EFFICACY AND TOXICITY OF BIOCIDES USED IN HANDLING STERILE PHARMACEUTICALS

Manita Mehmi

Doctor of Philosophy

Aston University

December 2009

ASTON UNIVERSITY

Efficacy and toxicity of biocides used in handling sterile pharmaceuticals

A thesis submitted by Manita Mehmi BSc

For the degree of Doctor of Philosophy

2009

SUMMARY

Spores produced by environmental bacteria are a major concern in hospital pharmacy aseptic areas. Current practice of many NHS hospitals in the UK employs 70% Industrial Methylated Spirit spray for surface disinfection of Grade A pharmaceutical environments. However, this biocide is not sporicidal. This thesis has sought to investigate other disinfection agents and procedures which may provide more effective sanitisation against bacterial spores. A hard-surface disinfection test method was designed to ascertain which combinations of biocide and application method were most effective against bacterial spores. A combination of spraying and wiping was the most effective method of disinfection against *Bacillus* spores, with wiping found to play a key role in spore removal. The most efficacious of the biocides investigated was the 6% hydrogen peroxide. Vaporised Hydrogen Peroxide (VHP) gassing was more effective than traditional disinfection.

In addition to efficacy, the toxic potential of the biocides to human airway epithelial cells *in vitro* was evaluated. Toxicity against human bronchial and nasal epithelial cells was assessed by determining cell viability, inflammatory status, protein oxidation and epithelial cell layer integrity. In addition the cell death mechanism following biocide exposure was investigated. There was a decrease in viable cells following exposure to all biocides when applied at practical concentrations. Almost all of the biocides tested elicited a pro-inflammatory response from the cells as measured by IL-8 production. All biocides increased protein oxidation as measured by thiol and carbonyl levels. Measurement of transepithelial electrical resistance and paracellular permeability indicated biocide-dependent decreases in epithelial cell barrier function. The cellular response was biased towards necrotic rather than apoptotic death.

The use of biocides, although efficacious to some effect against *Bacillus* spores, will require careful monitoring for adverse health effects on personnel.

Key words: *Bacillus* spores, disinfection, hydrogen peroxide, cell culture, *in vitro*

For my family

ACKNOWLEDGEMENTS

My sincere thanks go to my supervisors Prof. P. A. Lambert, Dr. L. J. Marshall and Dr. J. C. Smith for enabling me to undertake the studies presented in this thesis and for their guidance, encouragement and support. I am especially grateful to Prof. P. A. Lambert and Dr. L. J. Marshall for sharing their enthusiasm, expertise and research insight..

I would also like to thank members of the department especially Prof. H. R. Griffiths and Dr. A. Devitt for their advice. I would like to thank Dr. J-Y Maillaird and colleagues, Cardiff University for kindly allowing the use of their VHP gassing equipment. I am also grateful to Elizabeth Torr for her assistance with flow cytometry. Acknowledgement also goes to the EPSRC and Shield Medicare for sponsoring my PhD.

I would also like to thank the following for their support and for making the duration of this study a stimulating and pleasant experience, Jessica Rollason, Laura Wheeldon, Tarja Karpanen, Lauren Green, Gemma Slinn and Rachel Willetts.

I wish to thank my family for all their love, understanding, care and support. I would particularly like to mention my nan and my uncles whose memories I will cherish forever. I am especially grateful to Savita, Arun and Akaash for their love and support, as well as their humour. In addition I would like to thank my cousin Parminder for all her love and advice. A special mention should also go to Hazel and Angel, who gave me much joy throughout my PhD years. Most importantly, I wish to thank my parents, Charanjit Mehmi and Kashmiro Kaur Mehmi, who have always supported and encouraged me to do my best, and without whom I would not be where I am today.

LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AEC	Airway epithelial cell
AECGM	Airway epithelial cell growth medium
AGFK	Asparagine, glucose, fructose and potassium germinant
ALI	Air-liquid interface
ANOVA	One-way analysis of variance
AOAC	Association of Analytic Chemists
AP-1	Activating protein-1
ASL	Airway surface liquid
ATCC	American Type Culture Collection
AU	Arbitrary units
BCA	Bicinchoninic acid
BEAS-2B	Bronchial epithelial cells
BSA	Bovine serum albumin
BSEN	British Standard
CFU	Colony forming unit
CO_2	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
DEP	Diesel exhaust particles
DNA	Deoxyribonucleic acid
DNP	Dinitrophenyl
DNPH	Dinitrophenyl hydrazine
DTNB	5,5'-Dithio-bis(2-nitrobenzoic acid)
EDTA	Ethylenediamine-tetracetic acid

ELISA	Enzyme-linked immunosorbent assay
EV	Electronic volume
EVOM	Epithelial voltohmmeter
FCS/FBS	Foetal calf serum / Foetal bovine serum
FU	Fluorescence units
H_2O_2	Hydrogen peroxide
HBEC	Human bronchial epithelial cell
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HNEpC	Human nasal epithelial cells (primary)
IL	Interleukin
IMS	Industrial methylated spirit
IPA	Isopropyl alcohol
M1/M2	Morphology 1 / Morphology 2
Mw	Molecular weight
NaOH	Sodium hydroxide
NCTC	National Collection of Type Culture
Nf-κB	Nuclear factor-κB
nmoles	nanomoles
NTB	2-nitro-5-thiobenzoic acid
$OH^{\bullet}$	Hydroxyl radical
PBS	Phosphate buffered saline
pg	picograms
PI	Propidium iodide

PPM	Parts per million
Quats	Quaternary ammonium compounds
REACH	Registration, Evaluation and Authorisation of Chemicals
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SASPs	Small acid soluble proteins
SD	Standard deviation
SDW	Sterile distilled water
SEM	Standard error of the mean
SS	Side scatter
TER	Trans-epithelial electrical resistance
TNFα	Tumour necrosis factor alpha
TSA/TSB	Tryptone soya agar / Tryptone soya broth
UV	Ultra violet
VHP	Vaporised hydrogen peroxide
WFI	Water for injection

CHAPTER 1 INTRODUCTION

1.1 The importance of disinfection in aseptic transfer processes

The pharmacy cleanroom environment is constantly being challenged by microorganisms of human and environmental origin. Aseptic processes are vital in the hospital pharmacy environment, where sterile products are compounded to meet individual patients' specific needs. Aseptic dispensing is performed in a Grade A environment, such as a laminar flow cabinet or an isolator. Some pharmaceutical products undergo terminal sterilisation by heat or irradiation. However the majority of products are pharmaceutically active parenteral products such as Total Parenteral Nutrition (TPN) fluids, intravenous (IV) cytotoxic drugs, and sterile topical pharmaceutical preparations which, due to their composition, are heat-labile and so cannot be terminally sterilised. Ineffective transfer techniques can compromise the sterility of formulations produced under these conditions and can ultimately lead to nosocomial infection (Baird and Shooter, 1976).

1.2 Sources of microbial contamination in aseptic transfer processes

Within aseptic processing areas, microbial contamination can occur from a number of sources including; raw materials, water, personnel, packaging equipment, improper cleaning of air-lock items, or improper cleaning of the equipment and cleanroom surfaces. One of the major sources of contamination during hospital aseptic processing is surface bioburden, as demonstrated by studies investigating this bioburden on items passed into pharmaceutical isolators (Cockcroft *et al.*, 2001). There is very little data on the different *Bacillus* species and their orders of magnitude arising as contaminants of aseptic transfer items. Cockcroft *et al.* 2001 established that more than half of items ready for transfer into aseptic rooms were contaminated with *Bacillus* spores, with up to 60 *Bacillus* colonies being identified per item. These items included syringe packaging, glass vials and infusion bags. In a recent study carried out by Stubbs (2005), it was established that there was a high surface bioburden on a range of items (60% of items) that may be passed into pharmaceutical isolators. The inability to terminally sterilise such components is overcome by employing chemical biocides in the aseptic process.

Disinfection is a process used to *reduce* the number of microorganisms to acceptable levels (Ayliffe *et al.*, 1993) and is employed at transfer stages of aseptic processing. This is so that any potential contamination risks associated with transfer within the aseptic processing area are minimised. Consequently, all supplies, materials and products need to be disinfected before being passed into an aseptic production area via pass-throughs or air-locks.

1.3 Ideal biocide

There are many factors to consider when looking for an ideal biocide. Ideally it should possess the following attributes: sterile, broad spectrum of activity, bactericidal, sporicidal, non-toxic, non-corrosive, fast kill rates, leaves no residue on the product or item disinfected, easy to use, suitable for use on all types of hard surfaces in cleanrooms and safe to use. No biocide with all these properties is currently available.

1.4 Biocides currently in-use

A number of different chemical disinfectants are currently available and employed in hospital aseptic processing. Disinfectants widely used for cleaning hard-surfaces in hospital pharmacy and industrial cleanrooms are generally alcohol-based agents (Cockcroft *et al.*, 2001). Current practice in the majority of NHS hospitals is the use of 70% Industrial Methylated spirit (IMS) as sprays and wipes (Hiom, 2000; Hiom *et al.*, 2004; Beaney, 2006; Curnyn, 2005). These disinfectants have been found to rapidly kill vegetative organisms and exhibit broad-spectrum antimicrobial activity (McDonnell and Russell, 1999; Russell, 1990; Russell, 1995).

1.5 Bacterial spores

Spore-forming bacteria are a group of microorganisms that are capable of withstanding and adapting to environments that are unfavourable for continued growth as vegetative bacteria. Sporulation occurs inside a vegetative cell of some Gram positive bacteria as a response to an environmental stress. Stresses include nutrient starvation, lack of water, unfavourable pH changes and changes in temperature such as heat shock. By producing

endospores the bacteria overcome conditions of stress and spores are the most resistant life forms known in bacteria (Russell, 1995; Russell, 1999).

Table. 1.1 Properties of vegetative cells and endospores (adapted from Todar, 2008).

Property	Vegetative cells	Endospores
Surface coats	Typical Gram-positive peptidoglycan cell wall polymer; crystalline S-layer	Thick spore coat, cortex, and unique peptidoglycan core wall; no S-layer
Microscopic appearance	Non refractile	Refractile
Calcium dipicolinic acid	Absent	Present in core
Cytoplasmic water activity	High	Very low
Enzymatic activity	Present	Absent
Macromolecular synthesis	Present	Absent
Heat resistance	Low	High
Radiation resistance	Low	High
Resistance to chemicals and acids	Low	High
Sensitivity to lysozyme	Some sensitive; some resistant	Resistant
Sensitivity to dyes and staining	Sensitive	Some resistant

Some biocides target the same structures in both bacterial spores and cells, whereas other biocides are able to target structures specific to spores (McDonnell and Russell, 1999). Biocides can interact with three components of vegetative bacterial cells: the cell wall, cytoplasmic membrane and cytoplasm (Russell, 1990). Many factors determine the access and penetration of disinfectants to these regions particularly cell morphology and cellular chemical composition (Russell, 1990). In bacterial spores, biocides are able to act upon the spore coats, inner and outer membranes, protoplast, germ cell wall and cortex (Russell, 1990).

1.5.1 Sporulation

Sporulation is a complex process that occurs in seven stages (Russell, 1990):

Stage 0 – vegetative cell

Stage I – presporulation phase, in which DNA is present as an axial filament

Stage II – septation phase in which asymmetric cell formation occurs

Stage III – engulfment of the forespore occurs

Stage IV – cortex formation between the inner and outer forespore membranes

Stage V – synthesis of spore coats, dipicolinic acid, and uptake of Ca^{2+}

Stage VI – spore maturation, coat material becomes denser and refractility increases

Stage VII – lysis of the mother cell and liberation of the mature spore

1.5.2 Spore structure

The structure of a spore differs greatly to the structure of a vegetative cell of the same species. The structure of a bacterial spore is composed of (from the inner side to the outer side) the core (protoplast), plasma membrane, germ cell wall, cortex, inner and outer spore coat, and exosporium (only present in some species) (Lambert, 2004; Setlow, 2006). Figure 1.1 shows the structure of a typical bacterial endospore (Setlow, 2006). Spores are thus composed of several different layers and exhibit a higher resistance than their corresponding vegetative cells. Even though the state of low hydration (reduced water content in the core) plays a role in resistance, the difference in susceptibility to different biocides between vegetative organisms and spores is, to a great extent, due to fundamental differences in their structure. Of the layers that make up the spore, the inner and outer spore coats and the cortex appear to confer resistance to chemical, as well as physical, stresses.

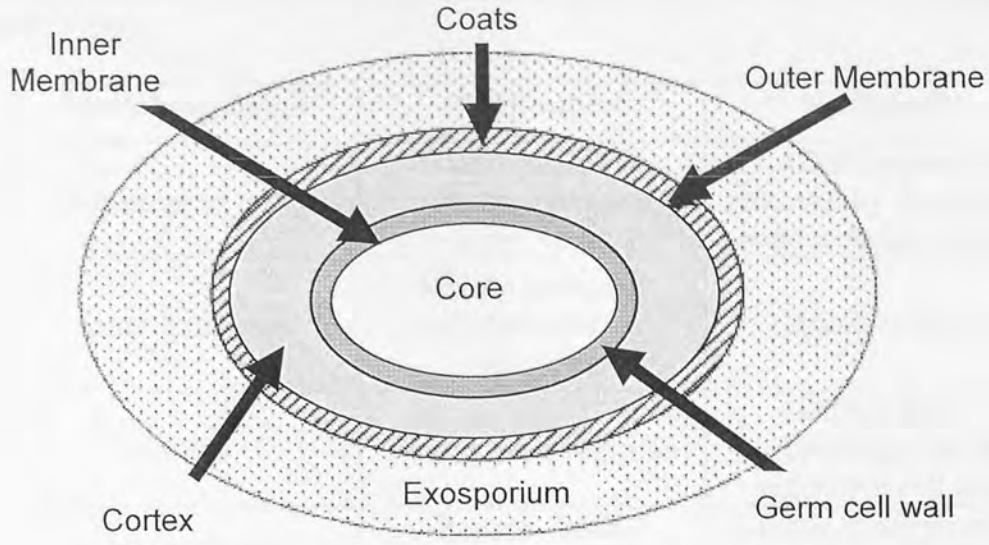


Figure 1.1 Schematical diagram of a bacterial spore to indicate the spatial arrangements of the different layers (adapted from Setlow, 2006).

The spore coat is comprised primarily of protein with smaller amounts of complex carbohydrates and lipids. The spore coat constitutes a large part of the spore and plays an important role in disinfectant resistance by restricting penetration to the core (Lambert, 2004). These spore coats contain alkali-soluble (inner spore coat) and alkali-resistant (outer spore coats) proteins which decrease permeability to hydrophilic agents (Russell, 1990). As well as providing mechanical protection for the spore, the cortex, consisting largely of peptidoglycan, also plays a role in maintaining the high level of dehydration of the spore protoplast (Setlow, 2003). The protoplast cell wall is a dense inner layer of the cortex that develops into the cell wall of the emergent cell when the cortex is degraded during germination (Setlow, 2003). The protoplast is the locality of RNA, DNA, dipicolinic acid (DPA) and most of the calcium, potassium, manganese and phosphorus in the spore (Russell 1995). Also within the protoplast are Small Acid Soluble Proteins (SASPs) which are low molecular weight basic proteins that protect the spores' DNA against damage and also help to repair damaged DNA during spore germination (Lambert, 2004; Setlow, 2006; Loshon *et al.,* 2001). An overview of the chemical composition of bacterial spores is given in table 1.2.

Table 1.2 Chemical composition of selected bacterial spore components (adapted from Russell, 1990)

Spore component	Composition	Comment
Outer spore coat	Mainly protein & carbohydrates and some lipids	Alkali resistant; removed by disulphide bond reducing agents
Inner spore coat	Mainly protein & carbohydrates and some lipids	Alkali soluble
Cortex	Mainly peptidoglycan	Differs from peptidoglycan of vegetative cell wall
Core (germ cell, protoplast)	Protein, DNA, RNA, calcium, dipicolinic acid (DPA)	Unique spore proteins associated with DNA (SASPs)

1.5.3 Germination of spores

Even though spores are metabolically dormant entities, they continuously monitor their environment through sensor proteins (Stephenson and Hoch, 2002), so that when conditions are favourable again the spore can germinate and convert back into a metabolically active cell.

Spores germinate in response to the presence of certain nutrients. These nutrients are known as germinants and are generally single amino acids, sugars or purine nucleosides (Setlow, 2003). Other non-nutrient germinants include lysozyme, salts, Ca^{2+}-DPA, cationic surfactants such as dodecylamine and also increases in pressure. These germinants can be species specific.

The process of germination occurs in 2 stages split into 5 steps (Setlow, 2003), with steps one to three comprising Stage 1 and steps four and five comprising Stage 2. Firstly (step 1), in response to a germinant the spore releases hydrogen ions, zinc and other monovalent cations from the spore core. This is followed by (step 2) the release of DPA and its associated cations such as calcium from the spore core. The DPA is then replaced by water via osmosis (step 3), thereby increasing core hydration which in turn decreases the wet-heat resistance of the spore. After core hydration, hydrolysis of the spore's peptidoglycan spore cortex (step 4) occurs, which is followed by the

swelling of the spore core (step 5) due to further water uptake. This results in the subsequent expansion of the core cell wall. After completion of stage 2, spore metabolism commences as enzyme action is initiated, which allows macromolecular synthesis to occur. When this happens, the germinated spore switches into a growing cell. Once a spore is triggered by a germinant, it will continue to germinate even when the germinant is removed (Setlow, 2003).

1.6 Spore resistance to chemical biocides

Spores produced by environmental bacteria are a major concern in hospital pharmacy aseptic areas (Cockcroft *et al.*, 2001; Stubbs, 2005; Hiom, 2000; Richard, 1988; Medina *et al.,* 1989; Boom *et al.,* 1991; Froud, 2005; Bounoure, 2006). The widespread use of biocides has lead to spores becoming increasingly resistant to the biocides currently available (McDonnell and Russell, 1999; Russell, 1995; Russell, 1999; Lambert, 2004). Although alcohols kill a broad range of vegetative organisms, they have no activity against bacterial spores (McDonnell and Russell, 1999; Russell, 1990; Russell 1995). Bacterial spores exhibit a higher resistance to both physical and chemical stresses than their respective vegetative cells. Despite their lack of sporicidal activity, alcohols are still used for hard-surface disinfection. Therefore the biocides currently being used in the aseptic transfer process are not fit for purpose since, although they are bactericidal, they do not possess sporicidal activity.

1.6.1 *Bacillus* as a test organism

Evaluating the effectiveness of different chemical disinfectants and disinfection methods against spores requires a good biological test organism, which accurately represents the bioburden of components. For instance, the study by Stubbs (2005) showed that 40% of the components ready for transfer into isolators were contaminated with *Bacillus* spores. *Bacillus* sp. are one of the most important genera of bacteria producing spores (Todar, 2008) and have been found to be part of the microbial bioburden in hospital environments (Cockcroft *et al.,* 2001). *Bacillus* spores are often used as test organisms when evaluating disinfection methods, because many of the microorganisms found to contaminate pharmaceutical preparations are, like *Bacilli,*

mesophilic, spore forming microorganisms representative of hospital microbial flora (Baird, 1981). Spores also present a more stern challenge to the disinfection process than vegetative bacteria.

1.6.2 Properties of *Bacillus* spores

Species in the genus *Bacillus* can be thermophilic, psychrophilic, acidophilic, alkaliphilic, halotolerant, or halophilic and are capable of growing at unfavourable pHs, temperatures, and salt concentrations where many others could not survive (Todar, 2008). Spores of *Bacillus subtilis* are found in soil and decomposing plant material. However they are found ubiquitously in the environment primarily due to aerobic transfer. *B. subtilis* is a rod-shaped (length of 1.5 – 1.8 μm and a diameter of 0.8 μm) (Deng *et al.*, 2005), Gram-positive, sporulating, aerobic or facultative anaerobic organism. Each bacterium creates only one spore which is resistant to heat, cold, radiation, desiccation, and biocides (McDonnell and Russell, 1999; Russell, 1990; Tennen *et al.*, 2000; Loshon *et al.*, 2001; Genest *et al.*, 2002; Cortezzo *et al.*, 2004; Setlow *et al.*, 2002; Young and Setlow, 2003). Due to their high level of resistance to biocides, *B. subtilis* spores are widely employed as indicators of effective chemical sterilization.

1.6.3 Pathogenicity of *Bacillus* sp.

Bacilli species can be non-pathogenic or pathogenic. Pathogenic *Bacilli* can cause a variety of infections ranging from ear infections to meningitis, and urinary tract infections to septicaemia (Richard, 1988). However, they occur mostly as secondary infections in immunodeficient or immunocompromised individuals. Therefore disinfection is essential, as spore contamination of pharmaceutically produced, non-terminally sterilised products could potentially lead to patient fatality as well as loss of integrity of the product itself. Infection with spores may also be responsible for the exacerbation of previous infections due to the production of tissue-damaging toxins or metabolites that interfere with treatment (Medina *et al.*, 1989).

1.7 Sporicidal biocides

Only a small number of biocides are active against both vegetative organisms and spores. These include glutaraldehyde, peroxygens and halogens (Russell, 1990; McDonnell and Russell, 1999; Russell, 1995; Russell, 1999; Tennen *et al.*, 2000). However, these agents are damaging to both humans and equipment. As there is currently no biocide that possesses all of the requirements of an effective sporicidal biocide, the obtainment of an effective kill rate becomes the necessary criterion. As a result, the search for an effective, non-hazardous sporicidal agent is of vital importance.

1.8 Biocides and their mechanisms of inactivation against microorganisms

Effective disinfection requires biocide uptake by bacteria, the subsequent transport of the absorbed biocides to the target sites of bacteria, and accumulation of disinfectants to damaging levels (Denyer and Stewart, 1998).

According to Denyer and Stewart (1998), disinfection action can cause damage at different levels by:

(1) disruption of the transmembrane proton motive force leading to an uncoupling of oxidative phosphorylation and inhibition of active transport across the membrane;
(2) inhibition of respiration or catabolic/anabolic reactions;
(3) disruption of replication;
(4) loss of membrane integrity resulting in leakage of essential intracellular constituents such as potassium cation, inorganic phosphate, pentoses, nucleotides and nucleosides, and proteins;
(5) lysis;
(6) coagulation of intracellular material.

The target sites of sporicidal biocides are believed to be within the spores (Russell, 1995) and include the inner and outer spore coats, the cortex, spore membranes and the

protoplast (McDonnell and Russell, 1999; Tennen *et al.*, 2000). Biocides can target a number of sites within spores, not necessarily just one site.

On the whole, in order for a biocide to reach its target which is usually the protoplast, the outer spore layers must be crossed. The surrounding protective layers protect the germ cell from any damage from chemical or physical stresses, with both the spore coat and cortex together ensuring that a robust physical barrier exists against and prevents the influx of any disinfectants (McDonnell and Russell, 1999; Young and Setlow, 2003; Cortezzo *et al.*, 2004). The low permeability of the inner membrane and the low water content within the spore core are also factors in the resistance of spores against chemicals (Genest *et al.*, 2002). Recently, the presence of SASPs in the spore core have also been found to play a role in the disinfectant resistance as they protect the spore DNA from damage that can be caused by some biocides (Setlow and Setlow, 1993; Loshon *et al.*, 1999; Setlow, 2006).

1.9 Mechanisms of biocide action

1.9.1 Alcohols

Ethanol is rapidly bactericidal at a concentration of 70% but is not sporicidal at any concentration. Isopropyl alcohol (IPA) is similar to ethanol, although IPA is considered to be slightly more efficacious against bacteria than ethanol (McDonnell and Russell, 1999). Alcohols exert their effects on bacterial cells by causing membrane damage and the rapid denaturation of proteins, resulting in interference with metabolism, and consequently cell lysis (Setlow *et al.,* 2002; McDonnell and Russell, 1999; Ali *et al.,* 2001). Due to their lack of sporicidal activity, use of alcohols for disinfection is not ideal, although alcohols are still used for general, as well as hard-surface disinfection.

1.9.2 Amphoteric agents

These are compounds composed of mixed anion-cation character. Amphoteric compounds are not sporicidal (McDonnell and Russell, 1999). They combine the detergent properties of anionic compounds with the bactericidal properties of the cationic lipopolysaccharide. They exert their effects mainly by hydrophobic interactions particularly against membrane lipid bilayers (Lambert, 2004; McDonnell and Russell, 1999).

1.9.3 Quaternary ammonium compounds (Quats)

Quaternary ammonium compounds (Quats) are chemically-active agents (cationic surfactants) that possess microbial properties and are used for hard-surface disinfection. Quats are commonly used for general sanitation and in the food industry (Frank and Chimielewski, 1997). They are known to be bactericidal at low concentrations, especially against Gram-positive organisms (Ioannou *et al.,* 2007) and are sporistatic but are not sporicidal (Russell *et al.,* 1995).

It is thought that Quats induce lysis of protoplasts by causing generalised membrane damage. Quats act by denaturing proteins and disrupting membranes via ionic

interactions. For example, positively charged Quats interact with negatively charged biomolecules i.e. phospholipids within the cytoplasmic membrane, causing membrane distortion and consequently bringing about protoplast lysis (McDonnell and Russell, 1999). They are also thought to work by inhibiting the outgrowth stage of spore development (McDonnell and Russell, 1999).

However, membrane damage is unlikely to occur if the spore cortex is present. This is because Quats are thought to combine to spore coats, but are unable to actually penetrate into the spore (Lambert, 2004). Therefore, spores are resistant to Quats.

1.9.4 Biguanides

Biguanides are used as general sterilising agents in the food industry and some are also used for the disinfection of swimming pools. Biguanides are bacteriostatic and bacteriocidal but are sporistatic rather than sporicidal (McDonnell and Russell, 1999). For example, chlorhexidine increases spore hydrophobicity (Shaker *et al.,* 1986) but is not sporicidal.

Biguanides are thought to damage the outer cell layers of spores but not sufficiently to cause their death. They are membrane-active and therefore compromise the integrity of membranes within the spore structure (McDonnell and Russell, 1999). Once through the outer cell layer, the biguanide crosses the cell wall and subsequently attacks the cytoplasmic or inner membrane. However, the cortex of the spore is responsible for the resistance of spores to biguanides such as chlorhexidine (McDonnell and Russell, 1999). Even at high concentrations of biguanide, at ambient temperatures *B. subtilis* spore viability is not compromised (Shaker *et al.,* 1988).

1.9.5 Chlorine dioxide

Chlorine compounds are used as sanitising agents in the food industry as well as in the disinfection of water. Chlorine compounds are both bactericidal and sporicidal. However, vegetative cells are more susceptible than bacterial spores.

Chlorine compounds remove spore coat protein, allowing accessibility to the cortex. They effectively separate the spore coat from the cortex, which is subsequently followed by lysis (McDonnell and Russell, 1999; Young and Setlow, 2003). It is the cortex which may be the key site of chlorine action, given that the removal of spore coats does not affect spore viability in the absence of chlorine (McDonnell and Russell, 1999; Tennen *et al.*, 2000; Setlow, 2006).

1.9.6 Hydrogen peroxide

Hydrogen peroxide (H_2O_2) in solutions of 35, 50 or 90% strengths is available for industrial use; however, 3-6% solutions are used as a general disinfectant. H_2O_2 is a peroxygen which is both bactericidal and sporicidal. H_2O_2 demonstrates slow sporicidal action which is increased by an increase in temperature. Concentration is also very important in terms of H_2O_2 activity. At a concentration of 6% H_2O_2 is bactericidal but exerts sporicidal activity very slowly (over hours) (Russell, 1990).

The production of hydroxyl radicals ($OH^{\bullet}$) from H_2O_2 is necessary for its sporicidal action (Melly *et al.*, 2002). The generation of hydroxyl radicals occurs on reaction of the H_2O_2 with calcium bound to the cortex peptidoglycan (Ando and Tsuzuki, 1986). The free radicals in turn damage proteins and DNA within the spore in an irreversible manner causing lysis (King and Gould, 1969). However, the presence of SASPs protects the spore from DNA damage. Therefore H_2O_2 is not thought to kill spores by targeting spore DNA (Setlow and Setlow, 1993). Enzymes present in the protoplast of the spore are also thought to be possible targets for H_2O_2 (Setlow and Setlow, 1993). H_2O_2 produces oxidizing effects upon proteins (Cortezzo *et al.*, 2004; Cortezzo and Setlow, 2005) especially upon thiol groups of cysteine residues (Lambert, 2004). It is thus able to cause the complete dissolution of spores by removing the spore coats, the cortex and ultimately degrading the core (King and Gould, 1969). It has also been found that H_2O_2 treatment results in spores which cannot swell properly during spore germination (Melly *et al.*, 2002) thereby preventing spore outgrowth (Setlow and Setlow, 1993).

1.10 Biocide application methods

A number of different chemical disinfection methods are employed in aseptic disinfection of pharmaceutical components. The main disinfection methods used are spraying with a biocide, wiping with a biocide impregnated wipe and combinations of either spraying followed by wiping, or wiping followed by spraying (Cockcroft *et al.*, 2001; Curnyn, 2005). One of the most commonly used hospital aseptic disinfection transfer systems consists of a combination of spraying with sterile 70% alcohol and wiping with sterile biocide impregnated wipes, e.g. IPA wipes (Curnyn, 2005; Hiom, 2000; Hiom *et al.*, 2004).

1.10.1 Methods used in disinfection processes

Spraying hard surfaces is one of the most commonly used disinfection methods which enables direct contact between the biocide and the surface to be disinfected. It is this direct contact that is thought to be responsible for the effective killing of the microorganisms. Wiping hard surfaces is also a method which is frequently used in hospital pharmacy cleanrooms. The wipe can exert its effect in two different manners (Cockcroft *et al.*, 2001; Curnyn, 2005). The first is the physical removal of microorganisms from the hard surface by the wipe's fibres. The second is the actual killing effect the biocide has on the microorganisms.

Contact time between biocides and surfaces to be disinfected is also an important factor involved in the disinfection process. Although the Association of Official Analytical Chemists (AOAC) recommends a 60 minute contact time with biocide to achieve a 3 log reduction in spore numbers (Lambert, 2004), often only a 2 minute contact time is employed. A 2 minute contact time is used because it represents the short period of time that components in the aseptic transfer disinfection process are likely to come into contact with a biocide (Hiom, 2000).

1.10.2 H_2O_2 gassing technology

Although the use of H_2O_2 in the liquid phase and also as a semi-solid (foam) is prevalent in numerous sterilisation products, vaporised H_2O_2 (VHP) decontamination

systems are becoming increasingly popular. Such systems are becoming the Gold standard and replacing the conventional hard-surface disinfection methods mentioned previously.

VHP decontamination is the process of disinfection via the generation of H_2O_2 vapour, which ultimately results in the obtainment of a sterile and residue free environment (Rogers *et al.*, 2005). VHP decontamination systems were first developed in the 1990's and have been employed as replacements for other disinfection methods such as steam, ethylene oxide, formaldehyde and dry heat. Of these, all were previously preferred as methods of sterilising biomedical devices, but are now less favourable fumigation techniques. This is because formaldehyde and ethylene oxide have been established as being carcinogenic and/or mutagenic (Dusseau, 2004; Cheney and Collins, 1995; McDonnell and Russell, 1999; Klapes and Velsey, 1990). In addition, steam and dry heat sterilisation are too time-consuming and therefore impractical in hospital pharmacy cleanroom sterilisation. Moreover, temperatures used in steam (121-134°C) and dry heat (160-180°C) sterilisation are too high for the end-processing of heat-labile products such as the parenterals. VHP is increasingly used in the pharmaceutical, healthcare and biotechnology sectors. It is currently used to decontaminate cleanrooms, medical devices, surgical instruments and surfaces, manufacturing equipment and packaging materials. VHP technology has also been employed in the food industry (Carlson, 1996) and has even been used as a method to sterilise chicken egg shells for use in vaccine production (Gruhn *et al.*, 1995).

There are over 700 different VHP systems in use worldwide (Steris®, 2008). All VHP generators operate in a similar way, essentially forming a closed loop with the area to be disinfected. The VHP is generated from a concentrated solution (30-35% H_2O_2), which is vaporised and introduced into a re-circulating airstream at a sufficient rate to achieve and maintain the required concentration. Once decontamination has occurred, the residual H_2O_2 vapour is catalytically decomposed into water and oxygen by recirculation through a destroyer. The process can be divided into 4 main phases:

(1) dehumidification – decreases the relative humidity of the area to be disinfected to less than 30%;

(2) conditioning – increases H_2O_2 concentration to the required concentration;

(3) decontamination – maintains the H_2O_2 concentration at the required concentration to allow decontamination to occur;

(4) aeration – removes H_2O_2 vapour from the decontamination chamber by catalytic conversion to the non-toxic products-water and oxygen (Steris®, 2008).

1.11 Validation of disinfection testing

Evaluation of chemical disinfectants usually involves the addition of a pre-determined concentration of microbial cells to a test solution of disinfectant. Following a designated contact time the solution is tested to quantify microbial viability (Lambert, 2004). There are 2 general categories of disinfectant tests: suspension tests and surface tests. Suspension tests involve the addition of a known concentration of microorganism to the test biocide in solution. Following a specified contact time, the disinfectant is neutralised (Sutton *et al.*, 2002) and the numbers of survivors are enumerated via either the culture method or by membrane filtration. Although suspension testing can be used to determine a disinfectant's efficacy, it is not entirely representative of the aseptic transfer process, where disinfectants are applied as sprays or wipes. Therefore, suspension testing is often only useful as a primary test to determine whether the biocide possesses any biocidal activity. The method usually associated with the aseptic transfer process is hard-surface testing which more accurately represents the in-use conditions of biocide application. Surface testing involves drying the organisms onto carriers which not only results in a decrease in the number of test bacteria, but also in the viability of the cell. Any biocide activity observed will be influenced by many factors, such as the drying time, the temperature and relative humidity of the air (Russell, 2004).

One simple method used to evaluate the efficacy of disinfectants against spores is the in-use dilution test. However this only establishes whether all spores inoculated onto a test surface are killed following exposure to a decontaminant. The problem with these results is that they can only be interpreted as being positive for the presence or negative for the absence of spores. An example of one carrier test is the Association of Analytical Chemists (AOAC) use-dilution test, whereby a carrier is artificially

contaminated with bacteria and dried. The carrier is usually immersed in the disinfectant at the test dilution (usually the in-use concentration) for a specified contact time and then checked for survivors on recovery following neutralisation (Ayliffe *et al.*, 1993; Cockcroft *et al.*, 2001; Lambert, 2004; Springthorpe and Sattar, 2005). A 5 minute contact time with biocides is recommended for vegetative bacteria and a 15 minute contact time is advised for sporicidal activity. However, these contact times are inappropriate for pharmacy cleanrooms in a hospital environment, where the production of medication and medical devices needs to occur continuously and in a short period of time (adapted from Lambert, 2004).

1.11.1 Current published standard methods of biocide efficacy testing

Some of the standards that have been published to determine biocide efficacy include the AOAC, the German Society for Hygiene and Microbiology (DGHM), the French Association of Normalisation (AFNOR) and the British Standards Institution (BSI). Methods currently used in Europe have been developed by the European Committee for Standardisation (CEN) in relation to the testing of chemical disinfectants and antiseptics. These British Standard European Normalisation (BS EN) methods include those listed in table 1.3.

Table 1.3 BS EN methods used to determine biocide efficacy.

BS EN Method	Description	Test for
BS EN 1276:1997 (suspension)	"Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas."	bactericidal activity
BS EN 1650:1997 (suspension)	"Quantitative suspension test for the evaluation of fungicidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas."	fungicidal activity
BS EN 13704:2002 (suspension)	"Quantitative suspension test for the evaluation of sporicidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas."	sporicidal activity
BS EN 13697:2001 (surface)	"Quantitative non-porous surface test for the evaluation of bactericidal and/or fungicidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas."	bactericidal activity

BS EN methods enable the determination of the efficacy of a disinfectant being tested under controlled conditions and give quantitative results in relation to disinfection efficacies. However these tests are usually only established for non-porous surface materials and not for the many porous surfaces that are encountered within the aseptic transfer process. These standards also do not take into consideration the growing concern in relation to resistant spores which are associated with dry conditions within aseptic units.

No standard methodology currently exists for the comparison of disinfectants and disinfection procedures against spores present on hard-surfaces. AOAC and BS EN standards are only available for the activity of biocides applied against spores in suspension (BS EN 13704:2002). Unfortunately this does not apply to hard-surface contamination by spores, which is the actual in-use problem that may occur within a hospital pharmacy cleanroom aseptic area (Stubbs, 2005; Cockcroft *et al.*, 2001). Therefore, although current standards are in place to validate biocides that claim to be

sporicidal, they can only be used to validate biocides when applied in suspension. As a result there is no standardisation in the number of bacterial spore log reductions required of the various biocide testing methods before a product is deemed sporicidal when applied to a hard-surface (adapted from Lambert, 2004).

1.12 The need for toxicity testing of biocides

Pharmaceutical operators who use disinfection agents are regularly and continuously exposed to biocides through inhalation of droplets and aerosols. It is therefore essential that the biocides are tested for any possible toxicity, to allow evaluation of the impact that these chemicals have on human health. By doing so the balance between a biocide having excellent biocidal activity coupled with relatively low toxicity in humans can be fully evaluated.

1.13 Occupational health studies

Studies on the effects of diesel exhaust fumes, cigarette smoke and ozone on the airway epithelium are well documented (Pandya *et al.*, 2002; Devalia *et al.*, 1999; Bayram *et al.*, 1998; Hiltermann *et al.*, 1998; Kawasaki *et al.*, 2001; Bonvallot *et al.*, 2001; Laan *et al.*, 2004; McCrea *et al.*, 1994; Rahman and MacNee, 1999; Sun *et al.*, 1995; Lannan *et al.*, 1994; Devlin *et al.*, 1994; Koren *et al.*, 1989), as are occupational work related asthma studies (Jaakkola and Jaakkola, 2006; Medina-Ramón *et al.*, 2003; Jeebhay and Quirce, 2007; Medina-Ramón *et al.*, 2005; Rosenman *et al.*, 2003). As in the present study, Zock *et al.* (2007) investigated biocide sprays (household sprays) which similarly to pharmacy cleanrooms are used frequently in cleaning and disinfection. The study found that there was an association between the use of cleaning products in spray form and incidence of asthma. Others, Blanc and Toren (1999) and Mannino (2000) have also looked into the risk factors and effects of occupational related asthma with regards to cleaning product usage. Mannino (2000) found that as many as 10-25% of asthma cases can be occupationally related. Whereas Blanc and Toren (1999) found that occupational factors in industrialised countries were implicated in 9-15% of all cases of adult asthma. Another study also found that there was a two-fold increased likelihood of asthma after entry into a US health care profession for those involved with using

general cleaning products (Delclos *et al.*, 2007). Therefore, these studies have established a link between both the use of biocide containing products such as cleaning agents and their possible effects on workers who encounter these biocides on a frequent basis.

Recently, interest into biocide toxicity has increased because of the Registration, Evaluation and Authorisation of Chemicals (REACH) legislation (see section 1.14) and updates to the biocidal products directives (Ahlers *et al.*, 2008; Greim *et al.*, 2006; Anon, 2008; BPD, 2009). However, the risks of biocide exposure are not well researched with only a small amount of data available (Wolkoff *et al.*, 1998; Nickmilder *et al.*, 2007; Bernard 2007; Pauluhn and Mohr, 2000; Kwon *et al.*, 2008; Vincent *et al.*, 2007). The fact that little research has been carried out on the risks of biocide use to human health is alarming as biocides are regularly used by a vast number of people working in the healthcare and pharmaceutical industries. For example, there are almost 1 million people working directly in healthcare in the NHS in England alone (NHS, 2009). As mentioned earlier, data is available on the impact of domestic cleaning products on asthma. Yet these data cannot be extrapolated to biocide exposure in the cleanroom environment, primarily due to the less frequent use of these biocides and their differing composition in the home. In contrast, in the cleanroom the biocides are often used many times during the course of a day. Another factor to consider is that the potential aggravational effect biocides may have on other respiratory disorders and diseases such as chronic bronchitis and chronic obstructive pulmonary disease (COPD) have not been extensively investigated. Consequently, it is essential that risk analysis studies of commonly used cleanroom biocides are carried out.

1.14 Chemical testing legislation

REACH is a European Union legislation that has been in place from 1st June 2007. This requires all chemicals to be toxicity tested and any chemicals that have not been tested cannot be used in new products being formulated. However, the implementation of REACH means that between 8 and 14 million animals will be needed to test approximately 30,000 chemicals (Anon, 2008). It is therefore desirable that alternative methods are established for use in the testing. The use of more physiologically relevant

and ethically acceptable test methods is a step forward in ensuring that any results obtained are applicable to humans.

The National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs UK) is leading the way in promoting, developing and implementing alternative methods of toxicity testing. Several scientifically robust *in vitro* cell culture models are in development for use to mimic human tissues, enabling them to be exposed and respond to chemicals in the same manner as they would *in vivo*. Therefore tissue culture models can be used as a means of testing any toxicity against cells representing a particular region of the human body that may come into contact with the biocides.

As well as being one step towards the reduction, refinement and replacement of animals in testing, airway epithelial cell culture models may provide useful for inhalation toxicology testing. The models could also be used in the near future as a method to register products with the European Biocidal Products Directive 98/8/EC (EU BPD 98/8/EC) (BPD, 2009) by examining the effects of biocide exposure to human cells. The EU BPD 98/8/EC requires biocide products to be risk assessed before they can be placed on the European market. This includes the risk assessment for humans comparing the toxic effects of a substance with a predicted dose (BPD, 2009). This could be tested by producing models of human airways *in vitro*, exposing them to the biocide and then testing for any cell damage or death.

1.15 Toxicity testing methods

Cell culture is one method that involves combining accurate physiological methods and relevant cell types to resemble conditions *in vivo*. Cell culture methods have been used to study drug delivery, metabolism, and transport studies as well as toxicity (Bakand *et al.*, 2005; Eisenbrand *et al.*, 2002; Lin *et al.*, 2007; Forbes *et al.*, 2003; Forbes and Ehrhardt, 2005; Foster *et al.*, 2000; Schmidt *et al.*, 1998; Gabrielson *et al.*, 1994). Previous methods of toxicology testing involved *in vivo* toxicology testing using whole animals (Bakand *et al.*, 2005; Eisenbrand *et al.*, 2002). However, the use of animals in scientific research is no longer popular or routinely advocated. Animal testing is

viewed as unethical, with its extrapolation to humans difficult and highly contested as conditions do not truly mimic those in humans. For example, many studies use mice as models to test for the efficacy of novel asthma drugs and drug combinations (Bakand *et al.*, 2005) even though mice do not suffer from asthma (Mestas and Hughes, 2004; Motta and van Oosterhout, 2006). As an alternative to whole animal models, human *in vitro* models of asthma have been developed. The advantages of these *in vitro* models is their close confirmation to the human condition. For example, goblet cell metaplasia and hypersecretion of mucus occurs in asthmatic airways (Rogers, 2003). *In vitro,* goblet cell hyperplasia is induced by chronic stimulation of air-liquid interface cultured epithelial cells with interleukin-13 (IL-13) (Rogers, 2003).

The *in vitro* cell culture method provides a number of advantages over *in vivo* testing (Bakand *et al.*, 2005; Eisenbrand *et al.*, 2002). Primarily, the data obtained is from human cells with no need for any data extrapolation as testing is carried out under controlled conditions. Toxicity testing using cell culture is a fairly quick and accurate method (Bakand *et al.*, 2005; Eisenbrand *et al.*, 2002). One essential aspect of single cell culture is that it provides the ability to focus on one particular cell type, organ or tissue of significance. However this is also a disadvantage as *in vivo* many cell types exist in synergy. So, cells cultured *in vitro* are not truly representative of all the epithelial cell types that exist *in vivo*. The cell culture system also lacks the complexity of an intact organism due to the absence of immunity, hormones and a nervous system. As well as with animal testing, *in vitro* cell culture is also a method in which it is very hard to tightly control all conditions, with variations likely to exist between individual animals as well as between different cell types. Consequently, alternative *in vitro* toxicology testing models based on human cells allow more physiologically relevant results to be obtained. However, these cell culture models currently being used cannot entirely replace animal testing.

1.16 The human airways

The anatomy of the human airways (figure 1.2) comprises two regions, the upper and lower airways. These regions differ in terms of their structure and function. The upper airways comprise the nasal and oral cavities which warm, humidify and filter inhaled

air. They also provide a passageway for air to enter and leave the body. In addition to this they guide air into the lower airways. The lower airways include the pharynx, and trachea which lead into the lungs, which are comprised of bronchi, bronchioles and alveoli. The trachea divides into two main bronchi at the tracheal bifurcation. The bronchi then lead to the bronchioles which finally lead to the alveoli. The lower airway is involved in respiratory exchange.

1.16.1 Human airway epithelial cell structure

The pseudo-stratified columnar epithelium of the airway is comprised of 3 major cell types. These are basal, ciliated and secretory (mucous, goblet, serous or clara) cells. Ciliated cells make up 50% of the human tracheal epithelium, with the other 50% being basal and secretory cells (Steimer *et al.,* 2005). Basal cells reside near the underlying

basement membranes and are interspersed between the taller ciliated and secretory cells. However, they do not contribute to the apical epithelial surface and are progenitor cells for differentiated ciliated and secretory cells.

Mucociliary clearance is a primary innate defence mechanism that protects airways from the unwanted effects of inhaled allergens, pollutants and pathogens. Both the ciliated and secretory cells along with an airway surface liquid (ASL) layer are involved in the protection of the epithelium as they play key roles in mucociliary clearance. Secretory cells produce the mucus blanket which covers the luminal surface of the epithelial cells (Steimer *et al.*, 2005). Ciliated cells are involved in the mucociliary clearance of inhaled exogenous particles via the nasal-pharyngeal pathway (figure 1.3). This is where, mucus traps inhaled exogenous particles or microorganisms, and the cilia transports the mucus either to the pharynx to be ingested or up to the nose to be expelled. Therefore, using an *in vitro* cell culture method that develops a differentiated cell population comprising all three cell types mentioned would be the most representative of the airway epithelium *in vivo*.

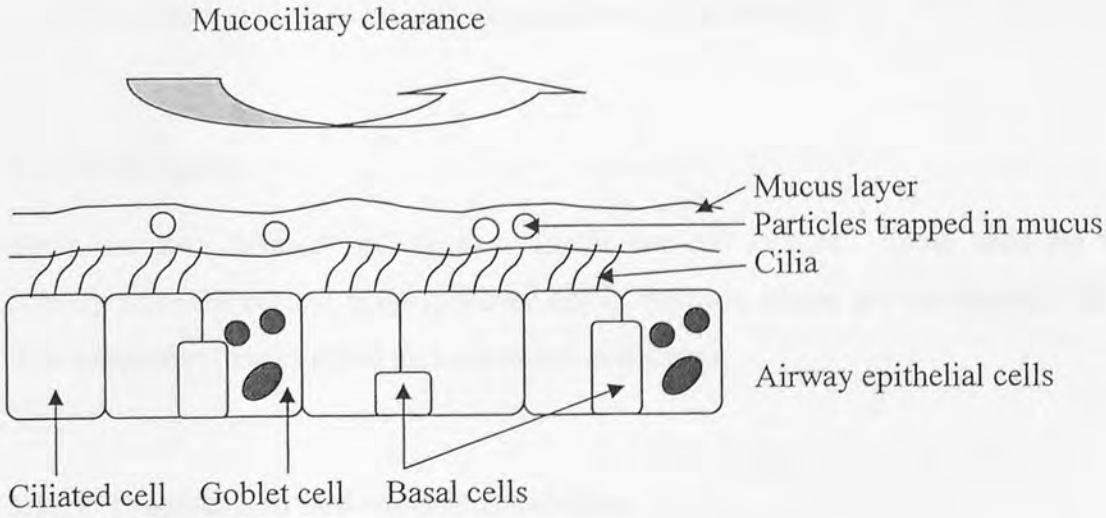


Figure 1.3 Mucociliary clearance in airway epithelial cells.

Although the skin is the largest organ at risk from any toxicity due to biocide exposure, airway epithelial cells are also at great risk of any toxic effects as humans are exposed to 500 millilitres of air with every breath (Silverthorn, 2001). This exchange of air upon breathing may therefore be toxic if humans are exposed to inhaled toxins. The

bronchial and nasal cells are the first point of contact of any potentially toxic inhaled substances. It is important that these cells are used when testing biocides that can be inhaled as they would be heavily exposed to inhalants. Thus, direct exposure to the biocides at concentrations known to be active against bacterial spores may cause adverse effects ranging from irritation to morbidity, due to acute intense or long-term, low-level repeated exposures. Studies carried out using the biocides on human airway epithelial cells are likely to be very informative and indicative of any potential toxicity that the biocides may be responsible for in the human airways.

1.16.2 Human airway epithelial cell culture models

Several cell lines are currently used as *in vitro* models of the respiratory epithelium. The most popular are Calu-3, 16HBE14o$^-$ and more recently, BEAS-2B. These three cell lines are representative of the epithelial cells of the upper airways and are often used for studying drug transport, metabolism and gene delivery (Florea *et al.,* 2003; Foster *et al.*, 2000; Forbes and Ehrhardt, 2005; Forbes, 2000; Noah *et al.,* 1996; Borchard *et al.,* 2002). They are used *in vitro* to predict and estimate the consequences of different factors such as biocide exposure on human airways.

1.17 Cell types

There are also different cell types available for cell culture. Some cells are taken directly from the human body (primary cells), whereas others are manipulated to alter their properties (transformed and cancerous cell-lines).

1.17.1 Transformed and cancerous cell-lines.

BEAS-2B cells are human airway epithelial cells that are derived from normal human bronchial epithelium and have been transformed (for immortality) with an SV40-adenovirus 12 hybrid virus (Reddel *et al.,* 1988). The BEAS-2B cells should therefore possess normal cellular proteins and components, but will be more robust than primary, non-transformed cells. BEAS-2B cells have been extensively used to investigate the effects of inhaled environmental pollutants such as dust, cigarette smoke and diesel

exhausts (Sakagami, 2006; Wilson *et al.*, 2000; Laan *et al.*, 2004; Kawasaki *et al.*, 2001; Takizawa *et al.*, 2000). This is because they are an easy airway epithelial cell line to maintain in culture and study the effects of chemicals on, and can also be passaged numerous times unlike the finite number of passages of normal primary, non-transformed cells (Sakagami, 2006).

However, although cell lines and transformed cells have longevity in culture, there is the possibility that phenotypic differences between the transformed cells and normal cells *in vivo* may exist and consequently the biological responses of the cells observed *in vitro* may differ compared to those *in vivo* (Forbes and Ehrhardt, 2005).

1.17.2 Primary cells

In contrast to immortalized and transformed cell lines, primary airway epithelial cells are comprised of mixed cell types, and as a result provide the closest *in vitro* representation of the airway epithelium (Lin *et al.*, 2007; Sakagami, 2006). Primary cells are not transformed and so should have the same genotype and phenotype as cells *in vivo*. Although they only have a limited number of passages, primary cells are truly representative of the cells *in vivo* and possess the same morphological and biochemical characteristics as cells of the respiratory epithelium. As it is very important that the phenotypic characteristics of the cells remain consistent throughout the experiment, the set numbers of passages need to be defined. However, one major disadvantage of primary cells is that in culture they have a finite replicative lifespan and consequently lack longevity in culture (Wan *et al.*, 2000). In some instances, primary cells can lose their differentiated properties over time (Eisenbrand *et al.*, 2001). Yet, there have been some studies that have used differentiated primary epithelial cells for over a month (Auger *et al.*, 2006). This longevity in culture observed with differentiated epithelial cells allows for the development of a model that has the potential for use in chronic as well as acute toxicity testing.

1.18 Cell culture methods

1.18.1 Submerged cell culture

Epithelial cells grown using submerged cell culture are grown on plastic with the culture medium completely covering them. The medium provides nutrients for the cells, with the time taken for the cell culture models to be produced ready for experimentation being relatively quick. But this method of cell culture is not truly representative of respiratory epithelial cells *in vivo* (Bakand *et al.,* 2005). This is because both the morphology and distribution of differentiation markers in the cells under submerged culture differ from those of the airway epithelium *in vivo* (Auger *et al.,* 2006; Florea *et al.,* 2003). However, because of the relatively short time required to produce a cell culture model, submerged culture is often used for experimental method development.

1.18.2 Culture at Air-Liquid Interface (ALI)

To ensure that the *in vitro* models used are comparable to native lung epithelia human airway epithelial cells may be cultures at an air-liquid interface (ALI). This promotes cellular differentiation to form a physiologically accurate model of human airways. ALI culture is increasingly popular, as it mimics the airway epithelial/nasal epithelial cells as they would exist *in vivo* (Auger *et al.,* 2006). This method of cell culture facilitates the expression of the differentiated characteristics found in native airway epithelium relating to cell morphology and cell functions (Yamaya *et al.,* 1992; Shen *et al.,* 1994; Ke *et al.,* 1988; Forbes and Ehrhardt, 2005). This is because only the apical surface of the cells is exposed to air (figure 1.4) as it would be in the lumen of the airways *in* vivo, whilst the basolateral surface is submerged. The reconstituted pseudostratified columnar epithelium then allows for the biocide to be deposited directly onto the apical cell surfaces, thereby reflecting inhalation exposure of the native lung surface.

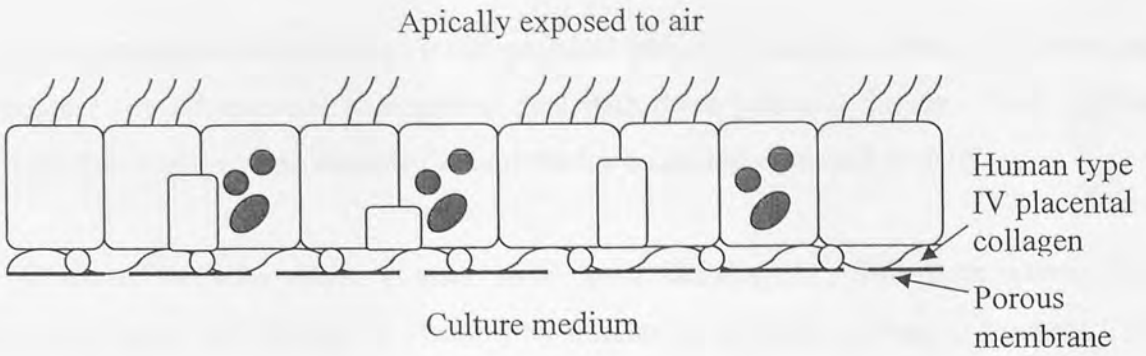


Figure 1.4 The *in vitro* ALI culture method employed to generate differentiated airway epithelial cells.

Culture at the ALI promotes the differentiation of cultures of human airway epithelia (Yamaya *et al.,* 1992; Shen *et al.*, 1994). For example, the differentiated morphology of Calu-3 cells is reduced when the cells are cultured in submerged culture conditions (Forbes and Ehrhardt, 2005). Calu-3 cells form a confluent mixed culture of ciliated and secretory cells when cultured at an ALI (Forbes and Ehrhardt, 2005). So, the growth of cells at an ALI allows direct comparability with native lung epithelia as the cells form a pseudo-stratified layer that exhibits all the characteristics of the respiratory epithelium *in vivo.* Other characteristics representing differentiation include the ability of ALI cultured bronchial and nasal epithelial cells to secrete mucus and generate cilia (Forbes and Ehrhardt 2005; Grainger *et al.,* 2006; Lee *et al.,* 2005; Auger *et al.,* 2006). ALI culture conditions also allow for the expression of proteins that may be expressed *in vivo* at an ALI but not under submerged conditions such as mucins (Bernacki *et al.,* 1999). So, by using an ALI cell culture method, the conditions for the growth of airway epithelial cells are optimised (Gruenert *et al.,* 1995) and allow the reproduction of *in vivo* conditions for contact between chemicals and the epithelial target cells (Bakand *et al.,* 2005; Chen *et al.*, 1993; Knebel *et al.,* 1998; Diabate *et al.,* 2002; Aufderheide *et al.,* 2003; Aufderheide, 2005; Gruenert, 1995).

1.19 Possible toxicity effects and markers of toxicity in epithelial cells

Airway epithelial cells form an initial physical barrier to inhaled airborne pollutants and possess several response strategies to deal with these potential threats. These cellular responses may provide suitable biomarkers for assessing chemical toxicity.

Numerous *in vitro* toxicity tests have been developed. The tests assess both morphological and biological changes by measuring different biological markers. The assays investigate a variety of different biological endpoints including; cell morphology, cell viability, cell metabolism, membrane integrity and release of pro-inflammatory cytokines (Bakand *et al.*, 2005; Eisenbrand *et al.*, 2001).

1.19.1 Morphology

Cellular morphology can be used as a marker of toxicity as any change from healthy cell morphology is indicative of toxicity. Any changes detected in the cell size, shape, cell-cell contacts, as well as nuclear or cytoplasmic vacuolisation are suggestive of toxicity (Bakand *et al.*, 2005). For example, the formation of cell-cell contacts between epithelial cells are important to maintain cell viability as well as a tight, impermeable epithelial barrier. Healthy, viable cells will remain in contact with neighbouring cells, whereas the loss of contact with neighbouring cells is indicative of cell death (Smit-de Vries *et al.*, 2007).

The processes of apoptosis and necrosis are two methods by which cell death occurs. Necrosis occurs when cells are exposed to extremely stressful conditions, such as upon infection, or as a result of a trauma injury. Whereas apoptosis is a controlled process which is often induced in response to an external stimulus, which in this case would be the presence of biocide. Apoptosis involves numerous biochemical events that ultimately result in cell death. Both processes have specific morphological characteristics. Necrosis is first marked by a loss in cell membrane integrity, followed by the swelling of the cytoplasm and mitochondria (Nemeth *et al.*, 2007). This ultimately results in the lysis of the cell and its internal organelles. However, programmed cell death, apoptosis, is first marked by cytoplasmic shrinkage and nuclear condensation. This is followed by the formation of membrane blebs, which eventually

separate from the cell, forming apoptotic bodies (Riss and Moravec, 2004). Careful observations of morphology can therefore distinguish between necrosis and apoptosis. This is important because the process of necrosis can result in increased damage to the cells' surroundings due to the lysis of its cellular contents into the surrounding environment and the initiation of an inflammatory response.

1.19.2 Viability

The toxicity of biocides can be determined by establishing the number of remaining viable cells following exposure. This is often ascertained by cell counting of dead/viable cells. A Trypan blue exclusion assay is one method used to detect viable cells which will not be stained by the reagent (Bakand *et al.,* 2005). However, this method of determining cell viability is time-consuming. One assay often used to determine cell viability is an MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, which is a colorimetric assay that measures the mitochondrial integrity of cells by determining the ability of cells (redox activity) to reduce MTT to MTT formazan (de Oliveira-Marques, 2007). A decrease in cellular MTT reduction could be an index of cell damage (Abe and Matsuki, 2000). Yet, the MTT assay also has some disadvantages. For example, the presence of glutathione-s-transferase (produced by cells to detoxify toxic compounds) can reduce the MTT independent of toxicity. The assay is also less effective in the absence of cell proliferation (Timmins, 2002). Another commercially available viability assay is the CellTiter-Blue™ reagent (Promega, Southampton, UK) which can be used to determine numbers of viable cells following chemical challenge. This endpoint assay provides a homogeneous, fluorometric method to monitor cell viability. Using this reagent cell viability is assessed by the ability of viable cells to metabolise the resazurin reagent into the fluorometrically detectable resorufin product. The CellTiter-Blue™ reagent is non-toxic to cells, easy to use and does not require the use of hazardous solvents (required for MTT-based assays) (Promega, 2008).

1.19.2.1 Distinguishing necrosis and apoptosis

Of the two most common cell death mechanisms, most *in vitro* cytotoxicity tests measure necrosis. Often propidium iodide (PI) staining of cells is used to determine the presence of necrotic cells in a cell population (Hampton and Orrenius, 1997). PI binds to the nuclei of the cells after the membrane becomes permeable. On damage to membranes the PI stain is able to pass into the cell, bind to the DNA and fluoresce. Consequently, increased cell fluorescence upon PI treatment is indicative of necrosis as necrotic cells have damaged cell membranes.

There are also numerous methods by which apoptosis may be detected. As there are many stages involved in the organised apoptotic process a number of factors can be used as markers to evaluate apoptosis. Along with morphological characteristics, molecular changes within the cells can also be investigated as biochemical markers of apoptosis. Assays used to detect apoptosis include the detection of caspase activity. The caspases are a family of cysteine proteases constitutively expressed in most cell types, once activated they cleave protein substrates within the cell (Grutter, 2000) which are associated with apoptosis (Oslund *et al.*, 2004; Nemeth *et al.*, 2007). The cytoplasmic presence of cytochrome c, which is an essential protein involved in mitochondrial respiration is also another marker of apoptosis (Truong-Tran *et al.*, 2001). In addition, phosphatidlyserine (PS) is also often used as a marker for apoptotic cells. PS is usually distributed asymmetrically in the inner and outer sides of the cell membrane, but is more extracellular following apoptosis. Changes in phospholipid asymmetry (and therefore apoptosis) can be detected via Annexin V binding. Annexin V is a phospholipid binding protein with a high affinity for PS. As a cell is dying, the fluorescein-isothiocyanate (FITC) fluorescent tag labelled-Annexin V which would not normally bind the cell membrane is able to bind the exposed PS and will therefore specifically label apoptotic cells with the fluorescent tag (Bucchieri *et al.*, 2002; Bossy-Wetzel and Green, 2000). Changes in fluorescence would then be detected using flow cytometry.

1.19.3 Pro-inflammatory cytokines

Cytokine expression and release by the airway epithelium can be upregulated or down-regulated in response to a variety of stimuli (Takizawa, 1998; Adler *et al.,* 1994; Martin *et al.,* 1997). Although biocide exposure may induce cytokine production, both the level and type of cytokines being produced may differ in response to different biocides. This is important because different cytokines would have different effects on the epithelium. For example, IL-1ß is a pro-inflammatory cytokine, whereas IL-10 is anti-inflammatory. Increased pro-inflammatory cytokine expression and release may result in an influx of inflammatory cells to the epithelium. For example, an increased release of the chemokine interleukin-8 (IL-8) is indicative of an alteration in the inflammatory status of the cells. Increased IL-8 secretion forms a chemotactic gradient which recruits inflammatory cells such as neutrophils and macrophages to the region (Laksminarayanan *et al.,* 1997). Normally, these cells would be required to deal with the invading pathogen that instigated the IL-8 response. However, if the influx is in response to biocides rather than bacteria, the inflammatory cells will release their arsenal of defences, including reactive oxygen species (ROS), into the surrounding host tissue. This type of event is prevalent in rheumatoid arthritis where inflammation occurs in the absence of an infection (Mirshafiey and Mohsenzadegan, 2008). In the case of biocide exposure, this would be an unwanted side effect that may be responsible for acute or chronic inflammation of the airways (Morio *et al.,* 2001; Oosting *et al.,* 1990; Shacter *et al.,* 1994).

Quantitation of cytokine release usually occurs via immunochemical enzyme-linked immunosorbent assays (ELISA) (Lakshminarayanan *et al.*, 1997; DeForge *et al.,* 1993; Nemeth *et al.,* 2007; Pelaia *et al.,* 2004).

1.19.4 Epithelial cell barrier integrity

Cell injury with loss of cell-cell contacts (Boardman *et al.,* 2004) will disrupt the epithelial cell barrier integrity. Transepithelial electrical resistance (TER) and paracellular permeability are used to measure cell barrier function (as described in Chapter 7, section 7.1). A decrease in TER and increase in paracellular permeability of

the cells is indicative of loss of barrier and possibly damage to epithelium (Waters *et al.*, 1997).

1.19.5 Oxidant/antioxidant status

The airways epithelium is constantly exposed to oxidants that are generated internally due to metabolism as well as from inhaling exogenous pollutants in the ambient air. Airway epithelial cells use numerous antioxidant systems that increase antioxidant production in response to such oxidant insults and aid in maintaining the oxidant/antioxidant balance. These antioxidant mechanisms can be split into two general categories; non-enzymatic and enzymatic. Non-enzymatic antioxidants such as vitamin E, vitamin C, ß-carotene, thiols, lactoferrin, albumin and transferrin act as radical scavengers and prevent radical formation (Wright *et al.*, 1994). Whereas enzymatic mechanisms (catalase, superoxide dismutase and glutathione peroxidase) function by transforming reactive oxygen species (ROS) into less potent entities (Rahman *et al.*, 2006).

The oxidant/antioxidant equilibrium is of vital importance in airway epithelial cell defence against biocides that may increase oxidative stress. If the equilibrium is challenged, in this particular case by biocide exposure resulting in oxidant release overwhelming antioxidant levels, damage may occur. Increased oxidant exposure can also occur during tissue injury and inflammation (Shacter *et al.*, 1994). Oxidative stress can lead to the oxidation of cells and their cellular constituents. This can result in the alteration of the cell at the molecular level. These effects are observed as exposure of cells to ROS can result in the modification of proteins, lipids, and DNA (Kazzaz *et al.*, 1996). Oxidation of proteins and lipids is therefore a potentially important effect of oxidant stress and oxidant mediated cell injury. Modification of key structural and functional proteins may be detrimental to the cell, resulting in cell injury and even cell death. As a result oxidised proteins, lipids and DNA damage are often used as endpoints in the study of oxidative stress.

The levels of protein thiols and carbonyls in cells prior to and following chemical insult are often used as markers of protein oxidation. A decrease in reduced protein thiols and

an increase in protein carbonyls are indicative of oxidation (Dalle-Donne *et al.,* 2006; Cockell and Belonge 2002).

Investigating a number of these biological endpoints following biocide exposure to the submerged and ALI cultured human airway epithelial cells *in vitro,* should provide insight into the potentially harmful/damaging effects of biocides on the airways of cleanroom personnel *in vivo.*

1.20 Objectives of study

The overall aims of this study were to determine the efficacy of commonly used cleanroom biocides against bacterial spores on hard-surfaces and to establish whether the biocides are likely to be toxic to cleanroom personnel. The specific aims were therefore:

- To develop a new method to accommodate the hard-surface testing of biocide efficacies against bacterial spores.
- To determine the efficacy of cleanroom biocides against *Bacillus* spores.
- To establish which traditional disinfection method of spraying, wiping or a combination of the two is the most effective in spore removal from hard-surfaces.
- To determine the efficacy of vaporised hydrogen peroxide (VHP) against *Bacillus* spores.
- To determine the toxicity of biocides against submerged cultured human bronchial epithelial cells *in vitro*.
- To determine the toxicity of biocides against submerged cultured human nasal epithelial cells *in vitro*.
- To determine the toxicity of biocides against air-liquid interface cultured human bronchial epithelial cells *in vitro*.
- To determine the toxicity of biocides against air-liquid interface cultured human nasal epithelial cells *in vitro*.

CHAPTER 2 BIOCIDE EFFICACY TEST METHOD DEVELOPMENT

2.1 Introduction

Validation of a disinfection process is important in hospital aseptic dispensing. This is because of difficulty in end-product testing of hospital aseptically prepared products. Current published standard methods include the BS EN and AOAC methods (Lambert, 2004). These methods enable the quantitative determination of the efficacy of a biocide tested under controlled conditions. Any potential biocides must be assessed for their efficacy using a method which reflects their use in practice. This is where BS EN 13697 proves to be the method of choice. Although it does not consider porous surfaces, the method does involve hard surface testing as would be the case in the aseptic transfer process in hospital pharmacy cleanrooms. It also does not specify the use of bacterial spores (resistant to most biocides) to detect the sporicidal activity of a biocide, but this test is used to evaluate bactericidal and fungicidal activity. In addition there is a lack of standardisation in the number of log reductions in viable bacterial spores required to be achieved by various biocide testing methods in order for a product to be deemed sporicidal. This is because a standard hard surface biocide testing method has not been established for bacterial spores, resulting in a lack of reproducibility in biocide efficacies.

For a standard method to be developed, testing procedures must be standardised. These include standardising conditions such as humidity, equipment, methods, solutions, replicate numbers, test temperature, contact time and type of spore forming organisms used (Lambert, 2004; Curnyn, 2005). By employing a standard method which allows the quantification of a chemical disinfection process, the efficacy of a particular biocide against a biological test organism can be established and verified. Thus allowing a particular log reduction in spore levels to be recognised as the standard required to be achieved in order for a biocide to be classified as a sporicidal agent. The efficacies of the biocides and disinfection methods can then be compared with each other, whereas before this would not have been possible.

Since there is no hard-surface test for assessing sporicidal activity of biocides, a combination of BS EN 13697:2001 and AOAC methods were used to determine the efficacy of biocides and biocide application methods against bacterial spores. This new method was developed to represent the working practice of a hospital aseptic dispensing unit. Alterations were made in order to encompass the use of bacterial spores rather than vegetative bacteria. Factors such as the organisms being used as test organisms, biocide contact times, effective neutralisers, incubation times and temperatures were also taken into consideration. In addition the method aims to reflect the high number of components regularly used in the pharmacy aseptic process as well as the composition of the product components and surfaces of cleanrooms and isolators.

This study evaluates the developed method by investigating the different biocides and disinfection methods often incorporated into aseptic transfer processes in hospital pharmacy aseptic units. This was carried out by applying a variety of biocides in various formats and combinations on contaminated hard surfaces, and then establishing any killing or removal of microorganisms from the "disinfected" surface. This enabled the effectiveness of each biocide and transfer disinfection method to be established.

2.2 Aim

The aim of this chapter was to develop current existing methods to accommodate the hard-surface testing of biocide efficacies against bacterial spores.

2.3 Materials and Methods

The method developed involved inoculation of a sterile surface with spores, drying, application of biocide for a limited contact time, recovery of spores from the surface, neutralisation of the biocide to prevent overkill, a germination stage, dilution, plating and enumeration.

A combination of the British Standard (BS EN) 13697:2001 and Association of Analytical Chemists (AOAC) methods were used to determine the effect of different biocide application methods on stainless steel carrier surfaces.

All tests were performed in triplicate, unless otherwise stated.

2.3.1 Materials

2.3.1.1 Test inoculum organisms

Non-pathogenic strains of *Bacillus subtilis subspecies subtilis* 168 spores (standard laboratory strain), *Bacillus subtilis* (ATCC 6633) spores and *Bacillus pumilis* ATCC 27142 spores were obtained from the Culture Collection in the Microbiology Department at Aston University, Birmingham, UK.

2.3.1.2 Biocide sprays and wipes used in study

All the biocides tested in this study (table 2.1) with the exception of the non-fleecing cotton face pads (Boots, Nottingham, UK), were manufactured by Shield Medicare (Surrey, UK) according to Good Manufacturing Practice (GMP). No preparation was required prior to use in the testing procedures, as the biocides were applied at their practical, in-use concentrations.

Table 2.1 Biocides used in this study.

Biocide	Product Components
Premier Klercide™-CR Sterile Biocide A	blend of a quaternary ammonium (Quat.) compound and a biguanide (Quat./biguanide)
Premier Klercide™-CR Sterile Filtered Biocide B	blend of stabilised chlorine dioxide and a Quat. compound (Quat./chlorine dioxide)
Premier Klercide™-CR Sterile Filtered Biocide C	6% v/v hydrogen peroxide
Premier Klercide™ 70/30 Sterile Denatured Ethanol	70% v/v denatured ethanol blended with deionised water
Premier – WFI Klercide™ 70/30 Sterile Denatured Ethanol	70% v/v denatured ethanol blended with water for injection
Klerwipe™ - CR Sterile Biocide A	polyester / cellulose wipe presaturated with a blend of a Quat. (Alkyl Dimethyl Benzyl Ammonium Chloride) and a biguanide (Polyhexamethylene Biguanide Hydrochloride)
Klerwipe™ - CR Sterile Filtered Biocide B	polyester / cellulose wipe presaturated with a stabilized blend of chlorine dioxide and a Quat. compound (Didecyl Dimethyl Ammonium Chloride)
Klerwipe™ - CR Sterile Filtered Biocide C	polyester / cellulose wipe presaturated with 6% v/v hydrogen peroxide
Klerwipe™ - CR Sterile Biocide D	polyester / cellulose wipe presaturated with an amphoteric surfactant (N, N-Bis (3-amino propyl) dodecylamine
Klerwipe™ Sterile 70% Denatured Ethanol	polyester / cellulose wipe presaturated with 70% v/v denatured ethanol blended with deionised water.
Klerwipe™ 70/30 WFI Sterile 70% IPA Polyester Wipe	polyester / cellulose wipe presaturated with 70% v/v Propan-2-ol (IPA) blended with water for injection
100% Non-fleecing Cotton Face Pads	100% pure cotton saturated with sterile distilled water

2.3.1.3 Solutions, reagents and media

Tryptone soya agar (TSA) and tryptone soya broth (TSB) were from Oxoid (Hampshire, UK). Sodium thiosulphate, L-histidine, asparagine and fructose were from Sigma (Dorset, UK). Glucose was from Fisher (Loughborough, UK). Modified Letheen broth was from Difco, Becton Dickinson (Oxford, UK) and saponin was from Fluka BioChemika (Steinheim, Germany). All other materials were obtained from Sigma (Dorset, UK) unless stated.

2.3.2 Methods

All methods were carried out in an aseptic manner.

2.3.2.1 Preparation of *Bacillus* spores

A loop of each *Bacillus* organism previously stored at -80°C was grown aerobically on TSA at 37°C for 48 hours. The resulting bacterial cells were harvested by scraping with an inoculating loop, suspended in 200 ml of TSB and incubated aerobically at 37°C for 12 days, until a high percentage of spores were produced, as detected by phase contrast microscopy using a 100x objective under oil immersion (Axioscope, Zeiss, Germany).

The spore suspensions were then heated in a water bath at 60°C for 10 minutes to kill any remaining vegetative cells. The spore suspensions were centrifuged (Beckman Coulter J-E centrifuge, USA) in sterile 50 ml centrifuge tubes at 10,000 rpm for 30 minutes at 5°C. The resultant supernatants were disposed of and the spore pellets collected. To ensure complete removal of the supernatants, the spore pellets were washed in 0.9% w/v saline, and once again centrifuged at 10,000 rpm for 30 minutes at 5°C. The supernatant was discarded and the pellets were resuspended in 100 ml of sterile distilled water (SDW). The final sporulated suspension was heated in a 60°C water bath for 30 minutes, with the purpose of killing all vegetative cells, and kept at 2-8°C until use. As the purpose of this study was to test the sensitivity of *Bacillus* spores to the different biocides, it was vital that all vegetative cells were removed from the inoculum. To ensure that this was the case, the two heating stages (aforementioned) were used to kill off any heat-resistant vegetative organisms surviving the first heating

stage and to ensure that any spores which may have germinated in the SDW were also removed.

A viable spore count was performed on the spore suspensions before use and spore concentrations in the range of 10^7 -10^8 colony forming units (CFU)/ml were used in the tests.

2.3.2.2 Gram staining of *Bacilli*

A drop of the suspended culture of vegetative organisms was transferred onto a slide with an inoculation loop. The culture was then air-dried and fixed over a gentle flame. The bacterial specimen was first stained with crystal violet for 1 minute before washing with water. It was then treated with an iodine solution for 1 minute before being rinsed with water. A few drops of ethanol were added for a few seconds followed by counterstaining with safranin for 30 seconds. The treated bacterial specimen was then rinsed with water and blotted dry. The slide was viewed under a light microscope and observed at a magnification of objective 100x. Gram-positive bacteria retain the violet stain of crystal violet, whereas gram-negative bacteria do not.

2.3.2.3 Phase contrast microscopy of *Bacillus* spores

To ensure that the majority of vegetative bacteria had undergone sporulation the cells were grown and observed at 2 day intervals using phase contrast microscopy. The cells were placed between a slide and a cover slip. A drop of oil was placed on top of the cover slip and the cells observed using the 40x phase light magnification (Axiozeiss). Spore formation was determined by the presence of phase bright dormant spores in comparison to the presence of phase-dark rods.

2.3.2.4 Total counts of *Bacilli*

Using a haemocytometer, a total count of the vegetative organisms or spores was carried out while counting under phase contrast microscopy.

2.3.2.5 Viability testing of *Bacillus* spores

Viability testing of the spore suspensions in the form of suspension testing comprising serial dilutions was carried out weekly to test the viability of the organisms. The aqueous spore suspension was diluted with TSB in volumes of 100 µl of spore suspension with 0.9 ml of TSB. 100µl of each dilution was plated onto TSA plates, and the plates were then aerobically incubated at 37°C overnight. Following incubation the CFUs were enumerated. An approximate spore concentration of 10^7 -10^8 spores per ml of spore suspension would be required in order to observe log reductions of around 5 - 6 logs. Therefore an initial inoculum size of 10^7-10^8 spores was used to inoculate the stainless steel carrier test surfaces.

2.3.2.6 Biocide neutraliser validation

Validation of the neutraliser was carried out to ensure that it arrested the activity of the biocides being tested and that it was not toxic against any spores that may have survived the disinfection process.

Modified Letheen neutralising broth (4.4 g/100 ml) containing sodium thiosulphate (0.5 g/100 ml), L-histidine monohydrochloride (0.1 g/100 ml) and saponin (3 g/100 ml) was used as a general neutraliser for each of the biocides (Appendix 11.1.1). The neutraliser had to be tested for its toxicity against all three species of the *Bacillus* test organisms, since neutraliser performance is species dependent (Sutton *et al.*, 2002) and interspecies variation may exist. The criteria for acceptable neutralisation evaluations is 75% of the inoculum count (Sutton *et al.*, 2002).

2.3.2.6.1 Neutraliser validation method

1 ml of biocide was added to 9 ml of Letheen neutralising broth and incubated for 10 minutes at room temperature. Biocide plus neutraliser, neutraliser alone and SDW (viability control) were inoculated with the *Bacillus* spore suspensions and incubated for 10 minutes at room temperature. Recovery of the organisms was performed by plating in triplicate 0.1 ml of each sample on TSA plates and incubating aerobically overnight

at 37°C. The plates were examined for recovery of colony forming units (CFUs), and the results analysed by comparing the number of spores recovered from the biocide/neutraliser and neutraliser alone, as a percentage of the spores recovered from the SDW controls (Sutton *et al.*, 2002):

Percentage of viable cells recovered (%) =

$$\frac{\text{CFUs recovered from biocide/neutraliser combinations}}{\text{CFUs recovered from SDW viability control}} \times 100$$

The results were analysed using an ordinary ANOVA to determine any significant differences from control. Data are presented as mean ± SEM. Differences were considered to be statistically significant when $P < 0.05$.

2.3.2.7 Treatment with biocides – Hard surface testing

2.3.2.7.1 Hard surface preparation

Stainless steel plates (8cm x 8cm) were used as carriers in the hard surface tests, to represent the components, such as ampoules, that are regularly used in hospital pharmacy aseptic dispensing. Prior to disinfection the carriers were washed and rinsed with distilled water and detergent applied to remove any soiling. They were then sterilised by autoclaving at 121°C for 15 minutes.

2.3.2.7.2 Spraying method

Stainless steel plates were wrapped in foil and pre-heated to 37°C for 30 minutes. TSA plates were also pre-heated to 37°C for 30 minutes. Following pre-heating, 100 µl of the stock spore suspensions were transferred to the stainless steel plates using a pipette, placing the suspension over the surface as small droplets. The spore suspensions were then spread evenly over the surface using a hockey stick spreader and allowed to dry aerobically, undisturbed for 15 minutes at 37°C. This drying of the spore suspension onto the carrier plates was carried out to represent how spores may be present in the pharmaceutical cleanroom environment, due to contamination of components used in

aseptic compounding as well as contamination of the isolator hoods and laminar air-flow units. By leaving the spore suspension to dry in an isolator the spores are exposed to an unfavourable environment devoid of any nutrients, substrates, water etc. This decreases the probability of any endospores converting back to their vegetative forms. After 15 minutes incubation at 37°C, the carriers were placed horizontally upon Petri dishes at room temperature and sprayed with the test biocide spray, ensuring that each spray was held 10 cm away from the surface and only one spray was expelled per sample. The stainless steel carrier was allowed to dry at room temperature for 2 minutes. A 2 minute contact time was used because of its feasibility in a busy hospital working environment, which would due to the sheer volume of patients not allow a longer contact time. Following the 2 minute exposure to the biocide spray, the stainless steel carriers were aseptically transferred into a sterile stomacher bag (Seward, Leicestershire) and 10 ml of sterile Letheen broth based neutralising solution added. The bag was then sealed and placed in a stomacher machine (Stomacher Lab-Blender 400, Seward, Leicestershire) where it was agitated for 2 minutes to release any spores that may have remained on the stainless steel carriers post-disinfection. After agitation for 2 minutes, 0.1 ml of the neutraliser-recovered spores were added to 0.9 ml of a 1:1 mixture of AGFK germinant and neutraliser, where AGFK germinant was made up of 0.33 mg/ml asparagine, 1 mg/ml glucose, 1 mg/ml fructose and 3.3 mg/ml potassium (Wax and Freese, 1968; Cabrera-Martinez *et al.*, 2003) and incubated at 37°C for 45 minutes. This enabled the germination of any recovered, sub-lethally damaged spores. Following this incubation period, 0.1 ml of the recovered AGFK/neutraliser treated spores were added to 0.9 ml of sterile Letheen neutraliser and a dilution series in neutraliser carried out. 0.1 ml of each dilution was then plated onto TSA in replicates of 3. The agar plates were incubated aerobically overnight and any CFU observed were enumerated and log reductions of spores calculated.

During the spraying process, inoculated carriers were placed on paper towels which were then processed in the same way to determine any displacement effects of spraying.

2.3.2.7.3 Wiping method

Carriers were inoculated with spores and dried as described in section 2.3.7.1.2. After which the carriers were placed at room temperature and wiped. The method of wiping with each biocide impregnated wipe involved each inoculated carrier being wiped using a biocide pre-impregnated wipe, ensuring that a new area of the wipe was used each time to prevent any possible cross-contamination. A 25% overlap was used to make sure that the whole of the stainless steel surface came into contact with the biocide. Following a 2 minute contact time with the biocide any remaining spores were neutralised, stomached, added to AGFK germinant and recovered as described in section 2.3.2.7.2.

After wiping, the wipe itself also underwent neutralisation, stomaching and recovery to determine the number of spores being released from the carrier on wiping.

2.3.2.7.4 Spraying and wiping method

Carriers were inoculated with spores, dried and sprayed with biocide as described in section 2.3.2.7.2. Following the 2 minute exposure to biocide spray, the wiping procedure was carried out using a corresponding biocide impregnated wipe (as described in section 2.3.2.7.3). The carriers were therefore sprayed for a contact time of 2 minutes, followed by wiping for a further contact time of 2 minutes. Following this, neutralisation, germination (AGFK) and recovery was carried out.

2.3.2.8 Treatment with biocides – Suspension testing

Suspension testing of the biocides was carried out in parallel with the hard surface testing, to measure biocide efficacy when the spores were in solution. This would allow comparison and subsequent evaluation between surface and suspension testing.

2.3.2.8.1 Suspension testing method

100 µl of the aqueous spore suspension was mixed with 0.9 ml of each biocide for 2 minutes at room temperature. After 2 minutes, 100 µl of the biocide/spore suspension was added to 0.9 ml of Letheen neutraliser and serial dilutions in neutraliser carried out. Each dilution was then plated in triplicate onto TSA plates which were incubated aerobically at 37°C overnight. Following incubation any CFUs observed (number of surviving spores) were counted and the log reductions in spore levels determined. A control of SDW was used along with the biocides.

2.3.2.9 Microbial enumeration

To determine the sporicidal activity of the biocides, the CFUs observed were enumerated and calculations were carried out to determine the log reductions in spores that had been exposed to the biocides. Log reductions of spores were calculated as follows:

Mean Log Reduction in Spores =

$\log_{10}$ (average number of CFUs recovered from positive control) – $\log_{10}$ (average number of CFUs recovered from disinfected coupons)

Positive controls were where the carriers were inoculated but no biocide was applied before neutralisation, germination and recovery was carried out. Negative controls were carriers without inoculum or biocide. The negative carriers were also subjected to neutralisation, germination and recovery.

A log reduction greater than 3 would render the biocide suitable for use as a biocide against bacterial spores i.e. sporicidal (BS EN, 2002; EPA, 2007).

2.3.2.10 Statistical analysis of data

A non-parametric statistical analysis was carried out on all data using an ordinary one-way ANOVA with Tukey–Kramer multiple comparisons test (GraphPad InStat, ver.

3.06; Graphpad Software Inc., La Jolla, USA) was used to compare methods of disinfection, as well as the biocides themselves. Differences were considered to be statistically significant when $P < 0.05$.

2.4 Results

2.4.1 *Bacillus* spores

2.4.1.1 *Bacillus* colony characteristics

B. subtilis subsp. subtilis 168 formed colonies that were creamy white, dry, non-mucoid, flat, and crusty with rough edges (figure 2.1). Both *B. subtilis* ATCC 6633 and *B. pumilis* ATCC 27142 spores also possessed these same characteristics.

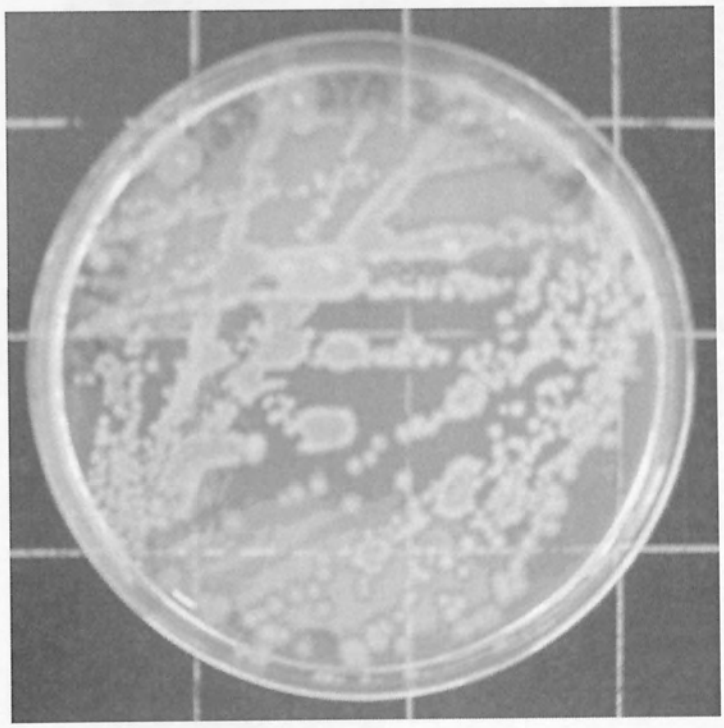


Figure 2.1 Colonies of *B. subtilis subsp. subtilis* 168

2.4.1.2 Gram staining of *B. subtilis subsp.subtilis* 168 organisms

When the bacilli were Gram stained, Gram positive bacilli were observed (figure 2.2).

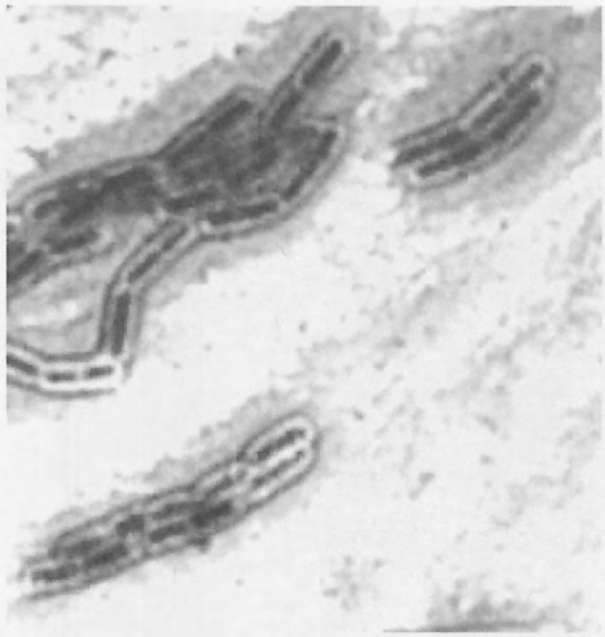


Figure 2.2 Gram stained Gram positive bacilli viewed under bright field microscope with 100x objective

2.4.1.3 Phase contrast microscopy of *Bacillus* spores

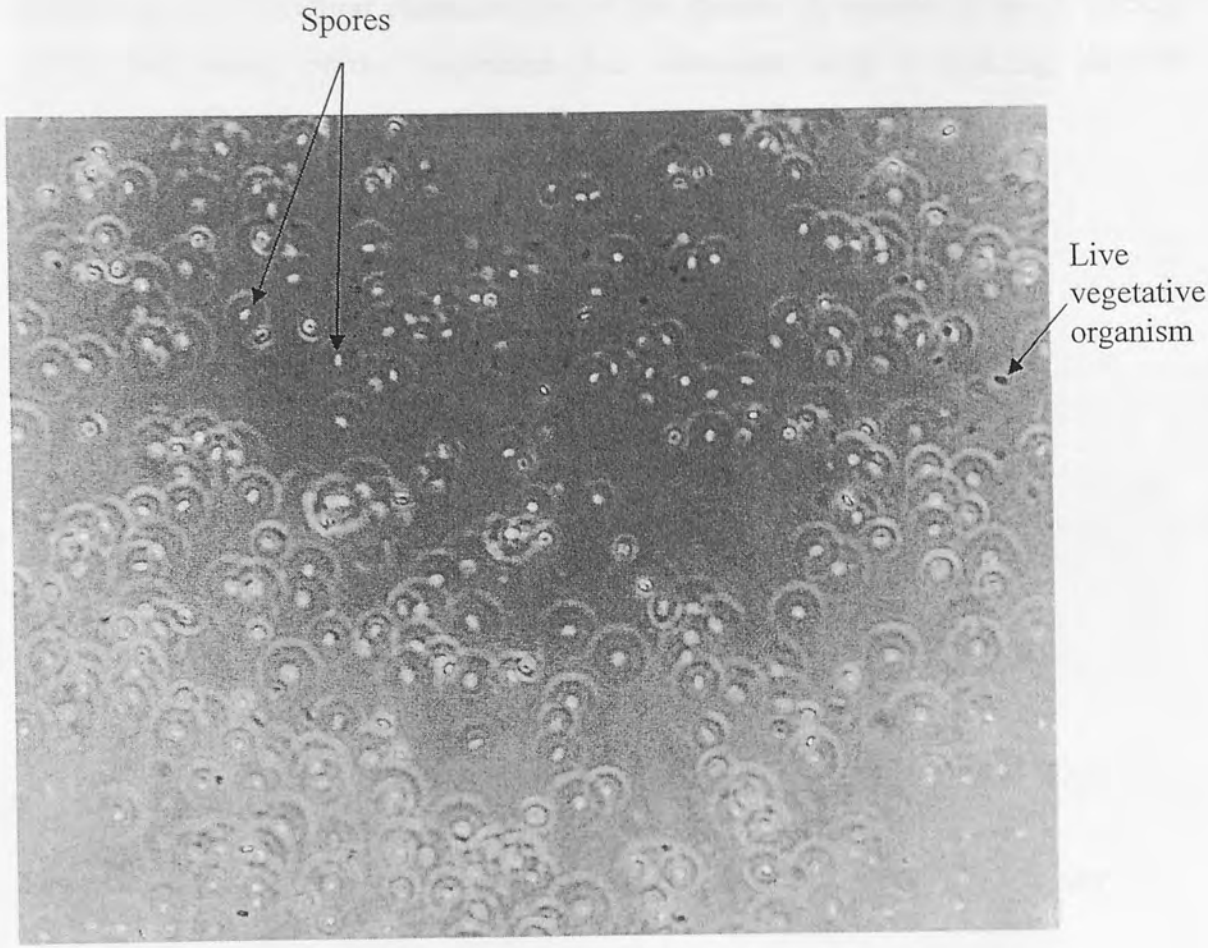


Figure 2.3 *Bacillus* spores as visualised under phase contrast microscopy (40x objective)

Phase bright round spores were observed on incubation of *B. subtilis subsp. subtilis* 168 spores following 12 days of incubation at 37°C (figure 2.3). Any vegetative organisms are visualised as phase-dark rods.

2.4.1.4 Scanning electron microscopy of *Bacillus subtilis* ATCC 6633 spores

Scanning electron microscopy was carried out on the *Bacillus* spores to determine the morphology and structural characteristics of the spores. A sample of the *B. subtilis* ATCC 6633 spore culture suspension was visualised using a scanning electron microscope (SEM taken by John Critchell).

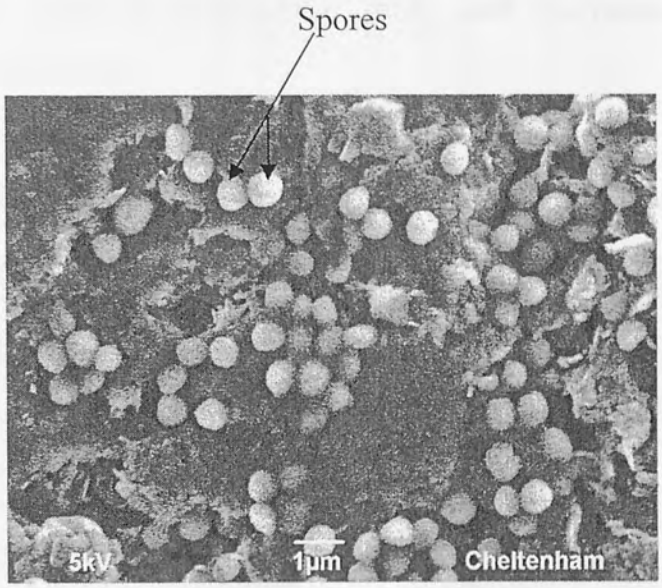


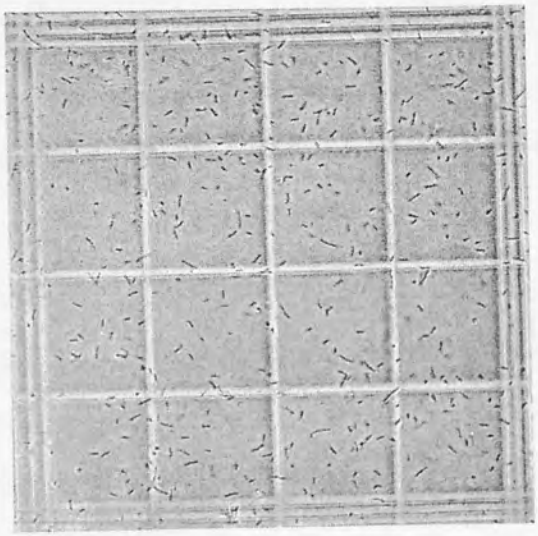
Figure 2.4 Scanning Electron Microscope image of *B. subtilis* ATCC 6633 spores

The SEM images indicated that *B. subtilis* ATCC 6633 spores were three-dimensional, round structures approximately 1µm in diameter (figure 2.4).

2.4.1.5 Total counts of *Bacilli*

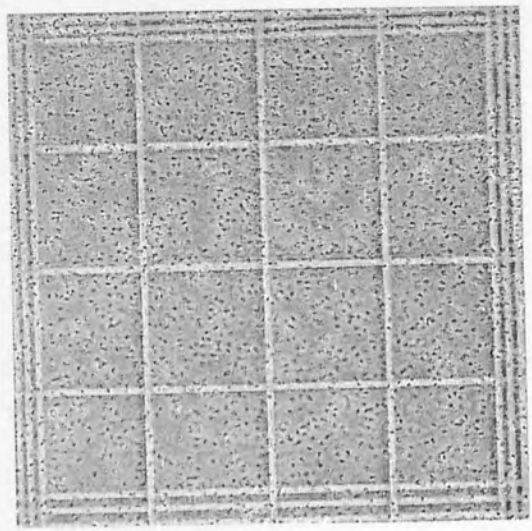
Organisms of *B. subtilis* ATCC 6633 in TSB transferred to a haemocytometer were observed as vegetative rods after 48 hours of incubation at 37°C (figure 2.5a), and as round spores (figure 2.5b) following 12 days of aerobic incubation at 37°C (Neubauer haemocytometer on light microscope using 40x objective). This enabled viability counts to be carried out on the vegetative organisms and allowed differentiation between the two states of unsporulated (rods) and sporulated (spores) *Bacillus* organisms to be established.

Bacillus rods (a)



48 hours

Bacillus spores (b)



12 days

Figure 2.5 *Bacillus* rods (a) and spores (b) as visualised under a haemocytometer (bright field microscopy, 40x objective)

2.4.1.6 Viability testing of *Bacillus* spores

Table 2.2. Viability counts of *Bacillus* spores. Data presented as mean CFU at 10^{-6} ± SD (n=3).

Bacillus spore solution	Viability Counts following incubation on TSA plates at 37°C for 24 hours (CFU)	
	Number of colonies from x10^{-6} dilution of suspension	Estimation of colonies per ml based on 10^{-6} counts (CFU/ml)
B. subtilis subspecies subtilis 168	325 ± 69	3.3 x 10^9
B. subtilis ATCC 6633	544 ± 110	5.4 x 10^9
B. pumilis ATCC 27142	65 ± 3	6.5 x 10^8

As there were more than 10^8 CFU/ml viable *Bacillus* spores in all three spore suspensions (table 2.2), this would allow 5-6 log reductions in spore numbers to be observed following treatment.

2.4.2 All biocides tested by spraying against all three *Bacillus* species

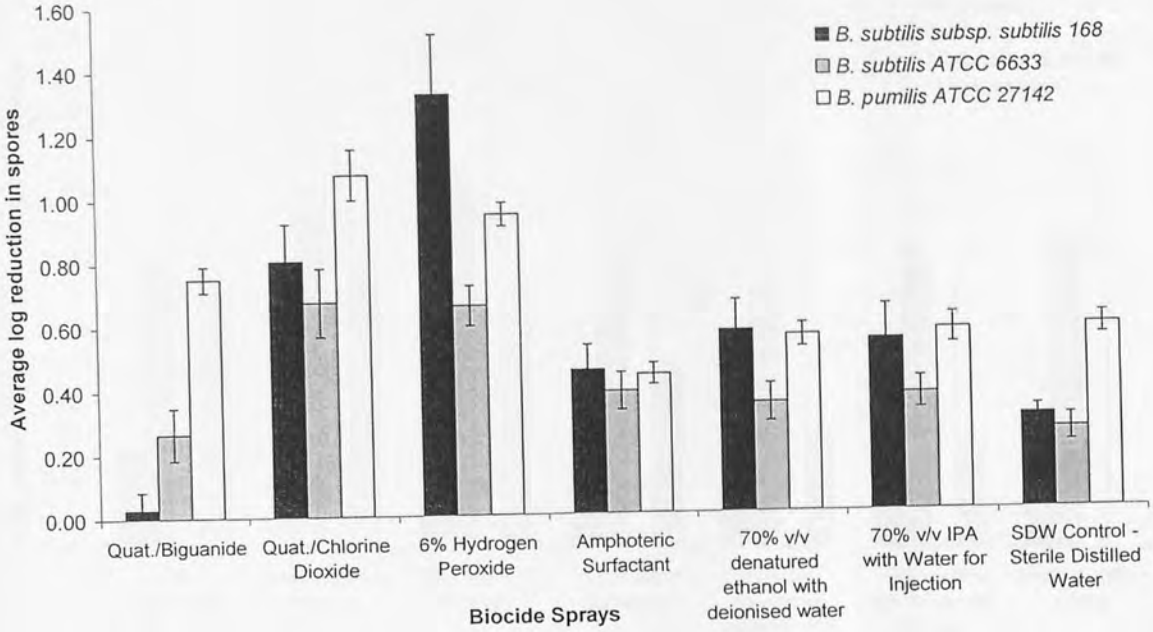


Figure 2.6 Log reductions of 6 different biocides on spraying against *B. subtilis* 168, *B. subtilis* ATCC 6633 and *B. pumilis* ATCC 27142 spores. Data are represented as mean ± SEM (n=9).

Figure 2.6 demonstrates that the log reductions obtained against the three test organisms on spraying with biocide ranged from between 0.03 log reductions with the Quat./biguanide formulation, to 1.32 log reductions with 6% H_2O_2. An ordinary ANOVA (Kruskal-Wallis) with post-hoc testing using Dunn's Multiple Comparisons indicated that the log reductions obtained by the 6 biocides were significantly different in relation to the three spore types tested ($P < 0.05$). Efficacy varied between the different biocides tested, indicating that spraying as an efficacy test method was able to distinguish between the effects of the different biocides. The efficacy of the biocide was also found to vary according to the *Bacillus* test organism, as the log reductions obtained on the use of the same biocide differed against the different *Bacillus* spores. This difference was significant for the Quat./biguanide and 6% H_2O_2 biocides ($P < 0.05$).

2.4.3 Disinfection of *B. subtilis* ATCC 6633 spores using 4 disinfection methods

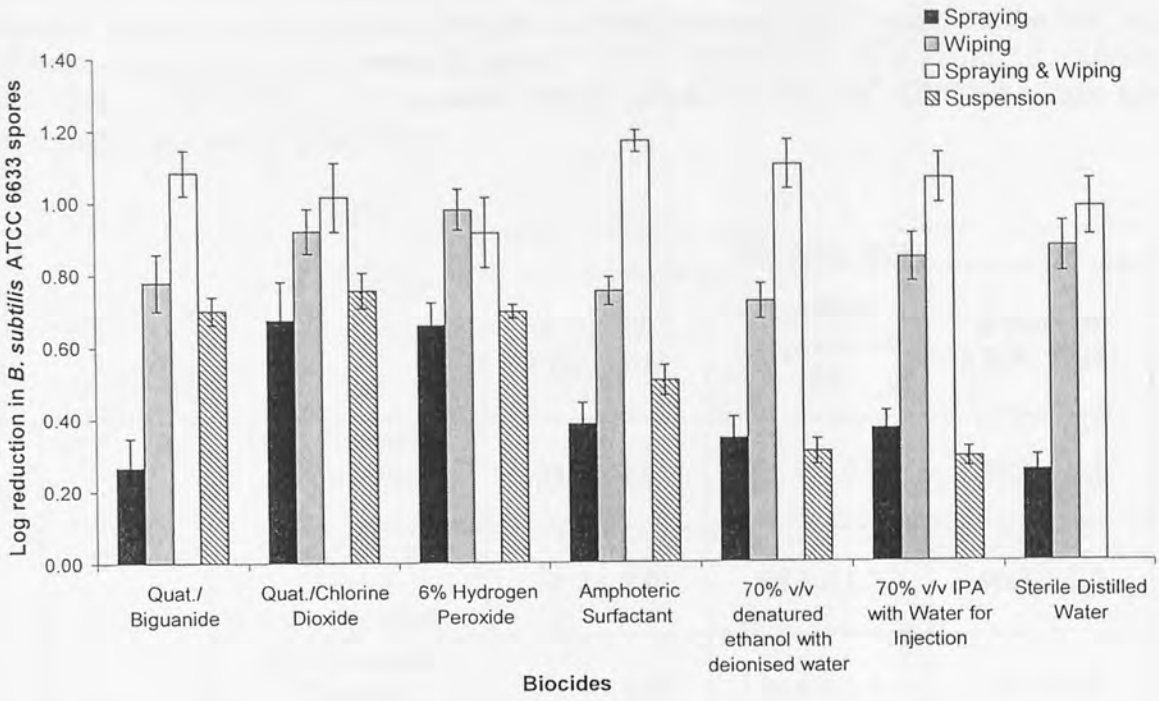


Figure 2.7 Comparisons between log reductions of *B. subtilis* ATCC 6633 spores following 1) Spraying, 2) Wiping, 3) Spraying and Wiping and 4) Suspension testing using 6 biocides and SDW. Data are represented as mean ± SEM (n=9).

Figure 2.7 demonstrates that the efficacy testing method used was able to differentiate between the different methods of disinfection used. This is because differences were observed between the efficacies obtained on spraying, wiping, both spraying and wiping and suspension testing against *B. subtilis* ATCC 6633 spores. With the *B. subtilis* ATCC 6633 spores, higher log decimal reductions in the range of 0.92 to 1.17 were achieved when spraying followed by wiping was used as a method of disinfection, rather than spraying or wiping alone. Suspension testing achieved lower log reductions in comparison to the hard-surface test results.

Overall the different log reductions obtained against the *B. subtilis* ATCC 6633 spores were found to be significantly different in relation to the four disinfection methods tested ($P < 0.05$).

2.4.4 Neutraliser efficacy and toxicity

Table 2.3. Percentage recovery of viable spores after neutralisation with modified Letheen broth based neutraliser. Percent recovery was calculated based on the number of spores inoculated which were: *B. subtilis* ATCC 6633: 3.3 x 10^9 CFU/ml, *B. subtilis* 168: 5.4 x 10^9 CFU/ml, *B. pumilis* ATCC 27142: 6.5 x 10^8 CFU/ml. Data are represented as mean ± SD (n=3).

		Test organism		
		B. subtilis ATCC 6633	*B. subtilis subsp. subtilis* 168	*B. pumilis* ATCC 27142
% Recovery of Viable Spores	Quat./Biguanide and neutraliser	98.7 ± 1.0	96.9 ± 0.9	96.3 ± 1.2
	Quat./Chlorine Dioxide and neutraliser	98.2 ± 1.0	96.6 ± 1.7	96.3 ± 1.3
	6% Hydrogen Peroxide and neutraliser	98.3 ± 0.3	96.6 ± 2.1	96.1 ± 0.3
	Amphoteric surfactant and neutraliser	97.8 ± 0.8	95.6 ± 1.1	97.4 ± 1.0
	70% v/v denatured Ethanol with deionised water and neutraliser	98.0 ± 0.7	95.7 ± 0.3	96.3 ± 0.9
	70% v/v IPA in WFI and neutraliser	97.4 ± 0.5	95.0 ± 0.6	97.6 ± 1.4
	Neutraliser only	98.5 ± 0.5	98.2 ± 0.0	97.6 ± 1.1

The results of the neutraliser evaluation procedure (table 2.3) indicate that the modified Letheen broth based neutraliser had a small effect on the number of viable cells, with up to 5% of the spores being killed in the presence of the biocide and neutraliser solution. The sensitivity observed is not entirely due to the biocide as the neutraliser alone resulted in a 1-3% reduction in spore levels.

The variation among the biocide/neutraliser and neutraliser alone is not significantly different to that of the SDW viability control ($P > 0.05$). Therefore although the neutraliser used was found to be slightly detrimental to spore viability, it was established that the extent to which the neutraliser was active against the spores (all organisms) was not significant ($P > 0.05$) when considering biocide efficacy for this study (*B. pumilis* spores $P = 0.85$, *B. subtilis subsp. subtilis* 168 spores $P = 0.36$, and *B. subtilis* ATCC 6633 spores $P = 0.87$). Incorporation of the H_2O_2 neutraliser catalase (1000 units/ml) to the neutraliser formulation did not have a significant effect on either neutraliser efficacy or toxicity (Appendix 11.1.2), and catalase was therefore omitted from the neutraliser.

2.5 Discussion

The developed hard-surface testing method used to determine the efficacy of different biocides and different disinfection methods achieved variable results. The method established differences between both the type of biocide and disinfection method used. The method enabled quantitative results to be obtained rather than just positive or negative results being designated; thereby allowing the measurement of log reductions in terms of pre- and post-disinfection results. Differences in log reductions were observed when different physical methods of disinfection were tested with a variety of biocides (figures 2.6 and 2.7). The different biocides used did not achieve the same results and therefore the efficacy testing method developed is selective, taking into account the activity of the biocides. The test method developed was also effective at determining the efficacy of the different biocides against a variety of *Bacillus sp.* spores. This was because the same biocides achieved different levels of log reductions against the different spores tested against (figure 2.6). Consequently the results obtained established that the efficacy testing method was sensitive to the test organism, and that validation of the method against all possible test organisms used to determine the efficacy of a particular biocide or disinfection method will be required.

As each stage of the efficacy testing method was carried out to represent hospital aseptic transfer disinfection, it is highly likely that the levels of log reductions obtained will be achieved in the cleanroom environment. By employing a high grade stainless steel plate as a carrier, the pharmaceutical components (with smooth surfaces) used in compounding are represented. The biocides tested were also applied in the manner in which they are applied in the aseptic process as sprays and wipes.

The spore drying process was important to validate as 90% of microorganisms can be lost leaving a small quantity of resistant organisms difficult to eradicate. The AOAC recommends a drying time of 30-40 minutes at 37°C (Lambert, 2004). However, a drying time of 15 minutes at 37°C in air was used for a 0.1 ml inoculum volume. This was adequate as this volume of spore suspension was dry after 15 minutes with the spores being stuck to the carriers. On drying of the spores onto the stainless steel carrier test surfaces approximately 1 log reduction in spore levels was observed. This indicates

that it is likely that some of the *Bacillus* spores have been killed by drying at 37°C and therefore the least resistant spores have been eliminated. However, it is thought that there is a 1-2 log loss in viability of the spores associated with this drying phase (Lambert, 2004). But this only serves to make the disinfection procedure a true reflection of the aseptic transfer process, because only the most resistant spores are liable to remain on hard surfaces.

The AOAC also recommends a 60 minute contact time with biocide to achieve a 3 log reduction in spore numbers (Lambert, 2004). As a high number of formulations need to be carried out in a short period of time, a 60 minute contact time is not feasible in such pharmacy cleanroom environments and is unlikely to be achieved. Therefore the 2 minute contact time employed was because of its feasibility and its representation of the true length of time that components in the aseptic transfer disinfection process encounter with a biocide (Hiom, 2000). Hence, the biocide efficacies observed in this time period are those likely to be used in practice.

The use of a neutralisation stage following treatment with the biocides was important in ensuring that the reduction in viable spores was not a result of a carry-over effect of the biocide on the agar, thereby preventing any overkill from occurring and ensuring that the level of kill is not artificially increased due to failure of the neutraliser (Sutton *et al.,* 2002; Johnston *et al.,* 2002). As there is a delay between the time that any recovered spores are plated onto TSA plates, there is a risk that during this time the biocides will continue to have an effect on the spores. As a result, the activity of the biocide had to be neutralised to ensure that the contact time between the spores and the biocides was adhered to. An ideal neutraliser should have no effect on the number of viable cells and should inactivate the challenge biocide in a given time (Sutton *et al.,* 2002). The Letheen based neutraliser tested is not ideal, because a reduction in the number of viable spores recovered after incubation of the biocides with the neutraliser was observed in comparison to the control (table 2.3). Although the neutraliser was found to be slightly sporicidal, it was established that the extent to which the neutraliser was active against the spores was not statistically significant ($P > 0.05$ for all the organisms tested) when considering biocide efficacy for this study. The variation among the biocide/neutraliser and neutraliser alone was not significantly different to that of the SDW viability control.

Any differences observed between the biocide/neutraliser and neutraliser alone were unlikely to affect the efficacy of the biocides when they were tested. As the neutraliser was validated for its efficacy against the biocides and relative non-toxicity towards the *Bacillus* test organisms, it can be established that neutralisation of the biocides was occurring. This indicates that the level of kill obtained was due to the 2 minute exposure of the spores to the biocide and not because of a prolonged exposure. Therefore a validated neutraliser formulation was used to ensure that biocide activity was arrested after the required contact time, without the neutraliser being toxic to the spores.

The use of a stomacher machine to recover any remaining spores from the stainless steel inoculated surfaces once they had been disinfected was also found to be effective. Spores were recovered from the carrier into the neutralising solution within the stomacher bags. Two minutes in the stomacher machine was sufficient to extract any surviving spores from the carrier test surface (data not shown). This is important because extracting any remaining spores from the stainless steel carriers ensures that the results observed are accurate and not exaggerated. If fewer spores were being recovered than were present, then the efficacy observed may actually have been lower than that obtained because of the poor recovery of the remaining spores.

Different active compounds within the biocides have differing abilities to cause the germination of spores (Cortezzo *et. al.*, 2004). This may contribute to the different number of recovered organisms being observed following application of different biocides. The recovered spores were therefore exposed to AGFK germinant solution (Appendix 11.1.3). This encourages germination of recovered *Bacillus* spores (Setlow, 2003; Cabrera-Martinez *et al.*, 2003) which may have been sub-lethally damaged during the disinfection process and so enables them to be detected as viable organisms. The presence of a germinant would encourage any remaining spores to germinate into vegetative cells and these would be observed as viable cells when plated onto agar. The number of spores recovered as viable cells following disinfection was found to differ in the presence and absence of the AGFK germinant (Appendix 11.1.4).

The development of an effective biocide efficacy test requires a number of parameters to be standardised. Only by standardising these parameters can results obtained be reproducible and therefore comparable. Disinfection methods need to be closely adhered to, reviewed and modified accordingly in order to ensure the removal of any potentially pathogenic bacteria. The method developed in this study is effective at determining the efficacy of the different biocides and disinfection methods on a hard-surface against a number of spores (an in depth analysis of biocide efficacy against *Bacillus* spores is presented in Chapter 3). It may therefore, after further investigation, be employed in the hard-surface efficacy testing of chemical biocides against different species of bacterial spores.

CHAPTER 3 EFFICACY TESTING OF BIOCIDES AND DISINFECTION METHODS

3.1 Introduction

Current practice in NHS hospitals is to use 70% Industrial Methylated Spirit (IMS) sprays as biocide for the surface disinfection of components to be transferred into Grade A pharmaceutical environments. This disinfection takes place before any manipulation occurs. Other agents that may provide more effective sanitisation of these components are available and include biguanides, chlorine based agents, H_2O_2 and Quats. Other potentially more effective disinfection methods are wiping and a combination of both spraying and wiping.

The purpose of this part of the study was to evaluate alternative biocides (as IMS is not sporicidal) and disinfection methods for transferring components into hospital pharmacy cleanrooms, and to determine which of the biocides and the disinfection methods tested have the greatest efficacy against *Bacillus* spores. Testing was based on the method developed in Chapter 2, whereby the biocides were applied to stainless steel carrier test surfaces inoculated with *Bacillus* spores, with the carriers representing the hard surfaces used in hospital pharmacy cleanroom environments. Different biocide formulations were tested using different disinfection methods, to see which biocide and method of disinfection consisting of suspension testing, spraying alone, wiping alone or a combination of spraying followed by wiping had the greatest efficacy against *B. subtilis subspecies subtilis* 168, *B. subtilis* ATCC 6633 and *B. pumilis* ATCC 27142 spores present on a stainless steel surface. Sporicidal activity was calculated as log reduction in CFU.

3.2 Aims

The aims of this chapter were:

- To assess the biocides that are currently used in aseptic transfer processes in order to identify the most effective biocide against spores of *Bacillus* species.

- To establish whether biocides currently available possess sporicidal activity.

- To determine whether current spore suspension testing, achieves similar results to hard-surface testing.

- To find out the best method of disinfection, comparing spraying, wiping, and a combination of spraying and wiping.

- To determine whether the methods of disinfection contribute to the level of efficacy that they achieve.

- To investigate whether any physical removal of spores occurs as a result of the spraying and wiping with biocide, and if this contributes to improving sporicidal efficacy.

3.3 Materials and Methods

3.3.1 Materials

Please refer to Chapter 2, Section 2.3.1.

3.3.2 Methods

The method involved inoculation of a sterile stainless steel surface with spores, drying, and application of biocide by spraying, wiping or a combination of spraying and wiping for a limited contact time, recovery of spores from the surface, neutralisation of the biocide to prevent overkill, a germination stage, dilution, plating and enumeration.

Please refer to detailed methods as described in Chapter 2, Section 2.3.2.

3.3.2.1 Recovery of spores released from carrier after spraying with biocide.

Spraying was carried out as described previously (Chapter 2, Section 2.3.7.2), but in order to recover any washed off spores, each inoculated, dried stainless steel square was placed onto a paper towel before any spraying took place. After the two minute contact time with biocide, both the stainless steel carrier and the underlying paper towel underwent neutralisation, stomaching and recovery. The percentage of spores washed off the carrier on spraying was calculated by comparing the number recovered from the paper towel with a positive control constituting an inoculated but non-disinfected carrier.

3.3.2.2 Recovery of spores wiped off carrier on wiping with biocide-impregnated wipe.

The method of wiping was carried out as described previously (Chapter 2, Section 2.3.7.3), but in order to quantify wiped-off spores, the biocide-impregnated wipes as well as the steel carriers were neutralised with Letheen neutraliser, stomached, treated

with AGFK germinant and any spores recovered. The percentage of spores wiped off the carrier on wiping was calculated by comparing the number recovered from the wipe with a positive control constituting an inoculated but non-disinfected carrier.

3.3.2.3 Statistical analysis of data

A non-parametric statistical analysis was carried out on all data using an ordinary one-way ANOVA with Tukey–Kramer multiple comparisons test (GraphPad InStat, ver. 3.06; GraphPad Software Inc., La Jolla, USA) was used to compare methods of disinfection, as well as the biocides themselves. Data are presented as mean ± SEM. Differences were considered to be statistically significant when $P < 0.05$.

3.4 Results

3.4.1 Efficacy of biocides

3.4.1.1 Efficacy of biocides against *B. subtilis subsp. subtilis* 168 spores

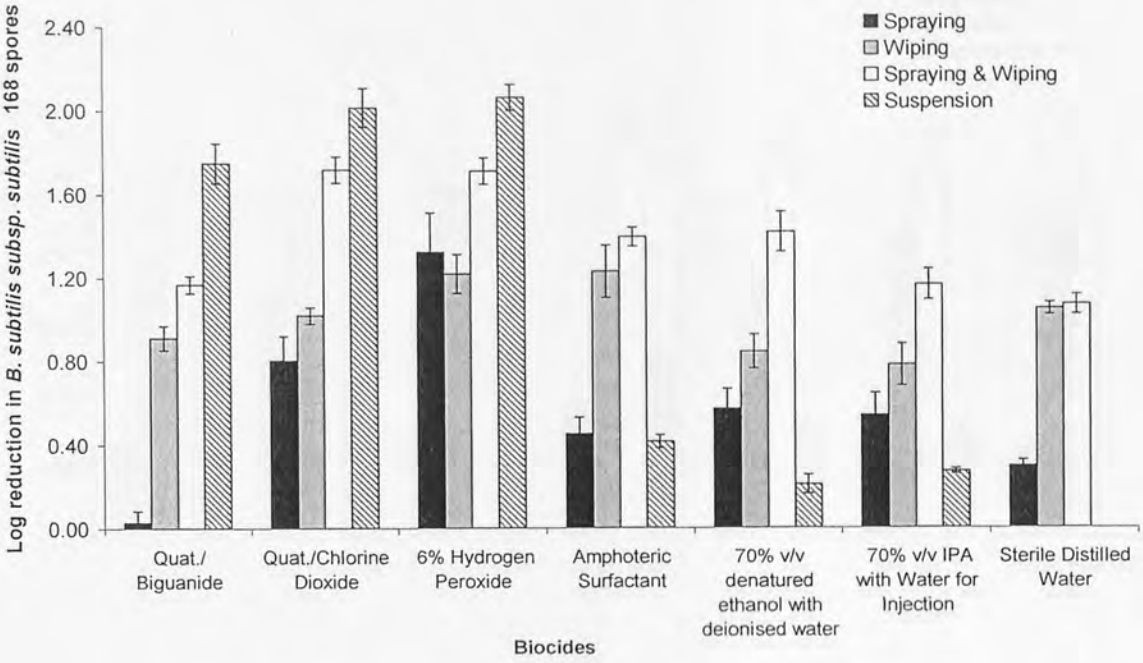


Figure 3.1 Efficacy of biocides against *B. subtilis subsp. subtilis* 168 spores. Comparisons between log reductions of *B. subtilis subsp. subtilis* 168 spores following 1) Spraying, 2) Wiping, 3) Spraying and Wiping and 4) Suspension testing using 6 biocides and SDW. Data are represented as mean ± SEM, n=9. Overall, all disinfection methods differed significantly from each other ($P < 0.05$). However, spraying was not significantly different from suspension testing ($P > 0.05$).

Suspension testing achieved higher log reductions for three of the biocides, ranging between 0.21 ± 0.04 to 2.07 ± 0.06 against *B. subtilis subsp. subtilis* 168 spores, than the hard surface testing (figure 3.1). This was followed by the spraying and wiping combination which achieved a highest log reduction of 1.72 ± 0.06 with the Quat./chlorine dioxide. The spraying alone method achieved the lowest log reductions in spore levels. Overall differences between all disinfection methods were significant ($P < 0.05$). This was with the exception of the differences between spraying alone and suspension testing with the amphoteric surfactant ($P > 0.05$), between the spraying and

wiping with the 6% H_2O_2, and the wiping alone and spraying/wiping combination with the SDW ($P > 0.05$).

3.4.1.2 Efficacy of biocides against *B. subtilis* ATCC 6633 spores

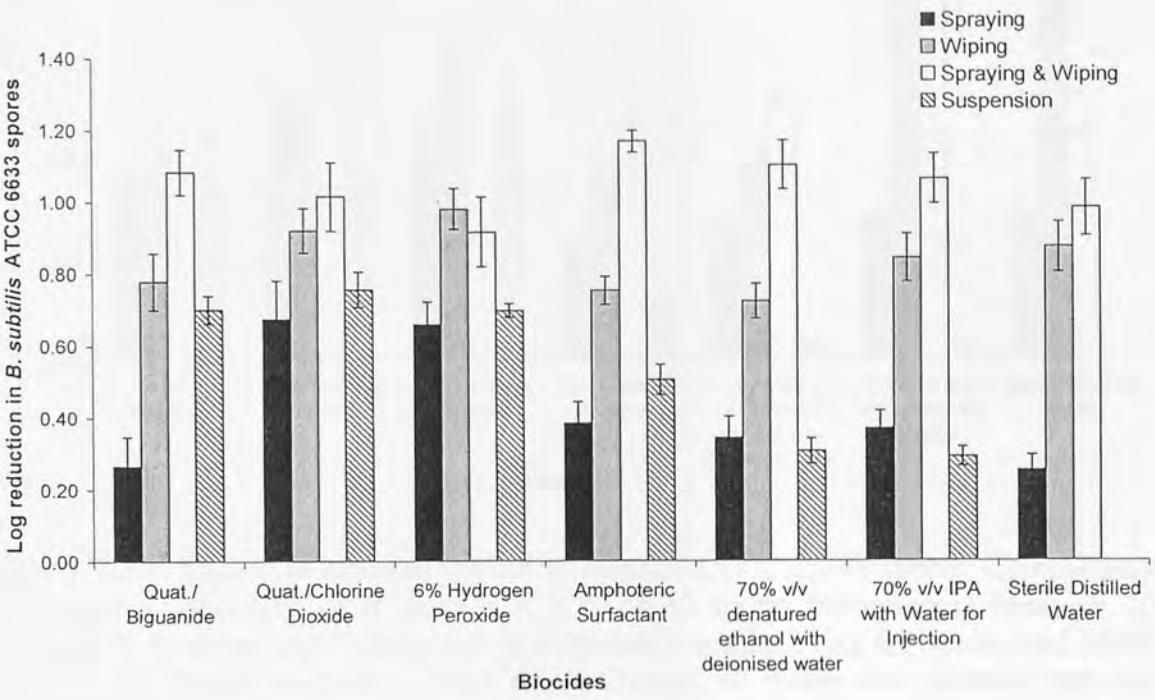


Figure 3.2 Efficacy of biocides against *B. subtilis* ATCC 6633 spores. Comparisons between log reductions of *B. subtilis* ATCC 6633 spores following 1) Spraying, 2) Wiping, 3) Spraying and Wiping and 4) Suspension testing using 6 biocides and SDW. Data are represented as mean ± SEM, n=9. Overall all differences observed between the disinfection methods were found to be significant ($P < 0.05$). However, even though spraying and wiping showed an increased level of efficacy in comparison to wiping alone, the difference between wiping alone and spraying & wiping was found to be non-significant ($P > 0.05$).

Higher log decimal reductions in the range of 0.92 ± 0.09 to 1.17 ± 0.03 were achieved against the *B. subtilis* ATCC 6633 spores when spraying followed by wiping was used as a method of disinfection, compared to spraying alone or wiping alone (figure 3.2). Suspension testing achieved less log reductions in comparison to the hard-surface test results. Overall, differences between all disinfection methods were significant ($P < 0.05$), with the exception of the difference between wiping alone and spraying and wiping against the *B. subtilis* ATCC 6633 spores.

3.4.1.3 Efficacy of biocides against *B. pumilis* ATCC 27142 spores

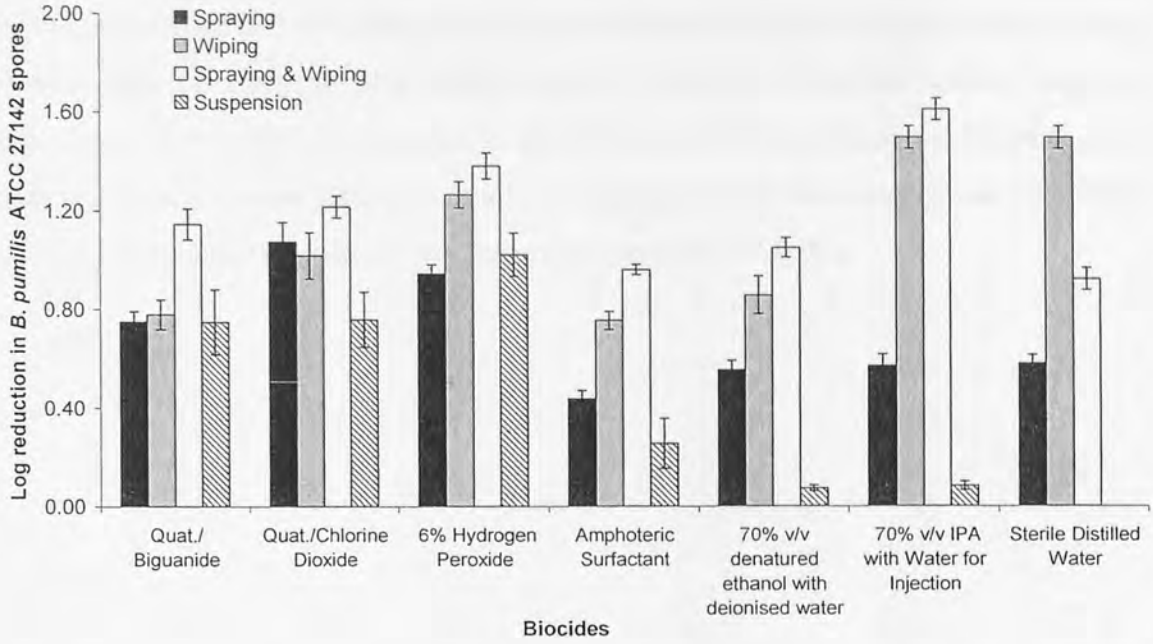


Figure 3.3. Efficacy of biocides against *B. pumilis* ATCC 27142 spores. Comparisons between log reductions of *B. pumilis* ATCC 27142 spores following 1) Spraying, 2) Wiping, 3) Spraying and Wiping and 4) Suspension testing using 6 biocides and SDW. Data are represented as mean ± SEM, n = 9. Overall, all differences observed between the disinfection methods were found to be significant ($P < 0.05$). Once again, even though spraying and wiping showed an increased level of efficacy in comparison to wiping alone, the difference between wiping and the combination of spraying & wiping was found to be non-significant ($P > 0.05$).

For the *B. pumilis* ATCC 27142 spores (figure 3.3) each biocide achieved higher log decimal reductions ranging between 0.92 ± 0.04 to 1.61 ± 0.04 when spraying followed by wiping was used as a method of disinfection, compared to spraying alone, wiping alone or suspension testing. Overall the differences between all disinfection methods were significant ($P < 0.05$), with the exception of the difference between wiping alone, and spraying and wiping against the *B. pumilis* ATCC 27142 spores.

Figures 3.1, 3.2 and 3.3 show that all three methods of disinfection gave rise to different levels of log reductions in spores. Overall, spraying alone achieved low levels of log reductions against the spores. Wiping and a combination of spraying and wiping achieved similar results against all three of the *Bacillus* spore species tested.

Disinfection by suspension also achieved reductions in spore levels. Overall the log reductions depended on the organism and the biocide.

It was determined that the difference in log reductions observed between wiping and the combination of spraying and wiping against the three *Bacillus* spores was non-significant ($P > 0.05$). In comparison the differences in log reductions in spore levels obtained on suspension testing in relation to spraying with the biocides was established as being significantly lower for the suspension testing ($P < 0.05$).

3.4.2 Efficacy of disinfection methods

3.4.2.1 Efficacy of disinfection methods using all biocides against all *Bacillus* spores

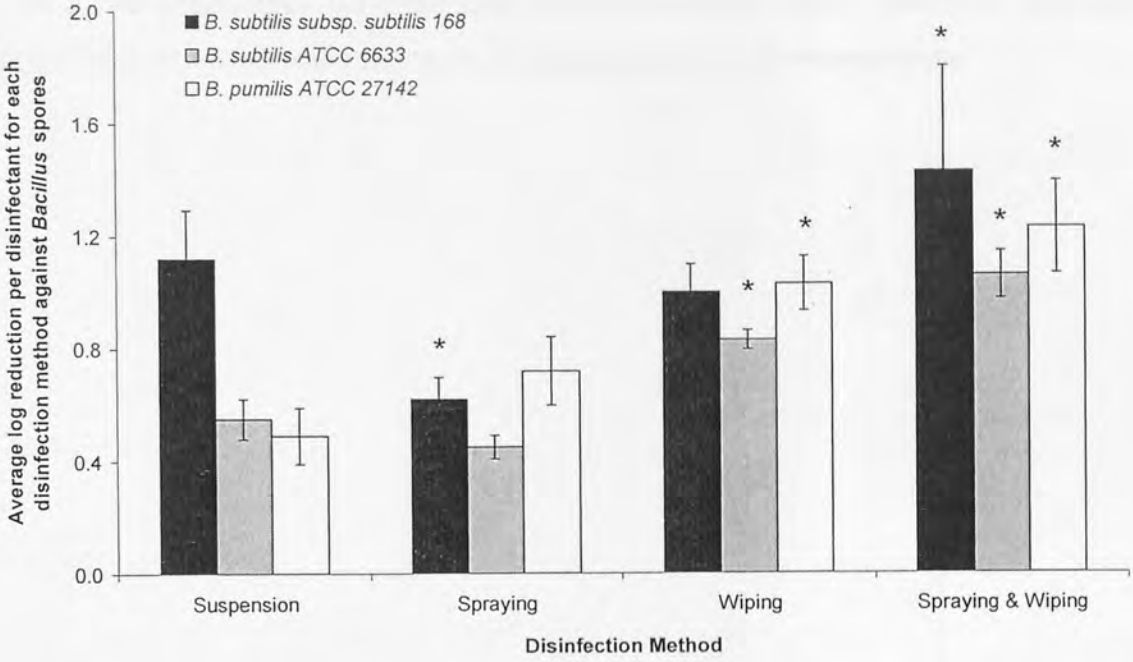


Figure 3.4. Efficacy of disinfection methods - all biocides against all *Bacillus* spores. Average log reduction achieved per biocide on spraying, wiping, spraying and wiping, and suspension testing disinfection methods against *B. subtilis* 168, *B. subtilis* ATCC 6633 and *B. pumilis* ATCC 27142 spores. Data are represented as mean ± SEM, n=9. *: Denotes significant differences between suspension testing and spraying, wiping or spraying and wiping ($P < 0.05$) for the *Bacillus* spore type indicated.

Figure 3.4 summarises the efficacies of the different disinfection methods against the 3 spore suspensions. It shows that the technique of spraying with a biocide was found to be the least effective of the disinfection procedures with an average 0.60 ± 0.08 log reductions in CFU being achieved. Slightly better than the spraying was the suspension testing which achieved an average 0.72 ± 0.20 log reductions against the *Bacillus* spores. Of the methods investigated spraying and wiping produced the best results against the *Bacillus* spores achieving an average 1.24 ± 0.10 log reductions. This was closely followed by wiping alone which achieved an average 0.95 ± 0.06 log reductions in spore numbers. Of the techniques investigated only the spraying and wiping

combination method achieved an average of at least a 1 log reduction against all three of the *Bacillus sp.* spores tested.

With the exception of the spraying alone and suspension testing methods ($P > 0.05$), the log reductions obtained by the disinfection methods against all the *Bacillus* spores were found to be significantly different from each other ($P < 0.05$). The most effective method of disinfection was spraying and wiping, followed by wiping alone.

3.4.2.2 Efficacy of disinfection methods using SDW against all *Bacillus* spores

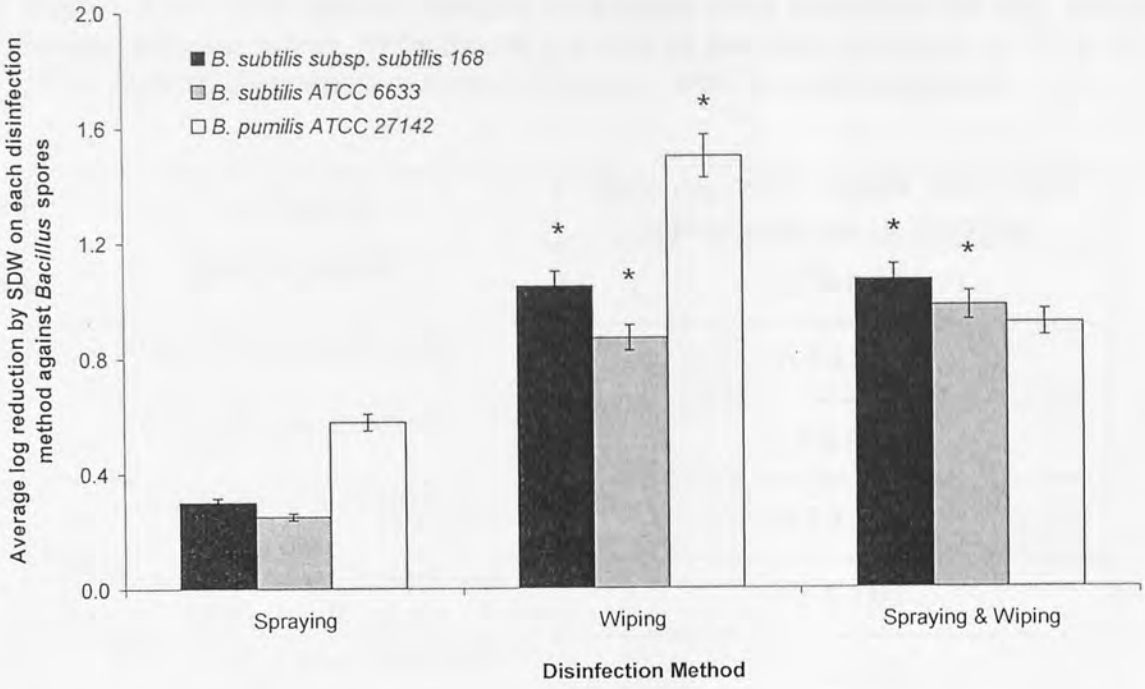


Figure 3.5. Efficacy of disinfection methods - SDW against all *Bacillus* spores. Average log reduction achieved using SDW on spraying, wiping, spraying and wiping, and suspension testing disinfection methods against *B. subtilis* 168, *B. subtilis* ATCC 6633 and *B. pumilis* ATCC 27142 spores. Data are represented as mean ± SEM, n=9.
*: Denotes significant differences between spraying and wiping alone or spraying and wiping ($P < 0.05$) for the *Bacillus* spore type indicated.

Spraying with SDW was the least effective of the disinfection procedures with an average log reduction of 0.37 ± 0.08 logs (figure 3.5). Of the methods investigated using SDW, spraying and wiping produced the best results against the *Bacillus* spores achieving at least an average 0.92 ± 0.04 log reduction in spore numbers. This was closely followed by wiping alone which achieved at least a 0.87 ± 0.18 log reduction in spore numbers. Both wiping alone and spraying and wiping achieved 1 log reductions against some of the three *Bacillus sp.* spores tested. The log reductions obtained by all 3 disinfection methods using SDW against all the *Bacillus* spores were found to be significantly different from each other ($P < 0.05$). This was with the exception of the wiping alone, and the spraying and wiping test methods ($P > 0.05$).

3.4.3 Release of spores from hard-surface carriers by washing

Table 3.1 Release of spores from hard-surface carriers by washing. Mean percentage of *B. subtilis* ATCC 6633 spores recovered from paper towel underlying the steel carrier following spraying, where 100% recovery would be the entire inoculum of 7.8×10^7 CFU/ml. Data are represented as mean ± SEM, n=2 with three replicates each.

Test Biocide	Percentage of *B. subtilis* ATCC 6633 spores removed on spraying
Quat./Biguanide	2.2 ± 0.00
Quat./Chlorine Dioxide	10.7 ± 0.01
6% Hydrogen Peroxide	2.5 ± 0.01
Amphoteric Surfactant	26.1 ± 0.22
70% v/v denatured Ethanol with Deionised Water	1.3 ± 0.00
70% v/v IPA with Water for Injection	1.7 ± 0.00
Sterile Distilled Water	20.8 ± 0.01

Spraying with the biocides released spores from the stainless steel inoculated carriers onto the underlying paper towel (table 3.1). The amphoteric surfactant and the SDW sprays removed the most spores (26.1 ± 0.22% and 20.8 ± 0.01% respectively). Spraying with the Quat./chlorine dioxide removed at 10.7 ± 0.01% of the *B. subtilis* ATCC 6633 spores. The Quat./biguanide, Quat./chlorine dioxide, 70% Ethanol and 70% IPA all achieved similar percentages of spores being washed off, ranging from 1.3 ± 0.00% to 2.5 ± 0.01%. It was found that $P > 0.05$ for all the biocides tested in comparison to the 100 % control. The ability of spraying to wash spores off the carrier onto the underlying surface did not differ significantly between each of the biocides tested ($P > 0.05$).

3.4.4 Spore transfer to wipes

Table 3.2 Spore transfer to wipes. Mean percentage of *B. subtilis* 168, *B. subtilis* ATCC 6633 and *B. pumilis* ATCC 27142 spores recovered from biocide wipes, following wiping. Data are represented as mean ± SEM, n=2 for both species of *B. subtilis* spores and n=3 for *B. pumilis* ATCC 27142 spores. Percent recovery is calculated based on the number of spores inoculated which were: *B. subtilis* ATCC 6633 (7.7×10^7 CFU/ml), *B. subtilis* 168 (7.6×10^7 CFU/ml) and *B. pumilis* ATCC 27142 (6.3×10^7 CFU/ml).

Test Biocide	Percentage of spores transferred to wipes		
	B. subtilis subsp. subtilis 168	*B. subtilis* ATCC 6633	*B. pumilis* ATCC 27142
Quat./Biguanide	15.6 ± 0.12	21.2 ± 0.02	79.0 ± 0.06
Quat./Chlorine Dioxide	14.8 ± 0.11	24.8 ± 0.12	80.7 ± 0.06
6% Hydrogen Peroxide	43.5 ± 0.17	20.4 ± 0.04	79.1 ± 0.02
Amphoteric Surfactant	19.7 ± 0.10	21.2 ± 0.00	80.3 ± 0.07
70% v/v denatured Ethanol with Deionised Water	27.1 ± 0.08	19.5 ± 0.04	41.3 ± 0.06
70% v/v IPA with Water for Injection	50.8 ± 0.11	50.4 ± 0.17	82.6 ± 0.12
Sterile Distilled Water	49.2 ± 0.11	25.7 ± 0.01	51.6 ± 0.03

The results in table 3.2 show that, on average, at least 14.8 ± 0.11% of *B. subtilis subsp. subtilis* 168 spores were wiped off from the inoculated stainless steel carriers onto the biocide impregnated wipes. Of the *B. subtilis* ATCC 6633 spores inoculated onto the stainless steel surfaces 19.5 ± 0.04% were recovered from the biocide wipes. The *B. pumilis* ATCC 27142 spores were found to be transferred in large numbers from the inoculated stainless steel surface onto the biocide wipes, with 41.3 ± 0.06% of spores recovered from wipes. Overall the 70% IPA with water for injection wipe was found to harbour the largest number of transferred spores with over 50% of spores being recovered from the wipes.

It was found that $P < 0.05$ for all the biocides tested in comparison to the 0% taken as control. Variation among the wipes in relation to the transfer of spores from the carrier surface to the wipe itself was not significantly different between each of the biocide wipes.

3.4.5 Evaluation of biocide efficacy

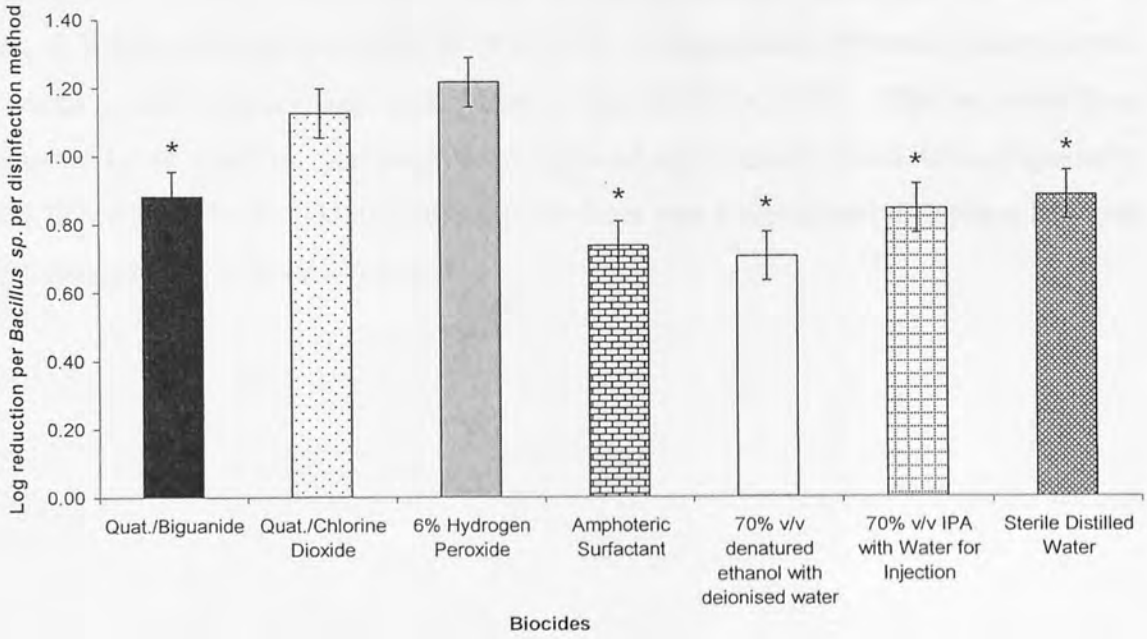


Figure 3.6 Evaluation of biocide efficacy. Average log reduction achieved by each biocide. Data are shown as the average log reduction for *Bacillus subtilis* 168, *B. subtilis* ATCC 6633 and *B. pumilis* ATCC 27142 spores for all disinfection methods used. Data are represented as mean ± SEM, n=9. *: $p < 0.05$ differences in log reductions versus 6% H_2O_2.

In order to compare the overall efficacy of the different biocides, the mean log reduction achieved for each of the disinfection procedures, including suspension testing, against all 3 spore suspensions was calculated (figure 3.6). Quat./chlorine dioxide and 6% H_2O_2 showed the highest average log reductions (1.13 and 1.22 respectively) against the *Bacillus* spores. Quat./biguanide, 70% IPA and SDW achieved similar average log reductions in spores (around 0.8). The amphoteric surfactant and 70% ethanol also

achieved similar log reductions of 0.74 and 0.71, respectively and these were the least effective of the biocides investigated.

A significant difference ($P < 0.05$) was observed between 6% H_2O_2 (highest average log reduction) and 70% ethanol (lowest average log reduction). Non-significant differences were observed between both the alcohols tested ($P > 0.05$). This was also true for the 6% H_2O_2 and Quat./chlorine dioxide. Both 6% H_2O_2 and Quat./chlorine dioxide, which was established as being the second most effective of the biocides against the *Bacillus* spores, were found to differ significantly to the amphoteric surfactant, 70% ethanol, 70% IPA, Quat./biguanide and SDW ($P < 0.05$). A significant difference was observed between Quat./biguanide and Quat./chlorine dioxide ($P < 0.05$). The log reductions achieved by amphoteric surfactant also differed significantly from Quat./biguanide, 70% IPA and SDW ($P < 0.05$). Furthermore there was a significant difference between 70% ethanol and SDW ($P < 0.05$).

3.5 Discussion

3.5.1 Disinfection methods

On investigating the different disinfection methods employed in the aseptic pharmaceutical environment it was determined that all three methods varied in their efficacy against the *Bacillus* spores. These results establish that both the biocide itself and its method of application can contribute to overall effectiveness.

3.5.1.1 Spraying

Of the three disinfection methods investigated, spraying alone was found to be the least effective against the *Bacillus* spores, achieving low levels of log reduction (figures 3.1 - 3.4). Cockcroft *et al.* (2001), albeit using a somewhat different method of testing whereby no germinant solution was used to recover any potentially sub-lethally injured spores, also found spraying to be an ineffective disinfection technique against environmental organisms such as *Bacillus.* However, although they also used a contact time of 2 minutes, their target organisms were vegetative bacilli and not the spore forms.

It is possible that some of the spores inoculated onto the carriers may have had little or no exposure to the biocide due to the delivery of the sprays as droplets, resulting in some areas of the test surface remaining uncovered. The level of coverage of the sprays is of considerable importance. If only 90% of the test surface were covered with droplets, even with a 100% kill rate, the 10% recovered from the edge would only enable achievement of a 1 log reduction. So any spores recovered may be due to lack of contact with the biocide, rather than the low efficacy of the biocide. However, the reverse could also occur as some of the biocides tested saturated the carrier. This may have resulted in some spores being physically released from the test surface as hypothesised by Cockcroft *et al.* (2001). Some spores were recovered from the underlying surface following spraying with up to 26% of the spores being washed off

(table 3.1), irrespective of the biocide used. Therefore the efficacy that was observed for each disinfection method was not entirely accurate as some spores presumed to have been killed, were simply washed off the carrier with up to 2.2×10^7 CFU/ml being released. This would also be expected with the other two *Bacillus* spore species and may explain why reductions in *Bacillus* spores were observed on treatment with the two alcohol-based sprays even though alcohols are non-sporicidal (Russell, 1990; Cortezzo *et al.*, 2004; McDonnell and Russell, 1999; Setlow *et. al.,* 2002; Setlow, 2006). However, adding the number of spores removed from the carriers to the number removed from the underlying paper towels as a result of physical washing of the spores, cannot account for all of the spores inoculated onto the carriers. This is because the data on spores present on the paper towels following spraying was obtained from carriers different to those used to determine the efficacy of the biocide sprays.

Although the method of spraying used in this study would require validation, the fact that only one spray was expelled per sample at a distance of 10 cm was an attempt to ensure that spray volumes were reproducible, with little variation existing between each application. It would also be useful to evaluate the extent to which spores adsorbed onto the paper towels to ensure that stomaching the paper towel would release all the spores that are attached to it.

3.5.1.2 Wiping

The use of biocides impregnated wipes was much more effective in spore eradication than spraying alone. This result was consistent with three previous studies (Hiom, 2000; Cockcroft *et. al.,* 2001; Panousi *et al.,* 2009).

Overall, wiping as a method of disinfection was found to be very effective, with all of the biocides achieving similar reductions in spore levels (figures 3.1 - 3.3). All biocides demonstrated at least a 1 log reduction against the *B. subtilis subsp. subtilis* 168 spores, with similar reductions being achieved by the two other *Bacillus* sp. spores tested. This may have occurred due to the increased contact between the spores and the biocides, as impregnated wipes allow direct contact in comparison to biocide sprays where coverage can be considerably less than 100%. Therefore the results achieved on wiping with the

biocides may be due to the physical removal of *Bacillus* spores, rather than the biocide impregnated in the wipe having an affect. This is supported by the observation that all of the biocide wipes tested achieved similar reductions in spore levels and were found to be almost equally effective in spore removal.

Panousi *et al.* (2009) demonstrated that alcohol wipes show poor antimicrobial efficacy (< 1 $\log_{10}$ reduction) against *Bacillus* spores. This leads to the possibility that, although the spores have been in direct contact with the biocide, they may not necessarily have been killed. Analysis of the wipes demonstrated that a large number of the *Bacillus* spores were transferred onto the wipe and were not killed by the biocide impregnated within the wipe (table 3.2). This was found to be the case with all the wipes tested. At least 14.8% of the *Bacillus sp.* spores that were dried onto the stainless steel carriers were picked up by the wipes on wiping. However, one biocide wipe proved to be better at the physical removal of the spores onto its wipe. This was the 70% v/v IPA in WFI wipe. Although not expected, the results with this wipe were better than 70% ethanol alone. These results were not statistically significant, but show that the wipe material composition may play an important role in spore reduction. The wipe impregnated with 70% v/v IPA in WFI which performed best, was composed of circular knit polyester, and this difference in texture (other wipes were polyester/cellulose) may be responsible for the improved spore removal. Similarly, the results observed with the SDW saturated wipe may have occurred because the wipe was composed of 100% cotton. Recently, the use of large pore-sized or thick wipes have been found to achieve better disinfection results against microorganisms in comparison to the use of small pore-sized and thin wipes (Lee *et al.*, 2007). Wiping with pre-impregnated wipes allows direct contact between the spores and the biocide. However, spraying biocide onto a wipe may result in some parts of the wipe becoming saturated, but, other areas may remain dry. Therefore spraying biocide onto wipes is not recommended compared to the use of pre-impregnated wipes (Panousi *et al.*, 2009).

The numbers of *Bacillus* spores transferred onto the wipes differed with different species tested. The *B. pumilis* spores were more easily removed by wiping than the two *B. subtilis* species tested. This may be partially explained by differences in spore coat surface characteristics. SEM analysis of the spore coat shows that the predominant

surface features are ridges, and these structures differ between and within species (Driks, 2004). The structure of *B. pumilis* spores is similar to *B. subtilis* spores, but *B. pumilis* spores are much smaller and in some cases the surface is nearly smooth (Bradley and Franklin, 1958). The smaller size and reduced presence of ridges (smoothness) of *B. pumilis* spores may play a role in the results observed because of the reduced friction between the wipe and the spores.

Recovery of viable spores from the wipes indicates that they were not killed by the biocide. These viable spores on the wipe can be transferred to the operator's gloves causing cross-contamination of any subsequent consumables being transferred into the aseptic area. A study using wipes against *Staphylococcus aureus* also established that wipes were a source of potential microbial transfer as bacteria were surviving on wipes (Williams *et al.,* 2007). The observation in the present study that spores remain viable after transfer to a biocide-impregnated wipe is crucially important. The implications of such findings are huge as using wipes may actually be worsening contamination by spreading spores to product components and other uninfected areas of the isolators if they are not used in a very strict aseptic manner. Consequently, the same portion of a wipe should not be used more than once as this may result in wiping amplifying contamination problems instead of alleviating them.

3.5.1.3 Spraying & Wiping

The combination of spraying and wiping achieved slightly higher log reductions (figures 3.1 - 3.4) than either spraying alone or wiping alone, with an average of at least 1.05 log reductions observed against the *Bacillus* spores. Cockroft *et al.* (2001) also found that a combination of spraying and wiping was more effective than spraying alone or wiping alone. The slight differences in log reductions obtained on use of the combination of spraying and wiping showed no significant advantage over wiping alone. However, both wiping alone and the combination of spraying and wiping were significantly better at reducing the number of spores than spraying alone and suspension testing.

The results observed were as expected (Hiom, 2000) as the contact time between the biocide and the surface to be disinfected is of great importance. This study employed a contact time of two minutes, although longer contact times generally achieve a higher reduction in spore levels (Fernando and Othman, 2006). By carrying out disinfection using a spray and wipe, the biocide exposure increases, allowing a longer contact time between biocide and spore organisms. Therefore some spores which may not have been killed on the application of a spray may be killed on prolonged exposure to the biocide when it is applied as a wipe. However, physical removal of the spores by wiping is the most important factor.

3.5.1.4 Suspension Testing

Suspension testing generally achieved a larger number of log reductions of *B. subtilis subsp. subtilis* 168 spores than the hard surface testing (figure 3.1). However, this was not the case with the spores of either *B. subtilis* ATCC 6633 or *B. pumilis* ATCC 27142 (figures 3.2 and 3.3). This may be due to the increased contact between the spores and the biocides, or as spores can differ in their susceptibility to biocides (Russell, 1999) the *B. subtilis subsp. subtilis* 168 spores are more susceptible to the biocides than the other *Bacillus* spores. Of the biocides tested, the alcohols (70% ethanol and 70% IPA) were not expected to have much effect against the *Bacillus* spores. This is because their thick spore coat prevents alcohols penetrating and acting upon the protoplast membrane (Russell, 1999). In this study the alcohols were found to exert some effect. Although the suspension test using alcohols achieved low log reductions (figures 3.1 – 3.3), increased reductions were achieved on wiping alone and on a combination of wiping and spraying with the alcohols. This indicates that the wiping can contribute to the removal of spores.

3.5.2 Biocide efficacy

The biocides tested differed in their efficacies against the *Bacillus* spores. Of the biocides tested, both the Quat./chlorine dioxide formulation and 6% H_2O_2 worked consistently better than the other biocides against all three spore species. The 6% H_2O_2 was the most effective, as it achieved higher than a 1 log reduction in each of the

disinfection methods tested on hard-surfaces (figures 3.1 - 3.3). The Quat./chlorine dioxide also achieved a higher than 1 log reduction against the spores in the suspension testing, wiping, and spraying and wiping. Only on application as a spray did it fail to achieve at least a 1 log reduction. However, this was as expected because although Quats are not sporicidal, both chlorine dioxide and H_2O_2 are known to possess sporicidal activity, including against spores of *B. subtilis* (Curnyn, 2005; Melly *et al.*, 2002; Setlow and Setlow, 1993; McDonnell and Russell, 1999; Setlow *et al.*, 1997; Young and Setlow, 2003).

The *Bacillus* spores may have been more susceptible to 6% H_2O_2 biocide if the temperature at which they sporulated (37°C) was altered. Sporulation temperature effects the resistance of *B. subtilis* spores against many inactivation agents including H_2O_2 (Melly *et al.*, 2002; Russell, 1990; Young and Setlow, 2003). Even though an increase in sporicidal activity may be achieved at higher temperatures, any activity not occurring at ambient temperatures is not relevant to a pharmacy cleanroom environment.

It is interesting that the Quat./biguanide and alcohol based biocides (70% ethanol and 70% IPA in WFI), which are considered sporistatic, were effective, to some extent, against spores in this study. Recently, work by Setlow *et al.* (2002) showed that a reduction in *B. subtilis* spores can be achieved by treatment with ethanol at 65°C. Alcohols have been found to inhibit germination (Cortezzo *et al.*, 2004). As the number of viable spores remaining is determined by germination of spores to CFUs, estimations of alcohol containing biocides by this method could over estimate their biocidal efficacy.

The biocide with the least efficacy against the *Bacillus* spores was 70% v/v denatured ethanol in deionised water. 70% v/v IPA in WFI also achieved fairly low spore reduction levels in comparison to the other biocides tested. As alcohols do not possess sporicidal activity (Russell, 1990; McDonnell and Russell, 1999) the results obtained re-enforce research that indicates the insufficiency of current disinfection protocols using alcohol sprays and wipes in the aseptic transfer process. Panousi *et al.* (2009) also demonstrated that alcohol wipes show poor antimicrobial efficacy (< 1 $\log_{10}$ reduction)

against *Bacillus* spores. Some reduction observed may have been due to the spores being physically removed by the washing (on spraying) or wiping (on wiping) process.

Quat./biguanide and SDW were equally effective in the removal of spores (figure 3.6). What was surprising was that on average the SDW was found to be a more effective biocide than the amphoteric surfactant and the two alcohol based biocides, although this difference was not found to be significant. However, the effectiveness of the SDW may be due partially to its application with an inert wipe which was found to achieve high log reductions in spore levels. All the biocides were found to attain very similar reductions in spore levels in both the "wiping alone" and "spraying and wiping" combination techniques.

The highest average number of log reductions observed by any of the biocides was only 1.22 logs (6% H_2O_2). This ineffective sporicidal activity is supported by BacLight™ viability assays carried out using 6% H_2O_2 treated *B. subtilis* ATCC 6633 spores (Appendix 11.2.1). These assays found that very few spores were killed following 2 minutes or 15 minutes contact with 6% H_2O_2. Indeed, the majority of *B. subtilis* ATCC 6633 spores remained viable. This finding correlates positively with the results obtained using traditional colony counting to show that the H_2O_2 is not very efficacious (no 3 log reductions achieved) against the *B. subtilis* ATCC 6633 spores. This also demonstrates that the number of viable spores counted following the tested disinfection methods is reflective of the level of kill being achieved, whereby the number of viable spores remained high following 6% H_2O_2 exposure. Also the relatively low log reduction levels of spores being recovered from the inoculated, disinfected carriers, indicates that large numbers of spores remain on the stainless steel plates even following spraying and wiping. This contradicts the results obtained in a previous study which was carried out on the same spore species (Curnyn, 2005). This researcher found no spores present on recovery following the wiping alone and spraying followed by wiping methods. None of the biocides tested gave a complete eradication of spores from the inoculated stainless steel carriers. Therefore it can be established that none of the biocides tested are 100% effective. Even though some reduction is observed, the required level of reduction to satisfy an aseptic sporicidal process is not obtained because log 3 or higher reductions were never achieved (Lambert, 2004).

The results achieved are probably due to the *Bacillus* spores requiring to be exposed to the biocides for a longer time in order for the biocide to bind, enter and take effect on the inner spore. The lower log reductions achieved at 2 minutes is attributable to the impermeability of the spore coat and the diffusion of the biocide across this multi-layered coat. The spore coat serves as a barrier to biocides and prevents penetration to the fundamental cellular components inside the spore. It is suggested that the biocide diffuses through the outer spore layers where it is tolerated by the cell in low concentrations. As the concentration of the biocide increases at the inner side of the outer membrane it reaches a critical level and the cell becomes irreversibly damaged and after a period of decay, eventually dies (Fernando and Othman, 2006). Therefore time is an important factor in the treatment of spores by biocides, as a longer exposure time is thought to allow further penetration of the biocide into the spore. A number of chemical compounds have been found to be sporicidal on longer contact times (Russell, 1990; Fernando and Othman, 2006). The results obtained on hard-surface testing in comparison to any suspension tests are deemed to be more constructive and therefore more reliable when calculating the efficacy of biocides used in hospital pharmacies. However, a longer contact time with biocide is not always practical within the confines of a hospital pharmacy based cleanroom environment.

Log reductions were found to differ against the three different *Bacillus* species tested. Higher log reductions were achieved by the biocides against *B. subtilis subsp. subtilis* 168 spores than against spores of *B. subtilis* ATCC 6633. Therefore, the biocides had different efficacy against different biological test organisms. No single biocide was the most effective against all three *Bacillus* spore species. Of the *Bacillus* spores tested, the *B. subtilis subsp. subtilis* 168 strain was the most susceptible to the biocides investigated. This was followed by the *B. pumilis* ATCC 27142 spores and *B. subtilis* ATCC 6633 spores, respectively.

Overall this study indicates that the method of disinfection employed is of great importance in a hospital pharmacy aseptic environment, as the efficacies of the biocides investigated differed between each disinfection method used. This demonstrates that the disinfection method contributes to the overall efficacy of a biocide. Thus it is important to validate the method of disinfection as well as the biocides themselves. In

addition, the use of alternative biocides to the standard alcohols currently being used should be considered in disinfection.

CHAPTER 4 HYDROGEN PEROXIDE GASSING DECONTAMINATION OF SPORES

4.1 Introduction

Alternative methods of disinfection, other than heat and chemical disinfection are becoming available. Although H_2O_2 in the liquid and semi-solid (foam) phases has been widely used for numerous sterilisation products, another method using H_2O_2 in the gas or vapour phase is becoming increasingly popular. This method is a frontrunner for replacement of conventional hard-surface disinfection methods such as those investigated in the previous chapter, where surfaces to be disinfected were sprayed, wiped or subject to a combination of both allowing for a 2 minute contact time with the biocides.

Therefore this chapter presents the results of vapourised H_2O_2 (VHP) gassing on the *Bacillus* spore viability. This would allow a direct comparison to be carried out between the conventional spraying and wiping techniques using 6% H_2O_2 and the relatively newer 'gassing' disinfection method. One aim is to compare the efficacy of VHP gassing technology for treating the *Bacillus* spores with the results obtained using conventional chemical disinfection methods in Chapter 3. Investigating both the level of log reduction achieved (thought to be at least 10^6-10^7 log reductions in spores (Otter and French, 2009; Meszaros, *et al.,* 2005) using biological indicators to validate the process) when a 30 minute exposure time is used and also the time in which the log reductions are achieved, would enable the efficacy of the H_2O_2 gassing process to be established. The use of time-kill experiments would determine the efficacy of the 30-35% VHP against the *Bacillus* spores in relation to exposure time, to see what levels of log reduction are obtained with the use of shorter gassing times. This would also determine whether the levels of kill obtained after the shorter exposure times are adequate for the disinfection of components which are regularly used in the compounding of pharmaceutical products. As a 3 log reduction is required to deem a liquid disinfectant a sporicide (BS EN, 2002; EPA, 2007), the exposure time taken to achieve this level of kill will be crucial to the possible modification

(reduction) of the VHP gassing process with regard to the time required to disinfect against robust *Bacillus* spores.

4.2 Aim

- To establish the efficacy of vaporised H_2O_2 against *Bacillus* spores.

4.3 Materials and Methods

4.3.1 Vaporised H_2O_2 gassing

Stainless steel coupons were inoculated with 25 µl of spore suspension (10^7 - 10^8 CFU/ml): *B. subtilis subsp. subtilis* 168, *B. subtilis* ATCC 6633 or *B. pumilis* ATCC 27142. The inoculated coupons were incubated aerobically at 37°C for 30 minutes to dry the spores onto the carriers. The coupons were then placed into a gassing chamber connected to a vaporised H_2O_2 generator (Steris® VHP 100) and the gassing cycle initiated. A VHP chemical indicator was used to determine that the gassing cycle reached the H_2O_2 concentration required.

After a 30 minute exposure time, the coupons were removed and placed in 10 ml of modified Letheen broth based neutraliser solution. The coupons were stored in the neutraliser for up to 24 hours (this was validated using a viability count carried out on untreated spores stored in neutraliser for a 24 hour period; Appendix 11.3.1.1). The coupons were then individually transferred along with the Letheen broth based neutraliser into stomacher bags (Seward, Leicestershire) and agitated in a stomacher machine for 2 minutes. 0.1 ml of the stomacher solution was added to 0.9 ml of AGFK germinant solution and incubated at 37°C for 45 minutes. After incubation the germinant treated solution was serially diluted in neutraliser (0.1 ml into 0.9 ml) and 0.1 ml of each dilution plated onto TSA plates. The plates were then incubated aerobically at 37°C overnight and CFU counted. CFU recovered from VHP exposed coupons were compared to those recovered from positive controls whereby spores were inoculated onto the coupons but were not exposed to VHP. The mean log reduction in spore number was calculated as follows:

Mean Log Reduction in Spores =

$\log_{10}$ (average number of CFU recovered from positive control) – $\log_{10}$ (average number of CFU recovered from disinfected coupons)

4.3.2 Statistical analysis of data

A non-parametric statistical analysis was carried out on all data using an ordinary one-way ANOVA with Tukey–Kramer multiple comparisons test (GraphPad InStat, ver. 3.06; Graphpad Software Inc., La Jolla, USA) was used to compare the VHP activity against the different spore species. Data are expressed as mean ± SEM. Differences were considered to be statistically significant when $P < 0.05$.

4.4 Results

4.4.1 Efficacy of 30 minute VHP exposure against *Bacillus* spores

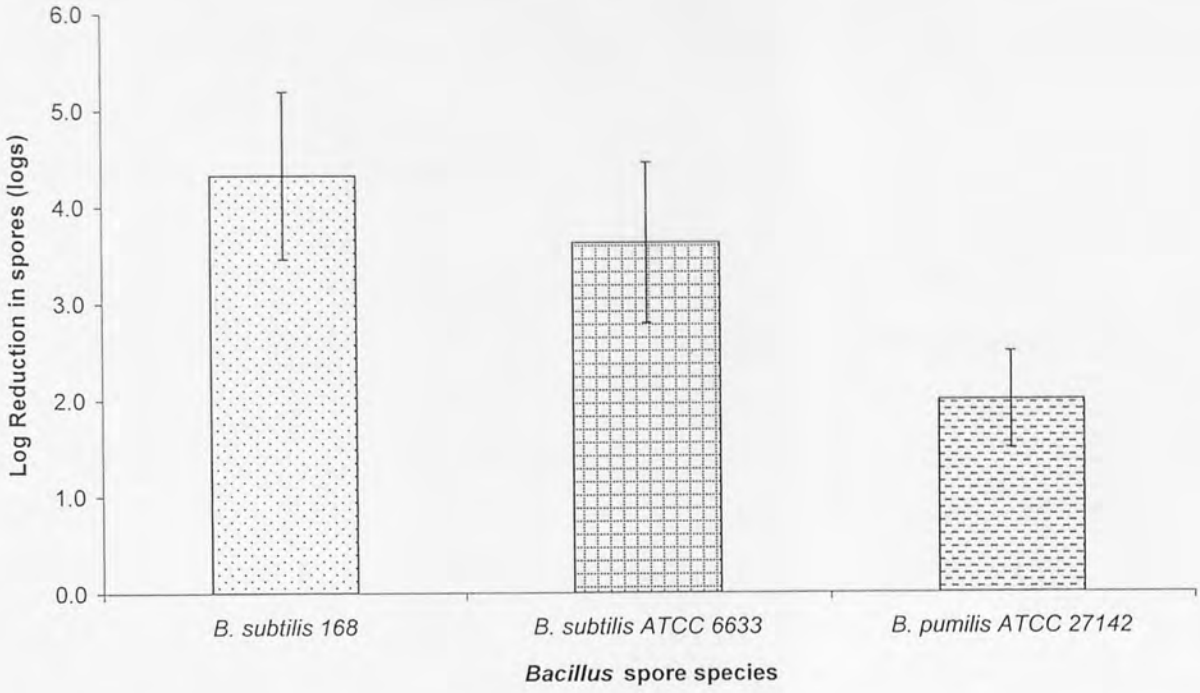


Figure 4.1 Efficacy of VHP against *Bacillus* spores. Log reduction in *Bacillus* spores following 30 minute treatment with VHP (n=3). Data are presented as mean log reduction ± SD for each *Bacillus* spore type.

A 2.0 - 4.3 log reduction in spore numbers of all three *Bacillus* spore types tested was observed (figure 4.1). A mean of 4.3 ± 0.8 log reductions were achieved with the 30 minute VHP gassing of the *B. subtilis* 168 spores. This was followed by a lower 3.6 ± 0.8 log reduction against the *B. subtilis* ATCC 6633 spores and a 2.0 ± 0.5 log reduction against the *B. pumilis* ATCC 27142 spores.

The 30 minute VHP exposure achieved an average of at least a 2 log reduction in *Bacillus* spore numbers. Log reductions were found to differ against the three different *Bacillus* species tested, whereby higher log reductions and therefore the highest efficacy was achieved by the VHP treatment against *B. subtilis subsp. subtilis* 168 followed by the *B. subtilis* ATCC 6633 spores. The VHP was least efficacious against the *B. pumilis* ATCC 27142 spores. Although different levels of log reduction

were achieved against the three different spore types, the differences between them were not significant ($P > 0.05$).

4.5 Discussion

H_2O_2 is a very powerful oxidiser that has been used as an antiseptic and antibacterial agent in residential, industrial and commercial applications for over 100 years. It is also used in commercial and industrial environments as a means of decontamination of sealed enclosures such as isolators, pass-through rooms, and cleanrooms.

The results achieved against the *Bacillus spores* are to some extent in contrast to the results of the conventional chemical disinfection methods as determined in chapter 3. Using the spraying and wiping techniques of disinfection, the H_2O_2 was found to be least efficacious against the *B. subtilis* ATCC 6633 spores with higher levels of log reduction being achieved against the other 2 spore species tested. Once again the *B. subtilis subsp. subtilis* 168 strain was found to be the most susceptible to the disinfection process tested; which in this case was the VHP treatment. The *B. pumilis* which was the least susceptible to the VHP exposure, was not the least susceptible in the chemical disinfection testing.

Overall, the higher log reduction levels in spore numbers achieved here demonstrate that decontamination against these *Bacillus* spores on stainless steel was much more effective using the VHP than the traditional spraying and wiping techniques. VHP gassing therefore met the standard 3 log reduction required of a liquid 'sporicide' when tested against two of the three *Bacillus* species. Yet, validation guidelines state that at least a 6 log reduction in spore levels should be reached in order to designate the VHP method as a validated sporicidal disinfection method (BS EN, 2002; EPA, 2007). The 6 log reduction is required to ensure overkill and is an increase that is a great deal higher in comparison to the 3 log reduction required by the spraying and wiping techniques. However the VHP method can be considered as being more sporicidal in relation to the chemical disinfection results, as it reaches the criteria required to classify a liquid disinfectant or disinfection method as being sporicidal (BS EN, 2002; EPA, 2007) for two of the three *Bacillus* species. Therefore marketing VHP gassing as being effective in the decontamination of bacterial spores on which the sprays and wipes would otherwise be fairly ineffective against, is reasonable when used in this context.

Although more than a 3 log reduction was achieved against two of the three spores tested following the 30 minute exposure to the VHP, a 6 log reduction was not achieved for any of the spore species. So once again it is very important to identify which particular spore types are problematic in specific laboratory environments and aseptic processing areas, departments and units.

The cleanroom bioburden level of a Grade A (Class 100) cleanroom is 1 CFU/cm^3 of air. This value is several orders of magnitude less than the 10^6-10^8 CFU of spores being inoculated onto the test coupons. Therefore by testing at 10^6 to 10^8 the bioburden is 10,000 to 1,000,000 times that of normal bioburden levels seen in the cleanroom. Consequently, the actual spore numbers being used to calculate the log reductions are far higher and unrepresentative of cleanroom bioburden. However, the majority of studies still require the use of a high bioburden level when testing for chemical efficacy (Lambert, 2004).

One factor to take into consideration is that previous researchers have often determined log reduction in spore counts, not by carrying out viable counts, but by determining the post-disinfection absence or presence of growth when coupons are placed in a nutrient broth over 7 days. Following neutralisation, these 'disinfected' coupons are often placed into nutrient broth to detect the presence of any remaining microorganisms. They are not recovered onto nutrient agar plates which would allow viable counts to be obtained, but are just designated as being either positive or negative for growth. In addition, the coupons are not always treated with a germinant solution to allow the recovery of any sub-lethally injured spores which may be viable should the right conditions arise. Therefore the application of an AGFK germinant treatment stage was used in all testing to encourage the growth of any remaining spores that may have become sub-lethally injured during the disinfection process.

The method used to determine VHP gassing efficacy was identical to that used in testing the efficacy of the biocides when used as sprays and wipes. The gassing was used in place of the spraying and/or wiping, with the only other difference being the time the post-treatment spores were stored in the neutraliser medium and the contact time. As the exposure of neutraliser to the spores for 24 hours was validated

(Appendix 11.3.1.1), it can be ascertained that the VHP gassing was the only contributing factor in the bio-decontamination results obtained.

The gassing method itself has been established as being a rapid method which demonstrates a broad biological efficacy against microorganisms, including bacteria, viruses, fungi, mycobacteria and bacterial spores (French *et al.,* 2004; Klapes and Vesley, 1990; Block, 2001; Heckert *et al.,* 1997; Kokubo *et al.,* 1998; Kahnert *et al.,* 2005; Meszaros *et al.,* 2005). The main mechanism of VHP action is likely to be through the production of hydroxyl radicals which then react with membrane lipids, proteins and DNA (McDonnell and Russell, 1999).

The efficacy of VHP gassing can be explained because gassing with H_2O_2 is a dry process that is able to achieve rapid antimicrobial activity at low concentration in contrast to liquid H_2O_2. The H_2O_2 concentration in biocides for surface wiping is generally 3% (30,000 ppm) with H_2O_2 found to be a more effective antimicrobial agent as a gas in comparison with a liquid (Block, 2001). VHP has been demonstrated as being sporicidal at concentrations as low as 1-2 mg/L at 25°C (Steris®, 2008). The H_2O_2 concentration in an aqueous solution is therefore over 20,000 fold greater than the concentration of H_2O_2 in a vapour-phase to achieve a similar kill. Liquid H_2O_2 concentrations of 200 ppm would require approximately 2-6 hours contact time to destroy anthrax spores (Block, 2001). Therefore H_2O_2 gassing is marketed as being effective in the decontamination of a wide variety of surfaces on which sprays and wipes would otherwise be ineffective against over a shorter time period.

Techniques using VHP have been found to achieve 6 log reductions in spore numbers. Otter and French (2009) found that within 90 minutes of exposure to VHP in a 100 m^3 test room, all spores and vegetative bacteria were inactivated. Jonston *et al.* (2005) found that for *Clostridium botulinum* spores dried onto stainless steel surfaces, the decimal reduction times or D-values (times required to kill 90% of the organisms) ranged from 1.41 to 4.38 min. Meszaros *et al.* (2005) established a VHP D-value for *Geobacillus stearothermophilus* spores of 42.3 seconds. It was only on a prolonged incubation of 60 minutes exposure to VHP that a 6 log reduction in *G. stearothermpohilus* spores was observed. This is in contrast to Steris® (2008)

whereby, *B. stearothermphilus* spores exposed to VHP at 1mg/L had a D-value of 150 seconds. So according to Steris® this method of disinfection is very effective, achieving a 90% reduction in spore bioburden on surfaces in less than 3 minutes exposure.

Furthermore, the efficacy of VHP has been shown to be compatible with a wide range of materials including carpet, glass, wood, concrete and metal (Rogers *et al.,* 2005). Therefore this H_2O_2 gassing technology allows the effective decontamination of a wide variety of surfaces on which the sprays and wipes would otherwise be ineffective against. The utilisation of gassing is preferred to liquid disinfection as it is highly effective in both small and large enclosed spaces such as cleanrooms, research areas and ambulances (Jahnke and Lauth, 1997; Malborg *et al.*, 2001; McDonnell, 2002; Krause *et al.,* 2001; Andersen *et al.,* 2006). As a result the technique can accommodate any sized environment required to be disinfected.

Although the use of H_2O_2 as a liquid disinfectant has the disadvantage of possibly leaving residues when used for a short period of time, gassing has the difficulty of ensuring that there is total coverage over an area. However, VHP is also able to penetrate porous materials. For example, H_2O_2 appears to have the ability to penetrate the porous texture of a sponge (Ikawa and Rossen, 1999). A 100% kill was reported after 3 hours for a spore suspension of 10^6 cfu/ml, and a 100% kill was reported after 6 hours for a stainless steel carrier coated with a spore suspension (Spotts-Whitney *et al.*, 2003). The penetration of porous paper is extremely important in the maintenance of a cleanroom environment because the consumables often used, such as syringes, are encased in a mixture of plastic and paper packaging which can be a route of contaminant introduction into the cleanroom process. Microbial resistance to oxidative stress is overcome by the higher concentrations of oxidative agents used for disinfection and sterilisation (McDonnell and Russell, 1999). In terms of sporicidal activity, H_2O_2 as a solution has been found to be much less sporicidal than the vaporised form at low concentrations (Carlson, 1996), although at lower temperatures its reaction time is considerably longer (Wagner and Yang, 2002). Also, unlike liquid peroxide, VHP has been found to be rapidly sporicidal at concentrations as low as 0.1 mg/L. A further advantage is that VHP has an excellent safety profile (McDonnell, 2002) even though it is able to achieve a higher concentration than when the H_2O_2 is

used as a liquid. This is also because unlike liquid peroxide, VHP is rapidly broken down into oxygen and water vapour, therefore presenting no significant environmental concerns.

Due to rapid gassing, the contact time between the VHP and the items to be decontaminated is very short and as a consequence any concerns regarding absorption of H_2O_2 into the solutions and liquids being decontaminated do not occur (Muller *et al.*, 2003). This is crucial as the possible contamination and resultant cross-reaction between such peroxide products and the oxidisable components of for example, the contents of a parenteral nutritional intravenous (IV) bag, could result in the formation of new dangerous compounds which could in turn compromise both product integrity and patient health.

Unfortunately, it was outwith the scope of the present study to test longer or shorter gassing exposure times. As a consequence, the VHP exposure time required to attain a 3 log reduction could not be established. In addition, only one of the two major gassing methods was tested, with the relative humidity being one of the key differences between the VHP gassing systems. Both these methods have been developed by Steris® and Bioquell®, the producers of such vapour phase generators. Bioquell® have developed the Clarus C™ generator which is a sterilisation unit with a potential application in the sterilisation of isolator surfaces. The two H_2O_2 gas systems that are available for use in bio-decontamination are similar, yet different. They are usually distinguished as being either 'wet' or 'dry' processes largely based on the physical chemistry of H_2O_2. Steris® (a dry process) maintains a low humidity (< 40%) at the start of disinfection and ensures that the VHP is maintained at a concentration below the condensation point, generally at 0.1 - 1.5mg/L at 25°C (Steris®, 2008). This is to ensure that the H_2O_2 remains as a vapour and does not condensate. However, in comparison, the wet process (as employed by Bioquell®) produces a gas which at a higher relative humidity results in micro-condensation, whereby a thin film of H_2O_2 (70%) is deposited over the surface to be biodecontaminated.

The testing carried out in this investigation against the three spore types used the Steris® technology dry exposure method. A comparison with the wet process as

employed by Bioquell® would also have determined the viability of an alternative VHP decontamination system that is used throughout the world. Bioquell® decontamination systems are currently being used in several NHS hospitals throughout the UK in the form of robots to tackle Methicillin Resistant *Staphylococcus aureus* (MRSA) and *Clostridium difficile* (Cowart, 2008). Yet another difference between the two processes is that Bioquell® use 30% liquid H_2O_2, whereas Steris® use 35% liquid H_2O_2. It can be established that the concentration of H_2O_2 employed by these processes is very high in comparison to those used in liquid disinfection, where H_2O_2 is used directly on the skin at 30,000 mg/L (3%), and in liquid biocides at 6% (60,000 mg/L) (McDonnell, 2002). As both of these systems offer different processes, they also vary in their antimicrobial efficacy, as well as their compatibility with surfaces.

There are numerous disadvantages of the use of gassing with H_2O_2 in relation to the more widely-used spraying and wiping techniques. Overall, VHP technology ensures the safe recovery of a sterile product while allowing complete control of the process. However, it is thought that the use of VHP can damage sensitive equipment due to micro-condensation of the H_2O_2 and therefore a 'dry' process is often required. From experience, the machinery can be unpredictable with equipment breaking down while a gassing cycle is occurring. One of the most important factors in relation to a busy pharmacy aseptic processing unit is the time taken for the entire gassing process. Although they are often described as rapid gassing methods, the time taken for disinfection to occur will vary with the number of products being disinfected, the type of product and the disinfection area size.

The varying results between the biocide solutions as sprays and wipes and the VHP reinforce the fact that the method of application of a biocide affects both its physical and biological properties. In general, the VHP gassing efficacy results against the *Bacillus* spores tested were better than that of the more traditional spraying and wiping methods, with greater log reductions being obtained against the robust endospores. However, other VHP exposure times need to be tested to determine the length of exposure time required to allow quick and effective processing of the products being disinfected. The labour intensive and time-consuming disinfection methods of spraying and wiping are known as traditional disinfection methods that are

regularly employed in the disinfection of aseptically produced sterile parenteral products. On the whole, as an alternative, VHP gassing is a method that in the future may altogether replace spraying and wiping due to its decreased labour dependency and higher antimicrobial efficacy. But, presently the use of such traditional disinfection techniques continue to be the most widely used methods of disinfection.

CHAPTER 5 SUBMERGED CULTURE OF BEAS-2B CELLS

5.1 Introduction

Human operators are likely to inhale biocides as they are regularly exposed to them. So the damage to the airways should be examined. Ultimately a balance between biocide efficacy and human risk is necessary, with a biocide having excellent biocidal activity but low toxicity.

5.1.1 Cell culture models

Cell culture models have been developed and used in toxicity testing (Bakand *et al.*, 2005; Eisenbrand *et al.*, 2002) as they enable toxicity to be directed against a particular region of the human body at the cellular level. This allows determination of the response of an exact, defined area of the body that is likely to come into contact with the toxic agent. An *in vitro* physiological model representing the native airway epithelium was used in the current study, as it is the epithelial cells of the upper airways (i.e. nasal and bronchial epithelium) that are the first points of contact of inhaled irritants and so at the most risk of exposure.

BEAS-2B cells are human airway epithelial cells derived from normal human bronchus (Oslund *et al.*, 2004) and transformed by infection with an SV40-adenovirus 12 hybrid virus (Reddel *et al.*, 1988). This cell line is representative of the epithelial cells of the upper airways and has been used to investigate the effects of inhaled environmental pollutants such as dust (Wilson *et al.*, 2000) cigarette smoke (Laan *et al.*, 2004) and diesel exhausts (Kawasaki *et al.*, 2001). As well as transformation occurring by transfection of single genes into epithelial cells (Reddel *et al.*, 1988), other immortalized human airway epithelial cells have been created through the stepwise introduction of genetic alterations using cellular oncogenes (Lundberg *et al.*, 2002). One example is the expression of the large T oncoprotein of the SV40 virus which enables cultured cells to bypass replicative senescence (Stewart and Bacchetti, 1991;

Lundberg *et al.*, 2002). Clonal cultures derived using such methods retain the characteristics of airway epithelial cells, as well as having extended i*n vitro* lifespans.

This chapter investigates the toxic effects of commonly used cleanroom biocides on BEAS-2B cells in submerged culture. The parameters investigated include the cytotoxic effects of the biocides upon the cells with regards to cell viability, changes in the pro-inflammatory status of the cells, potential oxidative effects of the biocides on the cells, and the mechanism of cell death.

Bronchial epithelial cells were exposed to the test chemicals suspended in culture medium. Dose-response experiments were used to estimate toxicity at different biocide concentrations. The cells were exposed to biocide for 24 hours before the biocide was removed and cells were allowed to recover for 48 hours. By allowing a recovery period, any cells that may have been sub-lethally damaged on exposure to the biocides would be able to repair themselves. Assessing the toxicity of the biocides at the epithelial cell surfaces will establish if biocide efficacy correlates with operator toxicity. This would help to determine whether a compromise can be found between biocide efficacy and low levels/absence of toxicity when trying to eliminate *Bacillus* spores.

5.1.2 Toxicity testing

There are many cytotoxicity assays available for use in determining the toxic effects of chemicals on different cells. The assays investigate a number of biological endpoints that, taken as a whole, can give an indication as to whether the chemical being tested is in fact toxic to the cells of interest.

5.1.3 Cell morphology

One of the biological endpoints examined here was cell morphology. BEAS-2B are adherent cells and show the cobblestone appearance of healthy normal epithelial cells (Reddel *et al.*, 1988: Ke *et al.*, 1988). Changes in the morphology of epithelial cells indicate changes in the health status of the cells. If changes occur in cell size, cell shape

or cell-cell contacts, then this suggests that alterations have occurred in the cell structure, with consequences for cell function, and viability.

5.1.4 Cell viability

Determining cell viability by establishing the number of remaining viable cells following exposure to chemicals is another method used to ascertain the toxic effects of biocides on cells. Decreased cell viability suggests that cells have been damaged and therefore may no longer have the ability to function effectively.

5.1.5 Chemokine release

Airway epithelial cells have the ability to express and release different types and quantities of cytokines, which are modulatory compounds that play an important role in inflammation (Mills *et al.,* 1999). The production of pro-inflammatory cytokines by airway epithelial cells may contribute to the pathological and clinical events that occur in a number of airway inflammatory diseases including chronic obstructive pulmonary disease (COPD) and cystic fibrosis (Repine *et al.,* 1997; Witko-Sarsat *et al.,* 1995, Brown *et al.,* 1996). Excessive production of pro-inflammatory mediators by the airway epithelium is proposed to have a key role in the development of tissue injury during acute and chronic inflammatory conditions, implicating the airway epithelium in the pathogenesis of inflammatory lung diseases (Rahman *et al.,* 2001).

Airway inflammation may be characterised by an increased infiltration of neutrophils to the tissue, and increased levels of cytokines and chemokines including IL-8 (Khabar *et al.,* 1997; Weiss, 1989; Weissmann *et al.*, 1980, Bédard *et al.,* 1993; Inoue, 1999; Martin *et al.*, 1997). IL-8 contributes to inflammatory responses associated with inflammatory lung diseases. Soluble IL-8 secretion from the airway epithelial cells in response to the biocides was measured here as an indicator of the pro-inflammatory status of the cells following biocide exposure.

5.1.6 Protein oxidation

Proteins can be targets of reactive oxygen species and detection of oxidatively modified proteins are often used as indicators of oxidative stress (Ischiropoulos and al-Mehdi, 1995). Protein oxidation is more often used as a measure of oxidation rather than lipid peroxidation, as the oxidised proteins are formed earlier and are relatively stable (Cockell and Belonge, 2002). Therefore, in the current study, protein oxidation rather than lipid peroxidation, was analysed as a measure of oxidative stress.

One indicator of oxidative stress is the protein thiol, which is a compound that contains the functional group composed of a sulphur atom and a hydrogen atom (-SH). The thiol moiety is very susceptible to oxidation. The oxidation of cysteine residues in proteins is often used as a method of determining the loss of protein thiol groups (Yan and Sohal, 2002). Thiol groups play an important role in defence against oxidative stress and healthy cells have a large number of free (reduced) thiol proteins (Smit-de Vries *et al.*, 2007). During oxidative stress, oxidants are able to bind to the thiol (-SH) moiety on the side chain in highly reactive cysteine residues rendering the thiol oxidised (Eaton, 2006). This thiol moiety is particularly sensitive to redox reactions and the interaction of thiols with a variety of oxidants, results in the formation of a reversible covalent modification. Whereas previously the thiol groups of two cysteine residues would form a disulphide bond during protein folding, direct oxidation of the cysteine residues destroys the ability of the protein to form disulphide bonds. Therefore, oxidation of free thiol groups can lead to the production of incorrectly folded proteins. In addition to this, the post-translational modification of proteins has been established as one of the major effects of oxidative stress (Ying *et al.*, 2007; Stadtman and Berlett, 1998). This post-translational modification is thought to occur in cytoplasmic proteins (Ghezzi and Bonetto, 2003).

By determining the level of free thiol groups present in the cell lysates prior to and following exposure to biocides, the oxidative effects of the biocide on intracellular proteins can be ascertained. Detection of free intracellular thiols can be achieved by measuring 5'5'-dithiobis-2-nitrobenzoic acid (DTNB) binding (Eaton 2006; Sanchez *et al.*, 2008). DTNB reacts with thiolate ions in a thiol-disulphide reaction, resulting in the generation of the chromogenic product 2-nitro-5-thiobenzoic acid (NTB). The

absorbance of the NTB can then be measured and used to calculate the free thiol content.

Oxidative stress and injury can also result in the formation of carbonyl moieties due to damage to proteins (Carty *et al.*, 2000). Protein carbonyls are biomarkers of protein oxidation mediated by reactive oxygen species. Carbonyl derivatives occur on direct oxidation of lysine, proline, arginine, histidine and threonine residues. These carbonyl groups represent an irreversible protein modification. Therefore increased levels of protein oxidation can be detected via an increase in the number of protein carbonyls.

There are several carbonyl assays available and used for the detection and quantitation of carbonyl content in oxidatively modified proteins (Yan and Sohal, 2002). One colorimetric method used to assay for cellular protein carbonyl content (Buss *et al.*, 1997, Robinson *et al.*, 1999) uses dinitrophenylhydrazine (DNPH) to derivatise isolated oxidised proteins. These are subsequently detected with an anti-dinitrophenyl (DNP) antibody and enzyme tagged to allow absorbance to be measured upon addition of an enzyme substrate.

Other methods used to detect carbonyl moietes in proteins employ High Pressure Liquid Chromatography (HPLC) and western blotting (Cockell and Belonge, 2002). There are also immunochemical, enzyme-linked immunosorbent assay (ELISA) methods available to detect carbonyl moieties (Alamdari *et al.*, 2005). The ELISA method of assessing oxidative damage is increasingly used and preferred as it is quick, specific, sensitive, accurate and reproducible. It also allows the quantitative analysis of protein oxidation using the protein carbonyls as a biomarker. In comparison to the colorimetric assay which requires at least 1mg of protein, the ELISA, which also relies on DNPH-derivatisation, only requires 60µg of the protein. The use of a primary antibody directed against the DNP labelled proteins allows for a high specificity for any carbonyl groups. One immunochemical assay (Robinson *et al.*, 1999) has been found to be approximately 1700-fold more sensitive than the conventional colorimetric assays while retaining a high level of specificity.

5.1.7 Cell death

Exposure of the cells to cytotoxic biocides may result in the cells undergoing necrosis or apoptosis as a result of the stress that the cells come under due to biocide-induced damage. Cells may be analysed for necrosis by staining with propidium iodide (PI), which is a nuclear DNA stain often used to differentiate between apoptotic, necrotic and normal cells (Hampton and Orrenius, 1997). PI binds to the nuclei of the cells only after the membrane becomes permeable. So if the permeability of a cell membrane is compromised due to damage, it allows the PI stain to pass through into the cell, bind to the DNA and fluoresce. Therefore the increased fluorescence of a cell on exposure to PI is indicative of necrosis, as necrotic cells have damaged cell membranes. Whether the cells show any necrotic phenotypes is important in understanding what happens to the cells following biocide exposure because necrotic cells can themselves cause oxidative damage to the surrounding cells.

5.2 Aims

- To determine whether the biocides, when applied at, and as a percentage of, their in-use concentrations, affect the viability of BEAS-2B grown in submerged culture.

- To ascertain whether biocides induce a pro-inflammatory response by BEAS-2B cells in submerged culture.

- To determine the thiol and carbonyl content of submerged cultured BEAS-2B cells when acutely exposed to biocides.

- To assess cell death and determine whether BEAS-2B cell death occurs by necrosis.

5.3 Materials and Methods

5.3.1 Materials

5.3.1.1 Cell culture materials

Promocell® Airway Epithelial Cell Growth Medium (AECGM) and SupplementMix were from Promocell (Heidelberg, Germany). Insulin, transferrin and selenium (ITS), Penicillin, Streptomycin, PBS, Tween®-20, Trypsin-EDTA, Trypan Blue Solution, Triton X-100, Bovine Serum Albumin, Sodium Dodecyl Sulfate, Sodium hydroxide, Cysteine, Carbonate bicarbonate buffer, Dinitrophenylhydrazine (DNPH), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), anti-DNP mouse immunogloubulin E (IgE), Peroxidase labelled rat anti-mouse IgE, Sulphuric acid, Hydrochloric acid, Tris buffer, SigmaFAST™ OPD substrate solution, Anisomycin, Staurosporine, Ionomycin, Etoposide, Greiner® 75cm^2 cell culture flasks, Nunc MaxiSorp™-96 well microtitre plates and Human placental type IV collagen were all from Sigma (Dorset, UK). 0.5 mm diameter needles were from Neolus, Terumo UK Ltd. (Surrey, UK). 2 ml syringes were from Becton Dickinson (Oxford, UK). BD Falcon™ 24-well multiwell culture plates were from BD Falcon (Oxford, UK). 5% Foetal Calf Serum (FCS) and Modified Eagles Medium was from PAA (Somerset, UK). 96-well microplates were from Fisher (Leicestershire, UK). Solid black mictrotitre plates were from Thermo Scientific (Basingstoke, UK). BCA Assay kit was from Pierce (Northumberland, UK). CellTiter-Blue™ reagent was from Promega (Southampton, UK). IL-8 ELISA Kit was from Peprotech EC Ltd. (London, UK).

5.3.1.2 Human bronchial epithelial cells

Epithelial cells used in the toxicity testing experiments were BEAS-2B normal human bronchial epithelial cells (adenovirus-12 SV40 hybrid virus transformed, non-tumorigenic human bronchial epithelial cells). These were obtained from the American

Type Culture Collection (ATCC) (CRL-9609). BEAS-2B were used between passage numbers 3 – 19.

5.3.2 Methods

5.3.2.1 Submerged epithelial cell culture

All cells were seeded on human placental type IV collagen-coated (10 $\mu g/cm^2$) 75 cm^2 tissue culture flasks. Collagen coating was carried out by adding the human placental type IV collagen (10 $\mu g/cm^2$) to the flasks and incubating for 30 minutes. The collagen was then removed and the flasks washed three-times with phenol-red containing Modified Eagles Medium (MEM) prior to seeding with cells. Human type IV collagen acts as a substitute basement membrane for cells *in vitro* and encourages them to maintain a representative, *in vivo* physiology (Fiedler *et al.*, 1991).

BEAS-2B were maintained in full medium comprising Promocell® Airway Epithelial Cell Growth Medium (AECGM) basal medium supplemented with 5% FCS and 1% penicillin (5,000 U/ml^{-1}) and streptomycin (5000 $\mu g/ml^{-1}$). A manufacturer's supplement was added containing the following growth factors: Bovine Pituitary Extract (0.4%), Epidermal Growth Factor (10 mg/ml), insulin (5 µg/ml), hydrocortisone (0.5 µg/ml), epinephrine (0.5 µg/ml), triiodothyronine (6.7 ng/ml), transferrin (10 µg/ml) and retinoic acid (0.1 ng/ml).

Cells were grown under standard cell culture conditions at 37°C and 5% CO_2. Cultures were viewed using an inverted microscope to assess the degree of confluency and confirm the absence of bacterial and fungal contaminants (healthy BEAS-2B cells were of typical cobble-stone appearance). The medium on the cells was removed every 2-3 days and replenished with fresh full medium (containing serum) until the cells were confluent.

5.3.2.2 Passaging and seeding cells onto well culture plates

Spent medium on the cells was removed. The cell monolayer was washed with PBS to remove traces of FCS, which would inhibit trypsin activity. 4 ml of 0.25% trypsin/EDTA was then added. The flasks were then returned to the incubator and left for 20-30 minutes until the cells had detached. Care was taken to ensure that the cells were only exposed to the trypsin/EDTA long enough to detach the cells as prolonged exposure could damage cell membranes and surface receptors. The cells were then examined using an inverted microscope to make sure that all the cells were detached and floating. The detached cells were resuspended by adding 5 ml of fresh full medium to each 75 cm^2 culture flask (to inactivate the trypsin). The cell suspension was centrifuged at 2000 rpm (380 x g) for 7 minutes. The resultant supernatant was discarded and the pellet resuspended in 5 ml full medium. The resuspended cells were completely dissociated by flushing through a 0.5 mm diameter needle attached to a 2 ml syringe. 10 µl of the cell suspension was then mixed with 90 µl of Trypan Blue Solution in order to determine the number of living cells/ml medium using an improved Neubauers haemocytometer. Cells were seeded into 24-well culture plates at 1×10^5 /ml and 1 ml added per well, and incubated at 37°C, 5% CO_2, until confluent. Medium was changed every 2-3 days, with confluence observed after 5-7 days.

5.3.2.3 Challenging submerged cultures of epithelial cells

Submerged cultured human airway epithelial cells were challenged with biocide and vehicles to ascertain any cytotoxicity of the biocides against the cells. Once a confluent monolayer was obtained the full medium was removed and replaced with 1 ml of quiescent medium (basal, serum-free medium containing only penicillin/streptomycin (5,000 µg/ ml^{-1}) and 1% insulin, transferrin and selenium (ITS) for 24 hours. This ensured that all of the cells in the monolayer had reversibly stopped dividing and entered a state of quiescence (G_0 phase of the cell cycle). This was to ensure that the cells would respond to any biocide or vehicle exposure in the same manner. After 24 hours, the quiescent medium was replaced with 1 ml of either the test vehicles (100%, 50%, 100% and 0%) or the biocides at a percentage of in-use concentrations (100%, 10%, 1% and 0%). These concentrations were such that 100% was the practical working concentration of the biocide. The test vehicles were PBS, HEPES, and SDW,

with quiescent medium used as the control. The biocides were Quat./biguanide, Quat./chlorine dioxide, 6% H_2O_2, amphoteric surfactant, 70% v/v denatured ethanol with deionised water and 70% v/v IPA with water for injection. Following 24 hours of contact with the vehicle or biocide, the cell supernatants were removed and centrifuged at 380 x g for 7 minutes. The cleared supernatants were then stored at -80°C for future analysis. The cells were replenished with 1 ml of full medium per well and left to recover over 48 hours, after which time the cells were either assayed for cell death using a commercial cell viability assay (CellTiter-blue™ viability assay, Promega) or cell supernatants were collected and measured for recovered IL-8 using an IL-8 ELISA. Finally, the cells were washed with 1 ml PBS (1%) and then treated with 500 µl Triton X-100 (1%) on ice for 15 minutes. The resultant cell lysates were stored at -80°C for future analysis.

Protein concentrations of each lysate were estimated using the colorimetric bicinchoninic acid (BCA) assay (Pierce, Northumberland, UK) according to the manufacturer's guidelines. Briefly, 5 µl of samples or bovine serum albumin (BSA) standards (125-2000 µg/ml) were mixed with 200 µl of working reagent (50 parts Reagent A to 1 part Reagent B) in triplicate wells of a 96-well microplate. Plates were incubated at 37°C for 30 minutes, then allowed to cool to room temperature before reading the absorbance at 570 nm using the Anthos microplate Reader 2001 (Anthos Labtec Instruments). A standard curve was constructed from the absorbance of the BSA (Appendix 11.4.1) and lysate protein concentrations calculated from this.

5.3.2.4 Determining cell morphology

Cell morphology was examined using light microscopy. The morphology of the cells was noted prior to, immediately after and following 1, 3, 6 and 24 hours of vehicle or biocide addition. The proportion of cells displaying either large, flat cells with visible nuclei (M1 cells) or small, shrunken cells which were no longer adherent (M2) were noted. In addition the numbers of cells displaying each morphology was also noted following the 48 hour recovery period.

5.3.2.5 Cell viability assay

Cell viability was assayed using the CellTiter-Blue™ assay. This endpoint assay provides a homogeneous, fluorometric method to monitor cell viability. In the CellTiter-Blue™ assay the fluorescence observed is proportional to the number of metabolically active, viable cells as only these cells retain the ability to reduce the redox dye, resazurin (blue with little intrinsic fluorescence activity) into resorufin (pink), which is highly fluorescent. There is a linear relationship between cell number and fluorescence (Promega, 2008).

After 48 hours of recovery in serum-containing medium, 200 µl of the CellTiter-Blue™ reagent was added directly to the cells cultured in 24-well plates and then cells were incubated at 37°C for 3 hours. For each experiment, a negative control was included, where cells were lysed with 1% Triton X-100 for 1 hour prior to incubation with CellTiter-Blue™. Following the 3 hours incubation, 100 µl of medium from the reagent treated wells were transferred to a solid black mictrotitre plate. The fluorescence signals were then recorded using a standard multiwell spectrofluorometer (Spectramax Gemini XS, Molecular Devices, Berkshire UK) with excitatory and emission wavelengths of 560 nm and 590 nm respectively.

5.3.2.6 Interleukin-8 ELISA of cell supernatants

A Human IL-8 ELISA Development kit, purchased from Peprotech EC Ltd. (London, UK), was used to quantify levels of the human IL-8 cytokine in the cell culture supernatant of biocide treated cells. All reagents unless otherwise stated were included in the kit and were prepared in accordance with the manufacturer's protocol. The ELISA method was performed in accordance with the manufacturer's guidelines, briefly; high affinity binding plates (Nunc MaxiSorp™-96 well microtitre plates) were coated with purified anti-human IL-8 capture antibody diluted in carbonate bicarbonate coating buffer by incubation overnight at 4°C. Wells were subsequently washed three times using 300 µl of wash buffer (PBS, 0.05% Tween®-20). Wells were blocked by incubation with 300 µl of block buffer (PBS, 1% BSA) for 1 hour at room temperature and washed 3 times as before with 300 µl of wash buffer. 100 µl of recombinant human IL-8 standards (0 pg/ml-2000 pg/ml) or experimental samples were added to the

appropriate wells in triplicate and incubated at room temperature for 2 hours. Wells were washed as before for a total of 3 washes and incubated with 100 µl anti-human IL-8 detection antibody at room temperature for 2 hours. Wells were again aspirated and washed 3 times and incubated with 100 µl of avidin-HRP enzyme solution for 30 minutes at room temperature. Wells were then aspirated and washed 5 times. 100 µl of SigmaFAST™ OPD substrate solution was added to each well and incubated for 5 minutes at room temperature, after which 50 µl per well of stop solution (1M hydrochloric acid) was added to each well. Plates were then read at 492 nm using an Anthos microplate Reader 2001 (Anthos Labtec Instruments). The IL-8 content of each sample was calculated using the recombinant human IL-8 standard curve (Appendix 11.4.2). IL-8 data were expressed relative to the number of viable cells as pg IL-8 released per 1000 BEAS-2B cells.

5.3.2.7 Thiol assay

The method used in this study was developed from Carty *et al.* (2000).

5 µl of NaOH (1N) and 5 µl of 15% SDS were added to the wells of a 96-well microtitre plate. This was followed by 40 µl of cell supernatant or lysate (at 20 mg/ml protein). 40 µl of cysteine (2.5 mM) standards (0-500 pg/mg protein) made up in 0.2 M Tris-HCl pH 8 buffer were added to the wells in triplicate (Appendix 11.4.3). Triton X-100 cell lysis buffer was used as a negative control. 150 µl of 5,5'-dithio-bis(2-nitrobenzoic acid), (DTNB / Ellmans reagent) made up in 2 M Tris-HCl (pH 8) was added to each of the wells, and the plate placed in the dark at room temperature for 5 minutes to allow the colour to develop. The absorbance was measured at 410 nm. The absorbance values of the standards were used to generate standard curves (Appendix 11.4.3) from which the thiol content (pg/µg protein) of the cell lysates was calculated.

5.3.2.8 Carbonyl ELISA

The carbonyl ELISA used was based on the methods of Carty *et al.* (2000). Standards and samples were diluted to 20 µg/ml in carbonate bicarbonate buffer. 50 µl of standards and samples were added to a 96-well microtitre plate and incubated for 1 hour

at 37°C. The wells were then washed 3 times with wash buffer (PBS – 0.05% (V/V) Tween®-20). After washing, 50 µl of DNPH (1mM in 2N HCl) was added per well and incubated at room temperature for 1 hour. The plate was washed as before. Any remaining binding sites were blocked with 200 µl/well of blocking buffer (PBS – 2% (V/V) Tween®-20) and plates incubated overnight at 4°C. Following this, the plate was washed again before adding 50 µl of anti-DNP antiserum, mouse immunogloubulin E (IgE) (diluted 1:1000 in blocking buffer) to each well and incubating for 2 hours at 37°C. Following another wash step, 50µl of peroxidase labelled rat anti-mouse IgE secondary antibody (diluted 1:1000 with blocking buffer) was added to the wells and left to incubate for 1 hour at 37°C. After a final washing 50 µl of SigmaFAST™ OPD substrate solution was added to the wells and left for 5 minutes in the dark at room temperature. To stop the reaction 50 µl of 2M H_2SO_4 was added to each well. The absorbance was then read at 492nm in a microtitre plate reader (Anthos microplate Reader 2001, Anthos Labtec Instruments) and readings from the standards used to generate a standard curve, (Appendix 11.4.4) from which the carbonyl content (nmoles/mg protein) in the cell samples was determined. Standards were produced by reducing and oxidising BSA to obtain a range of protein carbonyl standards as in Carty *et al.,* (2000). The carbonyl content of the standards ranged between 1.69 to 9.07 nmoles/µg protein,

5.3.2.9 Propidium iodide staining

BEAS-2B cells were seeded at 1 x 10^5 cells per well and grown to confluence in collagen (human type IV) coated 24-well culture plates. Once confluent the cells were exposed to one of the following for 24 hours: anisomycin (5 µg/ml), staurosporine (1 µM), ionomycin (1 µg/ml), etoposide (50 µm) or H_2O_2 (100%, 10% or 1%) made up in quiescent AECGM. Untreated cells were exposed to UV for 15 minutes. Following incubation, any remaining adherent cells were lifted off the culture plates by treating with 500 µl of PBS containing 5 mM EDTA. The cells were centrifuged at 300 g for 5 minutes and resuspended in 1 ml of PBS with 0.1% BSA. The cells were analysed in the presence and absence of 10 µM PI stain (2 µl per sample). The cells were passed through the flow cytometer (Cell Lab Quanta™ SC, Beckman Coulter) and the fluorescence determined. 10,000 events were analysed in each test sample.

5.3.2.10 Statistical analysis of data

Unless otherwise stated, statistical analysis was carried out on all data using an ordinary one-way ANOVA with Tukey–Kramer multiple comparisons test (GraphPad InStat, ver. 3.06; GraphPad Software Inc., La Jolla, USA). Data are presented as mean ± SEM. Differences were considered to be statistically significant when $P < 0.05$.

5.4 Results

5.4.1 BEAS-2B morphology following vehicle and biocide challenge

Morphological analysis of both vehicle and biocide exposed BEAS-2B cells was carried out under various conditions of exposure (tables 5.1 and 5.2). Changes were determined by comparing normal cellular morphology observed under control conditions (exposed to quiescent culture medium), with the morphology of cells following treatment with the biocides or vehicles.

Morphology 1 (M1) is used to describe flat cells which remain in contact with neighbouring cells, whereas morphology 2 (M2) indicates smaller cells that had shrunk in size, often losing contact with neighbouring cells or lifted off from the surface. A change in cellular morphology from M1 to M2 is indicative of cell death (Smit-de Vries *et al.*, 2007).

The cells were exposed to different concentrations of biocide vehicles to establish whether and to what extent the vehicles were contributing to the cytotoxicity of the biocides against the BEAS-2B cells. This would then help to determine the concentration of biocide at which any effects observed are due to the active components of the biocide, rather than the vehicle.

Table 5.1 BEAS-2B cell morphology following exposure to quiescent medium (QM) or 3 test vehicles: PBS, HEPES or SDW. Vehicles were mixed with quiescent medium to obtain 50% and 10% vehicle concentrations. Morphology was noted prior to exposure, immediately after exposure, and after 1, 3, 6 and 24 hours. The morphology was also noted following a 48 hour recovery period. (n=6). M1: Morphology 1 represents normal cell morphology of large, flat cells with visible nuclei and M2: Morphology 2 represents small cells that have shrunk and/or lifted off the plastic of the cell culture wells.

Time (Hour)	Vehicle									
	PBS			HEPES (0.5 mM)			SDW			QM
	100%	50 %	10%	100%	50%	10%	100%	50%	10%	100%
Prior to exposure	M1	M1	M1	M1	M1	M1	M1	M1	M1	M1
0	M1	M1	M1	M1	M1	M1	M1	M1	M1	M1
1	M1	M1	M1	20% M2	5% M2	M1	100% M2	10% M2	M1	M1
3	5% M2	5% M2	M1	30% M2	10% M2	5% M2	100% M2	10% M2	M1	M1
6	5% M2	5% M2	M1	50% M2	20% M2	10% M2	100% M2	10% M2	M1	M1
24	5% M2	5% M2	M1	100% M2	60% M2	30% M2	100% M2	10% M2	M1	M1
After recovery	5% M2	5% M2	M1	100% M2	60% M2	30% M2	100% M2	15% M2	M1	M1

All of the cells were M1 prior to and immediately after exposure to the three vehicles (table 5.1). The majority of quiescent medium alone exposed cells remained as M1 throughout the observation period. After 3 hours of exposure, some M2 cells were detected on exposure to 100% or 50% PBS. The proportion of these M2 cells remained the same throughout the 24 hour exposure and even after the 48 hours of recovery. With regards to the HEPES exposed cells, at 1 hour, some M2 cells were visible in the 100% and 50% exposed cells. The number of M2 cells increased as the exposure time increased. This was also true for the 10% HEPES exposed cells after 3 hours of exposure onwards. All of the cells were found to be M2 after 24 hours of contact with the 100% HEPES buffer with no change after 48 hours of recovery. The cells exposed to 100% SDW were all found to be M2 after just 1 hour. The SDW-dependent change from M1 to M2 was found to be irreversible. M2 cells were also observed after 50% SDW exposure for 1 hour but comprised only 15% of the cells following 48 hours recovery.

Table 5.2 BEAS-2B cell morphology following exposure to biocides: a) Quat./biguanide, Quat./chlorine dioxide or H_2O_2, or b) amphoteric surfactant, ethanol or IPA at 100%, 50%, 10% and 0% (quiescent medium control) of their practical concentrations. Morphology was noted prior to exposure, immediately after exposure, and after 1, 3, 6 and 24 hours. The morphology was also noted following a 48 hour recovery period. (n=3). M1: Morphology 1 represents normal cell morphology of large, flat cells with visible nuclei and M2: Morphology 2 represents small cells that have shrunk and/or lifted off the plastic of the cell culture wells.

a)

Time (Hour)	Biocide and concentration											
	Quat./Biguanide				Quat./Chlorine Dioxide				6% H_2O_2			
	100%	50%	10%	0%	100%	50%	10%	0%	100%	50%	10%	0%
Prior to exposure	M1	M1	M1	M1	M1	M1	M1	M1	M1	M1	M1	M1
0	M1	M1	M1	M1	M1	M1	M1	M1	M1	M1	M1	M1
1	100% M2	60% M2	25% M2	M1	70% M2	60% M2	30% M2I	M1	40% M2	30% M2	15% M2	M1
3	100% M2	60% M2	40% M2	M1	80% M2	70% M2	40% M2	M1	40% M2	30% M2	15% M2	M1
6	100% M2	90% M2	70% M2	M1	90% M2	80% M2	40% M2	M1	50% M2	50% M2	40% M2	M1
24	100% M2	100% M2	80% M2	M1	90% M2	80% M2	60% M2	M1	90% M2	70% M2	50% M2	M1
After recovery	100% M2	100% M2	80% M2	M1	90% M2	60% M2	60% M2	M1	100% M2	100% M2	50% M2	M1

b)

Time (Hour)	Biocide and concentration											
	Amphoteric Surfactant				70% Ethanol				70% IPA			
	100%	50%	10%	0%	100%	50%	10%	0%	100%	50%	10%	0%
Prior to exposure	M1	M1	M1	M1	M1	M1	M1	M1	M1	M1	M1	M1
0	M1	M1	M1	M1	M1	M1	M1	M1	M1	M1	M1	M1
1	80% M2	40% M2	10% M2	M1	M1	M1	M1	M1	M1	M1	M1	M1
3	80% M2	50% M2	20% M2	M1	M1	10% M2	20% M2	M1	M1	10% M2	20% M2	M1
6	100% M2	100% M2	90% M2	M1	15% M2	50% M2	20% M2	M1	15% M2	50% M2	20% M2	M1
24	100% M2	100% M2	100% M2	M1	15% M2	90% M2	20% M2	M1	15% M2	100% M2	20% M2	M1
After recovery	100% M2	100% M2	90% M2	M1	10% M2	70% M2	20% M2	M1	10% M2	100% M2	70% M2	M1

All cells were M1 prior to and immediately after exposure to the six biocides (table 5.2 a and b). All quiescent medium alone (0%) exposed cells also remained as M1 throughout the observations. Changes from M1 to M2 cells were observed after 1 hour of contact with 100% Quat./biguanide, Quat./chlorine dioxide, 6% H_2O_2 or amphoteric surfactant. The cells exposed to 100% Quat./biguanide were all found to be M2 after just 1 hour of exposure. After 24 hours of treatment, all of the 50% Quat./biguanide exposed cells were M2 and 80% of the 10% exposed cells were M2. Following 1 hour of exposure, 70% of the 100% Quat./chlorine dioxide treated cells were M2. The proportion of M2 cells increased as exposure time to the various concentrations of Quat./chlorine dioxide increased. With regards to the H_2O_2 exposed cells at 1 hour, some M2 cells were visible in the 100%, 50% and 10% exposed cells. After 24 hours of exposure and 48 hours of recovery nearly all of the 100% H_2O_2 exposed cells were M2. After 1 hour of exposure to 100%, 50% and 10% amphoteric surfactant, some M2 cells were detected. The proportion of these M2 cells increased throughout the 24 hour exposure, with the 100% and 50% resulting in all cells being M2. The 10% amphoteric surfactant treated cells were found to recover slightly following the recovery period as the proportion of M2 cells decreased from 100% to 90%.

Similar changes in cell morphology were observed in the ethanol and IPA exposed BEAS-2B cells. Surprisingly, no change in morphology was observed in the 100%-exposed cells, even after 3 hours of exposure, whereas some M2 cells were detected at the 50% and 10% concentrations. After 24 hours, the most M2 morphology cells were observed in the 50% concentrations of the ethanol and IPA. All cells were M2 following 24 hours exposure to Quat./biguanide, 6% H_2O_2 or amphoteric surfactant when tested at 100% of their in-use concentrations.

5.4.2 BEAS-2B cell viability - vehicle challenge

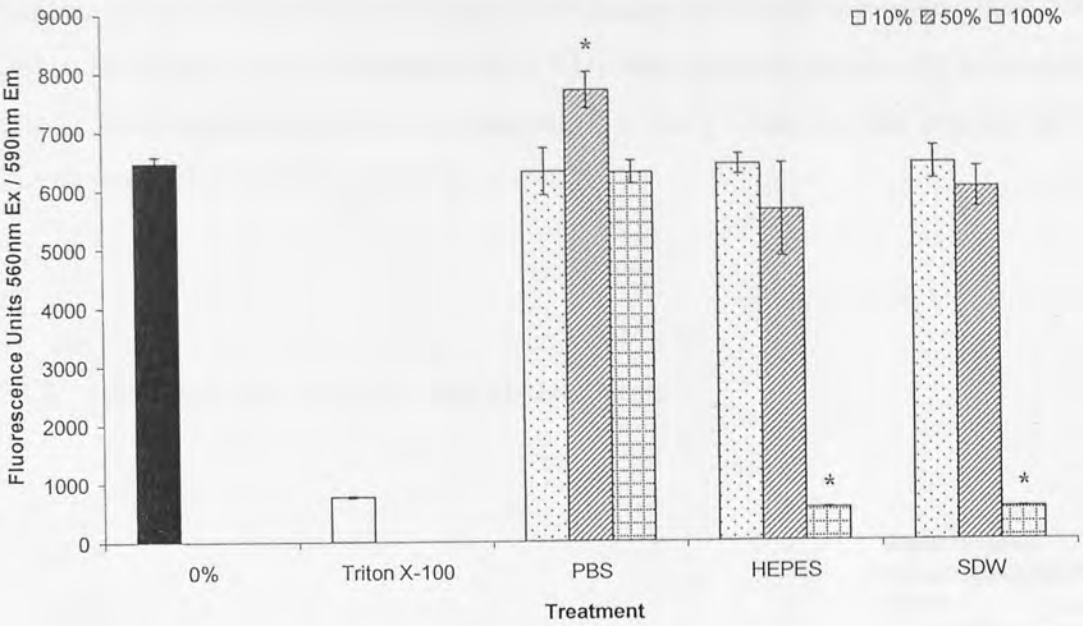


Figure 5.1 Viability of BEAS-2B cells exposed to vehicles (PBS, 0.5mM HEPES, SDW) for 24 hours and allowed to recover in full medium for 48 hours before viability was determined by CellTiter-Blue™ Assay. Black bar indicates baseline fluorescence of healthy cells under control conditions (quiescent medium) and clear bar indicates fluorescence of non-viable cells. (n=6). Data are presented as mean ± SEM (n=6). * $P < 0.05$ in relation to fluorescence at 0% vehicle concentrations.

The baseline fluorescence of the BEAS-2B cells when exposed to quiescent medium alone was 6469 ± 115 fluorescence units (figure 5.1) (fluorescence values in Appendix 11.4.5). The background fluorescence of the CellTiter-Blue™ reagent on its addition to PBS (256 ± 15 FU), HEPES (272 ± 17 FU) and SDW (271 ± 17 FU) in the absence of cells was low. In addition, the fluorescence of the CellTiter-Blue™ reagent with no additions in the absence of cells was low (251 ± 11). This did not differ significantly upon addition of the vehicles (Data not shown). The addition of 10% Triton X-100 did not alter FU of the medium alone (714 ± 13 FU) (Data not shown).

There was no change in the fluorescence of the cells exposed to any of the vehicles at 10% after 24 hours ($P > 0.05$). Fluorescence was decreased after exposure to 50%

HEPES (5654 ± 295 FU) and SDW (6027 ± 346 FU). However, the 50% PBS showed a significant increase in fluorescence (7708 ± 300 FU) in relation to the quiescent medium alone exposed cells ($P < 0.05$). After exposure of the BEAS-2B cells at 100% vehicle, a significant reduction between the fluorescence observed when 100% HEPES (561 ± 10 FU) and 100% SDW (555 ± 12 FU) were exposed to the cells in comparison to the culture medium control was observed ($P < 0.05$). This was not true for the 100% PBS exposed cells (6307 ± 192 FU).

5.4.3 BEAS-2B cell viability - biocide challenge

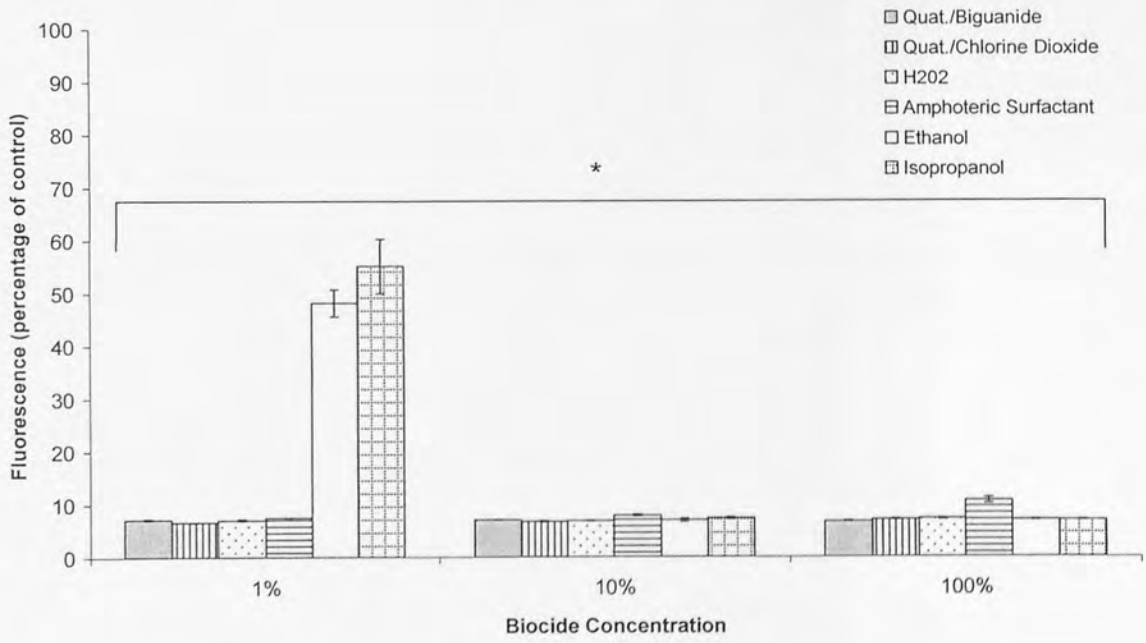


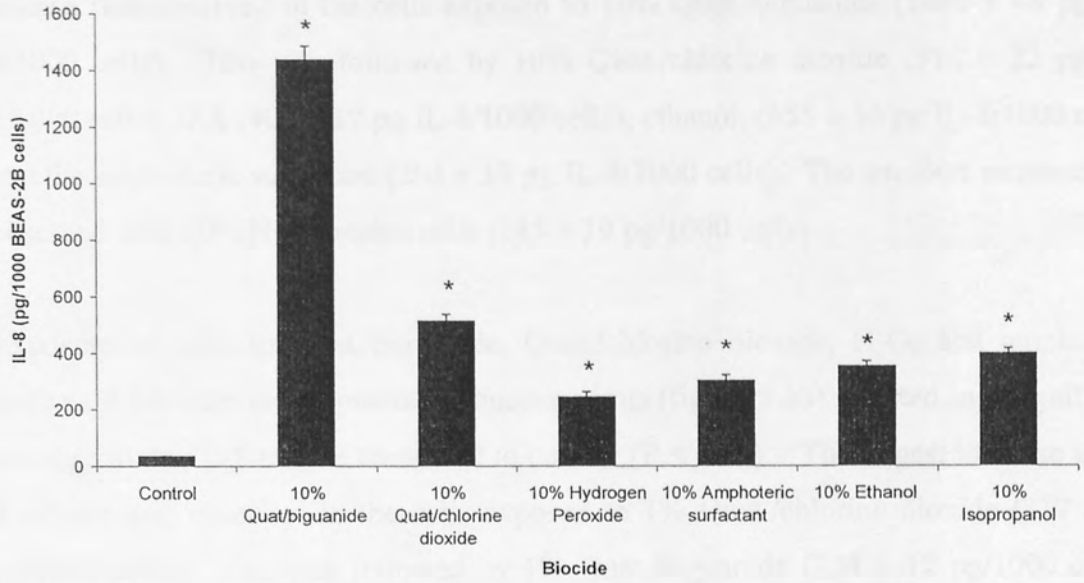
Figure 5.2 Viability of BEAS-2B cells exposed to biocides at 100%, 10% or 1% for 24 hours, with cell viability determined by CellTiter-Blue™ Assay after 48 hours of recovery. Data are presented as mean ± SEM (n=5), where fluorescence of unexposed, control cells was taken as 100% and other data expressed relative to this. * $P < 0.05$ in relation to fluorescence of control cells.

The fluorescence observed for healthy viable BEAS-2B cells (controls) was between 6,515 - 8,523 FU. The viability of the cells as a result of 24 hours biocide exposure was reduced in response to all of the biocides tested (figure 5.2) (fluorescence values in

Appendix 11.4.6). There was a significant ($P < 0.05$) decrease in the percentage of viable cells remaining when 100% and 10% Quat./biguanide, Quat./chlorine dioxide, 6% H_2O_2, amphoteric surfactant, ethanol or IPA were added to the cells (6.7% ± 0.1 to 10.7% ± 0.6) in comparison to the culture medium control (taken as 100%). The difference in viable cell numbers between both the alcohols at the 1% concentration was non-significant ($P > 0.05$). The percentage of viable BEAS-2B cells following exposure to 1% ethanol (48.2% ± 2.6) or 1% IPA (55.2% ± 5.1) was greater than the number of viable cells remaining following exposure to the other four biocides (6.6% ± 0.1 to 7.5% ± 0.1) at the same concentration ($P < 0.05$). The difference in the percentage of viable cells between all the biocides at 10% and 100% was found to be non-significant ($P > 0.05$).

5.4.4 IL-8 release from biocide exposed BEAS-2B cells

a)



b)

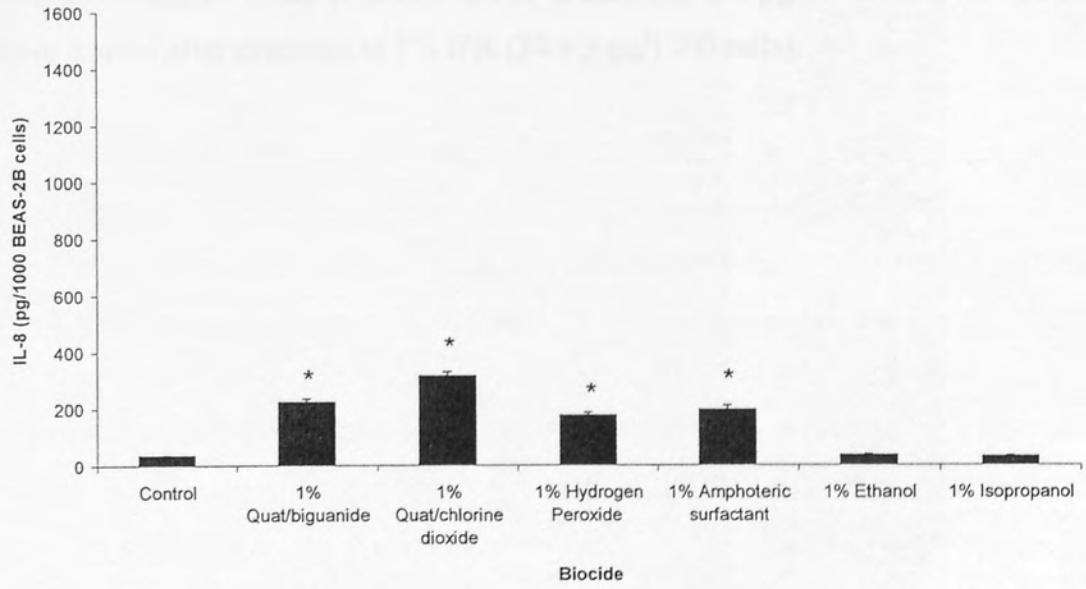


Figure 5.3 IL-8 released from BEAS-2B cells in response to 24 hours exposure to quiescent medium (control) or biocides at a) 10% and b) 1% of their practical in-use concentrations. IL-8 expressed as pg per 1000 viable BEAS-2B cells (n=5). Data are presented as mean ± SEM. * $P < 0.05$ compared to quiescent medium exposed control.

IL-8 data were expressed relative to the number of viable cells (Appendix 11.4.7). Exposure of BEAS-2B to all biocides at 10% practical concentrations (figure 5.3a), resulted in a significant ($P < 0.05$) increase in IL-8 release in comparison to the untreated, control (average 36 ± 1 pg IL-8/1000 cells). The largest increase in IL-8 release was observed in the cells exposed to 10% Quat./biguanide (1440 ± 48 pg IL-8/1000 cells). This was followed by 10% Quat./chlorine dioxide (516 ± 22 pg IL-8/1000 cells), IPA (400 ± 17 pg IL-8/1000 cells), ethanol, (355 ± 16 pg IL-8/1000 cells) and the amphoteric surfactant (304 ± 19 pg IL-8/1000 cells). The smallest increase was observed with 10% H_2O_2 treated cells (245 ± 19 pg/1000 cells).

Exposure of cells to Quat./biguanide, Quat./chlorine dioxide, H_2O_2 and amphoteric surfactant biocides at 1% practical concentrations (figure 5.3b) resulted in a significant increase in the IL-8 release compared to control ($P < 0.05$). The largest increase in IL-8 release was observed in the cells exposed to 1% Quat./chlorine dioxide (317 ± 13 pg/1000 cells). This was followed by 1% Quat./biguanide (224 ± 12 pg/1000 cells), amphoteric surfactant (196 ± 16 pg/1000 cells) and 1% H_2O_2 (177 ± 10 pg/1000 cells). In contrast there was a non-significant decrease in the IL-8 released by the BEAS-2B cells following 24 hours exposure to 1% ethanol (32 ± 3 pg/1000 cells) and no change from control after exposure to 1% IPA (36 ± 3 pg/1000 cells).

5.4.5 Thiol content of biocide-challenged BEAS-2B cells

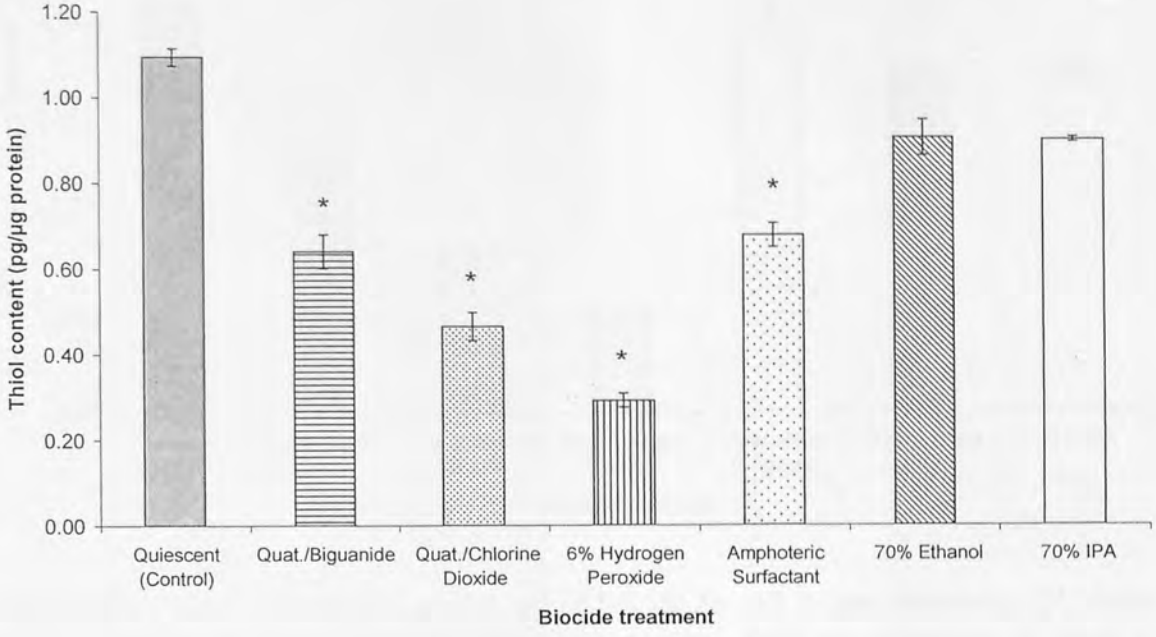


Figure 5.4 Thiol content (pg/µg protein) of BEAS-2B cell lysates following 24 hours of exposure to 10% biocide and 48 hours of recovery. Data are presented as mean ± SEM (n=6). * represents $P < 0.05$ comparing biocides and quiescent control.

Basal levels of free thiols in submerged cultured BEAS-2B cells were calculated as 1.09 ± 0.02 pg/µg of protein (figure 5.4). A reduction in comparison to this level was observed in the cell lysates on exposure to all 6 biocides when tested at 10% of their in-use concentrations. However, no significant differences in free thiol levels ($P > 0.05$) were observed between cell lysates on exposure of the BEAS-2B cells to the 70% ethanol (0.90 ± 0.04 pg/µg of protein) and 70% IPA (0.90 ± 0.01 pg/µg of protein). There was a significant reduction in free thiols in relation to the control on exposure to 10% H_2O_2 which reduced the thiol concentration from 1.09 ± 0.02 to 0.29 ± 0.02 pg/µg of protein ($P < 0.05$). There was also a significant reduction in free thiols on exposure to Quat./biguanide (0.64 ± 0.04 pg/µg of protein), Quat./chlorine dioxide (0.47 ± 0.03 pg/µg of protein) and amphoteric surfactant (0.68 ± 0.03 pg/µg of protein).

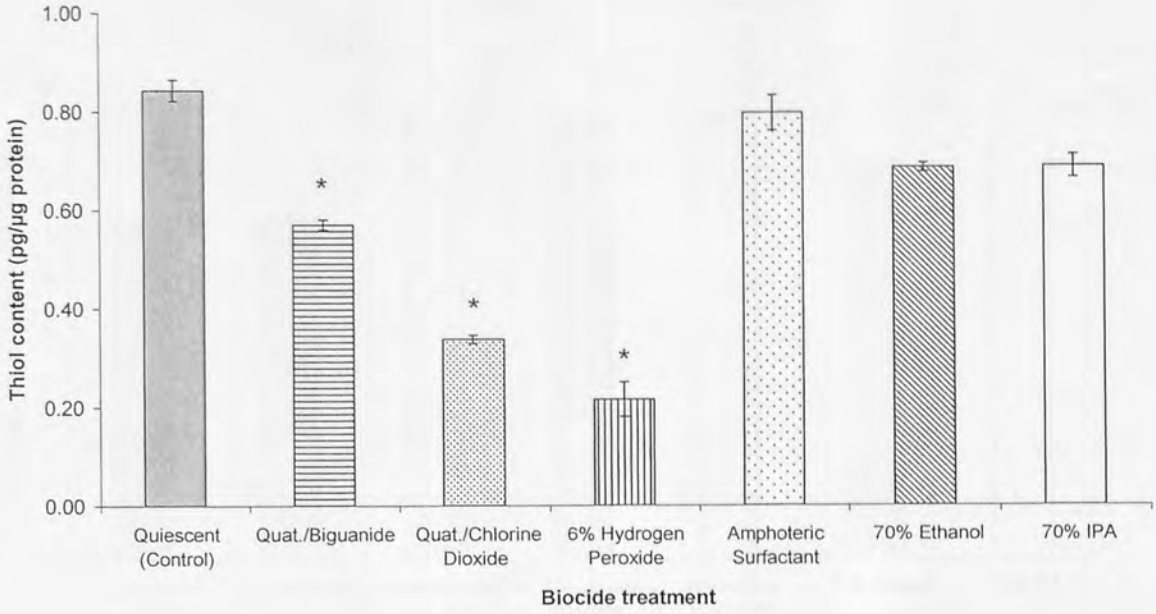


Figure 5.5 Thiol content (pg/µg protein) of BEAS-2B cell lysates following 24 hours of exposure to 1% biocide and 48 hours of recovery. Data are presented as mean ± SEM (n=6). * represents $P < 0.05$ comparing biocides and quiescent control.

Basal levels of free thiols in submerged cultured BEAS-2B cells were calculated as 0.84 ± 0.02 pg/µg of protein (figure 5.5). A reduction in comparison to this level was observed in the cell lysates on exposure to all 6 biocides when tested at 1% of their in-use concentrations. However, no significant differences in free thiol levels were observed between cell lysates on exposure of the BEAS-2B cells to the amphoteric surfactant (0.80 ± 0.04 pg/µg of protein), 70% ethanol (0.69 ± 0.01 pg/µg of protein) and 70% IPA (0.69 ± 0.02 pg/µg of protein). There was a significant reduction in free thiols in relation to the control on exposure to 1% H_2O_2 which reduced the thiol concentration from 0.84 ± 0.02 to 0.22 ± 0.04 pg/µg of protein ($P < 0.05$). There was also a significant reduction in free thiols on exposure to Quat./biguanide (0.57 ± 0.01 pg/µg of protein) and Quat./chlorine dioxide (0.34 ± 0.01 pg/µg of protein).

5.4.6 Carbonyl content of biocide-challenged BEAS-2B cells

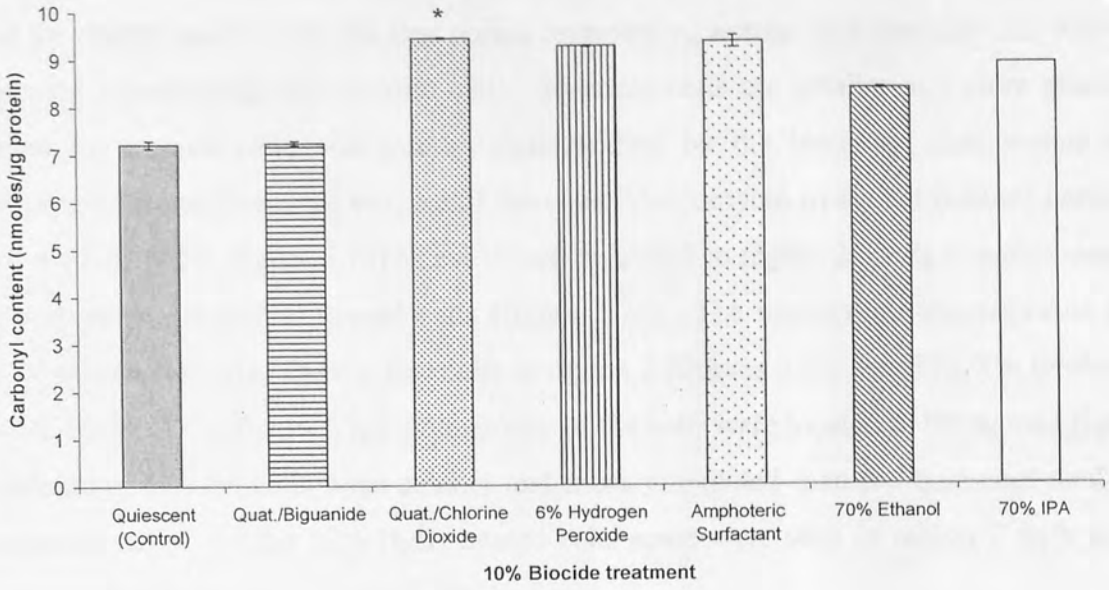


Figure 5.6. Carbonyl content (nmoles/µg protein) of BEAS-2B cell lysates following 24 hours of exposure to 10% in-use biocide concentrations after 48 hours of recovery. Results are expressed as mean ± SEM (n=6). * represents $P < 0.05$ comparing biocides and control.

Basal levels (control) of carbonyls in submerged cultured BEAS-2B cells were calculated as 7.21 ± 0.06 nmoles/µg of protein (figure 5.6). An increase in comparison to this level was observed in the cell lysates on exposure to all 6 biocides when tested at 10% of their in-use concentrations. Exposure of BEAS-2B cells to Quat./chlorine dioxide significantly increased the protein carbonyl concentration to 9.45 ± 0.04 nmoles/µg of protein from 7.21 ± 0.06 nmoles/µg of protein in unchallenged cells ($P < 0.05$).

5.4.7 Propidium iodide staining of challenged BEAS-2B cells

Cell samples were analysed for cell death by sorting the BEAS-2B cells according to electronic volume and side scatter. As shown in figure 5.7a, two regions were outlined in the scatter graphs, with the first region representing normal non-necrotic cells and the second representing any necrotic cells. Necrotic cells are smaller and more granular than the normal cells and can be distinguished by the increased side scatter and decreased mean electronic volume of the cells. Anisomycin treatment induced necrosis of the BEAS-2B (figure 5.7b) as evidenced by a shift to region 2. This was also clearly observed for etoposide treated cells (figure 5.7e). The ionomycin, staurosporine and UV treated cells also show a few cells in region 2 (figures 5.7c, d and f). On treatment with 100% H_2O_2 (figure 5.7g) the majority of the cells were located in the second region indicating that the cells were smaller and more granulated than the quiescent medium exposed cells. Of the 10% H_2O_2 treated cells some were seen in region 2 with some also present in region 1 (figure 5.7h).

Table 5.3 PI staining of challenged BEAS-2B cells. Percentage of necrotic cells and the mean fluorescence of the cells in the total cell population on exposure of the submerged cultured BEAS-2B cells to quiescent medium, anisomycin (5 μg/ml), staurosporine (1 μM), ionomycin (1 μg/ml), etoposide (50 μm), 100% H_2O_2 or 10% H_2O_2 for 24 hours, or UV for 15 minutes (n=1).

Cell treatment (24 hours)	Percentage of necrotic cells in total cell population (%)	Mean fluorescence of all cells in total population (Arbitrary Units)
Quiescent medium	2.71	2.61
Anisomycin	17.46	6.46
Staurosporine	2.40	2.15
Ionomycin	3.10	2.21
Etoposide	4.02	2.73
UV (15 min)	2.35	1.54
100% H_2O_2	89.63	25.0
10% H_2O_2	11.67	5.66

The percentage of necrotic cells in untreated quiescent medium was 2.71% (table 5.3). This value increased more than 30-fold when the cells were exposed to 100% H_2O_2 and

more than 4-fold when the cells were exposed to 10% H_2O_2 for 24 hours. The anisomycin (2.71% to 17.46%) and etoposide (2.71% to 4.02%) treated cells also had a higher percentage of necrotic cells in relation to the quiescent medium exposed cells. In contrast the percentage of necrotic cells present in the samples on exposure of the cells to ionomycin (2.71% to 3.10%), staurosporine (2.71% to 2.40 %) and the UV (2.71% to 2.35%) were not very different to the control.

Without PI, little auto-fluorescence of cells was occurring as the average mean fluorescence of the drug/biocide treated and untreated BEAS-2B cells was very low (1.02 arbitrary units to 2.19 AU). The greatest mean fluorescence of the BEAS-2B cells was observed after exposure to 100% H_2O_2 (table 5.3), whereby the fluorescence increased almost ten-fold in comparison to the quiescent medium exposed cells (2.61 AU to 25.0 AU). The fluorescence increased two-fold after exposure to 10% H_2O_2 (2.61 AU to 5.66 AU). The exposure of the cells to anisomycin resulted in an almost three-fold increase in the PI fluorescence of the cells (2.61 AU to 6.46 AU). The staurosporine (2.15 AU), ionomycin (2.21 AU), and UV (1.54 AU) treatments did not result in increased cell fluorescence. In fact, the fluorescence actually decreased in relation to the quiescent medium exposed control cells. However, an increase was observed in those cells exposed to etoposide (2.61 AU to 2.73 AU).

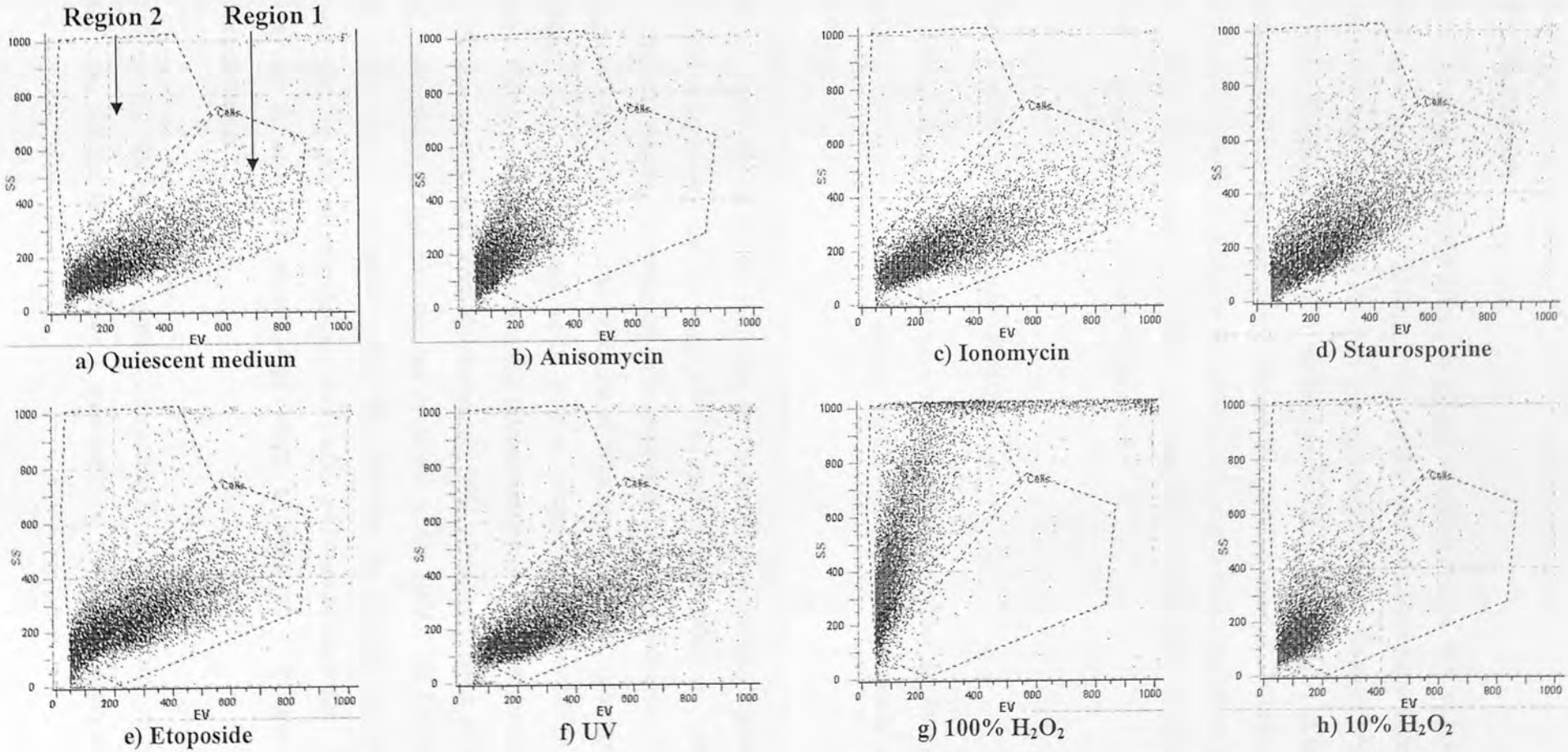


Figure 5.7 Scatter cytograms of submerged cultured BEAS-2B cells exposed for 24 hours to a) quiescent medium, b) anisomycin (5 µg/ml), c) ionomycin (1 µg/ml), d) staurosporine (1 µm), e) etoposide (50 µm), f) UV (15 min), g) 100% H_2O_2 and h) 10% H_2O_2. Y-axis (SS = side scatter) is representative of cell granularity and X-axis (EV = Electronic volume) represents the cell size. Region 1 represents the healthy cell population. Region 2 is representative of necrotic cells. Regions are outlined by a dashed line with gating set to overlap all cells (n=1). The most damaging treatment was 100% H_2O_2.

5.5 Discussion

Whilst biocides need to be effective against bacterial spores, it is important to consider their impact on operator health. The regular use of aerosolised biocides containing potentially harmful chemicals may put operators at risk through chronic inhalation exposure. In the current study focus was particularly paid to H_2O_2 as this was the most efficacious of the biocides tested against the *Bacillus* spores. Submerged cell culture models were used to investigate the effects of biocides on human airway epithelial cells. Cell morphology, viability, protein oxidation, the inflammatory status of the cells and the mechanism of cell death were examined.

5.5.1 Cell morphology and viability

The aim of the cytotoxicity testing was to determine whether the biocides reduced cell viability on exposure of the airway epithelial cells to commonly used cleanroom biocides.

The morphology of a cell indicates its integrity as a healthy epithelial cell, so an atypical morphology can be suggestive of cell damage (Smit de Vries *et al.*, 2007). Typical morphology of epithelial cells comprises large, flat, adherent cells with cell-cell contacts. Following biocide exposure of submerged cultures, cells shrunk in size, lost contact with neighbouring cells and/or became non-adherent. Some of these characteristic changes were observed in cells after just 1 hour of biocide exposure. At 100% concentrations at least approximately 90% of the cells were established as being M2, indicating that the biocides are cytotoxic to the BEAS-2B following 24 hours of exposure *in vitro*. In relation to the other biocides, the alcohols did not seem to have a great effect on the cell morphology, even after 24 hours of exposure. All biocides were observed to alter cell morphology when exposed at a fraction of their in-use concentrations.

Both the HEPES and SDW vehicles seemed to have an effect on cell morphology when they were applied at their practical concentrations (100%). In comparison the PBS did

not seem to do so (table 4.2.1). The extent of morphological damage caused by the vehicles generally decreased as the concentration exposed to the cells also decreased.

Challenging BEAS-2B cells with biocides at and as a proportion of their in-use concentrations was considered a more representative challenge when considering the effects of biocides in practical conditions. Of the 6 biocides tested, aside from H_2O_2, very few studies on their effects on human airway epithelial cell morphology have been undertaken (Olin *et al.*, 2002; Johansson *et al.*, 2005). However, the effects of H_2O_2 are relatively well characterised. Pelaia *et al.* (2004) found that exposure to 0.5 mM H_2O_2 dramatically altered the morphology of human bronchial epithelial cells (HBECs) from a confluent, intact monolayer to the cells becoming detached, rounded, granulated and having loosened cell-cell contacts. Treatment of human tracheobronchial derived cells with 100 μM H_2O_2 for 12 hours also changed cell morphology with apoptotic nuclear condensation and segmentation being observed (Chan and Goldkorn, 2000). Smit-de Vries *et al.* (2007) observed morphological changes in alveolar epithelial cells (A549) exposed to 0.5 mM and 1.0 mM H_2O_2. They found that basal, unstressed cells (controls) were mainly M1 but did contain some M2 cells. After 24 h of 0.5 mM H_2O_2 exposure all remaining cells were M2. However, most cells in the cultures incubated for 1 hour with 0.5 mM and 1.0 mM H_2O_2 showed an overall M1 morphology indicative of healthy cells. In agreement with the results obtained in the current study, as the time of incubation increased from 1 to 6 and 24 hours, the overall morphology changed from predominantly M1 to M2.

Overall, when submerged BEAS-2B cultures were exposed to the biocides, morphological differences were observed both immediately and after recovery of the cultures. The results obtained in the current study suggested that the majority of the cells were no longer healthy, viable cells after 24 hours contact with the biocides. Although morphology changes in cultures are common, it is often difficult to relate the results directly to the biocides as it is difficult to accurately quantify morphological changes. Consequentially, the Cell-Titer Blue™ viability assay (Promega) was used to determine whether the changes in morphology were reflected by changes in cell viability.

The results of the cell viability assays carried out on the cells confirmed that there was a loss in BEAS-2B cell viability following challenge with biocides at 100%, 10% and 1% of working concentrations. Application of the biocides at 100% was particularly damaging and resulted in almost total cell death. At this concentration it cannot be determined to what extent the biocides themselves were having an effect on the cells since 100% SDW or 100% HEPES also caused cell death. However, losses in cell viability were observed at 1% and 10% concentrations of the biocides, where no loss in cell viability was observed with the corresponding concentrations of vehicles. So, as the vehicles were found to be relatively non-toxic to the cells at 0%, 10% and 50% concentrations it can be established that any effect observed on the cells upon application of the biocides at these particular concentrations was due to biocide toxicity itself.

It was surprising that even the smallest concentrations of biocide investigated were significantly reducing BEAS-2B viability. At 100% and 10% biocide concentrations there was a 90% reduction in the relative fluorescence. However, at 1% working concentrations this loss in cell viability was not as great with the ethanol and IPA as it was with the other biocides, even though the alcohols dose dependently affected BEAS-2B viability. As in the present study, alcohols also caused a concentration dependent decrease in A549 viability (Kreja and Seidel, 2002).

At 100% concentrations the lack of medium available may explain why a loss in cell viability was observed. This is because the control data showed that BEAS-2B cell viability was much reduced with 100% SDW and HEPES. However, this was not the case with the PBS, as 100% PBS still showed the presence of viable cells. So the effects observed are likely to be due to the vehicle or biocides, and not due to the absence of quiescent medium. However, as 100% PBS is also medium deficient, the effects observed in the 100% SDW and HEPES challenged cells may be due to changes in osmolarity. As decreased osmolarity may have resulted in the cells being lysed upon exposure to the 100% SDW and HEPES.

The effects of biocides on human airway epithelial cells have not been well studied, with the exception of H_2O_2. Pelaia *et al.* (2004) found qualitatively that 0.25, 0.5 and 1.0 mM of H_2O_2 decreased HBEC viability. Mulier *et al.* (1998) reported 40% reduced

cellular viability of A549 and 16HBE140⁻ cells exposed to 0.1 mM and 1 mM H_2O_2 for 16 hours using a Lactate dehydrogenase (LDH) release assay. Smit-de Vries *et al.* (2007) investigated the effects of 24 hour treatment of A549 cells with 1 mM and 0.5 mM H_2O_2 and once again up to a 70% decrease in viable cell numbers, in comparison to unstressed basal cells, was observed. Other researchers have also established that H_2O_2 causes a decrease in the cell viability of submerged airway epithelial cells in a concentration dependent fashion (Gabrielson *et al.*, 1994; Spencer *et al.*, 1996). These findings correlate to some extent with the data from the present study, where cell viability was reduced more than 90% on exposure to H_2O_2. The results obtained in the present study used biocides at their practical concentrations. These are much higher than those that have been tested against epithelial cells in other studies. 6% H_2O_2 is 1.76 M, which is over 1000 times more concentrated than the highest concentration tested (1.0 mM) in the aforementioned studies. This is most likely the reason why a strong decrease in cell viability was observed in the BEAS-2B following biocide challenge.

There was very little correspondence between the morphology data and the Cell-Titer Blue™ viability data. Of these two methods of cell viability testing, the Cell-Titer Blue™ viability data would be more reliable as the results obtained are quantitative in comparison to the subjective morphology data.

Generally, a concentration dependent decrease in cell viability was observed, with the present study establishing that biocides at just 1% of their working concentrations were significantly toxic to the BEAS-2B.

5.5.2 IL-8 release from biocide-exposed BEAS-2B cells

Airway epithelial cells release biologically active compounds including lipid mediators, growth factors, cytokines and chemokines (Takizawa, 1998; Adler *et al.*, 1994; Martin *et al.*, 1997). IL-8 is one such pro-inflammatory chemokine which acts as a potent neutrophil chemoattractant (Standiford *et al.*, 1990; Huber *et al.*, 1991). If IL-8 production and release is increased in the absence of infection then the increased neutrophil recruitment to the airway epithelium may cause localised damage.

The decreased cell viability observed (section 5.4.3) was taken into consideration in calculating IL-8 release. This is because even though there may have been no increase in IL-8 compared to the control levels, the number of cells remaining viable following biocide exposure decreased, as established by the cytotoxicity testing results. So even though the treated wells may have lost a large number of cells, the remaining, viable cells may be producing relatively more IL-8 as only viable cells are able to release IL-8 (Oslund *et al.*, 2004).

In the present study BEAS-2B constitutively released 3.56 ± 0.11 pg/ml IL-8. Following biocide exposure of submerged cultured cells, higher levels of soluble IL-8 were detected in cell culture supernatants, however the cell viability indicated that a large proportion of the cells were dead. This indicates that the remaining viable cells were releasing very high levels of IL-8, with biocides inducing a pro-inflammatory effect in BEAS-2B. The only exceptions were the 1% ethanol and IPA, which were actually found to inhibit IL-8 release. Increases in IL-8 were biocide concentration dependent.

These data are confirmed by other studies which have also established that *in vitro* exposure of airway epithelial cells to an oxidant stress such as H_2O_2 stimulates IL-8 production (DeForge *et al.*, 1993; Pelaia *et al.*, 2004; Lakshminarayanan *et al.*, 1997; Roebuck, 1999; Biagioli *et al.*, 1999). The study from Pelaia *et al.* (2004) demonstrated that H_2O_2 stimulated IL-8 production in a dose-dependent manner in human bronchial epithelial cells, following 4 hours (1 mM H_2O_2: 200 – 240 pg/ml) and 8 hours (1 mM H_2O_2: 250 pg/ml) exposure. Although the concentrations and exposure times used in the present study were different to those investigated by Pelaia *et al.* (2004), a concentration dependent increase in IL-8 occurred following 24 hours exposure of the BEAS-2B to 0.6 % (176 mM) – 6% (1.76 M) H_2O_2. Biagioli *et al.* (1999) also found that there was a dose-dependent increase in IL-8 when BEAS-2B cells were stimulated for 6 hours with 0.0625 mM – 0.5 mM H_2O_2, with 176 ± 62 pg/ml detected on exposure to 0.5 mM H_2O_2. The IL-8 produced by 10% H_2O_2 (176 mM) exposed cells in the present study was established as 1680 pg/ml before IL-8 was calculated relative to the number of viable cells (Appendix 11.4.7). So, although a concentration dependent increase in IL-8 was observed by Biagioli *et al.* (1999), the H_2O_2 concentrations and

exposure times tested in their study were less than in the present study, in which IL-8 was calculated as pg/1000 cells.

Ovrevik *et al.* (2008) contrasts the results obtained in the present study. These workers found that 24 hours H_2O_2 exposure (up to 2 mM) did not significantly affect IL-8 release from BEAS-2B cells. They concluded that this was possibly because H_2O_2 has been found to reduce histone deacetylation but does not induce NF-κB activation in BEAS-2B, thereby only inducing a moderate increase in IL-8. These findings are in direct contrast to Biagioli *et al.* (1999) who investigated the same concentration over a shorter period of exposure. However, the concentration of H_2O_2 investigated in both these studies was less than tested in the present study. So the increase in IL-8 released by BEAS-2B cells in the present study may be due to the higher H_2O_2 concentrations used.

DeForge *et al.* (1993) found that the increased expression of IL-8 from A549 cells in response to H_2O_2 (0.5-2 mM) was due to oxidant stress. Oxidant stress can alter both the expression and activation of activating protein (AP-1) and nuclear factor-kB (NF-κB) which are redox-responsive transcription factors (Remick and Villarete, 1996). Both AP-1 and NF-κB regulate IL-8 expression (Lakshminarayanan *et al.*, 1998; Martin *et al.*, 1997; Rahman *et al.*, 2001; DeForge *et al.*, 1993; Mukaida *et. al.*, 1994). Sun and Oberley, (1996) established that reductants favoured AP-1 activity, whereas oxidants dramatically enhanced NF-κB activity. With regards to H_2O_2, it still remains unknown as to whether H_2O_2 itself activates NF-κB or if NF-κB activation is stimulated or inhibited by the H_2O_2 (de Oliveira-Marques *et. al.*, 2007). Lakshminarayanan *et al.* (1998) found that H_2O_2 induced IL-8 expression in the A549 and BEAS-2B epithelial cell lines through the action of the transcription factor AP-1 in a concentration dependent manner.

Other researchers have also established that H_2O_2 exposures (0.1 mM - 1 mM) are significantly toxic to human bronchial epithelial cells (Pelaia *et al.*, 2004; Mulier *et al.*, 1998: Smit-de Vries *et al.*, 2007; Gabrielson *et al.*, 1994; Spencer *et al.*, 1996). In the present study, H_2O_2 concentrations of 17.6 mM (a 1% working concentration) were toxic to the BEAS-2B with significant reductions in cell viability being observed alongside increases in IL-8 release.

5.5.3 Thiol levels of biocide-challenged BEAS-2B cells

Any protein oxidation effects observed must, first and foremost, be recognised as a change in the balance between protein oxidation rate and the oxidised protein degradation rate. If the protein oxidation rate is increased or degradation decreases, protein oxidation will be seen. Protein oxidation would be observed as a decrease in intracellular protein thiol levels due to the oxidation mediated formation of disulphides between the thiol groups on the cysteine residues of the proteins. The reduced level of free thiols in the cells can therefore be used as an indicator of oxidative stress.

Biocides reduced thiol content of submerged cultured BEAS-2B at both 1% and 10% of their in-use concentrations. However, the only significant decreases in thiol content were on exposure to the Quat./biguanides, Quat./chlorine dioxide, amphoteric surfactant and H_2O_2 at 10% concentrations. This was also true for these biocides at their corresponding 1% concentrations, with the exception of the amphoteric surfactant.

The results obtained with the H_2O_2 applications were as expected as H_2O_2 is a strong oxidising agent. Smit-de Vries *et al.* (2007) found that the resistance and recovery of A459 cells to H_2O_2 was determined by the pool of reduced thiol components, as cells with higher levels of reduced thiols following H_2O_2 exposure were more able to recover than those with few reduced thiols. In agreement with the present study they found that the cellular oxidative stress induced by the H_2O_2 was reflected in a lower free thiol content compared with basal unstressed values. This depletion of free thiols does not require cellular metabolism and can reduce the antioxidant capacities of the cells, thereby decreasing the protection of the epithelial cells against the resultant oxidative stress (Smit-de Vries *et al.*, 2007).

In comparison to 100% H_2O_2 the smallest decrease in free thiols was on exposure to 10% ethanol. In submerged culture, neither of the two alcohols tested produced a significant reduction in the protein thiol content of the cells in comparison to the control. However, as the previously mentioned biocides have not had their oxidative capacities established, a great decrease in thiols in relation to basal levels was not expected. Yet, the reduction in thiols by the ethanol can be explained because thiols are indirect markers of ethanol-induced free radical damage in tissues that have been

detected in rat animal models as well as *in-vitro* experimental systems (Mantle and Preedy, 1999).

Thiols have a number of roles within cells. These range from protein synthesis, where they are involved in the formation of disulphide bonds during protein folding, to the detoxification of ROS in the airways. A significant number of proteins involved in signalling have critical thiols, for example, receptors, protein kinases and some transcription factors can be altered by formation of mixed disulphides (Valko *et al.*, 2007). Free (reduced) thiols are also important because both AP-1 and NF-κB binding activity is mediated by the conserved redox-sensitive cysteine residues within the DNA binding domain (Lakshminarayanan *et al.*, 1998; Eaton, 2006; Deneke, 2000). Both AP-1 and NF-κB are oxidant-regulated transcription factors and therefore changes in intracellular ROS will alter both their expression and activation. NF-κB and AP-1, when activated, translocate to the nucleus and bind to their consensus sites within the promoters of many genes (Rahman *et al.*, 2001; Martin *et al.*, 1997) including pro-inflammatory chemokines and cytokines such as IL-8 and IL-6. This may explain the increases in IL-8 levels observed following exposure of the BEAS-2B to H_2O_2 and is important because the excessive production of such secondary pro-inflammatory mediators by airway epithelial cells plays a key role in the development of tissue injury during both acute and chronic inflammatory conditions (Rahman *et al.*, 2001).

Although the loss of free thiols may be as a result of the increased formation of disulphides, it should also be taken into account that the oxidants may also be having a direct effect on cellular protein levels. As well as being markers of oxidative stress and a protective mechanism against oxidative injury, the reduction in the number of intracellular free thiols present is also a causal factor of oxidative injury (Cockell and Belonge, 2002; Lambert, 2004). This is because, although the thiols are antioxidants, if they themselves are oxidised to disulphides, sulfoxides or disulfoxides, then a reduction in their antioxidant ability is observed (McDonnell and Russell, 1999). In addition, protein thiols may also serve as secondary mediators of inflammation as they can interact with oxygen radicals to form reactive oxysulfur radicals (Martin *et al.*, 1997; Włodek, 2002). It is vital that antioxidant activity is high, because if not, then insufficient decomposition of the H_2O_2 will result in the generation of the more toxic hydroxyl radicals (•OH).

As the majority of the cells were being killed on exposure to the biocides (section 5.4.3) it would be unexpected that any free thiols would be detected. However, the presence of free thiols following biocide exposure indicates that any remaining cells have responded to the oxidant challenge by increasing their thiol-redox status in order to re-assume the redox balance. In order to maintain their antioxidant activity, cells are able to restore the oxidised peptides or proteins. This occurs via enzymatic reduction and by increasing the expression of antioxidant peptides and proteins. Thiol antioxidants act through diverse mechanisms, including as components of the general thiol/disulfide redox buffer, as well as being metal chelators and radical quenchers (Deneke, 2000).

One point to consider is that the number of free thiols could actually be lower than found here, because the assay used only looks at the formation of mixed disulphide bonds as a result of oxidation, whereas the free thiol group cysteine readily undergoes reversible oxidation to form disulphides and sulfinic acids (Eaton, 2006).

5.5.4 Carbonyl content of biocide-challenged BEAS-2B cells

One other widely used measure of protein oxidation is protein carbonyl content, with protein oxidation measured by an increase in the number of DNP-reactive carbonyls.

Biocides increased carbonyl content of submerged cultured BEAS-2B. However, the only significant increase in carbonyl content was on exposure to the Quat./chlorine dioxide. This was as expected as the chlorine dioxide element of this biocide formulation is known to be a strong oxidiser. The smallest increase in protein carbonyl content was observed in the Quat./biguanide challenged cells. This was as expected, as neither Quats nor biguanides are oxidising agents. H_2O_2 is a very potent oxidiser, yet under submerged culture the cells exposed to it did not show any significant increase in the protein carbonyl content. Turi *et al.* (2002) found that incubation of primary human bronchial epithelial cells with lower concentrations of H_2O_2 (100 μM) did not result in an increase in protein carbonyl content. However, the concentrations used in the current study were much higher than 100 μM. Yet the effects of H_2O_2 are not well defined as although an increase in carbonyl content was observed it was not significant in relation to the control, thus indicating that no change in carbonyl content had been observed. In

the present study it is possible that no increase in protein carbonyls was observed because statistical significance was not reached due to the variability in the samples protein carbonyl content.

The increase in protein carbonyl content shows that biocides oxidise submerged cultured BEAS-2B cell proteins after 24 hours challenge *in vitro*. Carbonylation of proteins is an early event in oxidative stress *in vitro* (Cockell and Belonge, 2002). These carbonyl groups represent an irreversible protein modification, often leading to the inactivation of cellular proteins (Dalle-Donne *et al.*, 2006). Therefore an increase in carbonyl content reflects the increased oxidative stress under which the cells are put under on exposure to the biocides. In addition, carbonyl formation is irreversible, so oxidative changes could result in the loss of a host of key cellular processes and would be detrimental to the cells. The consequences of protein oxidation are varied and widespread with it often being associated with both respiratory and inflammatory diseases, as well as the aging process (Yan and Sohal, 2001). Oxidant stress has also been observed to increase secretion of mucin and depress ciliary beating efficiency (Adler *et al.*, 1994). So, increased protein oxidation as demonstrated by the increased carbonyl levels following biocide challenge, suggests that host defence will be impaired, resulting in harm to the operator. For example, impairment in the mucociliary clearance process due to increased mucus secretion and reduced ciliary beating may result in the exacerbation of any subsequent infections in the operator. Loss of protein function may result in alteration of cell processes, compromisation of the epithelium as a barrier and ultimately cell death.

Together thiols and carbonyls are known biological markers of protein oxidation. Individually these markers showed that the biocides were decreasing thiol levels and increasing carbonyl levels. However, the results did not entirely correlate with each other as variations occurred in the oxidising capability of the biocides, depending on whether the thiol content or carbonyl content of the cells was measured. This may be due to the biocides affecting thiols and carbonyls in a different manner. This may also be because of possible differences in the thiol and carbonyl groups in the cells themselves. This is such that the same biocide may have a different effect on thiols in comparison to carbonyls, as a result of the biocides reactive nature.

5.5.5 Propidium iodide staining of challenged BEAS-2B cells.

The mechanism of cell death in the airway epithelial cells following challenge with H_2O_2 was investigated as the H_2O_2 was the most efficacious against the bacterial spores, as well as being cytotoxic to the cells. PI staining of the cells was used to establish whether the BEAS-2B were necrotic following exposure to the H_2O_2. The results observed in the scatter cytograms (figure. 5.7) suggest that a change had occurred in the morphology of the cells due to the H_2O_2 exposure, as the H_2O_2 and drug exposed cells were of a smaller size and increased granularity in comparison to the unexposed basal cells. These findings were in agreement with the M1 and M2 morphological analysis of the cells. As a result the majority of the cells present in region 2 appeared to be typically necrotic.

The BEAS-2B cells had very little auto-fluorescence (1.02 - 2.19 AU). Consequently any PI fluorescence observed was due to changes in the cell membrane, brought about by the drugs/biocide which permitted PI entry into the cells interior. The mean fluorescence of the cells after biocide/drug treatment was compared with the mean fluorescence of the healthy cell population (quiescent medium alone exposed cells). It was expected that the fluorescence of a healthy population of cells would be low, as PI is unable to enter the cells and bind the DNA. In contrast, if all of the cells were dead then it would be expected that the fluorescence of the dead cell population would be high in relation to the healthy control. As fluorescent intensity is relative to necrosis, for BEAS-2B cells there were more necrotic cells in all the submerged biocide/drug exposed cells than those exposed to quiescent medium. This was with the exception of the ionomycin treatment which did not result in increased necrosis of the cells. This was as expected with the drugs employed here because they are cell death inducing drugs (Wu *et al.,* 2002; Lassota *et al.,* 1996; Seynaeve *et al.,* 1994; Kardalinou *et al.,* 1994; Shankaranarayanan and Nigam, 2003; Sergeev, 2004; Tombal *et al.,* 2002). Of the drugs tested, the greatest cell death was observed on exposure of the cells to the anisomycin. The fluorescence of the BEAS-2B following UV treatment may have been low because of the possible resistance of the cells to UV. However, it is important to consider that this was a preliminary experiment and therefore this possibility is not conclusive.

An H_2O_2 concentration dependent increase in necrotic cells was detected. The most necrotic cells were detected following exposure of the cells to the 100% H_2O_2, with the necrotic cells comprising nearly 89.63% of the cell population. This was a 30-fold increase in relation to the quiescent medium exposed control cells. In addition to this nearly 11.67% of the cells exposed to the 10% H_2O_2 were necrotic. It is important to note that this data is preliminary as the experiment was only carried out once.

The preliminary data obtained in the present study suggests that cell death on exposure to the 1.76 M (100%), 176 mM (10%) and 17.6 mM (1%) H_2O_2 is occurring via necrosis. In contrast, some researchers have found that H_2O_2 causes cell death by apoptosis (Misso and Thompson, 2005; Goldkorn *et al.*, 2005), whereas others have found that cell death occurs via necrosis. Tomita *et al.* (2002) did not detect any apoptosis in BEAS-2B cells exposed to 200 μM H_2O_2 for 4 hours. Oslund *et al.* (2004) also found very few BEAS-2B cells to be apoptotic following incubation with 0, 200, 400, or 600 μM H_2O_2 for 1 hour at 37°C. It is suggested that this is due to the ability of the H_2O_2 to nitrosylate proteins (Chandra *et al.*, 2000), resulting in them being capable of suppressing caspase activation and activity. However, in the present study, not all cell death may be taking place as necrosis as in addition to necrotic cells, there was also an indication that some cells may have undergone apoptosis because cell fragments (often associated with apoptosis) were observed in the scatter cytograms.

It is reported that the concentration of H_2O_2 to which the cells are exposed is a key factor, with higher concentrations likely to act upon cells by necrosis rather than lower concentrations which are likely to induce apoptosis (Tomita *et al.*, 2002). Smit-de Vries *et al.* (2007) demonstrated that A549 cells went into a state of necrosis without any pronounced signs of apoptosis when exposed to 0.5 or 1 mM H_2O_2. They also made the point that H_2O_2 exposed A549 cells may be undergoing necrosis; as such cells have a low free thiol content. It is likely that the decreased thiol content observed here may be the causal event resulting in BEAS-2B cell necrosis as Smit-de Vries *et al.* (2007) found that cells entering necrosis have a low free thiol/protein ratio. The 100% in-use concentration of H_2O_2 used in these experiments (more than a 1M concentration) is a very high concentration and therefore cells are likely to undergo necrosis, as high levels of necrotic cells were observed.

One important point to consider is that the cells were exposed to the H_2O_2 for 24 hours. This may mean that some cells in the samples were already dead and so apoptosis may have already occurred before the analysis of the samples by flow cytometry. The presence of cell fragments in the cell samples as observed in the scatter cytograms (events at the top of the graphs) support this possibility. This is because the cells have fragmented and apoptosis may have occurred, as the cells may have disintegrated resulting in the formation of apoptotic bodies. These would not allow the apoptotic cells to be detected, as the cells are no longer intact and consequently cannot be stained by PI. The clumping of the BEAS-2B cells was also seen in the cell samples (on the top right hand side of the scatter cytograms), making it likely that some cells in the centre of the clumps may not be accessible to the PI. So once again this demonstrates that there may be more necrotic cells than were being detected on exposure of the cells to the H_2O_2.

The possibility of necrosis being the method by which the biocide exposed cells die is likely, because of the results observed with the PI staining experiments and also the previous results which showed very quick reductions in cell viability (< 3 hrs) by way of changes in cell morphology (table 5.2) which were observed on exposure to the various concentrations of H_2O_2. This is indicative of necrosis, as necrosis is associated with rapid depletion of cellular adenosine triphosphate (ATP) (Nemeth *et al.*, 2007). However, the percentage of necrotic cells being detected in the H_2O_2 challenged cell cultures was not found to be 90%. This was in contrast to the cell viability assay carried out on the cells, where there was an approximate 90% reduction in viable cells following exposure to H_2O_2. This suggests that cell death following biocide challenge may also be occurring by a method additional to necrosis. So it is possible that upon exposure to the H_2O_2, some cells may have undergone apoptosis, whereas the majority of cells may have undergone necrosis.

Due to the presence of cell fragments, cell clumping and the relatively long (24 hour) exposure time, additional methods of apoptosis and necrosis detection would be required in addition to the PI staining (necrosis), to conclude whether H_2O_2 (at its in-use concentration) induces apoptosis and/or necrosis in the BEAS-2B cells. Taking into consideration the results to the preliminary cell death experiment, it is possible to contemplate that the major mode of cell death occurring in the BEAS-2B cells exposed

to biocides is likely to be necrosis. It is unsure as to whether apoptosis also occurs initially, followed by necrosis, or whether solely necrosis occurs as is being detected in this study. A two-stage cell death may be occurring in the cells with the first stage being immediate and representative of necrosis and the second stage being delayed and causing cell detachment which leads to apoptosis (Tomita *et al.*, 2002).

Generally, the H_2O_2 exposed submerged culture BEAS-2B were found to have more necrotic cells than the control. The mean fluorescence of the cells corresponded with the percentage of necrotic cells, with a higher mean fluorescence being observed when more of the cells were necrotic. So, although increased necrosis was occurring in the submerged cultured BEAS-2B, not all cells were necrotic.

Overall, this study found that exposure to biocides induces the release of IL-8 from submerged cultured human bronchial epithelial cells. Biocides also caused protein oxidation and cell death. This suggests that biocide exposure *in vivo* may contribute to the pathogenesis of airway disease in bronchial epithelial cells.

CHAPTER 6 SUBMERGED CULTURE OF HUMAN NASAL EPITHELIAL CELLS

6.1 Introduction

One of the first points of contact on inhalation of biocides are the nasal passages. The nose protects the lower airways by efficiently filtering and 'scrubbing' inhaled air, removing water-soluble reactive gases and vapours, and inhaled particles (Harkema *et al.,* 2006). Passage through the nasal tract warms, and humidifies inhaled air before it enters the tracheobronchial airways (Harkema *et al.,* 2006). The nasal epithelium comprises mainly of a ciliated pseudostratified columnar epithelium with many goblet cells (Harkema *et al.,* 2006). The goblet cell produces mucus that covers most of the luminal surfaces of the nasal passages and acts as a defence mechanism against any inhaled toxicants, by trapping particles and some gases or vapours. In addition, mucus has antioxidant activity as it scavenges oxidants (Cross *et al.,* 1984). If this first line of defence against inhaled toxicants is compromised, the host becomes more vulnerable to increased nasal infections and more susceptible to lower respiratory tract diseases (Harkema *et al.,* 2006).

Human nasal epithelial cells are a relatively underused cell type, partly because they are not commercially available, immortalised, transformed cell lines. As such, primary cells have been used to represent human nasal epithelium in this study. Primary cells are cells which have not been transformed by a virus or oncogenes.

Compared with immortalised cell lines, primary airway epithelial cells are composed of mixed types (goblet, basal, ciliated, non-ciliated epithelial cells) and are consequently more representative of airway epithelial cells *in vivo* (Lin *et al.,* 2007; Sakagami, 2006). In addition, primary cells have the same morphological and biochemical characteristics as cells of the respiratory epithelium (Sakagami, 2006). However, primary cells also have their disadvantages as they do not have longevity in culture (Wan *et al.,* 2000) and are also likely to lose their differentiated characteristics over time (Eisenbrand *et al.,* 2001).

In the last 20 years, the study of nasal toxicology has increased (Harkema *et al.*, 2006; Devalia *et al.*, 1991). HNEpC have often been used to carry out drug transport studies and have also been used to study the effect of pollutants with regard to nasal mucosal permeability and metabolism (Schmidt *et al.*, 1998; Lee *et al.*, 2005: Yoo *et al.*, 2003; Lin *et al.*, 2005; Mills *et al.*, 1999). Many studies have been undertaken investigating the vulnerability of nasal epithelium to short-term exposures of diesel exhaust particles (DEP) (Boland *et al.*, 1999). Often only short-term exposures are carried out due to the limited life-span of the primary human nasal epithelial cells.

One of the responses to DEP reported by Boland *et al.*, (1999) is the upregulation of IL-8 by nasal epithelial cells. IL-8 is constitutively expressed by human nasal epithelium (Takeuchi *et al.*, 2001; Adler *et al.*, 1994; Becker *et al.*, 1993). The increase in IL-8 release was found to contribute to the inflammatory response of the epithelium by increasing the inflammatory cell influx (mainly neutrophils) to the region. This in turn can have wider implications because the neutrophils chemotactically recruited to the epithelium can cause localised damage to the airways. This is likely to occur as in the absence of an infection, the epithelial cells become targets of the ROS released by the neutrophils (Wright *et al.*, 1994; Gabrielson *et al.*, 1994; Barnes, 1990; Takeuchi *et al.*, 2001; Auger *et al.*, 1994; Cohn and Adler, 1992).

DEPs themselves may induce the production of ROS including superoxides and hydroxyl radicals in response to adsorbed organic molecules on DEPs (Sagai *et al.*, 1993; Pandya *et al.*, 2002). Therefore it is likely that nasal epithelial cells are also subjected to oxidative stress. Although ROS are known to be responsible for protein oxidation in epithelial cells (Kazzaz *et al.*, 1996), protein oxidation effects in the nose have not been investigated. The nasal epithelial cells also have antioxidant capabilities. For example, an increased level of ascorbic acid in nasal airway lining fluid was observed immediately after DEP exposure (300mg/m^3 for 1 hour) (Blomberg *et al.*, 1998). These data indicate that the antioxidant defences of nasal epithelial lining fluid is modified in response to DEP exposure.

Although nasal cancer is rare in humans, some studies have established that there is a risk with inhaled chemicals such as formaldehyde (Luce *et al.*, 1993). Tobacco smoking has also been established as being a risk factor for sinonasal squamous cell

carcinoma ('t Mannetje *et al.*, 1999). Occupational exposure to complex chemical mixtures such as wood dust, textile dust, chromium- and nickel-containing materials and leather dust have also been associated with nasal cancer in humans. (Kuper *et al.*, 1997; Nylander and Dement, 1993; Boysen *et al.*, 1984). In addition, ozone has been shown to cause nasal epithelial inflammatory responses in humans (Nikasinovic *et al.*, 2003). Therefore it is possible that inhalation of biocides used by cleanroom operators may result in inflammation and/or injury occurring in nasal epithelial cells. Consequently, it is important to establish the possible toxic effects of biocides on nasal epithelial cells to ascertain possible occupational exposure risks.

The parameters established for measuring the effects of biocide challenge on BEAS-2B cells were used to assess the biocides effects on HNEpC. The cytotoxic effects of the biocides were determined by observing changes in morphology and viability (using the Cell Titer- Blue™ assay). The change in pro-inflammatory status of the cells following biocide challenge was studied by measuring IL-8 secretion. The oxidative effects of the biocides on the HNEpC were determined by establishing the protein thiol and protein carbonyl levels in biocide exposed cells. The cell death mechanism of necrosis (flow cytometry) was also investigated following biocide exposure.

6.2 Aims

- To determine whether the biocides cause a loss in cell viability of HNEpC grown in submerged culture when applied at, and as a percentage of, their in-use concentrations.

- To ascertain whether biocides induce a pro-inflammatory response in HNEpC in submerged culture.

- To determine the thiol and carbonyl content of submerged cultured HNEpC when acutely exposed to biocides.

- To determine whether HNEpC death occurs by necrosis.

6.3 Materials and Methods

6.3.1 Materials

6.3.1.1 Cell culture materials

Promocell® Airway Epithelial Cell Growth Medium (AECGM) and SupplementMix were from Promocell (Heidelberg, Germany). Insulin, transferrin and selenium (ITS), Penicillin, Streptomycin, PBS, Tween®-20, Trypsin-EDTA, Trypan Blue Solution, Triton X-100, Bovine Serum Albumin, Sodium Dodecyl Sulfate, Sodium hydroxide, Cysteine, Carbonate bicarbonate buffer, Dinitrophenylhydrazine (DNPH), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), anti-DNP mouse immunogloubulin E (IgE), Peroxidase labelled rat anti-mouse IgE, Sulphuric acid, Hydrochloric acid, Tris buffer, SigmaFAST™ OPD substrate solution, Anisomycin, Staurosporine, Ionomycin, Etoposide, Greiner® 75cm^2 cell culture flasks, Nunc MaxiSorp™-96 well microtitre plates and Human placental type IV collagen were all from Sigma (Dorset, UK). 0.5 mm diameter needles were from Neolus, Terumo UK Ltd. (Surrey, UK). 2 ml syringes were from Becton Dickinson (Oxford, UK). BD Falcon™ 24-well multiwell culture plates were from BD Falcon (Oxford, UK). 5% Foetal Calf Serum (FCS) and Modified Eagles Medium was from PAA (Somerset, UK). 96-well microplates were from Fisher (Leicestershire, UK). Solid black mictrotitre plates were from Thermo Scientific (Basingstoke, UK). BCA Assay kit was from Pierce (Northumberland, UK). CellTiter-Blue™ reagent was from Promega (Southampton, UK). IL-8 ELISA Kit was from Peprotech EC Ltd. (London, UK)

6.3.1.2 HNEpC

Human nasal epithelial cells (non-transformed, primary cells) were from Promocell, (Heidelberg, Germany). HNEpC were used between passages 2-7.

6.3.2 Methods

6.3.2.1 Submerged epithelial cell culture

Cells were seeded on human placental type IV collagen-coated (10 μg/cm^2) (as described in chapter 5, section 5.3.2.1) 80 cm^2 tissue culture flasks at a seeding density of 1 x 10^5 cells/ml and maintained in full medium comprising Promocell® Airway Epithelial Cell Growth Medium basal medium supplemented with 5% FCS and 1% penicillin (5,000 U/ml^{-1}) and streptomycin (5000 μg/ ml^{-1}). The manufacturer's supplement was added, this contained the following growth factors: Bovine Pituitary Extract (0.4%), Epidermal Growth Factor (10 mg/ml), insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), epinephrine (0.5 μg/ml), triiodothyronine (6.7 ng/ml), transferrin (10 μg/ml) and retinoic acid (0.1 ng/ml).

Cells were grown under standard cell culture conditions at 37°C and 5% CO_2. The medium on the cells was removed every 2-3 days and replenished with fresh full medium (containing serum) until the cells were confluent.

All other methods used are the same as described in Chapter 5.3.2.

6.4 Results

6.4.1 HNEpC morphology following vehicle and biocide challenge

Table 6.1 HNEpC morphology following exposure to quiescent medium (QM) and 3 test vehicles: PBS, HEPES or SDW. Vehicles were mixed with quiescent medium to obtain 50% and 10% vehicle concentrations. Morphology was noted prior to exposure, immediately after exposure, and after 1, 3, 6 and 24 hours. The morphology was also noted following a 48 hour recovery period (n=6). M1: Morphology 1 represents normal cell morphology of large, flat cells with visible nuclei and M2: Morphology 2 represents small cells that have shrunk and/or lifted off the plastic of the cell culture wells.

Time (Hour)	Vehicle									
	PBS			HEPES (0.5 mM)			SDW			QM
	100%	50 %	10%	100%	50%	10%	100%	50%	10%	100%
Prior to exposure	M1	M1	M1	M1	M1	M1	M1	M1	M1	M1
0	M1	M1	M1	M1	M1	M1	M1	M1	M1	M1
1	M1	M1	M1	25% M2	15% M2	M1	100% M2	10% M2	M1	M1
3	5% M2	5% M2	M1	30% M2	20% M2	5% M2	100% M2	10% M2	M1	M1
6	5% M2	5% M2	M1	50% M2	30% M2	10% M2	100% M2	15% M2	M1	M1
24	5% M2	5% M2	M1	100% M2	70% M2	30% M2	100% M2	15% M2	M1	M1
After recovery	5% M2	5% M2	M1	100% M2	70% M2	30% M2	100% M2	15% M2	M1	M1

The majority of the cells were of an M1 morphology prior to and immediately after exposure to the three vehicles (table 6.1). Some, but very few M2 cells were seen. The majority of quiescent medium alone exposed cells were also M1 throughout the observations. After 3 hours of exposure, some M2 cells were detected when exposed to the 100% or 50% PBS. The proportion of these M2 cells remained the same throughout the 24 hour exposure and even after the 48 hours of recovery. With regards to the HEPES exposed cells at 1 hour, some M2 cells were visible in cultures exposed to 100% and 50% concentrations. The number of M2 cells increased as the exposure time increased. This was also true for the 10% HEPES exposed cells after 3 hours of exposure onwards. All of the cells were found to be M2 after 24 hours of contact with the 100% HEPES buffer with no change after 48 hours of recovery. The cells exposed

to 100% SDW were all M2 after just 1 hour. This change from M1 to M2 was found to be irreversible in the case of the 100% SDW exposed cells. Around 15% M2 cells were also observed in the 50% SDW exposed cells.

Table 6.2 HNEpC morphology following exposure to biocides: a) Quat./biguanide, Quat./chlorine dioxide or H_2O_2, or b) amphoteric surfactant, ethanol or IPA at 100%, 50%, 10% and 0% (quiescent medium control) of their practical concentrations. Morphology was noted prior to exposure, immediately after exposure, and after 1, 3, 6 and 24 hours. The morphology was also noted following a 48 hour recovery period (n=3). M1: Morphology 1 represents normal cell morphology of large, flat cells with visible nuclei and M2: Morphology 2 represents small cells that have shrunk and/or lifted off the plastic of the cell culture wells.

a)

Time (Hour)	Biocide and concentration											
	Quat./Biguanide				Quat./Chlorine Dioxide				6% H_2O_2			
	100%	50%	10%	0%	100%	50%	10%	0%	100%	50%	10%	0%
Prior to exposure	M1	M1	M1	M1	M1	M1	M1	M1	M1	M1	M1	M1
0	M1	M1	M1	M1	M1	M1	M1	M1	M1	M1	M1	M1
1	90% M2	50% M2	20% M2	M1	70% M2	50% M2	25% M2	M1	50% M2	30% M2	20% M2	M1
3	90% M2	60% M2	30% M2	M1	80% M2	60% M2	40% M2	M1	50% M2	40% M2	20% M2	M1
6	100% M2	80% M2	50% M2	M1	90% M2	80% M2	50% M2	M1	60% M2	50% M2	40% M2	M1
24	100% M2	100% M2	70% M2	M1	90% M2	80% M2	60% M2	M1	90% M2	80% M2	60% M2	M1
After recovery	100% M2	100% M2	70% M2	M1	90% M2	80% M2	60% M2	M1	100% M2	100% M2	60% M2	M1

b)

Time (Hour)	Biocide and concentration											
	Amphoteric Surfactant				70% Ethanol				70% IPA			
	100%	50%	10%	0%	100%	50%	10%	0%	100%	50%	10%	0%
Prior to exposure	M1	M1	M1	M1	M1	M1	M1	M1	M1	M1	M1	M1
0	M1	M1	M1	M1	M1	M1	M1	M1	M1	M1	M1	M1
1	80% M2	40% M2	20% M2	M1	M1	M1	M1	M1	M1	M1	M1	M1
3	90% M2	60% M2	35% M2	M1	M1	20% M2	15% M2	M1	M1	20% M2	20% M2	M1
6	100% M2	80% M2	90% M2	M1	30% M2	40% M2	20% M2	M1	30% M2	40% M2	20% M2	M1
24	100% M2	100% M2	90% M2	M1	30% M2	50% M2	20% M2	M1	30% M2	50% M2	20% M2	M1
After recovery	100% M2	100% M2	80% M2	M1	20% M2	50% M2	20% M2	M1	20% M2	50% M2	30% M2	M1

All cells were of an M1 morphology prior to and immediately after exposure to the six biocides (tables 6.2 a and b). Some, but very few M2 cells were seen. All quiescent medium alone (0%) exposed cells remained M1 throughout the course of the experiment.

Changes from an M1 to M2 morphology were observed after 1 hour of contact with 100% concentrations of Quat./biguanide, Quat./chlorine dioxide, 6% H_2O_2 and amphoteric surfactant. All cells exposed to 100% Quat./biguanide were M2 after just 1 hour of exposure. After 24 hours of treatment, all of the 50% Quat./biguanide exposed cells were M2, and 70% of the 10% Quat./biguanide exposed cells were M2. Following 1 hour of exposure, 90% of the 100% Quat./chlorine dioxide treated cells were M2. The proportion of M2 cells increased as exposure time to the different concentrations of Quat./chlorine dioxide increased. With regards to the H_2O_2 exposed cells, at 1 hour some M2 cells were visible in the 100%, 50% and 10% exposed cells. After the 24 hours of exposure and 48 hours of recovery all of the 100% and 50% H_2O_2 cells were M2. Following 24 hours exposure to the biocides at 100% of their in-use concentration, the Quat./biguanide, 6% H_2O_2 and amphoteric surfactant exposed cells were all M2. After 1 hour of exposure, some M2 cells were detected when exposed to the 100%, 50% and 10% amphoteric surfactant. The proportion of M2 cells increased throughout the 24 hour exposure, with all of the cells becoming M2, with the exception of the 10% exposed cells (only 90% M2) at 24 hours. The 10% amphoteric surfactant treated cells were found to recover slightly following the recovery period as the proportion of M2 cells decreased from 90% to 80%.

Similar changes in cell morphology were observed between ethanol and IPA exposed cells. Surprisingly, no change in morphology was observed in the 100% concentrations until 6 hours exposure, whereas some M2 morphology cells were detected with 50% or 10% alcohol at 3 hours. After 24 hours, the largest proportion of cells of the M2 morphology were observed in the cultures exposed to 50% ethanol and IPA.

6.4.2 HNEpC viability - vehicle challenge

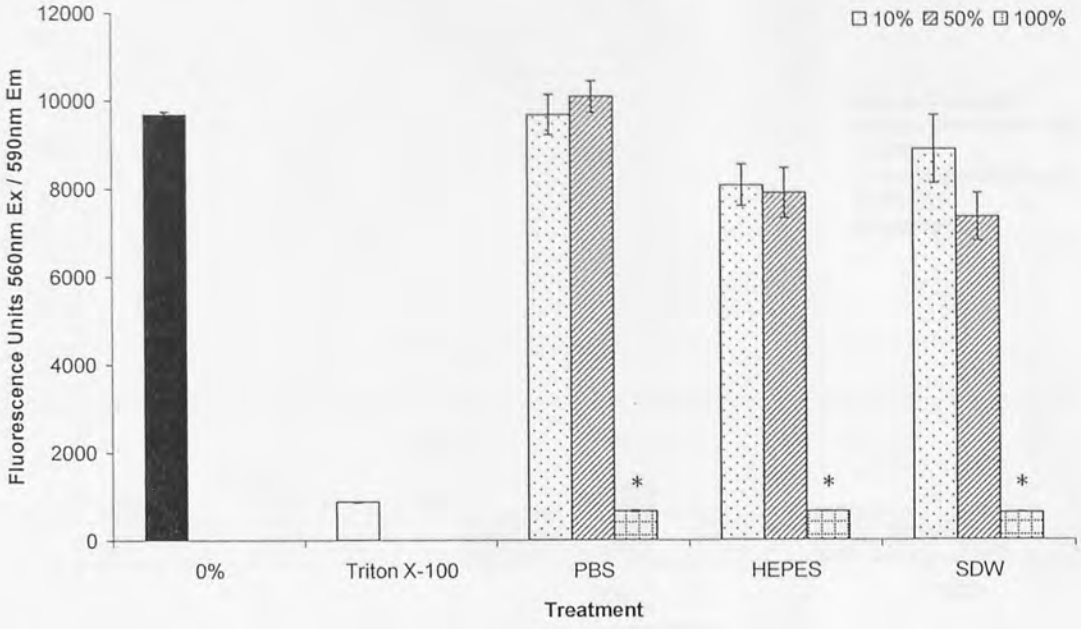


Figure. 6.1 Viability of HNEpC exposed to vehicles (PBS, 0.5mM HEPES, SDW) for 24 hours and allowed to recover in full medium for 48 hours before viability was determined by CellTiter-Blue™ Assay. Black bar indicates baseline fluorescence of healthy cells under control conditions (quiescent medium) and clear bar indicates fluorescence of non-viable cells. Data are presented as mean ± SEM (n=6). * $P < 0.05$ in relation to fluorescence at 0% vehicle concentrations.

The baseline fluorescence of HNEpC when exposed to quiescent medium alone was 9684 ± 65 fluorescence units (figure 6.1; fluorescence values are given in Appendix 11.5.1). The background fluorescence of the CellTiter-Blue™ reagent on its addition to PBS (256 ± 15 FU), HEPES (272 ± 17 FU) and SDW (271 ± 17 FU) in the absence of cells was low. The addition of 10% Triton X-100 did not alter FU of the medium alone (714 ± 13 FU [Data not shown]). After exposure of the HNEpC to 100% vehicle, a significant reduction in fluorescence was observed with HEPES (657 ± 11 FU), SDW (623 ± 6 FU) or PBS (664 ± 21 FU) in comparison to the culture medium control ($P < 0.05$). None of the 10% or 50% concentrations significantly altered fluorescence compared to quiescent medium control.

6.4.3 HNEpC viability - biocide challenge

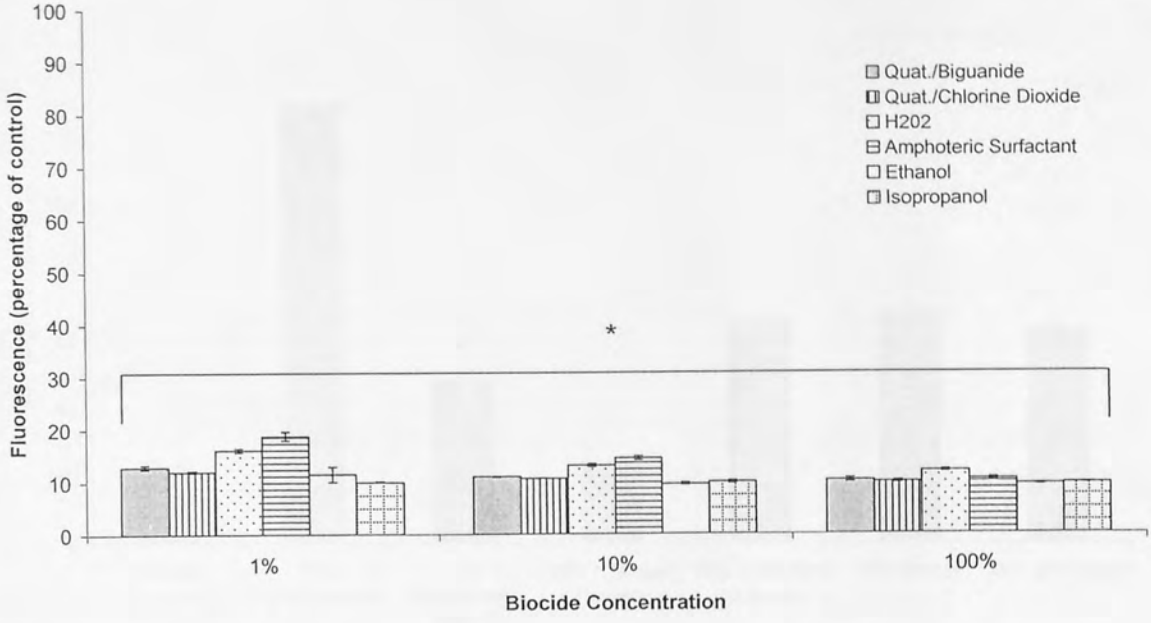
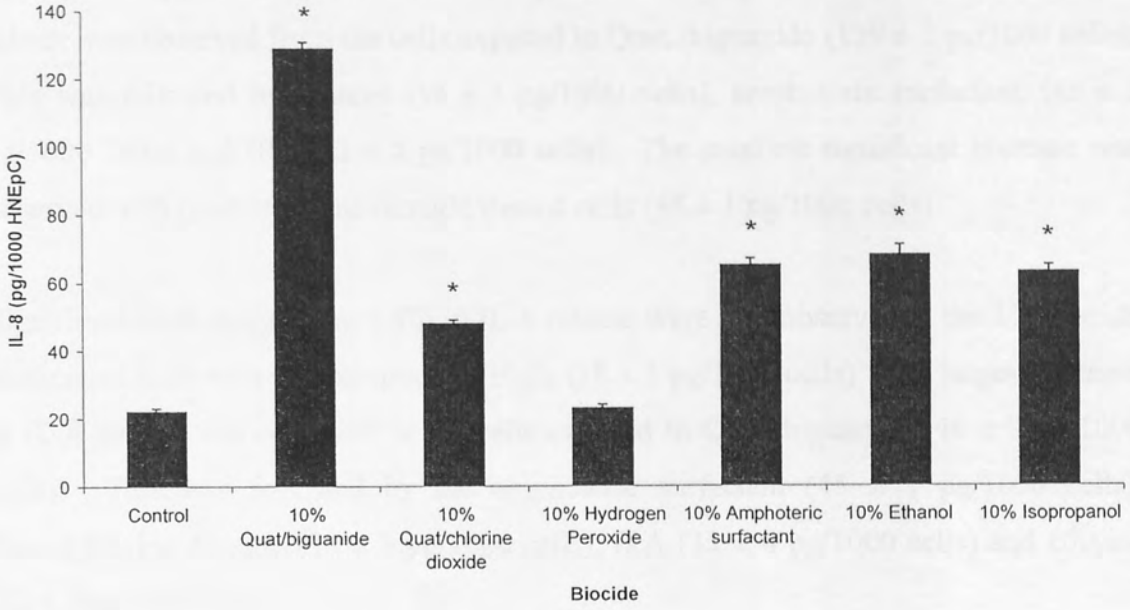


Figure 6.2 Viability of HNEpC exposed to biocides at 100%, 10% or 1% for 24 hours, with cell viability determined by CellTiter-Blue™ Assay after 48 hours of recovery. Data are presented as mean ± SEM (n=5), where fluorescence of unexposed, control cells was taken as 100% and other data expressed relative to this. * $P < 0.05$ in relation to fluorescence of control cells.

The fluorescence observed for healthy viable HNEpC (controls) was between 9,030-10,072 fluorescence units (fluorescence values in Appendix 11.5.2). The viability of the cells as a result of 24 hours biocide exposure was reduced in response to all of the biocides tested, regardless of the concentration (figure 6.2). All 6 biocides, even at 1% of in-use concentrations, significantly reduced cell viability, compared to control ($P < 0.05$).

6.4.4 IL-8 release from biocide exposed HNEpC

a)



b)

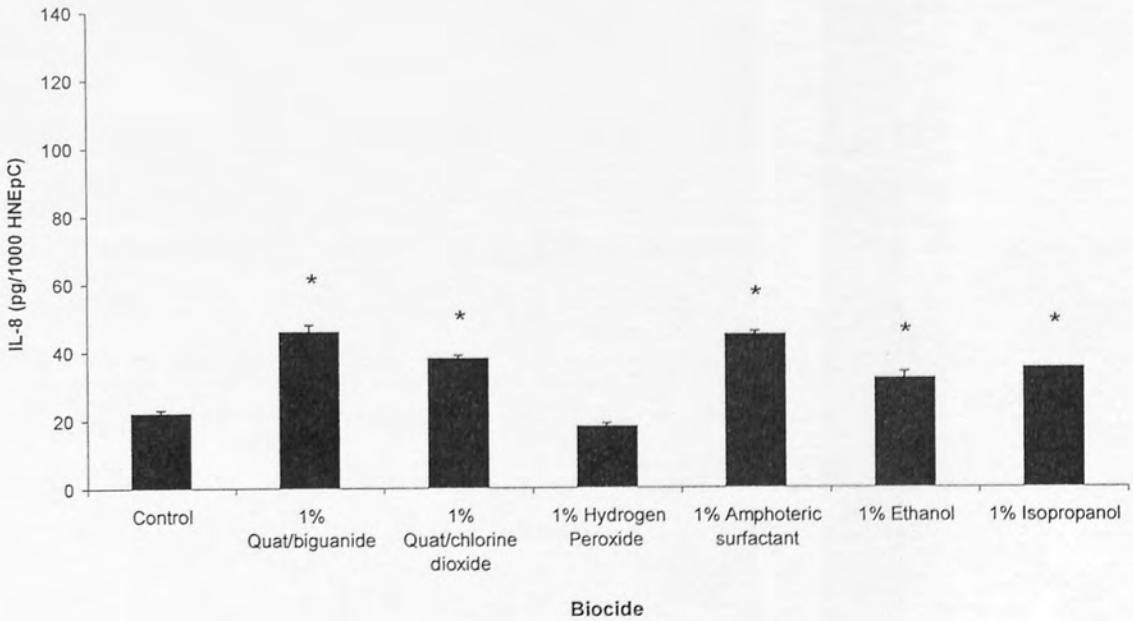


Figure 6.3 IL-8 released from HNEpC in response to 24 hours exposure to quiescent medium (control) or biocides at a) 10% and b) 1% of their practical in-use concentrations. IL-8 expressed as pg per 1000 viable HNEpC Data are presented as mean ± SEM (n=5). * $P < 0.05$ compared to quiescent medium exposed control.

IL-8 data was expressed relative to the number of viable cells (Appendix 11.5.3). Constitutive IL-8 release by HNEpC exposed to quiescent medium for 24 hours averaged 22 ± 1 pg/1000 cells. Exposure to 10% biocide (figure 6.3a) significantly increased IL-8 release compared to control ($P < 0.05$). This was with the exception of H_2O_2 (23 ± 1 pg/1000 cells) ($P > 0.05$ compared to control). The largest increase in IL-8 release was observed from the cells exposed to Quat./biguanide (129 ± 2 pg/1000 cells). This was followed by ethanol (68 ± 3 pg/1000 cells), amphoteric surfactant, (65 ± 2 pg/1000 cells) and IPA (63 ± 2 pg/1000 cells). The smallest significant increase was observed with Quat./chlorine dioxide treated cells (48 ± 1 pg/1000 cells).

Significant increases (figure 6.3b) in IL-8 release were also observed in the 1% biocide challenged cells with the exception of H_2O_2 (18 ± 1 pg/1000 cells). The largest increase in IL-8 release was observed in the cells exposed to Quat./biguanide (46 ± 2 pg/1000 cells). This was followed by the amphoteric surfactant (45 ± 1 pg/1000 cells), Quat./chlorine dioxide (38 ± 1 pg/1000 cells), IPA (35 ± 0 pg/1000 cells) and ethanol (32 ± 2pg/1000 cells).

6.4.5 Thiol content of biocide-challenged HNEpC

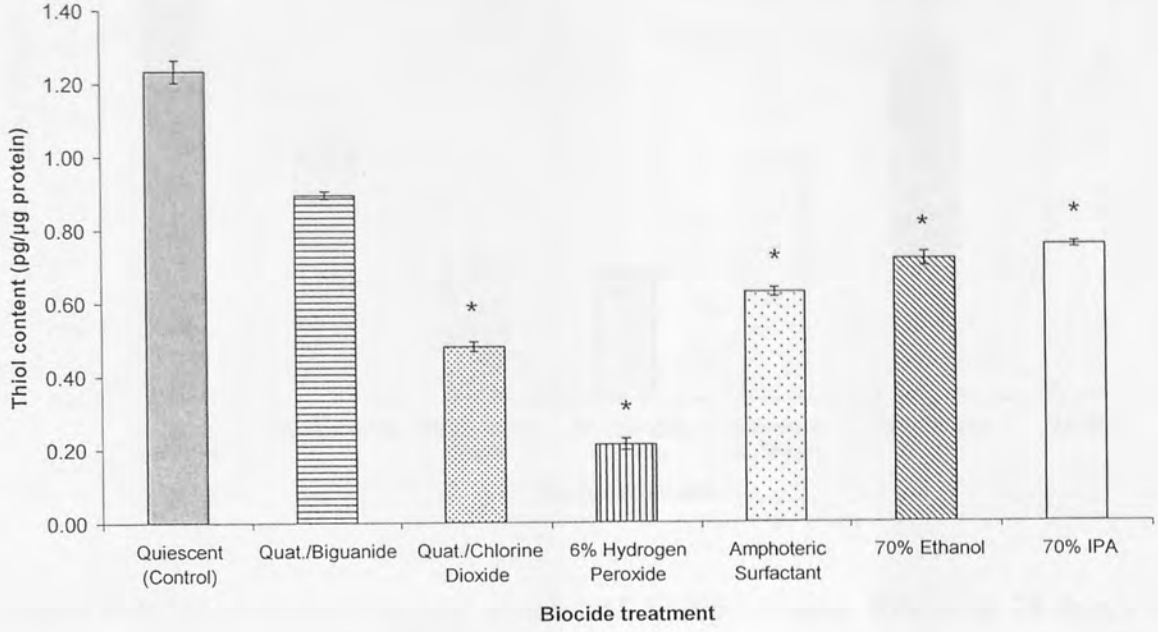


Figure 6.4 Thiol content (pg/µg protein) of HNEpC lysates following 24 hours of exposure to 10% biocide and 48 hours of recovery. Data are presented as mean ± SEM (n=6). * represents $P < 0.05$ comparing biocides and quiescent control.

The basal level of free thiols in submerged cultured HNEpC was 1.23 ± 0.03 pg/µg of protein (figure 6.4). This was reduced after exposure to the biocides at 10% of their in-use concentrations. However, no significant differences relative to control were observed on exposure of HNEpC to the Quat./biguanide (0.90 ± 0.01 pg/µg of protein). There was a significant reduction in free thiols in relation to the control on exposure to 10% H_2O_2 (0.21 ± 0.02 pg/µg of protein), Quat./chlorine dioxide (0.48 ± 0.01 pg/µg of protein), amphoteric surfactant (0.63 ± 0.01 pg/µg of protein), 70% ethanol (0.72 ± 0.02 pg/µg of protein) or 70% IPA (0.76 ± 0.01 pg/µg of protein) ($P < 0.05$).

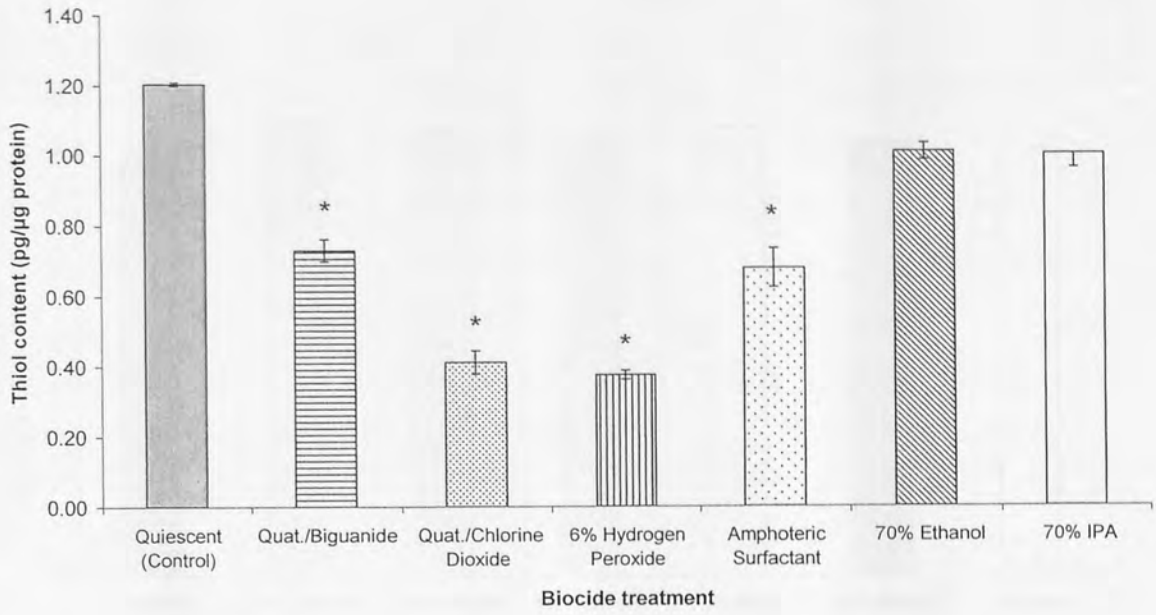


Figure 6.5 Thiol content (pg/µg protein) of HNEpC lysates following 24 hours of exposure to 1% biocide and 48 hours of recovery. Data are presented as mean ± SEM (n=6). * represents $P < 0.05$ comparing biocides and quiescent medium control.

The basal level of free thiols in submerged cultured HNEpC was 1.20 ± 0.01 pg/µg of protein (figure 6.5). A reduction in comparison to this level was observed in the cell lysates on exposure to all 6 biocides at 1% of their in-use concentrations. However, no significant differences relative to control were observed on exposure of the HNEpC to 70% ethanol (1.01 ± 0.02 pg/µg of protein) or 70% IPA (1.00 ± 0.04 pg/µg of protein). There was a significant reduction in free thiol content compared to control on exposure to 1% H_2O_2 (0.22 ± 0.04 pg/µg of protein), Quat./chlorine dioxide (0.41 ± 0.03 pg/µg of protein), Quat./biguanide (0.73 ± 0.03 pg/µg of protein) and amphoteric surfactant (0.68 ± 0.06 pg/µg of protein) ($P < 0.05$).

6.4.6 Carbonyl content of biocide-challenged HNEpC

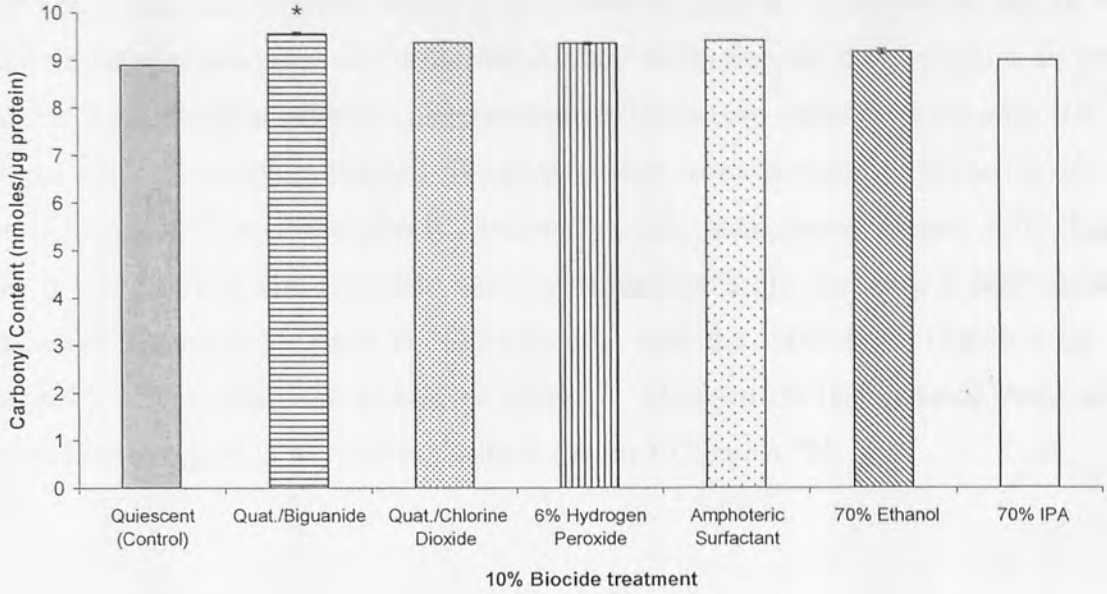


Figure 6.6 Carbonyl content (nmoles/µg protein) of HNEpC cell lysates following 24 hours of exposure to 10% in-use biocide concentrations after 48 hours of recovery. Results are expressed as mean ± SEM (n=6). * represents $P < 0.05$ comparing biocides and control.

Basal levels (control) of carbonyls in HNEpC in submerged culture was 8.90 ± 0.04 nmoles/µg of protein (figure 6.6). This was not changed following exposure to 10% 10% Quat./chlorine dioxide (9.38 ± 0.04 nmoles/µg of protein), H_2O_2 (9.36 ± 0.01 nmoles/µg of protein), amphoteric surfactant (9.41 ± 0.03 nmoles/mg of protein), ethanol (9.18 ± 0.01 nmoles/µg of protein) and IPA (9.00 ± 0.05 nmoles/µg of protein). Exposure to 10% Quat./biguanide significantly increased carbonyl content compared to control (9.56 ± 0.01 to 8.90 ± 0.04 nmoles per µg of protein, respectively; $P < 0.05$).

6.4.7 Propidium iodide staining of challenged HNEpC

Anisomycin, staurosporine, ionomycin and etoposide were used to induce cell death in HNEpC. The cells in gated region 1 (as shown in figure 6.7a) are viable cells of size and granularity characteristic of normal, healthy cells, whereas the population in gated region 2 represents smaller and more granular cells that are likely to be necrotic. Of the drugs used, Anisomycin induced the greatest shift towards region 2 (figure 6.7b). A shift to region 2 was also clearly observed in the staurosporine treated cells (figure 6.7d). The ionomycin, etoposide and UV treated cells did not show a shift towards region 2 (figures 6.7c, e and f). On treatment with the 100% H_2O_2 (figure 6.7g) the majority of the cells were located in region 2. Of the 10% H_2O_2 treated cells; some were seen in region 2, with the majority in region 1 (figure 6.7h).

Table 6.3 PI staining of challenged HNEpC. Percentage of necrotic cells and the mean fluorescence of the cells in the total cell population on exposure of the submerged cultured HNEpC to quiescent medium, anisomycin (5 μg/ml), staurosporine (1 μM), ionomycin (1 μg/ml), etoposide (50 μm), 100% H_2O_2 and 10% H_2O_2 for 24 hours, or UV for 15 minutes (n=1).

Cell treatment (24 hours)	Percentage of necrotic cells in total cell population (%)	Mean fluorescence of all cells in total population (Arbitrary Units)
Quiescent medium	5.92	2.73
Anisomycin	27.80	8.07
Staurosporine	3.26	1.40
Ionomycin	7.31	3.23
Etoposide	13.31	4.58
UV (15 min)	4.21	2.35
100% H_2O_2	61.24	27.8
10% H_2O_2	34.11	8.39

The percentage of necrotic cells in untreated quiescent medium was 5.92% (table 6.3). This value increased more than 10-fold when the cells were exposed to 100% H_2O_2 and more than 5-fold when the cells were exposed to 10% H_2O_2 for 24 hours. The anisomycin (5.92% to 27.80%), etoposide (5.92% to 13.31%) and ionomycin (5.92% to

7.31%) treated cells also had a higher percentage of necrotic cells in relation to the quiescent medium exposed cells. In contrast the percentage of necrotic cells present in the samples decreased on staurosporine exposure (5.92% to 3.26%) and the UV (5.92% to 4.21%).

The mean fluorescence of the cells was also determined, with increased fluorescence of cells on exposure to PI being indicative of necrosis. Without PI, little auto-fluorescence of HNEpC was occurring as the average mean fluorescence of the treated and untreated cells was very low (1.06 arbitrary units to 1.77 AU). The greatest mean fluorescence of the HNEpC was observed with the 100% H_2O_2 exposed cells (table 6.3), whereby the fluorescence increased more than 10-fold in comparison to the quiescent medium exposed cells (27.8 AU to 2.73 AU, respectively). The fluorescence increased three-fold with 10% H_2O_2 treatment (2.73 AU to 8.39 AU). The exposure of the cells to anisomycin resulted in an almost three-fold increase (2.73 AU to 8.07 AU) in fluorescence. The staurosporine (1.40 AU) and UV (2.35 AU) treatments did not result in increased cell fluorescence. Even more so, the level of fluorescence observed actually decreased in relation to the quiescent medium exposed control cells. However, an increase was observed in those cells exposed to ionomycin (2.73 AU to 3.23 AU) and etoposide (2.73 AU to 4.58 AU).

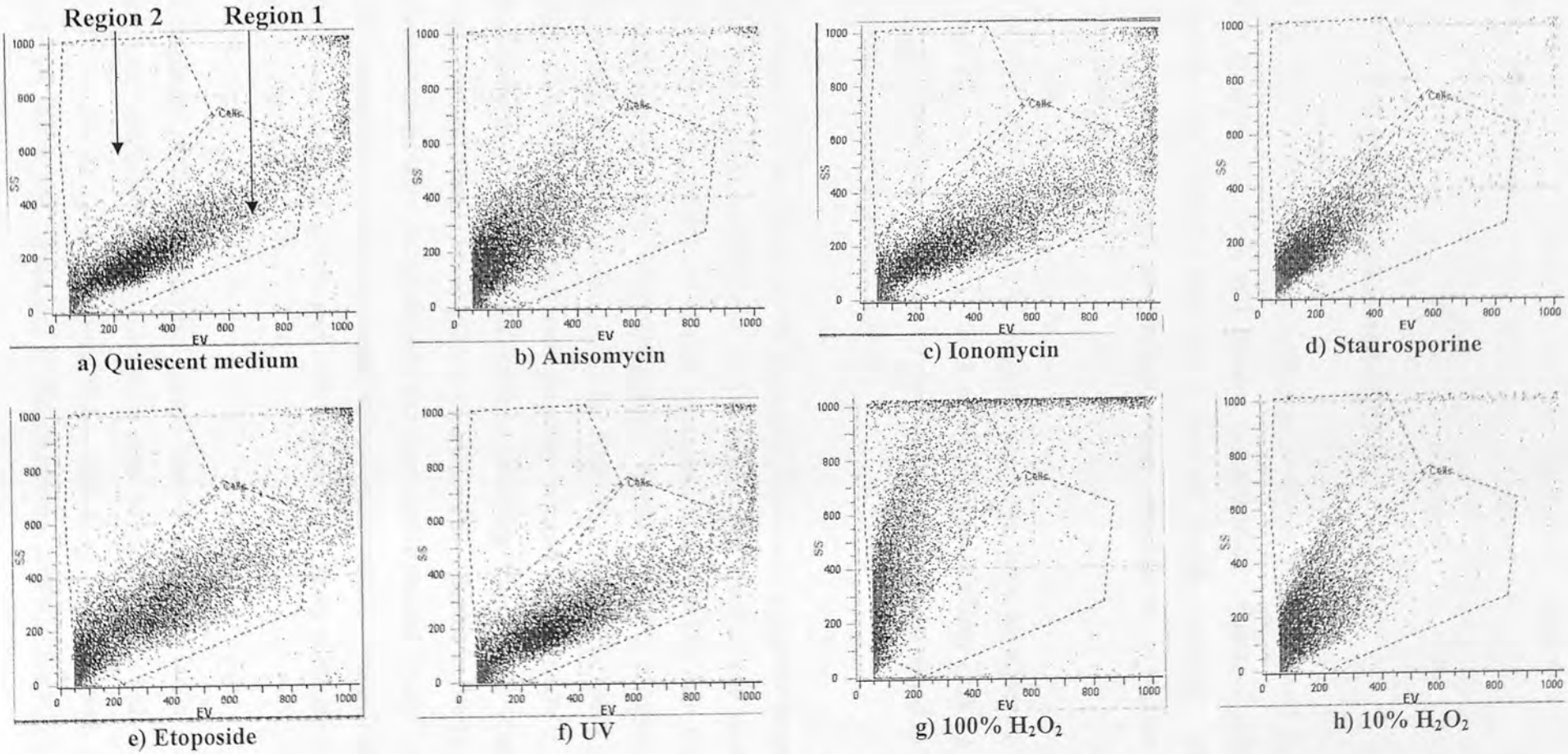


Figure 6.7 Scatter cytograms of submerged cultured HNEpC exposed for 24 hours to a) quiescent medium, b) anisomycin (5 µg/ml), c) ionomycin (1 µg/ml), d) staurosporine (1 µm), e) etoposide (50 µm), f) UV (15 min), g) 100% H_2O_2 and h) 10% H_2O_2. Y-axis (SS = side scatter) is representative of cell granularity and X-axis (EV = Electronic volume) represents the cell size. Region 1 represents the healthy cell population. Region 2 is representative of necrotic cells. Regions are outlined by a dashed line with gating set to overlap all cells (n=1). The most damaging treatment was the 100% H_2O_2.

6.5 Discussion

6.5.1 Cell morphology and viability

Following biocide exposure of submerged cultures, HNEpC shrunk in size, lost contact with neighbouring cells and became non-adherent. Some of these morphological changes were observed after just 1 hour of biocide exposure. All biocides were observed to alter cell morphology when exposed at a fraction of their in-use concentrations.

The PBS, HEPES and SDW vehicles seemed to have an effect on cell morphology when they were applied at their practical concentrations (100%). The extent of morphological damage caused by the vehicles generally decreased as the concentration exposed to the cells also decreased.

The results of the Triton X-100 exposure (figure 6.1) indicated fluorescence levels of less than approximately 873 ± 15 FU represented absolute cell death. Fluorescence levels following vehicle or biocide exposures that were less than this value indicated that the HNEpC were no longer viable. At 10% or 50%, all three vehicles had no significant effect on the viability of the HNEpC. However at 100% concentrations the PBS, SDW and HEPES buffer considerably reduced HNEpC viability in comparison to the quiescent medium exposed controls, achieving fluorescence levels lower than the complete cell death control (873 ± 15 FU). The vehicles at 100% of their practical concentrations were contributing to the cytotoxicity against HNEpC, whereas 50% and 10% concentrations were not contributing significantly to cytotoxic effects. Therefore the results observed at 50% and 10% exposures are likely to be due to the biocides and not the vehicles.

The results of the cell viability assays carried out on the cells following 24 hours exposure confirmed that biocides were toxic to the HNEpC, with only 9.4 ± 0.2% to 12.0 ± 0.2% of cells remaining viable following challenge with concentrations of biocides that would be applied in the cleanroom (100%). However, it is not entirely

sure at this 100% concentration whether the results observed are due to the biocides themselves or the biocide vehicles. Reductions in cell viability also occurred when biocides were applied at 10% (9.6 ± 0.3% to 14.6 ± 0.4%) or 1% (10.0 ± 0.1% to 18.8 ± 0.8%) of their concentrations.

It was important to also determine cell viability in addition to morphological changes because some changes in morphology upon challenge with HEPES were not reflected by changes in cell viability. Such that at a 50% concentration of HEPES, 70% of cells were of an M2 morphology (table 6.1) but cell viability data established that the majority of cells remained viable (figure 6.1).

Overall, at 1%, 10% and 100% of their in-use concentrations, all the biocides tested considerably reduced HNEpC viability. The viability data was also supportive of the morphology data. The general trend observed with all of the biocides was one of a concentration dependent decrease in HNEpC cell viability.

6.5.2 IL-8 release from biocide-exposed HNEpC

This study established that HNEpC under submerged culture produce IL-8, with an increase in IL-8 relative to the number of viable cells being observed in biocide exposed cells. This was with the exception of the 1% and 10% H_2O_2, which were found to inhibit IL-8 release. So, five of the six biocides tested were found to elicit a pro-inflammatory response in the submerged cultured HNEpC when exposed at 10% and 1% of their in-use concentrations. HNEpC released 2.17 ± 0.10 pg/ml under control conditions, with increases in IL-8 being biocide concentration dependent.

The present study found that H_2O_2 concentrations of 17.6 mM (a 1% working concentration) were toxic to the HNEpC with significant reductions in cell viability being observed, alongside no increase in IL-8 release. No studies have previously been carried out regarding the effects of biocides such as H_2O_2 on IL-8 release by HNEpC *in vitro*. However, the results with the H_2O_2 were unexpected as it is a strong oxidant which has been found in other cells types (BEAS-2B and A549 cells) to induce IL-8 expression by activation of the transcription factor AP-1 in a concentration dependent

manner (Lakshminarayanan *et al.*, 1998). Yet, unlike with the other biocides where a decrease in HNEpC viability generally correlated with increased IL-8 release, H_2O_2 did not induce IL-8 release.

Although it is unsure as to why no increase in IL-8 was observed with the H_2O_2 treated cells, the increased production of IL-8 as observed on exposure of the HNEpC to the other biocides can have several consequences on the nasal epithelium. This is because IL-8 is a key chemokine in recruiting neutrophils to sites of infection. In the absence of infection, this can cause localised damage to the airways as the surrounding cells and tissues become targets of the ROS released by the infiltrated neutrophils (Wright *et al.*, 1994; Gabrielson *et al.*, 1994; Barnes, 1990; Takeuchi *et al.*, 2001; Auger *et al.*, 1994; Cohn and Adler, 1992). As a consequence inflammation generates further oxidative stress.

6.5.3 Thiol levels of biocide-challenged HNEpC

Biocides reduced thiol content of submerged cultured HNEpC at both 1% and 10% of their in-use concentrations in a concentration dependent manner. However, non-significant decreases in thiol content were observed on exposure to 10% Quat./biguanide, 1% ethanol and 1% IPA. These findings were as expected as both chlorine dioxide and H_2O_2 are strong oxidising agents. The smallest decreases were observed in the ethanol exposed cells at the 1% exposure and the Quat./biguanide exposed cells at the 10% exposure. This was as expected, as these biocides are not strong oxidising agents.

It was unexpected that any free thiols would be detected due to the cell death observed. However, the detection of free thiols after biocide exposure where only 9.4 ± 0.2% to 18.8 ± 0.8% of cells remained viable suggests that any remaining damaged cells may have increased their thiol-redox status in order to restore the redox balance following the oxidant exposure (Deneke, 2000).

Very little research has been carried out regarding the redox potential of HNEpC, but it has been established in some studies that these cells are susceptible to oxidative

damage. One study investigated the effects of hydroxyl ions on HNEpC and found that they activated Ca^{2+}-activated chloride channels (Jeulin *et al.*, 2005). Activation of these channels can play a direct role in increased epithelial mucus secretion resulting in mucus plugging the lungs. This in turn can lead to impairment of the mucociliary clearance process as is characterised by CF lung disease (Clunes and Boucher, 2008). In addition it has been demonstrated that a disturbance of the balance between Na^+ absorption and Cl^- secretion by epithelial cells could also result in inflammation typical of CF lung disease (Chambers *et al.*, 2007).

The results in the present study also support the finding that HNEpC are susceptible to oxidative damage as a reduction in free thiols is indicative of an imbalance between oxidants and antioxidative defence. This change in cell redox potential is associated with the pathogenesis of several chronic inflammatory disorders of the respiratory tract.

6.5.4 Carbonyl content of biocide-challenged HNEpC

Biocides increased carbonyl content of submerged cultured HNEpC. However, the only significant increase in carbonyl content was on exposure to the Quat./biguanide. This was unexpected as this biocide does not possess any characteristic oxidising ability. The smallest increase in protein carbonyl content was observed in the 70% IPA challenged cells. This was as expected as alcohols are not potent oxidisers. Neither the H_2O_2 nor the Quat./chlorine dioxide significantly increased protein carbonyls. This was unexpected as both of these biocides contain potentially strong oxidising elements. Overall, the increase in protein carbonyl content shows that biocides oxidise submerged cultured HNEpC cell proteins after 24 hours challenge *in vitro*.

Although, there was a general correlation between the thiol and carbonyl levels, with a decrease in thiols and increase in protein carbonyls occurring upon exposure of the HNEpC to the biocides, a direct correlation between thiol and carbonyl levels was not established. The thiol results suggested that of the biocides investigated only H_2O_2 was oxidising cell proteins, whereas the carbonyl results suggested that only the Quat./biguanide was oxidising cell proteins. These observations may have occurred due to possible differences in sensitivity between the two methods. Whereas the thiol assay

only measures free thiol groups in cysteine residues, the formation of carbonyl derivatives only occurs upon direct oxidation of lysine, proline, arginine, histidine and threonine residues. Consequently, this may account for the absence of direct correlation between the two methods, as thiols and carbonyls may not have the same range of sensitivity with regards to cell protein oxidation in submerged cultured cells. As a result both methods could be used in conjunction with each other to get a wider view of any protein oxidation occurring in submerged cultured epithelial cells.

Overall, the oxidation of proteins following biocide treatment may result in the inactivation of cellular proteins (Dalle-Donne *et al.*, 2006). This could have a number of subsidiary effects on both key cellular processes, resulting in the possible impairment of host defence.

6.5.5 Propidium iodide staining of challenged HNEpC

The results observed in the scatter cytograms (figure 6.7) suggest that a change had occurred in the morphology of the cells due to the H_2O_2 exposure, as the H_2O_2 and drug exposed cells were of a smaller size and increased granularity in comparison to the unexposed basal cells. As a result the majority of the cells present in region 2 appeared to be typically necrotic. Finding that the cell morphology had changed following H_2O_2 exposure was in accordance with the morphology results obtained in table 6.2, which showed that cells had shrunk in size and lost contact with neighbouring cells. In addition, the HNEpC cells had very little auto-fluorescence (1.06 AU – 1.77 AU) and so any PI fluorescence observed was due to changes in the cell membrane, brought about by the drugs/biocide which permitted PI entry into the cells interior. As fluorescence in the presence of PI is relative to necrosis, the increased intensity observed indicates that there were more necrotic cells after biocide/drug exposure than in the quiescent medium exposed cells. This was with the exception of the staurosporine and UV which did not result in increased necrosis of the cells. It is hypothesised that this may have occurred due to the HNEpC possibly being resistant to staurosporine and UV exposures, resulting in a decreased level of necrosis occurring. However, no studies have demonstrated this. It is expected that the same results would occur on repetition of this preliminary experiment. Of the drugs tested, the greatest level of necrosis was observed on

exposure of the HNEpC to the anisomycin. So the anisomycin was used as the cell death inducing drug control for the HNEpC. However, H_2O_2 (100%) was twice as toxic to the HNEpC as the anisomycin. This result in itself emphasises the toxic capabilities of H_2O_2 against HNEpC *in vitro*.

A H_2O_2 concentration dependent increase in necrotic cells was detected. The most necrotic cells were detected following exposure of the cells to the 100% H_2O_2, with the necrotic cells comprising nearly 61.24% of the cell population. This was a 30-fold increase in relation to the quiescent medium exposed control cells. In addition to this nearly 34.11% of the cells exposed to the 10% H_2O_2 were necrotic. It is important to note that this data is preliminary as the experiment was only carried out once. Overall although all the cells were not necrotic, the H_2O_2 exposed submerged cultured HNEpC were found to possess more necrotic cells than the control.

This study found that exposure to biocides induces the release of inflammatory IL-8 from submerged cultured HNEpC. Biocides also caused protein oxidation and cell death. This study therefore suggests that biocides are generally pro-inflammatory, oxidising and cause HNEpC death after 24 hours exposure *in vitro*. This may result in compromisation of the human airway epithelium *in vivo* and consequently contribute to the pathogenesis of airway disease in nasal epithelial cells.

CHAPTER 7 AIR-LIQUID INTERFACE CULTURE OF BEAS-2B CELLS

7.1 Introduction

Air-liquid interface (ALI) culture is a method which allows the formation of a more physiologically accurate model of human airways than submerged culture techniques. Culture at ALI can produce a fully differentiated airway epithelium that forms a polarized monolayer with tight junctions separating apical and basolateral regions. In epithelial cells, tight junctions maintain the polarity between the apical and basolateral domains (Anderson and Van Itallie, 1995), such that the basolateral domain faces the endothelium and adjacent cells, whereas the apical domain is in contact with the lumen of the airways.

ALI culture has also been demonstrated to increase the number of ciliated cells in bronchial epithelial cells compared with submerged culture (Grainger *et al.*, 2006; Forbes *et al.*, 2003; Chapman *et al.*, 2002; Foster *et al.*, 2000) by promoting the process of ciliogenesis (de Jong *et al.*, 1994). The generation of a ciliated epithelium is important to obtain an accurate model of the bronchial epithelium, because cilia play a key role in the mucociliary clearance mechanism *in vivo*. In addition to ciliated cells, secretory goblet cells are also important as mucus hypersecretion and goblet cell hyperplasia is implicated in lung disease pathologies such as asthma and COPD (Aikawa *et al.*, 1992; Rogers, 2005a). In addition, mucus is able to trap inhaled toxicant particles and filter the inhalants (Steimer *et al.*, 2005). Increased mucus secretion in comparison to submerged cultured cells has also been observed in cells cultured at ALI (Grainger *et al.*, 2006). *In vitro* ALI culture models appear comparable to native lung epithelia as they are comprised of a heterogeneous population of both ciliated and secretory cells. *In vivo*, the apical surfaces of the cells are directly exposed to air but are nourished from the basolateral side and this is replicated in ALI culture (Bakand *et al.*, 2005; Chen *et al.*, 1993; Knebel *et al.*, 1998; Diabate *et al.*, 2002; Aufderheide *et al.*, 2003; Aufderheide, 2005). In addition, the use of ALI culture is advantageous as it

allows the cells to be exposed to the biocides on their apical side as would occur upon inhalation *in vivo*.

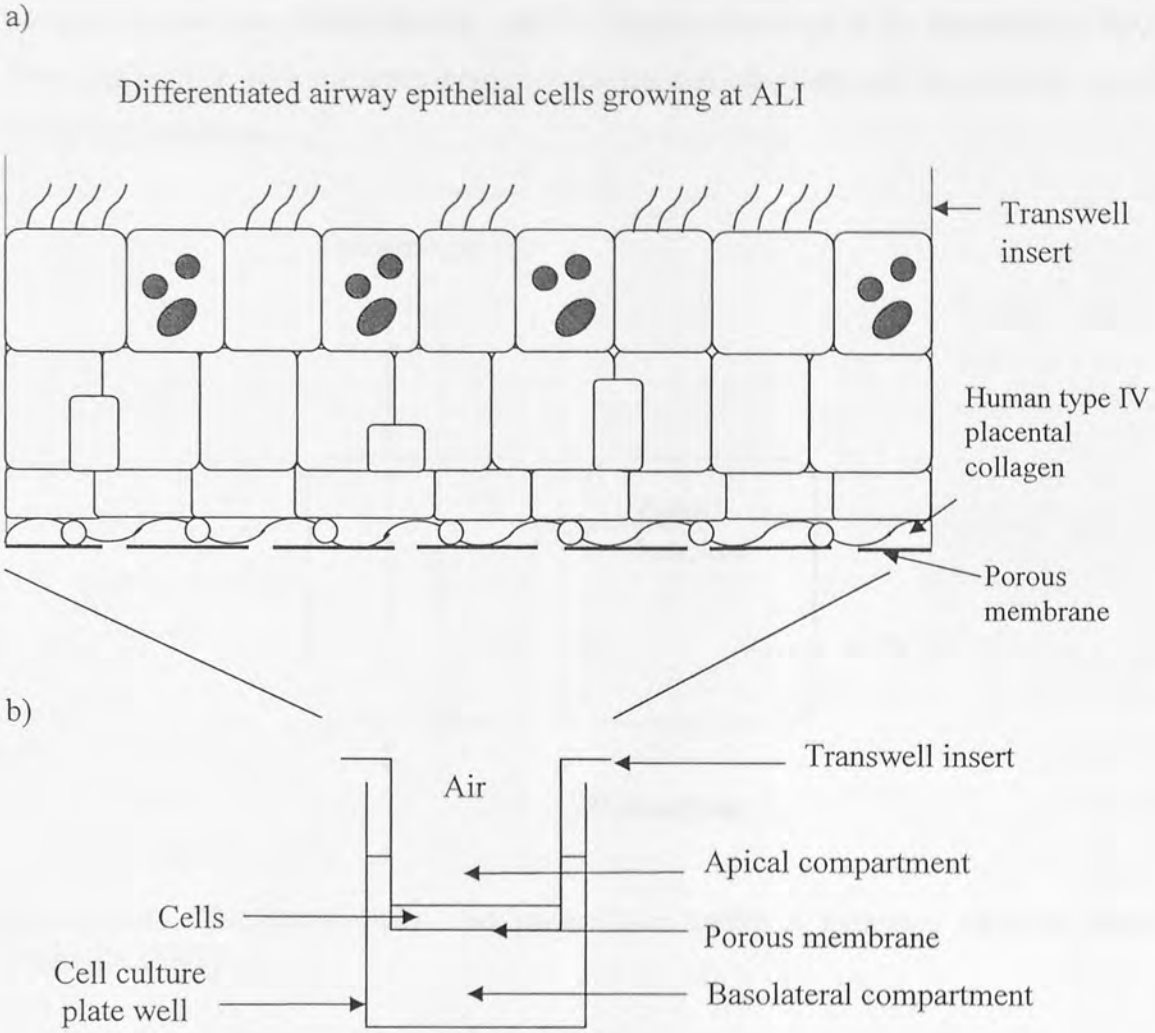


Figure 7.1. Diagram depicting the *in vitro* ALI culture of airway epithelial cells in a Transwell. a) Differentiated cells comprising ciliated, basal and secretory cells were cultured on the upper side of porous polycarbonate Transwells coated with human type IV collagen, b) A Transwell insert placed in a cell culture plate well forms apical and basolateral compartments separated by the collagen-coated porous membrane, upon which the cells are cultured. The cells are cultured at ALI in the apical compartment, while obtaining their nutrients from culture medium in the basolateral compartment, through the porous membrane.

Preservation of epithelium integrity is critical for vital physiological processes. Changes in the transcellular (movement of solutes across the epithelial cell layer through the cells) or paracellular (movement of materials across the epithelial cell layer

through the tight junctions between cells) transport pathways (figure 7.2) may indicate damage to the epithelium (Artursson *et al.*, 1996). Tight junctions constitute a barrier to the passive movement of fluid, electrolytes and macromolecules through the paracellular pathway (Ehrhardt *et al.*, 2002). Proteins involved in the formation of tight junctions include both transmembrane (occludins and claudins) and intracellular (zona occludens) proteins.

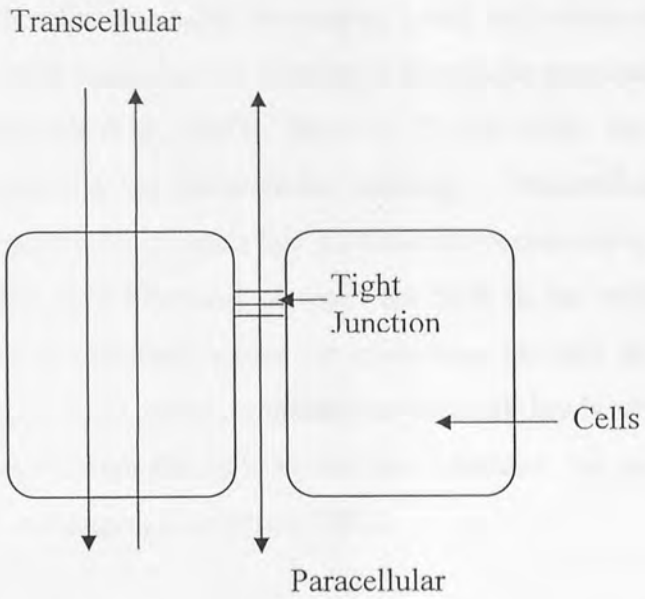


Figure 7.2. The transcellular and paracellular transport pathways (adapted from Anderson, 2001).

Transepithelial electrical resistance (TER) generated across an epithelium is a measure of the resistance to the movement of ions across the epithelium through the cells. TER is used to study alterations in organisation of tight junctions that influence the paracellular permeability of the epithelial cell layer (Ehrhardt *et al.*, 2002). Therefore TER is an indication that transepithelial permeability barriers such as tight junctions have become established (Hughson and Hirt, 1996). Thus, the TER of cells grown on Transwells can provide insight to the epithelium as a barrier (Kannan *et al.*, 2006; Chapman *et al.*, 2002). Therefore the TER of BEAS-2B cells cultured at ALI was measured at hourly intervals over a 24 hour period following application of either H_2O_2 (100%, 10% or 1%), quiescent medium or remaining unexposed.

Paracellular permeability of BSA was used alongside TER to investigate changes in epithelial cell membrane integrity during and after biocide exposure. So as well as determining the electrical properties of the tissues by measuring the electrical resistance of the cells, the permeability of the cell monolayers was assessed by measuring the apical to basolateral movement of BSA via the paracellular pathway. Whereas TER measures the ability of small ions to pass between the cell monolayer via the tight junctions (1.5 nm diameter) (Staehelin and Hull *et al.,* 1978), BSA is a 66 kDa protein (macromolecule) and therefore is unable to cross an intact epithelium via tight junction pores. BSA has previously been used as a probe to determine permeation pathways in human tracheal epithelia (Ma *et al.*, 1993). Ma *et al.* (1993) found that the BSA probe permeated predominantly via the paracellular pathway. Transcellular permeability differs to paracellular permeability, such that paracellular permeability is indicative of damage occurring to the tight junctions between the cells in the epithelium, thereby allowing the movement of materials across the epithelium through the damaged tight junctions between the cells. In contrast, transcellular permeability is associated with the movement of chemicals through the cells across the cytoplasm via plasma membrane channels, carriers, and exchangers (Anderson, 2001).

Increased transcellular permeability (movement through the cells) may result in an imbalance in the ion homeostasis which could have subsequent effects on the quantity and composition of airway surface liquid (ASL) and water regulation (Godfrey, 1997; Coyne *et al.,* 2002; Flynn *et al.,* 2009). ASL is important because its dehydration may result in the pathogenesis of mucociliary dysfunction and chronic airway disease (Chambers *et al.,* 2007). Increased epithelial paracellular permeability or damage to the barrier may allow inhaled microbial pathogens to breach the apical surface of the cells and move to the basolateral surface, thereby promoting infections in the host. In addition, paracellular permeability would allow other inhalants such as ozone and cigarette smoke which have oxidative capacities to access and oxidise underlying proteins which play many roles in the functioning of the cells. This could result in underlying endothelial cells losing some of their structural and/or functional integrity, which would have a subsequent impact on human health. For example, asthma and bronchitis sufferers have increased airway epithelial permeability, which is associated with bacterial infection and inflammation (Godfrey, 1997; Godfrey, 1993; Devalia *et al.,* 1994, Walker *et al.,* 1984).

Other parameters tested in this chapter included changes in the pro-inflammatory status of the ALI cultured BEAS-2B cells following biocide challenge. The polarity of the IL-8 secretion in response to biocide exposure was studied, since epithelial cells secrete cytokines in a polarised manner (Auger *et al.*, 2006). This was ascertained by the measurement of apical and basolateral IL-8 secretion using an IL-8 ELISA both following biocide challenge and then following recovery of the cells. By exposing the apical side of the cells to test compounds, it is possible to observe whether these cells express cytokines and other proteins in response to the biocides and whether any release of components is directed apically or basolaterally. The oxidative effects of the biocides on the cell proteins were determined by establishing the protein thiol (thiol assay) and protein carbonyl (carbonyl ELISA) levels in biocide exposed cells. The cell death mechanism of necrosis (flow cytometry) was also investigated following biocide exposure to the ALI cultured cells.

7.2 Aims

- To determine whether the integrity of the epithelial layer is compromised following biocide exposure.

- To ascertain whether biocides induce a pro-inflammatory status in BEAS-2B cells cultured at ALI and if the IL-8 release is directional.

- To determine the thiol and carbonyl content of BEAS-2B cells cultured at ALI when exposed to Quat./chlorine dioxide, 6% H_2O_2 and 70% ethanol.

- To assess cell death and determine whether BEAS-2B cell death occurs by necrosis.

7.3 Materials and Methods

7.3.1 Materials

BD Falcon™ Cell Culture Inserts (Transwells) and BD Falcon™ 24-well Cell Culture Insert Companion Plates were from BD Falcon (Oxford, UK). BSA and Human placental type IV collagen were from Sigma (Dorset, UK). Promocell® Airway Epithelial Cell Growth Medium (AECGM) and SupplementMix were from Promocell (Heidelberg, Germany). Modified Eagles Medium was from PAA (Somerset, UK). Epithelial Voltohmmeter was from EVOM, World Precision Instruments (Stevenage, UK).

For all other materials please refer to Chapter 5.3.1

7.3.2 Methods

7.3.2.1 Epithelial cell culture on Transwell inserts (ALI culture)

Cell culture Transwell inserts used have polyethylene terephthalate (PET) track-etched membranes. These membranes have a low pore density and are transparent. The effective diameter of the membrane is 6.4 mm with a growth area of 0.3 cm^2. The membrane comprised 1.6 $x10^6$ pores/square cm; pores were 0.4 microns in diameter. The cell culture inserts are aseptically handled with sterile forceps and placed into a 24-well cell culture insert companion plate.

Transwells were coated with human placental type IV collagen (10 $\mu g/cm^2$). Collagen coating was carried out by adding the human placental type IV collagen (10 $\mu g/cm^2$) to the Transwells and incubating for 30 minutes. The collagen was then removed and the Transwells washed three-times with phenol-red containing Modified Eagles Medium (MEM) prior to seeding with cells. All BEAS-2B (P3-P19) cells were seeded on the Transwells at a density of 1 x 10^5 cells/ml, in 300 µl Promocell® Airway Epithelial Cell Growth Medium supplemented with 5% FCS (PAA, Somerset, UK), 1% penicillin (5,000 U/ml^{-1}), streptomycin (5000 $\mu g/ ml^{-1}$) and the manufacturer's growth factor

supplement 600 µl medium was added to the basal compartment. The apical medium was removed three days after seeding to produce an ALI and promote cell differentiation. Cells were incubated at 37°C, 5% CO_2 and basolateral medium was changed every 3 days.

7.3.2.2 Determining TER of cell cultures at ALI in Transwells

The confluence of the BEAS-2B cells cultured at ALI was determined by measurement of the TER using an Epithelial Voltohmmeter. To measure the TER, 300 µl of serum-free medium was added to the apical compartments of the Transwells. The chopstick electrode was introduced to the Transwell and a current was passed between the apical and basal compartments. Overall TER measurements (Ohms x cm^2) were calculated by subtracting the value of a blank, cell-free collagen-coated Transwell from the experimental values and by multiplying by the surface area of the Transwell insert (0.33 cm^2). Measurements were taken every 2-3 days in triplicate for each well, until a TER of at least 300 Ohms x cm^2 was achieved.

Figure 7.3 An image of the EVOM - Epithelial Voltohmmeter, showing the chopstick electrode comprised of a fixed pair of double electrodes and the EVOM resistance reader (adapted from Anon A, 2008).

7.3.2.3 Challenging ALI cultures of epithelial cells.

Once the cells had reached a TER of ≥ 300 Ohms x cm^2, the basolateral medium was removed and replaced with 600 µl serum-free medium for 24 hours to quiesce the cells. Following this, 300 µl of the biocide (at 100%, 10%, 1% and 0% of practical concentrations diluted in quiescent medium) was added to the apical compartment. The biocides used were Quat./biguanide, Quat./chlorine dioxide, 6% H_2O_2, amphoteric surfactant, 70% ethanol and 70% IPA. After 24 hours, the apical and basal cell supernatants were removed and centrifuged separately at 380 x g for 7 minutes. The cleared supernatants were stored at -80°C. The cells were replenished with full medium (600 µl basal only) and left to recover for 48 hours, after which time the apical surface fluid was collected by washing with 300 µl PBS. Following this the basal cell supernatants were removed, centrifuged (380 x g for 7 minutes) and apical and basal collections were stored at -80°C for future analysis. The cells were then treated with 300 µl Triton X-100 (1%) on ice for 15 minutes. The resultant cell lysates were centrifuged at 380 x g for 7 minutes to remove any cell particles and stored at -80 °C.

7.3.2.4 Paracellular permeability

300 µl fresh medium containing 100 mg/ml of BSA was added apically to confluent BEAS-2B cells (TER of ≥ 300 Ohms x cm^2). One-tenth of the total volume of the medium in the basolateral chamber was removed at 1 hour time intervals until 6 hours, and then at 24 hours. To control for the basally reducing volume, an equal volume (60 µl) of quiescent medium was added to the basolateral chamber to replace the volume removed for BSA quantification. Paracellular permeability was measured with basolateral BSA expressed as a percentage of total BSA added apically.

7.3.2.5 IL-8 release from BEAS-2B cells cultured at ALI

IL-8 release was initially determined as pg/ml by ELISA, accounting for differences in apical and basolateral medium volumes. In order to take into account changes in cell number due to biocide treatment, IL-8 release was calculated relative to the protein content of cell lysates (Appendix 11.6.1) and is expressed as pg/µg protein. To

visualise any biocide-dependent changes in IL-8 release, the data are expressed as percentage of control. Control refers to IL-8 release in response to quiescent medium and is arbitrarily given the value of 100%.

Methods used to determine protein thiol content, protein carbonyl content and necrosis were described in Chapter 5.3.2.

7.4 Results

7.4.1 Transepithelial Electrical Resistance

7.4.1.1 TER of BEAS-2B cells cultured at ALI

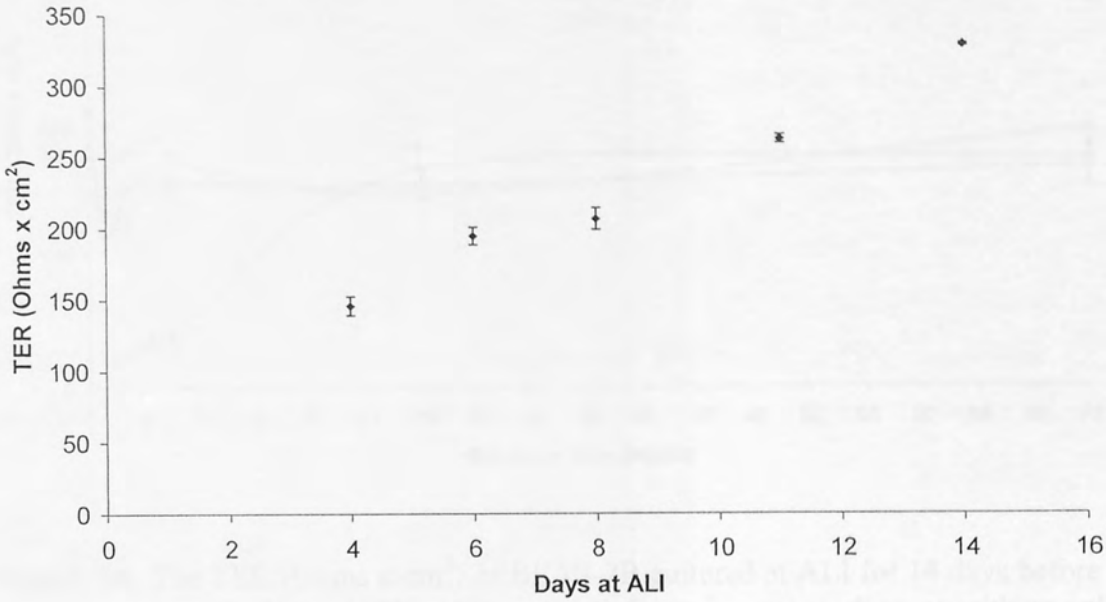


Figure 7.4 TER values of BEAS-2B cultured on Transwells at ALI for up to 14 days. Results are expressed as mean ± SEM. (n=12 with 6 of each passage 8 and passage 12).

Figure 7.4 indicates that a progressive increase in TER value was observed with time as the cells were grown at ALI. The cells reached a TER of 329 ± 2 Ohms x cm^2 after 14 days at ALI. It was at this point that the cells were used for biocide treatment.

7.4.1.2 TER of H_2O_2 treated BEAS-2B cells cultured at ALI

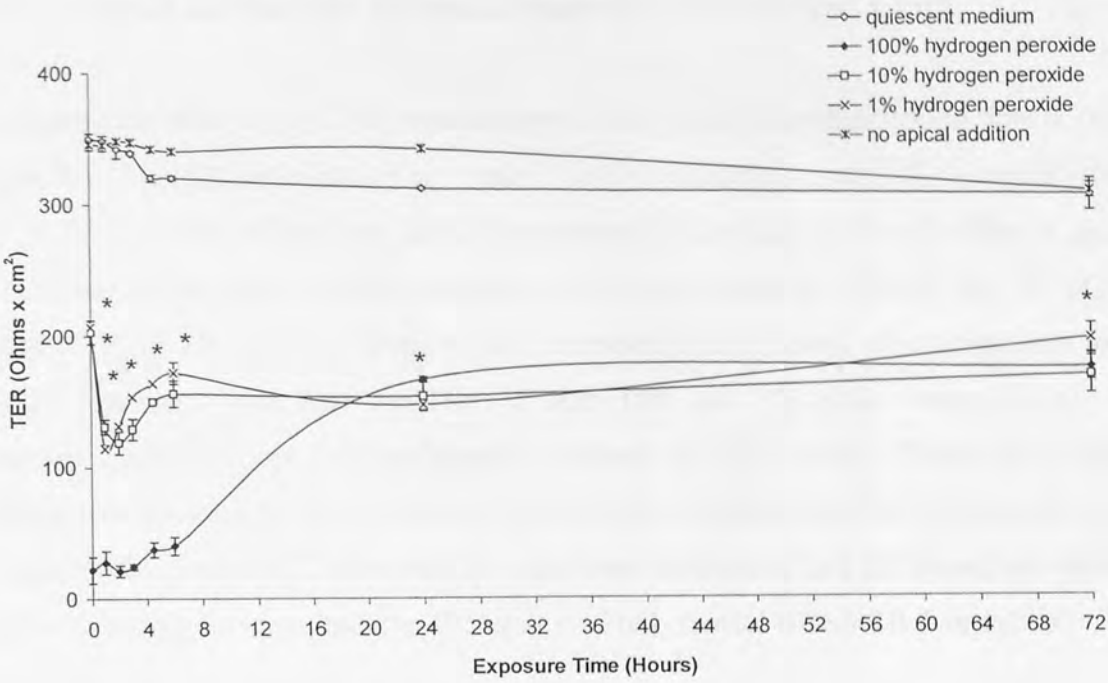


Figure 7.5 The TER (Ohms x cm^2) of BEAS-2B cultured at ALI for 14 days before 24 hours exposure apically to 100%, 10% or 1% H_2O_2, quiescent medium or with no apical addition. The TER of collagen-coated, cell-free Transwells exposed to H_2O_2 (control) were subtracted from the TER of BEAS-2B cells exposed to H_2O_2. Results are expressed as mean ± SEM (n=3). * represents $P < 0.05$ comparing biocide exposed and quiescent medium control cultures at that time-point.

The TER of the quiescent medium exposed control cells was initially 350 ± 4 Ohms x cm^2 (figure 7.5). This value decreased non-significantly over the 24 hours exposure time. All TER values were corrected for the TER of an empty Transwell which measured on average 15 ± 1 Ohms x cm^2. In addition, the effect of H_2O_2 on the performance of the voltohmmeter was examined with the TER of collagen-coated, cell-free Transwells exposed to H_2O_2 (Appendix 11.6.2) subtracted from the TER of H_2O_2 treated BEAS-2B.

On exposure to the 100% H_2O_2 there was a significant decrease in TER (22 ± 3 Ohms x cm^2) in comparison to the quiescent medium exposed cells ($P < 0.05$). The TER was observed to increase after 1 hour of exposure to 100% H_2O_2 (27 ± 3 Ohms x cm^2). However, the TER values decreased non-significantly after 2 hours of exposure (21 ± 4

Ohms x cm^2). This was followed by a non-significant increase at 3 hours (24 ± 2 Ohms x cm^2). In general the increased TER was sustained throughout the 24 hours of 100% H_2O_2 exposure and also after 48 hours of recovery (174 ± 10 Ohms x cm^2).

A significant reduction in TER was observed after 1 hour in comparison to time 0, with both the 10% (131 ± 6 Ohms x cm^2) and 1% (114 ± 3 Ohms x cm^2) H_2O_2 treated cells ($P < 0.05$). The TER of the 10% H_2O_2 treated BEAS-2B (119 ± 9 Ohms x cm^2) decreased further after 2 hours exposure. In comparison, the TER of the 1% H_2O_2 treated BEAS-2B (131 ± 2 Ohms x cm^2) increased after 2 hours, when compared to 1 hours exposure. But then the TER of both 10% and 1% H_2O_2 treated BEAS-2B increased after 3 hours and continued to increase up till 6 hours. There was a non-significant decrease in TER between 6 hours and 24 hours, with the TER observed to increase after recovery. There were no significant differences in TER throughout the 24 hours of testing between both the 10% and 1% H_2O_2 treated BEAS-2B Transwells.

Following 48 hours recovery, there was a non-significant increase in the TER of the 100% (174 ± 10 Ohms x cm^2), 10% (168 ± 14 Ohms x cm^2) and 1% (196 ± 11 Ohms x cm^2) H_2O_2 treated cells, when compared to the TER after 24 hours. However, these values were still significantly less than the TER of quiescent medium exposed control cells ($P < 0.05$ compared to quiescent medium control).

7.4.2 Paracellular permeability of BSA

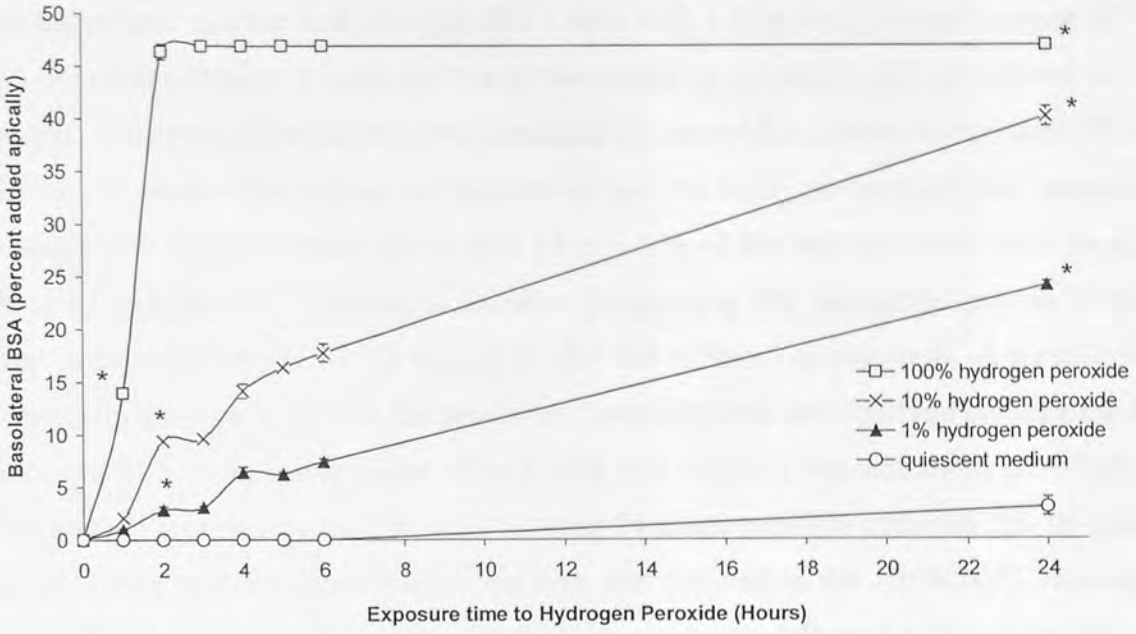


Figure 7.6 The paracellular permeability of BEAS-2B cells cultured at ALI to BSA over 24 hours of apical exposure to 100%, 10% or 1% H_2O_2, or quiescent medium. Results are expressed as mean basolateral BSA as a percentage of BSA added apically ± SD (n=3). Using ANOVA, * represents $P < 0.05$ comparing between biocide exposed and the quiescent medium exposed (control) at each time point.

No increase in protein levels in the basolateral compartments of BEAS-2B cells was observed after 6 hours of exposure to quiescent medium (figure 7.6). Only after 24 hours exposure to quiescent medium was a small increase in any paracellular permeability observed, with a 3 ± 0.9 % increase in the basolateral protein concentration. However, this increase in BSA paracellular permeability was not significant compared to time 0 ($P > 0.05$).

Following apical exposure of the BEAS-2B cells to 100% H_2O_2 containing 64 ± 3 mg/ml BSA (figure 7.6) a significant increase in the BSA passing paracellularly from the apical compartment of the Transwells through to the basolateral compartment was observed following just 1 hour of treatment ($P < 0.05$ compared to quiescent medium control at 1 hour). The level of BSA detected basolaterally also increased further after another hour (total 2 hrs) of exposure with 46 ± 0.7 % of the apical BSA now detected

in the basolateral medium. However, after 3 hours there was no further increase in the basolateral BSA level throughout the 24 hour experimental time-frame. On exposure of the cells to 10% H_2O_2, a small non-significant increase (2 ± 0.1 %) in protein levels in the basolateral medium was detected after 1 hour with a further significant increase (9 ± 0.3 %) observed after 2 hours ($P < 0.05$ compared to quiescent medium control at 2 hours). The level of protein observed remained the same after 3 hours of exposure (10 ± 0.1 %). However after 4 hours of exposure to the 10% H_2O_2, the level of BSA detected basolaterally increased once again, with 14 ± 0.6 % of the apically added BSA being detected basolaterally. Further consecutive increases in the basolateral protein levels were observed after 5 (16 ± 0.2 %) and 6 (18 ± 0.8 %) hours of exposure. A significant increase in the protein level in the basolateral compartments was observed between the 6 hour and 24 hour sampling stages of both 10% and 1% H_2O_2 concentrations ($P < 0.05$). The level of protein detected (40 ± 0.9 %) after 24 hours exposure of the BEAS-2B cells to 10% H_2O_2 was non-significantly less than that detected in the 100% H_2O_2 exposed cells (46 ± 0.7 %) ($P > 0.05$). The 1% H_2O_2 exposed cells followed a similar pattern to the 10% H_2O_2 exposed cells, whereby there was a continuous increase in the level of protein passing from the apical to basolateral compartments as exposure time increased. The BSA detected basolaterally increased at each sampling stage up till the 4 hour point (6 ± 0.5 %) and then remained the same at 5 hours (6 ± 0.2 %). The level then continued to increase after the 5 hour exposure stage. There was a significant increase in the level of protein detected after 24 hours (24 ± 0.4 %) in relation to 6 hours (7 ± 0.3 %) ($P < 0.05$). However the amount of protein detected basolaterally after 24 hours exposure to the 1% H_2O_2 (24 ± 0.4 %) was not higher than that observed with the 10% or 100% H_2O_2 exposed BEAS-2B cells. With the 10% and 1% H_2O_2, 46 ± 0.7 % and 24 ± 0.4 % of the apically added BSA was detected basolaterally after 24 hours of exposure, respectively.

As H_2O_2 may have oxidised the apically added BSA, a BCA protein assay (Chapter 5, section 5.3.2.3) was carried out after dissolving 100 mg/ml BSA in 100%, 10% and 1% H_2O_2. Some differences in the concentrations of proteins were detected with all three H_2O_2 concentrations. Therefore, even though 100 mg/ml BSA was dissolved in the H_2O_2, the actual protein levels able to be measured were less than 100 mg/ml due to the BSA being oxidised by the H_2O_2. Consequently, the concentrations of protein actually detected (for 100% H_2O_2: 64 ± 3 mg/ml, 10% H_2O_2: 73 ± 1 mg/ml and 1% H_2O_2: 85 ± 1

mg/ml), were arbitrarily given the value of 100%, with basolaterally detected BSA determined as a percentage of this concentration. The protein levels used to calculate percentage BSA permeability (figure 7.6) are in Appendix 11.6.3.

7.4.3 IL-8 release from biocide exposed BEAS-2B cells

BEAS-2B were cultured at ALI for 14 days before exposure to 100% or 10% concentrations of Quat./chlorine dioxide, H_2O_2 or ethanol. The Quat./chlorine dioxide and the H_2O_2 were chosen for further investigation as they were found to be the most efficacious against *Bacillus* spores (Chapter 3, section 3.4.5). The ethanol was chosen due to its prevalence of use for disinfection.

An Limulus Amoebocyte Lysate (LAL) assay carried out with the biocides determined that they were endotoxin free (Appendix 11.6.4) and therefore any increase in IL-8 release was not a response to endotoxin contamination of the biocides.

7.4.3.1 IL-8 released apically following 24 hours biocide exposure.

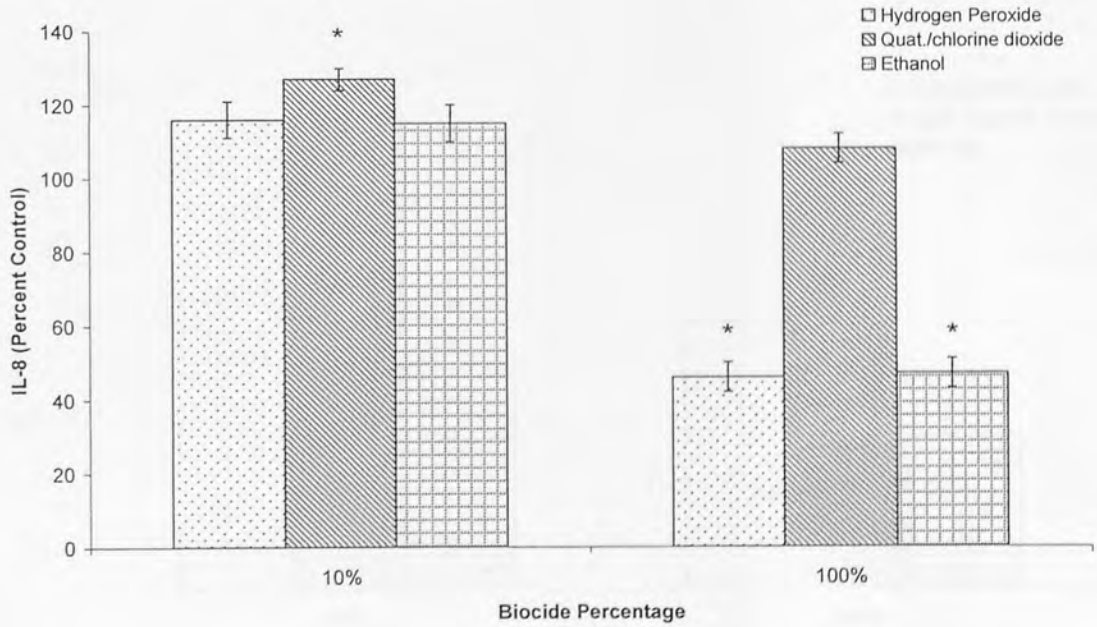


Figure 7.7 IL-8 released apically by BEAS-2B cells cultured at ALI and exposed to biocides at 10% or 100% of practical concentration for 24 hours, taking into account cell protein levels. Data are presented as mean percentage of control cells ± SEM (n=6), where IL-8 of quiescent medium exposed control cells was taken as 100%. * $P < 0.05$ in relation to control.

Non-significant increases in the IL-8 released apically by cells exposed to 10% H_2O_2 (116 ± 5 % control) or ethanol (115 ± 5 % control) were detected in comparison to quiescent medium exposed control cells (0.44 ± 0.05 pg/µg protein taken as 100%) (figure 7.7). A significant decrease in relation to the control apical IL-8 was observed when the cells were exposed to 100% H_2O_2 (46 ± 4 % control) and ethanol (47 ± 4 % control). In comparison, a significant increase (127 ± 3 % control) in the IL-8 released apically by the BEAS-2B cells was observed on exposure to Quat./chlorine dioxide at 10% of its working concentration ($P < 0.05$ compared to control). In contrast, a non-significant increase (108 ± 4 % control) was observed in the 100% Quat./chlorine dioxide exposure ($P < 0.05$ compared to control). The difference in IL-8 release was not significant between the 10% and 100% Quat./chlorine dioxide exposures ($P > 0.05$).

7.4.3.2 IL-8 released apically following 48 hours recovery from biocide exposure.

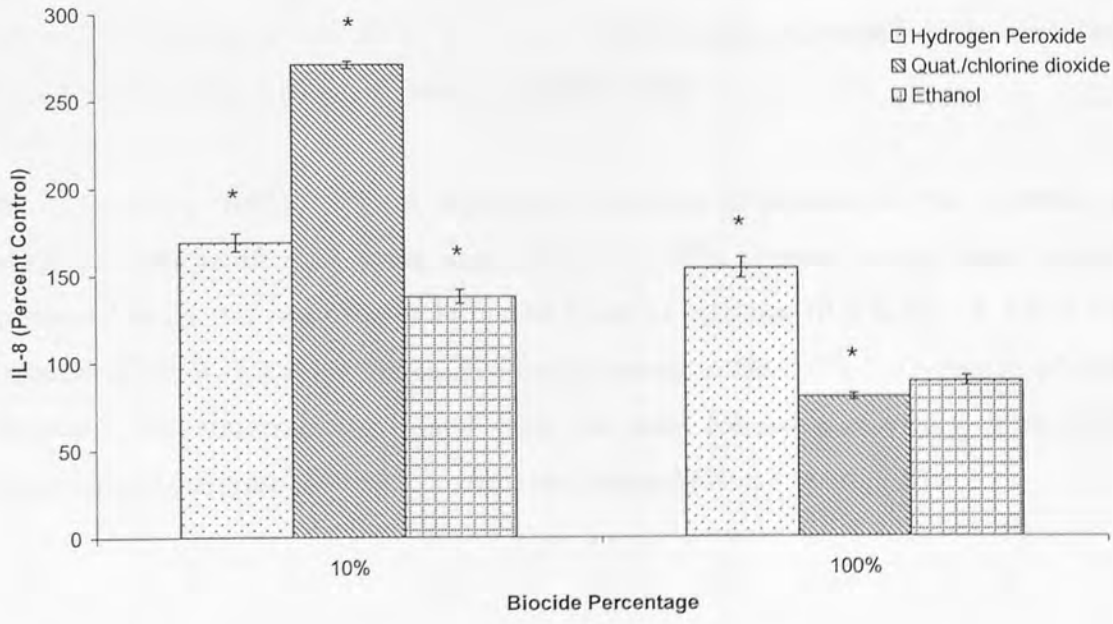


Figure 7.8 IL-8 released apically by BEAS-2B cells cultured at ALI and exposed for 24 hours to biocides at 10% or 100% of practical concentration and allowed to recover for 48 hours at ALI, taking into account cell protein levels. Data are presented as mean percentage of control cells ± SEM (n=6), where IL-8 of quiescent medium exposed control cells was taken as 100%. * $P < 0.05$ in relation to control.

With regards to the IL-8 released apically (figure 7.8), significant increases in IL-8 release in comparison to control levels (0.77 ± 0.14 pg/µg protein taken as 100%) were observed following exposure of the cells and after 48 hours recovery to all 10% biocides (H_2O_2: 169 ± 5 % control, Quat./chlorine dioxide: 271 ± 2 % control and ethanol: 138 ± 4 % control) ($P < 0.05$). A significant increase was also observed on exposure of the cells to the 100% H_2O_2 (154 ± 6 % control) ($P < 0.05$). In comparison a non-significant decrease in IL-8 released was observed after recovery of the cells to 100% ethanol exposure (89 ± 3 % control). A significant reduction in IL-8 released apically (80 ± 2 % control) was observed following exposure of the cells to 100% Quat./chlorine dioxide ($P < 0.05$ compared to control).

Some differences in IL-8 levels released by the cells were also detected between the cells following 24 hours of exposure to the biocides and after 48 hours of recovery of the biocide-exposed cells. IL-8 levels adjusted for the protein content of the cell lysates directly after exposure to the biocides at 10% were lower than after recovery. This was also found in the apical supernatants of the 100% Quat./chlorine dioxide treated BEAS-2B cells. However, all IL-8 levels in 10% biocide recovered cells were still significantly higher than in the control cells ($P < 0.05$).

At exposure to 100% H_2O_2, a significant decrease in relation to the control was observed directly after 24 hours exposure ($P < 0.05$), whereas a significant increase compared to control was observed after 48 hours of recovery ($P < 0.05$). A 154 ± 6 % increase of the IL-8 level of the control was observed in the 100% H_2O_2 recovered cells. However, the converse was found with the cells following recovery from 100% Quat./chlorine dioxide (80% ± 2 control) and ethanol (89 ± 3 % control).

7.4.3.3 IL-8 released basolaterally following 24 hours biocide exposure.

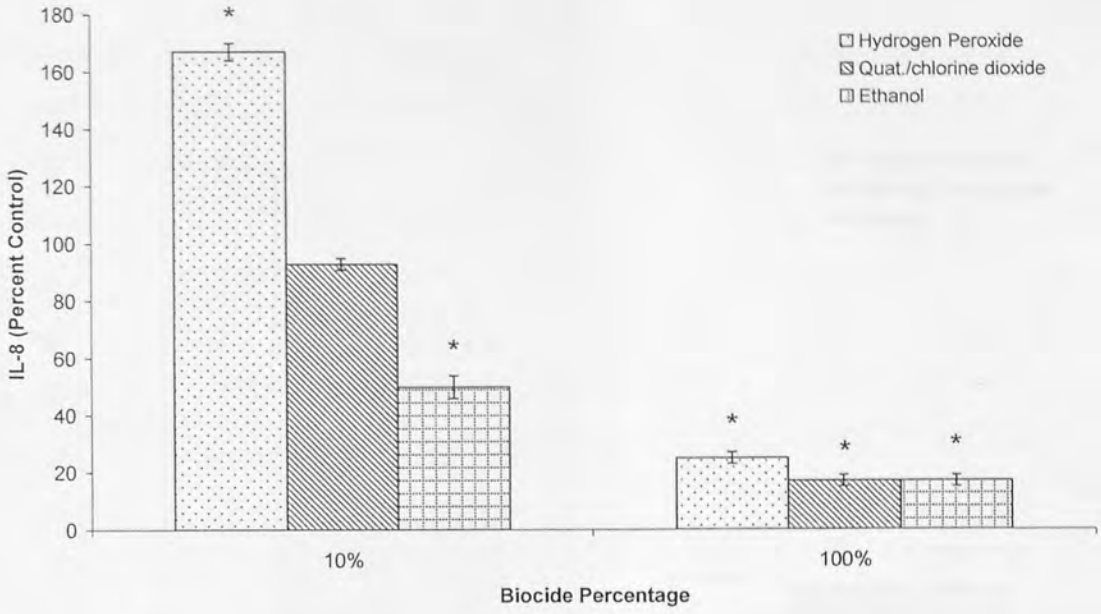


Figure 7.9 IL-8 released basolaterally by BEAS-2B cells cultured at ALI and exposed to biocides at 10% or 100% of practical concentration for 24 hours, taking into account cell protein levels. Data are presented as mean percentage of control cells ± SEM (n=6), where IL-8 of quiescent medium exposed control cells was taken as 100%. * $P < 0.05$ in relation to control.

A significant increase in levels of IL-8 basolaterally released (figure 7.9) were observed with 10% H_2O_2 exposed BEAS-2B cells (167 ± 3 % control) following 24 hours of exposure in comparison to quiescent medium exposed control cells (0.41 ± 0.02 pg/µg protein taken as 100%) ($P < 0.05$). In comparison, a decrease in IL-8 release was observed on exposure of the cells to the 10% Quat./chlorine dioxide (93 ± 2 % control). A significant reduction in basolateral IL-8 release (50 ± 4 % control) was observed following exposure of the cells to 10% ethanol ($P < 0.05$ compared to control). In addition, significant decreases in IL-8 release in comparison to control levels were observed following exposure of the cells to all three of the biocides (H_2O_2: 25 ± 2 % control, Quat./chlorine dioxide: 17 ± 2 % control and ethanol: 17 ± 2 % control), when tested at 100% of their in-use concentrations ($P < 0.05$).

7.4.3.4 IL-8 released basolaterally following 48 hours recovery from biocide exposure

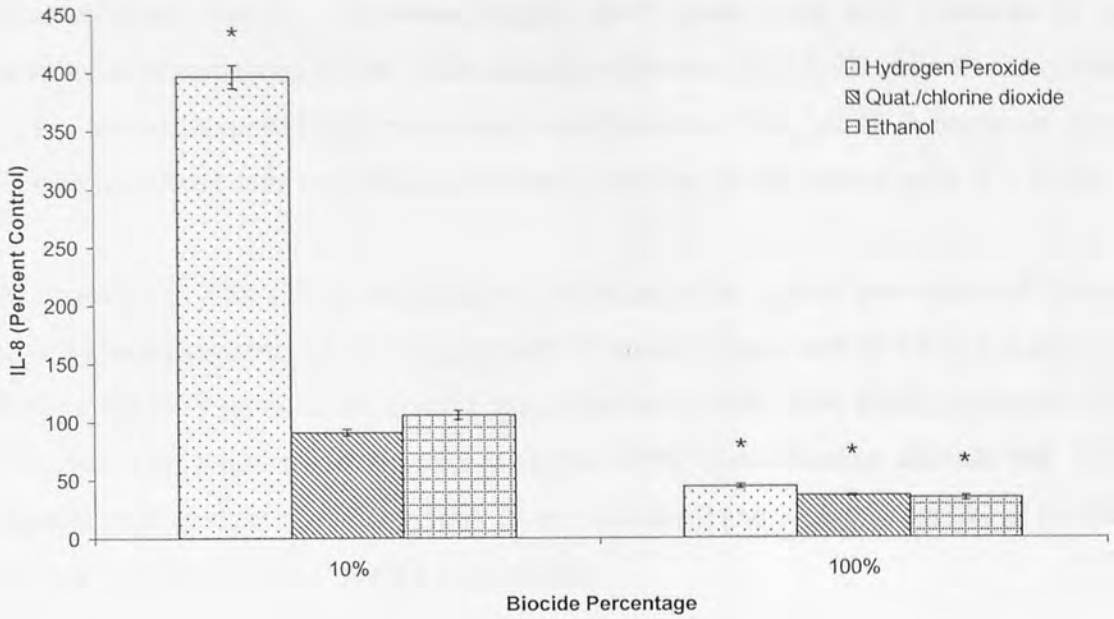


Figure 7.10 IL-8 released basolaterally from BEAS-2B cells cultured at ALI and exposed for 24 hours to biocides at 10% or 100% of practical concentration and allowed to recover for 48 hours at ALI, taking into account cell protein levels. Data are presented as mean percentage of control cells ± SEM (n=6), where IL-8 of quiescent medium exposed control cells was taken as 100%. * $P < 0.05$ in relation to control.

A non-significant decrease in IL-8 released basolaterally following recovery (figure 7.10) was observed with the 10% Quat./chlorine dioxide exposed BEAS-2B cells (91 ± 3 % control) in comparison to quiescent medium exposed control cells (0.96 ± 0.23 pg/μg protein taken as 100%). In addition, a non-significant increase in IL-8 released was observed on exposure of the cells to the 10% ethanol (106 ± 4 % control). However, a significant increase in IL-8 released basolaterally (397 ± 10 % control) was observed following exposure of the cells to 10% H_2O_2 ($P < 0.05$ compared to control). In addition, significant decreases in IL-8 release in comparison to control levels were observed following recovery exposure of the cells to 100% H_2O_2 (44 ± 2 % control) Quat./chlorine dioxide (36 ± 1 % control) and ethanol (34% ± 1 % control), when tested at 100% of their in-use concentrations ($P < 0.05$).

Some differences in IL-8 levels released basolaterally by the cells were also detected between the cells following 24 hours of exposure to the biocides and after 48 hours of recovery of the biocide-exposed cells. IL-8 levels were higher directly after exposure to the biocides at 10%, than after recovery. This was with the exception of the Quat./chlorine dioxide. However, higher IL-8 levels were also observed in the basolateral supernatants of the 100% biocide recovered BEAS-2B cells in comparison to the biocide exposed (not recovered) supernatants. Yet, all IL-8 levels in 100% biocide recovered cells were still significantly less than in the control cells ($P < 0.05$).

At exposure to 100% H_2O_2, a decrease in relation to the control was observed directly after 24 hours exposure ($P < 0.05$), and after 48 hours of recovery ($P < 0.05$). Only 44 ± 6 % of the IL-8 level of the control was observed in the 100% H_2O_2 recovered cells. This was also found to be the case with the 100% Quat./chlorine dioxide and 100% ethanol recovered cells whereby, only 36 ± 1 % control and 34 ± 1 % control of the IL-8 level of the control was observed, respectively.

Actual IL-8 levels (pg/μg protein) released apically and basolaterally, after 24 hours exposure and/or 48 hours recovery are in Appendix 11.6.5.

7.4.4 Thiol content of BEAS-2B cells

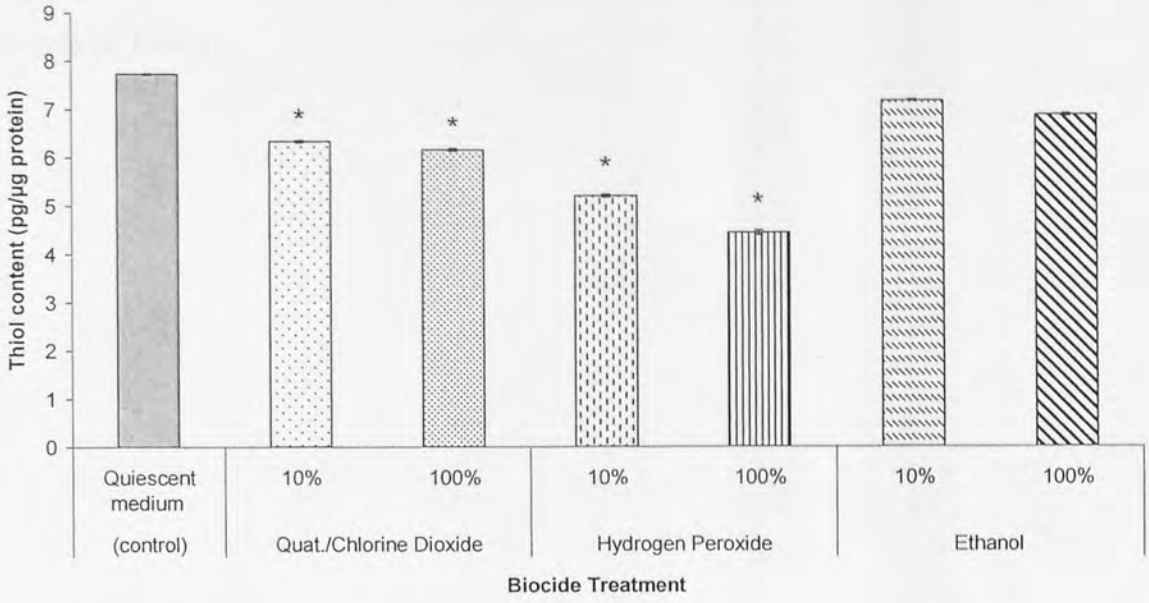


Figure 7.11 Thiol content (pg/µg protein) of BEAS-2B cell lysates following 24 hours of exposure to 10% and 100% in-use concentrations of biocide (Quat./chlorine dioxide, H_2O_2 or ethanol) and after 48 hours of recovery. Results are expressed as mean ± SEM (n=9). * represents $P < 0.05$, comparing between biocides and quiescent medium control.

Basal levels of free thiols in ALI cultured BEAS-2B cells were calculated as 7.71 ± 0.09 pg/µg of protein (figure 7.11). A reduction in comparison to this was observed on exposure to biocides at either 10% or 100% of their in-use concentrations. There was a significant reduction in free thiols on exposure to 10% or 100% Quat./chlorine dioxide or H_2O_2 ($P < 0.05$). No significant reduction ($P > 0.05$) was observed with ethanol at either 10% or 100% of its in-use concentration.

The greatest reduction in free thiols was seen in the cell lysates of the cells exposed to 100% H_2O_2, which reduced levels from 7.71 ± 0.09 to 4.44 ± 0.05 pg/µg of protein. This was followed closely by the 10% H_2O_2 which reduced thiol levels to 5.21 ± 0.03 pg/µg of protein.

A concentration dependent decrease in the level of free thiols was observed. Fewer free thiols per μg of protein were found in the 100% biocide treated BEAS-2B cells than the 10% biocide treated cells. This was observed in all of the biocides tested, with the exception of the ethanol, which was found to be non-significant in comparison to control ($P > 0.05$).

7.4.5 Carbonyl content of BEAS-2B cell lysates

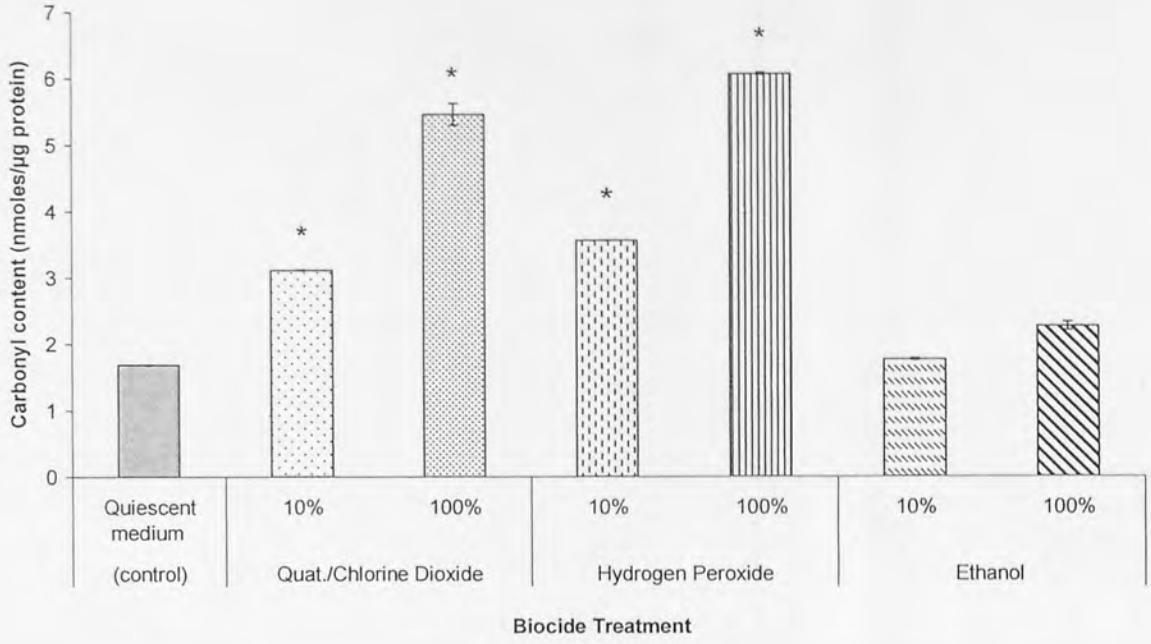


Figure 7.12 Carbonyl content (nmoles/µg protein) of BEAS-2B cell lysates following 24 hours of exposure to 10% and 100% in-use biocide concentrations (Quat./chlorine dioxide, H_2O_2 and ethanol) after 48 hours of recovery. Results are expressed as mean ± SEM (n=9). * represents $P < 0.05$, comparing between biocides and quiescent medium control.

Levels of carbonyls in quiescent medium exposed BEAS-2B cells were calculated as 1.69 ± 0.01 nmoles/µg of protein) (figure 7.12). All biocide exposures resulted in an increase in the carbonyl levels. A significant increase in carbonyl content in comparison to the control was observed on exposure to 100% Quat./chlorine dioxide or H_2O_2 ($P < 0.05$). Quat./chlorine dioxide or H_2O_2 at 10% induced a significant increase in carbonyl content in relation to the quiescent medium control ($P < 0.05$). No significant increase in the carbonyl content of the cell lysates was observed with either 10% or 100% ethanol exposed cells ($P > 0.05$).

The biggest increase in relation to the quiescent medium control was achieved on exposure of the BEAS-2B cells to 100% H_2O_2, which increased levels from 1.69 ± 0.01 (control) to 6.08 ± 0.02 nmoles/µg of protein). This was followed closely by the 100%

Quat./chlorine dioxide which increased levels to 5.47 ± 0.16 nmoles/μg of protein. The smallest increase in cellular carbonyl content occurred on exposure to 10% ethanol (1.77 ± 0.02 nmoles/μg of protein).

7.4.6 Propidium iodide staining of challenged BEAS-2B cells.

BEAS-2B cells in gated region 1 (figure 7.13) are defined as viable in terms of their reduced side scatter (SS) and high electronic volume (EV). The cells in gated region 2 are smaller (decreased EV) and more granular (increased side scatter). These characteristics are indicative of cell death. The majority of the cells exposed to quiescent medium were in region 1 with few present in region 2 (figure 7.13a). The anisomycin treated cells (figure 7.13b) shifted towards region 2. On treatment with the 100% H_2O_2 (figure 7.13c) a greater proportion of the cells were located in region 2 compared to quiescent medium exposed cells, indicating that the cells had shrunk in size and become more granular. This was also observed with the UV, 10% and 1% H_2O_2, but to a lesser extent, with the majority of the cells remaining in region 1 (figures 7.13d, 7.13e and 7.13f).

Table 7.1 PI staining of challenged BEAS-2B cells. Percentage of necrotic cells and the mean fluorescence of the cells in the total cell population on exposure of BEAS-2B cultured at ALI to quiescent medium, anisomycin (5 μg/ml), 100% H_2O_2, 10% H_2O_2, 1% H_2O_2 for 24 hours, or UV for 15 minutes (n=1).

Cell treatment (24 hours)	Percentage of necrotic cells in total cell population (%)	Mean fluorescence of all cells in total population (Arbitrary Units)
Quiescent medium	1.72	3.52
Anisomycin	25.05	3.69
100% H_2O_2	25.66	4.73
10% H_2O_2	19.25	4.31
1% H_2O_2	12.21	3.88
UV (15 min)	2.53	3.56

The percentage of cells determined as being necrotic in untreated, quiescent medium-exposed cells was 1.72% (table 7.1). This value increased 14-fold following 24 hours exposure to 100% H_2O_2 (1.72% to 25.66%) or anisomycin (1.72% to 25.05%). In addition, the percentage of necrotic cells present in the samples also increased following exposure to 10% H_2O_2 (1.72% to 19.25%), 1% H_2O_2 (1.72% to 12.21%) and UV (1.72% to 2.53%).

Without PI, little auto-fluorescence of the BEAS-2B cells was occurring as the average mean fluorescence of the drug/biocide treated and untreated cells was very low (1.84 arbitrary units to 2.38 AU). With PI, fluorescence of the BEAS-2B cells increased a little on exposure of the cells to 100% H_2O_2 in comparison to the quiescent medium exposed cells (3.52 AU to 4.73 AU respectively) (table 7.1). The fluorescence also increased with the 10% H_2O_2 (3.52 AU to 4.31 AU) and slightly with the 1% H_2O_2 (3.52 AU to 3.88 AU). Another small increase was observed with the anisomycin (3.52 AU to 3.69 AU) and UV (3.52 AU to 3.56 AU) treated cells.

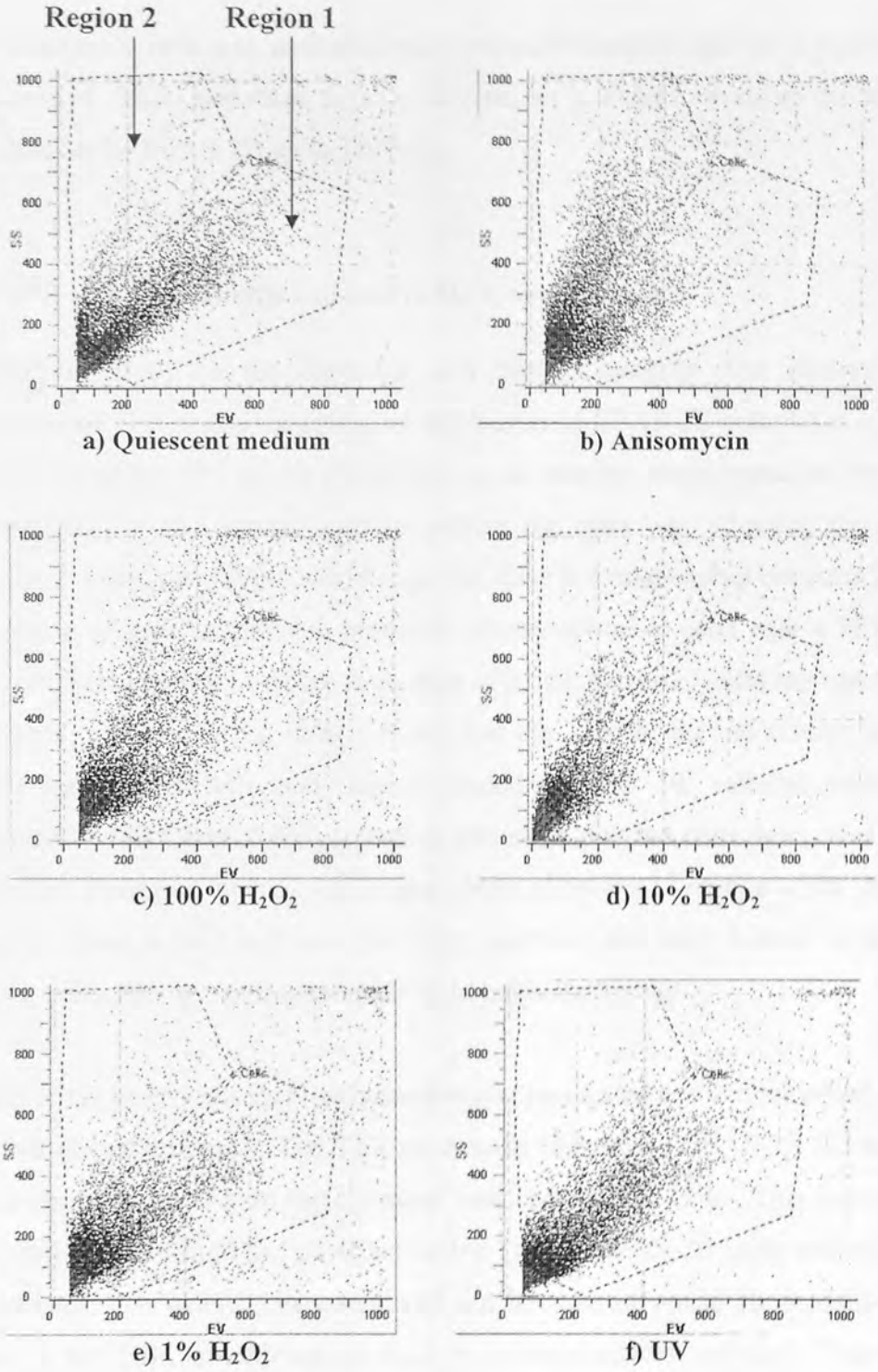


Figure 7.13 Scatter cytograms of BEAS-2B cells cultured at ALI and exposed for 24 hours to a) quiescent medium, b) anisomycin (5 µg/ml), c) 100% H_2O_2, d) 10% H_2O_2, e) 1% H_2O_2 and 15 minutes to f) UV. Y-axis (SS = side scatter) is representative of cell granularity and X-axis (EV = Electronic volume) represents the cell size. Region 1 represents the healthy cell population. Region 2 is representative of necrotic cells. Regions are outlined by a dashed line with gating set to overlap all cells (n=1).

7.5 Discussion

The ALI culture system was used to obtain well-differentiated cells as a model of the human airways. These were then exposed to biocides in order to evaluate the effects of the biocides on the BEAS-2B epithelial cells.

7.5.1 TER of BEAS-2B cells exposed to H_2O_2 over 24 hours.

The effect of H_2O_2 on the epithelial cell barrier integrity was determined by measurement of TER across the epithelial cell barrier of BEAS-2B cultured at an ALI *in vitro*. The baseline TER of the BEAS-2B in the present study increased over time, indicating that the ALI model used to culture the cells was allowing the cells to differentiate. Wan *et al.* (2000) established that there is a relationship between TER and the formation of tight junctions in bronchial airway epithelial cells with a TER $\geq$ 300 Ohms x cm^2 being indicative of the formation of a tight barrier of cells representative of *in vivo* cells. As Wan *et al.* (2000) found that the TER of normal human bronchial epithelial cells (16HBE14o- and Calu-3) seeded at 2.5 x 10^5 cells/ml peaked after reaching 300 - 350 Ohms x cm^2, it was at this stage that the cells were used in their experiments. Therefore the high TER value obtained upon ALI culture of the BEAS-2B cells ($\geq$300 Ohms x cm^2) indicated that tight junctions had been formed at the apical side of the cells, forming an impermeable tight epithelial barrier.

The TER of the Transwells that had quiescent medium added to them remained constant throughout the experiments. The TER of apically unexposed BEAS-2B did not differ significantly from the TER of the quiescent medium exposed cells. This indicated that the addition of apical fluid had no effect on the TER of BEAS-2B cells, and any effects observed were due to H_2O_2 exposure and not because of apical fluid addition. An increase in the TER on addition of H_2O_2 to collagen-coated, cell-free Transwells in comparison to quiescent medium exposed Transwells (Appendix 11.6.2) established that H_2O_2 interfered with the TER measurements. This was found to be a disadvantage of adding the fluid to the cells apically because as well as being non-physiological; the likely route of biocide exposure is via inhalation. As H_2O_2 is not an electrolyte, it is not known why an increase in TER on addition of the H_2O_2 to the cell-free Transwells was observed. As a result, the TER values of the H_2O_2 exposed collagen-coated, cell-free

Transwells were subtracted from the TER measurements of the H_2O_2 exposed BEAS-2B cells.

The TER of quiescent medium exposed cells remained stable over the 24 hour exposure period. However, a higher than 90% decrease in the TER of BEAS-2B cells was observed instantly following exposure to 100% H_2O_2, with an almost 50% decrease in TER observed following exposure to 10% and 1% H_2O_2. This instant decrease may actually be due to the H_2O_2 interfering with TER measurement, rather than being a truly instant effect of H_2O_2 on the cells. If the majority of cells were killed after 24 hours of exposure, it would be expected that the TER of the cells would be of a similar level to the TER of a cell-free Transwell. However, this did not occur. This may possibly be due to the presence of cell debris which may have been responsible for the TER of the cells remaining higher than a cell-free Transwell.

As epithelial cells form a tightly polarized barrier, and TER indicates the formation of intact, confluent layers of cells, a loss in confluency of the cells is reflected by a decreased TER (Chapman *et al.*, 2002; Waters *et al.*, 1997, Boardman *et al.*, 2004). ROS such as H_2O_2, superoxide radicals, and hydroxyl radicals have been shown to destroy the integrity of the epithelial barrier (Yamaya *et al.*, 1995; Waters *et al.*, 1997; Chapman *et al.*, 2002; Boardman *et al.*, 2004). H_2O_2-dependent increases in mannitol permeability and decreases in TER have been reported in airway epithelial cells (Waters *et al.*, 1997; Chapman *et al.*, 2002; Boardman *et al.*, 2004). The reduction in TER indicates that there may be a loss in the effectiveness of the cell tight junctions which regulate the barrier function of the epithelial cells (Waters *et al.*, 1997; Chapman *et al.*, 2002; Boardman *et al.*, 2004). Chapman *et al.* (2002) demonstrated that, depending on the dose, the TER of 16HBE14o- cells decreased in response to continuous perfusion with 0.1 mM and 0.5 mM H_2O_2. Upon observation of the cells after exposure, Chapman *et al.* (2002) determined that the reductions in TER were due to disruption of the cell tight junctions and not because of the denudation of cells from the filter membranes. This decrease was found to be irreversible. In contrast, the decrease in TER observed with the BEAS-2B in this study was reversible in those cells exposed to 100% H_2O_2. An increase in TER was observed after 3 hours exposure to 100% H_2O_2 and was sustained throughout the 24 hours. This was also the case with 1% and 10% H_2O_2 exposures, which also recovered partially (about 50%) after 48 hours of recovery.

Chapman *et al.* (2002) also found that the TER decreased almost immediately after exposure to 1.0 mM H_2O_2, but the decrease was delayed nearly 2 hours when cells were exposed to 0.5 mM H_2O_2. An immediate decrease was observed in the present study when BEAS-2B were exposed to 100% (1.76M), 10% (176 mM) and 1% (17.6 mM) H_2O_2. Yet, in contrast to Chapman *et al.* (2002), the decrease in TER of the BEAS-2B cells in the present study, was not delayed on exposure to the H_2O_2 additions. Boardman *et al.* (2004) found that after 2 hours of exposure to 0.5 mM H_2O_2, the normal smooth boundaries between Calu-3 cells became more tortuous. These findings are indicative of alterations occurring to the cellular tight junctions and intercellular channels following H_2O_2 treatment. Overall, it appears that the membrane integrity of the ALI cultured BEAS-2B is lost upon challenge with H_2O_2. Although recovery of the cells occurs, the recovery is not complete, as the TER even after 48 hours of recovery remains at 50% of the quiescent medium exposed control. Therefore H_2O_2 is likely to affect human bronchial epithelium permeability *in vivo*.

7.5.2 Paracellular permeability of BEAS-2B cells to BSA

In addition to the TER, the effects of biocide exposure on paracellular permeability of the BEAS-2B cells to BSA were investigated. BSA added apically to a collagen coated Transwell is detected at around 100% of the apical concentration within one hour (Anne Bielemeier, observation from laboratory). This indicates that BSA does not adhere to the Transwell membrane and is capable of moving through the collagen coated membrane to the basal compartment. This also indicates that upon culturing of the BEAS-2B cells on the Transwells a barrier is formed, as exposure of the cells to quiescent medium (control) had a non-significant effect on BEAS-2B cell permeability to BSA after 24 hours exposure.

At 10% and 1% practical concentrations of H_2O_2, a progressive increase in BSA paracellular permeability was observed over the 24 hours treatment. This indicates that the BEAS-2B cell barrier is disrupted upon exposure to 10% and 1% H_2O_2. Only up to 46% of apically added BSA passed through to the basolateral medium upon exposure to 100% H_2O_2. There was no further increase in basolateral BSA observed with the 100%

H_2O_2, after 2 hours of exposure. This indicates that there is a maximal 46% permeability of the epithelial cell barrier after 100% H_2O_2 treatment.

Other researchers have also found that H_2O_2 exposure alters barrier function. Waters *et al.* (1997) demonstrated that treatment of airway epithelial cells (Calu-3) by a single dose of 0.5 mM H_2O_2 increased permeability to fluorescein isothiocyanate-albumin. This was in correlation with the results obtained in the present study where an increase in paracellular permeability of BEAS-2B to BSA was observed following a single dose of 100%, 10% or 1% H_2O_2. However, with the 100% H_2O_2, there was not complete diffusion of the BSA over 24 hours indicating that the epithelium remained an effective barrier.

The results of the paracellular permeability experiments somewhat contradict those of the TER, which demonstrated an initial loss in TER and then its partial recovery as exposure time to the H_2O_2 increased. Although upon 100% H_2O_2 exposure, the concentration of BSA passing through the epithelium between the cells was not sustained after 2 hours exposure, this was not the case with the 10% and 1% H_2O_2. This was because the movement of BSA through the 10% and 1% H_2O_2 exposed epithelium was sustained throughout the 24 hours of exposure.

The increased protein detected in the basolateral compartment may be accounted for by the increases in basal IL-8 that were determined by IL-8 ELISA. However, the concentrations observed (pg) would not account entirely for the protein concentrations observed basally (mg) following apical BSA addition.

No further increase in BSA movement from apical to basolateral compartments after 2 hours exposure may have occurred due to the strong antioxidant defence capacity of airway epithelial cells (Boardman *et al.,* 2004), which could negate the effects of the apically added H_2O_2. It is therefore possible that at this point in time the cell antioxidant levels are upregulated following gene transcription, so no further damage occurs to the epithelial barrier. However, at the lower 10% and 1% H_2O_2 concentrations, there may not have been an upregulation of antioxidants to the extent possibly achieved by the 100% H_2O_2. The degradation of H_2O_2 by catalase and glutathione peroxidase, which are known to be produced by BEAS-2B (Park *et al.,*

2008; Kinnula *et al.*, 1994) may have occurred after 2 hours of contact with the BEAS-2B. Park *et al.* (2008) established that BEAS-2B cells treated with nanoparticles for 3 hours induced oxidative stress-related genes including catalase. This may have occurred in the H_2O_2 treated cells resulting in only an acute effect on the epithelial barrier, as the antioxidants upregulated by the BEAS-2B would neutralise the effects of the H_2O_2 following its initial addition to the cells. The cells could then recover to prevent any further disruption of the cell barrier which may otherwise allow the infiltration of inhaled pathogens across the airway epithelium. In addition, Boardman *et al.* (2004) found that Calu-3 cells metabolised 0.5 mM H_2O_2 within 60 minutes, so this rapid metabolism could also explain what was observed in the BEAS-2B cells in this study, where some increased BSA permeability was detected initially with the 100% H_2O_2 and was followed by decreased permeability after 2 hours of contact. Therefore, the metabolism of the apically added H_2O_2 may be responsible for the decreased permeability observed after 2 hours.

As H_2O_2 is a strong oxidising agent, any apically added BSA, which is often used as a standard reagent for oxidation (standard for determining the thiol content of cell samples as well as albumin-derived carbonyls), is likely to have been oxidised by the H_2O_2 (Di Simplicio *et al.*, 1991; Robinson *et al.*, 1999). The results to both the protein thiol and carbonyl assays in this chapter (sections 7.4.4 and 7.4.5) confirmed that oxidation of the BEAS-2B cell proteins was occurring upon exposure to H_2O_2. Such oxidation effects may possibly increase over time. The potential oxidation of BSA may explain why less protein was detected in the basolateral compartment of the Transwells after 2 hours exposure to the H_2O_2. .

In addition to this, the capacity of the damaged epithelium to heal after exposure to H_2O_2 (Chapman *et al.*, 2002) may explain the findings with regards to BSA paracellular permeability. Only up to 2 hours of 100% H_2O_2 exposure was there an increase in the level of BSA passing through the epithelial cell barrier. After 2 hours there was no further increase in BSA levels in the basolateral compartment of the Transwells. Although airway epithelial cell wound repair from exposure to chemicals such as NaOH have been studied (Herard *et al.*, 1996), no studies have been carried out on the repair of cells when treated with H_2O_2. Wound healing is one mechanism that may have been responsible for the restoration of the epithelium immediately after injury (Godfrey,

1997). It was between 2 - 3 hours following apical H_2O_2 (100% and 10%) addition that the level of basolateral BSA seemed to level off. This levelling off indicates that another mechanism may be responsible for the effects observed. It is thought that the effects of the H_2O_2 on the airway epithelial cells occurs as a result of tight junction disruption (Chapman *et al.*, 2002) with the paracellular permeability of the epithelial barrier being found to be dependent upon tight junction proteins and actin cytoskeletal integrity (Wan *et al.*, 2000). However, there is evidence to suggest that BEAS-2B do not express tight junction proteins (Reddel *et al.*, 1988) and so it is thought that H_2O_2-induced changes are indicative of a loss of actin cytoskeletal integrity (Waters *et al.*, 1997; Chapman *et al.*, 2002; Boardman *et al.*, 2004). Boardman *et al.* (2004), found that dissociation of the actin from the cytoskeleton was observed within 15 min of 0.5 mM H_2O_2 exposure. However, this was followed by recovery of these cytoskeletal components. This may explain the initial increase in BEAS-2B BSA permeability following H_2O_2 (100%) treatment and then the recovered integrity of the cell barrier, which could have occurred because of the possible acute damage to the cells immediately after exposure to H_2O_2, resulting in a loss in the tight junction integrity. Tight junctions are dynamic structures able to respond to H_2O_2 mediated stress (Godfrey, 1997). The cells may re-gain tight junction functioning after the H_2O_2 has been metabolised. So the recovery of tight junction functioning is feasible over the 24 hours time period for which the cells were exposed to the H_2O_2. This is because the H_2O_2 may have been metabolised by the BEAS-2B cells after just 2 hours of exposure. This would restore epithelial cell integrity preventing any BSA from further traversing the epithelial cells via the paracellular pathway into the basolateral compartment. This would indicate a reversible paracellular permeability barrier because a decrease in the functioning of tight junctions is parallel to an increase in paracellular permeability. However, at 10% and 1% H_2O_2 concentrations, due to the remaining presence of H_2O_2 some tight junctions may have become modified and so unable to restore the epithelial cell barrier. This may be reflected by the results to the PI staining of the H_2O_2 exposed cells (table 7.1), which indicated that not all cells were dead in terms of the cells being necrotic.

The perijunctional actin of tight junctions is also known to play a significant role in controlling paracellular permeability (Andersen and Van Itallie, 1995). Chapman *et al.* (2002) found that the initial, rapid permeability increase of Calu-3 cells was as a result

of a contraction of the peri-junctional actin of the cells tight junctions. This contraction of the actin cytoskeleton is a possible mechanism through which the H_2O_2 may be regulating the epithelial barrier function. Vermeer *et al.* (2003) suggested that a mechanism of tight-junction protein ligand-receptor segregation on either side of epithelial tight junctions may play a significant role in the rapid restoration of epithelium integrity following injury. Protein ligands on one side of the tight junction that have a corresponding receptor on the other side of the tight junction move apart upon injury but are rapidly restored as ligand-receptor complexes after injury. Therefore the recovery of the permeability barrier following exposure to H_2O_2 treatment as observed on exposure of the BEAS-2B to 100% H_2O_2 may be due to the rapid re-establishment of the protein ligand-receptor complexes.

Overall, concentration and time dependent increase in BSA paracellular permeability was observed with H_2O_2 treatment. At practical concentrations, H_2O_2 causes a temporary loss in cell barrier integrity. The cells exposed to 100% H_2O_2 demonstrated significant loss of cell barrier functioning measured as paracellular permeability after just 1 hour (with recovery occurring 2 to 3 hours after), with the 10% and 1% concentrations showing loss of barrier function after 4 hours.

Increased paracellular permeability generally did not correlate with a decrease in TER. However, the paracellular permeability of the BEAS-2B cells observed following 1 hour exposure to 100% H_2O_2 was in correlation to the TER results. As the TER decreased, the level of BSA protein passing through to the basolateral medium increased. The TER and paracellular permeability results therefore indicate that the cells exposed to 100% H_2O_2 for 1 hour, remained intact. However, as exposure times increased the paracellular permeability results demonstrated that the epithelium barrier was disrupted upon exposure to 10% and 1% H_2O_2. Whereas the TER results suggested that although the TER of these H_2O_2 exposed cells had decreased in comparison to unexposed cells, the cells were recovering. Increased paracellular permeability to BSA is reflective of a loss in the barrier function to macromolecules, whereas a decrease in TER is indicative of a loss of barrier function to ions. Studies using BSA as a probe to detect transepithelial permeability on exposure to histamine, have shown that although permeability can remain unaffected, the electrical resistance can be altered (Devalia *et al.*, 1994). As a result it is possible that even though the permeability of the cells

exposed to the 100% H_2O_2 was increased, the TER was not significantly affected. In addition, Boardman *et al.* (2004) suggested that the actin-cytoskeleton association was vital in maintaining the functioning of the epithelial barrier against large solutes, but did not have an effect on TER. Of the two methods tested, BSA permeability would be a better measure than the TER. This is because BSA permeability would be more physiologically relevant and informative of any possible movement of bigger molecules and pathogens through the epithelial barrier.

The loss of epithelial barrier integrity and function in the airways epithelium *in vivo* can have numerous consequences with regards to its role as a physical barrier and its regulation of ions and small molecules passing through the paracellular pathways. Associations have been established between altered epithelial barrier permeability and the development of the airway conditions: asthma and chronic bronchitis (Godfrey, 1997; Godfrey, 1993; Devalia *et al.,* 1994; Walker *et al.,* 1984). Overall, an increased permeability of the airways can affect lung defence mechanisms by exposing the underlying tissue to exogenous airborne toxins including bacterial and viral infections (Chapman *et al.,* 2002). The infections might occur both directly and due to the increased permeability to mucus which could have bacterial antigen associated with it. This could lead to inflammation and cell death.

The regulation of ASL electrolytes is another defence mechanism that could be affected as transepithelial and paracellular transport of electrolytes in airway epithelial cells controls the quantity and composition of the liquid (Flynn *et al.,* 2009). Disruption of electrolyte transport may have an effect on mucociliary clearance, ion secretion and water regulation in airway epithelium (Puchelle, 2000; Godfrey, 1997; Coyne *et al.,* 2002). Possible effects on mucociliary clearance include reduced mucus clearance and depression of ciliary activity. Loss of the mucociliary clearance mechanism could have a subsequent affect on the epithelial ability to eliminate microbial agents upon infection, as the airways will no longer be able to efficiently remove the pathogens from the airways. Consequently, respiratory infections may be prolonged. The proliferation and polarity of cells may also be regulated by cytoplasmic scaffolding molecules associated with tight junctions (Förster, 2008). Modification of these cytoplasmic scaffolding molecules may disrupt regulated diffusion in epithelial cells. Increased permeability may also allow carcinogens such as those present in cigarette smoke to access dividing

basal cells. This could result in an increase of the cells mutation rates which could ultimately lead to epithelium predisposition to carcinoma development (Godfrey, 1997).

7.5.3 IL-8 release in biocide exposed BEAS-2B cells

H_2O_2 and Quat./chlorine at 10% increased total (apical and basolateral) IL-8 release from the BEAS-2B cells post 24 hours challenge in comparison to control cells. The most IL-8 was secreted by cells exposed for 24 hours to 10% H_2O_2 (2.49 ± 0.03 pg/μg protein). This increase was sustained following 48 hours of recovery. Following recovery increased IL-8 was secreted by 10% H_2O_2 exposed cells (2.58 ± 0.06 pg/μg protein). With the 100% exposures there was no increase in total IL-8 in biocide exposed cells after challenge or after recovery, in comparison to control cell total IL-8 levels. Consequently, the 10% H_2O_2 was established to be inducing the greatest pro-inflammatory effect in BEAS-2B both after 24 hours exposure and 48 hours recovery.

The results to the present study show that BEAS-2B epithelial cells can release IL-8 in a directional manner. Unexposed cells released IL-8 apically (36% of the total IL-8) and basolaterally (64% of the total IL-8). The secretion of IL-8 towards the apical or basolateral region of the epithelium is potentially an important mechanism in controlling the inflammatory process. Polarised secretion of IL-8 by epithelial cells may provide the directed stimulus necessary for neutrophils to move towards the site of potential inflammation (Peralta and Casale 1998; Le Gall *et al.,* 1995). If IL-8 is released basolaterally, it serves to pass into the blood. On doing so, it activates and recruits neutrophils to the site of inflammation or injury. This occurs via the production of a chemotactic gradient which promotes the influx of inflammatory cells to the area. Chemotactic gradients of IL-8 are essential for neutrophils to migrate to the sub-epithelial matrix during bronchial inflammation and injury (Lakshminarayanan *et al.,* 1998; Oslund *et al.,* 2004). The neutrophils then move across the airway epithelium in the basolateral-to-apical direction into the lung lumen. On reaching the airway lumen, the neutrophils are able to remove any damaged, injured or dead cells.

The polarisation of IL-8 secretion either in the apical or basolateral direction is thought to be controlled by intrinsic signalling mechanisms that specifically targets IL-8 to the

apical or basolateral surface of the epithelial cell. It is hypothesised that the mechanisms are similar to those involved in regulating membrane trafficking to and from polarised membrane domains (Zegers and Hoekstra, 1998). This regulation of traffic in polarised epithelial cells is thought to play a central role in response to external signals, such that proteins may be targeted to either the basolateral or apical domains in response to targeting signals. In epithelial cells, specific proteins required to be secreted apically are likely to be sorted in the *trans*-Golgi network (TGN) and directly transported to the apical membrane. Sorted proteins in the TGN, are packed into vesicles and targeted directly to the basolateral or apical domain of the cell. Once the domain is reached, the proteins are released. Other studies have suggested that the polarised expression of key adhesion molecules and/or production of secondary peptide chemoattractants may play a role in ensuring that inflammatory mediators are secreted in a particular direction. It is probable that the biocides are able to alter the expression of such key adhesion molecules and/or secondary peptide chemoattractants in the time frame investigated, because the IL-8 detected was observed as being apically and basolaterally directed.

Following biocide exposure some reductions in both apical and basolateral IL-8 release were detected. This may have been due to the loss of cell viability; however IL-8 release was determined relative to viable cells. As in the submerged cultured cells, another explanation is that the biocides themselves may possibly have modified the IL-8 in some way which prevented it from being detected by the ELISA assay. As H_2O_2 and chlorine dioxide are strong oxidisers, they may have oxidised the IL-8, rendering it unable to be detected by the IL-8 antibody. H_2O_2 can spontaneously decompose to produce free-radicals capable of attacking the IL-8 protein which may result in the degradation of the IL-8. The observed reduction in IL-8 release may also be due to the oxidation of thiol groups in the IL-8. This may be due to the IL-8 possessing highly conserved thiol groups (Strieter, 2002) which act as potential oxidation targets. So the possible oxidation of the IL-8 may explain why less IL-8 was measured after H_2O_2 exposure.

In addition, Oslund *et al.* (2004) found that uninjured, oxidant-stressed BEAS-2B up-regulated IL-8 after oxidant injury. They found that when the H_2O_2 dose was sufficient to cause mitochondrial injury, IL-8 did not increase. This suggests that the production

of IL-8 is dependent on the type of stress or injury the cells have sustained during an oxidant insult and that there is a limit to the degree of injury that a cell could sustain and continue to produce IL-8. However, Oslund *et al.* (2004) found that necrotic BEAS-2B did not produce IL-8. Therefore in the present study the cells may have undergone necrosis in response to high concentrations of biocide exposure instead of releasing IL-8 and as a result low levels of IL-8 detected.

ELISA analysis of the supernatants of the biocide treated cells demonstrated that the IL-8 levels generally increased following recovery of the BEAS-2B in full serum-containing medium. Recovery would allow any sub-lethally injured cells to recover and also allow for the proliferation of any remaining viable cells. Following acute injury the airway epithelium is able to repair itself and regenerate through several mechanisms including the spreading and migration of basal cells, cell proliferation and differentiation (Puchelle *et al.*, 2006; Coraux *et al.*, 2005; Puchelle, 2000). In the present study, even under control conditions the total IL-8 increased after recovery in full medium (1.21 ± 0.16 pg/μg protein to 1.37 ± 0.25 pg/μg protein). Therefore the increase in IL-8 observed upon exposure of the cells to biocides was not because the control cells had stopped producing IL-8. An increase in IL-8 following recovery of 10% biocide exposed cells in comparison to directly after biocide exposure was also observed. This was probable because it would be likely that the numbers of viable cells are increased after recovery. This increase observed may be because airway epithelial cells constitutively release IL-8 (McDougall *et al.*, 2008; Marshall *et al.*, 2001) and so more cells will result in a higher level of IL-8 being detected. In addition, this increase in IL-8 post recovery may be due partly to the cells proliferating during the 48 hours recovery period, as proliferating epithelial cells increase IL-8 production in comparison to basal cells (Puchelle *et al.*, 2006; Coraux *et al.*, 2005). However, as IL-8 was determined relative to protein content, this indicates that more IL-8 was released by cells post-recovery. This was not therefore due to the recovery of cell numbers after 48 hours but due to an overall increased IL-8 release by the BEAS-2B cells.

The ethanol was not found to be pro-inflammatory in terms of epithelial cell-derived IL-8 released basolaterally following exposure to 10% or 100% biocide. However, an increase in IL-8 was detected in the apical supernatant of 10% ethanol challenged BEAS-2B cells. Like the Quats, alcohols also have the potential to act upon cells by

causing membrane damage and the rapid denaturation of proteins, resulting in interference with metabolism, and consequently cell lysis (McDonnell and Russell, 1999). If cells undergo necrosis as a result of biocide exposure, then ROS produced endogenously as a normal byproduct of cellular metabolism are released by the cells upon damage to lysosome membranes and are then able to act on neighbouring cells. These neighbouring cells then increase IL-8 release in response to the increased ROS in order to chemotactically recruit neutrophils to remove cell debris. IL-8 itself is an extremely stable protein which has pro-longed biological activity *in vivo* (DeForge *et al.*, 1993). However, its sustained expression can have a considerable impact on the cells with regards to increased neutrophil chemotaxis from the underlying tissues and blood vessels. This will result in inflammation of the airway and lead to tissue injury. If the cells are dying, activated phagocytic cells such as macrophages will release their granular content (proteases, lipases and nucleases), and remove cellular debris. In the absence of pathogenic organisms, the inflammatory reactions are directed against the normal host tissue of the airway epithelium, potentially resulting in tissue injury.

7.5.4 Thiol levels of biocide challenged BEAS-2B cells

At 10% and 100% of their in-use concentration, biocides reduced thiol content of BEAS-2B cultured at ALI, in a concentration-dependent manner. Decreases in protein thiol content in the biocide exposed BEAS-2B cell lysates in relation to the basal control were observed on exposure to the 10% and 100% Quat./chlorine dioxide and H_2O_2. The biggest decrease in thiol levels in comparison to the control was observed on exposure of the BEAS-2B cells to the 100% H_2O_2, followed by the 10% H_2O_2. This can be explained as H_2O_2 is thought to get its activity as an oxidant due to the formation of destructive hydroxyl free radicals ($OH^{\bullet}$) (Lambert, 2004; Rahman *et al.*, 2006; McDonnell and Russell, 1999), which have been found to indirectly oxidise the free thiol groups of cysteines in peptides and proteins within different types of human airway epithelial cells (Smit-de Vries *et al.*, 2007; Martin *et al.*, 1997; Stadtman and Berlett, 1998). The smallest decrease was observed in the 10% ethanol exposed cells. So, biocides oxidise BEAS-2B cell proteins after 24 hours challenge *in vitro*. No previous studies have investigated thiol levels in BEAS-2B cells cultured at ALI *in vitro*.

The correlation between thiols and their antioxidative capacity has been observed by many researchers (Lakshminarayanan *et al.*, 1997, Lakshminarayanan *et al.*, 1998, Martin *et al.*, 1997, Rahman *et al.*, 2001, Smit-de Vries *et al.*, 2007). The oxidation of membrane thiol groups has been found to alter the ability of the cells to transport amino acids (Pauluhn and Mohr, 2000; Deneke, 2000). As thiols are powerful antioxidants, it is possible to conclude that free thiol levels are likely to play a significant role in cell survival and protection against oxidative damage.

7.5.5 Carbonyl content of biocide challenged BEAS-2B cells

At 10% and 100% of their in-use concentration, all 3 of the biocides tested increased protein carbonyl content of ALI cultured BEAS-2B, in a concentration-dependent manner. The biggest increase in carbonyl levels in comparison to the control was observed on exposure of the cells to the 100% H_2O_2, followed by the 100% Quat./chlorine dioxide. This can be explained as both chlorine dioxide and H_2O_2 are powerful oxidisers and have the ability to oxidatively modify amino acid residues on proteins such as histidine, lysine, arginine, proline, and threonine. Consequently this modification results in the formation of protein carbonyl derivatives. H_2O_2, superoxides and hydroxyl radicals are amongst those molecules that can contribute to oxidant mediated airway damage as it has been established that such protein-damaging radicals can cause carbonyl formation (Turi *et al.*, 2002).

The increases in protein carbonyl levels may not be equivalent to those detected *in vivo* as the lung epithelial surface acts as a barrier to the external environment. In doing so, it is exposed to many inhaled, as well as endogenously produced, oxidants, but both enzymatic and scavenging antioxidant systems present in BEAS-2B cells have the ability to limit the level of oxidants and consequently play an important role in protecting the extent of oxidative damage to proteins (Carty *et al.*, 2000). Measurement of antioxidant enzymes such as catalase, glutathione peroxidase, and superoxide dismutase, whose function is to detoxify these oxidants (DeForge *et al.*, 1993) would aid in determining the extent to which these antioxidants are able to limit the effects of the biocides on the BEAS-2B.

The differences in protein oxidation following biocide exposure were more detectable and significant with the ALI cultured cells in comparison to submerged cultured cells (Chapter 5, sections 5.4.5 and 5.4.6). The decrease in free protein thiols and increase in protein carbonyls detected can result in the inactivation of proteins which can have numerous downstream effects on cell functioning. In addition, the modification of structural proteins can be detrimental to the cell, because cell structure is essential in maintaining the integrity of the epithelium as a whole. Overall, these results indicate that the human bronchial epithelial ALI cultured cells are being put under oxidative stress as a result of biocide exposure.

7.5.6 Propidium iodide staining of H_2O_2 challenged BEAS-2B cells.

Flow cytometry analysis of the cells following H_2O_2 exposure indicated that some of the ALI cultured BEAS-2B cells were typically necrotic. A H_2O_2 concentration dependent increase in necrotic cells was detected, with up-to a 14-fold increase observed in relation to the quiescent medium exposed control cells. In addition, the PI fluorescence intensity of the H_2O_2 treated cells increased in comparison to the control cells. As the PI fluorescence intensity is relative to cell death, this indicated that a concentration dependent increase in cell death was occurring upon exposure of the BEAS-2B cells to H_2O_2. The fluorescence intensity data obtained therefore correlates with the data on necrotic cells following H_2O_2 exposure.

Overall the BEAS-2B cells when cultured at an ALI and exposed to 100%, 10% or 1% H_2O_2 were found to possess more necrotic cells than the unchallenged control. However, even though not all of the cells were necrotic following exposure to the H_2O_2, any necrosis of epithelial cells is likely to have detrimental effects on the surrounding cells and their environment.

Little research has been undertaken on the mechanism of cell death upon exposure of well-differentiated BEAS-2B to H_2O_2. Although necrosis was detected following H_2O_2 exposure, apoptotic and necrotic cell death can occur simultaneously. So, it is likely that some cells were also undergoing cell death via apoptosis. Cells undergoing apoptosis do not release their intracellular contents. This is in contrast to necrotic cells,

whose cellular contents are released into the intracellular milieu upon damage to the plasma membrane. It is this release of intracellular content after plasma membrane rupture that is the cause of inflammation in necrosis. The release of these contents including ROS is able to cause further damage to the surrounding tissue by acting upon neighbouring cells as well as recruiting more pro-inflammatory cells to the region. As observed in the present study, the increase in IL-8 levels being basolaterally released by the cells in response to biocide exposure *in vitro* may be responsible for recruitment of ROS (from neutrophils) to the epithelium *in vivo*. This may result in further epithelial cell necrosis occurring.

In the present study, the majority of cells were not necrotic following a single dose of H_2O_2. This correlates with the TER and BSA paracellular permeability data, which found that although the integrity of the BEAS-2B epithelium was compromised immediately after exposure to H_2O_2, the cells were able to recover somewhat to partially restore the integrity of the epithelium as a barrier. The recovery of the barrier confirms that the majority of cells could not have been necrotic, as otherwise it is unlikely that any recovery of the TER and paracellular permeability would have occurred.

This study suggests that exposure to biocides induces the synthesis and release of inflammatory mediator IL-8 from human bronchial epithelial cells cultured at ALI. The TER results presented here are indicative of an injured epithelium, suggesting that H_2O_2 is able to immediately compromise the barrier function of airway epithelial cells. However, some recovery of the epithelial barrier is observed. Paracellular permeability indicates that the epithelial barrier is largely intact following 24 hours exposure to H_2O_2. The tight junctions of the cells may be responsible for these differing results possibly due to paracellular gaps formed by cytoskeleton contraction (Boardman *et al.*, 2004). However, it is also possible that the upregulation of antioxidants in response to the H_2O_2 may also reverse its effects, resulting in recovery of the tight junction integrity and consequently recovery of the epithelial cell barrier as observed with the TER data. The study also established that protein oxidation and some necrosis of the bronchial epithelial cells occurs following biocide treatment. Taken as a whole, these findings suggest that *in vivo* exposure to H_2O_2 may exacerbate the pathogenesis of airway disease.

CHAPTER 8 AIR-LIQUID INTERFACE CULTURE OF HNEpC

8.1 Introduction

This chapter investigates the toxicity of biocides against HNEpC cultured at an ALI. The cells were cultured at ALI, as such conditions have been shown to improve the differentiation of primary cultures of human airway epithelia as discussed in chapter 7 (Yamaya *et al.*, 1992; Shen *et al.*, 1994). Nasal epithelium cultured at an ALI constitutes a model similar to the *in vivo* intact airway epithelium with the formation of a polarised epithelium containing a heterogeneous population of both ciliated and secretory cells (Auger *et al.*, 2006; Guo *et al.*, 2004). Primary cultured cells are often used to model the nasal epithelium as these cells have the most similar characteristics to actual human nasal epithelial cells in their morphology, phenotype, and integrity (Yoo *et al.*, 2003).

The nasal epithelial cells are one of the first lines of defence against inhaled pathogens, dusts, allergens and irritants. Therefore a cell culture model of the nasal epithelium representative of nasal epithelial cells *in vivo* would consequently aid in determining the possible effects that biocide exposure may have on the upper airways of cleanroom operators.

Parameters tested in this chapter included changes in the pro-inflammatory status of the HNEpC cultured at ALI, following biocide challenge. This was ascertained by the measurement of apical and basolateral IL-8 secretion using an IL-8 ELISA. The oxidative effects of the biocides on the cell proteins were determined by establishing the protein thiol (thiol assay) and protein carbonyl (carbonyl ELISA) levels in biocide exposed cells. The cell death mechanism of necrosis was also investigated (using flow cytometry) following biocide exposure to the ALI cultured cells. The integrity of the epithelium as a barrier following/during biocide exposure was investigated by measuring the TER and paracellular permeability as described in Chapter 7.

8.2 Aims

- To determine whether the integrity of the epithelial layer is compromised following biocide exposure.

- To ascertain whether biocides induce a pro-inflammatory status in the HNEpC at ALI and if IL-8 release is directional.

- To determine the thiol and carbonyl content of HNEpC cultured at ALI when exposed to Quat./chlorine dioxide, 6% H_2O_2 and 70% ethanol.

- To assess cell death and determine whether any HNEpC death occurs by necrosis.

8.3 Materials and Methods

8.3.1 Materials

BD Falcon™ Cell Culture Inserts (Transwells) and BD Falcon™ 24-well Cell Culture Insert Companion Plates were from BD Falcon (Oxford, UK). BSA and Human placental type IV collagen were from Sigma (Dorset, UK). Promocell® Airway Epithelial Cell Growth Medium (AECGM) and SupplementMix were from Promocell (Heidelberg, Germany). Modified Eagles Medium was from PAA (Somerset, UK). Epithelial Voltohmmeter was from EVOM, World Precision Instruments (Stevenage, UK).

For all other materials please refer to Chapter 5.3.1

8.3.2 Methods

8.3.2.1 Epithelial cell culture on Transwell inserts (ALI culture)

Please refer to Chapter 7 Section 7.3.2.1

8.3.2.2 Determining TER of cell cultures at ALI in Transwells

Please refer to Chapter 7 Section 7.3.2.2

8.3.2.3 Challenging ALI cultures of epithelial cells.

Please refer to Chapter 7 Section 7.3.2.3

8.3.2.4 Paracellular permeability

Please refer to Chapter 7 Section 7.3.2.4

8.3.2.5 IL-8 expressed in HNEpC cultured at ALI

IL-8 release was initially determined as pg/ml by ELISA accounting for differences in apical and basolateral medium volumes. In order to take into account changes in cell number due to biocide treatment, IL-8 release was calculated relative to the protein content of cell lysates (Appendix 11.7.1) and expressed as pg/µg protein. To visualise any biocide-dependent changes in IL-8 release, the data are expressed as percentage of control. Control refers to IL-8 release in response to quiescent medium and is arbitrarily given the value of 100%.

Methods used to determine IL-8 production, protein thiol content, protein carbonyl content and necrosis are as described in Chapter 5.3.2

8.4 Results

8.4.1 Transepithelial Electrical Resistance (TER)

8.4.1.1 TER of HNEpC cultured at ALI

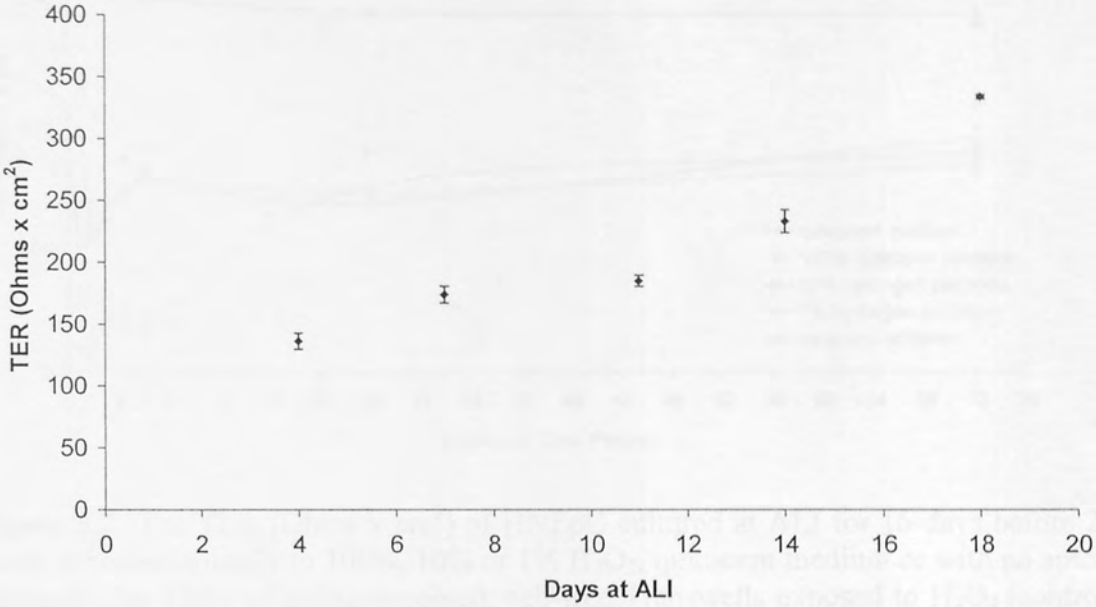


Figure 8.1 TER values of HNEpC cultured on Transwells at ALI for up to 18 days. Results are expressed as mean ± SEM. (n=12 with 6 of each passage 4 and passage 8).

Figure 8.1 indicates that the TER of HNEpC grown at an ALI increased with time with the cells reaching TER of 334 ± 2 Ohms x cm^2 after 18 days of incubation. A sharp increase in the TER was observed between day 14 and 18 of ALI culture. HNEpC with TER of ≥ 250 Ohms x cm^2 were used in experiments.

8.4.1.2 TER of H_2O_2 treated HNEpC cultured at ALI

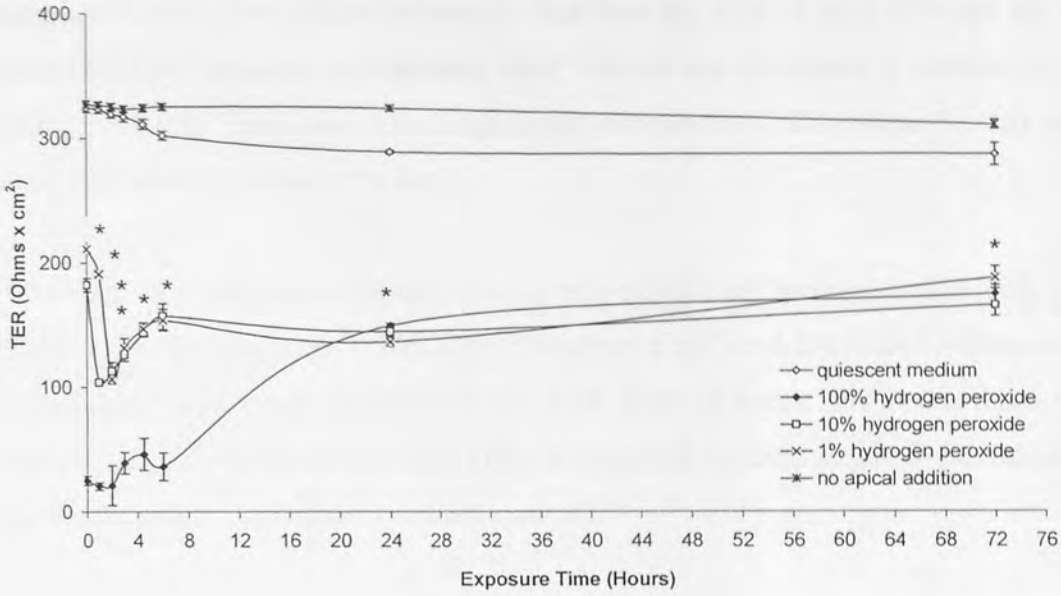


Figure 8.2 The TER (Ohms x cm^2) of HNEpC cultured at ALI for 16 days before 24 hours exposure apically to 100%, 10% or 1% H_2O_2, quiescent medium or with no apical addition. The TERs of collagen-coated, cell-free Transwells exposed to H_2O_2 (control) were subtracted from the TER of HNEpC exposed to H_2O_2. Results are expressed as mean ± SEM, (n=3). * represents $P < 0.05$ comparing biocide exposed and quiescent medium control cultures at that time-point.

The TER of quiescent medium exposed control cells was initially 324 ± 3 Ohms x cm^2. This value decreased to 287 ± 9 Ohms x cm^2 after 24 hours exposure (figure 8.2). All TER values were corrected for the TER of an empty Transwell which measured 15 ± 1 Ohms x cm^2. Data were also corrected for the effects of H_2O_2 on TER in the absence of cells (Appendix 11.6.2).

Immediately after exposure to 100% H_2O_2 there was a significant decrease in TER to 25 ± 3 Ohms x cm^2, in comparison to the quiescent medium exposed cells at time 0. TER continued to decrease at 1 hour to 20 ± 3 Ohms x cm^2 but increased to 21 ± 2 Ohms x cm^2 after 2 hours. This increased TER was sustained throughout the 24 hours of 100% H_2O_2 exposure. This was with the exception of a decrease in TER at 6 hours (36 ± 2 Ohms x cm^2).

After 1 hour of treatment, TER significantly decreases with either 10% (104 ± 5 Ohms x cm^2) or 1% (191 ± 2 Ohms x cm^2) H_2O_2 in comparison to control. The TER of the 10% (113 ± 7 Ohms x cm^2) and 1% (106 ± 3 Ohms x cm^2) H_2O_2 treated HNEpC increased non-significantly after 2 hours exposure. But then the TER of both 10% and 1% H_2O_2 treated HNEpC increased significantly after 3 hours and continued to increase up till 6 hours ($P < 0.05$). There was a non-significant decrease in TER between 6 hours and 24 hours exposure to 1% and 10% H_2O_2.

Following 48 hours recovery, there was a non-significant increase in the TER of the 100% (175 ± 7 Ohms x cm^2), 10% (166 ± 9 Ohms x cm^2) and 1% (188 ± 9 Ohms x cm^2) H_2O_2 treated cells, when compared to the TER after 24 hours. However, these values were still significantly less than the TER of quiescent medium exposed control cells ($P < 0.05$ compared to quiescent medium control).

8.4.2 Paracellular permeability of BSA

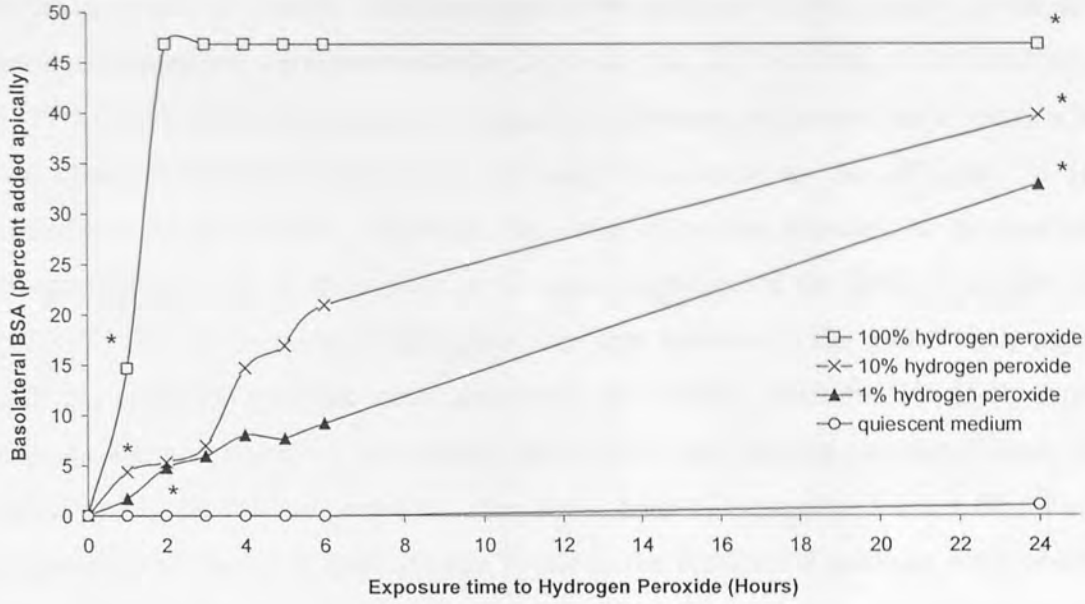


Figure 8.3 The paracellular permeability of HNEpC cultured at ALI to BSA over 24 hours of apical exposure to 100%, 10% or 1% H_2O_2 or quiescent medium. Results are expressed as mean basolateral BSA as a percentage of BSA added apically ± SD, (n=3). Using ANOVA, * represents $P < 0.05$ comparing between biocide exposed and the quiescent medium exposed (control) at each time point.

HNEpC exposed to quiescent medium (control) demonstrated no increase in basolateral compartment protein levels after 6 hours of exposure (figure 8.3). Only after 24 hours exposure to quiescent medium was a small increase in any paracellular permeability observed, with a 1 ± 0.1 % increase in the basolateral protein concentration. However, this increase in BSA paracellular permeability was not significant compared to time 0 ($P > 0.05$).

Following apical exposure of the HNEpC to 100% H_2O_2 containing 64 ± 3 mg/ml BSA (figure 8.3), a significant increase in the BSA passing from the apical compartments of the Transwells through to the basolateral compartment was observed following just 1 hour of exposure ($P < 0.05$ compared to quiescent medium control at 1 hour). A further increase in protein levels was detected after 2 hours of treatment with 47 ± 0.1 % of the apical BSA now detected in the basolateral medium. However, after 2 hours there was

no further increase in the basolateral BSA level throughout the 24 hour experimental time-frame. A small significant increase in basolateral protein levels was also observed after 1 hour of exposure to 10% H_2O_2 (4 ± 0.2 %) ($P < 0.05$ compared to quiescent medium control at 1 hour). Further consecutive increases in BSA protein levels in the basolateral medium were observed after 2 (5 ± 0.5 %), 3 (7 ± 0.1 %), 4 (15 ± 0.2 %) and 5 (17 ± 0.2 %) hours of exposure. A significant increase in the basolateral protein level was observed between 6 hours and 24 hours of exposure to the 10% and 1% H_2O_2 concentrations ($P < 0.05$). However, the level of protein detected in the basolateral compartments of the Transwells after 24 hours exposure of the HNEpC to 10% H_2O_2 (40 ± 0.2 %) was non-significantly less than that detected in the 100% H_2O_2 exposed cells (47 ± 0.1 %) over the same time-frame ($P > 0.05$). With the 1% H_2O_2 exposed cells its was also observed that apically added BSA was passing non-significantly from apical to basolateral compartments after just 1 hour of exposure (2 ± 0.1 %). Further consecutive increases in BSA protein levels in the basolateral medium were observed after 2 (5 ± 0.5 %), 3 (6 ± 0.1 %) and 4 (8 ± 0.7 %) hours of exposure. However, there was a small non-significant decrease in basolateral BSA protein levels after 5 hours of exposure (8 ± 0.7 %). There was a significant increase in the level of protein detected after 24 hours (33 ± 0.6 %) in relation to 6 hours (9 ± 0.3 %) ($P < 0.05$). Yet, the amount of protein detected basolaterally after 24 hours exposure to the 1% H_2O_2 (33 ± 0.6 %) was not higher than that observed with the 10% or 100% H_2O_2 exposed HNEpC. With the 100% and 10% H_2O_2, 47 ± 0.1 % and 40 ± 0.2 % of the apically added BSA was detected basolaterally after 24 hours of exposure, respectively. The protein levels used to calculate percentage BSA permeability (figure 8.3) are in Appendix 11.7.2.

8.4.3 IL-8 release from biocide exposed HNEpC

8.4.3.1 IL-8 released apically following 24 hours biocide exposure.

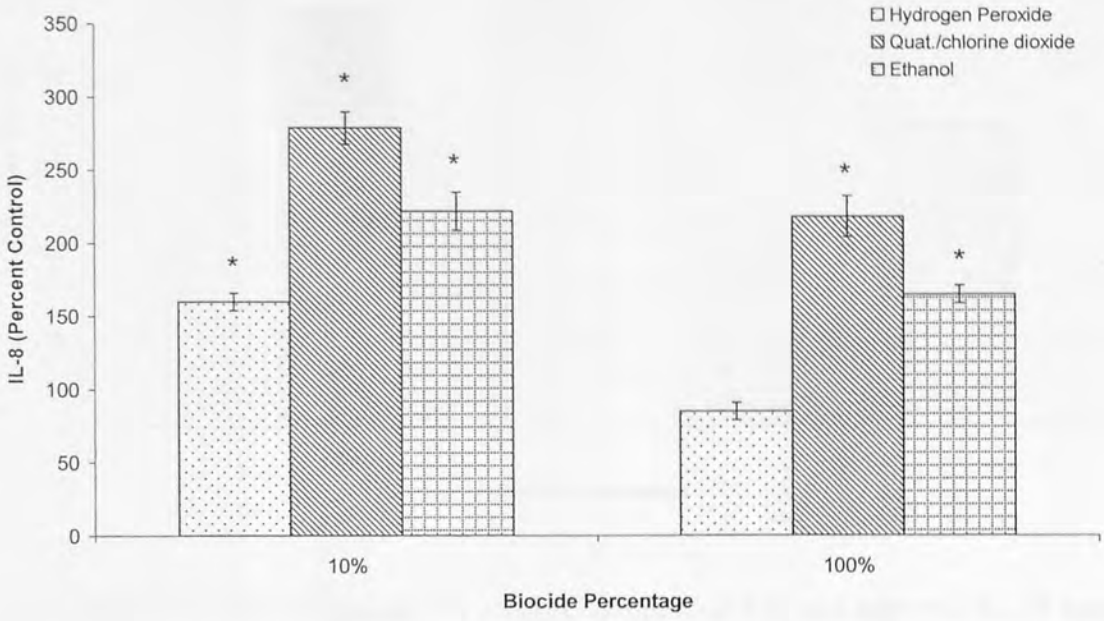


Figure 8.4 IL-8 released apically by HNEpC cultured at ALI and exposed to biocides at 10% or 100% of practical concentration for 24 hours, taking into account cell protein levels. Data are presented as mean percentage of control cells ± SEM (n=6), where IL-8 of quiescent medium exposed control cells was taken as 100%. * $P < 0.05$ in relation to control.

Significant increases in IL-8 released apically (figure 8.4) in comparison to quiescent medium exposed control levels (0.48 ± 0.03 pg/μg protein taken as 100%) were observed following exposure of the cells to all three of the 10% biocides (H_2O_2: 160 ± 6 % control, Quat./chlorine dioxide: 278 ± 11 % control and ethanol: 222 ± 13 % control) ($P < 0.05$). A non-significant decrease in the apical IL-8 released by the 100% H_2O_2 exposed cells was observed (85 ± 6 % control). By contrast significant increases in relation to the control were observed when the cells were exposed to 100% Quat./chlorine dioxide (218 ± 14 % control) and ethanol (165 ± 6 % control). Higher apical IL-8 levels were observed in relation to the control with the exception of the 100% H_2O_2 exposed cells.

8.4.3.2 IL-8 released apically following 48 hours recovery from biocide exposure.

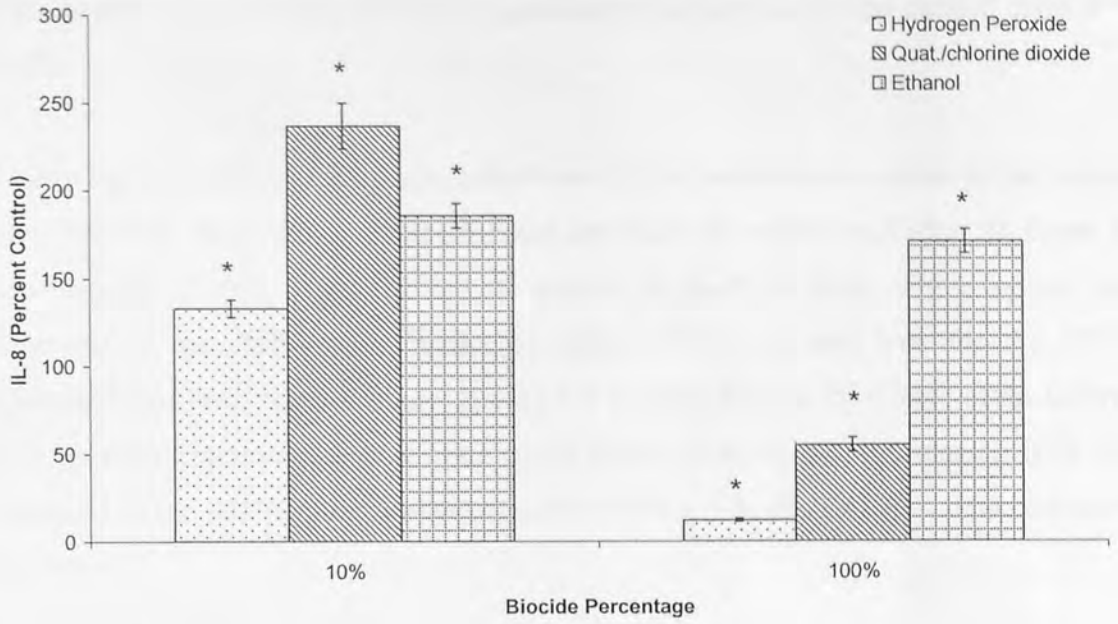


Figure 8.5 IL-8 released apically by HNEpC cultured at ALI and exposed for 24 hours to biocides at 10% or 100% of practical concentration and allowed to recover for 48 hours at ALI, taking into account cell protein levels. Data are presented as mean percentage of control cells ± SEM (n=6), where IL-8 of quiescent medium exposed control cells was taken as 100%. * $P < 0.05$ in relation to control.

Significant increases in IL-8 released apically (figure 8.5) in comparison to quiescent medium exposed control levels (1.30 ± 0.16 pg/μg protein taken as 100%) were observed following 48 hours recovery of the 10% biocide treated cells (H_2O_2: 133 ± 5 % control, Quat./chlorine dioxide: 237 ± 13 % control and ethanol: 186 ± 7 % control) ($P < 0.05$). A significant increase in relation to the control also observed when the cells were exposed to 100% ethanol (171 ± 7 % control) ($P < 0.05$). By contrast a significant decrease in the apical IL-8 released in response to either the 100% H_2O_2 (12 ± 1 % control) or Quat./chlorine dioxide (55 ± 4 % control) exposed cells was observed ($P > 0.05$ in relation to control).

Some differences in IL-8 levels released by the cells were also detected between the cells following 24 hours of exposure to the biocides and after recovery of the biocide-

exposed cells. IL-8 levels directly after exposure to the biocides at 10% were found to be higher than after recovery. This was also found in the apical supernatants of the 100% Quat./chlorine dioxide and ethanol treated HNEpC. However, all IL-8 levels in 10% biocide exposed cells were still significantly higher than in the control cells ($P < 0.05$).

Following exposure to 100% H_2O_2, a decrease in IL-8 secretion in relation to the control was observed, both directly after 24 hours exposure ($P > 0.05$) and after 48 hours of recovery ($P < 0.05$). Only 12 ± 1 % control of the IL-8 level of the control was observed in the 100% H_2O_2 recovered cells. This was also true for the 100% Quat./chlorine dioxide cells where only 55 ± 4 % control of the IL-8 level of the control was observed. In comparison a significantly higher level of apically released IL-8 was observed in the 100 % ethanol exposed cells (171% ± 7 % control) ($P < 0.05$ compared to control).

8.4.3.3 IL-8 released basolaterally following 24 hours biocide exposure.

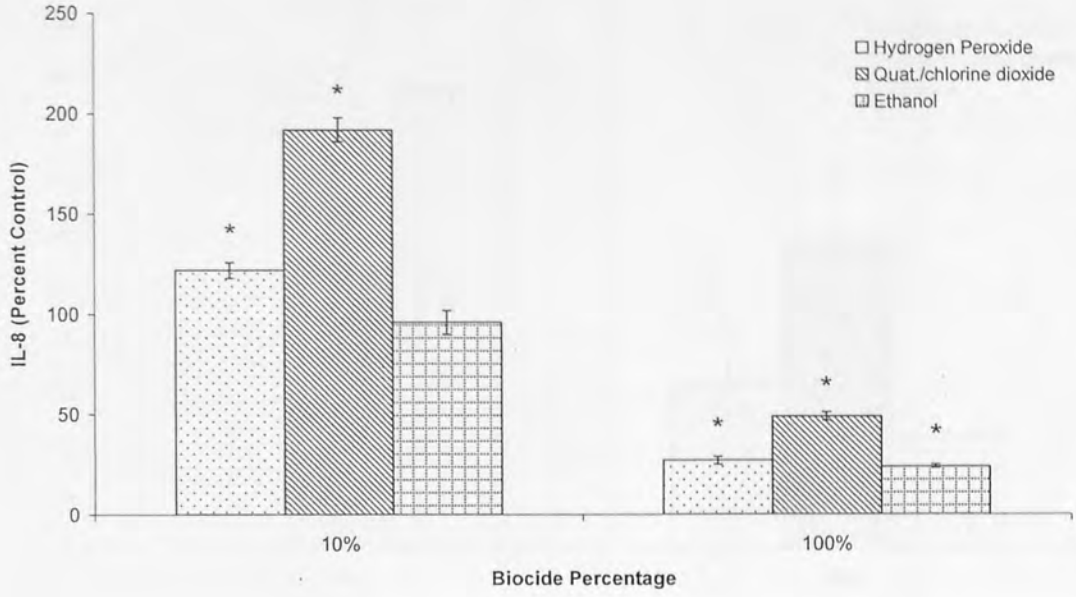


Figure 8.6 IL-8 released basolaterally by HNEpC cultured at ALI and exposed to biocides at 10% or 100% of practical concentration for 24 hours, taking into account cell protein levels. Data are presented as mean percentage of control cells ± SEM (n=6), where IL-8 of quiescent medium exposed control cells was taken as 100%. * $P < 0.05$ in relation to control.

A non-significant decrease in the IL-8 basolaterally released (figure 8.6) was observed with the 10% ethanol exposed HNEpC (96 ± 6 % control) following 24 hours of exposure in relation to the quiescent medium exposed control (0.51 ± 0.15 pg/μg protein taken as 100%) ($P > 0.05$). Significant increases in IL-8 release were observed on exposure of the cells to the 10% H_2O_2 (122 ± 4 % control) or Quat./chlorine dioxide (192 ± 6 % control) ($P < 0.05$ compared to control). In contrast, significant decreases in basolateral IL-8 release in comparison to control levels were observed following exposure of the cells to all three of the 100% biocides (H_2O_2: 27 ± 2 % control, Quat./chlorine dioxide: 49 ± 2 % control and ethanol: 24 ± 1 % control) ($P < 0.05$).

8.4.3.4 IL-8 released basolaterally following 48 hours recovery from biocide exposure

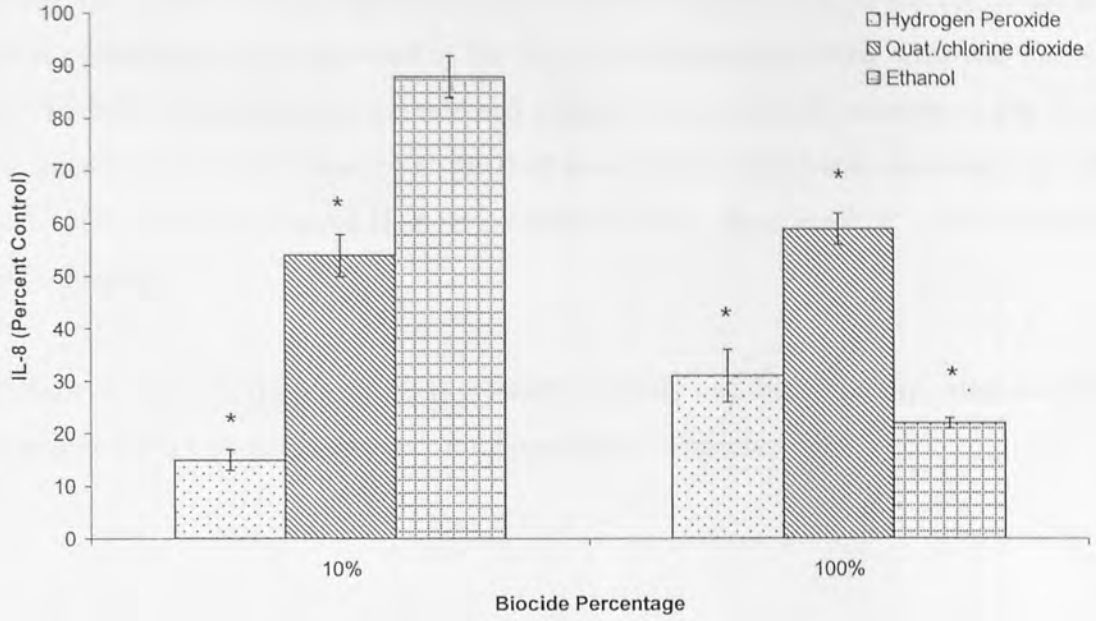


Figure 8.7 IL-8 released basolaterally from HNEpC cultured at ALI and exposed for 24 hours to biocides at 10% or 100% of practical concentration and allowed to recover for 48 hours at ALI, taking into account cell protein levels. Data are presented as mean percentage of control cells ± SEM (n=6), where IL-8 of quiescent medium exposed control cells was taken as 100%. (n=6). * $P < 0.05$ in relation to control.

Significant decreases in IL-8 released basolaterally in comparison to quiescent medium exposed control levels (1.46 ± 0.27 pg/µg protein taken as 100%) were observed following exposure of the cells to all 100% biocides (H_2O_2: 31 ± 5 % control, Quat./chlorine dioxide: 59 ± 3 % control and ethanol: 22 ± 2 % control) ($P < 0.05$) (figure 8.7). A significant decrease in relation to the control was also observed when the cells were exposed to 10% H_2O_2 (15 ± 2 % control) and Quat./chlorine dioxide (54 ± 4 % control) ($P < 0.05$). A non-significant decrease in the basolateral IL-8 released was observed following exposure of the cells to the 10% ethanol (88% ± 4 % control).

Some differences in IL-8 levels released by the cells were also detected between the cells following 24 hours of exposure to the biocides and after recovery of the biocide-exposed cells. IL-8 levels directly after exposure to the biocides at 10% were found to

be higher than after recovery. However, all IL-8 levels in 10% biocide recovered cells were significantly less than in the control cells ($P < 0.05$). At exposure to 100% H_2O_2, a decrease in relation to the control was observed, both directly after 24 hours exposure ($P < 0.05$) and after 48 hours of recovery ($P < 0.05$). Only 31 ± 5 % control of the IL-8 level of the control was observed in the 100% H_2O_2 recovered cells. This was also true for the 100% Quat./chlorine dioxide and ethanol recovered cells whereby, only 55 ± 4 % control and 82 ± 2 % control of the IL-8 level of the control was observed. All 10% and 100% biocide recovered IL-8 levels were less than the control ($P < 0.05$ except the 10% ethanol).

Actual IL-8 levels (pg/µg protein) released apically and basolaterally, after 24 hours exposure and/or 48 hours recovery are presented in Appendix 11.7.3.

8.4.4 Thiol content of HNEpC

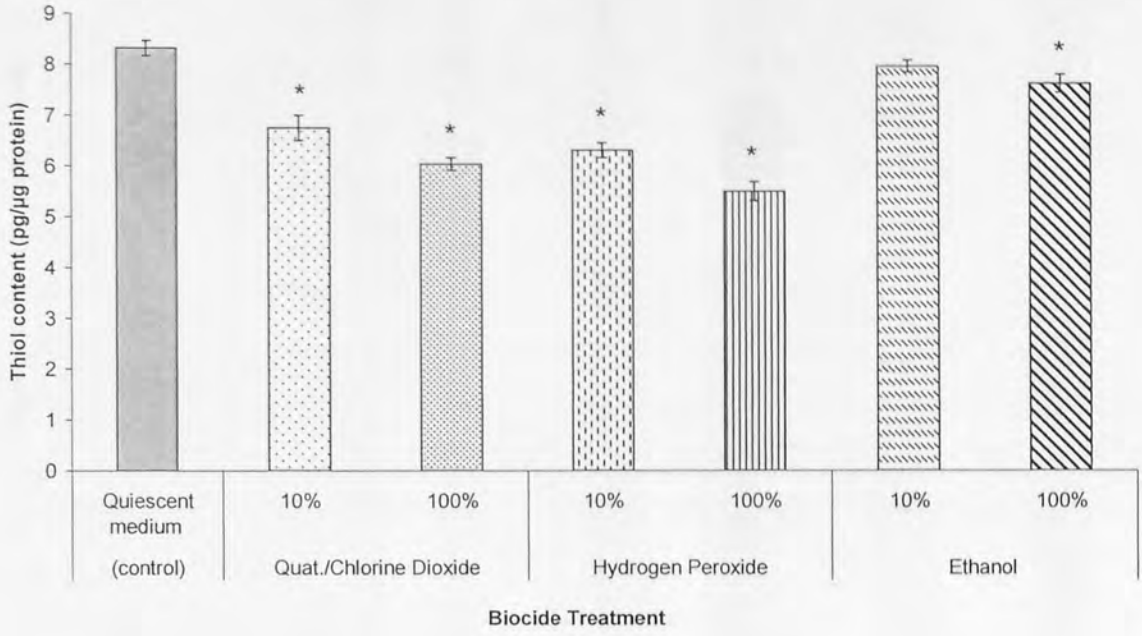


Figure 8.8 Thiol content (pg/µg protein) in HNEpC lysates following 24 hours of exposure to 10% and 100% in-use biocide concentrations (Quat./chlorine dioxide, H_2O_2 and ethanol) and 48 hours of recovery. Results are expressed as mean ± SEM (n=9). * represents $P < 0.05$, comparing between biocides and quiescent medium control.

Basal levels of free thiols in ALI cultured HNEpC were calculated as 8.32 ± 0.15 pg/µg of protein (figure 8.8). A reduction in comparison to the control was observed on exposure to all 3 biocides tested at either 10% or 100% of their in-use concentrations. There was a significant reduction in free thiols ($P < 0.05$) on exposure of the cells to 10% or 100% Quat./chlorine dioxide or H_2O_2 ($P < 0.05$). A significant reduction in the level of free thiols was observed with the cells at 100% of its in-use concentration ($P < 0.05$). This was not true for 10% ethanol exposed cell lysates.

The greatest reduction in free thiols was seen in the cell lysates of the cells exposed to 100% H_2O_2, which reduced levels from 8.32 ± 0.15 to 5.50 ± 0.19 pg/µg of protein. This was followed closely by the 100% Quat./chlorine dioxide which reduced thiol levels to 6.04 ± 0.12 pg/µg of protein. The lowest reduction in free thiols occurred on exposure of the cells grown at ALI to 10% ethanol (7.96 ± 0.12 pg/µg of protein).

8.4.5 Carbonyl content of HNEpC lysates

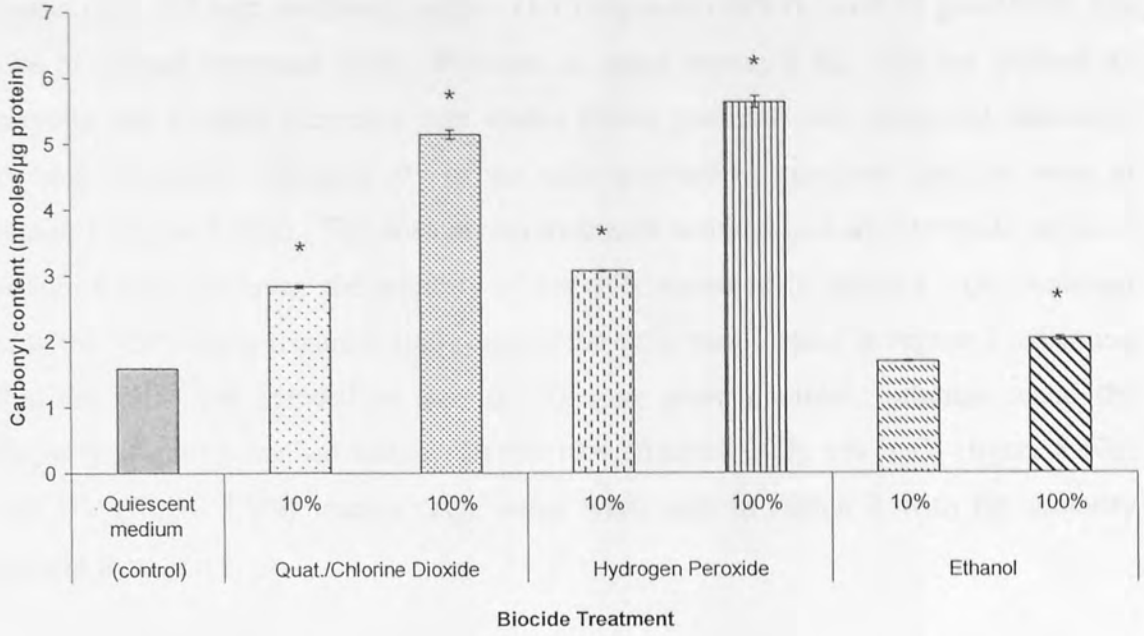


Figure 8.9 Carbonyl content (nmoles/µg protein) of HNEpC lysates following 24 hours of exposure to 10% and 100% in-use biocide concentrations (Quat./chlorine dioxide, H_2O_2 and ethanol) after 48 hours of recovery. Results are expressed as mean ± SEM (n=9). * represents $P < 0.05$, comparing between biocides and quiescent medium control.

Levels of carbonyls in quiescent medium exposed HNEpC lysates were 1.59 ± 0.01 nmoles/µg of protein (figure 8.9). A significant increase in carbonyl content in comparison to the quiescent medium control was observed on exposure to 100% Quat./chlorine dioxide, H_2O_2 or ethanol ($P < 0.05$). Quat./chlorine dioxide or H_2O_2 at 10% induced a significant increase in carbonyl content in relation to the quiescent medium control ($P < 0.05$). No significant increase in the carbonyl content of the cell lysates was observed in the 10% ethanol exposed cells ($P > 0.05$).

8.4.6 Propidium iodide staining of challenged HNEpC.

HNEpC in gated region 1 (figure 8.10) are defined as viable by virtue of their low side scatter (SS) and high electronic volume (EV) characteristics in terms of granularity and size of normal untreated cells. Whereas in gated region 2 the cells are defined as necrotic due to their increased side scatter (more granular) and decreased electronic volume (smaller). The majority of the cells exposed to quiescent medium were in region 1 (figure 8.10a). The anisomycin treatment resulted in a shift towards region 2 (figure 8.10b), however, the majority of the cells remained in region 1. On treatment with the 100% H_2O_2 (figure 8.10c) some of the cells were located in region 2 indicating that the cells had reduced in size and become more granular, although again the majority of cells remained viable. Of the 10% (figure 8.10d), 1% H_2O_2 (figure 8.10e) and UV (figure 8.10f) treated cells, some were seen in region 2 with the majority present in region 1.

Table 8.1 PI staining of challenged HNEpC. Percentage of necrotic cells and the mean fluorescence of the cells in the total cell population on exposure of HNEpC cultured at ALI to quiescent medium, anisomycin (5 μg/ml), 100% H_2O_2, 10% H_2O_2, 1% H_2O_2 for 24 hours, or UV for 15 minutes (n=1).

Cell treatment (24 hours)	Percentage of necrotic cells in total cell population (%)	Mean fluorescence of all cells in total population (Arbitrary Units)
Quiescent medium	1.46	2.47
Anisomycin	27.08	7.46
100% H_2O_2	34.57	8.68
10% H_2O_2	30.97	8.63
1% H_2O_2	18.82	6.43
UV (15 min)	2.32	2.64

The percentage of cells determined as necrotic in untreated, quiescent medium-exposed cells was 1.46% (table 8.1). This number increased more than 20-fold following 24 hours exposure to 100% H_2O_2 (34.57% necrotic) or 10% H_2O_2 (30.97% necrotic) for 24 hours. Compared to quiescent medium control, the percentage of necrotic cells increased 18-fold after treatment with anisomycin and 10-fold with the 1% H_2O_2. In

addition, the percentage of necrotic cells present in the samples also increased following exposure to the UV (1.46% to 2.32%).

Little auto-fluorescence of the HNEpC was observed and the average mean fluorescence of the drug/biocide treated and untreated cells were low (1.38 arbitrary units to 2.14 AU). The greatest PI-dependent mean fluorescence of the HNEpC was observed with the 100% H_2O_2 exposed cells (table 8.1), the fluorescence increased more than 3-fold in comparison to the quiescent medium exposed cells (2.47 AU to 8.68 AU). The fluorescence also increased three-fold with the 10% H_2O_2 (2.47 AU to 8.63 AU) and on exposure of the cells to anisomycin (2.47 AU to 7.46 AU). Another small increase was also observed with the UV (2.47 AU to 2.64 AU) treated cells.

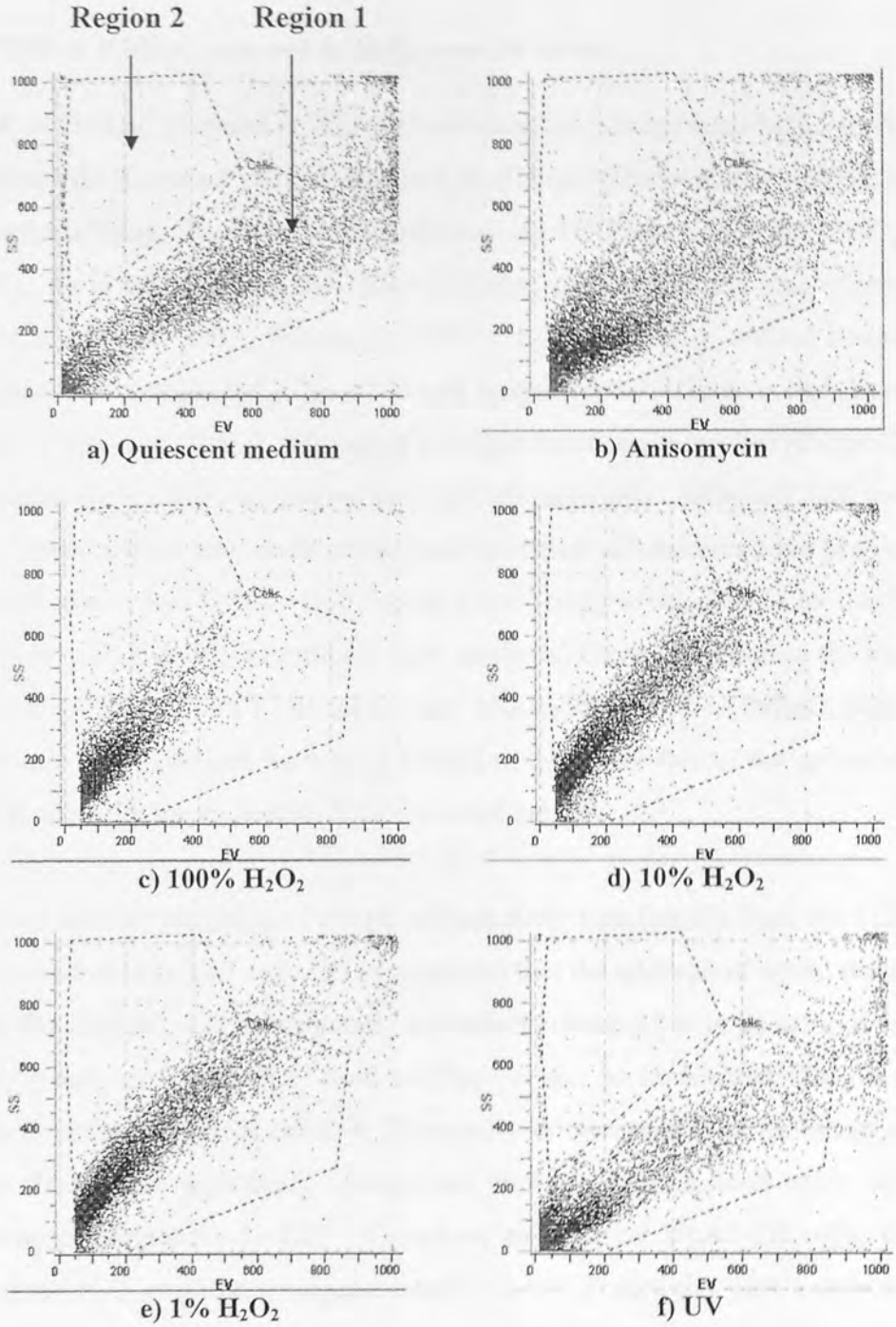


Figure 8.10 Scatter cytograms of HNEpC cultured at ALI and exposed for 24 hours to a) quiescent medium, b) anisomycin (5 µg/ml), c) 100% H_2O_2, d) 10% H_2O_2, e) 1% H_2O_2 and 15 minutes to f) UV. Y-axis (SS = side scatter) is representative of cell granularity and X-axis (EV = Electronic volume) represents the cell size. Region 1 represents the healthy cell population. Region 2 is representative of necrotic cells. Regions are outlined by a dashed line with gating set to overlap all cells (n=1).

8.5 Discussion

8.5.1 TER of HNEpC exposed to H_2O_2 over 24 hours.

The TER of HNEpC cultured at ALI was observed to increase with time. Increases in TER indicate development of tight junctions in HNEpC (Dimova *et al.*, 2005), with the tight junctions being characteristic of differentiated HNEpC (Yoo *et al.*, 2003; Lin *et al.*, 2007). As in other studies, the TER of HNEpC increased as the time of incubation increased (Lee *et al.*, 2005; Yoo *et al.*, 2003). TERs in freshly excised human nasal airways has been determined to be <100 and typically 60-75 Ohms x cm^2 (Boucher *et al.*, 1986; Coyne *et al.*, 2003), suggesting that tight junctions in *in vitro* HNEpC cultures are at least as electrically resistant as nasal epithelium *in vivo*. Although TER values for HNEpC *in vivo* are not well established, nasal epithelial cell cultures have been used for transport studies when TER > 250 Ohms x cm^2 (Agu *et al.*, 2001) as this TER is indicative of cells forming a confluent tight epithelial barrier. Therefore the high TER value ($\geq$ 250 Ohms x cm^2) obtained upon ALI culture of the HNEpC (figure 8.1) indicates that tight junctions have been formed at the apical side of the epithelial layer, forming a relatively impermeable, tight epithelial barrier.

The TER of apically unexposed HNEpC did not differ significantly from the TER of the quiescent medium exposed cells. This suggested that the addition of apical fluid had no effect on the HNEpC, and consequently any effects observed were because of exposure to the H_2O_2 and not due to apical fluid addition per se. An increase in TER on addition of H_2O_2 to collagen-coated, cell-free Transwells in comparison to quiescent medium exposed Transwells previously recognized that H_2O_2 interfered with the TER measurements (Appendix 11.6.2). Therefore, as with the BEAS-2B cells, the TER values of the H_2O_2 exposed collagen-coated, cell-free Transwells were subtracted from the TER measurements of the H_2O_2 exposed HNEpC.

The TER of quiescent medium exposed cells remained stable over the 24 hour exposure period. However, a higher than 90% decrease in the TER of HNEpC was observed instantly following exposure to 100% H_2O_2, with an almost 50% decrease in TER observed following exposure to 10% and 1% H_2O_2. The decreases in TER observed with the HNEpC exposed to 100%, 10%, 1% H_2O_2 in this study were established as being reversible. However, recovery of the TER was only partial after 48 hours.

Actin association with tight junctions has been established as indicative of the formation of a tight epithelial barrier in HNEpC (Lin *et al.*, 2007; Yoo *et al.*, 2003). In the present study, although the TER of the HNEpC was not restored to the level prior to any exposure, this may explain why the TER was found to increase following recovery of the cells after 1% and 10% H_2O_2 treatments. Recovery of tight junction function through the association of actin with the cytoskeleton may be responsible for the recovery of some of the TER of the epithelial cell barrier.

Herard *et al.* (1996) found that airway epithelial cell integrity *in vitro* was affected immediately after injury with sodium hydroxide. However, restoration of the epithelial barrier integrity was found to come about through the modification of tight junction function, with integrity being completely restored after 1-2 days. But they also found that if exposure to the chemical was maintained, then restoration of the epithelial barrier was prevented due to modification of the tight junctions. In some correlation with Herard *et al.* (1996), the present study established that HNEpC integrity was affected immediately upon exposure to H_2O_2. However, in contrast to Herard *et al.* (1996), the restoration of the epithelial barrier integrity with regards to the TER of the cells was not completely restored after 2 days. This could be explained by the possible H_2O_2-mediated oxidative modification of tight junction proteins that may have occurred over the 24 hours of exposure. Immunocytochemical techniques could be used to determine whether the expression of tight junction proteins was altered upon H_2O_2 treatment.

Tight junction integrity is thought to be maintained by the interactions between individual proteins, including occludins, claudins, functional adhesion molecules and zonula occludin-1 (ZO-1) (Wan *et al.*, 2000). Confluent monolayers of 16HBE14o- and Calu-3 cells have been found to show distinct staining of the tight junction protein occludin (Wan *et al.*, 2000). Wan *et al.* (2000) proposed that alterations in tight junction proteins may be significant in the airway epithelium, with regards to potential pathophysiological events. It has been suggested that some tight junction protein function may be regulated by phosphorylation. For example, the phosphorylated forms of occludin were found to be responsible for the tight junction seal as phosphorylation directed the protein to the tight junction (Wong, 1997). In addition to this, increased ZO-1 phosphorylation was found to result in decreased TER (Staddon *et al.*, 1995). It is these tight junctions that function as paracellular gates to close the paracellular space of

proximal cells (Sawada *et al.*, 2003). It is thought that H_2O_2 is able to act upon the cellular junctions. Chapman *et al.* (2002) found that the staining of multiple regions was disturbed on exposure of Calu-3 cells to 0.1 mM H_2O_2 for 350 minutes, indicating the disrupted localisation of the tight junction proteins ZO-1 and occludin.

Overall, with regards to TER, the membrane integrity of ALI cultured HNEpC is initially lost upon challenge with H_2O_2. Although recovery of the cells occurs, the recovery is not complete as the TER even after 48 hours of recovery remains at 50% of the quiescent medium exposed control. Therefore H_2O_2 is likely to affect human nasal epithelium permeability *in vivo*.

8.5.2 Paracellular permeability of HNEpC to BSA

BSA added apically to a collagen coated Transwell is detected at around 100% of the apical concentration within one hour (Anne Bielemeier, observation from laboratory), indicating that BSA is permeable through the Transwell membrane. The prevention of BSA movement upon culture of HNEpC on the Transwells indicates that a barrier of epithelial cells is formed. In this study, apical exposure of HNEpC to quiescent medium for 24 hours had no affect on permeability to BSA. A H_2O_2 concentration and time dependent increase in the paracellular permeability of the cells was detected following 2 hours of exposure to 1% and 10% H_2O_2. An increase in paracellular permeability to BSA was observed within 1 hour of exposure to 100% H_2O_2, with a further increase observed after 2 hours of treatment. After 2 hours, no further increase in the basolateral BSA concentration was observed. This indicates that there is a maximal 47% permeability of the HNEpC barrier to BSA after 2 hours treatment with 100% H_2O_2.

The results of the paracellular permeability experiments contrasted to some extent with those of the TER measurements. Although an initial decrease was observed in the TER upon H_2O_2 exposure, the TER partially recovered over time. In contrast, permeability of the cells to BSA increased as the time of exposure to 10% and 1% H_2O_2 increased but not with the 100% H_2O_2. Other studies have established a correlation between TER and permeability to mannitol or BSA (Yoo *et al.*, 2003; Lin *et al.*, 2007; Lee *et al.*,

2005). Here, reductions in TER generally correlated with increased paracellular permeability. However, in this study the paracellular permeability of the HNEpC observed following 24 hours exposure to 100% H_2O_2 was in contrast to the TER results. One study found that while airway epithelial cell permeability could remain unaffected, the electrical resistance of the cells could be changed (Devalia *et al.*, 1994). Consequently, it is possible that the permeability of the HNEpC exposed to the 100% H_2O_2, remained unaffected after 2 hours, while the TER was found to decrease further.

As no additional increase in BSA movement from apical to basolateral compartments occurs after 2 hours treatment to 100% H_2O_2, it is likely that some mechanism/s of cell defence have been activated in the HNEpCs. Otherwise, as with the BSA permeability of an empty Transwell, it would be expected that 100% BSA permeability be observed upon longer exposure to the 100% H_2O_2. Both the metabolism of H_2O_2 and production of antioxidants by HNEpC need to be taken into consideration. The degradation of H_2O_2 and/or its possible metabolism by HNEpC may be responsible for no further increase in apical to basolateral BSA movement being observed after the 2 hours. This may be due to the negation of the apically added H_2O_2's effects. HNEpC produce antioxidants such as catalase (Almagor *et al.*, 1985), which may reduce the effect the H_2O_2 has on the cells. In addition, the possible oxidation of the apical BSA by H_2O_2 may also be an explanation, as BSA may have become oxidatively modified and as a consequence no longer be able to be detected by the protein assay. The results to both the protein thiol and carbonyl assays in this study confirmed that oxidation of the HNEpC proteins was occurring upon exposure to H_2O_2.

As tight junctions are regulators of paracellular permeability (Yoo *et al.*, 2003), any changes in tight junction functioning could be responsible for the initial increase in permeability and then the recovery of epithelium barrier integrity. This may be attributable to perijunctional actin, which is known to play a significant role in the regulation of paracellular permeability in airway epithelial cells (Andersen and Van Itallie, 1995; Förster, 2008). Although no studies have been carried out on the effects of H_2O_2 treatment on HNEpC barrier function with regards to the contraction of the actin cytoskeleton, it is possible that this is the mechanism by which the H_2O_2 may be controlling barrier function. This is because initial, rapid permeability increases of H_2O_2 exposed airway epithelial cells (Calu-3) have been found to occur as a result of

contraction of the perijunctional actin of the cells tight junctions (Chapman *et al.*, 2002).

The nasal epithelium transports ions and metabolites via both the transcellular and paracellular pathways (Lee *et al.*, 2005). Loss of the polarised barrier function therefore produces an unregulated transport mechanism with the possible permeation of exogenous constituents through the epithelium. In addition to this, the transport of electrolytes by the epithelium is essential to maintain the required volume and salt composition of ASL (Jepsen *et al.*, 2000). Dazy *et al.* (2003) established that ionic homeostasis of HNEpC was altered on exposure to H_2O_2, such that there was an efflux of chloride ions into the surrounding environment and an increase in intracellular Ca^{2+}. They found that this was regularly accompanied by an efflux of water which may consequently reduce the viscosity of the fluid lining the epithelium surface (Dazy *et al.*, 2003). This would result in the impairment of the mucociliary clearance process as cilia are only able to transport mucus if it has appropriate viscoelasticity. If the mucus becomes too thin, gravity overcomes ciliary transport (Cone, 2005). Consequently the epithelium will become functionally impaired, making the nasal airways more susceptible to respiratory infections (Jepsen *et al.*, 2000).

Overall, these results indicate that H_2O_2 destructively disturbs the HNEpC barrier following 24 hours challenge *in vitro*. However, some recovery of the barrier is possible.

8.5.3 IL-8 release in biocide exposed HNEpC

Determining the direction of IL-8 release by HNEpCs helps to understand the communication between the epithelium and the cells of the immune system. The inflammatory status of the epithelium can determine the overall effect that the potential toxicants can have on the airway epithelium.

All biocides at 10% increased total IL-8 release (apical and basolateral) from the HNEpC immediately after 24 hours challenge in comparison to control cells. With the exception of the H_2O_2, this increase was sustained following 48 hours of recovery.

With the 100% exposures there was no increase in total IL-8 in biocide exposed cells after challenge or after recovery, in comparison to control total IL-8 levels. The 10% Quat./chlorine was established to be inducing the greatest pro-inflammatory effect in HNEpC immediately after exposure, with the 10% ethanol eliciting the greatest pro-inflammatory effect after 48 hours recovery.

The results to the present study show that HNEpC release IL-8 in a bidirectional manner. Control cells released IL-8 both apically (27% of the total IL-8) and basolaterally (73% of the total IL-8). Biocide challenge at 10% concentrations increased apical IL-8 release from HNEpC after 24 hours exposure and this was sustained for 48 hours after recovery. In comparison, the 100% biocide challenge increased apical IL-8 in only the Quat./chlorine dioxide and ethanol exposed cells immediately after challenge, with the increase being sustained in the ethanol challenged cells following recovery. The increases in IL-8 released apically are likely to up-regulate the IL-8-mediated recruitment and activation of neutrophils and macrophages to the airway lumen via a chemotactic gradient (Thompson *et al.,* 1995). This immune cell recruitment may become amplified as IL-8 is also produced by neutrophils themselves and the activation of macrophages is likely to provide positive feedback on epithelial cells due to macrophage production and release of pro-inflammatory mediators such as TNFα and IL-1 (Chung, 2001; Thompson *et al.,* 1995). As some of the increases in IL-8 released apically were observed as being sustained following recovery, it is likely that such biocide exposures are inducing inflammation in the airway epithelium that is sustained even when the biocide toxicant is removed. This may have great implications on the airway epithelium due to the increased recruitment of neutrophils, which will release their contents onto the surrounding cells. This will potentially cause cellular injury and may even cause cell death.

There was no increase in basolateral IL-8 post-recovery from both 10% and 100% biocide challenged cells. In fact, after recovery of the HNEpC a decrease in IL-8 was observed in basolateral supernatants. This was true for both 10% and 100% biocide exposures. An overall observation by Auger *et al.* (1996) was that constitutive IL-8 secretions from HNEpC occurred towards both compartments of ALI cultures but were more often directed towards the basolateral compartment. However, on exposure of the cells to DEPs the secretion of IL-8 was stimulated exclusively towards the basolateral

compartment of the Transwells. In the present study 10% H_2O_2 or Quat./chlorine dioxide increased basolateral IL-8 release from HNEpC immediately after 24 hours challenge. So, 24 hours exposure to 10% H_2O_2 or Quat./chlorine dioxide may recruit neutrophils to the region and result in inflammation of the airways due to the increased ROS presence. Under control conditions the total IL-8 increased after recovery in full medium (1.78 ± 0.17 pg/μg protein to 1.97 ± 0.26 pg/μg protein). Therefore the control cells had not stopped producing IL-8 following exposure to quiescent medium.

Apically and basolaterally there was a concentration dependent decrease in IL-8 levels released by HNEpC following exposure to Quat./chlorine dioxide or ethanol. Interestingly, cells exposed to 10% H_2O_2 released a higher level of IL-8 to the basolateral compartment following recovery than 100% H_2O_2 exposed cells. This suggests that the biocides may be less pro-inflammatory at 100% concentrations.

Generally, the initial IL-8 response was directed basolaterally (10% biocide concentrations) but this was not observed with the 100% biocides. However, IL-8 levels were reduced for both 10% and 100% exposures after 48 hours recovery. This implies that although inflammation may potentially occur *in vivo* in response to 24 hours biocide exposure, it may be resolved after 48 hours recovery. These findings seem to correlate with the time-course of IL-8 expression in human airways, as neutrophils are recruited and activated by IL-8 within 6-8 hours of an 'infection' (Goldsby *et al.*, 2000). Transcription of the IL-8 genes within the 24 hours exposure to biocide is likely to occur resulting in the expression and release of more IL-8. *In vivo* the recruitment of neutrophils will have a positive feedback on the epithelial cells further amplifying IL-8 levels. In addition to recruiting and stimulating inflammatory cells, epithelial cells may participate in the downregulation of inflammatory cells. The level of IL-8 being produced will be downgraded upon resolving inflammation. The decrease in IL-8 levels after recovery therefore suggests that inflammation was resolving after 48 hours in the absence of biocide. It is hypothesised that the reduced IL-8 response of the HNEpCs may be associated with low neutrophil transmigration after recovery. This may reflect either anti-inflammatory effects or cellular damage as antioxidants possess important properties that serve to interrupt or protect against the ongoing inflammatory process.

Secretion of IL-8 may play an important role in the recruitment and activation of leukocytes. Inflammation can become amplified because recruited neutrophils produce ROS (superoxide anions, H_2O_2, hydroxyl radicals). This triggers a potential mechanism for a positive feedback cycle of inflammation which recruits more neutrophils and ROS into the area (figure 8.11). The amplification of the neutrophil response has been established as being due partly to IL-8 expression in the recruited neutrophils themselves (Inoue, 1999). In response to infections, the ROS are able to act upon the infectious agents to effectively remove them from the airways. However, a potentially hazardous response may occur upon biocide exposure, as excess quantities of ROS can cause localised damage to the airway epithelium (Wright *et al.,* 1994; Chapman *et al.,* 2002; Gabrielson *et al.,* 1994; Barnes *et al.,* 1990). In addition to ROS, neutrophils also release proteases such as neutrophil elastase which can act upon cell proteins in the absence of any infectious organism. Neutrophil elastase cleaves structural proteins, leading to the stripping of the bronchial epithelium and reduction in ciliary beating, as well as stimulating epithelial cell IL-8 secretion which leads to further neutrophil recruitment (Stockley, 1995).

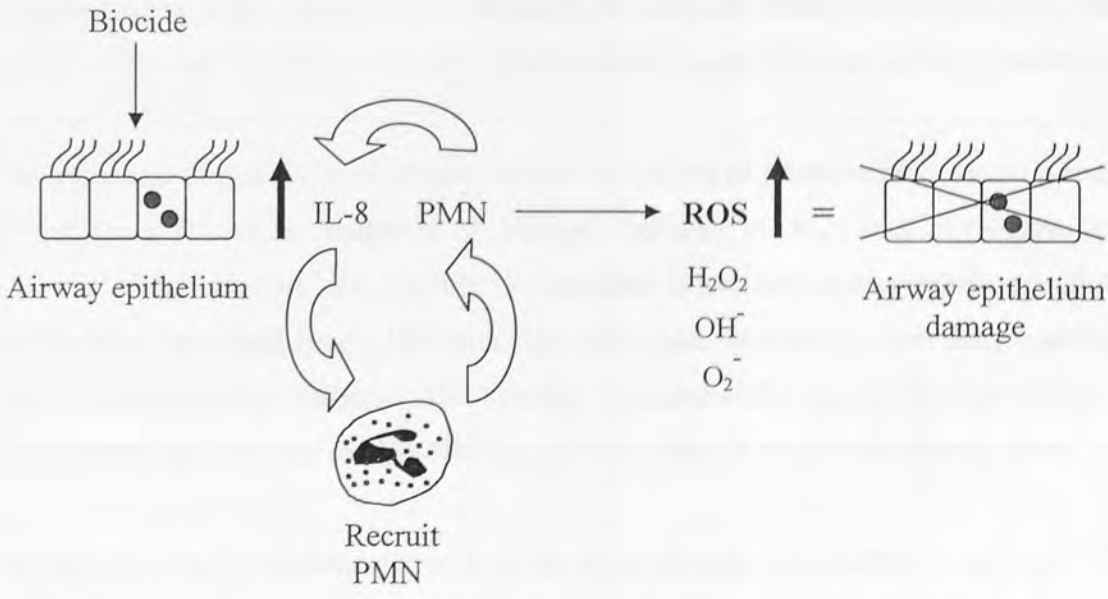


Figure 8.11 Cycle of inflammation and neutrophil recruitment to airway epithelium. Upon biocide exposure IL-8 secreted by the airway epithelial cells recruits polymorphonuclear leukocytes (PMN) to the epithelium. Recruitment of neutrophils may become amplified as IL-8 is also produced by neutrophils themselves. The neutrophils release ROS into the surrounding area causing damage to the airway epithelium.

The production of pro-inflammatory cytokines such as IL-8 by nasal epithelial cells may contribute to the pathologic effects observed in asthmatics (Adler *et al.*, 1994). Conversely, decreased IL-8 release may have an anti-inflammatory effect on the cells. The absence of IL-8 secretion towards the basolateral compartment could prevent the infiltration of neutrophils to the site of injury (Auger *et al.*, 2006). If IL-8 is released apically and at a lower level than that released basolaterally, the neutrophils in the vasculature will not be able to migrate down a chemotactic gradient to the site of injury. This could result in the down-regulation of the neutrophils in the airways and be anti-inflammatory preventing injury to the airway epithelium. To current knowledge, the kinetics of IL-8 secretion in response to H_2O_2 stress has before now not been studied in polarised, ALI-cultured HNEpC.

8.5.4 Thiol and carbonyl content of biocide challenged HNEpC

At 10% and 100% of their in-use concentration biocides reduced the thiol content and increased the carbonyl content of ALI cultured HNEpC, in a concentration-dependent manner. The biggest decrease in thiol levels in comparison to the control was observed on exposure of the HNEpC to 100% H_2O_2, followed by 100% Quat./chlorine dioxide. This ranking was also observed with increases in carbonyl content in comparison to the control. This was as expected as both chlorine dioxide and H_2O_2 are powerful oxidisers.

The oxidation of protein thiol groups and the formation of protein carbonyls as a result of oxidation following treatment of HNEpC cultured at ALI with H_2O_2 have not previously been studied. As a result, no literature is available that supports or refutes the findings described here. However, the thiol data obtained in this study correlate with and confirm the carbonyl data results. Consequently, this is the first study to demonstrate that biocides oxidise HNEpC proteins after 24 hours challenge *in vitro*.

As proteins play a fundamental role in the structure and functioning of all cells, the implications of biocide-induced protein oxidation can be widespread. Although protein thiol oxidation can be reversible, the formation of protein carbonyl derivatives is not. Consequently, the inactivation of proteins can have numerous downstream effects on many cellular processes. Modification of structural proteins may also have a significant

effect on cell viability as the structure of the cells aids in sustaining the integrity of the epithelium. The oxidation of protein thiol groups, which are powerful antioxidants often used in regulating cell processes through redox reactions, can also have a detrimental effect on cells by shifting the cellular redox balance. This would result in compromisation of the cell as free thiols protect against oxidative damage. However, if the free thiols themselves become oxidised as a result of biocide exposure, then they will be unable to protect the cells from further oxidation effects. In addition to further biocide exposure, such oxidation effects may include those arising from increased ROS presence. ROS will have accumulated in the epithelium as a consequence of increased IL-8 release from epithelial cells in response to biocides, as detected in the present study.

The differences in protein oxidation following biocide exposure were more detectable and significant with the ALI cultured HNEpC when challenged in comparison to submerged cultured cells (Chapter 6, sections 6.4.5 and 6.4.6). Overall, these results indicate that HNEpC cultured at ALI are being put under oxidative stress as a result of biocide exposure.

8.5.5 Propidium iodide staining of H_2O_2 challenged HNEpC

On treatment with H_2O_2 the ALI cultured HNEpC were of a typical necrotic morphology. A H_2O_2 concentration dependent increase in necrotic cells was detected. The highest proportion of necrotic cells was detected following exposure of the cells to 100% H_2O_2, with 34.57% of the cell population being necrotic. This was a 23-fold increase in relation to the quiescent medium exposed control cells. In addition to this, 30.97% of the cells exposed to the 10% H_2O_2 were necrotic. Even following challenge with 1% H_2O_2, 18.82% of the cells were found to be necrotic.

Little research has been undertaken on the mechanism of cell death upon exposure of well-differentiated HNEpC cultured at ALI to H_2O_2. However, Harkema *et al.* (2006) found that most chemical insults induce necrosis in nasal epithelium. Harkema *et al.* (1987) observed ciliated cell necrosis in the nasal respiratory epithelium of monkeys exposed to ozone (0.3 and 0.15 ppm, 8 hours a day, for 6 days). Formaldehyde

exposure in rodents (14.3 ppm for 6 hours) and monkeys (6 ppm, repeated 5 days per week, for 1-6 weeks) has also been found to result in necrosis of the nasal epithelium (Chang *et al.*, 1983; Monticello *et al.*, 1989). However, the level of necrosis was not quantified in these two studies. In the present study, the majority of cells were not necrotic following a single dose of H_2O_2. Overall the HNEpC exposed to the 100%, 10% and 1% H_2O_2 were found to have more necrotic cells than the control. As necrosis is a rapid mechanism of cell death, it would probably not allow closure of the basement membrane by neighbouring cells to occur (Tesfaigzi, 2006). This would render the epithelium susceptible to infection, resulting in the accumulation of inflammatory cells at the site. This hypothesis also correlates with the TER and BSA paracellular permeability data, which found that the integrity of the HNEpC epithelium was compromised immediately after exposure to H_2O_2. However, recovery of the epithelial barrier integrity was observed subsequent to biocide exposure and so supports the finding here that not all cells are necrotic.

This study suggests that exposure to biocides induces the synthesis and release of IL-8 from HNEpCs cultured at ALI. This study also demonstrates that the integrity of the human nasal epithelium as a barrier can be compromised immediately following H_2O_2 exposure. Although some recovery can occur on removal of the biocide, the recovery is not restored to control levels. In contrast, with regards to paracellular permeability the epithelium was found to remain largely intact when exposed to practical concentrations of H_2O_2. It was also established that protein oxidation and some necrosis of the nasal epithelial cells occurs following biocide treatment. Taken together, these findings suggest that *in vivo* exposure to H_2O_2 may result in alterations occurring in human nasal epithelial cells that could prove detrimental to the integrity of the nasal epithelium.

.

CHAPTER 9 GENERAL DISCUSSION

9.1 Summary and significance of findings

9.1.1 Sporicidal efficacy of biocides and relevance of findings with regards to aseptic processing

The results from this study are in general agreement with the current view of the efficacy of biocides against bacterial spores. The results presented here show that 70% IPA and 70% ethanol, biocides currently used in aseptic transfer disinfection, are not the most effective at killing *Bacillus* spores (McDonnell and Russell, 1999; Russell, 1990; Russell, 1995). The use of the alcohols (which are capable of killing vegetative organisms) could only be recommended in place of SDW to prevent the risk of microbial growth (e.g. *Pseudomonas*) occurring in water (Favero *et al.*, 1971). However, 6% H_2O_2, Quat./chlorine dioxide, Quat./biguanide and even SDW (as an inert wipe) were found to be better than either of the alcohols. 6% H_2O_2 was the most efficacious biocide against *Bacillus* spores, but only achieved an average 1 log reduction in viable spore numbers. A combination of spraying and wiping was the best method of disinfection, with the least effective being spraying alone. Wiping played a key role in the physical removal of spores from the contaminated surface. Spraying and wiping has previously been demonstrated to be an effective disinfection method (Cockroft *et al.*, 2001).

Although 3 log reductions were not achieved by any of the biocides tested, the total number of spores required to be eliminated should be taken into consideration. If relatively low numbers of spores are present, then the use of a sporicidal biocide which has a 1-2 log reduction efficacy is likely to be adequate for the majority of pharmaceutical grade cleanrooms. This is because the total bioburden, including spores, even for the lowest grade of cleanroom should be less than 200 per m^3 (Lambert, 2004). Therefore, even though a biocidal agent may not necessarily achieve the 3 log reductions required for it to be designated as a sporicidal agent, it will have some sporicidal capacity. As the majority of operators carry out aseptic transfer by spraying with IMS (70% v/v denatured ethanol) it can be concluded that the levels of disinfection

currently being achieved in cleanrooms can be increased by using alternative biocides. The results presented in this study support the use of 6% H_2O_2 or Quat./chlorine dioxide in place of alcohol for use against *Bacillus* spores when employing traditional disinfection. However, there are issues of concern regarding both of these agents. Although no residue of H_2O_2 is likely to persist due to its breakdown to water and oxygen, as is highlighted in this study toxicity issues surround its use as a biocide. There are concerns about residues of chlorine dioxide being left on surfaces and subsequently entering into components from packaging or trays, as well as toxicity issues. Chlorine sprays also tend to be corrosive and are capable of rusting stainless steel isolators. This problem may be overcome by employing a wiping with alcohol stage following initial disinfection with the Quat./chlorine dioxide formulated biocide. The use of an aqueous solution which is not volatile is also undesirable as it will remain on the surface of the transferred items for some considerable time. This may subsequently be transferred into isolators and could be a possible cause of contamination.

The findings in this study suggest that a wiping stage is essential to achieve high efficacy in the transfer process. This is largely due to the physical removal of spores, because alcohols and biguanides, which are not sporicidal, remove spores from carrier surfaces when applied as wipes. Regardless of which biocide was impregnated in a wipe, the number of log reductions achieved surpassed reductions observed in the absence of a wipe, i.e. on spraying alone. Consequently, it is recommended that a wiping stage be incorporated in hard-surface disinfection protocols during transfer of sterile products. These protocols should be followed with the greatest care and diligence as if not applied properly, the wipes can spread any contamination rather than eliminate it. The use of a wiping stage following a spraying stage is recommended because spores that may have been washed away on a surface on spraying can be picked up and removed on the use of a wipe.

The current standard method of using alcohol sprays should be replaced by a more efficacious method with regards to spore disinfection. The biocides and methods of biocide application recommended for disinfection of components associated with aseptic processing are; spraying with 6% H_2O_2 and wiping with a 6% H_2O_2 wipe when transferring items into pass-throughs or air-locks into a cleanroom. For the second

stage of transferring the items into the isolator hatch to transfer into the compounding area of the isolator, it is recommended that the items are sprayed and then wiped with IMS prior to any manipulation.

Although no disinfection process gave complete elimination of the spores, the VHP gassing achieved much better results against *Bacillus* spores in comparison to the traditional disinfection methods investigated. The results of this study support the use of VHP gassing for effective bacterial spore disinfection in agreement with other studies (Otter and French, 2009; Meszaros *et al.*, 2005; French *et al.*, 2004; Klapes and Vesley 1990; Block, 2001). In this study, following 30 minutes exposure to VHP > 2 log reduction in viable spore numbers was observed against all species tested. Despite the longer time taken to disinfect against the bacterial spores, the efficacy of the VHP gassing was superior to the conventional disinfection methods. However, this difference in efficacy was not due to the extended exposure time, as spraying for longer would probably not produce a 2 log reduction in spores, as determined by the BacLight™ assay carried out (Appendix 11.2.1). Even after 15 minutes exposure to liquid H_2O_2, the majority of the spores were still viable. Indeed, even though spraying for longer may produce higher log reductions, such contact times are not feasible in a busy pharmacy cleanroom environment, where each item is disinfected individually. By contrast, with VHP gassing items can be treated in bulk and so the time of disinfection in relation to individual items would be greatly reduced. VHP gassing should therefore be considered as an alternative disinfection method in aseptic transfer units as it would remove the need for cleanroom operators to be directly exposed to the biocides. Consequently, it is recommended that VHP be used to reduce, if not completely eliminate spore bioburden from pharmacy cleanrooms.

9.1.2 Testing biocide toxicity against airway epithelial cells

9.1.2.1 Submerged culture

In addition to their efficacy, biocides were also tested for their toxicity against airway epithelial cells by exposing submerged cultures of human bronchial and nasal epithelial cells to the biocides. The cell culture models used were representative of the airway epithelium as both bronchial and nasal epithelium would be impacted by the inhalation

of volatile biocides. It was determined that biocides decreased the viability of both submerged cultured human bronchial and nasal epithelial cells after 24 hours exposure. This loss in cell viability was occurring largely via necrosis, with higher levels of necrotic cells observed in the bronchial epithelial cells in comparison to the nasal epithelial cells. However, the nasal cells were more susceptible to the alcohol biocides at 1% concentrations, as a higher reduction in viable cells was observed in comparison to the bronchial cells. Biocides also increased oxidation of cell proteins, observed as a decrease in protein thiols and an increase in protein carbonyls. It is interesting to note that more free thiols were present in the submerged cultured nasal cells than the bronchial cells. This indicates that nasal epithelial cells are more protected with regards to oxidants, because free thiols play an important role in the defence against oxidative stress. Although levels of free protein thiols have yet to be established in nasal epithelial cells, the difference observed may probably be due to the greater protection required by this tissue. Nasal cells are situated more proximally, with regards to toxicant inhalation, than bronchial cells which are further down the respiratory tract. Therefore the bronchial cells may be less likely to be exposed to the high concentrations of inhaled toxicants that the nasal cells would encounter. It is tempting to speculate that the nasal cells require higher free thiol levels to counter the damaging effects of inhaled oxidants. Alveolar epithelial cell free protein thiols have been measured as less than observed in the bronchial and nasal cells of this study (Smit de-Vries *et al.*, 2007), supporting this hypothesis.

9.1.2.2 ALI culture

Biocides decreased the viability of both human bronchial and nasal epithelial cells following culture at ALI. This loss in cell viability was occurring via necrosis. Higher levels of necrotic cells were observed in the nasal cells in comparison to the bronchial cells. Biocides increased oxidation of cell proteins, observed as a decrease in protein thiols and an increase in protein carbonyls. So the biocides tested are a source of oxidative stress against both bronchial and nasal epithelial cells. However, nasal cells cultured at ALI were established to be more susceptible to the effects of biocide exposure than the bronchial cells, with epithelial barrier impairment and oxidation of proteins being greater in nasal than bronchial cells. The differences in protein oxidation following biocide exposure were more detectable and significant when ALI cultured

cells were challenged in comparison to submerged cultured cells. This is the first time to the authors' knowledge that these measurements have been undertaken on cells cultured at ALI. The decreased free thiol and directly corresponding increased carbonyl levels observed may be due to the differentiation of the ALI cultured cells. It is hypothesised that the cells are more susceptible to, and have the same range of sensitivity with regards to oxidation of protein thiols and other proteins which form carbonyl derivatives, when exposed to the biocides at ALI. Such that oxidation of thiols (in cysteines) may result in corresponding oxidation of proteins such as histidine, lysine, arginine, proline, and threonine, which result in the formation of protein carbonyls. The oxidation of free thiols can result in the production of incorrectly folded proteins. In addition, the oxidation mediated formation of carbonyl derivatives may result in irreversible damage to cell proteins. This collective damage to cell proteins may result in the alteration of cell processes, compromisation of host defence and the epithelium as a barrier, and ultimately cell death.

Biocides also elicited a pro-inflammatory response from human bronchial and nasal epithelial cells under both submerged and ALI culture as measured by IL-8 release. In biocide-exposed ALI cultured cells the release of IL-8 was generally basolaterally directed, indicating that exposure of the cells to the biocides is able to influence the chemotactic recruitment of leukocytes to the airway epithelium. Although the biocides tested are likely to exert pro-inflammatory effects in the bronchial and nasal epithelial cells, this effect is somewhat reduced following recovery of the cells. So, even if operators were exposed to the biocides for a short while, it is likely that their airways, given the opportunity, would recover from any potentially inflammatory or harmful effects. However, if operators were exposed to the biocides over the course of a day, every day for a 5-day working week then the potential damage to the airway epithelium is greater as recovery would not occur. In addition, day-to-day exposure may possibly result in increased concentrations in the airways resulting in chronic inflammation, which could then result in airway injury. Also, any initial damage caused by the biocides may become exacerbated if operators also live in a polluted area, or use household biocide cleaning agents such as those present in furniture polish and cleaning sprays, which have been determined by Zock *et al.* (2007) to be a risk factor in adult asthma. Although some recovery of airway epithelial integrity is probable with acute

exposures, chronic exposures would be much more damaging and so it is unlikely that complete recovery of the airways would occur upon chronic exposure.

This study also demonstrated that a practical working concentration of H_2O_2 instantly disrupts both human bronchial and nasal epithelial cell barrier function *in vitro*. Although some recovery can occur, if the exposure is continuous it is likely that the disruption may allow microbial pathogens which upon inhalation reach the apical surface of the cells to move to the basolateral surface, thereby promoting infections in the host. In addition, disruption would allow other inhalants such as ozone and cigarette smoke, which have oxidative capacities, to access and oxidise underlying proteins which play many roles in the functioning of the cells. This may result in underlying endothelial cells losing some of their structural and/or functional integrity, which would have a subsequent impact on human health. For example, asthma and bronchitis sufferers have increased airway epithelial permeability, which is associated with bacterial infection and inflammation (Godfrey, 1997; Godfrey, 1993; Devalia *et al.*, 1994, Walker *et al.*, 1984).

With regards to assessing the health of the ALI cultured bronchial and nasal epithelial cells, the most useful and reliable of the methods employed was the thiol assay. This is because the thiol assay results generally corresponded with the carbonyl assay results. In addition, the free thiol levels also corresponded directly to the results of the propidium iodide staining of the cells and were indicative of the necrotic status of the cells.

Some differences were observed between the results obtained with submerged cultured cells in comparison to those that were cultured by ALI. As ALI cultured cells constitute a differentiated phenotype, the results obtained using this method of cell culture are most probably more reflective of *in vivo* effects than the less physiological submerged culture results. ALI models are also more representative of how the cells would be challenged with a volatile inhalant such as H_2O_2 and also allow directed release of IL-8 to be detected. This directed release can be crucial in understanding the effects that IL-8 release is likely to have on the epithelium in response to biocide exposure.

9.1.3 Relevance of findings with regards to operative exposure

The consequences of exposure to occupational hazards such as the development of mesothelioma as a result of asbestos inhalation or asthma in cleaning operatives have been reported (Mohr *et al.*, 2005; Medina-Ramon *et al.*, 2005; Jaakola and Jaakola, 2006). As on exposure to the biocides used in the present study, exposure to asbestos has been demonstrated to catalyse the generation of ROS (Shukla *et al.*,2003) causing protein oxidation, as well as having a direct effect on lung epithelial barrier integrity (increased mannitol permeability) (Peterson *et al.*, 1993). Medina-Ramon *et al.* (2005) measured the frequency of cleaning and types of cleaning agents used (especially bleach) by women cleaners, establishing that there was a correlation between cleaning and an increased risk of asthma. In some similarity to Medina-Ramon *et al.* (2005) the present study also indicates that the use of the biocides tested may result in an increased risk of developing asthma-like symptoms due to the likely impairment of pulmonary function. However, safety issues of biocide exposure for pharmacy cleanroom operatives have not previously been explored. Taking into account the findings of this thesis, it can be concluded that biocides are cytotoxic to human airway epithelial cells *in vitro*, with reductions in cell viability being observed in cells when they are exposed to as little as 1% of their working concentrations. Changes in cell viability in response to biocides were also dependent on the cell type, with nasal epithelial cells appearing more vulnerable.

The human nasal and bronchial airway epithelial cells are also susceptible to the oxidative stress generated by the biocides. The modification of key cellular structural proteins due to protein oxidation can be detrimental to the cell, because cell structure is essential in maintaining the integrity of the epithelium as a whole. As the epithelial integrity of the cells is also disturbed, any inhaled biocide may possibly penetrate further into the endothelium and even into the bloodstream where it may be distributed throughout the body. In addition, epithelial barrier disruption will make operatives susceptible to microbial infection, as inhaled pathogens would be able to penetrate the epithelium. Increased oxidation may also result in the impairment of the mucociliary clearance mechanism due to increased mucus and reduced ciliary beating, which may result in the exacerbation of any subsequent infections in the operator. ROS can also exacerbate inflammation of a tissue as the epithelial cells produce chemokines such as

IL-8, which can recruit inflammatory cells to the region which produce yet more ROS. As biocides caused necrosis of airway epithelial cells *in vitro*, the release of ROS from the necrotised airway epithelial cells may cause further oxidation of proteins in neighbouring cells. This ROS production cycle can be especially damaging to the region by further compounding airway injury, inflammation and/or cell death (Wright *et al.*, 1997). Although the inflammatory response is key in host defence, such excessive or persistent inflammation could contribute to the pathogenesis of disease. Moreover, human airway epithelial cells from patients with airway disorders and diseases such as asthma and cystic fibrosis have shown increased levels of cytokines, including IL-8 (Lukacs and Tekkanat, 2000; Conese *et al.*, 2003; Douglass *et al.*, 1994; Bonfield *et al.*, 2000), as well as a massive influx of neutrophils into the airways which may play a role in the maintenance of disease pathophysiology. A possible increase in the amount or viscosity of mucus being secreted into the airways due to changes in epithelial transport to ions (and as a consequence water), might also obstruct the smaller airways and hinder gas exchange.

Limited information is available on the effects of inhalation exposures of H_2O_2 by humans or animals. However, in general, acute exposure studies have shown that airborne H_2O_2 can be irritating to the respiratory tract with symptoms including throat irritation, nasal irritation and discharge, oedema in the lungs and necrosis of bronchial tissue (ECETOC, 1992; Oberst *et al.*, 1954; Punte *et al.*, 1953). Chronic exposure studies involving rats exposed to 67 ppm of an aerosol/vapour mixture generated from 90% H_2O_2, over 7 weeks comprising 30 exposures established that nasal irritation and nasal discharge occurred after 2 weeks. Upon necropsy, the lungs of all the exposed rats were found to be congested. In addition, exposure of 10 rats to 33 mg/m^3 H_2O_2 for 6 hours per day, 5 days per week, for a period of 28 days resulted in respiratory tract irritation. Clinical signs included concentration related necrosis and inflammation of the epithelium of anterior regions of the nasal cavity, and perivascular neutrophil infiltration in the lungs (SCCP, 2007). These findings somewhat correlate with the present study which indicates that both necrosis of airway epithelial cells and inflammation of the airway are likely to occur upon biocide exposure. In addition, the lethal concentrations causing death in 50% of the subjects (LC_{50} values) have been established for both the alcohols and the H_2O_2. In rats, the LC_{50} for ethanol and IPA ranges between 16,000 to 20,000 ppm following 8-10 hours of exposure, whereas, in

rats the LC_{50} of H_2O_2 is <2000 ppm after 4 hours exposure. This may also be reflected upon human exposure, as in the present study the H_2O_2 was more toxic than the alcohol biocides tested against the human bronchial and nasal epithelial cells.

Together, all these factors suggest that the biocides investigated in this study are not toxicologically safe, either at the molecular or cellular level in relation to possible toxic effects in human airway epithelial cells. Exposure to biocides may result in compromising the human airway epithelium *in vivo* and consequently contribute to the pathogenesis of airway disease in airway epithelial cells. Consequently, cleanroom operators should be cautious, as under physiological conditions (24 hours exposures) even after recovery (48 hours) the airway epithelium remains compromised.

Very few studies have been carried out regarding the inhalation of biocides by humans, even though such data is essential for assessment of occupational exposure risk. Only recently more interest has been shown due to legislation such as REACH. The findings to this study are from biocide concentrations greater than usually tested in most studies. However, the concentrations investigated are representative of the concentrations used in practice, as it was important to expose cells to biocides *in vitro* correlating with the exposure expected *in vivo*. An average human inhales approximately 12,000 litres of air a day. Taking in 500 ml of air with every breath, an average 6 litres of air is inhaled per minute (Silverthorn, 2001). Yet it is not known what concentrations of the biocides are actually inhaled and reach the bronchial and nasal epithelium *in vivo*. Numerous critical factors have been established to play a role in biocide inhalation. These include the absorption, metabolism, and elimination of biocides following their inhalation.

Firstly, there are variations between personnel, in terms of airway dimensions such as nasal and lung volumes and surface areas, with differences also existing between males and females. For example, the average pulmonary volume for females is 4200 ml and for males is 5800 ml (Silverthorn, 2001). Consequently the average male will have a larger surface area which can come into contact with the inhaled biocide. Differences will also exist between people who breathe primarily through the nose and those who breathe through their mouth. Airflow patterns also play a role. Due to the turbinates of the human nasal cavity, airflow over the nasal regions is streamlined resulting in inefficient delivery of the chemical to the mucosal surface (Bogdanffy and Sarangapani,

2003). However, the branching pattern in humans is symmetric creating an airflow pattern that results in increased chemical deposition at bronchial branch points (Bogdanffy and Sarangapani, 2003). So the deposition (impact of chemical on mucus or tissue surfaces) and uptake (absorption of chemicals into tissues) of inhaled vapours may also play a key role in any toxicity effects. Inhaled materials which are not metabolised and are sufficiently water soluble are expected to be extracted and absorbed in the upper airways (Bogdanffy and Sarangapani, 2003). As mucus is approximately 90% to 95% water, it can act as a sink for vapours (Medinsky and Bond, 2001). After vapours are dissolved in the mucus, the vapour molecules can diffuse into the underlying epithelial cells of the respiratory tract.

Organic solvents such as the alcohols are expected to be readily absorbed by inhalation as they are highly soluble in water (Fiserova-Bergerova, 1985; Jones, 1983) and can rapidly equilibrate between airway fluids and respiratory air. A recent study using levels of ethanol exposure (25–1000 ppm) representative of current occupational exposure levels, reported that between 70 to 80% of inhaled ethanol was absorbed (Tardif *et al.,* 2004). Previously, during prolonged inspiration of ethanol vapour containing air, about 55% was found to be absorbed by human adults (Kruhøffer, 1983). So a large proportion of inspired ethanol is deposited in the airway lining. Zhang *et al.,* (2006) also found that ethanol vapours deposited substantially in the upper airways, with increased doses observed in this region. This suggests that ethanol vapour depositions can become concentrated following repeated exposure. This is likely because there is always a residual volume of air (approximately 1200 ml) remaining in the lungs (Silverthorn, 2001; Fox, 1993). Overall, inhaling even small levels of biocide may be detrimental to the cells as this may result in a possible build up of the biocide concentration in the lungs following inhalation. One potential point for the concentration of biocide vapour within the respiratory airway in addition to mucus may be the surfactant film at the ALI. The volume of ASL in the lungs with a surface area of 150 m^2 and an ASL depth of at least 5 microns (Widdicombe and Widdicombe, 1995) is estimated as 750 ml. The volume of ASL in the nasal cavity with a surface area of 18.1 m^2 (Montgomery, 1979) and an ASL depth of at least 5 microns is estimated as being 91 ml. So a potentially low-level repeated exposure could result in a higher concentration being achieved in the respiratory airways if any inhaled biocide becomes concentrated in the ASL.

In contrast other studies have shown that water soluble vapours such as ethanol are nearly completely scrubbed by the nose on inspiration (Dahl *et al.,* 1991; Gerde and Dahl, 1991). One point to consider is that during exhalation, vapours are capable of desorbing from the respiratory tract mucosa into the lumen of the airway and are exhaled (Snipes *et al.*, 1991). This process observed with water-soluble chemicals is known as the "wash-in-wash-out effect" (Johanson, 1991). On exhalation, up to 30% of the inhaled vapour was found to desorb and exhaled out (Dahl *et al.,* 1991; Gerde and Dahl, 1991). Whilst this effect restricts the overall dose of the biocide to the respiratory tract, it also highlights the protective effect that the nose has in dealing with toxicants. Aside from ethanol inhalation, no data exists in relation to the inhalation levels of the other 5 biocides investigated in this study, although several human studies have examined the toxicity of inhaled chlorine dioxide (Gloemme and Lundgren, 1957; Exner-Freisfeld *et al.*, 1986; Meggs *et al.*, 1996). However these studies are limited by the fact that actual exposure levels of chlorine dioxide were not known. It is expected that like the ethanol, the IPA would achieve similar inhaled concentrations in the airways. All other biocides investigated in this study are also soluble in water and so it is hypothesised that high levels of the biocides would be absorbed upon their inhalation. However, it is unlikely that the concentrations of biocide investigated against the airway epithelial cells here would be inhaled by personnel. Yet, any inhalation is likely to be potentially injurious to cells as was observed in this *in vitro* study. Consequently, the potential human exposure to vapours from biocides needs to be further researched.

In this study only the upper airways of the respiratory tract were investigated as modelling studies have proposed that inhaled vapour from the atmosphere, which has high water solubility, should not reach the alveoli of lower lungs (Wexler and Sarangapani, 1998). Other potential sites that may come into direct contact with the biocides are the skin, eyes and mouth.

9.2 Future work

It would have been useful to see exactly which proteins in the cells were being oxidised using proteomics studies so that the mechanism of any possible oxidative damage could be hypothesised. This would also show if the oxidative changes were occurring in key cell proteins and so could have a potential subsidiary effect throughout the cell. This would also demonstrate as hypothesised, whether IL-8 released by the bronchial and nasal cells was not detectable in higher amounts because it was being oxidised upon its release.

Only soluble levels of IL-8 were investigated on exposure of the epithelial cells to the biocides. Investigating the expression as well as secretion of IL-8 could be used to see whether the IL-8 is being produced in higher quantities but is being broken down before or straight after it is released from the cells. This would determine whether the IL-8 release in response to biocide exposure observed in this study is actually higher, and therefore eliciting a potentially more pro-inflammatory response from the cells. In addition, determining the presence or upregulation of antioxidants in the cells post-biocide exposure could show any response that the cells are producing against the biocides. This is important as antioxidants have been found to inhibit NF-κB activation in cells (DeForge *et al.*, 1993), which could have a direct effect on IL-8 production.

Chemotaxis assays could be carried out to detect if the secreted IL-8 is biologically active. If increased neutrophil chemotaxis is observed on exposure of the cells to the biocides, this would support the finding that the IL-8 levels are increased following exposure of the cells to the biocides. Controls using neutralising, blocking antibodies would establish whether the IL-8 was responsible for the neutrophil chemotaxis.

An exposure time of 24 hours was used to allow for protein transcription and translation to occur. However, this may be too late to detect significant IL-8 release as the majority of cells were no longer viable at this point and therefore no longer released IL-8. Whereas at shorter time-points of exposures, cells do not die immediately and so increased IL-8 mRNA or transcription factors could be investigated. In doing so, this could also help to establish whether any IL-8 is released gradually or acutely. Also,

carrying out repeated exposure of the cells to the biocides would see if the cells inflammatory response is the same following chronic exposure as with acute exposure.

Another point to consider was whether the effects observed were due to cell number/density rather than the biocide application having an effect. This could be established by removing the cells from the Transwell membranes by trypsin treatment and counting the cell numbers allowing IL-8 per cell to be calculated. IL-8 has been found to be presented in the sputum of patients with inflammatory airway diseases, and accounts in large part for the chemo-attractant activity present (Inoue, 1999). Ideally, determining any levels in the sputum of cleanroom personnel may give an additional indication as to the inflammatory phenotype of their airways.

Other inflammatory factors could also be investigated via ELISAs to ascertain if similar levels of other pro-inflammatory mediators such as IL-1 and TNFα, which are responsible for the upregulation and release of cytokines (Takizawa, 1998; Adler *et al.,* 1994) are produced and secreted by the cells on treatment with the biocides. In addition to pro-inflammatory mediators, anti-inflammatory molecules could also be assayed for. The impairment of mucociliary clearance which is a protective innate immune response of the airway may also occur on exposure to the biocides. The measurement of ciliary beat frequency and ultrastructural analyses may aid in determining whether mucociliary clearance is compromised. In addition, as ciliary movement, in turn depends on mucus secretion, enhanced expression and production of MUC5AC, which is a major goblet cell mucin found in airway secretions that confers viscoelasticity on mucus (Rogers, 2003) could also be investigated. Determining any levels in the sputum of cleanroom personnel may also provide an indication of possible detrimental effects occurring on mucocilliary clearance.

In addition, the potential metabolism of the biocides by the cells also needs to be taken into consideration in relation to the actual levels of exposure. This is due to the strong antioxidant defence capacity of airway epithelial cells (Boardman *et al.,* 2004). Consequently determining the level of antioxidants produced by the cells in response to biocide exposure may provide an indication of the actual biocide exposure level, rather than the biocide concentration initially added to the cells.

Investigating both early and late markers of apoptosis would also provide a better understanding of the mechanism by which cell death occurs in the cells. Further assays such as determining the presence of activated caspases, Bax levels, Bax activation, DNA fragmentation/laddering (terminal deoxynucleotidyl transferase - TUNEL assay) and cytochrome c release from mitochondria would aid in determining the actual effect that the H_2O_2 has on the cells concerning apoptosis. Examining cell morphology, as well as nuclear morphology would also aid in determining whether following biocide exposure the cells are of a typical necrotic or apoptotic morphology. This could be carried out on the cells once they have been purified and sorted by fluorescence activated cell sorting (FACS). Cell death via autophagy is another cell death process, whereby intact organelles and/or large fractions of the cell cytoplasm are engulfed within autophagic vacuoles for degradation via lysosomes (Galluzzi *et al.*, 2008). Recently, cigarette smoke extract (of which H_2O_2 is a constituent) was demonstrated to induce morphological and biochemical markers of autophagy in BEAS-2B cells accompanied with autophagosome formation (Kim *et al.*, 2008). It is thought that the build up of such autophagic vacuoles may signify this alternative cell death pathway. However, autophagy can also lead to apoptosis or necrosis with some cells even being found to switch between autophagic and apoptotic features of cell death (Fazi *et al.*, 2008; Galluzzi *et al.*, 2008). Consequently, it would be beneficial to carry out Western blots on the cell lysates to detect any autophagic proteins in comparison to controls. In addition, it would also have been useful to see whether the cells were proliferating after the recovery period. This could have been determined with the use of 5-bromo-2'-deoxyuridine (BrdU), which is a marker of epithelial cell proliferation (Smit-de Vries *et al.*, 2007). This could establish whether the cells that remained viable following biocide exposure and found to be unchanged in their appearance (remaining in region 1 of the scatter cytograms) were indeed capable of proliferating, and were as a result truly viable.

The method of biocide delivery was the only major difference in this study with regards to how the biocides would be encountered in practice. Yet, applying the biocides as a fluid was not a major factor in determining the effects of the biocides on the epithelial cells as quiescent medium exposed cells were not compromised in any manner. The inhalation of biocides as vapours may be more destructive due to the volatility and consequent facilitation of the biocides into the respiratory cells. So rather than

submerging the cells in liquid biocides, challenging the cells with the biocides in an aerosol format would be more reflective of biocide delivery methods used in cleanrooms.

The results of repeat doses representing chronic exposures that truly reflect the expected exposure of pharmaceutical personnel to biocides would be valuable in gauging any long-term effects that the biocides may be having on human airway epithelial cells. The use of co-culture models would further aid in understanding the effects of the biocides against airway epithelial cells as they would represent a more physiologically accurate model of the airway as it would exist *in vivo*. Co-culture cell systems are more refined as they have at least two different cell types representative of the *in vivo* cellular architecture. In doing so the system would correspond better to both the structural and functional characteristics of the airway epithelium. Consequently, the system would be of greater biological relevance than culture systems that use one cell type alone.

Although spraying and wiping was observed as the most effective method of spore disinfection, inhalation of biocides would result in chronic exposure, and could cause damage to the airways. Therefore, it would also be ideal to see the effects of 30% VHP on the ALI cultured primary nasal epithelial cells to ascertain the possible harmful effects that the gassing would have, if a person was accidently exposed to the VHP. The use of ALI cultured primary HNEpCs would be recommended as in this study they were the most representative and most biocide susceptible cell model used to mimic the *in vivo* epithelial cells of human operators. Also, since the upper airway is one of the first lines of defence against inhaled toxicants including pathogens, biocide-induced compromises in its defensive capacity could result in increased nasal infections and increased susceptibility to lower respiratory tract diseases.

9.3 Concluding remarks

Validation and standardisation of biocides and disinfection processes is of immense importance due to the lack of end-product testing associated with the aseptic transfer process. Aseptic manufacturing involving the transfer of items into an isolator and the disinfection of surfaces is thus essential for reducing the risk of final product contamination. This study aimed to evaluate currently available cleanroom biocides using the different methods of biocide application. This would enable the effectiveness of each biocide and transfer disinfection method to be established against representative bacterial spores. In addition, the potential health risks associated with biocide exposure were investigated.

Although biocides are efficacious against *Bacillus* spores, they display unwanted toxic, oxidative and pro-inflammatory effects in both human bronchial and nasal epithelial cells *in vitro*. Thus, the biocides currently available and used in aseptic transfer processes in pharmacy cleanrooms are likely to have unwanted effects on the airways of the cleanroom personnel. In addition, the *in vitro* damage may be time-dependent, whereby repeated use results in a higher level of exposure and consequently an increased concentration, which may result in increased cell toxicity.

As a result VHP gassing should be considered as a viable alternative to the traditional and commonly used disinfection methods of spraying and wiping. This would ensure that cleanroom personnel are not at risk from inhalation of the biocides, whilst ensuring the effective killing of bacterial spores. VHP gassing combines both excellent efficacy against bacterial spores with reduced risk and increased safety to the cleanroom workers. This would improve disinfection against bacterial spores and in doing so reduce the possibility of products such as the parenterals becoming contaminated with such spores. This is unlike with the use of liquid H_2O_2, which in addition to not being as effective in spore removal may via inhalation infer a cytotoxic, oxidative and pro-inflammatory effect in the nose and lungs of personnel.

Moreover, these findings may be extrapolated not just to pharmaceutical cleanrooms, but to all healthcare and research environment settings where biocides are regularly used to disinfect surfaces.

REFERENCES

Abe, K. and Matsuki, N. Measurement of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction activity and lactate dehydrogenase release using MTT. *Neurosci Res. 38(4):* 325–329, (2000).

Adler, K. B., Fischer, B. M., Wright, D. T., Cohn, L. A., and Becker, S. Interactions between respiratory epithelial cells and cytokines: relationships to lung inflammation. *Ann N Y Acad Sci.. 725:* 128-45, (1994).

Agu, R. U., Jorissen, M., Willems, T., Augustijns, P., Kinget, R., and Verbeke, N. In-vitro nasal drug delivery studies: comparison of derivatised, fibrillar and polymerised collagen matrix-based human nasal primary culture systems for nasal drug delivery studies. *J Pharm Pharmacol. 53(11):* 1447-56, (2001).

Ahlers, J., Stock, F., and Werschkun, B. Integrated testing and intelligent assessment-new challenges under REACH. *Environ Sci Pollut Res Int. 15(7):* 565-72, (2008).

Aikawa, T., Shimura, S., Sasaki, H., Ebina, M., and Takishima, T. Marked goblet cell hyperplasia with mucus accumulation in the airways of patients who died of severe acute asthma attack. *Chest 101(4):* 916–921, (1992).

Alamdari, D. H., Kostidou, E., Paletas, K., Sarigianni, M., Konstas, A. G., Karapiperidou, A., and Koliakos, G. High sensitivity enzyme-linked immunosorbent assay (ELISA) method for measuring protein carbonyl in samples with low amounts of protein. *Free Radic Biol Med. 39(10):* 1362–1367, (2005).

Ali, Y., Dolan, M. J., Fendler, E. J., and Larson, E. L. Alcohols. In Disinfection, sterilization, and preservation, edited by Block, S.S. p.229–254. (5th ed.), Lippincott Williams and Wilkins: Philadelphia 2001.

Almagor, M., Kahane, I., Wiesel, J. M., and Yatziv, S. Human ciliated epithelial cells from nasal polyps as an experimental model for Mycoplasma pneumoniae infection. *Infect Immun. 48(2):* 552-5, (1985).

Andersen, B. M., Rasch, M., Hochlin, K., Jensen, F.-H., Wismar, P., and Fredriksen, J.-E. Decontamination of rooms, medical equipment and ambulances using an aerosol of hydrogen peroxide disinfectant. *J Hosp Infect. 62(2):* 149-155, (2006).

Anderson, J. M. Molecular structure of tight junctions and their role in epithelial transport. *News Physiol Sci. 16:*126-30, (2001).

Anderson, J. M. and Van Itallie, C. M. Tight junctions and the molecular basis for regulation of paracellular permeability. *Am J Physiol. 269(4 Pt 1):* G467-475, (1995).

Ando, Y., and Tsuzuki, T. The effect of hydrogen peroxide on spores of Clostridium perfringens. *Lett. Appl. Microbiol. 2*: 65-68, (1986).

Anon, (2008). What is REACH? Accessed 12-12-08. (World Wide Web page) http://ec.europa.eu/environment/chemicals/reach/reach_intro.htm

Anon, A. (2008). EVOM – Epithelial Voltohmmeter. Accessed 11-11-08. (World Wide Web page) http://www.pharmaceutical-int.com/images/companies/1529/wpi17.jpg

Anon, (2009). Basic respiratory anatomy. Accessed 21-02-09. (World Wide Web page) www.tracheostomy.com/images/resp.gif

Artursson, P., Karlsson, J., Ocklind, G., and Schipper, N. (1996). Studying transport processes in absorptive epithelia. *In Epithelial Cell Culture – A Practical Approach,* edited by Shaw, A. J. p. 111-134. Oxford University Press: Oxford 1996.

Aufderheide, M. Direct exposure methods for testing native atmospheres. *Exp Toxicol Pathol. 57(S1):* 213-26, (2005).

Aufderheide, M., Knebel, J. W., and Ritter, D. An improved in vitro model for testing the pulmonary toxicity of complex mixtures such as cigarette smoke. *Exp. Toxicol. Pathol. 55(1):* 51–57, (2003).

Auger, F., Gendron, M. C., Chamot, C., Marano, F., and Dazy, A. C. Responses of well-differentiated nasal epithelial cells exposed to particles: role of the epithelium in airway inflammation. *Toxicol Appl Pharmacol. 215(3):* 285-94, (2006).

Ayliffe, G. A. J., Coates, D., and Hoffman, P.N. (1993). Chemical Disinfection In Hospitals. Public Health Laboratory Service. Blackmore Press: Shaftesbury.

Baird, R. M. (1981). Cleaning and disinfection of the hospital pharmacy. In *Disinfectants: their use and evaluation of effectiveness*, edited by Collins, C. H., Allwood M. C, Bloomfield, S. F. and Fox, A. p.149-157. Academic Press: London 1981.

Baird, R. M., and Shooter, R. A. Pseudomonas aeruginosa infections associated with use of contaminated medicaments. *Br Med J. 2(6031):* 349, (1976).

Bakand, S., Winder, C., Khalila, C., and Hayes, A. Toxicity assessment of industrial chemicals and airborne contaminants: transition from in vivo to in vitro test methods: a review. *Inha. Toxicol. 17(13):* 775-87, (2004).

Barnes, P. J. Reactive oxygen species and airway inflammation. *Free Radic Biol Med. 9(3):* 235-43, (1990).

Bayram, H., Devalia, J. L., Sapsford, R. J., Ohtoshi, T., Miyabara, Y., Sagai, M., and Davies, R. J. The effect of diesel exhaust particles on cell function and release of inflammatory mediators from human bronchial epithelial cells *in vitro. Am. J. Respir. Cell Mol. Biol. 18:* 441–448, (1998).

Beaney, A.M. Monitoring. In Quality Assurance of Aseptic Preparation Services, edited by Beaney, A. M. p.47–52. Pharmaceutical Press: London 2006.

Becker, S., Koren, H., and Henke, D. Interleukin-8 expression in normal nasal epithelium and its modulation by infection with respiratory syncytial virus and cytokines tumor necrosis factor, interleukin-1, and interleukin-6. *Am J Respir Cell Mol Biol.* 8: 20-27, (1993).

Bédard, M., McClure, C. D., Schiller, N. L., Francoeur, C., Cantin, A., and Denis, M. Release of interleukin-8, interleukin-6, and colony-stimulating factors by upper airway epithelial cells: implications for cystic fibrosis. *Am J Respir Cell Mol Biol. 9(4):* 455-62, (1993).

Bernacki, S. H., Nelson, A. L., Abdullah, L., Sheehan, J. K., Harris, A., Davis, C. W., Randell, S. H. Mucin gene expression during differentiation of human airway epithelia in vitro. Muc4 and muc5b are strongly induced. *Am J Respir Cell Mol Biol. 20(4):* 595-604, (1999).

Bernard, A. Chlorination products: emerging links with allergic diseases. Curr *Med Chem. 14(16):* 1771-82, (2007).

Biagioli, M. C., Kaul, P., Singh, I., and Turner, R. B. The role of oxidative stress in rhinovirus induced elaboration of IL-8 by respiratory epithelial cells. *Free Radic Biol Med. 26(3/4):* 454–462, (1999).

Blanc, P. D. and Toren, K. How much asthma can be attributed to occupational factors? *Am J Med. 107*: 580–587, (1999).

Block, S. S. Peroxygen compounds. In Disinfection, sterilization, and preservation, edited by Block, S.S. p.185-204. (5th ed.), Lippincott Williams and Wilkins: Philadelphia 2001.

Blomberg, A., Sainsbury, C., Rudell, B., Frew, A., Holgate, S., Sandstrom, T., and Kelly, F. J. Nasal cavity lining fluid ascorbic acid concentrations increases in healthy human volunteers following short term exposure to diesel exhaust. *Free Radical Res. 28:* 59–67, (1998).

Boardman, K. C., Aryal, A. M., Miller, W. M., and Waters, C. M. Actin re-distribution in response to hydrogen peroxide in airway epithelial cells. *J Cell Physiol. 199(1):* 57-66, (2004).

Bogdanffy, M. S. and Sarangapani, R. Physiologically-based kinetic modeling of vapours toxic to the respiratory tract. *Toxicol Lett. 138(1-2):* 103-17, (2003).

Boland, S., Baeza-Squiban, A., Fournier, T., Houcine, O., Gendron, M. C., Chévrier, M., Jouvenot, G., Coste, A., Aubier, M., and Marano, F. Diesel exhaust particles are taken up by human airway epithelial cells in vitro and alter cytokine production. *Am J Physiol. 276(4 Pt 1):* L604-13, (1999).

Bonfield, T. L., Konstan, M. W., and Berger, M. Altered respiratory epithelial cell cytokine production in cystic fibrosis. *J. Allergy Clin. Immunol. 104(1):* 72–78, (1999).

Bonvallot, V., Baeza-Squiban, A., Baulig, A., Brulant, S., Boland, S., Muzeau, F., Barouki, R., and Marano, F. Organic compounds from Diesel exhaust particles are mainly responsible for the proinflammatory response induced in human airway epithelial cells and induce cytochrome P450 1A1 expression. *Am. J. Respir. Cell Mol. Biol. 25:* 1–7, (2001).

Boom, F. A., Van der Heijden Van Beek, M. A., Paalman, A. C., and Stout-Zonneveld, A. Microbiological aspects of heat sterilization of drugs. III. Heat resistance of spore-forming bacteria, isolated from large-volume parenterals. *Pharm Weekbl Sci. 13(3):* 130-136, (1991).

Borchard, G., Cassara, M. L., Roemele, P. E., Florea, B. I., and Junginger, H. E. Transport and local metabolism of budesonide and fluticasone propionate in a human bronchial epithelial cell line (Calu-3). *J Pharm Sci., 91(6):* 1561–1567, (2002).

Boucher, R. C., Stutts, M. J., Knowles, M. R., Cantley, L., and Gatzy, J. T. Na+ transport in cystic fibrosis respiratory epithelia. Abnormal basal rate and response to adenylate cyclase activation. *J. Clin. Investig. 78:* 1245-1252, (1986).

Bounoure, F., Fiquet, H., and Arnaud, P. Comparison of hydrogen peroxide and peracetic acid as isolator sterilization agents in a hospital pharmacy. *Am J Health Syst Pharm. 63(5):* 451-455, (2006).

Bossy-Wetzel, E., and Green, D. R. Detection of apoptosis by annexin V labelling. *Methods Enzymol. 322:* 15-8, (2000).

Boysen, M., Solberg, L. A., Torjussen, W., Poppe, S., and Hogetveit, A. C. Histological changes, rhinoscopical findings and nickel concentration in plasma and urine in retired nickel workers. *Acta Otolaryngol. 97:* 105–15, (1984).

BPD. (2009). Biocidal Products Directive 98/8/EC. Health and Safety Executive. Issue No.4. Accessed 05-01-09. (World Wide Web page). http://www.hse.gov.uk/biocides/bpd/

Bradley, D. E. and Franklin, J. G. Electron microscope survey of the surface configuration of spores of the genus *Bacillus*. *J Bacteriol. 76(6):* 618-630, (1958).

Brown, R. K., Wyatt, H., Price, J. F., and Kelly, F. J. Pulmonary dysfunction in cystic fibrosis is associated with oxidative stress. E*ur. Respir. J. 9(2):* 334-339, (1996). BS EN. (2002). BS EN 13704:2002.

Bucchieri, F., Puddicombe, S. M., Lordan, J. L., Richter, A., Buchanan, D., Wilson, S. J., Ward, J., Zummo, G., Howarth, P. H., Djukanović, R., Holgate, S. T., and Davies. D. E. Asthmatic bronchial epithelium is more susceptible to oxidant-induced apoptosis. *Am J Respir Cell Mol Biol. 27(5):* 179-85, (2002).

Buss, H., Chan, T. P., Sluis, K. B., Domigan, N. M., and Winterbourn, C. C. Protein Carbonyl measurement by a sensitive ELISA method. *Free Radic Biol Med. 23(3):* 361–366, (1997).

Cabrera-Martinez, R.M., Tovar-Rojo, F., Vepachedu, V.R., and Setlow, P. Effects of overexpression of nutrient receptors on germination of spores of *Bacillus subtilis. J. Bacteriol. 185(8):* 2457-2464, (2003).

Carlson, V. R. Chapter 7: Food Packaging Equipment. In Aseptic Processing and Packaging of Food: A Food Industry Perspective, edited by David, J. R., Graves, R. H., and Carlson, V. R. p.127-146. (Illustrated edition), CRC Press: London 1996.

Carty, J. L., Bevan, R., Waller, H., Mistry, N., Cooke, M., Lunec, J., and Griffiths, H. R. The effects of Vitamin C supplementation on protein oxidation in healthy volunteers. *Biocheml Biophys Res Commun. 273(2*): 729-735, (2000).

Chambers, L. A., Rollins, B. M., and Tarran, R. Liquid movement across the surface epithelium of large airways. *Respir Physiol Neurobiol 159(3):* 256-70, (2007).

Chan, C. and Goldkorn, T. Ceramide Path in Human Lung Cell Death. *Am. J. Respir. Cell Mol. Biol. 22(4):* 460–468, (2000).

Chandra, J., Samali, A., and Orrenius, S. Triggering and modulation of apoptosis by oxidative stress. *Free Radic. Biol. Med. 29(3-4):* 323–333, (2000).

Chang, J. C., Gross, E. A., Swenberg, J. A., and Barrow, C. S. Nasal cavity deposition, histopathology, and cell proliferation after single or repeated formaldehyde exposures in B6C3F1 mice and F-344 rats. *Toxicol Appl Pharmacol. 68(2):* 161-76, (1983).

Chapman, K. E., Waters, C. M., and Miller, W. M. Continuous exposure of airway epithelial cells to hydrogen peroxide: protection by KGF. *J Cell Physiol., 192(1):* 71-80, (2002).

Chen, L. C., Fang, C. P., Qu, Q. S., Fine, J. M., and Schlesinger, R. B. A novel system for the in vitro exposure of pulmonary cells to acid sulfate aerosols. *Fundam. Appl.Toxicol. 20(2):* 170–176, (1993).

Cheney, J. E. and Collins, C. H. Formaldehyde disinfection in laboratories: limitations and hazards. *Br. J. Biomed. Sci. 52(3):* 195– 201, (1995).

Chung, K. F. Cytokines in chronic obstructive pulmonary disease. *Eur Respir J Suppl. 34:* 50s-59, (2001).

Clunes, M. T., and Boucher, R. C. Front-runners for pharmacotherapeutic correction of the airway ion transport defect in cystic fibrosis. *Curr Opin Pharmacol. 8(3):* 292-9, (2008).

Cockell, K. A., and Belonje, B. The Carbonyl Content of Specific Plasma Proteins Is Decreased by Dietary Copper Deficiency in Rats. *J Nutr. 132(9):* 2514-8, (2002).

Cockroft, M. G., Hepworth, D., Rhodes, J. C., Addison, P., and Beaney, A. M. Validation of Liquid Disinfection Techniques for Transfer of Components into Hospital Pharmacy Cleanrooms. *Hospital Pharmacist. 8:* 226-232, (2001).

Cohn, L. A., and Adler, K. B. Interactions between airway epithelium and mediators of inflammation. *Exp Lung Res. 18(3):* 299-322, (1992).

Cone, R. A. (2005). Mucus. In *Mucosal Immunology*, edited by Mestecky, J., Lamm, M. E,, Strober, W., Bienenstock, J., McGhess, J. R., and Maye, L. p.49-72. (3rd ed.), Academic Press: London 2005.

Conese, M., Copreni, E., Di Gioia, S., De Rinaldis, P., and Fumarulo, R. Neutrophil recruitment and airway epithelial cell involvement in chronic cystic fibrosis lung disease. *J Cyst Fibros. 2(3):* 129-35, (2003).

Coraux, C., Martinella-Catusse, C., Nawrocki-Raby, B., Hajj, R., Burlet, H., Escotte, S., Laplace, V., Birembaut, P., and Puchelle, E. Differential expression of matrix metalloproteinases and interleukin-8 during regeneration of human airway epithelium in vivo. *J Pathol. 206(2):* 160-9, (2005).

Cortezzo, D. E., Koziol-Dybe, D., Setlow, B., and Setlow, P. Treatment with oxidizing agents of spores of *Bacillus subtilis* and sensitizes spores to subsequent stress. *J Appl Microbiol. 97:* 838-852, (2004).

Cortezzo, D. E., and Setlow, P. Analysis of factors that influence the sensitivity of spores of Bacillus subtilis to DNA damaging chemicals. *J Appl Microbiol. 98(3):* 606-17, (2005).

Cowart, G. (2008). Robots set to tackle killer hospital bugs. Accessed 12-09-08. (World Wide Web page) http://bioquell.idmpreview.co.uk/new/Resources/PR_ImpCollege_20080908.pdf)

Coyne, C. B., Vanhook, M. K., Gambling, T. M., Carson, J. L., Boucher, R. C., and Johnson, L. G. Regulation of airway tight junctions by proinflammatory cytokines. *Mol Biol Cell. 13(9):* 3218-34, (2002).

Coyne, C. B., Gambling, T. M., Boucher, R. C., Carson, J. L., and Johnson, L. G. Role of claudin interactions in airway tight junctional permeability. *Am. J. Physiol. Lung Cell Mol. Physiol. 285:* L1166-L1178, (2003).

Cross, C. E., Halliwell, B., and Allen, A. Antioxidant protection: a function of tracheobronchial and gastrointestinal mucus. *Lancet.1(8390):* 1328-30, (1984).

Curnyn, E. Evaluation of aseptic transfer techniques; Survey of Disinfectant use in hospital pharmacy cleanrooms. MSc Thesis. Aston University, Bimingham. UK (2005).

Dahl, A. R., Snipes, M. B., and Gerde, P. Sites for uptake of inhaled vapors in Beagle dogs. *Toxicol. Appl. Pharmacol. 109:* 263–275, (1991).

Dalle-Donne, I., Aldini, G., Carini, M., Colombo, R., Rossi, R., and Milzani, A. Protein carbonylation, cellular dysfunction, and disease progression. *J. Cell. Mol. Med. 10(2):* 389-406, (2006).

Dazy, A. C., Auger, F., Bailbé, D., Blouquit, S., Lombet, A., and Marano, F. The toxicity of H2O2 on the ionic homeostasis of airway epithelial cells in vitro. *Toxicol In Vitro. 17(5-6):* 575-80, (2003).

de Jong, P. M., van Sterkenburg, M. A., Hesseling, S. C., Kempenaar, J. A., Mulder, A. A., Mommaas, A. M., Dijkman, J. H., and Ponec, M. Ciliogenesis in human bronchial epithelial cells cultured at the air-liquid interface. *Am J Respir Cell Mol Biol. 10(3):* 271-7, (1994).

de León, L. and Moujir, L. Activity and mechanism of the action of zeylasterone against *Bacillus subtilis*. *J Appl Microbiol. 104(5):* 1266-74, (2008).

de Oliveira-Marques, V., Cyrne, L., Marinho, H.S., and Antunes, F. A quantitative study of NF-kappaB activation by H2O2: relevance in inflammation and synergy with TNF-alpha. *J Immunol. 178(6):* 3893-902, (2007).

DeForge, L. E., Preston, A. M., Takeuchi, E., Kenney, J., Boxer, L. A., and Remick, D. G. Regulation of interleukin 8 gene expression by oxidant stress. *J Biol Chem. 268(34):* 25568-76, (1993).

Delclos, G. L., Gimeno, D., Arif, A. A., Burau, K. D., Carson, A., Lusk, C., Stock, T., Symanski, E., Whitehead, L. W., Zock, J. P., Benavides, F. G., and Antó, J. M. Occupational risk factors and asthma among health care professionals. *Am J Respir Crit Care Med. 175(7):* 667-75, (2007).

Deneke, S. M. Thiol-based antioxidants. *Curr Top Cell Reg. 36*: 151-80, (2000).

Deng, X. T., Shi, J. J., Shama, G., and Kong, M. G. Effects of microbial loading and sporulation temperature on atmospheric plasma inactivation of *Bacillus subtilis* spores. *Appl. Phys. Lett. 87(15):* 153901, (2005).

Denyer, S. P., and Stewart, G. S. A. B. Mechanisms of action of disinfectants. *Int Biodeter Biodegrad. 41:* 261-268, (1998).

Devalia, J. L., and Davies, R. Human nasal and bronchial epithelial cells in culture: an overview of their characteristics and function. *J. Allergy Proc. 12(2):* 71-9, (1991).

Devalia, J. L., Godfrey, R. W. A., Sapsford, R. J., Severs, N. J., Jeffery, P. K., and Davies, R. J. No effect of histamine on human bronchial epithelial cell permeability and tight junctional integrity *in vitro*. *Eur Respir J. 7:* 1958-1965, (1994).

Devalia, J. L., Bayram, H., Abdelaziz, M. M., Sapsford, R. J., and Davies, R. J. Differences between cytokine release from bronchial epithelial cells of asthmatic patients and nonasthmatic subjects: Effects of exposure to diesel exhaust particles. *Int Arch Allergy Immunol. 118:* 437–439, (1999).

Devlin, R. B., McKinnon, K. P., Noah, T., Becker, S., and Koren H. S. Ozone-induced release of cytokines and fibronectin by alveolar macrophages and airway epithelial cells. *Am. J. Physiol. 266 (Lung Cell. Mol. Physiol. 10):* L612–L619, (1994).

Diabate, S., Mulhopt, S., Paur, H. R., and Krug, H. F. Pro-inflammatory effects in lung cells after exposure to fly ash aerosol via the atmosphere or the liquid phase. *Ann. Occup. Hyg. 46(1):* 382–385, (2002).

Dimova, S., Brewster, M. E., Noppe, M., Jorissen, M., and Augustijns, P. The use of human nasal in vitro cell systems during drug discovery and development. *Toxicol In Vitro. 19(1):* 107-22, (2005).

Di Simplicio, P., Cheeseman, K. H., and Slater, T. F. The reactivity of the SH group of bovine serum albumin with free radicals. *Free Radic Res Commun. 14(4):* 253-62, (1991).

Douglass, J. A., Dhami, D., Gurr, C. E., Bulpitt, M., Shute, J. K., Howarth, P. H., Lindley. I. J., Church, M. K., and Holgate, S. T. Influence of interleukin-8 challenge in the nasal mucosa in atopic and nonatopic subjects. *Am J Respir Crit Care Med. 150(4):* 1108-13, (1994).

Driks, A. From Rings to Layers: Surprising patterns of protein deposition during bacterial spore assembly. *J Bacteriol. 186(14):* 4423-4426, (2004).

Dusseau, J-Y., Duroselle, P., and Freney, J. (2004). Gaseous Sterilization. In *Russell, Hugo & Ayliffes Principles and Practices of Disinfection, Preservation and Sterilization*, edited by Fraise, A. P., Lambert, P. A. and Maillard J-Y. p.401-435. (4th ed.), Blackwell Scientific Publications: Oxford 2004.

Eaton, P. Protein thiol oxidation in health and disease: Techniques for measuring disulfides and related modifications in complex protein mixtures. *Free Radic. Biol. Med. 40(11):* 1889–1899, (2006).

ECETOC. Hydrogen peroxide CAS No. 7722-84-1. Joint assessment of commodity chemicals no. 22. European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) (1992). (World Wide Web page) http://www.intox.org/databank/documents/chemical/hydroper/cie198.htm

Ehrhardt, C., Kneuer, C., Fiegel, J., Hanes, J., Schaefer, U. F., Kim, K. J. and Lehr, C. M. Influence of apical fluid volume on the development of functional intercellular junctions in the human epithelial cell line 16HBE14o-: implications for the use of this cell line as an in vitro model for bronchial drug absorption studies. *Cell Tissue Res. 308(3):* 391-400, (2002).

Eisenbrand, G., Pool- Zobel, B. P., Baker, V., Balls, M., Blaauboer, B. J., Boobis, A., Carere, A., Kevekordes, S., Lhuguenot, J. C., Pieters, R., and Kleiner, J. Methods of *in vitro* toxicology. *Food Chem. Toxicol. 40(2 -3):* 193–236, (2002).

EPA. (2007) EPA Guidance on Test Methods for Determining the Efficacy of Antimicrobial Products for Inactivating *Bacillus anthracis* Spores on Inanimate Surfaces. Accessed 21-05-06 (World Wide Web page) http://www.epa.gov/scipoly/SAP/meetings.2007/july/backgrounddoc6_18_2007.pdf)

Exner-Freisfeld, H., Kronenberger, H., Meier-Sydow, J., and Nerger, K. H. Intoxication from bleaching with sodium chlorite. The toxicology and clinical course. *Dtsch Med Wochenschr. 111(50):* 1927-1930, (1986).

Favero, M. S., Carson, L. A., Bond, W. W., and Petersen, N. J. Pseudomonas aeruginosa: growth in distilled water from hospitals. *Science. 173(999):* 836-8. (1971).

Fazi, B., Bursch, W., Fimia, G.M., Nardacci R., Piacentini, M., Di Sano, F., and Piredda, L. Fenretinide induces autophagic cell death in caspase-defective breast cancer cells. *Autophagy. 4(4):* 435-441, (2008).

Fernando, W. J. and Othman, R. Relevance of diffusion through bacterial spore coats/membranes and the associated concentration boundary layers in the initial lag phase of inactivation: a case study for Bacillus subtilis with ozone and monochloramine. *Math Biosci. 199:* 175-87, (2006).

Fiedler, M. A., Kaetzel, C. S., and Davis, P. B. Sustained production of secretory component by human tracheal epithelial cells in primary culture. *Am J Physiol. 261(4 Pt 1):* L255-61, (1991).

Fiserova-Bergerova, V. Toxicokinetics of organic solvents. *Scand J Work Environ Health. 11(S1):* 7-21, (1985).

Florea, B. I., Cassara, M. L., Junginger, H. E., and Borchard, G. Drug transport and metabolism characteristics of the human airway epithelial cell line Calu-3. *Journal Control Release. 87(1-3):* 131–138, (2003).

Flynn, A. N., Itani, O. A., Moninger, T. O., and Welsh, M. J. Acute regulation of tight junction ion selectivity in human airway epithelia. *Proc Natl Acad Sci U S A. 106(9):* 3591-6, (2009).

Forbes, I. I. Human airway epithelial cell lines for in vitro drug transport and metabolism studies. *Pharm Sci Technolo Today. 3(1):* 18-27, (2000).

Forbes, B. and Ehrhardt, C. Human respiratory epithelial cell culture for drug delivery applications. *Eur J Pharm Biopharm. 60(2):* 193-205, (2005).

Forbes, B., Shah, A., Martin, G. P., and Lansley, A. B. The human bronchial epithelial cell line 16HBE14o_ as a model system of the airways for studying drug transport. *Int J Pharm. 257:* 161–167, (2003).

Förster C. Tight junctions and the modulation of barrier function in disease. *Histochem Cell Biol. 30(1):* 55-70, (2008).

Foster, K. A., Avery, M. L., Yazdanian, M., and Audus, K. L. Characterization of the Calu-3 cell line as a tool to screen pulmonary drug delivery. *Int J Pharm. 208(1-2):* 1–11, (2000).

Fox, S. I. Human Physiology (4th ed.). Wm. C. Brown Publishers: London 1993.

Frank, J. F. and Chimielewski, R. A. Effectiveness of sanitation with quaternary ammonium compound or chlorine on stainless steel and other domestic food-preparation surfaces. *J Food Prot. 60:* 43-47, (1997).

French, G. L., Otter, J. A., Shannon, K. P., Adams, N. M., Watling, D., and Parks, M. J. Tackling contamination of the hospital environment by methicillin-resistant *Staphylococcus aureus* (MRSA): a comparison between conventional terminal cleaning and hydrogen peroxide decontamination. *J. Hosp. Infect. 57(1):* 31-37, (2004).

Froud, G. (2005). Reducing risks from everyday items. The European Journal of Contamination Control:Cleanroom-Technology. Accessed 14-04-08 (World Wide Web page) http://www.cleanroom-technology.co.uk/story.asp?storyCode=34883

Gabrielson, E. W., Yu, X. Y., and Spannhake, E. W. Comparison of the toxic effects of hydrogen peroxide and ozone on cultured human bronchial epithelial cells. *Environ Health Perspec. 102(11):* 972-4, (1994).

Galluzzi, L., Vicencio, J. M., Kepp, O., Tasdemir, E., Maiuri, M. C., and Kroemer, G. To die or not to die: that is the autophagic question. *Curr. Mol. Med. 8(2):* 78-91, (2008).

Genest, P.C., Setlow, B., Melly, E., and Setlow, P. Killing of spores of *Bacillus subtilis* by peroxynitrite appears to be caused by membrane damage. *Microbiology. 148:* 307-314, (2002).

Gerde, P. and Dahl, A. R. A model for the uptake of inhaled vapors in the nose of the dog during breathing. *Toxicol. Appl. Pharmacol. 109:* 276–288, (1991).

Ghezzi, P. and Bonetto, V. Redox proteomics: identification of oxidatively modified proteins. *Proteomics. 3(7):* 1145-53, (2003).

Gloemme, J. and Lundgren, K. D. Health hazards from chlorine dioxide. *Arch Ind Health. 16(2):* 169-176, (1957).

Godfrey, R. W. Human airway epithelial tight junctions. *Microsc Res Tech. 38(5):* 488-99, (1997).

Godfrey, R. W. A., Severs, N. J., and Jeffery, P.K. Structural alterations of airway epithelial tight junctions in cystic fibrosis: Comparison of transplant and post-mortem tissue. *Am. J. Respir. Cell Mol. Biol., 9(2):* 148–156. (1993).

Goldkorn T., Ravid T., and Khan E. M. Life and death decisions: ceramide generation and EGF receptor trafficking are modulated by oxidative stress. *Antioxid and Redox Signal. 7(1-2):* 119-28, (2005).

Goldsby, R. A., Kindt, T. J., and Osborne, B. A. Kuby Immunology (4th ed.). W. H. Freeman and Company: New York 2000.

Grainger, C. I., Greenwell, L. L., Lockley, D. J, Martin, G. P., and Forbes, B. Culture of Calu-3 cells at the air interface provides a representative model of the airway epithelial barrier. *Pharm Res. 23(7):* 1482-90, (2006).

Greim, H., Arand, M., Autrup, H., Bolt, H. M., Bridges, J., Dybing, E., Glomot, R., Foa, V., and Schulte-Hermann, R. Toxicological comments to the discussion about REACH. *Arch Toxicol. 80(3):* 121-4, (2006).

Gruenert, D. C., Finkbeiner, W. E., and Widdicombe, J. H. Culture and transformation of human airway epithelial cells. *Am J Physiol. 268(3 Pt 1):* L347-60, (1995).

Gruhn, R., Bassler, H.-J., and Werner, U. J. Sterilization of Chicken Eggs for Vaccine Production with Vaporized Hydrogen Peroxide. *Pharm. Ind. 57(10):* 873-877, (1995).

Grutter, M. G. Caspases: key players in programmed cell death. *Curr. Opin. Struct. Biol. 10(6):* 649–55. (2000).

Guo, Y., Zhao, X., and Yang, Z. Ciliogenesis in human nasal epithelial cells cultured at the air-liquid interface. *Lin Chuang Er Bi Yan Hou Ke Za Zhi. 18(2):* 88-90, (2004).

Hampton, M. B. and Orrenius, S. Dual regulation of caspase activity by hydrogen peroxide: implications for apoptosis. *FEBS Lett. 414(3):* 552-6, (1997).

Harkema, J. R., Plopper, C. G., Hyde, D. M., St George, J. A., Wilson, D. W., and Dungworth, D. L. Response of the macaque nasal epithelium to ambient levels of ozone. A morphologic and morphometric study of the transitional and respiratory epithelium. *Am J Pathol. 128:* 29–44, (1987).

Harkema, J. R., Carey, S. A., and Wagner, J. G. The nose revisited: a brief review of the comparative structure, function, and toxicologic pathology of the nasal epithelium. *Toxicol Pathol. 34(3):* 252-69, (2006).

Hashimoto, T., Frieben, W. R., and Conti, S. F. Germination of single bacterial spores. *J Bacteriol. 98(3):* 1011-1020, (1969).

Heckert, R. A., Best, M., Jordan, L. T., Dulac, G. C., Eddington, D. L., and Skerritt, W. G. Efficacy of vaporized hydrogen peroxide against exotic animal viruses. *Appl Environ Microbiol. 63(10):* 3916-3918, (1997).

Herard, A. L., Zahm, J. M., Pierrot, D., Hinnrasky, J., Fuchey, C., and Puchelle, E. Epithelial barrier integrity during in vitro wound repair of the airway epithelium. *Am J Respir Cell Mol Biol. 15(5):* 624-32, (1996).

Hiltermann, T. J., Stolk, J., van der Zee, S. C., Brunekreef, B., de Bruijne, C. R., Fischer, P. H., Ameling, C. B., Sterk, P. J., Hiemstra, P. S., and van Bree, L. Asthma severity and susceptibility to air pollution. *Eur. Respir. J. 11:* 686–693, (1998).

Hiom, S. J. Validation of disinfection techniques in hospital aseptic dispensing units. *Pharm J. 265(7110):* 277-278, (2000).

Hiom, S. J., Lowe, C., and Oldcome, M. Development and validation of a method to assess alcohol transfer disinfection procedures. *The Pharm J. 272:* 611-614, (2004).

Huber, A. R., Kunkel, S. L., Todd, R. F. 3rd, and, Weiss, S. J. Regulation of transendothelial neutrophil migration by endogenous interleukin-8. *Science. 254(5028):* 99-102, (1991).

Hughson, E. J., and Hirt, R. P. (1996). Assessment of cell polarity. *In Epithelial Cell Culture – A Practical Approach*, edited by Shaw, A. J. p. 37-66. Oxford University Press: Oxford 1996.

Ikawa J. Y. and Rossen, J. S. Reducing bacteria in household sponges. *Environ. Health. 7(4):*18-22, (1999).

Inoue, H. Interleukin-8 and airway inflammation. *Nihon Kokyuki Gakkai Zasshi. 37(9):* 673-9, (1999).

Ioannou, C. J., Hanlon, G. W., and Denyer, S. P. Action of disinfectant quaternary ammonium compounds against *Staphylococcus aureus. Agents and Chemother 51(1):* 296-306, (2007).

Ischiropoulos, H. and al-Mehdi, A. B. Peroxynitrite-mediated oxidative protein modification. *FEBS Lett. 364(3):* 279–282, (1995).

Jaakkola, J. J. and Jaakkola, M. S. Professional cleaning and asthma. *Curr Opin Allergy Clin Immunol. 6(2):* 85-90, (2006).

Jahnke, M., and Lauth, G. Bio-decontamination of a large volume filling room with hydrogen peroxide. *Pharn. Eng. 17:* 96-108, (1997).

Jeebhay, M. F. and Quirce, S. Occupational asthma in the developing and industrialised world: a review. *Int J Tuberc Lung Dis. 11(2):* 122-33, (2007).

Jepsen, M., Graham, S., Karp, P. H., and Zabner, J. Effect of topical nasal pharmaceuticals on sodium and chloride transport by human airway epithelia. *Am J Rhinol. 14(6):* 405-9, (2000).

Jeulin, C., Guadagnini, R., and Marano, F. Oxidant stress stimulates Ca2+-activated chloride channels in the apical activated membrane of cultured nonciliated human nasal epithelial cells. *Am J Physiol Lung Cell Mol Physiol. 289(4):* L636-46, (2005).

Johanson, J. Modelling of respiratory exchange of polar solvents. *Ann. Occup. Hyg. 35(3):* 323–339, (1991).

Johansson, A. S., Lidén, J., Okret, S., and Palmblad, J. E. Effects of ethanol on cytokine generation and NFkappaB activity in human lung epithelial cell. *Biochem Pharmacol. 70(4):* 545-51, (2005).

Johnston, M. D., Lambert, R. J. W., Hanlon, G. W., and Denyer, S. P. A rapid method for assessing the suitability of quenching agents for individual biocides as well as combinations. *J Appl Microbiol. 92(4):* 784-789, (2002).

Jones, A. W. Role of rebreathing in determination of the blood-breath ratio of expired ethanol. *J Appl Physiol. 55(4):* 1237-41, (1983).

Jonston, M. D., Lawson., S., and Otter, J. A. Evaluation of hydrogen peroxide vapour as a method for the decontamination of surfaces contaminated with *Clostridium botulinum* spores. *J Microbiol Methods. 60(3):* 403-411, (2005).

Kahnert, A., Seller, P., Stein, M., Aze, B., McDonnell, G., and Kaufmann, S. H. E. Decontamination with vaporized hydrogen peroxide is effective against *Mycobacterium tuberculosis. Lett Appl Microbiol. 40(6):* 448-452, (2005).

Kannan, R., Zhang, N., Sreekumar, P. G., Spee, C. K., Rodriguez, A., Barron, E., and Hinton, D. R. Stimulation of apical and basolateral VEGF-A and VEGF-C secretion by oxidative stress in polarized retinal pigment epithelial cells. *Mol Vis. 12:* 1649-59, (2006).

Kardalinou, E., Zhelev, N., Hazzalin, C. A., and Mahadevan, L. C. Anisomycin and rapamycin define an area upstream of p70/85S6k containing a bifurcation to histone H3-HMG-like protein phosphorylation and c-fos-c-jun induction. *Mol Cell Biol. 14(2):* 1066-74, (1994).

Kawasaki, S., Takizawa, H., Takami, K., Desaki, M., Okazaki, H., Kasama, T., Kobayashi, K., Yamamoto, K,, Nakahara, K., Tanaka, M., Sagai, M. T., and Ohtoshi, T. Benzene-extracted components are important for the major activity of diesel exhaust particles—effect on interleukin-8 gene expression in human bronchial epithelial cells. *Am. J. Respir. Cell Mol. Biol. 24:* 419–426, (2001).

Kazzaz, J. A., Xu, J., Palaia, T., Mantell, L., Fein, A. M., and Horowitz, S. Cellular oxygen toxicity. Oxidant injury without apoptosis. *J Biol Chem. 271(25):* 15182-6, (1996).

Ke, Y., Reddel, R. R., Gerwin, B. I., Miyashita, M., McMenamin, M., Lechner, J. F., and Harris, C. C. Human bronchial epithelial cells with integrated SV40 virus T antigen genes retain the ability to undergo squamous differentiation. *Differentiation. 38(1):* 60–66, (1988).

Khabar, K. S., Al-Zoghaibi, F., Al-Ahdal, M. N., Murayama, T., Dhalla, M., Mukaida, N., Taha, M., Al-Sedairy, S. T., Siddiqui, Y., Kessie, G., and Matsushima, K. The alpha chemokine, interleukin 8, inhibits the antiviral action of interferon alpha. *J Exp Med. 186 (7):* 1077-85, (1997).

Kim, H. P, Wang, X., Chen, Z. H, Lee, S. J., Huang, M. H., Wang, Y., Ryter, S. W., and Choi, A. M. Autophagic proteins regulate cigarette smoke-induced apoptosis: protective role of heme oxygenase-1. *Autophagy. 4(7):* 887-95, (2008).

King, W. L. and Gould, G. W. Lysis of bacterial spores with peroxide. *J Appl Bacteriol. 32:* 481-490, (1969).

Kinnula, V. L., Yankaskas, J. R., Chang, L., Virtanen, I., Linnala, A., Kang, B. H., and Crapo, J. D. Primary and immortalized (BEAS 2B) human bronchial epithelial cells have significant antioxidative capacity in vitro. *Am J Respir Cell Mol Biol. 11(5):* 568-76, (1994).

Klapes, N. A. and Velsey, D. Vapor-Phase Hydrogen Peroxide as a Surface Decontaminant and Sterilant. *Appl Environ Microbiol. 56(2):* 503-506, (1990).

Knebel, J. W., Ritter, D., and Aufderheide, M. Development of an in vitro system for studying effects of native and photochemically transformed gaseous compounds using an air/liquid culture technique. *Toxicol. Lett. 96–97:* 1–11, (1998).

Kokubo, M., Inoue, T., and Akers, J. Resistance of Common Environmental Spores of the Genus *Bacillus* of Vapor Hydrogen Peroxide. *PDA J Pharm Sci Technol. 52(5):* 228-231, (1998).

Koren, H. S., Devlin, R. B., Graham, D. E., McMenamin, M. G., Lechner, J. F., Su, R. T., Brash, D. E., Park, J. B., Rhim, J. S., and Harris, C. C. Ozone induced inflammation in the lower airways of human subjects. *Am Rev Respir Dis. 139:* 407-415, (1989).

Krause, J., McDonnell, G., and Riedesel, H. Biodecontamination of animal rooms and heat-sensitive equipment with vaporized hydrogen peroxide. *Contemp Top Lab Anim Sci. 40(6):* 18-21, (2001).

Kreja, L. and Seidel, H. J. On the cytotoxicity of some microbial volatile organic compounds as studied in the human lung cell line A549. *Chemosphere. 49(1):* 105-10, (2002).

Kruhøffer, P. W. Handling of inspired vaporized ethanol in the airways and lungs (with comments on forensic aspects). *Forensic Sci Int. 21(1)* :1-17, (1983).

Kuper, C. F., Woutersen, R. A., Slootweg, P. J., and Feron, V. J. Carcinogenic response of the nasal cavity to inhaled chemical mixtures. *Mutat. Res. 380:* 19–26, (1997).

Kwon, K. D., Jo, W. K., Lim, H. J., and Jeong, W. S. Volatile pollutants emitted from selected liquid household products. *Environ Sci Pollut Res Int. 15(6):* 521-6, (2008).

Laan, M., Bozinovski, S., and Anderson, G. Cigarette smoke inhibits lipopolysaccharide-induced production of inflammatory cytokines by suppressing the activation of activator protein-1 in bronchial epithelial cells. *J. Immunol. 173:* 4164–4170, (2004).

Laflamme C, Lavigne, S., Ho, J., and Duchaine, C. Assessment of bacterial endospore viability with fluorescent dyes. *J Appl Microbiol. 96(4):*,684-92, (2004).

Lakshminarayanan, V., Beno, D. W., Costa, R. H., and Roebuck, K. A. Differential regulation of interleukin-8 and intercellular adhesion molecule-1 by H2O2 and tumor necrosis factor-alpha in endothelial and epithelial cells. *J Biol Chem. 272(52):* 32910-8, (1997).

Lakshminarayanan V., Drab-Weiss E. A., and Roebuck K. A. H2O2 and tumor necrosis factor-alpha induce differential binding of the redox-responsive transcription factors AP-1 and NF-kappaB to the interleukin-8 promoter in endothelial and epithelial cells. *J Biol Chem. 273(49):* 32670-8, (1998).

Lambert, R. J. W. (2004). Evaluation of antimicrobial efficacy. In *Russell, Hugo & Ayliffes Principles and Practices of Disinfection, Preservation and Sterilization*, edited by Fraise, A. P., Lambert, P. A. and Maillard J-Y. p.345-360 (4th ed.), Blackwell Scientific Publications: Oxford 2004.

Lannan, S., Donaldson, K., Brown, D., and MacNee, W. Effect of cigarette smoke and its condensates on alveolar epithelial cell injury *in vitro. Am J Physiol. 266:* L92–L100, (1994).

Lassota, P., Singh, G., and Kramer, R. Mechanism of topoisomerase II inhibition by staurosporine and other protein kinase inhibitors. *J Biol. Chem. 271(42):* 26418-23, (1996).

Le Gall, A. H., Yeaman, C., and Boulan, E. R. Epithelial cell polarity: new perspectives. *Semin Nephrol. 15:* 272–284, (1995).

Lee, M. K., Yoo, J. W., Lin, H., Kim, Y. S., Kim, D. D., Choi, Y. M., and Roh, H. J. Air-liquid interface (ALI) culture of serially passaged human nasal epithelial cell monolayer for in vitro drug transport studies. *Drug Deliv. 12(5):* 305–311, (2005).

Lee, J., Lopes, J. A., and Pascall, M. A. Development of a sanitizing fabric wipe for use on food contact surfaces. *J Food Sci. 72(9):* M375-81, (2007).

Lin, H., Yoo, J. W., Roh, H. J., Lee, M. K., Chung, S. J., Shim, C. K., and Kim, D. D. Transport of anti-allergic drugs across the passage cultured human nasal epithelial cell monolayer. *Eur J Pharm Sci. 26:* 203–210, (2005).

Lin, H., Li, H., Cho, H-J., Bian, S., Roh, H-J., Lee, M-K., Kim, J. S., Chung, S. J., Shim, CH-K., and Kim, D-D. Air-Liquid Interface (ALI) Culture of Human Bronchial Epithelial Cell Monolayers as an In Vitro Model for Airway Drug Transport Studies. *J Pharm Sci. 96(2):* 341-350, (2007).

Loshon, C.A., Melly, E., Setlow, B., and Setlow, P. Analysis of killing of spores of *Bacillus subtilis* by a new disinfectant, Sterilox®. *J Appl Microbiol. 91:* 1051-1058, (2001).

Luce, D., Gerin, M., Leclerc, A., Morcet, J. F., Brugere, J., and Goldberg, M. Sinonasal cancer and occupational exposure to formaldehyde and other substances. *Int J Cancer. 53:* 224–31, (1993).

Lukacs, N. W. and Tekkanat, K. K. Role of chemokines in asthmatic airway inflammation. *Immunol Rev. 177:* 21-30, (2000).

Lundberg, A. S., Randell, S. H., Stewart, S. A., Elenbaas, B., Hartwell, K. A., Brooks, M. W., Fleming, M. D., Olsen, J. C., Miller, S. W., Weinberg, R. A., and Hahn, W. C. Immortalization and transformation of primary human airway epithelial cells by gene transfer. *Oncogene. 21(29):* 4577-86, (2002).

Ma, T. Y., Hollander, D., Riga, R., and Bhalla, D. Autoradiographic determination of permeation pathway of permeability probes across intestinal and tracheal epithelia. *J Lab Clin Med. 122(5):* 590-600, (1993).

Malborg, A., Wingren, M., Bonfield, P., and McDonnell, G. (2001). Room decontamination with vaporized hydrogen peroxide. CleanRooms. Accessed 25-07-08. (World Wide Web page) http://cr.pennnet.com/display_article/125641/15/ARTCL/none/none/1/VHP-takes-its-place-in-room-decontamination.

Mannino, D. M. How much asthma is occupationally related? *Occup Med. 15(2):* 359-68, (2000).

Mantle, D. and Preedy, V. R. Free radicals as mediators of alcohol toxicity. *Adverse Drug React Toxicol Rev. 18(4):* 235-52, (1999).

Marshall, L. J., Perks, B., Ferkol, T., and Shute, J. K. IL-8 released constitutively by primary bronchial epithelial cells in culture forms an inactive complex with secretory component. *J Immunol. 167(5):* 2816-23, (2001).

Martin, L. D., Rochelle, L. G., Fischer, B. M., Krunkosky, T. M., and Adler, K. B. Airway epithelium as an effector of inflammation: molecular regulation of secondary mediators. *Eur Respir J. 10:* 2139–2146, (1997).

McCrea, K. A., Ensor, J. E, Nall, K., Bleecker, E. R., and Hasday, J. D. Altered cytokine regulation in the lungs of cigarette smokers. *Am J Respir Crit Care Med. 150:* 696–703, (1994).

McDonnell, G. (2002). Decontamination of workstations and enclosed areas with Vapor Phase Hydrogen Peroxide (VHP®). Hospital Pharmacy Europe. Accessed 21-05-08. (World Wide Web page) http://www.vhp.pl/pliki_uploadowane/VHPHospitalDecon.pdf

McDonnell, G. and Russell, A. D. Antiseptics and disinfectants: activity, action, and resistance. *Clin Microbiol Rev. 12(1):* 147-179, (1999).

McDougall, C. M., Blaylock, M. G., Douglas, J. G., Brooker, R. J., Helms, P. J., and Walsh, G. M. Nasal epithelial cells as surrogates for bronchial epithelial cells in airway inflammation studies. *Am J Respir Cell Mol Biol. 39(5):* 560-8, (2008).

Medina, T. V., Herrero, C. G. M., Curiel, A. G., and Atienza. M. F. A microbial growth study in parenteral nutrition. *Nutr Hosp. 4(5):* 283-289, (1989).

Medina-Ramón, M., Zock, J. P., Kogevinas, M., Sunyer, J., and Antó, J. M. Asthma symptoms in women employed in domestic cleaning: a community based study. *Thorax. 58(11):* 950-4, (2003).

Medina-Ramón, M, Zock, J. P., Kogevinas, M., Sunyer, J., Torralba, Y., Borrell, A., Burgos, F., and Antó, J. M. Asthma, chronic bronchitis, and exposure to irritant agents in occupational domestic cleaning: a nested case-control study. *Occup Environ Med. 62(9):* 598-606, (2005).

Medinsky, M. A. and Bond, J. A. Sites and mechanisms for uptake of gases and vapors in the respiratory tract. *Toxicology. 160(1-3):* 165-72, (2001).

Meggs, W. J., Elsheik, T., Metzger, W. J., Albernaz, M., and Bloch, R. M. Nasal pathology and ultrastructure in patients with chronic airway inflammation (RADS and RUDS) following an irritant exposure. *Clin Toxicol. 34(4):* 383-396, (1996).

Melly, E., Genest, P. C., Gilmore, M. E., Little, S., Popham, D. L., Driks, A., and Setlow, P. Analysis of the properties of *Bacillus subtilis* prepared at different temperatures. *J Appl Microbiol. 92:* 1105-1115, (2002).

Mestas, J. and Hughes, C. C. Of mice and not men: differences between mouse and human immunology. *J Immunol. 172(5):* 2731-8, (2004).

Meszaros, J. E., Antloga, K., Justi, C., Plesnicher, C., and McDonnell, G. Area Fumigation with Hydrogen Peroxide Vapor. *Applied Biosafety. 10(2):* 91-100, (2005).

Mills, P. R., Davies, R. J., and Devalia, J. L. Airway Epithelial Cells, Cytokines, and Pollutants. *Am J Respir Crit Care Med. 160(5 Pt 2):* S38–43, (1999).

Mirshafiey, A., and Mohsenzadegan, M. The role of reactive oxygen species in immunopathogenesis of rheumatoid arthritis. *Iran J Allergy Asthma Immunol. 7(4):* 195-202, (2008).

Misso, N. L. and Thompson, P. J. Oxidative stress and antioxidant deficiences in asthma: potential modification by diet. *Redox Rep. 10(5):* 247-55, (2005).

Mohr, S., Keith, G., and Rihn, B. Asbestos and malignant pleural mesothelioma: molecular, cellular and physiopathological aspects. *Bull Cancer. 92(11):* 959-76, (2005).

Montgomery, W., Vig, P., Stabb, E., and Matteson, S. Computed tomography: a three-dimensional study of the nasal airway. *Am. J. Orthod. 76:* 363-375, (1979).

Monticello, T. M., Morgan, K. T., Everitt, J. I., and Popp, J. A. Effects of formaldehyde gas on the respiratory tract of rhesus monkeys: pathology and cell proliferation. *Am. J. Pathol. 134(4):* 515-527, (1989).

Morio, L. A., Hooper, K. A., Brittingham, J., Li, T. H., Gordon, R. E., Turpin, B. J., and Laskin, D. L. Tissue injury following inhalation of fine particulate matter and hydrogen peroxide is associated with altered production of inflammatory mediators and antioxidants by alveolar macrophages. *Toxicol Appl Pharmacol. 177(3):* 188-99, (2001).

Motta, A. C. and van Oosterhout, A. J. M. T cells in asthma: Lessons from mouse models. *Drug Discovery Today: Disease Models. 3(3):* 199-204, (2006).

Mulier, B., Rahman, I., Watchorn, T., Donaldson, K., MacNee, W., and Jeffery, P. K. Hydrogen peroxide-induced epithelial injury: the protective role of intracellular nonprotein thiols (NPSH). *Eur Respir J. 11(2):* 384-91, (1998).

Muller, H.-J., Sommermeyer, K., and Cech, F. Permeation of gaseous hydrogen peroxide and peracetic acid into IV bags during their surface sterilisation. *Hospital Pharmacist. 10:* 84-86, (2003).

Mukaida, N., Okamoto, S., Ishikawa, Y., and Matsushima, K. Molecular mechanism of interleukin-8 gene expression. *J Leukoc Biol. 56:* 554–558, (1994).

Nemeth, E, Halasz, A., Barath, A., Domokos, M., and Galfi, P. Effect of hydrogen peroxide on interleukin-8 synthesis and death of Caco-2 cells. *Immunopharmacol Immunotoxicol. 29(2):* 297-310, (2007).

NHS, (2009). NHS Careers. Accessed 23-03-09. (World Wide Web page) http://www.nhscareers.nhs.uk/notSure.shtml

Nickmilder, M., Carbonnelle, S., and Bernard, A. House cleaning with chlorine bleach and the risks of allergic and respiratory diseases in children. *Pediatr Allergy Immunol. 18(1):* 27-35, (2007).

Nikasinovic, L., Momas, I., and Seta, N. Nasal epithelial and inflammatory response to ozone exposure: a review of laboratory-based studies published since 1985. *J Toxicol Environ Health B Crit Rev. 6(5):* 521-68, (2003).

Noah, T. L., Wortman, I. A., Hu, P. C., Leigh, M. W., and Boucher, R. C. Cytokine production by cultured human bronchial epithelial cells infected with a replication-deficient adenoviral gene transfer vector of wild-type adenovirus type 5. *Am J Respir Cell Mol Biol. 14(5):* 417-24, (1996).

Nylander, L.A. and Dement, J. M. Carcinogenic effects of wood dust: review and discussion. *Am J Ind Med. 24(5):* 619-47, (1993).

Oberst, F. W., Comstock, C. C., and Hackley, E. B. Inhalation toxicity of ninety percent hydrogen peroxide vapor. *AMA Arch Ind Health.10(4):* 319-27, (1954).

Olin, A. C., Granung, G., Hagberg, S., Adriansson, M., Brisman, J., Dalander, O., Karlsson, B., and Torén, K. Respiratory health among bleachery workers exposed to ozone and chlorine dioxide. *Scand J Work Environ Health. 28(2):* 117-23, (2002).

Oosting, R. S., van Bree, L., van Iwaarden, J. F., van Golde, L. M., and Verhoef, J. Impairment of phagocytic functions of alveolar macrophages by hydrogen peroxide. *Am. J. Physiol. 259(2 Pt 1):* L87–L94, (1990).

Oslund, K. L., Miller, L. A., Usachenko, J. L., Tyler, N. K., Wu, R., and Hyde, D. M. Oxidant- injured airway epithelial cells upregulate thioredoxin but do not produce interleukin-8. *Am J Respir Cell Mol Biol. 30(5):* 597-604, (2004).

Otter, J. A., and French, G. L. Survival of nosocomial bacteria and spores on surfaces and inactivation by hydrogen peroxide vapour (HPV). *J Clin Microbiol., 47(1):* 205-7, (2009).

Ovrevik, J., Refsnes, M., Schwarze, P., and Låg. M. The ability of oxidative stress to mimic quartz-induced chemokine responses is lung cell line-dependent. *Toxicol Lett. 181(2):* 75-80, (2008).

Pandya, R. J., Solomon, G., Kinner. A., and Balmes, J. R. Diesel Exhaust and Asthma: Hypotheses and Molecular Mechanisms of Action. *Environ Health Perspect. 110(s1):* 103–112, (2002).

Panousi, M. N., Williams, G. J., Girdlestone, S., Hiom, S. J., and Maillard, J. Y. Evaluation of alcohol wipes used during aseptic manufacturing. *Lett Appl Microbiol. 48(5):* 648-51, (2009).

Park, E. J., Yi, J., Chung, K. H., Ryu, D. Y., Choi, J., and Park, K. Oxidative stress and apoptosis induced by titanium dioxide nanoparticles in cultured BEAS-2B cells. *Toxicol Lett. 180(3):* 222-9, (2008).

Pauluhn, J., and Mohr, U. Inhalation studies in laboratory animals--current concepts and alternatives. *Toxicol Pathol. 28(5):* 734-53, (2000).

Pelaia, G., Cuda, G., Vatrella, A., Gallelli, L., Fratto, D., Gioffrè, V., D'Agostino, B., Caputi, M., Maselli, R., Rossi, F., Costanzo, F. S., and Marsico, S. A. Effects of hydrogen peroxide on MAPK activation, IL-8 production and cell viability in primary cultures of human bronchial epithelial cells. *J Cell Biochem. 93(1):* 142-52, (2004).

Peralta, F. M., and Casale, T. B. Orientation and presence of epithelium are key to endotoxin-induced neutrophil migration. *Eur Respir J. 11(5):* 1053-9, (1998).

Peterson, M. W., Walter, M. E., and Gross, T. J. Asbestos directly increases lung epithelial permeability. *Am J Physiol. 265(3 Pt 1):* L308-17, (1993).

Promega, (2008). Promega CellTiter-Blue™ Cell Viability Assay – Technical Bulletin. Accessed Jan 2008. (World Wide Web page) http://www.promega.com/tbs/tb317/tb317.pdf

Puchelle, E. Airway epithelium wound repair and regeneration after injury. *Acta Otorhinolaryngol Belg. 54(3):* 263–270, (2000).

Puchelle, E., Le Simple, P., Hajj, R., and Coraux, Ch. Regeneration of injured airway epithelium. *Ann Pharm Fr. 64(2):* 107-13, (2006).

Punte, C. L., Saunders, L. Z., and Krackow, E. H. The inhalation toxicity of aerosols of 90% hydrogen peroxide. Chemical Corps Medical Laboratories, Report 189. Army Chemical Center, Maryland, 1-11, (1953). (World Wide Web page) ecb.jrc.it/classlab/4599a23_FIN_hydrogenperoxide.doc

Rahman, I. and MacNee W. Lung glutathione and oxidative stress: implications in cigarette smoke-induced airway disease. *Am J Physiol. 277(6 Pt 1):* L1067–L1088, (1999).

Rahman, I., Mulier, B., Gilmour, P. S., Watchorn, T., Donaldson, K., Jeffery, P. K., and MacNee, W. Oxidant-mediated lung epithelial cell tolerance: the role of intracellular glutathione and nuclear factor-kappaB. *Biochem Pharmacol. 62:* 787–794, (2001).

Rahman, I., Biswas, S. K., and Kode, A. Oxidant and antioxidant balance in the airways and airway diseases. *Eur J Pharmacol. 533(1-3):* 222-39, (2006).

Reddel, R. R., Ke, Y., Gerwin, B. I., McMenamin, M. G., Lechner, J. F., Su, R. T., Brash, D. E., Park, J. B., Rhim, J. S., and Harris, C. C. Transformation of human bronchial epithelial cells by infection with SV40 or adenovirus-12 SV40 hybrid virus, or transfection via strontium phosphate coprecipitation with a plasmid containing SV40 early region genes. *Cancer Res. 48(7):* 1904-1909, (1988).

Remick, D. G. and Villarete, L. Regulation of cytokine gene expression by reactive oxygen and reactive nitrogen intermediates. *J. Leukocyte Biol.* ***59:*** 471–475, (1996).

Repine, J. E., Bast, A., and Lankhorst. I. Oxidative stress in chronic obstructive pulmonary disease: oxidative stress study group. *Am. J. Respir. Crit.Care Med. 156 (2 Pt 1):* 341-357, (1997).

Richard, V., Van der Auwera, P., Snoeck, R., Daneau, D., and Meunier, F. Nosocomial bacteremia caused by Bacillus species. *Eur J Clin Microbiol and Infect Dis. 7(6):* 783-385, (1988).

Riss, T. L. and Moravec, R. A. Use of multiple assay endpoints to investigate the effects of incubation time, dose of toxin, and plating density in cell-based cytotoxicity assays. *Assay Drug Dev Technol. 2(1):* 51-62, (2004).

Robinson, C. E., Keshavarzian, A., Pasco, D. S., Frommel, T. O., Winship, D. H., and Holmes, E. W. Determination of Protein Carbonyl Groups by Immunoblotting. *Anal Biochem. 266(1):* 48–57, (1999).

Roebuck, K. A. Oxidant stress regulation of IL-8 and ICAM-1 gene expression differential activation and binding of the transcription factors AP-1 and NF-κB. *Int. J. Mol. Med. 4:* 223, (1999).

Rogers, D. F. The airway goblet cell. *Int J Biochem Cell Biol. 35(1):* 1-6, (2003).

Rogers, D. F. The role of airway secretions in COPD: pathophysiology, epidemiology and pharmacotherapeutic options. *COPD. 2(3):* 341-53, (2005a).

Rogers, J. V., Sabourin, C. L., Choi, Y. W., Richter, W. R., Rudnicki, D. C., Riggs, K. B., Taylor, M. L., and Chang, J. Decontamination assessment of Bacillus anthracis, Bacillus subtilis, and Geobacillus stearothermophilus spores on indoor surfaces using a hydrogen peroxide gas generator. *J Appl Microbiol. 99(4):*,739-48, (2005).

Rosenman, K. D., Reilly, M. J., Schill, D. P., Valiante, D., Flattery, J., Harrison, R., Reinisch, F., Pechter, E., Davis, L., Tumpowsky, C. M., and Filios, M. Cleaning products and work-related asthma. *J Occup Environ Med. 45(5):* 556-63, (2003).

Russell, A. D. Bacterial spores and chemical sporicidal agents. *Clin Microbiol Rev. 3(2):* 99–119, (1990).

Russell, A. D. Mechanisms of bacterial resistance to biocides. *International Biodeterioration and Biodegradation. 36:* 247-265, (1995).

Russell, A.D. Bacterial resistance to disinfectants: present knowledge and future problems. *J. Hosp. Infect. 44:* S57-68, (1999).

Russell, A. D. Bacterial adaptation and resistance to antiseptics, disinfectants and preservatives is not a new phenomenon. *J Hosp Infect. 57:* 97–104, (2004).

Sagai, M., Saito, H., Ichinose, T., Kodama, M. and Mori, Y. Biological effects of diesel exhaust particles. I. In vitro production of superoxide and in vivo toxicity in mouse. *Free Radic Biol Med. 14(1):* 37-47, (1993).

Sakagami, M. *In vivo, in vitro* and *ex vivo* models to assess pulmonary absorption and disposition of inhaled therapeutics for systemic delivery. *Adv Drug Deliv Rev. 58(9-10):* 1030–1060, (2006).

Sanchez, R., Riddle, M., Woo, J., and Momand, J. Prediction of reversibly oxidized protein cysteine thiols using protein structure properties. *Protein Sci. 17(3):* 473-81, (2008).

Sawada, N., Murata, M., Kikuchi, K., Osanai, M., Tobioka, H., Kojima, T., and Chiba, H. Tight junctions and human diseases. *Med Electron Microsc. 36(3):* 147-56, (2003).

SCCP. Scientific Committee on Consumer Products. Opinion on Hydrogen peroxide, in its free form or when released, in oral hygiene products and tooth whitening products SCCP/1129/07 (2007). (World Wide Web page) http://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_o_122.pdf

Schmidt, M. C., Peter, H., Lang, S. R., Ditzinger, G., and Merkle, H. P. In vitro cell models to study nasal mucosal permeability and metabolism. *Adv Drug Deliv Rev. 29:* 51–79, (1998).

Sergeev, I. N. Calcium as a mediator of 1,25-dihydroxyvitamin D3-induced apoptosis. *J Steroid Biochem Mol Biol. 89-90(1-5):* 419-25 (2004).

Setlow, P. Spore germination. *Curr Opin Microbiol. 6:* 550-556, (2003).

Setlow, P. Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. *J Appl Microbiol. 101(3):* 514-525, (2006).

Setlow, B and Setlow, P. Binding of small, acid-soluble spore proteins to DNA plays a significant role in the resistance of *Bacillus subtilis* spores to hydrogen peroxide. *Appl Envriron Microbiol. 59:* 3418-3423, (1993).

Setlow, B., Setlow, C. A., and Setlow, P. Killing bacterial spores by organic hydroperoxides. *J Ind Microbio Biotechnol. 18(6):* 384-388, (1997).

Setlow, B., Loshon, C. A., Genest, P. C., Cavan, A. E., Setlow, C., and Setlow, P. Mechanisms of killing spores of *Bacillus subtilis* by acid, alkali and ethanol. *J Appl Microbiol. 92:* 362-375, (2002).

Seynaeve, C. M., Kazanietz, M. G., Blumberg, P. M., Sausville, E. A., and Worland, P. J. Differential inhibition of protein kinase C isozymes by UCN-01, a staurosporine analogue. *Mol. Pharmacol. 45(6):* 1207-14, (1994).

Shacter, E., Williams, J. A., Lim, M., and Levine, R. L. Different susceptibility of plasma proteins to oxidative modification: Examination by Western blot immunoassay. *Free Radical Biol. Med. 17(5):* 429–437, (1994).

Shankaranarayanan, P. and Nigam, S. IL-4 Induces Apoptosis in A549 Lung Adenocarcinoma Cells: Evidence for the Pivotal Role of 15-Hydroxyeicosatetraenoic Acid Binding to Activated Peroxisome Proliferator-Activated Receptor γ Transcription Factor. *J Immunol.* 170(2): 887-894, (2003).

Shaker, L. A., Russell, A. D., and Furr, J. R. Aspects of the action of chlorhexidine on bacterial spores. *Int J Pharm. 34:* 51-56, (1986).

Shaker, L. A., Furr, J. R., and Russell, A. D. Mechanism of resistance of *Bacillus subtilis* spores to chlorhexidine. *J Appl Bacteriol. 64:* 531–539, (1988).

Shen, B. Q, Finkbeiner, W. E., Wine, J. J., Mrsny, R. J., and Widdicombe, J. H. Calu-3: A human airway epithelial cell line that shows cAMPdependent Cl-secretion. *Am J Physiol.,266(5 Pt 1):* L493–L501, (1994).

Shukla, A., Gulumian, M., Hei, T. K., Kamp, D., Rahman, Q. and Mossman, B. T. Multiple roles of oxidants in the pathogenesis of asbestos-induced diseases. *Free Radic Biol Med. 34(9):* 1117-29, (2003).

Silverthorn, D. U. Human physiology: an integrated approach (2nd ed.). Prentice-Hall International (UK) Limited: London 2001.

Smit-de Vries, M. P., van der Toorn, M., Bischoff, R., and Kauffman, H. F. Resistance of quiescent and proliferating airway epithelial cells to H_2O_2 challenge. *Eur Respir J. 29(4):* 633–642, (2007).

Snipes, M. B., Spoo, J. W., Brookins, L. K., Jones, S. E., Mauderly, J. L., Orwat, T. B., Stiver, J. H., and Dahl, A. R. A method for measuring nasal and lung uptake of inhaled vapour. *Fundam Appl Toxicol. 16(1):* 81-91, (1991).

Spencer, J. P. E., Jenner, A., Aruoma, O. I., Cross, C. E., Wu, R., and Halliwell, B. Oxidative DNA damage in human respiratory tract epithelial cells. Time course in relation to DNA strand breakage. *Biochem. Biophys. Res. Commun. 224(1):* 17–22, (1996).

Spotts-Whitney, E. A., Beatty, Me., Taylor, T. H Jr, Weyant, R., Sobel, J., and Arduino, M. J. Ashford, D. A. Inactivation of *Bacillus anthracis* spores. *Emerging Infect Dis. 9(6):* 623-627, (2003).

Springthorpe, V. S. and Sattar, S. A. Carrier tests to assess the microbicidal activities of chemical disinfectants for use on medical devices and environmental surfaces. *J AOAC Int. 88:* 182-201, (2005).

Staddon, J. M., Herrenknecht, K., Smales, C., and Rubin, L. L. Evidence that tyrosine phosphorylation may increase tight junction permeability. *J Cell Sci. 108(Pt 2):* 609–619, (1995).

Stadtman, E. R. and Berlett, B. S. Reactive oxygen-mediated protein oxidation in aging and disease. *Drug Metab Rev. 30(2):* 225-43, (1998).

Staehelin, L. A., and Hull, B. E. Junctions between living cells. *Sci Am. 238(5):* 140-52, (1978).

Standiford, T. J., Kunkel, S. L., Basha, M. A., Chensue, S. W., Lynch, J. P. 3rd, Toews, G. B., Westwick, J., and Strieter, R. M. Interleukin-8 gene expression by a pulmonary epithelial cell line: a model for cytokine networks in the lung. *J Clin Invest. 86(6):* 1945-1953, (1990).

Steimer, A., Haltner, E., and Lehr, C. M. Cell culture models of the respiratory tract relevant to pulmonary drug delivery. *J Aerosol Med. 18(2):* 137-82, (2005).

Stephenson, K. and Hoch, J. A. Evolution of signalling in the sporulation phosphorelay. *Mol Microbiol. 46(2):* 297-304, (2002).

Steris®, (2008). Accessed 06-06-08. (World Wide Web page) http://www.steris.com/explore/view_product_page.cfm?productid=395).

Stewart, N. and Bacchetti, S. Expression of SV40 large T antigen, but not small t antigen, is required for the induction of chromosomal aberrations in transformed human cells. *Virology. 180(1):* 49-57, (1991).

Stockley, R. A. Role of inflammation in respiratory tract infections. *Am J Med. 99(6B):* 8s-13, (1995).

Stocks, S. M. Mechanism and use of the commercially available viability stain, BacLight. *Cytometry A. 61(2):* 189-95, (2000).

Strieter, R. M. Interleukin-8: a very important chemokine of the human airway epithelium. *Am J Physiol Lung Cell Mol Physiol. 283(4):* L688-9, (2002).

Stubbs, S. Step-by-step selection of cleanroom disinfectants. *Hospital Pharmacy Europe. 18:* 25-26, (2005).

Sun, Y. and Oberley, L. W. Redox regulation of transcriptional activators. *Free Radic Biol Med. 21(3):* 335-48, (1996).

Sun, W., Wu, R., and Last, J. A. Effects of exposure to environmental tobacco smoke on a human tracheobronchial epithelial cell line. *Toxicology. 100:* 163–174, (1995).

Sutton, S. V. W., Proud, D. W., Rachui, S., and Brannan, D. K. Validation of microbial recovery from disinfectants. *PDA J Pharm Sci Technol. 56(5):* 255-266, (2002).

't Mannetje, A., Kogevinas, M., Luce, D., Demers, P. A., Bégin, D., Bolm-Audorff, U., Comba, P., Gérin, M., Hardell, L., Hayes, R. B., Leclerc, A., Magnani, C., Merler, E., Tobías, A., and Boffetta, P. Sinonasal cancer, occupation, and tobacco smoking in European women and men. *Am J Ind Med. 36:* 101–107, (1999).

Takeuchi, K., Kishioka, C., Ishinaga, H., Sakakura, Y., and Majima, Y. Histamine alters gene expression in cultured human nasal epithelial cells. *J Allergy Clin Immunol. 107(2):* 310-4, (2001).

Takizawa, H. Airway epithelial cells as regulators of airway inflammation. *Int J Mol Med. 1(2):* 367-78, (1998).

Takizawa, H., Ohtoshi ,T., Kawasaki, S., Abe, S., Sugawara, I., Nakahara, K., Matsushima, K., and Kudoh, S. Diesel exhaust particles activate human bronchial epithelial cells to express inflammatory mediators in the airways: a review. *Respirology. 5(2):* 197-203, (2000).

Tardif, R., Liu, L., and Raizenne, M. Exhaled ethanol and acetaldehyde in human subjects exposed to low levels of ethanol. *Inhal.Toxicol. 16(4):* 203–207, (2004).

Tennen, R., Setlow, B., Dans K. L., Loshin, C. A., and Setlow, P. Mechanisms of killing spores of *Bacillus subtilis* by iodine, glutaraldehyde and nitrous acid. *J Appl Microbiol. 89:* 30-338, (2000).

Tesfaigzi Y. Roles of apoptosis in airway epithelia. *Am J Respir Cell Mol Biol. 34(5):* 537-47, (2006).

Thompson, A. B., Robbins, R. A., Romberger, D. J., Sisson, J. H., Spurzem, J. R., Teschler, H., and Rennard, S. I. Immunological functions of the pulmonary epithelium. *Eur Respir J. 8(1):* 127-49, (1995).

Timmins, M. BD Bioisciences Discovery Labware Technical Bulletin #449: BD™ Oxygen Biosensor System vs. Existing Methods of Measuring Toxicology, (2002).

Todar, K. (2008). Todar's Online Textbook of Bacteriology. Accessed 15-12-08. (World Wide Web page) http://www.textbookofbacteriology.net/Bacillus_3.html

Tombal, B., Denmeade, S. R., Gillis, J. M., and Isaacs, J. T. A supramicromolar elevation of intracellular free calcium ([Ca(2+)](i)) is consistently required to induce the execution phase of apoptosis. *Cell Death Differ. 9(5):* 561-73, (2002).

Tomita, K., Caramori, G., Lim, S., Ito, K, Hanazawa, T., Oates, T., Chiselita, I., Jazrawi, E., Chung, K. F., Barnes, P. J., and Adcock, I. M. Increased $p21^{CIP1/WAF1}$ and B Cell Lymphoma Leukemia-x_L Expression and Reduced Apoptosis in Alveolar Macrophages from Smokers. *Am. J. Respir. Crit. Care Med. 166(5):* 724–731, (2002).

Truong-Tran, A. Q., Carter, J., Ruffin, R. E., and Zalewski, P. D. The role of zinc in caspase activation and apoptotic cell death. *Biometals. 14(3-4):* 315-30, (2001).

Turi, J. L., Jaspers, I., Dailey, L. A., Madden, M. C., Brighton, L. E., Carter, J. D., Nozik-Grayck, E., Piantadosi, C. A., and Ghio, A. J. Carbonyl Oxidative stress activates anion exchange protein 2 and AP-1 in airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol. 283(4):* L791-L798, (2002).

Valko, M., Leibfritz, D., Moncola, J., Cronin, M. T. D., Mazura, M., and Telser, J. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol. 39:* 44–84, (2007).

Vermeer, P. D., Einwalter, L. A., Moninger, T., Rokhlina, T., Kern, J. A., Zabner, J., and Welsh, M. Segregation of receptor and ligand regulates activation of epithelial growth factor receptor. *Nature. 422:* 322–326, (2003).

Vincent, G., Kopferschmitt-Kubler, M. C., Mirabel, P., Pauli, G., and Millet, M. Sampling and analysis of quaternary ammonium compounds (QACs) traces in indoor atmosphere. *Environ Monit Assess. 133(1-3):* 25-30, (2007).

Wagner, G. W. and Yang, Y-C. Rapid Nucleophilic/Oxidative Decontamination of Chemical Warfare Agents. *Ind. Eng. Chem. Res.* 41*(8):* 1925–1928, (2002).

Walker, D. C., MacKenzie, A., Wiggs, B. R., Hulbert, W. C., and Hogg, J. C. The structure of tight junctions in the tracheal epithelium may not correlate with permeability. *Cell Tiss Res. 235(3):* 607–613, (1984).

Wan, H., Winton, H. L., Soeller, C., Stewart, G. A., Thompson, P. J., Gruenert, D. C., Cannell, M. B. and Garrod, D. R., and Robinson, C. Tight junction properties of the immortalized human bronchial epithelial cell lines Calu-3 and 16HBE14o-. *Eur Respir J. 15(6):* 1058-1068, (2000).

Waters, C. M., Savla, U., and Panos, R. J. KGF prevents hydrogen peroxide-induced increases in airway epithelial cell permeability. *Am J Physiol. 272(4 Pt 1):* L681–689, (1997).

Wax, R. and Freese, E. Initiation of the Germination of *Bacillus subtilis* Spores by a Combination of Compounds in Place of L-Alanine. *J Bacteriol. 95(2):* 433-8, (1968).

Wax, R., Freese, E., and Cashel, M. Separation of two Functional Roles of L-Alanine in the Initiation of *Bacillus subtilis* Spore Germination. *J Bacteriol. 94:* 522-529, (1967).

Weiss, S. J. Tissue destruction by neutrophils. *N. Engl. J. Med. 320(6):* 365-376, (1989).

Weissmann, G., Smolen, J. E., and Korchak, H. M. Release of inflammatory mediators from stimulated neutrophils. *N. Engl. J. Med. 303(1):* 27-34, (1980).

Wexler, A. S. and Sarangapani, R. Particles do not increase vapour deposition in human airways. *J. Aerosol Sci. 29:* 197–204, (1998).

Wheeldon, L. J., Worthington, T., Hilton, A. C., Elliott, T. S., and Lambert, P. A. Physical and chemical factors influencing the germination of Clostridium difficile spores. *J Appl Microbiol. 105(6):* 2223-30, (2008).

Widdicombe, J. H. and Widdicombe, J. G. Regulation of human airway surface liquid. *Respir Physiol. 99:* 3–12, (1995).

Williams, G. J., Denyer, S. P., Hosein, I. K., Hill, D. W., and Maillard, J. Y. The development of a new three-step protocol to determine the efficacy of disinfectant wipes on surfaces contaminated with Staphylococcus aureus. *J Hosp Infect. 7(4):* 329-35, (2007).

Wilson, M. R., Stone, V., Cullen, R. T., Searl, A., Maynard, R. L., and Donaldson, K. In vitro toxicology of respirable Montserrat volcanic ash, *Occup. Environ. Med. 57:* 727–733, (2000).

Witko-Sarsat, V., Delacourt, C., Rabier, D., Bardet, J., Nguyen, A. T., and Descamps-Latscha, B. Neutrophil-derived long-lived oxidants in cystic fibrosis sputum. *Am. J. Respir. Crit. Care Med. 152(6 Pt 1):* 1910-1916, (1995).

Wlodek, L. Beneficial and harmful effects of thiols. *Pol. J. Pharmacol. 54(3):* 215–223, (2002).

Wolkoff, P., Schneider, T., Kildesø, J., Degerth, R., Jaroszewski, M., and Schunk, H. Risk in cleaning: chemical and physical exposure. *Sci Total Environ. 215(1-2):* 135-56, (1998).

Wong, V. Phosphorylation of occludin correlates with occluding localization and function at the tight junction. *Am J Physiol. 273(6 Pt 1):* C1859–C1867, (1997).

Wright, D. T., Cohn, L. A., Li, H., Fischer, B., Li, C. M., and Adler, K. B. Interactions of oxygen radicals with airway epithelium. *Environ Health Perspect. 102(S10):* 85-90, (1994).

Wu, D., Ingram, A., Lahti, J. H., Mazza, B., Grenet, J., Kapoor, A., Liu, L., Kidd, V. J., and Tang, D. Apoptotic release of histones from nucleosomes. *J Biol. Chem. 277(14):* 12001-8, (2002).

Wuytack E. Y., Soons, J., Poschet, F., and Michels, C. W. Comparative study of pressure- and nutrient-induced germination of *Bacillus subtilis* spores. *Appl Environ Microbiol. 66:* 257-261, (2000).

Yamaya, M., Finkbeiner, W. E., Chun, S. Y., and Widdicombe, J. H. Differentiated structure and function of cultures from human tracheal epithelium. *Am J Physiol. 262:* L713–L724, (1992).

Yamaya, M., Sekizawa, K., Masuda, T., Morikawa, M., Sawai, T., and Sasaki, H. Oxidants affect permeability and repair of the cultured human tracheal epithelium. *Am J Physiol. 268(2 Pt 1):* L284–L293, (1995).

Yan, L. J. and Sohal, R. S. Analysis of oxidative modification of proteins. *Curr Protoc Protein Sci., Chapter 14:* Unit 14.4. (2001).

Yan, L. J. and Sohal, R. S. Analysis of oxidative modification of proteins. *Curr Protoc Cell Biol. Chapter 7:* Unit 7.9, (2002).

Ying, J., Clavreul, N., Sethuraman, M., Adachi, T., and Cohen, R. A. Thiol oxidation in signaling and response to stress: detection and quantification of physiological and pathophysiological thiol modifications. *Free Radic Biol Med. 43(8):* 1099-108, (2007).

Yoo, J. W., Kim, Y. S., Lee, S. H., Lee, M. K., Roh, H. J., Jhun, B. H., Lee, C. H., and Kim, D. D. Serially passaged human nasal epithelial cell monolayer for in vitro drug transport studies. *Pharm Res. 20:* 1690–1696, (2003).

Young, S.B. and Setlow, P. Mechanisms of killing of *Bacillus subtilis* spores by hypochlorite and chlorine dioxide. *J Appl Microbiol. 95:* 54-67, (2003).

Zegers, M. M. and Hoekstra, D. Mechanisms and functional features of polarized membrane traffic in epithelial and hepatic cells. *Biochem J. 336(Pt 2):* 257-69, (1998).

Zhang, Z., Kleinstreuer, C., and Kim, C. S. Transport and uptake of MTBE and ethanol vapors in a human upper airway model. *Inhal Toxicol. 18(3):* 169-84, (2006).

Zock, J. P., Plana, E., Jarvis, D., Antó, J. M., Kromhout, H., Kennedy, S. M., Künzli, N., Villani, S., Olivieri, M., Torén, K., Radon, K., Sunyer, J., Dahlman-Hoglund, A., Norbäck, D., and Kogevinas, M. The use of household cleaning sprays and adult asthma: an international longitudinal study. *Am J Respir Crit Care Med. 176(8):* 735-41, (2007).

APPENDICES

11.1 Chapter 2 Appendices

11.1.1 Sterile letheen broth neutraliser.

Sterile Letheen broth neutraliser was used in Chapter 2: sections 2.3.6, 2.3.7 and 2.3.8; and Chapter 3: section 3.3.2. The use of an effective neutraliser is important to avoid any carry over of residual biocide which would exaggerate efficacy, while not compromise the germination of any surviving spores (Russell, 1990; Sutton *et al.*, 2002). Adequate neutralising of the test biocide must also be ascertained to prevent sporostasis occurring and thereby preventing the obtainment of false positives and false negatives. Validation of the neutraliser determines the effectiveness of the neutralizer and is vital as artificial results will be obtained; leading to the exaggeration of the efficacy of the biocide being tested due to the neutraliser not effectively neutralizing the activity of the biocide. The neutraliser used in these experiments allows the various active components of the biocides to be neutralised with the use of just one neutraliser for all biocides tested.

Sterile Letheen Broth Neutralising Solution (Curnyn, 2005):

Letheen Broth Modified 22g (Difco, Becton Dickinson)- compound (contains Letheen broth, Tryptone, Proteose Peptone No 3, Yeast Extract, Sodium Bisulfite)
Sodium thiosulphate 2.5g (Sigma)
L-Histidine Monohydrochloride 0.5g (Sigma)
Saponin 15g (Fluka, Biochemika)
Sterile Distilled Water to 500ml.

22g of Letheen Broth Modified media was dissolved in 500 ml SDW. Once the other components of the neutralising agent formulation had been added, the solution was heated for 1 minute in the microwave to ensure that the product had completely dissolved. Once dissolved to form a solution the neutraliser was autoclaved at 121°C for 15 minutes.

11.1.2 Neutraliser efficacy and toxicity with catalase

The efficacy and toxicity of the neutraliser with both the presence and absence of catalase was tested against the *Bacillus* spores (table 11.1). This was to determine whether the addition of catalase to the neutraliser was required in order to quench the activity of the H_2O_2 containing biocide.

Table 11.1. Percentage recovery of viable spores after neutralisation (with the addition of catalase to the neutraliser). Data are presented as mean percentage reduction in CFU ± standard deviation (SD), (n=3).

Spore test organisms	Percentage recovery of viable spores (%)		
	6% Hydrogen Peroxide and catalase neutraliser	Neutraliser only (no catalase)	Viability control - SDW
B. subtilis ATCC 6633 spores	96.3 ± 0.3	98.0 ± 0.5	100 ± 2.3
B. subtilis subsp. subtilis 168	95.5 ± 2.1	96.8 ± 0.0	100 ± 1.0
B. pumilis ATCC 27142	96.0 ± 0.3	97.0 ± 1.1	100 ± 2.8

The percentage reduction in spores did not differ significantly between the neutraliser solution without the addition of catalase or when catalase was added to the neutraliser ($P > 0.05$, 2-way non-parametric ANOVA). Therefore there is no requirement to add catalase to the neutraliser each time it is to be used, with the dilution effect likely to be responsible for the neutralisation of any H_2O_2 activity.

11.1.3 Germinant solution

Germinant solution was used in Chapter 2: sections 2.3.6, 2.3.7 and 2.3.8; and Chapter 3: section 3.3.2. In order to initiate germination of the *Bacillus* spores, certain compounds can be added to the spore suspension acting as germinants. Spores of some strains can be initiated by L-alanine alone (Wuytack *et al.*, 2000), others require D-glucose either alone or in addition to the L-alanine and some have quite different requirements (Wax *et al.*, 1967; Wax and Freese, 1968). In *B. subtilis* , the two best-known germination pathways are those induced by the chemical effectors alanine and the combination of asparagine, glucose, fructose and potassium ions known as AGFK (Setlow, 2003; Wax and Freese, 1968; Wuytack *et al.*, 2000) which have been found to cause germination of *B. subtilis* spores within seconds. This results in the spores germinating and therefore any recovered spores following exposure to biocide are now, more likely to be detectable. A germinant was also used as biocides may possess differing abilities to cause germination, due to their differing active compounds.

Asparagine Glucose Fructose and Potassium (AGFK) germinant solution (Wax and Freese, 1968; Setlow, 2003):

Asparagine = 0.33 mg/ml
Glucose = 1 mg/ml
Fructose = 1 mg/ml
Potassium Chloride = 3.3 mg/ml
0.1 M Tris Chloride Buffer

Components were dissolved in 50 ml SDW (pH 7.4).

11.1.4 Effects of AGFK germinant on *B. subtilis subsp. subtilis* 168 spores

The AGFK germinant was used because initially the numbers of spores being recovered from the positive controls were found to be lower than the number recovered following disinfection. In some cases log gains rather than log reductions were observed following disinfection. Table 11.2 shows the log reductions or log gains in *B. subtilis subsp. subtilis* 168 spores following disinfection with 5 biocides in both the absence and presence of AGFK germinant solution.

Table 11.2 Log reductions in *B. subtilis subsp. subtilis* 168 spore levels without AGFK germinant solution and with AGFK germinant solution.

Biocide	Without AGFK germinant			With AGFK germinant		
	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3
Quat./Biguanide	0.38	-0.02	-0.06	1.93	1.43	1.71
Quat./Chlorine Dioxide	0.79	0.04	0.10	1.73	1.97	2.01
6% Hydrogen Peroxide	0.81	0.56	0.27	2.23	2.27	1.84
Amphoteric Surfactant	-0.13	-0.23	0.01	0.36	0.20	0.06
70% Ethanol	-0.12	0.07	0.002	0.29	0.27	0.25

Statistical analysis was carried out on this data to determine whether use of AGFK germinant had an effect on the number of spores recovered following disinfection. A non-parametric, 1-tailed Mann-Whitney analysis was carried out on this data and showed that there was a significant difference ($P < 0.05$) between the number of spores recovered following the disinfection process (by way of log reductions) in the presence and absence of AGFK germinant. Therefore, the AGFK germinant was found to encourage the germination of the spores which may have been viable but sub-lethally injured during disinfection. By incorporating the AGFK germinant into the test method it is possible to avoid obtaining any false positive results that may exaggerate the efficacy of a biocide or disinfection method.

11.2 Chapter 3 Appendices

11.2.1 LIVE/DEAD® BacLight™ spore viability assay

To establish that the level of spore killing observed by traditional colony counting was valid, a LIVE/DEAD® BacLight™ (Invitrogen-Molecular Probes, Paisley, UK) viability fluorescence assay was carried out on one of the spore types previously tested.

11.2.1.1 BacLight™ spore viability assay method

The method used was adapted from Hashimoto *et al.*, (1969). 100 µl of *B. subtilis* ATCC 6633 spores (10^7) were mixed with 900 µl of 6% H_2O_2 for 2 or 15 minutes. Following this the spores were centrifuged at 5,000 rpm and the supernatant discarded. The pellet was washed three times by centrifugation at 5,000 rpm in 0.9 % saline. The spore pellet obtained was then resuspended in 1 ml of SDW. 5 µl of the 6 % H_2O_2 treated spore suspension was then spread on a glass coverslip and allowed to dry at room temperature. Following which the spores were fixed twice in 20 µl of acetone (validation for non-kill in Appendix 11.2.1.1.1) for 15-20 seconds. One microlitre of the LIVE/DEAD® BacLight™ stain (containing SYTO 9 (1.67 mM) and Propidium Iodide (18.3 mM) (Invitrogen-Molecular Probes, Paisley, UK) was placed onto a glass microscope slide and the coverslip of the dried spore film was inverted (contaminated side, face down) and then placed onto the slide to allow direct contact between the spores and the BacLight™ stain. The spores were then mounted using Live/Dead BacLight™ mounting oil and visualised using epifluorescence microscopy with a Zeiss Axioskop microscope (100 x / 1·30 Plan NEOFLUAR oil immersion objective) fitted with a Zeiss AxioCam HRc and AxioVision 3·1 software (Carl Zeiss Ltd, Welwyn Garden City, UK). As well as the fluorescence, the spores were also visualised under phase contrast microscopy.

11.2.1.1.1 Acetone testing

A control test was carried out to determine whether the acetone was itself toxic to the spores. This involved adding and drying 5 µl of the spore suspension onto 2 coverslips. Twenty microliters of acetone was added to add to one cover slip, allowed to dry and then another 20 µl of acetone added to aid fixing of the spores to the cover slip. Whereas the spores on the other cover slip were not exposed to any fixing agent. The cover slips were then placed in individual stomacher bags containing 5 ml of Letheen-based neutraliser and agitated for 2 minutes to ensure that all of the spores were detached from the surface of the cover slip and into the neutraliser solution. 100 µl of the neutraliser was then added to 0.9 ml of AGFK germinant and incubated at 37°C for 45 minutes. Following incubation, 100 µl of the germinant treated spores were added to 0.9 ml of neutraliser and a dilution series in the neutraliser carried out. 100 µl of each dilution was then plated out in triplicate onto TSA plates and incubated at 37°C

overnight. Following which the number of CFU observed were noted, with any difference established between spores exposed to acetone and those not.

Table. 11.3 Validation of the viability of *B. subtilis* ATCC 6633 spores following two fixing stages in acetone. Averages are shown as mean ± SD.

Treatment of spores	Number of CFU observed following incubation at 37°C overnight		
	Dilution		
	10^{-5}	10^{-6}	10^{-7}
Non-acetone exposed	TMTC TMTC TMTC	175 171 167 Avg. = 171 ± 4	14 13 12 Avg. = 13 ± 1
Acetone exposed	TMTC TMTC TMTC	178 175 174 Avg. = 176 ± 22	16 13 13 Avg. = 14 ± 2

The number of spores being recovered from the non-acetone exposed and the acetone exposed cover slips were found to be very similar (table 11.3). The differences between the two treatments were non-significant (P = 0.15, unpaired 2-sided t-test) indicating that fixing the spores in acetone did not significantly affect their viability.

11.2.1.2 BacLight™ spore viability assay results

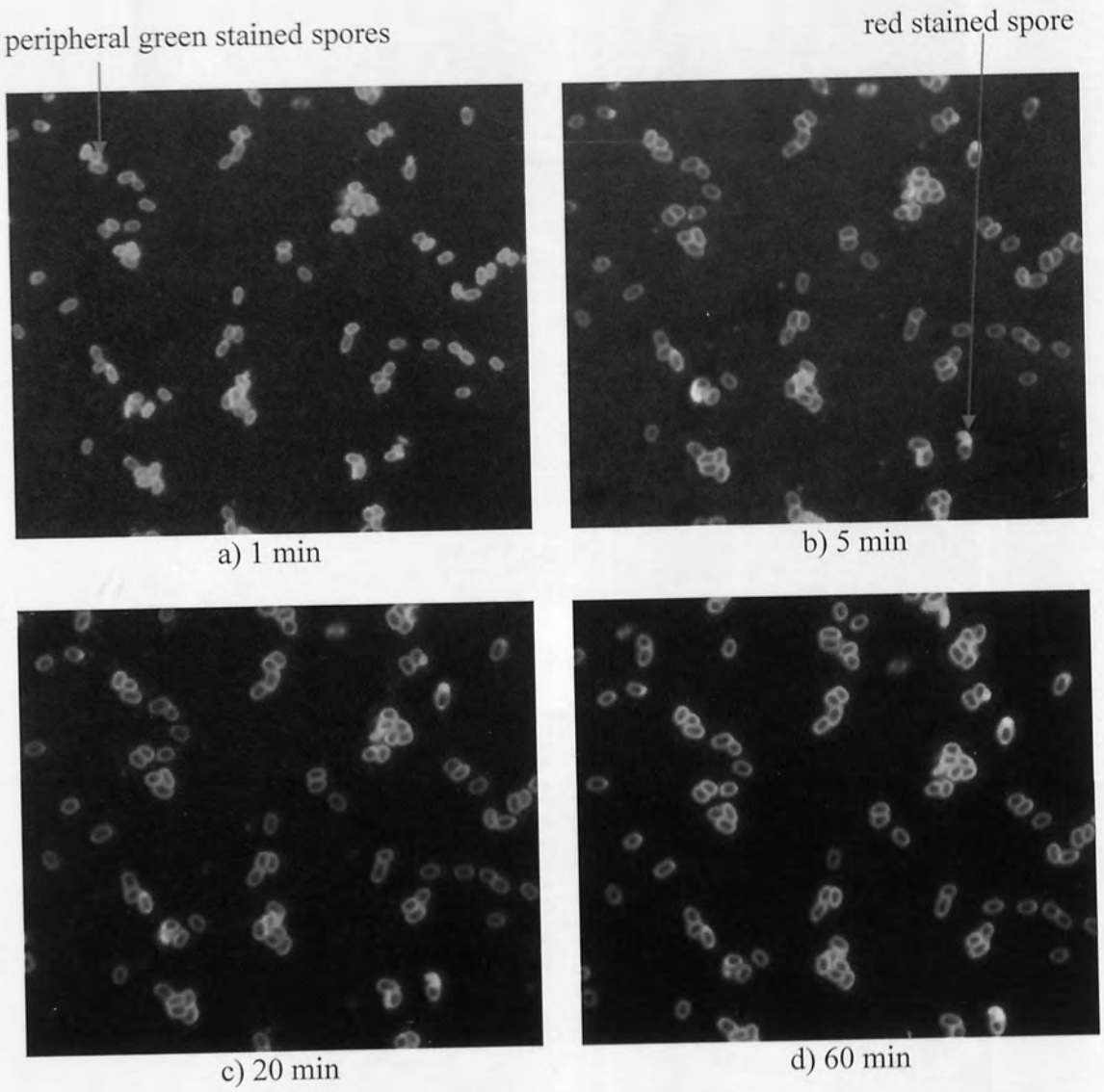
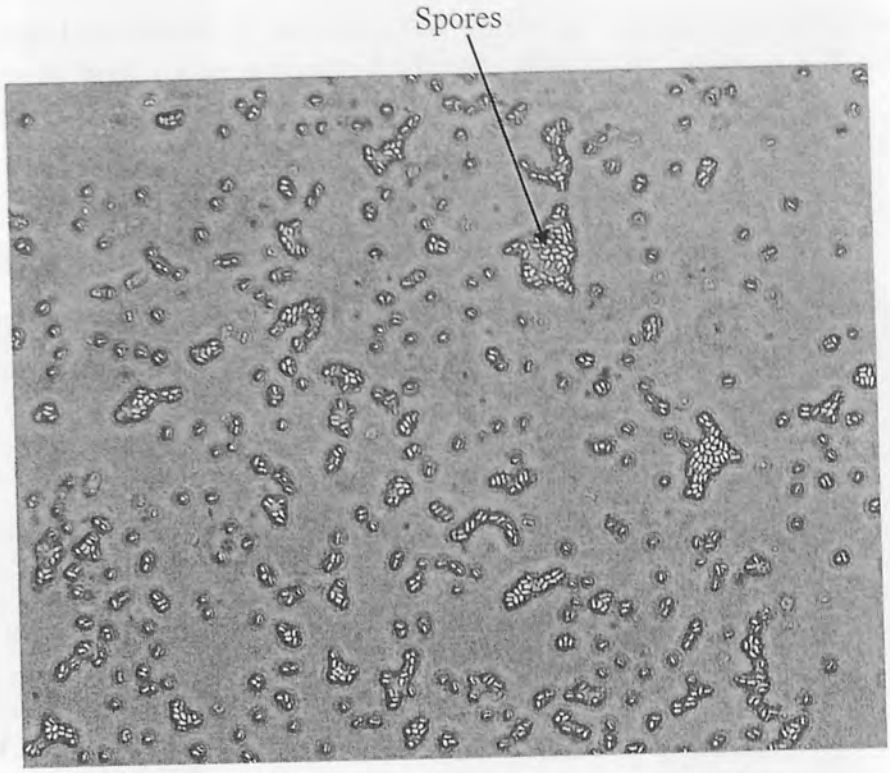
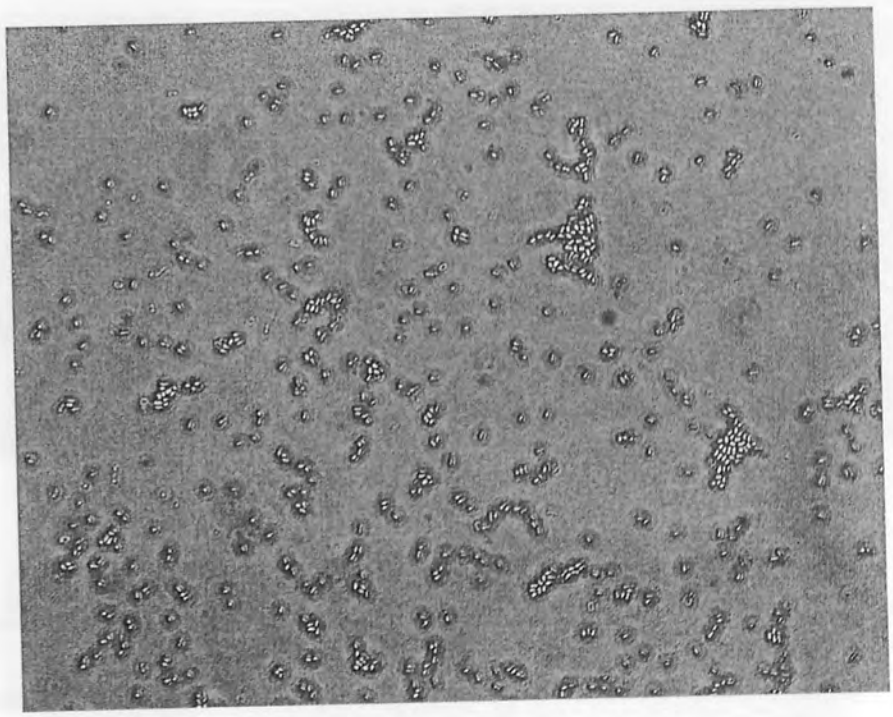


Figure 11.1 *B. subtilis* ATCC 6633 spores exposed to 6% H_2O_2 for 2 minutes, washed and then exposed to BacLight™ reagent for a) 1 min, b) 5 min, c) 20 min and d) 60 min. A fluorescence exposure rate of 3718 ms (milliseconds) was employed throughout the experiment (100x objective).

The majority of the 2 minute H_2O_2 treated spores were peripherally stained green when exposed to the BacLight™ reagent, with some of the spores fluorescing red (Figure 11.1). As time progressed from 1 minute (figure 11.1a) to 60 minutes (figure 11.1d) of exposure, no change in the fluorescence of the spores was observed.



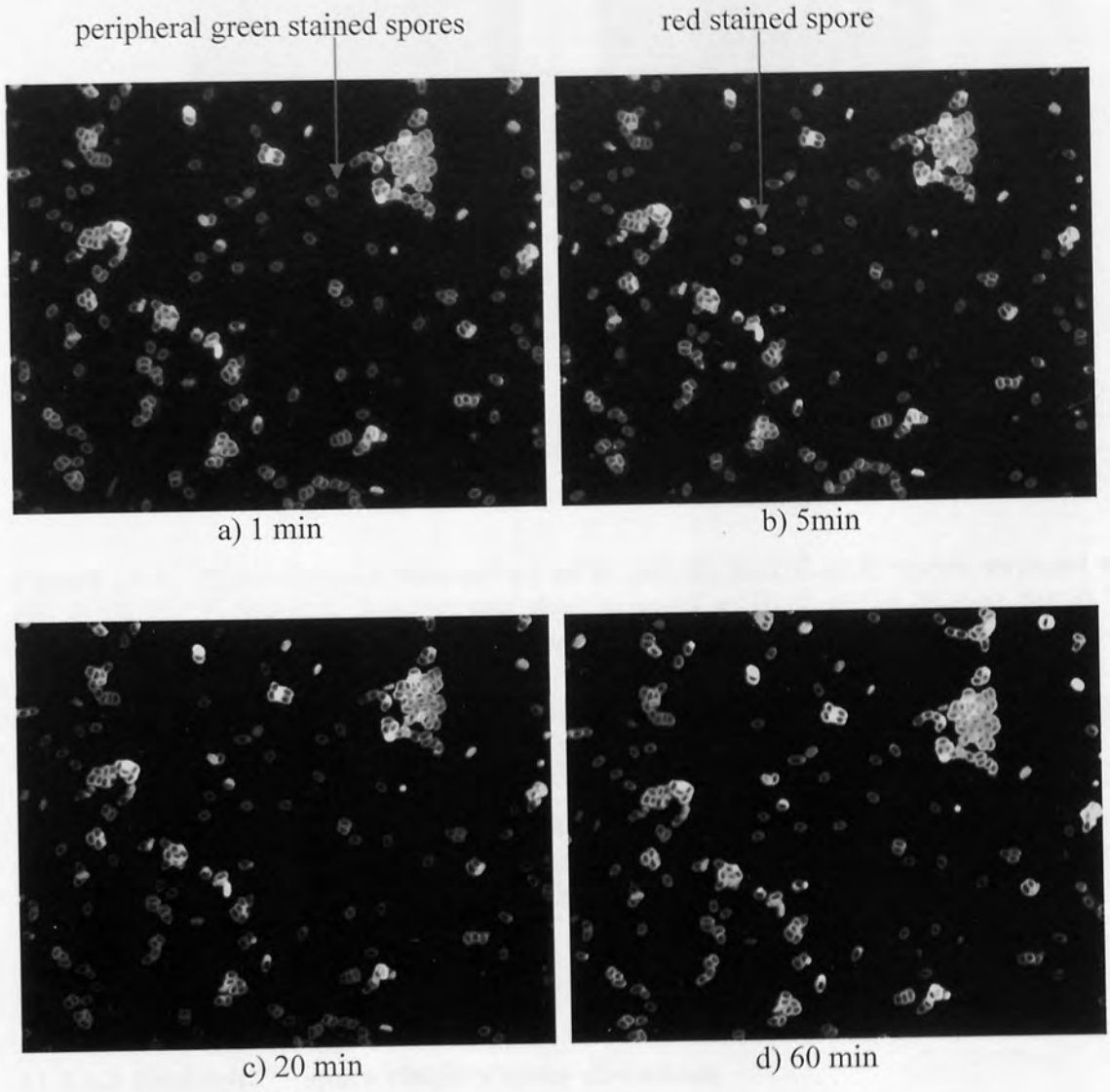
a) 1 min



b) 60 min

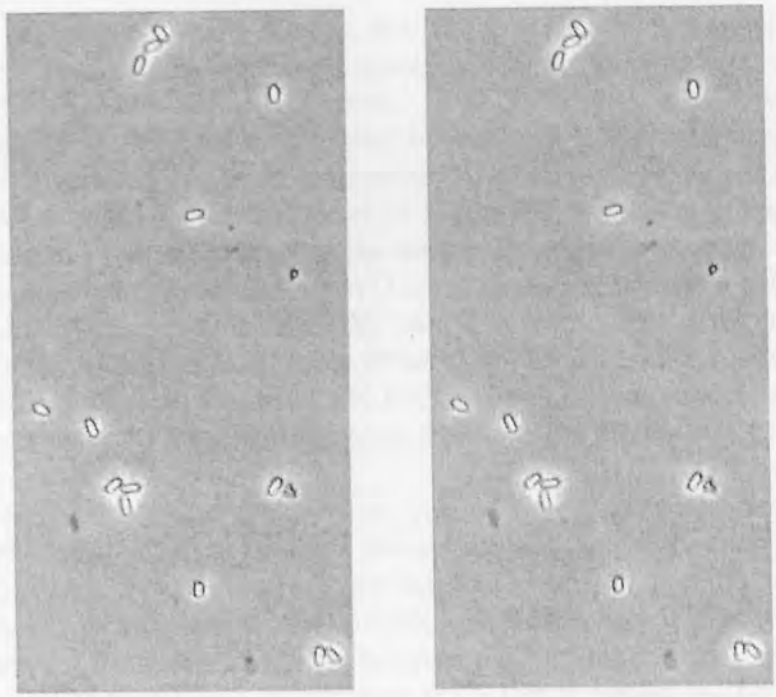
Figures 11.2 Phase-Contrast Microscopy of *B. subtilis* ATCC 6633 spores exposed to 6% H_2O_2 for 2 minutes, washed and then exposed to BacLight™ reagent for a) 1 min and b) 60 min. A phase contrast exposure rate of 1406 ms was employed throughout the experiment. (40x objective). (Note: pictures not taken in the same field of view).

The 2 minute H_2O_2 treated *B. subtilis* ATCC 6633 were visualised as short phase-bright rods following both 1 minute (figure 11.2a) and 60 minutes (figure 11.2b) of exposure to BacLight™ reagent.



Figures 11.3. *B. subtilis* ATCC 6633 spores exposed to 6% H_2O_2 for 15 minutes, washed and then exposed to BacLight™ reagent for a) 1 min, b) 5 min, c) 20 min and d) 60 min. A fluorescence exposure rate of 3863 ms was employed throughout the experiment (100x objective).

The majority of the 15 minute H_2O_2 treated spores were peripherally stained green when exposed to the BacLight™ reagent, with some of the spores fluorescing red (figure 11.3). Some spores were also observed as being entirely fluorescent green. As time progressed no change in the fluorescence of the spores was observed.



a) 1 min b) 60 min

Figure 11.4. Phase-Contrast Microscopy of *B. subtilis* ATCC 6633 spores exposed to 6% H_2O_2 for 15 minutes, washed and then exposed to BacLight™ reagent for a) 1 minute and b) 60 minutes. A phase contrast exposure rate of 1457 ms was employed throughout the experiment (100x objective).

The 15 minute H_2O_2 treated *B. subtilis* ATCC 6633 were visualised as short phase-bright rods following both 1 minute (figure 11.4a) and remained so after 60 minutes of exposure to BacLight™ reagent (figure 11.4b).

11.2.1.3 BacLight™ spore viability assay discussion

The BacLight™ assay is a simple, rapid assay able to distinguish between live and dead bacteria (Stocks, 2004; Laflamme *et al.,* 2004) and has also been used to look at membrane disruption in spores (de León and Moujir, 2008; Wheeldon *et al.,* 2008). The BacLight™ viability reagent is a mixture of 2 nucleic acid stains; the green-fluorescent SYTO 9 stain readily enters both live and dead cells while the red-fluorescent PI stain is only able to enter dead cells. Regarding fluorescence, dormant spores are only peripherally stained green with the BacLight™ reagent with no staining of the spore core (Setlow *et al.,* 2002; Melly *et al.*, 2002). With regards to the 2 minute, 6% H_2O_2 exposure; following 1 minute of contact with the BacLight™ stain both fluorescent red spores (dead) and spores with a green fluorescent rim (viable) were observed. This suggests that a few spores had been killed following 2 minutes contact with the 6% H_2O_2, with the majority of the *B. subtilis* ATCC 6633 spores remaining

viable. With regards to the 15 minute, 6% H_2O_2 exposure; following 1 minute of contact with the BacLight™ stain once again both fluorescent red spores and spores with a green fluorescent rim were observed. This indicates that a small number of spores had been killed on 15 minutes contact with the 6% H_2O_2 with the majority of the spores remaining viable. However, the number of dead spores (stained red) in the 15 minute 6% H_2O_2 exposed spores seemed to be higher in comparison to the 2 minute 6% H_2O_2 exposed cells. This indicates that the longer the contact time with the 6% H_2O_2, the greater the effect on the *B. subtilis* ATCC 6633 spores. However, longer contact with the BacLight™ stain did not alter the results observed after 1 minute. Therefore the 6% H_2O_2 is not seen to have a lasting effect upon the spores after its removal. This was true for both the 2 and 15 minutes 6% H_2O_2 exposed spores, as the number of dead spores did not increase on longer contact with the BacLight™ stain.

Under phase contrast microscopy the live, viable spores are phase-bright with dead spores being phase-dark. So, any change that is observed as a spore changes from being phase-bright to phase-dark would indicate that that particular previously viable spore has been killed on its exposure to the biocide. However, this was not observed after either 2 minutes or 15 minutes of contact between the *B. subtilis* ATCC 6633 spores and the 6% H_2O_2 over the 60 minutes of exposure to BacLight™ stain. So both the 2 minute and 15 minute exposed spores remained phase-bright after 60 minutes and therefore the spores remained viable.

Overall, the 6% H_2O_2 does not kill very many spores following a 2 minute contact time as determined by these BacLight™ experiments. This finding correlates positively with the results obtained using traditional colony counting to show that the H_2O_2 is not very efficacious (no 3 log reductions achieved) against the *B. subtilis* ATCC 6633 spores and that the number of viable spores counted following the tested disinfection methods is reflective of the level of kill being achieved, whereby the number of viable spores remained high following the 6% H_2O_2 exposure.

11.3 Chapter 4 Appendices

11.3.1 VHP gassing

11.3.1.1 Neutraliser test – up to 24 hours

Table 11.4. Recovery of viable bacteria after neutralisation in neutraliser (a) and incubation in SDW (b) for 24 hours. Data presented as mean CFU recovered ± SD (n=3).

a)

Spore Type	CFU (10^6)			
	10 minutes	3 hours	6 hours	24 hours
Bacillus subtilis 168	123 142 134	130 128 133	127 134 129	128 127 120
	Avg.= 133 ± 10	Avg.= 130 ± 3	Avg.= 130 ± 4	Avg.= 125 ± 4
Bacillus subtilis ATCC 6633	119 107 112	111 109 106	114 107 104	105 105 101
	Avg.= 113 ± 6	Avg.= 109 ± 3	Avg.= 108 ± 5	Avg.= 104 ± 2
Bacillus pumilis ATCC 27142	18 15 16	16 16 15	16 15 14	15 14 13
	Avg.= 16 ± 2	Avg.= 16 ± 1	Avg.= 15 ± 1	Avg.= 14 ± 1

b)

Spore Type	CFU (10^6)			
	10 minutes	3 hours	6 hours	24 hours
Bacillus subtilis 168	138 132 135	131 136 130	124 130 132	132 131 130
	Avg.= 135 ± 3	Avg.= 132 ± 3	Avg.= 129 ± 4	Avg.= 131 ± 1
Bacillus subtilis ATCC 6633	117 115 110	112 112 113	109 111 114	116 115 111
	Avg.= 114 ± 4	Avg.= 112 ± 1	Avg.= 111 ± 3	Avg.= 114 ± 3
Bacillus pumilis ATCC 27142	14 15 16	12 14 16	15 14 14	16 15 14
	Avg.= 15 ± 1	Avg.= 14 ± 2	Avg.= 14 ± 1	Avg.= 15 ± 1

There was no significant difference ($P > 0.05$, parametric 2-way ANOVA) between the number of spores present after 10 minutes, in comparison to after 24 hours of incubation in neutraliser (table 11.4a). This was true for all of the spore types the neutraliser was tested against. The difference between the CFUs obtained at 10 minutes and after the 3 hours, 6 hours and 24 hour time-points was also not statistically significant ($P > 0.05$) for all 3 spore types. These results are also reflected in the recovery of the spores when kept in SDW (table 11.4b). Therefore the neutraliser is non-toxic to all of the spores tested. This enables the storage of spores in neutraliser to be feasible up to at least 24 hours. So, if any recovered spores have to be stored in neutraliser for up to 24 hours, their will be no significant loss in spores during that 24 hour period of storage.

11.4 Chapter 5 Appendices

11.4.1 BSA standard curve for protein concentrations

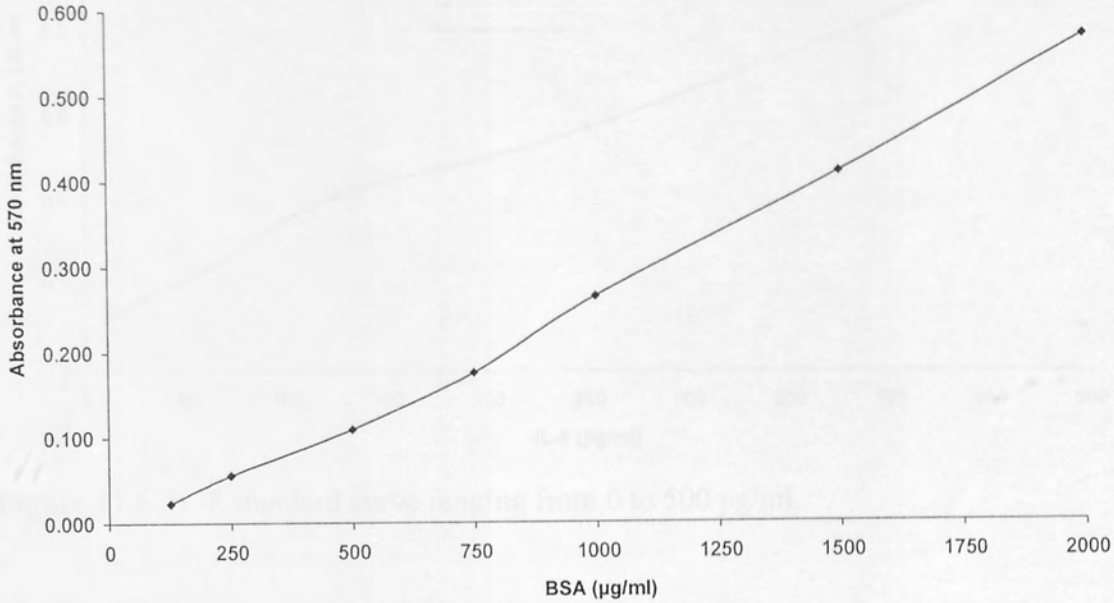


Figure 11.5 BSA standard curve for protein concentrations ranging from 125 to 2000 µg/ml using a colorimetric bicinchoninic acid (BCA) assay kit (Pierce, Northumberland, UK).

11.4.2 IL-8 standard curve

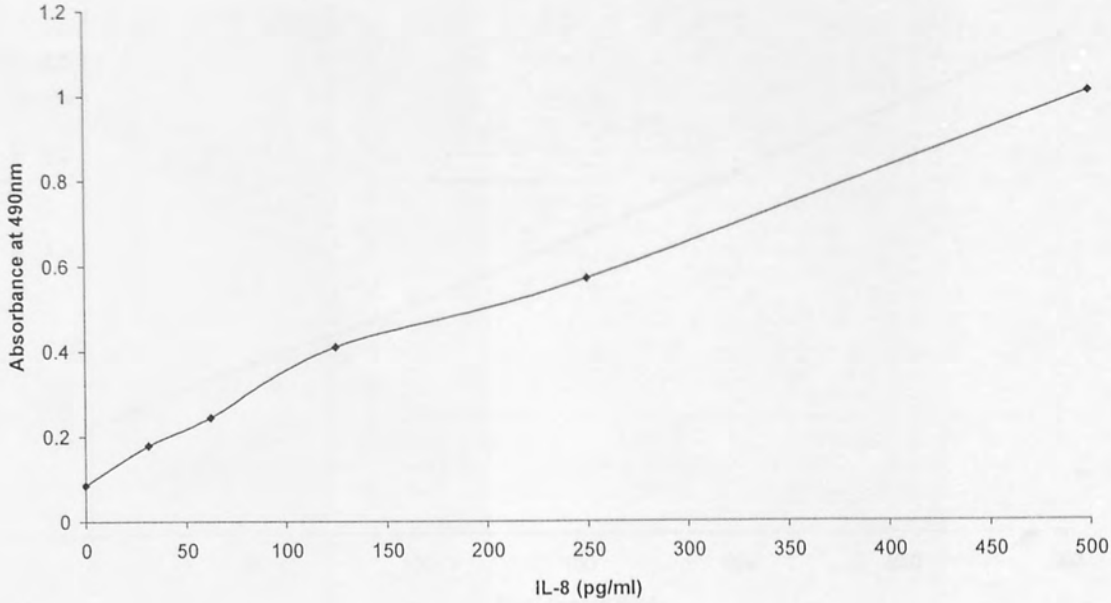


Figure 11.6 IL-8 standard curve ranging from 0 to 500 pg/ml.

11.4.3 Thiol assay standards and standard curve

Table 11.5 Reagent concentrations for thiol standard curve using 8 cysteine standards (values are in µl unless stated otherwise).

Final concentration of thiols (pg)	Volume of cysteine	Volume of DTNB	Volume of pH 8 Buffer	Volume of NaOH	Volume of SDS
0	0	150	40	5	5
50	4	150	36	5	5
100	8	150	32	5	5
200	16	150	24	5	5
300	24	150	16	5	5
400	32	150	8	5	5
500	40	150	0	5	5
500 reagent blank	40	0	150	5	5

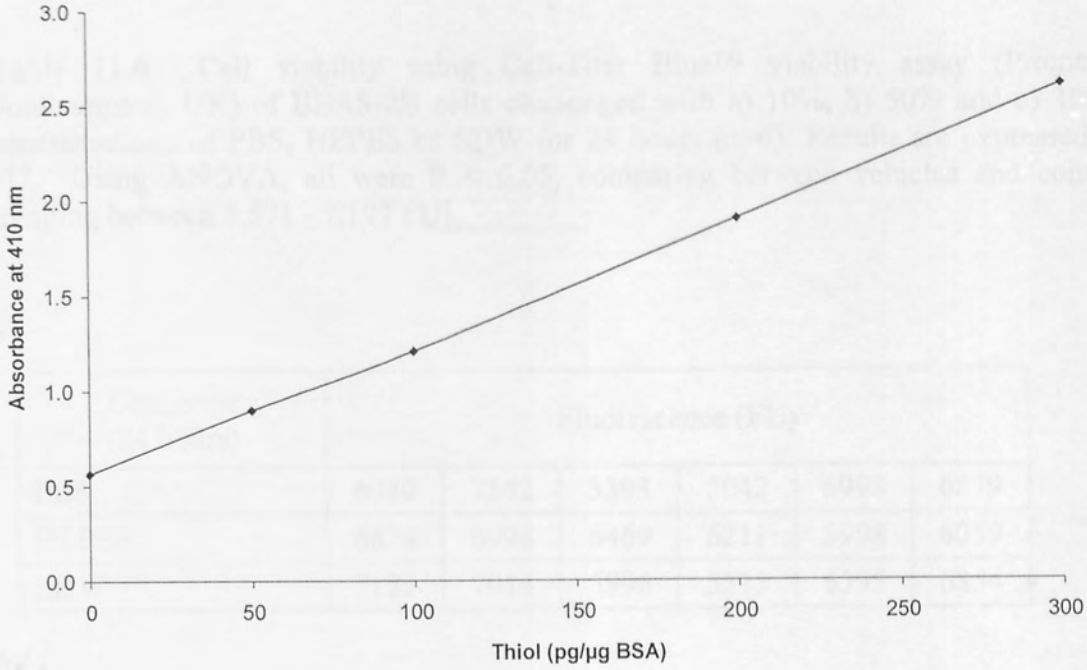


Figure 11.7 Thiol standard curve ranging from 0 to 300 pg/µg BSA.

11.4.4 Carbonyl standard curve

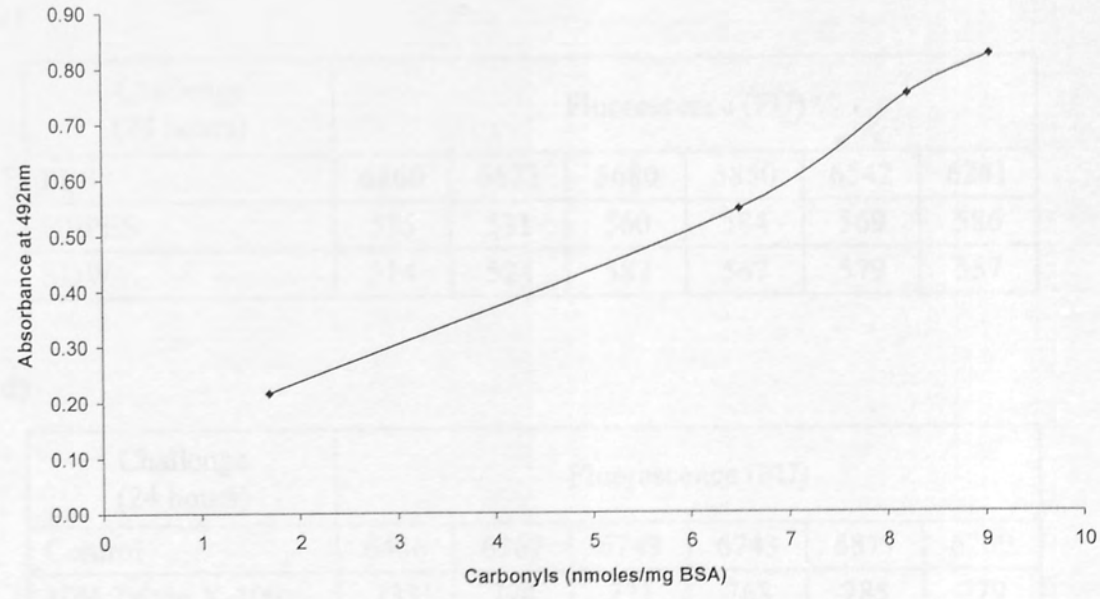


Figure 11.8 Carbonyl standard curve ranging from 1.69 to 9.07 nmoles/µg BSA.

11.4.5 Cell viability of vehicle exposed BEAS-2B cells

Table 11.6 Cell viability using Cell-Titer Blue™ viability assay (Promega, Southampton, UK) of BEAS-2B cells challenged with a) 10%, b) 50% and c) 100% concentrations of PBS, HEPES or SDW for 24 hours (n=6). Results are expressed as FU. Using ANOVA, all were $P < 0.05$, comparing between vehicles and control (ranging between 5,571 - 7,197 FU).

a)

Challenge (24 hours)	Fluorescence (FU)					
PBS	6080	7542	5393	5042	6998	6879
HEPES	6874	6996	6469	6211	5998	6059
SDW	7122	7014	5996	5313	6395	6854

b)

Challenge (24 hours)	Fluorescence (FU)					
PBS	8230	8872	7677	7658	6954	6862
HEPES	6351	8799	3214	4044	5621	5897
SDW	6566	6263	5164	4799	6952	6422

c)

Challenge (24 hours)	Fluorescence (FU)					
PBS	6860	6673	5680	5850	6542	6241
HEPES	535	531	560	584	569	586
SDW	514	524	587	567	579	557

d)

Challenge (24 hours)	Fluorescence (FU)					
Control	6446	6367	6749	6743	6877	6269
10% Triton X-100	733	774	771	768	785	779

11.4.6 Cell viability of biocide exposed BEAS-2B cells

Table 11.7 Cell viability using Cell-Titer Blue™ viability assay (Promega, Southampton, UK) of BEAS-2B cells challenged with a) 1%, b) 10% and c) 0% concentrations of Quat./biguanide, Quat./chlorine dioxide, H_2O_2, amphoteric surfactant, ethanol or IPA for 24 hours (n=5). Results are expressed as FU. Using ANOVA, all were $P < 0.05$, comparing between biocides and control (ranging between 6,515 - 8,523 FU).

a)

Challenge (24 hours)	Fluorescence (FU)				
Quat./biguanide	509	550	526	517	540
Quat./chlorine dioxide	477	499	491	499	481
H_2O_2	505	542	507	522	514
Amphoteric surfactant	551	561	542	552	551
Ethanol	3172	3726	3748	3456	3641
IPA	3601	4810	3776	4129	3995

b)

Challenge (24 hours)	Fluorescence (FU)				
Quat./biguanide	504	526	527	519	517
Quat./chlorine dioxide	478	510	514	510	491
H_2O_2	518	510	491	508	503
Amphoteric surfactant	550	599	595	586	579
Ethanol	470	525	549	510	520
IPA	519	530	574	546	538

c)

Challenge (24 hours)	Fluorescence (FU)				
Quat./biguanide	480	506	502	498	495
Quat./chlorine dioxide	505	525	539	525	518
H_2O_2	540	547	506	528	532
Amphoteric surfactant	695	833	827	810	762
Ethanol	537	514	503	518	517
IPA	497	514	524	508	515

11.4.7 IL-8 release from 10% and 1% biocide exposed BEAS-2B cells

Table 11.8 IL-8 released by submerged cultured BEAS-2B cells exposed to a) 1% and b) 10% concentrations of Quat./biguanide, Quat./chlorine dioxide, H_2O_2, amphoteric surfactant, ethanol and IPA for 24 hours (n=5). Results were expressed as IL-8 pg/1000 BEAS-2B cells in Chapter 5 section 5.4.4.

a)

Challenge (24 hours)	IL-8 (pg/ml)	Percentage viable cells (%)	IL-8 per viable cell	IL-8 per 1000 cells
Control	3559 ± 114	100 ± 0.0	0.04 ± 0.00	36 ± 1
Quat./biguanide	1602 ± 81	7.2 ± 0.1	0.22 ± 0.01	224 ± 12
Quat./chlorine dioxide	2106 ± 66	6.7 ± 0.1	0.32 ± 0.01	317 ± 13
H_2O_2	1248 ± 48	7.0 ± 0.1	0.18 ± 0.01	177 ± 10
Amphoteric surfactant	1464 ± 119	7.5 ± 0.0	0.20 ± 0.02	196 ± 16
Ethanol	1698 ± 104	48.2 ± 1.5	0.04 ± 0.00	36 ± 3
IPA	1726 ± 71	55.2 ± 2.8	0.03 ± 0.00	32 ± 3

b)

Challenge (24 hours)	IL-8 (pg/ml)	Percentage viable cells (%)	IL-8 per viable cell	IL-8 per 1000 cells
Control	3559 ± 114	100 ± 0.0	0.04 ± 0.00	36 ± 1
Quat./biguanide	10130 ± 272	7.0 ± 0.1	1.44 ± 0.05	1440 ± 48
Quat./chlorine dioxide	3500 ± 99	6.8 ± 0.1	0.52 ± 0.02	516 ± 22
H_2O_2	1680 ± 127	6.9 ± 0.1	0.24 ± 0.02	245 ± 19
Amphoteric surfactant	2398 ± 140	7.9 ± 0.1	0.30 ± 0.02	304 ± 19
Ethanol	2478 ± 110	7.0 ± 0.2	0.36 ± 0.02	355 ± 16
IPA	2940 ± 150	7.4 ± 0.1	0.40 ± 0.02	400 ± 17

11.5 Chapter 6 Appendices

11.5.1 Cell viability of vehicle exposed HNEpC

Table 11.9 Cell viability using Cell-Titer Blue™ viability assay (Promega, Southampton, UK) of HNEpC challenged with a) 10%, b) 50% and c) 100% concentrations of PBS, HEPES, SDW, or d) 10% Triton X-100 for 24 hours (n=6). Results are expressed as FU. Using ANOVA, all were $P < 0.05$, comparing between vehicles and control (ranging between 8,342 - 11,854 FU).

a)

Challenge (24 hours)	Fluorescence (FU)					
PBS	9152	9702	10721	9620	11011	7921
HEPES	8509	5768	8482	8757	8793	8160
SDW	7675	6326	9581	11452	8054	10259

b)

Challenge (24 hours)	Fluorescence (FU)					
PBS	10321	9741	10245	8807	11328	10421
HEPES	8238	6028	9694	8512	6429	8495
SDW	5760	8189	5747	7730	9066	7599

c)

Challenge (24 hours)	Fluorescence (FU)					
PBS	603	641	623	743	677	695
HEPES	692	656	664	651	665	612
SDW	615	623	634	633	645	604

d)

Challenge (24 hours)	Fluorescence (FU)					
Control	9749	9543	9877	10269	9429	10404
10% Triton X-100	871	859	856	883	876	895

11.5.2 Cell viability of biocide exposed HNEpC

Table 11.10 Cell viability using Cell-Titer Blue™ viability assay (Promega, Southampton, UK) of HNEpC challenged with a) 1%, b) 10% and c) 100% concentrations of Quat./biguanide, Quat./chlorine dioxide, H_2O_2, amphoteric surfactant, ethanol and IPA for 24 hours (n=5). Results are expressed as FU. Using ANOVA, all were $P < 0.05$, comparing between biocides and control (ranging between 9,030-10,072 FU).

a)

Challenge (24 hours)	Fluorescence (FU)				
Quat./biguanide	892	814	810	856	821
Quat./chlorine dioxide	793	798	757	776	790
H_2O_2	1094	1017	1030	1046	1047
Amphoteric surfactant	1115	1252	1295	1240	1201
Ethanol	936	661	642	800	693
IPA	665	638	646	651	649

b)

Challenge (24 hours)	Fluorescence (FU)				
Quat./biguanide	715	722	703	717	711
Quat./chlorine dioxide	688	696	688	691	691
H_2O_2	884	853	819	866	836
Amphoteric surfactant	922	920	992	963	927
Ethanol	900	608	653	622	620
IPA	679	638	623	650	644

c)

Challenge (24 hours)	Fluorescence (FU)				
Quat./biguanide	692	613	675	675	645
Quat./chlorine dioxide	614	663	650	644	640
H_2O_2	769	802	757	782	769
Amphoteric surfactant	710	666	646	660	686
Ethanol	587	618	623	616	604
IPA	630	625	612	622	622

11.5.3 IL-8 release from 10% and 1% biocide exposed HNEpC

Table 11.11 IL-8 released by submerged cultured HNEpC exposed to a) 1% and b) 10% concentrations of Quat./biguanide, Quat./chlorine dioxide, H_2O_2, amphoteric surfactant, ethanol or IPA for 24 hours (n=5). Results were expressed as IL-8 pg/1000 HNEpC in Chapter 6 section 6.4.4.

a)

Challenge (24 hours)	IL-8 (pg/ml)	Percentage viable cells (%)	IL-8 per viable cell	IL-8 per 1000 cells
Control	2170 ± 98	100 ± 0.0	0.02 ± 0.00	22 ± 1
Quat./biguanide	588 ± 10	12.9 ± 0.2	0.05 ± 0.00	46 ± 2
Quat./chlorine dioxide	457 ± 9	12.1 ± 0.1	0.04 ± 0.00	38 ± 1
H_2O_2	297 ± 8	16.1 ± 0.2	0.02 ± 0.00	18 ± 1
Amphoteric surfactant	850 ± 8	18.8 ± 0.5	0.05 ± 0.00	45 ± 1
Ethanol	360 ± 9	11.5 ± 0.8	0.03 ± 0.00	32 ± 2
IPA	353 ± 4	10.0 ± 0.1	0.04 ± 0.00	35 ± 0

b)

Challenge (24 hours)	IL-8 (pg/ml)	Percentage viable cells (%)	IL-8 per viable cell	IL-8 per 1000 cells
Control	2170 ± 98	100 ± 0.0	0.02 ± 0.00	22 ± 1
Quat./biguanide	1413 ± 19	11.0 ± 0.1	0.13 ± 0.00	129 ± 2
Quat./chlorine dioxide	513 ± 15	10.6 ± 0.0	0.05 ± 0.00	48 ± 1
H_2O_2	296 ± 11	13.1 ± 0.2	0.02 ± 0.00	23 ± 1
Amphoteric surfactant	947 ± 13	14.6 ± 0.2	0.07 ± 0.00	65 ± 2
Ethanol	649 ± 20	9.6 ± 0.1	0.07 ± 0.00	68 ± 3
IPA	527 ± 14	10.0 ± 0.1	0.06 ± 0.00	63 ± 2

11.6 Chapter 7 Appendices

11.6.1 Protein content in 10% and 100% biocide exposed BEAS-2B cells

Table 11.12 Protein content of BEAS-2B cells cultured at ALI and exposed to 10% or 100% Quat./chlorine dioxide, H_2O_2 or ethanol for 24 hours and recovered for 48 hours. Results are expressed as mean ± SEM (n=6).

	Protein content (µg/ml protein)		
	H_2O_2	Quat./chlorine dioxide	Ethanol
0% (control)	327 ± 13	341 ± 12	322 ± 37
10%	117 ± 3	125 ± 2	154 ± 6
100%	91 ± 3	118 ± 3	136 ± 14

This protein content data was used to calculate IL-8 release from 10% and 100% biocide exposed BEAS-2B cells cultured at ALI (Chapter 7 section 7.4.3) as determined in Appendix 11.6.3.

11.6.2 TER of collagen-coated, cell-free Transwells exposed to H_2O_2

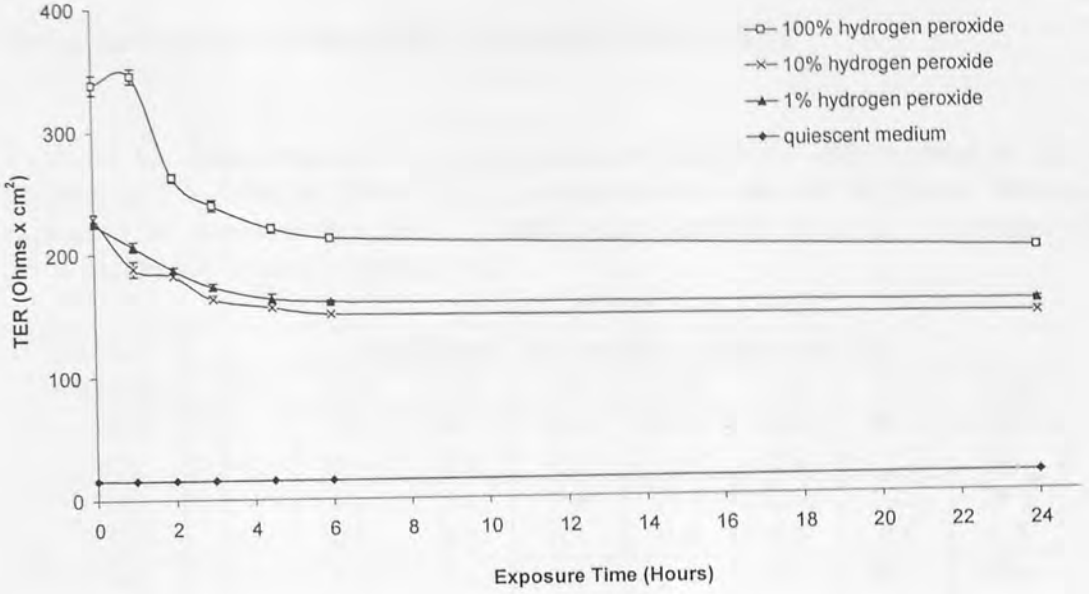


Figure 11.9 The TER (Ohms x cm^2) of collagen-coated, cell-free Transwells over 24 hours of apical exposure to 100%, 10% or 1% H_2O_2, and quiescent medium. Results are expressed as mean ± SD (n=3). TER was significantly higher with H_2O_2 compared with the quiescent medium control ($P < 0.05$).

Prior to any TER measurements upon H_2O_2 treated BEAS-2B, the effect of H_2O_2 on the performance of the voltohmmeter was examined, as exposure of the voltohmmeter electrode to H_2O_2 alone resulted in an increase in TER reading. Consequently, the TER of collagen-coated, cell-free Transwells exposed to H_2O_2 was determined.

The TER of the Transwells that had just quiescent medium added to them averaged 15 ± 1 Ohms x cm^2 throughout the 24 hours (figure 7.4). On addition of the 100% H_2O_2 to the collagen-coated, cell-free Transwells, an average TER of 338 ± 8 Ohms x cm^2 was observed. This value increased to 345 ± 6 Ohms x cm^2 following 1 hour of its addition and then steadily decreased to 212 ± 3 Ohms x cm^2 after 24 hours. The 10% and 1% H_2O_2 addition Transwells had a TER of 163 ± 4 and 151 ± 2 Ohms x cm^2, respectively. The TER of both these sets of Transwells gradually decreased with time.

None of the Transwells that the H_2O_2 additions were added to came back down to the TER of the quiescent medium exposed Transwell, as after 6 hours of exposure the 100%, 10% and 1% exposed Transwells had an average TER of 212 ± 3 Ohms x cm^2 150 ± 1 Ohms x cm^2 and 160 ± 2 Ohms x cm^2, respectively. There was a significant increase in the TER on addition of 10% and 1% H_2O_2 to the Transwells, in relation to the quiescent medium exposed readings ($P < 0.05$). The Transwells with the 100% H_2O_2 additions remained at a level that was significantly higher than the quiescent medium exposed control Transwells, even after 6 hours exposure ($P < 0.05$). After 24

hours of exposure, the TER of the 100%, 10% and 1% H_2O_2 Transwells remained significantly higher than the quiescent medium control Transwells ($P < 0.05$).

11.6.3 Paracellular permeability of BEAS-2B cells to BSA

Table 11.13 Basolateral BSA protein content of BEAS-2B cells cultured at ALI and exposed to 1%, 10% or 100% H_2O_2, or quiescent medium for 24 hours. Results are expressed as mean ± SD (n=3). Data were used to calculate percentage BSA permeability in Chapter 7 section 7.4.2.

Cell challenge	Basolateral BSA protein content (mg/ml)							
	0 hr	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr	24 hr
100% H_2O_2	0 ± 0.0	9 ± 0.4	30 ± 0.0	30 ± 0.0	30 ± 0.0	30 ± 0.1	30 ± 0.1	30 ± 0.1
10% H_2O_2	0 ± 0.0	2 ± 0.0	7 ± 0.3	7 ± 0.1	10 ± 0.6	12 ± 0.1	13 ± 0.6	29 ± 0.7
1% H_2O_2	0 ± 0.0	1 ± 0.1	2 ± 0.3	3 ± 0.2	5 ± 0.6	5 ± 0.2	6 ± 0.2	20 ± 0.3
Quiescent medium	0 ± 0.0	0 ± 0.3	0 ± 0.1	0 ± 0.2	0 ± 0.1	0 ± 0.0	0 ± 0.1	2 ± 0.2

11.6.4 Biocide endotoxin test – Limulus Amoebocyte Lysate assay

An *in vitro* end-product endotoxin assay was carried out on the three biocides that were tested more in-depth: the Quat./chlorine dioxide, 6% H_2O_2 and 70% ethanol. The assay was carried out to ensure that the biocides did not contain any gram negative bacterial endotoxin that would induce an immune response in the airway epithelial cells. Endotoxin-dependent responses may lead to an underestimation of any pro-inflammatory effects of biocides on the cells.

A modified Limulus Amoebocyte Lysate (LAL) test kit (Cambrex, Walkersville, USA) was used to test for endotoxin. The quantitative assay is based on the clotting reaction of horseshoe crab blood to endotoxin. Endotoxin catalyses the activation of a proenzyme in LAL which consequently cleaves a colourless substrate to produce a coloured end product. This can then be measured spectrophotometrically and compared to a standard curve.

Reagents:

LAL Reagent Water for reconstitution
LAL Reagent (lyophilized lysate prepared from the circulating amebocyte and the horseshoe crab *Limulus polyphemus*.
Escherichia coli Endotoxin standard
Chromogenic substrate
Stop reagent (Acetic acid)

A microtitre plate was pre-equilibrated at 37°C in a heating block. 50 µl of each biocide or standard was added to a well. 50 µl of LAL was added to the each well and incubated at 37°C for 10 min. After 10 min, 100 µl of substrate solution (pre-warmed to 37°C) was then mixed with the LAL-treated samples and standards and allowed to incubate at 37°C for a further 6 minutes. The reaction was stopped by adding 100 µl of stop reagent to each well. The presence of endotoxin was indicated by the development of a yellow colour. The absorbance of the standards and biocide samples was then determined spectrophotometrically at 410 nm. The mean absorbance of 4 standards (1.0, 0.5, 0.25 and 0.1 EU/ml) was determined.

Table 11.14 Endotoxin concentration of biocides following LAL assay (n=3).

	Endotoxin Concentration (Endotoxin Units/ml)			Average EU/ml
Quat./chlorine dioxide	-0.055	-0.060	-0.061	-0.06
H_2O_2	-0.078	-0.078	-0.076	-0.08
Ethanol	-0.041	-0.036	-0.036	-0.04
Positive Control - *Pseudomonas aeruginosa* Lipopolysaccharide (LPS)	3.415	3.399	3.483	3.432

Negative values of endotoxin concentration were observed for the biocides. These were taken as 0 and therefore all three sample biocides were found to be free from endotoxin.

11.6.5 IL-8 release from 10% and 100% biocide exposed BEAS-2B cells

Table 11.15 IL-8 released apically and basolaterally by BEAS-2B cells cultured at ALI and exposed to 10% or 100% Quat./chlorine dioxide, H_2O_2 or ethanol for 24 hours and recovered for 48 hours. Data was used to calculate IL-8 release as a percent of control (Chapter 7 section 7.4.3). Results are expressed as mean ± SEM (n=6). Using ANOVA, * represents $P < 0.05$, comparing between biocides and quiescent medium control.

		IL-8 (pg/μg protein)		
		H_2O_2	Quat./chlorine dioxide	Ethanol
Post Challenge (24 hrs)	Apical 0% (control)	0.39 ± 0.03	0.49 ± 0.02	0.45 ± 0.06
	Apical 10%	0.57 ± 0.03	0.62 ± 0.02 *	0.56 ± 0.02
	Apical 100%	0.22 ± 0.02 *	0.53 ± 0.02	0.23 ± 0.02 *
	Basolateral 0% (control)	0.52 ± 0.04	0.79 ± 0.03	1.01 ± 1.12
	Basolateral 10%	1.92 ± 0.03 *	1.08 ± 0.03	0.57 ± 0.04 *
	Basolateral 100%	0.28 ± 0.02 *	0.19 ± 0.02 *	0.20 ± 0.02 *
Post-Recovery (48 hrs)	Apical 0% (control)	0.40 ± 0.02	0.43 ± 0.02	0.40 ± 0.04
	Apical 10%	0.68 ± 0.02 *	1.16 ± 0.01 *	0.55 ± 0.02 *
	Apical 100%	0.61 ± 0.02 *	0.34 ± 0.01 *	0.36 ± 0.01
	Basolateral 0% (control)	0.48 ± 0.04	1.10 ± 0.04	1.31 ± 0.13
	Basolateral 10%	1.91 ± 0.05 *	1.01 ± 0.03	1.39 ± 0.05
	Basolateral 100%	0.21 ± 0.01 *	0.39 ± 0.01 *	0.44 ± 0.02 *

11.7 Chapter 8 Appendices

11.7.1 Protein content in 10% and 100% biocide exposed HNEpC

Table 11.16 Protein content of HNEpC cultured at ALI and exposed to 10% or 100% Quat./chlorine dioxide, H_2O_2 or ethanol for 24 hours and recovered for 48 hours. Results are expressed as mean ± SEM (n=6).

	Protein content (μg/ml protein)		
	H_2O_2	Quat./chlorine dioxide	Ethanol
0% (control)	308 ± 16	310 ± 18	303 ± 9
10%	108 ± 4	113 ± 8	151 ± 10
100%	92 ± 4	106 ± 7	120 ± 3

This protein content data was used to calculate IL-8 release from 10% and 100% biocide exposed HNEpC cultured at ALI (Chapter 8, section 8.4.3) as determined in Appendix 11.7.3.

11.7.2 Paracellular permeability of HNEpC to BSA

Table 11.17 Basolateral BSA protein content of HNEpC cultured at ALI and exposed to 1%, 10% or 100% H_2O_2, or quiescent medium for 24 hours. Results are expressed as mean ± SD (n=3). Data used to calculate percentage BSA permeability in Chapter 8, section 8.4.2.

Cell challenge	Basolateral BSA protein content (mg/ml)							
	0 hr	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr	24 hr
100% H_2O_2	0 ± 0.0	9 ± 0.2	30 ± 0.0	30 ± 0.0	30 ± 0.1	30 ± 0.1	30 ± 0.1	30 ± 0.1
10% H_2O_2	0 ± 0.0	3 ± 0.1	4 ± 0.4	5 ± 0.1	11 ± 0.2	12 ± 0.2	15 ± 0.1	29 ± 0.2
1% H_2O_2	0 ± 0.0	1 ± 0.1	4 ± 0.2	5 ± 0.1	7 ± 0.6	7 ± 0.6	8 ± 0.3	28 ± 0.5
Quiescent medium	0 ± 0.0	0 ± 0.1	0 ± 0.0	0 ± 0.1	0 ± 0.1	0 ± 0.1	0 ± 0.1	1 ± 0.1

11.7.3 IL-8 release from 10% and 100% biocide exposed HNEpC

Table 11.18 IL-8 apically and basolaterally released by HNEpC cultured at ALI and exposed to 10% and 100% concentrations of Quat./chlorine dioxide, H_2O_2 or ethanol for 24 hours and recovered for 48 hours. Data was used to calculate IL-8 release as a percent of control (Chapter 8 section 7.4.3). Results are expressed as mean ± SEM (n=6). Using ANOVA, * represents $P < 0.05$, comparing between biocides and quiescent medium control.

		IL-8 (pg/μg protein)		
		H_2O_2	Quat./chlorine dioxide	Ethanol
Post Challenge (24 hrs)	Apical 0% (control)	0.49 ± 0.03	0.51 ± 0.03	0.45 ± 0.01
	Apical 10%	0.78 ± 0.03 *	1.37 ± 0.06 *	1.09 ± 0.07 *
	Apical 100%	0.42 ± 0.03	1.09 ± 0.07 *	0.81 ± 0.03 *
	Basolateral 0% (control)	1.15 ± 0.07	1.47 ± 0.09	1.29 ± 0.05
	Basolateral 10%	1.40 ± 0.05 *	1.80 ± 0.07 *	1.11 ± 0.07
	Basolateral 100%	0.31 ± 0.03 *	0.56 ± 0.03 *	0.28 ± 0.01 *
Post-Recovery (48 hrs)	Apical 0% (control)	0.38 ± 0.03	0.46 ± 0.03	0.68 ± 0.02
	Apical 10%	0.50 ± 0.02 *	1.09 ± 0.06 *	1.26 ± 0.06 *
	Apical 100%	0.04 ± 0.00 *	0.25 ± 0.02 *	1.16 ± 0.05 *
	Basolateral 0% (control)	1.32 ± 0.07	1.77 ± 0.09	1.29 ± 0.04
	Basolateral 10%	0.20 ± 0.02 *	0.95 ± 0.07 *	1.14 ± 0.05
	Basolateral 100%	0.41 ± 0.06 *	1.04 ± 0.06 *	0.29 ± 0.01 *

CONFERENCES ATTENDED

October 2008	Parenteral Drug Association's 3rd Annual Global Conference on Pharmaceutical Microbiology, Chicago, USA
April 2008	Society for Applied Microbiology Spring Meeting: Broadening Microbiology Horizons, Birmingham, UK
April 2007	17th European Congress of Clinical Microbiology and Infectious Diseases, Munich, Germany
July 2006	Society for Applied Microbiology Summer Meeting: Living Together – Polymicrobial Communities, Edinburgh, UK

LIST OF PUBLICATIONS

Full Papers

Mehmi, M., Marshall, L. J., Lambert, P. A., and Smith, J. C. Evaluation of disinfecting procedures for aseptic transfer in hospital pharmacy departments. *PDA J Pharm. Sci. Technol.* 63(2): 123-138, (2009).

Abstracts

Mehmi, M., Marshall, L. J., Lambert, P. A., and Smith, J. C. Evaluation of disinfecting procedures for aseptic transfer in hospital pharmacy departments. (2008).
(PDA's 3rd Annual Global Conference on Pharmaceutical Microbiology Meeting; published in proceedings)

Mehmi, M., Smith, J. C., Lambert, P. A., and Marshall, L. J. Evaluation of the efficacy of disinfectants used in hospital aseptic dispensing against Bacillus spores. (2007).
(17th European Congress of Clinical Microbiology and Infectious Diseases Meeting; published in proceedings)