Biocide impregnated surface materials for use in clinical areas – under what conditions do they work?

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Summary

The survival of microorganisms on surfaces is well documented, potentially acting as a reservoir for the dissemination of healthcare-associated infections (HCAIs). Antimicrobial surfaces aim to control surface bioburden and lower HCAI rates. The existing antimicrobial surface efficacy test (JIS Z 2801) is an initial screening test; however, its set up (35°C, >90% relative humidity (RH)) bears little relationship to conditions in practice. This study aimed to develop new surface efficacy tests using wet and dried microbial inocula, reflecting conditions within a healthcare setting.

Changes in surface RH, temperature and bioburden were measured over one year at a hospital, allowing realistic parameters to be set for the new tests. Wet and dry inocula tests were developed and validated to mimic aerosol deposition and dry-touch contamination on surfaces, respectively. Aerosols of *S. aureus, A. baumannii* and *B. subtilis* spores and dry inocula of *S. aureus* and *A. baumannii* were tested against copper alloys and control stainless steel surfaces. Surviving bacteria were enumerated after varying contact times, and under in-use and JIS Z 2801 test conditions. FACS experiments were conducted to understand the mechanism of action of copper against dried microbial inocula.

Wet inoculum testing showed copper alloys presented significantly reduced activity against *S. aureus* aerosols at in-use conditions (>4 $\log_{10}$ after 60 min) compared to JIS Z 2801 test conditions (>4 $\log_{10}$ after 30 min). A >4 $\log_{10}$ reduction in *A. baumannii* was observed within 30 min but copper alloys were not sporicidal at in-use conditions. Dry inoculum testing showed a <2 $\log_{10}$ reduction in *S. aureus* and *A. baumannii* after 24 h at in-use conditions with potential mechanisms of action including; membrane damage, DNA damage and arrested cellular respiration.

The new tests developed provide realistic, second-tier tests to the JIS Z 2801. Copper was antimicrobial against both wet and dry inocula but was overall more efficacious against a wet inoculum, which suggests a liquid interface enhanced antimicrobial activity. It is recommended that antimicrobial surfaces are tested under in-use conditions against both wet and dry inocula to confidently predict their performance in practice.

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List of Abbreviations

ACC	Adult Critical Care
ACP	Acetyl-Acyl Carrier Protein
ANOVA	Analysis of Variance
ASTM	American Society for Testing and Materials
ATP	Adenosine Triphosphate
ATPase	Adenosine Triphosphatase
BHI	Brain Heart Infusion
BNF	British National Formulary
BOX	Bis (1,3-dibarbituric acid) trimethine oxanol
BSA	Bovine Serum Albumin
CA-MRSA	Community-Acquired Methicillin-Resistant *Staphylococcus aureus*
CDA	Copper Development Association
CDAD	*Clostridium difficile*-Associated Diarrhoea
CDMN	Clostridium Difficile Moxalactam Norfloxacin
cfu	colony forming units
cfu/cm^2	colony forming units per centimetre squared
cfu/mL	colony forming units per millimetre
CI	Confidence Interval
CTC	5-cyano-2,3-ditolyl tetrazolium chloride
DH	Department of Health
DMSO	Dimethyl Sulfoxide
DPA	Dipicolinic acid
EMRSA	Epidemic-Methicillin-Resistant *Staphylococcus aureus*

ENR	Enoyl-Acyl-Carrier Protein Reductase
FACS	Fluorescence-Activated Cell Sorting
HA-MRSA	Hospital-Acquired Methicillin-Resistant *Staphylococcus aureus*
HCAI	Healthcare-Associated Infection
HCW	Healthcare Worker
HEPA	High-Efficiency Particulate Air
HME	Heavy Metal Efflux
HPA	Health Protection Agency
HPV	Hydrogen Peroxide Vapour
ICU	Intensive Care Unit
ISO	International Organisation for Standardisation
ITU	Intensive Therapy Unit
LAAA	Light-Activated Antimicrobial Agent
LB	Luria-Bertani
MDR	Multidrug Resistant
MIC	Minimum Inhibitory Concentration
MMS	Moisture Measurement System
MRD	Maximum Recovery Diluent
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MSDS	Material Safety Data Sheets
MSSA	Methicillin-Sensitive *Staphylococcus aureus*
NAD+	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide plus Hydrogen
NAO	National Audit Office
NCIMB	National Collection of Industrial Marine and Food Bacteria

NCTC	National Collection of Type Cultures
NHS	National Health Service
nm	Nanometre
OD	Optical Density
OECD	Organisation and Economic Co-operation and Development
PBP	Penicillin Binding Protein
PBS	Phosphate Buffered Saline
PDT	Photodynamic Therapy
PHE	Public Health England
PI	Propidium Iodide
ppm	parts per million
QAC	Quaternary Ammonium Compounds
RH	Relative Humidity
RLU	Relative Light Unit
RND	Resistance Nodulation Division
SCCS	Scientific Committee on Consumer Safety
SD	Standard Deviation
spp.	Species
T_{AIR}	Air Temperature
T_{DEW}	Dew Point Temperature
T_{DIFF}	Temperature Difference
T_{S}	Surface Temperature
TSA	Tryptone Soya Agar
TSB	Tryptone Soya Broth
TSC	Tryptone Sodium Chloride

UHW	University Hospital of Wales
UK	United Kingdom
US EPA	United States Environmental Protection Agency
UVGI	Ultraviolet Germicidal Irradiation
VRE	Vancomycin Resistant Enterococci

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List of Publications

Results from this study have been published as follows:

Ojeil, M., Jermann, C., Holah, J., Denyer, S. and Maillard, J.-Y. (2013) Evaluation of new *in vitro* efficacy test for antimicrobial surface activity reflecting UK hospital conditions. *Journal of Hospital Infection*. 85(4):274-281

Biocide impreganted surface materials – under what conditions do they work? Manufacturing Technologies Panel, May 2012. Campden BRI, Chipping Campden, UK. Oral presentation

Ojeil, M., Holah, J., Jermann, C., Denyer, S. and Maillard, J-Y. (2012) Biocide impreganted surfaces – under what conditions do they work? Society for Applied Microbiology, Summer Meeting, July 2012. Edinburgh, UK. Poster presentation

Development of a new antimicrobial surface efficacy test reflective of "*in situ*" conditions. Microbiology Infection and Translational Research Group (MITReG) Interdisciplinary Postgraduate Research Day, September 2012. Cardiff, UK. Oral presentation

Ojeil, M., Holah, J., Jermann, C., Denyer, S. and Maillard, J-Y. (2012) The use of antimicrobial surfaces in the healthcare setting: can they work? Infection Prevention Society, Annual Conference, October 2012. Liverpool, UK. Poster presentation

Ojeil, M., Holah, J., Jermann, C., Denyer, S. and Maillard, J-Y. (2013) Development of a realistic *ex situ* surface test for determining the efficacy of antimicrobial surfaces. American Society for Microbiology. General Meeting, May 2013. Denver, USA. Poster presentation

Antimicrobial surfaces and the impact of water levels. NHS Rapid Review Panel meeting, April 2014. Department of Health, London, UK. Oral presentation

CHAPTER 1 GENERAL INTRODUCTION

1.1 Healthcare-associated infections

Healthcare-associated infections (HCAIs), or nosocomial pathogens, are 'any infectious agent acquired as a consequence of a person's treatment by a healthcare provider, or which is acquired by a healthcare worker in the course of their duties' (National Audit Office (NAO), 2009). It is likely a nosocomial pathogen has been acquired if symptoms develop 48 hours after admission to hospital (Murray *et al.*, 2007). HCAIs were previously referred to as 'hospital-acquired infections' but the term 'healthcare-associated infections' is now preferred since many medical procedures are not limited to the hospital setting (Website 1, publication date unavailable).

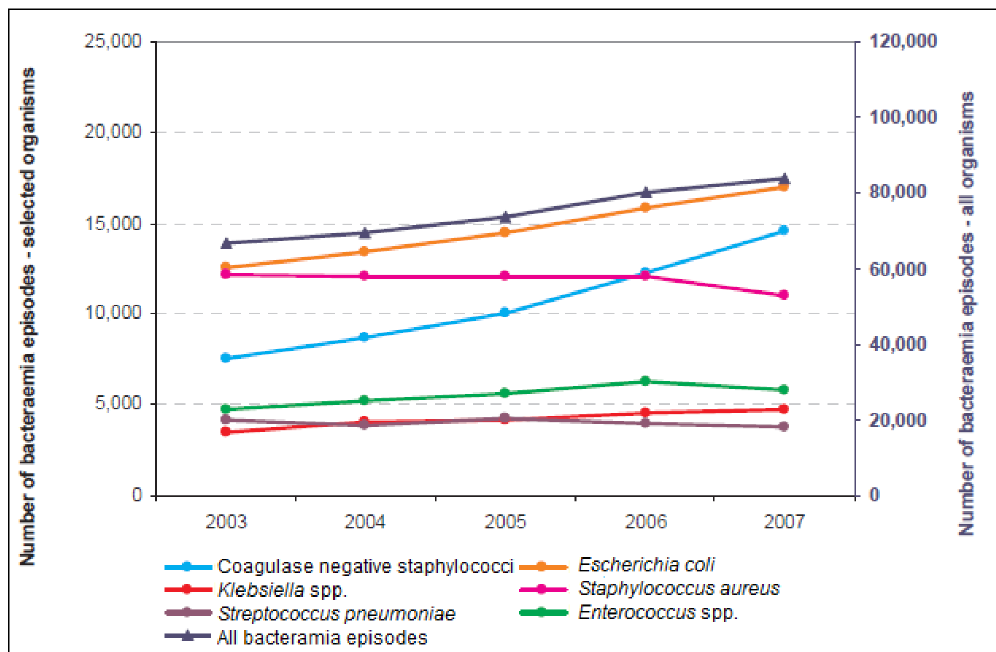
Common HCAIs include methicillin-resistant *Staphylococcus aureus* (MRSA), *Clostridium difficile* (*C. difficile*), *Acinetobacter* species (spp.), *Pseudomonas aeruginosa* (*P. aeruginosa*), coagulase negative staphylococci, *Klebsiella* spp. and *Enterococcocus* spp. (NAO, 2009).

HCAIs have a great impact on the economy and pose a financial burden on the National Health Service (NHS) costing around £1 billion a year (NAO, 2008). This high cost is associated with the length of hospital stay, cost of treating the infected patient and additional laboratory costs in the diagnosis of an HCAI (Shorr, 2007). It has been reported that each HCAI costs the NHS £4300 (NAO, 2009). In addition, the NHS has paid out £17 million between 2004 and 2008 in litigation costs (NAO, 2009).

The NHS previously stated one of its five main national priorities to be 'improving cleanliness and reducing healthcare-associated infections' (Website 2, 2009). In June 2009 'The Revised Healthcare Cleaning Manual' published by The National Patient Safety Agency, was published to help NHS Trusts provide a safe and clean working environment and to control HCAIs (National Patient Safety Agency, 2009). An updated version was published in 2012, although this manual is not as detailed and in depth, thus for this chapter the 2009 manual will be referred to. In addition, The Health Act 2006: Code of Practice for the Prevention and Control of Health Care Associated Infections outlines the policies and duties of NHS bodies in reducing HCAIs.

Figure 1.1 shows an increase in the total number of cases of bacteraemia in England between 2003 and 2007. Notable increases in the number of bacteraemia were in *E. coli* and coagulase negative staphylococci. Between 2006 and 2007 there was a slight decrease in the number of bacteraemia cases of *Staphylococcus aureus* (*S. aureus*) and *Enterococcus* spp. Overall, the data indicates the historical need to focus on the management of HCAIs within the clinical environment and explore all avenues of practical control measures, either at the patient level or via potential transmission routes.

Figure 1.1- Graph showing the number of cases of bacteraemia episodes due to coagulase negative staphylococci, *Klebsiella* spp., *Streptococcus pneumonia*, *E. coli*, *S. aureus* and *Enterococcus* spp. (HPA, 2009)

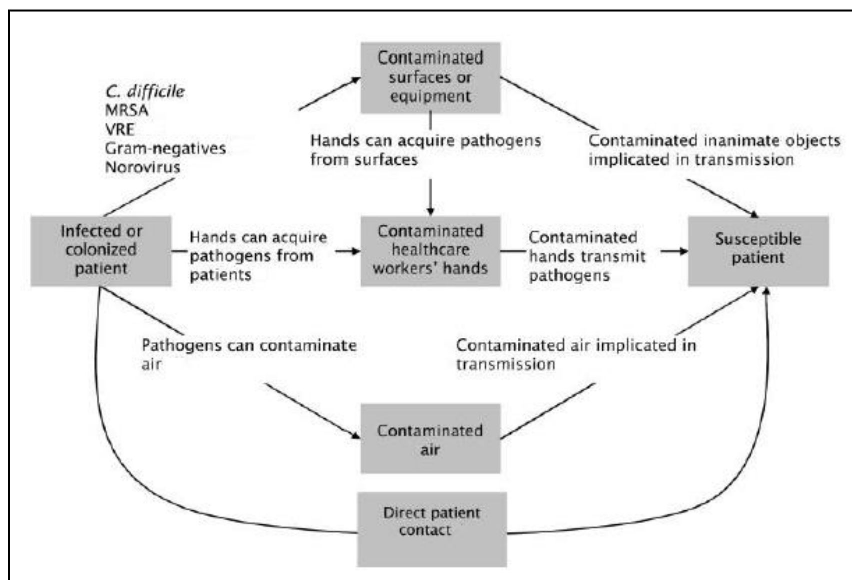


For the purpose of this study the focus was geared towards three HCAIs; *S. aureus*, *A. baumannii* (*A. baumannii*) and *C. difficile*. In addition, *B. subtilis* was used as a spore-forming model organism.

1.1.1 Routes of infection

Figure 1.2 is a diagram showing the transmission routes of infections in a healthcare setting from a review article by Otter *et al*. (2011). Patients can become infected directly by direct contact between patients or indirectly via contaminated surfaces, air or via healthcare workers (HCW) (Otter *et al*., 2011).

Figure 1.2 – Routes of transmission of microorganisms in healthcare environments (Otter *et al*. 2011)



1.2 *Staphylococcus aureus*

S. aureus belongs to the *Staphylococcus* genus of bacteria. *S. aureus* is a Gram-positive, catalase-positive, non-motile cocci (Murray *et al*., 2007). *S. aureus* colonises the anterior nares in around 20 % of the population (Gordon and Lowy, 2008). As a pathogen *S. aureus* is associated with skin and soft tissue infections, urinary tract infections, toxic shock syndrome, lung infections, deep-site infections (bones, joints, heart valves) and food poisoning (Murray *et al*., 2007).

1.2.1 Methicillin-resistant *Staphylococcus aureus* (MRSA)

1.2.1.1 Origin

MRSA is not susceptible to the β-lactam antibiotic methicillin (Hiramatsu *et al*., 2001). The β-lactam class of antibiotics functions by binding to penicillin-binding proteins (PBP) on the cell wall to prevent elongation of the bacterial peptidoglycan (Deurenberg *et al*., 2007). This will no longer result in cross-linking of the peptidoglycan. Penicillin, a β-lactam antibiotic, was first introduced in the 1940s, however, the emergence of resistance to this drug in *S. aureus* in 1942 prompted the development of methicillin in 1960 (Woodford, 2005; Deurenberg *et al*., 2007). In 1961 the first strain of MRSA was identified in the United Kingdom (UK) (Hiramatsu *et al*., 2001; Deurenberg *et al*., 2007). The development of methicillin-resistance in *S. aureus* is due to the acquisition of a large genetic element, the staphylococcal chromosomal cassette *mec* (SCC*mec*) (Hiramatsu *et al*., 2001; Woodford, 2005). This fragment of DNA integrates at a highly conserved site known as *attBscc orfX* found near *oriC*. Located on this cassette is a gene of 2.1 kb in size called *mecA*. This gene is responsible for the coding of a new penicillin-binding protein 2a (PBP2a) (Deurenberg *et al*., 2007). β-lactams are not effective against MRSA due to their low binding affinity to PBP2a, thus peptidoglycan synthesis is maintained (Hiramatsu et al., 2001). There are five types of SCC*mec* (I-V) of varying size. Types I, IV and V are responsible for β-lactam resistance only whereas Types II and III carry further antibiotic resistance genes (Deurenberg *et al*., 2007).

1.2.1.2 Community-acquired-MRSA

Community-acquired (CA)-MRSA has emerged in patients lacking the normal risk factors associated with MRSA including no hospitalisation, intravenous drug use or recent surgery (Hawkey, 2009). CA-MRSA is normally more susceptible to antibiotics than hospital-acquired (HA)-MRSA. In terms of SCC*mec* type, CA-MRSA possesses Type IV compared to Type II present in HA-MRSA (Weber, 2005). CA-MRSA infection can result in necrotising pneumonia, skin infections and sepsis due to the presence of the genes *lukS-PV* and *lukF-PV* that code for Panton-Valentine Leukocidin, a necrotising cytotoxin that lyses white blood cells (Weber, 2005; Boyle-Vavra and Daum, 2006; Elliot *et al*., 2007).

1.2.1.3 Treatment of MRSA infection

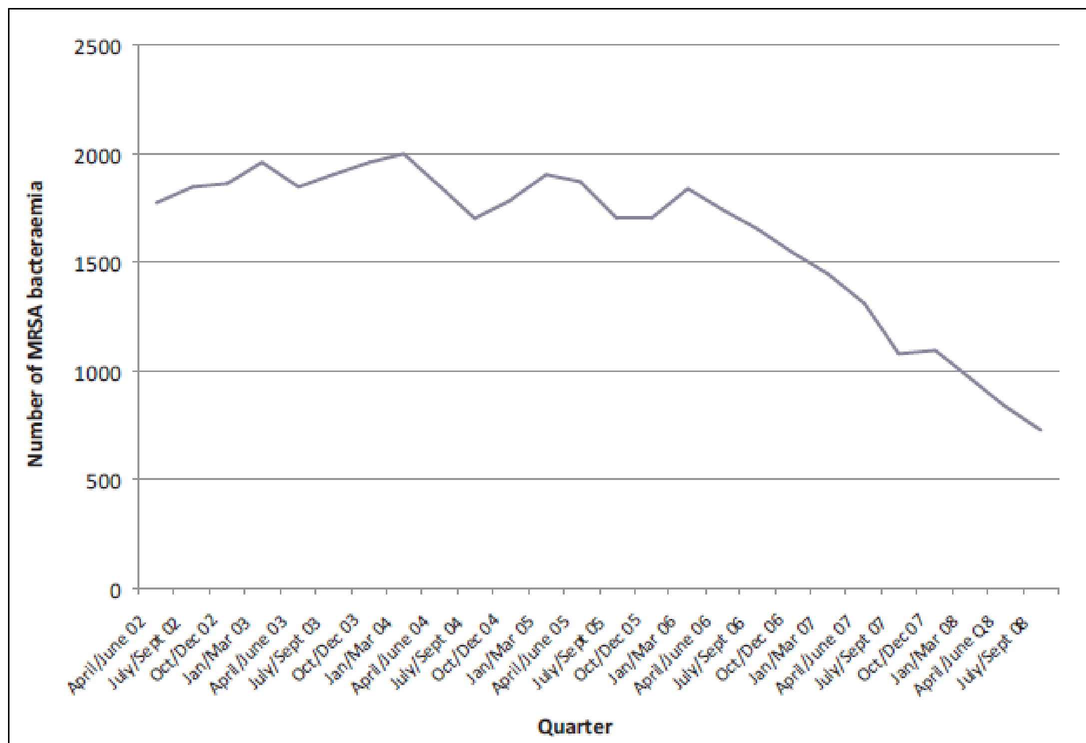
According to the British National Formulary (BNF) the recommended guidelines for MRSA treatment depends on the sensitivity of the strain. Tetracyclines are recommended for skin and soft tissue infections and urinary tract infections (British National Formulary, 2013). Glycopeptides are recommended for pneumonia and septicaemia (British National Formulary, 2013). Other factors influencing treatment include local policy, the patient's risk to others, the patient's own risk factors (for example, immunosuppression) and whether a patient is infected or colonised with MRSA (Royal College of Nursing, 2005).

1.2.1.4 Surveillance of MRSA bacteraemia

Figures 1.3 - 1.6 show data released by the Health Protection Agency (HPA, now Public Health England, PHE) regarding the number of MRSA bacteraemia cases over the past several years. The latest data to date are shown. Since April 2001 the Department of Health (DH) instructed all English acute NHS hospital Trusts to annually publish the number of cases of MRSA bloodstream infections (NAO, 2009). In 2004 the DH set a target of reducing MRSA infections by 50 % by 2008 and extended surveillance across all acute and foundation NHS Trusts (HPA, 2009). In 2005 the DH requested monthly figures of MRSA bacteraemia cases to be released to provide comprehensive data on MRSA and encourage high infection control. (Website 3, 2005). Currently data are published quarterly.

Figure 1.3, taken from a 2009 HPA report to the NAO, shows the number of MRSA bacteraemia cases collected by mandatory surveillance from 2002 to 2008. The number of bacteraemia cases peaked in the October/December 2003 period at 2000. The average number of bacteraemia cases in 2003/4 was 1925; this figure was used by the DH as a baseline figure for reduction targets. By the first quarter of 2008 there was a 57 % reduction in reported bacteraemia cases; thus meeting the DH's target of 50 % (HPA, 2009).

Figure 1.3 – Graph showing the number of MRSA bacteraemia cases reported to the mandatory surveillance system from April 2002 to September 2008 (HPA 2009b)



Quarterly counts of Trust-apportioned reports and all other reports of MRSA bacteraemia cases from the third quarter of 2008 to the third quarter of 2010 are shown in Figure 1.4. Over this reporting period there was a 46 % decrease in the overall number of MRSA bacteraemia cases. There was a 52 % decrease in Trust-apportioned reports and a 38 % reduction in all other reports (HPA, 2010).

Figure 1.4 - Quarterly counts of Trust-apportioned and all other reports of MRSA bacteraemia cases from July 2008 to September 2010 (HPA 2010)

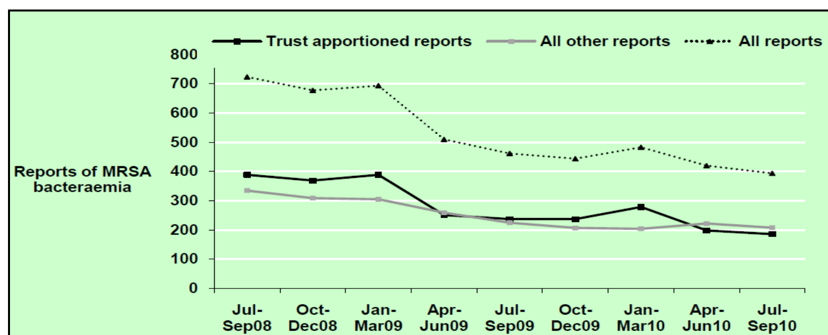


Figure 1.5 shows the continued general trend is a decrease in the number of MRSA bacteraemia cases between the reporting period of July 2009 to September 2011. Overall the HPA states there has been a 56.1 % decrease in the number of Trust-apportioned reported cases of MRSA bacteraemia between July 2009 to September 2011. All other reports presented a 28.4 % decrease in reported cases (HPA, 2011).

Figure 1.5 - Graph showing the number of Trust-apportioned reports and all other reports of MRSA bacteraemia cases between July 2009 and September 2011 (HPA 2011)

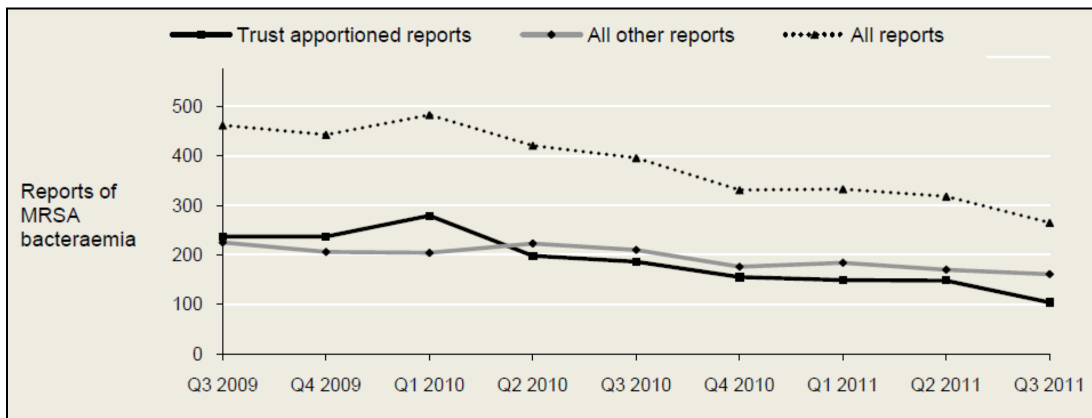
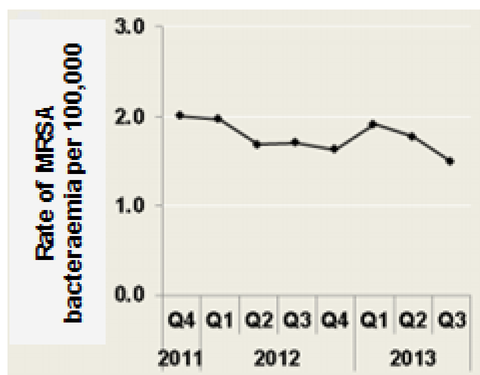


Figure 1.6 shows the reported cases of MRSA bacteraemia per 100,000 population, as published by PHE between the final quarter of 2011 to the third quarter of 2013. As between 2008 - 11 the continued trend is a decrease in the number of cases of MRSA bacteraemia. From the third quarter of 2012 to the third quarter of 2013 there was a 12 % decrease in MRSA bacteraemia cases (PHE, 2013a)

Figure 1.6 – Graph showing all reports of MRSA bacteraemia cases from the final quarter of 2011 to third quarter of 2013 (PHE, 2013a)

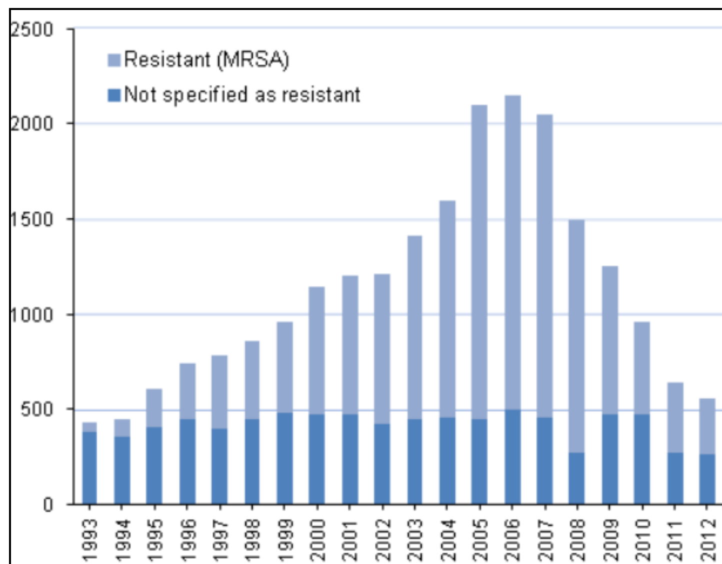


The HPA states it is difficult to determine exactly what has caused the fall in MRSA bacteraemia but they believe improved screening and decolonisation of patients, individual Trust guidelines and national campaigns such as ‘cleanyourhands’ (see section 1.6.1.7) are all contributing factors (HPA, 2009).

1.2.2 Deaths related to *S. aureus* including MRSA

Figure 1.7, produced by The Office for National Statistics, reports the number of deaths where *S. aureus* is mentioned on death certificates in England and Wales between 1993 and 2012. The graph includes data for *S. aureus* and MRSA. The highest number of deaths involving *S. aureus* or MRSA was in 2006. From 2007 to 2011 a five-fold decrease in the number of deaths involving MRSA occurred (Website 4, 2012). In 1993 12 % of deaths were due to MRSA, however by 2012 this figure rose to 52 %. Between 2011 and 2012 there was 20 % decrease in deaths due to MRSA. Overall between 2008 and 2012 only 0.2 % of hospital deaths were related to MRSA (Website 5, 2012).

Figure 1.7 – Number of deaths in England and Wales between 1993 and 2012 due to *S. aureus* (Website 5, 2012)



1.3 *Acinetobacter baumannii*

A. baumannii belongs to the *Acinetobacter* genus, which are short, strictly aerobic, non-motile, Gram-negative bacilli (Elliot *et al*., 2007; Perez *et al*., 2007). Natural habitats for *Acinetobacter* spp., such as *Acinetobacter johnsonii* and *Acinetobacter lwoffii* include human skin flora, water, soil and vegetables (Peleg *et al*., 2008). There are misconceptions that *A. baumannii* can be found in the above mentioned environments, however, its natural habitat is not yet fully understood (Peleg *et al*., 2008).

Due to its multidrug-resistant (MDR) nature *A. baumannii* is a nosocomial pathogen and the source of major outbreaks in healthcare settings (Perez *et al*., 2007). Risk factors for *A. baumannii* colonisation and infection include ICU (intensive care unit) stay, surgery, invasive procedures, antimicrobial therapy, prolonged hospital stay and mechanical ventilation (Perez *et al*., 2007). *A. baumannii* infection can result in urinary tract infections, bacteraemia, pneumonia, meningitis and wound infections (Maragakis and Perl, 2008). Other studies have shown that central nervous system, skin and soft tissue and bone infections are associated with *A. baumannii* infection (Peleg *et al*., 2008).

1.3.1 Resistance of *A. baumannii* to antibiotics

The resistance of *A. baumannii* to many antibiotics (MDR) has made its control and treatment difficult (Maragakis and Perl, 2008). *A. baumannii* is resistant to a wide range of antimicrobial classes. Its mechanisms of antibiotic resistance include the production of β-lactamases to breakdown β-lactam antibiotics such as pencillin, cephalosporins and carbapenems (Maragakis and Perl, 2008). Changes in outer membrane proteins, such as CarO, a 29-kDa protein, have been linked to imipenem and meropenem resistance (Limansky *et al*., 2002; Mussi *et al*., 2005; Siroy *et al*., 2005). The modification of target binding sites is another mechanism of resistance. For example, *gyrA* mutations, which codes DNA gyrase and *parC* mutations in the ParC subunit of topoisomerase IV affect the binding of quinolone antibiotics (Vila *et al*., 1997; Seward and Towner, 1998; Hamouda and Amyes, 2004). Another example of altered target site is 16S rRNA methylation by aminoglycoside-modifying enzymes that inhibit aminoglycoside

antibiotics such as gentamicin from binding to their target (Doi and Arakawa, 2007). Aminoglycosides normally impair protein synthesis by binding to 16S rRNA regions within 30S ribosomal subunits (Doi and Arakawa, 2007). *A. baumannii* encodes a resistance nodulation division (RND) efflux pump called AdeABC that confers multidrug resistance to β-lactams, aminoglycosides, and tetracyclines (Marchand *et al*., 2004; Nemec *et al*., 2007; Peleg *et al*., 2007).

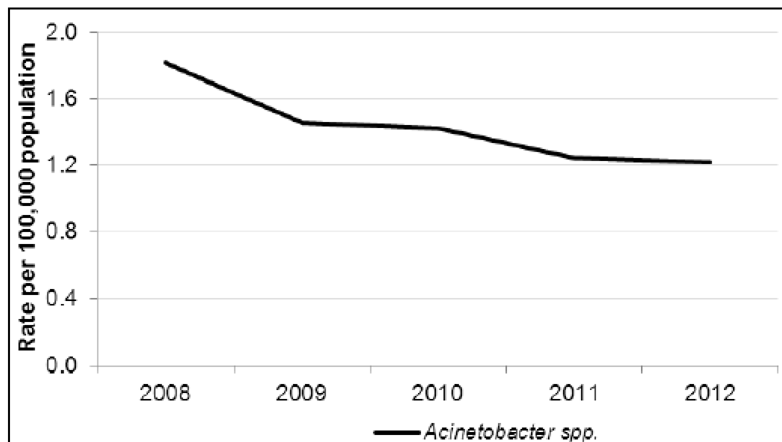
1.3.2 Treatment of *A. baumannii* infection

There are currently no guidelines for the treatment of *A. baumannii* infection in the BNF. Peleg *et al*. (2008) suggest carbapenems are used for the treatment of serious *A. baumannii* infections. However, due to the emergence of carbapenem-resistance they recommend newer approaches. Sulbactam is a β-lactamase inhibitor that is normally combined with β-lactams such as ampicillin or cefoperazone. Polymixins, for example colistin, are antimicrobial peptides that are normally administered via a nebuliser. Their target site is anionic lipopolysaccharide molecules in the outer membrane resulting in membrane damage and osmotic imbalance. Combination therapy, including the administration of colistin with other antimicrobials that act in synergy, is also recommended (Peleg *et al*., 2008).

1.3.3 Surveillance of *Acinetobacter* spp. bacteraemia

Unlike for MRSA, surveillance of *Acinetobacter* spp. is voluntary. Figure 1.8, produced by PHE, shows the rate per 100,000 population of reported *Acinetobacter* spp. bacteraemia in England, Wales and Northern Ireland from 2008 to 2012. Over this reporting period there was a 33 % decrease in the number of cases. In 2008 66 % of cases were identified according to their species. By 2012 72 % of cases were species-identified. The majority of species each year were *Acinetobacter calcoaceticus/baumannii* (31 % in 2008, 23 % in 2012) (PHE, 2013b).

Figure 1.8 – Graph showing rate per 100,000 population of *Acinetobacter* bacteraemia in England, Wales and Northern Ireland from 2008 to 2012 (PHE, 2013a)



1.4 *Clostridium difficile*

C. difficile belongs to the genus *Clostridium*. *C. difficile* is an anaerobic, spore-forming, Gram-positive bacillus (Murray *et al*., 2007). It was first isolated from healthy newborn babies in 1935 and known as *Bacillus difficilis*, however, it was not until the late 1970s that its clinical manifestations were recognised (Aktories and Wilkins, 2000; Voth and Ballard, 2005).

1.4.1 *C. difficile* infection

In the majority of cases *C. difficile* infection is linked to antibiotic usage, which disrupts the normal gut flora (Borriello, 1998). Upon *C. difficile* exposure and colonisation toxins are released and cause damage to the colonic musoca (Kelly and LaMont, 1998). Two toxins are responsible for clinical disease; Toxin A and Toxin B (Borriello, 1998). *C. difficile* is associated with hospital-acquired diarrhoea; ranging from mild to severe watery diarrhoea with abdominal pain (Borriello, 1998). In serious cases of infection a life-threatening condition called antibiotic-associated pseudomembranous colitis may develop (Murray *et al*., 2007). The onset of symptoms may occur around 4 - 10 days from the start of antibiotic treatment. *C. difficile* may be transmitted via the faecal-oral route or by contaminated surfaces (Murray *et al*., 2007).

Risk factors associated with *C. difficile* infection include increasing age, intensive therapy unit (ITU) stay, length of hospital stay and multiple antibiotic usage (Bignardi, 1998; Aktories and Wilkins, 2000). Other drugs related to *C. difficile* infection include cytotoxic drugs, antacids and stool softeners (Aktories and Wilkins, 2000).

1.4.2 Treatment of *C. difficile* infection

If possible *C. difficile* infection can be treated by discontinuing antibiotics in order to restore the body's normal gut flora (Kelly and LaMont, 1998). The administration of drugs to treat *C. difficile* is based on the severity of the infection. Metronidazole is given to treat mild and moderate *C. difficile* infection and vancomycin is administered for the treatment of severe infections (HPA, 2009a; British National Formulary, 2013). Metronidazole is the preferred first line treatment due to costs and the emergence of VRE (vancomycin resistant Enterococci) (Al-Nassir *et al.*, 2008). One method used to restore the body's flora is faecal bacteriotherapy, also known as faecal transplant; microflora from a healthy donor is transplanted to an infected patient (Floch, 2010). This procedure was first tested in 1958 by Eiseman *et al.* (1958) and produced a 100 % success rate. There are several advantages of faecal bacteriotherapy; the treatment requires minimal technology, it is cheaper than antimicrobial therapy and eliminates the risk of the antibiotic resistance developing (Bakken, 2009).

1.4.3 Surveillance of *C. difficile* infection

As with MRSA it is mandatory for NHS Trusts to publish quarterly the number of cases of *C. difficile* infections. Similarly to MRSA the number of reported cases of *C. difficile* infection is decreasing. Surveillance of *C. difficile* began in 2004 based on toxin-positive stool samples in patients aged 65 or above. Enhanced surveillance in 2007 included *C. difficile* toxin-positive results in patients aged 2 and above (HPA, 2009a). More recent guidance for reporting *C. difficile* infection were outlined in 2012 and include; toxin-positive diarrhoeal stools, toxin-positive toxic megacolon or ileostomy, pseudomembranous colitis, colonic histopathology showing typical signs of *C. difficile*

infection and samples collected post-mortem including toxin-positive stool (Website 6, 2012).

Figure 1.9 shows the data collected from voluntary and mandatory surveillance between 2000 and 2008 (HPA, 2009b). Voluntary surveillance showed an overall increase in *C. difficile* infections up to 2007 before a decrease in the number of cases. Since the introduction of mandatory surveillance in 2004 the number of cases has fluctuated; seasonal peaks are apparent during winter months. Between 2006 and 2008 there was a 41 % decrease in the number of reported *C. difficile* infections (HPA, 2009b).

Figure 1.9 – Voluntary and mandatory surveillance data of *C. difficile* infections from 2000 to 2008 (HPA, 2009b)

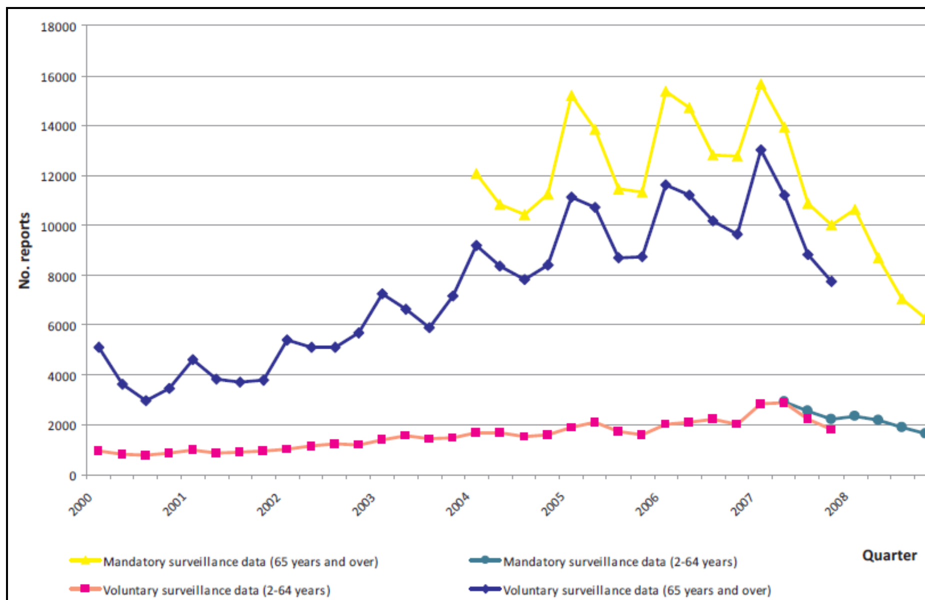


Figure 1.10 shows the quarterly number of reported cases of *C. difficile* infection between 2008 and 2011. Overall the number of reports has decreased over time, but seasonal increases are apparent (HPA, 2011).

Figure 1.10– Graph showing Trust-apportioned reported and all other reported cases of *C. difficile* infection (HPA 2011)

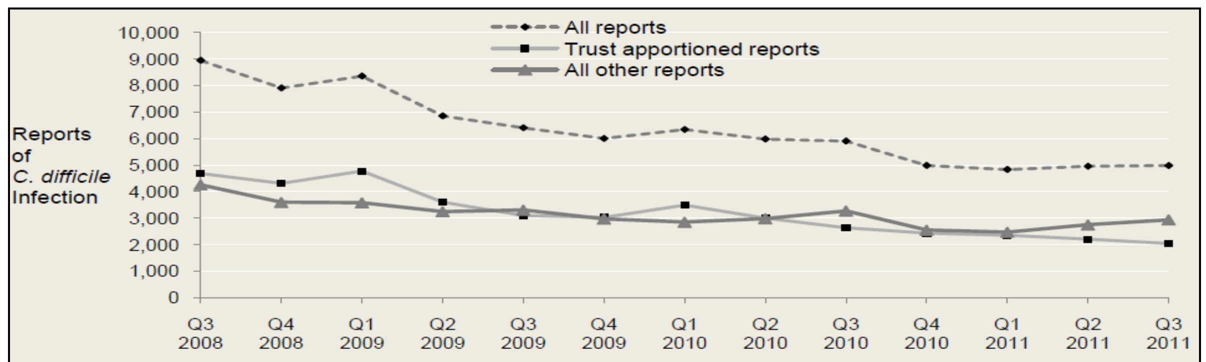
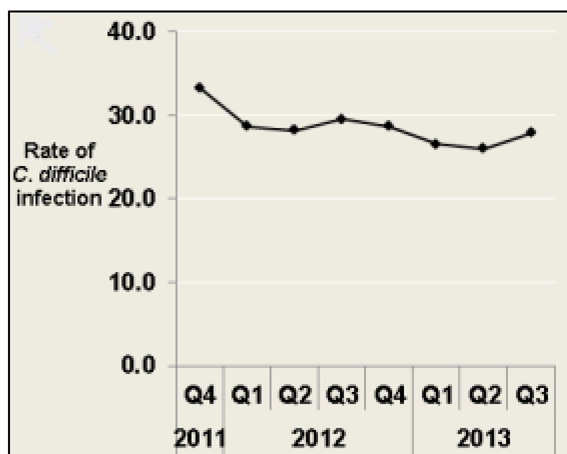


Figure 1.11 shows the surveillance data, from the final quarter of 2011 to the third quarter of 2013, published by PHE. Over this surveillance period cases of *C. difficile* infection decreased by 27.2 %, despite peaks in the third quarter of both 2012 and 2013 (PHE, 2013a).

Figure 1.11 - Graph showing all reports of *C. difficile* infections from end July 2011 to September 2013 (PHE, 2013a)

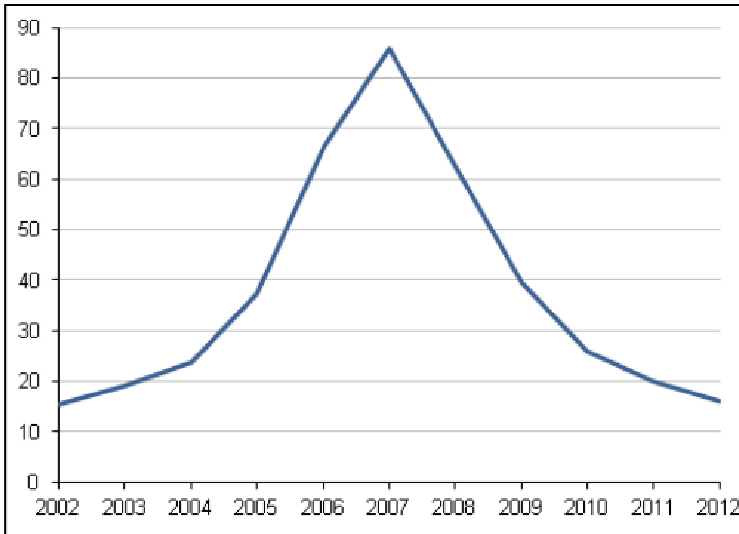


1.4.4 Deaths related to *C. difficile*

Figure 1.12 shows the mortality rate per 1,000,000 population for deaths that mentioned *C. difficile* on death certificates. The number of deaths increased between 2004 and 2007 from 2238 to 8324. Since its peak in 2007 the number of deaths relating to *C.*

difficile is continuously decreasing with 1646 deaths recorded in 2012 (Website 7, 2013).

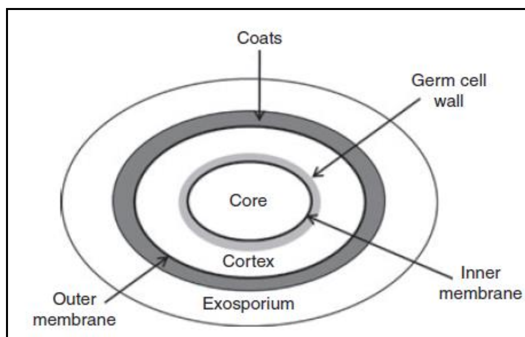
Figure 1.12 – Mortality rate per 1,000,000 in England and Wales related to *C. difficile* infection between 2002 and 2012 (Website 7, 2013)



1.4.5 *C. difficile* spores

The production of spores aids the transmission and survival of *C. difficile* (Burns *et al*., 2010). Spores are highly resistant to antimicrobial agents, which is due to their morphological phenotype (Russell, 1990). The structure of a bacterial spore is represented in Figure 1.13.

Figure 1.13 – Diagram showing bacterial spore structure (Leggett *et al*. 2012)



Most research regarding sporulation and germination has been studied in *Bacillus subtilis* (*B. subtilis*) but the basic processes are applicable to *C. difficile* and are described in section 1.5.1 The process of sporulation involves the transition of a vegetative cell to a spore (Russell, 1990). Germination is the converse; a spore irreversibly loses it properties and becomes a vegetative cell (Burns *et al*., 2010).

1.5 *Bacillus subtilis*

B. subtilis is a member of the *Bacillus* genus. *B. subtilis* is a Gram-positive, rod-shaped, spore-forming, aerobic microorganism (Elliot *et al*., 2007). *B. subtilis* can be found in the air, water and soil (Website 8, 1997). The US Environmental Protection Agency (US EPA) considers *B. subtilis* a safe microorganism and not toxigenic or pathogenic to humans, animals and plants (Website 8, 1997). The spore structure outlined in Figure 1.13 is applicable to spores of *B. subtilis.* Other members of the *Bacillus* genus that are notable pathogens include *B. cereus*, a food poisoning agent and *B. anthracis*, which is the causative agent of anthrax (Elliot *et al*., 2007).

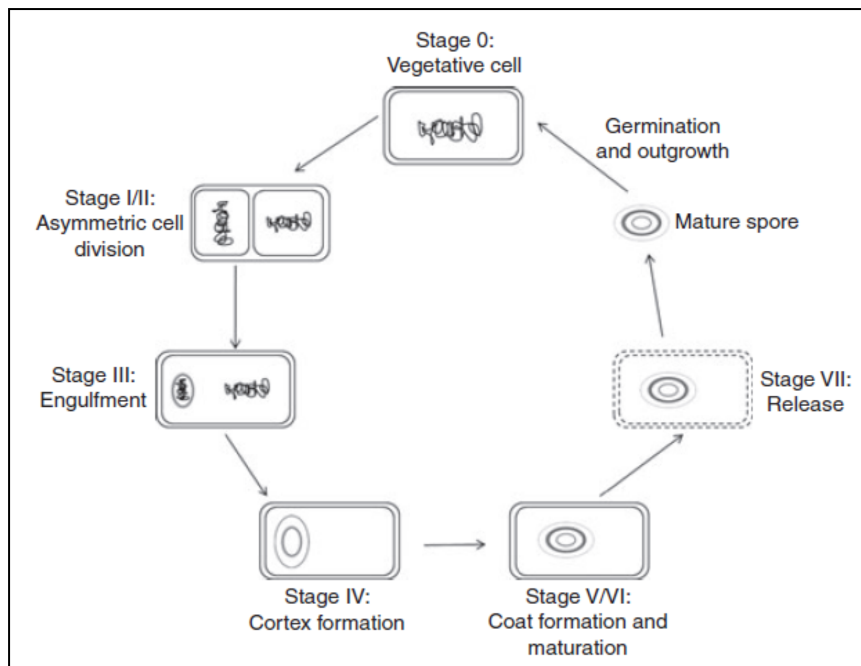
1.5.1 Sporulation and germination

Sporulation is triggered by nutrient starvation (Errington, 1993). The process of sporulation has most widely been studied in *B. subtilis* and the stages of the process are described in Figure 1.14, taken from a review article by Leggett *et al*. (2012). At Stage 0 the cell is vegetative. It then goes through asymmetric cell division; splitting into two cells that are separated by a septum (Stage I/II). The smaller of the two cells is called the prespore. During the next stage (Stage III) the prespore is engulfed by the larger, mother cell to become a forespore. At this stage there are distinct inner and outer membranes. Next, the spore cortex (Stage IV) forms between the inner and outer membranes. After this the spore coat forms and matures (Stages V and VI), before release from the mother cell (Stage VII) (Leggett *et al*., 2012).

The process of germination is triggered by nutrients named germinants, such as a 1:1 chelate of Ca^{2+} and dipicolinic acid (DPA), sugars, amino acids and purine nucleosides

(Paidhungat and Setlow, 2000; Setlow, 2003). Non-nutrients, such as high pressure, can also trigger germination (Paidhungat *et al*., 2002). Once in the presence of germinants spores begin germination and outgrowth (Leggett *et al*., 2012). During spore germination cations and Ca^{2+}-DPA are released, the cell becomes hydrated, the cortex is hydrolysed, the core expands and the resistant and dormant properties of the spore are lost (Setlow, 2003). During outgrowth spore metabolism, macromolecular synthesis, spore swelling, escape of spore coats and escape and growth of the new cell in the vegetative form take place (Setlow, 2003; Leggett *et al*., 2012).

Figure 1.14 – Diagram showing the stages of the sporulation process of vegetative cells (Leggett *et al*. 2012)



1.6 Infection control in UK hospitals

The DH states the main aims of cleaning are ‘to remove surface soil to achieve an aesthetically pleasing environment, contributing to patient safety and confidence, to remove the microorganisms that spread disease and to remove harmful chemical residues, and the dust, dirt and grime upon which microorganisms may feed and grow’

(DH, 2007a).

The NHS produced a guidance document in 2007, ‘The national specifications for cleanliness in the NHS: a framework for setting and measuring performance outcomes’ detailing its cleanliness standards in NHS hospitals. It states ‘providing a clean and safe environment for healthcare’ is a key priority. The comprehensive guidance document details areas and items in hospital wards that necessitate cleaning, the standard to which they should be cleaned and how frequently. The document lists items of close or direct contact to patients, fixed assets, flooring, electrical items, furnishing, kitchen items and bathrooms that should be cleaned completely showing no visible blood, body substances, dust, dirt, debris or spillages. The guidelines highlight the minimum cleaning frequency of very high-risk, high-risk, significant-risk and low-risk areas. For example, if a door is considered a very high- to significant-risk, daily cleaning is recommended compared to weekly recommended cleaning if considered low-risk (DH, 2007a).

In addition to these guidelines an NHS guidance document titled ‘Standard Infection Control Precautions’ details more instructions for hospitals regarding infection control. It describes areas that require cleaning, referred to as the ‘environment’, to include frequently touched surfaces in all rooms (e.g. treatment, physiotherapy, sluice, store, patient changing and dental/doctor surgeries), any horizontal surfaces in the patients environment, furniture (e.g. beds, trolleys, chairs, bedside televisions), toilets and commodes, items in shower rooms (e.g. sinks, baths, showers), floors, doors and door handles, paintwork, curtains, screens, window blinds, light fittings and switches and kitchen areas (NHS, 2010).

1.6.1 Current cleaning and infection control measures in UK hospitals

Cleaning schedules and cleaning agents are normally determined by each hospital Trust under its local policies. The NHS recommends cleaning takes place in accordance with local policy and when surfaces are visibly dirty, after spillages and when a patient is discharged (NHS, 2010). Described in sections 1.6.1.1 to 1.6.1.6 are currently used

surface cleaning measures across NHS hospitals. The NHS recommends audits are carried out regularly to monitor cleaning in hospitals and assess performance (NHS, 2007). The proposed recommendation, after cleaning, for surface contamination on hospital surfaces is <5 colony forming units per centimetre squared (cfu/cm^2) (Dancer, 2004) but others have suggested <2.5 cfu/cm^2 (Griffith *et al*., 2000).

1.6.1.1 Liquid disinfection

Liquid disinfection is the most commonly used method of surface disinfection and is normally applied to surfaces via mops, cloths and wipes. As with general cleaning guidelines, the cleaning agent of choice is normally determined by individual Trusts, however the NHS does have basic recommendations. It states 'the choice of cleaning agent that best meets overall needs is important and should be included in local cleaning procedure' (NHS, 2010). In addition, it recommends general detergents for routine cleaning as opposed to disinfectants. It discourages the use of chlorhexidine for disinfection and alcohol and detergent wipes for large surface cleaning (NHS, 2010). The guidance document states cleaning solutions must be prepared according to manufacturers' guidelines and kept for a maximum of 12 hours (NHS, 2010).

A disinfectant can be defined as 'a product capable of chemical disinfection, which is a reduction of the number of microorganisms in or on an inanimate matrix, achieved by the irreversible action of a product on their structure or metabolism, to a level judged to be appropriate for a defined purpose'. This definition is taken from EN 14885:2006 'Chemical disinfectants and antiseptics - Application of European Standards for chemical disinfectants and antiseptics', which is the standard to which products have to conform to in order to support claims for antimicrobial activity.

Whilst liquid disinfection has its obvious advantages in that it is quick, effective and easy, there are some disadvantages too. Firstly, the product needs to be compatible with the intended surface of use as some products, particularly chlorine-containing ones may have corrosive properties (Otter *et al*., 2011). Secondly, some products may be hazardous (e.g. chlorine-based products), thus they need to be handled with care and used appropriately according to Material Safety Data Sheets (MSDS) and local rules

(Otter *et al*., 2011). The shelf-life and stability of products varies so products must be used carefully according to manufacturers' guidelines. In addition, since some products need to be prepared in solution it is important staff are properly trained to ensure that products are prepared correctly and at the required concentration. Finally, a limitation of liquid disinfection is the potential for transfer of bacteria from one surface area to another, depending on what is used to apply the disinfectant to a surface. A study by Williams *et al*. (2009) highlighted this as a potential problem and suggest a 1 wipe, 1 application per surface policy.

1.6.1.2 Microfibre cloths and mops

Microfibre cloths and mops are composed of synthetic fibres and are used for general cleaning (DH, 2007a). In comparison to woven cloths they are very fine and have a much larger surface area, which allows cleaning inside crevices and cleaning of microscopic debris (DH, 2007a; National Patient Safety Agency, 2009). The natural static charge on microfibre cloths allows for the removal of dirt by electrostatic attraction and more is removed than by normal cloths and mops (DH, 2007a; National Patient Safety Agency, 2009). Advantages of microfibre cloths are they are strong and do not tear, snag or lint when used correctly (DH, 2007a). They are ergonomic too due their low weight and are machine washable (75-100 washes) making them re-usable (DH, 2007a; Sattar, 2010). In addition, hospitals have reported a decrease in cleaning time due to the use of microfibre cloths (National Patient Safety Agency, 2009). A study by Smith *et al*. (2011) found microfibre cloths were effective at removing microorganisms. Ten different microfibre cloths were tested against a range of surfaces (stainless steel, white ceramic glazed tiles and furniture laminate) inoculated with *E. coli*, MRSA and *C. difficile*; a range of approximately >1 – 3 $\log_{10}$ reductions were observed (Smith *et al*., 2011). A disadvantage of microfibre cloths is they are only to be used dry or with dampened water; biocidal agents must not be added since they can degrade the fibres (DH, 2007a). Therefore, they should only be used for general cleaning and not for disinfection or removal of bodily fluids (DH, 2007a). Another disadvantage is the cloths will snag if used over an old or damaged surface (National Patient Safety Agency, 2009). The Revised Healthcare Cleaning Manual suggests they are used for general maintenance of surfaces (National Patient Safety Agency, 2009).

Finally, microfibre cloths must be used in a manner that reduces the spread of bacteria to cleaned areas, for example, it is recommended the least contaminated area should be wiped first (Bergen *et al*., 2009).

1.6.1.3 Antimicrobial wipes

Antimicrobial wipes are wipes that are pre-soaked with disinfectant (Sattar and Maillard, 2013). A range of factors influence the efficacy of surface cleaning by antimicrobial wipes, such as; type and frequency of wiping, pressure applied to the wipe, types of target microorganisms and the ratio between the disinfectant and the wipe. Studies have shown that whilst antimicrobial wipes were effective at reducing microbial burden on surfaces, they may be implicated in microbial transfer if re-used (Williams *et al*., 2009). Similarly to microfibre mops and cloths use, 'one wipe – one application – one direction' is recommended for antimicrobial wipes to prevent bacterial transfer (Siani *et al*., 2011).

1.6.1.4 Fumigation

Hydrogen peroxide vapour (HPV) fumigation is a relatively new cleaning technology. It works by vapourising 30 % w/w aqueous hydrogen peroxide that reaches a concentration of 0.2 mg/L (DH/NHS, 2009; National Patient Safety Agency, 2009). Vapourised hydrogen peroxide is deposited on to surfaces then converted to oxygen and water (Boyce *et al*., 2008). The system used in NHS hospitals in the Bioquell model (DH/NHS, 2009). The Revised Cleaning Manual suggests HPV is used during periods of infection outbreaks (National Patient Safety Agency, 2009).

An advantage of using HPV is that it is has proved to be effective against pathogens and is sporicidal against *C. difficile* spores, which are normally resistant to routinely used cleaning products (Boyce *et al*., 2008; National Patient Safety Agency, 2009; Otter and French, 2009). A disadvantage of HPV is that rooms need to be emptied and sealed before treatment since the vapour is toxic (National Patient Safety Agency, 2009). Since wards need to be evacuated prior to cleaning HPV is most likely only suitable for terminal cleaning (Weber and Rutala, 2012). In addition surfaces must be positioned correctly to receive the vapour (DH/NHS, 2009). Another disadvantage is the process is

time-consuming. For example, a patient room can take 3 - 4 hours, whilst an entire ward up to 12 hours (Boyce *et al.*, 2008). In addition, surfaces must be cleaned beforehand since the vapour cannot pass through organic loads, thus HPV treatment must take place alongside normal cleaning (National Patient Safety Agency, 2009). Finally, the cost of the equipment and engineers is high; for example, a managed service by Bioquell is £15,000/month for the use of three vapourisers, assistance from engineers and consumables (DH/NHS, 2009).

A study by Malinowska and Holah (2010) found the efficacy of HPV depends on the hydrogen peroxide concentration utilised; at low concentrations (10 - 20 g/m^3) HPV was less effective than at higher concentrations (30 - 40 g/m^3) against both *Listeria monocytogenes* and *P. aeruginosa* (≤3 log_{10} reductions compared to >4 log_{10} reductions). However, an increase in hydrogen peroxide concentration had no significant effect on *S. aureus* viability, which the authors suggest may be due to aggregation. In addition, all microorganisms tested in this study are catalase-positive, which may explain the resistance observed at low hydrogen peroxide concentrations (Malinowska and Holah, 2010).

1.6.1.5 Steam cleaning

Steam cleaning works by delivering superheated, pressurised dry steam to surfaces. The steam acts as both a cleaning and disinfectant product. Steam is a good cleaner as it is able to remove dirt and grease that is later vacuumed and the high temperature (>140 °C) is effective at killing pathogens (DH, 2007a; National Patient Safety Agency, 2009). In Scotland steam cleaners have been issued to every NHS Board for the cleaning of beds and curtains (NHS Scotland, 2009). The DH recommends steam cleaning for periodic deep-cleaning as opposed to daily, routine cleaning (DH, 2007a). An advantage of steam cleaning is that it can be used to disinfect areas that are normally difficult to clean, for example, between bed rails and crevices (DH, 2007a). A disadvantages of the use of steam cleaners includes the cost of the machinery; it is estimated that steam cleaners cost up to £2200 (DH, 2007a). In addition, staff training is essential for the correct use of the machinery (DH, 2007a).

1.6.1.6 Combined detergents and disinfectants

The use of products containing both detergent and disinfectant agents are more recently being used in favour of two separate products. A surfactant is added to a detergent combined with a chlorine-based product to allow chlorine release. Chlorine concentration is usually at 1000 parts per million (ppm) (National Patient Safety Agency, 2009). These dual function products are mostly used for terminal cleaning but some hospitals utilise them for normal cleaning (National Patient Safety Agency, 2009). A major advantage of this technology is that is it less time consuming than using a detergent first followed by a disinfectant. A disadvantage is the health hazards associated with chlorine. The Revised Cleaning Manual suggests rooms are well ventilated prior to cleaning (National Patient Safety Agency, 2009).

As an example, the Cardiff and Vale University Health Board's policy is as follows; Actichlor Plus (a dual product containing detergent and a disinfectant; sodium hypochlorite at 1000 ppm) is used daily on all inpatient wards or twice daily if there are infected patients or outbreaks. This product is also used for cleaning patient equipment when patients are known to have an infection and on all commodes and beds after patient discharge. For all other areas a general purpose detergent is used (personal communication from Senior Nurse for Infection Prevention and Control at University Hospital of Wales, Cardiff).

1.6.1.7 Control of airborne microorganisms

The control of airborne microorganisms is described in detail in Chapter 4, section 4.1.4.

1.6.1.8 Other infection control measures

The importance of good hand hygiene to staff, patients and visitors is strongly emphasised as a major part of infection control and as a vital factor in reducing HCAIs (NAO, 2009). The NHS Standard Infection Control Precautions guidance document states most contamination from surfaces to patients and HCWs is by hand (NHS, 2010). Many national campaigns have raised awareness of the importance of good hand hygiene. The National Patient Safety Agency introduced two campaigns;

‘cleanyourhands’ in 2004 and ‘Clean Hands Saves Lives’ in 2008. The former was to install alcohol hand gels in all wards and promote good hand hygiene amongst HCWs (NAO, 2009). The NAO considers the ‘cleanyourhands’ campaign to be successful in two ways; it has been a cost-effective in terms of improving hand hygiene and it states there has been a subsequent reduction in the number of MRSA cases. The ‘Clean Hands Save Lives’ campaign was launched in 2008 to re-emphasise the importance of hand hygiene, particularly at the point of patient care (National Patient Safety Agency, 2010).

An MRSA screening programme is present in all NHS hospitals; all patients for elective and emergency admissions are screened (Website 9, 2008). Swabs are collected from patients (primarily from the nose, however the armpit or groin are other areas of *S. aureus* colonisation) and sent to laboratories for analysis. If a MRSA-positive result is observed the patient is normally treated and kept in isolation or separated from MRSA-negative patients (NHS, 2006).

For *C. difficile* infections the HPA and DH recommend patients are kept in isolation until they do not present diarrhoea for a minimum of 48 hours (HPA, 2009a). The HPA also recommends that rooms or bed spaces of patients infected with *C. difficile* are cleaned thoroughly and daily with cleaning products containing at least 1000ppm available chlorine (HPA, 2009a).

Fomites, from the Latin *fomes*, is a term used to describe inanimate objects with the ability to carry infection (Salcido, 2007; Tacconelli, 2011). In the healthcare environment objects such as doctors’ white coats, stethoscopes, pagers, patient charts, curtains to separate beds and tables have been identified as vectors for the transmission of pathogens (Huang *et al.*, 2006; Treakle *et al.*, 2009; Pimental, 2011; Tacconelli, 2011). Mobile phones and other new technological devices also have the potential to transmit microorganisms (Tacconelli, 2011). Surfaces are another example of fomites; contaminated surfaces are a cause of cross-contamination between patients and HCWs (Treakle *et al.*, 2009; Tacconelli, 2009). A study by Desai *et al.* (2011) investigated the survival and transmission of MRSA from fomites. Porous fomites such as towels, shoulder pads and bed sheets and non-porous fomites including ceramic, plastic toys and vinyl were subject to the test. The study showed MRSA can survive on fomites and

is quickly transmitted from fomites to humans. Transmissibility was greater from non-porous fomites than from porous fomites; surfaces were still contaminated up to eight weeks after initial contamination (Desai *et al*., 2011). In the UK in 2007 new guidelines by the DH discouraged clinical workers from wearing long sleeves, jewellery, watches and rings in order to promote good hygiene and prevent contamination of such objects. The new policy introduced became widely known as 'bare below the elbows' (DH, 2007b, 2010).

1.6.2 Role of cleaning in infection control

It is difficult to suggest a 'dirty' hospital ward is linked to an increase in infections due to the lack of comprehensive investigations into the cleanliness of hospitals (Dancer, 2004). Some investigations show there may be an association between contaminated surfaces and the dissemination of microorganisms. For example, a clinical study in a university hospital showed HCWs to be indirectly contaminated with MRSA after touching contaminated surfaces (Boyce *et al*., 1997).

However, there is evidence cleaning and disinfection of surfaces contributes to reduced surface contamination and transmission of microorganisms. For example, the use of HPV, as mentioned in section 1.6.1.4, possibly contributed to reduced *C. difficile* transmission; the number of cases of *C. difficile*-associated diarrhoea (CDAD) was significantly lower following the use of HPV than the period before without HPV decontamination (Boyce *et al*., 2008). In another study the use of hypochlorite solutions instead of quaternary ammonium compounds (QACs) resulted in a significant decrease of CDAD from 8.6 cases to 3.3 cases per 1000 patient-days. A reverse back to QACs resulted in an increase in CDAD rates to 8.1 cases per 1000 patient-days. The authors believe the switch to hypochlorite caused a decrease in surface contamination and subsequent transmission (Mayfield *et al*., 2000).

Dancer *et al*. (2009) suggest enhanced cleaning is sufficient to control infection rates; they found the employment of an extra cleaner on two surgical wards with endemic MRSA resulted in a 26.6 % reduction in new MRSA cases. There were nine cases of

MRSA during normal cleaning periods compared to four during the enhanced cleaning period. There was also a 32.5 % reduction in surface contamination on ten hand-touched sites including patient lockers, overbed tables and bed frames (all within patient vicinity), computer keyboard, desk and patient notes (by nurses station), patient hoist and infusion pump and blood pressure stands (clinical equipment) and door handles. The extra cleaner was instructed to clean hand-touch sites within patient vicinity two to three times a day, equipment by the nurse's station 1 - 2 times a times a day, clinical equipment 1 - 2 times a day and door handles 2 - 3 times a day. This cleaning took place alongside routine cleaning carried out by nurses. The authors suggest the addition of an extra cleaner within the two wards was cost-effective; the salary of the cleaner was less than the cost of treating MRSA infected patients throughout the period of this study (Dancer *et al*., 2009). In a different study the effect of enhanced cleaning to reduce VRE in an ICU at a university hospital was investigated. The study showed enhanced cleaning of accessible surfaces with a QAC disinfectant, education, monitoring of housekeeping and a hand hygiene campaign resulted in a significant reduction of VRE surface contamination, a decrease in VRE hand contamination amongst HCWs and a decrease in the number of patients acquiring VRE (Hayden *et al*., 2006).

Despite the above mentioned examples it is sometimes difficult to determine the link between hospital cleanliness and infection control due to a lack of standard methods (Mulvay *et al*., 2011). Current methods for determining levels of surface contamination include adenosine triphosphate (ATP) bioluminescence monitors and microbiological screening (Mulvay *et al*., 2011). ATP monitors are increasingly being used in NHS hospitals for monitoring and how they work is described in detail in Chapter 3, section 3.1.3. Briefly, a surface is swabbed, the swab is placed inside the monitor and a reading (relative light unit, RLU) is given that corresponds to the amount of ATP collected on the swab. Visual screening is also used to monitor cleanliness during audits in UK hospitals, however, this method is unreliable due to the microscopic nature of microorganisms and the fact that their presence does not necessarily equate to apparent dirt (Griffith *et al*., 2000; Dancer, 2009). For example, a study by Griffith *et al*. (2000) evaluated different methods for assessing environmental contamination and suggested visual screening is insufficient. The authors sampled 113 sites before and after cleaning across an operating theatre and surgical ward by three methods; visual screening, ATP

bioluminesence and microbiological assessment. Readings taken after cleaning showed 18 % of surfaces were unacceptable by visual screening whereas the percentages of sites deemed unacceptable by microbiological screening and ATP bioluminescence sampling were 70 % and 76 %, respectively. The authors suggest visual screening of surfaces should be undertaken more stringently, for example, if a site appears clean but is of high-risk then assessment by other means including ATP bioluminescence is recommended (Griffith *et al*., 2000).

Advantages of ATP monitors are that they provide quantitative, real-time results, they are good for staff training and their use can improve standards of cleanliness (Boyce *et al*., 2009; National Patient Safety Agency, 2009). Disadvantages include the fact that the monitor cannot differentiate between bacterial ATP and organic soiling, or between dead and live microorganisms (National Patient Safety Agency, 2009). In addition, the criteria for a 'pass' or 'fail' are not clear (National Patient Safety Agency, 2009). However, Mulvey *et al*. (2011) do recommend a value of 100 RLU to correspond with <2.5 cfu/cm^2.

1.7 Application of antimicrobial surfaces in the healthcare setting

Antimicrobial surfaces, or biocide-impregnated surfaces, are surfaces coated or impregnated with a biocide. A biocide can be defined as 'an active chemical molecule to control the growth of or kill bacteria in a biocidal product' (SCENIHR, 2009). These substances are not antibiotics since they are not intended for use during antimicrobial therapy, they provide more of a preventative measure to limit the spread of infection (Levy, 2001). In contrast to antibiotics that target specific structures or process, biocides tend to have an unspecific, multiple mode of action (Meyer and Cookson, 2010).

Common biocides incorporated into surfaces include copper, triclosan and silver and these are detailed in sections 1.8.1, 1.8.2 and 1.8.3, respectively. In addition, the use of light-activated antimicrobial agents and photosensitisers has been explored. These are discussed in section 1.9.

Antimicrobial surfaces potentially have an important role in infection control in clinical settings by controlling surface bioburden and reducing the transfer of microorganisms from surfaces to patients or HCWs (see Figure 1.15, the ‘nosocomial infection loop’) (Page *et al*., 2009).

Table 1.1 shows examples of antimicrobial surfaces and the incorporated biocide. Also, if known, the antimicrobial surface efficacy test used for testing the antimicrobial activity of surfaces is noted. The table shows antimicrobial surfaces within a clinical setting can be found in numerous areas of a typical ward or theatre room. The standard test utilised by the majority of manufacturers for determining antimicrobial activity is the ISO22196 (International Organisation for Standardisation), which derives from the Japanese Industry Standard (JIS Z 2801). The ISO22196 and JIS Z 2801 tests are described in detail in section 1.11.1.

Medical devices such as catheters, endotracheal tubes and surgical masks can be coated with biocides to prevent the dissemination of pathogens. Silver nanoparticles are an example of an effective antimicrobial coating used on such devices (Li *et al*., 2006; Roe *et al*., 2008; Monteiro *et al*., 2009). For the purpose of this study antimicrobial surfaces on hard, environmental surfaces in the healthcare setting will only be discussed.

Table 1.1 -Examples of antimicrobial surfaces, supplier name, antimicrobial agent utilised and efficacy test

Surface	Supplier	Biocide	Efficacy test (if known)	Reference
Door handle	Allgood (London, UK)	BioCote Silver Technology	ISO22196	Website 10, 2006
	Biomaster (Stafford, UK)	Silver	ISO22196	Website 11, 2011
	Ingersoll Rand (Co. Dublin, Ireland)	Silver	-	Website 12, 2008
	Microban (Huntersville, US)	Triclosan	-	Website 13, 2014
Door push plate	Aalco Ltd (Birmingham, UK)	Copper (70%)	-	Website 14, 2014
Grab rail	Allgood (London, UK)	BioCote Silver Technology	ISO22916	Website 15, publication date unavailable
Hand dryer	Dyson (Mamelsbury, UK)	BioCote Silver Technology	ISO22916	Website 16, 2006
Light switch	KME (Firenze, Italy)	Copper		Website 17, 2012
	Iles (UK)	BioCote Silver Technology	ISO22916	Website 15, publication date unavailable
Waste bin	Environmental Hygiene Products Ltd (Ross-Shire, UK)	BioCote Silver Technology	ISO22916	Website 15, publication date unavailable
Locker	Link Lockers (Telford, UK)	BioCote Silver Technology	ISO22916	Website 15, publication date unavailable
Radiator guard	Contour Castings (Telford, UK)	BioCote Silver Technology	ISO22916	Website 15, publication date unavailable

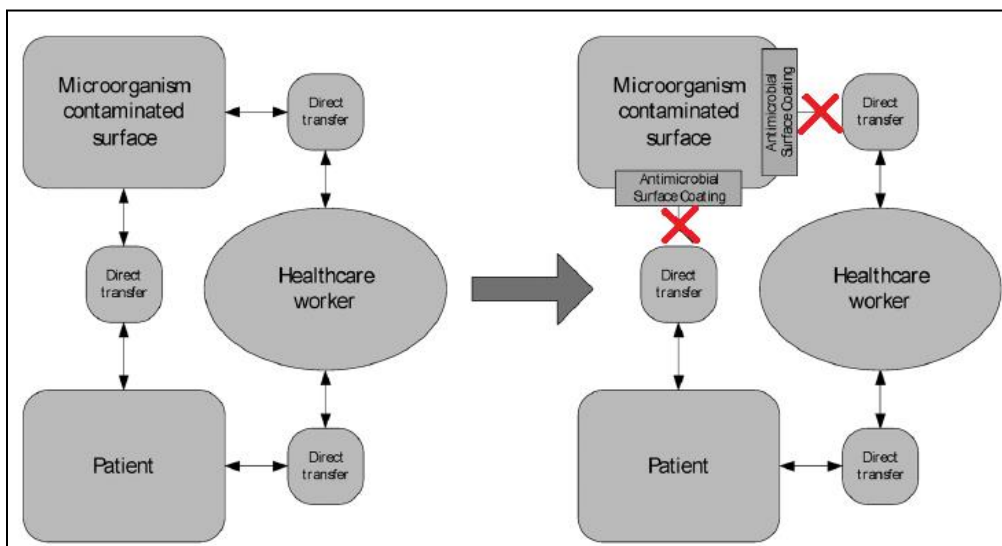
Table 1.1 continued -Examples of antimicrobial surfaces, supplier name, antimicrobial agent utilised and efficacy test

Surface	Supplier	Biocide	Efficacy test (if known)	Reference
Ceiling and wall cladding	Alvo (Śmigiel, Poland)	BioCote Silver Technology	ISO22916	Website 15, publication date unavailable
Walls	Suspended Ceiling Restoration Ltd. (Sheffield, UK)	BioCote Silver Technology	ISO22916	Website 15, publication date unavailable
Tap	Rada (Cheltenham, UK)	BioCote Silver Technology	ISO22916	Website 15, publication date unavailable
	Avilion Ltd (Birmingham, UK)	Copper (60%)	-	Casey *et al*. 2010
Toilet seat	Mercury Composite LLC (CA, USA)	Copper (70%)	-	Casey *et al*. 2010
Computer keyboard	BioSafe (Pittsburgh, UK)	Triclosan, silver	-	Website 18, 2014
Computer keyboard wrist support	Fellowes (Itasca, US)	Triclosan	-	Website 15, publication date unavailable
Over-bed table	Sidhil (Halifax, UK)	BioCote Silver Technology	ISO22916	Website 19, 2014
Healthcare furniture	Copper Development Association (Hemel Hempstead, UK)	Copper/copper alloys	-	Website 20, 2014

NB. All websites accessed February 2014

The diagram on the left hand side of Figure 1.15 represents the ‘nosocomial infection loop’, as termed by Page *et al* (2009). It shows that microorganisms can be directly transferred to and from surfaces and HCWs. If a surface is contaminated then microorganisms can be transmitted to a patient then on to a HCW or vice versa. The idea of an antimicrobial surface is that the spread of microorganisms can be controlled. The red crosses on the diagram on the right hand side of Figure 1.15 represent the desired effect of introducing antimicrobial surfaces; the transmission of microorganisms from surfaces to HCWs and patients is prevented and the ‘nosocomial infection loop’ is interrupted (Page *et al*., 2009).

Figure 1.15 – The nosocomial infection loop and the role of antimicrobial surfaces (Page *et al.* 2009)



Studies have shown that MRSA can persist on dry surfaces from anytime between seven days to seven months, and *C. difficile* spores can survive up to five months (Kramer *et al*., 2006). Indeed surfaces have been described as an ideal ‘reservoir’ for pathogens (Dancer, 2004). Antimicrobial surfaces aim to provide continuous disinfection alongside routine cleaning practices (Page *et al*., 2009; Weber and Rutala, 2013).

The advantages and disadvantages of introducing antimicrobial surfaces in healthcare settings are discussed in detail in Chapter 6, section 6.5.

1.8 Antimicrobial surfaces currently being explored for potential future use in UK hospitals

This section will discuss three antimicrobial surfaces noted in Table 1.1; copper, triclosan and silver. It can be seen that in terms of product numbers, the majority of example surfaces in Table 1.1 contain BioCote® silver antimicrobial technology; its mechanism of action is described later (section 1.9.3.1).

1.8.1 Copper

Copper, Cu, a transition metal element is a well-known antimicrobial. Copper has a wide range of domestic, industrial and agricultural uses and is in high demand for applications such as plumbing and wiring (Elguindi *et al*., 2011). It is thought that around 10 % of copper use is based solely on the antimicrobial properties of the metal (Elguindi *et al*., 2011). The Egyptians noted the use of copper for wound healing around 1500 BC in the Ebers Papyrus, an ancient Egyptian book of medical recordings (Sipos et al., 2004). Many other ancient civilisations including the Greeks and the Romans explored the antimicrobial properties of copper (Dollwet and Sorenson, 1985). Copper is used as an antimicrobial agent in agriculture; it is added to bactericides and fungicides to protect fruits and vegetables (Elguindi *et al*., 2011). Copper is utilised as an antimicrobial in water distribution and irrigation systems to prevent water-borne infections. It is also used to control algae levels in swimming pools, rivers, lakes and canals; studies have shown algae are susceptible to copper salts (Elguindi *et al*., 2011). Copper and copper alloy surfaces have recently been subject to extensive laboratory and clinical investigations to assess the biocidal efficacy of the metal as a suitable antimicrobial surface.

The Copper Development Association (CDA) is an organisation promoting the use of antimicrobial copper surfaces in healthcare settings (Website 20, 2014).

1.8.1.1 Copper-containing antimicrobial surfaces – laboratory findings

Noyce *et al.* (2006a) found pure copper is an effective antimicrobial surface in comparison to stainless steel. After 45 min exposure MRSA (NCTC 10442) was not detected on copper. Epidemic-MRSA-1 (EMRSA) and EMRSA-16 were also killed after 60 and 90 min exposure times, respectively, whereas all strains were viable after 72 h on the stainless steel surfaces. These tests were carried out at room temperature (Noyce *et al*., 2006a).

In the same study Noyce *et al.* (2006a) also investigated the use of copper alloys as antimicrobial surfaces using the same three MRSA strains mentioned above. Brass (containing 80 % copper) was compared to pure copper and stainless steel. Brass did produce an antimicrobial effect on the strains at room temperature but a significant decrease in activity was only apparent after 3 h. Complete kill of MRSA and EMRSA-1 occurred after 4.5 h suggesting that copper alloys present slower antimicrobial activity than pure copper. Additionally, EMRSA-16, the most epidemic strain in the UK, appeared to show tolerance to brass (Noyce *et al*., 2006a). Copper and copper alloys have also shown to be effective against *P. aeruginosa*, another important nosocomial pathogen (Elguindi *et al*., 2009). Complete kill was achieved after 120 min on 99.9 % and 88.6 % copper. There was no link between copper content and antimicrobial activity; an alloy containing 94.8 % copper presented the slowest activity requiring 240 min for complete kill (Elguindi *et al*., 2009).

The survival of *C. difficile* on surfaces has also been documented, although information is limited. Weaver *et al.* (2008) have shown *C. difficile* can survive on stainless steel for up to one week without a significant decrease in viability. The same study investigated the survival of *C. difficile* vegetative cells and spores (total cells) and purified spores on pure copper and copper alloy surfaces; 100 % kill was observed on pure copper in less than 24 h and on copper alloy (95 % Cu) within 48 h for both total cells and purified spores (Weaver *et al*., 2008).

As mentioned in section 1.3.1 the emergence of MDR bacteria such as *A. baumannii* is a major cause for concern. The efficacy of copper surfaces against five clinical isolates of carbapenemase-producing *A. baumannii* showed 99 % copper was bactericidal in

under 2 h and 63 % copper in 6 h. Other nosocomial isolates including *K. pneumoniae*, *P. aeruginosa*, *E. coli* and *Enterococcus* spp. were also tested. Antimicrobial activity of 99 % copper was quickest against *A. baumannii* isolates, with other complete kill times ranging from 3 to 6 h. The authors of this study suggest the use of copper antimicrobial surfaces in high-touch areas near high-risk patients may, in addition to existing infection control, potentially reduce surface bioburden and prevent the acquisition of nosocomial infections (Souli *et al*., 2013).

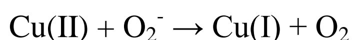
All the above mentioned findings involved the inoculation of a 20 or 25 μL droplet of bacterial suspension on to test and control surfaces. This makes all these tests 'wet inoculum' tests. The efficacy of copper surfaces against dry bacterial inocula is described later in Chapter 5, section 5.1.3.

Copper-containing surfaces may be useful in the food industry to help reduce food-borne infections. *Salmonella* is a common food-borne pathogen that can cause self-limiting gastroenteritis (Foley and Lynne, 2008). A study tested the antimicrobial efficacy of copper alloy surfaces against copper-resistant and –sensitive strains of *Salmonella*. The surfaces were tested against a wet inoculum and a wet inoculum allowed to dry. Both wet and dry inocula were killed quicker by alloys containing a high copper content than by a low copper content; although kill was slower on moist surfaces. This study also found copper alloys were more effective in the absence of organic load; it was proposed organic compounds bind to copper ions thus decreasing their bioavailability causing a reduced toxic effect on bacterial cells (Zhu *et al*., 2011).

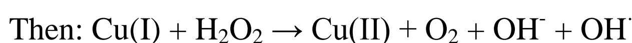
1.8.1.2 Mechanism of action

It is thought that there are several mechanisms of action of antimicrobial copper. At low concentrations, copper is an important nutrient for prokaryotic and eukaryotic organisms; it acts as a catalytic cofactor for enzymes (Pena *et al*., 1999; Faúndez *et al*., 2004; Santo *et al*., 2010). At higher concentrations, copper is toxic to living organisms as it produces highly reactive oxidative species that in turn results in damage to lipid membranes by peroxidation, conformational change to nucleic acids and proteins, alteration of osmotic homeostasis and the displacement of vital metals from their native

binding sites (Pena *et al*., 1999; Faúndez *et al*., 2004). Copper is active in two oxidative states; Cu(I) (Cu_2O, cuprous) and Cu(II) (CuO, cupric). Aerobically, the formation of hydroxyl radicals responsible for cell toxicity is represented in the following two equations (Santo *et al*., 2008):



$Cu(II) + O_2^- \rightarrow Cu(I) + O_2$



Then: $Cu(I) + H_2O_2 \rightarrow Cu(II) + O_2 + OH^- + OH^\cdot$

In terms of copper action on MRSA, the metal is thought to act on the cell membrane; the site of lipid membrane peroxidation (Weaver *et al*., 2010). It has been suggested that carotenoids to counteract the action of copper free-radicals, may contribute to bacterial resistance to copper at the lipid membrane (Weaver *et al*., 2010). Weaver *et al*. (2010) found that after 18 h exposure of MRSA to copper surfaces the lipid membrane was not disrupted but rather the degradation of genomic DNA and damage to cellular respiration were responsible for cytotoxicity. Copper has shown to bind to the double-helix of DNA at two different sites with differing affinities and high specificity (Sagripanti *et al*., 1991).

More information of the mechanism of action of copper is detailed in Chapter 5, section 5.1.3.

1.8.1.3 Trials in clinical areas

A trial at Selly Oak Hospital in Birmingham, UK explored the use of copper as an antimicrobial surface in a clinical setting. Toilet seats, tap handles and door push plates in an acute ward were coated with pure copper/resin composite of ~70 % Cu, 60 % Cu and 70 % Cu, respectively. The surfaces were installed six months before the trial was carried out to enable HCWs to accustom to the fittings. The surfaces were sampled for microbial contamination once a week for 10 weeks at the same time of the day on each occasion. The trial produced promising results; a significant difference was observed between the number of microorganisms detected on the copper-containing surfaces and on the control surfaces. The control surfaces were in the same ward, which according to the authors standardised the microbial challenge presented to both test and control

surfaces. There was a 90 – 100 % reduction in the number of microorganisms compared to control surfaces. In addition, MRSA, *C. difficile*, methicillin-sensitive *Staphylococcus aureus* (MSSA), VRE and *E. coli* were not detected on the copper alloy surfaces over the 10-week period (Casey *et al*., 2010).

Following on from this trial, a crossover study investigated the antimicrobial efficacy of copper alloy surface fittings in relation to standard fittings in an acute medical ward at a university hospital. Copper alloy surfaces were installed in high-contact areas such as toilet handles, door push plates, grab rails and commodes for 12 weeks. After the trial period copper alloy fittings were switched over with stainless steel standard fittings. The study showed reduced microbial counts on the copper alloy fittings; with more than half showing significantly decreased counts (Karpanen *et al*., 2012).

Another clinical trial was conducted by the same team from Birmingham, UK that compared microbial contamination on the surfaces of pens used by nurses in two critical care units at University Hospital Birmingham. Nurses were provided with copper-alloy coated or stainless steel pens for the duration of their 12.5 hour shifts. The initial level of contamination was determined and microbial load was measured immediately after the shift was over. In addition, a different set of pens was distributed but pens were stored for 11 hours at room temperature after the 12.5 hour shift to take into account non-use between shifts. Pens were chosen as the object of study as they are handled frequently and between users yet rarely decontaminated. The study showed that a significant reduction in microbial load was apparent with copper-containing pens than stainless steel pens in the samples collected immediately once the shift was over. The copper-containing pens sampled 11 hours after the shift ended also presented lower microbial counts than stainless steel pens after 11 hour storage. The authors of this study stated the copper-containing pens significantly reduced microbial load, however, more studies are required to prove a link between copper and reduced infection rates (Casey *et al*., 2011).

Another trial was conducted in a primary healthcare clinic in a rural area in South Africa rather than a hospital ward. Two consulting rooms were chosen; one as a test containing copper fittings on touch surfaces including desks, trolleys, window sills and

cupboards and a control room with standard stainless steel, wood and tile finishings for comparison. Overall, a 71 % reduction in microbial load was observed on copper surfaces compared to control surfaces. All copper surfaces presented a statistically significant decrease in microbial load (Marais *et al*., 2010).

A large trial over a 43-month period in three hospitals in the United States assessed the role of copper in reducing microbial burden. The large study was divided into two parts; first the microbial burden of six high-touch surfaces from 16 rooms across three hospitals was monitored weekly by microbiological screening. At month 23 the six high-touch surfaces in half the test rooms were surfaced with copper or copper alloys. The study found copper significantly reduced microbial burden by 83 % in comparison to control surfaces (Schmidt *et al*., 2012).

1.8.1.4 Copper antimicrobial surfaces in practice

In 2008 the US EPA first approved the use of copper and copper alloys as antimicrobial surfaces by registering five copper alloy products for potential use in healthcare and other settings (Website 21, 2008). Currently there are 479 copper alloys with US EPA approval (Website 22, 2012).

In January 2010, St. Francis hospital, a private hospital in County Westmeath, Ireland was the first hospital worldwide to permanently install copper door handles throughout and since then many healthcare establishments (both public and private) around the world, have installed copper and copper alloy fittings to potentially control environmental contamination and reduce the spread of microorganisms (Website 23, 2012).

1.8.1.5 Bacterial resistance to copper

A major concern with the increasing use of biocides in the healthcare environment is the emergence of bacterial resistance to the antimicrobial agent. The removal of copper ions by efflux pumps is one resistance mechanism. Gram-positive and -negative bacteria both express efflux pumps; channels utilised to expel toxic substances from the cell (Webber and Piddock, 2003). There are five families of efflux pumps; ATP binding

cassette (ABC), multidrug and toxic efflux (MATE), major facilitator superfamily (MFS), resistance nodulation division (RND) and small multidrug resistance (SMR) (Webber and Piddock, 2003). Efflux is a common resistance mechanism used by many bacteria and also contributes to the development of MDR (Piddock, 2006). A subfamily of RND efflux pumps has been identified as heavy-metal efflux (HME) (Long *et al*., 2010). An example of an HME pump is CusCBA; it can expel both copper and silver ions. Studies on the structure of the pump show in an inactive state it is closed but opens once bound by copper or silver ions due a change in conformation of the periplasmic and membrane domains of the pump (Long *et al*., 2010). Another method of copper resistance is the removal of copper ions by putative copper-translocating P-type ATPases (Adenosine Triphosphatase). P-type ATPases contributing to copper resistance in *E. coli* are encoded by the gene *copA* (Rensing *et al*., 2000). CopA P-type ATPase pumps out Cu(I) from the cytosol to the periplasm (Rensing *et al*., 2000). It is thought *copA* originates from *Pseudomonas syringae* and is found on an operon containing three other genes, *copABCD* (Cooksey, 1993).

1.8.2 Triclosan

Triclosan, 2,4,4'-trichloro-2'-hydroxydiphenylether, is a synthetic, broad spectrum biocide that inhibits the synthesis of bacterial fatty acids (Heath *et al*., 1999; Levy *et al*., 1999). Triclosan is a heat-stable compound, soluble in organic solvents, and insoluble in acidic, aqueous solutions (Schweizer, 2001). It is considered to be mainly an antibacterial agent, but it also used as an antiviral or antifungal (Glaser, 2004).

Microban is a company that manufactures products with a triclosan-containing coating (Website 24, 2013).

1.8.2.1 Mechanism of action

Bacteria utilise the type II fatty acid synthase system; a multi-step process to synthesise fatty acids (Rock and Jackowski, 2002). There are four main steps in fatty acid elongation. To begin with acetyl-acyl carrier protein (ACP) is converted to acetoacetyl-ACP and CO_2 by interacting with malonyl-ACP (Campbell and Cronan Jr, 2001). This

is a condensation reaction and is catalysed by FabK, β-ketoacyl ACP synthase III (Rock and Jackowski, 2002). Following this, β-ketoacyl-ACP is reduced by FabG. Next β-hydroxyacyl-ACP is dehydrated to form *trans*-2-enoyl-ACP. This reaction is catalysed by FabA or FabZ. Finally, FabI, an enoyl-acyl-carrier protein reductase (ENR), is involved in the nicotinamide adenine dinucleotide plus hydrogen (NADH)-dependent concluding step of elongation. There are four ENR isozymes present in bacteria; FabI, FabL, FabK and FabV. FabI, FabK and FabL are members of the short-chain dehydrogenase/reductase family (Zhu *et al*., 2010).

At low concentrations triclosan prevents bacterial fatty acid synthesis by binding to ENR reductase, FabI. Inhibition is achieved by the binding of the biocide to the active site of enoyl-ACP reductase (Schweizer, 2001); in turn this will prevent the final step of the elongation cycle of fatty acid synthesis. The association of triclosan with FabI-nicotinamide adenine dinucleotide (NAD+) was first determined in *E. coli* by x-ray crystallography (Heath *et al*., 1999). The interaction is strong and the structure forms a stable ternary complex (Heath *et al*., 1999; Campbell and Cronan Jr, 2001; Scweizer, 2001). FabI has been identified in *S. aureus* (saFabI) and showed similarity to that of *E. coli* (Heath *et al*., 2000). At higher concentrations triclosan acts at non-specific sites to exert its bactericidal effect (Gilbert and McBain, 2002). Membrane damage to cells is thought to occur after exposure to high concentrations of triclosan (Villalaíin *et al*., 2001).

1.8.2.2 Uses of triclosan

Originally triclosan was used in hospitals as a surgical scrub in 1972 (Glaser, 2004). Since then it has been added to many consumer products and within the household triclosan can be found in toothpaste, liquid soaps, bar soaps, creams, shower gel, cosmetics, deodorant, and on the surfaces of pizza-cutters, cutting boards, mattresses, sheets and pillows (Levy, 2001; Scweizer; 2001; Webber *et al*., 2008). Møretrø *et al*., (2011) have demonstrated an approximate 1.5 $\log_{10}$ reduction in *Salmonella* on triclosan-containing cutting boards after 24 h at 70 % relative humidity. In NHS hospitals triclosan-containing antimicrobial surfaces can be found on door handles; these were first introduced in 2006 (Page *et al*., 2009). Table 1.1 shows triclosan is not

commonly impregnated into surfaces in a clinical setting anymore, perhaps due to the emergence of bacterial resistance to the biocide (Page *et al*., 2009). The EU Cosmetics Directive 76/768/EEC advises the highest concentration of triclosan for use in cosmetics is 0.3 % (Scientific Committee on Consumer Safety (SCCS, 2010). Triclosan concentration in food contact materials is limited to 5 mg/kg of food (SCENIHR, 2009). Issues regarding the safety of triclosan have been raised, particularly as it is included in many products intended for personal use. Triclosan is non-toxic in humans but cases of skin irritation and sunlight photoallergic contact dermatitis as a result of both triclosan and sunlight exposure to skin have been highlighted (Glaser, 2004). Triclosan is readily absorbed into the body and has a half-life of 21 hours in plasma (Allmyr *et al*., 2006). It is not thought to present a long-term threat to human health; as of yet it has not proven to be carcinogenic, mutagenic or teratogenic; however, studies *in vitro* showed triclosan to inhibit phase I metabolising enzymes in the liver and possibly have an effect on thyroid hormone metabolism in rats and mice, respectively (Bhargava and Leonard, 1996; Glaser, 2004; Allmyr *et al*., 2006).

1.8.2.3 Triclosan-antibiotic cross-resistance

There are concerns biocide resistance may select for antibiotic resistance; this is primarily due to the similarity in their resistance mechanisms (Fraise, 2002). Recently the SCCS produced a guidance document on the safety of triclosan. The Committee identified four major hazards associated with triclosan use; triclosan can trigger resistance genes in bacteria, it can promote cross-resistance to biocides and antibiotics, high concentrations of triclosan have been identified in the environment and triclosan is resistant to bacterial biofilms (SCCS, 2010). In *Mycobacterium smegmatis* triclosan targets a FabI homologue, InhA, which is also an enoyl reductase and the target of the drug isoniazid. Mutations in *inhA* which encodes for InhA resulted in resistance to triclosan and isoniazid (McMurry *et al*., 1999). Another example of cross-resistance is in *Mycobacterium tuberculosis* (*M. tuberculosis*) that also contains InhA, whereby resistance to isoniazid by means of *inhA* mutation conferred resistance to the antibiotic rifampicin; a mutation in the *rpoB* gene, the target of rifampicin, is likely to occur directly as a consequence of isoniazid resistance (Heym *et al*., 1994).

1.8.3 Silver

Silver, Ag, is a transition metal element. As with copper the antimicrobial properties of silver have been explored for millennia. Silver nitrate ($AgNO_3$) was first used to treat ulcers in the 18th century and later in 1884 by K.S.F Crédé, a German physician, who used silver nitrate as a prophylactic to gonorrhoeal infections in newborns (Silver *et al*., 2006; Chopra, 2007). Nowadays, silver is commonly incorporated into wound dressings and is used to treat burns and diabetic ulcers (Silver *et al*., 2006). BioCote® is a commercially available antimicrobial surface coating that incorporates silver ion technology (Website 25, publication date unavailable). Numerous examples of environmental surfaces in the healthcare setting incorporating this technology are listed in Table 1.1.

1.8.3.1 Mechanism of action

Silver is active as an antimicrobial in the form of an ion, Ag^+ (Edwards-Jones, 2009). It is thought silver acts at several targets within a bacterial cell. At the cell membrane low concentrations of Ag^+ can cause cell death by inhibiting the proton motive force and inducing the loss of protons from the cell by affecting the permeability of the membrane (Percival *et al*., 2005; Edwards-Jones, 2009). In *E. coli* Ag^+ has been shown to uncouple the respiratory electron transport chain from oxidative phosphorylation (Percival *et al*., 2005; Edwards-Jones, 2009).

1.8.3.2 Trials in clinical areas

A trial at the Heart of England NHS Trust in Birmingham, UK investigated the antimicrobial efficacy of BioCote® treated surfaces compared to untreated surfaces. Similarly to the copper trial at Selly Oak, the surfaces were fitted well in advance of the trial commencing. Swabs were taken regularly over 16 weeks from treated and untreated surfaces including doors, door handles, furniture, electrical switches and waste bins. The results of the trial showed a 95.8 % reduction in the number of viable bacteria on surfaces coated with BioCote® compared with those left untreated (Taylor *et al*., 2009).

1.8.3.3 Bacterial resistance to silver

An issue with the use of silver in medicine is the emergence of bacterial resistance to the biocide. The first report of silver resistance was observed in Gram-negative bacilli in burns patients treated with silver nitrate (Cason *et al*., 1966). Another case of resistance was observed in *Salmonella enterica* serovar Typhimurium in a burns unit in the USA (Silver, 2003). Resistance was plasmid-mediated, by IncH1 on the plasmid pMG101, which also conferred MDR (Chopra, 2007). Following sequencing of this plasmid nine genes were identified that conferred bacterial resistance to silver (Silver, 2003). The resistance genes code for numerous products, for example, *silCBA* that encodes an RND efflux pump with homologues to that of AcrB in *E. coli* (Silver, 2003). As a result the cell can expel silver ions to render the bacterium resistant. The plasmid also contains the gene *silE*, which codes for the periplasmic silver-binding protein, SilE (Silver *et al*., 2006). This protein is thought to bind Ag^+ with high specificity causing a conformational change in the structure of SilE thus resulting in silver resistance (Silver, 2003).

1.9 Light-activated antimicrobial agents (LAAAs)

An alternative approach in reducing microbial surface contamination is the use of light-activated antimicrobial agents (LAAAs) coatings. LAAAs are utilised in photodynamic therapy (PDT) (Zolfaghari *et al*., 2009). PDT is commonly used for the treatment of cancer to kill cancerous cells (Jori *et al*., 2006; Page *et al*., 2009). The therapy works as follows; a photosensitiser is injected into the bloodstream and accumulates in cancerous tissues. A visible light of a suitable wavelength is placed over the area of interest, which results in the transition of the photosensitiser to an excited singlet state (Hamblin and Hasan, 2004). The excited state singlet may then enter a triplet excited state and from then there are two possible pathways, Type I and Type II, for the reaction to proceed down (Hamblin and Hasan, 2004). The Type I pathway involves electron transfer; radical ions react with oxygen which results in the formation of superoxide and hydroxyl radicals (Hamblin and Hasan, 2004). The Type II pathway consists of energy transfer from the excited triplet state to a ground state that results in the production of an excited singlet oxygen (Hamblin and Hasan, 2004). It is the formation of reactive

radical species and singlet oxygen that can target and kill cancerous cells (Page *et al*., 2009).

1.9.1 LAAAs – laboratory findings

The use of PDT to target microorganisms is an emerging field in antimicrobial therapy and antimicrobial surfaces (Jori *et al*., 2006). There have been numerous *in vitro* studies investigating the efficacy of LAAAs as potential antimicrobial surfaces. LAAAs are normally organic or inorganic dyes (Piccirillo *et al*., 2009). Examples of dyes tested in research include methylene blue, rose bengal and toluidine blue O. These can be incorporated into polymers and still retain their antimicrobial properties (Decraene *et al*., 2006). A study by Decraene *et al.* (2006) tested a variety of microorganisms including *S. aureus*, MRSA, *C. difficile* and *Candida albicans* against a cellulose acetate coating containing rose bengal and toluidine blue O upon exposure to white light. The results showed a 99.6 % reduction in *S. aureus* viable bacteria after 2 h and a 100 % reduction after 6 h. *C. difficile* was also eliminated completely after 6 h. Piccirillo *et al.* (2009) reported the antimicrobial activity of methylene blue and toluidine blue O when covalently attached to separate silicone surfaces that had been modified to allow the binding of the photosensitisers. The organisms tested were *E. coli* and *Staphylococcus epidermidis* (*S. epidermidis*). Microbial suspensions were placed over the silicone surface and irradiated with a laser light. Silicone surfaces bound to toluidine blue O resulted in a greater decrease in viable count of *E. coli* than surfaces bound to methylene blue. However, both were significantly more effective in reducing the viable count of *S. epidermidis* than of *E. coli* (Piccirillo *et al*., 2009).

In general Gram-positive bacteria are more susceptible to treatment by PDT due to the presence of pores within the peptidoglycan and lipoteichoic acid layers that allow the entry of photosensitisers into the cell (Jori *et al*., 2006). This was shown by Decraene *et al* (2006); *E. coli* was less susceptible to PDT than *S. aureus* was, as shown by the 100 % kill times of 16 h and 6 h, respectively.

Studies have also shown the addition of gold nanoparticles to a methylene blue-containing polymer can enhance the activity of the photosensitiser. Gold itself does not possess any antimicrobial properties but is thought to aid the formation of additional radical species other than singlet oxygen (Perni *et al*., 2009).

1.9.2 Advantages and disadvantages of LAAAs

There are several advantages of using LAAA-coated surfaces in the healthcare environment. Firstly LAAAs are effective against a range of microorganisms including bacteria, fungi, yeast and parasitic protozoa (Jori *et al*., 2006). The radical species that target microbial cells do not act at a specific site within a cell therefore bacterial resistance is not likely to develop to render the LAAA ineffective (Page *et al*., 2009). In addition, the cost of the light sources that excite the photosensitisers is low (Jori *et al*., 2006).

A concern with the long-term use of photosensitisers as antimicrobial surfaces is the photobleaching of the photosensiters. It has been suggested this can be prevented by regularly renewing the surface with a fresh coating containing a volatile solvent (Decraene *et al*., 2006). The aesthetics of antimicrobial surfaces containing photosensitisers dyes may be an issue in hospital wards. Piccirillo *et al.* (2009) suggest the use of polymers with covalently-bound dyes rather than physically-absorbed dyes; the colour of the dye is less obvious. In addition, their experimental work showed covalently-bound dyes were efficient at low concentrations and did not leach (Piccirillo *et al*., 2009).

1.10 Factors affecting the efficacy of biocides

Factors affecting the efficacy of biocides, including antimicrobial surfaces, are outlined in this chapter and further in Chapter 6, section 6.4.

1.10.1 Concentration

Concentration has been identified as an important factor affecting the efficacy of biocides. It is important a biocide is prepared at a concentration sufficient enough to produce a bactericidal or bacteriostatic effect. For example, triclosan is bacteriostatic at low concentrations (minimum inhibitory concentration (MIC) 0.05 -3.1 µg/mL against *S. aureus*) but bactericidal at high concentrations (MIC of 25 – 100 µg/mL against *S. aureus*) (Russell and McDonnell, 2000; Maillard, 2002). The concentration exponent (η) is a measure of the antimicrobial activity of biocides (Russell and McDonnell, 2000). The activity of biocides with a high η-value is affected by changes in concentration compared to biocides with a low η-value that are less affected (Russell and McDonnell, 2000). Biocides with a $\eta<2$ tend to bind strongly to their target by chemical or ionic binding whereas biocides with a $\eta>4$ interact weakly (Russell and McDonnell, 2000). It is important a suitable concentration of biocide is used in clinical settings; too high a concentration may present a hazard to staff and too low a concentration may not be sufficient for bacterial killing (Murtough *et al.*, 2001). It has been suggested biocide usage in hospitals is rotated frequently to prevent the emergence of biocide and antibiotic resistance (Murtough *et al.*, 2001).

In terms of antimicrobial surfaces, the effect of copper concentration is discussed in section 1.8.1.1 and further in Chapter 6, section 6.4.5.

1.10.2 Temperature and relative humidity

Temperature and relative humidity are important factors influencing the efficacy of biocides.

The antimicrobial efficacy of biocides generally increases with increasing temperature (Maillard, 2005a). The temperature co-efficient, θ, reflects the effect of a 1 °C rise in temperature on the efficacy of a biocide (Russell *et al.*, 2003). As this value tends to normally be in the range of 1.0 to 1.5 the Q_{10} (θ^{10}) value is used to determine the effect

of a 10 °C increase in temperature on biocide activity (Russell *et al*., 2003). The Q_{10} can be defined as:

$$Q_{10} = \frac{\text{Time to kill at T °C}}{\text{Time to kill (T + 10) °C}}$$

(Website 26, 2005).

Relative humidity is expressed as a percentage of water vapour in the air and is calculated using the following equation:

Relative Humidity = (Actual Vapour Density/Saturation Vapour Density) x 100 %
(Website 27, publication date unavailable).

An increase in relative humidity is generally associated with increased antimicrobial efficacy of copper-containing surfaces (Grass *et al*., 2011).

Michels *et al.* (2009) have studied the effect of temperature and relative humidity on the efficacy of copper and silver surfaces against *S. aureus*. The study was carried out under the parameters of the Japanese Industry Standard (JIS Z 2801) (see section 1.11.1) and also under conditions typical of an indoor environment. The study showed copper and copper alloys were effective in reducing bacterial load at both high and low relative humidities (>90 % (high) and 20 and 24 % (low) respectively) and temperatures (35 °C (high) and 20 °C (low) respectively). In contrast, silver ions only caused a significant reduction in microbial load at > 90 % relative humidity and 35 °C suggesting silver may not be an ideal antimicrobial in hospitals where the relative humidity and temperature are much lower (Michels *et al*., 2009). A study investigating the efficacy of copper alloys showed copper was more efficient and faster-acting at room temperature than at 4 °C when tested against strains of *P. aeruginosa* (Elguindi *et al*., 2009).
More examples describing the effect of temperature and relative humidity on antimicrobial surface activity are detailed in Chapter 4, section 4.1.5.

1.10.3 Type and number of microorganisms

When considering the choice of biocide it is important to take into account the target microorganisms (Maillard, 2005b). In general, bacterial spores, Gram-negative bacteria and mycobacteria present lower susceptibility to biocides than Gram-positive bacteria. This may be due to the differences in the composition of the outer cell wall (Maillard, 2002).

The number of bacterial cells can also influence the efficacy of a biocide. Generally, biocides tend to be more effective against a lower number of microorganisms (Russell *et al.*, 2003).

1.10.4 Antimicrobial surface properties

It is important to take into account the chemical and physical properties of a surface when considering it for use in clinical settings. Stainless steel is used throughout hospitals on surfaces as it is long-lasting and, in comparison to copper does not oxidise upon exposure to air (Gould *et al.*, 2009; Page *et al.*, 2009). Oxidation may prevent the release of copper ions thus rendering the surface ineffective (Sattar, 2010). This should be taken into account when surfaces are disinfected as some disinfectants may contain oxidising agents (Sattar, 2010). Warnes and Keevil (2011) found copper(I) and copper(II) were important for cell toxicity affecting bacterial DNA and cell respiration in VRE. They recommend cleaning agents used for surface disinfection do not contain chelating substances that could impede the release of copper ions (Warnes and Keevil, 2011). In addition pure copper may not be a favourable choice over copper alloys as it is highly corrosive and easily tarnished (Weaver *et al.*, 2008).

1.10.5 Organic load

Organic load, or interfering materials, or soiling, can affect the efficacy of biocides (Maillard, 2005b). Organic load can include blood, serum, pus and dirt (Russell, 2003). Organic load may decrease the activity of biocides via two mechanisms; it may react

with the biocide resulting in a lower concentration of biocide, or organic load may protect the microorganism from biocide action by adsorbing the antimicrobial agent (Lambert and Johnson, 2001; Russell *et al*., 2003).

Organic load may contribute to surface conditioning thus reducing the effectiveness of antimicrobial surfaces as investigated by Airey and Verran (2007). They looked at the role of interfering materials on the biocidal efficacy of copper. Copper, copper alloys and stainless steel (for comparison) were inoculated with *S. aureus* containing organic load (bovine serum albumin, BSA), dried for 5 min, then incubated for 24 h. Surfaces were then cleaned with wipes used in NHS hospitals. The cycle of soiling and cleaning was performed five times. After each procedure the levels of soil and live cells were determined by epifluorescence microscopy. After several cycles a high level of soil and cells was observed on copper surfaces whereas stainless steel surfaces were easily cleaned. From this is was concluded the cleaning agent used caused the organic load and bacteria mix to bond to the copper surfaces, resulting in resistance to the cleaning product (Airey and Verran, 2007).

A different study investigating the survival of HA- and CA-MRSA on copper alloy coins found bacteria survived in the presence of soiling. The addition of pus and blood, examples of soiling in a hospital environment, to surfaces significantly enhanced the survival of bacterial cells (Tolba *et al*., 2007).

1.11 Antimicrobial surface efficacy test protocols

For the purpose of this study antimicrobial surface efficacy tests for non-porous materials only are discussed.

1.11.1 Japanese Industry Standard - JIS Z 2801

At present the most widely used and accepted standard test for determining antimicrobial surface activity and efficacy is the Japanese Industry Standard (JIS Z 2801). This test is designed to provide quantitative data on the efficacy of surfaces. The

test protocol is described briefly in an official Organisation and Economic Co-operation and Development (OECD) 2007 document titled ‘Analysis and Assessment of Current Protocols to Develop Harmonised Test Methods and Relevant Performance Standards for the Efficacy Testing of Treated Articles/Treated Materials’.

Firstly a cell suspension of 10^6 colony forming units per millilitre (cfu/mL) cells is prepared and 400 μL is inoculated on to three test surfaces (50 x 50 mm). The surface is then covered with a 40 x 40 mm sterile polyethylene film and samples are incubated for 24 hours at 35 °C and high relative humidity (>90 %).The viable count of the initial inoculum on surfaces is determined by removing the plastic film from the surface then placing the film and surface into a stomacher bag with a validated neutraliser to prevent further antimicrobial activity by the test surface. A suitable dilution plate count method is utilised for determining total viable counts. Viable inocula are enumerated in the same way for 24 h surfaces. A surface is antimicrobial if there is a >2 $\log_{10}$ difference between the viable counts of control and test materials (OECD, 2008).

This test is the accepted efficacy test for testing antimicrobial-treated surfaces and is used by many manufacturers marketing surfaces as antimicrobial. Whilst it is a good screening test, it has been described as inappropriate for predicting the performance of surfaces in a healthcare setting. These points are considered in detail in Chapter 4, section 4.1.6 and Chapter 6, sections 6.2 and 6.3.

1.11.2 International Organisation for Standardisation - ISO22196

The ISO22196 test is an international standard for measuring antimicrobial activity on plastic surfaces. The test protocol is essentially the same as for the JIS Z 2801; indeed the ISO22196 is based on the JIS Z 2801 (OECD, 2008).

1.11.3 American Society for Testing and Materials - ASTM E2180-01

The ASTM E2180-01 test is a ‘standard test for determining the activity of incorporated antimicrobial agents in polymeric or hydrophobic materials’. In brief the test is carried

out as follows. A bacterial cell suspension of approximately 10^8 cfu/mL is prepared. Then 1 mL of bacterial suspension is added to 100 mL molten agar and from this 500 - 1000 µL is inoculated on to test surfaces. The reason for adding bacterial suspension to agar is that an 'artificial biofilm' is formed over the hydrophobic material. Surfaces are incubated for 24 h under humid conditions and at a temperature suitable for the test surfaces' intended use. As for the JIS Z 2801 viable bacteria are determined on control and test surfaces; surfaces are transferred to neutraliser and sonicated to break away the biofilm from the surface then total viable counts performed (OECD, 2008).

1.11.4 ASTM E2149-01

The ASTM E2149-01 is a 'standard test method for determining the antimicrobial activity of immobilised antimicrobial agents under dynamic contact conditions'. This test differs to the above mentioned standards in that test surfaces are placed directly into a bacterial cell suspension and agitated. The test surface is placed in the suspension for a specific time and the bacterial population is compared before and after (OECD, 2008).

1.11.5 XP G 39-010

This test is officially titled 'Propriétés des étoffes - Étoffes et surfaces polymériques àpropriétés antibactériennes Caractérisation et mesure de l'activité antibactérienne', which translates to 'Properties of textiles – textile and polymeric surfaces having antibacterial properties - characterisation and measurement of antibacterial activity'. For this test four test surfaces are placed on an agar plate containing a specific volume of bacteria. Each surface is applied on to the agar plate for 1 min with a 200 g weight. Next, surfaces are incubated for 24 h and 37 °C under humid conditions. Duplicate samples are utilised for determining the initial viable count. The viable count of the 24 h surfaces is determined and the difference between initial and 24 h viable counts calculated (OECD, 2008).

1.11.6 Three-tier approach to evaluate the effectiveness of antimicrobial surfaces

The OECD has suggested that a three-tiered approached is needed to precisely evaluate the effectiveness of antimicrobial surfaces and support antimicrobial claims. Tier 1 is a proof of principle test to compare treated materials with untreated materials. All the tests mentioned in this section are proof of principle tests. Tier 2 is a laboratory test and incorporates modified parameters that are more realistic and a reflection of in-use conditions. Factors such as strain selection, inoculum concentration, humidity, temperature, soiling and exposure time can be altered according to requirements. Finally, Tier 3 is an in-use evaluation of the efficacy claims. Field tests are performed to observe whether or not there is a reduction in microbial bioburden by antimicrobial surfaces (OECD, 2008). This draws parallels with Standards testing; Phase 1 tests are screening tests, Phase 2 tests predict antimicrobial action in simulated in-use conditions and Phase 3 are field trials. The tests described in sections 1.11.1 – 1.11.5 are all examples of Phase 1 tests.

1.12 Aims and objectives

The aims of this project were to address the following questions:

- Is the JIS Z 2810 an appropriate test for evaluating the efficacy of antimicrobial surfaces?
- Do the antimicrobial surfaces deliver antimicrobial activity when exposed to microbial aerosols?
- Do the antimicrobial surfaces deliver antimicrobial activity when presented with dried microbial inocula?
- What are the key factors affecting the efficacy of antimicrobial surfaces?

In addition the main objectives of this project were to:

- Provide robust *in vitro* data on the activity of antimicrobial surfaces used in clinical settings.
- Establish an efficacy test method for testing antimicrobial surfaces using realistic parameters to provide to the NHS and industry.

CHAPTER 2 GENERAL MATERIALS AND METHODS

2.1 Bacterial strains

Gram-positive, Gram-negative and spore-forming bacteria were utilised as test microorganisms. Table 2.1 details the strains used throughout this study.

Table 2.1 - Bacterial strains tested throughout this project

Type	Strain
Gram-positive	*Staphylococcus aureus* NCIMB 9518
Gram-negative	*Acinetobacter baumannii* NCIMB 9214
Spore-forming	*Clostridium difficile* NCTC 12726
	Bacillus subtilis NCTC 10400

NCIMB = National Collections of Industrial Marine and Food Bacteria,
NCTC = National Collection of Type Cultures

2.2 Media

Media were purchased from Oxoid (Basingstoke, UK) and Fisher Scientific (Loughborough, UK) unless otherwise stated.

Tryptone Soya Broth (TSB) (17 g/L pancreatic digest of casein, 3 g/L enzymatic digest of soya bean, 5 g/L sodium chloride, 2.5 g/L dipotassium hydrogen phosphate, 2.5 g/L glucose) and Tryptone Soya Agar (TSA) (15 g/L pancreatic digest of casein, 5 g/L enzymatic digest of soya bean, 5 g/L sodium chloride, 15 g/L agar) were used throughout as media for bacterial growth of *S. aureus* and *A. baumannii*.

C. difficile was grown in Brain Heart Infusion broth (12.5 g/L brain infusion solids, 5 g/L beef heart infusion solids, 10 g/L proteose peptone, 2 g/L glucose, 5 g/L sodium chloride, 2.5 g/L disodium phosphate) and agar (same composition as broth plus 10 g/L agar).

B. subtilis was grown in Luria-Bertani (LB) broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride) and agar (as broth plus additional 15 g/L agar).

Tryptone Sodium Chloride (TSC) was used as a buffer throughout initial experiments and prepared by adding 0.4 g tryptone and 3.4 g sodium chloride to 400 mL deionised water. Maximum Recovery Diluent (MRD) (8.5 g/L sodium chloride, 1 g/L peptone) was the buffer of choice for the majority of later testing and is indicated in the text when used.

All media were prepared according to manufacturer's guidelines in deionised water and autoclaved at 121 °C for 15 min before use based on British Pharmacopeia guidelines (British Pharmacopeia Commission, 2006). Once opened, all media were used within their recommended shelf-life.

2.3 Preparation of bacterial cultures

2.3.1 Recovery of strains from freezer stocks

All master stocks of strains were stored at -80 °C in glycerol stocks. Stocks for experimental use were stored on protect beads (Fisher Scientific, Loughborough, UK). A single bead was transferred to TSA for recovery of *S. aureus* and *A. baumannii* and to LB agar for *B. subtilis*. *S. aureus* and *B. subtilis* were grown at 37 °C; *A. baumannii* was grown at 25 °C. Cultures were stored at 4 °C and utilised within two weeks of recovery. *C. difficile* recovery from freezer stocks is detailed in section 2.6.3.

2.3.2 Preparation of bacterial broth cultures

Bacterial broth cultures of *S. aureus* and *A. baumannii* were prepared for experimental procedures. A loopful of culture (as grown and described in section 2.3.1) was transferred to the required volume of TSB for *S. aureus* and *A. baumannii*. In general cultures were grown in 10 mL TSB for 24 h at the required temperature (37 °C for *S. aureus*, 25 °C for *A. baumannii*), unless otherwise stated. Every time a broth culture was prepared a control containing just TSB was also incubated to check for the sterility of the TSB batch utilised.

2.3.3 Bacterial suspension for testing

This methodology applied to *S. aureus* and *A. baumannii* cultures. *C. difficile* and *B. subtilis* spore suspensions for testing were prepared as described in sections 2.6.4 and 2.7.1, respectively. A broth culture was prepared as described in 2.3.2. After incubation for a maximum of 24 h the culture was centrifuged for 15 min at 2500 *g* at room temperature. The supernatant was discarded and the remaining pellet was re-suspended in either TSC or MRD.

2.4 Bacterial viable counting using the drop count and spread plate methods

The drop count method was utilised to enumerate bacteria from a 24 h grown culture (Miles and Misra, 1938). A culture was prepared in 10 mL TSB according to 2.3.2. The culture was centrifuged for 15 min at 2500 *g*, re-suspended in 10 mL TSC or MRD and serially diluted to a dilution factor of 10^{-7}. Ten µL of each dilution was plated on to a TSA plate in triplicate. Plates were incubated for 24 h at 37 °C and the number of cfu/mL was determined. Enumeration was performed in triplicate.

The spread plate method was also utilised for enumeration of bacteria. This method was used mainly in Chapters 4 and 5. A bacterial culture was prepared in 10 mL TSB as described in 2.3.2, centrifuged for 15 min at 2500 g and re-suspended in either TSC or MRD. The suspension was serially diluted down to 10^{-7}. Next, 100 µL from each dilution was plated on to a TSA plate and spread with a sterile, plastic spreader. This was performed in triplicate. Plates were then incubated for 24 h at 37 °C and the number of cfu/mL was determined.

2.4.1 Validation of drop count method

In order to validate the drop count method as a suitable method for enumerating bacteria, ten separate dilution series were performed and the number of cfu/mL was calculated. Validation was performed with *S. aureus*.

An one-way analysis of variance (ANOVA) test showed there was no statistically significant difference between the number of counts of the ten repeats (P=0.326), which suggests the drop count method can be performed accurately and is a suitable and reproducible method for enumerating bacteria from suspension. Table 2.2 shows the average viable count with standard deviation (SD) per repeat.

Table 2.2 –Validation of drop count method

Repeat	Log_{10} cfu/mL ± SD
1	9.32 ± 0.04
2	9.24 ± 0.09
3	9.25 ± 0.09
4	9.29 ± 0.06
5	9.16 ± 0.08
6	9.23 ± 0.03
7	9.26 ± 0.04
8	9.23 ± 0.06
9	9.27 ± 0.04
10	9.26 ± 0.08

2.4.2 Validation of spread plate method

As with the drop count method, the spread plate method for enumerating bacteria was validated with *S. aureus*. Ten separate dilutions were performed and the amount of cfu/mL was determined, see Table 2.3. Statistical analysis by a one-way ANOVA confirmed the suitability of the spread plate method for enumerating bacteria, P=0.163.

Table 2.3 – Validation of spread plate method

Repeat	Log_{10} cfu/mL ± SD
1	9.18 ± 0.04
2	9.26 ± 0.03
3	9.23 ± 0.05
4	9.17 ± 0.03
5	9.19 ± 0.05
6	9.21 ± 0.03
7	9.22 ± 0.01
8	9.26 ± 0.09
9	9.21 ± 0.03
10	9.23 ± 0.02

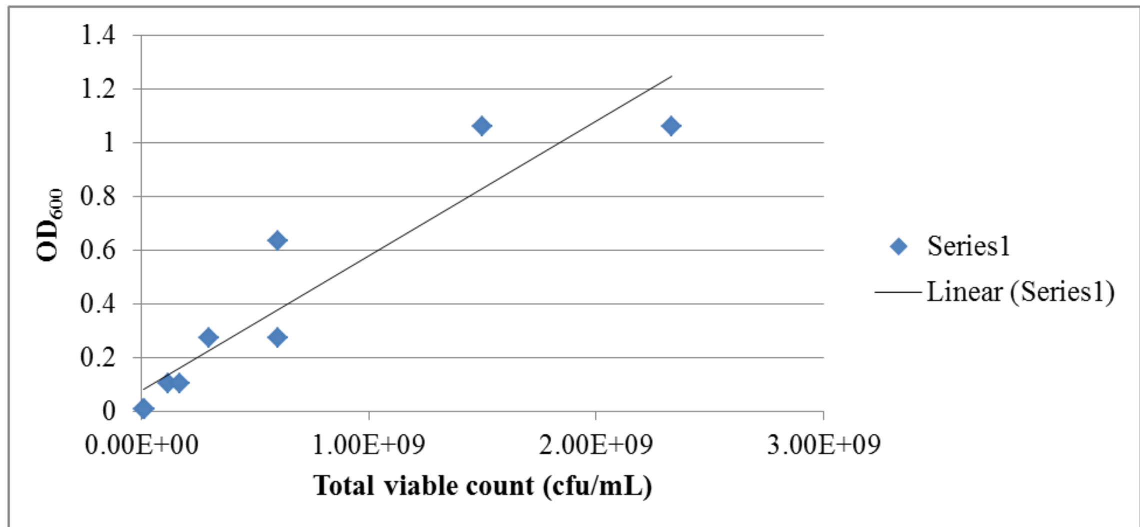
2.5 Alteration of bacterial concentration using a spectrophotometer

An optical density (OD) *vs*. total viable count graph for *S. aureus* was produced to help determine the OD required to obtain a particular bacterial concentration. A broth culture of *S. aureus* was prepared as described in 2.3.2. The culture was centrifuged at 2500 *g* for 15 min, re-suspended in 10 mL TSC and the OD measured at 600 nm (OD_{600}) with an Ultrospec 3100 spectrophotometer (Amersham Biosciences, UK). This was serially diluted in TSC to a dilution factor of 10^{-4}. The OD_{600} of each dilution was determined. In addition, 1 in 2 and 1 in 4 dilutions of the original culture were carried out to provide additional points to plot on the graph (see Figure 2.1). Each dilution (Neat to 10^{-4}, 1 in 2 and 1 in 4) was then serially diluted in TSC in order for total viable counts to be determined. Figure 2.1 shows the approximate OD_{600} required to obtain a particular bacterial concentration. The graph was a useful starting point for identifying what OD_{600} reading was needed to yield a certain concentration of bacterial cells. Generally a reading of around 0.45 provided 10^8 cfu/mL cells for *S. aureus*.

An OD against total viable count graph was not produced for *A. baumannii*; an OD_{600} of approximately 0.5 was sufficient to give 10^8 cfu/mL. This was first achieved by trial and error then utilised throughout for all *A. baumannii* suspensions required at 10^8

cfu/mL. The concentration of *C. difficile* spore and *B. subtilis* spore suspensions were not determined by optical density readings.

Figure 2.1 – Optical density against total viable count graph for *S. aureus* NCIMB 9518



2.6 *C. difficile*

2.6.1 Anaerobic growth requirement for *C. difficile*

C. difficile is an anaerobic organism therefore growth is required in anaerobic conditions. An anaerobic incubator, Bugbox Plus (Ruskin Technology Limited, Leeds, UK), was utilised to grow cultures in media. The conditions in the incubator were as follows; 95 % N_2, 10 % CO_2, 5 % H_2. Anaerobic indicator strips were placed inside the cabinet daily to ensure an anaerobic environment was maintained.

2.6.2 Recovery from freezer stocks

Master stocks of *C. difficile* NCTC 12726 were stored at -80 °C in glycerol stocks and on protect beads for experimental use.

The selective media of choice for the recovery of *C. difficile* from freezer stocks was *Clostridium difficile* moxalactam norfloxacin (CDMN). This consisted of the culture media supplements cysteine hydrochloride, norfloxacin and moxalactam (Oxoid, Basingstoke, UK) combined with *Clostridium difficile* Agar Base (Oxoid, Basingstoke UK) and 7 % (v/v) defibrinated horse blood (Oxoid, Basingstoke UK). The agar base was autoclaved at 121 °C for 15 min, cooled to 50 °C and the media supplements were added. Plates were then de-gassed for 24 h. Next, a single protect bead was transferred to CDMN selective agar and incubated for 48 h at 37 °C under anaerobic conditions.

2.6.3 Preparation of *C. difficile* spores using the Clospore method

Spores of *C. difficile* NCTC 12726 were prepared using a slightly adapted method by Perez *et al*. (2011), which the authors have named the 'Clospore' method. A freezer protect bead of *C. difficile* NCTC 12726 was streaked on to a de-gassed CDMN agar plate and incubated anaerobically for 48 h at 37 °C (as in 2.6.2). From this a few colonies were inoculated into de-gassed 4x 25 mL BHI broth and incubated anaerobically for 24 h. Next, 5 mL of culture was transferred to 500 mL Clospore media (10 g/L special peptone mix, 10 g/L yeast extract, 0.6 g/L $(NH_4)_2SO4$, 0.12 g/L $MgSO_4.7H_20$, 0.08 g/L $CaCl_2.2H_20$, 3.48 g/L K_2CO_3, 2.6 g/L KH_2PO_4, pH 7.9) for up to 7 days. Spores were harvested by aliquoting the culture into 50 mL Falcon tubes, centrifugated at 5000 *g* for 15 min at 4 °C, washed three times in 30 mL cold, sterile, deionised water then pooled into one Falcon tube and stored at 4 °C for three days. Next, cultures were centrifuged at 5000 *g* for 15 min at 4 °C and re-suspended in 25 mL 0.1 M sodium phosphate buffer, pH 7. Spore preparations were treated with two enzymes; lysozyme (20 µg/mg of wet weight of pellet) and trypsin (15 µg/mg of wet weight of pellet) to lyse vegetative cells and were then sonicated at 45 °C for 10 min every 2 h over a 6 h period. Prepared spore stocks were stored at 4 °C.

2.6.4 Viable count of *C. difficile* spore suspensions

Viable bacteria were enumerated by serial dilution in sterile, deionised water and plated via the drop count method on to BHI agar containing 0.1 % sodium taurocholate (Oxoid, Basingstoke, UK) to aid spore germination. Spores were grown under anaerobic

conditions as described in 2.6.1. To be confident the purified cells were spores and not vegetative cells, 600 µL of culture was heated at 60 °C for 25 min (Heeg *et al.*, 2012). This heat treatment killed any vegetative cells. The viable count of the spore suspension was compared before and after heat treatment. Table 2.4 shows the $\log_{10}$ viable count of five spore stocks before and after heat treatment. The $\log_{10}$ difference between the two counts is shown and is very small for all five stocks before and after heat treatment suggesting the stocks were mostly spore and not vegetative cells, therefore suitable for future testing.

Table 2.4 – Viable count of *C. difficile* spore stock suspensions pre- and post-heat treatment

Spore Stock	Viable count pre-heat treatment ($\log_{10}$ cfu/mL)	Viable count post-heat treatment ($\log_{10}$ cfu/mL)	Difference
1	7.33	7.58	-0.25*
2	7.05	7.01	0.04
3	7.10	7.13	-0.03
4	7.99	8.00	-0.01
5	7.99	7.98	0.01

* negative difference denotes an increase in viable count.

2.7 *B. subtilis*

2.7.1 Preparation of *B. subtilis* spores

Master stocks of *B. subtilis* NCTC 10400 were stored in glycerol and experimental stocks were stored on protect beads at -80 °C.

The method used for preparing *B. subtilis* spores was adapted from an existing method (Nicholson and Setlow, 1990). A freezer bead of *B. subtilis* was streaked on to LB agar and incubated for 16 h at 37 °C. Then a single colony was inoculated into LB broth and incubated for 3 - 3.5 h at 37 °C with shaking until an OD_{600} of 1 - 2 was reached. Once the correct OD_{600} was obtained, 200 µL of culture was spread on to 2 x SG agar (16 g/L

nutrient broth, 13 mL/L 2M KCl, 2 mL/L 1 M $MgSO_4$, 100 µL 1 M $MnCl_2$, 3 µL/L 0.36 M $FeSO_4$, 15 g/L agar, 970 mL/L deionised water and 20 mL 50x $Ca(NO_3)_2$ (1.18 g $Ca(NO_3)_2.4H_2O$, 5 g glucose, 100 mL deionised water)). Plates were incubated in a plastic bag to maintain high humidity for 3 days at 37 °C. This period was sufficient for spore release from the sporangia. Next, spores were scraped from plates using a sterile, plastic loop. Spores from 3 plates were placed into 30 mL cold, sterile water, sonicated for 1 min then cooled on ice for 1 min. This was repeated once. Spores were then centrifuged for 15 min at 5000 *g* and 4 °C, washed twice in cold, sterile water and re-suspended in a final volume of 30 mL water. Spore stocks were stored at 4 °C. A viable count before and after heat treatment (60 °C, 25 min) was performed as with *C. difficile* spores (see section 2.6.4). Spores were grown on LB agar for 24 h under aerobic conditions. Table 2.5 shows the $\log_{10}$ viable count of five spore stocks before and after heat treatment. Log_{10} differences were minimal therefore spore stocks were suitable for testing.

Table 2.5 – Viable count of *B. subtilis* spore stock suspensions pre- and post-heat treatment

Spore Stock	Viable count pre-heat treatment ($\log_{10}$ cfu/mL)	Viable count post-heat treatment ($\log_{10}$ cfu/mL)	Difference
1	9.01	8.99	0.02
2	9.08	9.02	0.06
3	9.07	9.07	0
4	9.15	9.14	0.01
5	9.10	9.09	0.01

2.8 Bacterial recovery from stainless steel discs by the carrier test method

This test was carried out based on an adapted version of British Standard EN13697 (Chemical disinfectants and antiseptics. Quantitative non-porous test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial,

domestic and institutional areas. Test methods and requirements without mechanical action) to look at the survival of microorganisms on stainless steel over a period of 48 h. Stainless steel discs of grade 2B finish (see section 2.10 for composition) were obtained from Goodfellows Cambridge Ltd (Huntington, UK). Discs were cleaned following a general laboratory protocol; discs were soaked with 5 % Decon90 (Decon Laboratories Limited, Hove, UK) in deionised water for 60 min, rinsed, dried then autoclaved before use.

2.8.1 Method

S. aureus, *A. baumannii*, *C. difficile* spores and *B. subtilis* spores were tested. Cultures of *S. aureus* and *A. baumannii* were prepared as in 2.3.2 and standardised with TSC to produce approximately 10^9 cfu/mL. *C. difficile* and *B. subtilis* spores (approximately 10^9 cfu/mL) were prepared as in sections 2.6.3 and 2.7.1, respectively. Next, stainless steel discs were placed in a Petri dish, inoculated with 10 µL of bacterial or spore suspension and left to dry for 30 min at 37 °C. Once visibly dry, discs were incubated for 0 min, 30 min, 60 min, 120 min, 24 h and 48 h at room temperature. Following the required contact time discs were transferred to a 100 mL glass bottle containing 5 g of 3 mm glass beads and 10 mL TSC (for *S. aureus* and *A. baumannii*) or sterile, deionised water (for spores). Bottles were shook for 1 min at 150 rpm to aid the removal of bacterial cells from the discs. Viable counts were then determined using the drop count method (see section 2.4). This was repeated in triplicate per contact time.

The test was also carried out in the presence of organic load to represent dirty conditions. BSA was utilised as an organic load at a final concentration of 3 g/L. To achieve dirty conditions, 1 mL of the bacterial or spore suspension and 1 mL 0.6 % BSA were mixed and from this, 10 µL was inoculated on to the stainless steel discs. The test conditions were at room temperature and room relative humidity.

2.8.2 Results

Table 2.6 shows the survival of the four tested microorganisms on stainless steel discs up to 48 h incubation at room temperature in the presence and absence of organic load. All statistical analysis was carried out by a General Linear Model using SPSS software.

There was a 1.77 $\log_{10}$ cfu/mL reduction between the initial inoculum (amount inoculated on to the disc) and the amount of *S. aureus* with no organic load recovered at 0 h. At 30, 60 and 120 min recovery was constant; between 5.40 and 5.89 $\log_{10}$ cfu/mL. There was a 1.29 and a 1.36 $\log_{10}$ cfu/mL reduction at 24 and 48 h respectively, in fact there was a significant difference ($P<0.001$) between the count at 0 h and 24 h and between 0 h and 48 h. No significant difference between the inoculum recovered at 24 h and at 48 h ($P=0.565$) was observed. In the presence of organic load there was also a significant difference between the counts at 0 h and at 24 h ($P=0.015$) and 48 h ($P<0.001$). There was a 2.33 and 2.78 $\log_{10}$ cfu/mL reduction after 24 h and 48 h, respectively, suggesting more of an effect with organic load. Indeed, there was a significant difference between the two conditions, $P=0.021$.

For *A. baumannii* there was a difference of <3 $\log_{10}$ cfu/mL between the initial viable count and the amount recovered at 0 h, both with and without organic load. Up to 120 min there was <1 $\log_{10}$ cfu/mL $\log_{10}$ reduction in relation to the 0 h count. After 48 h there was a 3.21 and 2.87 $\log_{10}$ cfu/mL reduction in the absence and presence of organic load, respectively. With organic load there was a significant difference between the counts from 0 h and both 24 h and 48 h (both $P<0.001$). In the presence of organic load there was a significant difference between in the counts at 0 h and all contact times ($P<0.001$), except 30 min ($P=0.647$). Between the two conditions, organic load or no organic load, there was overall a significant difference ($P<0.001$).

The difference between the initial inoculum of *C. difficile* spores and the amount recovered at 0 h was 2.2 $\log_{10}$ cfu/mL and 1.85 $\log_{10}$ cfu/mL, with and without organic load, respectively. After 48 h there was a 1.21 $\log_{10}$ cfu/mL reduction without organic load and a 0.9 $\log_{10}$ cfu/mL reduction with organic load present. In the absence of organic load there was no significant difference between 0 h counts and all other contact

time counts except at 48 h (P=0.039). In the presence of organic load a significant difference was observed between 0 h and 30 min counts (P=0.036) but with no other contact times. Overall, there was no significant difference between both conditions, P=0.486.

Over the 48 h experimental period there was very little change *B. subtilis* spore viability. Between 0 h and 48 h there was less than a 0.2 log_{10} cfu/mL reduction both in the presence and absence of organic load; differences that were not significantly different (no organic load P=0.25, with organic load P=0.07). However, there was an overall significant difference between the counts with organic load and the counts without (P<0.001).

It was apparent that *S. aureus*, *A. baumannii*, *C. difficile* spores and *B. subtilis* spores were able to survive on stainless steel for a long period of time; here at least up to 48 h. The results from this experiment were useful for future experiments where stainless steel was used as a negative control surface for antimicrobial surface testing.

Table 2.6 – Survival of *S. aureus*, *A. baumannii*, *C. difficile* and *B. subtilis* on stainless steel up to 48 h (n=3)

	S. aureus		*A. baumannii*	
Contact time	**Log_{10} cfu/mL ± SD without organic load**	**Log_{10} cfu/mL ± SD with organic load**	**Log_{10} cfu/mL ± SD without organic load**	**Log_{10} cfu/mL ± SD with organic load**
Initial viable count	7.38 ± 0.64	7.16 ± 0.32	9.01 ± 0.06	8.79 ± 0.13
0 min	5.61 ± 0.17	5.69 ± 0.13	6.54 ± 0.11	5.93 ± 0.07
30 min	5.40 ± 0.41	5.81 ± 0.03	6.18 ± 0.24	5.75 ± 0.08
60 min	5.79 ± 0.13	5.97 ± 0.11	5.95 ± 0.14	5.19 ± 0.20
120 min	5.86 ± 0.09	6.00 ± 0.11	6.03 ± 0.06	4.90 ± 0.13
24 h	4.57 ± 0.18	4.83 ± 0.54	4.63 ± 0.62	3.77 ± 0.22
48 h	4.25 ± 0.26	4.38 ± 0.24	3.33 ± 0.40	3.06 ± 0.08

Table 2.6 continued - Survival of *S. aureus*, *A. baumannii*, *C. difficile* and *B. subtilis* on stainless steel up to 48 h (n=3)

	C. difficile		***B. subtilis***	
Contact time	**Log_{10} cfu/mL ± SD without organic load**	**Log_{10} cfu/mL ± SD with organic load**	**Log_{10} cfu/mL ± SD without organic load**	**Log_{10} cfu/mL ± SD with organic load**
Initial viable count	6.41 ± 0.03	6.14 ± 0.05	9.40 ± 0.16	9.01 ± 0.02
0 min	4.21 ± 0.58	4.29 ± 0.35	6.97 ± 0.02	6.89 ± 0.02
30 min	3.96 ± 0.54	3.10 ± 0.17	6.96 ± 0.02	6.72 ± 0.09
60 min	4.16 ± 0.38	3.76 ± 0.62	6.89 ± 0.02	6.77 ± 0.04
120 min	3.68 ± 0.43	4.12 ± 0.46	6.90 ± 0.04	6.87 ± 0.03
24 h	3.43 ± 0.30	3.19 ± 0.18	7.06 ± 0.04	6.91 ± 0.08
48 h	3.00 ± 0.14	3.39 ± 0.48	6.91 ± 0.03	6.73 ± 0.04

2.9 General characterisation of microorganisms

Cell aggregation measurements and hydrophobicity tests were carried out to characterise the different bacteria used in this study.

2.9.1 Particle size

The particle size of all bacterial strains was determined using an N4Plus Dynamic Light Scattering machine (Beckman Coulter, Fullerton, USA). Particle size was measured in order to gain an insight to the approximate size of the bacterial strains in this study and to see if there was any bacterial cell or spore aggregation. Aggregation may act as a protective mechanism against biocidal activity.

Cultures of *S. aureus* and *A. baumannii* were prepared as in section 2.3.2. Bacterial concentrations were adjusted to 10^8 cfu/mL using water. Water was preferred to TSC to prevent interference in the determination of particle size. *C. difficile* and *B. subtilis* spores were already suspended in water following spore preparation (see sections 2.6.3 and 2.7.1). One mL of each suspension was transferred to an UV cuvette. Samples were placed in the light scattering machine; each cycle ran for 200 sec at a laser angle of 90 °. This was repeated in triplicate. The machine determined the mean particle size in nanometres (nm) of the particles in suspension. The experiment was repeated under 'dirty' conditions by adding BSA to the suspension at a final concentration of 3 g/L.

Table 2.7 shows the mean particle size of *S. aureus*, *A. baumannii*, *C. difficile* spores and *B. subtilis* spores determined by light scattering. The diameter of *S. aureus* is normally between 0.5 – 1.5 µm, the data obtained from light scattering fits in this size range (Harris *et al*., 2002). *A. baumannii* is usually 0.9 - 1.6 µm in diameter (Vaneechoutte *et al*., 2011); the mean diameter of the strain in this study was greater than this range, which may suggest aggregation of cells. A study has shown *C. difficile* spores range in diameter from 0.5 - 0.7 µm (Snelling *et al*., 2012). The mean particle sizes of *C. difficile* spores in the presence and absence of organic load were greater than this suggesting spore aggregation. It has been reported *B. subtilis* spores are in the size

region of 0.41 - 0.67 µm in diameter (Carrera *et al.*, 2007). *B. subtilis* spore diameters here were slightly higher than the top end of this range, both with and without BSA.

Statistical analysis by means of a paired *t*-test using SPSS software showed there was no statistical difference (see Table 2.7) between the mean diameter of cells or spores with and without organic load. The P-values were determined by comparing the particle size for each strain in the presence and absence of BSA. No significant difference suggests the addition of organic load did not influence cell aggregation for these particular strains.

Table 2.7 – Mean particle size of microorganism with and without organic load (3 g/L BSA) (n=3)

Microorganism	Mean diameter (nm) ± SD (no BSA)	Mean diameter (nm) ± SD (with BSA)	P-value
S. aureus	1332 ± 375	1251 ± 565	0.64
A. baumannii	2612.6 ± 856.4	3288.0 ± 1032.4	0.26
C. difficile	1730.4 ± 578.2	1500.2 ± 495.7	0.07
B. subtilis	916.4 ± 277	858.9 ± 371.9	0.38

2.9.2 Cell hydrophobicity

The surface hydrophobicity of the microorganisms was tested using the Microbial Adhesion to Hydrocarbon (MATH) test based on that of Rosenberg *et al.* (1980). Hydrophobicity is an important factor affecting the efficacy of antimicrobial surfaces since the hydrophobicity of a bacterial suspension may affect its interaction with a surface (OECD, 2008). The hydrocarbon used in this study was hexadecane ($C_{16}H_{34}$). The MATH test determined the percentage of bacterial cells adhered to a hydrocarbon; the higher the percentage the greater the hydrophobicity.

S. aureus and *A. baumannii* cultures were prepared as in section 2.3.2, centrifuged for 10 min at 940 *g* and pellets washed twice with phosphate buffered saline (PBS). Pellets were re-suspended in 10 mL PBS to yield approximately 10^8 cfu/mL. *C. difficile* and *B.*

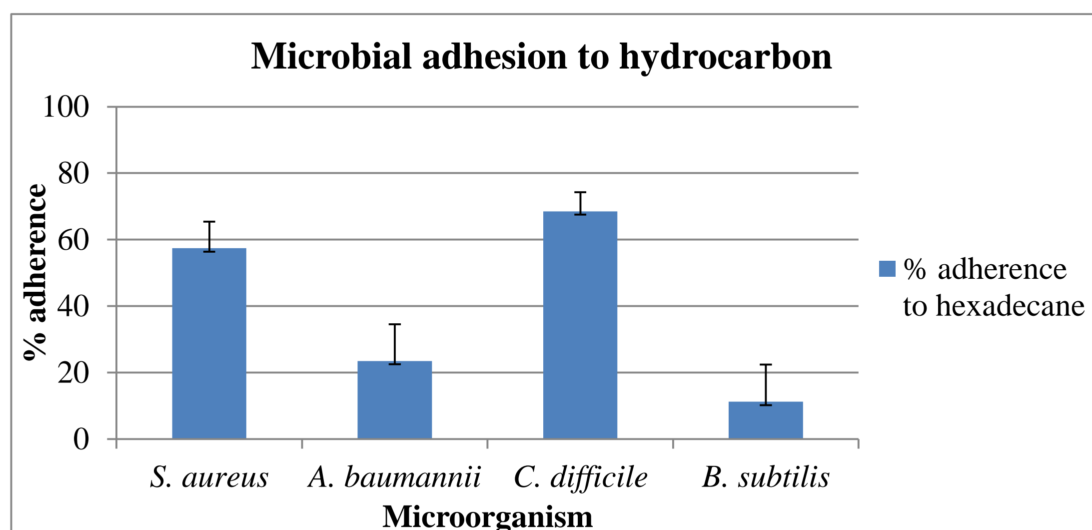
subtilis spore suspensions were prepared as in 2.6.3 and 2.7.1, respectively. Three mL of suspension was transferred to a McCartney bottle to which 800 μL hexadecane was added. Bottles were incubated for 10 min at 30 °C then vortexed for 1.5 min. The suspension was left to stand for 15 min then 1 mL of the aqueous layer was extracted with a Pasteur pipette and the OD_{600} was measured. The percentage adherence was calculated as follows:

$$\% \text{ adherence} = ((OD_{initial} - OD_{hexadecane}) / OD_{initial}) \times 100$$

The percentage of bacterial cells' adherence to hexadecane correlates to the level of hydrophobicity. A percentage adherence of >75 % means bacterial cells were hydrophobic. Adherence between 25 – 75 % suggests an intermediate hydrophobic/hydrophilic profile and adherence below 25 % suggest bacteria are hydrophilic (Cheeseman, 2010).

Figure 2.2 shows the hydrophobicity profile (% adherence ± SD) of *S. aureus*, *A. baumannii*, *C. difficile* spores and *B. subtilis* spores. *S. aureus* and *C. difficile* spores presented an average intermediate percentage adherence. *A. baumannii* and *B. subtilis* spores showed an average hydrophilic profile.

Figure 2.2 – Hydrophobicity profiles of *S. aureus*, *A. baumannii*, *C. difficile* spores and *B. subtilis* spores. Error bars represent the standard deviation.



2.10 Antimicrobial surfaces

Copper alloy test surfaces were kindly provided by the CDA, Hemel Hempstead, UK. The copper alloys were as follows; CuSn5, CuZn30, CuDHP and CuNi10Fe1Mn. Coupons measured 22 mm by 22 mm. Table 2.8 shows the chemical composition of the copper alloys. After testing, surfaces were disinfected by immersion in 70 % ethanol, dried then stored in a sterile Petri dish to prevent contamination. Control experiments confirmed disinfection by 70 % ethanol was sufficient to ensure surfaces were not contaminated post-disinfection. (NB. Surfaces were not re-used after bacterial spore testing).

Grade 2B finish stainless steel (Goodfellow Cambridge Ltd, Huntington, UK) with a composition of: C 0.08 %, Mn 2 %, P 0.045 %, S 0.03 %, Si 0.75 %, Cr 18-20 %, Ni 8-12 %, N 0.1 %, Fe balance was used as a control surface throughout antimicrobial surface testing. Discs were cleaned as described in 2.8. Discs measured 1 cm^2 in surface area.

Table 2.8 – Chemical composition of copper alloys

Metal type	**Formula**	**Composition**					
		% Cu	% Zn	% Sn	% Ni	% Fe	% Mn
Copper	CuDHP	99.99					
Brass	CuZn30	70	30				
Bronze	CuSn5	95		5			
Copper nickel	CuNi10Fe1Mn	86-89.7			9.0-11.0	1.0-2.0	0.3-1.0

2.11 Neutraliser toxicity and efficacy tests

2.11.1 Neutraliser toxicity

A neutraliser was utilised throughout antimicrobial surface testing (see Chapters 4 and 5) to neutralise the antimicrobial activity of surfaces. The neutraliser consisted of 3 g/L lecithin, 30 mL/L Tween 80, 5 g/L sodium thiosulphate, 1 g/L L-histidine, 10 mL phosphate diluent (34 g/L K_2HPO_4) and 30 g/L saponin all dissolved in 1 L deionised water. In order to be confident the neutraliser had no toxic effect on the tested microorganisms, a neutraliser toxicity test was initially carried out. *S. aureus* and *A. baumannii* were grown as described in 2.3.2 and re-suspended in 10 mL MRD to give a bacterial concentration of 10^9 cfu/mL. *B. subtilis* and *C. difficile* spore stocks were also tested. One mL of bacterial suspension was transferred into a 9 mL mix of neutraliser and MRD (9 mL MRD with 1 mL neutraliser). Water was used as a control; 1 mL of approximately 10^9 cfu/mL bacterial suspension was transferred to 9 mL deionised water. Mixtures were left for 5 min, and then viable bacteria were enumerated via the drop count method. This was repeated in triplicate. Table 2.9 shows the results from the toxicity test and counts are represented by $\log_{10}$ cfu/mL values. Statistical analysis by means of a one-way ANOVA using SPSS software showed there were no significant differences between the use of water and neutraliser (all P-values comparing water *vs.* neutraliser counts were greater than 0.05). It was therefore safe to assume the tested neutralisers had no toxic effect on *S. aureus*, *A. baumannii*, *C. difficile* spores and *B. subtilis* spores.

Table 2.9 – Neutraliser toxicity test results (n=3)

Microorganism	**Water ($\mathbf{Log_{10}}$ cfu/ml)**	**Neutraliser ($\mathbf{Log_{10}}$ cfu/mL)**	**P-value**
S. aureus	8.47 ± 0.17	8.42 ± 0.06	0.57
A. baumannii	8.42 ± 0.11	8.55 ± 0.09	0.133
C. difficile	7.94 ± 0.02	7.99 ± 0.02	0.098
B. subtilis	7.94 ± 0.03	7.99 ± 0.00	0.337

2.11.2 Neutraliser efficacy

To be certain the neutraliser of choice neutralised the antimicrobial activity of test copper surfaces, a neutraliser efficacy test was performed using the method described by Wheeldon *et al*. (2008). In brief, 50 µL of 10^8 cfu/mL *S. aureus* and 50 µL of neutraliser (1 mL neutraliser and 9 mL MRD) were mixed and inoculated on to CuDHP in triplicate for 30 min. CuDHP was chosen for testing as this surface contained the highest copper content of the four provided copper alloys. Five min was the maximum neutralisation time during surface testing (see Chapters 4 and 5), however, 30 min was tested in this experiment in order to observe antimicrobial activity by copper. Water was used as a control in place of the neutraliser for each test. After 30 min exposure surfaces were transferred to a 100 mL bottle containing 5 g glass beads and 10 mL MRD. Viable bacteria were determined by serial dilution using the drop count method. *A. baumannii* and *B. subtilis* spores were also tested in the same way. *C. difficile* spores were not tested as they were not utilised during antimicrobial surface testing (see Chapter 4, section 4.2.3).

Table 2.10 shows the viable counts of the initial suspension inoculated on to surfaces and the amount recovered from surfaces after exposure to bacteria containing neutraliser or water. There was less <1 $\log_{10}$ reduction between the bacterial counts after neutralisation with neutraliser and the initial viable count, confirming the suitability of the neutraliser as a neutraliser for antimicrobial copper surface testing. When water was used in place of neutraliser it is apparent there was antimicrobial activity by copper against *S. aureus* and *A. baumannii* but not against *B. subtilis* spores after 30 min.

Table 2.10 – Neutraliser efficacy test results (n=3)

Microorganism	Test	Average Log_{10} cfu/mL ± SD
S. aureus	Initial viable count	6.82 ± 0.06
	Neutraliser	7.09 ± 0.10
	Water	5.35 ± 0.34
A. baumannii	Initial viable count	7.54 ± 0.10
	Neutraliser	7.35 ± 0.02
	Water	5.60 ± 0.13
B. subtilis	Initial viable count	8.18 ± 0.27
	Neutraliser	8.21 ± 0.04
	Water	8.29 ± 0.03

CHAPTER 3 HOSPITAL SAMPLING

3.1 Introduction

The JIS Z 2801 test, as described in detail in Chapter 1, section 1.11.1 and Chapter 4, section 4.1.6, is the current recognised antimicrobial surface efficacy test. Its test conditions (35 °C and 100 % relative humidity (RH)) bear little relationship to in-use conditions, such as a UK hospital environment. Thus, the performance of antimicrobial surfaces in the laboratory under JIS Z 2801 test conditions does not guarantee the same antimicrobial efficacy in indoor conditions, where temperature and relative humidity are lower in the UK. Examples of varying efficacy of antimicrobial surfaces under varying relative and humidity temperature conditions have been reported and support the need for a new antimicrobial surface test using more appropriate environmental parameters (see Chapter 1, section 1.10.2).

3.1.1 Recommended temperature and relative humidity conditions in NHS hospitals

In the UK there are guidelines for minimum temperature conditions in the workplace. Although not a legal obligation, Workplace Regulations recommend the minimum temperature inside the workplace must be at least 16 °C or 13 °C if the indoor nature of the work involves physical activity. However, legally work places must be of 'reasonable' temperature, which depends on the nature and environment of the work. There is no maximum indoor temperature limit. The Health and Safety Executive states this is due to other influences such as radiant temperature, relative humidity and air velocity that affect ones comfort (Website 28, publication date unavailable).

Regarding temperature within the hospital environment the DH recommends a temperature range of 16 to 25 °C, which is flexible according to local needs. Ideally, temperatures in patient areas during summer should not be higher than 28 °C for 50 hours per year. In terms of relative humidity, due to the high costs associated with running and maintaining humidifiers and de-humidifiers, humidification is normally only provided when necessary. If humidifiers are used output is normally set at 70 %. Incoming air, if higher than 70 % relative humidity, may not necessarily be reduced by the humidifier. The upper relative humidity limit is set to 70 % to reduce condensation

on surfaces, particularly in winter. Overall, the DH suggests a relative humidity between 35 % and 70 % in hospital wards. In operating theatres the DH recommends stable conditions with temperature ranging from 19 - 23 °C and relative humidity from 35 - 60 % (DH, 2007c).

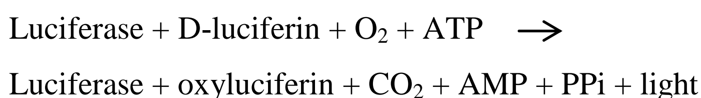
3.1.2 Defining high touch surfaces

If antimicrobial surfaces are to be utilised in healthcare settings they are most likely to be placed in areas of high touch. High touch surfaces within a hospital environment are surfaces that are touched frequently (Huslage *et al*., 2010). Since such surfaces are touched frequently they may potentially harbour microorganisms and also play a role in their transmission (Huslage *et al*., 2010). As described in Chapter 1, section 1.6, the NHS has produced several guidance documents identifying surfaces in a hospital ward that require cleaning and the recommended frequency of cleaning. However, areas of high-touch are not clearly defined. Huslage *et al*. (2010) set out to identify, by quantitative assessment, which surfaces are high-touch. Over an 18 month period five ICUs and seven medical-surgical floors were observed in a large teaching hospital in North Carolina, USA. Common surfaces to all five ICUs and all seven medical-surgical floors were identified then observed for contact frequency. Overall five surfaces were found to be high-touch and they were; bed rails, bed surfaces, supply carts, over-bed tables, and intravenous pumps. In the ICUs bed rails, bed surfaces and supply carts were high touch. Bed rails, over-bed tables, intravenous pumps, and the bed surface were found to be high touch in the medical-surgical wards (Huslage *et al*., 2010).

3.1.3. Surface bioburden

An aspect considered in this chapter is surface bioburden in hospital wards. One way to measure levels of surface contamination is with ATP bioluminescence monitors. The use of these as part of infection control measures and their advantages and disadvantages are introduced in Chapter 1, section 1.6.2. ATP bioluminescence monitors work by providing a Relative Light Unit (RLU) reading that corresponds to the amount of ATP collected on a swab used to swab a surface of interest. The swab

contains a liquid-stable luciferin-substrate/luciferase-enzyme system that emits light in direct proportion to the amount of ATP collected on the swab. Light is emitted by the oxidation of luciferin by luciferase, which requires oxygen and ATP. Once luciferin is oxidised photons are released, which are detected by the ATP monitor and quantified as RLU (Website 29, 2014 and Website 30, publication date unavailable). The chemical reaction that takes place is as follows (Boyce *et al*., 2009):



Luciferase + D-luciferin + O_2 + ATP $\rightarrow$
Luciferase + oxyluciferin + CO_2 + AMP + PPi + light

NB. AMP = adenosine monophosphate
PPi = inorganic pyrophosphate

The RLU reading provided can be classed as a 'pass' or 'fail' in relation to surface cleanliness, depending on the set benchmarks.

3.1.5 Aims and Objectives

The aim of this section was to gain information about the relative humidity, temperature and bioburden on a number of surfaces in a hospital environment by regular environmental sampling. This information was necessary to develop the *in vitro* surface efficacy tests described in Chapters 4 and 5 to investigate the efficacy of antimicrobial surfaces using appropriate physical parameters that mimic conditions found in practice. This will help assess the performance and suitability of surfaces for potential use in a UK hospital environment.

3.2 Materials and Methods

3.2.1 Selection of wards and surfaces for environmental sampling

The University Hospital of Wales (UHW), Cardiff, UK was the hospital site of choice for sampling of surfaces. Gastroenterology and Adult Critical Care Services (ACC)

wards were selected for surface temperature, relative humidity and bioburden sampling. In addition, surfaces in one of the hospital's theatre rooms were sampled. It was not possible to sample the same theatre room on each occasion. As a local hospital rule, one operating theatre room is free at any given time to accommodate emergency procedures, and this room was normally allocated for sampling. Sampling took place every two months over a one year period to allow for any seasonal variations. In total there were six sampling sessions. Sampling took place in the morning after normal cleaning. On one occasion (fourth sampling session, October 2011) sampling in theatre was not possible at all due to restrictions by theatre staff.

On each occasion surfaces sampled in gastroenterology included door handles (n=2), door push plates (n=2), computer keyboard (n=1) and mouse (n=1), trolleys (n=2), light switch (n=1), tap (n=1), wall panels (n=2), waste bin (n=1), bed grab rails (n=3), chairs (n=3) and tables (n=2).

In ACC the surfaces sampled were; door handles (n=2), door push plates (n=2), computer keyboard (n=1) and mouse (n=1), bed grab rails (n=2), chairs (n=3), trolleys (n=2), tables (n=2), light switch (n=1), wall panels (n=2) and waste bin (n=1).

In theatre, door handles (n=2), door push plates (n=2), computer keyboard (n=1), trolleys (n=3), anaesthetists stand (n=1), light switch (n=1), tap (n=1), wall panels (n=2) and waste bin (n=1) were sampled.

When possible the same surface was sampled on each occasion. These surfaces were selected based on general knowledge of frequently touched areas, surfaces within patient vicinity and from the study by Huslage *et al.* (2010).

3.2.2 Surface relative humidity and temperature measurements

A Protimeter Moisture Measurement System (MMS) (GE Sensing, Taunton, UK) was used to take relative humidity and air, surface and dew point temperature readings according to the manufacturer's guidelines. An image of the device is shown in Figure 3.1. Relative humidity is defined earlier (see Chapter 1, section 1.10.2). The dew point

temperature can be described as ‘the temperature to which the air would have to cool (at constant pressure and constant water vapour content) in order to reach saturation’. The dew point temperature is always lower than the air temperature. If the dew point is equal to the air temperature the air is saturated. If there is a small difference in the air temperature and dew point temperature then the relative humidity is high. If the difference between the two temperatures is great the relative humidity is low (Website 31, 2010).

The Protimeter MMS device has three measurement modes, all of which were utilised. Firstly the instrument was used in search mode; relative moisture readings of surfaces were measured. A reading of 60 – 1000 and an indication of whether a surface was in a dry, wet or at risk condition were provided. For this mode the Protimeter MMS was placed directly on the surface of interest.

Next the hygrometer mode of the device was used to take readings of the mixing ratio (AbS), surface relative humidity (% RH), surface air temperature (T_{AIR}, °C) and surface dew point temperature (T_{DEW}, °C). Here a hygrostick attachment was added to the Protimeter MMS and this was placed directly on the sampled surface. The hygrometer mode has a relative humidity measurement range of 30 - 100 %.

The condensator mode provided an indication of whether there was a risk of condensation on a surface. Surface relative humidity (% RH), surface air temperature (T_{AIR}, °C), surface dew point temperature (T_{DEW}, °C), surface temperature (T_S, °C) and temperature difference (T_{DIFF}, °C) were measured with the condensator mode. For this mode a surface temperature probe was attached to the Protimeter MMS and placed over the area of interest. The temperature difference reading was helpful as this reading indicated whether a surface is at risk of condensation. The temperature difference reading is the difference between the dew point temperature and the surface temperature. If the difference is more than 3 °C a ‘no condensation’ reading is given. If the difference is 3 °C or less an ‘at risk, no condensation’ message is given. Finally, if the surface temperature equals or is less than the dew point temperature a ‘condensation’ message is given. These are the guidelines provided in the device’s instruction manual.

Figure 3.1 – Picture of Protimeter MMS

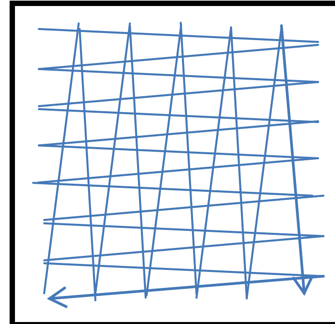
3.2.3 Surface bioburden measurements

Levels of surface bioburden were determined using a SystemSURE Plus ATP Hygiene Monitoring System (Hygiena, Watford, UK), which measured ATP levels (Figure 3.2a). The device was utilised according to the manufacturer's guidelines. The swabs used, Ultrasnap, (Hygiena, Watford, UK) contained a liquid-stable luciferase/luciferin agent that emitted light in direct proportion to the amount of ATP collected on the swab. The user is then provided with one of three readings; pass, caution or fail depending on the cleanliness of a surface. According to the device's instruction manual, a reading of less than 10 indicated that the surface was clean (pass). A reading between 11 - 29 indicated that the surface was not adequately clean (caution) and a reading over 30 suggested a surface was dirty (fail). The minimum possible RLU value was 0. Where possible a 10 cm x 10 cm area of the sampled surface was swabbed in a zig-zag motion, as shown in Figure 3.2b.

Figure 3.2 – ATP bioluminescence monitor (a) and the zig-zag pattern used for swabbing surfaces (b)

a) ATP bioluminescence monitor

b) zig-zag motion for swabbing surfaces



3.2.4 Statistical analysis

Data were analysed statistically by ANOVA with SPSS software. Post-hoc analysis by means of a Tukey test allowed identifying specifically where, if any, significant differences were. P-values, mean differences and 95 % confidence intervals (CI) are provided with the analysis.

3.3 Results

A comprehensive data set of surface temperature, relative humidity and bioburden was obtained from six sampling sessions over a period of one year. Sampling in theatre was not possible on the fourth sampling occasion due to hospital restrictions.

Table 3.1 is a summary of data collected showing average and range readings from the surfaces sampled in each ward on each sampling occasion. The average readings from the three wards combined per sampling session (numbers in bold) are also noted. The data shown are average and range relative humidities and surface air temperatures measured by the hygrometer mode. Dew point temperatures, surface temperatures and temperature differences measured using the condensator mode of the Protimeter MMS are also shown. Average and range RLU readings from the surfaces sampled in each

ward on each occasion are also shown Table 3.1. For raw data see Appendix 1, Tables 1 - 17.

Surface relative humidity, surface air temperature, surface temperature and bioburden data were analysed statistically. Statistical analysis of data was carried out to see if there were significant differences in the data collected across wards and across sampling sessions; one-way ANOVAs were used to analyse data across wards and across sampling sessions. P-values and 95 % CIs for the mean differences between wards or sampling sessions are provided, with a $P<0.05$ showing significance. Post-hoc analysis by means of a Tukey analysis allowed for identifying specific differences between sessions or wards. Differences in surface relative humidity, surface air temperature, surface temperature and surface bioburden across sampling sessions were analysed. Tables 3.4, 3.7, 3.10 and 3.14 show the post-hoc Tukey analysis results that allowed for identifying differences between the six sampling sessions, with significant differences highlighted in red.

Analysis of differences in relative humidity, surface air temperature, surface temperature and surface bioburden across wards over the entire sampling period was also conducted. Post-hoc analysis results are shown in Tables 3.3, 3.6, 3.9 and 3.13 and significant differences are highlighted.

Table 3.1 – Data collected from environmental sampling of surfaces at UHW

		HYGROMETER MODE			
Date	**Ward**	**Mean RH ±SD (%)**	**RH range (%)**	**Mean T_{AIR} ±SD (°C)**	**T_{AIR} range (°C)**
19/04/2011	Gastroenterology	34.6 ± 3.8	30.4 – 41.2	24.6 ± 1.7	21.0 – 28.5
	ACC	31.4 ± 0.5	30.5 – 32.6	24.3 ± 0.5	23.6 – 25.5
	Theatre Room	41.4 ± 2.3	37.2 – 44.7	22.3 ± 1.4	20.6 – 25.1
	Average	**35.8 ± 5.1**		**23.8 ± 1.3**	
17/06/2011	Gastroenterology	50.6 ± 2.4	46.7 – 58.2	22.8 ± 1.3	19.9 – 24.5
	ACC	45.4 ± 1.7	42.0 – 48.2	22.3 ± 0.7	21.2 – 23.7
	Theatre Room	46.5 ± 3.2	43.8 – 56.9	21.8 ± 0.7	21.1 – 23.1
	Average	**47.5 ± 2.7**		**22.3 ± 0.5**	
16/08/2011	Gastroenterology	63.6 ± 0.4	62.6 – 64.2	22.4 ± 0.6	20.4 – 23.1
	ACC	58.0 ± 1.8	54.2 – 61.4	24.2 ± 0.5	23.2 – 25.3
	Theatre Room	54.4 ± 4.2	47.7 – 61.8	20.9 ± 1.3	19.0 – 23.3
	Average	**58.7 ± 4.6**		**22.5 ± 1.7**	
18/10/2011	Gastroenterology	44.8 ± 3.3	39.2 – 52.1	21.8 ± 0.7	20.5 – 22.6
	ACC	38.5 ± 3.4	33.8 – 44.2	22.9 ± 0.5	22.1 – 23.8
	Average	**41.7 ± 4.5**		**22.4 ± 0.8**	
15/12/2011	Gastroenterology	42.1 ± 2.7	36.7 – 45.7	23.1 ± 1.0	21.2 – 24.6
	ACC	33.7 ± 2.2	31.3 – 38.1	23.1 ± 0.8	21.7 – 24.1
	Theatre Room	34.4 ± 0.7	32.9 – 35.1	20.2 ± 0.5	19.8 – 21.4
	Average	**36.7 ± 4.7**		**22.2 ± 1.7**	

Table 3.1 continued – Data collected from environmental sampling of surfaces at UHW

		HYGROMETER MODE			
Date	**Ward**	**Mean RH ±SD (%)**	**RH range (%)**	**Mean T_{AIR} ±SD (°C)**	**T_{AIR} range (°C)**
14/02/2012	Gastroenterology	37.6 ± 4.5	31.3 – 42.9	22.9 ± 0.9	21.8 – 25.2
	ACC	36.8 ± 3.8	31.3 – 43.0	24.0 ± 1.1	22.9 – 28.0
	Theatre Room	46.2 ± 0.9	43.8 – 47.6	21.8 ± 0.5	21.1 – 23.1
	Average	**40.2 ± 5.2**		**22.9 ± 1.1**	

Table 3.1 continued – Data collected from environmental sampling of surfaces at UHW

		CONDENSATOR MODE					
Date	**Ward**	**Mean T_{DEW} ±SD (°C)**	**T_{DEW} range (°C)**	**Mean T_S±SD (°C)**	**T_S range (°C)**	**Mean T_{DIFF} ±SD (°C)**	**T_{DIFF} range (°C)**
19/04/2011	Gastroenterology	7.1 ± 2.1	4.3 – 11.7	24.3 ± 1.4	22.0 – 26.8	17.2 ± 1.6	13.3 – 19.4
	ACC	6.1 ± 0.3	5.5 – 6.7	23.8 ± 0.3	23.3 – 24.3	18.0 ± 0.2	17.7 – 18.3
	Theatre Room	8.3 ± 0.4	7.7 – 8.7	22.4 ± 1.2	20.9 – 24.3	14.1 ± 1.0	13.0 -15.9
	Average	**7.2 ± 1.1**		**23.5 ± 1.0**		**16.5 ± 2.1**	
17/06/2011	Gastroenterology	11.7 ± 0.5	11.0 – 12.6	23.2 ± 0.7	21.9 – 24.2	11.4 ± 0.5	10.3 – 12.2
	ACC	9.6 ± 0.3	9.2 – 10.0	22.0 ± 0.9	20.9 – 23.2	12.4 ± 0.8	11.4 – 13.6
	Theatre Room	9.3 ± 0.2	9.0 – 9.6	20.9 ± 0.4	20.2 – 21.3	11.5 ± 0.6	10.2 – 12.2
	Average	**10.2 ± 1.3**		**22.0 ± 1.1**		**11.8 ± 0.6**	
16/08/2011	Gastroenterology	15.3 ± 0.2	15.0 – 15.9	22.4 ± 0.5	21.6 – 24.0	7.0 ± 0.4	6.3 – 8.2
	ACC	15.3 ± 1.9	15.0 – 15.9	23.2 ± 0.2	22.8 – 23.6	7.7 ± 0.4	7.0 – 8.3
	Theatre Room	11.5 ± 0.4	11.1 – 12.7	19.7 ± 1.0	18.2 – 21.3	8.2 ± 0.9	7.0 – 9.7
	Average	**14.1 ± 2.2**		**21.8 ± 1.1**		**7.6 ± 0.5**	
18/10/2011	Gastroenterology	9.5 ± 1.7	6.2 – 11.7	22.3 ± 0.4	21.6 – 23.2	13.5 ± 1.2	10.4 – 15.1
	ACC	7.9 ± 1.5	5.6 – 10.6	22.2 ± 0.4	21.5 – 22.9	13.9 ± 1.7	11.0 – 16.7
	Average	**8.7 ± 1.2**		**22.3 ± 0.0**		**13.7 ± 0.3**	
15/12/2011	Gastroenterology	9.4 ± 2.0	6.1 – 11.4	23.9 ± 1.0	21.9 – 25.3	14.4 ± 1.7	11.7 – 17.4
	ACC	5.8 ± 1.0	4.9 – 9.3	22.7 ± 0.3	21.9 – 23.1	16.9 ± 1.1	13.7 – 18.0
	Theatre Room	3.7 ± 0.4	3.2 - 4.4	20.2 ± 0.6	19.2 – 21.4	16.5 ± 0.8	15.5 – 18.5
	Average	**6.3 ± 2.9**		**22.3 ± 1.9**		**15.9 ± 1.3**	

Table 3.1 – Data collected from environmental sampling of surfaces at UHW

		CONDENSATOR MODE					
Date	**Ward**	**Mean T_{DEW} ±SD (°C)**	**T_{DEW} range (°C)**	**Mean T_S±SD (°C)**	**T_S range (°C)**	**Mean T_{DIFF} ±SD (°C)**	**T_{DIFF} range (°C)**
14/02/2012	Gastroenterology	8.5 ± 1.6	6.0 – 10.7	23.1 ± 1.1	20.7 – 24.6	14.6 ± 3.1	7.8 – 17.8
	ACC	8.5 ± 1.8	5.3 – 10.9	23.8 ± 0.4	23.3 – 24.7	15.3 ± 1.6	13.5 – 18.1
	Theatre Room	10.4 ± 0.7	9.9 – 12.6	22.6 ± 0.3	22.2 – 23.0	12.9 ± 0.4	12.2 – 13.3
	Average	**9.1 ± 1.1**		**23.2 ± 0.6**		**14.3 ± 1.2**	

Table 3.1 continued – Data collected from environmental sampling of surfaces at UHW

		BIOBURDEN	
Date	**Ward**	**Mean RLU**	**RLU range**
19/04/2011	Gastroenterology	110 ± 146	0 - 601
	ACC	149 ± 241	4 - 890
	Theatre Room	87 ± 78	1 – 199
	Average	**115 ± 31**	
17/06/2011	Gastroenterology	46 ± 79	0 - 365
	ACC	68 ± 76	2 - 249
	Theatre Room	19 ± 25	1 - 96
	Average	**44 ± 24**	
16/08/2011	Gastroenterology	165 ± 359	0 - 1456
	ACC	30 ± 26	1 -87
	Theatre Room	26 ± 17	4 - 64
	Average	**74 ± 79**	
18/10/2011	Gastroenterology	30 ± 38	2 - 77
	ACC	26 ± 22	0 - 140
	Average	**28 ± 3**	
15/12/2011	Gastroenterology	103 ± 121	9 - 431
	ACC	45 ± 67	1 - 281
	Theatre Room	52 ± 35	7 - 117
	Average	**67 ± 32**	
14/02/2012	Gastroenterology	54 ± 65	0 - 277
	ACC	78 ± 151	6 – 615
	Theatre Room	42 ± 27	5 – 95
	Average	**58 ± 18**	

Key

RH – relative humidity

$\mathbf{T_{AIR}}$ – air temperature

$\mathbf{T_{DEW}}$ – dew point temperature

$\mathbf{T_{S}}$ – surface temperature

$\mathbf{T_{DIFF}}$ – temperature difference

RLU – relative light units

3.3.1 Surface relative humidity findings

Surface relative humidity observations were measured using the hygrometer mode of the Protimeter MMS. Mean ± SD and median values are shown in Table 3.2. Mean surface relative humidity readings were similar in gastroenterology and theatre. Mean relative humidity in ACC was slightly lower at 40.6 ± 9.3 %. Across the six sampling sessions surface relative humidity ranged from 30.4 – 64.2 % in gastroenterology, 30.5 - 61.4 % in ACC and 32.9 – 61.8 % in theatre, see Table 3.2. The ranges are approximately similar. For all three wards the overall range in surface relative humidity readings from all six sampling session was high, which suggests there was great variability between sample sessions.

Table 3.2 – Range, mean and median relative humidity readings from each ward across the six sampling sessions

Ward	RH range (%)	Mean RH ± SD (%)	Median RH (%)
Gastroenterology (n=124)	30.4 – 64.2	45.4 ± 10.1	44
ACC (n=114)	30.5 – 61.4	40.6 ± 9.3	38.2
Theatre (n=69)	32.9 – 61.8	44.6 ± 7.1	45.4

Surface relative humidity ranges per ward, per sample session ranges are shown in Table 3.1. From all six sampling sessions in gastroenterology the highest relative humidity of 64.2 % was recorded from a bed rail (August 2011) and the lowest, 30.4 %, from a wall panel (April 2011). The highest surface relative humidity of 61.4 % in ACC was from a wall panel (August 2011) and the lowest, 30.5 %, was observed on a door push plate (April 2011). In theatre, the highest surface relative humidity, 61.8 %, was from a wall panel (August 2011) and the lowest, 32.9 %, from a door handle (December 2011).

In gastroenterology, the greatest range in surface relative humidity readings was observed during the fourth sampling session (39.2 – 52.1 %) and the smallest on the third sampling occasion (62.6 – 64.2 %). The greatest range in surface relative humidity in ACC was observed on the sixth sampling occasion (31.3 – 43 %) and the lowest on the first occasion (30.5 – 32.6 %). Finally in theatre, the greatest range in surface relative humidity was on

the third sampling occasion (47.7 – 61.8 %) and the smallest from the fifth occasion (32.9 – 35.1 %).

In terms of average surface relative humidity observations, overall averages from individual wards ranged from 31.4 ± 0.5 % (ACC, April 2011) to 63.6 ± 0.4 % (gastroenterology, August 2011). Average relative humidity readings of all the three wards from each session ranged from 35.7 ± 5.1% (April 2011) to 58.7 ± 4.6 % (August 2011).

Surface relative humidity readings recorded from the hygrometer mode were analysed statistically. Overall, over the sampling period the ward type had a significant effect on surface relative humidity observations (ANOVA, P=0.044). However, comparisons by a post-hoc Tukey test showed no significant difference between wards, shown in Table 3.3. All P-values are >0.05, suggesting no significant differences in surface relative humidity between wards.

Table 3.3 – Surface relative humidity comparisons across wards over the six sampling sessions

Ward comparison	P-value	Mean difference	95 % CI
Gastroenterology *vs*. ACC	0.061	2.77	-0.10 – 5.64
ACC *vs*. Theatre	0.127	2.80	-0.58 – 6.18
Gastroenterology *vs*. Theatre	>0.999	0.03	-3.38 – 3.32

Overall there was a significant difference in surface relative humidity between the sampling sessions in all three wards combined (ANOVA, P<0.001). Post-hoc analysis allowed identification of differences in surface relative humidity readings between sampling sessions. Significant differences were observed between all sessions except between April 2011 and December 2011, October 2011 and February 2012 and between December 2011 and February 2012, see Table 3.4.

Table 3.4 – Post-hoc analysis to show comparisons in surface relative humidity between sampling sessions. Significant differences ($P<0.05$) are highlighted in red.

Session comparison	P-value	Mean difference	95 % CI
April 2011 *vs*. June 2011	P<0.001	12.49	9.98 – 15.00
April 2011 *vs*. August 2011	P<0.001	24.29	21.79 – 26.81
April 2011 *vs*. October 2011	P<0.001	6.70	3.99 – 9.41
April 2011 *vs*. December 2011	0.250	1.91	-0.60 – 4.42
April 2011 *vs*. February 2012	P<0.001	4.41	1.91 – 6.91
June 2011 *vs*. August 2011	P<0.001	11.80	9.29 – 14.31
June 2011 *vs*. October 2011	P<0.001	5.79	3.08 – 8.50
June 2011 *vs*. December 2011	P<0.001	10.58	8.07 – 13.09
June 2011 *vs*. February 2012	P<0.001	8.09	5.59 – 10.58
August 2011 *vs*. October 2011	P<0.001	17.60	14.89 – 20.30
August 2011 *vs*. December 2011	P<0.001	22.39	19.88 – 24.90
August 2011 *vs*. February 2012	P<0.001	19.89	17.39 – 22.39
October 2011 *vs*. December 2011	P<0.001	4.79	2.09 – 7.50
October 2011 *vs*. February 2012	0.146	2.29	-0.40 – 4.99
December 2011 *vs*. February 2012	0.05	2.50	-0.00 -5.00

3.3.2 Air and surface temperature findings

3.3.2.1 Air temperature

Air temperature readings of surfaces were recorded using the hygrometer mode of the Protimeter MMS. Mean ± SD and median values are shown in Table 3.5. Mean surface air temperature was highest in ACC and lowest in theatre. Across the six sampling sessions surface air temperature ranged from 19.9 – 28.5 °C in gastroenterology, 21.2 – 28.0 °C in ACC and 19.0 – 25.1 °C in theatre.

Table 3.5 – Range, mean and median air temperature readings from each ward across the six sampling sessions

Ward	Mean air temp. range (°C)	Mean air temp. ± SD (°C)	Median air temp. (°C)
Gastroenterology (n=124)	19.9 – 28.5	22.9 ± 1.4	22.8
ACC (n=114)	21.2 – 28.0	23.5 ± 1.0	23.5
Theatre (n=69)	19.0 – 25.1	21.4 ± 1.2	21.4

Table 3.1 shows surface air temperature ranges per ward, per sample session. Taking into account all six sampling sessions, the highest air temperature in gastroenterology, 28.5 °C was taken from a tap (April 2011) and the lowest, 19.9 °C from a door handle (June 2011). The highest surface air temperature of 28.0 °C in ACC was obtained from a computer keyboard (February 2011) and the lowest, 21.2 °C, from a table (June 2011). In theatre the highest surface air temperature reading, 25.1 °C, was taken from a door handle (April 2011) and the lowest, 19.0 °C, from a wall panel (December 2011).

In gastroenterology, the greatest range in surface air temperature readings was observed in the first sampling session (21.0 – 28.5 °C) and the smallest on the fourth sampling occasion (20.5 – 22.6 °C). The greatest range in ACC was observed on the sixth session (22.9 – 28.0 °C) and the lowest on the fourth occasion (22.1 – 23.8 °C). The greatest range in surface air temperature observed in theatre was during the first sampling session (20.6 – 25.1 °C) and the smallest on the fifth occasion (19.8 – 21.4 °C).

Also noted in Table 3.1 are mean surface air temperatures values. Overall mean surface air temperatures from individual wards ranged from 20.2 ± 0.5 °C (theatre, December 2011) to 24.6 ± 1.7 °C (gastroenterology, April 2011). Average temperature readings of all the three wards from each sampling session ranged from 22.2 ± 1.7 °C (December 2011) to 23.8 ± 1.3 °C (April 2011).

Over the one year period there was a significant difference in air temperature across the three wards sampled (ANOVA, P<0.001). Post-hoc analysis by means of a Tukey test to identify differences between groups showed there were significant differences between all three wards (all comparisons P<0.05), as shown in Table 3.6.

Table 3.6 – Air temperature comparisons across wards over the six sampling sessions

Ward comparison	P-value	Mean difference	95 % CI
Gastroenterology *vs*. ACC	0.002	0.54	0.16 – 0.91
ACC *vs*. Theatre	<0.001	2.09	1.65 – 2.53
Gastroenterology *vs*. Theatre	<0.001	1.55	1.12 – 2.00

Overall there were significant differences in surface air temperature between the six sampling sessions (ANOVA, P<0.001). Post-hoc analysis of surface air temperature data allowed for comparisons between the different sampling sessions, as shown in Table 3.7. Statistically there were significant differences between the first sampling session (April 2011) and all other sampling occasions. No other significant differences were observed.

Table 3.7 – Post-hoc analysis to show comparisons in air temperature between sampling sessions. Significant differences (P<0.05) are highlighted in red.

Session comparison	P-value	Mean difference	95 % CI
April 2011 *vs.* June 2011	<0.001	1.61	0.86 – 2.36
April 2011 *vs.* August 2011	<0.001	1.29	0.55 – 2.04
April 2011 *vs.* October 2011	<0.001	1.60	0.79 – 2.41
April 2011 *vs.* December 2011	<0.001	1.59	0.84 – 2.34
April 2011 *vs.* February 2012	0.004	0.95	0.21 – 1.70
June 2011 *vs.* August 2011	0.882	0.32	-0.43 – 1.07
June 2011 *vs.* October 2011	>0.999	0.01	-0.80 – 0.82
June 2011 *vs.* December 2011	>0.999	0.02	-0.73 – 0.77
June 2011 *vs.* February 2012	0.116	0.66	-0.09 – 1.41
August 2011 *vs.* October 2011	0.880	0.31	-0.50 – 1.11
August 2011 *vs.* December 2011	0.860	0.30	-0.45 – 1.05
August 2011 *vs.* February 2012	0.776	0.34	-0.40 – 1.08
October 2011 *vs.* December 2011	>0.999	0.01	-0.80 – 0.82
October 2011 *vs.* February 2012	0.191	0.65	-0.15 – 1.46
December 2011 *vs.* February 2012	0.140	0.64	-0.11 – 1.39

3.3.2.2 Surface temperature

Surface temperature observations were recorded using the condensator mode of the Protimeter MMS as this mode allows one to identify if a surface was at risk of condensation. It is worth noting that the sample size for surfaces tested for surface temperature was lower than the other parameters; this was due to difficulties experienced with the surface temperature probe. For this reason surface air temperature readings obtained with the hygrometer mode were also analysed, (see previous section, 3.3.2.1). Over the six sampling sessions surface temperatures ranged from 20.7 – 26.8 °C in gastroenterology, 20.9 – 24.7 °C in ACC and 18.2 – 24.3 °C in theatre, see Table 3.8. Mean and median values are also shown in Table 3.8. The lowest mean surface temperature reading was obtained in theatre, and the highest in gastroenterology.

Table 3.8 – Range, mean and median surface temperature readings from each ward across the six sampling sessions

Ward	Surface temp. range (°C)	Mean surface temp. ± SD (°C)	Median surface temp. (°C)
Gastroenterology (n=110)	20.7 – 26.8	23.2 ± 1.1	22.9
ACC (n=95)	20.9 – 24.7	22.9 ± 0.9	22.9
Theatre (n=56)	18.2 – 24.3	21.2 ± 1.4	21.2

Surface temperature ranges per ward, per sample session are shown in Table 3.1. Across all six sampling sessions the highest surface temperature measured in gastroenterology was 26.8 °C, on a bed rail (April 2011), and the lowest, 20.7 °C from a chair (February 2012). The highest surface temperature reading of 24.7 °C in ACC was taken from a chair (February 2011) and the lowest, 20.9 °C, from a bed rail (June 2011). In theatre, the highest surface air temperature reading, 24.3 °C, was observed on a door handle (April 2011) and the lowest, 18.2 °C, on a waste bin (August 2011).

In gastroenterology, the greatest range in surface temperature readings was seen on the first sampling session (22.0 – 26.8 °C) and the smallest on the fourth (21.0 – 23.2 °C). The greatest range in ACC was seen during the second session (20.9 – 23.2 °C) and the smallest on the third occasion (22.8 – 23.6 °C). Finally, in theatre the greatest range in

surface temperature readings was from the first sampling session (20.9 – 24.3 °C) and the smallest from the sixth occasion (22.2 – 23.0 °C).

Average surface temperature ranged from 19.7 ± 1.0 °C (Theatre room, August 2011) to 24.3 ± 1.4 °C (gastroenterology, April 2011), see Table 3.1. Overall average readings from the three wards from each sampling session ranged from 21.8 ± 1.1 °C (August 2011) to 23.5 ± 1.0 °C (April 2011).

There was a significant difference in surface temperature across the three wards over the one year sampling period (ANOVA, $P<0.001$). Post-hoc analysis showed comparisons between the three wards, as shown in Table 3.9. Significant differences in surface temperatures were observed between theatre and all other wards (both $P<0.05$) but not between gastroenterology and ACC ($P=0.150$).

Table 3.9 - Surface temperature comparisons across wards over the six sampling sessions

Ward comparison	P-value	Mean difference	95 % CI
Gastroenterology *vs*. ACC	0.150	0.30	-0.07 – 0.67
ACC *vs*. Theatre	<0.001	1.68	1.23 – 2.13
Gastroenterology *vs*. Theatre	<0.001	1.99	1.54 – 2.41

One-way ANOVA analysis of differences in surface temperature over the sampling period showed an overall significance difference between sessions, (ANOVA, $P<0.001$). Post-hoc Tukey analysis showed significant differences between particular sessions, highlighted in red, in Table 3.10.

Table 3.10 – Post-hoc analysis to show comparisons in surface temperature between sampling sessions. Significant differences ($P<0.05$) are highlighted in red.

Session comparison	P-value	Mean difference	95 % CI
April 2011 *vs*. June 2011	<0.001	1.35	0.59 – 2.13
April 2011 *vs*. August 2011	<0.001	1.89	1.13 – 2.64
April 2011 *vs*. October 2011	<0.001	1.42	0.62 – 2.22
April 2011 *vs*. December 2011	<0.001	1.11	0.38 – 1.85
April 2011 *vs*. February 2012	0.478	0.45	-0.28 – 1.18
June 2011 *vs*. August 2011	0.335	0.53	-0.23 – 1.29
June 2011 *vs*. October 2011	>0.999	0.07	-0.73 – 0.87
June 2011 *vs*. December 2011	0.937	0.24	-0.50 – 0.99
June 2011 *vs*. February 2012	0.006	0.90	0.17 – 1.63
August 2011 *vs*. October 2011	0.545	0.46	-0.33 – 1.26
August 2011 *vs*. December 2011	0.033	0.78	0.04 – 1.51
August 2011 *vs*. February 2012	<0.001	1.43	0.71 – 2.16
October 2011 *vs*. December 2011	0.866	0.31	-0.47 – 1.09
October 2011 *vs*. February 2012	0.005	0.97	0.20 – 1.73
December 2011 *vs*. February 2012	0.085	0.66	-0.05 – 1.36

3.3.2.2.1 Temperature difference data

Temperature difference data is how many degrees a surface is above or below its dew point temperature. Range, mean and median temperature difference data are shown in Table 3.11. The highest mean temperature difference was in ACC and the lowest in theatre. The greatest range in temperature difference data was observed in gastroenterology; 6.3 – 19.4 °C. The smallest range was measured in theatre 13.0 – 18.5 °C.

Table 3.11 – Range, mean and median temperature difference readings from each ward across the six sampling sessions

Ward	Temp. difference range (°C)	Mean temp. difference ± SD (°C)	Median temp. difference (°C)
Gastroenterology (n=109)	6.3 – 19.4	13.0 ± 3.7	13.1
ACC (n=95)	7.0 – 18.3	14.3 ± 3.2	14.4
Theatre (n=54)	13.0 – 18.5	12.6 ± 2.9	13.0

Overall the lowest temperature difference value was 6.3 °C (gastroenterology, August 2011). This reading was taken from a wall panel. The highest temperature difference value observed was 19.4 °C, from a table in gastroenterology (April 2011). Other surfaces that showed relatively low temperature difference values included; a trolley (6.5 °C), waste bin (6.6 °C), bed rail (6.7 °C) and wall panel (6.7 °C), all gastroenterology, August 2011. Average temperature difference readings from all three wards on each sampling occasion ranged from 7.6 ± 0.5 (August 2011) to 16.5 ± 2.1 (April 2011), see Table 3.1. When ranking average surface temperatures and average temperature difference values from each sampling session the general trend was the higher the surface temperature the higher the temperature difference reading.

Data obtained confirmed all surfaces sampled were not at risk of condensation as all surface temperatures exceeded their dew point temperatures by more than 3 °C.

3.3.3. Surface bioburden findings

Surface bioburden measurements were recorded with an ATP bioluminescence monitor. Mean and median values for each ward are shown in Table 3.12. The lowest mean RLU observation was in theatre and the highest in gastroenterology. Over the one year sampling period, surface bioburden ranged from 0 - 1456 RLU in gastroenterology, 0 - 890 RLU in ACC and 1 – 199 in theatre.

Table 3.12 – Range, mean and median surface bioburden readings from each ward across the six sampling sessions

Ward	RLU range	Mean ± SD (RLU)	Median (RLU)
Gastroenterology (n=124)	0 – 1456	84 ± 175	30
ACC (n=113)	0 – 890	66 ± 127	23
Theatre (n=70)	1 - 199	45 ± 46	28

In gastroenterology, the greatest range in surface bioburden readings was from the third sampling session (0 – 1456 RLU) and the smallest on the fourth sampling occasion (2 – 77 RLU). The greatest range in RLU readings in ACC was seen in the second session (4 – 890 RLU) and the lowest on the third occasion (1 – 87 RLU). Finally, in theatre the greatest range in surface bioburden was from the first sampling session (1 – 199 RLU) and the smallest from the third occasion (4 – 64 RLU).

Surface bioburden ranges per ward, per sample session are shown in Table 3.1. The highest surface bioburden reading measured in gastroenterology was 1456 RLU from a trolley (August 2011). The lowest RLU, 0, was observed on several sampling occasions. Surfaces that presented an RLU value of 0 included a waste bin, chairs, wall panel, door handle and computer keyboard. The highest surface bioburden reading in ACC was 890 RLU from a table (April 2011) and the lowest, 0 RLU, from a wall panel (October 2011). In theatre, the highest surface bioburden reading was 199 RLU, this was taken from a door push plate (April 2011). The lowest RLU, 1, was recorded from a tap on two occasions (April 2011 and June 2011).

Table 3.1 also shows the average surface bioburden measurements for each ward on each sampling occasion. The standard deviations of the means for each ward are great showing the high variability in bioburden on surfaces. Surface bioburden averages from individual wards ranged from 19 ± 25 (theatre, June, 2011) to 165 ± 359 (gastroenterology, August, 2011). Overall averages from all three wards on each sampling occasion ranged from 28 ± 3 (October 2011) to 115 ± 31 (April 2011).

Over the sampling period there was no significant difference in RLU values across the three wards (ANOVA, P=0.156). Post-hoc analysis of the test by Tukey analysis to find specific, if any, differences between groups showed no significant differences between all wards (all P>0.05), as shown in Table 3.13.

Table 3.13 – Surface bioburden comparisons across wards over the six sampling sessions

Ward comparison	P-value	Mean difference	95 % CI
Gastroenterology *vs*. ACC	0.569	18.07	-23.95 – 60.09
ACC *vs*. Theatre	0.564	21.31	-27.78 – 70.45
Gastroenterology *vs*. Theatre	0.135	39.37	-8.93 – 87.68

Overall there was a significant difference in RLU across the six sampling sessions (ANOVA, P=0.035), however, post-hoc Tukey analysis showed there was only a significant difference between sessions on one occasion; April 2011 and October 2011 (P=0.026). Between all other sessions there were no significant differences (all P>0.005), see Table 3.14.

Table 3.14 – Post-hoc analysis to show comparisons in surface bioburden between sampling sessions. Significant differences (P<0.05) are highlighted in red.

Session comparison	P-value	Mean difference	95 % CI
April 2011 *vs*. June 2011	0.095	69.30	-6.47 – 145.07
April 2011 *vs*. August 2011	0.778	34.52	-40.90 – 109.94
April 2011 *vs*. October 2011	0.026	88.20	6.50 – 169.90
April 2011 *vs*. December 2011	0.474	47.28	-28.49 – 123.05
April 2011 *vs*. February 2012	0.261	56.72	-18.70 – 132.14
June 2011 *vs*. August 2011	0.772	34.78	-40.64 – 110.20
June 2011 *vs*. October 2011	0.986	18.90	-62.79 – 100.59
June 2011 *vs*. December 2011	0.961	22.02	-53.75 – 97.79
June 2011 *vs*. February 2012	0.997	12.58	-62.84 – 88.00
August 2011 *vs*. October 2011	0.409	53.68	-27.69 – 135.05
August 2011 *vs*. December 2011	0.997	12.76	-62.65 – 88.18
August 2011 *vs*. February 2012	0.958	22.20	-52.86 – 97.27
October 2011 *vs*. December 2011	0.705	40.92	-40.78 – 122.61
October 2011 *vs*. February 2012	0.877	31.48	-49.89 – 112.85
December 2011 *vs*. February 2012	0.999	9.44	-84.86 – 65.98

3.4 Discussion

Hospital sampling of surfaces for observing changes in surface relative humidity, temperature and bioburden was completed over a one year period. These sessions provided an insight into environmental conditions inside wards in a UK hospital in terms of surface relative humidity, surface temperature and surface bioburden. To date there have been no studies assessing surface relative humidity and surface temperature conditions in a UK hospital.

As mentioned in section 3.1.1, it is recommended that the minimum temperature in a workplace should be at least 16 °C or 13 °C if one's work involves physical activity. The air temperatures of surfaces sampled in all wards on all occasions were above 16 °C; in compliance with the HSE recommendations. The DH does not have specific recommendations for surface temperatures within a hospital environment but does suggest a temperature of 16 – 25 °C in hospital wards. When taking into account the overall average air (measured by hygrometer mode of Protimeter MMS) and surface temperatures (measured by the condensator mode) recorded per ward and per sampling session all averages were within this recommended guideline (see Table 3.1) (DH, 2007c). With an overall surface air temperature (hygrometer mode) range of 19.9 - 28.5 °C recorded in both gastroenterology and ACC, a total of 14 of the 237 surfaces sampled in total in the two wards were above 25 °C. The surface temperatures recorded (condensator mode) ranged from 20.7 - 26.8 °C in gastroenterology and ACC; 6 surfaces of the total sampled surfaces presented a surface temperature >25 °C. A lower temperature range guideline for theatre rooms of 19 – 23 °C is recommended. Again, this is a general guideline and not specific to surface temperatures. However, average surface air and surface temperatures per theatre room and per sample session did fall within this range (see Table 3.1) (DH, 2007c). Overall surface air temperatures (hygrometer mode) in theatre ranged from 19 - 25.1 °C; 6 of the 68 total sampled surfaces were >23 °C. Surface temperatures (condensator mode) recorded in theatre ranged from 18.2 - 24.3 °C; 2 surfaces were below and 5 were above the recommended temperature limit. Significant differences in surface air and surface temperatures in wards combined between sampling sessions showed that temperature was variable over the one year sampling period.

In general hospital wards the DH recommends a relative humidity in the range of 35 – 70 % (DH, 2007c). As with temperature guidelines, these recommendations are generic and there are no specific surface guidelines. Nevertheless, all surfaces sampled in both gastroenterology and ACC were within this range. In theatre a relative humidity between 35 – 60 % is advised (DH, 2007c). Only one of the total number of surfaces sampled presented a relative humidity greater than 60 %. Significant differences in relative humidity between sampling sessions and the large range of surface relative humidities observed in each ward warrants the testing of antimicrobial surfaces, for use in hospital environments, under a range of varying relative humidities. It is apparent there are seasonal variations in relative humidity, which should be accounted for.

Temperature difference data showed all surfaces tested were not at risk of condensation since surface temperatures exceeded their dew point temperatures by more than 3 °C. The risk of a surface to condensation is worth considering in terms of the introduction of antimicrobial surfaces in the healthcare setting. For example, if a particular surface is at risk of condensation, and is in an area of high-touch then this surface could be an ideal candidate as an antimicrobial surface. The presence of condensation on the surface will likely boost the surfaces' antimicrobial activity. In this study no surfaces were at risk of condensation; however, of all the surfaces sampled those at the lower end of the range and thus relatively close to being at risk included wall panels, trolleys, waste bins and bed rails. Despite all surfaces tested in this study not presenting a risk of condensation they should not be dismissed for potential replacement with antimicrobial surfaces, as a range of other contributing factors to a surface's efficacy are important too (see Chapter 6, section 6.4).

According to the manufacturer guidelines of the ATP monitor a RLU reading of <11 is a 'pass', 11-29 a 'caution' and >30 a 'fail', in terms of surface cleanliness. With these guidelines in mind, overall in gastroenterology 25 % of surfaces passed the cleanliness test, 25 % were 'caution' and 50 % failed. In ACC the same proportion passed, 35 % of surfaces were 'caution' and 40 % failed. Finally in theatre 20 % of surfaces sampled passed, 31 % were 'caution' and 49 % failed. In the hospital environment <250 RLU has been identified as a 'pass' (Lewis *et al.*, 2008). Previously <500 RLU was regarded a 'pass' (Griffith *et al.*, 2000). If the <250 RLU pass/fail benchmark were to be applied

to this study then 92 % of surfaces in gastroenterology, 94 % of surfaces in ACC and 100 % of surfaces in theatre would have passed. Thus, it is important when using ATP bioluminescence monitors to set pass/fail thresholds according to the type of environment that is being tested. ATP monitoring systems are useful for applying clean, caution and fail criteria according to RLU values obtained immediately after cleaning. ATP bioluminescence monitors are convenient for screening surfaces after cleaning as results can be achieved very quickly (<2 min) compared to standard microbiological screening (>24 h) (Griffith *et al*., 2000).

On each occasion sampling of surfaces commenced at around 9 am in gastroenterology, at around 10.30 am in ACC and at approximately 12 pm in theatre. All wards at UHW are cleaned in the morning, usually starting between 8 - 9 am (personal communication, Senior Infection Control Nurse, UHW). Thus, it is likely the values obtained from gastroenterology represented cleaned surfaces. Values obtained from ACC and theatre were likely to be taken a short while after cleaning, therefore, do not represent cleaned surfaces. With this in mind, despite statistical analysis showing no significant differences between wards, the fact that the range of values represented different times on the cleaning/soiling cycle should be taken into account. The nature of sampling in this study across the three wards was at random points during the soiling/cleaning cycle thus the data obtained only provided a rough indication of the level of surface bioburden, not an indication of the efficacy of surface cleaning. This may also explain the high variability of the results observed. Nevertheless, the level of surface bioburden in the hospital environment from this study has been established, which will allow for setting up a test surface with similar RLU bioburden values for experimental work.

When considering the potential use of antimicrobial surfaces in healthcare settings it is important to test surfaces under in-use conditions. Michels *et al*. (2009) and Ojeil *et al*. (2013) observed reduced antimicrobial activity of silver and copper, respectively under in-use relative humidity and temperature conditions. The influence of temperature and humidity on biocidal activity are described in detail elsewhere, see Chapter 1, section 1.10.2 and Chapter 4, section 4.1.5.

Despite gaining a comprehensive data set of surface relative humidity and surface temperature conditions within a UK hospital there were limitations to this study. Firstly, sampling was limited to one hospital site in the UK. It is possible that different regions in the UK may present different surface relative humidity and surface temperature data. Initially, approval was granted for sampling at a second site; a university hospital in London, but unfortunately this was eventually not followed through. A second data set may have provided comparable results to those obtained at UHW. Secondly, the data obtained in this study should not be used to set parameters for antimicrobial surface efficacy testing for predicting the performance of antimicrobial surfaces in non-UK hospitals where climate and weather may vary greatly, in turn influencing indoor hospital conditions. For example, a study in Sweden to investigate relative humidity and temperature in an orthopaedic ward showed an average relative humidity of 22 % in winter and 46.2 % in summer. The measurements were recorded next to a patient bed (Skoog, 2006). In this study the surface relative humidities recorded were comparatively higher in both winter and summer. Finally, this was a small study limited to a one year period. More comprehensive sampling, for example at a more frequent rate than once every two months, may provide a greater insight into environmental conditions within hospitals.

Nevertheless, the data obtained was very useful for setting parameters in the antimicrobial surface efficacy tests developed in Chapters 4 and 5. Based on mean, median and range observations from the six sampling sessions it was decided that that relative humidities 40 % and 50 % and a temperature of 20 °C ([20°C-50% RH] and [20°C-40% RH]) would be used in experiments to reflect conditions observed at UHW.

The work in this thesis was focused on the potential use of antimicrobial surfaces in clinical areas. However, the potential use of such surfaces is not limited to the hospital environment. This project was carried out in collaboration with Campden BRI, a food and drinks research company. In this collaboration their research was geared towards the potential use of antimicrobial surfaces in food factories. As with this study Campden BRI believe the test conditions of the JIS Z 2801are not always reflective of the environment of intended antimicrobial surface use. Thus, environmental sampling of surfaces in food factories to gain an insight into environmental conditions within a

variety of food factories was carried out. Five different food factories were sampled on one occasion. Surfaces sampled and relative humidity and temperature observations are provided in Appendix 3a, Tables 1-5. Additionally, Table 6 (Appendix 3a) summarises overall findings in each food factory.

There was an apparent difference in surface relative humidity and surface temperature between hospital and food factory sites. Although comparisons are limited since only one hospital site was sampled compared to five food factories it appears that, overall, surface relative humidities were higher and surface temperatures were lower in food factory settings. The exception is the brewery factory; the average surface temperature was greater than the average surface temperatures observed from each of the six sampling sessions at UHW.

The lower end of surface relative humidity ranges from dairy 1, dairy 2 and the meat processing factories showed measurements observed in the hospital setting. Likewise the top end values of surface temperature ranges in dairy 1, dairy 2 and the meat processing factories were not dissimilar to surface temperature observations at UHW. In comparison to the conditions within UHW, surface temperature variations were greater in the food factories, particularly in dairy 2, meat processing and the brewery. Surface relative humidity ranges in each factory were also great, which suggests conditions were variable in different areas within the factory. This was not observed in the three wards sampled at UHW. Variations, particularly in surface relative humidity, were apparent over the one year hospital sampling period; however, variations within each session were low. In addition, compared to hospital conditions, surface relative humidities in food factories were higher; in two of the factories some surfaces presented a relative humidity of 100 %. This was not observed at UHW. From their environmental sampling Campden BRI decided the following conditions were appropriate for Phase 2 testing of their antimicrobial surfaces of interest:

- 25 °C and 33 % relative humidity
- 25 °C and 75 % relative humidity
- 10 °C and 33 % relative humidity
- 10 °C and 75 % relative humidity

- 4 °C and 75 % relative humidity

The potential use of antimicrobial surfaces is not limited to the hospital environment; their introduction in the food industry and other settings is discussed in Chapter 6, section 6.6. Campden BRI's findings re-emphasise that the test conditions (relative humidity and temperature) in antimicrobial surface efficacy tests need to be flexible.

CHAPTER 4 THE ANTIMICROBIAL EFFICACY OF ANTIMICROBIAL SURFACES WHEN EXPOSED TO MICROBIAL AEROSOLS

4.1 Introduction

4.1.1 Airborne transmission of microorganisms

A multidisciplinary review defined the airborne transmission of pathogens as 'passage of microorganisms from a source to a person through aerosols, resulting in infection of the person with or without consequent disease' (Li *et al*., 2007).

There are several well-known airborne pathogens including *M. tuberculosis, Mycoplasma pneumoniae*, *Streptococcus* spp., *Bordetella pertussis*, *Neisseria meningitidis, S. aureus* and *Aspergillus* spp. (Siegel *et al*., 2007). Such pathogens can be transmitted directly from an infected patient to another person, or indirectly via contaminated surfaces (HPA, 2012). It is thought the incidence rate of airborne infections in hospitals is around 10 %, though more recent studies are lacking (Schaal, 1991).

There is evidence MRSA is an airborne pathogen; a study in an ITU found MRSA infected six patients, four whom were in the same bed, one after the other. A patient in a single occupancy side-room was isolated due to MRSA infection. In an adjacent room, was a ventilation system, which at the time of the study had a faulty exhaust grille. This meant airborne pathogens, from the infected patient in isolation, passed through the ventilation system and were released into the nearby main ward area through an opened window. This window was situated above the bed where four of the six infected patients were positioned. The authors suggested the faulty ventilation system became contaminated with MRSA and dust carrying the MRSA was released into the air and then travelled into the ward via the opened window (Cotterill *et al*., 1996). A later study by Gehanno *et al*. (2009) also found MRSA present in the air in a hospital environment. The study showed that of 24 MRSA-infected patient rooms sampled, 21 were positive for MRSA in the air. In each room there was at least one identical match between the patients' MRSA sample and that collected in the environment (Gehanno *et al*., 2009).

Acinetobacter was first identified as an airborne pathogen in 1985, during an infection outbreak at a district general hospital. Before this *Acinetobacter* was not known to be

transmitted by air and the authors suggested this airborne transmission contributed to the outbreak (Allen and Green, 1987). A recent article by Munoz-Price *et al.* (2013) found *A. baumannii* to be present in an aerosolised state in a trauma ICU unit in the United States. During the study 53 patient bed areas were air sampled and 12 of these were *A. baumannii* positive. 91.6 % of the 12 *A. baumannii* positive air samples came from bed areas occupied by *A. baumannii* positive patients (Munoz-Price *et al*., 2013).

A clinical study has identified *C. difficile* as an airborne pathogen. Spores were detected in the air on two different occasions in an elderly care bay in a UK hospital and it was proposed *C. difficile* spores can disseminate aerially (Roberts *et al*., 2008). The authors suggested better ventilation in hospitals may contribute to the control of *C. difficile*-associated diarrhoea.

The monitoring of bacterial aerosols is important in healthcare settings, particularly during infection outbreaks, to help with future control of airborne pathogens (Li and Hou, 2003)

4.1.2 Sources of airborne microorganisms

Aerosols are generated by humans when breathing, talking, coughing, sneezing and vomiting (Cole and Cook, 1998; Eames *et al*., 2009). Coughing can produce between 5 x 10^1 - 1 x 10^4 droplets (Fiegel *et al*., 2006). Sneezing can generate 40,000 droplets and particles can range from 0.01 to 500 µm in size, and in infected patients from 0.05 to 500 µm (Cole and Cook, 1998; Gralton *et al*., 2011). Breathing produces aerosols which can be projected up to 1 m in the air whilst during sneezing, a stronger activity, aerosols can be projected several metres (Tang *et al*., 2006).

Other sources of airborne microorganisms in hospitals include fabrics, textiles, dry skin, hair, floors, furniture, nebulisers and ventilation and air-conditioning systems (Schaal, 1991). The abrasion created during activities such as preparing beds, showering and dressing can generate aerosols in a hospital environment; thus desquamated skin is a source of staphylococcal aerosols (Spendlove and Fannin, 1983). It is estimated HCWs

and patients shed approximately 3 x 10^8 skin squame a day (Noble, 1975). A study showed MRSA aerosols were generated during bed making at a hospital and subsequently contaminated the hospital environment (Shiomori *et al*., 2002). A study in 2005 found bacterial and viral aerosols can be disseminated by the simple action of flushing a toilet (Barker and Jones, 2005).

Bacterial aerosols are a particular concern in dental surgeries as they are easily formed from instruments used in routine procedures (Rautemaa *et al*., 2006). Bacteria form biofilms on surfaces that may produce aerosols; *P. aeruginosa* and *Pseudomonas cepacia* are examples of microbial aerosols formed in this manner (Rautemaa *et al*., 2006).

4.1.3 Survival of microbial aerosols in the air and deposition on surfaces

The fate of an aerosol in the air is dependent upon many factors including gravity, air friction, electrical forces and Brownian motion (Morawska, 2006; Tang *et al*., 2006). Droplet transmission of particles is the transmission of large particles (>100 µm) that are likely to settle on the ground whereas small particles (<100 µm) evaporate, become droplet nuclei and suspend in the air to become airborne. These are the key findings from Wells' study on airborne pathogens and are widely accepted by the scientific community (Wells and Stone, 1934; Eames *et al*., 2009). Droplet nuclei are defined as <5 µm in size (Siegel *et al*., 2007). Droplet nuclei can stay airborne for a long time and are able to travel considerable distances, dependent on air movements (Beggs, 2003).

The transmission of bacterial aerosols can be divided into two classes; short-range and long-range, in terms of the distance an infectious aerosol travels (Tang *et al*., 2006). Short-range is classed as <1 m from source to host and an example includes the use of oxygen-masks that may result in aerosol release from a patient to others individuals within the vicinity (Hui *et al*., 2006). Long-range is >1 m from source to host and generally applies to droplet nuclei (Tang *et al*., 2006).

Bacterial aerosols are deposited on to surfaces by gravitational sedimentation or simple diffusion (Morawska, 2006; Meschke *et al*., 2009). Gravitational sedimentation of bacterial aerosols occurs when aerosols are produced close to a surface for deposition. Deposition of particles by diffusion can occur at a further distance from the source, as particles are suspended in the air and travel (Morawska, 2006). The electrostatic charge on surfaces has an important role in aerosol deposition on to surfaces. Bacterial cells are electrically charged both naturally and by the dispersion process (Mainelis *et al*., 2002). The nature of a surface can influence the amount of attracted bacterial aerosols; for example a polyethene plastic apron worn by HCWs attracts more bacteria than aluminium foil aprons. This is due to a static charge on the plastic apron that attracts bacterial aerosols (Allen *et al*., 2006). Becker *et al.* (1996) found the simple action of a surgeon wearing gloves and pointing to within 4 cm of a video recording monitor during an operation resulted in contamination of the gloves. This was due to the electrostatic field generated by the video monitor, which led to the transfer of microorganisms to the surgeons' gloves (Becker *et al*., 1996).

The survival of aerosols in the air and on surfaces depends on a number of environmental factors including temperature, relative humidity and the nature of the surface. For example, relative humidity can influence the size of a droplet and consequently the speed it falls to the ground. In dry conditions, droplets evaporate quickly thus become smaller and take longer to deposit on surfaces, whereas increased relative humidity may decrease the rate of evaporation (Tang *et al*., 2006; Gralton *et al*., 2011). Reduced relative humidity is thought to affect the trajectory of a particle (Gralton *et al*., 2011). Survival on surfaces under varying relative humidity conditions is detailed in section 4.1.5. In terms of the effect of temperature on airborne microorganisms, a temperature greater than 24 °C has been known to reduce survival (Tang, 2009).

4.1.4 Control of transmission of airborne microorganisms

The transmission of bacterial aerosols can be controlled to prevent the dissemination of airborne pathogens. The HPA has published its own guidelines regarding measures to reduce the transmission of respiratory pathogens from an infected patient to other

susceptible patients. These include patient isolation, control of the movement of patients in isolation and information for infected patients (such as the Catch it, Bin it, Kill it NHS campaign). If single occupancy rooms are unavailable, the HPA recommends infected patients are grouped together and beds are separated by >1 m. The guidance document also emphasises the importance of hand hygiene for HCWs. Basic hygiene such as covering one's nose when sneezing and using a tissue when coughing or sneezing can control transmission from infected patients. HCWs can protect themselves from infected patients by wearing Personal Protective Equipment, such as gloves and facemasks (HPA, 2012).

In the case of infected patients, such as tuberculosis carriers, the isolation of patients in a negative-pressure room may help reduce transmission (Cole and Cook, 1998). Other interventions include placing patients in laminar flow beds or encouraging the wearing of respirators (Cole and Cook, 1998).

The proper cleaning and disinfection of medical equipment, such as nebulisers used by patients, is encouraged to also help prevent transmission (Schultsz *et al*., 2013).

Ventilation systems help control the dissemination of aerosols and work by 'diluting the concentration of droplets in the air by removing the circulating droplets via air exchange' (Fiegel *et al*., 2006). Ventilation can be mechanical, natural or a mix of the two (World Health Organisation, 2007). It is recommended the air around infected patients is changed 10 - 12 times an hour (Eames *et al*., 2009). The quality of outdoor air (if outdoor air is used for dilution) is important and air should flow from clean to dirty areas within a hospital (Eames *et al*., 2009). Advantages of mechanical ventilation include the environment can be controlled and it can be used in all climates (World Health Organisation, 2007). Advantages of natural ventilation are the lower associated costs and high ventilation rates (World Health Organisation, 2007). A drawback of mechanical ventilation is the cost of mechanical equipment, and of natural ventilation is the unpredictable nature of weather, for example too hot or too cold (Fiegel *et al*., 2006; World Health Organisation, 2007).

Air purification, by using high-efficiency particulate air (HEPA) filters can remove around 99.97 % of airborne particles of $\geq$0.3 μm in size as the air they are in passes through such filters (Fiegel *et al*., 2006; British Standards Institution (BSI), 2010). HEPA filters are a very effective form of air disinfection, however, a drawback is they need to cleaned or replaced if they become blocked (Aliabadi *et al*., 2011).

Ultraviolet germicidal irradiation (UVGI) is another method of air purification that is mainly found in upper areas of rooms and is sometimes used in combination with filtration (Mermarzadeh *et al*., 2010). The UV light emitted is responsible for killing microorganisms (Fiegel *et al*., 2006). Advantages of UVGI include it is relatively cheap and easier to install and maintain, relative to filtration methods (Fiegel *et al*., 2006). In addition, small bacterial particles that are too small for filtration may be susceptible to UVGI (Mermarzadeh *et al*., 2010). Limitations include bacterial spores may be resistant to UVGI, room air must be mixed for UVGI to effectively work and UV lamps and light bulbs must be checked regularly as age and cleanliness can influence efficacy (Mermarzadeh *et al*., 2010). Also, the distance and level of irradiation influence the activity of UVGI against microorganisms (Mermarzadeh *et al*., 2010).

Negative air ionisation is also an example of an airborne disinfection system used within the NHS. One study found the use of negative air ionisation units resulted in repulsion of *Acinetobacter* spp. to plastic surfaces. These surfaces would normally hold an electrostatic charge but the negative air ioniser causes surfaces to develop a negative charge (Shepherd *et al*., 2010).

Following a study investigating *C. difficile* aerosol production after the flushing of a toilet, Best *et al.* (2012) highlighted the importance of closing toilet lids during flushing to reduce microbial aerosol transmission, since surface contamination by *C. difficile* was detectable after 90 min of flushing when the lid was open as opposed to closed (Best *et al*., 2012). *C. difficile* was detected in the air 25 cm above the toilet when the toilet was flushed with its lid open (Best *et al*., 2012).

4.1.5 Microbial aerosol studies

A study investigating the survival of *S. epidermidis* by a Goldberg drum found aerosols could be recovered when subject to a range of relative humidities; <20 %, 40 – 60 %, 70 – 80 % and >90 %. A Goldberg drum is a large, aluminium drum that rotates (in this study at 3.5 rpm). It contains baffles that ensure aerosols remain airborne within the drum. At 76 % relative humidity aerosols survived for up to 5 days (Thompson *et al*., 2011).

A study of the survival of *Enterococcus faecalis (E. faecalis)* on stainless steel surfaces found that lethality was directly proportional to relative humidity. Bacteria survived on surfaces at relative humidities of 0 and 31 % but were completely inactivated at 85 % relative humidity (Robine *et al*., 2002).

A study by Decraene *et al.* (2008) showed *S. aureus* aerosols could be significantly reduced in number when deposited on LAAAs containing toluidine blue O and rose bengal.

4.1.6 JIS Z 2801 – a wet inoculum antimicrobial surface efficacy test

The main aim of this project was to suggest alternatives to the current JIS Z 2801 test procedure, also referred to as the ISO22196 test, to incorporate more realistic temperature and relative humidity conditions. The JIS Z 2801 assesses the antimicrobial activity of surfaces at 35 °C and >90 % relative humidity. Many antimicrobial surface manufacturers utilise the test on their products to confirm antimicrobial activity before making them commercially available (see Chapter 1, section 1.7, Table 1.1). However, this test has been described as inappropriate to validate in-use performance since the parameters used (temperature, relative humidity, and presentation of the inocula) do not reflect conditions found in practice in UK hospitals. The JIS Z 2801 is a useful screening test for surfaces with antimicrobial claims but the NHS Rapid Review Panel, who has the responsibility for the preliminary evaluation of product claims before recommending infection control technologies to NHS practitioners, recognises the

absence of a suitable test for predicting clinical efficacy (personal communication, Rapid Review Panel).

4.1.7 Aims and Objectives

The aim of this chapter was to develop a method for delivering and depositing microbial aerosols on to antimicrobial surfaces. The antimicrobial efficacy of these surfaces could then be determined under parameters reflecting indoor hospital conditions based on observations from sampling sessions at UHW.

4.2 Materials and Methods

4.2.1 JIS Z 2801 testing

The JIS Z 2801 is considered the accepted international test for assessing the activity and efficacy of products with antimicrobial claims. The method carried out in this study was a slightly modified version of the original test procedure. Here the test was based on the method utilised by Campden BRI.

A TSA slope of *S. aureus* was prepared and stored for a maximum of one month at 4 °C. From this a fresh slope was prepared before the test was carried out by transferring a loopful of culture from the master slope to the new slope and incubating for 24 h at 37 °C. This was the first sub-culture. The day before testing another fresh TSA slope was inoculated with culture from the first sub-culture and incubated for 24 h at 37 °C. This was the working culture. On the day of testing 9 mL of MRD and 5 g 3 mm glass beads were added to the slope to recover the microorganism. The bottle was shook and vortexed for three minutes. The OD_{600} of the suspension was determined and adjusted to give a bacterial concentration of 2 – 8 x 10^8 cfu/mL. This was then serially diluted to give 2 – 8 x 10^6 cfu/mL. The viable count of the suspension was determined using the pour plate method in duplicate. One mL of the dilution was transferred to a Petri dish, 20 mL TSA was added and the plates were left to set. Plates were incubated for 24 h at 37 °C and cfu on dilutions containing 30 – 300 cfu was recorded.

The test surfaces used were copper alloys; CuSn5, CuZn30, CuDHP and CuNi10Fe1Mn. The control surface was stainless steel. All surfaces were tested in triplicate. Each surface was inoculated with 50 µL of bacterial suspension then covered completely with a plastic film. Test and control surfaces were incubated in a box for 24 h at 37 °C and a relative humidity of 100 %. This high percentage of relative humidity was achieved by covering surfaces with a plastic film and by also placing several beakers of saturated zinc sulphate solution in the box.

The inoculum levels at 0 h on the stainless steel control were next determined. Surfaces were placed in an individual stomacher bag to which 9 mL MRD and 1 mL neutraliser were added. The film over the surface was removed (by hand through the stomacher bag) and both were massaged by hand for 30 sec to recover the inoculum. One mL of this solution was serially diluted to 10^{-4} in MRD and bacterial counts were enumerated via the pour plate method. This was carried out in duplicate for each dilution. Plates were incubated at 37 °C for 24 h and the cfu/mL determined. This was repeated for the 24 h test surfaces.

Although the JIS Z 2801 does not test the activity of microbial aerosols against test surfaces; for the purpose of this study the JIS Z 2801 and the new antimicrobial test developed in this chapter were both 'wet' tests so were therefore comparable. The new test developed (section 4.4.2) was assessed in relation to the JIS Z 2801.

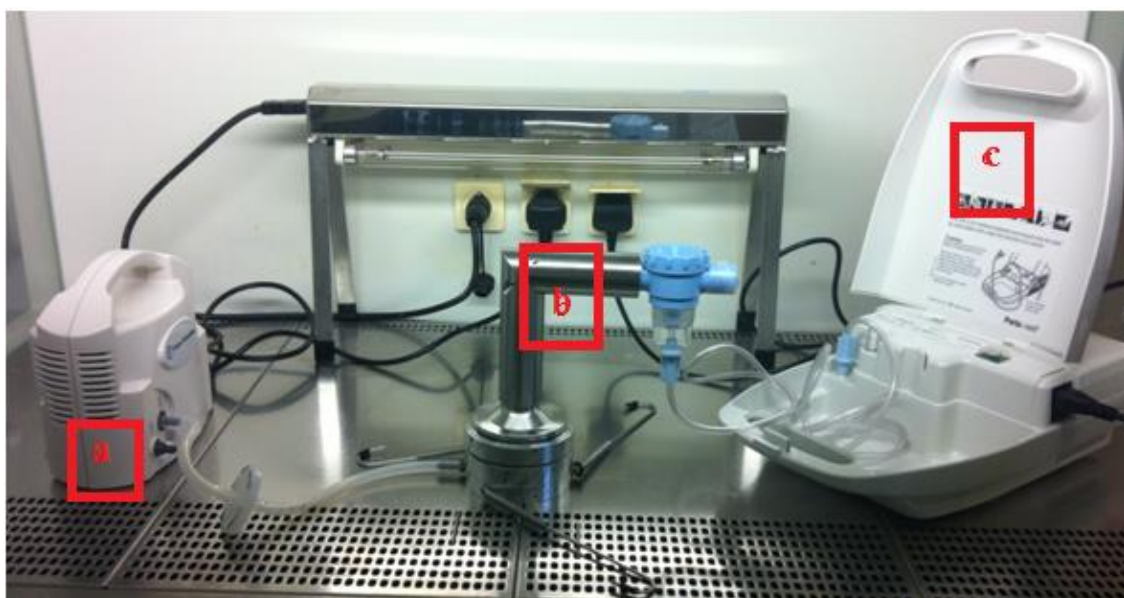
4.2.2 Development of a new antimicrobial surface efficacy test based on exposure of microbial aerosols

4.2.2.1 Use of a nebuliser to generate microbial aerosols

A Porta-neb VentStream Nebuliser (Philips Respronics, Best, The Netherlands) was used to generate bacterial aerosols (Figure 4.1). Nebulisers are medical devices commonly used to medicate drugs in an aerosolised state to patients with respiratory diseases. The nebuliser was connected to an Andersen cascade impactor (Westech Instrument Services Ltd, Bedfordshire, UK), of which Stages 0 and F only were used, as shown in Figure 4.1. A vacuum pump was connected to the cascade impactor. In

addition, a Copley Scientific DFM2000 (Nottingham, UK) flow meter, placed between the cascade impactor and vacuum pump (not shown) measured flow rate. Ten runs of the set-up presented an average flow rate of 4.86 ± 0.44 L/min.

Figure 4.1– Image of test set-up for delivering microbial aerosols on to antimicrobial surfaces



a=vacuum pump, b=Andersen cascade impactor, c=nebuliser

4.2.2.2 Recovery of microbial aerosols from stainless steel discs

An overnight culture of *S. aureus* NCIMB 9518 was prepared as in 2.3.2 and re-suspended in 10 mL TSC as described in 2.3.3. The OD_{600} of the suspension was adjusted to give a bacterial cell concentration of approximately 10^8 cfu/mL.

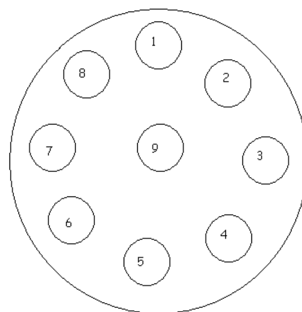
The recovery of microbial aerosols from stainless steel discs was determined in cfu/cm^2 to be able to compare different sized surfaces with each other in future experiments. In order to find out how many bacterial cells can be recovered from the discs the nebuliser was switched on for three test periods; 10, 20 and 30 min. Three stainless steel discs were placed on a collecting plate that was positioned over Stage F of the cascade impactor (see Figure 4.2) and the nebuliser ran for 10 min. Stainless discs were immediately transferred to a 100 mL bottle containing 5 g 3 mm glass beads and 10 mL

TSC. Bottles were placed on a shaking platform for 1 min and left to stand for 5 min. Next 100 μL of the suspension was added to 900 μL TSC and serially diluted to 10^{-4}. The spread plate method was utilised to enumerate viable bacteria; 100 μL of each dilution was placed onto pre-poured TSA plates, spread with a sterile spreader and incubated at 37 °C for 24 h. This was carried out in triplicate for each dilution. The process was repeated after the nebuliser when switched on for 20 min and 30 min.

Figure 4.2 – Position of three stainless steel discs within cascade impactor

It was decided the nebuliser would run for 30 min for all future experiments to enable maximal bacterial deposition on to the disc surface (see Table 4.2). The method of recovering bacterial aerosols was validated by depositing bacterial aerosols of *S. aureus* NCIMB 9518 on to nine stainless steel discs (Figure 4.3) and determining viability. The discs were arranged as follows:

Figure 4.3 – Position of stainless steel discs on collecting plate within the cascade impactor for validation of recovery of microbial aerosols



4.2.2.3 Development of method used to recover viable bacteria on surfaces

In order to make this protocol similar to that of the JIS Z 2801, MRD plus neutraliser was used as a recovery medium. The neutraliser was previously validated for its toxicity and efficacy (see Chapter 2, section 2.11).

Six stainless steel discs were placed on the collecting plate. An overnight culture was centrifuged and re-suspended in MRD to an OD_{600} that provided approximately 10^8 cfu/mL. Ten mL of the suspension was placed in the nebuliser and aerosolised for 30 min. Next, three discs were transferred individually to a 100 mL bottle containing 10 mL TSC and 5 g 3 mm glass beads. The remaining three discs were transferred to a bottle with 9 mL MRD, 1 mL neutraliser and 5 g 3 mm glass beads. Bottles were shook for 1 min at 150 rpm and viable bacteria were enumerated by the spread plate method.

4.2.3 New antimicrobial surface efficacy test method

After the set-up had been established the efficacy of all four test copper alloy surfaces against microbial aerosols of *S. aureus*, *A. baumannii* and *B. subtilis* spores was tested. Aerosols generated were exposed to copper alloy surfaces and subjected to a range of temperatures and relative humidities at varying contact times. This was to determine whether temperature and relative humidity had an effect on antimicrobial activity. Stainless steel was utilised as a control surface.

All surfaces were tested at 0 h (i.e. immediately after 30 min nebulisation) and after 30 min, 60 min and 24 h incubation at the required temperature and relative humidity conditions. Surfaces were exposed to the following conditions; [37°C-100% RH], [20°C-50% RH] and [20°C-40% RH]. The lower relative humidities (40 % and 50 %) and temperature (20 °C) were chosen to simulate in-use conditions observed at UHW throughout hospital sampling sessions (see Chapter 3, section 3.3).

S. aureus NCIMB 9518 was grown as described in 2.3.2 and prepared for testing as in 2.3.3 with MRD used as the re-suspending medium. The OD_{600} of the culture was

adjusted to give a bacterial concentration of 10^8 cfu/mL. Ten mL of culture was placed in the nebuliser. Nebulisation time was 30 min. The combination of surfaces tested was random within each run; stainless steel and copper alloy surfaces were mixed. Six surfaces were placed around the edge of a large stainless steel collecting plate, which was placed over Stage F of the cascade impactor, allowing aerosols to be passed through Stage 0. Immediately after nebulisation (0 h) all 0 h test surfaces were transferred to bottle containing 9 mL MRD, 1 mL neutraliser and 5 g 3 mm glass beads. Bottles were shaken at 150 rpm for 1 min then left to stand for 5 min. Viable bacteria were enumerated by serial dilution in MRD to 10^{-4} and plated via the spread plate method; 100 µl of each dilution was spread in triplicate on pre-poured TSA plates. All other surfaces were placed in individual Petri dishes and incubated at the required temperature, relative humidity and contact time. Following incubation at the required contact time viable bacteria were recovered and enumerated as described above. Each surface was tested in triplicate for each contact time and relative humidity and temperature condition. As with the JIS Z 2801 test high relative humidity was created by covering the test surface with a plastic film and by placing beakers of saturated solutions of zinc sulphate in the incubating box. PROsorb™ silica gel cassettes (Conservation by Design Ltd., UK) were used to stabilise relative humidity at 40 % and 50 %, no film was placed over the surfaces.

The experiment was repeated at [37°C-100% RH] and [20°C-40% RH] in the presence of organic load for *S. aureus*. A higher initial concentration (5 x 10^8 - 1 x 10^9 cfu/mL) of bacterial suspension was prepared and mixed with 0.6 % BSA in a 1:1 ratio resulting in a final concentration of 0.3 % BSA.

A. baumannii NCIMB 9214 was also tested. *A. baumannii* was prepared as in 2.3.2 and 2.3.3 with MRD used as the re-suspending buffer. Viable bacteria recovered from surfaces were grown at 25 °C for 24 h on TSA. *C. difficile* NCTC 12726 spores, as an example of an HCAI, were initially tested. However, recurrent issues with spore aggregation resulted in uneven deposition of the inoculum on to surfaces. This was unacceptable for further testing. Therefore *B. subtilis* NCTC 10400 spores were tested instead. *B. subtilis* spores were prepared as in 2.7.1 (NB. Spores were in suspension in water, not MRD). Viable bacteria recovered from surfaces were grown at 37 °C for 24 h

on LB agar. Surfaces exposed to *A. baumannii* and *B. subtilis* spore aerosols were incubated at [20°C-40% RH], the most realistic condition based on hospital sampling, for 30 min, 60 min and 24 h as previously described.

4.2.4. Statistical analysis

Statistical analysis was carried using SPSS or R software. In general, a one-way ANOVA or a General Linear Model were used to analyse data statistically.

4.3 Results

4.3.1 JIS Z 2801 results

The antimicrobial efficacy of test surfaces was determined by the JIS Z 2801 test using a method adapted by Campden BRI.

The log_{10} reductions (Table 4.1) represent the amount of viable bacteria on surfaces at 24 h subtracted from the 0 h count on stainless steel. All copper alloy surfaces presented a >4 log_{10} cfu/mL reduction after 24 h at [37°C-100% RH], suggesting they possessed antimicrobial properties. In contrast, the stainless steel control surfaces produced a 0.31 log_{10} increase after 24 h. This negative log_{10} reduction means there was a slight increase in viable bacteria on the surface after 24 h. The very little change in viable count indicates stainless steel had no antimicrobial activity.

Table 4.1– JIS Z 2801 test results showing the amount of viable bacteria on each surface at 24 h subtracted from the 0 h viable count on stainless steel (n=3)

Surface	Log_{10} reductions cfu/mL
Stainless steel	-0.31 ± 0.07*
CuSn5	>5.11 ± 0.35
CuDHP	>4.91 ± 0.00
CuZn30	>5.11 ± 0.00
CuNi10Fe1Mn	>4.85 ± 0.00

*denotes an increase in number rather than log_{10} reduction

4.3.2 Development of a new antimicrobial surface efficacy test based on the exposure of microbial aerosols

4.3.2.1 Recovery of microbial aerosols from stainless steel discs

To decide how long the nebuliser should run for to deposit a suitable concentration of bacterial cells, the nebuliser was tested for 10, 20 and 30 min, and recovery from stainless steel discs was determined. Table 4.2 shows the $\log_{10}$ cfu/cm^2 recovered from stainless steel discs after nebulisation times of 10, 20 and 30 min. A 10 min nebulisation time was not chosen for future experiments because the amount of bacteria recovered was too low to allow for adequate log reductions for disinfection studies. The longest time was selected to allow recovery of the highest inoculum to enable the elucidation of antimicrobial differences between surfaces. Therefore, 30 min was chosen as the designated time length for the nebuliser to run in all future experiments.

Table 4.2 - Testing nebuliser for 10, 20 and 30 min to assess the concentration of microbial aerosols deposited on surfaces (n=3)

	Repeat	$\log_{10}$ cfu/cm^2 ± SD	Mean ± SD
10 min	1	3.25 ± 0.02	
	2	3.40 ± 0.14	3.46 ± 0.24
	3	3.73 ± 0.04	
20 min	1	4.22 ± 0.27	
	2	4.18 ± 0.05	4.24 ± 0.10
	3	4.39 ± 0.11	
30 min	1	4.70 ± 0.04	
	2	4.66 ± 0.04	4.59 ± 0.15
	3	4.42 ± 0.00	

The effect of the position of the stainless steel discs within the large collecting plate in the cascade impactor on the amount of bacteria recovered was assessed (see Figure 4.3). The lowest bacterial count was that recovered from disc 9 (Table 4.3). The $\log_{10}$ cfu/cm^2 range of recoveries from discs 1 to 8 was 4.62 – 5.26. The average recovery of discs 1 –

8 was 5.40 $\log_{10}$ cfu/cm^2 with a standard deviation of 0.24. From these preliminary results surfaces will not be placed in the centre for future experiments (position 9, see Figure 4.3) as recovery was the lowest here. Statistical analysis by a one-way ANOVA showed there was no significant difference in the counts obtained when discs were placed in positions 1 - 8, P=0.684.

Table 4.3 - Validation of recovery of microbial aerosols from stainless steel discs (n=1)

Disc	$\mathbf{Log_{10}}$ cfu/cm^2 ± SD
1	5.26 ± 0.05
2	5.45 ± 0.02
3	4.97 ± 0.01
4	4.84 ± 0.00
5	5.20 ± 0.11
6	4.86 ± 0.02
7	5.18 ± 0.08
8	4.62 ± 0.09
9	4.09 ± 0.28

4.3.2.2 Use of MRD plus neutraliser vs. TSC for recovery of viable bacteria

Although it appears the recovery was slightly lower when the MRD and neutraliser mix was utilised compared to TSC (Table 4.4), there was no significant difference (P=0.672) when tested by an one-way ANOVA. From this point onwards it was decided MRD plus neutraliser was to be utilised for future experiments, and to keep in line with the JIS Z 2801 test protocol.

Table 4.4 – Amount of viable bacteria recovered from stainless steel discs when using TSC or MRD plus neutraliser as a recovery medium (n=3)

Recovery medium	$\mathbf{Log_{10}}$ cfu/cm^2
TSC	5.93 ± 0.26
MRD plus neutraliser	5.75 ± 0.59

4.3.3 New antimicrobial surface efficacy test results

4.3.3.1 **S. aureus** *results*

The copper alloy (test) and stainless steel (control) surfaces were exposed to bacterial aerosols of *S. aureus* then incubated for 30 min, 60 min and 24 h at [37°C-100% RH], [20°C-50% RH] and [20°C-40% RH]. Tables 4.5 - 4.7 show the $\log_{10}$ reductions calculated as the counts at 30 min, 60 min and 24 h subtracted from the 0 h count for each surface.

After 30 min nebulisation 6.56 ± 0.05 to 6.71 ± 0.14 $\log_{10}$ cfu/cm^2 was recovered from stainless steel discs. The amount of deposited aerosols recovered from copper alloys at 0 h was around 1 $\log_{10}$ lower, which might suggest antimicrobial activity by copper over the 30 min deposition period. After 24 h all copper alloy surfaces showed a >4 $\log_{10}$ reduction in viable bacteria at all temperature and relative humidity conditions. CuSn5, CuDHP and CuZn30 all displayed a >4 $\log_{10}$ reduction after 30 min incubation at [37°C-100% RH]. At [20°C-50% RH] and [20°C-40% RH] a >4 $\log_{10}$ reduction was only observed after 60 min by CuSn5, CuDHP and CuZn30 and CuDHP and CuZn30, respectively. At both [20°C-50% RH] and [20°C-40% RH] CuNi10Fe1Mn $\log_{10}$ reductions were >3 but <4 $\log_{10}$ after 60 min. At [20°C-40% RH] 24 h incubation was required for a >4 $\log_{10}$ reduction by CuSn5; although a >3.65 $\log_{10}$ reduction at 60 min was apparent. Stainless steel worked well as a control presenting a <1 $\log_{10}$ reduction after 30 min and 60 min at all conditions. After 24 h the greatest $\log_{10}$ reduction on stainless steel was 2.17 at [37°C-100% RH].

Data were transformed (natural log + 1) and analysed by a General Linear Model using R software. In terms of incubation conditions, there were significant differences between [20ºC-40% RH] and [37ºC-100% RH] and between [20ºC-50% RH] and [37ºC-100% RH] (both P<0.001). There was no significant difference between [20ºC-50% RH] and [20ºC-40% RH] (P=0.27). There was a significant difference between all contact times for all copper alloys (P<0.001) when all three test conditions were analysed together. The counts on all copper alloy surfaces were significantly different to the counts on stainless steel (P<0.001). No significant differences were observed between the copper alloy surfaces, except between CuZn30 and CuNi10FeMn (P=0.04).

When analysing each condition alone, at [37ºC-100% RH] there was a significant difference between stainless steel and all copper alloys. Between the copper alloys there were significant differences between CuNi10Fe1Mn and CuDHP ($P=0.004$), CuNi10Fe1Mn and CuZn30 ($P<0.001$) and CuNi10Fe1Mn and CuSn5 ($P<0.001$). There was a significant difference between each contact time. All P-values were <0.001 except between 30 min and 60 min ($P=0.015$) and between 60 min and 24 h ($P=0.003$). For both [20ºC-50% RH] and [20ºC-40% RH] there were no significant differences between the copper alloys (all $P>0.05$). There was a difference between stainless steel and each copper alloy ($P<0.001$). In terms of contact time, there were significant differences between all contact times (all $P<0.001$).

Table 4.5 – Recovery of *S. aureus* from deposited aerosols at 0 h and $\log_{10}$ reductions after 30 min, 60 min and 24 h incubation at [37°C-100% RH] (n=3)

	Stainless steel $\mathbf{Log_{10}}$ **cfu/cm²**	**CuSn5** $\mathbf{Log_{10}}$ **cfu/cm²**	**CuDHP** $\mathbf{Log_{10}}$ **cfu/cm²**	**CuZn30** $\mathbf{Log_{10}}$ **cfu/cm²**	**CuNi10Fe1Mn** $\mathbf{Log_{10}}$ **cfu/cm²**
0 h	6.56 ± 0.05	5.33 ± 0.29	5.75 ± 0.36	5.05 ± 0.67	5.01 ± 0.38
$\mathbf{Log_{10}}$ reductions					
30 min	0.23 ± 0.19	> 4.49 ± 0.00	> 4.91 ± 0.00	> 4.21 ± 0.00	2.34 ± 1.12
60 min	0.54 ± 0.60	> 4.49 ± 0.00	> 4.91 ± 0.00	> 4.21 ± 0.00	3.63 ± 0.47
24 h	2.17 ± 0.21	> 4.49 ± 0.00	> 4.91 ± 0.00	> 4.21 ± 0.00	> 4.17 ± 0.00

Table 4.6 - Recovery of *S. aureus* from deposited aerosols at 0 h and $\log_{10}$ reductions after 30 min, 60 min and 24 h incubation at [20°C-50% RH] (n=3)

	Stainless steel	**CuSn5**	**CuDHP**	**CuZn30**	**CuNi10Fe1Mn**
	Log_{10} cfu/cm^2	**Log_{10} cfu/cm^2**	**Log_{10} cfu/cm^2**	**Log_{10} cfu/cm^2**	**Log_{10} cfu/cm^2**
0 h	6.60 ± 0.20	5.67 ± 0.38	5.70 ± 0.35	5.52 ± 0.08	5.45 ± 0.02
Log_{10} reductions					
30 min	0.12 ± 0.35	2.97 ± 0.48	2.98 ± 0.39	2.86 ± 0.24	2.60 ± 0.19
60 min	0.57 ± 0.49	> 4.29 ± 0.57	4.26 ± 0.30	4.01 ± 0.18	3.54 ± 0.25
24 h	1.38 ± 0.36	> 4.83 ± 0.00	> 4.76 ± 0.17	> 4.68 ± 0.00	4.25 ± 0.10

Table 4.7 - Recovery of *S. aureus* from deposited aerosols at 0 h and $\log_{10}$ reductions after 30 min, 60 min and 24 h incubation at [20°C-40% RH] (n=3)

	Stainless steel	**CuSn5**	**CuDHP**	**CuZn30**	**CuNi10Fe1Mn**
	Log_{10} cfu/cm^2	**Log_{10} cfu/cm^2**	**Log_{10} cfu/cm^2**	**Log_{10} cfu/cm^2**	**Log_{10} cfu/cm^2**
0 h	6.71 ± 0.14	5.45 ± 0.15	5.59 ± 0.40	5.96 ± 0.42	5.13 ± 0.70
Log_{10} reductions					
30 min	0.44 ± 0.42	2.91 ± 0.51	3.79 ± 0.35	3.64 ± 1.04	2.73 ± 0.71
60 min	0.26 ± 0.15	> 3.65 ± 0.83	> 4.36 ± 0.36	> 4.96 ± 0.28	3.23 ± 0.13
24 h	1.23 ± 1.07	> 4.61 ± 0.00	> 4.75 ± 0.00	> 5.12 ± 0.00	> 4.29 ± 0.00

Tables 4.8 and 4.9 display results from experiments where copper alloy and stainless steel surfaces were exposed to microbial aerosols containing organic load then incubated for 30 min, 60 min and 24 h at [37°C-100% RH] and [20°C-40% RH]. The addition of BSA to the aerosolised suspension to mimic dirty conditions showed surprising results; some surfaces presented faster antimicrobial activity when exposed to aerosols containing BSA than in the absence of BSA.

After 24 h all copper alloy surfaces presented a >4 $\log_{10}$ reduction at both conditions. Activity at [37°C-100% RH] was quick with three copper alloys showing a >4 $\log_{10}$ reduction after 30 min and all showed this reduction after 60 min. At [37°C-100% RH] CuNi10Fe1Mn presented greater antimicrobial activity in the presence of organic load in comparison to in the absence of organic load, with reductions of >4 $\log_{10}$ and 2.34 ± 1.12, respectively. At [20°C-40% RH] three of the four copper alloys displayed a >4 $\log_{10}$ reduction after 60 min. CuSn5, like CuNi10Fe1Mn at [37°C-100% RH] was more antimicrobial in the presence of organic load. A >4 $\log_{10}$ reduction was observed after 30 min compared to after 24 h in the absence of organic load.

At [37°C-100% RH] there was no significant difference in the recovered amount of deposited aerosols between in the absence or in the presence of BSA ($P=0.653$). In contrast, at [20°C-40% RH] there was a significant difference ($P<0.001$).

Table 4.8 - Recovery of *S. aureus* from deposited aerosols at 0 h and log_{10} reductions after 30 min, 60 min and 24 h incubation at [37°C-100% RH] in the presence of organic load (n=3)

	Stainless steel $\mathbf{Log_{10}}$ **cfu/cm²**	**CuSn5** $\mathbf{Log_{10}}$ **cfu/cm²**	**CuDHP** $\mathbf{Log_{10}}$ **cfu/cm²**	**CuZn30** $\mathbf{Log_{10}}$ **cfu/cm²**	**CuNi10Fe1Mn** $\mathbf{Log_{10}}$ **cfu/cm²**
0 h	6.36 ± 0.16	5.31 ± 0.32	5.40 ± 0.20	5.44 ± 0.34	5.48 ± 0.34
$\mathbf{Log_{10}}$ **reductions**					
30 min	0.28 ± 0.36	> 4.47 ± 0.00	> 4.56 ± 0.00	> 3.79 ± 0.19	> 4.38 ± 0.24
60 min	0.40 ± 0.25	> 4.47 ± 0.00	> 4.46 ± 0.00	> 4.60 ± 0.00	> 4.64 ± 0.00
24 h	1.04 ± 0.74	> 4.47 ± 0.00	> 4.56 ± 0.00	> 4.60 ± 0.00	> 4.64 ± 0.00

Table 4.9 - Recovery of *S. aureus* from deposited aerosols at 0 h and log_{10} reductions after 30 min, 60 min and 24 h incubation at [20°C-40% RH] in the presence of organic load (n=3)

	Stainless steel Log_{10} **cfu/cm**^{2}	**CuSn5** Log_{10} **cfu/cm**^{2}	**CuDHP** Log_{10} **cfu/cm**^{2}	**CuZn30** Log_{10} **cfu/cm**^{2}	**CuNi10Fe1Mn** Log_{10} **cfu/cm**^{2}
0 h	6.08 ± 0.15	5.46 ± 0.10	5.41 ± 0.40	5.50 ± 0.13	5.40 ± 0.38
Log_{10} reductions					
30 min	0.23 ± 0.57	> 4.32 ± 0.75	2.96 ± 0.30	2.88 ± 0.51	3.30 ± 0.58
60 min	0.80 ± 0.32	> 4.62 ± 0.00	> 4.41 ± 0.28	> 4.46 ± 0.35	3.56 ± 0.23
24 h	1.82 ± 0.2	> 4.62 ± 0.00	> 4.57 ± 0.00	> 4.66 ± 0.00	> 4.56 ± 0.00

4.3.3.2 **A. baumannii** ***results***

To confirm the newly developed antimicrobial surface test worked against a range of microorganisms, aerosols of *A. baumannii* were exposed to the copper alloy surfaces. Results are displayed in Table 4.10. After 30 min nebulisation the initial count recovered from the copper alloys was 4.77 ± 0.27 to 5.24 ± 0.27 log_{10} cfu/cm^2. In contrast, a higher bacterial amount (6.14 ± 0.16 log_{10} cfu/cm^2) was recovered from stainless steel 0 h. After 30 min at [20°C-40% RH] high log_{10} reductions were observed on the copper alloy surfaces with three of the four displaying a >4 log_{10} reduction. For CuSn5, the log_{10} reduction after 24 h was not >4 log_{10}, due to the low initial count onto surfaces at 0 h in comparison to the other three copper alloys.

Data were transformed (natural log + 1) and analysed by a General Linear Model using SPSS software. Statistically, there were no significant differences between the four copper alloys ($P>0.05$). There were significant differences between all copper alloys and stainless steel ($P<0.001$). Regarding contact time, there were significant differences in counts between all time points except between 30 and 60 min ($P=0.672$).

Table 4.10 - Recovery of *A. baumannii* from deposited aerosols at 0 h and $\log_{10}$ reductions after 30 min, 60 min and 24 h incubation at [20°C-40% RH] (n=3)

	Stainless steel $\mathbf{Log_{10}}$ **cfu/cm^2**	**CuSn5** $\mathbf{Log_{10}}$ **cfu/cm^2**	**CuDHP** $\mathbf{Log_{10}}$ **cfu/cm^2**	**CuZn30** $\mathbf{Log_{10}}$ **cfu/cm^2**	**CuNi10Fe1Mn** $\mathbf{Log_{10}}$ **cfu/cm^2**
0 h	6.14 ± 0.16	4.77 ± 0.27	5.03 ± 0.71	5.05 ± 0.19	5.24 ± 0.27
$\mathbf{Log_{10}}$ reductions					
30 min	0.07 ± 0.56	>3.83 ± 0.17	>4.09 ± 0.17	>4.21 ± 0.00	>4.40 ± 0.00
60 min	0.57 ± 0.17	>3.93 ± 0.00	>4.19 ± 0.17	>4.21 ± 0.00	>4.40 ± 0.00
24 h	2.24 ± 0.55	>3.93 ± 0.00	>4.19 ± 0.17	>4.21 ± 0.00	>4.40 ± 0.00

4.3.3.3 **B. subtilis** ***spore results***

The antimicrobial activity of copper alloys against *B. subtilis* spores was very low (see Table 4.11). Even after 24 h exposure, <1 log_{10} reductions on all copper alloys were evident. Data were transformed (natural log + 1) for statistical analysis by a General Linear Model using SPSS software. There was a significant difference between all copper alloys (P<0.05), except between CuSn5 and CuZn30 (P=0.999). In fact, of the copper surfaces CuSn5 and CuZn30 presented the lowest reduction and highest log_{10} reductions after 24 h, respectively. The counts on all copper alloys, bar CuDHP (P<0.001), were not significantly different than the counts on stainless steel (P>0.05). In terms of contact time, overall there were significant differences between all time points (all P>0.05) except between 0 h and 30 min (P>0.999).

Table 4.11 – Recovery of *B. subtilis* spores from deposited aerosols at 0 h and $\log_{10}$ reductions after 30 min, 60 min and 24 h incubation at [20°C-40% RH] (n=3)

	Stainless Steel	**CuSn5**	**CuDHP**	**CuZn30**	**CuNi10Fe1Mn**
	$\log_{10}$ cfu/cm^2	**$\log_{10}$ cfu/cm^2**	**$\log_{10}$ cfu/cm^2**	**$\log_{10}$ cfu/cm^2**	**$\log_{10}$ cfu/cm^2**
0 h	7.29 ± 0.15	7.06 ± 0.46	6.26 ± 0.45	7.50 ± 0.20	6.79 ± 0.53
$\log_{10}$ reductions					
30 min	0.08 ± 0.42	-0.35 ± 0.17*	0.08 ± 0.27	0.40 ± 0.42	-0.21* ± 0.62
60 min	0.68 ± 0.35	-0.33 ± 0.11*	0.21 ± 0.60	0.48 ± 0.18	0.01 ± 0.04
24 h	0.66 ± 0.34	0.45 ± 0.40	0.60 ± 0.28	0.70 ± 0.37	0.46 ± 0.99

*denotes an increase in number rather than $\log_{10}$ reduction

4.4 Discussion

All copper alloy surfaces presented a >4 $\log_{10}$ reduction in viable *S. aureus* indicating they were antimicrobial under JIS Z 2801 test conditions. The JIS Z 2801 served as an initial, basic screening test to confirm antimicrobial activity. However, the test methodology contains several drawbacks. Firstly, the incubation conditions of 35 °C and >90 % relative humidity (NB. 37 °C and 100 % relative humidity conditions were utilised as JIS Z 2801 tests conditions in this study) are not reflective of an indoor, environment. They are too high, particularly for UK hospital conditions. Antimicrobial activity by surfaces observed in the laboratory may not necessarily translate to in-use antimicrobial activity. Michels *et al.* (2009) found this to be the case when testing silver-containing surfaces; surfaces were antimicrobial under JIS Z 2801 test conditions but when tested under conditions reflecting an indoor environment the surfaces did not present the same antimicrobial activity. Secondly, since test surfaces are covered with a plastic film over the entire contact period, surfaces tend to remain wet and this allows for optimum conditions of biocide diffusion (e.g. copper ions in this study) from the surface to the bacterial cells. Again, this is not indicative of real-life conditions, surfaces are ostensibly dry and if wet, do not remain wet for up to 24 h. This essentially makes the JIS Z 2801 a 'wet' surface efficacy test. The new antimicrobial surface test developed here was based on the exposure of surfaces to bacterial aerosols, to mimic actions such as sneezing, coughing, talking etc. In comparison to the JIS Z 2801, where a large volume inoculum is presented on to surface, the use of aerosolised bacteria in this new antimicrobial efficacy test might be a better reflection of how a wet inoculum, such as cough or sneeze particles, is deposited on to surfaces (Ojeil *et al*., 2013).

The JIS Z 2801 test and the test developed in this chapter were simple to carry out and allow one to determine the antimicrobial efficacy of surfaces with antimicrobial claims (Ojeil *et al*., 2013). In this study copper has shown to be an effective antimicrobial against Gram-positive and Gram-negative bacteria but not bacterial spores. The new test here was more discriminatory than the JIS Z 2801 test at other conditions enabling the distinction of efficacy between different copper alloys. For *S. aureus* there was no significant difference (at all conditions) between the activities of the tested copper

alloys but except for between CuZn30 and CuNi10Fe1Mn. This suggests a copper content as low as 70 % is just as efficacious as a copper content of 99.99 %, which is promising for practical reasons; copper alloys are generally stronger and less prone to oxidation than pure copper surfaces.

At present there is very little information in the scientific literature on the effect of antimicrobial surfaces against microbial aerosols. A study by Robine *et al.* (2002) showed *E. faecalis* aerosol viability on pure copper surfaces decreased with increasing relative humidity; aerosols were completely killed after 24 h at 100 % relative humidity but survived at 0 % relative humidity up to 96 h (Robine *et al.*, 2002). This shows some resemblance to the new test developed in this chapter; the quickest kill by copper alloys was at [37°C-100% RH]. The data from this chapter and from Robine *et al.*'s (2002) study suggests high relative humidity and an aqueous inoculum aids the diffusion of copper towards the bacterial cell.

MRD, which contains peptone (1 g/L), was used to act as soiling to the test and reflect the level of bioburden observed from hospital sampling sessions (see Chapter 3) (Ojeil *et al.*, 2013). The ATP levels measured on surfaces was a measure of all organic matter; not just microbial bioburden (Chapter 3, section 3.2). MRD alone and MRD with organic load (BSA) were used to reflect different levels of organic load. The addition of 3 g/L BSA to the bacterial suspensions is referred to as 'dirty' conditions in many standards. The quick rate of copper antimicrobial activity against an inoculum containing organic load was unusual; normally organic matter acts as a barrier between biocides and microorganisms (Lambert and Johnston, 2001). The study by Robine *et al.* (2002) highlighted the importance of surface fouling which enhanced bacterial survival regardless of the relative humidity, which they hypothesised to act as a barrier to copper release. In this chapter the addition of organic load (at the concentration tested) did not appear to protect the microbial aerosols from the antimicrobial activity of copper, which suggests that copper is effective even in the presence of organic matter.

At in-use conditions the majority of antimicrobial activity against *S. aureus* and *A. baumannii* occurred within 60 min, which correlated to the drying time of microbial aerosols since surfaces were wet at 0 h and at 30 min. Up to this time point surfaces

were still moist; the aerosols had not completely dried. This further suggests copper is antimicrobial in the presence of a liquid on a surface.

The little antimicrobial activity against *B. subtilis* was not surprising due to the highly resistant nature of spores. Galeano *et al.* (2003) also found little activity against *Bacillus* spores when exposed to a surface coated with silver- and zinc-containing zeolite formulation (Galeano *et al.*, 2003). On the other hand Weaver *et al.* (2008) and Wheeldon *et al.* (2008) have demonstrated antimicrobial copper activity against *C. difficile* spores. The former observed activity after 24 - 48 h whilst the latter only saw significant reductions in spore viability upon the addition of a spore germinant (Weaver *et al.*, 2008; Wheeldon *et al.*, 2008). Additionally, the relatively similar level of survival after deposition at 0 h for all surface types for *B. subtilis* may suggest there is death of *S. aureus* and *A. baumannii* during the 30 min nebulisation period.

In conclusion, the new antimicrobial surface test developed in this chapter provides a realistic, second-tier test that simulates in-use conditions. The test conditions of the JIS Z 2801 bear little relationship to an indoor environment, such as a hospital, thus it is not an ideal surface efficacy test method. In addition, the presentation of the inoculum and the 'wet' nature of the JIS Z 2801 test do not reflect how a surface could become contaminated in practice. The new test in this chapter presents the inoculum to a surface in a more realistic manner. It also allows for discrimination between surfaces, in turn allowing for better selection of surfaces for potential use in healthcare settings (Ojeil *et al.*, 2013).

CHAPTER 5 THE ANTIMICROBIAL ACTIVITY OF ANTIMICROBIAL SURFACES WHEN PRESENTED WITH DRIED MICROBIAL INOCULA

5.1 Introduction

5.1.1 Dry microbial inoculum

Most antimicrobial surface studies are performed with bacteria in suspension; i.e. as a wet inoculum. In this chapter the focus was geared towards the efficacy of antimicrobial surfaces against a dry inoculum; inoculum without the presence of liquid. A dry surface is a surface free from visible moisture. Environmental monitoring of the relative humidity of surfaces in a hospital setting (Chapter 3) were taken from visibly dry surfaces; however observations did indicate a surface relative humidity range of 30.4 – 64.2 %.

Contamination of surfaces within the hospital environment is described in Chapter 1, section 1.6. The persistence of microorganisms on surfaces is well documented and is described in Chapter 1, section 1.7. Many microorganisms including *S. aureus*, *A. baumannii* and *C. difficile* spores are known to survive well, up to 7 months, in a dry environment (Kramer *et al*., 2006; Otter *et al*., 2011). Thus, if antimicrobial surfaces are to be utilised in hospitals it is important to assess their efficacy against dry bacterial inocula.

Environmental studies have shown that in a hospital environment the amount of bacteria on dry surfaces in patient areas, such as bed rails, trolleys and door handles, ranged from 0 – 80 cfu/cm^2 (Griffith *et al*., 2000; Airey and Verran, 2007). Other studies have found loads of <10 cfu/cm^2 on a nurse workstation in an ICU and <1 - >250 cfu/cm^2 across 27 high-touch surfaces in both paediatric and surgical wards (Cooper *et al*., 2007; Hardy *et al*., 2007).

Jawad *et al*. (1996) investigated the effect of relative humidity on the survival of *Acinetobacter* spp. on surfaces. A range of species were inoculated on to glass cover slips then placed in boxes of varying relative humidity; 10 %, 31 % and 93 %. The inocula dried quickest at 10 % (3 – 4 h) and slowest at 93 % (10 – 16 h). Survival of *A. baumannii* was greatest at relative humidities of 31 % or greater (11 days) compared to at 10 % relative humidity (4 days). In addition, the influence of organic load on survival

was investigated. BSA increased survival from 11 days to 60 days at 23 – 34 % relative humidity (Jawad *et al*., 1996).

5.1.2 Dry microbial inoculum surface efficacy tests

Many studies that have reported the antimicrobial activity of copper usually inoculated a volume (e.g. 20 µL) of bacterial suspension on to test surfaces; thus testing the effectiveness of copper against a wet inoculum (Grass *et al*., 2011). However, it has been noted that this does not reflect the nature of surfaces in a hospital environment, thus more recent studies have geared their focus towards the performance of dry copper surfaces. Currently there is no international standard test method for testing the efficacy of surfaces against a dry microbial inoculum. Santo *et al.* (2008) and Warnes and Keevil (2011) have both developed dry inoculum surface efficacy tests, with reduced inocula volume to assist faster drying, which are described in section 5.1.3.

Despite the stringency of the newly developed antimicrobial test based on the exposure of microbial aerosols in this thesis (Chapter 4), it is also essentially a wet test. The use of dry inocula would be ideal to allow simulation of the antimicrobial performance of test surfaces when contaminated under dry conditions and will help confirm whether wet contact is required for the antimicrobial activity of copper. It is important that the efficacy of antimicrobial surfaces is tested against dry inocula as this would simulate dry-touch contamination in a healthcare setting.

5.1.3 Efficacy of antimicrobial surfaces against a dry microbial inoculum and mechanism of action of copper

To date there are very few published studies describing the presentation of antimicrobial surfaces with a dry microbial inoculum. Santo *et al.* (2008) have developed a test method for dry inocula using a swab moistened with bacterial suspension. The swab is spread on to a test surface and the inoculum dries within 5 s. Warnes and Keevil (2011) have also developed a dry surface efficacy test. They inoculate a low volume (1 µL),

high concentration drop on to surfaces, which they describe also dried within 5 s (Warnes and Keevil, 2011).

Santo *et al.* (2008) showed *E. coli* (at a high initial concentration of 9 log_{10}) exposed to 99 % copper was inactivated after 1 min at 23 °C and within 5 min at 5.5 °C when presented as a dry inoculum. Stainless steel was utilised as a control. The authors dismissed the role of desiccation or osmotic stress due to the survival of *E. coli* on stainless steel. However, this statement is questionable as the authors later described an initial 10-fold decrease in viable *E. coli* observed on stainless steel that occurred within the first 2 min, which they state may be due to plating stress. Copper-nickel-zinc alloy and brass were also tested. An initial 2-3 log_{10} reduction was observed, followed by a plateau, then a 6 log_{10} reduction within 2 min that killed all remaining viable cells. The authors stated a similar biphasic decrease in viability was observed on 99 % copper, but within a shorter time frame. Santo *et al*. (2008) believe the rapid reduction in viability in the last 2 min may be due to a stressor, such as copper ion, that needs to reach a lethal concentration before exuding its effect.

In another study Santo *et al*. (2011) investigated the efficacy of dry metallic copper surfaces compared to moist copper surfaces against *E. coli*. Surfaces were presented with a dry inoculum using the method described above, and with a 40 µL droplet to represent a wet inoculum. They found cells accumulated copper ions faster in cells when *E. coli* was presented as a dry rather than wet inoculum; $3.8 \pm 1.3 \times 10^9$ Cu atoms/cell accumulated within 5 sec (time 0 h, drying time) compared to $1.8 \pm 0.5 \times 10^4$ Cu atoms/cell at 0 h. After 1 min exposure of dry inoculum to copper the amount of copper accumulation remained constant at $4.1 \pm 2.9 \times 10^9$ Cu atoms/cell, which correlated with a 99.99 % reduction in viable cells. For cells exposed to copper as a dry inoculum, the authors claim cell death was caused by membrane and cell envelope damage and copper ion accumulation but cannot conclude whether the fast copper ion accumulation observed was the main cause of cell death or as a result of already damaged membranes. Membrane damage was confirmed by Live/Dead staining of copper exposed cells. Stainless steel was utilised as a control surface; Live/Dead staining showed intact membranes thus the authors suggest desiccation did not cause membrane damage to copper exposed cells. They suggest accumulation of copper was

quicker in dry rather than wet *E. coli* due to the absence of a buffering medium. The study also suggested no DNA damage to *E. coli* occurred on dry copper surfaces since *Deinococcus radiodurans*, a bacterium possessing many DNA repair mechanisms, was susceptible to dry copper surfaces. This suggests no DNA damage was induced since the bacterium would be able to repair such damage (Santo *et al*., 2011).

A later study by Santo *et al*. (2012) suggests copper acts at the cell membrane and does not target genes to induce cell damage in Staphylococci. *Staphylococcus haemolyticus* was investigated in this study. Cells were found to accumulate a high concentration of copper within 5 min exposure (Santo *et al*., 2012). In addition, the authors stated that when cells were exposed to media containing D-cycloserine, which interferes with cell wall biosynthesis, they did not develop an *aapA* gene mutation required for growth on the media. This gene codes for the protein AapA, a D-serine/D-alanine/glycine transporter that takes up D-cycloserine. However, the authors state that if the gene is inactivated, D-cycloserine is not taken up and cells do not grow unless they acquire mutations. In this study, the number of resistant mutants detected on stainless steel (control) and copper surfaces was similar suggesting cells exposed to copper did not develop mutations (Santo *et al*., 2012).

Warnes and Keevil (2011) demonstrated that copper alloy surfaces were able to produce a 6 $\log_{10}$ reduction in viable VRE (*Enterococcus faecalis* (*E. faecalis*) or *Enterococcus faecium* (*E. faecium*)) within 10 min when presented as a dry inoculum (1 µL, high concentration inoculum, dries within 5 s). Stainless steel was utilised as a control; less than a 0.5 $\log_{10}$ reduction was observed after 3 h. In contrast, a wet inoculum of similar concentration was killed after 1 h exposure to copper alloys (Warnes and Keevil, 2011). In terms of the mechanism of action of copper Warnes and Keevil (2011) found membrane depolarisation did not occur until after cell death, which is a discrepancy from Santo *et al*.'s (2011) findings. Copper ions and the generation of superoxide were found to be primarily responsible for cell death resulting in DNA damage and arrested cell respiration; again the DNA damage is something *Santo et al*. (2011) did not observe. These findings were obtained by staining cells exposed to control and test surfaces with a variety of fluorescent dyes that indicate membrane depolarisation, DNA damage and arrested cell respiration.

In a similar study Warnes *et al*. (2012) investigated the mechanisms of copper toxicity in *E. coli* and *Salmonella* Typhimurium as Gram-negative model organisms, compared to previous work with Gram-positive Enterococci. They utilised the same dry inoculum method as Warnes and Keevil (2011), described in section 5.1.2. *E. coli* was killed within 10 min (laboratory strain) and 20 min (pathogenic strain). *Salmonella* was killed within 5 min. Similar methods were used to Warnes and Keevil's (2011) methods to identify the mechanisms of action of copper. It was found that unlike in Enterococci, membrane depolarisation occurred immediately followed by a slower rate of DNA damage. Outer membrane disruption and arrested respiration were also apparent. Copper (I) and copper (II) ionic species and hydroxyl radicals were found to be responsible for the cellular damage observed (Warnes *et al*., 2012).

5.1.4 Aims and Objectives

The aim of this chapter was to develop a new test method for delivering dried microbial inocula on to surfaces to assess their antimicrobial efficacy against a dry inoculum. In comparison to Santo *et al*.'s (2008) and Warnes and Keevil's (2011) methods, the new test will aim to inoculate surfaces with a completely dried inoculum. The test developed by Warnes and Keevil (2011) was carried out as it is a published method and acted as a comparison to the new antimicrobial surface efficacy test developed in this chapter. In addition, the mechanisms of action of copper as an antimicrobial were explored to understand how copper acts against a dried microbial inoculum.

5.2 Materials and Methods

5.2.1 Preliminary testing for development of a new dry inoculum antimicrobial surface efficacy test

Preliminary experiments were carried out to develop a new test method for delivering a dry inoculum on to surfaces. Two methods were explored and are described in sections 5.2.1.1 and 5.2.1.2. The final test set-up is described in section 5.2.2.

5.2.1.1 Method One: Preparation of dried microbial inoculum by freeze-drying and use of an inhaler for delivery of inoculum on to surfaces

5.2.1.1.1 Principle of method

This method was based on the production of a dried microbial inoculum by freeze-drying. Freeze-drying, or lyophilisation, is defined as 'a controllable method for dehydrating labile products by vacuum desiccation' (Day and Stacey, 2007). Freeze-drying is commonly used for the long-term preservation and storage of culture collections (Day and Stacey, 2007). Cryoprotectants are required when freezing microorganisms to protect the microorganism from damage during the freezing process. They can prevent osmotic injury during freezing and thawing and reduce the development of ice crystals (Cleland *et al*., 2004).

The principle of this method was that a freeze-dried inoculum was prepared and loaded into a capsule placed in an inhaler, which was connected to a cascade impactor. The inoculum is then deposited on to surfaces. Starch was chosen as a protective medium in favour of more obvious choices including 10 % skimmed milk as the latter may act as organic load. Starch was prepared in deionised water and autoclaved at 121 °C for 15 min then cooled before use.

5.2.1.1.2 Method of preparing a freeze-dried inoculum and delivery on to surfaces using an inhaler

Dried bacterial inoculum was prepared as follows; an overnight culture of *S. aureus* NCIMB 9518 was prepared in triplicate. The following day the culture was centrifuged at 2500 *g* for 15 min at room temperature. The pellet was washed twice with buffer. MRD and TSC were both tested as suitable buffers. Next, the pellet was re-suspended in 10 mL starch, which was tested at 0.5 %, 1 %, 2 % and 5 % (v/w). A viable count was performed by serial dilution then the culture was snap-frozen by liquid nitrogen and stored for a maximum of two hours at -20 °C. The samples were freeze-dried in an EF 4 Modulyo freeze-dryer (Edwards, West Sussex, UK) for 24 h. Samples were rehydrated

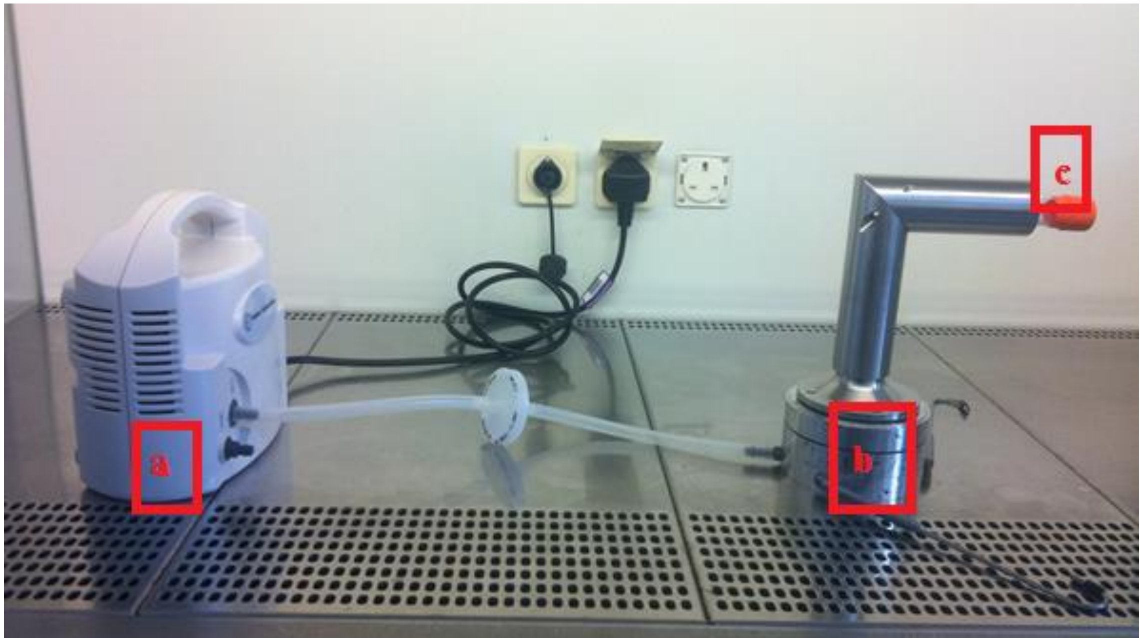
to their original volume and enumerated by serial dilution. Deionised water, TSB and MRD were tested as rehydration media. Samples were re-hydrated in either deionised water or MRD for 1 h at 20 °C, or in TSB for 15 min at 20 °C. Survivability was determined as follows:

Viability % = ($\log_{10}$ cfu/mL after freeze-drying / $\log_{10}$ cfu/mL before freeze-drying) x 100

A similar set-up to that of delivering microbial aerosols on to antimicrobial surfaces was considered for the delivery of dried microbial inocula. An inhaler was used in place of the nebuliser. The inhaler utilised, Flowcaps (Hoviove, Loures, Portugal) is a single-dose dry-powder inhaler. One capsule containing freeze-dried inoculum was placed inside the inhaler. The set-up is shown in Figure 5.1a and a close-up image of the inhaler in Figure 5.1b.

Figure 5.1 – Initial test set-up for delivering dried microbial inocula on to antimicrobial surfaces (a) and close-up photo of inhaler (b)

a) Initial test set-up for delivering dried microbial inocula on to antimicrobial surfaces



a=vacuum pump, b=Andersen cascade impactor, c=Flowcaps inhaler

b) Close-up photo of inhaler

5.2.1.1.3 Results

TSC and MRD were both tested as buffers during the wash steps of preparing samples for freeze-drying (see Table 5.1). Starch was tested at 0.5 % (v/w). The same buffer was utilised for rehydration after freeze-drying. The survival percentage was greater when MRD was utilised for the wash steps and rehydration. There was a significant difference in the survival percentages when MRD was used as opposed to TSC (one-way ANOVA, P=0.023). Thus it was decided MRD would be used for the wash steps.

Table 5.1 – Testing different buffers for wash steps and rehydration during the preparation of freeze-dried *S. aureus* (n=3)

Buffer	Survival percentage (%) ± SD
MRD	97.00 ± 0.84
TSC	92.51 ± 2.60

Three different rehydration media were tested for rehydrating samples after freeze-drying. Samples were rehydrated to their original volume in MRD, deionised water or TSB and viable counts were performed and compared to the viable counts prior to freeze-drying. Starch was utilised at 0.5 % (v/w). Results are shown in Table 5.2. The survival percentage was lowest when cells were rehydrated in TSB and highest when rehydrated in deionised water. As survivability was slightly higher when the dried inoculum was rehydrated with deionised water than in MRD it was decided water would be utilised for rehydration for subsequent experiments. Statistical analysis by a one-way

ANOVA, however, showed there was no significant difference in survival percentage of freeze-dried cells when rehydrated in MRD, deionised water or in TSB (P=0.195).

Table 5.2 – Testing different rehydration media for the rehydration of freeze-dried *S. aureus* (n=3)

Rehydration medium	Survival percentage (%) ± SD
MRD	97.00 ± 0.84
Deionised water	98.08 ± 0.96
TSB	95.43 ± 2.97

Next, different starch concentrations were tested; 0.5 %, 1 %, 2 % and 5 % (v/w). Survival percentages ranged from 92.19 % to 98.08 % with varying starch concentrations. There was no apparent correlation between starch concentration and survival percentage (see Table 5.3). The highest survival percentages were observed with starch at 0.5 % and 2 %, whereas the lowest survival percentages were seen when starch was utilised at 1 % and 5 %. Statistical analysis by a one-way ANOVA confirmed concentration did not have an effect on survivability, P>0.05. Therefore, since 0.5 % starch provided the highest survival percentage, starch was utilised at this concentration for subsequent testing. In addition when starch was autoclaved it gelatinised: the higher the starch concentration the greater the level of gelatinisation, which made preparation difficult.

Table 5.3 – Effect of starch concentration on the survivability of freeze-dried *S. aureus* (n=3)

Starch concentration (w/v %)	Survival percentage (%) ± SD
0.5	98.08 ± 0.96
1	92.19 ± 2.88
2	97.67 ± 1.85
5	92.53 ± 3.91

5.2.1.1.4 Discussion

A freeze-dried inoculum of *S. aureus* NCIMB 9518 was prepared using 0.5 % starch as a cryoprotectant. Trial runs of delivering the dried inoculum on to surfaces from an inhaler connected to an Andersen cascade impactor proved unsuccessful. The dried inoculum did pass through the neck of the impactor, however, it did not fully pass through Stage 0 of the impactor. Instead, the inoculum collected in the middle of the Stage 0 plate and thus did not pass through the holes through to the next plate where the stainless steel sample collecting plate was placed. The most likely explanation for this was that aggregation of the particles affected the flow rate within the test set-up (personal communication, Glyn Taylor and James Birchall, Cardiff School of Pharmacy and Pharmaceutical Sciences). This method was not developed further.

5.2.1.2 Method Two: Generation of dry microbial aerosols using a nebuliser

5.2.1.2.1 Principle of method

Microbial aerosols generated by a nebuliser are dried before deposition on to control and antimicrobial surfaces.

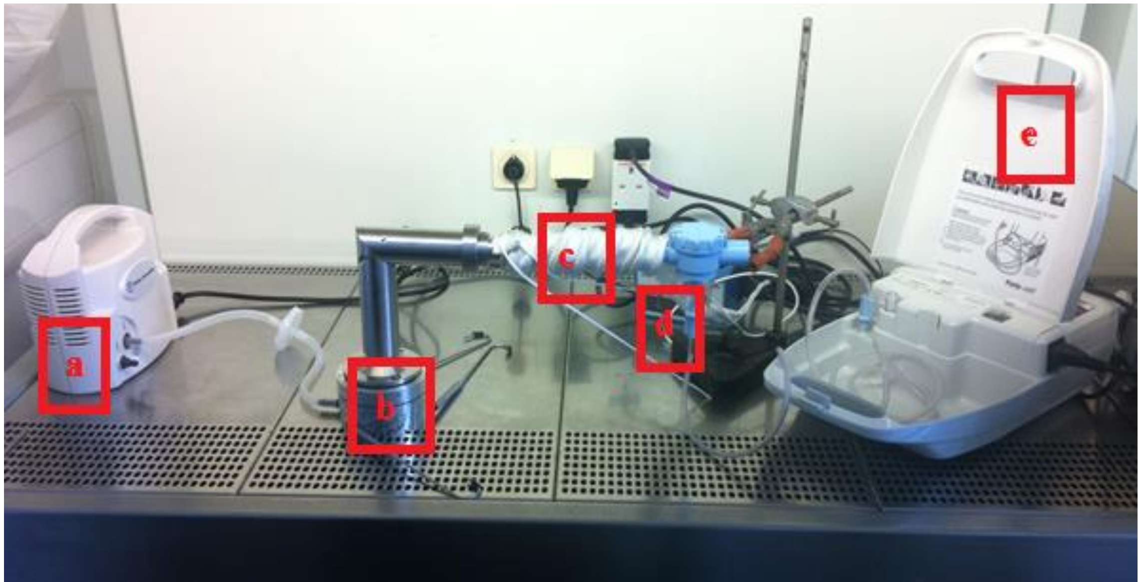
5.2.1.2.2 Method development

Step 1 - Initial test set-up

A *S. aureus* NCIMB 9518 culture was grown as described in Chapter 2, section 2.3.2. The culture was centrifuged for 15 min at 2500 *g* and re-suspended in MRD to provide a bacterial concentration of 10^9 cfu/mL. Ten mL of the suspension was placed in a nebuliser (Philips Respronics, Best, The Netherlands) to aerosolise the suspension. The nebuliser was connected to a 15 cm stainless steel tube wrapped with a 0.9 m heating tape (HT9 Fibre Glass Heating Tape, Electrothermal, Essex, UK). The temperature of the heating tape was maintained with a digital temperature controller (MC810B Digital Heating Controller, Electrothermal, Essex, UK). The heating tape was heated to a temperature high enough to dry the aerosols passing through, which were then passed

through an Andersen cascade impactor and deposited on to sample surfaces (as with the wet aerosol experiments, Chapter 4). Figure 5.2 shows the experimental test set-up.

Figure 5.2 – Test set-up for delivery of dried microbial aerosols on to antimicrobial surfaces



a=vacuum pump, b=Andersen cascade impactor, c=heating tape, d=temperature controller, e=nebuliser

Step 2 - Experiments to find a suitable temperature to dry aerosols

Tables 5.4 – 5.7 show the temperature the temperature controller was set to, the amount of inoculum recovered from stainless steel discs and the state of surfaces (‘dry’, ‘wet’ or ‘condensation’). A ‘dry’ surface indicates surfaces were visibly dry after 30 min of inoculum nebulisation, drying and deposition. Thus a dry inoculum has been deposited on to surfaces. ‘Wet’ means aerosols had not dried during the drying process and wet droplets were visible. ‘Condensation’ indicates a slight layer of condensation was present on surfaces after 30 min of inoculum nebulisation, drying and deposition. This layer of condensation, in most cases, evaporated immediately to then give the appearance that a dry inoculum had been deposited on to surfaces. However, a surface with condensation was considered wet. It was not possible to take a picture of the condensation layer present on surfaces as the condensation evaporated quickly.

The original length of the stainless steel tube placed between the cascade impactor and nebuliser was 15 cm. The original length of heating tape was 0.9 m. A bacterial suspension of *S. aureus* NCIMB 9518 was prepared and adjusted to a final concentration of 10^9 cfu/mL in MRD. The suspension was nebulised and passed through the stainless steel tube wrapped with heating tape and particles were deposited on to three stainless steel discs. The heating tape was tested once at a range of temperatures (60 - 100 °C). The temperature was set using the temperature controller shown in Figure 5.2 to see what temperature was required to completely dry the inoculum. After deposition, discs were transferred to a 100 mL bottle containing 5 g glass beads, 1 mL neutraliser and 9 mL MRD, shaken for 1 min at 150 rpm and viable bacteria enumerated. Table 5.4 shows the tested temperatures did not completely dry the nebulised bacterial suspension, as the discs were visibly wet after the inoculum was nebulised, dried and deposited.

Table 5.4 – Dry inoculum method optimisation: original stainless steel tube (15 cm) and heating tape (0.9 m) with starting inoculum 10^9 cfu/mL

Temperature (°C)	Recovery from stainless steel (n=3) ($\log_{10}$ cfu/cm^2)	Dry, wet or condensation?
60	5.45 ± 0.32	Wet
70	5.03 ± 0.14	Wet
80	4.72 ± 0.24	Wet
90	4.73 ± 0.32	Wet
100	4.96 ± 0.59	Wet

Step 3 - New test set-up with longer stainless steel tube

It was decided a longer stainless steel tube, 23 cm in length, would be placed between the cascade impactor and nebuliser to increase the opportunity for aerosols to dry. As before, the heating tape was heated to a range of temperatures to determine which was sufficient to dry aerosolised particles. Each temperature was tested in three separate runs. A 10^9 cfu/mL *S. aureus* suspension was prepared in MRD, the suspension was nebulised, passed through the stainless steel tube and the inoculum was deposited over

three stainless steel discs. Viable bacteria on discs were enumerated as previously described.

The use of a longer stainless steel tube produced a dried inoculum on surfaces. 60 °C was not sufficient to dry aerosolised particles (see Table 5.5). At 70 °C and 80 °C inconsistent results were produced; wet and dry inocula, in addition to condensation were apparent on surfaces. At 90 °C two of the three repeats presented a dry inoculum on surfaces, one run presented a fine layer of condensation. The discs were checked every 5 min during the 30 min nebulisation process. It was during the final check at 30 min that condensation on previously dry surfaces (between 0 - 25 min) developed. In terms of cfu/cm^2 of recovered dried inoculum from discs there was no significant difference in counts when the inoculum was dried at 90 °C (one way ANOVA, P=0.957).

Table 5.5 – Dry inoculum method optimisation: longer stainless steel tube (23 cm) and original heating tape (0.9 m) with starting inoculum 10^9 cfu/mL (n=3)

Temperature (°C)	**Recovery from stainless steel (n=3) ($\log_{10}$ cfu/cm^2)**	**Dry, wet or condensation?**
60	4.77 ± 0.14	Wet
	4.48 ± 0.09	Wet
	5.11 ± 0.20	Wet
70	4.54 ± 0.23	Dry
	4.18 ± 0.09	Wet
	4.14 ± 0.10	Condensation
80	4.73 ± 0.03	Dry
	5.14 ± 0.65	Wet
	4.02 ± 1.17	Condensation
90	3.74 ± 0.51	Condensation
	3.74 ± 1.34	Dry
	3.53 ± 0.80	Dry

Figure 5.3 shows images of wet and dry inocula deposits. It is apparent that the inoculum deposited in Figure 5.3a had not completely dried thus was a wet inoculum. The image in Figure 5.3b is of a dry inoculum; there were no visible wet aerosols.

Figure 5.3 – Image of wet (a) and dry (b) microbial inocula deposits

a) Wet

b) Dry

Step 4 - New test set-up with longer stainless steel tube, longer heating tape and addition of silica bead sachets

Since a longer stainless steel tube was used, the heating tape, when wrapped around the tube, did not fully and tightly cover the entire length of the tube. This might perhaps explain the inconsistent results observed previously (Table 5.5). Therefore, a longer heating tape (2.4 m long) was obtained, which fitted nicely around the stainless steel tube (see Figure 5.2). In addition, to overcome the problem of condensation that developed on surfaces within the final few minutes of nebulisation, five 1 g silica bead sachets were placed below the collecting plate within the cascade impactor (see Figure 5.4a). One sachet was also placed in the centre of Stage 0 of the cascade impactor (see Figure 5.4b). As before, a 10^9 cfu/mL suspension of *S. aureus* NCIMB 9518 was prepared, a range of temperatures tested and the amount of bacteria recovered from stainless steel discs was enumerated.

Figure 5.4 – Arrangement of silica bead sachets on a) Stage F and b) Stage 0 of cascade impactor

a) Stage F

b) Stage 0

Setting the temperature controller at 50 °C was not high enough to dry aerosolised particles; thus it was tested only once. It was apparent the temperature controller at 60 °C was sufficient to dry aerosolised bacteria; however there was still condensation on surfaces (see Table 5.6). A range of 4.17 to 5.15 log_{10} cfu/cm^2 of inocula (see Table 5.6) was recovered from stainless steel discs from these three runs; there was a significant difference in the amount of bacteria recovered from stainless steel discs from the three runs (one-way ANOVA, P=0.017)

Table 5.6 - Dry inoculum method optimisation: Longer stainless steel tube (23 cm), longer heating tape (2.4 m) and silica bead sachets to aid removal of moisture. Starting inoculum 10^9 cfu/mL

Temperature (°C)	Recovery from stainless steel (n=3) (log_{10} cfu/cm^2)	Dry, wet or condensation?
50	5.24 ± 0.51	Wet
60	5.15 ± 0.13	Dry
	4.17 ± 0.23	Condensation
	4.94 ± 0.45	Condensation

Step 5 - Modification of new test set-up – holding cascade impactor at 40 °C before nebulisation

In addition to the use of silica bead sachets, the cascade impactor was held at 40 °C for up to one hour before the nebulisation process to further overcome condensation issues. This was to reduce the temperature difference between the stainless steel tube and cascade impactor thus preventing the build-up of condensation within the cascade impactor. 60 °C was tested in triplicate and all three runs presented a dried inoculum without the presence of condensation. Recovery from stainless steel discs ranged from 4.34 to 4.57 $\log_{10}$ cfu/cm^2, which showed no significant difference between counts obtained from the three repeats (one-way ANOVA, P=0.727) (see Table 5.7).

Table 5.7 - Dry inoculum method optimisation: longer stainless steel tube (23 cm), longer heating tape (2.4 m), silica bead sachets and holding cascade impactor and discs for approx. 45 min at 40 °C before nebulisation. Starting inoculum 10^9 cfu/mL (n=3)

Temperature (°C)	Recovery from stainless steel (n=3) ($\log_{10}$ cfu/cm^2)	Dry or wet?
60	4.50 ± 0.44	Dry
	4.56 ± 0.16	Dry
	4.34 ± 0.33	Dry

Step 6 - Modification of new test set-up – higher starting inoculum concentration

At this stage the amount of dried bacterial inocula recovered from stainless steel was between 4.34 and 4.57 $\log_{10}$ cfu/cm^2. Ideally recovery should be higher. During wet inoculum testing the amount recovered from stainless steel discs and copper alloy surfaces at 0 h were around 6 and 5 $\log_{10}$ cfu/cm^2, respectively. For comparison to wet inoculum testing and to have a similar level of bacterial recovery from stainless steel discs at 0 h (immediately after nebulisation and drying of particles) the starting inoculum of *S. aureus* was increased from 10^9 cfu/mL to 10^{10} cfu/mL.

An overnight culture of *S. aureus* NCIMB 9518 was grown in 100 mL TSB for 24 h with shaking, centrifuged at 5000 *g* then re-suspended in 10 mL MRD. The suspension was nebulised; particles dried and deposited over three stainless steel discs. Setting the heating tape to 70 °C was sufficient to dry the aerosolised suspension. Table 5.8 confirms the higher starting inoculum of 10^{10} cfu/mL presented a higher level of bacterial recovery from stainless steel discs.

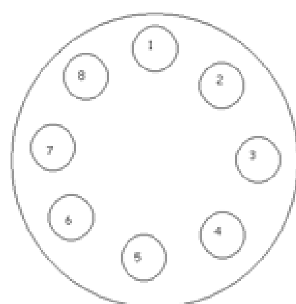
Table 5.8 – Recovery of dried inoculum from stainless steel discs using a longer stainless steel tube (23 cm), longer heating tape (2.4 m), silica bead sachets, holding cascade impactor and discs for approx. 45 min at 40 °C before nebulisation and high starting inoculum of 10^{10} cfu/mL

Temperature (°C)	Recovery from stainless steel (n=3) ($\log_{10}$ cfu/cm^2)	Dry or wet?
70	6.39 ± 0.31	Dry

5.2.1.2.3 Validation of recovery of dried microbial inoculum from stainless steel discs

The method of depositing and recovering dried bacterial aerosols was validated by depositing dried bacterial aerosols of *S. aureus* on to eight stainless steel discs, as positioned in Figure 5.5, and determining viability. The amount of viable bacteria recovered from each surface is shown in Table 5.9. This was performed once.

Figure 5.5 – Position of stainless steel discs on collecting plate within the cascade impactor for validation of recovery of dried microbial inoculum



Statistical analysis by means of a one-way ANOVA showed the positioning of a stainless steel disc had no effect on the amount of viable bacteria recovered. Table 5.9 shows the amount of viable bacteria recovered from each disc, which was calculated in triplicate. There was no significant difference in the amount of bacteria recovered between the eight discs, P=0.472.

Table 5.9 – Validation of recovery of dried microbial inoculum from stainless steel discs (n=1)

Disc	Log_{10} cfu/cm^2 ± SD
1	7.40 ± 0.02
2	7.27 ± 0.06
3	7.12 ± 0.03
4	7.32 ± 0.08
5	7.73 ± 0.04
6	7.74 ± 0.02
7	7.82 ± 0.03
8	7.70 ± 0.05

5.2.1.2.4 Discussion

This method of presenting a dried microbial inoculum was acceptable. A temperature setting of 70 °C was established to be sufficient to dry nebulised aerosols. The deposition of dried inoculum was validated with *S. aureus*. This test set-up and method was next used for assessing test and control surfaces against a dried inoculum (complete, final set-up and method described in section 5.2.2).

5.2.2 New antimicrobial surface test for testing dried microbial inocula – use of a nebuliser to generate dry microbial aerosols

5.2.2.1 Final test set-up

Test equipment was set-up as shown in Figure 5.2. A 2.4 m heating tube was wrapped around a 23 cm stainless steel tube that was connected to the nebuliser at one end and to the cascade impactor at the other end. Test and control surfaces were placed on the

collecting plate within the cascade impactor. In addition, five silica bead sachets were placed under the collecting plate on Stage F and one sachet was placed in the centre of Stage 0. Before testing commenced, the cascade impactor was held at 40 °C for up to one hour to help prevent the build-up of condensation during the dry inoculum deposition period. The temperature controller attached to the heating tape was set to 70 °C during the process. The actual temperature inside the stainless steel tube was measured using temperature probes described in section 5.2.2.3. A Copley Scientific DFM2000 (Nottingham, UK) flow meter was placed between the cascade impactor and vacuum pump (not shown in Figure 5.2). Ten runs of the set-up presented an average flow rate of 2.18 ± 0.54 L/min.

5.2.2.2 Final test method

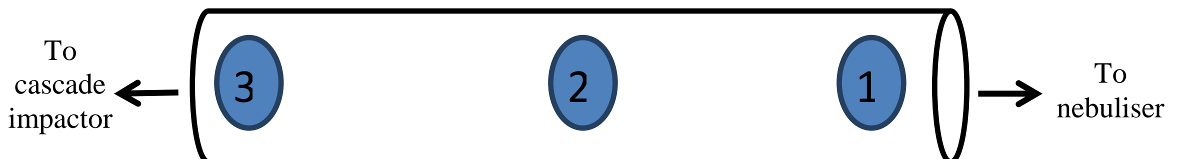
An overnight culture of *S. aureus* NCIMB 9518 was grown in 100 mL TSB at 37 °C with shaking. The culture was centrifuged at 5000 *g* and re-suspended in 10 mL MRD, nebulised, dried and deposited over a total of six control stainless steel discs and test copper alloys surfaces. The combination of surfaces tested was random within each run; stainless steel and copper alloy surfaces were mixed After 30 min inoculum nebulisation, drying and deposition (0 h), surfaces were transferred to a 100 mL bottle with 5 g 3mm glass beads containing 1 mL neutraliser and 9 mL MRD and shook for 1 min at 150 rpm. Viable bacteria were then determined by the spread plate method. As with wet inoculum testing, surfaces were incubated for 30 min, 60 min and 24 h after deposition of dried inoculum and viable bacteria then determined as mentioned above. Unlike wet inoculum testing, surfaces were only subject to one incubation condition, [20°C-40% RH]. This was the most realistic incubation condition reflective of an indoor hospital environment based on conditions observed during hospital sampling sessions (see Chapter 3). Each surface and contact time were tested in triplicate.

Copper alloy and control surfaces were also tested at [20°C-40% RH] against a dried inoculum of *A. baumannii* NCIMB 9214 as a Gram-negative model. The inoculum was prepared by growing an overnight culture of *A. baumannii* in 100 mL TSB for 24 h at 25 °C with shaking. The culture was then centrifuged at 5000 *g* and re-suspended in 10 mL MRD for testing.

5.2.2.3 Temperature and relative humidity conditions within test set-up

To gain information regarding temperature and relative humidity conditions within the test set-up, probes were placed within the cascade impactor and stainless steel tube that was wrapped with heating tape. Three SL54TH probes (Signatrol.com, Gloucestershire, UK) measuring 17 mm in diameter and 6 mm in thickness were positioned on the collecting plate, where test and control surfaces are normally placed. This probe measured temperature and relative humidity. Three SL53T probes (Signatrol.com, Gloucestershire, UK) of the same size as the SL54TH model were placed inside the stainless steel tube that connects the nebuliser to the cascade impactor. This probe recorded temperature only, up to a maximum of 125 °C. One probe was placed at the nebuliser end of the tube (position 1), a second was placed half way across the tube (position 2) and the third probe was placed at the cascade impactor end of the tube (position 3), see schematic diagram Figure 5.6. All three probes were set to log data every 30 sec. The test was set up and carried out as usual as described in 5.2.2.1 and 5.2.2.2 to mimic a normal run of the procedure. *S. aureus* was utilised as the test microorganism. This was repeated in triplicate.

Figure 5.6 – Schematic diagram of position of temperature probes (1, 2, and 3) within stainless steel tube



5.2.3 Efficacy of antimicrobial surfaces against a dry microbial inoculum – use of an existing, published method

5.2.3.1 Method

A modified method of Warnes and Keevil's (2011) test, which aims to simulate dry-touch contamination, for testing the efficacy of antimicrobial surfaces against a dry microbial inoculum was carried out. The idea of this test was that a low volume, high concentration inoculum was inoculated on to surfaces, which dried quickly. Surfaces

were then incubated under the required conditions and time points and viable bacteria were recovered from surfaces. This test was carried out using *S. aureus* only. Firstly *S. aureus* NCIMB 9518 was grown in 10 mL TSB for 24 h at 37 °C. The grown culture was centrifuged at 2500 *g* for 15 min and re-suspended in 500 µL MRD (yielding approximately 10^{10} cfu/mL). From this 1 µL (around 10^{7} cfu) was inoculated and spread evenly using the end of the pipette tip on to control stainless steel and test copper alloy surfaces. The inoculum was bench dried within 5 s; no visible moisture was apparent on the surface. To keep in line with the contact times used throughout this thesis, the contact times tested were 30 min, 60 min and 24 h. Surfaces were then incubated for these times at [20°C-40% RH]; the most realistic conditions based on observations from hospital sampling sessions (Chapter 3). The level of inoculum on surfaces at 0 h was determined by transferring surfaces to a 100 mL bottle containing 5 g 3 mm glass beads, 9 mL MRD and 1 mL neutraliser. Bottles were placed on a shaking platform for 1 min then left to stand for 5 min. Viable bacteria were determined by serial dilution down to 10^{-4}; 100 µL of each dilution was spread on to a TSA plate in triplicate and incubated for 24 h at 37 °C and cfu counted. Viable bacteria on surfaces incubated for the other contact times were recovered in the same way as at 0 h. Each surface and contact time were tested in triplicate.

5.2.3.2 Temperature and relative humidity conditions of test

Temperature and relative humidity conditions on surfaces during this test method were determined using a S154TH temperature and relative humidity probe. One probe was placed on a test surface and then the surface was inoculated with 1 µL of 10^{7} *S. aureus*, as prepared in 5.2.3.1. This was repeated in triplicate. The probes were set to record temperature and relative humidity at 0 min (before inoculation). Surfaces were inoculated at 1.5 min and probes were removed after 5 min. Measurements were recorded every 30 sec, which was the minimum interval possible.

5.2.4 Understanding the mechanism of action of copper against a dry microbial inoculum

To understand the antimicrobial effect, if any, of copper against a dry microbial inoculum, fluorescence-activated cell sorting (FACS) experiments were carried out to determine the physiological state of cells after copper exposure. In addition, the effect of the drying process on bacteria inocula, and any damage that may have been amplified by copper, was determined.

5.2.4.1 Dyes and surfaces tested

Propidium iodide (PI) and Bis (1,3-dibarbituric acid) trimethine oxanol (BOX) (both from Sigma-Aldrich, Poole, UK) were utilised in combination to assess membrane damage and changes in membrane potential, respectively. PI stains the DNA of cells with damaged membranes and BOX stains cells with collapsed membrane potentials. PI was prepared in sterile water at 200 μg/mL) to be used at a working concentration of 5 μg/mL. BOX was prepared in dimethyl sulfoxide (DMSO) at 10 mg/mL and diluted in phosphate buffered saline (PBS) to a working concentration of 10 μg/mL. 100 μL ethylenediaminetetraacetic acid (EDTA) at 4 mM was added to 9.9 mL working concentration of BOX to help with the staining of cells.

5-cyano-2,3-ditolyl tetrazolium chloride (CTC) purchased from Sigma-Aldrich, Poole, UK) was a dye used to detect respiring cells. During electron transport respiring cells reduce CTC to insoluble formazan, which fluoresces. SYTO9 (Invitrogen, UK) was used to assess DNA damage in cells; SYTO9 stains cells with intact DNA. CTC was prepared in sterile water at a stock concentration of 100 mM and used at 5 mM final concentration. SYTO9 was prepared at 5 mM in DMSO and diluted in sterile water for use at 5 μM final concentration. These two dyes were used in combination.

The effect of copper on dried inocula of *S. aureus* and *A. baumannii* was investigated at 0 h and after 24 h at [20°C-40% RH]. *S. aureus* was exposed to stainless steel (control surface), CuSn5 (presented lowest $\log_{10}$ reduction after 24 h, see Table 5.10) and CuZn30 (presented highest $\log_{10}$ reduction after 24 h, see Table 5.10). *A. baumannii*

was exposed to stainless steel (control surface), CuSn5 (presented lowest log_{10} reduction after 24 h, see Table 5.11) and CuNi10Fe1Mn (the counts from this surface were significantly different to the other three copper alloy surfaces, see Table 5.11).

5.2.4.2 Method of preparing bacterial cells for FACS analysis

Dry inoculum testing was carried out as described in section 5.2.2 for *S. aureus* and *A. baumannii*. Surfaces were tested at 0 h and after 24 h incubation at [20°C-40% RH]. After the required contact time surfaces were transferred to a 100 mL glass bottle containing glass beads, 9 mL MRD and 1 mL neutraliser and shook for 1 min at 150 rpm. The 10 mL mixture was then divided into 2x 5 mL and transferred to Universal tubes. Tubes were then centrifuged at 2500 *g* for 10 min. One 5 mL test sample was re-suspended in 500 µL PBS for PI and BOX staining, and the other 5 mL test sample was re-suspended in 500 µL sterile water for CTC and SYTO9 staining. 50 µL of each sample was added to 1 mL FACSFlow buffer (BD, UK) and the dyes were added at the required concentration as described in section 5.2.4.1.

PI and BOX were added together and left for 5 min in the dark before FACS analysis using a FACS ARIA II (BD, UK) machine. CTC was added first to samples and samples were incubated for 90 min in the dark at 37 °C. SYTO9 was then added; samples were incubated for 30 min in the dark at room temperature before FACS analysis.

Ethanol was used as a positive control to show membrane damage. Ethanol, heat shock and hydrogen peroxide (H_2O_2) treatment of cells were also used as positive controls to show arrested cellular respiration and DNA damage. Cultures of *S. aureus* and *A. baumannii* were prepared as in Chapter 2, section 2.3.2. One mL of each culture was centrifuged for 1 min at 13,000 *g* and re-suspended in 500 µL of 100 % ethanol for 10 min, or 500 µL of PBS for heat shock treatment at 100 °C for 30 min, or 500 µL of 10 % H_20_2 for 30 min. After each treatment the suspension was centrifuged for 1 min at 13,000 *g* and the pellet re-suspended in 500 µL PBS for PI and BOX staining or 500 µL sterile water for CTC and SYTO9 staining. As above, 50 µL was transferred to 1 mL FACSFlow buffer, dyes added and samples loaded into the FACS machine.

Additionally, a sample containing just cells from an overnight culture that had been centrifuged and re-suspended in PBS (no treatment) was stained with the dyes as another control.

Cell samples were loaded individually into the FACS Aria II illuminated with a 488 nm laser and data from 10,000 particles were collected. Fluorochromes already calibrated to the FACS Aria II were used. PI fluorescence (red) was collected at an excitation-max 482 nm/emission-max 678 nm and BOX fluorescence (green) was at an excitation-max 494 nm/emission-max 519 nm. CTC fluorescence (green) was collected at an excitation-max 494 nm/emission-max 519 nm and SYTO9 fluorescence (yellow) at an excitation-max 496 nm/emission-max 578 nm.

The combination of testing PI and BOX together to assess membrane damage was based on the methods described by Whitehead *et al*. (2011). The use of CTC and SYTO9 was based on several publications by Warnes and co-workers (Warnes *et al*., 20120, Warnes and Keevil, 2011).

Each experiment was repeated twice on different occasions.

5.2.5 Relative humidity of surfaces following hand-touch

A small experiment to gain an insight into the effect of hand-touch on surface relative humidity was carried out. Sl54TH probes mentioned in section 5.2.2.3, which measure relative humidity were utilised as test surfaces. The probes were set to record relative humidity every 30 sec. Eight participants were asked to pick up a probe and hold it for 1 min. This time frame is longer than a typical contact between a person and a surface (e.g. a person gripping a door handle in a hospital ward), however, since the probes can only log data at a minimum of 30 sec intervals, it was decided that a 1 min contact time would be tested. Each participant performed the experiment in triplicate. This experiment was performed over a 24 h period in a biosafety level 2 cabinet.

5.2.6 Statistical analysis

Statistical analysis was carried using SPSS software. In general, a General Linear Model or chi-squared test were used to analyse data statistically.

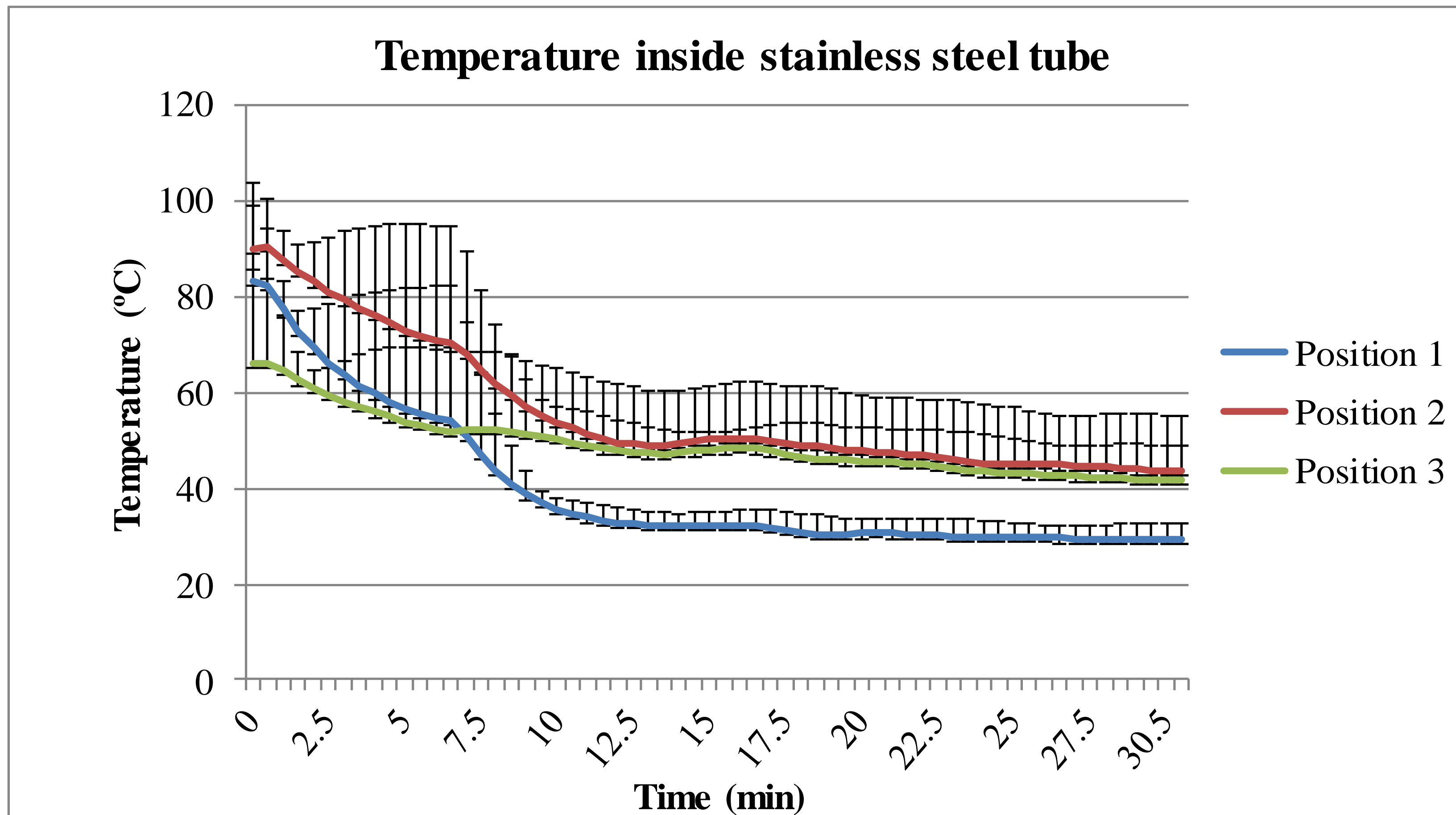
5.3 Results

5.3.1 Temperature and relative humidity conditions within the new dry inoculum test set-up

5.3.1.1 Temperature distribution within the stainless steel drying tube wrapped with heating tape

Figure 5.7 shows the temperature profile inside the stainless steel tube during the 30 min aerosol drying process. The probe at position 1 was placed inside the tube at the end connected to the nebuliser. The probe at position 2 was situated in the middle of the tube and the probe at position 3 was placed at the end of the tube connected to the cascade impactor. It is apparent that at position 1 the temperature decreased over approximately the first 10 min then remained constant for the final 20 min. At positions 2 and 3 there was a similar decrease in the first 10 min followed by approximately a 10 °C decrease during the last 20 min. At position 1 the highest temperature recorded was 95.7 °C at 0 min. The lowest recorded temperature was 26.8 °C during the 23rd min. The highest temperature recorded at position 2 was 90.7 °C, also at 0 min. The lowest temperature was 37.9 °C recorded within the 30th min. At position 3 the highest and lowest temperatures recorded were 88.5 °C at 0 min and 27.3 °C within the 30th min, respectively. The average temperatures recorded over three repeats at position 1 was 40.0 ± 17.3 °C, at position 2 was 56.0 ± 16.0 °C and at position 3 was 48.9 ± 12.9 °C. Overall, across all three positions, the average temperature inside the tube was 48.3 ± 14.0 °C.

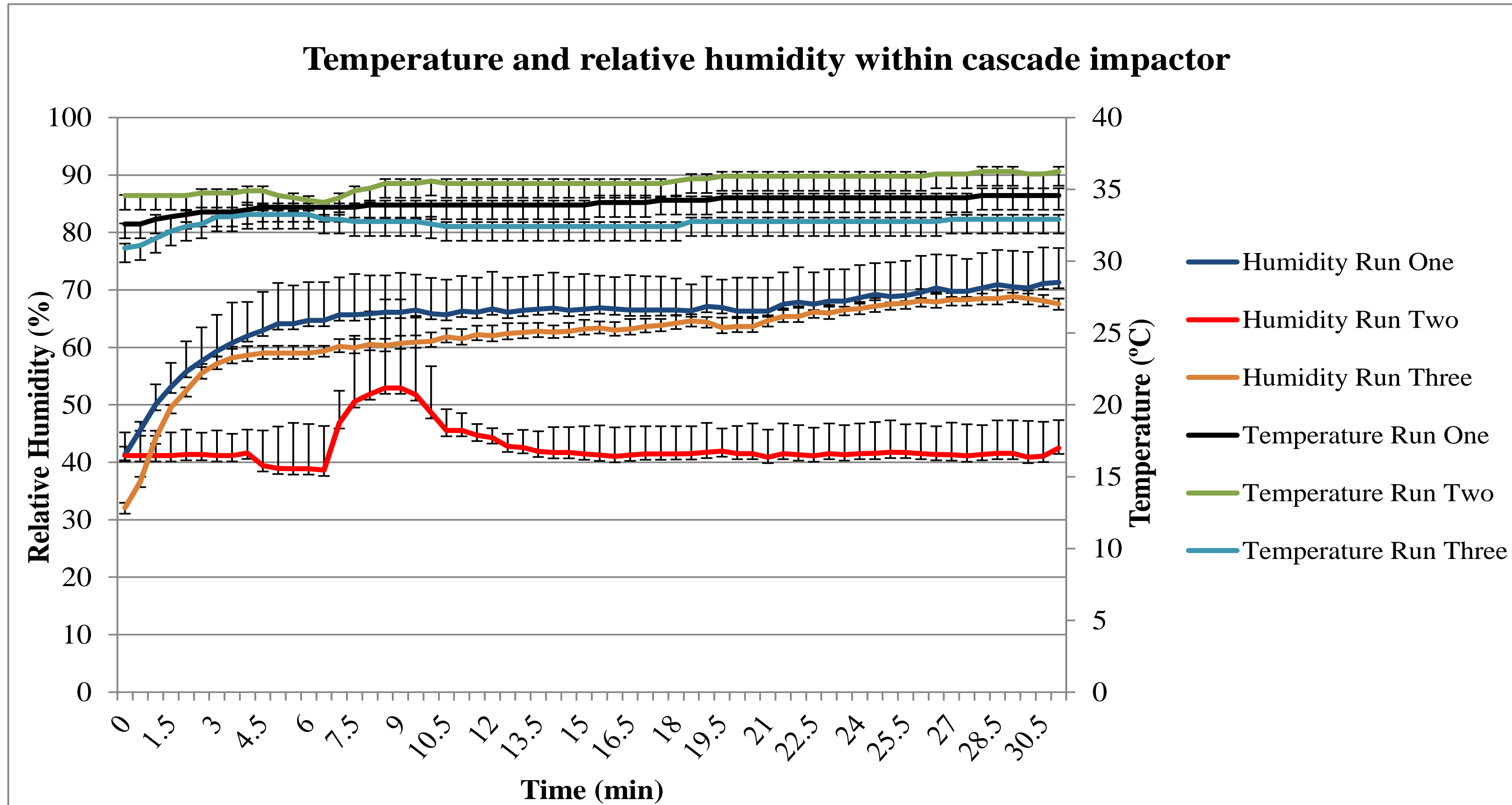
Figure 5.7 – Temperature profile within the stainless steel tube during nebulisation and drying of dry microbial inoculum (n=3). Error bars represent the standard deviation.



5.3.1.2 Temperature and relative humidity within the cascade impactor

Figure 5.8 shows the temperature and relative humidity conditions within the cascade impactor during the 30 min inoculum nebulisation, drying and deposition period. Three probes that measured both temperature and relative humidity were placed on a collecting plate, where test and control surfaces would normally be positioned. Three runs were performed. Over the three runs the highest temperature recorded was 36.3 °C during the 30th min. The lowest temperature was 30.9 °C recorded within the 1st min. The average temperature over the 30 min was 34.0 ± 1.2 °C. The relative humidity recorded ranged from 32.1 to 71.3 %, recorded during the 1st and 30th min, respectively. The average relative humidity was 56.6 ± 11.4 % over the 30 min. It appears the temperature profile within the cascade impactor is quite similar from three repeat runs. The three lines on the graph (see Figure 5.8) follow a similar patter, particularly from the 6th min onwards, showing only slight variability in the temperature recorded. On the other hand, the relative humidity profile showed more variability. Two runs (runs 1 and 3) showed a similar pattern, relative humidity rose sharply between 0 min to approximately the 5th min, then rose slowly and remained constant to approximately the 20th min before rising again during the final 10 min. In contrast, the relative humidity readings recorded during run 2 showed a very different pattern. Firstly the overall average was lower at 42.6 % compared to 65.3 % and 62.0 % for runs 1 and 2, respectively. Here, relative humidity stayed constant until approximately the 4th min, the decreased before peaking around the 8th min. Relative humidity then decreased until the 12th min before remaining constant until the 30th min. The variation observed during run 2 may be due to differences in ambient conditions on that particular day of testing. Relative humidity readings from run 2 were very different from runs 1 and 3; run 2 appears to have been an anomaly. If relative humidity readings from run 2 were to be dismissed the average relative humidity from runs 1 and 3 would be 63.7 ± 2.3 %.

Figure 5.8 – Temperature and relative humidity within cascade impactor during nebulisation, drying and deposition of dry inoculum (n=3). Error bars represent the standard deviation.



5.3.2 Antimicrobial efficacy of copper alloy surfaces against a dried microbial inoculum

The method described in section 5.2.2 was the final protocol developed for testing a dried microbial inoculum against test and control surfaces.

5.3.2.1 **S. aureus** *results*

Table 5.10 summarises the log_{10} reductions observed after 30 min, 60 min and 24 h exposure of dried *S. aureus* aerosols to copper alloy and stainless steel discs at [20°C-40% RH]. Dry bacterial recovery from stainless steel over the three replicates averaged at 7.02 ± 0.28 log_{10} cfu/cm^2 at 0 h. In contrast, recovery from copper alloy surfaces was 1.61-1.85 log_{10} cfu/cm^2 lower. Within 30 min at [20°C-40% RH] the log_{10} reductions presented by copper alloys were <1 $log_{10.}$ For CuDHP and CuNi10Fe1Mn the average log_{10} reductions at 30 min were less than the log_{10} reduction observed on stainless steel at the same time point. After 60 min incubation two copper alloys, CuDHP and CuZn30, presented a >1 log_{10} reduction. CuSn5 and CuNi10Fe1Mn showed lower log_{10} reductions of 0.99 ± 0.07 and 0.73 ± 0.45, respectively. These reductions were greater than that presented by stainless steel at 60 min. After 24 h CuSn5 and CuNi10Fe1Mn displayed a >1 log_{10} reduction. CuDHP and CuZn30 showed continued antimicrobial activity but reductions remained >1 and <2 log_{10}.

Data were analysed statistically using SPSS software. Data were transformed (natural log + 1) and analysed by a General Linear Model. There were significant differences between all copper alloy surfaces and stainless steel (P<0.001). CuSn5 was significantly different to all other copper alloys (P<0.001). There was no significant difference between CuDHP and CuZn30 (P>0.999), CuDHP and CuNi10Fe1Mn (P=0.771) and CuZn30 and CuNi10Fe1Mn (P=0.817). Overall, there were significant differences between all time points (P<0.001).

Table 5.10 - Recovery at 0 h and $\log_{10}$ reductions of a dried *S. aureus* inoculum after 30 min, 60 min and 24 h incubation at [20°C-40% RH] (n=3)

	Stainless steel	**CuSn5**	**CuDHP**	**CuZn30**	**CuNi10Fe1Mn**
	Log_{10} cfu/cm^2	**Log_{10} cfu/cm^2**	**Log_{10} cfu/cm^2**	**Log_{10} cfu/cm^2**	**Log_{10} cfu/cm^2**
0 h	7.02 ± 0.28	5.20 ± 0.50	5.37 ± 0.40	5.41 ± 0.15	5.17 ± 0.21
Log_{10} reductions					
30 min	0.45 ± 0.13	0.53 ± 0.09	0.34 ± 0.20	0.43 ± 0.40	0.33 ± 0.22
60 min	0.56 ± 0.19	0.99 ± 0.07	1.24 ± 0.20	1.19 ± 0.28	0.73 ± 0.45
24 h	0.70 ± 0.38	1.29 ± 0.11	1.67 ± 0.21	1.80 ± 0.22	1.59 ± 0.10

5.3.2.2 **A. baumannii** ***results***

Table 5.11 shows the $\log_{10}$ reductions observed after exposure of *A. baumannii* dried inoculum to stainless steel and copper alloy surfaces. Recovery of dried inoculum from stainless steel averaged 5.28 ± 0.25 $\log_{10}$ cfu/cm^2 at 0 h. Recovery from copper alloy surfaces was lower ranging from 3.38 ± 0.03 to 4.07 ± 0.23 $\log_{10}$ cfu/cm^2 at 0 h. After 30 min there was little additional antimicrobial activity by copper; all four alloys presented a <1 $\log_{10}$ reduction. The reduction observed on stainless steel after 30 min was lower than on the copper alloys, although the standard deviation was high. After 60 min CuDHP presented the highest $\log_{10}$ reduction. The average $\log_{10}$ reduction by CuZn30 remained constant after 60 min. The $\log_{10}$ reduction for CuNi10Fe1Mn and CuSn5 remained <1 $\log_{10}$ after 60 min. The stainless steel $\log_{10}$ reduction of 0.59 ± 0.75 at 60 min was lower than all copper alloys except for that of CuSn5. After 24 h all copper alloys presented a >1 $\log_{10}$ reduction. CuDHP presented the greatest reduction (1.46 ± 0.59 cfu/cm^2) and CuSn5 the lowest (1.01 ± 0.38 cfu/cm^2). The $\log_{10}$ reduction on stainless steel after 24 h was lower than on all copper alloys but the high standard deviation shows the variability in data obtained across the replicates.

Data were transformed (natural log + 1) and analysed by a General Linear Model using SPSS software. There were significant differences between all copper alloys and stainless steel ($P<0.001$). CuNi10Fe1Mn was significantly different to all other copper alloys (CuSn5 $P<0.001$, CuDHP $P<0.001$ and CuZn30 $P=0.011$). There was no significant difference between CuSn5 and CuDHP ($P=0.996$), CuSn5 and CuZn30 ($P=0.302$) and CuDHP and CuZn30 ($P=0.149$). There were significant differences between all time points ($P<0.001$) except between 30 min and 60 min ($P=0.639$).

Table 5.11 - Recovery at 0 h and $\log_{10}$ reductions of a dried *A. baumannii* inoculum after 30 min, 60 min and 24 h incubation at [20°C-40% RH] (n=3)

	Stainless steel	**CuSn5**	**CuDHP**	**CuZn30**	**CuNi10Fe1Mn**
	Log_{10} cfu/cm^2	**Log_{10} cfu/cm^2**	**Log_{10} cfu/cm^2**	**Log_{10} cfu/cm^2**	**Log_{10} cfu/cm^2**
0 h	5.28 ± 0.25	3.38 ± 0.03	3.67 ± 0.67	3.83 ± 0.39	4.07 ± 0.23
Log_{10} reductions					
30 min	0.39 ± 0.72	0.48 ± 0.34	0.75 ± 0.11	0.90 ± 0.15	0.52 ± 0.47
60 min	0.59 ± 0.75	0.38 ± 0.11	1.03 ± 0.44	0.90 ± 0.25	0.64 ± 0.10
24 h	0.99 ± 0.93	1.01 ± 0.38	1.46 ± 0.59	1.29 ± 0.05	1.44 ± 0.25

5.3.3 Antimicrobial efficacy of copper alloy surfaces against a dry microbial inoculum using an existing test method

5.3.3.1 Results

The dry inoculum test method described by Warnes and Keevil (2011) was modified and carried out. Within 30 min all copper alloy surfaces presented a >4 $\log_{10}$ cfu/cm^2 reduction in bacteria after incubation at [20°C-40% RH]. Two surfaces, CuSn5 and CuDHP, presented a >5 $\log_{10}$ cfu/cm^2 reduction after 30 min. After 24 h incubation all surfaces showed a >5 $\log_{10}$ cfu/cm^2 reduction. The stainless steel count only decreased by 0.79 ± 0.23 $\log_{10}$ cfu/cm^2 after 24 h (see Table 5.12)

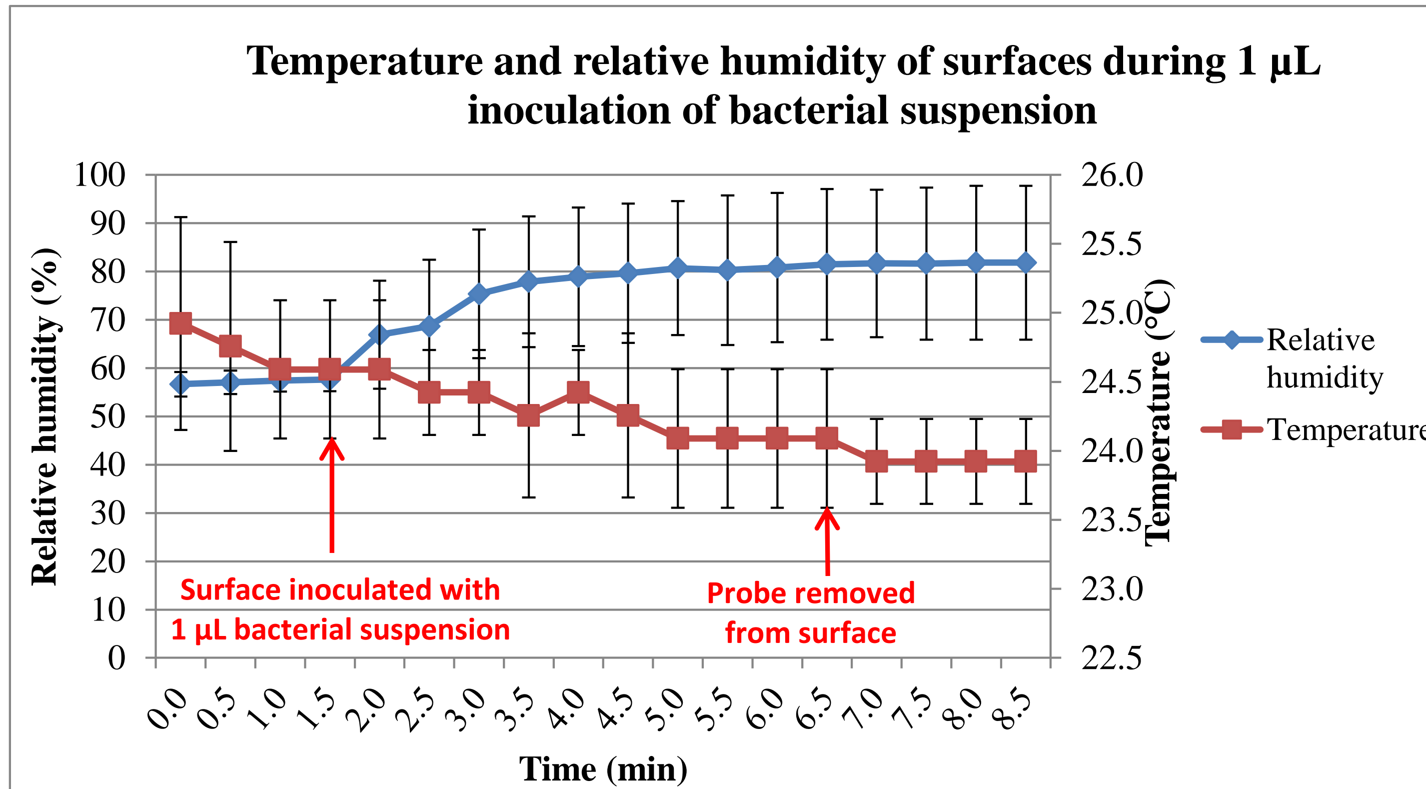
Table 5.12 – Recovery of dry *S. aureus* from low volume inoculum at 0 h and log_{10} reductions after 30 min, 60 min and 24 h incubation at [20°C-40% RH] (n=3)

	Stainless steel	**CuSn5**	**CuDHP**	**CuZn30**	**CuNi10Fe1Mn**
	Log_{10} cfu/cm^2	**Log_{10} cfu/cm^2**	**Log_{10} cfu/cm^2**	**Log_{10} cfu/cm^2**	**Log_{10} cfu/cm^2**
0 h	6.96 ± 0.17	6.50 ± 0.31	6.60 ± 0.09	6.14 ± 0.45	6.85 ± 0.04
Log_{10} reductions					
30 min	0.51 ± 0.06	> 5.66 ± 0.00	5.33 ± 0.74	4.77 ± 0.92	4.41 ± 0.79
60 min	0.50 ± 0.17	> 5.66 ± 0.00	5.76 ± 0.00	4.72 ± 0.50	5.30 ± 0.21
24 h	0.79 ± 0.23	> 5.66 ± 0.00	5.76 ± 0.00	5.30 ± 0.00	5.28 ± 0.68

5.3.3.2 Temperature and relative humidity conditions of test

Figure 5.9 shows the temperature and relative humidity conditions of Warnes and Keevil's (2011) dry inoculum surface efficacy test. Surfaces were inoculated with 1 µL bacterial suspension at 1.5 min and the relative humidity and temperature probe was removed at 6.5 min after 5 min of logging data. Despite variations between triplicate results it appears that relative humidity increased upon surface inoculation. At the inoculation time point (1.5 min) the average relative humidity of the three surfaces was 57.6 ± 2.4 %. Within 30 sec it increased to 66.9 ± 11.2 %. Over the 5 min recording period the average relative humidity was 74.7 ± 7.9 %. In terms of temperature, a small decrease was apparent upon surface inoculation. At the inoculation time point (1.5 min) the temperature was 24.6 ± 0.5 °C. During the 5 min recording period the temperature decreased slightly to 24.1 ± 0.5 °C.

Figure 5.9 – Temperature and relative humidity of surfaces following inoculation of 1 μL bacterial suspension. Error bars represent the standard deviation



5.3.4 FACS analysis of dry microbial inocula exposed to test and control surfaces

Data were analysed using FACS BD software. For each repeat a sample of bacterial cells recovered from surfaces and stained with the appropriate dyes was placed in the FACS machine. 10,000 events were recorded and a plot of forward scatter against side scatter was produced. Within this plot the area of highest intensity was gated for cells only to eliminate any small debris particles from any analysis. This gating was then applied for subsequent samples to select cells for FACS analysis. Despite setting the FACS machine to record 10,000 events, the actual number of cells counted per sample was less than this. This may be due to there being less than 10,000 events to count, or due to cells falling outside the gated area set previously, since removing any background noise was likely to decrease the number of events. The numbers of events recorded per sample are shown next to each FACS plot (see Appendix 2a, Figures 1-8).

5.3.4.1 Division of FACS plots into quadrants

Plots were produced for each dye combination tested. Figure 5.10 is a template of a PI *vs.* BOX plot. The lower-left quadrant represents 'healthy' cells (PI^-BOX^-) with an intact membrane and membrane potential. Cells in the upper-left quadrant have an intact membrane but interrupted membrane potential (PI^-BOX^+). The upper-right quadrant represents 'damaged' cells since they have a disrupted membrane and membrane potential (PI^+BOX^+). Cells in the lower-right quadrant (PI^+BOX^-) signify a disrupted membrane but maintained membrane potential.

Figure 5.10 – PI *vs*. BOX plot template

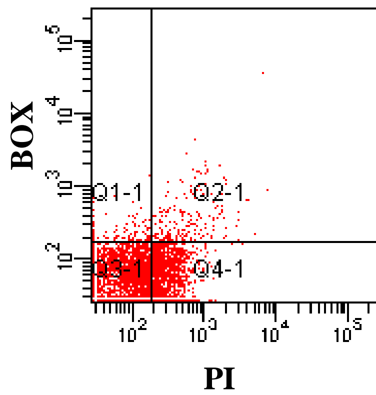
BOX		
	PI^-BOX^+ Intact membrane, disrupted membrane potential	**PI^+BOX^+** Membrane damaged, Disrupted membrane potential DAMAGED
	PI^-BOX^- Intact membrane, unaffected membrane potential HEALTHY	**PI^+BOX^-** Membrane damaged, unaffected membrane potential
	PI	

A positive control was used to aid the division of FACS plots into quadrants. The arbitrary separation of plots into quadrants provided an indication of the state of cells (i.e. healthy or damaged) according to their fluorescence profile based on the dyes utilised. This aided analysis of data. Ethanol was utilised as a control for PI and BOX stained cells.

Figure 5.11 shows the effect of ethanol treatment on *S. aureus*; cells shifted from the lower-left, healthy cells quadrant (Figure 5.11a) to the upper-right, damaged cells quadrant (Figure 5.11b). Cells with no treatment were 76.5 % healthy but after ethanol treatment for 10 min 96.8 % were damaged.

Figure 5.11 – Example of separation of quadrants based on ethanol treatment of *S. aureus* subsequently stained with PI and BOX

a) no treatment



b) ethanol treatment

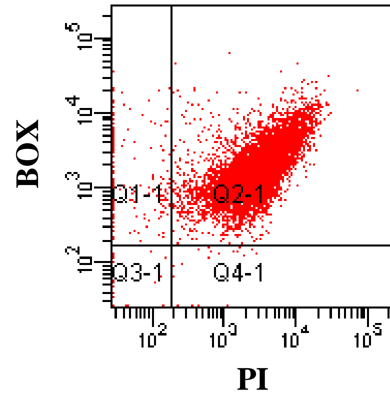
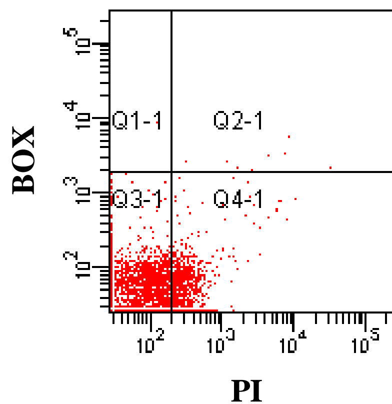


Figure 5.12 shows the effect of ethanol treatment on *A. baumannii*. 85.6 % of cells with no treatment were in the lower-left, healthy cells quadrant (Figure 5.12a). After ethanol treatment (10 min) 99.3 % of cells were in the upper-right, damaged cells quadrant (Figure 5.12b).

Figure 5.12 – Example of separation of quadrants based on ethanol treatment of *A. baumannii* subsequently stained with PI and BOX

a) no treatment



b) ethanol treatment

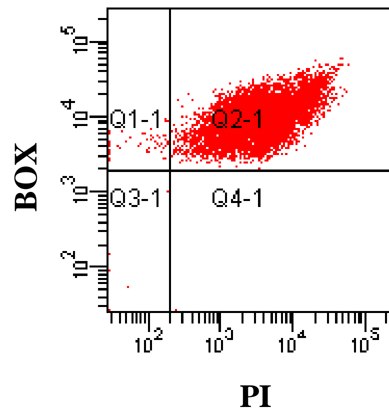


Figure 5.13 is a template of a CTC *vs.* SYTO9 plot. The lower-left quadrant (CTC^{-}SYTO9^{-}) represents 'damaged' cells; cells negative for both CTC and SYTO9, suggesting respiration and DNA integrity are compromised. The upper-left quadrant (CTC^{+}SYTO9^{-}) indicates respiring cells with DNA damage. Cells represented in the upper-right quadrant (CTC^{+}SYTO9^{+}) are 'healthy' as they are respiring and their DNA is intact. Finally, cells in the lower-right quadrant (CTC^{-}SYTO9^{+}) have intact DNA but are not respiring.

Positive controls were also used to help the separation of FACS plots into quadrants. Ethanol was utilised as a control. H_2O_2 and heat shock were also tested but did not provide sufficient damage to be utilised as positive controls.

Figure 5.13 – CTC *vs.* BOX plot template

	CTC^{+}SYTO9^{-} Normal respiration, damaged DNA	**CTC^{+}SYTO9^{+}** Normal respiration, intact DNA HEALTHY
CTC	**CTC^{-}SYTO9^{-}** Arrested respiration, damaged DNA DAMAGED	**CTC^{-}SYTO9^{+}** Arrested respiration, intact DNA
	SYTO9	

Figure 5.14 shows the shift of cells from the upper-right, healthy cells quadrant to the lower-left, damaged cells quadrant after 10 min ethanol treatment of *S. aureus*. 99.7 % of cells (no treatment) were in the upper-right quadrant (Figure 5.14a). Exposure to ethanol resulted in 15 % of cells being placed in the lower-left quadrant (Figure 5.14b).

Figure 5.14 – Example of separation of quadrants based on ethanol treatment of *S. aureus* subsequently stained with CTC and SYTO9

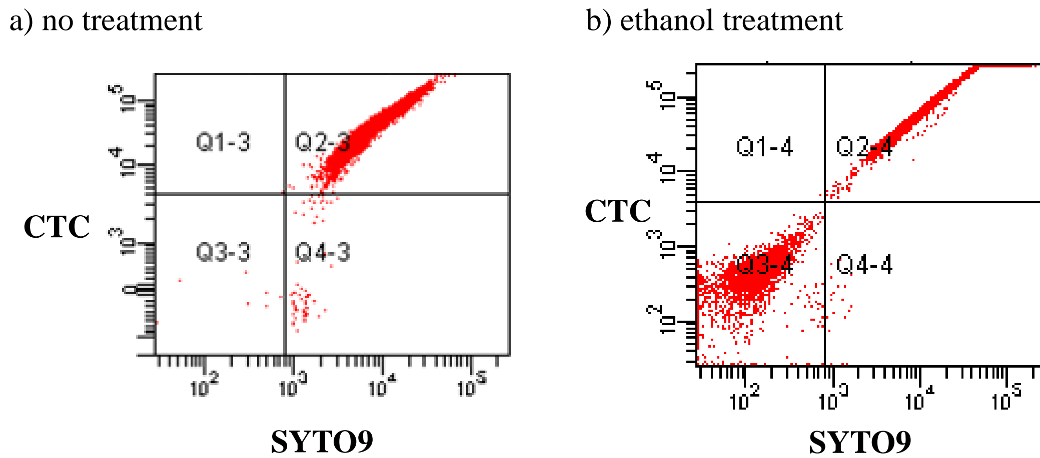
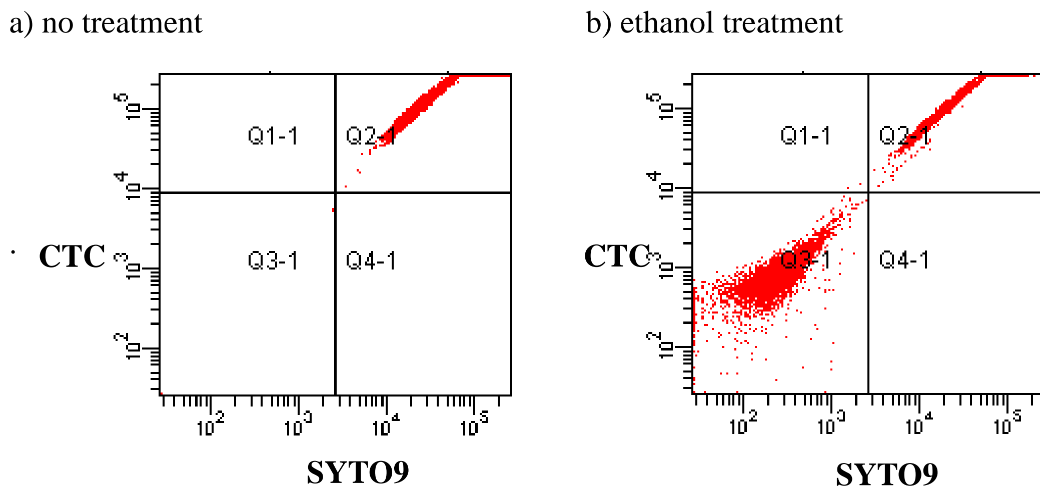


Figure 5.15 also shows the shift of cells from the upper-right, healthy to lower-left, damaged quadrant following ethanol treatment. In Figure 5.15a 100 % of cells are healthy. After ethanol treatment for 10 min 59.8 % of cells are damaged (Figure 5.15b).

Figure 5.15 – Example of separation of quadrants based on ethanol treatment of *A. baumannii* subsequently stained with CTC and SYTO9



5.3.4.2 FACS results

Results presented and analysed in this section are from a combination of the two repeats carried out. For each surface, time point and dye combination the average percentage of cells in each FACS plot quadrant was calculated. All FACS plots are shown in Appendix 2a. Tables 5.13 – 5.16 show the average percentage of healthy and damaged cells for each bacterial strain and dye combination. For raw data from each repeat see Appendix 2b, Tables 1 - 4.

Data were analysed statistically by a chi-squared test using SPSS software. The test looked for a relationship between surfaces and the physiological state of cells (i.e. healthy or damaged). For PI and BOX stained cells those showing only a collapsed membrane potential were not analysed statistically; only healthy or completely damaged cells were. It is worth noting that overall data between repeats 1 and 2 showed great variability. Average percentages are highlighted (with a *) when repeats 1 and 2 values displayed variability (Tables 5.13 – 5.16). Since only two repeats were performed and variability between results was evident, results from the statistical analysis of data need to be approached with caution.

5.3.4.2.1 *S. aureus* results

Table 5.13 shows the average percentage of healthy (membrane intact, membrane potential unaffected, damaged (membrane damaged and collapsed membrane potential) and collapsed membrane potential cells from PI and BOX staining of dried *S. aureus* inoculum exposed to stainless steel, CuSn5 and CuZn30 at 0 h and then for 24 h at [20°C-40% RH]. From the two repeats the average values show an increase in the percentage of damaged cells over 24 h for all surfaces; although this increase was small (<10 %). The percentage of damaged cells was greater at both 0 h and 24 h on copper alloy surfaces than on stainless steel. For stainless steel and CuSn5 the percentage of PI^- BOX^+ cells was mostly unchanged. For CuSn5 at both 0 h and 24 h there were large differences between repeats 1 and 2, as indicated by the asterisk.

Table 5.13 – Summary of *S. aureus* PI and BOX results

Surface and time	$PI^{-}BOX^{-}$ (healthy) (% of cells)	$PI^{+}BOX^{+}$ (damaged) (% of cells)	$PI^{-}BOX^{+}$ (collapsed membrane potential) (% of cells)
SS 0 h	50.0	22.5	34.3*
SS 24 h	46.9	27.0	34.8
CuSn5 0 h	30.1*	42.0*	26.2
CuSn5 24 h	23.3*	48.3*	20.5
CuZn30 0 h	34.9	35.0	27.1
CuZn30 24 h	35.4	43.2	13.8

At 0 h there was a significant relationship between surface and the effect on membrane damage (combined membrane damage and collapsed membrane potential) (chi squared with two degrees of freedom = 12.599, P=0.002). At 24 h there was also a significant association between surface and membrane damage (combined membrane damage and collapsed membrane potential), (chi-squared with two degrees of freedom = 14.320, P=0.001).

Table 5.14 shows the average percentage of healthy (unaffected cellular respiration, DNA intact) and damaged (arrested cellular respiration, DNA damaged) cells from CTC and SYTO9 staining of dried *S. aureus* inoculum exposed to stainless steel, CuSn5 and CuZn30 at 0 h and then for 24 h at [20°C-40% RH]. It is apparent that there was great variation between repeats 1 and 2; many average values are labelled with an asterisk to signify this. The percentage of healthy cells remained constant on the copper alloys but increased on stainless steel over 24 h. In turn, the percentage of damaged cells on the copper alloys remained unchanged and decreased on stainless steel over 24 h. The highest percentage of damaged cells at 24 h was on CuSn5 and the lowest on stainless steel.

Table 5.14 – Summary of *S. aureus* CTC and SYTO9 results

Surface and time	$CTC^{+}SYTO9^{+}$ (healthy) (% of cells)	$CTC^{-}SYTO9^{-}$ (damaged) (% of cells)
SS 0 h	50.9*	42.4*
SS 24 h	79.0	15.7*
CuSn5 0 h	62.7*	34.6*
CuSn5 24 h	62.8*	32.0*
CuZn30 0 h	67.6*	31.6*
CuZn30 24 h	70.0*	26.5*

There was no significant association between surface and the effect on DNA and cellular respiration at 0 h (chi squared with two degrees of freedom = 3.755, P=0.153). Conversely, at 24 h there was a significant relationship between surface and the state of cells (chi-squared with two degrees of freedom = 7.200, P=0.027).

5.3.4.2.2 *A. baumannii* results

Table 5.15 shows the average percentage of healthy, damaged and membrane potential collapsed cells from PI and BOX staining of dried *A. baumannii* inoculum exposed to stainless steel, CuSn5 and CuNi10Fe1Mn at 0 h and then for 24 h at [20°C-40% RH]. The percentage of healthy and collapsed membrane potential cells decreased after 24 h for all surfaces. This resulted in an increased in the percentage of damaged cells on all surfaces over 24 h. At 24 h the percentage of damaged cells were similar on all surfaces ranging from 50.1 – 59.5 %; the lowest from CuNi10Fe1Mn and the greatest from stainless steel. All surfaces presented variable results between repeats 1 and 2, as indicated by the asterisks next to average percentage values.

Table 5.15 – Summary of *A. baumannii* PI and BOX results

Surface and time	PI^-BOX^- (healthy) (% of cells)	PI^+BOX^+ (damaged) (% of cells)	PI^-BOX^+ (collapsed membrane potential) (% of cells)
SS 0 h	30.9	17.1	24.3*
SS 24	7.2	59.5	21.2*
CuSn5 0 h	18.7*	33.8*	22.6*
CuSn5 24 h	9.6*	58.6*	1.1
CuNi10Fe1Mn 0 h	30.7	31.4*	26.4*
CuNi10Fe1Mn 24 h	24.2	50.1*	3.1

At both 0 h and 24 h there were significant relationships between surface and the effect on membrane damage (membrane damage and collapsed membrane potential). At 0 h; chi-squared with two degrees of freedom = 8.323, P=0.016. At 24 h; chi-squared with two degrees of freedom = 12.472, P=0.002).

The average percentage of healthy and damaged cells from CTC and SYTO9 staining of dried *A. baumannii* inoculum exposed to stainless steel, CuSn5 and CuNi10Fe1Mn at 0 h and then for 24 h at [20°C-40% RH] are shown in Table 5.16. The percentage of healthy and damaged cells at both 0 h and 24 h were very similar for CuNi10Fe1mN. The percentage of healthy cells increased after 24 h on stainless steel and CuSn5; CuSn5 presented the greatest increase. At 24 h the greatest damage was observed on stained cells exposed to CuNi10Fe1Mn and the least damage on CuSn5. Variable results between repeats 1 and 2 were apparent for stainless steel at 0 h and 24 h and for CuSn5 at 0 h (as noted by the asterisks).

Table 5.16 – Summary of *A. baumannii* CTC and SYTO9 results

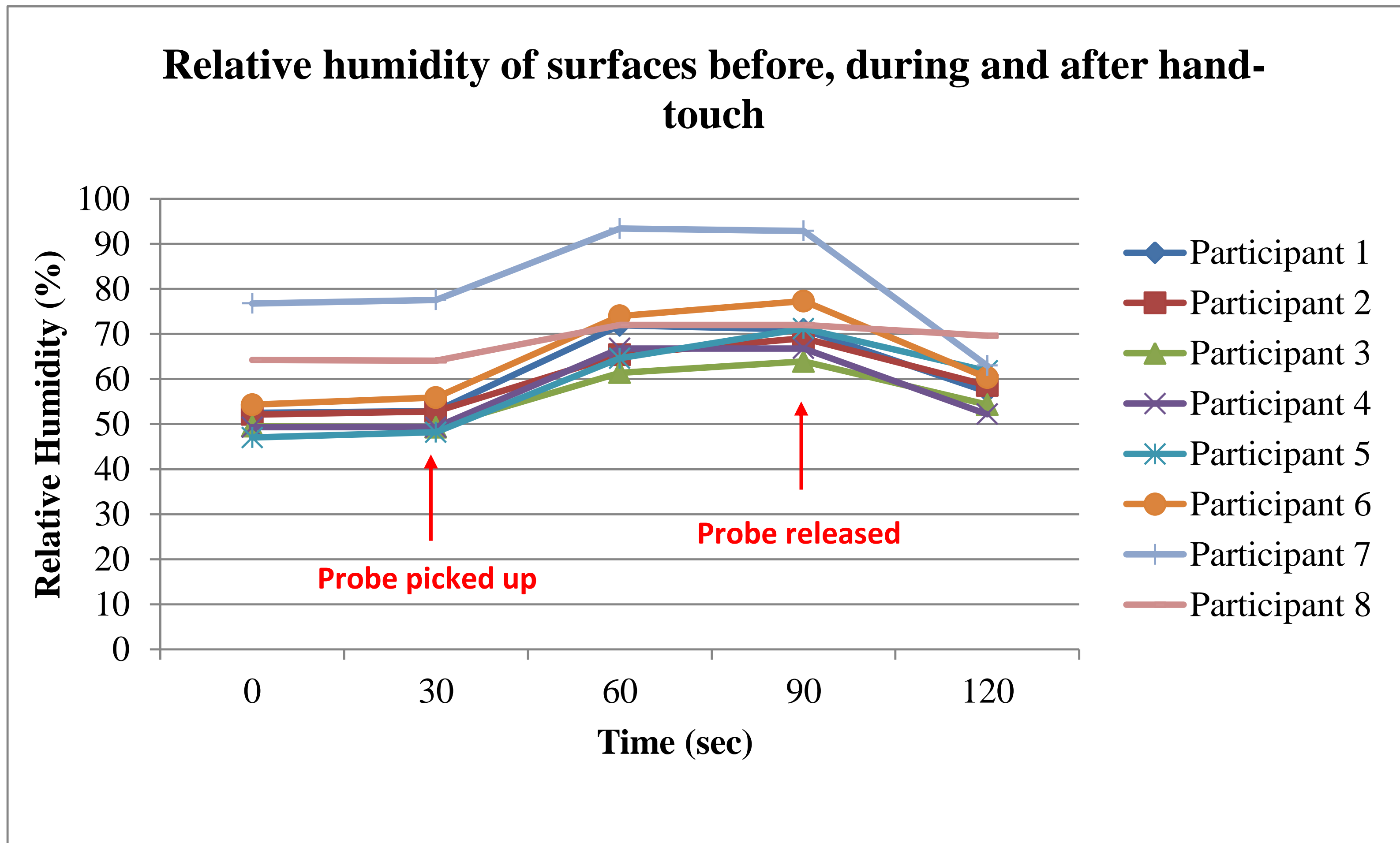
Surface and time	$CTC^{+}SYTO9^{+}$ (healthy) (% of cells)	$CTC^{-}SYTO9^{-}$ (damaged) (% of cells)
SS 0 h	48.9*	50.4*
SS 24 h	59.9*	38.6*
CuSn5 0 h	44.6*	55.0*
CuSn5 24 h	68.9	29.2
CuNi10Fe1Mn 0 h	29.5	69.5
CuNi10Fe1Mn 24 h	28.7	68.1

At 0 h there was a significant association between surface and the effect on DNA damage and arrested cellular respiration (chi-squared with two degrees of freedom = 8.560, P=0.014). At 24 h there was also a significant relationship between surface and the effect on DNA and cellular respiration (chi-squared with two degrees of freedom = 35.011, P<0.001).

5.3.5 Relative humidity of surfaces following hand-touch

Eight participants were asked to hold a Sl54TH probe that measured relative humidity every 30 sec. The probe was used to represent a person touching a surface in real-life. Figure 5.16 shows the average relative humidity profile from three probes handled by each participant. At 0 sec the probes were issued to start logging data. At 30 sec participants held the probe and at 90 sec they released the probe. It is apparent that for each probe between 0 sec and 30 sec (before hand-touch) relative humidity remained constant. Between 30 sec and 60 sec there was an increase of between 7.9 - 19 % in relative humidity. During the next 30 sec interval relative humidity increased slightly. Finally, during the last 30 sec (after hand-touch), relative humidity decreased. Overall, at the 60 sec time point (half-way point of hand contact) the highest relative humidity recorded by all participants was 93.4 % and the lowest was 61.4 %. The average relative humidity at 60 sec was 71.2 ± 10.0 %.

Figure 5.16 – Relative humidity of surfaces before, during and after hand-touch.



5.4 Discussion

There is a need for a standard test to assess the antimicrobial activity of surfaces against a dried microbial inoculum to simulate touch contamination in the healthcare setting under in-use conditions. Several protocols were employed in this chapter to develop such a method. The use of a nebuliser to generate aerosols that were subsequently dried and deposited was the method of choice for presenting a dry inoculum on to surfaces. This method was validated and provided a novel way of testing the antimicrobial efficacy of a dry inoculum deposited on to a surface. Due to the inactivity of copper alloys against a wet inoculum of *B. subtilis* spores (see Chapter 4, section 4.3.3.3) and the slow activity presented against dried inocula of *S. aureus* and *A. baumannii*, it was decided not to test copper against dry *B. subtilis* spores. It is highly likely copper would present no antimicrobial activity against the highly resistant nature of spores.

In this study the antimicrobial activity of copper against a dry inoculum was slow compared to the activity observed against a wet inoculum described in Chapter 4. A <2 log_{10} reduction in both *S. aureus* and *A. baumannii* after 24 h exposure at [20°C-40% RH] was observed. Despite a potential loss of almost 2 log_{10} through nebulisation as a copper effect, the overall effect due to copper was still much lower than that achieved by Santo *et al*. (2008) or Warnes and Keevil (2011), who observed very rapid action by copper using their respective dry surface test methods. Santo *et al*. (2008) observed a 9 log_{10} reduction within 1 min against *E. coli*, and Warnes and Keevil (2011) saw a 6 log_{10} reduction of VRE in less than 10 min. It could be argued that both of these were not strict 'dry' inoculum surface tests; Santo *et al*. (2008) presented the inoculum on to surfaces via a moistened swab, and Warnes and Keevil (2011) as a 1 µL drop. In both cases the initial inoculum presented on to the surface is wet, despite drying in a matter of seconds, whereas the inoculum presented on to surfaces in the new method developed in this chapter was dry. Their results may suggest that the initial wet contact between the inoculum and the copper surface is crucial for fast-acting antimicrobial action, something that was not observed in this study. Thus, the test developed in this chapter is a genuine 'dry' surface efficacy test.

The low volume inoculum test method by Warnes and Keevil (2011) was carried out in this study and based on their results, the results against the four copper alloys tested here were not surprising; >4 $\log_{10}$ reductions were observed after 30 min at [20°C-40 RH%]. This is in agreement with Warnes and Keevil's (2011) findings, who observed quick antimicrobial activity, although a direct comparison of results cannot be made due to differing contact times and incubation conditions. In comparison to the dry inoculum test method developed in this chapter, Warnes and Keevil's (2011) method was tested and demonstrated greater activity by all four copper alloys. Interestingly, in contrast to the wet inoculum test method in Chapter 4, all four copper surfaces performed better against a low volume, high concentration inoculum than against microbial aerosols under the same conditions (see Chapter 4, section 4.3.3.1).

Despite the low antimicrobial activity by copper, there was an obvious difference between the amount of dry inocula (for both *S. aureus* and *A. baumannii*) recovered from stainless steel and from the four copper alloys at 0 h. For *S. aureus* the amount recovered from stainless steel was 7.02 ± 0.28 $\log_{10}$ cfu/cm^2 compared to a range of 5.17 ± 0.21 to 5.41 ± 0.15 $\log_{10}$ cfu/cm^2 from copper alloy surfaces. Interestingly, for *A. baumannii*, the amount recovered from both stainless steel and the copper alloys was lower. The amount recovered from stainless steel averaged 5.28 ± 0.25 $\log_{10}$ cfu/cm^2 and was even lower on the copper alloys, ranging from 3.38 ± 0.03 to 4.07 ± 0.23 $\log_{10}$ cfu/cm^2. These low initial counts could possibly be due to cell aggregation or perhaps *A. baumannii* was more susceptible to drying. Monitoring of temperature and relative humidity conditions within the cascade impactor during the 30 min nebulisation period showed average readings of 34.0 ± 1.2 °C and 56.6 ± 11.4 %, respectively. It could be hypothesised that the high temperature and high relative humidity observed within the cascade impactor may have increased copper's antimicrobial activity, which may possibly have contributed to differences in the 0 h counts between stainless steel and the copper alloy surfaces. Inside the cascade impactor the average temperature and relative humidity recorded were higher than the conditions ([20°C -40% RH]) that surfaces were subsequently exposed to when assessing antimicrobial activity at varying contact times. Coupled with the high temperatures nebulised particles pass through (through the stainless steel tube) during the drying process, these conditions were likely to damage some cells thus potentially making them more sensitive to antimicrobial copper. It is

worth noting that the conditions within the cascade impactor during the 30 min nebulisation period were lower than that during the inoculation of surfaces using Warnes and Keevil's (2011) dry inoculum method. Over the 5 min recording period of Warnes and Keevil's (2011) test method the average relative humidity on surfaces was 74.7 ± 7.9 %. However, since the recording probes were only capable of taking measurements every 30 sec it was difficult to ascertain the exact conditions between the point of surface inoculation and the 5 s drying period. The differences in relative humidity between the test developed in this chapter (both during inoculum deposition and subsequent incubation conditions) and Warnes and Keevil's (2011) test may possibly have contributed to the differences in antimicrobial activity observed between the two methods.

Since only a >1 log_{10} reduction was achieved by all copper alloys against *S. aureus* and *A. baumannii*, FACS analysis was carried out to understand copper's antimicrobial activity in a dry state or whether simply drying of microorganisms in the experimental set-up resulted in reductions in viable bacteria. Warnes and Keevil (2011) suggest DNA damage and arrested cell respiration are the initial stages of cell death, followed by membrane damage in Gram-positive bacteria, whereas membrane depolarisation was observed before DNA damage in Gram-negative bacteria (Warnes *et al*., 2012). This agrees with findings by Santo *et al*. (2008) who also observed membrane damage to be the first stage of cell death in *E. coli*. These studies suggest bacterial morphology influences the mechanism of action of copper.

In this study the aim of FACS analysis was geared more towards the role of desiccation and to find out if copper was antimicrobial against dried microbial inocula, rather than investigating the sequence of events that lead to cell death. FACS experiments were carried out in duplicate for both *S. aureus* and *A. baumannii*. Unfortunately, results did not provide a clear explanation of the exact mechanism of action of copper due to variability between repeats for each microorganism. It was apparent that the drying process had an effect on the nebulised bacterial suspension; membrane damage, arrested bacterial respiration and DNA damage were all evident at 0 h on all surfaces. The level of damage during the drying process can be assessed by looking at the percentage of damaged cells on stainless steel at 0 h. The highest average percentage of membrane

damaged cells was 22.5 % and the highest average percentage of DNA damaged/arrested cellular respiration cells was 50.4 %. However, once the effect of drying was teased out, using stainless steel 0 h results, it was possible to assess the activity of copper. In addition, the percentage of damaged cells at 24 h on copper alloys *vs*. the percentage of damaged cells at 24 h on stainless steel provided an indication, if any, of antimicrobial activity by copper.

Taking into account average results from repeats 1 and 2 it is likely that exposure of *S. aureus* dried inoculum to copper alloys resulted in loss of membrane integrity and a collapsed membrane potential after 24 h at [20°C-40% RH]. Despite all surfaces showing an increase in the average percentage of damaged cells after 24 h, the average percentage of damaged cells from copper alloys were greater than stainless steel, probably due to the antimicrobial properties of copper. The effect of copper on bacterial respiration and DNA integrity was perhaps limited; the percentage of damaged cells at 0 h and 24 h was lower than the percentage of healthy cells. However, damage at 24 h was greater on copper alloys than on stainless steel, which might imply some antimicrobial activity by copper.

Exposure of dried *A. baumannii* to stainless steel and copper alloy surfaces showed an increase in cells showing membrane damage after 24 h. As the average percentage of damaged cells was similar on stainless steel and on the copper alloy surfaces at 24 h it cannot be concluded that copper alone resulted in membrane damage. It should be noted that for this experiment variability between repeats was high for the majority of surfaces and time points. In terms of the effect of copper on bacterial respiration and DNA integrity, it is likely that copper did have an antimicrobial effect. Whilst the average percentage of damaged cells was greater on stainless steel than on CuSn5 at 24 h, the average percentage of damaged cells on CuNi10Fe1Mn was even greater. The antimicrobial activity of CuNi10Fe1Mn was apparent once the effect of drying on cells was teased out.

Statistical analysis to look for associations between surfaces and the percentage of healthy and damaged cells showed a significant relationship for all bacterial strains, dye combinations and time points (except *S. aureus*, CTC-SYTO9, 0 h). However, due to

the variability between repeats 1 and 2, the statistical analysis must be taken with caution.

Keevil and co-workers observed membrane depolarisation, DNA damage and arrested cellular respiration in terms of the mechanism of action of copper (Warnes and Keevil, 2011; Warnes *et al.*, 2012). Some individual results from the FACS work carried out in this study agreed with Keevil and co-worker's findings (results in this study assessed complete membrane damage as well as membrane depolarisation). These are shown in Table 5.17 (see Appendix 2 for complete data set). Loss of membrane integrity and a collapsed membrane potential are apparent in *S. aureus* and *A. baumannii*. Arrested bacterial respiration and DNA damage by copper were also evident in *A. baumannii*. For all of the examples shown in Table 5.17 there was an increase in damaged cells after 24 h and the damage was greater than that observed on stainless steel at the same time point (see Appendix 2b, Tables 1-4).

Table 5.17 – Individual FACS results showing loss of membrane integrity and a collapsed membrane potential (PI-BOX staining) and DNA damage and arrested respiration (CTC-SYTO9 staining) by copper alloys

Microorganism	Surface and time	Dye combination	Repeat	% healthy cells	% damaged cells
S. aureus	CuSn5 0 h	PI-BOX	2	55.7	15.4
	CuSn5 24 h	PI-BOX	2	24.3	51.9
S. aureus	CuZn30 0 h	PI-BOX	2	60.2	14.4
	CuZn30 24 h	PI-BOX	2	46.1	39.4
A. baumannii	CuSn5 0 h	PI-BOX	2	2.3	54.5
	CuSn5 24 h	PI-BOX	2	3.2	94.8
A. baumannii	CuNi10Fe1Mn 0 h	PI-BOX	2	20.1	55
	CuNi10Fe1Mn 24 h	PI-BOX	2	16.5	74.3
A. baumannii	CuNi10Fe1Mn 0 h	CTC-SYTO9	1	38.7	59.6
	CuNi10Fe1Mn 24 h	CTC-SYTO9	1	20.4	74.9

A limitation of the FACS work presented here was that only two repeats were performed. Additional repeats would be necessary to fully understand the mechanism of action of copper against a dried microbial inoculum. Some variability in the results might have been generated from the arbitrary separation of FACS plots into four separate quadrants based on the use of a positive control. Although ethanol worked well when used with PI and BOX, it was clear that ethanol was not an ideal positive control for CTC and SYTO9 (see Appendix 2a, Figure 6d). The FACS protocol would benefit from optimisation of the positive control.

Despite the potential for cell damage during the test set-up, both *S. aureus* and *A. baumannii* were still viable after 24 h on all copper alloys as shown by low $\log_{10}$ reductions. This implies copper alloy surfaces do not provide quick kill against a dry inoculum. This also brings in the important question of whether wet contact between an inoculum and copper-containing surface is vital for antimicrobial activity. The results from this study suggest a liquid interface is crucial. The small investigation in this study to see the effect of hand-touch on the relative humidity of surfaces showed relative humidity increased upon contact. However, it is worth noting that the length of contact was exaggerated during this study. In practice, contact is likely to be variable depending on the surface, its use and how frequently it is touched. In turn, this may influence any changes in relative humidity due to hand contact and consequently the antimicrobial activity of surfaces.

Upon contact with air copper oxidises to form copper oxide. There are very few studies assessing the effect of copper oxide formation on the antimicrobial efficacy of copper/copper alloy surfaces. Most investigations have focused on the efficacy of copper oxide nanoparticles. However, a study by Hans *et al*. (2013) found that found that CuO, which is the common copper oxide formed under wet conditions, was less antimicrobial against *Enterococcus hirae* than Cu_2O that forms normally under dry, ambient conditions. Cu_2O was found to be as efficacious as pure copper (however, this was with a wet inoculum). In terms of copper ion release from surfaces, the copper oxides released less ions than pure copper but of the two oxides, release was higher from Cu_2O than CuO. They suggested the reason for high antimicrobial activity by Cu_2O was due to the release of Cu^{+} ions, which are more antimicrobial than Cu^{2+} (Hans

et al., 2013). In a different study Santo *et al*. (2008) believed copper oxidation may have resulted in copper ion release from surfaces and the generation of reactive oxidative species, in particular hydroxyl radicals, which led to cell toxicity in *E. coli*. These findings were observed with the previously described dry inoculum method developed by Santo *et al*. (2008).

To conclude, a novel method for presenting a dry inoculum on to surfaces has been developed and validated. Culture and FACS analysis have shown evidence of copper toxicity against *S. aureus* and *A. baumannii*. FACS analysis did show that damage occurred during the drying process; however, once this effect was teased out the antimicrobial activity of copper was evident. Overall culture analysis showed a >90 % to <99 % in reduction in viable bacteria on copper over 24 h; a significant difference to stainless steel. Whilst FACS analysis did show cell damage by copper, culture work showed that this damage was not sufficient for complete kill. This study has highlighted the importance of testing the efficacy of surfaces against dried inocula to assess the performance of such surfaces for potential use in healthcare settings.

CHAPTER 6 GENERAL DISCUSSION

6.1 HCAI rates in the UK are decreasing but further exploration of control measures is required

HCAIs are a continuous cause for concern for the NHS. Surveillance, both mandatory and voluntary, of certain bacteraemia cases including MRSA, *C. difficile* and *A. baumannii* has shown a general decrease in their number since their peak in the mid-2000s. However, there is still a need to explore control measures; this will not only improve patient outcome but also decrease the heavy financial burden HCAIs pose to the NHS. Many national campaigns have targeted the role of hand hygiene to promote improved infection control; however, interestingly there has been no focus on the need to control the indirect infection routes via surfaces.

The survival of microorganisms on surfaces is well documented and may act as a reservoir for the dissemination of HCAIs (Dancer, 2004; Kramer *et al.*, 2006). Consequently, a number of antimicrobial surfaces have been commercialised that claim to reduce surface bioburden. The potential use of antimicrobial surfaces, primarily copper and copper alloys, has been extensively tested in the laboratory and numerous trials in clinical settings have been reported (Chapter 1, section 1.8.1). This study also focused on copper alloys.

6.2 Is the JIS Z 2801 an appropriate antimicrobial surface efficacy test?

This study first addressed whether the JIS Z 2801 test is an appropriate test for testing the efficacy of antimicrobial, hard surfaces. The high relative humidity (>90 %) and high temperature (35 °C) of the JIS Z 2801 are ideal for increasing the activity of any antimicrobial effect of surfaces as they allow maximum biocide diffusion. The JIS Z 2801 test is thus an effective first-tier screening test, to determine any antimicrobial activity in optimal environmental conditions.

Sampling of high-touch surfaces over a one year period at a UK hospital in three areas (gastroenterology, ACC and theatre) showed a surface relative humidity range of 30.4 – 64.2 % and a surface temperature range of 18.2 – 26.8 °C. Significant differences in

surface relative humidity were observed between different sampling occasions; however, all surfaces sampled throughout the entire sampling period were not at risk of condensation. Thus, perhaps the variations in relative humidity may not be important practically. In addition, experiments in this study showed that the relative humidity of surfaces increased upon hand-touch. Sampling of surface bioburden in the three wards selected was performed at random; not directly after cleaning. The results showed varying bioburden levels, though not limited to microbial contamination, which adds to the potential usefulness of antimicrobial surfaces in clinical settings.

Environmental sampling of surfaces in a UK hospital confirmed the relative humidity and temperature parameters of the JIS Z 2801 are not realistic; indoor conditions were much lower (Chapter 3, section 3.3). The lack of a current surface efficacy test standard that incorporates realistic parameters to simulate in-use conditions is a concern. In addition, surfaces are covered with a plastic film throughout the test to maintain high relative humidity (i.e. > 90%), thus surfaces remain wet; in practice a wet inoculum would dry well within 24 h. From this study and others it can be concluded that the JIS Z 2801 is not an appropriate test for screening the antimicrobial efficacy of surfaces for use in healthcare settings (Michels *et al.*, 2009; Ojeil *et al.*, 2013). It should only be used during product development to determine whether a surface might have some potential antimicrobial activity, but under no circumstances should it be used to support a claim for activity in the field (such as those listed in Table 6.1).

Thus it appears important that these *in vivo* parameters are reflected in an efficacy test protocol. This led to the development of second-tier tests that utilise in-use conditions.

Table 6.1 – Examples of antimicrobial claims by companies based on JIS Z 2801 testing of antimicrobial surfaces/coatings

Company	Biocide	Applications	Antimicrobial claim	Reference
Corning Gorilla Glass	Silver ion	Glass doors and windows, protective screen cover glass (mobile phones, laptops, medical displays)	**> 99.9 % reduction**	Website 32, 2014
Resco	BioCote Silver Technology	Laminate panel	**up to 99.99 % reduction**	Website 33, 2013
SustainHygen	Silver	Coating for use in hospital patient areas, food manufacturing areas, assisted care facilities	**> 99.9 % reduction**	Website 34, publication date unavailable
CuTouch	Copper	Healthcare furniture e.g door handles, bedrails, switches	**> 5 log reduction**	Website 35, 2012
Lamitech	Nano silver ions	Laminate for use in hospitals, gyms, kitchens, bathrooms	**99.99 % reduction**	Website 36, publication date unavailable

6.3 Development of new antimicrobial surface efficacy tests to measure activity against microbial aerosols and dry microbial inocula

It appeared logical to test the deposition of microbial contaminants following aerosolisation or as dried contaminants. Thus, the next aim of this study was to assess the antimicrobial activity of antimicrobial surfaces when exposed to microbial aerosols (wet inoculum) and a dry microbial inoculum. Two new surface efficacy tests were developed providing second-tier tests that incorporated more realistic parameters, based on those reflective of hospital conditions. It is crucial to develop antimicrobial surface efficacy tests for surfaces using parameters that simulate the intended environment (e.g. healthcare, food, public buildings, see Table 6.5) to confidently predict their efficacy. The two tests developed in this study were flexible; parameters can be easily changed according to the intended in-use conditions of surfaces thus making the tests suitable for a wide range of applications.

Table 6.2 is a summary table that lists the pros and cons of some of the existing antimicrobial surface efficacy tests. This includes standards mentioned in Chapter 1 section 1.11, current dry inoculum published methods and the two new antimicrobial surface efficacy tests developed in this study.

The wet inoculum test developed was based on the exposure of microbial aerosols to test and control surfaces. The use of a nebuliser to produce aerosols aimed to mimic actions such as coughing, sneezing and flushing of toilets. Aerosols dried within 60 min at in-use conditions and were more realistic than the high volume inoculum of the JIS Z 2801. The testing of surfaces against microbial aerosols under specific parameters reflecting in-use conditions allowed the distinction of activity between copper alloys. Activity was significantly reduced at in-use conditions compared to JIS Z 2801 test conditions, which highlighted the importance of testing surfaces under realistic parameters. In turn, this will allow for the better selection of surfaces for use in clinical areas. The test set-up was not as cheap as the JIS Z 2801 in terms of the equipment required; however, overall this new test is more appropriate for predicting the efficacy of antimicrobial surfaces in real-life settings.

The development of an antimicrobial surface efficacy test to incorporate a dry bacterial inoculum aimed to mimic hand-touch contamination. All existing antimicrobial surface efficacy tests, described in Chapter 1, sections 1.11.1 – 1.11.5, are based on the presentation of a wet inoculum to surfaces. It has been acknowledged that antimicrobial surface efficacy tests utilising a wet inoculum are not suffice to test for the potential efficacy of antimicrobial surfaces in clinical areas (Santo *et al*., 2008; Warnes and Keevil, 2011; Ojeil *et al*., 2013). The method developed in this study was unique and based on the presentation of a dried aerosol on to surfaces, compared to other attempts that utilise an initial low volume inoculum (see Table 6.2). Dry inoculum testing highlighted the difference between the activity of copper against wet and dry inocula; activity was slower and reduced against a dry inoculum.

The drying process of the dry inoculum method inevitably caused some damage to bacterial cells (confirmed by FACS analysis of cells collected from test and control surfaces at 0 h); however, this did not hinder the ability to assess damage caused by copper. Another drawback of this new method was that a lot of equipment was required for the test set-up, some of which was expensive.

Table 6.2 – Table showing the pros and cons of antimicrobial surface efficacy tests

Antimicrobial surface efficacy test	Pros	Cons	Comments
JIS Z 2801 (Japan) /ISO22196 (International)	• Quantitative • Ideal for screening surfaces – 'proof of principle test' • Simple, inexpensive set-up	• Incubation conditions (35 °C and >90 % relative humidity) not reflective of in-use conditions • Surfaces covered with a plastic film thus remain wet during entire incubation period – not reflective of practice	• In this study all copper alloys presented a >4 log_{10} reduction in *S. aureus* after 24 h
ASTM E2180-01 (USA)	• Quantitative • Incorporates an artificial biofilm • Surfaces are incubated under temperatures suitable for test surfaces' intended use	• Surfaces incubated under humid conditions – may not reflect practice	
ASTM E2149-01 (USA)	• Quantitative • Dynamic contact test ideal for testing surfaces where prolonged wet contact is expected	• Not appropriate for testing surfaces for use in healthcare settings	
XP G 39-010 (France)	• Quantitative	• Surfaces are placed on an agar plate containing a specific volume of bacteria – not reflective of practice (e.g. healthcare setting) • Surfaces incubated under humid conditions at 37 °C – not reflective of in-use conditions	

Table 6.2 continued - Table showing the pros and cons of antimicrobial surface efficacy tests

Antimicrobial surface efficacy test	Pros	Cons	Comments
Santo *et al.*'s dry inoculum test (2008)	• Quantitative • Simple • Cheap • Dry inoculum mimics hand-touch contamination	• Initial inoculum presented to surfaces is wet but claimed to dry within 5 s	• Santo *et al*. (2008) showed 9 $\log_{10}$ *E. coli* reduction in 1 min at 23 °C
Warnes and Keevil's dry inoculum test (2011)	• Quantitative • Simple • Cheap • Dry inoculum mimics hand-touch contamination	• Initial inoculum presented to surfaces is wet but claimed to dry within 5 s	• Warnes and Keevil showed 6 $\log_{10}$ VRE reduction in 10 min • In this study, copper alloys presented a >4 $\log_{10}$ reduction in *S. aureus* within 30 min at [20°C-40% RH]
Wet inoculum test in this study (Chapter 4)	• Quantitative • Second-tier test to JIS Z 2801 • Use of microbial aerosols mimics coughing, sneezing etc. • Incubation parameters reflective of in-use conditions	• Lot of equipment required • Set-up expensive • 30 min deposition time required to deposit sufficient amount of bacteria on to surfaces	• >4 $\log_{10}$ reduction in *S. aureus* within 30 min at [37°C-100% RH] At in-use conditions: ▪ >4 $\log_{10}$ reduction in *S. aureus* within 60 min ▪ >4 $\log_{10}$ reduction in *A. baumannii* in 30 min ▪ <1 $\log_{10}$ reduction in *B. subtilis* spores after 24 h

Table 6.2 continued - Table showing the pros and cons of antimicrobial surface efficacy tests

Antimicrobial surface efficacy test	Pros	Cons	Comments
Dry inoculum test in this study (Chapter 5)	• Quantitative • Second-tier test to JIS Z 2801 • Inoculum deposited on to surfaces is visibly dry • Dry inoculum mimics hand-touch contamination • Incubation parameters reflective of in-use conditions	• Lot of equipment required • Set-up expensive • Optimisation necessary to establish temperature sufficient enough to dry nebulised inoculum • 30 min deposition time required to deposit sufficient amount of bacteria on to surfaces • Drying process likely to cause some damage to bacterial cells	• >1 log_{10} reduction observed in *S. aureus* and *A. baumannii* within 60 min at [20°C-40% RH] • >1 but <2 log_{10} reduction in *S. aureus* and *A. baumannii* after 24 h at [20°C-40% RH]

6.4 What are the key factors affecting the efficacy of antimicrobial surfaces?

This study has naturally led to the understanding of the key factors that affect the activity of antimicrobial surfaces. Such factors taken into consideration in this study included temperature, relative humidity, contact time, the presentation of a wet or dry bacterial inoculum to surfaces, bacterial cell type, organic load and copper concentration. These factors and how they affect biocides and/or antimicrobial surface efficacy are introduced in Chapter 1, section 1.10.

6.4.1 Temperature and relative humidity

The effects of temperature and relative humidity on the efficacy of antimicrobial surfaces are described previously in Chapter 1, section 1.10.2 and Chapter 4, section 4.1.5. In general, increased temperature and increased relative humidity favours increased antimicrobial surface activity (Grass *et al*., 2011).

In this study it was apparent that copper alloys were more effective at the high temperature and relative humidity conditions of the JIS Z 2801 than at hospital in-use conditions, which were considerably lower. Indeed, significant differences in viability of *S. aureus* deposited aerosols between the different conditions suggested a higher temperature and relative humidity increased copper's efficacy. Michels *et al*. (2009) did not observe a difference in copper's efficacy under JIS Z 2801 conditions and under in-use conditions, however, the only contact time tested was 24 h. On the other hand, other studies are in agreement with the findings from this study; increased temperature (room temperature compared to 4 °C) favoured increased antimicrobial copper activity (Noyce *et al*., 2006a; Elguindi *et al*., 2009).

Copper alloy surfaces in this study were tested under conditions reflective of a UK hospital environment. It is important that when tests and ultimately standards are developed (e.g. British or European) that they incorporate test conditions that simulate in-use conditions for the targeted environment. For parameters such as surface temperature and relative humidity in hospital settings, variability between countries is

likely; therefore, a British standard would perhaps be more appropriate in the UK. For EU standard tests, differences in different country's environmental conditions are usually incorporated into the standard on a worst case scenario. Therefore, an EU antimicrobial surface test would have to be based on the lowest relative humidity and temperature found in hospitals throughout Europe.

6.4.2 Contact time

In practice, antimicrobial surfaces differ from other biocides, such as liquid disinfectants, in that they provide continuous disinfection. However, generally, the longer the contact time between a biocide and a microorganism, the greater the antimicrobial efficacy (Maillard, 2005b).

In this study, the overall general trend observed was that increased contact time correlated with increased microbial reduction for both wet and dry inocula, except for some surfaces against wet inocula of *S. aureus* and *A. baumannii*. In such instances complete kill was observed at the shortest contact time tested.

6.4.3 Wet *vs*. dry bacterial inoculum

Overall, the activity of copper alloy surfaces was reduced at in-use conditions when presented with a dry inoculum ($<2\ log_{10}$ after 24 h) compared to microbial aerosols ($>4\ log_{10}$ after 24 h). It is likely that the wet contact between the inoculum and the copper alloy surfaces was crucial for fast antimicrobial activity; in fact the majority of antimicrobial activity by copper against microbial aerosols took place within 60 min whilst surfaces were still visibly wet. This is in contrast to Warnes and Keevil (2011), who found copper alloys were more efficacious against a dry rather than wet inoculum. However, Warnes and Keevil (2011) used different test microorganisms and wet and dry inoculum testing methods to those in this study.

Whilst it could be argued that the log_{10} reductions presented by copper against a dry inoculum were low relative to a wet inoculum; in clinical trials copper alloys have

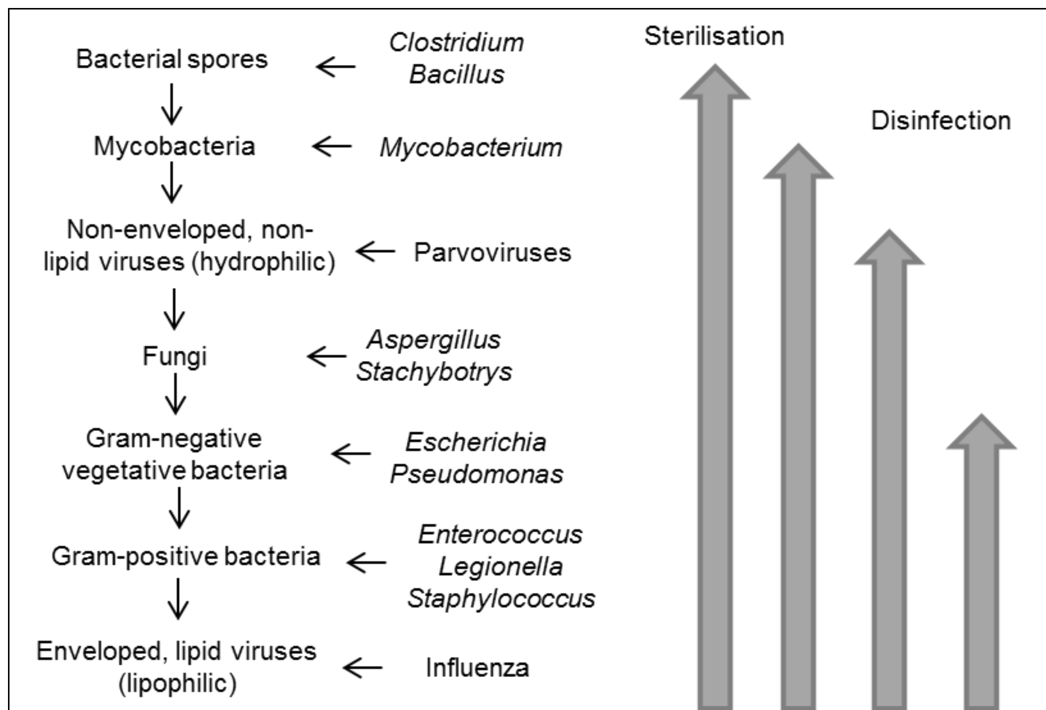
mostly only presented a 1 - 2 log_{10} reduction compared to standard fittings (Weber and Rutala, 2013). In real-life settings surface contamination is likely to be a mix of wet (e.g. aerosol) and dry contamination.

6.4.4. Type of microorganism

The Spaulding classification is a categorisation of surfaces, such as surgical and medical devices into critical, semi-critical and non-critical items. The classification also incorporates the subsequent minimum disinfection requirements per category. Critical items include those that make contact with a sterile area of the human body. Since these devises pose a high risk of infection it is advised that they are sterilised to kill all types of microorganisms present on a surface. Semi-critical items make contact with mucous membranes or broken skin, thus present a lower infection risk. High-level disinfection, sufficient to kill bacterial spores, mycobacteria and vegetative bacteria, is recommended for semi-critical devices. Finally, non-critical items pose the lowest risk since contact with intact skin is made. Low- or intermediate-level disinfection is recommended for such devices, which are not effective against bacterial spores. Intermediate-level disinfection is sufficient to kill mycobacteria and vegetative bacteria, whereas low-level disinfection is only suitable for vegetative bacteria (McDonnell and Burke, 2011).

Nowadays a similar classification is utilised as outlined in Figure 6.1. The diagram shows that with increasing resistance of microorganisms to biocides, increasing sterilisation or disinfection is required. Important hospital pathogens, such as *C. difficile* spores, require high level disinfection. On the other hand other hospital pathogens such as *Enterococcus* and *Staphylococcus* require low-level disinfection. In the food industry, important microorganisms such as *E. coli* would also require low-level disinfection.

Figure 6.1 – Diagram showing the susceptibility profile of microorganisms to biocides and the level of disinfection required (adapted from McDonnell and Burke, 2011)



This study has emphasised the highly resistant nature of bacterial spores to antimicrobials. *B. subtilis* spores were tested in the form of a wet inoculum as it was not possible to test *C. difficile* spores due to aggregation of spores, which resulted in uneven deposition of aerosols on to surfaces. Copper alloys did not present antimicrobial activity after 24 h against *B. subtilis* spores, in agreement with Santo *et al*. (2010), who showed *Bacillus* spores persisted and germinated on pure copper surfaces one month after exposure, whereas *Bacillus* vegetative cells were killed following less than 24 h exposure to copper. In addition, the fact that three of the four copper alloys tested in this study showed no significant differences between stainless steel perhaps indicates a limited role for copper as an effective sporicidal surface.

At in-use conditions copper alloy surfaces were more efficacious against microbial aerosols of *A. baumannii*, a Gram-negative microorganism than of *S. aureus*, a Gram-positive bacterium. This is unusual because Gram-positive bacteria are considered more sensitive to biocides, according to Figure 6.1. However, similar activity by copper alloys was observed against both microorganisms in the dried form.

6.4.5 Copper concentration

It has widely been reported the greater the copper content of copper alloy surfaces, the greater the antimicrobial efficacy (Grass *et al*., 2011). In general, copper alloys containing between 55 - 100 % have shown significant antimicrobial efficacy (O'Gorman and Humphreys, 2012).

In this study, at in-use conditions no significant differences were observed between copper alloy surfaces of ranging copper contents when presented with microbial aerosols of *S. aureus* and *A. baumannii*. When presented with *S. aureus* dried inoculum, CuSn5 (95 % Cu) was significantly different to all other alloys, however, this surface contained neither the highest nor lowest copper content of all four tested. Similarly, CuNi10Fe1Mn (86 – 89.7 % Cu) was significantly different to all other copper alloys after exposure to *A. baumannii* dried inoculum, however this alloy contained the second lowest copper concentration. Overall, there was no apparent link between copper concentration and antimicrobial efficacy, which agrees with findings by Elguindi *et al.* (2009) but disagrees with others who reported that the higher the copper content the greater the antimicrobial efficacy (Wilks *et al*., 2005; Noyce *et al*., 2006a, b).

6.4.6 Organic load

Organic load is added to bacterial suspensions in antimicrobial efficacy tests to mimic ‘dirty’ conditions, which aims to simulate organic matter found in environments such as hospitals (see Chapter 1, section 1.10.5) .

Examples of the effect of organic matter on the activity of copper are detailed in Chapter 1, section 1.10.5. Most findings to date have shown organic matter (e.g. BSA, blood, pus) enhanced the survival of microorganisms on copper-containing surfaces (Airey and Verran, 2007; Tolba *et al*., 2007; Zhu *et al*,. 2012). The addition of organic load to the wet inoculum in this study resulted in significant differences in viable bacteria at in-use conditions of [20°C-40% RH] but not at [37°C-100% RH]. Here organic load did not appear to have a detrimental effect on copper’s antimicrobial effect.

In fact, copper was more antimicrobial upon the addition of soiling. Although this may seem unusual and may require further investigation, Wheeldon *et al*. (2008) also observed no reduced activity by copper against *C. difficile* spores in the presence of a germinant and organic load. However, the germinant may have contributed to the observed copper activity. No other examples of enhanced antimicrobial activity by copper in the presence of organic load have been reported to date.

6.4.7 Recommendations

Based on the known factors affecting biocide efficacy and key findings from this study, the recommended parameters to be tested for potential surfaces in healthcare settings are outlined in Table 6.3. These recommended parameters are applicable to many environments where antimicrobial surfaces may be introduced; such as in the food industry or in public areas (see Table 6.5). The actual conditions, for example temperature and relative humidity, can be modified according to the intended application.

Table 6.3 Recommended parameters for antimicrobial surface efficacy testing

Parameters
• Temperature (e.g. 20 °C in a UK hospital) • Relative humidity (e.g. 40 %, 50 % in a UK hospital) • Contact time • Inoculum type (i.e. wet and dry) • Microorganism/s of interest • Biocide concentration • Organic load (e.g. 3 g/L BSA)

6.5 Future of antimicrobial surfaces in the healthcare setting

Antimicrobial surfaces, in particular copper-containing surfaces have proven to present antimicrobial activity in a wide range of laboratory studies. Many factors need to be taken into account before antimicrobial surfaces are introduced in clinical settings.

There is evidence that improved surface cleaning in hospital wards and intervention, particularly during infection outbreaks, can reduce surface contamination and may also have a role in reducing HCAIs (Donskey, 2013). There is also increasing evidence that copper can reduce microbial loads in clinical settings (Casey *et al*., 2010; Karpanen *et al*., 2012; Schmidt *et al*., 2012). However, the role of antimicrobial surfaces in reducing HCAI rates needs to be explored further. To date, there has been one published pilot study investigating the association between antimicrobial surfaces and infection rates. In a large trial across three hospitals in the US, patients admitted to ICUs were randomly placed in rooms containing EPA-registered copper alloy surfaces or in control rooms with no copper alloy surfaces. Six copper alloy surfaces were introduced in high-touch areas (selected on the basis of findings from previous studies) in all three ICUs. Four surfaces were common to all four hospitals; bed rails, overbed tables, IV poles and arms of the visitor's chair. Other surfaces included nurses' call button, computer mouse, bezel of touchscreen monitor and palm rest of laptop computer. The trial was conducted over one year and copper alloy surfaces were introduced nine months prior. The study showed a significantly reduced incident rate of HCAIs and/or MRSA or VRE colonisation in rooms containing copper alloys than in rooms without copper alloys (Salgado *et al*., 2013). The authors claimed that copper alloy surfaces decreased the risk of an HCAI by more than 50 % but stated more trials were required, particularly to assess the role of reduced surface contamination in controlling HCAI rates (Salgado *et al*., 2013). However, others have criticised the trial by stating the majority of HCAI-causing bacteria are endogenous, whereas copper alloy surfaces are more likely to kill exogenous bacteria (Harbarth *et al*., 2013; Humphreys, 2014). In addition, validity of the authors claims have been questioned due to the fact that only 10 % of surfaces in the ICUs were copper alloys and that 13 % of patients in control rooms had some exposure to copper alloy surfaces (Harbarth *et al*., 2013). It is certainly clear that more trials are

necessary to fully understand whether or not antimicrobial surfaces can contribute to reducing HCAI rates.

A major advantage of antimicrobial surfaces is that they provide continuous disinfection and since they are self-disinfecting their activity is not reliant upon effective surface cleaning (Weber and Rutala, 2013). To date the majority trials in clinical areas have been conducted with concurrent, non-copper-containing control surfaces or as a crossover study (Weber and Rutala, 2013). Examples are mentioned in Chapter 1, section 1.8.1.3. In all the cases described copper alloys presented significantly reduced microbial counts compared to control surfaces. In addition, trials in clinical settings have shown copper to be effective against a wide range of microorganisms including MRSA, MSSA, VRE, *E. coli* and coliforms (Casey *et al*., 2010; Karpanen *et al*., 2012; Schmidt *et al*., 2012). Finally, the use of antimicrobial surfaces as part of infection control does not require staff training that is necessary for other technologies (e.g. HPV) (Humphreys, 2014).

Although trials have shown promising results regarding the continuous disinfection by copper alloy surfaces, it is important that HCWs are aware of the limitations of antimicrobial surfaces. Firstly, they must not replace, but rather work in conjunction, with current cleaning methods (Page *et al*., 2009). Indeed, all trials conducted to date have tested the efficacy of copper alloy surfaces alongside normal cleaning regimes. Good hand hygiene must be maintained too at all times as antimicrobial surfaces do not reduce the direct transmission of microorganisms between individuals (Page *et al*., 2009).

An important factor that has not been reported in any trials is the cost of purchasing and installing antimicrobial surfaces (Weber and Rutala, 2013). It would not be feasible to replace all surfaces in a hospital ward with an antimicrobial coating or surface (Weber and Rutala, 2013), therefore, more studies are required to determine which wards or areas within wards would benefit most from their introduction. In light of the current reduction in bacteraemia rates of HCAIs such as MRSA and *A. baumannii*, the cost of installing copper fittings needs to be justified. For example, if copper alloys can reduce surface contamination and subsequently further decrease infection rates, the question of

what additional decrease in infection rates by copper would make their installation a viable option needs to be addressed. In addition, based on findings in this study, copper alloys are not likely to be antimicrobial against *C. difficile* spores, whereas others have demonstrated slow (24 – 48 h) activity (Weaver *et al*., 2008). Thus, the introduction of copper alloy surfaces may not influence *C. difficile* infection rates. Also, whilst activity against a wide range of microorganisms has been reported in clinical settings, it could be argued that the reductions in microbial bioburden (1 - 2 $\log_{10}$) produced by copper alloys compared to standard fittings are small (Weber and Rutala, 2013).

The York Health Economics Consortium has developed a cost-benefit model to assess the cost of installing copper surfaces against the benefits from reduced HCAI rates. It is based on the installation of copper surfaces including over-bed table, bed rail, chair, call button, data device and IV stand in a 20-bed ICU. The findings of a 5 year model are summarised in Table 6.4. The cost of fittings, number of infections, cost of infections and total cost were taken into account and showed a saving of £1,926,600 if copper surfaces were to be introduced. The model also claims 390 bed days would be saved a year (cost of one bed day £78.41). Overall, it is estimated that the cost of copper fittings would be recovered within two months (Website 37, 2013).

Table 6.4 - Cost-benefit model analysis of copper *vs*. standard fittings in a 20-bed ICU over a 5 year period (Website 37, 2013)

	Copper	**Standard**
Cost of fittings	£105,000	£74,400
Estimated number of infections	1301	1626
Cost of infections	£7,804,800	£9,756,000
Total cost of intervention	£7,905,000	£9,830,400
Saving	£1,926,600	

It is assumed that the cleaning costs (including time, staff numbers and chemicals) of copper surfaces are the same as non-antimicrobial surfaces; however, this is something that should be determined in cost-benefit analyses.

It is important too that any cleaning products used for routine disinfection do not contain chemicals that may interfere with or reduce the antimicrobial efficacy of surfaces (Warnes and Keevil, 2011). The properties of the antimicrobial surface should be taken into account; copper is corrosive and oxidises. Studies have shown that lowered corrosion and an oxide layer resulted in decreased antimicrobial activity against *E. coli* and *E. faecium* (Elguindi *et al*., 2011). In addition, the long-term efficacy and durability of copper alloy surfaces has not been fully explored, although surface conditioning from repeated soiling/cleaning has been observed (see Chapter 1, 1.11.5) (Airey and Verran, 2007; Weber and Rutala, 2013). It is vital that antimicrobial surfaces are durable and are not affected by repeated soiling and cleaning (Humphreys, 2014).

The emergence of bacterial resistance to antimicrobials is a challenge to infection control (Touati *et al*., 2010). The exact mechanisms of action of copper are not fully understood, however, it is thought to act at several different sites within the bacterial cell. Having multiple target sites reduces the likelihood of resistance. Nevertheless, prolonged survival of copper-ion resistant *E. faecium* (2 - 3 $\log_{10}$) has been observed after repeated inoculations every 3 h over a 24 h period with recurrent culture analysis. In contrast, copper-ion resistant *E. coli* was not detectable over the 24 h period (Elguindi *et al*., 2011). In a different study in a clinical setting, 62 isolates of Enterobacteriaceae were recovered from 428 surfaces from three hospitals over a four month period. From these isolates 16 were found to be extended spectrum β-lactamase-producing and all were copper-resistant too with MICs of >1600 µg/mL (Touati *et al*., 2010). The authors state this resistance may limit the antimicrobial efficacy of copper-containing surfaces against such strains (Touati *et al*., 2010).

Ultimately the decision regarding the widespread use of antimicrobial surfaces in clinical areas will be down to a range of factors. Worthington *et al*. (2012) recommend a pragmatic approach in the selection of areas where antimicrobial surfaces would be useful alongside routine infection control procedures. Within the NHS the cost of antimicrobial surfaces is likely to be a major limiting factor. Cost-benefit analyses should be conducted and more studies carried out to assess the clinical efficacy of antimicrobial surfaces. If the overall outcome is positive, there may be a future role for

antimicrobial surfaces to control surface contamination and work alongside other infection control measures to reduce HCAI rates.

6.6 Application of antimicrobial surfaces in food factories and other settings

This project was carried out in collaboration with Campden BRI. Campden BRI's focus in this collaboration was geared towards antimicrobial surfaces in food settings. Phase 1 – 3 tests were carried out to assess the potential use of antimicrobial surfaces in food factories. Environmental sampling of surfaces in food factories similar to the hospital sampling in this study was carried to set parameters for phase 2 testing, which also indicated the temperature and relative humidity conditions of the JIS Z 2801 test are not appropriate.

As in healthcare settings, the majority of hard, metal surfaces in food settings are composed of stainless steel since they can be easily cleaned (Wilks *et al.*, 2005). The use of antimicrobial surfaces in food factory settings has been considered. In one study pure copper produced complete kill of *E. coli* O157:H7, a foodborne pathogen, after 90 min at 20 °C (to mimic room temperature) and after 270 min at 4 °C (to mimic refrigeration temperature) (Wilks *et al.*, 2005). The authors of this study suggest pure copper should not be used in food settings as it corrodes and tarnishes easily. They recommend that copper alloys are utilised, which are less corrosive and more durable (Wilks *et al.*, 2005). In an environment such as a food factory, food safety is a key issue during preparation. It is important that if antimicrobial surfaces were to be introduced in food settings that they do not have an effect on food safety. One study found copper ions were released into meat after 50 min contact (Faúndez *et al.*, 2004). Food contact with copper surfaces should be controlled to ensure minimal copper release. Examples of antimicrobial surfaces found in the food industry are given in Table 6.5.

Campden BRI tested a range of surfaces under JIS Z 2801 conditions and four (copper nickel, grapefruit extract, silver and photo-oxidative), that presented a >4 $\log_{10}$ reduction in *E. coli* and/or *S. aureus*, proceeded to phase 2 testing against *S. aureus*. Phase 2 conditions were reflective of food factory conditions (4, 10 and 25 °C at 33 and 75 % relative humidity). Overall, only copper nickel presented a >4 $\log_{10}$ reduction after

24 h under all conditions except at 4 and 10 °C at 33 % relative humidity. During phase 3 testing the same four surfaces were introduced into food factories. They were tested over 83 days in a dairy factory to test the long-term efficacy of surfaces, and over 24 h in a potato processing environment to mimic every day cleaning in factories. Overall, all surfaces were not significantly different to standard fittings in terms of total aerobic count. See Appendix 3 for complete results.

Camdpen BRI stated their study highlighted the importance of not only testing surfaces under in-use conditions in the laboratory but also *in situ* under the real conditions of use. Their results suggested the use of antimicrobial surfaces in the specific food factory environment conditions tested is limited (personal communication, Colette Jermann, Campden BRI).

The potential use of antimicrobial surfaces is not just limited to healthcare and food settings. Examples of areas that may benefit from their introduction include public transport, public buildings and sports facilities amongst others. Table 6.5 highlights the large range of existing applications for antimicrobial surfaces.

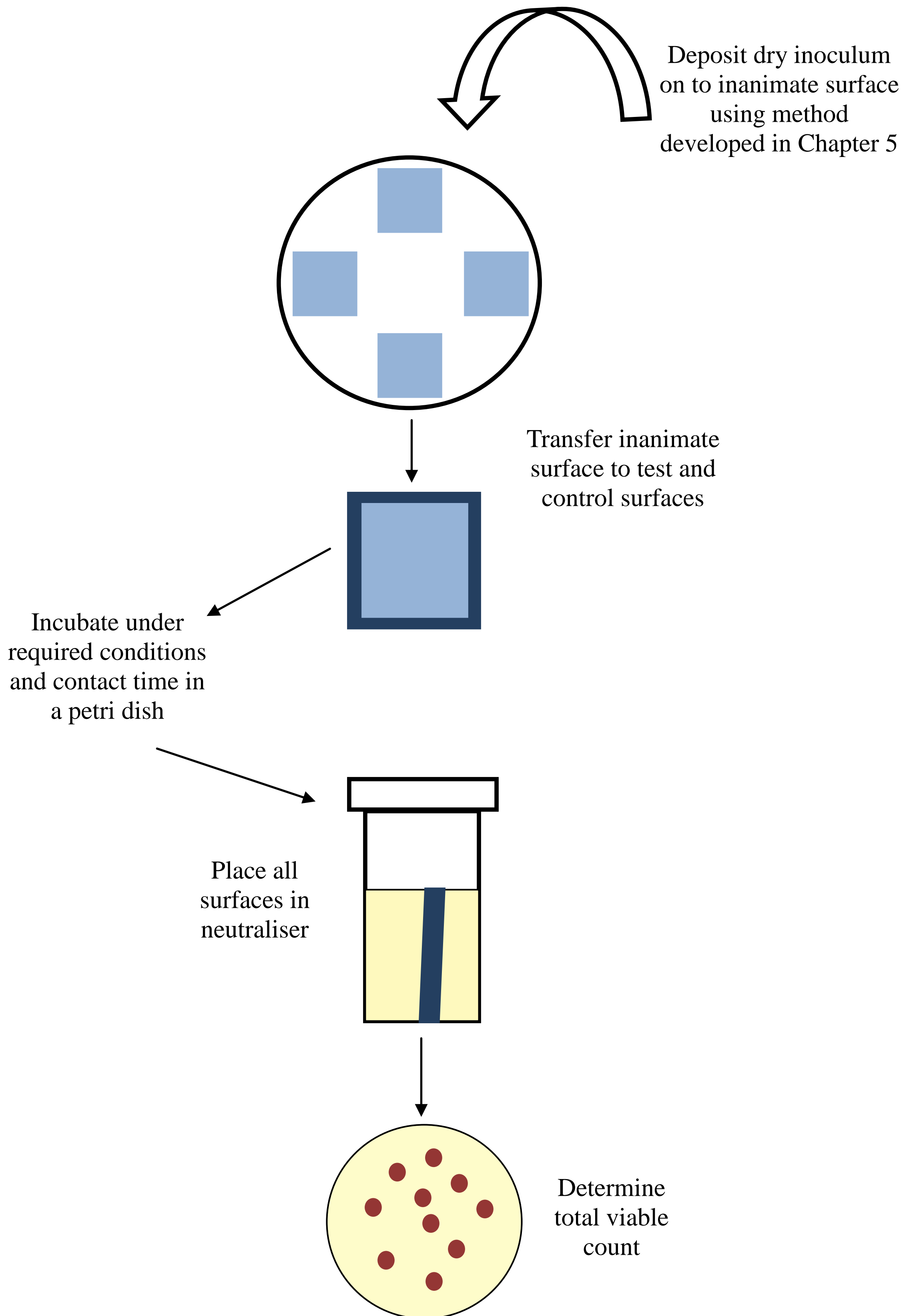
Table 6.5 – Examples of various antimicrobial surface applications

Application	Example surfaces	Reference
Food industry and hospitality	Copper kitchen drainer trough	Website 38, 2014
	Copper table tops and air conditioning units in a South Korean restaurant	Website 39, 2013
Public transport	Copper handrails and poles on trains in Chile	Website 40, 2013
	Copper handrails in subways in Chile	Website 41, 2011
	Copper handrails, counter tops and elevator guards at an airport in Brazil	Website 42, 2012
Public buildings	Copper door handles, rails and banisters at a school in Greece	Website 43, 2012
	Copper taps, handrails, door handles and push plates at a kindergarten in Japan	Website 44, 2012
	Copper desks at a school in Chile	Website 45, 2012
Veterinary	Copper tables, light switches, vaccine fridge door, operation light handles and table handles at a practice in South Africa	Website 46, 2012
	Copper horseshoes	Website 47, 2012
Personal items	Copper mobile phone cover	Website 48, 2013
	Protective screen glass for mobile phones, laptops etc. (silver ions)	Website 32, 2014
Sports facilities	EPA-approved antimicrobial coating for gym equipment	Website 49, 2014
	Laminate for use in gyms (nano silver ions)	Website 36, publication date unavailable
Miscellaneous	Copper door furniture in a laboratory that assists companies that develop disinfectants	Website 50, 2013

6.7 Limitations of this study and future work

Both antimicrobial surface efficacy tests developed in this study required a long nebulisation time of 30 min to recover a sufficient amount of bacteria from surfaces. During this 30 min differences were observed between the amount recovered from stainless steel and the amount from copper alloys, for both wet and dry inocula. The most likely explanation for this observation against the wet inoculum is that the copper alloy surfaces were antimicrobial during the nebulisation process due to continuous wet contact. For the dry inoculum the reasons are uncertain. It could be that the drying process combined with the high temperature and relative humidity conditions within the set-up rendered some cells more sensitive to copper, in addition to the existing antimicrobial activity of copper. To overcome this issue for the dry inoculum set-up, the inoculum could be deposited on to an inanimate surface (e.g. latex) then transferred to test and control surfaces. The test and control surfaces could then be incubated at the required conditions and viability determined after necessary contact times (see Figure 6.2). This transfer from a latex surface to a copper alloy surface would mimic, for example, hand touch contamination by a HCW. The key points would be to ensure the reproducibility of the inoculum deposited and transfer to the antimicrobial surface.

Figure 6.2 – Schematic diagram showing possible modifications to the dry inoculum test method developed in Chapter 5



More work should be carried out to further investigate the mechanisms of action of copper against both wet and dry inocula. In this study the mechanisms of action of copper were only assessed after exposure to dry inocula and only two repeats were carried. Studies utilising FACS to determine the mechanism of action of copper are limited in number. Similar to this study, Quaranta *et al*. (2011) utilised the fluorescent dye BOX to assess changes in membrane potential following copper exposure and observed rapid membrane depolarisation in yeast. FACS protocols should be optimised, particularly the positive control to confidently enable the separation of FACS plots into quadrants. Other techniques could be utilised too, scanning electron microscopy could be used to visualise the bacterial inocula deposited on to surfaces and the effect caused to individual cells after incubation under parameters representing conditions found *in situ*. In addition, antimicrobial release from copper alloy surfaces should be assessed to compare activity against wet and dry inocula using methods from Santo *et al*. (2011). They utilised Coppersensor11, a membrane-permeable fluorescent dye that increases in fluorescence when bound to Cu(I) to determine copper ion uptake in cells. Further studies should attempt to measure the available concentration of a biocide at the surface

As mentioned in section 6.5 the durability of copper alloy surfaces has not been fully assessed; existing techniques should be used to accelerate wear of surfaces. Aged surfaces should then be used re-tested to observe, if any, loss of antimicrobial efficacy.

More work should be carried out to determine the role of water in the antimicrobial efficacy of copper. Warnes and Keevil (2011) demonstrated quick kill when a low volume, 1 µL bacterial inoculum was presented on to surfaces. Since relative humidity appears to be an important factor affecting antimicrobial surface and copper alloys have shown to elicit an antimicrobial effect against a low volume inoculum, further studies should attempt to look at the role of water levels at a micro scale. Measuring water levels on surfaces should also confirm that the dry aerosol test developed in Chapter 5 is dryer on the surface than the dry inoculum method by Warnes and Keevil (2011).

Finally, this study has been limited to assessing the antimicrobial efficacy of copper alloys. The new antimicrobial surface efficacy tests could be tested against different

antimicrobial surfaces; many commercial products exist containing a variety of antimicrobial coatings.

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APPENDICES

Appendix 1 – Hospital sampling data

NB. NR = no reading

MR = missing reading

Table 1 – April 2011, Gastroenterology

	MOISTURE MODE		HYGROMETER MODE			
Surface	**Relative reading**	**Dry, at risk or wet?**	**% RH**	**T_{AIR}**	**T_{DEW}**	**AbS**
Door handle	166	Dry	30.5	23	4.7	5.31
Door handle	173	At risk	33.4	23.3	5.5	5.58
Door push plate	216	Wet	38.1	21	4.4	5.34
Door push plate	238	Wet	33.8	21.8	4.5	5.2
Computer keyboard	75	Dry	38.2	22.8	7.3	6.25
Computer mouse	75	Dry	31.4	24.4	6.2	5.97
Bed rail	64	Dry	40.6	26.1	11	8.61
Bed rail	63	Dry	38.2	26.9	10.7	8.11
Bed rail	63	Dry	38.8	25.7	10.4	7.87
Chair	112	Dry	32.9	24.3	6.2	5.91
Chair	65	Dry	31.4	24.8	6.4	5.96
Chair	65	Dry	39.4	24.4	9.1	7.19
Trolley	74	Dry	32.6	24.1	6.1	5.85
Trolley	217	Wet	38.1	24.2	7.9	6.66
Table	73	Dry	31.2	24.6	6.1	5.81
Table	111	Dry	41.2	24.2	10.1	7.72
Light switch	134	Dry	31.1	25.1	7	6.24
Tap	179	At risk	31.4	28.5	7.2	6.42
Wall panel	146	Dry	30.4	24.4	5.9	5.78
Wall panel	176	At risk	31.9	26.4	8.3	7.07
Waste bin	236	Wet	31.5	26.2	7.4	6.4

Table 1 continued – April 2011, Gastroenterology

Surface	CONDENSATOR MODE					BIOBURDEN	
	% RH	T_{AIR}	T_{DEW}	T_S	T_{DIFF}	RLU	Pass, caution or fail?
Door handle	29.9	23.8	5.1	22.4	17.3	17	Caution
Door handle	31.2	23.2	5.1	22.2	17.3	17	Caution
Door push plate	32.3	21.8	4.4	22.2	17.7	156	Fail
Door push plate	31.6	21.7	4.3	22	17.3	86	Fail
Computer keyboard	32.3	23.8	6.3	24.2	18	75	Fail
Computer mouse	32.2	24.2	6.3	24.1	18	86	Fail
Bed rail	41.3	25.5	11.7	25.1	13.3	150	Fail
Bed rail	37.4	26.6	10.5	26.8	16.4	2	Pass
Bed rail	37.5	26	10.4	26.1	15.6	9	Pass
Chair	30.3	24.9	6.2	24.1	18	367	Fail
Chair	30.4	24.5	6	24.1	18.2	12	Caution
Chair	39.4	24.1	9	23.9	15	252	Fail
Trolley	32.2	24	6.1	24.1	18.2	95	Fail
Trolley	35.9	24.4	8	24.4	17.2	173	Fail
Table	29.9	24.4	5.5	24.8	19.4	601	Fail
Table	38.3	24.1	9.6	24.6	14.5	55	Fail
Light switch	30.6	25.4	6.8	24.8	18.2	66	Fail
Tap	31.4	25.7	7.6	26.5	18.8	72	Fail
Wall panel	30.1	24.8	6.1	NR	NR	2	Pass
Wall panel	33.2	26.1	8	24.6	18.6	10	Pass
Waste bin	30	25.5	6.7	NR	NR	0	Pass

Table 2 – April 2011, ACC

	MOISTURE MODE		HYGROMETER MODE			
Surface	**Relative reading**	**Dry, at risk or wet?**	**% RH**	**T_{AIR}**	**T_{DEW}**	**AbS**
Door handle	155	Dry	31.1	23.6	5.6	5.64
Door handle	166	Dry	31.9	23.7	5.9	5.82
Door push plate	206	Wet	30.5	25.5	6.6	6
Door push plate	206	Wet	31.4	24.1	6.1	5.86
Computer keyboard	70	Dry	31	24.3	5.9	5.77
Computer mouse	84	Dry	31.4	23.6	5.6	5.66
Bed rail	105	Dry	31.3	24	5.9	5.82
Bed rail	106	Dry	32.6	24.5	6.4	6
Chair	68	Dry	31.4	24.4	6.2	5.92
Chair	70	Dry	31.5	24.8	6.6	6.08
Chair	195	At risk	31.3	24.1	5.8	5.79
Trolley	73	Dry	30.9	24.1	5.8	5.8
Trolley	90	Dry	31.3	24.2	6	5.9
Table	63	Dry	32.4	24.1	6.1	5.84
Table	78	Dry	31	24.1	5.8	5.8
Light switch	108	Dry	31.4	24.4	6.2	5.92
Wall panel	171	At risk	30.9	24.3	6	5.84
Wall panel	143	Dry	31.7	24.9	6.7	6.13
Waste bin	186	At risk	31.5	25.1	6.5	6.05

Table 2 continued – April 2011, ACC

	CONDENSATOR MODE					BIOBURDEN	
Surface	**% RH**	**T_{AIR}**	**T_{DEW}**	**T_{S}**	**T_{DIFF}**	**RLU**	**Pass, caution or fail?**
Door handle	30.7	23.9	5.5	23.5	18.1	13	Caution
Door handle	31.9	23.8	6	24.3	18.3	11	Caution
Door push plate	30.4	24.4	5.7	23.3	17.7	20	Caution
Door push plate	31.9	23.8	6.3	24.1	17.9	46	Fail
Computer keyboard	30.4	24	5.6	23.9	18.1	388	Fail
Computer mouse	30.9	23.8	5.6	23.7	18.1	25	Caution
Bed rail	30.9	24.2	5.9	23.9	18	27	Caution
Bed rail	31.8	24.5	6.7	23.7	17.7	4	Pass
Chair	31.1	24.5	6.3	NR	NR	393	Fail
Chair	30.9	24.8	6.4	NR	NR	32	Fail
Chair	MR	MR	MR	MR	MR	MR	MR
Trolley	30.9	24.4	6	NR	NR	21	Caution
Trolley	30.9	24.2	6.1	23.9	17.7	247	Fail
Table	31.2	24.3	6.1	23.9	17.9	78	Fail
Table	31.4	24.2	6	23.9	18.2	890	Fail
Light switch	30.6	24.5	6.3	23.9	17.7	11	Caution
Wall panel	30.8	24.5	6.1	NR	NR	7	Pass
Wall panel	30.9	24.8	6.3	NR	NR	4	Pass
Waste bin	31.3	24.8	6.4	NR	NR	464	Fail

Table 3 – April 2011, Theatre

	MOISTURE MODE		HYGROMETER MODE			
Surface	**Relative reading**	**Dry, at risk or wet?**	**% RH**	**T_{AIR}**	**T_{DEW}**	**AbS**
Door handle	153	Dry	38.1	25.1	9	7.06
Door handle	176	At risk	37.2	24.1	8.6	7.08
Door push plate	209	Wet	41.5	22.1	8.3	6.9
Door push plate	405	Wet	43.1	21.7	8.6	7.03
Computer keyboard	155	Dry	41.3	21.8	8.2	6.84
Trolley	1000	Wet	MR	MR	MR	MR
Trolley	219	At risk	41.3	22.4	8.5	6.96
Trolley	186	At risk	38.2	24.4	8.4	6.77
Anaesthetics stand	65	Dry	44.1	20.7	8	6.7
Light switch	1000	Wet	41.6	21.8	8.1	6.79
Tap	179	At risk	42	21.7	8.2	6.88
Wall panel	178	At risk	42.1	22.4	8.3	6.81
Wall panel	146	Dry	43.1	20.6	7.7	6.65
Waste bin	NR	NR	44.7	20.6	8	6.7

Table 3 continued – April 2011, Theatre

	CONDENSATOR MODE					BIOBURDEN	
Surface	**% RH**	**T_{AIR}**	**T_{DEW}**	**T_S**	**T_{DIFF}**	**RLU**	**Pass, caution or fail?**
Door handle	37.4	24.2	8.2	24.1	15.9	128	Fail
Door handle	38.4	23.7	8.7	24.3	15.5	191	Fail
Door push plate	42.3	21.7	8.4	21.3	13.1	199	Fail
Door push plate	44.1	21.3	8.6	22.2	13.4	181	Fail
Computer keyboard	38.1	23.1	8.1	23.2	14.7	107	Fail
Trolley	38.8	23	8.6	23.3	13.8	35	Fail
Trolley	41.6	22.3	8.6	23.3	14.9	12	Caution
Trolley	39.6	22.5	8.2	NR	NR	4	Pass
Anaesthetics stand	44.1	20.6	7.9	21.2	13	127	Fail
Light switch	40.7	22.3	8.6	NR	NR	12	Caution
Tap	42	22.3	8.6	NR	NR	1	Pass
Wall panel	40.7	21.9	7.9	22.2	14.2	7	Pass
Wall panel	41.6	21.3	7.7	20.9	13.2	33	Fail
Waste bin	44.4	20.5	7.8	21.4	13.5	127	Fail

Table 4 – June 2011, Gastroenterology

	MOISTURE MODE		HYGROMETER MODE			
Surface	**Relative reading**	**Dry, at risk or wet?**	**% RH**	**T_{AIR}**	**T_{DEW}**	**AbS**
Door handle	173	At risk	58.2	19.9	11.5	8.48
Door handle	166	At risk	56.4	20.4	11.6	8.44
Door push plate	220	At risk	53.6	20.9	11.1	8.25
Door push plate	211	At risk	52.3	21.1	10.9	8.32
Computer keyboard	78	Dry	51.8	22.1	11.5	8.49
Computer mouse	65	Dry	48.8	22.7	11.3	8.39
Bed rail	65	Dry	48.9	23.8	12.2	9.01
Bed rail	NR	NR	47.7	23.8	12.1	8.68
Bed rail	65	Dry	48	22.9	11.2	8.4
Chair	63	Dry	48.7	23	11.5	8.5
Chair	65	Dry	49.6	23.5	12.5	9.07
Chair	187	At risk	50.2	22.5	11.8	8.68
Trolley	241	Wet	46.7	23.5	11.2	8.44
Trolley	99	Dry	48.2	24.5	12.6	9.15
Table	112	Dry	48.7	23.5	11.8	8.76
Table	499	Wet	51.5	24.1	12.9	9.3
Light switch	152	Dry	50.2	23.5	12.5	9.06
Tap	174	At risk	51.5	23.7	12.7	9.13
Wall panel	156	Dry	48.6	23.1	11.8	8.65
Wall panel	181	At risk	49.2	22.7	11.4	8.4
Waste bin	173	At risk	58.2	19.9	11.5	8.48

Table 4 continued – June 2011, Gastroenterology

	CONDENSATOR MODE					**BIOBURDEN**	
Surface	**% RH**	**T_{AIR}**	**T_{DEW}**	**T_S**	**T_{DIFF}**	**RLU**	**Pass, caution or fail?**
Door handle	48.3	22.9	11.3	22	48.3	31	Fail
Door handle	48.1	22.8	11.1	22.3	48.1	5	Pass
Door push plate	47.9	22.7	11.2	22.3	47.9	41	Fail
Door push plate	48.4	22.6	11	22.3	48.4	29	Caution
Computer keyboard	50.8	22.8	12.1	24.1	50.8	36	Fail
Computer mouse	49.3	22.6	11.7	23.6	49.3	106	Fail
Bed rail	45.4	24.9	12.1	23.9	45.4	7	Pass
Bed rail	45.6	24.8	11.9	23.9	45.6	25	Caution
Bed rail	48.8	22.5	11.3	22.9	48.8	33	Fail
Chair	48.8	23	11.7	23.6	48.8	0	Pass
Chair	46.4	23.3	11.1	23.2	46.4	8	Pass
Chair	50.7	22.4	11.8	22.6	50.7	365	Fail
Trolley	46.2	23.1	11.2	NR	46.2	11	Caution
Trolley	48.8	23.7	12.2	23.4	48.8	67	Fail
Table	47.9	23.6	12	23.2	47.9	43	Fail
Table	47.9	24.2	12.2	23.3	47.9	17	Caution
Light switch	50.7	24	12.6	24.2	50.7	41	Fail
Tap	50.6	23.5	12.5	23.9	50.6	13	Caution
Wall panel	48.7	23.3	11.8	23.2	48.7	13	Caution
Wall panel	49.9	22.4	11.5	21.9	49.9	21	Caution
Waste bin	48.3	22.9	11.3	22	48.3	31	Fail

Table 5 – June 2011, ACC

	MOISTURE MODE		HYGROMETER MODE			
Surface	**Relative reading**	**Dry, at risk or wet?**	**% RH**	T_{AIR}	T_{DEW}	**AbS**
Door handle	158	Dry	46.6	21.8	9.9	7.64
Door handle	158	Dry	47.9	21.6	9.9	7.61
Door push plate	211	Wet	44.1	23.2	9.7	7.48
Door push plate	209	Wet	47.3	21.6	9.9	7.62
Computer keyboard	76	Dry	44.8	22.7	10	7.65
Computer mouse	78	Dry	48.2	22.4	10.6	7.84
Bed rail	68	Dry	47.2	21.3	9.3	7.5
Bed rail	215	Wet	44.1	22.5	9.6	7.48
Chair	176	At risk	46.5	21.4	9.3	7.34
Chair	125	Dry	44.1	22.8	9.7	7.52
Chair	96	Dry	43.5	22.5	9.4	7.4
Trolley	99	Dry	44.4	22.3	9.4	7.38
Trolley	84	Dry	45.8	22	9.8	7.53
Table	148	Dry	46.4	21.2	9.2	7.32
Table	73	Dry	44.1	22.4	9.6	7.49
Light switch	925	Wet	44.9	23	10.2	7.7
Wall panel	1000	Wet	46.7	22	9.8	7.57
Wall panel	186	At risk	44.8	22.6	9.9	7.61
Waste bin	65	Dry	42	23.7	9.6	7.44

Table 5 continued – June 2011, ACC

	CONDENSATOR MODE					BIOBURDEN	
Surface	**% RH**	**T_{AIR}**	**T_{DEW}**	**T_S**	**T_{DIFF}**	**RLU**	**Pass, caution or fail?**
Door handle	46.1	22	10	21.3	11.6	7	Pass
Door handle	47.6	22.1	9.9	21.3	11.4	6	Pass
Door push plate	43.7	22.5	9.4	21.1	11.8	8	Pass
Door push plate	45.3	21.8	9.3	20.9	11.6	3	Pass
Computer keyboard	46.5	21.8	9.7	22.9	13.1	123	Fail
Computer mouse	46.4	21.8	9.8	22.9	13.2	177	Fail
Bed rail	46.4	21.3	9.4	20.9	11.5	34	Fail
Bed rail	43.6	22.7	9.8	23.2	13.6	140	Fail
Chair	46.2	21.3	9.3	21.3	12.1	129	Fail
Chair	43.3	22.8	9.7	22.5	12.8	27	Caution
Chair	43.4	22.5	9.6	22.9	13.4	82	Fail
Trolley	44.3	21.6	9.2	21.3	12	187	Fail
Trolley	45.6	22.1	9.9	NR	NR	38	Fail
Table	45.5	21.6	9.2	21.3	12	18	Caution
Table	44.8	22.5	9.7	23.2	13.6	32	Fail
Light switch	43.8	22.6	9.7	NR	NR	3	Pass
Wall panel	45.8	21.8	9.6	22.3	12.5	26	Caution
Wall panel	45.1	22.3	9.8	NR	NR	2	Pass
Waste bin	42.2	23.1	9.4	21.9	12.6	249	Fail

Table 6 – June 2011, Theatre

	MOISTURE MODE		HYGROMETER MODE			
Surface	**Relative reading**	**Dry, at risk or wet?**	**% RH**	**T_{AIR}**	**T_{DEW}**	**AbS**
Door handle	173	At risk	44.8	23.1	10	7.63
Door handle	155	Dry	46.5	21.1	9.1	7.24
Door push plate	191	At risk	47.4	21.4	9.7	7.46
Door push plate	181	At risk	47.1	21.3	9.6	7.44
Computer keyboard	89	Dry	44.8	22.1	9.5	7.43
Trolley	1000	Wet	46.2	21.4	9.4	7.43
Trolley	1000	Wet	44.3	22.5	9.6	7.5
Trolley	1000	Wet	45.7	21.7	9.4	7.53
Anaesthetics stand	1000	Wet	47.1	21.4	9.6	7.52
Light switch	225	At risk	44.6	22.3	9.6	7.51
Tap	214	At risk	43.8	22.7	9.8	7.55
Wall panel	178	At risk	45.3	21.8	9.5	7.43
Wall panel	153	Dry	56.9	21.1	9.3	7.33
Waste bin	NR	NR	46.3	21.1	9.5	7.42

Table 6 continued – June 2011, Theatre

	CONDENSATOR MODE					BIOBURDEN	
Surface	**% RH**	**T_{AIR}**	**T_{DEW}**	**T_{S}**	**T_{DIFF}**	**RLU**	**Pass, caution or fail?**
Door handle	48.4	21	9.6	21.1	11.3	4	Pass
Door handle	46.7	21	9.1	20.7	11.5	8	Pass
Door push plate	46.1	21.1	9	21.3	12.2	2	Pass
Door push plate	46.7	21	9.1	20.9	11.7	16	Caution
Computer keyboard	47.5	21.1	9.4	20.2	10.2	18	Caution
Trolley	46.5	21.2	9.2	21.2	12	24	Caution
Trolley	47.2	21	9.2	NR	NR	96	Fail
Trolley	48.3	20.6	9.2	NR	NR	15	Caution
Anaesthetics stand	46.9	20.8	9.1	NR	NR	24	Caution
Light switch	47.1	21.1	9.6	21.2	11.4	8	Pass
Tap	48.1	21.1	9.6	NR	NR	1	Pass
Wall panel	47.3	21.1	9.3	NR	NR	12	Caution
Wall panel	46.8	21.1	9.3	NR	NR	41	Fail
Waste bin	47.6	20.7	9	NR	NR	3	Pass

Table 7 – August 2011, Gastroenterology

	MOISTURE MODE		HYGROMETER MODE			
Surface	**Relative reading**	**Dry, at risk or wet?**	**% RH**	**T_{AIR}**	**T_{DEW}**	**AbS**
Door handle	178	At risk	63.7	20.4	15	10.92
Door handle	173	At risk	63.6	21.3	13.5	11.04
Door push plate	266	Wet	63.4	21.8	15.4	10.98
Door push plate	402	Wet	63.3	21.7	15.4	11.02
Computer keyboard	87	Dry	63.2	22.3	15.9	11.42
Computer mouse	117	Dry	63	22.3	15.7	11.21
Bed rail	112	Dry	62.8	23	15.7	11.23
Bed rail	70	Dry	64	22.5	15.5	11.18
Bed rail	74	Dry	64.2	22.5	15.4	11.01
Chair	202	Wet	63.4	23	15.9	11.27
Chair	199	Wet	63.8	22.8	15.5	10.9
Chair	186	At risk	63.5	22.5	15.2	10.93
Trolley	1000	Wet	63.5	23	15.3	10.89
Trolley	249	Wet	63.6	22.5	15.4	10.98
Table	119	Dry	63.5	22.6	15.2	10.9
Table	118	Dry	62.6	23.1	15.7	11.21
Light switch	768	Wet	63.5	22.8	15.2	10.88
Tap	190	At risk	63.9	22.5	15.5	11.11
Wall panel	156	Dry	64	22.5	15.2	10.89
Wall panel	151	Dry	64.1	22.5	15.4	10.98
Waste bin	NR	NR	63.9	22.4	15.3	10.9

Table 7 continued – August 2011, Gastroenterology

	CONDENSATOR MODE					BIOBURDEN	
Surface	**% RH**	T_{AIR}	T_{DEW}	T_S	T_{DIFF}	**RLU**	**Pass, caution or fail?**
Door handle	64.1	22.4	15.3	22.1	6.9	4	Pass
Door handle	64	22.5	15.3	22.2	6.9	4	Pass
Door push plate	63.5	22.5	15.3	22.2	6.9	104	Fail
Door push plate	63.4	22.5	15.2	22.3	7.2	67	Fail
Computer keyboard	59.6	23.5	15.5	NR	NR	0	Pass
Computer mouse	59.7	23.5	15.4	NR	NR	15	Caution
Bed rail	62.6	23.4	15.8	24	8.2	862	Fail
Bed rail	66.1	22.8	15.9	22.6	6.7	87	Fail
Bed rail	63.1	22.7	15.3	22.4	7.1	129	Fail
Chair	62.5	22.8	15.3	22.5	7.2	25	Caution
Chair	62.8	22.7	15.2	22.5	7.1	8	Pass
Chair	63.3	22.4	15.1	22.3	7	0	Pass
Trolley	63.5	22.4	15.1	22.2	6.9	459	MR
Trolley	64.3	22.3	15.4	22.2	6.5	1456	MR
Table	60.6	23	15	22.5	7.3	16	Caution
Table	62.6	23.1	15.5	22.9	7.2	13	Caution
Light switch	61.6	22.9	15.1	22.6	7.4	11	Caution
Tap	64.6	22.7	15.6	22.6	7	14	Caution
Wall panel	63.4	22.6	15.3	21.6	6.3	6	Pass
Wall panel	63.6	22.5	15.2	21.9	6.7	0	Pass
Waste bin	63.8	22.4	15.3	21.9	6.6	182	Fail

Table 8 – August 2011, ACC

	MOISTURE MODE		HYGROMETER MODE			
Surface	**Relative reading**	**Dry, at risk or wet?**	**% RH**	**T_{AIR}**	**T_{DEW}**	**AbS**
Door handle	125	Dry	56.6	24.8	15.7	11.27
Door handle	214	At risk	54.4	24.7	14.9	10.65
Door push plate	193	At risk	54.2	25.3	15.5	11.19
Door push plate	240	Wet	58.2	24.6	15.8	11.39
Computer keyboard	93	Dry	59.3	23.8	15.3	10.98
Computer mouse	70	Dry	59.2	23.8	15.3	10.9
Bed rail	61	Dry	57.2	24.2	15.4	10.97
Bed rail	90	Dry	58.2	24.1	15.4	10.98
Chair	97	Dry	58.4	24.2	15.5	11.09
Chair	63	Dry	57.9	24.6	15.7	11.3
Chair	141	Dry	57.8	24.5	15.7	11.21
Trolley	1000	Wet	57.3	24.2	15.2	11.08
Trolley	75	Dry	58.4	24.2	15.6	11.14
Table	118	Dry	58.8	24	15.4	11.01
Table	90	Dry	60.5	23.5	15.3	10.95
Light switch	235	Wet	56.8	24.6	15.3	10.89
Wall panel	162	Dry	58.4	24.1	15.4	11.03
Wall panel	148	Dry	61.4	23.2	15.3	10.91
Waste bin	1000	Wet	59.2	24	15.8	11.34

Table 8 continued – August 2011, ACC

	CONDENSATOR MODE					BIOBURDEN	
Surface	**% RH**	**T_{AIR}**	**T_{DEW}**	**T_{S}**	**T_{DIFF}**	**RLU**	**Pass, caution or fail?**
Door handle	61.9	23.1	15.3	NR	NR	10	Pass
Door handle	57.8	23.9	15.2	NR	NR	6	Pass
Door push plate	57.2	24.2	15.2	NR	NR	2	Pass
Door push plate	61.9	23.1	15.3	NR	NR	9	Pass
Computer keyboard	60.8	23	15	23.6	8.3	45	Fail
Computer mouse	60.7	23.1	15.1	23.3	8.1	60	Fail
Bed rail	58.5	23.5	15.9	23.2	7.9	24	Caution
Bed rail	59.2	23.6	15.2	23.2	7.9	49	Fail
Chair	60.6	23.5	15.4	23.3	7	22	Caution
Chair	60.7	23.4	15.4	23.2	7.8	65	Fail
Chair	62.1	23.1	15.4	NR	NR	26	Caution
Trolley	60.4	23.2	15.2	22.8	7.1	12	Caution
Trolley	61	23.3	15.3	23.2	7.7	26	Caution
Table	62.5	23	15.4	NR	NR	31	Fail
Table	62.2	23	15.3	NR	NR	87	Fail
Light switch	59.2	23.5	15.4	22.9	7.7	14	Caution
Wall panel	61.4	23.4	15.4	NR	NR	11	Caution
Wall panel	60.4	23.1	15.1	23.2	7.7	1	Pass
Waste bin	61.6	23	15.2	23	7.9	70	Fail

Table 9 – August 2011, Theatre

	MOISTURE MODE		HYGROMETER MODE			
Surface	**Relative reading**	**Dry, at risk or wet?**	**% RH**	**T_{AIR}**	**T_{DEW}**	**AbS**
Door handle	166	Dry	47.7	23.3	11.8	8.74
Door handle	162	Dry	49.6	19.7	11.6	8.68
Door push plate	482	Wet	57.5	20.4	12.2	8.84
Door push plate	778	Wet	57.8	20.1	11.5	8.55
Computer keyboard	166	Dry	53.3	20.9	11.1	8.29
Trolley	1000	Wet	54.7	21.4	11.7	8.62
Trolley	209	Wet	56.9	20.2	11.5	8.54
Trolley	825	Wet	58.9	19.6	12.4	9.06
Anaesthetics stand	74	Dry	56	20	11.2	8.43
Light switch	657	Wet	49.4	22.9	11.9	8.93
Tap	276	Wet	50.4	22.5	11.8	8.79
Wall panel	182	At risk	50.9	22	11.5	8.51
Wall panel	161	Dry	61.8	19	11.5	8.56
Waste bin	NR	NR	56.4	20.4	11.4	8.52

Table 9 continued – August 2011, Theatre

	CONDENSATOR MODE					BIOBURDEN	
Surface	**% RH**	**T_{AIR}**	**T_{DEW}**	**T_S**	**T_{DIFF}**	**RLU**	**Pass, caution or fail?**
Door handle	58.7	20.6	12.7	20.9	7.8	17	Caution
Door handle	60.9	19.2	11.5	19.5	7.8	42	Fail
Door push plate	61.9	19.2	11.6	19.6	8.1	15	Caution
Door push plate	61	19.2	11.6	19.4	7.7	10	Pass
Computer keyboard	59.8	19.1	11.4	19.3	7.8	64	Fail
Trolley	56.1	20.3	11.2	19	7.2	22	Caution
Trolley	57.1	19.9	11.3	18.9	NR	13	Caution
Trolley	59.1	19.6	11.4	NR	NR	17	Caution
Anaesthetics stand	60.4	19.3	11.6	19.3	7.8	53	Fail
Light switch	52.1	21.7	11.5	21.3	9.7	23	Caution
Tap	53.5	21.7	11.9	21.2	9.6	4	Pass
Wall panel	52.3	21.3	11.1	20.7	9.6	28	Caution
Wall panel	61.5	18.9	11.3	19.3	7.9	37	Fail
Waste bin	53.8	20.7	11.2	18.2	7	23	Caution

Table 10 – October 2011, Gastroenterology

	MOISTURE MODE		HYGROMETER MODE			
Surface	**Relative reading**	**Dry, at risk or wet?**	**% RH**	**T_{AIR}**	**T_{DEW}**	**AbS**
Door handle	186	At risk	44.9	20.5	8.7	7.05
Door handle	181	At risk	43.7	20.7	7.9	6.67
Door push plate	207	At risk	41.4	21	7.3	6.45
Door push plate	240	At risk	40	21	7	6.13
Computer keyboard	105	Dry	44.9	22.3	10.2	7.73
Computer mouse	93	Dry	45.9	22.3	10.2	7.73
Bed rail	68	Dry	50	22	11.5	8.47
Bed rail	63	Dry	49.7	22.6	11.9	8.71
Bed rail	73	Dry	42.2	22.4	11.5	8.56
Chair	75	Dry	46.8	22	10.5	8.05
Chair	167	Dry	42.3	22.4	8.9	7.26
Chair	73	Dry	42.2	22.4	8.9	7.19
Trolley	355	Wet	44	21.3	8.1	6.71
Trolley	207	Wet	44.3	22.3	9.6	7.42
Table	141	Dry	46.5	21.6	9.7	7.66
Table	148	Dry	42.1	22.5	8.9	7.23
Light switch	592	Wet	39.2	21	7.2	6.3
Tap	158	Dry	52.1	22.3	11.8	8.82
Wall panel	161	Dry	47.2	21.8	9.9	7.61
Wall panel	179	At risk	45.7	21.8	11.2	6.33
Waste bin	208	Wet	46.3	21.5	9.9	7.67

Table 10 continued – October 2011, Gastroenterology

	CONDENSATOR MODE					BIOBURDEN	
Surface	**% RH**	T_{AIR}	T_{DEW}	T_S	T_{DIFF}	**RLU**	**Pass, caution or fail?**
Door handle	39.7	22.8	8.8	21.8	15.1	4	Pass
Door handle	38.8	22.8	7.8	22	15	6	Pass
Door push plate	37.2	22.7	7.4	22.3	14.6	75	Fail
Door push plate	35.7	22.5	6.2	22.2	14.6	29	Caution
Computer keyboard	42.3	22.4	8.8	22.9	14	42	Fail
Computer mouse	41.7	22.5	8.7	22.9	14.5	9	Pass
Bed rail	49.9	22.5	11.7	NR	NR	2	Pass
Bed rail	49.8	22.5	11.6	NR	NR	12	Caution
Bed rail	50.4	22.4	11.7	22	10.4	36	Fail
Chair	44.4	23	10.6	22.6	14.7	6	Pass
Chair	40.3	22.3	8	22.2	13.5	77	Fail
Chair	41.2	22.4	8.7	21.9	13.6	32	Fail
Trolley	46	22.8	11	21.7	12.7	35	Fail
Trolley	44.9	23	10.8	21.6	12.7	42	Fail
Table	44.1	22.5	9.6	22.3	13.7	52	Fail
Table	41.6	22.5	9	23.2	13.4	23	Caution
Light switch	38.2	22.3	7.2	NR	NR	4	Pass
Tap	48.7	23	11.7	22.2	12.3	14	Caution
Wall panel	45.1	22.5	10.2	22.2	13.2	15	Caution
Wall panel	47.4	22.6	11.2	22.6	12.2	13	Caution
Waste bin	43.4	22.3	9.3	NR	NR	17	Caution

Table 11 – October 2011, ACC

	MOISTURE MODE		HYGROMETER MODE			
Surface	**Relative reading**	**Dry, at risk or wet?**	**% RH**	T_{AIR}	T_{DEW}	**AbS**
Door handle	227	Wet	39.4	23.3	8.8	7.06
Door handle	148	Dry	42.6	22.8	9.3	7.17
Door push plate	209	Wet	40.8	22.4	8.8	7.28
Door push plate	139	Dry	43.3	22.5	9.7	7.34
Computer keyboard	68	Dry	33.8	22.1	5.3	5.57
Computer mouse	73	Dry	34.2	22.3	5.4	5.68
Bed rail	96	Dry	34.9	23.3	7.6	6.62
Bed rail	87	Dry	34.9	23.1	6.3	5.98
Chair	186	At risk	36.7	23.8	8.7	6.9
Chair	96	Dry	34.8	22.5	7.8	6.31
Chair	78	Dry	36.8	22.5	8.1	6.79
Trolley	150	Dry	40.2	23.4	9.4	7.31
Trolley	65	Dry	38.2	23.5	8.9	7.46
Table	161	Dry	44.2	23.5	10.4	7.86
Table	115	Dry	43	23.6	10.2	7.74
Light switch	397	Wet	39.3	22.6	9.5	7.2
Wall panel	143	Dry	34.5	22.5	5.8	5.73
Wall panel	90	Dry	39.5	22.5	8.1	6.74
Waste bin	NR	NR	40	23.5	11.5	7.71

Table 11 continued – October 2011, ACC

	CONDENSATOR MODE					BIOBURDEN	
Surface	**% RH**	**T_{AIR}**	**T_{DEW}**	**T_S**	**T_{DIFF}**	**RLU**	**Pass, caution or fail?**
Door handle	43.6	23	10.4	22.2	12.2	16	Caution
Door handle	46.1	22.5	10.6	22.2	11	8	Pass
Door push plate	39.9	22.5	9.2	21.9	12.4	5	Pass
Door push plate	43.6	22.6	9.7	22.2	11.9	7	Pass
Computer keyboard	35.8	22.5	6.2	22.9	16.6	24	Caution
Computer mouse	35.8	22.6	6.7	22.9	16.7	6	Pass
Bed rail	34.3	22.8	6.6	22.6	14	15	Caution
Bed rail	34.1	22.6	5.9	22.5	16.7	12	Caution
Chair	33.9	22.4	5.6	22.7	16.3	10	Pass
Chair	33.1	22.3	7.9	22.2	13.9	23	Caution
Chair	37.5	22.8	7.2	21.9	14.8	51	Fail
Trolley	37.4	22.3	6.7	22.2	15.1	30	Caution
Trolley	38.1	22.4	7.5	22.2	14.2	96	Fail
Table	43.6	22.5	9.4	22.6	12.9	140	Fail
Table	37.5	22.5	7.2	21.9	14.4	87	Fail
Light switch	40.3	22.2	8.8	21.9	12.8	15	Caution
Wall panel	43.3	22.5	8.2	22.2	12.6	0	Pass
Wall panel	38.2	22.7	7.7	21.9	12.6	0	Pass
Waste bin	38.9	22.5	8.1	21.5	13.8	22	Caution

Table 12 – December 2011, Gastroenterology

	MOISTURE MODE		HYGROMETER MODE			
Surface	**Relative reading**	**Dry, at risk or wet?**	**% RH**	**T_{AIR}**	**T_{DEW}**	**AbS**
Door handle	203	Wet	41.3	21.2	7.4	6.47
Door handle	193	At risk	41.3	21.4	7.6	6.62
Door push plate	257	Wet	40.1	21.7	7.2	6.3
Door push plate	240	Wet	38.1	22	7.1	6.14
Computer keyboard	84	Dry	36.7	24.5	8.8	7.03
Computer mouse	73	Dry	38.2	24.4	9.1	7.04
Bed rail	NR	NR	44.8	24.6	11.8	8.64
Bed rail	64	Dry	44.9	24.5	11.6	8.57
Bed rail	73	Dry	44.2	23.2	10.4	7.82
Chair	65	Dry	44.2	23.2	10.4	7.81
Chair	61	Dry	44	23.1	10.1	7.74
Chair	200	Wet	43.5	23.1	10	7.72
Trolley	182	At risk	44.9	23.4	10.4	7.94
Trolley	141	Dry	44.1	22.8	9.9	7.59
Table	139	Dry	40.5	23.6	9.3	7.33
Table	1000	Wet	40.2	23.4	9.4	7.43
Light switch	162	Dry	45.7	23.4	11.5	8.62
Tap	178	At risk	40	22.4	8.1	6.91
Wall panel	131	Dry	40.2	22.5	8.3	6.86
Wall panel	249	At risk	44.3	23.3	10.5	7.92
Waste bin	203	Wet	41.3	21.2	7.4	6.47

Table 12 continued – December 2011, Gastroenterology

	CONDENSATOR MODE					BIOBURDEN	
Surface	**% RH**	T_{AIR}	T_{DEW}	T_{S}	T_{DIFF}	**RLU**	**Pass, caution or fail?**
Door handle	33.6	23.5	6.5	22.9	15.4	13	Caution
Door handle	35.5	23.5	7	23	16.5	23	Caution
Door push plate	32.6	23.5	6.1	23.3	17.1	49	Fail
Door push plate	32.7	23.5	6.1	23.5	17.4	410	Fail
Computer keyboard	37.5	23.8	9.4	25.3	16.3	431	Fail
Computer mouse	37.4	24	9.7	25.3	15.4	120	Fail
Bed rail	45.9	23.6	11.4	24.4	13.1	147	Fail
Bed rail	46.4	23.8	11.4	24.4	12.8	33	Fail
Bed rail	43	24.2	11.3	24.6	11.7	27	Caution
Chair	43.3	24.2	11.2	24.4	13.3	36	Fail
Chair	44.4	23.8	11.1	24.1	13	60	Fail
Chair	41.3	24.2	10.3	24.3	13.6	147	Fail
Trolley	36.7	24.2	7.6	NR	NR	190	Fail
Trolley	40.7	24.4	10.4	24.3	14	118	Fail
Table	39.3	23.8	9.2	21.9	12.9	107	Fail
Table	44.1	23.9	10.8	24.1	13.5	25	Caution
Light switch	44.5	23.8	11	24.8	14	12	Caution
Tap	37.4	23.7	8.4	22.3	14.2	9	Pass
Wall panel	35.8	23.6	7.1	22.5	15.8	18	Caution
Wall panel	43.1	24.5	11.1	NR	NR	81	Fail
Waste bin	33.6	23.5	6.5	22.9	15.4	13	Caution

Table 13 – December 2011, ACC

	MOISTURE MODE		HYGROMETER MODE			
Surface	**Relative reading**	**Dry, at risk or wet?**	**% RH**	**T_{AIR}**	**T_{DEW}**	**AbS**
Door handle	158	Dry	32.5	24.1	6.5	6.04
Door handle	207	Wet	34.3	22.1	5.5	5.63
Door push plate	640	Wet	32.2	24	6.2	5.85
Door push plate	229	Wet	31.5	23.8	5.9	5.88
Computer keyboard	73	Dry	32.5	23.7	5.6	5.72
Computer mouse	75	Dry	31.5	23.4	5.4	5.58
Bed rail	65	Dry	32.7	24	6.2	5.93
Bed rail	73	Dry	31.4	23.4	5.5	5.66
Chair	70	Dry	35.9	23	6.5	6.17
Chair	150	Dry	35.2	22.9	6.7	6.11
Chair	65	Dry	33.8	21.7	4.9	5.37
Trolley	237	Wet	37.8	22.6	7	6.37
Trolley	139	Dry	35.7	22.7	6.6	6.06
Table	121	Dry	38.1	22.5	7.5	6.31
Table	150	Dry	33.8	22.1	4.8	5.32
Light switch	150	Dry	35.6	22.3	5.9	5.81
Wall panel	121	Dry	32	24	6	5.79
Wall panel	142	Dry	31.3	23.5	5.6	5.85
Waste bin	65	Dry	31.7	23.4	5.4	5.59

Table 13 continued – December 2011, ACC

	CONDENSATOR MODE					BIOBURDEN	
Surface	**% RH**	**T_{AIR}**	**T_{DEW}**	**T_S**	**T_{DIFF}**	**RLU**	**Pass, caution or fail?**
Door handle	32.7	22.5	5.3	22.9	17.4	21	Caution
Door handle	33.5	21.8	4.9	21.9	16.9	2	Pass
Door push plate	32.6	22.7	5.3	23	17.8	1	Pass
Door push plate	33	22.8	5.5	22.9	17.5	3	Pass
Computer keyboard	31.6	23.1	5.1	22.9	17.6	55	Fail
Computer mouse	32.4	22.8	5.2	22.9	17.7	14	Caution
Bed rail	33.2	22.5	6.4	23	17.1	281	Fail
Bed rail	32.9	22.5	5.3	22.6	17	113	Fail
Chair	32.8	23	5.6	22.7	17.2	24	Caution
Chair	34.3	22.1	5.7	NR	NR	89	Fail
Chair	35.6	21.7	5.3	22.5	17.3	14	Caution
Trolley	33.5	22.9	6.2	22.6	15.2	17	Caution
Trolley	37.4	23.1	6.9	23.2	16.6	70	Fail
Table	36.5	22.8	6.6	22.2	15.5	26	Caution
Table	35	22.8	9.3	22.3	13.7	13	Caution
Light switch	35.8	22.1	6.1	22.9	16.1	5	Pass
Wall panel	32.6	22.4	5.2	23.2	18	10	Pass
Wall panel	32.4	22.4	5	22.6	17.7	5	Pass
Waste bin	32.7	22.4	5.2	22.7	17.6	100	Fail

Table 14 – December 2011, Theatre

	MOISTURE MODE		HYGROMETER MODE			
Surface	**Relative reading**	**Dry, at risk or wet?**	**% RH**	**T_{AIR}**	**T_{DEW}**	**AbS**
Door handle	176	At risk	32.9	21.4	4.4	5.16
Door handle	164	At risk	33.5	21	4.1	5.05
Door push plate	408	Wet	33.5	20.9	4	5.03
Door push plate	238	Wet	34.2	20.3	3.9	5.06
Computer keyboard	121	Dry	34.3	20.1	4.1	5.24
Trolley	202	Wet	34.9	20	4	5.09
Trolley	900	Wet	35	20	3.9	5.12
Trolley	152	Dry	34.8	19.8	4	5.11
Anaesthetics stand	173	At risk	35.1	20	4.1	5.12
Light switch	156	Dry	34.8	20	4.2	5.11
Tap	275	Wet	34.7	19.8	4.1	5.13
Wall panel	159	Dry	34.8	20.1	3.9	5.15
Wall panel	134	Dry	34.9	20.1	4	5.03
Waste bin	1000	Dry	34	19.9	3.6	4.94

Table 14 continued – December 2011, Theatre

	CONDENSATOR MODE					BIOBURDEN	
Surface	**% RH**	**T_{AIR}**	**T_{DEW}**	**T_S**	**T_{DIFF}**	**RLU**	**Pass, caution or fail?**
Door handle	34.9	19.8	4	20.3	16.3	113	Fail
Door handle	34.7	19.8	3.9	20.3	16.3	41	Fail
Door push plate	35.2	19.8	3.9	20.3	15.7	74	Fail
Door push plate	36.3	19.7	4.4	20.4	15.5	59	Fail
Computer keyboard	35.8	19.7	4.1	20.2	16.4	117	Fail
Trolley	35.1	19.6	3.5	19.4	16	19	Caution
Trolley	34.6	19.6	3.6	20.1	16.8	28	Caution
Trolley	37.4	19.7	4.4	20.4	16.5	52	Fail
Anaesthetics stand	34.4	19.7	3.3	20.2	17	46	Fail
Light switch	34.2	19.6	3.2	NR	NR	54	Fail
Tap	34.3	19.3	3.4	21.4	18.5	23	Caution
Wall panel	34.3	19.4	3.3	19.2	NR	7	Pass
Wall panel	34.4	19.6	3.4	NR	NR	11	Caution
Waste bin	34.2	19.5	3.2	NR	NR	79	Fail

Table 15 – February 2012, Gastroenterology

	MOISTURE MODE		HYGROMETER MODE			
Surface	**Relative reading**	**Dry, at risk or wet?**	**% RH**	T_{AIR}	T_{DEW}	**AbS**
Door handle	193	At risk	38.2	22.8	7.1	6.09
Door handle	186	At risk	34.8	22.8	6.4	5.91
Door push plate	496	Wet	33	22.8	5.4	5.55
Door push plate	240	Wet	32.1	22.8	5.3	5.52
Computer keyboard	96	Dry	33.1	23.1	5.8	5.79
Computer mouse	121	Dry	36.5	23	6.2	5.85
Bed rail	200	At risk	31.6	25.2	6.9	6.21
Bed rail	1000	Wet	31.3	24.9	6.8	6.17
Bed rail	107	Dry	31.4	24.7	6.8	6.17
Chair	70	Dry	42.9	21.8	8.6	7.02
Chair	146	Dry	35.3	22.8	6.8	6.14
Chair	156	Dry	39.4	22.9	8.6	7.03
Trolley	100	Wet	42.1	22	8.7	7.08
Trolley	179	At risk	32.9	22.8	5.9	6.11
Table	171	At risk	42.5	21.8	8.5	7.03
Table	121	Dry	41.5	22.4	8.5	6.96
Light switch	163	Dry	42.4	22.2	8.8	7.17
Tap	147	At risk	42.6	22.3	9.4	7.42
Wall panel	155	Dry	41.4	22.8	9.2	7.43
Wall panel	156	Dry	42.2	22.8	9.2	7.22
Waste bin	255	At risk	41.4	22.4	8.6	7.06

Table 15 continued – February 2012, Gastroenterology

	CONDENSATOR MODE					BIOBURDEN	
Surface	**% RH**	**T_{AIR}**	**T_{DEW}**	**T_S**	**T_{DIFF}**	**RLU**	**Pass, caution or fail?**
Door handle	38.2	23.5	9.7	24.6	16.9	2	Pass
Door handle	34.8	23.8	8.1	23.9	16.5	0	Pass
Door push plate	32.4	23.8	6.7	24.4	17.7	28	Caution
Door push plate	35.6	23.9	7.5	24	17.8	60	Fail
Computer keyboard	32.9	23.8	7.4	24.3	17.7	72	Fail
Computer mouse	31.5	23.8	6	24.3	16.8	105	Fail
Bed rail	31.4	24.1	6.3	22.7	16.4	52	Fail
Bed rail	31.7	24.1	6.1	22.6	16.6	13	Caution
Bed rail	31.9	24.1	6.2	22.9	16.8	144	Fail
Chair	42.9	22.9	10	20.7	10.3	32	Fail
Chair	39.1	23.7	8.6	NR	NR	1	Pass
Chair	35.3	23.7	7.3	24.1	17.6	56	Fail
Trolley	42.7	23.5	10.3	22.4	11.9	66	Fail
Trolley	39.4	23.7	7.9	NR	NR	29	Caution
Table	43.3	23	9.8	21.6	11.9	53	Fail
Table	40.5	24	9.6	NR	NR	277	Fail
Light switch	42.9	23.1	10.1	22.2	7.8	10	Pass
Tap	41.8	23.5	10.1	22.6	12.4	10	Pass
Wall panel	41.9	23.7	10	22.9	12.9	3	Pass
Wall panel	40.1	23.8	9.4	23.3	12.8	5	Pass
Waste bin	41.8	23.8	10.7	23.1	12.6	115	Fail

Table 16 – February 2012, ACC

	MOISTURE MODE		HYGROMETER MODE			
Surface	**Relative reading**	**Dry, at risk or wet?**	**% RH**	**T_{AIR}**	**T_{DEW}**	**AbS**
Door handle	155	Dry	43	23.8	10.5	7.89
Door handle	179	At risk	42.3	23.8	10.7	7.81
Door push plate	243	Wet	38.2	23.3	8.6	6.94
Door push plate	213	At risk	39.7	23.5	9.4	7.36
Computer keyboard	73	Dry	32	28	7.8	6.57
Computer mouse	73	Dry	32.4	22.9	5.4	5.59
Bed rail	90	Dry	31.9	24.2	6.2	5.95
Bed rail	108	Dry	31.7	24.1	6	5.84
Chair	63	Dry	40	23.3	8.9	7.14
Chair	68	Dry	34.7	24.1	8	6.6
Chair	64	Dry	36.7	24.1	8.3	6.76
Trolley	200	Wet	32.9	25.2	7.3	6.32
Trolley	134	Dry	31.3	23.6	5.6	5.67
Table	141	Dry	38.2	23.4	8.3	6.91
Table	139	Dry	39.4	23.3	8.8	7.08
Light switch	211	Wet	38.8	23.5	8.2	6.72
Wall panel	167	Dry	38.2	23.5	7.7	6.36
Wall panel	158	Dry	37.3	24.4	8.1	6.67
Waste bin	241	Wet	40.4	23.3	8.6	6.82

Table 16 continued – February 2012, ACC

	CONDENSATOR MODE					BIOBURDEN	
Surface	**% RH**	**T_{AIR}**	**T_{DEW}**	**T_S**	**T_{DIFF}**	**RLU**	**Pass, caution or fail?**
Door handle	37.4	22.8	6.2	23.3	17.3	47	Fail
Door handle	43.4	23.4	9.6	24.3	14.2	6	Pass
Door push plate	41.8	23.1	9.7	23.9	13.6	19	Caution
Door push plate	42.2	23.5	9.6	24.1	14.5	86	Fail
Computer keyboard	33.2	23.4	6.1	23.4	17.6	18	Caution
Computer mouse	32.6	23.3	5.8	23.3	17.5	39	Fail
Bed rail	32.5	22.7	5.4	23.6	18.1	14	Caution
Bed rail	32.8	22.5	5.3	23.6	17.9	35	Fail
Chair	40.9	23.5	9.1	23.3	14.7	30	Caution
Chair	37.4	23.5	8.8	23.5	14.6	49	Caution
Chair	44.1	23.3	10.6	24.7	14.3	25	Caution
Trolley	33.3	22.5	8.2	23.3	14.8	615	Fail
Trolley	44.5	23.4	10.9	24.3	13.8	349	Fail
Table	42.4	23.5	9.8	24.1	14.2	20	Caution
Table	43.3	23.4	10.1	23.6	13.5	58	Fail
Light switch	38.4	23.5	8.4	24.3	15.4	13	Caution
Wall panel	38	23.6	8.9	23.6	15.4	18	Caution
Wall panel	37.4	23.7	8.6	23.6	15.5	19	Caution
Waste bin	43.3	23.1	9.9	24.3	14.1	14	Caution

Table 17 – February 2012, Theatre

	MOISTURE MODE		HYGROMETER MODE			
Surface	**Relative reading**	**Dry, at risk or wet?**	**% RH**	**T_{AIR}**	**T_{DEW}**	**AbS**
Door handle	183	At risk	47.1	21.1	9.3	7.35
Door handle	179	At risk	46.3	21.8	9.7	7.62
Door push plate	267	At risk	46.2	21.8	9.7	7.55
Door push plate	218	At risk	46	21.8	9.6	7.49
Computer keyboard	173	At risk	45.8	21.8	9.6	7.46
Trolley	114	Dry	45.7	21.9	9.6	7.47
Trolley	1000	Wet	46.1	21.8	9.6	7.49
Trolley	1000	Wet	45.4	22	9.6	7.46
Anaesthetics stand	69	Dry	43.8	23.1	9.9	7.58
Light switch	1000	Wet	47.6	21.4	9.6	7.46
Tap	197	At risk	46.9	21.2	9.4	7.45
Wall panel	159	Dry	46.9	21.6	9.6	7.45
Wall panel	161	Dry	45.8	21.8	9.6	7.45
Waste bin	NR	NR	46.7	21.7	9.7	7.49

Table 17 continued – February 2012, Theatre

	CONDENSATOR MODE					BIOBURDEN	
Surface	**% RH**	**T_{AIR}**	**T_{DEW}**	**T_S**	**T_{DIFF}**	**RLU**	**Pass, caution or fail?**
Door handle	46.3	22.4	12.6	22.3	12.9	51	Fail
Door handle	44.6	22.6	10.3	23	13.1	34	Fail
Door push plate	45.9	22.5	11	22.5	12.2	31	Fail
Door push plate	46	22.5	10.6	22.6	12.6	27	Caution
Computer keyboard	43.3	23	9.9	22.3	12.5	30	Caution
Trolley	41.3	24.2	10	22.2	12.7	10	Pass
Trolley	43.4	23.3	10.1	22.2	13.3	72	Fail
Trolley	46.2	22.3	9.9	22.9	13.2	95	Fail
Anaesthetics stand	45.7	22.4	10.4	22.6	12.7	5	Pass
Light switch	42.7	23.7	10.3	22.7	12.5	88	Fail
Tap	46	23.3	10	22.6	12.7	50	Fail
Wall panel	43.3	24.1	10.6	22.6	13.2	45	Fail
Wall panel	40.8	23.8	10.3	22.9	13.2	23	Caution
Waste bin	46	22.1	10.1	22.3	13.3	33	Fail

Appendix 2 - FACS data

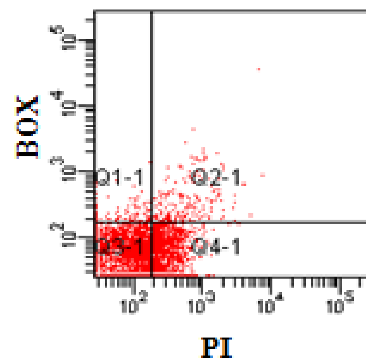
Appendix 2a – FACS plots

Appendix 2b – Tabulated FACS results

Appendix 2a – FACS plots

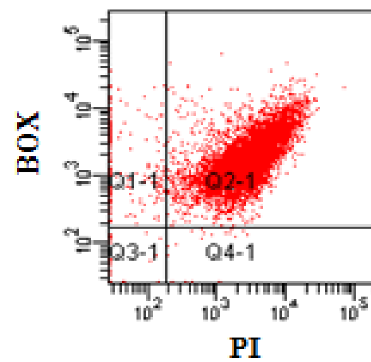
Figure 1 – *S. aureus* PI vs BOX plots (repeat 1)

a) No treatment



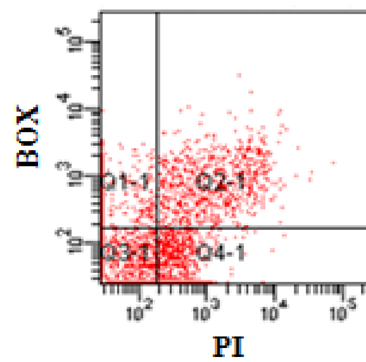
No. of events	9339
Quadrant	% of cells
PI^-BOX^+	1.5
PI^-BOX^-	76.5
PI^+BOX^+	1.9
PI^+BOX^-	20

b) Ethanol



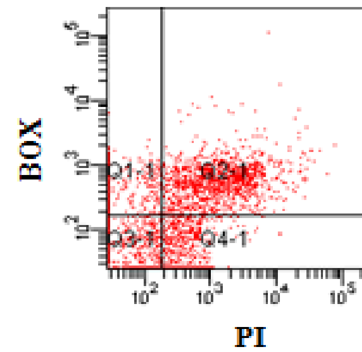
No. of events	8594
Quadrant	% of cells
PI^-BOX^+	2.7
PI^-BOX^-	0.3
PI^+BOX^+	96.8
PI^+BOX^-	0.3

c) SS 0 h



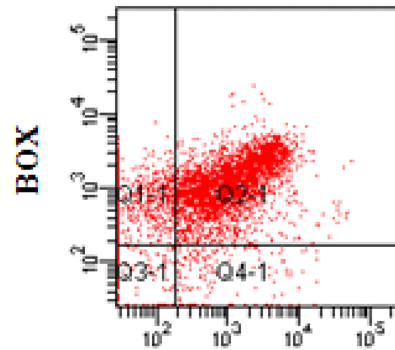
No. of events	5546
Quadrant	% of cells
PI^-BOX^+	7.8
PI^-BOX^-	55.9
PI^+BOX^+	15.4
PI^+BOX^-	20.9

d) SS 24 h



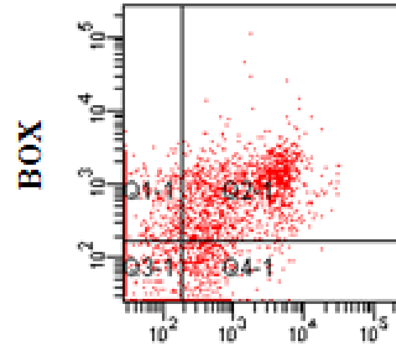
No. of events	4449
Quadrant	% of cells
PI^-BOX^+	12.3
PI^-BOX^-	40.6
PI^+BOX^+	29.7
PI^+BOX^-	17.4

e) CuSn5 0 h



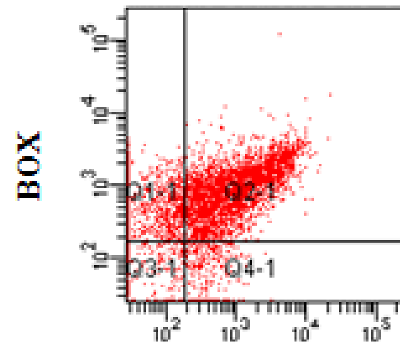
No. of events	5118
Quadrant	% of cells
PI^-BOX^+	23.9
PI^-BOX^-	4.4
PI^+BOX^+	68.6
PI^+BOX^-	3.1

f) CuSn5 24 h



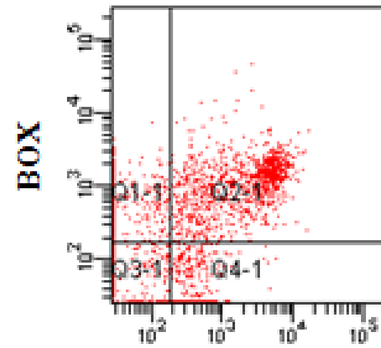
No. of events	3275
Quadrant	% of cells
PI^-BOX^+	19.0
PI^-BOX^-	22.3
PI^+BOX^+	44.7
PI^+BOX^-	14.0

g) CuZn30 0 h



No. of events	5961
Quadrant	% of cells
PI^-BOX^+	29.0
PI^-BOX^-	9.6
PI^+BOX^+	55.6
PI^+BOX^-	5.8

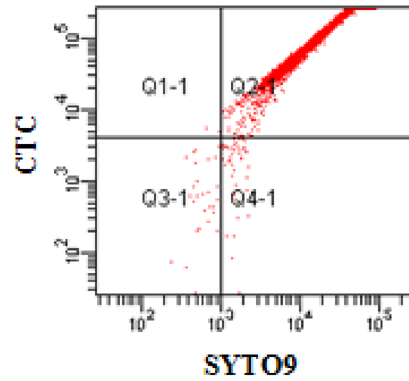
h) CuZn30 24 h



No. of events	2447
Quadrant	% of cells
PI^-BOX^+	16.3
PI^-BOX^-	24.7
PI^+BOX^+	47.0
PI^+BOX^-	12.0

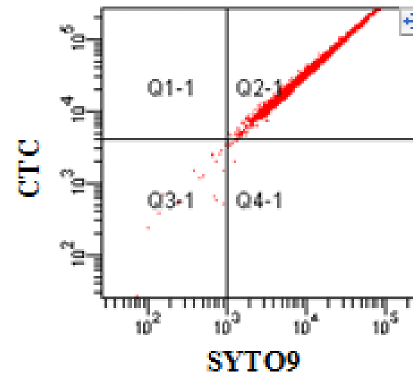
Figure 2 – *S. aureus* CTC vs SYTO9 plots (repeat 1)

a) No treatment



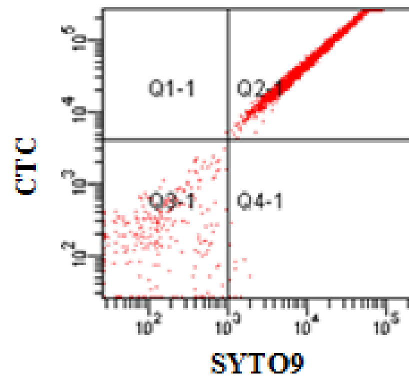
No. of events	7825
Quadrant	**% of cells**
$CTC^{+}SYTO9^{-}$	0
$CTC^{+}SYTO9^{+}$	99.0
$CTC^{-}SYTO9^{-}$	0.4
$CTC^{-}SYTO9^{+}$	0.6

b) Heat shock



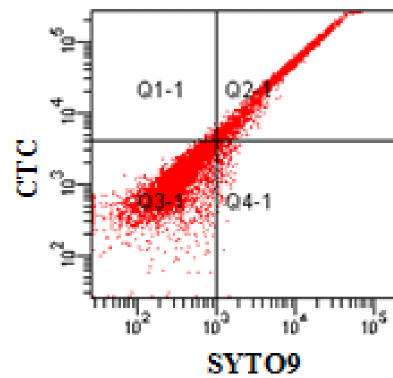
No. of events	8032
Quadrant	**% of cells**
$CTC^{+}SYTO9^{-}$	0
$CTC^{+}SYTO9^{+}$	99.6
$CTC^{-}SYTO9^{-}$	0.3
$CTC^{-}SYTO9^{+}$	0.1

c) H_2O_2



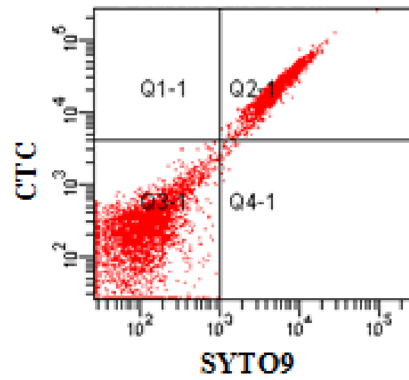
No. of events	8893
Quadrant	**% of cells**
$CTC^{+}SYTO9^{-}$	0
$CTC^{+}SYTO9^{+}$	96.9
$CTC^{-}SYTO9^{-}$	3.0
$CTC^{-}SYTO9^{+}$	0.1

d) Ethanol



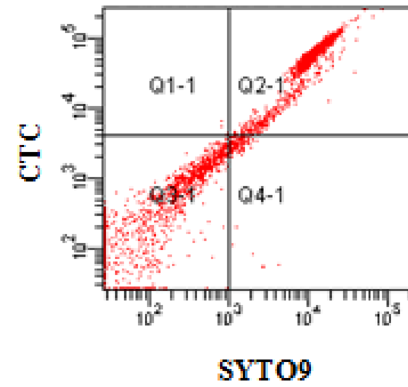
No. of events	8659
Quadrant	**% of cells**
$CTC^{+}SYTO9^{-}$	1.3
$CTC^{+}SYTO9^{+}$	30.7
$CTC^{-}SYTO9^{-}$	65.1
$CTC^{-}SYTO9^{+}$	2.9

e) SS 0 h



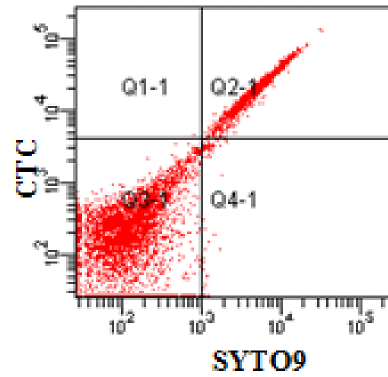
No. of events	8003
Quadrant	**% of cells**
$CTC^{+}SYTO9^{-}$	0
$CTC^{+}SYTO9^{+}$	34.7
$CTC^{-}SYTO9^{-}$	64.9
$CTC^{-}SYTO9^{+}$	0.4

f) SS 24 h



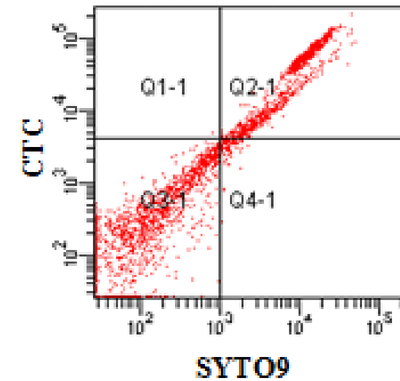
No. of events	4692
Quadrant	**% of cells**
$CTC^{+}SYTO9^{-}$	0.1
$CTC^{+}SYTO9^{+}$	68.8
$CTC^{-}SYTO9^{-}$	28.1
$CTC^{-}SYTO9^{+}$	3.0

g) CuSn5 0 h



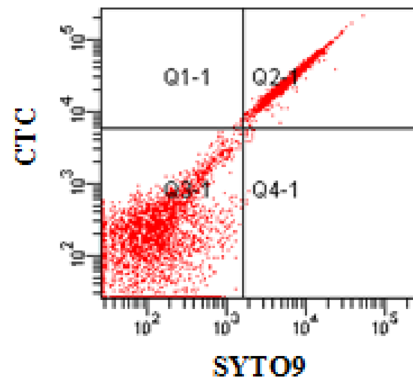
No. of events	8003
Quadrant	**% of cells**
$CTC^{+}SYTO9^{-}$	0
$CTC^{+}SYTO9^{+}$	32.7
$CTC^{-}SYTO9^{-}$	66.5
$CTC^{-}SYTO9^{+}$	0.8

h) CuSn5 24 h



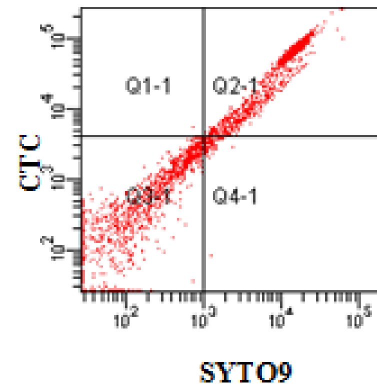
No. of events	3111
Quadrant	**% of cells**
$CTC^{+}SYTO9^{-}$	0.1
$CTC^{+}SYTO9^{+}$	44.0
$CTC^{-}SYTO9^{-}$	52.0
$CTC^{-}SYTO9^{+}$	3.9

i) CuZn30 0 h



No. of events	7859
Quadrant	**% of cells**
$CTC^{+}SYTO9^{-}$	0.1
$CTC^{+}SYTO9^{+}$	50.3
$CTC^{-}SYTO9^{-}$	49.3
$CTC^{-}SYTO9^{+}$	0.2

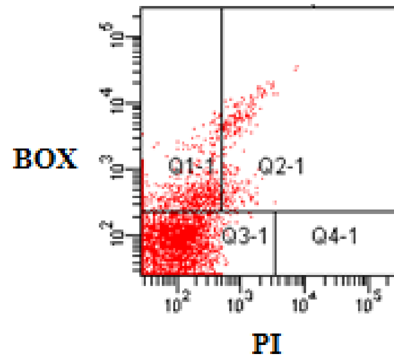
j) CuZn30 24 h



No. of events	2620
Quadrant	**% of cells**
$CTC^{+}SYTO9^{-}$	0.2
$CTC^{+}SYTO9^{+}$	47.1
$CTC^{-}SYTO9^{-}$	49.0
$CTC^{-}SYTO9^{+}$	3.8

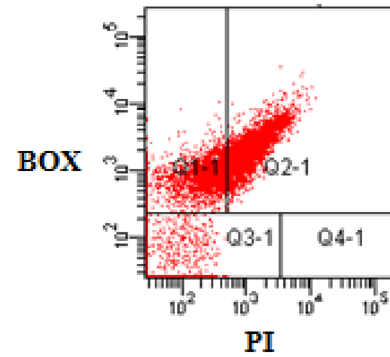
Figure 3 – *S. aureus* PI vs BOX plots (repeat 2)

a) No treatment



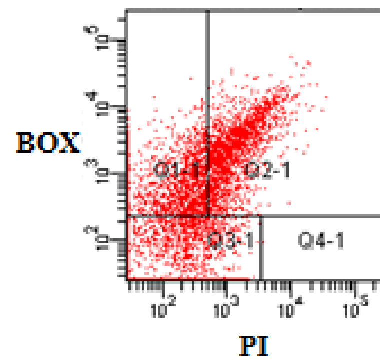
No. of events	8803
Quadrant	**% of cells**
PI^-BOX^+	2.1
PI^-BOX^-	97.9
PI^+BOX^+	0
PI^+BOX^-	0

b) Ethanol



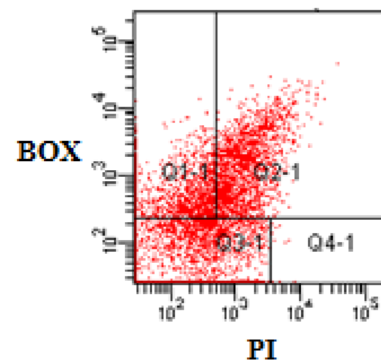
No. of events	8145
Quadrant	**% of cells**
PI^-BOX^+	34.9
PI^-BOX^-	15.3
PI^+BOX^+	49.8
PI^+BOX^-	0

c) SS 0 h



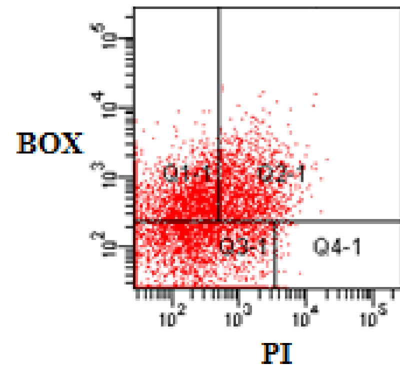
No. of events	7225
Quadrant	**% of cells**
PI^-BOX^+	26.5
PI^-BOX^-	44.0
PI^+BOX^+	29.5
PI^+BOX^-	0

d) SS 24 h



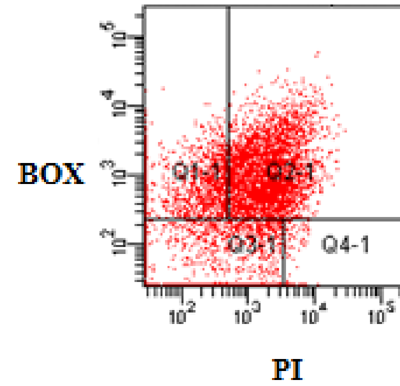
No. of events	7827
Quadrant	**% of cells**
PI^-BOX^+	22.5
PI^-BOX^-	53.1
PI^+BOX^+	24.2
PI^+BOX^-	0.2

e) CuSn5 0 h



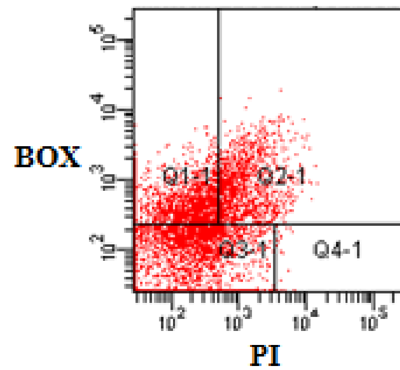
No. of events	8320
Quadrant	% of cells
PI^-BOX^+	28.4
PI^-BOX^-	55.7
PI^+BOX^+	15.4
PI^+BOX^-	0.5

f) CuSn5 24 h



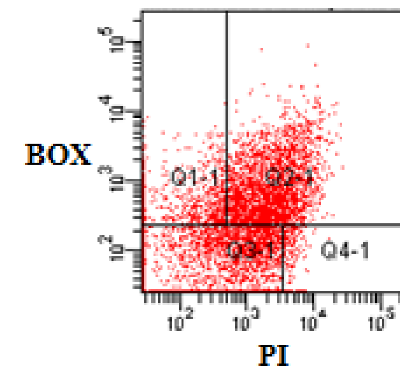
No. of events	7198
Quadrant	% of cells
PI^-BOX^+	21.9
PI^-BOX^-	24.3
PI^+BOX^+	51.9
PI^+BOX^-	1.8

g) CuZn30 0 h



No. of events	8733
Quadrant	% of cells
PI^-BOX^+	25.1
PI^-BOX^-	60.2
PI^+BOX^+	14.4
PI^+BOX^-	0.3

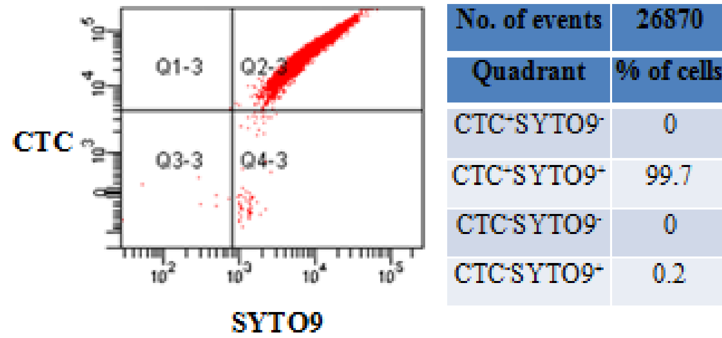
h) CuZn30 24 h



No. of events	7095
Quadrant	% of cells
PI^-BOX^+	11.2
PI^-BOX^-	46.1
PI^+BOX^+	39.4
PI^+BOX^-	3.3

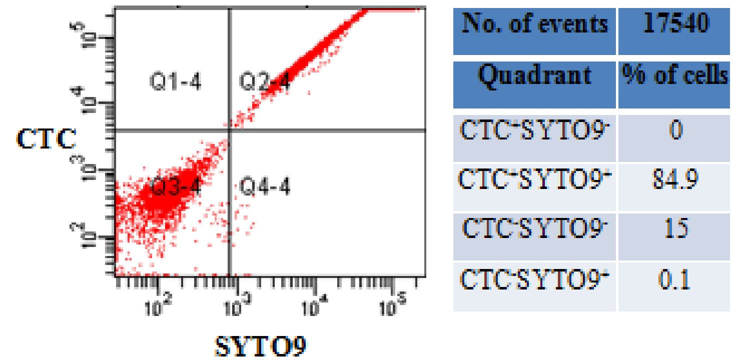
Figure 4 – *S. aureus* CTC vs SYTO9 plots (repeat 2) NB. No of events >10,000.

a) No treatment



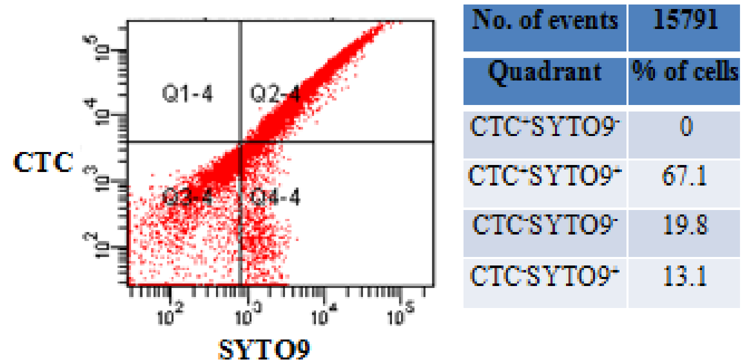
No. of events	26870
Quadrant	**% of cells**
CTC^+SYTO9^-	0
CTC^+SYTO9^+	99.7
CTC^-SYTO9^-	0
CTC^-SYTO9^+	0.2

b) Ethanol



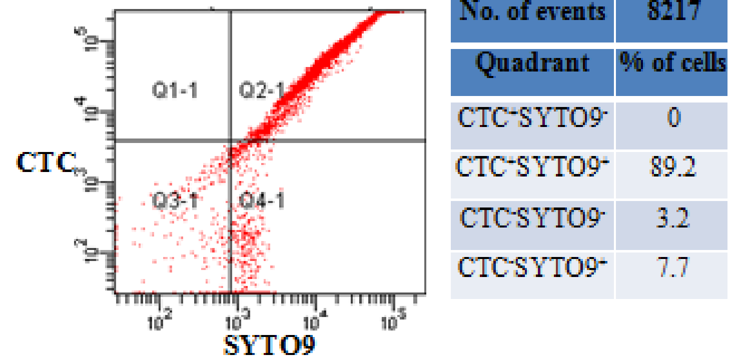
No. of events	17540
Quadrant	**% of cells**
CTC^+SYTO9^-	0
CTC^+SYTO9^+	84.9
CTC^-SYTO9^-	15
CTC^-SYTO9^+	0.1

c) SS 0 h



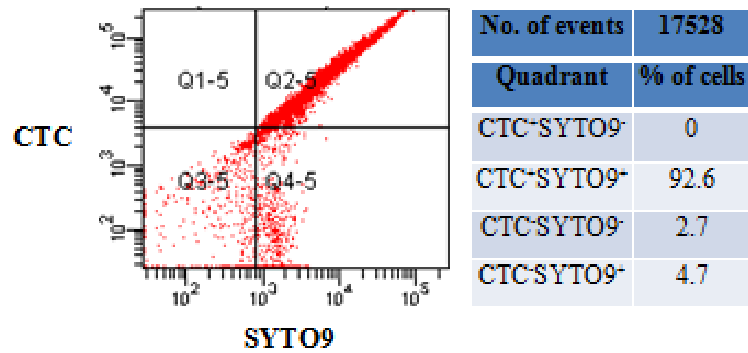
No. of events	15791
Quadrant	**% of cells**
CTC^+SYTO9^-	0
CTC^+SYTO9^+	67.1
CTC^-SYTO9^-	19.8
CTC^-SYTO9^+	13.1

d) SS 24 h



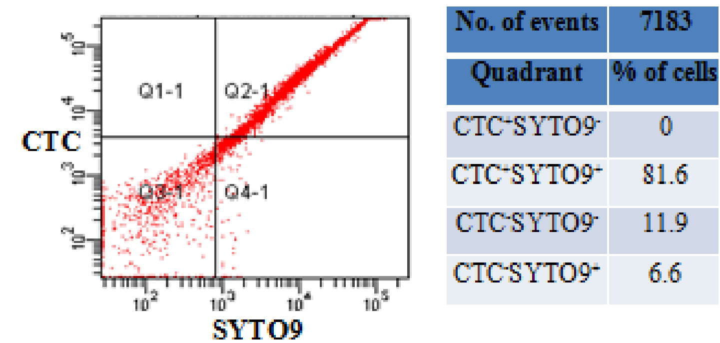
No. of events	8217
Quadrant	**% of cells**
CTC^+SYTO9^-	0
CTC^+SYTO9^+	89.2
CTC^-SYTO9^-	3.2
CTC^-SYTO9^+	7.7

e) CuSn5 0 h



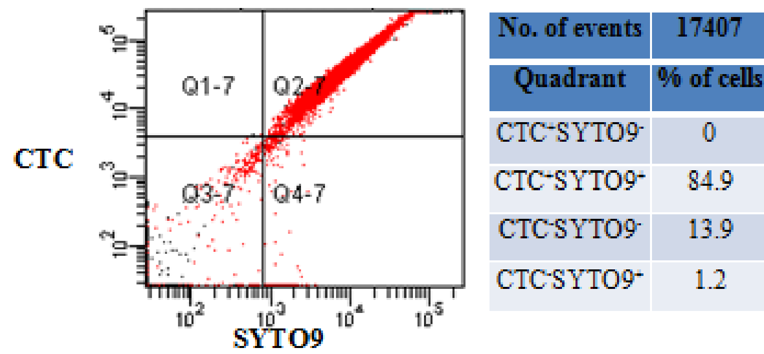
No. of events	17528
Quadrant	% of cells
$CTC^{+}SYTO9^{-}$	0
$CTC^{+}SYTO9^{+}$	92.6
$CTC^{-}SYTO9^{-}$	2.7
$CTC^{-}SYTO9^{+}$	4.7

f) CuSn5 24 h



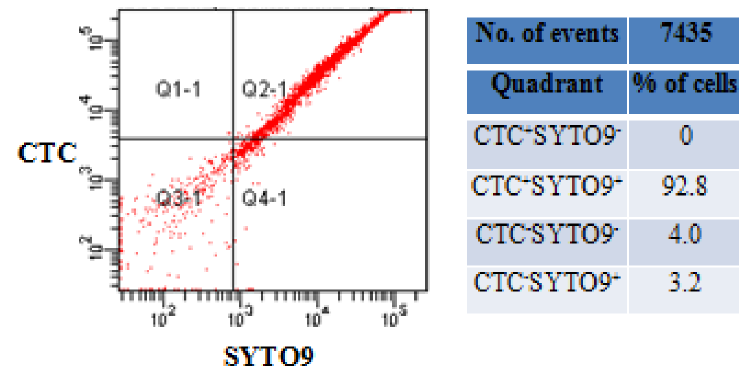
No. of events	7183
Quadrant	% of cells
$CTC^{+}SYTO9^{-}$	0
$CTC^{+}SYTO9^{+}$	81.6
$CTC^{-}SYTO9^{-}$	11.9
$CTC^{-}SYTO9^{+}$	6.6

g) CuZn30 0 h



No. of events	17407
Quadrant	% of cells
$CTC^{+}SYTO9^{-}$	0
$CTC^{+}SYTO9^{+}$	84.9
$CTC^{-}SYTO9^{-}$	13.9
$CTC^{-}SYTO9^{+}$	1.2

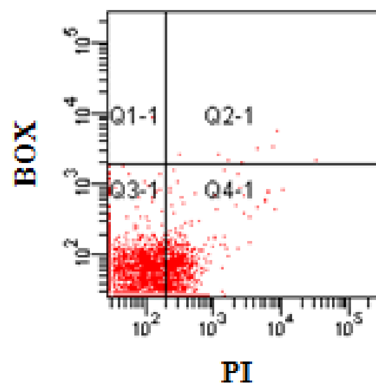
h) CuZn30 24 h



No. of events	7435
Quadrant	% of cells
$CTC^{+}SYTO9^{-}$	0
$CTC^{+}SYTO9^{+}$	92.8
$CTC^{-}SYTO9^{-}$	4.0
$CTC^{-}SYTO9^{+}$	3.2

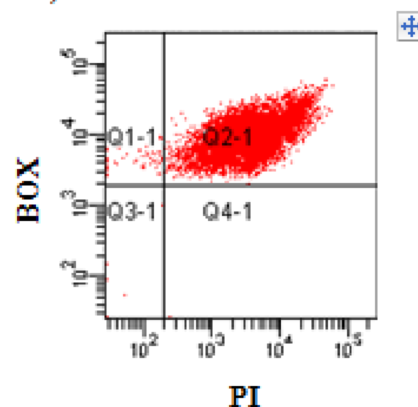
Figure 5 – *A. baumannii* PI vs BOX plots (repeat 1)

a) No treatment



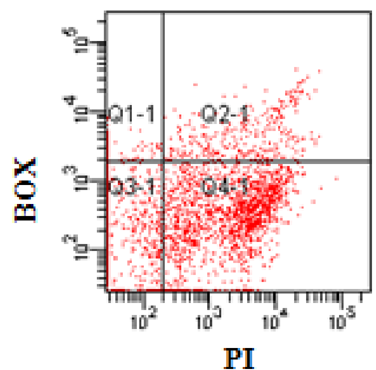
No. of events	9213
Quadrant	**% of cells**
PI^-BOX^+	0
PI^-BOX^-	85.6
PI^+BOX^+	0.1
PI^+BOX^-	14.3

b) Ethanol treatment



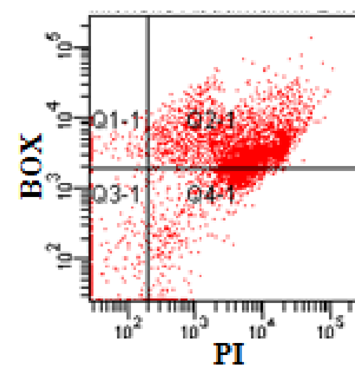
No. of events	8959
Quadrant	**% of cells**
PI^-BOX^+	0.6
PI^-BOX^-	0.1
PI^+BOX^+	99.3
PI^+BOX^-	0

c) SS 0 h



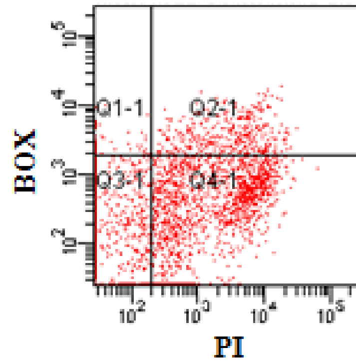
No. of events	3123
Quadrant	**% of cells**
PI^-BOX^+	2.3
PI^-BOX^-	31.8
PI^+BOX^+	10.3
PI^+BOX^-	55.6

d) SS 24 h



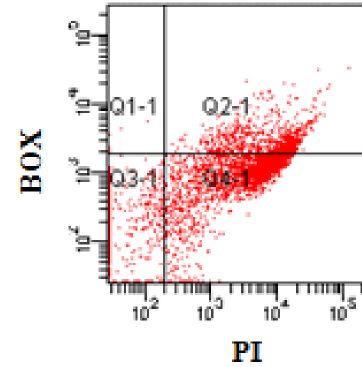
No. of events	4598
Quadrant	**% of cells**
PI^-BOX^+	2.3
PI^-BOX^-	6.4
PI^+BOX^+	66.8
PI^+BOX^-	24.5

e) CuSn5 0 h



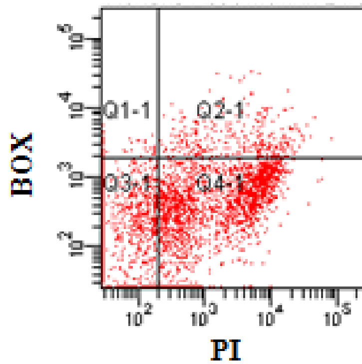
No. of events	2963
Quadrant	% of cells
PI^-BOX^+	2.0
PI^-BOX^-	35.0
PI^+BOX^+	13.1
PI^+BOX^-	49.8

f) CuSn5 24 h



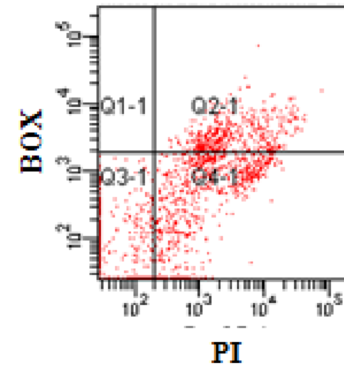
No. of events	4216
Quadrant	% of cells
PI^-BOX^+	0.1
PI^-BOX^-	15.9
PI^+BOX^+	22.4
PI^+BOX^-	61.6

g) CuNi10Fe1Mn 0 h



No. of events	4110
Quadrant	% of cells
PI^-BOX^+	1.6
PI^-BOX^-	41.2
PI^+BOX^+	7.7
PI^+BOX^-	49.4

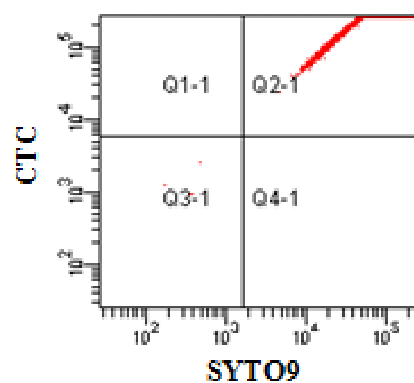
h) CuNi10Fe1Mn 24 h



No. of events	1829
Quadrant	% of cells
PI^-BOX^+	0
PI^-BOX^-	31.9
PI^+BOX^+	25.8
PI^+BOX^-	42.3

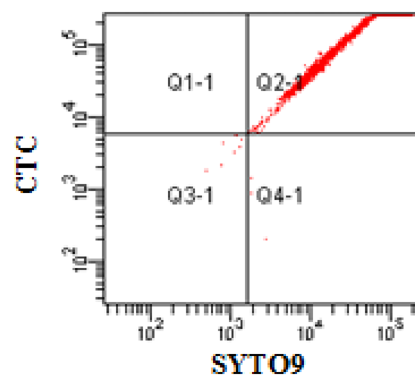
Figure 6 – *A. baumannii* CTC vs SYTO9 plots (repeat 1)

a) No treatment



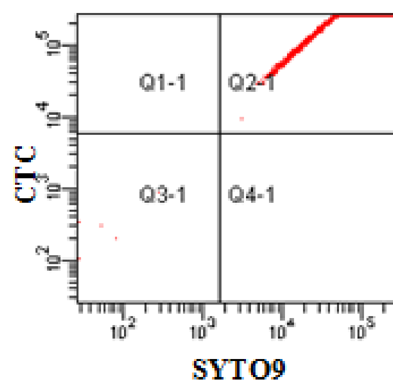
No. of events	9073
Quadrant	**% of cells**
$CTC^{+}SYTO9^{-}$	0
$CTC^{+}SYTO9^{+}$	100
$CTC^{-}SYTO9^{-}$	0
$CTC^{-}SYTO9^{+}$	0

b) Heat shock



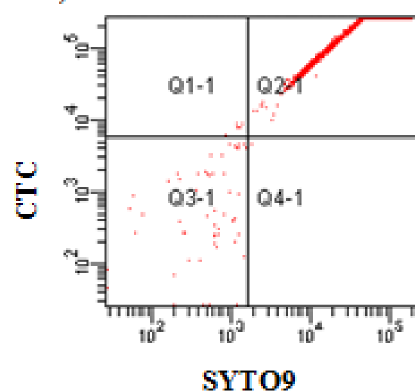
No. of events	8914
Quadrant	**% of cells**
$CTC^{+}SYTO9^{-}$	0
$CTC^{+}SYTO9^{+}$	99.9
$CTC^{-}SYTO9^{-}$	0.1
$CTC^{-}SYTO9^{+}$	0

c) H_2O_2



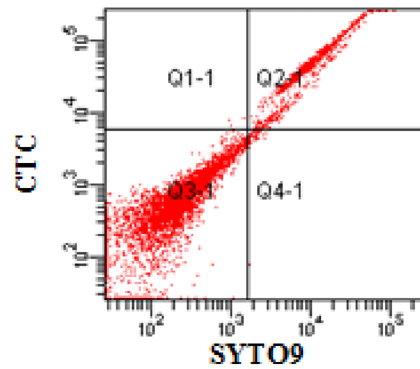
No. of events	9028
Quadrant	**% of cells**
$CTC^{+}SYTO9^{-}$	0
$CTC^{+}SYTO9^{+}$	99.9
$CTC^{-}SYTO9^{-}$	0.1
$CTC^{-}SYTO9^{+}$	0

d) Ethanol



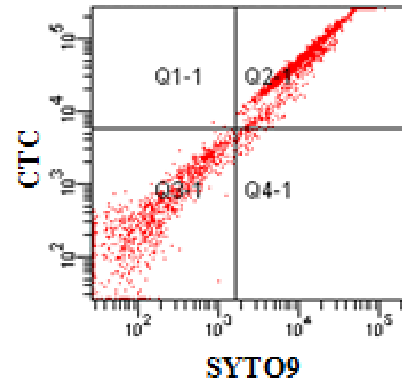
No. of events	9073
Quadrant	**% of cells**
$CTC^{+}SYTO9^{-}$	0
$CTC^{+}SYTO9^{+}$	99.9
$CTC^{-}SYTO9^{-}$	0.1
$CTC^{-}SYTO9^{+}$	0

e) SS 0 h



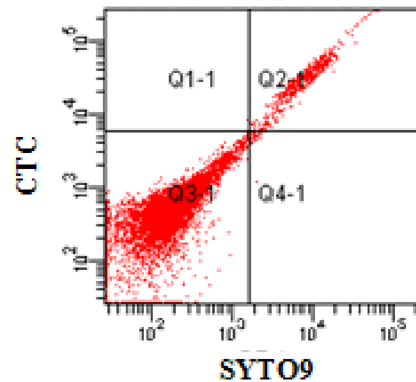
No. of events	6119
Quadrant	% of cells
CTC⁺SYTO9⁻	0
CTC⁺SYTO9⁺	15.0
CTC⁻SYTO9⁻	83.8
CTC⁻SYTO9⁺	1.1

f) SS 24



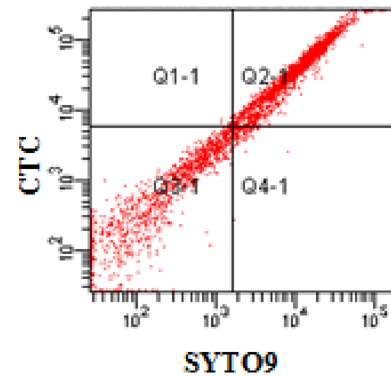
No. of events	5354
Quadrant	% of cells
CTC⁺SYTO9⁻	0.1
CTC⁺SYTO9⁺	79.0
CTC⁻SYTO9⁻	19.8
CTC⁻SYTO9⁺	1.1

g) CuSn5 0 h



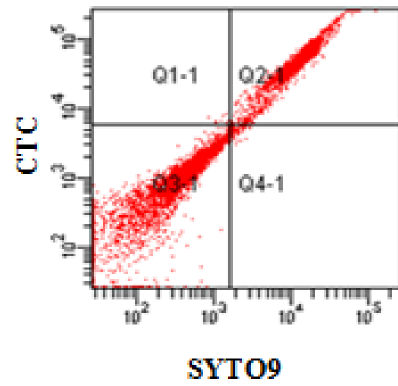
No. of events	7358
Quadrant	% of cells
CTC⁺SYTO9⁻	0.1
CTC⁺SYTO9⁺	7.8
CTC⁻SYTO9⁻	91.7
CTC⁻SYTO9⁺	0.5

h) CuSn5 24 h



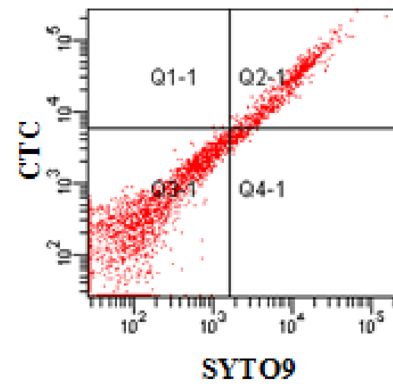
No. of events	4770
Quadrant	% of cells
CTC⁺SYTO9⁻	0.4
CTC⁺SYTO9⁺	69.2
CTC⁻SYTO9⁻	28.3
CTC⁻SYTO9⁺	2.1

i) CuNi10Fe1Mn 0 h



No. of events	6693
Quadrant	**% of cells**
CTC$^+$SYTO9$^-$	0.2
CTC$^+$SYTO9$^+$	38.7
CTC$^-$SYTO9$^-$	59.6
CTC$^-$SYTO9$^+$	1.5

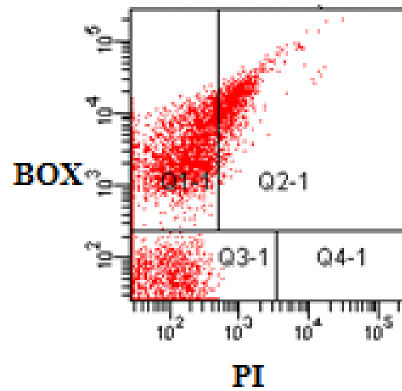
j) CuNi10Fe1Mn 24 h



No. of events	2930
Quadrant	**% of cells**
CTC$^+$SYTO9$^-$	0.1
CTC$^+$SYTO9$^+$	20.4
CTC$^-$SYTO9$^-$	74.9
CTC$^-$SYTO9$^+$	4.6

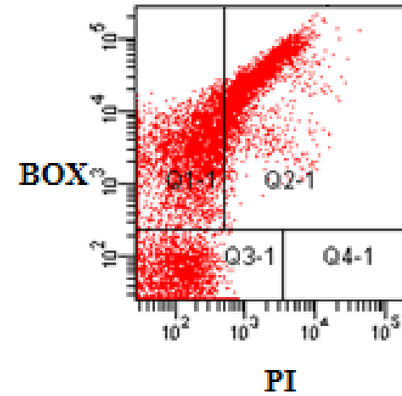
Figure 7 – *A. baumannii* PI vs BOX plots (repeat 2)

a) No treatment



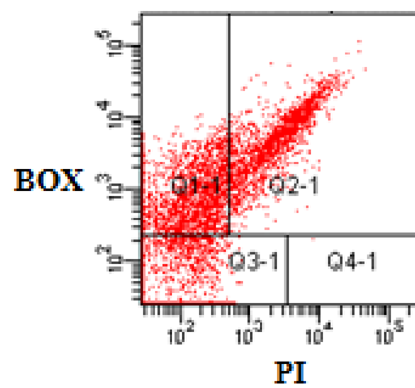
No. of events	8325
Quadrant	% of cells
PI^-BOX^+	36.6
PI^-BOX^-	52.8
PI^+BOX^+	10.6
PI^+BOX^-	0

b) Ethanol



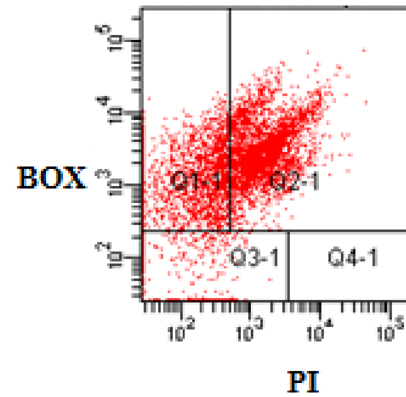
No. of events	7103
Quadrant	% of cells
PI^-BOX^+	27.8
PI^-BOX^-	51.8
PI^+BOX^+	20.3
PI^+BOX^-	0

c) SS 0 h



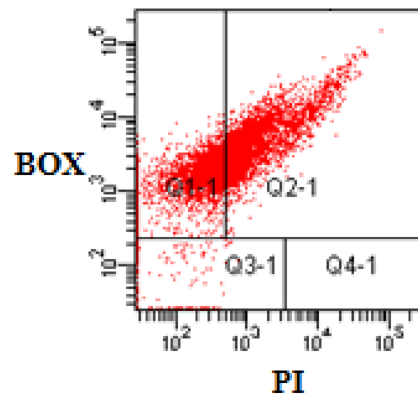
No. of events	7726
Quadrant	% of cells
PI^-BOX^+	46.2
PI^-BOX^-	29.9
PI^+BOX^+	23.8
PI^+BOX^-	0

d) SS 24 h



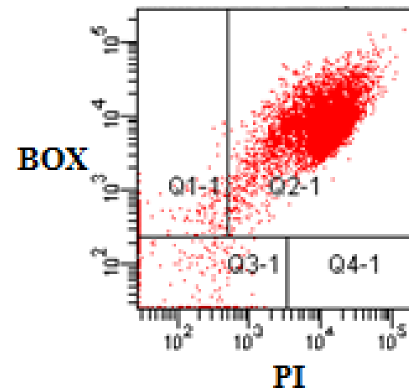
No. of events	6772
Quadrant	% of cells
PI^-BOX^+	40
PI^-BOX^-	7.9
PI^+BOX^+	52.1
PI^+BOX^-	0

e) CuSn5 0 h



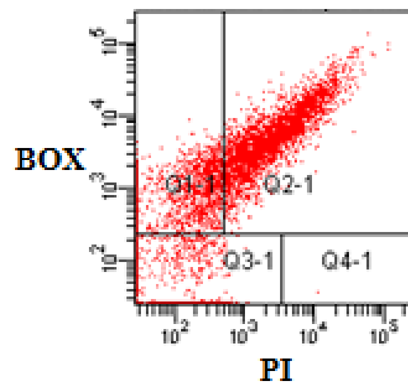
No. of events	7367
Quadrant	% of cells
PI^-BOX^+	43.2
PI^-BOX^-	2.3
PI^+BOX^+	54.5
PI^+BOX^-	0

f) CuSn5 24 h



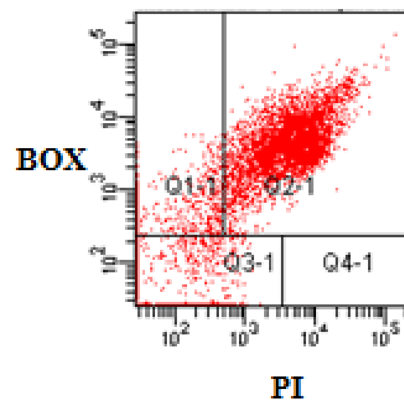
No. of events	7840
Quadrant	% of cells
PI^-BOX^+	2.0
PI^-BOX^-	3.2
PI^+BOX^+	94.8
PI^+BOX^-	0

g) CuNi10Fe1Mn 0 h



No. of events	6252
Quadrant	% of cells
PI^-BOX^+	24.8
PI^-BOX^-	20.1
PI^+BOX^+	55.0
PI^+BOX^-	0

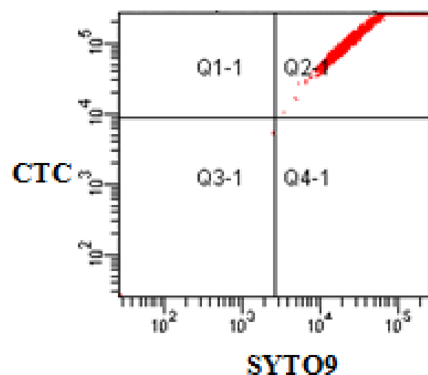
h) CuNi10Fe1Mn 24 h



No. of events	6656
Quadrant	% of cells
PI^-BOX^+	9.1
PI^-BOX^-	16.5
PI^+BOX^+	74.3
PI^+BOX^-	0

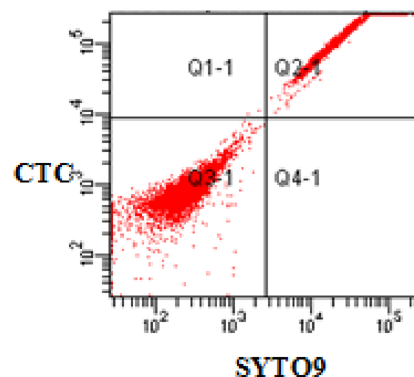
Figure 8 – *A. baumannii* CTC vs SYTO9 plots (repeat 2)

a) No treatment



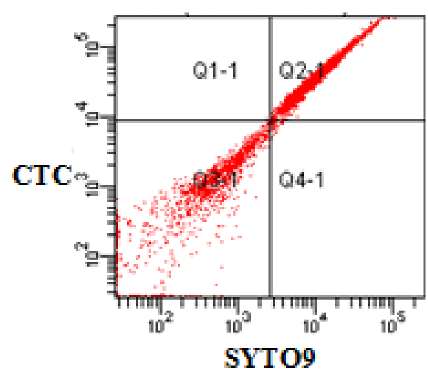
No. of events	8431
Quadrant	% of cells
$CTC^{+}SYTO9^{-}$	0
$CTC^{+}SYTO9^{+}$	100
$CTC^{-}SYTO9^{-}$	0
$CTC^{-}SYTO9^{+}$	0

b) Ethanol



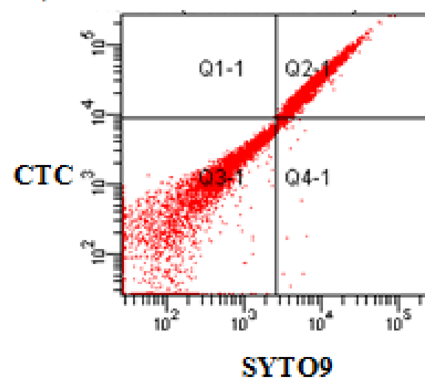
No. of events	8874
Quadrant	% of cells
$CTC^{+}SYTO9^{-}$	0
$CTC^{+}SYTO9^{+}$	40.2
$CTC^{-}SYTO9^{-}$	59.8
$CTC^{-}SYTO9^{+}$	0

c) SS 0 h



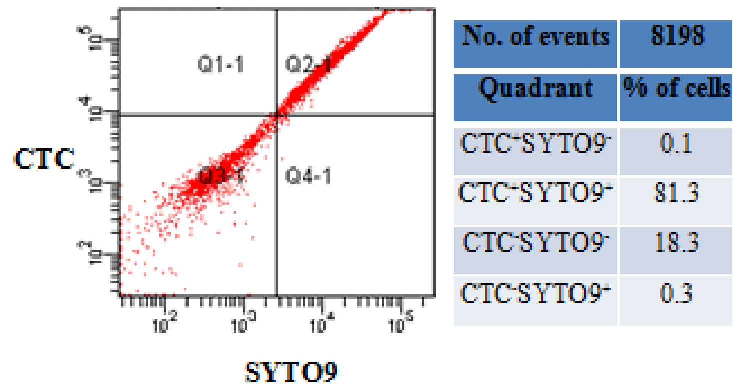
No. of events	8610
Quadrant	% of cells
$CTC^{+}SYTO9^{-}$	0
$CTC^{+}SYTO9^{+}$	82.8
$CTC^{-}SYTO9^{-}$	16.9
$CTC^{-}SYTO9^{+}$	0.4

d) SS 24 h



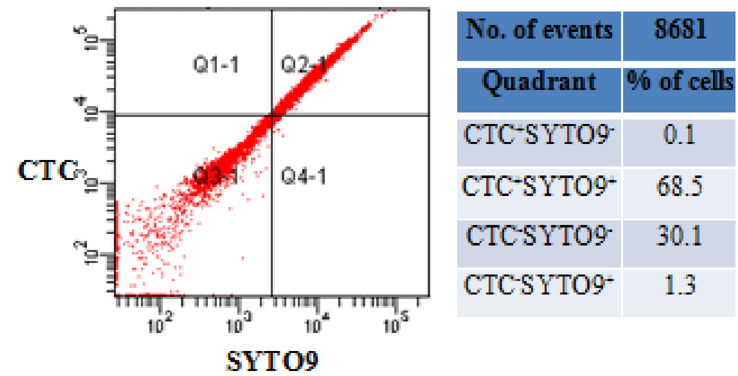
No. of events	8388
Quadrant	% of cells
$CTC^{+}SYTO9^{-}$	0
$CTC^{+}SYTO9^{+}$	40.8
$CTC^{-}SYTO9^{-}$	57.3
$CTC^{-}SYTO9^{+}$	0

e) CuSn5 0 h



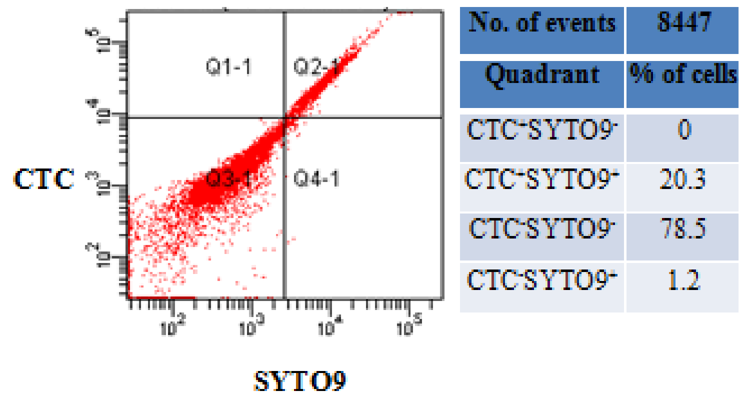
No. of events	8198
Quadrant	% of cells
$CTC^{+}SYTO9^{-}$	0.1
$CTC^{+}SYTO9^{+}$	81.3
$CTC^{-}SYTO9^{-}$	18.3
$CTC^{-}SYTO9^{+}$	0.3

f) CuSn5 24 h



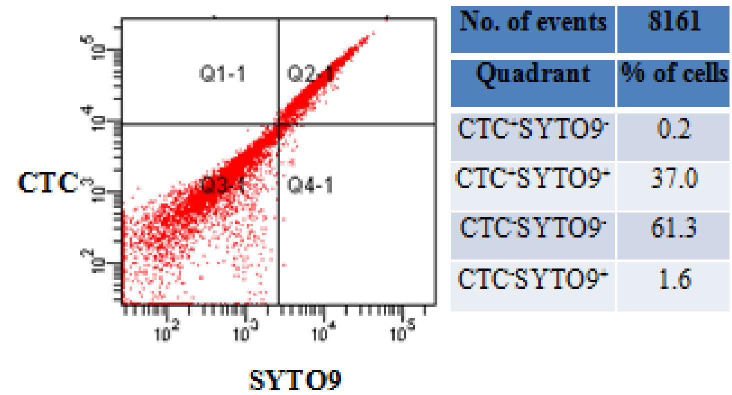
No. of events	8681
Quadrant	% of cells
$CTC^{+}SYTO9^{-}$	0.1
$CTC^{+}SYTO9^{+}$	68.5
$CTC^{-}SYTO9^{-}$	30.1
$CTC^{-}SYTO9^{+}$	1.3

g) CuNi10Fe1Mn 0 h



No. of events	8447
Quadrant	% of cells
$CTC^{+}SYTO9^{-}$	0
$CTC^{+}SYTO9^{+}$	20.3
$CTC^{-}SYTO9^{-}$	78.5
$CTC^{-}SYTO9^{+}$	1.2

h) CuNi10Fe1Mn 24 h



No. of events	8161
Quadrant	% of cells
$CTC^{+}SYTO9^{-}$	0.2
$CTC^{+}SYTO9^{+}$	37.0
$CTC^{-}SYTO9^{-}$	61.3
$CTC^{-}SYTO9^{+}$	1.6

Appendix 2b – Tabulated FACS results

Table 1 – *S. aureus* PI and BOX

Surface and time	Repeat	PI^-BOX^- (healthy) (% of cells)	PI^+BOX^+ (damaged) (% of cells)	PI^-BOX^+ (collapsed membrane potential) (% of cells)
SS 0 h	**1**	55.9	15.4	7.8
	2	44	29.5	26.5
	Average	**50.0**	**22.5**	**34.3**
SS 24 h	**1**	40.6	29.7	12.3
	2	53.1	24.2	22.5
	Average	**46.9**	**27.0**	**34.8**
CuSn5 0 h	**1**	4.4	68.6	23.9
	2	55.7	15.4	28.4
	Average	**30.1**	**42**	**26.2**
CuSn5 24 h	**1**	22.3	44.7	19
	2	24.3	51.9	21.9
	Average	**23.3**	**48.3**	**20.5**
CuZn30 0 h	**1**	9.6	55.6	29
	2	60.2	14.4	25.1
	Average	**34.9**	**35.0**	**27.1**
CuZn30 24 h	**1**	24.7	47	16.3
	2	46.1	39.4	11.2
	Average	**35.4**	**43.2**	**13.8**

Table 2 – *S. aureus* CTC and SYTO9

Surface and time	Repeat	CTC^+SYTO9^+ (healthy) (% of cells)	CTC^-SYTO9^- (damaged) (% of cells)
SS 0 h	**1**	34.7	64.9
	2	67.1	19.8
	Average	**50.9**	**42.4**
SS 24 h	**1**	68.8	28.1
	2	89.2	3.2
	Average	**79.0**	**15.7**
CuSn5 0 h	**1**	32.7	66.5
	2	92.6	2.7
	Average	**62.7**	**34.6**
CuSn5 24 h	**1**	44	52
	2	81.6	11.9
	Average	**62.8**	**32.0**
CuZn30 0 h	**1**	50.3	49.3
	2	84.9	13.9
	Average	**67.6**	**31.6**
CuZn30 24 h	**1**	47.1	49
	2	92.8	4
	Average	**70.0**	**26.5**

Table 3 – *A. baumannii* PI and BOX

Surface and time	Repeat	PI^-BOX^- (healthy) (% of cells)	PI^+BOX^+ (damaged) (% of cells)	PI^-BOX^+ (collapsed membrane potential) (% of cells)
SS 0 h	**1**	31.8	10.3	2.3
	2	29.9	23.8	46.2
	Average	**30.9**	**17.1**	**24.3**
SS 24 h	**1**	6.4	66.8	2.3
	2	7.9	52.1	40.0
	Average	**7.2**	**59.5**	**21.2**
CuSn5 0 h	**1**	35	13.1	2.0
	2	2.3	54.5	43.2
	Average	**18.7**	**33.8**	**22.6**
CuSn5 24 h	**1**	15.9	22.4	0.1
	2	3.2	94.8	2.0
	Average	**9.6**	**58.6**	**1.1**
CNi10Fe1Mn 0 h	**1**	41.2	7.7	1.6
	2	20.1	55	24.8
	Average	**30.7**	**31.4**	**26.4**
CNi10Fe1Mn 24 h	**1**	31.9	25.8	0
	2	16.5	74.3	9.1
	Average	**24.2**	**50.1**	**3.1**

Table 4 – *A. baumannii* CTC and SYTO9

Surface and time	Repeat	CTC^+SYTO9^+ (healthy) (% of cells)	CTC^-SYTO9^- (damaged) (% of cells)
SS 0 h	**1**	15	83.8
	2	82.8	16.9
	Average	**48.9**	**50.4**
SS 24 h	**1**	79	19.8
	2	40.8	57.3
	Average	**59.9**	**38.6**
CuSn5 0 h	**1**	7.8	91.7
	2	81.3	18.3
	Average	**44.6**	**55.0**
CuSn5 24 h	**1**	69.2	28.3
	2	68.5	30.1
	Average	**68.9**	**29.2**
CNi10Fe1Mn 0 h	**1**	38.7	59.6
	2	20.3	78.5
	Average	**29.5**	**69.1**
CNi10Fe1Mn 24 h	**1**	20.4	74.9
	2	37	61.3
	Average	**28.7**	**68.1**

Appendix 3 – Campden BRI's results

Appendix 3a – Environmental sampling in food factories

Campden BRI selected five sites for environmental sampling (surface temperature and surface relative humidity); two dairy factories, one fruit and vegetable processing factory, one brewery and one meat processing factory. Sampling was conducted on one occasion at each site. The Protimeter MMS was used to take surface relative humidity and surface temperature measurements. The condensator mode of the Protimeter MMS was utilised.

Table 1 – Campden BRI's environmental sampling of surfaces in a fruit and vegetable processing factory.

Surface	Temperature (°C)	Relative humidity (%)
Worktop	10.6	78.6
Metal rejects	10.8	100 (wet)
Scale	10.7	/
Horizontal bar next to platform mixer	8.7	76
Vertical bar next to platform mixer	8.7	76
Surface of mixer	9.4	88.2
Control panel	10.4	88.6
Fridge wall	6.2	82.1
Main coleslaw room – surface of the table with packaging on top. Line 2	7.4	88.3
Main coleslaw room - scale	7.5	87.2
Dairy line room - depositor top	9.2	90.8
Dairy line room - can opener	9	93.6
Panel feeder	7.9	87.1
Mayonnaise lid top	7.2	85.7
Plastic curtain in mayonnaise room	7.2	84.3

Table 2 – Campden BRI's environmental sampling of surfaces in a dairy factory (1)

Surface	Temperature (°C)	Relative humidity (%)
Open product zone - conveyor belt	14.3	65
Line 1 cutter blade	15.3	67.1
Line 1 surface before cutter blade	14.8	66.3
Line 1 door after cutter blade	15.4	66.8
Line 1 PFM infeed	15.5	65.1
Machine top	19.3	66.8
Transparent door before PFM infeed	17.5	68.4
Packaging part of food line 1	18.3	67.5
Close to the heat sealer line 1	17.9	66.6
Top of control panel	19.7	56.3
Platform under leak tester	14.9	78.3
Glove stand	14.8	78.9
Control panel	14.1	78.7
Side of conveyor belt	13.9	79
Wall beside sink	13.8	77.9
Screen beside a sink	13.6	77.8

Table 3 – Campden BRI's environmental sampling of surfaces in a dairy factory (2)

Surface	**Temperature (°C)**	**Relative humidity (%)**
Ingredient room preparation and weighing - entry of frozen products area	12.5	69.5
Ingredient room preparation and weighing - balance	13	72.5
Ingredient room preparation and weighing - table	13.2	76.6
Ingredient room preparation and weighing - control screen area of computer	20.5	57
Ingredient room preparation and weighing – sink	14.3	86.5
Under control panel of a machine	16.3	75.6
Line 2 table	12.3	73.6
Transport box	13.2	78.2
Line 2 horizontal surface of machine	17.3	69
Bottom shelf of a cheese trolley	13.8	72.6

Table 4 – Campden BRI's environmental sampling of surfaces in a meat processing factory

Surface	Temperature (°C)	Relative humidity (%)
Ham log entry stage	8.3	83.8
Trolley holding ham logs	9.2	73.4
Outside wall of ham filler	9.8	69.8
Metal part (handle) of guard	10	68.2
Plastic guard near slicer	10.6	68.2
Table	10	64.9
Scale	10.2	65.3
Metal under blue belt	11.4	66.7
Metal waste catch under cut ham belt	14.8	55
Beside packing where ham is placed in plastic trays	19.4	45.4
Vertical surface	16.9	50.3
Control panel to move trays on to be sealed	15.6	52.4
Wall above sink	11	65.8

Table 5 – Campden BRI's environmental sampling of surfaces in a brewery factory

Surface	Temperature (°C)	Relative humidity (%)
Ceiling of hot loft	27.5	37
Lid of ike tipping point	40.5	28
Ceiling of ike tipping room	38.8	27
Grain milling sampling point	27	35
Hot can dosing point	19.8	100 (in fluid)
Filter powder dosing point	23.5	Dry (no measure)
Fermantation room - valve of GYV17	20.6	Dry (no measure)
Process room - under parts of pipes	17.5	100 (wet)
Drain	17.5	100 (wet)
Green part of inverted keg belt	16.3	>66 (higher during operation)

Table 6 – Summary table showing relative humidity and temperature profile of surfaces sampled in five food factories

Factory	Surface relative humidity range (%)	Mean surface relative humidity (%)	Median surface relative humidity (%)	Surface temperature range (°C)	Mean surface temperature (°C)	Median surface temperature (°C)
Fruit and vegetable	76.0 - 100	86.2 ± 6.6	87.2	6.2 - 10.8	8.7 ± 1.5	8.7
Dairy 1	56.3 - 79.0	70.4 ± 7.0	67.3	13.6 - 19.7	15.8 ± 2.0	15.1
Dairy 2	57.0 - 86.5	73.1 ± 7.6	73.1	12.3 - 20.5	14.6 ± 2.6	13.5
Meat processing	45.4 - 83.8	63.8 ± 10.8	65.8	8.3 - 19.4	12.1 ± 3.4	10.6
Brewery	27.0 - 100	56.1 ± 32.7	37.0	16.3 - 40.5	25.7 ± 8.8	23.5

Appendix 3b – Phase 1 results

Fifteen test surfaces were tested under ISO22196 conditions against *S. aureus* NCIMB 8625 and *E. coli* NCIMB 8545. Log_{10} reductions are shown after 24 h, in relation to a stainless steel control surface. The log_{10} reductions are a mean of two tests for each microorganism.

Table 1 – Campden BRI's Phase 1 ISO22196 results

Active agent	***S. aureus*** **(log_{10} cfu/cm^2 reduction)**	***E. coli*** **(log_{10} cfu/cm^2 reduction)**
Silver	1.85	4.68
Silver	0.90	2.16
Silver	0.11	0.61
Silver	4.65	4.71
"Nitrogen spears"	< control	< control
"Nitrogen spears"	< control	< control
Zinc complex	5.09	5.30
Zinc complex	4.62	5.40
Copper	0.86	1.61
Coppernickel	1.69	-0.16
SilverNickel	1.16	0.98
Photo-oxidative surface	4.12	4.00
Liquid glass	< control	< control
Grapefruit extract	>4	>4
Water soluble quaternary ammonium compound	>4	>4

NB: Log reduction calculated in comparison to stainless steel control surface
>4 log reduction = pass, can proceed to phase 2 testing
<4 log reduction = fail
<control = increase in cfu/cm^2 from 0 h samples (fail)
Grapefruit extract is a surface coating

Appendix 3c – Phase 2 results

Copper nickel, grapefruit extract, silver and photo-oxidative surface were tested against S. *aureus* NCIMB 8625 (with 0.03 % BSA) at a range of relative humidities and temperatures reflective of conditions in food factories. Stainless steel, plastic and glazed tile surfaces were utilised as control surfaces.

Table 1 – Campden BRI's Phase 2 results – ISO22196 with modified parameters

Relative Humidity: 75 %, *S. aureus* (0.03 % BSA)							
	Controls	**4 °C**		**10 °C**		**25 °C**	
	0h	**5h**	**24h**	**5h**	**24h**	**5h**	**24h**
Stainless steel control (counts)	5.90	5.58	5.81	5.63	6.02	5.83	5.77
Copper nickel (log_{10} reduction)	0.01	0.41	>4.81	1.36	>4.28	2.57	>4.77
Grapefruit extract (log_{10} reduction)	0.10	0.09	0.14	0.01	0.38	-0.29	0.22
	Controls	**4 °C**		**10 °C**		**25 °C**	
	0h	**5h**	**24h**	**5h**	**24h**	**5h**	**24h**
Plastic control (counts)	4.72	4.92	4.35	4.84	4.23	4.92	5.01
Silver (log_{10} reduction)	-0.13	-0.95	-0.96	-1.00	-1.67	-1.03	0.09
	Controls	**4 °C**		**10 °C**		**25 °C**	
	0h	**5h**	**24h**	**5h**	**24h**	**5h**	**24h**
Glazed tile control (counts)	4.95	5.99	5.05	5.89	5.70	6.25	5.66
Photo-oxidative (log_{10} reduction)	-1.43	0.31	-0.77	-0.05	-0.33	-0.05	-0.59

Table 1 continued - Campden BRI's Phase 2 results – ISO22196 with modified parameters

	Relative Humidity: 33 %, *S. aureus* (0.03 % BSA)						
	Controls	4 °C		10 °C		25 °C	
	0h	5h	24h	5h	24h	5h	24h
Stainless steel control (counts)	4.72	4.92	4.35	4.84	4.23	4.92	5.01
Copper nickel (log_{10} reduction)	-0.04	0.72	>3.29	>1.69	1.52	>1.95	>4.01
Grapefruit extract (log_{10} reduction)	-0.07	0.24	0.72	1.02	0.94	0.40	2.28

Appendix 3d - Phase 3 results

The final phase of tests involved placing surfaces in a factory and regularly swabbing them for total aerobic bacteria count.

Long-term tests were carried out in dairy factory 1, where conditions ranged from 39 – 74 % relative humidity and 13.4 - 16.5 °C during phase 2 testing. Short-term tests were carried out in a potato processing environment where the relative humidity varied between 56 % and 72.3 % and temperature maintained at 21.2 °C throughout the day.

Table 1 – Surfaces used during phase 3 testing

Surface	**Control used**	**Long-term test**	**Short-term test**
Silver	Plastic A (provided)	√	√
Copper nickel	Stainless steel	√	√
Photo-oxidative surface	Uncoated glazed tile	√	√
Grapefruit extract on stainless steel	Uncoated stainless steel	√	√

Samples were tested at different time intervals depending on the test performed (see Table 2). For the long term test, the total experimental time was 83 days and samples were tested 12 times during this period. The short term tests were testing samples 5 times over a period of 24 hours. There were two sets of times due to tests being performed over two different days: one day silver and grapefruit extract coating and the following day with copper nickel and photo-oxidative. Surfaces were swabbed in duplicate for the long-term tests and in triplicate for the short-term tests.

Table 2 – Sampling times for each test

Long term test (Days)	Short term test (silver and grapefruit extract coating) (hours)	Short term test (copper nickel and photo-oxidative) (hours)
0	0	0
1	2	1
2	4	3.5
6	6	5
8	24	24
12		
16		
22		
34		
48		
64		
83		

Results

Short-term tests

All graphs (Figures 1- 7) show the total aerobic bacteria count on test surfaces and control surfaces over the testing period (short- and long-term). The means and ranges are shown on the graphs.

Over 24 h there was no statistical difference between the silver surface and its standard, see Figure 1 (P=0.878).

Figure 1 Total aerobic bacteria count on silver and on control surface

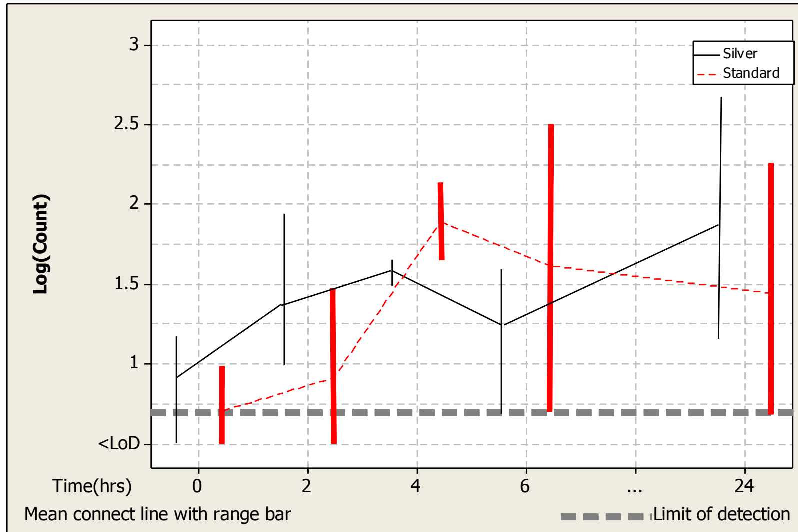
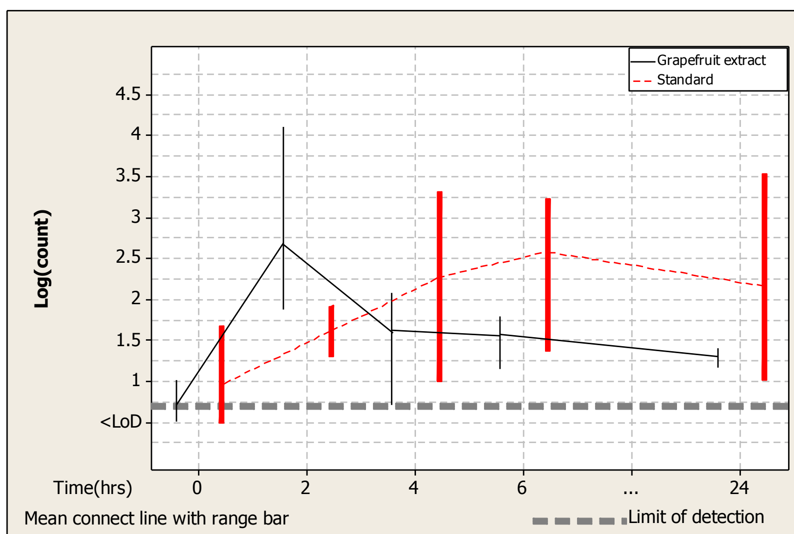


Figure 2 shows the total aerobic bacteria count on the grapefruit extract coated surface compared to the standard surface. There was no statistical difference between the antimicrobial surface and its standard (P=0.818).

Figure 2 Total aerobic bacteria count on grapefruit extract coated surface and on control surface



Over 24 h there was no statistical difference between the copper nickel surface and its standard, see Figure 3 (P=0.259).

Figure 3 Total aerobic bacteria count on copper nickel and on control surface

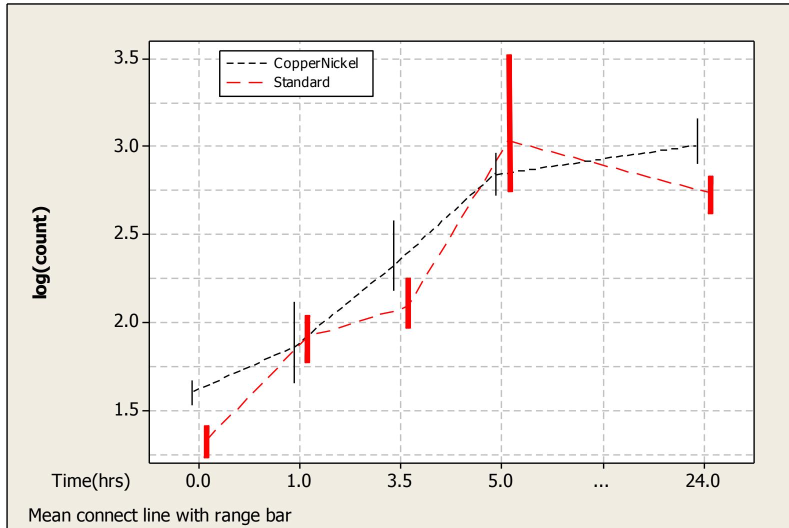
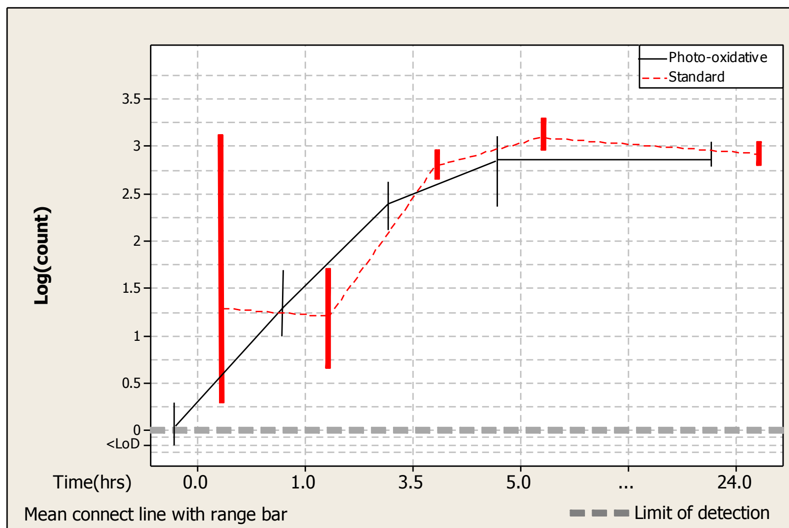


Figure 4 shows the total aerobic bacteria count on the photo-oxidative surface compared to the standard surface. There was no statistical difference between the photo-oxidative surface and its standard (P=0.201).

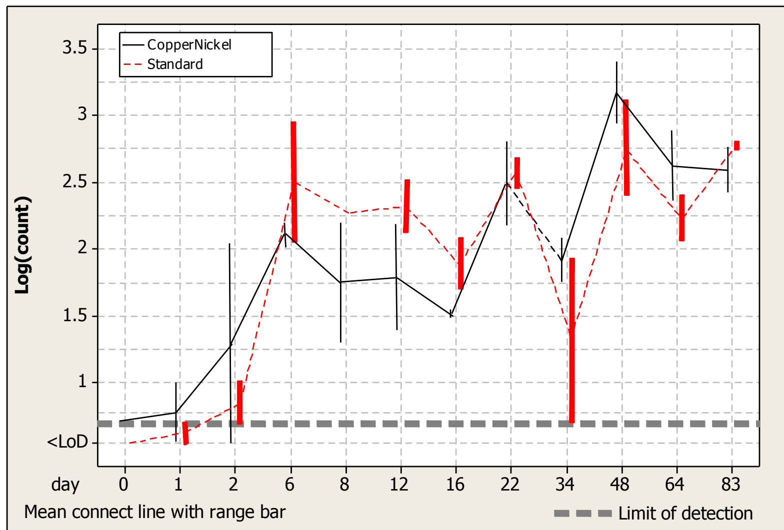
Figure 4 Total aerobic bacteria count on photo-oxidative surface and on control surface



Long-term tests

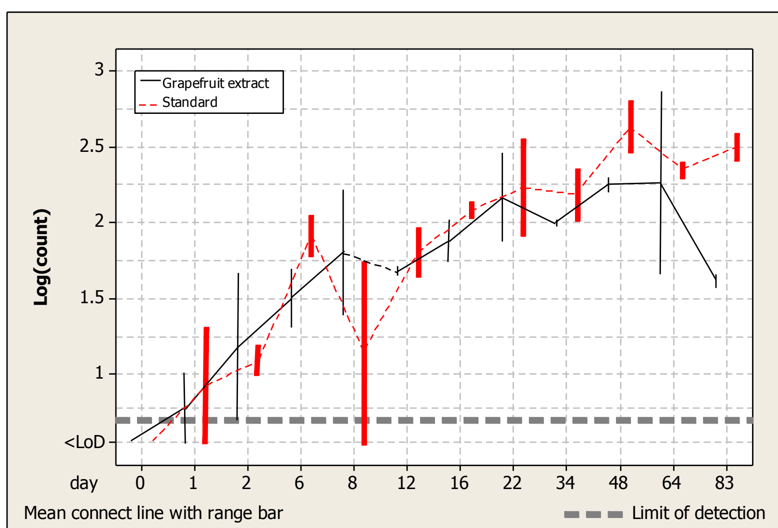
Over 83 days there was no statistical difference between the copper nickel surface and its standard, see Figure 5 (P=0.709).

Figure 5 Total aerobic bacteria count on copper nickel and on control surface



Over 83 days there was no statistical difference between the grapefruit extract coated surface and its standard (P=0.787).

Figure 6 Total aerobic bacteria count on grapefruit extract coated surface and on control surface



There was no statistical difference between the silver surface and its standard over 83 days of testing (P=0.849), see Figure 7.

Figure 7 Total aerobic bacteria count on silver and on control surface

There was no statistical difference between the photo-oxidative surface and its standard over 83 days of testing (P=0.703), see Figure 7.

Figure 8 Total aerobic bacteria count on photo-oxidative and on control surface