cm^2 (adapted from Moorhead *et al*., 2016a). The true value is actually much higher at 736.4 J/cm^2, due to natural resistance of bacterial spores.

Additionally, it appears that Hessling *et al*. (2017) calculated $\log_{10}$ reduction compared to the starting population, whereas in this study, $\log_{10}$ reduction was compared to equivalent non-exposed control population, as often as possible. This was evident when comparing the average dose for *B. cereus*. Hessling *et al.* (2017) calculated the dose for 1 $\log_{10}$ reduction using a 3.6 $\log_{10}$ reduction value however the study by Maclean *et al*. (2013) states that there was a 1 $\log_{10}$ reduction of the control, so the true reduction is actually 2.6 $\log_{10}$. Comparing data on *E. coli* inactivation from a study by McKenzie *et al*. (2016), highlighted additional differences. McKenzie *et al*. (2016) achieved 3.3 $\log_{10}$ and 7.7 $\log_{10}$ reductions of 10^7 and 10^9 CFU/ml populations of *E. coli* following doses of 234 J/cm^2 and 707 J/cm^2 405 nm light, respectively. However Hessling *et al*. (2017) stated that a 10^7 CFU/ml population received an applied dose of 702 J/cm^2, which is not actually stated anywhere in the original research paper.

The discrepancies with Hessling *et al*. (2017) are likely due to variation in the methods of analysis used or user error considering the large amount of data being processed. However, these differences may have altered the subsequent analysis performed by Hessling *et al*. (2017). This study used the correct values (stated above), reviewed more papers on bacterial inactivation by violet-blue light than Hessling *et al.* (2017) (including those by Kawada *et al*., 2002; Kotoku *et al*., 2009; Gupta *et al*., 2015; Barneck *et al*., 2016; Decarli *et al*., 2016; Gillespie *et al*., 2017; Kim *et al*., 2016; Hope *et al*., 2016; Fila *et al*., 2017; O'Donoghue *et al*., 2017), and also included inactivation data on fungi, viruses and yeasts. Therefore, it is possible to argue that this review is a more thorough analysis as a greater number of studies have been included, and represents a more accurate representation of the efficacy of violet-blue light between 380-480 nm.

Several other interesting comparisons in respect of temperature and anaerobic conditions during exposure, can be made using the data from the systematised review (Appendix A). Decreased or elevated temperatures during violet-blue light treatment had a varied effect on bacterial inactivation. In the case of 405 nm light exposure in low temperatures ($\leq$ 10°C) there was little change in inactivation of *B. cereus*, *Lactobacillus planetarium* and *P. aeruginosa* (Kumar *et al*., 2015; 2016), however there was enhanced inactivation of *E. coli*, and *L. monocytogenes* (McKenzie *et al*., 2014). Additionally, inactivation of *S. aureus* was reduced, with approximately 50% less inactivation than that achieved at room temperature (2.1 vs 4 $\log_{10}$ reduction) (Kumar *et al*., 2015, 2016). When temperature was increased ($\geq$ 37°C) during violet-blue light exposure, there was enhanced inactivation of *E. coli, L. monocytogenes and P. aeruginosa* (De Lucca *et al*., 2012; McKenzie *et al*., 2014).

This was particularly striking in *L. monocytogenes* with half the dose required (42 vs 84 J/cm^2) for a 5 $\log_{10}$ reduction following 405 nm light exposure at an irradiance of 70 mW/cm^2 (McKenzie *et al*., 2014). McKenzie *et al*. (2014) hypothesised that the enhanced inactivation seen during exposure in these stressed conditions may be a result of structural or metabolic stresses (due to the temperature) which when combined with 405 nm light, increased microbial susceptibility to ROS and subsequent oxidative damage.

As it is known that oxygen plays an essential role in the photoinactivation of microorganisms using violet-blue light (Maclean *et al*., 2009) it was interesting to compare inactivation of microorganisms exposed in anaerobic environments. Under these conditions there was little to no inactivation of bacteria, including *S. aureus, E. coli* and *E. faecalis* and significantly reduced inactivation (1-5 $\log_{10}$ less) of fungi and yeasts, including *A. niger, C. albicans* and *S. cerevisiae* (Murdoch *et al*., 2013; Hope *et al*., 2016). However up to 4 $\log_{10}$ inactivation of *Prophyriomonas gingivalis, Prevotella intermedia, Prevotella nigrescens* and *Propionibacterium acnes* could be achieved in anaerobic conditions following violet-blue light exposure (Ashkenzei *et al*., 2003; Hope *et al*., 2013, 2016). These results further demonstrate that environmental pathogens such as *S. aureus* require oxygen for violet-blue light inactivation (Maclean *et al*., 2009), however they also indicate that anaerobic oral bacteria such as *P. gingivalis* may not require oxygen for inactivation during violet-blue light exposure. Hope *et al.* (2016) hypothesised that inactivation of these oral bacteria may be due to Type I reactions occurring within bacterial cells, producing ion radicals which could cause damage to cellular structures without requiring oxygen as an intermediate. This demonstrates that, in certain oxygen depleted scenarios, violet-blue can still be used for microbial inactivation.

As discussed in Chapter 2, 405 nm light inactivation of bacteria is thought to occur through excitation of intracellular porphyrin molecules, resulting in oxidative damage, promoting cellular damage and cell death. This inactivation mechanism has benefits over many other current chemical and technological antimicrobial treatments, in that it is unlikely to induce DNA damage, making organisms less likely to develop genetic mutations and acquire resistance, making it a safer alternative for human exposure. Additionally, the non-specific oxidative damage exerted on exposed microorganisms enables effective inactivation of a wide range of microbial species. However, very few of the papers reviewed attempted to investigate the mechanism of inactivation of violet-blue light, through use of ROS scavengers; porphyrin identification; or by detecting bacterial damage. Therefore, there is a definite requirement for the inactivation mechanism to be fully established.

For many of the bacterial species (Appendix A) only one strain has been studied, therefore it would be interesting for future research to investigate a wider range of strains within a bacterial species. Additionally, as antimicrobial violet-blue light is being developed for a range of clinical applications, it is important to expand the number of clinical isolates investigated as there can be large variations in dose requirements. This was demonstrated by Halstead *et al*. (2016) who showed variations in the inactivation of isolates from an English hospital exposed to 400 nm light. Variation in dose requirements was particularly notable in clinical isolates of *Stenotrophomonas maltophilia,* with between 2.97-7.33 $\log_{10}$ reduction achieved following a dose of 108 J/cm^2 (Halstead *et al*., 2016). Very few of the organisms tested in the 76 studies reviewed have also been MDR strains, however dose requirements do seem to be similar between antibiotic sensitive and antibiotic resistant organisms. For example, an average dose of 7.14 J/cm^2 and 7.85 J/cm^2 was required for 1 $\log_{10}$ reduction of drug sensitive and multidrug resistant *P. aeruginosa* (Fila *et al.,* 2017). As these pathogens are an increasing problem in the hospital environment with very few treatment options (Pendelton *et al*., 2013), it is very important to continue to establish their susceptibility to violet-blue light. Successful demonstration of the reduction of MDR organisms would certainly support the use of 405 nm light for environmental decontamination in hospitals.

It was also apparent when comparing the data collected during the systematised review that there is a lack of evidence regarding the antimicrobial effect of violet-blue light on fungi, yeasts, protozoa and viruses. Only 10 of the studies reviewed investigated fungi and yeasts with the majority focussing on Gram positive and Gram negative bacteria. Although work has been carried out on *C. albicans*, *A. niger* and *Fusobacterium* spp., further work is required to investigate inactivation of additional hospital acquired pathogens such as *Candida* spp. (*C. glabrata, C. parapsilosis; C. tropicalis*), *Aspergillus* spp. (*A. fumigates; A. flavus*), *Mucorales*, *Fusarium* spp. (*F. moniliforme*, *F. solani,* and *F. oxysporum*), and *Scedosporium* spp. (*S. apiospermum* and *S. prolificans*) (Perlroth *et al*., 2007).

Evidence on the potential for violet-blue light inactivation of other microorganisms such as protozoa and viruses is also limited. In all the studies reviewed, only one investigated the inactivation of protozoa (namely *Acanthamoeba polyphaga*, ATCC 30461), with inactivation measured in fluorescence (Decarli *et al*., 2016). After doses of 300 J/cm^2 of 450 nm light, there was a 42% reduction in fluorescence of a 10^6 CFU/ml population compared to the un-exposed control (De Carli *et al*., 2016). Future work could investigate other protozoa which are harmful to human health such as *Blastocystis hominis, Cryptosporidium parvum, Entamoeba histolytica* and *Giardia lamblia*

(Sandokji *et al*., 2009). The published results from Chapter 4 are also the only evidence of the virucidal efficacy of 405 nm light against mammalian viruses (Tomb *et al*., 2016). Therefore, additional studies are required to support these findings and investigate efficacy against nosocomial viruses such as Adenovirus, Influenza and Rotavirus (Gleizes *et al*., 2006; Eibach *et al*., 2014; Ganime *et al*., 2016).

To further support the clinical application of violet-blue light it would be important to begin to standardise microbial inactivation studies between differing research groups. One way of doing so would be to distribute a standardised test panel of organisms, suspended on a 96-well plate, to research groups working with violet-blue light or hospitals utilising the HINS-light EDS. The results could then be collected and analysed to ensure that all groups involved are achieving similar levels of inactivation. Therefore, outcomes from future studies would be considered more robust and could be directly compared between groups.

As a final observation, it was clear from the systematised review that there is little known about the potential for microorganisms to become tolerant to violet-blue light inactivation. Only 3 of the studies reviewed investigated the potential for tolerance development in bacteria, with differing results (Amin *et al*., 2016; Guffey *et al*., 2013a; Zhang *et al*., 2014). It is particularly important to continue research on the potential for tolerance development, to ensure that evidence is generated from several research groups, using different experimental arrangements, allowing accumulation of data so users can form unbiased opinions. Investigating tolerance will also help to ensure that violet-blue light is effectively used within the clinical environment, utilised in a way that in unlikely to result in resistance and provide reassurance to end-point users.

Chapter Seven

Investigating Potential for Bacterial Tolerance to 405 nm Light

7.0 Overview

As discussed in Section 2.5.3, the bactericidal efficacy of 405 nm light is well established. However, a key question to be addressed for this, and indeed, all new decontamination technologies, is whether there is potential for bacteria to become tolerant to its antimicrobial effects. This chapter investigates the likelihood of tolerance development under two distinct scenarios: after culture in low-intensity 405 nm light (proliferating cells), and after repeated sub-lethal exposure to 405 nm light (non-proliferating cells).

7.1 Introduction

Over the past twenty years there has been an increase in bacterial resistance to antibiotics and a reduction in development of new antibiotics. The 'Review on Antimicrobial Resistance' by the UK government estimates that currently there are 700,000 deaths per annum worldwide due to antimicrobial-resistant organisms including those which cause tuberculosis and malaria, as well as nosocomial pathogens (O'Neill 2016). It is estimated that if resistance levels continue to rise, then by 2050 there will be 10 million deaths annually (O'Neill 2016).

Bacteria have been able to develop various resistance mechanisms primarily due to misuse of antibiotics, including: over-prescription; failure of patients to complete courses; and their over-use in farming and aquafarming (Laxminarayan *et al*. 2013; Cabello *et al*., 2016). Inconsistent hygiene practices in hospitals and low levels of personal hygiene also contribute (Laxminarayan *et al*. 2013). The two main resistance mechanisms in bacteria are known as intrinsic and acquired resistance. Intrinsic resistance occurs when bacteria have the natural ability to decrease sensitivity to a particular agent, whereas acquired resistance involves mutations of normal genes or acquisition of foreign genetic material, which allow bacteria to survive (most common in Gram negative species) (Russel 1999). Mechanisms which

bacteria employ to evade antibiotics include: cellular efflux pumps; impermeable cellular membranes; and the production of neutralizing intracellular enzymes (Frère 1995; Delcour 2009).

As discussed in Section 2.1 antibiotic resistant organisms in the clinical environment include methicillin-resistant Gram positive bacteria such as *S. aureus* and MDR Gram negative bacteria such as *A. baumannii, E. coli, K. pneumoniae* and *P. aeruginosa.* Antibiotic choices to treat these pathogens are becoming limited with resistance to the last resort drug, colistin, recently discovered via a transferable, plasmid-mediated, colistin-resistant gene (*mcr-1*) in countries including China, the UK and the USA (McGann *et al.,* 2016).

Therefore, as we approach the end of the so called 'antibiotic era', alternative decontamination strategies are required to prevent the spread of these MDR organisms. These organisms are posing greater strain on healthcare systems in a number of ways. Patients require longer hospital stays due to increased morbidity; infections are harder to treat as there are reduced antibiotic choices for clinicians (with alternatives being expensive drug combinations which can lead to higher patient mortality); and greater precautions are required to prevent spread amongst patients and staff (Cosgrove 2006).

405 nm light is one such technology designed to reduce transmission of these pathogenic organisms, aiding environmental decontamination and clinical treatment. As discussed in Section 2.5.3, 405 nm light has extensive antimicrobial action (Guffey and Wilborn 2006; Maclean *et al*., 2009, 2013b; McKenzie *et al*., 2013; Murdoch *et al*., 2012, 2013; Tomb *et al*., 2014, 2016); can be used at levels which are non-detrimental to mammalian cells (McDonald *et al*. 2013; Dai *et al*. 2013a, b; Ramakrishnan *et al*. 2016); can be used for continuous decontamination of occupied environments (Maclean *et al*., 2010, 2013a, 2014; Bache *et al*., 2012); and can be effectively used for wound decontamination (Dai *et al*., 2013b). However, little is known about the potential for the development of bacterial tolerance to violet-blue 405 nm light inactivation.

It is hypothesised that tolerance is unlikely due to the mechanism of inactivation (Dai *et al.,* 2013a; Maclean *et al*., 2014; Amin *et al*., 2016). Similar to that of PDI, which makes use of an additional photosensitizer, the mechanism of inactivation is thought to be non-selective, as ROS and $^1O_2$ produced cause unspecific damage to a wide spectrum of targets within bacterial cells (Tavares *et al.,* 2010; Dai *et al.,* 2013a, Maclean *et al.,* 2014; Amin *et al*., 2016). Nevertheless, there is little evidence to support this. Several studies have investigated tolerance formation following repeated PDI in a range of microorganisms

including *Actinobacillus actinomycetes, E. coli, Peptostreptococcus micros, P. aeruginosa, S. aureus* and *Vibrio fischeri*, in which none of the aforementioned species were found to become tolerant (Lauro *et al*., 2002; Pedgio *et al*., 2009; Giulinani *et al*., 2010; Tavares *et al*., 2010; Bartolomeu *et al*., 2016). At the time of literature review only three studies had been identified which focussed on tolerance formation in bacteria following exposure to violet-blue light alone. An initial study by Guffey *et al*. (2013a) demonstrated potential for *S. aureus* to become tolerant to 405 nm light, as after 7 repeated exposures there was a decrease in the kill rate. Conversely, two studies using 415 nm light demonstrated no evidence of tolerance formation following 10 sub-lethal exposures in *A. baumannii and P. aeruginosa* (Zhang *et al*., 2014; Amin *et al*., 2016).

As current results are conflicting, this study was carried out to investigate if tolerance to 405 nm light could be evidenced in the nosocomial pathogen *S. aureus*. The first stage of the study was designed to investigate whether differences could be detected in the 405 nm light sensitivity or stress response of *S. aureus* after culture under different lighting conditions (dark, white, low-level blue). The study then progressed to investigate the effect of repeated sub-lethal exposure of *S. aureus* to high-intensity 405 nm light. To further investigate the likelihood of tolerance, the inactivation kinetics and antibiotic susceptibility of survivors after sub-lethal exposure, were analysed and compared.

Finally, in order to evaluate the potential for tolerance development, it is important to fully understand the mechanism of 405 nm light microbial inactivation. As discussed in Section 2.5.1, inactivation is thought to be due to the excitation of endogenous porphyrins. To provide additional evidence on the inactivation mechanism of violet-blue light, High Performance Liquid Chromatography (HPLC) was carried out to identify and quantify the endogenous porphyrins within *S. aureus*.

To summarise, this chapter examines the potential for bacterial tolerance to 405 nm light and is divided into three main subsections as follows:

- Determining the potential for tolerance development following culture under different lighting conditions, to understand the effect of low-level light stress when bacteria are proliferating, such as those present in nutritious areas such as wounds.
- Determining the potential for tolerance development following repeated sub-lethal exposure to high-intensity 405 nm light, to model how repeated exposure would occur in stationary, non-proliferating bacteria such as those in the environment.
- Analysis of the presence of endogenous photosensitive porphyrins within *S. aureus.*

7.2 Determining Potential for Tolerance Development when Cultured under Different Lighting Conditions

This section of work investigates the differences in the 405 nm light susceptibility of *S. aureus* following culture in different lighting conditions: white, dark and low-intensity 405 nm light. The aim was to determine whether culture in low-level 405 nm light will subsequently affect the stress response and inactivation kinetics of *S. aureus*.

7.2.1 Bacterial Preparation and Cultivation in Different Lighting Conditions

Methicillin-sensitive *S. aureus* NCTC 4135 was inoculated in 100 ml nutrient broth and cultivated under the following lighting conditions:

- complete darkness (flasks wrapped in aluminium foil);
- white light (normal laboratory fluorescent lighting), at approximately 200 Lux, measured using a Light Level Meter (Labfacility, UK);
- 3 levels of low-intensity 405 nm light: 0.15, 0.5 & 1 mW/cm^2, using a 9-LED array, as detailed in Section 3.5.2.

When using low level 405 nm lighting, the LED array and power supply were placed in a 37 °C incubator and allowed to stabilise before setting the irradiance; this ensured that the current would not fluctuate during temperature increase. The voltage was set to 9.01 V, 9.33 V and 9.63 V to achieve irradiances of 0.15 mW/cm^2, 0.5 mW/cm^2 & 1 mW/cm^2, respectively. The irradiance was then measured 30 cm from the LED array, where the centre of the 500 ml flask would be positioned during bacterial cultivation. This was repeated with the power meter positioned inside the empty flask against the LED-facing surface, and there was no change in the irradiance levels, with 0.15, 0.5 and 1 mW/cm^2 recorded at the 3 settings. Measuring at this central point correlated with the irradiance of 405 nm light which would transmit through the flask to the broth.

Bacteria were cultured in these lighting conditions under rotary incubation (120 rpm) at 37 °C for 18-24 hours (Fig 7.1). The broths were then centrifuged at 3939 ×*g* for 10 min and the pellets re-suspended in 100 ml PBS. Bacterial suspensions were then diluted in PBS to an experimental starting population of 1-2 × 10^5 CFU/ml.

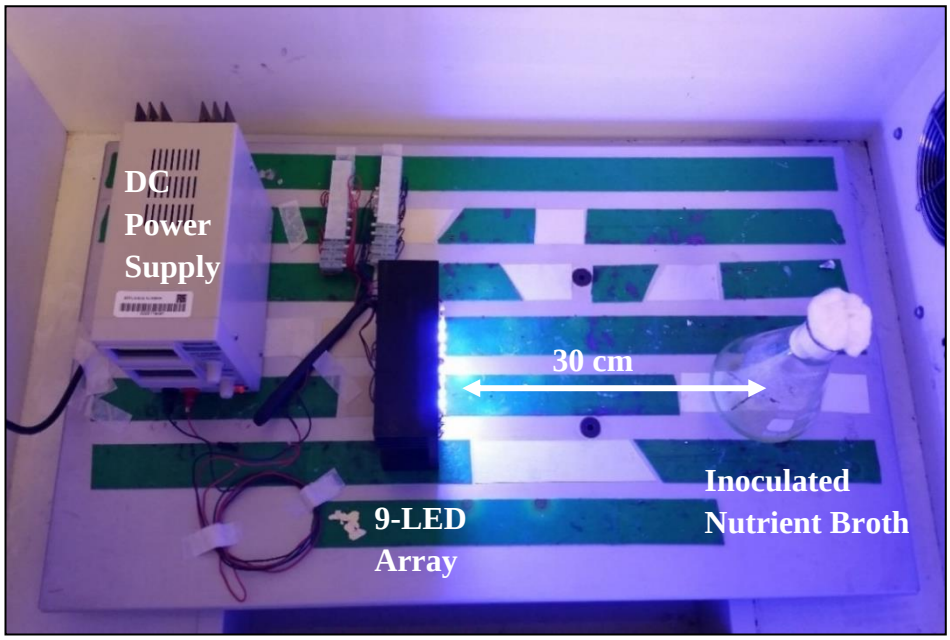


Figure 7.1 Experimental arrangement of the cultivation of Staphylococcus aureus under low-irradiance 405 nm light. Samples were exposed at an irradiance of 0.1, 0.5 or 1.0 mW/cm^2. The experimental set-up was housed in a rotary incubator (120 rpm), at 37 °C for 18-24 hours.

7.2.2 Exposure to High-Irradiance 405 nm Light Following Cultivation under Different Lighting Conditions

Following cultivation, the bacteria were exposed to high-irradiance 405 nm light to establish if their susceptibility to 405 nm light had been affected by pre-culture in the different lighting conditions. The light source used for inactivation was a high-power 405 nm LED array, as detailed in Section 3.5.1.

The LED array was placed in a PVC housing approx. 6 cm from the surface of bacterial samples. 5 ml bacterial samples were held in the central wells of 6-well plates then exposed to increasing doses of 405 nm light, using an irradiance of 60 mW/cm^2, under constant agitation using a magnetic stirrer (Fig 7.2). Control plates were held under identical conditions but subjected to normal laboratory lighting.

Samples (100 µl, 200 µl) were taken at 15 minute intervals during high-intensity 405 nm light exposure, and spread onto NA plates, with 500 µl samples also plated after final time

points. The plates were incubated at 37 °C for 24 hours and then enumerated, with results reported as CFU/ml as a function of dose (J/cm^2).

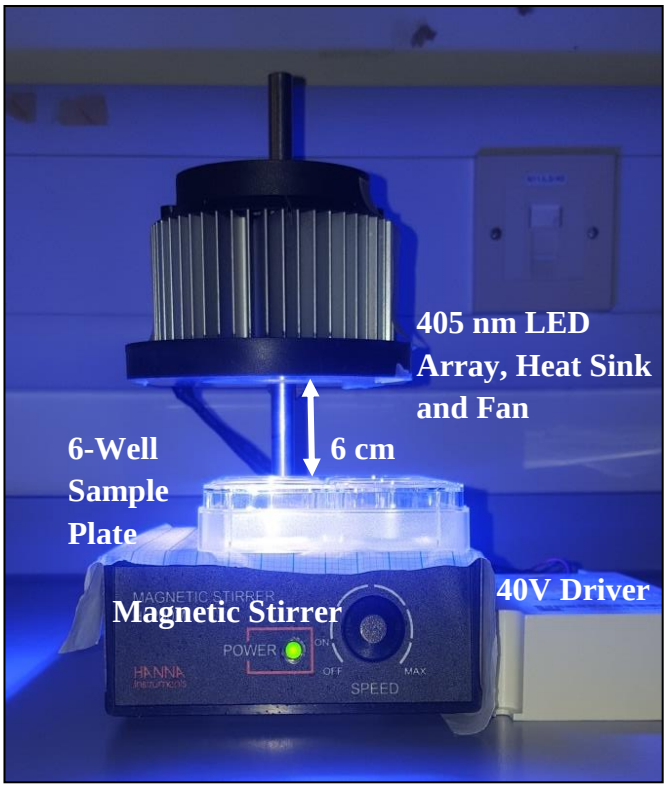


Figure 7.2 Experimental arrangement of the exposure of 5 ml samples of Staphylococcus aureus to high-intensity 405 nm light. Samples were exposed at an irradiance of 60 mW/cm^2, under constant agitation (using a magnetic stirrer).

7.2.3 Investigating the Minimum Inhibitory Concentration of Hydrogen Peroxide

The minimum inhibitory concentration (MIC) assay of hydrogen peroxide (H_2O_2) was based upon that used by Lipovsky *et al*. (2009). H_2O_2 dilutions were prepared by performing 2-fold dilutions of 6 % H_2O_2 in NB until the final dilution contained 1.8×10^{-3} % H_2O_2. Following 4-5 hours of cultivation in white light, complete darkness or 1 mW/cm^2 405 nm light, 100 µl *S. aureus*, at a density of 10^6 CFU/ml, was added to two-fold dilutions of H_2O_2 in NB. The H_2O_2 dilutions were then incubated at 37 °C for 24 hours and the MIC identified as the lowest concentration of H_2O_2 which caused visible inhibition of bacterial growth.

7.2.4 Carotenoid (Staphyloxanthin) Extraction

Carotenoid extraction was adapted from the methodology used by Bartolomeu *et al.* (2016). *S. aureus* was cultured in white light, complete darkness or 1 mW/cm^2 405 nm light for 24 hours. After cultivation, the cultures were washed thrice in sterile H_2O, re-suspended in 5 ml 99.9% methanol and incubated in a 55 °C water bath for 15 mins until the cells had been bleached. Following extraction, the samples were centrifuged at 8000 ×*g* for 10 mins, the supernatant removed and re-centrifuged at 10,000 ×*g* for 15 mins to ensure removal of any residual biomass. The carotenoid content was then measured by recording the absorbance at 465 nm, using a Biomate 5 Spectrophotometer.

7.2.5 Results: Bacterial Inactivation Kinetics following Single Culture

As can be seen in Figure 7.3, *S. aureus* cultivated in white light or complete darkness, showed relatively linear inactivation when exposed to high-intensity 405 nm light, with 5 $\log_{10}$ reductions achieved after a final dose of 216 J/cm^2. No significant differences in inactivation kinetics between *S. aureus* cultures cultivated in white light or darkness were detected at any of the tested sampling points using a (two-Sample T-test; $P \geq 0.05$).

When cultured in the presence of low-intensity 405 nm light, (0.15, 0.5 or 1 mW/cm^2), some slight differences in the inactivation kinetics were observed. Although trends appear similar (Fig 7.4), differences in the susceptibility of bacteria cultured in low intensity 405 nm light versus dark cultured can be seen at certain doses. Following exposure to 108 and 162 J/cm^2 high-intensity 405 nm light, *S. aureus* cultured in 0.15, 0.5 and 1 mW/cm^2 405 nm light showed significantly less inactivation than that cultured in complete darkness ($P < 0.05$). By a final dose of 216 J/cm^2, the inactivation achieved between the dark cultured and low-irradiance 405 nm light cultured *S. aureus* was similar (~4-5 $\log_{10}$ reduction), with the exception of 1 mW/cm^2 405 nm light: when cultured under this lighting condition, only 3.3 $\log_{10}$ reduction was achieved compared to 5 $\log_{10}$ reduction for dark-cultivated *S. aureus* ($P = 0.045$).

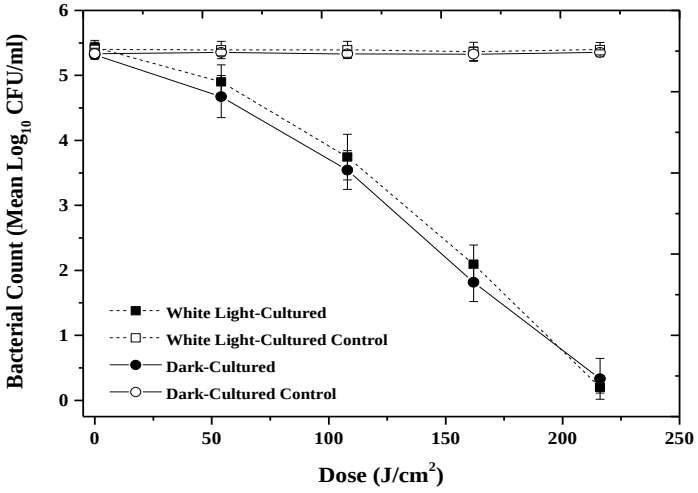


Figure 7.3 Inactivation of methicillin-sensitive Staphylococcus aureus by exposure to 60 mW/cm^2 405 nm light after culture in white light or complete darkness. Data points show the mean counts (n ≥ 6) ± SD. No significant differences were found between the susceptibility of the light and dark cultured *S. aureus* ($P \geq 0.05$), using two-sample T-test.

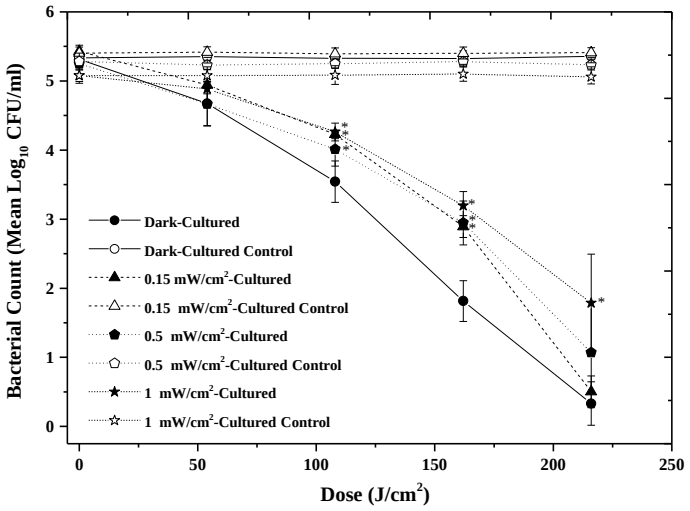


Figure 7.4 Inactivation of methicillin-sensitive Staphylococcus aureus by exposure to 60 mW/cm^2 405 nm light after overnight culture in low-level 405 nm light (0.15, 0.5 and 1 mW/cm^2). The inactivation kinetics of dark-cultured *S. aureus* have been included for reference. Data points show the mean counts (n ≥ 6) ± SD. Asterisks '*' indicate 405 nm light-cultured samples that were significantly different to those which had been dark-cultured ($P < 0.05$), using one-way ANOVA.

7.2.5.1 Investigating the Minimum Inhibitory Concentration of H_2O_2

To detect changes in the ability of *S. aureus* to tolerate oxidative stress after growth in 1 mW/cm^2 405 nm light compared to white light or complete darkness, a MIC assay using H_2O_2 was carried out, as well as measurement of the carotenoid content of each culture. Analysis was carried out using bacteria which had been cultivated in 1 mW/cm^2 405 nm light, as this culture demonstrated the greatest tolerance to high-intensity 405 nm light compared to bacteria grown in darkness, and was therefore likely to demonstrate greatest levels of oxidative stress tolerance. Results in Table 7.1 demonstrate that the MIC of H_2O_2 is significantly higher for *S. aureus* grown in 1 mW/cm^2 405 nm light compared to when grown in white light or complete darkness.

Table 7.1 Differences in the minimum inhibitory concentration (MIC) of hydrogen peroxide (H_2O_2) and carotenoid content of methicillin-sensitive Staphylococcus aureus when cultivated in different lighting conditions. Data points represent the mean counts (n = 3) ± SD. * represents a significant change ($P < 0.05$), using one-way ANOVA.

Growth Conditions	Average Minimum Inhibitory Concentration of H_2O_2 (%) ± SD	Average Carotenoid Absorbance at 465 nm ± SD
Complete Darkness	0.00293 ± 0.00000	0.90 ± 0.22
White Lighting	0.00513 ± 0.00146	0.82 ± 0.24
Low irradiance (1 mW/cm^2) 405 nm Light	0.01831 ± 0.01025*	0.17 ± 0.12*

7.2.5.2 Carotenoid (Staphyloxanthin) Extraction

Figure 7.5 visually demonstrates that the presence of the carotenoid pigment, staphyloxanthin, is significantly lower in *S. aureus* grown in 1 mW/cm^2 405 nm light compared to that grown in white light. The *S. aureus* pellet is completely bleached after growth in low-level 405 nm light and no longer has the golden pigmentation typical of when cultivated in white light.

This is supported by the quantitative results shown in Table 7.1, which demonstrate that staphyloxanthin levels are significantly lower in *S. aureus* cultured in 1 mW/cm^2 405 nm light (P = 0.009) compared to that grown in white light (P = 0.015) or complete darkness (P = 0.008).

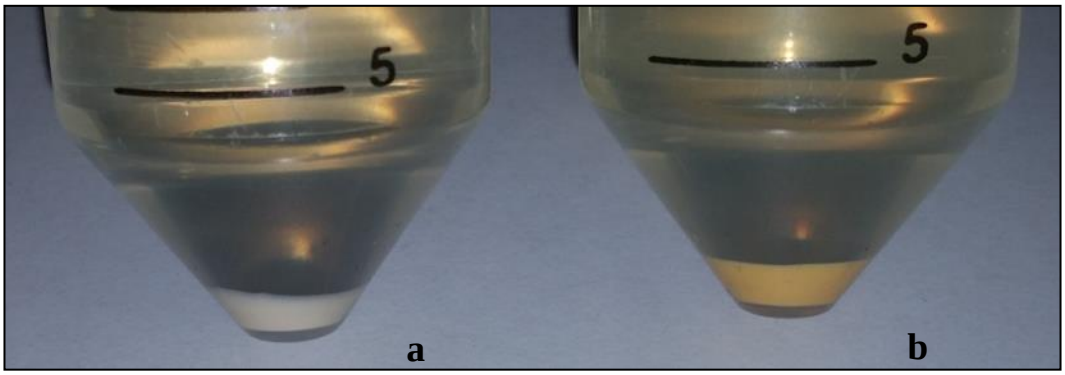


Figure 7.5 Appearance of Staphylococcus aureus cell pellets following 18-24 hour cultivation in (a) 1 mW/cm^2 violet-blue light and (b) white light, (200 Lux).

7.2.6 Results: Bacterial Inactivation Kinetics following Multiple Cultures in 1 mW/cm^2 405 nm Light

Further investigations were carried out to investigate if growth in low-intensity 405 nm light was selective for *S. aureus* which was able to adapt to a greater level of oxidative stress. Bacteria were grown in 1 mW/cm^2 405 nm light, sub-cultured onto NA plates, then re-cultured in (a) complete darkness once or (b) 1 mW/cm^2 a further two times. As can be seen in Figure 7.6a, when cultivated in 1 mW/cm^2 followed by cultivation in complete darkness, the level of inactivation returns to that of cultivation in darkness alone, with no significant difference in the level of inactivation (P = 0.395). Similarly, the level of inactivation was significantly greater than that of *S. aureus* grown in 1 mW/cm^2 405 nm light (P = 0.043). Figure 7.6b also demonstrates that triplicate cultivation in 1 mW/cm^2 does not select for more tolerant cultures. The sensitivity of *S. aureus* to high-intensity 405 nm light returns to a similar level of that when cultivated in complete darkness alone, with an average 0.7×10^1 CFU/ml population surviving following a dose of 216 J/cm^2. This was not significantly different to the average 0.3×10^1 CFU/ml surviving population which had been grown in darkness (P = 0.230).

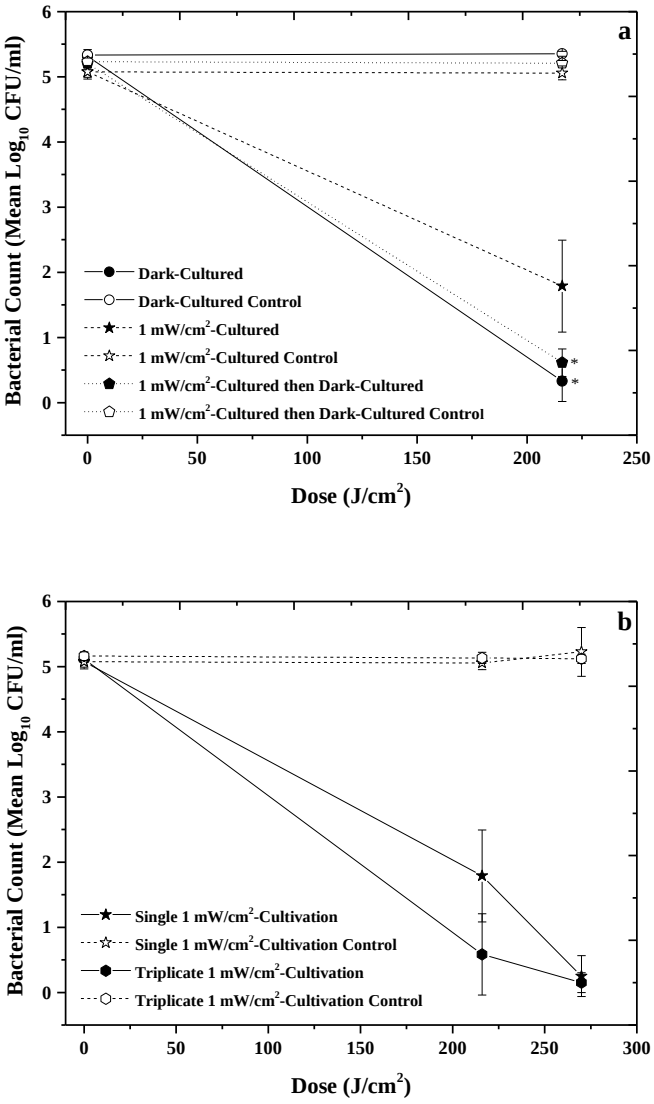


Figure 7.6 Comparison of the susceptibility of methicillin-sensitive Staphylococcus aureus to high-intensity 405 nm light after cultivation under different conditions. *(*a) *S. aureus* was cultivated in low intensity (1 mW/cm^2) 405 nm light followed by cultivation in complete darkness, and (b) *S. aureus* was subjected to repeated (three times) cultivation in low-intensity (1 mW/cm^2) 405 nm light. Each cultivation was at 37 °C for 18 hours. Following these cultivation conditions, bacteria were exposed to increasing doses of high intensity 60 mW/cm^2 light to establish the inactivation kinetics. Inactivation kinetics of bacteria which had been pre-cultured once in darkness or low intensity 405 nm light are given as a comparison. Data points show the mean counts ($n \geq 6$) ± SD. Asterisks '*' indicates cultured samples that were significantly different to those which had been cultured once in 1 mW/cm^2 405 nm light ($P < 0.05$), using one-way ANOVA.

7.3 Determining Potential for Tolerance Development Following Repeated Sub-Lethal Exposure to High-Intensity 405 nm Light

This section of work investigates if bacterial tolerance is likely to develop if non-proliferating bacteria are repeatedly exposed to sub-lethal levels of 405 nm light.

7.3.1 Bacterial Preparation

Methicillin-sensitive *S. aureus* (MSSA; NCTC 4135), and methicillin-resistant *S. aureus* (EMRSA-15) were inoculated, in triplicate, in 100 ml NB, and dark-cultivated at 37 °C for 18-24 hours under rotary conditions (120 rpm). Cultures were re-suspended in PBS and diluted to a 10^5 CFU/ml population.

7.3.2 Repeated Sub-Lethal Exposure

Following cultivation, bacterial samples were exposed to 405 nm light at a dose of 108 J/cm^2 (60 mW/cm^2 for 30 min) using the high-intensity 24-LED array (as described in Section 7.2.2.)

Following exposure, samples were plated onto nutrient agar, using an automated spiral plater (Section 3.3.4) and incubated at 37 °C for 18-24 hours. Surviving colonies were enumerated and termed as survivors from 'Run 1'. Surviving colonies from 'Run 1' were then used to inoculate NA broths, in triplicate, and the above process of exposure-subculture-exposure was repeated until 15 sub-lethal exposures had occurred (Fig 7.7). Unexposed controls were plated in the same way and enumerated, to check that no decrease in control populations occurred. Survivors from each run were tested using Staphaurex Plus Latex Agglutination (Thermo Fisher Scientific, UK) and Penicillin Binding Protein (PBP2a) Latex **(**Oxoid, UK) to confirm that no contamination had occurred. As an additional negative control, *S. aureus* was cultivated in complete darkness, sub-cultured onto NA plates and re-cultured in darkness for 15 cycles.

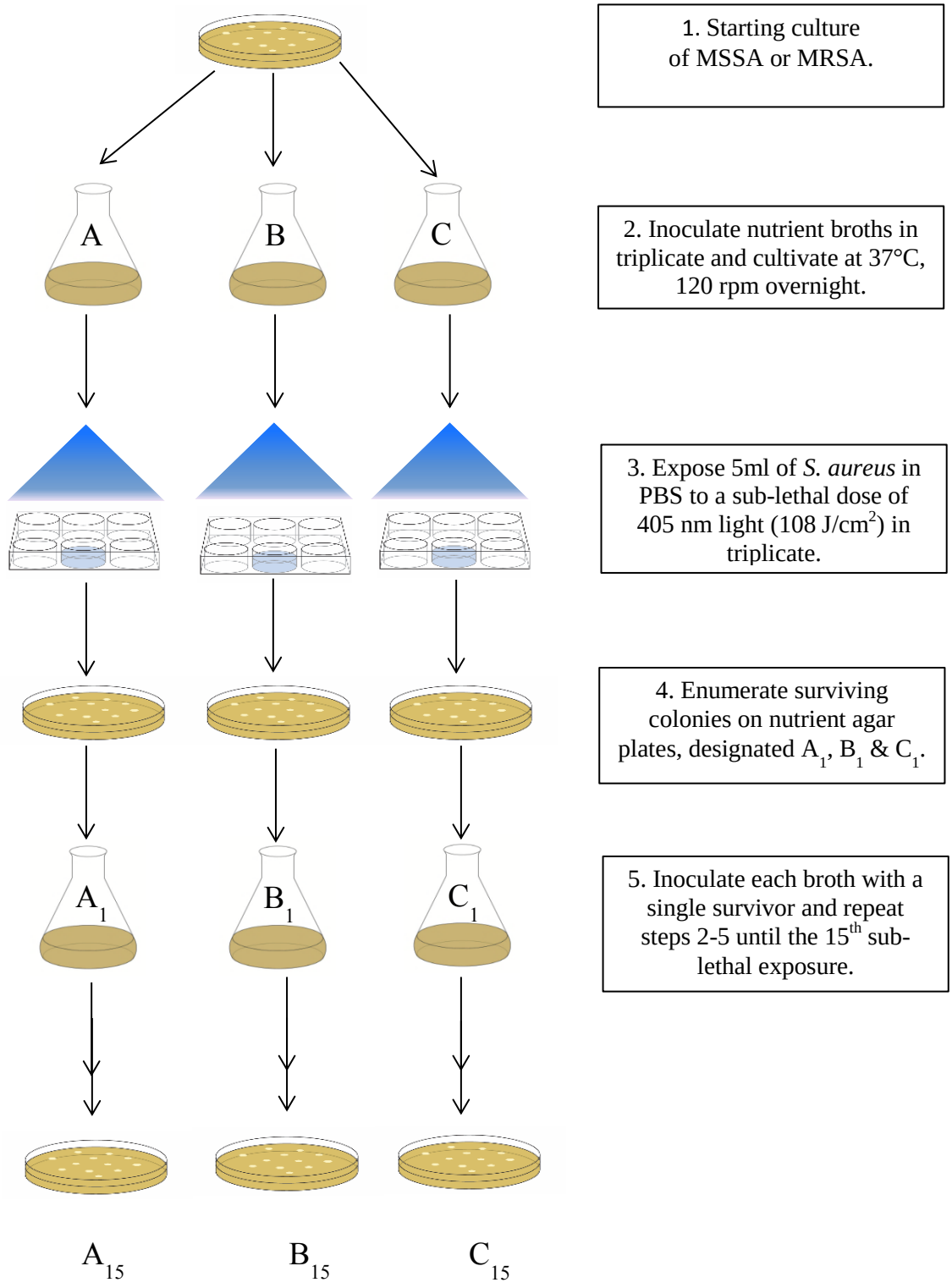


Figure 7.7 Flow diagram of the experimental procedure used during repeated sub-lethal 405 nm light exposure of Staphylococcus aureus. MSSA and MRSA were exposed to 15 cycles of a dose of 108 J/cm^2, using an irradiance of 60 mW/cm^2.

7.3.3 Inactivation Kinetics of Survivors following Sub-Lethal Exposure

Full inactivation kinetics were established using the initial cultures and survivors after 5, 10 and 15 sub-lethal exposures. The inactivation kinetics of the non-exposed control, which was sub-cultured in darkness 15 times, were also determined. Maximum doses of 270 J/cm^2 and 324 J/cm^2 were delivered for MSSA and MRSA respectively, as scoping experiments indicated that MRSA required higher doses for complete inactivation. This was expected, as dose requirements often vary between different strains of bacteria (Bartolomeu *et al*., 2016).

7.3.4 Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was carried out following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) disc diffusion methodology (Matuscheck *et al*., 2014) as closely as possible (variations included the use of 90 mm plates, stacking during incubation, and inoculation with only one antibiotic disc/plate to prevent overlap if bacterial susceptibility increased). The 11 antibiotics used were oxacillin, erythromycin, tetracycline, chloramphenicol, fusidic acid, gentamicin, ampicillin, ciprofloxacin, mupirocin, vancomycin and rifampicin, representing a range of clinical antibiotics with different mechanisms.

Fresh bacterial cultures from the initial stocks, isolates after 5, 10 & 15 sub-lethal exposures, and the non-exposed controls (15 sub-cultures in darkness), were grown in NB overnight in complete darkness. The following day the broths were centrifuged and diluted to a 10^8 CFU/ml population. The bacteria were spread onto MH agar plates using sterile swabs in three directions (Fig 7.8a). A single antibiotic disc (Mast Group, UK) was placed into the centre of the plate and immediately the plates were incubated at 35 ± 2°C for 18-20 hours. Following incubation, the diameter of the zone of inhibition was measured in normal laboratory lighting and recorded to the nearest mm (Fig 7.8b).

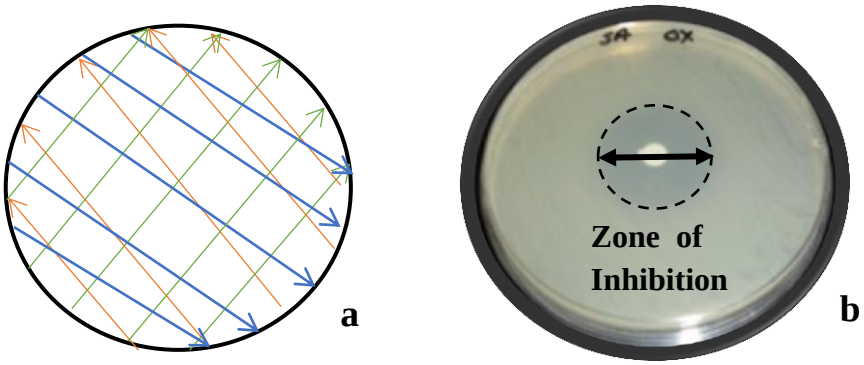


Figure 7.8 Methodology for zone of inhibition studies. (a) The three directions in which Mueller Hinton agar plates were swabbed with *Staphylococcus aureus*, and (b) the zone of inhibition surrounding an antibiotic disc on a *Staphylococcus aureus* lawn, with the arrow indicating the diameter of the zone of inhibition.

7.3.5 Results: Potential for Tolerance following Repeated Sub-Lethal Exposure to 405 nm Light

In order to investigate the likelihood of tolerance development when non-proliferating *Staphylococcus aureus* was repeatedly exposed to 405 nm light, bacteria were repeatedly exposed to a sub-lethal dose of 108 J/cm^2 405 nm light. This dose was selected as it had previously shown to cause approximately 98% (1.6-1.8 $\log_{10}$) inactivation (Fig 7.3).

As can be seen from Figure 7.9a and 7.10a, after repeated sub-lethal exposure there were fluctuations in MSSA inactivation. Figure 7.9a highlights the inactivation of each individual run A, B & C of MSSA, which generally followed a similar pattern of susceptibility throughout the 15 sub-lethal cycles. The level of bacterial inactivation achieved was greatest for isolates of run C on 8 occasions however a similar number of occurrences of the least inactivation was seen for runs A, B & C on 5, 6 and 4 occasions respectively. When the inactivation efficiency was analysed (Fig 7.10a) it can be seen that the maximum inactivation occurred following 7 sub-lethal exposures (1.5 $\log_{10}$ reduction), and minimum after 4 sub-lethal exposures (1.1 $\log_{10}$ reduction). However, there was no significant difference (P = 0.242) in MSSA inactivation, compared to equivalent non-exposed controls, after 1 sub-lethal exposure compared to those after 15 sub-lethal exposures, with 1.3 $\log_{10}$ and 1.2 $\log_{10}$ inactivation achieved respectively. Additionally, one-way ANOVA and Dunnett's post-hoc analysis, using Run 1 as the control group, indicated that there was no significant difference in the bactericidal efficiency of 405 nm light ($log_{10}\ N_0/N$) between the

sub-lethal exposures. No significant change was seen in the equivalent non-exposed controls (P = 0.198).

As a comparison, a clinical isolate of MRSA was also repeatedly exposed to sub-lethal levels of 405 nm light. Figures 7.9b and 7.10b demonstrate that similarly to MSSA, there were fluctuations in inactivation of MRSA. Figure 7.9b highlights that the inactivation of isolates of run B & C follow a similar pattern, however greater inactivation of run A was seen on 8 occasions, and was particularly higher following 3, 4, 7, 9 & 14 sub-lethal exposures. However, when the average inactivation efficiency of MRSA was analysed (Fig 7.10b) maximum inactivation of MRSA was achieved after 14 sub-lethal exposures (1.8 $\log_{10}$ reduction) and minimum after 10 sub-lethal exposures (1.1 $\log_{10}$ reduction). Furthermore, there was also no significant difference (P = 0.943) between the 1.3 $\log_{10}$ and 1.4 $\log_{10}$ reduction after 1 and 15 sub-lethal exposures, respectively. Similarly to MSSA, one-way ANOVA and Dunnett's post-hoc analysis, using Run 1 as the control group, indicated that there was no significant difference in the bactericidal efficiency of 405 nm light ($log_{10}\ N_0/N$) between the sub-lethal exposures. No significant change was seen in the equivalent non-exposed controls (P = 0.116).

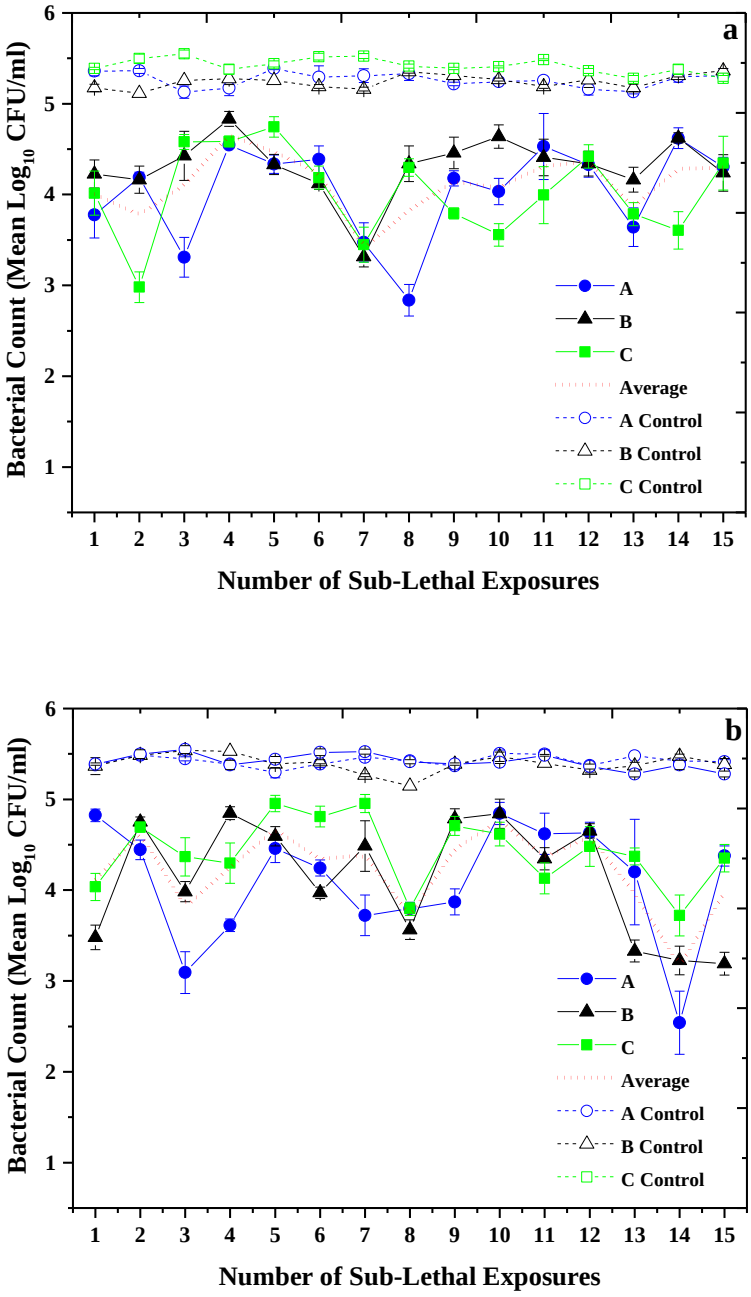


Fig 7.9 Repeated sub-lethal exposure of MSSA and MRSA to 405 nm light. MSSA (a) and MSSA (b) were exposed to 15 sub-lethal exposure cycles of a dose of 108 J/cm^2 405 nm light at an irradiance of 60 mW/cm^2. Exposed and non-exposed (control) data were enumerated after each cycle of exposure. Data points show the mean counts (n ≥ 2) ± SD.

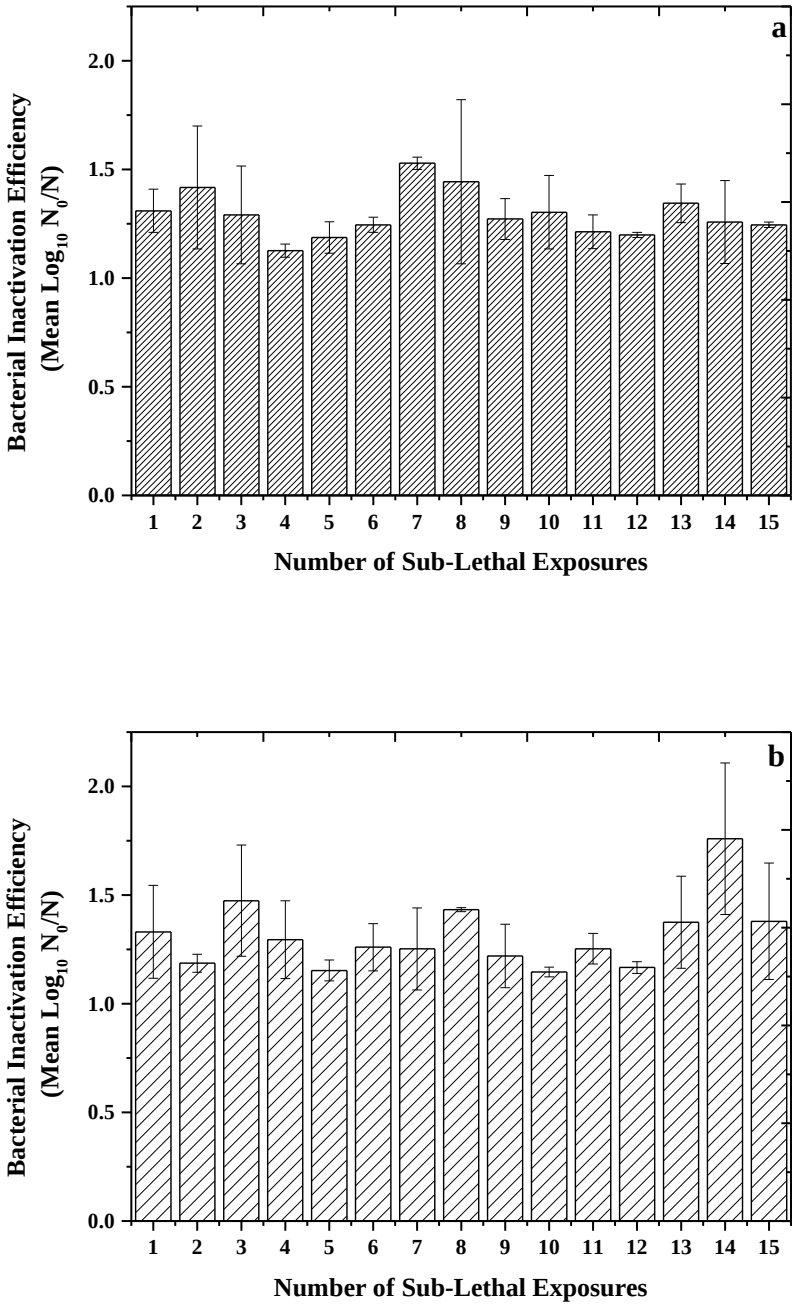


Figure 7.10 Average 405 nm light inactivation efficiency following repeated sub-lethal exposure of MSSA and MRSA. MSSA (a) and MRSA (b) were exposed to 15 sub-lethal exposure cycles of a dose of 108 J/cm^2, at an irradiance of 60 mW/cm^2. Repeated sub-lethal exposure results are reported as bacterial inactivation efficiency ($\log_{10}$ N_0/N), with N representing the light-exposed population, and N_0 the equivalent non-exposed control population. Data points show the mean counts ($n \geq 6$) ± SD. No statistical differences were seen using one-way ANOVA with Dunnett's post-hoc analysis.

7.3.6 Results: Inactivation Kinetics of Survivors following Sub-Lethal Exposure to 405 nm Light

The inactivation kinetics of surviving MSSA and MRSA isolates after 5, 10 and 15 sub-lethal exposures to 60 mW/cm^2 light were also determined.

Figure 7.11a demonstrates the average inactivation kinetics for MSSA and MRSA, with the inactivation kinetics for each individual run (designated A, B & C) detailed in Appendix B1.1 and B1.2, respectively. Following a dose of 270 J/cm^2, there was no significant difference in the level of MSSA inactivation after 5, 10 and 15 sub-lethal exposures, with 5.2 (P = 0.895), 5.2 (P = 0.795) and 4.8 (P = 0.583) $\log_{10}$ reduction achieved, compared to non-sub-lethally exposed MSSA. Similarly, MRSA showed no significant difference in inactivation after a dose of 324 J/cm^2, with 5.4 (P = 0.193), 5.4 (P = 0.259) and 5.3 (P = 0.081) $\log_{10}$ reductions of MRSA after 5, 10 and 15 sub-lethal exposures, respectively (Fig 7.11b).

Interestingly, for both MSSA and MRSA there was also significantly less inactivation seen in the negative control population which was sub-cultured in darkness 15 times. This occurred following a dose of 162 J/cm^2 and 216 J/cm^2 for the MSSA and MRSA isolates respectively, compared to the inactivation level seen in the equivalent initial starting population (P = 0.047, 0.039).

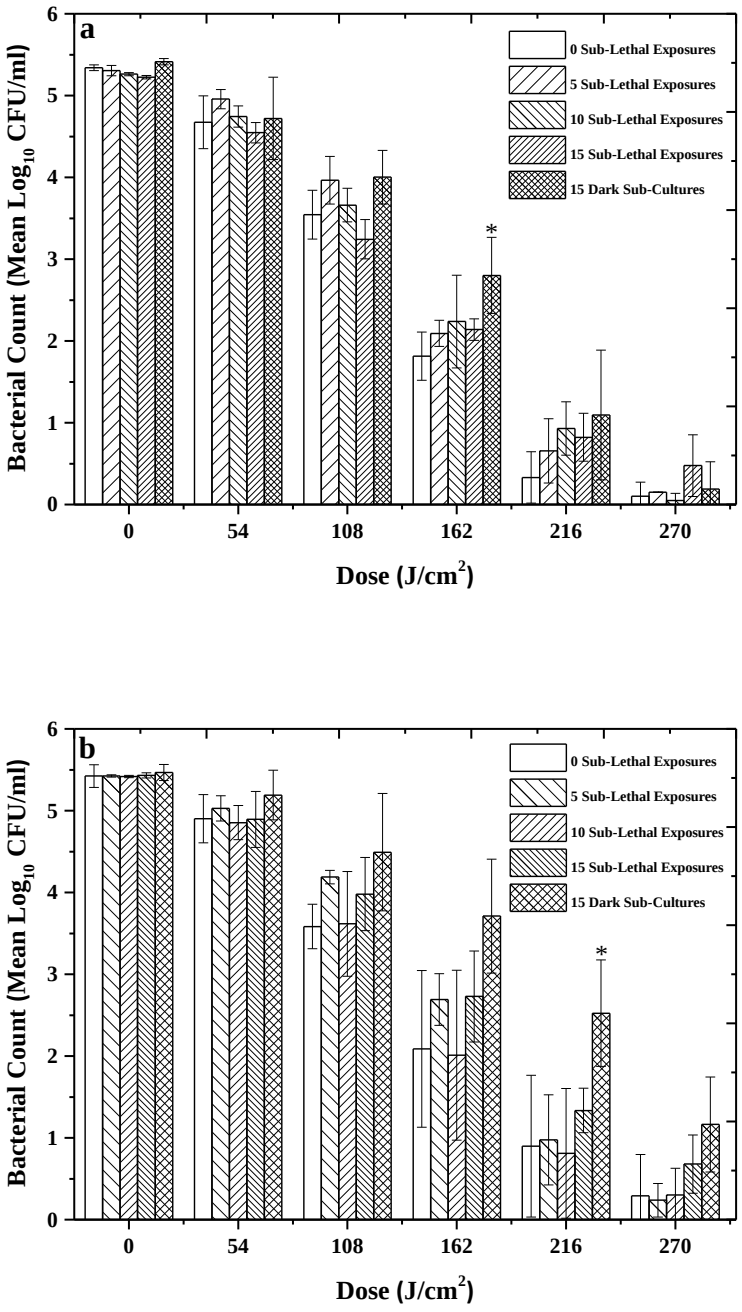


Figure 7.11 Average inactivation kinetics of isolates of MSSA and MSSA after 5, 10 and 15 sub-lethal exposures of 405 nm light. Sub-lethal exposures of (a) MSSA and (b) MRSA were conducted using a dose of 108 J/cm^2 (at an irradiance of 60 mW/cm^2), and inactivation kinetics were based on exposure to 60 mW/cm^2 for increasing time periods. Results are compared to non-sub-lethally exposed bacteria as a control. Data points show the mean counts ($n \geq 6$) ± SD. Asterisks '*' indicate significantly different inactivation compared to the 0 sub-lethal exposures control ($P < 0.05$), using one-way ANOVA.

7.3.7 Results: Antibiotic Susceptibility Following Repeated Sub-Lethal Exposure to 405 nm Light

Although little change was seen in bacterial susceptibility to high-intensity 405 nm light after repeated sub-lethal exposure, to investigate if stress hardening had occurred, antibiotic susceptibility testing was also carried out.

The average diameter of the zone of inhibition of MSSA and MRSA can be seen in Table 7.2, with the results for the individual runs (A, B & C) detailed in Appendix B2.1 and B2.2, respectively. There were no significant decreases in the average diameter of the zone of inhibition between the initial non sub-lethally exposed MSSA and survivors of 15 sub-lethal exposures ($P > 0.05$). There was a significant ($P = 0.007$) decrease in the average diameter of the zone of inhibition caused by mupirocin after 5 sub-lethal exposures, however after 10 and 15 sub-lethal exposures, inhibition was comparable to non-stressed cultures ($P > 0.05$). Interestingly, there were significant increases in the diameter of the zone of inhibition for fusidic acid after 10 sub-lethal exposures and rifampicin after 15 sub-lethal exposures

With regards to the average antibiotic susceptibility of MRSA isolates after sub-lethal exposure (Table 7.2), there were no significant decreases between the initial non-sub-lethally exposed populations and those after 15 sub-lethal exposures, except for ampicillin. There was also a significant increase in the diameter of the zone of inhibition using gentamicin after 15 sub-lethal exposures, compared to initial non sub-lethally exposed populations.

With regards to the negative control populations which were sub-cultured in darkness 15 times, there were several significant differences in antibiotic susceptibility (Table 7.2). In the case of MSSA there was an increase in resistance to oxacillin and ampicillin, however a decrease in resistance to erythromycin and gentamicin. Similarly, in the MRSA isolates, there was a significant decrease in the zone of inhibition due to ampicillin, but an increase in sensitivity to gentamicin.

Table 7.2 Average antibiotic susceptibility of MSSA and MRSA after 5, 10 and 15 sub-lethal exposures compared to equivalent non-sub-lethally exposed controls. Susceptibility was measured using Disc Diffusion Method. Data points show the mean value ($n \geq 3$) ± SD. Asterisks '*' indicates diameter of zone of inhibition that was significantly different to equivalent 0 sub-lethal exposures control ($P \leq 0.05$), using one-way ANOVA.

Antibiotic	Conc (µg)	Mean Diameter of Zone of Inhibition of MSSA (mm)					Mean Diameter of Zone of Inhibition of MRSA (mm)				
		0 Ct	5	10	15	15 Dark Ct	0 Ct	5	10	15	15 Dark Ct
Oxacillin	5	30 ± 0.0	29 ± 0.9	30 ± 0.6	30 ± 0.9	**20 ± 0.6***	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0.0 ± 0.0
Erythromycin	5	21 ± 0.6	21 ± 0.6	22 ± 0.8	21 ± 0.4	**23 ± 0.6***	23 ± 1.0	22 ± 0.8	23 ± 1.0	23 ± 1.2	23 ± 1.0
Tetracycline	25	28 ± 1.2	28 ± 1.0	28 ± 1.4	29 ± 1.3	27 ± 0.6	27 ± 1.0	27 ± 0.7	27 ± 1.0	26 ± 0.9	26 ± 0.6
Chloramphenicol	25	21 ± 1.5	21 ± 0.8	21 ± 1.2	21 ± 0.8	24 ± 0.6	24 ± 1.0	24 ± 1.1	23 ± 1.4	23 ± 1.1	22 ± 0.6
Fusidic Acid	10	30 ± 0.0	30 ± 0.8	**32 ± 1.0***	31 ± 1.8	29 ± 0.6	31 ± 0.6	30 ± 0.5	30 ± 0.7	30 ± 0.9	30 ± 0.6
Gentamicin	10	21 ± 0.6	21 ± 0.5	21 ± 0.5	20 ± 0.5	**25 ± 0.6***	21 ± 0.6	21 ± 0.5	22 ± 1.3	**23 ± 0.6***	**22 ± 0.6***
Ampicillin	25	40 ± 1.0	39 ± 1.4	40 ± 1.6	40 ± 1.3	**36± 0.6***	15 ± 0.6	15 ± 1.2	15 ± 1.1	**13 ± 1.0***	**11 ± 0.6***
Ciprofloxacin	5	22 ± 1.2	21 ± 0.5	22 ± 0.7	22 ± 1.3	21 ± 0.6	26 ± 0.6	26 ± 0.4	26 ± 1.0	26 ± 1.1	25 ± 0.6
Mupirocin	5	28 ± 0.6	**26 ± 0.5***	28 ± 1.4	28 ± 1.1	27 ± 0.6	26 ± 0.6	27 ± 1.1	26 ± 1.2	26 ± 1.3	26 ± 0.0
Vancomycin	5	15 ± 0.0	14 ± 0.7	15 ± 0.7	15 ± 0.5	14 ± 0.6	14 ± 0.6	15 ± 0.6	15 ± 0.5	14 ± 0.5	15 ± 0.6
Rifampicin	5	32 ± 1.0	32 ± 0.7	33 ± 1.8	**34 ± 0.6***	32 ± 0.6	31 ± 0.6	30 ± 0.7	31 ± 1.0	31 ± 0.9	31 ± 0.0

7.4 Analysis of Bacterial Porphyrin Content

Violet-blue light inactivation is thought to be due to the photoexcitation of intracellular porphyrins, with several studies demonstrating the presence of porphyrins within violet-blue light susceptible organisms including *A. baumannii*, *B. cereus*, *E. coli*, *H. pylori*, *L. monocytogenes*, *P. acnes*, *P. aeruginosa*, *S. aureus*, *S. epidermidis*, *S. faecalis* and *S. typhimurium* (Nitzan and Kauffman 1999; Romiti *et al*., 2000; Ashkenazi *et al*., 2003; Nitzan *et al*., 2004; Hamblin *et al*., 2005; Kumar *et al*., 2015; Amin *et al*., 2016; Wang *et al*., 2016). HPLC was carried out to identify the endogenous porphyrins present within *S. aureus* and provide further evidence to support the hypothesis that excitation of porphyrins is the main mechanism of the germicidal action of 405 nm light.

7.4.1 Porphyrin Extraction

Several different methodologies were tested in order to establish the best method for extracting endogenous porphyrins from *S. aureus*, and these are summarised in Appendix C. The method selected for use in this study was as follows:

S. aureus was cultivated in three 100 ml volumes of nutrient broth at 37 °C for 24 hours. Following growth, each broth was centrifuged at 3939 ×*g* for 10 minutes, and the pellets re-suspended in 2 ml PBS. The re-suspended pellets were then combined and washed thrice in PBS. The combined pellet was re-suspended in 1 ml of 0.1 M sodium hydroxide (NaOH)-1% sodium dodecyl sulphate (SDS). The suspension was vortexed for 30 seconds, followed by cell wall disruption by sonication for 15 mins in an ice bath. The samples were then held at room temperature overnight in complete darkness.

The following day the sample was centrifuged at 8000 ×*g* for 10 minutes. The supernatant was immediately removed and filtered through a 0.22 µm filter to remove cellular debris (to prevent damage to the HPLC column).

7.4.2 HPLC Instrumentation

For HPLC analysis an ACE 5 C18 column (Hichrom Ltd, UK) was used alongside a Dionex 680 HPLC system (Thermo Fisher Scientific, UK), with the results recorded on Chromelion Software v 6.7. The HPLC system consisted of an AS1-100 automated sample injector, P680 HPLC pump, RF2000 fluorescence detector, and UVD340U photodiode array detector (All Dionex, Thermo Fisher Scientific, UK). The column was heated using a column block heater (Jones Chromatography Ltd, USA). The conditions for the HPLC analysis process are

detailed in Table 7.3 and were adapted from previous methodologies which had demonstrated successful porphyrin analysis (Xie *et al*., 2001; Fyrestam *et al*., 2015).

Table 7.3 High Performance Liquid Chromatography (HPLC) conditions used for staphylococcal porphyrin analysis. Adapted from Xie *et al*. (2001) and Fyrestam *et al*. (2015).

HPLC Conditions for Porphyrin Analysis	
Column:	C18 -5µm (4.6 × 150mm, ACE 5, Hichrom Ltd, UK)
Wavelength:	Excitation 404 nm, Emission 620 nm
Solvents:	A: Acetic Acid: Acetonitrile: 50mM Ammonium Acetate (7:80:13) B: Acetic Acid: Acetonitrile: 50mM Ammonium Acetate (4:10:86)
Gradient:	A/B = 30/70% to 90/10% over 14 minutes, then hold Isocratic at 100/0% for 14 minutes
Flow Rate:	1 ml/min
Column Temperature:	40°C
Standards:	Free Porphyrin Kit (CMK-1A-KIT, Frontier Scientific, USA)
Injection Volume:	25µl

7.4.3 Preparation of Standards

The Free Porphyrin Kit (CMK-1A-KIT, Frontier Scientific, USA) was used as a standard kit for evaluation and quantification of the endogenous porphyrins within *S. aureus*. The freeze-dried porphyrins within the kit were dissolved in 1 ml 0.1M NaOH–1% SDS, which resulted in a concentration of 10 nmol/ml for each of uroporphyrin I (UPI), 7-carboxylporphyrin (7PI), 6-carboxylporphyrin (6PI), 5-carboxylporphyrin (5PI), coproporphyrin (CPI) and mesoporphyrin IX (MPIX). By running a neat sample of the porphyrin standards and two-fold dilutions through the column, and measuring the fluorescence intensities, the amounts of the related porphyrins in the tested samples could be calculated.

7.4.4 Results: Analysis of Endogenous Porphyrins within *S. aureus*

The first stage of the HPLC analysis was to run the extraction reagent 0.1M NaOH-1% SDS through the column, to identify if it would generate any peaks in the chromatograph which would interfere with the porphyrin analysis. Only one peak was seen as a result of 0.1M NaOH-1% SDS at a retention time of 18 mins (and an area of 0.769 mAU*min): any peaks in subsequent chromatographs at this time point would be considered to be due to the 0.1M NaOH-1% SDS.

The porphyrin standards were then run through the HPLC column with the elution order of UPI, 7PI, 6PI, 5PI, CPI and MPIX, corresponding to the decreasing number of carboxylic acid groups attached to the basic porphine structure (Fyrestam *et al*., 2015). The chromatogram provided with the porphyrin kit can be seen in Figure 7.12a with the equivalent peaks achieved when running the standards through the 'ACE 5 C18' column displaying a similar pattern in Figure 7.12b. The retention times slightly differ to those provided by Frontier Scientific, with the first 5 porphyrins having a shorter elution time and a slightly longer elution time for the final porphyrin. Analysis of the retention times of the porphyrin standards run in this study can be seen in Table 7.4 and indicates which porphyrins each of the main peaks refers to.

Figure 7.12c displays the results of HPLC analysis of *S. aureus*. Peaks were detected after 3.3 min (0.082 mAU*min) and 9.5 min (0.084 mAU*min), and upon comparison of retention times with the porphyrin standards in Table 7.5, it is likely that they represent 7PI and CPI, respectively. The peak at 18 min (0.079 mAU*min) represents the 0.1M NaOH-1% SDS extraction reagent.

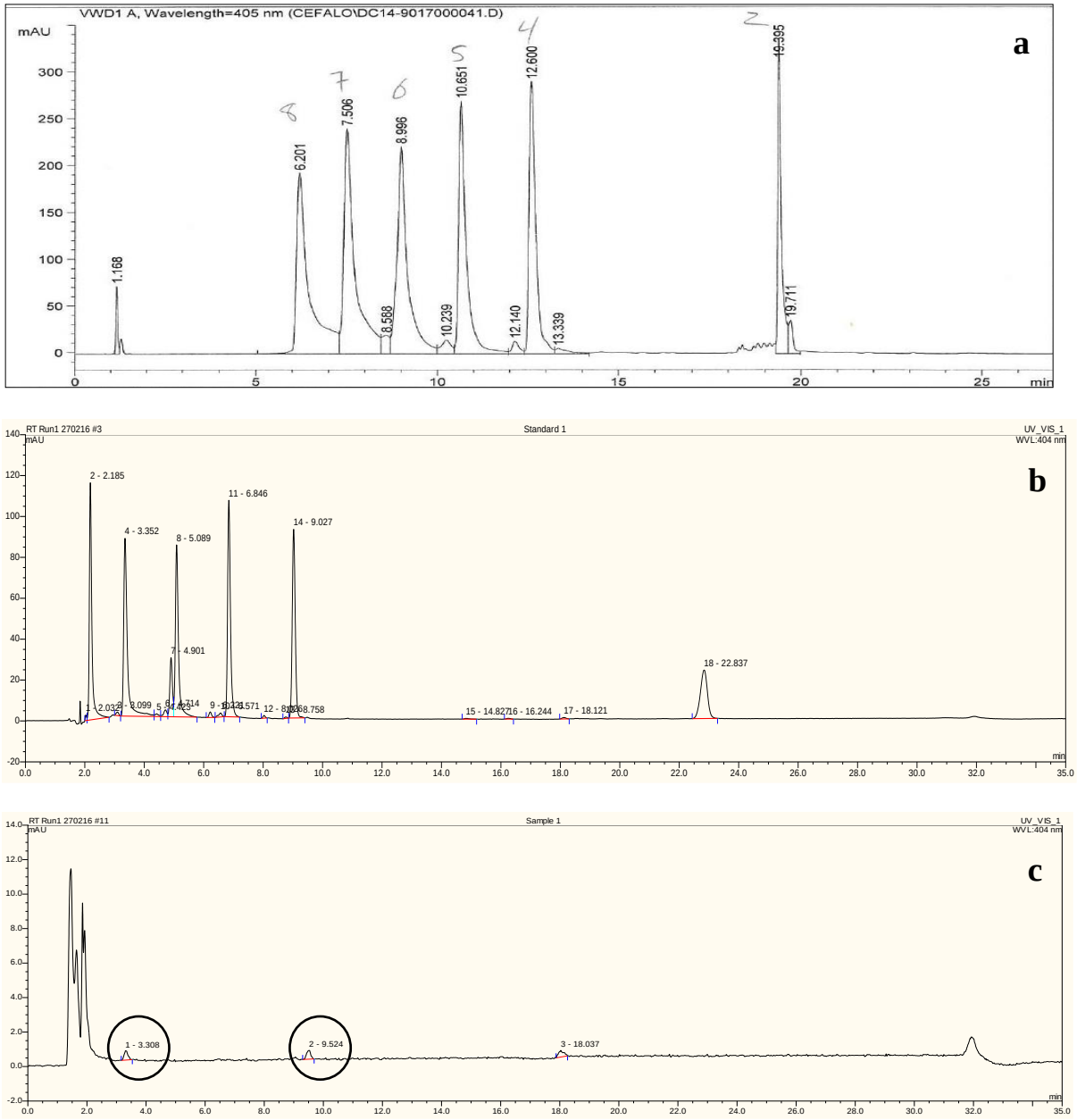


Figure 7.12 Chromatograms from High Performance Liquid Chromatography (HPLC) analysis of Staphylococcus aureus. (a) Porphyrin Standards, provided by Frontier Scientific Inc., with the written numbers referring to the specific carboxyl porphyrins: 8 refers to uroporphyrin I, 7 to 7-carboxylporphyrin, 6 to 6-carboxylporphyrin, 5 to 5-carboxylporphyrin, 4 to coproporphyrin I and 2 to mesoporphyrin IX. (b) Porphyrin standards dissolved in 1 ml of 0.1M NaOH-1%SDS, with an equivalent concentration of 10 nmol/ml, run through the ACE 5 C18 column. (c) *Staphylococcus aureus* sample suspended in 0.1 M NaOH-1% SDS, run through the ACE 5 C18 column.

Table 7.4 Analysis of the chromatogram data of the porphyrin standards dissolved in 1 ml of 0.1M NaOH-1%SDS. The chromatogram can be seen in Figure 7.12b. Peaks likely due to impurities in the sample are marked with a '/'.

Peak Number	Retention Time (min)	Area (mAU*min)	Identification
1	2.030	0.1551	/
2	2.186	15.7513	**Uroporphyrin I**
3	3.111	1.366	**Isomer of 7-Carboxylporphyrin**
4	3.361	21.0997	**7-Carboxylporphyrin**
5	4.418	0.7572	/
6	4.723	0.9899	/
7	4.909	4.5902	**Isomer of 6-Carboxylporphyrin**
8	5.093	18.0791	**6-Carboxylporphyrin**
9	6.225	0.3635	/
10	6.570	0.3488	/
11	6.844	21.4451	**5-Carboxylporphyrin**
12	8.030	0.1356	/
13	8.763	0.1220	/
14	9.016	20.5024	**Coproporphyrin I**
15	9.483	0.0620	/
16	10.832	0.1124	/
17	14.840	0.0975	/
18	16.280	0.1044	/
19	18.107	0.1791	SDS
20	19.386	0.0693	/
21	22.857	11.6867	**Mesoporphyrin IX**

As discussed in Section 7.4.3, several dilutions of the porphyrin standards were run through the column so that concentration curves of each porphyrin could be produced (Fig 7.15). Following this, the linear regression for each porphyrin standard was calculated using Microsoft Excel Software. The linear regression equation for each porphyrin was then used to calculate the approximate concentration of the equivalent porphyrin in the sample extracted from *S. aureus*. As can be seen in Table 7.5, using the linear regression equations for the porphyrin standards 7PI and CPI, the equivalent concentration of these porphyrins in the sample extracted from *S. aureus* was 0.8263 and 0.9616 nmol/ml respectively.

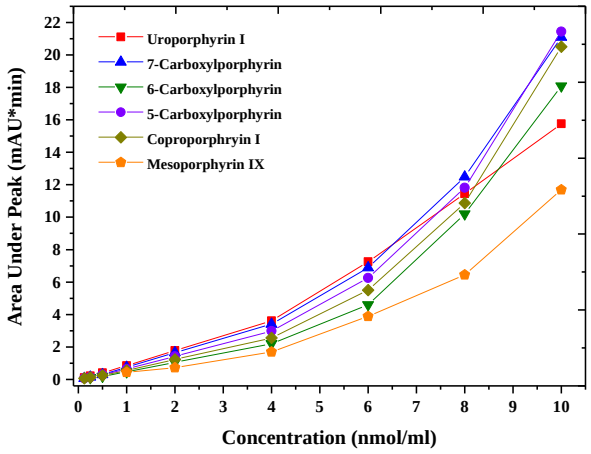


Figure 7.13 Concentration curves of the porphyrin standards

Table 7.5 Calculating the concentration of endogenous porphyrins extracted from Staphylococcus aureus. Calculated using the linear regression equation of the equivalent porphyrin standards. y = Area (mAU*min) and $\boldsymbol{x}$ = concentration (nmol/ml).

Porphyrin	Linear Regression Equation of Porphyrin Standard	Retention Time of Porphyrin Standard (Min)	Retention Time of Sample (Min)	Area of Sample Peak (mAU*min)	Approximate Concentration in Sample of *S. aureus* (nmol/ml)
7PI	y = 1.8846 x - 1.4752	3.352	3.308	0.0821	0.8263
CPI	y = 1.7611 x - 1.6097	9.027	9.524	0.0843	0.9619

7.5 Discussion

This study has investigated the potential for *S. aureus* to become tolerant to antimicrobial 405 nm violet-blue light, and has provided significant new information under carefully controlled experimental test conditions.

The results from the first stage of the study, showed that bacteria cultivated in 1 mW/cm^2 405 nm light appeared to exhibit a degree of tolerance to high-intensity 405 nm light, compared to when cultured in dark, white light or lower-level 405 nm light (0.15, 0.5 mW/m^2)-cultured. This suggests that there may be a critical level of 405 nm light which bacteria must be exposed to before potential stress responses are initiated. Exposure to 1 mW/cm^2 405 nm light during culture, may result in this critical level of exposure, which will in turn cause increased up-regulation of bacterial oxidative stress responses. Bacteria have developed several mechanisms to overcome oxidative stress, including enzymes to detoxify ROS such as catalase, peroxidase and superoxide dismutase (Maisch 2015). To detect changes in the ability of MSSA to tolerate oxidative stress after culture in the presence of 1 mW/cm^2 405 nm light, an MIC assay using H_2O_2 was carried out, and carotenoid content was also measured. Results determined that the average MIC of H_2O_2 was significantly higher for the organism when cultured in low-level (1 mW/cm^2) 405 nm light compared to white light or complete darkness. This demonstrates that during growth in low-irradiance 405 nm light there is likely an increase in the expression of protective enzymes, e.g. an increase in catalase enzymes which would act as H_2O_2 scavengers. An increase in catalase enzyme, KatA, following photodynamic inactivation of *S. aureus* (using blue light and exogenous porphyrin) was also demonstrated by Dosselli *et al*. (2012), which further suggests this enzyme is up-regulated to try to help protect the bacteria against oxidative stress.

The presence of the carotenoid staphyloxanthin was also found to be significantly lower in MSSA cultured in low level (1 mW/cm^2) 405 nm light compared to when grown in white light or darkness. As carotenoid pigments have anti-oxidative properties, in particular for protecting bacteria against $^1O_2$ stress (Liu *et al*., 2005), it would be expected that MSSA cultivated in 1 mW/cm^2 405 nm light would be more sensitive to subsequent high-intensity 405 nm light exposure, due to the lower levels of staphyloxanthin present. However as tolerance is increased upon exposure to high-intensity 405 nm light, it is likely that there is indeed an up-regulation of other oxidative stress responses within the bacteria and that the

carotenoid pigment provided protection against ROS during growth in low-intensity 405 nm light.

Although the inactivation achieved at 216 J/cm^2 (Fig 7.4) was less when the bacteria had been pre-cultured in low irradiance (1 mW/cm^2) 405 nm light, it is important to note that when exposed to a higher dose of 270 J/cm^2 a complete 5 $\log_{10}$ inactivation was still able to be achieved (Fig 7.6b). It is likely that complete inactivation is still able to occur due to the level of ROS produced being greater than the level that the basal bacterial oxidative stress defence systems are able to scavenge (Maisch 2015).

It was important to consider the possibility that bacteria may become less sensitive to high-irradiance 405 nm light after pre-culture in low-intensity, sub-lethal stress levels of 405 nm light, as this could have implications when blue light is used to inactivate bacteria in nutritious environments where the bacteria are able to replicate, for example within wounds or surgical sites. In these cases it would be important to ensure a bactericidal dose was administered: if the dose administered or irradiance used is too low, the bacteria may not be completely inactivated and cause pathogenic organisms to potentially become more tolerant to subsequent applications of blue light. Additionally, if the dose delivered is too low there may actually be an increase in population concentration, as visible light can encourage proliferation of microorganisms when used on nutrient rich areas such as wounds (Lipovsky *et al*., 2009).

Further investigations were carried out to investigate if growth in low-intensity 405 nm light was selective for MSSA which was able to adapt to a greater level of oxidative stress. Results demonstrated that this was not the case, with the sensitivity of MSSA returning to a similar level to that when cultivated in complete darkness alone. These results additionally suggest that the increased tolerance to high-intensity 405 nm light is caused by an up-regulation in bacterial stress response rather than selection for violet-blue light tolerant colonies due to growth conditions.

In the future, studies should investigate tolerance following cultivation in higher levels of violet-blue light. In this study, cells were no longer viable following cultivation in 405 nm light > 1mW/cm^2, however one suggestion would be to reduce the rpm during growth and thus the level of oxygen entering the flask. This may result in a reduced level of ROS produced and allow cells to successfully grow. It would then be interesting to establish if there is indeed a critical point whereby bacterial cells grown in low-level 405 nm light initiate stress responses, resulting in increased tolerance to high-intensity 405 nm light, or if

tolerance builds in-line with increased irradiances used during culture, and what effect this has on H_2O_2 tolerance and carotenoid content.

Additionally, work should be carried out to investigate the exact oxidative stress response following growth in low-intensity 405 nm light fully, such as the levels of superoxide dismutase, which has been previously shown to be up-regulated in *S. aureus* sensitive to PDI (Nakonieczna *et al*., 2010). Additionally, it is known that there is a heat shock protein cascade after PDI (Maisch 2015), and a study by St Denis *et al*. (2011) demonstrated that *E. coli* exposed to external stress before PDI showed a 2 $\log_{10}$ less reduction in bacterial inactivation compared to normal PDI inactivation levels. Therefore, it would be interesting to investigate if there was a heat shock protein cascade during growth in 1 mW/cm^2 violet-blue light, and if this up-regulation of proteins before high-intensity exposure may contribute to the stress tolerance seen. Investigations should also explore why the increased level of tolerance is not seen after repeated growth in the low-level stress conditions. However, as the bacteria can still be completely inactivated, and as results indicated repeated growth in low-irradiance light is unlikely to be a selective process, MSSA should continue to be susceptible to high-irradiance 405 nm light after growth in the presence of a low-level stressor.

However, it is important to note that the changes in susceptibility observed here are in response to the organisms being pre-cultured in low level 405 nm light. This is unlikely to occur in non-proliferating bacteria, such as is the case with environmental contamination (Maclean *et al*., 2014). As these organisms are stressed and not actively growing, up-regulation of stress responses is unlikely to occur, and actually when in a stationary stressed state, bacteria should become more susceptible to 405 nm light inactivation (McKenzie *et al*., 2014).

The next stage of this study was to investigate the likelihood of tolerance development when non-proliferating bacteria were repeatedly exposed to a sub-lethal dose of high intensity 405 nm light. MSSA and MRSA were subjected to 15 exposure-subculture-exposure cycles of 60 mW/cm^2 405 nm light, resulting in a dose of 108 J/cm^2 per sub-lethal exposure. Results demonstrated no significant change in the level of inactivation achieved, with 1.2 $\log_{10}$ and 1.4 $\log_{10}$ inactivation achieved for MSSA and MRSA after 15 sub-lethal exposures respectively, compared to an initial 1.3 $\log_{10}$ inactivation achieved for both.

The results in this study indicate negligible change in susceptibility to 405 nm light inactivation however this conflicts with those of Guffey *et al*. (2013a) who reported that

S. aureus may become less sensitive to 405 nm inactivation after repeated exposure to a dose of 9 J/cm^2. Guffey *et al*. (2013a) demonstrated an initial increase in kill rate from 32.92% to 59.49% after the 1st to 5th sub-lethal exposures, followed by a decline to 18.04% after the 7th repeated sub-lethal exposure, thought to be due to resistance. However, as shown in the present study, which used more than double the number of sub-lethal exposures, and higher bacterial populations, natural variation in the level of inactivation can occur after repeated sub-lethal exposure, and this variation will be particularly apparent if low population densities are used.

Additionally, in the present study *S. aureus* was repeatedly sub-lethally exposed whilst suspended in PBS. This allowed investigation of the sole effect that repeated antimicrobial violet-blue light exposure would have on bacterial cultures in a stationary, non-proliferating, state, representative of how bacteria would be found in the clinical environment. In the study by Guffey *et al.* (2013a), *S. aureus* was exposed to 405 nm light whilst seeded onto mannitol salt agar plates. Not only is mannitol known to be a ROS scavenger, which could have protected bacteria against ROS generated through violet-blue light exposure (Santos *et al*., 2012), in this scenario the bacteria were likely to be in a metabolic state, tolerating the high salt conditions (7.5% NaCl) and fermenting mannitol, and therefore these processes may have affected the subsequent bacterial stress response to violet-blue light. Consequently, the increased tolerance seen may be more relative to results in the earlier phase of this study, where low-level exposure to 405 nm light in nutritious conditions resulted in higher dose requirements for complete inactivation.

In keeping with the present study, Zhang *et al.* (2014) and Amin *et al.* (2016) found tolerance formation to be unlikely. Results demonstrated no evidence of tolerance to 415 nm light inactivation in 10^8 CFU/ml populations of *A. baumannii* and *P. aeruginos*a after 10 repeated sub-lethal exposures to a dose of 70.2 J/cm^2 and 36 J/cm^2 respectively (Zhang *et al.,* 2014; Amin *et al.,* 2016). These previous studies, along with the results in the present study, support the hypothesis that due to the broad spectrum of non-specific intracellular targets, tolerance is not likely to easily occur.

Interestingly in the study by Zhang *et al*. (2014) a significant increase in the sensitivity of *A. baumannii* was seen between the 1st exposure (4.52 $\log_{10}$ reduction) and the 10th exposure (6.28 $\log_{10}$ reduction) and inactivation curves revealed an increase in inactivation between 1, 6 and 9 sub-lethal exposures. These results were thought to indicate that a favourable mutation had occurred, increasing bacterial susceptibly to blue light inactivation (Zhang *et al.,* 2014). To investigate if this phenomenon would also occur in *S. aureus*, the inactivation

kinetics of surviving isolates after 5, 10 and 15 sub-lethal exposures were established, and all followed similar trends. Although these results do not indicate an increase in sensitivity of MSSA and MRSA to 405 nm light after repeated sub-lethal exposure, they do demonstrate consistent staphylococcal sensitivity to high-irradiance 405 nm light, further supporting the hypothesis that tolerance to 405 nm light inactivation is unlikely. However, the potential for tolerance should be evaluated in other microorganisms normally susceptible to 405 nm light inactivation, including MDR organisms which are currently a great problem in healthcare settings (Pendleton *et al*., 2013; Vasoo *et al*., 2015).

Although little change was seen in bacterial susceptibility to high-intensity 405 nm light after repeated sub-lethal exposure, antibiotic susceptibility was also investigated. Antibiotic susceptibility was analysed to ensure that repeated 405 nm light exposure did not give rise to 'stress hardening', whereby as a result of continued exposure to this sub-lethal stress, the bacteria would be able to adapt and develop protection mechanisms against other applied stresses (Koutsoumanis *et al*., 2003; McMahon *et al*., 2007; Pendleton *et al*., 2013).

Little significant variation was seen with the average antibiotic susceptibility for both *S. aureus* strains (Table 7.2). With regards to MSSA, there were no significant decreases in the diameter of the zones of inhibition between the non-sublethally exposed MSSA and survivors of 15 sub-lethal exposures ($P > 0.05$). There was a slight decrease in the average diameter of the zones of inhibition caused by mupirocin after 5 sub-lethal exposures, however following increased sub-lethal exposures to 10 or 15 cycles, inhibition was comparable to non-stressed cultures ($P > 0.05$). It is worth noting that although the concentration of the mupirocin antibiotic disc (5μg) was below the recommended concentration used by EUCAST (200μg), the zone of inhibition measured was still far greater (26 mm) than the EUCAST breakpoints for resistance (18 mm) (EUCAST, 2017). This indicates that although significantly different to the initial non sub-lethally exposed MSSA, after 5 sub-lethal exposures MSSA is still sensitive to mupirocin.

With regards to the average antibiotic susceptibility of MRSA isolates after sub-lethal exposure, there were no significant decreases between the initial non-sub-lethally exposed populations and those after 15 sub-lethal exposures, except a significantly smaller zone surrounding those exposed to ampicillin. This may have occurred as a result of increased oxidative stress responses, due to repeated sub-lethal exposure, which may have promoted antibiotic resistance (Poole 2012). However, as ampicillin is a β-lactam antibiotic this would not be clinically used to treat MRSA as it has a similar mode of action as oxacillin. Future studies should involve a greater range of commonly used antibiotics and use broth

microdilution studies to enable the quantification of the MIC of each antibiotic before and after sub-lethal exposure, to ensure that reduced susceptibility is not likely to occur following oxidative stress.

The differences in the inactivation kinetics and antibiotic susceptibility of the non-exposed control which was sub-cultured in darkness 15 times, compared to the initial control (Fig 7.11 and Table 7.2) does however demonstrate that variation can occur regardless of stress. As these variances occurred without 405 nm light exposure, this may indicate that the slight differences seen in the inactivation kinetics and antibiotic susceptibility of the sub-lethally exposed MSSA and MRSA isolates were due to natural variation, rather than being due to violet-blue light exposure. However, it should be noted that 15 sub-cultures of the non-exposed control, were only repeated once, and if repeated in triplicate, the calculated averages may actually reveal no significant differences compared to the initial control population.

To the best of the author's knowledge these are the first results comparing repeated sub-lethal 405 nm light exposure and antibiotic susceptibility, and they indicate that sub-lethal exposure is unlikely to result in the development of antibiotic resistance, thus supporting the practical application of 405 nm light for decontamination applications within the clinical environment. These findings are further supported by those of Pedigo *et al*. (2009), who demonstrated no antibiotic resistance occurring in MSSA after 25 repeat exposures to PDI, using a methylene blue photosensitizer and 670 nm light, compared to antibiotic resistance in MSSA after only 11 exposures to a 1µg/ml oxacillin disk. Similarly, Grinholc *et al*. (2007) found no change in antibiotic susceptibility in MRSA before and after exposure to PDI (using protoporphyrin diarginate and 624 nm light) with the 26 different antibiotics tested.

As a final investigation within this study, porphyrin analysis was carried out using HPLC to attempt to identify the endogenous porphyrins within *S. aureus* which are likely to play a role during photo-inactivation (Maclean *et al.,* 2008b). Several studies have indicated the presence of porphyrins such as uroporphyrin, coproporphyrin I/III and protoporphyrin IX within bacterial species which are sensitive to violet-blue light inactivation including *S. aureus, S. epidermidis; Bacillus cereus; A. baumannii; E. coli and L. monocytogenes* (Nitzan and Kauffman 1999; Romiti *et al*., 2000; Ashkenazi *et al.,* 2003; Nitzan *et al*., 2004; Hamblin *et al.,* 2005; Kumar *et al*., 2015; Amin *et al*., 2016; Wang *et al*., 2016).

The experimental plan when using HPLC, was to analyse the porphyrin content of *S. aureus*, by identifying and quantifying the porphyrins present and comparing the levels in isolates of

MSSA and MRSA before sub-lethal exposure and following 5, 10 & 15 sub-lethal exposures. Unfortunately, due to time constraints establishing the best methodology for extraction, only the porphyrin content of the original un-exposed culture of *S. aureus* could be analysed. Future work could investigate the porphyrin content of sub-lethally exposed isolates to investigate if there were any changes in concentration after repeated exposure to 405 nm light.

Results from this study indicate the presence of two porphyrins within *S. aureus;* 7-carboxylporphyrin at a concentration of 0.8263 nmol/ml, and coproporphyrin I at 0.9616 nmol/ml (Fig 7.12). There are conflicting reports of what the major porphyrin molecule found within *S. aureus* is. Several studies have used Aminolaevulinic acid (ALA), a naturally occurring metabolite during haeme bio-synthesis pathway whereby uroporphyrin, protoporphyrin and coproporphyrin are produced, to induce intracellular porphyrin production (Fotinos *et al.,* 2008). In a study by Nitzan and Kauffman (1999), using ALA, *S. aureus* produced 89% uroporphyrin and 1.8% coproporphyrin with a small amount of protoporphyrin (0.85%) also produced. However, a more recent study by the same research group, indicated that ALA induced porphyrins within *S. aureus* resulted in 25% uroporphyrin, 68.3% coproporphyrin, 1.8% 7-carboxylporphyrin, 1.2% 6-carboxylporphyrin and 3.7% 5-carboxylporphyrin. These differences in porphyrin concentration could be due to using different strains of *S. aureus*, which could have differing expression patterns of porphyrins. This was demonstrated by Romiti *et al.* (2000) who demonstrated two different patterns of porphyrin expression in *P. acnes*, with some strains producing protoporphyrin and coproporphyrin III whereas others produced uroheptacarboxylporphyrin and pentacarboxyporphyrins I/III, therefore demonstrating distinct genotypes within a bacterial species can produce different porphyrins.

It is therefore likely that the main porphyrin within *S. aureus* may differ between strains, however it is likely to be coproporphyrin or uroporphyrin. The results in this study have provided further evidence to suggest the main porphyrin present within *S. aureus* (NCTC 4135) is coproporphyrin. It is likely this porphyrin plays a role in 405 nm light inactivation by becoming excited, producing ROS, which damages multi-cellular targets and results in cell death. Therefore, damage is unlikely to cause genetic changes or structural changes to which bacteria can become tolerant, rather it is likely there are multiple targets of oxidative damage, to which tolerance development is unlikely.

However, new evidence has come to light that may suggest additional mechanisms are involved in 405 nm light inactivation alongside porphyrin excitation. In a recent study by

Kumar *et al*. (2015), HPLC analysis of porphyrins revealed greater levels of coproporphyrin within *B. cereus* (~5 ag/CFU) compared to *S. aureus* (~3 ag/CFU), however *S. aureus* was more sensitive to 405 nm light inactivation than *B. cereus*. This phenomenon was also seen in Gram negative bacteria with *S. typhimurium* found to contain higher concentration of porphyrins than *E. coli*, but was less susceptible to 405 nm light inactivation. Additionally, *P. aeruginosa* had the highest concentration of porphyrins of all the Gram negative bacteria analysed but was not inactivated by 405 nm light. Furthermore, Kim and Yuk (2017) indicated no significant difference in the level of coproporphyrin within different strains of *Salmonella* spp., but differing levels of inactivation. Following a dose of 576 J/cm^2 there was 5.6 $\log_{10}$ reduction of *S. enteritidis* but only 1.7 $\log_{10}$ reduction of *S. saintpaul.*

These results suggest that although excitation of porphyrins within bacteria may play a role in 405 nm light inactivation, there may be additional endogenous photosensitizers involved such as flavins and cytochromes (Feurestein *et al*., 2005). Other factors may also affect inactivation, including metabolic burden and bacterial stress response to ROS produced (Kumar *et al*., 2015). It is clear that additional experiments are required to establish a successful, reproducible and standardised methodology to analyse the porphyrins within bacteria, and investigate any other mechanism of 405 nm light inactivation which may have an impact on the potential for tolerance development.

Chapter Eight

Conclusions and Future Work

8.0 Overview

This study was conducted to investigate two of the key antimicrobial considerations relating to the potential application of 405 nm light for environmental decontamination within hospitals: antiviral efficacy and bacterial tolerance. A systematised reviewed was also carried out to compare the antiviral efficacy of violet-blue light with the broader antimicrobial effects. This chapter will summarise the main findings from each section of work and also discuss ideas for future work, which may benefit the application of this antimicrobial light within the clinical environment.

8.1 Conclusions

8.1.1 Proof-of-Concept of the Antiviral Efficacy of 405 nm Light

The first stage of this study investigated if high-intensity 405 nm light could inactivate viruses, using FCV, a model for NoV. The use of FCV as a surrogate was successful, as it could be propagated in FEA cells, the virucidal inactivation was able to be measured via a plaque assay technique, and results were clear and reproducible.

The initial results indicated that 405 nm light had virucidal efficacy (≥ 4 $\log_{10}$ reduction) following exposure in minimal media, however high doses of 405 nm light were required (2.8 kJ/cm^2). As viruses do not contain porphyrins, it was hypothesised that the slow inactivation was likely due to the low level of near-UV and > 410 nm wavelengths emitted from the light source, rather than direct effect of 405 nm light. Viral inactivation was enhanced when FCV was suspended in organically-rich media (DMEM), with 85% less dose required for near complete inactivation. This enhanced inactivation was thought to be due to the excitation of photosensitive components within DMEM (riboflavin, tryptophan, tyrosine, pyridoxine and folic acid).

As viral inactivation was enhanced in organically-rich media, the next stage of the study investigated the effect of 405 nm light when FCV was suspended in biologically-relevant

media. Results were significant, with near complete inactivation of a 10^5 PFU/ml population of FCV achieved when suspended in artificial saliva and human blood plasma, using doses between 420-560 J/cm^2. Enhanced inactivation of FCV was also achieved when suspended in artificial faeces compared to minimal media, with 50% less dose required for inactivation (1.4 kJ/cm^2). Fluorescent spectrophotometry revealed that these biologically-relevant media contained components which were excited by 405 nm light, with emission peaks seen at 461 nm, 510 nm and 519 nm for artificial saliva, blood plasma and artificial faeces, respectively, and these were likely responsible for the enhanced inactivation observed.

These results demonstrated the proof-of-concept of the virucidal efficacy of 405 nm light and mirrored those of the recent study by the author which used a bacteriophage as a viral surrogate (Tomb *et al*., 2014). Findings suggested that when viruses are suspended in nutritious and biologically-relevant media, components of the media may act as exogenous photosensitizers and upon exposure to 405 nm light, will produce ROS or other toxic photoproducts that could impart damage to the viruses. As viruses can be expelled from the body surrounded by bodily fluids rich in photosensitive components (e.g. in droplet nuclei from coughing and sneezing, in blood, and in faeces), this natural situation provides ideal conditions to enhance the susceptibility of viruses to 405 nm light inactivation. This is particularly significant in the case of NoV which is excreted in vomit and faeces, and is highly transmissible.

8.1.2 Viral Inactivation using Low-Irradiance 405 nm Light

Following successful viral inactivation when exposed to the high-intensity 405 nm light source, the next step was to investigate whether the same effect could be achieved using low-irradiance, using the HINS-light EDS as the light source. Bacteriophage, ϕC31, was initially used as a viral surrogate, in order to establish the experimental parameters required for viral inactivation using the EDS. FCV was then exposed to the EDS whilst suspended in minimal media with various levels of organic soiling (FBS), as well as in organically-rich and biologically-relevant media. FCV contamination on clinically-relevant surfaces was also evaluated.

Results supported those in Chapter 4 and indicated enhanced viral inactivation when in photosensitive and biologically-relevant media, following exposure to the EDS. Exposure of ϕC31 to doses > 600 J/cm^2 only resulted in 1 $\log_{10}$ reduction whereas when in organically-rich media, 60% less dose (259 J/cm^2) was required for a far greater 5 $\log_{10}$ reduction. Similar results were evidenced with FCV, with 1.3-1.4 $\log_{10}$ reduction requiring doses

> 600 J/cm^2 when the virus was suspended in minimal media whereas only a dose of 43 J/cm^2 was required when in organically-rich media. Results also indicated significant viral inactivation when surrounded by organic soiling (FBS), however this was to a lesser extent than when in minimal media alone, due to reduced transmission through the suspending media. Enhanced inactivation of FCV was also evidenced when dried onto surface coupons of vinyl, stainless steel and PVC. Exposure to the EDS on coupons in artificial faeces resulted in up to 2 × greater $\log_{10}$ inactivation than when exposed dried in DPBS.

Although reported results only focussed on inactivation of the bacteriophage, ϕC31, and FCV, the NoV surrogate, it is anticipated that the key findings could apply to a wide range of pathogenic viruses commonly found within the hospital environment. It is also reasonable to speculate that the inactivation seen will be similar when the virus is suspended in actual bodily fluids such as faecal matter. Further work is required to investigate the antiviral efficacy against other NoV surrogates as well as other nosocomial viruses, helping to provide a more informed picture of the likelihood of viral inactivation in hospitals. The mechanism of virucidal inactivation must also be investigated, with comparisons made between inactivation when exposed in minimal and organically-rich media.

Overall, results suggest that the use of the HINS-light EDS in the hospital environment could additionally provide long-term protection against nosocomial viral pathogens in liquids and on surfaces, alongside normal cleaning practices, particularly on hard to reach surfaces and those which are irregularly cleaned.

8.1.3 Comparison of the Virucidal Efficacy of Violet-Blue Light with the Wider Antimicrobial Efficacy

To compare how the antiviral efficacy of violet-blue light fits within its broader antimicrobial activity, a systematised review was performed. This allowed comparison of the inactivation data from the present research study and the wider research carried out at the ROLEST laboratory using specifically 405 nm light, but also with published literature on microbial inactivation using 380-480 nm violet-blue light.

Results, which compared the dose for 1 $\log_{10}$ reduction of a range of microorganisms, indicated relative similarities in requirements for Gram positive bacteria, Gram negative bacteria and yeast cells, with < 200 J/cm^2 required. The dose for 1 $\log_{10}$ reduction of mycobacteria and germinating fungal conidia had a broader range, spanning between 100-1000 J/cm^2, and the mean doses for fungal conidia and hyphae were similar at

~ 500 J/cm^2. Analysis also revealed that the least sensitive organisms were bacterial spores and viruses, with doses ranging from 600-1000 J/cm^2, required to achieve a 1 $\log_{10}$ reduction, respectively.

Data gathered also allowed for analysis of the antimicrobial efficacy to be compared when using different irradiances of light, exposing different starting populations and also between the different wavelengths of violet-blue light used, as well as highlighting areas of violet-blue light research which need further work. Analysis indicated that population density and irradiance of violet-blue light used is unlikely to have an effect on the average dose requirements for Gram positive and Gram negative bacteria. Interestingly, results demonstrated that higher doses are required for inactivation when longer wavelengths of violet-blue light are used, particularly in the case of *E. coli* and *L. monocytogenes*. This finding supports the use of light in the lower region of 405 nm light for inactivation of microorganisms. However, the review demonstrated the lack of published data on inactivation of MDR isolates, fungi, viruses and protozoa. As well as highlighting a requirement for further evidence on the mechanism of inactivation and potential for bacterial tolerance.

8.1.4 Potential for Bacterial Tolerance to 405 nm Light

As the systematised review indicated the need for further research on the potential for bacterial tolerance to violet-blue light, Chapter 7 attempted to address this question by investigating the potential for tolerance in both proliferating and non-proliferating *S. aureus* populations. Additionally, HPLC was carried out to analyse the porphyrin content within *S. aureus*, to demonstrate the presence of endogenous porphyrins which may play a role in the inactivation mechanism.

Results established several key findings surrounding the question of whether there is potential for *S. aureus* to develop tolerance to antimicrobial 405 nm violet-blue light. The results from the first stage of the study indicated that cultivation in low-irradiance (~1 mW/cm^2) 405 nm light may amplify oxidative stress responses, including catalase production, which in turn may increase bacterial tolerance to high-irradiance 405 nm light exposure. However, as these bacteria were still able to be completely inactivated, even following repeated growth in low-irradiance light, this is unlikely to be a selective process, and *S. aureus* should continue to be susceptible to high-irradiance 405 nm light after growth in the presence of a low-level stressor.

Results in this study also indicate that tolerance is unlikely in non-proliferating antibiotic sensitive and antibiotic resistant *S. aureus*. Inactivation kinetics remained similar even after 15 sub-lethal exposures to a dose of 108 J/cm^2 405 nm light. Furthermore, to the best of the author's knowledge, these are the first results comparing repeated sub-lethal 405 nm light exposure and antibiotic susceptibility, and they indicate that sub-lethal exposure is unlikely to result in antibiotic resistance.

HPLC was also used to analyse the intracellular contents of *S. aureus*, and results indicated the presence of coproporphyrin and 7-carboxylporphyrin. The presence of porphyrins within violet-blue light sensitive *S. aureus* upholds the hypothesis that porphyrins, and coproporphyrin in particular, may play a role in the inactivation mechanism.

Results from this study therefore provide additional support for the safe and effective use of the 405 nm antimicrobial light systems in the clinical setting. Whilst previous studies have established that 405 nm light can be used safely for continuous decontamination in occupied environments and that it can inactivate a wide range of microorganisms, our current findings indicate that bacterial tolerance is unlikely to occur even after repeated use.

8.2 Future work

8.2.1 Further Antiviral Studies

Further work should be carried out to establish the virucidal efficacy of 405 nm light on other NoV surrogates, such as MNV and TuV, which may be more resistant to decontamination. This will ensure that the antiviral efficacy of 405 nm light is not over or underestimated and allow a more accurate quantification of the doses required for NoV inactivation. Additionally, other clinically relevant viruses such as Influenza, Rotavirus and Adenovirus should be investigated, ensuring that successful inactivation against a range of single and double stranded DNA and RNA viruses can be established.

It will also be important to investigate the efficacy of 405 nm light when virions are suspended in human stool samples and vomitus to check that viral inactivation still occurs. However, as both these fluids are packed with micro and macro molecules, it is likely these would fluoresce in a similar manner to the human plasma used in this study, and exert a similar photoinactivation effect.

Furthermore it would be important to investigate the activity against pathogenic viruses not only when deposited on surfaces, but also when dispersed via the aerial route, using the low-irradiance EDS applied continuously over long periods, similar to that employed in clinical decontamination evaluations (Maclean *et al*., 2010, 2013a; Bache *et al*., 2012). Further studies could lead to the beneficial application of 405 nm light for viral decontamination of air, surfaces and equipment in healthcare settings, as well as in other indoor locations, where transmission of viral pathogens is a significant occurrence.

8.2.2 Understanding the Inactivation Mechanism of 405 nm Light

Results in this study have brought to light that further work is required to investigate not only the viral inactivation, but also the bacterial inactivation mechanism. With regards to viral inactivation, it would be interesting to expose FCV to specific wavelengths of near UV and visible light using a laser, or a broadband source coupled with narrowband filters. This may help to elucidate which wavelengths < 400 nm or > 410 nm may be responsible for viral inactivation when in minimal media, or in fact demonstrate that 405 nm light itself has an antiviral effect. It will also be important to elucidate where viral damage is occurring using techniques such as electron microscopy, SDS-PAGE, MALDI-TOF and OxyELISA^{TM} analysis (as discussed in Chapter 4), and compare results before and after 405 nm light exposure. It would be interesting to discover if light exposure results in complete destruction/disintegration of viral particles or if more localised damage is occurring, for example to viral cell receptors preventing attachment to host cells. Comparisons of the inactivation mechanism should also be made between viral particles exposed in minimal and organically-rich media to discover if mechanisms differ.

Additionally, Chapter 6 highlighted the lack of knowledge on the exact mechanism of 405 nm light inactivation of bacteria. Most studies, including this one, only use fluorescence spectrophotometry or HPLC analysis to confirm the presence of porphyrins by comparing emission patterns and chromatograms. There is still much further work required to understand and demonstrate the excitation mechanism of endogenous porphyrins, to ensure they act as hypothesised (Chapter 2). It would be interesting to compare inactivation of a range of bacterial knockouts which are not able to produce endogenous porphyrins. If inactivation was unsuccessful this would prove the germicidal effect of 405 nm light relies upon endogenous porphyrins (Grinholc *et al*., 2015). Furthermore, it is important to continue studying why cells are no longer viable post exposure, by investigating both the potential for genetic damage as well as well as cellular membrane and organelle damage, using a range of techniques such as DNA damage assays, flow cytometry and TEM. Analysis following

different exposure levels may also reveal how inactivation unfolds. These studies would ultimately provide a greater understanding of 405 nm light and help to determine if differing susceptibilities between microorganisms are due to genetically encoded stress responses, differing biological structures, or both.

8.2.3 Future Reviews of Antimicrobial Violet-Blue Light

With regards to the systematised review, future work could include expanding the review into a systematic review, which would require at least one additional reviewer to analyse the current literature on violet-blue light inactivation. Carrying out a review in this manner would allow registration and adherence to guidelines such as PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses). Following PRISMA guidelines and checklists, would ensure a more transparent and higher impact study. Additionally, results in a systematic review may be helpful when producing standardised procedures for violet-blue light exposure experiments and use in the clinical setting in the future (Moher *et al*., 2009).

As the systematised review only focussed on laboratory inactivation studies, future reviews could compare the use of violet-blue light for: wound decontamination (Zhang *et al*., 2014; Amin *et al*., 2016); dental hygiene (Genina *et al*., 2012; Song *et al*., 2012); acne treatments (Papageorgiou *et al*., 2000; Elman *et al*., 2003); prevention of food spoilage and disinfection of food (Guffey *et al*., 2016; Srimagal *et al.*, 2016; Sommers *et al*., 2017); and environmental decontamination purposes (Maclean *et al*., 2010, 2013a; Bache *et al*., 2012; Rutala *et al*., 2016; Sandhu *et al*., 2016; Sutton *et al*., 2016).

In the future it is also likely that research papers will become more transparent as research councils require data to be deposited in accessible online databases, such as the UK Data Archive. It may therefore be possible to retrieve the majority of the raw data from studies and apply mathematical models to provide more accurate estimates of microbial inactivation using violet-blue light, which would in turn improve the value of any systematic reviews published.

8.2.4 Bacterial Tolerance Studies

As results have demonstrated in this study, it is unlikely that 405 nm light will result in tolerance following repeated sub-lethal exposure. However, there are several research questions which need to be considered to provide additional evidence that tolerance will not develop.

As bacteria were more tolerant to high-intensity light after growth in low levels of violet-blue light, future work should investigate the exact mechanism of oxidative stress protection when bacteria are grown in the presence of low-level 405 nm light. Is tolerance due to an increase in H_2O_2 or $^1O_2$ activation of transcription regulator OxyR, which in turn activates stress response genes to express catalases, peroxidases and superoxide, or rather expression of other stress responses, such as heat shock proteins? (Wei *et al*., 2012; Maisch 2015). Furthermore, upon optimisation of the HPLC protocol, comparisons should be made between the porphyrin content within bacterial isolates before and after repeated sub-lethal exposure. Analysis would reveal if sub-lethal exposure selected for bacteria with reduced levels of endogenous porphyrins, or resulted in alternative porphyrin production.

Although other studies have investigated the potential for tolerance in *A. baumannii* and *P. aeruginosa* it would be important to investigate the potential for tolerance in other clinically relevant organisms which can exhibit multi-drug resistance such as *E. coli* and *K. pneumoniae* (Amin *et al*., 2016; Cerceo *et al*., 2016; Zhang *et al*., 2014). Additionally, it will be important to question whether tolerance can occur in other microorganisms such as fungi and viruses. However, this may also be unlikely as a recent study demonstrated no development of tolerance in *C. albicans* following repeated sub-lethal exposure to 415 nm light (Zhang *et al*., 2016).

Future studies should also investigate how variable factors such as population density, differing doses of 405 nm light and increasing numbers of sub-lethal exposures may affect the potential for tolerance. It would be interesting to investigate if bacterial tolerance would develop when exposed to sub-lethal levels of 405 nm light whilst also being exposed to sub-lethal levels of antibiotics or disinfectants, and if sensitivity to either light or/and the additional stressor would increase or decrease.

Another question which requires addressing is whether sub-lethally exposed bacteria, have increased secretion of virulence factors such as toxins and adhesion molecules. Although Fila *et al*. (2016) demonstrated that a single sub-lethal exposure of *P. aeruginosa* to 405 nm light did not affect virulence factor production or activity, it would be important to investigate if there were any differences following repeated sub-lethal exposure. The effect of repeated sub-lethal exposure on staphylococcal virulence factors could be investigated by analysing the activity/production of β-haemolysin, lipase, lecithinase, thermonuclease, coagulase and enterotoxins (Bartolomeu *et al*., 2016). Additionally, cytotoxicity assays of bacterial supernatants would determine if the toxicity of extracellular fractions increased following repeated sub-lethal exposure (Kossakowska *et al*., 2013; Fila *et al*., 2017).

Lastly, future work should also investigate if repeated sub-lethal exposure gives rise to small colony variants (SCVs) or viable but non culturable (VBNC) cells. SCVs are bacterial variants which have slower growth rates, atypical colony morphology and unusual biochemical characteristics (Proctor *et al*., 2006). These could be problematic as they have a greater ability to cause latent or recurrent infections due to their ability to persist in mammalian cells and reduced susceptibility to antibiotics (Proctor *et al*., 2006). Additionally, bacteria such as *E. coli, K. pneumoniae* and *P. aeruginosa* may also enter the VBNC state when responding to environmental stresses (such as 405 nm light), whereby they are still viable but no longer able to grow on cell culture media (Oliver 2010). Although these VBNC cells are unlikely to cause infectious disease they can be revived and regain the ability to cause infections (Oliver 2010). If repeated-sub-lethal exposure encouraged bacteria to enter either of these phenotypic states, this could cause additional clinical problems such as recurrent infections due to SCVs or environmental reservoirs of VBNC cells, when users have assumed that 405 nm light had reduced environmental contamination.

8.2.5 Potential Application of the HINS-Light EDS in Hospitals

This study has provided further evidence to support the use of the EDS in hospitals for continuous environmental decontamination purposes, with results indicating the potential for virucidal efficacy and little evidence of bacterial tolerance development. However further studies in the clinical environment are required to understand the overall benefit of using this technology.

Clinical evaluations to date, have used contact plate sampling to investigate the reduction of staphylococcal counts or total CFU (Maclean *et al*., 2010, 2013a; Bache *et al*., 2012; Rutala *et al*., 2016; Sandhu *et al*., 2016; Sutton *et al*., 2016). Future evaluations should investigate reduction of a wider range of clinical pathogens such as *C. difficile* and NoV by carrying out additional sampling using sponge swabbing (and RT-PCR in the case of NoV) (Ali *et al*., 2015; Park *et al*., 2015). Additionally, investigations should evaluate the potential for using the HINS-light EDS in different areas of the hospital, not only in burns wards and ICU, but also surgery, oncology and haematology and dialysis units. Most importantly studies are required to demonstrate if the long-term use of the EDS will help to reduce the number of HAIs and in turn reduce the number of outbreaks within the clinical environment, which could be carried out through before-after, cross-over or randomised control trials (Woodward 2013).

Further investigations regarding bacterial tolerance to the low-irradiance output of the HINS-light EDS are also required. Assessment of 405 nm light sensitivity of bacterial isolates collected during a clinical evaluation of the EDS, in the ICU at Glasgow Royal Infirmary (concurrent large research study, also funded by SIRN/CSO), demonstrated that tolerance following potentially prolonged exposure to low-level 405 nm light from the EDS, is unlikely (data not shown). Staphylococcal isolates collected from around the patient environment, using contact plate sampling, did not demonstrate tolerance to high-intensity 405 nm light, with complete inactivation of 10^5 CFU/ml populations achieved using doses < 400 J/cm^2. It could however be argued that the samples collected may have been a result of recent environmental inoculation events rather than isolates which had existed on surfaces for several weeks. Sensitive typing such as next generation sequencing would help to resolve this issue in future studies. Additionally, studies should investigate the effect of long term exposure of a wider range of clinical isolates which are both antibiotic resistant and sensitive, and compare inactivation kinetics, pre-, during, and post- long term exposure. It would also be important to investigate if there are differing likelihoods of tolerance when these organisms exist as vegetative cells or biofilms within the clinical environment.

As the EDS can be used continuously to enhance decontamination within hospitals, it would also be interesting to investigate if synergy could occur when used with terminal decontamination technologies, as has been demonstrated with chlorinated disinfectants and 405 nm light (Moorhead *et al*., 2016a). If an enhanced kill of microorganisms could be achieved when 405 nm light was used alongside CAPP, HPV or ozone, then this could lead to improved terminal decontamination processes and likely reductions in acquisition of nosocomial pathogens.

On a final note, the application of 405 nm light, and subsequent operational requirements, should be investigated out with the hospital environment. This could include the use of high-intensity 405 nm light for decontamination during manufacture of high-risk products such as medical supplies, food and drink. One prime example would be decontamination of foods associated with food poisoning, including shellfish, which can be contaminated with viruses such NoV and HEV (Crossan *et al*., 2012; Woods *et al*., 2016), or raw poultry, which can become contaminated with bacterial pathogens including *Campylobacter*, *Salmonella, E. coli* and *L. monocytogenes* (Cook *et al*., 2012). Studies would require to investigate an acceptable exposure process and if there are any detrimental effects on food quality following exposure. The low-irradiance HINS-light EDS could also be utilised in other settings in which microbial outbreaks are a common occurrence. This could include schools, gyms, hotels,

conference centres, cruise ships and other locations in which large numbers of people gather, allowing transmission to be rapid amongst those in close contact. It would be interesting to investigate if the EDS systems could also be used in the agriculture, aquaculture and veterinary sectors; how this would affect animal behaviour, and if it could result in reduced requirement for antibiotics in animal feed. Therefore 405 nm light could additionally be beneficial to global health out with the clinical environment, and long-term use could reduce the financial impact of outbreaks within the community.

8.3 Overall Summary

Overall, the results obtained within this study provide further evidence to support the application of 405 nm light within clinical environments, with long term use likely to reduce microbial bioburden which is not achieved by regular decontamination practices alone. As the results demonstrated that 405 nm light has antiviral efficacy and that bacterial tolerance is unlikely, there is potential to integrate this technology in other domestic and industrial settings. For example, high-irradiances could be used for short term decontamination of medical products whilst using low-irradiances for longer durations, may be of particular benefit in food preparation areas. In all cases, antimicrobial 405 nm light is likely to be of benefit to others by decreasing contamination levels. This reduction of pathogenic organisms is likely to reduce transmission, and in turn infections, in both the medical and wider community.

References

Aboubakr, H.A., Williams, P., Gangal, U., Youssef, M.M., El-Sohaimy, S.A., Bruggeman, P.J. & Goyal, S.M. (2015) Virucidal effect of cold atmospheric gaseous plasma on feline calicivirus, a surrogate for human norovirus. *Applied and Environmental Microbiology*. 81 (11), 3612-3622.

Aboubakr, H.A., Gangal, U., Youssef, M.M., Goyal, S.M. & Bruggeman, P.J. (2016) Inactivation of virus in solution by cold atmospheric pressure plasma: identification of chemical inactivation pathways. *Journal of Physics D: Applied Physics*. 49 (20), 204001.

Ahlfeld, B., Li, Y., Boulaaba, A., Binder, A., Schotte, U., Zimmermann, J.L., Morfill, G. & Klein, G. (2015) Inactivation of a foodborne norovirus outbreak strain with nonthermal atmospheric pressure plasma. *mBio*. 6 (1), e02300-e023014.

Aitken, C. & Jeffries, D.J. (2001) Nosocomial spread of viral disease. *Clinical Microbiology Reviews.* 14 (3), 528-546.

Ali, S., Muzslay, M. & Wilson, P. (2015) A novel quantitative sampling technique for detection and monitoring of *Clostridium difficile* contamination in the clinical environment. *Journal of Clinical Microbiology*. 53 (8), 2570-2574.

Allegranzi, B. & Pittet, D. (2009) Role of hand hygiene in healthcare-associated infection prevention. *Journal of Hospital Infection*. 73 (4), 305-315.

Allwood, P.B., Malik, Y.S., Maherchandani, S., Hedberg, C.W. & Goyal, S. M. (2005) Effect of temperature on the survival of F-specific RNA coliphage, feline calicivirus, and Escherichia coli in chlorinated water. *International Journal of Environmental Research and Public Health*. 2 (3), 442-446.

Almeida, A., Cunha, A., Faustino, M.A.F., Tomé, A.C. & Neves, M.G.P.M.S. (2011) Porphyrins as antimicrobial photosensitizing agents. *Photodynamic Inactivation of Microbial Pathogens: Medical and Environmental Applications*. 11, 83-160.

Amin, R.M., Bhayana, B., Hamblin, M.R. & Dai, T. (2016) Antimicrobial blue light inactivation of *Pseudomonas aeruginosa* by photo- excitation of endogenous porphyrins: In vitro and in vivo studies. *Lasers in Surgery and Medicine*. 48 (5), 562-568.

Anderson, R.E., Young, V., Stewart, M., Robertson, C. & Dancer, S.J. (2011) Cleanliness audit of clinical surfaces and equipment: who cleans what? *Journal of Hospital Infection*. 78 (3), 178-181.

Anderson, D.J., Gergen, M.F., Smathers, E., Sexton, D.J., Chen, L.F., Weber, D.J. & Rutala, W.A. (2013) Decontamination of targeted pathogens from patient rooms using an automated ultraviolet-C-emitting device. *Infection Control and Hospital Epidemiology.* 34 (5), 466-471.

Anderson, D., Chen, L.F., Weber, D.J., Moehring, R.W., Lewis, S.S., Triplett, P., Blocker, M., Becherer, P., Schwab, J.C., Knelson, L.P., Lokhnygina, Y., Rutala, W. & Sexton, D.J. (2015) The BETR-disinfection study. Presented at ID Week, San Diego, USA, October 2015. Available from: https://idsa.confex.com/idsa/2015/webprogram/Paper53062.html [Accessed March 2017]

Arnaud, M.J. (2003) Mild dehydration: a risk factor of constipation? *European Journal of Clinical Nutrition.* 57 (2), S88-S95.

Ashkenazi, H., Malik, Z., Harth, Y. & Nitzan, Y. (2003) Eradication of *Propionibacterium acnes* by its endogenic porphyrins after illumination with high intensity blue light. *FEMS Immunology and Medical Microbiology*. 35 (1), 17-24.

Atmar, R.L. & Estes, M.K. (2006). The epidemiologic and clinical importance of norovirus infection. *Gastroenterology Clinics of North America.* 35, 275–290.

Atmar, R.L., Opekun, A.R., Gilger, M.A., Estes, M.K., Crawford, S.E., Neill, F.H. & Graham, D.Y. (2008) Norwalk virus shedding after experimental human infection. *Emerging Infectious Diseases.* 14 (10), 1553-1557.

Atmar, R.L., Opekun, A.R., Gilger, M.A., Estes, M.K., Crawford, S.E., Neill, F.H., Ramani, S., Hill, H., Ferreira, J. & Graham, D.Y. (2013) Determination of the human infectious dose-50% for Norwalk virus. *Journal of Infectious Diseases*. 209 (7), 1016-1022.

AuBuchon, J.P. (2011) Update on the status of pathogen inactivation methods. *International Society of Blood Transfusion Science Series*. 6 (1), 181-188.

Bache, S.E., Maclean, M., MacGregor, S.J., Anderson, J.G., Gettinby, G., Coia, J.E. & Taggart, I. (2012) Clinical studies of the High-Intensity Narrow-Spectrum light Environmental Decontamination System (HINS-light EDS), for continuous disinfection in the burn unit inpatient and outpatient settings. *Burns*. 38, 69-76.

Bache, S.E. (2013) Clinical Evaluation of the HINS-light EDS for the continuation light based decontamination of the burns unit inpatient and outpatient settings. PhD Thesis. University of Strathclyde, Glasgow, UK.

Bache, S.E., Maclean, M., Gettinby, G., Anderson, J.G., MacGregor, S.J. & Taggart, I. (2015) Airborne bacterial dispersal during and after dressing and bed changes on burns patients. *Burns*. 41 (1), 39-48.

Bancroft, E.A., English, L., Terashita, D. & Yasuda, L. (2013) Outbreak of *Escherichia coli* infections associated with a contaminated transoesophageal echocardiography probe. *Infection Control and Hospital Epidemiology*. 34 (10), e1121-e1123.

Barbut, F., Menuet, D., Verachten, M. & Girou, E. (2009) Comparison of the efficacy of a hydrogen peroxide dry- mist disinfection system and sodium hypochlorite solution for eradication of *Clostridium difficile* spores. *Infection Control and Hospital Epidemiology*. 30 (6), 507-514.

Barclay, L., Park, G.W., Vega, E., Hall, A., Parashar, U., Vinjé, J. & Lopman, B. (2014) Infection control for norovirus. *Clinical Microbiology and Infection*. 20 (8), 731-740.

Barker, J., Vipond, I.B. & Bloomfield, S.F. (2004) Effects of cleaning and disinfection in reducing the spread of Norovirus contamination via environmental surfaces. *Journal of Hospital Infection*. 58 (1), 42-49.

Barneck, M.D., Rhodes, N.L., de la Presa, M., Allen, J.P., Poursaid, A.E., Nourian, M.M., Firpo, M.A. & Langell, J.T. (2015) Violet 405-nm light: a novel therapeutic agent against common pathogenic bacteria. *Journal of Surgical Research*. 206 (2), 316-324.

Bartolomeu, M., Rocha, S., Cunha, Â., Neves, M.G.P.M.S., Faustino, M.A. & Almeida, A. (2016) Effect of photodynamic therapy on the virulence factors of *Staphylococcus aureus. Frontiers in Microbiology*. 7, 267.

Bartsch, S.M., Lopman, B.A., Ozawa, S., Hall, A.J. & Lee, B.Y. (2016) Global economic burden of norovirus gastroenteritis. *PLOS One*. 11 (4), e0151219.

Bekele, A.Z., Gokulan, K., Williams, K. M. & Khare, S. (2016) Dose and Size-Dependent Antiviral Effects of Silver Nanoparticles on Feline Calicivirus, a Human Norovirus Surrogate. *Foodborne Pathogens and Disease*. 13 (5), 239-244.

Bennett, S., MacLean, A., Miller, R.S., Aitken, C. & Gunson, R.N. (2013) Increased norovirus activity in Scotland in 2012 is associated with the emergence of a new norovirus GII. 4 variant. *Eurosurveillance*. 18 (2), 20349.

Ben Said, M., Hassen, A. & Ackermann, H.W. (2009) Study of the relationship between bacteriophage and selective host cells according to different conditions of UV-C irradiation. *Desalination.* 246, 397-408.

Bentley, K., Dove, B.K., Parks, S.R., Walker, J.T. & Bennett, A.M. (2012) Hydrogen peroxide vapour decontamination of surfaces artificially contaminated with norovirus surrogate feline calicivirus. *Journal of Hospital Infection.* 80 (2), 116-121.

Bhalla, A., Pultz, N.J., Gries, D.M., Ray, A.J., Eckstein, E.C., Aron, D.C. & Donskey, C.J. (2004) Acquisition of nosocomial pathogens on hands after contact with environmental surfaces near hospitalized patients. *Infection Control and Hospital Epidemiology*. 25 (2), 164-167.

Bhella, D., Gatherer, D., Chaudhry, Y., Pink, R. & Goodfellow, I.G. (2008) Structural insights into calicivirus attachment and uncoating. *Journal of Virology*. 82 (16), 8051-8058.

Bidawid, S., Malik, N., Adegbunrin, O., Sattar, S.A. & Farber, J.M. (2003) A feline kidney cell line-based plaque assay for feline calicivirus, a surrogate for Norwalk virus. *Journal of Virology Methods.* 107 (2), 163-167.

Bihl, F., Castelli, D., Marincola, F., Dodd, R.Y. & Brander, C. (2007) Transfusion-transmitted infections. *Journal of Translational Medicine*. 5 (1), 1.

Birmpa, A., Vantarakis, A., Paparrodopoulos, S., Whyte, P. & Lyng, J. (2014) Efficacy of Three Light Technologies for Reducing Microbial Populations in Liquid Suspensions. *BioMed Research International.* 673939. Doi: 10.1155/2014/673939

Bogusz, A., Stewart, M., Hunter, J., Yip, B., Reid, D., Robertson, C. & Dancer, S.J. (2013) How quickly do hospital surfaces become contaminated after detergent cleaning? *Healthcare Infection*. 18 (1), 3-9.

Boone, S.A. & Gerba, C.P. (2007) Significance of fomites in the spread of respiratory and enteric viral disease. *Applied and Environmental Microbiology*. 73, 1687–1696.

Bourouiba, L., Dehandschoewercker, E. & Bush, J.W. (2014) Violent expiratory events: on coughing and sneezing. *Journal of Fluid Mechanics*. 745, 537-563.

Boyce, J.M., Havill, N.L., Otter, J.A. & Adams, N.M. (2007) Widespread environmental contamination associated with patients with diarrhoea and methicillin-resistant *Staphylococcus aureus* colonization of the gastrointestinal tract. *Infection Control and Hospital Epidemiology*. 28 (10), 1142-1147.

Boyce, J.M., Havill, N.L., Otter, J.A., McDonald, L.C., Adams, N.M., Cooper, T., Thompson, A., Wiggs, L., Killgore, G., Tauman, A. & Noble-Wang, J. (2008) Impact of hydrogen peroxide vapour room decontamination on *Clostridium difficile* environmental contamination and transmission in a healthcare setting. *Infection Control and Hospital Epidemiology*. 29 (8), 723-729.

Boyce, J.M. (2009) New approaches to decontamination of rooms after patients are discharged. *Infection Control and Hospital Epidemiology*. 30 (6), 515-517.

Boyce, J.M., Havill, N.L. & Moore, B.A. (2011) Terminal decontamination of patient rooms using an automated mobile UV light unit. *Infection Control and Hospital Epidemiology*. 32 (8), 737-742.

Boyce, J.M. (2016) Modern technologies for improving cleaning and disinfection of environmental surfaces in hospitals. *Antimicrobial Resistance and Infection Control*. 5 (10). Doi: 10.1186/s13756-016-0111-x

Brady, R.R., Fraser, S.F., Dunlop, M.G., Paterson-Brown, S. & Gibb, A.P. (2007) Bacterial contamination of mobile communication devices in the operative environment. *Journal of Hospital Infection*. 66 (4), 397-398.

Brown, B.E. & Duquette, D.J. (1993) The Effects of Dissolved Ozone on the Corrosion Behaviour of 304 Stainless Steel, Monel 400 and Naval Brass in Artificial Sea Water. Rensselaer Polytechnic Institute, Troy, New York, Department of Materials Engineering. ADA272271. Available from: www.dtic.mil/get-tr-doc/pdf?AD=ADA272271 [Accessed March 2017]

Bull, R.A., Tanaka, M.M. & White, P.A. (2007) Norovirus Recombination. *Journal of General Virology*. 88: 3347-3359.

Bull, R.A., Eden, J.S., Rawlinson, W.D. & White, P.A. (2010) Rapid evolution of pandemic noroviruses of the GII.4 lineage. *PLOS Pathogens*. 6 (3), e1000831.

Bumah, V.V., Masson-Meyers, D.S., Cashin, S.E & Enwemeka, C.S. (2013). Wavelength and Bacterial Density Influence the bactericidal Effect of Blue Light on Methicillin-Resistant *Staphylococcus aureus*. *Photomedicine and Laser Surgery.* 31 (11), 547-553.

Bumah, V.V., Masson- Meyers, D.S., Cashin, S. & Enwemeka, C.S. (2015a) Optimization of the antimicrobial effect of blue light on methicillin-resistant *Staphylococcus aureus* (MRSA) in vitro. *Lasers in Surgery and Medicine*, 47 (3), 266-272.

Bumah, V.V., Masson- Meyers, D.S. & Enwemeka, C.S. (2015b) Blue 470 nm light suppresses the growth of *Salmonella enterica* and methicillin-resistant *Staphylococcus aureus* (MRSA) in vitro. *Lasers in Surgery and Medicine*. 47 (7), 595-601.

Bumah, V.V., Whelan, H.T., Masson-Meyers, D.S., Quirk, B., Buchmann, E. & Enwemeka, C.S. (2015c) The bactericidal effect of 470-nm light and hyperbaric oxygen on methicillin-resistant *Staphylococcus aureus* (MRSA). *Lasers in Medical Science*. 30 (3), 1153-1159.

Bures, S., Fishbain, J.T., Uyehara, C.F., Parker, J.M. & Berg, B.W. (2000) Computer keyboards and faucet handles as reservoirs of nosocomial pathogens in the intensive care unit. *American Journal of Infection Control*. 28 (6), 465-471.

Cabello, F.C., Godfrey, H.P., Buschmann, A.H. & Dölz, H.J. (2016) Aquaculture as yet another environmental gateway to the development and globalisation of antimicrobial resistance. *The Lancet Infectious Diseases*. 16 (7), e127-e133.

Cahill, O.J., Claro, T., O'Connor, N., Cafolla, A.A., Stevens, N.T., Daniels, S. & Humphreys, H. (2014) Cold air plasma to decontaminate inanimate surfaces of the hospital environment. *Applied and Environmental Microbiology*. 80 (6), 2004-2010.

Cannon, J.L., Papafragkou, E., Park, G.W., Osborne, J., Jaykus, L.A. & Vinjé, J. (2006) Surrogates for the study of norovirus stability and inactivation in the environment: a comparison of murine norovirus and feline calicivirus. *Journal of Food Protection.* 69 (11), 2761-2765.

Carling, P.C., Parry, M.F. & Von Beheren, S.M. (2008) Identifying opportunities to enhance environmental cleaning in 23 acute care hospitals. *Infection Control and Hospital Epidemiology*. 29 (1), 1-7.

Caul, E.O. (1994). Small round structured viruses: airborne transmission and hospital control. *The Lancet.* 343 (8908), 240-1242.

Cerceo, E., Deitelzweig, S.B., Sherman, B.M. & Amin, A.N. (2016) Multidrug-resistant gram-negative bacterial infections in the hospital setting: overview, implications for clinical practice, and emerging treatment options. *Microbial Drug Resistance*. 22 (5), 412-431.

Ceri, H., Olson, M.E., Stremick, C., Read, R.R., Morck, D. & Buret, A. (1999) The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *Journal of Clinical Microbiology*. 37 (6), 1771-1776.

Chambers, H.F. & DeLeo, F.R. (2009) Waves of resistance: *Staphylococcus aureus* in the antibiotic era. *Nature Reviews Microbiology*. 7 (9), 629-641.

Chan, H.T., White, P., Sheorey, H., Cocks, J. & Waters, M.J. (2011) Evaluation of the biological efficacy of hydrogen peroxide vapour decontamination in wards of an Australian hospital. *Journal of Hospital Infection.* 79 (2), 125-128.

Chander, Y., Johnson, T., Goyal, S.M. & Russell, R.J. (2012) Antiviral activity of Ecasol against feline calicivirus, a surrogate of human norovirus. *Journal of Infection and Public Health.* 5 (6), 420-424.

Cheng, C.W., Chen, L.Y., Chou, C.W. & Liang, J.Y. (2015) Investigations of riboflavin photolysis via coloured light in the nitro blue tetrazolium assay for superoxide dismutase activity. *Journal of Photochemistry and Photobiology B.* 148, 262–267.

Cheung, N., Nagra, P. & Hammersmith, K. (2016) Emerging trends in contact lens-related infections. *Current Opinion in Ophthalmology*. 27 (4), 327-332.

Chey, W.D. & Wong, B.C. (2007) American College of Gastroenterology guideline on the management of *Helicobacter pylori* infection. *The American Journal of Gastroenterology*. 102 (8), 1808-1825.

Cho, M., Kim, J., Vinjé, J. & Park, G.W. (2010) Development of fluorinated TiO_2 film and evaluation of its potential application to control human norovirus on environmental surfaces. *American Journal of Infection Control.* 38, e16.

Chiu, S., Skura, B., Petric, M., McIntyre, L., Gamage, B. & Isaac-Renton, J. (2015) Efficacy of common disinfectant/cleaning agents in inactivating murine norovirus and feline calicivirus as surrogate viruses for human norovirus. *American Journal of Infection Control*. 43 (11), 1208-1212.

Chui, C., Aoki, A., Takeuchi, Y., Sasaki, Y., Hiratsuka, K., Abiko, Y. & Izumi, Y. (2013) Antimicrobial effect of photodynamic therapy using high-power blue light-emitting diode and red-dye agent on *Porphyromonas gingivalis*. *Journal of Periodontal Research*. 48 (6), 696-705.

Cieplik, F., Späth, A., Leibl, C., Gollmer, A., Regensburger, J., Tabenski, L., Hiller, K.A., Maisch, T. & Schmalz, G. (2014) Blue light kills *Aggregatibacter actinomycetemcomitans* due to its endogenous photosensitizers. *Clinical Oral Investigations.* 18 (7), 1763-1769.

Coia, J.E., Duckworth, G.J., Edwards, D.I., Farrington, M., Fry, C., Humphreys, H., Mallaghan, C. & Tucker, D.R. (2006) Guidelines for the control and prevention of methicillin-resistant *Staphylococcus aureus* (MRSA) in healthcare facilities. *Journal of Hospital Infection*. 63 (S1), S1-S44.

Coia, J.E., Ritche, L., Adisesh, A., Makison Booth, C., Bradley, C., Bunyan, D., Carson, G., Fry, C., Hoffmann, P., Jenkins, D., Phin, N., Taylor, B., Nguyen-Van-Tam, J.S. & Zuckerman, M. (2013) Guidance on the use of respiratory and facial protection equipment. *Journal of Hospital Infection*. 85 (3), 170-182.

Coia, J.E. (2016) MRSA–seeing the bigger picture. *Journal of Hospital Infection*. 93 (4), 364-365.

Colón, J., Forbis-Stokes, A.A. & Deshusses, M.A. (2015) Anaerobic digestion of undiluted simulant human excreta for sanitation and energy recovery in less-developed countries. *Energy for Sustainable Development.* 29, 57–64.

Cook, A., Odumeru, J., Lee, S. & Pollari, F. (2012) *Campylobacter, Salmonella, Listeria monocytogenes*, verotoxigenic *Escherichia coli*, and *Escherichia coli* prevalence, enumeration, and subtypes on retail chicken breasts with and without skin. *Journal of Food Protection*, 75 (1), 34-40.

Cosgrove, S.E. (2006) The relationship between antimicrobial resistance and patient outcomes: mortality, length of hospital stay, and health care costs. *Clinical Infectious Diseases*. 42, S82-S89.

Costa, L., Faustino, M.A.F., Neves, M.G.P., Cunha, Â. & Almeida, A. (2012) Photodynamic inactivation of mammalian viruses and bacteriophages. *Viruses.* 4 (7), 1034-1074.

Cromeans, T., Park, G.W., Costantini, V., Lee, D., Wang, Q., Farkas, T., Lee, A. & Vinjé, J. (2014). Comprehensive comparison of cultivable norovirus surrogates in response to different inactivation and disinfection treatments. *Applied and Environmental Microbiology*. 80 (18), 5743-5751.

Crossan, C., Baker, P.J., Craft, J., Takeuchi, Y., Dalton, H.R. & Scobie, L. (2012) Hepatitis E virus genotype 3 in shellfish, United Kingdom. *Emerging Infectious Diseases*. 18 (12), 2085-2087.

Daeschlein, G., Scholz, S., Ahmed, R., von Woedtke, T., Haase, H., Niggemeier, M., Kindel, E., Brandenburg, R., Weltmann, K.D. & Juenger, M. (2012) Skin decontamination by low-temperature atmospheric pressure plasma jet and dielectric barrier discharge plasma. *Journal of Hospital Infection*. 81 (3), 177-183.

Dai, T., Gupta, A., Murray, C.K., Vrahas, M.S., Tegos, G.P. & Hamblin, M.R. (2012). Blue light for infectious diseases: *Propionibacterium acnes, Helicobacter pylori*, and beyond? *Drug Resistance Updates*. 15 (4), 223-236.

Dai, T., Gupta, A., Huang, Y.Y., Yin, R., Murray, C.K., Vrahas, M.S., Sherwood, M.E., Tegos, G.P. & Hamblin, M.R. (2013a) Blue light rescues mice from potentially fatal *Pseudomonas aeruginosa* burn infection: efficacy, safety, and mechanism of action. *Antimicrobial Agents and Chemotherapy*. 57 (3), 1238-1245.

Dai, T., Gupta, A., Huang, Y.Y., Sherwood, M.E., Murray, C.K., Vrahas, M.S., Kielian, T. & Hamblin, M.R. (2013b) Blue light eliminates community-acquired methicillin-resistant *Staphylococcus aureus* in infected mouse skin abrasions. *Photomedicine and Laser Surgery*. 31 (11), 531-538.

Dancer, S.J. (2008) Importance of the environment in MRSA acquisition: the case for hospital cleaning. *The Lancet Infectious Diseases*. 8, 101-113.

Dancer, S. J., White, L. & Robertson, C. (2008) Monitoring environmental cleanliness on two surgical wards. *International Journal of Environmental Health Research*. 18 (5), 357-364.

Dancer, S.J. (2009) The role of environmental cleaning in the control of hospital-acquired infection. *Journal of Hospital Infection.* 73 (4), 378-385.

Dancer, S.J. (2014) Controlling hospital-acquired infection: focus on the role of the environment and new technologies for decontamination. *Clinical Microbiology Reviews.* 27 (4), 665-690.

Danial, J., Cepeda, J.A., Cameron, F., Cloy, K., Wishart, D. & Templeton, K.E. (2011) Epidemiology and costs associated with norovirus outbreaks in NHS Lothian, Scotland 2007–2009. *Journal of Hospital Infection.* 79 (4), 354-358.

Davies, A., Pottage, T., Bennett, A. & Walker, J. (2011) Gaseous and air decontamination technologies for *Clostridium difficile* in the healthcare environment. *Journal of Hospital Infection. 77* (3), 199-203.

de Boer, H.E., van Elzelingen-Dekker, C.M., van Rheenen-Verberg, C.M. & Spanjaard, L. (2006) Use of gaseous ozone for eradication of methicillin-resistant *Staphylococcus aureus* from the home environment of a colonized hospital employee. *Infection Control*. 27 (10), 1120-1122.

Decarli, M.C., Carvalho, M.T., Corrêa, T.Q., Bagnato, V.S. & de Souza, C.W.O. (2016) Different photoresponses of microorganisms: from bioinhibition to biostimulation. *Current Microbiology*. 72 (4), 473-481.

Delcour, A.H. (2009) Outer membrane permeability and antibiotic resistance. *Biochimica et Biophysica Acta - Proteins and Proteomics*. 1794 (5), 808-816.

De Lucca, A.J., Carter-Wientjes, C., Williams, K.A. & Bhatnagar, D. (2012) Blue light (470 nm) effectively inhibits bacterial and fungal growth. *Letters in Applied Microbiology*. 55 (6), 460-466.

Denton, M., Wilcox, M.H., Parnell, P., Green, D., Keer, V., Hawkey, P.M., Evans, I. & Murphy, P. (2004) Role of environmental cleaning in controlling an outbreak of *Acinetobacter baumannii* on a neurosurgical intensive care unit. *Journal of Hospital Infection*. 56 (2), 106-110.

de Roda Husman, A.M., Bijkerk, P., Lodder, W., Van Den Berg, H., Pribil, W., Cabaj, A., Gehringer, P., Sommer, R. & Duizer, E. (2004) Calicivirus inactivation by nonionizing (253.7-nanometer-wavelength [UV]) and ionizing (gamma) radiation. *Applied and Environmental Microbiology*. 70 (9), 5089-5093.

Devasagayam, T.P.A., Tilak, J.C., Boloor, K.K., Sane, K.S., Ghaskadbi, S.S. & Lele, R.D. (2004) Free radicals and antioxidants in human health: current status and future prospects. *Journal of Association of Physicians India.* 52 (10), 794-804.

Diez-Valcarce, M., Kovač, K., Raspor, P., Rodríguez-Lázaro, D. & Hernández, M. (2011) Virus genome quantification does not predict norovirus infectivity after application of food inactivation processing technologies. *Food and Environmental Virology*. 3 (3-4), 141-146.

Doan, L., Forrest, H., Fakis, A., Craig, J., Claxton, L. & Khare, M. (2012) Clinical and cost effectiveness of eight disinfection methods for terminal disinfection of hospital isolation rooms contaminated with *Clostridium difficile* 027. *Journal of Hospital Infection*. 82 (2), 114-121.

Donlan, R.M. & Costerton, J.W. (2002) Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews*. 15 (2), 167-193.

Dosselli, R., Millioni, R., Puricelli, L., Tessari, P., Arrigoni, G., Franchin, C., Segalla, A., Teardo E. & Reddi. E. (2012) Molecular targets of antimicrobial photodynamic therapy identified by a proteomic approach. *Journal of Proteomics.* 77, 329-343.

Doultree, J.C., Druce, J.D., Birch, C.J., Bowden, D.S. & Marshall, J.A. (1999) Inactivation of feline calicivirus, a Norwalk virus surrogate. *Journal of Hospital Infection.* 41 (1), 51-57.

Drees, M., Snydman, D.R., Schmid, C.H., Barefoot, L., Hansjosten, K., Vue, P.M., Cronin, M., Nasraway, S.A. & Golan, Y. (2008) Prior environmental contamination increases the risk of acquisition of vancomycin-resistant enterococci. *Clinical Infectious Diseases*. 46 (5), 678-685.

D'Souza, D.H., Sair, A., Williams. K., Papafragkou, E., Jean, J., Moore, C. & Jaykus, L. (2006) Persistence of caliciviruses on environmental surfaces and their transfer to food. *International Journal of Food Microbiology*. 108, 84–91.

D'Souza, D.H. & Su, X. (2010) Efficacy of chemical treatments against murine norovirus, feline calicivirus, and MS2 bacteriophage. *Foodborne Pathogens and Disease*. 7 (3), 319-326.

Duizer, E., Schwab, K.J., Neill, F.H., Atmar, R.L., Koopmans, M.P. & Estes, M.K. (2004a) Laboratory efforts to cultivate noroviruses. *Journal of General Virology.* 85 (1), 79-87.

Duizer, E., Bijkerk, P., Rockx, B., De Groot, A., Twisk, F. & Koopmans, M. (2004b) Inactivation of caliciviruses. *Applied and Environmental Microbiology.* 70 (8), 4538-4543.

Eames, I., Tang, J.W., Li, Y. & Wilson, P. (2009) Airborne transmission of disease in hospitals. *Journal of the Royal Society Interface*. Doi: 10.1098/rsif.2009.0407.focus

Eden, J.S., Hewitt, J., Lim, K.L., Boni, M.F., Merif, J., Greening, G., Ratcliff, R.M., Holmes, E.C., Tanaka, M.M. & Rawlinson, W.D. (2014) The emergence and evolution of the novel epidemic norovirus GII. 4 variant Sydney 2012. *Virology*. 450-451, 106-113.

Edgar, M., Dawes, C. & O'Mullane, D. (2004) Saliva and Oral Health. 3rd Edition. UK, British Dental Journal.

Egyeki, M., Turóczy, G., Majer, Z., Tóth, K., Fekete, A., Maillard, P. & Csík, G. (2003) Photosensitized inactivation of T7 phage as surrogate of non-enveloped DNA viruses: efficiency and mechanism of action. *Biochimica et Biophysica Acta.* 1624 (1), 115-124.

Eibach, D., Casalegno, J.S., Bouscambert, M., Bénet, T., Regis, C., Comte, B., Kim, B.A., Vanhems, P. & Lina, B. (2014) Routes of transmission during a nosocomial influenza A (H3N2) outbreak among geriatric patients and healthcare workers. *Journal of Hospital Infection*. 86 (3), 188-193.

Elman, M., Slatkine, M. & Harth, Y. (2003) The effective treatment of acne vulgaris by a high-intensity, narrow band 405–420 nm light source. *Journal of Cosmetic and Laser Therapy*. 5 (2), 111-117.

Emmert, S., Brehmer, F., Hänßle, H., Helmke, A., Mertens, N., Ahmed, R., Simon, D., Wandke, D., Maus-Friedrichs, W., Däschlein, G. & Schön, M.P. (2013) Atmospheric pressure plasma in dermatology: Ulcus treatment and much more. *Clinical Plasma Medicine*. 1 (1), 24-29.

Endarko, E., Maclean, M., Timoshkin, I.V., MacGregor, S.J. & Anderson, J.G. (2012) High-Intensity 405 nm Light Inactivation of *Listeria monocytogenes*. *Photochemistry and Photobiology*. 88 (5), 1280-1286.

Enwemeka, C.S., Williams, D., Hollosi, S., Yens, D. & Enwemeka, S.K. (2008) Visible 405 nm SLD light photo-destroys methicillin-resistant *Staphylococcus aureus* (MRSA) in vitro. *Lasers in Surgery and Medicine.* 40, 734-737.

Enwemeka, C.S., Williams, D., Enwemeka, S.K., Hollosi, S. & Yens, D. (2009) Blue 470-nm light kills methicillin-resistant *Staphylococcus aureus* (MRSA) in vitro. *Photomedicine and Laser Surgery*. 27 (2), 221-226.

Espinal, P., Marti, S. & Vila, J. (2012) Effect of biofilm formation on the survival of *Acinetobacter baumannii* on dry surfaces. *Journal of Hospital Infection*. 80 (1), 56-60.

Esseili, M.A., Saif, L.J., Farkas, T. & Wang, Q. (2015) Feline calicivirus, murine norovirus, porcine sapovirus, and Tulane virus survival on postharvest lettuce. *Applied and Environmental Microbiology*, 81 (15), 5085-5092.

Ettayebi, K., Crawford, S.E., Murakami, K., Broughman, J.R., Karandikar, U., Tenge, V.R., Neill, F.H., Blutt, S.E., Zeng, X.L., Qu, L., Kou, B., Opekun, A.R., Burrin, D., Graham, D.Y., Ramani, S., Atmar, R.L. & Estes, M.K. (2016) Replication of human noroviruses in stem cell–derived human enteroids. *Science*. 353 (6306), 1387-1393.

European Centre for Disease Prevention and Control. (2008) Annual Epidemiological Report on Communicable Diseases in 2008. ECDC, Stockholm, Sweden. Available from: http://ecdc.europa.eu/en/publications/Publications/0812_SUR_Annual_Epidemiological_Report_2008.pdf [Accessed March 2017]

European Centre for Disease Prevention and Control. (2015) Annual Epidemiological Report 2014. Antimicrobial resistance and healthcare-associated infections. ECDC, Stockholm, Sweden. Available from: http://ecdc.europa.eu/en/publications/Publications/antimicrobial-resistance-annual-epidemiological-report.pdf [Accessed March 2017]

European Committee on Antimicrobial Susceptibility Testing. (2017) Breakpoint tables for interpretation of MICs and zone diameters, Version 7.1. EUCAST, Växjö, Sweden. Available from: http://www.eucast.org [Accessed March 2017]

Fernstrom, A. & Goldblatt, M. (2013) Aerobiology and its role in the transmission of infectious diseases. *Journal of Pathogens*. 493960. Doi: 10.1155/2013/493960

Feuerstein, O., Persman, N. & Weiss, E.I. (2004) Phototoxic Effect of Visible Light on *Porphyromonas gingivalis* and *Fusobacterium nucleatum*: An *In Vitro* Study. *Photochemistry and Photobiology*. 80 (3), 412-415.

Feuerstein, O., Ginsburg, I., Dayan, E., Veler, D. & Weiss, E.I. (2005) Mechanism of visible light phototoxicity on *Porphyromonas gingivalis* and *Fusobacterium nucleatum*. *Photochemistry and Photobiology*. 81 (5), 1186-1189.

Fila, G., Kawiak, A. & Grinholc, M.S. (2017) Blue light treatment of *Pseudomonas aeruginosa*: Strong bactericidal activity, synergism with antibiotics and inactivation of virulence factors. *Virulence*. 1-21. Doi: 10.1080/21505594.2016.1250995

Filice, G.A., Nyman, J.A., Lexau, C., Lees, C.H., Bockstedt, L.A., Como-Sabetti, K., Lesher, L.J. & Lynfield, R. (2010) Excess costs and utilization associated with methicillin resistant for patients with S*taphylococcus aureus* infection. *Infection Control and Hospital Epidemiology*. 31 (4), 365-373.

Fontana, C.R., Song, X., Polymeri, A., Goodson, J.M., Wang, X. & Soukos, N.S. (2015) The effect of blue light on periodontal biofilm growth in vitro. *Lasers in Medical Science.* 30 (8), 2077-2086.

Fotinos, N., Convert, M., Piffaretti, J., Gurny, R. & Lange, N. (2008) 5-aminolevulinic acid and 5-aminolevulinic acid derivatives mediated effects on Gram-negative and Gram-positive bacteria. *Antimicrobial Agents and Chemotherapy*. 52, 1366–1373.

Frère, J.M. (1995) Beta- lactamases and bacterial resistance to antibiotics. *Molecular Microbiology.* 16 (3), 385-395.

Fu, T.Y., Gent, P. & Kumar, V. (2012) Efficacy, efficiency and safety aspects of hydrogen peroxide vapour and aerosolized hydrogen peroxide room disinfection systems. *Journal of Hospital Infection*. 80 (3), 199-205.

Fyrestam, J., Bjurshammar, N., Paulsson, E., Johannsen, A. & Östman, C. (2015) Determination of porphyrins in oral bacteria by liquid chromatography electrospray ionization tandem mass spectrometry. *Analytical and Bioanalytical Chemistry*. 407 (23), 7013-7023.

Gaillot, O., Maruéjouls, C., Abachin, É., Lecuru, F., Arlet, G., Simonet, M. & Berche, P. (1998) Nosocomial outbreak of *Klebsiella pneumoniae* producing SHV-5 extended-spectrum β-lactamase, originating from a contaminated ultrasonography coupling gel. *Journal of Clinical Microbiology*. 36 (5), 1357-1360.

Ganime, A.C., Leite, J.P.G., da Silva Figueiredo, C.E., Carvalho-Costa, F.A., Melgaço, F.G., Malta, F.C., Fumian, T.M. & Miagostovich, M.P. (2016) Dissemination of human adenoviruses and rotavirus species A on fomites of hospital paediatric units. *American Journal of Infection Control*. 44 (11), 1411-1413.

Ganz, R.A., Viveiros, J., Ahmad, A., Ahmadi, A., Khalil, A., Tolkoff, M.J., Nishioka, N.S. & Hamblin, M.R. (2005) *Helicobacter pylori* in patients can be killed by visible light. *Lasers in Surgery and Medicine.* 36 (4), 260-265.

Gardete, S. & Tomasz, A. (2014) Mechanisms of vancomycin resistance in *Staphylococcus aureus*. *The Journal of Clinical Investigation*. 124 (7), 2836-2840.

Garvey, M.I., Bradley, C.W., Tracey, J. & Oppenheim, B. (2016) Continued transmission of *Pseudomonas aeruginosa* from a wash hand basin tap in a critical care unit. *Journal of Hospital Infection*. 94 (1), 8-12

Genina, E.A., Titorenko, V.A., Belikov, A.V., Bashkatov, A.N. & Tuchin, V.V. (2015) Adjunctive dental therapy via tooth plaque reduction and gingivitis treatment by blue light-emitting diodes tooth brushing. *Journal of Biomedical Optics.* 20 (12), 1280041-1280047.

Gerba, C.P. (1984) Applied and theoretical aspects of virus adsorption to surfaces. *Advances in Applied Microbiology*. 30, 133-168.

Ghantoji, S.S., Stibich, M., Stachowiak, J., Cantu, S., Adachi, J.A., Raad, I.I. & Chemaly, R.F. (2015) Non-inferiority of pulsed xenon UV light versus bleach for reducing environmental *Clostridium difficile* contamination on high-touch surfaces in *Clostridium difficile* infection isolation rooms. *Journal of Medical Microbiology*. 64 (2), 191-194.

Ghate, V.S., Ng, K.S., Zhou, W., Yang, H., Khoo, G.H., Yoon, W.B. & Yuk, H.G. (2013) Antibacterial effect of light emitting diodes of visible wavelengths on selected foodborne pathogens at different illumination temperatures. *International Journal of Food Microbiology*. 166 (3), 399-406.

Ghate, V., Leong, A.L., Kumar, A., Bang, W.S., Zhou, W. & Yuk, H.G. (2015) Enhancing the antibacterial effect of 461 and 521 nm light emitting diodes on selected foodborne pathogens in trypticase soy broth by acidic and alkaline pH conditions. *Food Microbiology*. 48, 49-57.

Gillespie, J.B., Maclean, M., Given, M.J., Wilson, M.P., Judd, M.D., Timoshkin, I.V., & MacGregor, S.J. (2017) Efficacy of pulsed 405-nm light-emitting diodes for antimicrobial photodynamic inactivation: effects of intensity, frequency, and duty cycle. *Photomedicine and Laser Surgery*. 35 (3), 150-156.

Girard, P.M., Francesconi, S., Pozzebon, M., Graindorge, D., Rochette, P., Drouin, R. & Sage, E. (2011) UVA-induced damage to DNA and proteins: direct versus indirect photochemical processes. *Journal of Physics: Conference Series*. 261 (1), 012002.

Giuliani, F., Martinelli, M., Cocchi, A., Arbia, D., Fantetti, L. & Roncucci, G. (2010) *In vitro* resistance selection studies of RLP068/Cl, a new Zn(II) phthalocyanine suitable for antimicrobial photodynamic therapy. *Antimicrobial Agents and Chemotherapy*. 54, 637–642.

Glass, R.I., Parashar, U.D. & Estes, M.K. (2009) Norovirus gastroenteritis. *New England Journal of Medicine.* 361 (18), 1776-1785.

Gleizes, O., Desselberger, U., Tatochenko, V., Rodrigo, C., Salman, N., Mezner, Z. & Grimprel, E. (2006) Nosocomial rotavirus infection in European countries: a review of the epidemiology, severity and economic burden of hospital-acquired rotavirus disease. *The Paediatric Infectious Disease Journal*. 25 (1), S12-S21.

Goldoni, A. (2001). Porphyrins: fascinating molecules with biological significance. *ELETTRA Laboratory, Research Highlights*, 64-65. Available from: http://oldweb.elettra.trieste.it/science/highlights/2001-2002/index.html [Accessed March 2017]

Gonzalez, M.D., Langley, L.C., Buchan, B.W., Faron, M.L., Maier, M., Templeton, K., Walker, K., Popowitch, E.B., Miller, M.B., Rao, A., Liebert, U.G., Ledeboer, N.A., Vinjé, J. & Burnham, C.A.D. (2016) Multicenter evaluation of the Xpert Norovirus assay for detection of norovirus genogroups I and II in faecal specimens. *Journal of Clinical Microbiology*. 54 (1), 142-147.

Goodrich, R.P., Edrich, R.A., Li, J. & Seghatchian, J. (2006) The Mirasol™ PRT system for pathogen reduction of platelets and plasma: an overview of current status and future trends. *Transfusion and Apheresis Science.* 35 (1), 5-17.

Goyal, S.M., Chander, Y., Yezli, S. & Otter, J.A. (2014) Evaluating the virucidal efficacy of hydrogen peroxide vapour. *Journal of Hospital Infection*. 86 (4), 255-259.

Grant, M.J. & Booth, A. (2009) A typology of reviews: an analysis of 14 review types and associated methodologies. *Health Information and Libraries Journal*. 26 (2), 91-108.

Grayson, M.L., Jarvie, L.J., Martin, R., Johnson, P.D., Jodoin, M.E., McMullan, C., Gregory, R.H., Bellis, K., Cunnington, K., Wilson, F.L. & Quin, D. (2008) Significant reductions in methicillin-resistant *Staphylococcus aureus* bacteraemia and clinical isolates associated with a multisite, hand hygiene culture-change program and subsequent successful statewide roll-out. *Medical Journal of Australia*. 188 (11), 633-640.

Grinholc, M., Szramka, B., Olender, K. & Graczyk, A. (2007) Bactericidal effect of photodynamic therapy against methicillin-resistant *Staphylococcus aureus* strain with the use of various porphyrin photosensitizers. *Acta Biochimica Polonica-English Edition.* 54 (3), 665-670.

Grzelak, A., Rychlik, B. & Bartosz, G. (2001) Light-dependent generation of reactive oxygen species in cell culture media. *Free Radical Biology and Medicine.* 30 (12), 1418-1425.

Guerrero, D.M., Nerandzic, M.M., Jury, L.A., Jinno, S., Chang, S. & Donskey, C.J. (2012) Acquisition of spores on gloved hands after contact with the skin of patients with *Clostridium difficile* infection and with environmental surfaces in their rooms. *American Journal of Infection Control*. 40 (6), 556-558.

Guffey, J.S. & Wilborn, J. (2006) *In vitro* bactericidal effects of 405-nm and 470-nm blue light. *Photomedicine and Laser Surgery*. 24, 684-8.

Guffey, J.S., Payne, W., Jones, T. & Martin, K. (2013a) Evidence of resistance development by *Staphylococcus aureus* to an *in vitro*, multiple stage application of 405 nm light from a supraluminous diode array. *Photomedicine and Laser Surgery*. 31 (4), 179-182.

Guffey, J.S., Payne, W. & James, L. (2013b) Inactivation of *Mycobacterium smegmatis* following exposure to 405-nanometer light from a supraluminous diode array. *Wounds: A Compendium of Clinical Research and Practice*. 25 (5), 131-135.

Guffey, J.S., Payne, W.C., Motts, S.D., Towery, P., Hobson, T., Harrell, G., Meurer, L. & Lancaster, K. (2016) Inactivation of *Salmonella* on tainted foods: using blue light to disinfect cucumbers and processed meat products. *Food Science and Nutrition.* 4 (6), 878-887.

Gupta, S., Maclean, M., Anderson, J.G., MacGregor, S.J., Meek, R.M.D. & Grant, M.H. (2015) Inactivation of micro-organisms isolated from infected lower limb arthroplasties using high-intensity narrow-spectrum (HINS) light. *Bone and Joint Jouranl*. 97 (2), 283-288.

Ha, J.H., Kim, M.L., Choi, C., Choi, I.S., Myoung, J. & Ha, S.D. (2015) Recovery of structurally intact norovirus from food-contact surfaces. *Food Control*. 47, 564-568.

Haas, J.P., Menz, J., Dusza, S. & Montecalvo, M.A. (2014) Implementation and impact of ultraviolet environmental disinfection in an acute care setting. *American Journal of Infection Control.* 42 (6), 586–90.

Hall, A.J., Lopman, B.A., Payne, D.C., Patel, M.M., Gastañaduy, P.A., Vinjé, J. & Parashar, U.D. (2013) Norovirus disease in the United States. *Emerging Infectious Diseases.* 19 (8), 1198-205.

Hall-Stoodley, L., Costerton, J.W. & Stoodley, P. (2004) Bacterial biofilms: from the natural environment to infectious diseases. *Nature Reviews Microbiology*. 2 (2), 95-108.

Halstead, F.D., Thwaite, J.E., Burt, R., Laws, T.R., Raguse, M., Moeller, R., Webber, M.A. & Oppenheim, B.A. (2016) Antibacterial activity of blue light against nosocomial wound pathogens growing planktonically and as mature biofilms. *Applied and Environmental Microbiology.* 82 (13), 4006-4016.

Hamamoto, A., Mori, M., Takahashi, A., Nakano, M., Wakikawa, N., Akutagawa, M., Ikehara, T., Nakaya, Y. & Kinouchi, Y. (2007) New water disinfection system using UVA light-emitting diodes. *Journal of Applied Microbiology*. 103 (6), 2291-2298.

Hamblin, M.R. & Hasan, T. (2004) Photodynamic therapy: a new antimicrobial approach to infectious disease? *Photochemical and Photobiological Sciences*. 3 (5), 436-450.

Hamblin, M.R., Viveiros, J., Yang, C., Ahmadi, A., Ganz, R.A. & Tolkoff, M.J. (2005) *Helicobacter pylori* accumulates photoactive porphyrins and is killed by visible light. *Antimicrobial Agents and Chemotherapy.* 49, 2822- 2827.

Hamblin, M.R. (2016) Antimicrobial photodynamic inactivation: A bright new technique to kill resistant microbes. *Current Opinion in Microbiology*. 33, 67-73.

Harbarth, S., Sudre, P., Dharan, S., Cadenas, M. & Pittet, D. (1999) Outbreak of *Enterobacter cloacae* related to understaffing, overcrowding, and poor hygiene practices. *Infection Control and Hospital Epidemiology*. 20 (9), 598-603.

Hardy, K.J., Gossain, S., Henderson, N., Drugan, C., Oppenheim, B.A., Gao, F. & Hawkey, P.M. (2007) Rapid recontamination with MRSA of the environment of an intensive care unit after decontamination with hydrogen peroxide vapour. *Journal of Hospital Infection.* 66 (4), 360-368.

Harriott, M.M. & Noverr, M.C. (2009) *Candida albicans* and *Staphylococcus aureus* form polymicrobial biofilms: effects on antimicrobial resistance. *Antimicrobial Agents and Chemotherapy*. 53, 3914–3922.

Hartmann, B., Benson, M., Junger, A., Quinzio, L., Röhrig, R., Fengler, B., Färber, U.W., Wille, B. & Hempelmann, G. (2004) Computer keyboard and mouse as a reservoir of pathogens in an intensive care unit. *Journal of Clinical Monitoring and Computing*. 18 (1), 7-12.

Haughton, P.N., Grau, E.G., Lyng, J., Cronin, D., Fanning, S. & Whyte, P. (2012) Susceptibility of *Campylobacter* to high intensity near ultraviolet/visible 395±5nm light and its effectiveness for the decontamination of raw chicken and contact surfaces. *International Journal of Food Microbiology*. 159 (3), 267-273.

Havill, N.L., Havill, H.L., Mangionem, E., Dumigan, D.G. & Boyce, J.M. (2011) Cleanliness of portable medical equipment disinfected by nursing staff. *American Journal of Infection Control*. 39, 602-604.

Havill, N.L., Moore, B.A. & Boyce, J.M. (2012) Comparison of the microbiological efficacy of hydrogen peroxide vapor and ultraviolet light processes for room decontamination. *Infection Control and Hospital Epidemiology*. 33 (5), 507-512.

Hayden, M.K., Bonten, J.M., Blom, D.W., Lyle, E.A., van de Vijver, D. & Weinstein, R. (2006) Reduction in acquisition of vancomycin-resistant Enterococcus after enforcement of routine environmental cleaning measures. *Clinical Infectious Diseases*. 42 (11), 1552-1560.

Health Protection Scotland. (2012) Scottish National Point Prevalence Survey of Healthcare Associated Infection and Antimicrobial Prescribing 2011. HPS, Glasgow, UK. Available from: http://www.hps.scot.nhs.uk/resourcedocument.aspx?id=5536 [Accessed March 2017]

Health Protection Scotland. (2016) Healthcare Associated Infections Annual Report 2015. HPS, Glasgow, UK. Available from: http://www.hps.scot.nhs.uk/resourcedocument.aspx?id=4089 [Accessed March 2017]

Herruzo, R., Vizcaíno, M.J. & Herruzo, I. (2014) Quantifying Glosair™ 400 efficacy for surface disinfection of American Type Culture Collection strains and micro-organisms recently isolated from intensive care unit patients. *Journal of Hospital Infection.* 87 (3), 175-178.

Hessling, M., Spellerberg, B. and Hoenes, K. (2017) Photoinactivation of bacteria by endogenous photosensitizers and exposure to visible light of different wavelengths–A review on existing data. *FEMS Microbiology Letters*. 364 (2), fnw270.

Hockberger, P.E., Skimina, T.A., Centonze, V.E., Lavin, C., Chu, S., Dadras, S., Reddy, J.K. & White, J.G. (1999) Activation of flavin-containing oxidases underlies light-induced production of H_2O_2 in mammalian cells. *National Academy of Sciences*. 96 (11), 6255-6260.

Hoelzer, K., Fanaselle, W., Pouillot, R., Van Doren, J.M. & Dennis, S. (2013) Virus inactivation on hard surfaces or in suspension by chemical disinfectants: systematic review and meta-analysis of norovirus surrogates. *Journal of Food Protection*. 76 (6), 1006-1016.

Hoenes, K., Stangl, F., Sift, M. & Hessling, M. (2015) Visible optical radiation generates bactericidal effect applicable for inactivation of health care associated germs demonstrated by inactivation of E. coli and B. subtilis using 405 nm and 460 nm light emitting diodes. Proceedings of European Conference on Biomedical Optics, Munich, Germany, July 2015. 95400T. Doi: 10.1117/12.2183903

Hoenes, K., Stangl, F., Gross, A. & Hessling, M. (2016) Improved contact lens disinfection by exposure to violet radiation. *Technology and Health Care*. 24 (1), 145-151.

Holmdahl, T., Walder, M., Uzcátegui, N., Odenholt, I., Lanbeck, P., Medstrand, P. & Widell, A. (2016) Hydrogen Peroxide Vapor Decontamination in a Patient Room Using Feline Calicivirus and Murine Norovirus as Surrogate Markers for Human Norovirus. *Infection Control and Hospital Epidemiology*. 37 (5), 561-566.

Hope, C.K., Hindley, J.A., Khan, Z., de Jong, E.D.J. & Higham, S.M. (2013) Lethal photosensitization of *Porphyromonas gingivalis* by their endogenous porphyrins under anaerobic conditions: an in vitro study. *Photodiagnosis and Photodynamic Therapy*. 10 (4), 677-682.

Hope, C.K., Strother, M., Creber, H.K. & Higham, S.M. (2016) Lethal photosensitisation of Prevotellaceae under anaerobic conditions by their endogenous porphyrins. *Photodiagnosis and Photodynamic Therapy*. 13, 344-346.

Horan, T.C., Andrus, M. & Dudeck, M.A. (2008) CDC/NHSN surveillance definition of health care–associated infection and criteria for specific types of infections in the acute care setting. *American Journal of Infection Control*. 36 (5), 309-332.

Hosein, I., Madeloso, R., Nagaratnam, W., Villamaria, F., Stock, E. & Jinadatha, C. (2016) Evaluation of a pulsed xenon ultraviolet light device for isolation room disinfection in a United Kingdom hospital. *American Journal of Infection Control*. 44 (9), e157-e161.

Hota, S., Hirji, Z., Stockton, K., Lemieux, C., Dedier, H., Wolfaardt, G. & Gardam, M.A. (2009) Outbreak of multidrug-resistant *Pseudomonas aeruginosa* colonization and infection secondary to imperfect intensive care unit room design. *Infection Control and Hospital Epidemiology*. 30 (1), 25-33.

Huang, S.S., Datta, R. & Platt, R. (2006) Risk of acquiring antibiotic-resistant bacteria from prior room occupants. *Archives of Internal Medicine*. 166 (18), 945-1951.

Hudson, J.B., Sharma, M. & Petric, M. (2007) Inactivation of norovirus by ozone gas in conditions relevant to healthcare. *Journal of Hospital Infection.* 66, 40–45.

Imada, K., Tanaka, S., Ibaraki, Y., Yoshimura, K. & Ito, S. (2014) Antifungal effect of 405-nm light on *Botrytis cinerea*. *Letters in Applied Microbiology*. 59 (6), 670-676.

Indigo-Clean (2016). Continuous Environmental Disinfection for Surgical Suites. Available from: http://www.kenall.com/Kenall-Files/Product-Files/Literature/Kenall-Indigo-Clean-Brochure.pdf [Accessed March 2017]

Inweregbu, K., Dave, J. & Pittard, A. (2005) Nosocomial infections. *Continuing Education in Anaesthesia, Critical Care and Pain*. 5 (1), 14-17.

Irving, D., Lamprou, D.A., Maclean, M., MacGregor, S.J., Anderson, J.G. & Grant, M.H. (2016) A comparison study of the degradative effects and safety implications of UVC and 405 nm germicidal light sources for endoscope storage. *Polymer Degradation and Stability*. 133, 249-254.

Isbary, G., Heinlin, J., Shimizu, T., Zimmermann, J.L., Morfill, G., Schmidt, H.U., Monetti, R., Steffes, B., Bunk, W., Li, Y. & Klaempfl, T. (2012) Successful and safe use of 2 min cold atmospheric argon plasma in chronic wounds: results of a randomized controlled trial. *British Journal of Dermatology*. 167 (2), 404-410.

Isbary, G., Stolz, W., Shimizu, T., Monetti, R., Bunk, W., Schmidt, H.U., Morfill, G.E., Klämpfl, T.G., Steffes, B., Thomas, H.M. & Heinlin, J. (2013a) Cold atmospheric argon plasma treatment may accelerate wound healing in chronic wounds: Results of an open retrospective randomized controlled study *in vivo*. *Clinical Plasma Medicine*. 1 (2), 25-30.

Isbary, G., Köritzer, J., Mitra, A., Li, Y.F., Shimizu, T., Schroeder, J., Schlegel, J., Morfill, G.E., Stolz, W. & Zimmermann, J.L. (2013b) *Ex vivo* human skin experiments for the evaluation of safety of new cold atmospheric plasma devices. *Clinical Plasma Medicine*. 1 (1), 36-44.

Jarrett, O., Laird, H.M. & Hay, D. (1973) Determinants of the Host Range of Feline Leukaemia Viruses. *Journal of General Virology*. 20, 169-175.

Jean, J., Morales-Rayas, R., Anoman, M. N. & Lamhoujeb, S. (2011) Inactivation of hepatitis A virus and norovirus surrogate in suspension and on food-contact surfaces using pulsed UV light (pulsed light inactivation of food-borne viruses). *Food Microbiology*. 28 (3), 568-572.

Jevons, M.P., Coe, A.W. & Parker, M.T. (1963) Methicillin resistance in staphylococci. *The Lancet*. 1, 904–907.

Jiang, X., Wang, M., Wang, K. & Estes, M.K. (1993) Sequence and genomic organization of Norwalk virus. *Virology*. 195 (1), 51-61.

Jinadatha, C., Quezada, R., Huber, T.W., Williams, J.B., Zeber, J.E. & Copeland, L.A. (2014) Evaluation of a pulsed-xenon ultraviolet room disinfection device for impact on contamination levels of methicillin-resistant *Staphylococcus aureus*. *BioMed Central Infectious Diseases*. 14 (1), 187.

Jinadatha, C., Villamaria, F.C., Restrepo, M.I., Ganachari-Mallappa, N., Liao, I.C., Stock, E. M., Copeland, L.A. & Zeber, J.E. (2015) Is the pulsed xenon ultraviolet light no-touch disinfection system effective on methicillin-resistant *Staphylococcus aureus* in the absence of manual cleaning? *American Journal of Infection Control*. 43 (8), 878-881.

Jones, E.L., Kramer, A., Gaither, M. & Gerba, C.P. (2007) Role of fomite contamination during an outbreak of norovirus on houseboats. *International Journal of Environmental Health Research.* 17, 123–131.

Jones, M.K., Watanabe, M., Zhu, S., Graves, C.L., Keyes, L.R., Grau, K.R., Gonzalez-Hernandez, M.B., Iovine, N.M., Wobus, C.E., Vinjé, J. & Tibbetts, S.A. (2014) Enteric bacteria promote human and mouse norovirus infection of B cells. *Science*. 346 (6210), 755-759.

Joshi, P.C. (1985) Comparison of the DNA-damaging property of photosensitised riboflavin via singlet oxygen ($^1O_2$) and superoxide radical (O^{2-}) mechanisms. *Toxicology Letters*. 26 (2), 211-217.

Judge, C., Galvin, S., Burke, L., Thomas, T., Humphreys, H. & Fitzgerald-Hughes, D. (2013) Search and You Will Find: Detecting Extended-Spectrum β-Lactamase–Producing *Klebsiella pneumoniae* from a Patient's Immediate Environment. *Infection Control and Hospital Epidemiology*. 34 (5), 534-536.

Kambhampati, A., Koopmans, M. & Lopman, B.A. (2015) Burden of norovirus in healthcare facilities and strategies for outbreak control. *Journal of Hospital Infection.* 89 (4), 296-301.

Kampf, G., Grotheer, D. & Steinmann, J. (2005) Efficacy of three ethanol-based hand rubs against feline calicivirus, a surrogate virus for norovirus. *Journal of Hospital Infection*, 60 (2), 144-149.

Karst, S.M. (2010) Pathogenesis of Noroviruses, emerging RNA viruses. *Viruses*. 2 (3), 748-781.

Kawada, A., Aragane, Y., Kameyama, H., Sangen, Y. & Tezuka, T. (2002) Acne phototherapy with a high-intensity, enhanced, narrow-band, blue light source: an open study and *in vitro* investigation. Journal of Dermatological Science. 30 (2), 129–135.

Keshishyan, E.S., Zaporozhtseva, Z.V., Zenina, O.M. & Zrodnikov, V.S. (2015) Photodynamic Inactivation of Bacteria *In Vitro* Under the Effect of Blue Light. *Bulletin of Experimental Biology and Medicine.* 158 (4), 475.

Kim, S., Kim, J., Lim, W., Jeon, S., Kim, O., Koh, J.T., Kim, C.S., Choi, H. & Kim, O. (2013) *In vitro* bactericidal effects of 625, 525, and 425 nm wavelength (red, green, and blue) light-emitting diode irradiation. *Photomedicine and Laser Surgery*. 31 (11), 554-562.

Kim, M.J., Mikš-Krajnik, M., Kumar, A., Ghate, V. & Yuk, H.G. (2015) Antibacterial effect and mechanism of high-intensity 405±5nm light emitting diode on *Bacillus cereus*, *Listeria monocytogenes*, and *Staphylococcus aureus* under refrigerated condition. *Journal of Photochemistry and Photobiology B: Biology*. 153, 33-39.

Kim, M.J., Miks-Krjnik, M., Kumar, A. & Yuk, H.G. (2016) Inactivation by 405 nm ± 5 nm light emitting diode on *Escherichia coli*, *Salmonella Typhimurium*, and *Shigella sonnei* under refrigerated condition might be due to the loss of membrane integrity. *Food Control.* 59, 99-107.

Kim, M.J., Bang, W.S. & Yuk, H.G. (2017) 405±5 nm light emitting diode illumination causes photodynamic inactivation of *Salmonella* spp. on fresh-cut papaya without deterioration. *Food Microbiology*. 62, 124-132.

Kim, M.J. & Yuk, H.G. (2017) Antibacterial Mechanism of 405-Nanometer Light-Emitting Diode against *Salmonella* at Refrigeration Temperature. *Applied and Environmental Microbiology*. 83 (5), e02582-e02616.

Kirby, W.M. (1944) Extraction of a highly potent penicillin inactivator from penicillin resistant staphylococci. *Science*. 99 (2579), 452-453.

Klämpfl, T.G., Isbary, G., Shimizu, T., Li, Y.F., Zimmermann, J.L., Stolz, W., Schlegel, J., Morfill, G.E. & Schmidt, H.U. (2012) Cold atmospheric air plasma sterilization against spores and other microorganisms of clinical interest. *Applied and Environmental Microbiology*. 78 (15), 5077-5082.

Kniel, K.E. (2014) The makings of a good human norovirus surrogate. *Current Opinion in Virology*. 4, 85-90.

Knipe, D.M. & Howley, P.M. (2013) Fields Virology. 6th Edition. Philadelphia, USA, Wolters Kluwer Health/Lippincott Williams & Wilkins.

Kossakowska, M., Nakonieczna, J., Kawiak, A., Kurlenda, J., Bielawski, K. P. & Grinholc, M. (2013). Discovering the mechanisms of strain-dependent response of *Staphylococcus aureus* to photoinactivation: oxidative stress toleration, endogenous porphyrin level and strain's virulence. *Photodiagnosis and Photodynamic Therapy*. 10 (4), 348-355.

Kotoku, Y., Kato, J., Akashi, G., Hirai, Y. & Ishihara, K. (2009) Bactericidal effect of a 405-nm diode laser on *Porphyromonas gingivalis*. *Laser Physics Letters*. 6 (5), 388.

Kotwal, G. & Cannon, J.L. (2014) Environmental persistence and transfer of enteric viruses. *Current Opinion in Virology*. 4, 37-43.

Koutsoumanis, K.P., Kendall, P.A. & Sofos, J.N. (2003) Effect of food processing related stresses on acid tolerance of *Listeria monocytogenes*. *Applied and Environmental Microbiology*. 69, 514-7516.

Kowalski, W. (2010) Ultraviolet germicidal irradiation handbook: UVGI for air and surface disinfection. Heidelberg, Germany, Springer Science.

Kramer, A., Schwebke, I. & Kampf, G. (2006) How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. *BioMed Central Infectious Diseases*. 6 (1), 130.

Kühn, K.P., Chaberny, I.F., Massholder, K., Stickler, M., Benz, V.W., Sonntag, H.G. & Erdinger, L. (2003) Disinfection of surfaces by photocatalytic oxidation with titanium dioxide and UVA light. *Chemosphere*. 53 (1), 71-77

Kumar, S., Subhadra, S., Singh, B. & Panda, B.K. (2013) Hepatitis E virus: the current scenario. *International Journal of Infectious Diseases*. 17 (4), e228-e233.

Kumar, A., Ghate, V., Kim, M.J., Zhou, W., Khoo, G.H. & Yuk, H.G. (2015) Kinetics of bacterial inactivation by 405 nm and 520 nm light emitting diodes and the role of endogenous coproporphyrin on bacterial susceptibility. *Journal of Photochemistry and Photobiology B: Biology*. 149, 37-44.

Kumar, A., Ghate, V., Kim, M.J., Zhou, W., Khoo, G.H. & Yuk, H.G. (2016) Antibacterial efficacy of 405, 460 and 520 nm light emitting diodes on Lactobacillus plantarum, *Staphylococcus aureus* and *Vibrio parahaemolyticus*. *Journal of Applied Microbiology*. 120 (1), 49-56.

Lai, C.C., Wang, Y.H., Wu, C.Y., Hung, C.H., Jiang, D.D.S. & Wu, F.T. (2013) A norovirus outbreak in a nursing home: norovirus shedding time associated with age. *Journal of Clinical Virology*. 56 (2), 96-101.

Lam, B.C., Lee, J. & Lau, Y.L. (2004) Hand hygiene practices in a neonatal intensive care unit: a multimodal intervention and impact on nosocomial infection. *Paediatrics*. 114 (5), e565-e571.

Lambrechts, S.A.G., Aalders, M.C.G. & Van Marle, J. (2005) Mechanistic study of the photodynamic inactivation of *Candida albicans* by a cationic porphyrin. *Antimicrobial Agents and Chemotherapy*. 49 (5), 2026-2034.

Lamont, Y., Rzeżutka, A., Anderson, J.G., MacGregor, S.J., Given, M.J., Deppe, C. & Cook, N. (2007) Pulsed UV- light inactivation of poliovirus and adenovirus. *Letters in Applied Microbiology*. 45 (5), 564-567.

Lauro, F.M., Pretto, P., Covolo, L., Jori, G. & Bertoloni, G. (2002) Photoinactivation of bacterial strains involved in periodontal diseases sensitized by porphycene-polylysine conjugates. *Photochemical and Photobiological Science*. 1, 468-470.

Lavi, R., Sinyakov, M., Samuni, A., Shatz, S., Friedmann, H., Shainberg, A., Breitbart, H. & Lubart, R. (2004) ESR detection of $^1O_2$ reveals enhanced redox activity in illuminated cell cultures. *Free Radical Research*. 38 (9), 893-902.

Lawes, T., López-Lozano, J.M., Nebot, C., Macartney, G., Subbarao-Sharma, R., Dare, C.R., Edwards, G.F. & Gould, I.M. (2015) Turning the tide or riding the waves? Impacts of antibiotic stewardship and infection control on MRSA strain dynamics in a Scottish region over 16 years: non-linear time series analysis. *BMJ Open*. 5 (3), e006596.

Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A.K., Wertheim, H.F., Sumpradit, N., Vlieghe, E., Hara, G.L., Gould, I.M., Goossens, H. & Greko, C. (2013) Antibiotic resistance—the need for global solutions. *The Lancet Infectious Diseases*. 13 (12), 1057-1098.

Leclerc, H., Edberg, S., Pierzo, V. & Delattre, J.M. (2000) Bacteriophages as indicators of enteric viruses and public health risk in groundwaters. *Journal of Applied Microbiology*. 88 (1), 5-21.

Lee, N., Chan, M.C., Wong, B., Choi, K.W., Sin, W., Lui, G., Chan, P.K.S., Lai, R.W.M., Cockram, C.S., Sung, J.J.Y. & Leung, W.K. (2007) Faecal viral concentration and diarrhoea in norovirus gastroenteritis. *Emerging Infectious Diseases*. 13 (9), 1399-1401.

Lee, K.W.K., Periasamy, S., Mukherjee, M., Xie, C., Kjelleberg, S. & Rice, S.A. (2014) Biofilm development and enhanced stress resistance of a model, mixed-species community biofilm. *The ISME Journal*. 8, 894–907.

Lembo, A.J., Ganz, R.A., Sheth, S., Cave, D., Kelly, C., Levin, P., Kazlas, P.T., Baldwin, P.C., Lindmark, W.R., McGrath, J.R. & Hamblin, M.R. (2009) Treatment of *Helicobacter pylori* infection with intra-gastric violet light phototherapy: A pilot clinical trial. *Lasers in Surgery and Medicine*. 41 (5), 337-344.

Lemon, S.M. (1997) Type A viral hepatitis: epidemiology, diagnosis, and prevention. *Clinical Chemistry*. 43 (8), 1494-1499.

Levin, P.D., Shatz, O., Sviri, S., Moriah, D., Or-Barbash, A., Sprung, C.L., Moses, A.E. & Block, C. (2009) Contamination of portable radiograph equipment with resistant bacteria in the ICU. *CHEST Journal*. 136 (2), 426-432.

Li, C.S. & Wang, Y.C. (2003) Surface germicidal effects of ozone for microorganisms. *American Industrial Hygiene Association Journal*. 64 (4), 533-537.

Liebmann, J., Born, M. & Kolb-Bachofen, V. (2010) Blue-light irradiation regulates proliferation and differentiation in human skin cells. *Journal of Investigative Dermatology*. 130 (1), 259-269.

Lim, M.Y., Kim, J.M., Lee, J.E. & Ko, G. (2010) Characterization of ozone disinfection of murine norovirus. *Applied and Environmental Microbiology*. 76 (4), 1120-1124.

Lindsay, D. & von Holy, A. (2006) Bacterial biofilms within the clinical setting: what healthcare professionals should know. *Journal of Hospital Infection*. 64 (4), 313-325.

Linley, E., Denyer, S.P., McDonnell. G., Simons, C. & Maillard, J.Y. (2012) Use of hydrogen peroxide as a biocide: new consideration of its mechanisms of biocidal action. *Journal of Antimicrobial Chemotherapy*. 67 (7), 1589-1596.

Lipovsky, A., Nitzanm Y., Friedmann, H. & Lubart, R. (2009) Sensitivity of *Staphylococcus aureus* Strains to Broadband Visible Light. *Photochemistry and Photobiology*. 85, 255-260.

Lipovsky, A., Nitzan, Y., Gedanken, A. and Lubart, R. (2010) Visible light-induced killing of bacteria as a function of wavelength: Implication for wound healing. *Lasers in Surgery and Medicine*. 42 (6), 467-472.

Liu, G.Y., Essex, A., Buchanan, J.T., Datta, V., Hoffman, H.M., Bastian, J.F., Fierer, J. & Nizet, V. (2005) *Staphylococcus aureus* golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. *Journal of Experimental Medicine.* 202, 209-215.

Lopman, B.A., Adak, G.K., Reacher, M.H. & Brown, D.W. (2003) Two epidemiologic patterns of Norovirus outbreaks: surveillance in England and Wales, 1992-2000. *Emerging Infectious Diseases*. 9 (1), 71-77.

Lopman, B., Gastañaduy, P., Park, G.W., Hall, A.J., Parashar, U.D. & Vinjé, J. (2012) Environmental transmission of norovirus gastroenteritis. *Current Opinion in Virology*. 2 (1), 96-102.

Lubart, R., Lipovski, A., Nitzan, Y. & Friedmann, H. (2011) A possible mechanism for the bactericidal effect of visible light. *Laser Therapy*. 20 (1), 17-22.

Lui, G.Y., Roser, D., Corkish, R., Ashbolt, N.J. & Stuetz, R. (2016) Point-of-use water disinfection using ultraviolet and visible light-emitting diodes. *Science of the Total Environment*. 553, 626-635.

Luksienė, Z. & Zukauskas, A. (2009) Prospects of photosensitization in control of pathogenic and harmful micro- organisms. *Journal of Applied Microbiology*. 107 (5), 1415-1424.

Maclean, M., MacGregor, S.J., Anderson, J.G. & Woolsey, G. (2008a) High-intensity narrow-spectrum light inactivation and wavelength sensitivity of *Staphylococcus aureus*. *FEMS Microbiology Letters.* 285, 227-232.

Maclean, M., MacGregor, S.J., Anderson, J.G. & Woolsey, G.A. (2008b) The role of oxygen in the visible-light inactivation of *Staphylococcus aureus*. *Journal of Photochemistry and Photobiology B.* 92, 180-184.

Maclean, M., Murdoch, L.E., Lani, M.N., MacGregor, S.J., Anderson, J.G. & Woolsey, G.A. (2008c) Photoinactivation and Photoreactivation Responses by Bacterial Pathogens after Exposure to Pulsed UV-light. Proceedings of the 2008 IEEE International Power Modulators and High Voltage Conference, Las Vegas, USA, May 2008. 326-329. Doi: 10.1109/IPMC.2008.4743649

Maclean, M., MacGregor, S.J., Anderson, J.G. & Woolsey, G. (2009) Inactivation of bacterial pathogens following exposure to light from a 405-nanometer light-emitting diode array. *Applied and Environmental Microbiology.* 75, 1932-1937.

Maclean, M., Macgregor, S.J., Anderson, J.G., Woolsey, G.A., Coia, J.E., Hamilton, K., Taggart, I., Watson, S.B., Thakker, B. & Gettinby, G. (2010) Environmental decontamination of a hospital isolation room using high-intensity narrow-spectrum light. *Journal of Hospital Infection.* 76, 247-251.

Maclean, M., Booth, M.G., Anderson, J.G., MacGregor, S.J., Woolsey, G.A., Coia, J.E., Hamilton, K. & Gettinby, G. (2013a) Continuous decontamination of an intensive care isolation room during patient occupancy using 405 nm light technology. *Journal of Infection Prevention.* 14 (5), 176-181.

Maclean, M., Murdoch, L.E., MacGregor, S.J. & Anderson, J.G. (2013b) Sporicidal effects of high-intensity 405 nm visible light on endospore-forming bacteria. *Photochemistry and Photobiol*ogy. 89, 120-126.

Maclean, M., McKenzie, K., Anderson, J.G., Gettinby, G. & MacGregor, S.J. (2014) 405 nm light technology for the inactivation of pathogens and its potential role for environmental disinfection and infection control. *Journal of Hospital Infection.* 88 (1), 1-11.

Maclean, M., McKenzie, K., Moorhead, S., Tomb, R.M., Coia, J.E., MacGregor, S.J. & Anderson, J.G. (2015) Decontamination of the Hospital Environment: New Technologies for Infection Control. *Current Treatment Options in Infectious Diseases*. 1-13. Doi:10.1007/s40506-015-0037-5

Maclean, M., Anderson, J.G., MacGregor, S.J., White, T. & Atreya, C.D. (2016) A New Proof of Concept in Bacterial Reduction: Antimicrobial Action of Violet-Blue Light (405 nm) in *Ex Vivo* Stored Plasma. *Journal of Blood Transfusion*, 2920514. Doi: 10.1155/2016/2920514.

Mahida, N., Vaughan, N. & Boswell, T. (2013) First UK evaluation of an automated ultraviolet-C room decontamination device (Tru-D™). *Journal of Hospital Infection*. 84 (4), 332-335.

Mahida, N. & Boswell, T. (2016) Non-slip socks: a potential reservoir for transmitting multidrug-resistant organisms in hospitals? *Journal of Hospital Infection*. 94 (3), 273-275.

Maisch, T. (2009) A new strategy to destroy antibiotic resistant microorganisms: antimicrobial photodynamic treatment. *Mini Reviews in Medicinal Chemistry*. 9 (8), 974-983.

Maisch, T. (2015) Resistance in antimicrobial photodynamic inactivation of bacteria. *Photochemical and Photobiological Science*. 14, 1518-1526.

Manian, F.A., Griesenauer, S., Senkel, D., Setzer, J.M., Doll, S.A., Perry, A.M. & Wiechens, M. (2011) Isolation of *Acinetobacter baumannii* complex and methicillin-resistant *Staphylococcus aureus* from hospital rooms following terminal cleaning and disinfection: can we do better? *Infection Control and Hospital Epidemiology*. 32 (7), 667-672.

Manoil, D., Filieri, A., Schrenzel, J. & Bouillaguet, S. (2016) Rose bengal uptake by *E. faecalis* and *F. nucleatum* and light-mediated antibacterial activity measured by flow cytometry. *Journal of Photochemistry and Photobiology B: Biology*. 162, 258-265.

Margomenou, L., Birkmyre, L., Piggott, J.R. & Paterson, A. (2000) Optimisation and validation of the "Strathclyde simulated mouth" for beverage flavour research. *Journal of the Institute of Brewing*. 106 (2), 101-106.

Maunula, L. (2005) Norovirus Outbreaks from Drinking Water. *Emerging Infectious Disease-CDC*. 11 (11), 1716-1721.

Marschner, S. & Goodrich, R. (2011) Pathogen reduction technology treatment of platelets, plasma and whole blood using riboflavin and UV light. *Transfusion Medicine and Hemotherapy.* 38 (1), 8-18.

Masson-Meyers, D.S., Bumah, V.V., Biener, G., Raicu, V. & Enwemeka, C.S. (2015) The relative antimicrobial effect of blue 405 nm LED and blue 405 nm laser on methicillin-resistant *Staphylococcus aureus in vitro*. *Lasers in Medical Science.* 30 (9), 2265-2271.

Matoba, A.Y., Peterson, J.R. & Wilhelmus, K.R. (2016) Dendritiform keratopathy associated with exposure to polyquarternium-1, a common ophthalmic preservative. *Ophthalmology*. 123 (3), 451-456.

Matsumura, Y. & Ananthaswamy, H.N. (2004) Toxic effects of ultraviolet radiation on the skin. *Toxicology and Applied Pharmacology.*195 (3), 298-308.

Matuschek, E., Brown, D.F.J. & Kahlmeter, G. (2014) Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. *Clinical Microbiology and Infection*. 20 (4), O255-O266.

Mayfield, J.L., Leet, T., Miller, J. & Mundy, L.M. (2000). Environmental control to reduce transmission of *Clostridium difficile*. *Clinical Infectious Diseases*. 31 (4), 995-1000.

McDonagh, A.F. (2008) Photolysis and photoisomerization of bilirubin in serum specimens exposed to room lighting. *Clinica Chimica Acta*. 393 (2), 130.

McDonald, K.F., Curry, R.D., Clevenger, T.E., Brazos, B.J., Unklesbay, K., Eisenstark, A., Baker, S., Golden, J. & Morgan, R. (2000) The development of photosensitized pulsed and continuous ultraviolet decontamination techniques for surfaces and solutions. *IEEE Transactions on Plasma Science*. 28 (1), 89-96.

McDonald, R., MacGregor, S.J., Anderson, J.G., Maclean, M. & Grant, M.H. (2011) Effect of 405-nm high-intensity narrow-spectrum light on fibroblast-populated collagen lattices: an in vitro model of wound healing. *Journal of Biomedical Optics.* 16 (4), 048003.

McDonald, R.S., Gupta, S., Maclean, M., Ramakrishnan, P., Anderson, J.G., MacGregor, S.J., Meek, R.M. & Grant, M.H. (2013) 405 nm light exposure of osteoblasts and inactivation of bacterial isolates from arthroplasty patients: potential for new disinfection applications. *European Cells and Materials*. 25, 204-214.

McGann, P., Snesrud, E., Maybank, R., Corey, B., Ong, A.C., Clifford, R., Hinkle, M., Whitman, T., Lesho, E. & Schaecher, K.E. (2016) *Escherichia coli* harbouring mcr-1 and blaCTX-M on a novel IncF plasmid: first report of mcr-1 in the United States. *Antimicrobial Agents and Chemotherapy*. 60 (7), 4420-4421.

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

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

McKenzie, K. (2014) Inactivation of Foodborne pathogenic and spoilage organisms by 405 nm light: An investigation into potential decontamination applications. PhD Thesis. University of Strathclyde, Glasgow, UK.

McKenzie, K., Maclean, M., Grant, M.H., Ramakrishnan, P., MacGregor, S.J. & Anderson, J.G. (2016) The effects of 405 nm light on bacterial membrane integrity determined by salt and bile tolerance assays, leakage of UV-absorbing material and SYTOX green labelling. *Microbiology*. 162 (9), 1680-1688.

McLean, J.S., Lombardo, M.J., Ziegler, M.G., Novotny, M., Yee-Greenbaum, J., Badger, J. H., Tesler, G., Nurk, S., Lesin, V., Brami, D., Hall, A.P., Edlund, A., Allen, L.Z., Durkin, S., Reed, S., Torriani, F., Nealson, K.H., Pevzner, P.A., Friedman, R., Venter, J.C. & Lasken, R.S. (2013) Genome of the pathogen *Porphyromonas gingivalis* recovered from a biofilm in a hospital sink using a high-throughput single-cell genomics platform. *Genome Research*. 23 (5), 867-877.

McMahon, M.A., Xu, J., Moore, J.E., Blair, I.S. & McDowell, D.A. (2007) Environmental stress and antibiotic resistance in food-related pathogens. *Applied and Environmental Microbiology*. 73, 211-217.

Memarzadeh, F., Olmsted, R.N. & Bartley, J.M. (2010) Applications of ultraviolet germicidal irradiation disinfection in health care facilities: effective adjunct, but not stand-alone technology. *American Journal of Infection Control*. 38 (5), S13-S24.

Merigo, E., Conti, S., Ciociola, T., Fornaini, C., Polonelli, L., Lagori, G., Manfredi, M. & Vescovi, P. (2017) Effect of different wavelengths and dyes on *Candida albicans*: *In vivo* study using Galleria mellonella as an experimental model. *Photodiagnosis and Photodynamic Therapy*. 18, 34-38.

Messina, G., Ceriale, E., Burgassi, S., Russo, C., Defranceschi, C., Mariani, L., Taddei, L., Lenzi, D. & Manzi, P. (2013) Impact of a disinfecting technique on microbial contamination of computer keyboards and telephone handsets. *Journal of Hospital Administration*. *2* (4), 1-6.

Mitchell, B.G., Digney, W. & Ferguson, J.K. (2014) Prior room occupancy increases risk of methicillin-resistant *Staphylococcus aureus* acquisition. *Healthcare infection*. 19 (4), 135-140.

Mitsuoka, T., Torikai, A. & Fueki, K. (1993) Wavelength sensitivity of the photodegradation of poly (methyl methacrylate). *Journal of Applied Polymer Science*. 47 (6), 1027-1032.

Moat, J., Cargill, J., Shone, J. & Upton, M. (2009) Application of a novel decontamination process using gaseous ozone. *Canadian Journal of Microbiology.* 55 (8), 928-933.

Mohr, H., Lambrecht, B. & Selz, A. (1995). Photodynamic virus inactivation of blood components. *Immunological Investigations.* 24, 73-85.

Moher, D., Liberati, A., Tetzlaff, J., Altman, D. G. & Prisma Group. (2009) Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *PLOS Med*. 6 (7), e1000097.

Moorhead, S., Maclean, M., Coia, J.E., MacGregor, S.J. & Anderson, J.G. (2016a) Synergistic efficacy of 405 nm light and chlorinated disinfectants for enhanced decontamination of *Clostridium difficile* spores. *Anaerobe.* 37, 72-77.

Moorhead, S., Maclean, M., MacGregor, S.J. & Anderson, J.G. (2016b) Comparative sensitivity of Trichophyton and Aspergillus conidia to inactivation by violet-blue light exposure. *Photomedicine and Laser Surgery*. 34 (1), 36-41.

Morgan, D.J., Rogawski, E., Thom, K.A., Johnson, J.K., Perencevich, E.N., Shardell, M., Leekha, S. & Harris, A.D. (2012) Transfer of multidrug-resistant bacteria to healthcare workers' gloves and gowns after patient contact increases with environmental contamination. *Critical Care Medicine*. 40 (4), 1045-1051.

Mori, M., Hamamoto, A., Nakano, M., Akutagawa, M., Takahashi, A., Ikehara, T. and Kinouchi, Y., 2007. Effects of ultraviolet LED on bacteria. Proceedings of World Congress on Medical Physics and Biomedical Engineering, Seoul, Korea, August-September 2006. 1327-1330. Doi: 10.1007/978-3-540-36841-0_323

Morter, S., Bennet, G., Fish, J., Richards, J., Allen, D.J., Nawaz, S., Iturriza-Gómara, M., Brolly, S. & Gray, J. (2011) Norovirus in the hospital setting: virus introduction and spread within the hospital environment. *Journal of Hospital infection*. 77 (2), 106-112.

Murdoch, L.E., Maclean, M., MacGregor, S.J. & Anderson, J.G. (2010) Inactivation of *Campylobacter jejuni* by Exposure to High-Intensity 405-nm Visible Light. *Foodborne Pathogens and Disease.* 7 (10), 1211-1216.

Murdoch, L.E., Maclean, M., Endarko, E., MacGregor, S.J. & Anderson, J.G. (2012) Bactericidal effects of 405 nm light exposure demonstrated by inactivation of *Escherichia, Salmonella, Shigella, Listeria,* and *Mycobacterium* species in liquid suspensions and on exposed surfaces. *The Scientific World Journal*. 137805. Doi: 10.1100/2012/137805

Murdoch, L.E., McKenzie, K., Maclean, M., MacGregor, S.J. & Anderson, J.G. (2013) Lethal effects of high intensity violet 405-nm light on *Saccharomyces cerevisiae, Candida albicans* and on dormant and germinating spores of *Aspergillus niger*. *Fungal Biology.* 117, 519–527.

Murray, P.R., Rosenthal, K.S. & Pfaller, M.A. (2008a). Medical Microbiology. 6th Edition. Philadelphia, USA, Mosby Elseveir.

Murray, B.K., Ohmine, S., Tomer, D.P., Jensen, K.J., Johnson, F.B., Kirsi, J.J., Robison, R.A. & O'Neill, K.L. (2008b) Virion disruption by ozone-mediated reactive oxygen species. *Journal of Virology Methods.* 153 (1), 74-77.

Nakano, R., Ishiguro, H., Yao, Y., Kajioka, J., Fujishima, A., Sunada, K., Minoshima, M., Hashimoto, K. & Kubota, Y. (2012) Photocatalytic inactivation of influenza virus by titanium dioxide thin film. *Photochemical and Photobiological Sciences*. 11 (8), 1293-1298.

Nakonieczna, J., Michta, E., Rybicka, M., Grinholc, M., Gwizdek-Wiśniewska, A. & Bielawski, K.P. (2010) Superoxide dismutase is upregulated in *Staphylococcus aureus* following protoporphyrin-mediated photodynamic inactivation and does not directly influence the response to photodynamic treatment. *BioMed Central Microbiology*. 10, 323.

Naimi, T.S., LeDell, K.H., Como-Sabetti, K., Borchardt, S.M., Boxrud, D.J., Etienne, J., Johnson, S.K., Vandenesch, F., Fridkin, S., O'Boyle, C., Danila, R.N. & Lynfield, R. (2003) Comparison of community-and health care–associated methicillin-resistant *Staphylococcus aureus* infection. *Jama.* 290 (22), 2976-2984.

Nishimuta, M., Inoue, N., Kodama, N., Morikuni, E., Yoshioka, Y.H., Matsuzaki, N., Shimada, M., Sato, N., Iwamotot, T., Ohki, K., Takeyama, H. & Nishimuta, H. (2006). Moisture and Mineral Content of Human Feces—High Fecal Moisture Is Associated with Increased Sodium and Decreased Potassium Content—. *Journal of Nutritional Science and Vitaminology*. 52 (2), 121-126.

Neely, A.N., Maley, M.P. & Warden, G.D. (1999) Computer keyboards as reservoirs for *Acinetobacter baumannii* in a burn hospital. *Clinical Infectious Diseases*. 29 (5), 1358-1359.

Nerandzic, M.M., Cadnum, J.L., Pultz, M.J. & Donskey, C.J. (2010) Evaluation of an automated ultraviolet radiation device for decontamination of *Clostridium difficile* and other healthcare-associated pathogens in hospital rooms. *BioMed Central Infectious Diseases.* 10 (1), 197.

Nerandzic, M.M., Thota, P., Sankar, T.C., Jencson, A., Cadnum, J.L., Ray, A.J., Salata, R.A., Watkins, R.R. & Donskey, C.J. (2015) Evaluation of a pulsed xenon ultraviolet disinfection system for reduction of healthcare-associated pathogens in hospital rooms. *Infection Control and Hospital Epidemiology*. 36 (2), 192-197.

NHS National Services Scotland. (2016) Quality Assurance and Regulatory Compliance. NSS, Edinburgh, UK. Available from: https://nhsnss.org/services/blood-tissues-and-cells/quality-assurance-and-regulatory-compliance/ [Accessed March 2017]

NHS Greater Glasgow and Clyde. (2016) Infection Prevention and Control Manual, 2016. NHS GGC, Glasgow, UK. Available from: http://www.nhsggc.org.uk/your-health/infection-prevention-and-control/prevention-and-control-of-infection-manual-policies-sops-guidelines/ [Accessed March 2017]

Niendorf, S., Jacobsen, S., Faber, M., Eis-Hübinger, A.M., Hofmann, J., Zimmermann, O., Höhne, M. & Bock, T. (2017) Steep rise in norovirus cases and emergence of a new recombinant strain GII. P16-GII. 2, Germany, winter 2016. *Eurosurveillance*. 22 (4), S1-4.

Nims, R. & Plavsic, M. (2013) Inactivation of caliciviruses. *Pharmaceuticals*. 6 (3), 358-392.

Nitzan, Y. & Kauffman, M. (1999) Endodgenous porphyrin production in bacteria by δ-Aminolaevulinic acid and subsequent bacterial photoeradication. *Lasers in Medical Science*. 14 (4), 269-277.

Nitzan, Y., Salmon-Divon, M., Shporen, E. & Malik, Z. (2004) ALA induced photodynamic effects on Gram positive and negative bacteria. *Photochemical and Photobiological Sciences*. 3 (5), 430-435.

Nseir, S., Blazejewski, C., Lubret, R., Wallet, F., Courcol, R. & Durocher, A. (2011) Risk of acquiring multidrug-resistant Gram-negative bacilli from prior room occupants in the intensive care unit. *Clinical Microbiology and Infection*. 17 (8), 1201-1208.

Nuanualsuwan, S., Mariam, T., Himathongkham, S. & Cliver, D.O. (2002) Ultraviolet inactivation of feline calicivirus, human enteric viruses and coliphages. *Photochemistry and Photobiology*. 76 (4), 406-410.

O'Connor, N., Cahill, O., Daniels, S., Galvin, S. & Humphreys, H. (2014) Cold atmospheric pressure plasma and decontamination. Can it contribute to preventing hospital-acquired infections? *Journal of Hospital Infection*. 88 (2), 59-65.

O'Donoghue, B., NicAogáin, K., Bennett, C., Conneely, A., Tiensuu, T., Johansson, J. and O'Byrne, C. (2016) Inhibition of *Listeria monocytogenes* growth by blue light is mediated by reactive oxygen species: a role for sigma B and the blue light sensor, Lmo0799. *Applied and Environmental Microbiology*. AEM-00685-16. Doi: 10.1128/AEM.00685-16

Oliver, J. D. (2010) Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiology Reviews*. 34 (4), 415-425.

O'Neill, J. (2016) Tackling drug-resistant infections globally: final report and recommendations. Welcome Trust/HM Government, London, UK. Available from: https://amr-review.org/sites/default/files/160518_Final%20paper_with%20cover.pdf [Accessed March 2017]

Ormerod, E. & Jarrett, O. (1978) A classification of feline calicivirus isolates based on plaque morphology. *Journal of General Virology*. 39 (3), 537-540.

Otter, J.A. & French, G.L. (2009) Survival of nosocomial bacteria and spores on surfaces and inactivation by hydrogen peroxide vapor. *Journal of Clinical Microbiology.* 47 (1), 205-207.

Otter, J.A. & French, G.L. (2011) Community-associated methicillin-resistant *Staphylococcus aureus* strains as a cause of healthcare-associated infection. *Journal of Hospital Infection*. 79 (3), 189-193.

Otter, J.A., Yezli, S., Perl, T.M., Barbut, F. & French, G.L. (2013) The role of 'no-touch' automated room disinfection systems in infection prevention and control. *Journal of Hospital Infection.* 83 (1), 1-13.

Page, K., Wilson, M. & Parkin, I.P. (2009) Antimicrobial surfaces and their potential in reducing the role of the inanimate environment in the incidence of hospital-acquired infections. *Journal of Material Chemistry*. 19, 3819–3831.

Palmore, T.N. & Henderson, D.K. (2013) Managing transmission of carbapenem-resistant Enterobacteriaceae in healthcare settings: a view from the trenches. *Clinical Infectious Diseases*. 57 (11), 593-1599.

Papafragkou, E., Hewitt, J., Park, G.W., Greening, G. & Vinjé, J. (2013) Challenges of culturing human norovirus in three-dimensional organoid intestinal cell culture models. *PLOS One*. 8 (6), e63485.

Papageorgiou, P., Katsambas, A. & Chu, A. (2000) Phototherapy with blue (415 nm) and red (660 nm) light in the treatment of acne vulgaris. *British Journal of Dermatology*. 142 (5), 973-978.

Park, G.W., Boston, D.M., Kase, J.A., Sampson, M.N. & Sobsey, M.D. (2007). Evaluation of liquid-and fog-based application of Sterilox hypochlorous acid solution for surface inactivation of human norovirus. *Applied and Environmental Microbiology*. 73 (14), 4463-4468.

Park, G.W., Barclay, L., Macinga, D., Charbonneau, D., Pettigrew, C.A., & Vinjé, J. (2010) Comparative efficacy of seven hand sanitizers against murine norovirus, feline calicivirus, and GII. 4 norovirus. *Journal of Food Protection.* 73 (12), 2232-2238.

Park, G.W., Linden, K. G. & Sobsey, M.D. (2011) Inactivation of murine norovirus, feline calicivirus and echovirus 12 as surrogates for human norovirus (NoV) and coliphage (F+) MS2 by ultraviolet light (254 nm) and the effect of cell association on UV inactivation. *Letters in Applied Microbiology*. 52 (2), 162-167.

Park, G.W., Cho, M., Cates, E.L., Lee, D., Oh, B.T., Vinjé, J. & Kim, J.H. (2014) Fluorinated TiO_2 as an ambient light-activated virucidal surface coating material for the control of human norovirus. *Journal of Photochemistry and Photobiology B: Biology*. 140, 315-320.

Park, G.W., Lee, D., Treffiletti, A., Hrsak, M., Shugart, J. & Vinjé, J. (2015) Evaluation of a new environmental sampling protocol for detection of human norovirus on inanimate surfaces. *Applied and Environmental Microbiology*. 81 (17), 5987-5992.

Park, D., Shahbaz, H.M., Kim, S.H., Lee, M., Lee, W., Oh, J.W., Lee, D.U. & Park, J. (2016) Inactivation efficiency and mechanism of UV-TiO_2 photocatalysis against murine norovirus using a solidified agar matrix. *International Journal of Food Microbiology*. 238, 256-264.

Passaretti, C.L., Otter, J.A., Reich, N.G., Myers, J., Shepard, J., Ross, T., Carroll, K.C., Lipsett, P. & Perl, T.M. (2013) An evaluation of environmental decontamination with hydrogen peroxide vapor for reducing the risk of patient acquisition of multidrug-resistant organisms. *Clinical Infectious Diseases*. 56 (1), 27-35.

Patel, M.M., Hall, A.J., Vinjé, J. & Parashar, U.D. (2009) Noroviruses: a comprehensive review. *Journal of Clinical Virology*. 44 (1), 1-8.

Pattison, D.I., Rahmanto, A.S. & Davies M.J. (2012) Photo-oxidation of proteins. *Photochemical and Photobiological Sciences*. 11, 38-53.

Peacock, S.J. & Paterson, G.K. (2015) Mechanisms of methicillin resistance in *Staphylococcus aureus*. *Annual Review of Biochemistry*. 84, 577-601.

Pedigo, L.A., Gibbs, A.J., Scott, R.J. & Street, C.N. (2009) Absence of bacterial resistance following repeat exposure to photodynamic therapy. Proceddings of 12th World Congress of the International Photodynamic Association, Seattle, USA, July 2009. 73803H-73803H. Doi: 10.1117/12.822834

Pendleton, J.N., Gorman, S.P. & Gilmore, B.F. (2013) Clinical relevance of the ESKAPE pathogens. *Expert Review in Anti-infective Therapy.* 11, 297-308.

Perlroth, J., Choi, B. & Spellberg, B. (2007) Nosocomial fungal infections: epidemiology, diagnosis, and treatment. *Medical Mycology*. 45 (4), 321-346.

Pessoa-Silva, C. L., Hugonnet, S., Pfister, R., Touveneau, S., Dharan, S., Posfay-Barbe, K. & Pittet, D. (2007) Reduction of health care–associated infection risk in neonates by successful hand hygiene promotion. *Paediatrics*. 120 (2), e382-e390.

Piskin, N., Celebi, G., Kulah, C., Mengeloglu, Z. & Yumusak, M. (2011) Activity of a dry mist-generated hydrogen peroxide disinfection system against methicillin-resistant *Staphylococcus aureus* and *Acinetobacter baumannii*. *American Journal of Infection Control*. 39 (9), 757–62.

Pittet., D., Hugonnet, S., Harbarth, S., Mourouga, P., Sauvan, V., Touveneau, S. & Perneger, T.V. (2000) Effectiveness of a hospital-wide programme to improve compliance with hand hygiene. *The Lancet*. 356 (9238), 1307-1312.

Pittet, D., Allegranzi, B., Sax, H., Dharan, S., Pessoa-Silva, C.L., Donaldson, L. & Boyce, J.M. (2006) Evidence-based model for hand transmission during patient care and the role of improved practices. *The Lancet Infectious Diseases*. 6 (10), 641-652.

Poole, K. (2012) Bacterial stress responses as determinants of antimicrobial resistance. *Journal of Antimicrobial Chemotherapy*. 67 (9), 2069-2089.

Pottage, T., Richardson, C., Parks, S., Walker, J.T. & Bennett, A.M. (2010) Evaluation of hydrogen peroxide gaseous disinfection systems to decontaminate viruses. *Journal of Hospital Infection*. 74 (1), 55-61.

Predmore, A., Sanglay, G., Li, J. & Lee, K. (2015) Control of human norovirus surrogates in fresh foods by gaseous ozone and a proposed mechanism of inactivation. *Food Microbiology*. 50, 118–125

Proctor, R.A., Von Eiff, C., Kahl, B.C., Becker, K., McNamara, P., Herrmann, M. & Peters, G. (2006) Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nature Reviews Microbiology*. 4 (4), 295-305.

Raj, P., Tay, J., Ang, L.W., Tien, W.S., Thu, M., Lee, P., Pang, Q.Y., Tang, Y.L., Lee, K.Y., Maurer-Stroh, S. & Gunalan, V. (2017) A large common-source outbreak of norovirus gastroenteritis in a hotel in Singapore, 2012. *Epidemiology and Infection*. 145 (3), 535-544.

Ramakrishnan, P., Maclean, M., MacGregor, S.J., Anderson, J.G., & Grant, M.H. (2014) Differential sensitivity of osteoblasts and bacterial pathogens to 405-nm light highlighting potential for decontamination applications in orthopaedic surgery. *Journal of Biomedical Optics*. 19 (10), 105001-105001.

Ramakrishnan, P., Maclean, M., MacGregor, S.J., Anderson, J.G. & Grant, M.H. (2016) Cytotoxic responses to 405nm light exposure in mammalian and bacterial cells: Involvement of reactive oxygen species. *Toxicology in Vitro*. 33, 54-62.

Ramm, L., Siani, H., Wesgate, R. & Maillard, J.Y. (2015) Pathogen transfer and high variability in pathogen removal by detergent wipes. *American Journal of Infection Control*. 43 (7), 724-728.

Rammelkamp, C.H. & Maxon, T. (1942) Resistance of *Staphylococcus aureus* to the Action of Penicillin. *Experimental Biology and Medicine*. 51 (3), 386-389.

Randle, J., Arthur, A. & Vaughan, N. (2010) Twenty-four-hour observational study of hospital hand hygiene compliance. *Journal of Hospital Infection*, 76 (3), 252-255.

Rhodes, N.L., de la Presa, M., Barneck, M.D., Poursaid, A., Firpo, M.A. & Langell, J.T. (2016) Violet 405 nm light: A novel therapeutic agent against β-lactam-resistant *Escherichia coli*. *Lasers in Surgery and Medicine*. 48, 311–317

Richards, G.P. (2012) Critical review of norovirus surrogates in food safety research: rationale for considering volunteer studies. *Food and Environmental Virology*. 4 (1), 6-13.

Richardson, T.B. & Porter, C.D. (2005) Inactivation of murine leukaemia virus by exposure to visible light. *Virology*. 341 (2), 321-329.

Risović, D., Maver-Bišćanin, M., Mravak-Stipetić, M., Bukovski, S. & Bišćanin, A. (2014) Quantitative investigation of efficiency of ultraviolet and visible light in eradication of Candida albicans in vitro. *Photomedicine and Laser Surgery*. 32 (4), 232-239.

Robilotti, E., Deresinski, S. & Pinsky, B.A. (2015) Norovirus. *Clinical Microbiology Reviews*. 28 (1), 134-164.

Roh, H.J., Kim, A., Kang, G.S. & Kim, D.H. (2016) Photoinactivation of major bacterial pathogens in aquaculture. *Fisheries and Aquatic Sciences*. 19 (1), 28.

Romiti R., Schaller, M., Jacob, K. & Plewig, G. (2000) High-performance liquid chromatography analysis of porphyrins in *Propionibacterium acnes*. *Archives of Dermatological Research*. 292, 320-322.

Russell, A.D. (1999) Bacterial resistance to disinfectants: present knowledge and future problems. *Journal of Hospital Infection*. 43, S57–S68

Rutala, W.A., Peacock, J.E., Gergen, M.F., Sobsey, M.D. & Weber, D.J. (2006) Efficacy of hospital germicides against adenovirus 8, a common cause of epidemic keratoconjunctivitis in health care facilities. *Antimicrobial Agents and Chemotherapy*. 50 (4), 1419-1424.

Rutala, W.A., Gergen, M.F. & Weber, D.J. (2010) Room decontamination with UV radiation. *Infection Control and Hospital Epidemiology.* 31 (10), 1025-1029.

Rutala, W.A., Gergen, M.F. & Weber, D.J. (2012) Efficacy of Improved Hydrogen Peroxide against Healthcare-Associated Pathogens. *Infection Control and Hospital Epidemiology.* 33 (11), 1159-1161.

Rutala, W.A. & Weber, D.J. (2013a) Disinfectants used for environmental disinfection and new room decontamination technology. *American Journal of Infection Control*. 41 (5), S36-S41.

Rutala, W.A., Gergen, M.F., Tande, B.M. & Weber, D.J. (2013b) Rapid hospital room decontamination using ultraviolet (UV) light with a nanostructured UV-reflective wall coating. *Infection Control and Hospital Epidemiology*. 34 (5), 527-529.

Rutala, W.A., Gergen, M.F., Tande, B.M. & Weber, D.J. (2014) Room decontamination using an ultraviolet-C device with short ultraviolet exposure time. *Infection Control and Hospital Epidemiology*. 35 (8), 1070-1072.

Rutala, W., Kanamari, H., Gergen, M., Sickbert-Bennett, E., Sexton, D. & Anderson, D.R. (2016) Antimicrobial Activity of a Continuous Visible Light Disinfection System. Poster presented at ID Week, New Orleans, USA, October 2016. Available from: https://idsa.confex.com/idsa/2016/webprogram/Paper57395.html [Accessed March 2017]

Sabrià, A., Pintó, R.M., Bosch, A., Bartolomé, R., Cornejo, T., Torner, N., Martínez, A., de Simón, M., Domínguez, A. & Guix, S. (2016) Norovirus shedding among food and healthcare workers exposed to the virus in outbreak settings. *Journal of Clinical Virology*. 82, 119-125.

Sadowski, R., Strus, M., Buchalska, M., Heczko, P.B. & Macyk, W. (2015) Visible light induced photocatalytic inactivation of bacteria by modified titanium dioxide films on organic polymers. *Photochemical and Photobiological Sciences*. 14 (3), 514-519.

Salama, M.F., Jamal, W.Y., Al Mousa, H., Al-AbdulGhani, K.A. & Rotimi, V.O. (2013) The effect of hand hygiene compliance on hospital-acquired infections in an ICU setting in a Kuwaiti teaching hospital. *Journal of Infection and Public Health*. 6 (1), 27-34.

Sandhu, R., Murillo, M., Wyatt, D., Bhanot, N., Min, Z. & Thomas, J. (2016) Environmental Decontamination of Medical ICU Suites using High Intensity Narrow Spectrum Light. Poster presented at ID Week, New Orleans, USA, October 2016. Available from: https://idsa.confex.com/idsa/2016/webprogram/Paper57152.html [Accessed March 2017]

Sandokji, A.M., Murshid, K.R., El-Badry, A.A., Al-Ali, K.H. & Shalaby, S.A. (2009) Infectious nosocomial diarrhoea in the surgical wards: role of parasites and microbes imply stool analysis. *Journal of Taibah University Medical Sciences*. 4 (1), 73-81.

Santos, A.L., Gomes, N.C.M., Henriques, I., Almeida, A., Correia, A. & Cunha, Â. (2012) Contribution of reactive oxygen species to UV-B_induced damage in bacteria. *Journal of Photochemistry and Photobiology B: Biology*. 117, 40-46.

Sato, J., Miki, M., Kubota, H., Hitomi, J., Tokuda, H., Todaka-Takai, R. & Katayama, K. (2016) Effects of disinfectants against norovirus virus-like particles predict norovirus inactivation. *Microbiology and Immunology*. 60 (9), 609-616.

Sattar, S.A., Ali, M. & Tetro, J.A. (2011) *In vivo* comparison of two human norovirus surrogates for testing ethanol-based handrubs: the mouse chasing the cat! *PLOS One*. 6 (2) e17340.

Schagen, F.H.E., Moor, A.C.E., Cheong, S.C., Cramer, S.J., Van Ormondt, H., Van der Eb, A.J., Dubbelman, T.M.A.R. & Hoeben, R.C. (1999) Photodynamic treatment of adenoviral vectors with visible light: an easy and convenient method for viral inactivation. *Gene therapy*. 6 (5), 873-881.

Schorn, R., Höhne, M., Meerbach, A., Bossart, W., Wüthrich, R.P., Schreier, E., Müller, N.J. & Fehr, T. (2010) Chronic norovirus infection after kidney transplantation: molecular evidence for immune-driven viral evolution. *Clinical Infectious Diseases.* 51 (3), 307-314.

Seal, D.V., Kirkness, C.M., Bennett, H.G. & Peterson, M. (1999) Population-based cohort study of microbial keratitis in Scotland: incidence and features. *Contact Lens and Anterior Eye*. 22 (2), 49–57.

Seki, M., Machida, N., Yamagishi, Y., Yoshida, H. & Tomono, K. (2013) Nosocomial outbreak of multidrug-resistant *Pseudomonas aeruginosa* caused by damaged transoesophageal echocardiogram probe used in cardiovascular surgical operations. *Journal of Infection and Chemotherapy*. 19 (4), 677-681.

Sergent, A.P., Slekovec, C., Pauchot, J., Jeunet, L., Bertrand, X., Hocquet, D., Pazart, L. & Talon, D. (2012) Bacterial contamination of the hospital environment during wound dressing change. *Orthopaedics & Traumatology: Surgery & Research*. 98 (4), 441-445.

Sharma M. & Hudson J.B. (2008) Ozone gas is an effective and practical antibacterial agent. *American Journal of Infection Control.* 36 (8), 559-563.

Shaughnessy, M.K., Micielli, R.L., DePestel, D.D., Arndt, J., Strachan, C.L., Welch, K.B. & Chenoweth, C.E. (2011) Evaluation of hospital room assignment and acquisition of *Clostridium difficile* infection. *Infection Control and Hospital Epidemiology*. 32 (3), 201-206.

Shionoiri, N., Nogariya, O., Tanaka, M., Matsunaga, T. & Tanaka, T. (2015) Capsid protein oxidation in feline calicivirus using an electrochemical inactivation treatment. *Journal of Hazardous Materials*. 283, 410-415.

Sickbert-Bennett, E.E., DiBiase, L.M., Willis, T.M.S., Wolak, E.S., Weber, D.J & Rutala, W.A. (2016) Reduction of Healthcare-Associated Infections by Exceeding High Compliance with Hand Hygiene Practices. *Emerging Infectious Diseases*. 22 (9), 1628.

Siebenga, J.J., Vennema, H., Renckens, B., de Bruin, E., van der Veer, B., Siezen, R.J. & Koopmans, M. (2007) Epochal evolution of GII. 4 norovirus capsid proteins from 1995 to 2006. *Journal of Virology*. 81 (18), 9932-9941.

Siebenga, J.J., Duizer, E. & Koopmans, M.P.G. (2010) Norovirus Epidemiology. In Hansman, G.S., Jiang, X.J. & Green, K.Y. (Editors) Caliciviruses: Molecular and Cellular Virology. Norfolk, UK: Caister Academic Press. Pages 1-24

Sievert, D.M., Rudrik, J.T., Patel, J.B., McDonald, L.C., Wilkins, M.J. & Hageman, J.C. (2008). Vancomycin-resistant *Staphylococcus aureus* in the United States, 2002-2006. *Clinical Infectious Diseases*. 46 (5), 668–674.

Sigstam, T., Gannon, G., Cascella, M., Pecson, B.M., Wigginton, K.R. & Kohn, T. (2013) Subtle differences in virus composition affect disinfection kinetics and mechanisms. *Applied and Environmental Microbiology*. 79 (11), 3455-3467.

Slomka, M.J. & Appleton, H. (1998) Feline calicivirus as a model system for heat inactivation studies of small round structured viruses in shellfish. *Epidemiology and Infection.* 121 (2), 401-407.

Smith, K. & Hunter, I.S. (2008) Efficacy of common hospital biocides with biofilms of multi-drug resistant clinical isolates. *Journal of Medical Microbiology*. 57, 966-973.

Smith, K.C., Inns, T., Decraene, V., Fox, A., Allen, D.J. & Shah, A. (2017) An outbreak of norovirus GI-6 infection following a wedding in North West England. *Epidemiology and Infection*. 145 (6), 1-7.

Soehnge, H., Ouhtit, A. & Ananthaswamy, O.N. (1997) Mechanisms of induction of skin cancer by UV radiation. *Frontiers in Bioscience*. 2, D538-D551.

Sommers, C., Gunther, N.W. & Sheen, S. (2017) Inactivation of *Salmonella* spp., pathogenic *Escherichia coli*, *Staphylococcus* spp. or *Listeria monocytogenes* in chicken purge or skin using a 405-nm LED array. *Food Microbiology*. 64, 135-138.

Song, H.H., Lee, J.K., Um, H.S., Chang, B.S., Lee, S.Y. & Lee, M.K. (2013) Phototoxic effect of blue light on planktonic and biofilm state of anaerobic periodontal pathogens. *Journal of Periodontal and Implant Science.* 43, 72-78.

Soto-Giron, M.J., Rodriguez-R, L.M., Luo, C., Elk, M., Ryu, H., Hoelle, J., Santo Domingo, J.W. & Konstantinidis, K.T. (2016) Characterization of biofilms developing on hospital shower hoses and implications for nosocomial infections. *Applied and Environmental Microbiology*, AEM-03529. Doi: 10.1128/AEM.03529-15

Soukos, N.S., Som, S., Abernethy, A.D., Ruggiero, K., Dunham, J., Lee, C., Doukas, A.G.& Goodson, J.M. (2005) Phototargeting oral black-pigmented bacteria. *Antimicrobial Agents and Chemotherapy*. 49 (4), 1391-1396.

Srimagal, A., Ramesh, T. & Sahu, J.K. (2016) Effect of light emitting diode treatment on inactivation of *Escherichia coli* in milk. *LWT- Food Science and Technology.* 71, 378-385.

St Denis, T.G., Huang, L., Dai, T. & Hamblin, M.R. (2011) Analysis of the bacterial heat shock response to photodynamic therapy-mediated oxidative stress. *Photochemistry and Photobiology.* 87, 707-713.

Steinmann, J. (2004). Surrogate viruses for testing virucidal efficacy of chemical disinfectants. *Journal of Hospital Infection*. 56, 49-54.

Stevenson, R. (2009) The LED's Dark Secret. *IEEE Spectrum*. 46 (8). Doi: 10.1109/MSPEC.2009.5186552

Stibich, M., Stachowiak, J., Tanner, B., Berkheiser, M., Moore, L., Raad, I. & Chemaly, R F. (2011) Evaluation of a pulsed-xenon ultraviolet room disinfection device for impact on hospital operations and microbial reduction. *Infection Control and Hospital Epidemiology*. 32 (3), 286-288.

Stibich, M. & Stachowiak, J. (2016) The microbiological impact of pulsed xenon ultraviolet disinfection on resistant bacteria, bacterial spore and fungi and viruses: review. *Southern African Journal of Infectious Diseases*. 31 (1), 12-15.

Stiefel, U., Cadnum, J.L., Eckstein, B.C., Guerrero, D.M., Tima, M. A. & Donskey, C.J. (2011) Contamination of hands with methicillin-resistant *Staphylococcus aureus* after contact with environmental surfaces and after contact with the skin of colonized patients. *Infection Control and Hospital Epidemiology*. 32 (2), 185-187.

Straub, T.M., Bartholomew, R.A., Valdez, C.O., Valentine, N.B., Dohnalkova, A., Ozanich, R.M., Bruckner-Lea, C.J. & Call, D.R. (2011) Human Norovirus Infection of Caco-2 Cells Grown as a 3-Dimensional Tissue Structure. *Journal of Water and Health*. 9, 225–240.

Sutton, J., Cardinale, E., Epstein, S., Nummi, L., Bissinger, C. & Holder, C. (2016) Utilizing Passive Light Emitting Diode (LED) Disinfection Technology to Effectively Reduce Microbial Contamination in a Trauma Room. Poster presented at ID Week, New Orleans, USA, October 2016. Available from: https://idsa.confex.com/idsa/2016/webprogram/Paper57102.html [Accessed March 2017]

Takanashi, S., Saif, L.J., Hughes, J.H., Meulia, T., Jung, K., Scheuer, K.A. & Wang, Q. (2014) Failure of propagation of human norovirus in intestinal epithelial cells with microvilli grown in three-dimensional cultures. *Archives of Virology*. 159 (2), 257-266.

Tanner, B.D. (2009) Reduction in infection risk through treatment of microbially contaminated surfaces with a novel, portable, saturated steam vapor disinfection system. *American Journal of Infection Control*, 37 (1), 20-27.

Tavares, A., Carvalho, C., Faustino, M.A., Neves, M.G., Tomé, J.P., Tomé, A.C., Cavaleiro, J.A., Cunha, Â., Gomes, N., Alves, E. & Almeida, A. (2010) Antimicrobial photodynamic therapy: study of bacterial recovery viability and potential development of resistance after treatment. *Marine Drugs*. 8 (1), 91-105.

Terpstra, F.G., Van Den Blink, A.E., Bos, L.M., Boots, A.G.C., Brinkhuis, F.H.M., Gijsen, E., Van Remmerden, Y., Schuitemaker, H. & Van't Wout, A.B. (2007) Resistance of surface-dried virus to common disinfection procedures. *Journal of Hospital Infection*. 66 (4), 332-338.

Teunis, P.F.M., Moe, C.L., Liu, P., Miller, S.E., Lindesmith, L., Baric, R.S., Le Pendu, J. & Calderon, R.L. (2008) Norwalk virus: how infectious is it? *Journal of Medical Virology*. 80 (8), 1468-1476.

Thorne, L.G. & Goodfellow, I.G. (2014) Norovirus gene expression and replication. *Journal of General Virology*. 95 (2), 278-291.

Thurlow, L.R., Joshi, G.S. & Richardson, A.R. (2012) Virulence strategies of the dominant USA300 lineage of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA). *FEMS Immunology and Medical Microbiology*. 65 (1), 5-22.

Thurston-Enriquez, J.A., Haas, C.N., Jacangelo, J., Riley, K. & Gerba, C.P. (2003) Inactivation of feline calicivirus and adenovirus type 40 by UV radiation. *Applied and Environmental Microbiology*. 69 (1), 577-582.

Tomb, R.M., Maclean, M., Herron, P.R., Hoskisson, P.A., MacGregor, S.J. & Anderson, J.G. (2014) Inactivation of *Streptomyces* phage ϕC31 by 405 nm light: Requirement for exogenous photosensitizers? *Bacteriophage.* 4 (3): e32129.

Tong, S.Y., Davis, J.S., Eichenberger, E., Holland, T.L. & Fowler, V.G. (2015) *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management. *Clinical Microbiology Reviews.* 28 (3), 603-661.

Tsen, S.W.D., Kingsley, D.H., Kibler, K., Jacobs, B., Sizemore, S., Vaiana, S.M., Anderson, J., Tsen, K.T. & Achilefu, S. (2014) Pathogen reduction in human plasma using an ultrashort pulsed laser. *PLOS One*. 9 (11), e111673.

Tuladhar, E., Terpstra, P., Koopmans, M. & Duizer, E. (2012) Virucidal efficacy of hydrogen peroxide vapour disinfection. *Journal of Hospital Infection*. 80 (2), 110-115.

Ulger, F., Esen, S., Dilek, A., Yanik, K., Gunaydin, M. & Leblebicioglu, H. (2009) Are we aware how contaminated our mobile phones with nosocomial pathogens? *Annals of Clinical Microbiology and Antimicrobials*. 8 (1), 7.

Vandeputte, D., Falony, G., Vieira-Silva, S., Tito, R.Y., Joossens, M. & Raes, J. (2016) Stool consistency is strongly associated with gut microbiota richness and composition, enterotypes and bacterial growth rates. *Gut*. 65, 57-62.

Vasickova, P., Pavlik, I., Verani, M. & Carducci, A. (2010) Issues concerning survival of viruses on surfaces. *Food and Environmental Virology*. 2 (1), 24-34.

Vasoo, S., Barreto, J.N. & Tosh, P.K. (2015) Emerging issues in Gram-negative bacterial resistance: an update for the practicing clinician. *Mayo Clinical Proceedings.* 90, 395-403.

Vatansever, F., Melo, W.C., Avci, P., Vecchio, D., Sadasivam, M., Gupta, A., Chandran, R., Karimi, M., Parizotto, N.A., Yin, R. & Tegos, G.P. (2013) Antimicrobial strategies centered around reactive oxygen species–bactericidal antibiotics, photodynamic therapy, and beyond. *FEMS Microbiology Reviews*. 37 (6), 955-989.

Veenhuis, J. (2017) Efficacy of a multiple emitter UV-C whole room disinfection system. Presented to Infection Control and Prevention Conference, London, UK, Feb 2017. Available from: https://www.infectioncontrol2017.co.uk/programme [Accessed March 2017]

Veerabadran, S. & Parkinson, I.M. (2010) Cleaning, disinfection and sterilization of equipment. *Anaesthesia and Intensive Care Medicine*. 11 (11), 451-454.

Verhaelen, K., Bouwknegt, M., Rutjes, S., de Roda Husman, A. M. & Duizer, E. (2014) Wipes coated with a singlet-oxygen-producing photosensitizer are effective against human influenza virus but not against norovirus. *Applied and Environmental Microbiology*. 80 (14), 4391-4397.

Vickery, K., Deva, A., Jacombs, A., Allan, J., Valente, P. & Gosbell, I.B. (2012) Presence of biofilm containing viable multiresistant organisms despite terminal cleaning on clinical surfaces in an intensive care unit. *Journal of Hospital Infection*. 80 (1), 52-55.

Vivancos, R., Keenan, A., Sopwith, W., Smith, K., Quigley, C., Mutton, K., Dardamissis, E., Nichols, G., Harris, J., Gallimore, C. & Verhoef, L. (2010) Norovirus outbreak in a cruise ship sailing around the British Isles: investigation and multi-agency management of an international outbreak. *Journal of Infection.* 60 (6), 478-485.

Vohra, A., Goswami, D.Y., Deshpande, D.A. & Block, S.S. (2005) Enhanced photocatalytic inactivation of bacterial spores on surfaces in air. *Journal of Industrial Microbiology and Biotechnology*. 32 (8), 364-370.

Vohra, P. & Poxton, I.R. (2011). Efficacy of decontaminants and disinfectants against *Clostridium difficile*. *Journal of Medical Microbiology*. 60 (8), 1218-1224.

Wagenvoort, J.H.T., Sluijsmans, W. & Penders, R.J.R. (2000) Better environmental survival of outbreak vs. sporadic MRSA isolates. *Journal of Hospital Infection*. 45 (3), 231-234.

Walker, J. T., Jhutty, A., Parks, S., Willis, C., Copley, V., Turton, J.F., Hoffman, P.N. & Bennett, A.M. (2014). Investigation of healthcare-acquired infections associated with *Pseudomonas aeruginosa* biofilms in taps in neonatal units in Northern Ireland. *Journal of Hospital Infection*. 86 (1), 16-23.

Wang, R., Kalchayanand, N., Schmidt, J.W. & Harhay, D. M. (2013a) Mixed biofilm formation by Shiga toxin-producing *Escherichia coli* and *Salmonella enterica* serovar Typhimurium enhanced bacterial resistance to sanitization due to extracellular polymeric substances. *Journal of Food Protection.* 76, 1513–1522.

Wang, Q., Hirneisen, K.A., Markland, S.M. & Kniel, K.E. (2013b) Survival of murine norovirus, Tulane virus, and hepatitis A virus on alfalfa seeds and sprouts during storage and germination. *Applied and Environmental Microbiology*. 79 (22), 7021-7027.

Wang, D. & Tian, P. (2014) Inactivation conditions for human norovirus measured by an *in situ* capture-qRT-PCR method. *International Journal of Food Microbiology*. 172, 76-82.

Wang, Y., Wu, X., Chen, J., Amin, R., Lu, M., Bhayana, B., Zhao, J., Murray, C.K., Hamblin, M.R., Hooper, D.C. & Dai, T. (2016) Antimicrobial blue light inactivation of gram-negative pathogens in biofilms: in vitro and in vivo studies. *Journal of Infectious Diseases*. 213 (9), 1380-1387.

Warnes, S.L., Summersgill, E. N. & Keevil, C.W. (2015) Inactivation of murine norovirus on a range of copper alloy surfaces is accompanied by loss of capsid integrity. *Applied and Environmental Microbiology*. 81 (3), 1085-1091.

Wasson, C.J., Zourelias, J.L., Aardsma, N.A., Eells, J.T., Ganger, M.T., Schober, J.M. & Skwor, T.A. (2012) Inhibitory effects of 405 nm irradiation on *Chlamydia trachomatis* growth and characterization of the ensuing inflammatory response in HeLa cells. *BioMed Central Microbiology*. 12 (1), 176.

Webb, A.R. (2006) Considerations for lighting in the built environment: Non-visual effects of light. *Energy and Buildings*. 38 (7),721-727.

Weber, D.J., Rutala, W.A., Miller, M.B., Huslage, K. & Sickbert-Bennett, E. (2010) Role of hospital surfaces in the transmission of emerging health care-associated pathogens: norovirus, *Clostridium difficile*, and *Acinetobacter* species. *American Journal of Infection Control*. 38 (5), S25-S33.

Weber, D.J., Rutala, W.A., Anderson, D.J., Chen, L.F., Sickbert-Bennett, E.E. & Boyce, J.M. (2016). Effectiveness of ultraviolet devices and hydrogen peroxide systems for terminal rom decontamination: Focus on clinical trials. *American Journal of Infection Control.* 44, e77-e84.

Weedon, K.M., Rupp, A.H., Heffron, A.C., Kelly, S.F., Zheng, X., Shulman, S.T., Gutman, P., Wang, D., Zhou, Y., Noskin, G.A. & Anderson, E.J. (2013) The impact of infection control upon hospital-acquired influenza and respiratory syncytial virus. *Scandinavian Journal of Infectious Ddiseases*. 45 (4) 297-303.

Wei, Q., Le Minh, P.N., Dötsch, A., Hildebrand, F., Panmanee, W., Elfarash, A., Schulz, S., Plaisance, S., Charlier, D., Hassett, D. & Häussler, S. (2012) Global regulation of gene expression by OxyR in an important human opportunistic pathogen. *Nucleic Acids Research*. 40 (10), 4320-4333.

Wenzl, H.H., Fine, K.D., Schiller, L.R. & Fordtran, J.S. (1995) Determinants of decreased faecal consistency in patients with diarrhoea. *Gastroenterology*. 108 (6), 1729-1738.

Wertheim, H.F., Melles, D.C., Vos, M.C., van Leeuwen, W., van Belkum, A., Verbrugh, H. A. & Nouwen, J.L. (2005) The role of nasal carriage in *Staphylococcus aureus* infections. *The Lancet Infectious Diseases*. 5 (12), 751-762.

Wilcox, M.H., Fawley, W.N., Wigglesworth, N., Parnell, P., Verity, P. & Freeman, J. (2003) Comparison of the effect of detergent versus hypochlorite cleaning on environmental contamination and incidence of *Clostridium difficile* infection. *Journal of Hospital Infection*. 54 (2),109-114.

Williams, H.C., Dellavalle, R.P. & Garner, S. (2012) Acne vulgaris. *The Lancet*. 379 (9813), 361-372.

Woods, J.W., Calci, K.R., Marchant-Tambone, J.G. & Burkhardt, W. (2016) Detection and molecular characterization of norovirus from oysters implicated in outbreaks in the US. *Food Microbiology*. 59, 76-84.

Woodward, M. (2013) Epidemiology: study design and data analysis. 3rd Edition. Florida, USA, CRC press.

World Health Organisation. (2009) WHO guidelines on hand hygiene in health care: first global patient safety challenge. Clean care is safer care. WHO, Geneva, Switzerland. Available from: http://apps.who.int/iris/bitstream/10665/44102/1/9789241597906_eng.pdf [Accessed March 2017]

World Health Organisation. (2011) Report on the endemic burden of healthcare-associated infection worldwide. WHO, Geneva, Switzerland. Available from: http://apps.who.int/iris/bitstream/10665/80135/1/9789241501507_eng.pdf [Accessed March 2017]

World Health Organisation (2014) Antimicrobial resistance: Global Report on Surveillance. WHO, Geneva, Switzerland. Available from: http://apps.who.int/iris/bitstream/10665/112642/1/9789241564748_eng.pdf?ua=1 [Accessed March 2017]

Wu, H.M., Fornek, M., Schwab, K.J., Chapin, A.R., Gibson, K., Schwab, E., Spencer, C. & Henning, K. (2005) A norovirus outbreak at a long-term-care facility: the role of environmental surface contamination. *Infection Control and Hospital Epidemiology.* 26 (10), 802-810.

Wurtmann, E. J. & Wolin, S. L. (2009) RNA under attack: cellular handling of RNA damage. *Critical Reviews in Biochemistry and Molecular Biology*. 44 (1), 34-49.

Wyatt, R.G., Dolin, R., Blacklow, N.R., DuPont, H.L., Buscho, R.F., Thornhill, T.S., Kapikian, A.Z. & Chanock, R.M. (1974) Comparison of three agents of acute infectious nonbacterial gastroenteritis by cross-challenge in volunteers. *The Journal of Infectious Diseases.* 129 (6), 709-714.

Xie, Y., Kondo, M., Koga, H., Miyamoto, H. & Chiba, M. (2001) Urinary porphyrins in patients with endemic chronic arsenic poisoning caused by burning coal in China. *Environmental Health and Preventive Medicine.* 5 (4), 180.

Xu, H., Lin, Q., Chen, C., Zhang, J., Zhang, H. & Hao, C. (2013) Epidemiology of norovirus gastroenteritis outbreaks in two primary schools in a city in eastern China. *American Journal of Infection Control.* 41 (10), e107-e109.

Xu, H., Fu, Y.N., Li, T.L. & Wang, R. (2017) Effects of different LED light wavelengths on the resistance of tomato against *Botrytis cinerea* and the corresponding physiological mechanisms. *Journal of Integrative Agriculture*. 16 (1), 106-114.

Yamamoto, H. & Shimojo, H. (1971) Multiplicity reactivation of human adenovirus type 12 and simian virus 40 irradiated by ultraviolet light. *Virology*. 45 (2), 529-531.

Yang, E.S., Tan, J., Eells, S., Rieg, G., Tagudar, G. & Miller, L.G. (2009) Body site colonization in patients with community- associated methicillin- resistant *Staphylococcus aureus* and other types of *S. aureus* skin infections. *Clinical Microbiology and Infection*. 16 (5), 425-431.

Yang, P., Wang, N., Wang, C., Yao, Y., Fu, X., Yu, W., Cai, R. & Yao, M. (2017) 460nm visible light irradiation eradicates MRSA via inducing prophage activation. *Journal of Photochemistry and Photobiology B: Biology*. 166, 311-322.

Yeh, N., Ding, T.J. & Yeh, P. (2015) Light-emitting diodes light quality and their corresponding scientific applications. *Renewable and Sustainable Energy Reviews*. 51, 55-61.

Yin, R., Dai, T., Avci, P., Jorge, A.E.S., de Melo, W.C., Vecchio, D., Huang, Y.Y., Gupta, A. & Hamblin, M.R. (2013) Light based anti-infectives: ultraviolet C irradiation, photodynamic therapy, blue light, and beyond. *Current Opinions in Pharmacology.* 13, 731-62.

Young A.R. (2006) Acute effects of UVR on human eyes and skin. *Progress in Biophysics and Molecular Biology*. 92 (1), 80-85.

Zan, L., Fa, W., Peng, T. & Gong, Z.K. (2007) Photocatalysis effect of nanometer TiO_2 and TiO_2-coated ceramic plate on Hepatitis B virus. *Journal of Photochemistry and Photobiology B: Biology*. 86 (2), 165-169.

Zhang, Y., Zhu, Y., Gupta, A., Huang, Y., Murray, C.K., Vrahas, M.S., Sherwood, M.E., Baer, D.G., Hamblin, M.R. and Dai, T. (2014) Antimicrobial blue light therapy for multidrug-resistant *Acinetobacter baumannii* infection in a mouse burn model: implications for prophylaxis and treatment of combat-related wound infections. *Journal of Infectious Diseases*. 209 (12), 1963-1971.

Zhang, Y., Zhu, Y., Chen, J., Wang, Y., Sherwood, M.E., Murray, C.K., Vrahas, M.S., Hooper, D.C., Hamblin, M.R. & Dai, T. (2016) Antimicrobial blue light inactivation of *Candida albicans*: *In vitro* and *in vivo* studies. *Virulence*. 7 (5), 536-545.

Zheng, D.P., Ando, T., Fankhauser, R.L., Beard, R.S., Glass, R.I. & Monroe, S.S. (2006) Norovirus classification and proposed strain nomenclature. *Virology*. 346 (2), 312-323.

Zhu, H., Kochevar, I.E., Behlau, I., Zhao, J., Wang, F., Wang, Y., Sun, X., Hamblin, M.R. & Dai, T. (2017) Antimicrobial Blue Light Therapy for Infectious Keratitis: *Ex Vivo* and *In Vivo* Studies of Blue Light for Keratitis. *Investigative Ophthalmology and Visual Science*. 58 (1), 586-593.

Zonta, W., Mauroy, A., Farnir, F. & Thiry, E. (2016) Comparative virucidal efficacy of seven disinfectants against murine Norovirus and feline calicivirus, surrogates of human norovirus. *Food and Environmental Virology*. 8 (1), 1-12.

Zoutman, D., Shannon, M. & Mandel, A. (2011) Effectiveness of a novel ozone-based system for the rapid high-level disinfection of health care spaces and surfaces. *American Journal of Infection Control*. 39 (10), 873-879.

Appendix A: Systematised Review Data

Data from the systematised review of 380-480 nm violet-blue light inactivation of microorganisms, as detailed in Chapter 6.

Table A1.1 details inactivation of Gram positive and Gram negative bacteria, Gram positive endospores and mycobacteria.

Table A1.2 details inactivation of yeasts and fungal conidia, germinating conidia and hyphae.

Table A1.3 details inactivation of a bacteriophage and viruses.

Key:

- '**CI**' indicates an unspecified clinical isolate;
- '**A**' indicates microorganisms exposed under anaerobic conditions;
- '**-**' indicates bacteria exposed at low temperatures (≤ 10°C);
- '**+**' indicates bacteria exposed at elevated temperatures (≥ 37°C);
- '**P**' indicates microorganisms exposed on agar plates;
- '**E**' indicates $\log_{10}$ reduction values extracted from figures;
- '**V**' indicates dose for 1 $\log_{10}$ reduction values provided by paper

Table A1.1 Inactivation of bacteria using 380-480 nm light, collected during the systematised review.

Microorganism	Strain Number(s)	Wavelength (nm)	Irradiance (mW/cm^2)	Dose (J/cm^2)	Time (min)	Exposed Population (CFU/ml or CFU/plate)	Log_{10} reduction (CFU/ml)	Dose for 1 log_{10} reduction	Reference
Acinetobacter baumannii (Gram –ve)	ACI: 616; 618; 642; 648; 659; 665; 671; 672; 698. AYE: C60; 19606	400	60	108	30	10^6	7.06/ 5.78/ 6.73/ 6.14/ 6.55/ 6.14/ 6.34/ 6.22/ 6.39/ 6.7/ 6.76/ 6.81	15.3/ 18.7/ 16/ 17.6/ 16.5/ 17.6/ 17/ 17.4/ 16.9/ 16.1/ 16/ 15.9	Halstead *et al*., 2016
	LMG 1041	405	5	4.5	15	$1.5\text{-}3 \times 10^{2\ P}$	2.2	2	Ramakrishnan *et al*., 2014
	NCTC 12156		10	108	180	$10^4\text{-}10^5$	4.2	25.7	Maclean *et al*., 2009
	MDR CI	415	19.5	70.2	60	1×10^8	$4.5^{\ E}$	15.5	Zhang *et al*., 2014
Aggregatibacter actinomycetemcomitans (Gram -ve)	ATCC 43718	460	1260	150	2	1×10^8	5	30	Cieplik *et al*., 2014
Bacillus atrophaeus (Gram +ve)	-	470	80	$300/ 300/ 300^+$	62.5	$3 \times 10^{4\ P}$	2/2.3/2.2	130.4/150/136.4	De Lucca *et al*., 2012
Bacillus cereus (Gram +ve, Vegetative)	ATCC 14579	405	9.5	$306/ 306^-$	540	10^6	2.3/ 2.3	133/ 133	Kumar *et al*., 2015
			18	486^-	450	10^8	1.9	255.8	Kim *et al*., 2015
			40	108	45	$10^4\text{-}10^5$	4	27	Maclean *et al*., 2013b

Microorganism	Strain Number(s)	Wavelength (nm)	Irradiance (mW/cm^2)	Dose (J/cm^2)	Time (min)	Exposed Population (CFU/ml or CFU/plate)	Log_{10} reduction (CFU/ml)	Dose for 1 log_{10} reduction	Reference
Bacillus cereus (Gram +ve, Endospore)	NCTC 11143	405	40	1730	720	10^4-10^5	2.6	665.4	Maclean *et al.*, 2013b
Bacillus megaterium (Gram +ve, Endospore)	UoS CC	405	40	1150	480	10^4-10^5	1.8	638.9	Maclean *et al.*, 2013b
Bacillus subtilis (Gram +ve, Vegetative)	DSM 402	405	21	75.6	60	1×10^6	2	37.8	Hoenes *et al.*, 2016
		408	33.5	300	150	1×10^6	4.3 [E]	100	Hoenes *et al.*, 2015
		451	33.5	300	150	1×10^6	3 [E]	69.8	Hoenes *et al.*, 2015
Bacillus subtilis (Gram +ve, Endospore)	UoS CC	405	40	1150	480	10^4-10^5	1.7	676.5	Maclean *et al.*, 2013
Camylobacter coli (Gram -ve)	DF: 1140; 1662; 2124	395	7	2.1	5	10^7	7 [E]	0.3	Haughton *et al.*, 2012
Campylobacter jejuni (Gram -ve)	BC 323, DF 1136	395	7	4.2	10	10^7	7 [E]	0.6	Haughton *et al.*, 2012
	DF: 1135; 1146; 1147; 1354. NCTC 11168	395	7	2.1	5	10^7	7 [E]	0.3	Haughton *et al.*, 2012
	LMG 8841	405	10	18	30	10^5	5.25	3.4	Murdoch *et al.*, 2010

Microorganism	Strain Number(s)	Wavelength (nm)	Irradiance (mW/cm^2)	Dose (J/cm^2)	Time (min)	Exposed Population (CFU/ml or CFU/plate)	Log_{10} reduction (CFU/ml)	Dose for 1 log_{10} reduction	Reference
Clostridium difficile (Gram +ve, Vegetative)	NCTC 11204	405	40	48	20	10^4-10^5	3.7	13	Maclean *et al.*, 2013b
			70	252	60	3.3×10^3	3.3	76.7	Moorhead *et al.*, 2016a
Clostridium difficile (Gram +ve, Endospore)	NCTC 11204	405	40	1150	480	10^4-10^5	2.7	425.9	Maclean *et al.*, 2013b
			225	2430	180	3.5×10^3	3.3	736.4	Moorhead *et al.*, 2016a
Clostridium perfringens (Gram +ve)	ATCC 13124	405	10	45	75	10^4-10^5	4.4	10.2	Maclean *et al.*, 2009
Corynebacterium striatum (Gram +ve)	CI	405	71	128	30	$1\text{-}3 \times 10^2$ [P]	2	64	McDonald *et al.*, 2013
			123	221.4	30	10^5	5	4.3	Gupta *et al.*, 2015
Elizabethkingia meningoseptica (Gram -ve)	502	400	60	54	15	10^6	6.8	8	Halstead *et al.*, 2016
Enetrobacter cloacae (Gram -ve)	ENTCL: 525; 801; 804	400	60	360/ 648/ 576	100/ 180/ 160	10^6	6.76/ 6.61/ 6.24	53.3/ 98/ 92.3	Halstead *et al.*, 2016
Enterococcus faecalis (Gram +ve)	ATCC 19433	385	49	81	27.6	10^6	1 [V]	81	Lui *et al.*, 2016
	-	405 [A]	19.1	5.73	5	1×10^8	No Inactivation	-	Hope et al., 2016

Microorganism	Strain Number(s)	Wavelength (nm)	Irradiance (mW/cm^2)	Dose (J/cm^2)	Time (min)	Exposed Population (CFU/ml or CFU/plate)	Log_{10} reduction (CFU/ml)	Dose for 1 log_{10} reduction	Reference
Enterococcus faecalis (Gram +ve)	NCTC 00775	405	10	216	360	10^4-10^5	2.6	96	Maclean *et al.*, 2009
	ATCC 19433		65	130	33.3	10^6	1 [V]	130	Lui *et al.*, 2016
	CI		71	128	30	1-3 × 10^2 [P]	2 [E]	64	McDonald *et al.*, 2013
			123	885.6	30	10^5	4.72	187.6	Gupta *et al.*, 2015
	ATCC 19433	430	21	190	150.8	10^6	1 [V]	190	Lui *et al.*, 2016
	ATCC 23112	450	520	93.6	3	5 × 10^6	No Inactivation	-	Feuerstein *et al.*, 2004
	CI	455	37	410	184.7	10^6	1 [V]	410	McDonald *et al.*, 2013
Enterococcus faecium (Gram +ve)	513	400	60	648	180	10^6	1.86	348.4	Halstead *et al.*, 2016
Escherichia coli (Gram -ve)	EC: 73; 42	400	60	108	30	10^6	1.55/ 4.71	22.9/ 69.7	Halstead *et al.*, 2016
	-	405 [A]	19.1	5.73	5	2 × 10^8	No Inactivation	-	Hope *et al.*, 2016
	NCTC 9001	405	5	27	90	1.5-3 × 10^2 [P]	2.2 [E]	12.3	Ramakrishnan *et al.*, 2014
			10	180	300	10^4-10^5	3.1	58.1	Maclean *et al.*, 2009

Microorganism	Strain Number(s)	Wavelength (nm)	Irradiance (mW/cm^2)	Dose (J/cm^2)	Time (min)	Exposed Population (CFU/ml or CFU/plate)	Log_{10} reduction (CFU/ml)	Dose for 1 log_{10} reduction	Reference
Escherichia coli (Gram -ve)	NCTC 9001	405	65	234/ 702	60/ 180	10^7 / 10^9	3.3/ 7.7	70.9/ 60.8	McKenzie *et al.*, 2016
			70	378/ 378^+/ 252^-	90/ 90/ 60	10^5	5 [E]	75.6/ 75.6/ 50.4	McKenzie *et al.*, 2014
	CI		71	192	45	$1\text{-}3 \times 10^2$ [P]	2 [E]	96	McDonald *et al.*, 2013
	NCTC 9001		100	450	75	10^5	4.5	100	Maclean *et al.*, 2016
	CI		123	2214	300	10^5	5.01	441.9	Gupta *et al.*, 2015
	CI 1313	415	100	60	10	-	3	20	Lipovsky *et al.*, 2010
	ATCC 25922	450	2	117^+/ 117^-	975	3×10^1	0.85/ 1.15	137.7/ 101.7	Keshishyan *et al.*, 2015
	CI 1313	455	100	120	20	-	1	120	Lipovsky *et al.*, 2010
	ATCC 25922	460	1260	151	2	10^8	No Inactivation	-	Cieplik *et al.*, 2014
***Escherichia coli* K12** (Gram -ve)	ATCC W3110	385	49	61	20.7	10^6	1 [V]	61	Lui *et al.*, 2016
	DSM 1607	395	1.21	2.83	39	10^8	0.52	5.7	Birmpa *et al.*, 2014

Microorganism	Strain Number(s)	Wavelength (nm)	Irradiance (mW/cm^2)	Dose (J/cm^2)	Time (min)	Exposed Population (CFU/ml or CFU/plate)	Log_{10} reduction (CFU/ml)	Dose for 1 log_{10} reduction	Reference
***Escherichia coli* K12** (Gram -ve)	DSM 1607	405	6.5	2.83	7.25	10^8	0.6 [E]	4.7	Birmpa *et al.*, 2014
			32.2	36	18.6	10^8	1.37	26.3	Birmpa *et al.*, 2014
			8.71	130.6	250	1×10^2 [P]	6.27	20.8	Barneck *et al.*, 2016
	-		9.2	132.1	250	- [P]	6.27	21.1	Rhodes *et al.*, 2016
	DSM 498		21	151.2	120	10^6	3.5	43.2	Hoenes *et al.*, 2016
	ATCC W3110		65	86	22	10^6	1 [V]	86	Lui *et al.*, 2016
	DSM 498	408	33.5	600	300	10^6	3.8	157.9	Hoenes *et al.*, 2015
	ATCC W3110	430	21	100	79.4	10^6	1 [V]	100	Lui *et al.*, 2016
	DSM 498	451	33.5	600	300	10^6	1.2	500	Hoenes *et al.*, 2015
	ATCC W3110	455	37	300	135	10^6	1 [V]	300	Lui *et al.*, 2016
***Escherichia coli* O157:H7** (Gram -ve)	EDL 933	405	9.5	306/ 306[-]	540	10^6	0.5/ 1.3	612/ 235.4	Kumar *et al.*, 2015
	NCTCC 12900		10	288	480	$1\text{-}2 \times 10^5$/ 10^5	3.5/ 5.3	54.3/ 82.3	Murdoch *et al.*, 2010; 2012

Microorganism	Strain Number(s)	Wavelength (nm)	Irradiance (mW/cm^2)	Dose (J/cm^2)	Time (min)	Exposed Population (CFU/ml or CFU/plate)	Log_{10} reduction (CFU/ml)	Dose for 1 log_{10} reduction	Reference
***Escherichia coli* O157:H7** (Gram -ve)	EDL 933	405	18	486[-]	450	10^8	1	486	Kim *et al*., 2016
	NCTC 12900		71	270	45	$2.3–2.4 \times 10^5$ [P]	2.2	123.9	Murdoch *et al*., 2012
			85.6	554.7	108	10^5	4.52	122.7	Endarko *et al*., 2012
	EDL 933	461	22.1	596.7	450	10^6	4.9/ 1.2	121.8/ 497.3	Ghate *et al*., 2013; 2015
Fusobacterium nucleatum (Gram -ve)	ATCC: 1594; 25586	450	520	62/ 94	2/ 3	5×10^6	0.7/ 0.3	88.6/ 312	Feuerstein *et al*., 2004; 2005
	ATCC 25586	455	80	4.8	1	10^8	0.27	17.8	Fontana *et al*., 2015
Fusobacterium nucleatum* ss *polymorphum (Gram -ve)	ATCC 10953	455	80	4.8	1	10^8	1.2	4	Fontana *et al*., 2015
Fusobacterium nucleatum* ss *vincentii (Gram -ve)	ATCC 49256	455	80	4.8	1	10^8	0.49	9.8	Fontana *et al*., 2015
Fusobacterium periodonticum (Gram -ve)	ATCC 33692	455	80	4.8	1	10^8	0.46	10.4	Fontana *et al*., 2015

Microorganism	Strain Number(s)	Wavelength (nm)	Irradiance (mW/cm^2)	Dose (J/cm^2)	Time (min)	Exposed Population (CFU/ml or CFU/plate)	Log_{10} reduction (CFU/ml)	Dose for 1 log_{10} reduction	Reference
Helicobacter pylori (Gram -ve)	ATCC: 49503; 43904. CI 2	405	100	10	1.7	-	5/ 6/ 3.75 [E]	2/ 1.7/ 2/7	Hamblin *et al.*, 2005
	ATCC 700824. CI: 1; 3; 4		100	20	3.3	-	4.5/ 5.5/ 5/ 4.5	4.4/ 3.6/ 4/ 4.4	Hamblin *et al.*, 2005
	ATCC 43504		200	32	5.3	-	5 [E]	6.4	Ganz *et al.*, 2005
Klebsiella pneumoniae (Gram -ve)	MDR-A, MDR-B	400	60	504	140	10^6	6.61/ 6.88	73.3/ 76.2	Halstead *et al.*, 2016
	NCTC 09633	405	5	36	120	$1\text{-}3 \times 10^{2}$ [P]	2.2 [E]	16.4	Ramakrishnan *et al.*, 2014
			10	180	300	$10^4\text{-}10^5$	3.9	46.2	Maclean *et al.*, 2009
	CIs		71	128	30	$1\text{-}3 \times 10^{2}$ [P]	2	64	McDonald *et al.*, 2013
			123	1328.4	180	$10^4\text{-}10^5$	5.05	263	Gupta *et al.*, 2015
Lactobacillus plantarum (Gram +ve)	ATCC 8014	405	24	374/ 394[-]	260/ 274	10^6	1 [V]	374/ 394	Kumar *et al.*, 2016
		460	74	1121/ 1800[-]	251.5/ 274	10^6	1 [V]/ No Inactivation	1121/ -	Kumar *et al.*, 2016
Leuconostoc mesenteroids (Gram +ve)	-	470	80	-	-	3×10^{4} [P]	No inactivation	-	De Lucca *et al.*, 2012

Microorganism	Strain Number(s)	Wavelength (nm)	Irradiance (mW/cm^2)	Dose (J/cm^2)	Time (min)	Exposed Population (CFU/ml or CFU/plate)	Log_{10} reduction (CFU/ml)	Dose for 1 log_{10} reduction	Reference
Listeria innocua (Gram +ve)	NCTC 11288	395	1.21	2.832	39	10^8	1.1	2.57	Birmpa *et al*., 2014
			6.5	2.832	7.25		1.25 [E]	2.3	
			32.2	36	18.6		2.74	13.1	
Listeria ivanovii (Gram +ve)	NCTC 11846	405	85.6	184.9	35	10^5	4.12	44.9	Endarko *et al*., 2012
Listeria monocytogenes (Gram +ve)	NCTC 1194	400	7.5	123.3	274	10^5	1.05	117	Endarko *et al*., 2012
	NCTC 1194	405	8.6	154.1	299	10^5	2.5	61.6	Endarko *et al*., 2012
	BAA 679		9.5/ 9.5[-]	306	540	10^6	1.9	284.2	Kumar *et al*., 2015
	LMG 19944		10	108	180	$1\text{-}2 \times 10^5$	5.18	20.85	Murdoch *et al*., 2012
	BAA 679		17[-]	486	450	10^8	2.1	231.4	Kim *et al*., 2015
	LMG 19944		20	108	90	$1\text{-}2 \times 10^5$	5.05	21.4	Murdoch *et al*., 2012
	LMG 19944		30	108	60	$1\text{-}2 \times 10^5$	4.9	22	Murdoch *et al*., 2012
	NCTC 1194		44.7	154.1	57.5	10^5	3.5 [E]	44	Endarko *et al*., 2012

Microorganism	Strain Number(s)	Wavelength (nm)	Irradiance (mW/cm^2)	Dose (J/cm^2)	Time (min)	Exposed Population (CFU/ml or CFU/plate)	Log_{10} reduction (CFU/ml)	Dose for 1 log_{10} reduction	Reference
Listeria monocytogenes (Gram +ve)	NCTC 1194	405	66.1	154.1	39	10^5	3.25 [E]	47.4	Endarko *et al.*, 2012
	LMG 19944		70/ 70^-/ 70^+	84/ 63/ 42	20/ 15/ 10	10^5	5 [E]	16.8/ 12.6/ 8.4	McKenzie *et al.*, 2014
			71	108	30	$2.3\text{-}2.4 \times 10^2$	2.25	80	Murdoch *et al.*, 2012
	NCTC 1194		85.6	184.9	35	10^5	3.72	49.7	Endarko *et al.*, 2012
	NCTC 11994	410	7.2	123.3	285.4	10^5	1.23	100.2	Endarko *et al.*, 2012
		420	7.5	123.3	274	10^5	0.51	241.8	Endarko *et al.*, 2012
		430	7.7	123.3	266.9	10^5	0.25	493.2	Endarko *et al.*, 2012
		440	7.3	123.3	281.5	10^5	0.19	648.9	Endarko *et al.*, 2012
		450	8.3	123.3	-	10^5	0.11	1120.9	Endarko *et al.*, 2012
	BAA 679	461	22.1	596.7	450	10^6	4.3/ 1.4	138.8/ 426.2	Ghate *et al.*, 2013; 2015
	-	470	8	345.6	726	4×10^7	0.5	691.2	O'Donoghue *et al.*, 2016
Listeria seeligeri (Gram +ve)	NCTC 11856	405	8.3	184.9	371	10^5	3.31	55.9	Endarko *et al.*, 2012

Microorganism	Strain Number(s)	Wavelength (nm)	Irradiance (mW/cm^2)	Dose (J/cm^2)	Time (min)	Exposed Population (CFU/ml or CFU/plate)	Log_{10} reduction (CFU/ml)	Dose for 1 log_{10} reduction	Reference
Micrococcus spp (Gram +ve)	CI	405	71	85.2	20	$1\text{-}3 \times 10^2$	2 [E]	42.6	McDonald *et al.*, 2013
	CI		123	442.8	60	10^5	4.85	91.3	Gupta *et al.*, 2015
Mycobacterium massiliense (Gram *)	INCQS 00594	450	36.1	300	138.5	10^8	0.32	937.5	Decarli *et al.*, 2016
Mycobacterium smegmatis (Gram *)	-	405	160	120	12.5	10^3 [P]	1.8	66.4	Guffey *et al.*, 2013b
Mycobacterium terrae (Gram *)	LMG 10394	405	10	288	480	$1\text{-}2 \times 10^5$	5	57.6	Murdoch *et al.*, 2012
Porphyromonas gingivalis (Gram -ve)	ATCC 33277	405 [A]	11.4	3.42	5	10^7	1.22	2.8	Hope *et al.*, 2013
			328.5	98.5	5	10^7	1.26	78.2	Hope *et al.*, 2013
	ATCC 33277	405	200	4	0.33	10^2	1	4	Kotoku *et al.*, 2009
			400	8	0.33	10^2	1.1	7.3	Kotoku *et al.*, 2009
			800	16	0.33	10^2	2	8	Kotoku *et al.*, 2009
	KCTC 5352	425	10	288	480	2×10^8	8 [E]	36	Kim *et al.*, 2013

Microorganism	Strain Number(s)	Wavelength (nm)	Irradiance (mW/cm^2)	Dose (J/cm^2)	Time (min)	Exposed Population (CFU/ml or CFU/plate)	Log_{10} reduction (CFU/ml)	Dose for 1 log_{10} reduction	Reference
Porphyromonas gingivalis (Gram -ve)	ATCC 33277	448	100	60	1	10^8	1.1	49.5	Chui *et al*., 2013
		450	520	47/ 62.4	1.5-2	5×10^6	1	47/ 62.4	Feurestein *et al*., 2004; 2005
		455	80	4.8	1	10^8	0.1	50	Fontana *et al*., 2015
Prevotella intermedia (Gram -ve)	ATCC 25611	405 [A]	19.1	5.73	5	7.5×10^7	2.6	2.2	Hope *et al*., 2016
			346.2	20.77	1	7.5×10^7	4.4	4.7	Hope *et al*., 2016
		455	80	4.8	1	10^8	0.34	14.1	Fontana *et al*., 2015
Prevotella melaninogenica (Gram -ve)	ATCC 25845	455	80	4.8	1	10^8	0.67	7.16	Fontana *et al*., 2015
Prevotella nigrescens (Gram -ve)	ATCC 25261	405 [A]	19.1	5.73	5	7.5×10^7	1.2	4.8	Hope *et al*., 2016
	ATCC 33563	455	80	4.8	1	10^8	0.49	9.8	Fontana *et al*., 2015
Propionibacterium acnes (Gram +ve)	ATCC 211827	405	160	15	1.6	1.5×10^2 [P]	No Inactivation	-	Guffey & Wilborn, 2006
	ATCC 6919	414 [A]	20	225	180	2×10^8	5	45	Ashkenazi *et al*., 2003

Microorganism	Strain Number(s)	Wavelength (nm)	Irradiance (mW/cm^2)	Dose (J/cm^2)	Time (min)	Exposed Population (CFU/ml or CFU/plate)	Log_{10} reduction (CFU/ml)	Dose for 1 log_{10} reduction	Reference
Propionibacterium acnes (Gram +ve)	CIs	414	90	324	60	5.1×10^9	No Inactivation	-	Kawada *et al*., 2002
	ATCC 211827	470	150	15	1.7	$2 \times 10^{2\ P}$	No Inactivation	-	Guffey & Wilborn, 2006
Proteus vulgaris (Gram -ve)	CI 329	405	10	144	240	10^4 - 10^5	4.7	30.6	Maclean *et al*., 2009
Pseudomonas aeruginosa (Gram -ve)	568, PA01, 6749, 1054, 1586	400	60	108	30	10^6	6.48/ 5.59/ 6.55/ 6.01/ 6.07	16.7/ 19.3/ 16.5/ 18/ 17.8	Halstead *et al*., 2016
	LMG 9009	405	5	27	90	$1.5\text{-}3 \times 10^{2\ P}$	$2.2^{\ E}$	12.3	Ramakrishnan *et al*., 2014
	ATCC 27853		7.84	117.55	250	$-^{\ P}$	5.2	22.6	Barneck *et al*., 2016
	ATCC 10145		9.5/ 9.5	306	540	10^6	$0.3^{\ E}$	1020	Kumar *et al*., 2015
	NCTC 9009		10	180	300	10^4-10^5	4.2	42.9	Maclean *et al*., 2009
	PAO1, PA14, PAK		15.7	50	53	10^8	$7^{\ E}$/ $6^{\ E}$/$6.5^{\ E}$	7.14/ 8.3/ 7.7	Fila *et al*., 2017
	1959/o, 146/s, 133/k, 23/k, 3752/sz, 153/s, 2284/p		15.7	50	53	10^8	$7^{\ E}$	7.14	Fila *et al*., 2017

Microorganism	Strain Number(s)	Wavelength (nm)	Irradiance (mW/cm^2)	Dose (J/cm^2)	Time (min)	Exposed Population (CFU/ml or CFU/plate)	Log_{10} reduction (CFU/ml)	Dose for 1 log_{10} reduction	Reference
Pseudomonas aeruginosa (Gram -ve)	3404/p	405	15.7	30	32	10^8	7^{E}	4.3	Fila *et al.*, 2017
	556/k, 4190/pA, 3109/o, 143/p (MDR)		15.7	50	53	10^8	7^{E}/ 7^{E}/ 7^{E}/ 5^{E}	7.14/ 7.14/ 7.14/ 10	Fila *et al.*, 2017
	CI		71	53.25	12.5	$1\text{-}3 \times 10^{2\,P}$	2^{E}	26.7	McDonald *et al.*, 2013
	CI		123	664.2	90	105	5	132.8	Gupta *et al.*, 2015
	ATCC 27853		160	10	1.05	$1 \times 10^{2\,P}$	1.3^{E}	7.7	Guffey & Wilborn, 2006
	ATCC 19660	415	19.5	109.9	96	10^8	7.64	14.4	Dai *et al.*, 2013a
			20	48	40	10^8	3.45	13.9	Amin *et al.*, 2016
	ATCC 27853	450	2	117^{+}/ 117^{-}	975	3×10^1	0.81/ 1.18	144.4 / 99.2	Keshishyan *et al.*, 2015
			36.1	300	138.5	10^8	0.7	428.6	Decarli *et al.*, 2016
	-	470	80/ 80/ 80^{+}	50/ 50/ 10		3×10^4	0.7-0.8/ 2.1	62.5/ 71.4/ 4.8	De Lucca *et al.*, 2012
	ATCC 27853		150	5	0.55	$1 \times 10^{2\,P}$	0.46^{E}	10.9	Guffey & Wilborn 2006

Microorganism	Strain Number(s)	Wavelength (nm)	Irradiance (mW/cm^2)	Dose (J/cm^2)	Time (min)	Exposed Population (CFU/ml or CFU/plate)	Log_{10} reduction (CFU/ml)	Dose for 1 log_{10} reduction	Reference
Pseudomonas stutzeri (Gram -ve)	CI	405	50	240	80	10^4	4	60	Bache, 2013
Salmonella agona (Gram -ve)	BAA 707	405	35^-	900	420	10^9/ 10^{11}	2 [E]	450	Kim *et al*., 2017
Salmonella enterica serovar enteritidis (Gram -ve)	NCTC 4444	405	71	270	45	2.3-2.4 × 10^2 [P]	2.28	118.4	Murdoch *et al*., 2012
			10	252/ 288	420/ 480	1-2 × 10^5	2.96/ 3.5 [E]	72/ 97.3	Murdoch *et al*., 2010; 2012
	ATCC 13076		20^-	576	480	10^6	5.6	102.9	Kim & Yuk 2017
	NCTC 4444		85.6	739.6	144	10^5	1.36	543.8	Endarko *et al*., 2012
Salmonella enterica serovar heidelburg (Gram -ve)	ATCC 8326	470	30	110	31.1	10^6 [P]	0.94	117	Bumah *et al*., 2015b
Salmonella enterica serovar typhimurium (Gram -ve)	ATCC 14028	405	9.5/ 9.5^-	306	540	10^6	0.6/ 0.3 [E]	510/ 1020	Kumar *et al*., 2015
			18^-	486	450	10^8	2	243	Kim *et al*., 2016
			20^-	576	480	10^6	1.7	338.8	Kim & Yuk 2017
			35^-	900	420	10^9/ 10^{11}	2.7/ 2.4 [E]	333.3/ 375	Kim *et al*., 2017

Microorganism	Strain Number(s)	Wavelength (nm)	Irradiance (mW/cm^2)	Dose (J/cm^2)	Time (min)	Exposed Population (CFU/ml or CFU/plate)	Log_{10} reduction (CFU/ml)	Dose for 1 log_{10} reduction	Reference
Salmonella enterica serovar typhimurium (Gram -ve)	ATCC 14028	461	22.1	596.7	450	10^6	5/ 0.4	113.9/ 1491.8	Ghate *et al*., 2013, 2015
		470	30	110	31.1	$10^{6\ P}$	1.15	95.7	Bumah *et al*., 2015b
Salmonella saintpaul (Gram -ve)	ATCC 9712	405	20^-	576	480	1×10^6	1.7	338.8	Kim & Yuk 2017
Serratia marcescens (Gram -ve)	CI	405	71	256	60	$1\text{-}3 \times 10^{2\ P}$	$2^{\ E}$	128	MacDonald *et al*., 2013
			123	1771.2	240	10^5	5.26	336.7	Gupta *et al*., 2015
Shigella sonnei (Gram -ve)	NCTC 12984	405	10	180	300	$1\text{-}2 \times 10^5$	5	36	Murdoch *et al*., 2012
	ATCC 29031		18^-	486	450	10^8	0.8	607.5	Kim *et al*., 2016
	NCTC 12984		71	270	45	$2.3\text{-}2.4 \times 10^{2\ P}$	2.1	128.6	Murdoch *et al*., 2012
	LMG 10473		85.6	554.7	108	10^5	3.9	142.2	Endarko *et al*., 2012

Microorganism	Strain Number(s)	Wavelength (nm)	Irradiance (mW/cm^2)	Dose (J/cm^2)	Time (min)	Exposed Population (CFU/ml or CFU/plate)	Log_{10} reduction (CFU/ml)	Dose for 1 log_{10} reduction	Reference
Staphylococcus aureus (methicillin-sensitive) (Gram +ve)	NCTC 4135	400	3.27	23.5	120	2×10^5	1.5	15.7	Maclean *et al.*, 2008
	10788, F77. ATCC: 29213; 33807. NCTC: 4163; 10442		60	288/ 108/ 108/ 288/ 288/ 108	80/ 30/ 30/ 80/ 80/ 30	10^6	7.07/ 6.76/ 6.76/ 7.01/ 6.07/ 6.69	40.7/ 16/ 16/ 40.1/ 47.4/ 16.1	Halstead *et al.*, 2016
	-	405 [A]	19.1	5.73	5	9×10^7	0.1	57.3	Hope *et al.*, 2016
	NCTC 4135	405	3.27	23.5	120	2×10^5	2.4	9.8	Maclean *et al.*, 2008
			5	18	60	$1\text{-}3 \times 10^2$ [P]	1.72/ 2.2	10.5/ 8.2	McDonald *et al.*, 2013; Ramakrishnan *et al.*, 2014
			7.6	41	100	10^9	3	13.7	Maclean *et al.*, 2009
	ATCC 29213		8.32	124.81	250	- [P]	6.1	20.5	Barneck *et al.*, 2016
	ATCC 35932		9.5/ 9.5[-]	306	540	10^6	4/ 2.1	75.6/ 145.7	Kumar *et al.*, 2015
	NCTC 4135		10	36	60	10^3/ 10^4-10^5/ 10^6	3/ 5/ 3	12/ 7.2/ 12	Maclean *et al.*, 2009
			16	115.2	120	10^3	1.4 [E]	82.3	Gillespie *et al.*, 2017

Microorganism	Strain Number(s)	Wavelength (nm)	Irradiance (mW/cm^2)	Dose (J/cm^2)	Time (min)	Exposed Population (CFU/ml or CFU/plate)	Log_{10} reduction (CFU/ml)	Dose for 1 log_{10} reduction	Reference
Staphylococcus aureus (methicillin-sensitive) (Gram +ve)	ATCC 6538	405	18^{-}	486	450	10^8	0.9	540	Kim *et al.*, 2015
			24/ 24^{-}	600	416.7	10^6	0.6/ 0.5	1000/ 1200	Kumar *et al.*, 2016
	NCTC 4135		40	72	30	10^4-10^5	5.5	13.1	Maclean *et al.*, 2013
	NCTC 4135		60	216	60	10^5	5	43.2	Tomb *et al.*, 2017
			65	234/ 468	60/ 120	10^7/ 10^9	3.8/ 7.7	61.6/ 60.8	McKenzie *et al.*, 2016
	CI		71	42.6	10	1-3 × $10^{2\ P}$	2^{E}	21.3	McDonald *et al.*, 2013
	NCTC 4135		100	60	10	10^5	5	12	Maclean *et al.*, 2016
	CI		123	147.6	20	10^5	5.16	28.6	Gupta *et al.*, 2015
	ATCC 25923		160	9/ 15	0.93/ 1.57	$10^{3\ P}$/ 1.5 × $10^{2\ P}$	0.17/ 0.92	52.9/ 16.3	Guffey & Wilborn, 2006; Guffey *et al.*, 2013a
	NCTC 4135	410	3.27	23.5	120	2 × 10^5	1.1	21.4	Maclean *et al.*, 2008

Microorganism	Strain Number(s)	Wavelength (nm)	Irradiance (mW/cm^2)	Dose (J/cm^2)	Time (min)	Exposed Population (CFU/ml or CFU/plate)	Log_{10} reduction (CFU/ml)	Dose for 1 log_{10} reduction	Reference
Staphylococcus aureus (methicillin-sensitive) (Gram +ve)	NCTC 4135	415	3.27	23.5	120	2×10^5	0.5	47	Maclean *et al.*, 2008
	ATCC 5923		100	120	20	-	1	120	Lipovsky *et al.*, 2010
	NCTC 4135	420	3.27	23.5	120	2×10^5	0.3	78.3	Maclean *et al.*, 2008
	NCTC 4135	430	3.27	23.5	120	2×10^5	0.1	235	Maclean *et al.*, 2008
	ATCC 25923	450	2	117^+/ 117^-	975	3×10^1	0.3/ 1.2	390/ 97.5	Keshishyan *et al.*, 2015
			36.1	300	138.5	10^8	1	300	Decarli *et al.*, 2016
	ATCC 5923	455	100	120	20	-	0.3	400	Lipovsky *et al.*, 2010
	ATCC 6538	460	74/ 74^-	1800	240	10^6	No Inactivation	-	Kumar *et al.*, 2016
	ATCC 6538	461	22.1	596.7	360	10^6	5.2	114.8	Ghate *et al.*, 2013
	ATCC 25923	470	150	15	1.67	$1.5 \times 10^{2\ P}$	0.42	35.7	Guffey & Wilborn 2006

Microorganism	Strain Number(s)	Wavelength (nm)	Irradiance (mW/cm^2)	Dose (J/cm^2)	Time (min)	Exposed Population (CFU/ml or CFU/plate)	Log_{10} reduction (CFU/ml)	Dose for 1 log_{10} reduction	Reference
Staphylococcus aureus (methicillin-resistant) (Gram +ve)	508, 520, 531	400	60	108/ 54/ 108	30/ 15/ 30	10^6	6.17/ 6.82/ 6.41	17.5/ 7.9/ 16.8	Halstead *et al.*, 2016
	EMRSA-16a	405	10	45	75	10^4-10^5	5	9	Maclean *et al.*, 2009
	EMRSA-15		60	270	75	105	5.24	51.5	Tomb *et al.*, 2017
	ATCC BAA1680		100	50/ 60/ 121/ 240	40/ -/ -/ 20.2	5×10^5/ $3 \times 10^{6\,p}$/ $5 \times 10^{6\,p}$/ $7 \times 10^{6\,p}$	1.2^{E}/ 1.4^{E}/ 1.4^{E}/ 1.57	100.8/ 35.7/ 42.9/ 152.9	Bumah *et al.*, 2015a; Masson-Meyers *et al.*, 2015
	USA 300, IS853		100	55.2/ 50.4	9.2/ 8.4	5×10^6	1.1/ 1.2	50.2/ 42	Enwemeka *et al.*, 2008
	USA 300 LAC	415	19.5	168.3	144	10^7	4.92	34.2	Dai *et al.*, 2013b
	8325-4, 252	460	133.3	240	30	10^6	3^{E}	80	Yang *et al.*, 2017
	ATCC BAA 1680	470	30	60	33.3	-	0.48	124.6	Bumah *et al.*, 2013

Microorganism	Strain Number(s)	Wavelength (nm)	Irradiance (mW/cm^2)	Dose (J/cm^2)	Time (min)	Exposed Population (CFU/ml or CFU/plate)	Log_{10} reduction (CFU/ml)	Dose for 1 log_{10} reduction	Reference
Staphylococcus aureus (methicillin-resistant) (Gram +ve)	ATCC BAA 1680	470	30	55	30.6	$3 \times 10^{6\ P}$/ $5 \times 10^{6\ P}$/ $5 \times 10^{6\ P}$/ $7 \times 10^{6\ P}$/ $7 \times 10^{6\ P}$/ $8 \times 10^{6\ P}$/ $1.2 \times 10^{7\ P}$	1.5/ 1.1/ 1^{E}/ 0.5/ 0.7^{E}/ 0.6^{E}/ 0.7^{A}	35/ 50/ 55/ 110/ 78.6/ 91.7/ 78.6	Bumah *et al*., 2015b; 2015c
	USA300, IS854		30	55	30.6	$5 \times 10^{6\ P}$	1.01/ 1.01	54.5/ 54.5	Enwemeka *et al*., 2009
	ATCC BAA 1680		100	13/ 50/ 240	-/ -/ 133	$3 \times 10^{6\ P}$/ $5 \times 10^{6\ P}$/ $7 \times 10^{6\ P}$	0.8^{E}/ 1.15^{E}/ 2^{E}	16.25/ 43.5/ 120	Bumah *et al*., 2015a
Staphylococcus epidermidis (Gram +ve)	NCTC: 11964; 10956, LMG 10474	405	5	18	60	$1–3 \times 10^{2\ P}$/ 10^{3}/ $1–3 \times 10^{2\ P}$	0.8/ 3/ 2.2^{E}	22.5/ 2.7/ 8 .2	McDonald *et al*., 2011, 2013; Ramakrishnan *et al*., 2014
	NCTC 11964		10	42	70	10^{4}-10^{5}	4.6	9.1	Maclean *et al*., 2009
	LMG 10474		15	324	360	10^{9}	7	46.3	Ramakrishnan *et al*., 2016
	CI		71	32	7.5	$1\text{-}3 \times 10^{2\ P}$	2^{E}	16	McDonald *et al*., 2013
	NCTC 11964		100	60	10	10^{5}	5	12	Maclean *et al*., 2016

Microorganism	Strain Number(s)	Wavelength (nm)	Irradiance (mW/cm^2)	Dose (J/cm^2)	Time (min)	Exposed Population (CFU/ml or CFU/plate)	Log_{10} reduction (CFU/ml)	Dose for 1 log_{10} reduction	Reference
Staphylococcus epidermidis (Gram +ve)	CI	405	123	118.1	16	10^5	5.12	23.1	Gupta *et al.*, 2015
	CI	414	90	324	60	3.8×10^9	No Inactivation	-	Kawada *et al.*, 2002
Stenotrophomonas maltophilia (Gram -ve)	529, 551, 558	400	60	108	30	10^6	7.21/ 2.97/ 7.33	15/ 36.4/ 14.7	Halstead *et al.*, 2016
Streptococcus mutans (Gram +ve)	ATCC 27351	450	520	93.6	3	5×10^6	No Inactivation	-	Feuerstein *et al.*, 2004
Streptococcus pneumoniae (Gram +ve)	ATCC 49616	405	8.05	123.41	250	- [P]	6.13	20.1	Barneck *et al.*, 2016
Streptococcus pyogenes (Gram +ve)	NCTC 8198	405	10	54	90	10^4-10^5	5	10.8	Maclean *et al.*, 2009
Vibrio parahaemolyticus (Gram -ve)	ATCC 17802	405	24/ 24[-]	170/ 147	118/ 102	10^6	1	170/ 147	Kumar *et al.*, 2016
		460	74/ 74[-]	717/ 958	161.5/ 215.8	10^6	1	717/ 958	Kumar *et al.*, 2016

* Mycobacteria cannot be classified using Gram staining

Table A1.2 Inactivation data of fungi and yeasts using 380-480 nm light, collected during the systematised review

Microorganism	Strain Number(s)	Wavelength (nm)	Irradiance (mW/cm^2)	Dose (J/cm^2)	Time (min)	Exposed Population (CFU/ml or CFU/plate)	Log_{10} reduction (CFU/ml)	Dose for 1 log_{10} reduction	Reference
Aspergillus niger (spores)	MUCL 38993	405 [A]	63	2300	608.5	2×10^5	0.1	23000	Murdoch *et al.*, 2013
		405	50	1440	480	10^3	1.5	960	Moorhead *et al.*, 2016b
		405	63	2300	608.5	2×10^5	5.24	439	Murdoch *et al.*, 2013
Aspergillus niger (hyphae)	MUCL 38993	405	50	1440	480	10^3	3	480	Moorhead *et al.*, 2016b
Aspergillus niger (germinating spore suspension)	MUCL 38993	405	63	454	120	2×10^5	2.5	181.6	Murdoch *et al.*, 2013
Aspergillus niger (microconidia)	MUCL 38993	405	35	504	240	- [P]	No growth inhibition	-	Moorhead *et al.*, 2016b
Botrytis cinerea (spores)	CI	405	50	1080-3780	360-1260	10^5	Germination significantly inhibited	-	Imada *et al.*, 2014

Microorganism	Strain Number(s)	Wavelength (nm)	Irradiance (mW/cm^2)	Dose (J/cm^2)	Time (min)	Exposed Population (CFU/ml or CFU/plate)	Log_{10} reduction (CFU/ml)	Dose for 1 log_{10} reduction	Reference
Botrytis cinerea (mycelia)	CI	405	50	4320 / 17280	1440 / 5760	- [P]	Inhibited growth	-	Imada *et al.*, 2014
			-		720	- [P]	Reduced mycelial growth (20mm less than control)	-	Imada *et al.*, 2014
	CI	415	50	17280	5760	- [P]	Reduced mycelial growth [E] (22.5mm less than control)	-	Imada *et al.*, 2014
	CI	450	50	17280	5760	- [P]	No growth inhibition [E]	-	Imada *et al.*, 2014
	CI	455	-	-	720	- [P]	Reduced mycelial growth (10mm less than control)	-	Xu *et al.*, 2017

Microorganism	Strain Number(s)	Wavelength (nm)	Irradiance (mW/cm^2)	Dose (J/cm^2)	Time (min)	Exposed Population (CFU/ml or CFU/plate)	Log_{10} reduction (CFU/ml)	Dose for 1 log_{10} reduction	Reference
Candida albicans (yeast cells)	MUCKL 29903	405 [A]	40	576	240	2×10^5	1.76	327.3	Murdoch *et al.*, 2013
	MUCKL 29903	405	40	576	240	2×10^5	5	115.2	Murdoch *et al.*, 2013
	SC5314		-	30	-	5×10^7	No growth Inhibition	-	Merigo *et al.*, 2017
	CI		123	332.1	45	10^5	4.52	73.5	Gupta *et al.*, 2015
	ATCC 90028	406	-	80	-	$10^{4\,P}$	2.7 [E]	29.6	Risović *et al.*, 2014
	CEC 749	415	19.5	70.2	60	10^7	5.42	13	Zhang *et al.*, 2016
	ATCC 90028	420	-	5	-	$10^{4\,P}$	2.5 [E]	2	Risović *et al.*, 2014
	ATCC 20011	460	36.1	200	138.5	10^6	0.2	1000	Decarli *et al.*, 2016

Microorganism	Strain Number(s)	Wavelength (nm)	Irradiance (mW/cm^2)	Dose (J/cm^2)	Time (min)	Exposed Population (CFU/ml or CFU/plate)	Log_{10} reduction (CFU/ml)	Dose for 1 log_{10} reduction	Reference
Fusarium Graminearium (spores)	-	470	80	100	21.5	3×10^4	0.22	454.5	De Lucca *et al.*, 2012
Fusarium Graminearium (germinated spores)	-	470	80	100	21.5	3×10^4	0.28	357.1	De Lucca *et al.*, 2012
Penicillunum digitalum (spores)	-	470	80	100	21.5	3×10^4	No Inactivation	-	De Lucca *et al.*, 2012
Penicillunum digitalum (germinated spores)	-	470	80	100	21.5	3×10^4	0.097	1030.9	De Lucca *et al.*, 2012
Saccharomyces cerevisae (yeast cells)	MUCL 28749	405 [A]	40	288	95	2×10^5 [P]	4.37	65.9	Murdoch *et al.*, 2013
	MUCL 28749	405	40	288	95	2×10^5 [P]	5	57.6	Murdoch *et al.*, 2013
Trichophyton mentagrophytes (microconidia)	MUCL 9823	405	35	504	240	- [P]	Germination inhibited	-	Moorhead *et al.*, 2016b

Microorganism	Strain Number(s)	Wavelength (nm)	Irradiance (mW/cm^2)	Dose (J/cm^2)	Time (min)	Exposed Population (CFU/ml or CFU/plate)	Log_{10} reduction (CFU/ml)	Dose for 1 log_{10} reduction	Reference
Trichophyton rubrum (spores)	MUCL 11954	405	50	360	120	- [P]	2.3	156.5	Moorhead *et al.*, 2016b
Trichophyton rubrum (microconidia)	MUCL 11954	405	35	504	240	-	Germination inhibited	-	Moorhead *et al.*, 2016b

Table A1.3 Inactivation data of bacteriophage and viruses using 380-480 nm light, collected during the systematised review

Microorganism	Strain Number(s)	Wavelength (nm)	Irradiance (mW/cm^2)	Dose (J/cm^2)	Time (min)	Exposed Population (CFU/ml or CFU/plate)	Log_{10} reduction (CFU/ml)	Dose for 1 log_{10} reduction	Reference
Feline calicivirus (in MM) (Virus, ss RNA)	F9	405	10^5	155.8	2800	300	3.9	717.9	Tomb *et al*., 2016
Feline calicivirus (in ORM) (Virus, ss RNA)	F9	405	10^5	155.8	421	45	5	84.2	Tomb *et al*., 2016
Murine Leukemia Virus (in ORM) (Virus, ss RNA)	-	420-430	-	-	10080	-	-	-	Richardson & Porter 2005
φC31 (in MM) (Bacteriophage, ds DNA)	φC31cΔ25	405	56.7	306	90	10^3	0.3	1020	Tomb *et al*., 2014
φC31 (in ORM) (Bacteriophage, ds DNA)	φC31cΔ25	405	56.7	306/ 510/ 1430	90/ 150/ 420	10^3/ 105/ 10^7	2.7/ 5.4/ 7.1	113.3/ 94.4/ 201.4	Tomb *et al*., 2014

Appendix B1: Inactivation Kinetics Following Repeated Sub-Lethal Exposure

This appendix details the inactivation kinetics of *S. aureus*, from each individual run (A, B & C) that underwent repeated sub-lethal exposure to 405 nm light, as detailed in Chapter 7.

Table B1.1 details inactivation of methicillin-sensitive *Staphylococcus aureus*

Table B1.2 details inactivation kinetics of methicillin-resistant *Staphylococcus aureus*

Table B1.1 Inactivation kinetics of isolates of MSSA from triplicate runs A, B & C after 5, 10 & 15 sub-lethal exposures of 405 nm light. Sub-lethal exposures were conducted using a dose of 108 J/cm^2 (at an irradiance of 60 mW/cm^2), and inactivation kinetics were based on exposure to 60 mW/cm^2 for increasing time periods. Comparisons are made with the inactivation kinetics of the initial starting population. Each data point is a mean count ($n \geq 2$) ± SD. Asterisks '*' indicate bacterial inactivation level which was significantly different to equivalent 0 sub-cultures control ($P \leq 0.05$), using one-way ANOVA.

MSSA Inactivation Kinetics (Log_{10} reduction CFU/ml)										
Dose (J/cm^2)	**0 Sub-Cultures (Control)**	**5 Sub-Lethal Exposures**			**10 Sub-Lethal Exposures**			**15 Sub-Lethal Exposures**			**15 Dark Sub-Cultures (Control)**
		A	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**	
54	0.68 ± 0.36	0.47 ± 0.29	**0.15 ± 0.15***	0.28 ± 0.20	0.43 ± 0.31	0.66 ± 0.49	0.50 ± 0.02	0.77 ± 0.24	0.62 ± 0.23	0.68 ± 0.34	0.66 ± 0.50
108	1.79 ± 0.35	1.61 ± 0.76	0.95 ± 0.29	1.36 ± 0.46	1.39 ± 0.52	1.74 ± 0.75	1.67 ± 0.51	1.97 ± 0.67	1.74 ± 0.65	2.22 ± 1.06	1.39 ± 0.31
162	3.51 ± 0.40	3.32 ± 1.84	3.11 ± 0.27	3.40 ± 1.56	2.70 ± 0.63	3.64 ± 1.44	2.71 ± 0.49	3.07 ± 0.46	2.90 ± 0.86	3.21 ± 1.17	**2.56 ± 0.48***
216	5.02 ± 0.16	4.56 ± 1.22	4.96 ± 0.46	4.58 ± 0.56	4.36 ± 0.81	4.64 ± 0.51	**3.96 ± 0.58***	4.46 ± 0.75	4.08 ± 0.30	4.63 ± 1.03	4.30 ± 0.78
270	5.35 ± 0.12	5.04 ± 0.25	5.13 ± 0.13	5.36 ± 0.21	5.36 ± 0.07	4.89 ± 0.17	5.47 ± 0.03	5.13 ± 0.21	**4.52 ± 0.14***	4.82 ± 0.53	4.93 ± 0.71

Table B1.2 Inactivation kinetics of isolates of MRSA from triplicate runs A, B & C after 5, 10 & 15 sub-lethal exposures of 405 nm light. Sub-lethal exposures were conducted using a dose of 108 J/cm^2 (at an irradiance of 60 mW/cm^2), and inactivation kinetics were based on exposure to 60 mW/cm^2 for increasing time periods. Comparisons are made with the inactivation kinetics of the initial starting population. Each data point is a mean count ($n \geq 2$) ± SD. Asterisks '*' indicate bacterial inactivation level which was significantly different to 0 sub-cultures control ($P \leq 0.05$), using one-way ANOVA.

MRSA Inactivation Kinetics (Log_{10} reduction CFU/ml)										
Dose (J/cm^2)	**0 Sub-Cultures (Control)**	**5 Sub-Lethal Exposures**			**10 Sub-Lethal Exposures**			**15 Sub-Lethal Exposures**			**15 Dark Sub-Cultures (Control)**
		A	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**	
54	0.62 ± 0.32	0.35 ± 0.19	0.19 ± 0.24	0.55 ± 0.56	0.81 ± 0.28	0.47 ± 0.13	0.34 ± 0.11	**0.25 ± 0.01***	0.77 ± 0.20	0.66 ± 0.55	0.26 ± 0.22
108	1.95 ± 0.41	1.25 ± 0.40	**1.13 ± 0.37***	**1.33 ± 0.18***	**1.57 ± 0.27***	**2.44 ± 0.17***	**1.32 ± 0.25***	**1.13 ± 0.17***	1.83 ± 0.59	1.43 ± 0.44	0.96 ± 0.64
162	3.45 ± 1.06	**2.78 ± 0.45***	2.36 ± 0.92	2.98 ± 0.45	2.85 ± 0.36	4.51± 0.35	2.81 ± 0.61	**2.15 ± 0.63***	3.09 ± 1.02	2.89 ± 0.85	1.75 ± 0.55
216	4.59 ± 1.02	4.63 ± 0.49	3.78 ± 0.86	4.86 ± 0.24	4.64 ± 0.18	5.34 ± 0.06	3.80 ± 1.24	4.01 ± 1.53	4.32 ± 1.42	3.96 ± 1.39	**2.91 ± 0.63***
270	5.24 ± 0.58	5.12 ± 0.35	4.97 ± 0.07	5.34 ± 0.04	5.21 ± 0.04	5.33± 0.05	4.70 ± 0.76	4.42 ± 1.1.3	4.73 ± 0.82	5.14 ± 0.54	4.26 ± 0.51
324	5.30 ± 0.05	5.38 ± 0.01	5.31 ± 0.05	5.51 ± 0.05	5.53 ± 0.003	5.26 ± 0.01	5.41 ± 0.04	5.29 ± 0.36	5.35 ± 0.01	5.40 ± 0.004	5.12 ± 0.38

Appendix B2: Antibiotic Susceptibility Following Repeated Sub-Lethal Exposure

This appendix details the antibiotic susceptibility of *S. aureus,* from each individual run (A, B, C) that underwent repeated sub-lethal exposure to 405 nm light, as detailed in Chapter 7.

Table B2.1 details the antibiotic susceptibility of methicillin-sensitive *Staphylococcus aureus*

Table B2.2 details the antibiotic susceptibility of methicillin-resistant *Staphylococcus aureus*

Table B2.1 Antibiotic susceptibility of isolates of MSSA from individual runs A, B & C after 5, 10 & 15 sub-lethal exposures of 405 nm light. Susceptibility was measured using Disc Diffusion Method. Comparisons are made with the antibiotic susceptibility of the initial starting population. Each data point is a mean value (n = 3) ± SD. Asterisks '*' indicate diameter of zone of inhibition that was significantly different to equivalent 0 sub-cultures control ($P \leq 0.05$), using one-way ANOVA.

Antibiotic	Conc (µg)	Mean Diameter of Zone of Inhibition for MSSA (mm)										
		0 Sub-Cultures (Control)	5 Sub-Lethal Exposures			10 Sub-Lethal Exposures			15 Sub-Lethal Exposures			15 Dark Sub-Cultures (Control)
			A	B	C	A	B	C	A	B	C	
Oxacillin	**5**	30.0 ± 0.0	29.3 ± 1.2	29.0 ± 1.0	29.7 ± 0.6	30.0 ± 0.0	30.0 ± 1.0	29.7 ± 0.6	29.7 ± 0.6	30.0 ± 1.0	29.0 ± 1.0	**19.7 ± 0.6***
Erythromycin	**5**	21.3 ± 0.6	21.3 ± 0.6	21.3 ± 0.6	20.7 ± 0.6	21.3 ± 1.2	22.0 ± 0.0	22.3 ± 0.6	21.7 ± 0.6	21.0 ± 0.0	21.0 ± 0.0	**22.7 ± 0.6***
Tetracycline	**25**	27.7 ± 1.2	27.3 ± 1.2	28.0 ± 1.0	27.7 ± 1.2	29.0 ± 1.7	29.3 ± 0.6	27.0 ± 0.0	29.0 ± 1.0	28.7 ± 1.5	29.7 ± 1.5	26.7 ± 0.6
Chloramphenicol	**25**	21.3 ± 1.5	21.7 ± 0.6	20.7 ± 0.6	20.3 ± 0.6	22.3 ± 0.6	21.3 ± 1.5	20.7 ± 1.2	21.7 ± 0.6	20.3 ± 0.6	20.7 ± 0.6	23.7 ± 0.6
Fusidic Acid	**10**	30.0 ± 0.0	29.7 ± 1.2	30.3 ± 0.6	30.3 ± 0.6	31.0 ± 1.0	31.3 ± 1.2	**32.3 ± 0.6***	30.0 ± 2.0	30.7 ± 0.6	**33.0 ± 1.0***	29.3 ± 0.6
Gentamicin	**10**	20.7 ± 0.6	20.0 ± 0.0	21.0 ± 0.0	21.0 ± 0.0	20.3 ± 0.6	21.0 ± 0.0	20.7 ± 0.6	20.0 ± 0.0	20.3 ± 0.6	20.7 ± 1.0	**24.7 ± 0.6***
Ampicillin	**25**	40.0 ± 1.0	38.3 ± 0.6	38.3 ± 1.5	40.3 ± 1.2	39.7 ± 1.5	40.3 ± 0.6	38.7 ± 2.3	40.0 ± 0.0	38.7 ± 2.3	40.0 ± 0.0	**35.7± 0.6***
Ciprofloxacin	**5**	22.3 ± 1.2	21.3 ± 0.6	21.0 ± 0.0	21.7 ± 0.6	21.3 ± 0.6	22.0 ± 1.0	21.7 ± 0.6	23.3 ± 0.6	21.0 ± 1.0	23.0 ± 1.0	20.7 ± 0.6
Mupirocin	**5**	27.7 ± 0.6	26.3 ± 0.6	26.3 ± 0.6	26.7 ± 0.6	28.0 ± 1.0	27.3 ± 2.1	28.3 ± 1.2	27.7 ± 0.6	28.0 ± 1.7	26.7 ± 0.6	27.3 ± 0.6
Vancomycin	**5**	15.0 ± 0.0	14.0 ± 1.0	14.3 ± 0.6	14.3 ± 0.6	14.7 ± 0.6	15.7 ± 0.6	15.7 ± 0.6	15.0 ± 0.0	14.3 ± 0.6	14.7 ± 0.6	14.3 ± 0.6
Rifampicin	**5**	32.0 ± 1.0	31.7 ± 0.6	32.0 ± 1.0	31.7 ± 0.6	34.3 ± 1.2	32.7 ± 0.6	33.0 ± 3.0	**34.0 ± 0.0***	34.0 ± 1.0	33.7 ± 0.6	32.3 ± 0.6

Table B2.2 Antibiotic susceptibility of isolates of MRSA from individual runs A, B & C after 5, 10 & 15 sub-lethal exposures of 405 nm light. Susceptibility was measured using Disc Diffusion Method. Comparisons are made with the antibiotic susceptibility of the initial starting population. Each data point is a mean value (n = 3) ± SD. Asterisks '*' indicate diameter of zone of inhibition that was significantly different to equivalent 0 sub-cultures control ($P \leq 0.05$), using one-way ANOVA.

Antibiotic	Conc (µg)	Mean Diameter of Zone of Inhibition for MRSA (mm)										
		0 Sub-Cultures (Control)	5 Sub-Lethal Exposures			10 Sub-Lethal Exposures			15 Sub-Lethal Exposures			15 Dark Sub-Cultures (Control)
			A	B	C	A	B	C	A	B	C	
Oxacillin	5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Erythromycin	5	23.0 ± 1.0	22.7 ± 0.6	22.0 ± 1.0	21.7 ± 0.6	22.3 ± 1.5	22.3 ± 0.6	23.3 ± 0.6	23.0 ± 1.0	23.0 ± 1.0	24.3 ± 1.2	23.0 ± 1.0
Tetracycline	25	27.0 ± 1.0	27.3 ± 0.6	27.0 ± 0.0	27.3 ± 1.2	27.3 ± 1.2	27.0 ± 1.0	26.0 ± 0.0	25.7 ± 0.6	26.0 ± 1.0	26.7 ± 1.2	26.3 ± 0.6
Chloramphenicol	25	24.0 ± 1.0	22.7 ± 1.5	23.7 ± 0.6	24.3 ± 0.6	22.7 ± 1.5	23.3 ± 2.1	23.3 ± 0.6	21.7 ± 0.6	23.3 ± 0.6	23.7 ± 0.6	22.3 ± 0.6
Fusidic Acid	10	30.7 ± 0.6	30.7 ± 0.6	30.7 ± 0.6	30.0 ± 0.0	29.3 ± 0.6	30.3 ± 0.6	30.3 ± 0.6	29.7 ± 0.6	30.3 ± 0.6	31.0 ± 1.0	29.7 ± 0.6
Gentamicin	10	20.7 ± 0.6	20.7 ± 0.6	20.7 ± 0.6	20.3 ± 0.6	20.3 ± 0.6	21.7 ± 0.6	**23.0 ± 1.0***	**23.3 ± 0.6***	**23.3 ± 0.6***	**22.7 ± 0.6***	**22.3 ± 0.6***
Ampicillin	25	15.3 ± 0.6	14.7 ± 0.6	16.3 ± 1.5	15.3 ± 0.6	16.3 ± 0.6	14.3 ± 0.6	14.3 ± 0.6	**12.0 ± 1.0***	**12.3 ± 0.6***	**13.7 ± 0.6***	**10.7 ± 0.6***
Ciprofloxacin	5	25.7 ± 0.6	26.3 ± 0.6	26.3 ± 0.6	26.0 ± 0.0	26.7 ± 0.6	24.7 ± 0.6	25.7 ± 0.6	24.3 ± 1.2	26.3 ± 0.6	25.3 ± 0.6	24.7 ± 0.6
Mupirocin	5	26.3 ± 0.6	26.3 ± 0.6	27.7 ± 1.5	27.3 ± 0.6	25.7 ± 1.5	25.7 ± 0.6	26.3 ± 1.5	25.0 ± 1.0	27.3 ± 0.6	25.3 ± 0.6	26.0 ± 0.0
Vancomycin	5	14.3 ± 0.6	15.0 ± 0.0	**15.7 ± 0.6***	14.7 ± 0.6	14.3 ± 0.6	14.7 ± 0.6	14.7 ± 0.6	14.7 ± 0.6	14.0 ± 0.0	14.3 ± 0.6	14.7 ± 0.6
Rifampicin	5	30.7 ± 0.6	30.3 ± 0.6	30.7 ± 0.6	30.0 ± 1.0	30.0 ± 1.0	30.7 ± 0.6	31.7 ± 0.6	31.0 ± 0.0	30.7 ± 1.5	31.3 ± 0.6	31.0 ± 0.0

Appendix C: Porphyrin Extraction Methods

Table C1.1 The growth methods and regents used to extract porphyrins from Staphylococcus aureus for HPLC analysis. *S. aureus* was cultured for 24 hours in nutrient broth or for 48 hours on nutrient agar plates. Following cultivation and centrifugation, the bacterial pellet was incubated with either 1ml of 0.1M Sodium Hydroxide (NaOH)-1% Sodium Dodecyl Sulphate (SDS) or 0.1 M Ammonium Hydroxide (NH_4OH):Acetone (1:9 v/v). Samples were then vortexed and in some instances sonicated for 15 mins, before overnight incubation in complete darkness.

Method No.	Bacterial Growth Method	Extraction Reagent	Vortex and/or Sonication
1	1 × 100 ml Broth	0.1M of NaOH-1% SDS	Vortex
2	3 × 100 ml Broth	0.1M of NaOH-1% SDS	Vortex
3	Agar Plates × 10	0.1M of NaOH-1% SDS	Vortex
4	1 × 100 ml Broth	0.1M of NaOH-1% SDS	Vortex & Sonicate
5	**3 × 100 ml Broth**	**0.1M of NaOH-1% SDS**	**Vortex & Sonicate**
6	Agar Plates × 10	0.1M of NaOH-1% SDS	Vortex & Sonicate
7	6 × 100 ml Broth	0.1M of NaOH-1% SDS	Vortex & Sonicate
8	1 × 100 ml Broth	0.1 M NH_4OH:Acetone (1:9 v/v)	Vortex
9	3 × 100 ml Broth	0.1 M NH_4OH:Acetone (1:9 v/v)	Vortex
10	Agar Plates × 10	0.1 M NH_4OH:Acetone (1:9 v/v)	Vortex
11	1 × 100 ml Broth	0.1 M NH_4OH:Acetone (1:9 v/v)	Vortex & Sonicate
12	3 × 100 ml Broth	0.1 M NH_4OH:Acetone (1:9 v/v)	Vortex & Sonicate
13	Agar Plates × 10	0.1 M NH_4OH:Acetone (1:9 v/v)	Vortex & Sonicate

Appendix D: Publications

Peer Reviewed Journal Publications:

- Tomb, R.M., Maclean, M., Coia, J.E., Graham, E., McDonald, M., Atreya, C.D., MacGregor, S.J. & Anderson, J.G. (2016). New proof-of-concept in viral inactivation: virucidal efficacy of 405 nm light against feline calicivirus as a model for norovirus decontamination. *Food and Environmental Virology*. 9 (2), 159-167, June 2017. Doi:10.1007/s12560-016-9275-z
- Maclean, M., McKenzie, K., Moorhead, S., Tomb, R.M., Coia, J.E., MacGregor, S.J. & Anderson, J.G. (2015) Decontamination of the hospital environment: new technologies for infection control. *Journal of Current Treatment Options in Infectious Diseases.* 7 (1), 39-51, March 2015. Doi: 10.1007/s40506-015-0037-5
- Tomb, R.M., Maclean, M., Herron, P.R., Hoskisson, P.A., MacGregor, S.J. & Anderson, J.G. (2014) Inactivation of *Streptomyces* phage ϕC31 by 405 nm light: requirement for exogenous photosensitizers? *Bacteriophage*. 4 (3), e32129, July 2014. Doi: 10.4161/bact.32129 (Written during PhD)

Research Papers in Preparation:

- Tomb, R.M., Maclean, M., Coia, J.E., MacGregor, S.J. & Anderson, J.G. Blue-light exposure of proliferating and non-proliferating *Staphylococcus aureus* for assessment of potential tolerance development.

Conference Poster Publications:

- Moorhead*, S.M., Tomb*, R.T., Coia, J.E., Anderson, J.G., Graham, E., McDonald, M., MacGregor, S.J. & Maclean, M. Enhanced visible light inactivation of *Clostridium difficile* and Norovirus within simulated faecal contamination. *Microbiology Society Annual Conference*, 3-6 April 2017, Edinburgh, Scotland. * Equal contribution
- Tomb, R.M., Maclean, M., Coia, J.E., MacGregor, S.J. & Anderson, J.G. Can repeated sub-lethal exposure to antimicrobial 405 nm light induce tolerance in *Staphylococcus aureus*? *IV International Conference on Antimicrobial Research*, 29 June-1 July 2016, Malaga, Spain.

- Tomb, R.M., Maclean, M., Coia, J.E., MacGregor, S.J. & Anderson, J.G. Can *Staphylococcus* Develop Tolerance to Antimicrobial 405 nm Light? *Microbiology Society Annual Conference*, 21-24 March 2016, Liverpool, England.
- Tomb, R.M., Maclean, M., Coia, J.E., MacGregor, S.J. & Anderson, J.G. Potential for Bacterial Resistance to Antimicrobial 405 nm Light. *The Federation of Infection Societies (FIS) Conference 2015*, 21-23 November, Glasgow, Scotland.
- Tomb, R.M., Maclean, M., Coia, J.E., Graham, E., McDonald, M., MacGregor, S.J. & Anderson, J.G. Efficacy of 405 nm Light for Inactivation of Feline Calicivirus: A Surrogate for Norovirus. *The 9th Healthcare Infection Society (HIS) International Conference 2014*, 16-18 November 2014, Lyon, France.

Conference Grant Support:

- Healthcare Infection Society Travel Grant awarded to attend HIS 2014 conference, Lyon, France (£700).
- Microbiology Society Travel Grant awarded to attend the Microbiology Society Annual Conference 2016, Liverpool, UK (£320).

Oral Presentations:

- Tomb, R.M., Maclean, M., Coia, J.E., Graham, E., McDonald, M., MacGregor, S.J. & Anderson, J.G. 405 nm Light Inactivation of Feline Calicivirus. Presentation at a *Clinical Seminar at Glasgow University Veterinary School*, 7 December 2014.
- Tomb, R.M., Maclean, M., Coia, J.E., Graham, E., McDonald, M., MacGregor, S.J. & Anderson, J.G. 405 nm Light Inactivation of Feline Calicivirus: A Surrogate for Norovirus. *The Scottish Infection Research Network (SIRN) Autumn 2014 Research Event*, Glasgow, 13 November 2014.