Studies on Enhanced Surface Disinfection and Skin Antisepsis using Chlorhexidine and Eucalyptus Oil

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Doctor of Philosophy

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March 2011

Aston University

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Emma Hendry for the Degree of Doctor of Philosophy

2011

Healthcare associated infections may arise from many sources, including patient's own skin flora and the clinical environment, and inflict a significant burden within the health service. Adequate and effective skin antisepsis and surface disinfection are therefore essential factors in infection control. Current EPIC guidelines recommend 2 % chlorhexidine (CHG) in 70 % isopropyl alcohol (IPA) for skin antisepsis however poor penetration has been reported. Eucalyptus oil (EO) is a known permeation enhancer, producing synergistic antimicrobial activity when combined with CHG. In this current study, the antimicrobial efficacy of EO and its main constituent 1,8-cineole were assessed against a panel of clinically relevant microorganisms, alone and in combination with CHG. The superior antimicrobial efficacy of EO compared with 1,8-cineole, and synergistic effects with CHG against planktonic and biofilm cultures, confirmed its suitability for use in subsequent studies within this thesis. Impregnation of EO, CHG and IPA onto prototype hard surface disinfectant wipes demonstrated significantly improved efficacy compared with CHG/IPA wipes, with clear reductions in the time required to eliminate biofilms. Optimisation of the EO/CHG/IPA formulation resulted in the development of Euclean® wipes, with simulated-use and time kill studies confirming their ability to remove microbial surface contamination, prevent cross contamination and eliminate biofilms within 10 minutes. The employment of isothermal calorimetry provided additional information on the type and rate of antimicrobial activity possessed by Euclean® wipes. A clinical audit of the Euclean® wipes at Birmingham Children's Hospital, Birmingham, U.K. revealed divided staff opinion, with the highest cited advantage and disadvantage concerning the odour. Finally, skin penetration and cell toxicity studies of EO/CHG biopatches and Euclean® solution developed during this study, revealed no permeation into human skin following biopatch application, and no significant toxicity. These current studies enhance the knowledge regarding EO and its potential applications.

Key words: Eucalyptus Oil, Chlorhexidine, Biofilm, Antimicrobial Activity, Synergy.

"Obtaining my Ph.D. could be compared to a fairytale, unbelievable and curious at times yet a great source of entertainment and enjoyment. I find it particularly comparable to Snow White and the Seven Dwarfs. During my Ph.D., I have been each of the seven dwarfs. I was dopey and bashful at first, but by my second year became grumpy, sleepy and sick (sneezy). Progression through my third year made me happy with completion resulting in me being Doc! Now I can sleep like Snow White before 'Hi Ho Hi Ho, it's off to work I go' to my dream job and my own happy ever after. Thank you to everyone who shared in my fairytale."

Acknowledgements

Completion of this thesis would not have been possible without the selflessly given support and guidance from countless colleagues, family and friends during my studies; therefore I take this opportunity to express my sincere appreciation.

Firstly, I bestow my deepest gratitude upon my supervisors with whom it has been both a privilege and pleasure to work; genuine thanks Dr Tony Worthington, Professor Pete Lambert and Professor Barbara Conway, for your seemingly endless knowledge, inspiring enthusiasm and unfaltering belief in me. I have found your passion for science infectious, your wisdom invaluable and your friendship reassuring.

For specialist advice, I pay regards to Dr Mike O'Neill for your comprehension of isothermal calorimetry and assistance during our collaborative studies within chapter 5 of this thesis. I also express my gratitude to Dr Anthony Hilton for your profound statistical knowledge, Dr Chris Langley for your in-depth grasp of questionnaire writing, and Mrs Karen Farrow for your technical skills and expertise relating to gas chromatography.

In addition, I give a special mention to Insight Health Ltd. for financially contributing to my EPSRC studentship funding for this project, along with personal recognition of John Rawlinson for your valued opinions, vibrant ideas and loyal devotion to my research.

To Judith Room, the ward managers and their staff at Birmingham Children's Hospital, I send my kindest regards for your participation in the Euclean® Clinical Audit within chapter 6 of this thesis.

For sharing both the good and challenging times, I pay wholehearted tributes to Anne, Leanne, Lois, Charlie, Connie, Nai, Kamila, Rachel, Lynn, Zita, Jo and Adam. Our daily laughs have created too many fond memories to recall, and your encouragement, understanding and sincerity has built friendships I treasure greatly.

Finally, to my family: Mum, Dave, Debbie and Dom, Nan and Grandad. You have been my boulders. Your strength and solidarity are awe-inspiring and have driven me beyond belief. Kindly accept my loving thanks for your faultless tolerance, remarkable motivation and encouraging positivity. To you all, I am indebted. Thank you.

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Abbreviations

°C	Degrees Celsius
µg	Microgram
µL	Microlitre
µm	Micrometer
ADHD	Attention Deficit Hyperactivity Disorder
AEC	Airway Epithelial Cells
AEM	Airway Epithelial Cell Growth Medium
ATCC	American Type Culture Collection
ATP	Adenosine Tri-Phosphate
ATP-MR	Adenosine Tri-Phosphate Monitoring Reagent
BSI	Bloodstream Infections
CDC	Centres for Disease Control and Prevention
CF	Cystic Fibrosis
cfu	Colony Forming Units
CHG	Chlorhexidine Digluconate
cm	Centimetre
cm^2	Centimetre Squared
CNS	Coagulase Negative Staphylococci
CRBSI	Catheter-Related Bloodstream Infection
CRI	Catheter-Related Infection
CVC	Central Venous Catheter

Da	Dalton
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DoH	Department of Health
DSC	Differential Scanning Calorimetry
EDTA	Ethylenediaminetetraacetic Acid
EO	Eucalyptus Oil
EPIC	Evidence-Based Practice in Infection Control
FDA	Food and Drug Administration
FGM2	Fibroblast Growth Medium 2
FIC	Fractional Inhibitory Concentration
FICI	Fractional Inhibitory Concentration Indices
FID	Flame Ionization Detector
Flow-TAM	Flow Thermal Activity Monitor
g	Gram
GC	Gas Chromatography
GRAS	Generally Regarded as Safe
gsm	Grams per Square Metre
HAI	Healthcare Associated Infection
HEPA	High Efficiency Particulate Air
HII	High Impact Interventions
HPA	Health Protection Agency
HPF	Human Pulmonary Fibroblast

HPLC	High Performance Liquid Chromatography
IC	Isothermal Calorimetry
ICU	Intensive Care Unit
IPA	Isopropyl Alcohol
IS	Internal Standard
L	Litre
LOD	Level of Detection
LOQ	Level of Quantification
M	Molar
MBC	Minimum Bactericidal Concentration
MFC	Minimum Fungicidal Concentration
mg	Milligram
MHA	Mueller-Hinton Agar
MHB	Mueller-Hinton Broth
MIC	Minimum Inhibitory Concentration
mL	Millilitre
mm	Millimetre
MRSA	Methicillin-Resistant *Staphylococcus aureus*
Mw	Molecular Weight
n	Number
NAO	National Audit Office
NCTC	National Collection of Type Cultures
NHS	National Health Service

nm	Nanometre
NRES	National Research Ethics Service
OD	Optical Density
P	Probability
PBS	Phosphate Buffered Saline
PEG	Polyethylene Glycol
pH	Potential of Hydrogen
PIA	Polysaccharide Intercellular Adhesin
PNAG	Poly-N-acetyl-glucosamine
psi	Pound-Force per Square Inch
PVA	Polyvinyl Alcohol
PVP-I	Povidone-Iodine
QUATS	Quaternary Ammonium Compounds
RLU	Relative Light Units
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RRP	Rapid Review Panel
SC	Stratum Corneum
SD	Standard Deviation
SDA	Sabouraud Dextrose Agar
SDB	Sabouraud Dextrose Broth
SSI	Surgical Site Infection
TAM	Thermal Activity Monitor

TDDS	Transdermal Drug-Delivery System
TSA	Tryptone Soya Agar
TSB	Tryptone Soya Broth
TTO	Tea Tree Oil
UTI	Urinary Tract Infection
UV	Ultraviolet
v/v	Volume per Volume
VAP	Ventilator-Associated Pneumonia
VRE	Vancomycin-Resistant Enterococcus
w/v	Weight per Volume
w/w	Weight per Weight
WHO	World Health Organisation

Suppliers of Chemicals

1,8-cineole (Sigma-Aldrich, Dorset, U.K.)

Acetic acid (Fisher Scientific, Leicestershire, U.K.)

Acetic acid glacial HPLC grade (Fisher Scientific, Leicestershire, U.K.)

Acetone (Sigma-Aldrich, Dorset, U.K.)

Agar bacteriological (agar no.1) (Oxoid, Basingstoke, U.K.)

Airway epithelial cell growth medium (PromoCell, Heidelberg, Germany)

Alcian blue 8 XG (Sigma-Aldrich, Dorset, U.K.)

Cell titre-blue (Promega, Southampton, U.K.)

Chlorhexidine digluconate solution 20 % (v/v) (Sigma-Aldrich, Dorset, U.K.)

Chloroform (Sigma-Aldrich, Dorset, U.K.)

Congo red (Hopkins & Williams Limited, Essex, U.K.)

Cryospray (Bright Instruments, Cambridgeshire, U.K.)

Crystal violet (Reactifs RAL, France)

D-glucose (Fisher Scientific, Leicestershire, U.K.)

Dimethyl sulfoxide (Sigma-Aldrich, Dorset, U.K.)

Embedding compound (Bright Instruments, Cambridgeshire, U.K.)

Eucalyptus oil (Sigma-Aldrich, Dorset, U.K.)

Fibroblast growth medium 2 (PromoCell, Heidelberg, Germany)

Hexane HPLC grade (Sigma-Aldrich, Dorset, U.K.)

HPLC grade methanol (Fisher Scientific, Leicestershire, U.K.)

Isopropyl alcohol (Sigma-Aldrich, Dorset, U.K.)

Lecithin, Acros Organics (Fisher Scientific, Leicestershire, U.K.)

Letheen broth (Oxoid, Basingstoke, U.K.)

Methanol HPLC grade (Fisher Scientific, Leicestershire, U.K.)

Muller-hinton agar (Oxoid, Basingstoke, U.K.)

Muller-hinton broth (Oxoid, Basingstoke, U.K.)

N-tetradecane (Sigma-Aldrich, Dorset, U.K.)

Phosphate buffered saline (Sigma-Aldrich, Dorset, U.K.)

Sabouraud dextrose agar (Oxoid, Basingstoke, U.K.)

Sabouraud dextrose broth (Oxoid, Basingstoke, U.K.)

Sodium 1-heptane sulfonate (Sigma-Aldrich, Dorset, U.K.)

Sodium thiosulphate pentahydrate (BDH Ltd, Poole, U.K.)

Triton X-100 (Sigma-Aldrich, Dorset, U.K.)

Trypsin ethylenediaminetetraacetic acid (Sigma-Aldrich, Dorset, U.K.)

Tryptone soya agar (Oxoid, Basingstoke, U.K.)

Tryptone soya broth (Oxoid, Basingstoke, U.K.)

Tween-80 (Sigma-Aldrich, Dorset, U.K.)

ViaLight MDA Plus detection kit (Cambrex, U.S.A.)

Virkon® (Fisher Scientific, Leicestershire, U.K.)

Chapter 1

Introduction to Thesis

1.1. Healthcare associated infections

1.1.1. Types, prevalence and implications

Healthcare associated infections (HAI) are a major concern within the health service, and are defined as infections acquired either within hospitals, or as a direct result of a healthcare intervention (Department of Health, 2008*a*). They inflict a significant burden on the healthcare system due to increased morbidity and mortality rates, prolonged hospital occupancy and intensified treatment regimes to include repeated surgery and prolonged antibiotic therapy, all of which impose both financial and time constraints on trusts (Vilela *et al*., 2007; Weigelt *et al*., 2010). In 2008, the Department of Health (DoH) reported that the incidence of HAI within England was 8.2 %, comfortably within the range found across industrialised countries of between 5 and 10 % (Department of Health, 2008*a*).

There are many types of HAI, with a few in particular accounting for the majority of all reported cases, these include; skin and soft tissue which account for 10.8 % of cases, primary bloodstream infections (BSI) often associated with indwelling medical devices make up 6.8 %, gastrointestinal account for 22 %, pneumonia causes 13.9 % and urinary tract infections (UTI) are responsible for 19.7 % of cases (Hospital Infection Society, 2007). Surgical site infections (SSI) are recognised as one of the most significantly important types of HAI, and according to the Health Protection Agency (HPA) are to blame for around 15 % of all HAI (Heath Protection Agency, 2010). It has been estimated that the development of an SSI leads to a patient incurring an average additional hospital stay of 6.5 days and doubled hospital costs (Plowman *et al*., 2001), with the drain on NHS funds an estimated 1 billion pounds per annum (Bagnall *et al*., 2009).

1.1.2. Catheter-related infections

The management of critically ill patients is often accompanied by the insertion of one or more implanted prosthetic devices such as cerebrospinal fluid shunts or urinary, peritoneal or intravascular catheters, alongside other specialised prostheses including replacement joints, prosthetic valves, pacemakers and intraocular lenses (Piette & Verschraegen, 2009; Schierholz & Beuth, 2001). These devices are without doubt

crucial and often life saving, however patients are at increased risk from infections while these devices are *in situ* (Saginur *et al*., 2006), with intravascular catheters such as central venous catheters (CVC) often causing an array of catheter-related infections (CRI), ranging in severity from localised to systemic (Worthington & Elliott, 2005).

There are four distinct types of CVC-related infection causing some of the most common detrimental effects of all such implanted devices; colonisation of the catheter, wound infection around the exit site, infection of the tunnel and systemic bloodstream infection known as septicaemia (Boersam & Schouten, 2010). It has been estimated that 10,000 CVCs are utilised annually in the U.K., and that around 78 % of critically ill patients undergo insertion of a CVC during their treatment regime (Elliott, 2007; Vincent *et al*., 1995), and that of all catheter-related bloodstream infections (CRBSI), 90 % of cases are linked to CVC (Wang *et al*., 2010), with the Health Service financial expenditure costing the equivalent of between £2,500 and £36,000 per patient with a CRBSI (Maki *et al*., 2006). The greatest risk of CRI arises from short-term, single- or multi-lumen central venous catheters that are non-cuffed and inserted into the subclavian or internal jugular vein (Worthington & Elliott, 2005). Chen *et al*. (2006) illustrated that the risk of a CVC-related infection developing is increased by a variety of factors, including catheterisation of greater than 7 days, total parental nutrition being administered, a single CVC having greater than 3 infusion lines, and insertion being performed on a ward rather than in surgery. Similarly, others have identified risk factors including CVC insertion into the femoral vein causing significantly more localised and bloodstream infections than insertion into the subclavian or jugular veins, with the subclavian being the vein of choice to use (Lorente *et al*., 2005). Prevention of CRI is therefore, far more desirable than treatment of an established infection, as this often requires a lengthy course of antimicrobials, with the possibility that removal of the contaminated implant will be necessary (Wu *et al*., 2003).

1.1.3. Microorganisms

There are many microorganisms associated with HAI, and whilst some are typically linked with particular sites or types of HAI, others are related less frequently. SSI causative microorganisms include both Gram-positive and -negative bacteria such as *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), coagulase

negative staphylococci (CNS), *Enterococcus* spp., *Streptococcus* spp., *Klebsiella* spp. and *Pseudomonas aeruginosa*, as well as yeasts such as *Candida albicans*, fungi and anaerobic microorganisms (Hospital Infection Society, 2007; Weigelt *et al.*, 2010). In September 2010, the HPA published figures on 1,332 SSI cases recorded by 256 hospitals over a 12 month period. It was established that *S. aureus* was the causative microorganism in 31 % of cases, and of those, 32 % were methicillin resistant. This is a proportion reduction from four years previous, when HPA surveillance reported *S. aureus* responsible for 53 % of SSIs, of which 64 % were resistant to methicillin (Health Protection Agency, 2006). The quarterly counts of MRSA bacteraemia infections displayed in Figure 1.l support these findings, and shows an overall reduction in cases over the 3 years of monitoring, with slight fluctuations around December and January; possibly due to increased hospital admissions during the winter of those more susceptible to infection including the elderly and very young (Health Protection Agency, 2010*a*).

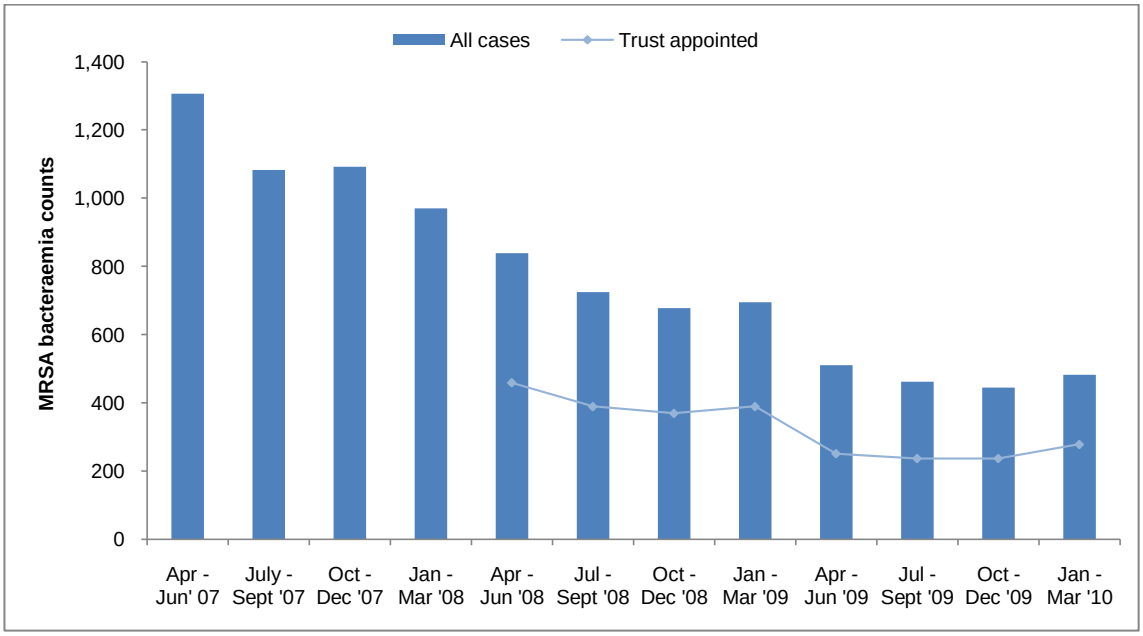

Figure 1.1. Quarterly counts of all reported MRSA bacteraemia cases from all NHS hospitals within the U.K. (April 2007 to March 2010), and trust appointed cases (April 2008 to March 2010), published by the HPA, March 2010*a*.

The majority of microorganisms responsible for CVC-related infections are skin related, in particular CNS such as *Staphylococcus epidermidis*, which is now recognised as a

major nosocomial pathogen and thus is becoming more important in modern medicine (Piette & Verschraegen, 2009).

1.1.4. *Staphylococcus epidermidis* and biofilms

S. epidermidis is an opportunistic pathogen that is thought to account for over 90 % of the normal aerobic cutaneous and mucous membrane microflora, this allows diffusion onto the surface of an implanted device to occur easily during implantation (Cogen *et al*., 2008; Vuong & Otto, 2002). Indeed, this *Staphylococcus* is responsible for more foreign body and device related infections than any other microorganism, therefore is of particular research interest (von Eiff *et al*., 2005; Rohde *et al*., 2010). This is largely due to the ability of some strains to produce biofilms following phenotypic and enzymatic changes, allowing them to become attached, irreversibly onto living or nonliving surfaces, as well as to each other (Cho *et al*., 2002; Curtin & Donlan, 2006; Eftekhar & Speert, 2009).

The biofilm, which is characterised by Saginur *et al*. (2006) as "high concentrations of organisms with little turnover," acts protectively, containing the bacteria making them less susceptible to the effects of antimicrobial agents and host defence mechanisms, resulting in an increase of the organism's pathogenicity (Mack *et al*., 2004; Qin *et al*., 2006). A biofilm, which may also be referred to as slime, is an extracellular matrix composed of many microcolonies, through which water channels pass (Schoenfelder *et al*., 2010). The biofilm matrix causes the embedded microorganisms to demonstrate altered phenotypes with regards to transcription of genes, rate of growth and sensitivity to antimicrobials (Costerton *et al*., 1995). The growth kinetics of a biofilm are controlled by clusters of cells and the growth rate is often slower in bacterial biofilms compared with that of planktonic cells due to the limited availability of nutrients (Kuehn *et al*., 1998; Olson *et al*., 2002). Microorganisms within a biofilm that are slow growing or not growing at all are more likely to display increased resistance to antimicrobial agents by virtue of their inactivity (Galanakos *et al*., 2009).

1.1.5. Biofilm formation

There are a number of phases to the formation of a biofilm on an implanted device. The initial phase involves the primary attachment of the bacteria to the biomaterial after it has been exposed to the surface. Forces including active forces (e.g. chemotaxis), passive forces (e.g. gravity), non-specific forces (e.g. surface hydrophobicity, electrostatic forces and van der Waals' forces) and fluid flow play a role in exposure, and for *S. epidermidis*, attachment is initiated by polysaccharide and protein factors (Liu *et al*., 2008; Schoenfelder *et al*., 2010; Vuong & Otto, 2002). Accumulative growth of multiple layers and the formation of glycocalyx then follow during colonisation of the implant (Mack *et al*., 1999). Finally, the unbound daughter cells drift away from the initial site and repeat the colonisation process at a new site.

The key molecule responsible for biofilm formation in *S. epidermidis* is poly-N-acetyl-glucosamine (PNAG). It is also termed polysaccharide intercellular adhesin (PIA) and is coded for by the *ica* operon; the most comprehended biofilm development instigator (Fitzpatrick *et al*., 2005; Maira-Litran *et al*., 2002; McKenney *et al*., 1998). Extracellular polymers such as polysaccharides, glycoproteins and proteins are also present, with the polysaccharides comprising a mixture of homo- and heteropolysaccharides, mostly of fructose, pyruvate, mannose, galactose and glucose (Costerton *et al*., 1995). The many different bonds that are possible between these saccharides produce a diverse range of polysaccharides (Johansen *et al*., 1997; Olson *et al*., 2002).

The amount of biofilm that is produced by a bacterial strain can vary considerably, with, among others, environmental factors influencing the quantity. This can therefore have an effect on the severity of the resultant infection (Mack *et al*., 2004). Thus, it is important that the complex mechanisms behind biofilm formation continue to be explored and understood, to allow for the possibility of aspects of the process to be targeted in order to prevent their development.

1.1.6. Catheter care and resistance

Catheters, particularly CVCs used to be maintained by twice daily washing with the anticoagulant heparin flushing solution but over time, preference changed to antimicrobial prophylaxis, however this lead to the development of increasing numbers

of resistant strains of microorganisms (Root *et al*., 1988). Antimicrobial lock therapy is sometimes used to remove bacteria suspected of growing inside the catheter by locking high concentrations of antibiotics within the catheter for a period of time while simultaneously delivering systemic antimicrobial agents (Bookstaver *et al*., 2010).

Treatment of *S. epidermidis* biofilm-associated infection is particularly difficult, as bacteria growing in biofilms demonstrate an increased level of resistance to antibiotics when compared to the corresponding planktonic form (Schoenfelder *et al*., 2010). Saginur *et al*. (2006) found that clinical isolates of *S. epidermidis* and *S. aureus* were considerably more resistant to both bacteriostatic and bactericidal antibiotics when in a biofilm than planktonic mode of growth, and that the same was true whether the drugs were used alone or in combination. The exact cause is unclear, although it may be due to a range of factors including high bacterial density, slower growth rate, poor penetration, changes in cell wall composition, altered surface structures, or even that the antibiotic simply binds to the slime and so does not reach the bacterial cells (Rupp & Hamer, 1998; Saginur *et al*., 2006). Whatever the reason, which is likely to be multi-factorial, the degree of increased resistance varies considerably between publications. Wu *et al*. (2003) reported that the resistance was 1,000 to 1,500 times greater in a biofilm-producing strain than a non-biofilm producing strain. Jabra-Rizk *et al*. (2006) stated resistance was enhanced 50 to 500 times, whilst Mah & O'Toole (2001) reported a 10 to 1,000-fold increase. It is therefore obvious to see that antimicrobial agents effective against planktonic bacteria may have limited therapeutic value in patients with an infected prosthetic implant (Saginur *et al*., 2006).

1.1.7. Biofilm treatment

Due to the increasing prevalence of many methicillin-resistant CNS, including some *S. epidermidis* strains, guidelines still recommend the use of vancomycin to treat catheter-related infections (Mermel *et al*., 2009). However, when used to treat *S. epidermidis* growing in a biofilm on a device, it has been unsuccessful in providing complete eradication of the organism (Wiederhold *et al*., 2005). Further to this, Monzon *et al*. (2002) reported that the age of the biofilm is also of significance, with drugs such as vancomycin reducing in efficacy against an aged biofilm of 48 hours, compared with a younger one of 24 hours for example. It is therefore vital that the appropriate drug

treatment be chosen if biofilms are to be eliminated from devices, thus preventing further infection complications.

Antimicrobial agents may be used in combination to combat biofilm infections as they may act synergistically or reduce the risk of the causative organism developing multi-drug resistance, which is an ever-increasing problem. Recent studies performed by Olson *et al*. (2010) demonstrated rifampicin to be efficacious against *S. epidermidis* biofilms, showing significantly reduced cell viability than vancomycin. Furthermore, combining rifampicin with daptomycin or vancomycin could further enhance biofilm removal of *S. epidermidis* due to the ability of the second antimicrobial to eliminate any subpopulation to develop rifampicin resistance.

Susceptibility tests may be carried out to determine which antibiotic is optimal to use against a particular microorganism. However standard susceptibility test values are based on planktonic growth of organisms in suspension and do not include biofilms (Amorena *et al*., 1999; Monzon *et al*., 2001). The minimum inhibitory concentration (MIC) is a measure of a specific concentration of a given chemotherapeutic agent that is required to inhibit growth of bacteria, whilst the minimum bactericidal concentration (MBC) is that required to kill (Olson *et al*., 2002). However, as these measures are for planktonic microorganisms and the antibiotic resistance of individual cells, they do not give true representations of bacteria growing in biofilms (Monzon *et al*., 2001). The MIC and MBC are important indicators for the successful treatment of many life-threatening infections, though treatment is often unsuccessful if employed to device related infections associated with a biofilm-producing microorganism. In recent years, some models have been made available for biofilm antibiotic susceptibility testing, although isolates that can be tested are severely restricted to a few strains of limited microorganisms, including *P. aeruginosa*, *E. coli*, *S. aureus* and *S. epidermidis* (Clutterbuck *et al*., 2007; Monzon *et al*., 2002). At present there is still no standard method for testing biofilm susceptibility to antibiotics.

1.1.8. Novel strategies for biofilm treatment

Many new approaches are being considered for the treatment of biofilm infections resulting from implanted devices. Antimicrobial lock therapy has long been used with heparin, as well as a wide range of antibiotics, in particular vancomycin, however new

combinations of antimicrobials are frequently being tested, and also alcohols such as ethanol (Qu *et al*., 2009). Other suggested strategies include combining the use of antimicrobials with biofilm matrix-cleaving enzymes which are capable of breaking apart polysaccharides, therefore reducing the structural integrity of the biofilm, allowing the antimicrobial to eliminate the infection (Estrela & Abraham, 2010). Also along these lines, is the possibility of using antimicrobials mixed with fatty acid compounds naturally present within biofilm-producing cells, that are responsible for cell signalling chances. The changes insight the reversal of the recipient cell state from biofilm-producing to planktonic, by means of dispersing their slime, and has been effectively demonstrated for a range of microorganisms including *S. aureus*, *E. coli* and *C. albicans* (Estrela & Abraham, 2010).

1.1.9. Prevention rather than cure

Failure to achieve successful treatment due to biofilm resistance against many commonly used antimicrobial agents, often leads to the contaminated medical implant being removed. This often requires an already ill patient to undergo further surgery, which in turn can then lead to further complications (Wu *et al*., 2003). However, if left in place an indwelling device colonised with *S. epidermidis* can lead to chronic infections, with the implant acting as a septic focus, initiating complications such as osteomyelitis, acute sepsis and in the most severe cases, particularly if the patient is immuno-compromised, death can result (Kaplan *et al*., 2004).

Preventing the initial colonisation and growth of biofilms on catheters and other implanted devices is a much more favoured approach to eliminate infection than persevering with a lengthy course of antibiotics, in an often unsuccessful attempt to treat an already existing infection (Polonio *et al*., 2001). It is in this direction that new technological advances are frequently focussed and have been for a number of years.

One recent study conducted into the effect of chlorhexidine-impregnated wound dressings to reduce CRI concluded their use to significantly reduce infection rates amongst chemotherapy patients (Ruschulte *et al*., 2009). Another study by Maki (2010) reported internal biofilm occurrence within needleless connectors being prevented after incorporation of nanoparticle silver into the lining. A similar but older study by Logghe *et al*. (1997) combined chlorhexidine (CHG) and silver-sulfadiazine but found there was

no benefit after 20 days catheterisation. It is therefore possible that the effects were optimal for a maximum of around 10 days, after which time the anti-infective properties reduced. However, when designing anti-infective catheters, the consequences must be well thought out to ensure that whilst inhibiting bacterial growth, they do not permit nor promote fungal growth, such as that of *Candida* spp., which would prove just as problematic (Raad *et al*., 1995). They must also be able to overcome the inhibitory effect of the slime on antimicrobial agents while ensuring resistance does not develop.

Many suggestions of antimicrobial agents that could be used as preventative measures against skin microorganisms responsible for CVC related infections have been made and studied. These include a range of polyantibiotic creams, biocides such as chlorhexidine, as well as many complementary and alternative medicines such as essential oils, which are regarded as mixtures of compounds that produce a flavour or aroma, and are from natural sources such as flowers, spices and aromatic herbs (Crnich & Maki, 2002; Loza-Tavera, 1999; Ruschulte *et al*., 2009).

1.2. Laboratory testing methods

1.2.1. ATP bioluminescence

Adenosine triphosphate (ATP) bioluminescence enables the detection of ATP, the energy source from within viable cells, to be converted into a measurement of fluorescence providing an indicator of cleanliness levels from surfaces. Hand-held kits capable of providing rapid, real-time, reproducible results are widely available, and contain a swab that is wiped over a surface and inserted into the hand-held terminal where the enzyme luciferase catalyses the reactions between luciferin and the cellular ATP (Sykes & Avery, 2009). This reaction produces light which the terminal converts into a numerical value of fluorescence. The fluorescence reading is therefore directly proportional to the ATP concentration. ATP bioluminescence is a well established method for detecting microbial contamination that has been used within the food industry for many years, and has in recent years increased in popularity amongst healthcare settings following an escalation in clinical trials using the method as a means of illustrating baseline and improved hygiene levels (Carling & Bartley, 2010; Whitehead *et al*., 2008). There are however some limitations of these rapid detection kits, including an inability to attribute the source of the ATP to either microbial or other contamination (Moore *et al*., 2010). Also, it generates qualitative data as the readings generated do not equate to a specific number of microorganisms, therefore an initial baseline reading is required prior to any cleaning intervention in order for subsequent readings to provide information regarding improvements made by the intervention (Sherlock *et al*., 2009).

1.2.2. Isothermal calorimetry

Isothermal calorimetry (IC) is a technique that enables the study of the thermodynamics within a liquid. It is performed in an isothermal calorimeter which generates accurate readings, in real time of the heat changes within the system being investigated (O'Neill *et al*., 2003). IC is frequently used for pharmaceutical applications such as aiding development of new drugs by assessing long term stability profiles (Beezer *et al*., 2001). However, applications for microbiological purposes have until now been limited, with only a small number of studies using this technique as a means of establishing the efficacy of agents against microorganisms (von Ah *et al*., 2009; Morgan *et al*., 2001).

Calorimetry is based upon the principle that all processes involve a change in heat to or from their surroundings; it is these temperature changes that the IC monitors and records. Within the calorimeter there are two ampoule chambers, one contains the sample and the other is for reference. Both are surrounded by water set to an appropriately predetermined constant temperature in which the experiment can be facilitated (Jelesarov & Bosshard, 1999). Recordings of exothermic or endothermic heat changes between the ampoules are recorded as the units of power required to maintain the reference ampoule at the same temperature as the sample one while the reactions or processes inside the ampoule occur. As the external environment can be controlled, IC is capable of generating precise results with great sensitivity (Salim & Feig, 2009). Furthermore, the readings are taken every few seconds therefore if connected to graphical software, can be visualised almost instantaneously and attributed to a particular instigating event (O'Neill *et al*., 2003; Trampuz *et al*., 2007).

1.2.3. Toxicity testing

Assessing the potential toxicity risk to humans is an important part of early product development for drugs, medications, inhalants and general skin-contact devices such as impregnated bandages (Collins *et al*., 2008). It is vital to ensure that no excessive, adverse reactions occur at a cellular level to chemical exposure. There are *in vivo* and *in vitro* methods of assessing toxicity risks, as well as more preliminary means whereby predictions are made based upon the properties of known toxicants and their use, or similarity to those used in the test product (Collins *et al*., 2008). *In vitro* assays offer greater benefits over *in vivo* trials, as they are often faster and less expensive to perform, and also eliminate products of especially high toxicity therefore reducing unnecessary testing on animals of products destined to breach regulation limits (Sayes *et al*., 2007). This was supported in 2007 by the United States National Research Council who proposed that toxicity testing should move towards tissue culture techniques and away from whole animal testing (Villeneuve & Garcia-Reyero, 2011). However, careful considerations need to be taken into account during the planning stages of toxicity testing to ensure suitable cell lines, doses and time parameters are used that represent those to be created by clinical use of the final product.

1.3. Antimicrobial antiseptic agents

1.3.1. Alcohols

Alcohols are volatile, flammable organic compounds that consist chemically of a functional hydroxyl group joined to a carbon atom. Whilst there are many types, aliphatic, short chain alcohols such as ethanol (ethyl alcohol), isopropyl alcohol (propan-1-ol) and n-propanol (propan-2-ol) are the most commonly employed alcohols for skin antisepsis (Guthery *et al*., 2005; Kampf & Kramer, 2004; Reichel *et al*., 2009).

The first studies to suggest the use of ethanol as a potential skin disinfectant were published by Furbringer (1888) and explained his investigation of colonies observed in gelatine from physician's hands washed with potash soap and 80 % alcohol. In the 21st century, alcohols are recommended for multiple uses within the clinical environment; alcohol-based hand rubs are advised for general antisepsis, and alcoholic chlorhexidine gluconate solution is encouraged for cutaneous decontamination prior to catheter insertion (Pratt *et al*., 2007).

Alcohol use for skin antisepsis has become more favoured over recent years due to their water soluble properties, reduced skin irritation compared with traditional washing using soaps and lack of microbial resistance reported to date (Kampf & Kramer, 2004; Loffler *et al*., 2007). However, contradictory publications reiterate the knowledge that ethanol acts as a permeation enhancer when applied topically to human skin, therefore may induce irritation and furthermore, may also possess carcinogenic properties (Lachenmeier, 2008). In addition to this, the flammability of alcohols makes them potentially hazardous within operating rooms; it is therefore paramount that any alcohol has completely evaporated from the skin prior to any surgical intervention (Hemani & Lepor, 2009). Weber *et al*. (2006) described one example in which second and third degree burns were suffered to the chest, shoulders and neck of a 62 year old male patient undergoing an awake-tracheostomy after DuraPrep^{TM}, an isopropyl alcohol-based skin antiseptic, was used for preoperative skin preparation. The patient was hirsute, and when electrocautery was used for incision, a fire ignited as a result of the hair impeding the antiseptic drying.

Alcohols are non specific in their mode of action and deliver rapid antimicrobial efficacy against a wide range of bacteria, fungi and enveloped viruses, though are

generally regarded as not possessing any sporicidal activity (Messina *et al*., 2008; Rotter, 2001). They denature proteins and cause membrane damage, which in turn leads to cell lysis (McDonnell & Russell, 1999). The optimal concentration for ethanol efficacy spans 60 % to 95 %, with the range 60 % to 90 % deemed best in general for any other alcohol concentration. At higher concentrations the lack of water makes protein denaturing difficult, however at concentrations below 50 %, significant reductions in efficacy are observed (Bloomfield *et al*., 2007; McDonnell & Russell, 1999).

Of the three main alcohols, the antimicrobial efficacy of ethanol has been concluded to be less than that of isopropyl alcohol, which in turn is less than that exhibited by *n*-propanol (Reichel *et al*., 2009; Rotter, 2001). Determination of which alcohol to use has been shown by Reichel *et al*. (2009) to be the most important factor in achieving optimum skin antisepsis, with the concentration and contact time of application following sequentially. However there are disadvantages to alcohol use for skin antisepsis. Alcohols do not possess prolonged endurance on the skin, therefore if used alone, are unable to deliver continued skin antisepsis over the periods of time required within the hospital or clinical environment. It is therefore, recommended that they are used in combination with preservatives such as chlorhexidine gluconate or iodine, with the result being enhanced longevity of antimicrobial efficacy (Guthery *et al*., 2005).

1.3.2. Povidone-iodine

Povidone-iodine (PVP-I) has been used for skin disinfection for over 50 years, and is a water soluble mixture of iodine and polyvinylpyrrolidone (Chiang *et al*., 2010; Shelanski & Shelanski, 1956). It possesses rapid, broad spectrum antimicrobial activity against bacteria, fungi and viruses by destroying proteins and DNA; however its bactericidal activity is inactivated by the presence of protein-rich biomaterials including blood and serum, as well as inhibited by pus, fat and even glove powder (Hemani & Lepor, 2009; Zamora *et al*., 1985).

Like many antimicrobial agents, PVP-I has not managed to evade reports of disadvantages. Chiang *et al*. (2010) published a case study of a 2 year old female who developed chemical burns following application of PVP-I soaked padding underneath tourniquets applied to her arms, and in place for just 2.5 hours.

There are two common preparations of PVP-I for surgical use, these are 10 % aqueous, and 70 % alcohol; however, whilst rare in occurrence, testimonies of burns have been recorded from both (Lowe *et al*., 2006). Recent comparisons between 10 % aqueous PVP-I and 2 % CHG have demonstrated PVP-I to be inferior to CHG for the disinfection of venipuncture sites prior to blood sample collection, determined by comparisons of culture contamination rates (Suwanpimolkul *et al*., 2008). However, Guzel *et al*. (2009) suggested that pre-surgery skin preparation with CHG followed by PVP-I proved not only safe but also effective enough to potentially become the standard procedure for neurosurgical skin disinfection, as microbial flora were detected before and after CHG cleaning but following a subsequent PVP-I cleanse, no microbial growth was observed.

1.3.3. Chlorhexidine

Chlorhexidine is a found in many disinfectants and antiseptics as the main, active ingredient and is a cationic, chlorophenyl bisbiguanide that has been used for over 30 years (Hibbard *et al*., 2002; Hope & Wilson, 2004). It can bind to skin and mucous membranes with little toxicity and possesses broad spectrum bactericidal activity against Gram-positive and -negative bacteria, as well as yeasts such as *C. albicans*, by causing enzyme inhibition and cell membrane damage which leads to a loss of structural integrity, resulting in irreversible discharge of cytoplasmic components (Hemani & Lepor, 2009; Hope & Wilson, 2004). The mechanism of action of chlorhexidine against fungi is primarily the same as that against bacteria, whereby cell plasma membrane disruption leads rapidly to increased permeation and cell rupture (Suci & Tyler, 2002), and unlike povidone-iodine, chlorhexidine is resistant to inactivation by proteinaceous products (Hemani & Lepor, 2009). The severity of cell damage caused escalates with increases in CHG concentration, with 0.2 % being deemed the most effective (Jones, 1997).

The chlorhexidine molecule (Figure 1.2) consists of a central hydrophobic hexamethylene chain, with a hydrophilic biguanidide group located at either end just prior to a hydrophobic phenyl ring at each terminus (Zeng *et al*., 2009). Chlorhexidine is available in base and salt forms, with chlorhexidine digluconate being a salt derived following dissolution of chlorhexidine into aqueous gluconic acid, which demonstrates

greatly enhanced water solubility properties compared to the base form (Lboutounne *et al*., 2002). The resultant molecule (Figure 1.3) consists of one chlorhexidine and two gluconic acid molecules, generating the chemical formula $C_{12}H_{30}Cl_2N_{10} \cdot 2(C_6H_{12}O_7)$ (Farkas *et al*., 2007).

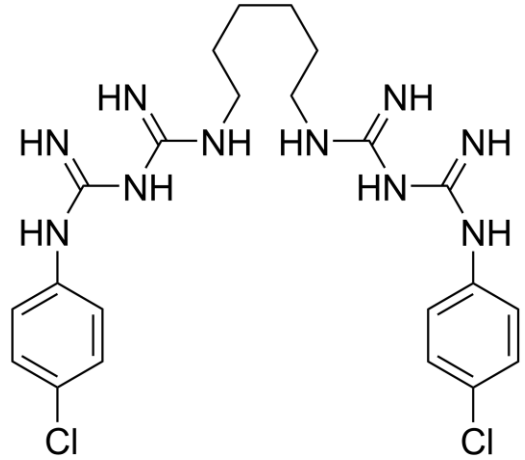

Figure 1.2. Chemical structure of chlorhexidine, with the molecular formula $C_{22}H_{30}Cl_2N_{10}$.

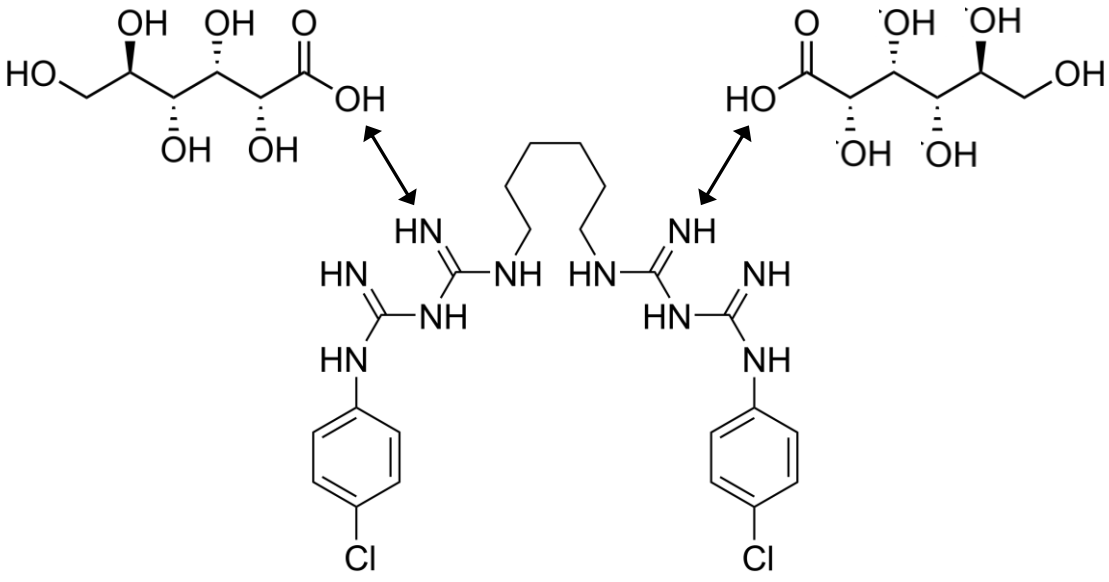

Figure 1.3. Chemical structure of chlorhexidine digluconate illustrating bonding sites between chlorhexidine molecule and two gluconic acid molecules, with the resulting molecular formula $C_{22}H_{30}Cl_2N_{10} \cdot 2C_6H_{32}O_7$.

Chlorhexidine is most commonly used in hand washing solutions, sanitiser gels and mouth washes, but has also been impregnated onto wet-wipes, and even applied to a sponge biopatch to suppress cutaneous flora around catheter insertion sites (Kaiser *et al*., 2009; Timsit *et al*., 2009). A variety of skin antiseptics are currently available to staff, visitors and patients for topical hand and preoperative use, with hospitals often using 0.5 % CHG in 70 % isopropyl alcohol (IPA) on skin (Adams & Elliott, 2007). However, guidelines for the Evidence-Based Practice in Infection Control (EPIC) recommend the use of 2 % CHG in 70 % IPA for skin antisepsis prior to insertion of intravascular devices, as well as for injection port disinfection (Pratt *et al*., 2007).

Soothill *et al*. (2009) reported a significant reduction in the incidence of CRBSI at Great Ormond Street Hospital, from 12 per 1000 catheter days when 70 % isopropanol was used alone for catheter connection antisepsis, to just 3 per 1000 catheter days when the antiseptic was replaced with 2 % CHG in 70 % isopropanol. Furthermore, chlorhexidine has also been shown to elicit superior efficacy when compared with alcohol-based povidone-iodine in a study conducted by Mimoz *et al*. (2007), in which a 50 % reduction in catheter colonisation incidence was observed. These results may be due in part to the residue left by chlorhexidine solutions which may illustrate persistence of efficacy after use therefore continuing to be of antimicrobial benefit to potential contaminants following disinfection.

Although chlorhexidine is an ideal skin antiseptic, its permeation into the deeper layers of the skin is poor (Hibbard *et al*., 2002; Karpanen *et al*., 2008*a*), therefore some cutaneous flora may remain in pores and hair follicles, allowing infection to develop as a result of catheter insertion, even after skin antisepsis has been performed. There is therefore a need for novel agents that can penetrate the deeper layers of the skin or act as carriers to direct antimicrobial agents to these sites.

1.3.4. Essential oils

Plants, herbs and flowers, along with their products have been used medicinally for thousands of years, with initial development and use thought to have originated in China as early as 2700 BC, before subsequently spreading across India, Egypt, the Eastern Mediterranean and finally Europe (Halm, 2008; Jones, 1996). Plant-derived essential oils offer an alternative approach to conventional pharmaceutical strategies of

infection control by means of antibiotic, antifungal and antiviral agents, as many have shown to possess an array of antimicrobial properties. Essential oils such as lemongrass, sage, coriander, peppermint, clove and cinnamon have elicited antimicrobial efficacy against both Gram-positive and -negative bacteria, as well as *Candida* spp., with other oils including lavender and grapefruit efficacious against one or more types of microorganism (Hili *et al*., 1997; Prabuseenivasan *et al*., 2006; Warnke *et al*., 2009).

Nowadays, essential oils are used for an array of functions, from botanical fungicides to mouthwashes, to cosmetic ingredients and food preservatives, while also frequently used for both traditional and modern medicinal purposes to include skin antisepsis and treatments of wounds, aches, sore throats, inflammation, atherosclerosis and even cancer (De Corato *et al*., 2010; Edris, 2007; Eyob *et al*., 2008; Fisher & Phillips, 2008; Kejlova *et al*., 2010). Throughout the literature, much research has been undertaken to explore the antimicrobial aspect of essential oils, helped in part by the seemingly endless combinations possible between the large numbers of microorganisms and vast assortment of essential oils.

Phenolic compounds are responsible for most of the antimicrobial activity of essential oils derived from herbs and spices, whereas secondary metabolites such as tannins, terpenoids, alkaloids and flavonoids possess the antimicrobial properties found in plants (de Sousa Araujo *et al*., 2008; Weerakkody *et al*., 2010). However, the quantities of these antimicrobial compounds within essential oils may vary with season or strain of plant increasing the complexity of generating reproducible batches of oil with the same antimicrobial efficacy (Delaquis *et al*., 2002).

Essential oils used for antimicrobial purposes, in particular spray formulations, are frequently diluted in ethanol, which itself is a known antimicrobial. This often leads to disapproving critique about the true efficacy of the oil as the ethanol enhances volatility. This has been somewhat dispelled by Warnke *et al*. (2009), whom when testing a range of essential oils against a panel of microorganisms, used a 70 % ethanol control, and successfully demonstrated that the oils possessed superior efficacy over the solvent, thus concluding whilst ethanol alone was antimicrobial, the oil accounted for the majority of activity observed. Nonetheless, adjunctive use of essential oils appears to be advocated throughout the literature concerning dermal wound care and healing, however comprehension of the systemic pharmacodynamics and toxicity of such oils is not yet complete. Furthermore, the resistance potential of essential oils is unclear, as is

the mechanism by which oils and their components elicit antimicrobial effects (Halcon & Milkus, 2004; Hili *et al*., 1997; Warnke *et al*., 2009). Therefore, when this is combined with the knowledge that essential oils are volatile, have poor solubility and potentially seasonal variation in efficacy, common use of them in the healthcare profession will require further research to enable limitations in clinical application to be overcome (Delaquis *et al*., 2002; Halcon & Milkus, 2004).

1.3.5. Eucalyptus oil

The Myrtaceae family contains the large genus *Eucalyptus*, of which there are over 700 species (Maciel *et al*., 2010). While *Eucalyptus* surpasses other tree populations within its native Australia, where it dominates landscapes throughout the country, it is now intensively managed on temperate climate plantations in Argentina, Chilli and Portugal, or in sub-tropical climes of Brazil, South Africa and India (Forrester *et al*., 2010).

Eucalyptus oil (EO), a plant-derived essential oil is contained within the leaves of *Eucalyptus* and can be extracted using steam- or hydro-distillation (Batish *et al*., 2008). The oil can account for up to 5 % of the leaf mass and contains in the region 58 constituents (Canhoto & Graca, 1996; Wildy *et al*., 2000). These can vary greatly between species and seasons but the main component is undoubtedly 1,8-cineole which is also known as eucalyptol, and has been reported to account for anything up to 92 % of the oil (Wildy *et al*., 2000). Other components often found in high quantities include camphene, α- and β-pinene, and *p*-cymene (Batish *et al*., 2008; Cimanga *et al*., 2002). Essential oils are volatile, and in the case of eucalyptus oil, this is due to alcohols, ketones, aldehydes, monoterpene hydrocarbons and terpenoids, of which the main constituents are terpinen-4-ol, α-terpineol and 1,8-cineole (Lee & Shibamoto, 2001). Terpenoids are derived from natural products of five-carbon isoprene units, and are divided into groups according to their carbon structures. They are also the most prevalent compounds in most essential oils (Trombetta *et al*., 2005). Terpinen-4-ol belongs to the monoterpenes group of terpenoids as it has ten carbons, and is a terpenoid that has been modified by cyclization reactions and affects bacterial cell walls (Papadopoulos *et al*., 2008; Warnke *et al*., 2009).

Eucalyptus oil is known to possess antibacterial and antifungal properties, however for *Eucalyptus* leaves to be of medicinal use, they must contain a minimum cineole content of 70 % (Goodger & Woodrow, 2008).

The first reports documenting people benefiting from *Eucalyptus* told of Aborigines who had crushed leaves to heal wounds and fungal infections, and made broths with soaked leaves to heal sore throats (Ayepola & Adeniyi 2008; Gilles *et al*., 2010). In modern medicine, eucalyptus oil is often applied directly to the skin as a balm or rub, used in mouthwashes or inhaled as a spray or vapour. It has been, and still is used to treat or relieve symptoms of all manner of complaints including burns, neuralgia, coughs, ringworm, diabetes, asthma, genital herpes, cancer and arthritis (Tovar & Petzel, 2009).

The mechanisms behind the antibacterial properties of EO are not clearly understood, but it is thought that the hydrophobicity of the oil causes the lipids of microbial cell wall, membrane and mitochondria to separate, leading to the structural stability of the cell being damaged, which in turn leads to an increase in membrane fluidity and permeability, and eventually cell rupture (Prabuseenivasan *et al*., 2006). Whilst the antimicrobial properties of eucalyptus oil are still being studied, the efficacy of the eucalyptus fruit oil has yet to be investigated, though it may prove to be a valuable oil as 1,8-cineole is abundant in the fruit oil, which is known to harbour antimicrobial efficacy (Mulyaningsih *et al*., 2010).

1.3.6. 1,8-Cineole

A potent monoterpene, containing only carbon, hydrogen and oxygen atoms, 1,8-cineole is a cyclic ether and a known permeation enhancer (Anjos *et al*., 2007; Williams *et al*., 2006). Acting on the stratum corneum (SC) of the skin, 1,8-cineole temporarily affects both the polar head groups and lipid alkyl tails of the intercellular lipids, causing disruption from an organised state to a less ordered state of packaging, by mechanisms as yet not fully understood, allowing entry into the skin of otherwise impenetrable substances (Aqil *et al*., 2007; Williams *et al*., 2006). Such substances include medicated drugs, with which 1,8-cineole can be combined with an extensive repertoire as it is suitable for coupling with hydrophilic and lipophilic drugs (Aqil *et al*., 2007).

1,8-cineole is found in most plant- and herb-derived essential oils and is a major component within rosemary, lemon, lavender, grapefruit and peppermint oils (Yang *et al*., 2010). Antimicrobial studies of 1,8-cineole have confirmed broad spectrum efficacy against Gram-positive and -negative bacteria, as well as *C. albicans* when grown in planktonic cultures, furthermore efficacy was maintained when challenged with cultures in biofilm modes of growth (Hendry *et al*., 2009).

The physiochemical properties of different types of drugs needs to be considered when pairing with terpenes for transdermal drug delivery, as this can alter the permeation enhancing effects (Kang *et al*., 2007). The boiling point of 1,8-cineole is 173 °C therefore it is in a liquid phase at room temperature rather than solid, and it is a monoterpene (Figure 1.4), meaning it contains two isoprene units rather than a tetri- or tetra-terpene which contain three and four respectively. These particular physiochemical factors were determined by Kang *et al*. (2007) as likely to be possessed by terpenes with greater permeability coefficients.

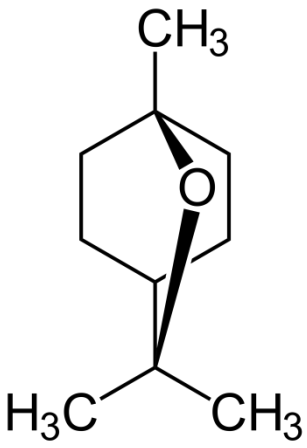

Figure 1.4. Chemical structure of the monoterpene 1,8-cineole, with the molecular formula $C_{10}H_{18}O$.

Registered by the Food and Drug Administration (FDA) on the Generally Recognised As Safe (GRAS) list, many terpenes such as 1,8-cineole, are regarded as eliciting low levels of cutaneous irritation, with no skin toxicity, and have been permitted for use in cosmetics and hair colorants for many years (Aburjai & Natsheh, 2003; Aqil *et al*., 2007). In particular, 1,8-cineole is reported to alter the side chain rather than the backbone of the SC proteins, which prevents conformational changes of these proteins

resulting in the observation of such low irritancy levels (Anjos *et al*., 2007). This makes them suitable for all manner of potential uses within the healthcare system related to enhanced skin permeation and antimicrobial efficacy.

Koalas (*Phascolarctos cinereus*) and greater gliders (*Petauroides volans*) consume *Eucalyptus* leaves almost exclusively, with rare additions to their diet including the occasional consumption of tea tree or paperbark (Lunney *et al*., 2004). *Eucalyptus* leaves have shown to possess large amounts of 1,8-cineole, a substance that in such consistently high concentrations would be particularly toxic to humans if ingested in similar quantities to that by the koala, this has naturally raised questions about the toxicity threat posed to eucalypt folivores. Studies by Boyle *et al*. (2001) ascertained that a highly specific metabolic pathway, resulting in detoxification by oxidation on carbons 7 and 9, eliminates ingested 1,8-cineole, with 7-hydroxy-9-cineolic acid the main metabolite produced from a total of seven recovered in excrement. Further to this, the antimicrobial effects of 1,8-cineole do not appear to pose problems for the koala either, with microbial flora abundant within the gut and colon, and tannin-protein complex degrading bacteria particularly prevalent on the cecum and proximal colon epithelial surface (Stevens & Hume, 1998).

1.4. Human skin

1.4.1. Properties of skin

The skin is the largest organ in the human body, covering an area of around 2 m^2 and accounting for around 4 % of the total bodies weight (Cevc & Vierl, 2010). It contains many specialised cells and creates a physical barrier between the external environment and the inside of the body, acting as the first line of defence against chemical and physical damage, dehydration, heat loss, and infection. However, breach of this protective barrier function can leave the body exposed, and in particular vulnerable to microbial attack (Cevc & Vierl, 2010). The structure and physiology of the skin varies from person to person, with ethnicity, age and gender all affecting hair growth, hormone metabolism, sweat rate and surface pH, which is usually just above pH 5 (Cevc & Vierl, 2010; Giacomoni *et al*., 2009).

The skin harbours many resident microorganisms, including *Staphylococcus* spp., *Corynebacterium* spp., *Pseudomonas* spp., *Streptococcus* spp. and *Propionibacterium* spp. (Cogen *et al*., 2008). The normal microbial density differs greatly with body site, and can range from 10^2 microorganisms per cm^2 on the forearm where it is dry, to 10^5 per cm^2 in the moist underarms, and can even reach as high as 10^7 per cm^2 on areas of the skin that are covered, such as under surgical patches (Cevc & Vierl, 2010). The surface of the skin contains many niches ideal for microorganisms including pores, hair follicles and sweat glands, all of which provide suitable environments of pH level, temperature and moisture content for microbial survival (Grice *et al*., 2008).

1.4.2. Structure and layers

There are three main layers that make up the full thickness of human skin; the SC, the epidermis and the dermis. The SC is the outermost layer of the skin and varies between 5 and 20 µm in thickness. It is the first defence barrier to drug absorption and penetration, and is comprised of a multilamellar lipid domain, embedded into which are corneocytes; dead cells filled with keratin (Anjos *et al*., 2007). These overlapping dead cells provide the waterproofing of the skin, whilst adding durability (Cevc & Vierl, 2010).

The epidermis is the second layer of the skin and is around 2 mm in thickness (Cevc & Vierl, 2010). It is comprised of several sub-layers including the uppermost granular cell layer, the spinous cell layer in the centre, and the basal layer at the bottom. It is this basal layer that is responsible for replenishment of skin surface cells, as the basal cells undergo stages of differentiation during progressive movement up through the layers towards the SC, ultimately replacing the dead SC cells that have been shed (Blanpain & Fuchs, 2009). As the cells of the basal layer proliferate, some detach and begin to rise. Undergoing changes, they synthesise new keratins; the main structural protein of the epidermis. The keratins assemble together, forming intermediate filaments which link to form the cytoskeleton. The keratinocytes responsible for synthesising keratin also produce cytokines, which are triggered by injury and are important in inflammation, repair and growth (Powell, 2006). Cell to cell joining creates spiny desmosomes as the cells move up through the spinous layer. Finally, enzymes present within lysosomes of the granular cell layer, instigate cell death by destroying organelles including the nucleus. The squames that remain are the terminal differentiation of the cells and are subsequently shed once they have reached the surface of the SC (Alonso & Fuchs, 2003). The entire process from basal layer to SC shedding takes around 28 days (Powell, 2006).

The deepest layer of the skin is the dermis which is 2 to 4 mm thick and is separated from the epidermis by the epidermal-dermal junction or basement membrane, which is a convoluted layer of interconnected proteins, separating the two distinct cellular compartments and providing integrity whilst allowing nutrients to pass through from the dermis (Myung & Marinkovich, 2010). The dermis consists of two sub-layers, the papillary on the top and the reticular dermis below which contain mainly collagen (Cevc & Vierl, 2010). Vascular, lymphatic and nervous systems reside in the dermis, providing plentiful supplies of blood and nutrients to glands and hair follicles, as well as drainage and sensory provisions (Powell, 2006).

Below the dermis is the panniculus adiposus known more commonly as the subcutaneous layer, and comprises largely of fat cells and loose connective tissue (Zolfaghari *et al*., 2009). Together, the dermis and subcutaneous fat provide insulation and protective cushioning (Powell, 2006).

1.4.3. Penetration into the skin

Effective skin antisepsis is required prior to many medical procedures in order to effectively prevent infection, and applies to both patients and clinicians. Current EPIC guidelines recommend the use of 2 % CHG in 70 % IPA for adequate skin antisepsis (Pratt *et al*., 2007), however more concentrated solutions are often used for surgical scrubs. Present antiseptics are effective at removing microorganisms from the surface of skin, with some even leaving residual activity preventing recolonisation over a course of hours. However, it is known that microbial skin flora reside in pores and hair follicles, and therefore pose a great threat to skin antisepsis as many products used regularly within the clinical setting are inadequate at reaching these deeper layers. One such example is CHG, which whilst demonstrating broad spectrum antimicrobial activity, has shown poor penetration into the deeper layers of human skin, therefore allowing microbial survival despite completion of antiseptic practices (Karpanen *et al*., 2008*a*). In surgical theatres, additional measures including scrubbing with nail brushes used to be employed routinely with the aim of reducing microbial skin colonisation. However a study by Tanner *et al*. (2009) concluded the use of nail brushes or picks in combination with chlorhexidine caused no significant reduction in the number of colony forming units residing on hands 1 hour post procedure, compared with using chlorhexidine alone. Furthermore, the results suggested that CHG alone may actually provide better skin decontamination due to the potential of brushes and picks to draw out additional microorganisms from within the skin. Despite this, antiseptics capable of providing complete skin antisepsis, both on the surface and within the deeper layers of the skin, are still desired.

Use of penetration enhancers is the most commonly employed technique for increasing percutaneous absorption, and is a suitable means of delivering antimicrobial agents into the deeper layers of skin, as well as administering transdermal medication. Such enhancers cause temporary and reversible disruption of the intercellular lipids of the SC barrier, as well as tight junctions and keratin domains thus diminishing the barrier properties of the SC (Sapra *et al*., 2008). Topical delivery of drugs that are not ordinarily skin-permeable confers numerous advantages, including avoidance of premature metabolism, low risk of systemic side effects and ease of use (Ghafourian *et al*., 2004). Furthermore, they are non-invasive and often able to release slowly over time periods of up to a week (Prausnitz & Langer, 2008). Often these drugs are packaged as

creams and lotions; however as the process and kinetics of applying medication to a coating that will later allow for release is becoming more understood, use of transdermal patches for delivery of various drugs is becoming used more widespread.

1.4.4. Transdermal patches

The first transdermal patch was approved in America in 1979, for the systemic delivery of the motion sickness drug scopolamine. This was followed ten years later by the development of the much publicised, nicotine patches for smokers, which contain permeation enhancers to aid dermal passage (Prausnitz & Langer, 2008). Between 2002 and 2007, it was estimated that every 7.5 months, authorisation was given for a new transdermal delivery patch, with recent estimates in the region of over a billion patches manufactured each year for transdermal delivery, many of which contain one or more permeation enhancers to aid delivery through the skin (Prausnitz & Langer, 2008).

Transdermal patches are used frequently for the delivery of hormones including; estrogen to relieve symptoms of menopause, parathyroid and human growth hormones to maintain endocrine regulation, and testosterone for deficiency treatment (Kumar & Philip, 2007). Combinations of hormones are also used, though largely for contraceptive purposes (Jick *et al*., 2007). Along with hormones, transdermal patches have proved especially successful for delivery, via the skin, of pain relief as they can be used in replacement of intravenous or oral medication therefore removing dosing confusion, replacing the use of needles and evading metabolism by the liver (Ghafourian *et al*., 2004). First synthesised in 1960, fentanyl is a synthetic opioid analgesic that has, since the early 1990s, been incorporated into transdermal patches for the treatment of moderate to severe pain (Stanley, 1992). However, concerns about misuse of the fentanyl from within the reservoir patch led to the removal the original Durogesic® patch from the market, before alternative delivery mechanisms using matrices were developed (Kress *et al*., 2010). Finally, a number of transdermal patches have been developed incorporating nitroglycerin, and methylphenidate also known as Ritalin, for the prophylactic treatment of angina pectoris, and control of attention deficit hyperactivity disorder (ADHD) respectively (Keane, 2008; Wei *et al*., 2011).

There have been many chemicals deemed suitable for use as permeation enhancers, ranging from alcohols to fatty acids and components of essential oils such as the terpene

1,8-cineole (Williams *et al*., 2006). 1,8-cineole has been used in many skin permeation studies involving both hydrophilic and lipophilic drugs, as it causes disruption to the stratum corneum allowing penetration, as well as other studies involving antimicrobial drugs although to a lesser extent, however as yet 1,8-cineole appears to have not been used in commercial transdermal patches (Liu *et al*., 2011).

1.5. Hand hygiene

1.5.1. Purpose and compliance

Another important component in infection control and reduction of HAI is effective hand hygiene. From childhood, hand hygiene becomes instilled in the majority of us, as part of daily life; however the multitudes of recent clinical studies highlighting the lack of compliance would imply this basic task is not encouraged to the same extent in all upbringings (Nicol *et al*., 2009). The knowledge that hand hygiene can have immense effects on the likelihood of infection prevalence and spread is comprehensive, particularly within the healthcare system, yet noncompliance remains a persistent issue. One study conducted within a neonatal unit of a Children's Hospital in Geneva, showed incidence of clonally related bacteraemia in very low birth weight babies was reduced by 60 % with compliance to a hand hygiene promotion, reinforcing the idea that hand washing breaks the cycle of transmission between patients (Pessoa-Silva *et al*., 2007). Despite this, adherence to hand hygiene protocols reveals inconsistencies between nursing staff, with one study by Burnett (2009) citing complexities in human behaviour as an explanation for nurse appreciation of infection dissemination.

1.5.2. Campaigns for clean practice

Over recent years, many local and national initiatives have been introduced in a bid to improve good hand hygiene practice within the National Health Service (NHS), including the 'Clean Your Hands' campaign in 2004 which saw the introduction of alcohol-based hand rubs to patient bedsides and ward entrances; an undertaking costing £2.5 million, subsequently independently deemed by the National Audit Office (NAO) to have been a success (National Audit Office, 2009). In 2007, Scotland began the 'Germs, Wash Your Hands of Them' campaign, at the same time as Wales commenced the '1000 Lives Campaign' which has since been followed up by '1000 Lives Plus' launched in May this year (Health Protection Scotland, 2007; Jewell & Wilkinson, 2008). Unfortunately, compliance with hand hygiene regimes often drops shortly after the trial or incentive ceases; this may explain the reasoning behind the slightly different approach taken by the World Health Organisation (WHO). In 2006, WHO orchestrated one of the largest campaigns called 'My Five Moments for Hand Hygiene' throughout all healthcare buildings, not just hospitals (Sax *et al*., 2007; World Health Organisation,

2009). This improvement strategy emphasises five fundamental but crucial instances when healthcare workers need to clean their hands and is designed to be used in combination with current infection control programmes. It appears to be aiming ultimately, to transform the daily practice and habits of healthcare workers, as well as provide education to explain why hand washing is required before or after a particular task, rather than simply asking them, as so many previously promoted studies have done before, to increase their hand washing frequency. This combination approach has been used in other, similar studies where it was concluded to be more effective than campaigns which rely solely upon increasing awareness (Forrester *et al*., 2010). The five moments when hands should be cleaned are defined by WHO (2009) as:

- Before touching a patient
- Before aseptic procedures
- After exposure to bodily fluids
- After touching a patient
- After touching patient surroundings

1.5.3. Products and agents

There are many different skin antiseptics available to staff, visitors and patients for use on hands, with the most commonly seen within hospitals comprising alcoholic hand rubs, often in a gel formulation. These agents are largely accepted as easier to use than soap washes, and are often more easily accessible than sinks and soap, as they can be easily located near beds, patients and doors, or even tied individually to nursing staff uniforms by elasticated cords attached to portable bottles (Goroncy-Bermes *et al*., 2010; Tavolacci *et al*., 2006). Whilst this seems to suggest alcoholic hand rubs are a perfect substitution for traditional soap washing, there are many factors that can reduce the efficacy of this practice, such as wearing jewellery, having long nails and using a reduced volume, which have demonstrated significant negative impacts on the effectiveness of hand rubbing (Hautemaniere *et al*., 2010). Furthermore, the first reports of burns from alcoholic gels have started to appear in the literature. One reported an unfortunate incident where a care worker suffered burns to her hands after a fire ball

was generated following her igniting a cigarette lighter she had just cleaned with alcoholic hand gel (O'Leary & Price, 2010). However, another reported a burn obtained through less careless actions, whereby a nurse burnt her hand when static electricity from an electronic call system sparked with the alcohol gel she had just applied as she answered a call (Davis, 2008).

1.5.4. Is hand hygiene alone sufficient to control infection transmission?

Patient to patient transmission of infections has been shown in countless studies to be dramatically reduced with improved hand hygiene. However, one opinion that is becoming more widely acknowledged is that hand hygiene alone is not enough, as people, put simply, are dirty. Judah *et al*. (2010) concluded that amongst five U.K. cities, a total of 28 % of commuters had faecal-origin microorganisms on their hands. The incidence rate observed increased the further north the city, with the highest rates derived from Newcastle males, followed by males then females from Liverpool. Within the clinical environment, inadequate hand hygiene may be further exacerbated by the use of gloves. One study by Girou *et al*. (2004) focussed on the potential cross infection risks posed by improper glove use. They reported that nearly 65 % of all contacts between the gloved hand of a staff member and a patient were subsequently followed by further contact elsewhere before the glove was removed. This equated to 18.3 % of all contacts requiring aseptic handling potentially inducing microbial transfer.

Despite continued efforts by individuals, healthcare trusts and governing bodies to encourage, bribe or even force staff to perform adequate hand hygiene procedures, HAIs remain a persistent concern. This is likely to be, as argued so comprehensively by Dancer (2008), that hand hygiene is not enough if not in conjunction with surface cleaning and disinfection, and that no matter how clean hands are in practice, if surfaces are contaminated, it is only a matter of time before transition to a patient occurs; thus highlighting the importance of effective surface cleaning and disinfection practices.

1.6. Surface disinfection

1.6.1. Reasons and rationale

Hospitals and other healthcare premises provide copious sites for microbial survival and multiplication. The spillage of bodily secretions and excretions onto healthcare surfaces instantly introduces microbial contamination, and furthermore then acts as a reservoir for potential pathogens; a problem that is further embellished by corners and crevices in which microorganisms can reside undisturbed (French *et al*., 2004; Rebmann *et al*., 2009). At present, there is no standard method for measuring cleaning efficacy, and with microbial transfer thought to occur largely as a result of hand transmission, most efforts are focussed on improving hand hygiene rather than surface cleaning, which also provides the added benefit of being financially preferable (Dancer, 2004).

Surfaces within the food industry must comply with strict regulations with regards to hygiene levels, however there is no such standard for hospital surfaces to suggest what may be an acceptable level of microbial presence. Dancer (2004) proposed that a total aerobic count of less than 5 colony forming units (cfu) per cm^2 would be acceptable for hospital surfaces commonly touched by hands, and that a count of less than 1 cfu/cm^2 should be adopted for indicator organisms; that is those which pose a potential infection control risk to patients and included MRSA and *Clostridium difficile*. Despite such suggestions, there are to date still no legislative regulations concerning appropriate surface contamination levels, only guidelines to suggest that suitable monitoring should be upheld to maintain a sanitary environment (Siegel *et al*., 2007).

On average, a patient is 73 % more likely to contract an infection from MRSA, *C. difficile*, vancomycin-resistant Enterococcus (VRE) or *Acinetobacter baumannii* if the previous patient to occupy that room was infected, compared with patients of another room (Carling & Bartley, 2010). This emphasises the need for satisfactory surface cleaning and disinfection in order to reduce the risk, something that the government has tried to address in recent years.

1.6.2. National incentives

In September 2007, the government launched ‘The Deep Clean Programme’. Costing £57 million, the programme was aimed at encouraging hospitals to improve the

experiences of patients, by assessing the present environment to ascertain areas in need of a deep clean or restoration (Department of Health, 2008*b*). Completion of the deep clean would then give trusts a fresh starting point, upon which future cleaning protocols, having been amended and updated, could be more easily upheld and therefore effective. Subsequent surveys frequently reported improved patient perceptions of cleanliness, whilst many infection rates were shown to decrease, no doubt due in part to the completion of refurbishment works to floors, walls and window frames, as well replacement purchases of items such as commodes, basins and bedside lockers (Department of Health, 2008*b*). Upon cessation of 'The Deep Clean Programme', it was hoped that trusts would continue to monitor and improve their environment and cleaning practices in a bid to limit potential occurrence of infection outbreaks.

1.6.3. Disinfection and sterilisation products

There are many products available to hospitals within the U.K. for cleaning purposes however there is no standardisation in which product to use. The choice of surface cleaning or disinfection product to use is often a decision left to individual wards, or the cleaning company contracted to the hospital. Agents are available in a range of forms, to include both concentrated and pre-diluted solutions, as well as in tablet form requiring dilution. These products are often focussed towards one of the two main cleaning purposes; disinfection or sterilisation. Disinfection refers to microbial removal of pathogenic organisms from objects and hard surfaces; it does not however include spore removal. Conversely, sterilisation eradicates all microbial contamination, including bacterial spores and is often facilitated by physical or chemical means including pressurised steam or gaseous hydrogen peroxide (Rutala *et al*., 2008). Products relating mainly to disinfection will be focussed on here, though this is by no means an exhaustive list.

1.6.4. Alcohols and iodophors

Alcohols such as ethyl alcohol and isopropyl alcohol and iodophors such as povidone iodine (all discussed previously) are rapidly bactericidal against Gram-positive and -negative bacteria, as well as possessing fungicidal and virucidal activity. Alcohols and iodophors are used for surface disinfection within the clinical environment, however

this is seldom the case due to preference and suitability lending better to skin antisepsis. Furthermore, inactivation of iodine solutions by proteinaceous substances and limited surface material suitability e.g. incompatibility with fabrics, limits potential uses, therefore they will not be discussed further here.

1.6.5. Chlorine

Chlorine-based products possess antimicrobial activity against bacteria, fungi, viruses, mycobacteria and spores, however they are inactivated by the presence of organic matter (Rutala *et al*., 2008; Wheeldon *et al*., 2008). The most common chlorine-based disinfectants used within the healthcare profession are hypochlorites, with sodium formulations remaining suitable for use up to a month after preparation, unlike aqueous hypochlorites which deteriorate over such time periods. Their exact mode of action has not yet been fully clarified, though it is thought to be instigated by denatured proteins disrupting metabolic processes, as a result of multiple sites being targeted; this may explain the lack of resistance observed towards biocides (Fraise, 1999; McDonnell & Russell, 1999; Rutala *et al*., 2008). Hypochlorite disinfectants are used on furniture, patient equipment and surfaces to include bed frames, basins, drip stands, shelves and curtain rails, whilst general chlorine-based disinfectants are frequently used for damp-mopping floors (National Patient Safety Agency, 2009). However they are not pleasant to use as the smell may be thought of as noxious and if inappropriately diluted can lead to respiratory irritation.

1.6.6. Aldehydes

Formaldehyde and glutaraldehyde are the main aldehydes used within the health service; however despite its wide range of efficacy, formaldehyde is now only used for disinfection of high risk areas due to toxicity regulations (Fraise, 1999). Glutaraldehydes can be acidic or alkaline and are particularly useful within the healthcare setting due to their biocidal properties, even in the presence of a soil load (Rutala *et al*., 2008). They cause deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and protein synthesis to be changed providing efficacy against bacteria, fungi, mycobacteria, viruses and spores, however alkalising agents can be added to aqueous, acidic glutaraldehyde to activate the solution and convert it to an alkaline solution,

which then possess sporicidal activity (Rutala *et al*., 2008). Glutaraldehyde is often used for equipment disinfection such as endoscopes as it does not cause corrosion to metal, or damage to plastic or rubber. However, glutaraldehyde is toxic and a known irritant, and contamination on enemas or equipment used for endoscopy has in some instances induced chemical colitis; presenting with rectal bleeding and abdominal pain, and treated generally by supportive care alone (Ahishali *et al*., 2009). For this reason, orthophthalaldehyde and hydrogen peroxide are increasingly replacing the use of glutaraldehyde within the health service, yet knowledge concerning the potential of these agents to also induce sensitisation, remains limited (Rideout *et al*., 2005).

1.6.7. Quaternary ammonium compounds

Quaternary ammonium compounds (QUATS) were discovered in 1856, with the first germicidal properties reported in 1916; they were then introduced to the medical field in 1933 for use in clinical practice (Russell, 2004; Russell 2002). They are one of the most important biocides used in hospital disinfection and are efficacious through cytoplasmic membrane targeting (Sutterlin *et al*., 2008). The pH at which QUATS are used is known to affect their efficacy, with optimum activity in alkaline rather than acidic pH (Russell, 2004). QUATS are not sporicidal however are active against bacteria, enveloped viruses and fungi and are often used for floor cleaning, though it is recommended that they are used only on the day of reconstitution and not reused subsequently (Beilenhoff *et al*., 2008; Rutala *et al*., 2008).

1.6.8. Chlorhexidine

Chlorhexidine (as discussed previously) possesses broad antimicrobial efficacy and therefore is often incorporated into disinfectant products. However, such products are frequently intended for the disinfection of device and equipment surfaces including dental implants (Kandaswamy *et al*., 2010). Furthermore, in recent years, chlorhexidine has been used largely for skin antisepsis rather than disinfection of hospital floors and hard surfaces therefore will not be discussed further here.

1.7. Care bundles

High impact interventions (HII) were introduced into the NHS in June 2005 under the 'Saving Lives Campaign', and comprise bundles of care, intended for use by all staff within the clinical setting. Bundles are structured care packages, designed to improve the treatment process and final patient outcomes from the cumulative effect of multiple evidence-based improvement interventions, with significant reduction in HAI the ultimate goal (Department of Health, 2006). There are many different bundles to cover completion of specific procedures such as central- or peripheral venous catheter insertion, upkeep and maintenance of surgical sites, and preventative measures for infections such as ventilator-associated pneumonia (VAP) and CRBSI (Bonello *et al*., 2008). Introduction of care bundles has been shown to reduce hospital mortality rates. Robb *et al*. (2010) reported a significant reduction in the number of deaths following an intervention which included the implementation of eight care bundles, specifically targeted to diagnoses accounting for the greatest mortality rates in a North West London, acute hospital trust. Furthermore, Guerin *et al*., (2010) made the discovery that in a trust where care bundles were used for central venous catheter insertion with a high rate of conformity, a significant reduction in CRBSI could still be achieved with the introduction of post insertion care bundle. Measurement of bundle compliance is by way of 'all or none' meaning that all aspects of the bundle need completion for recognition of accomplishment; failure to fulfil even one aspect condemns the bundle to failure (Vogeli *et al*., 2009).

1.8. New ideas

1.8.1. Antibiotic approaches

In order to continue reducing HAIs, new strategies for infection control are constantly being considered, including novel antimicrobial agents, cleaning products and methods. Modification of antibiotics already in existence has proved a useful means of generating more drugs, in particular, semi-synthetic ones capable of targeting microorganisms demonstrating resistance to the original, unmodified form; one such example is the conversion of tetracycline to the semi-synthetic tigecycline (Livermore, 2005). Furthermore, because many antibiotics are produced as secondary metabolites by microorganisms, genetic manipulation of the actual organism has lead to the development of altered antibiotics, boosting the total number of drug varieties available (Walsh & Fischbach, 2009). Finally, there are still many undiscovered and untested microorganisms, especially soil microbes that may also produce metabolites of potential antimicrobial use.

1.8.2. Targeting surfaces

New cleaning products, solutions and chemicals are continually being brought to market, each claiming to kill more microorganisms and last longer than the competitors. However, one method of improving cleaning practice within the healthcare setting is based on feedback rather than products and monitors cleaning efficacy using ATP bioluminescence. Surfaces can be swabbed and a numerical value for ATP obtained almost instantly following insertion of the swab into a hand-held terminal containing reagents that react with any ATP present. There are however, a number of limitations to this method as ATP is present not only in microorganisms, but also in bodily fluids and food therefore high readings may not necessarily indicate microbial contamination due to the inability to distinguish the source (Moore *et al*., 2010). Furthermore, readings need to be taken in context as there is no 'cut-off' point at which a surface would be deemed acceptably clean or not, therefore readings need to be monitored over time to establish baselines, often meaning ATP bioluminescence is used as a means of highlighting how changes to disinfection regime can affect cleanliness levels. This method is often employed in the food industry and rarely used within the clinical environment, in part due to the limitations, however the 3M™ Clean-Trace™ Clinical

Hygiene Monitoring System has recently been approved for use in the clinical setting by the rapid review panel (Department of Health and NHS Purchasing and Supply Agency, 2009*a*). Advanced surface development is another means of potentially reducing HAI by preventing viable cells surviving on surfaces they come into contact with. Many different antimicrobials have been incorporated into surfaces including zinc, copper, silver, biocides and antibiotics however resolutions that can withstand cleaning protocols and general wear and tear have proved harder to find; furthermore any potential product must be cost effective if it is to replace existing surfaces within the healthcare setting. One example of a disinfectant surface covering is photocatalytic coatings, which are also known as self cleaning and consist of a photo-excited semiconductor, frequently titanium dioxide. This semiconductor causes redox reactions which create reactive oxygen species (ROS), in turn these attack structural cellular proteins and result in cell death (Dunlop *et al*., 2010). Due to the mechanism of action, development of microbial resistance to photocatalytic coatings is thought to be unlikely, providing additional benefits to a system that already shows clinical potential from favourable efficacy results against an array of microorganisms, to include biofilm cultures (Dunlop *et al*., 2010; Goulhen-Chollet *et al*., 2009).

1.8.3. Using metal

Silver and copper have long been known to possess antimicrobial properties, and both have been, and continue to be, exploited for use within the clinical setting in a variety of properties. Huang *et al*. (2008) reported that addition of copper or silver ions to water demonstrated antimicrobial efficacy against a number of organisms known to cause HAI such as *P. aeruginosa*, and that moreover, if added together copper and silver ions generated synergistic results, however testing against biofilm cultures was not undertaken. Silver dressings for application to wound sites are available from many companies including world leaders such as Johnson & Johnson and Smith & Nephew. The silver can be easily impregnated into films or layered between sheets providing a sustained release of particles which eradicate wound microorganisms, however residual efficacy is limited and presence of organic matter can greatly reduce efficacy (Atiyeh *et al*., 2007). Other silver products include creams, solutions and even silver-impregnated fabrics such as pyjamas and bed sheets (O'Hanlon & Enright, 2009). Copper products have also been trialled. Casey *et al*. (2010) replaced a number of high touch surfaces

including door push plates, tap handles and toilet seats within an acute medical ward with copper-containing substitutes. Throughout the trial comparisons were made between the microbial counts on the newly installed copper-containing surfaces and those on non-copper-containing controls with significant differences being found on tap handles and push plates indicating that the use of copper surfaces may aid infection control within the healthcare setting (Casey *et al*., 2010). One recent study investigated the use of a copper-based biocide applied to microfiber cloths and mops and showed a total viable count reduction of 56 % on surfaces, compared with a 30 % reduction by the water control. Furthermore, they observed residual activity of the copper-based biocide for up to two weeks (Hamilton *et al*., 2010). Titanium dioxide treated polyester fabrics have also shown to be antimicrobial and of potential use within the clinical setting to aid infection control (Dastjerdi *et al*., 2010).

1.8.4. Hospital design

Air, water, surfaces and equipment can potentially harbour many microorganisms and are therefore capable of acting as reservoirs, introducing microorganism into the healthcare setting and inducing transmission of infections. The possibility of microbial spread is now an aspect that is taken into account during the designing of modern hospitals. Sinks within each patients' room, additional to those in the en-suite, is one of the most commonly introduced features to newly designed facilities (Bartley *et al*., 2010). Along with this, more single-patient rooms are often incorporated into the design. Ventilation and air flow considerations, including the installation of high efficiency particulate air (HEPA) filters and appropriate direction of air into, around and out of wards and rooms to minimise infection spread of microbial spores in particular, are also made during the design stage (Qian *et al*., 2010). Small details must also be taken into account when planning hospital layout with HAI minimisation in mind, including locations for placement of alcoholic hand rubs, designated areas for dirty linen and waste bins, flooring and surface material that allows for easy cleaning and even hinge direction on doors so they can be pushed rather than pulled if hands are contaminated (Stockley *et al*., 2006). Design features that allow for changes to occur throughout the lifetime of the hospital are beneficial, for example more single rooms will provide flexibility should an outbreak scenario occur as they can be easily transformed into isolation units (Lateef, 2009).

1.9. Aims of thesis

The aims of this thesis were to:

- Determine the antimicrobial efficacy of eucalyptus oil and 1,8-cineole both alone and in combination with chlorhexidine, against a panel of clinically relevant microorganisms grown in planktonic and biofilm cultures. The outcome from this part of the research would be used to determine the most efficacious agent with which subsequent studies of this thesis would be undertaken.

- Investigate the antimicrobial efficacy of 10 % (v/v) eucalyptus oil-containing wipes for hard surface disinfection against a panel of microorganisms grown in planktonic and biofilm cultures. Time kill studies, microbial removal from surfaces, and potential of wipes to induce cross contamination would be assessed.

- Study of the antimicrobial efficacy of novel hard surface disinfectant wipes comprising either 5 % or 2 % (v/v) eucalyptus oil with 2 % (v/v) chlorhexidine digluconate and 70 % (v/v) isopropyl alcohol designated Euclean® wipes, against a panel of microorganisms grown in planktonic and biofilm cultures. Time kill studies, microbial removal from surfaces and potential of wipes to induce cross contamination would be assessed.

- Explore the potential of isothermal calorimetry as an alternative to standard laboratory culture methods for assessment of antimicrobial efficacy of Euclean® hard surface disinfectant wipes against a panel of clinically relevant microorganisms.

- Assess the opinions of NHS staff at Birmingham Children's Hospital, Birmingham, U.K., towards current cleaning products and Euclean® hard surface disinfectant wipes by means of a clinical audit and questionnaire.

- Develop a prototype transdermal biopatch containing 5 % (v/v) eucalyptus oil and 2 % (v/v) chlorhexidine digluconate, then assess its antimicrobial efficacy against a panel of clinically relevant microorganisms, release and skin penetration of the antimicrobial agents using Franz diffusion cells, high performance liquid chromatography (HPLC) and gas chromatography (GC), and establish the associated potential toxicity using tissue culture techniques.

Chapter 2

Antimicrobial Efficacy of Eucalyptus Oil, 1,8-Cineole and Chlorhexidine Digluconate, Alone and in Combination against a Panel of Microorganisms in Planktonic and Biofilm Modes of Growth

2.1. Introduction

Chlorhexidine (CHG) possesses rapid antimicrobial efficacy against both Gram-positive and -negative bacteria, yeasts and lipophilic viruses, and is the antimicrobial of choice recommended by EPIC guidelines for skin antisepsis (Pratt *et al*., 2007). However, penetration studies have demonstrated its less favourable attribute of poor permeation into the deeper layers of human skin (Karpanen *et al*., 2008*a*). This can result in incomplete skin antisepsis from microorganisms residing in pores and hair follicles, as well as those existing as biofilm-like microcolonies within the skin. During surgical procedures such as CVC insertion, the barrier properties of the skin become breached, allowing infectious complications to arise from these deeply resident microorganisms (Health Protection Agency, 2010*c*). Therefore, there is a need to develop a means of achieving effective skin antisepsis throughout all layers of the skin, potentially by combining current agents with other antimicrobial agents, and/or permeation enhancers.

For many years, essential oils have been recognised as possessing a broad array of antimicrobial properties, furthermore some are now recognised to contain components that are known permeation enhancers (Edris, 2007). The addition of eucalyptus oil (EO) to CHG has recently shown to significantly enhance skin penetration of CHG into the epidermis and dermis (Karpanen *et al*., 2010). Eucalyptus oil has been studied by a number of groups investigating its antimicrobial properties, however it still remains unknown which, of the almost 60 constituents within the oil, are responsible for this efficacy and whether it is due to one component alone or the synergistic properties of many (Wildy *et al*., 2000).

The main constituent within EO is 1,8-cineole; a potent terpene and notorious permeation enhancer (Chandra *et al*., 2009). Causing temporary disruption to the intercellular lipids of the stratum corneum, 1,8-cineole has been used in many penetration studies and is thought to aid delivery of some drugs through the skin via the 'pull' effect, whereby it forms a complex with the drug and drags it through at the same rate (Heard *et al*., 2006). However, studies into the antimicrobial efficacy of 1,8-cineole are less readily available.

Due to the potential enhanced permeation offered by EO and 1,8-cineole, it is possible that combining them with CHG will result in improved antimicrobial activity in the

deeper layers of the skin, however prior to this, the antimicrobial efficacy of the agents alone and in combination needs to be studied.

2.2. Aims of the study

The aims of this study were to:

- Assess the antimicrobial activity of EO, 1,8-cineole and CHG using zones of inhibition and determination of minimum inhibitorary concentrations (MIC) and minimum bactericidal concentrations (MBC) through microbroth dilution and the ‘scrape and wash’ technique, against a panel of clinically relevant microorganisms, grown in both planktonic and biofilm cultures.
- Determine the antimicrobial activity of EO and 1,8-cineole in combination with CHG, using a checkerboard method and adenosine triphosphate (ATP) bioluminescence, against a panel of clinically relevant microorganisms, grown in both planktonic and biofilm cultures.

2.3. Materials

2.3.1. Microbial cultures

National Collection of Type Cultures (NCTC) and American Type Culture Collection (ATCC) strains of microorganisms were selected. *Staphylococcus hominis* ATCC 35982, *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* RP62A, MRSA N315, *Pseudomonas aeruginosa* ATCC 15442, *Escherichia coli* NCTC 10418 and *Candida albicans* ATCC 76615 were stored on MicroBank beads (Pro-Lab Diagnostics, Cheshire, U.K.) at -70 °C until required.

2.3.2. Microbiological media

Mueller-Hinton agar (MHA), Mueller-Hinton broth (MHB), Sabouraud dextrose agar (SDA) and Sabouraud dextrose broth (SDB) were purchased from Oxoid (Basingstoke, U.K.), prepared as per manufacturer's instructions and sterilised at 121 °C for 15 minutes. Agar was cooled to 50 °C before pouring and broths were stored at 4 °C until required.

Letheen broth and agar bacteriological (agar no.1) were purchased from Oxoid (Basingstoke, U.K.). Neutralising letheen agar was made from letheen broth prepared as per manufacturer's instructions with the addition of 1 % (w/w) agar no.1, then sterilised at 121 °C for 15 minutes and cooled to 50 °C before pouring.

Congo red agar was prepared initially in two parts with a ratio of 80:20 part one to two. Part one contained 3.75 % (w/v) MHB or SDB (Oxoid, Basingstoke, U.K.), 1.25 % (w/v) D-glucose (Fisher Scientific, Leicestershire, U.K.) and 1.875 % (w/v) agar number 1 (Oxoid, Basingstoke, U.K.) in double distilled water. Part two consisted of 0.4 % (w/v) Congo red (Hopkins & Williams Limited, Essex, U.K.) in double distilled water. The two parts were autoclaved separately and cooled to 50 °C before part two was aseptically transferred into part one and plates were poured.

2.3.3. Chemicals

2.3.3.1. Eucalyptus oil stock solution

Stock solutions of EO (Sigma-Aldrich, Dorset, U.K.) were made fresh on the day of use, to a concentration of 512 mg/mL then further diluted as required. The concentration of EO was 0.910 g/mL; equivalent to 910 mg/mL, thus the stock solution consisted of 56.26 % (v/v) EO, 5 % (v/v) dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Dorset, U.K.) in sterile, double distilled water.

2.3.3.2. 1,8-Cineole stock solution

Stock solutions of 1,8-cineole (Sigma-Aldrich, Dorset, U.K.) were made fresh on the day of use, to a concentration of 512 mg/mL and further diluted as required. The concentration of 1,8-cineole was 0.924 g/mL; equivalent to 924 mg/mL, thus the stock solution consisted of 55.41 % (v/v) 1,8-cineole, 5 % (v/v) DMSO (Sigma-Aldrich, Dorset, U.K.) in sterile, double distilled water.

2.3.3.3. Chlorhexidine digluconate stock solution

Stock solutions of CHG (Sigma-Aldrich, Dorset, U.K.) were made fresh on the day of use, to a concentration of 256 µg/mL and further diluted as required. The concentration of the CHG solution obtained from Sigma was 200,000 µg/mL, thus the stock solution consisted of 12.8 % (v/v) CHG in sterile, double distilled water to give a concentration of 25,600 µg/mL, which then required a 1:100 dilution to achieve the desired stock solution concentration of 256 µg/mL.

2.3.3.4. Alcian blue solution

Alcian blue stain was made with 1 % (w/v) Alcian blue 8 XG (Sigma-Aldrich, Dorset, U.K.) and 3 % (v/v) acetic acid (Fisher Scientific, Leicestershire, U.K.) in sterile, double distilled water.

2.3.4. Equipment

Optical density (OD) readings were taken using a Pharmacia LKB visible spectrophotometer, Novaspec II (Pharmacia, Freiburg, Germany).

Fluorescence and OD of microtitre plates were measured using an EL808IU-PC microplate reader with Gen5 software (BioTek Instruments Ltd, Leeds, U.K.).

Microscopy was performed with a Zeiss Axio Scope microscope fitted with a Zeiss AxioCam HRc camera and AxioVision software version 3.1 (Carl Zeiss Ltd, Hertfordshire, U.K.).

2.4. Methods

2.4.1. Antimicrobial activity of eucalyptus oil, 1,8-cineole and chlorhexidine digluconate against microorganisms in planktonic mode of growth

2.4.1.1. Standard curves of colony forming units using optical density

Overnight cell suspensions of *S. aureus*, *S. epidermidis*, MRSA, *P. aeruginosa* and *E. coli* were prepared in MHB and incubated at 37 °C. *C. albicans* was grown at 30 °C in SDB. A range of serial and double dilutions were made of each cell suspension in either MHB or SDB as required in duplicate, and the OD of each was read in a spectrophotometer set to 570 nm following appropriate broth blanking. Cell suspensions were plated onto MHA or SDA using the drop count method (Miles & Misra, 1938) and incubated overnight at 37 and 30 °C respectively. The colony forming units (cfu) were determined for each microorganism and equated to cfu per millilitre (cfu/mL), then plotted against OD.

2.4.1.2. Microbial inhibition by eucalyptus oil and 1,8-cineole solutions using agar diffusion

Based on methods by Smith-Palmer *et al.* (1998), cell suspensions of *S. aureus* and *E. coli* in MHB were prepared containing 10^4 cfu/mL and spread with a sterile cotton swab over MHA plates made from 25 mL to give a uniform thickness. Sterile glass pipettes were used to cut three wells from each plate. Neat, 50, 40, 30, 20 and 10 % (v/v) solutions of EO and 1,8-cineole were made using sterile, double distilled water and 5 % (v/v) DMSO. Sixty microlitres of each EO or 1,8-cineole dilution was added to triplicate wells cut from a single plate. Control plates containing EO alone, 1,8-cineole alone and 5 % (v/v) DMSO in water were also prepared. Plates were stored at 4 °C for 1 hour then incubated at 37 °C overnight. Following incubation, inhibition zone sizes were measured as the distance between the edge of the well and visible growth.

2.4.1.3. Microbial inhibition by eucalyptus oil and 1,8-cineole solutions following direct application

MHA plates were inoculated with *S. aureus* and *E. coli* as previously described (Section 2.4.1.2) and the same dilutions of EO and 1,8-cineole were prepared, along with the controls. Duplicate 25 µL drops of each test antimicrobial or control were spotted onto each microorganism. Plates were dried at 4 °C for 1 hour then incubated at 37 °C overnight. Inhibition zone diameters were measured after incubation.

2.4.1.4. Minimum inhibitory and bactericidal concentrations of eucalyptus oil, 1,8-cineole and chlorhexidine using microbroth dilution

Double dilutions of EO and 1,8-cineole ranging from 512 to 0.25 mg/mL were made in MHB and SDB, from stock solutions (Sections 2.3.3.1 and 2.3.3.2). Dilutions of CHG were made from 128 to 0.0625 µL/mL also in MHB and SDB. Triplicate wells were set up in a clear, round bottom, 96-well microtitre plate (Fisher Scientific, Leicestershire, U.K.) (Figure 2.1) with 100 µL of test antimicrobial and 100 µL of 10^6 cfu/mL cell suspension, diluted from overnight broth inocula of all six microorganisms. Phosphate buffered saline (PBS) (Sigma-Aldrich, Dorset, U.K.) and DMSO controls ranging 20 % (v/v) to 0.625 % (v/v) were also set up for each microorganism. All plates were incubated overnight at 37 ºC; *C. albicans* at 30 ºC. The minimum inhibitory concentration (MIC) was determined as the lowest concentration to have no clearly visible button of growth in the well. The solution from each MIC well and those of higher all concentrations were plated onto neutralising letheen agar, spread with a wedge-shaped spreader and incubated overnight, as were the controls. Growth controls of each microorganism were also prepared on letheen agar. The minimum bactericidal/fungicidal concentration (MBC/MFC) was determined as the lowest concentration to show a 99.9 % reduction in cfu/mL from that of the control.

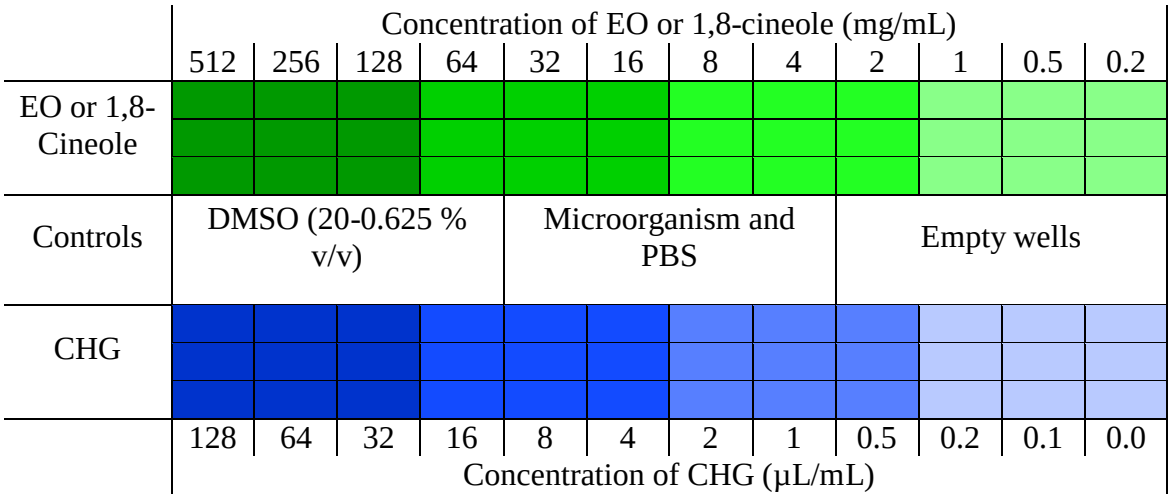

Figure 2.1. Diagram showing microtitre plate set up for the method entitled 'MIC and MBC using microbroth dilution'. A range of concentrations of EO, 1,8-cineole or CHG are added to equal volumes of cell suspensions of each microorganism to determine MIC and MBC values.

2.4.1.5. Checkerboard assay combining eucalyptus oil and 1,8-cineole with chlorhexidine using optical density

Double dilutions of EO and 1,8-cineole were made in MHB or SDB ranging from 512 to 0.5 mg/mL, and CHG from 256 to 0.125 µg/mL. For each of the six microorganisms, duplicate clear, round bottom, 96-well microtitre plates (Fisher Scientific, Leicestershire, U.K.) were set up (Figure 2.2) with 100 µL of CHG and 100 µL of EO per well, as well as duplicate plates of 100 µL of CHG and 100 µL of 1,8-cineole. For each plate, the highest concentration of CHG was added to the first column, the next concentration to the second column etc. The EO or 1,8-cineole was added in reducing concentrations to the first 9 wells of each row. Controls were set up of CHG alone, EO or 1,8-cineole alone, 5 % (v/v) DMSO in MHB or SDB as appropriate, and PBS alone. All wells were inoculated with 10 µL of a 10^6 cfu/mL cell suspension. *C. albicans* plates were incubated overnight at 30 ºC, all others were incubated at 37 ºC. The MIC of each agent alone and in combination was determined as the lowest concentration to show no visible growth, and was verified with OD using a microplate reader set to 570 nm, then analysed using Gen5 software. Pour plates of molten MHA and SDA cooled to 50 °C were made containing the contents of the well determined as the MIC, and all those containing test agents of a higher concentration. The MBC was determined as the lowest concentration to show 99.9 % reduction in cfu/mL from that of the control. The

effects of the combinations were then determined as synergistic, indifferent or antagonistic by calculating the fractional inhibitory concentration (FIC) for the agents, and then the fractional inhibitory concentration indices (FICI) value for each combination (White *et al*., 1996).

$$\text{FIC} = \frac{\text{MIC of antimicrobial in combination}}{\text{MIC of antimicrobials alone}}$$

$$\text{FICI} = \text{FIC of oil} + \text{FIC of chlorhexidine}$$

Where: FICI $\leq$ 0.5 shows synergism

FICI > 0.5 or $\leq$ 4.0 shows indifference

FICI > 4.0 shows antagonism

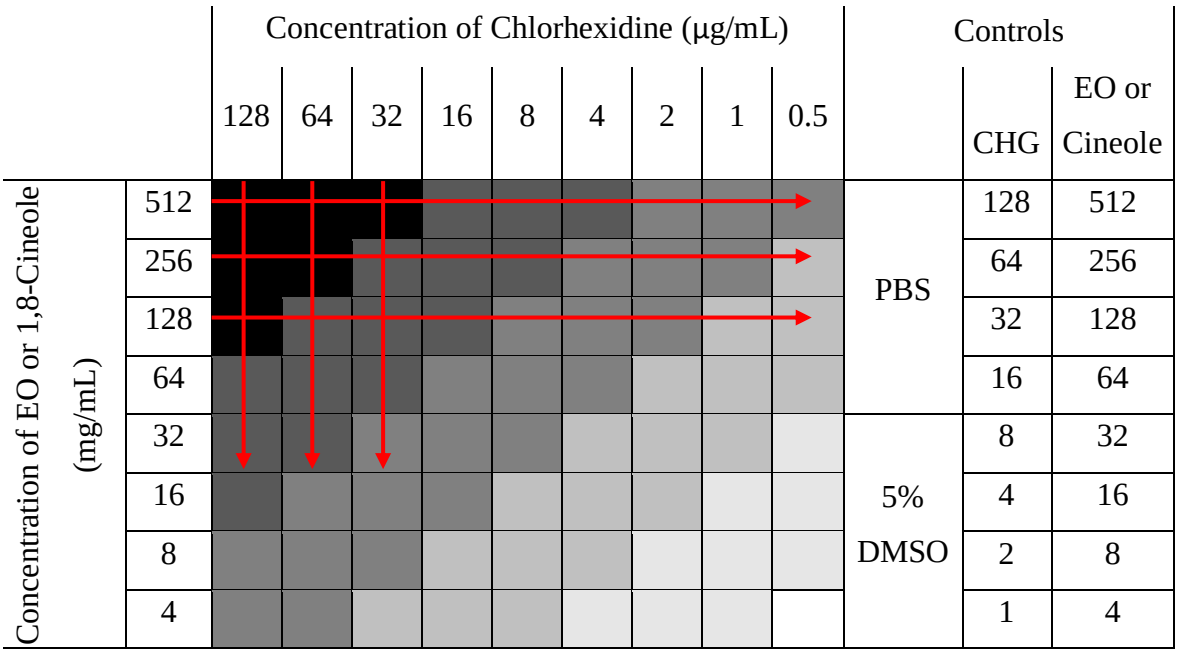

Figure 2.2. Diagram showing microtitre plate set up for the method entitled 'Checkerboard assay'. Reducing concentrations of EO or 1,8-cineole are added to the rows, while reducing concentrations of CHG are added in equal volume to the columns. A small inoculum of concentrated cell suspensions of each microorganism is added to determine synergism, indifference or antagonism.

2.4.2. Antimicrobial activity of eucalyptus oil, 1,8-cineole and chlorhexidine digluconate against microorganisms in biofilm mode of growth

2.4.2.1. Verification of biofilm production

Overnight cell suspensions of all six microorganisms were quantified using previously established OD/cfu standard curves (Section 2.4.1.1), and diluted in MHB or SDB to 10^5 cfu/mL. Three millilitres of each microorganism were inoculated into four wells of a clear plastic, flat bottom, 6-well, tissue culture treated plate (Appleton Woods, Birmingham, U.K.). All plates were incubated for 48 hours at 37 ºC; 30 ºC for *C. albicans*. Following incubation, the broth was discarded and all wells were washed once with PBS. For each 6-well tissue culture treated plate, three wells (duplicate inoculated wells and one empty) were stained with 3 mL Alcian blue for 15 minutes, while the remaining three wells (also two inoculated and one empty) had 3 mL crystal violet stain (Reactifs RAL, France) for 5 minutes. Dyes were removed after the allocated exposure time and wells washed again with PBS. The wells of the 6-well plates were left to dry in air, then immersion oil was added and biofilm presence verified microscopically, using the phase plate and x40 objective of a Zeiss Axio Scope microscope fitted with a Zeiss AxioCam HRc camera (Carl Zeiss Ltd, Hertfordshire, U.K.).

Congo red plates were inoculated with each microorganism from five identical colonies grown on MHA or SDA, and spread for single colonies using a sterile, metal loop. A negative control was also set up in the same manner using *S. hominis*. Plates were incubated overnight at 37 ºC; *C. albicans* at 30 ºC. A positive result was observed as black, crusty colonies. Pink colonies represented a negative result for biofilm production.

2.4.2.2. Quantification of microbial cell numbers within a biofilm

Wells of a clear plastic, flat bottom, 96-well microtitre plate (Thermo Labsystems, Franklin, U.S.A.) were inoculated with 200 µL of an overnight cell suspension of each microorganism, diluted in MHB or SDB to 10^4 cfu/mL. Plates were incubated for 48 hours at 37 ºC; *C. albicans* at 30 ºC. Following incubation, all broth was removed and wells were washed once with PBS. Into each well, 250 µL of PBS was added and plates

were sonicated in a 50 Hz water bath sonicator (Kerry Ultrasonics, Hertfordshire, U.K.) for 30 minutes. A sterile pipette tip was used to dislodge any remaining bound cells and the contents of 4 wells compiled into one eppendorf tube to produce 1 mL from four initial biofilms. Eppendorf tubes were mixed for 1 minute then serial dilutions made in MHB or SDB, and plated onto MHA or SDA using the drop count method (Miles & Misra, 1938). Following overnight incubation, colonies were counted and converted into cfu/mL of biofilm. The assay was performed in quadruplicate.

2.4.2.3. Minimum inhibitory and bactericidal concentrations of eucalyptus oil, 1,8-cineole and chlorhexidine using the scrape and wash technique

Biofilms of all six microorganisms were grown in microtitre plates as previously described for 48 hours (Section 2.4.2.2). After incubation, all broth was removed and wells were washed once with PBS. Double dilutions of EO and 1,8-cineole covering the range 512 to 0.25 mg/mL were made in MHB and SDB, from stock solutions. Dilutions of CHG were made from 128 to 0.125µl/mL. Wells were treated in triplicate with 100 µL of double the required final concentration of test agent, and 100 µL of MHB or SDB. Controls of PBS and 5 % (v/v) DMSO were also set up for each microorganism. The plates were incubated overnight at 37 ºC; 30 ºC for *C. albicans*. The test antimicrobial agents were removed and the wells washed once with PBS before adding 250 µL fresh PBS to each well and subjecting the plate to 30 minutes sonication in a 50 Hz water bath sonicator (Kerry Ultrasonics, Hertfordshire, U.K.). All wells were scraped and washed (Adams, 2006) once to loosen the biofilm, then plated into molten MHA or SDA cooled to 50 °C and incubated overnight. The MIC was determined as the lowest concentration to show reduced growth from that of the control plate, and the MBC as the lowest concentration to show a 99.9 % reduction in the cfu/mL compared with that of the control.

2.4.2.4. Standard growth curves using ATP bioluminescence

Overnight cell suspensions of all six microorganisms were diluted to 10^5 cfu/mL then 200 µL of each microorganism inoculated into 15 wells of a white wall, clear bottom, tissue treated, 96-well microtitre plate (Fisher Scientific, Leicestershire, U.K.).

Following 48 hours incubation at 37 ºC or 30 ºC for *C. albicans*, the broth was removed and wells were washed once with PBS. Each well had 250 µL fresh PBS added then the plates were sonicated for 30 minutes in a 50 Hz water bath sonicator (Kerry Ultrasonics, U.K.). Wells were scraped and washed (Adams, 2006) once into a sterile universal bottle containing 1 g sterile, borosilicate solid glass beads, 3 mm (Sigma-Aldrich, Dorset, U.K.), and vortexed for 1 minute. This neat solution was serially diluted in MHB or SDB as appropriate, with double dilutions of these also prepared. Samples of 1 mL of each dilution was added to a petri dish and pour plates made using molten MHA or SDA cooled to 50 °C. Duplicate wells of a fresh white wall, clear bottom, tissue treated, 96-well microtitre plate (Fisher Scientific, Leicestershire, U.K.) were inoculated with 100 µL of each dilution. Fifty microlitres of bactolyse from the ViaLight MDA plus detection kit (Cambrex, U.S.A.) was added to each well and left as described in the kit for 10 minutes. One hundred microlitres of adenosine tri-phosphate monitoring reagent (ATP-MR) was added to every well, left for 2 minutes then fluorescence was read on the microplate reader. Following overnight incubation of the pour plates, the colonies were counted and converted to cfu/mL, then plotted against the relative light units (RLU) of luminescence.

2.4.2.5. Checkerboard assay combining eucalyptus oil and 1,8-cineole with chlorhexidine using ATP bioluminescence

White wall, clear bottom, tissue treated, 96-well microtitre plates (Fisher Scientific, Leicestershire, U.K.) were inoculated with 200 µL per well of a 10^5 cfu/mL cell suspension, diluted in MHB or SDB from an overnight cell suspension; three wells per plate were left empty. Plates were incubated for 48 hours at 37 ºC; 30 ºC for *C. albicans*. Following incubation, double dilutions of stock solutions were made of EO or 1,8-cineole and CHG ranging from 512 to 1 mg/mL and 128 to 0.5 µg/mL respectively. All broth was removed from the microtitre plates and wells were washed once with PBS. Wells were set up with 125 µL EO or 1,8-cineole and 125 µL CHG such that EO or 1,8-cineole concentration reduced with each column and CHG concentration reduced with each row. Controls were also set up with 125 µL EO, 1,8-cineole or CHG and 125 µL MHB or SDB. PBS and 5 % (v/v) DMSO controls were also made. Plates were returned to the appropriate incubator overnight. After incubation, all test antimicrobial agents were removed from the plates and wells were washed once with PBS. To each

well, 150 µL PBS and 50 µL bactolyse from the ViaLight MDA plus detection kit (Cambrex, U.S.A.) were added, then the plate was sonicated at for 30 minutes in a 50 Hz water bath sonicator (Kerry Ultrasonics, Hertfordshire, U.K.). After sonication, 100 µL ATP-MR was added to each well and left as required for 2 minutes before fluorescence was read on the microplate reader.

2.5. Results

2.5.1. Antimicrobial activity of eucalyptus oil, 1,8-cineole and chlorhexidine digluconate against microorganisms in planktonic mode of growth

2.5.1.1. Standard curves of colony forming units using optical density

The standard curves for cell suspension quantification of each microorganism using OD produced linear graphs with R^2 values of 0.9822 for *S. aureus*, 0.9986 for *S. epidermidis*, 0.9939 for MRSA, 0.9798 for *P. aeruginosa*, 0.9899 for *E. coli* and 0.9935 for *C. albicans* (Figures A1-A6).

2.5.1.2. Microbial inhibition by eucalyptus oil and 1,8-cineole solutions using agar diffusion

Both EO and 1,8-cineole demonstrated antimicrobial activity against *S. aureus* and *E. coli* (Table 2.1). Overall however, EO gave significantly larger ($P<0.001$, Paired t-test, Instat3 GraphPad) zones of inhibition than 1,8-cineole, while significantly greater ($P<0.001$, Paired t-test, Instat3 GraphPad) sensitivity was observed by *S. aureus* than *E. coli* towards EO. The mean zone sizes and standard deviation (SD) for EO were 12.39 and 7.33; for 1,8-cineole these were 0.53 and 1.06 respectively when data were compiled regardless of microorganism. The DMSO controls all had zones of <1 mm diameter, therefore were deemed as not contributing to any antimicrobial activity observed.

Table 2.1. Antimicrobial efficacy of neat and diluted eucalyptus oil and 1,8-cineole against *S. aureus* and *E. coli* expressed as mean (n=3) sizes of inhibition zones observed using the agar diffusion method.

	Inhibition zone (mm) for eucalyptus oil/1,8-cineole					
	Neat	50 %	40 %	30 %	20 %	10 %
S. aureus	12/3	12/<1	11/<1	8/<1	6/<1	3/<1
E. coli	7/2	6/1	5/<1	5/<1	2/<1	1/<1

2.5.1.3. Microbial inhibition by eucalyptus oil and 1,8-cineole solutions following direct application

Overall, EO showed significantly greater (P<0.005, Paired t-test, Instat3 GraphPad) efficacy than 1,8-cineole when applied directly to *S. aureus* and *E. coli* (Table 2.2). The mean zone sizes and SD for EO were 10.96 and 8.46; for 1,8-cineole these were 3.63 and 5.24 respectively when data were compiled regardless of microorganism. The DMSO controls all had zones of <1 mm diameter, therefore were deemed as not contributing to any antimicrobial activity observed.

Table 2.2. Antimicrobial efficacy of neat and diluted eucalyptus oil and 1,8-cineole against *S. aureus* and *E. coli* expressed as mean (n=2) sizes of inhibition zones observed following direct application.

	Inhibition zone (mm) for eucalyptus oil/1,8-cineole					
	Neat	50 %	40 %	30 %	20 %	10 %
S. aureus	23/<1	20/<1	18/<1	14/<1	<1/<1	<1/<1
E. coli	18/11	15/11	13/11	12/11	<1/<1	<1/<1

2.5.1.4. Minimum inhibitory and bactericidal concentrations of eucalyptus oil, 1,8-cineole and chlorhexidine using microbroth dilution

CHG demonstrated significantly greater (P<0.005, Wilcoxon signed rank test, Instat) antimicrobial activity than EO or 1,8-cineole against all six microorganisms tested, with the MIC and MBC values of CHG being a magnitude of 1000-times less than that obtained for EO and 1,8-cineole (Figure 2.3). The median MIC and MBC for EO were 6 and 8 mg/mL respectively, for 1,8-cineole these were 48 and 192 mg/mL, and for CHG values of 2 and 2 µg/mL were recorded. *P. aeruginosa* demonstrated reduced sensitivity to EO and 1,8-cineole compared with the other microorganisms tested, with the highest concentration tested (256 mg/mL) of 1,8-cineole failing to inhibit growth. The MIC and MBC data were categorised into high or low using the midpoint of the observed range as the cut off value (Table A1), which showed the MBC values of EO to be significantly lower (P<0.05) than that of 1,8-cineole when applied to a Chi squared 2 by 2 contingency table (Instat3 GraphPad). Collating the MIC and MBC results demonstrated that overall, there were significant differences (P<0.05, Chi squared 2 by 2 contingency table, Instat3 GraphPad) between EO and 1,8-cineole, as well as between

both of these and CHG. The 5 % (v/v) DMSO control showed no antimicrobial activity against any of the microorganisms tested.

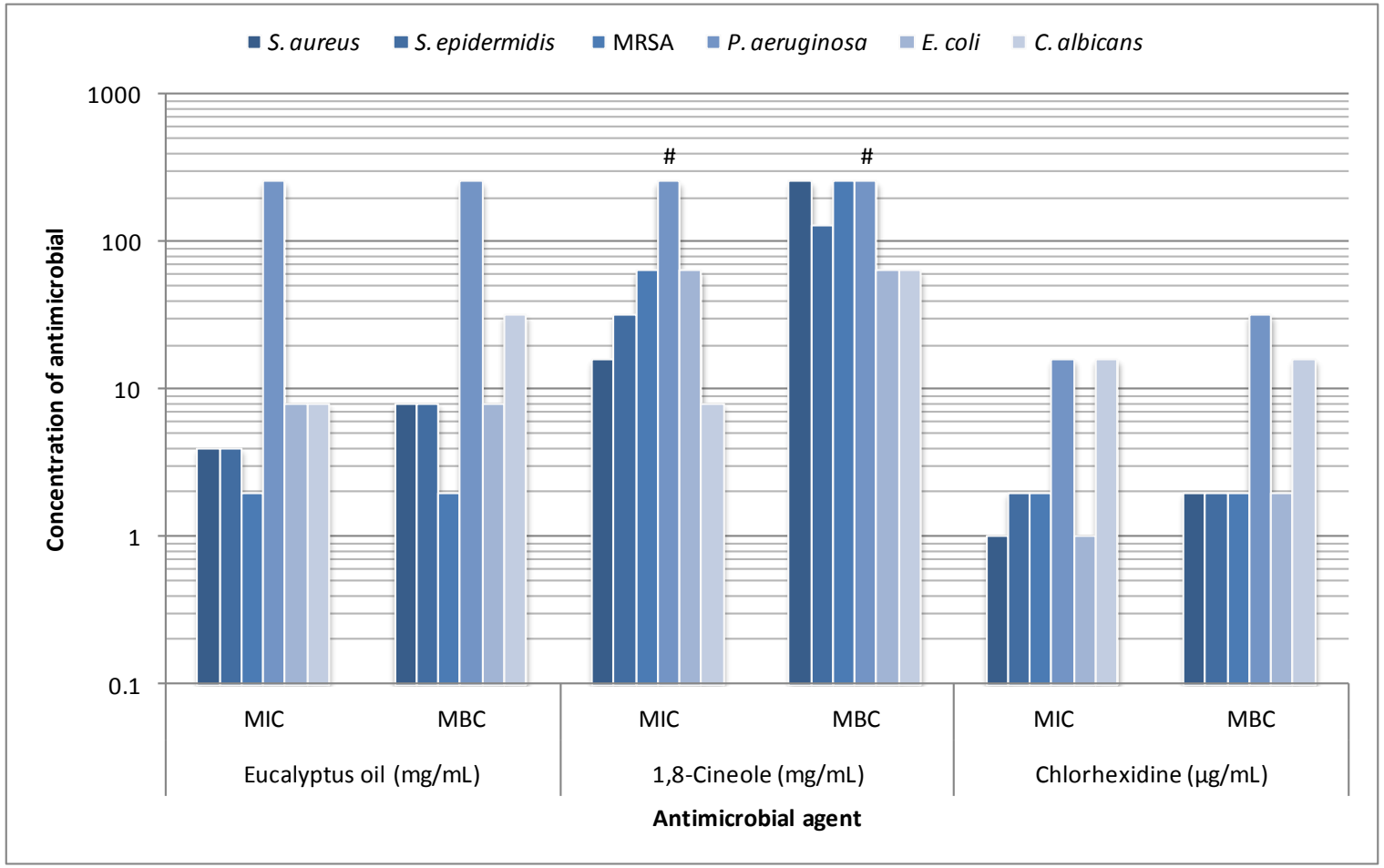

Figure 2.3. MIC and MBC median values (n=3) of EO, 1,8-cineole and CHG against a panel of six microorganisms, obtained using the microbroth dilution method. (# represents MIC or MBC > 256 mg/mL).

2.5.1.5. Checkerboard assay combining eucalyptus oil and 1,8-cineole with chlorhexidine using optical density

All except three combinations of EO or 1,8-cineole with CHG resulted in synergistic antimicrobial activity, with EO/CHG for *S. epidermidis* demonstrating indifference (neither synergistic nor antagonistic) with an FICI value of 0.625, along with both EO/CHG and 1,8-cineole/CHG for *P. aeruginosa* with FICI values of 0.750 and 0.516 respectively (Table 2.3).

Table 2.3. Resultant antimicrobial efficacy of EO and 1,8-cineole when combined with CHG against planktonic growth of a panel of six microorganisms, using a checkerboard assay. The results are shown in duplicate and expressed as Fractional Inhibitory Concentration (FIC) and FIC Index (FICI), where FICI ≤ 0.5 shows synergy, FICI > 0.5 or ≤ 4.0 shows indifference, and FICI > 4.0 shows antagonism.

Microorganism/ Combination Tested	MIC of oil (mg/mL) alone/in combination		Mean FIC of oil	MIC of CHG (µg/mL) alone/in combination		Mean FIC of CHG	Mean FICI	Result
S. aureus								
EO + CHG	32/8	32/4	0.188	1/0.125	1/0.125	0.125	0.313	Synergy
Cineole + CHG	128/4	128/4	0.031	1/0.25	1/0.25	0.250	0.281	Synergy
S. epidermidis								
EO + CHG	8/1	8/1	0.125	2/1	2/1	0.500	0.625	Indifference
Cineole + CHG	32/8	32/8	0.250	2/0.125	2/0.125	0.063	0.313	Synergy
MRSA								
EO + CHG	16/4	16/4	0.250	2/0.25	2/0.25	0.125	0.375	Synergy
Cineole + CHG	128/8	128/4	0.047	0.5/0.125	0.5/0.125	0.250	0.297	Synergy
P. aeruginosa								
EO + CHG	16/4	16/8	0.375	4/2	4/1	0.375	0.750	Indifference
Cineole + CHG	256/4	256/4	0.016	4/2	4/2	0.500	0.516	Indifference
E. coli								
EO + CHG	8/1	8/1	0.125	0.5/0.25	0.5/0.125	0.375	0.500	Synergy
Cineole + CHG	32/8	32/8	0.250	0.5/0.125	0.5/0.125	0.250	0.500	Synergy
C. albicans								
EO + CHG	8/2	8/2	0.250	4/1	4/1	0.250	0.500	Synergy
Cineole + CHG	32/4	64/4	0.094	2/0.5	2/1	0.375	0.469	Synergy

2.5.2. Antimicrobial activity of eucalyptus oil, 1,8-cineole and chlorhexidine digluconate against microorganisms in biofilm mode of growth

2.5.2.1. Verification of biofilm production

Crystal violet (Figure 2.4), Alcian blue (Figure 2.5) and Congo red agar (Figure 2.6) confirmed biofilm and slime production by all microorganisms.

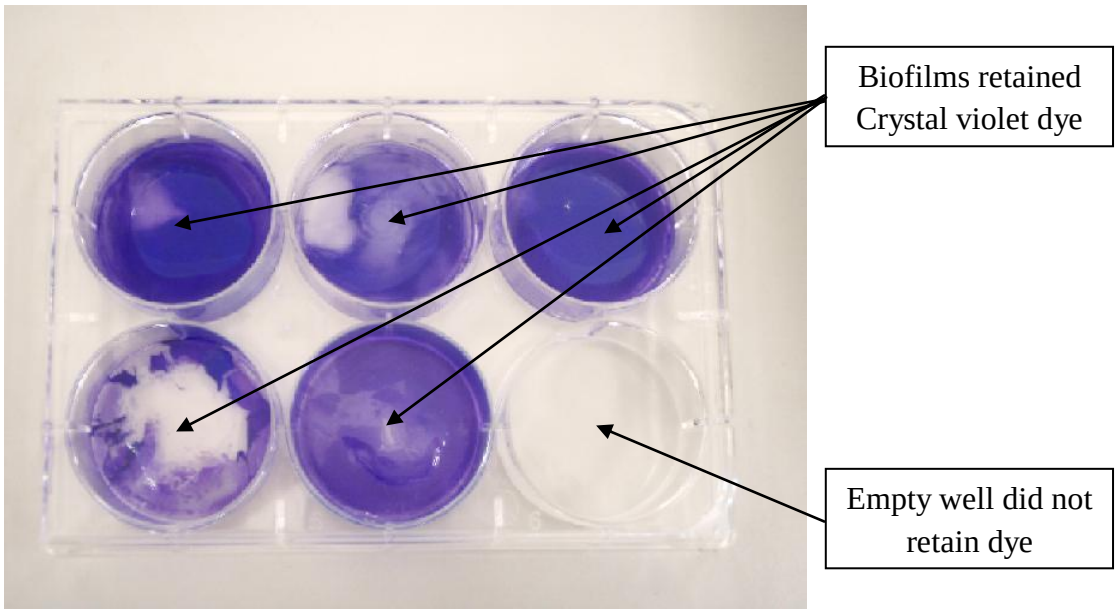

Figure 2.4. Biofilm production confirmed by each microorganism in tissue culture treated plates with Crystal violet dye, control (bottom right) observed as unable to retain dye.

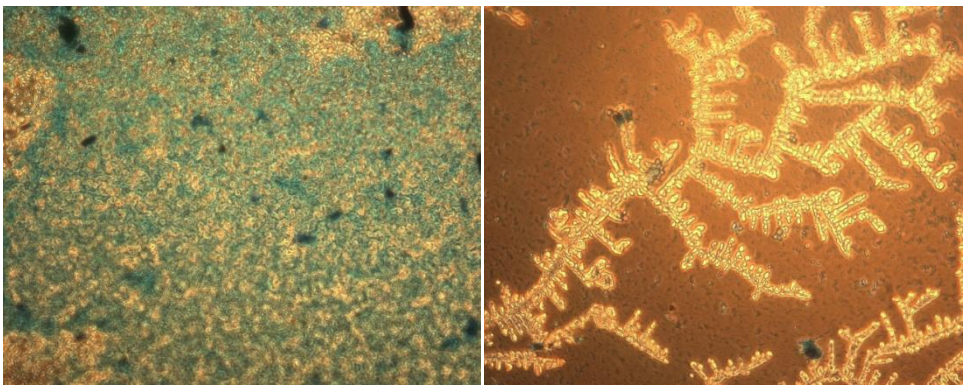

Figure 2.5. Biofilm of *S. epidermidis* (left) visible under x40 objective of light microscope (Zeiss Axio Scope microscope with AxioCam HRc camera, Carl Zeiss Ltd, Hertfordshire, U.K.) after Alcian blue staining, compared with control (right).

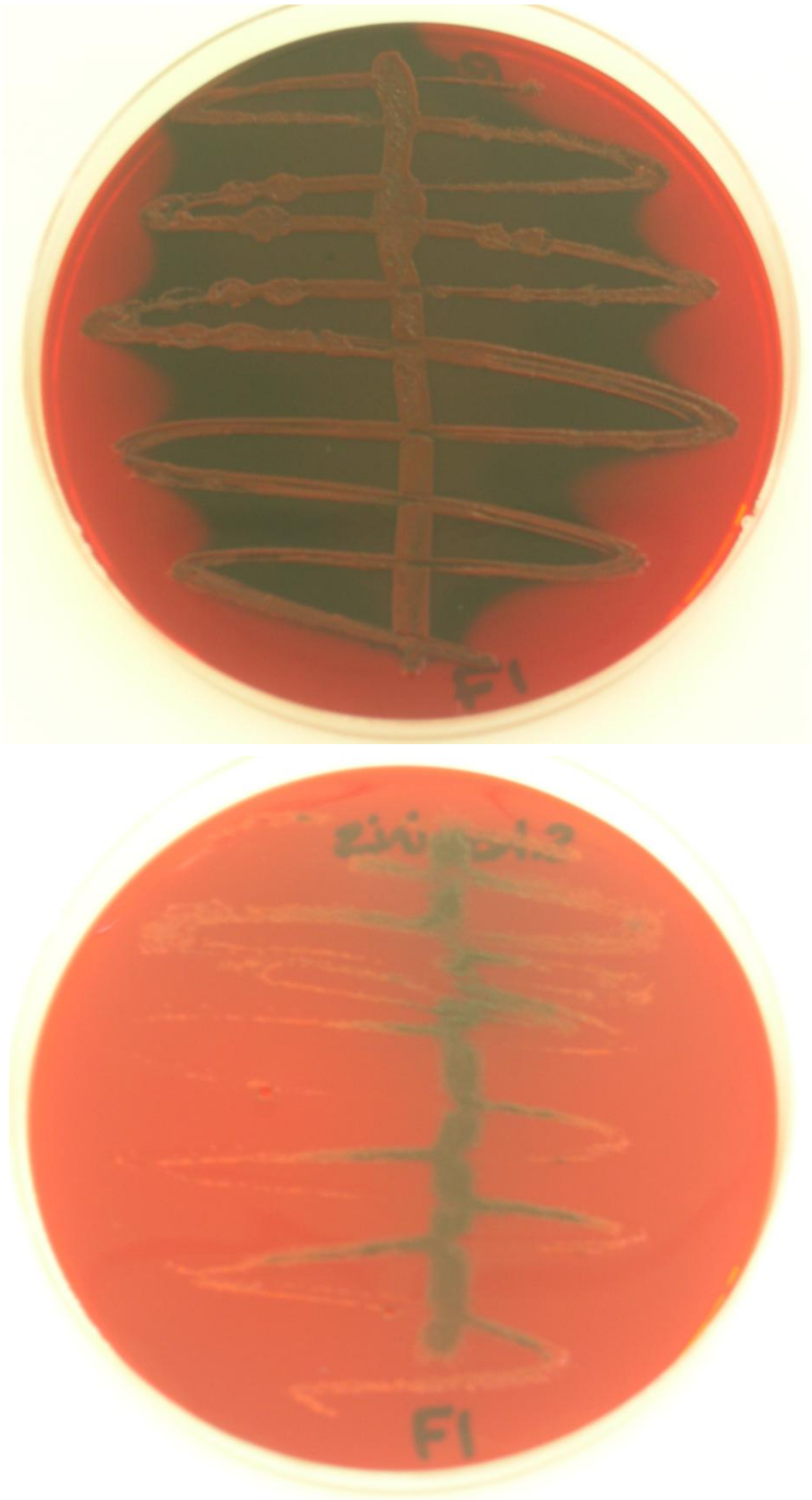

Figure 2.6. Slime production verified by growth of black, crusty colonies shown here by *S. epidermidis* on Congo red agar (top), compared with control of *S. hominis* (bottom) which is a known non-slime producer.

2.5.2.2. Quantification of microbial cell numbers within a biofilm

Confluent biofilms were produced and quantified using the Miles and Misra (1938) method for all six microorganisms following 48 hour growth of 200 µL inocula in 96-well microtitre plates (Figure 2.7). The mean biofilm per well ranged from 3.5×10^5 cfu for *C. albicans* to 1.3×10^{10} cfu for *P. aeruginosa*.

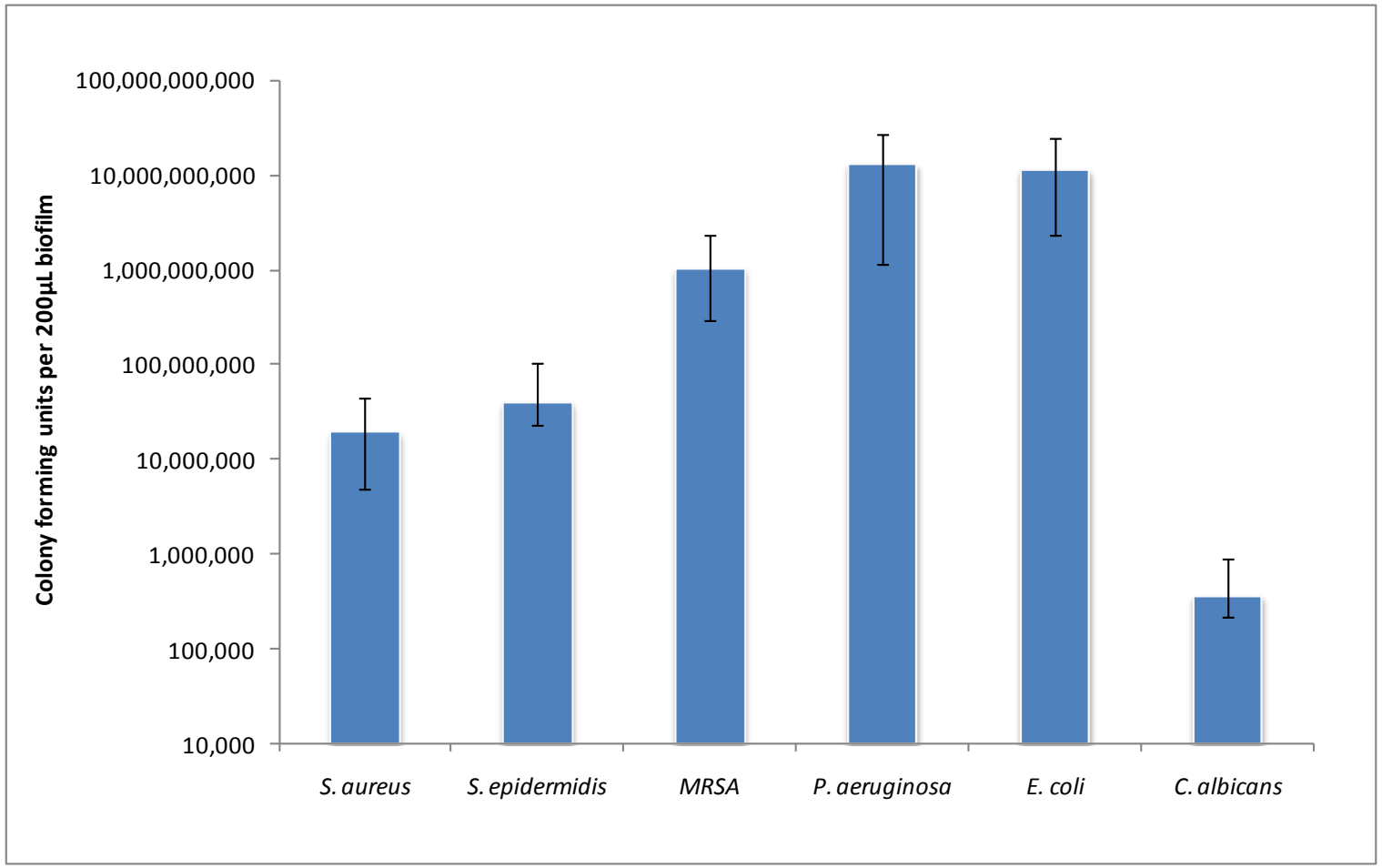

Figure 2.7. Mean (n=4) cfu (and range) per 200 µL biofilm of a panel of microorganisms after 48 hours growth in microtitre plates, as determined using the Miles and Misra (1938) method.

2.5.2.3. Minimum inhibitory and bactericidal concentrations of eucalyptus oil, 1,8-cineole and chlorhexidine using the scrape and wash technique

EO, 1,8-cineole and CHG exhibited some antimicrobial activity against a panel of microorganisms grown in biofilms (Figure 2.8). As previously observed with MIC and MBC results for planktonic cells (Section 2.5.1.4), the antimicrobial efficacy of CHG was significantly greater ($P<0.005$, Wilcoxon signed rank test, Instat) than that observed by EO or 1,8-cineole, with the results differing between mg/mL for EO and 1,8-cineole and µg/mL for CHG. However, no significant difference was observed in the efficacies of EO and 1,8-cineole ($P>0.5$ Wilcoxon signed rank test, Instat). The median MIC and MBC for EO against biofilms were 256 and 512 mg/mL respectively, for 1,8-cineole these were 512 and >512 mg/mL, and for CHG values of 80 and >128 µg/mL were recorded (Figure 2.8). When categorised (Table A1), there was a significant difference ($P<0.05$, Chi squared 2 by 2 contingency table, Instat3 GraphPad) between the MIC results of EO and CHG. The 5 % (v/v) DMSO control showed no antimicrobial activity against any of the microbial biofilms tested.

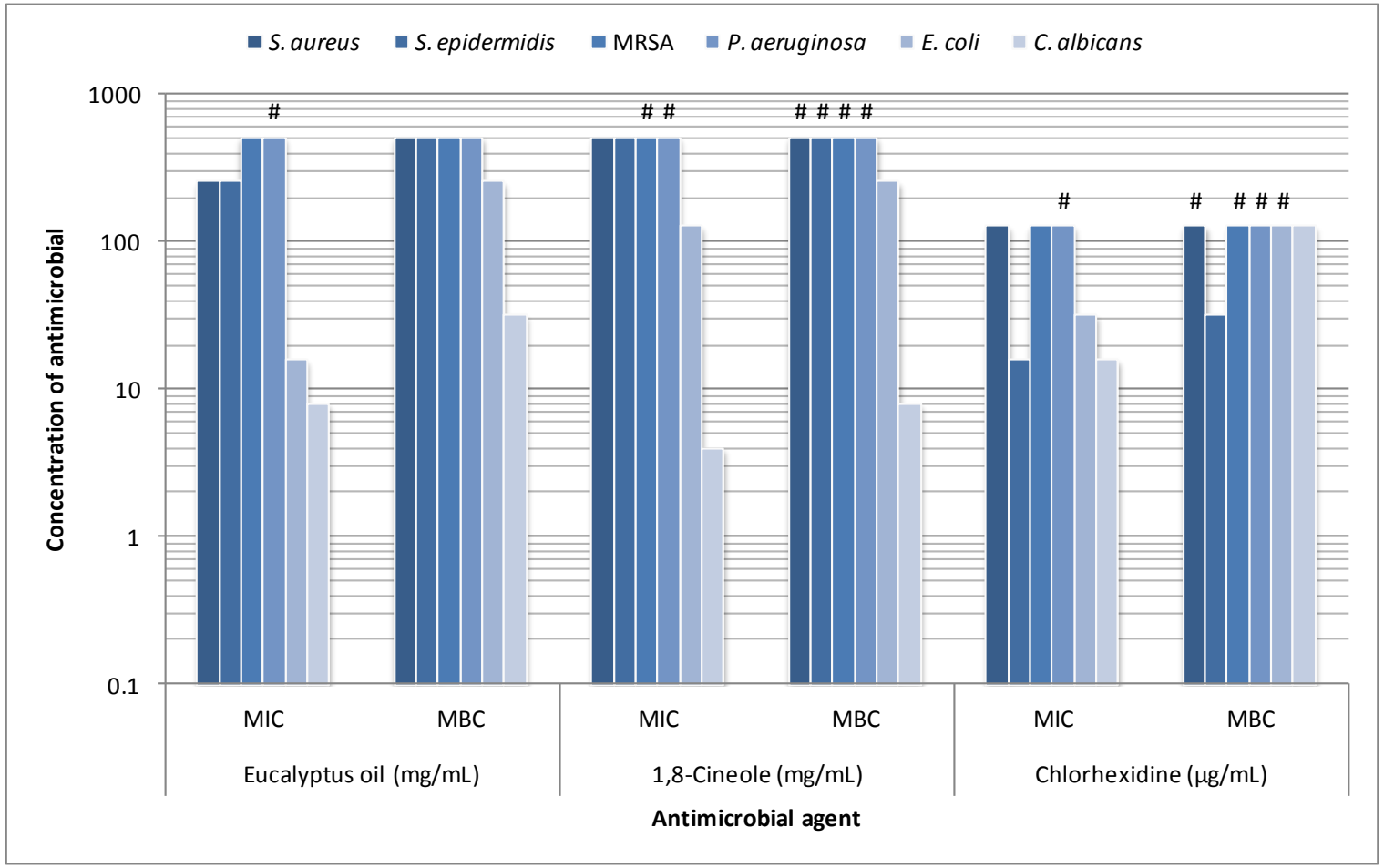

Figure 2.8. MIC and MBC median values (n=3) of EO, 1,8-cineole and CHG against a panel of six microorganisms, obtained using the microbroth dilution method. (# represents MIC or MBC > 256 mg/mL for EO or 1,8-cineole, and > 128 µg/mL for CHG).

2.5.2.4. Standard growth curves using ATP bioluminescence

The standard curves for biofilm quantification of each microorganism using fluorescence generated linear graphs with the following R^2 values: 0.9982 for *S. aureus*, 0.9951 for *S. epidermidis*, 0.9983 for MRSA, 0.9980 for *P. aeruginosa*, 0.9998 for *E. coli* and 0.9914 for *C. albicans* (Figures A7-A12).

2.5.2.5. Checkerboard assay combining eucalyptus oil and 1,8-cineole with chlorhexidine using ATP bioluminescence

Combinations of EO/CHG resulted in synergistic antimicrobial activity for *S. aureus*, MRSA, *P. aeruginosa* and *C. albicans*. When 1,8-cineole was combined with CHG, synergy was determined for *S. epidermidis*, MRSA, *P. aeruginosa* and *E. coli*. Indifference was observed for EO/CHG against *S. epidermidis* and *E. coli* with FICI values of 0.725 and 0.750 respectively. For 1,8-cineole/CHG combinations, indifference was concluded for *S. aureus* and *C. albicans* with FICI values of 0.625 and 1.125 respectively (Table 2.4).

Table 2.4. Resultant antimicrobial efficacy of EO and 1,8-cineole when combined with CHG against biofilm growth of a panel of six microorganisms, using a ATP bioluminescence. The results are shown in duplicate and expressed as Fractional Inhibitory Concentration (FIC) and FIC Index (FICI), where FICI ≤ 0.5 shows synergy, FICI > 0.5 or ≤ 4.0 shows indifference, and FICI > 4.0 shows antagonism.

Microorganism/ Combination Tested	MIC of oil (g/L) alone/in combination		Mean FIC of oil	MIC of CHG (mg/L) alone/in combination		Mean FIC of CHG	Mean FICI	Result
S. aureus								
EO + CHG	128/4	128/4	0.031	16/4	16/4	0.250	0.281	Synergy
Cineole + CHG	256/32	256/32	0.125	16/8	16/8	0.500	0.625	Indifference
S. epidermidis								
EO + CHG	256/128	256/128	0.500	16/4	16/4	0.250	0.725	Indifference
Cineole + CHG	256/16	256/16	0.063	16/4	16/4	0.250	0.313	Synergy
MRSA								
EO + CHG	256/4	256/4	0.016	8/1	8/1	0.125	0.141	Synergy
Cineole + CHG	128/4	128/4	0.031	16/4	16/4	0.250	0.281	Synergy
P. aeruginosa								
EO + CHG	>256/32	>256/32	0.125	64/16	64/16	0.250	0.375	Synergy
Cineole + CHG	>256/4	>256/4	0.016	16/4	16/4	0.250	0.266	Synergy
E. coli								
EO + CHG	16/4	16/4	0.250	16/8	16/8	0.500	0.750	Indifference
Cineole + CHG	64/4	64/4	0.063	16/0.5	16/0.5	0.031	0.094	Synergy
C. albicans								
EO + CHG	64/4	64/4	0.063	16/0.5	16/0.5	0.031	0.094	Synergy
Cineole + CHG	16/16	16/16	1.000	16/2	16/2	0.125	1.125	Indifference

2.6. Discussion

The therapeutic properties of essential oils have been recognised for thousands of years, with modern day thinking now challenging the impeding practicalities of use within the clinical setting. Assessment of the obstacles preventing realistic, daily use is underway, with research striving to gain comprehensive knowledge about the therapeutic potentials of oils to include detailed antimicrobial profiles, along with information regarding toxicity, longevity and residual activity, drug/chemical combination prospects, modes of action and resistance potential.

This study adds to the current knowledge about prospective antimicrobial use of essential oils by testing microorganisms commonly associated with HAI, in particular CVC related infections, against eucalyptus oil and its main component 1,8-cineole in comparison with CHG; currently recommended by EPIC guidelines for skin antisepsis (Pratt *et al*., 2007). EO, 1,8-cineole and CHG were tested alone and in combination against planktonic and biofilm cultures of six clinically significant microorganisms in order to determine their potential for use within the healthcare system, as well as to establish if crude EO is really needed or if the efficacy observed is due to 1,8-cineole, in which case this simplified, single agent would go forth for further studies.

The results of this study demonstrated that CHG, EO and 1,8-cineole possess antibacterial and antifungal activity, proving efficacious against Gram-positive *S. aureus*, *S. epidermidis*, and MRSA; Gram-negative *P. aeruginosa* and *E. coli*, as well as the yeast *C. albicans*. Furthermore, activity was not limited to just planktonic forms of microbial growth, but was observed against the more resilient form of growth within protective biofilm cultures.

The use of essential oils within the healthcare setting has proved previously to be limited, with more attention having been focussed on the conceptual idea of using tea tree oil for the decontamination of MRSA-colonised skin (Thompson *et al*., 2008). However, there is an abundance of investigations focused on their potential antimicrobial use, with a number of studies similar to those of this chapter, in which the efficacy of essential oils has been trialled against a panel of microorganisms. One in particular by Warnke *et al*. (2009) focused on 13 different essential oils to include EO, which were tested against multiple strains of *Staphylococcus* spp., *Streptococcus* spp. and *Candida* spp. Furthermore, they also used CHG as one of their controls. However, unlike the results described in the present study, they found the CHG control gave larger

zones of inhibition than EO against *S. aureus*, *S. epidermidis* and *C. albicans*. This is possibly due to easier release of the agents from preloaded discs, compared with the methods described here whereby the agents needed to diffuse through the agar medium from wells cut within it, therefore inducing higher resistance and smaller zones of inhibition.

The primary criticism regarding using essential oils in therapeutic practice centres upon their dilution in many studies being in the customary used ethanol, provoking dispute over the genuine source of efficacy. This was taken into account by Warnke *et al*. (2009) who reported its use as one of many controls, to be less efficacious than the oils in question, residing the observed efficacy to be derived from the oil not the solvent into which it is diluted. In this series of experiments, EO and 1,8-cineole were prepared in DMSO as it aids dissolution thus preventing precipitation which can lead to inconsistent and irreproducible results. Furthermore, the use of DMSO also allows the mixture to remain transparent, allowing results such as growth in microtitre plate wells from microbroth dilution to be more clearly interpreted.

CHG demonstrated significantly greater antimicrobial efficacy compared with EO and 1,8-cineole, when applied to planktonic cultures of the six microorganisms using a microbroth dilution technique. Furthermore, the differences between EO and 1,8-cineole were also concluded to be significantly different, with EO more efficacious. In one instance where testing was against *P. aeruginosa*, 1,8-cineole failed to achieve inhibition, confirming the contribution by other constituents within EO to efficacy. Significant differences between the efficacy of CHG and essential oils have also been observed in other studies, a large proportion of which have focussed on dental applications involving novel mouthwashes, toothpastes or denture adhesives for the control of plaque and gingivitis (Gunsolley, 2010; Van Leeuwen *et al*., 2010). However, the majority of studies appear to focus on either chlorhexidine, or one or more essential oil, making valid comparisons between the two difficult due to the vast discrepancies in results obtained from differences in experimental design (Fine, 2010).

Despite the significant differences observed in this study, any MIC and MBC data must however be taken in context, as one study by Dryden *et al*. (2004) showed that tea tree oil (TTO) was more successful at removing MRSA from wounds and superficial skin sites than CHG, despite published MIC and MBC results proclaiming CHG efficacy to be greater than that of TTO (Karpanen *et al*., 2008*b*). The environment and structure of

the skin is likely to favour the penetration properties offered by the lesser efficacious TTO over CHG, suggesting that studies need to consider and simulate the intended use rather than focus solely on numerical figures.

The decreased levels of susceptibility recorded for *P. aeruginosa* are not unusual. It is widely reported and accepted that this Gram-negative bacilli is characteristically problematic; repeatedly demonstrating increased tolerances to many antibiotics as well as chemical disinfectants including Triclosan, formaldehyde and QUATS (Chuanchuen *et al*., 2001; Meyer & Cookson, 2010). One study by Prabuseenivasan *et al*. (2006) reported findings highlighting EO to possess no antimicrobial activity at all against *P. aeruginosa* when tested using disc diffusion. Complications involving *P. aeruginosa* tolerance levels may be further exacerbated by the ability of many clinical strains to produce biofilms, in which the levels of resistance are known to rise. In addition to this, is the potential for many strains to become multi-drug resistant through the acquisition of chromosomally encoded or imported genetic mutations, or over expression of efflux pumps (Lister *et al*., 2009; Tam *et al*., 2010). In the clinical setting, this would create grave difficulties for infection treatment, making it all the more vital for new agents to be discovered or synthesised with efficacy against *P. aeruginosa*, to alleviate use of drugs to which resistance is beginning to emerge.

Synergistic antimicrobial activity was demonstrated when CHG was combined with EO or 1,8-cineole against all microorganisms, with just three combinations being calculated as indifferent. These results concur with a previous study in which EO and CHG combined were concluded to give an indifferent result against planktonic cultures of *S. epidermidis* (Karpanen *et al*., 2008*b*). As previously mentioned, it was no surprise that *P. aeruginosa* showed less susceptibility to EO and 1,8-cineole than other microorganisms tested. One study by Cox & Markham (2007) focussed on the tolerance of *Pseudomonas* spp. to essential oils and concluded some strains were tolerant to numerous volatile plant components due to an ATP-dependant efflux mechanism responsible for ejecting toxic substances from inside the cell to the extracellular environment. Efflux pumps of many varieties are known to play a significant role in antibiotic resistance in numerous microorganisms, and are likely to be of possible concern with regards resistance to environmental biocides (Webber & Piddock, 2003).

Healthcare associated infections caused by biofilm growth of skin related microorganisms on implanted, prosthetic devices such as CVCs are a major concern

within the healthcare system, and pose added complications to patient management, whilst creating extra financial burdens. Eradication of such infections would undoubtedly save lives, however accomplishment of such a feat is not proving to be simple. In the second part of this study, assessment of six microbial biofilm cultures was undertaken, as previously with planktonic cultures, in order to determine the antimicrobial efficacy or EO and 1,8-cineole alone and in combination with CHG.

Initial verification of biofilm production by all six microorganisms was confirmed using culture onto Congo red agar and staining with Crystal violet and Alcian blue. The number of cells within a typical biofilm was also quantified prior to antimicrobial assessment of EO, 1,8-cineole and CHG.

Congo red agar is a selective media that provides easy distinction between slime-producing microorganisms commonly associated with biofilms, and non slime-producing ones by creating visual differences in colony morphology, giving results that are both sensitive and reproducible (Freeman *et al*., 1989). Congo red is an acidic dye as the coloured portion of the dye is a negatively charged ion. Slime of *S. aureus* for example, is also negatively charged, causing it to repel the dye in the agar, pushing it away from the centre of the colony generating a build up around the edge of the slime (Cangelosi *et al*., 1999). This accumulation of dye creates a black, crusty appearance highly distinguishable from a negative control such as *S. hominis* which produces pink to red colonies. However, as reported by Zmantar *et al*. (2010), there is not always a correlation between slime production as seen on Congo red agar and actual biofilm formation. Therefore, the staining techniques employed aided confirmation as Crystal violet and Alcian blue are positively charged so bind to the negative surface of the cells and biofilm exopolysaccharides respectively (Serra *et al*., 2008).

Biofilms are the most commonly found mechanism of microbial growth in nature and have only been recognised as a concern within hospitals in relatively recent years (Prakash *et al*., 2003). In nature, the number of cells within a biofilm varies greatly, and often there are multitudes of different microorganisms present in the same biofilm. However, laboratory growth of biofilms can generate pure, single-organism cultures, of relatively reproducible microbial quantity due to capabilities of nutrient and temperature management. This said, there were noticeable differences in the number of cells present in the 48 hour biofilms of each microorganism, having increased from the initial inoculum of 10^4 cfu, to between 10^5 and 10^{10} cfu. Furthermore, the number of cells

within biofilms of Gram-negative microorganisms was more than within Gram-positive biofilms, this is in line with findings by Ceri *et al*. (1999) and Olson *et al*. (2002), and may be due to differences in growth rates or nutrient acquisition. There is no standard number of cells per biofilm of a specific microorganism as many factors such as water availability, space and age of biofilm can alter the rate of growth of such a dynamic system (Wang & Zhang, 2010). This may go some way to explaining the conflicting reports about exactly how many times more resistant biofilms are to agents, compared with corresponding planktonic growth.

The results for the biofilm MIC and MBC showed a clear increase in resistance compared with those of their planktonic counterparts. This is widely supported within the literature through the knowledge that biofilms act as a physical barrier, protective of the cells within. As previously observed, the diminishing efficacy of the agents showed the same arrangement; CHG, EO then 1,8-cineole. There was however, no significant difference between EO and 1,8-cineole this time proposing that even known permeation enhancers may be hindered somewhat by biofilm presence. The efficacy of EO being greater than 1,8-cineole is in line with results published in 2001 by Inouye *et al*. who reported that of 6 respiratory tract microorganisms tested, the minimum gaseous dose of EO required for microbial inhibition was less than that of 1,8-cineole for 4 microorganisms, the same for one and more for the final microorganism, indicating that other constituents in the oil aid efficacy. There were limitations to the scrape and wash method employed for the MIC/MBC determination which could potentially lead to inaccuracies in cfu counts. The cells within biofilms coagglutinate, making it possible for one colony on agar to have originated from either a single cell or a clump of microorganisms, with no means of distinguishing which.

ATP bioluminescence is frequently used in the food industry as a means of assessing hygiene levels, although it has recently increased in popularity within hospitals following a number of trials using disposable ATP kits to measure cleaning standards (Carling & Bartley, 2010; Vilar *et al*., 2008). The ViaLight MDA plus ATP detection kit used in this study consisted of a bactolyse solution which lysed cells, causing the release of ATP. In turn, this cellular ATP then reacts with the luciferin and the enzyme luciferase within the ATP monitoring reagent, producing luminescence in relative light units proportional to the amount of ATP. This method is both rapid and reliable, giving sensitive and quantified results as confirmed by the almost perfectly linear relationship

between cfu and relative light units observed in the standard curve of each microorganism.

Combination therapy is often used to combat the emergence of multi-drug resistant microorganisms. It may also be used to create synergistic effects whereby the effect of an antimicrobial used in isolation, is enhanced if used in combination. The biofilm checkerboard results in the latter part of this study supported earlier planktonic results confirming combinations of CHG with either EO or 1,8-cineole can lead to synergistic antimicrobial activity. CHG, EO and 1,8-cineole are all thought to target cell membranes thus possibly explaining the synergistic observations. The mechanisms behind this combined efficacy are as yet not fully understood, however it is possible that EO or 1,8-cineole alone are sufficient only to cause partial cell damage from lipid disruption hence the higher MIC and MBC results. When combined with CHG however, the partially damaged cells are more vulnerable to attack by that second combined agent, resulting in substantial irreparable damage to the cell membrane, resulting in loss of cellular components. This idea is supported findings by Carson *et al*. (2006) who noted that 1,8-cineole could induce higher antimicrobial properties in combinations as a result of its ability to facilitate entry for other, more active components.

Microbial colonisation is not exclusive to living entities such as human skin, and often resides on surfaces particularly within the healthcare system where biofilms are prevalent. CHG is currently used routinely within the health service however the results of this study indicate that EO and CHG may provide a useful combined agent for improved antimicrobial decolonisation, including biofilm removal, to enhance skin antisepsis and potentially surface disinfection.

2.7. Conclusion

EO, 1,8-cineole and CHG successfully demonstrated antimicrobial activity against a panel of six clinically important microorganisms, including Gram-positive and -negative bacteria, as well as yeast, in both planktonic and biofilm modes of growth. The concentration required to kill by CHG was an order of magnitude less than that for EO and 1,8-cineole, whilst comparisons between the oil and its major constituent, concluded EO to possess better overall activity than 1,8-cineole against both modes of microbial growth. The synergistic antimicrobial efficacy observed with EO and 1,8-cineole in combination with CHG may induce development of potentially beneficial applications for use within the healthcare setting. However, the increased efficacies presented by the combination of EO and CHG, bestow greater potential interest for clinical and therapeutic application, for example development of a surface disinfectant for possible enhanced removal and kill of microorganisms residing in biofilms, and improvement of current skin antisepsis procedures.

Chapter 3

Antimicrobial Efficacy of Prototype Eucalyptus Oil-Containing Wipes for Hard Surface Disinfection against a Panel of Microorganisms in Planktonic and Biofilm Modes of Growth

3.1. Introduction

Combining EO with CHG has demonstrated within chapter 2 of this thesis, synergistic antimicrobial efficacy against a panel of microorganisms, in both planktonic and biofilm modes of growth (Hendry *et al*., 2009). This offers potential for the development of a novel skin antiseptic with enhanced efficacy, as well as improved surface disinfection, in a new, combined-agent product.

Enhanced environmental cleaning and disinfection within the healthcare setting is becoming increasingly more appreciated as an integral component of infection control practice, and is often incorporated into care bundles (Griffith & Dancer 2009; Webber *et al*., 2010). Hospital cleaning and disinfection have dual purposes, firstly they preserve functionality and reinstate original appearance, and secondly they reduce microbial load therefore infection risk. Effective removal of microbial colonisation from surfaces is essential if the cycle of cross-contamination, which can lead to HAIs, is to be broken (Dancer, 2008). Further to this, any potentially pathogenic microorganisms adhering to cleaning products after removal from a surface should ideally be killed by the product in order to prevent deposition onto subsequently touched surfaces otherwise they would act solely as an inducer of cross-contamination (Bergen *et al*., 2009; Williams *et al*., 2007).

There are many cleaning products available for use within hospitals, with disposable wet-wipes frequently opted for due to ease of use. Those used most often within hospitals for high touch surfaces tend to contain alcohol as their basic active ingredient, such as Sani-Cloth® products, which comprise a 70 % (v/v) alcohol base, with and without 2 % (v/v) CHG or a detergent.

Whilst a mixture of agents may possess antimicrobial efficacy in a solution, there are many factors that can alter the activity once it has been converted into a product, especially if it is applied to a fabric or required for use over long time periods, as is the case with wet wipes (Bloss *et al*., 2010). Once applied to a fabric wipe and stored in a tub, the efficacy of a solution can change. The active ingredients may adhere, irreversibly to the fabric of the wipe through adsorption, hindering release and potentially eliminating all previously observed efficacy (Bloss *et al*., 2010). Furthermore, once a sealed tub has been opened, drying can occur, which along with degradation of agents, can result in diminishing activity over time. It is therefore

necessary that any agent intended for application onto, or conversion into a product, be thoroughly tested for efficacy in the desired, final product form.

The enhanced antimicrobial efficacy against microbial biofilms achieved by combining EO and CHG, demonstrated in chapter 2 of the thesis, suggests that such combinations could be suitable for incorporation into wet-wipes designed for hard surface disinfection.

3.2. Aims of the study

The aims of this series of experiments were to:

- Investigate the antimicrobial efficacy of various combinations of 10 % (v/v) EO, 2 % (v/v) CHG and 70 % (v/v) IPA when impregnated onto viscose/ polypropylene (50:50) wipes.
- Determine the bactericidal or bacteriostatic mode of action of the wipes as well as the associated time necessary for the wipes to exert such effect against a panel of clinically relevant microorganisms in planktonic and biofilm modes of growth.
- Assess the potential of the wipes to induce cross-contamination between surfaces.

3.3. Materials

3.3.1. Microbial cultures

Staphylococcus aureus ATCC 6538, *Staphylococcus epidermidis* RP62A, MRSA N315, *Pseudomonas aeruginosa* ATCC 15442, *Escherichia coli* NCTC 10418 and *Candida albicans* ATCC 76615 were stored on MicroBank beads (Pro-Lab Diagnostics, Cheshire, U.K.) at -70 °C until required.

3.3.2. Microbiological media

Mueller-Hinton agar (MHA), Mueller-Hinton broth (MHB), Sabouraud dextrose agar (SDA), Sabouraud dextrose broth (SDB), Tryptone soya agar (TSA) and Tryptone soya broth (TSB) were purchased from Oxoid (Basingstoke, U.K.), prepared as per manufacturer's instructions and sterilised at 121 °C for 15 minutes. Agar was cooled to 50 °C before pouring and broths were stored at 4 °C until required.

3.3.3. Chemicals

3.3.3.1. Neutralising solution

Neutralising solution was prepared with 1.17 % (w/v) lecithin, granular, molecular weight (Mw) 750.00 Acros Organics (Fisher Scientific, Leicestershire, U.K.), 2 % (v/v) tween-80 (Sigma-Aldrich, Dorset, U.K.), 0.785 % (w/v) sodium thiosulphate pentahydrate (BDH Ltd, Poole, U.K.) and 0.1 % (v/v) triton X-100 (Sigma-Aldrich, Dorset, U.K.), made up to 1 L with double distilled water. The solution was sterilised at 121 °C for 15 minutes and stored at 4 °C until required.

3.3.4. Equipment

Optical density (OD) readings were taken using a Pharmacia LKB visible spectrophotometer, Novaspec II (Pharmacia, Freiburg, Germany).

3.4. Methods

3.4.1. Wipe impregnation process and combinations

The solutions for wipe impregnation were each prepared to a total volume of 384 mL and contained various combinations of 10 % (v/v) EO, 2 % (v/v) CHG, 70 % (v/v) IPA and 0.1 % (v/v) tween-80 all purchased from Sigma-Aldrich (Dorset, U.K.) in sterile, double distilled water. In accordance with methods used by the wipe manufacturer PAL International (Leicestershire, U.K.), each solution was poured into a dispensing tub containing 200 blank, 23 grams per square metre (gsm), viscose/polypropylene (50:50) wipes provided by PAL International, and left for a minimum of 48 hours to allow saturation of the wipes before use. The combinations made were:

- 10 % (v/v) EO / 2 % (v/v) CHG / 70 % (v/v) IPA / 0.1 % (v/v) tween-80
- 10 % (v/v) EO / 2 % (v/v) CHG / 0.1 % (v/v) tween-80
- 10 % (v/v) EO / 70 % (v/v) IPA / 0.1 % (v/v) tween-80
- 2 % (v/v) CHG / 70 % (v/v) IPA
- 10 % (v/v) EO / 0.1 % (v/v) tween-80
- 2 % (v/v) CHG
- 70 % (v/v) IPA
- Sterile, double distilled water (control)

3.4.2. Verification of extraction methods for release of agents from eucalyptus oil-containing wipes

Duplicate sections measuring 4 cm diameter were cut from the EO/CHG/tween-80 wipes, and added to two universal bottles containing 10 mL PBS and 1 g sterile, borosilicate solid glass beads, 3 mm (Sigma-Aldrich, Dorset, U.K.) and were then mixed for 2 minutes. Control wipes were also set up in the same way. Sterile, cotton wool swabs were then dipped into cell suspensions of each microorganism at a concentration of 10^4 cfu/mL and used to inoculate TSA or SDA plates as appropriate. For each microorganism, two separate 20 µL drops of the PBS solution from the

EO/CHG/tween-80 and control universal bottles were placed onto the previously inoculated TSA or SDA plates. Plates were stored at 4 ºC for 1 hour, and then incubated overnight at 37 ºC; 30 ºC for *C. albicans*. Following incubation, presence or absence of an inhibition zone was observed.

3.4.3. Neutraliser efficacy within agar and broth media

Following methods by Williams *et al*. (2007), TSA and SDA plates containing 10 % (v/v) neutralising solution were prepared with sections of wipe measuring 4 cm^2 placed within the agar. A cell suspension of each microorganism was prepared in TSB or SDB from five identical colonies grown on MHA or SDA plates. A sterile cotton swab was used to inoculate the suspension over the agar to generate a semi-confluent lawn of growth. All plates were incubated overnight at either 37 °C or 30 °C for *C. albicans*, and then neutraliser efficacy was validated by the presence of microbial growth over the entire surface. In parallel to this, 4 cm^2 sections of the wipes were added to TSB and SDB containing 10 % (v/v) neutralising solution and 1 mL of cell suspension containing 10^7 cfu/mL. Broths were incubated overnight at either 37 or 30 °C then microbial viability was confirmed visually following subculture onto TSA or SDA using the Miles & Misra (1938) technique. Both experiments were repeated in triplicate. The wipes tested were:

- 10 % (v/v) EO / 2 % (v/v) CHG / 70 % (v/v) IPA / 0.1 % (v/v) tween-80
- 10 % (v/v) EO / 2 % (v/v) CHG / 0.1 % (v/v) tween-80
- 10 % (v/v) EO / 70 % (v/v) IPA / 0.1 % (v/v) tween-80
- 70 % (v/v) IPA
- Sterile, double distilled water (control)

3.4.4. Neutraliser toxicity to microorganisms

Based on methods described by Williams *et al*. (2007), a 4 cm^2 section of EO/CHG/tween-80 wipe was added to duplicate universal bottles, each containing 1 g sterile, borosilicate solid glass beads, 3 mm (Sigma-Aldrich, Dorset, U.K.) and 10 mL neutralising solution. Control wipes were also set up in parallel. All universal bottles

were mixed for 2 minutes and after 30 minutes of contact, the wipes were removed and discarded. The OD of overnight cell suspensions of all six microorganisms was determined and adjusted to 10^7 cfu/mL using previously established OD/cfu standard curves (Section 2.4.1.1), then 100 µL was added to each universal bottle of neutralising solution and mixed. After 30 minutes, serial dilutions were made in TSB or SDB as appropriate and pour plates made with molten TSA or SDA cooled to 50 °C. Following overnight incubation in air at 37 °C or 30 °C for *C. albicans*, toxicity was assessed by comparing the initial cfu/mL added and that recovered for each wipe.

3.4.5. Microbial inhibition by eucalyptus oil-containing wipes using agar diffusion

Using methods described by Williams *et al*. (2007), all six organisms were inoculated onto TSA or SDA plates using cotton wool swabs inoculated from a cell suspension containing 10^4 cfu/mL. Squares measuring 20 mm by 20 mm were cut from impregnated wipes and applied to triplicate agar plates of each microorganism. Plates were incubated in air overnight at 37 ºC; 30 ºC for *C. albicans*, then inhibition zone sizes were measured as the distance between the edge of the wipe and visible growth. The wipes tested were:

- 10 % (v/v) EO / 2 % (v/v) CHG / 70 % (v/v) IPA / 0.1 % (v/v) tween-80
- 10 % (v/v) EO / 2 % (v/v) CHG / 0.1 % (v/v) tween-80
- 2 % (v/v) CHG / 70 % (v/v) IPA
- 10 % (v/v) EO / 0.1 % (v/v) tween-80
- 2 % (v/v) CHG
- 70 % (v/v) IPA
- Sterile, double distilled water (control)

3.4.6. Bacteriostatic/bactericidal testing of eucalyptus oil-containing wipes

Following completion of the previous experiment (Section 3.4.5), the wipes were removed from the surface of the agar and a section of agar measuring 10 mm by 10 mm was cut from underneath the wipe and inserted into a bijou bottle containing 1 mL neutralising solution. The bijou bottle was mixed for 1 minute, then after 30 minutes contact with the neutralising solution, 1 mL was removed into a petri dish and a pour plate made with molten TSA or SDA containing 10 % (v/v) neutralising solution, cooled to 50 °C. Plates were incubated in air at 37 ºC or 30 ºC for *C. albicans* and microbial viability used to determine bacteriostatic or bactericidal mode of action.

3.4.7. Time-kill study of antimicrobial wipe solutions against microorganisms in planktonic mode of growth

The following solutions were prepared as described previously (Section 3.4.1):

- 10 % (v/v) EO / 2 % (v/v) CHG / 70 % (v/v) IPA / 0.1 % (v/v) tween-80
- 10 % (v/v) EO / 2 % (v/v) CHG / 0.1 % (v/v) tween-80
- 2 % (v/v) CHG / 70 % (v/v) IPA
- Sterile, double distilled water (control)

Overnight cell suspensions of each microorganism were diluted to 10^8 cfu/mL (10^7 cfu/mL for *C. albicans*) and 1 mL of each added to 9 mL of each of the test and control solutions listed above, then mixed. After 30 seconds, 1, 2, 5, 10, 30 and 60 minutes, 1 mL of the inoculated test agent and control was removed and mixed with 9 mL of neutralising solution. Serial dilutions of all neutralising solutions were made in TSB or SDB and pour plates of TSA or SDA made. Plates were incubated overnight at 37 ºC or 30 ºC as necessary. The time to kill was determined as the time resulting in a 99.9 % reduction in cfu/mL from that of the control.

3.4.8. Time-kill study of eucalyptus oil-containing wipes against microorganisms in biofilm mode of growth

Overnight suspensions were prepared of each microorganism in MHB or SDB as required, then diluted to 10^4 cfu/mL. Petri dishes were set up with a double thickness layer of a sterile cloth in the base, moistened with sterile, double distilled water. Stainless steel discs were cut to 1.5 cm^2 and placed on the moistened cloth. Biofilms were grown on the discs following inoculation of 100 µL of the diluted cell suspension onto each disc, before the petri dishes were sealed with Sellotape® to prevent drying, and incubated for 48 hours at 37 °C; 30 ºC for *C. albicans* to allow biofilm formation in conditions verified as appropriate in chapter 2 (Section 2.5.2.1). Following incubation, the excess broth was discarded from the discs and each was washed twice with PBS (Sigma-Aldrich, Dorset, U.K.). A sterile cotton swab dipped into 70 % (v/v) ethanol water was used to wipe the reverse of the disc before being washed once more with PBS and allowed to dry. The following wipes were tested:

- 10 % (v/v) EO / 2 % (v/v) CHG / 70 % (v/v) IPA, 0.1 % (v/v) tween-80
- 10 % (v/v) EO / 2 % (v/v) CHG / 0.1 % (v/v) tween-80
- 2 % (v/v) CHG / 70 % (v/v) IPA
- 70 % (v/v) IPA
- Sterile, double distilled water (control)

Sections of each wipe were added to the base of fresh petri dishes and the discs placed on top such that the biofilm was in contact with the wipe. A 10 g weight was then placed over the discs to ensure constant contact. After minute intervals up to 10 minutes, 30 minutes, 1, 2, 4 and 6 hours, the discs were removed and added to 10 mL neutralising solution containing 1 g sterile, borosilicate solid glass beads, 3 mm (Sigma-Aldrich, Dorset, U.K.) and mixed. Following 30 minutes contact, serial dilutions were made in MHB or SDB and pour plates of MHA or SDA made. Plates were incubated overnight at 37 ºC or 30 ºC as necessary. The time to kill was determined as the time resulting in a 99.9 % reduction in cfu/mL from that of the control. The experiment was repeated in duplicate.

3.4.9. Microbial transfer from wipes onto subsequently touched surfaces

Based on methods described by Williams *et al*. (2007), 20 µL drops of overnight cell suspensions of *S. epidermidis*, *E. coli* and *C. albicans*, diluted to 10^8 cfu/mL were added to 4 cm^2 sections of the following wipes:

- 10 % (v/v) EO / 2 % (v/v) CHG / 70 % (v/v) IPA / 0.1 % (v/v) tween-80
- 10 % (v/v) EO / 2 % (v/v) CHG / 0.1 % (v/v) tween-80
- 10 % (v/v) EO / 70 % (v/v) IPA / 0.1 % (v/v) tween-80
- 70 % (v/v) IPA
- Sterile, double distilled water (control)

Each wipe was then pressed consecutively onto eight TSA or SDA plates containing 10 % (v/v) neutralising solution with approximately 5 seconds between plates, before being added to 10 mL TSB or SDB with 10 % (v/v) neutralising solution. All plates and broths were incubated in air overnight at either 37 or 30 °C as required. The wipes were deemed as positive or negative for inducing surface cross-contamination dependent upon the presence or absence of growth on the TSA or SDA plates. Meanwhile, serial dilutions were preformed on the broths and then plated onto TSA or SDA using the Miles & Misra (1938) technique. Following a further overnight incubation, microbial viability was used to confirm neutraliser efficacy. The experiment was performed in triplicate.

3.4.10. Removal and spread of microbial surface contamination by eucalyptus oil-containing wipes

Following the methods of Williams *et al*. (2007), 20 µL drops of overnight suspensions of each microorganism diluted to 10^8 cfu/mL were inoculated onto stainless steel discs cut to 1.5 cm^2, and allowed to dry in air. The discs were then systematically wiped back and forth five times by hand to assess surface removal; the discs were subsequently added to neutralising solution containing 1 g sterile, borosilicate solid glass beads, 3 mm (Sigma-Aldrich, Dorset, U.K.). After mixing for 1 minute and a total of 30 minutes

in contact with the neutraliser, serial dilutions were made in TSB or SDB and pour plates made with molten TSA or SDA cooled to 50 °C. Meanwhile, the contaminated wipes were pressed onto eight consecutive TSA or SDA plates containing 10 % (v/v) neutralising solution then added to TSB or SDB with 10 % (v/v) neutralising solution. The plates from the surface removal and adpression tests were incubated overnight at 37 °C; 30 °C for *C. albicans* along with the broths containing the contaminated wipes. Following incubation, viable colony counts determined the number of cells that had failed to be removed from the discs by wiping therefore assessing the wipes' ability to physically remove microbial contamination from surfaces. Positive or negative growth results from the adpression plates were used to determine the potential of each wipe to induce surface cross-contamination, while the broths containing the wipes were subcultured into TSA or SDA plates using the Miles & Misra (1938) technique and further incubated overnight before microbial viability could be determined. Experiments were performed in triplicate for the following wipes:

- 10 % (v/v) EO / 2 % (v/v) CHG / 70 % (v/v) IPA / 0.1 % (v/v) tween-80
- 2 % (v/v) CHG / 70 % (v/v) IPA
- Sterile, double distiller water (control)

3.5. Results

3.5.1. Verification of extraction methods for release of agents from eucalyptus oil-containing wipes

Antimicrobial activity was demonstrated by the PBS extraction solution, thus confirming the efficacy of the extraction method. Inhibition zones were observed for each microorganism with a mean radius of 20 mm (range 9 - 28 mm). The control did not show any antimicrobial activity.

3.5.2. Neutraliser efficacy within agar and broth media

The neutraliser was effective against all wipes in both broth and agar, for all microorganisms tested. Semi-confluent growth was observed over the surface of all agar plates, with no visible difference between the growth above the wipe and elsewhere on the agar. Microbial viability was visually confirmed from all subcultured broths.

3.5.3. Neutraliser toxicity to microorganisms

The neutraliser was not toxic to any of the microorganisms tested as there was no significant difference ($P>0.05$, ANOVA, Instat3, GraphPad) between the number of cells initially added and that recovered. This was the case for the EO/CHG/tween-80 wipes as well as the control wipes.

3.5.4. Microbial inhibition by eucalyptus oil-containing wipes using agar diffusion

All wipes impregnated with a combination of agents as well as the CHG wipes, demonstrated antimicrobial activity (Table 3.1), however, the EO/tween-80 and the IPA wipes resulted in zones of <1 mm diameter, showing no difference in antimicrobial efficacy from that of the control wipes, which were deemed as not contributing to any antimicrobial activity observed. For all microorganisms, growth was clearly visible underneath the EO/tween-80, IPA and control wipes. Significant differences were observed between the antimicrobial efficacy of the CHG and the CHG/IPA wipes

(P<0.01, Paired t-test, Instat3 GraphPad) as well as between the CHG/IPA and the EO/CHG/IPA/tween-80 wipes (P<0.001, Paired t-test, Instat3 GraphPad).

Table 3.1. Antimicrobial efficacy of test and control wipes against a panel of six microorganisms expressed as mean (n=3) sizes of inhibition zones observed using the agar diffusion method, and the standard deviation.

	Inhibition zone (mm) for wipes/standard deviation						
	EO/CHG /IPA	EO/ CHG	CHG/ IPA	EO	CHG	IPA	Control
S. aureus	9/1.0	6/1.7	9/1.5	<1/0	6/0	<1/0	<1/0
S. epidermidis	8/1.0	7/1.2	12/2.0	<1/0	8/1.0	<1/0	<1/0
MRSA	8/0.6	4/1.2	11/1.5	<1/0	7/0	<1/0	<1/0
P. aeruginosa	1/0.6	<1/0	2/1.2	<1/0	3/1.2	<1/0	<1/0
E. coli	5/1.0	6/3.0	7/2.5	<1/0	5/0	<1/0	<1/0
C. albicans	6/1.7	<1/0	6/1.5	<1/0	7/0.6	<1/0	<1/0

3.5.5. Bacteriostatic/bactericidal testing of eucalyptus oil-containing wipes

Bacteriostatic activity was determined following observation of microbial growth on agar by all six microorganisms when challenged by EO/tween-80 wipes, IPA wipes and control wipes. Bactericidal antimicrobial activity was demonstrated by CHG wipes against all six microorganisms, as well as by EO/CHG/tween-80 wipes against *S. aureus*, *S. epidermidis* and *E. coli*. The EO/CHG/IPA/tween-80 wipes showed bactericidal activity against all microorganisms except *P. aeruginosa*, for which bacteriostatic activity was concluded.

3.5.6. Time-kill study of antimicrobial wipe solutions against microorganisms in planktonic mode of growth

The CHG/IPA wipes, EO/CHG/IPA/tween-80 wipes, and the EO/CHG/tween-80 wipes killed planktonic cells of each microorganism in under 30 seconds. The control wipes did not possess any antimicrobial activity against any of the microorganisms.

3.5.7. Time-kill study of eucalyptus oil-containing wipes against microorganisms in biofilm mode of growth

Variations in the time required to kill the cells within biofilms of each microorganism were observed (Table 3.2), however overall the EO/CHG/IPA/tween-80 wipes demonstrated significantly enhanced ($P<0.05$, ANOVA, Instat3, GraphPad) antimicrobial activity compared with EO/CHG/tween-80 wipes, and also control wipes, with considerably less time required. Whilst the difference between the efficacy of EO/CHG/IPA/tween-80 wipes and either CHG/IPA or IPA wipes was not statistically significant, there is an obvious distinction between their respective times, with the MRSA result for EO/CHG/IPA/tween-80 potentially affecting the statistical analysis as the CHG/IPA and IPA results are largely in terms of hours, while EO/CHG/IPA/tween-80 is in minutes.

Table 3.2. Antimicrobial efficacy of test and control wipes against a panel of six microorganisms grown in biofilms on stainless steel discs, expressed as mean (n=2) time to eradicate the biofilm.

	Time for wipe to eradicate biofilm				
	EO/CHG/IPA	EO/CHG	CHG/IPA	IPA	Control
S. aureus	< 10 min	< 6 hr	> 6 hr	> 6 hr	> 6 hr
S. epidermidis	< 3 min	> 6 hr	< 9 min	< 10 min	> 6 hr
MRSA	< 4 hr	< 4 hr	> 6 hr	> 6 hr	> 6 hr
P. aeruginosa	< 1 min	> 6 hr	< 4 hr	< 6 hr	> 6 hr
E. coli	< 2 min	< 4 hr	< 10 min	< 10 min	> 6 hr
C. albicans	< 10 min	< 6 hr	< 4 hr	< 6 hr	> 6 hr

3.5.8. Microbial transfer from wipes onto subsequently touched surfaces

Cross contamination was demonstrated across all eight consecutively touched surfaces by all wipes tested, with all three microorganisms (Table 3.3).

Table 3.3. Potential for microbial transfer of three microorganisms, between surfaces by test and control wipes, expressed as mean (n=3) number of surfaces positive for contamination following adpression by an inoculated wipe over an approximate time period of 40 seconds.

	Number of positively cross contaminated surfaces by wipes				
	EO/CHG/IPA	EO/CHG	CHG/IPA	IPA	Control
S. epidermidis	8	8	8	8	8
E. coli	8	8	8	8	8
C. albicans	8	8	8	8	8

3.5.9. Removal and spread of microbial surface contamination by eucalyptus oil-containing wipes

Compared with the initial inoculum, all wipes demonstrated at least a 1-log reduction in the number of cells remaining on the discs after wiping, except for CHG/IPA against MRSA which showed only a 0.2-log reduction (Figure 3.1). Overall, EO/CHG/IPA/tween-80 wipes possessed significantly enhanced ($P<0.05$, AVONA, Instat3, GraphPad) removal ability against microorganisms dried onto a surface than both the CHG/IPA and control wipes, with a mean cfu reduction of 6.3-log compared with the 2.3-log reduction observed by CHG/IPA and control wipes. The adpression and viability results (Table 3.4) demonstrated that both test and control wipes possess the ability to induce cross-contamination between surfaces, with microbial viability remaining in many instances after all eight surfaces had been touched. Some differences however were observed in which cross-contamination had not persisted over all 8 surfaces and microbial viability had not persisted.

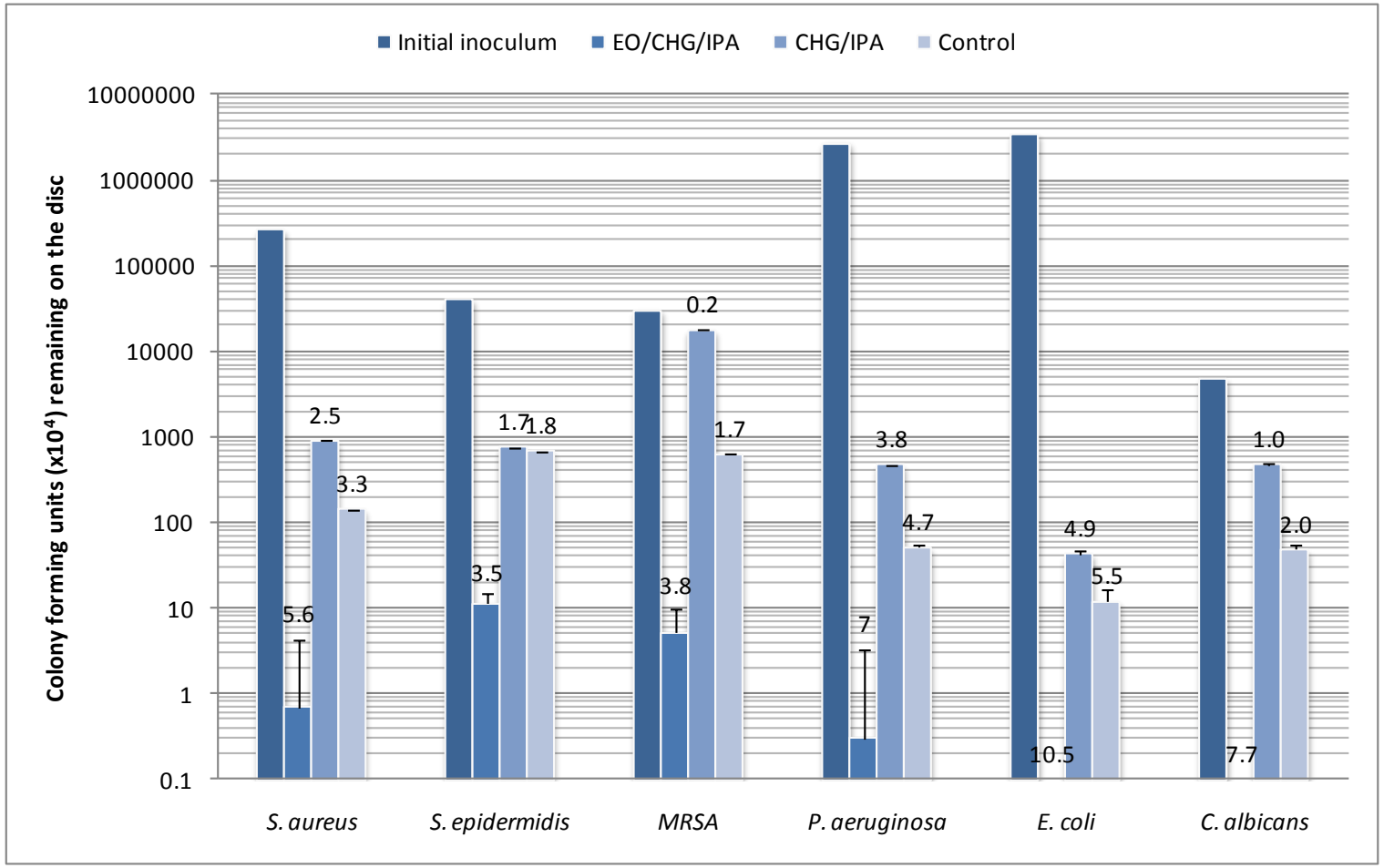

Figure 3.1. Reduction in mean (n=3) cfu/disc of a panel of planktonic microorganisms following wiping with test and control wipes, with the log reduction from the initial inoculum and standard deviation.

Table 3.4. Mean number (n=3) of surfaces positive for cross-contamination following adpression onto agar of wipes soiled with microorganisms (from the previous experiment), with microbial viability after broth culture of the wipes indicated by + for viable, and - for not viable.

	Surfaces positive for cross-contamination/Microbial viability		
	EO/CHG/IPA	CHG/IPA	Control
S. aureus	8/+	8/+	8/+
S. epidermidis	8/+	8/+	8/+
MRSA	8/+	0/-	8/+
P. aeruginosa	8/+	0/-	8/+
E. coli	4/-	8/+	8/+
C. albicans	0/-	0/-	8/+

3.6. Discussion

Infections resulting from hospitalisation or patients undergoing a healthcare intervention remain a persistent concern for both medical staff and patients alike. Whilst there have been many incentives to encourage hand hygiene particularly over recent years, cleanliness of surfaces often remains largely overlooked. Controversy continues over the contribution of environmental surfaces to HAI however it is clear that ensuring cleanliness of high touch surfaces can have a positive effect at reducing nosocomial infections (Hota, 2004; Webber *et al*., 2010). Many studies have confirmed the potential hazards posed by microorganisms on hospital surfaces, including Boyce *et al*. whom as early as 1997, brought to attention the discovery that staff entering the room of an MRSA positive patient, could subsequently be found with MRSA contamination of their gloves, despite having had no direct contact with that patient. Thus confirming a residing microbial population on environmental surfaces can lead to cross-contamination and ultimately provide potential for infection dissemination.

Within the healthcare system, cleaning regimes are set in place that suggest the frequency and degree with which objects and surfaces should be cleaned, such as daily cleaning of rooms and equipment, or weekly cleaning of patient care devices (Rutala *et al*., 2008). These guidelines take into consideration the likelihood of contamination occurring as well as the residual activity of the product with which cleaning is suggested. Research into the duration of microbial persistence on surfaces has shown that *C. albicans* can survive on dry surfaces, such as glass and stainless steel for up to 3 days, and on fabric for over 14 days (Traore *et al*., 2002). Furthermore, the same study demonstrated recovery of 20 % of the original *C. albicans* inoculum applied to skin, for up to an hour after inoculation, allowing sufficient time for contaminated hands to transfer microorganisms to a surface. Another study by Otter & French (2009) confirmed viability and desiccation resistance of some other hospital pathogenic inocula including dried MRSA, VRE, *C. difficile* spores and *Acinetobacter* spp. after 6 weeks.

It is assumed that there is an inversely proportional relationship between the survival time of a microorganism on a surface and the surrounding air temperature, whereby at lower temperatures such as around 6 °C, cells can remain viable longer than at higher ones of around 22 °C (Traore *et al*., 2002). This may explain differences observed between long viability durations on surfaces compared with shorter ones on skin. With such potentially long durations of microbial survival on surfaces, it is paramount that

good hand hygiene is continually enforced and that cleaning protocols are not only employed regularly, but that they are sufficient to remove and kill any pathogenic contaminants.

The results of this study provide an indication of the potential efficacy of a biocide wipe developed at Aston University and designed for hard surface disinfection, and based upon the combination of agents found to show antimicrobial synergy in the previous chapter. In a solution, the combination of EO and CHG has previously demonstrated enhanced antimicrobial efficacy against a panel of microorganisms both in planktonic and biofilm cultures. The results of this study support the previous findings, with the added benefit of efficacy remaining following adaptations of the solution to include IPA and application of it onto a viscose/polypropylene wipe.

The different wipes developed and tested in this series of experiments demonstrated mixed efficacy against the panel of microorganisms chosen for their clinical importance. EPIC guidelines by Pratt *et al*. (2007) currently recommend the use of 2 % CHG in 70 % IPA for skin antisepsis due to its potent antimicrobial efficacy, something that was supported in the early stages of this study by the significant differences observed between this and CHG alone in the agar diffusion test. The addition of EO to CHG/IPA wipes appeared to slightly reduce the zone of inhibition. This may be due to slightly uneven diffusion of agents through the wipes during the impregnation process caused by partitioning of the oil from the mixture, impeded release of EO from the fabric of the wipes, or the heightened sensitivity of *S. epidermidis* and MRSA to wipes comprising CHG/IPA, compared with EO/CHG/IPA or CHG wipes, creating skewed data when tested statistically in groups of wipe components rather than microorganism and wipe components. It is also possible that EO is unable to diffuse through the agar to the same extent as the other agents, therefore restricting CHG and IPA movement and resulting in a reduced zone of inhibition.

During formulation of the wipes, the solution of EO and CHG used in chapter 2 was amended to incorporate IPA and a replacement of DMSO with tween-80 was made. This addition of tween-80 may be responsible for observations of reduced efficacy by the EO-containing wipes compared with the CHG/IPA wipes, noticeable in the agar diffusion experiment. Tween-80 is frequently used as an ingredient within neutralising solutions and therefore potentially could have partially or entirely inactivated the CHG present within the EO-containing wipes, leading to reduced efficacy. The effect would

not have been observed in the CHG/IPA wipes however as the absence of EO prevented solution precipitation and therefore avoided the requirement for tween-80 addition. However, throughout the literature, the use of tween-80 alone as a neutraliser appears to not be common practice, with other agents and lecithin in particular, frequently added to the neutralising solution. Furthermore, one study by Kampf *et al*. (1998) reported that the combination of tween-80, cysteine, histidine and saponine were insufficient at quenching the activity of CHG, therefore supporting the probability that whilst the addition of tween-80 may have had a reducing effect upon the wipes' activity, it is unlikely to have been a significant impact.

In line with common knowledge and previous findings, *P. aeruginosa* demonstrated decreased susceptibility to the bactericidal activity of EO/CHG/IPA wipes conferred by the other microorganisms tested with bacteriostatic activity being concluded. Yet again, the presence of EO led to a reduction in the overall efficacy against the panel compared with the CHG/IPA wipes however as the same wipes were used in the agar diffusion test, the possible justifications of outcome remain the same as previously described.

Biofilm presence is a serious complication of any surgical procedure due to the extra resiliencies and resistances portrayed during physical or antimicrobial elimination. This can be highlighted somewhat if comparisons between the two time kill experiments are made. When applied to planktonic cultures of six microorganisms, the solutions used to impregnate the wipes were capable of achieving complete eradication of all cells within 30 seconds. However, when biofilms of the same microorganisms were challenged with wipes impregnated with those same solutions, viable cells of some microorganisms persisted even after 6 hours of exposure to the wipes. This may be partly due to the change in form, from a liquid solution to a wipe in which the solution may be slightly contained, either delaying or preventing release. However, previous results confirmed the wipes do possess efficacy therefore the reminder of the justification may be attributed to the biofilm itself.

Microfibre wipes are now used frequently within hospitals and are reported to possess superior cleaning properties compared with wipes and cloths of other materials such as cotton, sponge and paper towels (Diab-Elschahawi *et al*., 2010). Their surface area can be up to 40 times greater than that of a conventional cotton wipe due to their composition which is assembled from strands of synthetic fibre, less than one hundredth the thickness of a human hair, woven together (Diab-Elschahawi *et al*., 2010).

Furthermore, a study by Wren *et al*. (2008) concluded the ability of ultra-microfibre wipes, which contain even thinner fibres, to remove microbial contamination so triumphant, that complete or almost complete removal of bacteria and spores from rough tile, laminate and stainless steel surfaces could be achieved when only wetted with water. This recognises the importance held by cleaning product material however as yet, there are no guidelines for standards of cleaning equipment for clinical use despite many recommendations covering disinfectants (Diab-Elschahawi *et al*., 2010). All of the wipes used in this study were non-woven, and not microfibre therefore if produced in a different material, could potentially show improved results to those witnessed here.

Hospital cleaning is aimed largely at removing contamination of microbial origin, to enable management of infection without risk of inanimate objects acting as reservoirs for transmission. However, poor cleaning practice can promote rather than conquer this by collecting, and then depositing elsewhere, any potential microbial pathogens picked up. In order to eliminate this, improvements to staff awareness of microbial transmission routes needs addressing through education. Along with this, advances in product development can ease the burden by simplifying how it is used. The results for the final parts of this study indicated that all wipes tested possessed some capability to transmit collected microorganisms onto multiple, subsequently touched surfaces. This may be due to a time aspect exclusive to the laboratory conditions in which the surfaces were touched in reasonably quick succession, therefore potentially failing to provide adequate time between wiping to permit successful biocidal activity. In clinical practice, this time period may be increased therefore provide adequate time for the wipes to kill collected microbial contamination between consecutive surface contacts. There are however, many studies that support the findings here whereby cloths act as carriers, increasing spread of microorganisms around multitudes of surfaces, and even some that report cloths to encourage microbial multiplication within them (Bartz *et al*., 2010). In the clinical setting, this could lead to infection outbreaks, as without bactericidal action the wipes would require folding to allow a fresh side to be the contact surface; however this adds potential complications for the user (Moore & Griffith, 2006). Therefore these EO-containing wipes would need to be aimed at single use so they are only used on one surface before being discarded.

The final outcomes of the study appear promising with significant increases in efficiency demonstrated by the EO/CHG/IPA wipes compared with the CHG/IPA

wipes, when removal of dried microorganisms from surfaces was investigated. It is possible that the penetration properties of EO may have influenced this by helping with cell detachment from the surface, and would suggest a commercially produced product with such improved qualities from those currently available may have a place in the market within healthcare hard surface disinfectants.

3.7. Conclusion

The results of this study confirm development of an EO-containing wipe, could have potential as a hard surface disinfection product, designed for the healthcare service. The enhanced antimicrobial efficacy of the EO/CHG combined solution from the previous study has remained present in this series of experiments, following adaptation of the solution to include IPA, and progression from a liquid into a wet-wipe format. The CHG wipes demonstrated bactericidal activity against all six microorganisms, and the CHG/IPA wipes killed planktonic cultures within the same time period as the EO/CHG/IPA wipes. However, the EO/CHG/IPA wipe demonstrated significant improvements over currently used CHG/IPA wipes for the removal of microbial contamination from surfaces, as well as a clear reduction in the time required to eradicate microbial biofilms from a surface. The clinical implications of microbial contamination on inanimate surfaces, and biofilm modes of growth, are substantial, therefore the prototype EO-containing wipe developed and assessed within this study demonstrated more suitability for clinical use and could provide an adjunct to current infection control procedures if optimised further as a hard surface disinfectant wipe.

Chapter 4

Antimicrobial Efficacy of Novel Euclean® Wipes Comprising Eucalyptus Oil, Chlorhexidine Digluconate and Isopropyl Alcohol, for Enhanced Hard Surface Disinfection against a Panel of Microorganisms

4.1. Introduction

Microbial contamination of environmental surfaces within the healthcare setting can impose additional risks to those potentially most at risk, such as already ill patients, with possible increased susceptibility to infection from pre-existing conditions or treatment regimes. Effective cleaning and disinfection is therefore of great importance within the entire package governing patient care, wellbeing and recovery. Furthermore, increases in prevalence of multidrug resistant microorganisms, drives the need for novel cleaning products with enhanced antimicrobial activity to aid combat of HAIs.

Objects within the clinical environment that come into contact with intact skin are deemed low-level, non-critical items, and include bed rails, bedside tables, crutches and floors. Such items are often cleaned with IPA, sodium hypochlorite, QUATS or diluted iodophor detergents (Rutala *et al*., 2008). Whilst the expectation of these items to cause infection is lower than objects that come into contact with broken skin or mucous membranes, such as endoscopes and respiratory apparatus, the capability remains therefore such articles must not be overlooked during development of cleaning products.

Eucalyptus oil, when combined with chlorhexidine, has demonstrated synergistic antimicrobial efficacy against planktonic and biofilm modes of growth (Hendry *et al*., 2009). Moreover, studies in the previous chapter involving a prototype hard surface disinfectant wipe containing this combination of agents have shown promising results in their ability to remove microorganisms adhering to surfaces similar to those frequently observed on low-level, non-critical objects.

New ideas for surface disinfectants are constantly coming to light however conversion from an initial prototype to an actual product, through the process of commercial up-scaling can introduce unforeseen problems with formulation or processing, for example. It therefore follows that antimicrobial testing of the final product is required in addition to preliminary studies, to ensure adequate efficacy is maintained.

4.2. Aims of the study

The aims of this study were to:

- Develop further the preliminary research in chapter 3 to assess the antimicrobial efficacy of EO and CHG combined within a hard surface disinfectant wipe called Euclean® wipes, developed with Insight Health Ltd, Wembley, U. K. The wipes contain a reduced concentration of EO and increased concentration of tween-80 than used previously, to resolve problems arising from the oil and other agents partitioning.

- Assess the potential of 5 % Euclean® wipes which comprise 5 % (v/v) EO, 2 % (v/v) CHG, 70 % (v/v) IPA and 1 % (v/v) tween-80, and 2 % Euclean® wipes comprising 2 % (v/v) EO, 2 % (v/v) CHG, 70 % (v/v) IPA and 1 % (v/v) tween-80 to induce cross-contamination between surfaces.

- Determine the bactericidal or bacteriostatic mode of action of the wipes as well as the time necessary for them to exert an antimicrobial effect against a panel of clinically relevant microorganisms in biofilm modes of growth.

- Compare the antimicrobial efficacy of 5 % Euclean® wipes and 2 % Euclean® wipes to determine potential formulation optimisations.

4.3. Materials

4.3.1. Microbial cultures

Staphylococcus aureus ATCC 6538, *Staphylococcus epidermidis* RP62A, MRSA N315, *Pseudomonas aeruginosa* ATCC 15442, *Escherichia coli* NCTC 10418 and *Candida albicans* ATCC 76615 were stored on MicroBank beads (Pro-Lab Diagnostics, Cheshire, U.K.) at -70 °C until required.

4.3.2. Microbiological media

Mueller-Hinton agar (MHA), Mueller-Hinton broth (MHB), Sabouraud dextrose agar (SDA), Sabouraud dextrose broth (SDB), Tryptone soya agar (TSA) and Tryptone soya broth (TSB) were purchased from Oxoid (Basingstoke, U.K.), prepared as per manufacturer's instructions and sterilised at 121 °C for 15 minutes. Agar was cooled to 50 °C before pouring and broths were stored at 4 °C until required.

4.3.3. Chemicals

4.3.3.1. Neutralising solution

Neutralising solution was prepared with 1.17 % (w/v) lecithin, granular, Mw 750.00 Acros Organics (Fisher Scientific, Leicestershire, U.K.), 2 % (v/v) tween-80 (Sigma-Aldrich, Dorset, U.K.), 0.785 % (w/v) sodium thiosulphate pentahydrate (BDH Ltd, Poole, U.K.) and 0.1 % (v/v) triton X-100 (Sigma-Aldrich, Dorset, U.K.), made up to 1 L with double distilled water. The solution was sterilised at 121 °C for 15 minutes and stored at 4 °C until required.

4.3.4. Equipment

Optical density (OD) readings were taken using a Pharmacia LKB visible spectrophotometer, Novaspec II (Pharmacia, Freiburg, Germany).

4.3.5. Euclean® disinfectant wipes

4.3.5.1. 5 % Euclean® wipes

The 5 % Euclean® wipes were produced by PAL International (Leicestershire, U.K.) by application of 5 % (v/v) EO, 2 % (v/v) CHG, 70 % (v/v) IPA and 1 % (v/v) tween-80 in water sprayed into tubs containing 200 blank, 23 gsm, viscose/polypropylene (50:50) non-woven wipes. Tubs were left standing for a minimum of 48 hours to allow saturation of the wipes before being delivered for use.

4.3.5.2. 2 % Euclean® wipes

The 2 % Euclean® wipes were produced by PAL International (Leicestershire, U.K.) by application of 2 % (v/v) EO, 2 % (v/v) CHG, 70 % (v/v) IPA and 1 % (v/v) tween-80 in water sprayed into tubs containing 200 blank, 23 gsm, viscose/polypropylene (50:50) non-woven wipes. Tubs were left standing for a minimum of 48 hours to allow saturation of the wipes before being delivered for use.

4.4. Methods

4.4.1. Wipe impregnation process and combinations

4.4.1.1. Chlorhexidine digluconate and isopropyl alcohol wipes

The solution for wipe impregnation was prepared to a total volume of 384 mL and contained 2 % (v/v) CHG and 70 % (v/v) IPA both purchased from Sigma-Aldrich (Dorset, U.K.) in sterile, double distilled water. In accordance with methods used by the wipe manufacturer PAL International (Leicestershire, U.K.), the solution was poured into a tub containing 200 blank, 23 gsm, viscose/polypropylene (50:50) non-woven wipes provided by PAL International, and left for a minimum of 48 hours to allow saturation of the wipes before use.

4.4.1.2. Control wipes

The solution for wipe impregnation was prepared to a total volume of 384 mL and contained only sterile, double distilled water. In accordance with methods used by the wipe manufacturer PAL International (Leicestershire, U.K.), the solution was poured into a tub containing 200 blank, 23 gsm, viscose/polypropylene (50:50) non-woven wipes provided by PAL International, and left for a minimum of 48 hours to allow saturation of the wipes before use.

4.4.2. Removal and spread of microbial surface contamination by Euclean® wipes

Following the methods of Williams *et al*. (2007), 20 µL drops of overnight cell suspensions of MRSA, *E. coli* and *C. albicans* diluted to 10^8 cfu/mL were inoculated onto stainless steel discs cut to 1.5 cm^2, and allowed to dry in air. The discs were then systematically wiped back and forth five times by hand with either a 5 % Euclean® wipe, a 2 % Euclean® wipe or a control (sterile, double distilled water) wipe to assess surface removal; the discs were subsequently added to neutralising solution containing 1 g sterile, borosilicate solid glass beads, 3 mm (Sigma-Aldrich, Dorset, U.K.). After mixing for 1 minute and a total of 30 minutes in contact with the neutraliser, serial dilutions were made in TSB or SDB and pour plates made with molten TSA or SDA

cooled to 50 °C. Meanwhile, the contaminated wipes were pressed onto eight consecutive TSA or SDA plates containing 10 % (v/v) neutralising solution then added to TSB or SDB with 10 % (v/v) neutralising solution. The plates from the surface removal and adpression tests were incubated overnight at 37 °C, and 30 °C for *C. albicans* along with the broths containing the contaminated wipes. Following incubation, viable colony counts were undertaken to determine the number of cells that had failed to be removed from the discs by wiping therefore assessing the wipes' ability to physically remove microbial contamination from surfaces. Positive or negative growth results from the adpression plates were used to determine the potential of each wipe to induce surface cross-contamination, while the broths containing the wipes were subcultured into TSA or SDA plates using the Miles & Misra (1938) technique and further incubated overnight before microbial viability could be determined. Experiments were performed in triplicate.

4.4.3. Microbial inhibition by Euclean® wipes using agar diffusion

Using methods described by Williams *et al*. (2007), all six organisms were inoculated onto TSA or SDA plates using cotton wool swabs inoculated from a cell suspension containing 10^4 cfu/mL. Squares of 20 mm by 20 mm were cut from impregnated wipes and applied to triplicate agar plates of each microorganism. Plates were incubated overnight at 37 ºC, and 30 ºC for *C. albicans*, and inhibition zone sizes were measured as the distance between the edge of the wipe and visible growth. The wipes tested were:

- 5 % Euclean®
- 2 % Euclean®
- Sterile, double distiller water (control)

4.4.4. Bacteriostatic/bactericidal testing of Euclean® wipes

Following completion of the previous experiment (Section 4.4.3), the wipes were removed from the surface of the agar and a section of agar measuring 10 mm by 10 mm was cut from underneath the wipe and inserted into a bijou bottle containing 1 mL

neutralising solution. The bijou bottle was mixed for 1 minute, then after 30 minutes contact with the neutralising solution, 1 mL was removed into a petri dish and a pour plate made with molten TSA or SDA containing 10 % (v/v) neutralising solution, cooled to 50 °C. Plates were incubated and microbial viability used to determine bacteriostatic or bactericidal mode of action.

4.4.5. Time-kill study of Euclean® wipes against microorganisms in biofilm mode of growth

Overnight cell suspensions were prepared of each microorganism in MHB or SDB as required, then diluted to 10^4 cfu/mL. Petri dishes were set up with a double thickness layer of a sterile cloth in the base, moistened with sterile, double distilled water. Stainless steel discs were cut to 1.5 cm^2 and placed on the moistened cloth. Each disc was then inoculated with 100 µL of the diluted cell suspension before the petri dishes were sealed with Sellotape® and incubated for 48 hours at 37 °C; 30 ºC for *C. albicans*. Following incubation, the excess broth was discarded from the discs and each was washed twice with PBS (Sigma-Aldrich, Dorset, U.K.). A sterile cotton swab dipped into 70 % (v/v) ethanol water was used to wipe the reverse of the disc before being washed once more with PBS and allowed to dry. The following wipes were tested:

- 5 % Euclean®
- 2 % Euclean®
- 2 % (v/v) CHG / 70 % (v/v) IPA

Sections of each wipe were added to the base of fresh petri dishes and the discs placed on top such that the biofilm was in contact with the wipe. A 10 g weight was then placed over the discs to ensure constant contact. At time zero, and every 5 minutes up to 30 minutes, the discs were removed and added to 10 mL neutralising solution containing 1 g sterile, borosilicate solid glass beads, 3 mm (Sigma-Aldrich, Dorset, U.K.) and mixed. Following 30 minutes contact, serial dilutions were made in MHB or SDB and pour plates of MHA or SDA made. Plates were incubated overnight at 37 ºC or 30 ºC as necessary. The time to kill was determined as the time resulting in a 99.9 % reduction in cfu/mL from that of the control. The experiment was performed in triplicate.

4.5. Results

4.5.1. Removal and spread of microbial surface contamination by Euclean® wipes

Both 5 % and 2 % Euclean® wipes induced a minimum 2-log reduction in the number of cells remaining on the disc after wiping, compared with that of the initial inoculum (Figure 4.1). Whilst there was no significant difference ($P>0.05$, ANOVA, Instat3, GraphPad) between the overall log reductions demonstrated by the two different percentage Euclean® wipes and the control wipe, there was a significant difference ($P<0.05$, Paired t-test, Instat3 GraphPad) for each microorganism/wipe combination, between the cfu in the initial inoculum and that remaining after wiping. The adpression and viability results (Table 4.1) demonstrated that unlike the control wipes, neither 5 % Euclean® nor 2 % Euclean® wipes induced cross-contamination onto successively touched surfaces; furthermore, they had not permitted endurance of microbial viability.

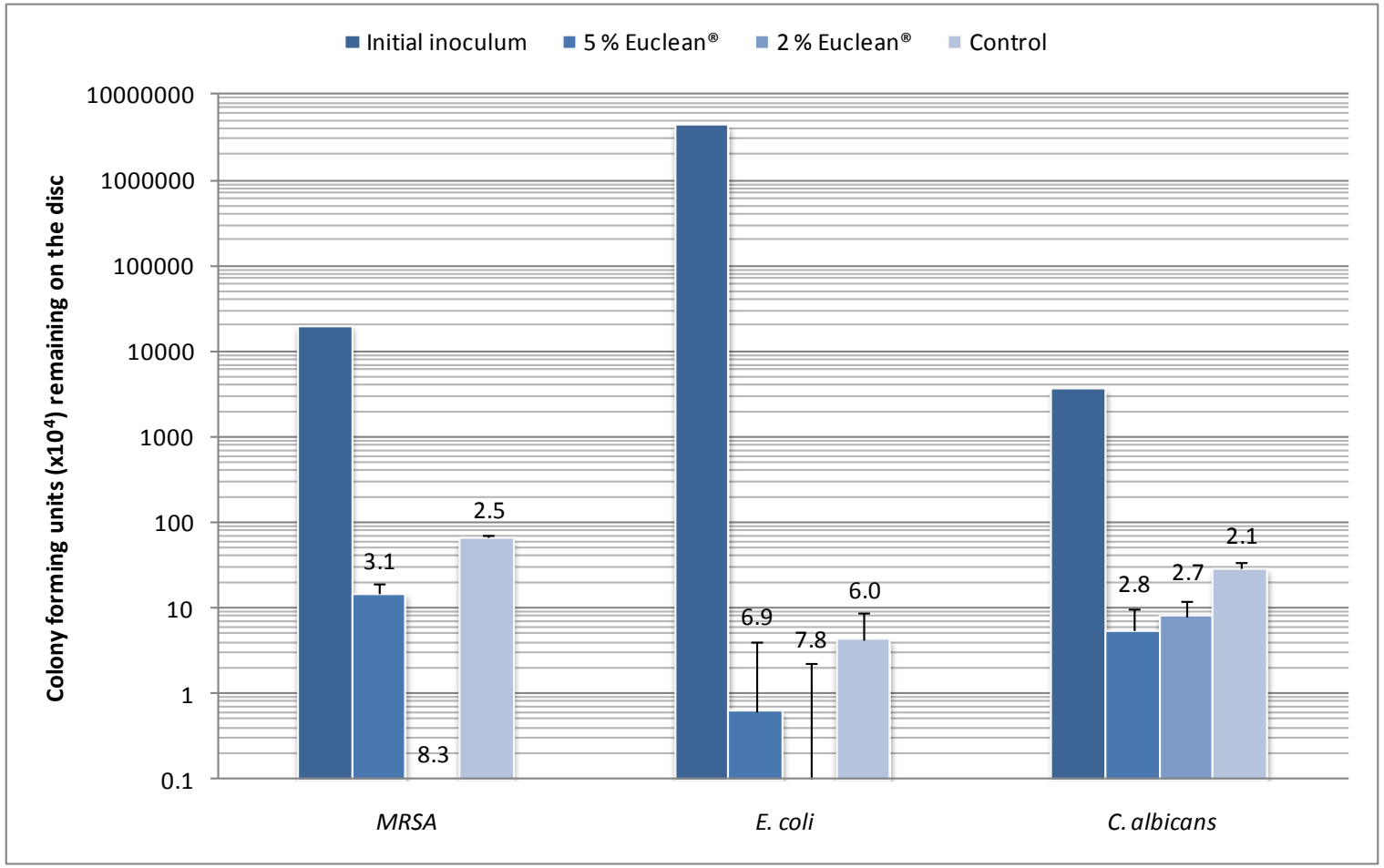

Figure 4.1. Reduction in mean (n=3) cfu/disc of a panel of dried-on microorganisms following wiping with test and control wipes, with the log reduction from the initial inoculum and standard deviation.

Table 4.1. Mean number (n=3) of surfaces positive for cross-contamination following adpression onto agar of wipes soiled with microorganisms (from the previous experiment), with microbial viability after broth culture of the wipes indicated by + for viable, and - for not viable.

	Surfaces positive for cross-contamination/Microbial viability		
	5 % Euclean®	2 % Euclean®	Control
MRSA	0/-	0/-	8/+
E. coli	0/-	0/-	8/+
C. albicans	0/-	0/-	8/+

4.5.2. Microbial inhibition by Euclean® wipes using agar diffusion

Both the 5 % and 2 % Euclean® wipes demonstrated antimicrobial efficacy by the presence of inhibition zones against a panel of microorganisms (Table 4.2). There was no significant difference ($P>0.05$, Paired t-test, Instat3 GraphPad) between the efficacy of the two Euclean® wipes of 5 % and 2 % EO, however both were significantly more efficacious ($P<0.05$, Paired t-test, Instat3 GraphPad) than the control wipes which were deemed as not antimicrobial due to zone measurements of <1 mm diameter.

Table 4.2. The antimicrobial efficacy of test and control wipes against a panel of six microorganisms expressed as mean (n=3) sizes of inhibition zones observed using the agar diffusion method.

	Inhibition zone (mm) for wipes		
	5 % Euclean®	2 % Euclean®	Control
S. aureus	7	7	<1
S. epidermidis	7	7	<1
MRSA	7	7	<1
P. aeruginosa	4	5	<1
E. coli	7	6	<1
C. albicans	5	4	<1

4.5.3. Bacteriostatic/bactericidal testing of Euclean® wipes

The 5 % Euclean® and 2 % Euclean® wipes exhibited bactericidal antimicrobial activity against all six microorganisms tested as no microbial growth was recovered. Control wipes showed no inhibition of microbial growth.

4.5.4. Time-kill study of Euclean® wipes against microorganisms in biofilm mode of growth

Euclean® wipes in both 5 % and 2 % concentrations demonstrated significantly quicker ($P<0.05$, ANOVA, Instat3, GraphPad) biofilm removal compared with the CHG/IPA wipes. The 5 % Euclean® wipes killed biofilms of every microorganism in under 5 minutes, except *P. aeruginosa* which took under 10 minutes (Table 4.3). The 2 % Euclean® wipes took under 10 minutes to eliminate biofilms of *S. epidermidis*, *P. aeruginosa* and *E. coli*, and under 5 minutes for the other microorganisms, compared with CHG/IPA wipes which killed *C. albicans* in under 25 minutes but then failed to eradicate any other microbial biofilm in the 30 minute test time.

Table 4.3. The mean (n=3) time required for test and control wipes to remove microbial biofilms of six microorganisms from stainless steel surfaces.

	Time for wipes to eliminate microbial biofilms (mins)		
	5 % Euclean®	2 % Euclean®	Control
S. aureus	<5	<5	>30
S. epidermidis	<5	<10	>30
MRSA	<5	<5	>30
P. aeruginosa	<10	<10	>30
E. coli	<5	<10	>30
C. albicans	<5	<5	<25

4.6. Discussion

In this part of the research, the antimicrobial efficacy of 5 % Euclean® and 2 % Euclean® wipes was assessed. Despite uncertainties surrounding the number of HAIs stemming from environmental contamination on surfaces, it is now appreciated that surface colonisation is a contributing factor in infection occurrence. With many different products available and recommendations of cleaning processes differing between healthcare establishments, and sometimes even between wards or departments of the same hospital, it is easy to appreciate how inconsistencies in cleanliness levels can occur between locations. This can therefore have an obvious impact on the infection rates within that unit.

The results of the first experiment within this study confirmed the physical wiping action to be largely responsible for the removal of microorganisms dried onto a surface, rather than the antimicrobial agent impregnated onto the wipes, which concurs with findings by Mehmi *et al*. (2009). The physical abrasion applied to the dried contamination, resulted in a significant reduction in the microbial load remaining on the discs for the control wipes and both of the Euclean® wipes. However no significant difference was observed between the control wipes wetted solely with water and either of the antimicrobial Euclean® wipes. Along with physically removing cells, the wetness of the wipes may have also contributed to the wipes ability to remove microorganisms. Studies have confirmed the wetness of a wipe to be important in its ability to remove microbes from surfaces, with one study by Diab-Elschahawi *et al*. (2010) concluding wet paper towels, microfibre, cotton and sponge cloths all showed significantly improved decontamination capabilities compared with their dry counterparts.

The results of chapter 3 illustrated that the EO-containing wipes were capable of inducing cross-contamination between surfaces, however, in conjunction with the first experiment of this study, the subsequent results demonstrated that unlike the control wipes, both compositions of Euclean® wipes did not induce any microbial cross-contamination onto surfaces subsequently touched after the wipes had been used. Furthermore, no viable cells were recovered from the wipes. This difference in findings may be due to the altered formulation impregnated onto the Euclean® wipes which comprised a greater tween-80 content, thus creating more uniform diffusion throughout the wipes as a result of a better mixed initial solution. It is also possible that the Euclean® wipes were wetter as their commercial packaging consisted of an airtight seal

absent on the EO-containing wipes, designed to prevent desiccation. Both of these factors may have resulted in the Euclean® wipes being more efficacious, despite the reduced concentration of EO, therefore inducing microbial death before collected microbial contamination could be deposited onto subsequent surfaces. When compiled with the inhibition zone presence in the agar diffusion and fully bactericidal results, the collection of data in this study confirms that both 5 % and 2 % Euclean® wipes effectively cleared microbial contamination from a surface, and then killed the remains preventing deposition onto subsequent surfaces. In addition, there were no significant differences in the efficacy of the 5 % and 2 % Euclean® wipes suggesting that the reduction in EO concentration had not compromised the efficacy of the antimicrobial combination.

As discussed in chapter 3, the presence of tween-80 in the wipe solution may have impacted on the antimicrobial potential of the wipes through its possible neutralisation of CHG. Furthermore, to improve mixing and therefore solution diffusion through the wipes, the percentage content of tween-80 was raised from 0.1 % in the EO-containing wipes (chapter 3), to 1 % in the 5 % and 2 % Euclean® wipes. One study has illustrated the use of tween-80 as a neutralising agent for CHG, however the CHG concentration used was 0.5 %, compared with the 2 % in the two compositions of the Euclean® wipes, and the subsequent neutralisation of this CHG was performed by a great dilution of 1 in 1000 into a neutraliser comprising both 1 % tween-80 and 1 % tween-20 (Jayakumar *et al*., 2011). It therefore follows that whilst CHG neutralisation was effectively carried out using tween-80 in the literature, the dilutions and concentrations involved differed greatly from those used within this study, therefore alone, may not truly represent the neutralisation activity by tween-80 within the Euclean® wipes. Furthermore, another study by Zamany & Spangberg (2002) reported effective neutralisation of 2 % CHG was possible with 3 % tween in combination with 0.3 % lecithin, however 3 % tween-80 alone was insufficient to quench the activity of 2 % CHG thus further supporting the likelihood that the 1 % tween-80 incorporated within the Euclean® wipes, three times less than confirmed ineffective, would not result in inactivation of the 2 % CHG present.

In 2004, a Rapid Review Panel (RRP) of specialists and clinicians affiliated with infection control was convened to impart guidance on new technologies that could, if introduced to the health service, reduce HAIs. Invitations were then extended for companies to come forward if they believed they had an idea that could aid reduction in

HAI rates. Subsequently, in 2008, the NHS and Department of Health collaborated and launched the Healthcare Associated Infection Technology Innovation Programme, to facilitate development and adoption of the new technologies previously assessed and deemed beneficial. Ideas granted RRP 'category 1' were instantly implemented in showcase hospitals, and available throughout the NHS, those obtaining categories 2 or 3 were provided assistance with further development and testing before deciding if it should be made widely available (Department of Health, 2010). Alongside these, ran the Smart Ideas Programme in autumn 2007 which involved NHS staff and aimed to gather opinion about what they felt could positively influence HAI rates. The response was immense with 157 ideas divulged, from which 10 believed to be the most promising were prioritised and in turn, underwent trials in pilot studies within showcase hospitals throughout eight U.K. trusts; the same hospitals RRP category 1 ideas were installed into for real-use examination. As a result of this entire collaborative programme encouraging creativity, many new products have been developed with the list of those becoming available to NHS trusts continually increasing. Some ideas arising from these programmes include amongst others a Hydrogen Peroxide Vapour System from Bioquell® for room decontamination, and a hand held device for measuring microbial surface contamination called Clean Trace ATP Monitoring System by 3M®. Both of which have since been concluded as successful aids in reducing HAIs causing reductions in microbial presence as well as being favourable amongst staff (Department of Health and NHS Purchasing and Supply Agency, 2009*a*; Department of Health and NHS Purchasing and Supply Agency, 2009*b*). The knowledge that these ideas are believed by a whole panel including specialists from the health service and infection control leaders, to possess potential benefits for reducing HAIs, confirms that surfaces are increasingly being taken more seriously in the fight for controlling infection transmission. However, none of these new ideas focuses specifically on biofilms which are, as discussed previously, of significant concern within the healthcare system.

Both the 5 % and 2 % Euclean® wipes removed biofilm cultures from stainless steel discs significantly quicker than the CHG/IPA wipes, which permitted some biofilms to still remain after 30 minutes contact. However, there was a slight difference observed in this experiment between the 5 % Euclean® wipes and the 2 % Euclean® wipes. Five of the six tested microorganisms were successfully eradicated in under 5 minutes contact with the 5 % Euclean® wipes, and the remaining microorganism (*P. aeruginosa*) was

killed within 10 minutes; compared with the 2 % Euclean® wipes which destroyed three microorganisms within 5 minutes, and the remaining 3 within 10 minutes.

EO is a known permeation enhancer and has previously presented increased potency against biofilm cultures when combined with CHG (Hendry *et al*., 2009). The permeation properties of EO are likely to be partly responsible for aiding biofilm removal, by penetrating deeper into the extracellular matrix than CHG or IPA alone, allowing the contained cells to be targeted by the EO, along with the CHG and IPA. This hypothesis is supported by research which has shown that EO can carry CHG into the deeper layers of human skin therefore is it possible the same pulling effect could occur in biofilm penetration (Karpanen *et al*., 2010). This permeation attribute would account for the difference in time taken by the 5 % and 2 % Euclean® wipes to eliminate the biofilm as with less EO present, the permeation effect could be reduced therefore require longer time to achieve the same result.

The results from this part of the study confirm that development from an idea into a prototype developed at Aston University and subsequently into a commercial product has, for this combination of agents, retained viability and antimicrobial efficacy. Some efficacy against biofilm removal was lost with the reduction in EO concentration however, even with this reduced efficacy, the 2 % Euclean® wipes demonstrated significantly more antimicrobial efficacy than the CHG/IPA wipes similar to those often used throughout the health service at present. Due to the increased prevalence of biofilms, compared with planktonic cells on hard surfaces, these results bear significance to the application of the wipes and therefore the 5 % Euclean® wipes will be used for future studies within this thesis.

4.7. Conclusion

Development from a prototype into a commercial product can create unforeseen complications and result in an altered final outcome. However, development from an EO-containing wet wipe assessed in chapter 3, into Euclean® hard surface disinfectant wipes has proved successful. Both 5 % and 2 % varieties of Euclean® wipes have demonstrated antimicrobial efficacy against a panel of six clinically important microorganisms adhered to surfaces, with no reduction in efficacy as a result of decreased EO concentration. Furthermore, they both possessed greater efficacy against microbial biofilms than that of the currently popular wipe containing CHG and IPA. However, the reduction in EO concentration did result in a slightly increased time period required to kill biofilms of *S. epidermidis*, *P. aeruginosa* and *E. coli*; prevalent nosocomial pathogens. As biofilms are more true to the mode of growth observed most commonly on hard surfaces, with recognition dating to the early fossil records (Branda *et al*., 2005; Chandra *et al*., 2001), the 5 % Euclean® wipes show more suitability for use within the clinical setting than the 2 % Euclean® wipes, and shall therefore be put forward for a clinical audit to assess opinion of them.

Chapter 5

The Use of Isothermal Calorimetry as an Alternative Method for Determination of the Antimicrobial Activity of Eucalyptus Oil-Containing Wipes and Commercially Produced Euclean® Hard Surface Disinfectant Wipes, against a Panel of Microorganisms in Moderate and High Density Suspensions

5.1. Introduction

There is a vast repertoire of conventional laboratory methods employed for testing new antimicrobial products such as disinfectants, prior to reaching clinical trials and ultimate commercialisation (Williams *et al*., 2007). These can involve microbial exposure tests, time-kill experiments, studies in the presence of soil loads and resistance development potentials. An alternative method for testing the efficacy of a new agent, involves the use of calorimetry, however its application for assessment of antimicrobial agents, to date, has been limited.

Advances in calorimetry have progressed immensely since first being used for biological applications in the 1780's when guinea pig metabolism was studied, with microcalorimeters now widely available (Chaires, 2008; Lavoisier & Laplace, 1780). Calorimetry provides information on the energy of a thermodynamic system, known as enthalpy, measured as 'power' and converted into the SI units of Joules (Chaires, 2008). The two main types of calorimetry used are differential scanning calorimetry (DSC), and isothermal calorimetry (IC). Both types contain a sample and reference ampoule with the temperature difference between them monitored, however in DSC, the external temperature is increased at steady, actuate intervals whereas in an IC, it is kept constant (Jelesarov & Bosshard, 1999).

In recent years, IC has been frequently used by the pharmaceutical industry to aid discovery, and development of drug products. In particular, the use of calorimetry can provide data on the long term stability of drugs from the highly sensitive results it generates over continued time periods (Beezer *et al*., 2001).

Calorimeters rely on the knowledge that all processes, chemical and physical, involve an exchange of heat energy to or from their surroundings; it is this variable which is recorded with great sensitivity within the calorimeter. They are also non-specific in their operation, therefore will monitor and record all changes that are observed within a given test. Furthermore, the rate of heat exchange within the IC is measured in real time allowing the dynamic response of the system being challenged, to be monitored directly (O'Neill *et al*., 2003). Some other advantages conferred by IC include superior sensitivity and versatility over many other classical analytical techniques (Salim & Feig, 2009). They are capable of controlling the operating environment, which in turn results in any temperature changes being instantly observed if linked with a computer, and

attributed to an instigating event, such as microbial growth or death (Trampuz *et al*., 2007).

IC has previously been deemed effective in monitoring the bactericidal efficacy of other complex microbial systems, where traditional culturing techniques would have been difficult to employ accurately. Two previous studies have been undertaken exploring the efficacy of silver-containing wound dressings following addition of dressing sections to suspensions of *S. aureus* and *P. aeruginosa*. They confirmed the method to be more suitable for this type of assessment, as it eliminates plate counting complications whilst providing the additional benefits of real time results from a more accurately controlled system (Gaisford *et al*., 2009; O'Neill *et al*., 2003). Another study used IC to determine the MIC of 12 antibiotics against five microorganisms, describing the method as accurate and reproducible, with its passivity allowing samples to remain undamaged and untouched, allowing for subsequent processing if required (von Ah *et al*., 2009).

Previous research within chapter 2 of this thesis has confirmed that EO and CHG possess synergistic antimicrobial activity when combined together against both planktonic and biofilm modes of growth (Hendry *et al*., 2009). Following adaptation of the combined solution into the commercially produced 5 % Euclean® hard surface disinfectant wipes (chapter 4), conventional laboratory experiments have confirmed efficacy to remain, with suitability for use within the clinical environment established, in particular against biofilms (Hendry *et al*., 2010). Employing isothermal calorimetry as an accompanying technique for the assessment of 5 % Euclean® wipes, may provide additional information about their antimicrobial efficacy, specifically with regards to their speed of action and potential to permit or even encourage microbial growth.

5.2. Aims of the study

The aims of this study were to:

- Investigate the potential use of isothermal calorimetry for assessing antimicrobial activity, as an alternative or accompanying method to conventional laboratory culturing techniques and traditional time-kill assays.
- Assess the antimicrobial efficacy of 10 % EO-containing wipes (chapter 3) and the commercially produced 5 % Euclean® wipes (chapter 4), against a panel of clinically important microorganisms in planktonic cultures of both a moderate and high density suspension, using isothermal calorimetry.

5.3. Materials

5.3.1. Microbial cultures

Staphylococcus aureus ATCC 6538, *Staphylococcus epidermidis* RP62A, MRSA N315, *Pseudomonas aeruginosa* ATCC 15442, *Escherichia coli* NCTC 10418 and *Candida albicans* ATCC 76615 were stored on MicroBank beads (Pro-Lab Diagnostics, Cheshire, U.K.) at -70 °C until required.

5.3.2. Microbiological media

Mueller-Hinton agar (MHA), Mueller-Hinton broth (MHB), Sabouraud dextrose agar (SDA) and Sabouraud dextrose broth (SDB) were purchased from Oxoid (Basingstoke, U.K.), prepared as per manufacturer's instructions and sterilised at 121 °C for 15 minutes. Agar was cooled to 50 °C before pouring and broths were stored at 4 °C until required.

5.3.3. Chemicals

5.3.3.1. Neutralising solution

Neutralising solution was prepared with 1.17 % (w/v) lecithin, granular, Mw 750.00 Acros Organics (Fisher Scientific, Leicestershire, U.K.), 2 % (v/v) tween-80 (Sigma-Aldrich, Dorset, U.K.), 0.5 % (w/v) sodium thiosulphate pentahydrate (BDH Limited, Dorset, U.K.) and 0.1 % (v/v) triton-X 100 (Sigma-Aldrich, Dorset, U.K.), made up to 1 L with double distilled water. The solution was sterilised at 121 °C for 15 minutes and stored at 4 °C until required.

5.3.4. Equipment

All static calorimetry experiments were performed in a TA Instruments 2277 Thermal Activity Monitor (TAM) (TA Instruments, Delaware, U.S.A.) set to 37 °C. The electrical substitution method was used to calibrate the instrument which was then verified using a chemical test and reference reaction (Beezer *et al*., 2001). On each occasion, the ampoules were allowed to reach thermal equilibrium in the calorimeter (±

10 minutes) before being lowered into the measuring position. Data were collected using the dedicated software package Digitam®, and analysed using Microcal Origin® Software Version 7.0.

Flow calorimetry experiments were performed in a TA Instruments 2277-Flow Thermal Activity Monitor (flow-TAM) (TA Instruments, Delaware, U.S.A.) set to 30 °C. The instrument underwent simple calibration prior to each flow-through calorimetric experiment, and on each occasion, both the system and media were allowed to reach thermal equilibrium (± 10 minutes) before measurement commenced. It should be noted that movement of fluid in flow calorimetry introduces frictional heat, whilst the outward flowing fluid carries heat out of the system therefore calibration was required, however as the intention of these studies was for distinguishing between live and dead microorganisms, the thermodynamics of the system such as enthalpy of reaction, were not in this instance calculated. Data were collected using the dedicated software package Digitam®, and analysed using Microcal Origin® Software Version 7.0.

5.3.5. 5 % Euclean® wipes

The 5 % Euclean® wipes were produced by PAL International (Leicestershire, U.K.) by application of 5 % (v/v) EO, 2 % (v/v) CHG, 70 % (v/v) IPA and 1 % (v/v) tween-80 in water sprayed into tubs containing 200 blank, 23 gsm, viscose/polypropylene (50:50) non-woven wipes. Tubs were left standing for a minimum of 48 hours to allow saturation of the wipes before being delivered for use.

5.4. Methods

5.4.1. Wipe impregnation process and combinations

The solutions for wipe impregnation were each prepared to a total volume of 384 mL for two sets of wipes:

- 10 % (v/v) EO / 2 % (v/v) CHG / 70 % (v/v) IPA / 0.1 % (v/v) tween-80 all purchased from Sigma-Aldrich (Dorset, U.K.) in sterile, double distilled water made in sterile, double distilled water
- Sterile, double distilled water (control wipes)

In accordance with methods used by the wipe manufacturer PAL International (Leicestershire, U.K.), each solution was poured into a tub containing 200 blank, 23 gsm, viscose/polypropylene (50:50) non-woven wipes provided by PAL International, and left for a minimum of 48 hours to allow saturation of the wipes before use.

5.4.2. Assessment of the antimicrobial efficacy of Euclean® solution against *Candida albicans* using flow calorimetry

An overnight cell suspension of *C. albicans* was prepared in SDB and diluted using previously established OD/cfu standard curves to provide a 50 mL culture of 10^4 cfu/mL. A magnetic stirring bar was added to the culture then it was placed in a heated water bath set at 33 °C to keep the culture maintained at 30 °C. The calorimeter used was a flow-TAM (TA instruments, Delaware, U.S.A.) calorimeter through which the culture was cycled for a minimum of 20 hours to generate a growth curve. The antimicrobial efficacy of the Euclean® solution was then assessed by the addition of 2 % (v/v) Euclean® solution (comprising 5 % (v/v) EO, 2 % (v/v) CHG, 70 % (v/v) IPA and 0.1 % (v/v) tween-80, in sterile, double distilled water) to the growth culture just prior to the maximum peak of growth occurring. The culture was cycled through the calorimeter a further 18 hours before triplicate pour plates were made from 1 mL samples into molten SDA containing 10 % (v/v) neutralising solution to assess microbial viability. A control experiment was carried out with 2 % (v/v) Euclean® solution (made as described above) added to SDB circulating around the flow

calorimeter without microbial inoculum, to determine whether any effects observed in the earlier experiment were caused by fiction as the two agents combined.

5.4.3. Antimicrobial efficacy of Euclean® wipes against moderate and high density microbial suspensions using isothermal calorimetry

Overnight cell suspensions of each microorganism were prepared in MHB or SDB from five identical colonies grown on MHA or SDA plates. Using previously established OD/cfu standard curves (Section 2.4.1.1), all cultures (excluding MRSA) were diluted in MHB or SDB as required to give final concentrations of 10^4 cfu/mL and 10^7 cfu/mL, while MRSA was diluted in MHB to 10^5 cfu/mL and 10^7 cfu/mL. Using the lower density cell cultures of each microorganism, growth curves were generated following inoculation of 3 mL microbial suspension into 4 mL stainless steel ampoules (TA Instruments, Delaware, U.S.A.). Ampoules were then hermetically sealed using a stainless steel closure, and following achievement of thermal equilibrium, were lowered fully into the recording position of the calorimeter. To determine the effect of wipes on microbial growth, 3 mL of cell suspension was added to the ampoules as described and a 4 cm^2 section of wipe was rolled up and added to the ampoule prior to sealing and loading. The wipes tested were:

- 10 % (v/v) EO / 2 % (v/v) CHG / 70 % (v/v) IPA / 0.1 % (v/v) tween-80
- 5 % Euclean®
- Sterile, double distilled water (control)

Further testing of 5 % Euclean® wipes was carried out using the same methods as described but with the higher density cell suspension containing 10^7 cfu/mL. All experiments were carried out for a minimum of 20 hours. Finally, microbial viability was confirmed or refuted once the calorimeter process had concluded, by making pour plates from mixing 1 mL of the ampoule contents with molten MHA or SDA containing 10 % (v/v) neutralising solution, cooled to 50 °C. Plates were incubated overnight at 37 °C; 30 °C for *C. albicans*. All experiments were repeated in triplicate.

5.4.4. Time-kill study of Euclean® wipes against moderate and high density microbial suspensions

Overnight cell suspensions of each microorganism were diluted in MHB or SDB to the same final concentrations as used previously (Section 5.4.3). To replace the data lost by equilibrating the ampoules, time kill studies were carried out over a 30 minute time period for the scenarios tested in the calorimeter, with the conditions replicated by using the same temperature, volume and density of cell suspensions, and size of wipe for the 5 % Euclean® wipes. Samples were taken at time zero, minute intervals up to 5 minutes, and then every 5 minutes up to half an hour. Each sample was cultured into molten MHA or SDA containing 10 % (v/v) neutralising solution, cooled to 50 °C. Plates were incubated overnight at 37 °C; 30 °C for *C. albicans* and microbial viability determined by a 99.9 % reduction in cfu from the time zero control.

5.5. Results

5.5.1. Assessment of the antimicrobial efficacy of Euclean® solution against *Candida albicans* using flow calorimetry

The growth curve produced by *C. albicans* in the flow calorimeter reached a maximum power output signal of around 350 µW (Figure A13), however the addition of 2 % (v/v) of Euclean® (5 %) solution into a growing cell suspension resulted in almost instantaneous cell death, subsequently confirmed by no visible growth following agar culture after the completion of the flow calorimetry experiment. The control experiment showed addition of Euclean® solution into broth caused a small power output signal of around 75 µW, which accounted for only one quarter of the total power output of 300 µW created when the solution was added to growing cells (Figure A14). However, due to recurring contamination issues with the flow method, static calorimetry was adopted for future experiments.

5.5.2. Antimicrobial efficacy of Euclean® wipes against moderate and high density microbial suspensions using isothermal calorimetry

The power-time growth curves for each microorganism (Figures 5.1 to 5.6) initially showed escalating power output over time as the cell numbers increased producing heat, up to a maximum cell density. At which point, density peaked and began to fall due to a reduction in the rate of cell turnover, causing a decrease in power output as heat output reduced. The presence of a control wipe had little effect on the power-time curves, compared with the respective growth curve. However, in all instances, the addition of a 10 % EO-containing wipe or 5 % Euclean® wipe resulted in a flat line showing no power output equating to no heat output, thus eradication of growth. The same was observed when the wipes were added to the higher density cell suspensions. The viability testing of the ampoule contents concluded no growth at all, confirming 5 % Euclean® wipes to be bactericidal in their mode of action, and verifying that a power output of zero is indicative of a bactericidal effect rather than bacteriostatic.

Figure 5.1. Power-time curves showing the calorimetric response of 10^4 cfu/mL *S. aureus* cell suspension when exposed to control (red), 5 % Euclean® (green) and 10 % EO-containing wipes (purple), as well as a 10^7 cfu/mL cell suspension exposed to 5 % Euclean® wipes (blue), compared with a growth curve (black) in the absence of any wipe.

Figure 5.2. Power-time curves showing the calorimetric response of 10^4 cfu/mL *S. epidermidis* cell suspension when exposed to control (red), 5 % Euclean® (green) and 10 % EO-containing wipes (purple), as well as a 10^7 cfu/mL cell suspension exposed to 5 % Euclean® wipes (blue), compared with a growth curve (black) in the absence of any wipe.

Figure 5.3. Power-time curves showing the calorimetric response of 10^4 cfu/mL MRSA cell suspension when exposed to control (red), 5 % Euclean® (green) and 10 % EO-containing wipes (purple), as well as a 10^7 cfu/mL cell suspension exposed to 5 % Euclean® wipes (blue), compared with a growth curve (black) in the absence of any wipe.

Figure 5.4. Power-time curves showing the calorimetric response of 10^4 cfu/mL *P. aeruginosa* cell suspension when exposed to control (red), 5 % Euclean® (green) and 10 % EO-containing wipes (purple), as well as a 10^7 cfu/mL cell suspension exposed to 5 % Euclean® wipes (blue), compared with a growth curve (black) in the absence of any wipe.

Figure 5.5. Power-time curves showing the calorimetric response of 10^4 cfu/mL *E. coli* cell suspension when exposed to control (red), 5 % Euclean® (green) and 10 % EO-containing wipes (purple), as well as a 10^7 cfu/mL cell suspension exposed to 5 % Euclean® wipes (blue), compared with a growth curve (black) in the absence of any wipe.

Figure 5.6. Power-time curves showing the calorimetric response of 10^4 cfu/mL *C. albicans* cell suspension when exposed to control (red), 5 % Euclean® (green) and 10 % EO-containing wipes (purple), as well as a 10^7 cfu/mL cell suspension exposed to 5 % Euclean® wipes (blue), compared with a growth curve (black) in the absence of any wipe.

5.5.3. Time-kill study of Euclean® wipes against moderate and high density microbial suspensions

The results for the suspension time-kill demonstrated that insertion of the 5 % Euclean® wipes into the cell suspensions killed all microorganisms tested, including the higher density cell suspensions, within a contact time of less than 1 minute.

5.6. Discussion

The 5 % Euclean® wipes have previously demonstrated enhanced antimicrobial efficacy against planktonic and biofilm cultures of a variety of clinically important microorganisms, with the penetration properties of EO the most likely justification for facilitating the improved microbial elimination (Hendry *et al*., 2009; Hendry *et al*., 2010). Furthermore, the results of this study have reemphasised the suitability demonstrated in chapter 4 of 5 % Euclean® wipes for use within the healthcare setting, following confirmation of their rapid antimicrobial action. While conventional microbiological techniques have been employed previously to assess the antimicrobial properties of these wipes, in this study, the use of isothermal calorimetry was employed.

Calorimetry allows continuous measurement of microbial metabolic activity over time without the need for external sampling. The TAM used in this study precisely controlled the experimental environment, therefore any temperature changes could not only be observed as they occurred, but credited to a triggering incident such as cell growth or death. This particular TAM measured changes in the power needed to match the temperature of the control ampoule, to that of the sample. Therefore, the use of 'power' and 'heat' become almost interchangeable; as microbial growth caused the heat in the sample ampoule rises, more power is needed to heat the reference up to the same temperature, as cells die heat reduces thus less power is needed to match the heat of the sample ampoule (Salim & Feig, 2008).

The calorimeter is not destructive to the sample under observation, it is also non-invasive hence the course of the experiment is not influenced by the calorimeter. This is a significant advantage conferred by isothermal calorimetry as it allows experiments to be performed on a test substance directly, regardless of its form or diversity. Such capabilities are particularly useful for the study of microbiological systems, which by their very nature, are complex. Having previously been confirmed as an effective means of monitoring the bactericidal properties of other similarly complex systems, the use of IC for this study was perceived as suitable, whilst providing additional beneficial data, unobtainable by conventional laboratory culture methods (Gaisford *et al*., 2009; O'Neill *et al*., 2003).

Microorganisms are known to grow at different rates, as confirmed in this study by the application of isothermal calorimetry. All of the six microorganisms investigated here showed a growth curve with at least two stages of growth. The double peaking of

growth curves found in this study, is supported by those obtained in other studies, and results from changes to the utilisation of a different metabolic pathway (von Ah *et al*., 2008; Hou *et al*., 2010; O'Neill *et al*., 2003).

The results clearly demonstrate that both the EO-containing wipes and Euclean® wipes possessed antimicrobial activity against all six microorganisms with the power outputs represented by flat lines rather than the curves previously witnessed. This was true irrespective of Gram classification or microbial origin such as bacteria or yeast. Furthermore, the antimicrobial efficacy of the wipes was maintained in the presence of a particularly challenging, increased microbial load. Subsequent agar culture confirmed the wipes to be bactericidal in their mode of action, eliminating complications seen with some wipes where the fabric can not only harbour collected microbial contamination, but induce growth within them (Diab-Elschahawi *et al*., 2010). This is an important feature for wipes intended for clinical applications, as cleaning surfaces with contaminated cloths that disperse microorganisms, could prove more harmful in the dissemination of potential pathogens than not cleaning in the first instance. These findings support previous research where EO and CHG have been shown to act synergistically to induce a bactericidal effect, possibly as a result of both targeting the cytoplasmic membrane leading to structural damage of the cell, and consequently increased permeability (Filoche *et al*., 2005; Prabuseenivasan *et al*., 2006).

The sensitivity of modern calorimeters is such that a bacterial culture containing 10^4 cfu/mL is sufficient to generate a detectable signal through heat production (von Ah *et al*., 2009). Therefore, as growing microorganisms generate detectable heat, any antimicrobial effect generated in this study by 5 % Euclean® wipes against any of the six microorganisms resulted in a reduced heat output. If the microorganisms are killed, there is no detectable signal from heat output. This was observed for all six microorganisms tested when EO-containing wipes and 5 % Euclean® wipes were present, with the result being a flat-line graph, subsequently verified by culturing onto agar, as no viable cells remaining. There is however, one limitation of this method. Whilst it was possible to confirm bactericidal action by 5 % Euclean® wipes, it was not possible to determine how rapidly this occurred following contact with the wipes. After loading the ampoules into the TAM and allowing 30 minutes for thermal equilibrium to be achieved, the baseline must return to zero before death can be confirmed, often resulting in no data for the first hour of contact. However, conventional microbiological testing used to replace this missing data has verified that 5 % Euclean® wipes

sufficiently kill all the microbial suspensions described in this study with a contact time of less than 1 minute. This is in line with previous results whereby 10 % EO-containing wipes killed planktonic cells within 30 seconds (chapter 3), and results of the subsequent chapter in which 5 % Euclean® wipes killed biofilms of all microorganisms within 5 minutes except *P. aeruginosa* which took under 10 minutes (chapter 4).

The control wipes did not show any antimicrobial efficacy when compared with the growth curve for each microorganism. They did however result in a small reduction in the maximum heat output over the growth period, and in some instances, a slightly delayed growth curve was generated. This was possibly due to the very nature of the wipes physically containing and restricting cells, reducing their exposure to nutrients and space.

Decontamination of hard surfaces within the healthcare setting plays an important role in reducing the spread of HAIs. Interventions, such as changes in disinfection product, education and feedback, applied to cleaning procedures have proved to induce reductions in microbial recovery from surfaces harbouring pathogenic contamination, including MRSA and VRE (Goodman *et al*., 2008). However, the cleaning method and protocol used need careful consideration if maximum efficacy is to be achieved. A recent study by Diab-Elschahawi *et al*. (2010) highlighted the importance of using moist cloths for cleaning due to the significantly reduced microbial removal by a dry cloth. It has also been noted that the fabric of the wipe has an effect on its efficacy, with microfibre cloths being recognised by many, as superior to cotton cloths (Rutala *et al*., 2007). However, another recent study reported differences in efficacy between microfibre cloths, concluding most to be not significantly better than a paper towel at removing dried microorganisms in the presence of a soil load. This emphasised that using a microfibre cloth is not always indicative of superior performance, leading to questions regarding the justification of the extra cost, if current methods offer similar performance (Moore & Griffith, 2006). Euclean® wipes are moist but not microfibrous, therefore their physical characteristics may contribute positively towards their efficacy but could potentially be improved. Furthermore, the unique combination of EO and CHG/IPA within the wipe allows for enhanced synergistic antimicrobial efficacy when compared with CHG/IPA alone (Hendry *et al*., 2009).

Alcohol wipes are widely used within the health service at present however other types of cleaning products are also available. Research has confirmed that the method

employed can have an effect on the cleaning merit; this has been highlighted in a previous study by Panousi *et al*. (2008) who concluded that alcohol impregnated wipes performed much better when removing microbial contamination from surfaces than a dry wipe sprayed with alcohol and subsequently used.

The results obtained in this study not only support, but also extend the information previously established regarding the antimicrobial efficacy and mode of action of EO-containing wipes (chapter 3) and 5 % Euclean® wipes (chapter 4).

5.7. Conclusion

The results from this study support and extend those gained previously using conventional laboratory culture techniques. Here, Euclean® wipes have again been confirmed to possess significant antimicrobial activity against both Gram-positive and -negative bacteria, as well as yeast, which was successfully maintained when challenged with an increased microbial load. Furthermore, when combined with the use of more familiar culture methods, confirmation was obtained that Euclean® wipes induced complete extermination of all viable cells for all six microorganisms investigated, including those of the higher cell density, in less than 1 minute, therefore further verifying their suitability as hard surface disinfectant wipes for use within the healthcare setting. Moreover, this study has highlighted the use of IC as a viable alternative to classical methods of analysis for the detection of wipe efficacy and microbial viability in cell suspensions of this nature.

Chapter 6

Clinical Audit of Euclean® Hard Surface Disinfectant Wipes Undertaken on Five Wards at Birmingham Children's Hospital

6.1. Introduction

The responsibility of cleaning and disinfection within the healthcare service falls upon a number of different staff members within the health service. External, contracted cleaning firms are recruited sporadically for intermittent deep cleans, however the duties of daily cleaning are usually split between ward cleaners responsible for large areas such as walls and floors, and nurses, whose remit includes high touch surfaces such as drip stands, bedside tables and lockers, along with intricate clinical equipment (Dancer, 2009). However, overlaps in obligation can easily lead to confusion with some surfaces potentially remaining missed. Where cleaning is concerned, understaffing can exacerbate the burden imposed on others, increasing the chances of executing rushed technique or last minute reallocation of responsibilities, creating disorder and potentially omitting cover entirely for some areas. As concluded by Hugonnet *et al*. (2007) and Dancer *et al*. (2006), staff shortages, which are all too frequent within many hospitals, are linked to the undesirable resultant outcome of increased infection risk in critically ill patients. Add to this cost cutting measures, and a keen emphasis on reducing waiting lists which can often lead to overcrowding, and the problem of infection spread is further intensified (Dancer, 2008).

Within the literature, there appears a shortage of information covering proven benefits of cleaning in the U.K., with many studies only instigated following an infection outbreak (Dancer, 2009). Furthermore, there appears to be nothing in the way of opinion from healthcare workers regarding their thoughts on the cleaning products and methods they employ daily. This is surprising as opinions have been sought on other important issues concerning infection control, such as hand hygiene policies and procedures (Harris *et al*., 2000). Furthermore, dislike of a product is more likely to induce evasive behaviour towards it, therefore it should be imperative that staff are not only asked their thoughts concerning a new product, but that their feedback is listened to and acted upon.

Following stringent and extensive testing using both conventional laboratory culturing techniques and isothermal calorimetry, Euclean® wipes have demonstrated great potential for use within the clinical setting. However, cleaning of high touch surfaces requires regular repetition therefore products need to fulfil a number of criteria in order to ease this process. Favourable attributes include ease of use, suitable moistness/drying

time and skin friendly, i.e. they must not induce irritation. However the weighting of importance attributed to each characteristic may differ from one person to the next.

6.2. Aims of the study

The aims of this audit were to:

- Design a questionnaire to collate the opinions of healthcare workers regarding the use of cleaning and disinfection products.
- Liaise with ward staff and infection control managers at Birmingham Children's Hospital to facilitate administration of a cross-over study to ascertain their opinion, by means of a questionnaire, on their current cleaning and disinfection products, followed by commercially produced Euclean® hard surface disinfection wipes.

6.3. Materials

The 5 % Euclean® wipes were provided by PAL International (Leicestershire, U.K.) and comprised 5 % (v/v) EO, 2 % (v/v) CHG, 70 % (v/v) IPA and 1 % (v/v) tween-80 in water, sprayed into tubs containing 200 blank, 23 gsm, viscose/polypropylene (50:50) non-woven wipes. Tubs were left standing for a minimum of 48 hours to allow saturation of the wipes before being delivered and subsequently distributed to five wards at Birmingham Children's Hospital.

6.4. Methods

6.4.1. Ethical approval

Prior to undertaking the Euclean® clinical audit, ethical approval was obtained by the Chair of the Aston University's Ethics Committee (Image A1), and clearance was granted by the Research and Development Department of Birmingham Children's Hospital (Image A2). Contact with the National Research Ethics Service (NRES) confirmed ethical approval at trust level was not required due to the questionnaire being classified as an audit.

6.4.2. Questionnaire design and structure

The questionnaire (Image A3) was designed in collaboration with Dr Chris Langley (Senior Lecturer in Pharmacy Practice, Aston University) and consisted of 28 questions, split into two sections; the first included the demographics and questions regarding hard-surface disinfection products currently being used on that ward. The second concentrated on Euclean® wipes. The majority of questions were closed-ended; denoted by tick boxes, restricting the answers to a list of possible options. However, there were also some open-ended questions where opinion was requested, as well as some filter questions where further explanation was only required following a specified answer being given previously. The combination of question styles used would therefore generate both quantitative and qualitative data.

6.4.3. Questionnaire format and appearance

In order to maximise response rate, the overall format and appearance of the questionnaire was carefully considered. The questionnaire was completely anonymous so as to avoid receiving distorted answers through staff displaying feelings of their work or ability being under investigation or scrutiny which can lead to biases (Boynton, 2004). Both the title and completion instructions were clear and concise, using basic vocabulary to avoid confusion or initial reluctance to partake (Uher & Goodman, 2010). The wording of questions has been shown to impact on the quality of responses (McColl *et al*., 2001), therefore, the language used was simple and direct, with guidance given on how to answer e.g. tick one, tick all, with listed answers being alphabetised to

avoid biased emphasis being placed on particular answers. The length of the questionnaire was kept to the minimum required to obtain the desired information, as lengthy questionnaires generate lower response rates than shorter ones (Nakash *et al*., 2006). Finally, while the colour of the paper has been summarised as unimportant by some researchers (Booth, 2003), others have found it can impact significantly on response rates. Pucel *et al*. (1971) found a 7.1 % increase in response rate when using a green questionnaire compared with a white one. It was therefore decided that pastel green paper would be used for the clinical audit of Euclean® wipes, as it represented the green colour of Myrtaceae trees leaves from which eucalyptus oil is derived, while on a practical level, improved visibility of the questionnaire to staff if filed amongst other papers, thus hopefully increasing compliance, and therefore response rate.

6.4.4. Sample population

Ward participation within the Euclean® audit was on a voluntary basis; however some wards such as the intensive care unit (ICU) were prohibited from inclusion due to infection control policies. Of eight potential wards, the following five wards took part in the audit:

- Ward 4; Neonatal Surgery
- Ward 5; Burns Centre
- Ward 6; Medical Ward
- Ward 10; Neuro-Surgery
- Ward 11; Heart Unit

As any member of staff from these wards was eligible to complete the audit and questionnaire, a maximum of 167 replies could be achieved.

6.4.5. Data collection

The guidelines on the front of the questionnaire and on the informative poster (Image A4) instructed staff to complete the audit in two phases. Phase 1 of the cross-over study would last one week and required no changes to be made to their current practices of

hard-surface disinfection. At the end of the week they were asked to complete the first 16 questions. Phase 2 of the cross-over study would then begin with the replacement of all previous hard-surface cleaning products on the ward with Euclean® wipes. This phase would also last one week, during which, only Euclean® wipes would be used, with the final 12 questions then being answered at the end of that week. In this study, the term cross-over referred to a controlled trial in which there was balance between the number and duration of treatments, however the sequence of those treatments was not random.

6.5. Results

6.5.1. Data collection

A total of 167 staff members from 5 wards at Birmingham Children's Hospital were eligible to complete the Euclean® clinical audit. Sixty-two (37 %) out of the 167 responded. Of the 62 questionnaire replies received, 8 were discounted due to partial completion, while the remaining 54 responses were ascertained useable; this represents a final response rate of 32 %. The demographics highlighted that 53 responses were from female participants, with only one from a male participant, the ages and job roles of the participants are shown in Figure 6.1. The duration spent working in their current role varied greatly from 1 month to 20 years, with the mean and median time periods as almost 4 yrs and exactly 2 years respectively.

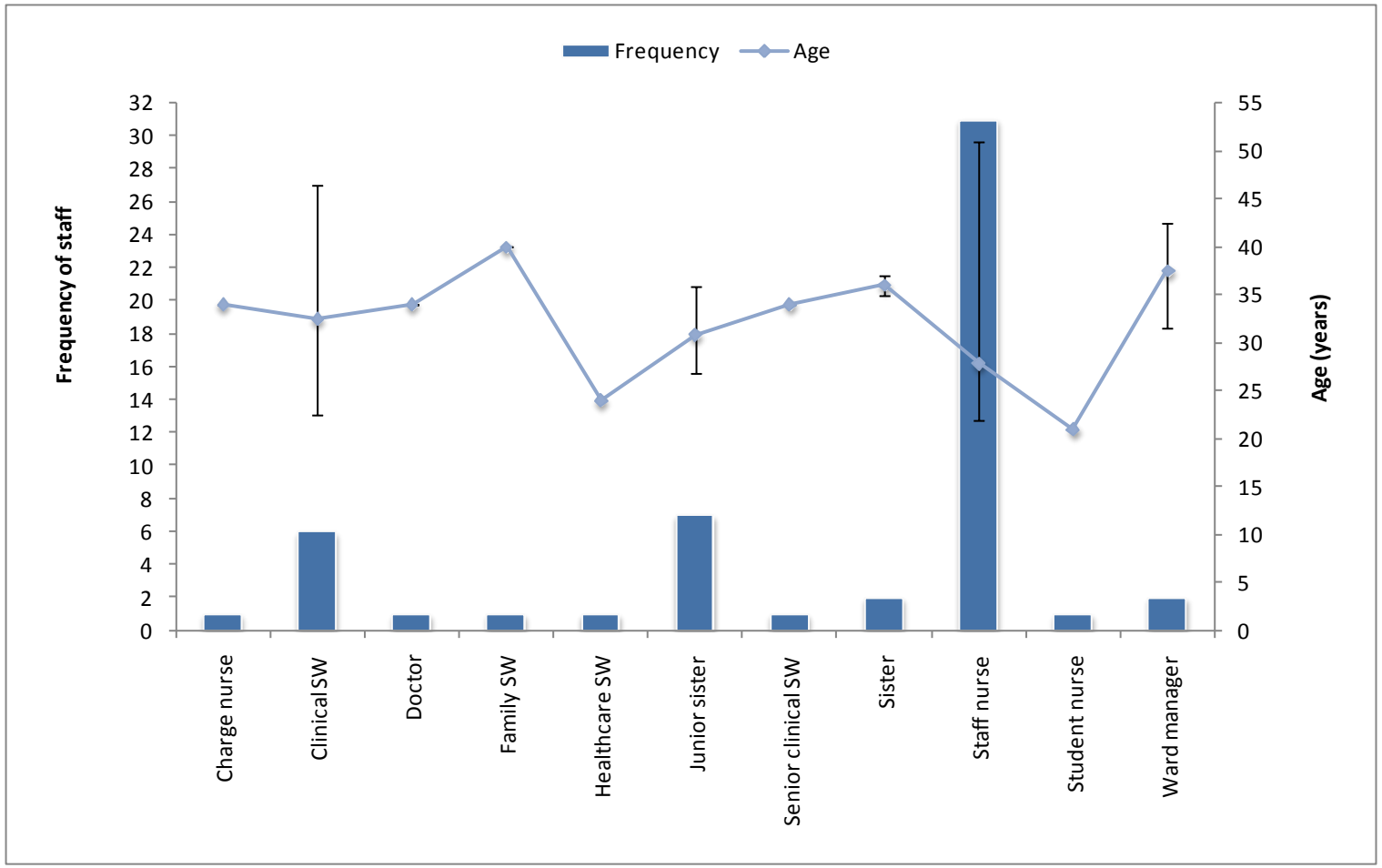

Figure 6.1. Frequency of staff with each job title, plus mean (n=1 to 31) age and age range of staff at Birmingham Children's Hospital who completed the Euclean® clinical audit. SW= support worker.

The final demographic result concluded the individual wards' response rate to the Euclean® clinical audit varied from 13 % to 27.8 % as demonstrated in Table 6.1.

Table 6.1. Euclean® clinical audit response rates of individual wards at Birmingham Children's Hospital.

Ward Number and Speciality	Response rate (% of ward)
4 - Neonatal Surgery	14.8
5 - Burns Centre	22.2
6 - Medical Ward	27.8
10 - Neuro-Surgery	13.0
11 - Heart Unit	22.2

6.5.1.1. Phase 1 of the cross-over study: Current cleaning and disinfection wipes used within Birmingham Children's Hospital

The Euclean® clinical audit revealed that disinfectant wet wipes are used daily by 98.1% participants (n=53), and monthly by 1.9 % as part of their routine work in practice, with a total of eight different wipes identified as being in-use within the hospital. Sanicloth-70 was by far the most common, used by almost 48 % of participants (Figure 6.2), however 37 % of those questioned reported using more than one type of wet wipe for surface disinfection.

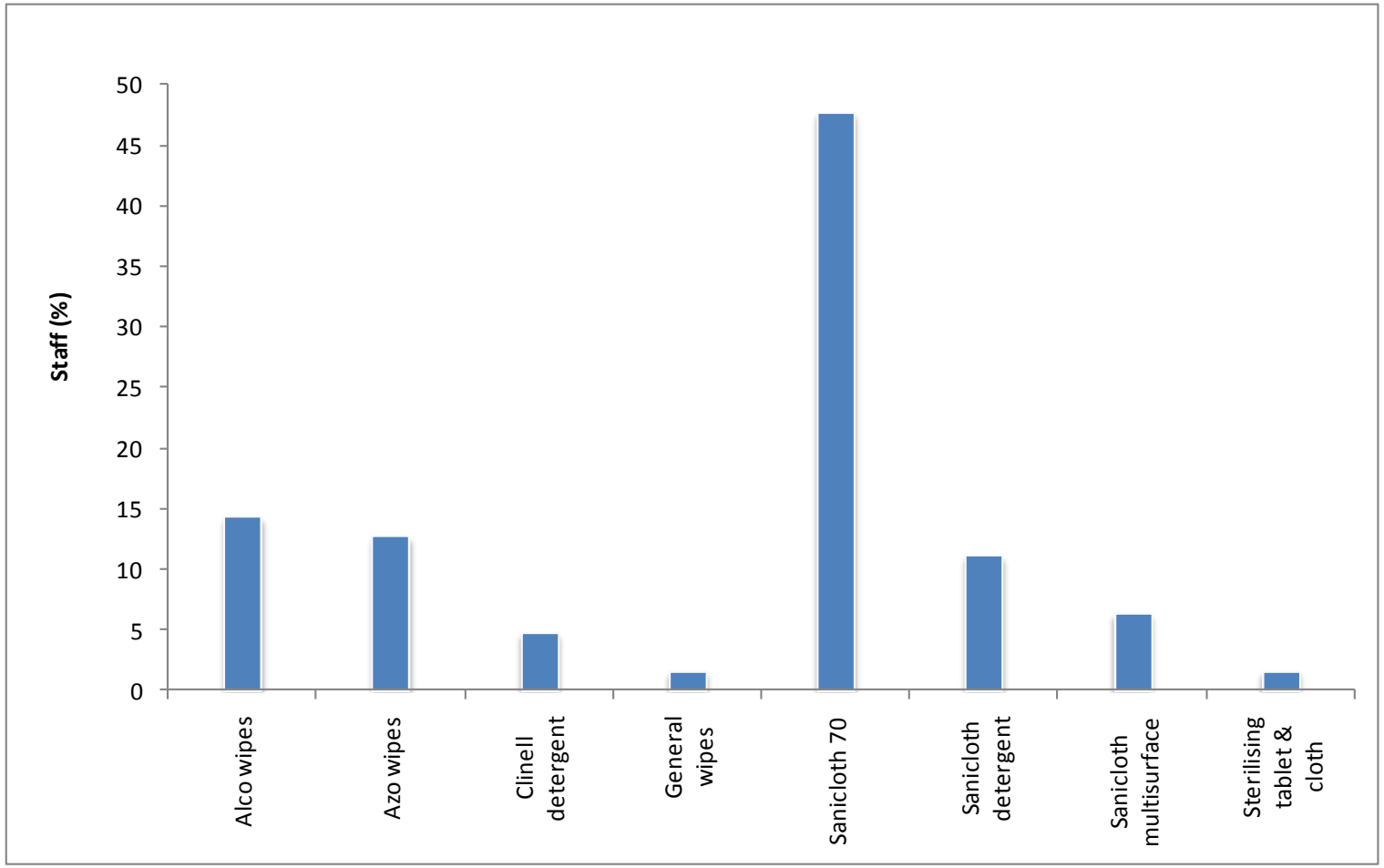

Figure 6.2. Disinfectant wet wipes currently used as part of daily disinfection practice by staff at Birmingham Children's Hospital, shown as the percentage of staff to use each particular type of wipe.

Disinfectant wet wipes were used by participants to clean every surface option from a list of 30 suggestions, and whilst some surfaces were ticked by almost every participant such as cots and bedside cabinets, others were only indicated by one or two; these included toilets, windows and floors (Figure 6.3). The option of 'other' invited responses which included intravenous trays (n=9), dressing trolleys (n=5), trolleys (n=4), drip stands (n=2), couches (n=1) and baths (n=1).

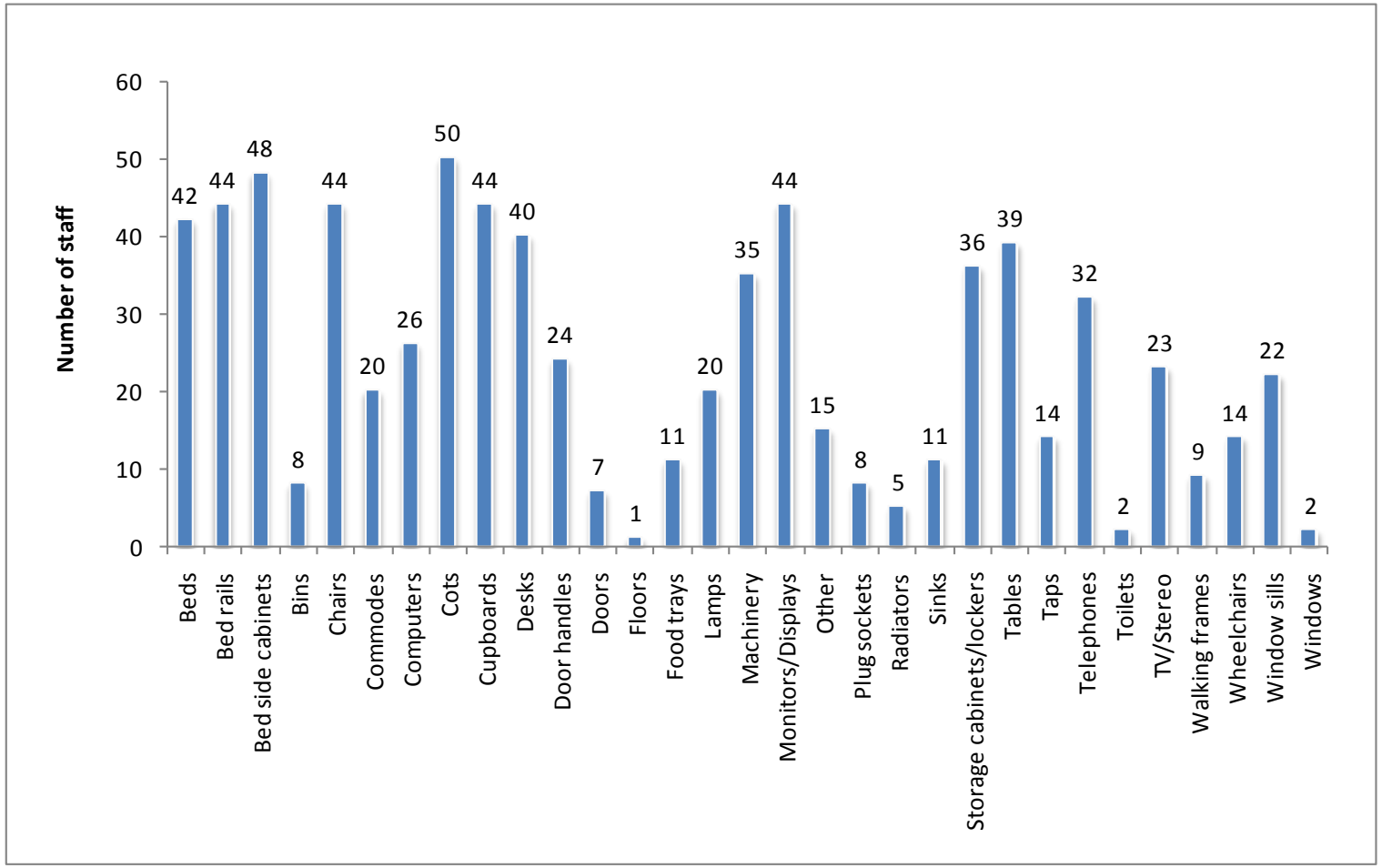

Figure 6.3. Objects and surfaces cleaned by staff at Birmingham Children's Hospital with their usual disinfectant wet wipes, labelled with the number of staff responses.

The most common materials to make up the wiped surfaces were plastic and metal with 52 and 41 participant responses respectively, conversely copper was not recorded by any respondent (Figure 6.4). The option of 'other' brought about the responses of laminate from two participants and cloth from one another, the fourth did not specify a material. The size of surface that could be cleaned before the disinfectant wet wipe needed replacing produced a rather large range of responses from 4.5 cm^2 to 300 cm^2. The mean size was 89 cm^2 and the mode and median were both 40 cm^2. Sixteen participants did not enter a size measurement, and 5 stated 'it varies'.

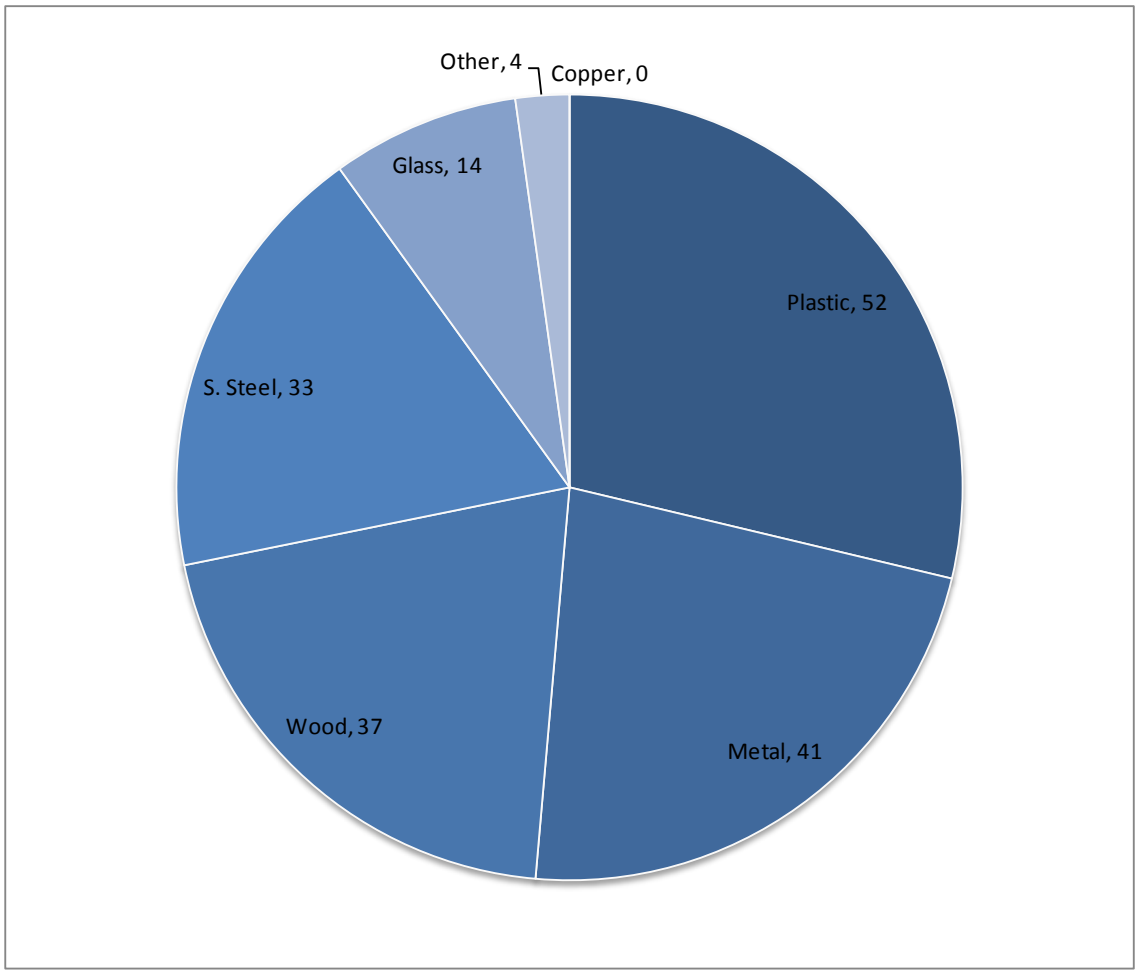

Figure 6.4. Number of responses by staff at Birmingham Children's Hospital when asked what material the surfaces wiped by disinfectant wet wipes are made from.

Skin irritation from the disinfectant wet wipes was experienced by 3 (5.6 %) of the 54 participants, with the symptoms reported consisting of redness, stinging, eczema and hands cracking. When asked for their opinion on the disinfectant wet wipes, participants

suggested 9 advantageous themes and 6 disadvantageous (Table 6.2). Overall, when asked to rate the wipes on a 1 to 10 scale, a mean of 7.5 was achieved covering the range 4 to 10. The median and mode were both 8 with a resultant final total 'score' of 403. However, 85.2 % of participants said they would be prepared to change from their current disinfectant wet wipes to an alternative wipe.

Table 6.2. Staff comments of the advantages and disadvantages of their current disinfectant wet wipes for surface cleaning within Birmingham Children's Hospital, followed by the number of staff to suggest each.

Advantages	Disadvantages
▪ Ease of use (13) ▪ They clean well (9) ▪ Smell either nice or fragrance free (6) ▪ High alcohol content makes them good for disinfection (2) ▪ Remove stubborn stains (1) ▪ No need for soap and water (1) ▪ Does not leave smears (1) ▪ Stay wet (1) ▪ Does not leave hands rough (1)	▪ Smell (7) ▪ Dry out quickly (6) ▪ Difficult to get out of dispenser (4) ▪ Does not get rid of tough stains (3) ▪ Use a lot so not as environmentally friendly (1). ▪ Sting if hands cut (1)

6.5.1.2. Phase 2 of the cross-over study: Use of Euclean® hard surface disinfection wipes used within Birmingham Children's Hospital

Euclean® wipes had been used daily by 98.1 % of participants during the second week of the audit, and only once by 1.9 % (n=53:1). There was no difference in the types of surfaces cleaned by Euclean® wipes compared to those previously recorded in the first half of the questionnaire. Bedside cabinets and cots were again the most frequently declared with 46 responses each, whilst toilets, windows and floors were again the least (Figure 6.5). The option of 'other' invited the following responses: intravenous trays (n=7), trolleys (n=5), dressing trolleys (n=4), drip stands (n=1) and examination couches (n=1).

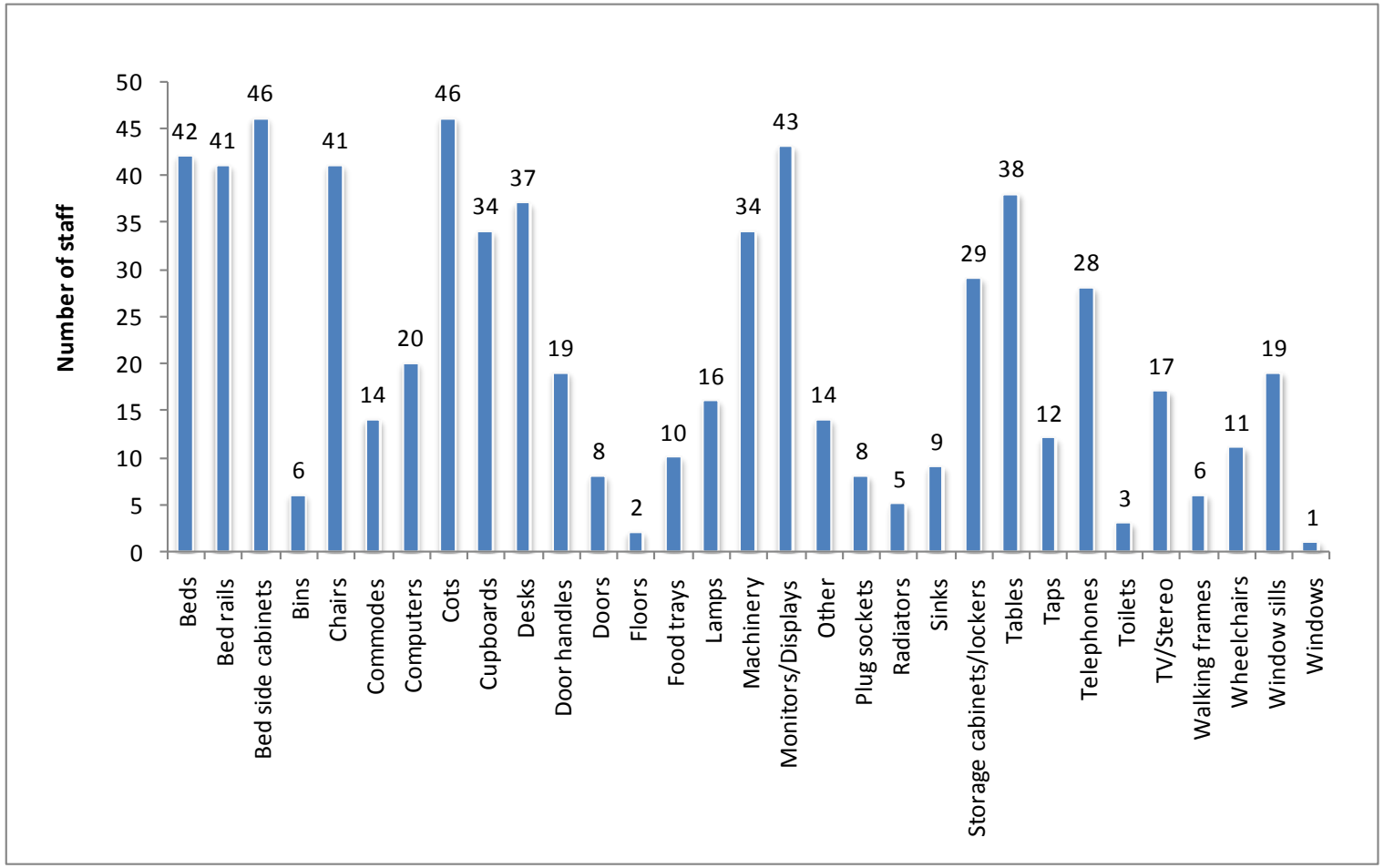

Figure 6.5. Objects and surfaces cleaned by staff at Birmingham Children's Hospital with Euclean® wipes, labelled with the number of staff responses.

As was found in the responses from the first half of the questionnaire, the most common materials to make up the wiped surfaces were plastic and metal with 53 and 46 participant responses respectively, on the contrary copper was only recorded by one respondent (Figure 6.6). The option of 'other' brought about the same responses as previous (laminate, cloth and not specified). As previously demonstrated with the disinfectant wet wipes, the size of surface that could be cleaned before the Euclean® wipe needed replacing produced a vast range of responses from 20 cm^2 to 300 cm^2. The mean surface size was 82 cm^2 while the mode and median were both 40 cm^2. Twenty-two participants did not enter a size measurement, and 4 stated 'it varies'.

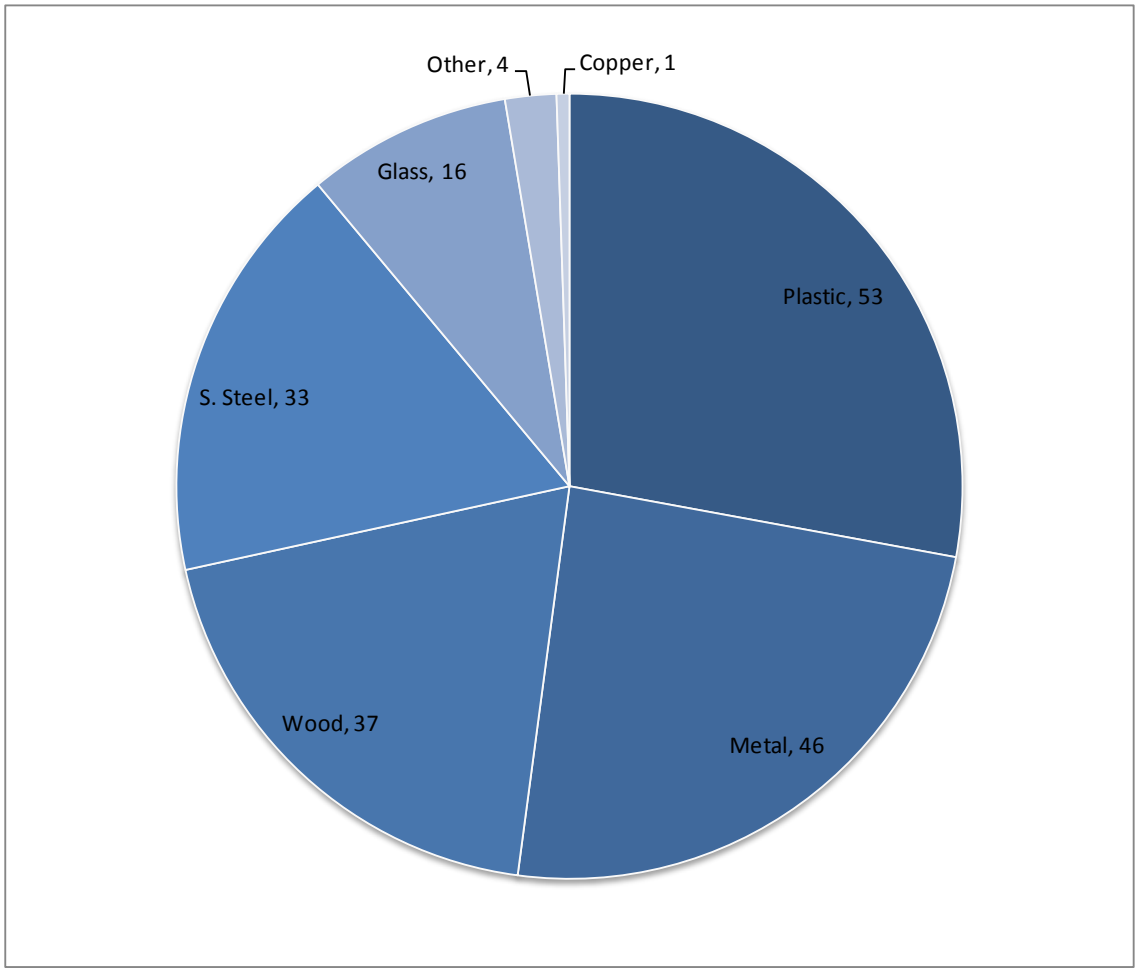

Figure 6.6. Number of responses by staff at Birmingham Children's Hospital when asked what material the surfaces wiped by Euclean® wipes are made from.

Participants preferred the existing packaging of Euclean® wipes with 46 choosing the current tub as a preference (Table 6.3). Larger tubs and flexible re-sealable pouches also

demonstrated some popularity with 11 and 10 votes respectively. The single 'other' response simply requested two sizes of packaging to be available. The size of the Euclean® wipes was categorised as 'just right' by 85.2 % of participants, with 11.3 % and 3.7 % describing them as 'too small' and 'too big' respectively. The thickness was also deemed to be 'just right' by the majority with a 77.8 % response rate, whilst the remaining 22.2 % concluded the wipes to be 'too thin'. None of the participants thought the Euclean® wipes were too thick.

Table 6.3. The number of staff at Birmingham Children's Hospital in favour of various types of packaging for Euclean® wipes.

Packaging	Number
Flexible plastic pouch with re-sealable peal back opening	10
Individually wrapped sachets	4
Larger tubs	11
Other	1
Smaller tubs	5
Tubs (as currently packaged)	46

Skin irritation and discomfort from the Euclean® wipes was experienced by 7 (13 %) of the 54 participants, with an array of symptoms reported including itchy and watery eyes, wheezing, rash, stinging eyes and throat, sore hands and headache. When asked for their opinion on the disinfectant Euclean® wipes, participants suggested 10 advantageous themes and 7 disadvantageous (Table 6.4). Overall, when asked to rate the wipes on a 1 to 10 scale, a mean of 6.3 was achieved covering the full range (1-10). The median was 6 and the mode was 9, with a resultant final total 'score' of 341.

Table 6.4. Staff comments of the advantages and disadvantages of Euclean® wipes for surface cleaning within Birmingham Children's Hospital, followed by the number of staff to suggest each.

Advantages	Disadvantages
▪ Smell nice (16) ▪ Cleaned well (3) ▪ Quick and easy to use (2) ▪ Dry quickly (2) ▪ Stays wet longer (2) ▪ Stronger wipe (1) ▪ Easy to remove from tub (1) ▪ Removed sticky patches (1) ▪ Did not leave streaks (1) ▪ No need for soap and water (1)	▪ Smell (30) ▪ Dried out too quickly (7) ▪ Irritant/caused rash (3) ▪ Needed more than normal to clean an area (1) ▪ Left smears (1) ▪ Does not clean as well (1) ▪ Not easy to open wipe fully (1)

Finally, participants were invited to leave any general feedback or comments with regards to Euclean® wipes; of the many that did, a large proportion commented either for or against the smell, or on the inability of the wipes to remain moist over long periods of time; however a selection of some of the more interesting statements are listed below.

"I felt the wipes did the same job as the wipes already used, but as stated earlier the general feeling on the ward was that the Euclean® wipes had a nasty smell to them. Unless cost is an issue I see no advantages in changing over. It would be interesting to see research on bacteria on surfaces following the use of present wipes vs. Euclean®."

"The wipes had an overall better cleaning ability; however on some tough stains extra scrubbing was needed (stain not known). The fragrance of the wipes is more pleasant and acceptable in a ward environment."

"Smell much nicer than original alcohol smell. Also good for clearing blocked nose! Takes away from other smells on ward e.g. if used to clean bed after child vomits as it over powers the vomit smell."

“The smell is very strong and they seem to be quite dry, but they still do the job, so if they have fewer chemicals than the other wipes they are ok.”

“It's very good, does not leave any streaks on surfaces. It's not as moist as the other ones we used. Too strong of eucalyptus but leaves a fresh scent/smell after.”

“Overwhelming odour, this can be quite strong to small babies especially when used in heated baby incubators.”

“I liked the smell more than others, didn't smell so "hospital" and "clinical" as others.”

6.6. Discussion

Over recent years, the use of disinfectant wet wipes for cleaning hard surfaces within the healthcare setting has increased vastly, with an assortment of brands now available. There are a number of advantages of using these types of wet wipes for disinfection over conventional products. Already impregnated with antimicrobial solutions, wet wipes require no additional preparation such as dissolving tablets in liquid, therefore are easier to use and can be available for instant use whenever needed. Furthermore, their compatibility with neat packaging means they can be stored handily in pocket sachets or in wall mounted tubs, encouraging use. However, wet wipes are, like so many other disinfectant products, not without their drawbacks. Uncertainty regarding longevity of either an individual wipe or an opened packet can lead to wipes dying out and therefore losing efficacy. Furthermore, some evidence has pointed out the potential of some wipes to induce surface cross contamination if they are not bactericidal or used on multiple surfaces when design favours a single use application (Bartz *et al*., 2010).

This study aimed to ascertain healthcare workers opinions towards the wipes they currently use during their daily practice, and determine how they compared to Euclean® wipes. As a result of this study, the opinions gathered could assist and guide further development of the wipes, to enable production to be of a product favoured by those for whom it has been designed.

The response rate for this study, defined as the percentage of useable, returned questionnaires from the number of eligible respondents, was 32 %, which compared poorly to other voluntary studies involving health professionals, where rates of over 50 % have been reported (Aiken *et al*., 2002). However, a review published by Asch *et al*. (1997) noticed how the response rates published in medical journals differed between physicians and non-physicians, with mean values of 54 % and 68 % respectively. As the majority of those eligible to partake in this study were non-physicians, this further disparages the response rate obtained. Response rates are important in questionnaires, as the higher the response rate, the more serious a study is taken as it offers a better representation of the thoughts of the entire group.

One study by Nakash *et al*. (2006) concluded that shorted questionnaires of between 7 and 47 questions resulted in a 9 % increase in response rates compared with longer ones of between 36 and 123 questions. In addition to this, they also noted that implementing follow up efforts were likely to increase response rate yet surprisingly, the use of

incentives bore no significance. The Euclean® questionnaire contained only 28 questions and follow up calls and visits were conducted, therefore it would be right to assume more replies should have been generated. However, the two surgical wards caring for critically ill patients generated the lowest rates, therefore it is possible that prioritisation simply detracted attention away from the questionnaire.

The demographics concluded that the typical respondent would be a 32 year old, female nurse with around 4 year's service in that post. All but one member of staff declared daily use of wet wipes for surface disinfection, with the only exception being a doctor whose job role, unlike that of the nurses, is unlikely to include ward cleaning duties. This emphasises the commercialisation potentials for developing a product chosen by hospitals for use on their wards. Furthermore, the results of preferred packaging type suggested that a variety of small, resealable pouches and larger tubs would make suitable additions to the current sized tub, therefore generating a wider range and increasing the consumer's choice.

The results of this study showed that Euclean® wipes almost completely divided opinion; with a nearly fifty-fifty split in those apparent to love and loathe them. The wipes found to be most commonly used by staff at Birmingham Children's Hospital, were Sanicloth-70, which are impregnated with 70 % alcohol and 2 % CHG; the same composition as in Euclean® wipes, but without the EO. As these are the wipes of choice, it can be assumed that they are generally well-liked. Therefore, the division in opinion towards the Euclean® wipes suggests that the presence of EO is the source of the unfavourable feedback.

Almost 40 % of staff admitted they used more than one type of disinfectant wipe therefore, whilst three people mentioned they had suffered from some degree of skin irritation, it cannot be assumed that the Sanicloth-70 wipes were the cause. The numbers reporting skin irritation rose to seven during the week of the Euclean® wipe use. There are two possible reasons for this; one being that they have sensitive skin and therefore normally use more delicate wipes thus would be most likely to suffer irritation upon changing product, and the second could be linked with the presence of the eucalyptus oil in the Euclean® wipes. EO is a known permeation enhancer and is capable of causing temporary, reversible disruption of the intercellular lipids in the stratum corneum. This can permit entry of otherwise impenetrable substances into the skin,

which can in turn lead to drying of the skin and therefore increased risk of irritation occurring (Aqil *et al*., 2007; Williams *et al*., 2006).

The replacement from their regular wipes to Euclean® wipes, did not result in any great change in the types of surfaces cleaned, in particular there was no observation of any surfaces becoming completely excluded. Nor did they results in a significant change to the size of the area cleaned before needing replacing. Furthermore, the ergonomics including size and thickness of the wipes were rated by most as 'just right'. Therefore, it can be assumed that Euclean® wipes were considered suitable by staff for use on all the usual items requiring disinfection and would prove favourable if large scale production were to commence. The most frequently reported surfaces cleaned (those depicted by 30 or more staff), can be classed as high touch surfaces. These are often very close to patients such as at their bedside, and are touched repeatedly by the patients themselves, healthcare workers and visitors. Therefore, such surfaces are prone to higher levels of microbial colonisation than objects less regularly touched by potentially contaminated hands, such as plug sockets and radiators (Dancer *et al*., 2009). Thus it follows that cleaning protocols would include these objects in daily, if not more frequent cleaning and so more staff would be likely to clean them at some stage.

When asked to give a numerical score of the disinfectant wipes currently used and the Euclean® wipes, staff scored the Euclean® wipes slightly less, with a mean of 6.3 compared with 7.5. However, this alone does not show the entire picture as the most common scores given for the original and Euclean® wipes were 8 and 9 respectively, illustrating an opposing preference to that obtained by comparisons of the means.

Within the questionnaire, staff were invited to offer any advantages or disadvantages of both current and Euclean® wipes, as well as any further comments at the end of the survey. Interestingly, when grouped into themes, the subject of smell which generated the most responses as an advantage also generated the greatest result in the disadvantage category. Respondent opinion was divided with some finding the smell extremely pleasant, while others found it overwhelming, especially if used for long periods of time or in a confined space. Similar attitudes were noted by Tovey & McDonnald (1997), who, while discussing the use of eucalyptus oil in washing detergents in Australia, mentioned that it is considered 'pleasant' by most people, however the residual scent which can last up to 3 days may be a source of annoyance to some.

Finally, some interesting comments made reference to the cost effectiveness and chemical content of Euclean® wipes, revealing some practical issues that could be considered in future development. One respondent had no preference for the current or Euclean® wipes, but stated that if Euclean® wipes were more cost effective, they would be more likely to convert. At present, it is likely that Euclean® wipes will be slightly more expensive than those currently used therefore cost reducing mechanisms could be investigated. Euclean® wipes contain eucalyptus oil which is a natural product therefore, depending on personal opinion, it may not necessarily be considered an added chemical, but as a natural, organic substance, thus on the contrary, Euclean® wipes could signify a more holistic product with similar advantages to those offered by alternative and complimentary therapies.

Whilst the data generated by the Euclean® wipe audit was overall, encouraging, some areas for potential improvement of the product were highlighted. The major disadvantage expressed by some staff was the smell which indicates that further improvements could be made in this area possibly by reducing the EO concentration, providing it is not to the detriment of the antimicrobial efficacy. This could also provide an additional benefit for commercialisation, as the reduction in EO content would not only reduce the intensity of the smell, but also the manufacturing costs related to Euclean® wipes. Furthermore, a decrease in EO could reduce the additional reports of skin irritation generated by Euclean® wipe use, yet further investigations into skin toxicity would be required regardless.

6.7. Conclusion

The Euclean® wipe audit at Birmingham Children's Hospital generated data which could be used in further development and refinement of the product. The most significant result came from the division in opinion towards Euclean® wipes, with the smell being described as the major advantage, as well as the greatest disadvantage. Wet wipes were used daily by all-but-one of the healthcare workers on an array of surfaces, with alcohol based wipes used more so than any other disinfectant wipes. The conversion to Euclean® wipes did not instigate any changes to the objects cleaned or the typical area that could be wiped before replacing. The overall rating of Euclean® wipes generated a mean numerical score of 6.3 out of 10, compared with 7.5 for the currently used wipes, however the result generated most frequently for Euclean® wipes was 9/10 compared to 8/10 for the initial wipes. The frequent comment regarding the 'overwhelming' smell suggests further formulation developments to reduce the EO content would be favoured, potentially reducing the intensity of the smell, manufacturing costs and skin irritation, providing the antimicrobial efficacy is not compromised.

Chapter 7

Development of Eucalyptus Oil-Containing Polyvinyl Alcohol and Chitosan Films with Subsequent Release Studies, Skin Penetration Assessment and Potential Cell Toxicity Testing

7.1. Introduction

Infectious complications arising from implanted medical devices inflict additional complications on patient care. Devices such as central venous catheters (CVC) puncture the skin, breaching its barrier properties and immediately providing a point of entry for microorganisms. The most commonly observed microorganisms colonising these sites are associated with the skin and are often opportunistic pathogens of previously resident microflora such as *S. epidermidis* (Rohde *et al*., 2010). Additional complications can arise from the capability of these microorganisms to grow within a biofilm, which provides a physical barrier preventing penetration of antimicrobial agents and immune system detection (Mack *et al*., 2004; Qin *et al*., 2006). Adequate skin antisepsis prior to catheter insertion is therefore required however recent studies have shown poor penetration of the EPIC recommended solution, 2 % CHG in 70 % IPA, into the skins' deeper layers, thus allowing some viable microorganisms to remain in pores and hair follicles even after following the recommended directions for antisepsis (Karpanen *et al*., 2008*a*). It therefore follows that there is a need for enhanced antiseptic activity or performance that can eliminate all surface and deeper-residing microorganisms, to cease development of many preventable infections.

The use of essential oils for medicinal purposes has been long standing; however recent research has demonstrated that some combinations of oil and CHG can result in synergistic antimicrobial activity, with the results for eucalyptus oil appearing particularly promising (Karpanen *et al*., 2008*b*). Subsequent studies have verified antimicrobial synergism by EO/CHG combination, against a panel of skin-associated microorganisms, with particular focus on its enhanced biofilm removal; furthermore the combination of EO/CHG/IPA has also demonstrated significantly enhanced skin penetration compared with CHG/IPA (Hendry *et al*., 2009; Karpanen *et al*., 2010).

The delivery of agents into the skin has often been overcome with the application of creams or lotions however their use can induce complications including difficulty in accurately measuring dosage and accidental removal by washing. These issues can be circumvented by the use of transdermal patches, with many varieties now widely available. These are capable of delivering sustained and controlled release of medication into or across the skin and are frequently supplemented with permeation enhancers (Prausnitz & Langer, 2008). One patch currently available from Johnson & Johnson incorporates CHG into impregnated sponges, and is designed for use as a dressing to

surround intravascular catheters. Results obtained from clinical trials cited reductions in bloodstream infection rates related to central venous catheters following introduction of these CHG biopatches to a number of intensive care units (Timsit *et al*., 2009).

EO is a recognised permeation enhancer, largely due to its major component 1,8-cineole, which has been used in previous studies for enhancing dermal delivery (Liu *et al*., 2011). Therefore, bringing together the knowledge that EO and CHG provides deeper skin penetration than current recommendations, possesses synergistic antimicrobial activity against a number of skin microorganisms and acts synergistically against biofilms, it is possible that application of this combination into a dermal patch, could provide enhanced skin antisepsis prior to insertion of an intravascular device, and furthermore, could continue to impart antimicrobial protection around the insertion site to prevent and eliminate biofilm infection. However, application into a dermal patch would require development as well as appropriate, comprehensive testing to include release studies, verification of antimicrobial efficacy, compatibility testing with CVC plastic and assessment of skin toxicity using tissue culture techniques.

7.2. Aims of the study

The previous chapters within this thesis have focused upon the antimicrobial activity of EO and CHG with emphasis on the development of a novel hard surface disinfectant. The aims of this final series of experiments were to:

- Expand the concepts of this thesis further, by assessing the potential of polyvinyl alcohol and chitosan as biopatch films, into which EO and CHG could be incorporated.
- Investigate the antimicrobial efficacy of polyvinyl alcohol and chitosan biopatch films.
- Study the release of EO and CHG from a polyvinyl alcohol biopatch through skin penetration studies, using high performance liquid chromatography and gas chromatography for analysis.
- Determine to potential toxicity of 5 % and 2 % Euclean® solutions using tissue culture techniques.

7.3. Materials

7.3.1. Microbial cultures

Staphylococcus aureus ATCC 6538, *Staphylococcus epidermidis* RP62A, MRSA N315, *Pseudomonas aeruginosa* ATCC 15442, *Escherichia coli* NCTC 10418 and *Candida albicans* ATCC 76615 were stored on MicroBank beads (Pro-Lab Diagnostics, Cheshire, U.K.) at -70 °C until required.

7.3.2. Cell lines

Airway epithelial cells (AEC) C38 CF-corrected cells were purchased from the American Type Cultures Collection (ATCC), and human pulmonary fibroblasts (HPF) were purchased from PromoCell (Heidelberg, Germany).

7.3.3. Microbiological media

Airway epithelial cell growth medium (AEM) and fibroblast growth medium 2 (FGM2) were purchased from PromoCell (Heidelberg, Germany).

7.3.4. Chemicals

7.3.4.1. Chlorhexidine digluconate stock solution

Stock solutions of chlorhexidine digluconate (CHG) (Sigma-Aldrich, Dorset, U.K.) were made fresh on the day of use, to a concentration of 256 µg/mL and further diluted as required. The concentration of CHG was 200,000 µg/mL, thus the stock solution consisted of 12.8 % (v/v) CHG in PBS (Sigma-Aldrich, Dorset, U.K.) to give a concentration of 25,600 µg/mL, which then required a 1:100 dilution to achieve the desired stock solution concentration of 256 µg/mL.

7.3.4.2. Neutralising solution

Neutralising solution was prepared with 1.17 % (w/v) lecithin, granular, Mw 750.00 Acros Organics (Fisher Scientific, Leicestershire, U.K.), 2 % (v/v) tween-80 (Sigma-

Aldrich, Dorset, U.K.), 0.785 % (w/v) sodium thiosulphate pentahydrate (BDH Ltd, Poole, U.K.) and 0.1 % (v/v) triton X-100 (Sigma-Aldrich, Dorset, U.K.), made up to 1 L with double distilled water. The solution was sterilised at 121 °C for 15 minutes and stored at 4 °C until required.

7.3.4.3. Mobile phase 75:25 for high performance liquid chromatography

Isocratic mobile phase containing methanol: water in a ratio of 75:25 was prepared with 75 % (v/v) HPLC grade methanol (Fisher Scientific, Leicestershire, U.K.), 0.2 % (v/v) diethylamine (Sigma-Aldrich, Dorset, U.K.) and 0.005 M sodium 1-heptane sulfonate (Sigma-Aldrich, Dorset, U.K.) made up to 1 L with double, distilled water. Glacial acetic acid HPLC grade (Fisher Scientific, Leicestershire, U.K.) was added gradually to lower the pH to pH 4. The mobile phase solution was then degassed with a vacuum pump, and stored in a brown jar for use that week.

7.3.4.4. Mobile phase 65:35 for high performance liquid chromatography

Isocratic mobile phase containing methanol: water in a ratio of 65:35 was prepared with 65 % (v/v) HPLC grade methanol (Fisher Scientific, Leicestershire, U.K.), 0.2 % (v/v) diethylamine (Sigma-Aldrich, Dorset, U.K.) and 0.005 M sodium 1-heptane sulfonate (Sigma-Aldrich, Dorset, U.K.) made up to 1 L with double, distilled water. Glacial acetic acid HPLC grade (Fisher Scientific, Leicestershire, U.K.) was added gradually to lower the pH to pH 4. The mobile phase solution was then degassed with a vacuum pump, and stored in a brown jar for use that week.

7.3.4.5. Methanol water for high performance liquid chromatography

Flush for the HPLC was made with 50:50 HPLC grade methanol (Fisher Scientific, Leicestershire, U.K.) in double distilled water then degassed with a vacuum pump, and stored in a brown jar for use that week.

7.3.4.6. Euclean® and chlorhexidine digluconate/isopropyl alcohol solutions for tissue culture

Euclean® solutions were prepared to 5 % (v/v) and 2 % (v/v) concentrations, in both airway epithelial cell growth medium (AEM) (PromoCell, Heidelberg, Germany) and fibroblast growth medium 2 (FGM2) (PromoCell, Heidelberg, Germany). Euclean® solutions contained 5 % (v/v) or 2 % (v/v) EO with 2 % (v/v) CHG, 70 % (v/v) IPA and 1 % (v/v) tween-80 in AEM and FGM2. The CHG/IPA solution was also prepared in both cell growth media and comprised 2 % (v/v) CHG and 70 % (v/v) IPA in AEM and FGM2.

7.3.5. Polyvinyl alcohol film preparation

Polyvinyl alcohol (PVA) powder was purchased from Sigma-Aldrich (Dorset, U.K.) in three different molecular weights; low Mw 47,000 Dalton (Da), medium Mw 125,000 Da and high Mw 205, 000 Da. The powder was dissolved in sterile, double distilled water while stirring for 4 hours at 95 °C. It was then cooled to 30 °C and EO and/or CHG and/or tween-80 were added as required before casting onto acrylic boards (Perspex Distribution, Tamworth, U.K.) to a thickness of 1000 µm using an Erichsen Film Applicator, Multicator 411 (Erichsen, Hemer, Germany).

7.3.6. Chitosan film preparation

Chitosan powder of a medium Mw was purchased from Sigma-Aldrich (Dorset, U.K.). The powder was dissolved in sterile, double distilled water and varying concentrations of HPLC grade glacial acetic acid (Fisher Scientific, Leicestershire, U.K.) while stirring overnight at room temperature as described in methods by Chi *et al*. (2006). It was then cooled to 30 °C and EO and/or CHG and/or tween-80 were added as required before casting onto acrylic boards (Perspex Distribution, Tamworth, U.K.) to a thickness of 1000 µm using an Erichsen Film Applicator, Multicator 411 (Erichsen, Hemer, Germany).

7.3.7. Skin samples

The skin samples used were provided by The Stephen Kirby Skin Bank (Queen Mary's Hospital, London, U.K.) and were full thickness human skin, acquired from patients undertaking breast reduction surgery. The skin samples were frozen on the day of excision and stored at -70 °C until required. Prior to the study commencing, full ethical approval was obtained (REC 2002/169).

7.3.8. Equipment

High performance liquid chromatography (HPLC) was performed using an Agilent 1200 series high performance liquid instrument (Agilent Technologies, West Lothian, U.K.) with ultraviolet (UV) detection. The HPLC was fitted with a CPS-2 Hypersil reverse phase chromatography column (Thermo Scientific, Northumberland, U.K.) measuring 150 x 4.6 mm, with 5 µm particle size. The other columns used during optimisation were an ODS-2 Hypersil reverse phase chromatography column (Thermo Scientific, Northumberland, U.K.) measuring 150 x 4.6 mm, with 5 µm particle size, and a Pinnacle DB Cyano chromatography column (Restek International, Buckinghamshire, U.K.) measuring 250 x 4.6 mm with 5 µm particle size.

Gas chromatography (GC) was performed using an HP5890 series II, capillary gas chromatograph with flame ionization detector (FID) and split injector (Hewlett Packard, Berkshire, U.K.) programmed with injector and detector temperatures at 200 and 250 ºC respectively. Samples were run through an HP-INNOWax column, measuring 30 m in length with a film thickness of 0.25 µm and column ID 0.32 mm (Hewlett Packard, Berkshire, U.K.). The internal standard used was based on methods described by Milthorpe *et al*. (1998) and consisted of spiking samples with 50 µL of a solution made with 40 % (w/v) n-tetradecane (Sigma-Aldrich, Dorset, U.K.) dissolved in chloroform (Sigma-Aldrich, Dorset, U.K.). Data were analysed using HP Chemstation Software Rev.A.04.01 (Hewlett Packard, Berkshire, U.K.).

Polyvinyl alcohol and chitosan biopatch films were cast to a thickness of 1000 µm using an Erichsen Film Applicator, Multicator 411 model (Erichsen, Hemer, Germany).

Franz diffusion cells (made in-house) were connected to a Techne Circulator C-85A circulating water-heating pump (Bibby Scientific Limited, Staffordshire, U.K.) and stirred using an IKAMAG® RO10 power magnetic stirrer (IKA® Werke, Staufen,

Germany) for the skin penetration studies. Prior to use, the Franz diffusion cell receptor chambers and sampling ports were washed thoroughly; twice with 70:30 acetone (Sigma-Aldrich, Dorset, U.K.):water, twice with Virkon® (Fisher Scientific, Leicestershire, U.K.), twice with PBS (Sigma-Aldrich, Dorset, U.K.) then a final wash with double distilled water. A moisture resistant film (Parafilm M®, Alcan Packaging, U.S.A.) was then used to cover the receptor chambers and sampling ports.

The fluorescent plate reader used for the cell toxicity experiments was a Spectra Max Gemini XS (Molecular Devices, California, U.S.A.) set up for a 24 well plate, with excitation and emission wavelengths of 560 nm and 590 nm respectively, auto-mix on, and machine temperature set to 37 °C. Data were analysed using Softmax Pro V5 (Molecular Devices, California, U.S.A.).

7.4. Methods

7.4.1. High performance liquid chromatography

7.4.1.1. Development of optimum high performance liquid chromatography methodology

A range of triplicate CHG dilutions were made in PBS (Sigma-Aldrich, Dorset, U.K.) from triplicate stock solutions (Section 7.3.4.1) covering the range 256 µg/mL to 0.0039 µg/mL. Each was filtered through a 0.45 µm nylon filter (Kinesis, Cambridgeshire, U.K.) into 8 mm screw cap HPLC vials (Kinesis, Cambridgeshire, U.K.). Samples were analysed using an Agilent 1200 series HPLC instrument (Agilent Technologies, West Lothian, U.K.) under a number of conditions to determine the most suitable for use. Three different columns were tested; an ODS-2 Hypersil reverse phase chromatography column (Thermo Scientific, Northumberland, U.K.) measuring 150 x 4.6 mm, with 5 µm particle size; a Cyano chromatography column (Restek International, Buckinghamshire, U.K.) measuring 250 x 4.6 mm with 5 µm particle size; and a CPS-2 Hypersil reverse phase chromatography column (Thermo Scientific, Northumberland, U.K.) measuring 150 x 4.6 mm, with 5 µm particle size. The ODS-2 and CPS-2 columns were tested using MP at 75:25 and 65:35 ratios of methanol: water, while only 65:35 was used with the Cyano column. On each occasion, the baselines of temperature, pH, pressure, flow rate and wavelength were monitored during the preliminary flushing with MP to ensure stability prior to sample running. The flow rate used in all experiments was 1.2 mL/minute for a duration of up to 12 minutes depending upon the elution time observed under each condition, with ultraviolet detection at 254 nm.

7.4.1.2. Standard curve of chlorhexidine digluconate concentration and peak area generated by high performance liquid chromatography

A range of triplicate CHG dilutions were prepared and filtered as described above (Section 7.4.1.1) from triplicate stock solutions. The samples were analysed on Agilent 1200 series HPLC instrument (Agilent Technologies, West Lothian, U.K.) at room temperature through a CPS-2 Hypersil reverse phase chromatography column (Thermo Scientific, Northumberland, U.K.) measuring 150 x 4.6 mm, with 5 µm particle size. The flow rate used was 1.2 mL/minute for 9 minutes with ultraviolet detection at 254

nm. The isocratic MP was a 75:25 ratio of methanol:water with 0.2 % (v/v) diethylamine (Sigma-Aldrich, Dorset, U.K.), 0.005 M sodium 1-heptane sulfonate (Sigma-Aldrich, Dorset, U.K.) in double, distilled water, adjusted to pH 4 with HPLC grade glacial acetic acid (Fisher Scientific, Leicestershire, U.K.). Each sample was analysed using double injection and the results of peak area plotted against CHG concentration.

7.4.1.3. Level of detection and quantification for high performance liquid chromatography

The level of detection (LOD) and level of quantification (LOQ) were calculated from the standard curve using equations 8 and 11 described by Epshtein (2004).

$$\text{Level of detection} = \frac{3 \times \text{standard deviation of intercept}}{\text{gradient}}$$

$$\text{Level of quantification} = \frac{10 \times \text{standard deviation of intercept}}{\text{gradient}}$$

7.4.2. Gas chromatography

7.4.2.1. Suitability of column

Solutions were prepared of undiluted EO and 1,8-cineole, 0.1 % (v/v) EO or 1,8-cineole in HPLC grade hexane (Sigma-Aldrich, Dorset, U.K.), and hexane alone. One microlitre samples of each were injected into the GC (Hewlett Packard, Berkshire, U.K.) to determine retention time and good resolution between the solvent front and the EO/1,8-cineole peak.

7.4.2.2. Preliminary extraction of eucalyptus oil using hexane

Sterile universal bottles were set up each containing a section of 10 % EO with 2 % CHG wipe measuring 4 cm^2. At set time intervals, 1 mL of HPLC grade hexane (Sigma-Aldrich, Dorset, U.K.) was added to a universal bottle and mixed. The samples were set up such that the wipes were in hexane for 30 minutes, and 1, 2, 4, 6, 8, 10, 12, 18 and 24 hours. After exposure to hexane, 1 µL samples of each were injected into the HP5890 series II, capillary GC (Hewlett Packard, Berkshire, U.K.) and data analysed using HP Chemstation Software Rev.A.04.01 (Hewlett Packard, Berkshire, U.K.). Differences in the quantity of EO extracted from the wipes at each time point were observed by measuring the peak of 1,8-cineole as it passed through the column.

7.4.2.3. Standard curve of eucalyptus oil concentration and peak area generated by gas chromatography

From triplicate stock solutions of EO in HPLC grade hexane (Sigma-Aldrich, Dorset, U.K.) at a concentration of 512 mg/mL, a range of serial dilutions were made also in hexane to a concentration of 0.0078 mg/mL. Internal standard (IS) was spiked into each sample to give 0.02 g of IS per sample before they were filtered through a 0.45 µm nylon filter (Kinesis, Cambridgeshire, U.K.) into 8 mm screw cap HPLC vials (Kinesis, Cambridgeshire, U.K.). Controls of IS, EO and n-tetradecane each dissolved in hexane were prepared. Each sample was then injected into the HP5890 series II, capillary gas chromatograph fitted with an HP-INNOWax column (Hewlett Packard, Berkshire, U.K.) connected to an FID. Helium was the carrier gas and the flow rate was set to 10 pound-force per square inch (psi). The oven temperature was pre-programmed to 150 °C for 3 minutes, while the injector and detector temperatures were 200 and 250 °C respectively, with an injection of 1 µL and a 50:1 split ratio. Data were analysed using HP Chemstation Software Rev.A.04.01 (Hewlett Packard, Berkshire, U.K.). The experiment was repeated in triplicate and the results were plotted with peak area against EO concentration.

7.4.2.4. Level of detection and quantification for gas chromatography

The level of detection (LOD) and level of quantification (LOQ) were calculated from the standard curve using equations 8 and 11 described by Epshtein (2004). (Section 7.4.1.3).

7.4.3. Biopatch film development and antimicrobial testing

7.4.3.1. Polyvinyl and chitosan film optimisation

Optimisation of PVA and chitosan films was carried out in an ordered and methodical manner, encompassing a range of powder concentrations and glacial acid content, with eliminations occurring at each stage based upon the resulting biopatch properties. Films were initially made from powder and water of each concentration. CHG, IPA and EO all purchased from Sigma-Aldrich (Dorset, U.K.) were then added alone and in combination to the more suitable films following assessment of their properties, and finally the quantity of HPLC grade glacial acetic acid within the chitosan films was reduced. Based upon methods described by Gal & Nussinovitch (2009), each of the three PVA powders were prepared in sterile, double distilled water to concentrations of 1 % intervals, ranging 1 % (w/w) to 12 % (w/w) then dissolved with 4 hours of heated stirring at 95 °C. Once dissolved, the solutions were cooled to 30 °C and a variety of 5 % (v/v) EO and/or 2 % (v/v) CHG and/or 1 % (v/v) tween-80 were added as required, before casting onto acrylic boards (Perspex Distribution, Tamworth, U.K.) to a thickness of 1000 µm using an Erichsen Film Applicator, Multicator 411 (Erichsen, Hemer, Germany). Chitosan films were prepared in sterile, double distilled water to concentrations of 0.5 % intervals, ranging 1 % (w/w) to 5 % (w/w) both without and with HPLC grade glacial acetic acid (Fisher Scientific, Leicestershire, U.K.) added in equal, corresponding concentrations at 0.5 % intervals from 1 % (v/v) to 5 % (v/v). The powder was allowed to dissolve at room temperature overnight before 5 % (v/v) EO, 2 % (v/v) CHG and 1 % (v/v) tween-80 were added alone and in combination, and cast onto acrylic boards as previously described for PVA. The most suitable films for consideration in future studies were decided upon by a number of characteristics including flexibility, and the ability of powder to fully dissolve and antimicrobials to mix without separation.

7.4.3.2. Release study of eucalyptus oil and chlorhexidine digluconate from a polyvinyl alcohol biopatch film using Franz diffusion cells

Biopatches were made from 7 % (w/w) PVA with Mw 47,000 Da containing 5 % (v/v) EO, 2 % (v/v) CHG and 1 % (v/v) tween-80; controls were absent of EO and tween-80. Verification of EO and CHG release was observed using Franz diffusion cells. The receptor chambers were filled with 29 mL of PBS (Sigma-Aldrich, Dorset, U.K.) which was held at 37 °C by heated water circulating through water jackets. A magnetic stirring bar was added to each receptor chamber before the chambers and sampling ports were sealed with moisture resistant film (Parafilm M®, Alcan Packaging, U.S.A.) until the temperature of the PBS was as required. Once at temperature, a section of biopatch was placed over each receptor chamber, triplicate contained CHG alone and acted as controls, another three contained EO and CHG. Samples of 1 mL receptor chamber fluid were taken through needles via the sampling port at time zero, 30 minutes, 1, 2, 4, 6 and 24 hours, and replaced with 1 mL warmed PBS each time. Each sample was filtered, spiked with IS if required and processed by the HPLC or GC as appropriate to verify release of both agents from the patches and determine if the presence of EO has any effect on CHG release.

7.4.3.3. Microbial inhibition by eucalyptus oil and chlorhexidine digluconate-containing polyvinyl alcohol and chitosan films using agar diffusion

The following patches were made as described previously (Section 7.4.3.1), each was prepared with, and without 5 % (v/v) EO, 2 % (v/v) CHG and 1 % (v/v) tween-80;

- 7 % (w/w) PVA, Mw 47,000
- 9 % (w/w) PVA, Mw 47,000
- 7 % (w/w) PVA, Mw 125,000
- 7 % (w/w) PVA, Mw 205,000
- 9 % (w/w) PVA, Mw 205,000
- 2 % (w/w) chitosan with 2 % (v/v) HPLC grade glacial acetic acid

Cell suspensions containing 10^4 cfu/mL of each microorganism were prepared in MHB or SDB from five identical colonies grown on MHA or SDA. Cotton wool swabs were used to inoculate the surfaces of MHA or SDA plates before squares of each biopatch measuring 20 mm by 20 mm were applied to triplicate plates. Plates were incubated overnight at 37 ºC; 30 ºC for *C. albicans*, and inhibition zone sizes were measured as the distance between the edge of the patch and visible growth.

7.4.3.4. Bacteriostatic/bactericidal testing of eucalyptus oil and chlorhexidine digluconate-containing polyvinyl alcohol and chitosan films

Following completion of the previous experiment (Section 7.4.3.3), the biopatches were removed from the surface of the agar and a section of agar measuring 10 mm by 10 mm was cut from underneath the patch and inserted into a bijou bottle containing 1 mL neutralising solution. The bijou bottle was mixed for 1 minute, then after 30 minutes contact with the neutralising solution, 1 mL was removed into a petri dish and a pour plate made with molten MHA or SDA containing 10 % (v/v) neutralising solution, cooled to 50 °C. Plates were incubated and microbial viability used to determine bacteriostatic or bactericidal mode of action.

7.4.4. Catheter tolerance to Euclean® solution

A Hydrocath Assure™ catheter and hub (Beckton Dickinson, Oxford, U.K.) were fully submerged into Euclean® solution (5 %) and assessed visually every 24 hours over a 5 day period for evidence of fragmentation or disintegration. A control was also set up with an identical catheter and hub submerged in PBS (Sigma-Aldrich, Dorset, U.K.). After 5 days, the devices were removed and their texture assessed for signs of becoming sticky or softened.

7.4.5. Polyvinyl alcohol films on human skin

7.4.5.1. Penetration of eucalyptus oil and chlorhexidine digluconate from a polyvinyl alcohol film into human skin

The penetration of EO and CHG through human skin from the biopatch was assessed with the use of Franz diffusion cells. The receptor chambers were filled with 29 mL of PBS (Sigma-Aldrich, Dorset, U.K.) which was retained at 37 °C by heated water circulating through water jackets. A magnetic stirring bar was added to each receptor chamber before the chambers and sampling ports were sealed with moisture resistant film (Parafilm M®, Alcan Packaging, U.S.A.) until the temperature of the PBS was as required. Full thickness human skin was partially thawed at room temperature and cut into circular discs measuring 3 cm diameter. The moisture resistant film (Parafilm M®, Alcan Packaging, U.S.A.) was removed from the receptor chambers of the Franz diffusion cells, and the skin mounted in its place such that the stratum corneum (SC) was upward facing, and the underside was in contact with the PBS in the receptor chamber, having removed any air bubbles. The donor chambers were clamped on top of the skin and sealed with moisture resistant film (Parafilm M®, Alcan Packaging, U.S.A.) for around 30 minutes to allow the surface of the skin to reach 32 °C prior to commencement of an experiment. Once the skin had reached temperature, the donor chamber was temporarily removed and a section of biopatch placed over the skin, ensuring full contact, before the donor chamber was replaced. The biopatches tested were 7 % (w/w) PVA, Mw 47,000 Da, containing 5 % (v/v) EO, 2 % (v/v) CHG and 1 % (v/v) tween-80, with triplicate skin discs used for the EO and CHG patch, and triplicate used with a CHG only patch as a control. Samples of 1 mL receptor chamber fluid were taken through needles via the sampling port at time zero, 30 minutes, 1, 2, 4, 8 and 24 hours, and replaced with 1 mL warmed PBS each time. Each sample was split with half being filtered and processed by the HPLC for detection of CHG, and the other half spiked with IS, filtered and processed on the GC for EO presence.

7.4.5.2. Detection of eucalyptus oil and chlorhexidine digluconate in layers of human skin

Following 24 hour exposure of full thickness human skin to biopatches containing CHG with and without EO as described above (Section 7.4.5.1), the patches were removed

and discarded, and the six discs of skin were removed and frozen with cryospray (Bright Instruments, Cambridgeshire, U.K.) then stored at -20 °C until sliced. Duplicate punch biopsies measuring 8 mm diameter were cut from each of the discs and mounted onto cork with embedding compound (Bright Instruments, Cambridgeshire, U.K.). Each biopsy was sectioned horizontally using a Bright OFT cryotome (Bright Instruments, Cambridgeshire, U.K.) into five slices of 20 µm, five of 40 µm and five of 60 µm, then added to pre-weighted eppendorf tubes such that the duplicate punch biopsies from each disc were combined allowing the weight of each skin sample to be determined. PBS (Sigma-Aldrich, Dorset, U.K.) was added to each eppendorf tube and mixed before the samples were split and filtered as previously described (Section 7.4.5.1), and analysed using HPLC and GC.

7.4.6. Testing of Euclean® solutions using tissue culture

7.4.6.1. Tissue culture technique

Airway epithelial cells (AEC) and human pulmonary fibroblasts (HPF) were grown for 48 hours at 37 °C, in 10 mL airway epithelial cell growth medium (AEM) and fibroblast growth medium 2 (FGM2) (both from PromoCell, Heidelberg, Germany) respectively, on the base of triplicate, 75 cm^2 tissue culture flasks (Corning, Amsterdam, Netherlands). The medium was discarded and each flask washed once with PBS (Sigma-Aldrich, Dorset, U.K.) to prevent the medium inhibiting trypsin activity later on. The PBS was then also discarded. Cells were lifted from the base of the flasks by adding 2.5 mL of trypsin ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, Dorset, U.K.) to each flask. After 2 minutes contact at 37 °C, 6.5 mL AEM or FGM2 was added as appropriate, to the flasks before being mixed gently to dislodge any remaining bound cells. The triplicate AEC and HPF cell suspensions were combined in universal bottles to give one large suspension of each cell line. Cell counts were performed using an improved, Neubauer double cell, standard chamber, haemocytometer (Fisher Scientific, Leicestershire, U.K.). Dilutions were performed in AEM or FGM2 accordingly and 500 µL seeded into wells of triplicate 24-well, tissue culture test plates (SPL Life Sciences Inc., Korea) such that the cell suspension gave 10^5 cfu/well. Plates were then incubated in air at 37 °C overnight before toxicity testing could begin.

7.4.6.2. Cell toxicity of Euclean® solution to human airway epithelial cells and human pulmonary fibroblasts

Triplicate 24-well tissue culture test plates (SPL Life Sciences Inc., Korea) were prepared as described previously (Section 7.4.6.1) with cell suspensions of AEC and HPF at a concentration of 10^5 cfu/well and incubated overnight to allow the cells to adhere to the base of the wells. Following incubation, medium was removed and replaced with either 500 µL of the 5 % Euclean® solution, 2 % Euclean® solution or the CHG/IPA solution. Growth control wells were exposed to 500 µL fresh AEM or FGM2 accordingly while negative controls containing 10 % (v/v) triton X-100 (Sigma-Aldrich, Dorset, U.K.) in AEM or FGM2 were also made. Cells were exposed to test and control solutions for time periods of zero, 10 and 30 minutes, 1, 2 and 4 hours with plates incubated at 37 °C between samples. After the allotted time period, 100 µL cell titre-blue, cell viability assay (Promega, Southampton, U.K.) was added to the wells then plates were returned to the incubator for a further 90 minutes. Cell viability was determined by measuring fluorescence with a Spectra Max Gemini XS (Molecular Devices, California, U.S.A.).

7.5. Results

7.5.1. High performance liquid chromatography

7.5.1.1. Development of optimum high performance liquid chromatography methodology

The CPS-2 Hypersil column with 75:25 MP was determined as most suitable for use with CHG samples in future experiments, as other column/MP combinations resulted in various complications such as limitations on detection of lower concentration samples, solvent front and peak too close together and irregular sized or shaped peaks.

7.5.1.2. Standard curve of chlorhexidine digluconate concentration and peak area generated by high performance liquid chromatography

The standard curve for CHG measured using HPLC produced a linear graph with an R^2 value of 1.0 over the range 0.03125 to 256 µg/mL CHG (Figure A15).

7.5.1.3. Level of detection and quantification for high performance liquid chromatography

The LOD and LOQ were calculated as 0.1588 µg/mL and 0.5293 µg/mL respectively.

7.5.2. Gas chromatography

7.5.2.1. Suitability of column

The HP-INNOWax column (Hewlett Packard, Berkshire, U.K.) column was established as suitable for future use as the preliminary peaks revealed good resolution between the solvent front which had a mean retention time of 1.488 minutes, and the EO or 1,8-cineole (Figures A16 and A17). The mean retention time for 1,8-cineole was 1.765 minutes. EO generated many small peaks with retention times between 1.6 and 1.9 minutes, however the largest peak, highlighted on Figures A16 and A17, was identified by retention time as being 1,8-cineole and was used to represent EO in future experiments.

7.5.2.2. Preliminary extraction of eucalyptus oil using hexane

EO was extracted from wipes in hexane and quantified using GC, however there was no correlation between the duration in hexane and the quantity recovered from any sample, therefore future experiments will use the minimum extraction time tested of 30 minutes.

7.5.2.3. Standard curve of eucalyptus oil concentration and peak area generated by gas chromatography

The standard curve for EO in hexane measured by gas chromatography in relation to an IS produced a linear graph with an R^2 value of 0.9973 over the range 0.078 mg/mL to 256 mg/mL (Figure A18).

7.5.2.4. Level of detection and quantification for gas chromatography

The LOD and LOQ were calculated as 2 mg/mL and 6 mg/mL respectively.

7.5.3. Biopatch film development and antimicrobial testing

7.5.3.1. Polyvinyl and chitosan film optimisation

The following six biopatches with 5 % (v/v) EO, 2 % (v/v) CHG and 1 % (v/v) tween-80, were considered to have suitable properties required for use in future studies;

- 7 % (w/w) PVA, Mw 47,000
- 9 % (w/w) PVA, Mw 47,000
- 7 % (w/w) PVA, Mw 125,000
- 7 % (w/w) PVA, Mw 205,000
- 9 % (w/w) PVA, Mw 205,000
- 2 % (w/w) chitosan with 2 % (v/v) HPLC grade glacial acetic acid

7.5.3.2. Release study of eucalyptus oil and chlorhexidine digluconate from a polyvinyl alcohol biopatch film using Franz diffusion cells

The GC and HPLC confirmed release of EO and CHG respectively from the biopatches over the 24 hour period. Both agents were detected over the entire 24 hour period, with a peak of EO and CHG release at 1 hour, and a second CHG peak at 4 hours (Figure 7.1). Overall and at any given individual time point, there was no significant difference ($P>0.05$, ANOVA, Instat3, GraphPad) between the amount of CHG released by the biopatch containing EO and CHG, and the control containing CHG alone, therefore the presence of EO in the biopatch had no impact on the amount of CHG released. However, using the previously established LOD and LOQ results (Section 7.5.2.4) the concentration of EO release were established as accurately unquantifiable from 4 hours, and undetectable at 24 hours.

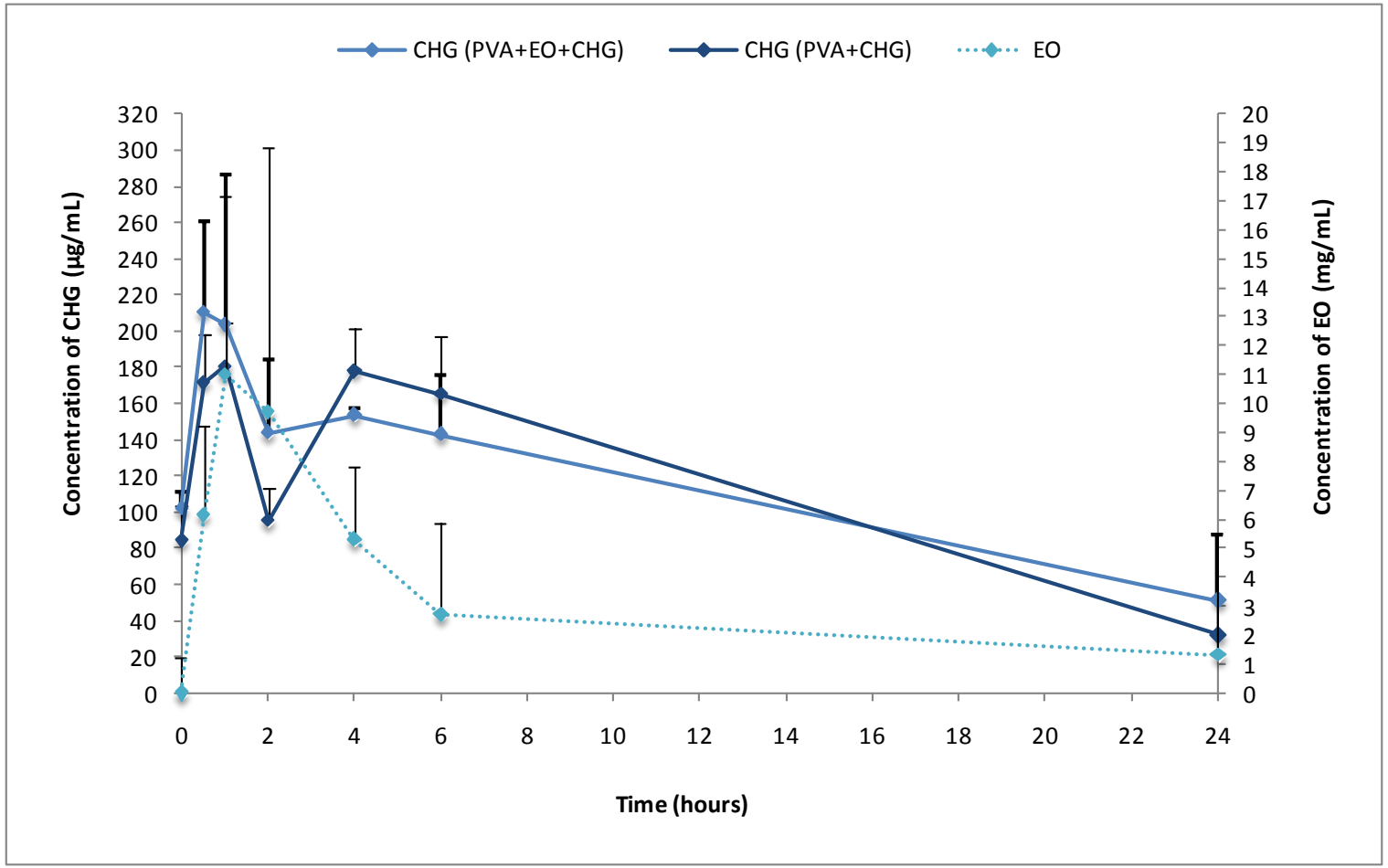

Figure 7.1. Mean (n=3) concentration and standard deviation of CHG and EO released over time from biopatches of 7 % PVA with Mw of 47,000 containing 2 % (v/v) CHG, with and without 5 % (v/v) EO, using Franz diffusion cells.

7.5.3.3. Microbial inhibition by eucalyptus oil and chlorhexidine digluconate-containing polyvinyl alcohol and chitosan films using agar diffusion

Zones of inhibition were present around all biopatches containing 5 % EO and 2 % CHG for all microorganisms tested, however no visible zones were displayed by biopatches without EO and CHG, furthermore, microbial growth was clearly visible underneath many of the control biopatches (Table 7.1). Overall, the 9 % PVA of Mw 205,000 demonstrated significantly enhanced ($P<0.05$, ANOVA, Instat3, GraphPad) antimicrobial efficacy against the panel of microorganisms, compared with the other biopatches tested.

Table 7.1. The antimicrobial efficacy of various biopatches with and without 5 % (v/v) EO and 2 % (v/v) CHG, against a panel of six microorganisms expressed as mean (n=3) sizes of inhibition zones observed using the agar diffusion method.

	Inhibition zone (mm) for biopatches containing 5 % EO and 2 % CHG/without EO or CHG					
	7 % PVA, Mw 47,000	9 % PVA, Mw 47,000	7 % PVA, Mw 125,000	7 % PVA, Mw 205,000	9 % PVA, Mw 205,000	2 % Chitosan + 2 % acetic acid
S. aureus	9/<1	10/<1	8/<1	10/<1	15/<1	8/<1
S. epidermidis	11/<1	8/<1	13/<1	17/<1	20/<1	9/<1
MRSA	8/<1	10/<1	11/<1	9/<1	17/<1	8/<1
P. aeruginosa	7/<1	5/<1	6/<1	7/<1	15/<1	6/<1
E. coli	13/<1	9/<1	10/<1	7/<1	7/<1	7/<1
C. albicans	8/<1	10/<1	4/<1	5/<1	11/<1	8/<1

7.5.3.4. Bacteriostatic/bactericidal testing of eucalyptus oil and chlorhexidine digluconate-containing polyvinyl alcohol and chitosan films

Bactericidal antimicrobial activity was demonstrated against all six microorganisms by all biopatches containing EO and CHG, with no microbial growth revealed by culture. The control PVA biopatches (without EO and CHG) did not possess antimicrobial

activity, however no microbial growth was recovered from underneath the chitosan control biopatch therefore it was determined to possess bactericidal activity.

7.5.4. Catheter tolerance to Euclean® solution

No visible change was observed between the Hydrocath Assure™ catheter and hub submerged in Euclean® solution and immersed in PBS over the 5 day period. Furthermore, at the end of the 5 days, no stickiness or softening of the CVC was apparent.

7.5.5. Polyvinyl alcohol films on human skin

7.5.5.1. Penetration of eucalyptus oil and chlorhexidine digluconate from a polyvinyl alcohol film into human skin

No EO or CHG was detected in the receptor fluid of the Franz diffusion cells at any point during the 24 hour exposure of human skin to biopatches.

7.5.5.2. Detection of eucalyptus oil and chlorhexidine digluconate in layers of human skin

No EO or CHG were detected in sliced sections of human skin following 24 hour contact with biopatches.

7.5.6. Testing of Euclean® solutions using tissue culture

7.5.6.1. Cell toxicity of Euclean® solution to human airway epithelial cells and human pulmonary fibroblasts

The 5 % Euclean®, 2 % Euclean® and CHG/IPA solutions all exhibited toxicity towards both airway epithelial cells and human pulmonary fibroblasts when viable cells were measured with fluorescence after staining with cell titre-blue (Figures 7.2 and 7.3). For each cell line tested, there was a significant reduction ($P<0.05$, ANOVA, Instat3,

GraphPad) in the number of viable cells remaining after just 10 minutes exposure to the solutions, however there was no further significant decline ($P>0.05$, ANOVA, Instat3, GraphPad) in viability between this point and any other over the 4 hour exposure time. For the AEC, the reduction in viability observed by contact with the control solution demonstrated significance ($P<0.05$, ANOVA, Instat3, GraphPad) from the initial time zero value, at each time point except for the 1 hour measurement, which was not sufficient to be deemed significant. The decrease in cell viability between the control at time zero and subsequent time points was also significant ($P<0.05$, ANOVA, Instat3, GraphPad) for the HPF cell line. It can therefore be assumed that the process of removing old media and replacing with new, accounted for some loss in cell viability. Finally, whilst toxicity was observed, there was no significant difference ($P>0.05$, ANOVA, Instat3, GraphPad) between that induced by either of the Euclean® solutions and the CHG/IPA solution; already commercially available for use on human skin as ChloraPrep®.

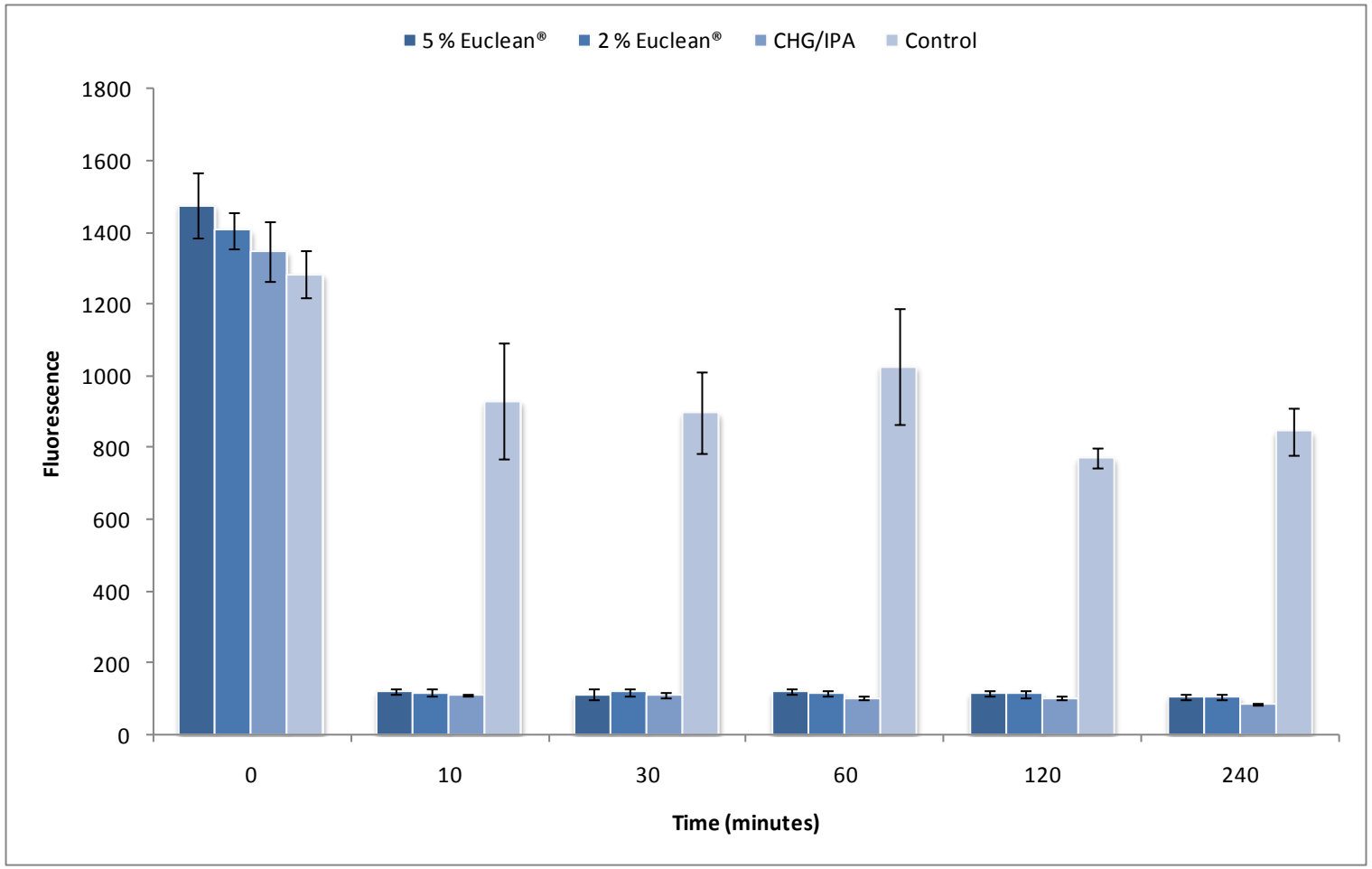

Figure 7.2. Mean (n=3) toxicity and standard deviation of 5 % Euclean®, 2 % Euclean® and CHG/IPA solutions to AEC during 4 hours exposure, measured by fluorescence (arbitrary units) of viable cells using cell titre-blue.

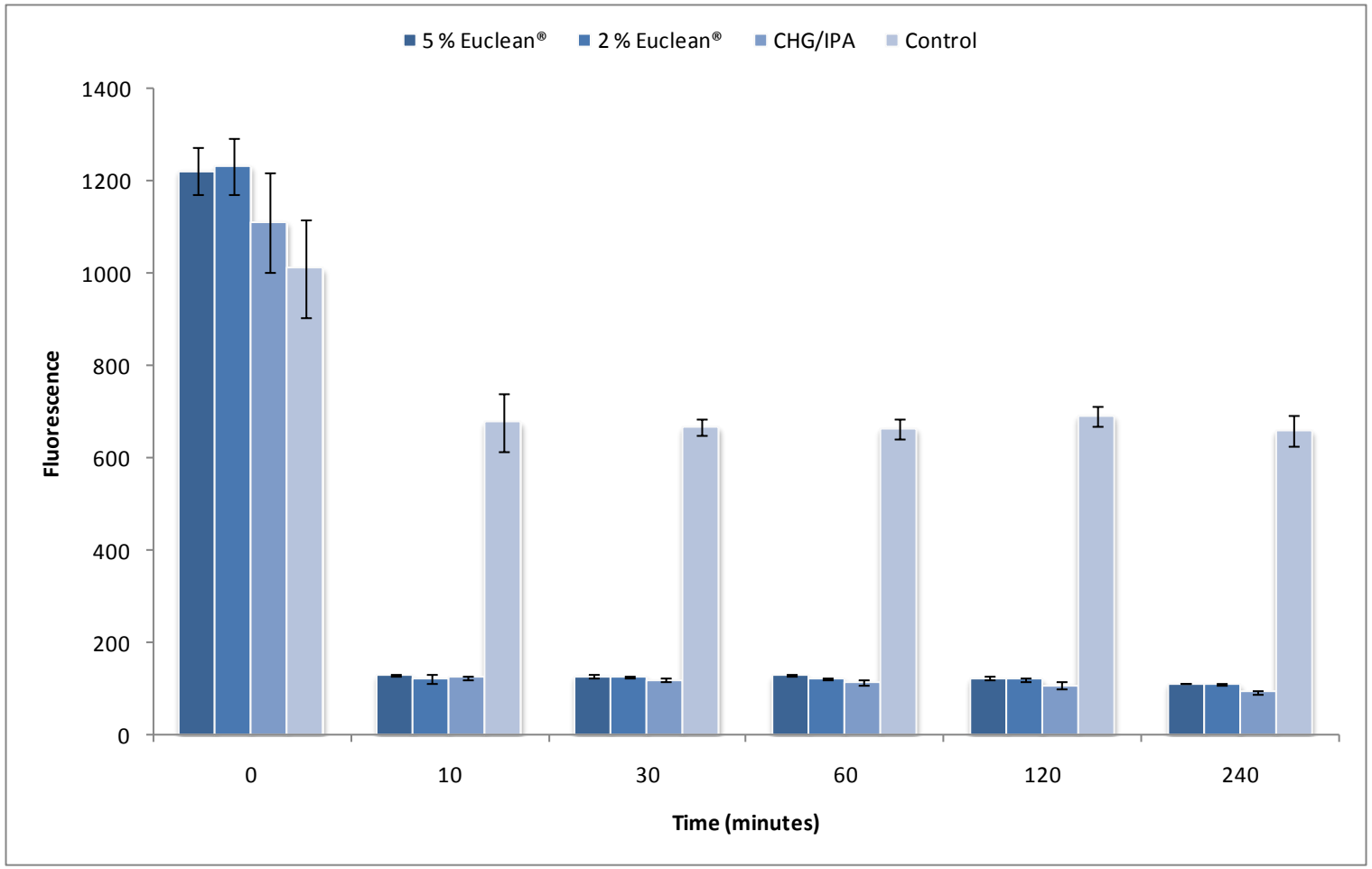

Figure 7.3. Mean (n=3) toxicity and standard deviation of 5 % Euclean®, 2 % Euclean® and CHG/IPA solutions to HPF during 4 hours exposure, measured by fluorescence (arbitrary units) of viable cells using cell titre-blue.

7.6. Discussion

Previous research within this thesis has used an array of methods to assess the properties of, and subsequently confirm the superior benefits achieved by, the addition of EO to CHG and CHG/IPA. Current EPIC guidelines suggest the use of 2 % CHG in 70 % alcohol for skin antisepsis however recent findings suggest that while this may be adequate for the surface of the skin, the deeper layers can persist in harbouring microbial colonisation within pores and hair follicles which remain unreached (Karpanen *et al*., 2008*a*; Pratt *et al*., 2007). Therefore, enhanced penetration is required. However new research has confirmed that the presence of EO can induce deeper penetration of CHG into the skin (Karpanen *et al*., 2010). This is possibly due to the 'pull' effect whereby CHG may be carried into the deeper layers of the skin by EO, as a result of its permeation properties, however application of the EO/CHG/IPA combination in the study by Karpanen *et al*. (2010) was means of a solution, therefore potentially maximising the resultant depth to which the agents could permeate. Irrespective of the underlying mechanisms, this research offers additional benefits, as it is now clear that the EO/CHG combination not only results in synergistic antimicrobial efficacy and improved biofilm removal, but also provides enhanced skin penetration.

Biopatches or transdermal drug-delivery systems (TDDS) are a highly developed means of delivering many different agents through the skin via the application of an agent-containing patch to the skin. They provide many advantages over conventional delivery mechanisms, to include evasion of liver metabolism which can reduce the final circulating concentration, controlled release eliminating dosing errors or complications regarding use, and reduced systemic side effects (Ghafourian *et al*., 2004). Their slow release over time periods of up to a week reduces the need for hourly, or daily attention therefore often improves patient compliance (Tanner & Marks, 2008). There is however some important considerations for using TDDS, of which one central but often overlooked aspect, is the adhesive properties, which are often reported within TDDS information leaflets as not yet tested (Wokovich *et al*., 2006). Effective delivery relies upon complete contact between the patch and the skin. Breaches in contact can lead to inadequate dosing, as the results of any laboratory efficacy testing and therefore the quantity of drug contained, are based upon the entire surface making full contact. Furthermore, poor adhesion can ultimately result in the TDDS falling off, which itself can lead to additional complications from under dosing or breaches in safety following accidental contact between a patch and persons other than the intended patient. Reports

of injury and even death have followed improper dispensing/administration of, or accidents involving, fentanyl-containing patches intended for chronic pain relief, including reports of patients who have fatally injected the patch contents, and a one-year old child killed following ingestion of a discarded patch (Teske *et al*., 2007; Tharp *et al*., 2004). However, reports of this nature are fortunately rare and few medications used in TDDS are as lethal as fentanyl, with delivery more frequently consisting of nicotine replacement therapy and hormonal contraceptives (Tanner & Marks, 2008).

There are a number of ways in which TDDS can be composed however all are compiled from layers. Reservoir and matrix systems are the two main types used for large scale production, with two types of matrix systems produced, either with, or without a membrane to control release rate (Wokovich *et al*., 2006). All have a basal backing and an adhesive surface covered by a removable protective liner, however the inner layers differ. The major difference is that reservoir patches contain drug mixed with excipients in the layer above the backing. The excipients carry the drug, releasing it through the rate-controlling membrane and adhesive into the skin. Matrix patches contain the drug within the adhesive however if there is a rate-controlling membrane present, a second layer of drug-containing adhesive is applied over its surface (Padula *et al*., 2007; Wokovich *et al*., 2006).

This study focused on a simplified variation of a TDDS in which EO and CHG were added to polyvinyl alcohol and chitosan before being cast into film biopatches, and tested for skin antisepsis suitability. The ultimate aim of the biopatch was to provide dermal antisepsis from the delivery of synergistic antimicrobial agents to the deeper layers of skin where microorganisms reside in pores and hair follicles, rather than transdermal delivery, as systemic distribution of the agents was not desirable. PVA is a transparent, nontoxic and biocompatible polymer, and one of the most frequently used polymers for skin application and its development into PVA hydrogels has been widely employed (Kim *et al*., 2008; Valentia & Auner, 2004). Chitosan is also a polymer, naturally derived from chitin, which has previously been studied as an aid for dermal delivery in particular its combination with essential oils has been explored in both microcapsules and films (Pedro *et al*., 2009; Zivanovic *et al*., 2005). The natural properties of chitosan lend it favourably to this type of application as it is a permeation enhancer, antimicrobial and mucoadhesive, therefore forms optimal attachment to mucosal surfaces while aiding drug delivery and preventing microbial contaminants.

The results of this study concluded a number of PVA concentrations and one chitosan formulation possessed potential as a biopatch for enhanced skin antisepsis, displaying suitable qualities of thickness, flexibility and compatibility with EO and CHG. By varying the molecular weight and the quantity of PVA used, it was possible to formulate films exhibiting different qualities, ranging from thin and electrostatically charged, to thick and brittle. Release studies subsequently confirmed that EO and CHG were discharged from the PVA biopatch tested, with both agents reaching a peak of release after 1 hour which reduced at 2 hours. While the concentration of EO continued to reduce reaching a plateau at around 6 hours, the CHG showed a further release peak after 4 hours before gradually declining up to 24 hours. However, it appears that the presence of EO within the biopatch, led to slightly slower release of CHG, with twice as much CHG in the receptor fluid of the EO/CHG patch as in that of the patch containing CHG alone. Contact between the PBS of the receptor chamber and the patch instigated swelling and therefore increased release of EO and CHG, demonstrated by the first peak. While most of the EO appeared to have been released here, the second peak of CHG may be due to early stages of disintegration as the biopatches began to dissolve into the receptor chamber. EO is volatile and light sensitive therefore alterations occurring after release may cause it to become undetectable by the GC. This may also explain the low concentrations of EO being classed as accurately unquantifiable from 4 hours and undetectable at 24 hours, as established from the LOD and LOQ calculations performed previously, something that was unexpected as the 5 % EO added to the biopatch was above the limit of detectable and quantifiable concentrations from the GC, as well as above that of all the MIC and MBC results established previously (chapter 2). The presence of EO in the biopatch created variation in the stability of the film compared to those without EO; this could possibly reduce breakdown of the PVA structure and prolong the ability of the EO/CHG biopatch to release CHG. The manipulation of quantity and molecular weight can alter the extent to which films of the same polymer swell once moistened, therefore result in differences in permeation profiles (Padula *et al*., 2007). It is therefore possible that this manner of testing could produce differences between the release profiles of the six films, however it remains unclear if the 2 % variation in PVA composition would be sufficient to cause noticeable differences to the findings.

When tested against the panel of microorganisms, it was clear that the PVA biopatches alone did not elicit antimicrobial efficacy however there was an obvious improvement

with the addition of EO and CHG, further supported by the bactericidal activity of all the EO/CHG-containing biopatches. As discussed previously (chapters 3 & 4), it is possible that some inactivation of CHG may have occurred from the presence of tween-80 to aid in EO dissolution. Although unlikely due to the concentrations involved, this occurrence would act only to provide improved results if tween-80 were substituted with another mixing agent. Whilst PVA alone exhibited no antimicrobial efficacy, the chitosan-only film did possess bactericidal properties. This is no surprise as chitosan is well known to possess antimicrobial activity against Gram-positive and -negative bacteria, as well as fungi, and has been used within the food industry for many years as edible, biodegradable, nontoxic, protective coatings for food (Dutta *et al*., 2009). With its positively charged amino group, chitosan targets the membranes of cells, which are negatively charged, however one study has noted that increases in the molecular weight of the chitosan favour targeting of Gram-positive microorganisms, and decreases favourability towards Gram-negative (Zheng & Zhu, 2003). In clinical practice it cannot always be predicted which microorganisms are most likely to infect a wound site, therefore unless a compromise could be established, the development of two opposing biopatches may be needed; one for each Gram type.

The combination of EO and CHG has demonstrated enhanced penetration into the deeper layers of the skin when applied to the surface as a solution (Karpanen *et al*., 2010). However, the results of this study established that neither EO nor CHG were detected in the receptor fluid of the Franz diffusion cells therefore had not passed through the full thickness of the skin. Subsequent slicing of the surface layers also revealed absence of EO and CHG within the tissue. It is possible that release issues ensued however this is doubtful as the prior experiments confirmed these capabilities. Inadequate concentration on or in the skin to permit detection is a more likely cause, though this is also dubious as the antimicrobial efficacy experiments proved sufficient EO and CHG were released to kill microorganisms, therefore would be of detectable and quantifiable concentrations. The most probable cause lies with imperfect contact between the biopatch and the skin. The biopatch studied here became more adhesive when dampened; something that has been previously reported for other patches and aids patch to skin contact therefore improves delivery (Gal & Nussinovitch, 2009; Wokovich *et al*., 2006). However in this study, the skin was almost completely dry increasing the possibility of only partial contact thus breaking the transportation route from biopatch to skin. The improved release and flexibility provided by moisture could form a basis for

future developments of the biopatch by coupling it with an adhesive, conductive solution or adding a gel layer to provide this function. Alternatively, plasticisers could be added to the biopatch to increase flexibility therefore adhesion and delivery, but their molecular weight is known to alter the physical properties of the final product, especially in chitosan films therefore their inclusion may not always the best option (Srinivasa *et al*., 2007).

The final investigation within this series, examined toxicity of the solutions incorporated into the biopatch developed here and impregnated onto the Euclean® wipes discussed in the preceding chapters. Application of any agent to the skin must undergo strict testing to ensure the safety of the user. Absence of allergy and toxicity risks need to be verified before clearance of wide-scale use can be given. Chlorhexidine is EPIC-recommended for skin antisepsis, and whilst reported incidents of adverse reactions to it are extremely low, some incidents of delayed hypersensitivity including contact dermatitis have been made, but of more concern are the fortunately infrequent cases depicting instant responses such as asthma and anaphylactic shock (Krautheim *et al*., 2004; Sachs *et al*., 2007). EO is quite different by comparison with frequency and array of undesirable consequences greater than those related to CHG. Exposure to EO can lead to widely ranging effects from skin irritation, drowsiness, and seizures, to even fatality if more than 3.5 mL is ingested. Furthermore, prolonged exposure can have toxic outcomes (Tovar & Petzel, 2009).

Combining EO and CHG would potentially induce irritation for a user of Euclean® wipes or a patient exposed to an EO-containing biopatch therefore it was imperative that initial toxicity testing be carried out. The results of tissue culture confirmed that the all the solutions tested resulted in a significant reduction in the number of viable cells remaining after exposure. However, there was no difference observed between the CHG/IPA which is the same formulation as ChloraPrep® and EPIC recommended for skin antisepsis, and either of the two Euclean® solutions. Therefore it can be assumed that the level of irritancy would be similar to that reported for ChloraPrep®. Furthermore, the results concluded that prolonged exposure had no additional adverse effects.

The airway epithelial cells used during this study were human bronchial in origin and isolated from a cystic fibrosis (CF) sufferer. However, they are corrected from CF-type by transfection of the deficient cyclic AMP-mediated protein kinase-A responsible for

activation of chloride conductance. Epithelial cells are the interface between the external environment and the inside of the body and line the respiratory tract. They also cover the outside of the body however the use of AEC in this study will depict the worst possible outcome of application of the EO-containing biopatch, as these cells, unlike external epithelial cells, are offered no protection by the keratinised cells of the stratum corneum (Crystal *et al*., 2008). The second cell line chosen were human pulmonary fibroblasts isolated from human lung tissue. Fibroblasts make collagen which is another component of skin therefore provide a realistic representation of the likely effect of skin contact with the Euclean® solution on Euclean® wipes and in the EO/CHG biopatch. These results suggest favourable findings regarding the potential skin toxicity of the biopatch, and imply that its application to skin would be unlikely to cause any greater level of irritation than the most currently used and recommended skin antiseptic. However some studies have confirmed 1,8-cineole, which is present in EO, is capable of inducing apoptosis in KB cells though the exposure time was more than double that tested here and the difference in cell line may bestow alterations in susceptibility therefore highlighting that the experiments explained here could benefit from further expansion (Cha *et al*., 2010).

7.7. Conclusion

In this study, previous work has been elaborated upon, and led to the development and testing of a prototype dermal biopatch containing EO and CHG, for enhanced skin antisepsis. Optimising formulations of PVA and chitosan resulted in a panel of six potential compositions for biopatch testing. Release of EO and CHG from a PVA biopatch was confirmed using GC and HPLC respectively, and bactericidal activity of all shortlisted biopatches, against six clinically important microorganisms was verified. Whilst compatibility of the Euclean® solution with CVC plastic was determined, skin studies highlighted the failure of EO or CHG to be recovered from any depth skin slice following 24 hours biopatch contact, therefore bringing awareness to possibilities of release, contact or detection flaws. Finally, tissue culture techniques confirmed that while the Euclean® solution induced some cell death amongst human epithelial and fibroblast cells, the extent of the toxicity was no more severe than that revealed by CHG in alcohol; the current recommended solution for skin antisepsis. Overall, this study confirms that the combination of EO and CHG could have potential for use within a biopatch aimed at delivering enhanced skin antisepsis however further studies are warranted to develop and refine the ideas trialled in this chapter.

Chapter 8

Final Discussion and Further Work

Healthcare associated infections (HAI) exert significant financial burdens and time constraints upon health service resources through the need for additional medication and treatment procedures, extended patient hospitalisation, and increased morbidity and mortality rates (Vilela *et al*., 2007; Weigelt *et al*., 2010). Responsible for an estimated 15 % of HAI, surgical site infections (SSI) represent a considerable proportion of all nosocomial infections, and are the most common infection occurring in surgical patients (Stevens, 2009). Whilst post operative care can reduce infection rates, an effective pre-operative procedure is crucial to avoid development of many preventable infections (Heath Protection Agency, 2010).

Skin associated microorganisms such as *S. aureus* and *S. epidermidis* are amongst the most frequent causative pathogens of infections derived from surgical sites, as breach of the skins' defences during insertion provides a point of entry for otherwise harmless commensal microorganisms, providing opportunity for infection to commence (Stevens, 2009; Weigelt *et al*., 2010). Furthermore, the ability of these microorganisms to grow within biofilms, both in the environment and within the skin, creates additional complications due to their reduced susceptibility to antimicrobial agents. It is therefore vital that adequate environmental disinfection and skin antisepsis be delivered prior to commencement of any surgical procedure to remove skin microflora and aid in preventing infection.

Current EPIC and Centres for Disease Control and Prevention (CDC) guidelines for skin antisepsis prior to insertion of any intravascular device, recommend the use of 2 % CHG in 70 % IPA for the U.K. and U.S.A. respectively (O'Grady *et al*., 2002; Pratt *et al*., 2007). Both CHG and IPA are potent antimicrobial agents with broad spectrum efficacy (Hemani & Lepor, 2009). However, recent studies have highlighted concerns over the poor penetration properties exhibited by this combination, with awareness concentrated towards the potential persistent viability of remaining microorganisms within the deeper layers of skin after antisepsis has been performed (Karpanen *et al*., 2008*a*). Therefore, the development of novel antiseptic agents and formulations with enhanced penetration and antimicrobial properties is desired to prevent onset of such infections.

In this study, the antimicrobial efficacies of eucalyptus oil, its major component 1,8-cineole and chlorhexidine digluconate were assessed alone and in combinations. The initial aim of this research was to select the more efficacious agent between EO and 1,8-

cineole, with which to continue further research. The results given in chapter 2 of this thesis demonstrate that whilst both agents possessed antimicrobial activity, EO possessed significantly greater ($P<0.05$) antimicrobial efficacy than 1,8-cineole against planktonic cultures, however no difference was observed between the two against biofilm modes of growth. In addition to this, the subsequent results of chapter 2 confirmed synergistic antimicrobial efficacy could be achieved against planktonic and biofilm cultures when EO or 1,8-cineole were combined with CHG; these results corroborate with previous findings by Karpanen *et al*. (2008*b*) who also reported the synergistic activity when EO and CHG were combined.

Comparisons between crude EO and its most abundant constituent, 1,8-cineole, established that crude EO possessed superior antimicrobial activity, in particular against biofilm modes of growth, and therefore was determined as the better agent with which to progress future studies. This may offer additional benefits as it is possible that EO would be less likely to permit development of microbial resistance due to the presence of numerous constituents which may target cells in a variety of ways. These findings suggest the collaborative presence of other constituents within EO has an impact on the resultant properties and activity. Other research supports this hypothesis having arisen to the same conclusion, including a study by Delaquis *et al*. (2002) who established synergistic antimicrobial effects could be accomplished by combining different fractions of crude oils, and another by Santana-Rios *et al*. (2001) who speculated that synergistic actions between the major and minor constituents within different varieties of tea, are responsible for enhanced anti-mutagenic and anti-chemotherapeutic activity.

Records of biofilm presence date back to the early fossil records, though appreciation of them as clinical complications is a relatively modern concept, with their significance only recently being fully appreciated (Asikainen, 2009; Hall-Stoodley *et al*., 2004). Biofilm development around surgical sites can lead to potentially severe complications, however not all infections originate from the flora of patient's skin; the hands of healthcare workers are known to aid dissemination of microorganisms within the healthcare setting, therefore satisfactory levels of hand hygiene are fundamental in the prevention of HAI (Messina *et al*., 2008). Within the clinical environment, hands can act as vectors, picking up microorganisms from one location and depositing them at another, providing a link in the cycle of infection transmission between patients, and surfaces or objects (Bjerke, 2004). Therefore it follows that one colonised patient can easily bring about microbial contamination of a nearby surface, which can consequently

colonise or infect subsequent patients, healthcare workers or visitors, resulting in rapid and considerable spread as witnessed in infection outbreak cases. Cleanliness of surfaces is therefore also important in infection control.

Progressing from the studies focussing on the antimicrobial efficacy of EO, 1,8-cineole and CHG within chapter 2, the subsequent research in this thesis began to develop the potential applications for the EO and CHG combination, into a prototype, and then a commercial wet wipe intended for hard surface disinfection (chapters 3 and 4 respectively).

In chapter 3, antimicrobial testing against a panel of clinically important microorganisms (*S. aureus*, *S. epidermidis*, MRSA, *P. aeruginosa*, *E. coli* and *C. albicans*) revealed the wipe solution comprising 10 % EO, 2 % CHG and 70 % IPA possessed rapid (within 30 seconds) bactericidal activity against all planktonic cultures, but that large variations existed in the time required for the impregnated wipes to eliminate microbial biofilms, from less than 2 minutes for *E. coli*, to within 4 hours for MRSA. The subsequent experiment in this chapter revealed these wipes demonstrated significantly better ($P<0.05$) removal of all microorganisms from the surfaces of stainless steel discs compared with wipes devoid of EO, with the achievement of complete elimination of *E. coli* and *C. albicans* from the discs. However, the capability of the 10 % EO, 2 % CHG and 70 % IPA wipes to induce microbial transfer on up to eight successive surfaces led to concerns about suitability for clinical use. These findings were in line with those reported by Williams *et al*. (2007) whose methods were adapted for the purpose of this experiment, and cited that grapefruit extract-containing disinfectant wipes removed significantly more cells from surfaces than control wipes, but that they too demonstrated microbial transfer onto eight subsequently contacted agar surfaces.

Formulation development of the prototype 10 % EO, 2 % CHG and 70 % IPA disinfectant wipe (chapter 3) produced improvements in the mixing of the individual agents within the solution and therefore potentially a more even distribution over the Euclean® wipes (chapter 4). This modification of the formulation involved increasing the tween-80 content from 0.1 % to 1 %, and decreasing the EO content from 10 % to 5 % and 2 %, and resulted in enhanced antimicrobial efficacy of the Euclean® wipes compared to the prototype. Similar to results in chapter 3, the 5 % and 2 % Euclean® wipes removed significantly more microorganisms ($P<0.05$) from the stainless steel

discs than the control. However, one obvious improvement conferred by the Euclean® wipes became apparent through confirmation via the adpression test, that they did not induce cross contamination between surfaces and moreover, the viability of microorganisms removed from the surface, was not sustained. In addition to these findings, improvements were also apparent in their rapid, bactericidal activity which extended across all microbial biofilms. A maximum of 5 minutes contact time was required by the 5 % Euclean® wipes to completely eradicate all biofilms, except *P. aeruginosa* which required up to 10 minutes. The 2 % Euclean® wipes demonstrated bactericidal and fungicidal activity against *S. aureus*, MRSA and *C. albicans* biofilms in less than 5 minutes, and the remaining three in fewer than 10 minutes. All of these properties make Euclean® wipes suitable as an adjunct to environmental cleaning and disinfection in the healthcare setting, thus aiding the prevention of nosocomial infections. However the presence of spills and soil loads within the health service can prove particularly challenging for cleaning and disinfection products, in particular PVP-I which is inactivated by proteinaceous products, therefore future studies are warranted to establish the efficacy of Euclean® wipes under these conditions.

Spills within the healthcare setting of bodily secretions and excretions are common place, and not only introduce microorganisms into the environment, but also provide a source of nutrients while the very presence of a soil load acts to trap further potential pathogens (French *et al*., 2004; Rebmann *et al*., 2009). Therefore, the ability of Euclean® wipes to remain efficacious against microorganisms in planktonic and biofilm modes of growth whilst a soil load is present, is more than simply desirable. Investigations using artificial soil loads added to cell suspensions, whereby the simulated spills are produced with bovine serum albumin and bovine mucin (Perez *et al*., 2005), would provide an understanding of the impact posed by bodily fluids upon the efficacy of Euclean® wipes, particularly if the same series of experiments as performed within chapters 1 to 5, were repeated with the soil load addition. Cleaning and disinfection of environmental surfaces within the healthcare environment plays an important role in the prevention of nosocomial infections (Dancer, 2008). However, there are as yet no standards outlining which products should be used and no guidelines on the numbers or types of microorganisms permissible before a surface is considered ‘dirty’, though five or more colony forming units per square centimetre of high touch surface, could indicate an increased risk of infection (Dancer *et al*., 2009; Diab-Elschahawi *et al*., 2010). Suggestions made by Dancer (2009) promote the conversion

of healthcare cleaning to an ‘evidence-based science’ whereby standards can be set and outcomes monitored. As a result of no legislative policies, the choice of which cleaning and disinfection products to use is often left up to the individual hospital or ward, resulting in many different agents being used within the same trust. Over recent years, this has led to the development of numerous novel products and solutions, as well as many ideas and trials involving alternative strategies for preventing microbial contamination of surfaces.

A number of smart surfaces have been developed and subsequently tested, which either repel soiling thus preventing initial microbial attachment, or contain antimicrobials built into their surface that are capable of eliminating any deposited microorganisms (Bartley *et al*., 2010). Those which repel and prevent adhesion of microorganisms include, amongst other non-stick surfaces, diamond-like carbon films which have high water resistance and low friction coefficients reducing the adhesion potential (Page *et al*., 2009). These can also be coated with silver or copper to instil antimicrobial properties (Kwok *et al*., 2007). However, use of polyethylene glycol (PEG) polymeric coatings is one of the most renowned methods for preventing microbial attachment, and is based upon repulsive osmotic interactions (Page *et al*., 2009). Antimicrobial agents used to infuse surfaces, include light activated agents such as titanium dioxide photocatalysts, and metals including silver and copper. Also in this category is triclosan, which is possibly the most commonly marketed agent and has been widely used within hospitals for a number of applications. Some studies however, have reported poor efficacy, including one in which the antimicrobial properties of a triclosan-containing floor cleaner used within a poultry processing plant was reported to be ‘very low’ (Moretro *et al*., 2006).

Some other approaches for eliminating microbial surface contamination have also been tested and include replacing high touch surfaces such as taps and door handles with silver or copper ones, using antimicrobial moulded plastics to cover keyboards, and even copper paints for walls which have been shown to successfully reduce microbial counts of *S. aureus*, *E. coli*, *P. aeruginosa* and *Enterococcus faecalis* within 24 hours (Casey *et al*., 2010; Cooney, 1995; Markarian, 2009). However, none of these ideas have as yet, been implemented throughout all U.K. health services as a permanent feature therefore, at present, stringent cleaning regimes must be upheld as the major means of reducing surface contamination of a microbial nature.

Whilst development of novel strategies to eliminate microbial contamination persist, the increasing emergence of multi-drug resistant microorganisms poses great concern to the health service and has created an escalating need for new antimicrobial drugs and cleaning agents. Traditional development of new antimicrobial drugs relies upon microbiological testing, often encompassing conventional laboratory techniques such as determination of MIC and MBC, to establish the ability of the agent to inhibit microbial multiplication (Coates *et al*., 2002). The use of genomic studies to find targeted antimicrobials has also been employed following the successful sequencing of over 100 microorganisms. It is considered a more modern and advanced technique however this has only generated the oxazolidinone group of antibiotics within the past 30 years (Norrby *et al*., 2005). Surface disinfection development follows a different testing protocol, with genomic techniques surplus to requirement. The systems employed within Europe for surface disinfection development and subsequent testing, involve three stages which incorporate a number of suspension tests initially, followed by simulated-condition experiments and finally field trials (Luppens *et al*., 2002).

In chapter 5 of this thesis, isothermal calorimetry (IC) was used as an alternative to the conventional laboratory techniques and culture methods described above, to assess the antimicrobial efficacy of 5 % Euclean® wipes. This method has been used for determination of antimicrobial activity in only a small number of published papers, despite its potential value in such applications (von Ah *et al*., 2009; O'Neill *et al*., 2003). The precise sensitivity, real time monitoring and passivity of IC are well suited to the study of microorganisms, enabling accurate determination of changes to growth rates almost instantaneously, without damage to the sample under observation (O'Neill *et al*., 2003). The results of this study supported previous findings from chapter 4, that Euclean® wipes possess bactericidal activity against all six clinically important microorganisms tested (*S. aureus*, *S. epidermidis*, MRSA, *P. aeruginosa*, *E. coli* and *C. albicans*), and furthermore, it established that such occurrences were rapid, taking place in less than 30 minutes. However, one fundamental downfall to using this method was highlighted. The duration required for the ampoules to reach equilibrium with the surrounding temperature, resulted in the loss of the initial data covering roughly the first 30 minutes. As a result, conventional culture techniques were required to simulate the environment within the IC in order to determine the time needed by 5 % Euclean® wipes to instigate bactericidal activity, and fill in the missing data up to 30 minutes. A possible solution would be to investigate the activity using a flow calorimeter whereby

the wipe could be added to a continually circulating microbial suspension. This would provide data on the speed of activity from the time the wipe is inserted, however continued complications with microbial contamination during the use of this method in the first experiments of chapter 5, eliminated its use on the grounds of unreliability of results.

IC has also been used to establish the effect application of sub-inhibitory concentrations of antimicrobials, has upon microbial growth rates. In a recent study by von Ah *et al*. (2009), *E. coli* and *S. aureus* were subjected to concentrations of 12 antibiotics below that of the MIC value. The resultant heat production profiles of the curves generated were compared with growth curves absent of antibiotics, to determine the mode of action of each drug. Using findings by Antoce *et al*. (1997) they were able to attribute delays to the instigation of a heat curve, followed by the resultant slope shape matching that of the control, to the antimicrobial under examination reducing the number of viable cells initially, therefore possessing a bacteriostatic mode of action. This method could be used in future studies to extend the current knowledge regarding the antimicrobial activity of EO, by establishing the effects generated by exposing microorganisms to subinhibitory concentrations, and therefore possibly provide an insight into the potential development of microbial resistance to EO or other antimicrobials as a result of EO tolerance. This could prove particularly important for EO use within the clinical setting as a study by Walsh *et al*. (2003*b*) cited that creating microbial mutants of *E. coli* with induced tolerance to the essential oils thymol and eugenol, could result in cross-resistance to other biocides and reduced susceptibility to some antibiotics. In addition, soil loads could be incorporated into the suspension to assess the impact of bodily fluids and spills on the activity of Euclean® wipes.

Essential oils have previously demonstrated potential for application within the clinical setting, including tea tree oil, which has been exhaustively studied for its antimicrobial efficacy against MRSA (Carson *et al*., 2006). The results from the first four chapters of this thesis confirmed that the antimicrobial activity and microbial removal properties of EO and Euclean® wipes, lend favourably towards their use within the healthcare environment. It therefore followed that clinical audit of the 5 % Euclean® wipes was necessary to determine the opinions of healthcare workers who would use the final product daily.

The results from the Euclean® wipe clinical audit (chapter 6) undertaken at Birmingham Children's Hospital, brought about mixed opinions towards the products currently used for cleaning and disinfection, as well as Euclean® wipes. The cross-over audit investigated opinions on current cleaning and disinfection products following their use for one week, then repeated the questions following a second week of exclusive Euclean® wipe use. This style of cross-over study is frequently used within scientific research as it enables direct comparisons to be made between treatments however it is most commonly used to assess patient opinion rather than that of staff. In this study the term cross-over referred to a controlled trial in which there was balance but the sequence of treatments was not random.

Hospital cleaning and disinfection includes the use of many different chemicals and agents, usually designed for specific surfaces and requiring diverse preparations; however it became apparent during the Euclean® audit that healthcare workers value ease of use products. Mixing and dilution of cleaning agents is often required prior to use, with a range of concentrations recommended for different uses, for example chlorine-based agents are frequently diluted for floor mopping and managing spillages, however the concentration suggested for blood spills is ten-times greater than that for urine (Health Protection Agency, 2010*b*). This is not only time consuming, but can easily result in inappropriate dilution, and therefore inadequate efficacy due to sub-lethal concentrations. Numerous previous studies have demonstrated that microbial exposure to subinhibitory concentrations of biocide can alter microbial growth and induce tolerance to not only the agent being used, but also bring about cross-resistance to other biocides and antibiotics (Maillard, 2007; Walsh *et al*., 2003*a*; Walsh *et al*., 2003*b*). Disinfectant wipes, such as Euclean® wipes, eliminate complications posed by dilution and preparation as are pre-impregnated with the desired concentrations of cleaning agents.

The daily use of disinfectant wet wipes within Birmingham Children's Hospital encompassed all five wards partaking in the audit, with staff nurses accounting for the majority of users (chapter 6). Alcohol based wipes, in particular Sanicloth-70, were identified as the most abundant used during clinical practice, with equipment and furniture within close proximity to the patient most frequently cleaned. Such surfaces, including bed rails, bedside cabinets, chairs, cots and machinery are classified as hand contact, or high touch surfaces and are often found harbouring microbial contamination, including MRSA and VRE (Boyce, 2007). In both phases of the trial, there was

relatively little difference between the cleaning frequency of plastic, metal, wood and stainless steel surface materials. The material used for surfaces is known to have an impact on the number of microorganisms it can potentially harbour and the degree of efficiency with which it can be cleaned. Plastic and plastic laminate surfaces are particularly prone to harbouring microorganisms for long durations, whilst glass and nonporous surfaces provide less hospitable environments therefore reduced contamination survival (Hota, 2004). One study by Milling *et al*. (2005) reported that microbial survival on wood and plastic was in part due to ambient temperature and humidity levels. In addition they added that different types of wood permitted survival to a greater or lesser extent, with pine and oak being significantly more hygienic than spruce, larch, maple, beech and poplar, having shown complete reduction in *E. coli* cell numbers over a seven day period.

Cleaning interventions can reduce microbial contamination on surfaces and have also demonstrated cost effective benefits, with a recent study by Dancer *et al*. (2009) citing savings of between £30,000 and £70,000 following the addition of one extra cleaner reducing the incidence of infection. This supports the hypothesis that surfaces bear significance in the dissemination of infectious microorganisms and that cleaning is therefore a crucial part of its regulation. However, improved levels of cleaning can create alternative problems. As discussed by Chapman (2003), numerous reports have cited the increased observations over recent years in prevalence of disinfectant resistant microorganisms, with microbial tolerances reported towards chlorine, glutaraldehyde, iodophor, peroxides, phenols and QUATS. Furthermore, contaminated disinfectants have resulted in reports of infection outbreaks (Webber *et al*., 2007). In addition to microorganisms resistant to disinfectants, antiseptic-resistant microorganisms have also caused outbreaks with increased occurrence, including alcohol, chlorhexidine and povidone iodine (Bouallegue *et al*., 2004; Nasser *et al*., 2004; Tena *et al*., 2005). However, Meyer & Cookson (2010) concluded that the risks posed to the delivery of healthcare by biocide-related microbial resistance are low.

The Euclean® clinical audit raised awareness of potential skin irritation induced by currently used products, as well as the Euclean® wipes. A known permeation enhancer, EO causes disruption to the intercellular lipids of the stratum corneum yet conversely, its use could potentially reduce skin irritation for some healthcare workers (Aqil *et al*., 2007). Skin irritation has been linked with frequency of performing hand hygiene practices whilst carrying out patient care (Larson *et al*., 2006). The repeated washing

causes depletion of lipids, allowing sanitisers to penetrate further into the skin layers, both of which lead to, and then exacerbate drying of the skin on the hands. Essential oils such as EO possess some residual activity which could, if sufficient, prevent microbial contamination for a period of time after use, therefore increasing their favourability for skin disinfection amongst healthcare workers by means of reducing the frequency of hand hygiene required. CHG is also recognised as possessing residual activity thus providing prolonged antimicrobial protection on the skin for a longer duration after use than some other agents including povidone iodine (Milstone *et al*., 2008). One study evaluated disinfectants with residual activity for use on inanimate surfaces, and concluded that such products could offer considerable advantages over current products, with additional potential cost effective benefits (Rutala & Webber, 2001). In addition to this, hand sanitisers containing residual activity have also been tested including 'PureHands' which is an alcohol-based antiseptic containing a mixture of herbs, plants and essential oils and has demonstrated significant reductions in microbial colonisation on the hands of healthcare workers over a seven day period of use (Das *et al*., 2005; Kavathekar *et al*., 2004). Investigations exploring the residual properties of EO and CHG alone and in combination are warranted to enhance their potential applications, as their ability to provide antimicrobial effects after use could prove a useful property for both skin antisepsis and surface disinfection.

After each phase of the Euclean® audit, participants were asked to provide an overall numerical rating of the wipes they currently use and the Euclean® wipes. The final scores generated were 7.5 and 6.5 for the currently used and Euclean® wipes respectively, however the highest frequency scores for each were 8/10 and 9/10 respectively. This suggests that those in favour of the Euclean® wipes scored them extremely highly, however the scores from the remaining participants were lower for the Euclean® wipes than the current wipes, having the result of lowering the overall average score. This was further supported by the final questions of the audit which invited advantages and disadvantages, followed by other comments and feedback on the Euclean® wipes. The most frequently mentioned advantage and disadvantage related to the smell of the wipes, clearly dividing opinion of the healthcare workers, for and against. The request for comments and feedback provoked some interesting points, with one comment mentioning they could be enticed into changing to Euclean® wipes only if it was cost effective, while another comment revealed that whilst the smell was strong, the presence of fewer chemicals within the Euclean® wipes was favourable.

The final studies of this thesis (chapter 7) assessed the potential for incorporating 5 % EO and 2 % CHG into PVA and chitosan biopatch films, and then investigated the ability of the one biopatch, containing 7 % PVA (Mw 47,000 Da), 5 % EO and 2 % CHG, to deliver enhanced skin antisepsis using a Franz diffusion cell model. The use of transdermal patches for delivery of agents via the skin has increased over recent years with many varieties now widely available for delivery of hormones and various medications (Kumar & Philip, 2007). However, prior to any product being considered safe for commercial production, thorough skin testing including assessment of the potential toxicity and irritancy, is required (Collins *et al*., 2008). Furthermore, whilst the addition of permeation enhancers to a transdermal biopatch can aid delivery of the incorporated agent through the skin, their presence can also instigate irritation from the temporary disruption they cause to the intercellular lipids of the SC (Sapra *et al*., 2008). EO is known to cause some degree of skin irritation however, many terpenes including 1,8-cineole, the main constituent of EO, are generally regarded as safe, possessing no toxicity and low levels of irritancy (Aburjai & Natsheh, 2003; Aqil *et al*., 2007; Tovar and Petzel, 2009). This may however, not be the case if applied to damaged or broken skin, although knowledge concerning this is extremely limited with more research urgently required if application of such agents were to become routine within the health service. One recent study published by Karpanen *et al*. (2010) demonstrated that the addition of EO to an alcoholic CHG preparation significantly enhanced the depth of CHG penetration into human skin when applied to the surface as a liquid solution for 2 minutes, therefore reiterating its potential benefits for dermal delivery and further signifying the need for further information regarding irritancy and toxicity.

Implantation of a CVC can frequently result in the development of infection either around, or originating from, the site of insertion, with additional complications from biofilm development common (Frasca *et al*, 2010). The best preventative method involves the use of adequate skin antisepsis prior to insertion (Frasca *et al*, 2010). However, alternative options including antimicrobial-impregnated patch dressings which surround the insertion site, have been studied, with an assortment of products now commercially available. Examples of such dressings containing silver include the Algidex Ag® silver alginate wound dressing from DeRoyal®, and the Silverlon® 'lifesaver™ Ag' catheter dressing (Bhende & Rothenburger, 2007). In addition to these, a number of chlorhexidine-impregnated catheter surrounds are available including the Ethicon® BIOPATCH® protective disk produced by Johnson & Johnson (Timsit *et al*.,

2009). However, one study comparing the efficacy of four silver-containing dressings with a chlorhexidine gluconate covering, reported improved and sustained antimicrobial efficacy was conferred over a 7-day period by the chlorhexidine dressing (Bhende & Rothenburger, 2007).

In chapter 7 of this thesis, 5 % EO and 2 % CHG were incorporated into PVA and chitosan biopatch films for potential use as CVC insertion site dressings. The results from this study confirmed that both 5 % EO and 2 % CHG could be successfully mixed into, and subsequently released from both types of biopatch. Furthermore, conventional laboratory culture techniques confirmed all PVA and chitosan biopatches containing EO and CHG possessed bactericidal antimicrobial activity against a panel of clinically important microorganisms (*S. aureus, S. epidermidis*, MRSA, *P. aeruginosa, E. coli* and *C. albicans*). This was not the case for the PVA biopatches without EO and CHG, however bactericidal activity was confirmed from the chitosan biopatch devoid of EO and CHG. Further studies could incorporate the use of isothermal calorimetry as used in chapter 5 of this thesis, to investigate the mode of activity of the biopatch further, as well as establish its rate of activity against microorganisms. In addition to this, concerns throughout chapters 3, 4 and 7 regarding the potential of tween-80 incorporated in the EO-containing wipes, Euclean® wipes and biopatches to inactivate CHG could have reduced the antimicrobial properties of them, therefore investigations to determine the actual effect, if any, posed by the presence of tween-80 would be justified in future work.

Polyvinyl alcohol is frequently used for applying agents to the skin, with adaption into PVA hydrogels widely utilised for their temperature stable, non-toxic, biodegradable and flexible properties (Kim *et al*., 2008). Furthermore, these transdermal patches enable a constant rate of medication to be released over prolonged time periods, reducing the need for regular dosing (Kulkarni *et al*., 2010). In addition to the use of PVA for transport of medication via the skin, chitosan has also been scrutinised in several investigations exploring transdermal drug delivery and wound dressings (Bhattarai *et al*., 2010; Jayakumar *et al*., 2011). One recent study discussed its use for transdermal delivery of insulin following encapsulation of insulin within nanoparticles of chitosan (Wong, 2009). It possesses a number of properties that could prove beneficial if used as a transdermal biopatch around CVC insertion sites, including the ability to reduce pain sensation by blocking nerve endings and aid natural blood clotting. Furthermore, gradual depolymerisation of chitosan releases N-acetyl-b-D-

glucosamine which brings about ordered collagen deposition therefore promoting faster wound healing with reduced scaring (Paul & Sharma, 2004).

In order for a transdermal patch to be suitable for commercialisation, it must provide the user with adequate levels of agent at the required site, for an appropriate duration. For example, a sufficient dose must be released continually, at a constant rate, and permeate through partial or full thickness of the skin depending upon the requirement for local, or bloodstream and therefore systemic targeted treatment. In chapter 7 of this thesis, analysis of sliced human skin sections following 24 hour contact with an EO and CHG-containing PVA biopatch, failed to detect EO or CHG using GC and HPLC respectively, on either the surface or in the deeper layers of the skin. A recent study by Karpanen *et al*. (2010) has confirmed combining these two antimicrobials can result in enhanced penetration into the deeper layers of skin however there were two potentially significant differences in the design of their study compared to that of this thesis. The EO and CHG they used were made using an alcoholic base, and then applied directly onto the skin as a liquid preparation, however in this study an aqueous mixture was used and bound into a PVA polymer patch before being applied to the skin. It is possible that the presence of IPA in the solution had an effect on the solutions penetration into the skin; however one study reported increases to the permeation barrier of the SC when high concentrations of alcohol are applied to skin as a result of lipid extraction (Van der Merwe & Riviere, 2005). As the previous studies within this chapter confirmed the antimicrobial efficacy of the PVA biopatch, as well as the release and detection of EO and CHG from the biopatch, it is likely the most probable cause of the deficient penetration, was inadequate skin-to-biopatch contact. Therefore the properties of this PVA biopatch would benefit from further investigation and possible modification. Plasticisers could be used to reduce the brittleness of the biopatch, with the increase the flexibility providing improved contact over the contours of the skin. However, whilst their inclusion in a study by Gal & Nussinovitch (2009) demonstrated little impact on the roughness of a skin bioadhesive patch surface, alternative drawbacks including lower adhesion properties, diminished tensile strength, reduced pealing force and increased strain at failure were reported. In addition to this, alternative methods could be tested, including spray drying with microencapsulation of EO within small particles to overcome sinking of EO during drying of films, and oxidisation in air. Failing advances using such methods, alternative strategies for biopatch development could be

considered, include exploration of both reservoir and matrix patches, along with the addition of an adhesive layer to improve patch-skin adhesion.

To conclude chapter 7, toxicity studies were performed on human airway epithelial and human pulmonary fibroblast cell lines to establish the potential degree of skin irritation induced by the combination of 5 % or 2 % EO, 2 % CHG and 70 % IPA. Comparisons were made with 2 % CHG in 70 % IPA which is the current EPIC recommended solution for skin antisepsis (Pratt *et al*., 2007). The results confirmed that whilst the EO-containing solution resulted in some cell death, it was not significantly different from the effects generated from the EIPC-recommended solution, therefore suggesting that skin irritation from the Euclean® wipe solution (chapter 4) and the EO-containing biopatch (chapter 7) is likely to be no greater than that of the nationally approved agent. This confirms that whilst further development is required for the EO and CHG biopatch to obtain the required physical properties, the principle shows potential without the likelihood of skin irritation causing complications.

The research within this thesis has confirmed the possession of antimicrobial efficacy by EO and CHG against planktonic and biofilm cultures. Through incorporation of 10 % EO and 2 % CHG into wipes, and subsequent optimisation of the formulation into 5 % and 2 % Euclean® wipes, their potential for hard surface disinfection was assessed. Isothermal calorimetry was employed as an alternative method to conventional culture techniques for determination of antimicrobial efficacy of 5 % Euclean® wipes, which were then audited at Birmingham Children's Hospital. Finally, 5 % EO, 2 % CHG and 70 % IPA were incorporated into PVA and chitosan biopatches with penetration of the agents into human skin subsequently assessed for a 7 % PVA (Mw 47,000 Da) biopatch. Tissue culture techniques were then employed on two human cell lines to determine the potential toxicity posed by the 5 % EO, 2 % CHG and 70 % IPA solution used to impregnate the Euclean® wipes and incorporated into the biopatch. Further studies are warranted to improve the penetration capabilities and physical properties of the PVA biopatch.

Chapter 9

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A

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Chapter 10

Appendix

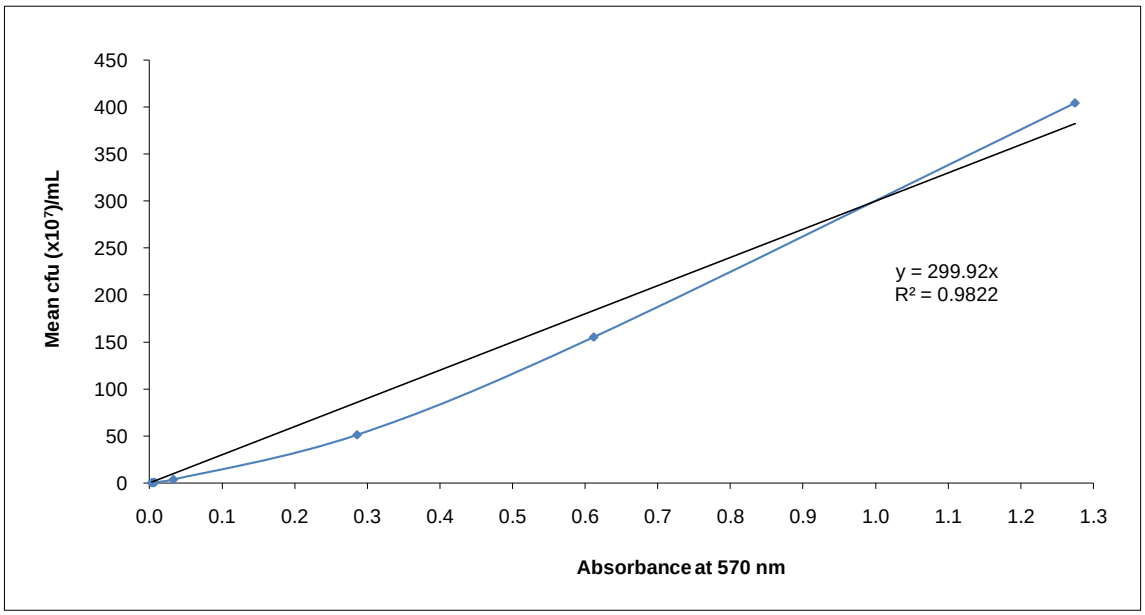

Figure A1. Mean (n=2) colony forming units (cfu) of *S. aureus* cell suspension correlated to turbidity measured at an absorbance of 570 nm.

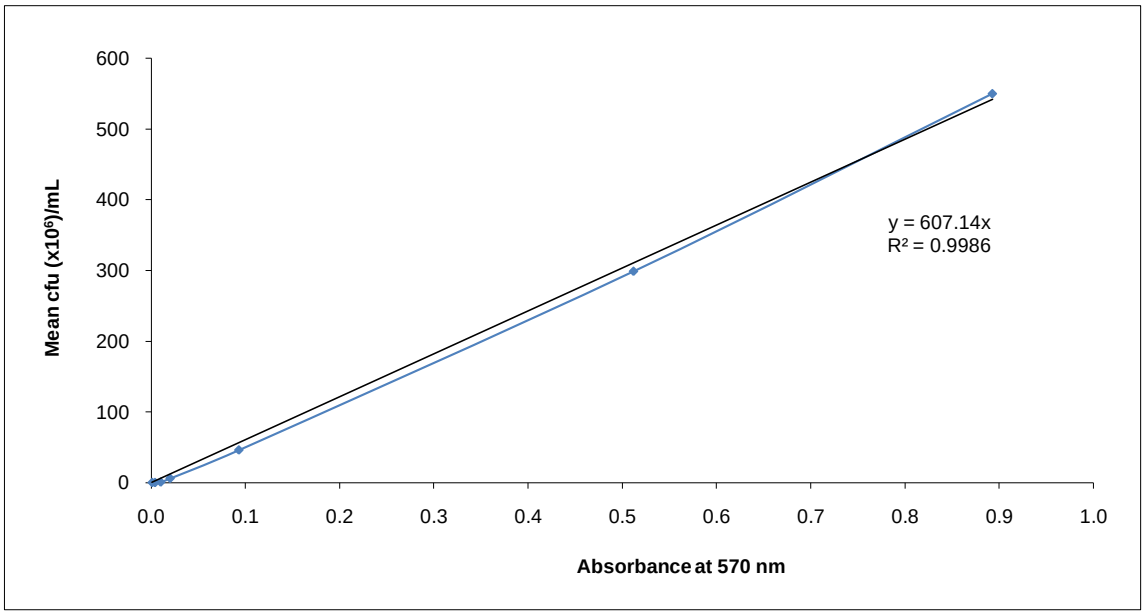

Figure A2. Mean (n=2) colony forming units (cfu) of *S. epidermidis* cell suspension correlated to turbidity measured at an absorbance of 570 nm.

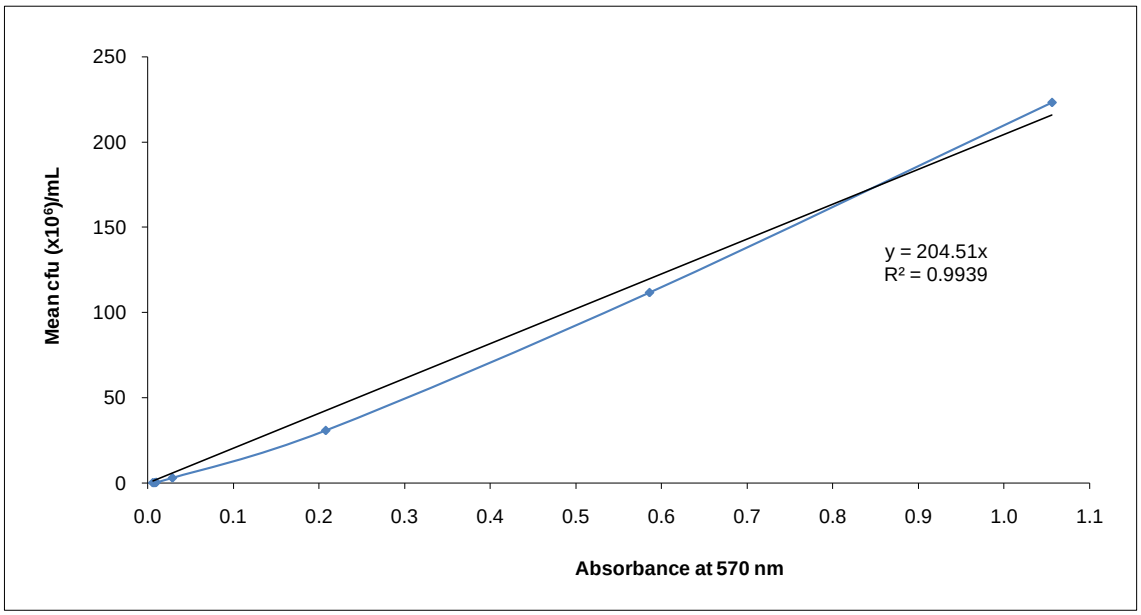

Figure A3. Mean (n=2) colony forming units (cfu) of MRSA cell suspension correlated to turbidity measured at an absorbance of 570 nm.

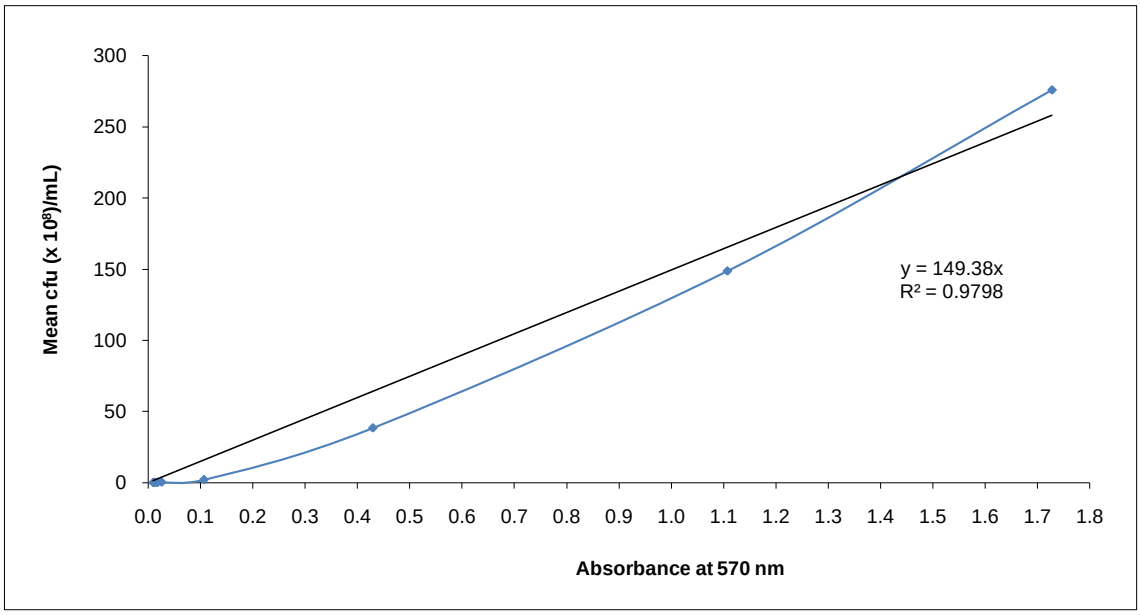

Figure A4. Mean (n=2) colony forming units (cfu) of *P. aeruginosa* cell suspension correlated to turbidity measured at an absorbance of 570 nm.

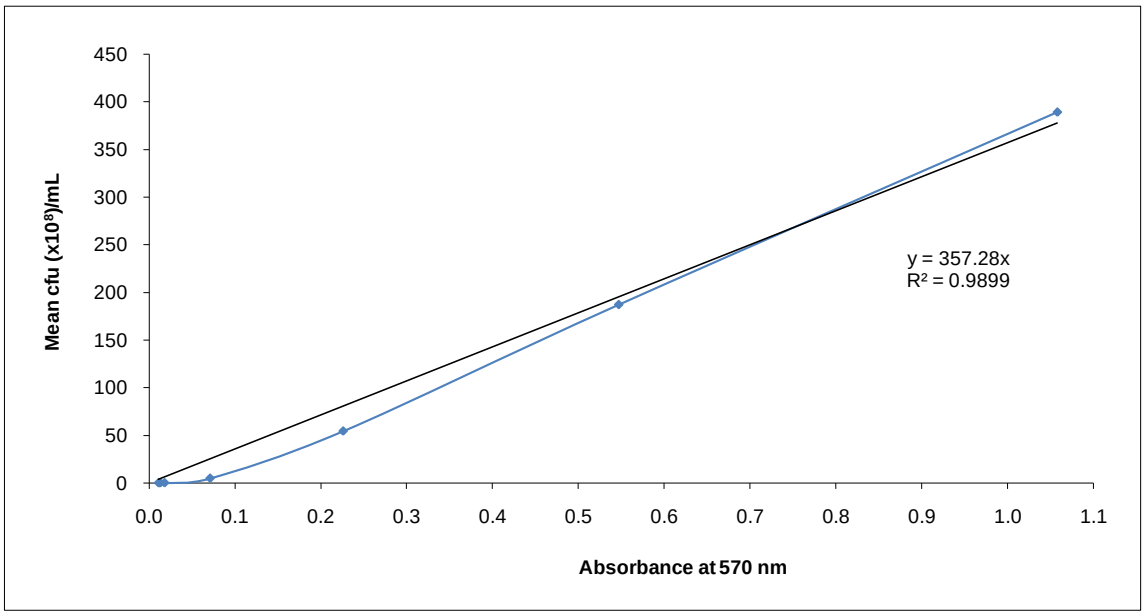

Figure A5. Mean (n=2) colony forming units (cfu) of *E. coli* cell suspension correlated to turbidity measured at an absorbance of 570 nm.

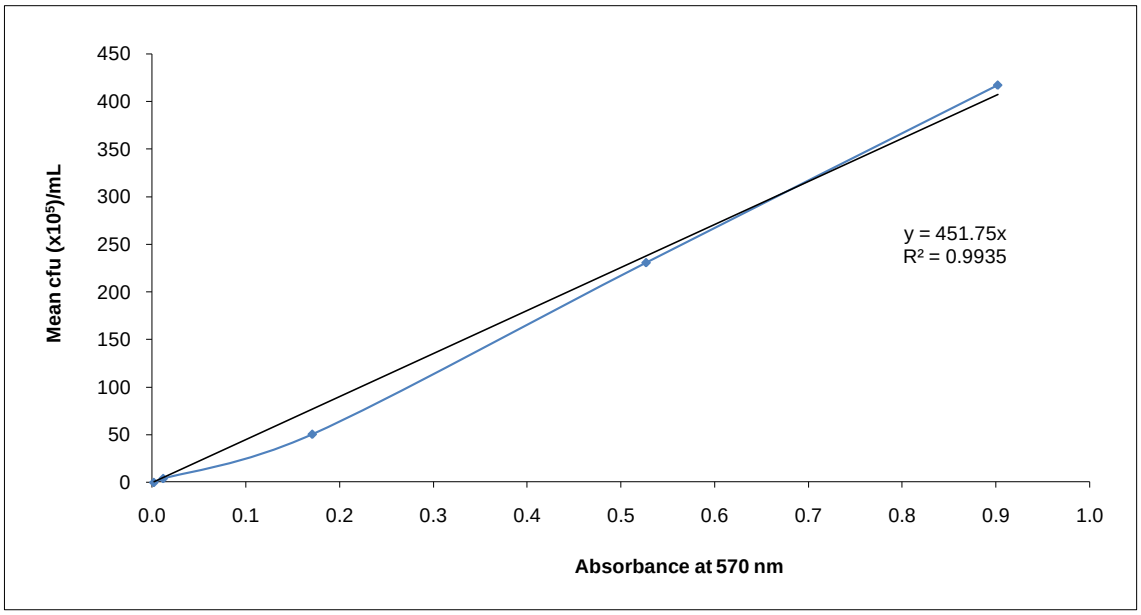

Figure A6. Mean (n=2) colony forming units (cfu) of *C. albicans* cell suspension correlated to turbidity measured at an absorbance of 570 nm.

Table A1. All MIC and MBC data categorised as high or low for statistical analysis, using the midpoint of the observed range as the cut off.

Organism	Eucalyptus Oil											
	MIC						MBC					
	Planktonic			Biofilm			Planktonic			Biofilm		
S. aureus	4	4	4	256	256	256	8	8	8	512	512	512
S. epidermidis	4	4	4	256	256	256	8	8	8	512	512	512
MRSA	2	2	2	512	512	512	2	2	2	512	512	512
P. aeruginosa	256	256	256	>512	>512	>512	256	256	256	>512	>512	>512
E. coli	8	8	8	16	16	16	8	8	8	256	256	256
C. albicans	8	8	8	8	8	8	32	32	32	32	32	32

Organism	1,8-Cineole											
	MIC						MBC					
	Planktonic			Biofilm			Planktonic			Biofilm		
S. aureus	16	16	16	512	512	512	256	256	256	>512	>512	>512
S. epidermidis	32	32	32	512	512	512	128	128	128	>512	>512	>512
MRSA	64	64	64	>512	>512	>512	256	256	256	>512	>512	>512
P. aeruginosa	>256	>256	>256	512	512	512	>256	>256	>256	>512	>512	>512
E. coli	64	64	64	128	128	128	64	64	64	256	256	256
C. albicans	8	8	8	4	4	4	64	64	64	8	8	8

Organism	Chlorhexidine											
	MIC						MBC					
	Planktonic			Biofilm			Planktonic			Biofilm		
S. aureus	1	1	1	128	128	128	2	2	2	>128	>128	>128
S. epidermidis	2	2	2	16	16	16	2	2	2	32	32	32
MRSA	2	2	2	128	128	128	2	2	2	>128	>128	>128
P. aeruginosa	16	16	16	>128	>128	>128	32	32	32	>128	>128	>128
E. coli	1	1	1	32	32	32	2	2	2	>128	>128	>128
C. albicans	16	16	16	16	16	16	16	16	16	>128	>128	>128

Low MIC/MBC
High MIC/MBC

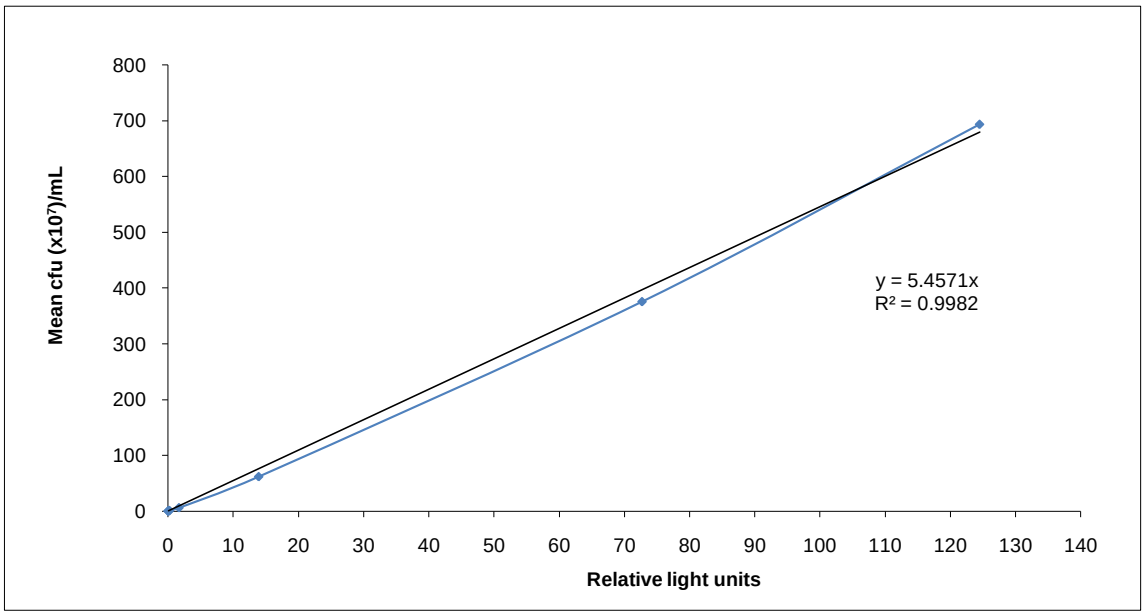

Figure A7. Mean (n=2) colony forming units (cfu) of *S. aureus* biofilms correlated relative light units measured using ATP bioluminescence.

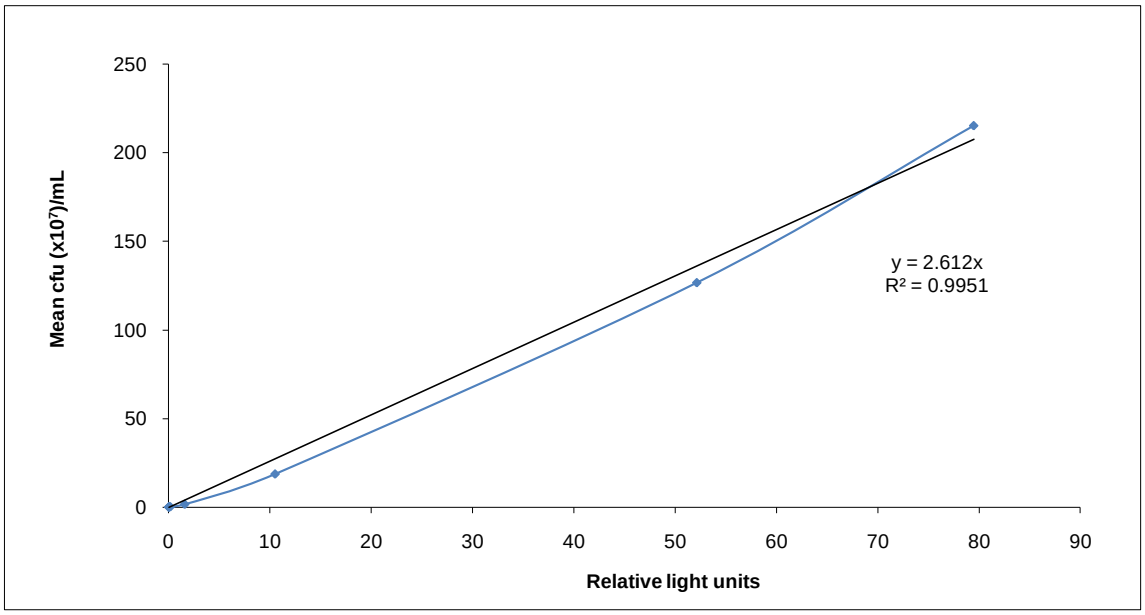

Figure A8. Mean (n=2) colony forming units (cfu) of *S. epidermidis* biofilms correlated relative light units measured using ATP bioluminescence.

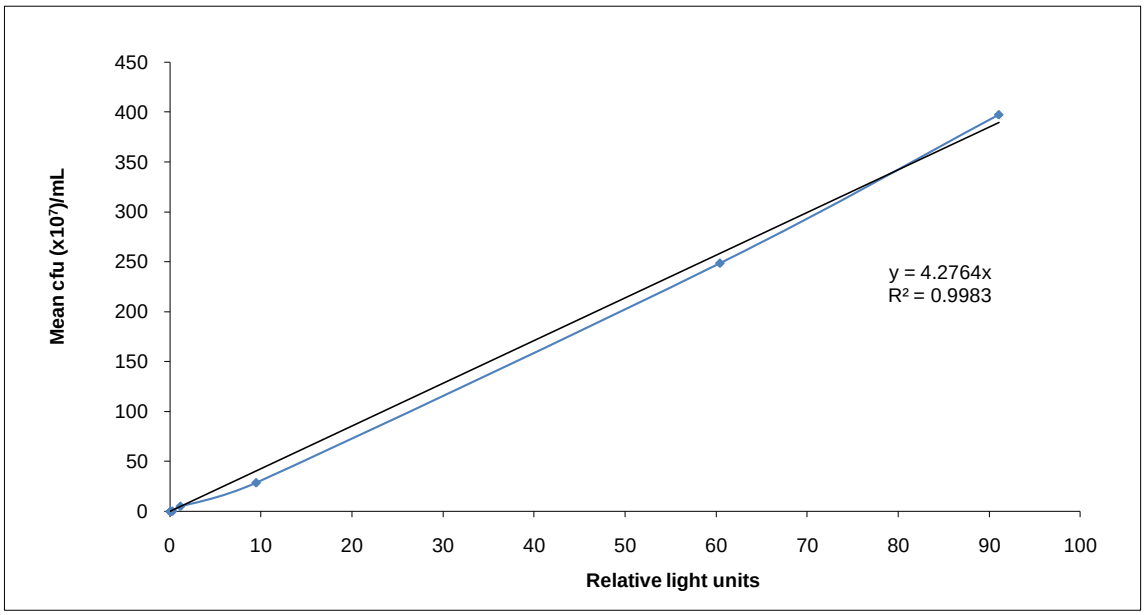

Figure A9. Mean (n=2) colony forming units (cfu) of MRSA biofilms correlated relative light units measured using ATP bioluminescence.

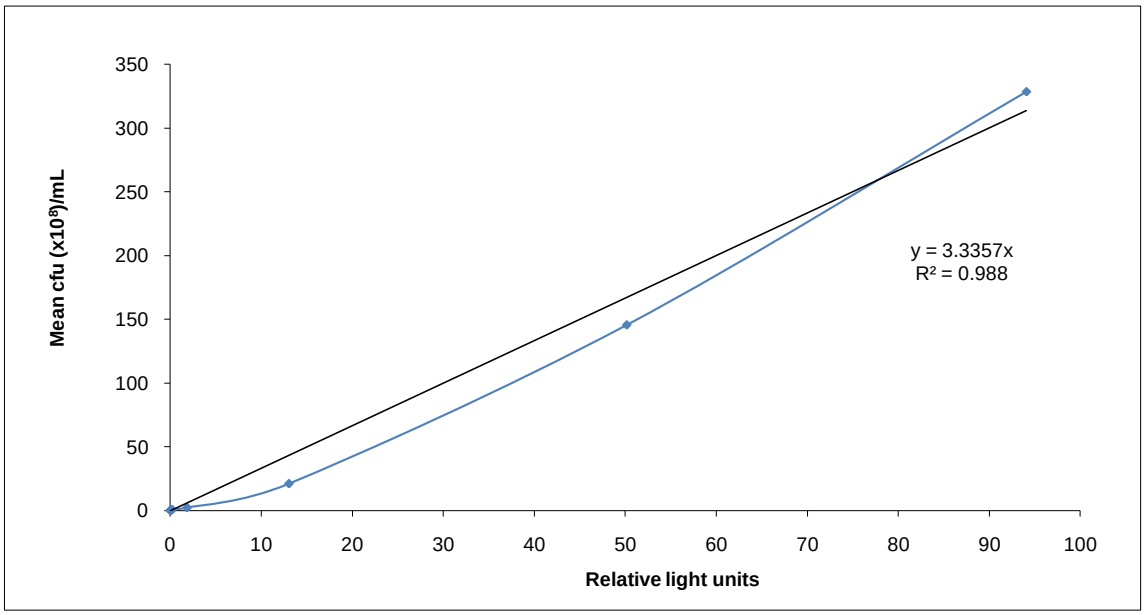

Figure A10. Mean (n=2) colony forming units (cfu) of *P. aeruginosa* biofilms correlated relative light units measured using ATP bioluminescence.

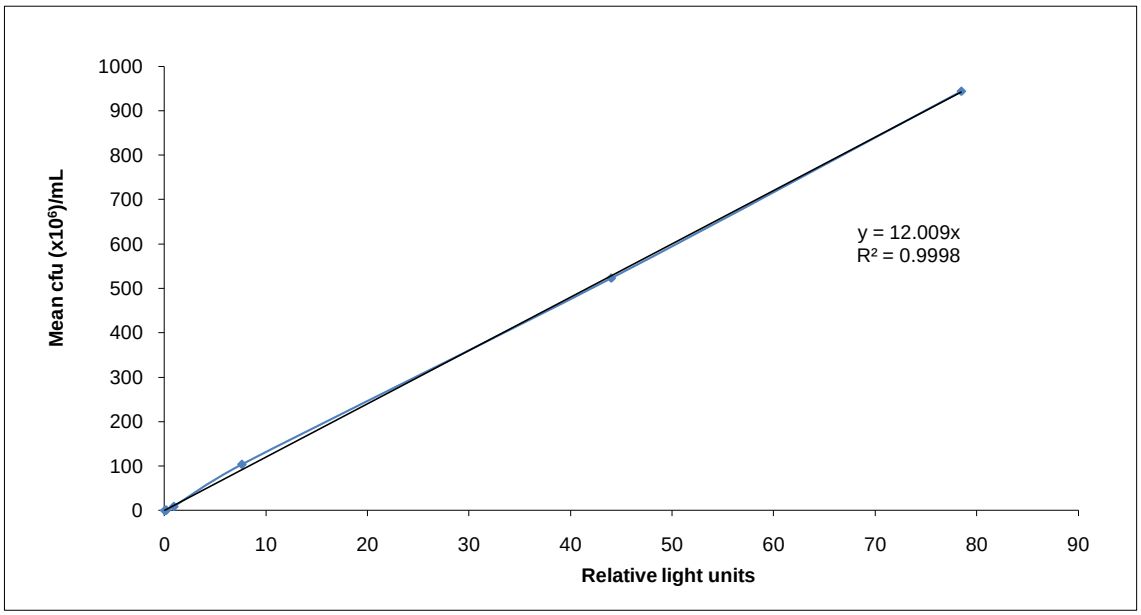

Figure A11. Mean (n=2) colony forming units (cfu) of *E. coli* biofilms correlated relative light units measured using ATP bioluminescence.

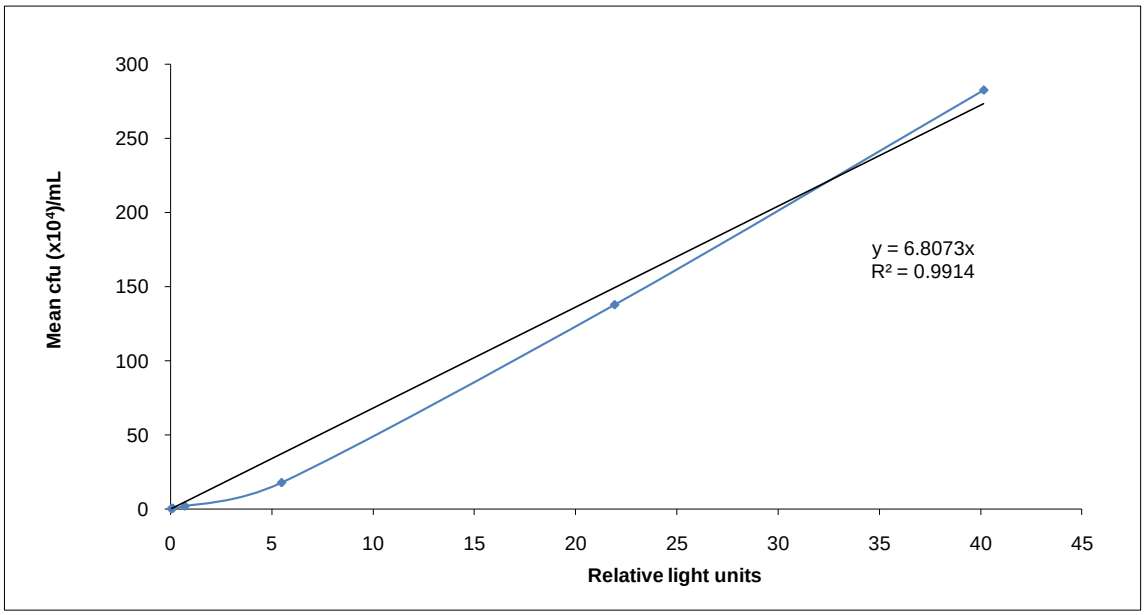

Figure A12. Mean (n=2) colony forming units (cfu) of *C. albicans* biofilms correlated relative light units measured using ATP bioluminescence.

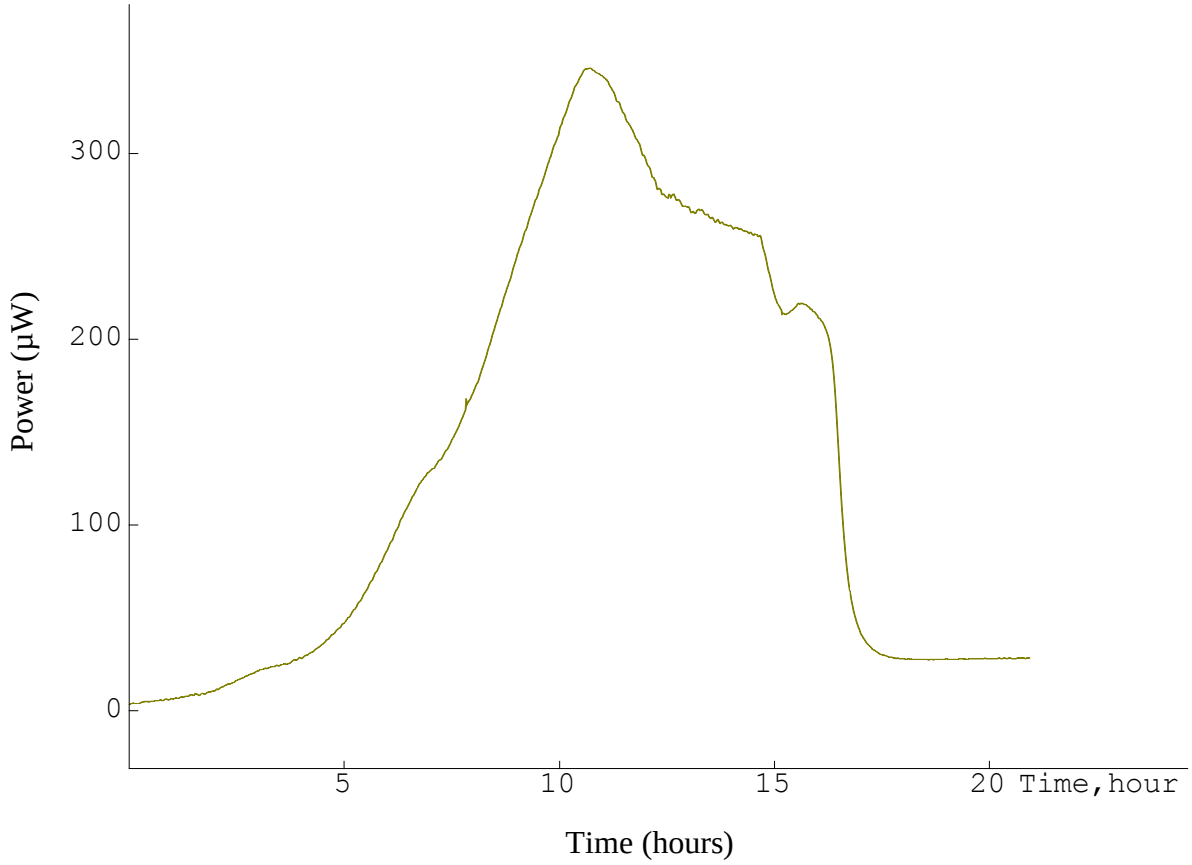

Figure A13. Calorimetric response of *C. albicans* growth curve represented as power-time data from flow calorimetry.

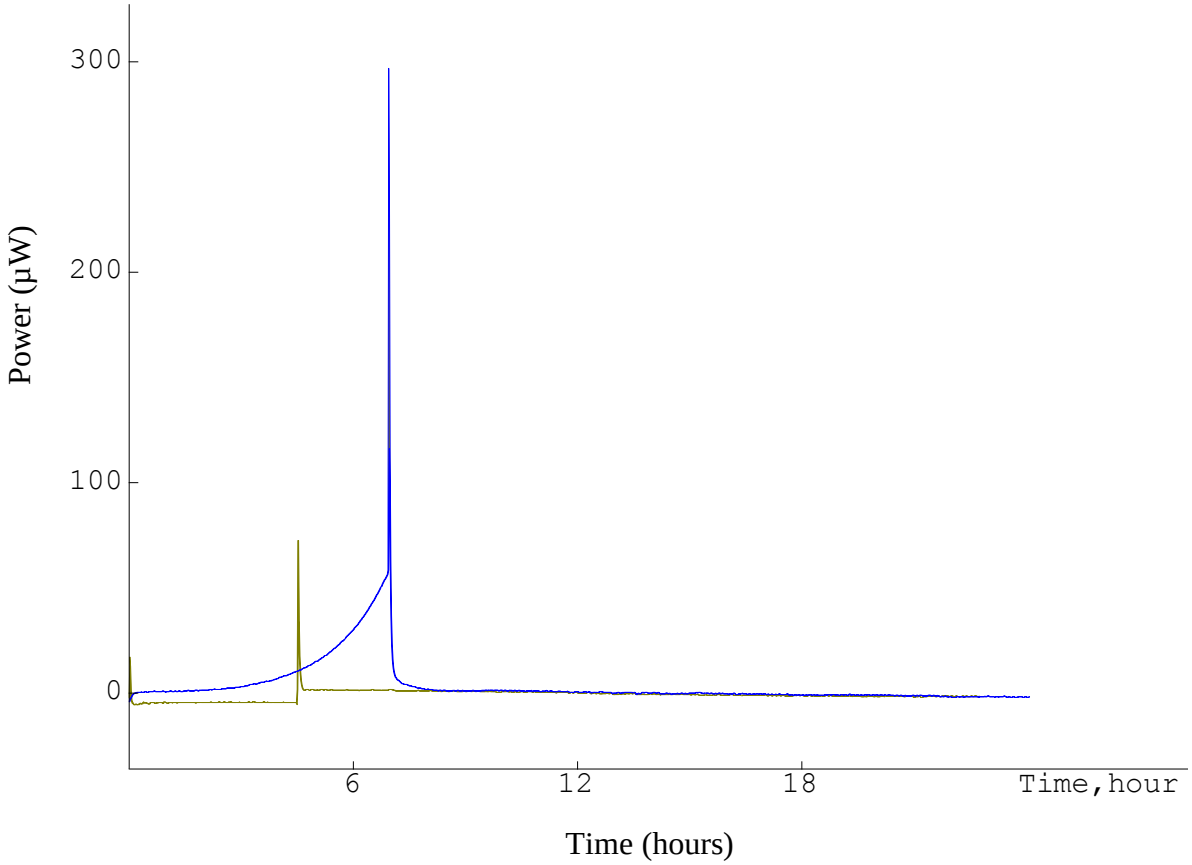

Figure A14. Calorimetric response of *C. albicans* growth curve (blue line) and broth only control (green line) interrupted by 2 % (v/v) addition of 5 % Euclean® solution during the log phase of growth for *C. albicans* and similar time period for the control, represented as power-time data from flow calorimetry.

Image A1. Ethical approval granted by the Chair of Aston University's Ethics Committee for the Euclean® clinical audit to be completed at Birmingham Children's Hospital.

Image A2. Ethical approval for the completion of the Euclean® clinical audit from the Research and Development department, and infection control teams at Birmingham Children's Hospital.

-----Original Message-----
Subject: RE: EuClean wipe questionnaire

Dear Jim, Michelle and Judith

Please see the attached email and letter from a PhD student at Aston requesting help with her research.

The research in question would not require research ethics approval, but would need appropriate approvals from with BCH.

Please let me know your thoughts from the infection control and nursing point of view. I did wonder whether nurses were the right staff group.

Emma - the study/questionnaire would clearly need to be designed to have minimal impact in terms of work time. It would also be useful if you could let me know who funds your Phd, the supervisor and also about any commercial involvement in the project.

Best wishes

Carole (Research and Development)

-----Original Message-----
Subject: RE: EuClean wipe questionnaire

Dear all,

I have no problem in principle with using this wipe. The only proviso is that I don't think it should be used to clean patient devices (such as lines) unless there are data to show compatibility.

I wonder also whether the study might also have to include healthcare assistants, who will often be responsible for cleaning of hard surfaces.

Jim (Consultant Microbiologist)

-----Original Message-----
Subject: RE: EuClean wipe questionnaire

Dear all,

I have just spoken with Emma and it appears that her study is for an environmental disinfectant rather than a medical device wipe.

I am happy to meet with Emma in relation to suggested areas, timescales etc I think the healthcare assistants/ care support workers will be key as they are the main users of the hard surface environmental disinfectant wipes.

Judith (Infection Control)

Image A3. Questionnaire used in the Euclean® disinfectant wipe clinical audit at Birmingham Children's Hospital.

Aston University
Life & Health Sciences

CLINICAL AUDIT OF EUCLEAN® DISINFECTANT WET-WIPES

About This Audit

Disinfectant wet-wipes are used in many hospitals for the removal of potentially harmful microorganisms from hard surfaces. Research based at Aston University and in conjunction with Insight Health Limited has lead to the development of Euclean® disinfectant wet wipe for use on hard surfaces, which has shown to possess enhanced antimicrobial activity compared with some currently available wipes.

The Euclean® disinfectant wipes contain 2% chlorhexidine, 70% isopropyl alcohol and 10% eucalyptus oil. They have shown to possess enhanced antimicrobial activity against a range of microorganisms including *Staphylococcus aureus*, *Staphylococcus epidermidis*, MRSA, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*.

DO NOT USE EUCLEAN® WIPES ON SKIN. The questions in this survey relate ONLY to wipes used for hard surface cleaning and disinfection.

What To Do

All responses you give will be anonymous.

This survey is in two parts, and requires the use of wet wipes for a total of two weeks;

1) Use your usual hard-surface disinfectant wipes during the first week, then answer questions 1-16.
2) Use the Euclean® disinfectant hard-surface wipes for the second week and answer questions 17-28.

The whole questionnaire can be completed in less than 15 minutes.

When completed, please return the questionnaire to the collection box located on each ward no later than.....

If you require further information about this questionnaire, please contact:

Emma Hendry on hendryer@aston.ac.uk

Many thanks for taking the time to complete this questionnaire.

Section 1: General Information

1. Gender ☐ Male ☐ Female
2. Age _____ years
3. What is your job title? _____

   _____
4. How long have you worked in your current role? _____ years _____ months
5. Which department/ward do you currently work in? _____

   _____

Section 2: Information in relation to current disinfection practice

The questions in this section relate to the disinfectant wet wipes you currently use during the working day within the hospital.
Please only answer the questions in this section after using your usual disinfectant wipes for one week.

Start Date: _____/_____/_____ End Date: _____/_____/_____

6. Do you use disinfectant wipes as a part of your routine work in practice? Tick one.

☐ Yes ☐ No

7. Name all the wipes you currently use for disinfecting hard surfaces _____

_____

_____

8. How often do you use disinfectant wipes for cleaning surfaces at work? Tick one.

☐ Daily ☐ Weekly ☐ Monthly ☐ Yearly ☐ Never

9. What objects/surfaces do you clean with your current disinfectant wipes? Tick all that apply and add others if needed.

☐ Bed
☐ Bed rails
☐ Bed side cabinets
☐ Bins
☐ Chairs
☐ Commodes
☐ Computers
☐ Cots
☐ Cupboards
☐ Desks
☐ Door handles
☐ Doors
☐ Floors
☐ Food trays
☐ Lamps
☐ Machinery
☐ Monitors/Displays
☐ Plug sockets
☐ Radiators
☐ Sinks
☐ Storage cabinets/lockers
☐ Tables
☐ Taps
☐ Telephones
☐ Toilets
☐ TV/stereo
☐ Walking frames
☐ Wheelchairs
☐ Window sills
☐ Windows
☐ Others _____

_____

10. What materials are the objects/surfaces in question 9 made of? Tick all that apply and add others if needed.

☐ Copper ☐ Glass ☐ Metal ☐ Plastic ☐ Stainless steel ☐ Wood
☐ Others _____

11. On average, what size surface area do you clean with your current disinfectant wipe, before needing to replace the wipe? Enter a rough estimate.

_____ cm by _____ cm.

12. Do you suffer any skin irritation/discomfort as a result of using your current disinfectant wipe(s)? Tick one.

☐ Yes ☐ No

If yes, please specify which wipes and symptoms experienced with each _____

_____

_____

13. What if any, are the advantages of your current disinfectant wipe(s)? _____

_____

_____

14. What if any, are the disadvantages of your current disinfectant wipe(s)? _____

_____

_____

15. Overall, how would you rate the wipes you currently use? Circle one number.

1 2 3 4 5 6 7 8 9 10

(Poor) (Average) (Excellent)

16. Would you be prepared to change using from your current disinfectant wipes to using a novel wipe? Tick one.

☐ Yes ☐ No

If no, please explain _____

_____

Section 3: Information in relation to use of Euclean® disinfectant wipes

The questions in this section relate to the Euclean® disinfectant wet wipes.

Please only answer the questions in this section after using the Euclean® disinfectant wipes

for one week.

Start Date: _____/_____/_____ End Date: _____/_____/_____

17. How often did you use the Euclean® disinfectant wipes for cleaning surfaces at work? Tick one.

☐ Daily ☐ Weekly ☐ Never

18. What objects/surfaces did you clean with the Euclean® disinfectant wipes? Tick all that apply and add others if needed.

☐ Bed
☐ Bed rails
☐ Bed side cabinets
☐ Bins
☐ Chairs
☐ Commodes
☐ Computers
☐ Cots
☐ Cupboards
☐ Desks
☐ Door handles
☐ Doors
☐ Floors
☐ Food trays
☐ Lamps
☐ Machinery
☐ Monitors/Displays
☐ Plug sockets
☐ Radiators
☐ Sinks
☐ Storage cabinets/lockers
☐ Tables
☐ Taps
☐ Telephones
☐ Toilets
☐ TV/stereo
☐ Walking frames
☐ Wheelchairs
☐ Window sills
☐ Windows
☐ Others _____

_____

19. What materials were the objects/surfaces in question 18 made of? Tick all that apply and add others if needed.

☐ Copper ☐ Glass ☐ Metal ☐ Plastic ☐ Stainless steel ☐ Wood

☐ Others _____

20. On average, what size surface area did you clean before needing to replace the Euclean® disinfectant wipe? Enter a rough estimate.

_____ cm by _____ cm.

21. What type of packaging would you prefer for the Euclean® wipes? Tick all that apply.

☐ Flexible plastic pouch with re-sealable peal back opening

☐ Individually wrapped sachets

☐ Larger tubs

☐ Smaller tubs

☐ Tubs (as currently packaged)

☐ Other _____

22. What did you think of the size of the individual Euclean® wipes? Tick one.

☐ Too small ☐ Just right ☐ Too Big

23. What did you think of the thickness of the individual Euclean® wipes? Tick one.

☐ Too thin ☐ Just right ☐ Too thick

24. Did you suffer any skin irritation/discomfort as a result of using the Euclean® disinfectant wipes? Tick one.

☐ Yes ☐ No

If yes, please specify the symptoms experienced _____

_____

_____

25. What if any, were the advantages of the Euclean® disinfectant wipe? _____

_____

_____

26. What if any, were the disadvantages of the Euclean® disinfectant wipe? _____

_____

_____

27. Overall, how would you rate the Euclean® disinfectant wipes? Circle one number.

1 2 3 4 5 6 7 8 9 10

(Poor) (Average) (Excellent)

28. Please provide any general feedback, comments or thoughts about the Euclean® disinfectant wipe. _____

_____

_____

_____

_____

_____

_____

_____

_____

_____

If you would be willing to partake in future studies of a similar kind, please provide your contact details below (these details will not be used in conjunction with this questionnaire).

Image A4. Poster detailing completion instructions for the Euclean® disinfectant wipe clinical audit questionnaire, and suitable uses for the wipes.

Aston University
Life & Health Sciences

INSIGHT
HEALTH LIMITED

EUCLEAN® DISINFECTANT WIPE TRIAL

What to do

1) Use your normal cleaning methods for one week, then answer questions 1-16.
2) Use Euclean® wipes for the second week, then answer questions 17-28.

Safe for use on:

- Any hard surfaces (e.g. desks, window sills, chairs, doors, lockers etc.)
- Bathroom surfaces (e.g. sinks, showers, taps, toilets, floors etc.)
- Beds (including mattresses)
- Machinery/Equipment (e.g. monitors, trays etc.)

Do not use on:

- Objects that penetrate the skin
- Patient's skin

Many thanks for taking part in this trial, which will form part of my PhD research.

If you have any questions regarding this trial, please contact:
Emma Hendry BSc. (Microbiology PhD Student, Aston University)
Email: hendryer@aston.ac.uk
Phone: 07866 371167

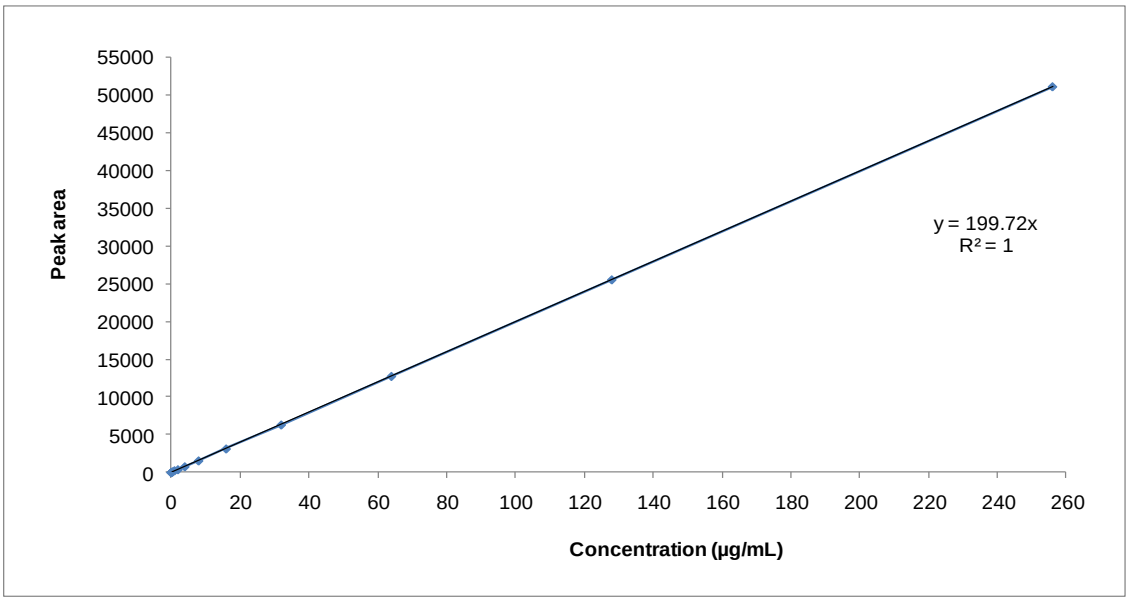

Figure A15. Mean (n=3) peak area from double dilutions of chlorhexidine covering the range 0.03125 µg/mL to 256 µg/mL measured using HPLC.

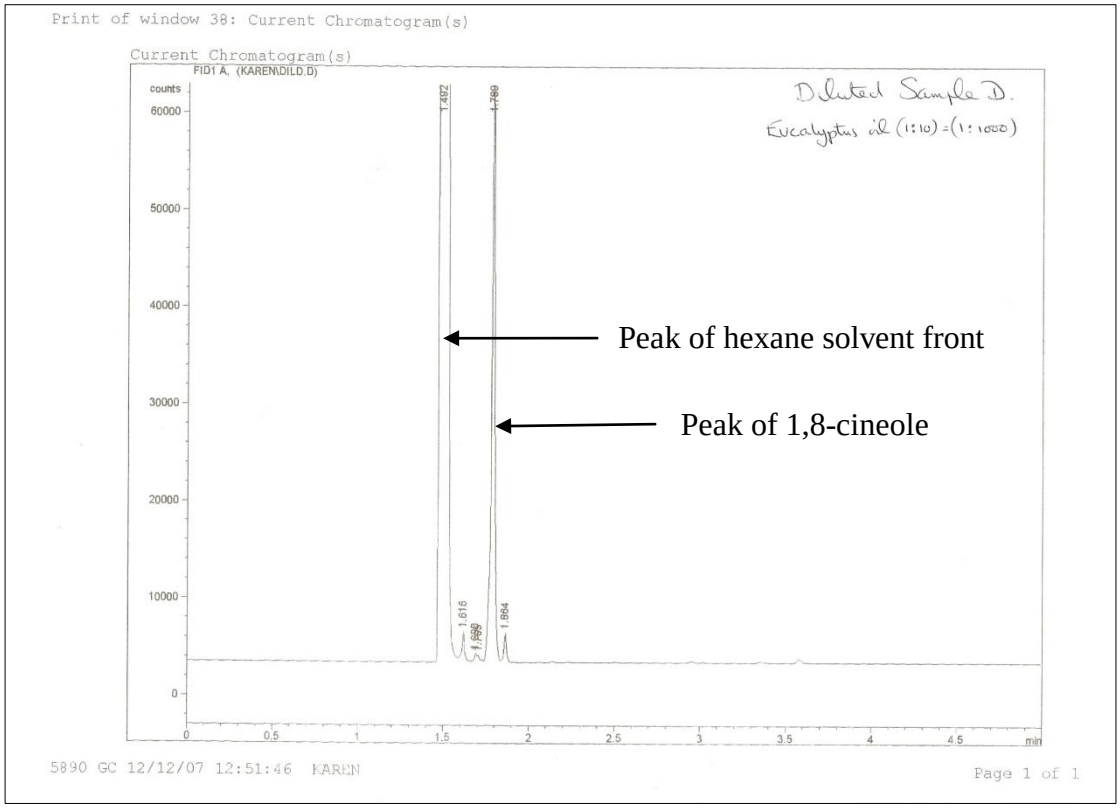

Figure A16. Chromatogram of hexane solvent front eluted from gas chromatograph with a retention time of 1.492 minutes, and the various small and one large peak (identified as 1,8-cineole) of eucalyptus oil with retention times of between 1.616 and 1.864 minutes.

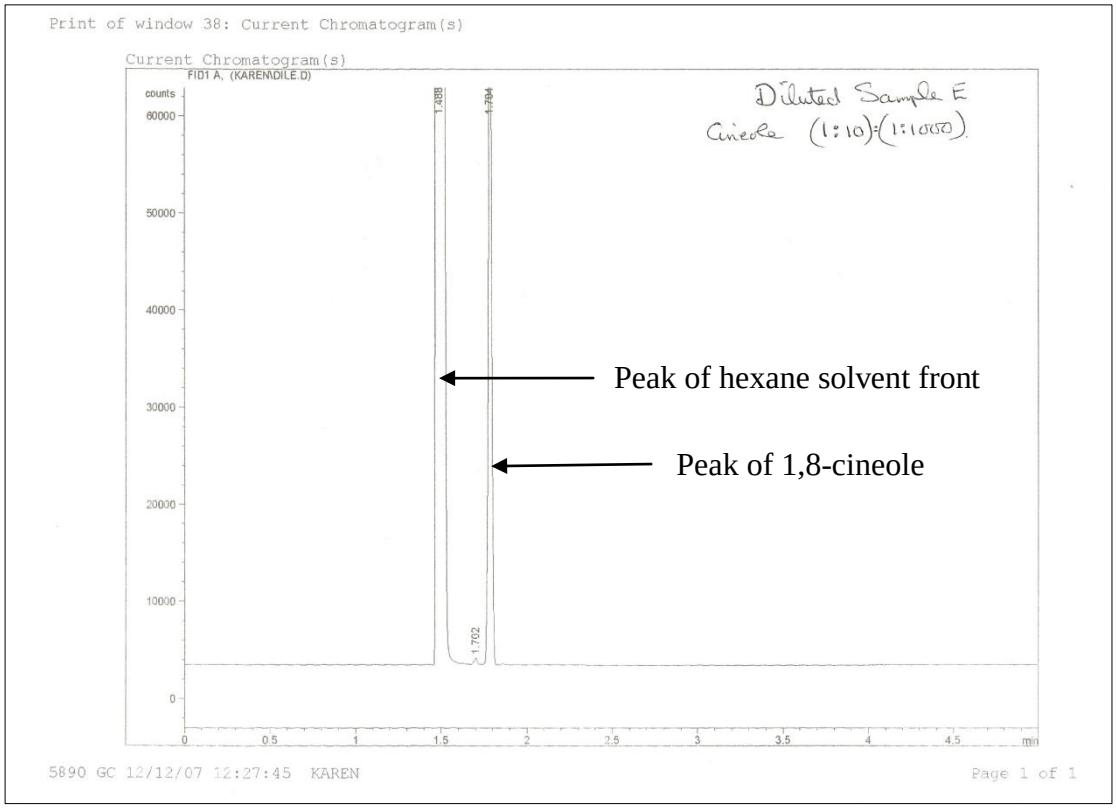

Figure A17. Chromatogram of hexane solvent front eluted from gas chromatograph after a retention time of 1.492 minutes, followed by 1,8-cineole with retention times of 1.764 minutes.

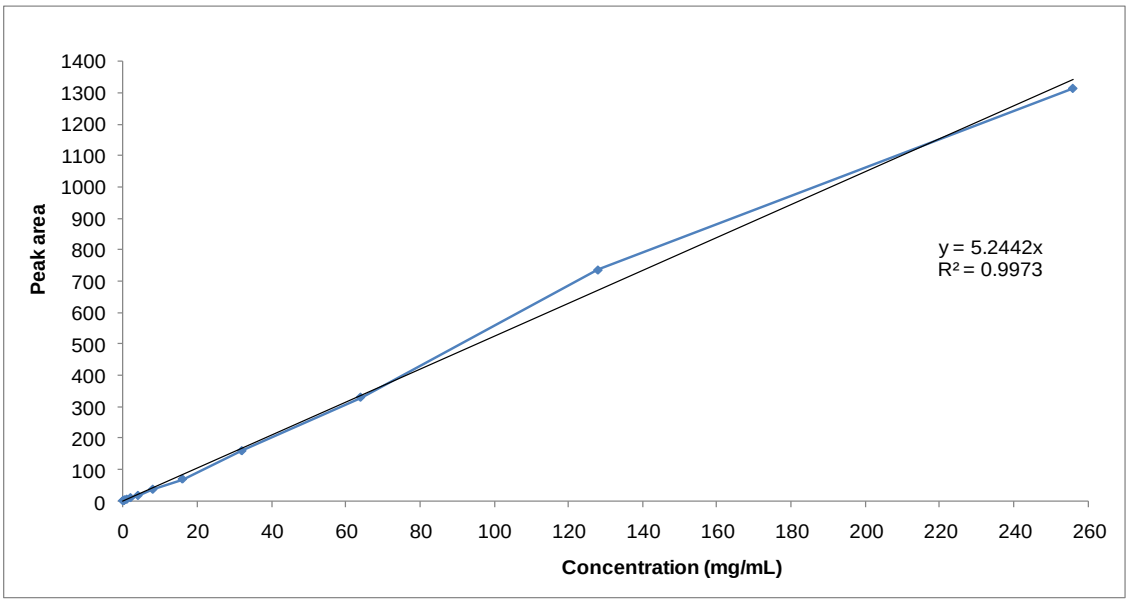

Figure A18. Mean (n=3) peak area comparative to internal standard, from double dilutions of eucalyptus oil in hexane covering the range 0.0078 mg/mL to 256 mg/mL, measured using gas chromatography.

Chapter 11

Publications and Professional Activities

Publications

Hendry, E. R., Worthington, T., Conway, B. R. and Lambert, P. A. Euclean® hard surface disinfectant wipes: antimicrobial efficacy against MRSA, *E. coli* and *C. albicans*, and potential to induce cross surface contamination. In preparation.

Hendry, E. R., O'Neill, M. A. A., Worthington, T., Conway, B. R. and Lambert, P. A. Isothermal calorimetry as an alternative method to traditional viable count for assessment of antimicrobial activity. In preparation.

Hendry, E. R., Worthington, T., Conway, B. R. and Lambert, P. A. (2010). P08.17 Antimicrobial efficacy of novel Euclean® hard surface disinfectant wipes. *J Hosp Infect*, **76** (1): S27.

Karpanen, T. J., Hendry, E. R., Worthington, T., Lambert, P. A. and Conway, B. R. (2010). Chlorhexidine: Skin permeation and antisepsis. *Ind Pharm*, **26**: 11-4.

Hendry, E. R., Worthington, T., Conway, B. R. and Lambert, P. A. (2009). Antimicrobial efficacy of eucalyptus oil and 1,8-cineole alone and in combination with chlorhexidine digluconate against microorganisms grown in planktonic and biofilm cultures. *J Antimicrob Chemother*, **64**: 1219-25.

Karpanen, T. J., Worthington, T., Hendry, E. R., Conway, B. R., Lambert, P. A. (2008). Antimicrobial efficacy of chlorhexidine digluconate alone and in combination with eucalyptus oil, tea tree oil and thymol against planktonic and biofilm cultures of *Staphylococcus epidermidis*. *J Antimicrob Chemother*, **62**: 1031-6.

Poster presentations

Hendry, E. R., Mistry, P., Davies, M. and Chaudhry, R. (2011). Microbiology: Infectious disease, investigation and prevention. Aston University Postgraduate Poster Day, 16th March, Aston University, Birmingham, U.K.

Hendry, E. R., Worthington, T., Conway, B. R. and Lambert, P. A. (2010). (Antimicrobial efficacy of novel) Euclean® hard surface disinfectant wipes. 7th International Conference of the Hospital Infection Society, 10-13th October, Liverpool, U.K.

Hendry, E. R., Worthington, T., Conway, B. R. and Lambert, P. A. (2010). Antimicrobial efficacy of novel Euclean® hard surface disinfectant wipes. Life and Health Sciences Postgraduate Research Day, 2nd July, Aston University, Birmingham, U.K.

Hendry, E. R., Worthington, T., Conway, B. R., Hilton, A. C. and Lambert, P. A. (2009). Antimicrobial activity of crude eucalyptus oil against *Staphylococcus aureus*, MRSA, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans* grown in planktonic and biofilm cultures. Life and Health Sciences Postgraduate Research Day, 3rd July, Aston University, Birmingham, U.K.

Hendry, E. R., Worthington, T., Conway, B. R., Hilton, A. C. and Lambert, P. A. (2009). Antimicrobial activity of crude eucalyptus oil against *Staphylococcus aureus*, MRSA, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans* grown in planktonic and biofilm cultures. 19th European Congress of Clinical Microbiology and Infectious Diseases, 16-19th May, Helsinki, Finland.

Oral presentations

Hendry, E. R. Studies on enhanced skin antisepsis. (2010). Research in Progress Seminar, 28th April, Aston University, Birmingham, U.K.

Hendry, E. R. Eucalyptus oil and 1,8-cineole as potential antimicrobial agents against microorganisms grown in planktonic and biofilm cultures. (2008). Research in Progress Seminar, 30th October, Aston University, Birmingham, U.K.

Hendry, E. R. Antimicrobial efficacy of eucalyptus oil and 1,8-cineole against planktonic and biofilm cultures of S*taphylococcus aureus*, *Escherichia coli* and *Candida albicans*. (2008). Life and Health Sciences Postgraduate Research Day, 30th June, Aston University, Birmingham, U.K.

Magazine articles

Hendry, E. R. (2009). Essential oils for improved skin antisepsis: the way forward? *The Microbiologist, the magazine of the Society for Applied Microbiology, September 2009*, **10** (3): 49-50.

Funding

Aston University Interdepartmental Fund for equipment and conference attendance, October 2009 (£1740).

The Society for Applied Microbiology President's fund, April 2008 (£855).

Awards

2010: Dean's Prize for the best Published Research Paper, School of Life and Health Sciences Postgraduate Research Day, 2nd July 2010. School of Life and Health Sciences, Aston University.

2007: Greenshields Prize for the best Undergraguate Dissertation within Biology (2007). School of Life and Health Sciences, Aston University.

Related professional activities

Jan 2010 - Aug 2010: Reviewed papers for the refereed journals Letters in drug design and discovery, Journal of medical devices: evidence and research, and Proceedings of the Pakistan academy of sciences.

Oct 2008 - Mar 2011: Designed and supervised undergraduate dissertation projects.

Jun 2008: Participated in interviews and filming for the Midland Health Academy biosciences videos project.

Mar 2008 - Oct 2010: Conducted community engagement and outreach work; volunteered at the British science festival, mentored school placement projects and assisted at convocation and open days.

Jan 2008 - Dec 2010: Worked as a teaching assistant, examination grader and laboratory demonstrator for undergraduate medical biochemistry, biotechnology, food microbiology, biology key skills and IT modules.

Nov 2007 - May 2010: Attended and participated in scientific conferences both in the U.K. and Europe.

Oct 2007 - Mar 2011: Undertook training courses including Research ethics, Teaching and assessment, Safe procedures and best practice, Endnote bibliographic software Microsoft Excel and Microsoft Word, at Aston University.


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1.

Aston University
Birmingham

Studies on Enhanced Skin Antisepsis

Emma Hendry

2.

Setting the Scene

- Hospital acquired infections (HAI) include bloodstream, urinary tract and surgical site infections, and are a major problem:
  - Morbidity (prevalence of disease).
  - Mortality (prevalence of death).
  - Cost.
- "It has been estimated that each patient with a surgical site infection requires an additional hospital stay of 6.5 days and hospital costs are doubled." (1)
- Often associated with inadequate skin antisepsis.

Aston University

1. Health Protection Agency. 2008. Surveillance of Surgical Site Infection in English Hospitals 1997-2002.

3.

The Skin

- The skin covers an area of approx $2m^2$. (2)
- Number of resident bacteria known as normal flora on skin varies with site:
  - Few hundred per cm^2 on forearm. (3)
  - Tens of thousand per cm^2 in groin. (3)
- Normal flora helps protect the skin from pathogens:
  - Compete for nutrients.
  - Compete for space.
- If given the opportunity, normal flora can become pathogens.

Disposable central venous catheter set

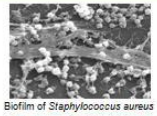
Aston University

2. Hedgraft, J. (2001). Skin, The Final Frontier. *International Journal of Pharmaceutics* 224, p1-10.
3. Mims, C., Dockrell, H. M., Goering, R. V., Roitt, I., Wakelin, D., Zuckerman, M. (2004). Medical Microbiology 3rd edition. Elsevier Limited, Spain.

4.

The Cause of the Problem

- Skin microorganisms can be held most accountable:
  - *Staphylococcus epidermidis*
  - *Staphylococcus aureus*
  - Gram negative bacilli
  - *Candida* species
- Biofilms have increased resistance.
- Hospitals often use 0.5% (w/v) chlorhexidine (CHG) in 70% isopropyl alcohol (IPA), recent EPIC recommendations for 2% CHG in 70% IPA.(4)



Biofilm of *Staphylococcus aureus*

Exit site wound

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4. Pratt, R. J., Pellowe, C. M., Wilson, J. A., Loveday, H. P., Harper, P. J., Jones, S. R. L. J., McDougall, C., Wilcox, M. H. (2007). epic2: National Evidence-Based Guidelines for Preventing Healthcare-Associated Infections in NHS Hospitals in England. *Journal of Hospital Infection* 65, p1-S49.

5.

Eucalyptus Oil

- Novel antiseptics are needed to improve skin sterilisation.
- Oil from leaves of eucalyptus trees (Myrtaceae).
- Shown antimicrobial properties:
  - On skin as a balm.
  - Used in mouthwashes.
  - Added to inhalation sprays.
  - Hydrophobicity possibly separates bacterial cell membrane lipids causing damage and increased permeability. (5)
- Main constituent 1,8-cineole (eucalyptol):
  - Also has antimicrobial properties.
  - Known to be an effective permeation enhancer.
  - Disrupts stratum corneum barrier and inter-cellular lipid interactions.

Aston University

5. Prabuseenivasan, S., Jayakumar, M., Ignacimuthu, S. (2006). *In Vitro* Antibacterial Activity of Some Plant Essential Oils. *BMC Complementary and Alternative Medicine* 6, p39.

6.

Initial Aim

- Investigate the antimicrobial efficacy of EO and 1,8-cineole, alone and in combination with CHG, against planktonic and biofilm cultures of six clinically relevant microorganisms:
  - *Staphylococcus aureus*
  - MRSA
  - *Staphylococcus epidermidis*
  - *Escherichia coli*
  - *Pseudomonas aeruginosa*
  - *Candida albicans*
- Use the antimicrobial with greatest efficacy for further studies of skin antisepsis.

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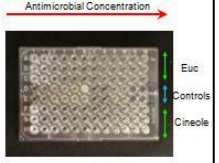
7.

Methods: Planktonic Cultures

- Cultures stored on beads at -70°C.
- Suspension cultures
  - Overnight broth diluted to 10^6cfu/ml.
  - Stocks of eucalyptus oil and 1,8-cineole diluted to give range of concentrations (512-0.25mg/ml).
  - 100µl of cell suspension and 100µl of antimicrobial added to wells in triplicate.
  - Incubated for 24 hours.
  - MIC determined visually and optical densities of wells read.
  - Well contents plated out using Miles & Misra [8] and MBC determined.



Staphylococcus aureus

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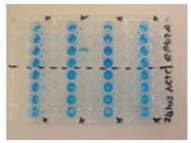
6. Miles, A. A. and Misra, S. S. (1938). The Estimation of the Bactericidal Power of the Blood. Journal of Hygiene, p732-749.

8.

Methods: Biofilm Cultures

- Optimum conditions for biofilm growth established.
- Biofilm cultures
  - 200µl of 10^4cfu/ml added and biofilm grown for 48 hours.
  - Wells washed with PBS, triplicate wells subjected to eucalyptus oil and 1,8-cineole (512-0.25mg/ml) for 24 hours.
  - Wells washed.
  - OD read and 250µl PBS added, plates sonicated for 30 minutes, 50Hz.
  - Biofilm scraped from wells, diluted and plated using Miles & Misra. [8]
  - MIC and MBC determined.



Alcian blue verified biofilm was produced

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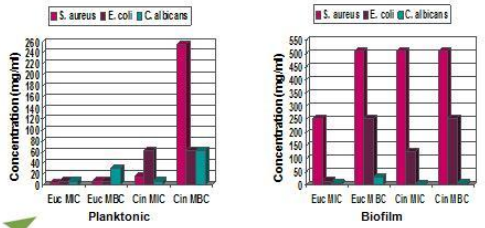
6. Miles, A. A. and Misra, S. S. (1938). The Estimation of the Bactericidal Power of the Blood. Journal of Hygiene, p732-749.

9.

Results: Planktonic and Biofilm

Graphs showing the minimum inhibitory and bactericidal concentrations (MIC/MBC) of eucalyptus oil and 1,8-cineole against *S. aureus*, *E. coli* and *C. albicans*.



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10.

Results: Planktonic and Biofilm

- The antimicrobial activity of crude eucalyptus oil was significantly better than 1,8-cineole against planktonic cultures of *S. aureus*, *E. coli* and *C. albicans* ($p<0.05$).
- There was no significant difference between the activity of the two antimicrobials when tested against biofilms ($p>0.05$).

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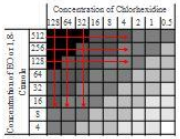
11.

Results: Checkerboard



Microorganism	Planktonic		Biofilm	
	EO/CHG	cin/CHG	EO/CHG	cin/CHG
S. aureus	Synergy	Synergy	Synergy	Indifference
S. epidermidis	Synergy	Synergy	Synergy	Synergy
MRSA	Synergy	Synergy	Synergy	Synergy
P. aeruginosa	Indifference	Indifference	Synergy	Synergy
E. coli	Synergy	Synergy	Indifference	Synergy
C. albicans	Synergy	Synergy	Synergy	Indifference

All combinations resulted in the same or enhanced antimicrobial activity of agents alone.

Crude eucalyptus oil was adopted for future research into skin antisepsis and hard surface disinfection.

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12.

Subsequent Aims

- Develop hard surface disinfectant wipes containing EO, CHG and IPA.
- Assess antimicrobial efficacy of wipes against clinically important pathogens.
- Trial wipes within the clinical setting and assess by means of a questionnaire.
- Develop a biopatch for skin application, containing EO, +/- CHG, +/- IPA.
- Investigate permeation of biopatch components into the skin.

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13.

Antimicrobial Surface Wipes

- Tubs of 200 viscose/polypropylene (50:50) wipes, 20 cm^2.
- Wipes impregnated with 384ml per tub:
  - Eucalyptus-Chloraprep wipes: 10% (v/v) EO, 2% (v/v) CHG, 70% (v/v) IPA and 0.1% Tween-80.
  - Eucalyptus-Chlorhexidine wipes: 10% (v/v) EO, 2% (v/v) CHG and 0.1% Tween-80.
  - Chloraprep wipes: 2% (v/v) CHG and 70% (v/v) IPA.
  - Alcohol wipes: 70% (v/v) IPA.
  - Control wipes: distilled water.
- Rested for 24-hour diffusion prior to use.

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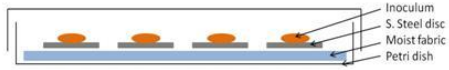
14.

Methods: Time Kill Study

- Stainless steel discs inoculated with 100µl of a 10^4cfu/ml suspension.



- Biofilm were established after 48 hr incubation.
- Unbound cells were washed off and disc underside cleaned.
- Discs were upturned onto wipes and weighted with 10g for time points (every minute up to 10 min, then 30 min, 1 hr, 2 hr, 4 hr and 6 hr).
- Discs were neutralised and the remaining biofilm removed.
- Time point of microbial kill was determined following serial dilution, agar culture and overnight incubation.

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15.

Results: Time Kill

- Eucalyptus-Chloraprep wipes required significantly less time to remove biofilms of all six microorganisms ($P<0.05$) than the other wipes tested.

Microorganism	Wipe	EO/Chloraprep	EO/CHG	Chloraprep	IPA
	S. aureus	< 10 min	< 6 hr	> 6 hr	> 6 hr
	MRSA	< 4 hr	< 4 hr	> 6 hr	> 6 hr
	S. epidermidis	< 4 min	> 6 hr	< 9 min	< 10 min
	E. coli	< 2 min	< 4 hr	< 10 min	< 10 min
	P. aeruginosa	< 1 min	> 6 hr	< 4 hr	< 6 hr
	C. albicans	< 10 min	< 6 hr	< 4 hr	< 6 hr

<1 min-30 min | 10 min-4 hours | 4-6 hours | Over 6 hours

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16.

Change of Formulation

- Eucalyptus-Chloraprep wipes:
  - Strong smell.
  - Difficulties of large scale production.
  - EO separation from solution.
  - Cost; EO expensive ingredient.
  - 10% EO was reduced to 5% EO.
  - Tween 80 increased from 0.1% to 1%.
- Euclean® wipes:
  - 5% (v/v) EO, 2% (v/v) CHG, 70% (v/v) IPA and 1% Tween-80.

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17.

Euclean® Wipes

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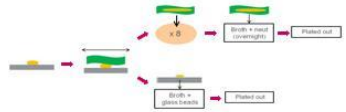
18.

Methods: Three-Step Protocol



- MRSA, *E. coli* and *C. albicans*.
- 20µl of a 10^8 cfu/ml suspension was dried onto a stainless steel disc.
- The disc was then wiped.
- Remaining cells recovered from disc.
- The contaminated wipe was pressed onto 8 consecutive agar plates.
- The wipe was neutralised overnight and viable cells recovered.

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7. Williams, G. J., Denyer, S. P., Hosein, I. K., Hill, D. W. and Maillard, J. Y. (2007). The Development of a Three-Step Protocol to Determine the Efficacy of Disinfectant Wipes on Surfaces Contaminated with *Staphylococcus aureus*. *Journal of Hospital Infection*, 67(4), pp29-35.

19.

Results: Three-Step Protocol

- Difference in cfu remaining on disc after Euclean® wipe:
  - 2 log reduction in MRSA.
  - 4 log reduction in *E. coli*.
  - 3 log reduction in *C. albicans*.
- Cross contamination by soiled Euclean® wipe:
  - No plates contaminated with MRSA.
  - No plates contaminated with *E. coli*.
  - No plates contaminated with *C. albicans*.
- Cell viability on Euclean® wipe after overnight incubation:
  - No viable MRSA cells remaining.
  - No viable *E. coli* cells remaining.
  - No viable *C. albicans* cells remaining.

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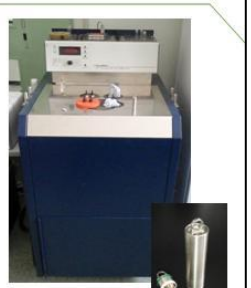
20.

Methods: Calorimetry

- Growth curves established for all six microorganisms (min 20 hrs).
- 3ml stainless steel ampoules inoculated with 2ml of 10^4 cfu/ml culture.
- 2cm x 2cm section of (10%) Eucalyptus-Chloraprep, Euclean® or control wipe added to ampoule.
- Increased inoculum to 10^7 cfu/ml.



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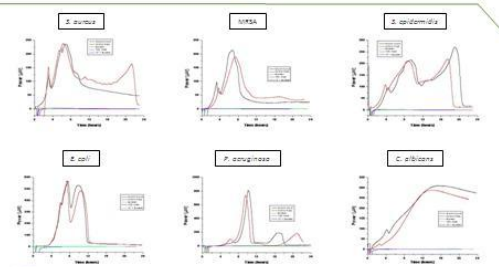
21.

Results: Calorimetry



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22.

Methods: Questionnaire

- Euclean® wipe trial in 5 wards at Birmingham Children's Hospital (BCH).
- Two week assessment:
  - Week 1- Use current cleaning procedure then complete the first half of the questionnaire.
  - Week 2- Use Euclean® wipes and complete the second half of the questionnaire.
- Questions included:
  - What wipes are currently used and how often?
  - What objects/surfaces are wiped?
  - What size surface area is cleaned before the wipe is replaced?
  - Do you suffer skin irritation?
  - Are there any advantages/disadvantages?
  - Overall score and feedback.
  - Euclean® wipes; packaging container/size, wipe size and thickness.

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23.

Results: Questionnaire (Week 1)

- Average response rate 20% (wards ranged from 13% to 27.8%).
- 98.1% of staff questioned use wipes daily during routine cleaning work.
- Most commonly used wipes (47.6%) were Sanicloth 70 (contains 70% alcohol), followed by Alco-wipes (14.3%).
- Surfaces cleaned with wipes most often include cots, bed side cabinets, bed rails, chairs, cupboards, monitors and beds.
- Surprisingly, bins, sinks, taps and food trays were amongst the least often cleaned with wipes.
- Less than 6% suffered irritation from their current wipes.
- 'Ease of use' rated the highest advantage of current wipes.
- 'Smell' was the biggest disadvantage of current wipes.
- 85.2% of staff would be prepared to change the wipes they use.

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24.

Results: Questionnaire (Week 2)

- Tubs (currently used) were the preferential packaging of 60% of staff.
- Individual wipe size was thought 'just right' by 85.2%.
- Wipe thickness was also thought 'just right' by 77.8%.
- Irritation was suffered by 12.9%.
- The overwhelming advantage was 'smell', but so was the main disadvantage.
- General comments included:
  - 'Smelled fresh and clean, they weren't so 'hospital' or 'clinical' as other wipes '
  - 'Unless cost is an issue, I see no reason to change'
  - 'The smell is very strong'
  - 'The wipes had an overall better cleaning ability'
  - 'Good for clearing a blocked nose, and detracting from other smells such as child's vomit, it overpowers vomit smell!'

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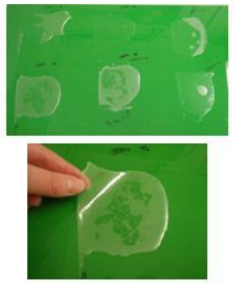
25.

Biopatch Development

- Polyvinyl alcohol (PVA)
  - Low, medium and high molecular weight.
  - Dissolved in water for 4 hr at 80°C.
- Chitosan
  - Dissolved in water with acetic acid overnight at room temperature.
- Range of concentrations made.
- Added EO and CHG.
- Films cast on acrylic boards.
- Set up to 24 hrs.
- Compromise between thin and flexible, or thick and brittle.



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26.

Skin Permeation

- PVA+CHG film vs. PVA+CHG+EO film applied to skin on Franz cell.
- Receptor chamber samples taken over 24 hrs and run on HPLC and GC.
- Skin removed and sliced on cryostat, also put through HPLC and GC.
- No permeation of CHG or EO into skin: quantification, release or contact issue?

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27.

Conclusions

- EO combined with CHG, has demonstrated enhanced antimicrobial against planktonic and biofilm cultures.
- Euclean® wipes have shown to increase microbial removal from surfaces, prevent cross contamination and kill residual organisms on the wipe, further verified by calorimetry.
- The opinion of hospital staff seemed divided on Euclean® wipes, 2% now being studied.
- The biopatch release studies require further investigation and possibly more optimisation of the material to allow skin permeation assessment.

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28.

Acknowledgements

- Many thanks to Dr Tony Worthington, Dr Barbara Conway and Prof. Pete Lambert for their continued support and enthusiasm.
- Thanks to Dr Mike O'Neill for calorimetry guidance and advice.
- Funding: EPSRC CASE studentship (Insight Health Limited).
- Birmingham Children's Hospital, and Roy Mckenzie for Euclean® trial.
- Thank you to all in lab 327 for their help and encouragement.
- Thank you to all in lab 331 for their endless supplies of gossip and laughs.

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29.

Many Thanks

Aston University

1.

Aston University
Birmingham

Eucalyptus Oil and 1,8-Cineole As Potential Antimicrobial Agents Against Microorganisms Grown in Planktonic and Biofilm Cultures

Emma Hendry

2.

Setting the Scene

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  - Morbidity (prevalence of disease).
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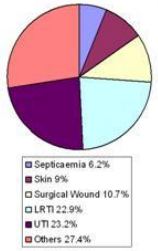

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Disposable central venous catheter set

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The Cause of the Problem

- Skin microorganisms can be held most accountable:
  - *Staphylococcus epidermidis*
  - *Staphylococcus aureus*
  - Gram negative bacilli
  - *Candida* species
- Biofilms have increased resistance.
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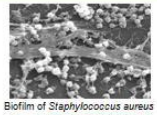

Biofilm of *Staphylococcus aureus*

Exit site wound

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- Novel antiseptics are needed to improve skin permeation.
- Oil from leaves of eucalyptus trees (Myrtaceae).
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- Main constituent 1,8-cineole (eucalyptol):
  - Also has antimicrobial properties.
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Birmingham

5. Prabuseenivasan, S., Jayakumar, M., Ignacimuthu, S. (2006). In Vitro Antibacterial Activity of Some Plant Essential Oils. *BMC Complimentary and Alternative Medicine* 6, p39.

6.

Aims of this Research

- Determine MIC and MBC/MFC of eucalyptus oil and 1,8-cineole against planktonic and biofilm cultures of;

Escherichia coli | *Staphylococcus aureus* | *Candida albicans*

- Decide which antimicrobial will be continued with for future work.

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Birmingham

7.

Methods: Planktonic Cultures

- Cultures stored on beads at -70°C.
- Suspension cultures
  - Overnight broth diluted to 10^6cfu/ml.
  - Stocks of eucalyptus oil and 1,8-cineole diluted to give range of concentrations (512-0.25mg/ml).
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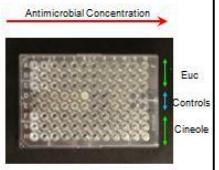

Staphylococcus aureus

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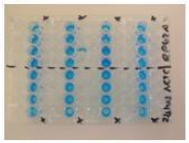

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Aston University

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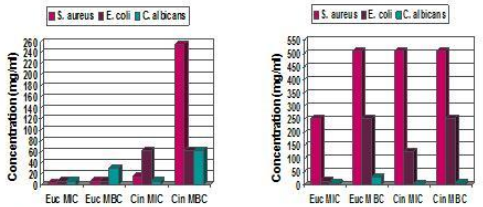
Results: Planktonic and Biofilm

Graphs showing the minimum inhibitory and bactericidal concentrations (MIC/MBC) of eucalyptus oil and 1,8-cineole against *S. aureus*, *E. coli* and *C. albicans*.



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10.

Results Continued

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- Crude eucalyptus oil was adopted for future research into skin antiseptics or hard surface disinfectants.

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11.

Discussion

- Both eucalyptus oil and 1,8-cineole disrupt the lipid packing of cells increasing their permeability. [7]
- Cineole makes up around 70% of eucalyptus oil and gave higher MIC/MBC values than the eucalyptus oil; other constituents may be contributing to its activity when in the crude oil form.
- Other main components of eucalyptus oil with antimicrobial activity include α-Pinene (14%), Limonene (3.6%), Terpinen-4-ol (3.1%), and Globulol (3%). [8]
- Reasons for indifference against biofilms is still unclear.

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7. Williams, A. C., Edwards, H. G. M., Lawson, E. E., Barry, B. W. (2009). Molecular Interactions Between the Penetration Enhancer 1,8-Cineole and Human Skin. *Journal of Raman Spectroscopy* 37, p981-988.
8. Silvestre, A. J. D., Cavaleiro, J. A. S., Desmond, B., Filliatre, C., Bourgeois, G. (1997). Analysis of the Variation of the Essential Oil Composition of *Eucalyptus globulus* Labill. from Portugal Using Multivariate Statistical Analysis. *Industrial Crops and Products* 6, p27-33.

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Conclusion

Eucalyptus oil and 1,8-cineole have both shown to be antimicrobial against a range of organisms, with it being concluded that eucalyptus oil has the highest efficacy and thus future work has continued with crude eucalyptus oil.

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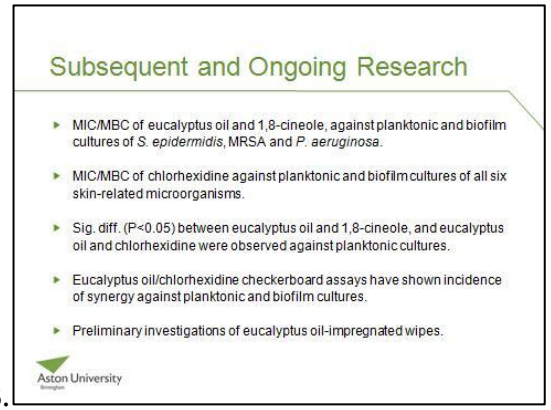
13. 
Subsequent and Ongoing Research
MIC/MBC of eucalyptus oil and 1,8-cineole, against planktonic and biofilm cultures of S. epidermidis, MRSA and P. aeruginosa.
MIC/MBC of chlorhexidine against planktonic and biofilm cultures of all six skin-related microorganisms.
Sig. diff. (P<0.05) between eucalyptus oil and 1,8-cineole, and eucalyptus oil and chlorhexidine were observed against planktonic cultures.
Eucalyptus oil/chlorhexidine checkerboard assays have shown incidence of synergy against planktonic and biofilm cultures.
Preliminary investigations of eucalyptus oil-impregnated wipes.
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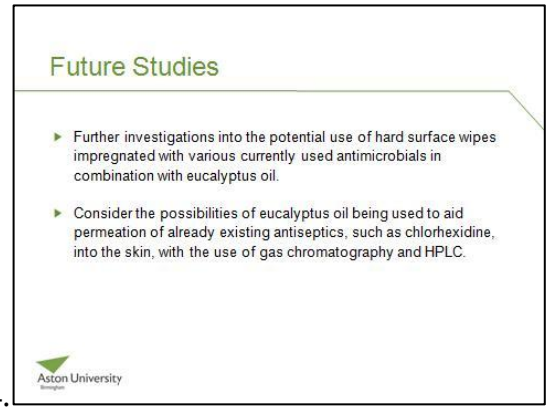
14. 
Future Studies
Further investigations into the potential use of hard surface wipes impregnated with various currently used antimicrobials in combination with eucalyptus oil.
Consider the possibilities of eucalyptus oil being used to aid permeation of already existing antiseptics, such as chlorhexidine, into the skin, with the use of gas chromatography and HPLC.
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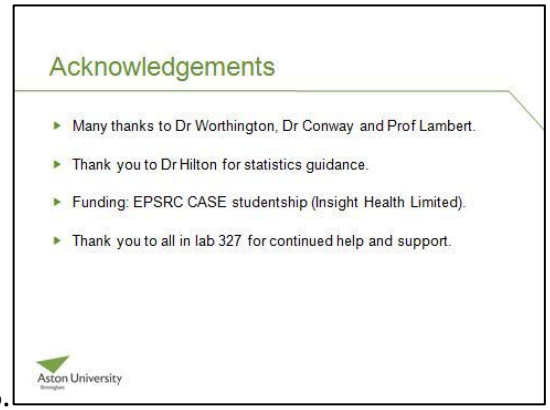
15. 
Acknowledgements
Many thanks to Dr Worthington, Dr Conway and Prof Lambert.
Thank you to Dr Hilton for statistics guidance.
Funding: EPSRC CASE studentship (Insight Health Limited).
Thank you to all in lab 327 for continued help and support.
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1.

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Antimicrobial Efficacy of Eucalyptus Oil and 1,8-Cineole Against Planktonic and Biofilm Cultures of *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans.*

Emma Hendry

2.

Welcome

- Began at Aston University in 2003 studying Applied and Human Biology.
- Undertook a microbiology placement at Birmingham's Children's Hospital in 2005.
- Graduated in 2007 with BSc Hons Applied and Human Biology.
- I am now a member of the Inflammation and Infection Research Group, after returning to Aston in October 2007 to study for a PhD entitled Studies on Enhanced Skin Antisepsis.

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3.

Background Information

- Hospital acquired infections (HAI) include bloodstream, urinary tract and surgical site infections, and are a major problem:
  - Morbidity (prevalence of disease).
  - Mortality (prevalence of death).
  - Cost.
- "It has been estimated that each patient with a surgical site infection requires an additional hospital stay of 6.5 days and hospital costs are doubled." (1)
- Often associated with inadequate skin antisepsis.

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1. Health Protection Agency. 2008. Surveillance of Surgical Site Infection in English Hospitals 1997-2002.

4.

The Skin

- The skin covers an area of approx $2m^2$. (2)
- Number of resident bacteria known as normal flora on skin varies with site:
  - Few hundred per cm^2 on forearm. (3)
  - Tens of thousand per cm^2 in groin. (3)
- Normal flora helps protect the skin from pathogens:
  - Compete for nutrients.
  - Compete for space.
- If given the opportunity, normal flora can become pathogens.

Disposable central venous catheter set

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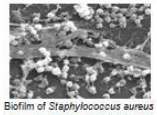
2. Hadgraft, J. (2001). Skin, The Final Frontier. *International Journal of Pharmaceutics* 224, p1-18.
3. Mims, C., Dockrell, H. M., Goering, R. V., Roitt, I., Wakelin, D., Zuckerman, M. (2004). Medical Microbiology 3rd edition. Elsevier Limited, Spain.

5.

The Cause of the Problem

- Skin microorganisms can be held most accountable:
  - *Staphylococcus epidermidis*
  - *Staphylococcus aureus*
  - Gram negative bacilli
  - *Candida* species
- Biofilms have increased resistance.
- Hospitals often use 0.5% (w/v) chlorhexidine (CHG) in 70% isopropyl alcohol (IPA), recent EPIC recommendations for 2% CHG in 70% IPA.(4)



Biofilm of *Staphylococcus aureus*

Exit site wound

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4. Pratt, R. J., Pellowe, C. M., Wilson, J. A., Loveday, H. P., Harper, P. J., Jones, S. R. L. J., McDougall, C., Wilcox, M. H. (2007). epic2: National Evidence-Based Guidelines for Preventing Healthcare-Associated Infections in NHS Hospitals in England. *Journal of Hospital Infection* 65, p1-S69.

6.

Eucalyptus Oil

- Novel antiseptics are needed to improve skin permeation.
- Oil from leaves of eucalyptus trees (Myrtaceae).
- Shown antimicrobial properties:
  - On skin as a balm.
  - Used in mouthwashes.
  - Added to inhalation sprays.
  - Hydrophobicity possibly separates bacterial cell wall, membrane and mitochondria lipids causing damage and increased permeability. (5)
- Main constituent 1,8-cineole (eucalyptol):
  - Also has antimicrobial properties.
  - Known to be an effective permeation enhancer.
  - Disrupts stratum corneum barrier and inter-cellular lipid interactions.

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5. Prabuseenivasan, S., Jayakumar, M., Ignacimuthu, S. (2006). *In Vitro* Antibacterial Activity of Some Plant Essential Oils. *BMC Complimentary and Alternative Medicine* 6, p39.

7.

Aims of this Research

- Determine MIC and MBC/MFC of eucalyptus oil and 1, 8-cineole against planktonic and biofilm cultures of;

Escherichia coli | *Staphylococcus aureus* | *Candida albicans*

- Decide which antimicrobial will be continued with for future work.

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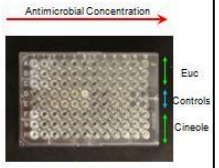
8.

Methods: Planktonic Cultures

- Cultures stored on beads at -70°C.
- Suspension cultures
  - Overnight broth diluted to 10^6cfu/ml.
  - Stocks of Eucalyptus oil and 1,8-cineole diluted to give range of concentrations (512-0.25mg/ml).
  - 100µl of cell suspension and 100µl of antimicrobial added to wells in triplicate.
  - Incubated for 24 hours.
  - MIC determined visually and optical densities of wells read.
  - Well contents plated out using Miles & Misra [6] and MBC determined.



Staphylococcus aureus

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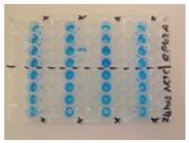
6. Miles, A. A. and Misra, S. S. (1938). The Estimation of the Bactericidal Power of the Blood. *Journal of Hygiene*, p732-749.

9.

Methods: Biofilm Cultures

- Optimum conditions for biofilm growth established.
- Biofilm cultures:
  - 200µl of 10^4cfu/ml added and biofilm grown for 48 hours.
  - Wells washed with PBS, triplicate wells subjected to eucalyptus oil and 1,8-cineole (512-0.25mg/ml) for 24 hours.
  - Wells washed.
  - OD read and 250µl PBS added, plates sonicated for 30 minutes, 50Hz.
  - Biofilm scraped from wells, diluted and plated using Miles & Misra.[6]
  - MIC and MBC determined.



Alcian blue verified biofilm was produced

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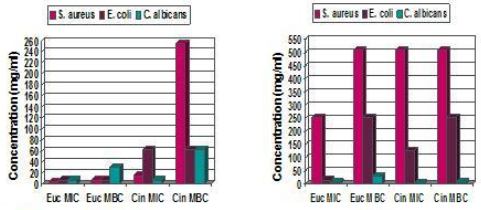

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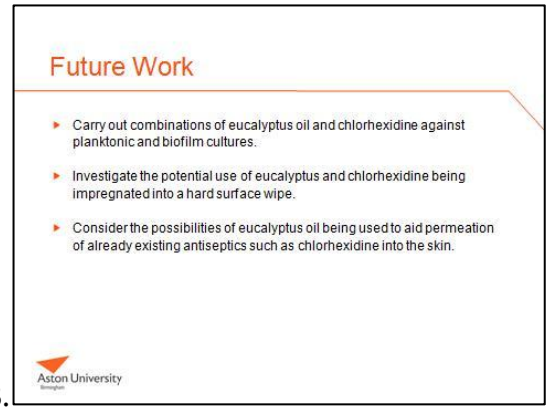
13. 
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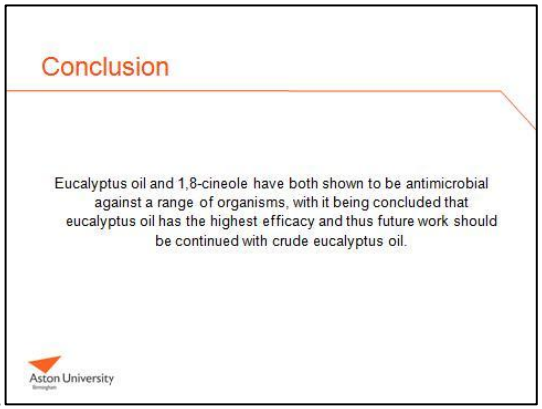
14. 
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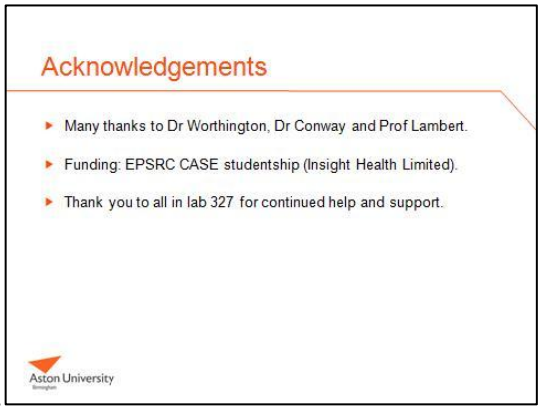
15. 
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