THE EFFECT OF BIOCIDAL RESIDUES ON RESISTANCE PHENOTYPE IN ESCHERICHIA COLI

Thesis presented for the Degree of Master of Philosophy

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SUMMARY

Antimicrobial resistance (AMR) poses a threat to worldwide health, in particular in relation to multi-drug resistant organisms. Hygienic cleaning and disinfection can contribute in the prevention of AMR. There is ample evidence to support the use of disinfectants (biocides) in the decrease of healthcare acquired infections (HCAIs) (Weinstein and Hota, 2004, Maillard, 2018, Webber *et al*., 2015). However, there is also evidence of instances where disinfectant efficacy may be impeded resulting in microbial survival and emerging resistance (A Rutala and J Weber, 2007). Biocides are said to act as a selective pressure that encourages the acquisition of resistance traits in bacterial cells (Qiu *et al*., 2012). Furthermore, selective pressure may result from the overexposure of very low concentrations of biocides over long periods of time (Andersson *et al*., 2012, Gullberg *et al*., 2014, Gullberg *et al*., 2011, Thomas *et al*., 2000). Some biocidal products make claims of "residual biocidal activity" whereas efficacy is usually imparted to a much higher concentration. Some microbial populations may survive exposure to low biocide concentrations, and show decreased susceptibility or resistance to a biocide or consequentially other antimicrobials.

This study aims to understand differences between bacterial selection and adaptation in *Escherichia coli* following exposure to realistic residual - during use - chlorhexidine (CHX) or benzalkonium chloride (BZC) concentrations. It was hypothesised that exposure to a high sub-biocide minimum inhibitory concentration (MIC) would exert a selective pressure enabling the least susceptible bacteria to survive resulting in a permanent change of susceptibility phenotype, whereas a low sub-MIC would be conducive to reactive metabolic shifts resulting in a transient change of susceptibility phenotype.

Baseline biocide (CHX and BZC) and antibiotic susceptibility of *E. coli* isolates was obtained using a standard micro-dilution broth protocol, and EUCAST protocol. “Residual” CHX concentration left on surface over a 168 hours period was measured by HPLC. The impact of a range of biocide concentrations (including residual CHX ones) on growth kinetics was investigated. Any changes in susceptibility profile was assessed for stability. Efflux activity and metabolic regulation during exposure to low and high sub-CHX MIC were investigated aiming to identify a link with observed changes in susceptibility phenotype. Finally the propensity for different levels of CHX exposure to influence genetic transfer via conjugation was explored.

It was demonstrated that a 0.006 ± 0.002 mg/mL is a realistic residual - during use exposure concentration of CHX. This concentration is 99% lower than the concentration initially applied (20 mg/mL). At this residual concentration, it was possible for CHX susceptible bacteria to survive the disinfection process. Five genotypically distinct strains (UCD-CFS ECP-1L3, UCD-CFS ECP-1L4, UCD-CFS ECP-1B2, UCD-CFS ECP-13P5, UCD-CFS ECP-13P4) demonstrated survival after a 5 min but not 24 hours CHX exposure. Surviving bacteria demonstrated elevated MIC and MBC values; the highest fold change was 32-fold (MIC) and 62-fold (MBC). The elevated MIC values obtained were higher than the average concentration of CHX found on surface. Decreases in MIC or MBC values were observed after residual BZC exposure. No stable changes in MIC and MBC were observed after exposure to residual CHX or BZC, but stable changes were observed for antibiotic resistance for amoxicillin/clavulanic acid, ampicillin, cefpodoxime and cephalothin. Efflux activity was observed during exposure to low (0.00005 mg/mL) but not for high (0.002mg/mL) sub-CHX and sub-BZC MIC. It was demonstrated that changes in susceptibility coincided with changes in the ability to metabolise certain substrates including salicin, L-alanine, betain, creatanine and

phenylethlalamine. These substances were linked to cell wall and stress signalling regulatory processes. It was surmised that *E. coli* was able to adapt through metabolic alterations to produce transient changes in CHX susceptibility and stable changes in antibiotic susceptibility. Furthermore, our results show that a transiently adapted population may be selected amongst less tolerant sub-populations at the established CHX-during use concentration.

Overall, this work suggests that the intended application concentration of a biocide may in fact be lower than the MIC of target organisms. It is concluded that residual concentrations of biocides do have the potential to drive resistance, particularly stable cross-resistance to antibiotics, through prolonged exposure to low level during use concentrations, driving metabolic modifications of the cell envelope. The potential risk of cross-resistance warrants further investigation.

LIST OF ABBRIVIATIONS

ABC Adenosine Triphosphate Binding Cassette

AG Augmentin

AK amikacin

AMC Amoxicillin/clavulanic acid

AMP Ampicillin

AMR Antimicrobial Resistance

ANOVA Analysis of Variance

A/S ampicillin/sulbactam

ASTM American Society for testing & materials

ATP Adenosine Triphosphate

AZ aztreonam

BPR Biocidal Product Regulation

BSAC British Society for Antimicrobial Chemotherapy

BZC Benzalkonium Chloride

C Chloramphenicol

CAZ Ceftazidime

CCCP Carbonyl cyanide *m*-chlorophenylhydrazone

CDF Cation Diffusion Facilitator

CFU Colony Forming Units

CHX Chlorhexidine digluconate

CIP Ciprofloxacin

CN Gentamicin

CLSI Clinical Laboratory Standards Institute

CPD Cefpodoxime

CTX Cefotaxime

D-ala D-alanine

di$H_2$0 Deionised Water

EARSS European Antimicrobial Resistance Surveillance System

ECHA European Chemical Agency

ECOFF Ecological Cut-off

EPI Efflux Pump Inhibitor

ESBL Extended-Spectrum Beta-Lactamase

EtBr Ethidium Bromide

EU European Union

EUCAST European Committee Antimicrobial Susceptibility Testing

FU Cefuroxime

FZ Cefazoline

HAI Hospital Acquired Infection

HELICS Hospitals in Europe Link for Infection Control through Surveillance

HGT Horizontal Gene Transfer

HPA Health Protection Agency

HPLC High-Performance Liquid Chromatography

IF Inoculating Fluid

IMP Imipenem

KEGG Kyoto Encyclopedia of Genes and Genomes

L-ala L-Alanine

LE Lag Extension

LEV levofloxacin

LPS Lipopolysaccharide

MATE Multidrug and Toxic Compound Extrusion

MBC Minimum Bactericidal Concentration

MDR Multiple Drug Resistance

MFS Multidrug Facilitator Superfamily

MGEs Mobile GElements

MIC Minimum Inhibitory Concentration

MSC Minimal Selective Concentration

MYSTIC Meropenem Yearly Susceptibility Test Information Collection

MW Molecular Weight

NOR Norfloxacin

OD Optical Density

OM Outer Membrane

OMP Outer Membrane Protein

PaβN Phenyl-arginine-β-napthylamide

PACE Proteobacterial Antimicrobial Compound Efflux

PBS Phosphate Buffered Saline

PIM Cefepime

PHE Public Health England

PM Phenotype Microarray

PMF Proton Motive Force

PR Piperacillin

QAC Quaternary Ammonium Compound

RND Resistance Nodule Division

SCENIHR Scientific Committee Emerging Newly Identified Health Risk

SD Standard Deviation

SMR Small Multidrug Resistance

SNP Small Nucleotide Polymorphism

SXT trimethoprim-sulfamethoxazole

T Transmitence

TB Tobramycin

TE Tetracycline

VBNC Viable But Not Countable

VGT Vertical Gene Transfer

W Trimethoprim

GLOSSARY

Biocide: a chemical agent that kills living organisms (Maillard, 2005)

Biocidal product: A biocidal product is any substance or mixture with the intention of preventing the action of, or otherwise exerting a controlling effect on, any harmful organism (BPR (EU) No. 528/2012; https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:62010CC0420)

Biocide resistance: a shift 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)

Biocide tolerance: Transient withstanding of exposure to toxic concentrations that would have otherwise been fatal (e.g. as a result of slowing in metabolic processes) (Trastoy *et al*., 2018)

Biocide persistence: Transient withstanding of exposure to otherwise fatal toxic concentrations, persistence is usually characterised by a subpopulation of tolerant cells as opposed to an entire tolerant population.

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

Phenotype: V**i**able and behavioral characteristic of an organism

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CHAPTER 1: GENERAL INTRODUCTION

1. INTRODUCTION

1.1. Background – antimicrobial resistance today

Antimicrobial resistance (AMR) is a natural phenomenon that occurs as a result of spontaneous genetic changes that result in microbes becoming less sensitive to antimicrobials (WHO, 2015). The year 2018 marked the end of a 5-year Public Health England (PHE) led collaborative initiative to i) improve knowledge and understanding of antimicrobial resistance (AMR), ii) preserve the efficacy of pre-existing treatments and iii) develop new therapies to replace those that are no longer effective (Department of Health and Department for Environment Food and Rural Affairs, UK; 2013; DH, PHE, Defra, and Veterinary Medicines Directorate, 2014). This initiative is preceded by a comprehensive review written by Jim O'Neill (2014), which outlines the global AMR crisis. O'Neill estimated that in the US and Europe alone, resistant infections cause at least 50,000 deaths per year. Exacerbated by this fact is the ever-impending decrease in the efficacy of our first line treatments due to the lack of regulated antibiotic stewardship. The world health organization (O'Neill, 2016) stated, "a high percentage of hospital-acquired infections are caused by highly resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) or multidrug-resistant Gram-negative bacteria". The annual health protection report published in 2015 by Public Health England (PHE) noted that the incidence of *Esherichia coli* bacteraemia increased by 16.7% from 2008 to 2015. Perhaps more worryingly, the report stated that the percentage of non-susceptibility of *E. coli* isolates to the antibiotic amoxicillin/clavulanate increased from 25.2% in 2010 to 40.9% in 2014 (PHE, 2014). Additionally, the World Health Organisation (WHO, 2015) stated that resistance to fluoroquinolone antibiotics is increasingly widespread with countries where ineffective treatment accounts for more than half of the patients. The European Union summary report on antimicrobial resistance (ECDC, 2019) collated

epidemiological cut-off values (ECOFF) data from European countries in 2017 and reported that percentage resistance values were high in fattening pigs (food producing animals) for tetracycline (52%), sulfamethoxazole (42%), ampicillin (38.5%) and trimethoprim (32.2%). As food producing animals act as a source of transmission of AMR organisms (Thanner *et al*., 2016) these data are valuable. The presence of antimicrobial drugs in farming and agriculture, food production, and healthcare all contribute to the perpetuation of the AMR crisis (O'Neill, 2016). Moreover, the use of biocidal products such as disinfectants and antiseptic formulations may also act to drive microbial resistance in similar modes to antibiotics (SCENIHR, 2010, Furi *et al*., 2016, Maillard, 2013, Oggioni *et al*., 2013). This use of biocides, their modes of action and their contribution to possible resistance will be discussed further in the next sections.

1.2. Biocidal products application

The word *biocide* means to "kill any living thing". This is a broad term that can often be misleading, as biocidal products are often efficacious or even targeted specifically against bacteriophage and virus. This thesis will refer to an active compound (non-formulated product) that is toxic to bacteria as either a "biocide" (kills bacteria) or a "biocidal product" (formulated product). For this thesis, the term biocidal product (a biocide that is intended for commercial use) encompasses chemicals and formulations that are disinfectants, preservatives and antiseptics with activity against all microorganisms. It excludes chemotherapeutic antibiotics (Maillard, 2013). The use of biocides have a dated history, as seen with the early use of salts and alcohol as food preservatives and antiseptics (Fraise *et al*., 2015). Biocides are used for the disinfection of medical device and surfaces, preservation of industrial and consumer products and are even incorporated into domestic and

personal hygiene products such as toothpaste and shampoo **(Table 1.1**). The use of biocides in healthcare settings has been established since the 1800's. The development of cationic biocides such as biguanides, quaternary ammonium compounds, phenolics, aldehydes and peroxygens lead to the increased applications of these in clinical antisepsis, disinfection and sterilisation (Russell, 2000, Maillard, 2005). The active chemical compounds within biocides commonly have a broad range of target sites (Denyer, 1995) (**Table 1.1**) and most are effective against a wide range of microorganisms, although efficacy depends on the chemistry of the biocide and the biology of the organism to be treated (Maillard, 2002). Gram-negative bacteria are less susceptible to some biocides due to the protection that the outer cell wall provides in comparison to Gram-positive bacteria (Denyer and Maillard, 2002). The same biocide may also be used effectively against different target organisms at differing concentrations (Sheldon, 2005). For example, CHX is bactericidal against most Gram-positive microorganisms at low concentrations, at high concentrations however it becomes effective against Gram-negative organisms too. Due to the wide range of organisms targeted and their in-use efficacy, biocides have become the primary method of controlling healthcare associated infections (HCAIs) within clinical settings.

Table 1.1 Biocidal compounds and their applications, microbial target site and mechanism of action.

Table adapted from Maillard JY, 2002, "Bacterial target sites for biocide action". Journal of Applied Microbiology, 92: 16S–27S. doi:10.1046/j.1365-2672.92.5s1.3.x

Type of biocide	Example	Application	Target site	Mechanism of action
Quaternary ammonium compounds	Benzalkonim chloride	Healthcare, domestic products, food production, pharmaceutical industry	Cell membrane, cytoplasmic membrane, cytoplasmic constituents	Increased permeability, membrane potential, electron transport chain, enzyme inhibition,
Biguanides	Chlorhexidine digluconate	Healthcare, domestic products	Cell membrane, cytoplasmic membrane, cytoplasmic constituents	Increased permeability, Adenosine triphosphate synthesis, enzyme inhibition, coagulation
Phenolic compounds	Triclosan	Healthcare, cosmetic products, domestic products, pharmaceutical industry	Cell membrane, cytoplasmic membrane, cytoplasmic constituents	Increased permeability, membrane potential, electron transport chain, enzyme inhibition, coagulation
Chlorine compounds	Sodium hypochlorite	Healthcare, Water treatment, domestic products	Cell membrane, cytoplasmic constituents, interaction with specific groups	Increased permeability, nucleic acid, thiol groups, sulphydryl groups
Peroxide compounds	Hydrogen peroxide	Healthcare, domestic products, Water treatment	Cytoplasmic constituents, interaction with specific groups, biocide-induced autocidal activity	Ribosomes, thiol groups, sulphydryl groups, accumulation of free radicals

1.3. Factors effecting biocidal efficacy and the relevance of residual activity claims

In order for a biocide to be effective, potential factors that hinder the delivery and activity of the active chemical compound should be minimised. In the case of ineffective biocide use, pathogenic microorganisms may persist and subsequently spread (Ramm *et al*., 2015, Tuladhar *et al*., 2012). There are several factors that can affect the efficacy of a biocide (**Table 1.2**)(Maillard, 2002). There are external factors such as active concentration, contact time, interfering substances, temperature and pH, which can all reduce or quench the activity of a biocide (Sheldon, 2005). Alternatively, biofilm formation and the number of organisms present are among some of the difficult biological factors that hinder the activity of a biocide. If the number of viable bacterial cells left after disinfection are not sufficiently reduced or inactivated, the remaining survivors have potential to adapt under selective pressure, in turn, this adaptive process gives rise to the development of antimicrobial resistance (Qiu *et al*., 2012). There is ample evidence to support the use of disinfectants in the decrease of HCAIs (Weinstein and Hota, 2004, Maillard, 2018, Webber *et al*., 2015). However, there is also evidence of instances where efficacy may be impeded resulting in potential failure (A Rutala and J Weber, 2007).

Table 1.2 The factors affecting the efficacy of a biocide

Maillard JY, 2005, "Antimicrobial biocides in the healthcare environment: efficacy, usage, policies, and perceived problems". *Therapeutics and Clinical Risk Management*. 2005;1(4):307-320.

Factors	Comments
Factors inherent to the biocide	
Concentration	Understand the concentration exponent (i.e., the effect of dilution upon activity)
Contact time	Longer contact time often associated with increased activity
Organic load	Quench the activity of a biocide or protect microorganisms
Formulation	Possible inactivation of biocide
Temperature	Important for some devices (e.g., endoscope washer)
pH	Affect both the biocide (stability and ionisation) and the microorganism (growth and electric charge)
Factors inherent to the cell	
Presence of biofilm	Dormant "persister" cells difficult to eradicate. Likely to be present on equipment, certain surfaces
Type of microorganisms	Will affect the choice of the agent to use. Bacterial spores: the most resistant; envelope viruses: the least resistant
Number of microorganisms	High number more difficult to eradicate

1.3.1. Concentration

One impinging factor that determines the efficacy of a biocide is the in-use concentration of the active chemical compound; this can be demonstrated using the "concentration exponent" (**η**) (Maillard, 2005; Ioannou *et al*., 2007). The concentration exponent describes the association of dilution and activity of a biocide and is calculated by the time taken to kill a pre-determined amount of a population at certain concentrations (Ioannou *et al*., 2007). Generally, biocides with a high concentration exponent are more susceptible to a decrease of activity upon dilution; those with low concentration exponent (i.e. biguanides and quaternary ammonium compounds) are less affected by dilution (Denyer and Stewart, 1998). A biocide can be used for different applications depending on the concentration of which it is used. An example of this is the biguanide chlorhexidine, it is used for the liquid disinfection of surfaces at 0.5%-4% (v/v), for antiseptics such as hand soaps at 0.2%-4% (v/v) and for the preservation of products at a concentration of 0.0025%-0.01% (v/v) (Larson and Laughon, 1987, Maillard, 2005). Although a high concentration can be the most efficacious, a balance between how well it works to kill bacteria and human toxicity of the chemical has to be found. For this reason there is a plethora of commercial and industrial products that claim efficacy and low toxicity with the application of low concentrations of a biocidal product. Although some biocides may be effective at low concentrations, further dilution of the product may end in a concentration below that which is required to kill most bacteria. This may result in sub-lethal concentrations of biocide and a proportion of a bacterial population that may survive the treatment (McDonnell and Russell, 1999a) It has been suggested that the use of even extremely low concentrations of biocides may potentially be adding to the proliferation of harmful microorganisms that carry resistance to both commonly used biocides and antibiotics(Gullberg *et al*., 2014, Gullberg *et al*., 2011, Maillard, 2013).

1.3.2. Contact time

Contact time is an underpinning factor when considering the efficacy of a biocide. Longer contact times are associated with increased activity, however the contact time required to kill bacteria varies for each biocide and is often dependent on the concentration of the active compound.

Prolonged exposure of bacteria to low concentrations of a biocide has been reported to result in the development of biocidal resistance. Wesgate *et al*. (2016) reported that after a 24 hour exposure of *E. coli* to triclosan at a sub-lethal concentration (0.0004%), there was a 32-fold increase in the minimal inhibitory concentration (MIC) that is the lowest concentration that is required to inhibit growth of the bacteria. Another study reported reduced susceptibility to triclosan, chlorhexidine diacetate and benzalkonium chloride (BZC) in *Campylobacter* spp. after repeated exposure to sub-lethal concentrations; this also resulted in cross-resistance to the antibiotics erythromycin and ciprofloxacin (European Food Safety *et al*., 2019).

1.3.3. Interfering substances

During the process of disinfection with biocides, the environment can influence antimicrobial activity. The activity of a biocidal product may be compromised by the presence of interfering substances such as biological materials. When considering healthcare environments surfaces may be burdened with wet or dry organic materials that have the potential to quench the activity of some biocides (Russell and Day, 1993). Organic materials present extra proteins that biocidal compounds have to contend with, these extra interactions mean that there is less biocide available to interact with bacteria (Otzen, 2017). Cationic biocides are essentially neutralized by negatively charged organic particulate, this has been

demonstrated particularly in QACs such as benzalkonium chloride (Jono *et al*., 1986). Araujo *et al*. (2013) showed that in the presence of an organic load (bovine serum albumin) the efficacy of two commonly used QACs was decreased against *Bacillus cereus*.

1.3.4. Temperature and pH

Both temperature and pH can have an effect on biocide efficacy. Some biocides are more efficacious within specific environmental parameters. The increase of pH has the potential to enhance biocide efficacy (Russell, 2004), for example chlorhexidine digluconate works in an ideal acidic range of pH5.5-7.0 (Karpinski and Szkaradkiewicz, 2015, Wiegand *et al*., 2015). In the Bronsted-Lowry definition of the acid-base relationship, an acidic solution is considered a "proton donor" and an alkaline solution is considered a "proton acceptor" (Brönsted, 1923, Lowry, 1923). The negative ionic charge of an alkaline solution effectively neutralises a positively charged cationic biocide through dissociation. This pH specificity is due to the cationic, positive charge of chlorhexidine and this relationship can be seen for other cationic biocides such as benzalkonium chloride.

Biocides may also require specific storage temperatures to maintain their quality; storage temperatures that are too low may degrade a biocide (Leung *et al*., 2004). When assessing the activity of a biocide or a biocidal product, standard efficacy testing protocols such as the BS EN1276 (2009) stipulate that testing temperatures must be kept constant throughout procedures to minimise variability in testing conditions. Gelinas *et al*. (1984) found that biguanide and QACs were most effected by a change in temperature within a range of 4-50 °C (GÉLinas *et al*., 1984). Moreover, Taylor *et al*., (1999) tested 10 and 20°C but found that

lower temperatures compromised activity of QACs against *Pseudomonas aeruginosa* and *Escherichia coli*.

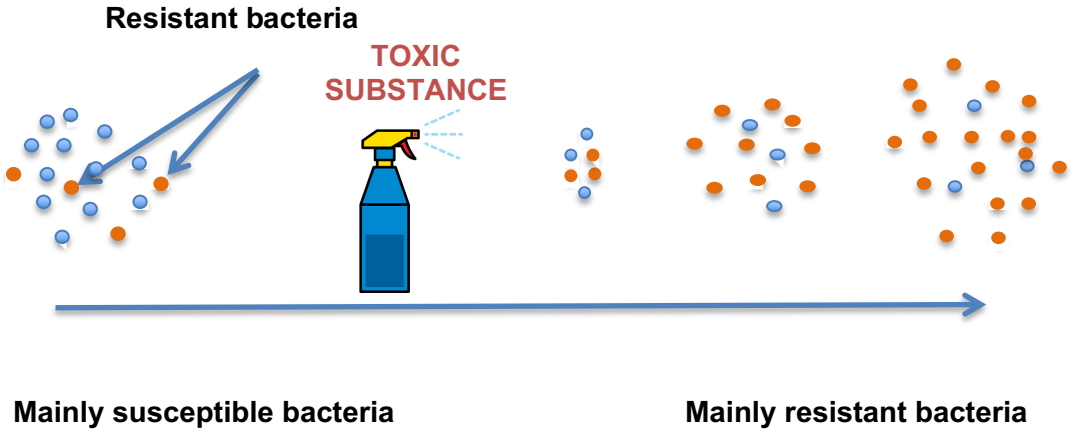
1.3.5. Residual biocidal activity and its role in microbial adaption and selection

Some biocidal product formulations make claims of "residual biocidal activity". CHX is most often incorporated into disinfectant products at 2% (20 mg/mL), and is often marketed with claims of residual activity of up to 6 hours, most recently 48 hours when applied to the skin surface (George *et al*., 2017). Efficacious biocide activity is dependant upon a relatively high stable concentration. Circumstances such as dilution, drying, microbial concentration and heavy bioburden may have a negative impact on biocidal products, driving them to sub-inhibitory concentrations, that is, concentrations that are below the Minimal inhibitory concentration (MIC). Subsequently some microbial populations will survive through the expression of mechanisms such as degradation, modification or extrusion of the toxic chemicals. These surviving populations may have decreased susceptibility or resistance to a biocide or consequentially an antimicrobial as a result of exposure at an inappropriate concentration. Microorganisms have been reported to survive on surfaces for prolonged periods of time, for instance some *Enterococcus* species have been reported to survive for up to 46 months (Kramer *et al*., 2006). If biocide products that possess long-lasting effects are not present at the required concentration to inactivate all microbial cells present, they will nonetheless impose a stress factor to the microbe and the induction and transmission of resistance may occur (Andersson *et al*., 2012, Gullberg *et al*., 2014, Gullberg *et al*., 2011, Maillard, 2013). Some studies have investigated the principle of microbial biocide adaption through culturing susceptible bacteria in a stepwise manner to gradually increasing concentrations of biocides. (Thomas *et al*., 2000) demonstrated through gradually

increasing the concentration of CHX in a *P. aeruginosa* culture, it is possible to decrease sensitivity to CHX. Furthermore, a number or cross-tolerances were observed with some antibiotics. More recently, a study by Gadea *et al*. (2017) showed that stepwise exposure to the QAC cetrimide and CHX resulted in transient decreases in susceptibility to the other biocides and antibiotics. This study also revealed a number of phenotypic changes in heat-tolerance as a result of exposure to both biocides.

Adaption and selection are closely related phenomenon. Adaption is the characteristic or phenotype that increases the probability of an organism's survival in adverse circumstances. Selection is the circumstance that the organisms must overcome; it is what drives the possibility of the adaptive characteristic being inherited in future generations. **Figure 1.1** demonstrates the process of natural microbial selection in the presence of a toxic substance.

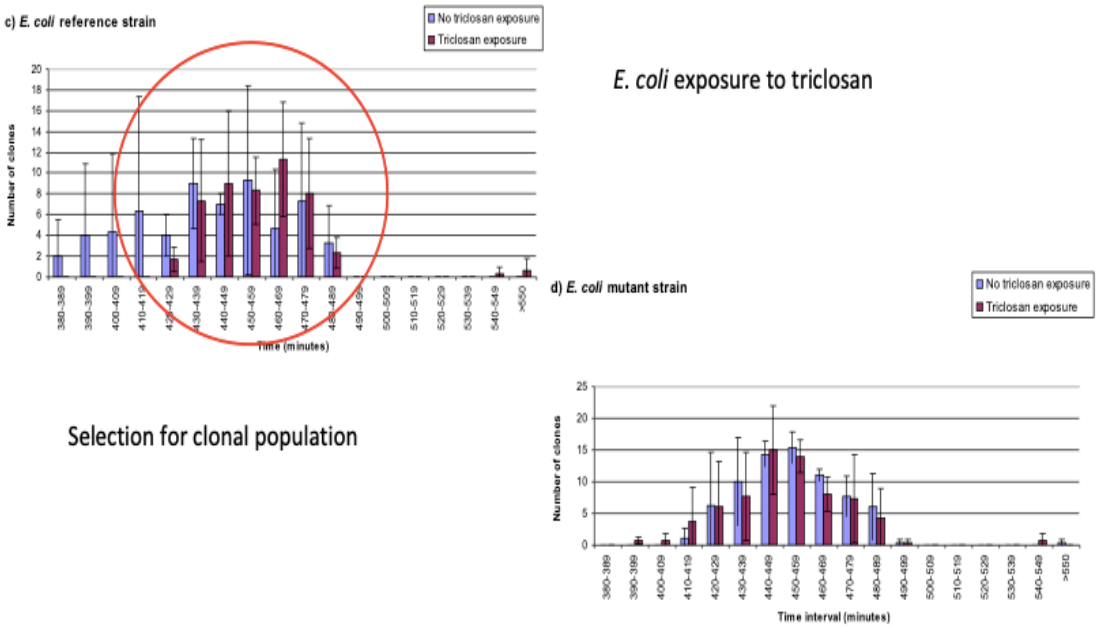
Figure 1.1 Natural selection of bacterial populations in the presence of a toxic substance



In the case of microbial selection through biocides exposure, sensitive populations are inactivated whereas less susceptible or resistant populations survive and

proliferate. This process of the selection of resistant populations succeeding the death of susceptible ones has been thought to occur only at higher biocide concentrations. However, it has been demonstrated that in the case of antibiotics, the selection process might be relevant at very low concentrations (Gullberg *et al*., 2014, Gullberg *et al*., 2011, Liu *et al*., 2011, Sandegren, 2014). Cottell (2006) investigated "time of detection" (t_d) for a reference and a mutant strain of *E. coli* when grown with or without Triclosan present (Gadea *et al*., 2017). Triclosan was included at $1/10^{th}$ of the MIC (0.020µg/mL for reference strain and 100.00µg/mL for mutant strain**). Figure 1.2** illustrates some results from this study. When observing the reference strain, histogram c in **Figure 1.2**, it can be noted that with no triclosan exposure the t_d is very low (i.e. 380mins), this is not the case for the triclosan-exposed reference cells. This was explained by the presence of a sub-population that was characterised by a high adaptive potential towards a new environment but a low adaptive potential when exposed to low concentrations of triclosan. It was concluded that the disappearance of the very low t_d values was due to the identified sub-population being killed by the presence of sub-inhibitory concentrations of triclosan. The presence of a sub-population with low t_d was not identified for the mutant strain.

Figure 1.2. Time detection values for cultures grown from single *E. coli* cells c) reference and d) mutant strains. Cottell (2006).



Mutant (specific mutation not mentioned; Cottell, 2006) strains were shown previously to have reduced susceptibility to Triclosan (Lear et al., 2000; Walsh et al., 2003; Gomez Escalada et al., 2005).

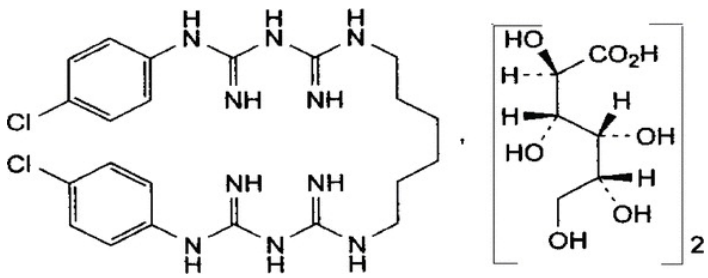
Building upon this theory, Witstand-Yuen *et al*. (2018) investigated the differences in acquired mutations after antibiotic selection at high (200 mg/L) and low (1 mg/L) level Streptomycin (Wistrand-Yuen *et al*., 2018). It was demonstrated that mutations selected for by streptomycin concentrations above MIC were mainly in the gene *rpsL*, encoding the 30S ribosomal subunit protein S12 (Gill and Amyes, 2004, Spagnolo *et al*., 2015b, Spagnolo *et al*., 2015a, Wistrand-Yuen *et al*., 2018). The study by Whitstand-Yuen *et al*. (2018) showed that the mutations selected at sub-minimal concentrations of strepromycin were not in the *rspL* gene but were related to mutations in 5 genes; *gidB, trkH, nuoG, cyoB*, and *znuA*. Through

deletion combinations of each of these genes this study showed that the addition of each mutation resulted in an increase in resistance to streptomycin, thus indicating that selection at lower concentrations of the antibiotic resulted in slower, step-wise adaption and selection of resistance mutations. This supports the principle of a distinct difference in the selection process of low and high concentrations of antibiotics however a similar comparative study has not yet been performed for biocides. The potential for CHX to select resistance at low concentrations has been investigated with inconclusive results. Thomas *et al*. (2000) produced "stable resistance" of *P. aeruginosa* to CHX after just one exposure. However, Wesgate *et al*. (2016) found no decrease in susceptibility of *E. coli* to CHX after 24 hours exposure. Further investigations into the differences between high and low level exposures of biocides have on selection and adaption processes will provide insight into the risk that they pose on future microbial resistance.

1.4. Chlorhexidine digluconate

Chlorhexidine digluconate (**Figure 1.3**) is the gluconate salt for of chlorhexidine. It is a bisbiguanide with a board spectrum of activity whose major application is the disinfection of microorganisms.

Figure 1.3 Chemical structure of chlorhexidine digluconate

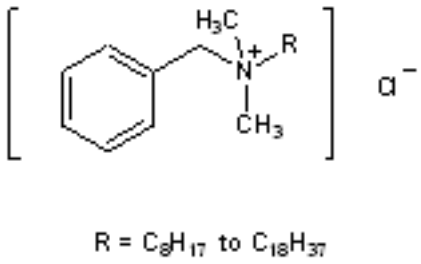


The chemical structure of CHX, specifically its biguanide chains, gives it an overall positive ionic charge. The target sites of CHX are the cell membrane, cytoplasmic membrane and cytoplasmic constituents (**Table 1.1**). When exposed to negatively charged microorganisms, CHX will destabilise bacterial cell membrane by binding to the phospholipid bilayer (El-Moug *et al*., 1985; Cheung *et al*., 2012). This binding results in generalised membrane damage. At low concentrations, this membrane active compound is considered microbistatic and will affect membrane integrity, membrane potential and an increase of cell permeability. However, at high levels CHX is fatal causing irreversible membrane damage leading to the congealing of the cytoplasm (McDonnell & Russell, 1999). After interfering with the outer structure of the cell membrane, a CHX molecule will interact with the cytoplasmic membrane. The subsequent collapse of the membrane potential and lysis of the cell allows for cytoplasmic leakage, interruption of adenosine triphosphate (ATP) synthesis, enzyme inhibition and eventual coagulation, occurrences that ultimately lead to cell death (Fitzgerald *et al*., 1992; Kuyyakanond & Quesnel, 1992; McDonnell & Russell, 1999; Cheung *et al*., 2012). It is worth noting that it is not ATP inactivation that is associated with the lethality of CHX but membrane disruption (Kuyyakanond *et al*., 1992; Barett-Bee *et al*., 1994).

1.5. Benzalkonium chloride

Benzalkonium chloride (**Figure 1.4**) is a positively charged derivative of an ammonium compound. BZC is utilised as a broad-spectrum disinfectant agent and is incorporated into biocidal product formulations. BZC interacts primarily with the cell surface, as its positive charge allows it to attach to a negatively charged cell wall.

Figure 1.4 Chemical structure of benzalkonium chloride



The amphiphilic properties of a BZC molecule allow the hydrophilic cationic region and the hydrophobic region to work together to effect cell death. The hydrophobic region of a BZC molecule firstly destabilises cell surface by forming an electrostatic interaction with the negatively charged cell (Coughlin *et al*., 1983, Fazlara and Ekhtelat, 2012, McDonnell and Russell, 1999b). The hydrophillic region of the BZC molecule then penetrates the hydrophobic bi-layer of the cell wall, this in turn causes the collapse of the membrane potential and leads to cell leakage and ultimately cell lysis. (Fazlara and Ekhtelat, 2012, Mangalappalli-Illathu and Korber, 2006, McDonnell and Russell, 1999b).

1.6. Biocide Product regulation

The European Biocidal Product Regulation (BPR, Regulation (EU) 528/2012) superseded the Biocidal Product Directive (98/8/EC). The legislation aims to standardise and monitor the introduction to market and the use of biocidal products

(BP). The definition of biocidal active substance, biocidal products and treated article is listed in the BPR as follows:

- ⇒ Active substance: a substance or a micro-organism that has an action on or against harmful organisms.
- ⇒ Biocidal products: any substance or mixture, consisting of, containing or generating one or more active substances, with the intention of destroying, deterring, rendering harmless, preventing the action of, or otherwise exerting a controlling effect on, any harmful organism by any means other than mere physical or mechanical action.
- ⇒ Treated article: Articles that have been treated with, or intentionally incorporating, one or more biocidal products.

Biocidal products that are incorporated into the BPR are classified into 22 product-types and are categorized in 4 main groups; 1. Disinfectants; 2. Preservatives; 3. Pest control; 4. Other products. The underlying condition of BPR regulation is that all biocidal products require an authorisation by European Chemicals Agency (ECHA) before they can enter the EU market. Included in the “conditions for granting an authorisation” section (BPR, EU, 2012, p21.), it is stipulated that the responsible regulatory body must be notified if the product owner is aware, or becomes aware that the BP is not sufficiently effective or that there is potential development of resistance to the active substance. It is also mandatory that the “biocidal product has no unacceptable effects on the target organisms, in particular unacceptable resistance or cross-resistance or unnecessary suffering and pain for vertebrates” and that the “chemical diversity of the active substances is adequate to minimise the occurrence of resistance in the target harmful organism”.

Standard efficacy testing protocols established from bodies such as the EN, OECD, ISO, AOAC and ASTM outline specific and stringent testing conditions,

including temperature, that ensure the rigorous comparative and standard efficacy testing. Wesgate *et al*. (2018) demonstrated that there were big differences in biocidal product efficacy depending on which standard testing protocol was applied.

1.7. Risks of biocide resistance, co-selection and cross-resistance in bacteria

Resistance has been defined as "*a change in susceptibility to a microbicide that renders it ineffective against a micro-organism that was previously susceptible to that microbicide*" (Maillard, 2013). It is imperative to distinguish between resistance and decreased susceptibility to a microbicide. Decreased susceptibility occurs when there is an increase in the lowest concentration that would previously kill or inhibit a microbe (minimal inhibitory (MIC) or bactericidal (MBC) concentration). Decreased susceptibility is not always clinically relevant as the change in MIC or MBC is still significantly lower than the concentration of microbicide or biocidal product used *in-situ*.

Multi-drug resistant organisms (MDROs) pose an issue to be overcome in clinical healthcare settings, agricultural industries, food and water production industries and even the domestic health care industry. Multidrug resistance in bacteria are therefore an increasingly ominous threat. In each of these areas of practice antimicrobial biocides are used to prevent and control levels of microbial contamination. Biocides are said to act as a selective pressure that encourages the acquisition of resistance traits in bacterial cells (Qiu *et al*., 2012). Furthermore this selective pressure may result from the overexposure of very low concentrations of biocides over long periods of time (Andersson *et al*., 2012, Gullberg *et al*., 2014,

Gullberg *et al*., 2011, Thomas *et al*., 2000). In the situation where a selective pressure in exerted on a bacterial population, subpopulations, which are less sensitive to a biocide, will survive to reproduce over those that remain sensitive, this gives those with resistance traits a selective advantage.

Empirically, antimicrobial resistance is considered either “intrinsic” or “acquired”. Intrinsic resistance is the innate insensitivity of a bacterial species to a particular antimicrobial agent. Intrinsic resistance occurs when bacterial genomes contain genes that have the potential to exhibit a resistance phenotype (Blair *et al*., 2014, Davies and Davies, 2010, Lister *et al*., 2009). Acquired resistance occurs when a microorganism obtains tolerance to an antimicrobial to which it was previously sensitive. Acquired resistance can result from the mutation of genes or from the acquisition of mobile genetic elements that carry foreign resistance genes through horizontal gene transfer (HGT) (Blair *et al*., 2014, Davies and Davies, 2010, Fernández and Hancock, 2012, Lister *et al*., 2009). Intrinsic and acquired resistance traits differ in that those associated with acquired resistance are not present in all strains or sub-populations of a bacterial species.

Bacteria with antimicrobial resistance traits are not necessarily solely resistant to one type of antimicrobial. Cross-resistant bacteria are defined as “those that have developed survival methods that are effective against different types of antimicrobial molecules with similar mechanism(s) of action” (SCENIHR, 2010). Cross-resistance with antibiotics is a growing concern that stems from the use of biocidal products both in clinical and domestic settings alike. It has been said that some identified mechanisms for resistance are alike between biocides and antibiotics (SCENIHR, 2010). It is debated that the use of biocidal products may be a driving factor in antimicrobial resistance. Marco *et al*. (2015) demonstrated a correlation between Chlorhexidine (CHX) and Benzalkonium chloride (BZC) for *S. aureus* susceptibility to quinolones, β-lactams and macrolides antibiotics (Marco *et*

al., 2015). The study did not find a correlation between Triclosan and Sodium hypochlorite and the antibiotics tested.

Co-resistance may also occur as a result of acquired resistance in bacterial cells. Co-resistance "involves transfer of several genes into the same bacteria and/or the acquisition of mutations in different genetic loci affecting different antimicrobials" (Cantón and Ruiz-Garbajosa, 2011). Co-resistance of disinfectant-resistant bacteria to antibiotics has been documented to occur with bacteria that carry QAC-resistance genes (Chapman, 2003, LemaÎTre *et al*., 1998) that are carried on transmissible plasmids, these plasmids carry multiple resistance genes.

1.8. Current surveillance of biocide resistance

Biocides are strongly regulated when it comes to their chemical makeup, their efficacy and their toxicity. Regulations such as the EU biocidal products directives 98/8/EC (1998) and 528/2012 ensure the use of biocides in commercial products is stringently controlled. However, information about the fate of these products in our environment is limited and data to show the relationship between biocides in our environment and bacterial resistance is scarce. The Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR, 2010) stated, "Despite the regulatory requirements to study the environmental stability of individual products, data on the fate and concentrations of biocides in the environment are sparse". One organisation that aims to solve that problem is the Swedish Research Council. INTERACT is a large research program founded by the Swedish Research Council, which aims to gain understanding of how biocides and heavy metals, especially in combination, contribute to development of antibiotic resistance (http://interact.gu.se; accessed: 21.05.2019). It is important to grasp an understanding of the role of external factors on the emergence and dissemination

of antimicrobial resistant bacteria (SCENIHR, 2010). It is clear that better surveillance of the use, disposal and environmental fate of biocides is needed in order to control better the growing issue of antimicrobial resistance.

1.9. Aims and objectives

There is not yet a standardised way to predict the levels of biocide concentrations that are left on surfaces as a result of the use of disinfectants, nor is there a standardised way to predict the potential risk that these levels pose to microbial resistance. The difference in selection and adaption processes at high and low-level biocide exposure is something that has not been fully investigated to date. This study aims to investigate links between exposure effects of CHX and BZC at high and low concentrations, the resulting phenotypes of *E. coli* and how these findings relate to the risk of resistance. The growing need for standardising the way microbicides are assessed for their potential risk leading to microbicidal resistance is a driving force behind this project with the aim of understanding potential markers for the risk of resistance development.

CHAPTER 2: GENERAL MATERIALS AND METHODS

2. GENERAL METHODS

2.1 Sterilisation of media

All media was sterilised in accordance to British Pharmacopoeia methods of preparation of sterile products (BP, 2005). All products were sterilised with steam in an autoclave (Fisher, UK) at 121°C for 15 minutes and let to cool to room temperature (≈ 20°C) before use.

2.2 Bacterial strains and growth conditions

2.2.1 Justification of bacteria

When investigating bacterial resistance it is important to consider that the development of resistance is strictly strain dependant. Standard culture collection strains are not necessarily suitable for studying the mechanisms of resistance (Maillard, 2018). environmental or clinical isolates depict a more applicable insight into the expression of resistance mechanisms. For these reasons this study will investigate seven environmental faecal isolates of *Escherichia coli* compared to reference strain *Escherichia coli* ATCC 25922 (https://www.attc.org/) that was used as a control.

Isolates of *Escherichia coli* used in this study were obtained from the University College Dublin (**Table 2.1**). Their genetic background and content including specific genetic elements have been determined. Each of the strains has been fully sequenced and has a clean genetic background. The strains are known to carry

conjugative-plasmid borne ESBLs. The specific features of interest of the chosen isolates are highlighted in **Table 2.1**.

Table 2.1. Summary of ESBL producing *E. coli* environmental isolates obtained from the University College Dublin and of their resistance features.

ISOLATE	MLST	ESBLs	Plasmid(s) (kb) isolated from transconjugants
UCD-CFS ECP-1L3	ST23	CTX-M-14	200-, 120-
UCD-CFS ECP-1B2	ST1629	CTX-M-14	110-
UCD-CFS ECP-1L4	ST23	CTX-M-14	130-
UCD-CFS ECP-13P5	ST10	CTX-M-15	80-
UCD-CFS ECP-13P4	ST10	CTX-M-15	70-
UCD-CFS ECP-25OS1	ST34 (ST10 Cpix)	TEM-20	120-, 60-
UCD-CFS ECP-25P5	ST10	TEM-20	110-, 50-

2.2.2. Bacterial growth conditions

All strains were cultured in Muller Hinton broth (MHB; Fisher Scientific, Loughborough, UK) and incubated at 37 (±1)°C for 18-24h. When necessary all bacterial strains were cultured on Muller Hinton agar (MHA) plates and stored in the fridge at 4-6°C for up to one month.

One loopful of colonies was taken from a MHA plate containing bacterial growth incubated at 37± 2°C for 18-24h. Bacterial cultures were incubated overnight in centrifuge tubes (Fisher Scientific, Loughborough, UK) containing 10 mL MHB. All

overnight cultures were incubated at 37 ± 2°C for 18-24h in an orbital shaker (150rpm).

2.3 Storage of isolates

All isolates were stored in glycerol and on protect beads (Fisher Scientific, Loughborough, UK). To make a glycerol stock, 800 µL of an overnight bacterial culture was mixed with 200 µL of glycerol in a cryovial and stored in the freezer at -80°C (± 1°C) for long-term storage. For short-term storage of isolates, one loopful of colonies was added to a vial of protect beads, vortexed for 1 min and left for 1 min. The supernatant was removed from the vial and protect beads were stored for up to one year at -20°C. Working cultures of each strain were made by streaking one loopful of fresh overnight culture onto MHA plates and were stored at 4-6°C for up to one month and restricted to a maximum of 2 subcultures from the original freezer stock prior to exposure to a given antimicrobial. Streak culture plates were performed regularly on freezer stocks to ensure purity.

2.4 Preparation of test inoculum

Overnight MHB bacterial cultures were centrifuged at 5000xg for 15 min at 20°C (± 1°C). The supernatant was discarded and the bacterial pellet was re-suspended in 10 mL phosphate buffer saline (PBS; Fisher Scientific, Loughborough, UK). This procedure was performed twice to ensure that all cultures were adequately washed of toxic growth products and remaining media.

Washed bacterial suspensions were adjusted to contain the appropriate concentration of bacterial cells for testing. Turbidity of suspensions was adjusted

using sterile MHB to achieve a turbidity equivalent to that of a 0.5 McFarland standard which results in the suspension containing approximately 1 to 2 × 10^8 CFU/mL. Optical density values were read using a spectrophotometer (wavelength 625nm) to ensure accuracy and consistency. 0.5 McFarland standard was produced.

2.5 Bacterial propagation and enumeration

Viable counts of all bacterial suspensions were performed before and after each test to determine viable bacterial cell concentration. Enumeration was carried out using the drop counting method (Miles, 1938). This method involves the serial dilution of the bacterial suspension; 100 µL was taken from the neat suspension and added to a sterile microcentrifuge tube containing 900 µL of sterile PBS buffer and mixed with a vortex. Dilutions of 1:10 were made in succession until eight serial dilutions have been made. To determine the mean colony count, 10 µL of each dilution was plated onto a MHA plate in triplicate and left to dry. Plates were incubated at 37 ± 2°C for 18-24h in a static incubator and dilutions that produced between 3 and 50 colonies were read and recorded.

2.6 Biocide preparation

Two antimicrobial agents, chlorhexidine digluconate and benzalkonium chloride, were chosen for their consistent use in healthcare environments and their relevance in current issues surrounding antimicrobial resistance (**Chapter 1.**). All compounds were diluted to the required concentrations in sterile deionized water (di$H_2$0). Each antimicrobial agent was prepared at an initial stock concentration to ensure accuracy in dilution; 1 and 2% w/v chlorhexidine digluconate, and 1 and 2% w/v benzalkonium chloride.

2.7 Neutraliser preparation

A neutraliser was used to quench the activity of both biocides during experimental procedures. Dey-Engley neutralising broth (LabM, Haywood, UK) was chosen for its universal application. The powdered media was dissolved per manufacturer's instruction in diH_20 and sterilised before use. Neutraliser was kept at 4-6°C for up to one month and regular checks were made to ensure sterility.

2.8 Neutraliser efficacy and toxicity test

To determine bacterial toxicity of the Dey-Engley neutralising broth, a suspension test was carried out in accordance with the British Standard EN 1276 2009 protocol (BSI, 2009). One mL of the test suspension was added to 9 mL of neutralizer, mixed for 1 min using a vortex and left to dwell for 5 min. A suspension of 1 mL bacteria and 9 mL of just diH_2O was included as a negative control. All suspensions were enumerated with the drop counting method (**Section 2.5**). Recorded colony counts for test and control suspensions were compared to determine whether or not exposure to the neutraliser resulted in a significant decrease in viable bacterial cells. The neutraliser would be deemed toxic if there was a ≥ 1 $\log_{10}$ decrease in viability was observed (BSI, 2009). This procedure was performed in accordance with the BS EN1276 (2009) suspension testing protocol.

To ascertain the ability of the neutraliser to sufficiently quench activity of the antimicrobial compounds, a neutraliser efficacy test was performed. Briefly, 1 mL of antimicrobial compound at the highest concentration to be tested was added to 8 mL of neutraliser and mixed with a vortex for 1 min. After mixing, 1 mL of bacterial standardised suspension (1 x 10^8 CFU/mL) was added, mixed and dwelled for 5

min. A suspension of 1 mL bacteria (1 x 10^8 CFU/mL), 1 mL antimicrobial and 8 mL of diH_2O was also tested for 5 min and included as a negative control. Viable counts of test and control suspensions were performed using the drop counting method **(section 2.5)** and colony counts for test and control suspensions were compared. The neutraliser was considered sufficiently effective if there was ≤ 1 $\log_{10}$ decrease in CFU/mL observed between the initial count and those taken after exposure to antimicrobial / neutraliser mixed solution. This test was carried out in accordance with the BS EN1276 (2009) suspension testing protocol.

CHAPTER 3: BASELINE SUSCEPTIBILITY PROFILE

3. BASELINE SUSCEPTIBILITY PROFILE

3.1. Introduction

As previously expressed (Chapter 1, Section 1.2), antimicrobial products have either microbicidal or microbistatic effects on bacterial populations. Their efficacy depends on both environmental and biological conditions. There are a wide variety of protocols established in scientific studies to ascertain the efficacy of an antimicrobial product. As this study is interested in exploring how biocides may alter biological processes related to antimicrobial susceptibility, protocols that investigate bacterial growth kinetics and antimicrobial susceptibility were selected for consideration.

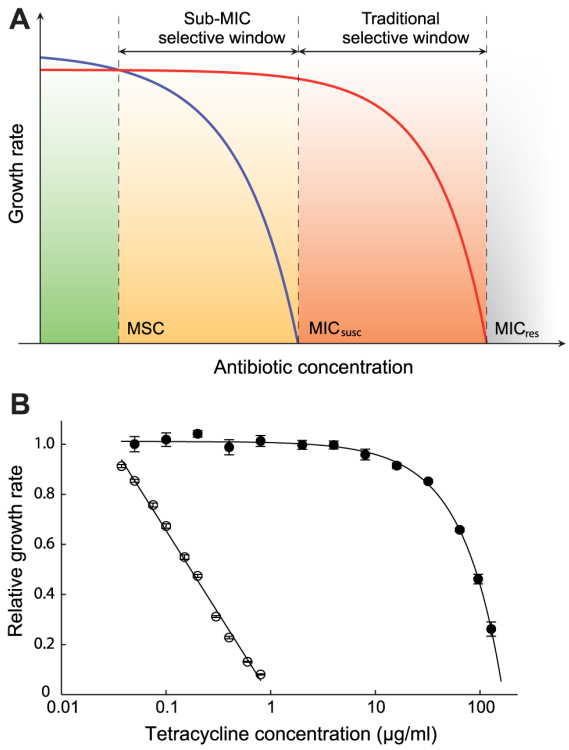
3.1.1. Minimal Inhibitory, Minimal bactericidal and Minimal selective concentrations

Microbroth dilution method (BSI, 2006) involves controlled concentrations of bacterial cells being exposed to a range of antimicrobial concentrations under set conditions in order to ascertain both the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC). The MIC is defined as the lowest concentration of a biocide that inhibits visible bacterial growth. The MBC is defined as the lowest concentration of a biocide that kills a bacterial population and is determined by culturing the wells surrounding the MIC to count viable bacterial cell growth.

In situ, biocides are typically applied in concentrations that are considerably above that needed to kill bacterial populations and are most commonly tested for efficacy

at the product's "active" or "in-use" concentration. For this reason, an increase in MIC or MBC after exposure to an antimicrobial does not inevitably result in failure to kill a microorganism when a biocide is used at "in-use" concentrations (Russell, 2000, Thomas *et al*., 2005). When assessing bacterial resistance to antibiotics, the MIC is the value, which is typically considered critical to the indication of change in susceptibility (Begot *et al*., 1996, Strotmann and Pagga, 1996). The MIC can be used as a marker for change in phenotypic behaviour after exposure to an antimicrobial (Maillard, 2013). It has been previously understood that concentrations of an antimicrobial above the MIC of a susceptible bacteria and below that of resistant bacteria are responsible for the selection of resistant populations (Gullberg *et al*., 2011). Resistant bacteria consequently outcompete susceptible bacteria, are selected and survive (Drlica, 2003). However, it is more recently understood that there is a lower concentration range in which the selection for resistant microorganisms can occur (**Figure 3.1**). This range is referred to as the Minimal Selective Concentration (MSC) (Gullberg *et al*., 2014, Gullberg *et al*., 2011, Liu *et al*., 2011). **Fig. 3.1A** demonstrates the antibiotic concentration range in which the MSC is now understood to select for resistant bacterial populations. **Fig. 3.1B** shows the difference in effect on growth rates the antibiotic MSC can have on susceptible and non-susceptible populations. Concentrations substantially below the antibiotic MIC of the susceptible strain decreased its growth rate, whereas these concentrations had no effect on the resistant strain. Bacterial populations selected within the MSC range, can have high-level resistance just as those selected above MIC (Gullberg *et al*., 2014, Wistrand-Yuen *et al*., 2018). Although the MSC in the examples given tackle the effects of selective pressure applied by antibiotics, this is an interesting concept to adapt to the use of biocides.

Figure 3.1. Growth rates as a function of antibiotic concentration. from Gullberg *et al*. (2011).



(**A**) Schematic representation of growth rates as a function of antibiotic concentration. Green indicates a concentration interval where the susceptible strain (blue line) will outcompete the resistant strain (red line). Orange (sub-MIC selective window) and red (traditional mutant selective window) indicate concentration intervals where the resistant strain will outcompete the susceptible strain. MIC_{susc} = minimal inhibitory concentration of the susceptible strain, MIC_{res} = minimal inhibitory concentration of the resistant strain and MSC = minimal selective concentration. (**B**). Relative exponential growth rates of susceptible (open circles) and resistant (closed circles) strains of *S. typhimurium* as a function of tetracycline concentration. Standard errors of the mean are indicated. A relative growth rate of 1.0 corresponds to approximately 1.8 hr^{-1}. Cells were grown in Mueller Hinton medium at 37°C.

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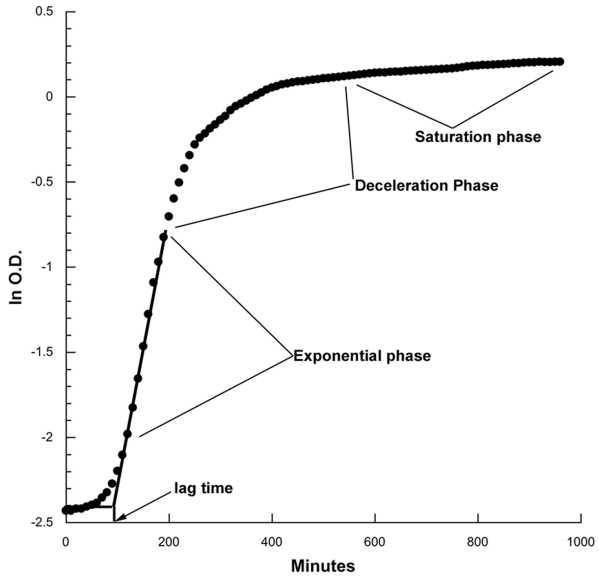
As discussed previously (Sundheim *et al*., 1998) it is possible that biocides are present in our environments in concentrations lower than intended whether as a result of incorrect use or dilution. Furthermore, it is possible that prolonged exposure to low residual antimicrobial concentrations could lead to less susceptible bacterial populations (Andersson *et al*., 2012, Gullberg *et al*., 2014, Gullberg *et al*., 2011, Thomas *et al*., 2000, Thomas *et al*., 2005, Wistrand-Yuen *et al*., 2018). It is therefore important to consider the significance of the effect that these lower concentrations have on bacterial populations (SCENIHR 2009).

3.1.2. Growth kinetics

Bacterial growth kinetics are usually visualised in the form of growth curves and are produced by using spectrophotometers measuring optical density values of a bacterial suspension over time (Begot *et al*., 1996, Hall *et al*., 2013). Growth curves are a representation of the natural growth phase of a bacterial strain, which is depicted in **Figure 3.2**. (Hall *et al*., 2013). Specific phases of growth are responsible for different biological processes that work to enable bacterial populations to overcome stresses, adapt to their environment and replicate to thrive. Growth curves can be useful for determining whether or not a growth phase is altered due to the presence of an antimicrobial substance. Alterations in growth kinetics after exposure to antimicrobials suggest either an inhibitory or an adaptive response of the microorganism.

Figure 3.2. A typical bacterial growth curve (Hall *et al*., 2013)



The comparison of non-biocide exposed and biocide-exposed bacteria using growth kinetics cannot be used alone to predict antimicrobial resistance but serve as an indication that a biocide is effecting a change. Inhibitory effects observed in the presence of an antimicrobial can be an indication of an environment that might favour or select for resistance phenotype. The lag phase is when microorganisms react to the environment around them and adapt to stressful influences (Rolfe *et al*., 2012). It is the phase of growth in which primary changes to increase drug tolerance occurs (Fridman *et al*., 2014). The investigation of (Li *et al*., 2016) into the lag phase of *E. coli* showed that understanding the lag phase was integral to predicting drug susceptibility. The study inferred that an elongated lag phase provided survival advantages and promoted regrowth upon the removal of an antibiotic. Sleight and Lenski (2017) found that shortened lag phases in some *E. coli* strains provided a selective advantage for growth resumption after stationary phase (Sleight and Lenski, 2007). However, several studies highlighted heterogeneity in growth resumption timings, and suggested that many *E. coli* cells

have extended lag phases even in favourable conditions (Levin-Reisman *et al*., 2010, Niven *et al*., 2008, Rolfe *et al*., 2012). Joers and Tenson (2016) found that heterogeneous growth resumption during lag phase could protect the population from adverse effect of stress, because stress-resilient dormant cells are always present (Jõers and Tenson, 2016). The presence of these so called "persister" cells that lay dormant whilst others cells resume growth during the lag phase can lead to decreased efficacy of an antimicrobial even though the cells remain susceptible (Allison *et al*., 2011, Balaban *et al*., 2004). "Persister" cells re-initiate growth when environmental conditions are more favourable. Persister cells commonly exhibit low proton motive force (PMF) activity enabling them to inhibit, for example, the activity of aminoglycoside antibiotics. Allison *et al*. (2011) demonstrated that this characteristic can be overridden in *E. coli* and *S. aureus* by the addition of certain carbon sources as persisters, although dormant are still "primed for metabolic uptake, central metabolism and respiration". To surmise, if the lag phase of a bacterial growth curve is extended in the presence of an antimicrobial, this can be indicative of adaption to its bactericidal activity, including the possible involvement of persister cells (Allison *et al*., 2011, Li *et al*., 2016, Whitehead *et al*., 2011). Furthermore, growth-phase dependant expression of various genes in *E. coli* has been denoted (Madar *et al*., 2013). Kobayashi *et al*., (2006) identified that at the early to late exponential phase (Kobayashi *et al*., 2006), the *emrE*, *mdfA*, and *acrA* genes showed relatively high expression levels; the deletion of these genes is known to increase antibiotic susceptibility (Sulavik *et al*., 2001). The study showed that expression of the MdtEF and the AcrAB drug exporter genes confer drug tolerance in the saturation or stationary phase phenotype. This knowledge affirms the value of growth kinetics in evaluating the potential for antimicrobial compounds to select resistance in microorganisms.

3.1.3. Antibiotic susceptibility determination

There are a number of methods available for measuring antibiotic susceptibility in bacteria. These include standardised testing protocols from the Clinical and Laboratory Standards Institute (CLSI, 2015), the British Society for Antimicrobial chemotherapy (BSAC) (Andrews, 2009) and the European Committee on Antimicrobial Susceptibility testing (EUCAST, 2015) methods. These protocols stipulate either micro-broth dilution or antibiotic disc susceptibility assays, and provide the user with set susceptibility breakpoints, which provide an indication of whether a bacterial population is clinically sensitive, intermediate or resistance against a certain antibiotic. These protocols are not always applicable as clinical breakpoints for certain bacteria are often unavailable. Epidemiological cut off (ECOFF) values are collected, collated and established by the European Committee on Antimicrobial Susceptibility Testing (2010). MIC distributions are collected from worldwide sources through published research articles, the pharmaceutical industry, veterinary institutions and surveillance programmes such as Brittish Society for Antimicrobial Chemotherapy (BSAC), ECO-SENS, European Antimicrobial Resistance Surveillance System (EARSS), Hospitals in Europe Link for Infection Control through Surveillance (HELICS), The Meropenem Yearly Susceptibility Test Information Collection (MYSTIC), NORM and the SENTRY Antimicrobial Surveillance Programme. These data are used to produce a distribution range of MICs for microorganisms with and without acquired resistance mechanisms. This type of epidemiological data may be more useful than single study MICs as it provides a broader depiction of the level of risk and can be applied to monitor advances in resistance. A similar set up for the surveillance of biocide susceptibility does not exist, Biocide MIC/MBC breakpoints are scarcely available. However there has been a recent study that intended to produce a testing protocol

that, aimed to measure the capability of biocides to confer cross-resistance to antibiotics using a combination of the micro-broth dilution and disc diffusion techniques. This method sought to use changes in biocide and antibiotic sensitivity as markers for predicting resistance and cross-resistance in micro-organisms (Knapp *et al*., 2015).

3.1.3. Antibiotic and biocide test correlation

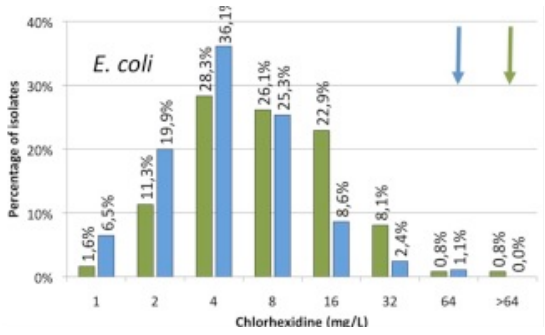
As antibiotic resistance is such a massive risk to worldwide health (O'Neil, 2016), it goes without saying that there is a wide variety of research that has investigated and supported the idea that the long-term use of antibiotics has exacerbated the issue of antibiotic resistance. However, research into the extent of biocide implication in antimicrobial resistance is still in its primary stages, no similar systems are currently established to monitor biocide-mediated antimicrobial resistance. It has been attempted to apply ECOFF (upper limit of normal MIC for a given species) values to commonly used biocides (Morrissey *et al*., 2014) in order to define realistic, applicable breakpoints. **Figure 3.3.** Illustrates findings from the Morrissey study (2014) with relation to *E. coli,* CHX and BZC. ECOFF MIC and MBC for CHX were 64mg/L and >64 mg/L respectively. ECOFF MIC and MBC for BZC were 64 mg/mL (0.064 mg/mL) and 128 mg/mL (0.128 mg/mL) respectively.

Figure 3.3. Populational susceptibility of *E. coli* to CHX and BZC.

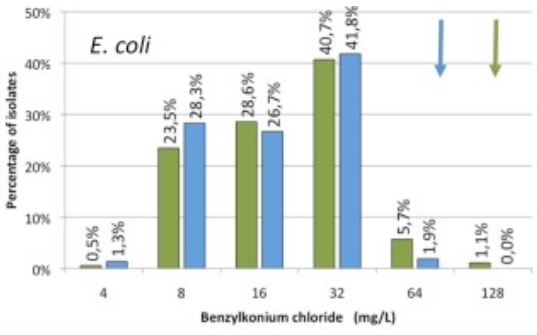
MIC - highlighted in blue, ECOFF MIC indicated with blue arrow

MBC – highlighted in green, ECOFF MBC indicated with green arrow

a)



b)



These data provide valuable information on the susceptibility trends of clinically relevant microorganisms to biocides.

3.1.4. Aims and objectives

This chapter aims to ascertain a baseline antimicrobial susceptibility profile of the isolates selected using the ISO: 20776-1 (BS EN ISO: 20776-1; ISO, 2006), growth kinetics and EUCAST (EUCAST, 2015) methods. This investigation used growth curves in conjunction with an attempt to apply to biocides, the minimal selective concentration determination procedure (Gullberg *et al*. 2011, 2014) to determine growth kinetics during exposure to high and low concentrations of biocides. Studies into the MSC have thus far focussed on antibiotic selectivity. The growth curves produced through this study will be used to identify potential minimal selective inhibitory concentrations of the chosen biocides. The determination of a minimal selective concentration will provide the study with a focal point for investigation of the effects of residual biocide concentrations on antimicrobial resistance.

3.2. Materials and methods

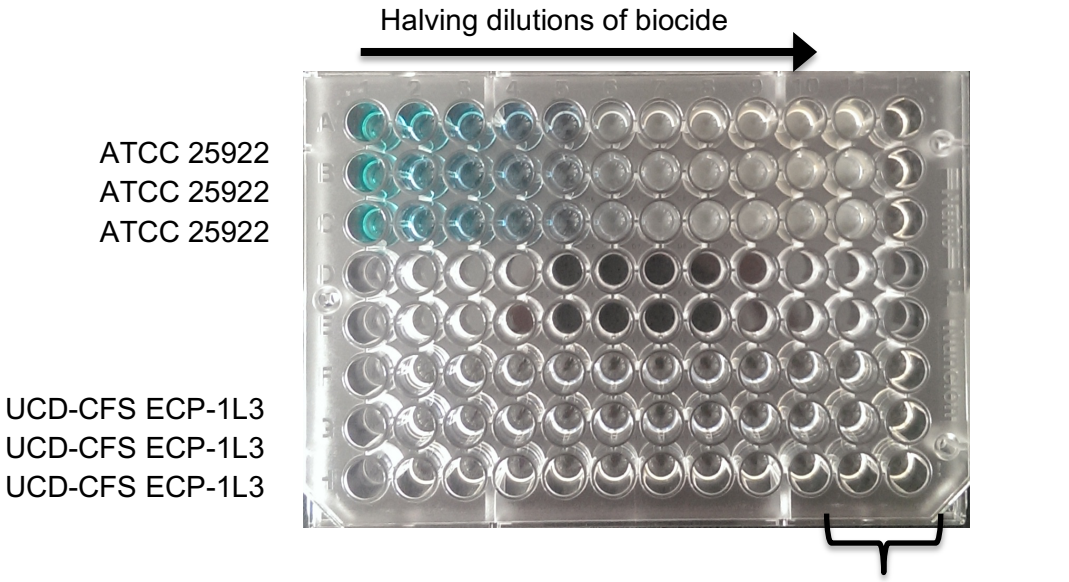
3.2.1. Minimal Inhibitory and Minimal bactericidal concentrations

The MIC of each biocide was determined following the BS EN ISO: 20776-1 (ISO, 2006) protocol. All bacterial strains (**Chapter 2: Section 2.2.1; table 2.1**) were tested including the reference strain *E. coli* ATCC 25922. To ensure that all dilutions were accurate and consistent the Viaflo ASSIST 4500 (Integra, UK) robot was used to load all microtiter plates. Fifty µL of MHB was added to wells 2-12 of a 96 well microtiter plate (Sterilin Ltd, Newport, UK) (**Figure 3.4**). Fifty µL of each antimicrobial agent was doubly diluted across wells 1-10. Each antimicrobial agent was placed in three consecutive rows in order to replicate the experiment in triplicate. Column 11 (**Figure 3.4**) was designated as a positive control test where only broth and bacteria were incubated without biocide. Column 12 was (**Figure 3.4**) designated as a negative control test where broth only was incubated without

antimicrobial agent or bacteria. Fifty µL of standardised washed bacterial culture (1 x 10^8 CFU/mL; **Chapter 2, section 2.4**) was individually added to all wells in the 3 rows allocated. The plate was covered with a sterile lid, wrapped in parafilm and incubated at 37 ± 2°C for 18-24 h in a shaking incubator at 150 rpm. The range of concentrations tested was as follows: 0.031 – 0.000061 % w/v chlorhexidine digluconate and 0.125 – 0.00024 % w/v benzalkonium chloride. The MIC was recorded as the lowest concentration at which no visible growth was observed visually.

The minimal bactericidal concentration of each biocide was determined following incubation of the plate used to determine the minimal inhibitory concentration. Twenty µL of test suspension was removed from each well of the microtitre plate where no bacterial growth was observed and the two lowest biocide concentrations at which growth was observed, plated onto a MHA plate containing 10 % v/v neutraliser **(Chapter 2; Section 2.7)** and incubated at 37 ±1°C for 24 h. The minimum bactericidal concentration was defined as the lowest antimicrobial concentration where no bacterial growth was observed on the agar plate.

Figure 3.4. An example of plate layout for MIC testing.



3.2.2. Growth kinetics

The Bioscreen C analyzer (Oy Growth Curves AB Ltd, Helsinki, Finland) was used to obtain growth curves from all isolates in the presence of a biocide at doubling concentrations. Overnight bacterial washed cultures were standardised to 1 x 10^6 CFU/mL, this was to ensure that all phases of growth were visually apparent. Three hundred and fifty µL of pre-prepared biocidal compound at appropriate concentrations was added to the appropriate wells of a honeycomb plate (Oy Growth Curves AB Ltd, Helsinki, Finland) as indicated in **Figure 3.5.** Fifty µL of standardized bacterial cell suspension was added to the appropriate wells (**Figure 3.5).** A negative control (biocide and broth only) was included succeeding each test column in order to account for any change of turbidity in the media due to the antimicrobial compound. The plates were incubated in the Bioscreen at 37 ± 1°C for 24 h and turbidity was recorded using a wideband filter (420– 580 nm) with readings taken every 4 min with continuous shaking. A total of three independent experiments were performed using fresh bacterial cultures.

Figure 3.5. Depiction of the layout of a honeycomb plate incubated in the Bioscreen C analyser. The first two columns are repeated with each different strain. Image adapted (Oy Growth Curves AB Ltd, Helsinki, Finland).

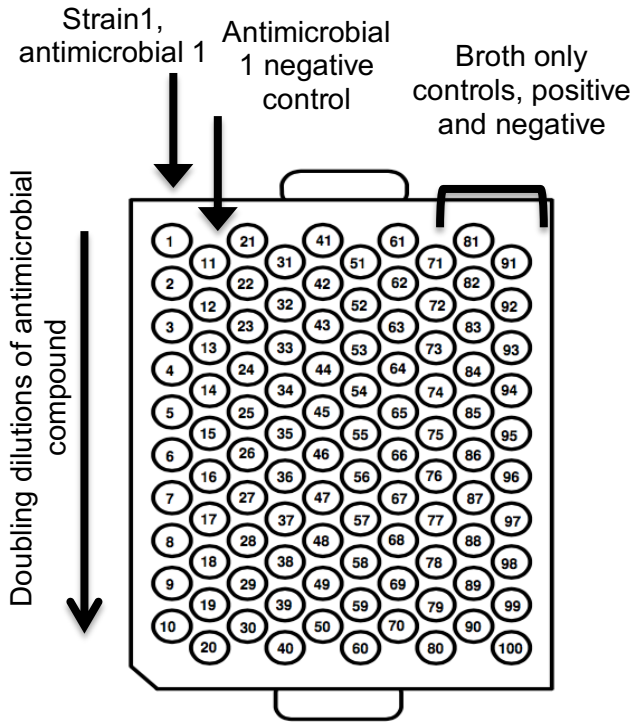


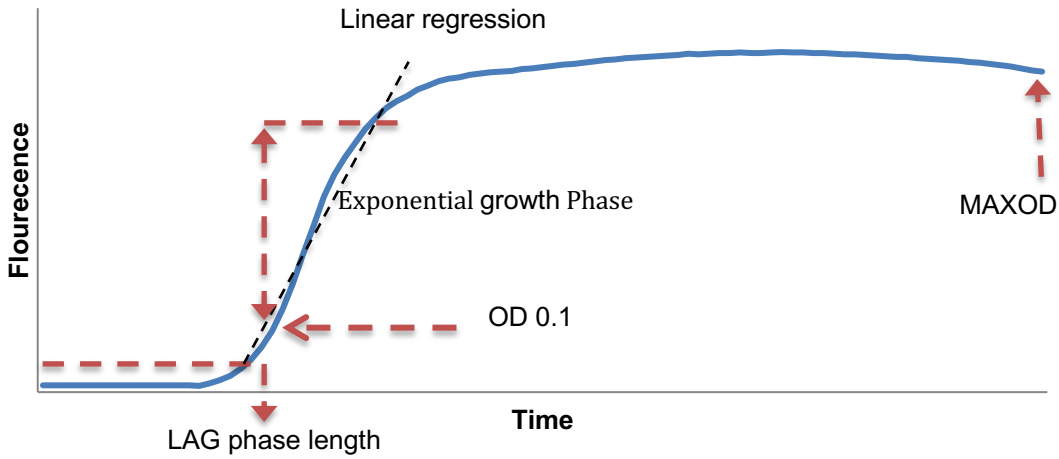
Figure 3.6 demonstrates the point at which each stage of the growth curve was analysed. Lag phase (λ) was defined as the length of time (min) taken to reach OD_{600} of 0.1. This is the point before the growth curves begin to enter exponential growth phase. Lag phase extension (LE) was measured in order to quantitatively evaluate the effects of biocides on the lag phase. This has previously been performed for antibiotics in order to evaluate the potential risk of resistance development to antibiotics (Li *et al.*, 2016). LE is expressed in **equation 3.1** where λ_c = lag phase duration in the presence of biocide at concentration C and λ_0 = lag phase length in the no biocide control.

Equation 3.1 Lag phase extension (LE)

$$LE = \lambda_c/\lambda_0$$

Maximum Optical Density (MAXOD) was recorded as the optical density value (OD_{600}) at the last time point (1440 minutes) for each instance. Specific growth rate (**μ**) was calculated as the linear regression of the slope of the exponential growth phase (**Figure 3.6).** OD_{600} values are an indirect reflection of the actual total cell count and were used instead of absolute cell number (Bergot *et al*., 1996). The coefficient of determination (R^2) values ranged from R^2=0.91 to R^2=0.1; these levels of variation were considered acceptable.

Figure 3.6. A depiction of the parameters assigned for the calculation of each growth phase related value (Log phase length, Maximum OD_{600} reached and Specific growth rate).



3.2.3. Antibiotic susceptibility

The susceptibility of each strain to all stipulated antibiotics was determined in accordance with the EUCAST protocol for antimicrobial disc susceptibility testing (EUCAST, 2015). Briefly, standardised overnight bacterial cultures (1 x 10^6 CFU/mL) of each strain were spread evenly onto MHA plates and left to dry for no

more than 15 min. Antibiotic discs were placed using sterile forceps, onto the bacterial lawn and plates were incubated at 37 ± 2°C for 18-24 h in a static incubator. The antibiotics chosen for their clinical relevance and in accordance with EUCAST (2015) were as follows: ampicillin (10 µg), amoxicillin-clavulanic acid (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), cefodoxime (10 µg), cefoxitin (30 µg), tetracycline (30 µg), streptomycin (10 µg), imipenem (10 µg), cephalothin (30 µg), nalidixic acid (30 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), trimethoprim (5 µg). All antibiotics were purchased from Beckton and Dickinson (Oxford UK). Clinical susceptibility of each strain was based on breakpoints stipulated in the EUCAST protocol and measured from the size of the zones of inhibition produced by the antibiotic. Breakpoint guidelines that were used from the EUCAST protocol are included in the in the appendices (File name: Appendices > appendix one > Breakpoint-Table-EUCAST).

3.2.4. Statistical analysis

A Two-Way analysis of variance (ANOVA) test was used when comparing MIC/MBC, Growth rate, Lag phase or maximum OD values between strains and between biocide concentrations. A one-Way ANOVA was used to compare MIC and MBC values. A students T-test was used to compare values of exposed and non-exposed bacteria regardless of CHX concentration or strain.

3.3. Results and discussion

3.3.1. Chlorhexidine digluconate MIC and MBC

MIC and MBC values were statistically different between the 8 *E. coli* strains *(P*<0.0001; Two-way ANOVA; Graphpad PRISM8*)*. Isolate UCD-CFS ECP-13P5

demonstrated the highest CHX MIC and MBC values at 0.01 mg/mL (**Table 3.1**). Morrissey *et al*., (2014) suggested that the ECOFF MIC and MBC values for *E. coli* and CHX were 0.064 mg/mL and >0.064 mg/mL, respectively. The highest MIC value obtained here was 6.4-fold less than the obtained ECOFF. The average MIC and MBC for CHX in this study were 0.005 mg/mL and 0.006 mg/mL compared to the MIC and MBC ECOFF which were 0.004 mg/mL and >0.004 mg/mL, respectively. There was a 5-fold difference between the highest and lowest MIC (I.e. 0.01 mg/mL and 0.002 mg/mL). However, there was no statistically significant difference found between MIC and MBC values (*P*=0.36*;* ONE-WAY ANOVA; Graphpad PRISM8*).*

Knowing that isolates UCD-CFS ECP-1L3 – 25051 carry ESBL resistance genes (Table 2.1; Chapter 2, section 2.2.1) might indicate that the slight variation of the baseline MIC and MBC phenotypes are related to these genes. UCD-CFS ECP-13P5 carries plasmid encoded CTX-M-15, an ESLB-type antibiotic resistance gene that has been related in resistance to ampicillin (AMP) piperacillin (PR), amoxiclavulanic acid/augmentin (AG), ampicillin/sulbactam (A/S), cefuroxime (FU), cefazoline (FZ), cefepime (PIM), cefotaxime (CTX), ceftazidime (CAZ) , gentamicin (CN), amikacin (AK), tobramycin (TB) aztreonam (AZ), ciprofloxacin (CIP), norfloxacin (NOR), levofloxacin (LEV), tetracycline (TE) and chloramphenicol (C) (Guiral *et al*., 2011). This is relevant as CHX and some antibiotics, for example aminoglyciosides (i.e gentimicin) share modes of entry into a bacterial cell via self-promoted uptake. Both enter the cell through the displacement of cations in the bacterial cell envelope and re-organisation of lipopolysaccharides (Hancock and Baddiley, 1985). The similarities in mode between aminoglycosides and CHX have led to a discussion as to whether reduced CHX (and other biguanide biocides) uptake is linked to reduced aminoglycoside susceptibility.

Table 3.1. Minimal inhibitory and bactericidal concentrations (mg/mL) for all test strains (ESBL type in brackets) in the presence of CHX (n=3).

± standard deviation of the mean of 3 replicates

	CHLORHEXIDINE DIGLUCONATE	
ISOLATE	**MIC**	**MBC**
ATCC 25922	0.005 ± 0.000	0.005 ± 0.000
UCD-CFS ECP-1L3 (CTX-M-14)	0.005 ± 0.000	0.005 ± 0.000
UCD-CFS ECP-1L4 (CTX-M-14)	0.005 ± 0.000	0.005 ± 0.000
UCD-CFS ECP-IB2 (CTX-M-14)	0.007 ± 0.003	0.007 ± 0.003
UCD-CFS ECP-13P5 (CTX-M-15)	0.010 ± 0.000	0.010 ± 0.000
UCD-CFS ECP-13P4 (CTX-M-15)	0.002 ± 0.000	0.005 ± 0.000
UCD-CFS ECP-25P5 (TEM-20)	0.005 ± 0.000	0.005 ± 0.000
UCD-CFS ECP-25OS1 (TEM-20)	0.005 ± 0.000	0.005 ± 0.020

3.3.2. Benzalkonium chloride MIC and MBC

The average MIC and MBC for BZC in this study were 0.018 mg/mL and 0.023 mg/mL compared to the MIC and MBC ECOFF which were 0.032 mg/ml and 0.032mg/mL, respectively. There was no statistically significant difference between *E. coli* strains when comparing their BZC MIC and MBC values (*P*=0.13*;* Two-way ANOVA; Graphpad PRISM8*)* (**Table 3.2**). These results suggest that although these strains carry different resistance genes (**Table 3.2),** these did not affect their baseline susceptibility to BZC. There was a 2-fold difference in MIC and MBC (i.e. 0.02 mg/mL to 0.04mg/mL) for isolates UCD-CFS ECP-1L3 and UCD-CFS ECP-25P5. The highest MIC and MBC values obtained were 0.02 mg/mL (UCD-CFS ECP-1L3, UCD-CFS ECP-1B2, UCD-CFS ECP-13P5, UCD-CFS ECP-13P4, UCD-

CFS ECP-25P5, UCD-CFS ECP-25051) and 0.04 mg/mL (UCD-CFS ECP-1L3, UCD-CFS ECP-25P5) respectively. Compared with ECOFF values obtained by Morrissey *et al*. (2014), MIC values here were 3.2-fold less and MBC values were 4.2-fold less.

Table 3.2 Minimal inhibitory and bactericidal concentrations (mg/mL) for all test strains (ESBL type in brackets) in the presence of BZC (n=3).

± Standard deviation of the mean of 3 replicates

	BENZALKONIUM CHLORIDE	
ISOLATE	**MIC**	**MBC**
ATCC 25922	0.01 ± 0.00	0.01 ± 0.00
UCD-CFS ECP-1L3 (CTX-M-14)	0.02 ± 0.00	0.04 ± 0.00
UCD-CFS ECP-1L4 (CTX-M-14)	0.01 ± 0.00	0.01 ± 0.00
UCD-CFS ECP-IB2 (CTX-M-14)	0.02 ± 0.00	0.02 ± 0.00
UCD-CFS ECP-13P5 (CTX-M-15)	0.02 ± 0.00	0.02 ± 0.00
UCD-CFS ECP-13P4 (CTX-M-15)	0.02 ± 0.00	0.02 ± 0.00
UCD-CFS ECP-25P5 (TEM-20)	0.02 ± 0.00	0.04 ± 0.00
UCD-CFS ECP-25OS1 (TEM-20)	0.02 ± 0.00	0.02 ± 0.00

3.3.3. Growth kinetics without biocides

After 24 hours incubation in the Bioscreen C analyser, OD_{600} values were plotted against time for all isolates without any biocide exposure to demonstrate baseline growth kinetics **(Figure 3.7).** In order to determine differences in growth kinetics the specific growth rate ($hour^{-1}$), lag phase length (min) and maximum OD_{600} reached were compared between isolates.

Table 3.3 shows the baseline specific growth rates ($hour^{-1}$) for eight *E. coli* isolates. When comparing between isolates, the difference between the values was statistically significant (*P*=0.003) (**Table 3.3**). It has been discussed that organisms that have slower growth rates are more able to survive adverse conditions (Williams, 1988; Brown & Williams, 1985; Brown *et al*., 1985). Studies have demonstrated the correlation between decreased growth rates and a decrease in susceptibility to a number of environmental stressors (Berney *et al*., 2006, Lindqvist and Barmark, 2014). Evans *et al*. (1990) demonstrated that a specific growth rate of ≤ 0.15 h^{-1} was linked to decreased susceptibility to cetrimide, a commonly used QAC. Wright & Gilbert (1987) however, found that the relationship between slow growth rate (ca 0.08 h^{-1}) and sensitivity of *E. coli* to CHX was inconclusive.

Table 3.3. shows baseline lag phase lengths (*λ)* for *E. coli* isolates. The isolate with the highest Lag phase was UCD-CFS ECP-25051 (140min ± 12.2), the lowest was UCD-CFS ECP-IB2 (83.0 ± 12.5 min). The lag phase was strain dependent (P=<0.0001; ONE WAY ANOVA; Graphpad PRISM8), suggesting that baseline lag phase is a strain unique physiological process.

Figure 3.7. Shows baseline growth curves for all isolates in the presence of broth only (n=3).

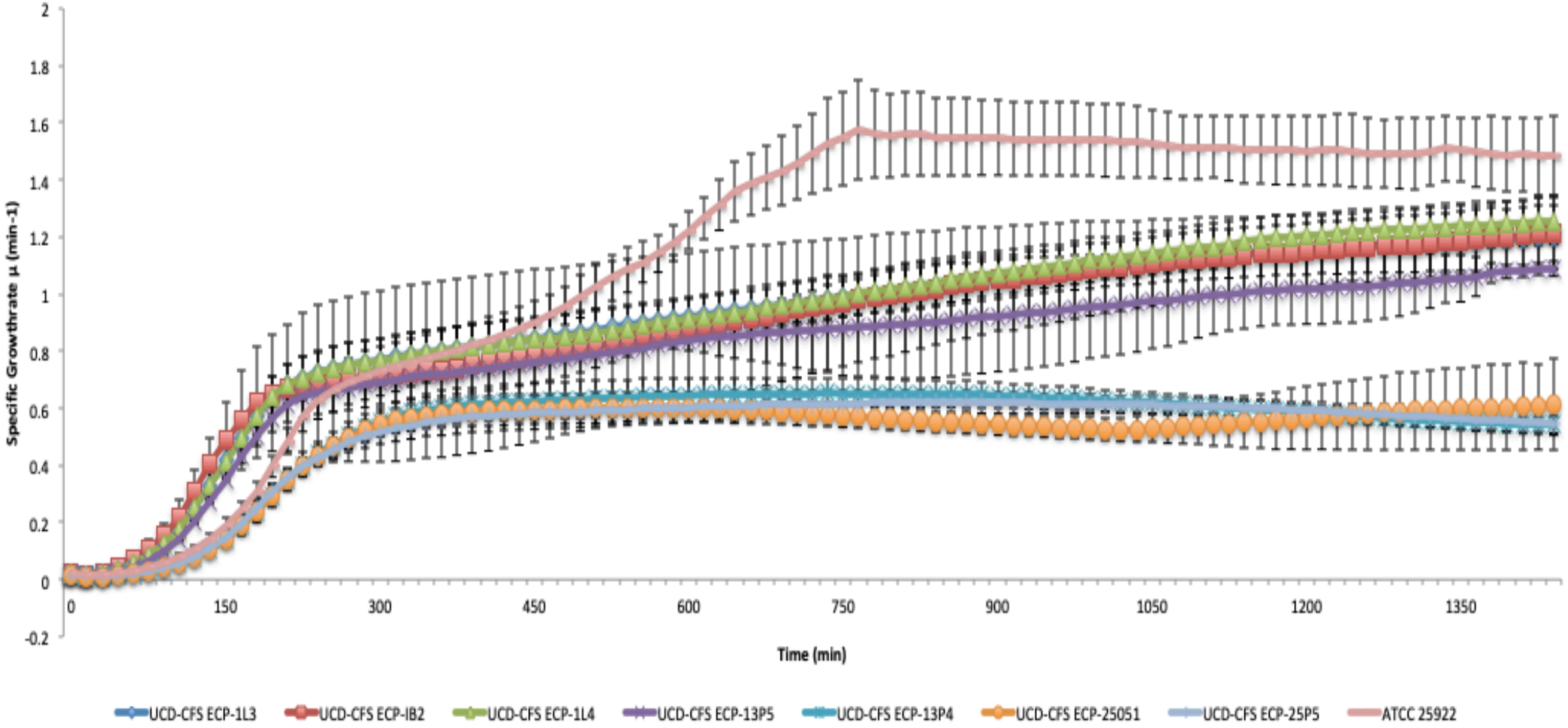


Table 3.3. Baseline values for specific growth rate (h^{-1}), Lag phase length (min) and Maximum OD_{600} for each isolate (ESBL type in brackets) (n=3).

± Standard deviation of the mean of 3 replicates

ISOLATE	SPECIFIC GROWTH RATE	LAG PHASE LENGTH	MAXIMUM OD_{600nm}
ATCC 25922	0.12 ± 0.01	128.0 ± 8.2	1.60 ± 0.16
UCD-CFS ECP-1L3 (CTX-M-14)	0.16 ± 0.04	88 .0± 11.3	1.20 ± 0.11
UCD-CFS ECP-1L4 (CTX-M-14)	0.15 ± 0.02	88.0 ± 11.3	1.26 ± 0.08
UCD-CFS ECP-IB2 (CTX-M-14)	0.17 ± 0.04	83.0 ± 12.5	1.22 ± 0.13
UCD-CFS ECP-13P5 (CTX-M-15)	0.12 ± 0.01	138.0 ± 11.3	0.67 ± 0.04
UCD-CFS ECP-13P4 (CTX-M-15)	0.16 ± 0.02	100.0 ± 7.7	1.38 ± 0.02
UCD-CFS ECP-25P5 (TEM-20)	0.13 ± 0.02	138.0 ± 11.3	0.63 ± 0.03
UCD-CFS ECP-25OS1 (TEM-20)	0.12 ± 0.01	140.0 ± 12.2	0.67 ± 0.12

3.3.3.1. Growth kinetics in the presence of CHX

3.3.3.1.1 Specific growth rate and the application of minimal selective concentrations

Growth rates and percentage increase and decrease growth rate in the absence of biocides were calculated (**Table 3.4**) (🡅= Increase; 🡇= Decrease). The addition of CHX at 0.00005 mg/mL, 0.0002 mg/mL and 0.0008 mg/mL triggered an increase in specific growth rate for all isolates (except for UCD-CFS ECP-IB2 at 0.0008 mg/mL; 1% 🡇. There was a significant difference in growth rates between non-exposed and exposed strains (P=<0.0001; T-TEST; Graphpad PRISM8). As previously mentioned Wright and Gilbert (1987) found that a few small, transient CHX susceptibility differences at low growth rate (ca 0.08 h^{-1}) in *E. coli* however these data were inconclusive (Wright and Gilbert, 1987). There was no significant difference between growth rates for CHX exposure at concentrations 0.00005 mg/mL and 0.0002 mg/mL (P=0.16 T-TEST; Graphpad PRISM8) and between 0.0002 mg/mL and 0.0008 mg/mL. (P=0.38: T-TEST; Graphpad PRISM8). Gomez Escalada *et al*. (2005) demonstrated that triclosan at sub-inhibitory concentrations affected the growth rate of *E. coli,* although this link was not concentration dependent (Gomez Escalada *et al*., 2005), the significant difference was found between non-exposed and exposed bacteria. Here, the specific growth rate decreased for most isolates that survive CHX 0.002 mg/mL. There was a significant difference between specific growth rates at concentrations 0.0008 mg/mL and 0.002 mg/mL (P=0.0001; T-TEST; Graphpad PRISM8). The decrease of specific growth rate has been associated with decreased sensitivity to antimicrobials and biocides. Here, it was demonstrated that low concentrations (0.00005 mg/mL, 0.0002 mg/mL and 0.0008 mg/mL) of CHX increased specific growth rate. On the contrary, higher concentrations of CHX (≥ 0.002 mg/mL)

decreased specific growth rate of *E. coli*. Furthermore, there was a statistically significant difference of growth rates between strains (Inclusive of ATCC25922 - P=<0.0001; Exclusive of ATCC25922 – P=<0.0001; ONE WAY ANOVA; Graphpad PRISM8). Our data suggest that the alteration of specific growth rate in response to CHX presence was strain dependent.

Specific growth rate was applied with the intention of investigating whether a Minimal Selective Concentration (MSC) or a "sub-MIC selective window" could be observed with biocide as was previously identified in the case for antibiotics (Gullberg *et al*., 2011, Sandegren, 2014). This method has been applied to free chlorine and monochloramine (Microorganisms: *Bacillus*, *Paenibacillus, Acidovarax* & *Micrococcus*) with success (Khan *et al*., 2017). **Figure 3.8** shows the specific growth rates of all *E. coli* isolates in halving concentrations of CHX. When analysing this data, MIC_{susc}= minimal inhibitory concentration of the susceptible strain, MIC_{res}= minimal inhibitory concentration of the resistant strain and MSC= minimal selective concentration.

Table 3.5. Minimal selective concentrations for CHX sensitive isolates UCD-CFE ECP-1L3 and UCD-CFE ECP-1B2 (ESBL type in brackets) when compared to CHX tolerant isolate UCD-CFE ECP-13P5.

STRAIN	**MSC**	**MIC*susc***	**MIC*susc*/MSC**
UCD-CFE ECP-1L3 (CTX-M-14)	0.0014	0.002	1.4
UCD-CFE ECP-1B2 (CTX-M-14)	0.00005	0.005	100

Isolates ATCC25922, UCD-CFE ECP-1L4, UCD-CFE ECP-13P5, UCD-CFE ECP-13P4, UCD-CFE ECP-25P5 and UCD-CFE ECP-25051 did not demonstrate an MSC. In this study, UCD-CFE ECP-13P5 had the highest MIC of 0.02 mg/mL (**Figure 3.9**). MSC values were identified for UCD-CFE ECP-13P5 when compared to UCD-CFE ECP-1L3 and UCD-CFE ECP-13P5 when compared to UCD-CFE ECP-1B2 (**Figure 3.9 and Figure 3.10**). **Table 3.5** shows the MSC and its ratio with MIC*susc.* The MSC for UCD-CFS ECP-1B2 is $1/100_{th}$ of the MIC*susc.*

Table 3.4. Specific growth rate (h^{-1}) and percentage (%) increase or decrease compared to non-exposed *E. coli* in varying concentrations of CHX (n=3).

	CHX CONCENTRATION (mg/mL)						
ISOLATE	**0.00005**	**0.0002**	**0.0008**	**0.002**	**0.005**	**0.01**	**0.02**
ATCC 25922	0.16 ± 0.03 36% ↑	0.15 ± 0.02 23% ↑	0.15 ± 0.03 25% ↑	0.06 ± 0.05 48% ↓	-	-	-
UCD-CFS ECP-1L3 (CTX-M-14)	0.17 ± 0.01 8% ↑	0.17 ± 0.03 4% ↑	0.17 ± 0.01 4% ↑	0.17 ± 0.05 6% ↑	-	-	-
UCD-CFS ECP-1L4 (CTX-M-14)	0.18 ± 0.02 21% ↑	0.17 ± 0.00 12% ↑	0.17 ± 0.01 10% ↑	0.02 ± 0.04 84% ↓	-	-	-
UCD-CFS ECP-IB2 (CTX-M-14)	0.19 ± 0.02 13% ↑	0.17 ± 0.01 3% ↑	0.17 ± 0.01 1% ↓	0.11 ± 0.13 37% ↓	-	-	-
UCD-CFS ECP-13P5 (CTX-M-15)	0.19 ± 0.02 22% ↑	0.18 ± 0.03 14% ↑	0.19 ± 0.02 17% ↑	0.13 ± 0.02 17% ↓	0.10 ± 0.03 37% ↓	0.07 ± 0.03 57% ↓	-
UCD-CFS ECP-13P4 (CTX-M-15)	0.16 ± 0.01 31% ↑	0.15 ± 0.01 25% ↑	0.15 ± 0.02 27% ↑	0.05 ± 0.08 60% ↓	-	-	-
UCD-CFS ECP-25P5 (TEM-20)	0.15 ± 0.01 15% ↑	0.14 ± 0.02 15% ↑	0.14 ± 0.00 13% ↑	-	-	-	-
UCD-CFS ECP-25OS1 (TEM-20)	0.15 ± 0.01 25% ↑	0.16 ± 0.01 30% ↑	0.16 ± 0.03 31□↑	-	-	-	-

± Standard deviation of the mean of 3 replicates ↑= Increase; ↓= Decrease - indicates no growth

Figure. 3.8 Specific growth rates (min^{-1}) of *E. coli* isolates incubated in the presence of varying concentrations of CHX (n=3).

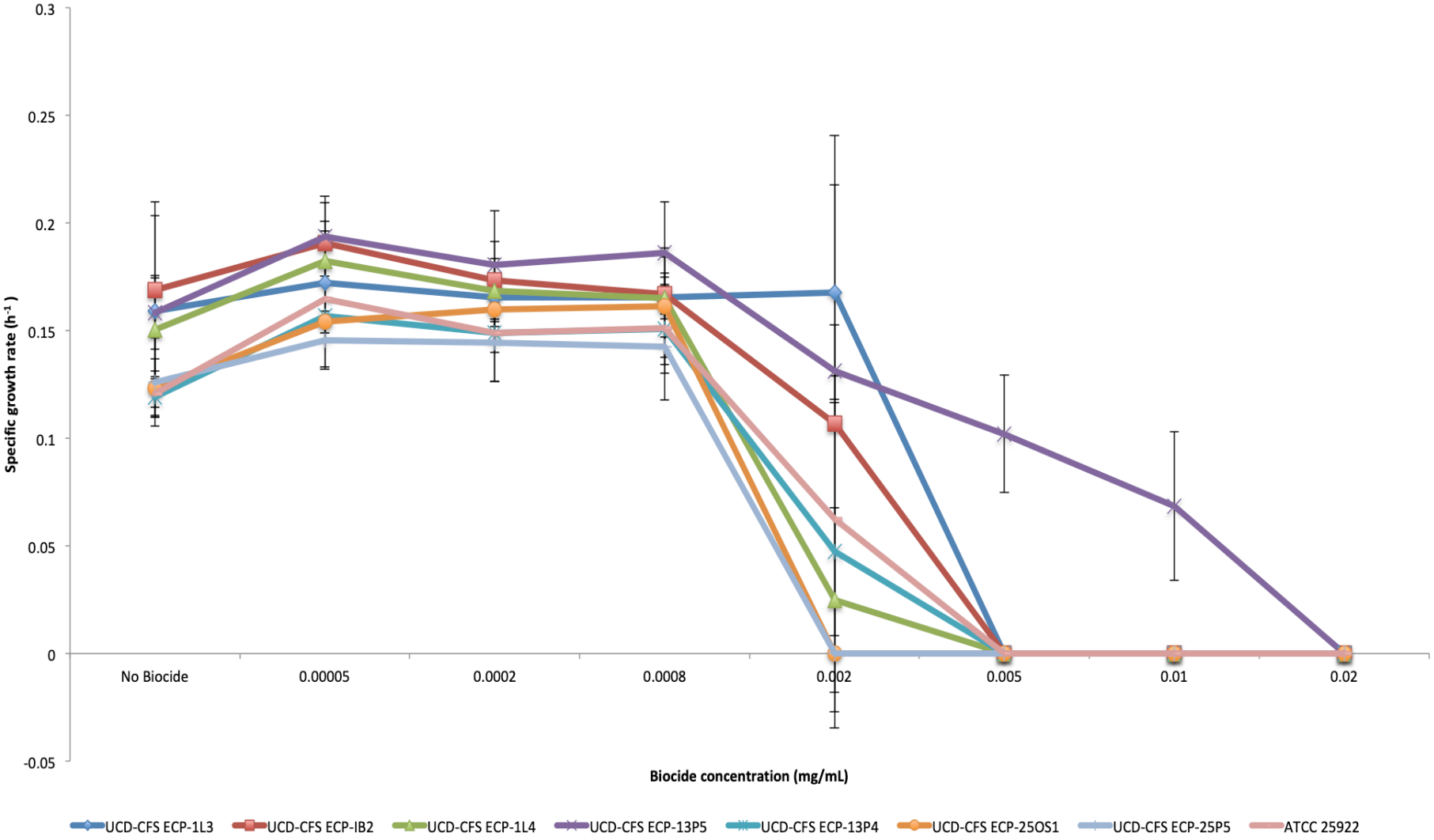


Figure 3.9. Minimal selective concentration (MSC) of UCD-CFE ECP-1L3 when compared with UCD-CFE ECP-13P5.

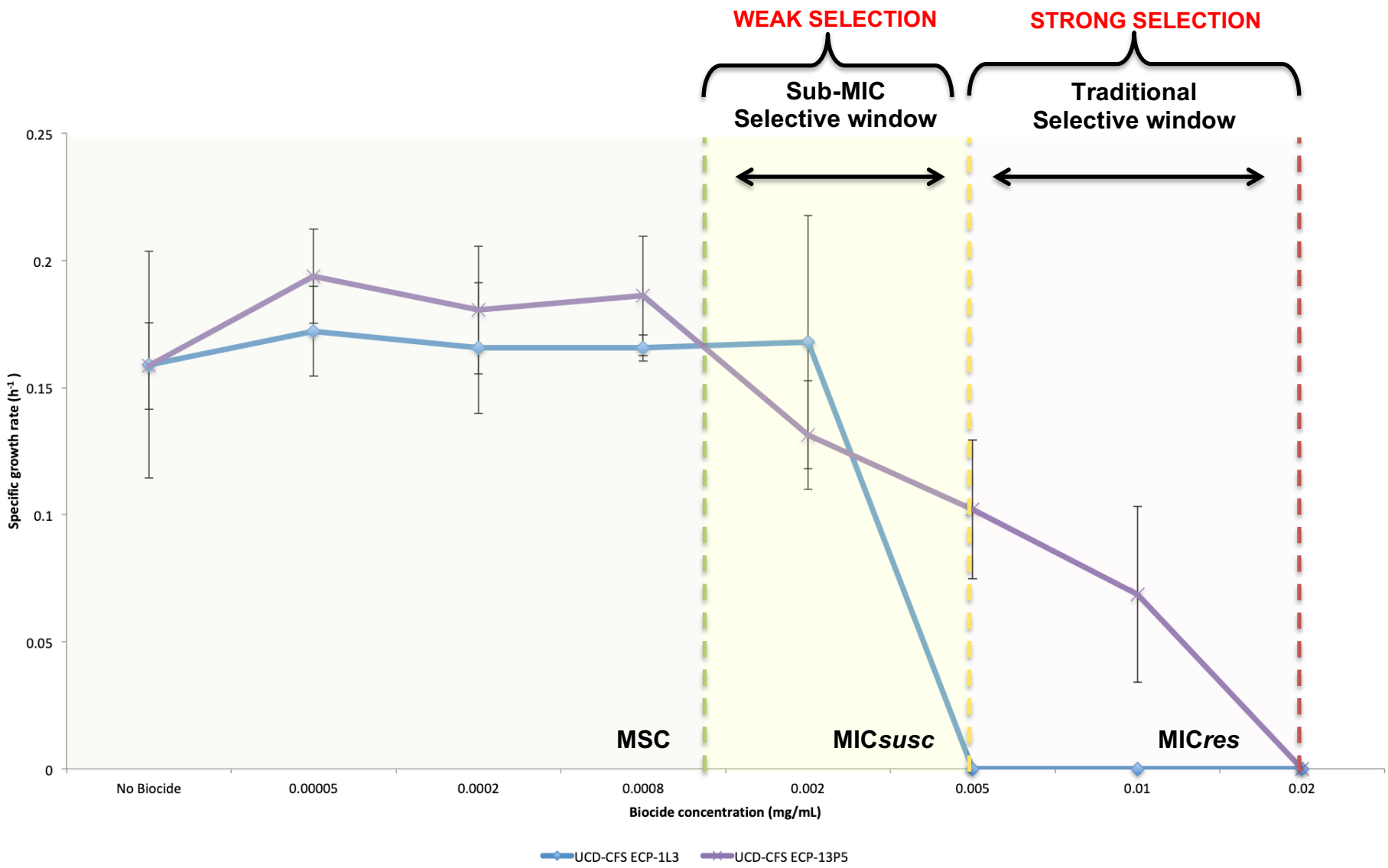
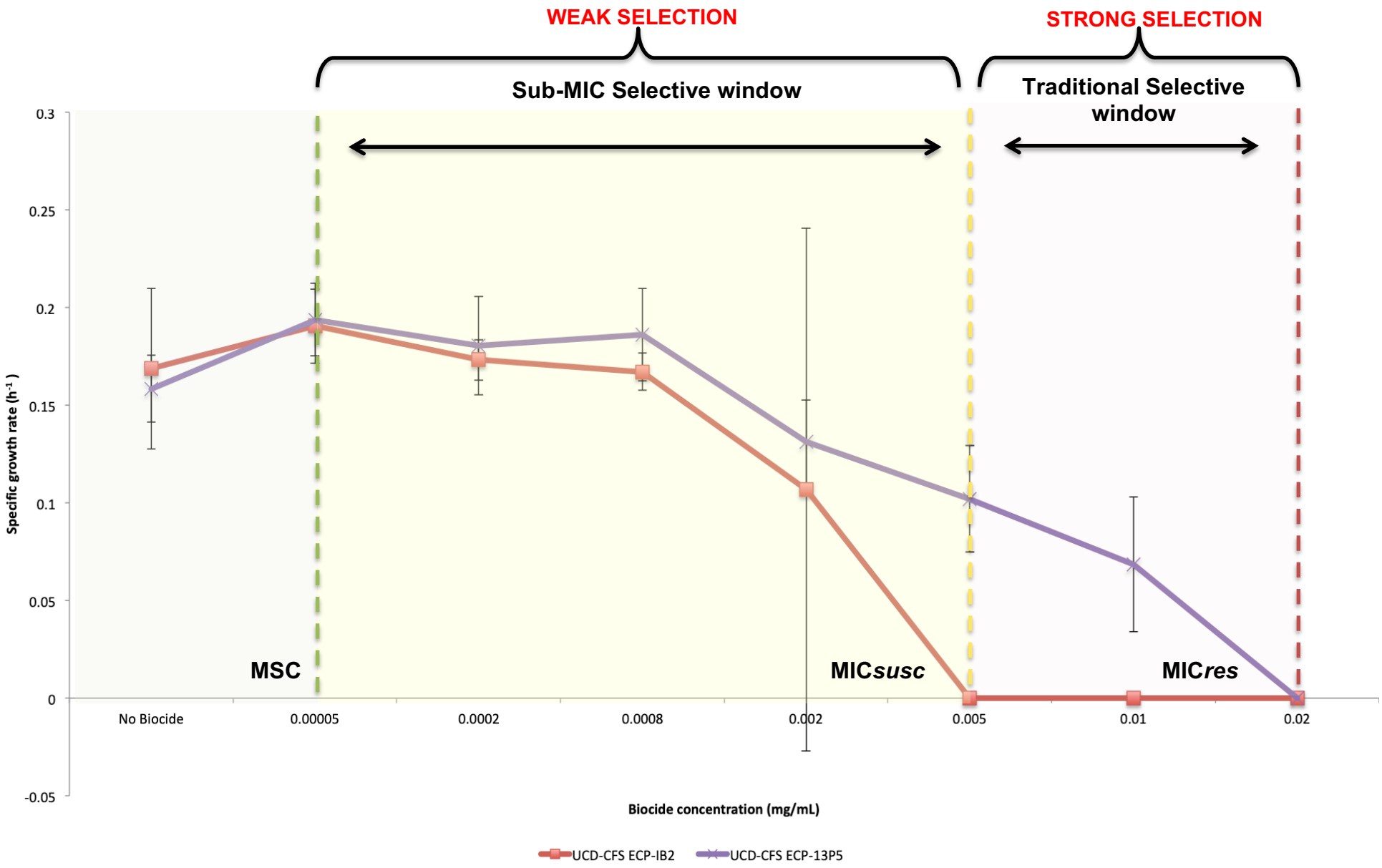


Figure 3.10. Minimal selective concentration (MSC) of UCD-CFE ECP-1B2 when compared with UCD-CFE ECP-13P5.



3.3.3.1.2 Lag phase extension

Table 3.6 shows the lag phase length (*λ*) of *E. coli* isolates in the presence of various concentrations of CHX. CHX concentration effected statistically significant increase in lag phase, a statistically (P=<0.0001; TWO WAY ANOVA; Graphpad prism8). As CHX concentration increases, so does the lag phase for isolates UCD-CFS ECP-1L3, UCD-CFS ECP-1B2, UCD-CFS ECP-13P5, UCD-CFS ECP-13P4 and UCD-CFS ECP-25051. There was a significant difference for lag phase length between strains (P=0.0001; TWO WAY ANOVA; Graphpad PRISM8), reflecting the baseline data obtained and indicating that this is likely due a strain dependent physiologically controlled process. UCD-CFS ECP-13P5 demonstrated the longest lag phase length overall (615 ± 584 min) at 0.01 mg/mL CHX, the concentration immediately below the MIC. However, this value has a high standard deviation suggesting that lag phase at this concentration is highly variable. Isolate UCD-CFS ECP-13P5 had an LE value of 12.00 at 0.01 mg/mL CHX indicating that Lag phase length is twelve times longer at this concentration. This isolate has the highest MIC, although LE12.00 is not the highest LE value observed, assuming that LE is a determinant of increased tolerance (Li *et al*., 2016) the rising LE value may be reflective of adaptation to CHX which lends UCD-CFS ECP-13P5 with an elevated MIC. UCD-CFS ECP-1L3 has the highest value (LE = 14.20 min) at 0.002 mg/mL CHX, which is the concentration immediately below the MIC for this isolate.

3.3.3.1.3 Maximum OD_{600}

Table 3.7 shows the maximum Optical Density values obtains after 24h incubation in the presence of halving concentrations of CHX. OD is based on turbidity of a cell suspension (Koch, 1961, Koch, 1970) and is applied as a measurement of cell growth and is implemented assuming that the OD value is directly proportional to

actual cell number (Stevenson *et al*., 2016). As CHX concentration increases, the maximum OD_{600} reached appeared to decrease with the exception of isolates ATCC25922 and UCD-CFS ECP-13P5 (**Table 3.7**). This trend however is not statistically significant between no biocide and 0.00005 mg/mL CHX (*P*=0.88; T-TEST; Graphpad PRISM8) but is between 0.0008 mg/mL and 0.002 mg/mL CHX (*P*=0.0004; T-TEST; Graphpad PRISM8). A lower maximum OD suggests a lower actual cell count, which indicates that a fitness cost has been present throughout cell replication (Hall *et al*., 2014).

Table 3.6. Lag phase length (min) and Lag phase extension (LE) after 24 hours incubation in varying concentrations of CHX (n=3).

	BIOCIDE CONCENTRATION (mg/mL)							
	No Biocide	**0.00005**	**0.0002**	**0.0008**	**0.002**	**0.005**	**0.01**	**0.02**
ATCC 25922	120 ± 0	130 ± 17 LE1.00	120 ± 0 LE1.00	135 ± 0 LE1.13	540 ± 476 LE7.63	-	-	-
UCD-CFS ECP-1L3 (CTX-M-14)	80 ± 9	95 ± 9 LE1.20	90 ± 0 LE1.20	95 ± 9 LE1.20	160 ± 277 LE14.20	-	-	-
UCD-CFS ECP-1L4 (CTX-M-14)	80 ± 9	85 ± 9 LE1.00	95 ± 9 LE1.00	100 ± 9 LE1.00	240 ± 416 *	-	-	-
UCD-CFS ECP-lB2 (CTX-M-14)	75 ± 15	80 ± 9 LE1.00	90 ± 0 LE1.00	85 ± 9 LE1.20	595 ± 548 LE14.20	-	-	-
UCD-CFS ECP-13P5 (CTX-M-15)	95 ± 9	100 ± 9 LE1.17	100 ± 9 LE1.11	100 ± 9 LE1.17	290 ± 109 LE2.50	560 ± 60.62 LE6.00	615 ± 548 LE12.00	-
UCD-CFS ECP-13P4 (CTX-M-15)	135 ± 15	145 ± 9 LE1.00	140 ± 17 LE1.00	150 ± 15 LE1.11	240 ± 416 *	-	-	-
UCD-CFS ECP-25P5 (TEM-20)	130 ± 9	150 ± 30 LE1.00	145 ± 23 LE1.00	145 ± 23 LE1.00	-	-	-	-
UCD-CFS ECP-25OS1 (TEM-20)	135 ± 15	145 ± 9 LE1.11	143.33 ± 19 LE1.00	151.66 ± 14 LE1.11	-	-	-	-

± Standard deviation of the mean of 3 replicates * Value not obtained - Indicates no growth

Table 3.7. Maximum optical density reached (OD_{600}) after 24 hours incubation in varying concentrations of CHX (n=3).

	BIOCIDE CONCENTRATION (mg/mL)						
ISOLATE	**No Biocide**	**0.00005**	**0.0002**	**0.0008**	**0.002**	**0.005**	**0.01**
ATCC 25922	1.60 ± 0.16	1.57 ± 0.24	1.41 ± 0.16	1.47 ± 0.21	0.57 ± 0.52	-	-
UCD-CFS ECP-1L3 (CTX-M-14)	1.20 ± 0.11	1.26 ± 0.29	1.09 ± 0.26	1.14 ± 0.22	0.24 ± 0.38	-	-
UCD-CFS ECP-1L4 (CTX-M-14)	1.25 ± 0.08	1.30 ± 0.27	1.19 ± 0.27	1.09 ± 0.25	0.15 ± 0.16	-	-
UCD-CFS ECP-IB2 (CTX-M-14)	1.21 ± 0.12	1.33 ± 0.26	1.16 ± 0.24	0.99 ± 0.18	0.23 ± 0.19	-	-
UCD-CFS ECP-13P5 (CTX-M-15)	1.37 ± 0.02	1.15 ± 0.33	0.82 ± 0.21	0.91 ± 0.35	0.64 ± 0.19	0.71 ± 0.26	-
UCD-CFS ECP-13P4 (CTX-M-15)	0.67 ± 0.12	0.91 ± 0.31	0.74 ± 0.25	0.66 ± 0.16	0.31 ± 0.51	-	-
UCD-CFS ECP-25P5 (TEM-20)	0.63 ± 0.03	0.95 ± 0.33	0.60 ± 0.11	0.58 ± 0.90	-	-	-
UCD-CFS ECP-25OS1 (TEM-20)	0.67 ± 0.12	0.91 ± 0.31	0.58 ± 0.07	0.57 ± 0.10	-	-	-

± Standard deviation of the mean of 3 replicates

3.3.4. Growth kinetics in the presence of BZC

3.3.4.1. Specific growth rate and the application of minimal selective concentrations

Table 3.8 shows the specific growth rate values and the percentage increase or decrease between BZC exposed and non-exposed cells. When evaluating the effect of BZC on the percentage increase or decrease of specific growth rate, it was notable that there was always a decrease at the concentration immediately below the BZC MIC (0.002 mg/mL) **(Table 3.8).** The lowest decrease was in the case of isolate UCD-CFS ECP-13P4 (3%) and the highest was that of 1L3 (69%). Although there was a difference for increase or decrease in specific growth rate and concentration, the correlation was not concentration dependent from 0.00005 mg/mL to 0.0008 mg/mL (P=<0.0001 ONE-WAY ANOVA; Graphpad PRISM8), neither was it dependent on strain (P=0.88 ONE-WAY ANOVA; Graphpad PRISM8). However, 0.002 mg/mL BZC caused a consistent decrease in growth rates for all isolates.

Whitehead *et al*. (2011) worked with *S. enterica*, which, in response to high-level QAC exposure (in-use concentration; 10 mg/mL) exhibited consistent, reduced growth rates and low-level decreased sensitivity to ciprofloxacin, tetracycline and chloramphenicol, although no change is sensitivity to the challenge biocide was observed. In response to a low dose QAC challenge (0.05 mg/mL) survivors demonstrated the same or reduced growth rates as the initial inoculum, and the same survival percentage when re-challenged, suggesting that adaptation under these conditions was a transient physiological process (Wales and Davies, 2015). Data from **Table 3.8** suggests similar pattern for *E. coli*, at BZC concentrations

below 0.002 mg/mL specific growth rate was not affected. However, at 0.002 mg/mL caused specific growth rate to decrease.

Figure 3.11 shows growth rates of *E. coli* isolates incubated in halving concentrations of BZC. A minimal selective concentration range was not observed. In previous studies based upon the MSC a known resistant strain is used as a comparison with a known susceptible strain. In this case, all isolates had the same MIC meaning that comparison could not be made.

3.3.4.2. Lag phase extension

Table 3.9 shows the lag phase length and lag phase extensions for *E. coli* in halving concentrations of BZC. There was an increase in lag phase length for all isolates at BZC 0.002 mg/mL. No statistically significant difference (P=>0.97 T-TEST; Graphpad PRISM8) was found between no biocide exposed isolates and exposure to BZC 0.00005 mg/mL, 0.0002 mg/mL and 0.0008 mg/mL. However, there was a significant difference (P= 0.002 T-TEST; Graphpad PRISM8) between lag phases at 0.0008 mg/mL and 0.002 mg/mL BZC. A difference was also observed between strains suggesting that the resistance genes carried by strains UDC-CFS ECP-1L3, UDC-CFS ECP-1L4, UDC-CFS ECP-1B2, UDC-CFS ECP-13P5, UDC-CFS ECP-13P4, UDC-CFS ECP-25P2 and UDC-CFS ECP-25051 do may affect this part of their growth kinetics. The highest lag phase length (610 ± 62.5 min) and LE (4.52) was observed for isolate ATCC25922. As previously discussed, all strains had the same MIC for BZC (**Table 3.2**).

3.3.4.3. Maximum OD_{600}

Table 3.10 shows the maximum Optical Density values obtains after 24 h incubation in the presence of halving concentrations of BZC. There were no concentration dependent changes in maximum OD (P=0.88 Two-Way ANOVA; Graphpad PRISM8). There were strain dependent changes in maximum OD observed (P=0.67 Two-Way ANOVA; Graphpad PRISM8). These data suggest that the lowest and (highest immediately below the MIC) concentration of BZC tested, had no consistent effect on maximum OD.

Table 3.8. Specific growth rate (h^{-1}) and percentage (%) increase or decrease compared to non-exposed *E. coli* in varying concentrations of BZC (n=3).

	BZC CONCENTRATION (mg/mL)						
ISOLATE	**0.00005**	**0.0002**	**0.0008**	**0.002**	**0.005**	**0.01**	**0.02**
ATCC 25922	0.16 ± 0.01 11% ↑	0.14 ± 0.01 2% ↓	0.16 ± 0.02 9% ↑	0.2 ± 0.01 31% ↓	-	-	-
UCD-CFS ECP-1L3 (CTX-M-14)	0.16 ± 0.01 19% ↓	0.18 ± 0.01 11% ↓	0.17 ± 0.01 17% ↓	0.06 ± 0.01 69% ↓	-	-	-
UCD-CFS ECP-1L4 (CTX-M-14)	0.18 ± 0.01 0.21% ↑	0.18 ± 0.01 3% ↑	0.16 ± 0.01 10% ↓	0.08 ± 0.03 57% ↓	-	-	-
UCD-CFS ECP-IB2 (CTX-M-14)	0.19 ± 0.01 2% ↓	0.18 ± 0.01 9% ↓	0.18 ± 0.01 7% ↓	0.11 ± 0.02 43% ↓	-	-	-
UCD-CFS ECP-13P5 (CTX-M-15)	0.17 ± 0.01 2% ↑	0.18 ± 0.01 8% ↑	0.19 ± 0.00 11% ↑	0.15 ± 0.00 7% ↓	-	-	-
UCD-CFS ECP-13P4 (CTX-M-15)	0.17 ± 0.01 26% ↑	0.15 ± 0.01 8% ↑	0.16 ± 0.00 18% ↑	0.13 ± 0.02 3% ↓	-	-	-
UCD-CFS ECP-25P5 (TEM-20)	0.15 ± 0.01 9% ↓	0.16 ± 0.00 0.25% ↑	0.15 ± 0.01 8% ↓	0.14 ± 0.01 15□↓	-	-	-
UCD-CFS ECP-25OS1 (TEM-20)	0.16 ± 0.01 6% ↓	0.15 ± 0.01 14% ↓	0.15 ± 0.01 5□↓	0.15 ± 0.02 10□↓	-	-	-

± Standard deviation of the mean of 3 replicates ↑= Increase; ↓= Decrease - indicates no growth

Figure 3.11. Specific growth rates (h^{-1}) of *E. coli* isolates incubated in the presence of varying concentrations of BZC (n=3).

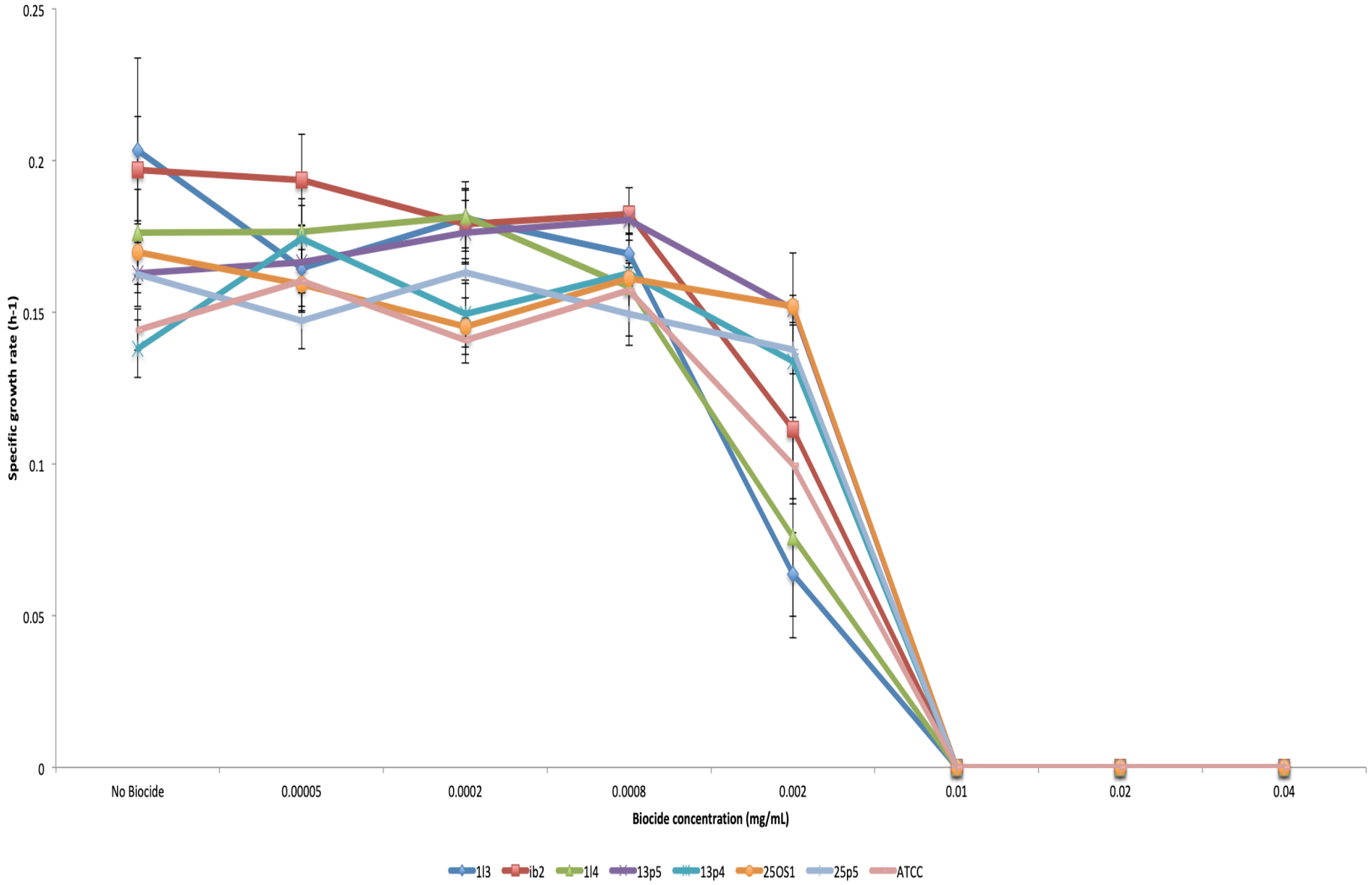


Table 3.9. Lag phase length (min) and Lag phase extension (LE) after 24 hours incubation in varying concentrations of BZC (n=3).

	BIOCIDE CONCENTRATION (mg/mL)							
ISOLATE	No Biocide	**0.00005**	**0.0002**	**0.0008**	**0.002**	**0.005**	**0.01**	**0.02**
ATCC 25922	135 ± 0	135 ± 0 LE1.00	135 ± 0 LE1.00	155 ± 34.64 LE1.15	610 ± 62.5 LE4.52	-	-	-
UCD-CFS ECP-1L3 (CTX-M-14)	95 ± 8.66	95 ± 8.66 LE1.00	100 ± 8.66 LE1.05	95 ± 8.66 LE1.00	315 ± 77.94 LE3.32	-	-	-
UCD-CFS ECP-1L4 (CTX-M-14)	95 ± 8.66	95 ± 8.66 LE1.00	100 ± 8.66 LE1.05	105 ± 15 LE1.11	370 ± 31.2 LE3.89	-	-	-
UCD-CFS ECP-IB2 (CTX-M-14)	90 ± 0	95 ± 8.66 LE1.06	95 ± 8.66 LE1.06	100 ± 8.66 LE1.11	200 ± 113.58 LE2.22	-	-	-
UCD-CFS ECP-13P5 (CTX-M-15)	105 ± 0	100 ± 8.66 LE0.95	100 ± 8.66 LE0.95	105 ± 0 LE1.00	125 ± 8.66 LE1.19	-	-	-
UCD-CFS ECP-13P4 (CTX-M-15)	140 ± 8.66	150 ± 15 LE1.07	145 ± 8.66 LE1.04	150 ± 15 LE1.07	165 ± 26 LE1.18	-	-	-
UCD-CFS ECP-25P5 (TEM-20)	145 ± 8.66	145 ± 8.66 LE1.00	145 ± 8.66 LE1.00	145 ± 8.66 LE1.00	165 ± 15 LE1.14	-	-	-
UCD-CFS ECP-25OS1 (TEM-20)	145 ± 8.66	140 ± 8.66 LE0.97	145 ± 8.66 LE1.00	140 ± 17.32 LE0.97	160 ± 17.32 LE1.10	-	-	-

± Standard deviation of the mean of 9 replicates * Value not obtained - Indicates no growth

Table 3.10. Maximum Optical density reached (OD_{600}) after 24 hours incubation in varying concentrations of BZC (n=3).

	BIOCIDE CONCENTRATION (mg/mL)						
ISOLATE	No Biocide	**0.00005**	**0.0002**	**0.0008**	**0.002**	**0.005**	**0.01**
ATCC 25922	1.60 ± 0.16	1.62 ± 0.08	1.62 ± 0.07	1.60 ± 0.07	0.75 ± 0.10	-	-
UCD-CFS ECP-1L3 (CTX-M-14)	1.20 ± 0.11	1.30 ± 0.13	1.23 ± 0.13	1.41 ± 0.04	1.25 ± 0.02	-	-
UCD-CFS ECP-1L4 (CTX-M-14)	1.25 ± 0.08	1.16 ± 0.16	1.39 ± 0.14	1.17 ± 0.26	1.14 ± 0.07	-	-
UCD-CFS ECP-IB2 (CTX-M-14)	1.21 ± 0.12	1.31 ± 0.23	1.33 ± 0.20	1.31 ± 0.12	1.18 ± 0.25	-	-
UCD-CFS ECP-13P5 (CTX-M-15)	1.37 ± 0.02	1.04 ± 0.29	1.20 ± 0.31	1.49 ± 0.14	1.25 ± 0.01	-	-
UCD-CFS ECP-13P4 (CTX-M-15)	0.67 ± 0.12	0.90 ± 0.28	0.79 ± 0.35	1.09 ± 0.12	1.06 ± 0.11	-	-
UCD-CFS ECP-25P5 (TEM-20)	0.63 ± 0.03	1.29 ± 0.12	0.72 ± 0.21	0.92 ± 0.33	0.75 ± 0.21	-	-
UCD-CFS ECP-25OS1 (TEM-20)	0.67 ± 0.12	0.96 ± 0.26	0.86 ± 0.43	0.97 ± 0.34	1.03 ± 0.09	-	-

± Standard deviation of the mean of 9 replicates

3.3.5. Baseline antibiotic susceptibility

Table 3.11. shows the baseline antibiotic susceptibility values based on the EUCAST disc diffusion method (EUCAST, 2015). Antibiotic resistance was highlighted in red. The standard reference strain ATCC25922 was susceptible to all antibiotics tested **(Table 3.11**). This identifies a distinct difference between this strain and the other isolates. None of the isolates tested presented resistance to imipenem. This supports the findings of Zhao *et al*., (2013) who identified activity against CTX-M ESBL producing *E. coli* (Zhao *et al*., 2014). Furthermore, that study identified resistance of CTX-M ESBL producing isolates to ampicillin (17 isolates), cefotoxin (3 isolates) and ciprofloxacin (12 isolates). Here, no isolates were found to be resistant to cefoxitin and ciprofloxacin, however all isolates except UCD-CFS ECP-1B2 were resistant to ampicillin.

A recent study undertaken by Xie *et al*. (2016) identified 37 CTX-M-type ESBL producing isolates that carried CTX-M-type resistance to naladixic acid, trimethoprim- sulfamethoxazole, chloramphenicol, and ciprofloxacin (Xie *et al*., 2016). Data in **Table 3.11** highlight resistance to trimethoprim and trimethoprim-sulfamethoxazole (UCD-CFS ECP-1L3, UCD-CFS ECP-1L4, UCD-CFS ECP-1B2 and UCD-CFS ECP-13P5). Isolates UCD-CFS ECP-1L3, UCD-CFS ECP-1L4 and UCD-CFS ECP-13P5 demonstrated the same antibiotic resistant phenotypes with resistance to amoxicillin, cefpodoxime, tetracycline, streptomycin, cephalothin, trimethoprim and trimethoprim-sulfamethoxazole. Isolates UCD-CFS ECP-13P4, UCD-CFS ECP-25P5 and UCD-CFS ECP-25051 are grouped together for their resistance to amoxicillin, cefpodoxime and cephalothin. Isolates UCD-CFS ECP-1B2 and UCD-CFS ECP-13P5 are resistant to streptomycin (**Table 3.11**),

Table 3.11. Antibiogram to show the zones of inhibition of all test strains when exposed to chosen antibiotics in the EUCAST disk diffusion assay (n=3).

ANTIBIOTIC	ATCC25922	UCD-CFS ECP-1L3	UCD-CFS ECP-1L4	UCD-CFS ECP-IB2	UCD-CFS ECP-13P5	UCD-CFS ECP-13P4	UCD-CFS ECP-25P5	UCD-CFS ECP-25O51
Ampicillin	19.5 (1.53)	0 (0)	0 (0)	19.5 (0.58)	0 (0)	0 (0)	0 (0)	0 (0)
Amoxicillin/clavulanic acid	21.5 (1)	19 (1.15)	19 (0.58)	21.5 (1)	20 (0)	22.5 (2.12)	22 (1.15)	23 (1.73)
Chloramphenicol	24.5 (1)	24 (1.15)	23.5 (1)	20.5 (1)	24.5 (0.58)	24.5 (2.12)	25.5 (1)	23.5 (2.52)
Ciprofloxacin	31.5 (1.53)	31 (1.53)	31 (1)	28 (1.53)	33.5 (4.04)	40.5 (0)	44 (4.58)	40 (0)
Cefpodoxime	24 (1.53)	0 (0)	0 (0)	21 (3.06)	0 (0)	0 (0)	0 (0)	0 (0)
Cefoxitin	26 (3.06)	22 (1.15)	22 (1.15)	21 (0.58)	24 (1)	31.5 (0.71)	32 (1.53)	31.5 (1)
Tetracycline	23.5 (0.58)	*0 (0)	*0 (0)	*0 (0)	*0 (0)	27.5 (0.71)	29.5 (1)	28.5 (1.73)
Streptomycin	13.5 (1)	12 (0.58)	11	*0 (0)	*0 (0)	17 (1.41)	17 (0)	17 (1.15)
Imipenem	29.5 (0.58)	28.5 (1)	28 (0.58)	28.5 (1)	31.5 (1)	33 (1.41)	32 (0.58)	31.5 (0.6)
Cephalothin	16 (1.15)	*0 (0)	*0 (0)	17.5 (1)	*0 (0)	*0 (0)	*0 (0)	*0 (0)
Nalidixic acid	25.5 (1)	21 (0)	22 (0.58)	20.5 (0.58)	24 (0.58)	22 (1.41)	23.5 (2.52)	22.5 (2.1)
Trimethoprim-sulfamethoxazole	23 (0.06)	0 (0)	0 (0)	0 (0)	0 (0)	34 (0.15)	33 (0.06)	32 (0.12)
Trimethoprim	22 (0.1)	0 (0)	0 (0)	0 (0)	0 (0)	34 (0.06)	33 (0.06)	34 (0.06)

± Standard deviation of the mean of 3 replicates

- No EUCAST definition data available, in the case of insusceptibility the value is considered resistant.

  Where EUCAST breakpoints determined resistance, values are highlighted in red.

Table 3.12 highlights the ESBL resistance characteristics of the isolates and the shared intrinsic antibiotic resistance that was identified in Table 3.10. The principal ESBL families of clinical importance include TEM and CTX-M (Bush and Jacoby, 2010). Pitout *et al*., (2004) identified that all isolates studied that presented CTX-M-14 and CTX-M-15 were resistant to CPD. This would support the findings in **Table 3.11** where isolates UCD-CFS ECP-13P5 and UCD-CFS ECP-13P4 who carry CTX-M-15 are CPD resistant. UCD-CFS ECP-1L3 and UCD-CFS ECP-1l4 which carry CTX-M-14 are also resistant to CPD, UCD-CFS ECP-1B2 is the only isolate with the CTX-M-14 gene to not have resistance to CPD (**Table 3.11**), AMP resistance has recently been discovered in *E. coli* isolated from rabbit farms in China containing TEM-type ESBLs (Zhao *et al*., 2018) which is in keeping with results from **Table 3.12** where isolates UCD-CFS ECP-25P5 and UCD-CFS ECP-25051 are AMP resistant.

Table 3.12. ESBL characteristics and shared antibiotic resistance observations

ISOLATE	ESBL	SHARED ANTIBIOTIC RESISTANCE
ATCC 25922	Information unattained	No resistance observed
UCD-CFS ECP-1L3 UCD-CFS ECP-1L4 UCD-CFS ECP-IB2	CTX-M-14	TE, W, SXT
UCD-CFS ECP-13P5 UCD-CFS ECP-13P4	CTX-M-15	AMP, CPD, CF
UCD-CFS ECP-25P5 UCD-CFS ECP-25OS1	TEM-20	AMP, CPD, CF

TE, tetracycline; W, Trimethoprim; SXT, trimethoprim-sulfamethoxazole; AMP, ampicillin; CPD, cefpodoxime; CF, cephalothin

Although isolate UCD-CFS ECP-13P5 (CTX-M-15) shares antibiotic resistance with UCD-CFS ECP-13P4 (CTX-M-15) as would be expected due to their common ESBLs, It also shares antibiotic resistance with all other ESBL carrying isolates which carry ESB-type CTX-M-14 and TEM-20. Previously demonstrated was that UCD-CFS ECP-13P5 has the highest CHX MIC and MBC of all of the isolates, this is significant as streptomycin and CHX both enter the cell via self-promoted uptake through the displacement of cations in the bacterial cell envelope and re-organisation of lipopolysaccharides (Hancock, 1981). The similarities in mode between streptomycin (and other aminoglycosides) and CHX have led to a discussion at to whether reduced CHX (and other biguanide biocides) uptake is linked to reduced aminoglycoside uptake.

3.4. Conclusion

Baseline biocide susceptibility profiles were obtained successfully using MIC/MBC determination of CHX and BZC. Differences in susceptibility were observed for CHX (**Table 3.1**). UCD-CFS ECP-13P5 had the highest MIC and MBC at 0.01 (± 0.00) mg/mL this level of susceptibility falls in the middle proportion of the ECOFF values demonstrated by Morrisey *et al*. (2014). MIC values for BZC ranged from 0.01-0.02 mg/mL for all isolates (**Table 3.2**); there was no significant difference between MICs between strains (P=0.13; TwoWay ANOVA; Graphpad PRISM8). The MIC and MBC values for BZC fell in the lower portion of the ECOFF values, 23.5% of isolates tested had an MIC and 28.3% had an MBC of 0.008 mg/mL (**Figure 3.3**). No clinically relevant resistance was found for any of the isolates to BZC or CHX according to the ECOFF values observed by Morrisey *et al*. (2014).

Baseline growth kinetics differed between strains with the exception of growth rate, which remained constant between isolates for BZC (P=0.008). The most variable differences following biocide exposure were found within lag phase length and maximum OD_{600} values. Li *et al*. (2016) suggested that lag phase extension (LE) is a determinant of decreased susceptibility in antibiotic and biocide exposed bacteria. UCD-CFS ECP-13P5 demonstrated the highest MIC and MBC to CHX (0.01mg/mL ± 0.00mg/mL), LE values in the presence of CHX for this isolate increased with concentration (**Table 3.6**). This relationship may be indicative of adaptive responses that lend to this isolate's elevated MIC (Li *et al*., 2016). However the highest LE value was observed for UCD-CFS ECP-1L3 (**Table 3.6**), which did not demonstrate an elevated MIC (0.000 ± 0.00 mg/mL) in the presence of CHX. For both CHX and BZC, a concentration of 0.002 mg/mL had the most altering effect on growth rate, lag phase length and maximum optical density reached. Baseline antibiotic susceptibility profiles are in keeping with expectations considering the drug exporter genes that we know our environmental isolates to express (**Table 3.12**)

Antibiotic susceptibility profiles were obtained, and breakpoints were assessed according to EUCAST (2015). The standard reference strain ATCC29522 was susceptible to all antibiotics tested (**Table 3.11**). Resistance was observed in isolates UCD-CFS ECP-1L3, UCD-CFS ECP-1L4, UCD-CFS ECP-1B2, UCD-CFS ECP-13P4, UCD-CFS ECP-13P5, UCD-CFS ECP-25P5 and UCD-CFS ECP-25051, Isolates shared resistance phenotype depending on the ESBL resistance gene they carry. Strains that possess CTX-M-14 (UCD-CFS ECP-1L3, UCD-CFS ECP-1L4 and UCD-CFS ECP-1B2) were resistant to tetracycline, trimethoprim and trimethoprim-sulfamethoxazole. Strains that have CTX-M-15 (UCD-CFS ECP-13P4 and UCD-CFS ECP-13P5) and TEM-20 (UCD-CFS ECP-25P5 and UCD-CFS ECP-25051) were resistant to ampicillin, cefpodoxime and cephalothin. The resistant phenotypes displayed by the isolates that carry ESBL resistance genes were in line with previous

findings (Pitout *et al*., 2004, Xie *et al*., 2016, Zhao *et al*., 2014). Isolate UCD-CFS ECP-13P5 has CTX-M-15, it shared common antibiotic resistance phenotypes with all other ESBL carrying isolates with ESBL-type CTX-M-14 and TEM-20. The combination of the antibiotic resistance profile and a higher MIC of UCD-CFS ECP-13P5 is significant as some antibiotics and CHX have a common mode of entry (Hancock,1981). It is debated that reduced CHX (and other biguanide biocides) uptake may be linked to reduced antibiotic uptake.

The 'minimal selective concentration' hypothesis, as first defined in relation antibiotics by Gullberg *et al*. (2011), was successfully applied to CHX using isolates UCD-CFS ECP-1L3, UCD-CFS ECP-1B2 and UCD-CFS ECP-13P5. Isolate UCD-CFS ECP-13P5 provided the MIC*res* (0.01 mg/mL) as it was less susceptible to CHX than the other isolates. UCD-CFS ECP-1L3 and UCD-CFS ECP-1B2 provided the MIC*susc,* 0.002 mg/ml and 0.005mg/mL respectively (**Table 3.4**). The MSC value for UCD-CFS ECP-1L3 was 0.0014mg/mL, 1.4 times lower than the MIC*susc* (**Figure 3.9**). The MSC value for UCD-CFS ECP-IB2 was 0.00005 mg/mL, 100 times lower than the MIC*susc* (**Figure 3.9**). The MSC could not be observed for any of the other strains, neither could it be observed for any of the strains when exposed to BZC. Previous studies based upon the MSC have employed a known resistant strain as a control comparison with known susceptible strains. Here, as MIC values were similar for other isolates for CHX further comparisons could not be observed. It is assumed that an MSC was not observed for BZC for the same reason.

The next chapter will explore the *in-situ* concentrations of biocides that remain on surfaces after disinfection. Using the baseline data gained from this chapter, it will investigate the impact of residual concentrations of CHX and BZC on the biocide and antibiotic susceptibility profiles. A modified carrier test will attempt to replicate *in-situ* disinfection situations and provide a method for evaluating real to life exposure of

bacteria to residual concentrations of biocidal actives. The concentrations of CHX found to be present on surfaces after application and surface drying will be compared to the MSC values observed for UCD-CFS ECP-1L3 and UCD-CFS ECP-1B2. Isolate UCD-CFS ECP-13P5 is of interest due to its elevated MIC and MBC values and multiple antibiotic resistance phenotypes, therefore this isolate will provide a focus with UCD-CFS ECP-1B2 and ATCC29522 for comparison. Additional study was needed to explore in depth the mechanisms that may be responsible for the changes in growth kinetics that have been observed in this chapter. Efflux was investigated with a focus on the difference of activity after exposure to high and low concentrations of BZC. The aim was to elucidate whether or not adaptation to biocides differs depending on the concentration of the biocide present, and to identify which mechanisms are responsible for changes in phenotype that may occur as a result of exposure.

CHAPTER FOUR: THE EFFECT OF EXPOSURE TO BIOCIDE RESIDUES ON ANTIMICROBIAL SUSCEPTIBILITY PROFILE, EFFLUX AND METABOLISM

4.1. Introduction

There is a growing intrigue as to how the cleaning and disinfection products that we use are influencing acute bacterial stress responses and ultimately their evolution (Maillard, 2018, Webber *et al*., 2015, White and McDermott, 2001). Information is a necessity in a climate in which our current lines of clinical treatments, namely antibiotics, are being made redundant to the elevating antibiotic resistance crisis (O'Neill, 2014). If we can understand how exposure to the biocides we use may potentiate microbial resistance, there is a possibility of developing new processes to ensure there are effective treatments available in the future. Generally, studies investigating changes after exposure focus on genotyping species to observe an up or down-regulation response of certain genes pertaining to potential susceptibility shifts (Weber *et al*., 2005). These data are useful, although a genotypic change does not always result in a phenotypic response (Bergmiller *et al*., 2017, Burga and Lehner, 2012, Orgogozo *et al*., 2015). Additional information about how phenotypic shifts occur as a result of biocide exposure is necessary to provide practical, clinically relevant data that can be applied to *in-situ* usage. Changes in phenotypic traits such as growth rate, biocide susceptibility, inactivation kinetics, efflux and metabolism are markers that can be used to predict shifts in tolerance (Knapp *et al*., 2015, Li *et al*., 2017). This information can be substantiated with phenotype stability testing to investigate how transient a change is (Knapp *et al*., 2015).

4.1.2 Efflux as a primary resistance mechanism to biocides in bacteria

When a bacterium is exposed to a toxic substance, such as a biocide, cellular stress responses are triggered via genetic expression, which result in a cascade of defence mechanisms that work to minimise cell damage and ultimately prevent cell death (Seier-Petersen *et al*., 2014). These mechanisms work to extrude, degrade or modify

noxious compounds so that normal cell growth can resume (Seier-Petersen, 2014). When considering antibiotics, target sites are usually substrate specific i.e. they inactivate one specific cellular process rendering a microbe incapable of functioning normally (Kapoor *et al*., 2017). When considering biocides, target site specificity is less common and usually multiple target sites are implicated. For this reason, it is less likely for bacteria to become resistant to a biocide, as there are more obstacles to overcome for survival. However, the induction of resistance mechanisms as a result of exposure may cause subsequent cross-resistance to unrelated compounds (Maillard *et al*., 2013).

For a biocide to be effective it must first penetrate the cell envelope**.** There are two principal pathways through the cell envelope and into a bacterial cell: diffusion through porins and through lipid mediated transport (Delcour, 2009). Toxic chemical compounds are prevented from entering and reaching their target site within the cell through altering functional porins located at the cell wall. Antibiotic and biocidal resistance mediated by changes in cell permeability has been identified in a number of species including *E. coli* (Hancock and Baddiley, 1985; Poole, 2002).

Functional changes in cell permeability has been closely linked to efflux mechanisms, with the cell wall acting as a gateway for the efflux pumps to expel unwanted substances (Fernández and Hancock, 2012). Efflux pumps are energy dependent transport mechanisms that in the case of cell defence expel toxic substances, preventing them from entering the cell and inflicting damage. The presence of efflux pumps are not an indication of resistance, both susceptible and non-susceptible bacteria carry efflux pumps, in fact they are present in all microorganisms (Blanco *et al*., 2016b). However, they are mechanisms through which decreased susceptibility or resistance may be mediated. It is debated that the origin of efflux pumps is ancient. This is supported by the fact that efflux pumps have the capability to transport a broad variety of substances aside from synthetic drugs and commonly

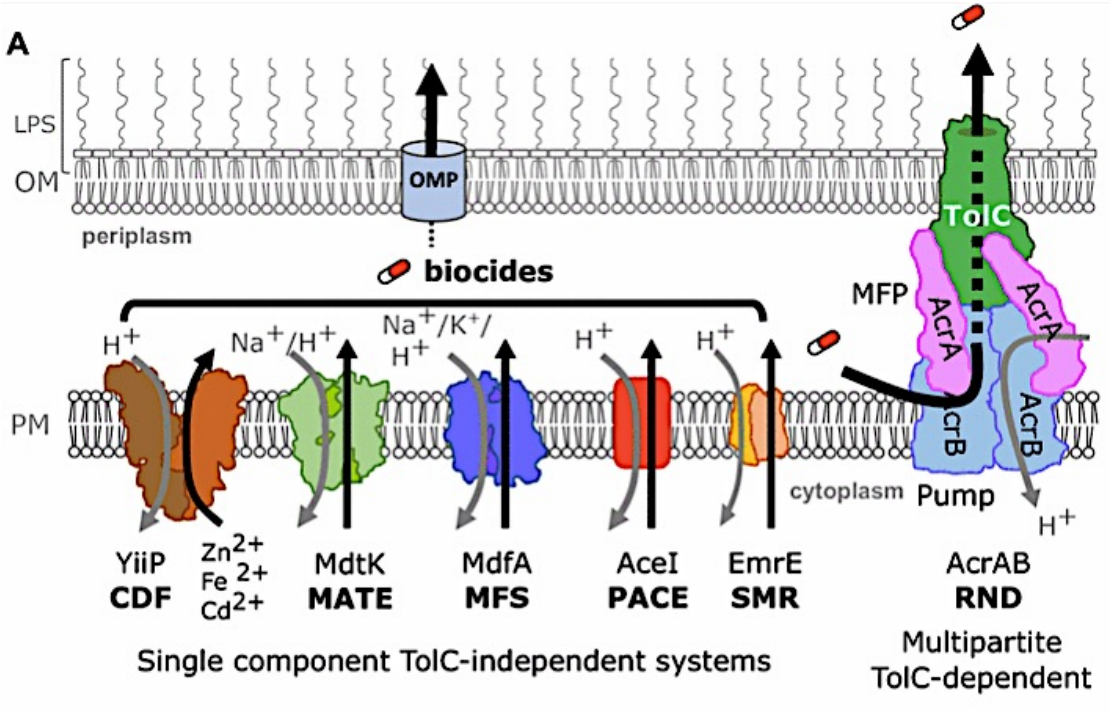
used antibiotics such as heavy metals and organic solvents, substances found in the natural environment. It is surmised that the primary purpose of efflux pumps is not solely to act as a defence mechanism, but to partake in the regulation of normal cell metabolic function (Blanco *et al*., 2016a). However, it is clear that efflux pumps are a vital determinant of resistance whether due to active efflux or the part that they play in cell regulation (Alibert *et al*., 2017; Maillard, 2018).

Figure 4.1 shows the principle families of efflux systems, the Resistant-Nodulation Superfamily (RND), Small Multidrug Resistance (SMR) family, Proteobacterial Antimicrobial Compound Efflux (PACE) family, Major Facilitator (MFS) family, Multidrug and Toxic Compound Extrusion (MATE) family and the Cation Diffusion Facilitator (CDF) family. The adenosine triphosphate (ABC) superfamily is not pictured in figure 4.1. SMR, MFS, MATE, CDF and ABC families can all be found in both Gram-negative and Gram-positive bacteria. The RND superfamily is specific to Gram-negative. The ABC superfamily family relies upon ATP hydrolysis as a source of energy (Lubelski *et al*., 2007). MATE pumps are driven by Na^+/H^+ drug antiport systems (Alvarez-Ortega *et al*., 2013) and the RND, SMR and MFS families rely on proton motive force (PMF) which in turn is dependent on pH. The effect of pH on efflux has been investigated previously in *E. coli* (Amaral *et al*., 2014, Martins *et al*., 2009). It was shown that *E. coli* is more efficient at effluxing ethidium bromide when at lower pH. RND transport systems are important efflux producers in *E. coli* and are providers of intrinsic multidrug transports. Unlike the SMR, PACE, MFS, MATE and CDF families, which are single component systems, RND systems are comprised of a tripartite structure consisting of the RND pump, found in the inner membrane, a periplasmic adapter protein and an outer membrane protein (OMP). This tripartite complex ensures that toxic compounds are transported outside of the cell and do not stay in the periplasmic space, as is such with the other families, making it more difficult for them to re-enter the cell. The AcrAB-TolC transporter is part of the

hydrophobic and amphiphilic (HAE-RND) RND subfamily and has the capability to transport an array of substrates. This family has previously been isolated in *E. coli*, the deletion of AcrB has been shown to result in the increased uptake of ethidium bromide (EtBr) and decreased efflux activity (Paixão *et al*., 2009).

Figure 4.1: Schematic representation of the principle classes of efflux systems*: Resistance-nodulation (RND) family, small multidrug resistance (SMR) family, proteobacterial antimicrobial compound efflux (PACE) family, major facilitator (MFS) family, multidrug and toxic compound extrusion (MATE) family and the cation diffusion facilitator (CDF) family.*

Image taken from Slipski et al. (2017).



Although a single family of efflux pumps can individually extrude a variety of different substrates, efflux pumps act in tangent, resulting in an enhanced plasticity and threat to biocide efficacy (Alcalde-Rico *et al*., 2016). Singlet pumps such as those belonging

to the MFS family pump substrates out of the cell into the periplasmic space (inactivation and detoxification processes occur here); substrates are then caught by the RND tripartite systems and pumped externally. This synergy was demonstrated in a study involving *E. coli,* undertaken by Tal and Schuldiner (Tal and Schuldiner, 2009). They described the overlapping of functionality of transporters such as AcrAB-TolC (RND family), *emrE* (SMR family) and *mdfA (MFS family)* efflux. The AcrAB efflux gene has been shown to be responsible for tolerance to several antibiotic substrates such as ciprofloxacin, tetracycline, chloramphenicol, erythromycin and ampicillin (Sulavik *et al*., 2001); levofloxacin (Opperman *et al*., 2014) and tigecycline (Hirata *et al*., 2004). Moreover, several studies have associated the overexpression of efflux pumps to clinical infections (Kosmidis *et al*., 2012, Pakzad *et al*., 2013, Sato *et al*., 2013).

Due to the widened substrate specificity of efflux pumps, the interaction between efflux mechanisms and disinfectant compounds is a warranted line of investigation. Overexpressed efflux pumps have been associated with changes in susceptibility phenotype to antimicrobials in several studies (Alonso-Calleja *et al*., 2015, Grande Burgos *et al*., 2016). Prolonged low-level exposure of chlorhexidine digluconate (CHX) or benzalkonium chloride (BZC) have been shown to induce activity of the MexCD-OprJ (RND family) of *Pseudomonas aeruginosa (Fraud et al., 2008, Morita et al., 2003)*. Furthermore low level exposure of these biocides have proven to result in both elevated MICs to the biocide and cross resistance to a number of antibiotics in *E. coli* (**Table 4.1**)**.** Buffet-Bataillon *et al*. (2015) showed that changes in susceptibility are sometimes linked to efflux pump activity, the study demonstrated that the overexpression of MDR efflux pumps induced by QAC exposure lead to MDR efflux pump mediated fluoroquinolone cross-resistance (Buffet-Bataillon *et al*., 2015). (Grande Burgos *et al*., 2016) highlighted that after a 12-day exposure of *E. coli* and non-typhoidal *Salmonella* strains to sub-MIC benzalkonium chloride and

glutaraldehyde (150 mg/mL; 150 mg/mL), strains produced a mean product MIC increase of 31%, which correlated with increases in MIC for all antibiotics tested. Furthermore, when strains were exposed to the efflux pump inhibitor PaβN MICs decreased rendering the strains more susceptible, suggesting that efflux pumps were responsible for the changes in susceptibility phenotype that were observed.

A member of the PACE family, denoted the ACEI pump has been recently identified as a chlorhexidine mediated extrusion system. ACEI was originally discovered in *A. baumannii* and was induced by chlorhexidine exposure (Hassan *et al*., 2012; 2013; 2015; 2018) resulting in efflux-facilitated resistance.

Table 4.1: Previously reported fold changes in MIC (mg/mL) for *E. coli* isolates after low-level exposure to CHX and BZC and concurrent antibiotic cross-resistance. Where –R is stated, an MIC could no longer be found and strain was considered resistant.

BIOCIDE	STRAIN	MIC FOLD CHANGE AFTER EXPOSURE	CROSS-RESISANCE (MIC fold change)	REFERENCE
CHX	NCIMB8545	≤ 6-fold	Tobramycin	Wesgate *et al*., 2016
	NCTC12900 (O157)	Approx. 50-fold	No cross-resistance reported	Braoudaki & Hilton, 2004
BZC	ATCC11776	6-fold	Ampicillin 5-fold; chloramphenicol 24-fold; gentamicin 2-fold; kanamycin 2-fold; nalidixic acid-4 fold; norfloxacin 3-fold; Penicillin 2-fold; tetracycline-8 fold	Langsrud *et al*., 2003
	DSM 682	6-fold	Ampicillin 6-fold; chloramphenicol 12-fold; erythromycin 1-fold; gentamicin 2-fold; nalidixic acid 8-fold; norfloxacin 300-fold; Penicillin 2-fold; tetracycline 2-fold	Langsrud *et al*., 2003
	ATCC47076	6/7-fold	Chloramphenicol 16-fold; lorfenficol 8-fold; ciprofloxacin 4-fold; nalidixic acid 8-fold; ampicillin 2-fold; cafotaxime 8-fold	Braoudaki & Hilton, 2004
	NCTC12900 (O157)	Approx. 100-fold	Amoxicillin-clavulanic acid – R; cmoxicillin – R; Chloramphenicol – R; colistin 10-fold; trimethoprim - R	Braoudaki & Hilton 2004

CHX: chlorhexidine; BZC: benzalkonium chloride

**Table adapted from Kampf, 2018*

4.1.3 Induction of resistance, phenotypic stability and the impact of exposure to biocidal residues

As mentioned previously, it is less likely for a microbe to become resistant to a biocide than it is to an antibiotic due to their broad activity and multiple cellular target sites (Maillard *et al*., 2013). However, decreased susceptibility to biocides does occur as a result of cells surviving exposure. The reduction of concentrations within the cell to below lethal levels allows the activation of resistance mechanisms via metabolic regulation or genetic mutation (Maillard and Denyer 2009). When disinfectant products fail or are used incorrectly there is potential for the active compound to be present in less than intended concentrations (Maillard, 2005). It is also the case that although products that claim to have residual or prolonged activity may not maintain the high level of activity that is necessary to prevent the survival of pathogens. A biocide at low residual levels might not efficiently kill an entire bacterial population, or will be present within bacterial cells at sub-lethal concentrations due to extrusion or degradation via cell resistance mechanisms. In conjunction to a products failure, low-level concentrations will still impose a stimuli or pressure upon bacteria, resulting in stress responses. The regulation of bacterial stress responses can be categorised into two main types:

(i) Global regulation genetic cascade – promotes processes such as expression of efflux pumps and down regulation of membrane permeability

(ii) Local regulation via direct activation of the promoter region.

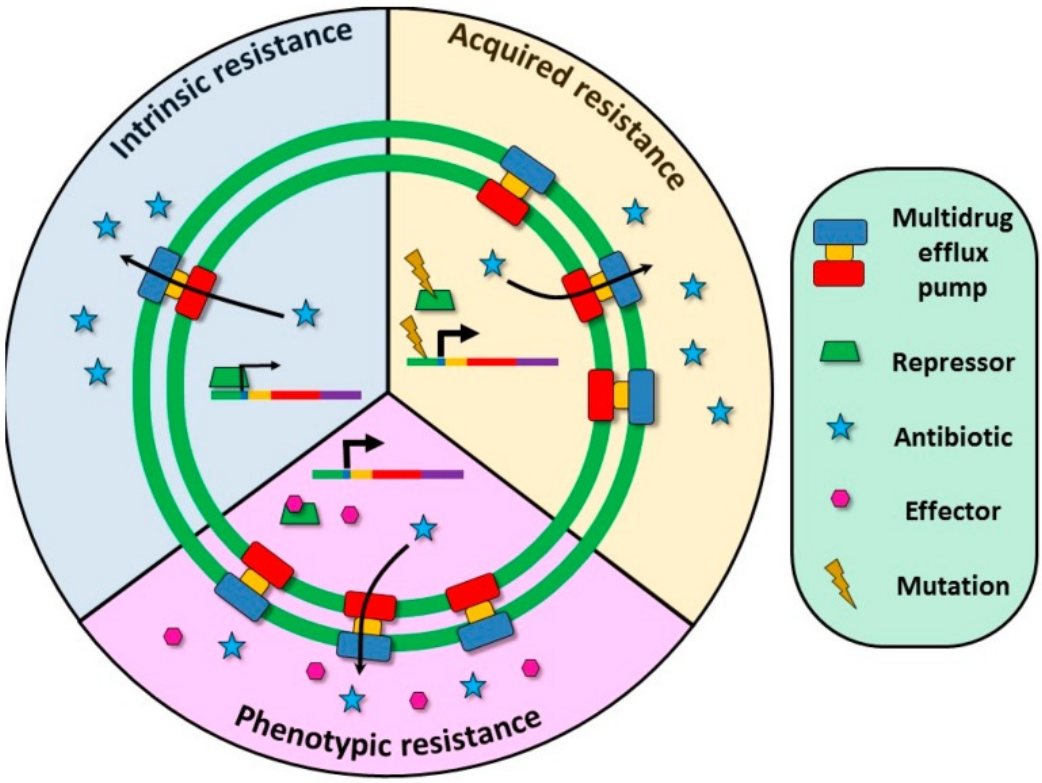
Global regulator genes such as *soxS, acr* and *mar* are responsible for controlling measures of cell defence and augment cells plasticity. One of these defences is the expression of efflux pumps.

When efflux pumps are not naturally expressed at a higher level constitutively their expression may be induced by prolonged exposure to toxic compounds (Buffet-Bataillon *et al*., 2016). **Figure 4.2** Demonstrates how the presence of a biocide can induce resistance via the regulation of efflux pump expression. In one instance of globally regulated *marA*-mediated antibiotic resistance was a result of increased expression of the AcrAB efflux pump in *E. coli* (Grande Burgos *et al*., 2016).

Figure 4.2: The role of efflux pumps in antibiotic resistance.

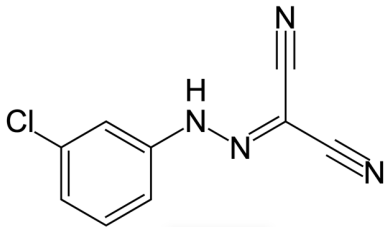
Intrinsic resistance: Some MDR efflux pumps such as E. coli AcrAB-TolC present a basal level of expression which results in intrinsic antimicrobial resistance (blue section). Acquired resistance: De-repression of the expression of the efflux pumps can be achieved by mutations at the regulatory proteins, rendering stable acquired resistance (yellow). Phenotypic resistance: The expression of efflux pumps can be triggered in the presence of specific inducers, rendering transient phenotypic resistance (pink).

Image taken from Blanco et al. 2016



Efflux pump inhibitors (EPIs) are used to impede efflux activity and prevent bacterial cells from expelling efflux pump substrates. A wide range of EPIs have been researched for their possible application in the re-instatement of antibiotics that are no longer effective due to clinically relevant pathogens that overproduce efflux pumps. Although there have been a plethora of studies investigating the relationships between antibiotics and efflux, there is not a lot of research that tackles that of biocides such as CHX and BZC. Consequently, this chapter will focus on the use of EPIs to isolate the origin of change in biocide susceptibility via the investigation of efflux pump activity. The use of EPIs for this purpose has been documented in the literature. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) depicted in **Figure. 4.3** is a proton conductor that works by disrupting proton motive force processes at the cell membrane. CCCP does not directly inhibit nor change the efflux pump itself but eliminates the source of energy that is available for activity of efflux mechanisms, rendering pumps inactive. CCCP has been used for inhibiting the activity of the RND and MFS family of efflux pumps (Xiong *et al*., 2000). The RND family work to efflux a wide range of compounds (Nikaido and Pagès, 2012).

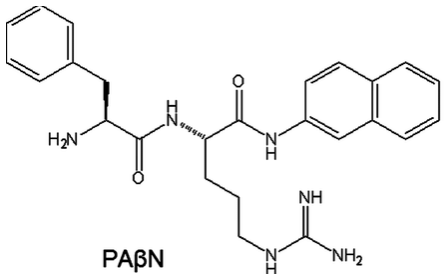
Figure 4.3: Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)



Phenylalanine-arginine-β-naphthylamide (PaβN; **Figure 4.4**) is an EPI of the *E. coli* AcrAB-Tolc efflux system (Kinana *et al*., 2016; Lomovskaya *et al*., 1996; Misra *et al*., 2015) and AcrEF pump (Misra *et al*., 2015*)* of the RND family. PaβN is a

substrate of these pumps and so blocks the pumps therefore inhibiting the extruding of other compounds.

Figure 4.4: Phenylalanine-arginine-β-naphthylamide (PaβN)



When choosing appropriate EPIs it is important to consider the concentration that is used. There are examples where the inhibitors are used at inappropriate concentration (LI, 2015). For example, at high concentrations PaβN, alongside its primary purpose to inhibit efflux pumps, also has an effect on the destabilisation of the cell wall membrane (Lamers *et al*., 2013). Misra *et al*. (2015) concluded that at 0.02 mg/mL PaβN had very weak membrane-destabilising action against *E. coli* but, within 60 s after addition, it inhibited efflux pump activities of AcrAB and AcrEF.

The process of resistance induction via stress-induced genetic mediation does not only apply to the expression of efflux pump activity. The regulatory system PhoE is responsible for the modulation of porins located at the outer membrane (OM) (Gehring and Nikaido, 1989). Porins enable the transfer of substances from one side of a membrane to the other (less than 1000Da in size). Changes in porins' shape and size are responsible for the level of permeability of a cell and have been associated with changes in drug susceptibility (Chollet *et al*., 2002). Furthermore, the finding of decreased permeability by decreased porins' expression was associated with the overexpression of AcrAB. There is currently still a gap in the knowledge of how compounds commonly used in disinfectant products can influence efflux activity and

other regulatory defence systems and how, if any, resulting phenotypic changes differ depending on exposure concentration and duration.

When assessing the risk of a biocide that can induce changes in susceptibility of a microorganism, it is important to determine whether or not this change is stable or transient. It is helpful to categorise levels of susceptibility and distinguish between resistance, tolerance and persistence. **Figure 4.5** depicts the differences in resistance, tolerance and persistence.

RESISTANCE

- ⇒ Intrinsic – Natural, inherent insusceptibility
- ⇒ Acquired – genetically defined, stable changes that arise from either mutation or acquisition of genetic material (e.g. via horizontal gene transfer (HGT) (Meyer and Cookson, 2010)

TOLERANCE

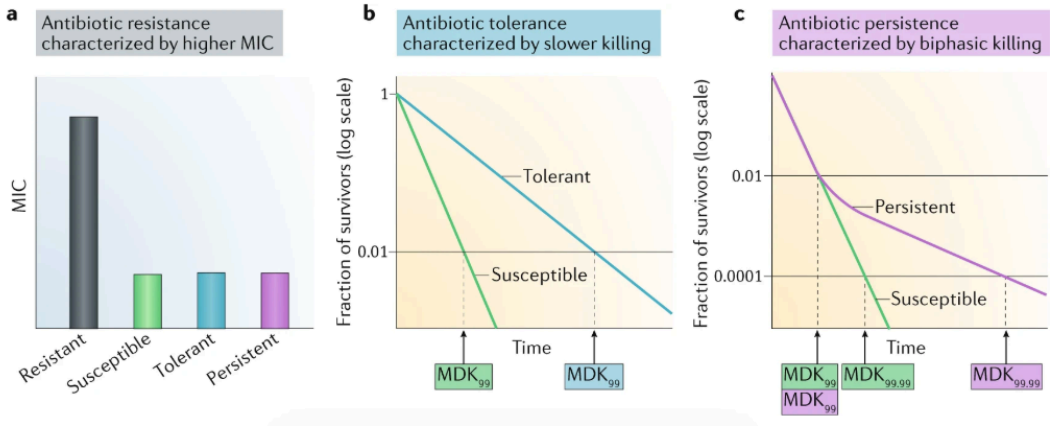
- ⇒ Phenotype adaption – Transient withstanding of exposure to toxic concentrations that would have otherwise been fatal (e.g. as a result of slowing in metabolic processes)

PERSISTENCE

- ⇒ Similar to tolerance in its transient nature, persistence is usually characterised by a subpopulation of tolerant cells as opposed to an entire tolerant population.

Figure 4.5: Resistance, tolerance and persistence

Image taken from Brauner et al & Balaban et al, 2019



*"Resistance, tolerance and persistence are distinct responses to antibiotic treatment that lead to increased survival compared with susceptible cells. **a** | To inhibit the growth of resistant bacteria, a substantially higher minimum inhibitory concentration (MIC) of the antibiotic is needed than for susceptible bacteria. Notably, persistence and tolerance do not lead to an increase in the MIC compared with susceptible bacteria. **b** | By contrast, tolerance increases the minimum duration for killing (MDK; for example, for 99% of bacterial cells in the population (MDK_{99})) compared with susceptible bacteria. **c** | Persistence leads to a similar MIC and a similar initial killing of the bacterial population compared with susceptible bacteria; however, the MDK for 99.99% of bacterial cells in the population ($MDK_{99.99}$) can be substantially higher owing to the survival of the persister cells. Note that pure exponential killing of the susceptible strain is rarely observed because most bacterial cultures have some level of persistence. The data shown are only illustrations and not actual measurements" (Brauner et al & Balaban et al, 2019)*

Resistance to a biocide can be quantified using markers such as MIC and MBC values however tolerance and persistence do not always result in a change in MIC (**Figure 4.5a**) where as resistance infers an increase in concentration, tolerance and persistence infer an increase in the minimal length of time the required for the cells to be inactivated (**Figure 4.5b and c**). Inactivation kinetics can be used as a tool for

observing interactions between a biocide and populations as well as identifying the nature of changes in susceptibility as a result of exposure (Maillard & Denyer, 2009).

There currently exist a number of practical (Knapp *et al*., 2013; Wesgate *et al*. 2016) and mathematical (Balaban *et al*, 2019) models to predict whether or not exposure of a biocide will result in resistance, tolerance or persistence of a microorganism. This practical information is invaluable, nevertheless if new processes are to be devised with the technology intended to prevent resistance, it is necessary that the mechanisms behind these outcomes be further understood.

4.1.4. Horizontal gene transfer, focusing on conjugation

Bacteria typically reproduce through the process of binary fission. During this process the mother cell undergoes physical changes that result in the direct replication of its chromosomal DNA. A copy of this DNA is allocated to each of the two daughter cells produced. The daughter cells that result through binary fission are identical copies of the mother cell. This process referred to as vertical gene transfer (VGT) allows genetic information to be passed down through a single lineage. With the exception of single point mutations this process does not present opportunities for the distribution of genetic diversity within microbial systems. There are three main independent mechanisms of gene transfer in prokaryotic cells that make genetic alteration and dissemination possible; transformation, transduction and conjugation (Bushman, 2002). These are referred to as methods of horizontal gene transfer (HGT). During the process of HGT, genetic information is passed from either the environment or from one bacterial cell to another and is integrated into its new hosts DNA.

The exchange of genetic materials via HGT is enabled through the mediation of mobile genetic elements (MGEs). MGEs are fragments of DNA that encode enzymes

and other proteins that mediate the movement of DNA within genomes (intracellular mobility) or between bacterial cells (intercellular mobility) (Frost *et al*., 2005). The term MGEs encompasses plasmids, bacteriophage, transposable elements and group I and II introns. The activity of MGEs can be noted in all prokaryotic genome sequences (Gogarten and Townsend, 2005) and these mobile pockets of genetic material add to the large accessible genetic diversity that can be seen within a bacterial species. *E. coli* ATCC 25922 possesses a genome size of 5.20-Mbp, which includes two plasmids of 48,488 and 24,185-bp, respectively (Minogue *et al*., 2015). The prokaryotic genome does not only contain requisite genes. Along with domestic genetic information that can be found in all members of a taxon (the core-genome) and that codes for fundamental cellular processes such as reproduction, are non-core genes. Non-core genes make up the accessory genome, are not present in all members of a taxon and are not essential for cellular function, however may offer other advantages such as acquired antibiotic resistance. The combination of the core genome and the non-core genome for an entire taxon is referred to as the pan-genome; it is usually the accessory genome that accounts for the majority. This was demonstrated in the case of 61 *E. coli* isolates whose genomes were sequenced and revealed that of the pan-genome, only 6% of gene families formed the shared core genome, meaning that 90% of the pan-genome was made up solely of accessory genes (Lukjancenko *et al*., 2010). This is suggestive that MGEs play a large role in the genetic makeup of microorganisms. The horizontal exchange of mobile genetic material is more likely to occur between closely related organisms rather than those that are distantly related (Skippington and Ragan, 2012). However, the pan-genome represents thousands of prospective novel genes that are available to the whole taxon. The size of the accessory genome is a testament to the amount of gene variability and motility within a bacterial species, it is through the exchange of these variations that bacterial populations adapt and consequently evolve.

HGT and the uptake of MGEs usually incur a large cost on the host bacterial cell population. This cost may arise from the various metabolic elements involved within the transfer process, through loss of functionality within the cell due to the genetic insertion or due to cell death as a result of the newly acquired genetic element (Baltrus, 2013). There is often a cost/benefit trade-off between VGT and HGT where in the case of the bacterial cell survival is its goal and in the case of the genetic element successful dissemination is the desired outcome. The transfer of MGEs appears to occur mostly in times of cell stress and toxic environments, in these situations selective pressure favours dissemination of advantageous genetic traits. This was demonstrated in the case of *E. coli* where a non-conjugative plasmid initially decreased fitness in the host population however, with the addition of antibiotics the hosts fitness was regained (Bouma and Lenski, 1988). Therefore, it is generally assumed that successfully transferred genetic material commonly provides a selective advantage to the recipient however, this is not strictly a necessary outcome, transferred genes may also be neutral (Gogarten and Townsend, 2005). MGEs may either be passed on directly through vertical genetic transfer or laterally through HGT, whether or not one pathway is used over the other is usually decided by the costs that they impose on their hosts (Turner 1998, Turner 2004, Haft 2009).

Conjugation is the transfer of genetic material from one bacterial cell to another via direct cell-to-cell contact. Joshua Lederberd and Edward Tatum who set out to investigate whether or not bacteria utilise reproductive pathways similar to sexual reproduction initially discovered this form of horizontal gene transfer in 1946. Through conjugative transfer, bacterial cells are able to exchange genetic information not only with cells of the same strain and species but also between other bacterial species (Giedraitiene *et al*., 2011). Conjugation requires independently replicating genetic elements called conjugative plasmids, or chromosomally integrated conjugative elements such as integrons or transposons (Frost *et al*., 2005). Conjugative plasmids

often carry genes that code for multidrug resistance. Large sized plasmids, usually considered >50kb (Carattoli, 2013), are often associated with the transport of genes, which code antibiotic resistance, an example being Extended-Spectrum Beta-Lactamases (Wang, *et al*., 2013). ESBL production in *E. coli* poses as a significant causative agent of nosocomial and community acquired infections in Europe and the United States (Coque *et al*., 2008). Plasmids have been identified as vehicles for genes that code for ESBLs (Garcillán-Barcia *et al*., 2011). Replicon typing of plasmids is seen as a method of surveillance of the acquisition and spread of antimicrobial resistance (Carattoli, 2013; Wang *et al*., 2012).

It is generally agreed that the use of antibiotics act to promote horizontal gene transfer in bacterial populations however there are counteracting arguments that insist there may be more to the complex interaction than that. Lopatkin *et al*. (2016) suggests that instead of promoting horizontal gene transfer, the relationship between the exposure of bacterial cells to antibiotics and the increase in prevalence of resistance was related more to cell death and population dynamics (Lopatkin *et al*., 2016). It is clear that although investigations into these processes are underway, little is still clear about the complex interactions between antimicrobials, bacterial populations and genetic transfer. Furthermore, the relationship between biocides and the spread of resistance through HGT needs to be investigated thoroughly. Jutkina *et al*., (2018) recently justified the need for further investigation of biocides and HGT with the demonstration that exposure of low concentrations of CHX (200 times below the MIC) and triclosan ($1/20_{th}$ below the MIC) can significantly increase frequencies of transfer of antibiotic resistance. Understanding the significance of the role that selective pressures such as biocide exposure play in the exchange of MGEs could be the key to slowing down plasmid mediated antibiotic resistance (Amábile-Cuevas and Heinemann, 2004; Jutkina *et al*., 2018)

4.1.5 Aims and objectives

The aims of this chapter were to firstly ascertain a realistic understanding of the concentration of remaining CHX and BZC residues deposited on a surface after application. Then it will investigate the effect that biocide drying over time has on biocide efficacy.

In parallel the risk of decreased bacterial susceptibility due to low-level residual biocidal concentrations will be determined with a focus on MIC, MBC and antibiotic susceptibility using standardised test protocol as well as looking at inactivation kinetics.

This chapter also aims to explicate the relationship between active efflux mechanisms and exposure to CHX and BZC at two sub-MIC concentrations. Ethidium Bromide (EthBr) accumulation assays will provide insight into the resistance mechanisms utilised in response to biocide toxicity and elucidate possible origins of changes in susceptibility after exposure. Emphasis will be placed on a comparison between efflux activities at exposure to very low-level residual concentrations and higher sub-MIC biocide concentrations (immediately below MIC).

Finally, changes of MIC/MBC antibiotic susceptibility phenotypes will be explored for their stability over a time period of 10 daily passages. Results will indicate whether or not changes in susceptibility profile are transient and only correspond to an exposure period.

4.2. Materials and Methods

4.2.1 Determination of biocide concentration remaining after surface drying

4.2.1.1. Application, drying and recovery of biocide

One mL of 20 mg/mL CHX (Sigma-Aldrich) or 4.5 mg/mL BZC (Sigma-Aldrich) was pipetted into a glass flat-bottomed McCartney bottle (Fisher scientific) ensuring that neither the liquid nor pipette touched the sides or rim of the bottle. The 1 ml of CHX was removed via pipette at 0 h and left to dry at room temperature (21°C) in a category two biological safety cabinet for 6, 24, 48, 96 or 168 hours. After the appropriate drying time 1 mL of sterile de-ionised water (diH_20) was added to the McCartney bottle and the remaining CHX was re-suspended using a vortex mixer and magnetic stirrer for 1 minute. This solution was aspirated and dispensed into a glass autosampler vial (Fisher scientific) for HPLC analysis. If required samples were refrigerated (2-4°C) and stored up to 1month before being discarded.

4.2.1.2. High-Performance Liquid Chromatography (HPLC) of CHX after drying on a surface

After application, drying and recovery of the biocide (Section 4.2.1.1) HPLC analysis was performed in order to quantify the amount of chlorhexidine remaining. The mobile phase was a 1:1 ratio of water and acetonitrile (HPLC grade, Sigma Aldrich, UK) with 0.5% trifluroacetic acid (HPLC grade, Sigma Aldrich, UK). Retention rate was 6 minutes. An initial calibration curve was performed with a CHX standard stock (20 mg/mL). Halving concentrations running from 0.5 mg/mL to 0.001 mg/mL were analysed (n=3). The regression coefficient (R^2) for the calibration-standardised curve

was 0.99874, this gives confidence that the data are not variable beyond validity. An R^2 value closer to +1 describes less variability in values, the further from +1 the more variability between values.

4.2.2 The effect of biocidal residues on microbial susceptibility profile after drying

4.2.2.1 Modified carrier test and cell survival after exposure

Modified carrier tests were performed to assess bacterial cell survival after exposure to surface dried, residual concentrations of CHX. Briefly, standardised bacterial suspensions were prepared (Section 2.4) and used within 15 min of preparation. One mL of 20 mg/mL CHX or 4.5 mg/mL BZC was pipetted into a glass flat-bottomed McCartney bottle as described in Section 4.2.1.1. The 1 ml was removed via pipette at 0 h and the bottle left to dry at room temperature (21°C). A separate glass bottle was used for each CHX drying time point of 6, 24 or 168 hours. After the appropriate drying time 20 µl of standardised inoculum (10^8cfu/mL) was added to the bottom of the McCartney bottle and left for an exposure time of either 5 min or 24 h. Following exposure, 1 mL of De-Engley neutraliser was added to the bottle and the remaining inoculum was re-suspended using a vortex mixer for 1 min. The suspensions were serially diluted and enumerated using the drop counting method (**Section 2**). Drops were placed onto a MHB agar plate (E&O, UK) and incubated for 16-24 hours. The log reduction was determined (**Equation 4.1**).

Equation 4.1.

Cfu/mL = number of colonies x dilution factor / volume plated

In addition, 100 µL suspension was removed from the test vial after neutralisation, placed into 10 mL MHB and incubated for 18-24 hours 37°C in order to ascertain whether survivors were present after biocide exposure and to perform additional susceptibility testing (Sections 4.2.2.; 4.2.3 and 4.2.4).

4.2.2.2 Minimal Inhibitory and minimal bactericidal concentrations after exposure

After exposure to either CHX or BZC in a modified carrier test **(Section 4.2.3.1)** 100 µl of test suspension was removed and added to 10 ml MHB and incubated for 24 h at 37°C. Suspensions were then tested using the BS EN ISO: 20776-1 (ISO, 2006) broth microdilution method as described in detail in **Section 3.2.1**. Briefly, cultures with positive growth after incubation were centrifuged at 5000g for 10 min and re-suspended in 10 mL PBS. Suspensions that grew were standardised to a viable cell concentration of 1 x 10^8 CFU/mL. A 96 well microtitre plate was prepared with halving dilutions of biocide (CHX/BZC) in 50 µL double strength MHB, finally 50 µL standardized bacterial cultures were added to each well. Each plate was incubated for 24 hours at 37°C and results were recorded based on positive or negative growth. The MIC was the lowest concentration in which no growth was visible. Twenty µL was taken out of each well and plated onto a DE neutralising agar plate and incubated for 24 hours at 37°C. The MBC was recorded as the lowest concentration that showed no growth.

4.2.2.3 Antibiotic susceptibility after exposure

After recovery following exposure (**Section 4.2.2.2**) viable bacteria (1 x 10^4 CFU/mL) were spread onto a MHB agar plate. Antibiotic susceptibility discs (BD) were placed

onto the agar surface as described in **section 3.** Zones of inhibition were read, and breakpoints calculated in accordance to the EUCAST (2016) protocol.

4.2.3 Effect of exposure to biocidal residues on efflux mechanisms

4.2.3.1 Ethidium Bromide use in the detection of efflux pump activity

Ethidium Bromide (EtBr) is a fluorescent dye that is able to enter a bacterial cell and bind to intracellular components via DNA intercalation; subsequently the dye produces a strong signal. When the dye is in an aqueous solution alone, it produces a much weaker, if no signal. If there is no efflux, EtBr will produce a strong signal, when efflux activity is high the dye will be pumped out of the cell, therefore the signal will be low. EthBr has been used extensively to investigate efflux mechanisms in *E. coli* and has worked particularly well when investigating AcrAB-TolC efflux systems (paixao *et al* 200, Pal *et al*., 2019).

4.2.3.2 Use of carbonyl cyanide m-chlorophenylhydrazone and phenylalanine-arginine-β-naphthylamide as efflux pump inhibitors

Prior to performing efflux activity assays in this chapter, MIC values were obtained for EPIs. Average MIC for CCCP was ≥ 0.010 ±0.009 mg/mL; for PaβN the MIC was ≥ 0.010 ±0.008 mg/mL. PaβN was used in this experiment at 0.005 mg/mL and CCCP at a concentration of 0.0002 mg/mL.

Overnight bacterial cultures of each *E. coli* strain were prepared in accordance with **Section 2.2.2.** Bacterial cultures were re-suspended and adjusted to an OD_{600} of 0.1

in 20 mL of sterile MHB (1 x 10^8 CFU/mL). Suspensions were then incubated at 37°C in a shaking incubator (120rpm) until the mid-log growth phase was reached (OD_{600} of 0.2-0.3; 2 to 3 hours approx.). Bacterial cells were washed with diH2O and centrifuged once to remove unwanted supernatant and spent media, as described in section 2**.** Suspension was adjusted to a final OD_{600} of 0.4. On mL of each strain suspension was removed and boiled (95°C) for 10 min to be used as a positive control. Fifty µL of an EtBr stock solution (10 mg/mL) was added to each well of a 96 well microtitre plate to give a final concentration of 0.005 mg/mL. Fifty µL of EPI was added to appropriate wells to give a final concentration of 0.1 mM CCCP and 0.5 mg/mL PABN. One hundred µL of either boiled bacteria cells or test bacteria cells were added to the plate and 50 µL of PBS was added to those wells without EPI. The final volume of all wells was 250 µL. Plates were read in the TECAN plate reader at 37°C for an initial 10 min to obtain baseline fluorescence values. After 10 min an injection of 50 µL CHX was added to each well. Half of the 96 well plate was exposed to 0.00005 mg/mL and the other half was exposed to 0.002 mg/mL. The plate was read in the plate reader for an additional 50 min (1 hour in total). Background controls consisting of biocides or EPI and EthBr with no bacteria, were run alongside experimental sets and used to normalise data accounting for any fluorescence omitted by inhibitory compounds used. The pH was kept constant throughout this experiment, as this can be a determining factor in the expression of efflux phenotype (Martins *et al*., 2009; Amaral *et al*., 2011).

4.2.4 Inactivation kinetics

One mL of standardised bacteria culture (1 x 10^9 CFU/mL) was mixed with 1 mL PBS. Eight mL of CHX at 20 mg/mL, 0.002 mg/mL or 0.007mg/mL was added to the

bacterial suspension and vortexed for 30 s. The *E. coli* strains UCD-CFS ECP-1B2 and UCD-CFS ECP13P5 were exposed to 20 mg/mL or 0.002 mg/mL CHX for contact times of 0, 0.5, 1, 3 and 5 min at room temperature. Contact time for strains with 0.007 mg/mL were 0, 0.5, 1, 3, 5, 10, 20, 30 and 60 min. This concentration was selected to compare the sub-MIC concentration (0.002 mg/mL) to the concentration of CHX found on a surface (Section 2): 0.006 mg/mL ± 0.002. Following these contact times 1 mL of each test suspension was added to 9mL DE neutralising agar and vortexed for 30 s. One hundred µL of the neutralised mixture was diluted in 900 µL PBS and surviving bacteria were enumerated in duplicate on MHB using the drop counting method. Plates were incubated at 37°C for 24 h and CFU/mL were calculated. Inactivation kinetics were plotted using the Log_{10} CFU/mL recovered over time.

4.2.5. Phenotype stability testing

The stability in biocide and antibiotic susceptibility changes observed after biocide exposure was assessed through successive passaging of surviving bacteria in biocide-free broth or broth supplemented with CHX (0.002 mg/mL). The protocol followed by Knapp *et al*. (2015) was adhered to. Ten daily passages were performed (24 h) and biocide MIC, MBC and antibiotic susceptibility values were reevaluated after passage 1, 5 and 10. Validation of culture and inspection of homogeneity was made after every passage by plating subculture onto a selective media plate and examining a Gram stain under the microscope.

4.2.6. Conjugation assay

To determine the conjugative transfer of AMP resistance, the liquid mating method was followed as described by (Lambrecht *et al*., 2017). For each test three

independent biological replicates were assayed. A single colony for each replicate was inoculated into 5 mL MHB (16-18h, 37°C). The donor strain, UCD-CFS ECP-13P5) was grown in the presence of ampicillin (100 µg/mL) and the recipient strain (*E. coli* J35R) was grown in the presence of rifampicin (100 µg/mL). Cultures were centrifuged (5000g), washed with PBS and re-suspended in 5 mL MHB. Strains were 10-fold diluted in MHB with CHX to obtain an exposure concentration of 0.00005 mg/mL or 0.002 mg/mL. A control was performed with no biocide exposure. Initial mating concentrations ranged from 2.30 x 10^7 CFU/mL to 7.30 x 10^7 CFU/mL for the donor strain (UCD-CFS ECP-13P5) and 1.17 x 10^8 CFU/mL to 7.30 x 10^8 CFU/mL for the recipient strain (*E. coli* J35R). Donor and recipient strains were mixed in a ratio of 1:5. Liquid mating was performed for 4 hours at room temperature (25°C). At the end of the 4 hours mating period donors, recipients and transconjugants were enumerated using the spread plating technique. Enumerated mating suspensions were plated onto media containing ampicillin (100 µg/mL) (donors & transconjugants), rifampicin (100 µg/mL) (recipient + transconjugants) or double selective plates containing ampicillin (100 µg/mL) and rifampicin (100 µg/mL) (transconjugants). Plates were incubated overnight 16-18h, 37°C) and colonies were counted. The limit of detection for enumeration was 1 CFU/mL. The limit of quantification was ≥10 colonies/plate. Transfer ratios were calculated as the number of transconjugants divided by the number of recipients, defined in **equation 4.2**.

Equation **4.2**.

$$Transfer\ ratio = \frac{number\ of\ transconjugants}{number\ of\ recipients}$$

4.2.7 Statistical analysis

Pearson's correlation analysis was used to determine the relationship between surface drying time and the concentration of CHX determined via HPLC. One-Way

and Two-way Analysis of Variance (ANOVA) were used when comparing differences between single and multiple factors respectively.

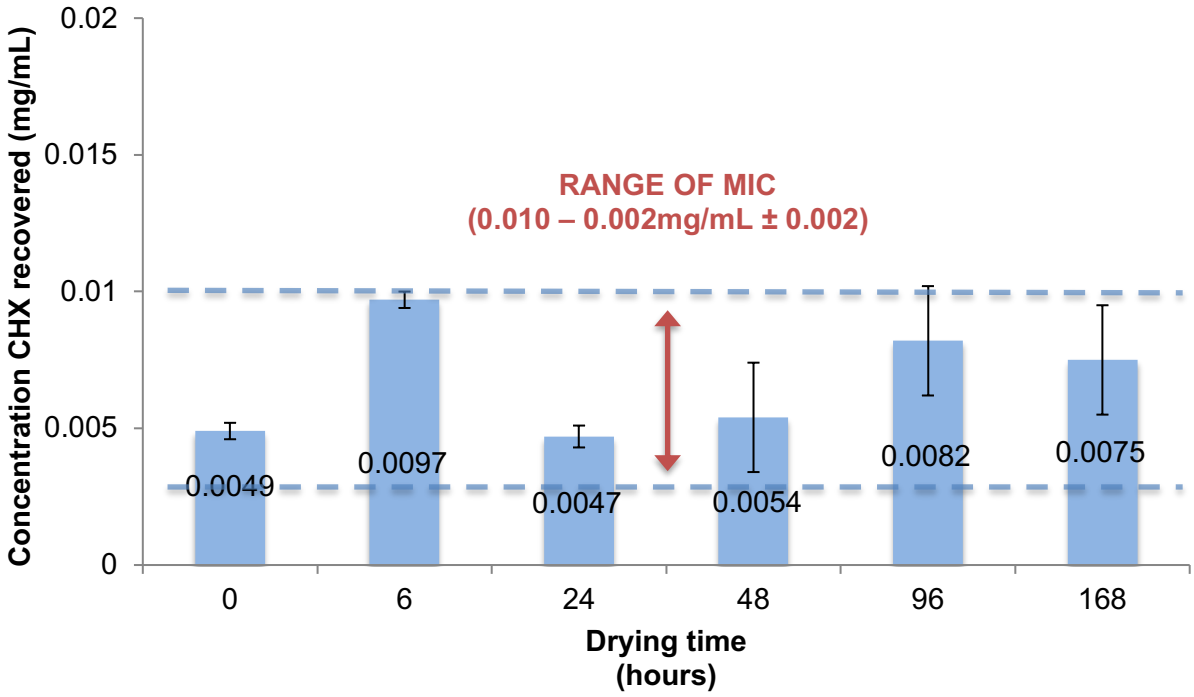
4.3 Results and discussion

4.3.1 Determination of biocide concentration remaining after drying via HPLC

4.3.1.1 Chlorhexidine digluconate

After the addition of 1 mL CHX (20 mg/mL) and its removal from, the bottom of a glass McCartney bottle **(**Section 4.2.1.1), the remaining concentration over time was quantified using the HPLC method. There was an average of 99.97% (± 0.01) decrease in concentration after the solution was removed at time zero and drying times of 6, 24, 48, 96 and 168 hours**.** Figure 4.6 shows the concentrations of CHX recovered after each drying time. This finding is in agreement with Thomas *et al*., (2004) where it was found that regardless of either the drying time or the amount of concentration initially added, the concentration of CHX applied to the bottle surface decreased by 98.8%.

Figure 4.6. The concentration of CHX (20 mg/mL) recovered after drying on a surface. Error bars are standard deviation of the mean of three replicates. Dashed lines depict the average MIC and MBC values for *E. coli* isolates in this study obtained in Section 3.3.1. (n=3)



CHX concentration did not decrease over time (0-168 h) after drying on a surface (P=0.62; Pearsons correlation analysis, r^2=0.07; Graphpad PRISM8). The capability of a substance to persist is termed its substantivity and is determined by the degree of physical and chemical bonding to the surface and resistance to removal or inactivation. It has been demonstrated that CHX has a strong substantivity. Khademi *et al*. (2006) demonstrated that after 5 min application of 2% CHX, the substantivity was up to 4 weeks (Khademi *et al*., 2006). This strength in prolonged activity is due to the protein bound nature of CHX, lending to persistent slow release efficacy (Mohammadi *et al*., 2009).

It is most important to note that these remaining biocidal concentrations are close to the MIC and MBC values that were obtained in **Section 3.3.1.** The highest baseline MIC and MBC values recorded were 0.01 mg/mL (UCD-CFS ECP-13P5). The lowest MIC and MBC values recorded were 0.002 mg/mL (UCD-CFS ECP-13P4) and 0.005 mg/mL (ATCC25922, UCD-CFS ECP-1L3, UCD-CFS ECP-1L4, UCD-CFS ECP-13P4, UCD-CFS ECP-25P5, UCD-CFS ECP-25015). These data suggest that *in-situ*

biocidal concentrations may not always be present at the intentionally excessive active concentration. Furthermore, the concentration may be lower than that needed to inhibit microbial growth, in this instance *E. coli*. This is demonstrated in **Figure 4.6,** for which the range of baseline MIC and MBC values obtained in **Section 3.3.1** are overlaid.

The average MIC and MBC for CHX against the isolates tested was 0.006 ± 0.002 mg/mL, the concentration recovered from the surface on average was 0.006 ± 0.002 mg/mL. The exact concentration needed to inhibit growth of the strains tested leaving no margin for error in case of product failure despite the initial application of 20 mg/mL. When comparing these findings with that of Morrissey *et al*., (2014), it falls around 10-fold below, ECOFF MIC and MCB for CHX were 0.064 mg/mL and >0.064 mg/mL respectively.

4.3.2 The effect of biocidal residues on microbial susceptibility profile after drying

4.3.2.1 Modified carrier test and cell survival after exposure

Eight *E. coli* isolates were exposed to surface dried residues of either CHX or BZC through a modified carrier test in order to ascertain whether or not the concentration present was still sufficient to produce total bacterial kill. Survivors were enumerated and biocidal efficacy of residual CHX and BZC was compared. Post exposure MIC, MBC and antibiotic susceptibility assays were carried out to determine whether or not survivors had undergone a phenotype change in susceptibility.

4.3.2.1.1 Chlorhexidine digluconate - 5 minutes and 24 hours exposure, range of surface drying times

When 1 mL of 20 mg/mL CHX solution was added to the bottom of a glass McCartney bottle and was removed after 0 h time point, the amount of CHX remaining was sufficient to prevent any microbial recovery of all isolates tested (**Table 4.2)**.

After 6 hours of drying CHX onto a glass surface, there was some recoverable growth for isolates UCD-CFS ECP-1L4 and UCD-CFS ECP-13P5 with Log_{10} reductions of 3.48 and 5.63 respectively (**Table 4.2**).

The majority of biocide testing standards specify ≥4Log_{10} or ≥5Log_{10} reduction as part of the pass criteria (EN13697: 2001; EN14561: 2006; EN14349: 2007; EN13727: 2012). There was no significant difference found for CHX activity between isolates (P=0.12; One-Way ANOVA; GraphPad PRISM 8).

There was no significant difference between drying time and Log_{10} reduction (P=0.34; Two-Way ANOVA; GraphPad PRISM 8) suggesting that CHX activity is stable after drying on a surface over time (168 hours). This result is complimentary to data from section 4.3.1.1 where drying time did not correlate with a decrease in CHX concentration. This finding would support the claims of residual activity made by some marketed biocidal products containing CHX (Jaiben *et al*., 2017).

Table 4.2: Log_{10} reductions after modified carrier test with residual concentrations of CHX for 5 min **(n=3).**

	LOG_{10} REDUCTION IN CFU/ML			
DRYING TIME (h)	**0**	**6**	**24**	**168**
CONCENTRATION (mg/ml)	**0.0049 mg/mL CHX**	**0.0097 mg/mL CHX**	**0.0047 mg/mL CHX**	**0.0075 mg/mL CHX**

ATCC 25922	>5.82 ± 0.15	>5.82 ± 0.15	>5.82 ± 0.15	>5.83 ± 0.08
UCD-CFS ECP-1L3	>5.81 ± 0.44	>5.81 ± 0.44	>5.81 ± 0.44	**5.34** ± 0.49
UCD-CFS ECP-1L4	>5.93 ± 0.29	**5.08** ± 0.41	>5.93 ± 0.29	>5.99 ± 0.05

	LOG_{10} REDUCTION IN CFU/ML			
UCD-CFS ECP-IB2	>6.00 ± 0.21	>6.00 ± 0.21	>6.00 ± 0.21	**5.60** ± 0.60
UCD-CFS ECP-13P5	>5.89 ± 0.13	**5.76** ± 0.28	>5.89 ± 0.13	>5.93 ± 0.05
UCD-CFS ECP-13P4	>5.67 ± 0.40	>5.67 ± 0.40	**5.55** ± 0.41	**5.49** ± 0.68
UCD-CFS ECP-25P5	>5.78 ± 0.26	>5.78 ± 0.26	>5.78 ± 0.26	>5.81 ± 0.01
UCD-CFS ECP-25OS1	>5.80 ± 0.30	>5.80 ± 0.30	>5.80 ± 0.30	>5.92 ± 0.30

The limit of detection was 1 x 10^2 CFU/mL (2 Log_{10}). Instances where recoverable growth was present are highlighted in bold text.

Twenty four hours exposure time proved enough for the CHX concentration remaining on the glass surface after drying (0-168 hours) to prevent any recoverable colonies of the eight *E. coli* isolates (**Table 4.3**). There was a significant difference found between Log_{10} reductions of all isolates exposed to CHX at either 5 min or 24 h (P=0.34; ONE-WAY ANOVA; GraphPad PRISM 8). Although CHX residues are lower than the intended 20 mg/mL application **(Section 4.3.1.1)**, they are still effective with a 24 h exposure. Some variability in results can be observed (**Table 4.2**) with some isolates. Such variability may be explained by the difference in residual CHX concentration left on surfaces after drying **(Figure 4.6).** In some instances, not all bacteria were killed, and one can therefore consider what adaptions and changes the remaining survivors may undergo in response to the low-level CHX exposure.

Table 4.3 Log_{10} reductions after modified carrier test with residual concentrations of CHX for 24h (n=3).

DRYING TIME (h) CONCENTRATION (mg/ml)	0 0.0049 mg/mL CHX	6 0.0097 mg/mL CHX	24 0.0047 mg/mL CHX	168 0.0075 mg/mL CHX
ATCC 25922	>5.73 ± 0.15	>5.73 ± 0.15	>5.73 ± 0.15	>5.73 ± 0.15
UCD-CFS ECP-1L3	>5.92 ± 0.05	>5.92 ± 0.05	>5.92 ± 0.05	>5.92 ± 0.05
UCD-CFS ECP-1L4	>5.97 ± 0.06	>5.97 ± 0.06	>5.97 ± 0.06	>5.97 ± 0.06
UCD-CFS ECP-IB2	>5.83 ± 0.15	>5.83 ± 0.15	>5.83 ± 0.15	>5.83 ± 0.15
UCD-CFS ECP-13P5	>5.91 ± 0.04	>5.91 ± 0.04	>5.91 ± 0.04	>5.91 ± 0.04
UCD-CFS ECP-13P4	>5.85 ± 0.02	>5.85 ± 0.02	>5.85 ± 0.02	>5.85 ± 0.02
UCD-CFS ECP-25P5	>5.71 ± 0.12	>5.71 ± 0.12	>5.71 ± 0.12	>5.71 ± 0.12
UCD-CFS ECP-25OS1	>5.70 ± 0.06	>5.70 ± 0.06	>5.70 ± 0.06	>5.70 ± 0.06

The limit of detection was 1 x 10^2 CFU/mL (2 Log_{10})

4.3.2.1.2 Benzalkonium chloride – 5 minutes and 24 hours exposure after range of surface drying times

In some instances, 5 min exposure to BZC after 0 hours drying was not sufficient to provide either total kill or 4 Log_{10} reduction **(Table 4.4)**. This can be observed for isolates UCD-CFS ECP-1L4, UCD-CFS ECP-IB2, UCD-CFS ECP-25P5 and UCD-CFS ECP-25OS1.

Table 4.4 Log_{10} reductions after modified carrier test with residual concentrations of BZC for 5 min (n=3).

	LOG_{10} REDUCTION IN CFU/ML

DRYING TIME (h)	0	6	24	168
ATCC 25922	**5.70** ± 0.00	**5.70** ± 1.09	**4.39** ± 1.80	**1.83** ± 0.27
UCD-CFS ECP-1L3	>5.83 ± 0.25	**0.81** ± 1.07	**2.45** ± 0.60	**1.87** ± 0.22
UCD-CFS ECP-1L4	**5.04** ± 1.36	**4.50** ± 2.29	**3.28** ± 1.80	**2.85** ± 2.05
UCD-CFS ECP-IB2	**5.92** ± 0.09	**1.32** ± 1.21	**3.78** ± 1.82	**2.81** ± 1.36
UCD-CFS ECP-13P5	>5.85 ± 0.11	**2.73** ± 1.44	**2.15** ± 1.97	**3.33** ± 1.98
UCD-CFS ECP-13P4	>5.66 ± 0.08	**2.45** ± 2.95	**4.51** ± 2.06	**1.80** ± 0.13
UCD-CFS ECP-25P5	**4.64** ± 1.58	**1.81** ± 1.17	**4.24** ± 1.45	**1.96** ± 0.05
UCD-CFS ECP-25OS1	**4.37** ± 1.34	**1.86** ± 1.66	**3.05** ± 1.85	**3.25** ± 2.20

The limit of detection was 1 x 10^7 CFU/mL (2 Log_{10}). Instances where recoverable growth was present are highlighted in bold text.

The difference between BZC surface concentration and Log_{10} reduction was statistically significant (P= 6.85654E-10; ONEWAY ANOVA; GraphPad PRISM8) suggesting that drying affects the efficacy of BZC activity. Although the concentration of BZC was not analysed over drying time, it may be suggested that concentration of BZC has decreased over time, making it less effective.

There was a difference (P=3.26076E-7; T-TEST; Graphpad PRISM8) between BZC activity at 0 hours and 6 hours drying, there was no statistically significant difference of BZC activity between 6 hours and 24 hours drying (P=0.05837; T-TEST; Graphpad PRISM8). However, there was a statistically significant difference (P=0.00896; T-Test; Graphpad PRISM8) in BZC activity between 24 h and 168 h drying of BZC. This suggests that the longer BZC is left to dry on the surface, the less efficacious it is against the *E. coli* isolates tested. This cannot be confirmed conclusively as BZC concentrations after drying could not be analysed.

Maybe not surprisingly, Log_{10} reductions after 24 h exposure to BZC residual concentrations were higher (P=<0.0001; One-Way ANOVA; Graphpad PRISM8) than after 5 min exposure (**Table 4.5**). There was no statistically significant difference (P=0.98; One-Way ANOVA; Graphpad PRISM8) found between the efficacies of BZC on the different isolates. Furthermore, the length of time that the BZC was left to dry did not produce a difference (P=0.54; One-way ANOVA; Graphpad PRISM8) in activity when bacteria were exposed for 24 hours.

Table 4.5 Log_{10} reductions after modified carrier test with residual concentrations of BZC for 24h **(n=3).**

	LOG_{10} REDUCTION IN CFU/ML			
DRYING TIME (h)	**0**	**6**	**24**	**168**
ATCC 25922	>5.63 ± 0.78	>5.24 ± 0.10	>5.63 ± 0.78	**2.99** ± 1.23
UCD-CFS ECP-1L3	>5.77 ± 0.14	**5.71** ± 0.09	>5.77 ± 0.14	**1.77** ± 0.14
UCD-CFS ECP-1L4	>5.76 ± 0.13	>5.76 ± 0.13	>5.76 ± 0.13	**1.23** ± 0.14
UCD-CFS ECP-lB2	**4.85** ± 1.44	>4.96 ± 0.09	>5.68 ± 0.09	**1.75** ± 0.67
UCD-CFS ECP-13P5	>5.52 ± 0.04	>5.52 ± 0.04	**4.36** ± 2.04	**1.62** ± 0.05
UCD-CFS ECP-13P4	>5.51 ± 0.96	**4.34** ± 2.03	>5.51 ± 0.96	**3.08** ± 2.32
UCD-CFS ECP-25P5	>5.52 ± 0.07	**3.76** ± 0.82	>5.52 ± 0.07	**2.37** ± 0.95
UCD-CFS ECP-25OS1	**4.54** ± 1.65	>5.48 ± 0.02	>5.49 ± 0.01	**2.30** ± 0.10

The limit of detection was 1 x 10^7 CFU/mL (2 Log_{10}). Instances where recoverable growth was present are highlighted in bold text.

4.3.2.3 Minimal inhibitory and minimal bactericidal concentrations of cells surviving exposure

4.3.2.3.1 Chlorhexidine digluconate - 5 minutes and 24 hours exposure after range of surface drying times

Table 4.6 shows the changes in MIC and MBC values after exposure to CHX. Recovered bacteria always demonstrated a change in MIC or MBC (expect for UCD-CFS ECP-13P5, 6 h dried CHX, repeat 1). The highest MIC fold change of 32-fold, corresponding to an increase in MIC from 0.005 mg/mL to 0.16 mg/mL, were observed for isolates ATCC 25922, UCD-CFS ECP-1L3, UCD-CFS ECP-1L4 and UCD-CFS ECP-1B2. These findings contradict those of Wesgate *et al*. (2016) who found that exposure to CHX did not render *E. coli* less sensitive to either CHX or a number of antibiotics.

After 0-168 h of CHX drying onto a glass surface, corresponding to the presence of residual concentration of 0.0049 mg/mL, 0.0097 mg/mL, 0.0047 mg/mL and 0.0075 mg/mL CHX, no viable bacteria were recovered after 24 h exposure **(Section 4.3.1.2.1)** precluding the investigation into MIC /MBC changes.

Table 4.6 MIC, MBC values (mg/mL) **and fold change** after modified carrier test with residual concentrations of CHX for 5 min **(n=3).**

- No recoverable growth post-exposure

		MIC					MBC				
		EXPOSURE TIME: 5 minutes									
	DRYING TIME (h) / CONCENTRATION (mg/ml)	Baseline	0 0.0049mg/mL CHX	6 0.0097mg/mL CHX	24 0.0047mg/mL CHX	168 0.0075mg/mL CHX	Baseline	0 0.0049mg/mL CHX	6 0.0097mg/mL CHX	24 0.0047mg/mL CHX	168 0.0075mg/mL CHX
	Repeat: 1	0.005	-	-	-	**0.16 (32-fold)**	0.005	-	-	-	**0.16 (32-fold)**
ATCC 25922	**2**	0.005	**0.04 (8-fold)**	-	**0.04 (8-fold)**	-	0.005	**0.04 (8-fold)**	-	**0.08 (16-fold)**	-
	3	0.005	-	-	-	-	0.005	-	-	-	-
	1	0.005	-	-	-	**0.16 (32-fold)**	0.005	-	-	-	**0.31 (62-fold)**
UCD-CFS ECP-1L3	**2**	0.005	-	**0.04 (8-fold)**	**0.04 (8-fold)**	-	0.005	-	**0.04 (8-fold)**	**0.04 (8-fold)**	-
	3	0.005	-	-	-	**0.16 (32-fold)**	0.005	-	-	-	**0.16 (32-fold)**
	1	0.005	-	-	-	**0.16 (32-fold)**	0.005	-	-	-	**0.16 (32-fold)**
UCD-CFS ECP-1L4	**2**	0.005	-	**0.04 (8-fold)**	-	-	0.005	-	**0.04 (8-fold)**	-	-
	3	0.005	-	-	-	-	0.005	-	-	-	-
	1	0.005	-	-	-	**0.16 (32-fold)**	0.005	-	-		**0.31 (62-fold)**
UCD-CFS ECP-IB2	**2**	0.005	-	-	**0.04 (8-fold)**	-	0.005	-	-	**0.08 (16-fold)**	
	3	0.01	-	-	**0.08 (8-fold)**	**0.16 (16-fold)**	0.01	-	-	**0.08 (8-fold)**	**0.16 (16-fold)**
	1	0.01	-	0.01	-	-	0.01	-	0.01	-	-
UCD-CFS ECP-13P5	**2**	0.01	-	-	-	-	0.01	-	-	-	-
	3	0.01	-	**0.04 (4-fold)**	**0.08 (8-fold)**	**0.1 (10-fold)**	0.01	**-**	**0.04 (4-fold)**	**0.08 (8-fold)**	**0.16 (16-fold)**
	1	0.002	-	-	-	-	0.005	-	-	-	-
UCD-CFS ECP-13P4	**2**	0.002	-	-	-	-	0.005	-	-	-	-
	3	0.002	-	-	-	-	0.005	-	-	-	-
	1	0.005	-	-	-	-	0.005	-	-	-	-
UCD-CFS ECP-25P5	**2**	0.005	-	-	-	-	0.005	-	-	-	-
	3	0.005	-	-	**0.04 (8-fold)**	-	0.005	-	-	**0.04 (8-fold)**	-
	1	0.005	-	-	-	-	0.005	-	-	-	-
	2	0.005	-	-	-	-	0.005	-	-	-	-
UCD-CFS ECP-25OS1	**3**	0.005	-	-	-	-	0.005	-	-	-	-

4.3.2.3.2 Benzalkonium chloride - 5 minutes and 24 hours exposure after range of surface drying times

Tables 4.7 and 4.8 show the changes in MIC and MBC values after exposure to BZC where incidences of post-exposure recovery were possible. There were no increases in MIC or MBC values following BZC exposure, however there were frequently incidences of decrease in MIC or MBC suggesting that the isolates become more susceptible to BZC following exposure to residues.

After neutralisation surviving bacteria were added to Muller-Hinton broth and incubated overnight. Muller-Hinton broth is a nutrient rich media; recovery of pre-treated organisms in media such as this can lead to an underestimation of recoverable organisms due to the phenomenon termed “nutrient shock” (Azevedo *et al*., 2012; Davis, 2014; Emerson *et al*., 2017). Through this method, only bacteria that are able to undergo damage repair and recover reproduction processes will be recovered, eliminating cells that are damaged but not dead, which are coined as viable but not countable (VBNC). It is difficult to isolate VBNC population, however, it is worth noting that they are of interest as they will have undergone significant change in cell metabolic regulation and given the chance have the potential to persist further exposure.

Table 4.7 MIC, MBC values **(mg/mL)** of *E. coli* isolates after modified carrier test with BZC exposure 5 minutes, after a range of BZC surface drying times. Fold change indications are also included **(n=3).**

		MIC					MBC				
		EXPOSURE TIME : 5 minutes									
	DRYING TIME (hrs)	**Baseline**	**0**	**6**	**24**	**168**	**Baseline**	**0**	**6**	**24**	**168**
	Repeat:		-			0.001		-			
	1	0.01		0.005	0.002		0.01		-	0.002	0.001
	2	0.01	-	-	-	0.001	0.01	-	-	-	0.001
ATCC 25922	**3**	0.01	-	-	0.005	0.001	0.01	-	-	0.005	0.001
	1	0.02	-	0.005	0.005	0.001	0.04	-	0.005	0.002	0.001
UCD-CFS	**2**	0.02	-	0.01	-	0.001	0.04	-	-	-	0.001
ECP-1L3	**3**	0.02	-	0.005	0.002	0.001	0.04	-	-	0.01	0.001
	1	0.01	-		0.002	0.001	0.01	-	-	0.002	0.001
UCD-CFS	**2**	0.01	-	0.01	0.002	0.001	0.01	-	-	0.002	0.002
ECP-1L4	**3**	0.01	0.01		0.005	0.001	0.01	0.01	-	0.005	0.001
	1	0.02	0.005	0.01	0.002	-	0.02	0.005	-	0.002	-
UCD-CFS	**2**	0.02	-	0.01	0.005	0.001	0.02	-	-	0.005	0.001
ECP-IB2	**3**	0.02	-	0.01	0.005	0.001	0.02	-	-	0.005	0.002
	1	0.02	-	0.01	0.005	0.001	0.02	-	-	0.01	0.002
UCD-CFS	**2**	0.02	-	0.005	0.002	0.001	0.02	-	-	0.002	0.001
ECP-13P5	**3**	0.02	-	0.01	0.005	0.002	0.02	-	-	0.01	0.002
	1	0.02	-	0.01	-	0.001	0.02	-	-	-	0.001
UCD-CFS	**2**	0.02	-	0.01	0.005	0.001	0.02	-	-	0.005	0.001
ECP-13P4	**3**	0.02	-	0.01	0.005	0.002	0.02	-	0.002	0.01	0.002
	1	0.02	-	0.01	-	0.001	0.04	-	0.002	-	0.001
UCD-CFS	**2**	0.02	0.01	0.005	0.005	0.002	0.04	0.01	0.005	0.005	0.002
ECP-25P5	**3**	0.02	-	0.01	0.005	0.002	0.04	-	0.002	0.005	0.002
	1	0.02	0.005	0.01	0.005	-	0.02	0.005	-	0.005	-
UCD-CFS	**2**	0.02	0.01	0.005	-	0.002	0.02	0.01	-	-	0.002
ECP-25OS1	**3**	0.02	-	0.01	0.01	0.002	0.02	-	-	0.01	0.002

- No recoverable growth post-exposure

Table 4.8 MIC, MBC values **(mg/mL)** of *E. coli* isolates after modified carrier test with BZC exposure 24 h, after a range of BZC surface drying times. Fold change indications are also included **(n=3).**

		MIC					MBC				
		EXPOSURE TIME: 24 hours									
	DRYING TIME (hrs)	**Baseline**	**0**	**6**	**24**	**168**	**Baseline**	**0**	**6**	**24**	**168**
	Repeat:		-	-	-	-		-	-		
	1	0.01					0.01			0.002	**-**
	2	0.01	-	-	-	0.001	0.01	-	-	-	0.001
ATCC 25922	**3**	0.01	-	-	-	0.001	0.01	-	-	-	0.001
	1	0.02	-	0.005	-	0.001	0.04	-	0.005	-	**0.001**
UCD-CFS	**2**	0.02	-	-	-	0.001	0.04	-	-	-	0.001
ECP-1L3	**3**	0.02	-	-	-	0.001	0.04	-	-	-	**0.001**
	1	0.01	-	-	-	0.001	0.01	-	-	-	0.001
UCD-CFS	**2**	0.01	-	-	-	0.001	0.01	-	-	-	0.001
ECP-1L4	**3**	0.01	-	-	-	0.001	0.01	0.01	-	-	0.001
	1	0.02	0.005	-	-	-	0.02	0.005	-	-	**-**
UCD-CFS	**2**	0.02	-	-	-	0.001	0.02	-	-	-	0.001
ECP-IB2	**3**	0.02	-	-	-	0.001	0.02	-	-	-	**0.001**
	1	0.02	-	-	-	0.001	0.02	-	-	-	0.002
UCD-CFS	**2**	0.02	-	-	0.002	0.001	0.02	-	-	-	0.001
ECP-13P5	**3**	0.02	-	-		0.001	0.02	-	-	0.002	**0.001**
	1	0.02	-	-	-	0.001	0.02	-	-	-	0.001
UCD-CFS	**2**	0.02	-	-	-	0.001	0.02	-	-	-	0.001
ECP-13P4	**3**	0.02	-	0.005	-	-	0.02	-	0.002	-	-
	1	0.02	-	0.005	-	0.001	0.04	-	0.002	-	0.001
UCD-CFS	**2**	0.02	-	0.005	-	0.001	0.04	-	0.005	-	0.001
ECP-25P5	**3**	0.02	-	-	-	0.001	0.04	-	0.002	-	0.001
	1	0.02	-	-	-	0.001	0.02	0.005	-	-	0.001
UCD-CFS	**2**	0.02	-	-	-	0.001	0.02	-	-	0.005	0.001
ECP-25OS1	**3**	0.02	-	0.005	-	-	0.02	-	-	-	-

- No recoverable growth post-exposure

4.3.2.4 Antibiotic susceptibility phenotype of cells surviving exposure

4.3.2.4.1 Chlorhexidine digluconate - 5 minutes and 24 hours exposure after range of drying times

A change in antibiotic susceptibility was considered clinically significant when the breakpoint according to the EUCAST (EUCAST, 2015) method was marked as resistant. There was one clinically significant change in antibiotic susceptibility phenotype after exposure to biocide residues of CHX (0.0049 mg/mL, surface dried 24h). This change was for tetracycline from sensitive to resistant (zone of inhibition = 0 mm) after 5 min exposure of isolate UCD-CFS ECP-25P5. Antibiotic susceptibility testing was not performed for 24h CHX exposure as all bacteria were inactivated (**Table 4.3**). CHX surface dried for 6, 12 and 168 hours did not appear to consistently result in a clinical change in antibiotic susceptibility phenotype after exposure to CHX residues of 0.0047 mg/mL and 0.0075 mg/mL. All recorded zones of inhibition and breakpoint classifications can be viewed in the appendices (File name: Appendices > appendix one > Breakpoint-Table-EUCAST).

4.3.2.4.1 Benzalkonium chloride - 5 minutes and 24 hours exposure after range of drying times

There were a number of clinically significant changes in susceptibility phenotype in 6 (ATCC 25922, UCD-CFS ECP-IB2, UCD-CFS ECP-13P5, UCD-CFS ECP-13P4, UCD-CFS ECP-25P5 and UCD-CFS ECP-25OS1) out of 8 of the *E. coli* isolates exposed to BZC (**Table 4.9).** Isolates UCD-CFS ECP-1L3 and UCD-CFS ECP-1L4 did not undergo any change in phenotype, therefore were not included in **Table 4.9.** However, all antibiotics susceptibility results are included the appendix. Five minutes exposure to BZC after 168 hours drying in a modified carrier test effected the most change including a shift from sensitive to resistant for antibiotics ampicillin, cefpodoxime, tetracycline, cephalothin, trimethoprim, trimethoprim-

sulfamethoxazole and streptomycin. Bore *et al*. (2007) demonstrated that exposure to BZC resulted in decreased susceptibility to ciprofloxacin, chloramphenicol, naladixic acid, cefotaxime and ampicillin. Here, the isolate that demonstrated the most consistent pattern of phenotype change was UCD-CFS ECP-13P4, which had 7 incidences of shift from sensitive to resistant for antibiotics tetracycline, streptomycin, amoxicillin-clavulanic acid, trimethoprim-sulfamethoxazole and trimethoprim most commonly the shift involved trimethoprim-sulfamethoxazole and trimethoprim.

There were three occurrences of a shift from previously resistant to sensitive for isolates UCD-CFS ECP-IB2, UCD-CFS ECP-I3P5 and UCD-CFS ECP-25051 with antibiotics tetracycline, streptomycin, trimethoprim-sulfamethoxazole, trimethoprim, ampicillin, cefpodoxime and cephalothin (**Table 4.9**). Tetracycline crosses the outer membrane of Gram-negative bacteria through the OmpF and OmpC porin channels (Chopra *et al*., 1992; Schnappinger & Hillen, 1996) and accumulates in the periplasm where it is able to diffuse through the lipid bilayer. The energy depended process of uptake is controlled through the pH component of the proton motive force process (Nikaido *et al*., 1993; Schnappinger & Hillen, 1996; Ghai *et al*., 2018) BZC causes increased permeability, and disruption of membrane potential and electron transport chain. Therefore, it was hypothesised that after 6 h drying, BZC concentration was high enough to cause substantial damage to bacterial cells, which results in alteration to the outer membrane allowing enhanced uptake of antibiotics such as tetracycline.

Table 4.9: Clinically relevant changes in antibiotic susceptibility phenotype according to EUCAST breakpoint values for eight *E. coli* isolates before and after exposure to BZC (4.5 mg/mL; 5 min and 24 h) via a modified carrier test.

		BZC 0 hours drying		BZC 6 hours drying		BZC 24 hours drying		BZC 168 hours drying	
	Exposure time	**5m**	**24h**	**5m**	**24h**	**5m**	**24h**	**5m**	**24h**
ATCC 25922	Repeat: 1								
	2							**AMP, CPD,TE,CF,W,SXT**	
	3								
UCD-CFS ECP-IB2	1	**TE,S,W,SXT**				**CPD**			
	2			**IPM**					
	3								
UCD-CFS ECP-13P5	1								
	2				**TE,S,SXT.W**				
	3								
UCD-CFS ECP-13P4	1							**SXT,W**	
	2			**TE,S,SXT,W**		**AMC,W,SXT**		**SXT,W**	
	3			**TE,S,SXT,W**		**SXT,W**		**SXT,W**	
UCD-CFS ECP-25P5	1								
	2								
	3							**TE,S,SXT,W**	
UCD-CFS ECP-25OS1	1								
	2	**AMP,CPD,CF**							
	3		**SXT,W**						

Green text represents a change in phenotype from intrinsically resistant to clinically sensitive in accordance with EUCAST breakpoints

Red text represents a change in phenotype from clinically sensitive to clinically resistant in accordance with EUCAST breakpoint. Ampicillin (AMP), amoxicillin (AMC), imipenem (IMP), tetracycline (TE), trimethoprim (W), trimethoprim/sulfamethoxazole (SXT), streptomycin (S), cefpodoxime (CPD), cephalothin (CF)

4.3.4 Effect of exposure to biocidal residues on efflux mechanisms

Figure 4.7 and 4.8 show the change in relative fluorescence with or without the inclusion of an EPI following the addition of two concentrations of CHX or BZC at 10 min incubation. The figures are examples of only three of the isolates (ATCC25922, UCD-CFS ECP-IB2 and UCD-CFS ECP-13P5) that underwent efflux assays. All efflux experiment results can be found in the appendices (File name: Appendices > appendix two >Efflux histograms).

With the addition of CHX or BZC at 0.00005 mg/mL, the relative fluorescence either remains minimal or decreases, inferring the extrusion of EthBr from within the cells, in turn suggesting the activation of efflux pump mechanisms. The inclusion of the EPI CCCP with CHX (0.00005 mg/mL) causes the level of fluorescence to rise (P=<0.0001; One-Way ANOVA; Graphpad PRISM8), confirming that efflux pumps were in fact responsible for the previously lower values. As CCCP has been found to work well with inhibition of the RND, MFS and SMR superfamilies due to its disruption of the PMF (Slipski *et al*., 2017), it is possible that these efflux systems may be responsible for the decrease in fluorescence following the addition of 0.00005 mg/mL CHX. The expression of MexXY (a homologue of the RND-type AcrAB pumps in *E. coli*) efflux systems (members of the RND superfamily) in *P. aeruginosa* have been linked to biocide exposure. Morita *et al*, (2003) found that the RND-type efflux pump MexCD-oprJ was induced by the addition of BZC (20 mg/L) and CHX (concentration not stated), allowing previously susceptible bacterial cells to grow in the presence of 1 mg/L norfloxacin. On the contrary, PaβN does not appear to work well (P=<0.0001; One-Way ANOVA; Graphpad PRISM8) to inhibit the efflux of EthBr at 0.00005 mg/mL CHX, which would suggest that the primary pump activity it is not orchestrated by RND-type pumps such as the AcrAB-TolC efflux system, specifically AcrAB and AcrEF. The literature suggests that this EPI is a primary inhibitor of these (Lomovskaya *et al*, 2001; Misra *et al*., 2015; Olliver ET AL 2005; Kinaria *et al*., 2016). Although the RND superfamily is well documented and prolific in its involvement with antimicrobial resistance (Russel et al.,

1993; Fralick, 1996) it is possible that other efflux pumps are active. Hassan *et al*. (2013, 2015, 2018) have described how the Proteobacterial Antimicrobial Compound Efflux (PACE) family is abundantly present in Gram-negative bacteria. Of particular interest here is an active CHX efflux protein, "AceI", Hassan *et al*. (2013, 2015, 2018) found that the over-expression of the AceI efflux pump in *E. coli* resulted in significant increase of resistance to CHX. Furthermore, Hassan *et al*. (2013, 2015, 2018) found the EPI CCCP to be a reliable inhibitor of AceI efflux activity, therefore supporting the theory that AceI efflux pumps may be involved in the efflux activity observed in **Figure 4.7** In order to confirm the source of efflux activity in the *E. coli* isolates in this study (**Figures 4.7, 4.8)** either specific pump targeted genetic analysis or whole genome sequencing could be undertaken.

The findings presented in **Figure 4.7** Illustrate a distinct difference between efflux involvement at high (0.002 mg/mL) and low (0.00005 mg/mL) concentrations of CHX exposure. The addition of the higher concentration of CHX 0.002 mg/mL resulted in a rise in relative fluorescence level, this is most noticeable for ATCC25922. The highest relative fluorescence value following the addition of CHX 0.002 mg/mL is 0.51 (**Figure 4.7**). It may be that at this concentration of CHX, changes in cell membrane permeability occur, inducing cytoplasmic leakage, meaning that Ethbr that is bound to nucleic acids is able to fluoresce outside of the cell, resulting in an increase of relative fluorescence. Here, inactivation kinetics experiments show that CHX at 0.002 mg/mL caused a 1.67 Log_{10} reduction in viable cells after 5 min CHX exposure meaning that 96% of the starting inoculum was damaged enough to prevent replication. **(Section 4.3.4.3.)** It is also possible that structural changes in the outer membrane as a result of either stress or damage impinge the activity of efflux pumps (Castillo *et al*, 2006; Knapp *et al*, 2016). Kuyyakanond *et al*. (1992) demonstrated that membrane disruption was the principle damaging effect that CHX had on *E. coli* K12. However, CHX does not affect ATP hydrolysis even at bactericidal concentrations

(Kuyyakanond *et al*.,1992), and as such it is unlikely that the increase in fluorescence is due to the disabling of ATP dependant efflux pumps such as those belonging to the MFS family.

When we observe the graphs demonstrating the inclusion of PaβN and 0.002 mg/mL CHX, fluorescence is clearly higher (P=<0.0001; One-Way ANOVA; Graphpad PRISM8) than that when an EPI is included. Kinana *et al*. (2016) described in depth the process of influx and efflux of PaβN and compared it to three homologs in the context of the inhibition of nitrocefin efflux in *E. coli*. PaβN works as a competitive inhibitor of substrates of the AcrAB-TolC efflux systems. It binds to promoter regions of the pump blocking the way for other molecules to be extruded. Kiniana *et al*. (2016) found that PaβN has a modest affinity to AcrB pumps and is pumped out rapidly when it interacts with them. It has been reported that two homologs of PaβN (Ala β-naphthylamide, Arg β-naphthylamide) act as efflux stimulators in the case of nitrocefin. It was suggested that as these molecules are effluxed so rapidly (Lomovskaya *et al*., 2001, Kiniana *et al*., 2016) they “sweep off” other substrates with them. Although this explanation cannot be conclusively applied to our findings on the efflux relationship of CHX with PaβN, it might provide a starting point for further investigation. These observations of the difference between EPI inclusion at the higher concentration of CHX could not be seen with exposure to BZC (**Figure 4.8**).

Figure 4.8 shows the change in relative fluorescence with or without the inclusion of an EPI following the addition of two concentrations BZC at 10 min incubation. Here, it was observed that PaβN is not an effective inhibitor of efflux pumps that are active during the presence of BZC at 0.00005 mg/mL and 0.002 mg/mL. Again, this would suggest that the primary pump activity it is not orchestrated by RND-type pumps such as the AcrAB-TolC efflux system (Lomovskaya *et al*, 2001, Misra *et al*., 2015, Olliver *et al*., 2005, Kinaria *et al*., 2016). CCCP does significantly inhibit efflux pump activity at 0.00005 mg/mL BZC. As was demonstrated with CHX (0.00005 mg/mL and 0.002 mg/mL), there is a distinct difference in efflux activity

at the low (0.00005 mg/mL) and high (0.002 mg/mL) concentration of BZC tested. As BZC is also a membrane active biocide, changes in membrane permeability and leakage of EthBr may also explain the difference in relative fluoresce values observed when comparing BZC concentration. We have previously seen a loss of resistance to tetracycline, streptomycin, trimethoprim-sulfamethoxazole and trimethoprim for UCD-CFS ECP-13P5 when exposed to BZC for 24 hours (6h surface dried), This was explained by changes in cell membrane permeability which would support the findings of the efflux assay.

Figure 4.9 demonstrates a change in efflux activity after previous exposure (5min) of *E. coli* to CHX (surface dried for 24 h, 0.0047 mg/mL). Exposure was undertaken through the modified carrier test. The relative fluorescence level of UCD-CFS ECP-IB2 spiked with the addition of CHX at both high and low concentrations. The difference in efflux activity seen in **Figure 4.7,** following the addition of 0.00005 mg/mL and 0.002 mg/mL, was no longer observed. It can be seen that exposure to residual CHX levels for 5 min incited a change in either the activity of *E. coli* efflux phenotype or the biochemical ability for the mechanisms to perform efficiently. In the first 10 min incubation of the efflux assay, normal efflux levels were demonstrated. The addition of CHX at even low (0.00005 mg/mL) concentration appeared to have a negative effect on efflux activity in UCD-CFS ECP-IB2 **(Figure 4.9).** Relative fluorescence values are generally higher for isolates that have been exposed to CHX (carrier test, 0.0047 mg/mL) before undergoing the efflux assay **(Figure 4.7.).** The reasons for this are inconclusive. However, it may be suggestive of accumulative damage and change in membrane permeability after continuous exposure of the biocide.

Figure 4.7: Relative fluorescence values of *E. coli* isolates ATCC 25922, UCD-CFS ECP-IB2 and UCD-CFS ECP-13P5 for 60 min with the addition of CHX (0.00005 mg/mL or 0.002 mg/mL) at 10 minutes incubation. CHX 0.00005 mg/mL CHX 0.00005 mg/mL + EPI CHX 0.002 mg/mL CHX 0.002 mg/mL +EPI

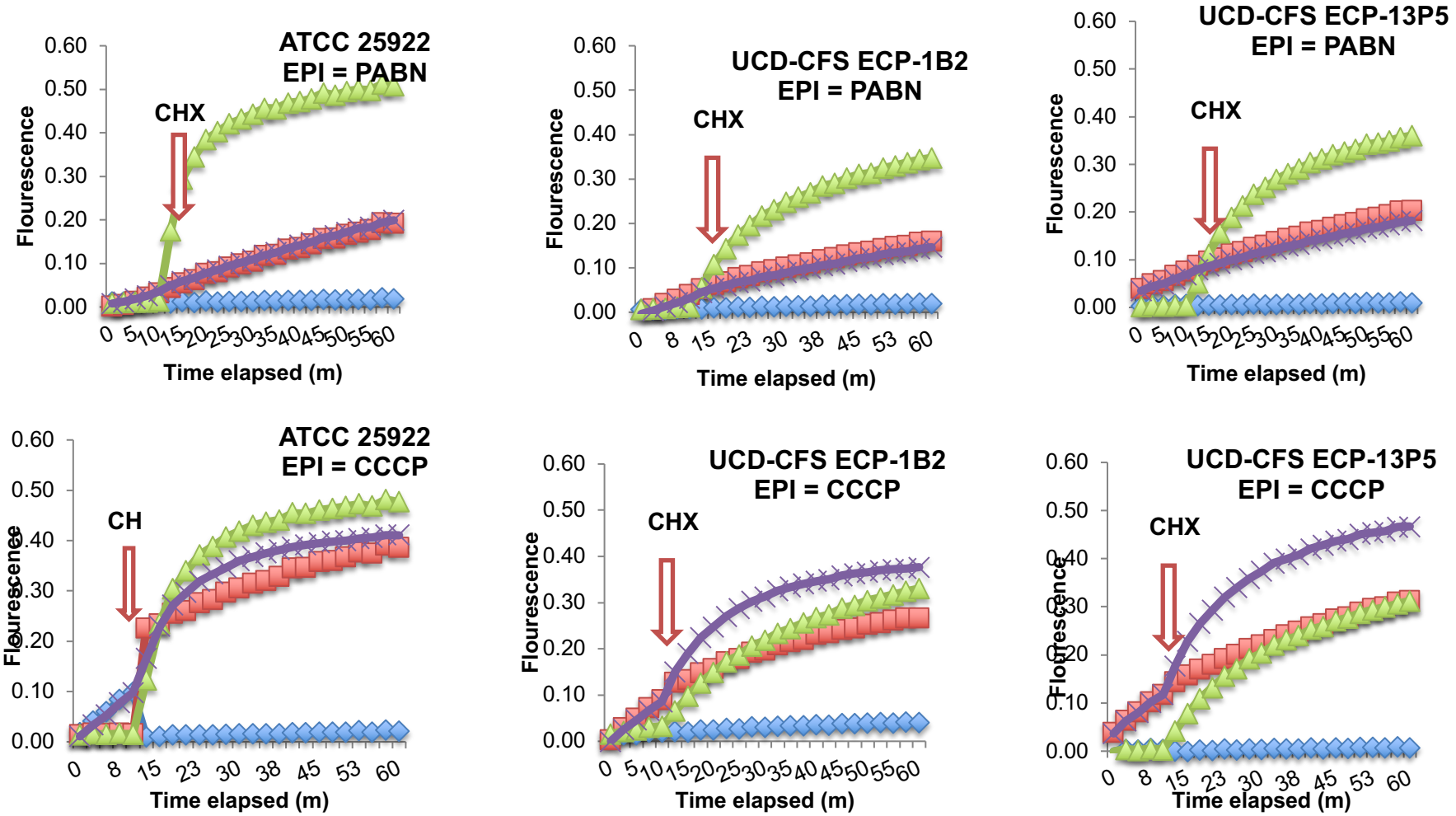


Figure 4.8: Relative fluorescence values of *E. coli* isolates ATCC 25922, UCD-CFS ECP-IB2 and UCD-CFS ECP-13P5 for 60 min with the addition of BZC (0.00005 mg/mL or 0.005 mg/mL) at 10 minutes incubation. BZC 0.00005 mg/mL BZC 0.00005 mg/mL + EPI BZC 0.002 mg/mL BZC 0.002 mg/mL +EPI

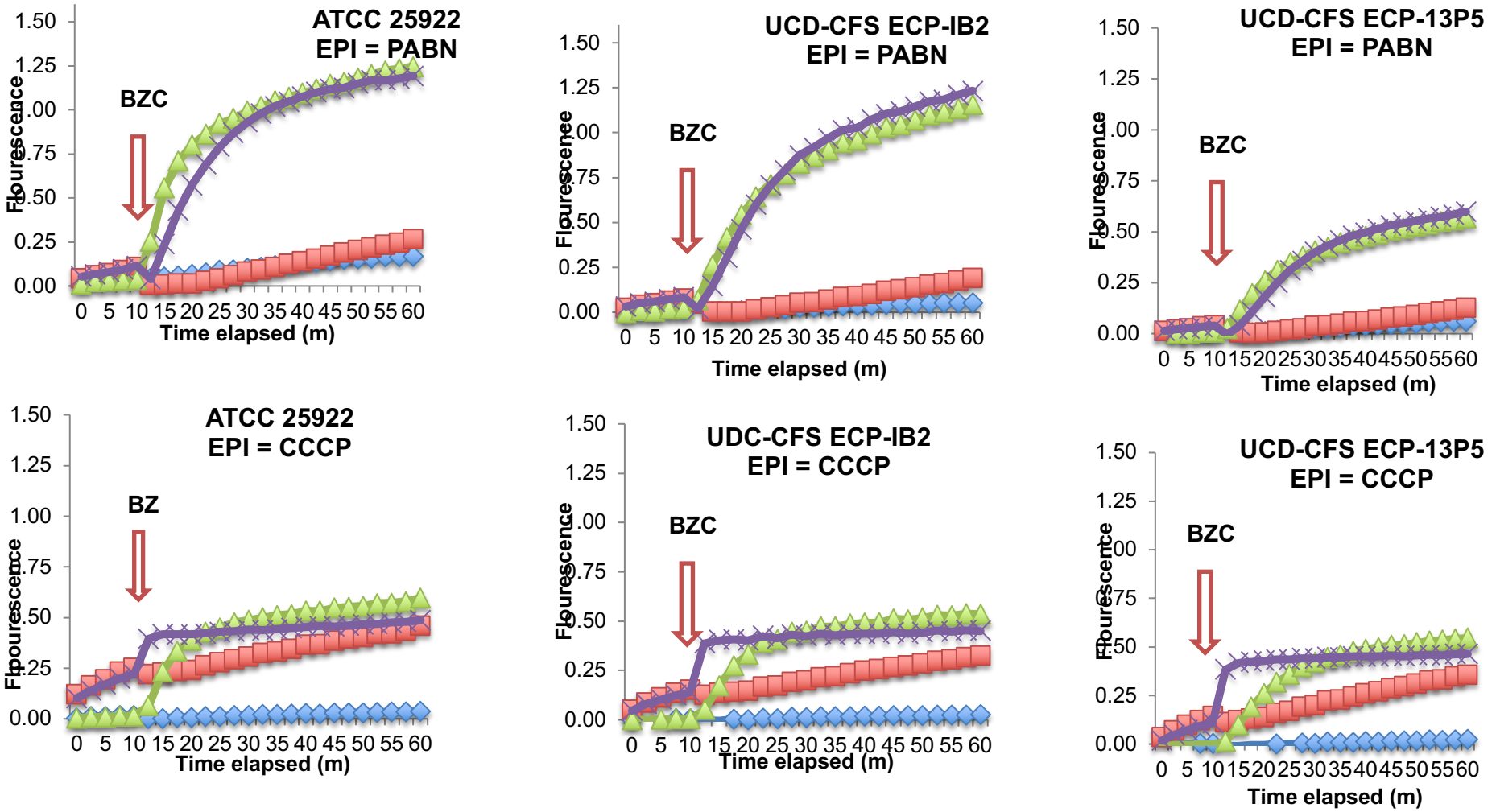
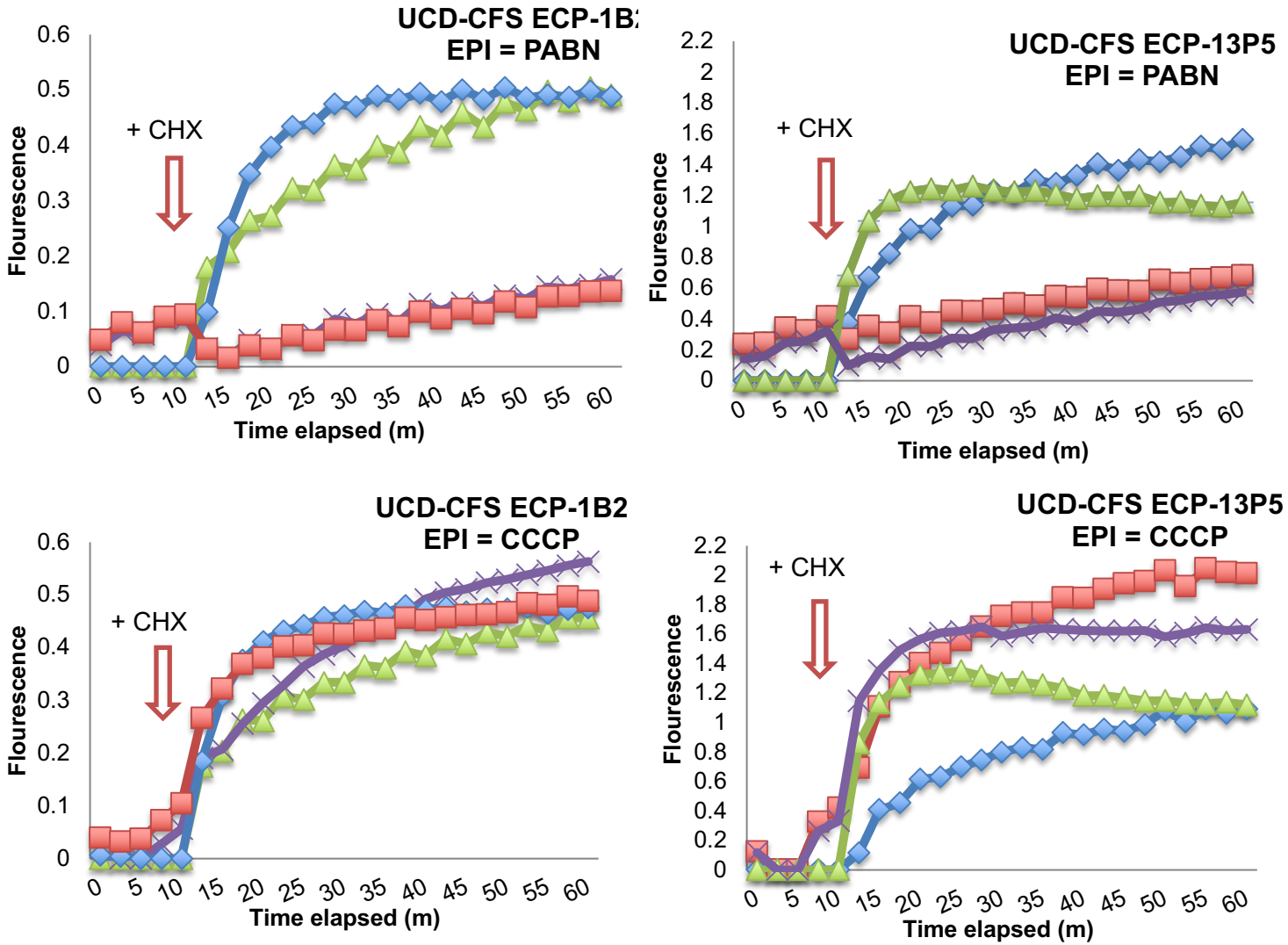


Figure 4.9: Relative fluorescence values of previously exposed *E. coli* isolates UCD-CFS ECP-IB2 and UCD-CFS ECP-13P5 for 60 min with the addition of CHX (0.00005 mg/mL or 0.002 mg/mL) at 10 minutes incubation. CHX 0.00005 mg/mL CHX 0.00005 mg/mL + EPI CHX 0.002 mg/mL CHX 0.002 mg/mL +EPI



4.3.5. Effect of exposure to biocidal residues on the stability of phenotypic change and inactivation kinetics

4.3.5.1. Choice of isolates to further study

In all instances after exposure, although the Log_{10} reductions were substantial, survivors were recoverable **(Table 4.10**). Isolates UCD-CFS ECP-13P5 and UCD-CFS ECP-IB2 were selected for further investigation due to their ability to recover and grow after exposure to 5 min CHX (dried on surface for 24 and 168 hours) **(Section 4.3.2.1).** These isolates were also selected as they demonstrated changes to CHX in their susceptibility phenotype; in the case of UCD-CFS ECP-IB2 there was an 8-fold increase in MIC and a 16-fold increase in MBC **(Section 4.3.2.2).** Both isolates were subjected to a modified carrier test and survivors were kept for further testing.

Table. 4.10 Log_{10} reduction after 5 min CHX exposure (n=2).

		Log_{10} reduction	
		EXPOSURE TIME: 5 minutes	
	Repeats	**24**	**168**
		0.0047 mg/mL	**0.0075 mg/mL**
UCD-CFS ECP-1B2	**1**	5.49	5.44
	2	4.76	5.13
UCD-CFS ECP-13P5	**1**	5.22	4.48
	2	5.03	5.62

Changes in MIC and MBC phenotype were observed in recovered cultures following exposure to CHX (**Table 4.11**). The most notable change was an 8-fold increase in MIC observed for isolate UCD-CFS ECP-IB2 after 5 min exposure to CHX (surface dried for 168

hours before microbial exposure). There were also instances of MIC and MBC decrease, which was not seen with CHX exposure in section 4.3.2.2.

Table 4.11 MIC, MBC and fold changes after CHX exposure (n=2).

		MIC (mg/mL)			MBC (mg/mL)		
		EXPOSURE TIME: 5 minutes					
	Repeats	**Baseline**	**24 0.0047 mg/mL**	**168 0.0075 mg/mL**	**Baseline**	**24 0.0047 mg/mL**	**168 0.0075 mg/mL**
UCD-CFS ECP-1B2	**1**	0.02	0.04 (4-fold)	0.16 (8-fold)	0.02	0.02	0.04 (2-fold)
	2	0.02	0.01	0.04 (4-fold)	0.02	0.02	0.02
UCD-CFS ECP-13P5	**1**	0.02	0.005	0.02	0.02	0.005	0.01
	2	0.02	0.002	0.08 (4-fold)	0.02	0.005	0.04 (2-fold)

4.3.5.2. Phenotype stability

In order to ascertain whether or not a change in susceptibility was transient or permanent, the MIC, MBC and the antibiotic susceptibility was assessed for stability over time following exposure to 0.002 mg/mL CHX. Passages were performed successively over a ten-day period.

Figure 4.10 and 4.11 depict changes in MIC and MBC values over the course of 10 passages with or without CHX. There was no difference in MIC when compared to the pre-exposure value. After one passage, UCD-CFS ECP-13P5 demonstrated a 8-fold increase in MBC (from 0.02 to 0.16 mg/mL CHX) when kept in the presence of CHX. However, when the isolate was passaged without the biocide the MIC returned to the pre-exposure value (0.02 mg/mL) **(Figure 4.10).** This demonstrates that the phenotypic change in MIC was transient. Forbes *et al*. (2014) found no change in MIC and MBC after CHX exposure. Wesgate *et al*. (2016) also found no stable changes in MIC or MBC after *E. coli* exposure to CHX 0.0005 mg/mL. All changes to MBC for UCD-CFS ECP-13P5 were unstable and by passage 10 the MBC returned to the pre-exposure value (0.02 mg/mL) **(Figure 4.11).** Isolate UCD-CFS ECP-1B2 also demonstrated an 8-fold increase in MBC after one passage in the presence of CHX. Changes in MIC and MBC were not stable and returned to the pre-exposure value after 10 passages in broth only.

Table 4.12 presents clinically relevant changes in antibiotic susceptibility (UCD-CFS ECP-13P5) after ten passages in CHX (24h surface dried 0.0047 mg/mL or 168 h surface dried 0.0075 mg/mL) or in broth only. Changes in antibiotic susceptibility (from sensitive to resistant according to EUCAST (2014) that were stable over ten passages were demonstrated for amoxicillin-clavulanic acid and isolate UCD-CFS ECP-13P5. Such

changes were not observed previously in **section 4.3.2.3** where antibiotic susceptibility remained the same (sensitive) after CHX exposure. According to a report from Public Health Wales Healthcare Associated Infection, Antimicrobial Resistance & Prescribing Programme (HARP) on antimicrobial resistance in Wales from 2008-2017 (Heginbothom *et al*., 2018) there was a statistically significant increase in resistance rates for amoxicillin-clavulanic acid between 2016 and 2017 which would suggest that resistance to this antibiotic is an increasing issue. A transient change from sensitive to resistant was observed for cefoxitin (initial exposure) and imipenem at passage 5 after CHX exposure (168 h surface dried 0.0075 mg/mL). Carbapenems such as imipenem are usually reserved for severe infections and resistance is less prevalent, in England 0.1% of bloodstream infections were as a result of carbapenem resistant *E. coli* in 2017 (PHE, 2018). Resistance to carbapenems due to the presence of CTX-M15-type ESBL producing bacteria has been previously reported (Liang *et al*., 2018). There were also transient changes in MBC for UCD-CFS ECP-13P5 at passage one in the presence of CHX (**Figure 4.10**). Gram-negative bacteria alter cell membrane potential and porin size when exposed to certain stressors (Oliver *et al*., 2002; Koebnik *et al*., 2000), which can prevent antibiotics from reaching their target sites. As previously discussed, CHX targets and causes changes to the cell membrane. The reduction in membrane permeability and the consequential IMP resistance may be an adaption of UCD-CFS ECP-13P5 to the sub lethal concentration of CHX.

Table 4.12 shows clinically relevant changes in antibiotic susceptibility (UCD-CFS ECP-1B2) after ten passages in CHX (24 h surface dried 0.0047 mg/mL or 168 h surface dried 0.0075 mg/mL) or in broth only. Previously (**section 4.3.2.4**.), no change to antibiotic susceptibility was seen after initial exposure of UCD-CFS ECP-IB2 to CHX. Here changes to resistant were observed for amoxicillin-clavulanic acid after initial exposure (168 h surface dried CHX 0.0075 mg/mL). The resistance observed for amoxicillin-clavulanic acid

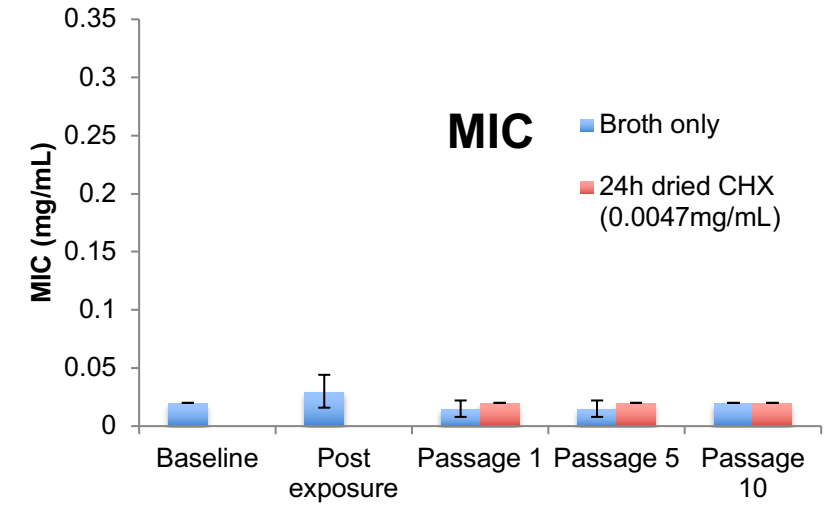
was no longer present after passage one however it re-appeared after passage 5 implying that this change was caused by physiological mechanisms of adaption such as porin regulation and efflux expression (Fernández & Hancock, 2019) and can therefore be switched on and off in an adaptive response to environmental stressors. Stable changes (from susceptible to resistant) were observed for ampicillin, amoxicillin-clavulanic acid, cefpodoxime and cephalothin from passage 5 until passage 10. These changes were observed both in the presence of CHX (0.002 mg/mL) and when passaged in broth only. This suggests that these changes are due to mutations. Mutational resistance can occur for both porin-encoding genes and efflux pumps (Lou *et al*., 2011; Fernández & Hancock, 2019).

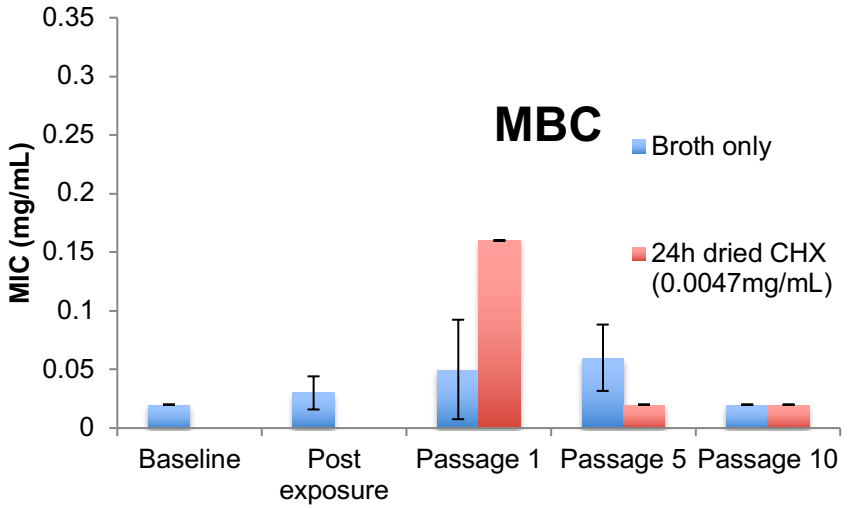
Previously (**section 4.3.2.4.),** no change to antibiotic susceptibility was seen after initial exposure of UCD-CFS ECP-IB2 to CHX. Cefpodoxime is a third-generation cephalosporin, PHE report that 13% of bloodstream infections in England (2017) were as a result of carbepenem resistant *E. coli* (PHE, 2018). The WHO global report on surveillance of antimicrobial resistance (2014) states that *E. coli* resistance to third-generation cephalosporin was reported in Africa, The Americas, Eastern Mediterranean region, Europe, South-East Asia and the Western Pacific. Oliver *et al*. (2002) demonstrated that low-level resistance to cefpodoxime in *E. coli* might be due to essential changes in major outer membrane proteins (OMP), which in turn lead to changes in porin regulation. This was shown to confer resistance phenotypes regardless of whether or not the isolate was an ESBL producer (Oliver *et al*., 2002). Fernández & Hancock (2019) describe how a synergy between efflux and low-permeability of the cell envelope in *E. coli* provide excellent defences against antimicrobials. **Table 4.13** presents studies that highlight examples of efflux pump activity and incidences of resistance to antibiotics relevant to this thesis. UCD-CFS ECP-1B2 demonstrated transient resistance to ciprofloxacin after passage 5 in broth only. Ciprofloxacin resistance was demonstrated in *E. coli* due to the up-regulation of the

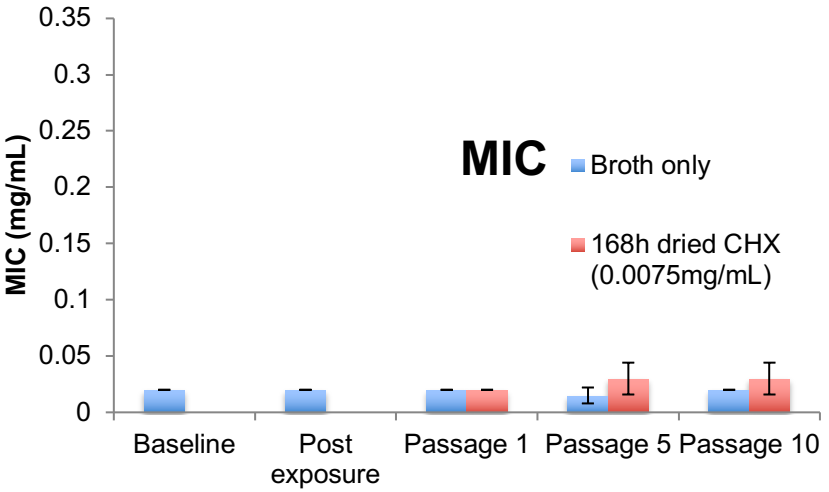
YdhE (MATE) efflux pump (**Table 4.13**) (Morita *et al*.,1988). Other fluroquinolones are shown to be effluxed by pumps from the ABC, MFS, and RND families (Table 4.13), suggesting that these efflux pumps may be active after previous exposure to CHX (24 h surface dried 0.0047 mg/ml and 168 h surface 0.0075 mg/mL). As seen in **Figure 4.9** after previous 5 min exposure to CHX (24h surface dried 0.0047 mg/mL) efflux activity appeared to cease where it was previously present (**Figure 4.7**). This would suggest that efflux may not be responsible for the changes in antibiotic susceptibility for UCD-CFS ECP-1B2 seen in this section. On the other hand, efflux activity did occur in UCD-CFS ECP-13P5 after previous exposure to CHX (24h surface dried 0.0047 mg/mL), indicating that increased efflux activity may be the cause of changes in antibiotic susceptibility towards amoxicillin-clavulanic acid, cefoxitin and Imipenem (**Table 4.11**).

Figure 4.10: Average MIC and MBC values of UCD-CFS ECP-13P5 before and after 1, 5 and 10 passages in CHX (n=3).

Standard deviation of the mean is shown







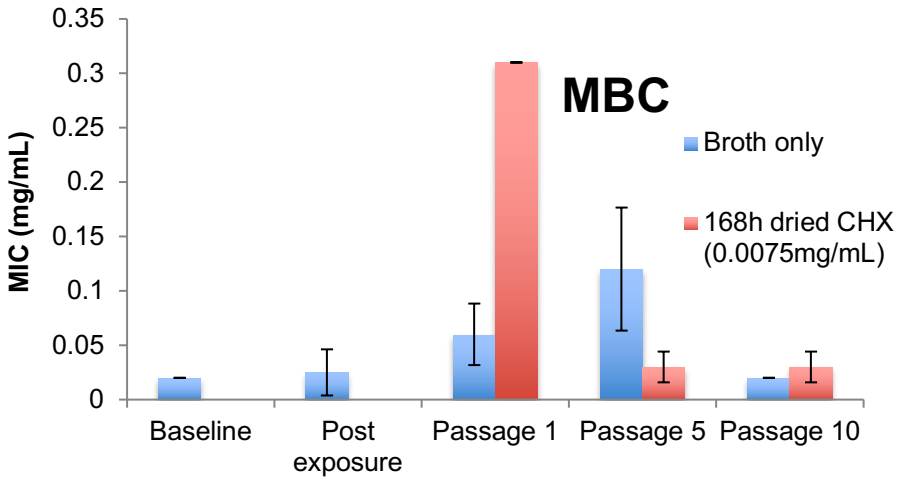
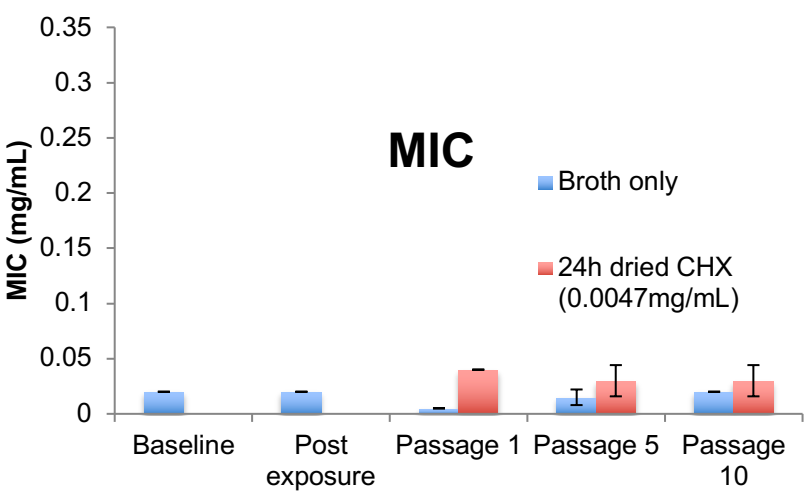
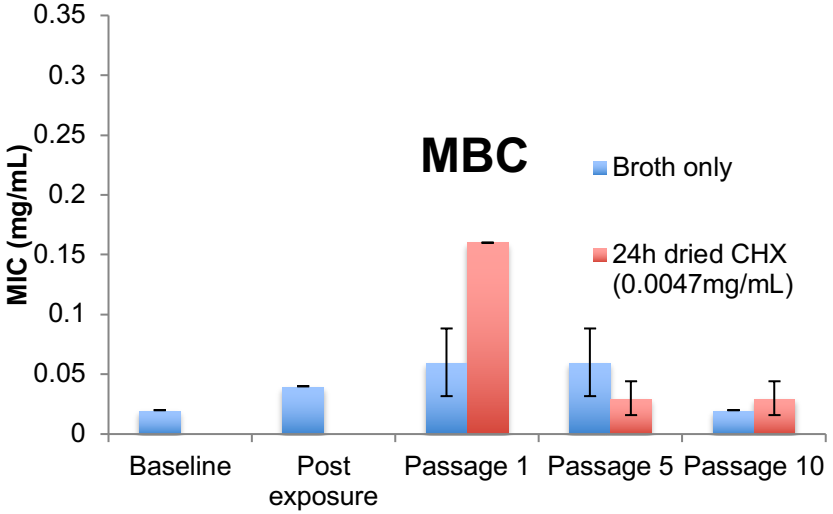
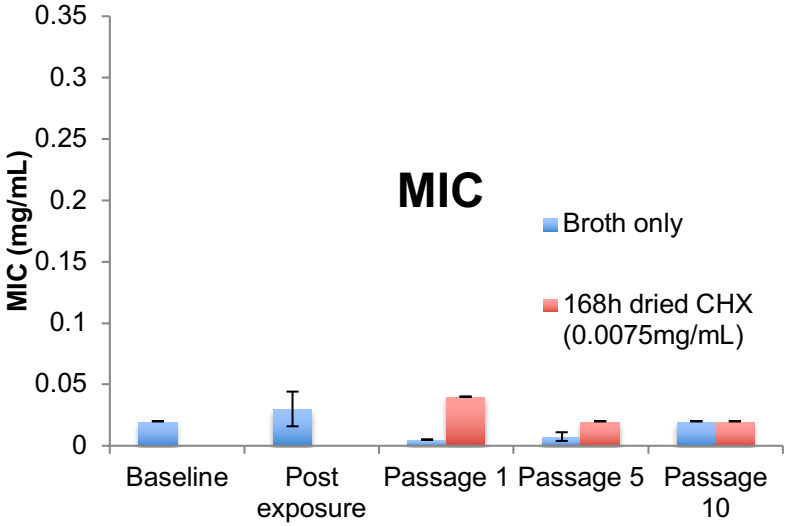


Figure 4.11: Average MIC and MBC values of UCD-CFS ECP-1B2 before and after 1, 5 and 10 passages in CHX (n=3).

Standard deviation of the mean is shown







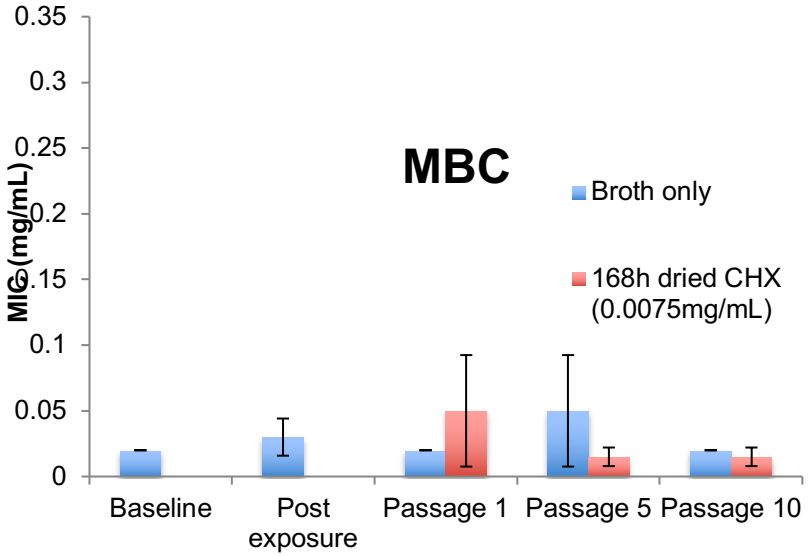


Table 4.11. Clinically relevant changes in antibiotic susceptibility phenotype according to EUCAST breakpoint values for *E. coli* isolate UCD-CFS ECP-13P5 before and after initial exposure to, and passage in, CHX or broth only (20 mg/mL; 5 min after CHX surface drying for 24 and 168 h) via a modified carrier test.

		CHX 24 hours drying (0.0047mg/mL)		**CHX 168 hours drying (0.0075mg/mL)**	
		CHX	**BROTH ONLY**	**CHX**	**BROTH ONLY**
	Exposure time	**5m**	**5m**	**5m**	**5m**
INITIAL EXPOSURE	Repeat: 1	**AMC**	-	**AMC, FOX**	-
	2	**AMC**	-	**AMC, FOX**	-
PASSAGE 1	1	**AMC**	*	**AMC**	*
	2	**AMC**	**AMC**	**AMC**	**AMC**
PASSAGE 5	1	**AMC**	**AMC**	**AMC**	**AMC**
	2	**AMC**	**AMC**	**AMC, IPM**	**AMC**
PASSAGE 10	1	**AMC**	**AMC**	**AMC**	**AMC**
	2	**AMC**	**AMC**	**AMC**	**AMC**

*- Not tested * No change in antibiotic susceptibility observed*

Red text represents a change in phenotype from clinically sensitive to clinically resistant in accordance with EUCAST breakpoints

amoxicillin/clavulanic acid (AMC), imipenem (IMP), cefoxitin (FOX)

Table 4.12. Clinically relevant changes in antibiotic susceptibility phenotype according to EUCAST breakpoint values for *E. coli* isolate UCD-CFS ECP-1B2 before and after initial exposure to and passage in CHX or broth only (20 mg/mL; 5 min after CHX surface drying for 24 and 168 h) via a modified carrier test.

		CHX 24 hours drying (0.0047mg/mL)		**CHX 168 hours drying (0.0075mg/mL)**	
		CHX	**BROTH ONLY**	**CHX**	**BROTH ONLY**
	Exposure time	**5m**	**5m**	**5m**	**5m**
INITIAL EXPOSURE	Repeat: 1	*	-	**AMC**	-
	2	*	-	**AMC**	-
PASSAGE 1	1	*	*	*	**AMC**
	2	*	**AMC**	**AMP**	**AMC**
PASSAGE 5	1	*	**AMP,CIP,CPD,CF**	**AMP,AMC,CPD,CF**	**AMP,AMC,CIP,CPD,CF**
	2	*	**AMP,AMC,CIP,CPD,CF**	**AMP,AMC,CPD,CF**	**AMP,AMC,CIP,CPD,CF**
PASSAGE 10	1	**CF**	**AMP,AMC,CPD,CF**	**AMP,AMC,CPD,CF**	**AMP,AMC,CPD,CF**
	2	**CF**	**AMP,AMC,CPD,CF**	**AMP,AMC,CPD,CF**	**AMP,AMC,CPD,CF**

- Not tested ** No change in antibiotic susceptibility observed*

Red text represents a change in phenotype from clinically sensitive to clinically resistant in accordance with EUCAST breakpoints

Ampicillin (AMP), amoxicillin/clavulanic acid (AMC), cefpodoxime (CPD), cephalothin (CF), ciprofloxacin (CIP)

Table 4.13. Examples of efflux pump activity and incidences of antibiotic resistance

FAMILY	PUMP	RESISTANCE IDENTIFIED	REFERENCE
ABC	MacAb-TolC	Fluoroquinolones	Garrido *et al*., 1988 Lomovskaya *et al*., 1996
MFS	QepA, QepA2	Fluoroquinolones	Cattoir *et al*., 2008 Yamane *et al*., 2007
	MdfA	Fluoroquinolones	Nilsen *et al*., 1996
MATE	NorE	Fluoroquinolones	Yang *et al*., 2003
	YdhE	(Ciprofloxacin)	Morita *et al*., 1988
RND	AcrAB-TolC	β-Lactam	Fralick, 1996
		Fluoroquinolone	Russel et al., 1993
	AcrEF-TolC	Fluoroquinolone	Russel et al., 1994

4.3.5.3 Inactivation kinetics

Following the modified carrier test, UCD-CFS ECP1B2 and UCD-CFS ECP-13P5 underwent inactivation kinetics assays. This was to ascertain whether previous exposure to CHX residue had an effect on the rate of re-introduced microbial reduction over time. UCD-CFS ECP-13P5 and UCD-CFS ECP-1B2 were exposed to CHX at 20 mg/mL, 0.007 mg/mL or 0.002 mg/mL. These concentrations were chosen to represent the in-use concentration (20 mg/mL), the concentration found left on a surface (0.006 ± 0.002 mg/mL; **Section 4.3.1.1**) and the concentration below the MIC value (0.002 mg/mL) in order to exert a selective pressure but not necessarily kill all microbial cells.

When exposed to 20 mg/mL for 30 s no viable bacteria were recovered for UCD-CFS ECP-13P5 or UCD-CFS ECP- 1B2 **Figures 4.11** and **4.12**). UCD-CFS ECP-13P5 exposure to 0.007 mg/mL CHX resulted in 2.56 ± 0.15 Log_{10} reduction after 30 s. There was no significant difference (P = <0.0001; T-Test; Graphpad PRISM8) in Log_{10} reduction between 30 s and 60 min exposure. There was a 1.67 Log_{10} reduction in viability after 30 s exposure to 0.002 mg/mL CHX. This loss of viability did not increase with a 5 min contact time. There was no statistical difference (P = <0.0001; T-Test; Graphpad PRISM8) in efficacy of CHX at 20 mg/mL or 0.002 mg/mL between UCD-CFS ECP-13P5 previously exposed to CHX residues (0.0047mg/mL or 0.0075mg/mL). However, pre-exposure to CHX dried on surface for 24 h and 168 h rendered the isolates more susceptible to 0.007 mg/mL CHX after 30 s. This may be explained by accumulative damage following repeated exposure of CHX, rendering them particularly susceptible to CHX. As 0.007 mg/mL CHX is a higher concentration than the MIC, the initial reduction followed by tolerance may be explained in terms of population persistence **(Figure 4.11)** (Balaban *et al*, 2019). This explanation may be investigated further through phenotypic microarrays in order to ascertain whether or not changes in metabolic regulation are present. When comparing these findings to those in **section 3,** it can be postulated that the slowing down in lag phase and the decrease of specific growth rate may be the decrease in metabolic processes that are usually attributed to population persistence (Brauner *et al*, Balaban *et al*, 2019).

Figure 4.12: Inactivation kinetics of exposed and non-exposed *E. coli* isolate UCD-CFS ECP-13P5 in the presence of CHX (20 mg/mL, 0.007 mg/mL and 0.002 mg/mL) over a 5 min contact time.

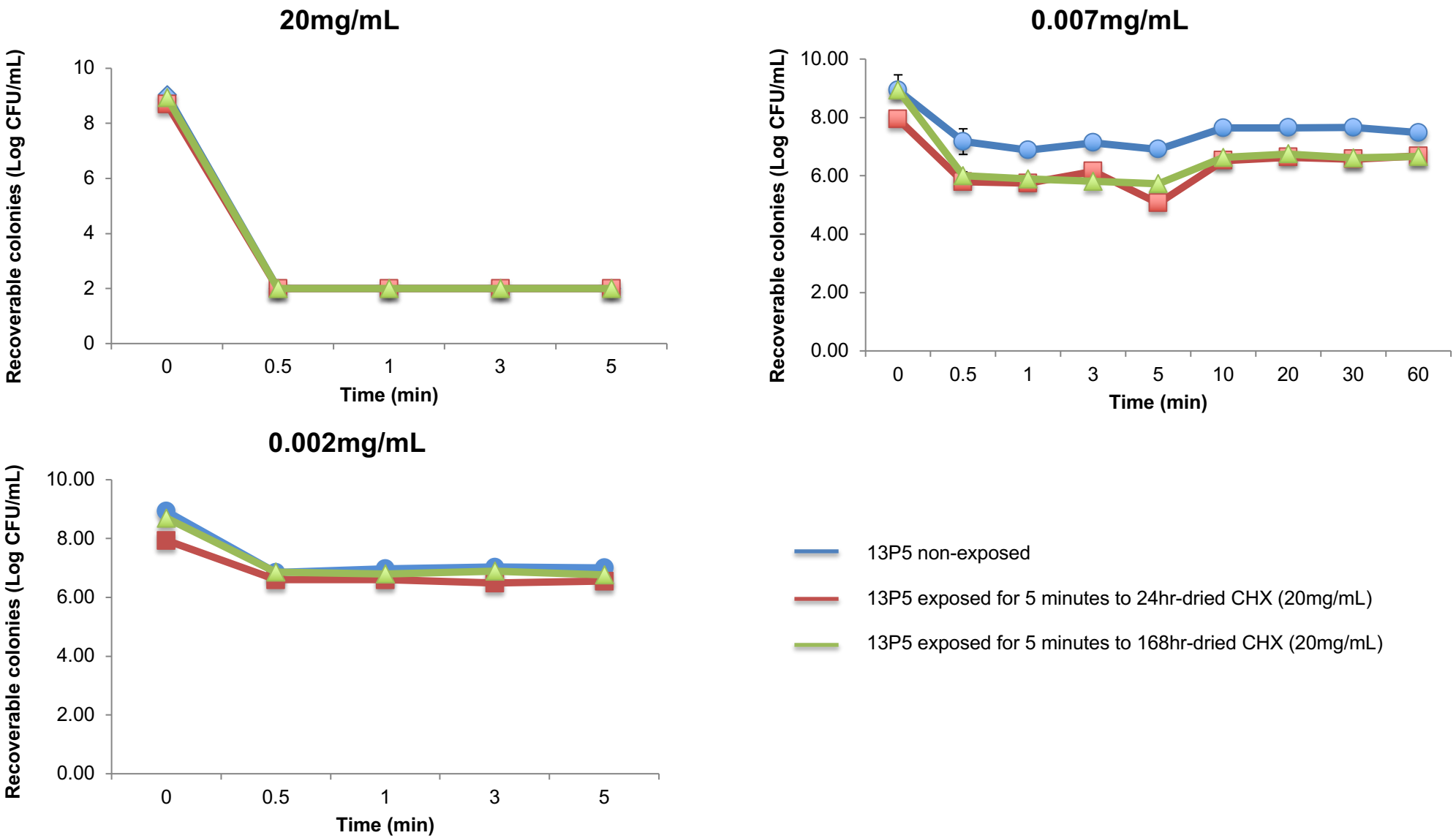
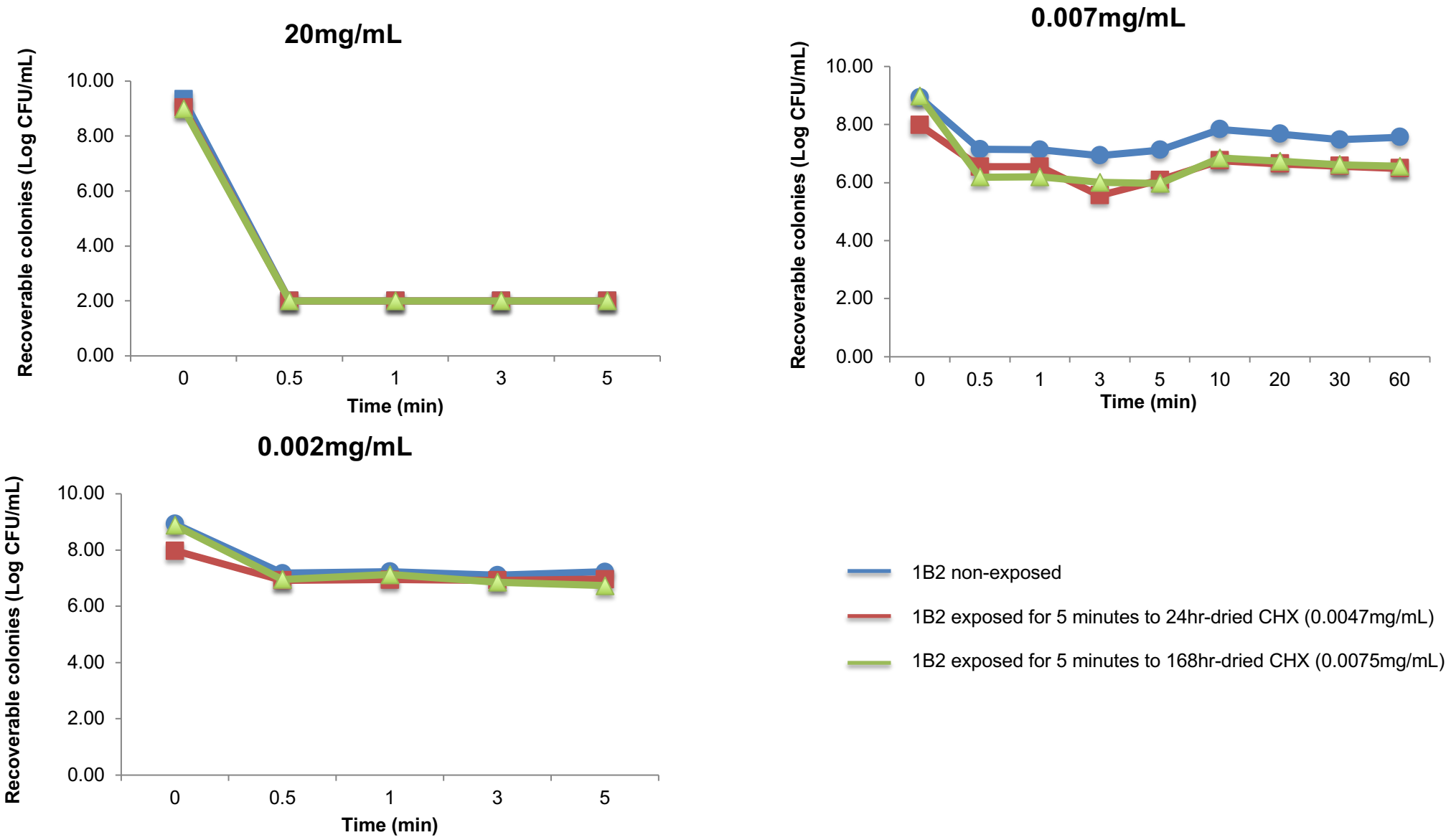


Figure 4.13: Inactivation kinetics of exposed and non-exposed *E. coli* isolate UCD-CFS ECP-1B2 in the presence of CHX (20mg/mL, 0.007mg/mL and 0.002mg/mL) over a 5 minute contact time.



4.3.6. Exploring the effect of exposure to biocide residues on gene transfer by conjugation

4.3.6.1. Choice of Isolates to further study

Isolate UCD-CFS ECP-13P5 was selected for further investigation due to its ampicillin resistance (**Section 3.3.5**) and its ability to recover and grow after exposure to 5 min CHX (dried on surface for 24 and 168 h) (**Section 4.3.2.1**). The recipient strain *E. coli* J35R was chosen for its chromosomally encoded rifampicin resistance.

4.3.6.2. Conjugation assay

Table 4.14 shows transfer ratios of ampicillin resistance from UCD-CFS ECP-13P5 to J35R before and after CHX exposure. UCD-CFS ECP-13P5 showed an ampicillin resistance transfer ratio in the range of 1.34 x 10^{-5}. The presence of 0.00005 mg/mL CHX did not demonstrate a statistically significant change in rate of transfer (P=0.730; T-Test; Graphpad PRISM8). Jutkina *et al*. (2018) demonstrated that 0.00002 mg/mL CHX increased transfer frequency rates of sulfamethoxazol resistance in *E. coli*. An increase in conjugative transfer of ampicillin resistance in *E. coli* has also been reported after exposure to sub-inhibitory concentrations of chlorine, chloramine and hydrogen peroxide (Zhang *et al*., 2017).

The presence of 0.002 mg/mL CHX caused a statistically significant decrease in conjugative transfer (P=0.009 T-TEST; Graphpad PRISM8). Conjugation appeared to stop altogether with a rate of transfer of 0 (**Table 4.14**). The halting of conjugative transfer might be explained by the effect that membrane active CHX has on the synthesis conjugative apparatus (Masaudi *et al*., 1991; Pearce *et al*., 1999). However, it was noted that the

number of donors and recipients also decrease after exposure at this concentration (0.002 mg/mL). The number of donors decreased from 7.86 Log_{10} to 4.60 Log_{10} which amounts to a 3.26 Log_{10} loss after exposure. The number of recipients decreases from 8.51 Log_{10} to 4.70 Log_{10}, a 3.81 Log_{10} loss. As conjugation relies upon cell-cell contact, fewer cells will result in less cell mating. Therefore the stopping of conjugative transfer may be due to the loss of viable cells through CHX exposure. This experiment was performed on growing cells as outlined by Lambrecht *et al*. (2017). It has been suggested and demonstrated for *E. coli* in relation to cefotaxime, that bacterial conjugation is defined by the phase of growth that they are undertaking; non-growing cells usually demonstrate higher rates of transfer than growing cells (Lampokowska *et al*., 2008; Headd & Bradford, 2018). Here, we used exponential growing cells, which might have impinged on conjugation efficiency.

Table 4.14 Log_{10} values of donors and recipients before and after mating and the rate of conjugative transfer (n=3).

	Number (Log_{10}) of donors in 1 mL		**Number (Log_{10}) of recipients in 1 mL**				
	Before mating	**After mating**	**Before mating**	**After mating**	**Donor to recipient ratio**	**Number (Log_{10}) of transconjugants in 1 mL**	**Rate of transfer**
No exposure	7.36	7.04	8.07	7.70	1:5	2.79	1.3417E-05
0.00005 mg/mL	7.86	8.72	8.51	8.53	1:5	3.58	1.55894E-05
0.002 mg/mL	7.86	4.60	8.51	4.70	1:5	0.00	0

4.4 Conclusion

The concentration of biocide that was recovered after drying was 98.8% lower than that initially applied. The significance of this result becomes apparent as the average remaining concentration (0.006 ± 0.002 mg/mL) was the same as the average MIC of the isolates tested 0.006 ± 0.002 mg/mL). This allowed for bacterial survivors (UCD-CFS ECP-1L3, UCD-CFS ECP-1L4, UCD-CFS ECP-1B2, UCD-CFS ECP-13P5, UCD-CFS ECP-13P4) after a 5 min (**Table 4.2**) exposure CHX at a range of residual concentrations (0.0049-0.0097 mg/mL) but not after 24 hours (**Table 4.3**). These results obtained after 5 min CHX exposure were not consistent for each experimental repeat. Variability could also be seen for concentration on the surface after drying (**Figure 4.5**). However, the variations in bacterial recovery and CHX concentration were random and cannot be explained by the decrease in concentration over time as shown by pearsons correlation analysis (P=0.62; Pearsons correlation analysis, r^2=0.07; Graphpad PRISM8). The variance of the concentration recovered after 0-168 hours is indicative that there is a potential for failure of a biocide after application *in-situ*. The average concentration of CHX recovered from the surface between 0-168 hours drying (0.006 ± 0.002 mg/mL) was lower than the ECOFF value (0.064 mg/mL) obtained by Morissey *et al*. (2018), indicating that only 1.1% of *E. coli* isolates included in the study would not survive treatment of 0.006 mg/mL CHX. Furthermore, the average MIC value for the ECOFF study was 0.04 mg/mL. This encompasses 36.1% of the isolates studied, meaning these would also survive in the event of a residual surface concentration of 0.006 mg/mL as demonstrated in this chapter. The average CHX concentration that was recovered on the surface (0.006 ± 0.002 mg/mL) was above the MSC-MIC*susc* range **(Table 3.4; Figure 3.8; Figure 3.9)** for the two isolates analysed (UCDCFS ECP-1L3, 0.0014-0.002 mg/mL and UCD-

CFS ECP-1B2, 0.00005-0.005 mg/mL). However, both of these isolates demonstrated survival after 5 min exposure to CHX 0.0075 mg/mL (surface dried 168 h) (**Table 4.2**). Furthermore 0.0075 mg/mL is within the MIC*susc to* MIC*res* concentration range for both isolates (0.005-0.02 mg/mL). It has been discussed that at this higher range of concentrations, there is a stronger selection pressure, which may result in inactivation of susceptible sub-populations and the persistence of more tolerant cells (**Figure 4.3c**). The possibility of persistent cells was reflected in the results from CHX inactivation kinetics **(Section 4.3.5.3)** where a decrease in viable cells of 1.67 Log_{10} was demonstrated after exposure of UCD-CFS ECP-13P5 and UCD-CFS ECP-1B2 to CHX 0.002 mg/mL, followed by no further decrease but apparent persistence (**Figure 4.11; Figure 4.12**).

Exposure to residual levels of CHX (0.0049 mg/mL, 0.0097 mg/mL, 0.0047 mg/mL and 0.0075 mg/mL) resulted in elevated MIC and MBC values. The elevated MIC values obtained (**Table 4.6**) were higher than the average concentration of CHX found on surface (0.006 mg/mL). The determination of MIC values is an indicator of the ability for a biocide to alter the bacterial phenotype; the MBC is a more significant indication of the lethality of a biocide (Maillard & Denyer, 2009). No increases in MIC or MBC values were observed after residual BZC exposure (**Table 4.8; Table 4.9**). Instead, decreases in MIC and MBC values were demonstrated, possibly attributed to a cellular damage or the fitness cost of adaption (McBain *et al*., 2004). McBain *et al*. (2004) showed that when *E. coli* demonstrated triclosan resistance, it also demonstrated an increase in susceptibility to CHX. The reasons for this were inconclusive but it was surmised that bacteria underwent transient physiological changes during exposure to triclosan that rendered them more susceptible to CHX. Cross-susceptibility was also demonstrated with *E. coli* after repeated exposure to CHX, increased susceptibility to ciprofloxacin and ampicillin were observed (Forbes *et al*., 2019).

Antibiotic susceptibility changes were observed after one exposure to residual concentrations of CHX (0.0047 & 0.0075 mg/mL) for isolates UCD-CFS ECP-13P5 and UCD-CFS ECP-1B2 (**Table 4.11. & Table 4.12**) from susceptible to resistant for amoxicillin-clavulanic acid and cefoxitin. Changes in amoxicillin-clavulanic acid susceptibility were stable once appeared for UCD-CFS CEP-13P5 and appeared both in the presence of CHX (0.002 mg/mL) and when passaged in broth only. These stable changes of decreased susceptibility are suggestive of the selection of mutations that render the isolate more tolerant (Lou *et al*., 2011; Fernández & Hancock, 2019). Conversely, another type of susceptibility change was demonstrated, where amoxicillin-clavulanic acid resistance was observed after the first exposure to CHX (0.0075 mg/mL) but disappeared until passage 5 where it reappeared and maintained presence until passage 10, indicating that this change in antibiotic resistance phenotype was as a result of a transient physiological adaptation. These data imply that residual concentrations of CHX can induce changes in antibiotic susceptibility (from sensitive to resistant) that are mediated through selection of mutation and transient adaptive physiological changes.

There were distinct differences in efflux activity between high and low concentrations of CHX and BZC (**Figure 4.7, Figure 4.8**). High efflux activity was observable at the lower concentrations of CHX and BZC only (0.00005 mg/mL). CCCP appeared to be a good indicator of CHX mediated efflux activity for ATCC25922, UCD-CFS ECP-13P5 and UCD-CFS ECP-1B2. The likelihood of a recently discovered CHX efflux pump AceI, a member of the PACE super family was discussed as CCCP was proven to work as a successful indicator of this pump (Hassan *et al*., 2013; 2015; 2018). PAβN was not a good inhibitor of efflux pump activity of CHX or BZC which lead to the conclusion that RND efflux pumps were most likely not the primary source of efflux activity (Lomovskaya *et al*, 2001, Misra

et al., 2015, Olliver ET AL 2005, Kinaria *et al*., 2016). The presence of 0.002 mg/mL CHX and BZC efflux decreased dramatically. This was attributed to changes in the cell membrane and the possible leakage of EthBr due to structural cell damage. This may be supported by the previous finding of the loss of resistance to tetracycline, streptomycin, trimethoprim and after residual BZC exposure. The residual concentration of BZC left on a surface after drying was not determined; it is possible that this concentration is similar to the high concentration used in the efflux study (0.002 mg/mL). Pre-exposure to CHX dramatically changes efflux activity of UCD-CFS ECP13P5 and UCD-CFS ECP-1B2 via the efflux assay, which presents a second CHX challenge. There was no longer efflux activity at 0.00005 mg/mL CHX, the reasons for this were inconclusive but as it is known that CHX is a membrane active biocide (Maillad and Denyer 2002). Accumulative damage to the bacterial cell membrane may be preventing the efflux pumps from working. It was previously outlined (**Section 4.3.5.3)** that after 30s exposure to 0.007 mg/mL CHX there was a reduction in viable cells of 2.56 ± 0.15 Log_{10}. No further reduction was seen after 60 min exposure. 0.007 mg/mL is close to the average concentration of CHX recovered from the surface (0.006 ± 0.002 mg/mL), therefore the loss of efflux could be due to the loss of cell viability after two successive exposures (starting inoculum was ≈ 8.00Log_{10}). Viable counts were not performed after exposure before the efflux assay was performed, for this reason the explanation is inconclusive.

The propensity for biocides to induce the transfer of plasmid-mediated resistance is well documented (Zhang *et al*., 2017; Jutkina *et al*., 2018). This study aimed to investigate the difference of effect between low (0.00005 mg/mL) and high (0.002 mg/mL) residual concentrations of CHX. There was a difference in the effect of high and low residual concentrations of CHX on conjugal transfer of ampicillin

resistance from isolate UCD-CFS ECP-13P5. However, this difference could not be attributed to the pressures of the higher concentration of CHX alone as it was unclear whether the halting of conjugation was in part down to the loss of viable cells and therefore the loss of cell-to-cell contacts. It is possible that 0.002 mg/mL CHX damages conjugal apparatus in turn leading to a decrease in conjugal transfer, but this is currently inconclusive.

From the data obtained in this section it appears that the experimental design is a good representation of the *in-situ* circumstances of biocidal application and the estimation of residual concentrations left on surfaces. For example, in this study 1 mL of 20 mg/mL solution of standard CHX or 0.45 mg/mL BZC was first added, then immediately removed from a glass surface. In true to life circumstances, firstly the product would most likely be applied using a cloth or mop, which means that the liquid, including the biocide will be absorbed and removed from the surface rapidly. Moreover, a biocidal product based upon active CHX or BZC would be formulated with a number of other compounds including preservatives and surfactants. Susceptibility testing to biocides is usually performed in aqueous solutions containing only the biocide active. It is suggested that this might lead to an over-estimation of the real to life outcomes of biocides impact to microbial susceptibility (Forbes *et al*., 2019).

The next section will look further into physiological changes might lead to the changes in MIC, MBC, antibiotic susceptibility and efflux resistance phenotypes that have been observed in this section and will aim to understand how metabolic regulation is linked to these changes.

CHAPTER 5: CHANGES IN METABOLIC REGULATION AS A RESULT OF EXPOSURE TO CHX RESIDUAL CONCENTRATIONS

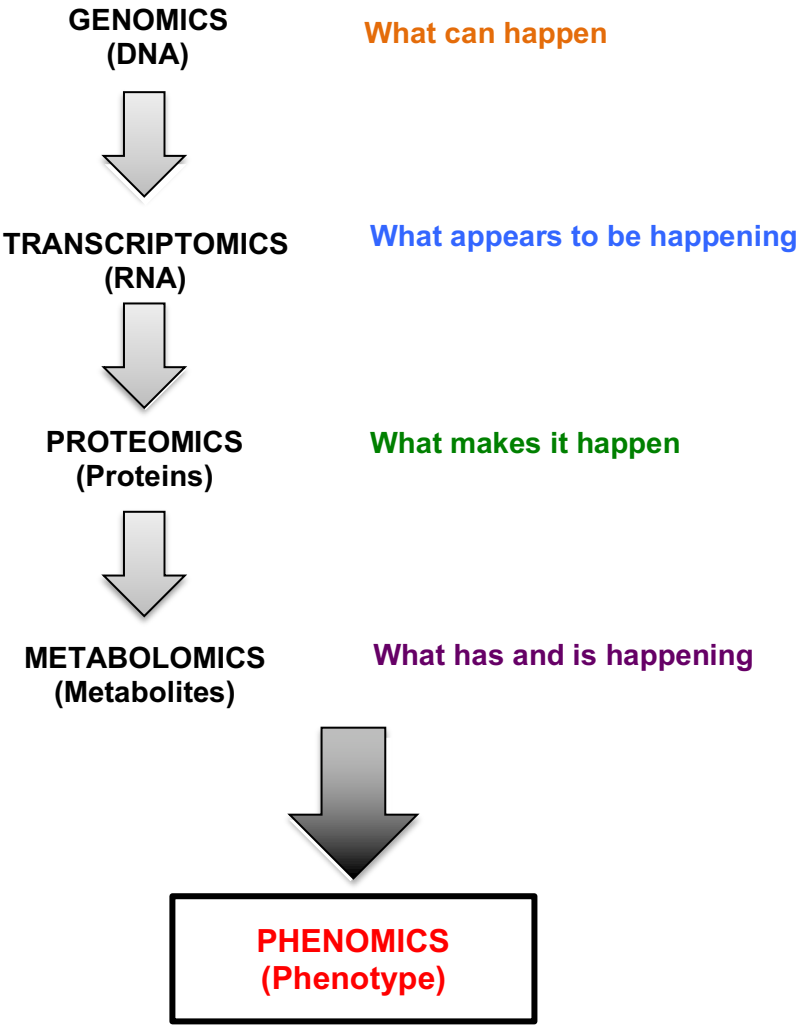
5.1. Assessing CHX Mediated Changes in *E. coli* Metabolism Using the Phenotypic Microarray

5.1.1. Information obtainable through the "Omics" cascade

The "omics" cascade (**Figure 5.1**), as described by (Dettmer *et al*., 2007) depicts the associations of the genome, transcriptome, proteome and the metabolome. The term "omics" denotes a global assessment of a group of molecules (Hasin *et al*., 2017).

Whilst genetics studies focus on single genes, genomics investigations delve into whole genomes. These results give an insight into the possibilities of bacterial phenotype dependent on the presence of certain genes. However, the presence of a gene does not always confer a phenotype. The transcriptome is the complete set of RNA transcripts that are produced by the genome, under specific circumstances. Transcriptomic assays explore and identify genome-wide RNA levels. What and how much of a transcript is expressed can be distinguished. The proteins responsible for genetic occurrences, peptide abundance, alteration and interactions can be unearthed through proteomic assays. The metabolome can be defined as a complete set of metabolites (i.e. amino acids, carbohydrates, fatty acids) in an organism or cell. Studying the metabolome provides an insight into how environmental fluctuations can result in biological changes. Metabolomic tests investigate what has and is happening as a result of a combination of genomic, transcriptomic and proteomic expressions (Schellenberger *et al*., 2010).

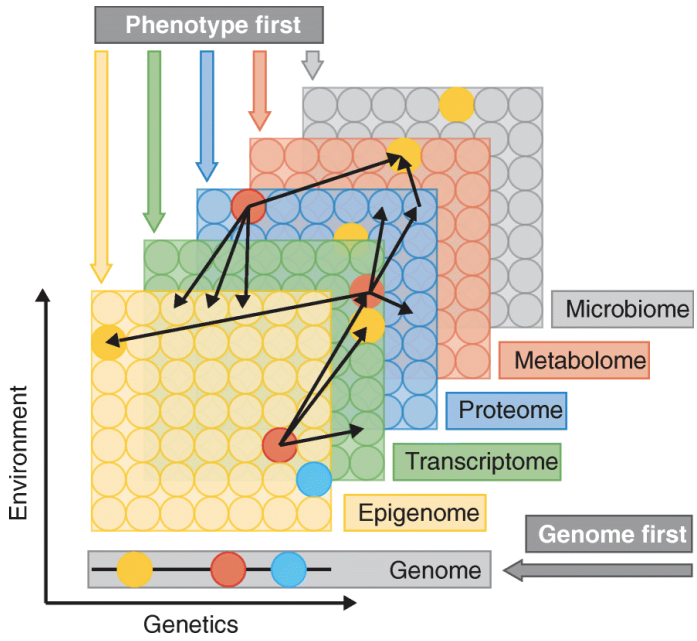
Figure 5.1 The "Omics cascade, modified and adapted from Dettmer *et al*., 2007



Encompassing, and at the forefront of the "omics" cascade is phenomics, the analysis of live cells. Using one form of "omics" assay (**Figure 5.2**) is useful for identifying differences in biological pathways and can provide markers for potential change. However, in the search for a comprehensive understanding of environment-mediated global regulation, and for confirmation of what genetic expression means in terms of phenotypic outcome, all of the "omics" faculties can be combined. The integration of several "omics" classes is referred to as "multiple-omics" and is depicted in **Figure 5.2**.

Figure 5.2 Multiple-omics approaches. Taken from Hasin *et al*. (2017).

"Omics data are collected on the entire pool of molecules, represented as circles. Except for the genome, all data layers reflect both genetic regulation and environment, which may affect each individual molecule to a different extent. The thin red arrows represent potential interactions or correlations detected between molecules in different layers—for example, the red transcript can be correlated to multiple proteins. Within layer interactions, although prevalent, are not depicted. Thicker arrows indicate different potential starting points or conceptual frameworks for consolidating multiple omics data to understand disease. The genome first approach implies that one starts from associated locus, while the phenotype first approach implies any other layer as the starting point. The environment first approach (not shown) examines environmental perturbations." (Hasin *et al*., 2017).



(Suzuki *et al*., 2014) combined phenotype and genotype mapping for drug resistance in *E. coli* and showed that expression levels of a small number of well-known resistance-related genes (i.e. *acrB* & *ompF*) can predict changes in resistance and susceptibility. (Mensah *et al*., 2019) combined genomic and metabolomics analysis to compare metabolic properties of pathogenesis in *Salmonella typhimurium* to commensal *E. coli* K12 in order to understand how

metabolism contributes towards niche adaption of bacteria. The extent of metabolic involvement in antimicrobial resistance is also a research topic that the "omics cascade" can help elucidate.

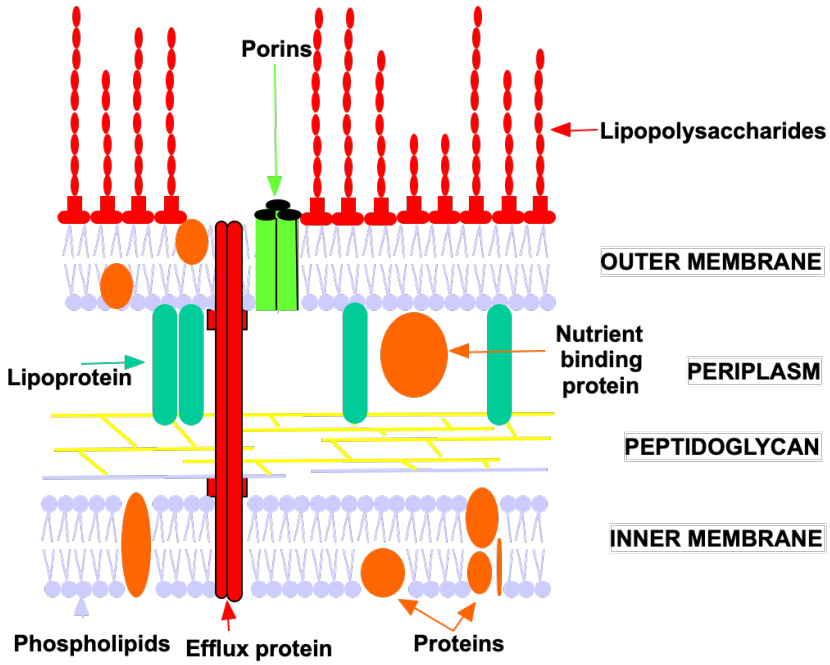
5.1.2. The Phenotype microarray

Phenotype microarray (Biolog, Inc., Hayward, CA, United States) is a high-throughput technique that can provide insight into phenotype changes by using cell respiration as a biomarker. A tetrazolium violet dye is used as a colorimetric reporter system; reduction of the dye results in the development of a purple colour, which accumulates over time providing a direct measurement of respiration levels. Mechanisms of resistance, such as mutations or gene regulation can entail a fitness cost due to excessive energy and resource demands (Maharjan and Ferenci, 2017). Furthermore, bacteria often employ less efficient metabolic mechanisms to counter the effect of a toxic substance such as a biocide (Maharjan and Ferenci, 2017). Examining changes in metabolic regulation can supplement information gained from growth kinetics and antimicrobial susceptibility profiling when investigating bacterial responses to stressors such as biocide exposure. Respiration does not only occur when a bacteria cell is growing, the phenotype microarray can detect phenotypes that do not lead to growth (Bochner *et al*., 2001). *E. coli* has been analysed as a model cellular system to validate the Biolog system (Bochner *et al*., 2001).

5.1.3. The bacterial cell wall and the effect of chlorhexidine digluconate on the membrane integrity

The cell wall of a Gram-negative bacterium lends itself to their intrinsic tolerance to some antibiotics and acts as a barrier to toxic substances (Whitfield and Roberts, 1999). It consists of an outer membrane (cell envelope), the periplasmic space and a peptidoglycan layer (**Figure 5.3**).

Figure 5.3 Gram-negative cell wall structures. Taken from Denyer & Maillard, (2002).



The outer membrane carries a negative net charge and is comprised of a bilayer of lipopolysaccharides and phospholipids, which is embedded with porins (Denyer and Maillard, 2002). Lipopolysaccharides are responsible for membrane impermeability characteristics of Gram-negative bacteria (Russell, 2003, Silhavy *et al*., 2010). Porins are hydrophilic channels that span the outer membrane through to the periplasmic space; they are responsible for the regulation of outer membrane permeability. Porins are classified into two groups: i) the general diffusion porins

(i.e. OmpC, OmpE and OmpF) are non-specific channels that allow diffusion of small hydrophilic molecules (Denyer and Maillard, 2002) and ii) the specific porins (i.e. PhoE) regulate the transport of particular solutes and are associated with the passage of negatively charged molecules. The loss of porins or the alteration of porin shape and size has been related to antimicrobial resistance (Gootz, 2006, Poole, 2002). The periplasmic space consists of polysaccharides that facilitate nutrition, transport and cell wall maintenance and oligosaccharides that are responsible for osmoregulation within the cell (Beveridge and Kadurugamuwa, 1996). The periplasm is a pertinent centre for metabolic processing (Denyer and Maillard, 2002). Lastly, the peptidoglycan wall that borders the inner membrane confers shape and provides the bacterium with mechanical strength (Salton & Kim 1996). Overall the role of the cell wall as a defence mechanism is to limit the uptake of noxious compounds into the cell and minimise cell damage.

Cationic compounds such as CHX and QACs are membrane active biocides that have a significant damaging effect to the cell membrane of microorganisms including Gram-negative bacteria such as *E. coli* (AD, 1999, Denyer and Stewart, 1998). Hugo & Longworth (1966) suggested that the direct effect of CHX is a disruption of the cytoplasmic membrane. CHX and QACs combine with phospholipids in the cell wall. Cationic peptides have a greater affinity for LPS than divalent cations in the cell envelope therefore they displace them and disrupt the normal permeability barrier of the outer membrane (Hancock *et al*., 1990). Changes in fatty acid composition in *P. aeruginosa* as a result of QAC exposure have been documented (Guerin-Mechin *et al*., 2000). The ability to alter membrane lipid composition is crucial for bacterial survival and adaptation in response to environmental stress (Rowlett *et al*., 2017). Rowlett *et al*. (2017) demonstrated that through the elimination of *E. coli* membrane proteins PE, CL or PG/CL phospholipid defects incur internal stress for the cell and in turn triggers the

activation of cell envelope and cytoplasmic stress responses. When a bacterial cell comes under stress such as the presence of a biocide, global metabolic rearrangements are coordinated via changes in the cell transcriptome, proteome and metabolome, coupled with cell wall restructuring (Martínez and Rojo, 2011, Needham and Trent, 2013, Needham *et al*., 2013, Rowlett *et al*., 2017, Silhavy *et al*., 2010). The cell wall acts to detect and instigate signalling cascades that lead to well known stress response pathways in order to protect bacterial fitness. For example, in *E. coli* at least five response pathways are induced in response to cell envelope stress (Bae, Cpx, Psp, Rcs, and σ^E) (Bury-Moné *et al*., 2009). Stress response pathways have been demonstrated to co-ordinate corresponding physiological functions, which in turn leads to distinct bacterial phenotypes. These comprehensive processes form an all-inclusive adaptive response (Needham and Trent, 2013, Ruiz and Silhavy, 2005) that may result in transiently reduced susceptibility to antimicrobials that is dependent on bacterial metabolic shift (Levin and Rozen, 2006).

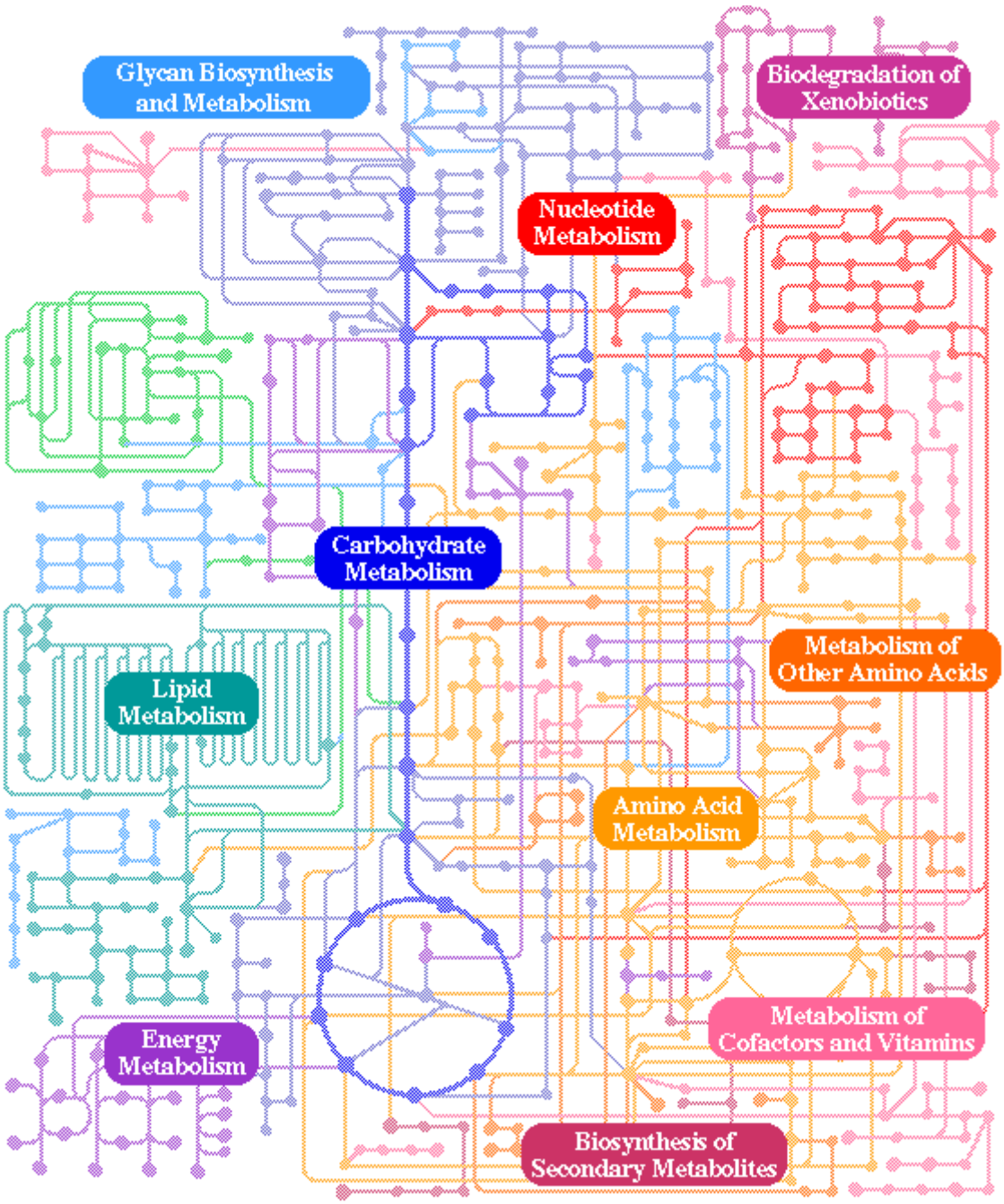
5.1.4. The Kyoto Encyclopedia of Genes and Genomes Database and the Mapping of Metabolic Pathways

Kyoto Encyclopedia of Genes and Genomes (KEGG) is a reference database for pathway mapping. Pathway maps represent acquired information on molecular interaction, reaction and relation networks for: metabolism; genetic information processing; cellular processes; organismal systems; human diseases; drug development (https://www.genome.jp/kegg; Accessed on: 20.07.2019). KEGG is an amalgamation of fifteen manually curated databases (Kanehisa *et al*., 2016), a list of these databases can be viewed in the appendices (File name: Appendices > appendix three > KEGG database List). **Figure 5.4** shows an example KEGG

metabolic pathway map and the sectioned areas of each type of metabolism. These maps allow intricate analysis of the relationship between different metabolic processes.

Figure 5.4 Metabolic pathways.

Taken from http://ibm4.life.nthu.edu.tw/KPST/ Accession date: 26.07.2019



5.1.5. Aims

This chapter focused on the ability of an *E. coli* isolate (UCD-CFS EFP-13P5) to metabolise substrates following growth in the presence of CHX at 2 sub-MIC concentrations (0.00005 mg/mL and 0.002 mg/mL). Phenotypic microarrays were performed in order to ascertain whether CHX exposure results in modifications in metabolic phenotype. Foremost, a comparative analysis was performed between bacteria grown on media only and that grown in the presence of CHX. Moreover, the effect that concentration of CHX has on the metabolic capabilities of *E. coli* UCD-CFS EFP-13P5 was explored.

5.2. Materials and methods

5.2.1. Justification of choice of isolate

E. coli isolate UCD-CFS EFP-13P5 has the highest MIC and MBC (0.01 mg/mL) values of all of the isolates tested in this study. Additionally, this isolate is clinically resistant to six of the antibiotics tested (ampicillin, cefpodoxime, tetracycline, streptomycin, trimethoprim and trimethoprim-sulfamethoxazole) (**Chapter 3: Section 3.3.5**) likely due to the presence of CTX-M-15 ESBLs (**Chapter 2; Section 2.2.1; Table 2.1**). Moreover, after exposure to residual levels of CHX UCD-CFS ECP-13P5 developed additional stable resistance to amoxicillin-clavulanic acid, cefoxitin and imipenem. UCD-CFS ECP-13P5 demonstrated efflux activity in the presence of CHX 0.00005 mg/mL, although efflux activity decreased significantly at 0.002 mg/mL CHX, highlighting a distinct difference in phenotype when exposed to low and high concentrations of CHX. For these reasons UCD-CFS ECP-13P5 will undergo phenotypic microarray analysis with the aim of understanding how metabolic processes interlink with biocide susceptibility.

5.2.2. Justification of residual concentrations of CHX

The lowest concentration of CHX tested in the previous investigations was 0.00005 mg/mL. This concentration affected growth kinetics of isolates (**Chapter 3: Section 3.3**) and produced changes in susceptibility (**Chapter 4: Section 4.3.2**). The use of 0.00005 mg/mL CHX provides a low toxicity level whilst providing an environment for adaptive tolerances. 0.002 mg/mL is a high sub-inhibitory concentration of CHX. This high sub-inhibitory concentration creates a sub-optimal environment that should provide selective advantage for more tolerant bacterial cells and exert a pressure for metabolic changes.

5.2.3. Bacterial preparation and exposure to CHX

E. coli UCD-CFS ECP-13P5 was grown to its 3rd generation with three subsequent sub-cultures onto MHA agar (incubated at 37°C; 16-18h). One loopful of colonies was taken from a MHA plate containing bacterial growth, streaked onto a new MHA plate and incubated at 37± 2°C for 18-24h. Two independent biological replicates were grown for each test; the two replicates were tested on a separate day (n=8). The 3rd generation was sub-cultured onto three MHA agar plates containing no CHX, 0.00005 mg/mL CHX or 0.002 mg/mL CHX. A preliminary test was performed in order to ensure that the isolate would grow on the agar plates with the CHX present. These sub-cultures were incubated at 37± 2°C for 16-18h.

5.2.4. Justification of phenotypic microarray plates

Phenotype microarray (PM) plates named PM1 to PM10 (excluding PM5) were selected for the assay. Each plate contains a defined set of small molecule substrates including carbon, nitrogen, phosphorous and sulphur, peptide nitrogen sources (Bochner *et al*., 2001). The individual integrated substrates that make up each plate for testing are included in the appendices (File name: Appendices > appendix three > Layout of Phenotype Microplates) where plate templates are shown. **Table 5.1** provides a brief list of details of the plates selected.

Table 5.1 A list of the PM microplates selected for use and their substrate class

PLATE IDENTIFICATION	SUBSTRATE CLASS
PM1	Carbon sources
PM2A	Carbon sources
PM3B	Nitrogen sources
PM4A	Phosphorous and sulfur sources
PM6	Peptide nitrogen sources
PM7	Peptide nitrogen sources
PM8	Peptide nitrogen sources
PM9	Osmolytes
PM10	pH

5.2.5. Phenotype microarray

Before being loaded into the PM microplates for experimentation, UCD-CFS ECP-13P5 was incubated for 16-18 h on MHA plates containing no CHX, 0.00005 mg/mL CHX or 0.002 mg/mL CHX. After incubation in the presence of CHX or not, several colonies were selected with a sterile plastic culture loop and suspended

into Inoculating Fluid-0 (IF-0; Biolog, Inc., Hayward, CA, United States) until a cell density of 42% transmittance ($T_{42\%}$) was reached in a turbidimeter (Biolog, Inc., Hayward, CA, United States). For plates PM1-PM2, 15 ml of $T_{42\%}$ cell suspension was mixed with 75 mL of Biolog redox dye mix A (1:5 dilution) in order to create a final cell suspension of $T_{85\%}$. For PM 3-8, 680 µL of a 2 M-sodium succinate/200 µM ferric citrate solution was added to 68 mL of the $T_{85\%}$ cell suspension. One hundred µL of each mixture was pipetted into each well of the appropriate microplate. All PM plates were incubated in an OmniLog reader at 37°C for 72 h. Readings were taken every 15 min and data were analysed in OmniLog PM software (Biolog, Inc). Each experiment was performed in duplicate on two separate days with independent bacterial cultures.

5.2.6. Analysis of Phenotype Microarray data

Data obtained from PM experiments were collated and analysed in OmniLog PM software. Well A1 for plates 2-8 were negative control wells where no metabolism was seen. Data were primarily "A1-zeroed", any noise obtained in well A1 was subtracted from the data in wells A2 to H12. **Figure 5.5** depicts an example data output formulated through the OmniLog PM software. It is possible for two strains or conditions to be compared at one time; each is allocated as either red (usually the reference) or green. Where the two data sets overlap it is coloured yellow. In the comparison example below (**Figure 5.5**) the reference strain is achieving higher levels of metabolism than the experiment.

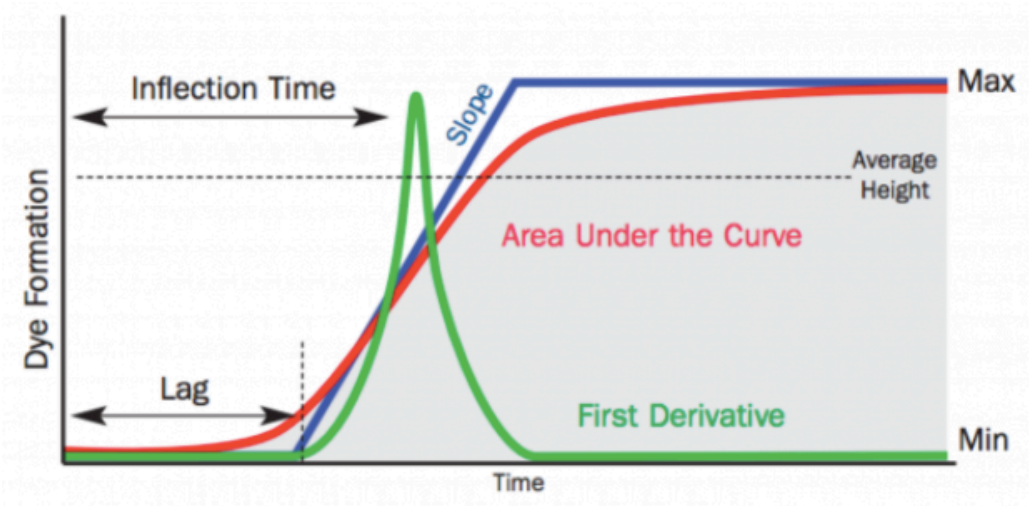
Figure 5.5 Data output from PM microarray.

Taken from: https://www.biolog.com/; Accessed on: 25.07.2919

Figure 5.6 shows the different parameters that can be calculated from the kinetic plots obtained through the PM experiment. The area under the curve was determined and compared when analysing the data obtained from the PM microarray. In order to define differences between data sets the "metabolic distance" is used. This is the "average height" of the curve and the threshold is arbitrarily set to 20,000 by the OmniLog PM software. The higher the metabolic distance, the more stringently selective it is for the difference between data sets. As the threshold value can be manually selected at whichever value necessary, it is a subjective analysis. For the purpose of the analysis in this chapter the threshold value for metabolic distance was set to 15,000 in order to highlight more metabolic differences. The OmniLog PM software does not offer an in-built statistical analysis. Biolog users have created software packages that perform statistical analysis (Henry *et al*., 2010). These statistical models were not employed for the purpose of this analysis.

Figure 5.6 Parameters derived and calculated from kinetic plots by OmniLog PM software. Taken from: https://www.biolog.com/; Accessed on: 25.07.2919.

Lag: period of time where the bacteria adjust to their environment before they will start to replicate. Slope: linear regression of the exponential growth phase. Inflection time: time taken to reach the end of exponential growth phase and the point at which the curve flattens and stationary phase begins. Area under the curve: measure of the complete metabolic activity over the time point measured.



5.3. Results and discussion

5.3.1. Phenotype microarray

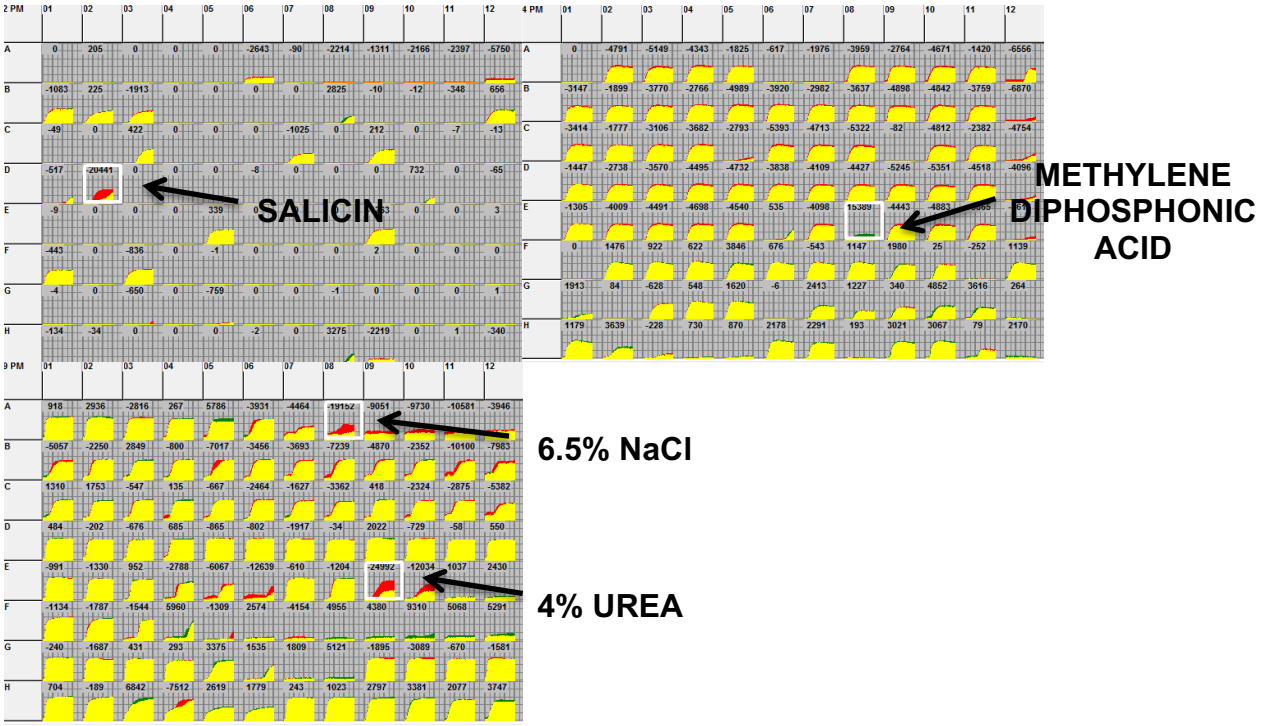
The effect of microbicidal concentrations on the metabolism of *E. coli* UCD-CFS ECP-13P5 was assessed using the OmniLog reader (Biolog, Inc., Hayward, CA, United States). The difference in dye intensity was a direct indication of the levels of metabolism involved. Time series charts that plot time against dye intensity were produced for each well and recorded by the OmniLog PM software. Differences in metabolism were compared with a focus on the effect of low (0.00005 mg/mL) and

high (0.002 mg/mL) sub-MIC CHX concentrations. As changes in CHX susceptibility phenotype were observed in **Chapter 4 (Section 4.3.2),** we hypothesise that changes to metabolic regulation will be observed when UCD-CFS ECP-13P5 has been exposed to CHX.

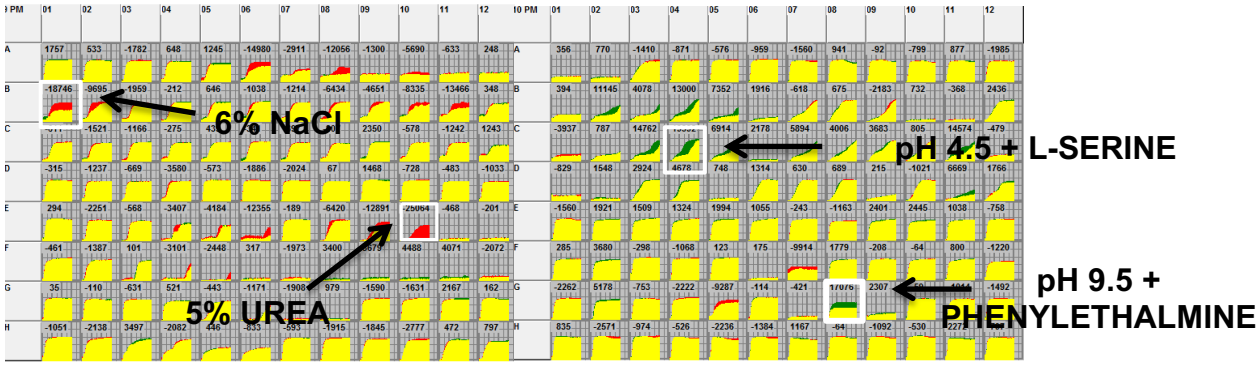
Figure 5.7 depicts phenotype microarray plates (PM) with metabolic distances of 15,000 or above. These data represent an average of two independent replicates. Growth in the presence of CHX 0.00005 mg/mL decreased the ability of UCD-CFS ECP-13P5 to metabolise salicin (PM2; Well D2; Mode: Carbon source), 6.5% NaCl (PM9; Well A8; Mode: Osmotic sensitivity) and 4% Urea (PM9; Well E9; Mode: Osmotic sensitivity) (**Figure 5.7 a**). The metabolism of methylene diphosphonic acid increased in the presence of 0.00005 mg/mL CHX.

Figure 5.7 UCD-CFS ECP-13P5 phenotype microarray output plots (n=2). **a)** Bacterial metabolism when exposed to no Biocide compared with 0.00005 mg/mL CHX (PM2, PM4 & PM9); **b)** Bacterial metabolism when exposed to no Biocide compared to 0.002 mg/mL CHX (PM9 & PM10); **c)** Bacterial metabolism when exposed to 0.00005 mg/mL compared to 0.002 mg/mL CHX (PM2)

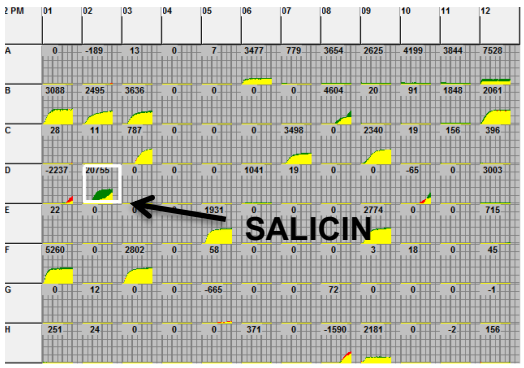
a)



b)



c)

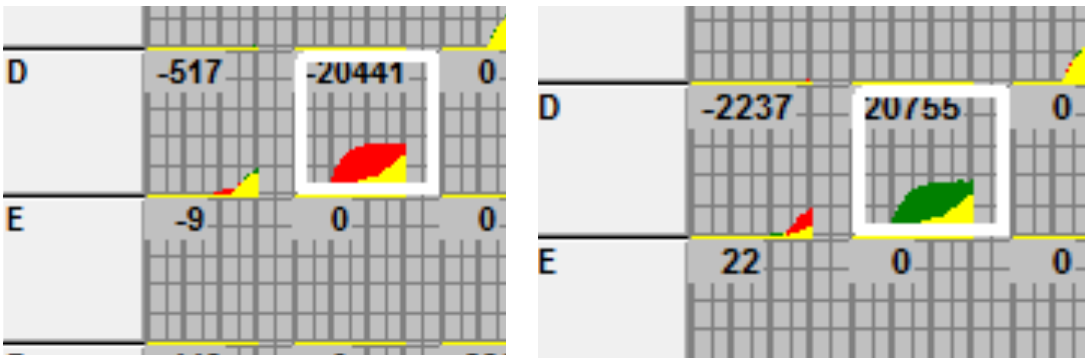


Salicin is a plant-derived β-glycoside and a secondary metabolite (Prasad and Schaefler, 1974). Demain and Fang defined it as a natural metabolic product that is not essential for vegetative growth of the organism, but is considered as a differentiation compound conferring adaptive roles (e.g. functioning as defence compounds; signalling molecules; metal transport) (Demain and Fang, 2000). The ability to metabolise salicin is a silent genetic mechanism regulated by the *bgl* operon of *E. coli.* The existence of "silent" and "cryptic" genes in microorganisms is a phenomenon that is recognised but not entirely understood. Although it is common for these two terms to be used in tandem, it is suggested that silent genes are distinct from cryptic genes. Although cryptic genes may also be silent, it is thought that they are retained due to the selection process, whereas silent genes are expected to have only a transient existence in the genome. As microorganisms commonly exist in conditions of limited resources and competition, the ability to utilise sources of energy where others cannot provides a fitness advantage. Salicin originates in the leaves of plants from the genus *Salix*. Harwani *et al*. (2012) and Madan *et al*. (2005) demonstrated that strains that carry an activated *bgl* operon outcompete the wild-type strain in competition experiments, even when β-glucosides are not supplemented in the medium (Harwani *et al*., 2012, Madan *et al*., 2005). Moreover, they postulate the possibility that the *bgl* operon exerts a regulatory effect on downstream target genes other than those implicated in β-glucoside catabolism, expression of which provides a fitness advantage in the stationary phase. When the group investigated upregulated proteins in the proteome of the Bgl^+ strain they were mainly participants in transport functions or enzymes involved in cellular metabolism. It was concluded that Bgl^+ cells are more adept at nutrient procurement and utilisation as a result of activating additional metabolic functions. The metabolism of methylene diphosphonic acid increased in the presence of 0.00005 mg/mL CHX.

It has been demonstrated that the elevated expression of the *bgl* operon can occur even in the absence of β-glycosides (Harwani *et al*., 2012). The oligonucleotide-peptide transporter gene *oppA* (a member of the ABC transporter super family) was found to be over-expressed in bgl^{+} *E. coli (*Harwani *et al*., 2012). *oppA* has been involved in functions related to oligonucleotide-peptide uptake and the recycling of cell wall peptides (Hiles *et al*., 1987). Furthermore, *oppA* is linked to RpoS (a stress activated sigma factor) expression, the combination of the *bgl* operon, *oppA* and RpoS expression were attributed to a growth advantage during stationary phase of *E. coli* (Maden *et al*., 2005). It has been demonstrated that RpoS-dependent gene expression leads to general stress resistance of cells (Battesti *et al*., 2011). **Figure 5.8** a) and b) show the data output from the OmniLog PM software after 72 hours of reading.

Figure 5.8 UCD-CFS ECP-13P5 phenotype microarray output plots (n=2) for salicin. Bacterial metabolism when exposed to **a)** 0.00005 mg/mL CHX and **b)** 0.002 mg/mL CHX

a) **b)**



It can be observed that where UCD-CFS ECP-13P5 has previously been exposed to CHX 0.00005 mg/mL the metabolism of salacin has an elongated lag and does not start to increase until 28.30 hours. For UCD-CFS ECP-13P5 that has been

exposed to 0.002 mg/mL or no CHX the metabolism of salacin starts at 17.96 hours.

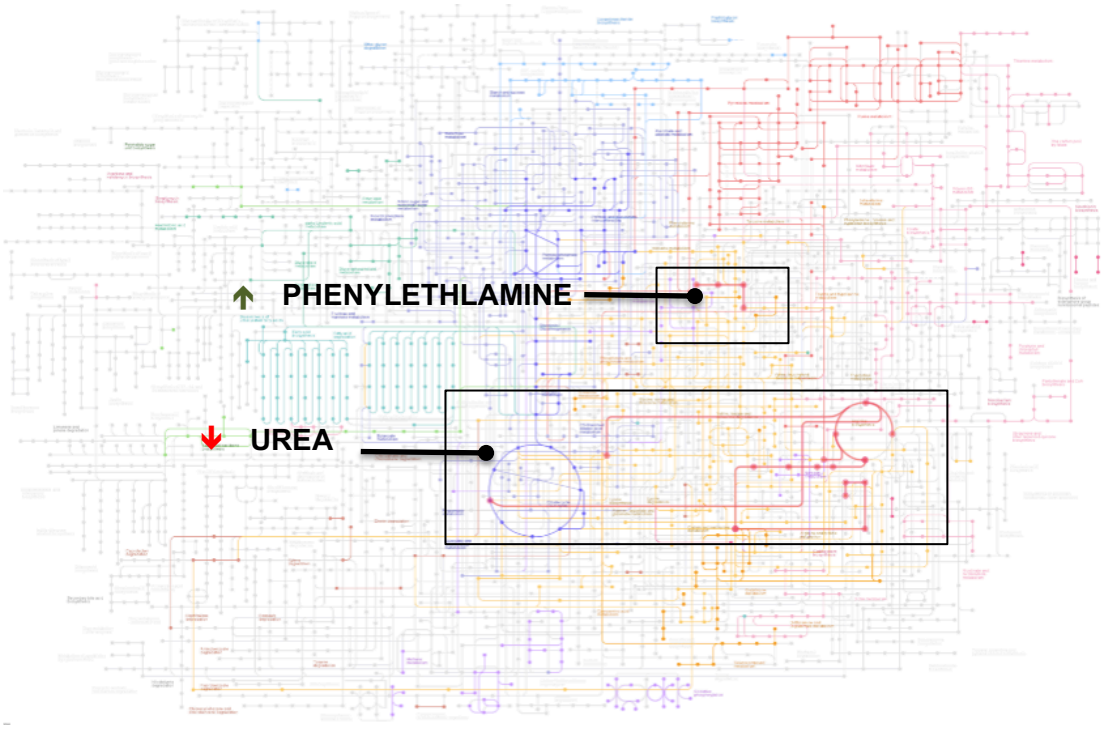
Growth in the presence of CHX 0.002 mg/mL decreased the metabolism of 6% NaCl (PM9; Well B1; Mode: Osmotic sensitivity) and 5% urea (PM9; Well E10; Mode: Osmotic sensitivity). However, the metabolism of pH4.5 + L-serine (PM10; Well C4) and pH + 9.5 phenylethlamine (PM10; Well G8; Mode: pH, decarboxylase) increased in the presence of 0.002 mg/mL CHX. The only difference in metabolism when comparing the two exposure concentrations (0.00005 mg/mL and 0.002 mg/mL) was with salicin where UCD-CFS ECP-13P5 was less efficient at metabolising salicin when exposed to 0.00005 mg/mL than when exposed to 0.002 mg/mL or not exposed to biocide.

Figure 5.9 shows the metabolic pathway mapping of *E. coli* K12 (produced with: https://www.genome.jp/kegg; accessed on: 24.07.19). Areas of metabolic change observed in this study are highlighted. All of the changes observed in this study were located in the areas of amino acid metabolism, carbohydrate metabolism, metabolism of co-factors and vitamins and the biosynthesis of secondary metabolites. There is a clear difference in amount of changes observed for 0.00005 mg/mL and 0.002 mg/mL CHX, the higher concentration producing more changes than the lower.

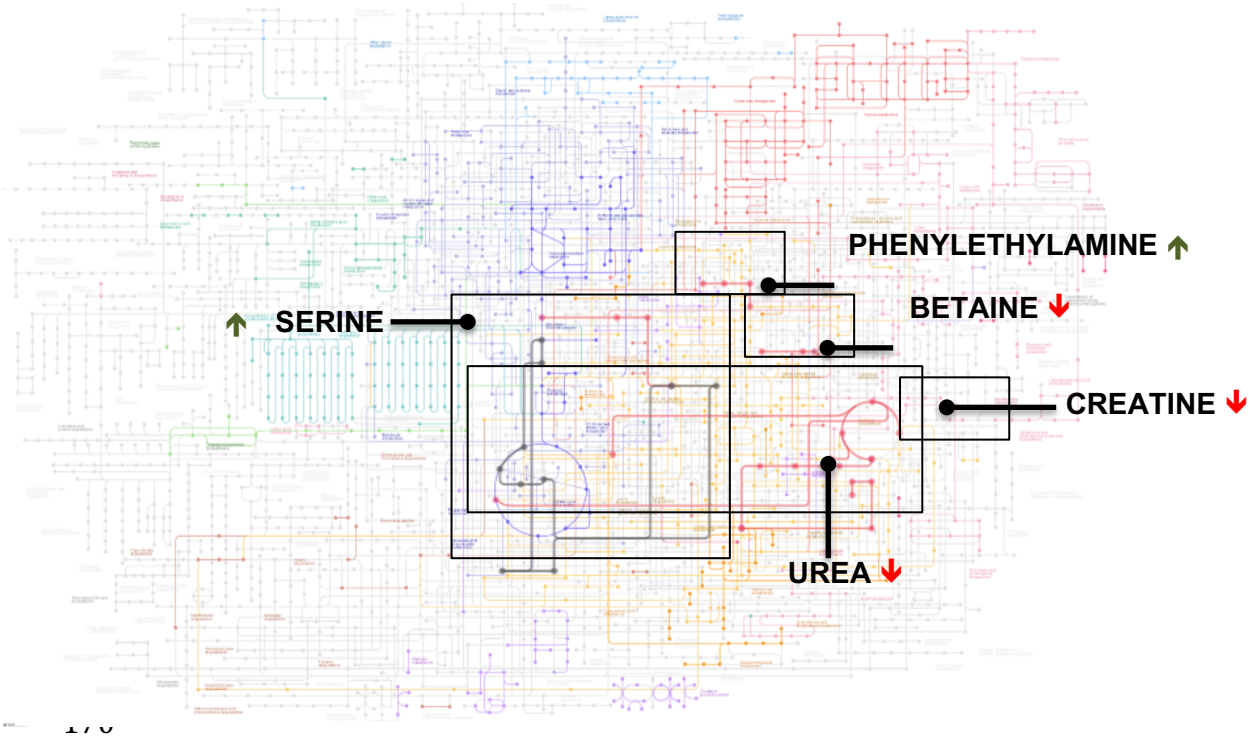
Figure 5.9 metabolic pathway mapping. Location of pathways that include substrates involved in observed metabolic changes (n=2). **a)** bacteria exposed to no biocide compared with exposure to 0.00005 mg/mL CHX. **b)** bacteria exposed to no biocide compared with exposure to 0.002 mg/mL CHX.

↑= increased metabolism of substrate ↓= decreased metabolism of substrate

a)



b)



When observing PM data from each individual replicate (n=2) more differences can be observed. All differences in output values for metabolic distance for both replicates can be observed in the appendices (File name: Appendices > appendix three > BIOLOG Data). **Table 5.2** highlights all increases, decreases and equality of metabolism during the phenotype microarray experiment. Growth in the presence of CHX (0.00005 and 0.002 mg/mL) increased the ability of UCD-CFS ECP-13P5 to metabolise pH 4.5 L-alinine (PM10; Well B2; Mode: pH, decarboxylase), pH 9.5 phenylethylamine (PM10; WellG8; Mode: pH, deaminase) and 6% NaCl + betaine (PM9; Well B2; Mode: osmolyte, betaine) when compared to no biocide exposure (**Table 5.2**).

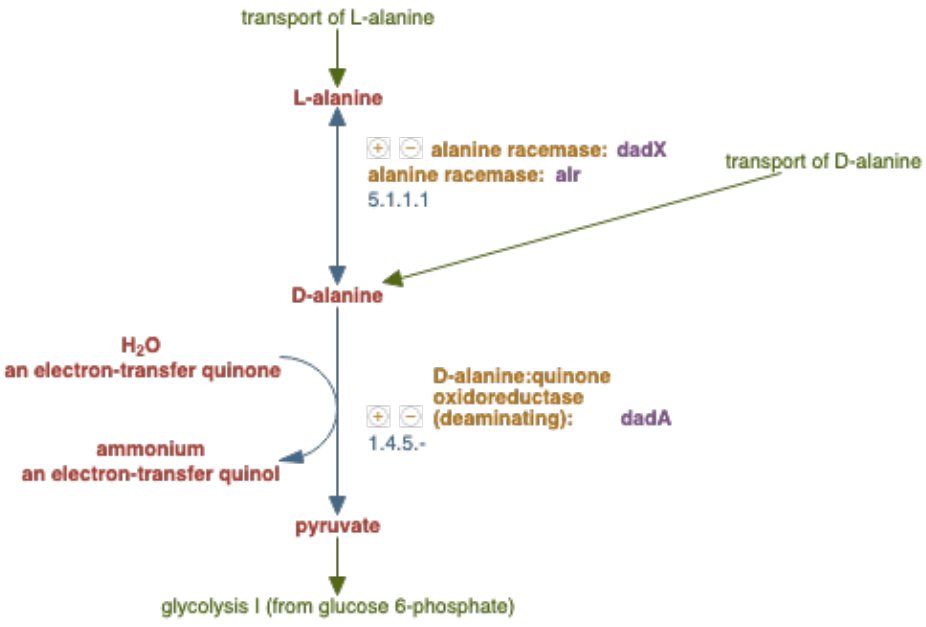
Table 5.2 All observed Increase **(↑)**, decrease (↓) or equality (=) of metabolism after exposure to CHX.

	Compared to No CHX		**Compared to each other**	
	0.00005 mg/mL	**0.002 mg/mL**	**0.00005 mg/mL**	**0.002 mg/mL**
Salicin	↓	=	↓	↑
Methylene diphosphonic acid	↑	=	↑	↓
pH 4.5 + L-Alanine	↑	↑	=	=
pH 4.5 + L-Serine	=	↑	=	=
pH 9.5 + Phenylethylamine	↑	↑	=	=
5.5%NaCl	=	↓	↑	↓
6%NaCl	=	↓	↑	↓
6% NaCl + Betaine	=	↓	=	=
6%NaCl + Creatinine	=	↓	=	=
4% Urea	↓	=	↓	↑
5% Urea	↓	↓	=	=

Alanine is a fundamental component of protein in the form of L-alanine (L-Ala). L-alanine and its isomer D-alanine (D-Ala) are small molecule amino acids that *E. coli* can utilise as the single source of carbon, nitrogen and energy (Kim *et al*., 2010). **Figure 5.10** Depicts the pathway through which L- and D-alanine are utilised through the glycolysis pathway to produce pyruvate. L-alanine is essential to the production of D-alanine. L- alanine is converted by racemase enzymes DadX and Alr into D-alanine, which is in turn converted into pyruvate. Aside from their role in the synthesis of pyruvate in the glycolysis pathway, L- and D-alanine participate in the biosynthesis of cross-links in the peptidoglycan cell wall. D-alanine and L-alanine are utilised in cell wall synthesis at a 3:1 mix. During the cross-linking of peptides to form the peptidoglycan wall, transpeptidation reactions cleave intrastrand peptide D-Ala-D-Ala bonds to form interstrand peptides DAP-ε-NH-D-Ala or –Glys-D-Ala (Gumbart *et al*., 2014, Walsh, 1989).

Figure 5.10 The role of L-alanine and D-alanine in the glycolysis pathway.

https://biocyc.org/ accessed on 23/06/2019



Trivedi *et al*. (2018) describes how an increase in alanine level interferes with the transcriptional regulation of peptidoglycan transpeptidases in *P. aeruginosa* (Trivedi *et al*., 2018). In this example, a *dadA* (loss-of-function) mutant demonstrated that as D-alanine levels increased, the expression of *ponA* and *dacC* (genes that encode cell wall enzymes) was inhibited; a decrease in cross-linking was observed leading to a lack of cell wall stiffness. The cell wall is an integral part of a Gram-negative bacterial cell's defence; it protects the cell from environmental stressors and regulates substrate import and export. In accordance with the knowledge gained from Trivedi *et al*. (2018) in *P. aeruginosa*, it is plausible that CHX incites an energy deficit or an increased necessity for the conversion of L-alanine to D-alanine in order to promote cell-wall synthesis, improve cross-linking and stiffen, or thicken the cell wall.

UCD-CFS ECP-13P5 was less efficient at metabolising 6% NaCl + betaine after exposure to 0.002 mg/mL CHX. The reason for the metabolic decrease is not known at this time, however betaine is involved in osmoregulation (Ly *et al*., 2004) Glycine-betaine is an osmo-protectant that confers tolerance to urea in *E. coli* by preventing denaturation of the cytoplasm (Benaroudj *et al*., 2001). CHX is a cationic biguanide that disrupts the bacterial membrane via displacement of associated divalent cations, ultimately resulting in a reduction of membrane fluidity and osmoregulation (Bay and Turner, 2009, Slipski *et al*., 2018).

5.4. Conclusion

All of the changes observed were located in the areas of amino acid metabolism, carbohydrate metabolism, metabolism of co-factors and vitamins, and the biosynthesis of secondary metabolites. Amino acids are essential for the biosynthesis of proteins and cell wall peptidoglycan. For this reason, the cell wall is a major source of amino acids. Proteins contain various combinations of twenty common L-amino acids and have regulatory or catalytic functions or are involved in binding and transport processes of the bacterial membrane (Fox *et al*., 1990). The increase of amino acid metabolism after CHX exposure would suggest that UCD-CFS ECP-13P5 is directing mechanisms primarily towards cell membrane processes such as changes in outer membrane structure or possibly signalling functions (Bury-Moné *et al*., 2009). The cell wall acts to detect and instigate signalling cascades that lead to well known stress response pathways in order to protect bacterial fitness (Guo and Gross, 2014). Stress response pathways regulated by sigma factors have been demonstrated to co-ordinate corresponding physiological functions, and ultimately bacterial phenotypes (Asmar *et al*., 2017, Bury-Moné *et al*., 2009, Fox *et al*., 1990, Guo and Gross, 2014, Needham and Trent, 2013, Ruiz and Silhavy, 2005). The resulting phenotypes may demonstrate transient reduced susceptibility to antimicrobials (Levin and Rozen, 2006). There is a clear disparity in the number of changes seen after pre-exposure to a low (0.00005 mg/mL) and high (0.002 mg/mL) sub-MIC concentration of CHX. 4% urea and salicin were metabolised equally with no previous biocide exposure and 0.002 mg/mL CHX (**Table 5.2**). This may be explained through the process of biocide-mediated microbial selection and adaptation. When UCD-CFS ECP-13P5 is exposed to the low concentration of CHX (0.00005 mg/mL), its presence may have an altering effect on metabolic regulation (Levin and Rozen, 2006). For example, we saw the increase of L-alanine utilisation (**Table 5.2**). However, it has been

previously demonstrated that 0.002 mg/mL CHX was a concentration high enough to reduce viable cell count by 1.67 Log_{10} after 5 min (**Chapter 4; Section 4.3.2**). It is therefore postulated that 0.002 mg/mL acts as a stronger selective pressure that resulted in the survival of a sub-population capable of the same level of salacin metabolism as non-exposed cells. This is interesting as the ability to utilise salicin as a carbon source has been previously demonstrated to provide a competitive advantage to capable *E. coli* populations (Harwani *et al*., 2012, Madan *et al*., 2005). In addition, a combination of the increased utilisation of amino acids and carbohydrates suggest that CHX induces a membrane related stress response of UCD-CFS ECP-13P5. This stress response is more apparent after exposure to 0.002 mg/mL, a higher sub-lethal concentration of CHX, demonstrating a distinct difference in responses to CHX concentrations.

CHAPTER 6: GENERAL DISCUSSION

6. General discussion

This study sought to investigate differences between the exposure effects of CHX and BZC at high (0.002 mg/mL) and low (0.00005 mg/mL) sub-MIC concentrations. Moreover, this study aimed to produce a realistic estimation of the residual concentration of CHX left on a surface after disinfection. Focus was placed on the resulting susceptibility phenotypes of *E. coli after* exposure*;* in what way these were related to metabolic regulation and how findings relate to the risk of resistance.

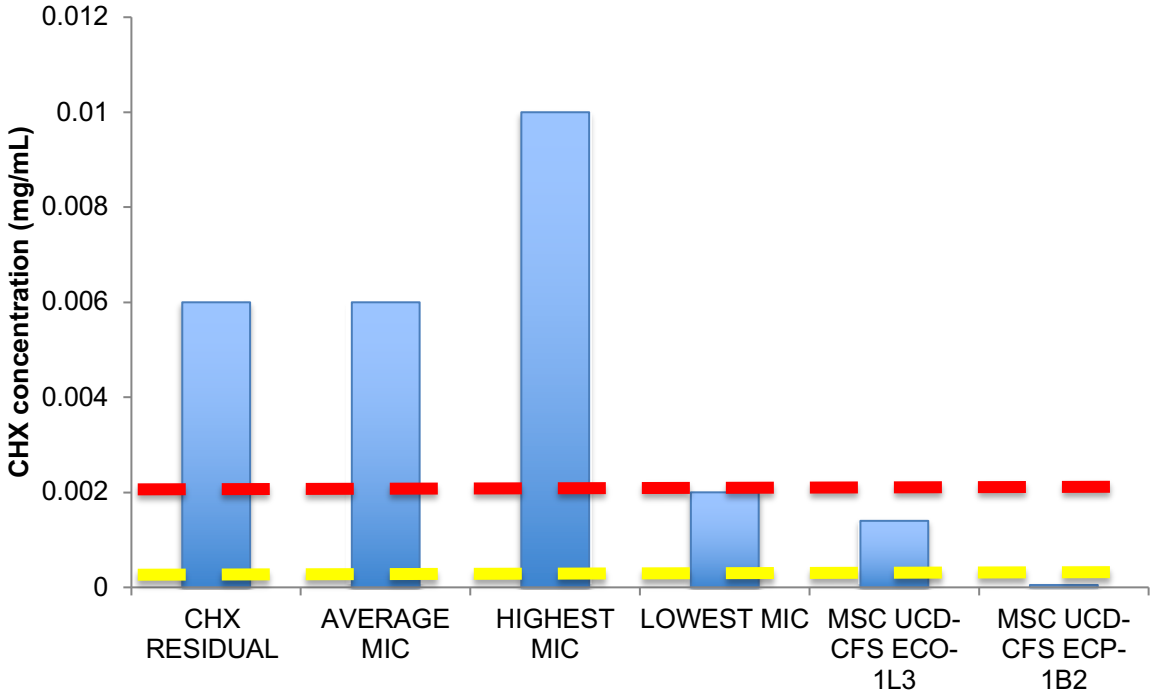
6.1. Chlorhexidine digluconate (CHX)

6.1.1. CHX residues left on a surface

It has been discussed that the microbicidal efficacy of a biocidal active is depended on environmental factors such as concentration, contact time, interfering substances, temperature and pH. These factors can all reduce or quench the activity of a biocide and may result in concentrations that is below the MIC (Maillard, 2002, A Rutala and J Weber, 2007). Wesgate el al. (2016) described the term “during use exposure” which represents the realistic condition (i.e. concentration, contact time, temperature, organic load) of product use during its application. During use exposure is different to “low concentration” which often reflects a concentration below the minimal inhibitory concentration, which is not necessarily representative of a concentration that occurs in practice. Some biocidal products make claims of a “residual biocidal activity”. CHX is most often incorporated into disinfectant products at 2% (20 mg/mL) and is often marketed with claims of residual activity of up to 6 hours, and most recently 48 hours when applied to the skin surface (George *et al.*, 2017). Furthermore, microorganisms

have been reported to survive on surfaces for prolonged periods of time, for instance some *Enterococcus* species have been reported to survive for up to 46 months (Kramer *et al*., 2006). If biocide products that possess long-lasting effects are not present at the required concentration, surviving bacteria have potential to adapt under selective pressure. In turn, this adaptive process gives rise to the development and transmission of antimicrobial resistance (Andersson *et al*., 2012, Gadea *et al*., 2017, Gullberg *et al*., 2014, Gullberg *et al*., 2011, Maillard, 2013, Qiu *et al*., 2012, Ramm *et al*., 2015, Tuladhar *et al*., 2012). In **Chapter 4, Section 4.3.1.1** the amount of CHX recovered on a surface after application and drying was assessed. The amount of CHX recovered is representative of the during use exposure concentration (Wesgate *et al*., 2016). The concentration of biocide that was recovered after drying was 98.8% lower than that initially applied with an average of 0.006 ± 0.002 mg/mL (**Figure 6.1**). This was the same concentration as the average MIC of the isolates tested (**Chapter 3: Section 3.3.1; Table 3.1**).

Figure 6.1. The concentration of CHX (20 mg/mL) recovered after drying on a surface, average, highest and lowest baseline MIC observed and obtained MSC values for *E. coli* isolates in this study obtained in Chapter 3; Section 3.3.1. (n=3). Dashed lines depict the high and low sub-MIC concentrations. – Yellow line: lower sub-MIC tested. Red line: highest sub-MIC tested



Following exposure to the residual concentration of CHX, survivors (UCD-CFS ECP-1L3, UCD-CFS ECP-1L4, UCD-CFS ECP-1B2, UCD-CFS ECP-13P5, UCD-CFS ECP-13P4) were recovered after a 5 min (**Chapter 4: Section 4.3.2: Table 4.2**) but not after 24 hours (**Chapter 4: Section 4.3.2: Table 4.3**), implying that there is a realistic potential for failure of a biocide after short in situ exposure in this case. In terms of clinical application, the ECOFF breakpoints collated by (Morrissey *et al*., 2014) for CHX indicated that only 1.1% of *E. coli* isolates included in the study would not survive treatment of 0.006 mg/mL CHX. As ECOFF values are

intended to be a realistic representative of clinical significance, the finding of residual CHX concentrations of around 0.006 mg/mL would imply that the majority of clinically important *E. coli* strains are potentially not being inactivated. Isolate UCD-CFS ECP-13P5 demonstrated a slightly elevated tolerance of CHX (MIC/MBC of 0.01 mg/mL; **Chapter 4: Section; Table 3.1**). Indeed the MIC/MBC of UCD-CFS ECP-13P5 was higher than that of the average residual concentration CHX found on the surface (0.006 ± 0.002 mg/mL) after application (**Figure 4.6**).

6.1.2. Minimal Selective Concentration of CHX and Resistance

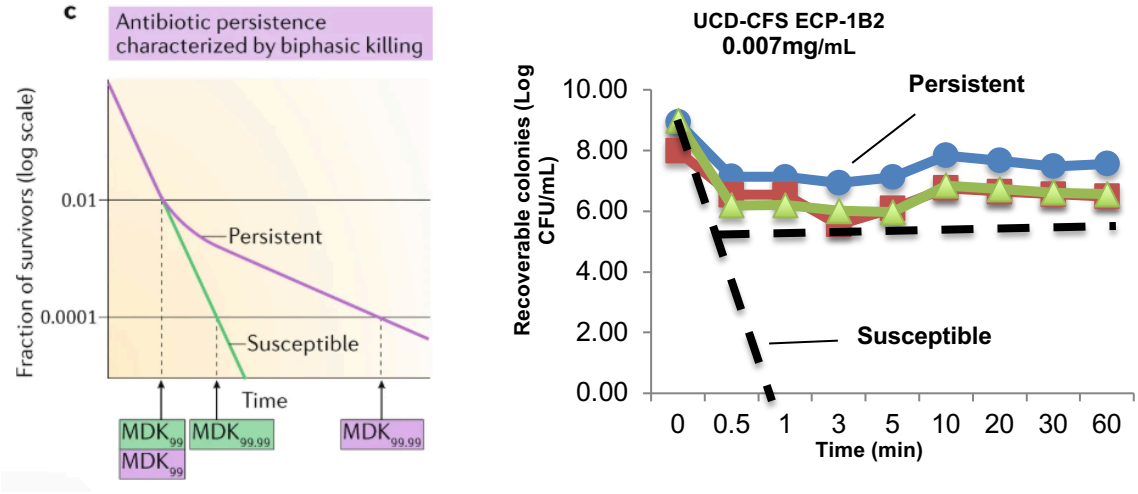
The 'minimal selective concentration' hypothesis (Gullberg *et al*., 2014, Gullberg *et al*., 2011, Liu *et al*., 2011, Sandegren, 2014) was applied successfully to CHX using isolates UCD-CFS ECP-1L3, UCD-CFS ECP-1B2 and UCD-CFS ECP-13P5 (**Chapter 3: Section: Table 3.4**). **Table 6.1** displays a summary of all of the relevant values and changes that were obtained throughout this thesis. The MSC value for UCD-CFS ECP-1L3 was 0.0014 mg/mL, 1.4 times lower than the MIC*susc* (**Table 6.1)**. The MSC value for UCD-CFS ECP-IB2 was 0.00005 mg/mL, 100 times lower than the MIC*susc* (**Table 6.1**). However, both of these isolates demonstrated survival after 5 min exposure to CHX 0.0075 mg/mL (surface dried 168 h) and transient increases in MIC and MBC. Furthermore, isolate UCD-CFS ECP-1B2 demonstrated stable changes in antibiotic susceptibility to amoxicillin/clavulanic acid, ampicillin, ciprofloxacin, cefpodoxime and cephalothin (**Table 6.1**).

Table 6.1. Summary of all of the relevant values and changes that were obtained throughout this thesis
MIC/MBC/MSC are recorded in mg/mL; NT – Not Tested; RC – Residual Concentration, this is an average of all of the concentrations recovered of the surface during the carrier test **=** no change in value before and after exposure; ↑ I□□; ↓ Decrease in previous value after exposure; cephalothin (CF); amoxicillin/clavulanic acid (AMC); cefoxitin (FOX); imipenem (IMP); ampicillin (AMP); ciprofloxacin (CIP); cefpodoxime (CPD).

				GROWTH KINETICS CHANGE AFTER EXPOSURE TO CHX					**TRANSIENT CHANGE**		**STABLE CHANGE**		
STRAIN	**ESBL**	**CHX CONC.**	**INITIAL MIC**	**GROWTH RATE**	**LAG PHASE**	**MAX OD**	**MSC**	**EFFLUX**	**MIC/ MBC**	**ABS**	**MIC/ MBC**	**ABS**	**METABOLIC CHANGE**
ATCC25922	NT	**0.00005**	□□□□□	↑	=	=	NT	↑	NT		=	NT	NT
		0.002		↓	↑	↓		↓					
		RC (0.006)		NT				NT	↑	=			
UCD-CFS ECP-1L3	**CTX-M-14**	**0.00005**	0.005	=	=	=	**0.0014**	NT	NT		=	NT	
		0.002		=	↑	↓							
		RC (0.006)		NT					↑	=			
UCD-CFS ECP-1L4		**0.00005**	□□□□□	↑	=	=	NT	NT	NT		=	NT	
		0.002		↓	↑	↓							
		RC (0.006)		NT					↑	=			
UCD-CFS ECP-1B2		**0.00005**	□□□□□	↑	=	=	**0.00005**	↑	NT		=	NT	
		0.002		↓	↑	↓		↓					
		RC (0.006)		NT				↓	↑	CF, AMC		AMC, AMP, CIP, CPD, CF	
UCD-CFS ECP-13P5	**CTX-M-15**	**0.00005**	□□□□	↑	=	=	NT	↑	NT		=	NT	↑ phenylethlamine, L-ala ↓ urea, salicin
		0.002		↓	↑	↓		↓					↑ phenylethlamine, L-serine, L-ala ↓ urea, creatinine, betain, NaCl
		RC (0.006)		NT				↓	↑	FOX, IPM			NT
UCD-CFS ECP-13P4		**0.00005**	0.002	↑	=	=	NT	NT	NT		=	NT	NT
		0.002		↓	↑	↓							
		RC (0.006)		NT					=	=			
UCD-CFS ECP-25P5	**TEM-20**	**0.00005**	□□□□□	↑	=	=	NT	NT	NT		=	NT	
		0.002		NT									
		RC (0.006)							↑	TE			
UCD-CFS ECP-25051		**0.00005**	□□□□□	↑	=	=	NT	NT	NT		=		
		0.002		NT									
		RC (0.006)							=				

It was discussed that at this higher sub-MIC range of concentrations, there is a stronger selection pressure, which may result in inactivation of susceptible sub-populations and the persistence of more tolerant cells (**Figure 6.2**).

Figure 6.2. A comparison between the theory of persistent bacterial populations and data obtained from inactivation kinetics (**Chapter 4: Section 4.3.5.3: Figure 4.12).** (Trastoy *et al*., 2018)



The possibility of persistent cells was reflected in the results from CHX inactivation kinetics (**Chapter 4: Section 4.3.5.3**) where a decrease in viable cells of 1.67 Log_{10} was demonstrated after exposure of UCD-CFS ECP-13P5 and UCD-CFS ECP-1B2 to CHX 0.002 mg/mL, followed by no further decrease but apparent persistence (**Figure 6.2**). It could be postulated that the concentration of CHX that is remaining on a surface after application (0.0047 - 0.0097 mg/mL) might be within the MSC range of a strain, (in this case UCD-CFS ECP-1B2; between 0.00005 mg/mL and 0.005 mg/mL CHX) (**Figure 6.1**). The next logical step was to investigate specific changes within the cells at low sub-MIC CHX concentrations.

6.1.3. Efflux of CHX and change in metabolism

The effect of CHX on the increase and decrease of efflux in isolates ATCC25922, UCD-CFS ECP-13P5 and UCD-CFS ECP-1B2 is shown in **Table 6.1**. There were distinct differences in efflux activity between high (0.002 mg/mL) and low (0.00005 mg/mL) sub-MIC concentrations of CHX (**Chapter 4: Section: Figure 4.7).** 0.00005 mg/mL CHX incited efflux activity whereas 0.002 mg/mL prevented it. The outer membrane has been previously linked to efflux activity in *E. coli*. In particular, it has been associated with the regulation of molecules in and out of the cell via direct cooperation with outer membrane porins (Krishnamoorthy *et al*., 2016, Krishnamoorthy *et al*., 2017, Fernández and Hancock, 2012). The loss of the outer membrane porin OmpF has been identified as a resistance mechanism for *E. coli* against β-lactams (Martinez-Martinez *et al*., 2000) and fluoroquinolones (Tavío *et al*., 1999). In Chapter 4, resistance to ciprofloxacin, cefoxitin and amoxicillin/clavulanic acid were observed. It is possible that these resistances are related to a change in outer membrane structure. This change could be related to changes in membrane porins and the efflux activity demonstrated after CHX exposure. Resistance of UCD-CFS ECP-13P5 to cefoxitin and imipenem was transient (**Table 4.12**), likewise with UCD-CFS ECP-1B2 resistance to amoxicillin/clavulanic acid (**Table 4.11**). Bornet *et al*. (2000) suggested that porin loss could be reversible (Bornet *et al*., 2000). It is plausible that the alteration or loss of porins is the reason for transient changes in MIC and MBC values after exposure to CHX.

Baseline growth kinetics differed between strains with the exception of growth rate, which remained constant between isolates for CHX. Lag phase extension (LE) was assessed as a determinant of decreased susceptibility in biocide exposed bacteria (Li *et al*., 2017). UCD-CFS ECP-13P5 showed the highest MIC and MBC to CHX

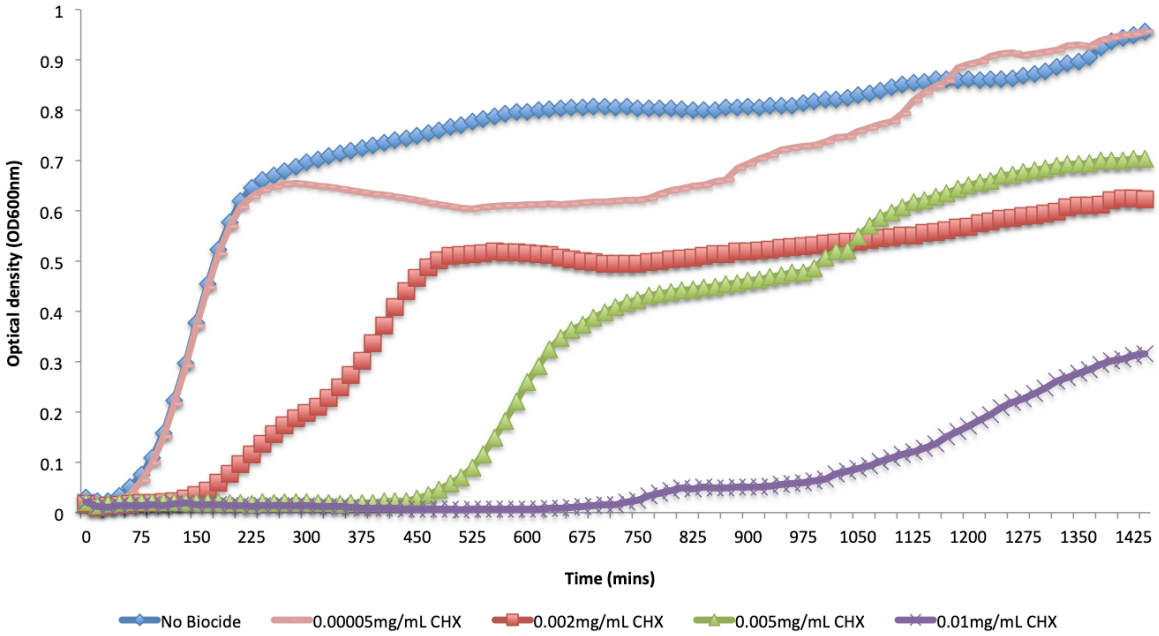
(0.01 mg/mL), which may suggest that it possesses a selective advantage that owes to decreased susceptibility to CHX. LE values in the presence of CHX for this isolate increased with concentration (**Table 6.1**). This may be indicative of adaptive responses that lend to this isolate's elevated MIC (Li *et al*., 2017). However the highest LE value was observed for UCD-CFS ECP-1L3 (**Table 6.1**), which did not demonstrate an elevated MIC (0.005 mg/mL). For both CHX and BZC, a concentration of 0.002 mg/mL had the most altering effect on growth rate, lag phase length and maximum optical density reached.

There are several examples throughout this study where results have indicated a distinct difference in the selection process of high and adaption of bacteria at low concentrations biocides, differences between effects of CHX 0.00005 mg/mL or 0.002 mg/mL on:

- Growth kinetics (**Chapter 3**).
- Efflux activity (**Chapter 4**).
- The selection of an adapted sub population of UCD-CFS ECP-13P5 during inactivation kinetics (**Chapter 4**).

Adaptation was also demonstrated when analysing the phenotype microarray and the ability for UCD-CFS ECP-13P5 to metabolise and utilise certain substrates. Salicin and 4% Urea were more effectively metabolised in the case of no CHX exposure and the highest concentration of CHX (0.002 mg/mL) (**Chapter 5: Section 5.3.1: Table 5.2**). The growth kinetics of UCD-CFS ECP-13P5 in no CHX, 0.002mg/mL CHX and 0.00005 mg/mL CHX are displayed in Fig**ure 6.3.**

Figure 6.3 Growth kinetics of UCD-CFS ECP-13P5 in the presence of CHX at increasing concentrations (n=3).



It is observable that the length of lag phase for no biocide and 0.00005 mg/ml CHX are not significantly different (95 ± 8.66 and 100 ± 8.66 respectively; P= 0.5185; T-TEST; Graphpad PRISM 8). However, when grown in the presence of the higher (sub-inhibitory) concentration of CHX (0.002 mg/mL), there was an extended lag phase of 290 ± 109 mins. As the concentration increases, the length of lag phase extends further as can be seen in the case of concentrations 0.005 mg/mL (560 ± 60.62) and 0.01 mg/mL (615 ± 548). An extended lag phase is associated with the adaption of microbial populations (Rolfe *et al*., 2012). All of the changes observed in the phenotype microarray were located in the areas of amino acid metabolism, carbohydrate metabolism, metabolism of co-factors and vitamins and the biosynthesis of secondary metabolites (**Table 6.3**). The increase of amino acid metabolism after 0.002 mg/mL CHX exposure, and the increase of lag phase length would suggest that UCD-CFS ECP-13P5 is adapting through regulatory mechanisms directed primarily towards cell membrane processes such as changes

in outer membrane structure or possibly signalling functions. Moreover, we know that at this concentration of CHX the population of cells able to adapt are selected for through persistence. We have therefore demonstrated that at an established “during use” residual concentration of CHX, it is possible for bacteria to survive the disinfection process, adapt to incur transient increases in susceptibility and stable cross-resistance to antibiotics and be selected amongst less tolerant sub-populations.

6.2. Benzalkonium Chloride (BZC)

6.2.1. BZC residues left on a surface

The remaining residual concentration of BZC after drying could not be determined during this study. It would be interesting to perform the same modified carrier test, as a significant difference in BZC efficacy was demonstrated between 24 h and 168 h drying (P=0.00896; test) (Chapter 4: Section 4.3.2) and owing the reported risks of bacterial resistance and cross-resistance associated with BZC (Curiao *et al*., 2016, Forbes *et al*., 2017, Langsrud *et al*., 2004, Nhung *et al*., 2016, Pagedar *et al*., 2012).

6.2.2. Minimal Selective Concentration of BZC

The MSC could not be obtained for BZC as there was no comparison between susceptible and resistant strains available. It might be useful in future to apply the comparison of strains that are susceptible and resistant, this may highlight more MSC relationships and better define the effect of a concentration range of biocides on bacterial growth.

6.2.3. Efflux of BZC and resistance

There was a distinct difference in efflux activity at the low (0.00005 mg/mL) and high (0.002 mg/mL) sub-MIC concentration of BZC tested (P=<0.001; T-TEST; Graphpad PRISM8). BZC is a membrane active biocide, like CHX, so concentration dependent changes in membrane permeability may also explain the difference in relative fluoresce values observed. We have previously seen a loss of resistance to tetracycline, streptomycin, trimethoprim-sulfamethoxazole and trimethoprim for UCD-CFS ECP-13P5 when exposed to BZC for 24 hours (6h

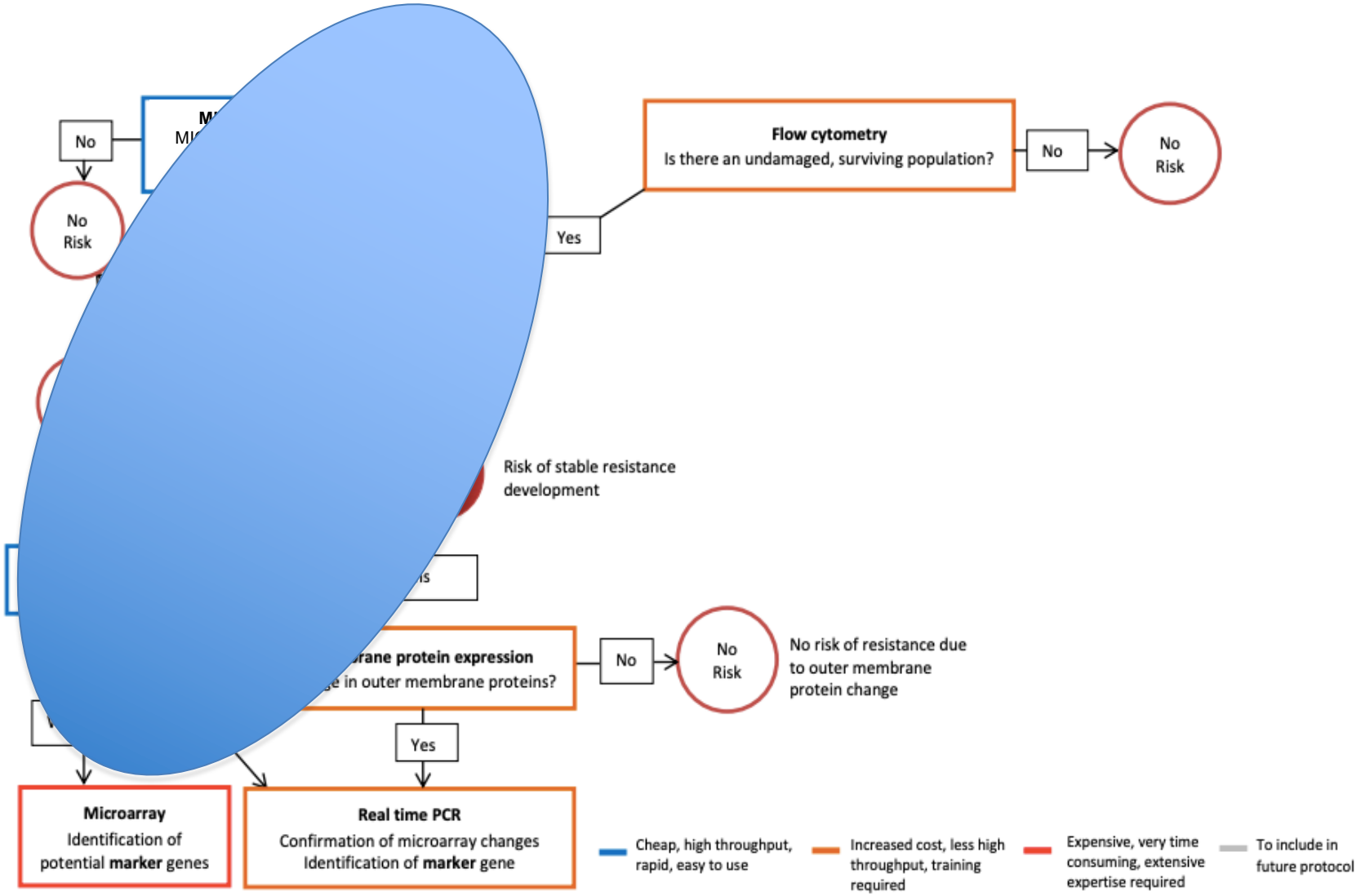
surface dried). This was explained by changes in cell membrane permeability which would support the findings of the efflux assay.

6.4 Biocidal product regulation the development of a predictive protocol to evaluate the risk of resistance

As previously discussed, there is no standard protocol to evaluate and predict the risk of the development of resistance to biocides. (Knapp *et al*., 2015) designed a decision tree based method that allows the step-by-step assessment of a biocides potential to cause resistance. This protocol is intended for use with formulated biocides in order to provide a realistic understanding of risk when a product is applied *in-situ*. **Figure 6.4** is the decision tree devised by Knapp *et al*. (2015). According to the decision tree CHX is at "risk of stable resistance development" to antibiotics (derived from stable antibiotic resistances seen in UCD-CFS ECP-1B2 & 13P5). The next logical step according to the decision tree is to undertake genomic characterisation of the test strains in order to uncover the mechanisms involved with the resistance risk posed. The scope of this study has not allowed for genotypic investigations. Furthermore, as this thesis unveiled a distinct difference in phenotype adaption between high and low biocide exposure it seems pertinent to suggest the inclusion of a range of biocide testing concentrations using the predictive protocol. Testing a high concentration of biocide through the predictive protocol will depict the "worst case scenario", however it will also exclude bacterial populations that also have the potential to develop resistance and will pose a strong selection pressure but will not provide information about adaptive potential. We suggested that "during use exposure" of realistic residual active concentrations should be included. The "conditions for granting an authorisation" section of the Biocidal Products Regulation (BPR, EU, 2012, p21.) stipulates that the responsible

regulatory body must be notified if the product owner is aware, or becomes aware that the BP is not sufficiently effective or that there is potential development of resistance to the active substance. The findings of this study suggest that combined with the possible application of the resistance risk decision tree by Knapp *et al*. (2015) would provide a structured platform that could be implemented before a product is allowed to go to market.

Figure 6.4 Decision tree and proposed protocol for the prediction of bacterial resistance of CHX after modified carrier test. Circled are the protocols performed through this thesis. ↓ Indicates the pathway undertaken as a result of susceptibility testing.



6.5. Key findings

This thesis aimed to:

- Obtain realistic residual surface concentrations of biocides
- Observe transient and stable changes in antimicrobial susceptibility as a result of biocide exposure
- Identify differences between selection and adaption processes at high and low sub-MIC biocide concentrations
- Relate metabolic alterations to observed susceptibility phenotype after biocide exposure

In answer to the aims of the thesis we have demonstrated that a 0.006 mg/mL is a realistic during use exposure concentration of CHX. At this residual concentration it is possible for CHX susceptible bacteria to survive the disinfection process, adapt through metabolic alterations, incur transient increases in susceptibility and stable cross-resistance to antibiotics. Furthermore, our results show that a transiently adapted population may be selected amongst less tolerant sub-populations. Using the seven distinct plasmid-mediated ESBL isolates has provided an additional opportunity to understand how high and low sub-MIC concentrations of CHX affect the transfer of resistance via conjugation. It was found that it was possible for the isolate UCD-CFS ECP-13P5 to transfer ampicillin resistance naturally and at 0.00005 mg/mL CHX at the same rate. However 0.002 mg/mL CHX prevented conjugative transfer.

6.6. Future experimentation

Further investigations are warranted based on the findings of this study in order to provide a more indicative representation of the application of formulated biocidal products. Testing was undertaken using simple aqueous solutions containing pure biocidal actives. It has been suggested that this might lead to an over-estimation of the real to life outcomes of biocides impact to microbial susceptibility (Forbes *et al*., 2019).

The information gained through the phenotype microarray (**Chapter 5: Section 5.3**) would be enhanced with a direct comparison to genotypic expression of the bacteria grown in the same conditions, such as the study undertaken by Chaudhuri *et al*. (2010). As an endorsement of this thesis it would be recommended that phenotypic microarray could be added to the decision tree as part of the protocols applied to investigate mechanism. Supplementary to the mandatory analysis software (Biolog, Inc., Hayward, CA, United States) that aids the data production of the phenotype microarray, additional structures such as the KEGG database and, duct Tape provide tools that combine both phenotypic and genetic expression in order to form a more complete picture of cellular processes. Mutational changes found within the resistant genes on plasmids or mutations that alter the function of chromosome-encoded genes might contribute to the antibiotic resistant mechanisms (Lister *et al*., 2009) Next generation sequencing (NGS) technology has the capability to analyse the whole genome, whole proteome and whole transcriptome (Ramanathan *et al*., 2017). Bacterial genomes within species are made up of both a commonly shared core genome and a so called “accessory genome” which introduces variability or genetic polymorphisms between strains of the same species (Schürch *et al*., 2018). This variability can originate from point mutations, homologous recombination and differences in genome content. Point

mutations, in particluar single-nucelotide polymorphisms (SNPs) and mutations in antibiotic resistance genes have been utilised to investigate clinically relevant antibiotic resistant strains (Petkau *et al*., 2017). The application of this technology would further enhance the findings of this study, would provide a comprehensive summary of the links between genotype and phenotype of my strains and provide insight into the selective advantage that was identified in isolate UCD-CFS ECP-13P5.

PUBLICATIONS

2018 *American Journal of Infection Control*

Impact of antimicrobial wipes compared with hypochlorite solution on environmental surface contamination in a health care setting: a double-crossover study *(Journal article)*

Authors: Harsha Siani, Rebecca Wesgate, Jean-Yves maillard

2016 *The Journal of Hospital Infection 93(3)*

Impact of standard test protocols on sporicidal efficacy *(Journal article)*

Authors: Rebecca Wesgate, Gaetan Rauwel, J. Criquelion, jean-Yves Maillard

2016 *American Journal of Infection Control 44(4)*

Use of a predictive protocol to measure the antimicrobial resistance risks associated with biocidal product usage *(Journal article)*

Authors: Rebecca Wesgate, Pierre Grasha, Jean-Yves Maillard

2015 *The Journal of Hospital Infection 91*

Disinfectant wipes are appropriate to control microbial bioburden from surfaces: Use of a new standardised test protocol to demonstrate efficacy *(Journal article)*

Authors: Syed A Sattar *et al*.

2015 *The Journal of Applied Microbiology 119(6)*

Bacillus subtilis vegetative isolate surviving chlorine dioxide exposure: An elusive mechanism of resistance *(Journal article)*

Authors: Deborah J H Martin *et al*.

2015 *American Journal of Infection Control 43(7)*

Pathogen transfer and high variability in pathogen removal by detergent wipes *(Journal article)*

Authors: Lauren Ramm, Rebecca Wesgate, Harsha Siani,

Jean-Yves Maillard

2014 *The Journal of Hospital Infection 89(1)*

Development of a sporicidal test method for *Clostridium difficile (Journal article)*

Authors: A P Fraise *et al*.

APPENDICES

All appendices data is stored on the included USB flash drive. Flash drive contents are as follows:

Appendix one

- File name: Breakpoint-Table-EUCAST.Pdf

  Contents: Contains Breakpoint cut-offs and method tips for EUCAST disk diffusion method

Appendix two

- File name: Antibiotic susceptibility data (EUCAST).xlsx

  Contents: Raw data from EUCAST antibiotic susceptibility testing (data relevant to chapter three and four)

- File name: Efflux histograms.xlsx

  Contents: Histograms from efflux assays that were not displayed in Chapter 4.

Appendix three

- File name: BIOLOG data.xlsx

  Contents: Collated raw data values (Metabolic distance) from the Phenotype Microarray experiment (Chapter 5)

- File name: KEGG database list.docx

  Contents: A reference list of all databases utilised to form the KEGG pathway-mapping package (Chapter 5)

- File name: Layout of Phenotype Microplates.pdf

  Contents: Phenotype Microarray plate layouts, including substrate identity and mode (Chapter 5)

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