Understanding microbial survival in, and the development of resistance to, high-level disinfection

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Parts of this work have been published in scientific abstracts and papers, and presented as detailed below.

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SUMMARY

High-level disinfection is employed throughout the health services in the disinfection of medical equipment, such as endoscopes, to prevent patient-to-patient infections. The likelihood of an endoscope transmitted infection occurring is rare, providing strict guidelines are followed for effective decontamination between procedures. Endoscopes are subjected to rigorous cleaning and high-level disinfection within washer-disinfectors. However, poor decontamination protocols and inappropriate use of disinfectants can lead to can lead to incomplete disinfection and resistance. A number of bacterial strains were isolated from endoscope washer-disinfectors on several occasions. The efficacy of high-level disinfectants (chlorine dioxide, peracetic acid and hydrogen peroxide-based) against these isolates was measured using standard efficacy tests. Resistance mechanisms involved in bacterial survival following biocide exposure were investigated using scanning and transmission electron-microscopy for gross-morphology changes, measurements of expression of detoxifying enzyme and RT-PCR for resistance genes expression, while the role of extracellular polysaccharide in decreasing biocide efficacy, was studied. Two bacterial isolates (*Bacillus subtilis* and *Micrococcus luteus*) were shown to have a high resistance to chlorine dioxide. Electron microscopy showed significant differences between isolates and reference strains. The *B. subtilis* isolate produced large quantities of extracellular polysaccharide, which may be interfering with biocide activity. Genes for catalase and superoxide dismutase were present in *B. subtilis* and enzyme activity varied between isolates and reference strains, indicating a potential involvement in resistance mechanisms, however the extent remains unclear. It was found that the isolate extracellular polysaccharide was not involved in conferring resistance to oxidising agents. This study demonstrated that bacteria can survive high-level disinfection with oxidising agents and that mechanisms conferring resistance are complex but might not be linked to impaired biocide penetration. Furthermore, the findings of this work show that surveillance programmes are essential for monitoring the incidence of biocide resistant isolates in the healthcare environment.

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ABBREVIATIONS

AER	Automated endoscope reprocessor
AOAC	Association of Official Analytical Chemists
ASGE	American Society for Gastrointestinal Endoscopy
ASTM	American Society for Testing and Materials
ATCC	American Type Culture Collection
BD	Becton Dickinson
BS	British Standards
BSA	Bovine serum albumin
BSG	British Society of Gastroenterology
CFU	Colony forming units
CJD	Creutzfeldt-Jacob disease
CMI	5-chloro-2-methyl-isothiazol-3-one
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EAW	Electrolyzed acid water
EDTA	Ethylenediaminetetraacetic acid
EGD	Esophagogastroduodenoscopy
EN	European Standards
EPS	Extracellular polymeric substances
g	Gravitational force
GTA	Glutaraldehyde
HCV	Hepatitis C virus
HLD	High-level disinfection
HTM	Heath Technical Memorandum
LPS	Lipopolysaccharide
MDA	Medical Devices Agency
MHRA	Medicines and Healthcare Products Regulatory Agency
MIC	Minimum inhibitory concentration
mRNA	messenger RNA
MRSA	Methicillin resistant *Staphylococcus aureus*

MSSA	Methicillin sensitive *Staphylococcus aureus*
NCBI	National Centre for Biotechnology Information
NHS	National Health Services
OD	Optical density
OPA	*Ortho*-phthalaldehyde
PAA	Peracetic acid
PCR	Polymerase chain reaction
QAC	Quaternary ammonium compounds
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT PCR	Reverse transcription polymerase chain reaction
RT	Reverse transcription
SD	Standard deviation
SEM	Scanning electron microscopy
SGNA	Society of Gastroenterology Nurses Association
SOD	Superoxide dismutase
TAE	Tris-acetate-EDTA
TEM	Transmission electron microscopy
TSA	Tryptone soya agar
TSB	Tryptone soya broth
TSC	Tryptone sodium chloride
USP	US Pharmacopoeia
vCJD	Variant Creutzfeldt-Jacob disease
w/v	Weight for volume
w/w	Weight for weight
WD	Washer-disinfector

Chapter 1:

Introduction

1 Introduction

1.1 History of Disinfection

Chemical control of microorganisms was established as a science around the early 1800s (Talaro *et al.*, 1997), although it has been practiced since at least as early as c. 450BC by the storing of water in vessels made from copper or silver (Fraise, 2004). Chemotherapeutic agents which were used more recently during the 1800s included cinchona bark, containing the active alkaloid quinine which was used as an anti-malarial agent, the use of mercury for treating syphilis (although this is, of course, now known to be toxic to humans too!) and chaulmoogra oil for treating leprosy (Greenwood, 1995).

Paul Ehrlich, often referred to as "the father of chemotherapy" (Greenwood, 1995), was one of the main contenders in the search for antimicrobials. His studies first started with dyes used for staining tissues for histological examination, and the possibility that such dyes could show selective toxicity. However his attentions later turned to arsenicals and in 1909 after testing the 606th derivative of atoxyl, he demonstrated a compound capable of curing animals of the spirochetes of syphilis, relapsing fever and chicken spirillosis (Greenwood 1995).

Alcohol has been used for disinfection purposes for over 2000 years, and in the 19th and 20th centuries compounds such as phenolics and

hypochlorites were used in disinfection (the first recorded use of other chemical disinfectants are listed in table 1.1).

Table 1.1 Chemical disinfection important years.

Year	Disinfectant Agent
1798	Bleaching powder first made
1872	Bleaching powder used in preparations as a deodorant and disinfectant.
1843	Introduction of chlorine to water
1839	Iodine used as a wound dressing
1843	Hydrogen peroxide first examined
1915	Chlorine releasing compounds reported
1916	Quaternary compounds introduced

(Table information sourced from Fraise, 2004).

Sterilization practices were one of the many interests of the Manchester physician, William Henry in the 1830s, whose name is taken for one of the gas laws. His studies include work on the effects of heat as a disinfectant. He placed contaminated clothes which had been worn by sufferers of typhus and scarlet fever in a pressure vessel and showed that if the clothes were then worn again by other individuals they did not contract the disease (Fraise, 2004).

1.2 Definitions

Antimicrobial agents are chemicals which are used to control or prevent infection. Biocides belong to this category, and this is the general term which is used to describe a chemical agent which can inactivate or kill microorganisms. The activities of biocides can be classified as –static (growth inhibitory) or –cidal (bacterial death) (McDonnell *et al.*, 1999). Biocides are currently in use as antiseptics, preservatives and disinfectants; these are used extensively in the healthcare setting frequently as part of hygiene measures, since hospital-acquired infections are a major concern. There are four different types of decontamination processes, which are described below.

1.2.1 Sterilization

Sterilization is the removal or killing of all microorganisms (with the possible exception of prions) from a particular surface, piece of equipment, product or culture medium. Sterilisation can be achieved by either physical (dry heat, steam under pressure (autoclave), irradiation, filtration) or chemical processes (ethylene oxide gas, hydrogen peroxide gas plasma) (Rutala and Weber, 1999a).

1.2.2 Disinfection

Disinfection reduces the number of microorganisms on inanimate objects to a level which is not harmful. However, it may not kill bacterial

endospores. Disinfection can be achieved through the use of liquid chemicals or wet pasteurisation. The disinfection process can be made less efficient through a number of factors, including poor cleaning prior to disinfection, organic or inorganic load, concentration or exposure time of the disinfectant, temperature and pH, and also the compatibility of the object that is being disinfected, for example endoscopes containing lumens, occluded ends and long channels challenge the disinfection process. Examples of commonly used chemical disinfectants include alcohols, oxidizing agents and quaternary ammonium compounds.

1.2.3 Cleaning

Cleaning is the process where the physical removal of contaminants occurs. The physical removal of contaminants can be facilitated with water, detergents or enzymatic cleaners. Cleaning is a pre-requisite to ensure that good disinfection or sterilization is achieved, especially in the decontamination of endoscopes (Medical Devices Agency Manual Part 1, 2002). Cleaning can play an important part in disinfection process by removing organic and inorganic soil which may inhibit the action of biocidal agent.

1.2.4 Antisepsis

Antisepsis is a form of disinfection, involving the destruction or inhibition of microorganisms on living tissue. Chemicals used as antiseptics are applied to the skin to prevent the possibility of infection, such as before surgical procedures (Hugo *et al.*, 1998). Antiseptics are distinguished from antibiotics which are usually used to eliminate micro-organisms from inside the body. In order to determine which biocide or type of process is required the category of decontamination must be chosen, as shown in table 1.2.

Table1.2. Category of decontamination.

Category	Example	Equipment	Measure
High Risk	Penetration of the skin, mucous membranes, sterile body parts.	Surgical instruments and needles.	Sterilization
Medium Risk	Use on mucous membranes.	Endoscopes, bedpans	Disinfection or sterilization
Low Risk	Use on un broken skin.	Wash pans, mattresses.	Cleaning

There are many different types of disinfectants which are utilized in order to control infection but not all disinfectants are sporicidal. There are different categories of action which these chemicals fall into which can be described as follows.

1.2.5 High-level disinfection

High-level disinfection can be achieved via heat or liquid chemical disinfection. Both have the ability to destroy all microorganisms but may not kill all bacterial spores present (Rutala *et al.*, 2004a). Examples of high level liquid disinfectants include glutaraldehyde, peracetic acid and hydrogen peroxide. Many compounds are not sporicidal but sporostatic, which inhibit germination or outgrowth. Examples of these types of compounds include phenols, QACs, biguanides and alcohols. Sporicidal agents include glutaraldehyde, peroxgens and formaldehyde (Russell, 2004).

1.2.6 Intermediate-level disinfection

Intermediate-level disinfection (through liquid disinfection) is the destruction of vegetative bacteria including *Mycobacterium tuberculosis* but not all viruses and fungi. Intermediate disinfection with liquid will have little or no effect against spores (Hugo *et al.*, 1998; Rutala *et al.*, 2004a).

1.2.7 Low-level disinfection

Low-level disinfection liquid contact can also kill most vegetative bacteria fungi and viruses, but low-level disinfection does not affect spores or mycobacteria (Hugo *et al.*, 1998; Rutala *et al.*, 2004a).

1.3 Disinfectants

Disinfectants are chemical agents used to kill microorganisms, although not necessarily all microorganisms present. The British Standards Institution further defines disinfection as not necessarily killing all microorganisms but reducing the level which is harmful (British Standards Institution 1986).

The ideal disinfectant would fit into the following categories: effective against a wide range of organisms; compatible with endoscopes, endoscope accessories, and washer-disinfectors; non-irritant and safe to use; environmentally friendly. However, not all disinfectants used fall into all of these categories.

Chemical disinfection is an ideal choice for use with endoscopes and washer-disinfectors as heat sterilisation cannot be used on these pieces of equipment. Several disinfectants can be used in conjunction with endoscope washer-disinfectors, which are the standard means of disinfecting endoscopes. It is always essential that these disinfectants are used in accordance with manufacturers' instructions and paired with the appropriate devices.

1.3.1 Oxidising agents

1.3.1.1 Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is an oxidizing agent which is used in disinfection, sterilization and antisepsis. It was first isolated by the French chemist Louis Jacques Thenard in 1818 (Lever and Sutton 1996). It is a clear, colourless biocide which can be degraded into oxygen and water; it is used commercially in concentrations ranging from 3% to 90% (McDonnell *et al.*, 1999). It works as a biocidal agent by producing destructive hydroxyl free-radicals which can attack areas of the bacterial cell components, such as membrane lipids and DNA. H_2O_2 is sporicidal and bactericidal, but is toxic if ingested and is especially dangerous if it comes into contact with the eyes so great care is taken when it is being used. The concentration of hydrogen peroxide employed is important in order to be effective; it has been shown that a concentration of 10% inactivates 10^6 vegetative *Bacillus* spp. cells in 60 minutes, whereas a concentration of 3% takes 150 minutes to inactivate the same number of organisms. These results were shown in 6 out of 7 trials (Rutala and Weber, 1999). It has also been shown that 7.5% of hydrogen peroxide is more efficient at eradicating *Bacillus* spores than 2% glutaraldehyde (Rutala and Weber, 1999). Hydrogen peroxide can be found at a 7.5% concentration under the trade name Sporox (Reckitt & Colman Inc.), and EndoSpor Plus Sterilizing and Disinfecting Solution (Cottrell limited), all disinfectants used in endoscope disinfection.

Hydrogen peroxide as an antiseptic at a concentration of 3% has many uses such as wound cleaning, bed sore care, mouth washing, and treatment of periodontal diseases and is highly effective on anaerobic bacteria where the breakdown product of oxygen, is lethal to these organisms (Talaro and Talaro, 1999). Although the catalase enzyme can protect cells from metabolically produced hydrogen peroxide the concentration used in practice overcomes this. Hydrogen peroxide as a disinfectant can be used to disinfect medical supplies and equipment, for example endoscopes, urinary drainage bags, renal dialysis equipment, and is also used to disinfect contact lenses (Lever *et al.*, 1996). Hydrogen peroxide can also be used in vapour form for the sterilization of larger items and rooms. Hydrogen peroxide can be found in disinfectant formulations with other biocides, such as Cidex PA which contains peracetic acid and 1% hydrogen peroxide (Shumaway and Broussard, 2003).

1.3.1.2 Peracetic Acid

Peracetic acid (PAA) is considered to be fundamentally more potent a biocide than hydrogen peroxide. This is due to its wider range of activity: it is sporicidal, bactericidal, virucidal and fungicidal at concentrations below 0.3% (McDonnell and Russell, 1999). The mechanism of action of peracetic acid is that it denatures proteins and disrupts the cell-wall leading to increased permeability. Peracetic acid decomposes into acetic acid and oxygen. Peracetic acid can be used in

the inhibition of fungal growth on fruit and in the sterilization of medical devices such as pacemakers. There are a number of products that contain peracetic acid in various formulations, e.g. Nu Cidex 0.35% (Johnson & Johnson), Steris 0.20% (Steris Corporation), Anioxyde 1000 (Anios) (Rey *et al.*, 2003).

Peracetic acid is found to have some advantages over other biocides. It is more effective than glutaraldehyde at penetrating and removing organic matter, and peracetic acid does not fix proteins and can dissolve organic matter (Rey *et al.*, 2003). It is claimed to be less of an irritant to staff using the product and it is thought to be safer to the environment when compared to glutaraldehyde. Also there is currently no evidence that microorganisms develop resistance to this biocide; this may be due to its broad spectrum of targets and activity (Rey *et al.*, 2003). Disadvantages include its corrosiveness (it corrodes copper, brass and steel) and its pungent vinegar like odour.

1.3.1.3 Chlorine dioxide

Chlorine dioxide (ClO_2) is also an oxidizing agent and is effective against non-sporing bacteria, viruses, and spores (Kruse and Neumann 2003). It was first isolated in the 19th century, and then later used as a disinfecting additive to the municipal water supplies of Europe after 1850 (Benarde *et al.*, 1965). The mode of action of chlorine dioxide is not by the inactivation of DNA (Roller *et al.*, 1980), but through the

oxidation of sulphydryl groups, which are essential for the activity of many enzymes (Roller *et al.*, 1980).

Chlorine dioxide formulations come in two parts: a base solution and an activator solution, which are then mixed together and diluted to the desired concentration using water. Examples of the chlorine dioxide disinfectants which are commercially available include Tristel 700-1000 ppm (Tristel) and Dexit (Genesis Medical Ltd).

Although chlorine dioxide is extremely effective as a disinfectant, constant use may cause damage to endoscopes and washer-disinfectors (Coates, 2001). Chlorine dioxide is compatible with titanium, stainless steel, silicone rubber, ceramics, PVC and polyethylene, however equipment should always be checked frequently for damage (Medical Device Agency device bulletin, 2002). The use of chlorine dioxide in automated washer-disinfectors is likely to require longer contact times (Rey *et al.,* 2003). Chlorine dioxide is also a respiratory irritant and causes irritation to skin and eyes, and it also has a strong chlorine odour (Rey *et al.,* 2003). It should always be stored in sealed containers.

1.3.1.4 Other Oxidizing Agents

1.3.1.4.1 Ozone

Ozone (O_3) is a strong oxidizing agent and causes cell wall and membrane damage and the inactivation of enzymes (McDonnell, 2007). Ozone can be applied to water and waste water as a disinfectant, but is primarily used for odour control fumigation at higher concentrations. It is effective against bacteria and viruses at concentrations ranging from 0.2 to 0.5 mg/litre, and resistance can be observed with spores and mycobacteria. It is as safe as it is environmentally friendly, so little time is needed for aeration after area decontamination (McDonnell, 2007).

1.3.1.4.2 Vapour-phase Hydrogen peroxide (VHP)

VHP has been offered as a method for the decontamination of rooms, sterilising of dental instruments and is an emerging technology for the sterilisation of endoscopes. Hydrogen peroxide gas has greater activity at a lower concentration compared to liquid hydrogen peroxide. Concentrations range from 0.0001 to 0.001% (McDonnell, 2007).

1.3.2 Alkylating agents

1.3.2.1 Glutaraldehyde

Glutaraldehyde is available commercially as a 2, 2.5 or 50% solution, and for disinfection purposes a concentration of 2% is generally used (e.g. Cidex, Asep, Totacide 28, Steranios) (Rey *et al.*, 2003). Various manufacturers list glutaraldehyde as being compatible with their endoscopes, including Olympus, Pentaxt and Fujinon (Shumway, 2003). However, manufacturer instructions should always be followed regarding the compatibility of disinfectants with products requiring disinfection.

Glutaraldehyde possesses high anti-microbial activity. It is active against fungi, vegetative bacteria and spores, some viruses (including human immunodeficiency virus and hepatitis B virus) and with longer contact times is effective against vegetative *Bacillus* spp. (Isomoto *et al.*, 2006). Glutaraldehyde can be effective within less than five minutes, although *Mycobacteria* are more resistant requiring longer contact times, and therefore it is recommended that medical devices such as endoscopes are immersed for 20 minutes in a 2% glutaraldehyde solution (British Society of Gastroenterology working party report, 2005).

Organic matter can affect the efficacy of disinfectants (discussed in section 1.3.4.4). However, glutaraldehyde can remain relatively active in the presence of high levels of organic matter, such as a 20% serum (Moore and Payne, 2004). Although it is stated above that glutaraldehyde can be effective against bacterial spores, this is not an easy task as it takes longer contact times, as demonstrated by the Association of Official Analytical Chemists (AOAC) who tested dried spores of *B. subtilis* against 2% alkaline glutaraldehyde, showing it required up to 10 hours to achieve sterilisation at 20°C (Rubbo *et al.*, 1967). Glutaraldehyde was once the preferred choice of high level disinfectant, especially in the disinfection of medical devices due to its non-corrosive nature against most materials. However, it has its problems. It can be hazardous towards humans and animals causing irritation to the skin, eyes, throat and lungs (Shumway, 2003). Problems also occur when devices are not rinsed completely after the disinfection process the residuals ultimately cause chemical colitis, pancreatitis and mucosal damage in patients (Shumway, 2003). Problems have arisen with its toxicity and due to its problems associated with dermatitis and asthma (Isomoto *et al.*, 2006). Another problem caused by glutaraldehyde is that it can fix proteins, which can then aid the proliferation of biofilms (Rey *et al.*, 2003). Therefore, other high-level disinfectants are now favoured, and those products based on glutaraldehyde (e.g. CIDEX Activated Glutaraldehyde solution, ASEP, Totacide) have been withdrawn from supply in the UK (Niven, 2007).

1.3.2.2 *ortho*-phthalaldehyde

Ortho-phthalaldehyde (OPA) is an aromatic aldehyde which has been shown to have bactericidal and virucidal activity (Walsh *et al.*, 1999a,b; Walsh *et al.*, 2001; Rutala *et al.*, 2001). OPA does not interact as effectively as glutaraldehyde with amino acids, proteins and microorganisms (Moore and Payne, 2004). OPA can be found in commercial formulations at a concentration of 0.55% (Cidex OPA, Johnson & Johnson's advanced sterilisation products), and is a fast acting disinfectant with tuberculocidal activity and superior anti mycobacterial activity to glutaraldehyde. OPA can also achieve partial elimination of vegetative *Bacillus* in the presence of organic matter (Rey *et al.*, 2003). Also it is more stable and has a lower vapour pressure which makes it less hazardous than glutaraldehyde. However it is a potential respiratory and dermal irritant, and should be handled as carefully as glutaraldehyde (Shumway, 2003). The main disadvantages of OPA are that there is not a lot of data available on the long term effects of exposure, and it also has some potential to fix proteins on surfaces, which can in turn allow the development of biofilms within devices (Rey *et al.*, 2003).

1.3.2.3 Other Alkylating Agents (Combinations)

Other aldehydes include those based on mixtures, e.g. Gigasept Rapid (a mixture of glutaraldehyde and formaldehyde, 4%), SEPTO DN (a

mixture of glyoxal and glutaraldehyde) and NewGenn®, which is a combination of 6% Sactimed-I-Sinald, a quaternary ammonium compound (QAC) and 0.5% *ortho*-phthalaldehyde (British Society of Gastroenterology working party report, 2003).

1.3.3 Other Disinfectants

1.3.3.1 Quaternary Ammonium Compounds (QAC)

QAC disinfectants include Sactimed Sinald, Dettol ED and Thermoton Endo. These are less effective against Gram-negative than Gram-positive bacteria and at low concentrations are only bacteriostatic. They are also fungicidal but not sporicidal (Medical Device Agency device bulletin, 2002). These types of disinfectants are not recommended for the disinfection of endoscopes. They are generally used for surface disinfection, used on table tops, walls and floors. They are also used in antiseptic applications and in preservation. However, due to no sporicidal activity of the products, formulated products are pre-sterilised (McDonnell, 2007).

1.3.3.2 Amine Compounds/Glucoprotamin

These products include Sekusept PLUS and Korsolex AF and are generally used for the disinfection of automated washer-disinfectors in Europe. One advantage of these products is that they do not support

protein fixation and the potential for proliferation of biofilm growth (Rey *et al.*, 2003).

1.3.3.3 Electrolyzed Acid Water (EAW)

The two main products in this category which are commercially available are Sterilox® and Cleantop WM-S®. EAW is produced by using water and salt under electrolysis with membrane separation (Rey *et al.*, 2003). Sterilox is a rapidly active biocide that is effective against vegetative bacteria, mycobacteria, spores, yeasts and viruses (Selkon *et al.*, 1999). Its advantages include a rapid and strong bactericidal effect, non-irritant, and safe for staff and environment. The activity of these products can be reduced in the presence of organic matter (Rey *et al.*, 2003).

1.3.4 Factors affecting disinfectant efficacy

There are many different factors which affect the ability of a disinfectant to inactivate microorganisms to a safe level. These factors include concentration, temperature, pH, organic matter and the bacterial species involved. These factors are further discussed below.

1.3.4.1 Concentration

Concentration can affect how, and how many, microorganisms are inactivated during disinfection. If the concentration of the disinfectant is too low then it may not kill sufficient of the microorganisms present within the area being disinfected to reach a safe level. This can also be the case if microorganisms become resistant to a particular concentration and can therefore withstand higher concentrations over a longer period of time than non- or less-resistant organisms. Kinetic studies are used to investigate the effect of concentration on the activity of biocides on microorganisms, and the symbol, η, is given as the concentration exponent, which is the measure of the effect of changes in concentration on cell death rate. In order to establish the value of η, a measurement is taken at a time point where different concentrations of disinfectant produce a comparable degree of death within a bacterial suspension (Russell, 2004). If a disinfectant has a high η value then a modest decrease in concentration will greatly increase the time which it takes to achieve a comparable kill (Russell, 2004). For example, alcohol has a high η value of 10, so if its concentration is reduced by one half it will take 1024 longer (2^{10}) to kill microorganisms, it follows therefore, that in order to neutralise alcohol all you need to do is dilute with water (Russell, 2004). The efficacy of alcohol is greatly compromised if the concentration is reduced.

1.3.4.2 Temperature

It has been shown that the activity of disinfectants or preservatives can be increased with an increase in temperature, for example with glutaraldehyde. Other examples of disinfectants which show an increased activity as temperature increases include isoascorbic acid (Mackey and Seymour. 1990), and chlorine dioxide (Benarde *et al.*, 1967).

Useful formula to measure the effects of temperature on the activity of a disinfectant are given as;

$\Theta^{(T2-T1)} = k2/k1$

or

$\Theta^{(T2-T1)} = t1/t2.$

In the equation k2 and k1 are the rate (velocity) constants at temperatures T2 and T1 or t2 and t1 are the times to bring about a complete kill at T2 and T1. Θ is the temperature coefficient and refers to the effect of temperature per 1°C rise (Russell, 2004). The Q_{10} value is normally more usually specified, which is the change in activity per 10°C rise in temperature (Russell, 2004).

New liquid sterilants like liquid peracetic acid (STERIS 20 used in the SYSTEM 1 to disinfect endoscopes) can be used at high temperatures to increase its sporicidal efficacy. For the spores of *G.*

stearothermophilus the D-value for the STERIS 20 formulation at 30°C is 1.4 min, whereas at 55°C it takes <10 s (McDonnell, 2007). The D-value is the time required to achieve inactivation of 90% of the population of a given microorganism under stated conditions (McDonnell, 2007). However, it should be stated that temperatures remain lower than that which would detrimentally affect the components of the endoscopes, as these are sensitive devices that cannot withstand very high temperatures.

1.3.4.3 pH

It has been shown that changes in pH can alter the activity of biocides. The ways in which pH can do this include: (1) Molecular changes; as with glutaraldehyde which is a more stable molecule at an acid pH, but a more potent disinfectant an alkaline pH. It is likely that interactions with amino groups are responsible for this and changes occur rapidly above pH 7 (Russell, 2004). (2) Cell surface interactions; when the pH is increased the number of negatively charged groups on the surface of the bacterial cell will increase, so that positively charged molecules can then attach easily, which can be advantageous in disinfection, for example QAC (quaternary ammonium compounds) (Hugo, 1991). (3) The partitioning of a compound between a product in which it is present and a cell.

1.3.4.4 Organic matter

In the clinical environment organic matter generally refers to serum, blood, food, faecal material, mucus and tissue. It has the ability to interfere with the efficacy of the disinfectant being used; frequently because a reaction occurs between the two and reduces the disinfectant concentration, so the disinfectant efficacy is then reduced in accordance with the concentration exponent, for example with the high level disinfectant chlorine dioxide (Russell, 2004). Organic matter is used in efficacy testing of disinfectants as an interfering substance; this is to show how effective the agents will be in practice, for example in an endoscopy unit, where endoscopes will be contaminated with a lot of organic matter following a particular clinical procedure.

There are various disinfectants that cannot tolerate the presence of organic matter; these include anionic acid, sodium hypochlorite and iodophor (Gelinas and Goulet, 1983). Glutaraldehyde does well in comparison with these in the presence of powdered milk, dried beef blood and fish meal (Gelinas and Goulet, 1983).

The activity of OPA has also be shown to be unaffected by the presence of organic matter (Fraud *et al.,* 2001). OPA has been marketed as an alternative to glutaraldehyde and has been shown to be as effective as glutaraldehyde (Walsh *et al.*, 1999ab).

1.3.4.5 Formulations

There are a large number of disinfectants manufactured but they are not always used on their own, but are often used in combination with others. The combined properties are exploited to greatly improve the efficacy of the formulated disinfectant product. One of the main considerations in formulating a product has to be which biocide is being formulated, and the optimal ranges of pH, temperature, concentration, stability, solubility and spectrum of activity (MacDonnell, 2007). The basic contents of formulations are water and other solvents. Other ingredients that are used in creating disinfectant formulations are buffers (stabilising pH), surfactants (which allow dispersal and aid biocide penetration) and chelating agents (preventing substances interfering with efficacy), and sometimes there is the need for solubilizing agents as biocides are not always soluble in water (McDonnell, 2007). This is an important factor to consider as the product may have to be diluted prior to use, as is the case with several disinfectants used in the healthcare industry (e.g. for endoscope disinfection). The various elements which go into creating formulations are listed in table 1.3, which illustrates some ingredients and their purposes.

Table 1.3 Various ingredients of formulated disinfectants

Ingredient	Purpose	Examples
Biocide	Antimicrobial or preservative	QACs, phenolics, biguanides
Solvent	Dissolution and dilution of the biocide and ingredients; vehicle for delivery	Isopropanol, urea, deionized water, isopropanol, propylene glycol.
Emulsifiers, surfactants	Allow formulation of stable mixtures of water and oil mixtures. Surfactants reduce surface tension, improve wettability , disperse contaminants, inhibit foam formation	Lecithin, sodium lauryl sulphate, potassium laurate, non-ionic and other surfactants.
Thickeners	Increase viscosity of formulation	Polyethylene glycol, pectin and alginates
Chelating agents or sequestrants	Binding metals to inhibit their precipitation, water softening, prevent mineral deposits. Protect activity.	Ethylenediamine: EDTA, EGTA
Alkali or Acid	pH stabilization	Alkali (NaOH KOH) Acids (acetic, citric)
Buffer	Maintain pH over time	Disodium phosphate
Corrosion inhibitory	Reduce corrosion and protect surface of metals	Nitrates, phosphates, molybdates
Others	Aesthetic qualities	Colours and fragrances

(adapted from McDonnell, 2007)

1.3.4.6 Types of micro-organisms

Different types of bacteria respond differently to different biocides. It has been shown that Gram-positive bacteria are more sensitive to disinfectants than Gram-negative bacteria. The reasons for this are due to the construction of the cell envelope: the Gram-positive bacterial cell wall is composed of peptidoglycan forming a thick fibrous layer which does not act as an effective barrier to biocides (McDonnell and Russell, 1999).

Mycobacteria stain as Gram-positive; however they are generally not as sensitive as Gram-positive bacteria, mainly due to their unusual cell wall, which contains high wall-lipid content increasing the hydrophobicity of the wall. Gram-negative bacteria are less susceptible to disinfectants, which is down to the outer membrane and the lipopolysaccharide (LPS) present. It is well known that *P. aeruginosa* has an ability to withstand biocidal and antibiotic treatments (Russell, 2004) and is a known hospital pathogen.

Bacterial spores also have a high resistance level to biocides, where the spore coat acts as an impermeable barrier. Sporicidal biocides do exist but longer contact times often need to be employed. Examples of disinfectants which are active against spores include glutaraldehyde, peroxygens, formaldehyde and ethylene oxide. Some biocides are not sporicidal but are sporostatic (McDonnell and Russell, 1999).

Not all antibacterial agents are effective against viruses, but an example of one that can be is glutaraldehyde (Russell, 1994). The potential for viruses to be transmitted via medical devices such as endoscopes is of major concern (Hanson, 1991; Chong, 2009). However, it has been established that the likelihood is low (Morris *et al.*, 2006) with Hepatitis C reported as being rarely transmitted and only a hand-full of identified cases of Hepatitis B. This transmission is largely down to inappropriate disinfection (Morris *et al.*, 2006).

1.3.4.7 Microbial biofilm

Bacterial populations which have developed in biofilm formation are more resistant to antibacterial agents (Stickler, 2004). The various reasons for this include the inability of the biocide to interact fully with all the bacterial cells within the biofilm, bacterial growth rate is slowed so this too protects the bacteria from the biocide action, and bacterial enzymes can be produced which can neutralise the biocide (Mah and O'Toole, 2001; Gilbert *et al.,* 2002). This will be discussed further in section 1.6.

1.4 Endoscopy

1.4.1 Introduction

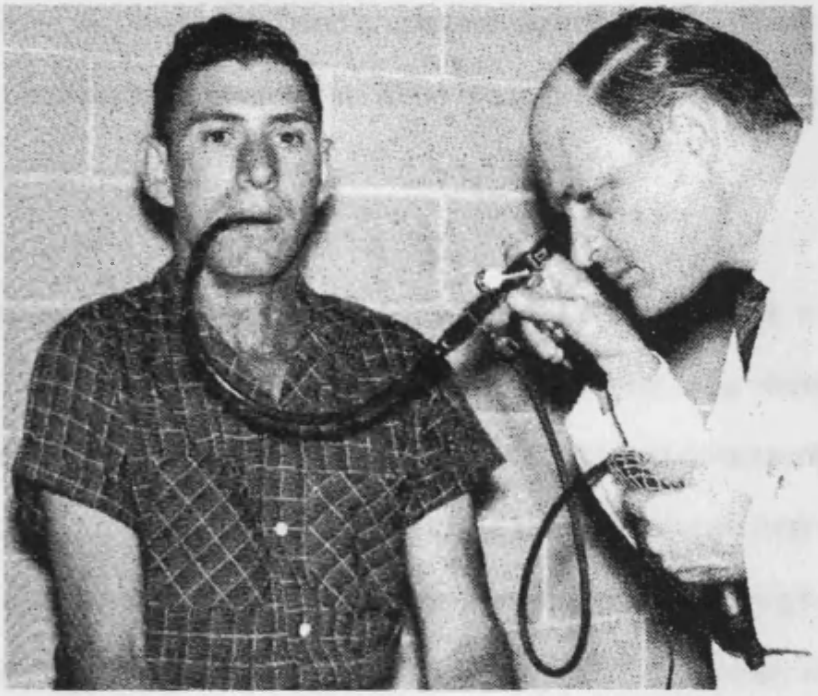
Endoscopes are used during clinical procedures in order to look into the interior of the body without the need for major surgery. They have many benefits and are used for diagnostic and therapeutic procedures, including the detection of ulcers, cancers and sites of internal bleeding. Tissue samples can also be taken through the biopsy channel of an endoscope in order to assist diagnosis of various pathologies within the body.

The first development of the fibre-optic endoscope for use in upper gastrointestinal (GI) investigations occurred in the mid 1950s (Alvarado *et al.*, 1999). Further developments were made around the 1960s as endoscopy instruments for colonoscopy, bronchoscopy and upper GI endoscopy were introduced into the healthcare setting. The video chip endoscopes replaced these around the 1990s (Cotton *et al.*, 1996). There is limited up to date data on the subject however It was estimated that 10 million endoscopy procedures were performed each year in the United States alone 10 years ago (Alvarado *et al.*, 1999).

Endoscopes were first used clinically in 1961, and described in a paper in the *Lancet* by Basil Hirschowitz on how the patient is prepared and how the endoscope imaging works (Figure 1.1) as it is passed through

a bundle of glass fibres. The paper also details various advisory comments and describes better imaging using the new endoscope. From this article, it can be seen how far endoscopy has progressed to the present day, with significant advancement in design, procedure, and disinfection.

Figure1.1 Endoscope Being Inserted Into Patients Stomach (Hirschowitz 1961).



1.4.2 History of endoscope disinfection

As the design of endoscopes has improved, so has the cleaning and disinfection procedures of them. In the mid 1960s, as the potential for the transmission of disease between patients undergoing endoscopy procedures was less well reported, endoscopes simply received brief flushing of the suction/accessory channel with alcohol between patient

uses (Antonucci, 2008). This cleaning procedure was improved in the 1970s by the flushing of disinfectants through the channels (Peterson 1999). It was well known by the 1980s that bacteria would grow in these channels overnight, which led to further increases in the levels of cleaning and disinfection of endoscopes. However, some endoscopes in use prior to 1983 were not suitably manufactured with cleaning or disinfection in mind - in fact, in some cases the elevator, air and water channels could not be accessed at all (Peterson 1999). At this time any instruments that had been used on potentially infectious patients were sent to be decontaminated by ethylene oxide gas sterilisation (Peterson 1999).

For these reasons, standards were established and published, with the earliest being the 1978 SGNA (Society of Gastroenterology Nurses and Associates) guidelines for the care of endoscopes and accessories (Antonucci, 2008). It had been noted in the United Kingdom that endoscope disinfection standards were inadequate and not regimentally adhered to. In 1981, a survey was conducted on 52 endoscopy units evaluating disinfection practices. It was established that 23 % of the units questioned never used effective disinfectant and admitted that they were unaware that this process was unsatisfactory (Axon *et al.*, 1981). This paper also highlighted problems with the use of the disinfectant glutaraldehyde, and the effects it had on the health of the staff coming into contact with it.

Such problems raised understandable concerns regarding the use of disinfectants, and the inadequate reprocessing of endoscopes led to the publication of multi-society guidelines (American Society for Gastrointestinal Endoscopy, ASGE) detailing procedures for the cleaning and disinfection of GI endoscopes (1988). These guidelines recommended the cleaning of all channels, the use of approved disinfectants, rinsing after disinfection, and drying after rinsing. In the same year the British Society of Gastroenterology (BSG) also submitted guidelines on the cleaning and disinfection of equipment for GI endoscopy (Weller et al., 1988). These guidelines emphasised how to disinfect when the endoscopy equipment was potentially infected with viruses, detailing disinfectant times and agents to use, and made the point that pre-cleaning of an endoscope is fundamental to the safe disinfection process. The ASGE updated its guidelines in 1999 and again in 2008, as did the BSG in 1998 and 2008. Various other disinfection guidelines for endoscopy equipment have been published and updated over the years, including the publication of the NHS Estates publication HTM 2030 in 1997 which offers guidance on the choice, specification, purchase, installation, periodic testing, operation and maintenance of washer-disinfectors (WDs) used for the disinfection of endoscopes.

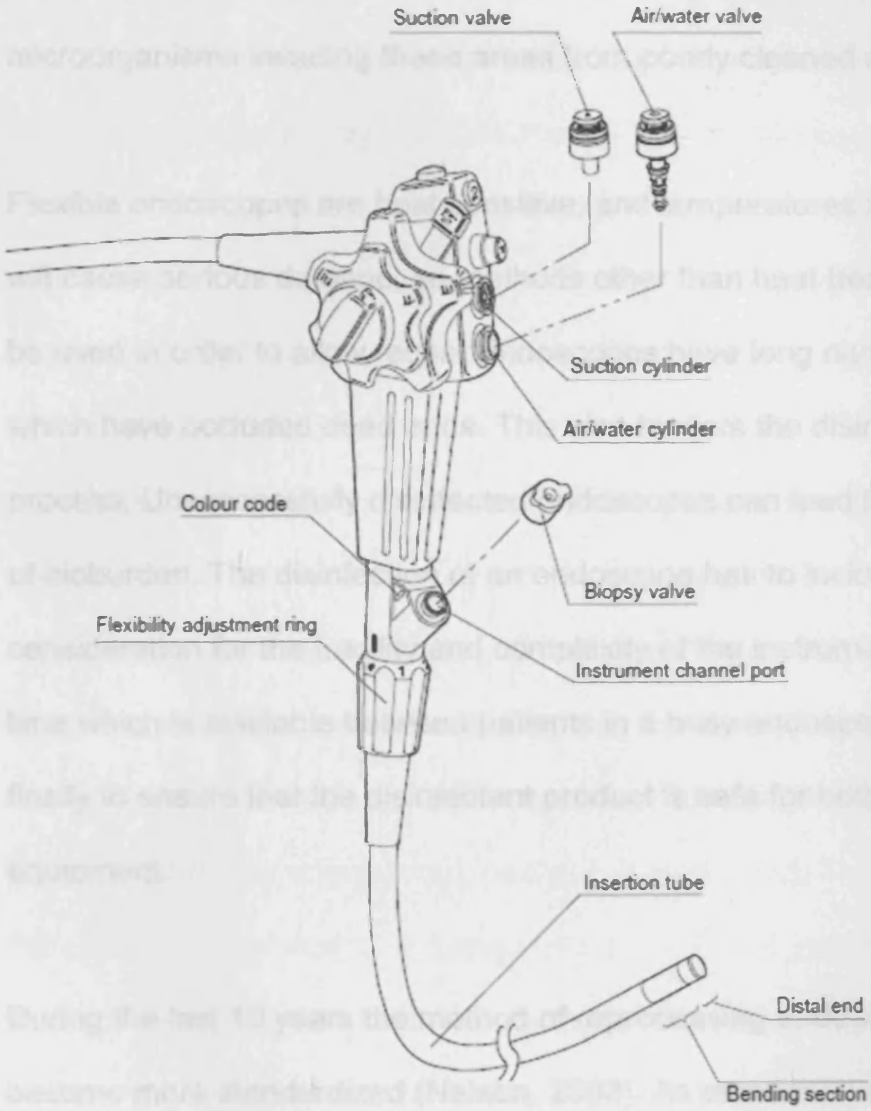
1.4.3 Types of Endoscopes

As briefly introduced above, there are various different types of endoscope, used either for viewing purposes only, or for more complex investigational and surgical procedures. There are 3 basic endoscope types: flexible, semi-rigid and rigid.

Flexible endoscopes include gastrointestinal endoscopes (used for investigating the stomach and intestinal regions) and bronchoscopes (for inspecting the interior bronchi). A flexible endoscope consists of a control head, and a flexible shaft which has a tip designed to be manoeuvrable (distal end). An example of a flexible endoscope can be seen in Figure 1.2. Semi-rigid scopes can be curved or straight, depending on their intended use. An example of a semi-rigid endoscope is an ureteroscope (Med Care) which has a maneuverable distal tip. Rigid endoscopes include bronchoscopes, laparoscopes and arthroscopes, all of which are used for invasive procedures and are generally easy to clean by steam, ethylene oxide or liquid chemical sterilants (Medical Devices Agency 1997). An endoscope also has many internal cavities, lumens and channels; one which is important is the suction channel which is used for the passage of procedural tools such as biopsy forceps. Flexible endoscopes become more heavily contaminated with bacteria than rigid endoscopes, as flexible endoscopes are used to probe areas of the body which have a high microbial content. Since endoscopes inevitably come into contact with

mucous membranes, the use of high-level disinfection between patients is paramount.

Figure1.2 Flexible Endoscope. (MDA, Device Bulletin 2002)



1.4.4 Endoscope disinfection

Out of all medical devices used, endoscopes are thought to be the ones which are most likely to be linked with hospital acquired infection outbreaks and pseudo-outbreaks (Rutala *et al.*, 2004a). In recent years

there has been a higher risk of spreading infections as the procedures are becoming more invasive. Endoscopes invade areas with mucous membranes, and whilst these areas are generally resistant to infection from bacterial spores they are susceptible to other organisms so high level disinfection must be achieved in order to prevent other microorganisms invading these areas from poorly cleaned endoscopes.

Flexible endoscopes are heat sensitive, and temperatures above 60°C will cause serious damage, so methods other than heat treatment must be used in order to allow reuse. Endoscopes have long narrow lumens which have occluded dead ends. This also hinders the disinfection process. Unsuccessfully disinfected endoscopes can lead to a build up of bioburden. The disinfection of an endoscope has to include special consideration for the fragility and complexity of the instrument, the short time which is available between patients in a busy endoscopy unit, and finally to ensure that the disinfectant product is safe for both user and equipment.

During the last 10 years the method of reprocessing endoscopes has become more standardized (Nelson, 2003). As mentioned above, these methods have been developed over the years to ensure optimum procedures to appropriately maintain the sterility of equipment and prevent the transmission of exogenous infections.

The current guidelines on the decontamination of flexible endoscopes according to the BSG can be broken down into various steps as follows. Prior to beginning any treatment of endoscopes staff must adequately protect themselves by putting on appropriate personal protective equipment including aprons, full face visors and single-use gloves. Also, all staff should be adequately trained for each procedure they will be doing, as emphasised by the 2006 Health Act, concerning staff training in decontamination processes and having appropriate competencies for their role (British Society of Gastroenterology working party report, 2008).

Firstly, decontamination should begin swiftly after the endoscopy procedure has finished, and this can and will occur when the endoscope is still attached to the light source. This involves the process of sucking water and detergent through the working channel, whilst the air and water channels should be irrigated with water. This process should ensure that any debris, blood or mucus has been removed from the channels. The shaft of the endoscope is then wiped with enzymatic cleaner and can be removed from the light source/ video processor. At this point the scope should be checked for any surface damage.

The second disinfection stage involves the removal of valves and water bottle inlets, and all detachable parts that are to be re-used should be reprocessed at the same time as the endoscope.

The third stage begins the process of manual cleaning of the endoscope. This is the most important stage, as this will further remove any organic matter which could potentially interfere with the disinfection process later on (Martin, 1994; Alvarado, 2000). Manual cleaning will also reduce the chances of biofilm formation inside the endoscope (ASGE Standards of Practice Committee 2008). All channels should be cleaned using a purpose build device (channel brush) regardless of whether or not they were used during the endoscopy procedure. The use of the enzymatic solution is important as it promotes protein lysis, which in turn enhances the efficacy of the brushing procedure (Shumway, 2003). It has been shown in some studies that this alone will kill viruses which could be present in the endoscope (ASGE Standards of Practice Committee, 2008) and also aids the removal of dried blood (Bloß and Kampf, 2004). The rinsing of all external surfaces and internal channels should take place in a separate sink filled with clean water.

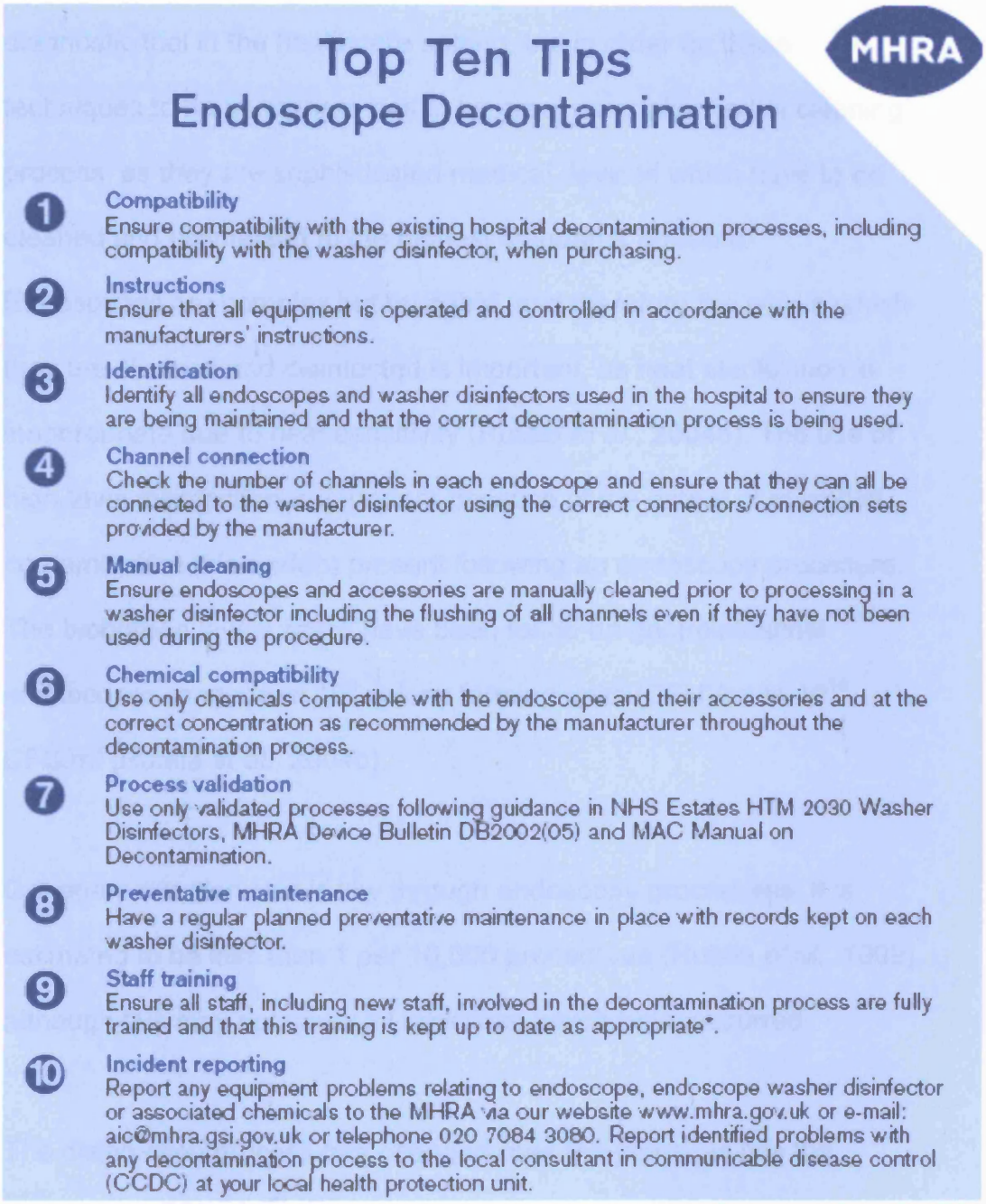
The endoscope should be thoroughly checked for any damage after the above procedure is finished and the scope has been brought into the disinfection area. The first procedure check is the manoeuvrability of the scope by deflecting the scope distal tip; this is done by rotating the angulation controls. Next is the leak test which is done by inflating the endoscope shaft under pressure (Cotton, 1996). The dry test is performed by observing the needle in a pressure gauge for rapid

movement. A wet test is also performed, which is done by checking for bubbles by submerging the endoscope fully in hot water.

The fourth stage is high level disinfection with an approved compatible disinfectant. This should happen within an automated endoscope reprocessor (AER). Manual disinfection is unacceptable and must not be undertaken. This process should be concluded by further rinsing with sterile or filtered water, followed by proper drying and storage of each endoscope. Storage of the endoscopes is an important part of the cleaning process, as it prevents the growth of microorganisms (Pineau *et al.*, 2008). A review by Muscarella (2006) shows that the drying of endoscopes after disinfection helps the prevention of disease transmission. If they are not stored by hanging up correctly moisture can collect in dead ends and bacterial growth could then occur (Cotton *et al.*, 1996).

The Medicines and Healthcare Products Regulatory Agency (MHRA) has outlined the top ten tips when decontaminating endoscopes, and this is reproduced in Figure 1.3.

Figure 1.3 Top Ten Tips for endoscope decontamination. (MHRA Device Bulletin DB2002 (05))



Top Ten Tips
Endoscope Decontamination

MHRA

1. **Compatibility**
   Ensure compatibility with the existing hospital decontamination processes, including compatibility with the washer disinfector, when purchasing.
2. **Instructions**
   Ensure that all equipment is operated and controlled in accordance with the manufacturers' instructions.
3. **Identification**
   Identify all endoscopes and washer disinfectors used in the hospital to ensure they are being maintained and that the correct decontamination process is being used.
4. **Channel connection**
   Check the number of channels in each endoscope and ensure that they can all be connected to the washer disinfector using the correct connectors/connection sets provided by the manufacturer.
5. **Manual cleaning**
   Ensure endoscopes and accessories are manually cleaned prior to processing in a washer disinfector including the flushing of all channels even if they have not been used during the procedure.
6. **Chemical compatibility**
   Use only chemicals compatible with the endoscope and their accessories and at the correct concentration as recommended by the manufacturer throughout the decontamination process.
7. **Process validation**
   Use only validated processes following guidance in NHS Estates HTM 2030 Washer Disinfectors, MHRA Device Bulletin DB2002(05) and MAC Manual on Decontamination.
8. **Preventative maintenance**
   Have a regular planned preventative maintenance in place with records kept on each washer disinfector.
9. **Staff training**
   Ensure all staff, including new staff, involved in the decontamination process are fully trained and that this training is kept up to date as appropriate*.
10. **Incident reporting**
    Report any equipment problems relating to endoscope, endoscope washer disinfector or associated chemicals to the MHRA via our website www.mhra.gov.uk or e-mail: aic@mhra.gsi.gov.uk or telephone 020 7084 3080. Report identified problems with any decontamination process to the local consultant in communicable disease control (CCDC) at your local health protection unit.

1.4.5 Transmission of infections during endoscopy.

Over the last 55 years flexible endoscopes have been a valuable diagnostic tool in the healthcare setting, but in order for these techniques to be used there has to be great care taken in the cleaning process, as they are sophisticated medical devices which have to be cleaned and disinfected to the highest standards possible. Endoscopes are complex but reusable, and therefore the way in which they are cleaned and disinfected is important, as heat sterilization is inappropriate due to heat sensitivity (Rutala *et al.*, 2004b). The use of high-level disinfection is important because of the extent of microbial contamination (bioburden) present following an endoscopy procedure. The bioburden levels which have been found on gastrointestinal endoscopes range from 10^5 colony forming units (CFU)/ml to 10^{10} CFU/ml (Rutala *et al.*, 2004b).

Generally infection rate is low through endoscopy procedures. It is estimated to be less than 1 per 10,000 procedures (Rutala *et al.*, 1999), although this may not cover all infections which have occurred.

The disinfection process has changed over the years, as has the endoscope design. Before 1983 endoscopes were not fully immersed in a disinfectant, and until late 1981 endoscopes were not disinfected between patients in the United Kingdom (Nelson, 2003). About 23% of hospitals in the UK in 1981 did not even use disinfectants on

endoscopes at all (Nelson, 2003). So, although the disinfection process for endoscopes has changed and improved over the years, cross-infection with pathogens through the use of endoscopes might still occur when the cleaning/disinfection chain is broken and the strict guidelines in the reprocessing of endoscopes are not followed correctly (Alvarado *et al.*, 1999; Nelson *et al.*, 2003).

Infection through endoscopes can be caused by both endogenous and exogenous microbes (Alvarado *et al.*, 1999). Endogenous infection is when microbial flora inhabiting the gastrointestinal tract can gain access to sterile areas of the body through mucosal trauma, for example when a biopsy is taken (Nelson, 2003a; ASGE Standards of Practice Committee 2008). Endocarditis can potentially occur as a result of an endoscopy procedure, however it has been noted by Nelson (2003) that only 7 esophagogastroduodenoscopy (EGD) procedures have resulted in endocarditis. Further data reveals 15 cases in total, showing that although infection during endoscopy procedures does occur, the rate of infection is low in comparison to the number of endoscopy procedures performed in a year. It has been proposed that in order to minimise the risk of infections occurring in immunocompromised patients, periprocedural antibiotic therapy could be used (ASGE Standards of Practice Committee 2008). High rates of endocarditis have been reported with oesophageal dilation and sclerotherapy. It is recommended that patients that have a GI endoscopy are not give

antibiotics prophylactically (ASGE Standards of Practice Committee 2008), however, patients with established GI-tract infections in which enterococci may be part of the infecting bacterial flora (such as cholangitis) and with cardiac conditions (i.e. a prosthetic cardiac valve) associated with the highest risk of an undesirable outcome from endocarditis can be given periprocedural antibiotic therapy (ASGE Standards of Practice Committee, 2008).

Exogenous infections involve the transmission of microorganisms between patients, the transfer of environmental microorganisms to patients, the spread of microorganisms between patient and endoscopy staff, or from staff to patient (ASEG Standards of Practice Committee 2008). When high level disinfection guidelines are followed accordingly, instances of the transmission of infections are zero (ASEG Standards of Practice Committee 2008). However, when these guidelines are breached there is the potential for transmission to occur by the above routes. The potential for the transmission of microorganisms will be the main focus of this part of the introduction.

The Technology Assessment Committee of the American Society for Gastrointestinal Endoscopy "found 28 reported cases of endoscopy related transmission of infections between 1988 - 1992" (Alvarado *et al.*, 1999). During this period 40 million endoscopy procedures were carried out and the incidence of transmission was approximately 1 in

1.8 million endoscopy procedures (Nelson *et al.*, 2003). From 1993 to 2003 there have only been five additional reported cases of transmission of pathogens in the United States (Nelson, 2003), although others have suggested that endoscope cross-infection is overlooked and there is more likely to be 270,000 infections transmitted by endoscopes a year (Moses *et al.*, 2003). However, estimating the true infection rate due to endoscopes alone is difficult, as other endoscope-associated procedures complicate separation of the data. For example, septicaemia following endoscopic retrograde cholangiopancreatography (ERCP) procedures could skew the data of exogenous infections which are not due to the endoscope itself, but a complication caused by the procedure (British Society of Gastroenterology working party report, 2008). Also, there is the potential that the patient may not experience any problems until they have been discharged from hospital, and/or the infective agent may have a long incubation period, for example vCJD (British Society of Gastroenterology working party report, 2008).

1.4.6 Microbial contaminants

Due to the nature of endoscopic probing within the body, heavy contamination with a variety of microorganisms is likely. If these microorganisms are not killed prior to the next use, the potential for microorganisms to be passed onto the next patient is high. The bioburden of a typical flexible gastrointestinal endoscope after use in a

procedure ranges from 10^5 cfu/ml to 10^{10} cfu/ml (Rutala *et al.*, 2004b; Chu and Favero, 2000; Vesley *et al.*, 1999). Pathogens which have been associated with gastrointestinal endoscopes include *Salmonella* spp. and *Pseudomonas aeruginosa*. Pathogens which are generally associated with bronchoscopy include *Mycobacterium tuberculosis*, atypical *mycobacteria* and *P. aeruginosa* (Rutala and Weber, 1999). Other bacteria which have been isolated from endoscopes include *Micrococcus luteus*, *Bacillus* species and *Enterococcus* species (Bisset *et al.*, 2006).

1.4.7 Microbial contaminants due to inadequate reprocessing

In 2004, an incident occurred in Northern Ireland in which there had been a failure to properly decontaminate endoscopes (Doherty *et al.*, 2004). It was found that the auxiliary channels had not been adequately cleaned. The concerns in this incident were the possibility of transmitting of blood-borne viruses. At the same time further investigations were conducted with 23 gastrointestinal endoscopes, which also showed failure in cleaning efficiency (Morris *et al.*, 2006). This shows that poor disinfection is not only a problem related to biocide activity, but also to endoscope pre-cleaning and human error. If, during the pre-cleaning procedure, bacterial debris remains then this itself may prevent the in-use disinfectant from working adequately. This

could in turn lead to channels becoming blocked, which may not become apparent for some time.

There was a major example of the transmission of infection through endoscopes in 1995, when an endoscope was contaminated with hepatitis C virus (HCV) from a patient with HCV which was transmitted to two other patients. This particular incident was due to the biopsy channel being improperly cleaned (Obee *et al.*, 2005). It was found in other cases too that this area is not always appropriately cleaned (Kinney *et al.*, 2002; Kirschke *et al.*, 2003; Srinivasan *et al.*, 2003). Other parts of the endoscope which cause concern include the air/ water channels. This was a problem particularly with older endoscopes where air/water channels were smaller, making them much more difficult to disinfect as brushes were often too big to fit.

Martiny and Floss (2001), provide a reviewed of a series of cases in which microbial residuals were present on endoscopes after reprocessing. Their review detailed that in 1999 (investigation done by Roth and team) that out of the 57 endoscopes examined, 42 of them had bacterial growth and 12 of the 42 had colony forming units (CFU) of 100 or more. Bacteria found to be present included Streptococci, *Enterococcus* spp. and *Pseudomonas* spp. (Martiny and Floss., 2001). These authors further discuss an instance where multidrug-resistant (MDR) *Mycobacterium tuberculosis* was transmitted to four people; two

out of the four patients died due to this incident. It was found that between patient examinations, the bronchoscope had not been immersed in the high-level disinfectant glutaraldehyde at any time (Martiny and Floss., 2001). The main conclusion in the review by Martiny and Floss (2001) was that the infections transmitted by endoscopy procedures were largely due to inadequate processing of the endoscopes.

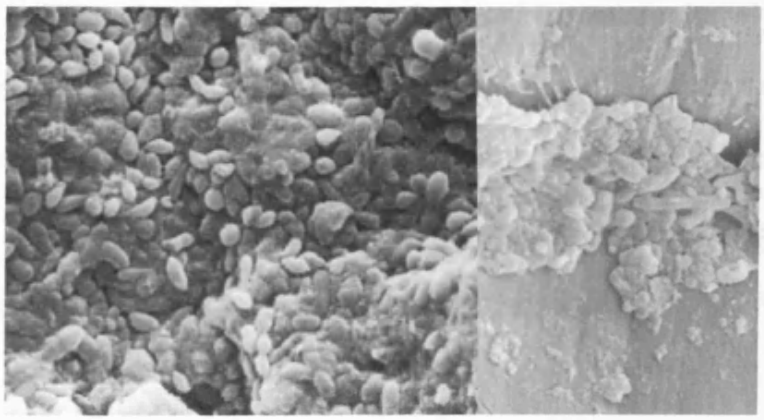
As inappropriate cleaning is a major problem associated with disinfection of endoscopes, an audit was done in 1997 on the adherence to guidelines of disinfection in a bronchoscopy department (Honeybourne *et al.*, 1997). In this particular study, 218 questionnaires were sent out to the different bronchoscopy units in the UK, with a response rate of 73%. The findings of this audit showed that out of this batch of units only 57% used sterile water in the disinfection process. When emergency procedures were performed by 65% of the units only 34% actually disinfected prior to the procedure. The audit also showed that only 53% of technicians and nurses in the unit had received technical training in the use and disinfection of endoscopes (Honeybourne *et al.*, 1997). In the safety area of the audit, only 7% of staff routinely wore all the protective clothing recommended (Honeybourne *et al.*, 1997). The issue of not wearing protective clothing does not only affect the welfare of the staff and patients, but could also detrimentally affect the disinfection of the endoscopes as contamination

could be transferred from the body or unsuitable clothing. Through this audit, it can be shown that when guidelines are put down in order to be followed, compliance is not complete. This is a major area of concern, especially if staff are not trained in the appropriate disinfection procedures, and incidents of infection become much more likely. Another area of potential contamination of endoscopes is through the use of non-disposable biopsy caps. This was reported in two separate cases in 2001 and 2002 in which *P. aeruginosa* was involved with an outbreak associated with bronchoscopes. These occurred as the biopsy ports were lost and had got contaminated during the cleaning process and when replaced within the endoscope to be disinfected the contaminated area was not in contact with the disinfectant in use (Kirschke *et al.,* 2003; Srinvasan, 2003). The design of endoscopes has changed since this problem in 2001 and 2002. It is now the recommendation of the BSG that biopsy port caps should be single-use and discarded after biopsies are taken during endoscopic procedures (British Society of Gastroenterology working party report, 2008).

A study was conducted by Pajkas *et al.* (2004) which investigated the accumulation of biofilms on endoscope tubing (shown in Figure 1.4) as a result of failure to properly clean endoscopes. The study involved sampling 13 endoscopes which had their tubing removed and examined. The study showed that biological deposits were present on all samples tested. Biofilm was also found on 5 out of the 13 samples

located on the suction/biopsy channels. Also, all 12 air/water channels examined showed biofilm which was found to be extensive in 9 of the 12 channels. Overall, the study showed that the presence of the biofilm decreased the efficacy of the disinfectants and also showed that disinfection procedures were far from adequate (Pajkos *et al.*, 2004). A study by Ishino *et al.* (2001) also highlighted this point. They took two groups (A and B) of staff and asked them to clean and then disinfect several endoscopes. Group A had the air/water channel cleaned and Group B did not have the air/water channel cleaned. The results of the study showed that there was no contamination of the air channel, and although in group B endoscope water channels were contaminated, there was no contaminated water channel in group A. The study shows that this is an important area to be cleaned. In addition, there could be problems with older endoscopes which cannot be cleaned extensively due to narrow channels (Ishino *et al.*, 2001).

Figure1.4 Biofilm formation on the inside of an endoscope channel. (Pajkos *et al.* 2004).



Reeves and Brown (1995) discussed the growth of bacteria in the suction channel and described several cases where contamination of bacteria resulted in cross-infection and contamination. In one particular case the contamination was due to the use of tap water to make up the disinfectant. The tap water was found to be contaminated with *Mycobacterium chelonae (Reeves et al., 1995)*.

1.4.8 Microbial contamination in Automated Washer-disinfectors

Cleaning the endoscope manually before putting it in the automated washer disinfector is important to remove large quantities of debris. One concern is that even if the endoscopes are being cleaned effectively, the water used in the rinse cycle may not be free from contaminants. In 1998, in a hospital in London after the installation of new washer-disinfectors as a health and safety concern, an outbreak of *P. aeruginosa* occurred, which affected eight ITU patients who had had bronchoscopy procedures. When investigated, the endoscopes were found to be contaminated, as was the washer disinfector which was covered in biofilm and limescale deposits. The main reasons behind the build up of microorganisms within the washer-disinfectors were down to poor maintenance and the lack of understanding of cleaning protocols. When other washer-disinfectors where investigated within the hospital

these too were found to have *Pseudomonas* spp. present within them (Schelez and French 2000).

Sometimes the washer disinfector itself can be a source of problems, which can potentially lead to build up of biofilm if certain areas in the machine do not come into contact with disinfectants. This was shown in 1988 when gastrointestinal endoscopes became infected with *P. aeruginosa*. Six months after purchase of washer-disinfectors the infections of *P. aeruginosa* went up by 36%. It was found during investigations that the detergent holding area was not disinfected and this resulted in the build up of biofilm within. This then contaminated the endoscopes leading to infections in patients (Alvarado *et al.,* 1991).

One microorganism of particular concern is *Mycobacterium chelonae.* This microbe grows rapidly and can be found in mains water supplies (Reeves *et al.*, 1995). The problem of eliminating this microorganism has now been solved by the installation of filters in the system. However, this may not always be the case and therefore problems with contamination are likely to still occur. A paper by Cooke *et al.* (1998) stated that bacteria-free rinse water "was an impossible dream" at that particular time. The paper investigated how the installation of automated washer-disinfectors systems fitted with water filtration systems affected water contaminants. Water can be contaminated with

bacteria such as *Mycobacterium*, *Legionellae* and other Gram-negative bacilli (Cooke *et al.*, 1998).

Cooke *et al.* (1998) tested the water from the disinfector and mains supply over a regular period in order to establish the level of contamination. They found that over a sixth-month period the water tanks were culture-positive. They also found that when the endoscopes had been processed they too were contaminated. The bacterial contaminant was *Mycobacterium tuberculosis*. The paper concluded that although there was a filter fitted the water was not bacteria-free, but there was no additional contamination of the bronchoscopes being cleaned (Cooke *et al.*, 1998).

In 2001, a further study (Pang *et al.*, 2002) was conducted on the subject of bacteria-free rinse water. This water was found to be contaminated with bacteria and investigations showed that the pipework was responsible for the problem, as it was contaminated with *Pseudomonas* spp. This was overcome by flushing the pipes with water at a temperature above 60°C for 60 minutes on a daily basis (Pang *et al.*, 2002). This decreased the amount of bacterial contaminants. It also showed that achieving bacteria-free rinse water was still a problem. Many washer-disinfectors now contain filters to prevent the presence of bacteria in the rinse water. Other outbreaks of *P. aeruginosa* contamination occurred in 2001 which also involved automated washer-

disinfectors, including the contamination of the disinfectant used as the concentration was too low (Antonucci *et al.,* 2008) and also with the use of inappropriate channel connectors (Sorin *et al.,* 2001).

1.4.9 Transmission of Viral infections through endoscopy

Due to the long incubation times of viral infections it is more difficult to screen for them. One of the main concerns is the potential for the transmission of hepatitis C virus (HCV). There have been reports of the transmission of HCV but this only happened when there was a breach in endoscope reprocessing, including a lack of mechanical cleaning before disinfection and the failure to sterilize biopsy forceps (Bronowicki *et al.*, 1997). It has been duly noted that when endoscope cleaning and disinfection occurs according to current guidelines, the risk of transmission is effectively eliminated (Ciancio *at al.*, 2005, Mikhail *et al.*, 2007).

Again, if breaches in endoscopy disinfection procedures occur, there is the potential for the transmission of Hepatitis B virus (Morris *et al*, 1975; Seefeld *et al.,* 1981; Birnie *et al.*, 1983). It has been shown by various studies that when endoscope cleaning guidelines are adhered to, transmission of hepatitis B virus to patients is avoided (McDonald *et al.,* 1976; McClelland, 1978).

The latest American Society for Gastrointestinal Endoscopy (ASGE) guidelines (2008) state that there are no reports of the transmission of HIV by endoscope and that manual cleaning with detergent eradicates > 99.0% of the virus and afurther 2 minutes contact with glutaraldehyde will eliminate it.

1.4.10 Other potential contaminants of endoscopes

1.4.10.1 Prions

Creutzfeldt-Jacob disease (CJD) is a neurological disease caused by the transmission of proteinaceous agents called prions. Gastrointestinal endoscopes do not come into contact with non-variant CJD prion infected tissue, so the disinfection of endoscopes can remain as per guidelines. The incubation period of the disease may vary from months to decades, but once symptoms develop the disorder is usually fatal within 1 year (Rutala and Weber, 2001). Currently there are no reports of CJD linked to endoscopic procedures (ASGE Standards of Practice Committee, 2008).

Variant Creutzfeldt-Jacob Disease (vCJD) can be found in the lymphoid tissue throughout the body, including the tonsils and the gut (ASGE Standards of Practice Committee, 2008). The incubation time is 10-30 years and the individual with vCJD could potentially pass on causal prions through endoscopic procedures due to the inability of

conventional sterilization or decontamination methods to remove or destroy the prions (BSG working party report, 2008). It is the recommendation of the European Society of Gastrointestinal Endoscopy that endoscopic procedures should be avoided in patients with vCJD (Axon *et al.*, 2001). There are, however, endoscopes available to use if an endoscopy procedure in unavoidable, through units such as the vCJD Surveillance Unit Edinburgh (BSG working party report, 2008). The report of a Working Party of the British Society of Gastroenterology Endoscopy Committee recommends further guidelines in this area.

Generally, if guidelines are followed to the letter in the disinfection of endoscopes then infection rates are low. However, as discussed above, if instances occur where infection control procedures break down and decontamination practices are not adhered to, then the transmission of potentially harmful microorganisms can take place. Fortunately, this is infrequent, as guidelines detail exactly what to do and how to do it, along with how to determine on a regular basis whether there are contaminants in equipment and accessories including washer-disinfectors.

1.5 Microbial resistance to disinfectants

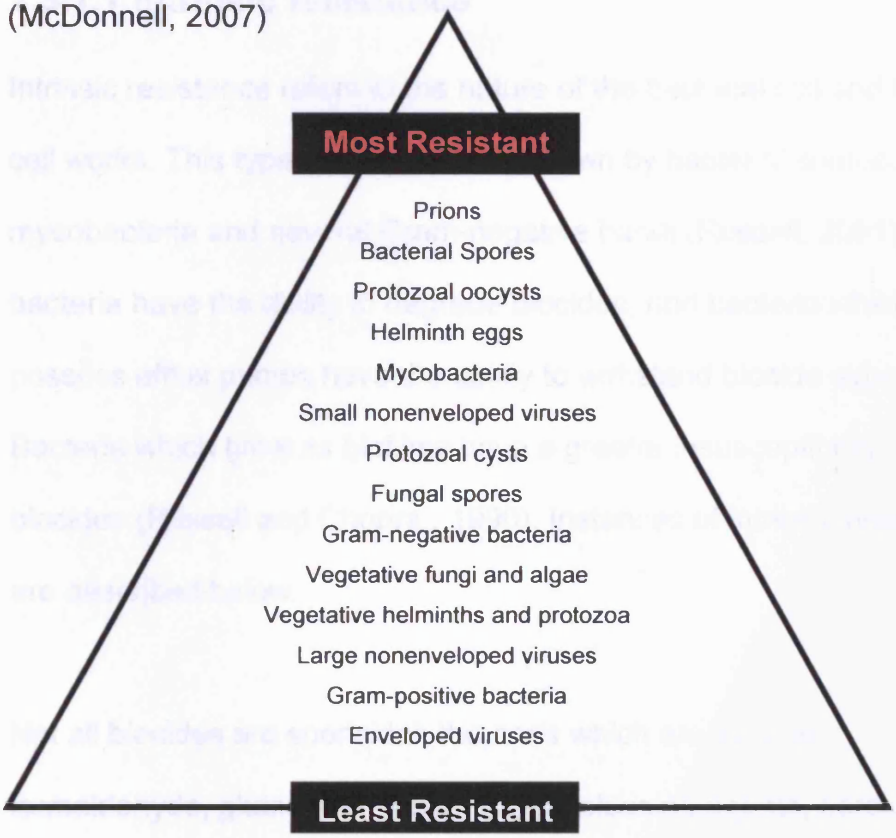
1.5.1 Resistance mechanisms

Microorganisms naturally differ in the ways in which they interact with disinfectants, sterilants and antiseptics. This interaction depends on several factors which have been discussed in section 1.3.3. A microorganism can be considered to be resistant to a biocide when it is not inactivated by an in-use concentration of that agent, or a biocide concentration that will inactivate other strains of the microorganism (Russell, 2003). Bacterial spores are generally considered the most resistant to antiseptics, disinfectants and sterilisation processes.

Microorganisms behave differently when they come into contact with various biocides, as shown in Figure 1.5 which details microorganisms from most resistant to least resistant types. Although this is true for the various groups of microorganisms, variations will exist within these groups with each under the various categories having higher or lower resistance status. For example, Gram-negative bacteria generally have a higher resistance status compared to others, but within that group there can be differences in resistance, such as between *P. aeruginosa* and *E. coli*. Also, it has been shown that prion inactivation with biocides is possible, although these are at the top of the most resistant category (Fichet *et al.*, 2004). There are also strains of mycobacteria that show

distinctive resistance to glutaraldehyde, associated with alterations in the cell wall components (Manzoor *et al.*, 1999).

Figure1.5 Microbial resistance to biocides, most to least resistant. (McDonnell, 2007)



Over recent years there has been growing concern over the increased resistance of antibiotics, with various multiple resistance microorganisms identified in the healthcare setting. Of equal concern is the increasing resistance of microorganisms to biocides, as they are used routinely throughout our daily lives in the healthcare situation and to clean our homes and workplaces. As the usage of biocidal agents continues to grow, the potential for biocide resistance to occur also increases, as well as the potential for cross-resistance.

Bacteria use two main mechanisms of resistance to biocides. These are intrinsic and acquired methods of resistance.

1.5.1.1 Intrinsic resistance

Intrinsic resistance refers to the nature of the bacterial cell and how the cell works. This type of resistance is shown by bacterial spores, mycobacteria and several Gram-negative bacilli (Russell, 2001). Many bacteria have the ability to degrade biocides, and bacteria which possess efflux pumps have the ability to withstand biocide exposure. Bacteria which grow as biofilms have a greater insusceptibility to biocides (Russell and Chopra., 1990). Instances of intrinsic resistance are described below.

Not all biocides are sporicidal; the ones which are include formaldehyde, glutaraldehyde, halogen-releasing agents, peroxygens and ethylene oxide (Russell, 1995). Bacterial spores of the genera *Bacillus*, *Geobacillus and Clostridium* are known to be amongst the most resistant types to biocides. A spore is constructed in the following manner: it has a central core (protoplast, germ cell) and a germ cell wall which is surrounded by a cortex and an inner and outer spore coat, and then the exosporium (McDonnell and Russell,1999). The roles of the coat and the cortex are extremely relevant to the induction of resistance. The layers of the spore can be removed to study this resistance by using chemical and enzymatic treatment (McDonnell,

2007). Firstly, the outer layers of the spore are known to be a barrier to the biocides, since when they are removed there is an increase in susceptibility (Russell, 1990; Knott, 1995). The coat and outer spore membrane protect spores against biocidal activity by some smaller chemical agents, including glutaraldehyde, iodine, and some oxidizing agents (Bloomfield, 1994). The development of glutaraldehyde resistance in spores occurs in the late stages of sporulation when the protein coat is deposited (Power and Russell, 1988). There is a low level of water in the spore core, and this helps the spore to hold resistance to wet-heat (Cortezzo, 2004). Spore DNA is protected by its saturation with specific DNA binding proteins (Cortezzo, 2004). *Bacillus* spores are used regularly to investigate the principles of their resistance.

Other types of bacteria that have higher intrinsic resistance are mycobacteria. The reason that this particular type of bacterium is less sensitive is due to the cell wall being relatively impermeable, so the uptake of biocides into the cell is limited (Russell, 1996). Also, the highly hydrophobic nature of the cell wall prevents hydrophilic biocides from penetrating efficiently and in a high enough concentration to have any effect (Russell and McDonnell, 1999). There are resistant strains to glutaraldehyde which have been isolated from endoscope washers, which have also shown cross-resistance to peracetic acid (van Klingeren and Pullen, 1993). There has been another instance of

mycobacterial resistance to peracetic acid (Griffiths, 1997). This is concerning as peracetic acid is currently used in the disinfection of endoscopes, and it is known that mycobacteria are a problem to avoid when performing endoscopies on immunocompromised individuals.

Gram-negative bacteria are also particularly resistant to biocides, including *P. aeruginosa*, *Burkholderia cepacia* and *Proteus* spp., to mention just a few (Russell, 1999; Stickler, 2004). *P. aeruginosa* shows enhanced resistance to various biocides including QACs and chlorhexidine (Stickler, 2004). This bacterium has a low permeability outer membrane and it is known that the high Mg^{2+} content in the outer membrane produces strong lipopolysaccharide links. When these links are altered by the use of the chelating agent EDTA, increased sensitivity to particular biocides will occur, for example QACs and chlorhexidine (Stickler, 2004). There are multidrug efflux systems present within the inner membrane of *P. aeruginosa* which can pump out triclosan from the cell (Schweizer, 2001). The genus of *Proteus* has been shown to be resistant to QACs, chlorhexidine, and EDTA (Russell 1999), and the resistance of *Proteus mirabilis* has been shown to be due to the high content of phosphate-linked 4-arabinose in its lipopolysaccharide (LPS). This has also been shown for *B. cepacia* (Cox and Wilkinson, 1991; Tattawasart *et al.*, 2000).

1.5.1.2 Acquired resistance

Acquired resistance is not an inherent part of the bacterial cell make-up, but happens after mutation or acquisition of genetic material from plasmids or transposons.

Plasmids are extra-chromosomal genetic elements which have the ability to replicate independently (Rosenberg *et al.*, 1983). Resistance which is determined by plasmids is far more dominant and widespread than chromosomal resistance (Russell *et al.*, 1990). This particular type of resistance can be passed on to other bacteria, as plasmids can be transferred through the process of transduction, conjugation or transformation. Transformation involves the cell naturally taking up plasmids from the environment. Conjugation involves direct cell to cell contact, which is encoded by the plasmid. Transduction involves the transfer of the plasmid or genetic material by bacteriophage transfer (McDonnell, 2007). Transposons are mobile genetic (DNA) sequences, and can insert into host chromosomes or plasmids. They are not capable of autonomous replication, they can shift from one part of the chromosome to another (jumping genes), and the overall effect is to scramble the genetic information. This can be beneficial or have an adverse effect to the bacteria (Talaro and Talaro 1997). Transposons are involved in creation of different genetic combinations, changes in traits such as colony morphology, replacement of damaged DNA and can carry genes conferring resistance (Greenwood 1995).

Mutations can also happen which will change the nucleotide sequence of the nucleic acid which can favour the bacteria and the environment which it is in. An example of this can be seen with *S. marcescens* and its ability to grow in 100,000 µg/ml of QACs, whereas it would normally survive only <100 µg/ml (Chaplin, 1952). It was shown that the resistant and sensitive cells had different surface characteristics; the resistant cells lost their resistance when cultured in medium containing no QACs. It has been suggested that a mutational change occurred when the bacteria where in the presence of the QACs, however further evidence would need to be obtained.

Examples of triclosan resistance have been shown to be due to genetic mutations of the FabI (enoyl reductase) enzyme, and increased expression in wild type *S. aureus* can give rise to low level resistance, whilst increased expression in mutated FabI produces higher resistance (Heath *et al*, 1998; McMurry *et al.*, 1998; Fan *et al.*, 2002). Other methods of acquired resistance against triclosan include increased efflux and decreased uptake (McDonnell, 2007).

It is known that resistance to mercury is plasmid born and this can be transferred via conjugation or transduction (Russell, 1997). Silver resistance has been found in hospital burns wards (Klasen, 2000), which has also been linked to plasmids (Percival et al., 2005). Other examples of acquired resistance can be seen in *S. aureus* involving

plasmid-mediated resistance and encoded by the *qac* series of genes (Tennent *et al.,* 1989). Plasmid-encoded resistance to antiseptics and disinfectants has been reported in *Pseudomonas spp.* and members of the *Enterobacteriaceae.* These resistance phenotypes were attributed to the expression of the multidrug transporter genes *qacE* or *qacE*Δ1 in *Pseudomonas* and *Enterobacteriaceae* (Kucken *et al.,* 2000).

Efflux mechanisms may also be present within the cell which can aid biocide resistance. Active efflux occurs when molecules entering the cell are pumped out by membrane-bound proteins (Paulsen 1996), and this mechanism can accommodate a variety of biocides and antibiotics (Paulsen 1996). Efflux resistance of QACs can be found in Gram-positive bacteria, and especially in *Staphylococcus* spp. (Poole, 2005). Some of the efflux determinates are plasmid encoded, for example the Small Multidrug Resistance (SMR) family exporters. However the QAC A/B is a major facilitator (MF) family efflux system, where resistance arose from plasmid acquisition (Poole, 2005). Silver resistance in *Salmonella* has been associated with two plasmid encoded efflux systems (Silver, 2003).

1.5.1.3 Biofilm

Bacteria in biofilm formation are generally more resistant to biocides than planktonic organisms. A bacterial biofilm is bacterial growth on a solid surface which becomes encased in exopolysaccharide matrix

(Morton *et al.*, 1998; Mah and O'Toole, 2001). Biofilms can contain a variety of different species of microorganisms or be of singular species. It has been show that dental biofilms can have > 500 different bacterial species within (Whittaker *et al.,* 1996). The disinfection of biofilms is important as they can grow on medical devices such as indwelling catheters (Stickler, 1987), joint prostheses (Hall-Stoodley *et al.,* 2004), and endoscopes (Pajkas *et al.,* 2004), and also in the industrial environment in tanks, pipelines, water circulation systems and filtration units (Wood *et al.*, 1996)

Biofilms present a significant challenge to disinfection and sterilization. Various mechanisms are thought to be employed by the biofilm to withstand the disinfection process (Mah and O'Toole, 2001; Gilbert *et al.*, 2002). These include reduced access to the cells within the biofilm, and it has been suggested that the embedding of the biofilm in the anionic polysaccharide matrix gives protection to the bacterial cells housed within (Costerton, 1984). Studies using chlorine have shown the difficulty of penetrating the biofilm of mixed populations, and have shown degradation of the chlorine within the biofilm (de Beer *et al.*, 1994). It has also been suggested that enzymes produced by the bacteria within the biofilm also aid in resistance. Stewart *et al.* (2000) investigated the effect of catalase on reducing hydrogen peroxide penetration into a biofilm. There is evidence showing that hydrogen peroxide could penetrate a thick biofilm of *P. aeruginosa* which lacked

the catalase-producing gene *kat* A (Stewart *et al.*, 2000). However, there have been instances reported where the penetration of antibiotics has not been inhibited by the biofilm (Anderl *et al.*, 2000), or a slower penetration has occurred (Nichols *et al.*, 1988). Biofilm resistance has also been associated with slow bacterial growth and stress response (Mah and O'Toole, 2001). Wentland *et al.* (1996) have visualised using acridine orange stain the areas of the biofilm with fastest growth and areas with slower growth at the substratum level (Mah and O'Toole, 2001). However, this cannot be the singular mechanism that aids survival as the bacteria that have the faster growth rate at the periphery of the biofilm are far more sensitive towards a biocide attack and are killed, which subsequently results in the overall increased growth of other cells deep within the biofilm as the lysed cells provide nutrition (Gilbert *et al.*, 2002).

It has been suggested that the induction of a stress response will aid in the survival of a biofilm, and that this is what is making the bacteria grow slowly rather than the lack of nutrients deep within the biofilm (Mah and O'Toole, 2001). A stress response will produce a cascade of physiological changes which will help protect the cells from nutrient limitations, changes in pH, heat or cold shock, and many chemical toxins (Stickler, 2004). The stress response is controlled by primary sigma (RpoS) factor, which is increased in cells growing at high densities as shown by the production of trehalose (naturally occurring

disaccharide) and catalase (Liu *et al.*, 2000). Foley *et al.* (1999) found strong mRNA expression of the RpoS-encoded sigma factor in sputum from cystic fibrosis patients with chronic *Pseudomonas aeruginosa* lung infection. The evidence for a stress response produced during biofilm production has been further enhanced by the findings of Adams and McLean (1999) in *E. coli* which cannot produce the RpoS factor and cannot form biofilms.

It is possible that there is a general biofilm phenotype which has resistance to antimicrobials, and it is this which ultimately also controls the resistance of biofilms to disinfectants and sterilants. However, it is likely there is a number and variety of mechanisms involved in the resistance of biofilms, since an individual mechanism, when isolated in the laboratory, does not always result in resistance.

1.5.1.4 Enzymatic resistance

In the investigation of bacterial resistance, it is understood that various mechanisms play a role in producing heightened levels of insusceptibility. Enzymatic mediated resistance occurs when a bacterial species produces enzymes that detoxify the biocide which is attacking it. This method may also play a role in biofilm resistance (Mah and O'Toole, 2001.).

Examples of enzyme mediated resistance in the literature include the production of catalase in response to oxidative biocides like chlorine, hydrogen peroxide and peracetic acid (Chapman, 2003b). One particular example is in the over-production of catalase or by the induction of the *oxy*R or *sox*RS (activators of stress response) regulators that occur in *E. coli* (Dukan and Touati, 1996a,b). There have been various studies looking at the production of catalase and the protection it gives a bacterial cell to hydrogen peroxide exposure (Elkinis *et al.* 1999; Stewart *et al.* 2000; Eiamphungporn *et al.*, 2003). It has been shown that *B. subtilis* produces the enzyme catalase, with the gene *kat*A encoding for the vegetative catalase. KatA production is essential for tolerance against oxidative stress in *B. subtilis* (Naclerio *et al.*, 1995) and removal of the *kat*A gene results in increased susceptibility to hydrogen peroxide. The antioxidants glutathione and thioredoxin are known to play a role in the tolerance of oxidative stress in bacteria (Takemoto *et al.*, 1998; McDonnell, 2007).

There is evidence that enzyme mediated resistance plays a role in the resistance of *Pseudomonas* species to formaldehyde by over-producing formaldehyde dehydrogenase (Sondossi *et al.*,1986; Sondossi, 1989). This has also been shown in the resistance mechanisms of *Enterobacteriaceae*, where it is known that the enzyme is plasmid-mediated (Kaulfers and Marquardt, 1991). *E. coli* also degrades formaldehyde by producing glutathione which reacts to form

hydroxymethylglutathione, which is then oxidized by the dehydrogenase to S-formyglutathione which can be then metabolised by the cell (Dorsey and Actis, 2003; McDonnell, 2007).

1.5.1.5 Extracellular polymeric substances (EPS)

Extracellular polymeric substances (EPS) are the building blocks for bacteria to form clumps and aggregates in their natural environment. EPS is responsible for the structure and functional integrity of a biofilm (Wingender *et al.*, 1999). In fact, it is 28 years since Costerton (1981) suggested that EPS produced by bacteria would give the bacteria a degree of protection. The encasing of bacteria in EPS will restrict the diffusion of biocides and it is possible that the EPS will react with biocides and neutralise them (Dodds *et al.*, 2000) or exclude them due to size and viscosity (Brown, 1995; Nichols, 1988).

Oxidising agents can be consumed by a reaction with EPS; this was demonstrated by Wingender *et al.* (1999) with chlorine and strains of *P. aeruginosa*, and alginate was shown to be involved. Other examples of this particular reaction have further been shown with chlorine (de Beer *et al.*, 1994; Chen and Stewart, 1996) and also hydrogen peroxide (Liu *et al.*, 1997; Stewart, 1998). Stewart *et al.* (1998b) also showed that transport limitation within alginate (type of EPS in *Pseudomonas aeruginosa*) was not just directed at oxidising agents as it also affected glutaraldehyde, isothiazolone, and QACs. EPS also has a role in the

enzyme reactions which bacteria elute into their environment. Chumak *et al.* (1995) studied these interactions between bacterial EPS and bacteriolytic enzymes, and showed that they were excreted into the culture medium of *Pseudomonas* bacteria, and that this occurred during the lag growth phase. Relationships between bacterial EPS and extracellular enzymes have also been shown in activated sludge studies (Wingender *et al.,* 1999).

Bacterial EPS can play a huge part in resistance to various disinfection methods applied, and this is an important area of investigation. However, as it has been noted time and time again it may not always be one single method of the resistance that results in bacterial survival, but rather multiple resistance mechanisms. Also, it can depend on the concentration of the substance in use, the particular biocide being used, and exactly where the bacteria are growing (Wingender *et al.,* 1999).

1.6 Aims and Hypothesis

The aim of this study is to investigate and increase the understanding of the survival and resistance of bacteria to high level disinfectants. This will be done in the following manner:

- Establish the level of microbial contaminants of water derived from washer-disinfectors in samples collected after endoscope disinfection.
- Identify the microbial contaminants found, by use of various microbiological identification techniques.
- Establish a susceptibility profile to high level disinfectants, by using various standard efficacy tests.
- Understand possible resistance mechanisms used by the various microbial contaminants to survive harsh environments, by examining the bacterial cells through electron microscopy techniques and investigating changes in expression of possible detoxification genes using molecular biology techniques.

The main hypothesis of the study is that microbial contaminants will be found within the water of washer-disinfectors, the microbial contaminates will not be susceptible to standard concentrations of high level disinfectants (as evidenced by their isolation) indicating resistance, and finally will posses a variety of mechanisms (rather than one single mechanism) to aid their survival in what is a harsh environment.

Chapter 2:

General Materials and Methods

2 General materials and methods

2.1 Materials

2.1.1 Chemicals

Tryptone (Oxoid, Basingstoke. UK) and sodium chloride (Fisher Scientific, Leicestershire, UK) were used to make tryptone sodium chloride (TSC). Sodium thiosulphate (Fisher Scientific, Leicestershire, UK) was used as a biocide neutralizer in the suspension and carrier test methods and was made according to British Standard BS EN 1279:1997. Glycerol (Fisher Scientific, Leicestershire, UK) was used for making frozen stock bacterial cultures.

For the staining of bacterial samples, safranin, crystal violet, Gram's iodine, malachite green and copper sulphate were all purchased from Fisher Scientific (Leicestershire, UK). Albumin from bovine serum was purchased from Fisher Scientific (Leicestershire, UK) for use as an organic load in the suspension and carrier tests.

2.1.2 Biocides

Tristel© (chlorine dioxide), Vaprox® (hydrogen peroxide), Steris 20® (peracetic acid and hydrogen peroxide plus formulation ingredients), Steris Hamo PAA® (peracetic acid) and Reliance HLD® (peracetic acid) were kindly supplied by Steris® Ohio, USA.

2.1.3 Growth media

Tryptone Soya Agar (TSA) and Tryptone Soya Broth (TSB) were purchased from Oxoid Ltd. (Basingstoke, UK). Middlebrook 7H9 broth and Middlebrook 7H11 agar were purchased from Becton Dickinson & Co. (Franklin Lakes, NJ, USA).

2.1.4 Sporulation media

Sporulation media was made in accordance with the EN 14347: 2005 (European Committee for Standardization 2005), which contained: 10 g peptone (Fisher Scientific, UK), 2 g yeast extract (Fisher Scientific, UK), 0.04 g manganese sulphate (Fisher Scientific, UK), 15 g agar (Fisher Scientific, UK) made up to 1,000 ml with deionized water. This was then sterilised for 15 min at 121 °C.

2.2 General methods

2.2.1 Biocides

All biocides were made to the desired concentrations using sterile deionized water, and made fresh on each day of use, adhering to the strict guidelines produced by Steris® as outlined bellow. Tristel (chlorine dioxide) was prepared in the following manner: 1.2 ml of solution A of the disinfectant was pipetted into a conical flask along with 1.2 ml of solution B, both solutions were mixed for a few minutes then 250 ml of

sterile deionized water was added. This gives a final concentration of 135 ppm ClO_2, which is the in-use concentration of this product. The mixed solution was then heated to 30°C prior to use.

Steris 20® (peracetic acid and hydrogen peroxide plus formulation ingredients) was prepared in the following manner: 0.82g of the dry component was dissolved in 50ml of sterile deionized water and heated to 53 °C, and then 350 µl of the 35% peracetic acid was added. This was used for testing immediately; activation of the disinfectant should be performed in a well-ventilated area or fume hood.

Reliance HLD® (peracetic acid) was prepared in the following manner: 0.5 g of Part A of the formulation was dissolved in 100 ml of deionized sterile water and heated to 53°C. Once this temperature was reached 0.28 g Part B was added and the solutions gently mixed at 53°C for 4 minutes, which is required for activation. Activation of the disinfectant should be performed in a well ventilated area or fume hood.

Steris Hamo PAA® (peracetic acid) was prepared in the following manner: disinfectant parts A and B were mixed together in equal quantities. (Further dilutions of this solution were done with deionized sterile water).

2.2.2 Micro-organisms

2.2.3 Bacterial Strains

Reference strains were purchased freeze-dried from the American Type Culture Collection (ATCC). Table 2.1 shows the strains used in this study. All other strains were environmentally isolated from endoscope washer disinfectors (see chapter 3, section 3.3).

2.2.4 Culture and Growth

Broth cultures were incubated for 24 hours at 30°C or 37°C, depending on the bacterial strain, in a shaking incubator (Lab Companion, SI-300, Jeio Tech., Seoul, Republic of Korea) at 70 rpm. Agar cultures were incubated for 24 hours at 30°C or 37°C, depending on the bacterial strain, in an appropriate incubator (Memmert IPP 400 (30°C) or Memmert INE 600 (37°C), Schwabach, Germany).

2.2.5 Maintenance of strains

Freeze dried ATCC reference strains were recovered through the recommended ATCC method. Aseptically, 0.3 - 0.4 ml of liquid medium was added to the freeze-dried material with a Pasteur pipette and mixed well. Then the mixture was transferred to the recommended broth medium (5 - 6 ml). Some of this suspension was also transferred to an

agar plate. Broth and agar plates were then incubated under the appropriate conditions.

Isolation of pure bacterial cultures was done through a streak plate method (Cappucino and Sherman, 1999). This was undertaken to check that the bacterial culture was of one culture and no contamination had occurred. Cultures were maintained on TSA slopes, in triplicate. The first slope was used to inoculate cultures, the second one as back-up, and the third one for additional sub-culturing when needed. Slopes were re-made every four weeks from freezer cultures.

2.2.5.1 Washed cultures

Bacterial cultures were grown overnight at a temperature required for the bacterial strain being used, centrifuged (MSE, Mistral 1000. London, UK) for 10 min at 5000 g. The pellet was then re-suspended by vortexing in TSC until it was fully suspended.

2.2.5.2 Frozen cultures

Washed cultures were added to 1.5 ml cryogenic vials (Fisher Scientific, Leicestershire, UK) which contained 20% glycerol. The vials were vortexed to ensure glycerol and bacterial cultures had mixed well. Vials were then frozen at −80°C.

Table 2.1 Bacterial strains used in the study.

Strain	Incubation temperature (°C)	Source
Micrococcus luteus ATCC 4698	30	ATCC
Bacillus subtilis ATCC 6051	30	ATCC
Micrococcus luteus	30	Environmental isolate
Bacillus subtilis	37	Environmental isolate
Streptococcus mutants	37	Environmental isolate
Streptococcus sanguis	37	Environmental isolate
Staphylococcus intermedius	37	Environmental isolate
Streptococcus gordonii	37	Environmental isolate
Bacillus licheniformis	30	Environmental isolate
Gardnerella vaginalis	37	Environmental isolate
Brevibacillus brevis	30	Environmental isolate
Leifsonia aquaticum	37	Environmental isolate

2.2.6 Viable count: Drop count method

The method used to calculate viable count as colony forming units per ml (CFU/ml) of bacterial culture was the drop count method (Maillard *et al.*, 1998). Bacterial samples were serially diluted (1/10) in TSC, then plated out in 3× 10 µl spots per TSA plates, for three replicate plates. Plates were left to dry on the bench at room temperature (22 - 25°C), inverted and incubated at the appropriate temperature. After 24 hours incubation, the CFU per plate were counted and the viable count in the culture (CFU/ml) calculated. The drop count technique was validated by performing 10 separate dilution series as described above. The data were then subjected to one way ANOVA in Minitab Statistical Software Package (release 14 software, Minitab Inc. PA, USA) to ensure no significant difference in the drops made. Validation was deemed successful when $p \geq 0.05$ and data was normally distributed according to the Anderson-Darling normality test.

2.2.7 Viable count: Pour plate method

The pour plate method was used as an alternative to the drop count method (2.2.6) for tests that required a higher degree of accuracy or for detection of lower concentrations of cells, due to the lower limit of detection. The bacterial suspensions were serially diluted (1/10) in TSC, and then 1 ml was pipetted into sterile Petri dishes for 3 replicate plates. Approximately 20 ml of molten agar at about 45°C was then poured into

the Petri dish. Plates were left to dry at room temperature for approximately 1 hour until solidified, then inverted and incubated at the appropriate temperature for 24 hours. Dilutions yielding counts between 30 and 300 CFU were recorded, and others were discarded.
The mean CFUs of the duplicate plates were calculated and from this the CFU/ml of the original suspension.

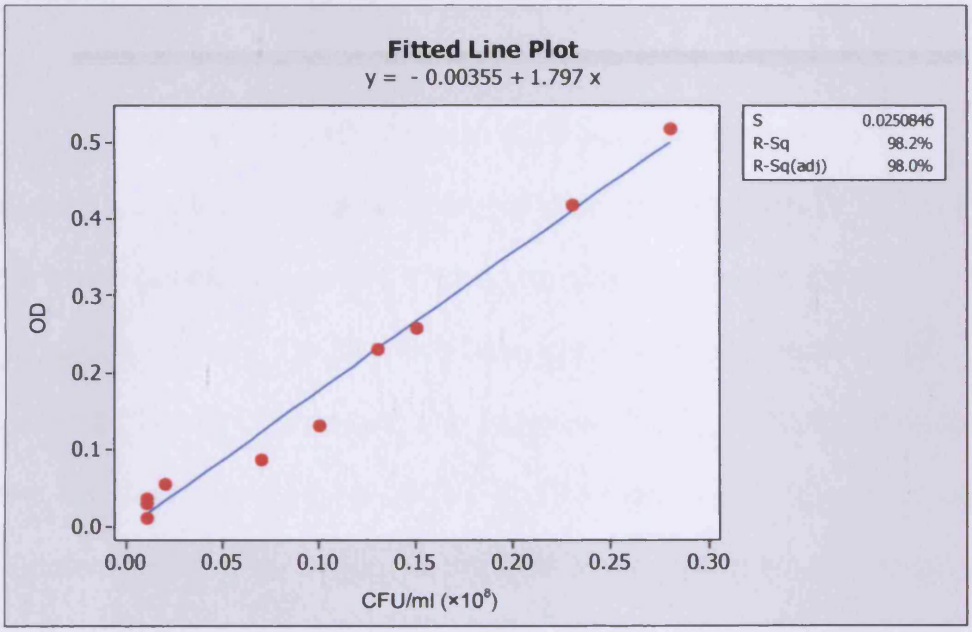
The data were then subjected to one way ANOVA in Minitab Statistical Software Package to make sure there was no significant difference in the pipetting. Validation was deemed successful when $p \geq 0.05$ and data were normally distributed according to the Anderson-Darling normality test.

2.2.8 Standardisation of Cultures

A bacterial suspension was prepared according to section 2.2.5.1 and the following dilution series was made with culture and TSC: 1/2, 1/4, 1/5, 1/10, 1/20, 1/40 and 1/100. The optical density for each suspension was measured at 500 nm using a spectrophotometer (Helios α spectrophotometer, Unicam, Cambridge, UK). Viable counts were performed using the drop count method for each dilution and the starting culture. The data produced for optical density were then plotted against the data produced from the viable count in a fitted line plot graph. This was achieved using Minitab® (release 14 software, Minitab Inc. PA, USA). From the plot a linear equation was used in order to

establish the optical density required to reach the desired bacterial concentration. Figure 2.1 shows an example of this type of plot for *Bacillus subtilis* 6051.

Figure 2.1 Optical density vs. CFU/ml graph for *B. subtilis* 6051. The curve represents the best fit by linear regression and the equation shown on the graph is y = c + mx.



2.2.9 Statistical Analyses

When statistical analysis was required, the results in this study are the mean of three replicates unless otherwise stated. Statistical analyses were carried out with Minitab® (release 14 software, Minitab Inc. PA, USA). The one-way analysis of variance (ANOVA) was used, with statistical significance set at the 0.05 level. Values are stated as mean ± standard deviation.

Chapter 3:

Microbial Contamination of Flexible Endoscopes

3 Microbial contamination of flexible endoscopes

3.1 Introduction

A recent enquiry has sparked new interest in potential endoscope decontamination failure. In 2004, the NHS in Northern Ireland had to acknowledge the potential risk of transmission of blood borne viruses, which then involved investigations in 1300 patients, many of whom required blood tests to assess potential infections (Doherty *et al.*, 2004). This investigation was started after an auxiliary channel in an endoscope was found not to have been cleaned or disinfected since purchase (Gamble, Duckworth and Ridgway, 2007). A medical device alert was then issued by the MHRA, and investigations into endoscope decontamination were begun. At the time of the incident in Northern Ireland, English Trusts were asked to review their decontamination procedures. Other examples of contamination of endoscopes have been shown to be caused by the endoscope washer disinfector problems. This has been shown with outbreaks of *Pseudomonas* in 2001, where contaminated disinfectants were used and the concentration being employed was deemed too low (Antonucci *et al.*, 2001). Also, the washer disinfector can have biofilm build-up inside if the machinery is not well maintained; this was shown to be the cause of patient infections with *Ps. aeruginosa* after endoscopy procedures (Alvarado *et al.*, 1991). Other outbreaks associated with washer

disinfectors have been linked to the contamination of water used in the machinery (Reeves *et al.,* 1995, Cooke *et al.,* 1998, Pang *et al.,* 2002).

Several procedures of endoscope decontamination in England over the period of 2003-2004 were shown to be questionable (Gamble *et al.*, 2007). The report showed that errors may have occurred with 23,338 patients and many of the patient numbers were unknown. Various errors were noted as occurring, including auxiliary channel decontamination not being achieved, endoscopes receiving manual decontamination only, failure with automated endoscope reprocessor (Gamble *et al.*, 2007) (which include alarms being removed from systems and the inappropriate use of disinfectants), and also staff training and ability came under scrutiny. These investigations continued to highlight the fact that even if all areas were assessed, thought to be under control and working effectively, then this may not always actually be the case. Therefore, the regulation and surveillance of disinfection should be investigated on a regular basis.

The aim of this chapter is to investigate endoscope washer disinfectors and to establish whether or not contaminants can survive high level disinfection within the working machines. If microorganisms can survive they potentially can be transferred between patients via endoscopes.

3.2 Materials and methods

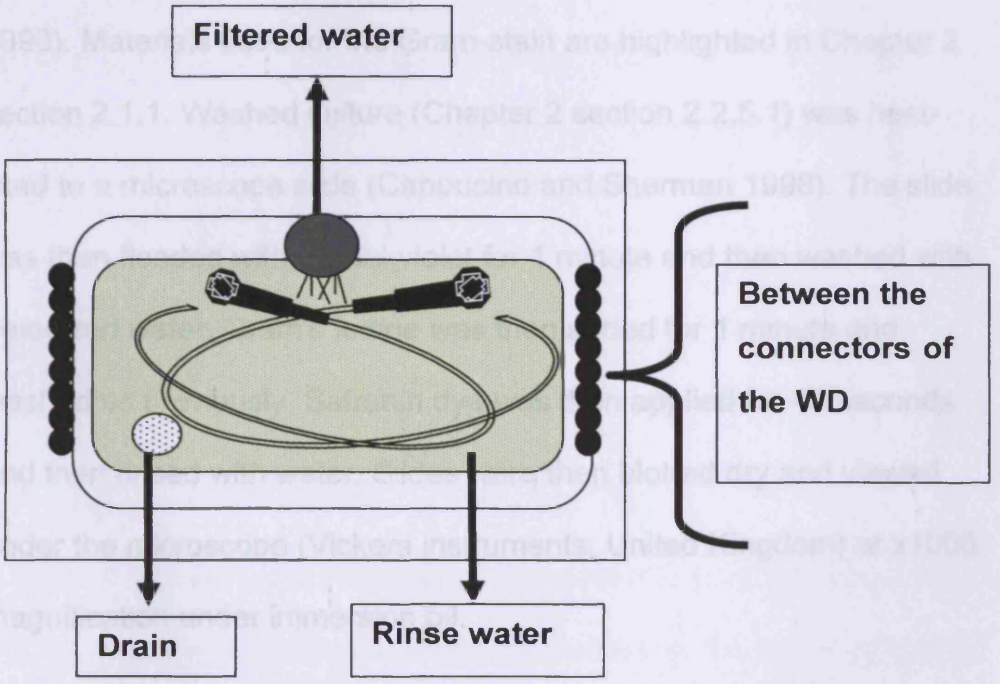
3.2.1 Sampling plan

Samples were taken from an Endoscopy Unit at a Hospital in the United Kingdom. Sampling took place on 25th January 2006, 3rd October 2006 and the 26th of April 2007 (Martin *et al.*, 2008). The type of endoscope washer disinfector (WD) used at the Endoscopy Unit was an Autoscope Guardian washer disinfector (Labcare, Clevedon, UK), using the disinfectant Tristel One-Shot® (Tristel Solutions Ltd., Cambridgeshire, UK). On the first day of sampling, samples were taken from the endoscope prior to disinfection by swabbing (swabs from Technical Service Consultants Ltd., Heywood, UK) the endoscope surfaces and brushes used to clean the endoscope channels. This was to get pilot data on contamination levels before disinfection. Samples were then taken after the endoscopes had been processed in the washer disinfector; the areas which where sampled are highlighted in Figure 3.1.

Samples could not be taken from the endoscopes after disinfection as they were needed for procedures. The rinse water was sampled by taking a 10 ml sample using a sterile syringe (when the disinfection process was in its final stage) and then a swab was made from this on to an agar plate. The water tank was also sampled using this method on the second and third date of sampling. The sampling was based on the

methods of testing used at the Endoscopy Unit. All sampling swabs were inserted into the accompanying sterile tube containing Amies medium and charcoal, placed on ice for transportation, and processed in the laboratory within the recommended 24 hours.

Figure 3.1 Areas sampled from automated washer disinfector.



The swabbed samples were inoculated onto TSA, and Middlebrook 7H11 agar. The plates where incubated at 37°C and 30°C for 24 and 48 hours and then re-incubated for a further 24 hours to identify any new growth. After incubation the bacteria where then picked on the basis of variations in colony morphology and pigmentation. Pure cultures were then made of each of the bacteria isolated.

3.2.2 Sample identification

3.2.2.1 Staining

3.2.2.1.1 Gram-stain

Gram-staining is a technique used to determine the difference between Gram-negative and Gram-positive bacteria (Cappuccino and Sherman 1998). Materials used for the Gram-stain are highlighted in Chapter 2 section 2.1.1. Washed culture (Chapter 2 section 2.2.5.1) was heat-fixed to a microscope slide (Cappucino and Sherman 1998). The slide was then flooded with crystal violet for 1 minute and then washed with deionized water. Gram's iodine was then added for 1 minute and washed as previously. Safranin dye was then applied for 45 seconds and then rinsed with water. Slides were then blotted dry and viewed under the microscope (Vickers instruments, United Kingdom) at x1000 magnification under immersion oil.

3.2.2.1.2 Spore Stain

Materials used for the spore stain are highlighted in Chapter 2 section 2.1.1 Chemicals. Washed cultures were heat fixed to a microscope slide, then flooded with malachite green and placed onto a hot plate and allowed to steam for 2-3 minutes. The slide was then removed and washed with deionized water and counter-stained with safranin dye for 30 seconds (Cappuccino and Sherman 1998). The slide was then

blotted dry and viewed under the microscope at x1000 magnification under immersion oil.

3.2.2.1.3 Capsule stain

Washed cultures were air-dried onto microscope slides, flooded with crystal violet for 5-7 minutes and washed with 20% copper sulphate solution (Cappuccino and Sherman 1998). Slides were then blotted dry and observed under the microscope at x1000 magnification under immersion oil.

3.2.2.2 Enzymatic assays

3.2.2.2.1 Catalase test

A drop of washed bacterial cultures was added to a microscope slide. A 3% hydrogen peroxide solution was then added to the bacterial culture on the slide. A positive reaction was observed by the formation of bubbling in catalase positive cells (Cappucino and Sherman 1998). *Micrococcus luteus* ATCC 4698 was used as a positive control.

3.2.2.2.2 Oxidase test.

Oxidase test sticks (Oxoid Limited, Hampshire, UK) were removed from the refrigerator five minutes before the start of the procedure. The impregnated end of the stick was rotated over a single colony. The stick

was examined after 30 seconds, if no colour change had occurred the stick was observed after a further 3 minutes. A positive reaction was shown with a blue-purple colour, with no colour change being negative. Positive and negative controls were used as recommended by Oxoid's instructions. Positive control *Pseudomonas aeruginosa* ATCC 27853 and negative control *Staphylococcus aureus* ATCC 25923. Both these bacterial strains were grown as described in chapter 2 section 2.2.4

3.2.2.3 BD BBL Crystal™ identification systems

A BBL Crystal kit (Becton Dickinson and Company, Maryland, USA) works by using modified classical methods to identify bacteria with fluorogenic and chromogenic substrates. The Gram-positive BBL crystal kits were used as all samples were Gram-positive. Table 3.1 summarizes the different tests within the BBL crystal Gram-positive kit.

The BBL crystal kit was used by following the manufacturer's instructions. Bacterial sample were incubated overnight, using the streak out method to achieve single colonies (Cappucino and Sherman). Single colonies were re-suspended in a tube of BBL crystal inoculum fluid (Becton Dickinson and Company, Maryland, USA). Inoculum turbidity was equivalent to a McFarland No. 0.5 standard (Becton Dickinson and Company, Maryland, USA). The inoculum was then poured into the base target area. The base was held in both hands then tilted to fill all wells in the base, and any excess fluid put back into

the target area. The lid was then clicked into place, and identification number was added. In order to show sterility a small drop of the inoculum fluid was inoculated on to an agar plate, and incubated. The plates were checked the following day.

The inoculated panels were incubated face down with a small drop of water on an incubation tray at 37°C for 24 hours and read within 30 minutes after the incubation period. The panels were read using the colour reaction chart. The fluorescent results were read with a BBL Crystal panel viewer (Becton Dickinson and Company, Maryland, USA). Once the profile number had been generated, it was then entered into the BBL Crystal MIND software (Becton Dickinson and Company, Maryland, USA).

One sample (environmental sample 48) was sent for identification verification to NCIMB Ltd., Aberdeen, UK using their MicroSeq™ service. This was done to confirm the identity of the bacterial sample.

Table 3.1 Test used in BBL Crystal kit.

Test Feature	Principle
Fluorescent negative control 4MU-β-D-glucoside L-valine L-phenylalanine 4MU-α-D-glucoside L-pyroglutamic acid L-tryptophan L-arginine 4MU-N-acetyl-β-D-glucosaminide 4MU-phosphate 4MU-β-D-glucuronide L-isoleucine	Control for fluorescent enzymatic hydrolysis of the amide or glycosidic bond results in the release of a fluorescent coumarin derivative
Trehalose Lactose Methyl-α & β-glucoside Sucrose Mannitol Maltotriose Arabinose Glycerol Fructose	Utilization of carbohydrate results in lower pH and a change in indicator
p-nitophenyl-β-D-glucoside p-nitophenyl-β-D-celloboside	Enzymatic hydrolsis of the colourless aryl substituted glycoside releases yellow p-nitrophenol
Proline & Leucine-p-nitroanilide	Enzymatic hydrolysis of the colourless amide substrate releases yellow p-nitroaniline
p-nitrophenyl-phosphate p-nitrophenyl-α-D-maltoside o-nitrophenyl-β-D-glactoside	Enzymatic hydrolysis of the colourless aryl substituted glycoside releases yellow p-nitrophenol
Urea	Hydrolysis of urea and the resulting ammonia change the pH indicator colour
Esculin	Hydrolysis of esculin results in a black precipitate in the presence of ferric ion
Arginine	Utilization of arginine results in pH rise

3.3 Results

3.3.1 Sampling

Sampling took place on three separate occasions. Details of samples retrieved and identified are shown in Tables 3.2-3.4. The first sampling took place on the 24/01/06 (Martin *et al.*, 2008). The first batch of sampling on that date was to investigate the overall contamination of the endoscopes prior to disinfection. The results showed that contamination levels were high as the agar plates were covered with a lawn of bacterial growth; no identification was performed on these samples.

Table 3.2 Samples isolated: first collection.

Date of sampling	WDs number	Area of sampling	Positive/ negative	Identification numbers
24/1/2006	1	Rinse water	+	47
	1	Drain	+	50
	1	Tube area	+	49
	2	Rinse water	+	48
	2	Drain	+	52
	2	Tube area	-	n/a

The second batch of sampling on this date examined contaminants from the washer disinfector, the samples retrieved here are detailed in table 3.2, and their identification is shown in table 3.6. The third batch of sampling done on this date involved looking at the rinse water which is used in the final disinfection stage in the washer disinfector. These

results showed that there was no contamination of the water entering the disinfection system.

The second date on which sampling took place was the 3/10/06. This second sampling was used to establish if the contaminants identified in the first sampling reoccurred, and specifically looked at the washer disinfector area of sampling as this was where the samples had been retrieved previously. These results are shown in table 3.3 with the identification of the bacteria in table 3.6.

Table 3.3 Samples isolated: second collection.

Date of sampling	WDs number	Area of sampling	Positive/ negative	Identification numbers
3/10/06	1	Rinse water	-	n/a
	1	Drain bottom	-	n/a
	1	Drain side	-	n/a
	1	Tube area	-	n/a
	1	Water tank	+	52
	2	Rinse water	-	n/a
	2	Drain bottom	+	54, 52.1, 55, 57.1
	2	Drain side	+	55.1, 56, 54.1
	2	Tube area	-	n/a
	2	Water tank	-	n/a
	4	Rinse water	-	n/a
	4	Drain bottom	-	n/a
	4	Drain side	-	n/a
	4	Tube area	+	53
	4	Water tank	-	n/a

The final date of sampling was on the 26/04/07, and once again this focussed on the washer disinfector to see if isolates which had been retrieved on sampling date one and two were retrieved again. This last sampling retrieved no positive results. This could show that the problem that was causing the persistence of isolates had been removed, or the washer disinfector had undergone maintenance and decontamination to resolve the problem.

Table 3.4 Samples isolated: third collection.

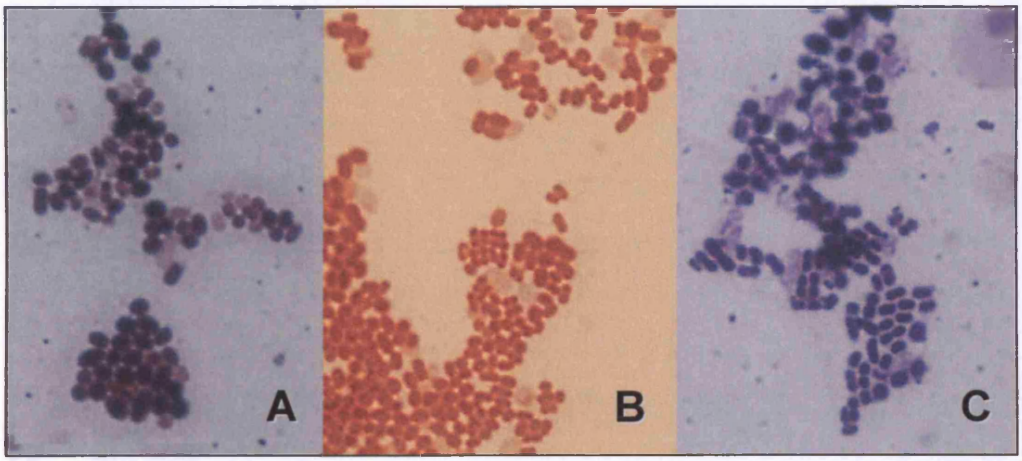
Date of sampling	WDs number	Area of sampling	Positive/ negative	Identification numbers
26/04/07	1	Rinse water	-	n/a
	1	Drain bottom	-	n/a
	1	Drain side	-	n/a
	1	Tube area	-	n/a
	1	Water tank	-	n/a
	2	Rinse water	-	n/a
	2	Drain bottom	-	n/a
	2	Drain side	-	n/a
	2	Tube area	-	n/a
	2	Water tank	-	n/a

3.3.2 Sample identification

The results from the basic microbiology identification tests are shown in Table 3.5. Firstly the staining of the samples were conducted to identify if the bacteria were Gram-negative or -positive and to establish if they could produce spores and had capsules. Examples of the staining

techniques used are seen in figure 3.2. This is the staining done for sample number 49 which was isolated from the tubing which connects the endoscope to the washer disinfector. This shows that it is a Gram positive organism, it does not produce spores and it does not have a capsule. Staining the bacteria also makes the morphology of the bacteria more visible.

Figure 3.2 Staining techniques used for identification. Showing examples of the Gram-stain (A), spore stain (B) and capsule stain (C).



Not all standard bacterial testing and staining was done on all bacteria isolated as identification was finalised with the use of the BBL crystal test. Using appropriate standards, *Pseudomonas aeruginosa* ATCC 27853 was positive in the oxidase test and *Staphylococcus aureus* ATCC 25923 was negative in the oxidase test. *Micrococcus luteus* ATCC 4698 was positive for catalase production. This showed that the identification test was working appropriately.

Table 3.5 Microbiological identification test results.

N°	Location	Staining			Enzyme test	
		Gram	Capsule	Spore	Oxidase	Catalase
47	Rinse Water	+	–	–	–	+
48	Rinse water	+	–	+	+	+
49	Tube area	+	–	–	–	–
50	Drain	+	–	–	–	–
51	Drain	+	–	–	–	+
52	Water tank	+	*	–	*	–
52.1	Drain bottom	+	*	–	*	–
53	Tube area	+	-	+	+	+
54	Drain bottom	+/–	–	–	–	–
54.1	Drain side	+/–	–	–	–	–
55	Drain bottom	+	*	+	*	+
55.1	Drain side	+	*	+	*	+
56	Drain side	+	*	–	*	+
57	Drain bottom	+	–	–	–	+

Key. +/– refer to Gram variable organisms

*** tests were not conducted**

The bacterial samples were fully identified using the BBL Crystal kits. The results are shown in table 3.6. From this table we can see that a variety of bacteria were isolated, including species recognised as part of the normal oral flora of humans, (*Streptococcus sanguis*, *Streptococcus mutans, Streptococcus gordonii*) and linked to dental plaque production

(Talaro and Talaro, 1997). They are also significant causative bacteria for infective endocarditis.

Table 3.6 Sample identification of WD isolates.

N°	BBL Crystal N°	Identification	Confidence*
47	1761000177	*Micrococcus luteus*	.9995
48	2704000653	*Bacillus subtilis*	.9998
49	3571000141	*Streptococcus sanguis*	.923
50	0460661702	*Streptococcus mutans*	.9846
51	0224777571	*Staphylococcus intermedius*	.9955
52	3465000561	*Streptococcus gordonii*	.9972
52.1	3465000561	*Streptococcus gordonii*	.9972
53	2735162767	*Bacillus licheniformis*	.851
54	1565000141	*Gardnerella vaginals*	.7782
54.1	1565000141	*Gardnerella vaginals*	.7782
55	3675000567	*Brevibacillus brevis*	.9999
55.1	3464000563	*Brevibacillus brevis*	.9579
56	3665000773	*Leifsonia aquaticum*	.9999
57	0761000165	*Micrococcus luteus*	.9986

* Confidence rating of 0.6000 to 1.0000 was considered a correct identification.

Bacillus licheniformis, *Bacillus subtilis* and *Brevibacillus brevis* can be found in the soil but have also been know to cause infections. *Leifsonia aquaticum* can be found in water samples, and have been found as biofilms in haemodialysis machinery. *Gardnerella vaginalis* it is most commonly recognized for its role as one of the organisms responsible for bacterial vaginosis but has been found in the pharynx.

3.4 Discussion

The sampling methods used were established through meetings with staff from the Infection, Prevention & Control Department at a Hospital in the United Kingdom. The methods used were extended beyond just sampling of rinse water (this was what was in place at the endoscopy unit at time of sampling) to looking at the various areas described in figure 3.1, in order to examine areas which have been cited in the literature review (chapter 1 section 1.5), as potential problem areas and areas which have been associated with bacterial contamination.

Sampling after high-level disinfection of the endoscopes would have been beneficial in order to establish if any contaminants from the washer disinfector had been transferred to the endoscope, or if there was indeed still bacterial biofilm within the endoscope channels. This was unfortunately not possible due to the high demand for endoscopes within the unit.

The diversity of bacterial samples obtained was interesting, although not necessarily the expected isolates. Bacteria which have been found in the past include *Pseudomonas*, and mycobacteria, which were not found within this study. However, *Bacillus subtilis* and *Micrococcus luteus* have been isolated previously (Bisset *et al.*, 2006) in a study looking at patient-ready endoscopes, and also now in this study.

The lack of mycobacterial isolation may have been arisen because of the short length of time agar plates were incubated, if the mycobacteria were present at all. It would be ideal for the experiments to be repeated with longer incubation periods and the use of minimal medium, as some bacteria may have not been able to grow as well on the rich nutrient agar provided.

Of the bacteria isolated, *Micrococcus luteus* was isolated on a second sampling date. The first time was from the rinse water showing the possibility of bacteria still being present within the endoscope, whilst the second time it was found present on the surface of the drain of the endoscope washer disinfector, surviving even after high-level disinfection. *Micrococcus luteus* is part of the normal flora and isolated from the environment; it has also been encountered as an opportunistic pathogen. It has been associated with clinical manifestations including catheter-related bacteremia and sepsis, endocarditis, central nervous system infection, and traumatic and post-operative endophthalmitis (Magee *et al.*, 1990, Oudiz *et al.,* 2004, Miller *et al.*, 2007). The fact that this microorganism apparently survived high-level disinfection leads to the question as to whether there were any isolated cases of bacteremia involving this microorganism in patients who had received endoscopy procedures around this period of time. It would be important here to identify if both these isolates were indeed the same strain, possibly

indicating that the bacteria was surviving within the machinery or it was introduced from an external source.

Other samples were found in similar areas, including rinse water, drain areas, water tank and connectors that attach the endoscopes to the machine. Drain areas should contain no bacterial samples, as these are areas which are always in contact with the disinfection solution, so this indicates that the disinfectant being used may be ineffective or incorrectly prepared.

Since the rinse water used after disinfection was filter sterilised no bacteria should have been present within this system. This suggests that there was a step within the disinfection programme that was not being followed, or that was missed out. It shows that the first part of manual cleaning was not being done effectively, or that the rinse water used was indeed not bacteria-free, therefore introducing bacteria into the system.

Some of the bacterial samples isolated were unsuspected. For example, *Staphylococcus intermedius* is a zoonotic organism which can be found in pigeons, cats and dogs. When found in humans, it is usually due to exposure to infected animals, such as from a canine bite (Pottumarthy *et al.,* 2004), so how this organism came to be found in the drain of a washer disinfector is baffling. This may have entered

through staff contact when cleaning endoscopes. Great care was taken when samples were being retrieved in order not to contaminate them (appropriate gowns, face mask and gloves were used in the endoscopy unit when sampling), and therefore contamination by the sampler is minimised.

On the final sampling of the washer disinfectors, (which took place in April of 2007) it showed that there were no samples showing bacterial growth. This indicates that there may have been a change in the disinfection protocols used within the washer disinfector. However, any changes made were not indicated to the researcher by the endoscope unit staff or management team.

3.4.1 Summary

This chapter has shown that bacteria can be isolated from areas that have been processed with high-level disinfectants (Martin *et al.*, 2008), and that these organisms may persist within the system and may be isolated again. It also highlights the areas where contaminants can be found and that the routine sampling of rinse water only may not be sufficient and other areas should also be sampled on a regular basis. Also, if sampling and culture methods are amended appropriately, other microorganisms might be identified.

The implementation of a monitoring procedure for the cleaning and disinfection process should be considered to ensure that staff are aware of each protocol and how to undertake the tasks of manual and chemical disinfection as required. The literature recognises that in some instances set guidelines are not always complied with. Failure to do so allows bacterial contaminants to survive in areas in which they would ordinarily be excluded following effective decontamination. The use of surveillance programmes should be initiated taking into account the issues raised by Cookson (2005). Such programmes should include monitoring of pathogens within the hospital setting, then testing microorganisms that are isolated against biocides being used. It is also important to identify the resistance mechanism in order to make changes to disinfection strategies to resolve any problems of re-occurring pathogens.

Chapter 4:

Efficacy of oxidising disinfectants against washer disinfector (WD) isolates

4 Efficacy of oxidising disinfectants against washer disinfector (WD) isolates

4.1 Introduction

Efficacy tests are used in order to establish the ability of a biocide to kill microorganisms within a specific exposure time and under particular physical conditions, such as on surfaces, different temperatures, and concentrations, etc. (Reybrouck, 2004). There are three fundamental stages in investigating disinfection efficacy: 1, primary testing or screening (e.g. use of MICs), 2, laboratory tests (suspension and carrier tests); and 3, in-loco tests (investigating how a disinfectant would work in the conditions under which it is used) (Lambert, 2004). There are various types of testing available including carrier tests, suspension tests, and capacity tests, which will be discussed later on in this chapter. There are practical tests which replicate real-life conditions looking at the testing of instrument, surface and textile disinfection (Reybrouck, 2004). These practical tests follow investigations establishing time/concentration relationships of the disinfectant; they are used to establish if the disinfectant will work under conditions which will influence the outcome, for example, in the presence of organic matter (Reybrouck, 2004). Instrument disinfection testing can be based on the carrier test with a standard piece of metal as the inoculated carrier. A higher load of organic matter is used and disinfectants can be diluted in hard water (Reybrouck, 2004). In-use tests are also used to

establish the effectiveness of a disinfectant product in the field of use and under actual conditions used for that product (Reybrouck, 2004). In-use testing has been used to evaluate the effectiveness of endoscope disinfection (Coates, 2001). There are various testing protocols that can be used for particular types of bacteria, for instance in investigating the activity of disinfectants against mycobacteria, bacterial spores, fungi (Reybrouck, 2004) and viruses (Valot *et al.*, 2000; McDonnell, 2007). There are diverse tests for the specific activity of disinfectants and various organisations have published a number of standard protocols (McDonnell, 2007), and the various standards for suspension and carrier test methods are listed in Tables 4.1 and 4.2.

There is no single universal test which can be implemented for all disinfection testing as situations vary and there are benefits and disadvantages in each of the methods used. When conducting the various tests on disinfection and evaluating a particular disinfectant reference strains are available to challenge the disinfectants. In the standard efficacy test particular test organisms have been indicated for evaluating efficacy that might challenge the disinfectant.

Table 4.1 Examples of standard suspension test methods.

Standard suspension test methods, European and international methods

Group + Standard Number	Title
AOAC official method 955.11	Testing disinfectants against *Salmonella typhi*
ASTM E1052-96 (2002)	Standard test method for efficacy of antimicrobial agents against viruses in suspension
ASTM E1891-97 (2002)	Standard guide for determination of a survival curve for antimicrobial agents against selected microorganisms and calculation of a D-Value and concentration coefficient.
USP XXIII	Antimicrobial preservatives- effectiveness protocol
EN 1040: 1997	Chemical disinfects and antiseptics. Basic bactericidal activity. Test method and requirements (Phase 1)
EN 1650:1997	Chemical disinfects and antiseptics. Quantitative suspension test for the evaluation of fungicidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas. Test method and requirements (Phase 2 step 1)
EN 13610:2002	Chemical disinfectants. Quantitative suspension test for the evaluation of viricidal activity against bacteriophage of chemical disinfectants used in food and industrial areas. Test method and requirements (Phase 2 step 1)

Table 4.2 Examples of standard carrier test methods.

Standard carrier test methods, European and international methods

Group + Standard Number	Title
AOAC Official methods 991.47, 991.48, 991.49	Hard surface carrier test
AOAC Official method	Tuberculocidal activity of disinfectants
AOAC Official method 966.04	Sporicidal test method
ASTM E1053	Standard test method for efficacy of viricidal agents intended for inanimate environmental surfaces
ASTM E2111	Standard quantitative carrier test method to evaluate the bactericidal, fungicidal, mycobactericidal and sporicidal potencies of liquid chemical germicides
EN 13607	Chemical disinfectants and antiseptics. Quantitative non-porous surface test for the evaluation of bactericidal and/ or fungicidal activity of chemical disinfectants used in food, industrial, domestic and institutional areas. Test method and requirements

4.1.1 Suspension testing

Suspension testing would be the preliminary and secondary methods used to establish the efficacy of a disinfectant. The simplest form of efficacy testing using a suspension based method is in the determination of MICs establishing the lowest concentration preventing the growth of an organism (Andrews, 2001; Kampf *et al.,* 2003; Vieira *et al.,* 2005). A series of different dilutions of a biocide from high to low is employed, and into each tube the same concentration of test microorganism is added. This would then be incubated overnight and the MIC is determined by examining for the last tube with no growth (Baron, 1996). This method can be limiting as biocides (notably oxidising agents and aldehydes) can react with the organic and inorganic compounds of growth media and therefore this is in frequently used to establish biocide efficacy (McDonnell, 2007) and is not recommended (Plat and Bucknall, 1988).

Other variations of the suspension test can give either quantitative or qualitative results. The quantitative suspension test involves counting the bacterial survivors either by direct culture or membrane filtration (Lambert, 2004). It involves mixing a bacterial inoculum with a given biocide at a specific concentration in suspension for a defined period of time. The treated inoculum is then removed and the biocide activity neutralised. The mixture is then plated out and incubated to recover survivors, and the bacteria are then counted to establish the bacterial

kill at that concentration and contact time (Lambert, 2004; Messenger *et al.,* 2004; McDonnell, 2007). Qualitative results only assesses whether or not there is growth; with the end point determined visually by the presence of growth (Lambert, 2004). This is a useful method to establish quickly the concentration range for activity of the biocide, although it will never be an accurate measurement of a surviving population (Lambert, 2004). Suspension testing can vary to take into consideration the presence of organic matter and water hardness, thus making the test more rigorous (Lambert, 2004).

Capacity tests are another form of suspension test involved in the second testing phase of a disinfectant (Lambert, 2004). An example of a capacity test would involve a mop head being placed into a bucket which contains a disinfectant solution. As the mop would contain organic matter that is then added to the disinfectant solution, the ability of the disinfectant to remain active would be tested (Reybrouck, 1998). By culturing the solution each time the mop is place in the bucket, this can ultimately determine the capacity of the disinfectant by the point at which the activity of the biocide is exhausted (Lambert, 2004; Reybrouck, 1998). The best known capacity test is the Kelsey-Sykes test (Kelsey and Sykes, 1969; Mattila, 1987).

In the study used in the thesis, a quantitative suspension test was employed as a primary test to establish if any isolated bacteria from

endoscope washers survived treatment with oxidising agents within a 5 minute exposure time, as this is the time emplyed in the endoscope washer disinfector. The test used was based on the European standard, EN 1276: 1997.

4.1.2 Surface Testing

Surface tests are used to investigate the antimicrobial activity of a biocide on surfaces, and can include carriers such as metal coupons, fabrics, and skin (Sattar *et al.*, 2000; Sattar *et al.*, 2003; Hernandez *et al.*, 2005; Maillard *et al.*, 1998; Malik *et al.*, 2006; Williams *et al.*, 2007). The surface test is used to establish whether a given concentration of biocide can reduce a microbial bioburden to a particular level within a specified time period. The microbial sample is not in suspension but dried onto a surface which can represent surfaces found *in situ*. The carrier is then subjected to contact with a disinfectant for a period of time. The disinfectant is then neutralised and the microorganisms are recovered from the surface and counted. A $\log_{10}$ kill of the microorganism is then established to assess the efficacy of the disinfectant. Stainless steel is frequently used as the carrier, and is used in the European Standard (EN 13697:2001). Testing can be performed with commercially available products used in the manufacturing of medical devices such as endoscopes (Marion *et al.*, 2006) or by using endoscope models to test new disinfectants (Vizcaino-Alcaide *et al.*, 2003). Surface tests can assess how effective

a disinfectant would be in practice, for example in the disinfection of an endoscope (Foliente *et al.,* 2001; Vizcaino-Alcaide et al. 2003). Another adaptation of the surface test involves the investigation of antiseptics by using sections of skin as a carrier (surface) (Graham *et al.,* 1996; Maillard *et al.,* 1998).

In this investigation, the surface test was based on the EN 13697:2001. It was used to investigate the susceptibility of bacterial isolates that survive a biocide contact time of 5 minutes or more in the suspension test. This surface test particularly mimics the situations within the washer disinfectors as there is a potential that biofilm could be attached to the surface of the machine, and potentially the endoscope.

4.1.3 Biofilm testing

It is important to investigate the efficacy of disinfectants on biofilms, as in the natural environment this is how bacteria would be attached to surfaces (Donlan and Costerton, 2002). Bacterial biofilm disinfection is important as it is known that biofilms are far more resistant to disinfectants than planktonic bacteria (Donlan and Costerton, 2002), so laboratory tests which will specifically look at the disinfection of biofilms are useful. This is especially important as biofilms can be found in the hospital situations, for example in endoscopes (Pajkos *et al.*, 2004). The test has to take into consideration all the biological, chemical, and analytical components of conventional suspension or dried surface

tests, but also involves a biofilm reactor for growing a reproducible biofilm which should represent the naturally-occurring biofilm (Buckingham-Meyer et al., 2007) so that the test can accurately show how the disinfectant would work in the setting in which it would be used. A variety of methods for testing disinfectants against biofilms are available, including the use of microtitration trays, on which biofilms are developed (Pitts et al., 2003; Augustin and Ali-Vehmas, 2004), and the use of various methods with biofilm reactors, CDC biofilm reactors and drip flow reactors, or with static biofilms (Buckingham-Meyer et al., 2007). Disinfection of biofilms has also been studied by looking at dried biofilms in haemolysis glass tubes (Henoun et al., 2004) and on glass slides (Tachikawa et al., 2005). As there are numerous methods for testing biofilm, it is important that the one which represents the environmental conditions where the disinfectant is used is employed to establish disinfectant efficacy otherwise the results will not translate to the practical setting. The study by Buckingham-Meyer *et al.* (2007) looked at various ways of making biofilms and concluded that biofilms grown under liquid flow conditions was most like the biofilms found in the environment and showed that the CDC biofilm reactor produced the smallest $\log_{10}$ reduction, giving the most rigorous efficacy test (Buckingham-Meyer *et al.*, 2007). Currently there are no written standards for the testing of biofilm disinfection to establish how effective disinfectants would be in the working environment.

4.1.4 Sporicidal testing

Bacterial endospores are most resilient to disinfection and sterilization processes, withstanding high temperatures, ionizing radiation and extreme atmospheric pressures (Nicholson 2002). Bacterial endospores can survive in extream environments for extended periods of time. Spores have been isolated from salt crystals 250 million years old (Vreeland *et al.,* 2000). Biocides that can kill vegative cells may not necessarily be sporicidal (Russell, 1998). There are only a few biocides which can be classed as sporicidal, which include aldehydes, halogen releasing agents, peroxides and ethylene oxide (Russell, 1998). Some biocides used for endoscope disinfection are sporicidal, for example hydrogen peroxide, glutaraldehyde, peracetic acid and chlorine dioxide (Rey *et al.*, 2003). It is important to establish the sporicidal activity of disinfectants. Sporicidal tests are almost the same as bactericidal tests as previously described, however it has to be taken into account that spores must go through germination and outgrowth before producing countable colonies, so longer incubation periods are required (Russell, 1998). An example of a sporicidal test is the EN 14347: 2005 (Block, 2004; European Committee for Standardization 2005; García-de-Lomas *et al.*, 2008).

Here, a sporicidal test was performed to establish the effect the biocides would have on the spores of the *B. subtilis* isolate and ATCC reference strain. There was the possibility that the bacteria surviving

within the washer disinfector has occurred because of the presence of spores. The European standard EN 14347: 2005 was used for spore production whilst the sporicidal testing method was based on the carrier test (EN 13697:2001) method in order to compare the results from the carrier test and the spore test together.

4.1.5 Aims and objectives

This chapter aimed to establish the efficacy of different oxidising biocides against the bacterial species isolated from the washer disinfectors (Chapter 3; Section 3.3).

The biocides which were tested in this investigation include a chlorine dioxide formulation (Tristel), hydrogen peroxide (VHP, Steris®), peracetic acid (Hamo, Steris), Steris 20 and Reliance HLD (Steris). Three standard efficacy tests were performed for each of the disinfectants: suspension, carrier and sporicidal tests.

4.2 Materials and Methods

4.2.1 Bacterial culture and growth

All bacterial cultures used in this chapter were grown and cultured following methods described in chapter 2 section 2.2.4.

4.2.2 Investigation of spore formation

Prior to the use of washed bacterial cells (of either the washer disinfector isolate *B. subtilis* or the ATCC reference strain *B. subtilis*) in the disinfection efficacy test, the samples were checked for the presence of spores, as this would interfere with the results obtained (due to spores being more resistant to disinfectants (McDonnell, 2007)). Methods included using the spore stain on each sample (Chapter 3 section 3.2.2.1.2). Slides were observed under the microscope; 20 fields containing a average of 10 cells were observed on each occasion. Bacterial samples were also boiled for 10 min, (this was to shown that any surviving bacteria would not be vegetative cell but spores) bacterial numbers (cfu/ml) were checked before and after boiling. On average 1 $\times 10^8$ cfu/ml of bacterial cells were added prior to boiling.

4.2.3 Suspension testing

4.2.3.1 Neutraliser toxicity test

A neutraliser toxicity test was performed to demonstrate that the chosen neutraliser was not toxic to the investigated bacterial isolates and standard strains. Sodium thiosulphate (5 g/L) was used to quench the activity of hydrogen peroxide, chlorine dioxide and peracetic acid (Stanley, 1999). The protocol for the neutraliser toxicity test was adapted from the British Standard BS EN 1279:1997 (British Standards, 1997). Briefly, washed cells (1 ml; approx 1×10^8 cfu/ml) were added to 9 ml of sodium thiosulphate (5 g/L), and incubated at room temperature (25 °C) for 30 min. The control bottles contained 9 ml of deionized sterile water and 1 ml washed bacterial suspension. A 100 µl sample was then removed and serially dilution (1/10) in TSC. Each diluted suspension was thoroughly vortexed and 3 × 10 µl samples of each dilution were plated out on either TSA or 7H11 depending upon the sample. This drop-counting method was adapted from the Miles-Misra dilution protocol (Miles *et al.*, 1938). All plates where left to dry on the bench and then incubated overnight (24hrs) in the inverted position at 30 °C. After the incubation period colonies were counted and recorded observations were made if there was any difference between control and test bottles.

4.2.3.2 Neutraliser efficacy test

The neutraliser efficacy test was used to ensure that the neutraliser effectively quenches the activity of the biocide. A washed bacterial suspension was re-suspended in TSC. The test bottle contained 8 ml of neutraliser to which 1 ml of biocide (at the highest concentration tested in the efficacy tests) and then 1 ml of washed bacterial culture (approx 1 x 10^8 cfu/ml) was added. The control bottle contained 8 ml of sterile deionized water, 1 ml of biocide at the highest concentration used in the efficacy test, and 1 ml of bacterial suspension. After 15 min at room temperature (25°C) a 100 µl sample was removed and serially diluted in TSC as previously described. Each dilution was plated on TSA plates as described previously and incubated at 30°C. Colony forming units were counted the following day. Neutraliser was deemed effective if there was not a significant $\log_{10}$ reduction between the test bottle and original inoculum.

4.2.3.3 Suspension testing

The suspension test was adapted from BS EN 1276 (1997). Test bottles for the suspension test contained 9 ml of biocide plus 0.3 g/L of bovine serum albumin (BSA; Acros Organics, NJ, USA) to represent clean conditions or 3 g/L for dirty conditions. Control bottles contained no biocide (deionised water only). 1 ml of washed bacterial suspension (approx 1 × 10^8 cfu/ml) was added to the test and control bottles. After

the required contact time 1 ml was removed from the test bottle and added to a neutraliser bottle which contained 9 ml of neutraliser. Then the 100 µl sample was removed, serially diluted in TSC and 3 × 10 µl drops from the different dilutions were plated onto TSC as described above. Colonies from control and biocide treatment plates were then counted after 24h incubation at 30°C.

4.2.4 Carrier Testing

4.2.4.1 Neutralizer validation

Stainless steel discs (grade 2B finish, 2 cm diameter, Goodfellows Cambridge Ltd, Huntingdon, UK) were aseptically added to a 6-well plate (Corning, NY, USA), which was inoculated with 20 µl of a washed bacterial suspension (1×10^8 cfu/ml). The bacterial suspension was dried for 30 min in an incubator at 37°C. A 100 ml glass bottle was prepared containing 10 ml of neutraliser and 5 g of glass beads (3 mm diameter, Sigma). In order to test the neutraliser 100 µl of the biocide solution was added to one bottle (test) and 100 µl of deionised sterile water was added to another bottle (control). The contents of the bottles were mixed and left for 5 min. Inoculated discs were then aseptically transferred into the neutraliser or control bottles, which were shaken at 150 rpm for 1 min. A serial dilution was then performed by taking 400 µl aliquots and diluting them in 3600 µl of TSC, and this was repeated down to the serial dilution needed (normally 10^{-5}), vortexing in between.

1 ml was added to TSA pour plates in triplicate (Chapter 2 section 2.2.7). Plates were incubated at 37°C and cfu counted after 24 hours.

4.2.4.2 Interpretation of neutraliser validation

The toxicity of the neutraliser was expressed as a reduction in viable number compared to a control. The $\log_{10}$ reduction in bacterial number was calculated using the following equations based on the European standard EN 13697 (2001):

$$Log_{10}\ N - Log_{10}\ NC$$

where N is the number of cfu/20 µl in the original inoculum and NC the cfu/disc in the neutralisation control, and

$$Log_{10}\ NC - Log_{10}\ NT$$

where NC is the number of cfu/disc in test surface and NT neutraliser control test solutions. The neutralisation medium was valid if $Log_{10}N - Log_{10}$ NC was not greater than 2, and Log_{10} NC – Log_{10} NT was not greater than 0.3.

4.2.4.3 Carrier testing

Stainless steel discs were aseptically added to 6 well-plates (Corning, NY, USA). Each well was then inoculated with 20 µl of a washed bacterial suspension (1×10^8 cfu/ml). The inoculum either contained 0.06 g/100 µl of BSA for clean conditions or 0.6 g/100 µl for dirty conditions. The discs were then air dried for 30 min in an incubator at 37°C. 100 µl of the biocidal solution was then added to the surface of the disc, on top of the dried inoculum. Water instead of biocide was used for the control experiments. Once a pre-determined contact time (0.5, 1, 5, 10, 30 min) had been reached, discs were then placed in 100 ml bottles containing 10 ml of neutraliser plus beads. The bottles were then shaken on an orbital shaker for 1 min at 150 rpm. A serial dilution was then performed and 1 ml of each dilution was added to 3 separate Petri dishes and approximately 20 ml of molten TSA (40°C) was poured. The plates were then incubated at 37°C and colonies counted after 24 hours. Each experiment was performed in triplicate.

4.2.4.4 Spore tests

The protocol to produce spores was based on EN 14347: 2005 (European Committee for Standardization 2005). Overnight cultures of *B. subtilis* WD isolate and *B. subtilis* ATCC 6051 reference strain were prepared in TSB, in 100 ml flasks and incubated in a shaking incubator for 24 hrs. 10 ml of the overnight culture was then added to 1000 ml

Roux-bottles containing sporulation agar (Chapter 2 section 2.1.4). The culture was distributed over the surface of the agar using 5 g of sterile 3 mm glass beads (Fisher scientific, UK). The flasks were then incubated at 36°C for 2 days and then for 21 days in the 30°C. In order to harvest the spores, 10 ml of sterile deionised water was pipetted into the Roux-bottles using 10 ml serological pipettes. Spores were then re-suspended and washed off the agar surface with the glass beads. The suspension was then transferred aseptically into sterile 100 ml glass flasks. This process was repeated twice to retrieve more spores. The spore suspension was then filtered through two layers of sterile gauze (Fisher scientific, UK) over a sterile funnel. The filtrate was dispensed into 50 ml screw cap centrifuge tubes (Fisher scientific, UK), and centrifuged at 3000 g for 30 min at 10°C. The supernatant was then removed and the pellet was re-suspended in 65% propan-2-ol for 3 hours, to kill the remaining vegetative cells (EN 14347: 2005 European Committee for Standardization 2005). After 3 hours, the propan-2-ol was diluted with the same volume of water and the suspension was then centrifuged at 3000 g for 30 min at 10°C. A smear slide was then prepared in order to check that there were no vegetative cells left; this was done using the spore stain method (Chapter 3 section 3.2.2.1.2). The spore suspension was adjusted to the appropriate concentration (approx. 1×10^8 cfu/ml) and was left in the refrigerator (4°C) for 4 weeks before being used in the carrier test. Carrier testing was conducted as previously described (section 4.2.4.3) substituting 20 µl

washed bacterial cells for 20 µl spore suspension. A longer incubation period of 4 days was used following the protocol of standard EN 14347: 2005 (European Committee for Standardization 2005). Spore testing was conducted clean conditions only, due to time limitations.

4.2.4.5 Statistical analysis

One way analysis of variance was used at the 95% level of significance to test differences between the means of data sets. Minitab® (release 14 soft ware, Minitab Inc., PA, USA) was used to perform the calculations and to test normality of distribution of the residuals and homogeneity of variances of the data. This has a null hypothesis that there is no significant difference between the means of two or more groups of quantitative data. If any assumptions of ANOVA were violated the Kurskal-Wallis test was performed on the medians of the data sets. Where appropriate the standard deviations (SD) of means are indicated in this study.

4.3 Results

4.3.1 Investigation of spore formation

B. subtilis strains used in suspension and carrier tests were tested to determine if any bacterial spores were present, as this would alter the efficacy of the disinfectants. The absence of spores, following boiling of the test inoculum was demonstrated with the use of spore stain. The boiled samples retrieved no colony forming units, and the spore stain did not show any bacterial spores. Both experiments showed that there were no spores present in the bacterial samples prior to testing the disinfectant efficacy; therefore resistance to the disinfectants did not occur through the presence of bacterial spores.

4.3.2 Suspension test

4.3.2.1 Neutralizer efficacy

Neutraliser efficacy was established to show that the oxidising biocides were effectively quenched by sodium thiosulphate. All biocides used in the suspension test were neutralised by 5 g/L sodium thiosulphate (Table 4.3). The data shows that the neutraliser was able to quench the activity of the biocide. There was no significant difference ($p > 0.05$) between the initial inoculum and the test bottle, and the control bottle shows that without neutraliser there is still a log reduction in bacterial number.

Table 4.3 Neutraliser efficacy using suspension test method. *B. subtilis* reference strain 6051.

Biocide	Initial inoculum log_{10}	Test log_{10}	Control log_{10}
Chlorine dioxide 0.3%	5.87 ± 0.10	5.74 ± 0.50	<3.00 ± 0.00
Hydrogen peroxide 7.5 %	7.12 ± 0.50	7.09 ± 0.50	<3.00 ± 0.00
Peracetic acid 2.25 %	8.34 ± 0.04	8.27 ± 0.07	<3.00 ± 0.00

4.3.2.2 Neutraliser toxicity

Sodium thiosulphate (5 g/L) was not toxic against the test bacteria (Table 4.4). Using the premise that the carrier test neutraliser is not toxic if the log_{10} reduction from the control bottle (without neutraliser) and test bottle (with neutraliser) is not greater than 0.3 (British Standards 1997), then the neutraliser was not deemed to be toxic to the bacterial cells.

Table 4.4 Neutraliser toxicity suspension test.

Sample	Control log_{10}	Test log_{10}	log_{10} reduction
M. luteus isolate	5.54 ± 0.17	5.40 ± 0.44	0.14
M. luteus ATCC 4698	8.42 ± 0.04	8.34 ± 0.05	0.08
B. subtilis isolate	6.78 ± 0.54	6.69 ± 0.66	0.09
B. subtilis ATCC 6051	7.94 ± 0.05	7.91 ± 0.02	0.03
S. sanguis	7.20 ± 0.01	7.10 ± 0.10	0.10
S. mutans	7.40 ± 0.00	7.36 ± 0.05	0.04
S. intermedius	7.40 ± 0.17	7.33 ± 0.05	0.07

4.3.2.3 Biocide efficacy

The results from the suspension test for all the WD isolates (*B. subtilis*, *M. luteus*, *S. intermedius*, *S. mutans* and *S. sanguis*) and the reference strains (*B. subtilis* ATCC 6051 and *M. luteus* ATCC 4698) are shown in tables 4.5 to 4.7 and presented for each bacterial genus in the sub-sections below.

4.3.2.3.1 Bacillus subtilis

The results of the suspension efficacy tests for the *B. subtilis* WD isolates and reference strains are presented in table 4.5. It was observed that the reference strain was significantly ($p < 0.05$) more susceptible to the biocides tested than the WD isolates. With the exception of chlorine dioxide in the presence of organic load (3 g/L BSA), the reference strain was completely inactivated (> 5 $\log_{10}$) within 1 min contact time. After 60 min exposure, chlorine dioxide failed to inactivate the WD isolates in the presence of organic load (3 g/L BSA) as a 5 $\log_{10}$ reduction was not achieved. With the exception of hydrogen peroxide, the presence of organic load severely restricted the activity of the oxidising agents. Peracetic acid was the most effective oxidising agent tested (Martin *et al.*, 2008).

Table 4.5 Suspension test results for *B. subtilis* (vegetative Cells).

Biocides	Time (min)	Mean log_{10} reduction in bacterial number (± S.D.)			
		ATCC 6051 *B. subtilis*		WD isolate *B. subtilis*	
		Organic load		Organic load	
		0.3 g/L BSA	3 g/L BSA	0.3 g/L BSA	3 g/L BSA
ClO_2 (0.03%)	0.5	4.16 (0.50)	—	—	—
	1	> 5.28 (0.10)	—	1.50 (0.90)	—
	5	—	2 .74 (0.10)	> 5.00 (0.00)	—
	30	—	> 5.12 (0.10)	—	—
	60	—	—	—	0.22 (0.20)
H_2O_2 (7.5%)	0.5	> 5.04 (0.10)	3.39 (0.10)	—	—
	1	—	> 5.49 (0.04)	—	—
	30	—	—	0.30 (0.30)	1.15 (0.06)
	60	—	—	> 5.02 (0.05)	5.07 (0.09)
PAA (2.25%)	0.5	> 5.18 (0.40)	> 5.15 (0.30)	—	—
	1	—	—	2.62 (0.04)	—
	5	—	—	> 5.00 (0.00)	2.78 (0.9)
	30	—	—	—	> 5.08 (0.10)

— : not tested; BSA, bovine serum albumin.

4.3.2.3.2 Micrococcus luteus

The results of the suspension efficacy tests for the *M. luteus* WD isolate and its reference strain counterpart are shown in Table 4.6. The reference strain was readily killed within 30 sec exposure by all biocides in the presence of soiling or not. These results contrast with the WD isolate which was inactivated by chlorine dioxide in the presence of organic load (3 g/L BSA) only following 30 min exposure, although the other two oxidising agents produced a > 5 log_{10} reduction within 30 s.

Table 4.6 Suspension test results from *M. luteus*.

Biocides	Time (min)	Mean log_{10} reduction in bacterial number (± S.D.)			
		ATCC 4698 *M. luteus*		WD isolate *M. luteus*	
		Organic load		Organic load	
		0.3 g/L BSA	3 g/L BSA	0.3 g/L BSA	3 g/L BSA
ClO_2 (0.03%)	0.5	> 5.53 (0.03)	> 5.26 (0.09)	> 5.02 (0.04)	—
	5	—	—	—	1.40 (0.60)
	30	—	—	—	> 5.00 (0.40)
H_2O_2 (7.5%)	0.5	> 5.40 (0.07)	> 5.44 (0.07)	> 5.02 (0.00)	> 5.16 (0.08)
PAA (2.25%)	0.5	> 5.40 (0.03)	> 5.39 (0.09)	> 5.14 (0.10)	> 5.02 (0.04)

— : not tested

4.3.2.3.3 Other isolated bacteria

Three other bacterial strains which were isolated from the washer disinfector (first sampling) were also tested against the oxidising biocides using the suspension test method (Table 4.7). Once again chlorine dioxide performed the worst, taking 30 min in dirty conditions to kill (> 5 log_{10}) the other three isolates (Table 4.7). There was no real difference in biocide susceptibility between these bacterial isolates (Martin *et al.*, 2008).

4.3.3 Carrier test

4.3.3.1 Neutraliser efficacy

The biocide neutraliser efficacy tests were conducted to establish if the neutraliser could quench the biocide effects when applied on a surface, Results of the neutralising efficacy tests on surfaces are shown in Table 4.8. Sodium thiosulphate was effective at quenching the biocide activity, as none of the $\log_{10}$ results were above 2. According to the European Standard EN 13697 (2001), the results also showed that the neutraliser did not affect the viability of the bacterial cells as none of the $\log_{10}$ results were greater than 0.3.

Table 4.7 Suspension test results for the other WD isolates.

Biocides	Mean $\log_{10}$ reduction in bacterial number (± S.D.)								
	Staphylococcus intermedius			*Streptococcus mutans*			*Streptococcus sanguis*		
	Time	Organic load		Time	Organic load		Time	Organic load	
	(min)	0.3 g/L BSA	3 g/L BSA	(min)	0.3 g/L BSA	3 g/L BSA	(min)	0.3 g/L BSA	3 g/L BSA
ClO_2 (0.03%)	0.5	> 5.80 (0.04)	—	0.5	> 5.00 (0.00)	—	0.5	> 5.02 (0.04)	—
	5	—	0.60 (0.40)	1	—	0.40 (0.50)	5	—	1.32 (0.30)
	30	—	> 5.40 (0.01)	5	—	> 5.28 (0.10)	30	—	>5.00 (0.00)
H_2O_2 (7.5%)	0.5	> 5.42 (0.01)	> 5.36 (0.10)	0.5	> 5.02 (0.04)	> 5.02 (0.05)	0.5	> 5.00 (0.00)	> 5.35 (0.05)
PAA (2.25%)	0.5	> 5.88 (0.20)	> 5.36 (0.20)	0.5	> 5.08 (0.10)	> 5.08 (0.10)	0.5	> 5.00 (0.00)	> 5.06 (0.10)

— : not tested

Table 4.8 Efficacy and toxicity of sodium thiosulphate used in carrier test.

Biocide	Log_{10} N – log NC *M. luteus* ATCC (±) SD		Log_{10} NC – log NT *M. luteus* ATCC (±) SD		Log_{10} N – log NC *B. subtilis* ATCC (±) SD		Log_{10} NC – log NT *B. subtilis* ATCC (±) SD	
	0.3 g/L BSA	3 g/L BSA	0.3 g/L BSA	3 g/L BSA	0.3 g/L BSA	3 g/L BSA	0.3 g/L BSA	3 g/L BSA
R HLD 0.12%	0.45 (0.10)	0.45 (0.05)	0.003 (0.03)	0.14 (0.10)	—	—	—	—
Steris 20 0.2%	0.27 (0.02)	0.09 (0.10)	0.02 (0.01)	0.09 (0.10)	—	—	—	—
ClO_2 0.3 %	0.09 (0.03)	—	0.14 (0.02)	—	0.40 (0.01)	0.97 (0.06)	0.01 (0.03)	0.04 (0.07)
H_2O_2 7.5 %	0.05 (0.03)	—	0.05 (0.04)	—	0.87 (0.10)	0.93 (0.20)	0.01 (0.20)	0.02 (0.30)
PAA 2.5%	0.10 (0.10)	—	0.03 (0.09)	—	—	—	—	—

—: not tested

4.3.3.2 Carrier test results

Carrier test results are shown in Tables 4.9 and 4.10, for all biocides, they also show the results with WD bacterial isolates and ATCC reference strains for *B. subtilis* and *M. luteus*.

4.3.3.2.1 Bacillus subtilis

The results from the carrier test for *B. subtilis* are show in Table 4.9. The results clearly showed how ineffective chlorine dioxide was at killing both the *B. subtilis* reference strain and WD isolate. A 60 min exposure in clean conditions produced a > 4 $\log_{10}$ reduction with the reference strain. Chlorine dioxide failed to produce 1 $\log_{10}$ reduction with the WD isolates even after 60 min. The formulations Steris 20 and Reliance HLD were the most effective and produced a complete kill (> 4 $\log_{10}$) within 30 sec. There was no significant difference ($p > 0.05$) in the activity of both formulations against the two strains in clean or dirty conditions. Both of these biocides were used at the increased temperature of 53°C, which might have increased their effectiveness. Peracetic acid was the most effective biocide. The unformulated peracetic acid showed a good activity against the reference strain which was inactivated within 30 sec. The WD isolate was more resilient notably in the presence of 3 g/L BSA and it took 30 and 60 min to achieve a > 4 $\log_{10}$ reduction in clean and dirty conditions, respectively. Hydrogen peroxide inactivated the reference strain within 30 min

exposure regardless of the presence of soiling (p = 0.192). A 60 min exposure was not sufficient to produce a > 4 $\log_{10}$ reduction in number with the WD isolates. Again with the WD isolate the presence of organic load did not affect the activity of the oxidising agent (p = 0.236). In this section the absence of spores was determined using a number of methods (Chapter 4, section 4.2.2).

Table 4.9 Carrier test results for *B. subtilis*.

Biocides	Time (min)	Mean log_{10} reduction in bacterial number (± S.D.)			
		B. subtilis ATCC 6051		WD Isolate B. subtilis	
		Organic load		Organic load	
		0.3 g/L BSA	3 g/L BSA	0.3 g/L BSA	3 g/L BSA
ClO_2 (0.03%)	0.5	0.24 (0.26)	0.17 (0.24)	0.31 (0.14)	0.24 (0.09)
	1	0.71 (0.16)	0.41(0.03)	0.15 (0.09)	0.07(0.03)
	5	0.81 (0.07)	0.58 (0.22)	0.31 (0.11)	0.46 (0.16)
	30	3.19 (0.08)	1.95 (0.02)	0.25 (0.15)	0.23 (0.06)
	60	> 4.21 (0.02)	3.86 (0.20)	0.97 (0.14)	0.28 (0.05)
H_2O_2 (7.5%)	0.5	1.82 (0.30)	1.59 (0.20)	—	—
	1	1.89 (0.10)	0.54 (0.40)	—	—
	5	2.50 (0.10)	2.76 (0.10)	—	—
	30	> 4.92 (0.02)	> 4.32 (0.02)	—	—
	60	—	—	1.13 (0.60)	0.62 (0.10)
PAA (2.25%)	0.5	> 4.21 (0.02)	3.86(0.20)	—	—
	1	—	—	0.79 (0.09)	0.19 (0.50)
	5	—	—	> 4.50 (0.05)	0.69 (0.60)
	30	—	—	—	> 4.59 (0.05)
Steris 20 (0.2%)	0.5	> 4.70 (0.10)	> 4.62 (0.10)	> 4.53 (0.05)	> 4.53 (0.10)
R HLD (0.12%)	0.5	> 4.13 (0.01)	> 4.13 (0.01)	> 4.47 (0.30)	> 4.50 (0.30)

—: not tested

4.3.3.2.2 Micrococcus luteus

The carrier test results for *M. luteus* reference strain 4698 and WD isolate are shown in Table 4.10. The formulations Steris 20 and Reliance HLD once again were the most efficacious together with the unformulated peracetic acid solution. Both biocides produced a > 4 log_{10} reduction with both the reference strain and WD isolate within 30 sec. There was no significant difference ($p > 0.05$) in the activity of both formulations against the two in clean or dirty conditions. The increased temperature at which the formulations were tested might have contributed to an increase in activity. Hydrogen peroxide readily killed the reference strain 4698 within 30 sec exposure in both clean and dirty conditions, but a 1 min and 5 min exposure were necessary to produce a > 4 Log_{10} reduction with the WD isolate in clean and dirty conditions respectively.

Longer contact times were needed for chlorine dioxide to show some activity in the presence of organic load (3 g/L BSA). Although the reference strain 4698 and WD isolate were rapidly inactivated when no soiling was present, the WD isolate was significantly ($p < 0.05$) more resistant to chlorine dioxide than the reference strain when soiling was present.

The efficacy of chlorine dioxide in the presence of soiling further highlighted the problem some disinfectants might have when used

within endoscopes, where organic matter remains within the channels if these devices are not firstly rigorously cleaned.

Table 4.10 Carrier test results for *M. luteus*.

Biocides	Time (min)	Mean log_{10} reduction in bacterial number (± S.D.)			
		ATCC 4698 *M. luteus*		WD Isolate *M. luteus*	
		Organic load		Organic load	
		0.3 g/L BSA	3 g/L BSA	0.3 g/L BSA	3 g/L BSA
ClO_2 (0.03%)	0.5	> 4.70 (0.00)	2.89 (1.80)	> 4.50 (0.30)	2.92 (0.90)
	1	—	> 4.52 (0.02)	—	—
	5	—	—	—	2.92 (0.60)
	30	—	—	—	>4.7 (0.00)
H_2O_2 (7.5%)	0.5	> 4.79 (0.08)	> 5.05 (0.05)	—	—
	1	—	—	> 4.76 (0.06)	2.76 (0.70)
	5	—	—		> 4.70 (0.00)
PAA (2.25%)	0.5	> 4.78 (0.05)	> 4.77 (0.06)	> 4.86 (0.20)	> 4.71 (0.00)
Steris 20 (0.2%)	0.5	> 5.11 (0.05)	> 5.33 (0.02)	> 5.00 (0.03)	> 5.34 (0.09)
R HLD (0.12%)	0.5	> 5.35 (0.01)	> 5.43 (0.01)	> 5.18 (0.01)	> 5.20 (0.01)

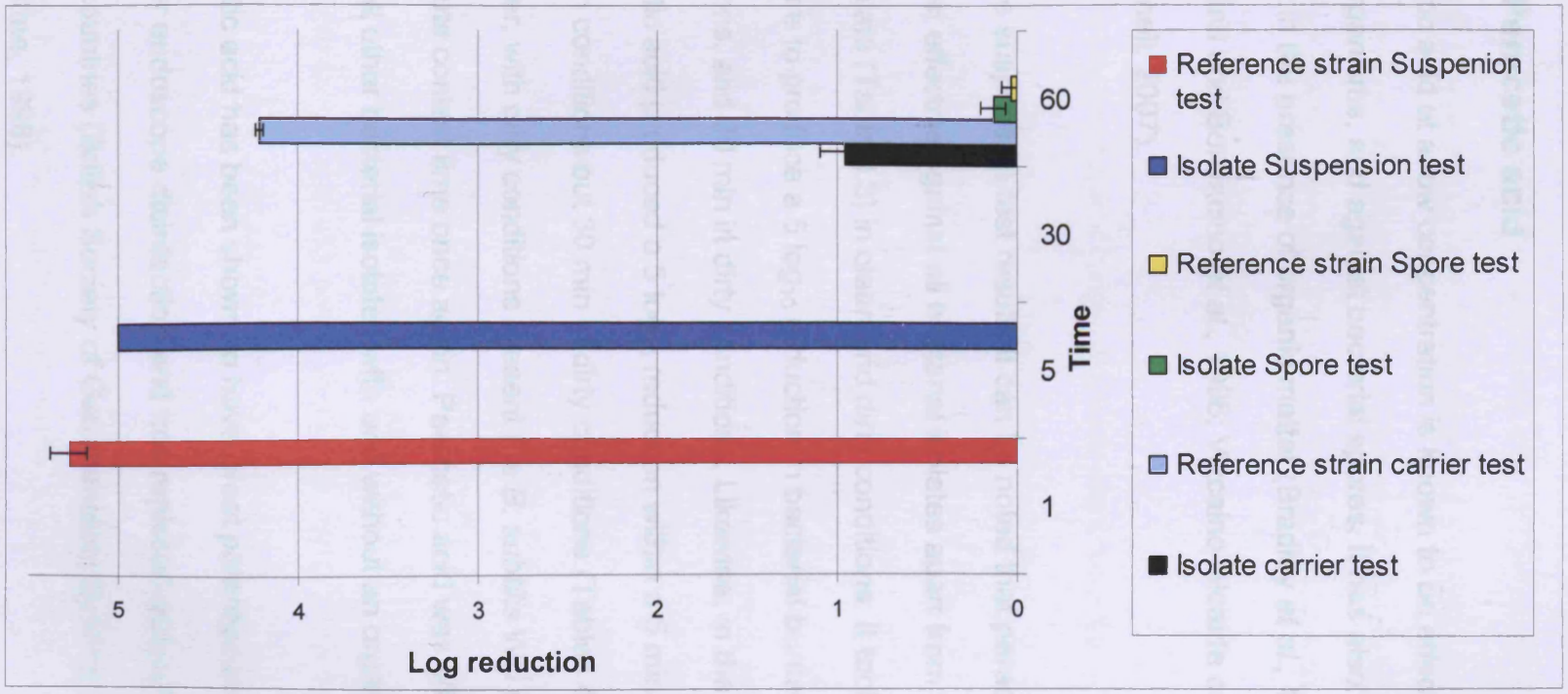
—: not tested

4.3.3.3 Sporicidal test results

The sporicidal test was performed to investigate the susceptibility of the spores to the oxidising agents. Indeed, although the carrier test described above was performed against vegetative bacteria, the presence and survival of spores within the endoscope washer could further influence bacterial contamination following high-level disinfection. The results from the sporicidal test with chlorine dioxide are shown in Figure 4.1 together with the suspension and carrier test results presented above.

Chlorine dioxide failed to inactive the spore suspension within 60 min. A 0.03 and 0.13 $\log_{10}$ reduction in viable spores was observed with the reference and WD isolate respectively. There was no significant difference ($p = 0.297$) in the susceptibility of spores from the different strains to chlorine dioxide. This contrasts with the results obtained with the other two efficacy tests. There was a significant difference ($p < 0.05$) between the inactivation results from the carrier and suspension test but not with the spore ($p < 0.05$) and carrier efficacy test. These results are not surprising since spores are generally considered to be more resilient to disinfection than vegetative bacteria (McDonnell, 2007). The absence of spores during the suspension and carrier tests was thoroughly checked with a number of protocols (Chapter 4, section 4.3.1).

Figure 4.1 Efficacy of chlorine dioxide against spores and vegetative bacteria of *B. subtilis* WD isolate and ATCC reference strain. Graph shows the contact times and bacterial kill $\log_{10}$ kill at these times.



4.4 Discussion

4.4.1 Peracetic acid

Peracetic acid at a low concentration is known to be effective against all microorganisms, and against bacterial spores. It has also been shown to work in the presence of organic matter (Bradley *et al.,* 1995; Sagripanti and Bonifacino *et al.,* 1996; Vizcaino-Alcaide *et al,* 2003; McDonnell, 2007).

With the suspension test results it can be noted that peracetic acid is the most effective against all bacterial isolates apart from the *B. subtilis* WD isolate (Table 4.3) in clean and dirty conditions. It took a 5 min exposure to produce a 5 $\log_{10}$ reduction in bacterial burden in clean conditions, and 30 min in dirty conditions. Likewise, in the carrier test peracetic acid produced a 5 $\log_{10}$ reduction within a 5 minute timeframe in clean conditions but 30 min in dirty conditions (Tables 4.5 and 4.6). However, with dirty conditions present the *B. subtilis* WD isolate took a 30 minute contact time once again. Peracetic acid was effective in killing all other bacterial isolates with and without an organic load.

Peracetic acid has been shown to have great potential as a biocide used for endoscope disinfection and has replaced glutaraldehyde in some countries (British Society of Gastroenterology Endoscopy Committee, 1998).

However, peracetic acid has also faced problems during usage, notably in the fixation of blood. Kampf *et al.* (2004) observed that between 19% and 78% of dried blood became fixed after a 15 minute contact with peracetic acid and could not be removed by a cleaning process that showed removal efficacy of >99%. This has also been shown with the disinfectant glutaraldehyde. This again highlights the importance of thorough cleaning prior to disinfection.

Two other peracetic acid based products were also tested. These were Steris 20 and Reliance HLD (high level disinfectant). Steris 20 contains a concentrated peracetic acid and separate dry mixture which contains surfactants, buffers and anticorrosive (McDonnell, 2007). The two components are then mixed with sterile water given a final concentration of 0.2% PAA. It is used at a temperature of between 50 and 56 °C. Steris 20 is sporicidal and temperature can dramatically increase it efficacy, for example the D-value of *G. stearothermophilus* spores for Steris 20 at 30°C is 1.4 min, compared to < 10 s at 55°C (McDonnell, 2007). This biocide has also been shown to retain its efficacy in the presence of soiling (Justi *et al.,* 2001). This is in agreement with the results presented in this study (Tables 4.6 to 4.7). Following the use of Steris 20, both *B. subtilis* and *M. luteus* (reference strain and WD isolate) were inactivated (> 4 $\log_{10}$ reduction) within a contact time of 0.5 min. The other peracetic acid based biocide Reliance HLD has two dried components. They are both mixed with

sterile water to produce an active concentration of 0.12% PAA. This biocide was shown to kill various microorganisms (*Ps .aeruginosa*, glutaraldehyde resistant *Mycobacterium chelonae,* MRSA and *E. faecalis*) and spores (*C. difficile*) (Sattar, 2006). In this study, Reliance HLD was also shown to be effective (> 4 $\log_{10}$ reduction) against both *B. subtilis* and *M. luteus* (reference strain and WD isolate) within 0.5 min exposure time.

4.4.2 Hydrogen peroxide

The suspension and carrier tests showed that hydrogen peroxide could not kill the *B. subtilis* WD vegetative isolate. Bacterial survivors were still present following a 60 minute contact time. This result was observed for both clean and dirty conditions. The reference strain of *B. subtilis* needed a contact time of 30 min to reduce the bacterial burden by 4 $\log_{10}$ in the carrier test. This contrasts with the suspension test for which only 1 min exposure was sufficient to obtain the same level of kill. The other bacterial isolates *M. luteus* (WD isolate and reference strain), *Staphylococcus intermedius, Streptococcus mutans and Streptococcus sanguis* were all inactivated (> 5 $\log_{10}$ reduction) within 0.5 minute contact time in clean and dirty conditions. These results provide evidence that the bacteria are affected in different ways by the same biocide. When compare to the suspension tests, results from the carrier tests demonstrate that dried bacterial inoculum (as in the carrier test) are more difficult for disinfectants to eliminate. This again highlights the

need for good manual processing before disinfection (Babb and Bradley, 1995; Martiny and floss 2001; Vizcanino Alcaid, *et al.* 2003).

Hydrogen peroxide was the second least effective biocide at reducing the bacterial burden during the efficacy test, particularly *B. subtilis* reference strain and WD isolate in both suspension and carrier test. One reason for this resistance may be a problem with biocide penetration. It is well know that if bacteria form a biofilm on surfaces; this can offer some degree of protection against biocides and antibiotics (Costerton, 1987; Gilbert and McBain, 2001; Mah and O'Toole, 2001). Among the resistance mechanisms associated with biofilm, penetration of biocides might be an area for concern (Hall-Stoodley, 2004), however, it is thought that this is not a singular mechanism (Mah and O'Toole, 2001; Gilbert *et a.l*, 2002). Nevertheless bacterial glycocalyx (exopolysaccharides, EPS) found during biofilm formation and cell attachment to surfaces (Wingender *et al.*, 1999) might impinge on biocide penetration (Costerton *et al.*, 1987), in addition to the presence of extracellular enzymes within bacterial glycocalyx biocide degradation (Gilbert *et al.,* 2002). Bacteria produce a variety of enzymatic processes that can neutralize the effects of oxygen species (McDonnell, 2007). This is done in defence to naturally occurring hydrogen peroxide in order to prevent damage to the cell (McDonnell, 2007). In particular, the presence of catalase and peroxidases neutralize hydrogen peroxide (McDonnell, 2007). The production of catalase has the effect of aiding

bacterial survival when presented with low concentrations of hydrogen peroxide, and when bacteria are catalase-deficient they become more susceptible to hydrogen peroxide (Loewen, 1984). *B. subtilis* has been shown to have various levels of production of catalase, and this enzyme can be found in both vegative and spores of the bacteria (Loewen, 1987). Catalase has also been shown also in *P. aeruginosa* biofilms to give some protection against hydrogen peroxide (Elkins, 1999; Stewart, 2000).

The resistance to hydrogen peroxide in this particular study may be caused by over-production of catalase, or the restricted penetration of the biocide through bacterial EPS. These aspects are investigated in Chapter 5.

4.4.3 Chlorine dioxide

Chlorine dioxide is used in the disinfection of endoscopes and for water decontamination (Rey *et al.,* 2003; Coates, 2001). There are several chlorine dioxide based instrument disinfectants commercially available including the following under the trade names of Dexit, Tristel and Medicle. They are all based on two part activation, which is generally which is generally achieved by mixing prior to disinfection. One of the drawbacks of chlorine dioxide is that it can cause damage to the instrument which is being disinfected (McDonnell, 2007).

Chlorine dioxide is a powerful oxidising agent and can rapidly kill bacterial spores (McDonnell 2007). Previous studies have shown chlorine dioxide to be a highly successful biocide against bacterial contamination. Chlorine dioxide has been shown to be effective against many microorganisms including MRSA, MRSE, (α)-*haemolytic Streptococcus*, *E. coli*, *E. faecalis*, *K. pneumoniae*, *E. cloacae*, *Ps. aeruginosa*, *S. marcescens*, *H. pylori* and *C. albicans* within a 10s contact time (Isomoto *et al.,* 2006). The work by Isomoto *et al.* (2006) also showed that chlorine dioxide had the ability to kill *B. subtilis* within a 5 minute contact time at concentrations as low as 0.003%. Coates (2001) showed that *B. subtilis* could be reduced by > 5 $\log_{10}$ with the presence of organic load within a contact time of 2.5 min using chlorine dioxide as low as 0.009%. Coates (2001) highlighted a problem with the generation of chlorine dioxide: the desired concentration needed was 0.023%, but the concentration generated was actually between 0.021-0.025%, showing that there is the potential for the wrong concentration of chlorine dioxide to be generated by WD machine. If a low concentration was used, then less susceptible bacteria could survive disinfection, and remain within the WD.

In our study the efficacy of chlorine dioxide was conducted using both suspension and carrier tests with and without the presence of organic load. The results from the suspension tests (Tables 4.3. and 4.4) showed that with dirty conditions (3 g/L BSA) chlorine dioxide (0.03%)

needed a contact time of 30 minutes to produce > 5 log_{10} reduction in all but one of the WD isolates. At a 60 minute contact time the average log_{10} reduction was 0.22 ± 0.2 for *B. subtilis* WD isolate, so even a prolonged contact time of 60 min was still ineffective (0.22 ± 0.2 log_{10} reduction) against the *B. subtilis* WD isolate .

When dried on a surface the *B. subtilis* isolate showed that it could withstand a prolonged contact time with chlorine dioxide (0.3%) in the presence of soiling (Table 4.6). The *B. subtilis* reference also needed longer exposure times (i.e. 60 min) when dried on a surface in both clean and dirty conditions. Therefore, not only the environmental isolate has the ability to survive chlorine dioxide exposure when dried on a surface.

M. luteus (Table 4.7) was the other bacterial isolate tested with the carrier efficacy test. The reference strain was inactivated by > 4 Log_{10} within 30 sec (clean) and 1 min (dirty) by a concentration of chlorine dioxide of 0.03%. The WD isolate needed a longer contact time (i.e. 30 min) in the presence of soiling.

Chlorine dioxide has been shown to be efficacious against bacteria (Coates, 2001), and bacterial endospores of *B. subtilis*. However, the presence of organic matter affects its activity (Isomoto, 2006). Our

results clearly agree with the literature and especially with the detrimental effect of soiling upon effectiveness.

In addition the WD isolates were shown to be far less susceptible than the reference strains, notably in the presence of organic load. There are a number of possibilities that could explain emerging resistance or decrease susceptibility to chlorine dioxide. The main one is the development and selection for resistant isolates through repetitive exposure to an inappropriate (low) concentration in the WD. In addition, the resistance displayed by these isolates was stable as it did not disappear following subculturing.

The survival of the bacterial isolates exposed to high concentrations and long contact time might be associated with the presence of BSA, which interferes with the efficacy of the biocide (Isomoto, 2006). The isolation of these resistant isolates to chlorine dioxide is possibly associated with an inadequate exposure to the appropriate concentration of the oxidiser. This may have been due to occlusion (Gamble *et al.*, 2007), inadequate exposure under normal use conditions (Gamble *et al.*, 2007), protection from soil (inadequate cleaning) (Pajkos *et al.,* 2004) or the use of an inappropriate regimen (Honeybourne *et al.*, 1997).

4.4.4 Cross-resistance

Cross-resistance can occur when different antimicrobial agents attack the same target, and co-resistance occurs when the genes specifying resistant phenotypes are located together on a mobile genetic constituent (Chapman, 2003b). They both have the same end result: resistance of one antibacterial agent is accompanied by resistance to another agent (Chapman, 2003b). The areas where cross- and co-resistance can occur include changes in bacterial outer membranes and changes in activity of efflux pump (Russell, 2000).

There are different references to cross-resistance in the literature; examples include resistance mediated by efflux. This has been shown with triclosan resistance in *P. aeruginosa* (Chuanchuen *et al.*, 2001) and pine-oil resistance of *E. coli,* which also showed resistance to multiple antibiotics (Moken *et al.*, 1997). It has been proposed that the acquisition of a *qac* gene by staphylococci results in selection for antibiotic resistant bacteria and it has been related to the widespread use of chlorhexidine - strains containing *qac*A show multiple antibiotic resistance, and resistance to quaternary ammonium compounds and benzalkonium chloride (Paulsen et al 1996; Paulsen 1998). *Mycobacterium chelonae* has shown resistance to ethambutol, and has also been shown to be resistant to glutaraldehyde, which is linked to changes in composition of the cell wall (Manzoor *et al.,* 1999).

It is apparent in the literature that these changes and the development of cross- and co-resistance are linked to the use of biocides at low concentration which may be below the in-use concentration (Russell, 2000).

This may be an important problem, as there are instances where biocides are diluted to below their effective in-use concentration, and the potential for the development of resistance and cross-resistance to other agents is increased, for example, in the home where strict guidelines are not present, or when these guidelines are present and not adhered to. If these situations continuously occur, there is potential for the bacteria to be subjected to low concentrations of agents and the development of resistance to these agents.

The potential for cross-resistance in these isolates was investigated. The results clearly demonstrate cross-resistance between chlorine dioxide, hydrogen peroxide and peracetic acid in the vegetative *B. subtilis* WD isolate. Cross-resistance has the potential to occur when antimicrobials have the same or similar modes of action (Chapman, 2003b). All three of these biocides are oxidising agents. The biocides stimulate electron removal from macromolecules leading to a loss of structure and function. Other examples where cross resistance occurs with hydrogen peroxide include bacteria resistant to formaldehyde and 5-chloro-2-methyl-isothiazol-3-one (CMI) (Chapman, 1998).

4.4.5 Testing protocols

When disinfectants are tested to show efficacy, suspension tests are generally the method used, as there can be tailored to look at various conditions that a disinfectant may encounter when used *in-situ*, and can include organic matter, water hardness and temperature differences (Gibson *et al.,* 1995).

However, this does not test bacteria as they grow in their natural environment as it is known that bacteria naturally aggregate in films or flocs (planktonic biofilms) and sludges tightly adhered with EPS rather than as individual planktonic organisms (Fleming and Wingender, 2001). This is important when looking at the testing of disinfectants, as the areas they are going to be used in a healthcare situation, on surfaces, must be taken into account. Surfaces where there is the potential for bacteria growth to occur, or in the disinfection of medical devices, such as endoscopes, can contain biofilms (Pajkos *et al.,* 2004). It has also been shown by a number of researchers (detailed by Gibson *et al.*, 1995) that when bacteria are attached to surfaces they are more resistant to various biocides than when bacteria are grown in suspension.

Here, it was also shown that when the bacterial cells were attached to the surface of a stainless steel disc it took a longer time to kill the bacteria. For example, in the suspension test for *B. subtilis* ATCC

reference strain, with the disinfectant chlorine dioxide in clean conditions it took 1 min for the population to be reduced to a satisfactory level, whereas in the carrier test it took 60 min to achieve the same log reduction kill. This was also shown with the *B. subtilis* WD isolate a 5 min contact time (4 $\log_{10}$ reduction) in the suspension test compared to 60 min contact time in the carrier test, which only achieved a 1 $\log_{10}$ reduction in bacterial cell count.

This reiterates the fact that when bacteria are grown on surfaces the time needed to achieve the desired log kill is considerably increased. Therefore, a combination of carrier and suspension test should be used to establish biocide efficacy. However, within the healthcare setting this process should be taken a step further and disinfectants should be tested on growing biofilms to establish the efficacy. This would be fundamental for the efficacy of disinfectant used within endoscope disinfection. This has been done in previous studies (Vickery *et al.*, 2004; Augustin and Ali-Vehamas, 2004).

As the disinfection of endoscopes involves various steps it is also important to validate each of these steps, including the removal of debris from the endoscopes (Bloß and Kampf, 2004; Zuhlsdorf *et al.*, 2004), and the disinfection process within washer disinfectors (Coates, 2001; Kircheis and Martiny, 2007). European standards are also being developed for testing of chemical washer disinfectors including

automatic endoscope re-processors, in the EN15883 which is divided into five parts (Fraise, 2008). These tests will cover the efficacy of cleaning and the removal of soil, and look at water purity and levels of contaminants, for example *Legionella*, *P. aeruginosa* and *mycobacteria* (Fraise, 2008). This guidance can also be found in the HTM 2030.

4.4.6 Summary

It appears that vegetative Gram-positive bacteria surviving within the WDs had built up resistance to chlorine dioxide, and for the *Bacillus* WD isolate, stable cross-resistance to other oxidising agents. Since *B. subtilis* was isolated, it may be expected that resistance was a result of spore formation within culture preparation, necessitating a longer contact time for sporicidal activity. However, the results from these investigations (Chapter 4, section 4.3.1) showed that the vegetative forms alone were capable of withstanding high (in-use) concentrations of the oxidising agents tested.

The investigation of the various biocides and their efficacy towards bacterial isolates from washer disinfectors show that there are various levels of biocide efficacy. Peracetic acid, Steris 20 and Reliance HLD all showed high efficacy towards most bacterial isolates. These particular disinfectants are formulated to increase their efficacy and are also used at higher temperatures. These features will play an integral part in these biocides being more effective.

Chlorine dioxide proved to be the biocide with the least efficacy toward some of the bacterial isolates; mainly the *B. subtilis* WD isolate and reference strain. This could be down to these particular bacteria possessing resistance to chlorine dioxide, which had built up over a period of time within the washer disinfector, as chlorine dioxide was the disinfectant used. Inappropriate concentration may have been used in the disinfection process resulting in singling out of these resistant bacterial isolates.

In addition, the presence of organic load (dirty conditions) further limited the efficacy of both chlorine dioxide and peracetic acid when used against the *B. subtilis* WD isolate. The presence of an organic load in disinfection tests is often considered to be required to mimic worst-case exposure conditions (Russell, 2004). Organic matter can affect the efficacy of most biocides, including oxidising agents (Russell, 2004). Although rigorous cleaning should take place before disinfection of endoscopes and organic matter (blood and faeces) should no longer be present in practice (Babb and Bradley, 1995). The mechanisms of resistance of these isolates are clearly different, since one showed evidence of cross-resistance to other oxidising agents, while the other only showed resistance to chlorine dioxide.

Chapter 5:

Investigations of potential resistance mechanisms to oxidising agents

5 Investigations of potential resistance mechanisms to oxidising agents

5.1 Introduction

Chapter 4 demonstrated that the WD isolates from the washer disinfectors and the ATCC reference strain (*B. subtilis* in particular) can withstand prolonged contact times with the biocides tested at an in-use concentration or higher.

Bacterial resistance has been described as a bacterial strain surviving exposure to a biocide concentration that would kill the rest of the bacterial population (Russell, 2003). Maillard (2007) has described resistance in a laboratory setting as a bacterial strain that can survive concentrations of a biocide that would kill its standard strain, and also refers to it in a practical situation where resistance would mean bacterial survival after an in-use concentration of biocide has been applied.

There is a problem when resistance is not defined correctly and pseudo-resistance is assumed; this often relates to mistakes made in the application of a disinfectant (Heinzel 1998). Just because the disinfectant no longer produces a complete kill this does not necessarily mean that the treated bacteria are resistant (White *et al.,* 2001). Several reasons can lead to a biocide becoming ineffective, including the use of

biocides with a low spectrum of activity; not using the biocidal product as directed by the manufacturer; insufficient contact of the biocide with the surface being treated; and insufficient availability of active agents (Heinzel 1998). However, over time such miss-applications could help the bacteria to adapt to the biocidal product.

True resistance can be achieved through bacteria having or gaining mechanisms to aid survival or through growth in biofilm where the production of glycocalyx can prevent the agent from reaching the bacterial cell. Biofilm growth inside complex machinery like endoscopes and WDs can prevent biocides from actively killing all the microorganisms present. Microbial populations can survive when exposed to sub-lethal concentrations where the microorganisms can tolerate and/or develop resistance to the biocide (Moken *et al.,* 1997; Gilbert and McBain, 2001; Maillard, 2002; Braoudaki and Hilton, 2004; McMahon *et al.*, 2007).

Secretions from bacterial cells also will aid survival. Increased resistance to chlorine has been shown in the organism *Vibrio cholera* which produces an amorphous exopolysaccharide causing cell aggregation (McDonnell, 2007). Further examples of chlorine being consumed by the biofilm before it could react with the bacterial cells can be shown in work done by Chen and Stewart (1996).

Physical inactivation can also occur. Biocides can be detoxified by overproduction of metabolites which can neutralise the biocide chemically, or by the expression of detoxifying/degrading enzymes. An example of this would be in the production of catalase in the response to oxidative biocides (Ellkinis *et al.,* 1999; Stewart *et al.*, 2000; Chapman, 2003a). Detoxification/degradation may take time to render a high concentration of a biocide ineffective. However, if a sub-lethal concentration is used, the efficacy of a biocide might be decreased to a bacteriostatic level or below, enabling the micro-organism to adapt and change its mechanism of metabolism (Heinzel 1998). This has been shown with oxidizing agents like chlorine, hydrogen peroxide and peracetic acid. Resistance to these oxidizing agents has occurred with the over-production of catalase (Bol and Yasbin 1991; Engelmann and Hecker, 1996; Elkins 1999). Resistance to catalase depended on the expression of a functional *katA* gene (Bol and Yasbin 1990).

Since bacteria in biofilm have increased resistance to biocides, methods for enhancing biocide efficacy against biofilms are important to investigate (Hall-Stoodley *et al.,* 2004). One approach includes the use of ultrasonic treatment of biofilms. This treatment has been shown to be effective in reducing the amount of viable bacterial in a biofilm (Rediske *et al.*,1999) and has been shown to remove bacterial biofilm attached to the inside of glass tubes (Mott, 1998). Such an approach might be usefully employed in increasing the efficacy of disinfection of bacterial

biofilm, which can be present inside endoscopes and washer disinfectors. Other approaches for the removal of biofilm in the industrial setting have been demonstrated. For example the use of enzymes (Johansen *et al.*, 1997; Orgaz *et al.*, 2006), electric fields (Blenkinsopp *et al.,* 1992) and catalyst modified surfaces (Wood *et al.,* 1996). Investigations into the effects that proteinase have on bacterial biofilm have shown that they can completely remove the biofilm (Espinosa-Urgel *et al.*, 2000; Rohde *et al.,* 2005; Boles and Horswill, 2008).

Preventing the build-up of bacterial biofilm is key, through the rigorous cleaning and/or disinfection of the area in question (Meyer, 2003), and the use of bacterial growth inhibiting surfaces has also been looked into (Meyer, 2003). Rogers *et al.* (1994) looked at various surfaces which would be present in the plumbing of water systems and found that out of the samples tested, stainless steel supported the least abundant biofilm. However, this could change depending on the type of bacterial biofilm.

5.1.1 Aims and objectives

The aim of this chapter is to investigate the presence of a number of mechanisms that might explain the decreased susceptibility of the environmental isolates described in Chapter 4 to oxidising agents. These mechanisms include the presence and effect of extracellular

polysaccharides (EPS) in resistance to the oxidisers, and the presence, activity and expression of detoxifying enzymes. In addition, the potential use of sonic treatments to increase the activity of the oxidiser against these isolates will be investigated.

The presence of EPS-associated with bacterial isolates from the washer disinfectors was evaluated with electron microscopy and light microscopy techniques. The effect of the presence of bacterial EPS on the efficacy of chlorine dioxide was established by the removal of EPS and the use of surface carrier efficacy testing. The ATCC reference strains will be used as a negative control.

The presence of detoxifying enzymes that could inactivate the oxidisers was investigated using standardised enzymatic assays. In addition, the expression of detoxifying enzymes following biocide challenge in these WD isolates and reference ATCC strains will be investigated by PCR methods. These experiments aim to establish a better understanding of the survival mechanisms of the environmental isolates from washer disinfectors to the oxidising treatments. Finally, preliminary experiments were conducted to establish the potential for the use of ultrasonic treatment to increase the efficacy of the biocide chlorine dioxide against *B. subtilis*. The test protocol was based on a standard suspension test.

5.2 Material and Methods

5.2.1 Bacterial strains

Washer disinfector isolates *B. subtilis*, *M. luteus* and the ATCC reference strains (Chapter 3, section 3.3.2) were used in this section. Bacterial culture was conducted in the manner outlined in Chapter 2.

5.2.2 Electron microscopy

5.2.2.1 Initial bacterial cell preparations

Scanning electron microscopy (SEM) was used to observe the structure of microbial isolates taken from the washer disinfector at the endoscopy unit (Chapter 3, Section 3.3.2) and the ATCC reference strains. The effect of chlorine dioxide treatment on the bacterial cells was also investigated with transmission electron microscopy (TEM).

All strains were cultured on agar as describe in Chapter 2 (section 2.2.2). Bacterial cells were harvested and centrifuged as normal (Chapter 2 section 2.2.5.1) and then re-suspended in sterile deionized water at a concentration of 1×10^8 cfu/ml. Bacterial isolates prepared for SEM observation were not treated with biocides. TEM samples were treated with chlorine dioxide at the following concentration: 0.01% (in-use concentrations) and 0.03% (used in suspension and carrier test). Untreated bacteria were used as a control. Biocide exposure was

conducted immediately prior to EM preparations. A contact time of 30 s was used for *B. subtilis* as this was shown not to cause complete kill (Chapter 4; Section 4.3.2.3.1). The same contact time was used to treat *M. luteus*. A 30 s exposure with chlorine dioxide (0.03%) was shown to produce a > 5.53 $\log_{10}$ reduction in viable number in the suspension test (Chapter 4; Section 4.32). Since, the aim of this work was to identify damage caused by the oxidising agents, only the complete degradation would have a negative effect on observation. A lower concentration (0.01%) was also used. Following exposure to chlorine dioxide bacterial samples were then treated with 5 g/L of sodium thiosulphate as this was the neutraliser validated for efficacy testing (Chapter 4, Section 4.2.4.1).

5.2.2.2 Initial EM preparations

The initial preparation for SEM and TEM samples were identical and are detailed as follows. 2 ml of 2.5% glutaraldehyde (GTA) in 0.1 M sodium cacodylate buffer (pH 7.4) was mixed with the same volume of bacterial cells in suspension in a plastic test tube. Samples were then centrifuged at 2,200 g (Micro S centrifuge, MSE, Crawley, UK) for 5 min and the supernatant was removed. 5 ml of 2.5% glutaraldehyde (GTA) in sodium cacodylate buffer was added to the bacterial cell pellet which was resuspended. The sample was then left to fix for 1 hr at room temperature, centrifuged as before, washed twice for 5 min in 0.1 M cacodylate buffer, and stained for 1 hr in 1% osmium tetroxide in 0.1 M cacodylate buffer in a fume hood. This was followed by 3 × 5 min

washes in 0.1 M cacodylate buffer. The samples were then separated for SEM and TEM for further preparation.

5.2.2.3 SEM preparation

The SEM samples were placed in purpose-made filtration capsules with 0.2 µm polyester membrane filters (Nucleopore, Oxshott, UK) and centrifuged at 2,200 g for 5 min. All samples were then progressively dehydrated in 30% alcohol for 5 min, 50% for 5 min, 70% for 5 min, 90% for 5 min, and finally 3 × 10 min in 100% alcohol. Samples were then dried in a sample dryer (Samdri 780, Maryland, USA). The filter membranes were then removed from their filtration capsule and attached with double-sided tape to brass SEM specimen carriers. Samples were then gold coated with a gold sputter coater (EMScope, UK).

5.2.2.4 TEM preparation

Bacterial samples were embedded in 3% agar; once set, samples were placed in holders, cut using a razor blade and dehydrated progressively with 5 min washes with 30, 50, 70, 80, 90 and 100% ethanol. Samples were then washed in propylene oxide for 10 min. Resin (5 g Araldite CY212, 5 g dodecyl succinic anhydride and 0.15 g N-benzyldimethylamine) infiltration was then carried out overnight at room temperature. Resin embedding then took place for 2 days at 60°C.

Sections were cut using a Reichert-Jung Ultracut Microtome (Wien, Austria) and placed onto copper grids.

Samples were stained with 2% uranyl acetate for 10 min, rinsed twice in deionized water and then immersed in Reynolds lead citrate for 5 min. Grids were dried with filter paper between each step.

5.2.2.5 SEM and TEM imaging

SEM samples were examined at 5,000× and 10,000× magnification using a XL20 scanning electron microscope, (Philips, Croydon, UK). Ten randomly selected fields of vision were looked at, and representative images were recorded and photographed.

TEM samples were examined at 13,000×, 20,000×, 40,000×, 50,000×, 80,000× and 100,000× magnification with an EM 280 transmission electron microscope (Philips, Croydon, UK). Images were taken with the aid of inbuilt software (Philips, UK) and examined visually for cellular differences between reference strains and washer disinfector isolates, pre-exposed or not to chlorine dioxide (0.01%) for 1 min.

5.2.3 Role of EPS

5.2.3.1 Visualisation of bacterial EPS

The possible presence of bacterial EPS in the wild-type clinical isolates from the endoscopy unit was demonstrated by a simple staining protocol. Overnight broth cultures were grown in a shaken incubator at 70 rpm. Glass cover slips (Fisher Scientific, UK) were washed in ethanol and placed into six-well plates (Fisher Scientific, UK). Bacterial samples (100 µl) were vortexed then added to the wells together with 900 µl TSB. The plates were incubated at 37°C for 6 hr in order to allow attachment to the glass coverslip. The coverslip was then removed, and the underneath side of the slide was washed to aid attachment. The coverslip was attached to the microscope slide, allowed to dry and then fixed by passing the slide through a Bunsen burner. Once cooled the slide was stained with a 2:1 mixture of aqueous Congo Red (Fisher Scientific, UK) and 10% Tween 80 for 15 min. The slide was rinsed with deionized water, stained with Zeihl Carbol Fuschin (Fisher Scientific, UK) for 6 min and then washed with deionized water and air dried. The slides were observed under 1000× magnification (Olympus, Bx50 Microscope Olympus, UK). Bacteria appear purple/pink, and EPS if present as pale pink (Harraison-Balestra 2003). Images were made using an Olympus DP10 camera (Olympus, UK). Ten randomly selected fields of vision were observed, and representative images were recorded and photographed.

5.2.3.2 EPS removal

In order to establish what effect EPS had on the efficacy of chlorine dioxide, EPS was removed and the efficacy of the oxidiser tested in surface carrier efficacy tests.

Standardised cultures were prepared as described in Chapter 2 (section 2.2.51). 1 ml of a standardised culture was added onto a TSA plate and incubated for 24 hours at 37°C. 5ml of sterile deionised water was pipetted onto the surface of the agar plate. Then using a sterile glass spreader the bacteria was removed from the surface, and the sample was added to a 50 ml polypropylene copolymer centrifuge tube (Fisher Scientific, UK) and vortexed. A total viable count was then performed using methods described in Chapter 2, section 2.2.6. The sample was centrifuged at 13,500 g at 15°C for 30 min and the supernatant removed and retained. The bacterial pellet was re-suspended in 5 ml of sterile deionised water and centrifuged again at 13,500 g at 15°C for 30 min. The supernatants from both centrifugation steps were combined and centrifuged at 13,500 g for 15 minutes at 4°C. The resulting supernatant was seeded with 0.1 g sodium acetate and three volumes of 95% ethanol. The sample was stored at -20°C until analysed.

5.2.4 Disruption of bacterial aggregates

Bacterial cells have the potential to clump together and this mechanism can aid bacterial survival when treated with biocides. Clumping is often observed when EPS is produced (Wolfaardt *el al.*, 1999). The propensity of the bacterial isolates to clump was investigated by using sonication to disrupt the bacterial aggregates and improve the effectiveness of the oxidising agent.

To verify the presence of aggregates the following experiment was carried out with a high frequency 23 KHz generator with piezoelectric transducer sonicator (Soniprep 150). An overnight culture of *B. subtilis* WD isolate was used in this experiment (Chapter 2; Section 2.5.1). Bacteria were washed, adjusted to 5×10^8 cfu/ml and 2.5 ml aliquots were placed onto the shelf of the sonicator. Sonication took place over a period of 30 min. At the following time intervals 1, 5, 10, 15, 20, 25 and 30 min, a 100 µl sample was removed and viable count was performed using the drop count method (see Chapter 2; Section 2.2.6) to establish if there were any differences in bacterial cell count after sonication.

To assess the effect of clumping on the efficacy of chlorine dioxide, the following experiment was carried out. An overnight culture of *B. subtilis* WD isolate was used in this experiment (Chapter 2; Section 2.5.1). Bacteria were washed and adjusted to 5×10^8 cfu/ml and separated

into 2.5 ml aliquots. Aliquots were placed onto the shelf of a high frequency 23 KHz generator with piezoelectric transducer sonicator (Soniprep 150). The probe was lowered into the sample and sonication took place over a period of 30 min. Contact times from 30 s to 30 min were used on the sample and after each contact time a sample was taken and used in the suspension efficacy test described in Chapter 4, Section 4.2.3.3.

5.2.5 Expression of detoxifying genes

5.2.5.1 Primer design

Oligonucleotide primers for catalase in both *M. luteus* (*catA*) and *B. subtilis* (*katA*), and superoxide dismutase in *B. subtilis* (*sodA*) were designed as follows. The National Centre for Biotechnology Information's (NCBI) nucleotide database was searched for the above named nucleotide sequences in the bacterial target genome. mRNA sequences were copied into the 'source sequence box' of the web-based Primer3 primer design software (Rozen and Skaletsky 2000). The Primer3 software selected forward and reverse primers without modification of the default software setting. Primers (see Table 5.1) were obtained from Invitrogen (Paisley, UK).

Table 5.1 Oligonucleotide primer details.

Primer	Sequence	Product size	Location	Acc. No.
catA	F: CGAGGACGTGTCGAAGTACA R: AGAAGATCGGGGTGTTGTTG	194	224-417	AJ438208
katA	F: GGCGTGAAAAACCTTGATGT R: TGACATCAAACGGATCGAAA	178	826-1003	AB046412
sodA	F: GCTTTACTCGCTGGGAATTG R: TCTCCGGCTAAAAGCACACT	231	1490-1720	D86856
gyrB	F: ACGGCATTACGGTTGAAGTG R: TCATCTCCGCTTAGGTTTGG	199	4866-6779	NC000964

The *gyr*B primer (which was used as control in RT-PCR) information was obtained from Prof. Colin Harwood, Newcastle University, UK and then also ordered from Invitrogen.

5.2.5.2 DNA extraction

DNA was extracted from washed overnight bacterial cultures (see Chapter 2, section 2.2.5.1) using Trizol ® (Invitrogen) following the manufacturer's protocol.

5.2.5.3 RNA extraction

RNA extraction was carried out using the Ambion RiboPure™-Bacteria Kit (Applied Biosystems/Ambion, Austin, USA). The operation guide was followed and is detailed as follows:

Washed cells from an overnight culture (see Chapter 2; section 2.2.5.1 were adjusted to 5 × 10^8 cfu/ml (Chapter 2, section 2.2.8). Bacterial suspension was centrifuged at 13,000 g for 60 s at 4°C. The supernatant was discarded. The bacterial cells were disrupted by resuspending the pellet in RNAwiz and placing into tubes that contain zirconia beads (Applied Biosystems/Ambion, Austin, USA). The tubes were then vortexed for 10 min using a vortex mixer (Genie 2, Scientific Industries Inc, USA) with adapter (Applied Biosystems/Ambion, Austin, USA) at maximum speed.

Zirconia beads were pelleted by centrifuging at 13,000 g for 5 min at 4°C. The bacterial lysate was transferred to a fresh tube. A 0.2 volume of chloroform was added to the lysate, shaken for 30 s and incubated for 10 min at room temperature. The sample was then centrifuged at 13,000 g for 5 min at 4°C, and the aqueous phase was transferred to a fresh tube.

The final RNA purification continued as follows: a 0.5× volume of 100% ethanol was added to the sample and mixed thoroughly. A filter cartridge (Applied Biosystems/Ambion, Austin, USA) was then placed inside a 2 ml collection tube. The sample was then transferred to the filter cartridge, lid closed and centrifuged at 13,000 g for 1 min. The filtrate was discarded and the filter returned to the collection tube. The filter was then washed by adding 700 µl of wash solution 1 and

centrifuged for 1 min at 13,000 g at 4°C. The filtrate was discarded, the filter returned to the collection tube then washed with 500 µl of wash solution 2/3, centrifuged as before and the filtrate again discarded. This step was repeated with 500 µl of wash solution 2/3. The filter was centrifuged for 1 min at 13,000 g at 4°C to remove excess wash and transferred to a fresh collection tube.

The RNA was then eluted by applying the desired volume of elution solution needed depending on how much was eluted in the first instance (see manufacturer's instruction for Ambion RiboPure™), which had been preheated to 95-100°C and applied to the centre of the filter. The filter was centrifuged for 1 min at 13,000 g at 4°C. The elution step was repeated in order to retrieve the maximum total RNA. Eluted RNA was stored at −80°C until used. The RNA samples were treated with DNase following the Ambion RiboPure™-Bacteria Kit instructions..

RNA was then quantified by absorption at 260 nm and 280nm. An absorption ratio of at least 1.6 was taken to indicate high quality nucleic acid, sample measurements were above the 1.6 value. RNA samples were diluted when necessary with nuclease-free H_2O (Fisher Scientific, UK) to a maximum concentration of 1 µg/µl. All RNA samples were stored at −80°C.

5.2.5.4 PCR

Amplification of the DNA and the cDNA from the RT reaction (Section 5.2.6.5) took place in 25 µl reactions using oligonucleotide primers obtained from Invitrogen Ltd. (see Section 5.2.5.1 for primer details). PCR was conducted using the GoTaq® Flexi DNA Polymerase kit (Promega) and control reactions were carried out either with sterile nuclease-free water instead of cDNA, or using the no-AMV-RT reaction samples instead of cDNA. The standard composition of PCR reactions is shown in Table 5.2 and PCR conditions are shown in Table 5.3, the same annealing temperature was used for all PCRs. The PCR amplification products were analysed by gel electrophoresis and stained with ethidium bromide, in 1× TAE buffer (40 mM Tris-Acetate, 1 mM EDTA) and visualised under UV light. Gene expression was measured qualitatively by observing the brightness of the band signal.

Table 5.2 PCR reaction composition.

Components	**Final concentrations**	**Volumes**
5× Green GoTaq® Flexi Buffer	1×	5.0 µl
$MgCl_2$, 25 mM	1.5 mM	1.5 µl
PCR nucleotide (dNTP) mixture	200 µM each dNTP	1.3 µl
Forward primer (sense)	0.4 µM	1.0 µl
Reverse primer (antisense)	0.4 µM	1.0 µl
GoTaq® DNA polymerase (5 u/µl)	0.3125 u	0.1 µl
cDNA (or H_2O, or No-AMV.RT)		1.0 µl
Nuclease-free H_2O		to 25 µl

Table 5.3 PCR reaction conditions.

Step	Temperature °C	Time	No. of cycles
Initial denaturation	95	10 min	1
Denaturation	95	30 s	
Annealing	55	45 s	30
Extension	72	1 min	
Final extension	72	10 min	1
Soak	4	Indefinite	

5.2.5.5 RT-PCR

Bacterial samples used for RT-PCR experiments were pre-treated with chlorine dioxide for 1 min (using suspension test method, Chapter 4 section 4.2.3.3) prior to RNA extraction which was done following the protocol described earlier in section 5.5.2.3. Control samples had no pre-treatment with chlorine dioxide.

Reverse transcription (RT) was carried out with the Promega Improm-II Reverse Transcription System, using 1 µg RNA in a 20 µl reaction (see Table 5.4 for composition) alongside a negative control omitting the enzyme AMV reverse transcriptase. The RNA template, primer and nuclease-free H_2O initial 5 µl reaction mixture was run at 70°C for 5 min then held on ice until needed. The RT was run at 25°C for 5 minutes annealing time, 42°C for 60 minutes extension time, and 70°C for 15 minutes to heat inactivated the AMV-reverse transcriptase, in a

Techgene thermal cycler (Jencons-PLS, UK). The cDNA product was stored at −20°C until used, and cDNA products were analysed as described in Section 5.2.5.4.

Table 5.4 Composition of reverse transcription reaction.

Components	**Final concentrations**	**Volumes**
RNA template	1 µg	
Random Primers	0.5 µg	1 µl
Nuclease-free H_2O		to 5 µl
Improm-II™ 5× Reaction Buffer	1×	4 µl
$MgCl_2$ (25 mM)	3 mM	2.4 µl
dNTP mixture (5 mM each)	0.5 mM each dNTP	2 µl
Recombinant RNasin® Ribonuclease Inhibitor	1 u/µl	0.5 µl
Improm-II™ AMV Reverse Transcriptase*	15 u/µg	1 µl
Nuclease-free H_2O		to 20 µl total volume

* omitted from negative control reaction

5.2.6 Enzyme assays

5.2.6.1 Catalase

Catalase production was established using the standard catalase test outlined in Chapter 3 section 3.2.2.2.1. In order to establish the effect of the production of catalase on biocide efficacy, the level of catalase production in the bacterial isolates and standard culture counterparts had to be first established. The disc flotation method was used to establish this (Gagnon et al., 1959). A dilution series of catalase (Bovine Liver, Sigma-Aldrich, US), in potassium phosphate buffer was made to obtain the following concentrations: 0.01, 0.0025, 0.001, 0.00025 and 0.0001%. Filter discs (Fisher scientific, UK) were soaked in the different catalase solution (one at a time) for 30 s (until the disc was no longer visibly dry). The discs were then added to test tubes containing 5 ml of hydrogen peroxide (3%) and the time which lapsed from the disc touching the surface of the hydrogen peroxide then floating back to the surface again was recorded (Gagnon *et al.,* 1959). This was repeated in triplicate for each concentration. A calibration curve was then made from the data retrieved.

Bacterial supernatant samples (see Chapter 2 section 2.2.5.1) were then tested in the same manner. Using the calibration curve the production of catalase (% w/v) was calculated.

5.2.6.2 Proteinase K

The presence of EPS is fundamental to the surface attachment of bacteria and EPS is also know to protect the microorganism and be involved with the production of bacteriolytic enzymes (Wolfaardt et al 1999) and may play a role in decreased bacterial susceptibility to oxidising agents. Proteinase K breaks down proteins and peptides into amino acids (Petsch *et al.,* 1998). Here proteinase K was employed in an attempt to degrade bacterial EPS (Patterson *et al.*, 2007; Boles *et al.,* 2008) and any biocide detoxifying enzymes excreted outside the isolates.

In the first instance the effect of Proteinase K on the growth and survival of *B. subtilis* was investigated using the Bioscreen C Microbial Growth Analyser. A range of concentrations was employed to select the appropriate concentration to use in conjunction with an oxidising agent in a surface carrier efficacy test.

Varying concentrations (2, 10, 20, 50, 100, 200 µg/ml) of Proteinase K were tested to see what effects it had on *B. subtilis* reference strain and on the WD isolate, using the bioscreen (Bioscreen C Microbial Growth Analyser) in order to establish which concentration should be used in the carrier test. Bacterial samples of *B. subtilis* reference strain and WD isolate were grown as previously described in Chapter 2 (section 2.24 and 2.2.5.1). 50 µl of each concentrations of Proteinase K was added to

the wells of the bioscreen together with 300 µl TSB and 50 µl of bacterial sample in TSB. Bioscreen plates were incubated in the Bioscreen for 14 h taking readings every 15 min. The plates were shaken before each reading. Readings were recorded using a PC with EZExperiment (Oy Growth Curves Ab Ltd, Finland) and Excel Software. Data was then analysed and the appropriate concentration for the carrier efficacy test was selected.

The carrier efficacy test was conducted in the manner outlined previously in Chapter 4 (Section 4.2.4). However, the bacterial suspension (environmental isolate) was pre-treated with Proteinase K (200 µg/ml) for 4 h at 37°C prior to being added to the stainless steel disc and dried. The carrier test was then conducted as previously described (Chapter 4 section 4.2.4), using the biocide chlorine dioxide.

5.3 Results

5.3.1 Electron Microscopy

5.3.1.1 SEM - observations of cultures grown on TSA plates

SEM (Figures 5.1 – 5.4) was used compare the WD isolates from the Endoscopy Unit and with reference strains. The reference strain and WD isolate of *B. subtilis* (Figure 5.1- 5.2) had morphology as follows: rod shaped bacteria of a size approximately 2 µm in length. However the general appearance of each isolate was different. The reference strain could be described as having a smooth surface, whereas the WD isolate was shown to be heavily embedded into EPS. The reference strain of *M. luteus* (Figure 5.3) had morphology as follows: small cocci of < 1 µm diameter associated in clusters. This contrasted sharply with the WD isolate (Figure 5.4) they were a lot larger (2 µm in size) and were appearing to form groups of four cocci. The surface of the WD isolate appeared to be rough and uneven. This appearance might be caused by the presence of EPS. All micrographs were taken at 10,000× magnification (Martin *et al.*, 2008).

Figure 5.1 *Bacillus subtilis* ATCC reference strain 6051.

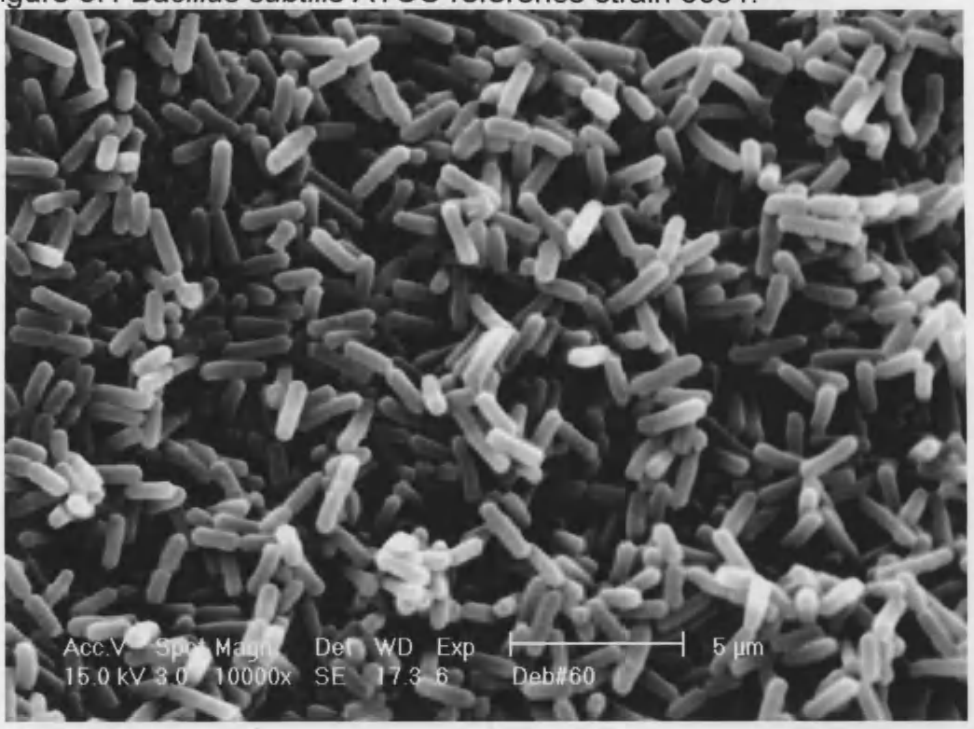


Figure 5.2 *Bacillus subtilis* WD isolate from endoscopy unit.

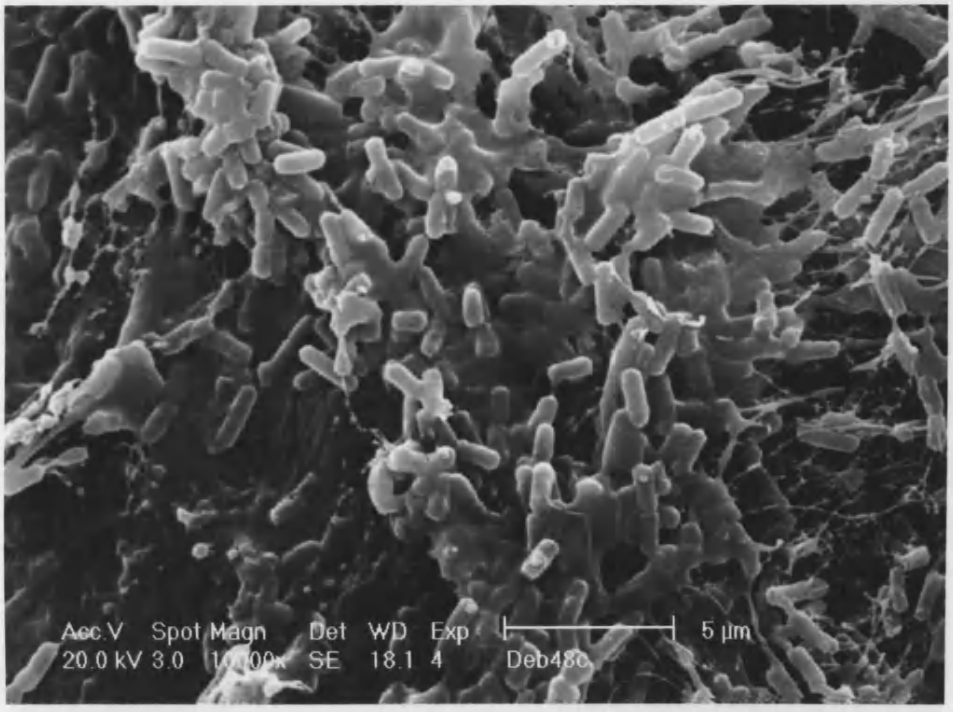


Figure 5.3 *Micrococcus luteus* ATCC reference strain 4698.

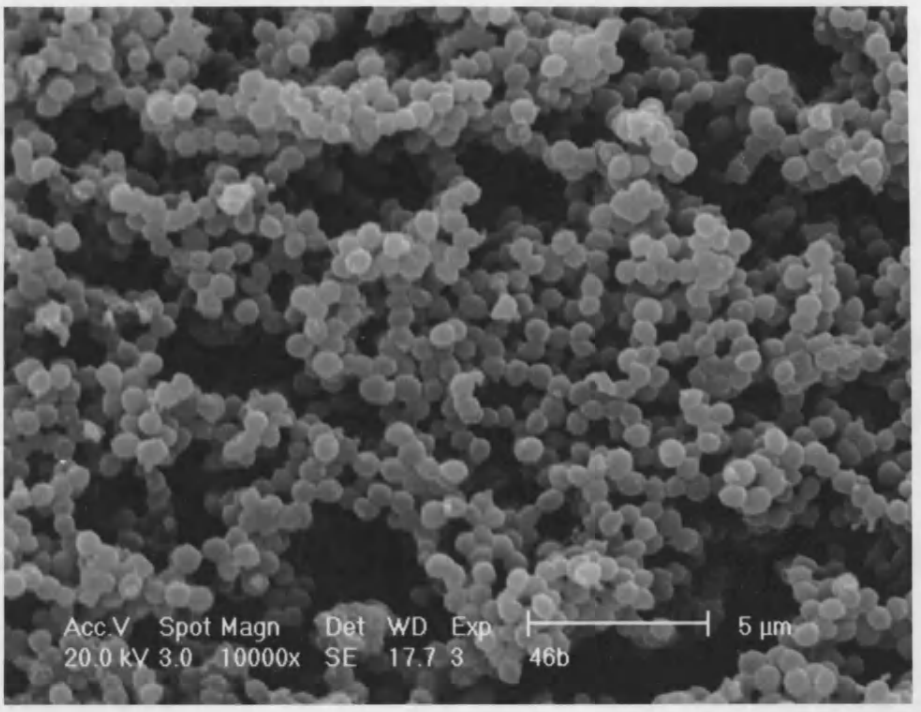


Figure 5.4 *Micrococcus luteus* WD isolate from endoscopy unit.

5.3.1.2 TEM – observations following biocide pre-exposure

TEM (Figures 5.5 - 5.13) provided further comparison between WD isolates and reference strains. TEM micrographs of *M. luteus* WD isolate and its reference strain (Figures 5.5 and 5.6) showed that the bacterial cells were similar in size unlike the observation from the SEM micrographs. Indeed, the size of fifty bacterial cells of each strain was measured. The average cell diameter for *M. luteus* ATCC reference strain and WD isolates were 0.54 µm (SD 0.15) and 0.48 µm (SD 0.08) respectively. Although appearing slightly smaller, the size of the environmental isolate was not significantly different from that of the reference strain ($p = 0.14$). The different observations between SEM and TEM micrographs provide further evidence that EPS is most probably encasing the WD isolate. In addition, TEM and SEM micrographs of *M. luteus* WD isolate clearly showed a clustering of cocci in groups of four. Whether or not such clustering might provide some protection against chlorine dioxide as observed in the surface carrier efficacy test (Chapter 4, Section 4.3.3) remained to be determined.

The TEM micrographs for *B. subtilis* are shown in Figures 5.7 and 5.8. From these, both reference strain and WD isolate are shown to have a similar size. The size of fifty bacterial cells of each strain was measured. The average cell length for *B subtilis* ATCC reference strain

and WD isolate was 1.41 µm (SD 0.26) and 1.6 µm (SD 0.27) respectively. The difference in size was not significant ($p = 0.29$). More importantly, the micrographs also show that there is no difference in the fine structure of the cell wall between the two strains, although the external structure of the WD isolates appeared to be less defined. This might be due to the presence of remnant EPS.

Figure 5.5 TEM micrograph of *M. luteus* reference ATCC strain. (representation from 20 fields of view).

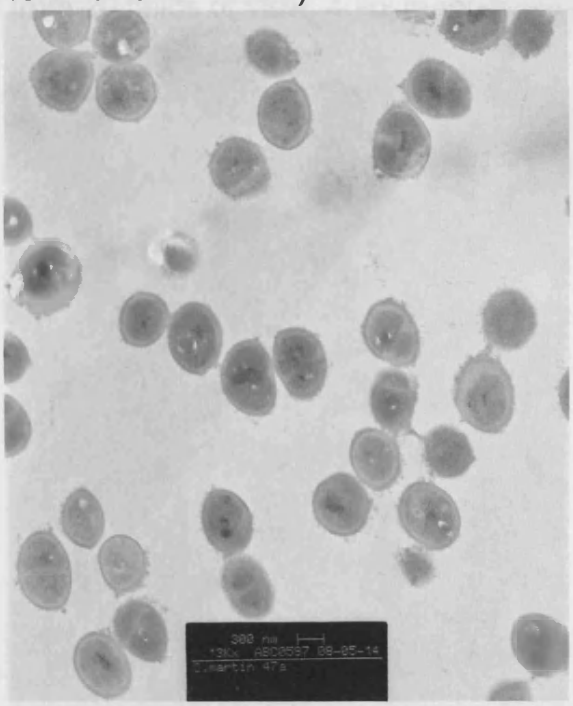


Figure 5.6 TEM micrograph of *M. luteus* WD isolate (representation from 20 fields of view).

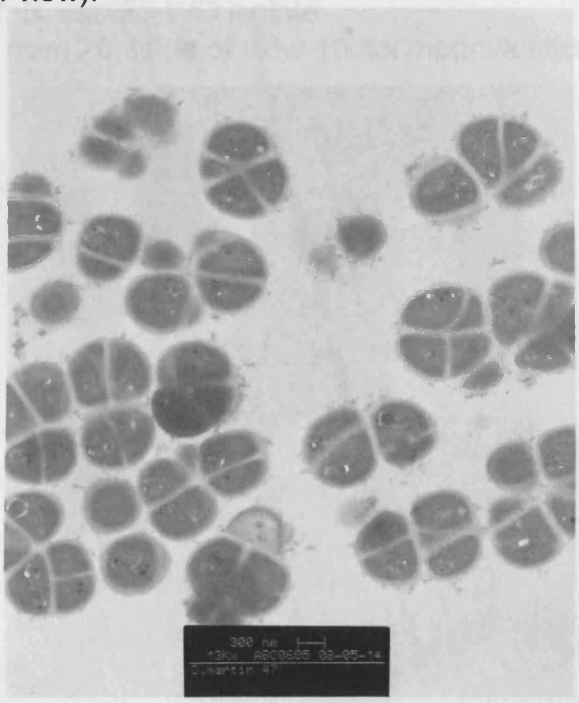


Figure 5.7 TEM: *B. subtilis* 6051 ATCC reference strain.
(representation from 20 fields of view, 40kx magnification).

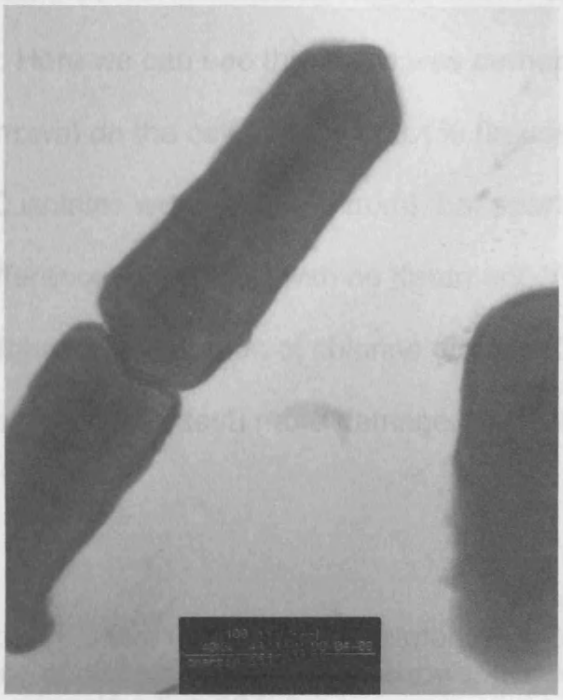
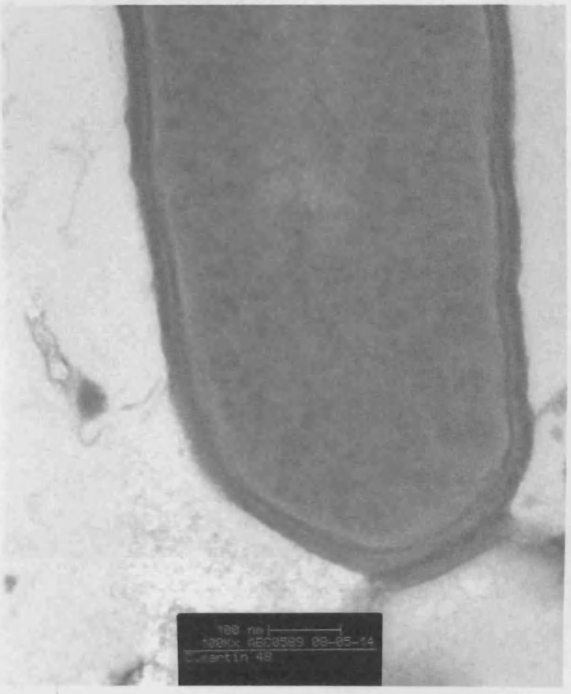


Figure 5.8 TEM: *B. subtilis* WD isolate
(representation from 20 fields of view 100kx magnification).



TEM micrographs (looking at 20 fields of view) (Figures 5.9 and 5.10) showed the effects that chlorine dioxide had on the ATCC reference strain *B. subtilis*. Here we can see that there was perhaps some blebbing (see arrows) on the cell surface at 0.1% (in-use concentration used, where WD isolates were retrieved from), but apart from that there was really no difference to the TEM with no treatment. With the treatment of a higher concentration of chlorine dioxide (0.3%, concentration used in carrier test) more damage (see arrow) to the cell was apparent.

Figure 5.9 TEM: ATCC 6051 *B. subtilis*, treatment with chlorine dioxide at 0.1%. Evidence of blebbing is shown by arrows. (representation from 20 fields of view, magnification 32 kx).

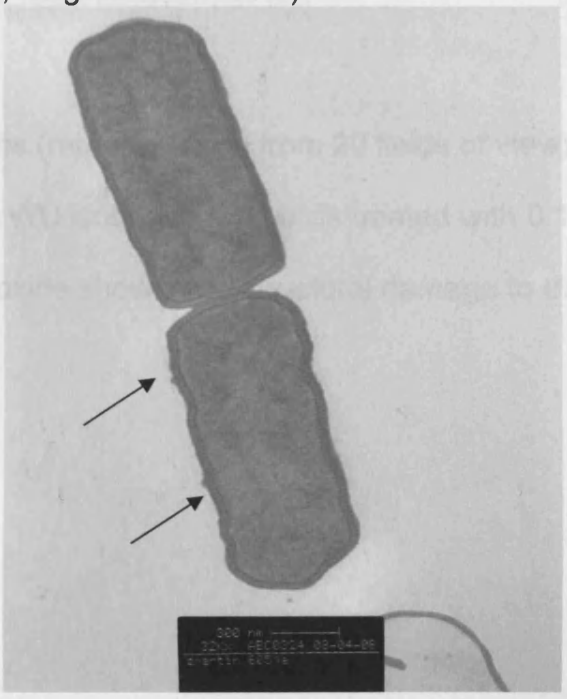
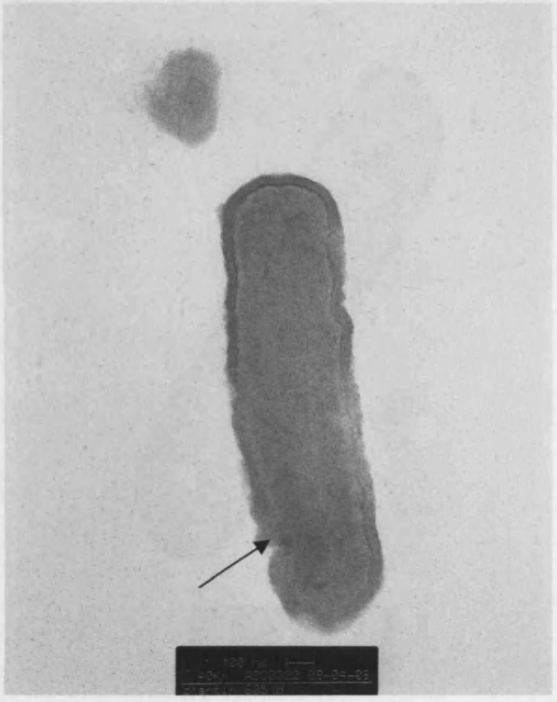


Figure 5.10 TEM: ATCC 6051 *B. subtilis*, treatment with chlorine dioxide at 0.3%. Damage to cell is shown by arrows. (representation from 20 fields of view, magnification 40 kx).



TEM micrographs (representation from 20 fields of view) (Figure 5.11 and 5.12) of the WD isolate of *B. subtilis* treated with 0.1% and 0.3%chlorine dioxide showed no structural damage to the cells or blebbing.

Figure 5.11 TEM: WD isolate of *B. subtilis*, treatment with chlorine dioxide 0.1%, (representation from 20 fields of view, magnification 40 Kx).

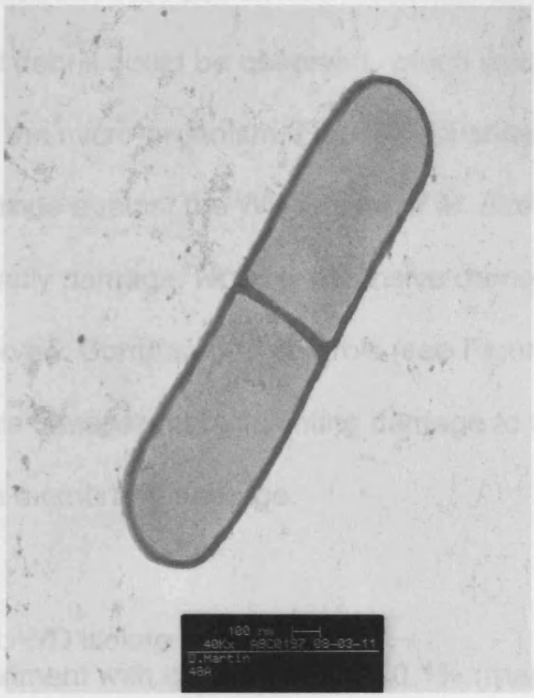
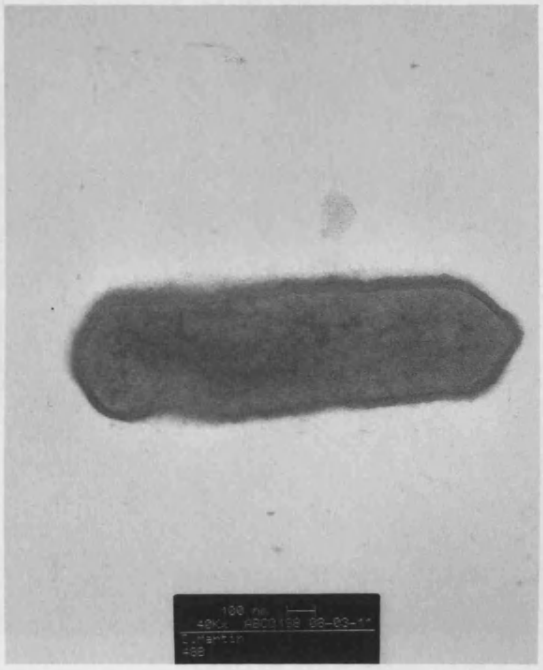
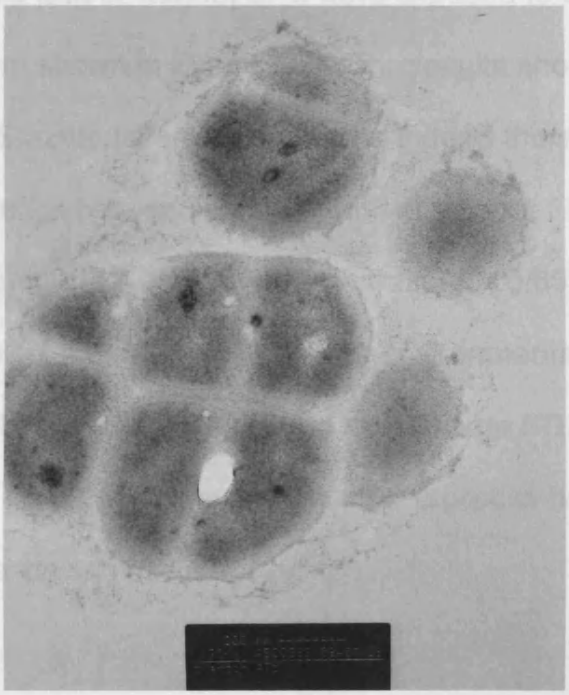


Figure 5.12 TEM: WD isolate of *B. subtilis*, treatment with chlorine dioxide 0.3%.(representation from 20 fields of view, magnification 40 Kx).



The effects of chlorine dioxide (0.1 and 0.3%) on the structure of *M. luteus* ATCC reference strain could not be established by EM. Indeed, only bacterial cell debris could be observed, which would indicate a complete lysis of the micro-organism. Figure 5.13 shows the effects of 0.1% chlorine dioxide against the WD isolate *of M. luteus*. The bacterial cells appeared badly damage. Notably extensive damage to the cell wall can be observed. Compared to controls (see Figure 5.6) the cells appear to be more transparent highlighting damage to the bacterial cell wall and possible membrane damage.

Figure 5.13 TEM: WD isolate of *M. luteus*, treatment with chlorine dioxide 0.1% (magnification 32 Kx).



5.3.2 Role of EPS

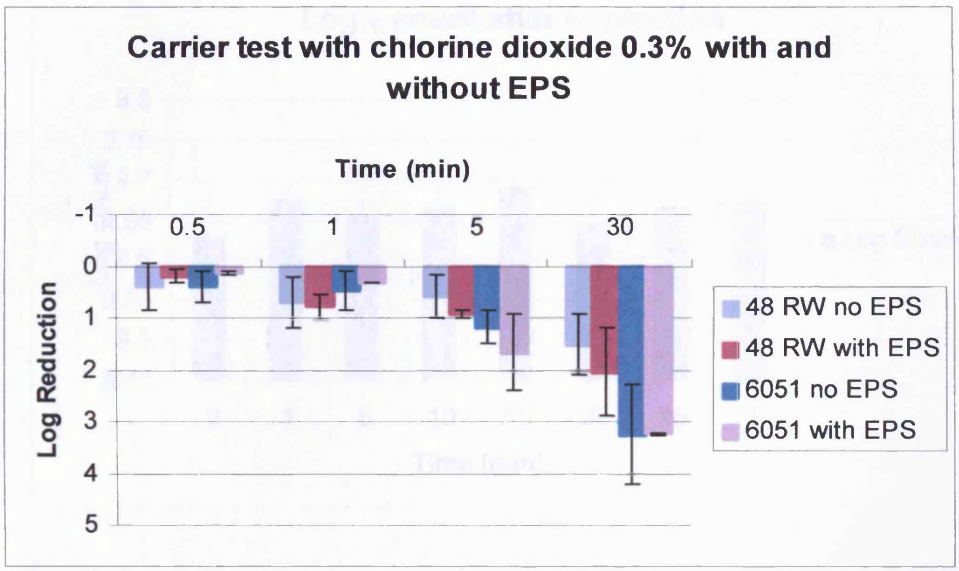
5.3.2.1 Visualisation of EPS

Observation of stained bacterial samples under the light microscope showed no appearance of pink stained EPS (data not shown). These results contradict EM observation where a high amount of EPS can be observed with the WD isolate (e.g. Figure 5.2).

5.3.2.2 Removal of EPS

The investigation as to whether EPS was affecting the action of the oxidising agents or not was based on carrier efficacy testing where bacterial samples with or without EPS were exposed to chlorine dioxide 0.3%. Results are shown in Figure 5.14. The results showed that the presence of EPS made little difference, and indeed there was no significant difference between samples with or without EPS for the WD isolate ($p = 0.06$) or the ATCC reference strain ($p = 0.85$) in activity of the oxidiser against the reference strain or environmental isolate. There was some variability in results indicated by the large SD. Such variability might be explained by the inability to predict how much EPS was present in each sample.

Figure 5.14 Carrier test results with and without EPS. This graph shows *B. subtilis* ATCC reference strain (labelled 6051) and WD isolate (labelled 48 RW).



5.3.3 Disruption of cells

Bacteria clumping together can reduce the activity of a biocide. Clumping often results from the production of EPS by the bacterial cell. The effect of bacterial aggregation on oxidiser was investigated by the application of sonication prior to measuring efficacy with a suspension efficacy test. The results from sonication can be seen in Figures 5.15 and 5.16. Sonication made no significant difference in increasing the number of cells in suspension, (p =.0.88) (Figure 5.15). Sonication did not increase the activity of chlorine dioxide, (p > 0.05 for clean and dirty conditions) (Figure 5.16).

Figure 5.15 Viable count for *B. subtilis* WD isolate after treatment with sonication. Average count shown in cfu/ml converted to $\log_{10}$.

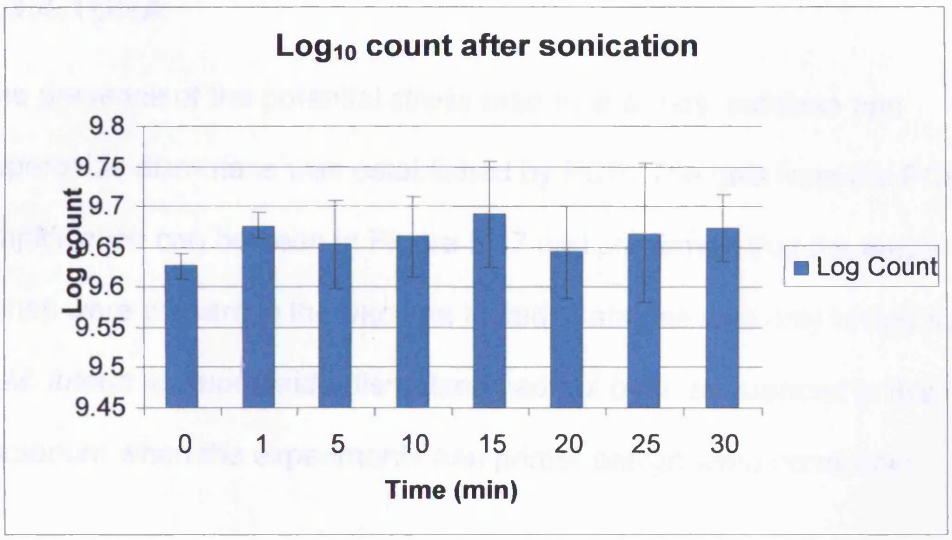
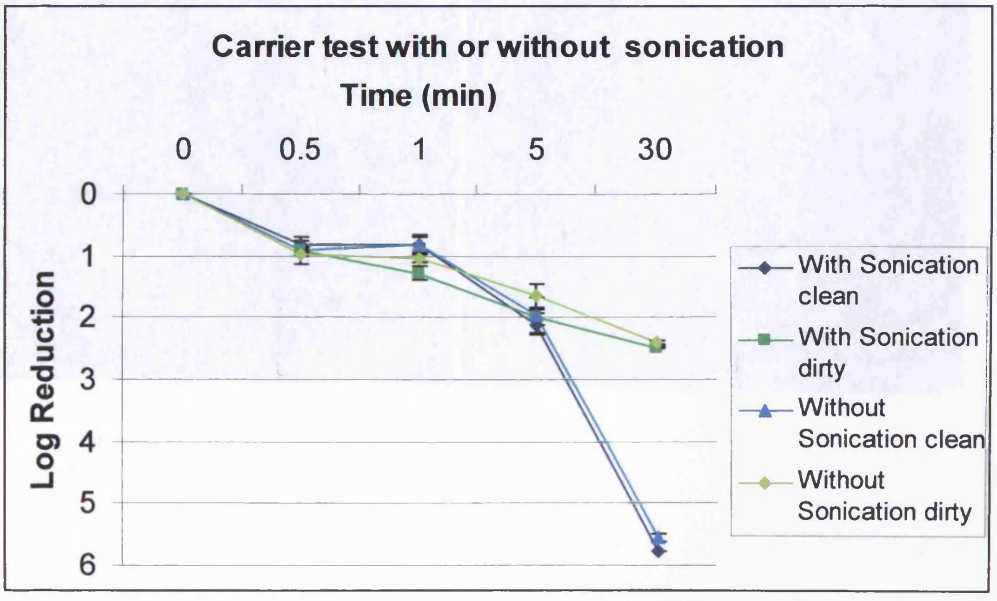


Figure 5.16 Carrier test: Results for *B. subtilis* WD isolate after sonication treatment. Average log reduction after pre-treatment of sonication for 1 min.

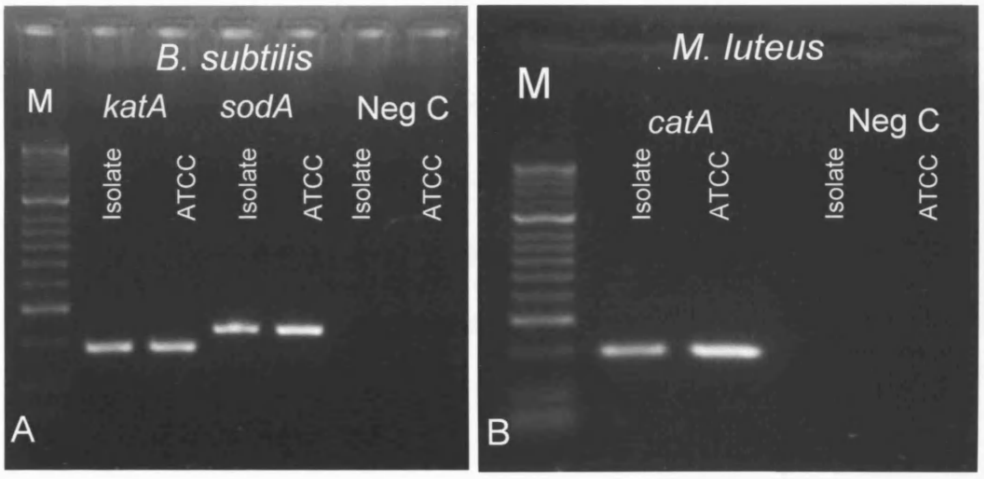


5.3.4 PCR investigations

5.3.4.1DNA

The presence of the potential stress response genes, catalase and superoxide dismutase was established by PCR. The gels from the PCR amplification can be seen in Figure 5.17 and confirmed that the target genes were present in the bacteria tested. Catalase was only tested for in *M. luteus* as superoxide dismutase had not been sequenced in this bacterium when the experiments and primer design were conducted.

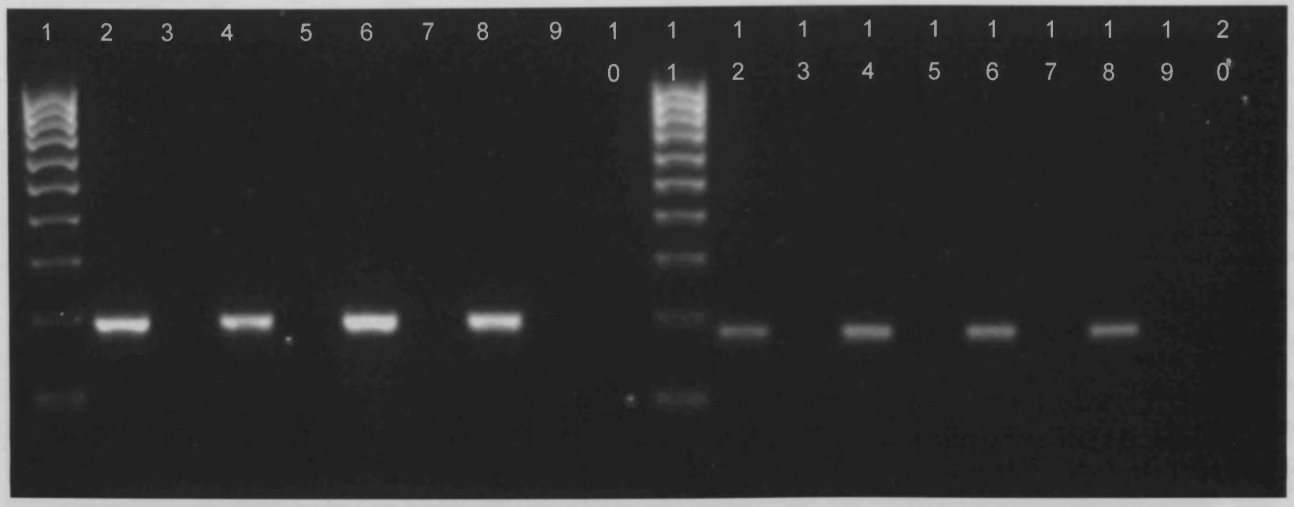
Figure 5.17 DNA PCR results for *M. luteus* and *B. subtilis*, for genes of catalase and superoxide dismutase. Gel A shows the genes in the *B. subtilis* isolate and reference strain and gel B shows only catalase for the WD isolate and reference strain. Negative controls omitted DNA.



5.3.4.2 RT-PCR

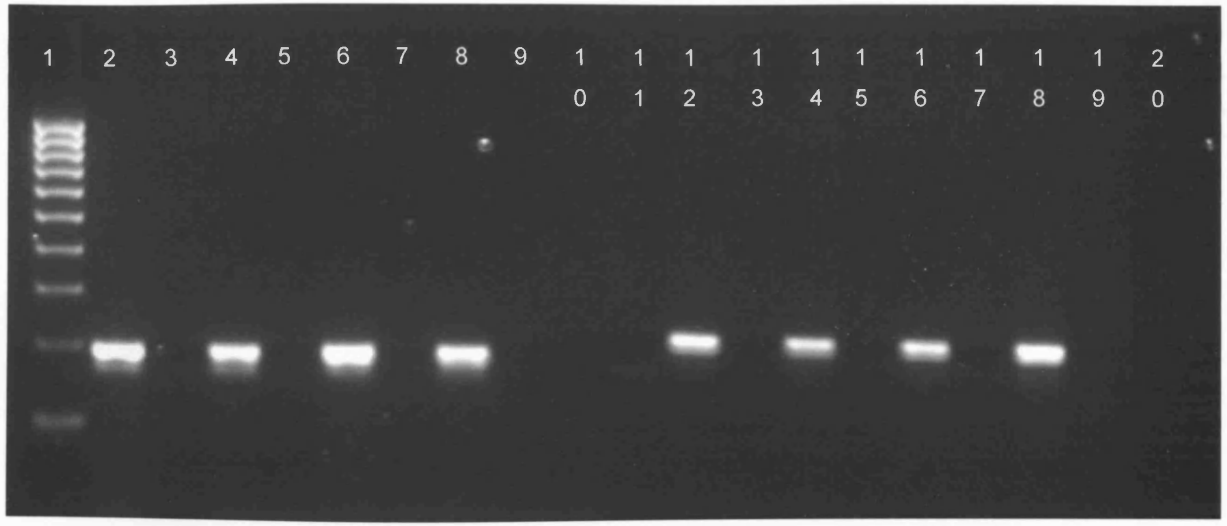
RT-PCR was used to establish if gene expression was altered in the presence of the oxidising agent chlorine dioxide. These experiments were only conducted on the *B. subtilis* reference strain and WD isolate. Bacterial samples used for RT-PCR experiments were pre-treated with chlorine dioxide for 1 min (using suspension test method, Chapter 4 section 4.2.3.3) prior to RNA extraction. Results are presented in figure 5.18 and 5.19. All genes investigated remained expressed after exposure. There was no evidence that the house-keeping gene *gyr*B was up-regulated following exposure to the oxidising agent. The gene *kat*A seems to be up-regulated in the environmental isolate but not in the reference strain following oxidiser treatment. The gene *sod*A did not seem to be up-regulated in the environmental isolate, but a brighter band was observed with the reference strain, possibly indicating some degree of up-regulation. It has to be noted that gene expression was measured qualitatively by observing the brightness of the band signal, and not truly quantitatively. Further experiments would therefore have to be carried out using real-time quantitative PCR (q-PCR) in order to establish the extent of any up- or down- regulation of specific genes.

Figure 5.18 RT-PCR results for *B. subtilis* for the gene catalase. 48 is the WD isolate and 6051 is the ATCC reference strain. *kat*A is catalase and *gyr*B is the control gene for *B. subtilis*. RT is in reference to the sample containing the AMV-Reverse Transcriptase enzyme. Control is without chlorine dioxide pre-treatment, test is with chlorine dioxide pre-treatment.



1 Lane	Marker
2	*gyr*B RT 48 Control
3	*gyr*B No RT 48 control
4	*gyr*B RT 48 test
5	*gyr*B No RT test
6	*gyr*B RT 6051 control
7	*gyr*B No RT 6051 control
8	*gyr*B RT 6051 test
9	*gyr*B No RT test
10	Water control
11	Marker
12	*kat*A RT 48 control
13	*kat*A No RT 48 control
14	*kat*A RT 48 test
15	*kat*A No RT 48 test
16	*kat*A RT 6051 control
17	*kat*A No RT 6051 control
18	*kat*A RT 6051 test
19	*kat*A N No RT 6051 test
20	Water control

Figure 5.19 RT-PCR results *B. subtilis* for the gene superoxide dismutase. 48 is the WD isolate and 6051 is the ATCC reference strain. *sod*A is the gene for superoxide dismutase and *gyr*B is the control gene for *B. subtilis*. RT is in reference to the sample containing the AMV-Reverse Transcriptase enzyme.



1 lane	Marker
2	*gyr*B RT 48 Control
3	*gyr*B No RT 48 control
4	*gyr*B RT 48 test
5	*gyr*B No RT test
6	*gyr*B RT 6051 control
7	*gyr*B No RT 6051 control
8	*gyr*B RT 6051 test
9	*gyr*B No RT test
10	Water control
11	Marker

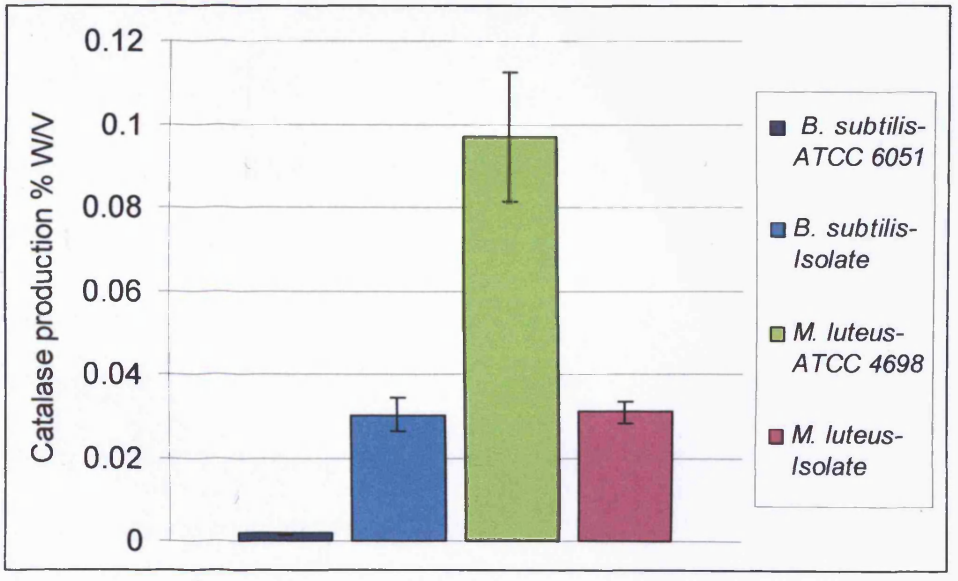
12	*sod*A RT 48 Control
13	*sod*A No RT 48 Control
14	*sod*A RT 48 Test
15	*sod*A No RT 48 Test
16	*sod*A RT 6051 Control
17	*sod*A No RT 6051 Control
18	*sod*A RT 6051 Test
19	*sod*A No RT 6051 Test
20	*sod*A Water Control
21	Marker Hyper ladder VI

5.3.5 Enzyme assays

5.3.5.1 Catalase

Catalase activity was measured as this has been known to interfere with oxidising agents, mainly related to hydrogen peroxide treatment (Elkins *et al.*, 1999; Stewart *et al.*, 2000; McDonnell, 2007). The results of catalase production for the bacterial strains are shown in figure 5.20. It was observed that the WD isolate of *B. subtilis* produced significantly more catalase ($p < 0.05$) than its ATCC reference strain. However, with *M. luteus* strains, the ATCC reference strain produced significantly more catalase ($p < 0.05$) than the WD isolate, and produced the highest amount over all. Unfortunately, with the disc flotation method used here, it was not possible to test the production of catalase during exposure to oxidising agents.

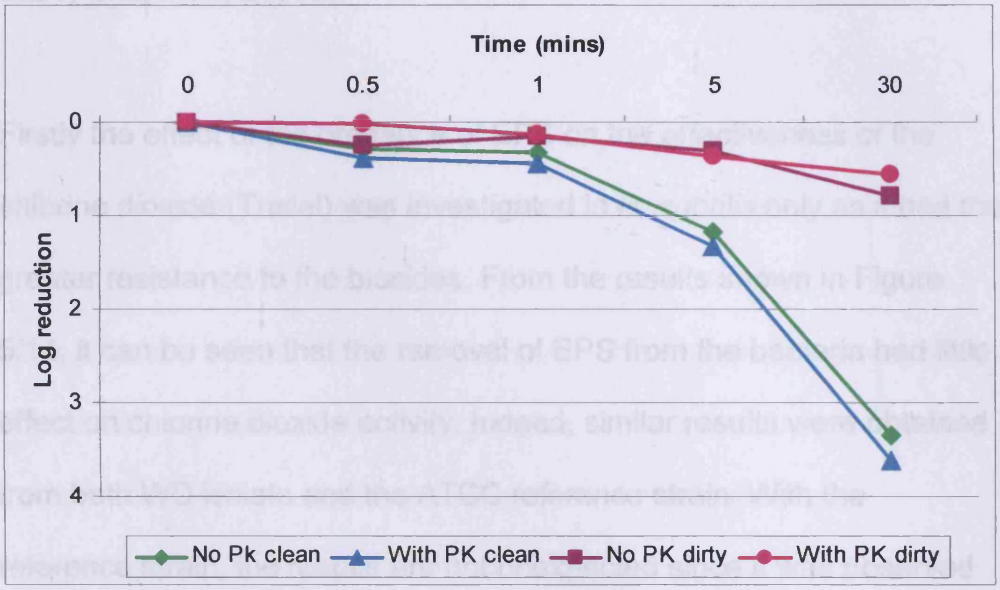
Figure 5.20 Production of catalase from WD isolates and reference strains.



5.3.5.2 Proteinase K

Proteinase K was used to digest any EPS and extracellular enzymes that may be produced by the bacterial cell which might have helped in the degradation of oxidising agents. It was established using the bioscreen which concentration of proteinase K did not affect the growth of *B. subtilis*, and this concentration was 200 µl/ml. *B. subtilis* WD isolates were treated with proteinase K for 2 hours prior to being used in the carrier test to establish the effect of chlorine dioxide in clean or dirty conditions. The results of this experiment are shown in Figure 5.21. There was no increase in bacterial inactivation by chlorine dioxide with or without pre-exposure to proteinase K. The contact time needed for a 4 $\log_{10}$ reduction in clean conditions was still 2 hours with pre-exposure to the proteinase K.

Figure 5.21 Carrier test results with and without treatment of proteinase K (PK) with *B. subtilis* WD isolate. Test was conducted in clean and dirty conditions.



5.4 Discussion

Scanning electron microscopy enabled visualisation of the external cell structure of the bacteria investigated and shaped the direction of investigation into the mechanisms of resistance to oxidising agents (Chapter 4).

SEM micrographs of WD isolate *B. subtilis* showed the potential for the presence of bacterial EPS. EPS has been shown to interfere with the effectiveness of biocides (Flemming and Wingender, 2001) by preventing the biocide from gaining contact with the bacteria cell target (the glycocalyx preventing penetration) (Chen and Stewart, 1996), by increasing clumping and thus protecting cells within the aggregate or by

decreasing the concentration of biocide, notably through the production of extracellular detoxifying enzymes (Heinzel, 1998; Stewart *et al.*, 2000; Chapman, 2003a).

Firstly the effect of the presence of EPS on the effectiveness of the chlorine dioxide (Tristel) was investigated in *B. subtilis* only as it had the greater resistance to the biocides. From the results shown in Figure 5.14, it can be seen that the removal of EPS from the bacteria had little effect on chlorine dioxide activity. Indeed, similar results were obtained from both WD isolate and the ATCC reference strain. With the reference strain, the results are not unexpected since it was observed that the reference strain had little EPS (Figure 5.1). With the WD isolate, it was difficult to quantify the amount of EPS removed prior to exposure to the oxidiser.

It is known that sonication can increase the effectiveness of biocides by breaking up bacterial clumps (Duckhouse, 2004). This allows a greater surface to be presented to the disinfectant. In addition, the bacterial cell wall might be weakened (Duckhouse, 2004). Breaking potential clumps to increase efficacy was investigated using a sonicator at low frequencies. It is known that sonication has two effects on *Bacillus subtilis*: the first is the breaking up of bacterial clumps (this is generally found to happen at low power sonication 20-40 kHz), and the second is that bacterial killing increases (at high frequencies of 850 kHz, bacterial

kill rate predominates) (Joyce *et al.,* 2003a). The effect of bacteria declumping from sonication occurs in the first 10 minutes of sonication, and after this bacterial kill becomes increasingly apparent (Joyce *et al.*, 2003b).

The result from the use of sonication prior to biocide treatment showed no effect on increasing the number of cfu (i.e. no effect on de-clumping), nor on increasing the activity of chlorine dioxide (Figures 5.20 and 5.21). Such results could be explained by a number of factors, such as ineffective frequency used (20 kHz) or time period of sonication. Perhaps longer times should have been used or the use of sonication throughout the contact with the biocide. In this case, other factors such as heat generated could have had an effect on the efficacy of the biocide. However, it is known that constant sonication allows the generation of heat in suspension, temperatures increasing form 27-44°C over a period of 120 s (Salleh-Mack and Roberts, 2007). The increase in temperature readily increases the inactivation of bacteria (Salleh-Mack and Roberts, 2007). Further investigations into the use of ultrasound in conjunction with disinfectants would be useful, as this has been shown to be effective at increasing the efficacy of hypochlorite (Duckhouse *et al.*, 2004) and in combination with UV treatment of waste water (Blume and Neis, 2004).

The presence of genes encoding for detoxifying enzymes that degrade oxidising agents was then investigated by PCR. The presence of two specific genes encoding for catalase and superoxide dismutase was investigated in particular. Catalase and superoxide dismutase have been shown to play a part in the regulation of oxidative stress (Brioukhanov *et al.*, 2006). Results clearly indicated that catalase and superoxide dismutase genes were present in *B. subtilis* and catalase in *M. luteus*. RT-PCR results highlighted that these genes were expressed in both organisms and that exposure to chlorine dioxide might result in an overexpression of these enzymes, although this cannot be categorically determined without further quantitative methods such as qPCR (Huet *et al.*, 2008; Drevinek *et al.,* 2008).

Catalase activity in both micro-organisms was then investigated. The production of catalase is well known in the involvement in detoxifying oxidising agents (MacDonnell 2007). The production of catalase has been shown to increase the survival of bacteria in the presence of hydrogen peroxide (Engelmann and Hecker, 1996; Rochat *et al.,* 2005), and affect the penetration of hydrogen peroxide into bacterial biofilm (Stewart *et al.,* 2000). Firstly, the investigation looked at how much catalase was being produced from the WD isolates and ATCC reference strains. The results indicated that the most catalase produced was by the *M. luteus* ATCC reference strain (Figure 5.20). However, results from the suspension and carrier test (Chapter 4; Section 4.3.2-

4.3.3) indicate that this bacterium was killed within the 30 s contact time. Hence, the production of larger amounts of catalase might not be a mechanism used by the cell to survive exposure to the oxidising agents. The WD isolates *B. subtilis* and *M. luteus* both produced relatively similar catalase concentrations, whereas the *B. subtilis* ATCC reference strain produced the lowest amount.

It has been previously shown by others that proteinase K could disrupt biofilm formation through interfering and destroying the EPS produced by bacteria (Patterson *et al.*, 2007; Boles *et al.,* 2008). Proteinase K was used to degrade any such enzymes present. From the results in Figure 5.21, there was no significant difference in the effectiveness of the biocide following bacterial pre-exposure to proteinase K. This enzyme was shown not to damage the cell at high concentration (200 µg/ml) and it is possible that proteinase K was not specific enough to degrade such extracellular enzymes. Likewise, contact times or concentration of proteinase K may not have been sufficient to affect extracellular enzymes.

5.4.1 Summary

Investigations into resistance mechanisms of *B. subtilis* towards chlorine dioxide have shown that the large quantities of extracellular polysaccharides may not be involved in interfering with biocide activity.

Genes for catalase and superoxide dismutase were present in *B. subtilis* and enzyme activity varied between WD isolates and reference strains, indicating a potential involvement in resistance mechanisms. However, any up-regulation of detoxifying genes in the presence of chlorine dioxide remains unclear. The involvement of bacterial clumping preventing the biocide activity also remains unclear; this would be an area for further work looking at the effect of combining sonication and biocide agents. It has been shown that mechanisms conferring resistance are complex, but might not be linked to impaired biocide penetration.

Chapter 6:

General Discussion

6 General Discussion and Conclusions

6.1 General Discussion

6.1.1 Contamination of Endoscope WDs

Since the first clinical use of endoscopes 48 years ago (Hirschowitz, 1961), the benefits endoscopy have given modern health care services have been well established. The use of endoscopes is covered in an array of procedures which can identify and prevent diseases, including diagnosis of intestinal tumours (Kawaguchi *et al.*, 1997), investigations in biliary and pancreatic diseases (Cotton and Williams 1996) and diagnosis of gastric cancer (Yanai *et al.,* 1999).

However, it is also recognised that the cleaning and disinfection of endoscopes is always at the forefront of media interest, rather than the benefits which the procedures of endoscopy offer. Most recently in the headlines was an incident at the University Hospital of Leicester involving a mechanical error of a washer disinfector (Metro, Saturday, June 28, 2008), although the risk of infection from this incident was deemed low by the Health Protection Agency (HPA).

Fortunately the transmission of infections via endoscopes is unlikely, due to the rigorous guidelines in place on the decontamination of endoscopes. Nevertheless the potential remains for an outbreak when these guidelines are not adhered to. There have been various instances

throughout the years where a breakdown in decontamination procedures has led to the transmission of infections to patients (Gamble *et al.*, 2007).

In this thesis, the identification of microorganisms from washer disinfectors has highlighted that in some instances bacteria can survive the decontamination procedure for endoscopes (Martin *et al.*, 2008). These microorganisms can potentially be transferred to other endoscopes processed in the equipment. This could, of course, ultimately lead to the infection being transferred to a patient who has undergone an endoscopy procedure with a contaminated endoscope. The risk to the patient then depends on the species of bacteria that survive the decontamination procedures and the type of procedure being conducted.

Here, a number of bacterial species were isolated, and one of particular epidemiological interest was the survival of *Micrococcus luteus*. This microorganism was isolated from the same washer disinfector on two separate occasions in an eight-month period, and has been isolated by others (Bisset *et al.*, 2006). It was not established if the two were identical however the use of bacterial DNA fingerprinting (RAPD technique) could have established this (Cheeseman *et al.*, 2007). *Micrococcus luteus* is a commensal organism however it is also an opportunistic pathogen which can cause septic shock, pneumonia, and

urinary tract infections particularly in an immunocompromised individual (Miller, 2007).

It was not established in this thesis if there were any instances of *Micrococcus luteus* infection following endoscopy procedures performed during the times that the bacteria were isolated from the endoscope washer disinfector. This could be because these may not be linked to the procedure which had taken place, as healthcare staff and patients might consider that decontamination had been carried out successfully. The other microorganism which was isolated and found to be highly resistant to the biocides tested was *Bacillus subtilis*. It is often referred to as a non-pathogenic bacterium and few infections have been attributed to this bacterium (Sietske de Boer and Diderichsen, 1991).

There are guidelines for procedures to establish whether contamination is occurring in the process of endoscopy disinfection and tests should be carried out on a weekly, monthly and yearly basis. The HTM 2030 (1997) gives guidance in the validation and verification of endoscopy washer disinfectors to ensure equipment is working safely and effectively, and detailing what microbiological testing should be done. However, these tests have in the past been forgotten at some endoscopy units within the NHS Trusts in England (Gamble *et al.*, 2007). It should be noted that any survey data from testing in Wales was not available. If instances are occurring where the correct

procedures for decontamination and validation of decontamination procedures are being left to chance, then undoubtedly there will come a time when an incident associated with endoscope decontamination failure will have drastic consequences and the risk of transmission of infection to patients will be deemed more than low by the HPA (Weber and Rutala, 2001; Nelson, 2003).

Table 6.1 summarises the bacteria isolated from the washer disinfectors in this study, and compares with organisms isolated from patient ready endoscopes (Bisset *et al.*, 2006) and those which have been involved in outbreaks associated with endoscope decontamination failures (Nelson, 2003; Antonucci *et al.,* 2008). This study primarily isolated Gram-positive microorganisms, compared with the study by Bisset *et al.* (2005) and the organisms which have been involved in infection outbreaks (Nelson, 2003; Antonucci *et al.,* 2008). In these instances, it was mostly Gram-negative bacteria which were isolated. This could be linked to Gram-negative bacteria being regarded has being more resistant to biocides than Gram-positive ones. The natural bioburden of gastrointestinal endoscopes after the cleaning process has found to be primarily Gram-positive bacteria (Chu *et al.*, 1998). This may account for the type of bacteria being isolated from the WDs (especially if there were breakdowns in the disinfection protocol), as it was gastrointestinal endoscopes being disinfected on the days of sampling.

Table 6.1 Summary of bacteria isolated from this study and others.

Bacteria isolates	Endoscopy Units		Caused Outbreaks	
	This study	Bisset *et al.*, 2006	Antonucci *et al.*, 2008	Nelson, 2003
Micrococcus luteus	✓	✓		
Bacillus subtilis	✓	✓ (*Bacillus* spp.)		
Bacillus licheniformis	✓			
Brevibacillus brevis	✓			
Streptococcus sanguis	✓			
Streptococcus mutans	✓			
Streptococcus gordonii	✓			
Staphylococcus intermedius	✓			
Staphylococcus epidermidis		✓		
Staphylococcus saprophyticus		✓		
Staphyloccocus aureus		✓		
Enterococcus spp.		✓		
Enterococcus cloacae		✓		
Gardnerella vaginals	✓			
Leifsonia aquaticum	✓			
Pseudomonas aeruginosa		✓	✓	✓
Pseudomonas fluorescens		✓	✓	
Proteus mirabilis			✓	
Proteus vulgaris			✓	
Klebsiella pneumoniae			✓	✓
Enterobacter aerogenes		✓		
Escherichia coli		✓		
Serratia marcescens		✓		✓
Salmonella typhi				✓
Helicobacter pylori				✓
Mycobacterium tuberculosis			✓	
Mycobacterium chelonae			✓	

Thankfully the bacteria isolated on this occasion are not routinely associated with infection transmission. The isolation of the slow growing mycobacteria was not established in this study due to the short incubation times used, following sampling of the WDs.

6.1.2 Efficacy of oxidising agents

Oxidising agents are used routinely endoscopy disinfection, and are found to be highly effective in the killing of microbial populations present in endoscopes and washer disinfectors (Coates, 2001; Vizcaino *et al.*, 2003; Isomoto *et al.*, 2006; Sattar *et al.*, 2006).

In this thesis, bacterial isolates sampled from endoscope washer disinfectors were shown to be less susceptible to oxidising agents than ATCC reference strains (Martin *et al.*, 2008). Different strains of a bacterial species commonly have varying degrees of susceptibility to biocidal agents and antibiotics. This has also been shown in the susceptibilities of *P. aeruginosa* clinical isolates, which were resilient to antibiotics being tested compared to reference strains being used (Lambert *et al.,* 2001). The antimicrobial susceptibilities between MRSA, MSSA strains and reference strains are also a classic example (Hammond *et al.*, 1987; Suller and Russell, 1999). The importance of challenging biocides with the relevant microorganisms is fundamental, as it can be shown that a biocidal agent is effective at killing bacteria in an efficacy test, but when the disinfectant is used in practice the

organisms may not be as susceptible as the reference strains used(Gebel *et al.*, 2002).

The susceptibility testing of the WD isolates and reference strains was carried out with suspension, carrier and sporicidal tests (*B. subtilis* only). These tests were chosen as they measure different levels of disinfectant efficacy. The suspension test is a test used to establish basic bactericidal activity at a range of contact times. Table 6.2 gives a summary of the efficacy of chlorine dioxide in the suspension test compared with other studies in clean conditions. It has been shown in other studies that shorter contact times at lower concentrations are needed for a bactericidal effect (Coates, 2001; Isomoto et al., 2006).

Table 6.2 Summary results from suspension in clean conditions.

	Time (min) to achieve a 10^5–fold reduction (suspension test) BSA 0.3 g/L				
	This study	Coates, 2001		Isomoto *et al.*, 2006	
Concentration (ppm)	310	97	146	600	30
Bacteria					
B. subtilis ATCC 6051	**1**	N/C	N/C	N/C	N/C
B. subtilis WD isolate	**30**	N/C	N/C	N/C	N/C
B. subtilis ATCC 6633	N/C	N/C	N/C	**5**	**5**
B. subtilis NCTC 10073	N/C	**2.5**	**2.5**	N/C	N/C

N/C- not conducted.

This highlights that the use of different reference strains can establish various levels of biocide efficacy, thus the use of a variety of reference strains and/or wild-type isolates should be fundamental in establishing the efficacy of disinfectants being used in a clinical environment.

Table 6.3 shows the variability of the suspension test when conducted under dirty conditions. However, one study showed that the presence of organic matter did not interfere with the biocide efficacy (Coates, 2001), once again highlighting the various reactions of bacteria to biocide depending on bacterial type and environment which experiments are conducted within.

Table 6.3 Summary of suspension test in dirty conditions

	Time (min) to achieve a 10^5–fold reduction (suspension test) BSA 3 g/L		
	This study	Coates, 2001	
Concentration (ppm)	310	97	146
Bacteria			
B. subtilis ATCC 6051	**30**	N/C	N/C
B. subtilis WD isolate	**>60**	N/C	N/C
B. subtilis NCTC 10073	N/C	**2.5**	**2.5**

N/C- not conducted.

The carrier tests are more stringent and more closely represents in-use conditions since the efficacy of the disinfectants is being tested on

surfaces; microorganisms attached to surfaces may have different biocide susceptibility levels (Gibson et al., 1995). The sporicidal tests are used to measure specifically spore inactivation when attached to a surface. It is essential to use an appropriate test when investigating biocide efficacy, as this allows testing to be done under realistic terms which will mimic the circumstances that the biocide will be under when in-use. The efficacy of disinfectants should be established using published protocols by the national standards. The disinfectant standards are classified into phase 1, phase 2 and phase 3 tests, with phase 2 tests divided into step 1 and step 2. Phase 1 tests use suspension tests to look for basic antimicrobial activity of active agents whereas phase 2 tests are designed to mimic practical conditions for finished products. Phase 2, step 1 suspension tests use a wider range of organisms and phase 2, step 2 tests simulate the practical conditions under which the disinfectant will be used. Phase 3 tests are field tests under practical conditions although the methodology for these tests has not yet been established (Fraise, 2008).

Biocidal agents that are to be used in endoscope disinfection should go through various testing methods to establish if they are all of the following; bactericidal, sporicidal, viricidal, tuberculocidal, mycobactericidal, fungicidal and yeasticidal (Wendt and Kampf, 2008). These should also include testing the biocidal agents under various soiling conditions. It was shown in this thesis that the addition of organic

matter (BSA) changed the outcome of bactericidal action, resulting in the bacteria being able to withstand longer contact time with the biocides, especially when exposed to chlorine dioxide. The effect of soiling has been shown to interfere in the disinfection with various biocides (Russell, 1992; Lambert and Johnston, 2001; Isomoto *et al.*, 2006).

Realistic interfering substance should be used when determining the integrity of biocidal agents used within endoscopy disinfection. In the healthcare setting, various interfering substances will be present on the surfaces to be disinfected (Bloß and Kampf, 2004; Sattar *et al.*, 2006). As table 6.3 shows, increased contact times were needed within this study to achieve a bactericidal effect in the presence of organic matter compared to clean conditions (see Table 6.2). It has been suggested by the HTM 2030 (1997) that the test soil should contain water, glycerol, horse serum, dehydrated hog mucin, plain flour and safranine solution, when investigating the cleaning efficacy of washer disinfectors. When testing disinfection efficacy, the use of organic material (serum or blood) and/or inorganic material (mineral salts causing water hardness) is recommended (HTM 2030, 1997). When investigation the testing of chemical washer disinfectors including automatic endoscope re-processors the EN15883 use be used.

It is important to use a variety of microorganisms and endoscopes when testing the efficacy of washer disinfectors, including organisms that are nosocomial pathogens and bacterial isolates which have greater resistance to disinfection, for example the use of glutaraldehyde-resistant mycobacteria (Sattar *et al.,* 2006). Tables 6.2 and 6.3 show there is a difference between the efficacy of chlorine dioxide when tested with different reference strains and the WD isolate. These are not different species however; there is a difference in biocide efficacy. This highlights that even bacterial within the same species will react differently to biocides. So when testing biocide efficacy a wide range of microorganisms should be used to include reference strains and WD isolates. Investigating the disinfection of various types of endoscopes within a disinfection system is also important due to the wide range and differences in complexity between endoscopes. This would ensure that the disinfection equipment can be implemented in units with different endoscopes without affecting the outcome of disinfection (Sattar *et al.,* 2006).

6.1.3 Mechanisms of bacterial resistance to oxidising agents

It has been established and reported in various reviews on endoscope decontamination that incidents occur through the inappropriate use of disinfectants, by not preparing the disinfectant precisely as instructed, either by adding the wrong volume of water, or not using the correct

solutions of the disinfectants (Nelson, 2003; Gamble *et al.*, 2007). The disinfectant is then at a concentration which will not kill all microorganisms present after endoscopy procedures (Griffiths *et al.* 1997). If these microorganisms are left to establish a biofilm within the equipment, decontamination failure will become a regular occurrence.

Low concentrations of a disinfectant can give the microorganism a chance to become accustomed to these concentrations and become resistant to in-use concentrations (Griffiths *et al.,* 1997). This has been demonstrated by Thomas *et al.* (2000) when investigating the influence of residual concentrations of chlorhexidine on the bacterium *P. aeruginosa*. It was shown that *Ps. aeruginosa* had an increase in MIC towards chlorhexidine after prior exposures at a low concentration of the biocide; however this increased MIC was still lower than that for the in-use concentration (Thomas *et al.*, 2000).

The production of spores by *B. subtilis* is a well-known mechanism of survival against biocides (McDonnell, 2007). Formation of spores within a WD water system is important as it will allow the bacteria to survive for extended periods of time (Marrow *et al.*, 2008). However, in this study the production of spores by *B. subtilis* was not the reason behind the higher contact times needed for the biocides to achieve biocidal activity. Table 6.4 summarises the various resistance mechanisms to oxidising agents present within this study, and compares with other

studies which have been conducted in the past. Reasons for the resistance of the WD isolate of *B. subtilis* were considered to be due to various mechanisms. It was at first thought that the presence of high levels of EPS was the reason behind the elevated contact time of the biocides against this particular isolate (Martin *et al.*, 2008). EPS has been shown to interfere with the effectiveness of biocides (Stewart and Chen, 1998; Flemming and Wingender 2001). EPS can provide a defence against oxidising agents that can be consumed by the reaction with EPS (Wingender *et al.,* 1999). This has also been demonstrated with limited diffusion of chlorine into artificial biofilms, where it was speculated that chlorine was neutralised by the organic constituents of the biofilm (Chen and Stewart, 1996).

However, in this study there were no differences in the susceptibility patterns to chlorine dioxide when EPS was removed (Chapter 5, Section 5.3.2). This would not have explained the resistance in the reference strain either, as there was no EPS present in the electron micrographs taken of this particular bacterial sample (Chapter 5, Section 5.3.1). The methods used to remove the bacterial EPS could be criticised as it was not established if the method had removed the EPS. This method was down to physical removal whereas chemical removal would have been more efficient. However, the bacteria may have the ability to switch on and off the production of EPS, like *Ps. aeruginosa*, which activates EPS production at high cell density (Davies et al.,

1998). Varying expression of bacterial EPS has also been shown in other bacteria, for example *Vibrio cholerae* (Nadell *et al.*, 2008).

A brief investigation was carried out using proteinase K to disrupt any bacterial EPS, and potentially any enzymes that the bacteria could be producing which may reduce the activity of the oxidising agent; this did not increase the efficacy of chlorine dioxide. It had been previously shown by others that proteinase K could disrupt biofilm formation through interfering and destroying the EPS produced by bacteria (Patterson *et al.*, 2007; Boles *et al.*, 2008)

The next step was to look at the production of enzymes which may detoxify the biocide (Heinzel, 1998; Stewart *et al.*, 2000; Chapman, 2003a). Catalase and superoxide dismutase were investigated and it was shown by PCR that genes for these enzymes were present in the bacterial isolates (Chapter 5, section 5.34). However, it could not be fully determined by RT-PCR whether the genes for these enzymes were up-regulated in the presence of chlorine dioxide. Further investigations with q-PCR would be required to establish this.

Table 6.4 Mechanisms of resistance to oxidising agents, this study and others, looking at *B. subtilis*.

Resistance mechanism	This study	Elkins 1999	Engelmann *et al.*, 1996	Stewart *et al.*, 1998	Wingender *et al.*, 1999
EPS	x*				✓
Catalase	✓	✓	✓		
SOD	✓				
Biofilm		✓		✓	

*x: no evidence that EPS was involved; SOD: superoxide dismutase

It may be possible that there was cross-resistance occurring between the oxidising agents tested. In this study, the bacterial isolates had been sampled from washer disinfectors that used the high-level disinfectant chlorine dioxide, whilst it was indicated that this biocide may not have been used at the appropriate in-use concentration. This could have led to the failure of the oxidising agents to inactivate the microorganisms at longer contact times, but also could be the reason that one of the bacteria isolated (*B. subtilis*) was also resistant to hydrogen peroxide (Chapter 4; section 4.3.3). It is possible that this is due to the similarities of the two biocides to interact with the bacterial cell, disruption of cell walls and membranes, which concludes with cell death. Changes in the bacterial cell wall could ultimately affect the efficacy of the biocide along with associated bacterial EPS interacting with the biocide, which can lead to reduced activity at lower biocide concentration. All this can aid bacterial survival.

It has been discussed frequently within the literature that there is the potential for cross-resistance to occur between biocides and antibiotics (Russell *et al.*, 1999; Russell, 2000;; Levy, 2000; Ng *et al.*, 2001; Fraise, 2002; Russell, 2003). Cross-resistance between biocides and antibiotics in a hospital environment is of great concern and should be meticulously investigated as a priority.

6.2 Conclusions

The hypothesis of this thesis was that (i) microbial contaminants would be found in WDs; (ii) the contaminants would be insusceptible to high-level disinfectants and (iii) they would possess a variety of resistance mechanisms. Parts (i) and (ii) of the hypothesis were found to be true, whilst at present there is insufficient data to determine whether part (iii) is true or false. In summary, this investigation has highlighted the presence of bacterial isolates within endoscope washer disinfectors and their ability to survive high-level disinfection with oxidising agents. Constant monitoring of microorganisms in washer disinfectors is essential as it is apparent in the literature that infectious outbreaks can happen when there is a breakdown in decontamination protocols. A further understanding of the bacteria present within these systems could allow the introduction of measures to minimise the risk of infectious outbreaks and increase the knowledge of how particular microorganisms survive these harsh environments.

The use of surveillance programmes should be initiated taking into account the issues raised by Cookson (2005). These should include looking at the monitoring of pathogens within the hospital setting, then testing microorganisms that are isolated against biocides being used, looking for the potential of cross-resistance. It is also important to identify the resistance mechanism so if need be changes can be made to disinfection strategies to solve the problems with re-occurring pathogens. Also, the education of staff should be taken into account within areas where disinfection occurs, so they know exactly the procedures needed to get disinfection done effectively (Cookson, 2005). It may be essential to have regular audits of the process within hospital disinfection to ensure guidelines are being followed.

6.3 Future Work

Even though the possibility for passing exogenous infections via endoscopes is regarded as low by the American Society for Gastrointestinal Endoscopy (ASGE Standards of Practice Committee, 2008) and the British Society for Gastroenterology (The Report of a Working Party of the British Society of Gastroenterology Endoscopy Committee, 2008), it is clear from this investigation that microorganisms can survive the disinfection process. It would be of further interest to continue to investigate bacterial presence in the endoscope washer disinfectors and on the endoscopes. If there are contaminates, then the susceptibility levels of the microorganism towards the disinfectants

being used should be tested. Mechanisms of resistance of the bacteria should be investigated further since this study did not identify prominent mechanisms involved in bacterial survival following exposure to high-level disinfectants. Quantitative-PCR should be done to ultimately establish whether there is up-regulation of genes which encode for the enzymes catalase and SOD in the presence of the biocides (Brioukhanov *et al.*, 2006).

Investigations are suggested into the way that bacteria use repair mechanisms to survive a biocide attack (Chang *et al.*, 2005). It would be of interest to use DNA microarray analysis of antimicrobial resistance genes and any other genes up- or down-regulated, producing responses by the bacteria in the presence of each of the individual biocides (Frye *et al.*, 2006; Chang *et al.*, 2006; Allen *et al.*, 2006). This may also show instances where the potential of cross-resistance may occur if the bacteria respond similarly to each biocide. DNA microarray analysis has showed significant increases in mRNA levels of catalase in *P. aeruginosa* in the presence of hydrogen peroxide (Chang *et al.*, 2005).

Future studies might leave behind the investigations of *B. subtilis* resistance and look instead at the survival of the bacterium *M. luteus,* which is of more epidemiological value in the hospital setting. This particular isolate of *M. luteus* was shown to be oversized in comparison

under SEM with the culture collection reference strain. However, the TEM images showed WD isolate to be approximately the same size (see Chapter 5, Figure 5.4) as the reference strain and the outer cell structure to be the same. This indicates the large size observed in SEM might be due to the presence of EPS. The outer cell wall structure is of importance, since in MRSA the thickness of the cell wall compared to MSSA is deemed to be responsible in part for the lower susceptibility to antimicrobials (Raju *et al.,* 2007). It would therefore be of interest to investigate the presence of a bacterial capsule and EPS in this bacterium, and how this may play a part in resistance to chlorine dioxide.

As the biocides are thought to interact directly with the bacterial cell, it would be beneficial to investigate these interactions to determine how this may play a part in resistance to the biocides (Liaqat and Sabri, 2008). As biocides have to cross the cell wall, manipulation on this area can either enhance or decrease the activity of the biocides. It would also be of interest to look into the interaction of these biocides with the WD isolates grown as a biofilm. Biofilm growth would represent the association of environmental isolates with surfaces mimicking bacterial survival on the inner channels of endoscopes and inside the inner workings of endoscope disinfection equipment (Stewart *et al.,* 2000; Tachikawa *et al.*, 2005; Smith and Hunter, 2008).

Chapter 7:

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7 References

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Appendix I:

Publications relevant to this thesis

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Resistance and cross-resistance to oxidising agents of bacterial isolates from endoscope washer disinfectors

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Summary Bacteria isolated from washer disinfectors using chlorine dioxide as a high-level disinfectant were exposed to peracetic acid, chlorine dioxide and hydrogen peroxide to investigate their susceptibility and possible bacterial cross-resistance to these highly reactive oxidising biocides. A standard suspension test was used to establish a rate of kill of these biocides against two stable isolates (*Bacillus subtilis* and *Micrococcus luteus*). Suspension tests demonstrated that 'in use' concentrations were not always effective to provide the required disinfection efficacy within recommended exposure times and in some instances a 60 min exposure was necessary to achieve a reduction in number by a factor of 10^5. It appears that vegetative Gram-positive isolates can become resistant to oxidising agents in vitro, and that cross-resistance to related compounds can occur. Since these bacteria are deemed to be susceptible to highly reactive biocides, there should be further study of the resistance mechanisms in these isolates to explain their survival.

Introduction

Of all the medical devices used, flexible endoscopes are the most likely to be linked to the transmission of hospital-acquired infections.[1] These appliances are used for investigative and

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therapeutic purposes, being regarded as semi-critical devices. They should be thoroughly decontaminated between clinical uses, including rigorous cleaning and, at a minimum, high-level disinfection.[2,3] Due to their intolerance of high temperatures, high-level chemical disinfectants are used. High-level disinfection of endoscopes in the UK usually involves the use of washer disinfectors (WDs), which can reduce the potential risk of disinfectant exposure to endoscopy staff and ensure reliable disinfection efficacy.[4] However, WDs can themselves be the source of microbial contaminants resulting in cross-infection in some instances.[5–8] The sources of contamination can be varied and include inadequately filtered rinse water, biofilm development, blocked internal device channels or malfunction of the machinery.[9] Endoscope reprocessing has changed over the years but it still remains a particular challenge to consistently remove microbial contamination, since endoscopes are complex and highly sensitive devices.[1] At present there are no general guidelines that recommend checking the contamination levels in endoscopes between patients. However, guidelines (such as HTM 2030 in the UK) and standards (EN ISO 15883-1) do offer information on the maintenance and stringent efficacy testing with the use of WDs.[10,11] Unfortunately these testing regimens are not always adhered to or applied.[12] This study aimed to isolate microbial contaminants from WDs used for the reprocessing of endoscopes and to assess their susceptibility to commonly used oxidising agent-based high-level disinfectants.

Methods

Sampling

Samples were taken from two identical washer disinfectors (Autoscope Guardian, Labcare, Clevedon, UK) in an endoscopy unit within the UK. The WDs used a chlorine dioxide (0.01%) formulation. Swabs (Technical Service Consultants Limited, Heywood, UK) were used to sample several areas of the WDs, including the drain, between the connector tubes and the final rinse water. These areas have been described as problematic in the literature.[5,6,9] All swabbed samples were transported in charcoal and stored at 4 °C until processing. Samples were inoculated onto tryptone soya agar (TSA) (Oxoid Ltd, Basingstoke, UK) and incubated at 30 °C and 37 °C for 24 h. The WDs were repeatedly sampled on three different occasions.

Identification of WD contaminants

Any isolates identified were subcultured to ensure purity and investigated for microbial identification. The Becton Dickinson BBL crystal identification system (Becton Dickinson, Franklin Lakes, MD, USA) was used to identify the bacterial contaminants after preliminary microbial identification methods (Gram stain, spore stain, oxidase and catalase test) were performed. Results of laboratory identification of the isolates were then verified with 16S rDNA sequence analysis (NCIMB Ltd, Aberdeen, UK).

Investigation of spore formation

In order to establish whether or not spores of *Bacillus subtilis* isolates were present in the prepared suspensions, random bacterial suspensions were boiled for 10 min. Bacterial suspensions were prepared from agar slope according to the BS EN 1276 (European Standard).[13] Bacterial numbers (cfu/mL) were checked before and after boiling. In addition, a spore stain (Malachite Green) was used to screen the presence of spores in all cultures used within the experiments. A 24 h smear of a washed bacterial culture was fixed to a glass slide. The slides were then flooded with Malachite Green (Pro-Lab Diagnostics, Neston, UK) and then placed on a hot plate, steamed for 2–3 min. The slides were then removed, cooled and washed with water. They were counterstained with safranin (Pro-Lab Diagnostics) for 30 s, washed with water and dried. Slides were observed under the microscope; 20 fields containing on average 10 cells were observed on each occasion.

Disinfection studies

Bacterial suspensions were prepared from agar slope according to the BS EN 1276 (European Standard).[13] Three high-level disinfectants were used: 7.5% hydrogen peroxide, 2.5% peracetic acid and 0.03% chlorine dioxide formulations. A higher concentration of chlorine dioxide was subsequently investigated as isolates were found to be insensitive to the recommended in-use concentration. Disinfectant efficacy at 0.5, 1, 5, 30 and 60 min exposure at room temperature (~20 °C) was tested with a standard suspension test method.[13] When reduction by a factor of 10^5 was achieved, no further contact time was investigated. The neutralising agent used for all three biocides was 5 g/L of sodium thiosulphate (Fisher Scientific, Loughborough, UK).

One mL of a 1–5 × 10^8 cfu/mL washed bacterial suspension (*B. subtilis* and *Micrococcus luteus* WD

isolates and their standard counterpart strains *B. subtilis* American Type Culture Collection (ATCC) 6051 and *M. luteus* ATCC 4698, and *Staphylococcus intermedius*, *Streptococcus mutans* and *Streptococcus sanguis*) in tryptone sodium chloride [TSC, tryptone (1 g/L; Oxoid), sodium chloride (8.5 g/L; Fisher Scientific)] was added to 1 mL of an organic load (bovine serum albumin; Acros Organic, Fair Lawn, NJ, USA) at 3 g/L for 'dirty' conditions or 0.3 g/L for 'clean' conditions and 8 mL of biocide. After the appropriate contact time, 1 mL was removed and added to 9 mL of the neutraliser. Serial dilution was performed in TSC and 3 × 10 µL drops were placed on to TSA and incubated for 24 h at 37 °C. Colonies were then counted and the reduction factor in viable bacteria calculated. Controls consisted of 1 mL of a washed bacterial suspension added to 1 mL of organic load which was then added to 8 mL of sterilised water. In addition, a neutraliser efficacy test and toxicity tests were performed.[13]

Scanning electron microscopy

An aliquot (20 µL) of the isolate suspension (*B. subtilis* and *M. luteus* WD isolates and their standard counterpart strains ATCC 6051 and ATCC 4698 respectively) was attached to a 0.2 µm membrane filter and fixed by 2.5% glutaraldehyde in 0.1 M of sodium cacodylate buffer for 1 h. Samples were then washed in 0.1 M of cacodylate buffer twice for 5 min. Postfixation was then achieved by adding 1% osmium tetroxide in 0.1 M of cacodylate buffer for 30 min. Samples were then washed three times for 5 min, dehydrated in ethanol and then dried in a critical-point drier (Samdri, Rockville, MD, USA). The filters were then removed and placed on a stainless steel SEM specimen carrier with double-sided tape, gold-coated with a sputter coater (EMscope, Ashford, UK). All steps were performed at room temperature. The SEM samples were viewed with an XL20 scanning electron microscope (Phillips, Eindhoven, The Netherlands).

Results

Sampling and identification

Sampling was repeated on three separate occasions (7–9 months apart). On the first and second visits, samples for positive growth were isolated at 30 and 37 °C on TSA. The third visit did not yield any isolates as it emerged subsequently that the disinfection regimen was modified. The bacterial samples isolated from WDs following chlorine dioxide disinfection after the first and/or second visit are shown in Table I. This study focused on two bacterial isolates in particular that showed remarkable resistance ability to oxidising agents. *M. luteus* was isolated in two consecutive occasions and *B. subtilis* during the first visit. Other bacteria were isolated (Table I) but they did not show any increased resistance to the oxidising agents used. Their identification was confirmed by 16S rDNA sequence analysis (data not shown). All isolates were subsequently used for disinfection efficacy studies although only the *B. subtilis* and *M. luteus* WD isolates and their relevant standard counterpart strains *B. subtilis* ATCC 6051 and *M. luteus* ATCC 4698 were studied further (EM investigation).

Disinfection studies

Control experiments showed that the neutraliser was non-toxic to the test bacteria and efficiently quenched the oxidising agents tested (data not shown). Results from the disinfection efficacy tests are summarised in Tables II–IV. When *B. subtilis* strains were tested (Table II), the WD isolates were often less susceptible than their standard control strains to the oxidising agents. Both *B. subtilis* strain samples used in the disinfection tests were confirmed not to contain any spores at the time of testing (data not shown). The *B. subtilis* (vegetative cells) WD isolate was highly resistant to twice the in-use concentration of chlorine dioxide for up to 60 min compared with 30 min for the standard ATCC 6051 strain in the presence of organic load (Table II). Hydrogen peroxide and peracetic acid were also less effective against the *B. subtilis* WD isolate. A 60 min and a 30 min exposure were necessary to achieve reduction by a factor of 10^5 at 20 °C following treatment with hydrogen peroxide and peracetic acid, respectively, whereas the standard strain was eliminated within 30 s by both oxidisers (Table II). Finally, the

Table I Bacterial strains isolated from washer disinfectors after high-level disinfection with chlorine dioxide (0.01%)

Bacterial strains	Locations
Bacillus subtilis	Rinse water
Micrococcus luteus[a]	Rinse water
Streptococcus sanguis	Connectors
Streptococcus mutans	Drain
Staphylococcus intermedius	Drain

The bacterial isolates were isolated from the first or second visit except for *M. luteus* which was isolated in two consecutive visits.

[a] Isolated on two separate occasions.

Table II Efficacy of oxidising agents against *Bacillus subtilis* washer disinfector (WD) isolate and standard strain

Biocides	Log_{10} reduction in bacterial number					
	ATCC 6091			WD isolate		
	Time (min)	Organic load		Time (min)	Organic load	
		0.3 g/L BSA	3 g/L BSA		0.3 g/L BSA	3 g/L BSA
ClO_2 (0.03%)	0.5	>4.16	–	1	1.50	–
	1	>5.28	–	5	>5.00	–
	30	–	>5.00	60	–	0.22
H_2O_2 (7.5%)	0.5	>5.40	3.92	30	0.39	1.20
	1	–	>5.29	60	>5.07	>5.00
PAA (2.25%)	0.5	>4.98	>5.15	1	2.62	–
				5	>5.00	2.78
				30	–	>4.69

ATCC, American Type Culture Collection; BSA, bovine serum albumin; PAA, peracetic acid; –, not tested.

efficacy of the chlorine dioxide in particular but also of peracetic acid with the *B. subtilis* WD isolate was further compromised in the presence of a high organic load (Table II).

The *M. luteus* WD isolate was only resistant to chlorine dioxide under soil conditions, which took 30 min at twice the in-use concentration to achieve reduction by a factor of 10^5 (Table III). However, the other two oxidising agents produced a complete kill of both the WD isolate and standard ATCC strains within 30 s irrespective of soiling (Table III).

The other WD isolates, *S. intermedius*, *S. mutans* and *S. sanguis*, were isolated from the WDs only once. These isolates proved to be less susceptible to chlorine dioxide in the presence of heavy soiling (Table IV) and did not show any cross-resistance to the other two oxidising agents.

Scanning electron microscope

Examples of micrographs from SEM analysis of the strains are shown in Figure 1. SEM images of the *B. subtilis* WD isolate showed that the bacterial cells were heavily embedded in an exopolysaccharide (EPS) matrix compared with the control strain (Figure 1a,b). *M. luteus* SEM micrographs showed that the WD isolates (Figure 1d) were grossly oversized and formed a tightly packed group when compared with the standard strain (Figure 1c).

Discussion

The presence of Gram-positive bacterial isolates from the WDs tested following high-level disinfection highlighted a problem with the disinfection process. Failure of the process may have been caused by the use of an inappropriate concentration of the disinfectant, inappropriate disinfection contact times, not following decontamination protocols or insufficient washing–disinfection within the endoscope washer disinfectors.[12,14,15] The bacteria isolated from the WDs showed the ability to withstand long exposures to twice the recommended in-use concentration of the biocide (chlorine dioxide) used. It is most probable that these isolates persisted in the WDs following an

Table III Efficacy of oxidising agents against *Micrococcus luteus* washer disinfector (WD) isolate and standard strain

Biocides	Log_{10} reduction in bacterial number					
	ATCC 4698			WD isolate		
	Time (min)	Organic load		Time (min)	Organic load	
		0.3 g/L BSA	3 g/L BSA		0.3 g/L BSA	3 g/L BSA
ClO_2 (0.03%)	0.5	>5.35	>5.00	0.5	>5.02	1.40
				30	–	>5.00
H_2O_2 (7.5%)	0.5	>5.40	>5.40	0.5	>5.00	>5.20
PAA (2.25%)	0.5	>5.40	>5.39	0.5	>5.14	>5.20

ATCC, American Type Culture Collection; BSA, bovine serum albumin; PAA, peracetic acid; –, not tested.

Table IV Efficacy of oxidising agents against other washer disinfector isolates

Biocides	Log_{10} reduction in bacterial number								
	Staphylococcus intermedius			*Streptococcus mutans*			*Streptococcus sanguis*		
	Time (min)	Organic load		Time (min)	Organic load		Time (min)	Organic load	
		0.3 g/L BSA	3 g/L BSA		0.3 g/L BSA	3 g/L BSA		0.3 g/L BSA	3 g/L BSA
ClO_2 (0.03%)	0.5	>5.80	–	0.5	>5.00	–	0.5	>5.04	–
	5	–	0.60	1	–	0.60	5	–	0.60
	30	–	>5.40	5	–	>5.00	30	–	>5.00
H_2O_2 (7.5%)	0.5	>5.36	>5.42	0.5	>5.02	>5.02	0.5	>5.00	>5.26
PAA (2.25%)	0.5	>5.88	>5.36	0.5	>5.08	>4.88	0.5	>5.00	>4.86

BSA, bovine serum albumin; PAA, peracetic acid; –, not tested.

inadequate exposure to the appropriate concentration of oxidiser. This may have been due to occlusion, inadequate exposure under normal use conditions or protection from soil. In addition, the vegetative cells of *B. subtilis* showed cross-resistance to other oxidizing agents (hydrogen peroxide and peracetic acid). These biocides are all used for the disinfection of endoscopes within hospital environments.[1,16,17] The control strain of *B. subtilis* was shown to be more susceptible to all three oxidising agents, compared with the environmental isolate. It is not usual to identify strains that persist in environmental conditions but revert to being susceptible when grown under laboratory

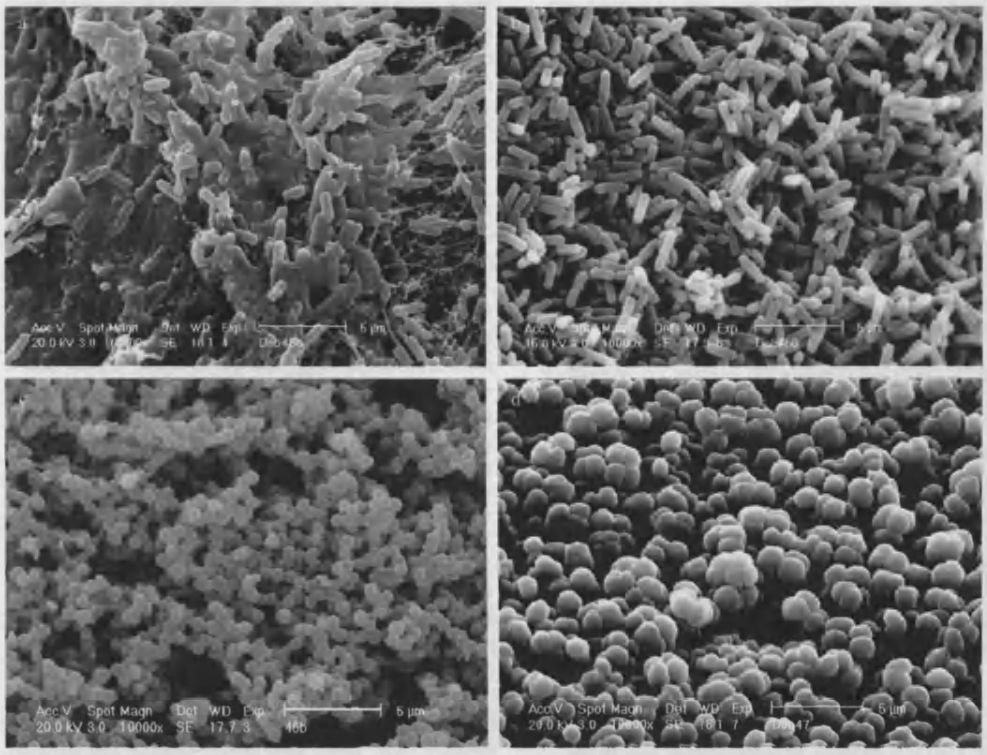

Figure 1 Scanning electron micrographs of *B. subtilis* washer disinfector (WD) isolate (a) and (b) American Type Culture Collection (ATCC) 6051, and of *M. luteus* (c), ATCC 4698 and (d) WD isolate.

conditions; however, in this case all WD isolates showed a less susceptible phenotype to at least one oxidiser following subculturing. It appears that Gram-positive bacteria surviving within the WDs had built up resistance to chlorine dioxide, and, for the Bacillus strain, stable cross-resistance to other oxidizing agents. Since *B. subtilis* was isolated, it may be expected that resistance was a result of spore formation within culture preparation, necessitating a longer contact time for sporicidal activity. However, the results from these investigations showed that the vegetative forms alone were capable of withstanding high (in-use) concentrations of the oxidising agents tested. In addition, the presence of organic load ('soiled' conditions as defined by the EN test method) further limited the efficacy of both chlorine dioxide and peracetic acid when used against the *B. subtilis* WD isolate.[13] The presence of an organic load is often considered to be required to mimic worst-case exposure conditions within a washer disinfector process. Organic matter can affect the efficacy of most biocides, including oxidising agents.[18] Although rigorous cleaning should take place before disinfection of endoscopes and organic matter (blood and faeces) should no longer be present in practice, this study showed that the organic load did not substantially affect the efficacy of the high concentration of the oxidising agents against the susceptible micro-organisms.

The *B. subtilis* WD isolate was shown to be embedded in EPS, which probably affected the penetration of the biocides.[19–21] EPS can play a part in the disruption of biocides.[22] It is also conceivable that the highly reactive biocide hydrogen peroxide is deactivated within the EPS, thus reducing its effective concentration.[23] As for the *M. luteus* WD isolate, the SEM clearly showed much larger-sized bacterial cells compared with the control strain. This could also indicate possible penetration, exclusion and/or deactivation mechanisms for the biocide resistance. At this stage the presence of an EPS matrix around the individual bacteria or an irregular cell wall structure cannot be ruled out and is under further investigation.

Sampling of the WDs showed that some micro-organisms can survive exposure to high-level disinfection processes. This study highlighted that these isolates may accumulate powerful mechanisms of resistance to oxidising agents to explain their survival after exposure to such high concentrations. The mechanisms of resistance of these isolates are clearly different, since one showed evidence of cross-resistance to oxidising agents, while the other only showed resistance to chlorine dioxide. Work is in progress to further elucidate these mechanisms of resistance.

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Conflict of interest statement
None declared.

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