Bacterial resistance to biocides: development of a predictive protocol

Thesis presented for the Degree of Philosophiae Doctor by

Laura Jane Knapp

Cardiff School of Pharmacy & Pharmaceutical Science

March 2014

Acknowledgements

I would like to express my heartfelt thanks to the following:

My supervisors Professor Jean-Yves Maillard & Professor Eshwar Mahenthiralingam for their continued support, guidance and encouragement throughout my PhD.

Dr Laura Rushton for her invaluable training and assistance with all the real-time PCR work.

Dr Mark Webber and all members of the antimicrobial agents research group at the University of Birmingham for lending their equipment and expertise for both the microarray and flow cytometry work.

Alejandro Amezquita, Peter McClure and Sara Stewart at Unilever for their expertise, advice and support.

All my lab colleagues past and present in Cardiff School of Pharmacy & Pharmaceutical Science: Jenny Hawkins, Rebecca Wesgate, Dr Mark Leggett, Michelle Ojeil, Harsha Siani, James Blaxland, Chris Hatton, Kerry Guest, Dr Ezra Linley, Dr William McCully, Dr Tina Joshi, Dr Abdullah Alyouself, Team Latino, Dr Leon O'Malley and Dr Bettina Schelkle. Thank you for your friendship, help and for making life as a PhD student so enjoyable.

My family and friends (particularly Anna Stevens and Anita Lewis) for cheering me up during the more tedious moments of the last three years!

Summary

In the last 10 years biocides have been used increasingly and questions have been raised about their contribution to the reported increase in biocide and antibiotic resistance in pathogenic bacteria. The EU Biocidal Product Regulation (BPR) now requires information on the risk of resistance development in organisms targeted by the biocidal product. There is no current protocol available to predict the likelihood of bacteria becoming resistant to a biocidal product or biocides contained therein.

This study aimed to identify useful markers of biocide resistance and develop a step-by-step protocol predictive of bacterial biocide resistance and antibiotic cross-resistance following biocide exposure.

A range of experimental techniques with the potential to generate markers of biocide resistance were explored. These included minimum inhibitory concentration (MIC)/minimum bactericidal concentration (MBC)/antibiotic susceptibility determination, flow cytometry, efflux activity measurements, outer membrane protein changes, real-time PCR and microarrays. *Salmonella enterica* serovar Typhimurium strains SL1344 and 14028S, and *Burkholderia lata* strain 383 were exposed to low concentrations of chlorhexidine gluconate and benzalkonium chloride as test biocides. Baseline and post-exposure data were then compared. Techniques used to understand any change in antimicrobial susceptibility were assessed in terms of practicality, cost and ease of use, and a step-by-step protocol was put together accounting for each of these factors.

Increases in biocide MIC and MBC of up to 100 fold were observed in SL1344 and 14028S after exposure to both biocides. However these changes were not stable after subculture of surviving organisms in the absence of either biocide. No such dramatic changes were observed within *B. lata*. Up-regulation of efflux activity was observed as a result of CHG/BZC exposure and the efflux regulatory gene *acrR* underwent a >100 fold down-regulation in both *Salmonella* strains after CHG exposure. Flow cytometry experiments performed using SL1344 and 14028S indicated that at low CHG/BZC concentrations (0.0001 – 0.0004 %) greater than 50 % of the population were not killed and that these organisms could be sorted and further investigated to determine the mechanisms behind their survival. Reduction in the expression of two outer membrane proteins was observed in strain SL1344 after exposure to 0.0004 % CHG but further protein sequencing would be required to identify these.

Changes in phenotype and genotype of biocide-exposed bacteria were identified using different experimental techniques. Some of these changes e.g. increased MIC/MBC values, altered antibiotic susceptibility, up-regulated efflux activity, alterations in the expression of specific genes and surviving organisms identified by flow cytometry represent useful markers of biocide resistance. A preliminary step by step protocol incorporating these techniques was successfully developed and allows for the rapid identification of biocide resistance and antibiotic cross-resistance as a result of biocide exposure, and will prove particularly useful in light of the recent changes to the BPR.

List of abbreviations

1-(1-NP)	1-(1-napthylmethyl) piperazine
ABC	ATP-Binding Cassette
AMP	Ampicillin
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
BC	Benzethonium Chloride
BCA	Bicinchoninic Acid
Bcc	*Burkholderia cepacia* complex
BIT	Benzisothiazolinone
BOX	Bis (1,3-dibarbituric acid) Trimethine Oxanol
BPR	Biocidal Product Regulation
BSAC	British Society for Antimicrobial Chemotherapy
BSA	Basal Salts Agar
BVSA	Bovine Serum Albumin
BSB	Basal Salts Broth
BZC	Benzalkonium Chloride
CEF	Ceftriaxone
CEFT	Ceftazidime
CF	Cystic Fibrosis
CFU	Colony Forming Units
CHG	Chlorhexidine digluconate
CHL	Chloramphenicol
CIP	Ciprofloxacin
CLSI	Clinical Laboratory Standards Institute
diH_20	Deionised Water
DMSO	Dimethyl Sulfoxide
dNTP	Deoxynucleotide triphosphates
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
EPI	Efflux Pump Inhibitor
EU	European Union
FDA	Food and Drug Administration
G	Gram(s)
H	Hour(s)
HAI	Hospital Acquired Infection
HIV	Human Immunodeficiency Virus
HPA	Health Protection Agency
HPC	Home and Personal Care
IFH	International Scientific Forum on Home Hygiene
IMI	Imipenem
LPS	Lipopolysaccharide

MATE	Multidrug and Toxic Compound Extrusion
MBC	Minimum Bactericidal Concentration
M-CMIT	Methylisothiazolinone-chloromethylisothiazolinone
MDR	Multiple Drug Resistance
MEM	Meropenem
MFS	Multidrug Facilitator Superfamily
MIC	Minimum Inhibitory Concentration
MIT	Methylisothiazolinone
MLST	Multi-Locus Sequence Typing
MG	Milligram(s)
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MW	Molecular Weight
NFW	Nuclease Free Water
OD	Optical Density
OMP	Outer Membrane Protein
PAβN	Phenyl-arginine-β-napthylamide
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PI	Propidium Iodide
PIP	Piperacillin
QAC	Quaternary Ammonium Compound
QRDR	Quinolone Resistance Determining Region
RNA	Ribonucleic Acid
RND	Resistance Nodule Division
RPM	Revolutions Per Minute
RT	Reverse Transcriptase
SC	Subculture
SEC	Second(s)
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SMR	Small Multidrug Resistance
spp	Species
TSA	Tryptone Soya Agar
TSB	Tryptone Soya Broth
TSC	Tryptone Sodium Chloride
TOB	Tobramycin
TVC	Total Viable Count
µG	Microgram(s)
µL	Microlitre(s)

Glossary

Biocide: a chemical agent used to kill living organisms (Maillard, 2005)

Biocidal product: any substance or mixture consisting of/containing/capable of generating one or more active substances with the intention of destroying/rendering harmless/preventing the action of any harmful organism (BPR (EU) 528/2012 http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2012:167:0001:0123:EN:PDF accessed 11_03_2014)

Biocide resistance: a change in susceptibility to a biocide that renders it ineffective against a micro-organism that was previously susceptible to that biocide (Maillard *et al.*, 2013)

Reduced biocide susceptibility: an increase in the minimum inhibitory or minimum bactericidal concentration of a biocide where the biocide still remains effective for its intended purpose (Maillard *et al.*, 2013)

Antimicrobial: an agent that kills/inhibits the growth of microorganisms – can be a biocide or antibiotic

Table of Contents

List of figures

List of tables

Chapter One: General Introduction

1.1 General biocide use

Biocides have been widely used in the control of bacteria for decades, and despite their increasing use, bacteria generally remain susceptible to biocides when they are used correctly. However, the indiscriminate use of biocides in human medicine, cosmetics, agriculture, livestock farming, food production, personal care products and household products has raised concerns about the development of bacterial biocide resistance and potential cross resistance to antibiotics as a result of biocide exposure. Despite the establishment of the European Union (EU) biocidal product regulation (528/2012 – discussed in section 1.3), to regulate the authorisation and use of biocidal products throughout the EU, the total amount of biocide use in the EU remains unknown (SCENIHR, 2010). Concerns have also been raised over the release of biocides in to waste water and the surrounding environment as a result of their increased use, and the potential effect of this on microbial populations in soil and aquatic habitats. Concentrations present in the environment may be sufficient to select for bacterial strains with reduced antimicrobial susceptibility, and furthermore could result in increased exposure of the human and animal population to biocides which could lead to alterations in biocide susceptibility of micro-flora on humans and animals.

1.2 The EU biocidal product regulation (BPR)

The aim of the BPR is to improve the free movement of biocidal products throughout the EU whilst protecting human, animal and environmental health. Any product used to protect humans/animals/other articles against harmful organisms such as bacteria or pests must be authorised for use. The new BPR (EU 528/2012) which came in to effect on 01/09/2013 replaced the previous directive (98/8/EC). The active substance contained within a biocidal product must now be authorised for use at both Union level and member state level, whereas previously only member state authorisation was required. A further important

amendment to the BPR is the requirement by biocidal product manufacturers to provide information on the likelihood of resistance development to their product in target organisms (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2012:167:0001:0123:EN:PDF accessed 11_03_2014)

> "(13) Active substances can, on basis of their intrinsic hazardous properties, be designated as candidates for substitution with other active substances, whenever such substances considered as efficient towards the targeted harmful organisms become available in sufficient variety to avoid the development of resistances amongst harmful organisms…
>
> (25) … The use of low-risk biocidal products should not lead to a high risk of developing resistance in target organisms.
>
> (33) When biocidal products are being authorized, it is necessary to ensure that, when properly used for the purpose intended, they are sufficiently effective and have no unacceptable effect on the target organisms such as resistance, … When deciding whether a biocidal product should be authorized, due consideration should be given to the benefits from its use."

In addition the US Food and Drug Administration (FDA) have issued a proposed rule to determine the safety and efficacy of antibacterial soaps (http://www.fda.gov/newsevents/newsroom/pressannouncements/ucm378542 accessed 11/03/14).

> "The U.S. Food and Drug Administration today issued a proposed rule to require manufacturers of antibacterial hand soaps and body washes to demonstrate that their products are safe for long-term daily use and more effective than plain soap and water in preventing illness and the spread of certain infections. Under the

proposal, if companies do not demonstrate such safety and effectiveness, these products would need to be reformulated or relabelled to remain on the market."

"…some data suggest that long-term exposure to certain active ingredients used in antibacterial products—for example, triclosan (liquid soaps) and triclocarban (bar soaps)—could pose health risks, such as bacterial resistance …"

1.3 Factors affecting biocide efficacy

Biocide efficacy is dependent on numerous factors discussed below.

1.3.1 Concentration

Biocides are formulated for broad-spectrum activity and use on both inanimate surfaces (disinfection) and animated surfaces (antisepsis). Biocide activity can be measured using a concentration exponent 'Ƞ'. Biocides that interact strongly with bacterial targets (e.g. membrane active agents such as quaternary ammonium compounds and chlorhexidine) have a low Ƞ-value (<2) and are more likely to be affected by alterations in concentration than those with a high Ƞ-value (>4), e.g. biocides that have a weak physical interaction with the lipophilic components of the bacterial cell envelope such as alcohols or phenolics (Russell and McDonnell, 2000). Despite the fact that biocides may be affected by changes in concentration, some are incorporated in to different products at varying concentrations and remain effective. For example chlorhexidine digluconate (CHG) can be used at concentrations as high as 4 % in liquid disinfectants, but is incorporated in to hand soaps at lower concentrations of approximately 2 % (Larson and Laughon, 1987). However dilution of a biocidal product could reduce biocide concentration to a level that is below the minimum inhibitory concentration (MIC) for some bacteria (i.e. the lowest concentration

required to inhibit bacterial growth). The MIC is a lot more variable between isolates than the minimum bactericidal concentration (MBC) (i.e. the lowest concentration required to kill bacteria). This suggests that biocide dilution to a sub-lethal concentration could result in growth inhibition of only a small proportion of a bacterial population (Russell and McDonnell, 2000) resulting in persistence of bacteria with reduced biocide susceptibility. Bacteria have also been found to possess the ability to grow and persist in the product itself with bacterial survival in biocidal formulations having been reported since the 1950s (Maillard, 2005).

Gilbert and McBain (2003) have proposed that there is likely to be a continuum of biocide concentration ranging from in-use to zero present in any environment . This suggests that at some point in any environment bacteria may be exposed to low and potentially sub-lethal concentrations of a particular biocide, creating a pressure that may select for bacteria with reduced biocide susceptibility. This may be particularly true for bacteria present within the depths of a biofilm found on a surface in the home as it is less likely that the same concentration of a biocidal product that reaches the surface of a biofilm will reach the deeper layers. For this reason it is important that the user complies with the recommendations of the manufacturer when using the product in order to minimise the development of reduced biocide susceptibility. It must also be stressed that biocide concentration is one of several factors that can affect biocide efficacy and must therefore be critically examined alongside other factors when testing the antimicrobial activity of biocidal products (SCENIHR, 2010, Russell and McDonnell, 2000).

1.3.2 Contact time

A longer contact time would usually be associated with increased efficacy of a biocide and the killing of a larger number of microorganisms. However Whitehead *et al*., (2011)

reported stable multi-drug resistance in *Salmonella enterica* serovar Typhimurium isolates after 5 hour exposure to the biocides Trigene and Superkill at the recommended in-use concentration (1 %). This lengthy exposure time may not have been expected to result in the emergence of mutant organisms particularly at a recommended in-use concentration. Depending on the application of the biocidal product, there may be a recommended minimal contact time when carrying out standard efficacy tests, which may be as short as 1 min for hand soaps or 5 min for surface disinfectants (Maillard, 2005). However some of these products may not be as effective as claimed, even when used for the recommended length of time, as demonstrated by Cheeseman *et al.,* (2009) when investigating the efficacy of alcoholic hand rubs used in hospitals. They reported that all three hand rubs tested did not produce a 4 $\log_{10}$ reduction in CFU/mL of *Staphylococcus aureus* when tested at contact times used in practice (approx 15 sec). Reports like this are concerning, particularly as the biocidal products tested were used in a hospital environment where it is of high importance to minimise the spread of microbial contamination and infection.

It is also important that concentration is taken in to account alongside contact time as prolonged exposure of bacteria to a very low concentration of a particular biocide has been reported to result in the development of biocide resistance in some bacteria. Mavri *et al.,* (2013) reported the *in vitro* development of reduced susceptibility to triclosan, chlorhexidine diacetate and benzalkonium chloride in *Campylobacter* spp. as a result of repeat exposure to sub-lethal concentrations of these biocides. This also resulted in cross-resistance to erythromycin and ciprofloxacin. Of further concern are *in vivo* reports of infection outbreaks as a result of repeat exposure of a particular bacterium to a biocidal product. Duarte *et al.,* (2009) reported an outbreak of post- surgical infections in 38 hospitals in Brazil caused by *Mycobacterium massiliense* that was resistant to 2 % glutaraldehyde. They speculated that repeat exposure of these organisms to 2 % glutaraldehyde used in the cleaning of surgical instruments, combined with insufficient

mechanical cleaning, may have created a selective pressure for glutaraldehyde resistance. This highlights the risk of repeat exposure of a particular bacterium to a biocidal product and highlights the importance of adherence to proper cleaning procedures in a hospital environment.

1.3.3 Interfering materials

The presence of organic materials or quenching agents may affect the activity of a biocidal product. Standard efficacy testing protocols such as the BS EN 1276 (2009) suspension testing protocol provide guidelines for testing a biocidal product under clean and dirty conditions so that comparisons can be made. Reduced biocide efficacy has been observed in the presence of other compounds. For example, Benson *et al.,* (1990) found that the use of anionic moisturising products on the hands after a chlorhexidine-based hand wash had been applied inhibited all residual antimicrobial activity of the hand wash. Otter *et al*., (2012) found that the presence of bovine serum albumin (used to represent dirty conditions) reduced the efficacy of hydrogen peroxide vapour against *S. aureus in vitro*, concluding that sub-optimal cleaning may reduce biocide efficacy. The clinical application of the biocidal product must therefore be considered and precautions must be taken to ensure the efficacy of the product is not affected.

1.3.4 Temperature and pH

Temperature and pH may also have an effect on the activity of a biocide. A specific temperature may be required for activation of the biocide in processes such as the sterilization of medical equipment (Maillard, 2005). Standard protocols such as the BS EN 1276 (2009) suspension testing protocol specify that temperature must be controlled for the duration of the test performed in order to minimise variability in results due to temperature fluctuations. Certain formulations may also have a specific storage

temperature required for preservation of the product. Leung *et al.,* (2004) investigated the effect of storage temperature on the efficacy of contact lens solutions against *Pseudomonas aeruginosa* and found that optimum storage temperature ranged between 25 – 30 °C and that solutions stored at fridge temperature showed reduced efficacy and reduced shelf-life. This highlights the importance of correct storage of biocidal products to ensure that antimicrobial activity is not reduced.

pH may affect the overall charge of the biocide which could alter its ability to interact with the target microorganism, particularly in the case of Gram-negative organisms that possess a cell wall with a negative charge. The activity of cationic biocides such as chlorhexidine can be enhanced if the pH is increased (Russell, 2004). The pH can also affect the availability of chlorine in biocides such as sodium hypochlorite (Guerreiro-Tanomaru *et al.*, 2011). Guerreiro-Tanomaru *et al.,* (2011) reported that lower pH resulted in a reduction in available chlorine, but that this did not affect the activity of sodium hypochlorite against *Enterococcus faecalis.* Despite the activity of sodium hypochlorite not being affected in this particular case, it is clear that pH should be controlled when testing the efficacy of a biocidal product, so that antimicrobial activity is not quenched and the test concentration remains accurate and false-negative results are not obtained.

The nature of the microorganism targeted may also affect the activity and efficacy of the biocide. Factors that contribute to bacterial resistance to biocides are discussed in section 1.4.

1.4 Mechanisms of bacterial resistance to biocides

Antibiotics usually have a specific bacterial cellular target, and antibiotic resistance therefore typically occurs due to modification of this particular target. Biocides have multiple target sites in bacterial cells and it is therefore less likely that bacteria can become

resistant to a particular biocide via the alteration of a target site although there are exceptions (Bailey *et al.*, 2009). Biocide resistance therefore generally occurs as a result of mechanisms that decrease the concentration of a particular biocide to a level that is not lethal to the bacterial cell or prevent the entry of the biocide in to the cell. Examples of biocide resistance mechanisms are discussed below.

1.4.1 Cell permeability

Bacterial intrinsic insusceptibility to biocides may be associated with changes to the outer layer that alter cell permeability and restrict the uptake of the biocide into the bacterial cell (Dubois-Brissonnet *et al.*, 2011, Ferreira *et al.*, 2011). Gram-negative bacteria such as *Salmonella* and *Burkholderia* spp. tend to be less susceptible to biocides than Gram-positive bacteria due to the presence of a lipid-rich outer membrane. This membrane is composed of phospholipids, fatty acids, lipopolysaccharide (LPS) and porins that aid in limiting the uptake of the biocide in to the cell (Mensa *et al.*, 2011). It also possesses a negative charge which may result in the repulsion of biocide molecules away from the bacterial cell. Figure 1.1 illustrates both the Gram-positive and Gram-negative bacterial cell walls. *Mycobacterium* spp. possess a lipid-rich cell envelope composed of mycolic acids (Portevin *et al.*, 2004) which have been associated with antimicrobial resistance. This layer prevents the Gram staining of this species, and maintains the structural integrity of the membrane and has been associated with resistance to oxidising agents such as hydrogen peroxide (Yuan *et al.*, 1995).

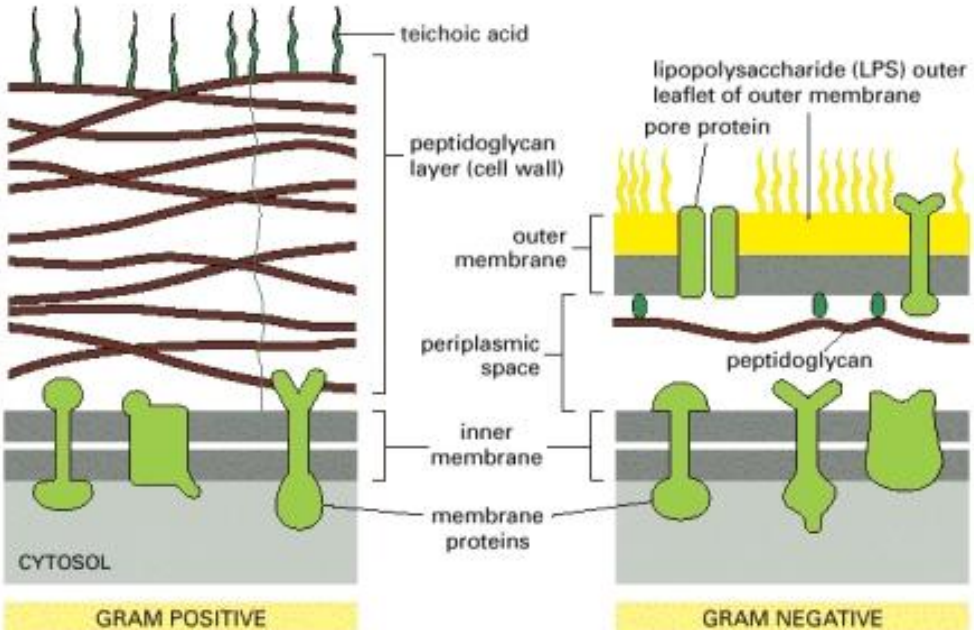


Figure 1.1: A comparison of the Gram-positive and Gram-negative bacterial cell walls
Image taken from (Alberts *et al.,* 2002)

Alteration and modification of the bacterial cell membrane in response to biocide exposure has been described on more than one occasion. Dubois-Brissonnet *et al.*, (2011) described the modification of membrane fatty acid composition resulting in increased tolerance to peracetic acid and dodecyl ammonium bromide in *Salmonella enterica* serovar Typhimurium. They found that exposure to sub-MIC concentrations of natural plant-derived terpenes used as chemical preservatives in the food industry resulted in the increased production of saturated fatty acids throughout all bacterial growth phases. This change was thought to stabilise the membrane in the presence of antimicrobials that exert their effects on the cell via the partitioning of the lipid membrane. Tattawasart *et al*., (2000) reported that chlorhexidine diacetate resistant *Pseudomonas stutzeri* had an altered outer membrane protein profile and found the expression of two additional protein bands. They also observed changes in LPS that were thought to contribute to cross- resistance to

other antimicrobial agents aside from chlorhexidine diacetate, such as polymyxin B sulphate and gentamicin.

Despite the increased biocide susceptibility observed in Gram-positive bacteria (compared to Gram-negatives), certain Gram- positive species of bacteria, such as *Clostridium* spp. and *Bacillus* spp. have the ability to form spores when under environmental stress such as nutrient starvation. Bacteria in spore form exist in a dormant state and can survive in this state for many years (Leggett *et al.*, 2012). The presence of a spore coat, composed of highly cross-linked proteins is thought to contribute to intrinsic resistance to antimicrobials, as treatment of spores with chemical disruptors of disulphide bonds has been shown to increase spore susceptibility to hydrogen peroxide and lysozyme (Gould, 1970).

1.4.2 Biofilm formation

It has been estimated that > 90 % of bacteria in nature exist as a biofilm (Baugh *et al.*, 2013). A biofilm is a structured community of bacteria attached to a surface by exopolymeric substances (Vickery *et al.*, 2012). Biofilms can consist of monocultures, of several diverse species, or of mixed phenotypes of a given species. An environment limited in nutrients has been shown to induce a 'stress' response where bacteria adopt a resting or dormant phenotype similar to that of endospores which are resistant to numerous chemical agents (Leggett *et al.*, 2012). Bacteria within a biofilm therefore typically exist in a slow growing, nutrient-depleted state, or non-growing state (Gilbert and McBain, 2003) and have an altered phenotype in comparison to planktonic (non-biofilm) species.
In a hospital environment biofilms are generally found on moist surfaces such as catheters, disinfecting soap dispensers, instruments regularly immersed in fluid, as well as other places including patients which can lead to the increased spread of infection (Vickery *et al.*, 2012). There are numerous examples of human infections caused by biofilms including

Burkholderia in the lungs of cystic fibrosis patients (Peeters *et al.*, 2008) and *Legionella pneumophilia* causing legionellosis (Baugh *et al.*, 2013).

Some biofilm-forming bacteria have been shown to be 10 - 100 fold more resistant to antimicrobials in comparison to their planktonic counterparts (Rose *et al.*, 2009, White and McDermott, 2001). Furthermore, the exchange of mobile genetic elements between bacteria within biofilms has been reported (Antonova and Hammer, 2011). Antonova & Hammer (2011) observed that *Vibrio cholera* present in a biofilm produce an autoinducer molecule that allows them to become naturally competent to take up extracellular DNA. This demonstrates the possibility of the acquisition of resistance genes amongst bacteria present in a biofilm, and may further contribute to the reduced biocide susceptibility of these bacteria. Wong *et al*., (2010b) tested the efficacy of benzalkonium chloride (BZC), CHG, citric acid, sodium hypochlorite and ethanol against planktonic *S. enterica* serovar Typhimurium cells and 3 day old *S. enterica* serovar Typhimurium biofilms at recommended in-use concentrations, and found that all biocides were able to reduce the number of biofilm cells, but still left some viable cells, whereas all planktonic cells were eliminated. This demonstrates the reduced biocide susceptibility of cells present in a biofilm and the potential for further spread of infection if a biofilm is not completely eliminated. It has also been reported that biofilm age has no effect on the efficacy of the antimicrobial and that older biofilms are no more or less susceptible to biocides than new biofilms (Wong *et al.*, 2010a). Wong *et al*., (2010b) suggested that an increase in concentration and contact time was the only way to ensure 100 % reduction of viable cells present in a biofilm. The need to increase biocide concentrations in order to kill biofilms could result in high environmental toxicity and increased costs, and may also create a selection pressure for increased biofilm development amongst bacterial species.

1.4.3 Metabolism

As biocides generally have multiple targets (e.g. cell wall, cytoplasmic membrane, DNA, proteins) in the bacterial cell it is unlikely that biocide resistance would occur due to biocide inactivation by bacteria. However, the break down and inactivation of quaternary ammonium compounds (QACs) has been reported. Nishihara *et al.,* (2000) showed that *Pseudomonas fluorescens* TN4 isolated from sludge was able to degrade didecyldimethylammonium chloride which is a QAC. This isolate was also able to degrade other QACs via an N-dealkylation process. The initial parent compound was broken down after 24 h and the first metabolite was then further broken down after a period of 7 days. This strain was found to be highly resistant to the compounds it could break down demonstrating the presence of bacteria that are able to degrade QACs in the environment. Biocide use in waste water treatment, industry, building materials and fuel may result in the release of biocide residues in to the environment, although the exact quantities are unclear (SCENIHR, 2010). Biocide residues in the environment may create a selective pressure for the clonal expansion of bacteria with the ability to degrade certain compounds, contributing to biocide resistance.

Although there are very few reports of bacteria that are able to directly break down biocides, there is evidence of bacteria making alterations to a specific metabolic pathway targeted by a biocide in order to prevent damage to the bacterial cell. Bailey *et al.,* (2009) described a triclosan-specific alteration in metabolism in *S. enterica* serovar Typhimurium that assisted in the protection of the bacterial cell from the biocide. They demonstrated through the use of DNA microarrays that the bacterium was able to down-regulate expression of the *fab* gene cluster associated with fatty acid biosynthesis and up-regulate pyruvate synthesis genes in order to by-pass the inhibitory effects of triclosan, which inhibits fatty acid biosynthesis at low concentrations, preventing cell membrane synthesis.

Due to the specificity of this alteration in response to biocide exposure there is not yet any reported evidence of cross -resistance to antibiotics.

1.4.4 Efflux

Efflux is the pumping of a solute out of a cell, and efflux pumps are present in all organisms. In bacteria efflux pump genes can be chromosomally encoded or found on mobile genetic elements such as plasmids. Efflux pumps are proteins that span the bacterial cell membrane and can either transport a single, specific substrate or a range of structurally similar compounds (Nikaido and Pages, 2012). Examples of bacterial efflux pump substrates include dyes, detergents, antibiotics (e.g. quinolones, fluoroquinolones, chloramphenicol, and tetracycline) and biocides (e.g. cetrimide, triclosan). Many bacterial efflux pumps are now well characterised and have been associated with a multidrug resistant phenotype (Guo *et al.*, 2013, Buroni *et al.*, 2009, Smith and Hunter, 2008). There are five well-described efflux pump families in bacteria. These are shown in figure 1.2. Pumps are classified based on the number of components a pump has, the energy source required for the pump to transport substrates, the number of transmembrane spanning regions and the type of substrate (Piddock, 2006b). Different species of bacteria can express more than 1 type of efflux pump.

In Gram-negative bacteria, the type of efflux pump most commonly associated with multi-drug resistance is the resistance nodule division (RND) type pump. RND pumps are composed of a tripartite system. E.g. the AcrAB-TolC efflux pump in *E. coli* and *S*. *enterica* serovar Typhimurium is composed of an inner membrane transporter (AcrB), an accessory protein present in the periplasmic space (AcrA) and an outer membrane protein (TolC) (Hinchliffe *et al.*, 2013). Substrates of this pump include chloramphenicol, quinolones, tetracycline, triclosan, nalidixic acid and triton X-100 (Nikaido and Zgurskaya, 2001). Bailey *et al*., (2009) reported that deletion of this efflux system in *S. enterica* serovar Typhimurium

resulted in a 4 to 10-fold increase in triclosan susceptibility, whereas over-expression of this system lead to a 2 to 4-fold decrease in triclosan susceptibility. Guo *et al.,* (2013) also attributed BZC resistance in *S*. *enterica s*erovar Typhimurium BZC- resistant mutants to constitutive over-expression of AcrAB-TolC. Of further concern was that inactivation of AcrAB-TolC in these mutants resulted in increased expression of AcrEF-TolC in order to maintain BZC resistance.

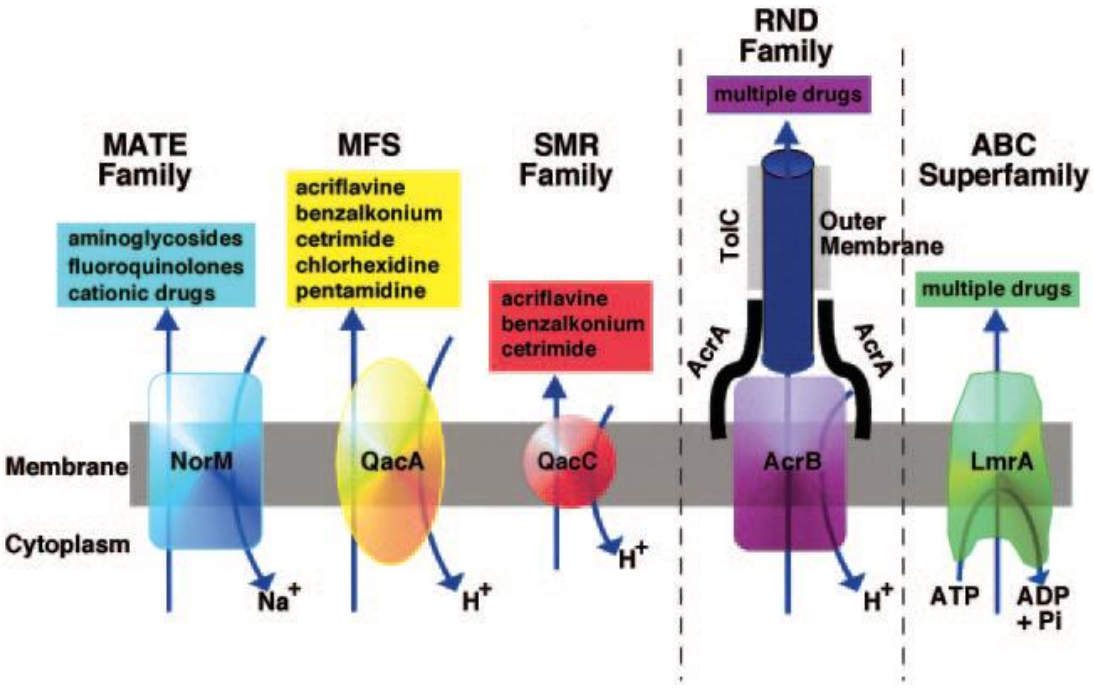


Figure 1.2: The 5 classes of bacterial efflux pump
MATE = multidrug and toxic compound extrusion. MFS = major facilitator superfamily. SMR = small multidrug resistance. RND = resistance nodule division. ABC = ATP-binding cassette. Figure taken from (Piddock, 2006a)

In Gram-positive bacteria the main class of efflux pump associated with multi-drug resistance is the major facilitator superfamily (MFS) (Saidijam *et al.*, 2006). An example of a well characterised efflux pump of this type is the chromosomally-encoded pump NorA present in *S. aureus*. Huet *et al.,* (2008) found that repeat exposure of *S. aureus* isolates to sub-lethal concentrations of several biocides including chlorhexidine and BZC resulted in mutants over-expressing *norA* and *norC* due to mutations in the promoter regions of these

genes. They highlighted the concern for hospitalised patients that are treated with antibiotics such as ciprofloxacin and norfloxacin that are also substrates for these pumps.

Bacterial efflux pump genes can also be acquired through horizontal gene transfer. Transformation, transduction and conjugation are all types of this process (Koraimann and Wagner, 2014). Plasmids and transposons are transferable between bacteria of the same species or bacteria of different species and can both carry resistance genes. An example of plasmid-borne efflux pump genes is *qac* genes. Qac efflux pumps confer resistance to quaternary ammonium compounds, such as BZC, hence their name but also have further substrates including chlorhexidine, intercalating dyes and triclosan (Smith and Hunter, 2008). Smith and Hunter (2008) reported the presence of multiple *qac* genes in clinical isolates of *S. aureus* and methicillin-resistant *S. aureus* (MRSA). These isolates had much higher MBCs for the biocides tested than isolates that did not possess *qac* genes. Plasmid transfer of *qac* genes is not exclusive to Staphylococci. *Salmonella* spp. can also acquire *qacE* which confers resistance to multiple biocides (White and McDermott, 2001). External chemical stress, such as biocide exposure may promote the maintenance of transferable resistance genes and increased transfer of these genes. However there is little evidence of this, and Pearce *et al.,* (1999) reported that exposure of *S. aureus* isolates to sub-lethal concentrations of chlorhexidine reduced the transfer of a resistance plasmid (pWG613) via conjugation or transduction.

Gilbert and McBain (2003) have speculated that the increased use of biocides could potentially create a selection pressure for mutant strains that hyper-express these multi-substrate efflux pumps when exposed. This hyper-expression could occur via a point mutation in the efflux gene promoter or a mutation in the global repressor (Baucheron *et al.*, 2004a). These mutations may result in over-expression of the gene in the absence of the substrate that induces its expression. This would result in reduced susceptibility to any

other substrates that come into contact with the bacterial cell, including antibiotics, which may lead to limited therapeutic options when treating an infected patient.

1.5 Biocide resistance in the clinical environment

Biocides have a range of clinical applications including disinfection of both hospital surfaces and pre-operative patient skin, sterilisation of medical equipment and general infection prevention via incorporation in to hospital bed linens and curtains. Examples of biocides commonly used in the hospital environment include cationic biocides such as QACs (e.g. BZC), chlorhexidine, cetrimide and triclosan (Bailey *et al.*, 2009, Smith and Hunter, 2008).

Biocides used to control the growth of pathogenic organisms in a clinical environment can be categorised based on the level of bacterial inactivation reached. For example, low-level disinfectants (e.g. isopropyl alcohol) may only eliminate vegetative bacteria whereas high-level disinfectants (e.g. hydrogen peroxide) inactivate many microorganisms including vegetative bacteria, mycobacteria, viruses and most fungi (Rutala and Weber, 2007). Low-level disinfectants are commonly used to disinfect 'non-critical' hospital devices that come into contact with intact skin (e.g. stethoscopes, electrocardiogram cables). These devices are unlikely to transmit infectious agents to patients and therefore do not require high-level disinfection (Dettenkofer *et al.*, 2004). 'Critical' devices that penetrate sterile tissues (e.g. catheters, needles) are sterilised using high-level disinfectants.

Hospital-acquired infections (HAIs) cause significant morbidity and mortality in the UK with approximately 6.4 % (3,360 out of 52,443) of hospital patients in the UK acquiring an infection whilst in hospital per year. *Enterobacteriaceae* are the most frequently reported organisms associated with HAI (English National Point Prevalence Survey on Healthcare-associated Infections and Antimicrobial Use, 2011

http://www.hpa.org.uk/servlet/Satellite?c=HPAweb_C&pagename=HPAwebFile&rendermode=previewnoinsite&cid=1317134304594 accessed 11/03/2014). This emphasises the requirement for effective disinfection procedures and the correct use of the appropriate biocidal products in order to prevent the spread of infection. One of the main issues is that the study of the efficacy, mode of action and mechanisms of resistance to biocides is fairly limited when compared to current knowledge of antibiotics. The efficacy of a particular biocidal product is usually tested *in vitro* and experimental conditions may not reflect the final application of the product (SCENIHR, 2010) which may result in inaccurate efficacy data. Standard tests and protocols that are available for testing bacterial susceptibility to antibiotics are not available for biocides and responses to different biocides vary enormously between species and strains.

Russell (2004) concluded that biocides are only really required in high risk areas where the spread of HAIs is high e.g. in the sterilisation of medical equipment but not perhaps in areas that are rarely heavily contaminated such as hospital walls and ceilings. With the increased use of biocides it can be assumed that bacteria in the clinical environment are being exposed to biocidal products more frequently. Regular exposure to biocide stress could lead to genetic, biochemical, functional or physiological changes in the bacterial cell that select for bacteria with greater tolerance to these conditions and biocide exposure (SCENIHR, 2010). For example Block and Furman (2002) found that in clinical areas of a hospital where chlorhexidine was used more intensely, micro-organisms isolated from patients showed decreased in susceptibility to this biocide.

It has been argued that bacterial strains showing reduced susceptibility to biocides are still not a major health concern as generally biocides are used at high concentrations that are lethal to these strains (Thomas *et al.*, 2005). However Duarte *et al*., (2009) reported an

epidemic of rapidly growing *Mycobacterium massiliense* in patients that had undergone surgery in one of 63 hospitals in Rio de Janeiro, Brazil. Five isolates belonging to a specific clonal group referred to as BRA100 were resistant to 2 % glutaraldehyde solution which had been commercially used in the sterilisation of surgical instruments. All isolates tested were also found to be clinically resistant to ciprofloxacin, cefoxitin and doxycycline. This finding contradicts the claim that much higher, in-use concentrations of biocides are highly effective.

Despite *in vitro* studies demonstrating reduced biocide susceptibility as a result of bacterial exposure to sub-lethal biocide concentrations, fewer *in vivo* reports of this exist and a concrete relationship between increased biocide use in the clinical environment and biocide resistance in bacteria is yet to be established. It remains essential for the users of biocidal products to comply with the manufacturers recommendations in terms of concentration, application and conditions of use in order for them to remain effective in the prevention of contamination and infection.

1.6 Biocide resistance in the domestic environment

It has been suggested that if the use of biocides in the clinical environment is affecting antimicrobial resistance, a similar situation could be seen in the domestic environment (Gilbert and McBain, 2003). Home hygiene is considered by the public as essential in the prevention of the spread of infectious diseases. Regular use of household products such as laundry detergents, cleaning products, pet disinfectants and general disinfectants are the major sources of bacterial exposure to biocides in home settings (Gilbert and McBain, 2003). In order to maintain hygiene in the home various cleaning procedures can be carried out. The responsible and correct use of biocidal products in the home will contribute to reducing the spread of infection. It is therefore important that the use of biocides is not discouraged in the home as it is largely beneficial.

The International Scientific Forum on Home Hygiene (IFH) (http://www.ifh-homehygiene.org/ accessed 11_03_14) collected data to determine the lead causes of transmission of pathogens in the home. Hands, hand contact surfaces, food contact surfaces and cleaning utensils were identified as critical points of transmission in the home, as well as clothing and household linens (Bloomfield *et al.*, 2012). The IFH describe a 'chain of pathogen transmission' and suggest the identification of critical points of intervention throughout the chain in order to prevent transmission. Intervention strategies included promotion of effective hand hygiene e.g. using alcohol hand gel, and instigating a change in behaviour towards home hygiene, e.g. hygienic disposal of household waste (Bloomfield *et al.*, 2012).

Biocidal products that are used to disinfect surfaces in the home, and those used as hand washes may leave a low residual concentration after their use (SCENIHR, 2010, Thomas *et al.*, 2000). Hand washes typically do not have a contact time of more than one minute with the skin before being washed off. It is therefore quite likely that a residual concentration of the biocide will remain on the skin. Exposure of bacteria to sub-lethal concentrations of a biocide may create a selective pressure for organisms with reduced antimicrobial susceptibility (Christensen *et al.*, 2011). However Jones (2000) reported that although the frequent use of antimicrobial hand wash products can bring about changes in the human skin micro-flora, it has not been associated with increased antimicrobial resistance. As a large number of studies focus on the relationship between biocide use and biocide resistance in the clinical environment, a concrete relationship between increased biocide use in the home and the isolation of biocide-resistant bacteria is yet to be established.

1.7 Test protocols to measure bacterial resistance to biocides

Different studies use different methodologies when testing bacterial susceptibility to biocides, but each of these alone provides limited information on the activity of the

biocide, and different microorganisms respond in different ways. Many factors need to be considered in biocide susceptibility testing including *in vivo* conditions such as pH and temperature, as well as the concentrations to be tested and their reflection of the recommended or in-use concentrations.

Biocides should be tested at concentrations recommended for use by the manufacturer. However in-use concentrations may vary from the recommended concentration intended for use due to biocidal product application. The presence of organic load (e.g. dirt) or product dilution may alter the in-use concentration and this must also be accounted for during susceptibility testing.

In addition, after biocide use there may be a residual concentration remaining. For example if a biocide is incorporated in to a surface cleaner, there may be a lower, residual concentration of that biocide remaining after the surface has been wiped clean. This remaining concentration may have a different effect on target microorganisms and biocide efficacy should also therefore be tested at these residual concentrations. Biocide manufacturers do not currently provide information on the effect of bacterial exposure to residual biocide concentrations, but it has been reported in the literature that this type of exposure can lead to the selection of isolates with reduced antimicrobial susceptibility (Christensen *et al.*, 2011, Whitehead *et al.*, 2011). Furthermore, information on the likelihood of resistance development now must be provided by product manufacturers according to the BPR (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2012:167:0001:0123:EN:PDF accessed 11_03_2014).

1.7.1. Non-molecular protocols

1.7.1.1 Minimum inhibitory and minimum bactericidal concentrations

Many reports on emerging bacterial resistance to biocides are based on the determination of minimum inhibitory concentrations (MICs) (Skovgaard *et al.*, 2013, Kastbjerg and Gram, 2012, Buffet-Bataillon *et al.*, 2011). Using MICs to measure biocide resistance is arguable since much higher concentrations of biocides are used in practice and resistance to a biocide due to elevated MICs has been deemed unlikely (Russell and McDonnell, 2000). Some studies have shown that bacterial strains showing a significant increase in the MIC for a particular biocide were nevertheless susceptible to higher (in-use) concentrations of the same biocide (Thomas *et al.*, 2005, Lear *et al.*, 2002) and others have shown no change in the MIC of a biocide after exposure to an in-use concentration, despite altered antibiotic MICs (Whitehead *et al.*, 2011). One study by Carson *et al*. (2008) found that bacteria with high QAC MICs isolated from the domestic environment in which antimicrobial products containing QACs had been used for a year, also had high triclosan MICs and were more likely to be resistant to an antibiotic. Despite providing limited information on resistance to in-use biocide concentrations, MICs may be indicative of a trend towards resistance and therefore may be useful in combination with other techniques used to measure biocide resistance such as determination of the MBC.

Concentration is central to biocide efficacy. It may therefore be more appropriate to measure bacterial lethality rather than bacterial growth after biocide exposure. Determination of the MBC may therefore be considered a more appropriate methodology that allows the comparison of lethality between a wild type (normally susceptible) strain and potentially resistant strains. It is important that a neutralising agent is used when determining the MBC to avoid getting an overestimation of the lethal concentration. Several studies have used the MBC as an indication of biocide resistance (Knapp *et al.*, 2013, Kawamura-Sato *et al.*, 2010, Rose *et al.*, 2009). For example Knapp *et al.,* (2013)

compared the MBC of CHG and BZC before and after exposure of *Burkholderia lata* to low concentrations of these biocides, in order to predict biocide resistance in this species. The MBC is a simple, straightforward measurement that allows a large number of strains/biocidal products to be tested at one time and in a 24 hour time period, allowing rapid data generation. It is also simple to compare MBC values and in-use biocide concentrations in order to identify resistant strains.

1.7.1.2 Suspension tests & surface disinfection tests

Suspension tests are a simple way to evaluate the antibacterial activity of a biocide allowing the user to determine the effect of concentration and contact time on biocide efficacy. There are standard protocols available for suspension tests, e.g. BS EN 1276 (2009) that provide guidelines on temperature and pH maintenance, biocide diluents and appropriate neutralising agents. One of the biggest limitations of a suspension test is that the neutralisation and enumeration steps result in dilution of the bacterial suspension which creates a limit of detection with regards to the enumeration of surviving organisms. It also must be taken in to account that biocides have a wide variety of applications, and that in many cases a suspension test alone may not be efficient in the evaluation of the bactericidal activity of a biocide, nor may it be an appropriate reflection of the *in vivo* conditions in which the biocide would be used.

Alternatively a surface disinfection test could be used to look at the bactericidal activity of a biocide against bacteria colonising surfaces rather than those held in suspension. This involves the drying of washed bacterial cells before exposure to a particular biocide, in order to represent in-use conditions (Ojeil *et al.*, 2013, Thomas *et al.*, 2005). For example Ojeil *et al*., (2013) used a surface disinfection test to determine the efficacy of copper alloy and stainless steel surfaces in removing bacterial bioburden. A surface test can also be used

to look at the effect of bacterial exposure to residual concentrations of biocide remaining after a cleaning procedure has taken place (Thomas *et al.*, 2005).

1.7.1.3 Growth kinetics

Experiments involving growth kinetics and looking at growth curves cannot directly measure bacterial resistance to biocides, but may give an indication of a trend towards decreased susceptibility. An extended lag phase before entry in to exponential phase in biocide-exposed bacteria may be indicative of the initial inhibitory effects of the biocide on the bacterial cell, followed by putative adaptation to the presence of the biocide and the ability to of the bacterium grow (Whitehead *et al.*, 2011). The comparison of growth kinetics between biocide-exposed and non-biocide-exposed bacteria can also give an indication of any inhibitory effects the biocide has on growth and the severity of these. Growth curve analysis can therefore be used as an indicator of a trend towards reduced susceptibility (Whitehead *et al.*, 2011) to a particular antimicrobial product but may be more useful when used in combination with other tests such as MIC and MBC determination.

1.7.1.4 Biofilm susceptibility tests

Bacteria frequently exist in biofilms *in situ.* Despite this there is no standard protocol for measuring the susceptibility of bacteria within a biofilm to biocides (SCENIHR, 2010). This is likely to be due to the fact that it is difficult to mimic *in situ* conditions in the laboratory and that there numerous available methods that can be used to grow biofilms (McBain, 2009). The use of different methods to grow biofilms is likely to result in variability in results obtained from biocide susceptibility tests, and the design of a standard protocol to measure the effect of biocide exposure on biofilms would therefore prove useful in

providing susceptibility data using parameters representative of *in vivo* conditions. This protocol could include; observation of biofilm development after biocide exposure, investigation into the role of persister cells within the biofilm and susceptibility of re-grown biofilms after initial biocide exposure (SCENIHR, 2010).

1.7.2 Molecular Protocols

Alternative methods used to measure or identify biocide resistance in bacteria involve looking at changes in the transcriptome or proteome. A change in the transcriptome, i.e. an increase or decrease in the amount of mRNA present, may be indicative of increased or decreased expression of a particular gene. However, changes in the amount of mRNA present do not always lead to changes in the proteome, as the amount of protein present is more reflective of the cell conditions and stability of the protein itself. Proteomics allows the validation of changes seen in the transcriptome and also allows the investigation of post-translational modification of proteins which may have an effect on their activity in the cell.

1.7.2.1 Microarrays

Microarrays allow the user to observe genome wide changes in gene expression and can uncover patterns of genetic activity, help provide new understanding of gene functions and, in the case of biocide resistance, generate insight into transcriptional processes and biological mechanisms (Ricke et al., 2013). Microarray technology involves the fluorescent labelling of control and treated cDNA samples that have been reversed transcribed from RNA isolated from the relevant organism/sample. The labelled cDNA is then hybridised to a microarray slide containing oligonucleotides representing all the genes present in the genome of a particular organism (in the case of bacteria) (Leveque et al., 2013). Fold

increases and decreases in gene expression in control and biocide-treated samples can be compared allowing the user to identify groups of genes associated with a particular response to biocide exposure. Microarray data may also provide an insight into putative mechanisms of biocide resistance which when combined with data from other susceptibility tests may provide more information on how and why a particular bacterium has become biocide resistant. Despite providing a large amount of data on genome-wide changes after biocide exposure, microarrays are expensive and time consuming procedures. Data analysis itself may also prove time consuming and reasons behind changes in certain groups of genes may be difficult to ascertain, particularly if the mechanism of action of the biocide tested is unknown and putative resistance mechanisms in the test organism have not been determined. It may therefore be useful to study selected groups of genes that have a potential association with observed biocide resistance, using techniques such as real-time and semi-quantitative polymerase chain reaction (PCR).

1.7.2.2 Real-time & semi-quantitative PCR

PCR can be used to validate microarray observations or to investigate changes in the expression of specific genes of interest. PCR involves the amplification of a particular gene using forward and reverse primers specific to that gene sequence, and a DNA polymerase that makes copies of the gene sequence. Semi-quantitative PCR products are electrophoresed on an agarose gel and stained with a dye such as ethidium bromide that binds DNA, appearing as bands on the gel (Davis, 2014). Semi-quantitative PCR therefore does not allow precise quantification of the product.

Real-time PCR involves the staining of a cDNA copy of extracted RNA with a dye that fluoresces when bound to double-stranded DNA (e.g. SYBR green) – i.e. fluorescence occurs

in real time, as the gene of interest is amplified by DNA polymerase. An increase in fluorescence is therefore associated with increased amplification of the gene. Real-time PCR reactions result in the production of Ct values – this is the point where fluorescence crosses a threshold level (set by the user) and becomes exponential (Davis, 2014). A melting point analysis of PCR products can also be carried out when all reaction cycles are complete, to check for contaminants or primer dimers. The PCR product will be a specific length and therefore have a specific melting temperature. Fold increases or decreases in gene expression can then be calculated, taking in to account the efficiency of the primers used (Pfaffl, 2001), therefore making real-time PCR a quantitative method of studying changes in gene expression.

1.7.2.3 Proteomics

Outer membrane or total proteins can be extracted from treated and untreated bacterial cells and electrophoresed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins can be separated by mass (one dimension) or mass and isoelectric point (2 dimensions). The staining of proteins with stains such as Coomassie stain or silver nitrate (binds cysteine) stain allows the visualisation of proteins on the gel. Comparisons can then be made between proteins present/absent in biocide- treated and untreated bacteria. This technique alone does not allow the identification of proteins. However when used in combination with mass spectrometry, proteins can be identified (Van Oudenhove and Devreese, 2013). Specific proteins can also be detected using Western blotting. This involves gel electrophoresis of proteins, before their transfer to a nitrocellulose membrane where they are stained with an antibody specific to the protein of interest (Patton, 2002). Western blotting is useful if one has knowledge of particular proteins associated with biocide resistance, whereas one or two dimensional SDS-PAGE is useful for the identification of changes in total protein.

1.7.3 Measuring susceptibility to antibiotics

There are several standard protocols available for the measurement of bacterial susceptibility to antibiotics. These are available from the British Society for Antimicrobial Chemotherapy (BSAC) (Andrews, 2009) and the Clinical and Laboratory Standards Institute (www.CLSI.org accessed 11_03_14), amongst others. These protocols include susceptibility breakpoints that give an indication of how clinically sensitive or resistant an individual bacterial species is to a particular antibiotic. The limitations of these guidelines are that susceptibility breakpoints are not always provided for certain species of bacteria (e.g. BSAC disk diffusion protocol does not provide breakpoints for *Burkholderia* spp.). There is at present no standard protocol available for measuring the capability of biocides to confer cross resistance to antibiotics, but such a protocol would be useful due to the numerous reports of antibiotic resistance as a result of biocide exposure (Knapp *et al.*, 2013, Christensen *et al.*, 2011, Whitehead *et al.*, 2011, Randall *et al.*, 2007).

1.8 Generating bacterial resistance to biocides

There is no standard protocol for the generation of bacteria that are resistant to a particular biocide. The generation of biocide-resistant mutants is useful as it allows the exploration of mechanisms behind resistance to a particular biocide, investigation in to the effect of the developed resistance on virulence or growth and the identification of any cross- resistance to other biocides or antibiotics.

A common *in vitro* method that has successfully generated biocide resistant bacteria involves the stepwise passaging of bacteria through gradually increasing concentrations of a particular biocide, either on agar or in broth. For example Pagedar *et al*., (2012) produced *E. coli* mutants adapted to BZC via 24 h subculture of isolates in nutrient broth supplemented with gradually increasing concentrations of BZC. They reported that

resistant isolates showed a significant increase in efflux pump activity and were better at biofilm formation than non-resistant isolates. This demonstrates how the *in vitro* generation of biocide resistant mutants provides useful information on the effects of biocide exposure and potential mechanisms behind the resistance, and highlights the usefulness of developing a standard protocol for the generation of biocide-resistant mutants. However it must be noted that the bacterial species and mechanism of action of the biocide used may have an effect on the successful generation of resistant mutants. This means that a single, universal method may not be possible, as ideally the method would be based on conditions under which the biocidal product is used (Maillard and Denyer, 2009). Parameters that could be kept consistent could include the preparation of the test inoculum, the number of repeats performed, appropriate neutralisation of the test product and investigation in to resistance to the in-use concentration of the product (Maillard and Denyer, 2009).

1.9 Biocides used in this study

1.9.1 Chlorhexidine digluconate (CHG)

Chlorhexidine has been used as a topical antiseptic for over 50 years, and is effective against Gram-positive, Gram-negative and non-spore forming bacteria, yeast and lipid envelope viruses including HIV (Edmiston *et al.*, 2013). Chlorhexidine is a cationic, biguanide molecule and is available in the acetate, gluconate and hydrochloride forms. As well as its use as a topical antiseptic it is incorporated in to household disinfectants, hand washes, mouthwashes and surgical scrubs at varying concentrations depending on the application. For example antibacterial mouthwashes contain 0.2 % w/v CHG whereas 'Hibiscrub' hand disinfectant contains 4 % w/v CHG (Thomas *et al.*, 2000).

The mode of action of chlorhexidine is concentration dependent. At lower concentrations it is bacteriostatic, causing the leakage of potassium and phosphorus from the bacterial cell and inhibiting bacterial growth (Hugo and Longworth, 1964). The positive charge of the cationic chlorhexidine molecule is thought to assist its interaction with the negatively charged bacterial cell though it is not known exactly how it partitions in to the bacterial cell membrane. It is thought to occur via the bending of the molecule which allows it to form a wedge shape and interact with the membrane lipids (Komljenovic *et al.*, 2010). This ultimately results in cleavage of the lipid matrix and leakage of the cell contents which is thought to occur within seconds of the biocide making contact with the cell. At much higher concentrations chlorhexidine causes cell death via the precipitation of cell cytoplasmic contents (Edmiston *et al.*, 2013).

There are an increasing number of *in vivo* reports of bacterial resistance to chlorhexidine at in-use concentrations in the clinical and domestic environments. Smith *et al.,* (2013) tested the efficacy of over the counter mouthwashes containing chlorhexidine against 28 clinical MRSA biofilm isolates from the oral cavity of patients. They found that none of the mouthwashes tested were able to completely eradicate MRSA biofilms and concluded that this observation may result in problems with infection control and the use of chlorhexidine. Contamination of chlorhexidine-containing products has also been reported and has resulted in outbreaks of bacterial infection. Heo *et al*., (2008) reported an outbreak of *Burkholderia stabilis* bacteraemia in haematological malignancy patients as a result of the contamination of a 0.5 % chlorhexidine-containing solution used to sterilise patient catheters. Lepainteur *et al*., (2013) reported that 12 % of staphylococci isolated from neonates with bloodstream *S. aureus* infections showed reduced susceptibility to chlorhexidine. Hassan *et al.,* (2013) recently reported a chlorhexidine-specific energy-dependent efflux protein (AceI) in *Acinetobacter* spp. which they stated has orthologs in

other proteobacteria such as *E. coli.* Reports such as these listed above are concerning, particularly due to the fact that the chlorhexidine-containing products in question do not appear effective at in-use concentrations.

1.9.2 Benzalkonium chloride (BZC)

BZC is a broad-spectrum, nitrogen-based QAC. QACs are effective against Gram-positive and Gram-negative bacteria and are sporistatic. They also cause membrane damage in yeast cells and are effective against lipid and enveloped viruses (Smith and Hunter, 2008). BZC is commonly used as a preservative in nasal, ophthalmic and otic products (Marple *et al.*, 2004). It is also incorporated in to disinfectants used for surface disinfection and hard surface cleaning and used in the disinfection of unbroken, preoperative skin (Nagai *et al.*, 2003). The concentration at which BZC is used is dependent on the application. Contact lens solutions commonly contain low BZC concentrations of 0.002 – 0.01 %. Concentrations of up to 0.5 % can be found in strong disinfectants and hard surface cleaning products. Concentrations of 10 % or greater are toxic to humans causing irritation to the skin and mucosa, and death if ingested (Bernstein, 2000).

Like chlorhexidine, BZC is a membrane-active biocide (Bragg *et al.*, 2014, Mc Cay *et al.*, 2010) . The following series of events is thought to occur when a bacterial cell is exposed to BZC: (i) cell wall adsorption and penetration; (ii) diffusion of the biocide through the cell wall; (iii) binding to the cytoplasmic membrane; (iv) membrane disorganisation; (v) leakage of cytoplasmic constituents and (vi) cell death (Nagai *et al.*, 2003).

There have been several reports of BZC-resistant bacteria. Of particular concern was the isolation of a strain of *Ps. fluorescens* by Nagai *et al*., (2003) from a 10 % stock solution of BZC. The resistance mechanisms were found to be a decrease in bacterial cell membrane negative charge to reduce biocide absorption, and activity of an energy- dependent efflux

system specific to certain QACs. Frank *et al*., (1976) and Geftic *et al.*, (1979) both reported the contamination of aqueous solutions containing BZC as a preservative agent. Furthermore Mc Cay *et al.,* (2010) commented on the theory described by Gilbert and McBain (2003) that a bacterium is likely to be exposed to a gradient of biocide concentration in any environment and therefore at a given time may be exposed to a sub-lethal biocide concentration. They reported that *Ps. aeruginosa* isolates adapted to BZC via exposure to sub-lethal concentrations were resistant to fluoroquinolone antibiotics, and raised concerns about the possibility of this *in vivo*.

1.10 Knowledge gaps and suggested studies

The Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) produce an annual report that identifies knowledge gaps in antimicrobial research, and suggests studies that should be carried out in order to develop knowledge on antimicrobial use and develop new ideas for combating antimicrobial resistance in bacteria and other microorganisms (SCENIHR, 2010). Some of these are discussed below.

1.10.1 Scientific gaps

Despite the large number of *in vitro* laboratory studies carried out investigating the effect of biocide exposure on biocide and antibiotic resistance, there are insufficient environmental studies being performed that provide epidemiological data on biocide resistance and antibiotic cross resistance as a result of biocide miss-use. The concentration of active biocidal substances present in any one environment e.g. soil, air, ground water, sludge, waste water should be investigated as well as the fate and bioavailability of these active substances under different environmental conditions. These investigations will

provide information on the concentrations of biocidal substances that target and non-target organisms are being exposed to.

A further gap in scientific knowledge is the effect of biocide exposure on the maintenance and transfer of extra-chromosomal elements, such as plasmids and transposons that may carry genes associated with biocide resistance. Exposure of bovine *S. aureus* isolates to QACs has been shown to favour the dissemination and maintenance of a plasmid carrying a *smr* gene which conferred resistance to a QAC (Bjorland *et al.*, 2001). This data suggests that biocide exposure may create a selective pressure for the maintenance and transfer of resistance genes between bacteria of the same species or between different species. Further investigation in to the long-term effects of biocide exposure on the dissemination of resistance genes must be carried out to prevent clonal expansion of resistant organisms.

1.10.2 Technical gaps

Few studies have been performed investigating the environmental stability of biocides under different conditions. Other chemical compounds present in a particular environment may bind and interact with biocides and alter the concentration of biocide that is available. This may result in exposure of bacteria to low biocide concentrations, creating a selective pressure for the survival of resistant organisms. Furthermore, the concentration of biocide metabolites present in the environment has not been investigated, nor has the effect of the type of environment on the metabolites present. There is also no information on the dose-response relationship between target and non-target organisms and biocide metabolites. This type of information may be difficult to obtain, but over a long period of time would help in preventing biocide resistance and potential harm to non-target organisms.

Despite the fact that bacterial biofilms are very common in the environment, the majority of laboratory biocide susceptibility studies are not carried out using biofilms. This may be

due to the fact that it is difficult to mimic *in situ* biofilms in the laboratory. One suggestion is the transfer of biofilms to a fermenter to sustain growth (McBain *et al.*, 2003). There is no current European standard for testing the efficacy of biocides used in a healthcare setting on biofilms. Such a protocol would prove useful as several studies emphasise the limited efficacy of biocides against bacterial biofilms compared to planktonic cells (Wong *et al.*, 2010a, Wong *et al.*, 2010b, Smith and Hunter, 2008).

There is no current standard protocol available for the prediction of bacterial resistance to biocides and potential cross-resistance to antibiotics as a result of biocide exposure. A change in the antimicrobial susceptibility of a bacterium after biocide exposure could provide an estimate of the risk of resistance development and this information now must be provided by product manufacturers according to the new BPR (528/2012) (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2012:167:0001:0123:EN:PDF accessed 11_03_2014). The use of test parameters reflective of the application of the biocidal product (concentration, temperature, formulation etc.) would provide realistic data that could assist in the prediction of bacterial biocide resistance. The generation of reproducible 'baseline' susceptibility data (e.g. MIC/MBC pre-exposure) from both environmental and laboratory strains would provide a useful comparison point for post-exposure MIC/MBC values. This type of protocol would allow biocidal product manufacturers to alter the formulation of a product or the concentration of the active biocide within the product if a high risk of resistance development was predicted.

1.11 Aim of this study

Having considered the knowledge gaps defined by SCENIHR (2010) and the new information on resistance development required by biocidal product manufacturers according to the BPR, the aim of this work was to design a protocol that allows the

prediction of bacterial resistance to biocides and potential cross-resistance to antibiotics after exposure to a particular biocidal product. This was to be achieved via the exploration of different techniques that could be used to identify practical markers of biocide resistance and antibiotic cross-resistance, with a view to compiling a step-by-step protocol that allows the user to predict bacterial resistance to a given biocidal product.

Chapter Two: General Materials and Methods

2.1 Bacterial samples and cultures

The bacterial strains used in this study and their origin are shown in table 2.1. These strains were selected because their genomes have been fully sequenced (Jarvik *et al.*, 2010, Vanlaere *et al.*, 2009) (http://www.sanger.ac.uk/resources/downloads/bacteria/salmonella.html accessed 11/03/2014) making it possible to carry out whole genome analysis.

Table 2.1: Bacterial strains

Strain	**Source**
Salmonella enterica serovar Typhimurium SL1344	Department of Infection and Immunity, The Medical School, University of Birmingham, UK
Salmonella enterica serovar Typhimurium 14028S	Department of Infection and Immunity, The Medical School, University of Birmingham, UK
Burkholderia lata 383	School of Biosciences, Cardiff University, Wales, UK

All culture media was purchased from Fisher Scientific (Loughborough, UK) unless otherwise stated. *Salmonella enterica* serovar Typhimurium strains were cultured on tryptone soya agar (TSA) and were incubated for 24 h at 37°C. *Burkholderia lata* strain 383 was cultured on Basal Salts agar (BSA - see table 2.2) and was incubated for 24 h at 30°C. To make BSA 15 g of agar was added to 1L of the basal salts media (table 2.2). Plates were stored at 4°C (± 1°C).

Table 2.2: Basal Salts Media

All components of the Basal Salts media were purchased from Sigma (Dorset, UK).

Stock	Components	Quantity per litre
Phosphate salts 20x stock	di-Potassium hydrogen orthophosphate trihydrate	85 g
	Sodium di-hydrogen orthophosphate monohydrate	20 g
Ammonium chloride 20x Stock	Ammonium chloride	40 g
Nitrilotriacetic acid 100x Stock	Nitrilotriacetic acid	10 g
Metal salts 100x Stock	Magnesium sulphate heptahydrate	20 g
	Ferrous sulphate heptahydrate	1.2 g
	Manganese sulphate monohydrate	0.3 g
	Zinc Sulphate heptahydrate	0.3 g
	Cobalt sulphate heptahydrate	0.1 g
CAS amino Acids 5% Stock	CAS amino Acids	50 g
Yeast extract 5% Stock	Yeast extract	50g
Glucose 200g/L Stock	Glucose	4 g

2.1.1 Freezer Storage

2.1.1.1 Salmonella enterica serovar Typhimurium

Salmonella enterica serovar Typhimurium strains were stored on protect beads (Fisher Scientific, Loughborough, UK) at -80 °C (± 1°C). Briefly, a large loopful of fresh growth was taken from an agar plate and added to the protect beads. All liquid was removed from the protect bead tube before freezing.

2.1.1.2 Burkholderia lata

Burkholderia lata strains were stored as follows. A swab of fresh growth was taken from an agar plate and re-suspended in 1 mL basal salts broth (BSB) (table 2.2) containing 8 % v/v dimethyl sulfoxide (Sigma, Dorset, UK) and stored at -80°C (± 1°C).

2.1.2 Overnight broth culture

All *Salmonella enterica* serovar Typhimurium strains were cultured in tryptone soya broth (TSB). Briefly, a loopful of fresh growth was taken from an agar plate and used to inoculate 10 mL of TSB, which was incubated for 24 h at 37°C (± 1°C).

Burkholderia strains were cultured in 3 mL BSB (pH 7 ± 0.2) and incubated horizontally in an orbital shaker (150 rpm) for 18 h at 30°C (± 1°C).

2.1.3 Preparation of a bacterial suspension from an overnight culture

All overnight cultures ($1 \times 10^9 – 1 \times 10^{10}$ CFU/mL) were centrifuged at 5000 x g for 15 minutes at 18°C (± 1°C). The supernatant was discarded and the pellet re-suspended in 10 mL of tryptone sodium chloride (TSC) buffer (0.4 g tryptone, 3.4 g sodium chloride, 400 mL deionised water (diH_20)) unless otherwise stated.

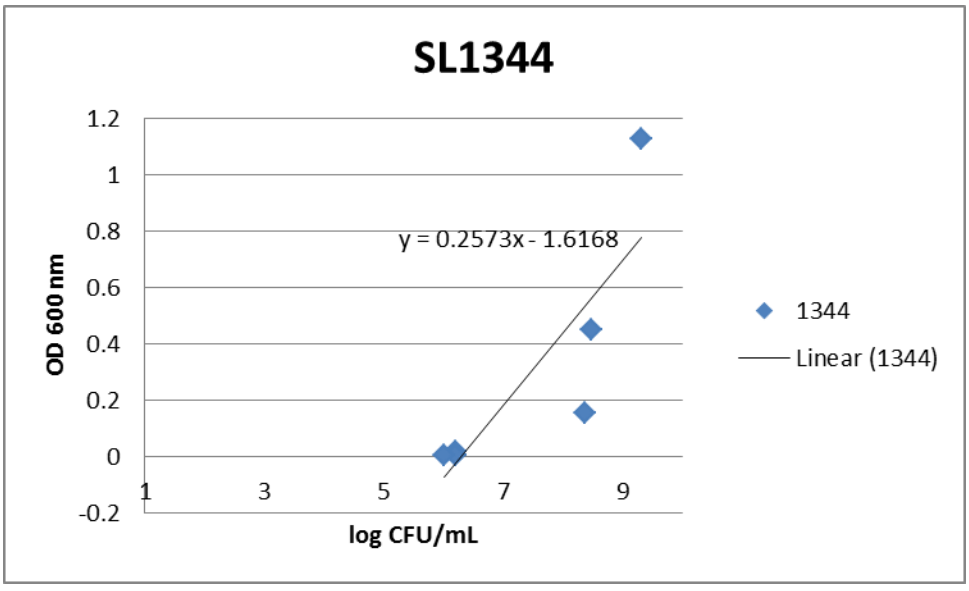
2.1.4 Viable Counts

Viable counts were performed before and after testing to determine the concentration of a bacterial suspension. This was done using the drop count method (Miles *et al.*, 1938)**.** Briefly, 100 µL of the neat bacterial suspension was added to a sterile microcentrifuge tube containing 900 µL of TSC buffer and vortex mixed. One hundred µL of this suspension was then transferred to another sterile microcentrifuge tube containing 900 µL of TSC buffer. This was repeated until ten serial dilutions had been performed. Ten µL of each dilution was plated on to the appropriate media in triplicate. Plates were left to dry before incubation for 24 h at the appropriate temperature. A mean colony count was determined for the dilution that produced between 3 and 50 colonies.

2.1.5 Optical density vs. Total viable count

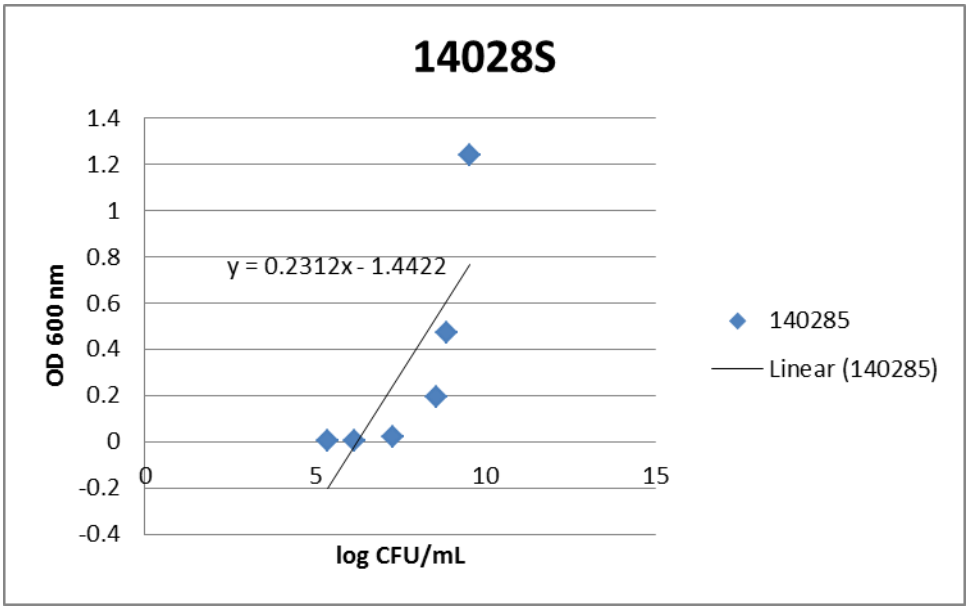
In order to determine at which optical density (600 nm) 1-5 x 10^8 CFU/mL were present, an optical density *vs*. total viable count (TVC) experiment was performed for each strain used. Briefly, bacterial suspensions were produced from overnight broth cultures and four 1 in 10 serial dilutions of these suspensions in TSC were prepared. The OD_{600} of each suspension was recorded using an Ultra*pro* 3000 spectrophotometer (GE Healthcare, UK). Viable counts of each suspension were then performed using the drop count method (2.1.4). OD_{600} *vs*. TVC graphs (shown in figures 2.1 a, b and c) for *S. enterica* strains SL1344 and 14028S and *B. lata* strain 383 were produced and the OD_{600} range was calculated using the equation from the line of best fit displayed on the graph, where y = OD_{600} and x = log CFU/mL.

Figure 2.1 (a): Optical density vs. total viable count for S. enterica strain SL1344



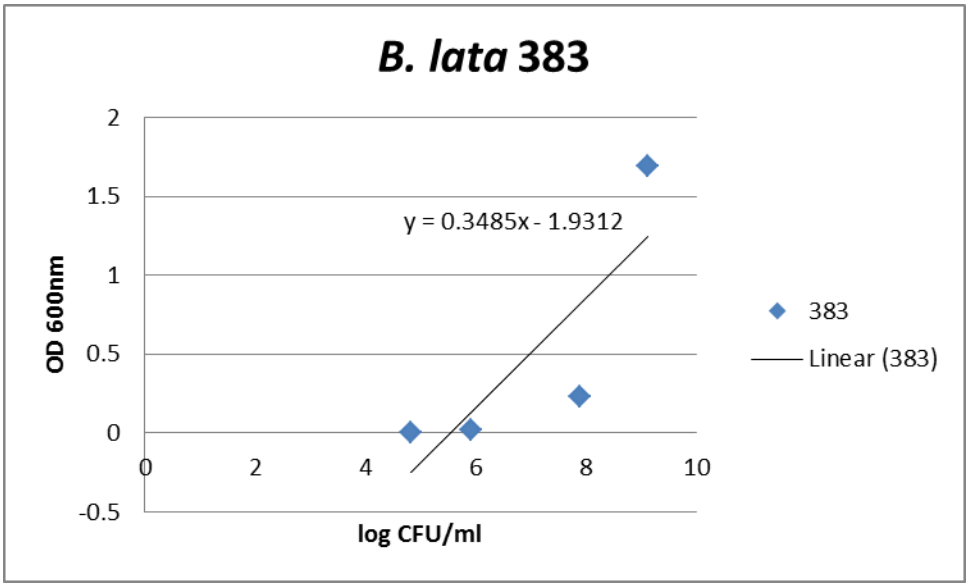
The OD_{600} range that gave 1-5 x 10^8 CFU/mL for strain SL1344 was calculated as 0.45 – 0.6.

Figure 2.1 (b): Optical density vs. total viable count for S. enterica strain 14028



The OD_{600} range that gave 1-5 x 10^8 CFU/mL for strain 14028S was calculated as 0.4 – 0.57.

Figure 2.1 (c): Optical density vs. total viable count for B. lata strain 383



The OD_{600} range that gave 1-5 x 10^8 CFU/mL for strain 383 was calculated as 0.85 – 1.1.

2.2 Biocides

The biocides used were chlorhexidine gluconate (CHG) (20 % solution in water) and benzalkonium chloride (solid crystals) (BZC) (Sigma, Dorset, UK).

2.3 Neutraliser

The neutraliser used was composed of Tween 80 (30 g/L) (Fisher Scientific, Loughborough, UK), azolectin (3 g/L) (Sigma, Dorset UK) and diH_20.

2.3.1 Neutraliser Toxicity

The toxicity of the neutraliser to all strains of bacteria was tested as follows. A bacterial suspension produced from an overnight culture was standardised to 1 x 10^8 CFU/mL. One mL of this suspension was added to 9 mL of neutraliser, vortex mixed and left for 5 min. A control experiment was performed alongside this where 1 mL of bacterial suspension was added to 9 mL of diH_20.

Viable counts were performed on test and control suspensions using the drop count method (2.1.4). Test and control counts were compared to determine if exposure to neutraliser caused any significant decrease in CFU/mL. The neutraliser was considered toxic if ≥ 1 $\log_{10}$ decrease was observed in the test colony count according to the BS EN 1276 (2009) suspension testing protocol.

2.3.2 Neutraliser Efficacy

The ability of the neutraliser to quench the activity of CHG and BZC was tested as follows: One mL of the biocide at the highest concentration used (CHG – 5%, BZC – 12.5%) was added to 8 mL of neutraliser and vortex mixed. One mL of a bacterial suspension containing

1 x 10^8 CFU/mL was then added and the suspension vortex mixed and left for 5 min. A control experiment was performed alongside this using 8 mL diH_20 instead of neutraliser. Viable counts of both control and test suspensions were performed using the drop count method (2.1.4). The neutraliser was considered effective if ≤ 1 $\log_{10}$ difference in CFU/mL was observed between initial counts and counts taken after bacterial exposure to biocide treated with neutraliser according to the BS EN 1276 (2009) suspension testing protocol.

2.4 Biocide minimum inhibitory concentration (MIC)

The MIC of CHG and BZC was determined for all bacterial strains, following the BS EN ISO: 20776-1 (2006) protocol.

In brief, 50 µL of the appropriate broth was added to wells 2-12 of a 96 well microtitre plate. Fifty µL of the chosen biocide was then double diluted across wells 1-11. Column 12 was a control column containing no biocide (see figure 2.2). The concentration range for CHG was 1 – 0.0009 % and for BZC was 0.25 – 0.00015 %. These incorporated in-use concentrations of both biocides (Berstein, 2000; Thomas *et al*., 2000).

Suspensions of all bacterial strains were standardised to 1 x 10^8 CFU/mL in TSB. Fifty µL of an individual strain was then added to all wells in three rows of the plate (i.e. in triplicate). The plate was then covered with a sterile lid and incubated for 24 h at the appropriate temperature with shaking at 150 rpm. The MIC was the lowest concentration at which no bacterial growth was observed on the microtitre plate. This was observed visually.

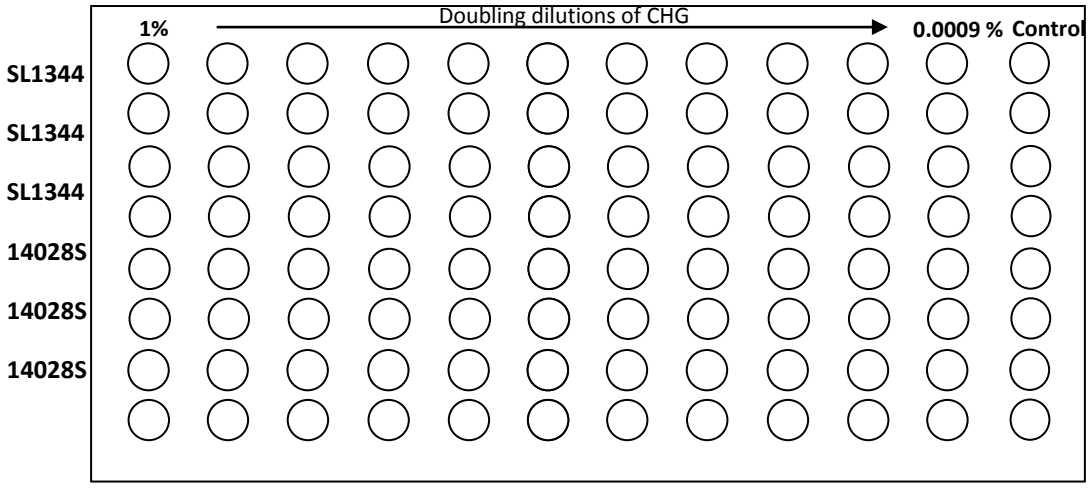


Figure 2.2: Example MIC plate for S. enterica strains SL1344 and 14028S

2.5 Minimum bactericidal concentration (MBC)

The MBC of CHG and BZC was determined as follows. Twenty µL of suspension was removed from each well of the MIC 96 well plate where no bacterial growth was observed and the two lowest concentrations at which growth was observed, and added to 180 µL of neutraliser. This was vortex mixed and 25 µL was spotted on to the appropriate agar. Plates were incubated for 24 h at the appropriate temperature before being observed for bacterial growth. The MBC was the lowest concentration where no bacterial growth was observed on the agar plate.

2.6 Antibiotic susceptibility testing

Susceptibility to clinically relevant antibiotics was determined following the BSAC Disc Diffusion Method for Antimicrobial Susceptibility Testing Version 9.1 (Andrews, 2009). All antibiotics were purchased from Oxoid, Basingstoke, UK. Briefly, bacterial suspensions in diH_20 were prepared from overnight cultures of each strain grown in Iso-Sensitest Broth and standardised to 1 x 10^8 CFU/mL. A 1:100 dilution of this suspension in diH_20 was then

made. A sterile swab was used to inoculate an Iso-Sensitest Agar plate with the diluted suspension. The plate was left to dry for no more than 15 min before being inoculated with discs containing the relevant antibiotics and incubated for 24 h at the appropriate temperature. Zones of inhibition around the antibiotic disks were then measured and values matched up to susceptibility breakpoints provided by the protocol.

2.7 Suspension Testing

2.7.1 Exposure to the minimum bactericidal concentration

To observe the effect of contact time on the activity of CHG and BZC, suspension tests were carried out following the British Standard EN 1276 protocol (2009). Briefly, bacterial suspensions in diH_20 produced from overnight cultures were standardised to 1 x 10^8CFU/mL. Viable counts were performed on these suspensions using the drop count method (2.1.4). One mL of standardised suspension was added to 9 mL of biocide (diluted in diH_20) at 1.25 times the minimum bactericidal concentration. After exposure for 1, 2.5, 4, 5, 10, 30, 40 and 60 minutes, 1 mL of this suspension was removed and added to 9 mL of neutraliser. Viable counts using the drop count method (2.1.4) were then carried out to enumerate surviving organisms.

2.7.2 Exposure to a range of low concentrations

Suspension tests were carried out as described in section 2.7.1 except the exposure time was limited to 5 min. This contact time was chosen in order to obtain a 2-3 $\log_{10}$ reduction in CFU/mL, leaving sufficient survivors for further testing. Concentrations ranged from 0.00001 – 0.0005 % CHG or BZC. The MIC, MBC and antibiotic susceptibility of test organisms were determined before and after 5 min exposure using the methods described in sections 2.4, 2.5 and 2.6.

2.8 Phenotype stability testing

The stability of any changes in biocide and antibiotic susceptibility observed after 5 min biocide exposure was determined through continuous subculture of surviving bacteria in biocide-free broth or broth supplemented with CHG or BZC. Subcultures were made every 24 h and biocide MIC/MBC and antibiotic susceptibility were determined after 1, 5 and ten passages. A culture purity check was performed after each subculture.

2.9 Efflux assays

The protocol for efflux assays was based on that used by Whitehead et al., (2011). One hundred and twenty µL from an overnight culture of each strain was added to 3 mL of fresh broth (either biocide-free or supplemented with a low concentration of CHG or BZC). These were then incubated at the appropriate temperature with shaking at 150 rpm until mid-logarithmic growth phase was achieved. Mid-log phase cells were harvested by centrifugation at 5000 x g for 10 min at room temperature and the supernatant discarded. The remaining cell pellet was re-suspended in 3 mL of TSC buffer. Samples were then adjusted to an approximate OD_{600} of 0.1 (approx 1 x 10^6 CFU/mL), using TSC as a diluent.

Hoechst 33342 dye (Sigma, Dorset, UK) was added to each of the diluted suspensions to a final concentration of 2.5 µM and suspensions were left for 5 min. Cells were then collected again by centrifugation at 5000 x g for 10 min at room temperature and the supernatant (excess dye) was removed. The cell pellet was then re-suspended in 3 mL TSC buffer or 3 mL of TSC buffer containing one of the following efflux pump inhibitors (EPIs) (all purchased from Sigma, Dorset, UK): phenyl-arginine-β-napthylamide (PaβN) (10 mg/L), verapamil (50 mg/L), 1-(1-napthylmethyl) piperazine (1-(1-NP) (10 mg/L). The toxicity of the inhibitors to each bacterial strain was tested prior to the experiment following the same method used for neutraliser toxicity testing (section 2.3.1). PaβN and 1-(1-NP) target RND type efflux

pumps and are broad spectrum inhibitors. Verapamil is a calcium ion blocker and inhibits ABC transporters. A range of inhibitors were used to cover several types of efflux pump. A positive control with no efflux activity (consisting of cells heated to 95 °C for 5 min) was included. One hundred and eighty µL of each suspension was then added to a 96 well black plate, and the fluorescence (Ex 340 nm, Em 510 nm) was measured using a Fluostar Optima (BMG Labtech, Aylesbury, UK).

2.10 Light scattering experiments

To assess the presence/absence of bacterial aggregates in biocide-treated and untreated samples, an N4 Plus Dynamic Light Scattering machine (Coulter, High Wycombe, UK) was used. Bacterial suspensions prepared from overnight cultures were standardised to 1 x 10^8 CFU/mL in diH_20. For untreated samples 1 mL of the suspension was transferred to a cuvette with 4 clear sides and placed in the N4 Plus machine. For biocide-treated samples 1 mL of the suspension was added to 9 mL of the biocide at 1.25 times the minimum bactericidal concentration before vortex mixing. After 1 min 1 mL of this suspension was transferred to a cuvette and placed in the machine. The N4 Plus measures different particle sizes within the sample and produces a mean particle diameter value (nm). Each run lasted approx 13 min, making total biocide exposure time 15 min.

2.11 RNA extraction for real-time PCR

2.11.1 Harvesting bacterial cells

Flasks containing 25 mL of the appropriate broth were inoculated with 2 x 10^8 CFU/mL of a bacterial strain and incubated at the appropriate temperature with shaking at 150 rpm. For biocide-exposed organisms broth was supplemented with a low concentration of CHG or

BZC. The OD_{600} nm was measured hourly and bacterial cells were harvested at mid-logarithmic growth phase and aliquoted in to microcentrifuge tubes before immediate snap-freezing in liquid nitrogen. Aliquots were then centrifuged at 20,000 x g at 4 ˚C for 1 min. The supernatant was discarded and pellets were frozen at -80 ˚C. In the case of bacterial cells exposed to biocides for 5 min (following the method described in section 2.7.2) tubes were centrifuged at 5000 x g for 10 min and the supernatant discarded. RNA was then extracted from the remaining pellet.

2.11.2 RNA extraction

Total RNA was extracted from bacterial cells using the Ribopure™ Bacteria Kit (Ambion, Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. This method includes disruption of bacterial cell walls by beating cells mixed with RNAwiz and Zirconia beads, a phenol extraction of the lysate and glass-fibre filter purification of the RNA. A 2 µL aliquot of extracted RNA was taken and the quantity and purity of the RNA present was analysed using a Nanovue spectrophotometer (GE Healthcare, Buckinghamshire, UK).

2.12 cDNA synthesis for real-time PCR

cDNA was synthesised from extracted RNA using an Improm-II™ Reverse Transcription System (Promega, Southampton, UK). The kit encompassed controls including a negative control (no-RNA) to check for template contamination and a positive control (included in the kit) to check the activity of the reverse transcriptase enzyme. For experimental samples 3 µL of template RNA were combined with 1 µL of random primers (supplied with kit) and made up to 5 µL with nuclease free water (NFW). Negative controls contained no RNA

template. Experimental reaction mixes were first denatured for 5 min at 70 °C and then chilled in ice water for 5 min. cDNA was then synthesised from RNA in the following reaction mixture: 4 µL of reaction buffer, 4.8 µL of 25 mM $MgCl_2$, 1 µL dNTP mix, 0.5 µL of RNasin ribonuclease inhibitor, 1 µL of Improm–II reverse transcriptase (or NFW for no-RT controls). This mixture was made up to 15 µL with NFW. Five µL of the denatured random primer/RNA mix was then added to the reaction mixture (except the no-RNA control) and placed in a thermal cycler. The programme used was as follows: 5 min at 25 °C, extension for 60 min at 42 °C and 15 min at 70 °C. cDNA was stored at -20 °C for further use.

2.13 Real-time PCR reaction conditions

Reactions were performed in triplicate using an Absolute QPCR SYBR Green Kit (ABgene, Epsom, UK). Two µL of cDNA (generated as described in section 2.12) was added to 10 µL of SYBR Green mix, 0.4 µL of forward and reverse primers (70 nM) and made up to 20 µL with NFW. A standard curve was generated to assess the efficiency of the amplification using 1 in 5, 1 in 25 and 1 in 50 dilutions of the cDNA template. No-RT and no-cDNA controls were included to control for genomic DNA contamination and for primer-dimers respectively. Expression levels of a house-keeping gene were used to normalise data. Reactions were run on a MJ Research PTC-200 thermal cycler (DNA Engine Opticon, Bio-Rad Laboratories, Hertfordshire, UK). The programme used was as follows: 15 min thermal activation of the modified Taq-polymerase at 95 °C, 50 cycles of 15 s at 95 °C, 30 s at the appropriate primer annealing temperature and 30 s at 72 °C. A melting curve analysis was performed at the end of the reaction to test for the specific PCR product.

2.14 Real-time PCR analysis – the Pfaffl method

The Pfaffl method (Pfaffl, 2001) takes in to account the efficiency of the real-time PCR reaction and therefore gives a more accurate quantification of mRNA present in the original sample compared with methods that assume 100% efficiency of the reaction (e.g. delta-delta Ct method). A standard curve was produced from Ct values obtained through performing real-time PCR reactions using a range of concentrations of cDNA template. The slope of the standard curve is used to calculate the efficiency using the following formula.

$$\text{Efficiency (E)} = 10^{-1/\text{slope}}$$

The difference in gene expression was then calculated using the following formula:

$$\text{Ration} = \frac{(E_{target})^{\Delta CT \text{ Target (control-test)}}}{(E_{reference})^{\Delta CT \text{ Reference (control-test)}}}$$

Here E_{target} is the amplification efficiency of the target gene transcript and $E_{reference}$ is the amplification efficiency of the reference gene transcript.

Chapter Three: Salmonella enterica serovar Typhimurium as a model organism for predicting biocide resistance

3.1 Introduction

Salmonella enterica is a Gram-negative bacterium commonly associated with salmonellosis or 'food poisoning' in the human population (Wang *et al.*, 2010). *Salmonella* infection was reported as the second most commonly identified gastrointestinal infection in the EU in 2012 (http://ecdc.europa.eu/en/publications/Publications/Annual-Epidemiological-Report-2012.pdf accessed 11/03/2014). Although *Salmonella* infection is not normally treated with antibiotics, severe systemic infections are treated using quinolone and fluoroquinolone antibiotics e.g. ciprofloxacin (Rushdy *et al.*, 2013). In the past 30 years there has been an increase in the number of multi-drug resistant *Salmonella* strains reported in the USA and worldwide (Kautz *et al.*, 2013).

3.1.1 Salmonella enterica in the clinical environment

In 2010, 102,323 confirmed cases of salmonellosis were reported by 29 EU countries (http://ecdc.europa.eu/en/publications/Publications/Annual-Epidemiological-Report-2012.pdf accessed 11/03/2014). When *Salmonella* spp. come into contact with human intestinal epithelial cells, they cause a ruffling of the cell membrane via stimulation of changes to the actin cytoskeleton that ultimately results in internalisation of the bacterium into the cell. Once inside the cell *Salmonella* use a type III secretion system to induce the activation of transcription factors that stimulate the production of inflammatory cytokines. They also induce the secretion of Cl- ions from the cell. This results in subsequent loss of water from the cell, and inflammatory diarrhoea that is characteristic of *Salmonella* infection (Galan, 1998).

There are over 2,463 serovars of *Salmonella enterica,* but the two most associated with food-borne illness are *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis (Wang *et al.*, 2010). Interestingly these two different serovars have been reported to be associated with different food types. *S. enterica* serovar Enteritidis infections are most often acquired from

contaminated eggs whereas *S. enterica* serovar Typhimurium is more frequently associated with the contamination of poultry meat and drinking water (Wang *et al.*, 2010). Figure 3.1 shows the different serovars of *Salmonella* reported to the Health Protection Agency (HPA) in England and Wales between 2000 and 2010.

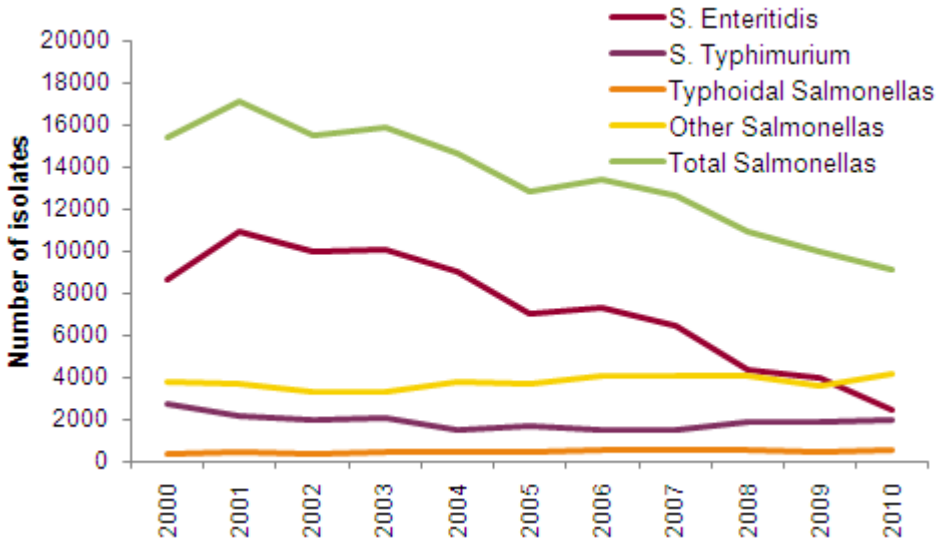


Figure 3.1: All human Salmonella isolates reported to the Health Protection Agency Centre for Infections. England and Wales, 2000 –2010

http://www.hpa.org.uk/Topics/InfectiousDiseases/InfectionsAZ/Salmonella/EpidemiologicalData/salmDataHuman/ (accessed: 11/03/2014).

From figure 3.1 it can be seen that the number of reported isolates has decreased over the ten year period. *S. enterica* serovar Enteritidis was responsible for the greatest number of reported infections, whereas *S. enterica* serovar Typhimurium was responsible for a much smaller number. Interestingly the number of *S. enterica* serovar Typhimurium infections has remained consistent over the ten year period. The general decline in reported salmonellosis cases is thought to be due to the implementation of control programs in the poultry industry, particularly in laying hens. This said the majority of cases of *Salmonella* infection in the EU are still associated with contaminated eggs

(http://ecdc.europa.eu/en/publications/Publications/Annual-Epidemiological-Report-2012.pdf accessed 11/03/2014). For this reason it is essential to control the spread of *Salmonella* amongst farmed livestock and in the food industry.

3.1.2 Salmonella enterica in the industrial environment

In order to limit the spread of multi-drug resistant isolates in the food chain, regulations have now been introduced that prevent the use of antibiotics as growth promoters in livestock (Karatzas *et al.*, 2007). This in turn has resulted in the increased use of biocidal products to clean/disinfect animal houses, as preservatives in animal feed and in all steps of the food production chain (Condell *et al.*, 2012a). This increased biocide use has led to concerns that biocide exposure may be selecting for antibiotic-resistant isolates. It has been reported that exposure of *S. enterica* to various biocides (e.g. Virkon, Trigene, triclosan) at the recommended in-use concentration has resulted in stable multi-drug resistance in surviving organisms (Whitehead *et al.*, 2011) and increased antibiotic MICs (Karatzas *et al.*, 2007). Despite these findings being obtained *in vitro*, there have been numerous reports of multi-drug resistant *Salmonella* spp. isolated from patients in the clinical environment (Rushdy *et al.*, 2013, Akiyama and Khan, 2012, Giraud *et al.*, 2012, Yoon *et al.*, 2009). Furthermore, outbreaks of serious infections have been observed due to improper biocide use (Duarte *et al.*, 2009) so it is possible that increased biocide use may increase the risk of isolating multi-drug resistant *Salmonella* isolates from an industrial environment.

3.1.3 Mechanisms of antimicrobial resistance in Salmonella enterica

Antibiotic resistance in *salmonella*, particularly resistance to quinolones and fluoroquinolones is often attributed to chromosomal mutations in ‘quinolone resistance-determining regions’

(QRDRs) in *gyrA/B* (encoding DNA gyrase) or *parC/E* (encoding topoisomerase IV) (Kautz *et al.*, 2013). These mutations prevent the inhibition of DNA gyrase and DNA topoisomerase IV by quinolone/fluoroquinolone antibiotics allowing DNA super-coiling and synthesis to continue to take place in the bacterial cell (Hawkey, 2003).

Aside from mutations in specific antibiotic cellular targets, multiple antibiotic resistance in *S. enterica* has been attributed to over-expression of efflux systems, particularly the well-characterised AcrAB-TolC RND type efflux system (Baugh *et al.*, 2013, Rushdy *et al.*, 2013, Giraud *et al.*, 2000). This efflux system has also been shown to confer resistance to biocides such as QACs and triclosan (Karatzas *et al.*, 2007, Whitehead *et al.*, 2011). Karatzas *et al.,* (2007) showed that over-expression of the *acrB* gene in *S. enterica* serovar Typhimurium resulted in a 4 fold increase in the MIC of chloramphenicol, tetracycline, ampicillin, acriflavine and triclosan, demonstrating that isolates with mutations in efflux-associated genes may be particularly difficult to eradicate if they cause infection in humans or animals. Mutations in genes associated with the regulation of efflux pumps, such as *ramA* and *ramR* have also been shown to confer fluoroquinolone resistance in *S. enterica* serovar Schwarzengrund (Akiyama and Khan, 2012). *Salmonella enterica* spp. have also been shown to alter outer membrane protein composition, and therefore cell permeability to reduce susceptibility to antibiotics (Giraud *et al.*, 2000). Due to the lack of specificity of this alteration to the cell, it is likely that changes in outer membrane composition may confer reduced susceptibility to multiple antimicrobials.

Salmonella enterica spp. also have the ability to form a biofilm, allowing them to persist in industrial and medical settings and on foodstuffs. Biofilms are more difficult to eradicate and concentrations of antibiotics and biocides of up to 1000 times greater than those required to kill planktonic cells may be required to kill cells in a biofilm (Baugh *et al.*, 2013). *S. enterica* serovar Typhi has be found to form biofilms on gallstones in the gall bladder, resulting in

persistent infection and a reservoir for re-infection (Vaishnavi *et al.*, 2005). *Salmonella* biofilms have also been associated with outbreaks of food poisoning linked to salad leaves (Baugh *et al.*, 2013).

3.1.4 Salmonella enterica serovar Typhimurium as a model organism for predicting biocide resistance

S. enterica serovar Typhimurium strains SL1344 and 14028S are wild-type strains of this species and were selected for use as a potential model organism to predict biocide resistance. These strains were chosen due to the fact that their genomes had been fully sequenced (Jarvik *et al.*, 2010)(http://www.sanger.ac.uk/resources/downloads/bacteria/salmonella.html accessed 11/03/2014) making whole genome analysis possible. These strains were also chosen due to the fact that *S. enterica* serovar Typhimurium (alongside *S. enterica* serovar Enteritidis) is most commonly associated with food industry contamination and human food poisoning, and many multi-drug resistant clinical isolates of this species have been observed. Furthermore, the numerous defined resistance mechanisms that *S. enterica* possesses make it a good potential model organism for predicting biocide resistance.

3.1.5 Aims

The principle aim of this chapter was to determine if short term exposure of *S. enterica* serovar Typhimurium strains SL1344 and 14028S to a low concentration of CHG or BZC resulted in a change in the antimicrobial susceptibility of surviving organisms. Further aims were to assess the suitability of strains SL1344 and 14028S as model organisms for predicting biocide resistance, and to assess to suitability of the techniques used in this chapter for predicting biocide resistance.

3.2 Materials and Methods

3.2.1 Suspension testing

In order to determine the effect of contact time on biocide efficacy, suspension tests were carried out following the British Standard EN 1276 protocol (2009) described in chapter 2 section 2.7. Biocides were tested at the minimum bactericidal concentration to investigate the length of time taken to kill all viable bacteria at this concentration.

3.2.2 Antimicrobial susceptibility testing

3.2.2.1 Baseline data

The MIC and MBC of CHG and BZC were determined for strains SL1344 and 14028S as described in chapter 2 sections 2.4 and 2.5. Susceptibility to the following antibiotics was also determined following the BSAC disk diffusion protocol (Andrews, 2009) described in chapter 2 section 2.6: ciprofloxacin (1 µg), chloramphenicol (30 µg), ampicillin (10 µg), ceftriaxone (30 µg) and piperacillin (75 µg).

3.2.2.2 Antimicrobial susceptibility of biocide-exposed organisms

Salmonella strains SL1344 and 14028S were exposed to a range of low CHG and BZC concentrations (0.00001 – 0.0005 %) for 5 min according to the BS EN 1276 (2009) suspension testing protocol described in chapter 2 section 2.7.2. This contact time resulted in a 2-3 $\log_{10}$ reduction in CFU/mL, leaving sufficient survivors for further testing. It was also chosen as it may reflect the in-use contact time of CHG/BZC (e.g. in surface cleaning). After neutralisation, the neutralised suspension was centrifuged at 5000 x g for 10 min and the resulting supernatant discarded. The remaining pellet was then re-suspended in 10 mL TSC buffer. This

suspension was then used in the determination of the MIC, MBC and antibiotic susceptibility of surviving organisms as described previously in chapter 2 sections 2.4 and 2.5.

3.2.2.3 Phenotype stability testing

In order to determine the stability of any changes in biocide susceptibility observed after 5 min biocide exposure, subculture of surviving bacteria through biocide-free TSB and TSB supplemented with CHG or BZC was performed (for SL1344: 0.0004 % CHG and BZC, for 14028S: 0.0001 % CHG and 0.0004 % BZC. Exposure to these concentrations resulted in the greatest changes in MIC and MBC in surviving organisms). Subcultures were made every 24 h and CHG and BZC MIC and MBC were determined after 1, 5 and ten subcultures following the method described in chapter 2 sections 2.4 and 2.5.

3.2.2.4 Data reproducibility

In order to determine the reproducibility of the MIC, MBC and antibiotic susceptibility data obtained from biocide-exposed bacteria, the experiment described in section 3.2.2.2 was performed on 3 separate occasions (each a month apart), each using 3 biological replicates. This resulted in 9 MIC, MBC and antibiotic susceptibility readings for each strain at each concentration tested.

3.2.3 Efflux assays

Efflux assays were carried out according to the protocol by Whitehead et al. (2011) described fully in chapter 2 section 2.9. Strains SL1344 and 14028S were grown to mid-log phase (OD_{600} 0.5-0.7) in TSB supplemented with a low concentration of CHG or BZC (for SL1344 0.0004 % CHG and BZC, for 14028S 0.0004 % BZC and 0.0001 % CHG) or biocide-free TSB. To ensure the

correct growth phase had been reached the optical density of a 1 mL aliquot of each culture was read at 600 nm using an Ultra*pro* 3000 spectrophotometer (GE Healthcare, Amersham, UK). The fluorescence was measured in biocide- treated and un-treated cells using a Fluostar Optima fluorescent plate reader as an indication of the level of efflux activity taking place in the cell. This was also measured in the presence/absence of 3 efflux pump inhibitors (EPIs): verapamil (50 mg/L), phenyl-arginine-beta-napthylamide (PAβN) (10mg/L) or 1-(1-napthylmethyl) piperazine (1-(1-NP)) (10 mg/L) to confirm efflux activity was taking place in the cells. The toxicity of the EPIs to both strains was tested prior to the efflux assays being performed (see chapter 2 section 2.9).

3.2.4 One Dimensional Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

One dimensional SDS-PAGE was performed in order to separate outer membrane proteins (OMPs) extracted from biocide-treated and untreated *S. enterica* and make a comparison between the OMP composition of biocide-treated and untreated bacteria.

3.2.4.1 Outer membrane protein preparation

3.2.4.1.1 Crude protein preparation

S. enterica strains SL1344 and 14028S were grown to mid-log phase (2.5 – 3 h) in TSB in the absence or presence of CHG (0.0004 % for SL1344, 0.0001 % for 14028S) or BZC (0.0004 %). Cells were harvested by centrifugation and re-suspended in 10 mL diH_20. A Complete Mini Protease Inhibitor Cocktail tablet (Roche Applied Science, Burgess Hill, UK) was added to this suspension. Cells were then broken apart by three cycles of 60 s sonication on ice separated by

30 s intervals. Cells suspensions were then centrifuged at 4000 x g for 15 min at 4 °C to remove debris, and the supernatant retained.

3.2.4.1.2 Protein preparation

OMPs were purified from the crude cell extract by addition of sarkosyl solution (Sigma, Dorset, UK) at a final concentration of 1 %. The suspension was then incubated for 30 min at room temperature with agitation every 10 min. Following incubation, suspensions were centrifuged at 26000 x g for 1 h at 4 °C. The resulting supernatant was discarded and the remaining pellet washed three times with 1 mL diH_20. The pellet was then dissolved in 150 µL collection buffer composed of 7 M urea, 1 % ASB-14, 40 mM Tris, 0.5 % ampholytes pH 3-10 (all purchased from Sigma, Dorset, UK). Dissolved protein was divided in to 50 µL aliquots and stored at -80 °C (± 1 °C).

3.2.4.1.3 Protein quantification

Extracted protein was quantified using a Bicinchoninic Acid (BCA) assay. A series of 11 standards containing 0 – 0.5 mg/mL bovine serum albumin (BVSA) (Fisher Scientific, UK) in phosphate buffered saline (PBS) (Fisher Scientific, UK) were prepared. A BCA working solution composed of 1 mL of 4 % (w/v) copper sulphate (Fisher Scientific, UK) and 49 mL BCA (Sigma, UK) was also prepared. Protein samples to be quantified were diluted 1 in 10 in PBS. Two hundred µL of BCA working solution was added to 25 µL of each standard or protein sample in a 96 well plate. The plate was then incubated for 30 min at 37 °C before being placed on ice for 5 min. Absorbance was then read at OD_{562} nm using a Fluostar Optima microplate reader (BMG LabTech, Aylesbury, UK) and a standard curve was constructed by plotting the mean absorbance of the standards against the concentration of protein. The final concentration of

protein samples was corrected for the dilution factor (1/10). All protein quantifications were performed in triplicate.

3.2.4.2 One Dimensional SDS-PAGE

Each protein sample was added to 2 x SDS-PAGE loading buffer (0.09 M Tris-HCL, pH 6.8, 20 % (v/v) glycerol, 2 % (w/v) SDS, 0.02 % (w/v) bromophenol blue, 0.1 M DTT (all purchased from Sigma, Dorset, UK)) at a final concentration of 20 µg in 20 µL. Samples were then heated for 7 min at 95 °C and 20 µL of each sample was loaded in to a 4 – 20 % Criterion™ Precast Mini gel (Bio-Rad, UK). Electrophoresis was carried out in 1 x running buffer (per litre: 14.4 g glycine, 3.03 g Tris Base, 1 g SDS (all purchased from Sigma, Dorset, UK)) at 150 V, 400 mA, 30 watts for approximately 1 h.

3.2.4.3 Colloidal Coomassie Staining

Following one dimensional SDS-PAGE, gels were washed in diH_20 for 2 x 5 min. Gels were then fixed in 100 mL of fixing solution (40 % (v/v) methanol, 10 % (v/v) glacial acetic acid (Fisher, Basingstoke, UK) in diH_20 for 1 h. A 1 x working solution of Brilliant Blue G-Colloidal Concentrate (Sigma, Dorset, UK) was prepared according to the manufacturer's instructions prior to staining and stored at 4 °C. Just before staining 4 parts working solution was combined with 1 part methanol. Gels were stained for 24 h before de-staining with 25 % methanol. Images were then taken using a ChemiDock XRS Plus System (Bio-Rad, Herts, UK) and analysed using ImageLab Software Version 3 (Bio-Rad).

3.2.5 Flow cytometry

3.2.5.1. Principle

Flow cytometry was used to separate biocide-treated bacteria according to their light scattering and fluorescent properties when stained with different fluorophores. This technique did not require bacterial growth on an agar plate and could therefore identify cells that were viable but non-culturable. It allowed the observation of 'shifts' in a bacterial population as a result of biocide exposure and allowed the quantification of bacterial cells that were alive, damaged or dead.

3.2.5.2 Fluorescent dyes

Biocide-treated and untreated bacteria were stained with the fluorescent dyes propidium iodide (PI) and Bis (1,3-dibarbituric acid) trimethine oxanol (BOX) (both from Sigma, Dorset, UK). PI stains the DNA of bacteria that have lost membrane integrity and BOX stains bacteria with collapsed membrane potential. A 200 µg/mL stock of PI in diH_2O was produced and stored at 4 ^{o}C. This was diluted to a working concentration of 5 µg/mL in Dulbecco's PBS (Gibco, Paisley, UK). BOX was made up in dimethyl sulfoxide (DMSO) (Sigma, Dorset, UK) to a stock of 10 mg/mL and stored at -20 ^{o}C. This was diluted to a working concentration of 10 µg/mL in Dulbecco's PBS. One hundred µl of 4 mM ethylenediaminetetraacetic acid (EDTA) (previous work carried out indicated no toxicity to cells from EDTA – Webber MA, personal communication 25/10/2013) (Sigma, Dorset, UK) was added to 9.9 mL of working concentration BOX to aid staining. Both PI and BOX working stocks were made on the day of use and sterilised by passing through a 0.22 µM filter.

3.2.5.3 Sample preparation and analysis

Briefly, strains SL1344 and 14028S were exposed to CHG (SL1344 0.0004 % v/v, 14028S 0.0001 %) and BZC (0.0004 %) for 5 min following the BS EN 1276 suspension testing protocol (2009) described in chapter 2 section 2.7, except no neutralisation step was carried out. SL1344 was also exposed to 80 % ethanol for 5 min for use as a positive control. After exposure, bacterial suspensions were centrifuged at 5000 x g for 10 min and the resulting supernatant discarded. The remaining pellet was re-suspended in 500 µL PBS. Fifty µL of this suspension was added to 1 mL of FACSFlow buffer (BD, Oxford, UK). Fifty µL of PI and 10 µL of BOX were then added to the tube and samples analysed using a FACS ARIA II (BD, Oxford, UK). Cells were illuminated with a 488 nm laser and data from 10,000 particles were collected. Forward- and side-scatter data were collected along with PI fluorescence (red, collected through an LP 565 mirror and BP 610/20 filter) and BOX fluorescence (green, collected through an LP 502 mirror and BP 530/30 filter). Data obtained were plotted on graphs using FACSDiva software version 6.0 (BD, Oxford, UK).

3.2.5.4 Further data analysis

Cells were defined from debris and other particles via the comparison of unstained cells with a PBS control. Once cells had been defined FACSDiva software was used to exclude events not defined as cells so that graphs displayed cells only. Quadrants were defined based on the position of stained un-treated cells and stained ethanol-killed cells on the graph; i.e. the position of untreated cells defined the 'alive' quadrant and the position of killed cells defined the 'dead' quadrant. Once defined, the quadrants remained in the same position throughout the analysis of all samples.

3.2.6 Light scattering experiments

The formation of bacterial aggregates can potentially affect the efficacy of a particular biocide. To determine if bacterial aggregates were present in biocide-treated or untreated cells a series of experiments measuring particle size were carried out, as described in chapter 2 section 2.10. Strain SL1344 was exposed to 0.01 % CHG and 0.003 % BZC and strain 14028S was exposed to 0.006 % CHG and 0.008 % BZC (MBCs). Particle size was measured using an N4 Plus dynamic light scattering machine.

3.2.7 Microarrays

3.2.7.1 RNA extraction

One hundred µL of an overnight culture of SL1344 and 14028S was used to inoculate fresh 25 mL TSB broths (containing 0.0004 % CHG and BZC for SL1344 and 0.0001 % CHG and 0.0004 % BZC for 14028S) and incubated at 37°C with shaking at 150 rpm until cells reached mid-logarithmic growth phase. Four biological replicates were grown for each treatment. An OD (600 nm) of 0.5-0.7 was considered an acceptable indication of mid-log phase. Cells were then harvested through centrifugation at 5000 x g for 10 min and the supernatant discarded. RNA was then extracted using a SV Total RNA Isolation System (Promega, UK) following the manufacturers' instructions. Briefly, samples were homogenised in RNA lysis buffer before transfer to a fresh tube and the addition of RNA dilution buffer. After centrifugation the supernatant was retained and 95 % ethanol was added. This sample was then transferred to a spin column and centrifuged to deposit RNA on to a filter. RNA was then washed before undergoing a DNase treatment to remove any DNA contamination, and then eluted in to a fresh tube. Purified RNA was then stored in the freezer at -80 °C.

3.2.7.2 RNA Bioanalysis

3.2.7.2.1 Gel electrophoresis

Three µL of the resulting RNA was electrophoresed on a 1 % agarose gel. Briefly, 5 µL of extracted RNA was added to 2 µL of 5 x sample loading buffer (Bioline, London, UK). This was then loaded in to a 1 % agarose gel (1 g agarose in 100 mL) containing 5 µL of ethidium bromide (Sigma, Dorset, UK). Seven µL of DNA hyperladder I (Bioline, London, UK) was also loaded in to the gel. RNA was then electrophoresed at 120 V for 45 min. RNA was visualised using GeneSys image acquisition software (Syngene, Cambridge, UK). (See appendix file name: Chapter 3 appendix data > microarray > concentration and purity of extracted RNA).

3.2.7.2.2 Further bioanalysis

One µL of extracted RNA from each treatment was examined using a Nanovue spectrophotometer (GE Healthcare, Buckinghamshire, UK) in order to determine the quality (A260/280) and quantity of RNA. Extracted RNA was also bioanalysed by the Functioning genomics Lab at The University of Birmingham, UK using an Agilent 2100 Bioanalyzer (Agilent Technologies, UK) which gave a more accurate determination of the quality and quantity of the RNA extracted for microarray analysis. (See appendix file name: Chapter 3 appendix data > microarray > concentration and purity of extracted RNA).

3.2.7.3 Array design and execution

The array used was an 8 x 15K Agilent eARRAY (Design ID: 029000, Design Name: Webber_Salmonella) consisting of 2 oligonucleotides of 60 base pairs for each gene. The array contained all coding sequences from *Salmonella enterica* serovar Typhimurium strains LT2, SL1344 and 14028S and was repeated 8 times on each array slide (see appendix file name:

Chapter 3 appendix data > Microarray, for full list of genes). cDNA synthesis, labelling and hybridisation (of each biological replicate) to the array was performed by the Functioning Genomics Lab at the University of Birmingham, UK. Arrays were analysed by Dr Ewan Hunter (University of Birmingham) to give lists of genes and pathways with statistically significantly changed expression values. Analysis was carried out using GeneSpring software (version 12). Further analysis was then carried out in Cardiff University where lists of significantly changed genes were compiled (based on a p value of ≤ 0.01) for each strain and treatment. (See appendix folder name: Chapter 3 appendix data > Microarray for raw microarray data from Dr Ewan Hunter and data analysed at Cardiff).

3.2.8 Real-time PCR

3.2.8.1 Genes of interest

Real time PCR reactions were carried out to investigate and confirm changes in the expression of specific genes selected from microarray data produced after the exposure of SL1344 and 14028S to low concentrations of CHG and BZC. Table 3.1 shows the list of genes that were selected to be investigated due to their significant up/down regulation observed in the microarray work carried out. Genes were also selected based on their possible association with results observed in other experiments performed throughout this chapter. *rrsH* was chosen as a reference gene as it has successfully been shown to maintain a consistent level of expression after exposure to multiple biocides (Whitehead *et al.*, 2011).

Table 3.1: List of S. enterica serovar Typhimurium genes investigated using real time PCR

Name	Function	Fold change in Microarray
ydgF	Multi-drug efflux system protein MdtJ	**2**-fold up-regulated in 14028S treated with 0.0001 % CHG
acrR	Transcriptional repressor and regulator of the AcrAB operon (which is involved in efflux)	**0.60**-fold down regulated in 14028S treated with 0.0004 % BZC
blc	Outer membrane lipoprotein	**2**-fold up-regulated in 1344 treated with 0.0004 % BZC and CHG
hybB	Hydrogenase 2 large subunit	**9.9**-fold up-regulated in 14028S treated with 0.0004 % BZC
hycA	Formate hydrogenlyase regulatory protein	**13.8**-fold up-regulated in 14028S treated with 0.0004 % BZC
ompW	Outer membrane protein	**6.8**-fold up-regulated in 14028S treated with 0.0004 % BZC
rrsH	16S ribosomal RNA – (reference gene)	-

3.2.8.2 Primers

Primers were designed using Primer 3 software (http://bioinfo.ut.ee/primer3-0.4.0/ accessed 11/03/2014) and their specificity for each *Salmonella* gene target was tested using the *In Silico* PCR tool (http://insilico.ehu.es/PCR accessed 11/03/2012). All primers were from Invitrogen, Paisley, UK. Primers sequences are listed in table 3.2.

Table 3.2: Primers for real-time PCR

Primer Name	Primer (Forward and Reverse) 5' – 3'	Product size (bp)	Annealing temperature (°C)
rrsH (house-keeping)	CAGAAGAAGCACCGGCTAAC	218	Multiple
	GACTCAAGCCTGCCAGTTTC		
blc	GTTACTGCGGCATTTTTGGT	150	55.2
	TTGTTCCAGTCCACGTTCAA		
hycA	TTGGGAAATAAGCGAAAAGG	173	55.2
	GCTCAAACAGGACAAAGCAA		
hybB	GGGCGGAAAAATCATCAGTA	153	55.2
	AGGTCAAAGGCAATCCAGAC		
acrR	GCCGCTTATTGATGGAGATT	152	56.2
	TTCAGGCAGCATTTTAGCATT		
ompW	GCGTGGGGGTGAACTACA	264	55.2
	AGCCTGCCGAGAACATAAAT		
ydgF	CTGGCTATCGCGACTGAAAT	184	56.2

3.2.8.3 Real time PCR reactions

Reactions were performed in triplicate under the conditions described in chapter 2 section 2.13. Data analysis was carried out using the Pfaffl method (Pfaffl, 2001) described in detail in chapter 2 section 2.14. (See appendix folder name: Chapter 3 appendix data > real_time_PCR for raw data).

3.2.9 Statistical tests

A Students t-test was used to compare MIC, MBC and zone of inhibition values before and after biocide exposure and to compare fluorescence values between biocide-treated and untreated cells in efflux assay experiments. A one-way analysis of variance (ANOVA) test was used when comparing zone of inhibition values obtained from the phenotype stability tests and particle size values from light scattering experiments.

3.3 Results

3.3.1 Suspension testing

Strains SL1344 and 14028S were exposed to CHG and BZC at the minimum bactericidal concentration (0.01 % CHG & 0.006 % BZC for SL1344 and 0.006 % CHG & 0.008 % BZC for 14028S) for a total time period of 60 min, in order to determine the effect of contact time on biocide efficacy. The toxicity and efficacy of the neutraliser was tested prior to the suspension test being carried out. Tables 3.3 and 3.4 show the neutraliser toxicity and efficacy data.

Table 3.3: Neutraliser toxicity data for strains SL1344 & 14028S. N=3

SD = standard deviation

Treatment	**Log CFU/mL ± SD**
Control (diH_20)	SL1344: 8.48 ± 0.00
	14028S: 8.70 ± 0.00
Neutraliser	SL1344: 8.48 ± 0.00
	14028S: 8.67 ± 0.02

Table 3.4: Neutraliser efficacy data for strains SL1344 & 14028S. N=3

Treatment	**Log CFU/mL ± SD**
Control (diH_20)	SL1344: 8.48 ± 0.00
	14028S: 8.70 ± 0.00
CHG (5 %)	SL1344: 0.00 ± 0.00
	14028S: 0.00 ± 0.00
CHG + Neutraliser	SL1344: 8.10 ± 0.04
	14028S: 8.47 ± 0.00
BZC (12.5 %)	SL1344: 0.00 ± 0.00
	14028S: 0.00 ± 0.00
BZC + Neutraliser	SL1344: 8.10 ± 0.05
	14028S: 8.47 ± 0.00

The neutraliser was considered non-toxic if ≤ 1 log_{10} reduction in CFU/mL (compared to control) was observed. The neutraliser was considered effective if ≤ 1 log_{10} reduction in CFU/mL was observed in the presence of biocide and neutraliser ((based on BS EN 1276 (2009) suspension testing guidelines)). Tables 3.3 and 3.4 show that the neutraliser was not toxic to either strain, and that it was effective in neutralising CHG and BZC.

The dilutions performed during the suspension tests resulted in a 4.4 log_{10} limit of detection for SL1344 and a 4.6 log_{10} limit of detection for 14028S. Figure 3.2 shows the effect of contact time on exposure of both strains to CHG.

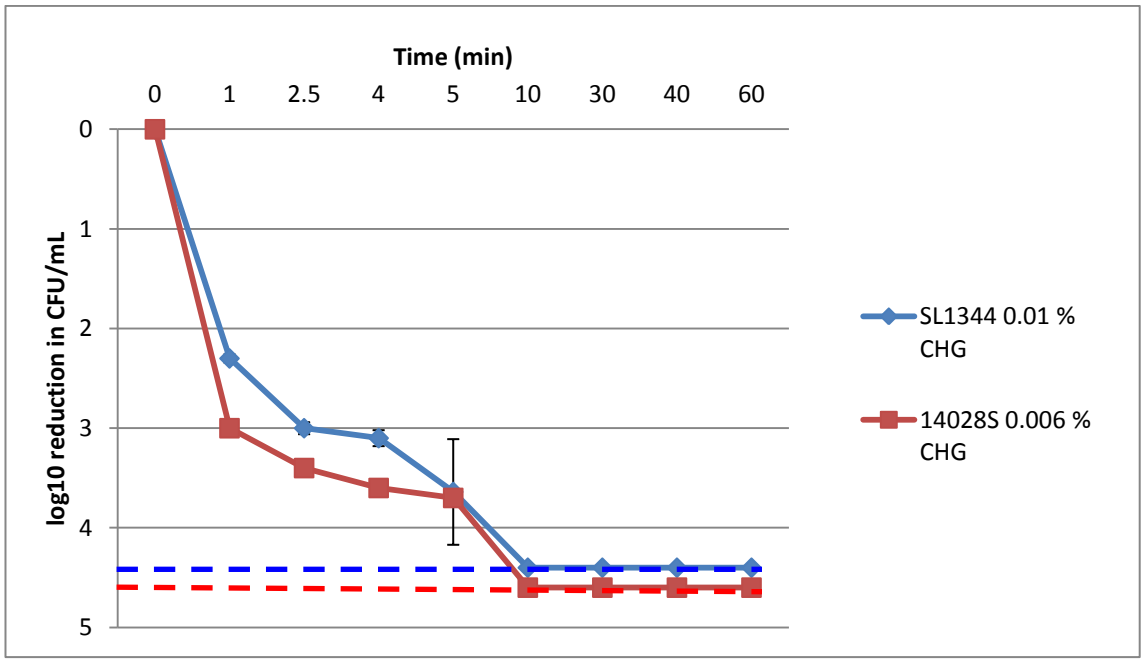


Figure 3.2: log_{10} reduction in CFU/mL of strains SL1344 & 14028S after exposure to CHG. Contact times of 1, 2.5, 4, 5, 10, 30, 40 and 60 min. N=3

- - - - limit of detection for SL1344 - - - - limit of detection for 14028S

After exposure of SL1344 to 0.01% CHG the greatest log_{10} reduction was observed in the first minute of exposure (~2.3 log_{10}). After 10 min a 4.4 log_{10} reduction was observed. This was the

maximum log_{10} reduction that could be determined due to the 4.4 log_{10} limit of detection. The 4.4 log_{10} reduction corresponded to a plate count of zero. The greatest log_{10} reduction also occurred in the first minute of exposure of strain 14028S to 0.006 % CHG (3 log_{10}). After 10 min exposure to 0.006 % CHG a 4.6 log_{10} reduction was observed in strain 14028S. This was the maximum log_{10} reduction that could be determined due to the limit of detection.

Figure 3.3 shows the effect of contact time on the efficacy of BZC against SL1344 and 14028S.

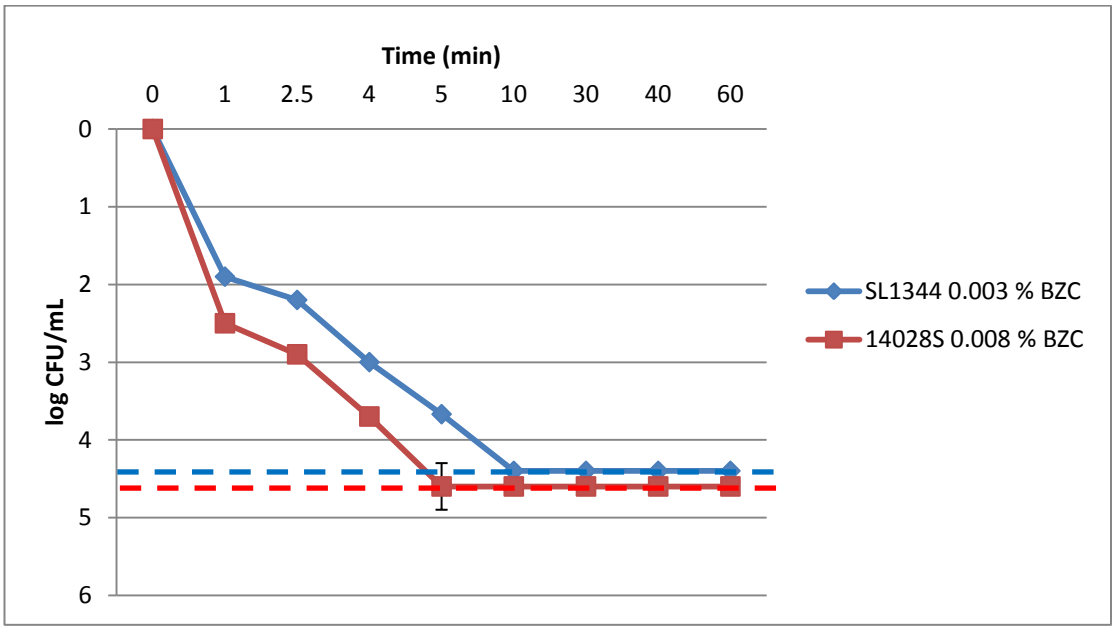


Figure 3.3: log_{10} reduction in CFU/mL of strains SL1344 & 14028S after exposure to BZC. Contact times of 1, 2.5, 4, 5, 10, 30, 40 and 60 min. N=3

- - - - limit of detection for SL1344 - - - - limit of detection for 14028S

In the first minute of exposure of SL1344 to 0.003 % BZC a 2 log_{10} reduction in log CFU/mL was observed. Between 1 and 10 min a further 2.4 log_{10} reduction was observed. At 10 min a 4.4 log_{10} reduction in CFU/mL was observed in SL1344. After exposure of 14028S to 0.008 % BZC for 1 min a 2.5 log_{10} reduction was observed. After 5 min a 4.6 log_{10} reduction was observed.

3.3.2 Antimicrobial susceptibility testing

The MIC and MBC of CHG and BZC, and susceptibility to the antibiotics ciprofloxacin, ceftriaxone, ampicillin, piperacillin and chloramphenicol were determined before and after 5 min exposure to a range of low CHG and BZC concentrations. Baseline and post-exposure values were compared in order to determine if short term exposure to a low concentration of CHG or BZC resulted in a change in antimicrobial susceptibility in surviving organisms.

3.3.2.1 Changes in minimum inhibitory and minimum bactericidal concentrations

Baseline and post-exposure MIC and MBC values for CHG and BZC were compared in order to determine if 5 min exposure to CHG or BZC resulted in an increase in the MIC or MBC for either of these biocides. Tables 3.5 and 3.6 show baseline and post-exposure MIC and MBC values for SL1344 and 14028S. In the case of both strains post-exposure MIC and MBC values for CHG and BZC were all significantly different from baseline MIC and MBC values ($p \leq 0.05$). For strain SL1344 the greatest increases in MIC and MBC were observed after 5 min exposure to 0.0004 % CHG and 0.0004 % BZC (table 3.5). For strain 14028S exposure to 0.0001 % CHG and 0.0004 % w/v BZC resulted in the greatest increases in MIC and MBC in surviving organisms (table 3.6). Increases in the MIC and MBC for BZC were observed after 5 min exposure to a low CHG concentration in both strains, indicating that short-term exposure to a low concentration of a particular biocide can affect the susceptibility of surviving organisms to other biocides. This was also true for the MIC and MBC of CHG after 5 min exposure to BZC. Both strains tested appeared to respond to 5 min exposure to a low biocide concentration in a similar way. The data also appear highly reproducible across all 9 repeats in the case of both strains, as indicated by the low standard deviation values.

Table 3.5: MIC and MBC values of CHG and BZC for strain SL1344 after 5 min exposure to a range of low CHG and BZC concentrations. N=9

	Biocide concentration (%)							
MIC/MBC (%)	**Baseline**	**0.0004 CHG**	**0.0001 CHG**	**0.00005 CHG**	**0.00001 CHG**	**0.0004 BZC**	**0.0001 BZC**	**0.00001 BZC**
CHG MIC	0.003	0.08	0.08	0.04	0.08	0.05	0.04	0.08
± SD	± 0.003	± 0.00	± 0.00	± 0.00	± 0.000	± 0.02	± 0.00	± 0.000
CHG MBC	0.010	0.20	0.20	0.04	0.10	0.30	0.20	0.20
± SD	± 0.006	± 0.09	± 0.00	± 0.00	± 0.040	± 0.00	± 0.00	± 0.100
BZC MIC	0.003	0.20	0.03	0.01	0.07	0.30	0.08	0.07
± SD	± 0.000	± 0.00	± 0.02	± 0.00	± 0.100	± 0.10	± 0.00	± 0.100
BZC MBC	0.003	0.20	0.05	0.20	0.13	0.80	0.20	0.30
± SD	± 0.003	± 0.00	± 0.02	± 0.200	± 0.200	± 0.00	± 0.00	± 0.200

Table 3.6: MIC and MBC values of CHG and BZC for strain 14028S after 5 min exposure to a range of low CHG and BZC concentrations. N=9

	Biocide concentration (%)				
MIC/MBC (%)	**Baseline**	**0.0005 CHG**	**0.0001 CHG**	**0.0015 BZC**	**0.0004 BZC**
CHG MIC ± SD	0.003 ± 0.003	0.01 ± 0.000	0.10 ± 0.000	0.04 ± 0.000	0.08 ± 0.000
CHG MBC ± SD	0.006 ± 0.003	0.10 ± 0.090	2.00 ± 0.000	0.50 ± 0.000	0.30 ± 0.000
BZC MIC ± SD	0.004 ± 0.003	0.08 ± 0.000	0.01 ± 0.000	0.08 ± 0.000	0.20 ± 0.000
BZC MBC ± SD	0.008 ± 0.002	0.10 ± 0.000	0.20 ± 0.060	0.10 ± 0.000	2.00 ± 0.900

3.3.2.2 Changes in antibiotic susceptibility

Clinical susceptibility to a range of antibiotics before and after 5 min exposure to a range of low CHG and BZC concentrations was determined following the BSAC disk diffusion protocol (Andrews, 2009). Tables 3.7 and 3.8 show the mean zone of inhibition sizes for a range of antibiotics observed before and after 5 min exposure to low CHG and BZC concentrations. Clinical susceptibility (i.e. sensitivity or resistance) was determined using the BSAC breakpoints for *Enterobacteriaceae* (Andrews, 2009).

Table 3.7 shows that despite the statistically significant decreases in the mean zone of inhibition size observed for the antibiotics ciprofloxacin and ceftriaxone, there were no changes in clinical susceptibility to any of the antibiotics tested after 5 min exposure to a

range of low CHG and BZC concentrations (according to BSAC susceptibility breakpoints for *Enterobacteriaceae* (Andrews, 2009)). There were no significant changes in mean zone of inhibition size for the remaining antibiotics tested. These data suggest that 5 min biocide exposure does not result in a change in clinical antibiotic susceptibility.

Table 3.7: Mean zone of inhibition sizes (mm) for a range of antibiotics after exposure of strain SL1344 to a range of low CHG and BZC concentrations. N=9

^{S} = clinically sensitive to antibiotic according to BSAC breakpoints for *Enterobacteriaceae*

* = significantly different to baseline value ($p \leq 0.05$)

Cip = ciprofloxacin Cef = ceftriaxone Chl = chloramphenicol Amp = ampicillin

Pip = piperacillin

	Mean zone of inhibition size (mm) ± SD							
Antibiotic	**Base-line**	**0.0004 % CHG**	**0.0001 % CHG**	**0.00005 % CHG**	**0.00001 % CHG**	**0.0004 % BZC**	**0.0001 % BZC**	**0.00001 % BZC**
Cip (1µg)	41.3^{S} ± 2.3	31.0^{S*} ± 2.0	31.0^{S*} ± 0.0	34.3^{S*} ± 0.6	34.2^{S*} ± 0.6	30.3^{S*} ± 1.5	30.0^{S*} ± 1.7	33.3^{S*} ± 0.1
Cef (30µg)	40.7^{S} ± 0.6	37.0^{S*} ± 2.0	30.0^{S*} ± 0.0	36.7^{S*} ± 0.6	35.2^{S*} ± 0.5	33.3^{S*} ± 2.1	30.0^{S*} ± 1.0	36.0^{S*} ± 0.0
Chl (30µg)	27.3^{S} ± 1.2	27.7^{S} ± 2.3	29.0^{S} ± 1.2	32.0^{S} ± 1.7	27.7^{S} ± 2.1	28.7^{S} ± 0.6	29.0^{S} ± 1.7	28.0^{S} ± 0.0
Amp (10µg)	32.0^{S} ± 2.0	29.7^{S} ± 0.6	28.0^{S} ± 0.5	32.7^{S} ± 1.5	28.7^{S} ± 1.5	28.7^{S} ± 1.5	29.7^{S} ± 2.1	31.0^{S} ± 0.0
Pip (75µg)	31.7^{S} ± 0.6	31.0^{S} ± 1.7	28.0^{S} ± 0.5	32.0^{S} ± 0.0	29.0^{S} ± 0.0	29.3^{S} ± 1.5	28.0^{S} ± 0.6	32.0^{S} ± 0.0

Table 3.8: Mean zone of inhibition sizes (mm) for a range of antibiotics after exposure of strain 14028S to a range of low CHG and BZC concentrations. N=9

^{S} = clinically sensitive to antibiotic according to BSAC breakpoints for *Enterobacteriaceae*

* = significantly different to baseline value ($p \leq 0.05$)

Cip = ciprofloxacin Cef = ceftriaxone Chl = chloramphenicol Amp = ampicillin

Pip = piperacillin

	Mean zone of inhibition size (mm) ± SD				
Antibiotic	**Baseline**	**0.0005% CHG**	**0.0001% CHG**	**0.0004% BZC**	**0.0001% BZC**
Cip (1µg)	43.3^{S} ± 1.2	30.3^{S*} ± 0.5	31.0^{S*} ± 0.6	30.3^{S*} ± 1.2	27.0^{S*} ± 0.0
Cef (30µg)	39.3^{S} ± 1.2	34.7^{S*} ± 1.2	30.0^{S*} ± 0.6	34.7^{S*} ± 2.1	34.7^{S*} ± 1.6
Chl (30µg)	26.7^{S} ± 2.3	27.7^{S} ± 0.6	26.7^{S} ± 1.5	27.7^{S} ± 2.1	26.7^{S} ± 1.5
Amp (10µg)	31.3^{S} ± 2.3	31.3^{S} ± 1.2	27.7^{S} ± 1.2	31.3^{S} ± 2.3	27.7^{S} ± 1.2
Pip (75µg)	31.7^{S} ± 1.5	29.7^{S} ± 1.7	29.0^{S} ± 1.7	29.7^{S} ± 2.5	29.0^{S} ± 0.0

As observed in strain SL1344 (table 3.7) there were no clinical changes in susceptibility to any of the antibiotics tested according to the BSAC breakpoints for *Enterobacteriaceae* (Andrews, 2009).

3.3.2.3 Phenotype stability testing

The stability of the increases in MBC observed after 5 min exposure to a range of low CHG and BZC concentrations was investigated via the 24 h subculture of surviving organisms through TSB +/- a low concentration of CHG or BZC. Tables 3.9 and 3.10 show the MBC values after 1, 5 and 10 subcultures of surviving organisms through TSB +/- CHG/BZC.

As shown in tables 3.9 and 3.10, the high MBC values observed after the initial 5 min exposure to CHG or BZC were lost after 1 subculture in the absence of CHG or BZC. The values obtained after 1 subculture in the absence of CHG or BZC were not significantly different from the baseline MBC value. This suggests that in the absence of any selective pressure (i.e. removal of biocide), the MBC values reverted to baseline level. These values remained stable and at baseline level throughout the remaining subcultures in the absence of CHG or BZC in the case of both strains.

After 1 and 5 subcultures of survivors of strain 14028S in the presence of 0.0001 % CHG or 0.0004 % BZC there was a significant difference between MBC values obtained and baseline MBC values. This indicates that in the presence of these low biocide concentrations an elevated MBC was maintained. However there was no significant difference between the MBC obtained after 10 passages of survivors of strain 14028S in the presence of 0.0001 % CHG or 0.0004 % BZC and the baseline MBC (table 3.10). This shows that despite the presence of a low biocide concentration, the elevated MBC values observed after 5 min exposure were lost after 10 subcultures. This was also observed in the case of SL1344 survivors subcultured in the presence of 0.0004 % BZC (table 3.9). The elevated MBC observed after the initial 5 min exposure to 0.0001 % CHG had reverted back to baseline level after 24 h, even in the presence of the low biocide concentration. The fact that the elevated MBCs were not maintained even in the presence of a low concentration of CHG or BZC may have been due to accumulative damage from the continuous presence of the

biocide. It could also have been due to the fact that the maintenance of an elevated MBC was detrimental to the bacterial cell.

Table 3.9: Mean MBC values for CHG and BZC after 1, 5 and 10 subcultures of surviving SL1344 through TSB +/- 0.0004 % CHG or BZC. N=3

SC = subculture * = significantly different from baseline (p≤0.05)

	Baseline MBC (%)	**5 min CHG 0.0004 %**	**1 SC**	**5 SC**	**10 SC**	**1 SC (CHG)**	**5 SC (CHG)**	**10 SC (CHG)**
CHG MBC (%)	0.010	0.500*	0.008	0.009	0.006	0.015	0.010	0.010
± SD	± 0.09	± 0.00	± 0.00	± 0.00	± 0.00	± 0.04	± 0.04	± 0.00
BZC MBC (%)	0.003	0.150*	0.004	0.006	0.006	0.019*	0.050*	0.006
± SD	± 0.00	± 0.00	± 0.00	± 0.00	± 0.00	± 0.00	± 0.02	± 0.00
	Baseline MBC (%)	**5 min BZC 0.0004 %**	**1 SC**	**5 SC**	**10 SC**	**1 SC (BZC)**	**5 SC (BZC)**	**10 SC (BZC)**
CHG MBC (%)	0.010	0.500*	0.020	0.010	0.009	0.08*	0.080*	0.010
± SD	± 0.09	± 0.00	± 0.03	± 0.00	± 0.00	± 0.04	± 0.04	± 0.00
BZC MBC (%)	0.003	0.300*	0.006	0.006	0.006	0.078*	0.060*	0.003
± SD	± 0.00	± 0.00	± 0.00	± 0.00	± 0.00	± 0.00	± 0.02	± 0.00

Table 3.10: Mean MBC values for CHG and BZC after 1, 5 and 10 subcultures of surviving 14028S through TSB +/- 0.0001 % CHG or 0.0004 % BZC. N=3

SC = subculture * = significantly different from baseline (p≤0.05)

	Baseline MBC (%)	**5 min CHG 0.0001 %**	**1 SC**	**5 SC**	**10 SC**	**1 SC (CHG)**	**5 SC (CHG)**	**10 SC (CHG)**
CHG MBC (%)	0.006	0.500*	0.001	0.006	0.009	0.080*	0.080*	0.006
± SD	± 0.003	± 0.00	± 0.00	± 0.00	± 0.00	± 0.04	± 0.04	± 0.00
BZC MBC (%)	0.008	0.300*	0.006	0.007	0.006	0.019*	0.020*	0.006
± SD	± 0.002	± 0.00	± 0.00	± 0.00	± 0.00	± 0.00	± 0.00	± 0.00
	Baseline MBC (%)	**5 min BZC 0.0004 %**	**1 SC**	**5 SC**	**10 SC**	**1 SC (BZC)**	**5 SC (BZC)**	**10 SC (BZC)**
CHG MBC (%)	0.006	0.500*	0.006	0.005	0.006	0.040*	0.070*	0.006
± SD	± 0.003	± 0.00	± 0.00	± 0.00	± 0.00	± 0.02	± 0.07	± 0.00
BZC MBC (%)	0.008	0.300*	0.007	0.004	0.006	0.019*	0.020*	0.006
± SD	± 0.002	± 0.00	± 0.00	± 0.00	± 0.00	± 0.00	± 0.00	± 0.00

3.3.3 Efflux assays

The accumulation of fluorescent Hoechst dye was measured in SL1344 or 14028S cells grown to mid-log phase in the presence/absence of CHG or BZC, and in the presence/absence of 3 different efflux pump inhibitors (EPIs). This gave an indication of the level of efflux activity taking place in the bacterial cell as a result of biocide exposure. The toxicity of the EPIs was tested prior to the efflux assays. Table 3.11 shows the toxicity testing data.

Table 3.11: EPI toxicity data for strains SL1344 & 14028S

EPI	**Log_{10} CFU/mL before exposure ± SD**	**Log_{10} CFU/mL after exposure ± SD**	**Log10 reduction in CFU/mL after EPI exposure**
Verapamil (50 mg/L)	SL1344: 8.26 ± 0.01	SL1344: 7.39 ± 0.13	0.87 ± 0.13
	14028S: 8.60 ± 0.02	14028S: 8.10 ± 0.09	0.50 ± 0.09
PAβN (10 mg/L)	SL1344: 8.26 ± 0.01	SL1344: 7.67 ± 0.15	0.59 ± 0.15
	14028S: 8.24 ± 0.01	14028S: 7.29 ± 0.06	0.95 ± 0.06
1-(1-NP) (10mg/L)	SL1344: 8.26 ± 0.01	SL1344: 7.31 ± 0.06	0.95 ± 0.06
	14028S: 8.23 ± 0.04	14028S: 7.28 ± 0.10	0.95 ± 0.10

The EPIs were considered non-toxic if ≤ 1 log_{10} reduction was observed after exposure (according to BS EN 1276 2009 protocol). Table 3.11 shows that none of the EPIs used were toxic to strains SL1344 and 14028S at the concentrations chosen.

Figure 3.4 shows mean fluorescence values in biocide-treated and untreated SL1344 cells in the presence/absence of verapamil (50 mg/L) or PAβN (10 mg/L) or 1-(1-NP) (10 mg/L).

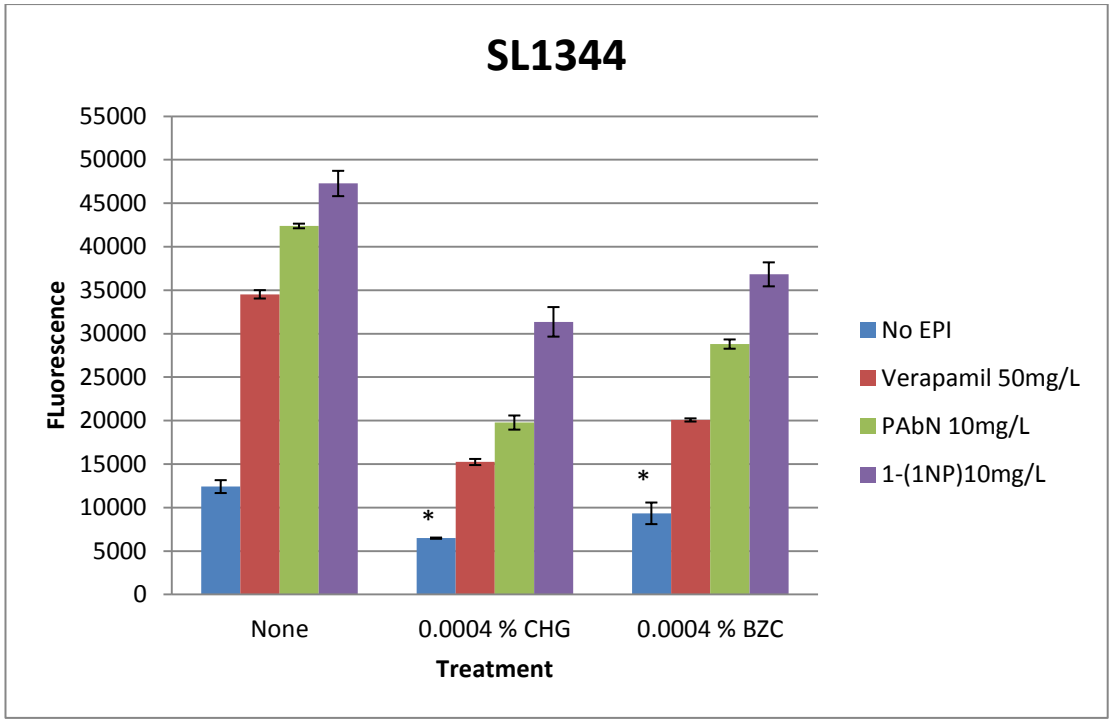


Figure 3.4: Mean fluorescence values observed in biocide-exposed and untreated SL1344 before and after the addition of different efflux pump inhibitors (EPIs). N=3

* = significantly different from untreated

CHG and BZC treated SL1344 accumulated significantly less ($p \leq 0.05$) Hoechst dye than untreated cells, as indicated by the lower fluorescence reading (figure 3.4 blue bars). This reduced accumulation of dye in biocide-treated cells may have been due to the up-regulation of efflux-associated genes or increased efflux pump activity. This suggests that biocide treatment may have had an effect on the efflux activity taking place in SL1344 cells. All three EPIs tested caused an increase in the fluorescence reading observed across all three treatments (figure 3.4). This suggests that the EPIs were inhibiting efflux activity, resulting in an increased accumulation of Hoechst dye in the cells. 1-(1-NP) and PAβN had a greater inhibitory effect on efflux, as indicated by the greater increase in fluorescence in figure 3.4 (green and purple bars). Both inhibit RND type efflux pumps, suggesting that a large proportion of the efflux pump activity taking place may have been due to this type of pump. Verapamil also caused an increase in the fluorescence reading across all treatments. Verapamil is an inhibitor of ABC type efflux pumps. This suggests that more than one type

of efflux pump was present and that Hoechst dye may be a substrate of more than one type of efflux pump present in SL1344.

Figure 3.5 shows mean fluorescence values in biocide-treated and untreated 14028S cells in the presence/absence of verapamil (50 mg/L) or PAβN (1 0mg/L) or 1-(1-NP) (10 mg/L).

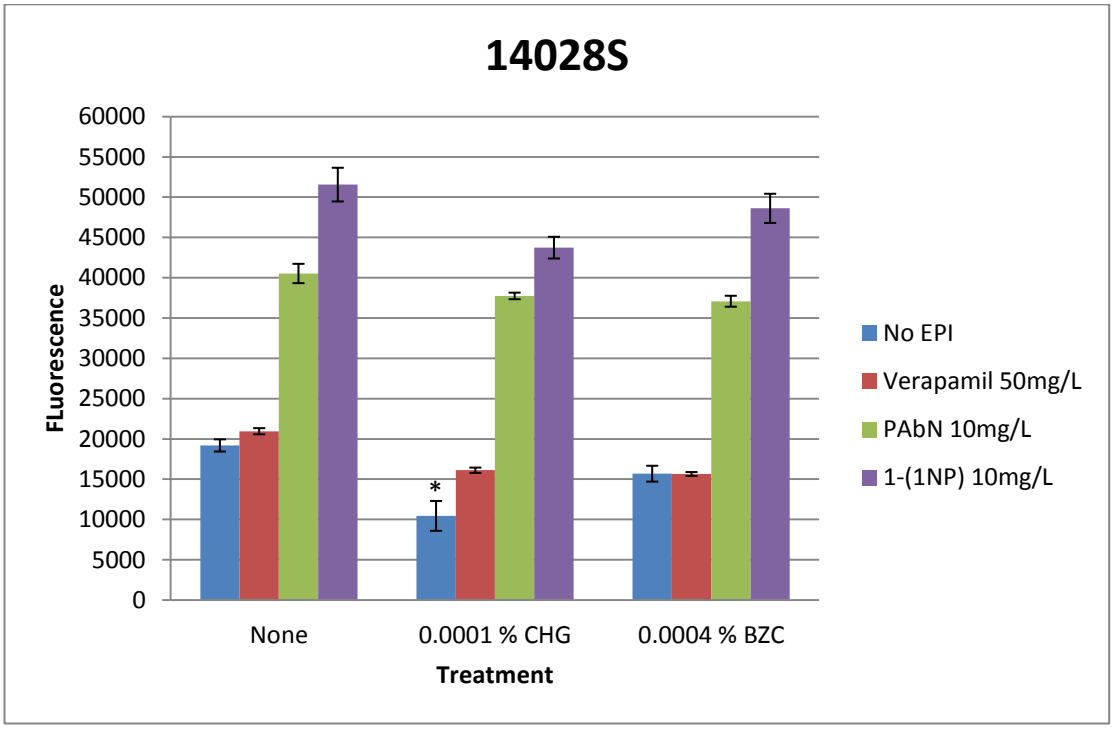


Figure 3.5: Mean fluorescence values observed in biocide-exposed and untreated 14028S before and after the addition of different efflux pump inhibitors (EPIs). N=3

* = significantly different from untreated

CHG treated 14028S cells accumulated significantly less ($p \leq 0.05$) Hoechst dye than untreated cells as indicated by the lower fluorescence reading in figure 3.5 blue bars. This response to CHG exposure was also observed in SL1344 and is suggestive of increased efflux pump activity as a result of biocide exposure. Exposure of 14028S to 0.0004 % BZC did not result a significant change in the fluorescence reading observed (blue bars figure 3.5) suggesting that BZC exposure did not result in a significant alteration in efflux pump

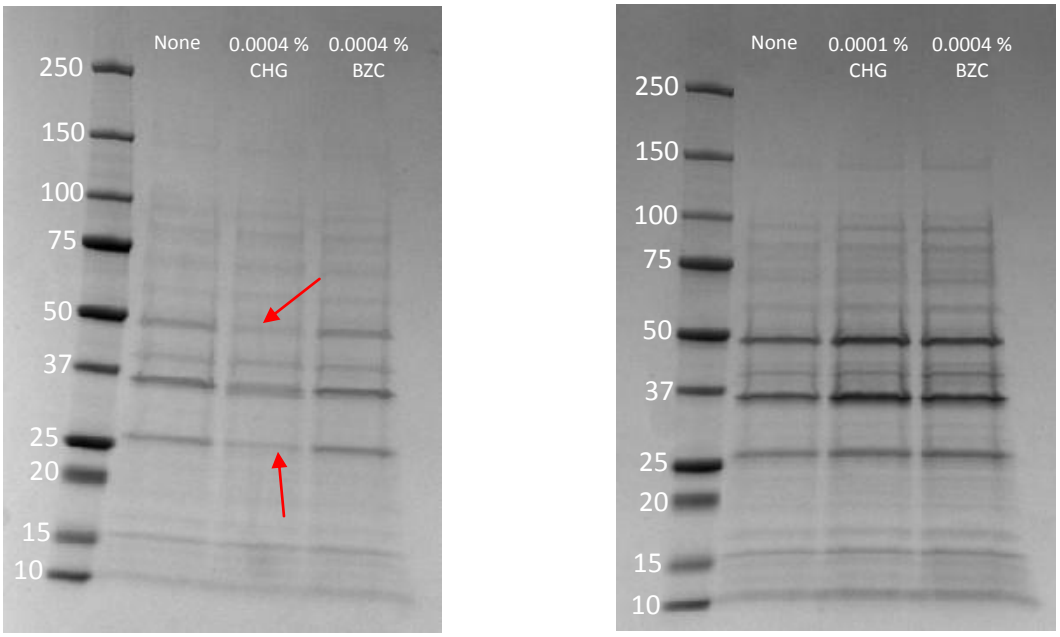
activity in this strain. The EPIs PAβN and 1-(1-NP) caused a large increase in the fluorescence reading across all treatments (figure 3.5 purple and green bars). As observed in SL1344 this suggests the presence and activity of RND type efflux pumps.

3.3.4 One Dimensional Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

One dimensional SDS-PAGE was performed on OMPs extracted from SL1344 and 14028S grown to mid-log phase in a low CHG/BZC concentration, in order to determine if biocide exposure caused alterations in OMP composition. Figure 3.6 shows the protein bands present in untreated and CHG/BZC treated SL1344 and 14028S cells. The same starting concentration (20 µg/ 20µL) of each sample was loaded on to the gel and protein bands were stained with colloidal coomassie stain.

Figure 3.6 shows that there was no definitive absence of existing bands or appearance of new bands in either strain after exposure to CHG or BZC. The intensity of two bands was reduced after SL1344 was grown to mid-log phase in the presence of 0.0004 % CHG (indicated by red arrows in figure 3.6). The molecular weights (MW) of the two bands showing reduced intensity are approximately 25 and 50 kDa. Proteins from the *omp* family present in *S*. Typhimurium vary in MW and are all around 40 kDa, with the exception of OmpW which has a MW of 22.96 kDa. This value is quite close in MW to the approximate MW of one of the OMPs observed here. It is possible that the 25 kDa band is representative of OmpW, a porin present in the outer membrane. Reduced expression of porins (i.e. reduced band intensity on a gel) may result in reduced accumulation of a biocide in the cell, and a possible increase in the MIC or MBC for that biocide. The outer membrane proteins SmvA (associated with acriflavine efflux) and TolC (an outer membrane efflux system protein) are 52.14 and 53.68 kDa in size respectively. They are therefore close in MW to the

50 kDa protein observed here. However it is not clear why efflux associated protein production would be reduced after biocide exposure, considering the fact that efflux assay data was indicative of increased efflux activity. It is possible that the protein band observed here does not represent either of these proteins. Further sequencing and proper identification of proteins with reduced band intensity would provide further information on the effect of biocide exposure on OMP composition.



SL1344

14028S

Figure 3.6: Outer membrane protein bands observed in SL1344 & 14028S either grown to mid-log phase in low concentrations of CHG/BZC or untreated

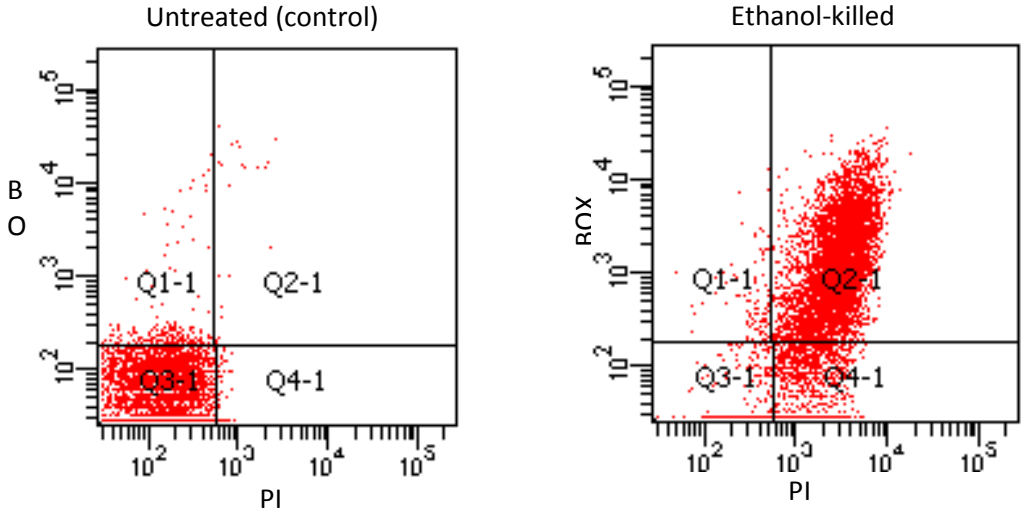
Red arrows indicate reduction in band intensity

3.3.5 Flow cytometry

Biocide-treated and untreated bacteria were stained with PI and BOX and separated according to their light scattering and fluorescent properties. The level of uptake of each dye and the corresponding fluorescent signal provided information on the amount of damage to the cell as a result of biocide exposure, and the number of cells alive, damaged

or dead. Figure 3.7 (a) shows the distribution of untreated and ethanol-killed (positive control) SL1344 cells. This figure demonstrates the position on the plot of 'live' (untreated) and 'dead' (ethanol-killed) cells and illustrates how the quadrants defining live, dead or damaged cells were chosen. Figures 3.7 (b) and (c) show the distribution of SL1344 (b) and 14028S (c) cells after 5 min exposure to a low concentration of CHG or BZC (0.0004 % CHG and BZC for SL1344 and 0.0001 % CHG and 0.0004 % BZC for 14028S) and staining with PI and BOX, and a table of statistics adapted from those calculated using FACSDiva software.

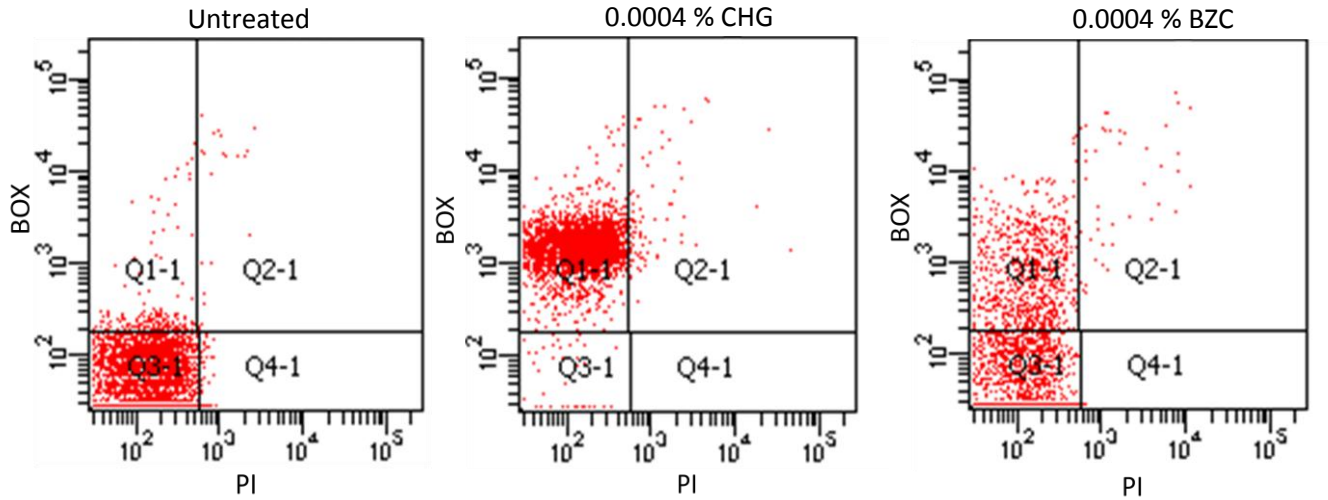
Figure 3.7 (b) shows that untreated SL1344 cells did not accumulate large amounts of PI or BOX indicating that they were undamaged. This was also reflected in the statistics that show that 97.0 % of cells appeared in Q3-1. A change in the population was clearly observed after 5 min exposure of SL1344 to 0.0004 % CHG. As shown in the statistics table in figure 3.7 (b), 96.0 % of cells appeared in Q1-1, indicating that a large proportion of the population was damaged after biocide exposure. However only 1.60 % of cells appeared in Q2-1 (dead), indicating that exposure to this concentration did not kill a large number of cells after 5 min. Exposure of SL1344 to 0.0004 % BZC also resulted in damage to the population with 23.8 % of cells appearing in Q1-1 after 5 min exposure. However 75.4 % of the population appeared in Q3-1 suggesting they were not damaged by 5 min exposure to 0.0004 % BZC. It is possible that this population that were exposed to the biocide but not damaged were responsible for the elevated MIC and MBC values observed in section 3.3.2. Sorting of these cells in to FACSflow buffer and determination of the MIC and MBC of CHG and BZC would confirm this. As observed in strain SL1344, exposure of 14028S to 0.0001 % CHG or 0.0004 % BZC for 5 min resulted in some damage to the population, but the majority of cells remained in Q3-1 (92.0 % and 88.8 % respectively) (figure 3.7c). As with strain SL1344, it is possible that biocide-exposed cells present in Q3-1 (undamaged) could be the population responsible for the elevated MIC and MBC values observed in section 3.3.2.



Treatment	% of events in Q1-1	% of events in Q2-1	% of events in Q3-1
Untreated	2.10	0.20	97.0
80 % Ethanol	1.40	78.6	3.5

Q1: Damaged cells (membrane potential lost, membrane intact)
Q2: Dead (ruptured membrane)
Q3: Alive (minimal uptake of dyes)

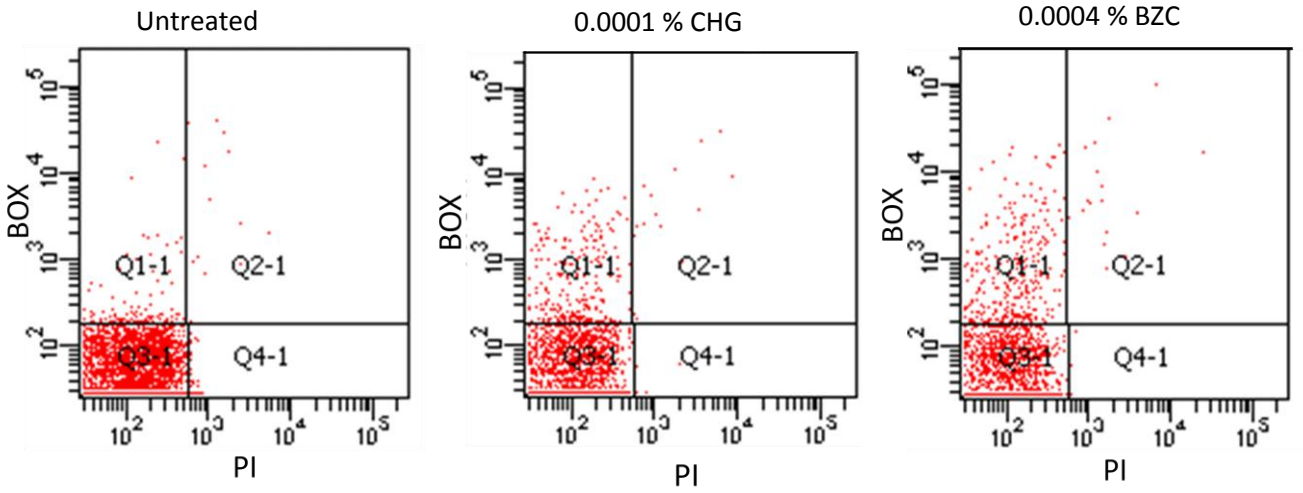
Figure 3.7 (a): Distribution of untreated or ethanol-killed SL1344 cells stained with PI and BOX



Treatment	% of events in Q1-1	% of events in Q2-1	% of events in Q3-1
Untreated	2.10	0.20	97.0
0.0004 % CHG	96.0	1.60	2.40
0.0004 % BZC	23.8	0.70	75.4

Q1: Damaged cells (membrane potential lost, membrane intact)
Q2: Dead (ruptured membrane)
Q3: Alive (minimal uptake of dyes)

Figure 3.7 (b): Distribution of untreated or 0.0004 % CHG/BZC treated SL1344 stained with PI and BOX



Treatment	% of events in Q1-1	% of events in Q2-1	% of events in Q3-1
Untreated	1.20	0.10	98.3
0.0001 % CHG	7.60	0.20	92.0
0.0004 % BZC	10.8	0.30	88.8

Q1: Damaged cells (membrane potential lost, membrane intact)
Q2: Dead (ruptured membrane)
Q3: Alive (minimal uptake of dyes)

Figure 3.7 (c): Distribution of untreated or 0.0001 % CHG/0.0004 % BZC treated 14028S stained with PI and BOX

3.3.6 Light scattering experiments

Biocide-treated and untreated cells were passed through an N4 Plus Dynamic Light Scattering machine to determine if bacterial aggregates were present in any samples. Table 3.12 shows the mean particle sizes in untreated SL1344 and 14028S cells and cells treated with CHG or BZC at the minimum bactericidal concentration.

Table 3.12: Mean particle size (nm) for untreated and biocide-treated SL1344 & 14028S cells. N=3

Strain	Treatment	Mean particle diameter (nm) (range)
SL1344	Untreated	749 (60.20)
	0.010 % CHG	978 (187.70)*
	0.003 % BZC	771 (231.60)
14028S	Untreated	700 (117.20)
	0.006 % CHG	902 (299.60)
	0.008 % BZC	771 (170.80)

* = significantly different ($p \leq 0.05$) from untreated

There was no significant difference ($p \leq 0.05$) in mean particle size after treatment of 14028S with 0.006 % CHG or 0.008 % BZC (table 3.12). This suggests that exposure to these biocides at the concentration tested for 15 min did not result in the formation of bacterial aggregates. Similarly, exposure of SL1344 to 0.003 % BZC did not result in any significant change in particle size ($p \leq 0.05$). However, exposure of SL1344 to 0.010 % CHG resulted in a significant increase in particle size. This suggests that exposure to 0.010 % CHG may cause the formation of bacterial aggregates in this strain. The approximate diameter of a single *Salmonella* bacterium is 0.5-0.7 µM (500-700 nm). This correlates with the mean particle diameter observed in untreated samples (~700 nm) in the case of both strains and suggests few, if any aggregates present in the untreated sample. The mean particle diameter of 978 nm observed after exposure of SL1344 to 0.010 % CHG is therefore unlikely to represent bacterial aggregates and is more

likely to represent the association of 1-2 cells that may be growing or dividing. Is it therefore also unlikely that CHG efficacy would be affected in this case.

3.3.7 Microarray

Microarray experiments were carried out after exposure of SL1344 and 14028S to CHG (0.0004 % for SL1344, 0.0001 % for 14028S and BZC (0.0004 %) with a view to finding a potential marker gene that was significantly up or down-regulated in both strains and both biocide treatments.

Table 3.13 shows the numbers of genes significantly up-regulated that were common to both strains (same treatment) and common to CHG and BZC (same strain).

Table 3.13: Numbers of genes significantly up/down-regulated (p≤0.01) for each treatment and number of genes common to both strains/treatments

Strain	Treatment	No. of genes significantly up-regulated	No. of genes significantly down-regulated	No. of genes common to other strain	No of genes common with other biocide
SL1344	0.0004 % CHG	109	91	4	31
	0.0004 % BZC	310	268	24	
14028S	0.0001 % CHG	84	76	4	18
	0.0004 % BZC	177	195	24	

SL1344 and 14028S responded quite differently to CHG and BZC treatment at the gene expression level, as they only had 4 significantly changed genes in common after CHG exposure and 24 significantly changed genes in common after BZC exposure (Table 3.13). Thirty one common genes were altered in expression after exposure of strain SL1344 to CHG and BZC, and 18 genes after exposure of strain 14028S to CHG and BZC. The relatively small numbers of genes common to both biocides suggests that the effect each biocide has on the bacterial cell is different. Figure 3.8 shows the distribution of significantly up or down-regulated genes for each treatment.

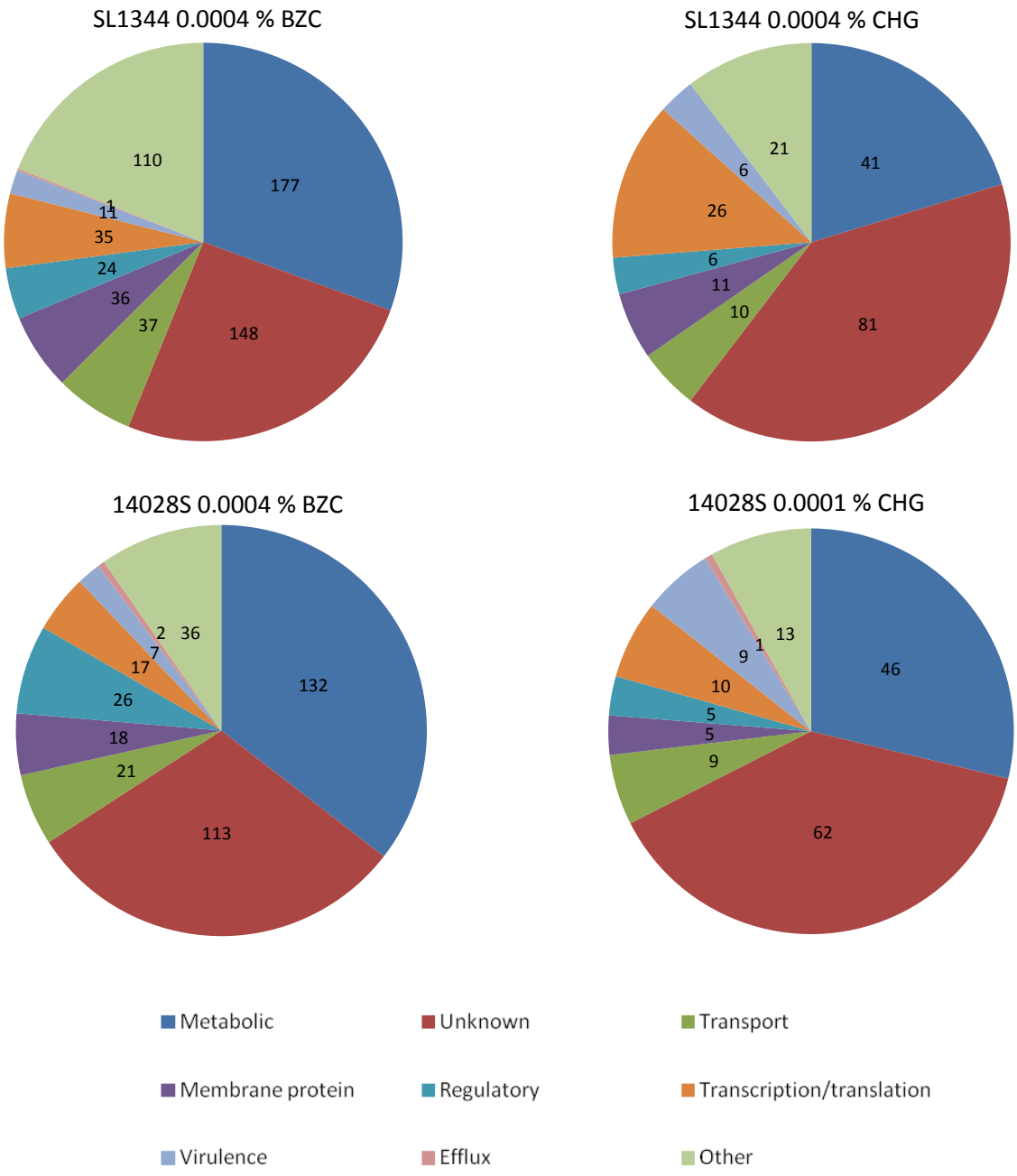


Figure 3.8: The distribution of genes significantly up or down-regulated after exposure of SL1344 and 14028S to a low concentration of CHG or BZC

Figure 3.8 shows that in both strains and both treatments, the largest proportion of genes with a significant change in expression were those involved in metabolic processes, e.g. kinases, glycotransferases, dehydratases, dehydrogenases. A large number of genes with unknown function were also significantly up or down-regulated. Exposure to BZC resulted in a greater overall number of significantly altered genes in comparison to CHG exposure (figure 3.8, table

3.13). The proportions of genes within each functional group did not differ substantially across strains and treatments, suggesting that the general response to each biocide by each strain may be similar, despite the individual genes responsible for this response differing between each strain and treatment.

Results from other experiments in this chapter indicated potential up-regulation of efflux activity as a result of biocide exposure, as well as potential formation of bacterial aggregates as a result of CHG exposure. Changes in the expression of genes that could potentially be associated with these responses were searched for in the microarray data. Changes in membrane associated genes were also searched for as CHG and BZC both primarily cause damage to the bacterial membrane. Table 3.14 shows key genes that were up or down-regulated and could possibly be associated with results observed in other experiments in this chapter.

Table 3.14: Fold changes in genes associated with transport, membrane, efflux, aggregation, metabolism and regulation

↑ = up-regulated ↓ = down-regulated

Gene name	Fold Change	Function	Possible explanation
Transport			
potE	9.06 ↑ in SL1344 BZC	Putrescine-ornithine antiporter	Putrescine is involved in cell proliferation and growth in *E. coli* (Tabor and Tabor, 1984)
potA	1.60 ↑ in 14028S CHG	Spermidine/putresene transport ATP-binding protein (ABC family)	
ybhR	1.64 ↑ in SL1344 BZC	Putative transport protein	Transport of compounds for metabolic processes
dcuA	4.50 ↑ in 14028S BZC	Anaerobic C4-dicarboxylate transporter	Anaerobic respiration – energy production
dcuB	6.74 ↑ in SL1344 BZC		

dcuC	4.56 ↑ in 14028S BZC		
ydeY	2.07 ↑ in SL1344 BZC	Putative sugar transport protein	Transport of sugars for respiration
ego	2.80 ↑ in SL1344 CHG	Putative ABC-type aldose transport ATPase component	
glpF	2.80 ↑ in 14028S CHG	Glycerol uptake facilitator protein	Uptake of glycerol as a source of carbohydrate for energy production
Membrane associated			
blc	2.00 ↑ in SL1344 CHG and BZC	Lipoprotein	Membrane integrity
spr	2.22 ↑in SL1344 BZC	Putative outer membrane lipoprotein	
nlpI	1.65 ↑ in SL1344 BZC	Lipoprotein	
ybfN	1.63 ↑ in SL1344 CHG	Putative lipoprotein	
rfaZ	1.35 ↑ in 14028S CHG	LPS core biosynthesis protein	Structural integrity and protection of membrane
wzzE	1.22 ↑ in 14028S CHG	LPS core biosynthesis protein	
tolA	0.79 ↑ in SL1344 CHG	Inner membrane integrity	Inner membrane integrity
ompW	6.80 ↑ in 14028S BZC	Outer membrane protein	Possible porin – biocide removal
yhbV	4.22 ↑ in 14028S BZC	Putative membrane protein	Membrane integrity/biocide removal
Efflux			
ybhS	1.80 ↑in SL1344 BZC	Putative ABC superfamily membrane protein	Removal of biocide from the cell
sfbB	1.27 ↑ in 14028S CHG	Putative ABC transport system ATPase component	
ydgF	2.00 ↑ in 14028S CHG	Multidrug efflux system protein MdtJ	
mdfA	1.40 ↑ in 14028S CHG 1.70 ↑ in 14028S BZC	Multidrug translocase – similar to *E. coli* PMF pump	

acrD	1.76 ↑ in 14028S BZC	Putative efflux pump	
emrD	0.75 ↓ in SL1344 BZC	Multidrug resistance protein D	
Aggregation			
fimZ	0.67 ↓ in SL1344 BZC	Regulator of FimA (major fimbriae protein)	De-repression of *fimA* expression and increase in fimbriae
Metabolic			
hycA	13.8 ↑ in 14028S BZC	Formate hydrogenlyase regulatory protein	Anaerobic respiration component – conversion of formate to CO2 and H2
hybB	9.93 ↑ in 14028S BZC	Hydrogenase-2 large subunit	Anaerobic metabolism, generation of transmembrane proton motive force
hybA	6.79 ↑ in 14028S BZC	Hydrogenase-2 small protein	
hybC	6.69 ↑ in 14028S BZC	Hydrogenase-2 large subunit	
hybD	6.74 ↑ in 14028S BZC	Hydrogenase-2 component protein	
adhE	6.44 ↑ in 14028S BZC 3.15 ↑ in SL1344 BZC	Alcohol dehydrogenase	Fermentation of pyruvate to generate NAD+ (i.e. energy)
pflF	1.68 ↑ in SL1344 CHG	Putative pyruvate formate lyase	
yhhX	1.45 ↑ in SL1344 CHG	Putative dehydrogenase	
crp	1.27 ↑ in SL1344 CHG	cAMP receptor protein	Energy production in the absence of glucose
glpA	6.13 ↑ in 14028S CHG	Anaerobic glycerol 3-phosphate dehydrogenase subunit A	Anaerobic respiration
garK	4.11 ↑ in 14028S CHG	Glycerate kinase I	Catalyses ADP production
dgoK	3.65 ↑ in 14028S CHG	2-oxo-3-deoxygalaconate kinase	Anaerobic metabolism components
dgoA	6.91 ↑ in SL1344 BZC	2-oxo-3-deoxygalaconate-6-phosphate	

		aldolase/galacatonate dehydratase	
Regulatory			
acrR	0.60 ↓ in 14028S BZC	*acrAB* operon repressor	De-repression of *acrAB* genes and increased efflux gene expression
soxS	0.40 ↓ in 14028S BZC	Regulator of oxidative stress genes	Up-regulation of oxidative stress genes
ynaF	1.45 ↑ in SL1344 BZC	Putative universal stress protein	Stress response

As shown in table 3.14 sugar transporters were up-regulated in both strains and treatments, suggesting that during CHG and BZC exposure there is an up-regulation of genes associated with the generation of energy for further cellular processes. Numerous efflux-associated genes also underwent significant changes in expression after exposure to both biocides, correlating with the efflux assay experiments which were suggestive of increased efflux activity. *acrR* - a regulator of the *acrAB* operon of efflux-associated genes was down-regulated, which may have resulted in de-repression of this operon, and increased expression of efflux associated genes to remove biocide from the bacterial cell. A large number of genes associated with anaerobic metabolism were up-regulated, suggesting that during biocide exposure, both strains switch to anaerobic respiration, and also suggesting that there was an increased requirement for energy production. Several genes encoding lipoproteins and membrane proteins were up-regulated. Considering the fact that CHG and BZC primarily cause damage to the bacterial cell membrane, genes encoding membrane proteins may have been up-regulated to improve membrane integrity in the presence of the biocide. However there did not appear to be any significant up-regulation of lipid synthesis genes. The regulatory gene *fimZ* was the only gene associated with aggregation that was significantly altered as a result of biocide exposure. Down-regulation of *fimZ* may have resulted in the increased expression of *fimA* and therefore the increased production of aggregative fimbriae. However this was only observed in strain 14028S exposed

to BZC. Down-regulation of *soxS* was observed in 14028S exposed to BZC. This may have resulted in de-repression of genes associated with counteracting oxidative stress inflicted on the cell as a result of biocide exposure. The up-regulation of efflux-associated genes, and membrane-associated genes may have contributed to the elevated MIC and MBC values observed in the biocide exposure experiments in tables 3.4 and 3.5.

The microarray experiments did not identify a marker gene that was common to both strains and both treatments, but did identify genes that may have contributed to the elevated MICs and MBCs observed, and the possible increased efflux activity observed. Six genes (*ydgF, blc, hycA, hybB, acrR, ompW*) were investigated using real-time PCR (Table 3.15).

3.3.8 Real-time PCR

Six genes were selected from microarray data and their expression in biocide-treated bacteria was compared with that in untreated bacteria. *ydgF, blc, hycA, hybB, acrR and ompW* were chosen either due to a large change in their expression after biocide exposure in the microarray experiments or due to possible association with other results obtained e.g. efflux up-regulation, elevated MICs/MBCs. Tables 3.15 and 3.16 show the changes in gene expression of the selected genes after growth of SL1344 (a) or 14028S (b) to mid-log phase in the presence of a low CHG/BZC concentration.

Table 3.15: Fold changes in gene expression after exposure of strain SL1344 to 0.0004 % CHG or BZC. N=3

↑ = up-regulated ↓ = down-regulated FC = Fold Change

Gene	Treatment	FC in microarray	FC qPCR ± SD
ydgF	0.0004 % CHG	1.58 ↑	1.33 ± 0.10 ↑
	0.0004 % BZC	1.80 ↑	2.52 ± 0.53 ↑
blc	0.0004 % CHG	1.82 ↑	4.64 ± 0.00 ↑
	0.0004 % BZC	2.00 ↑	6.83 ± 0.06 ↑
hycA	0.0004 % CHG	1.19 ↑	1.43 ± 0.22 ↑
	0.0004 % BZC	1.02 ↑	2.42 ± 1.41 ↑
hybB	0.0004 % CHG	1.95 ↑	1.20 ± 2.29 ↑
	0.0004 % BZC	2.97 ↑	0.97 ± 0.26 ↑
acrR	0.0004 % CHG	1.87 ↓	132. ± 0.22 ↓
	0.0004 % BZC	1.17 ↓	2.39 ± 0.71 ↓
ompW	0.0004 % CHG	2.14 ↑	3.20 ± 0.84 ↑
	0.0004 % BZC	2.34 ↑	4.14 ± 0.22 ↑

The greatest change in gene expression occurred in *acrR* after exposure of SL1344 to 0.0004 % CHG, where a 132 fold down-regulation of this gene was observed (table 3.15). *acrR* is a potential repressor of the *acrAB* operon which encodes genes associated with efflux. Down-regulation of this gene may therefore result in loss of repression of efflux, and possible increased efflux of the biocide from the cell. This level of down-regulation was not observed in *acrR* after exposure of SL1344 to 0.0004 % BZC. The greatest up-regulation in gene expression after biocide exposure was observed in *blc* (6.83 fold) after exposure of SL1344 to 0.0004 % BZC. Blc is a putative lipoprotein and may therefore be involved in the maintenance of membrane integrity or removal of biocide from the bacterial cell. The remaining changes in gene expression ranged between 4.64 and 1.33 fold.

Table 3.16: Fold changes in gene expression after exposure of 14028S to 0.0001 % CHG or 0.0004 % BZC. N=3

↑ = up-regulated ↓ = down-regulated FC = Fold Change

n/a – change in this gene was not provided in raw microarray data

Gene	Treatment	FC in microarray	FC qPCR ± SD
ydgF	0.0001 % CHG	2.00 ↑	1.35 ± 0.45 ↑
	0.0004 % BZC	2.88 ↑	2.14 ± 0.76 ↑
blc	0.0001 % CHG	1.21 ↑	2.76 ± 0.42 ↑
	0.0004 % BZC	1.10 ↑	1.94 ± 0.74 ↑
hycA	0.0001 % CHG	n/a	0.99 ± 0.87 ↑
	0.0004 % BZC	13.8 ↑	14.4 ± 0.16 ↑
hybB	0.0001 % CHG	5.10 ↑	11.8 ± 0.91 ↑
	0.0004 % BZC	9.90 ↑	10.5 ± 0.93 ↑
acrR	0.0001 % CHG	1.30 ↓	148.7 ± 0.53↓
	0.0004 % BZC	1.76 ↓	1.87 ± 1.19 ↓
ompW	0.0001 % CHG	4.44 ↑	6.80 ± 0.23 ↑
	0.0004 % BZC	5.76 ↑	9.95 ± 0.42 ↑

The greatest change in gene expression occurred in *acrR*, after exposure of strain 14028S to 0.0001 % CHG (Table 3.16). A 148.7 fold down-regulation of this gene was observed. This suggests that exposure to a low concentration of CHG can result in common responses between both strains. The greatest up-regulation was observed in *hybB* after exposure to both CHG and BZC (table 3.16). The *hybB* gene encodes the large subunit of the hydrogenlyase-2 enzyme. HybB is involved in anaerobic metabolism and its up-regulation may result in increased production of transmembrane proton motive force required for biocide efflux. *hybB* was not up-regulated to the same level in SL1344 (table 3.15). A 14.4 fold up-regulation of the *hycA* gene was also observed after 14028S exposure to 0.0004 % BZC (table 3.16). HycA is a

formate hydrogenlyase regulatory protein and may be associated with HybB. The remaining changes in gene expression ranged between 9.95 and 0.99 fold (table 3.16).

3.4 Discussion

The MIC and MBC values obtained for *S. enterica* strains SL1344 and 14028S were considerably lower than the concentrations currently used in commercial products. CHG is used in mouthwash at 0.2 % and in alcohol based hand rubs at 1 % (Lai *et al.*, 2012). The MICs obtained here were 0.003 % and the MBCs 0.01 % or less for SL1344 and 14028S. This suggests that the strains used here were highly susceptible to the in-use concentrations of CHG. Condell *et al*., (2012a) looked at the MIC of chlorhexidine for SL1344 using a broth dilution method, like the one used in the experiments in this chapter, and found that it was 0.0004 %. This is approximately 10-fold lower than the MIC determined here and confirms this strains' high susceptibility to the biocide. BZC is used at a concentration range of up to 0.5 % in strong cleaning products. The MICs and MBCs obtained for both *Salmonella* strains were considerably lower than this (0.003 – 0.008 %). This shows that both strains were highly susceptible to this biocide at the in-use concentration. Condell *et al.,* (2012a) also looked at the susceptibility of a different serovar of *Salmonella* (Hvittingfoss S41) to BZC and found that the MIC was 0.0015 %. This value was close to the MICs for BZC of the strains used here. Morrissey *et al.,* (2014) defined the 'cut-off' MIC for BZC- resistant *Salmonella* spp. as 128 mg/L (0.0128 %). This value is much greater than the MIC values observed here, suggesting SL1344 and 14028S are not resistant to BZC. The initial susceptibility of all strains tested provided a good comparison point for any reduced susceptibility observed after biocide exposure.

Exposure of strains SL1344 and 14028S to a range of low concentrations of CHG and BZC (0.00001 – 0.0004 %) resulted in an increase in the MIC and MBC of both biocides of up to 100 fold. These increases were highly reproducible and provided a useful initial marker of resistance that was obtainable within a 24 h period. The broth dilution method used also allowed for the testing of multiple strains and biocides at one time, making this technique very high throughput. Previous work has been carried out looking at single and repeat exposure of *Pseudomonas aeruginosa* to chlorhexidine diacetate (Thomas *et al.*, 2000). Thomas et al.,

(2000) reported that single exposure of *Ps. aeruginosa* to a residual (sub-lethal) concentration of chlorhexidine diacetate resulted in an increase in the MIC of this biocide. An increase in MIC has also been observed by Escalada *et al.,* (2005) in *E. coli* exposed to the biocide triclosan. However, neither of these studies report such an increase in MBC as here observed with *S. enterica* serovar Typhimurium. Thomas *et al.,* (2000) also reported that the increased MIC for chlorhexidine diacetate observed after a single exposure was not stable and did not result in any antibiotic cross-resistance. This correlates with the unstable increases in MIC and MBC observed here in strains SL1344 and 14028S. Mavri *et al*., (2013) reported adaptation to BZC and chlorhexidine diacetate in *Campylobacter jejuni* and *Campylobacter coli* that was stable for 10 passages in biocide-free broth. However this adaptation was achieved through step-wise subculturing of the bacteria through increasing concentrations of the biocide, rather than after a single exposure.

Exposure of *S. enterica* serovar Typhimurium to low CHG and BZC concentrations resulted in a significant decrease in the zone of inhibition size (mm) for the antibiotics ciprofloxacin and ceftriaxone. Koljalg *et al.,* (2002) reported a relationship between chlorhexidine susceptibility and decreased susceptibility/resistance to antibiotics. They investigated the susceptibility of clinical isolates of Gram-negative bacteria including *E. coli, Ps. aeruginosa* and *Klebsiella pneumoniae* to chlorhexidine and several antibiotics, one of which was ciprofloxacin. They reported that *K. pneumoniae* with decreased chlorhexidine susceptibility was also clinically resistant (according to the National Committee for Clinical Laboratory Standards) to several antibiotics including imipenem, cefotaxime, gentamicin and ciprofloxacin. This suggests that there may be a common mechanism responsible for reduced susceptibility to both chlorhexidine and ciprofloxacin. This mechanism may have been triggered here in strains SL1344 and 14028S during 5 min exposure to the range of low CHG concentrations tested, resulting in an increase in CHG and BZC MIC and MBC and changes in the zone of inhibition size

for the antibiotics ciprofloxacin and ceftriaxone. Koljalg *et al.,* (2002) did not speculate as to a possible mechanism associated with decreased susceptibility to multiple antimicrobial products. However the qPCR data generated in this chapter is suggestive of increased efflux activity as a result of down-regulation of the regulatory gene *acrR*. Mavri *et al.,* (2013) reported reduced susceptibility to ciprofloxacin as a result of BZC exposure in *Campylobacter* spp. which correlates with what was observed here in *Salmonella*, and they attributed this to the up-regulation of more than one type of efflux system, and also to thickening of the cell envelope.

Tattawasart *et al*., (1999) speculated that alterations to the bacterial cell envelope may be responsible for the resistance to triclosan and some antibiotics that they observed in chlorhexidine diactetate-resistant *Ps. stutzeri.* It is possible that changes to the cell envelope may have contributed to the decreased susceptibility to both CHG and BZC observed here in SL1344 and 14028S after a single exposure to these biocides. In contrast to this Braoudaki and Hilton (2004) suggested that mechanisms of resistance to more than one antimicrobial may not be non-specific like cell envelope changes. They found that BZC-resistant *Salmonella enterica* serovar Virchow were also resistant to chlorhexidine but chlorhexidine-resistant isolates were not resistant to BZC, suggesting the application of a specific mechanism involving alteration of a particular cellular target rather than a non-specific mechanism designed to remove the antimicrobial from the cell.

Changes to the cell envelope may also encompass alterations in OMP composition as a result of biocide exposure. Condell *et al*., (2012b) investigated total protein changes in triclosan-sensitive and triclosan-adapted (>1000 fold increase in MIC) *S. enterica* serovar Typhimurium using one and two dimensional SDS-PAGE. They did not observe any significant changes in protein composition when comparing the wild type and adapted strains using one dimensional SDS-PAGE. This correlates with what was observed here after exposure of SL1344 and 14028S

to a low concentration of CHG and BZC. However Condell *et al*., (2012b) did observe numerous changes in total protein composition when using two dimensional SDS-PAGE, suggesting that one dimensional SDS-PAGE may be limited in its sensitivity. Interestingly, a large number of proteins that were up-regulated were involved in glycolysis and the generation of energy (e.g. GarL AtpA, GapA). This was also observed here in genes associated with energy generation in the microarray work carried out (e.g *hybB, garL, agp, atpG*). Zhang *et al*.,(2011) looked at the effect of phenol exposure on *E. coli* and observed changes in 9 different proteins using SDS-PAGE and Western blotting, one of which was the porin OmpA. Rushdy *et al*., (2013) also used SDS-PAGE to identify changes in OMPs in 5 MDR clinical isolates of *S. enterica* and found loss of OmpF in all 5. This protein was however present in the reference strain (14028). OmpF is also a porin and may therefore have been lost to reduce the access of antimicrobials to the bacterial cell. Karatzas *et al*., (2008) also found reduced levels of OmpA, C and F in multiple antibiotic resistant *S. enterica* serovar Typhimurium isolates that had been exposed to 4 disinfectants. However Mavri *et al*., (2013) found that loss of OMPs in *C. coli* was actually associated with very weak adaptive resistance to the biocides tested, so it may not necessarily be associated with reduced biocide susceptibility. This may be the reason why there was no loss of protein expression observed in the one-dimensional SDS-PAGE carried out here. Microarray and qPCR work carried out here showed an increase in the expression of *ompW* after biocide exposure. It is possible that OmpW may be involved in the efflux of compounds from the cell, hence its increased expression. No significant alteration in the expression of any other *omp* genes was observed in the microarray work. However it is worth noting that an alteration in gene expression does not always directly correlate with the amount of the corresponding protein present. The limited exploration of OMP changes carried out here just using one dimensional SDS-PAGE means that protein identity was not determined, and detailed conclusions about alterations in the OMP profile after exposure to a low concentration of CHG/BZC cannot be

drawn. Further experiments using two-dimensional SDS-PAGE or Western blotting may provide more detail on the OMP profile.

Pagedar *et al*., (2011) reported adaptation to BZC and ciprofloxacin and consequent cross-resistance to other antimicrobials in *Ps. aeruginosa.* They speculated that efflux pump activity was responsible for the adaptation observed and confirmed this by reducing adaptation using the efflux pump inhibitor 2, 4 dinitrophenol. They also reported that efflux pump inhibition was more effective in isolates that were originally non-resistant to BZC and ciprofloxacin, again suggesting that efflux pump activity was likely to be responsible for the adaptation and cross-resistance. It is possible that the reduced susceptibility to CHG and BZC observed here in strains SL1344 and 14028S was due to efflux pump activity, especially due to the fact that decreased accumulation of Hoechst dye was observed in CHG and BZC-treated cells in the efflux assay experiments, and furthermore due to >100 fold down-regulation of *acrR* in the microarray experiments.

Pagedar et al., (2012) also used ethidium bromide accumulation and fluorescence to measure the efflux activity in BZC-resistant and non-BZC resistant *E. coli* strains. They found the same level of efflux up-regulation in both resistant and non-resistant strains after BZC exposure. This suggests that efflux up-regulation was not just a characteristic of strains adapted to a biocide but also a response observed in non-resistant strains as a result of exposure. This finding correlates with the work carried out here, as both SL1344 and 14028S were not biocide-adapted strains and demonstrated a potential increase in efflux activity as a result of biocide exposure. Pagedar *et al*., (2012) also investigated the level of efflux pump activity up-regulation in *E. coli* strains adapted to the antibiotic ciprofloxacin and found between 16 and 50 % up-regulation despite antibiotic resistance commonly being attributed to more specific mechanisms. The reduced antibiotic susceptibility observed in *S. enterica* strains SL1344 and 14028S after exposure to low CHG and BZC concentrations could therefore be attributed to

efflux pump up-regulation. In contradiction to this Naparstek *et al*., (2012) reported reduced susceptibility to chlorhexidine in *Klebsiella pneumoniae* but were not able to link it to efflux pump activity. They found that high chlorhexidine MICs were independent of the expression of *cepA, acrA* and *kdeA* efflux pump genes. This suggested that efflux activity was not necessarily the most likely reason for reduced biocide susceptibility, and this must be taken in to account when considering the increased MICs and MBCs observed here in SL1344 and 14028S.

The AcrAB-TolC tri-partite efflux system is the major multidrug efflux pump present in *Salmonella* species and has been shown to be over-expressed in response to low doses of biocides (Karatzas *et al.*, 2007, Randall *et al.*, 2007). The expression of other efflux systems such as AcrEF and AcrD can also mediate multidrug-resistance. Whitehead *et al.,* (2011) described the up-regulation of efflux activity in response to the biocides Trigene and Superkill at the in-use concentration (1%) using the same method used in this chapter. They attributed this up-regulation to the increased expression of the regulatory gene *marA,* and *acrF*. They concluded that the AcrEF-TolC efflux system was responsible for the up-regulated efflux activity. The biocide Superkill contains a mix of aldehydes and QACs. Considering the fact that BZC (tested here) is also a QAC, there is the possibility that the up-regulation of efflux observed in *Salmonella* strains SL1344 and 14028S could also be due to increased expression of the AcrEF-TolC efflux system. The efflux pump inhibitor PAβN blocks the function of RND transporters and its use resulted in the increased accumulation of Hoechst dye in strain SL1344. This again suggests that an RND transporter system such as AcrEF-TolC may be up-regulated in the presence of BZC. In further support of an RND transport system being involved in the potential increase in efflux activity observed here, the regulatory gene *acrR* (regulates the *acrAB* operon involved in efflux (Kumar and Schweizer, 2005) was significantly down-regulated in both SL1344 and 14028S after exposure to a low CHG concentration. Down-regulation of this gene may result in decreased regulation of the *acrAB* operon and increased efflux activity.

Exposure of *S. enterica* serovar Typhimurium strain SL1344 to CHG at the minimum bactericidal concentration (0.01 %) resulted in a significant increase in mean particle size (nm). However the observed particle size after CHG exposure was too small (978 nm) to correspond to the formation of bacterial aggregates. Aggregate formation in *Salmonella* species has been observed by other groups. White *et al*., (2008) investigated *Salmonella* morphotypes under conditions of stress and found that when under stress *Salmonella* form a red, dry and rough morphotype where they express an increased number of aggregative fimbriae. This morphotype has been associated with reduced susceptibility to the biocide sodium hypochlorite. An increase in the formation of bacterial aggregates could reduce the efficacy of a particular biocide. Bacteria frequently exist in biofilms *in vivo* and the aggregative phenotype associated with biofilms has been shown to cause a reduction in biocide efficacy (Wong *et al.*, 2010a, Wong *et al.*, 2010b). However in the case of CHG, exposure did not appear to result in the formation of bacterial aggregates and biocide efficacy was therefore unlikely to be reduced. It is also worth noting that a statistically significant change in particle size only occurred in SL1344 after exposure to CHG, but elevated CHG and BZC MICs and MBCs were observed in both SL1344 and 14028S after CHG and BZC exposure, so it is unlikely that aggregate formation is a key reason behind the level of MBC increases observed in this study. In addition, microarray experiments did not show significant changes in the expression of genes associated with aggregation (e.g. *fim* genes or *csg* (curli-associated) genes) after CHG exposure.

The flow cytometry work carried out indicated that 5 min exposure of SL1344 and 14028S to a low concentration of CHG and BZC resulted in damage to > 50 % of the population, but did not result in a large amount of cell death. The lack of cells present (< 3 %) in Q2-1 (dead) suggested that a significant proportion of the population may have survived, and that some of these surviving cells may be responsible for the high MICs and MBCs observed in section 3.3.2. It of course cannot be ascertained from the flow cytometry plots alone whether or not those cells

that were damaged would ultimately recover from the damage or die. Whitehead et al., (2011) used flow cytometry to look at *S. enterica* serovar Typhimurium cells exposed to a low concentration of the biocides Superkill, Trigene, AQAS and Virkon for 5 hours. They reported a mixture of live, damaged and dead cells after exposure – much like what was observed with SL1344 and 14028S here. Whitehead *et al*., (2011) sorted cells that maintained an intact membrane and membrane potential (i.e. alive) and found that these cells showed no alteration in susceptibility to antibiotics tested and that there was no significant difference in the number of cells in the 'live' quadrant after these survivors were re-challenged with the same concentration of biocide as before. This correlates with the phenotype stability test data obtained here, in that there were no stable changes observed in the susceptibility of surviving organisms. However due to the fact that cell sorting was not performed here, a direct comparison cannot be made. Whitehead *et al*., (2011) successfully used flow cytometry and cell sorting to identify mutants with stable antibiotic resistance after 5 h exposure of *S. enterica* serovar Typhimurium to Superkill and Trigene at the in-use concentration. This highlights the usefulness of the technique in the identification of organisms that survive biocide exposure, where techniques such as a suspension test may not as they require the recovery of organisms on rich media over a 24 h period.

A large number of experimental techniques were employed in order to determine if exposure of *S. enterica* strains SL1344 and 14028S to low concentrations of CHG and BZC resulted in a change in antimicrobial susceptibility of surviving organisms. Potential markers of biocide resistance identified included significant increases in the MIC and MBC of both CHG and BZC, increased efflux activity, up-regulation of genes associated with efflux (microarray, qPCR), survival of bacterial cells (i.e. cells in 'live' quadrant) after biocide exposure. The use of strains SL1344 and 14028S resulted in the acquisition of highly reproducible MIC, MBC and antibiotic susceptibility data, suggesting that *S. enterica* serovar Typhimurium may be a useful model

organism in the prediction of bacterial resistance to biocides and cross resistance to antibiotics.

Chapter Four: Burkholderia lata strain 383 as a model bacterium for predicting biocide resistance

4.1 Introduction

Burkholderia species are a common problem in both the clinical and industrial environments. Their intrinsic resistance to multiple antimicrobials makes *Burkholderia* infections in immunocompromised individuals (particularly those with cystic fibrosis) difficult to treat, and has also resulted in increasingly frequent isolation of numerous *Burkholderia* species from pharmaceutical and cosmetic products. The most commonly isolated *Burkholderia* species in both the clinic and pharmaceutical products are members of the *Burkholderia* cepacia complex (Bcc).

4.1.1 The Burkholderia cepacia complex

The Bcc currently comprises at least 17 closely related Gram-negative, non-fermenting, motile rods (Rushton *et al.*, 2013). An isolates position in the Bcc is determined via sequencing of the *recA* gene and through multi-locus sequence analysis. Bcc species have been isolated from numerous environments including freshwater, soil and plant rhizospheres (Mahenthiralingam *et al.*, 2008). They have been used as bio-pesticides to prevent the spread of fungal disease in plants in some cases (Torbeck *et al.*, 2011). However they are best known for their roles as opportunistic pathogens in disease development and as major contaminants of disinfectants, cosmetics and pharmaceuticals (Sousa *et al.*, 2011).

Some Bcc species have unique metabolic features that include the ability to degrade carcinogenic or toxic products such as polycyclic aromatic compounds and other constituents of crude oil (Torbeck *et al.*, 2011). This makes them a useful candidate for bioremediation processes but also makes them difficult to eliminate from pharmaceutical products as they are able to metabolise a range of carbon sources. They can also remain viable in stressful conditions, such as low nutrients and the presence of antimicrobials or organic solvents, and can survive in water for many months (Torbeck *et al.*, 2011). There is therefore a high risk to

patients/users if these organisms are present at any stage of the manufacturing process of pharmaceutical products (e.g. process water, manufacturing equipment).

4.1.2 Burkholderia in the clinical environment

Bcc bacteria cause infection in 2-8 % of cystic fibrosis (CF) patients (Lipuma, 2010) with *Burkholderia cenocepacia* and *Burkholderia multivorans* being responsible for 85-97 % of infections (Drevinek *et al.*, 2008). The primary route of transmission is through direct interpersonal contact or through contact with body perspiration, although Bcc can also be transmitted via contact with hard surfaces (Torbeck *et al.*, 2011). Nosocomial (hospital-acquired) *Burkholderia* infection in non-CF patients has been observed due to the use of contaminated ultrasound gels (Jacobson *et al.*, 2006) and the use of contaminated chlorhexidine solution (0.5 %) before blood transfusions (Garcia-Erce *et al.*, 2002).

Pulmonary colonisation by Bcc bacteria, via adhesion to lung epithelial cell receptors and invasion via intracellular vacuoles or epithelial translocation, may persist for years and can cause severe deterioration of patient health. Virulence factors such as lipases, metalloproteinases and LPS induce an inflammatory response, damage the epithelial cell layer and can inhibit proper formation of tight junctions between epithelial cells (McClean and Callaghan, 2009). Patients may develop 'Cepacia syndrome', characterised by severe necrotising pneumonia and acute pulmonary deterioration, which can cause death within weeks.

Antibiotic combination therapy (may include tobramycin, ciprofloxacin, ceftazidime, imipenem, meropenem, co-trimoxazole) is used to clear early infection in CF patients (Rose *et al.*, 2009) but is often ineffective due to the intrinsic resistance to multiple clinically used antibiotics that Bcc species possess (Jassem *et al.*, 2014, Aaron *et al.*, 2000). Multiple drug

resistance (MDR) in CF isolates is defined as resistance to all agents belonging to at least two of three antibiotic classes (Bazzini *et al.*, 2011).

4.1.3 Burkholderia in the industrial environment

As well as their role as opportunistic pathogens Bcc members are contaminants of home and personal care (HPC) products. Contaminated sterile solutions, pharmaceuticals, cosmetics, disinfectants and preservatives are considered major sources of Bcc infections acquired worldwide (Torbeck *et al.*, 2011, Jimenez, 2004). *B. cepacia* is one of the most frequently isolated organisms in pharmaceutical samples around the world (Jimenez, 2004) although many of these reports have not accurately determined the Bcc species identity. Bcc contamination has resulted in the recall of large numbers of cosmetic products. Between 1994 and 1998 *B. cepacia* contamination was the cause of 33 % (19/55) of recalled cosmetic products in the United States (Wong *et al.*, 2000) and 4 % (1/24) of recalled contaminated cosmetics in the EU between 2005 and 2008 (Lundov and Zachariae, 2008). Bcc species have also been isolated from fuel samples (White *et al.*, 2011). Contamination of fuels can lead to deterioration of the fuel due to accumulation of bacteria, degradation of fuel additives and production of corrosive metabolic by-products. *Burkholderia* spp. contamination has been attributed to a number of things by manufacturers including inadequate cleaning, use of unsuitable water, inadequate storage/sterilisation of products and incorrect efficacy testing (Torbeck *et al.*, 2011). Of further concern is that Bcc species have been found to contaminate products containing BZC, CHG, hydrogen peroxide, sodium hypochlorite, cetylpyridinium chloride, citric acid and more (Torbeck *et al.*, 2011). All of these compounds are usually employed due to their bacteriostatic or bactericidal activity. Considering the extent to which Bcc species are capable of growing in the presence of antimicrobials and considering the fact that finished product testing is not always reliable in determining product efficacy it would be very useful to establish a standard protocol to predict biocide resistance in these organisms.

4.1.4 Mechanisms of resistance in Burkholderia

Antimicrobial resistance in *Burkholderia* has frequently been attributed to the presence and expression of RND type efflux pumps (Rushton *et al.*, 2013, Bazzini *et al.*, 2011). These efflux pumps have contributed to resistance to chloramphenicol, quinolones and tetracyclines in planktonic *B. thailandensis* cells (Biot *et al.*, 2011), as well as chlorhexidine tolerance in planktonic and sessile *B. cenocepacia* cells (Coenye *et al.*, 2011).

Biofilm formation and cell wall impermeability (George *et al.*, 2009, Drevinek *et al.*, 2008) are further mechanisms that contribute to the intrinsic antimicrobial resistance observed in *Burkholderia* species. No correlation between antimicrobial susceptibility and the ability to form a biofilm has been found amongst Bcc species, although *Burkholderia* spp. present in biofilm formation have been found to be more tolerant to certain biocides (e.g. chlorhexidine, hydrogen peroxide) than their planktonic counterparts (Coenye *et al.*, 2011, Peeters *et al.*, 2008). It has also been reported that chlorhexidine concentrations as high as 0.1 - 0.2 % have had no effect on *B. cepacia* biofilms (Miyano *et al.*, 2003).

As a result of the presence of these intrinsic resistance mechanisms Bcc bacteria may need to be exposed to at least 25 times the MIC of an antimicrobial to achieve killing, and some in-use concentrations of biocides such as chlorhexidine (used at 0.1 – 4 %) may not be high enough to achieve killing (Rose *et al.*, 2009). It would therefore be particularly useful to be able to predict resistance to antimicrobial products in these bacteria.

4.1.5 Predicting antimicrobial resistance in Burkholderia lata

B. lata was identified as a new species within the Bcc in 2009 (Vanlaere *et al.*, 2009) through the use of multi-locus sequence typing (MLST) analysis and *recA* gene sequencing. It forms part of a distinct MLST cluster known as group K (figure 4.1). Strain 383 (used throughout this work) is the type strain and was originally recovered from forest soil in Trinidad in 1958

(Vanlaere *et al.*, 2009). A large proportion of *B. lata* isolates are recovered from industrial samples or the environment with fewer being clinical isolates (Vanlaere *et al.*, 2009). *B. lata* strain 383 was selected for use in this work due to the fact that its genome had been fully sequenced and that previous biocide susceptibility and gene expression work had been successfully carried out using this strain (Rushton *et al.*, 2013, Rose *et al.*, 2009).

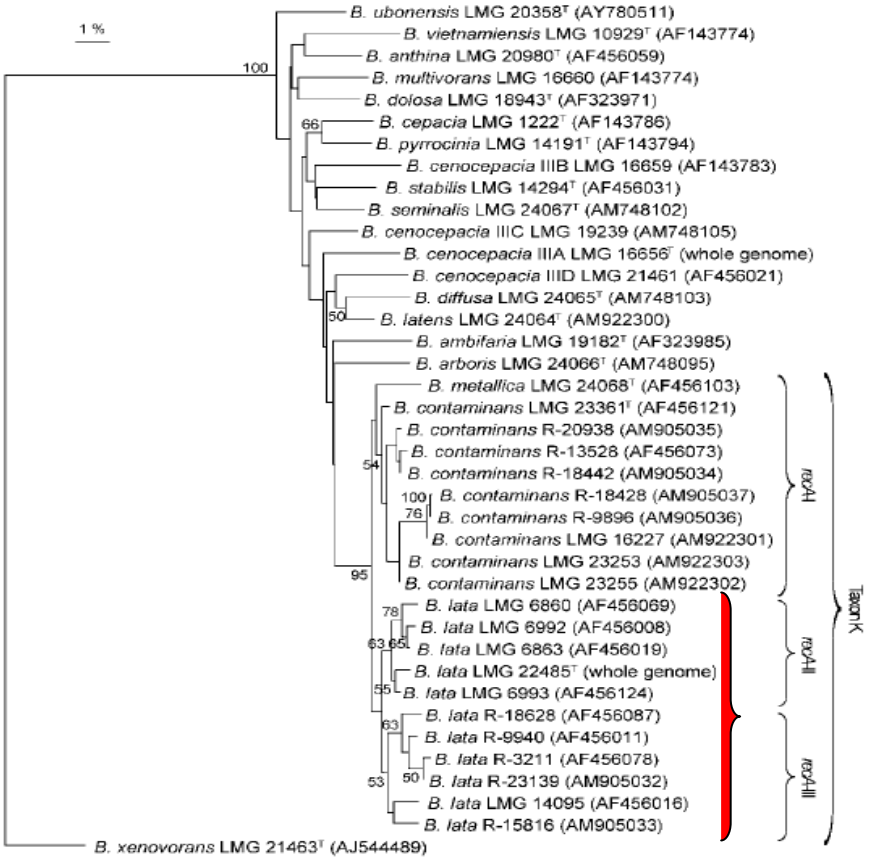


Figure 4.1: A phylogenetic tree derived from recA sequence analysis of established Bcc species and taxon K strains (B. lata in red bracket) adapted from (Vanlaere et al., 2009).

4.1.6 Aims

The principle aim of this chapter was to determine if short-term exposure of *B. lata* strain 383 to a low concentration of CHG or BZC resulted in a change in biocide/antibiotic susceptibility in surviving organisms. Further aims were to assess the suitability of *B. lata* strain 383 as a model

organism for the prediction of biocide/antibiotic resistance and to assess the efficacy/practicality of the chosen techniques employed to measure antimicrobial resistance.

4.2 Materials & Methods

4.2.1 Suspension testing

In order to determine the effect of contact time on biocide efficacy, suspension tests were carried out according to the BS EN 1276 (2009) protocol described in chapter 2 section 2.7.1. Biocides were used at the minimum bactericidal concentration (CHG – 0.4 %, BZC 0.05 %).

4.2.2 Antimicrobial susceptibility testing

4.2.2.1 Baseline data

The MIC and MBC of CHG and BZC were determined following the BS EN ISO: 20776-1 (2006) protocol as described in chapter 2 sections 2.4 and 2.5. Susceptibility to the following antibiotics was also determined following the BSAC disk diffusion protocol (Andrews, 2009) described in section 2.6: ciprofloxacin (1 μg), ceftazidime (30 μg), tobramycin (10 μg), imipenem (10 μg) and meropenem (10 μg). These antibiotics were selected due to their potential use in the treatment of *Burkholderia* infection (Rose *et al.*, 2009).

4.2.2.2 Antimicrobial susceptibility of biocide-exposed organisms

B. lata strain 383 was exposed to a range of low CHG and BZC concentrations (0.005 – 0.04 %) for 5 min according to the BS EN: 1276 (2009) suspension testing protocol described in chapter 2 section 2.7.2. A 5 min exposure time and a 0.005 – 0.04 % concentration range were chosen in order to leave sufficient surviving organisms for further susceptibility testing (i.e. a 2-3 $\log_{10}$ reduction in CFU/mL was observed). After neutralisation, the neutralised suspension was centrifuged at 5000 x g for 10 min and the resulting supernatant discarded. The remaining pellet was re-suspended in 10 mL TSC buffer. This suspension was then used in the determination of the MIC, MBC and antibiotic susceptibility of surviving organisms as described previously in chapter 2 sections 2.4, 2.5 and 2.6. In order to determine the

reproducibility of any changes in antibiotic susceptibility observed, the above experiment was performed on 4 separate occasions over a one year period.

4.2.2.3 Phenotype stability testing

The stability of any changes in antibiotic susceptibility observed after 5 min biocide exposure was determined via the continuous 24 h subculture of surviving organisms through TSB +/- a 0.005 % CHG or BZC. This concentration was chosen as it resulted in changes in antimicrobial susceptibility in survivors. The detailed method is described in chapter 2 section 2.8. Antibiotic susceptibility after 1, 5 and 10 subcultures was determined following the BSAC disk diffusion protocol (Andrews 2009), fully described in section 2.6.

4.2.3 Efflux assays

B. lata strain 383 was grown to mid-log phase (OD_{600} 0.5-0.6) in BSB supplemented with 0.005 % BZC or CHG, or in biocide-free BSB. To ensure the correct growth phase had been reached the OD_{600} of a 1 mL aliquot of each culture was read using an Ultra*pro* 3000 spectrophotometer (GE Healthcare, Buckinghamshire, UK). The accumulation of Hoechst dye was measured in treated and un-treated cells as an indication of the level of efflux activity taking place in the cell. This was also measured in the presence/absence of two EPIs: verapamil (50 mg/L) or phenyl-arginine-beta-napthylamide (PAβN) (10 mg/L) to confirm efflux activity was taking place in the cells. The method based on that used by Whitehead et al. (2011) is described fully in chapter 2 section 2.9. The toxicity of the EPIs was determined prior to performing the efflux assay (chapter 2, section 2.9).

4.2.4 Real-time PCR

4.2.4.1 Genes of interest

Real time PCR reactions were carried out to identify changes in the expression of specific genes after *B. lata* exposure to 0.005 % CHG and BZC, with a view to identifying a marker gene for biocide resistance. The genes selected had previously been identified to be up-regulated after exposure to antibiotics, preservatives or biocides (Sass *et al.*, 2011, Rose *et al.*, 2009). Table 4.1 shows a list of *Burkholderia* genes investigated. These genes were chosen due to their presence in the outer membrane or putative contribution to efflux of antimicrobial compounds from the bacterial cell.

Table 4.1 Genes investigated using real-time PCR

Gene Name	**Putative function**	**Response observed by global gene expression analysis using microarrays or gene mutagenesis studies**
B. cenocepacia antimicrobial resistance target genes		
BCAM_0925	Outer membrane protein that is part of an RND efflux pump	12-fold up-regulated after exposure to 0.05 mM chlorpromazine and mutation of the gene results in increased chlorhexidine susceptibility (Sass *et al.*, 2011); 8-fold up-regulated after exposure to chlorhexidine (Rose, H. and Mahenthiralingam, E., unpublished data)
BCAS_0081	ABC transporter	Up-regulated in an antibiotic resistant clinical clone of *B. cenocepacia* J2315 and mutation of the gene results in an increased chlorhexidine susceptibility (Sass *et al.*, 2011); 6.8-fold up-regulated after chlorhexidine exposure (Rose, H. and Mahenthiralingam, E.,unpublished data)
BCAM_2551	Multi-drug efflux transport protein CeoA	296-fold up-regulated in a derivative adapted to trimethoprim sulfamethoxazole (Sass *et al.*, 2011)
BCAS_0167	Squalene-hopene cyclase	Up-regulated 4.36 -fold after exposure to 0.05 mM chlorpromazine and mutation of the gene results in increased chlorhexidine susceptibility (Sass *et al.*,

		2011)
BCAL1663	PrkA family serine protein kinase	Up-regulated in stationary phase (8- fold), low oxygen (8- fold), and heat stress (2 -fold) (Sass *et al.*, 2013)
B. lata 383 antimicrobial target gene		
Bcep18194_B1327	MFS_1 transporter	4.7 - fold up-regulated in derivative adapted to blend of methylisothiazolinone/chloromethylisothiazolinone (M-CMIT) preservative (Rushton *et al.*, 2013)

4.2.4.2 Primers

Universal primers were designed so that the gene of interest could be amplified in numerous *Burkholderia* species including *B. lata, B. ambifaria, B. cepacia, B. cenocepacia, B. multivorans, B. phymatum* and *B. mallei*. The relevant gene sequence for each species was obtained from www.Burkholderia.com (accessed 11/03/2014) and sequences were aligned using molecular evolutionary analysis software (MEGA 5; http://www.megasoftware.net accessed 11/03/2014) Primers were designed to selected regions of homology using Primer 3 software (http://bioinfo.ut.ee/primer3-0.4.0 accessed 11/03/2014) and their specificity for each *Burkholderia* gene target tested using the In Silico PCR tool (http://insilico.ehu.es/PCR accessed 11/03/2014). The metabolism-associated gene *phaC* (BCAL1861) was selected as a reference control gene as it has been shown to remain stable under a variety of growth conditions (Sass *et al.*, 2013) and has also been successfully used in the validation of *B. lata* global gene expression in response to preservative exposure (Rushton *et al.*, 2013). All primers were synthesised by and purchased from Invitrogen, Paisley, UK. Primers sequences are listed in table 4.2.

4.2.4.3 Real-time PCR conditions

Reactions were performed in triplicate under conditions described in chapter 2 section 2.13 using the annealing temperatures listed in table 4.2. Data analysis was carried out using the Pfaffl method (Pfaffl, 2001) described in chapter 2 section 2.14. (See appendix file name: chapter 4 appendix data > real_time_PCR for raw data).

Table 4.2 Primers for real-time PCR

Primer Name	Primer (Forward and Reverse) 5' – 3'	Product size (bp)	Annealing temperature (°C)
phaC (control)	AAGCGTTCGACAAGGTCAAG	218	Multiple
	GTTCACCGACGAGATGTTGA		
BCAM_0925	CTGGCGCACGATGTTC	120	67.0
	ATGCCGTACTGCGCTTC		
BCAS_0081	TTCGACGGGCTGAACCT	214	59.4
	GCAGCAGCGAGGTATCCT		
BCAM_2551	TCGGTGTCGCCGATCTAC	338	65.2
	TCGACGACGAACACGAACT		
BCAS_0167	CCTGATGATGCATTTCATGGAC	368	67.0
	ACGCGACCTTGTACATCGAG		
BCAL1663	GTTCAAGGCGCCGATCA	162	67.0
	TCGTTGTTGCGGTTGTTG		
Bcep18194_B1327	GAGGTGGAGATGACCGAATC	206	63.2
	GAGGTGGAGATGACCGAATC		

4.2.5 Light scattering experiments

To determine if bacterial aggregates were present in biocide-treated or untreated samples a series of experiments measuring particle size were carried out using an N4 Plus dynamic light scattering machine that measured the diameter of different particles present in each sample. These experiments are described in detail chapter 2 section 2.10. Strain 383 was exposed to 0.4 % CHG and 0.05 % BZC for 15 min. Untreated cells were suspended in diH_20.

4.2.6 Statistical tests

A Students t-test was used to compare MIC, MBC and zone of inhibition values before and after biocide exposure and to compare fluorescence values between biocide-treated and untreated cells in efflux assay experiments. A one-way ANOVA test was used when comparing zone of inhibition values obtained from the phenotype stability tests and particle size values from light scattering experiments.

4.3 Results

4.3.1 Suspension testing

Strain 383 was exposed to CHG and BZC at the minimum bactericidal concentration to determine the effect of contact time on biocide efficacy and to determine the time period taken for all bacteria to be killed at this concentration. The efficacy and toxicity of the neutraliser to strain 383 was determined before the suspension tests were performed. Table 4.3 shows the neutraliser efficacy and toxicity data. The neutraliser was considered non-toxic if ≤ 1 $\log_{10}$ reduction in CFU/mL (compared to the control) was observed after exposure, and considered effective if ≤ 1 $\log_{10}$ reduction in CFU/mL was observed in the presence of biocide and neutraliser (according to the BS EN 1276 2009 suspension testing protocol).

Table 4.3: Neutraliser efficacy and toxicity data

Treatment	Mean log CFU/mL ± SD
Control (diH20)	8.56 ± 0.00
Neutraliser	8.52 ± 0.01
CHG	0.00 ± 0.00
BZC	0.00 ± 0.00
CHG + Neutraliser	8.20 ± 0.02
BZC + Neutraliser	8.20 ± 0.28

Table 4.3 shows that the neutraliser was both non-toxic to strain 383 and effective in neutralising CHG and BZC.

Figure 4.2 shows the effect of contact time on the efficacy of 0.05 % BZC and 0.4 % CHG. The dilutions performed throughout the suspension test resulted in a 4.2 $\log_{10}$ limit of detection.

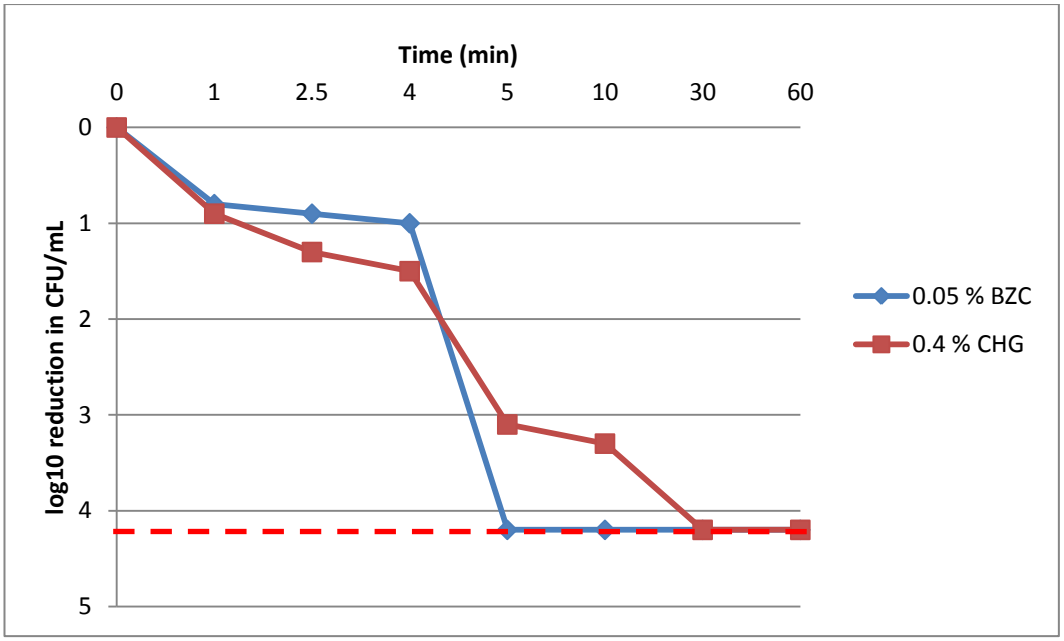


Figure 4.2: log$_{10}$ CFU/mL of B. lata strain 383 after exposure to 0.05 % BZC and 0.4 % CHG. Contact times of 1, 2.5, 4, 5, 10, 30 and 60 min. N=3

Red dashed line indicates the maximum $\log_{10}$ reduction which could be determined based on a 4.2 $\log_{10}$ limit of detection

Figure 4.2 shows that after 4 min exposure to 0.05 % BZC a 1 $\log_{10}$ reduction in CFU/mL was observed. The greatest reductions in $\log_{10}$ CFU/mL occurred between 4 and 5 min of exposure to 0.05 % BZC. After 5 min exposure a 4.2 $\log_{10}$ reduction was observed. This was the maximum $\log_{10}$ reduction that could be determined based on the limit of detection and corresponded to a plate count of zero. The greatest $\log_{10}$ reduction after exposure of strain 383 to 0.4 % CHG occurred between 4 and 5 min (~ 1.7). After 60 min exposure to 0.4 % CHG a 4.2 $\log_{10}$ reduction in CFU/mL was observed.

4.3.2 Antimicrobial susceptibility testing

The MIC and MBC of CHG and BZC were determined before and after exposure to a range of low CHG and BZC concentrations. These values were compared to determine whether short

term exposure to a low biocide concentration resulted in a change in susceptibility to that biocide and other antimicrobials in surviving organisms. Susceptibility to a range of clinically relevant antibiotics was also determined before and after biocide exposure.

4.3.2.1 Changes in minimum inhibitory and minimum bactericidal concentrations

The MIC and MBC values obtained before and after exposure to a range of low concentrations of CHG and BZC are shown in table 4.4(a) and 4.4 (b).

Table 4.4 (a): MIC and MBC values of CHG and BZC after 5 min exposure of B. lata strain 383 to a range of low CHG concentrations. N=3

* = value is significantly different from baseline value (p≤0.05)

	CHG concentration (%)			
MIC/MBC (%)	**Baseline**	**0.040**	**0.010**	**0.005**
CHG MIC ± SD	0.07 ± 0.00	0.15 ± 0.00*	0.30 ± 0.09*	0.30 ± 0.09
CHG MBC ± SD	0.40 ± 0.60	0.20 ± 1.00	0.40 ± 0.02	0.40 ± 0.20
BZC MIC ± SD	0.05 ± 0.05	0.20 ± 0.00*	0.10 ± 0.00*	0.30 ± 0.10*
BZC MBC ± SD	0.05 ± 0.05	0.50 ± 0.20*	0.60 ± 0.30*	0.50 ± 0.20*

Five min exposure of strain 383 to low concentrations of CHG resulted in small increases in the MIC and MBC for both biocides tested. The greatest increase was observed in the MBC for BZC after exposure to 0.01 % CHG, (0.60 %) indicating that exposure to one particular biocide can result in a change in the MBC of another biocide in the case of this strain (Table 4.4 (a)). The MBC for CHG did not increase after exposure to any of the low CHG concentrations tested suggesting that exposure of *B. lata* strain 383 to low CHG concentrations does not consistently result in an increase in the MIC and MBC of the biocide tested.

Table 4.4 (b): MIC and MBC values of CHG and BZC after 5 min exposure of B. lata strain 383 to a range of low BZC concentrations. N=3

*= value is significantly different from baseline (p≤0.05)

	BZC concentration (%)			
MIC/MCB (%)	**Baseline**	**0.045**	**0.010**	**0.005**
BZC MIC ± SD	0.05 ± 0.05	0.25 ± 0.00*	0.30 ± 0.10*	0.40 ± 0.00*
BZC MBC ± SD	0.05 ± 0.05	0.20 ± 1.00	0.78 ± 0.20*	0.26 ± 0.10*
CHG MIC ± SD	0.07 ± 0.00	0.08 ± 0.00	0.10 ± 0.00*	0.30 ± 0.09*
CHG MBC ± SD	0.40 ± 0.60	0.10 ± 0.20	0.30 ± 0.00	0.40 ± 0.00

The greatest increase was observed in the MBC for BZC after 5 min exposure to 0.01 % BZC (table 4.4 (b)). The MBC for CHG did not increase after exposure to any of the BZC concentrations tested. This was also observed after 5 min exposure to a range of low CHG concentrations (table 4.4 (a)). There did not appear to be a distinct relationship between the fold increase in MIC/MBC observed and the concentration of biocide tested.

4.3.2.2 Changes in antibiotic susceptibility

Clinical susceptibility to a range of antibiotics was determined before and after 5 min exposure of strain 383 to a range of low CHG and BZC concentrations. Table 4.5 shows the mean zone of inhibition sizes before and after biocide exposure and the clinical susceptibility to each antibiotic according to BSAC breakpoints for *Pseudomonas* spp. (Andrews, 2009) (none available for *Burkholderia* spp. so the breakpoints for the most closely related species were used).

Table 4.5: Mean zone of inhibition sizes (mm) for a range of clinically relevant antibiotics after exposure of B. lata strain 383 to low concentrations of CHG and BZC. N=3

S=sensitive I=intermediate R=resistant (based on clinical breakpoints for *Pseudomonas* species provided in the BSAC disk diffusion protocol (Andrews, 2009))

*value is significantly different from baseline (p≤0.05)

	Mean zone of inhibition size (mm) ± SD						
Antibiotic	**Baseline**	**0.04% CHG**	**0.01% CHG**	**0.005% CHG**	**0.045% BZC**	**0.01% BZC**	**0.005% BZC**
Ciprofloxacin	30.0^{S}	11.3^{R}*	17.3^{I}*	26.3^{S}*	12.0^{R}*	28.0^{S}	28.3^{S}
(1µg)	± 0.00	± 1.20	± 0.60	± 2.10	± 1.00	± 7.80	± 1.52
Tobramycin	7.30^{R}	9.00^{R}	4.30^{R}	11.6^{R}	8.00^{R}	0.00^{R}*	8.60^{R}
(10µg)	± 1.20	± 1.00	± 3.80	± 1.53	± 0.00	± 0.00	± 0.58
Ceftazidime	40.3^{S}	33.3^{S}*	30.0^{S}*	30.3^{S}*	39.0^{S}	36.0^{S}*	30.0^{S}*
(30µg)	± 0.60	± 1.20	± 0.00	± 3.10	± 1.00	± 4.60	± 2.64
Imipenem	24.0^{S}	15.0^{R}*	30.7^{S}*	19.3^{I}*	16.0^{R}*	18.0^{I}*	19.0^{I}*
(10µg)	± 0.00	± 3.0	± 2.10	± 1.20	± 1.00	± 0.00	± 2.00
Meropenem	40.7^{S}	37.0^{S}	35.3^{S}*	33.0^{S}*	35.5^{S}*	33.0^{S}*	34.0^{S}*
(15µg)	± 1.20	± 1.00	± 1.52	± 1.73	± 1.00	± 1.00	± 1.00

Clinical susceptibility (according to BSAC breakpoints for *Pseudomonas* spp.) to the antibiotics meropenem, tobramycin and ceftazidime did not change after 5 min exposure to a range of low concentrations of CHG or BZC despite significant decreases in the mean zone of inhibition size observed. After 5 min exposure to 0.04 % CHG and 0.045 % BZC strain 383 changed from sensitive to resistant to imipenem and ciprofloxacin (based on BSAC clinical susceptibility breakpoints for *Pseudomonas* spp. (Andrews, 2009)). Intermediate (neither sensitive nor resistant) susceptibility to imipenem was observed in strain 383 after 5 min exposure to 0.005 % CHG, 0.01 % BZC and 0.005 % BZC. Intermediate susceptibility to ciprofloxacin was observed after 5 min exposure to 0.01 % CHG.

4.3.2.3 Phenotype stability testing

The stability of the observed change (from sensitive to resistant) in susceptibility to imipenem (10 µg) and ciprofloxacin (1 µg) was tested via the continuous 24 h subculture of surviving organisms in the presence/absence of 0.005 % CHG and BZC. Tables 4.6 (a) and (b) show the mean zone of inhibition sizes observed for imipenem and ciprofloxacin after 1, 5 and 15 subcultures in TSB +/- 0.005 % CHG or BZC.

Table 4.6 (a): Mean zone of inhibition sizes (mm) for imipenem and ciprofloxacin after 5 min exposure of B. lata strain 383 to 0.005 % CHG and after 1, 5 and 10 subcultures through TSB +/- 0.005 % CHG. N=3

S=sensitive I=intermediate R=resistant (based on clinical breakpoints for *Pseudomonas* species provided in the BSAC disk diffusion protocol (Andrews, 2009))

*value is significantly different from baseline ($p \leq 0.05$) SC = subculture

	Mean zone of inhibition size (mm) ± SD							
Antibiotic	**Baseline**	**Initial 5 min exposure**	**1 SC (no CHG)**	**5 SCs (no CHG)**	**10 SCs (no CHG)**	**1 SC (CHG)**	**5 SCs (CHG)**	**10 SCs (CHG)**
Imipenem (10 µg)	24.0^{S} ± 0.00	15.6^{R}* ± 4.20	24.0^{S} ± 1.00	21.6^{S} ± 0.19	24.3^{S} ± 1.15	14.3^{R}* ± 0.58	18.6^{I}* ± 0.21	21.6^{S} ± 0.58
Ciprofloxacin (1 µg)	30.0^{S} ± 0.00	11.3^{R}* ± 2.31	28.0^{S} ± 1.00	31.0^{S} ± 0.22	30.0^{S} ± 4.36	7.60^{R}* ± 1.15	11.3^{R}* ± 1.15	11.6^{R}* ± 0.58

Table 4.6 (b): Mean zone of inhibition sizes (mm) for imipenem and ciprofloxacin after 5 min exposure of B. lata strain 383 to 0.005 % BZC and after 1, 5 and 10 passages through TSB +/- 0.005 % BZC. N=3

S=sensitive I=intermediate R=resistant (based on clinical breakpoints for *Pseudomonas* species provided in the BSAC disk diffusion protocol (Andrews, 2009))

*value is significantly different from baseline (p≤0.05) SC = subculture

	Mean zone of inhibition size (mm) ± SD							
Antibiotic	**Baseline**	**Initial 5 min Exposure**	**1 SC (no BZC)**	**5 SCs (no BZC)**	**10 SCs (no BZC)**	**1 SC (BZC)**	**5 SCs (BZC)**	**10 SCs (BZC)**
Imipenem (10 µg)	24.0^{S} ± 0.00	16.0^{R}* ± 1.00	24.0^{S} ± 1.53	24.0^{S} ± 4.00	25.3^{S} ± 3.06	13.6^{R}* ± 0.58	17.0^{I}* ± 3.45	25.6^{S} ± 1.15
Ciprofloxacin (1 µg)	30.0^{S} ± 0.00	11.3^{R}* ± 0.58	26.3^{S} ± 1.15	29.0^{S} ± 3.45	37.3^{S}* ± 4.04	7.30^{R}* ± 1.53	9.00^{R}* ± 1.00	13.0^{S}* ± 3.60

After 1 subculture (24 h) in the absence of CHG or BZC, strain 383 lost the resistance to both ciprofloxacin and imipenem initially observed after 5 min biocide exposure (tables 4.6 (a), (b)). After 5 subcultures in the presence of 0.005 % CHG and BZC, clinical resistance to ciprofloxacin was maintained and intermediate susceptibility to imipenem was observed. After 10 subcultures in the presence of 0.005 % CHG surviving bacteria remained resistant to ciprofloxacin, whereas resistance to imipenem was lost (table 4.6 (a)). After 10 subcultures of survivors in the presence of 0.005 % BZC resistance to both antibiotics was lost (table 4.6 (b)). All clinical antibiotic susceptibilities observed were based on the BSAC clinical breakpoints for *Pseudomonas* spp. (Andrews, 2009).

4.3.2.4 Data reproducibility

In order to determine the reproducibility of the changes in antibiotic susceptibility observed in strain 383 after 5 min biocide exposure, antibiotic susceptibility tests were performed in triplicate on four separate occasions over a one year time period. Table 4.7 shows the mean zone of inhibition sizes for 5 clinically relevant antibiotics observed after exposure to 0.005 % CHG or BZC on 4 separate occasions. A 5 min exposure of strain 383 to 0.005 % CHG or BZC resulted in clinical resistance to imipenem and ciprofloxacin (table 4.7, repeat 1) according to the BSAC disk diffusion protocol interpretation for *Pseudomonas* (Andrews, 2009) on only 1 of the 4 occasions on which the experiment was performed. There were no changes in clinical susceptibility to tobramycin, meropenem or ceftazidime observed on any of the 4 occasions, despite significant reductions in zone of inhibition size ($p \leq 0.05$) having been observed (table 4.7). These results suggest that a 5 min exposure to a low concentration of CHG and BZC can have an effect on susceptibility to imipenem and ciprofloxacin in *B. lata* strain 383. This change in susceptibility did not appear to be reproducible suggesting that any changes that do occur as a result of exposure to CHG or BZC at 0.005 % are not stable.

Table 4.7: Mean zone of inhibition sizes (mm) of a range of clinically relevant antibiotics after 5 min exposure of B. lata strain 383 to 0.005 % CHG and BZC

S = sensitive I = intermediate R= resistant *value is significantly different from baseline (p≤0.05)

Cip = ciprofloxacin Tob = tobramycin Ceft = ceftazidime Imi = imipenem Mem = meropenem

	Mean zone of inhibition size (mm) ± SD								
Antibiotic (separate repeats)	**Baseline**	**0.005% CHG (1)**	**0.005% CHG (2)**	**0.005% CHG (3)**	**0.005 % CHG (4)**	**0.005% BZC (1)**	**0.005% BZC (2)**	**0.005% BZC (3)**	**0.005 % BZC (4)**
Cip (1µg)	30.0^{S} ± 0.00	11.3^{R*} ± 2.31	24.0^{S} ± 0.00	20.0^{S*} ± 3.00	29.0^{S} ± 1.00	12.0^{R*} ± 1.53	24.0^{S*} ± 3.00	28.5^{S} ± 2.00	29.0^{S} ± 1.00
Tob (10µg)	7.30^{R} ± 1.20	9.00^{R} ± 0.00	4.30^{R} ± 1.70	11.6^{R} ± 2.50	8.0^{R} ± 0.00	8.00^{R} ± 0.00	0.00^{R*} ± 0.00	8.60^{R} ± 1.20	8.00^{R} ± 0.00
Cef (30µg)	40.3^{S} ± 0.60	33.3^{S*} ± 1.60	30.0^{S*} ± 3.00	30.3^{S*} ± 3.50	34.5^{S*} ± 4.38	39.0^{S} ± 1.00	36.0^{S*} ± 3.60	30.0^{S*} ± 4.40	32.5^{S*} ± 23.1
Imi (10µg)	24.0^{S} ± 0.00	15.0^{R} ± 4.20	27.3^{S} ± 2.50	25.0^{S} ± 0.00	21.0^{S} ± 2.00	16.0^{R} ± 0.58	30.0^{S*} ± 4.30	29.0^{S*} ± 2.00	24.0^{S} ± 0.00
Mem (15µg)	40.7^{S} ± 1.20	37.0^{S} ± 2.30	35.3^{S*} ± 1.15	33.0^{S*} ± 3.00	39.0^{S} ± 4.00	40.7^{S} ± 3.30	35.5^{S*} ± 4.00	34.0^{S*} ± 3.20	39.2^{R} ± 1.15

4.3.3 Efflux assays

The accumulation of fluorescent Hoechst dye was measured in *B. lata* strain 383 cells grown to mid-log phase in the presence/absence of 0.005 % CHG or BZC, and in the presence/absence of 2 different EPIs. This gave an indication of the level of efflux activity taking place in the bacterial cell as a result of biocide exposure. The toxicity of the EPIs was tested prior to their use in the efflux assay and both were found to be non-toxic to strain 383 at the concentrations used. Toxicity testing data is shown in table 4.8. An EPI was considered to be non-toxic if ≤ 1 $\log_{10}$ reduction in CFU/mL was observed after exposure

Table 4.8: Toxicity of EPIs to strain 383

EPI	**Concentration (mg/L)**	**log CFU/mL before**	**log CFU/mL after EPI**	**Log CFU/mL after diH20**
Verapamil	50	8.20 ± 0.00	7.20 **±** 0.44	7.95 **±** 0.35
PAβN	10	8.90 ± 0.00	8.10 **±** 0.24	8.87 **±** 0.77

Figure 4.3 shows the mean fluorescence values in biocide-treated and untreated cells in the presence/absence of verapamil (50 mg/L) or PAβN (10 mg/L).

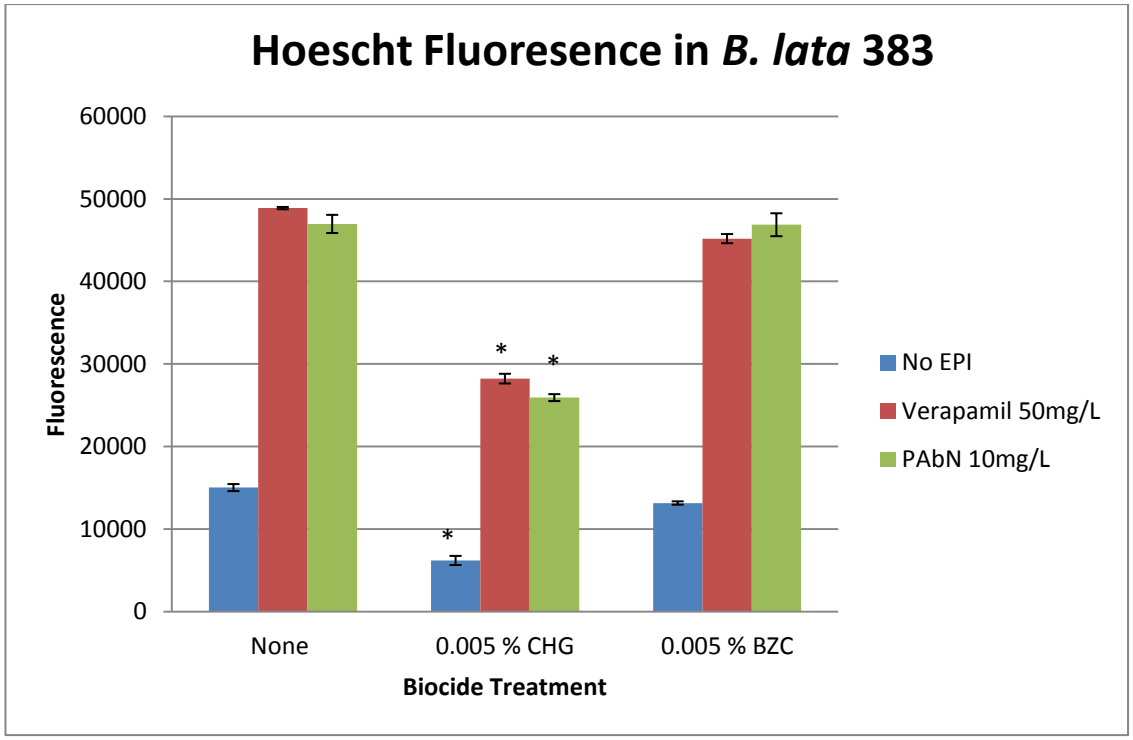


Figure 4.3: Mean fluorescence values observed in untreated and biocide-treated B. lata strain 383 in the presence/absence of 2 different efflux pump inhibitors. N=3

*CHG-treated significantly different from untreated ($p \leq 0.05$)

B. lata strain 383 cells grown to mid-log phase in the presence of 0.005 % CHG accumulated significantly less ($p \leq 0.05$) Hoechst dye than untreated cells, as indicated by the lower fluorescence reading (figure 4.3, blue bars). This reduced accumulation of Hoechst dye may have occurred as a result of increased efflux pump activity or up-regulation of efflux associated genes. Cells grown in the presence of 0.005 % BZC accumulated less Hoechst dye than untreated cells but there was no statistically significant difference in fluorescence observed (figure 4.3). This suggests that the presence of 0.005 % BZC may not have a significant effect on the efflux activity taking place in the cell. The addition of either verapamil (50 mg/L) or PAβN (10 mg/L) resulted in a large increase in fluorescence in both biocide-treated and untreated *B. lata* strain 383 cells, suggesting that some inhibition of the efflux of Hoechst dye

was taking place. The fact that this effect was observed in the presence of both EPIs suggests that more than one type of efflux pump may be contributing to Hoechst dye efflux, as verapamil specifically inhibits ABC transporters and PAβN inhibits RND type pumps. The increase in fluorescence observed after the addition of both EPIs in the case of 0.005 % CHG – treated cells was significantly lower ($p \leq 0.05$) than that observed in untreated cells (figure 4.3), again suggesting that greater efflux pump activity was taking place in the CHG-treated cells. There was no significant difference in fluorescence observed after the addition of EPIs when comparing untreated and BZC-treated cells.

4.3.4 Real-time PCR

Real time PCR reactions were carried out to identify changes in the expression of specific genes after growing *B. lata* strain 383 to mid-log phase in 0.005% CHG or BZC, with a view to identifying a marker gene for biocide resistance. Table 4.9 shows the mean fold changes in the expression of selected genes after biocide exposure. The greatest fold changes in expression were observed in an outer membrane protein (BCAM_0925) and in an ABC transporter (BCAS_0081) (table 4.9). Fourteen-fold changes in expression or less were observed in the remaining 4 genes investigated. Changes in the expression of genes BCAM_0925 and BCAS_0081 were further observed using RNA extracted from bacterial cells that were exposed to 0.005% CHG or BZC for 5 min. There were no changes in gene expression observed after 5 min exposure to 0.005 % CHG or BZC (table 4.9).

Table 4.9: Mean fold changes in gene expression after B. lata strain 383 was grown to mid-exponential phase in 0.005 % CHG or BZC (N=3) and mean fold change in expression in genes BCAM_0925 and BCAS_0081 after 5 min biocide exposure (N=3)

Gene Name	**Mean fold change in expression Control vs. 0.005 % CHG**	**Mean fold change in expression Control vs. 0.005 % BZC**	**Mean fold change in expression 0.005 % CHG (5min)**	**Mean fold change in expression 0.005 % BZC (5min)**
BCAM_0925	0.27	43.41	0.14	0.05
BCAS_0081	102.30	37.99	0.01	0.12
BCAM_2551	2.54	3.98	n/a	n/a
BCAS0167	0.44	0.97	n/a	n/a
Bcep18194_B1327	0.23	0.05	n/a	n/a
BCAL1663	14.72	3.68	n/a	n/a

4.3.5 Light scattering experiments

Biocide-treated and untreated cells were passed through an N4 Plus Dynamic Light Scattering machine to determine if bacterial aggregates were present in any samples. Table 4.10 shows mean particle sizes in untreated samples and cells treated with CHG or BZC at the minimum bactericidal concentration.

Table 4.10: Mean particle size (nm) for untreated and biocide-treated B. lata strain 383 cells. N=3

* = significantly different ($p \leq 0.05$) from untreated

Treatment	Mean particle diameter (nm) (range)
Untreated	722.50 (30.00)
0.40 % CHG	1129.8 (285.2) *
0.05 % BZC	611.60 (52.50) *

After exposure to 0.4 % CHG (15 min) there was a significant increase in mean particle diameter observed in comparison to untreated cells. There was also a much larger range (285.2) after exposure to CHG, suggesting a greater variance in particles sizes. It is possible that the increase in mean particle diameter may have been observed due to the aggregation of cells in response to biocide exposure. The formation of aggregates may restrict the access that a biocide or antibiotic has to the bacterial cell and may therefore result in reduced susceptibility to that biocide or antibiotic. This suggests a possible reason for the reduced susceptibility to ciprofloxacin and imipenem observed after 5 min biocide exposure. However after exposure to 0.05 % BZC there was a significant decrease in mean particle diameter in comparison to untreated cells. This may have been due to damage to the cells and release of cellular material. Despite the fact that both CHG and BZC primarily cause damage to the

bacterial cell membrane, the response of strain 383 in terms of aggregation appears to differ for each biocide.

4.4 Discussion

B. lata strain 383 was exposed to low concentrations of CHG and BZC in order to determine if short-term biocide exposure resulted in reduced antimicrobial susceptibility in surviving organisms. The reproducibility of the data obtained was assessed in order to determine the suitability of strain 383 as a model organism for predicting biocide resistance.

No significant change in the biocide susceptibility profile of strain 383 was observed after 5 min exposure to low concentrations of CHG and BZC. Thomas *et al.,* (2000) observed stable chlorhexidine diacetate resistance in *Ps. aeruginosa* after 24 h exposure to 1 μg^{-1} and speculated that contact time may affect the mechanisms employed by the bacterial cell to counteract the antimicrobial, and therefore may affect the susceptibility profile of any surviving organisms. Possible aggregate formation was observed as a result of exposure to CHG for 15 min, but not after BZC exposure. The formation of aggregates has been observed in *B. cenocepacia* as a result of high levels of free iron (Fe^{3+} , 10 and 100 µM) present in the sputum of CF patients (Berlutti *et al.*, 2005). A more stressful, iron-depleted environment was not associated with aggregate formation in this case. There does not appear to be a definitive relationship between biocide exposure and aggregate formation in *B. lata* strain 383, although a stressful, nutrient-depleted environment has been demonstrated to cause the formation of aggregates in other bacterial species such as *Salmonella* Typhimurium (White *et al.*, 2008, White *et al.*, 2006). Kaplan *et al.,* (2012) showed that exposure to low concentrations (up to 1 x MIC) of β-lactam antibiotics resulted in bacterial aggregation and biofilm formation in *Staphylococcus aureus,* which they suggested may promote antibiotic resistance in a clinical setting. The small changes observed in the MIC and MBC of CHG and BZC observed here after 5 min exposure to low biocide concentrations could be explained in part by the formation of aggregates in a response similar to that demonstrated in *S. aureus*.

Changes in susceptibility to the antibiotics imipenem and ciprofloxacin were observed after exposure to CHG and BZC. However these changes were not observed consistently across all four repeats of the experiment, nor were they stable in the absence of either biocide. This highlights the need to assess the reproducibility of the data obtained over time, as well as the stability of any altered susceptibility observed. It is also worth noting that the method used to determine antibiotic susceptibility may affect the data obtained. Although the BSAC methodology (Andrews, 2009) was strictly followed, Schuurmans *et a*l., (2009) found that antibiotic MIC values could vary by a factor of up to 8 if small alterations were made in the method used to determine them. This reiterates the requirement for a standard protocol for susceptibility measurement. Larsen *et al*., (1993) observed phenotypic variability when single colonies of *B. cepacia* were used experimentally. In the work carried out here the test inoculums were based on broth culture instead of single colonies which rules out this phenotypic variability. It was difficult to determine the clinical significance of the observed reduced zone of inhibition sizes due to the fact that the BSAC protocol does not provide susceptibility breakpoints for *Burkholderia* species. If one uses the breakpoints available for *Pseudomonas aeruginosa* (Andrews, 2009) clinical resistance to ciprofloxacin and imipenem would only have been observed on one out of the four occasions. The mechanisms of action of imipenem and ciprofloxacin differ considerably. Imipenem inhibits bacterial cell wall synthesis via the binding of penicillin binding proteins, preventing peptidoglycan formation (Sawasdidoln *et al.*, 2010), whereas ciprofloxacin inhibits DNA synthesis via the inhibition of the enzyme DNA gyrase that unwinds double-stranded DNA (Lunn *et al.*, 2010). This suggests that the mechanisms behind the reduced susceptibility observed are likely to be non-specific.

A very small number of studies have been carried out investigating the effect of biocide exposure on the antimicrobial susceptibility of surviving *Burkholderia* species. Rose *et al.,* (2009) investigated biocide and antibiotic susceptibility of 12 species of the Bcc complex including *B. lata* strain 383 and reported no correlation between CHG and BZC susceptibility

and antibiotic susceptibility. They did not however investigate the direct effect of biocide exposure on antibiotic susceptibility. Rushton *et al*., (2013) investigated the effect of adaptation of *B. lata* strain 383 to various preservatives on susceptibility to other antimicrobials. They reported that strain 383 adapted to methylisothiazolinone (MIT), methylisothiazolinone-chloromethylisothiazolinone (M-CMIT), benzisothiazolinone (BIT) and benzethonium chloride (BC) was cross-resistant to other preservatives and showed increased tolerance to chloramphenicol and fluoroquinolones. They attributed M-CMIT tolerance and fluoroquinolone resistance to up-regulated efflux pump activity. It is possible that the changes in susceptibility to ciprofloxacin and imipenem observed in the work carried out here could be due to up-regulated efflux pump activity as a result of CHG/BZC exposure.

A change in antibiotic susceptibility with no change in biocide susceptibility has been observed by Christensen *et al*., (2011) in *Listeria monocytogenes* after exposure to triclosan. They reported a 16-fold decrease in gentamicin susceptibility after exposure to a sub-lethal concentration of triclosan, despite observing no change in susceptibility to triclosan itself. Gentamicin resistant organisms were also resistant to other aminoglycosides. In contrast to this Birošová *et al*., (2009) reported that after 30 min exposure of *S. enterica* to sub-MIC concentrations of triclosan, there was no alteration in biocide or antibiotic susceptibility. Whitehead *et al*., (2011) observed that a single (5 h) exposure to cationic biocides (including a QAC and 'Superkill' – a mix of QACs) or triclosan at the in-use concentration (1 %) selected for multiple drug resistant surviving organisms. The above mentioned studies did not make a comparison between baseline susceptibility data and change in susceptibility profile after biocide exposure, therefore making the reproducibility of their observations difficult to determine.

It is important to consider the stability of a change in susceptibility as it raises questions about the selection effect of a given biocide (Knapp *et al.*, 2013). In the work carried out here the reduced antibiotic susceptibility was not maintained in the absence of either biocide. However

in the presence of 0.005 % CHG or BZC the decreased zones of inhibition did not revert back to baseline values after ten passages. It appears that the selective pressure for reduced antibiotic susceptibility (i.e. biocide exposure) was required for the maintenance of decreased zones of inhibition.

The greatest changes in gene expression after exposure of strain 383 to 0.005 % CHG or BZC were observed in genes BCAM_0925 and BCAS_0081. These encode an outer membrane protein and an ABC transporter respectively, both of which could contribute to the efflux of antimicrobial compounds from the bacterial cell (Buroni *et al.*, 2009). Efflux has been described as an important non-specific mechanism that can reduce the intracellular concentration of unrelated antimicrobials (Maillard and Denyer, 2009) and such is a likely candidate to explain a change in susceptibility profile. Bazzini *et al*., (2011) recently observed the effect of deleting the RND-4 and RND-9 efflux systems in *B. cenocepacia*. They reported that a double mutant had a 4 – 16 fold increase in susceptibility to antibiotics tested, including ciprofloxacin. This suggests a potential role for these efflux systems in antimicrobial susceptibility, and may in part explain what has been observed here with exposure of strain 383 to CHG and BZC, particularly as changes in susceptibility to more than one antimicrobial were observed. Sass *et al*., (2011) looked at global gene expression in *B. cenocepacia* strain J2315 in relation to spontaneous antibiotic resistance and exposure to the cationic antibiotic potentiator chlorpromazine. Up-regulation of BCAM_0925 was observed in response to chlorpromazine exposure, and its subsequent deletion resulted in increased susceptibility to azithromycin and chlorhexidine. BCAS_0081 is composed of an ATP binding cassette and transmembrane components that possess homology to the *Escherichia coli mdlB* gene which has been associated with multiple drug resistance. Deletion of BCAS_0081 resulted in increased susceptibility to tetracycline and chlorhexidine but not to other antibiotics tested, including ciprofloxacin and imipenem. The susceptibility profiles of strain 383 and *B.*

cenocepacia strain J2315 appear very different, making comparisons between the two difficult to make. The findings of Rushton *et al*., (2013) do however correlate well the work carried out here, as they demonstrated the role of efflux in preservative resistance in *B. lata* which also lead to stable, elevated fluoroquinolone resistance. It is worth noting that only a small number of genes that could have played a role in the change in susceptibility profile observed were investigated, and that perhaps a wider range of *B. lata* genes should be investigated in order to establish a genuine effect of biocide exposure.

When establishing a standard protocol predictive of bacterial biocide resistance it is essential to ensure reproducibility of the findings made. Despite following standard assays for the determination of the MIC, MBC and antibiotic susceptibility profile, the data obtained using *B. lata* strain 383 were not reproducible. This suggests that this bacterium may not be an appropriate model organism to use in the generation of predictive markers of biocide resistance.

Chapter Five: Protocol validation

5.1 Introduction

5.1.1 Background

The principle aim of this work was to design a standard protocol that allows the prediction of biocide resistance and antibiotic cross-resistance in bacteria. Chapters 3 and 4 involved the assessment of a variety of techniques in their ability to generate practical markers of biocide resistance and antibiotic cross-resistance in *S*. *enterica* serovar Typhimurium and *B. lata.* Important factors considered when assessing these techniques included test practicality (i.e. how high throughput the test was), cost of the test, usefulness of the technique (i.e. how much data was obtained) and most importantly the reproducibility of the data obtained. Table 5.1 summarises the advantages and disadvantages of each of the techniques explored in chapters 3 and 4, taking in to account the results obtained in these chapters.

Table 5.1: Advantages & disadvantages of techniques employed to identify resistance markers in survivors of biocide exposure (based on data obtained in chapters 3 and 4)

Experimental technique	Advantages	Disadvantages
Changes in MIC and MBC	High throughput, data within 24h, good initial indication of resistance, reproducible for *S. enterica* strains, low cost	Non-reproducible for *Burkholderia lata*– choice of model organism important
Changes in antibiotic susceptibility	High throughput, data within 24 h, disk diffusion method low in cost. Good initial marker of antibiotic cross-resistance	Disk diffusion zones of inhibition not always relevant to clinical change in susceptibility. Not reproducible for *B. lata,* no *Burkholderia* spp. breakpoints available
Phenotype stability tests	Give an idea of stability of changes observed – determines if susceptibility change is transient/stable	No details on what changes have taken place in cells present in population

Efflux assays	Good start point if one observes reduced biocide susceptibility– more likely to be a non-specific mechanism such as efflux rather than a specific mutation. Efflux pump inhibitors may give indication of which pumps responsible for efflux	Long preparation time
Real-time PCR	Useful if one has identified specific genes potentially involved in resistance already, cheaper than microarray	Relatively expensive, not useful if no knowledge of genes potentially involved in resistance. DNA/RNA prep time consuming if you have a large sample number
Microarray	See genome wide changes - most likely to identify marker gene(s)	Expensive, long preparation time, data analysis is time consuming, may require professional biostatistician
One dimensional SDS-PAGE	Good starting point for observation of outer membrane protein changes	Expensive, time consuming, 1 dimension does not provide info on protein identity. Semi-quantitative
Flow cytometry	Allows the observation of viable but non culturable cells that may not appear in suspension test due to dilution	Susceptibility of surviving cells not apparent until they are further sorted and tested, and some may not be recoverable

5.1.2 Principle of validation

In the literature, validation of a protocol often involves the comparison of data generated from the new protocol with data from an existing, validated standard (Ebentier *et al.*, 2013, Lofstrom *et al.*, 2012, McCabe *et al.*, 2011). However due to the fact that there is no existing

standard protocol for predicting biocide resistance that could be used as a comparison point, validation here focused on data repeatability (data variability between biological replicates from the same experiment) and reproducibility (variability between data generated from experiments performed on separate occasions) . Validation of a particular technique involved the execution of the experiment on at least 3 separate occasions (each using 3 biological replicates) over a 6 month period of time using a variety of biocidal products and bacterial strains. It was important that the technique could be employed to predict resistance to multiple biocidal products using several different bacterial species, as different products have different applications and target organisms.

In order for a technique to be validated for use in the prediction of bacterial biocide resistance and antibiotic cross-resistance, repeatable and reproducible data had to be obtained. For example, if a clinical change in antibiotic susceptibility was observed (i.e. a bacterium became clinically resistant to a particular antibiotic after exposure to a biocidal product), this change needed to be observed in all 3 biological replicates in that individual experiment, as well as on each separate occasion on which the experiment was carried out.

5.1.3 Techniques chosen for use in validation

Due to the low cost of pre and post- biocide exposure MIC and MBC determination (following the BS EN ISO: 20776-1 (2006) protocol), and the possibility of testing a large number of bacterial strains and biocidal products within a 24 h period (table 5.1), this technique was selected for use in validation experiments. Antibiotic susceptibility testing following the BSAC disk diffusion method (Andrews, 2009) was also selected for use in validation experiments as it was a useful and high throughput technique that allowed the determination of antibiotic cross-resistance as a result of biocide exposure. Both these techniques were also chosen due to the fact that they provided useful initial information on biocide and antibiotic resistance in

surviving organisms, particularly in the case of *S*. *enterica* serovar Typhimurium, that could then be followed up with more time consuming and costly techniques such as efflux assays or real-time PCR.

5.1.4 Aims

The principle aim of this chapter was to validate pre and post- biocide exposure MIC, MBC and antibiotic susceptibility determination as a practical technique in the prediction of biocide resistance and antibiotic cross-resistance in bacteria. Further aims were to determine if exposure of *S*. *enterica* serovar Typhimurium, *Ps. aeruginosa*, *Klebsiella pneumoniae* and *B. cepacia* to Corsodyl mouthwash, Dermax therapeutic shampoo or eye make-up remover resulted in a change in biocide and/or antibiotic susceptibility in surviving organisms.

5.2 Materials & Methods

5.2.1 Bacterial strains

In addition to *S. enterica* strains SL1344 and 14028S used in chapter 3, a further 3 strains were used in the validation experiments. These were *Burkholderia cenocepacia* (UL2P), *Klebsiella pneumoniae* (UL13) and *Pseudomonas aeruginosa* (UL-7P) and were selected in agreement with Unilever SEAC, Colworth, UK. All 3 were selected as challenge organisms due to their routine use, propagation and handling in Unilever laboratories. They were originally isolated from household product contamination. Tested strains are listed in table 5.2.

Table 5.2: Strains used in validation experiments

Strain	**Source**
Burkholderia cenocepacia (UL2P)	Unilever SEAC, Colworth, UK
Klebsiella pneumoniae (UL13)	Unilever SEAC, Colworth, UK
Pseudomonas aeruginosa (UL-7P)	Unilever SEAC, Colworth, UK
SL1344	See chapter 2 table 2.1
14028S	See chapter 2 table 2.1

5.2.2 Culture and storage of bacteria

All strains used in validation experiments were cultured on TSA or in TSB at 37°C (± 1 °C) as described in chapter 2 section 2.1. All strains were stored at -80 °C (± 1 °C) as described in chapter 2 section 2.1.

5.2.3 Biocides and neutraliser

As well as CHG and BZC (described in chapter 2 section 2.2), 3 further biocidal products were tested. These were Corsodyl Mouthwash (0.2 % w/v CHG), Eye Make-up Remover (0.1 % biocide (confidential)) and Dermax Therapeutic Shampoo (0.5 % w/w BZC). These products

were chosen following discussions and in agreement with Unilever SEAC. Their selection was based on the fact that they are commonly used home and personal care products and all contain either CHG or BZC, which were both tested in previous chapters. The neutraliser used was of the same composition as that described in chapter 2 section 2.3. Neutraliser efficacy for mouthwash, shampoo and eye make-up remover, and toxicity towards strains UL2P, UL13 and UL-7P was determined as described in chapter 2, sections 2.3.2 and 2.3.1 respectively.

5.2.4 Suspension testing

Suspension tests were performed to determine the efficacy of each product when tested at the lowest concentration attained during product use (I.e. the concentration of the product after any dilution by the consumer as a result of the product application). These concentrations were agreed with Unilever SEAC. Suspension tests were carried out according to the BS EN 1276 protocol (2009) described in detail in chapter 2 section 2.7. Further suspension tests were then performed in order to determine a concentration of each product that would leave sufficient surviving organisms for further antimicrobial susceptibility testing (i.e. a concentration that resulted in a 1-3 $\log_{10}$ reduction in CFU/mL).

5.2.5 Suspension test conditions

The suspension test conditions used were chosen in order to reflect parameters used in practice as closely as possible. Test (lowest attainable) concentrations were calculated using dilution factors for each product provided by Unilever SEAC. These dilution factors were based on the product application and how it was used by the consumer. A contact time of 1 min was chosen for all products as an estimation of the length of time spent using the product by the consumer in order to reflect in-use conditions as accurately as possible. Table 5.3 shows the

contact times and calculated test concentrations (based on dilution factors provided by Unilever SEAC) for each product tested.

Table 5.3: Initial & test (lowest attainable) concentrations & contact times for Corsodyl mouthwash, Dermax shampoo & eye make-up remover

Product	Concentration of active agent contained in product (%)	Dilution due to product use	Test (lowest attainable) concentration (%)	Contact time (min)
Corsodyl Mouthwash	0.20 CHG	1:40	**0.005**	1.00
Dermax Shampoo	0.50 BZC	1:100	**0.005**	1.00
Eye make-up remover	0.10 Biocide	None	**0.100**	1.00

5.2.6 Antimicrobial susceptibility testing

5.2.6.1 Baseline data

The MIC and MBC of each biocidal product were determined for all 5 strains following the BS EN ISO: 20776-1 (2006) protocol described in chapter 2 sections 2.4 and 2.5. The MIC and MBC of CHG and BZC were also determined for strains UL2P, UL13 and UL-7P following the same protocol. Clinical susceptibility to a range of antibiotics was determined for strains UL2P, UL13 and UL-7P following the BSAC disk diffusion protocol (Andrews, 2009) described in chapter 2 section 2.6. As previously stated in chapter 4, the BSAC protocol does not provide susceptibility breakpoints for *Burkholderia* spp. Breakpoints for *Pseudomonas* spp. were therefore used instead in the case of strain UL2P (*B. cenocepacia*). The antibiotics tested for each strain are shown in table 5.4. Selected antibiotics represented different antibiotic classes in order to determine the effect of biocide exposure on susceptibility to multiple classes of antibiotic.

Table 5.4: Antibiotics tested for strains UL2P, UL13, UL-7P, SL1344 & 14028S

Strain	Antibiotics tested
B. cenocepacia (UL2P)	ceftazidime (30 µg), ciprofloxacin (1 µg), meropenem (15 µg), imipenem (10 µg), tobramycin (10 µg)
K. pneumoniae (UL13)	ceftazidime (30 µg), ampicillin (10 µg), ciprofloxacin (1 µg), chloramphenicol (50 µg), ceftriaxone (30 µg)
Ps. aeruginosa (UL-7P)	imipenem (10 µg), ceftriaxone (30 µg), meropenem (15 µg), tobramycin (10 µg), aztreonam (30 µg)
SL1344	As described in chapter 3 section 3.2.2.1
14028S	As described in chapter 3 section 3.2.2.1

5.2.6.2 Antimicrobial susceptibility of biocide-exposed organisms

All 5 strains were exposed for 1 min (reflective of in-use conditions) to a concentration of each biocidal product that resulted in a 1-3 $\log_{10}$ reduction in CFU/mL, following the BS EN 1276 (2009) suspension testing protocol fully described in chapter 2 section 2.7.2. All 5 strains were also exposed to CHG and BZC for 1 min at the same concentration as that present in each biocidal product. Temperature was maintained at 20 °C throughout the experiment using a water bath.

After neutralisation the neutralised suspension was centrifuged at 5000 x g for 10 min, and the supernatant discarded. The remaining cell pellet was then re-suspended in 10 mL TSC buffer. This suspension was then used in the determination of the biocidal product MIC, MBC and antibiotic susceptibility of surviving organisms as described in chapter 2 sections 2.4, 2.5 and

2.6. In order to determine the reproducibility and repeatability of the data obtained, the above experiment was performed on 3 separate occasions (each using 3 biological replicates) over a 6 month period.

5.2.7 Statistical analysis

A Students t-test was used to compare MIC, MBC and antibiotic zone of inhibition sizes before and after biocide exposure.

5.3 Results

5.3.1 Neutraliser toxicity and efficacy

The toxicity of the neutraliser to strains UL13, UL-7P and UL2P, and efficacy of the neutraliser in neutralising Corsodyl mouthwash, Dermax shampoo and eye make-up remover were tested prior to the suspension tests being carried out. The neutraliser was considered non-toxic if ≤ 1 $\log_{10}$ reduction in CFU/mL was observed after exposure to the neutraliser (compared to control). The neutraliser was considered effective if ≤ 1 $\log_{10}$ reduction in CFU/mL was observed in the presence of biocide and neutraliser (according to BS EN 1276 2009 guidelines). Tables 5.5 and 5.6 show neutraliser toxicity and efficacy data for strains UL2P, UL13 and UL-7P (tests performed for SL1344 and 14028S in chapter 3) and mouthwash, shampoo and eye make-up remover.

Table 5.5: Neutraliser toxicity data for strains UL2P (B. cenocepacia), UL13 (K. pneumoniae) and UL-7P (Ps. aeruginosa). N=3

Treatment	**Mean Log CFU/mL ± SD**
Control (diH_20)	UL2P: 8.78 ± 0.00
	UL13: 9.07 ± 0.00
	UL-7P: 8.95 ± 0.00
Neutraliser	UL2P: 8.24 ± 0.24
	UL13: 8.93 ± 0.06
	UL-7P: 8.85 ± 0.06

Table 5.6: Neutraliser efficacy data for Corsodyl mouthwash, Dermax shampoo & eye make-up remover using strain SL1344. N=3

Treatment	Mean Log CFU/mL ± SD
Control (diH_20)	8.60 ± 0.00
Corsodyl mouthwash	0.00 ± 0.00
Corsodyl + neutraliser	8.09 ± 0.05
Dermax shampoo	6.09 ± 0.00
Shampoo + neutraliser	8.10 ± 0.07
Eye make-up remover	5.35 ± 0.40
Eye make-up remover + neutraliser	8.40 ± 0.04

Tables 5.5 and 5.6 show that the neutraliser was not toxic to any strain, and that it was effective in neutralising Corsodyl mouthwash, Dermax shampoo and eye make-up remover.

5.3.2. Suspension tests

Suspension tests were carried out in order to determine the efficacy of each biocidal product when tested at the lowest concentration attained during product use (0.1 % make-up remover, 0.005 % shampoo and mouthwash – see table 5.3) and a contact time of 1 min. Table 5.7 shows the log_{10} reduction in CFU/mL after exposure of all 5 strains to each biocidal product, and to CHG and BZC at the same concentration as that contained within the product. The dilutions performed during suspension testing resulted in a 4 log_{10} limit of detection.

As shown in table 5.7, 1 min exposure to Corsodyl mouthwash (0.005 %) resulted in a 4 log_{10} reduction in CFU/mL in all strains tested. This was the maximum log_{10} reduction that could be determined due to the 4 log_{10} limit of detection, and corresponded to a plate count of 0. One min exposure to CHG at the same concentration contained within the mouthwash (0.005 %) resulted in a ~ 3 log_{10} reduction in CFU/mL, leaving some surviving organisms in each strain.

This suggests that Corsodyl mouthwash was more effective after 1 min against each strain in comparison with CHG alone when tested at the same concentration. One min exposure to Dermax shampoo (0.005 %) only resulted in a 1.78-2.34 $\log_{10}$ reduction in CFU/mL in all strains tested, whereas exposure to BZC at the same concentration contained within the shampoo (0.005 %) resulted in a 4 $\log_{10}$ reduction in all strains except *B. cenocepacia* (1.92 $\log_{10}$ reduction after exposure to 0.005 % BZC). This suggests that the efficacy of the BZC present in the shampoo may be reduced by the other components of the shampoo, as BZC alone was much more effective against all strains (except *B. cenocepacia*). One min exposure of all strains to eye make-up remover resulted in a 2.26 – 4 $\log_{10}$ reduction across all strains. Exposure of all strains to 0.1 % biocide (contained within the eye make-up remover) resulted in a 1.97 – 3.17 $\log_{10}$ reduction in CFU/mL. There was very little difference between $\log_{10}$ reductions observed after exposure to the make-up remover and biocide alone suggesting that the efficacy of the biocide contained within the make-up remover was not affected by the other components of the product.

Table 5.7: log_{10} reductions in CFU/mL of strains SL1344, 14028S, K. pneumoniae, B. cenocepacia & Ps. aeruginosa after 1 min exposure to mouthwash, shampoo & eye make-up remover at the lowest concentration attained during product use. N=3

[1] 4.00 log_{10} reduction is the limit of detection and corresponds to a plate count of 0.

	Log_{10} reduction ± SD					
Strain	**Corsodyl (0.005 %)**	**CHG (0.005 %)**	**Shampoo (0.005 %)**	**BZC (0.005 %)**	**Make-up Remover (0.1 %)**	**Biocide (0.1 %)**
SL1344	**4.00**[1] ± 0.00	**3.20** ± 0.20	**2.08** ± 0.00	**4.00** ± 0.00	**3.12** ± 0.10	**2.33** ± 0.14
14028S	**4.00** ± 0.00	**3.20** ± 0.20	**2.34** ± 0.11	**4.00** ± 0.00	**3.36** ± 0.17	**2.20** ± 0.09
Klebsiella	**4.00** ± 0.00	**3.90** ± 0.60	**1.78** ± 0.04	**4.00** ± 0.00	**3.15** ± 0.08	**2.18** ± 0.17
B. cenocepacia	**4.00** ± 0.00	**3.80** ± 0.60	**2.27** ± 0.38	**1.92** ± 0.50	**2.26** ± 0.33	**1.97** ± 0.34
Ps. aeruginosa	**4.00** ± 0.00	**3.80** ± 0.02	**2.01** ± 0.03	**4.00** ± 0.00	**4.00** ± 0.00	**3.17** ± 0.34

Further suspension tests were then performed to identify concentrations of each biocidal product and CHG/BZC that resulted in a 1-3 log_{10} reduction in CFU/mL after 1 min exposure. A 1-3 log_{10} reduction in CFU/mL left a sufficient number of surviving organisms for use in post-exposure susceptibility tests. The chosen concentrations are shown in table 5.8.

Table 5.8: Concentrations of products and CHG/BZC for use in antimicrobial susceptibility testing

Biocidal product	**Concentration contained within product**	**Concentration of product/active biocide resulting in 1-3 log_{10} reduction (%)**	**Dilution due to product use**	**Test concentration (%) ***
Corsodyl mouthwash	0.2 % CHG	**0.0005**	1:40	**0.0000125**
Dermax Shampoo	0.5 % BZC	**0.0015**	1:100	**0.000015**
Eye make-up remover	0.1 % biocide	**0.1**	None	**0.1**

* Concentrations used in antimicrobial susceptibility testing

As shown in table 5.8 the test concentrations of each product used (column 5) are considerably lower than the concentrations contained within Corsodyl mouthwash and Dermax shampoo (column 2). However, concentrations needed to be reduced so that a sufficient number of surviving organisms remained for further susceptibility testing for the purpose of protocol development.

5.3.3 Antimicrobial susceptibility testing

The susceptibility of surviving organisms to each biocidal product or CHG/BZC alone and a range of antibiotics was tested after exposure of each strain to each biocidal product, in order to determine if exposure to any of the biocidal products resulted in a change in antimicrobial susceptibility in the survivors. Each experiment was performed on three separate occasions over a 6 month period in order to assess the repeatability and reproducibility of the data when using this technique in the identification of surviving organisms with altered antimicrobial susceptibility. Table 5.9 shows the baseline and post-exposure MIC and MBC values after 1 min exposure of all 5 strains to eye make-up remover (0.1 %).

Table 5.9: Baseline & post-exposure MIC and MBC values after 1 min exposure of SL1344, 14028S, K. pneumoniae, B. cenocepacia & Ps. aeruginosa to eye make-up remover (undiluted) carried out on 3 separate occasions over a 6 month period.

* = significantly different from baseline value ($p \leq 0.05$)

[1] each MIC/MBC value is the mean of 3 biological replicates

	MIC/MBC ± SD							
Strain	**Baseline MIC (%)**	**MIC 1[1] (%)**	**MIC 2 (%)**	**MIC 3 (%)**	**Baseline MBC (%)**	**MBC 1 (%)**	**MBC 2 (%)**	**MBC 3 (%)**
SL1344	0.003 ± 0.00	0.006 ± 0.00*	0.003 ± 0.00	0.003 ± 0.00	> 0.05 ± 0.00	> 0.05 ± 0.00	> 0.05 ± 0.00	> 0.05 ± 0.00
14028S	0.003 ± 0.00	0.006 ± 0.00*	0.006 ± 0.00*	0.006 ± 0.00*	> 0.05 ± 0.00	> 0.05 ± 0.00	> 0.05 ± 0.00	> 0.05 ± 0.00
K. pneumoniae	0.012 ± 0.00	0.012 ± 0.00	0.012 ± 0.00	0.012 ± 0.00	> 0.05 ± 0.00	> 0.05 ± 0.00	> 0.05 ± 0.00	> 0.05 ± 0.00
B. cenocepacia	0.012 ± 0.00	0.012 ± 0.00	0.012 ± 0.00	0.012 ± 0.00	> 0.05 ± 0.00	> 0.05 ± 0.00	> 0.05 ± 0.00	> 0.05 ± 0.00
Ps. aeruginosa	0.012 ± 0.00	0.012 ± 0.00	0.012 ± 0.00	0.012 ± 0.00	> 0.05 ± 0.00	> 0.05 ± 0.00	> 0.05 ± 0.00	> 0.05 ± 0.00

As shown in table 5.9, statistically significant changes ($p \leq 0.05$) in the MIC of eye make-up remover were observed on all 3 occasions for strain 14028S and on 1 occasion for strain SL1344. However despite being statistically significant, these changes in MIC were very small, particularly in comparison to those observed in SL1344 and 14028S after exposure to low concentrations of CHG/BZC (0.00001 – 0.0004 %) (Chapter 3, tables 3.5 & 3.6). It was not possible to ascertain if there were changes in the MBC of this product as viable cells from all 5 strains remained in the highest testable concentration of eye make-up remover (0.05 %). What is clear from table 5.9 is that the resulting mean MIC values were highly repeatable (indicated by low SD values) and that mean MIC values were reproducible across all 3 separate experiments. This suggests that determination of the MIC/MBC before and after biocide exposure is a useful and valid technique for use in the prediction of biocide resistance in the 5 organisms tested.

Table 5.10 shows the mean zone of inhibition sizes observed for a range of antibiotics before and after 1 min exposure of all 5 strains to make-up remover (0.1 %). As shown in table 5.10, there was no clinical change in susceptibility to any of the antibiotics tested after 1 min exposure to make-up remover, in the case of all 5 strains (according to BSAC susceptibility breakpoints for *Enterobacteriaceae/Pseudomonas* spp). In the case of some strains and antibiotics, statistically significant changes in the zone of inhibition size were observed but this did not correspond to a clinical change in susceptibility (e.g. ciprofloxacin and SL1344, 14028S; ceftazidime and *Klebsiella pneumoniae*). As previously discussed in detail in chapter 4, it was not possible to directly determine if clinical changes in susceptibility were observed in *B. cenocepacia*, as there were no available breakpoints provided for this species in the BSAC protocol, and clinical susceptibility was therefore based on *Pseudomonas* spp.

Carrying out this experiment on 3 separate occasions over a 6 month period also allowed for an assessment of the reproducibility of the results obtained. It is clear from table 5.10 that the

post-exposure antibiotic susceptibility data was reproducible across all three separate experiments, validating this technique for the determination of antibiotic cross-resistance as a result of biocide exposure in the 5 organisms tested here.

Biocidal product/biocide concentrations that resulted in a 1-3 $\log_{10}$ reduction in CFU/mL were 0.0005 % Corsodyl mouthwash, 0.0005 % CHG, 0.0015 % Dermax shampoo, 0.0015 % BZC and 0.1 % biocide (equivalent to biocide concentration in make-up remover). As shown in table 5.3, Corsodyl mouthwash and Dermax shampoo undergo a 1:40 and 1:100 dilution respectively as a result of product use by the consumer (eye make-up remover is not diluted). This means that final exposure concentrations of mouthwash/CHG and shampoo/BZC in this case were 0.0000125 % and 0.000015 % respectively (no dilution for eye make-up remover) (see table 5.8). The final concentrations of Corsodyl mouthwash and Dermax shampoo tested were therefore considerably lower than the original product concentration in table 5.8. This was due to the fact that lower biocide concentrations were required to leave a sufficient number of surviving organisms for post-biocide exposure testing. This had to be considered when analysing the data obtained.

The resulting MIC/MBC and antibiotic susceptibility values for each strain and each biocidal product and active biocide are shown in the appendix (file name: appendix data for chapter 5). No significant changes in clinical antibiotic susceptibility were observed after exposure of all 5 strains to the 3 biocidal products and equivalent concentrations of CHG or BZC (according to BSAC antibiotic susceptibility breakpoints for Enterobacteriaceae/*Pseudomonas* spp.) with the exception of tobramycin resistance observed in 1/3 occasions in *B. cenocepacia* after exposure to Dermax shampoo.

Table 5.10: Baseline & post-exposure mean zone of inhibition values (mm) after 1 min exposure of SL1344, 14028S, K. pneumoniae, B. cenocepacia & Ps. aeruginosa to eye make-up remover (undiluted) carried out on 3 separate occasions over a 6 month period.

Green = clinically sensitive Red = clinically resistant (according to BSAC antibiotic susceptibility breakpoints for *Enterobacteriaceae* (SL1344, 14028S, *Klebsiella*) or *Pseudomonas* spp. (*B. cenocepacia, P. aeruginosa*))

* = significantly different from baseline value $p \leq 0.05$

		Mean zone of inhibition size (mm) ± SD			
Strain	**Antibiotic**	**Baseline**	**Post- exposure 1**	**Post- exposure 2**	**Post- exposure 3**
SL1344	Chloramphenicol (50 µg)	28.7 ± 1.2	28.0 ± 0.0	28.0 ± 0.0	27.8 ± 1.2
	Ampicillin (10 µg)	31.7 ± 0.6	28.3 ± 1.5*	30.0 ± 0.0	31.0 ± 0.0
	Ciprofloxacin (1 µg)	32.0 ± 1.7	28.0 ± 0.0*	31.3 ± 0.6	30.0 ± 0.0
	Ceftriaxone (30 µg)	34.3 ± 2.1	36.3 ± 1.5	32.0 ± 0.0	34.0 ± 0.0
	Piperacillin (30 µg)	29.0 ± 1.0	29.7 ± 1.5	29.0 ± 0.0	29.0 ± 0.0
14028S	Chloramphenicol (50 µg)	27.7 ± 0.6	27.3 ± 0.6	27.5 ± 1.2	28.1 ± 0.8
	Ampicillin (10 µg)	31.0 ± 1.7	29.7 ± 0.6	30.1 ± 0.7	29.9 ± 1.3
	Ciprofloxacin (1 µg)	31.3 ± 1.2	27.0 ± 1.0*	28.5 ± 0.5	30.0 ± 0.0
	Ceftriaxone (30 µg)	33.7 ± 0.6	30.7 ± 0.6*	33.0 ± 0.0	31.4 ± 0.6
	Piperacillin (30 µg)	29.7 ± 0.6	29.8 ± 0.6	29.0 ± 0.0	29.0 ± 0.0

K. pneumoniae	Ceftriaxone (30 µg)	35.7 ± 0.6	34.7 ± 0.6	34.2 ± 0.6	33.6 ± 0.5
	Ampicillin (10 µg)	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
	Ciprofloxacin (1 µg)	27.3 ± 1.2	26.3 ± 0.6	27.0 ± 0.0	25.8 ± 0.6
	Chloramphenicol (50 µg)	25.3 ± 0.6	26.7 ± 0.4	26.3 ± 1.2	26.0 ± 0.0
	Ceftazidime (30 µg)	32.3 ± 0.6	31.0 ± 0.6*	30.0 ± 0.0*	31.4 ± 0.4
Ps. aeruginosa	Imipenem (10 µg)	26.7 ± 0.6	22.7 ± 0.6	25.0 ± 0.0	27.3 ± 0.3
	Ceftazidime (30 µg)	22.3 ± 1.2	23.0 ± 1.0	22.0 ± 0.0	23.0 ± 0.0
	Meropenem (15 µg)	33.0 ± 1.0	33.0 ± 2.0	32.0 ± 0.0	33.1 ± 1.3
	Tobramycin (10 µg)	28.3 ± 2.1	29.0 ± 2.6	28.5 ± 1.5	29.0 ± 0.0
	Aztreonam (30 µg)	12.0 ± 0.0	10.7 ± 1.5	10.3 ± 1.2	10.0 ± 0.0
B. cenocepacia	Imipenem (10 µg)	35.3 ± 1.2	34.0 ± 0.0	35.0 ± 0.0	34.2 ± 1.4
	Ceftazidime (30 µg)	25.3 ± 0.6	27.5 ± 0.5	26.5 ± 1.5	27.7 ± 0.9
	Meropenem (15 µg)	36.0 ± 0.0	35.0 ± 0.0	36.0 ± 0.0	36.0 ± 0.0
	Tobramycin (10 µg)	20.0 ± 1.0	19.5 ± 0.5	20.0 ± 0.0	20.5 ± 0.0
	Ciprofloxacin (1 µg)	19.0 ± 0.6	19.0 ± 0.0	19.0 ± 0.0	19.0 ± 0.0

5.4 Discussion

SL1344, 14028S, *Ps. aeruginosa*, *B. cenocepacia* and *K. pneumoniae* were exposed to Corsodyl mouthwash, Dermax shampoo, eye make-up remover and CHG or BZC at the same concentration contained within the biocidal product in order to validate the determination of the MIC/MBC and antibiotic susceptibility of surviving organisms as a practical technique in the prediction of biocide resistance.

Despite no significant changes in clinical antibiotic susceptibility as a result of biocide exposure in any strains tested, reduced antibiotic susceptibility has been observed as a result of biocide exposure in *Ps. aeruginosa* by other groups (Tandukar *et al.*, 2013, D'Arezzo *et al.*, 2012). Tandukar *et al.,* (2013) observed increased resistance to the antibiotics penicillin G, ciprofloxacin and tetracycline as a result of long-term exposure to BZC (added to growth media) which they attributed to degradation of the antibiotic (penicillin G) and efflux pump activity. It is worth noting that the data obtained by Tandukar *et al*., (2013) was as a result of step-wise, long-term BZC exposure whereas the data obtained in this chapter was as a result of a single exposure, taking in to account realistic parameters such as contact time and possible product dilution through product use. D'Arezzo *et al.*, (2012) observed cross- resistance to 6 antibiotics including ciprofloxacin in a triclosan-adapted strain of *Ps. aeruginosa*. However this data was also obtained as a result of step-wise adaptation of *Ps. aeruginosa* to triclosan, and is therefore not directly comparable to the data obtained in this chapter. When predicting bacterial biocide resistance it is important to consider the application of the product and potential in-use contact time in order to create realistic test parameters. This was considered here when testing Corsodyl mouthwash, Dermax shampoo and make-up remover and may provide more realistic susceptibility data in surviving organisms than stepwise adaptation experiments that do not necessarily consider realistic parameters when it comes to exposure time and concentration. MIC/MBC and antibiotic susceptibility values were highly repeatable and reproducible across separate experiments performed over a 6 month period suggesting

that determination of the MIC/MBC and antibiotic susceptibility following standard protocols (BS EN ISO: 20776-1 (2006) and BSAC protocol (Andrews, 2009) respectively)) before and after biocide exposure is a valid and practical technique for use in the prediction of bacterial biocide resistance.

Very little investigation in to the relationship between biocide and antibiotic susceptibility in *Klebsiella* spp. has been carried out. Data obtained in this chapter indicated that 1 min exposure to the three biocidal products tested here did not have an effect on the antibiotic susceptibility of surviving organisms, nor on the MIC/MBC of the biocidal product itself (appendix file name: appendix data for chapter 5). Abuzaid *et al.,* (2012) investigated the relationship between biocide and antibiotic susceptibility in *K. pneumoniae* isolates. They found that strains that carried the efflux pump genes *cepA* and *qacE* showed reduced susceptibility to the biocides CHG and BZC but were not resistant to any antibiotics tested. This suggests there may not be a common mechanism present that could confer cross-resistance to antibiotics after biocide exposure. The lack of investigation in to the effect of biocide exposure on antibiotic susceptibility in this species suggests that *Klebsiella* spp. may not be an ideal model organism for use in the prediction of biocide resistance. However the data obtained using this organism was highly reproducible and consistent across repeats, indicating that MIC/MBC and antibiotic susceptibility determination following standard protocols is a practical technique for biocide resistance determination.

Large increases (up to 100-fold) in MIC/MBC were observed in SL1344 and 14028S after exposure to low CHG/BZC concentrations (chapter 3, tables 3.5 & 3.6). Such increases were not observed in this chapter after 1 min exposure to Corsodyl mouthwash, Dermax Shampoo and eye make-up remover. However the concentrations and contact time used in this chapter differ from those used in chapter 3 (5 min, 0.00001 – 0.0004 % CHG/BZC) and therefore may not have resulted in the same response at the cellular level. Biocide exposure and antibiotic

cross resistance in *Salmonella* species has been described by Condell et al., (2012a), Whitehead *et al*., (2011), Randall *et al*., (2007) and Braoudaki and Hilton (2004) and has been discussed in detail in chapter 3 section 3.4. More importantly, the data obtained for both *Salmonella enterica* strains across experimental repeats in both chapter 3 and this chapter is highly reproducible, even when separate experiments were performed over a 6 month period of time. This confirms the validity of MIC/MBC and antibiotic susceptibility determination before and after exposure to a particular biocide for use in the prediction of biocide resistance. These data also further confirm that *Salmonella enterica* serovar Typhimurium may be a useful model organism in the prediction of biocide resistance.

Alterations in clinical susceptibility to the antibiotics ciprofloxacin and imipenem were observed in *B. lata* in chapter 4 (table 4.5) after 5 min exposure to 0.005 % CHG and BZC but no changes in clinical antibiotic susceptibility were observed here in *B. cenocepacia* after exposure to Corsodyl mouthwash or eye make-up remover. Susceptibility to tobramycin was altered from sensitive to resistant on 1 out of 3 occasions after exposure to Dermax shampoo (appendix file name: appendix data for chapter 5). This isolated change in susceptibility appears to confirm the lack of data reproducibility when using *Burkholderia* spp. as a model organism for predicting biocide resistance and antibiotic cross-resistance (Knapp *et al.*, 2013). Rose *et al.,* (2009) investigated biocide and antibiotic susceptibility of *B. cenocepacia* and found that despite being resistant to multiple antibiotics, there was no direct correlation between biocide susceptibility and antibiotic susceptibility in this species of *Burkholderia* which correlates with the findings in this chapter. The effect of biocide exposure on the susceptibility of *Burkholderia* species has been investigated by Knapp *et al*., (2013) and Rushton *et al*., (2013) and is discussed in detail in chapter 4 section 4.4. Data obtained using *B. cenocepacia* was generally reproducible across separate repeats with the exception of 1 clinical change in tobramycin susceptibility, whereas there was considerable variability in antibiotic susceptibility after biocide exposure was observed in *B. lata.* These findings suggest

that *Burkholderia spp.* may not be an appropriate model organism for use in the prediction of biocide resistance and antibiotic cross-resistance. Furthermore the lack of clinical antibiotic susceptibility breakpoints for *Burkholderia* spp. available in the BSAC protocol also makes this species a less favourable model organism for resistance prediction.

The principle aim of this chapter was to validate the use of MIC, MBC and antibiotic susceptibility determination before and after biocide exposure, for use in the prediction of bacterial biocide resistance. MIC, MBC and antibiotic susceptibility values for Corsodyl mouthwash, Dermax shampoo, eye make-up remover, CHG and BZC were repeatable between biological replicates and reproducible between separate repeat experiments at the concentrations tested in 4 out of 5 strains. The reproducibility of the data across all strains (except *B. cenocepacia*) and experiments indicates that MIC, MBC and antibiotic susceptibility determination (following standard protocols (BS EN ISO: 20776-1 (2006) and BSAC protocol (Andrews, 2009) respectively)) is a practical and high throughput technique that provides useful initial information in the prediction of bacterial biocide resistance.

Chapter Six: General discussion

The principle aim of this project was to use a variety of experimental techniques to explore a range of practical markers of biocide resistance and antibiotic cross-resistance with a view to developing a step-by-step protocol that allows the prediction of bacterial biocide resistance. With this in mind a number of techniques were used, including MIC/MBC/antibiotic susceptibility determination, efflux assays, SDS-PAGE, real-time PCR, and microarray, and their ability to generate practical markers of biocide resistance, their cost effectiveness and their ability to generate repeatable and reproducible data was determined.

6.1 Protocol design

As previously mentioned (chapter 1, section 1.3) the BPR now states that manufacturers of biocidal products must provide information on the likelihood of resistance development to their product. With this in mind there is a requirement for a standard protocol that allows the prediction of resistance, using high throughput and effective techniques that allow the manufacturer to provide this type of data promptly (SCENIHR, 2010).

6.1.1 Protocol consideration

The protocol designed here is based on the comparison of baseline (pre-biocide exposure) and post-biocide exposure data, and this principle was validated using MIC/MBC and antibiotic susceptibility determination (chapter 5). The protocol provides logical steps to take in order to firstly identify changes in biocide/antibiotic susceptibility as a result of biocide exposure (predict resistance) and secondly identify further resistance markers and possible mechanisms behind the observed changes. It also takes in to account how high throughput a technique is, how practical the technique is and the cost of each technique. Table 6.1 provides a comparison of the techniques explored in terms of practicality, cost, and ease of use (i.e. the amount of

training/knowledge required to successfully use the technique). The table helps provide the rationale behind the order of the techniques used in the step-by-step protocol.

Table 6.1: Comparison of practicality, cost and complexity of techniques explored throughout project

Technique	Practicality	Approximate/Predicted cost	Complexity/Ease of use
MIC/MBC determination	High throughput, data within 24 h, 96 well plate easy to set up Can test multiple species/biocides/concentrations at once	~£1 per 96 well MIC plate+ lid Media cost dependent on test bacterium. TSA/TSB ~£40/500g Approx cost per sample (MIC): £1.10 (plate + media). Note can run multiple samples per plate to reduce cost per sample Approx cost per sample (MBC): 50 p (based on 1 TSA plate per sample)	Straightforward, follow step by step ISO:20776-1protocol, data easy to interpret
Antibiotic susceptibility testing	High throughput, data within 24 h, disk diffusion protocol allows testing of 6 antibiotics per plate.	Antibiotic disks £11-15 for 50 depending on which antibiotic is required Iso-sensitest agar ~£200 per 500g. Approx cost per sample (testing 6 antibiotics on one plate): £2.00	Straightforward, follow step by step BSAC disk diffusion protocol, clinical breakpoints provided make data easy to interpret
Phenotype stability testing	New data generated after every subculture (every 24 h). High throughput, large data set obtained over chosen test period. Valuable info on stability of change. Subcultures + MIC/MBC tests easy to perform	Dependent on duration of stability testing/number of samples. E.g. One strain, 2 biocides uses approximately 14 96 well plates over a 7 day period - cost ~£14. Media cost dependent on test bacterium. TSA/TSB ~£40/500g Approx cost for one strain, 2	Simple technique, requires MIC/MBC and antibiotic determination every 24 h.

		biocides over 7 days (including MIC/MBC determination, antibiotic testing): £25	
Efflux assay	Simple but time consuming set-up (takes 3-6 h for bacteria to reach mid-log phase depending on species). Once set up, data obtained within 3 min from plate reader, little data manipulation required to generate meaningful data. Despite long set-up can read several plates rapidly, allowing multiple strains/biocides to be tested at once	Black 96 well plate for fluorescence reading ~ £1.50 for plate + lid (1 strain + 2 biocides per plate) Hoechst dye £36.60/100 mg – 2 tubes required for all reactions performed in this project (3 strains, 3 treatments per strain) Approx cost per sample (one strain untreated/CHG treated/BZC treated): £ 25 i.e. plate + dye assuming 3 biological replicates per treatment	Based on changes in fluorescence readings obtained from different samples – data easy to analyse. Simple biocide exposure followed by staining with fluorescent dye
Protein assay (SDS-PAGE)	Limited info on protein identity using one dimension only (used here). Long prep time – protein extraction, purification, quantification. Electrophoresis, staining, de-staining and visualisation also time consuming. Further work required for more informative data to be obtained (e.g. protein sequencing, western blot)	Extraction, electrophoresis and staining reagents are costly but can be purchased in bulk and used for multiple samples Pre-cast gel £13 – can run one strain + 3 treatments per gel Approx cost per individual sample (i.e. one strain untreated, CHG treated, BZC treated): £30-35, includes gel and all purification, electrophoresis and staining components	Previous training in gel electrophoresis, loading gels, staining and visualising gels may be required for efficient use of technique and ease of data interpretation (although not essential). Knowledge of approximate protein molecular weights may assist in data analysis

Real-time PCR	RNA extraction simplified by use of step-by-step kit, but time consuming especially if large sample number. A large number of PCR reactions can be run at once (max 96), run time approx 3.5 hours, so 2 -3 runs can be completed in one day. Data analysis straightforward.	Kits expensive but allow large number of samples to be processed Extraction kit: £322 (but variable depending on kit choice), allows 50 preps cDNA synthesis kit: £298 for 100 reactions SYBR Green kit: £140 for 200 25 µL runs In this project use of qPCR machine £5 per run Approx cost per sample: £15 -20 (includes extraction, cDNA synthesis, SYBR Green and use of machine and accounts for variability in cost of kits)	Previous training in primer design, RNA extraction and setting up PCR reactions essential as real-time PCR highly sensitive to contamination. Training in data interpretation, calculation of fold changes also essential as more than one method available – choice of method may affect accuracy of fold changes calculated e.g. Pfaffl method accounts for primer efficiency but delta-delta method does not
Microarray	Very time consuming prep – RNA extraction, bioanalysis, labelling. Generates a very large volume of raw data which requires extensive analysis. Not a practical choice if you require rapid data generation	SV RNA isolation system £204 for 50 preps Approximate cost of work carried out here (includes RNA prep and bioanalysis, microarray execution and primary data analysis by biostatistician: £3000 Approx cost per strain (i.e. untreated, CHG and BZC treated): £1500. Based on 8 x 15k array slides, i.e. 8 repeats of same gene set on one slide	Previous training in running a microarray essential, particularly labelling and hybridisation steps. Good theoretical knowledge of how a microarray works also required. Extensive training in data interpretation/analysis required, including training in the use of software for microarray data analysis e.g. GeneSpring. Basic knowledge of how biocidal product works/bacterium may respond could help with data

			interpretation
Flow cytometry	Preparation may be time consuming depending on biocide exposure times and bacterial growth requirements, but is simple. Instant generation of data by flow cytometer, analysis may be time consuming and data can be difficult to interpret. Useful starting point for looking at changes in a population as a result of exposure	Propidium iodide: £38.60/ 25 mg BOX dye: £147 / 25 mg Cost per sample (based on staining with PI (5 µg/mL) and BOX (10 µg/mL)): ~£1	Set up and staining of samples straightforward. However previous experience/training in the use of a flow cytometer is essential as well as training in the use of accompanying software e.g. FACSDiva in order to be able to analyse data effectively.

Figure 6.1 shows the decision tree for risk of emerging resistance. It is based on the protocol proposed for current use including the techniques to use and the order they are used, as well as points when the user can make an informed decision on the risk of resistance. As shown in figure 6.1 a decision on how high the risk of resistance development is can be made using the first 3 steps of protocol (MIC/MBC/antibiotic susceptibility, flow cytometry and phenotype stability testing). If no changes in the MIC/MBC or antibiotic susceptibility are observed after biocide exposure, and flow cytometry experiments do not identify undamaged, surviving cells, the risk of resistance development to the biocide is low. If an increase in the MIC or MBC or a clinical change in antibiotic susceptibility is observed the stability of this change can inform the user on the risk of resistance development. A stable change suggests a high risk of resistance development whereas an unstable change suggests a low risk. The steps that follow (efflux assay, protein assay, microarray, PCR) can then be used to identify further resistance markers and inform the user on potential mechanisms of resistance to the test biocide.

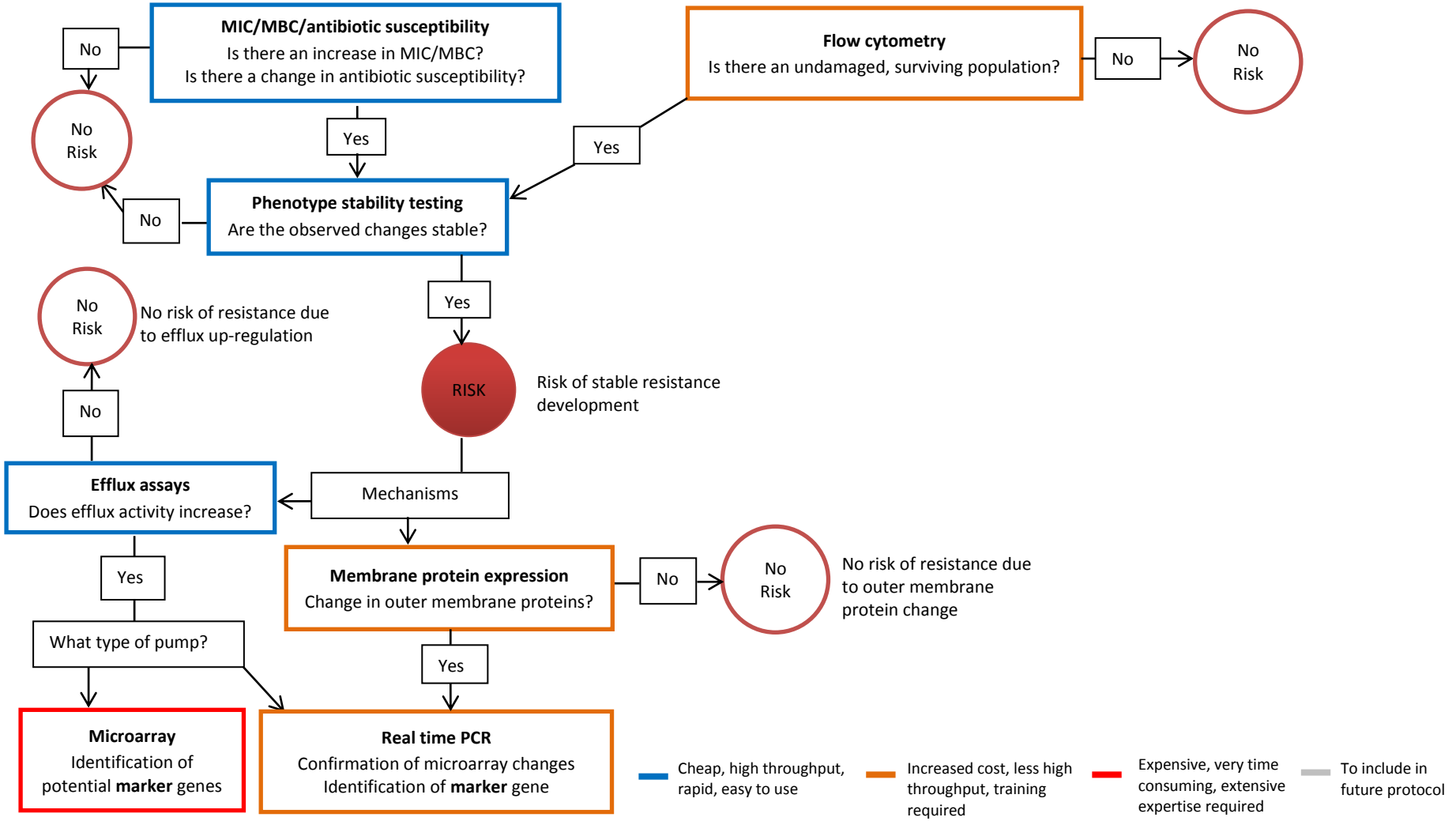


Figure 6.1: Decision tree and proposed protocol for the prediction of bacterial biocide resistance

6.1.2 Protocol rationale

Figure 6.2 is populated with data obtained from chapter 3 in order to demonstrate how the protocol can be used to predict resistance development. Techniques that are high throughput (i.e. generate a large volume of a data in a short period of time), low in cost per sample and simple to execute (i.e. minimal training/knowledge required, if any) are carried out before those that are more complex and costly as shown in figures 6.1 and 6.2. MIC, MBC and antibiotic susceptibility determination comprise the first step of the protocol as they are high throughput, cheap and simple to execute. Furthermore, the elevated MIC and MBC values observed when exposing *S*. *enterica* serovar Typhimurium strains SL1344 and 14028S to CHG and BZC (chapter 3) provided a useful initial resistance marker that could then be further explored using different techniques. There is also the potential to start the protocol using flow cytometry alongside MIC/MBC/antibiotic susceptibility determination to identify cells within a biocide- exposed population that are undamaged after exposure, as these cells may demonstrate reduced biocide susceptibility.

Phenotype stability testing follows MIC/MBC/antibiotic susceptibility determination as it establishes whether the increased MIC/MBC or change in antibiotic susceptibility is stable or transient. At this point in the protocol a decision can be made on the risk of resistance development, based on the stability of the changes observed. Phenotype stability testing is also low in cost and high throughput, and successfully established that the increased MIC and MBC values observed in SL1344 and 14028S were not stable (chapter 3).

The techniques that follow phenotype stability testing are less high throughput and may require some form of training/knowledge for effective execution. The suggested order shown in figure 6.1 was chosen to first identify a putative mechanism behind the increased MIC/MBCs (efflux assay, protein assay) and then to potentially identify a change in a specific gene or protein associated with a particular mechanism, thus generating multiple markers of

resistance. Efflux and protein assays were chosen as logical steps following increases in the MIC/MBC as bacterial biocide resistance is often associated with mechanisms that remove biocide from the cell (i.e. efflux up-regulation) or prevent biocide entering the cell (e.g. changes in porins). As discussed in table 6.1, further sequencing of proteins would be required to provide more information on protein changes observed. In the work carried out here a positive result was obtained for efflux up-regulation in strains SL1344 & 14028S as indicated by the decrease in fluorescence after biocide exposure, corresponding to less Hoechst dye accumulating within the bacterial cell. This represented a further resistance marker.

Efflux assays are followed by either real-time PCR reactions or microarrays, with a view to identifying a specific type of efflux pump or a marker gene associated with this efflux up-regulation. Real-time PCR would be recommended over microarrays as it is less costly, and data can be obtained faster. However the use of real-time PCR does require knowledge of specific genes that could be associated with resistance. If this information cannot be acquired by the user a microarray exploring genome-wide gene expression is recommended. However microarrays are costly and require training and expertise to be performed successfully. Microarray therefore appears as a last step in the protocol. A combination of microarray technology and real-time PCR was used to identify down-regulation of the *acrR* efflux pump regulatory gene in SL1344 and 14028S after CHG exposure (chapter 3) which may be responsible for the up-regulation in efflux observed in the efflux assays. Down-regulation of this gene across both strains made it a useful resistance marker.

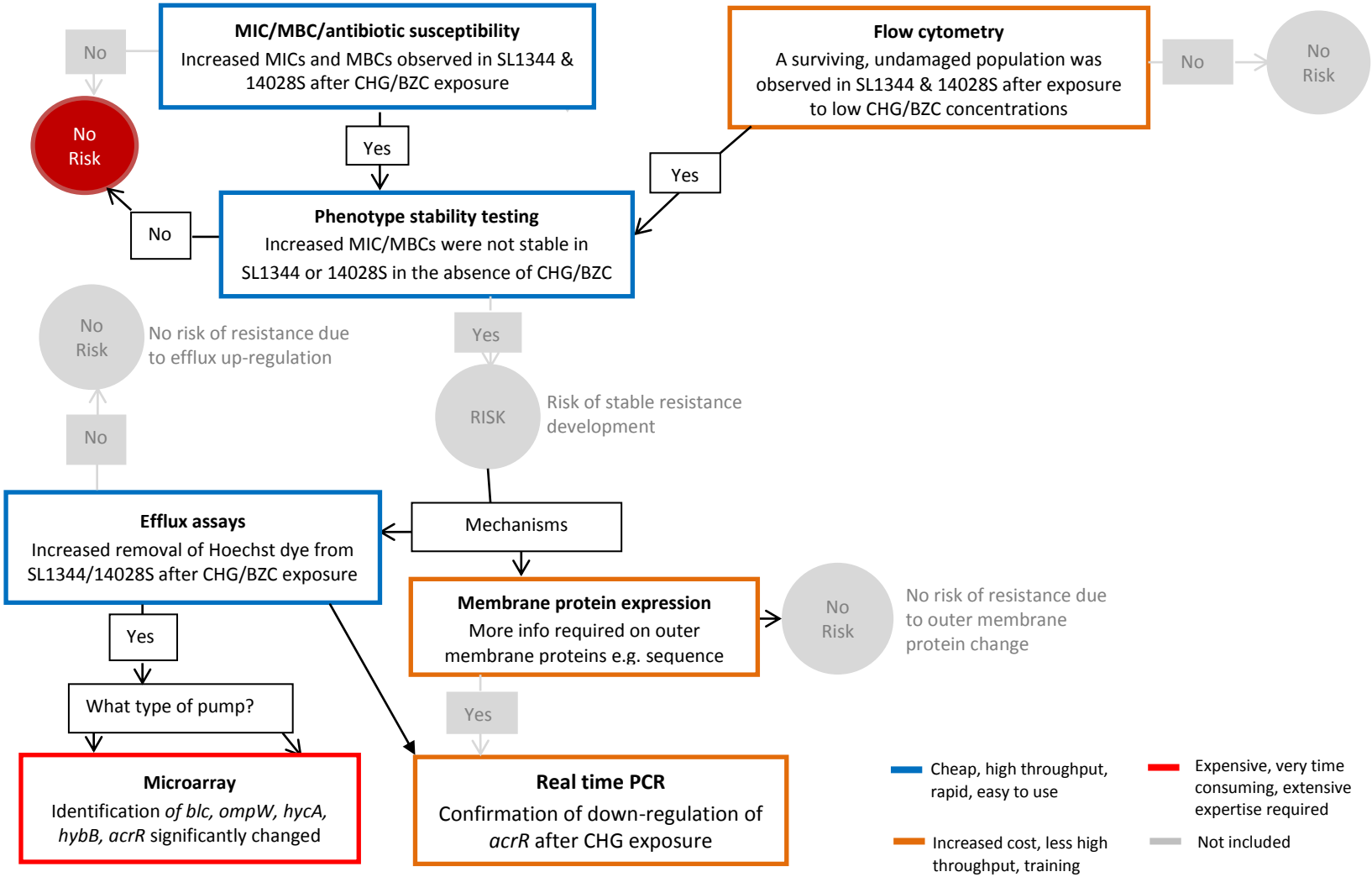


Figure 6.2: Decision tree and predictive protocol populated with data from chapter 3

6.3 Choice of model organism

Various species were used in this project including *S*. *enterica* serovar Typhimurium, *B. lata, B*. *cepacia*, *K*. *pneumoniae* and *Ps*. *aeruginosa*. It was important that the predictive protocol designed was suitable for use with multiple bacterial species as different biocidal products have different target organisms. As determined in chapters 4 and 5, *Burkholderia* spp. were not a particularly useful model organism for the prediction of biocide resistance and antibiotic cross-resistance due to the lack of repeatable and reproducible data obtained. This was observed in antibiotic susceptibility tests using *B. lata* strain 383 where resistance to imipenem and ciprofloxacin as a result of 5 min exposure to 0.005 % CHG/BZC was only observed in 1 out of 4 separate repeats of the experiment. Lack of data repeatability was also observed in chapter 5 in the validation work carried out using *B. cenocepacia,* where resistance to tobramycin as a result of exposure to Dermax shampoo was observed in only 1 of 3 separate repeat experiments. A further limitation to using the BSAC disk diffusion protocol for antibiotic susceptibility testing (Andrews, 2009) with *Burkholderia* spp. was that it does not provide clinical breakpoints for these organisms. However, the data generated from the three remaining species was both repeatable and reproducible. This indicates that the protocol (figure 6.1, 6.2) is suitable for use with different species of bacteria. *S*. Typhimurium in particular would make a good model organism as all the MIC/MBC and antibiotic susceptibility data generated using this organism was highly reproducible. Further tests used to identify possible biocide resistance mechanisms and additional markers in this species (efflux assays, real-time PCR, microarray) also generated some useful resistance markers, e.g. increased efflux of Hoechst dye, down-regulation of the *acrR* gene as illustrated in figure 6.2.

6.4 Using the protocol to determine the risk of resistance development to CHG and BZC

Changes in biocide susceptibility observed included an increase in the MIC and MBC of CHG and BZC for *S. enterica* serovar Typhimurium strains SL1344 and 14028S. However, phenotype stability tests carried out indicated that these increases were not stable in the presence or absence of low concentrations (0.0001 – 0.0004 %) of these biocides. A change that is stable in the absence of any biocide may indicate the presence of a permanent mutation in a particular gene or a permanent alteration to the bacterial cell physiology. A permanent/stable change in the bacterial cell as a result of biocide exposure would be associated with a greater risk of resistance development compared with a transient, unstable response. This suggests that despite the increases in MIC and MBC observed here, there is not a high risk of stable resistance development to CHG and BZC in either *Salmonella enterica* strain tested, due to the transient and unstable nature of the response. Despite the unstable changes observed, further tests were performed for the purpose of protocol development. Efflux assays, microarrays and real-time PCR indicated that efflux up-regulation (increased Hoechst dye efflux, down-regulation of *acrR*) was the putative mechanism behind the increased MIC and MBC values observed. This data indicated that despite CHG and BZC generally being used at higher concentrations than those tested here, much lower, potentially residual concentrations (0.00001 – 0.0004 %) can induce responses such as efflux up-regulation resulting in increases in biocide MIC and MBC. The protocol shown in figure 6.1 can therefore be used to test both in-use and residual biocide concentrations in order to determine if residual concentrations of biocide could be associated with resistance development. However due to the instability of the changes observed there is no risk of resistance development to CHG or BZC at the concentrations tested.

6.5 Protocol limitations and further considerations

6.5.1 Test conditions

The repeatability and reproducibility of the data obtained using any standard protocol can be affected by test conditions such as temperature, test inoculum preparation and pH. Existing protocols such as the BS EN 1276 (2009) suspension testing protocol specify that temperature must be maintained at 20 ± 1°C and provide specific instructions on the preparation of the diluents used for the test biocide and preparation of the test organism. The BSAC disk diffusion (2009) protocol for antibiotic susceptibility testing also specifies that the depth of the Iso-Sensitest agar used must consistently be 4 ± 0.5 mm so that all antibiotics diffuse evenly in to the agar and reproducible results are obtained. To ensure that data obtained using the protocol shown here (figure 6.1) were reproducible, the test inoculum was always prepared in the same way (see chapter 2 section 2.1) and temperature during biocide exposure was maintained at 20 ± 1 °C using a water bath. Controlling these parameters is essential for the successful use of the protocol developed here as it minimises the chance of data variability due to altered biocide efficacy, either due to temperature fluctuations or differences in the test inoculum e.g. variable cell count or presence of aggregates.

6.5.2. Biocide vs. Formulation

As identified by SCENIHR (2009, 2010) the vast majority of biocide susceptibility testing is carried out using individual biocides rather than formulations. It is important that formulations are tested as components of the formulation may affect the efficacy of the biocide contained within the product in comparison to the efficacy of the active biocide tested alone. This point was highlighted in preliminary suspension tests performed in chapter 5 (table 5.7) where Dermax shampoo (contains 0.5 % BZC) was much less effective in killing test organisms in comparison to BZC alone. Factors such as the viscosity of a formulation (e.g. shampoo) may

affect the data obtained when using certain techniques to predict resistance. The protocol designed here (figure 6.1) was validated testing both biocides (CHG, BZC) and formulations (mouthwash, shampoo, eye make-up remover) with reproducible and repeatable data obtained in both cases. To demonstrate the use of the protocol in testing formulations, figure 6.3 shows the use of the protocol in predicting the risk of resistance development to Dermax shampoo using *S. enterica* strains SL1344 and 14028S as model organisms. As shown in figure 6.3, there were no increases in the MIC or MBC for Dermax shampoo, nor were there any clinical changes in antibiotic resistance observed after exposure to Dermax shampoo. It can therefore be concluded that the risk of resistance development in SL1344 and 14028S is low.

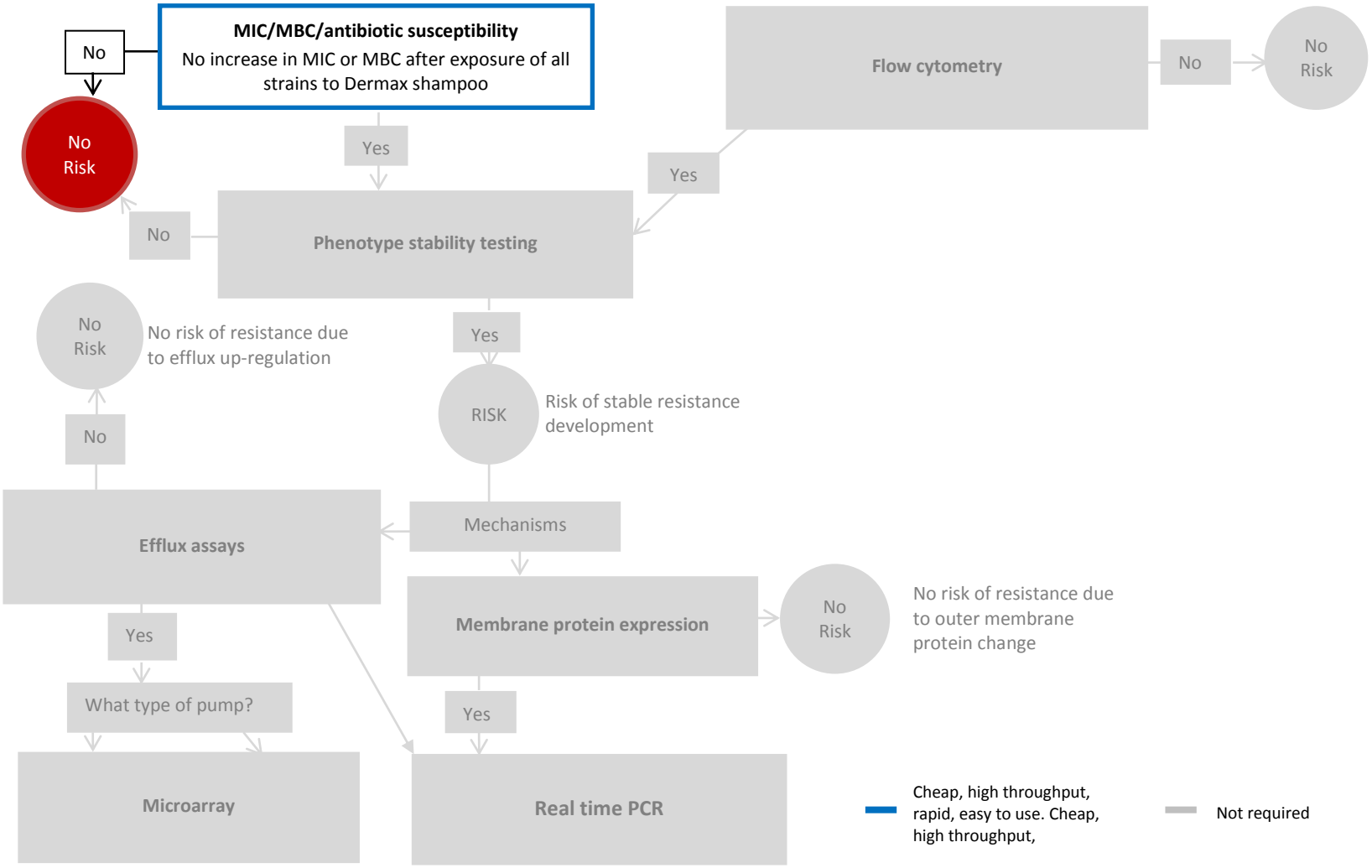


Figure 6.3: Use of the predictive protocol to determine the risk of resistance development to Dermax shampoo in S. enterica strains SL1344 & 14028S

6.5.3 Additional techniques and future work

6.5.3.1 Transferable and acquired resistance

Multiple drug resistance in bacteria is most often associated with genotypic changes. These can be in the form of mutations in genes associated with resistance, e.g. efflux pump genes, porins (Svetlikova *et al.*, 2009, Baucheron *et al.*, 2004b) and can also occur when bacteria acquire resistance genes present on plasmids or transposons (Popowska and Krawczyk-Balska, 2013). It is therefore important that predictive protocols are designed with this in mind, incorporating techniques that can inform the user of the effect of biocide exposure on gene exchange. The protocol designed here (figure 6.1) includes techniques such as real-time PCR and microarray which allow the exploration of changes in gene expression as a result of biocide exposure. Despite being costly, these techniques are well-established and have been successfully used in identifying changes in gene expression that have led to biocide resistance (Rushton *et al.*, 2013, Sass *et al.*, 2011, Whitehead *et al.*, 2011). However these techniques cannot provide information on the effect of biocide exposure on plasmid transfer and therefore the dissemination of resistance genes and acquired MDR. This is also poorly studied and is more difficult to incorporate in to a predictive protocol as there are no standard techniques available (SCENIHR, 2009). It also must be taken in to consideration that resistance genes can be transferred between different bacterial species present in a given environment, as well as between the same species (Frye *et al.*, 2011). The use of a particular biocide may therefore affect other bacterial species as well as the target organism. The method used must be suitable for the testing of multiple biocides and bacterial species and produce reproducible data. Such a method should be incorporated in to further developed versions of the protocol shown in figure 6.1.

6.5.3.2 Mutation

Mutations in specific genes are often more associated with antibiotic resistance. For example, mutations in the QRDRs of genes such as *gyrA* have been shown to result in resistance to quinolone antibiotics in *Salmonella* species (Webber *et al.*, 2013). However biocide exposure can result in the appearance of mutants with reduced biocide tolerance (Whitehead *et al.*, 2011). It would therefore be useful to include techniques that determine bacterial mutation frequency as a result of biocide exposure in future predictive protocols. Although there is no current standard technique for the determination of mutation frequency, it has been studied by several groups. The technique generally involves the exposure of the test organism to the biocide before surviving organisms are plated on to agar containing the same biocide at a concentration 2-4 times the MIC. The number of colonies on the plate can then be counted and the mutation frequency calculated (Christensen *et al.*, 2011, Randall *et al.*, 2004). The mutation frequency without biocide exposure should also be determined alongside this as a comparison point. It may also be useful to use a test organism for which the mutation frequency is already well-established, so an increase in the mutation frequency is easy to interpret. As this technique appears rapid and straightforward it would make a valuable addition to future predictive protocols.

6.5.3.3. Biofilms

As > 90 % of bacteria are thought to exist in biofilm formation *in vivo* (Baugh *et al.*, 2013), biocide susceptibility testing should be more frequently performed using biofilms (SCENIHR, 2009). Comparisons between the susceptibility of biofilm and planktonic cells to a particular biocide should also be made. The protocol designed here (figure 6.1) was developed using planktonic cells only, but tests such as MIC/MBC determination, antibiotic susceptibility testing, efflux assays, real-time PCR, SDS-PAGE and microarray have been successfully used

with biofilms previously (Baugh *et al.*, 2013, Coenye *et al.*, 2011, Pagedar *et al.*, 2011, Caraher *et al.*, 2007, Tabak *et al.*, 2007, Svensater *et al.*, 2001) so the protocol would be suitable for predicting resistance in biofilm cells although some additional techniques could be added. Of particular interest to investigate would be the effect of biocide exposure on the transfer of genetic material between cells present in a biofilm, as biofilms are often composed of numerous different bacterial species. The effect of biocide exposure on the ability of bacterial cells to form a biofilm should also be investigated as a biocide could both promote biofilm formation (as a mechanism to reduce biocide susceptibility) or reduce it (cell damage). Global gene expression analysis in biofilm cells would be also be of interest as biofilm cells often exist in a dormant state with reduced metabolic activity (Baugh *et al.*, 2013) and resistance marker genes in biofilm cells may therefore differ considerably from those in planktonic cells.

6.5.3.4 Fluorescence activated cell sorting (FACS)

Flow cytometry experiments performed in chapter 3 highlighted that some cells within the population exposed to a low concentration of CHG or BZC (0.0001 – 0.0004 %) appear to remain undamaged after exposure. FACS allows the sorting and isolation of surviving cells and therefore provides the opportunity for further experiments to be performed using these cells. Whitehead *et al.,* (2011) successfully used a combination of flow cytometry and FACS to identify and isolate multidrug resistant surviving *S*. *enterica* serovar Typhimurium isolates after biocide exposure. Flow cytometry and FACS could be incorporated in to future predictive protocols as a method for isolating surviving organisms before MIC/MBC determination and testing for further markers of resistance.

6.6 Concluding remarks

The protocol proposed in figure 6.1 is composed of a range of techniques explored throughout this project that successfully generated markers of biocide resistance. The protocol has been validated (chapter 5) and produces reproducible data when testing both biocides and biocidal formulations. The protocol shown in figure 6.1 is therefore proposed for current use in the prediction of bacterial biocide resistance and antibiotic cross- resistance.

Improvements can be made to this protocol via the incorporation of additional techniques including gene transfer analysis, mutation frequency experiments and FACS which will provide additional resistance markers. Figure 6.4 shows a possible future protocol incorporating these techniques. FACS should be carried out alongside flow cytometry as it allows the sorting and isolation of surviving populations observed in flow cytometry experiments. Mutation frequency and gene transfer experiments could be carried out following the observation of a stable increase in MIC, MBC, and/or antibiotic susceptibility.

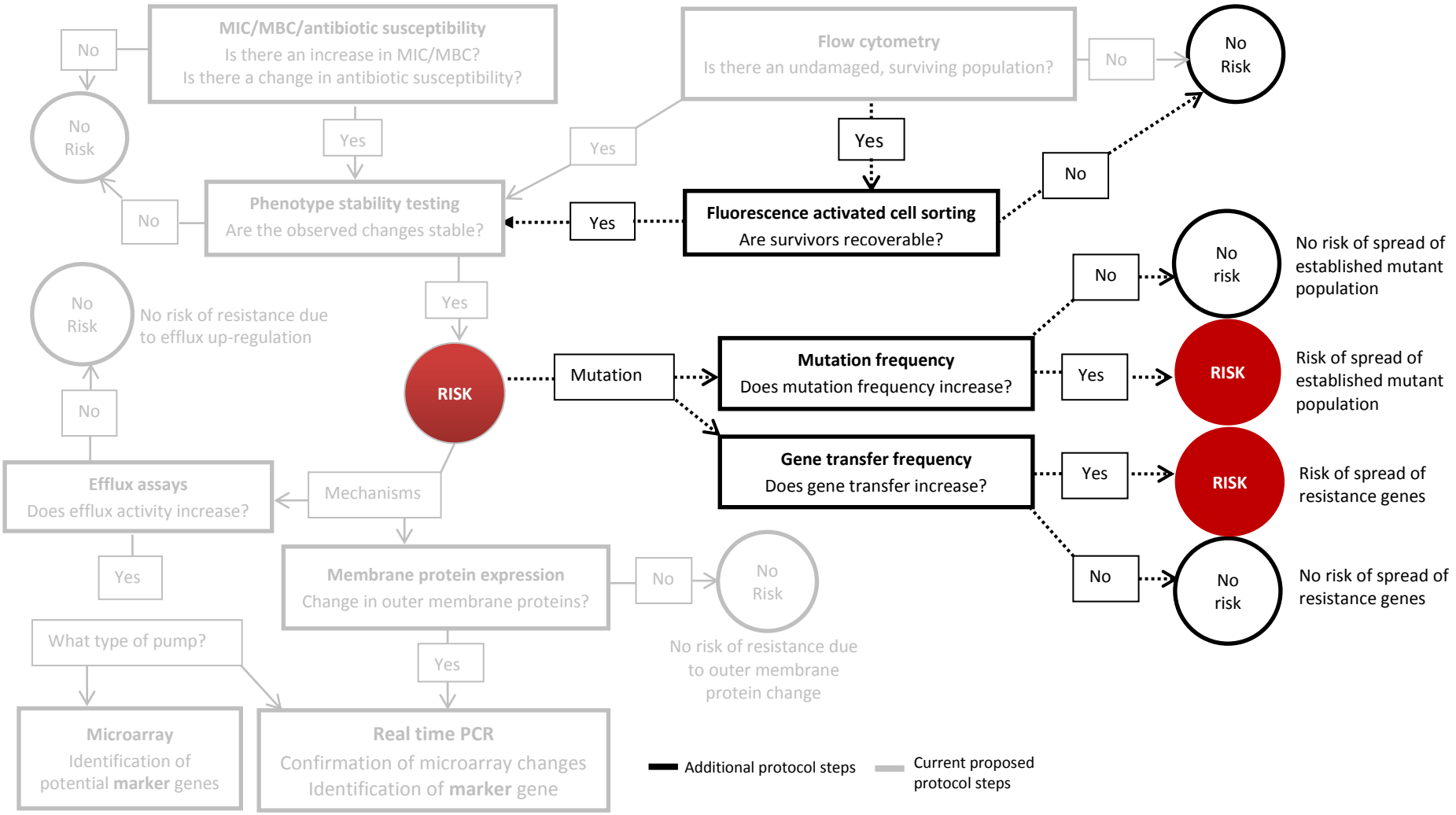


Figure 6.4: Proposed future predictive protocol incorporating techniques that allow determination of the effect of biocide exposure on gene transfer and mutation frequency, as well as the use of FACS to isolate potentially resistant populations

References

AARON, S. D., FERRIS, W., HENRY, D. A., SPEERT, D. P. & MACDONALD, N. E. 2000. Multiple combination bactericidal antibiotic testing for patients with cystic fibrosis infected with Burkholderia cepacia. *Am J Respir Crit Care Med,* 161**,** 1206-12.

ABUZAID, A., HAMOUDA, A. & AMYES, S. G. 2012. Klebsiella pneumoniae susceptibility to biocides and its association with cepA, qacDeltaE and qacE efflux pump genes and antibiotic resistance. *J Hosp Infect,* 81**,** 87-91.

AKIYAMA, T. & KHAN, A. A. 2012. Molecular characterization of strains of fluoroquinolone-resistant Salmonella enterica serovar Schwarzengrund carrying multidrug resistance isolated from imported foods. *J Antimicrob Chemother,* 67**,** 101-10.

ALBERTS, B., JOHNSON, A., LEWIS, J., M., R., K., R. & P, W. 2002. *Molecular Biology of the Cell,* New York, Garland Science.

ANDREWS, J. M. 2009. BSAC standardized disc susceptibility testing method (version 8). *J Antimicrob Chemother,* 64**,** 454-89.

ANTONOVA, E. S. & HAMMER, B. K. 2011. Quorum-sensing autoinducer molecules produced by members of a multispecies biofilm promote horizontal gene transfer to Vibrio cholerae. *FEMS Microbiol Lett,* 322**,** 68-76.

BAILEY, A. M., CONSTANTINIDOU, C., IVENS, A., GARVEY, M. I., WEBBER, M. A., COLDHAM, N., HOBMAN, J. L., WAIN, J., WOODWARD, M. J. & PIDDOCK, L. J. 2009. Exposure of Escherichia coli and Salmonella enterica serovar Typhimurium to triclosan induces a species-specific response, including drug detoxification. *J Antimicrob Chemother,* 64**,** 973-85.

BAUCHERON, S., CHASLUS-DANCLA, E. & CLOECKAERT, A. 2004a. Role of TolC and parC mutation in high-level fluoroquinolone resistance in Salmonella enterica serotype Typhimurium DT204. *J Antimicrob Chemother,* 53**,** 657-9.

BAUCHERON, S., TYLER, S., BOYD, D., MULVEY, M. R., CHASLUS-DANCLA, E. & CLOECKAERT, A. 2004b. AcrAB-TolC directs efflux-mediated multidrug resistance in Salmonella enterica serovar typhimurium DT104. *Antimicrob Agents Chemother,* 48**,** 3729-35.

BAUGH, S., EKANAYAKA, A. S., PIDDOCK, L. J. & WEBBER, M. A. 2013. Loss of or inhibition of all multidrug resistance efflux pumps of Salmonella enterica serovar Typhimurium results in impaired ability to form a biofilm. *J Antimicrob Chemother,* 67**,** 2409-17.

BAZZINI, S., UDINE, C., SASS, A., PASCA, M. R., LONGO, F., EMILIANI, G., FONDI, M., PERRIN, E., DECOROSI, F., VITI, C., GIOVANNETTI, L., LEONI, L., FANI, R., RICCARDI, G., MAHENTHIRALINGAM, E. & BURONI, S. 2011. Deciphering the role of RND efflux transporters in Burkholderia cenocepacia. *PLoS One,* 6**,** e18902.

BENSON, L., BUSH, L. & LEBLANC, D. 1990. Importance of neutralizers in the stripping fluid in a simulated healthcare personnel handwash. *Infect Control Hosp Epidemiol,* 11**,** 595-9.

BERLUTTI, F., MOREA, C., BATTISTONI, A., SARLI, S., CIPRIANI, P., SUPERTI, F., AMMENDOLIA, M. G. & VALENTI, P. 2005. Iron availability influences aggregation, biofilm, adhesion and invasion of Pseudomonas aeruginosa and Burkholderia cenocepacia. *Int J Immunopathol Pharmacol,* 18**,** 661-70.

BERNSTEIN, I. L. 2000. Is the use of benzalkonium chloride as a preservative for nasal formulations a safety concern? A cautionary note based on compromised mucociliary transport. *J Allergy Clin Immunol,* 105**,** 39-44.

BIOT, F. V., VALADE, E., GARNOTEL, E., CHEVALIER, J., VILLARD, C., THIBAULT, F. M., VIDAL, D. R. & PAGES, J. M. 2011. Involvement of the efflux pumps in chloramphenicol selected strains of Burkholderia thailandensis: proteomic and mechanistic evidence. *PLoS One,* 6**,** e16892.

BIROSOVA, L. & MIKULASOVA, M. 2009. Development of triclosan and antibiotic resistance in Salmonella enterica serovar Typhimurium. *J Med Microbiol,* 58**,** 436-41.

BJORLAND, J., SUNDE, M. & WAAGE, S. 2001. Plasmid-borne smr gene causes resistance to quaternary ammonium compounds in bovine Staphylococcus aureus. *J Clin Microbiol,* 39**,** 3999-4004.

BLOCK, C. & FURMAN, M. 2002. Association between intensity of chlorhexidine use and micro-organisms of reduced susceptibility in a hospital environment. *J Hosp Infect,* 51**,** 201-6.

BLOOMFIELD, S. F., EXNER, M., CARLO SIGNORELLI, C., NATH, K. J. & SCOTT, E. A. 2012. The chain of infection transmission in the home and everyday life settings, and the role of hygiene in reducing the risk of infection. *http://www.ifh-homehygiene.org/IntegratedCRD.nsf/111e68ea0824afe1802575070003f039/9df1597d90588986802572970061709 3?OpenDocument*.

BRAGG, R., JANSEN, A., COETZEE, M., VAN DER WESTHUIZEN, W. & BOUCHER, C. 2014. Bacterial Resistance to Quaternary Ammonium Compounds (QAC) Disinfectants. *Adv Exp Med Biol,* 808**,** 1-13.

BRAOUDAKI, M. & HILTON, A. C. 2004. Adaptive resistance to biocides in Salmonella enterica and Escherichia coli O157 and cross-resistance to antimicrobial agents. *J Clin Microbiol,* 42**,** 73-8.

BSI 2006. BS EN ISO 20776-1 Clinical laboratory testing and in vitro diagnostic test systems. Susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices. Reference method for testing the in vitro activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases.

BSI. 2009. BS EN 1276: Chemical disinfectants and antiseptics— Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas— Test method andrequirements (phase 2, step 1).

BUFFET-BATAILLON, S., BRANGER, B., CORMIER, M., BONNAURE-MALLET, M. & JOLIVET-GOUGEON, A. 2011. Effect of higher minimum inhibitory concentrations of quaternary ammonium compounds in clinical E. coli isolates on antibiotic susceptibilities and clinical outcomes. *J Hosp Infect,* 79**,** 141-6.

BURONI, S., PASCA, M. R., FLANNAGAN, R. S., BAZZINI, S., MILANO, A., BERTANI, I., VENTURI, V., VALVANO, M. A. & RICCARDI, G. 2009. Assessment of three Resistance-Nodulation-Cell Division drug efflux transporters of Burkholderia cenocepacia in intrinsic antibiotic resistance. *BMC Microbiol,* 9**,** 200.

CARAHER, E., REYNOLDS, G., MURPHY, P., MCCLEAN, S. & CALLAGHAN, M. 2007. Comparison of antibiotic susceptibility of Burkholderia cepacia complex organisms when grown planktonically or as biofilm in vitro. *Eur J Clin Microbiol Infect Dis,* 26**,** 213-6.

CARSON, R. T., LARSON, E., LEVY, S. B., MARSHALL, B. M. & AIELLO, A. E. 2008. Use of antibacterial consumer products containing quaternary ammonium compounds and drug resistance in the community. *J Antimicrob Chemother,* 62**,** 1160-2.

CHEESEMAN, K. E., DENYER, S. P., HOSEIN, I. K., WILLIAMS, G. J. & MAILLARD, J. Y. 2009. Evaluation of the bactericidal efficacy of three different alcohol hand rubs against 57 clinical isolates of S. aureus. *J Hosp Infect,* 72**,** 319-25.

CHRISTENSEN, E. G., GRAM, L. & KASTBJERG, V. G. 2011. Sublethal triclosan exposure decreases susceptibility to gentamicin and other aminoglycosides in Listeria monocytogenes. *Antimicrob Agents Chemother,* 55**,** 4064-71.

COENYE, T., VAN ACKER, H., PEETERS, E., SASS, A., BURONI, S., RICCARDI, G. & MAHENTHIRALINGAM, E. 2011. Molecular mechanisms of chlorhexidine tolerance in Burkholderia cenocepacia biofilms. *Antimicrob Agents Chemother,* 55**,** 1912-9.

CONDELL, O., IVERSEN, C., COONEY, S., POWER, K. A., WALSH, C., BURGESS, C. & FANNING, S. 2012a. Efficacy of biocides used in the modern food industry to control salmonella enterica, and links between biocide tolerance and resistance to clinically relevant antimicrobial compounds. *Appl Environ Microbiol,* 78**,** 3087-97.

CONDELL, O., SHERIDAN, A., POWER, K. A., BONILLA-SANTIAGO, R., SERGEANT, K., RENAUT, J., BURGESS, C., FANNING, S. & NALLY, J. E. 2012b. Comparative proteomic analysis of Salmonella tolerance to the biocide active agent triclosan. *J Proteomics,* 75**,** 4505-19.

D'AREZZO, S., LANINI, S., PURO, V., IPPOLITO, G. & VISCA, P. 2012. High-level tolerance to triclosan may play a role in Pseudomonas aeruginosa antibiotic resistance in immunocompromised hosts: evidence from outbreak investigation. *BMC Res Notes,* 5**,** 43.

DAVIS, C. 2014. Enumeration of probiotic strains: Review of culture-dependent and alternative techniques to quantify viable bacteria. *J Microbiol Methods*.

DETTENKOFER, M., WENZLER, S., AMTHOR, S., ANTES, G., MOTSCHALL, E. & DASCHNER, F. D. 2004. Does disinfection of environmental surfaces influence nosocomial infection rates? A systematic review. *Am J Infect Control,* 32**,** 84-9.

DREVINEK, P., HOLDEN, M. T., GE, Z., JONES, A. M., KETCHELL, I., GILL, R. T. & MAHENTHIRALINGAM, E. 2008. Gene expression changes linked to antimicrobial resistance, oxidative stress, iron depletion and retained motility are observed when Burkholderia cenocepacia grows in cystic fibrosis sputum. *BMC Infect Dis,* 8**,** 121.

DUARTE, R. S., LOURENCO, M. C., FONSECA LDE, S., LEAO, S. C., AMORIM EDE, L., ROCHA, I. L., COELHO, F. S., VIANA-NIERO, C., GOMES, K. M., DA SILVA, M. G., LORENA, N. S., PITOMBO, M. B., FERREIRA, R. M., GARCIA, M. H., DE OLIVEIRA, G. P., LUPI, O., VILACA, B. R., SERRADAS, L. R., CHEBABO, A., MARQUES, E. A., TEIXEIRA, L. M., DALCOLMO, M., SENNA, S. G. & SAMPAIO, J. L. 2009. Epidemic of postsurgical infections caused by Mycobacterium massiliense. *J Clin Microbiol,* 47**,** 2149-55.

DUBOIS-BRISSONNET, F., NAITALI, M., MAFU, A. A. & BRIANDET, R. 2011. Induction of fatty acid composition modifications and tolerance to biocides in Salmonella enterica serovar Typhimurium by plant-derived terpenes. *Appl Environ Microbiol,* 77**,** 906-10.

EBENTIER, D. L., HANLEY, K. T., CAO, Y., BADGLEY, B. D., BOEHM, A. B., ERVIN, J. S., GOODWIN, K. D., GOURMELON, M., GRIFFITH, J. F., HOLDEN, P. A., KELTY, C. A., LOZACH, S., MCGEE, C., PEED, L. A., RAITH, M., RYU, H., SADOWSKY, M. J., SCOTT, E. A., DOMINGO, J. S., SCHRIEWER, A., SINIGALLIANO, C. D., SHANKS, O. C., VAN DE WERFHORST, L. C., WANG, D., WUERTZ, S. & JAY, J. A. 2013. Evaluation of the repeatability and reproducibility of a suite of qPCR-based microbial source tracking methods. *Water Res,* 47**,** 6839-48.

EDMISTON, C. E., JR., BRUDEN, B., RUCINSKI, M. C., HENEN, C., GRAHAM, M. B. & LEWIS, B. L. 2013. Reducing the risk of surgical site infections: does chlorhexidine gluconate provide a risk reduction benefit? *Am J Infect Control,* 41**,** S49-55.

ESCALADA, M. G., HARWOOD, J. L., MAILLARD, J. Y. & OCHS, D. 2005. Triclosan inhibition of fatty acid synthesis and its effect on growth of Escherichia coli and Pseudomonas aeruginosa. *J Antimicrob Chemother,* 55**,** 879-82.

FERREIRA, C., PEREIRA, A. M., PEREIRA, M. C., MELO, L. F. & SIMOES, M. 2011. Physiological changes induced by the quaternary ammonium compound benzyldimethyldodecylammonium chloride on Pseudomonas fluorescens. *J Antimicrob Chemother,* 66**,** 1036-43.

FRANK, M. J. & SCHAFFNER, W. 1976. Contaminated aqueous benzalkonium chloride. An unnecessary hospital infection hazard. *JAMA,* 236**,** 2418-9.

FRYE, J. G., LINDSEY, R. L., MEINERSMANN, R. J., BERRANG, M. E., JACKSON, C. R., ENGLEN, M. D., TURPIN, J. B. & FEDORKA-CRAY, P. J. 2011. Related antimicrobial resistance genes

detected in different bacterial species co-isolated from swine fecal samples. *Foodborne Pathog Dis,* 8**,** 663-79.

GALAN, J. E. 1998. Interactions of Salmonella with host cells: encounters of the closest kind. *Proc Natl Acad Sci U S A,* 95**,** 14006-8.

GARCIA-ERCE, J. A., GRASA, J. M., SOLANO, V. M., GIMENO, J. J., LOPEZ, A., HERNANDEZ, M. J., MARCO, M. L., ARRIBAS, J. L. & GIRALT, M. 2002. Bacterial contamination of blood components due to Burkholderia cepacia contamination from clorhexidine bottles. *Vox Sang,* 83**,** 70-1.

GEFTIC, S. G., HEYMANN, H. & ADAIR, F. W. 1979. Fourteen-year survival of Pseudomonas cepacia in a salts solution preserved with benzalkonium chloride. *Appl Environ Microbiol,* 37**,** 505-10.

GEORGE, A. M., JONES, P. M. & MIDDLETON, P. G. 2009. Cystic fibrosis infections: treatment strategies and prospects. *FEMS Microbiol Lett,* 300**,** 153-64.

GILBERT, P. & MCBAIN, A. J. 2003. Potential impact of increased use of biocides in consumer products on prevalence of antibiotic resistance. *Clin Microbiol Rev,* 16**,** 189-208.

GIRAUD, E., BAUCHERON, S., VIRLOGEUX-PAYANT, I., NISHINO, K. & CLOECKAERT, A. 2012. Effects of natural mutations in the ramRA locus on invasiveness of epidemic fluoroquinolone-resistant Salmonella enterica serovar Typhimurium isolates. *J Infect Dis,* 207**,** 794-802.

GIRAUD, E., CLOECKAERT, A., KERBOEUF, D. & CHASLUS-DANCLA, E. 2000. Evidence for active efflux as the primary mechanism of resistance to ciprofloxacin in Salmonella enterica serovar typhimurium. *Antimicrob Agents Chemother,* 44**,** 1223-8.

GOULD, G. W. 1970. Mechanism of the inhibition of germination of bacterial spores by gamma-irradiation in the presence of iodoacetamide and iodate. *J Gen Microbiol,* 64**,** 301-9.

GUERREIRO-TANOMARU, J. M., MORGENTAL, R. D., FLUMIGNAN, D. L., GASPARINI, F., OLIVEIRA, J. E. & TANOMARU-FILHO, M. 2011. Evaluation of pH, available chlorine content, and antibacterial activity of endodontic irrigants and their combinations against Enterococcus faecalis. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod,* 112**,** 132-5.

GUO, W., CUI, S., XU, X. & WANG, H. 2013. Resistant Mechanism Study of Benzalkonium Chloride Selected Salmonella Typhimurium Mutants. *Microb Drug Resist*.

HASSAN, K. A., JACKSON, S. M., PENESYAN, A., PATCHING, S. G., TETU, S. G., EIJKELKAMP, B. A., BROWN, M. H., HENDERSON, P. J. & PAULSEN, I. T. 2013. Transcriptomic and biochemical analyses identify a family of chlorhexidine efflux proteins. *Proc Natl Acad Sci U S A,* 110**,** 20254-9.

HAWKEY, P. M. 2003. Mechanisms of quinolone action and microbial response. *J Antimicrob Chemother,* 51 Suppl 1**,** 29-35.

HEO, S. T., KIM, S. J., JEONG, Y. G., BAE, I. G., JIN, J. S. & LEE, J. C. 2008. Hospital outbreak of Burkholderia stabilis bacteraemia related to contaminated chlorhexidine in haematological malignancy patients with indwelling catheters. *J Hosp Infect,* 70**,** 241-5.

HINCHLIFFE, P., SYMMONS, M. F., HUGHES, C. & KORONAKIS, V. 2013. Structure and operation of bacterial tripartite pumps. *Annu Rev Microbiol,* 67**,** 221-42.

HUET, A. A., RAYGADA, J. L., MENDIRATTA, K., SEO, S. M. & KAATZ, G. W. 2008. Multidrug efflux pump overexpression in Staphylococcus aureus after single and multiple in vitro exposures to biocides and dyes. *Microbiology,* 154**,** 3144-53.

HUGO, W. B. & LONGWORTH, A. R. 1964. Some Aspects of the Mode of Action of Chlorhexidine. *J Pharm Pharmacol,* 16**,** 655-62.

JACOBSON, M., WRAY, R., KOVACH, D., HENRY, D., SPEERT, D. & MATLOW, A. 2006. Sustained endemicity of Burkholderia cepacia complex in a pediatric institution, associated with contaminated ultrasound gel. *Infect Control Hosp Epidemiol,* 27**,** 362-6.

JARVIK, T., SMILLIE, C., GROISMAN, E. A. & OCHMAN, H. 2010. Short-term signatures of evolutionary change in the Salmonella enterica serovar typhimurium 14028 genome. *J Bacteriol,* 192**,** 560-7.

JASSEM, A. N., FORBES, C. M. & SPEERT, D. P. 2014. Investigation of aminoglycoside resistance inducing conditions and a putative AmrAB-OprM efflux system in Burkholderia vietnamiensis. *Ann Clin Microbiol Antimicrob,* 13**,** 2.

JIMENEZ, L. 2004. *Microbial Contamination Control in the Pharmaceutical Industry*, Marcel Dekker New York.

KAPLAN, J. B., IZANO, E. A., GOPAL, P., KARWACKI, M. T., KIM, S., BOSE, J. L., BAYLES, K. W. & HORSWILL, A. R. 2012. Low levels of beta-lactam antibiotics induce extracellular DNA release and biofilm formation in Staphylococcus aureus. *MBio,* 3**,** e00198-12.

KARATZAS, K. A., RANDALL, L. P., WEBBER, M., PIDDOCK, L. J., HUMPHREY, T. J., WOODWARD, M. J. & COLDHAM, N. G. 2008. Phenotypic and proteomic characterization of multiply antibiotic-resistant variants of Salmonella enterica serovar Typhimurium selected following exposure to disinfectants. *Appl Environ Microbiol,* 74**,** 1508-16.

KARATZAS, K. A., WEBBER, M. A., JORGENSEN, F., WOODWARD, M. J., PIDDOCK, L. J. & HUMPHREY, T. J. 2007. Prolonged treatment of Salmonella enterica serovar Typhimurium with commercial disinfectants selects for multiple antibiotic resistance, increased efflux and reduced invasiveness. *J Antimicrob Chemother,* 60**,** 947-55.

KASTBJERG, V. G. & GRAM, L. 2012. Industrial disinfectants do not select for resistance in Listeria monocytogenes following long term exposure. *Int J Food Microbiol,* 160**,** 11-5.

KAUTZ, M. J., DVORZHINSKIY, A., FRYE, J. G., STEVENSON, N. & HERSON, D. S. 2013. Pathogenicity of dodecyltrimethylammonium chloride-resistant Salmonella enterica. *Appl Environ Microbiol,* 79**,** 2371-6.

KAWAMURA-SATO, K., WACHINO, J., KONDO, T., ITO, H. & ARAKAWA, Y. 2010. Correlation between reduced susceptibility to disinfectants and multidrug resistance among clinical isolates of Acinetobacter species. *J Antimicrob Chemother,* 65**,** 1975-83.

KNAPP, L., RUSHTON, L., STAPLETON, H., SASS, A., STEWART, S., AMEZQUITA, A., MCCLURE, P., MAHENTHIRALINGAM, E. & MAILLARD, J. Y. 2013. The effect of cationic microbicide exposure against Burkholderia cepacia complex (Bcc); the use of Burkholderia lata strain 383 as a model bacterium. *J Appl Microbiol*.

KOLJALG, S., NAABER, P. & MIKELSAAR, M. 2002. Antibiotic resistance as an indicator of bacterial chlorhexidine susceptibility. *J Hosp Infect,* 51**,** 106-13.

KOMLJENOVIC, I., MARQUARDT, D., HARROUN, T. A. & STERNIN, E. 2010. Location of chlorhexidine in DMPC model membranes: a neutron diffraction study. *Chem Phys Lipids,* 163**,** 480-7.

KORAIMANN, G. & WAGNER, M. A. 2014. Social behavior and decision making in bacterial conjugation. *Front Cell Infect Microbiol,* 4**,** 54.

KUMAR, A. & SCHWEIZER, H. P. 2005. Bacterial resistance to antibiotics: active efflux and reduced uptake. *Adv Drug Deliv Rev,* 57**,** 1486-513.

LAI, K. W., FOO, T. L., LOW, W. & NAIDU, G. 2012. Surgical hand antisepsis-a pilot study comparing povidone iodine hand scrub and alcohol-based chlorhexidine gluconate hand rub. *Ann Acad Med Singapore,* 41**,** 12-6.

LARSEN, G. Y., STULL, T. L. & BURNS, J. L. 1993. Marked phenotypic variability in Pseudomonas cepacia isolated from a patient with cystic fibrosis. *J Clin Microbiol,* 31**,** 788-92.

LARSON, E. L. & LAUGHON, B. E. 1987. Comparison of four antiseptic products containing chlorhexidine gluconate. *Antimicrob Agents Chemother,* 31**,** 1572-4.

LEAR, J. C., MAILLARD, J. Y., DETTMAR, P. W., GODDARD, P. A. & RUSSELL, A. D. 2002. Chloroxylenol- and triclosan-tolerant bacteria from industrial sources. *J Ind Microbiol Biotechnol,* 29**,** 238-42.

LEGGETT, M. J., MCDONNELL, G., DENYER, S. P., SETLOW, P. & MAILLARD, J. Y. 2012. Bacterial spore structures and their protective role in biocide resistance. *J Appl Microbiol,* 113**,** 485-98.

LEPAINTEUR, M., ROYER, G., BOURREL, A. S., ROMAIN, O., DUPORT, C., DOUCET-POPULAIRE, F. & DECOUSSER, J. W. 2013. Prevalence of resistance to antiseptics and mupirocin among invasive coagulase-negative staphylococci from very preterm neonates in NICU: the creeping threat? *J Hosp Infect,* 83**,** 333-6.

LEUNG, P., BOOST, M. V. & CHO, P. 2004. Effect of storage temperatures and time on the efficacy of multipurpose solutions for contact lenses. *Ophthalmic Physiol Opt,* 24**,** 218-24.

LEVEQUE, N., RENOIS, F. & ANDREOLETTI, L. 2013. The microarray technology: facts and controversies. *Clin Microbiol Infect,* 19**,** 10-4.

LIPUMA, J. J. 2010. The changing microbial epidemiology in cystic fibrosis. *Clin Microbiol Rev,* 23**,** 299-323.

LOFSTROM, C., HANSEN, F., MANSDAL, S. & HOORFAR, J. 2012. Detection of Salmonella in meat: comparative and interlaboratory validation of a noncomplex and cost-effective pre-PCR protocol. *J AOAC Int,* 95**,** 100-4.

LUNDOV, M. D. & ZACHARIAE, C. 2008. Recalls of microbiologically contaminated cosmetics in EU from 2005 to May 2008. *Int J Cosmet Sci,* 30**,** 471-4.

LUNN, A. D., FABREGA, A., SANCHEZ-CESPEDES, J. & VILA, J. 2010. Prevalence of mechanisms decreasing quinolone-susceptibility among Salmonella spp. clinical isolates. *Int Microbiol,* 13**,** 15-20.

MAHENTHIRALINGAM, E., BALDWIN, A. & DOWSON, C. G. 2008. Burkholderia cepacia complex bacteria: opportunistic pathogens with important natural biology. *J Appl Microbiol,* 104**,** 1539-51.

MAILLARD, J.-Y. & DENYER, S. P. 2009. Emerging bacterial resistance following biocide exposure: should we be concerned? *Chemica Oggi,* 27**,** 26-28.

MAILLARD, J. Y. 2005. Antimicrobial biocides in the healthcare environment: efficacy, usage, policies, and perceived problems. *Ther Clin Risk Manag,* 1**,** 307-20.

MAILLARD, J. Y., BLOOMFIELD, S., COELHO, J. R., COLLIER, P., COOKSON, B., FANNING, S., HILL, A., HARTEMANN, P., MCBAIN, A. J., OGGIONI, M., SATTAR, S., SCHWEIZER, H. P. & THRELFALL, J. 2013. Does microbicide use in consumer products promote antimicrobial resistance? A critical review and recommendations for a cohesive approach to risk assessment. *Microb Drug Resist,* 19**,** 344-54.

MARPLE, B., ROLAND, P. & BENNINGER, M. 2004. Safety review of benzalkonium chloride used as a preservative in intranasal solutions: an overview of conflicting data and opinions. *Otolaryngol Head Neck Surg,* 130**,** 131-41.

MAVRI, A. & SMOLE MOZINA, S. 2013. Development of antimicrobial resistance in Campylobacter jejuni and Campylobacter coli adapted to biocides. *Int J Food Microbiol,* 160**,** 304-12.

MC CAY, P. H., OCAMPO-SOSA, A. A. & FLEMING, G. T. 2010. Effect of subinhibitory concentrations of benzalkonium chloride on the competitiveness of Pseudomonas aeruginosa grown in continuous culture. *Microbiology,* 156**,** 30-8.

MCBAIN, A. J. 2009. *Chapter 4 In vitro biofilm models: An overview*, Elsevier.

MCBAIN, A. J., BARTOLO, R. G., CATRENICH, C. E., CHARBONNEAU, D., LEDDER, R. G. & GILBERT, P. 2003. Effects of triclosan-containing rinse on the dynamics and antimicrobial susceptibility of in vitro plaque ecosystems. *Antimicrob Agents Chemother,* 47**,** 3531-8.

MCCABE, E. M., BURGESS, C. M., WALSH, D., O'REGAN, E., MCGUINNESS, S., BARRY, T., FANNING, S. & DUFFY, G. 2011. Validation of DNA and RNA real-time assays for food

analysis using the hilA gene of Salmonella enterica serovars. *J Microbiol Methods,* 84**,** 19-26.
MCCLEAN, S. & CALLAGHAN, M. 2009. Burkholderia cepacia complex: epithelial cell-pathogen confrontations and potential for therapeutic intervention. *J Med Microbiol,* 58**,** 1-12.
MENSA, B., KIM, Y. H., CHOI, S., SCOTT, R., CAPUTO, G. A. & DEGRADO, W. F. 2011. Antibacterial mechanism of action of arylamide foldamers. *Antimicrob Agents Chemother,* 55**,** 5043-53.
MILES, A. A., MISRA, S. S. & IRWIN, J. O. 1938. The estimation of the bactericidal power of the blood. *J Hyg (Lond),* 38**,** 732-49.
MIYANO, N., OIE, S. & KAMIYA, A. 2003. Efficacy of disinfectants and hot water against biofilm cells of Burkholderia cepacia. *Biol Pharm Bull,* 26**,** 671-4.
MORRISSEY, I., OGGIONI, M. R., KNIGHT, D., CURIAO, T., COQUE, T., KALKANCI, A., MARTINEZ, J. L. & CONSORTIUM, B. 2014. Evaluation of epidemiological cut-off values indicates that biocide resistant subpopulations are uncommon in natural isolates of clinically-relevant microorganisms. *PLoS One,* 9**,** e86669.
NAGAI, K., MURATA, T., OHTA, S., ZENDA, H., OHNISHI, M. & HAYASHI, T. 2003. Two different mechanisms are involved in the extremely high-level benzalkonium chloride resistance of a Pseudomonas fluorescens strain. *Microbiol Immunol,* 47**,** 709-15.
NAPARSTEK, L., CARMELI, Y., CHMELNITSKY, I., BANIN, E. & NAVON-VENEZIA, S. 2012. Reduced susceptibility to chlorhexidine among extremely-drug-resistant strains of Klebsiella pneumoniae. *J Hosp Infect,* 81**,** 15-9.
NIKAIDO, H. & PAGES, J. M. 2012. Broad-specificity efflux pumps and their role in multidrug resistance of Gram-negative bacteria. *FEMS Microbiol Rev,* 36**,** 340-63.
NIKAIDO, H. & ZGURSKAYA, H. I. 2001. AcrAB and related multidrug efflux pumps of Escherichia coli. *J Mol Microbiol Biotechnol,* 3**,** 215-8.
NISHIHARA, T., OKAMOTO, T. & NISHIYAMA, N. 2000. Biodegradation of didecyldimethylammonium chloride by Pseudomonas fluorescens TN4 isolated from activated sludge. *J Appl Microbiol,* 88**,** 641-7.
OJEIL, M., JERMANN, C., HOLAH, J., DENYER, S. P. & MAILLARD, J. Y. 2013. Evaluation of new in vitro efficacy test for antimicrobial surface activity reflecting UK hospital conditions. *J Hosp Infect,* 85**,** 274-81.
OTTER, J. A., YEZLI, S. & FRENCH, G. L. 2012. Impact of the suspending medium on susceptibility of meticillin-resistant Staphylococcus aureus to hydrogen peroxide vapour decontamination. *J Hosp Infect,* 82**,** 213-5.
PAGEDAR, A., SINGH, J. & BATISH, V. K. 2011. Efflux mediated adaptive and cross resistance to ciprofloxacin and benzalkonium chloride in Pseudomonas aeruginosa of dairy origin. *J Basic Microbiol,* 51**,** 289-95.
PAGEDAR, A., SINGH, J. & BATISH, V. K. 2012. Adaptation to benzalkonium chloride and ciprofloxacin affects biofilm formation potential, efflux pump and haemolysin activity of Escherichia coli of dairy origin. *J Dairy Res,* 79**,** 383-9.
PATTON, W. F. 2002. Detection technologies in proteome analysis. *J Chromatogr B Analyt Technol Biomed Life Sci,* 771**,** 3-31.
PEARCE, H., MESSAGER, S. & MAILLARD, J. Y. 1999. Effect of biocides commonly used in the hospital environment on the transfer of antibiotic-resistance genes in Staphylococcus aureus. *J Hosp Infect,* 43**,** 101-7.
PEETERS, E., NELIS, H. J. & COENYE, T. 2008. Evaluation of the efficacy of disinfection procedures against Burkholderia cenocepacia biofilms. *J Hosp Infect,* 70**,** 361-8.
PFAFFL, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res,* 29**,** e45.
PIDDOCK, L. J. 2006a. Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. *Clin Microbiol Rev,* 19**,** 382-402.

PIDDOCK, L. J. 2006b. Multidrug-resistance efflux pumps - not just for resistance. *Nat Rev Microbiol,* 4**,** 629-36.
POPOWSKA, M. & KRAWCZYK-BALSKA, A. 2013. Broad-host-range IncP-1 plasmids and their resistance potential. *Front Microbiol,* 4**,** 44.
PORTEVIN, D., DE SOUSA-D'AURIA, C., HOUSSIN, C., GRIMALDI, C., CHAMI, M., DAFFE, M. & GUILHOT, C. 2004. A polyketide synthase catalyzes the last condensation step of mycolic acid biosynthesis in mycobacteria and related organisms. *Proc Natl Acad Sci U S A,* 101**,** 314-9.
RANDALL, L. P., COOLES, S. W., COLDHAM, N. G., PENUELA, E. G., MOTT, A. C., WOODWARD, M. J., PIDDOCK, L. J. & WEBBER, M. A. 2007. Commonly used farm disinfectants can select for mutant Salmonella enterica serovar Typhimurium with decreased susceptibility to biocides and antibiotics without compromising virulence. *J Antimicrob Chemother,* 60**,** 1273-80.
RANDALL, L. P., COOLES, S. W., PIDDOCK, L. J. & WOODWARD, M. J. 2004. Effect of triclosan or a phenolic farm disinfectant on the selection of antibiotic-resistant Salmonella enterica. *J Antimicrob Chemother,* 54**,** 621-7.
RICKE, S. C., KHATIWARA, A. & KWON, Y. M. 2013. Application of microarray analysis of foodborne Salmonella in poultry production: a review. *Poult Sci,* 92**,** 2243-50.
ROSE, H., BALDWIN, A., DOWSON, C. G. & MAHENTHIRALINGAM, E. 2009. Biocide susceptibility of the Burkholderia cepacia complex. *J Antimicrob Chemother,* 63**,** 502-10.
RUSHDY, A. A., MABROUK, M. I., ABU-SEF, F. A., KHEIRALLA, Z. H., ABDEL-ALL, S. M. & SALEH, N. M. 2013. Contribution of different mechanisms to the resistance to fluoroquinolones in clinical isolates of Salmonella enterica. *Braz J Infect Dis,* 17**,** 431-7.
RUSHTON, L., SASS, A., BALDWIN, A., DOWSON, C. G., DONOGHUE, D. & MAHENTHIRALINGAM, E. 2013. Key role for efflux in the preservative susceptibility and adaptive resistance of Burkholderia cepacia complex bacteria. *Antimicrob Agents Chemother,* 57**,** 2972-80.
RUSSELL, A. D. 2004. Bacterial adaptation and resistance to antiseptics, disinfectants and preservatives is not a new phenomenon. *J Hosp Infect,* 57**,** 97-104.
RUSSELL, A. D. & MCDONNELL, G. 2000. Concentration: a major factor in studying biocidal action. *J Hosp Infect,* 44**,** 1-3.
RUTALA, W. A. & WEBER, D. J. 2007. How to assess risk of disease transmission to patients when there is a failure to follow recommended disinfection and sterilization guidelines. *Infect Control Hosp Epidemiol,* 28**,** 146-55.
SAIDIJAM, M., BENEDETTI, G., REN, Q., XU, Z., HOYLE, C. J., PALMER, S. L., WARD, A., BETTANEY, K. E., SZAKONYI, G., MEULLER, J., MORRISON, S., POS, M. K., BUTAYE, P., WALRAVENS, K., LANGTON, K., HERBERT, R. B., SKURRAY, R. A., PAULSEN, I. T., O'REILLY, J., RUTHERFORD, N. G., BROWN, M. H., BILL, R. M. & HENDERSON, P. J. 2006. Microbial drug efflux proteins of the major facilitator superfamily. *Curr Drug Targets,* 7**,** 793-811.
SASS, A., MARCHBANK, A., TULLIS, E., LIPUMA, J. J. & MAHENTHIRALINGAM, E. 2011. Spontaneous and evolutionary changes in the antibiotic resistance of Burkholderia cenocepacia observed by global gene expression analysis. *BMC Genomics,* 12**,** 373.
SASS, A. M., SCHMERK, C., AGNOLI, K., NORVILLE, P. J., EBERL, L., VALVANO, M. A. & MAHENTHIRALINGAM, E. 2013. The unexpected discovery of a novel low-oxygen-activated locus for the anoxic persistence of Burkholderia cenocepacia. *ISME J,* 7**,** 1568-81.
SAWASDIDOLN, C., TAWEECHAISUPAPONG, S., SERMSWAN, R. W., TATTAWASART, U., TUNGPRADABKUL, S. & WONGRATANACHEEWIN, S. 2010. Growing Burkholderia

pseudomallei in biofilm stimulating conditions significantly induces antimicrobial resistance. *PLoS One,* 5**,** e9196.

SCENIHR 2009. Research strategy to address the knowledge gaps on the antimicrobial resistance effects of biocides. Available online http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_028.pdf accessed 11/03/2014.

SCENIHR 2010. Scientific Committee on Emerging and Newly Identified Health Risks: Research strategy to address the knowledge gaps on the antimicrobial resistance effects of biocides. Available online http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_028.pdf accessed 11/03/2014.

SCHUURMANS, J. M., NURI HAYALI, A. S., KOENDERS, B. B. & TER KUILE, B. H. 2009. Variations in MIC value caused by differences in experimental protocol. *J Microbiol Methods,* 79**,** 44-7.

SKOVGAARD, S., NIELSEN, L. N., LARSEN, M. H., SKOV, R. L., INGMER, H. & WESTH, H. 2013. Staphylococcus epidermidis isolated in 1965 are more susceptible to triclosan than current isolates. *PLoS One,* 8**,** e62197.

SMITH, K. & HUNTER, I. S. 2008. Efficacy of common hospital biocides with biofilms of multi-drug resistant clinical isolates. *J Med Microbiol,* 57**,** 966-73.

SMITH, K., ROBERTSON, D. P., LAPPIN, D. F. & RAMAGE, G. 2013. Commercial mouthwashes are ineffective against oral MRSA biofilms. *Oral Surg Oral Med Oral Pathol Oral Radiol,* 115**,** 624-9.

SOUSA, S. A., RAMOS, C. G. & LEITAO, J. H. 2011. Burkholderia cepacia Complex: Emerging Multihost Pathogens Equipped with a Wide Range of Virulence Factors and Determinants. *Int J Microbiol,* 2011.

SVENSATER, G., WELIN, J., WILKINS, J. C., BEIGHTON, D. & HAMILTON, I. R. 2001. Protein expression by planktonic and biofilm cells of Streptococcus mutans. *FEMS Microbiol Lett,* 205**,** 139-46.

SVETLIKOVA, Z., SKOVIEROVA, H., NIEDERWEIS, M., GAILLARD, J. L., MCDONNELL, G. & JACKSON, M. 2009. Role of porins in the susceptibility of Mycobacterium smegmatis and Mycobacterium chelonae to aldehyde-based disinfectants and drugs. *Antimicrob Agents Chemother,* 53**,** 4015-8.

TABAK, M., SCHER, K., HARTOG, E., ROMLING, U., MATTHEWS, K. R., CHIKINDAS, M. L. & YARON, S. 2007. Effect of triclosan on Salmonella typhimurium at different growth stages and in biofilms. *FEMS Microbiol Lett,* 267**,** 200-6.

TABOR, C. W. & TABOR, H. 1984. Polyamines. *Annu Rev Biochem,* 53**,** 749-90.

TANDUKAR, M., OH, S., TEZEL, U., KONSTANTINIDIS, K. T. & PAVLOSTATHIS, S. G. 2013. Long-term exposure to benzalkonium chloride disinfectants results in change of microbial community structure and increased antimicrobial resistance. *Environ Sci Technol,* 47**,** 9730-8.

TATTAWASART, U., MAILLARD, J. Y., FURR, J. R. & RUSSELL, A. D. 1999. Development of resistance to chlorhexidine diacetate and cetylpyridinium chloride in Pseudomonas stutzeri and changes in antibiotic susceptibility. *J Hosp Infect,* 42**,** 219-29.

THOMAS, L., MAILLARD, J. Y., LAMBERT, R. J. & RUSSELL, A. D. 2000. Development of resistance to chlorhexidine diacetate in Pseudomonas aeruginosa and the effect of a "residual" concentration. *J Hosp Infect,* 46**,** 297-303.

THOMAS, L., RUSSELL, A. D. & MAILLARD, J. Y. 2005. Antimicrobial activity of chlorhexidine diacetate and benzalkonium chloride against Pseudomonas aeruginosa and its response to biocide residues. *J Appl Microbiol,* 98**,** 533-43.

TORBECK, L., RACCASI, D., GUILFOYLE, D. E., FRIEDMAN, R. L. & HUSSONG, D. 2011. Burkholderia cepacia: This Decision Is Overdue. *PDA J Pharm Sci Technol,* 65**,** 535-43.

VAISHNAVI, C., SINGH, S., KOCHHAR, R., BHASIN, D., SINGH, G. & SINGH, K. 2005. Prevalence of Salmonella enterica serovar typhi in bile and stool of patients with biliary diseases and those requiring biliary drainage for other purposes. *Jpn J Infect Dis,* 58**,** 363-5.

VAN OUDENHOVE, L. & DEVREESE, B. 2013. A review on recent developments in mass spectrometry instrumentation and quantitative tools advancing bacterial proteomics. *Appl Microbiol Biotechnol,* 97**,** 4749-62.

VANLAERE, E., BALDWIN, A., GEVERS, D., HENRY, D., DE BRANDT, E., LIPUMA, J. J., MAHENTHIRALINGAM, E., SPEERT, D. P., DOWSON, C. & VANDAMME, P. 2009. Taxon K, a complex within the Burkholderia cepacia complex, comprises at least two novel species, Burkholderia contaminans sp. nov. and Burkholderia lata sp. nov. *Int J Syst Evol Microbiol,* 59**,** 102-11.

VICKERY, K., DEVA, A., JACOMBS, A., ALLAN, J., VALENTE, P. & GOSBELL, I. B. 2012. Presence of biofilm containing viable multiresistant organisms despite terminal cleaning on clinical surfaces in an intensive care unit. *J Hosp Infect,* 80**,** 52-5.

WANG, S., PHILLIPPY, A. M., DENG, K., RUI, X., LI, Z., TORTORELLO, M. L. & ZHANG, W. 2010. Transcriptomic responses of Salmonella enterica serovars Enteritidis and Typhimurium to chlorine-based oxidative stress. *Appl Environ Microbiol,* 76**,** 5013-24.

WEBBER, M. A., RICCI, V., WHITEHEAD, R., PATEL, M., FOOKES, M., IVENS, A. & PIDDOCK, L. J. 2013. Clinically relevant mutant DNA gyrase alters supercoiling, changes the transcriptome, and confers multidrug resistance. *MBio,* 4.

WHITE, A. P., GIBSON, D. L., GRASSL, G. A., KAY, W. W., FINLAY, B. B., VALLANCE, B. A. & SURETTE, M. G. 2008. Aggregation via the red, dry, and rough morphotype is not a virulence adaptation in Salmonella enterica serovar Typhimurium. *Infect Immun,* 76**,** 1048-58.

WHITE, A. P., GIBSON, D. L., KIM, W., KAY, W. W. & SURETTE, M. G. 2006. Thin aggregative fimbriae and cellulose enhance long-term survival and persistence of Salmonella. *J Bacteriol,* 188**,** 3219-27.

WHITE, D. G. & MCDERMOTT, P. F. 2001. Biocides, drug resistance and microbial evolution. *Curr Opin Microbiol,* 4**,** 313-7.

WHITE, J., GILBERT, J., HILL, G., HILL, E., HUSE, S. M., WEIGHTMAN, A. J. & MAHENTHIRALINGAM, E. 2011. Culture-independent analysis of bacterial fuel contamination provides insight into the level of concordance with the standard industry practice of aerobic cultivation. *Appl Environ Microbiol,* 77**,** 4527-38.

WHITEHEAD, R. N., OVERTON, T. W., KEMP, C. L. & WEBBER, M. A. 2011. Exposure of Salmonella enterica serovar Typhimurium to high level biocide challenge can select multidrug resistant mutants in a single step. *PLoS One,* 6**,** e22833.

WONG, H. S., TOWNSEND, K. M., FENWICK, S. G., MAKER, G., TRENGOVE, R. D. & O'HANDLEY, R. M. 2010a. Comparative susceptibility of Salmonella Typhimurium biofilms of different ages to disinfectants. *Biofouling,* 26**,** 859-64.

WONG, H. S., TOWNSEND, K. M., FENWICK, S. G., TRENGOVE, R. D. & O'HANDLEY, R. M. 2010b. Comparative susceptibility of planktonic and 3-day-old Salmonella Typhimurium biofilms to disinfectants. *J Appl Microbiol,* 108**,** 2222-8.

WONG, S., STREET, D., DELGADO, S. I. & KLONTZ, K. C. 2000. Recalls of foods and cosmetics due to microbial contamination reported to the U.S. Food and Drug Administration. *J Food Prot,* 63**,** 1113-6.

YOON, H. J., CHO, S. H. & KIM, S. H. 2009. A case of multidrug-resistant Salmonella enterica serovar Typhi treated with a bench to bedside approach. *Yonsei Med J,* 50**,** 147-51.

YUAN, Y., LEE, R. E., BESRA, G. S., BELISLE, J. T. & BARRY, C. E., 3RD 1995. Identification of a gene involved in the biosynthesis of cyclopropanated mycolic acids in Mycobacterium tuberculosis. *Proc Natl Acad Sci U S A,* 92**,** 6630-4.

ZHANG, D. F., LI, H., LIN, X. M., WANG, S. Y. & PENG, X. X. 2011. Characterization of outer membrane proteins of Escherichia coli in response to phenol stress. *Curr Microbiol,* 62**,** 777-83.

Publications

Knapp L, Rushton L, Stapleton H, Sass A, Stewart S, Amezquita A, McClure P, Mahenthiralingam E, Maillard J-Y (2013). The effect of cationic microbicide exposure against *Burkholderia cepacia* complex (Bcc); the use of *Burkholderia lata* strain 383 as a model bacterium. doi: 10.1111/jam.12320

Biocide exposure results in reduced antibiotic susceptibility in *Burkholderia lata* that may be due to up-regulation of efflux- associated genes. Society for Applied Microbiology (SFAM) summer meeting, Dublin 2011. Poster presentation.

The effect of cationic microbicide exposure against *Burkholderia cepacia* complex (Bcc); the use of *Burkholderia lata* strain 383 as a model bacterium. Microbiology & Infection Translational Research Group (MITREG) conference, Cardiff 2012.

Bacterial resistance to biocides: development of a predictive protocol. SFAM summer meeting, Edinburgh 2012 & Clostnet spring meeting, Nottingham 2013. Poster presentation.

Bacterial resistance to biocides: development of a predictive protocol. American Society of Microbiology AGM, Denver 2013. Poster presentation.

Appendix

All appendix data is stored on the attached CD. CD contents are as follows.

Chapter three appendix data

- Microarray
  - RAW DATA
    - SL1344 0.0004 % CHG
    - SL1344 0.0004 % BZC
    - 14028S 0.0001 % CHG
    - 14028S 0.0004 % BZC
  - Analysed data with a cut off p value of 0.01
    - SL1344 0.0004 % CHG
    - SL1344 0.0004 % BZC
    - 14028S 0.0001 % CHG
    - 14028S 0.0004 % BZC
  - List of all genes in microarray
  - Concentration and purity of RNA extracted

- Real-time PCR
  - Contains standard curves and fold change calculations for all *Salmonella* genes tested

Chapter four appendix data

- Real-time PCR
  - Contains standard curves and fold change calculations for all *Burkholderia* genes tested

Chapter five appendix data

- Contains tables 5.11 – 5.20
- Contains MIC, MBC and antibiotic susceptibility values before and after exposure of strains SL1344, 14028S, *K. pneumoniae, B. cenocepacia* & *Ps. aeruginosa* to Dermax shampoo, Corsodyl mouthwash, CHG or BZC.

Copy of: Knapp L, *et al*. (2013). The effect of cationic microbicide exposure against *Burkholderia cepacia* complex (Bcc); the use of *Burkholderia lata* strain 383 as a model bacterium. doi: 10.1111/jam.12320