Realism-based Approaches for Evaluating Bacterial Susceptibility to Antimicrobials used in Home and Personal Care Products

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List of Abbreviations

BAC	Benzalkonium Chloride
BIT	Benzisothiazolinone
CDFF	Constant-Depth Film Fermenter
CHX	Chlorhexidine
DDAC	Didecyldimethyl ammonium chloride
DGGE	Denaturing gradient gel electrophoresis
DMDM	1,3-Dimethylol-5,5-dimethylhydantoin
EPS	Extracellular Polymeric Substance
FDA	Food and Drug Administration
MBC	Minimum Bactericidal Concentration
MBEC	Minimum Biofilm Eradication Concentration
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
NGS	Next generation sequencing
PCR	Polymerase Chain Reaction
PHMB	Polyhexamethylene biguanide
PTFE	Polytetrafluoroethylene
QAC	Quaternary Ammonium Compound
SCV	Small Colony Variant

THE UNIVERSITY OF MANCHESTER

ABSTRACT OF THESIS submitted by Nicola Louise Cowley for the Degree of Doctor of Philosophy entitled Realism-based Studies for Evaluating Bacterial Susceptibility to Antimicrobials used in Home and Personal Care Products. May 2016.

Microbicides are used in consumer products worldwide to enhance their antibacterial potency in disinfection, for antisepsis or as preservatives. With the widespread use of these compounds, concerns have been expressed about their potential to select for reduced susceptibility. Whilst *in vitro* studies have reported the laboratory generation of bacterial insusceptibility for certain combinations of bacterium and microbicide, true microbicide resistance, which is defined as a change in susceptibility that is likely to affect the outcome of treatment, has not been frequently reported or observed in the environment. Importantly, risk assessments on the use of microbicides have been largely based on laboratory studies whereby pure cultures of bacteria are exposed to microbicides in aqueous solution. In use however, microbicides are formulated with various excipients and bacteria are exposed to them most commonly in complex biofilm communities. Work described in this doctoral thesis evaluates the effects of exposing bacteria to microbicides using exposure conditions intended to increase realism, with the ultimate aim of informing improved risk assessment methods that better reflect deployment of microbicides in the real-world, taking in to account the effects of formulation, growth in multi-species communities and potential reduced competiveness in adapted bacteria. Test bacteria (8 species, 7 genera) were repeatedly exposed to selected microbicides in aqueous solution and in various formulations reflecting their use in the domestic environment, such as general-purpose cleaners and laundry detergents. Minimum inhibitory concentrations, minimum bactericidal concentrations and minimum biofilm eradication concentrations were determined before and after 14 passages (P14) in the presence of microbicides (benzalkonium chloride (BAC), benzisothiazolinone (BIT), chlorhexidine (CHX), didecyldimethyl ammonium chloride (DDAC), Glydant (DMDM-hydantoin), polyhexamethylene biguanide (PHMB), thymol and triclosan) in aqueous solution or in formulation, using a previously validated gradient plating system. Bacteria were subsequently passaged a further 14 times in the absence of any antimicrobial agent to determine the stability of any adaptations (X14). In bacterial isolates that demonstrated marked changes in susceptibility, further phenotypic analysis was conducted to test for any induced alterations in antibiotic susceptibility, planktonic growth rate, biofilm formation, competitive fitness and relative pathogenicity. Exposure of microbial communities was carried out using a previously validated domestic drain biofilm simulator within constant depth film fermenters (CDFF). The CDFFs were exposed to increasing concentrations of BAC in aqueous solution or BAC formulation over a 32-week period. Changes in bacterial community composition and antimicrobial susceptibility distributions were assessed via replica plating onto selective and antimicrobial-containing agars as well as through the use of next generation sequencing technologies via the illumina Miseq platform and QIIME software. The formulation of microbicides significantly increased antibacterial and anti-biofilm potency and reduced the incidence and extent of the development of insusceptibility isolated bacteria (7 non-revertible bacteria in MBCs for microbicides, whilst 2 non-revertible bacteria in MBCs for formulations). In bacteria that develop marked changes in antimicrobial susceptibility after repeated exposure show changes to biofilm growth rates (10 increases and 6 decreases after microbicide exposure; 1 increase and 3 decreases after formulation exposure), as well as alterations in competitive fitness (6 decreases and 19 increases after microbicide exposure; whilst all exposed to formulation had decreased fitness) and virulence (9/13 decreased and 1/13 increased after microbicide exposure; 4/7 decreased and 2/7 increased after formulation exposure). In the multispecies microcosm system, long-term exposure to BAC or BAC formulation induced shifts in bacterial community dynamics and resulted in a decrease in BAC and various antibiotic susceptibilities (1 $\log_{10}$ reduction in the BAC system; 2.5 $\log_{10}$ reduction in the BAC formulation system of viable bacteria). Such shifts in community dynamics after antimicrobial treatment are theorised to be mainly due to clonal expansion of innately insusceptible bacteria (abundance of *Achromobacter* sp. increased by 39% in BAC system and 10% in BAC formulation system). Understanding the potential selectivity of microbicide-containing formulations is likely to better serve by testing formulations as well as actives in aqueous solutions. This highlights the need to conduct risk assessments of induced microbicide susceptibility changes using conditions that more accurately reflect their deployment.

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Research Contributions

List of Publications

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Chapter 1

General Introduction

1.1 General Overview

Antimicrobial compounds are chemicals that inhibit or kill microorganisms (Cowan, 1999). Microbicides (or biocides) have been defined as broad-spectrum antimicrobials that are bacteriostatic or bactericidal (Müller and Kramer, 2008) but which, for various reasons cannot be used systemically, a general feature which differentiates microbicides from antibiotics. Microbicides have a wide range of application including deployment in the domestic environment (Levy, 2001) for hygienic purposes or as preservatives in consumer products (Nychas and Arkoudelos, 1990); in hospitals for antisepsis and disinfection (Kampf and Kramer, 2004) and in industry (Holah et al., 2002) to control microbial growth. The increased development and use of consumer products containing microbicides has led to increased concern about their potential effects on bacteria susceptibility (Walsh et al., 2003b, Chuanchuen et al., 2001). Whilst previous research has demonstrated the development of reduced bacterial susceptibility to microbicides after repeated exposure *in vitro,* it remains unclear whether such observations are reflective of real life (Karatzas et al., 2007). Furthermore, it is uncertain whether the development of true microbicide resistance which can be defined as outcome changing reduction in susceptibility, occurs frequently outside laboratory settings (McBain and Gilbert, 2001, Karatzas et al., 2007, Moore et al., 2008, Maillard et al., 2013). There is concern, however, that the increasing deployment of microbicides and the potential for them to become diluted in the environment away from the point of application may lead to selective pressure potentially resulting in reductions in susceptibility towards the primary agent, as well as possibly decreasing susceptibility towards unrelated antimicrobials, such as other microbicides or antibiotics (Chuanchuen et al., 2001).

Microbicides are often formulated in consumer products at concentrations considerably higher than those required for growth inhibition. This is likely to significantly reduce the risk of resistance, at least in the primarily exposed bacterial populations (Chapman, 2003). However, following deployment, microbicides may undergo dilution which can result in exposure of bacteria to sub-effective concentrations, increasing the probability of bacterial adaptation. Few studies have examined the effects of residual concentrations of microbicides upon bacterial communities in the domestic environment and the effects of microbicides have upon the susceptibility and composition of bacterial communities, (McBain et al., 2003b,

McBain et al., 2004, Marshall et al., 2012) and are therefore poorly understood. A better understanding of how microbicides interact with bacteria in the real world is likely to facilitate the development of more realistic risk assessments for microbicide usage and the potential development of bacterial insusceptibility.

1.2 Antimicrobials, an overview

Antimicrobials are used to control the growth of microorganisms and are deployed in the healthcare, domestic and industrial environments. They can be naturally occurring agents, such as honey (Cooper et al., 1999) or essential oils found in plants (Burt, 2004) which can be used to treat bacteria commonly associated with wound infection (Edwards-Jones et al., 2004). The use of synthetic antimicrobials has increased over the past 60 years, with the majority being used for disinfection, preservation and as antibiotics (Russell, 2002). The use and misuse of antibiotics since their discovery has inevitably led to the evolution of antibiotic resistance, such as methicillin-resistant *Staphylococcus aureus* (MRSA) (Gosbell, 2004) and vancomycin resistant enterococci (VRE) (Cetinkaya et al., 2000) which has fuelled further research into the discovery and development of novel antimicrobial agents.

1.3 Microbicides

Microbicides generally have multiple target sites including the bacterial cellular membrane, cell wall synthesis (Richards and Cavill, 1976); destabilisation of cations (Gilbert and Moore, 2005); loss of ATP through membranotrophic activities or inhibition of enzymes (Kuyyakanond and Quesnel, 1992) including those involved in fatty acid synthesis (Russell, 2004). The microbicides investigated in this doctoral thesis are benzalkonium chloride (BAC), benzisothiazolinone (BIT), chlorhexidine (CHX), didecyldimethyl ammonium chloride (DDAC), polyhexamethylene biguanide (PHMB), thymol and triclosan. These are described in more detail in Sections 1.3.1; 1.3.1.1; 1.3.1.2; 1.3.1.3; 1.3.1.4; 1.3.1.5 and 1.3.1.6.

1.3.1 Mechanisms of Action of Microbicides

Microbicides usually have a broad-spectrum of activity and tend to have various mechanisms of action with low target specificity (Poole, 2002). Microbicides can effect bacteria differently depending on the mode of action and the concentration used, ranging from bacteriostatic to bactericidal effects (Bloomfield, 1991). The currently proposed modes of action will be discussed in the next section. The following section focuses on the classes of microbicides investigated in this thesis.

1.3.1.1 Quaternary Ammonium Compounds (QACs)

Quaternary ammonium compounds (QACs) are cationic surface-acting microbicides. Due to the net positive charge they are attracted to the negatively charged bacterial cell. On encountering the cell, the longer hydrophobic alkyl tail of the QAC can interdigitate into the bacterial cellular membrane, causing a loss of fluidity (Gilbert and Moore, 2005). The long alkyl group tail enables the microbicide to act like a detergent and causes cellular damage through cytoplasmic leakage, by dislodging the cell wall and causing breakdown of the lipid bilayer (Gilbert and Al-taae, 1985). Due to the positive charge of QACs, they have an ability to readily bind to anionic sites, such as the phospholipid head group on the surface of the bacterial membrane (Lambert and Hammond, 1973). Increases in antibacterial activity are seen with QACs at higher temperatures and increased pH (Hoogerheide, 1945), however QACs are relatively ineffective against spores due to their inability to penetrate the spore coating (Russell et al., 1985).

The QACs investigated in this doctoral thesis are benzalkonium chloride (BAC) and didecyldimethyl ammonium chloride (DDAC). BAC is commonly used in personal hygiene products and surface cleaners. Due to the flexibility of the BAC molecule, the microbicide is absorbed into the bacterial membrane between phospholipids making the cell more rigid (Salton, 1968). Micelles of combined BAC and phospholipid are produced from the solubilisation of proteins and phospholipids in the outer membrane, causing loss of fluidity, perturbed protein function and eventually cell lysis (Gilbert and Moore, 2005). DDAC is able to disrupt the bilayer of bacterial cells, as well as causing internal damage to the bacterium

(Gilbert and Al-taae, 1985). Similarly to BAC, DDAC causes disruption of membrane fluidity through absorption into the outer cellular membrane causing loss of fluidity and high cellular leakage of potassium and causing irreversible damage to the cell, and eventually cell death (Walsh et al., 2003b). DDAC is used to sterilise surgical equipment and is also used in antiseptics (Lankford et al., 2006).

1.3.1.2 Biguanides

Biguanides are cationic microbicides that can be both bacteriostatic and bactericidal (Rose and Swain, 1956). Biguanides are broad-spectrum microbicides, used in personal care products. Biguanides are relatively ineffective against *Pseudomonadaceae* and *Providentia* spp. due to the thicker outer membrane of Gram-negative bacteria and the mucoid layer of a number of *Pseudomonadaceae*, preventing entry into the bacterial cell (Rosin et al., 2002, McDonnell and Russell, 1999). The biguanides used in this study are polyhexamethylene biguanide (PHMB) and chlorhexidine (CHX).

PHMB is commonly used as a disinfectant in hard surface cleaners (Hansmann et al., 2004), for sanitising swimming pools (Gilbert and Moore, 2005) as well as being effective in mouthwash at inhibiting dental plague growth (Rosin et al., 2002). PHMB is a polymeric biguanide with repeating groups of biguanides joined by hydrocarbon chains (Gilbert et al., 1990). PHMB is believed to act on the bacterial cell envelope of bacterial organisms, in particular the phospholipids (Broxton et al., 1984). The inflexible structure of the PHMB molecule means it cannot interdigitate effectively into the bacterial cellular membrane and therefore relies upon altering the membrane integrity by bridging acidic phospholipids within the bacterial membrane (Ikeda et al., 1985). The negatively charged bacterial cells attract the positive PHMB which binds to the cell membrane, interacting with phospholipids present, competing and destabilising the positive ions (Ca^{2+} and Mg^{+}) which are present on the cell surface (Broxton et al., 1984). PHMB causes loss of membrane fluidity due to interactions with the acidic membrane components, leading to separation of individual lipids (Ikeda et al., 1985). PHMB reportedly aggregates to a hexagonal arrangement with the individual lipid domains, causing a complete loss of the outer membrane (Ikeda et al., 1985). As the cells become

susceptible due to the breakdown of the outer membrane, this leads to self-promoted uptake of PHMB, causing further damage to the cytoplasmic membrane (Gilbert et al., 1990). Potassium leakage occurs, which is followed by loss of cellular functions from the bacterial cell (McDonnell and Russell, 1999).

Chlorhexidine is frequently used in mouthwash and surface cleaners, as well as topical antiseptics creams (Rose and Swain, 1956). CHX is a bisbiguanide containing two cationic head groups separated by a hexamethylene group which is hydrophobic (Gilbert and Moore, 2005). CHX is bacteriostatic and bactericidal, as well as sporostatic, with antibacterial activity dependent upon pH (Russell, 1986). Similar to PHMB, CHX binds strongly to the acidic phospholipids and proteins on the cell membrane, displacing cations of Mg^{+} and Ca^{2+} (Davies, 1973) and causing damage to the cellular membrane. CHX then passes into the cell via passive diffusion, causing cellular leakage and cytoplasmic damage, as well as self-promoted uptake, whereby the damaged caused to the bacterial cells leads to further uptake of the microbicide (McDonnell and Russell, 1999). CHX is also thought to inhibit the activity of ATPase, at high concentrations (Harold et al., 1969), however inhibition of ATPase is not thought to lead to cell death; membrane disruption is considered the lethal event (Kuyyakanond and Quesnel, 1992).

1.3.1.3 Bisphenols

Bisphenols have broad-spectrum activity and are used in soap, detergents and a number of oral care products (Block, 2001). Bisphenols are relatively ineffective against *P. aeruginosa* due to the expression of multidrug efflux pumps such as MexCD-OprJ (Chuanchuen et al., 2001, Chuanchuen et al., 2003) and increased lipid content which can inhibit diffusion (Meincke et al., 1979). Triclosan is a bisphenol antimicrobial with a broad spectrum activity with the ability to inhibit bacteria via blocking fatty acid synthesis (Russell, 2004). The mechanism of action of triclosan reportedly involves inhibition of the fatty acid synthesis. Triclosan forms a complex with the enzyme FabI, by tightly binding to the enoyl substrate site, which inhibits the breakdown of NADPH into $NADP^{+}$ (Heath et al., 1999), inhibiting the fatty acid synthesis process and halting the production of fatty acids. High

concentrations of triclosan reportedly disrupts the cellular membrane, which leads to potassium leakage from the cell and eventually destruction of the cell. Triclosan is currently used in certain toothpastes and soaps, (Rodricks et al., 2010) and in various other applications. However this is under review by the food and drug administration (FDA) due to studies showing triclosan to be readily absorbed into the body, with evidence of it being found in urine (Sandborgh-Englund et al., 2006) and breast milk (Rodricks et al., 2010).

1.3.1.4 Essential Oils

Essential oils are hydrophobic antimicrobial agents derived from plant extracts and are often used in household products, primarily as flavouring and preservatives (Nychas and Arkoudelos, 1990). Their antimicrobial mechanism of action is incompletely understood, but research indicates that cellular membrane and intercellular energy generating processes are targeted (Hyldgaard et al., 2012). Thymol is a naturally occurring microbicide, which exhibits antibacterial properties and low toxicity (Falcone et al., 2005). Thymol is used as an active ingredient in oral hygiene and in some other personal care products (Stephenson et al., 1985). Thymol can gain entry into the bacterial cell through porins located on the outer membrane (Lambert et al., 2001). Thymol interacts with the phospholipid head group, integrating itself into the outer membrane, causing loss in fluidity of the bacterial cell (Di Pasqua et al., 2010). This causes membrane dysfunction and is theorised to increase the permeability of the bacterial membrane, which causes increased cellular leakage of potassium ions and ATP (Lambert et al., 2001). The loss of ATP leads to the inhibition energy dependent proton-pumps which are needed in the maintenance of pH homeostasis (Lambert et al., 2001).

Essential oils have been increasingly researched to examine the antimicrobial properties they may possess, including; aegle, ageratum, citronella, eucalyptus, geranium, lemongrass, orange palmarosa, patchouli and peppermint (Pattnaik et al., 1996) to name a few. Recent studies have examined the effects essential oils, such as eugenol have upon permeability of bacterial cellular membrane (Devi et al., 2010, Michiels et al., 2007), as well as carvacrol demonstrating reductions in pH gradient across the membrane and destabilisation of the cytoplasmic membrane (Ultee et al., 2002). Essential oils are naturally

occurring substances, which have been examined as potential mixed compounds with antibiotics, with studies showing essential oils to increase the activity of antibiotics (Borges et al., 2016, Fadli et al., 2012, Rodrigues et al., 2009, Rosato et al., 2007). Synergistic effects of essential oils and antibiotics may potentially lead to a new therapeutic method in the fight against antibiotic resistant bacteria.

1.3.1.5 Aldehydes

Aldehydes contain a formyl group and are primarily used as preservatives in consumer products, as well as for flavouring in a variety of food products (Paulus, 2012). 1,3-Dimethylol-5,5-dimethylhydantoin (DMDM) is used in skin care products, shampoos and conditioners (De Groot and Veenstra, 2010). DMDM hydantoin is bactericidal, bacteriostatic and sporicidal (Kunicka-Styczynska et al., 2009). DMDM hydantoin is theorised to enter the bacterial cell through porins due to the low molecular weight (Paulus, 2012). The agent is theorised to interact with the bacterial cell, causing cross-linking of DNA, RNA and proteins (McDonnell and Russell, 1999, Paulus, 2012). Cross-linking of DNA causes cell replication to stop by linking two amino acids, either on the same strand of DNA or complementary strands (Neely, 1963). It is also thought that formaldehyde-releasing agents have the ability to block methionine synthesis in bacterial cells (Paulus, 2012). Methionine is the amino acid which is coded by the initiation codon, which begins the translation process; blocking methionine will inhibit translation of proteins (Fowler, 2005). Cross-linking of proteins in the cell wall or cell membrane can also occur which inhibits the transport of nutrients into the cell, therefore leading to cell death due to starvation (Gorman et al., 1980). Other aldehydes have been seen to exhibit antimicrobial properties, such as, glutaraldehyde which are used as system disinfectants (Leers 1980) and are shown to inhibit the growth of antibiotic resistant Burkholderia cepacia (Li et al., 2013); as well as formaldehydes which demonstrate antimicrobial activity (Musher and Griffith, 1974).

1.3.1.6 Isothiazolinones

Benzisothiazolinone (BIT) is a sulphur containing compound used as a preservative in laundry detergents, dish soap, spray cleaner and sunscreen (Novick et al., 2013); as well as having antimicrobial properties (Alexander, 2002). BIT is thought to enter the cell through transport proteins, such as porins (Williams, 2007). BIT is an electrophile microbicide which interacts with the thiol group of the amino acid cysteine and the tripeptide glutathione (Chapman and Diehl, 1995). Cysteine and glutathione are nucleophiles and are important in enzymic processes, such as, ATP synthesis and protein structures; the breakdown of these structures leaves the bacterial cell susceptible to free-radical attacks (Chapman and Diehl, 1995). The breakdown of thiols leads to inhibition of enzymes, due to thiols being key active sites in dehydrogenase enzymes, which are needed for ATP synthesis; this in turn halts the process of the cell to make energy and therefore inhibits growth and repair functions of the cell (Williams, 2007).

There are a variety of antibacterial agents which are of equal importance to those mentioned; these include, halogen-releasing agents (chlorine- and iodine-based compounds) which are known to inhibit bacterial species and are potentially sporicidal (Williams and Russell, 1991); peroxygens which exhibit sporicidal and antimicrobial activities, with Gram-negative bacteria shown to be less susceptible due to the presence of catalase in the organism (Hassett et al., 1999, Reiter et al., 1976) and surfactants which are known to increase cellular permeability and cause cytoplasmic leakage of potassium ions (Moore et al., 2006). The thesis will examine the aforementioned microbicides in great detail and discuss the potential for microbicide resistant towards these agents.

1.3.2 Microbicide Resistance

The increased use of microbicides in recent years has led to a concern that prolonged exposure of bacteria to sub inhibitory concentrations of microbicide-containing products may select for bacterial populations which show decreased susceptibility. In pure cultures however, microbicide resistance has been attributed to both temporary phenotypic adaptation or through the selection of insusceptible mutants. Microbicides generally target multiple sites of bacterial

cells, in a concentration dependent manner; the lower concentrations usually lead to bacterial inhibition while increased concentrations can cause cell lysis (McDonnell and Russell, 1999). There have been concerns that cross-resistance between microbicides and other antimicrobials may occur, potentially due to induced phenotypic changes to the cell after microbicide exposure or as a result of alterations of shared target sites, making the bacteria less susceptible (Chuanchuen et al., 2001).

Studies have demonstrated that decreases in QAC susceptibility can occur when bacteria are exposed to sub-inhibitory QAC concentrations (Aase et al., 2000). Several mechanisms may be involved in this, with the increase in the expression or activity of efflux pumps being a key factor (Poole, 2005). An upregulation of the AcrAB efflux pump in *E. coli*, or the upregulation of MdrL efflux pump in *Listeria monocytogenes* has reportedly led to in decreases in susceptibility towards BAC (Okusu et al., 1996, Romanova et al., 2006). Another mechanism that can cause decreases in QAC susceptibility in bacteria, is alteration of the protein composition of the cell envelope, which has been found with *P. aeruginosa* and causes the bacteria to be relatively insusceptible towards QACs due to limited diffusion into the cell from the narrowing of porins (Campanac et al., 2002).

PHMB susceptibility in bacteria does not appear to be influenced by the over expression or increased activity of efflux pumps (Gilbert and Moore, 2005). It is theorized that biguanides do not become solubilized in the membrane core, limiting the protective effect of the efflux pumps (Davies, 1973). However, it was previously suggested that cation displacement and changes in the bridging of the phospholipid head groups within the cytoplasmic membrane can lead to minor susceptibility changes occurring in bacteria towards PHMB (Gilbert and Moore, 2005). This theory has since been challenged; with evidence that damage to the bacteria membrane caused by the exposure of microbicides such as biguanides may increase the number of efflux pumps (Fraud et al., 2008). Fraud *et al.,* (2008) demonstrated the reduction of bacterial susceptibility to other biguanides, such as CHX, due to the induction of efflux pumps, such as the multidrug efflux pump, MexCD-OprJ in *P. aeruginosa*.

As discussed, triclosan is known to target a specific enzyme, FabI, which is involved in the synthesis of fatty acids in bacteria (McMurry et al., 1998). McMurry *et al*. (1998) demonstrated the development of triclosan resistant *E. coli*, which was due to a mutation in the *fabI* gene or attributable to increased expression of the efflux pump, AcrAB or its positive regulators, recA. Chuanchuen *et al*. (2003) reported that low susceptibility to triclosan in *P. aeruginosa* was due to efflux pumps; whilst Fan *et al*. (2002) demonstrated that upregulation of the *fabI* gene in *S. aureus* after exposure to triclosan led to decreased triclosan susceptibility, suggesting that several mechanisms could lead to decreases in susceptibility towards this microbicide. Walsh *et al.,* (2003b) demonstrated that bacteria can become less susceptible towards thymol following sub-lethal exposure due to changes in the outer membrane which impede integration into the cell. However, Palaniappan and Holley (2010) reported that concomitant exposure to thymol may lower the risk of bacterial resistance towards a number of antibiotics. The study involved challenging bacterial species with other natural antimicrobials (cinnamaldehyde, eugenol and allyl isothiocyanate) as well as thymol, showing synergistic interactions with ampicillin, penicillin, tetracycline, bacitracin and novobiocin (Palaniappan and Holley, 2010).

1.3.2.1 Efflux Pumps

Efflux pumps are trans-membrane proteins that function as transporters of metabolites and xenobiotics including some antimicrobials (Figure 1). Expression of efflux pumps has been shown to contribute towards intrinsic or acquired bacterial resistance for numerous antimicrobial agents (Poole, 2002). In *S. aureus*, upregulation of the efflux pump NorA reportedly led to reductions in antimicrobial susceptibility (Gibbons et al., 2003). In this study, NorA expression was upregulated after antimicrobial exposure as part of a broad-range defense mechanism (Kaatz and Seo, 1995). This up-regulation is theorized to be short lived and when the antimicrobial pressure is removed bacteria are able to revert to the unexposed phenotype, which possesses lower numbers of NorA (Kaatz and Seo, 1995). In *E. coli,* increased expression of the efflux system, AcrAB, has been correlated to changes in

susceptibility to QACs such as BAC (Okusu et al., 1996), AcrAB is believed to be upregulated as part of a general stress-induced response (Ma et al., 1995).

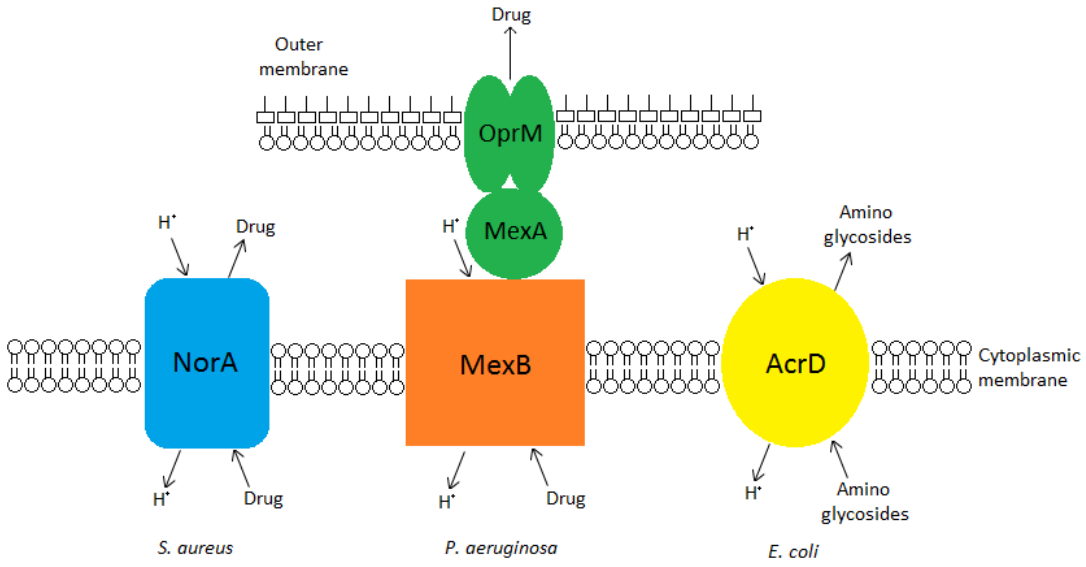

Figure 1.1 An illustration showing efflux systems in *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*. Shown are NorA efflux pump predominately found in *S. aureus*; MexAB-OprM efflux system found in *P. aeruginosa* and AcrD efflux pump found in *E. coli* (Schweizer, 2003).

1.3.2.2 Porins

Porins are pore-like proteins located on the outer membrane of bacteria which enable entry of small hydrophilic molecules into the cell (Livermore, 2001). Porins are either; (i) non-specific, which do not have a specified ligand-binding site, allowing diffusion of small molecules (ii) specific, which have specific binding sites which allow entry of specific molecules that are unable to enter the cell via diffusion (Nikaido, 1992). Evolutionary changes in the structure and expression level of many porins is evident in a number of bacterial species, as they are located on the outer membrane of bacteria and therefore are subjected to outside stimuli frequently. It is theorised that looped structures on the porin narrow the size of the porin when subjected to harmful agents (Nikaido, 1992). The narrowing of porins due to increased looped structures or the formation of strong bonds between lipopolysaccharides

has previously been seen in *P. aeruginosa*, which correlated with a reduction in microbicide susceptibility (Nikaido and Vaara, 1985). Reductions in the number of porins can also decrease susceptibility of certain bacterial species to microbicides, as seen with porin deficient *E. coli*, which exhibits silver resistance (Li et al., 1997).

1.4 Evidence of Bacterial Susceptibility Changes to Microbicides

A large number of studies have indicated that pure cultures can potentially become less susceptible to antimicrobials when exposed to sub-inhibitory concentrations of microbicides (Cookson et al., 1991, Walsh et al., 2003b, Walsh et al., 2003a). Walsh *et al.,* (2003a) suggested that exposure to thymol, eugenol, DDAC and C_{10}-C_{16} alkyl dimethyl amine *N*-ocides (ADMAO) led to susceptibility reductions of log 4 - log 5 in bacterial species; *Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa*. The study examined potassium leakage in bacteria after exposure to microbicides, indicating increased potassium leakage after exposure to DDAC, thymol, ADMAO and eugenol. This supports the assertion that the modes of action for these microbicides are membrane based. In a separate study, Walsh *et al.*, (2003b) examined the microbicide exposed bacterial species against antibiotics. The results showed a decrease in antibiotic susceptibility in the bacteria when exposed to sub-inhibitory concentrations of microbicides. In some cases, exposure to DDAC caused *E. coli* to become less susceptible towards chloramphenicol, ciprofloxacin, tetracycline, carbenicillin and piperacillin, when compared to the parent strain. While eugenol exposed *E. coli* became increasingly susceptible towards several antibiotics; gentamicin, tobramycin, amikacin, neomycin, kanamycin, ciprofloxacin, ceftazidime and polymyxin, when compared to the parent strain. The study demonstrates the potential of microbicide exposed bacteria to exhibit changes in sensitivity to antibiotics.

Relatively few studies have examined susceptibility changes in bacteria after exposure to sub-inhibitory concentrations of microbicides in commercial formulations which is how microbicides are normally deployed (Jones, 1999, McBain et al., 2004). The majority of studies on microbicide susceptibility have involved exposing pure cultures of bacteria to microbicides in aqueous solutions. Latimer *et al.,* (2012) examined the effects of triclosan

exposure on *S. aureus*, by determining phenotypic alterations in the bacteria that developed after growth in the presence of the microbicide. The results suggested the induction of a small colony variant phenotype, which are known to display reduced triclosan susceptibility, virulence and biofilm forming ability when compared to the unexposed parent strain (Bayston *et al*., 2007, Sifri *et al.*, 2006). Changes to antimicrobial susceptibility can also lead to alterations of fitness, potentially leading to fitness burdens of exposed bacteria. Rozen *et al.,* (2007) had demonstrated *Streptococcus pneumoniae* to express fitness burdens when fluoroquinolone resistance is acquired, showing considerable decreases to fitness after mutations to *parC, parE* and *gyrA* loci. Bacteria with reduced susceptibility to certain antimicrobials often exhibit reductions in fitness, virulence and growth, which may cause them to be readily outcompeted in a mixed species community.

1.5 Bacterial Fitness as a Potential Determinant of the Implication of Microbicide Adaptation

When studying the generation of antimicrobial susceptibility in bacteria, any alteration in the fitness of the resulting microorganism needs to be taken into consideration. Bacterial strains that have reduced susceptibility and a consequential reduction in fitness may not be able to compete with their fitter more susceptible congeners within a mixed species community. The fitness of the bacteria is usually defined as the success at which a species can survive and reproduce, whilst competing with other bacterial strains (Zhang *et al.*, 2006). Microbial fitness can change due to a variety of circumstances, such as, mutation, environmental factors or targeted substances (Reynolds, 2000). Previous studies demonstrate reductions in growth rate and defects in the electron transport chain due to reductions in susceptibility to microbicide (Besier et al., 2005). Exposure to microbicide can cause phenotypic alterations to the bacteria, which in turn can reduce planktonic growth rate and decreased susceptibility to the microbicide due to impaired cell division (Forbes et al., 2015, Latimer et al., 2012).

Bacterial species can evolve in a multitude of ways: mutation due to high selection pressures (Levin et al., 2000) or genetic transformation (Lorenz and Wackernagel, 1994), due

to transduction and conjugation (Davison, 1999). Bacteria can be affected by environmental factors, such as, changes in pH levels, depletion of oxygen or presence of antibacterial substances, all of which provide evolutionary pressures (Gagneux *et al*., 2006). Gene mutations and subsequent deletions may occur during bacterial replication, if the gene product is no longer a necessity to the cell (Baba *et al*., 2006). Exposing bacteria to a microbicidal pressure could result in decreases in microbicide susceptibility due to alteration in genes expression, possibly due to the acquisition of mutations in genes or their promoter regions (Allen *et al.*, 2006). The accumulation of genetic mutations may have a resulting cost to the bacteria's overall fitness. Previous studies show a variety of fitness costs that a bacterium may acquire during adaptation to selective pressures, although most of the aforementioned studies involve antibiotic selection, which shows mutations rendering bacteria antibiotic resistant but also leading to fitness costs, such as attenuation of virulence (Andersson and Levin, 1999).

1.5.1 Cross-resistance

The term cross-resistance refers to instances when adaptation to one antimicrobial compounds results in resistance to another. Cross-resistance between microbicides and antibiotics, if it occurs is obviously of concern because of the clinical importance of antibiotics. There is however little evidence to show that this occurs in the domestic environment. Marshall *et al.* (2012) assessed the effect of microbicide use on the microbicide susceptibility profiles of bacteria isolated from domestic kitchen drain bacterial communities. The study compared the microbial composition of various sample sites throughout the domestic environment taken from households that use or abstain from regular microbicide use. The study suggests that the data shows no significant differences in bacterial resistance to antibiotics between households. The study did however show that a multitude of domestic bacterial species were intrinsically antibiotic resistant. The study did not take into fact that antimicrobial substances are used is preservatives within a majority of products and therefore most domestic bacteria would often be subjected to small amounts of antimicrobials (Marshall *et al*., 2012). However, it is important to note that use of antimicrobial as preservatives differs in formulation, concentration and in many cases, the agent used.

Multidrug resistant bacterial strains are believed, to result mainly from the use or misuse of antibiotics (Yamamoto et al., 1988), with concern that overuse of microbicides could potentially lead to increased resistance towards antibiotics (Schweizer, 2001, Sakagami et al., 1989). Cross-resistance between an antibiotic and microbicide may occur due to the selection for a mutations in shared pharmacological target or through the induction of broad-range defence mechanisms such as efflux pumps, which can extruded antimicrobials from the bacterial cell (Fraud et al., 2008, Schweizer, 2003). Induced stress response of exposed bacteria can lead to changes in cell permeability or spore formation, causing decreases in susceptibility of bacteria to antimicrobials (Campanac et al., 2002, Walsh et al., 2003b). It is therefore important, when studying the effects of antimicrobials, to understand the way that microbicides and bacteria interact in the natural environment, such as in a biofilm and the potential for the induction of antibiotic cross-resistance. The next section will consider antibiotics used in this theses, examining modes of action, as well as previous evidence of cross-resistance.

1.5.2.1 Ampicillin

Ampicillin is a broad spectrum β-lactam antibiotic which has been used to treat enterobacteria, staphylococci, *Haemophilus* and *Neisseria* spp. (Livermore, 1995). Ampicillin disrupts cell wall synthesis, by inhibiting the enzyme transpeptidase which facilitates penicillin-binding proteins (PBPs), leading to cell lysis (Tomasz, 1979). The inhibition of transpeptidase and in turn cell wall synthesis, renders the cell unable to replicate properly leading to cell death. Resistance of ampicillin is thought to occur due to plasmid encoded enzymes; β-lactamases, which breaks down the β-lactam ring of the antibiotics through hydrolysis (Jacoby and Medeiros, 1991). Cross-resistance to ampicillin has also been reported for methicillin-resistant *Staphylococcus aureus* (MRSA) which, in the study, had been seen to exhibit resistance to BAC (Akimitsu *et al.,* 1999).

1.5.2.2 Cephalothin

Cephalothin is a cephalosporin antibiotic, (β-lactam) that is used predominately against Gram positive bacteria (Moellering *et al.,* 1974). It has the same mode of action as ampicillin; halting the peptidoglycan layer forming for the bacterial cell wall by mimicking the amino acid combination (D-Ala-D-Ala) for peptidoglycan precursors and binding to the PBPs active site. This disrupts the cross-linking of the peptidoglycan and therefore causing cell lysis and death (Tomasz, 1979). Cephalothin is less sensitive to β-lactamases; instead resistance involves the antibiotic acquiring a reduced affinity towards PBP components due to mutations of PBPs (Tipper, 1979). Cross-resistance of cephalothin in *E. coli* has been seen previously after exposure to microbicide sodium nitrite (Capita et al, 2013). Capita *et al.,* (2013) demonstrated reduced susceptibility of *E. coli* to cephalothin and kanamycin after prolonged exposure of sodium nitrite.

1.5.2.3 Ciprofloxacin

Ciprofloxacin is a broad-spectrum fluoroquinolone antibiotic used to treat enterobacteriaceae, gonococcal urtheritis, urinary tract infections and endocarditis (Chin and Neu, 1984), as well as exposure to anthrax, respiratory or sinuses infections (Peloquin et al., 1989). Ciprofloxacin inhibits cells division of bacterial cells by interacting with the type IV topoisomerase enzyme and DNA gyrase enzyme, which causes breakage of DNA. The breakage in DNA causes blocked replication forks, which in turn causes replication to halt, leading to bacteriostasis and eventually cell lysis (Smith, 1986, Kohanski et al., 2010). Mutations in the *gyrA* or *gyrB* gene can lead to an altered target site, reducing the affinity of the antibiotic to DNA gyrase (Pan *et al.,* 1996). Resistance can also occur when mutations in the *ParC* or *ParE* subunits of the topoisomerase IV enzyme lead to changes in the binding site (Pan *et al*., 1996). Cross-resistance to ciprofloxacin has been reported after exposure to tetracycline, which show *E. coli* demonstrating a ≥7 fold decrease in susceptibility to ciprofloxacin (Cohen et al., 1989). The induction of cross-resistance to ciprofloxacin in *E. coli*, is believed to occur due to reduction or loss of the OmpF porin, along with increases in the efflux system (Cohen et al., 1989). Cross-resistance to ciprofloxacin has also been

demonstrated in *P. aeruginosa*, where adaptive resistance was reported after exposure to BAC. This is believed to be due to an increase in efflux pumps (Pagedar et al., 2011).

1.5.2.4 Kanamycin

Kanamycin is an aminoglycoside which is used to treat *Staphylococcus*, *E. coli*, *H. pylori* and a number of Gram negative bacterial infections (Moore *et al.*, 1984). Kanamycin induces mistranslation of proteins through incorrect aminoacyl-tRNAs replacing correct ones in the translation process, leading to faulty protein production (Davis *et al.*, 1974). Resistance of kanamycin can occur via plasmid transfer of Tn5 (Jorgensen *et al.*, 1979), which contains the neomycin phosphotransferase I (*nptI*) and neomycin phosphotransferase II (*nptII*) gene, which encodes for the aminoglycoside 3'–phosphotransferase enzyme, inactivating kanamycin through phosphorylation (Leff *et al.*, 1993). Yamamoto *et al.,* (1988) demonstrated MRSA strains to exhibit cross-resistance to kanamycin, as well as BAC and CHX after plasmid pSAJ1 was integrated into the bacterial genome, which exhibits multidrug resistance.

1.5.2.5 Tetracycline

Tetracycline can be used to treat a number of infections including Lyme and rickettsial disease (Roberts, 1996); and has previously been used to treat gonorrhoea until resistance became widespread (Mendez *et al.*, 1980). Tetracycline may also be used to treat oral infections such as, gingivitis and periodontitis (Jeffcoat et al., 1982). Tetracycline is an effective antibiotic against both Gram negative and Gram positive organisms; halting protein synthesis in the bacterial cell by inhibiting aminoacyl-tRNA attachment to the ribosomal acceptor site (Chopra and Roberts, 2001). The binding of tetracycline to the aminoacyl-tRNA stops further amino acids being added to the polypeptide in translation, therefore halting any protein production by the bacterial cell, which is reversible once removed (Speer *et al.*, 1992). The uptake of tetracycline into the bacterial cells is energy dependant, based upon the ΔpH of the cell (Nikaido and Thanassi, 1993). Tetracycline remains in the cell as it is chelated to a magnesium-chelate form (THMg) which does not diffuse readily back through the phospholipid bilayer (Yamaguchi *et al.*, 1991). Resistance of tetracycline can occur through

a number of mechanisms, such as; transformation of the gene *tet*, which produces an enzyme with the ability to inactivate tetracycline (Speer and Salyers, 1989); ribosomal protection proteins Tet(M) or Tet(O) which either reduce binding of tetracycline to aminoacyl-tRNA or release tetracycline when in the aminoacyl-tRNA complex (Burdett, 1996); efflux pumps which transport substances out of the bacterial cell, rendering the cell undamaged (McMurry *et al.*, 1980). Evidence of cross resistance to tetracycline has been seen previously in *E. coli* after exposure to BAC (Langsrud et al., 2003). Langsrud *et al.,* (2003) demonstrated reduced sensitivity to tetracycline after exposure to BAC due to increased number of efflux pumps.

1.6 The Relevance of Biofilms to Microbicide Resistance

Biofilms are a naturally occurring accumulation of planktonic bacterial species which are ubiquitous. Studies into microbicide susceptibility changes in bacteria are typically undertaken using planktonic populations; however bacterial biofilms should be considered in investigations into microbicide susceptibility due to their recalcitrance. Biofilms reportedly occur in practically any non-sterile environment where sufficient water is present including domestic drains which harbour numerous bacterial species which are subjected to sporadic nutrients and wetting, as well as microbicides. Exposure to reduced concentrations of antimicrobial to the domestic drain biofilms have been suggested to represent an elevated risk for the selection of reduced microbicide susceptibility.

A biofilm is a complex microbial population that adheres to a surface and is encased in a protective matrix of extracellular polymeric substance (EPS). Biofilms normally form in a successive manner, initially planktonic bacterial cells attach to a surface by Van der Waals forces, the attachment is weak and if not developed further will revert back into planktonic cells (Kierek-Pearson and Karatan, 2005). The cells then become anchored on to the surface using cell adhesion structures, such as pili (Kierek-Pearson and Karatan, 2005), dividing and spreading upwards and outwards from the attachment point, creating micro-colonies. Further cells attach to the layer to form microcolonies which, if developed, can become a mature biofilm. Quorum sensing; the ability of the biofilm community to communicate with surrounding cells of the biofilm, may influence the development of the biofilm and, once the biofilm is stable

and large enough, cells begin to shed (Figure 1.2). In some situations and for certain species, the cells of the outer layer of the biofilm may disperse when conditions are no longer optimal for them, such as, nutrient depletion (Kierek-Pearson and Karatan, 2005). Biofilms are found in most non-sterile environments where water is present and are particularly problematic for hospital equipment (catheters) and cystic fibrosis suffers (Singh et al., 2000). Of relevance to this thesis, they are also found in kitchen drains (McBain et al., 2003b) and within washing machines (Legnani and Leoni, 1997).

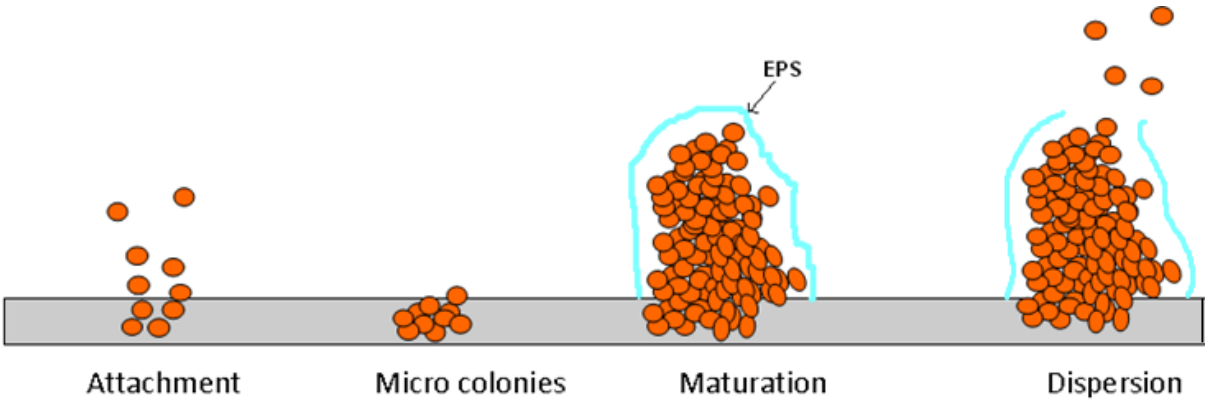

Figure 1.2 Biofilm formation, showing the mechanism of reversible and irreversible attachment, micro colonies formation, maturation and finally dispersion (Monroe, 2007). The orange ovals depict bacterial cells, while the blue line depicts EPS; the grey rectangle shows an attachment surface.

1.6.2 Biofilm: Extracellular Polymeric Substance (EPS)

During the attachment stage, biofilm structures produce substances which enable further planktonic cells to attach to the surface, extracellular polymeric substance (EPS) (Flemming and Wingender, 2001). It is believed this substance is extremely important when observing biofilm insusceptibility to antimicrobials. EPS is thought to act in a protective manner, shielding the bacterial cells within the biofilm and slowing the diffusion of antimicrobial substances to the cells and therefore the biofilm exhibits a resistance (Costerton and Ventullo, 1982). There is a longstanding theory that EPS may have the ability to inactivate antimicrobials when initially diffusing into the biofilm (Donlan, 2000), EPS may interact with the antimicrobial and possibly neutralise the bactericidal effects (Lindsay and von Holy, 2006). However, there is also evidence that catalase plays a role in diffusion of antimicrobials into

the bacterial cell (Stewart et al., 2000, Elkins et al., 1999). Stewart et al. (2000) demonstrated inability of hydrogen peroxide to penetrate *P. aeruginosa* cells which exhibited catalase activity, whilst mutants which did not demonstrate catalase activity, due to a knock-out of gene *katA*, were partially killed as the antimicrobial was able to penetrate the cells. The neutralisation of antimicrobials may also occur through the inhibition of enzymes, such as, β-lactamases and formaldehyde dehydrogenases, which have been shown to be concentrated around the anionic EPS and have the ability to deactivate β–lactam antibiotics and aldehydes (Giwercman et al., 1991). EPS enables the biofilm to exhibit varying physiological states, which could also have an effect on bacterial susceptibility towards microbicides.

1.6.3 Physiological Gradients in Biofilms

Biofilms are complex structures, which contain cells at various physiological states. Lack of nutrients and low oxygen levels can create survival pressures for the bacteria within the biofilm. Biofilms can contain several different bacterial species, as well as single species (Mah and O'Toole, 2001). Multi-species biofilms may exhibit varied rates of tolerance towards antimicrobials, due to the variation of species; for example dental plaque biofilms grown in the presences of sucrose can generate higher proportions of streptococci which are susceptible to chlorhexidine exposure, leading to a reduction in viable bacteria in comparison to biofilms grown in the absence of sucrose (Wilson et al., 1998). Susceptibility differences could be due to genotypic changes of the microorganisms when attaching onto a biofilm. Planktonic microorganisms express different proteins when compared to microorganisms in a biofilm colony; biofilm colonies up-regulate cell envelope proteins to help adhesion, which decreases microbicide permeation (Sakagami et al., 1989), while planktonic cells express proteins involved in proteases to aid with survival (Resch et al., 2005).

The bacteria residing in a biofilm may adapt to the external stimuli possibly creating a genetic trade-off that may delete or silence unwanted genome sequences (Gilbert *et al.*, 2002). Certain genetic traits are silenced or deleted in order to allow for more useful sequences to be expressed in the harsh environment of the biofilm (Davies and Geesey, 1995, Sauer et al., 2002). These changes can result in major changes to the biofilm, in order for it to

survive as a whole unit. The changes can also impact on susceptibility, enabling certain genes to be either down-regulated or upregulated affecting antimicrobial entrance to the cell (Maiques et al., 2006). The cellular organisation of a biofilm could mean that cells of the bacterial mass can grow at slower rates due to the challenging biofilm environment, and therefore the uptake of microbicides could decrease. Reduced nutrients and challenging environments can lead bacteria to remain dormant and halt all cellular activity, leading to a decrease in microbicide cellular entry. These dormant cells are known as persister cells, which are cells that do not actively divide (Keren et al., 2004). Keren *et al.* (2004) examined persister cells in biofilms and the underlying mutations that can lead to the development of persister cells. The study demonstrates that the mutant persisters were able to survive and exhibited a reduced susceptibility to ampicillin and ofloxacin than the wild type.

When planktonic bacteria are sub-lethally exposed to antimicrobials for a prolonged period of time, resistance may develop. This is theorised to be the same for biofilms, which exhibit changes in sensitivity towards antimicrobials. This concept has been challenged, with researchers showing that biofilms are merely more tolerant to antimicrobials due to their larger structure and limited nutrient diffusion, leading to a slower growth rate (Keren et al., 2004). Such phenotypic elements are believed to slow the uptake of antimicrobial substances by the biofilm, as it does not grow in the same logarithmic phase as planktonic cells do. The complex structure of biofilms allow protection against a variety of conventional disinfection methods (Yu and Mcfeters, 1994). Biofilms are less susceptible to naturally occurring bactericidal agents, such as, bacteriophages or antibiotics, as well as chemically made substances such as microbicides (Costerton et al., 1999).

1.6.5 Quorum Sensing

Quorum sensing is the communication of bacteria within a community via the release of secreted signalling molecules. *N*-acylhomoserine lactone (AHL) is an auto inducer molecule which is able to regulate quorum sensing within the biofilm in certain bacterial species (Dong et al., 2001). AHL is a gene regulator and when released it causes certain genes in the surrounding bacteria to become expressed or depressed, possibly leading to an

altered growth rate (Dong et al., 2001, Tuomanen et al., 1986). A slow growth rate is believed to be the reason biofilms exhibit tolerance towards many antimicrobial substances (Tuomanen et al., 1986). Quorum sensing effects biofilm development by producing signalling chemicals, which control the growth of the biofilm. This could therefore have an effect on biofilm susceptibility; if the microbicide is able to block, for example the production or detection of auto inducer molecules then biofilm maturation may be inhibited, in some cases, reduction of virulence factors of the bacteria, such as, azithromycin exposure to *P. aeruginosa* which causes blockage of quorum sensing (Hoffmann et al., 2007).

1.7 Effects of Microbicides on Biofilms

Since the turn of the 20th century, increasing studies have involved biofilms and the mechanism by which susceptibility changes towards antimicrobials take place. There has been research that shows bacteria to become decreasingly susceptible towards antimicrobials during prolonged sub-lethal exposure (Russell, 2000, Walsh et al., 2003b). Van der Veen and Abee (2011) demonstrated that both single and mixed species biofilms of *Listeria monocytogenes* and *Lactobacillus plantarum* exhibited reduced susceptibility to BAC, with mixed species biofilms demonstrating a higher level of resistance. Though the study did not focus on the mechanism of action of BAC, the resulting conclusion considered susceptibility of dual species biofilms to monospecies biofilms (Van der Veen and Abee, 2011). Mangalappalli-Illathu and Korber (2006) examined the effected continuous and intermitted exposure of sublethal concentrations of BAC have on single species biofilms of *Salmonella enterica* serovar Enteritidis. The results show that exposed biofilms retained viability after exposure to theoretically lethal concentrations of BAC (Mangalappalli-Illathu and Korber, 2006), demonstrating up-regulation of proteins used in defence enzymes, such as, thiol peroxidase and superoxide dismutase which are associated with oxidising stress response enabling resistance to oxidising microbicides (Cloete, 2003). Mah and O'Toole, (2001) have theorised that biofilms have the ability to become less susceptible towards antimicrobials than planktonic cells, due to the biofilm's complex structure (Mah and O'Toole, 2001), though this could be construed as a misconception.

Studies examining bacterial susceptibility changes towards microbicides have usually involved the exposure of pure cultures of bacteria to microbicides in aqueous solution. This is not comparable to the domestic environment where complex bacterial communities encounter microbicides in formulation with various excipients and surfactants. Domestic environments that are particularly densely colonised with bacteria and which are regularly exposed to microbicides include sinks and drains, and bacteria residing in these environments may be frequently exposed to microbicides in consumer products, possibly leading to the development of changes in susceptibility. Whilst the development of microbicide insusceptibility in bacteria has been shown in the laboratory, there is insufficient evidence to suggest that this will occur in the domestic environment. There have been few studies based around mimicking the realistic encounters that bacteria experience *in situ*, in terms of antimicrobial exposure. There are a limited number of studies that aim to provide a realism based approach to the development of microbicide insusceptibility, which are largely based around evaluating changes in bacteria that undergo microbicide exposure within a mixed species drain microcosm; however, these studies did not show a change in antimicrobial susceptibility in the domestic environment (McBain et al., 2003b). This differs from studies, which have shown single species laboratory grown biofilms to exhibit susceptibility changes after prolonged exposure (Fraud et al., 2008; Walsh et al., 2003a). These conditions are not representative of the conditions that the bacteria would be subjected to in nature; such as low nutrient levels, and constant variations in temperature or pH (Morikawa, 2006). However, the formation of biofilms *in vitro* within microcosms is a more realistic representation of bacteria in the domestic setting (Lopes *et al.,* 2012).

1.7.1 Biofilm Model Systems

The use of biofilm models has enabled a greater understanding of bacterial biofilms and the effects external stimuli have upon them. Methods of biofilm models can differ depending upon the nature of the project and the required results. A commonly used system is based upon microtiter plates, which enable bacterial biofilms to grow in the wells of the plate (Cerca et al., 2005) which can be used for crystal violet assays, on coupons (LeChevallier et

al., 1988) and on Calgary biofilm devices (CBDs) (Ceri et al., 1999) used for minimum biofilm eradication concentration methods. The microtiter systems are effective however, are subject to frequent changes due to nutrient depletion or accumulation of signalling molecules, if media is not changed regularly (Coenye and Nelis, 2010). The drip flow reactors are used to model system that have a close air-liquid interface and low fluid exposure, such as, catheters and oral cavity (Goeres et al., 2009). Also perfused biofilm fermenters which are used to examine growth rate and recalcitrance of bacterial biofilms (Gilbert et al., 2002).

Flow displacement systems allow media to be continuously fed to the bacteria, with waste being continuously removed. Such systems can comprise of: modified Robbins device (MRD) which have been used to analyse antimicrobial impregnated materials (van de Belt et al., 2001); Centres for Disease Control (CDC) biofilm reactor which have been previously used to examine high doses of antibiotics (daptomycin, moxifloxacin and vancomycin) against developed biofilms (Parra-Ruiz et al., 2010) and the constant depth film fermenter (CDFF). The CDFF enables growth of microcosms developed from pre-established drain biofilms (Wimpenny et al., 1983). Microcosms are laboratory models that simulate the physiological state of biofilms that are present *in situ* (Peters and Wimpenny, 1988). Microcosms are advantageous as they exhibit similar complexities of the original biofilm, as well as enabling the researcher the ability to manipulate specific aspects of the experimental model in the continuous culture model system. Continuous culture models, such as the CDFF, mimics environmental factors that *in situ* biofilms encounter; sporadic nutrients, irregular wetting and sub effective antimicrobial exposure (McBain et al., 2003c). The CDFF can be established to effectively mimic the environmental conditions found within a domestic drain (Figure 1.3). Media is fed through into the enclosed chamber, which is inoculated with bacterial biofilm isolated *in situ*. The bacteria grow into stable distinct microcosms on polytetrafluoroethylene (PTFE) pegs at a defined depth. CDFF has been used in a number of previous studies to produce consistent reproducible biofilm samples throughout the course of an antimicrobial exposure period (McBain et al., 2004, McBain et al., 2003b, McBain et al., 2003c).

McBain *et al.* (2003c) used CDFFs for growing drain microcosms from pre-established kitchen drain biofilms. The research from this paper led to further research into drain

microcosm susceptibility changes in relation to QACs (McBain et al., 2004). McBain *et al.,* (2004) demonstrated the improbability of antimicrobial resistance emerging from the use of QACs on domestic drain microcosms. The microcosm sample was taken from a pre-existing domestic drain biofilm that had infrequently been exposed to antimicrobial products, with the exception of bleach. The bacterial species residing in the microcosm were characterised and exposed to defined concentrations of the QAC over a prolonged period of time to determine the induction of any alteration in MIC. During pure culture experimentation, decreases in MICs were observed for *Ralstonia* sp., *Aranicola proteolyticus, and Chryseobacterium sp.,* whilst increases in MICs were seen for *Entercoccus saccharolyticus, Citrobacter* sp., *Pseudoxanthomonas* sp., and *Microbacterium phyllosphaerae*; which is believed to be due to cellular damage. The microcosms were subjected to sub-inhibitory concentrations of the QAC containing detergent for three months. The results did not indicate that the microcosms were developing significant changes in susceptibility to the microbicide, however suggested clonal expansion to lead to potential decreases in susceptibility.

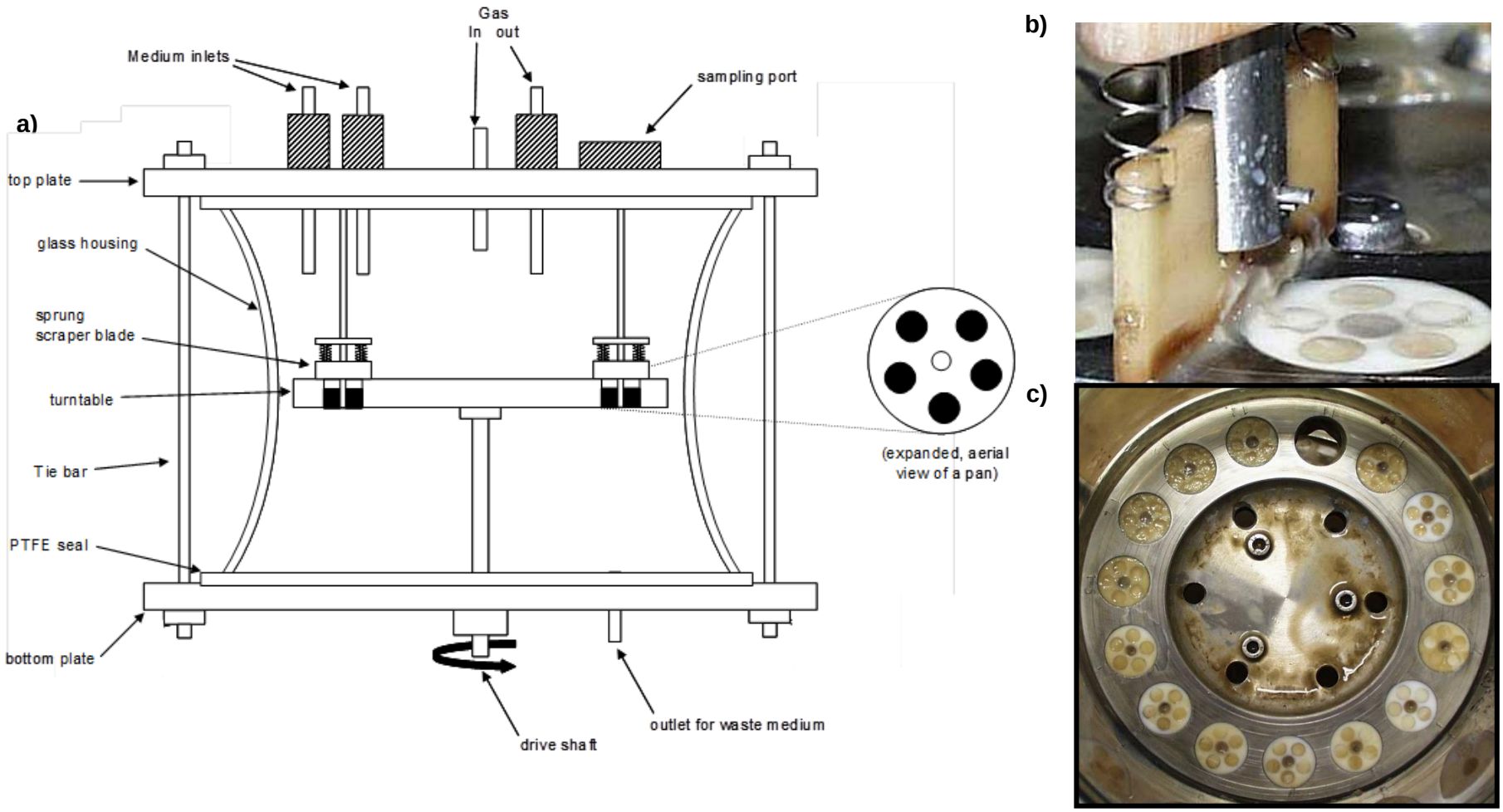

Figure 1.3 The Constant Depth Film Fermenter: (a) A diagrammatic depiction of the enclosed vessel, illustrating individual aspects of the model used for long-term, continuous culture. Biofilm depth can be approximately determined on the PTFE pans by adjusting the depth of the internal pegs. The model is inoculated through the sampling port and fed continuous media through medium inlets. PTFE scraper is used to remove over established microcosms to keep each distinct community at a constant depth (McBain, 2009); (b-c) microcosms forming on PTFE pegs, inserted in a rotating metal stage are kept at a constant depth by two scraper blades.

1.8 Formulation Effects

There have been a number of studies researching the influence of antimicrobials on bacterial resistance, especially in connection with antibiotics (Chuanchuen et al., 2001, Nordmann et al., 2007, Schweizer, 2001). It is well documented that the misuse of antibiotics has led to an increase in bacterial resistance (Nordmann et al., 2007). There is now an increasing concern that overuse of microbicides could cause the same effect on bacteria, if the modes of action are similar. The increasing overexposure of microbicides to microorganisms does increase the chances of mutation occurring, however with exposure concentrations of the majority of studies being lower than the recommended dosage or in-use concentration of microbicides (Suller and Russell, 2000), the likelihood of the microorganism becoming resistant is less likely than first considered.

The majority of household cleaning products are not a pure form of microbicide. The products contain: dyes, in order to make the formulation appealing towards consumers, as well as, surfactants and sequestrants which have antimicrobial properties and are often used as a preservative for products (Vareltzis et al., 1997). These additional chemical components are usually over-looked when considering antimicrobial susceptibility towards these antimicrobial containing consumer products. There are differences in pH levels between products, for example most hand sanitizers are pH 7, while toilet cleaners are more acidic and a few surface cleaners are alkali (Dyer et al., 1998, Jiliang, 2001). Such differences in pH levels can enable microbicides to work more effectively, such as CHX, which is more effective at an alkali pH (Russell, 1986).

Surfactants and sequestrants are compounds with the ability to preserve products and increase bioavailability in formulated products. Surfactants are used in a variety of products, such as; motor oil, medication and detergents (Rosen and Kunjappu, 2012). Surfactants, such as alcohol ethoxylates, are able to reduce surface tension of bacteria and potentially increase efficacy of microbicides (White, 1993). Alcohol ethoxylates increase the permeability of bacterial cells by disruption of the membrane, which leads to leakage of potassium ions (Moore *et al.*, 2006). Sequestrants have been used in toothpastes (Wulknitz, 1997), household

cleaners and in the food industry (Stanga, 2010). Ethylenediaminetetraacetate (EDTA) is a known sequestrant which has demonstrated antimicrobial properties, and is known as a chelating agent (Vareltzis et al., 1997). As a chelating agent, the bacterial membrane is disrupted due to the destabilisation of the outer membrane as a result of the binding and removal of cations from the membrane by the chelator (Haque and Russell, 1974). Considering formulation effects on bacteria would therefore enable a more detailed understanding of the effects of microbicide *in situ*. The sequestrant used in this thesis is sodium tripolyphosphate (0.5%), and the surfactant used in this thesis is Neodol 91-5 (3%).

1.9 Aims and Objectives

Previous studies have evaluated induced changes in bacterial susceptibility after exposure to microbicides (Cookson et al., 1991, Russell, 2004). However, this does not realistically recreate the complex exposure conditions that occur between bacteria and microbicides in the domestic environment. This doctoral project focuses on the effects of repeated antimicrobial usage on domestic bacterial communities *in vitro.* The impact of repeated bacterial exposure to antimicrobials and antimicrobial containing formulations will be assessed. This will include; analysis of changes in bacterial community dynamics, antimicrobial susceptibility profiles and bacterial fitness after antimicrobial exposure in both pure culture experiments and mixed consortium microcosms. Through this study the aim is to develop and validate methods to realistically assess the effects of exposing bacteria to microbicides in a domestic setting.

Chapter 2

General Experimental Methods

2.1 Growth Media

Unless otherwise stated, all growth media were purchased from Oxoid Ltd. (Basingstoke, UK) and chemicals from Sigma-Aldrich (Dorset, UK). Growth media included BBL™ Columbia Colistin Nalidixic acid agar (CNA; containing colistin at 0.015 g/L and nalidixic acid at 0.01 g/L) with 5% defibrillated sheep blood added (Scientific Laboratory Suppliers, Nottingham, UK); MacConkey agar (MK); *Pseudomonas* agar containing C-F-C supplement (cetrimide 10 mg/L, fucidin, 10 mg/L, cephalosporin 50 mg/L) (PA); Tryptone Soya broth (TSB); Tryptone Soya agar (TSA); Wilkins-Chalgren broth (WCB) containing 2.5% sucrose; Wilkins-Chalgren agar (WCA). Bacterial growth media was sterilized at 121°C and 15 psi for 15 min prior to use.

2.2 Antimicrobial Compounds

The antimicrobial compounds used were chlorhexidine acetate (CHX) (v/v), triclosan, thymol and benzalkonium chloride (BAC). These were purchased from Sigma-Aldrich (Dorset, UK). Didecyldimethyl ammonium chloride (DDAC) 50% solution in 2-propanol/water (2:3) was obtained from Merck Millipore (Durham, UK). Glydant (DMDM hydantoin) 54% (v/w) was obtained from Lonza Ltd. (Bishops Stortford, UK). Vantocil™ 20% (v/v) aqueous solution of polyhexamethylene biguanide (PHMB) was obtained from Arch Chemicals Inc. (Manchester, UK). A general purpose cleaner (GPC) contained 3% Neodol 91-5 (non-ionic surfactant), 0.5% sodium tripolyphosphate (STP-P) (sequestrant) and 0.03% Sodium Carbonate. Main Wash Laundry Detergent with benzisothiazolinone (BIT) and Main Wash Laundry Detergent (0.0066% triclosan) were obtained from Unilever (Port Sunlight, UK). All microbicides were tested in aqueous solution and in formulation, at concentrations reflective of their normal deployment in consumer products. BAC, CHX, DDAC, DMDM hydantoin, PHMB and thymol were prepared at 1% (v/v) in a general purpose cleaner. Triclosan was formulated into a laundry detergent at 0.0066% (w/v). Benzisothiazolinone (BIT) was formulated into a laundry detergent at 0.02% (v/v). Each of the actives were diluted using sterile distilled water and kept refrigerated for 14 days before the actives were remade. Thymol and triclosan were adjusted

to the correct concentration levels using 70% ethanol (1 ml) with 1 g of active and then diluted to the correct concentration using sterile distilled water.

2.3 Bacteria

Escherichia coli ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538 were obtained from Oxoid (Basingstoke, UK). *Enterococcus faecalis* MRBG15.6 was donated from Dr. Angela Oates at The University of Manchester isolated from an infected wound. The isolates obtained from the drain biofilm collected from a pre-established kitchen drain (Manchester, UK) were *Acinetobacter baumannii* MBRG15.1, *Pseudomonas putida MBRG15.2, Moraxella osloensis* MBRG15.3, *Escherichia coli* MBRG15.4*,* and *Cronobacter sakazakii* MBRG15*.* Species were identified using 16S rRNA gene sequencing. Bacterial cultures were grown overnight at 37^{o}C in WCB containing 2.5% sucrose for *A. baumannii, C. sakazakii* and *M. osloensis* and TSB for other bacteria. These were then placed into 2 ml Eppendorf tubes along with 50% glycerol and kept at -80^{o}C as stocks. *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538, and *Escherichia coli* ATCC 25922 were obtained from Oxoid (Basingstoke, United Kingdom). *Enterococcus faecalis* MBRG15.6 was provided by Angela Oates, The University of Manchester.

2.4 16S rRNA Gene Sequencing

Single pure colonies of morphologically distinct bacteria were aseptically removed from the isolation plates and added to 100 µl of sterile polymerase chain reaction (PCR) water in a sterile Eppendorf tube (0.2 ml PCR tube). The tubes were then heated to 100^{o}C (10 min) to lyse the cells. The tubes were centrifuged at 13,500 rpm (10 min) to pellet cell debris and 90 µl of supernatant was transferred to a sterile Eppendorf tube. A PCR mixture was made containing 25 µl of MyTaq DNA polymerase ready mix (Bioline Reagents Ltd, London, UK), 1 µl of 8 FLP1 (5’ – GAGTTTGATCCTGGCTCAG – 3’) (Weisburg et al., 1991) and 1 µl of 806R (5’ – GGACTACCAGGGTATCTAAT – 3’) (Wilson et al., 1990), 18 µl PCR water and 5 µl of the DNA template was placed into a PCR tube (0.2 ml). T Gradient Thermocycler (Biometra,

Glasgow, UK) was used to run 35 thermal cycles: 94°C (1 min), 53°C (1 min), 72°C (1 min) and the final cycle incorporates a final 15 minute chain elongation step. QIAquick PCR purification kit (Qiagen, West Sussex, UK) was used to purify the DNA, according to the manufactures protocol. A NanoDrop lite UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA), was used to quantify the amount of DNA present in each sample following the PCR. The samples were then transferred to a 1% agarose gel and ran against a Hyperladder marker IV (Bioline Reagents Ltd, London, UK) to ensure the samples are present and pure.

2.5 Agarose Gel Electrophoresis

A 1% agarose gel was prepared by using 80 ml of Tris-acetate-ethylenediaminetraacetic acid (TAE) buffer (50 x stock: 40 mM Tris base, 20 mM glacial acetic acid and 1 mM EDTA, pH 8) to dissolve 0.8 g of agarose powder. Visualisation of the sequences under ultra violet (UV) light (312 nm) is detected by the addition of 5 µl of GelRed™ (Biotium, Middlesex, UK). The mixture is poured into an electrophoresis tank (Bio-Rad, Hempstead, UK), which is allowed to set for approximately 1 hr. The samples from the PCR (3 µl) were loaded into the gel, with loading buffer (62.5 mM Tris-HCl, 2% (w/v) SDS, 10% glycerol and 0.01% (w/v) bromophenol blue) and run for approximately 1 hr at 70v. A Hyperladder marker IV (Bioline Reagents Ltd, London, UK) was used as a marker to determine the band sizes of the samples. Once the run was completed, the gel was examined under a UV light using a T2201 transilluminator (Sigma, Poole, UK).

2.6 Determining Minimum Inhibitory Concentrations (MICs)

MICs were determined as the lowest concentration whereby no visible bacterial growth appeared in broth. Bacterial cultures were grown on TSA for 18-24 h at 37^{o}C before single colonies were aseptically removed and grown in broth (TSB/ WCB with 2.5% sucrose) for 18-24 h at 37^{o}C. The optical density (OD) of all the bacteria were measured at 600nm and adjusted to an OD of 0.8 (3.2×10^{6} – 6.8×10^{8} cfu/ml). The cultures were then diluted 1:100 in fresh broth, 120 µl of which was placed into 96 well plates. A range of 0.2 - 1000 mg/L of

BAC, BIT, BIT formulation, CHX, DDAC, GPC, PHMB and triclosan (active and formulation); 94 - 12000 mg/L of thymol and glydant (in aqueous solution and in formulation) microbicides were made and placed into the first row (120 µl) with the bacterial culture. Doubling dilution series were carried out down the plate. Columns 1 and 2 were negative and positive controls respectively; negative controls consisted of fresh broth containing antimicrobial compound, while the positive control contained bacterial culture with broth. Thymol and triclosan contained ethanol controls, whereby 10% of ethanol was placed into broth and bacterial culture to enable the ethanol did not affect the bacteria contained in the cultures. Each bacterium was tested against each microbicide in triplicate, along with a biological replicate. The plates were placed into a shaker incubator for 18-24 h at 37^{o}C. Growth was viewed as turbidity (600nm) in comparison to an uninoculated well (negative control) and was detected using a microtiter plate reader (PowerWave XS; BioTek, Bedfordshire, UK).

2.7 Minimum Bacterial Concentrations (MBC)

MBCs were determined as the lowest concentration whereby no visible bacterial growth appeared in agar. Bacterial cultures were grown on TSA for 18-24 h at 37^{o}C before single colonies were aseptically removed and grown in broth (TSB/ WCB with 2.5% sucrose) for 18-24 h at 37^{o}C. The optical density (OD) of all the bacteria at 600 nm were measured and adjusted to an OD of 0.8 (3.2 x 10^{6} – 6.8 x 10^{8} cfu/ml). The cultures were then diluted 1:100 in fresh broth, 120 µl of which was placed into 96 well plates. A range of 0.2 - 1000 mg/L of BAC, BIT, BIT formulation, CHX, DDAC, GPC, PHMB and triclosan (active and formulation); 94 - 12000 mg/L of thymol and glydant (active and formulation) test microbicides were made and placed into the first row (120 µl) with the bacterial culture. Doubling dilutions were carried out down the plate. Negative controls consisted of fresh broth containing active, while the positive control contained bacterial culture with broth. Thymol and triclosan contained ethanol controls, whereby 10% of ethanol was placed into broth and bacterial culture. Each bacterium was tested against each microbicide in triplicate, along with a biological replicate. The plates were placed into a shaker incubator for 18-24 h at 37^{o}C. MBCs were determined by removing 5 µl from the microtitre plates and spotted onto TSA plates in

triplicate. This was grown aerobically for 4 days at 37°C. The MBC was defined as the lowest concentration of microbicide at which no growth occurred after 4 days of incubation.

2.8 Minimum Biofilm Eradication Concentrations (MBEC)

MBECs were determined as the lowest concentration whereby no visible bacterial growth appeared in broth. Calgary biofilm devices (CBDs) (Ceri et al., 1999) were used to create single bacterial biofilms. CBDs consist of plastic pegs, which protrude into NUNC Immunoassay Transferable Solid Phase (TSP) plates. Bacterial cultures were grown in broth (TSB/ WCB with 2.5% sucrose) for 18-24 h at 37°C. The OD_{600nm} of all the bacteria were taken and adjusted to an OD of 0.8 ($3.2 \times 10^6 – 6.8 \times 10^8$ cfu/ml). The cultures were then diluted 1:100 in fresh broth, 150 µl of which was placed into NUNC-TSP with the CBDs being placed on top of the plates. The plates were then left to grow aerobically for 48 h at 37°C, to allow the biofilms to form. After 48 h the lid was removed and the biofilms were rinsed twice in 200 µl of PBS in sterile wash plates to remove any remaining planktonic organisms before being exposed to the antimicrobials. A range of 0.2 - 1000 mg/L of BAC, BIT, BIT formulation, CHX, DDAC, GPC, PHMB and triclosan (active and formulation); 94 - 12000 mg/L of thymol and glydant (active and formulation) microbicides were made and placed into the first row (160 µl) with 160 µl of fresh broth, to account for the air/ surface interface of a fresh NUNC-TSP plate. Doubling dilution series was carried out down the plate. Negative controls consisted of fresh broth containing active, while the positive control contained bacterial culture with broth. Thymol and triclosan contained ethanol controls, whereby 10% of ethanol was placed into broth and bacterial culture to enable the ethanol did not affect the bacteria contained in the cultures. Each bacterium was tested against each microbicide in triplicate, along with a biological replicate. The CBDs were placed into the plates and placed into an incubator for 18-24 h at 37°C. Fresh broth (170 µl) was placed into fresh sterile plates and the CBDs were placed into these plates and for 72 h at 37°C.

2.9 Repeated Exposure of Bacteria to Microbicides

A Wasp II™ spiral plater (Whitley Automated Spiral Plater, Don Whitley Scientific, Shipley, UK) was used to dispense Archimedes spirals at a 100-fold concentration gradient across a rotating TSA plate. Microbicide solutions were made (1 mg/ml) and deposited onto the agar plates using the spiral plater; this was then left to dry for 1 h at room temperature. Bacteria were streaked across the diameter of the spiral, from the lowest concentration of microbicide to the highest. The plates were then inverted and grown aerobically for 72 h at 37°C. Growth occurring at the highest concentration was aseptically removed and streaked across a freshly spiralled plate. If growth had occurred across the whole diameter, the removed bacterial culture was placed onto a freshly spiralled plate with a 10-fold increase in concentration (10 mg/ml), up to 100 mg/ml. This process was repeated until 14 passages, or until sensitivity changes no longer occurred. Every 7 passages stocks of the bacterial cultures were taken and stored at -80°C, as well as carrying out further MICs and MBCs of the bacteria. MBECs were determined again at passage 14.

2.10 Repeated Exposure of Bacteria in the Absence of Microbicides

Bacterial species demonstrating a 2-fold change to MIC when trained, underwent detraining which consists of radially inoculating the bacteria across TSA plates in the absence of antimicrobials. The plates were placed into a 37°C incubator for 48 h. Bacteria was aseptically removed and streaked onto a fresh plate. This process was repeated for 14 passages, after which MICs, MBCs and MBEC of all bacteria was taken, as well as stocks.

2.11 Planktonic Growth Rate

The unexposed (P0), exposed (P14) and detrained (X14) bacteria were grown in broth cultures overnight. The OD_{600nm} of the bacteria was adjusted to 0.8 and diluted 1 in 100 in TSB/WCB (5 ml). The cultures were deposited into 96 well plates in triplicate and placed into a microplate spectrophotometer (PowerWave™ XS, BioTek, Swindon, UK). Using the Gen5™1.08 software (BioTek, Bedfordshire, UK), the plate was read every hour for 18 h to determine the planktonic growth rate of bacteria.

2.12 Crystal Violet Biofilm Assay

The assay was adapted from O'Toole (2011) to determine the growth of biofilms. Overnight bacterial cultures were diluted to an optical density of 0.8 and then further diluted 1:100 in broth (TSB/ WCB with 2.5% sucrose); aliquots of diluted inoculum (150 µl) were placed into 96 well microtitre plates and were incubated at 37^{o}C for 48 hrs (Corning Ltd., Corning, NY). After incubation media was aspirated and the wells were washed in 2 x 200 µl phosphate buffered saline (PBS) (Oxoid Ltd, Basingstoke, UK). A solution of 0.5% (200 µl) crystal violet was added to each well and plates were incubated at room temperature for 30 minutes. Plates were washed in 2 x 200 µl PBS and left to dry at room temperature for 1 h prior to solubilisation of the crystal violet in 250 µl of 70% ethanol using agitation (20 rpm for 1 h). OD_{600nm} was determined for each well relative to a sterile control using a plate reader (Powerwave XS) and Gen5™1.08 software (BioTek, Bedfordshire, UK) Biofilm formations were classed as an increase in OD_{600nm} relative to the sterile control. Replicates were done in triplicate, with 2 biological replications.

Chapter 3

The Effect of Formulation on Microbicide Potency and Mitigation of the Development of Bacterial Insusceptibility

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The Effect of Formulation on Microbicide Potency and Mitigation of the Development of Bacterial Insusceptibility

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3.0 Abstract

Background: Risk assessments of the potential for microbicides to select for reduced bacterial susceptibility have been based largely on data generated through the exposure of bacteria to microbicides in aqueous solution. Since microbicides are normally formulated with multiple excipients, we have investigated the effect of formulation on antimicrobial activity and the induction of bacterial insusceptibility.

Methods: We tested 8 species of bacteria (7 genera) before and after repeated exposure (14 passages), using a previously validated gradient plating system, for their susceptibilities to the microbicides benzalkonium chloride, benzisothiazolinone, chlorhexidine, didecyldimethyl ammonium chloride, DMDM-hydantoin, polyhexamethylene biguanide, thymol, and triclosan in aqueous solution (non-formulated) and in formulation with excipients often deployed in consumer products. Susceptibilities were also assessed following an additional 14 passages without microbicide to determine the stability of any susceptibility changes.

Results: Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBC) were on average 11-fold lower for formulated microbicides than for non-formulated microbicides. After exposure to the antimicrobial compounds, of 72 combinations of microbicide and bacterium there were 19 ≥4-fold (mean, 8-fold) increases in MIC for non-formulated and 8 ≥4-fold (mean, 2-fold) increases in MIC for formulated microbicides. Furthermore, there were 20 ≥4-fold increases in MBC (mean, 8-fold) for non-formulated and 10 ≥4-fold (mean, 2-fold) increases in MBC for formulated microbicides. Susceptibility decreases fully or partially reverted back to pre-exposure values for 49% of MICs and 72% of MBCs after further passage.

Conclusion: In summary, formulated microbicides exhibited greater antibacterial potency than unformulated actives and susceptibility decreases after repeated exposure were lower in frequency and extent.

3.1 Introduction

Microbicides are broad-spectrum chemical agents that inactivate microorganisms (Müller and Kramer, 2008, Escalada et al., 2005, McBain et al., 2004). They are widely deployed throughout healthcare (Kampf and Kramer, 2004, Brady et al., 1990, Zafar et al., 1995), domestic (Levy, 2001, Larson et al., 2004) and industrial environments (Pereira et al., 2001, Holah et al., 2002, Rosenthal, 1982) where their application includes antisepsis (Koburger et al.), hard surface disinfection (Rusin et al., 1998) and pharmaceutical product preservation (Patrone et al., 2010). They may also be incorporated into medical device coatings, for instance in sutures (Barbolt, 2002), wound dressings (Silver et al., 2006) and urinary catheters (Gaonkar et al., 2003) to inhibit bacterial adhesion and subsequent biofilm formation.

It has been hypothesized that the use of microbicides could select for bacterial adaptation, resulting in reduced efficacy of the primary agent, as well as potentially decreasing bacterial susceptibility to chemically-unrelated agents such as other microbicides and antibiotics (Chuanchuen et al., 2001). Whilst there have been reports documenting the laboratory selection of bacteria with decreased microbicide sensitivity following repeated exposure to microbicides in highly selective conditions, it remains unclear whether this commonly occurs in the environment (Karatzas et al., 2007, Moore et al., 2008, Mc Cay et al., 2010, Maillard et al., 2013, Walsh et al., 2003b, McBain and Gilbert, 2001).

The majority of studies reporting reductions in microbicide susceptibility have used the active compound in aqueous solution with or without the addition of co-solvents such as dimethyl sulfoxide (DMSO) (Forbes et al., 2014) or ethanol (Mechin et al., 1999, Ledder et al., 2006). In real use however, microbicides are deployed in formulated products with multiple excipients, which may enhance potency. The potential effect of the formulation of microbicides on reducing the development of bacterial insusceptibility has received little research attention. Furthermore, despite the research effort that has been directed toward the possible risk of induced microbicide insusceptibility, the stability of such susceptibility changes has been investigated infrequently (McBain and Gilbert, 2001).

With the ultimate aim of developing realism-based approaches to risk assessment, the current investigation evaluates the frequency, magnitude and reversibility of susceptibility changes that may be induced by the repeated exposure of a range of bacteria to microbicides in aqueous solution or in formulation. The microbicides selected reflect those frequently used in consumer products such as laundry detergents, hard surface disinfectants and personal care products. Planktonic susceptibilities (MIC, MBC) and minimum biofilm eradication concentrations (MBEC) were determined before and after repeated exposure to sub-lethal concentrations of the microbicides; benzalkonium chloride (BAC), benzisothiazolinone (BIT), chlorhexidine (CHX), didecyldimethyl ammonium chloride (DDAC), glydant (DMDM hydantoin), polyhexamethylene biguanide (PHMB), thymol, and triclosan in aqueous solutions and in formulation with commonly used sequestrants and surfactants. Bacteria were also passaged further in the absence of any antimicrobial to determine the stability of any observed change in susceptibility.

3.2 Materials and Methods

3.2.1 Bacteria

Pseudomonas aeruginosa ATCC 9027*, Staphylococcus aureus* ATCC 6538*,* and *Escherichia coli* ATCC 25922 were obtained from Oxoid (Basingstoke, United Kingdom). *Acinetobacter baumannii* MBRG15.1*, Pseudomonas putida* MBRG15.2, *Moraxella osloensis* MBRG15.3, *Escherichia coli* MBRG15.4, and *Cronobacter sakazakii* MBRG15.5*,* were isolated from a domestic kitchen drain biofilm. *Enterococcus faecalis* MRBG15.6 is a wound isolate provided by Angela Oates, The University of Manchester.

3.2.2 Chemical Reagents and Bacterial Growth Media

Bacteriological growth media were purchased from Oxoid (Basingstoke, UK). All other chemical reagents were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise stated. Bacterial growth media were sterilized at 121°C and 15 psi for 15 min prior to use. *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli* and *Enterococcus faecalis* were cultured on Tryptone Soya agar and broth. *Acinetobacter baumannii, Pseudomonas putida*, *Moraxella osloensis* and *Cronobacter sakazakii* were grown on Wilkins

Chalgren agar and broth containing 2% sucrose. All bacteria were incubated aerobically at 37˚C for 18h unless stated otherwise. Antimicrobial compounds consisted of benzalkonium chloride (BAC), chlorhexidine (CHX), thymol and triclosan were purchased from Sigma-Aldrich (Dorset, UK). Didecyldimethyl ammonium chloride (DDAC) (50% [v/v]) was purchased from Merck Millipore (Durham, UK). Vantocil (a 20% [v/v] aqueous solution of PHMB) was obtained from Arch Chemicals, Inc. (Manchester, UK). Glydant (DMDM hydantoin) was obtained from Lonza (Bishop's Stortford, UK). All microbicides were tested in aqueous solution or added to microbicide-free formulation chassis containing sequestrants and surfactants, at concentrations reflective of their normal deployment in consumer products. Specifically, BAC, CHX, DDAC, DMDM hydantoin, PHMB and thymol were prepared at 1% (v/v) in a general purpose cleaner. Triclosan and benzisothiazolinone (BIT) were formulated into a laundry detergent at 0.0066% and 0.02% (wt/vol), respectively.

3.2.3 Exposure of Bacteria to Sub-lethal Concentrations of Microbicides as active and formulation

A previously validated system (Moore et al., 2008, Forbes et al., 2014) was used to generate reproducible ~ 100-fold-concentration gradients of the antimicrobial compounds on Tryptone Soya agar plates using a spiral plater (Whitley Automated Spiral Plater, Don Whitley Scientific, Shipley, UK). Antimicrobials in aqueous solution or formulation (50µl) were deposited on the agar surface in an Archimedes spiral using the spiral plater. Plates were dried for 1 h at room temperature prior to radial deposition of bacterial pure cultures and then incubated (4 days; 37˚C) in an aerobic incubator. After incubation, growth observed at the highest microbicide concentration was aseptically removed and streaked onto a fresh plate containing the same antimicrobial concentration gradient. Where growth was observed across the whole antimicrobial gradient, a new plate produced with 5-fold-higher concentration was used (McBain and Gilbert, 2001). This process was repeated until 14 passages had occurred (P14). Bacteria that exhibited ≥4-fold changes in MIC, MBC or MBEC were then passaged a further 14 times in the absence of any antimicrobial compound (X14) to ascertain the stability of adaptation. Bacteria at P0 (unexposed), P14 and X14 were archived for subsequent MIC

and MBC testing. Susceptibility testing (MIC, MBC, MBEC) was performed in two separate experiments each with three technical replicates.

3.2.4 Determination of bacterial Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC)

MIC values were determined using the microdilution method as described previously (Stephens et al., 2012). Briefly, overnight bacterial cultures were adjusted to an optical density at 600 nm (OD_{600}) of 0.8 and diluted 1 in 100 in Tryptone Soya broth or Wilkins Chalgren broth with 2% sucrose in a 96-well microtiter plate containing doubling dilutions of the relevant microbicide. Plates were incubated at 37ºC (24 h) with agitation (100 rpm). The MIC was defined as the lowest concentration for which bacterial growth did not occur. Growth was viewed as turbidity (600 nm) in comparison to an uninoculated well (negative control) and was detected using a microtiter plate reader (PowerWave XS; BioTek, Bedfordshire, UK). MBCs were determined as described previously (Forbes et al., 2014), in brief aliquots (10µl) from wells exhibiting no turbidity were transferred to sterile Tryptone Soya agar or Wilkins Chalgren agar prior to 4 days incubation at 37ºC to determine the MBC (Forbes et al., 2014). The MBC was defined as the lowest concentration of microbicide at which no growth occurred after 4 days of incubation.

3.2.5 Determination of Minimum Biofilm Eradication Concentrations

Single species biofilms were grown on the pegs of a Calgary Biofilm Device (CBD) (Ceri et al., 1999). To produce inocula for biofilm susceptibility testing, single colonies of test bacteria were inoculated into 10ml of sterile Tryptone Soya Broth or Wilkins Chalgren Broth with 2% sucrose and incubated at 37˚C in a shaking aerobic incubator (100 rpm) for 18 h. Cultures were diluted to an OD_{600} of 0.8, then further diluted 1:100 using fresh growth medium. Next, 100µl of bacterial inoculum was added to each well of the CBD base, and the plates were then incubated at 37˚C and 30 rpm for 48 h to allow biofilm formation on the pegs. Doubling dilutions for microbicides (150µl) were prepared in sterile broth across a 96 well microtiter plate. Biofilms were exposed to antimicrobial compounds and incubated for 24 h at 37˚C and 100rpm. After incubation, the lid was transferred to a 96-well plate containing

200 µl of sterile broth and was incubated for 24 h at 37˚C and 100rpm. Minimum biofilm eradication concentrations (MBECs) were determined as the lowest concentration for which bacterial growth did not occur after 18 h of incubation. Growth was viewed as turbidity in comparison to an uninoculated well (negative control) and was detected using a microtiter plate reader at 600nm wavelength (BioTek, Bedfordshire, UK).

3.3 Results

Two main variables describe the data associated with the selection of decreased susceptibility by exposure to microbicides in the present study; (i) the frequency of susceptibility decreases of >2-fold (Forbes et al., 2015) for multiple test bacteria and microbicides and (ii) the extent of susceptibility changes for each combination of bacterium and microbicide.

Repeated exposure to the microbicide-containing formulations resulted in a lower frequency of susceptibility reductions than did exposure to the same microbicide in aqueous solutions, and where decreases in susceptibility did occur, these were generally smaller for formulated microbicides. All individual values for bacterial susceptibility before, during and after microbicide exposure have been given in Tables 3.1-8. However, due to the large number of combinations of bacterium and antimicrobial compounds that were tested, the extent of susceptibility has also been expressed as mean values in the following section.

After repeated exposure to unformulated microbicides there were 19 ≥4-fold increases in MIC (1 of which fully reverted back to pre-exposure values after subsequent passage in the absence of microbicide, 13 of which partially reverted and 5 which did not revert; average increase in MIC [P0 to P14] was 11-fold across the test panel of bacteria and microbicides). There were 20 increases in MBC (2 fully revertible, 11 partially revertible and 7 non-revertible; average 8-fold increase) and 17 increases in MBEC (7 fully revertible, 6 partially revertible and 4 non- revertible; average 4-fold increase) after microbicide exposure (Tables 3.1-8). After exposure to microbicide-containing formulations there were 8 ≥4-fold increases in MIC (2 fully revertible and 6 non-revertible; average 2-fold increase), 10 increases in MBC (3 fully revertible, 5 partially revertible and 2 non-revertible; average 2-fold increase) and 16 increases

in MBEC (5 fully revertible, 8 partially revertible and 3 non-revertible; average 3-fold increase) (Tables 1-8). In terms of antimicrobial potency, when comparing the formulated to non-formulated microbicides across the test panel of bacteria we saw ~ 11-fold lower MIC and MBC values and a 3-fold lower MBEC for the unexposed (P0) bacterial isolates. For the P14 isolates, we observed ~ 35-fold lower MIC, an ~ 36-fold lower MBC, and an ~ 4-fold lower MBEC (Tables 1-8).

3.3.1 Benzalkonium Chloride

All test bacteria, with the exception of *M. osloensis*, *C. sakazakii* and the *E. coli* drain isolate, exhibited a ≥4-fold increase in MIC after exposure to BAC (Table 3.1). Increases observed in MBC, whilst generally smaller than those in MIC, were also observed at ≥4-fold *for S. aureus, E. coli* and *P. aeruginosa*. Furthermore ≥4-fold increases in MBEC occurred for *S. aureus* and *E. faecalis* after BAC exposure. After growth in the absence of BAC, subsequent full or partial reversion in MIC, MBC or MBEC occurred for all test bacteria with the exception of *E. coli* and *P. aeruginosa* (MIC and MBC). In contrast, after exposure to the BAC formulation only *S. aureus*, *E. coli*, *P. aeruginosa* and *A. baumannii* showed a ≥4-fold increase in MIC with *S. aureus* and *E. coli* also demonstrating a ≥4-fold increase in MBC. *S. aureus*, *E. faecalis* and *P. aeruginosa* also exhibited a ≥4-fold increase in MBEC after exposure to BAC formulation. After recovery in the absence of BAC formulation only *S. aureus* demonstrated any reversion in susceptibility (MBEC). The MIC for BAC was reduced in *M. osloensis* at P14 compared to P0 when this bacterium was exposed to BAC in aqueous solution.

3.3.2 Benzisothiazolinone

No bacterium displayed a substantial change in susceptibility (≥4-fold MIC, MBC or MBEC) to BIT or to BIT formulation after long-term exposure to the respective agent (Table 3.2).

3.3.3 Chlorhexidine

After repeated exposure to chlorhexidine both *S. aureus* and *E. coli* showed ≥4-fold increases in MIC and MBC, which partially reverted in the absence of the microbicide (Table 3.3). *P. aeruginosa* demonstrated a ≥4-fold increase in MIC which did not revert after regrowth in a chlorhexidine free environment. *E. faecalis* and *M. osloensis* exhibited ≥4-fold increases in MBEC, which partially and fully reverted in the absence of chlorhexidine, respectively. In contrast, after exposure to chlorhexidine formulation no bacterium exhibited a ≥4-fold decrease in susceptibility at MIC, MBC or MBEC level.

3.3.4 Didecyldimethyl Ammonium Chloride

After repeated DDAC exposure *P. aeruginosa, A. baumannii* and the *E. coli* drain isolate exhibited a ≥4-fold increase in MBC, out of which *P. aeruginosa* fully reverted, whilst *A. baumannii* and *E. coli* partially reverted following repeated growth in the absence of DDAC. *S. aureus. E. coli, E. faecalis* and the *E. coli* drain isolate all exhibited a ≥4-fold increase in MBEC, out of which *E. faecalis* and the *E. coli* drain isolate partially reverted, *E. coli* fully reverted and *S. aureus* did not revert back to pre-exposure values after growth in the absence of the microbicide (Table 3.4). After exposure to the DDAC-containing formulation, *P. aeruginosa* and the *E. coli* drain isolate exhibited a ≥4-fold increase in MBC, out of which *E. coli* partially reverted and *P. aeruginosa* fully reverted after passage in an antimicrobial free environment. In agreement with the changes in MBEC observed after exposure to DDAC active, *S. aureus, E. coli, E. faecalis* and the *E. coli* drain isolate also showed a ≥4-fold increase in MBEC after exposure to DDAC formulation. MBEC values partially reverted for both *E. coli* isolates and for *E. faecalis* but did not revert for *S. aureus* after recovery in the absence of DDAC.

3.3.5 Glydant (DMDM Hydantoin)

The *E. coli* drain isolate exhibited a ≥4-fold increase in MBC after repeated exposure to DMDM hydantoin; this susceptibility decrease fully reverted in the absence of the microbicide (Table 3.5). Comparatively, after exposure to DMDM hydantoin formulation both

E. coli isolates and *C. sakazakii* showed a ≥4-fold increase in MBEC, all of which fully reverted in an antimicrobial-free environment.

3.3.6 Polyhexamethylene Biguanide

S. aureus, E. faecalis, M. osloensis and *A. baumannii* exhibited a ≥4-fold increase in MIC after PHMB exposure out of which *M. osloensis* and *A. baumannii* fully reverted and *S. aureus* and *E. faecalis* partially reverted after growth in the absence of PHMB (Table 3.6). *S. aureus, E. coli, P. aeruginosa, E. faecalis,* and the *E. coli* drain isolate demonstrated a ≥4-fold increase in MBC, out of which *S. aureus*, *E. faecalis* and the *E. coli* drain isolate showed partial reversion and *E. coli* and *P. aeruginosa* showed no reversion to pre-exposure values in the absence of PHMB. After PHMB exposure, *S. aureus, E. faecalis, A. baumannii, C. sakazakii* and the *E. coli* drain isolate also displayed a ≥4-fold increase in MBEC, which fully reverted for *S. aureus, A. baumannii* and *E. coli* drain isolate, and partially reverted for *E. faecalis* and *C. sakazakii* after regrowth in the absence of PHMB. After exposure to PHMB formulation *S. aureus, E. faecalis* and *P. aeruginosa* showed substantial changes in their PHMB susceptibility displaying ≥4-fold increases in MBC, all of which fully or partially reverted in the absence of the antimicrobial formulation. *S. aureus* and *E. faecalis* also exhibited a ≥4-fold increase in MBEC after exposure to PHMB formulation, all of which partially reverted back to pre-exposure values after regrowth in the absence of the formulation.

3.3.7 Thymol

After long-term thymol exposure, none of the bacterial isolates showed a ≥4-fold decrease in thymol susceptibility at MIC, MBC or MBEC level (Table 3.7). After exposure to the thymol-containing formulation, *E. coli* and *A. baumannii* both underwent ≥4-fold increases in MBC whilst *P. putida* demonstrated a ≥4-fold increase in MIC and MBC, all of which partially reverted in the absence of thymol formulation. Furthermore, both *E. coli* isolates showed a ≥4-fold increase in MBEC, which partially reverted after growth in the absence of thymol formulation. The MBC for thymol was reduced in *P. aeruginosa* at P14 compared to P0 when this bacterium was exposed to thymol in aqueous solution.

3.3.8 Triclosan

All bacterial isolates, with the exception of *E. faecalis, A. baumannii* and *P. aeruginosa*, which is non-susceptible to triclosan, demonstrated an increase in MIC after repeated triclosan exposure, none of which fully reverted back to pre-exposure levels after regrowth in the absence of triclosan (Table 3.8). All isolates apart from *P. aeruginosa, A. baumannii* and *P. putida* showed a ≥4-fold increase in MBC out of which *C. sakazakii* and the *E. coli* drain isolate showed partial reversion, whilst the others showed no reversion after passage in the absence of triclosan. Both *E. coli* isolates in addition to *C. sakazakii, E. faecalis* and *A. baumannii* showed ≥4-fold increase in MBEC after repeated triclosan exposure, out of which *C. sakazakii* and *E. faecalis* did not revert and both *E. coli* isolates completely reverted in the absence of the microbicide. In comparison, after exposure to triclosan formulation only the *E. coli* isolates and *P. aeruginosa* showed ≥4-fold increase in MIC, which fully reverted for *P. aeruginosa* but did not revert for either *E. coli* strain in the absence of triclosan formulation. MBECs increased ≥4-fold for *S. aureus* and *E. faecalis* but fully reverted for both bacteria after regrowth in the absence of triclosan formulation.

3.4 Discussion

The majority of investigations into the potential of microbicides to select for changes in bacterial susceptibility have been conducted by exposing pure cultures of bacteria to microbicides as pure actives in aqueous solution or in simple formulations (aqueous solutions containing the active, and in some studies, cosolvents such as DMSO (Forbes et al., 2014) or ethanol (Ledder et al., 2006)). It has been hypothesized that formulated products may interact with bacteria in a manner that is distinct from aqueous solutions (Condell et al., 2012, Stephens et al., 2012), potentially reducing the frequency and extent of susceptibility changes. Although numerous studies have evaluated the antimicrobial potency of formulated microbicides (McBain et al., 2003a, McBain et al., 2004, Cutter et al., 2001), to our knowledge there are no current studies in the literature that have compared the effects of repeated bacterial exposure to microbicides in aqueous solution and in complex formulation for a range of bacteria and microbicides. In the present investigation therefore, we evaluated the effect of

the formulation of microbicides on antimicrobial potency and on the mitigation of bacterial insusceptibility for a selection of bacterial isolates and microbicides encompassing biguanides, quaternary ammonium compounds, phenolics, isothiazolinones, formaldehyde releasers and essential oils. Microbicides were tested as aqueous solutions of the active compounds and in complex formulations with sequestrants and ionic/non-ionic surfactants to mimic their real-world use as hard-surface disinfectants (for BAC, chlorhexidine, DDAC, DMDM hydantoin, PHMB and thymol) and laundry detergents (for BIT and triclosan). The reversibility of any induced susceptibility changes was also investigated to ascertain the stability of adaptation.

Reductions in bacterial susceptibility to an antimicrobial compound can be influenced by several factors related to the antimicrobial or the microorganism. Bacterial susceptibility may be affected by the structural integrity of the bacterial cell envelope and its ability to function as an effective permeability barrier (Chuanchuen et al., 2003, Moore et al., 2006). Innate bacterial non-susceptibility toward an antimicrobial agent may occur due to effective barrier components of the bacterial cell, such as an outer membrane in Gram-negative bacteria (Chuanchuen et al., 2003) or the spore coat in bacterial endospores (Vareltzis et al., 1997). Changes in cell envelope permeability may therefore affect bacterial susceptibility, which can include alterations in lipopolysaccharide expression and structure (Vaara, 1992), reduction in the number of outer membrane porins (Haque and Russell, 1974) and alterations in membrane fatty acid composition (Kotra et al., 2000). The expression of efflux pumps has also been linked to decreases in microbicide susceptibility in bacteria, particularly towards membrane active compounds such as biguanides (Lee et al., 1994, Helander et al., 1998), (CHX and PHMB) and quaternary ammonium compounds (BAC and DDAC) (Cutter et al., 2001, Tassou et al., 2000). The increased expression of efflux pumps may therefore also potentially account for some of the susceptibility changes observed in many of our bacterial isolates.

Reversible susceptibility changes to microbicides may result from temporary phenotypic adaptations in bacteria, such as the induction of stress responses that revert once the bacteria recover in an antimicrobial free environment (Coenye and Nelis, Jordan et al., 2008). Equally, the development of microbicide insusceptibility may be attributable to the

selection of insusceptible mutants, for instance mutations in FabI are known to render bacteria insusceptible to triclosan (McMurry et al., 1998, Alekshun and Levy, 1997). However, the inherent stability of a particular mutation largely depends upon the overall fitness cost that it exerts on the host microorganism versus the competitive advantage that it provides in a particular environment (Kunz et al., 2012). Hence any mutation that renders a bacterium less susceptible towards an antimicrobial agent may be lost once the selective pressure is removed if the mutation results in a biologically significant reduction in the fitness of the microorganism (Maisnier-Patin et al., 2002).

Whilst previous studies have reported the induction of microbicide insusceptibility in bacteria, it should be noted that adapted bacterial isolates often remain susceptible to the microbicide at concentrations used in consumer products, and that true microbicide resistance is likely uncommon (Forbes et al., 2014). In the current investigation, the only test bacterium that was refractory to a microbicide was *P. aeruginosa* to triclosan. This was apparent before microbicide exposure and has previously been attributed to the physiology of the bacterium, including expression of efflux pumps (Chuanchuen et al., 2003). Interestingly, *P. aeruginosa* was comparatively susceptible to the triclosan formulation, illustrating marked differences in potency for the microbicide in aqueous solution compared to the formulated product.

Of all the microbicides in unformulated form, BAC and triclosan induced the highest frequency of ≥4-fold increases in MIC; with 6/9 bacterial isolates showing a reduction in susceptibility to both antimicrobials at this level. This was followed by PHMB (4 isolates) and CHX (3 isolates). Triclosan exposure resulted in the highest frequency of ≥4-fold increases in MBC (6 isolates) followed by PHMB (5 isolates), DDAC and BAC (3 isolates), then CHX (2 isolates) and DMDM hydantoin (1 isolate). In terms of the susceptibility of bacteria when grown as biofilms, PHMB adaptation resulted in the highest number of isolates showing ≥4-fold increases in MBEC (5 isolates) followed by triclosan and DDAC (4 isolates each) then BAC and CHX (2 isolates).

With respect to the formulated microbicides, BAC induced the highest number of ≥4-fold increases in MIC (4 isolates) followed by triclosan (3 isolates) and thymol (1 isolate). DMDM hydantoin-, thymol- and PHMB-containing formulations induced the largest number of ≥4-fold increases in MBC (3 isolates each) followed by BAC and DDAC (2 isolates each).

Exposure to the DDAC containing formulations resulted in the highest numbers of bacterial isolates exhibiting a ≥4-fold increase in MBEC (4 isolates), followed by BAC and DMDM hydantoin (3 isolates) then PHMB, thymol and triclosan formulations (2 isolates).

Although the current investigation demonstrates that induced reductions in susceptibility towards both microbicides and microbicide-containing formulations may occur, a substantially higher number of bacterial isolates underwent ≥4-fold increases in MIC, MBC or MBEC when exposed to microbicides in aqueous solution, in comparison to those in formulation. The only exception to this was thymol, for which changes in susceptibility were more frequent in bacteria exposed to the compound in formulation. Thymol is poorly soluble in water and when incorporated into formulation, solubility may have improved increasing bacterial exposure and thus selectivity. Furthermore, since incorporating microbicides into formulations frequently enhanced antimicrobial potency, the formulated microbicides often maintained higher antimicrobial activity in comparison to microbicides in aqueous solution, even after repeated exposure. The incorporation of non-ionic surfactants and sequestrants into microbicide-containing formulations therefore appears to increase antimicrobial potency, as well as mitigating the development of antimicrobial insusceptibility both in terms of frequency and magnitude of susceptibility change. Since excipients can interact with different cellular targets to the accompanying microbicide, formulations may have a cumulative antimicrobial effect that would require further multiple physiological adaptations to render the microorganism insusceptible.

Alcohol ethoxylates are a major class of non-ionic surfactants which are often used in household detergents, cleaners and personal care products and have previously shown bacteriostatic effects due to their direct impact on the bacterial cell membrane leading to the leakage of cytoplasmic components, indicating an increase in membrane permeability (Moore et al., 2006). An increase in membrane permeability would allow microbicides to more readily transverse the cytoplasmic membrane increasing their access to intracellular target sites. Therefore, combining microbicides and alcohol ethoxylates in formulation may enhance overall antimicrobial potency, when compared to the pure active. Sodium tripolyphosphate (STP-P), a chelating agent commonly used in domestic detergents, has previously shown antibacterial activity against several bacteria often found as food contaminants (Vareltzis et

al., 1997). Since STP-P is a chelating agent it is plausible, like other chelators such as Ethylenediaminetetraacetic acid (EDTA), that this antibacterial activity occurs by disruption of the bacterial cell envelope through the sequestration of stabilising divalent cations. Such cations normally link bacterial lipopolysaccharides to the outer membrane and interference with this process can destabilise the outer membrane in Gram negative bacteria, impairing barrier function (Vaara, 1992, Haque and Russell, 1974, Kotra et al., 2000). Furthermore, strong chelating agents may inhibit bacteria growth by sequestering trace minerals required for bacterial metabolism (Lee et al., 1994, Haque and Russell, 1974).

Essential oils, such as thymol, are often incorporated into antimicrobial formulation due to their inhibitory effects on bacterial growth. The antimicrobial activity of essential oils reportedly occurs through interaction with the bacterial cytoplasmic membrane, resulting in increased cell permeability and disruption of energy generation (Helander et al., 1998, Tassou et al., 2000). Compensatory adaptations may occur, but whether these would result in outcome-changing effects during deployment depends on the extent of any susceptibility decreases, the concentration used in the product and the antimicrobial potency of the formulation (i.e., the active compound and excipients in combination).

In a small number of cases bacterial susceptibility was increased following repeated exposure to microbicides. This could be due to knock-on effects of cellular damage caused by microbicide exposure.

3.5 Conclusion

With the ultimate aim of developing realistic approaches to risk assessment, it was observed that repeated exposure of 9 bacteria to 8 microbicides in aqueous solution or within complex formulations with sequestrants and ionic/non-ionic surfactants, induced reductions in bacterial susceptibility in a highly selective laboratory exposure system. Susceptibility changes varied in reversibility, possibly reflecting a range of underlying mechanisms including temporary phenotypic adaptation, such as the induction of stress responses, or the selection of stable mutations. Importantly, the formulation of microbicides markedly increased overall antimicrobial potency for the test microbicides against the

majority of the bacteria, as well as reducing the frequency and magnitude of susceptibility changes. Although it remains unclear how observations based on the *in vitro* exposure of bacteria to microbicides can be extrapolated to their deployment in the real world, understanding the potential selectivity of microbicide-containing formulations is likely to better served by testing formulations as well as actives in aqueous solution. This highlights the need to conduct risk assessments of induced microbicide susceptibility changes using conditions, which better reflect their deployment.

Table 3.1 Bacterial susceptibility towards benzalkonium chloride in planktonic and biofilm growth modes before, during and after repeated exposure to benzalkonium chloride in aqueous solution or in formulation

	MIC						**MBC**						**MBEC**					
	UF			**F**			**UF**			**F**			**UF**			**F**		
Bacterium	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14
S. aureus†	0.1	**3.9**	**2.0**	0.5	**2.0**	**2.0**	2.0	**15.6**	**7.8**	2.0	**7.8**	**7.8**	2.6 (1)	**31.3**	**15.6**	3.9	**125**	7.8
E. coli†	4.6 (1)	**31.3**	**31.3**	3.9	**31.3**	**31.3**	7.2 (2)	**41.7 (16)**	**62.5**	7.8	**31.3**	**62.5**	31.3	31.3	62.5	31.3	62.5	62.5
E. faecalis†	2.0	**7.8**	3.9	2.0	3.9	3.9	3.3 (1)	7.8	7.8	3.9	7.8	7.8	6.5 (1)	**31.3**	7.8	6.7 (2)	**46.9 (17)**	**46.9 (17)**
P. aeruginosa†	14.3 (2)	**62.5**	**62.5**	15.6	**62.5**	**125**	23.4 (9)	**125**	**125**	31.3	62.5	250	125	250	**500**	62.5	**250**	**500**
*M. osloensis**	3.9	2.0	na	1.0	1.0	na	7.8	15.6	na	2.0	2.0	na	7.8	na	na	7.8	2.0	na
*A. baumannii**	2.0	**62.5**	**31.3**	3.9	**31.3**	**31.3**	93.8 (34)	250	125	62.5	62.5	125	125	250	125	125	125	93.8 (34)
*P. putida**	15.6	**62.5**	31.3	15.6	15.6	na	125	125	62.5	62.5	31.3	na	125	na	62.5	125	31.3	na
*C. sakazakii**	62.5	52.1 (16)	na	31.3	31.3	na	125	125	na	31.3	31.3	na	31.3	na	na	31.3	62.5	na
*E. coli**	18.4 (7)	52.1 (16)	na	15.6	31.3	na	62.5	125	na	31.3	31.3	na	62.5	na	na	62.5	62.5	na

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; MBEC, minimum biofilm eradication concentration.
Before antimicrobial exposure (P0); during antimicrobial exposure (P14) and after passage in the absence of antimicrobial (X14) All values are in mg/L. †, non-drain isolates; *,drain isolates. UF, unformulated (microbicide in aqueous solution); F, formulated (microbicide in formulation). Organisms that underwent a ≥4-fold increase in MIC, MBC or MBEC (as indicated by bold text) were passaged a further 14 times in the absence of microbicide. na, bacteria that did not undergo a ≥4-fold change and were not assessed for reversibility. Data represents six replicates. Where data varied between biological replicates, standard deviations have been given in parentheses. In controls were bacteria were tested against formulations without microbicide, all bacteria were non-susceptible to in-use concentrations. + over concentration indicated.

Table 3.2 Bacterial susceptibility towards benzisothiazolinone in planktonic and biofilm growth modes before, during and after repeated exposure to benzisothiazolinone in aqueous solution or in formulation

Bacterium	MIC						MBC						MBEC					
	UF			F			UF			F			UF			F		
	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14
S. aureus†	7.8	15.6	na	1.0	2.0	na	31.3	62.5	na	15.6	15.6	na	62.5	62.5	na	31.3	62.5	na
E. coli†	15.6	15.6	na	7.8	7.8	na	31.3	62.5	na	31.3	31.3	na	250	187.5 (68)	na	125	125	na
E. faecalis†	7.8	15.6	na	0.5	1.0	na	7.8	7.8	na	0.5	1.0	na	250	41.7 (16)	na	125	125	na
P. aeruginosa†	125	250	na	15.6	31.3	na	250	500	na	62.5	125	na	500	500	na	125+	125+	na
*M. osloensis**	1.0	1.0	na	0.5	0.5	na	1.0	1.0	na	0.5	0.5	na	2.0	2.0	na	0.5	1.0	na
*A. baumannii**	31.3	31.3	na	7.8	15.6	na	31.3	62.5	na	31.3	62.5	na	250	250	na	62.5	125	na
*P. putida**	15.6	31.3	na	31.3	31.3	na	62.5	62.5	na	31.3	62.5	na	250	250	na	62.5	125	na
*C. sakazakii**	7.8	7.8	na	7.8	7.8	na	31.3	31.3	na	31.3	31.3	na	250	500	na	62.5	125	na
*E. coli**	15.6	31.3	na	15.6	15.6	na	62.5	62.5	na	15.6	31.3	na	250	187.5	na	125	125	na

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; MBEC, minimum biofilm eradication concentration.
Before antimicrobial exposure (P0); during antimicrobial exposure (P14) and after passage in the absence of antimicrobial (X14) All values are in mg/L. †, non-drain isolates; *,drain isolates. UF, unformulated (microbicide in aqueous solution); F, formulated (microbicide in formulation). Organisms that underwent a ≥4-fold increase in MIC, MBC or MBEC (as indicated by bold text) were passaged a further 14 times in the absence of microbicide. na, bacteria that did not undergo a ≥4-fold change and were not assessed for reversibility. Data represents six replicates. Where data varied between biological replicates, standard deviations have been given in parentheses. In controls were bacteria were tested against formulations without microbicide, all bacteria were non-susceptible to in-use concentrations. + over concentration indicated.

Table 3.3 Bacterial susceptibility towards chlorhexidine in planktonic and biofilm growth modes before, during and after repeated exposure to chlorhexidine in aqueous solution or in formulation

	MIC						MBC						MBEC					
	UF			F			UF			F			UF			F		
Bacterium	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14
S. aureus†	1.7 (1)	**7.8**	3.9	2.0	2.0	na	5.2 (2)	**46.9 (17)**	**31.3**	7.8	7.8	na	13 (4)	31.3	31.3	7.8	15.6	na
E. coli†	2.4 (1)	**11.7 (4)**	7.9	2.0	3.9	na	9.8 (5)	**62.5**	**31.3**	15.6	31.3	na	52.1 (16)	62.5	31.3	62.5	31.3	na
E. faecalis†	3.9	7.8	15.6	3.9	7.8	na	14.3 (3)	31.3	31.3	7.8	15.6	na	31.3	**125**	62.5	31.3	62.5	na
P. aeruginosa†	7.8	**31.3**	**31.3**	7.8	15.6	na	68.8 (34)	**250**	125	125	125	na	250	125	125	250	125	na
*M. osloensis**	3.9	2.0	2.0	1.0	1.0	na	31.3	15.6	3.9	1.0	1.0	na	31.3	**125**	15.6	15.6	31.3	na
*A. baumannii**	7.8	7.8	na	3.9	7.8	na	125	62.5	na	15.6	31.3	na	125	125	na	125	31.3	na
*P. putida**	7.8	7.8	na	4.6 (2)	3.9	na	93.8 (34)	62.5	na	7.8	7.8	na	62.5	125	na	62.5	62.5	na
*C. sakazakii**	7.8	7.8	na	3.9	3.9	na	62.5	125	na	7.8	15.6	na	62.5	125	na	31.3	10.4 (4)	na
*E. coli**	7.8	10.4 (4)	15.6	3.9	3.9	na	46.8 (17)	125	125	7.8	15.6	na	125	125	125	62.5	23.4 (9)	na

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; MBEC, minimum biofilm eradication concentration.
Before antimicrobial exposure (P0); during antimicrobial exposure (P14) and after passage in the absence of antimicrobial (X14) All values are in mg/L. †, non-drain isolates; *,drain isolates. UF, unformulated (microbicide in aqueous solution); F, formulated (microbicide in formulation). Organisms that underwent a ≥4-fold increase in MIC, MBC or MBEC (as indicated by bold text) were passaged a further 14 times in the absence of microbicide. na, bacteria that did not undergo a ≥4-fold change and were not assessed for reversibility. Data represents six replicates. Where data varied between biological replicates, standard deviations have been given in parentheses. In controls were bacteria were tested against formulations without microbicide, all bacteria were non-susceptible to in-use concentrations. + over concentration indicated.

Table 3.4 Bacterial susceptibility towards didecyldimethyl ammonium chloride in planktonic and biofilm growth modes before, during and after repeated exposure to didecyldimethyl ammonium chloride in aqueous solution or in formulation

	MIC						MBC						MBEC					
	UF			F			UF			F			UF			F		
Bacterium	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	*X14*
S. aureus†	0.5	1.0	1.0	0.5	0.5	0.5	2.0	3.9	3.9	2.0	0.5	0.5	3.9	**31.3**	**31.3**	3.9	**62.5**	**62.5**
E. coli†	7.8	11.7 (4)	7.8	3.9	7.8	3.9	3.9	11.7 (4)	15.6	3.9	7.8	3.9	31.3	**125**	15.6	7.8	**36.5 (13)**	15.6
E. faecalis†	1.0	2.0	2.0	2.0	2.0	2.0	1.0	2.0	2.0	2.0	3.9	3.9	2.0	**125**	**31.3**	2.0	**104.2 (32)**	**62.5**
P. aeruginosa†	14.3 (2)	31.3	15.6	15.6	31.3	15.6	31.3	**125**	31.3	31.3	**125**	31.3	125	125	250	62.5	125	62.5
*M. osloensis**	1.0	1.0	1.0	1.0	1.0	na	1.4 (0.5)	3.9	2.0	2.0	2	na	2.0	3.9	3.9	2.0	2.0	na
*A. baumannii**	15.6	31.3	15.6	3.9	7.8	na	15.6	**62.5**	31.3	62.5	62.5	na	62.5	125	31.3	62.5	62.5	na
*P. putida**	47.4 (17)	31.3	na	4.6(1)	3.9	na	62.5	41.7 (17)	na	31.3	62.5	na	62.5	62.5	na	62.5	62.5	na
*C. sakazakii**	7.2 (2)	15.6	15.6	7.8	15.6	na	15.6	31.3	31.3	7.8	15.6	na	31.3	62.5	62.5	15.6	31.3	na
*E. coli**	4.6 (2)	15.6	15.6	3.9	7.8	3.9	10.4 (4)	**41.7 (17)**	31.3	3.9	**15.6**	7.8	15.6	**62.5**	31.3	15.6	**62.5**	23.5 (9)

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; MBEC, minimum biofilm eradication concentration.
Before antimicrobial exposure (P0); during antimicrobial exposure (P14) and after passage in the absence of antimicrobial (X14) All values are in mg/L. †, non-drain isolates; *,drain isolates. UF, unformulated (microbicide in aqueous solution); F, formulated (microbicide in formulation). Organisms that underwent a ≥4-fold increase in MIC, MBC or MBEC (as indicated by bold text) were passaged a further 14 times in the absence of microbicide. na, bacteria that did not undergo a ≥4-fold change and were not assessed for reversibility. Data represents six replicates. Where data varied between biological replicates, standard deviations have been given in parentheses. In controls were bacteria were tested against formulations without microbicide, all bacteria were non-susceptible to in-use concentrations. + over concentration indicated.

Table 3.5 Bacterial susceptibility towards Glydant (DMDM-hydantoin) in planktonic and biofilm growth modes before, during and after repeated exposure to Glydant (DMDM-hydantoin) in aqueous solution or in formulation.

	MIC						MBC						MBEC					
	UF			F			UF			F			UF			F		
Bacterium	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14
S. aureus†	187.5	187.5	na	187.5	187.5	na	375	482 (183)	na	375	375	na	3000	3000	na	1500	3000	na
E. coli†	375	375	na	375	375	375	1500	1500	na	375	750	375	6000	6000	na	1500	**6000**	1500
E. faecalis†	187.5	187.5	na	187.5	187.5	na	1500	1500	na	1500	750	na	3000	3000	na	3000	6000	na
P. aeruginosa†	187.5	187.5	na	187.5	187.5	na	6000	6000	na	1500	1500	na	6000	6000	na	6000	12000	na
*M. osloensis**	375	375	na	46.9	62.5	na	325	375	na	187.5	187.5	na	750	1500	na	750	1500	na
*A. baumannii**	375	325	na	187.5	187.5	na	750	750	na	375	375	na	6000	6000	na	6000	6000	na
*P. putida**	375	375	na	375	375	na	750	750	na	750	375	na	6000	6000	na	3000	6000	na
*C. sakazakii**	375	375	na	187.5	187.5	375	3000	3000	na	375	750	375	6000	6000	na	1500	**6000**	1500
*E. coli**	187.5	466 (219)	187.5	187.5	375	187.5	375	**1500**	375	375	750	375	6000	6000	6000	1500	**12000**	1500

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; MBEC, minimum biofilm eradication concentration.
Before antimicrobial exposure (P0); during antimicrobial exposure (P14) and after passage in the absence of antimicrobial (X14) All values are in mg/L. †, non-drain isolates; *,drain isolates. UF, unformulated (microbicide in aqueous solution); F, formulated (microbicide in formulation). Organisms that underwent a ≥4-fold increase in MIC, MBC or MBEC (as indicated by bold text) were passaged a further 14 times in the absence of microbicide. na, bacteria that did not undergo a ≥4-fold change and were not assessed for reversibility. Data represents six replicates. Where data varied between biological replicates, standard deviations have been given in parentheses. In controls were bacteria were tested against formulations without microbicide, all bacteria were non-susceptible to in-use concentrations. + over concentration indicated.

Table 3.6 Bacterial susceptibility towards PHMB in planktonic and biofilm growth modes before, during and after repeated exposure to PHMB in aqueous solution or in formulation

	MIC						MBC						MBEC					
	UF			F			UF			F			UF			F		
Bacterium	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14
S. aureus†	3.9	**23.5 (9)**	**15.6**	3.9	3.9	3.9	3.9	**125**	**15.6**	3.9	**15.6**	7.8	15.6	**125**	15.6	15.6	**125**	31.3
E. coli†	15 (10)	31.3	15.6	7.8	15.6	na	15 (10)	**62.5**	**62.5**	15.6	31.3	na	62.5	62.5	62.5	62.5	31.3	na
E. faecalis†	7.8	**31.3**	15.6	5.9(1)	15.6	7.8	7.8	**125**	15.6	7.8	**31.3**	7.8	14.3 (3)	**125**	31.3	15.6	**125**	31.3
P. aeruginosa†	22.8 (15)	31.3	62.5	15.6	15.6	15.6	22.8 (15)	**125**	**125**	31.3	**125**	31.3	250	250	250	250	62.5	62.5
*M. osloensis**	7.8	**31.3**	3.9	1.0	1.0	na	62.5	31.3	31.3	7.8	7.8	na	62.5	62.5	31.3	31.3	62.5	na
*A. baumannii**	7.8	**31.3**	7.8	9.1 (3)	15.6	na	62.5	125	62.5	31.3	62.5	na	62.5	**250**	62.5	62.5	125	na
*P. putida**	28.9 (8)	31.3	na	15.6	15.6	na	62.5	62.5	na	31.3	62.5	na	125	125	na	125	125	na
*C. sakazakii**	7.8	15.6	15.6	31.2	15.6	na	104 (32)	125	125	15.6	31.3	na	62.5	**250**	125	62.5	125	na
*E. coli**	7.8	7.8	31.3	7.8	15.6	na	15.6	**250**	31.3	15.6	31.3	na	62.5	**250**	31.3	62.5	31.3	na

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; MBEC, minimum biofilm eradication concentration.
Before antimicrobial exposure (P0); during antimicrobial exposure (P14) and after passage in the absence of antimicrobial (X14) All values are in mg/L. †, non-drain isolates; *,drain isolates. UF, unformulated (microbicide in aqueous solution); F, formulated (microbicide in formulation). Organisms that underwent a ≥4-fold increase in MIC, MBC or MBEC (as indicated by bold text) were passaged a further 14 times in the absence of microbicide. na, bacteria that did not undergo a ≥4-fold change and were not assessed for reversibility. Data represents six replicates. Where data varied between biological replicates, standard deviations have been given in parentheses. In controls were bacteria were tested against formulations without microbicide, all bacteria were non-susceptible to in-use concentrations. + over concentration indicated.

Table 3.7 Bacterial susceptibility towards thymol in planktonic and biofilm growth modes before, during and after repeated exposure to thymol in aqueous solution or in formulation

	MIC						MBC						MBEC					
	UF			F			UF			F			UF			F		
Bacterium	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14
S. aureus†	187.5	187.5	na	187.5	187.5	na	375	375	na	375	750	na	416 (160)	375	na	375	750	na
E. coli†	1500	1500	na	187.5	375	375	1500	1500	na	375	**1500**	750	1500	1500	na	375	**3000**	1500
E. faecalis†	375	750	na	187.5	375	na	750	750	na	375	750	na	750	750	na	750	1500	na
P. aeruginosa†	3000	3000	na	1500	3000	na	6000	3000	na	3000	6000	na	6000	6000	na	6000	12000	na
*M. osloensis**	750	750	na	187.5	375	na	750	750	na	187.5	375	na	3000	1500	na	3000	375	na
*A. baumannii**	750	750	na	375	375	375	1500	3000	na	750	**6000**	**3000**	6000	6000	na	6000	6000	6000
*P. putida**	750	750	na	375	**3000**	375	1500	3000	na	1500	**6000**	3000	6000	6000	na	6000	6000	12000
*C. sakazakii**	750	750	na	375	375	na	2250 (822)	3000	na	375	750	na	6000	6000	na	3000	750	na
*E. coli**	665 (190)	750	na	187.5	375	na	3000	3000	na	375	750	na	6000	6000	na	750	**3000**	1500

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; MBEC, minimum biofilm eradication concentration.
Before antimicrobial exposure (P0); during antimicrobial exposure (P14) and after passage in the absence of antimicrobial (X14) All values are in mg/L. †, non-drain isolates; *,drain isolates. UF, unformulated (microbicide in aqueous solution); F, formulated (microbicide in formulation). Organisms that underwent a ≥4-fold increase in MIC, MBC or MBEC (as indicated by bold text) were passaged a further 14 times in the absence of microbicide. na, bacteria that did not undergo a ≥4-fold change and were not assessed for reversibility. Data represents six replicates. Where data varied between biological replicates, standard deviations have been given in parentheses. In controls were bacteria were tested against formulations without microbicide, all bacteria were non-susceptible to in-use concentrations. + over concentration indicated.

Table 3.8 Bacterial susceptibility towards triclosan in planktonic and biofilm growth modes before, during and after repeated exposure to triclosan in aqueous solution or in formulation

Bacterium	MIC						MBC						MBEC					
	UF			F			UF			F			UF			F		
	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14	P0	P14	X14
S. aureus†	0.2	**62.5**	**31.3**	0.1	0.1	0.1	3.9	**62.5**	**62.5**	0.1	0.1	0.1	65.1	125	125	2.0	**7.8**	2.0
E. coli†	2.0	**62.5**	**62.5**	0.1	**2.0**	**3.9**	2.0	**125**	**125**	7.8	7.8	3.9	125	**500**	125	62.5	15.6	15.6
E. faecalis†	62.5	62.5	62.5	0.1	0.1	0.1	62.5	125	125	0.1	0.1	0.1	15.6	**125**	**125**	2.0	**7.8**	2.0
P. aeruginosa†	ns	ns	ns	7.8	**62.5**	7.8	ns	ns	ns	62.5	62.5	7.8	ns	ns	ns	62.5	62.5	7.8
*M. osloensis**	1.0	**15.6**	**7.8**	1.0	1.0	na	7.8	**31.3**	**31.3**	3.9	3.9	na	125	125	125	3.9	3.9	na
*A. baumannii**	125	125	125	2.0	2.0	na	125	250	125	31.6	15.6	na	125	250	125	62.5	15.6	na
*P. putida**	15.6	**62.5**	**62.5**	1.0	2.0	na	62.5	125	125	15.6	15.6	na	125	250	500	62.5	15.6	na
*C. sakazakii**	7.8	**500**	**187.5**	2.0	2.0	na	7.8	**1000**	**250**	31.3	31.3	na	1.3 (0.5)	**125**	**125**	62.5	31.3	na
*E. coli**	1.0	**125**	**62.5**	0.1	**2.0**	**3.9**	2.0	**250**	**125**	15.6	15.6	15.6	125	**500**	125	62.5	15.6	15.6

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; MBEC, minimum biofilm eradication concentration.
Before antimicrobial exposure (P0); during antimicrobial exposure (P14) and after passage in the absence of antimicrobial (X14) All values are in mg/L. †, non-drain isolates; *,drain isolates. UF, unformulated (microbicide in aqueous solution); F, formulated (microbicide in formulation). Organisms that underwent a ≥4-fold increase in MIC, MBC or MBEC (as indicated by bold text) were passaged a further 14 times in the absence of microbicide. na, bacteria that did not undergo a ≥4-fold change and were not assessed for reversibility. Data represents six replicates. Where data varied between biological replicates, standard deviations have been given in parentheses. In controls were bacteria were tested against formulations without microbicide, all bacteria were non-susceptible to in-use concentrations. ns, not susceptible (MBC/MIC ≥1000 mg/L). + over concentration indicated.

Chapter 4

Phenotypic Analysis of Bacteria before and after Repeated Exposure to Microbicides and Microbicide-Containing Formulations

4.0 Abstract

Background: Whilst exposing bacteria to microbicides under laboratory conditions may result in changes in susceptibility, the effect of this on bacterial competitiveness and relevance to environmental exposure remain unclear. In this chapter planktonic growth rate, biofilm formation, competitive fitness, bacterial pathogenicity and antibiotic susceptibility were determined in bacterial isolates that had previously demonstrated ≥4-fold increase in minimum bactericidal concentration after repeated exposure to microbicide or microbicide in formulation. Whilst there have been previous investigations demonstrating reductions in bacterial susceptibility after exposure to microbicide or microbicide in formulation, few studies have collectively examined the effects of long-term microbicide exposure on bacterial susceptibility in parallel to biofilm formation, competitive fitness, pathogenicity or antibiotic susceptibility, with no studies analysing formulation effects.

Methods: Biofilm formation was determined using a crystal violet stain assay on unexposed (P0) bacteria; bacteria exposed to unformulated microbicide (P14); bacteria exposed to formulated microbicide (F14); bacteria grown in the absence of unformulated microbicide (X14) and bacteria grown in the absence of formulated microbicide (FX). Bacterial competitive fitness was determined using a pair-wise method to grow unexposed (P0) and exposed (P14) isolates in combination to observe competitive fitness of the isolates. Pathogenicity was determined using *Galleria mellonella* larvae to observe pathogenicity changes for the selected bacterial isolates. Antibiotic disc diffusion assays were used to determine antibiotic susceptibility.

Results: Biofilm formation analysis show 8/18 of unexposed bacteria (P0) to yield a higher density biofilm when compared to P14, whilst 8/18 exposed bacteria (P14) were seen to yield a higher density biofilm when compared to P0. In contrast, after exposure to the microbicides in formulation 3/10 unexposed bacteria (P0) yield a higher density biofilm when compared to F14, whilst 2/10 bacteria exposed to microbicide in formulation (F14) yield a higher density of biofilm compared with P0. Competitive fitness between P0 and P14 of microbicide exposed bacterial isolates show 2/19 of the isolates to have statistically significant increases in P14 fitness, whilst 14/19 isolates

were seen to be significantly fitter at P0. In contrast, all formula-exposed isolates became less fit after exposure. Pathogenicity assays indicated that 9/13 unexposed bacteria (P0) exhibited higher *Galleria* pathogenicity compared with P14 and X14; 1/13 microbicide exposed bacteria (P14) show higher pathogenicity then P0 and X14, whilst 3/13 bacteria grown in the absence of microbicide (X14) to have increased pathogenicity compared with P0 and P14. When examining formulation effects; P0 demonstrated 2/7 to have a higher pathogenicity when compared with F14 and FX; bacteria exposed to microbicide in formulation (F14) show 2/7 to demonstrate higher pathogenicity when compared with P0 and XF and 1/7 to show increase pathogenicity once grown in the absence of antimicrobial (FX). When comparing the antibiotic susceptibility of the isolates exposed to microbicide to the respective unexposed parent strain there were 21/90 increases in susceptibility and 22/90 decreases in overall susceptibility to antibiotics, whilst exposure to formulated microbicides showed 7/50 increases to susceptibility and 9/50 decreases to susceptibility.

Conclusion: Overall, this study shows phenotypic changes to occur to 24% of tested bacterial isolates when exposed to microbicides, however, exposed to microbicide in formulation 16% of bacterial isolates demonstrated changes, showing 8% fewer changes when compared to the bacteria exposed to microbicide.

4.1 Introduction

Microbicides have been used to control microbial growth in environments such as hospitals, households, and laboratories (Russell, 2002). Concern that microbicide use might be associated with decreases in microbicide susceptibility and cross-resistance to antibiotics has led to increased research into the potential for, and mechanisms of, bacterial adaptation (Russell et al., 1998). Previous studies have shown that prolonged sub-lethal exposure of bacteria to microbicides can lead to reductions in susceptibility (Walsh et al., 2003b, Cookson et al., 1991). In Chapter 3, selected bacteria were repeatedly exposed to microbicides in aqueous solution and microbicide in formulation. Results indicated that exposure to formulated microbicides were associated with reduced frequency and extent of susceptibility decreases. When considering any induced changes in bacterial susceptibility towards a particular antimicrobial it is important to take into account any resulting costs to competitive fitness and pathogenicity as well as any induced cross-resistance to third party agents such as antibiotics. In this current chapter we therefore aim to further evaluate the phenotypic alterations in selected bacterial isolates that demonstrated a ≥ 4-fold increase in MBC after repeated antimicrobial exposure (Cowley et al., 2015). This includes the assessment of changes in planktonic growth rate, biofilm formation, competitive fitness and pathogenicity.

4.1.1 Biofilm formation

As outlined in Chapter 1 (1.6), biofilms form when planktonic bacteria adhere to a surface, producing an initial sessile bacterial layer attached to a surface (Donlan, 2002). Extracellular polymeric substance (EPS) is produced from the sessile bacteria, enabling further planktonic cells to attach to the initial micro colonies (Flemming and Wingender, 2001). The microcolonies mature to form a biofilm and eventually begin to disperse (Kierek-Pearson and Karatan, 2005). The process provides multiple advantages for the bacteria within the biofilm, including shielding from environmental influences such as microbicides (Donlan, 2000). The production of the protective anionic EPS also helps shield the bacteria from any environmental stress, slowing the diffusion of

microbicides to the centre of the biofilm. Cells within the biofilm often have a slower growth rate, as a result of nutrient limitations, which may reduce their susceptibility to agents that target actively growing cells, such as certain antibiotics (Keren et al., 2004).

4.1.2 Competitive fitness

Previous investigations have demonstrated that the generation of antimicrobial resistance in bacteria may be associated with reduced fitness (Andersson and Hughes, 2010). It has been theorised that the fitness of a bacterium is associated with the rate of transmission and ability to compete with other bacteria within the host (Lindgren et al., 2005). Therefore, competitive fitness may be an important variable associated with clinical significance and potential for environmental persistence of a bacterium. For example, a bacterium acquiring a mutation that results in antimicrobial resistance but also in a fitness burden, may not establish in its environment due to reduced competitive fitness (Besier et al., 2005). Alternatively, it has been suggested that the bacterium could persist at low level for a prolonged period increasing the likelihood of transmission (Keren et al., 2004). However, if an adapted bacterium cannot compete with its congeners or has a markedly reduced specific growth rate then its pathogenic capability may be reduced. When considering the consequence of the development of antimicrobial susceptibility in bacteria it is important to also consider the fitness cost associated with this change.

4.1.3 Bacterial pathogenesis

Bacterial pathogenesis is an important factor to consider when investigating the impact of any antimicrobial susceptibility changes occurring within bacteria. A reduction in toxins produced by a bacterium for example may be associated with decreased pathogenicity, and been reported for certain strains of *S. aureus* that acquired methicillin-resistance (Collins et al., 2010). Decreases in pathogenicity after exposure to microbicide have been previously reported, with *S. aureus* expressing lower pathogenicity after prolonged exposure to triclosan, which has been shown to induced small colony variants (Latimer et al., 2012). Changes in bacterial pathogenicity

can occur due mutations affecting the production of pathogenicity factors such as a mutation affecting DNA adenine methylase (Dam) gene, causing a multidrug resistant *Salmonella typhimurium* to become avirulent (Björkman et al., 1998, Heithoff et al., 1999). Growth rate may also play a role in bacterial pathogenicity; for example slower growth rates may result in slower invasion of the host and therefore a lower pathogenesis (Gulig and Doyle, 1993).

4.1.4 Antibiotic cross-resistance

The extensive use of antibiotics in recent years has led to the development of bacterial strains that exhibit antibiotic resistance. This has fuelled further research into the identification of new antibiotics and has led to an increase in the use of alternative antimicrobial agents such as microbicides (Poole, 2002, Threlfall et al., 1997). However, previous investigations have reported on the generation of cross-resistance between microbicides and antibiotics (Chuanchuen et al., 2001, Levy, 2002). This may be due to the selection of bacteria with mutations in shared target sites or through the induction of broad range defence mechanisms (Lin et al., 2015). An increase in efflux pump expression is widely considered to be a main contributor to the generation of cross-resistance between microbicides and antibiotics (Langsrud et al., 2004). A study by Braoudaki and Hilton (2004), demonstrate passaged *E. coli* strains against triclosan to exhibit susceptibility changes occurring; in conjunction with changes in antibiotic susceptibility. The *E. coli* strains shows low level cross-resistance occurring when repeatedly exposed to triclosan against antibiotics chloramphenicol and trimethoprim (Braoudaki and Hilton, 2004b). It is therefore apparent that when considering an application for microbicide use that understanding the effects that prolonged microbicide/formulation exposure has on antibiotic susceptibility in bacteria is of potential significance.

Chapter 3 examined the effect of sub-lethal exposure to microbicides and formulations on selected bacteria. Subsequently, this chapter will further characterise the additional effects of repeated microbicide/formulation exposure in bacteria, through evaluating fitness costs, changes

in pathogenicity, changes to biofilm formation and by assessing the induction of antibiotic cross resistance.

4.2 Materials and Methods

4.2.1 Chemicals and Growth Media

See section 3.2.2

4.2.2 Bacterial Strains and Drain Isolates

The bacterial strains used in this study are stated in section 3.2.1 and were classed as unexposed (P0), trained (P14) and detrained (X14) as stated in section 3.2.3; and exhibited a ≥4-fold increase in MIC, MBC or MBEC after microbicide/formulation exposure.

4.2.3 Planktonic growth rate

The unexposed (P0), exposed (P14) and detrained (X14) bacteria were grown in broth cultures overnight. The $OD_{600\ nm}$ of the bacteria was adjusted to 0.8 and diluted 1 in 100 in TSB/WCB (5 ml). The cultures were deposited into 96 well microtitre plates in triplicate and placed into a plate reader (BioTek, Bedfordshire, UK). Using the Gen5™1.08 software and a microplate spectrophotometer (PowerWave™ XS, BioTek, Swindon, UK), the optical densities were read every hour for 19 h to determine the planktonic growth rate of bacteria. Replicates were performed in triplicate, with 2 biological replications. Statistical significance was determined using a Mann-Whitney test ($P < 0.05$). Statistical significance was determined using a T-test and a modified Gompertz equation (Zwietering et al., 1990):

$$A\exp(\exp(\mu me/A(\gamma - t)+1)$$

Where A is stationary phase, exp is the exponential phase, μme is specific growth rate, γ is lag phase and t is time.

4.2.4 Determining Biofilm Growth using Crystal Violet assay

The assay was adapted from O'Toole (2011). Overnight bacterial cultures were diluted to an optical density of 0.8 and then further diluted 1:100 in broth (TSB/ WCB with 2.5% sucrose); aliquots of diluted inoculum (150 µl) were placed into 96 well microtitre plates and were incubated at 37ºC for 48 h (Corning Ltd., Corning, NY). After incubation media was aspirated and the wells were washed in 2 x 200 µl phosphate buffered saline (PBS) (Oxoid Ltd, Basingstock, UK). A solution of 0.5% (200 µl) crystal violet was added to each well and plates were incubated at room temperature for 30 minutes. Plates were washed in 2 x 200 µl PBS and left to dry at room temperature for 1 h prior to solubilisation of the crystal violet in 250 µl of 70% ethanol using agitation (20 rpm for 1 h). OD_{600nm} was determined for each well relative to a sterile control using a microplate spectrophotometer (PowerWave™ XS, BioTek, Swindon, UK) and Gen5™1.08 software (BioTek, Bedfordshire, UK) Biofilm formations were classed as an increase in OD_{600nm} relative to the sterile control. Replicates were done in triplicate, with 2 biological replications. Statistical significance was determined using a Kruskal-Wallis test ($P < 0.05$).

4.2.5 Competitive Fitness assay

A competitive fitness assay was adapted from Leroi *et al.* (1994). Overnight cultures of P0 and exposed bacteria (P14) were adjusted to an OD_{600nm} of 0.8 and then further diluted 1:100 in combinations into 200 ml of sterile broth to provide a mixed planktonic culture. Flasks were incubated aerobically at 37˚C for 24 h. Bacterial counts (cfu/ml) were determined at 0 h and 24 h via spot plating (10^{-2} to 10^{-8}; 3 x 20 µl) onto TSA plates and TSA containing a selective concentration of microbicide (0, 5, 10, 100 µg/ml). Agar plates were incubated for 24 h aerobically at 37˚C (n=3 x 2). Plates containing no microbicide were used as total count plates, whilst plates containing microbicide were used to determine growth of P14 bacteria. Competitive fitness was assessed using the equation;

$W = \ln (RF/RI)/ \ln (SF/SI)$

Where competitive fitness is W, resistant (P14) and susceptible (P0) cells at 0 h are RI and SI respectively and resistant and susceptible cells at 24 h are RF and SF, respectively. Statistical significance for bacterial counts before and after was determining using a Student's t test ($P < 0.05$).

4.2.6 *Galleria mellonella* pathogenicity assay

The methodology was based on one described previously by Latimer *et al.* (2012). Larvae of *Galleria mellonella* (Live Foods Direct, Sheffield, UK) were stored at 4°C for less than 3 days. The larvae were placed into 37°C for 30 minutes prior to injection, with 12 larvae being randomly assigned to each treatment group. Overnight cultures of P0, P14, X14, F14 and XF14 were pelleted (3,102 g, 8 min) and suspended in PBS, this was repeated twice. Cultures were adjusted to an OD_{600} of 0.1 (2×10^4 to 8×10^7 CFU/ml, confirmed by colony counts on TSA) and injected (5 µl) into the hemocele of each *G. mellonella* using a Hamilton syringe (CFU per individual), with 5 µl PBS injected into the control group. All treatment groups were incubated at 37°C, with a separate no treatment control and death rates were recorded daily (n=3 x 2). Dead *G. mellonella* were placed into sterile universals and homogenised in sterile 10 ml PBS. They were then serial diluted (10^{-1} to 10^{-8}) and spot plated onto TSA to calculate bacterial load per individual larvae. The experiments were terminated once 2 of the control individuals had died or turned into a chrysalis. After the data was collected a log-rank test was carried out to determine the statistical significance of the data. The equation used was:

Log-rank statistic = $(O_2 - E_2)^2$ / Var $(O_2 - E_2)$

Log-rank = (Observed P0 – Expected P0)^2/Expected P0 + (Observed P14 (or X14) – Expected P14 (or X14)^2/Expected P14 (or X14)

4.2.7 Antibiotic disc diffusion assay

Using the CLSI (clinical and laboratory standards institute) standardised method (Andrews et al. 2001); overnight cultures of bacteria were adjusted to 0.8 $OD_{600\ nm}$ and diluted 1:100 in sterile broth. Cotton wool swabs (Sainsbury's, Manchester, UK) were sterilised and dipped into the inoculum, with excess liquid removed. The swab was spread evenly across a TSA plate, changing direction 3 times to ensure even coverage. Plates were left to dry (10-15 mins) before 3 antibiotic discs were placed onto the plate at 3 separate locations. The antibiotic discs used were ampicillin (20 mcg), cephalothin (30 mcg), ciprofloxacin (1 mcg), kanamycin (5 mcg) and tetracycline (10 mcg). Plates were inverted and incubated aerobically at 37^{o}C for 24 h. Antibiotic inhibition zones were measured in mm using a ruler. Antibiotic susceptibility of test bacteria was determined using two biological and three technical replicates. Statistical significance of data was carried out using a Mann-Whitney U test.

4.3 Results

4.3.1 Analysis of Planktonic Growth rate

After repeated exposure to BAC (P14), 1/3 bacterial isolates demonstrated a decrease in growth rate in comparison to the parent strain (P0) (Figure 4.1.A-C); the *P. aeruginosa* isolate did not revert back to pre-exposed levels in the absence of microbicide (P-value = 0.3) (X14) (Figure 4.1.C). After repeated exposure to BAC formulation (F14) no bacteria exhibited significant changes in growth rate in comparison to the parent strain (P0) (Figure 4.1.D-E). After repeated exposure to DDAC (P14) 1/3 bacterial isolates demonstrated a decrease in growth rate in comparison to the parent strain (P0) (Figure 4.1.F-H); the *A. baumannii* isolate did not revert back to pre-exposed levels in the absence of microbicide (P-value = 0.34) (X14) (Figure 4.1.F). After repeated exposure to DDAC formulation (F14) 2/2 bacterial isolates demonstrated decreases in growth rate in comparison to the parent strain (P0); the isolates did not revert back to pre-exposed levels in the absence of microbicide (X14) (Figure 4.2.A-B).

After repeated exposure to CHX (P14) none of the bacterial isolates exhibited a change in growth rate in comparison to the parent strain (P0) (Figure 4.2.C-D). After repeated exposure to DMDM hydantoin (P14) the *E. coli* drain isolate demonstrated an increase in growth rate in comparison to the parent strain (P0); the isolate did not revert back to pre-exposed levels in the absence of microbicide (P-value = 0.14) (X14) (Figure 4.2.E). After repeated exposure to PHMB (P14) 1/5 isolates demonstrated a decrease in growth rate in comparison to the parent strain (P0) (Figure 4.2.F-H; Figure 4.3.A-B); the *E. faecalis* isolate partially reverted back to pre-exposed levels in the absence of microbicide (P-value > 0.05) (X14) (Figure 4.2.H). After repeated exposure to PHMB formulation (P14) none of the bacterial isolates demonstrated a change in growth rate in comparison to the parent strain (P0) (Figure 4.3. C-E).

After repeated exposure to triclosan (P14) 2/4 bacterial isolates demonstrated decreases in growth rate in comparison to the parent strain (P0) (Figure 4.3.F-H; Figure 4.4.A); from these isolates, *E. coli* fully reverted back to pre-exposed levels in the absence of microbicide (P-value = 0.08) (X14) (Figure 4.3.G). After repeated exposure to thymol formulation (F14) *P. putida*

demonstrated a quicker transition into the log phase when exposed (P-value > 0.05) (F14), however entered into stationary phase much quicker than the parent strain (P0) (Figure 4.4.B). After exposure to thymol formulation *E. coli* and *A. baumannii* demonstrate no significant changes in planktonic growth rate, whilst growth rate of X14 for both bacterial isolates, show an increase in specific growth rate (P-value = o.4) (Figure 4.4.C-D).

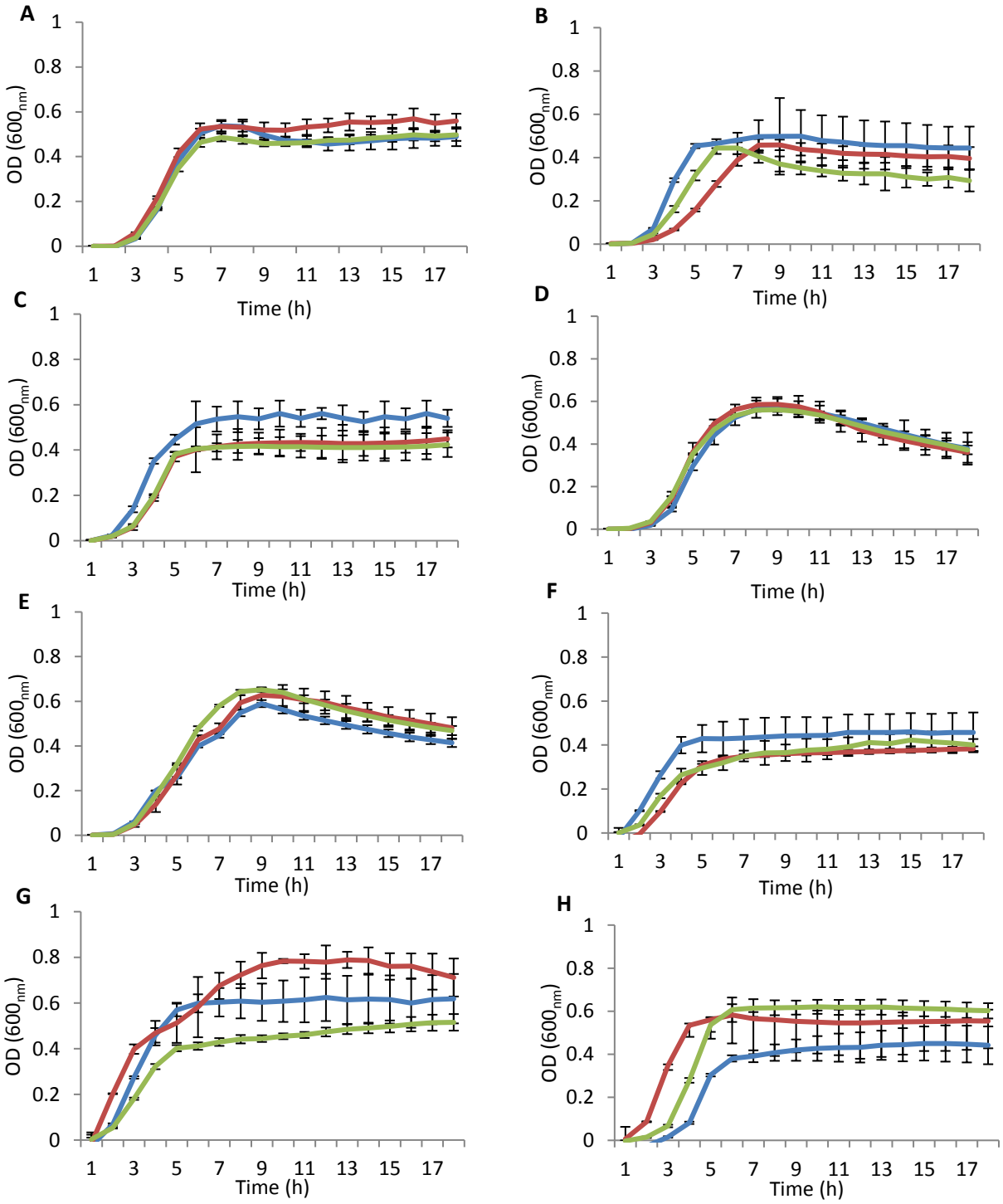

Figure 4.1 Bacterial planktonic growth rate of P0 (blue), P14/ F14 (red) and X14/ FX (green). A) *S.aureus* exposed to BAC; B) *E. coli* exposed to BAC; C) *P. aeruginosa* exposed to BAC; D) *S. aureus* exposed to BAC formulation; E) *E. coli* exposed to BAC formulation; F) *A. baumannii* exposed to DDAC; G) *E. coli* drain isolate exposed to DDAC; H) *P. aeruginosa* exposed to DDAC. Error bars show standard deviation of optical density of bacteria (n=3).

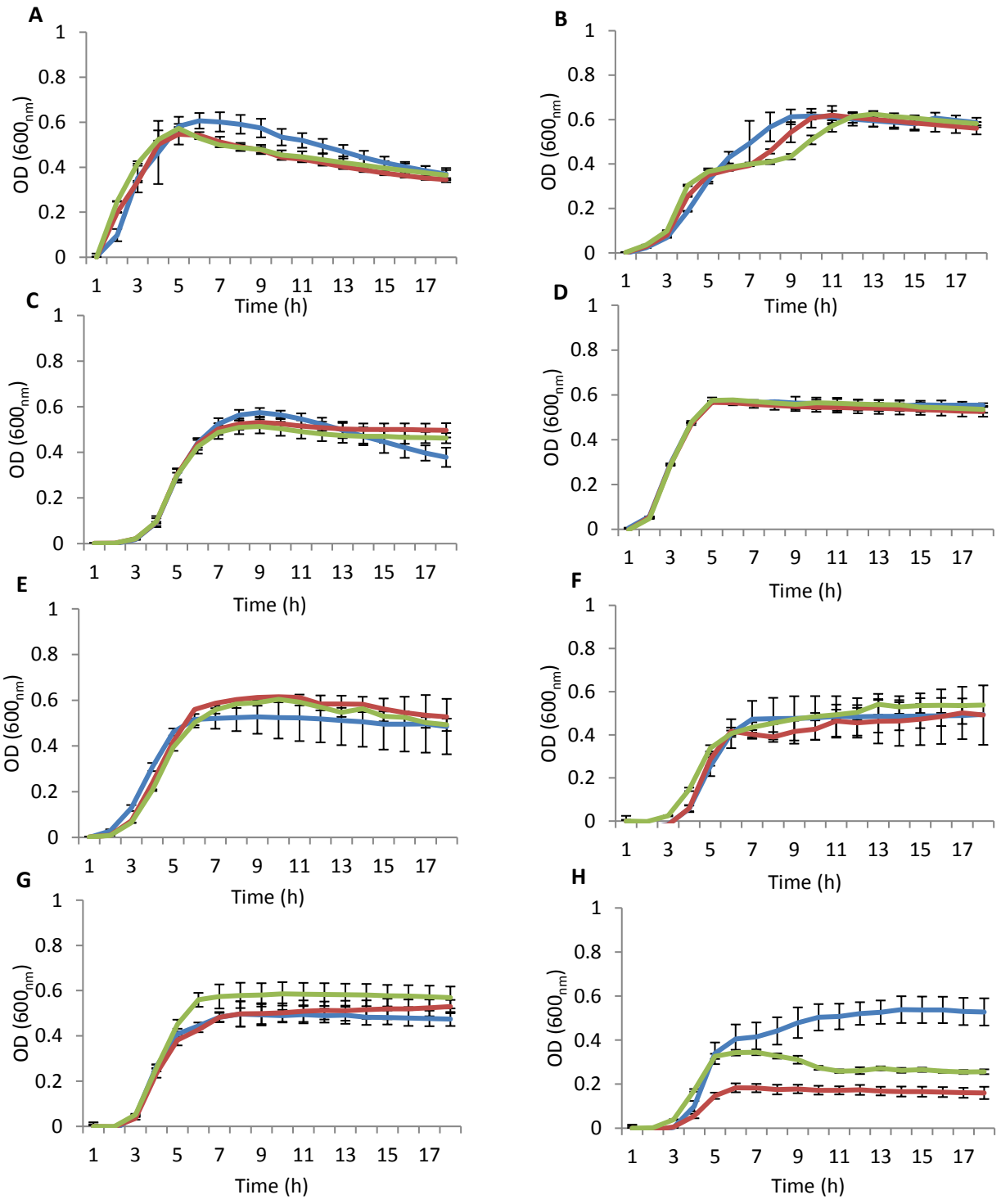

Figure 4.2 Bacterial planktonic growth rates of P0 (blue), P14/ F14 (red) and X14/ FX (green). A) the *E. coli* drain isolate exposed to DDAC formulation; B) *P. aeruginosa* exposed to DDAC formulation; C) *S. aureus* exposed to CHX; D) *E. coli* exposed to CHX.; E) *E. coli* drain isolate exposed to DMDM hydantoin; F) *S. aureus* exposed to PHMB; G) *E. coli* exposed to PHMB; H) *E. faecalis* exposed to PHMB. Error bars show standard deviation of optical density of bacteria (n=3).

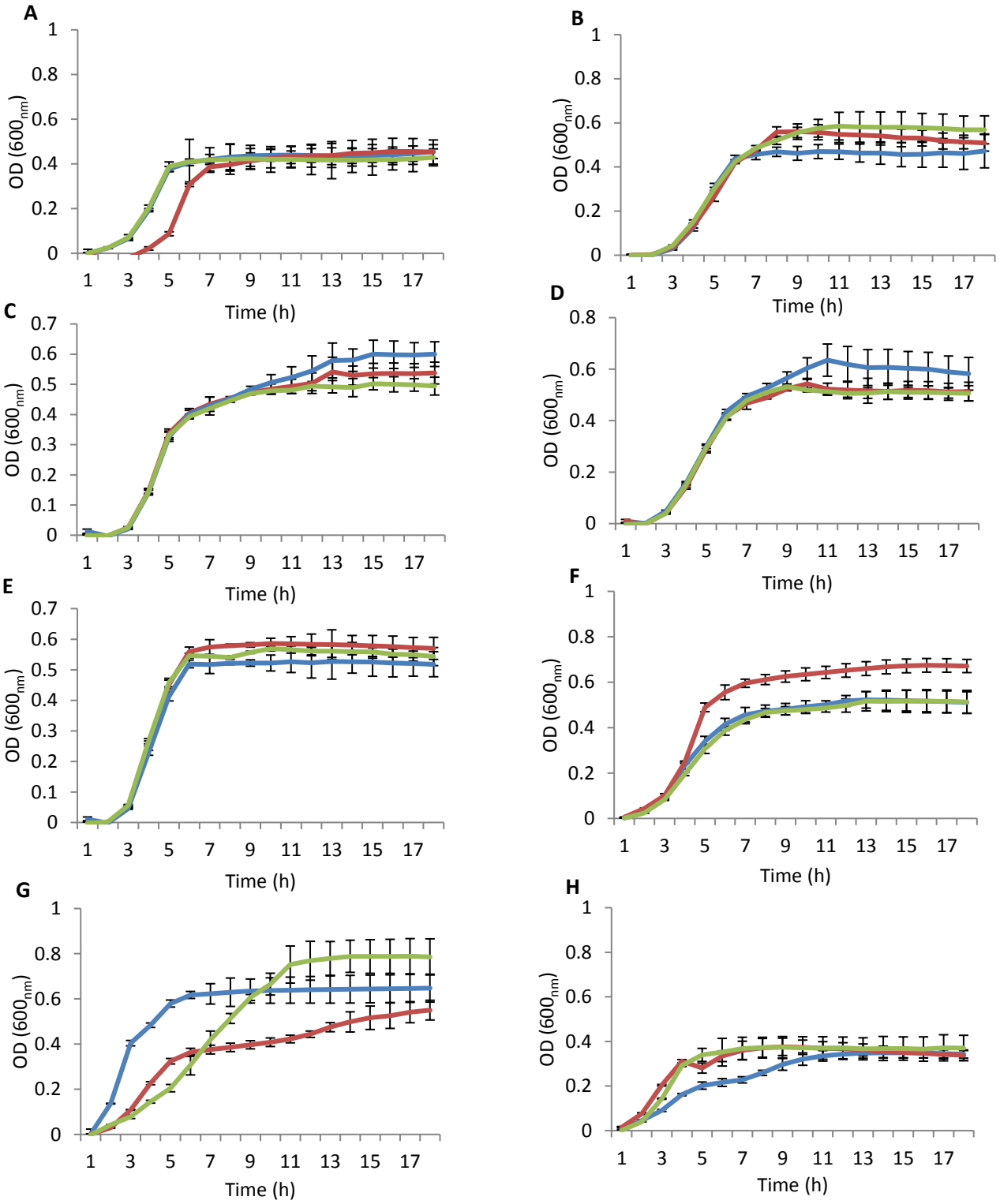

Figure 4.3 Bacterial planktonic growth rate of P0 (blue), P14 /F14 (red) and X14/ FX (green). A) *P. aeruginosa* exposed to PHMB; B) *E. coli* drain isolate exposed to PHMB; C) *S. aureus* exposed to PHMB formulation D) *E. coli* exposed to PHMB formulation; E) *P. aeruginosa* exposed to PHMB formulation; F) *S. aureus* exposed to triclosan; G) *E. coli* exposed to triclosan H) *C. sakazaki* exposed to triclosan. Error bars show standard deviation of optical density of bacteria (n=3).

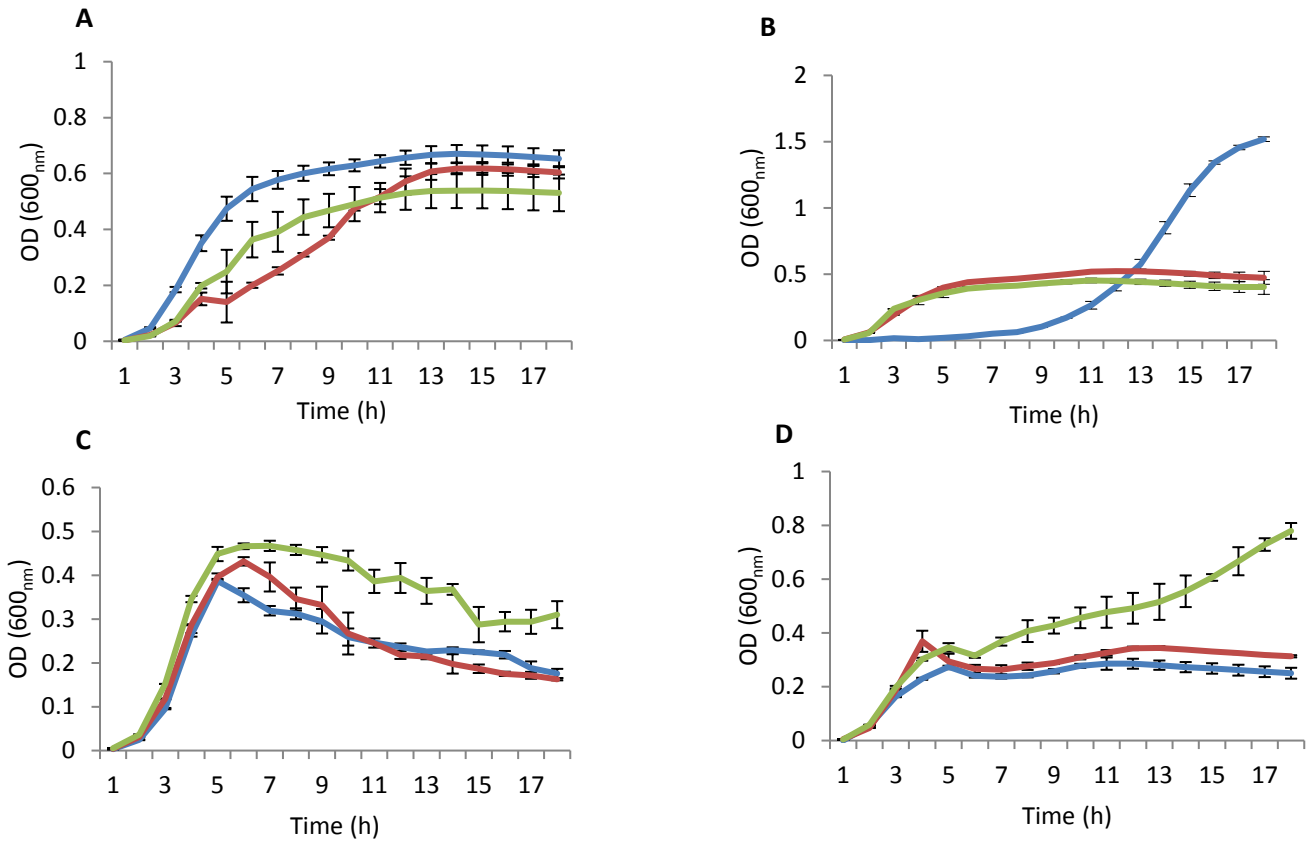

Figure 4.4 Bacterial planktonic growth rate of P0 (blue), P14/ F14 (red) and X14/ FX (green). A) the *E. coli* drain isolate exposed to triclosan; B) *P. putida* exposed to thymol formulation; C) *E. coli* exposed to thymol formulation; D) *A. baumannii* exposed to thymol formulation. Error bars show standard deviation of optical density of bacteria (n=3).

4.3.1 Biofilm growth analysis of pre-exposed bacteria

These data show that 8/18 of the unexposed bacteria (P0) produced a higher density of biofilm formation when compared to P14. In comparison 8/18 of the P14 bacteria were observed to produce a higher density of biofilm formation when compared to the parent strain (P0). Formulated actives show that 3/10 of the unexposed bacteria (P0) demonstrated a higher density of biofilm formation when compared to F14. In comparison 2/10 of the F14 bacteria were observed to produce a higher density of biofilm formation when compared to the parent strain (P0).

After repeated exposure to BAC (P14) *E. coli* demonstrated an increase in biofilm formation ($P < 0.05$); whilst *P. aeruginosa* demonstrated a decrease in biofilm formation ($P < 0.05$) in comparison to the parent strain (P0); from these isolates *E. coli* fully reverted back to pre-exposed levels, whilst *P. aeruginosa* partially reverted back to pre-exposed levels in the absence of microbicide (X14) (Figure 4.5.A). After repeated exposure to BAC formulation (F14) none of the bacterial isolates demonstrated a significant change in biofilm formation in comparison to the parent strain (P0) (Figure 4.5.A). After repeated exposure to DDAC (P14) *A. baumannii* and the *E. coli* drain isolate demonstrated an increase in biofilm formation ($P < 0.05$) in comparison to the parent strain (P0), whilst *P. aeruginosa* demonstrated a decrease ($P < 0.05$); from these isolates *A. baumannii* partially reverted back to pre-exposed levels in the absence of microbicide (X14) (Figure 4.5.B). After repeated exposure to DDAC formulation (F14) the *E. coli* drain isolate demonstrated an increase in biofilm formation ($P < 0.05$) in comparison to the parent strain (P0); the *E. coli* drain isolate partially reverted back to pre-exposed levels (XF) (Figure 4.5.B).

After repeated exposure to CHX (P14) *E. coli* demonstrated a decrease in biofilm formation ($P < 0.05$) in comparison to the parent strain (P0); the *E. coli* isolate fully reverted back to pre-exposed levels in the absence of microbicide (X14) (Figure 4.5.C). After repeated exposure to DMDM hydantoin (P14) the *E. coli* drain isolate did not demonstrate a significant change in biofilm formation in comparison to the parent strain (P0) (Figure 4.5.D). After repeated exposure to PHMB (P14) *E. coli* and *S. aureus* demonstrated a decrease in biofilm formation ($P < 0.05$) in comparison to the parent strain (P0), whilst *E. faecalis* and the *E. coli* drain isolate demonstrated an increase in biofilm formation ($P < 0.05$); from these isolates *E. faecalis* partially reverted back

to pre-exposed levels in the absence of microbicide (X14) (Figure 4.5.A). After repeated exposure to PHMB formulation (F14) *S. aureus* demonstrated a decrease in biofilm formation ($P < 0.05$), whilst *E. faecalis* demonstrated an increase in biofilm formation ($P < 0.05$) in comparison to the parent strain (P0); the *S. aureus* isolate fully reverted back to pre-exposed levels (XF) (Figure 4.6.A).

After repeated exposure to triclosan (P14) *S. aureus* and the *E. coli* drain isolate demonstrated a decrease in biofilm formation in comparison to the parent strain (P0); from these isolates *S. aureus* fully reverted back to pre-exposed levels in the absence of microbicide (X14) (Figure 4.6.B). After repeated exposure to thymol formulation (P14) *E. coli* demonstrated an increase in biofilm formation ($P < 0.05$), whilst *P. putida* and *A. baumannii* shows a decrease in biofilm formation ($P < 0.05$) in comparison to the parent strain (P0); the *E. coli* isolate did not revert back to pre-exposed levels in the absence of microbicide (X14) (Figure 4.6.C).

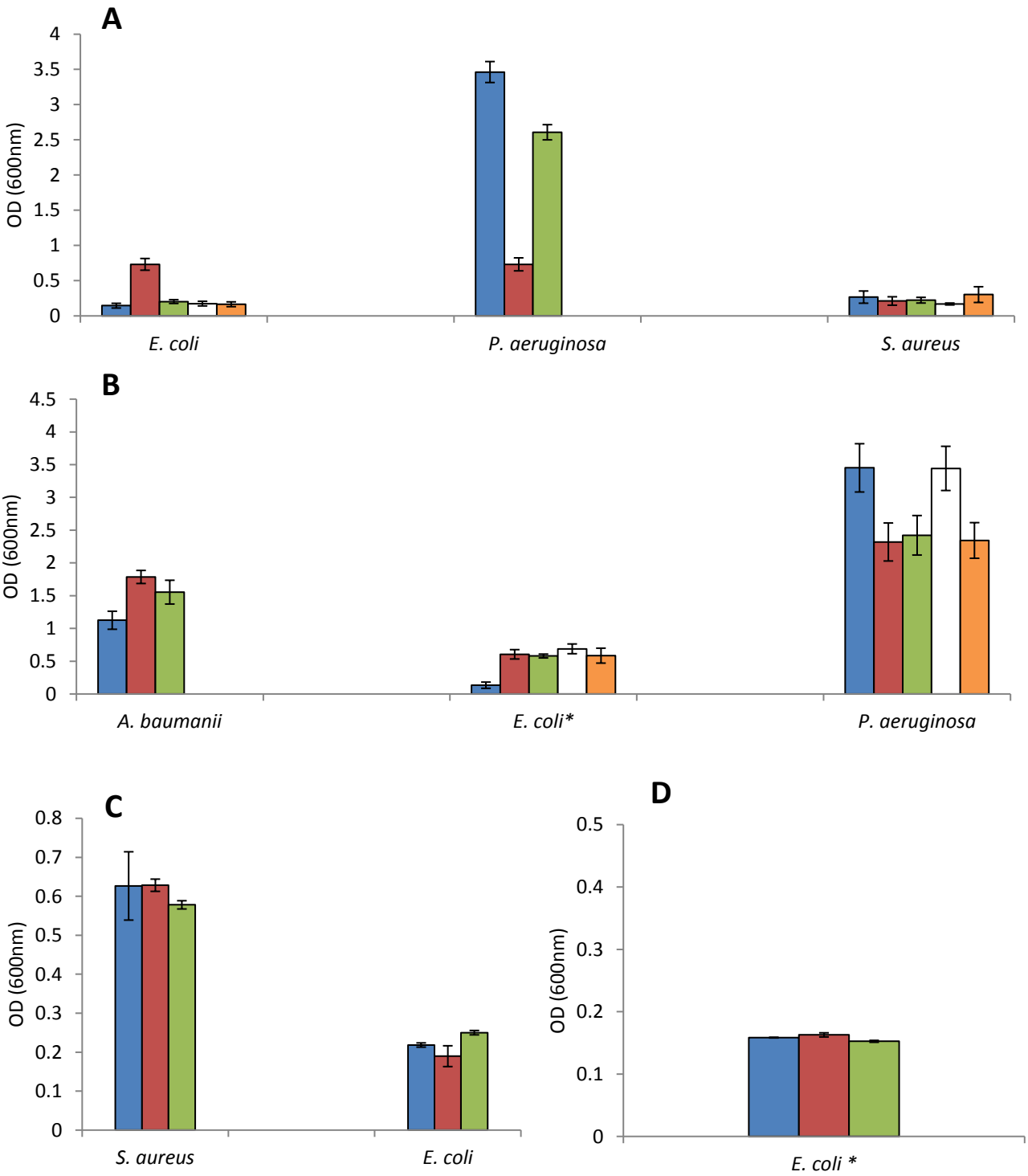

Figure 4.5 Bacterial biofilm formation of each organism expressing a ≥4-fold change, at pre-exposed (P0) (blue bar), exposed (P14) (red bar), detrained (X14) (green bar), exposed to formulation microbicides (F14) (white bar) and detrained formulation microbicides (FX) (orange bar). A) Organisms exposed to benzalkonium chloride; B) organisms exposed to didecyldimethyl ammonium chloride; C) organisms exposed to chlorhexidine; D) organisms exposed to DMDM hydantoin. Error bars show standard deviation of bacterial strains biofilm formation (n=3). * drain isolate

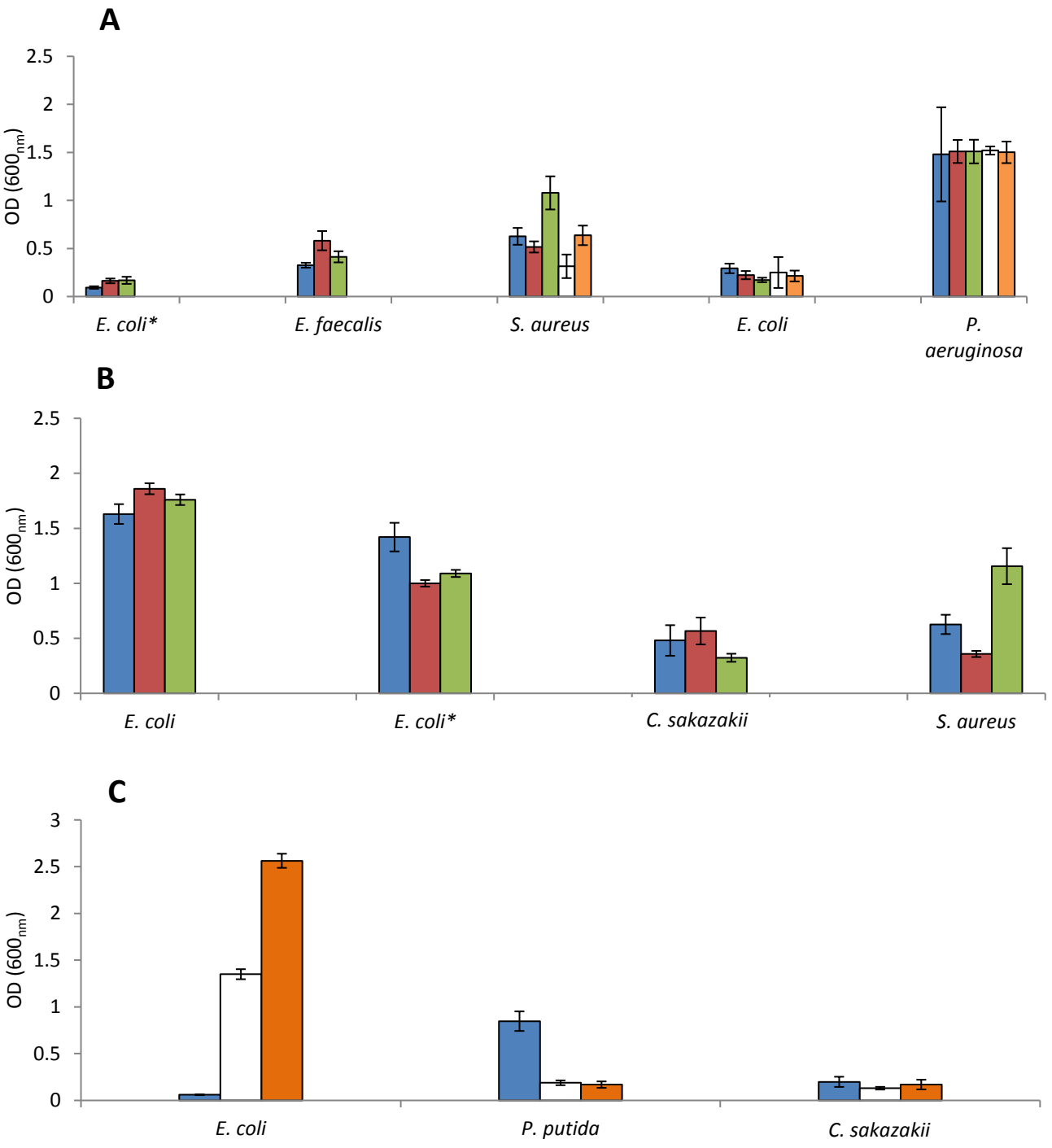

Figure 4.6 Bacterial biofilm formation of each organism expressing a ≥4-fold change, at pre-exposed (P0) (blue bar), exposed (P14) (red bar), detrained (X14) (green bar), exposed to formulation microbicides (F14) (white bar) and detrained formulation microbicides (FX) (orange bar). A) Optical density of organisms exposed to PHMB; B) Optical density of organisms exposed to triclosan; C) Optical density of organisms exposed to thymol formulation. Error bars show standard deviation of bacterial strains biofilm formation (n=3). * drain isolate.

4.3.2 Competitive Fitness Analysis of Unexposed/ Exposed Bacteria

According to the test system used, a relative fitness (W) of 1 indicates no fitness effect between bacterial strains, a value of below 1 implies impaired fitness and above 1 enhanced fitness (Paulander et al., 2012). When testing bacterial competitive fitness, thymol precipitated out of the agar and therefore was unable to be tested. After exposure to BAC (P14) *S. aureus* showed an increase in competitive fitness ($P < 0.05$) and *P. aeruginosa* ($P < 0.05$) and *E. coli* had showed a decrease in fitness (Figure 4.7.A). After exposure to BAC formulation (F14) both *S. aureus* ($P < 0.05$) and *E. coli* demonstrated a decrease in competitive fitness (Figure 4.7.A). After exposure to DDAC (P14) all bacterial isolates showed a decrease in competitive fitness ($P < 0.05$) (Figure 4.7.B).

After exposure to DMDM hydantoin (P14) the *E. coli* drain isolate showed a decrease in competitive fitness (Figure 4.7.C). After exposure to CHX (P14) *E. coli* shown an increase in competitive fitness ($P < 0.05$) and *S. aureus* demonstrated a decrease in fitness ($P < 0.05$) (Figure 4.7.D). After exposure to PHMB (P14) all bacterial isolates showed a decrease in competitive fitness ($P < 0.05$) (Figure 4.8.A). After exposure to PHMB formulation (F14) all bacterial isolates demonstrated a decrease in competitive fitness ($P < 0.05$) (Figure 4.8.A). After exposure to triclosan (P14) all bacterial isolates showed a decrease in competitive fitness ($P < 0.05$) (Figure 4.8.B).

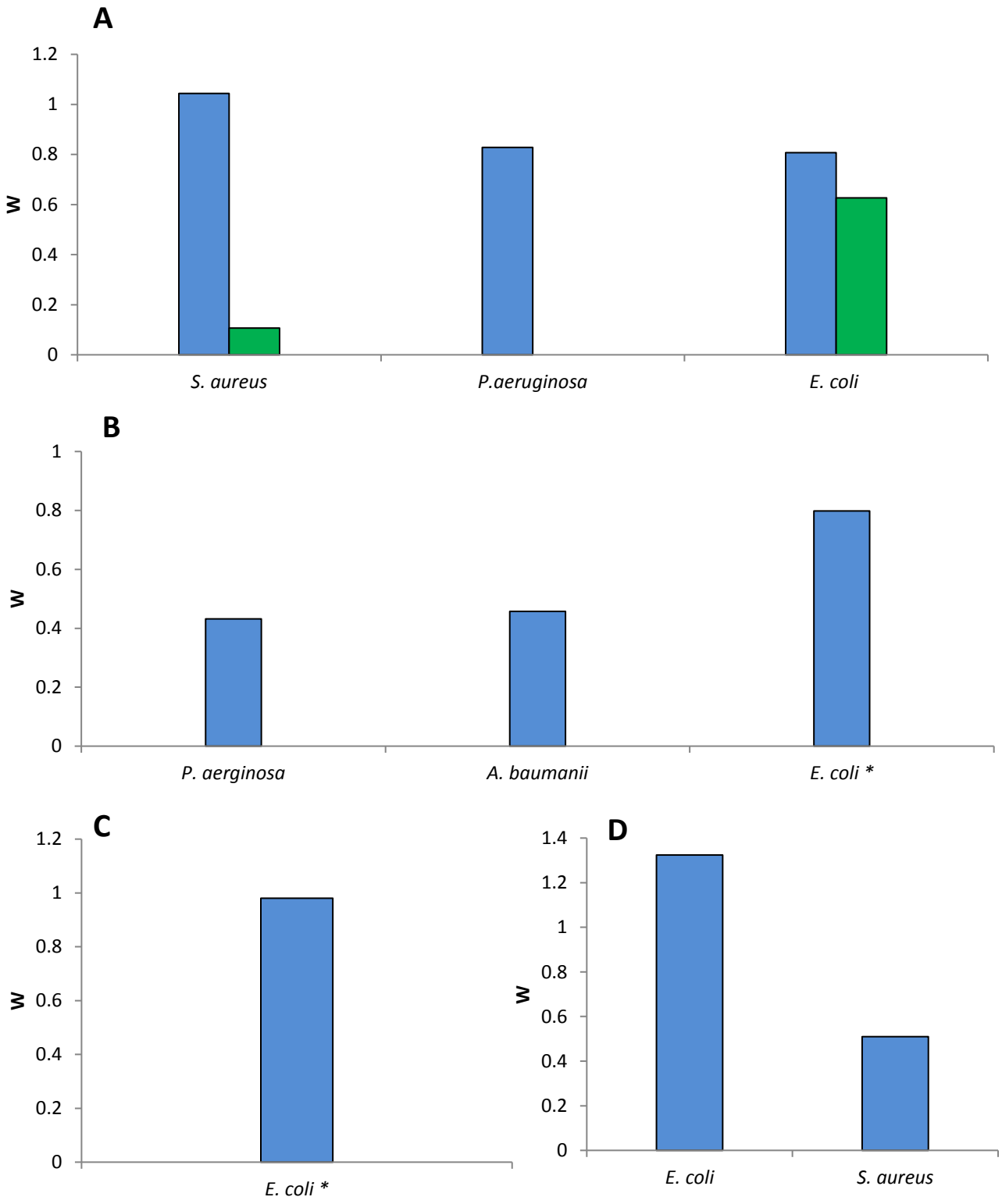

Figure 4.7 The competitive fitness of selected organisms against pure actives (blue bars) and formulation actives (green bar). Values over 1 show a higher fitness for P14; values below 1 show a higher fitness for P0. A) Fitness of bacteria exposed to benzalkonium chloride; B) fitness levels exposed to DDAC; C) fitness of bacteria exposed to DMDM hydantoin; D) fitness of bacteria exposed to CHX. * drain isolate.

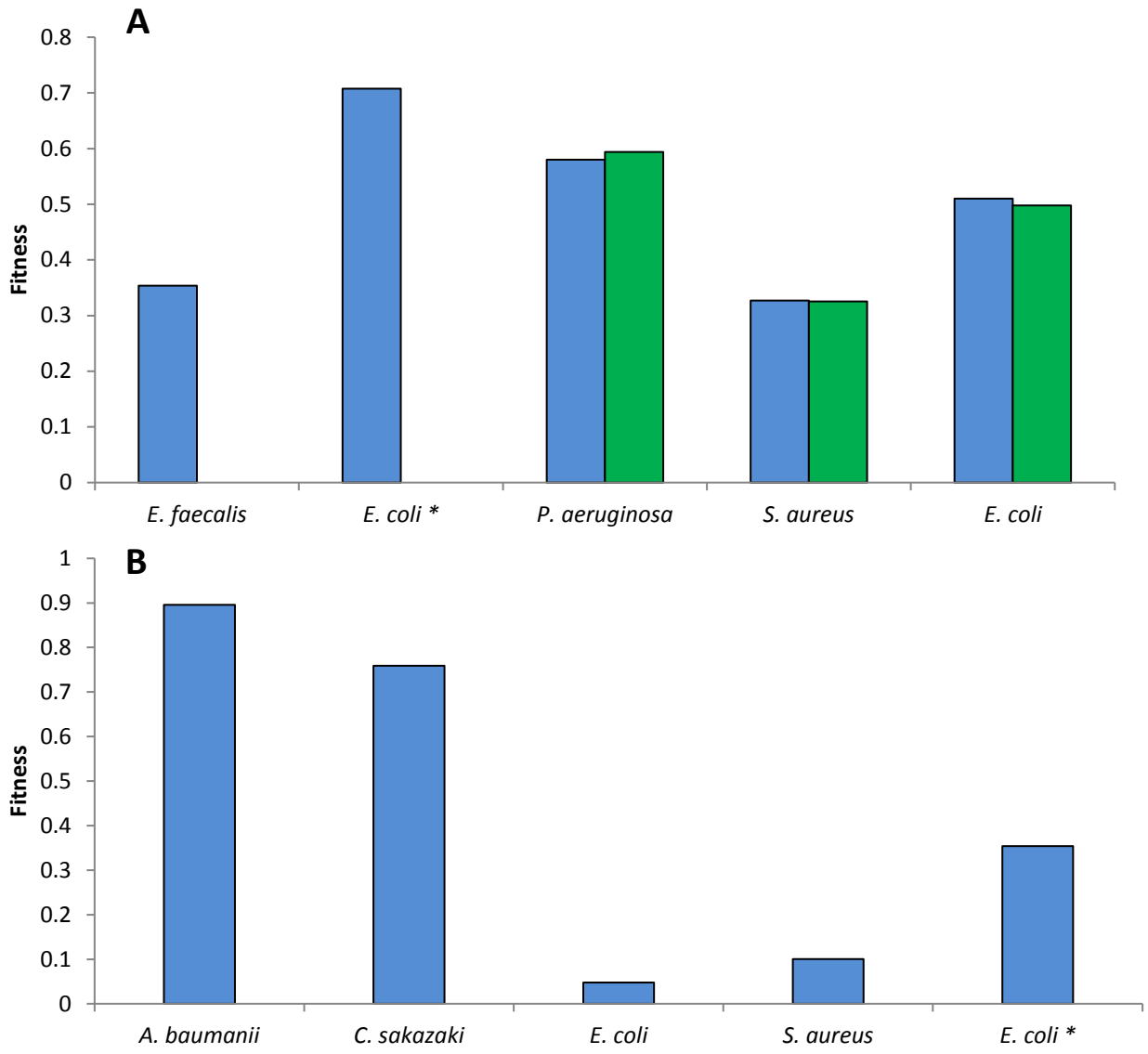

Figure 4.8 The competitive fitness of selected organisms against pure actives (blue bars) and formulation actives (green bar). Values over 1 show a higher fitness for P14; values below 1 show a higher fitness for P0. A) Fitness of bacteria exposed to PHMB; B) Fitness of bacteria exposed to triclosan. * drain isolate.

4.3.3 Analysis of Bacterial Pathogenicity using *Galleria mellonella* Model

Selected bacteria demonstrating 4 fold and above changes in MBC during pure culture training were examined for changes in pathogenicity, using the *G. mellonella* pathogenicity assay. Differences in pathogenicity, as determined through larvae death rate, were examined over a 7-day period, with the bacterial load of each waxworm calculated after death. Due to the high pathogenicity of *P. aeruginosa* (all isolates caused total larvae death before 24 hours) there is no analytical data for this species. In contrast, *C. sakazakiii* and *A. baumannii* demonstrated no larvae deaths and therefore no analytic data was available.

After exposure to BAC (P14) *E. coli* showed a decreased pathogenicity when compared to P0; with partial reversion in the absence of microbicide (X14). The bacterial load of *E. coli* showed P0 demonstrating the highest bacterial load (Figure 4.9.A). The log-rank for *E. coli* exposed to BAC for P0 against P14 was 36.39 ($P < 0.01$); P0 against X14 log-rank was 24.73 ($P < 0.01$); P14 against X14 log-rank was 11.97 ($P < 0.01$). After exposure to BAC (P14) *S. aureus* showed a decreased pathogenicity when compared to P0, with full reversion in the absence of microbicide (X14) (Figure 4.9.B). The log-rank for *S. aureus* exposed to BAC for P0 against P14 was 5.49 ($P < 0.05$); P0 against X14 log-rank was 7.08 ($P < 0.01$); P14 against X14 has no statistical significance. After exposure to BAC formulation (F14) *E. coli* demonstrated a decreased pathogenicity when compared to P0, with no reversion in the absence of microbicide (FX) (Figure 4.9.C). The log-rank for *E. coli* exposed to BAC formulation for P0 against F14 was 185.26 ($P < 0.01$); P0 against FX log-rank was 191.30 ($P < 0.01$); F14 against FX log-rank was 9.89 ($P < 0.01$). After exposure to BAC formulation (F14) *S. aureus* showed an increased pathogenicity when compared to P0, with no reversion in the absence of microbicide (FX) (Figure 4.9.D). The log-rank for *S. aureus* exposed to BAC formulation for P0 against F14 was 6.94 ($P < 0.01$); P0 against FX log-rank was 6.28 ($P < 0.01$); P14 against X14 has no statistical significance.

After exposure to DDAC (P14) the *E. coli* drain isolate demonstrated a decrease in pathogenicity when compared to P0, with full reversion in the absence of microbicide (X14) (Figure 4.10.A). The log-rank for the *E. coli* drain isolate exposed to DDAC for P0 against P14 was 167.5

(P < 0.01); P0 against X14 log-rank was not statistically significant. After exposure to DDAC formulation (F14) the *E. coli* drain isolate showed a decreased pathogenicity when compared to P0, with partial reversion in the absence of microbicide (FX) (Figure 4.10.B). The log-rank for the *E. coli* drain isolate exposed to DDAC formulation for P0 against F14 was 167.5 (P < 0.01); P0 against FX log-rank was 196.7 (P < 0.01).

After exposure to CHX (P14) *E. coli* showed no change in pathogenicity when compared to P0 (Figure 4.10.C). The log-rank for *E. coli* exposed to CHX was not attainable. After exposure to CHX (P14) *S. aureus* showed no change in pathogenicity when compared to P0 (Figure 4.10.D). The log-rank for *S. aureus* exposed to BAC for P0 against P14 was 0.34 (P = 0.56); P0 against X14 log-rank was 0.31 (P = 0.55); P14 against X14 log-rank was 0.12 (P = 0.72). After exposure to DMDM hydantoin (P14) the *E. coli* drain isolate showed a decreased pathogenicity when compared to P0, with no reversion in the absence of microbicide (X14) (Figure 4.11.A). The log-rank for the *E. coli* drain isolate exposed to DMDM hydantoin for P0 against P14 was 49.03 (P < 0.01); P0 against X14 log-rank was 52.59 (P < 0.01); P14 against X14 log-rank was 6.61 (P < 0.05).

After exposure to PHMB (P14) the *E. coli* drain isolate showed no significant pathogenicity change when compared to P0 (Figure 4.11.B). After exposure to PHMB (P14) *S. aureus* showed a decreased pathogenicity when compared to P0, with full reversion in the absence of microbicide (X14) (Figure 4.11.C). The log-rank for *S. aureus* exposed to PHMB for P0 against P14 was 8.83 (P < 0.01); P0 against X14 log-rank was 9.54 (P < 0.01). After exposure to PHMB (P14) *E. coli* showed no significant pathogenicity change when compared to P0 (Figure 4.11.D). After exposure to PHMB (P14) *E. faecalis s*howed a decreased pathogenicity when compared to P0, with partial reversion in the absence of microbicide (X14) (Figure 4.12.A). The log-rank for *E. faecalis* exposed to PHMB for P0 against P14 was 32.92 (P < 0.01); P0 against X14 log-rank was 31.17 (P < 0.01). After exposure to PHMB formulation (P14) *E. coli* showed no significant pathogenicity change when compared to P0 (Figure 4.12.B). After exposure to PHMB formulation (P14) *S. aureus* showed no significant pathogenicity change when compared to P0 (Figure 4.12.C).

After exposure to triclosan (P14) the *E. coli* drain isolate showed a complete absence of pathogenicity when compared to P0, with partial reversion in the absence of microbicide (X14) (Figure 4.12.D). The log-rank for the *E. coli* drain isolate exposed to triclosan for P0 against P14 was 163.72 ($P < 0.01$); P0 against X14 log-rank was 148.09 ($P < 0.01$); P14 against X14 log-rank was 17.01 ($P < 0.01$). After exposure to triclosan (P14) *S. aureus* showed a decrease in pathogenicity when compared to P0, with full reversion in the absence of microbicide (X14) (Figure 4.13.A). The log-rank for *S. aureus* exposed to triclosan for P0 against P14 was 6.34 ($P < 0.05$); P0 against X14 log-rank was 3.40 ($P < 0.01$). After exposure to triclosan (P14) *E. coli* showed a complete absence of pathogenicity when compared to P0, no reversion in the absence of microbicide (X14) (Figure 4.13.B). The log-rank for *E. coli* exposed to triclosan was not attainable.

After exposure to thymol formulation (F14) *E. coli* showed no significant pathogenicity change when compared to P0 (Figure 4.13.C). After exposure to thymol formulation (F14) *P. putida* showed an increase in pathogenicity when compared to P0, with full reversion in the absence of microbicide (FX) (Figure 4.13.D). The log-rank for *P. putida* exposed to thymol formulation for P0 against F14 was 515.22 ($P < 0.01$); P0 against FX log-rank was 20.94 ($P < 0.01$).

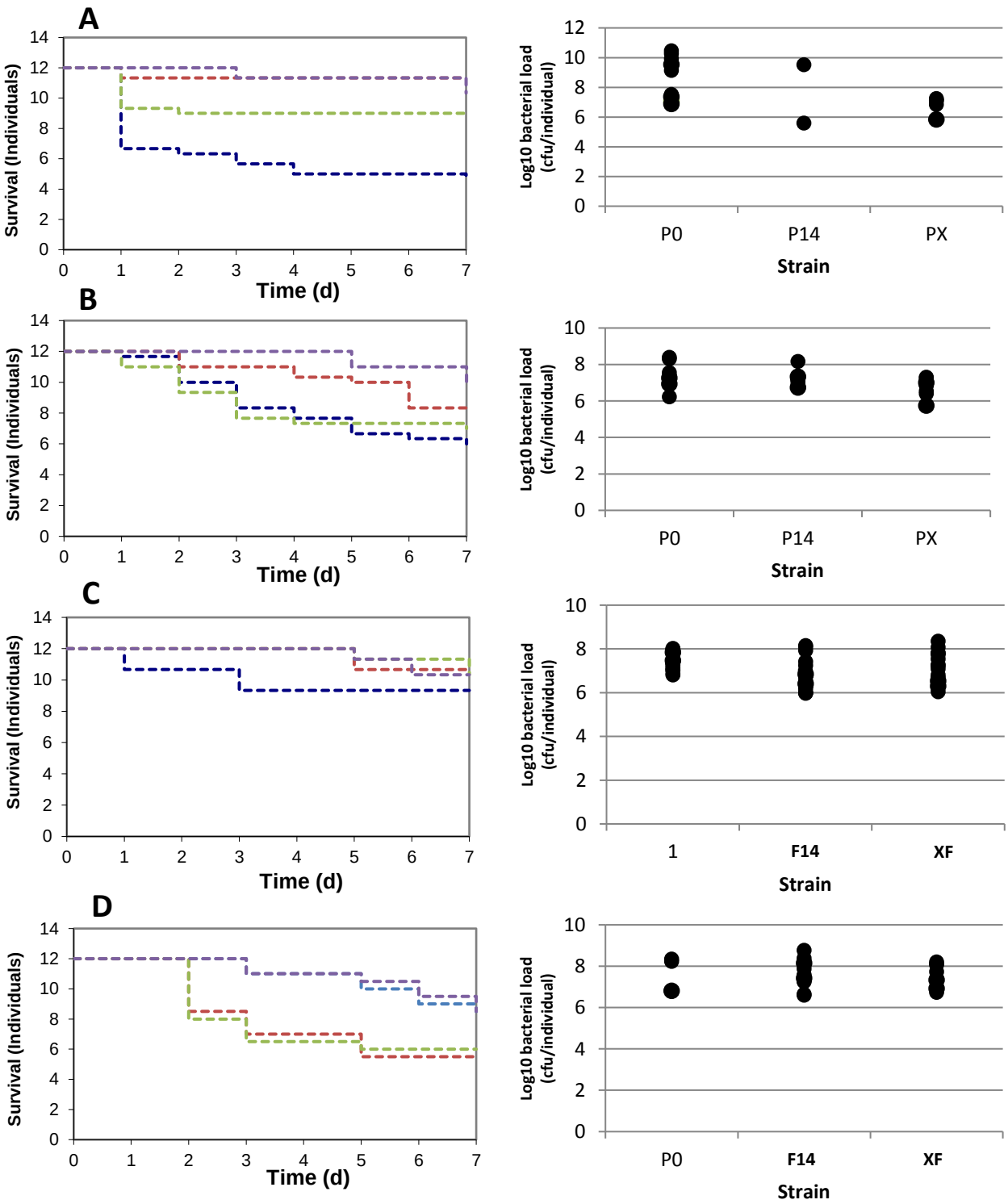

Figure 4.9 *Galleria mellonella* pathogenicity assay. Data shows survival curve, P0 (blue dashed line), P14/ F14 (red dashed line), X14/ FX (green dashed line) and control (purple dashed line) along with bacterial load at time of death (black dot indicates individual caterpillar). A) Survival curve and bacterial load of dead worms of *E. coli* after exposure to BAC; B) Survival curve and bacterial load of dead worms of *S. aureus* after exposure to BAC; C); Survival curve and bacterial load of dead worms of *E. coli* after exposure to BAC in formulation; D) Survival curve and bacterial load of dead worms of *S. aureus* after exposure to BAC in formulation.

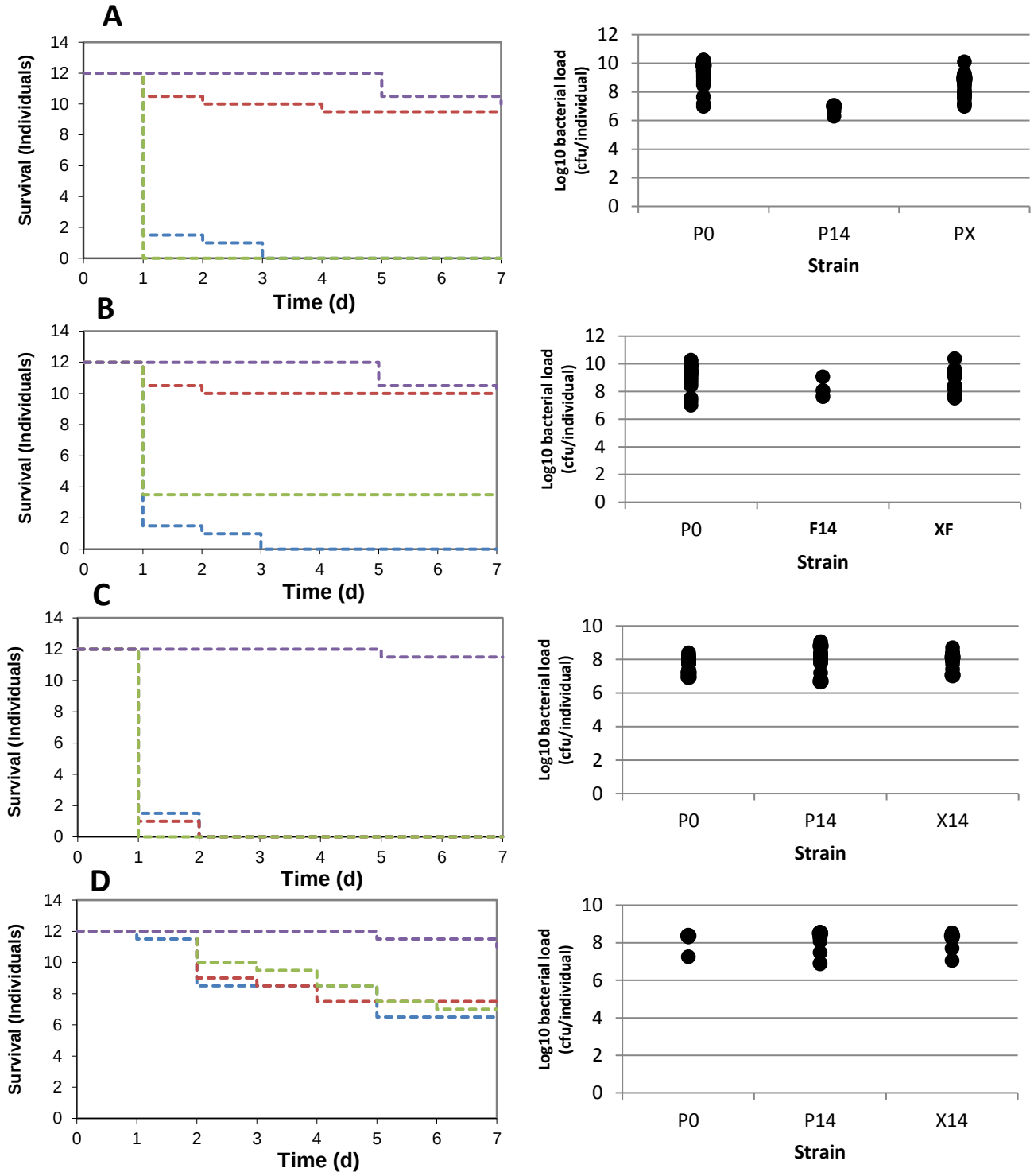

Figure 4.10 *Galleria mellonella* pathogenicity assay. Data shows survival curve, P0 (blue dashed line), P14/ F14 (red dashed line), X14/ FX (green dashed line) and control (purple dashed line) along with bacterial load at time of death (black dot indicates individual caterpillar). A) Survival curve and bacterial load of dead worms of the *E. coli* drain isolate after exposure to DDAC; B) Survival curve and bacterial load of dead worms of the *E. coli* drain isolate after exposure to DDAC formulation; C) Survival curve and bacterial load of dead worms of *E. coli* after exposure to CHX; D) Survival curve and bacterial cell density associated with dead worms of *S. aureus* after exposure to CHX.

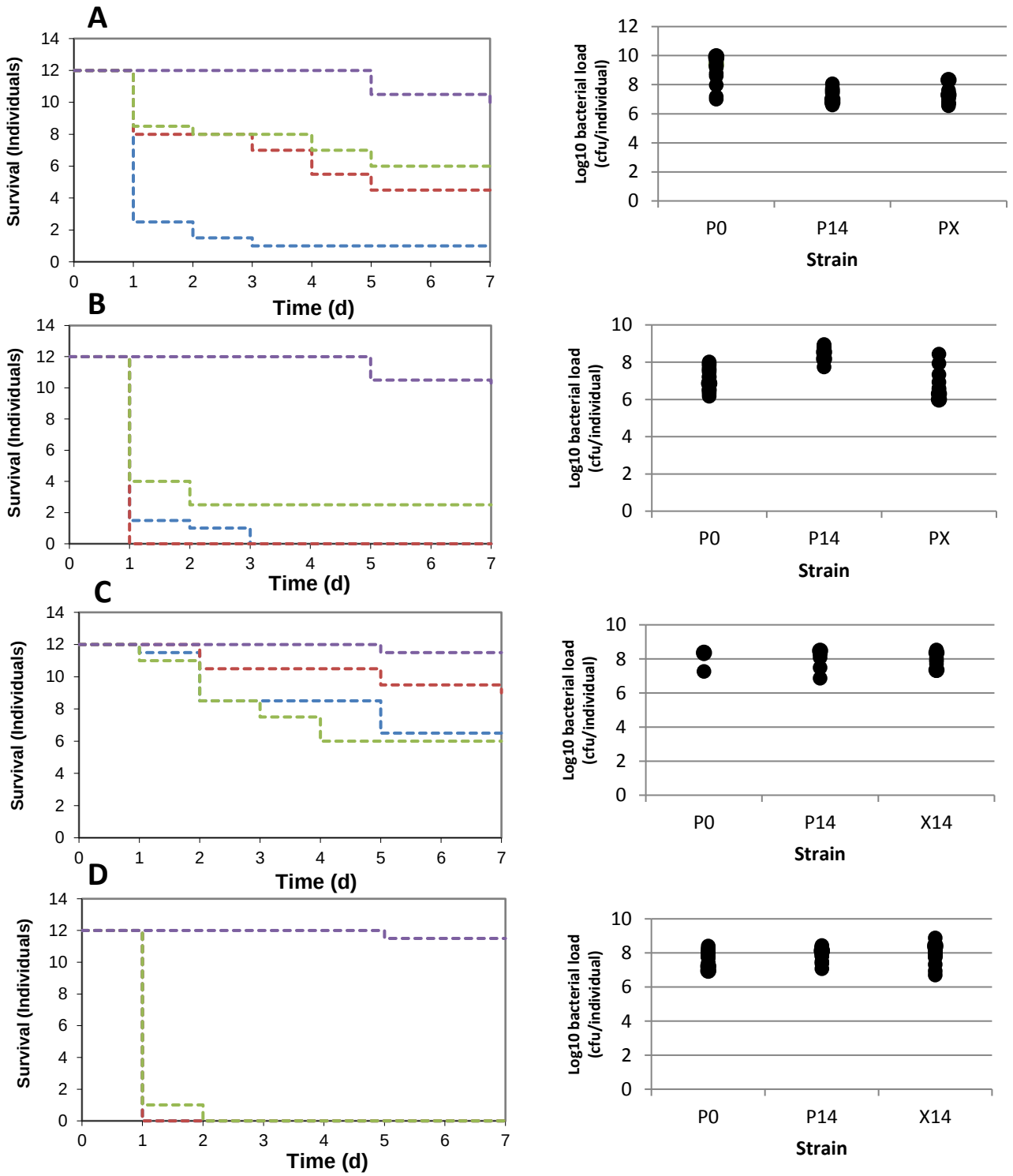

Figure 4.11 *Galleria mellonella* pathogenicity assay. Data shows survival curve, P0 (blue dashed line), P14 (red dashed line), X14 (green dashed line) and control (purple dashed line) along with bacterial load at time of death (black dot indicates individual caterpillar). A) Survival curve and bacterial load of dead worms of the *E. coli* drain isolate after exposure to DMDM hydantoin; B) Survival curve and bacterial load of dead worms of the *E. coli* drain isolate after exposure PHMB; C) Survival curve and bacterial load of dead worms of *S. aureus* after exposure to PHMB; D) Survival curve and bacterial load of dead worms of *E. coli* after exposure to PHMB.

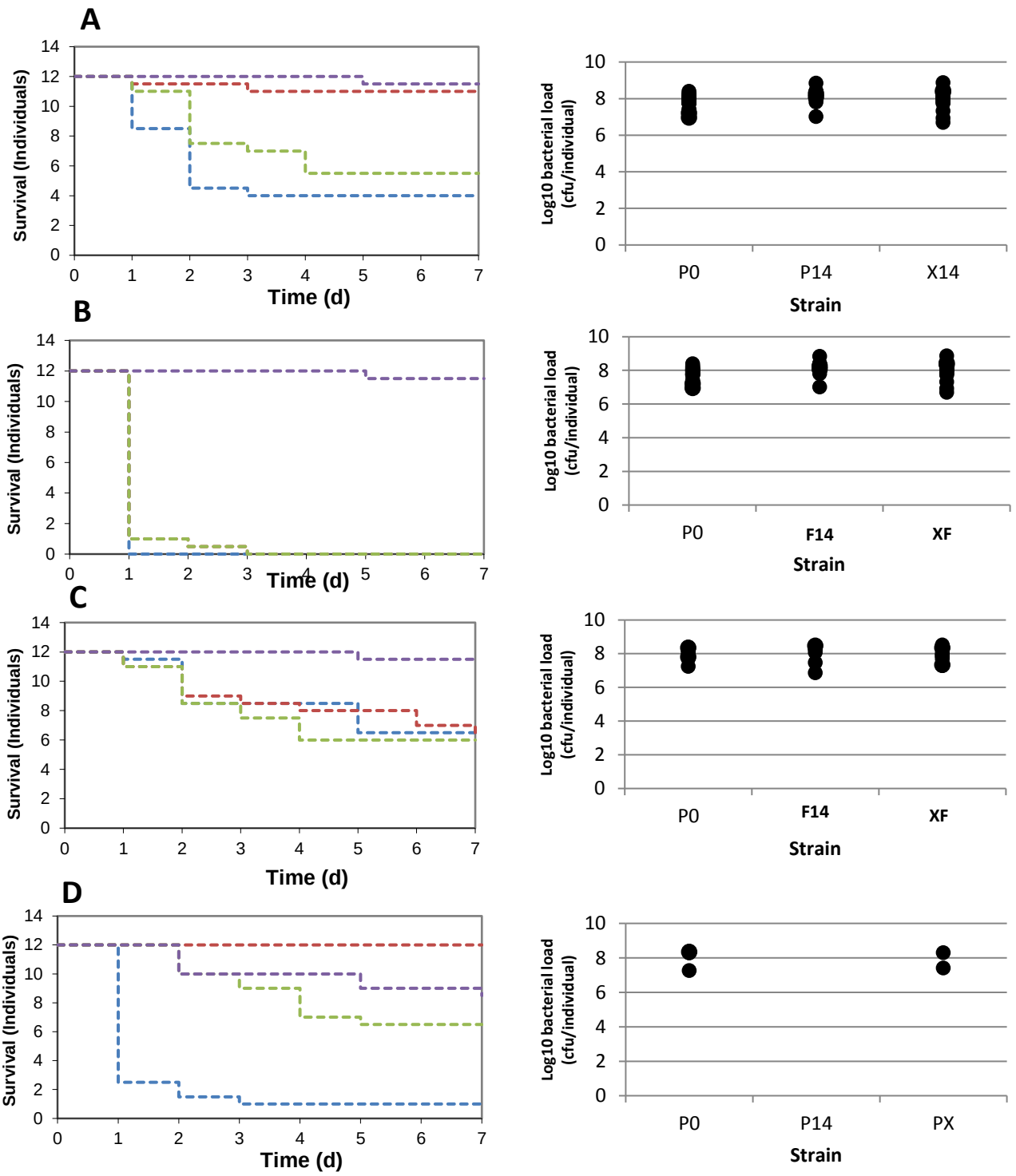

Figure 4.12 *Galleria mellonella* pathogenicity assay. Data shows survival curve, P0 (blue dashed line), P14/ F14 (red dashed line), X14/ FX (green dashed line) and control (purple dashed line) along with bacterial load at time of death (black dot indicates individual caterpillar). A) Survival curve and bacterial load of dead worms of *E. faecalis* after exposure to PHMB; B) Survival curve and bacterial load of dead worms of *E. coli* after exposure to PHMB in formulation; C) Survival curve and bacterial load of dead worms of S. aureus after exposure to PHMB in formulation; D) Survival curve and bacterial load of dead worms of the *E. coli* drain isolate after exposure to triclosan.

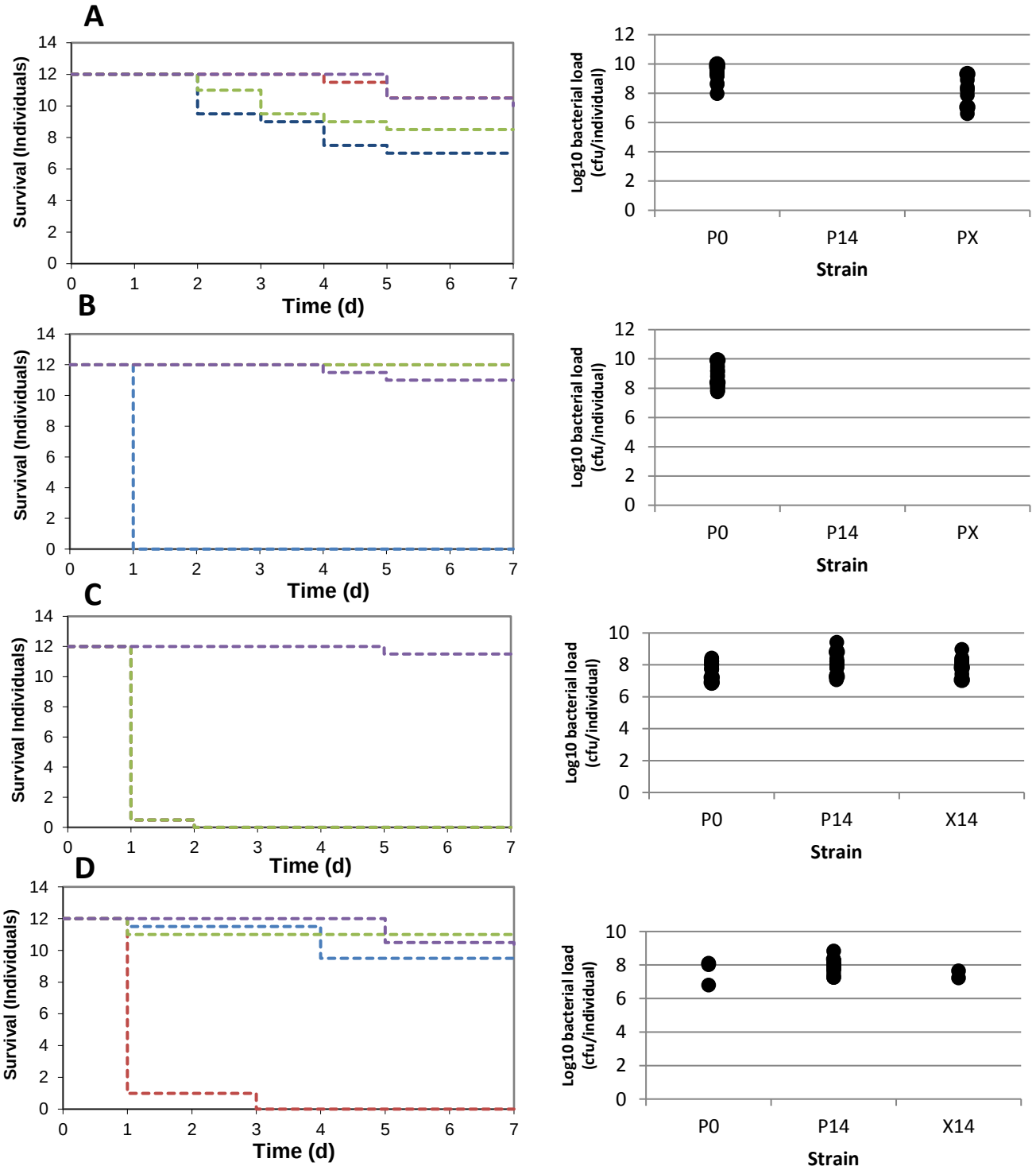

Figure 4.13 *Galleria mellonella* pathogenicity assay. Data shows survival curve, P0 (blue dashed line), P14 (red dashed line), X14 (green dashed line) and control (purple dashed line) along with bacterial load at time of death (black dot indicates individual caterpillar). A) Survival curve and bacterial load of dead worms of *S. aureus* after exposure to triclosan; B) Survival curve and bacterial load of dead worms of *E. coli* after exposure to triclosan; C) Survival curve and bacterial load of dead worms of *E. coli* after exposure to thymol formulation; D) Survival curve and bacterial load of dead worms of *P. putida* after exposure to thymol formulation.

4.3.4 Antibiotic Susceptibility Testing

Bacteria demonstrating 4 fold or above differences in MBC were tested against 5 different antibiotics in a disc diffusion assay. After repeated BAC exposure 5/15 bacterial isolates showed a decrease in overall antibiotic susceptibility (above or equal to 2 mm difference in inhibition zone size), whilst 1/15 bacterial isolates showed an increase; exposure to BAC formulation demonstrated 1/10 isolates to demonstrate a decrease in susceptibility and 1/10 isolate show an increase (Table 4.1). After repeated CHX exposure 2/10 bacterial isolates showed a decrease in susceptibility, whilst 5/10 bacterial isolates showed an increase (Table 4.2). Repeated exposure to DDAC shows 2/15 bacterial isolates to decrease in antibiotic susceptibility, whilst 5/15 bacterial isolates showed an increase in susceptibility; exposure to DDAC formulation demonstrated 4/10 isolates to show a decrease in susceptibility and 2/10 isolate show an increase (Table 4.3). The *E. coli* drain isolate exposed to DMDM hydantoin shows a decrease in 4/5 antibiotics and an increase in susceptibility to tetracycline (Table 4.4). After repeated PHMB exposure 4/25 bacterial isolates showed a decrease in overall antibiotic susceptibility, whilst 1/25 bacterial isolates showed an increase; exposure to PHMB formulation demonstrated 1/15 isolates to demonstrate a decrease in susceptibility with no isolates demonstrating a marked increase in susceptibility (Table 4.5). After repeated exposure to thymol formulation 3/15 bacterial isolates showed a decrease in susceptibility, whilst 4/15 bacterial isolates showed an increase (Table 4.6). Repeated exposure to triclosan shows 5/20 bacterial isolates to decrease in antibiotic susceptibility, whilst 8/20 bacterial isolates showed an increase in susceptibility (Table 4.7).

4.3.4.1 Antibiotic Susceptibility to Benzalkonium chloride Exposed Bacteria

When comparing the pre to the post-BAC adapted isolates, there was a substantial decrease in susceptibility for *S. aureus* towards ciprofloxacin ($P < 0.05$) and kanamycin (Table 4.1). *E. coli* showed a significant reduction in kanamycin susceptibility, however demonstrated increased ciprofloxacin susceptibility compared to that of the pre-adapted isolate. After repeated exposure to BAC formulation *S. aureus* showed a marked decreased susceptibility to ciprofloxacin

(P < 0.05). Comparatively, *E. coli* and *P. aeruginosa* showed increased ciprofloxacin susceptibility.

4.3.4.2 Antibiotic Susceptibility to Chlorhexidine Exposed Bacteria

S. aureus showed a substantial decrease in susceptibility towards ampicillin (P <0.05) and ciprofloxacin (P <0.05) after CHX exposure as well as an increased susceptibility towards tetracycline (P <0.05) (Table 4.2). *E. coli* demonstrated increased susceptibility to ciprofloxacin (P <0.05), cephalothin and ampicillin (P <0.05).

4.3.4.3 Antibiotic Susceptibility to Didecyldimethyl ammonium chloride Exposed Bacteria

After DDAC exposure, the *E. coli* drain isolate increased in susceptibility to kanamycin (P <0.05) and cephalothin (P <0.05) (Table 4.3). After exposure to DDAC formulation *A. baumannii* demonstrated reduced tetracycline susceptibility compared to the wild-type parent strain. In contrast *A. baumannii* and the *E. coli* drain isolate showed an increased susceptibility to ciprofloxacin (P <0.05). Furthermore, *A. baumannii* also showed increased susceptibility to kanamycin, whilst the *E. coli* drain isolate demonstrated a decreased to tetracycline susceptibility after exposure to DDAC and DDAC formulation (P <0.05), whilst *P. aeruginosa* demonstrated an increase in ciprofloxacin susceptibility after exposure to DDAC formulation.

4.3.4.4 Antibiotic Susceptibility to DMDM hydantoin Exposed Bacteria

After repeated exposure to DMDM hydantoin the *E. coli* drain isolate decreased in kanamycin (P <0.05), ciprofloxacin and ampicillin sensitivity (P <0.05) and increased in tetracycline sensitivity (P <0.05) (Table 4.4).

4.3.4.5 Antibiotic Susceptibility to Polyhexamine Biguanide Exposed Bacteria

After repeated PHMB exposure the *E. coli* drain isolate demonstrated a decrease in kanamycin susceptibility (Table 4.5). *S. aureus* demonstrated lower ampicillin susceptibility ($P < 0.05$) when compared to the unexposed parent strain. Additionally, PHMB adapted *S. aureus* increased in tetracycline susceptibility when compared to the pre-PHMB adapted strain.

4.3.4.6 Antibiotic Susceptibility to Thymol Exposed Bacteria

After exposure to thymol containing formulation, *P. putida* demonstrated a marked decrease in susceptibility to ciprofloxacin ($P < 0.05$), kanamycin and tetracycline (Table 4.6). *A. baumannii* showed a substantial increase in ampicillin sensitivity, whilst *E. coli* increased in susceptibility to ciprofloxacin ($P < 0.05$).

4.3.4.7 Antibiotic Susceptibility to Triclosan Exposed Bacteria

After long-term triclosan exposure *C. sakazakii* and the *S. aureus* exhibited reduced ampicillin susceptibility ($P < 0.05$) compared to the parent strain (Table 4.7). Comparatively *C. sakazakii* and *E. coli* showed a substantial increase in ciprofloxacin sensitivity ($P < 0.05$). *S. aureus* showed an increase to kanamycin ($P < 0.05$), cephalothin ($P < 0.05$) and tetracycline susceptibility ($P < 0.05$), whilst kanamycin susceptibility in *C. sakazakii* also increased ($P < 0.05$). *E. coli* exhibited increased susceptibility to ampicillin ($P < 0.05$) when compared to the parent strain.

Table 4.1 Antibiotic susceptibility towards bacterial isolates that showed a ≥ 4-fold decrease in microbicide/formulation susceptibility at MIC, MBC or MBEC level during the bacterial training procedure against benzalkonium chloride.

Bacteria	Ciprofloxacin			Kanamycin			Cephalothin			Ampicillin			Tetracycline		
		UF	F		UF	F		UF	F		UF	F		UF	F
	P0	P14	P14	P0	P14	P14	P0	P14	P14	P0	P14	P14	P0	P14	P14
S. aureus†	22	14 (0.6)	18 (0.6)	17 (1.5)	14 (0.6)	17 (0.6)	45 (0.6)	43	45	47 (0.6)	45 (0.6)	46	26 (0.6)	25 (0.6)	27 (0.6)
E. coli†	29 (1.5)	31	31 (0.6)	15 (1.2)	12 (0.6)	14 (0.4)	17 (0.6)	16 (2.1)	18	21	22 (0.6)	21	21 (0.6)	21 (0.6)	20 (0.6)
P. aeruginosa†	25 (1.5)	25	na	0	0	na	0	0	na	0	0	na	0	0	na
E. faecalis†	0	na	na	0	na	na	12	na	na	33	na	na	8	na	na
*A. baumannii**	19	na	na	19	na	na	0	na	na	0	na	na	15	na	na
*C. sakazakii**	28	na	na	17	na	na	11	na	na	25	na	na	17	na	na
*P. putida**	27	na	na	30	na	na	0	na	na	0	na	na	14	na	na
*E. coli **	37	na	na	14	na	na	19	na	na	25	na	na	20	na	na
*M. osloensis**	16 (1.2)	na	na	13 (0.8)	na	na	55 (0.5)	na	na	47 (0.6)	na	na	26 (0.6)	na	na

Data shows growth inhibition zones (mm) representative of antibiotic susceptibility before and after 14 passages in the presence of microbicide/formulation. Where data varied around the mean standard deviation is given in the parenthesis. Data represents two biological and three technical replicates (n=6). Before antimicrobial exposure (P0); during antimicrobial exposure (P14) and after passage in the absence of antimicrobial (X14) All values are in mg/L. †, non-drain isolates; *,drain isolates. UF, unformulated (microbicide in aqueous solution); F, formulated (microbicide in formulation). na, bacteria that did not undergo a ≥4-fold change and were not assessed. Data represents six replicates. Where data varied between biological replicates, standard deviations have been given in parentheses.

Table 4.2 Antibiotic susceptibility towards bacterial isolates that showed a ≥ 4-fold decrease in microbicide/formulation susceptibility at MIC, MBC or MBEC level during the bacterial training procedure against chlorhexidine.

Bacteria	Ciprofloxacin			Kanamycin			Cephalothin			Ampicillin			Tetracycline		
		UF	F		UF	F		UF	F		UF	F		UF	F
	P0	P14	P14	P0	P14	P14	P0	P14	P14	P0	P14	P14	P0	P14	P14
S. aureus†	22	19 (0.6)	na	17 (1.5)	18	na	45 (0.6)	45 (0.6)	na	47 (0.6)	29 (1.7)	na	26 (0.6)	35 (2.1)	na
E. coli†	29 (1.5)	35 (0.6)	na	15 (1.2)	16 (0.6)	na	17 (0.6)	20 (2.1)	na	21	24 (0.6)	na	21 (0.6)	23 (1.5)	na
P. aeruginosa†	25 (1.5)	na	na	0	na	na	0	na	na	0	na	na	0	na	na
E. faecalis†	0	na	na	0	na	na	12	na	na	33	na	na	8	na	na
*A. baumannii**	19	na	na	19	na	na	0	na	na	0	na	na	15	na	na
*C. sakazakii**	28	na	na	17	na	na	11	na	na	25	na	na	17	na	na
*P. putida**	27	na	na	30	na	na	0	na	na	0	na	na	14	na	na
*E. coli **	37	na	na	14	na	na	19	na	na	25	na	na	20	na	na
*M. osloensis**	13 (0.8)	na	na	13 (0.8)	na	na	55 (0.5)	na	na	47 (0.6)	na	na	26 (0.6)	na	na

Data shows growth inhibition zones (mm) representative of antibiotic susceptibility before and after 14 passages in the presence of microbicide/formulation. Where data varied around the mean standard deviation is given in the parenthesis. Data represents two biological and three technical replicates (n=6). Before antimicrobial exposure (P0); during antimicrobial exposure (P14) and after passage in the absence of antimicrobial (X14) All values are in mg/L. †, non-drain isolates; *,drain isolates. UF, unformulated (microbicide in aqueous solution); F, formulated (microbicide in formulation). na, bacteria that did not undergo a ≥4-fold change and were not assessed. Data represents six replicates. Where data varied between biological replicates, standard deviations have been given in parentheses.

Table 4.3 Antibiotic susceptibility towards bacterial isolates that showed a ≥ 4-fold decrease in microbicide/formulation susceptibility at MIC, MBC or MBEC level during the bacterial training procedure against didecyldimethyl ammonium chloride.

Bacteria	Ciprofloxacin			Kanamycin			Cephalothin			Ampicillin			Tetracycline		
		UF	F		UF	F		UF	F		UF	F		UF	F
	P0	P14	P14	P0	P14	P14	P0	P14	P14	P0	P14	P14	P0	P14	P14
S. aureus†	22	na	na	17 (1.5)	na	na	45 (0.6)	na	na	47 (0.6)	na	na	26 (0.6)	na	na
E. coli†	29 (1.5)	na	na	15 (1.2)	na	na	17 (0.6)	na	na	21	na	na	21 (0.6)	na	na
P. aeruginosa†	25 (1.5)	25	28 (0.6)	0	0	0	0	0	0	0	0	0	0	0	0
E. faecalis†	0	na	na	0	na	na	12	na	na	33	na	na	8	na	na
*A. baumannii**	19	27	na	19	21	na	0	0	na	0	0	na	15	13	na
*C. sakazakii**	28	na	na	17	na	na	11	na	na	25	na	na	17	na	na
*P. putida**	27	na	na	30	na	na	0	na	na	0	na	na	14	na	na
*E. coli **	37	42 (1.5)	40 (0.6)	14	18	11	19	24 (2.1)	15 (0.6)	25	26 (1.5)	21 (0.6)	20	11 (0.6)	11 (0.6)
*M. osloensis**	13 (0.8)	na	na	13 (0.8)	na	na	55 (0.5)	na	na	47 (0.6)	na	na	26 (0.6)	na	na

Data shows growth inhibition zones (mm) representative of antibiotic susceptibility before and after 14 passages in the presence of microbicide/formulation. Where data varied around the mean standard deviation is given in the parenthesis. Data represents two biological and three technical replicates (n=6). Before antimicrobial exposure (P0); during antimicrobial exposure (P14) and after passage in the absence of antimicrobial (X14) All values are in mg/L. †, non-drain isolates; *,drain isolates. UF, unformulated (microbicide in aqueous solution); F, formulated (microbicide in formulation). na, bacteria that did not undergo a ≥4-fold change and were not assessed. Data represents six replicates. Where data varied between biological replicates, standard deviations have been given in parentheses.

Table 4.4 Antibiotic susceptibility towards bacterial isolates that showed a ≥ 4-fold decrease in microbicide/formulation susceptibility at MIC, MBC or MBEC level during the bacterial training procedure against DMDM hydantoin.

Bacteria	Ciprofloxacin			Kanamycin			Cephalothin			Ampicillin			Tetracycline		
		UF	F		UF	F		UF	F		UF	F		UF	F
	P0	P14	P14	P0	P14	P14	P0	P14	P14	P0	P14	P14	P0	P14	P14
S. aureus†	22	na	na	17 (1.5)	na	na	45 (0.6)	na	na	47 (0.6)	na	na	26 (0.6)	na	na
E. coli†	29 (1.5)	na	na	15 (1.2)	na	na	17 (0.6)	na	na	21	na	na	21 (0.6)	na	na
P. aeruginosa†	25 (1.5)	na	na	0	na	na	0	na	na	0	na	na	0	na	na
E. faecalis†	0	na	na	0	na	na	12	na	na	33	na	na	8	na	na
*A. baumannii**	19	na	na	19	na	na	0	na	na	0	na	na	15	na	na
*C. sakazakii**	28	na	na	17	na	na	11	na	na	25	na	na	17	na	na
*P. putida**	27	na	na	30	na	na	0	na	na	0	na	na	14	na	na
*E. coli **	37	35	na	14	12 (1.5)	na	19	16	na	25	20 (0.6)	na	20	24	na
*M. osloensis**	13 (0.8)	na	na	13 (0.8)	na	na	55 (0.5)	na	na	47 (0.6)	na	na	26 (0.6)	na	na

Data shows growth inhibition zones (mm) representative of antibiotic susceptibility before and after 14 passages in the presence of microbicide/formulation. Where data varied around the mean standard deviation is given in the parenthesis. Data represents two biological and three technical replicates (n=6). Before antimicrobial exposure (P0); during antimicrobial exposure (P14) and after passage in the absence of antimicrobial (X14) All values are in mg/L. †, non-drain isolates; *,drain isolates. UF, unformulated (microbicide in aqueous solution); F, formulated (microbicide in formulation). na, bacteria that did not undergo a ≥4-fold change and were not assessed. Data represents six replicates. Where data varied between biological replicates, standard deviations have been given in parentheses.

Table 4.5 Antibiotic susceptibility towards bacterial isolates that showed a ≥ 4-fold decrease in microbicide/formulation susceptibility at MIC, MBC or MBEC level during the bacterial training procedure against polyhexamethylene biguanide.

Bacteria	Ciprofloxacin			Kanamycin			Cephalothin			Ampicillin			Tetracycline		
		UF	F		UF	F		UF	F		UF	F		UF	F
	P0	P14	P14	P0	P14	P14	P0	P14	P14	P0	P14	P14	P0	P14	P14
S. aureus†	22	20 (0.6)	21	17 (1.5)	17 (1.2)	16 (0.6)	45 (0.6)	45 (0.6)	45	47 (0.6)	35 (0.6)	45 (1.5)	26 (0.6)	36 (1.5)	25 (0.6)
E. coli†	29 (1.5)	29	na	15 (1.2)	16 (0.6)	na	17 (0.6)	18 (2.1)	na	21	20 (1.5)	na	21 (0.6)	22 (0.6)	na
P. aeruginosa†	25 (1.5)	25	25 (1)	0	0	0	0	0	0	0	0	0	0	0	0
E. faecalis†	0	0	0	0	0	0	12	13 (0.6)	12 (2.7)	33	33	33 (1.2)	8	8	9 (0.6)
*A. baumannii**	19	na	na	19	na	na	0	na	na	0	na	na	15	na	na
*C. sakazakii**	28	na	na	17	na	na	11	na	na	25	na	na	17	na	na
*P. putida**	27	na	na	30	na	na	0	na	na	0	na	na	14	na	na
*E. coli **	37	28 (0.6)	na	14	12 (1.5)	na	19	18 (2.1)	na	25	25 (0.6)	na	20	20 (0.6)	na
*M. osloensis**	13 (0.8)	na	na	13 (0.8)	na	na	55 (0.5)	na	na	47 (0.6)	na	na	26 (0.6)	na	na

Data shows growth inhibition zones (mm) representative of antibiotic susceptibility before and after 14 passages in the presence of microbicide/formulation. Where data varied around the mean standard deviation is given in the parenthesis. Data represents two biological and three technical replicates (n=6). Before antimicrobial exposure (P0); during antimicrobial exposure (P14) and after passage in the absence of antimicrobial (X14) All values are in mg/L. †, non-drain isolates; *,drain isolates. UF, unformulated (microbicide in aqueous solution); F, formulated (microbicide in formulation). na, bacteria that did not undergo a ≥4-fold change and were not assessed. Data represents six replicates. Where data varied between biological replicates, standard deviations have been given in parentheses.

Table 4.6 Antibiotic susceptibility towards bacterial isolates that showed a ≥ 4-fold decrease in microbicide/formulation susceptibility at MIC, MBC or MBEC level during the bacterial training procedure against thymol.

Bacteria	Ciprofloxacin			Kanamycin			Cephalothin			Ampicillin			Tetracycline		
		UF	F		UF	F		UF	F		UF	F		UF	F
	P0	P14	P14	P0	P14	P14	P0	P14	P14	P0	P14	P14	P0	P14	P14
S. aureus†	22	na	na	17 (1.5)	na	na	45 (0.6)	na	na	47 (0.6)	na	na	26 (0.6)	na	na
E. coli†	29 (1.5)	na	33 (0.6)	15 (1.2)	na	14 (1.2)	17 (0.6)	na	19	21	na	21	21 (0.6)	na	20
P. aeruginosa†	25 (1.5)	na	na	0	na	na	0	na	na	0	na	na	0	na	na
E. faecalis†	0	na	na	0	na	na	12	na	na	33	na	na	8	na	na
*A. baumannii**	19	na	20	19	na	22 (0.6)	0	na	0	0	na	7 (0.6)	15	na	16 (0.6)
*C. sakazakii**	28	na	na	17	na	na	11	na	na	25	na	na	17	na	na
*P. putida**	27	na	20 (0.6)	30	na	27 (0.6)	0	na	0	0	na	0	14	na	12 (2.1)
*E. coli **	37	na	na	14	na	na	19	na	na	25	na	na	20	na	na
*M. osloensis**	13 (0.8)	na	na	13 (0.8)	na	na	55 (0.5)	na	na	47 (0.6)	na	na	26 (0.6)	na	na

Data shows growth inhibition zones (mm) representative of antibiotic susceptibility before and after 14 passages in the presence of microbicide/formulation. Where data varied around the mean standard deviation is given in the parenthesis. Data represents two biological and three technical replicates (n=6). Before antimicrobial exposure (P0); during antimicrobial exposure (P14) and after passage in the absence of antimicrobial (X14) All values are in mg/L. †, non-drain isolates; *,drain isolates. UF, unformulated (microbicide in aqueous solution); F, formulated (microbicide in formulation). na, bacteria that did not undergo a ≥4-fold change and were not assessed. Data represents six replicates. Where data varied between biological replicates, standard deviations have been given in parentheses.

Table 4.7 Antibiotic susceptibility towards bacterial isolates that showed a ≥ 4-fold decrease in microbicide/formulation susceptibility at MIC, MBC or MBEC level during the bacterial training procedure against triclosan.

Bacteria	Ciprofloxacin			Kanamycin			Cephalothin			Ampicillin			Tetracycline		
		UF	F		UF	F		UF	F		UF	F		UF	F
	P0	P14	P14	P0	P14	P14	P0	P14	P14	P0	P14	P14	P0	P14	P14
S. aureus†	22	20 (0.6)	na	17 (1.5)	21 (0.6)	na	45 (0.6)	51 (2.5)	na	47 (0.6)	44 (0.6)	na	26 (0.6)	34 (0)	na
E. coli†	29 (1.5)	41 (1.5)	na	15 (1.2)	13 (0.6)	na	17 (0.6)	18 (0.6)	na	21	28 (0.6)	na	21 (0.6)	20 (1.5)	na
P. aeruginosa†	25 (1.5)	na	na	0	na	na	0	na	na	0	na	na	0	na	na
E. faecalis†	0	na	na	0	na	na	12	na	na	33	na	na	8	na	na
*A. baumannii**	19	na	na	19	na	na	0	na	na	0	na	na	15	na	na
*C. sakazakii**	28	32 (0.6)	na	17	20 (0.6)	na	11	12	na	25	21 (0.6)	na	17	17 (0.6)	na
*P. putida**	27	na	na	30	na	na	0	na	na	0	na	na	14	na	na
*E. coli **	37	35 (0)	na	14	15 (1.2)	na	19	20	na	25	24 (1.2)	na	20	23 (2.1)	na
*M. osloensis**	13 (0.8)	na	na	13 (0.8)	na	na	55 (0.5)	na	na	47 (0.6)	na	na	26 (0.6)	na	na

Data shows growth inhibition zones (mm) representative of antibiotic susceptibility before and after 14 passages in the presence of microbicide/formulation. Where data varied around the mean standard deviation is given in the parenthesis. Data represents two biological and three technical replicates (n=6). Before antimicrobial exposure (P0); during antimicrobial exposure (P14) and after passage in the absence of antimicrobial (X14) All values are in mg/L. †, non-drain isolates; *,drain isolates. UF, unformulated (microbicide in aqueous solution); F, formulated (microbicide in formulation). na, bacteria that did not undergo a ≥4-fold change and were not assessed. Data represents six replicates. Where data varied between biological replicates, standard deviations have been given in parentheses.

4.4 Discussion

Repeated exposure to microbicides may cause adaptation in bacteria resulting in decreases in microbicide susceptibility, either through the selection of insusceptible mutants or through temporary phenotypic adaptation (Latimer *et al.* 2012), such as the induction of reversible stress responses (Chapter 3). In a selective environment, such as in the presence of microbicides, any adaptation that renders a bacterium less microbicide susceptible may provide a competitive advantage, as long as the selective pressure is maintained (Walsh *et al.* 2003b). This would theoretically allow the less susceptible strain to outcompete its more susceptible congeners in a mixed species community in the presence of the microbicide. However, if the selective environment is not maintained than this adaptation may potentially become a fitness burden (Laurent et al., 2001). It is therefore important when evaluating changes in microbicide susceptibility in bacteria to fully consider not just the susceptibility change itself but also the reversibility of this change as well as the resulting effects that it may have on bacterial competitiveness and pathogenicity.

4.4.1 Use of Crystal Violet Assay to Determine Biofilm growth

The method is used to quantify biofilm formation of selected bacteria, before and after exposure to microbicide, as well as exposed bacteria grown in the absence of microbicide. The method of using crystal violet to stain biofilm growth has been used previously (O'Toole, 2011, Forbes et al., 2014, Junker and Clardy, 2007, Bonnekoh et al., 1989). Initially, the method used was similar to that of Forbes *et al.* (2014), which yielded good data. However, the paper by O'Toole (2011) had proved to be easier and less damaging to the cells, as using pipette tips to wash the cells with PBS was seen to cause some damage or cause the biofilm structure to float away in the solution. The crystal violet assay is a colorimetric assay, allowing the crystal violet stain to bind to the cell surface of bacteria, enabling a quantification of biofilm development (Junker and Clardy, 2007). Ethanol solubilises the biofilm giving a purple solution, which is used in a spectrophotometer to read the absorbance of the solution.

4.4.2 Use of Competitive Fitness assay

The method allows a clear fitness comparison between the two strains, as well as a statistical analysis to enable a distinction between the strains (Leroi et al., 1994). Other papers have used similar methods and calculations to determine fitness costs from a wild-type bacterial strain to a mutant (Rozen et al., 2007, Kunz et al., 2012); due to the low costing and visual results the method is a useful way to compare two strains.

4.4.3 Use of *Galleria mellonella* Model to Determine Bacterial Pathogenicity

Bacterial pathogenicity may be determined by its direct effects on the health of the host as well as its rate of clearance. The use of *Galleria mellonella* species as a model for this experiment enables an easy and clear distinction of pathogenicity between the exposed and unexposed strains. Latimer *et al.,* (2014) used this method effectively to show differences in pathogenicity after microbicide exposure. Previous papers (Tan et al., 1999, Miyazaki et al., 2012, Sellon et al., 1998) have used other models; nematodes, silkworm, mice to determine bacterial pathogenesis. Using *Galleria mellonella* is cost effective and have a short life span, which allows a quicker analysis process, as well as replications of the experiment (Mukherjee et al., 2010).

4.4.4 Use of Antibiotic Disc Diffusion assay to Determine Antibiotic Sensitivity

The use of antibiotic disc diffusion was based on the CLSI standardised method (Andrews *et al.* 2001). The method allows a visual analysis of bacterial susceptibility to the antibiotics, as well as clear distinction between strains. The CLSI method has comparable zones of inhibition for certain bacteria with specific antibiotics. This allows a comparison with the data collected in this study for resistant strains. The antibiotics selected in this study were adapted from the paper by Marshall *et al.* (2012), for the paper had examined bacteria residing in household drains and the potential for cross-resistance with household cleaning products; similar to the overall purpose of this thesis.

4.4.5 Biofilm Growth determination on pre-exposed bacteria

After repeated microbicide/ formulation exposure changes in biofilm formation were evident for the bacteria tested. Of the selected bacteria analysed, 44% of selected bacteria exposure to microbicide in aqueous solution demonstrated an increase in biofilm formation. This increase may be due to an increase in bacterial attachment, growth rate or EPS production (Lewis 2005) or possibly due to interference in processes involved in biofilm maturation such as quorum sensing (Lei et al., 2004). Similarly to our findings, Knobloch *et al.* (2002) showed increased biofilm expression when bacteria were exposed to alcohol disinfectants, theorising a gene loci, *icaADBC* which is thought to be responsible for the production of EPS in biofilm formations (Rogers et al., 2008), to be inserted into the bacterial genes. Similarly, Kumar and Ting (2013) showed an increase in biofilm production when bacteria were exposed to norfloxacin, showing a lowering of zeta potential and an increase in hydrophobicity, enabling the bacteria to attach to surfaces more readily. The research has shown similar findings to this study; stressful environments can lead to an increase in biofilm production in order to aid with survival. The increase in biofilm production enables a greater chance of bacterial cells surviving, by increasing attachment properties (Kumar and Ting, 2013) or changes in the bacterial genome (Knobloch et al., 2002b) .

Bacterial biofilms are of increasing interest to researchers due to their lower antibiotic susceptibility in comparison to the planktonic counterparts (Anderson and O'Toole, 2008). Biofilms are complex structures, which allows protection against microbicides or antibiotics (Suci et al., 1994, Bardouniotis et al., 2001), due to persister cells (Keren et al., 2004), matrix and slow growth rate (Suci et al., 1994). Bardouniotis *et al.* (2001) show that compared to planktonic cells, biofilms have a much lower susceptibility towards selected microbicides, therefore persisting in the environment. The structure and complexity of biofilms have led to persistent infections for a number of immunocompromised patients (Costerton et al., 1999, Singh et al., 2000).

The data shows changes occurring toward biofilm formation when the bacteria are exposed to microbicide or formulation. Increases in biofilm production were seen in and overall of 8 isolates when exposed to microbicide, comparatively only 2 increases were seen when exposed to formulation. Our study shows increases in biofilm production when bacteria

are placed under stressful conditions, similar to other studies (Knobloch et al., 2002b, Kumar and Ting, 2013). However, our study has shown less changes occurring when exposed to formulation, which could be linked to less changes occurring with susceptibility after prolonged exposure.

4.4.6 Competitive Fitness of selected pre- and post-exposed bacteria

Fitness costs can occur when new genes or plasmids are introduced into bacteria (Dahlberg and Chao, 2003, Bouma and Lenski, 1988). Bouma and Lenski (1988) showed reduced fitness of *E. coli*, compared with the parent strain, when an antibiotic resistant plasmid was taken into the cell. However, Marcusson *et al.* (2009) demonstrates *E. coli* to increase fitness with the acquisition of fluoroquinolone resistance mutation. These studies have shown an evolutionary affect occurring when bacteria acquire beneficial mutations or genes. Studies showed a compensatory effect on bacterial fitness associated with other beneficial mechanisms (Marcusson et al., 2009, zur Wiesch et al., 2010). Laurent *et al*. (2001) show an increase in competitive growth of MRSA susceptible to gentamicin in comparison to parent strains; demonstrating a cost associated with a beneficial mutation.

In the current chapter, the data frequently shows an overall decrease in competitive fitness after exposure to unformulated microbicides. BAC exposed bacteria shows the most organisms to demonstrate a higher fitness level at P14 than any other active. CHX exposed bacteria demonstrate 1/2 bacteria analysed to show P14 to be competitively fitter. Bacteria exposed to other actives and actives in formulation had shown P0 to out compete P14. The evidence suggests that a fitness cost is associated with prolonged exposure to unformulated microbicide, but not necessarily with microbicide in formulation. Formulations contain varies substances, such as, sequestrants, which have been known to increase microbicide activity by potentially allowing a higher solubility and surfactants, which contain a hydrophobic and hydrophilic group enabling the bacteria to attract to either group (Zoller, 2008). Therefore, bacteria are less able to adapt to formulations due to the chemical composition which can also cause increased efficiency of the microbicide, leading to less changes occurring. In the study,

bacteria were seen to decrease in fitness when exposed to microbicide, which correlates with previous studies mentioned.

4.4.7 Bacterial Pathogenicity Changes Pre- and Post-Exposure

Changes to susceptibility of bacteria to microbicides or antibiotics can often lead to phenotypic adaptations of the bacteria, which can include bacterial pathogenicity (Latimer et al., 2012, Heath et al., 1998). Studies have shown bacteria to exhibit lower pathogenicity when acquiring beneficial mutations (Li et al., 1998, Wilson et al., 1995). Wilson *et al.* (1995) demonstrates *Mycobacterium tuberculosis* with a mutation of the inhA gene to gain resistance to the antibiotic isoniazid. The gained mutation was seen to inadvertently cause a decrease in pathogenicity in comparison to the non-mutate strain. Li *et al*. (1998) had demonstrated decreased pathogenicity in isoniazid resistant *M. tuberculosis* to be explained by a loss of the gene katG, which is seen to enable growth and survival of the bacteria in infection.

The pathogenesis data shows an overall decrease in pathogenicity when bacteria are exposed to unformulated microbicide. The results suggest that long term exposure could cause mutations which could inadvertently affect bacterial pathogenicity. Few bacteria show a reversion of pathogenicity after recovery, with the majority of bacteria demonstrating P0 to be more potent and virulent than P14, X14, F14 and FX. Bacterial load for each caterpillar has shown minimal difference between exposures. Changes in bacterial load could be explained by the *G. mellonella* species immune response, potentially clearing the bacterial infection (Mohammadi et al., 1997). Our results correlate with previous studies showing decreases in pathogenicity in the majority of bacteria tested when exposed to microbicide (Li et al., 1998, Wilson et al., 1995).

4.4.8 Determination of Antibiotic Susceptibility of Selected Bacteria

Antibiotic resistance can be acquired through mutation, recombination or horizontal gene transfer (Martinez and Baquero, 2000). The increase in bacterial resistant to antibiotics has been a concern for a number of years, with particular interest in hospital environments (de Kraker et al., 2011). Studies have examined the presence of cross-resistance between

microbicides and antibiotics (Gilbert and McBain, 2003, Russell et al., 1998); which have shown varied results, with few of bacteria showing low level cross-resistance to occur when exposed to pure active (Walsh et al., 2003b). Out of the tested bacteria we observed, on average, a 23% increase and a 24% decrease in antibiotic susceptibility in bacteria exhibiting a ≥4-fold change in MBC after exposure to the microbicides alone. In comparison, in the bacterial isolates exhibiting a ≥4-fold change in MBC after exposure to the microbicide-containing formulations, on average 14% of isolates show an increase in antibiotic susceptibility and 18% showing a decrease. Whilst there were multiple changes in antibiotic susceptibility after microbicide/formulation exposure the clinical significance of these findings based on antibiotic disc diffusion results are difficult to ascertain. Furthermore, according to the guidelines set out by the BSAC no bacterial isolate changed from susceptible to resistant. However, these guidelines do not cover all combinations of bacterium and antibiotic tested in this current study.

The antibiotic disc diffusion results showed exposure to the antibiotic ciprofloxacin to exhibit the most changes of the antibiotics selected. As mentioned in the first chapter (1.5.2.3), ciprofloxacin inhibits topoisomerase type II and type IV, effectively inhibiting DNA gyrase (Fisher et al., 1989). Ciprofloxacin susceptibility has been seen to be linked with efflux pumps (Berlanga et al., 2004); therefore, changes to susceptibility could be an increase in production of efflux pumps on the bacteria. An increase in efflux pumps could explain decreases to antibiotic susceptibilities (Webber and Piddock, 2003), such as, cross-resistance between triclosan and certain antibiotics, which have been reported in *P. aeruginosa* and is largely believed to be due to an increased expression of the MexAB-OprM efflux system (Chuanchuen et al., 2001). Whilst there has been substantial research focus into the induction of cross-resistance between microbicides and antibiotics relatively few investigations have evaluated the effects that exposing bacteria to microbicide-containing formulations has on antibiotic susceptibility. In the current investigation, whilst the induction of true antibiotic resistance appears to be rare we have observed both decreases and increases in antibiotic susceptibility in bacteria after exposure to both microbicides and microbicide-containing formulations.

4.5 Conclusion

The overall data presented in this chapter indicates that phenotypic changes can occur with bacterial species when exposed to microbicide and formulation, but is more pronounced after microbicide exposure. Competitive fitness and pathogenicity of the exposed bacteria were, overall, seen to decrease in comparison to the parent strain after exposure to the pure microbicides. Increases in adaptation to microbicides may explain the decrease in both fitness and pathogenicity of the bacteria (Enne et al., 2004). Antibiotic susceptibility changes were seen with few of the bacteria; however, none demonstrated notable changes to suggest any cross-resistance had occurred. When comparing the outcome of bacteria exposure to a microbicide in aqueous solution to exposure to the same compound within a formulation, overall, less phenotypic adaptations occurred with biofilm formation, fitness, pathogenicity and antibiotic sensitivity. Overall, the bacteria exposed to microbicide in formulation had demonstrated similar properties to that of the unexposed bacteria or a slight decrease in the aspects tested.

Chapter 5

Analysis of Changes in Bacterial Community Dynamics and Antimicrobial Susceptibility Profiles in a Mixed Species Microcosm

5.0 Abstract

Background: Chapter 3 examines the effects long term exposure of antimicrobial has on susceptibility of bacterial isolates, whilst chapter 4 consider the phenotypic effects of the exposure of bacteria. Domestic drain bacterial biofilms are subjected to wetting, minimal nutrients and sub lethal exposure of antimicrobials; therefore, examining these biofilms can enable a realistic analysis of real world exposure of antimicrobials to bacterial biofilms. This chapter will examine the effects that long-term exposure of bacteria to BAC and BAC in formulation with a non-ionic surfactant and sequestrant has upon bacterial susceptibility within a mixed-species biofilm community.

Methods: Biofilm sampled from a domestic drain was used to inoculate constant depth film fermenter systems. Each system was subsequently dosed daily with BAC in formulation (system A) or BAC alone (system B), over a 32-week period. Concentrations of BAC and BAC formulation were added at increasing concentrations as follows; 0.01% (starting at week 12), 0.1% (week 16) and 1% (week 26). Samples were analysed for changes in susceptibility to antibiotics, using agar plates containing antibiotics and changes to BAC susceptibility, using increasing concentrations of BAC in agar plates. PCR-DGGE and next-generation sequencing (Illumina MiSeq platform), were used to elucidate the effects of exposure on microbial composition.

Results: Reductions of bacterial viability were evident in both systems resulting in a reduction in total approximately 1 $\log_{10}$ following exposure to BAC in aqueous solution compared to a 2.5 $\log_{10}$ during exposure to the BAC formulation. According to DGGE, biofilms were compositionally similar prior to antimicrobial exposure, with clustering being seen in weeks 1 - 16, weeks 20 - 24 and weeks 28 - 32 for BAC formulation exposed system and weeks 1 – 12, 16 – 20 and weeks 24 – 32 for BAC exposed system. However, according to high throughput sequencing there was a 39 % and a 29 % decrease in the relative abundance of *Elizabethkingia sp* in both BAC formulation and BAC systems, respectively. Abundance of *Achromobacter* sp. increased by 39% in BAC formulation system and 10% in the BAC system. Principle coordinate analysis (PCoA) plots show clustering with weeks 8 and 12 between both systems, showing phylogenetic similarities; further weeks show less clustering from initial weeks, with week 32 being the most distance phylogenetically to all other samples, suggesting

shifts in phylogenetically similar bacteria after exposure to either BAC formulation or BAC in aqueous solution. BAC susceptibility changes were demonstrated in the BAC exposed system, with an increase in population percentage grown on higher concentrations of agar containing BAC than with BAC formulation exposed system. Agar plates containing antibiotics show similar results with higher percentage population of BAC exposed communities growing on kanamycin, compared with BAC formulation exposed. After analysis of the bacterial strains, susceptible bacterial species were no longer detectable in the systems, with intrinsically insusceptible bacteria residing in both CDFF systems after prolonged exposure.

Conclusion: Bacterial communities exposed to BAC in aqueous solution or in formulation showed changes in community diversity and susceptibility, partially due to clonal expansion of intrinsically insusceptible bacteria. More substantial reductions in viable bacteria after exposure of the biofilm to BAC in formulation compared to exposure to the pure BAC microbicide provides further evidence of the increased efficacy of microbicides in formulation. Changes in community susceptibility profiles after antimicrobial exposure indicate that the formulation of microbicides may partly mitigate the development of antimicrobial insusceptibility.

5.1 Introduction

As previously mentioned in chapter 1 (sections 1.1; 1.3.1.1; 1.6); the frequent use of antimicrobials within the domestic environment has led to further research into microbicide effect upon bacterial strains, with recent studies demonstrating bacterial resistance occurring to microbicides (McMurry et al., 1998). Quaternary ammonium compounds (QACs) have been used extensively in medical environments (Smith et al., 2008), as well as in household cleaning products (Larson et al., 2004) for the purpose of antisepsis and disinfection. Due to the frequent use of microbicides in everyday environments and studies demonstrating reduce bacterial susceptibility to microbicides (McMurry et al., 1998, Walsh et al., 2003b), there is increasing interest in examining the effects that QAC exposure has upon domestic bacterial communities. Bacterial communities are highly diverse (Demeter et al., 2015) and are usually found in biofilms, which are shown to be less susceptible towards antimicrobials than planktonic bacteria (Keren et al., 2004). The domestic drain environment contains a large diversity of bacteria found in biofilms (McBain et al., 2003c), which are naturally abundant in the environment.

The domestic drain undergo constant selective environmental pressures due to the frequent use of microbicides (Russell et al., 1998). Microbicides are used in consumer products, for surface cleaning or hand washing (Larson et al., 2004), which may lead to bacterial communities residing in the drain to be exposed to residual concentrations of these products. The domestic kitchen drain provides bacteria with a hard surface for biofilm attachment (Lund and Ormerod, 1995, Holden et al., 1995), as well as constant nutrients from the water supply and residual amounts of food from close proximity of food preparations (McBain *et al.* 2003a).

There has been limited research using bacterial microcosms to look at the effects of antimicrobials on bacterial communities (McBain et al., 2004, Marshall et al., 2012). McBain *et al.* (2004) demonstrated that the exposure of biofilms to QACs within a microcosm system have little effect upon the susceptibility of the bacterial community. Similarly, McBain *et al.* (2003a) demonstrated no apparent susceptibility changes when drain microcosms were subjected to sub-lethal levels of triclosan. These studies examined biofilm communities within constant depth film fermenters (CDFF) that were subjected to sub inhibitory levels of

microbicide over a prolonged period. Marshall *et al.* (2012) carried out a similar study examining the effects of household products on domestic drains *'in situ'.* Marshall *et al.* (2012) showed that the drains sampled contained multidrug resistant bacteria; however, there was no apparent statistical correlation for households using microbicides, compared with households that did not use microbicides. Both studies show the considerable recalcitrance of the domestic biofilm community when it is exposed to microbicides.

Previous research has shown the effects that exposure to commonly used household microbicides has on bacteria (McBain et al., 2003b, McBain et al., 2004). As discussed, QACs, such as benzalkonium chloride (BAC), are used as hard surface cleaners in the home and are able to bind to the bacterial cell via phospholipids and proteins on the outer membrane (Maris, 1995), eventually causing irreversible damage to the cellular membrane, leading to cytoplasmic leakage (Gilbert et al., 2002). BAC has been shown to cause changes in bacterial susceptibility when the bacteria are exposed for prolonged periods at sub-lethal concentrations (Braoudaki and Hilton, 2004a). However, it has also been demonstrated that using BAC at in-use concentrations causes no changes in bacteria susceptibility (Houari and Di Martino, 2007). This highlights the variability of results between different methodologies and re-enforces the importance of using realistic experimental conditions.

Chapters 3 and 4 have examined the effects that exposing pure cultures of bacteria to microbicides and microbicide-containing formulations has on bacterial susceptibility and cell physiology. This included examining changes in bacterial fitness, biofilm formation, pathogenicity and antibiotic susceptibility (section 4.3). Investigations described in this chapter examined the effects that BAC and BAC in formulation have upon the community composition of bacterial biofilms, susceptibility changes to BAC, as well as the induced cross-resistance towards ciprofloxacin, kanamycin, tetracycline, ampicillin and cephalothin.

5.2 Methods

5.2.1 Chemicals and Growth Media

See section 3.2.2

5.2.2 Bacterial Microcosm Analysis

Methodology was based on the methods of McBain *et al.* (2003a). Samples of drain biofilm were collected from a pre-established kitchen drain (Manchester, UK) using a sterile 30 ml universal tube. The biofilm sample was weighed to 2.5g (w/v) and diluted 1 in 10 in phosphate buffered saline (PBS). The sample was homogenized for 1 min using a vortex, in the presence of five glass beads (3.5 – 5.5 mm diameter, VWR, Leicester, UK), with 1 ml (x 2) of the sample being used to inoculate the constant depth film fermenters (CDFFs). The remaining sample (7 ml) was diluted to 10^{-8} in PBS and plated on to; tryptone soya agar (TSA), Wilkins-Chalgren agar (WC), MacConkey agar (MK), Pseudomonas selective agar (C-F-C) (PA) and BBL™ Columbia CNA blood agar (CNA) (Scientific Laboratory Suppliers, Nottingham, UK) to give an overall view of the bacteria residing in the CDFF. TSA containing benzalkonium chloride (BAC) at 0.1 µl/ml, 1 µl/ml, 10 µl/ml, 100 µl/ml and 1000 µl/ml was used to analyse initial susceptibility or changes occurring in the bacterial consortium towards BAC, whilst TSA containing kanamycin (10 µg/ml^{-1}), ciprofloxacin (1 µg/ml^{-1}), cephalothin (25 µg/ml^{-1}), tetracycline (10 µg/ml^{-1}) and ampicillin (25 µg/ml^{-1}) (Marshall et al., 2012) was used to analyse initial susceptibility or changes occurring within the bacterial consortium towards selected antibiotics. Samples underwent 16S rRNA gene sequencing (section 5.2.4) for selected bacterial colonies, which were assigned according to relative similarity from BLAST.

5.2.3 Constant Depth-Film Fermenters

Samples of drain biofilm were used to inoculate two CDFFs. A portable incubator was set up at a temperature of 28°C, to keep both vessels at a consistent temperature. Artificial dish water (in grams per litre in distilled water: peptone, 0.5; tryptone, 0.5; yeast, 0.5; NaCl, 1; starch, 1; margarine (Flora; Van den Berg Foods, Ltd., Crawley, United Kingdom), 0.05;

domestic detergent (Fairy Original; Procter and Gamble, Newcastle Upon Tyne, United Kingdom), 0.05; hemin, 0.001; tomato ketchup (Heinz, Uxbridge, United Kingdom), 0.05) was added as media to the fermenters using a peristaltic pump (Gilson, Villers le Bel, France). Media was fed drop wise in a continuous manner throughout the experiment. The CDFFs contain 15 Teflon pans, which contain 5 pegs, all of which are set at a constant depth of 5 mm. The CDFFs were allowed to stabilise for 12 weeks before any microbicide was added, with antimicrobial concentrations being increased once the CDFF was deemed stable (2 consecutive weeks showing similar levels of bacteria counts). BAC in formulation (5 ml) was added drop wise to vessel A, whilst BAC alone was added to vessel B, once per day for 32 weeks. Concentrations of antimicrobial were as follows; 0.01% (start at week 12), 0.1% (start at week 16) and 1% (start at week 26). Sampling of the CDFFs was carried out using a sterile metal grip to pull out sample pans which had resided in the vessel for over 10 days in order to avoid sampling immature biofilms. Pegs (2 x 2 pegs for biological replication, while remaining peg was archived) from the pans were pushed out using a sterile metal prod and added to 10 ml PBS containing approximately 5 glass beads, vortexed and diluted to 10^{-8} in PBS and plated (100 µl) in triplicate on to selective agars. All agar plates were incubated at 37°C aerobically for 24 h, with the exception of WC agar plates, which were incubated at 37°C anaerobically for 48 hrs. Bacteria grown on plates were counted and used to plot changes in community diversity.

5.2.4 16S rRNA Gene Sequencing, NGS and PCR amplification

DNA from CDFF microcosms were extracted using QIAGEN QIAamp® kit (QIAGEN Ltd., Crawley, West Sussex, UK) in accordance with manufacturer's instructions and added to 100 µl of sterile polymerase chain reaction (PCR) nanopore water in an Eppendorf tube (1.5 ml). The reaction tubes were then heated to 100^{o}C (10 min) to lyse the cells. The tubes were then centrifuged at 8,150 x *g* (10 min) to pellet cell debris. 90 µl of supernatant was transferred to a sterile reaction tube. A PCR mixture was made containing 25 µl of MyTaq DNA polymerase ready mix (Bioline Reagants Ltd, London, UK), 1 µl of 8 FLP1 (5' – GAGTTTGATCCTGGCTCAG – 3') and 1 µl of 806R (5' – GGACTACCAGGGTATCTAAT – 3') (Wilson et al., 1990), 18 µl nanopore water and 5 µl of the DNA template. T Gradient

Thermocycler (Biometra, Glasgow, UK) was used to run 35 thermol cycles 94°C (1 minute), 53°C (1 minute), 72°C (1 minute) and the final cycle incorporates reductase 15 minute chain elongation step. Qiogen PCR purification kit (Bioline Reagents Ltd, London, UK) was used to purify the DNA, according to the manufactures protocol. A Nanodrop lite UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA), was used to quantify the amount of DNA present in each sample proceeding the PCR. The samples were then transferred to a 1 % agarose gel and ran against a Hyperladder marker IV (Bioline Reagents Ltd, London, UK) to ensure the samples were present and pure. Sequences retrieved from 16S rRNA gene sequencing were searched using BLAST, against sequences contained in the European Molecular Biology Laboratories (EMBL) prokaryote database; closest relative species were assigned to the sequence. Similar steps were carried out to establish amplicons for NGS, using primers; 16S Amplicon PCR Forward Primer = 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; 16S Amplicon PCR Reverse Primer = 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC, which were used to amplify the variable regions V3 and V4, amplifying approximately 460 bp. Due to the diversity of the metagenomic community, the samples were identified using next generation sequencing (NGS, Genomic Technologies Core Facility, Manchester, UK) on the Illumina MiSeq system (2 X 300 bp). The sequences retrieved were identified using the software QIIME 1.8.0 (quantitative insights into microbial ecology, scikit-bio) and run against Greengenes database (version 13_5). Sequences are filtered to delete attached barcodes and adapters (underlined), before being merged via the software PAired-eND Assembler for DNA sequences (PANDAseq, GitHub, San Francisco, CA, USA.) and analysed in a closed reference against the Greengenes database. Principle coordinate analysis (PCoA) plot is used to show phylogenetic similarities between samples. Shannon-Wiener diversity index analysis was carried out to examine the difference in biodiversity of the different treatments (formulation/ active) and various concentrations (0, 0.01%, 0.1% and 1%).

5.2.5 Denaturing Gradient Gel Electrophoresis

PCR products from the community were used in denaturing gradient gel electrophoresis (DGGE). Polyacrylamide (8%) gels (16 by 16 cm; 1 mm) were run with 1 X TAE buffer, diluted from a 50 X TAE buffer (40 mM Tris base, 20 mM glacial acetic acid and 1 mM EDTA). A 20% to 50% denaturation urea gradient for 5 h at 144 V was used for community analysis. SYBR Gold stain (diluted 10^{-4} in 1 X TAE buffer [Molecular probes (Europe), Leiden, The Netherlands] for 30 min.) was used to stain the gels; images were seen using a transilluminator (model T2201; Sigma, St. Louis, Mo.) and analysed using the software BioNumerics (Applied Maths, TX., USA). Bands of interest were excised, DNA re-amplified and 16S rRNA gene sequencing performed to determine the band identity.

5.3 Results

5.3.1 Cultured Bacteria of CDFF Exposed to BAC and BAC in Formulation

These data (Figures 5.1-5) show that the microbial community may undergo shifts in bacteriological composition and diversity after prolonged repeated exposure to BAC. BAC or antibiotic susceptibility was not measured in the initial first 3 weeks of biofilm development due to the biofilms still developing into stable communities. Exposure of the biofilm to 0.01% of BAC or BAC in formulation resulted in little change in viable bacteria (Figure 5.1). In terms of BAC susceptibility, there was an increase in the number bacteria able to grow in the presence of 1000 µg/ml BAC by week 18 (BAC) and week 20 (BAC formulation; Figure 5.2). Exposure of the biofilm to 1% BAC/formulation caused a decrease in total viable counts, Gram negative, Gram positive, anaerobic and Pseudomonads (Figure 5.1), this reduction in viability was more pronounced in the BAC formulation system, showing reductions in viable bacteria of 10-fold in the BAC treated system versus an approximate 2.5 log reduction in the BAC formulation treated system. Similarly decreases in viable bacteria were seen on all BAC plates and all antibiotic plates once dosing concentrations reached 1 % (Figure 5.2 and Figure 5.4; respectively). Due to the loss of total viable bacteria in both systems, the percentage of the total population that was able to be cultured on both BAC and antibiotic plates was determined to provide clearer insight into any resulting changes in the overall community susceptibility

after antimicrobial exposure (Figure 5.3 and 5.5; respectively). Figure 5.3.A shows a decrease in the total culturable bacteria that were able to grow in the presence of 10 - 100 µg ml of BAC before exposure (week 4) compared to after exposure (week 32) (Figure 5.3.A) in both systems. The percentage population of bacteria able to grow in the presence of BAC, increased across all concentration gradients (Figure 5.3.B). This trend was mimicked when examining the total viable bacteria in comparison to the percentage of the viable bacterial population that is able to grow in the presence of all test antibiotics after antimicrobial exposure. Comparatively, BAC exposed bacterial communities demonstrated an increase in population percentage able to grow on higher concentrations of BAC containing plates than BAC formulation. Agar plates containing antibiotics show similar results with more BAC exposed communities growing on kanamycin, compared with BAC formulation exposed. Examining the bacteria obtained from both systems through culturing techniques; *Pseudomonas* sp*., Bacillus cereus, S. pasteuri, Alcaligenes* sp. and *Micrococcus* sp. are prevalent throughout the experiment.

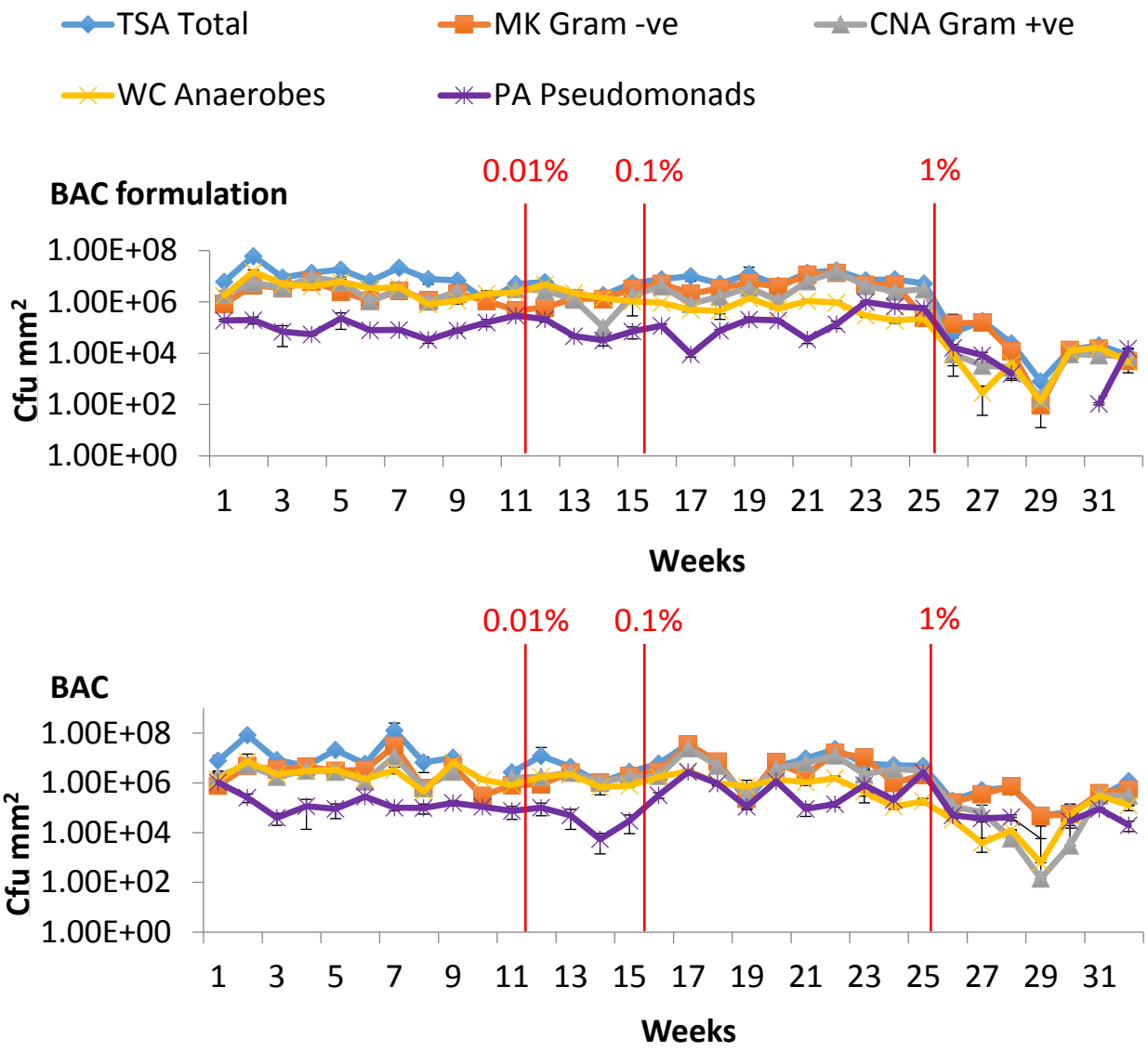

Figure 5.14 Total viable bacteria that were cultured in both models using TSA (blue line) for total counts; MacConkey agar (orange line) for Gram negative bacteria; Wilkins-Chalgren agar (yellow line) for anaerobic bacteria; Pseudomonas selective agar (purple line) for Pseudomonads; and BBL™ Columbia CNA blood agar (grey line) for Gram positive bacteria. Red lines indicate increased dose concentrations of respective microbicide; 0.01% (week 12); 0.1% (week 16); 1% (week 26).

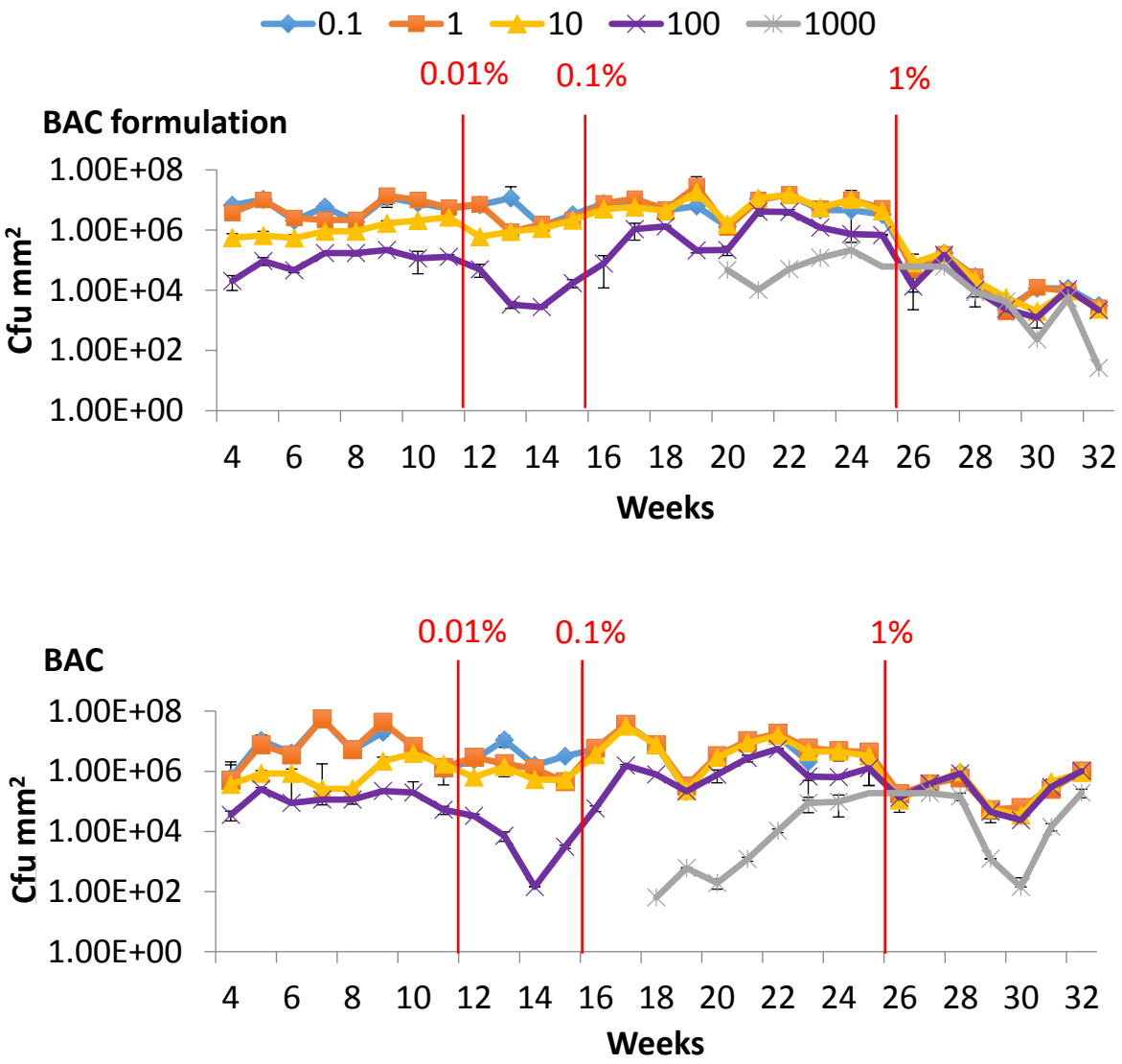

Figure 5.15 Total viable bacteria that were cultured in both models after exposure to 0.1 µg/ml BAC (blue line); 1 µg/ml BAC (orange line); 10 µg/ml BAC (yellow line); 100 µg/ml BAC (purple line); and 1000 µg/ml BAC (grey line). Red lines indicate increased dose concentrations of respective microbicide; 0.01% (week 12); 0.1% (week 16); 1% (week 26).

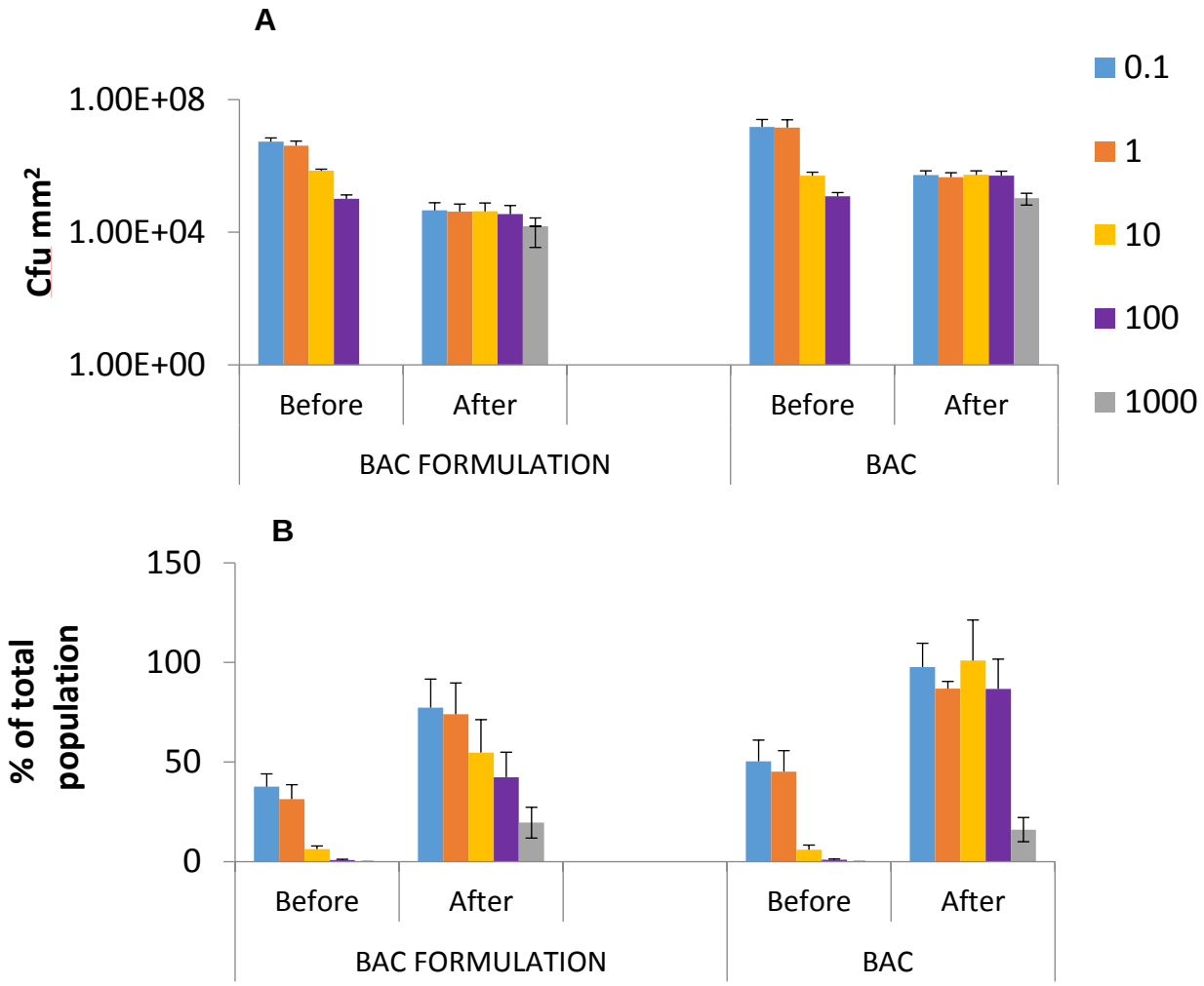

Figure 5.16 Graph A shows total counts of bacteria cultured on BAC plates (cfu mm2). Graph B shows percentage of total population cultured on BAC plates. 0.1 µg/ml BAC (blue); 1 µg/ml BAC (orange); 10 µg/ml BAC (yellow); 100 µg/ml BAC (purple); and 1000 µg/ml BAC (grey).

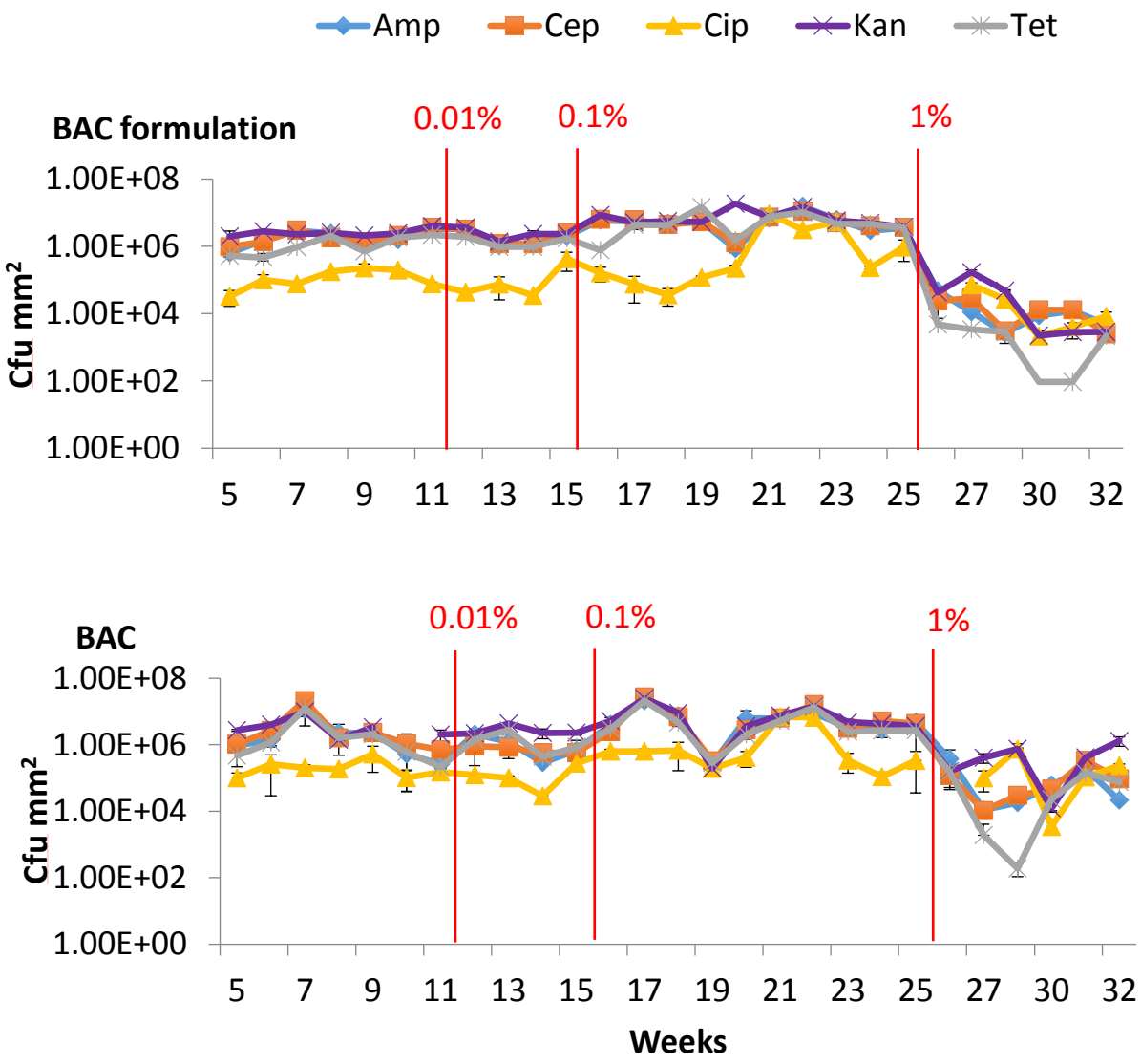

Figure 5.17 Total viable bacteria that were cultured in both models in the presence of ampicillin (blue line); cephalothin (orange line); ciprofloxacin (yellow line); kanamycin (purple line); and tetracycline (grey line). Red lines indicate increased dose concentrations of respective microbicide; 0.01% (week 12); 0.1% (week 16); 1% (week 26).

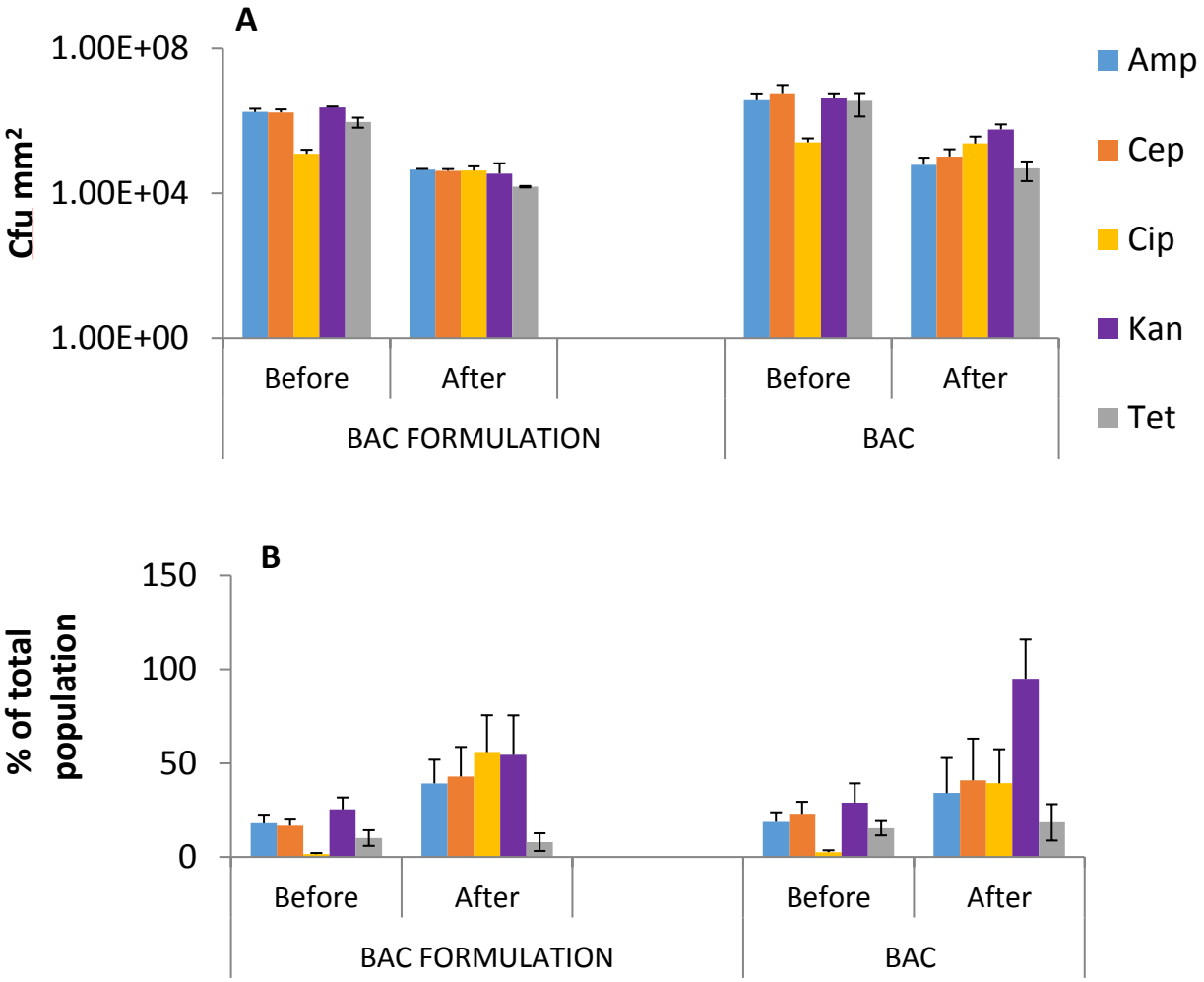

Figure 5.18 Graph A shows total counts of bacteria cultured on BAC plates (cfu mm2). Graph B shows percentage of total population cultured on BAC plates. Ampicillin (blue); cephalothin (orange); ciprofloxacin (yellow); kanamycin (purple); and tetracycline (grey).

5.3.2 DGGE Analysis

Due to the large amount of samples collected, DGGE analysis was used to pre-screen samples for next generation sequencing (NGS) analysis in order to determine samples that showed substantial changes in community composition. The isolates obtained were *Aeromonas* spp. (Accession number KC469704.1), *Bacteroidetes* spp. (Accession number DQ886166.1), *Bosea* spp. (Accession number KF730777.1), *Brucella* spp. (Accession number KJ634086.1), *Chryseobacterium* spp. (Accession number HF678230.1), *Clostridium* spp. (Accession number KJ722495.1), *Comomonas* spp. (Accession number JQ885562.1), *Dokdonella* spp. (Accession number AB851003.1), *Elizabethkingia* spp. (Accession number JX067927.1), *Enterobacter* spp. (Accession number KC191586.1), *Flavobacterium* spp. (Accession number AB498900.1), *Nilaparvata* spp. (Accession number GU124500.2), *Pedobacter* spp. (Accession number KF003152.1) and *Pseudomonas* spp. (Accession number KF803331.1), *Aeromonas* spp. (Accession number KC711626.1), *Bacteroidetes* spp. (Accession number KC302824.1), *Bosea* spp. (Accession number AB851222.1), *Brucella* spp. (Accession number KF756359.1), *Chryseobacterium* spp. (Accession number AJ746141.1), *Comomonas* spp. (Accession number KF214953.1), *Dysgonomonas* spp. (Accession number KF176996.1), *Elizabethkingia* spp. (Accession number JX067927.1), *Flavobacterium* spp. (Accession number KF951536.1), *Nonlabens* spp. (Accession number NR102491.1), *Ochrobacterium* spp. (Accession number JF293081.1), *Propionivibrio* spp. (Accession number JN713386.1), *Rhizobium* spp. (Accession number JQ659613.1) and *Sphingomonas* spp. (Accession number FM202724.1). Weeks 1 – 9 were analysed (Figure 5.6) to show similarities within the systems during the initial stages of biofilm development. Subsequently, samples were tested from every 4 weeks during the BAC exposure period and were analysed using DGGE (Figure 5.7 and 5.8). The BAC formulation system showed distinct bands of bacteria throughout weeks 1 – 32 (Figure 5.7.A), which were excised and re-amplified for 16S rRNA gene sequencing. The isolates obtained were *Aeromonas* spp., *Bacteroidetes* spp., *Bosea* spp., *Brucella* spp., *Chryseobacterium* spp., *Clostridium* spp., *Comomonas* spp., *Dokdonella* spp., *Elizabethkingia* spp., *Enterobacter* spp., *Flavobacterium* spp., *Nilaparvata* spp., *Pedobacter* spp. and *Pseudomonas* spp. The system demonstrates soft clustering between weeks 1 – 16 (Figure 5.7.B), with similarities seen with initial weeks and weeks 20 –

24. Weeks 28 – 32 are clustered and show less similarities with initial weeks. The BAC system shows distinct bands of bacteria throughout weeks 1 – 32 (Figure 5.8.A), which were excised and re-amplified for 16S rRNA gene sequencing. The isolates obtained were *Aeromonas* spp., *Bacteroidetes* spp., *Bosea* spp., *Brucella* spp., *Chryseobacterium* spp., *Comomonas* spp., *Dysgonomonas* spp., *Elizabethkingia* spp., *Flavobacterium* spp., *Nonlabens* spp., *Ochrobacterium* spp., *Propionivibrio* spp., *Rhizobium* spp. and *Sphingomonas* spp. The system demonstrates clustering between weeks 1 – 12 (Figure 5.8.B), with similarities seen with initial weeks and weeks 16 – 20. Weeks 24 – 32 are clustered and show less similarities with initial weeks.

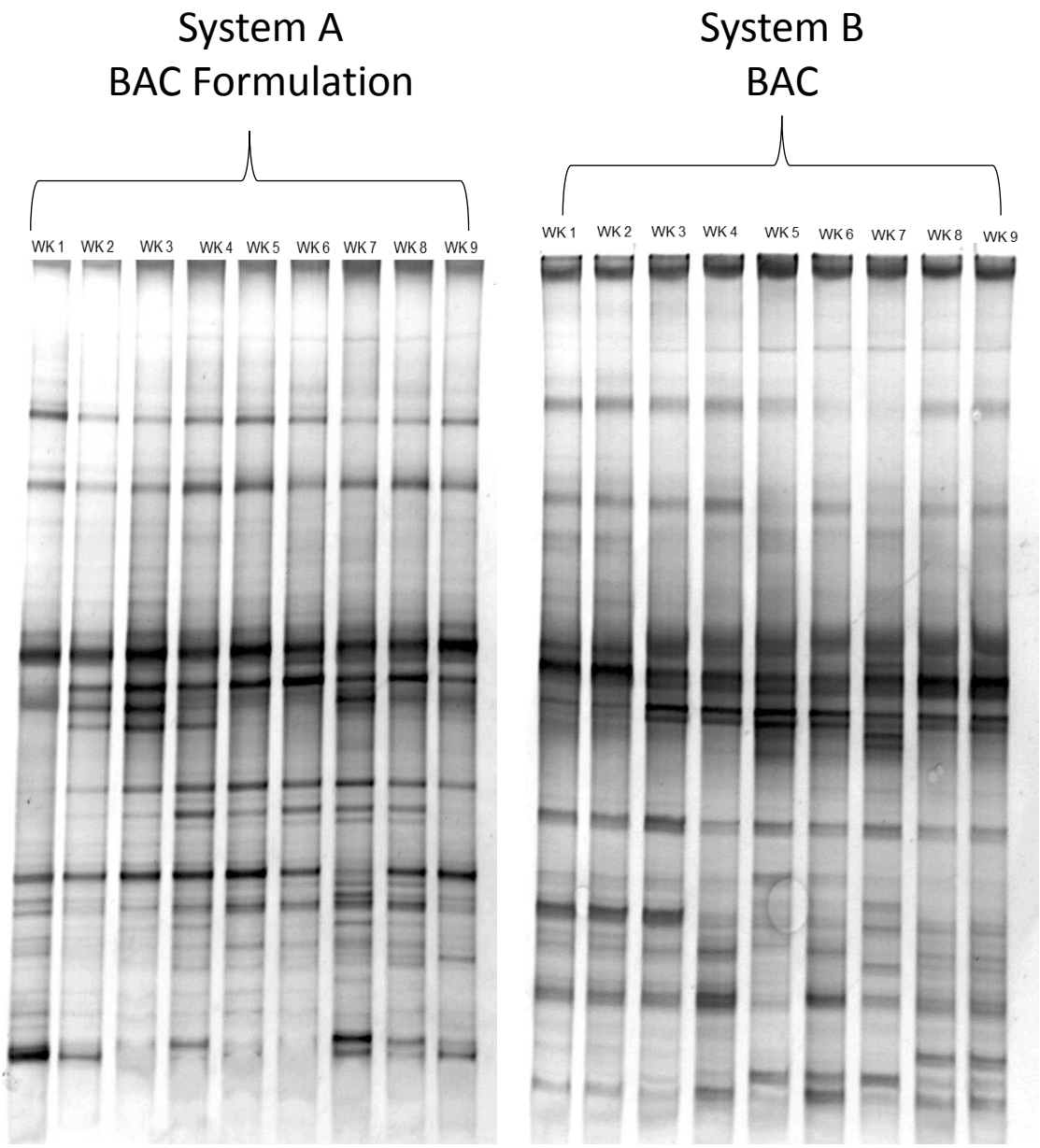

Figure 5.19 DGGE dendrogram from system A (BAC formulation) and system B (BAC), showing separation of the various different bacterial species identified with bands for weeks 1 - 9.

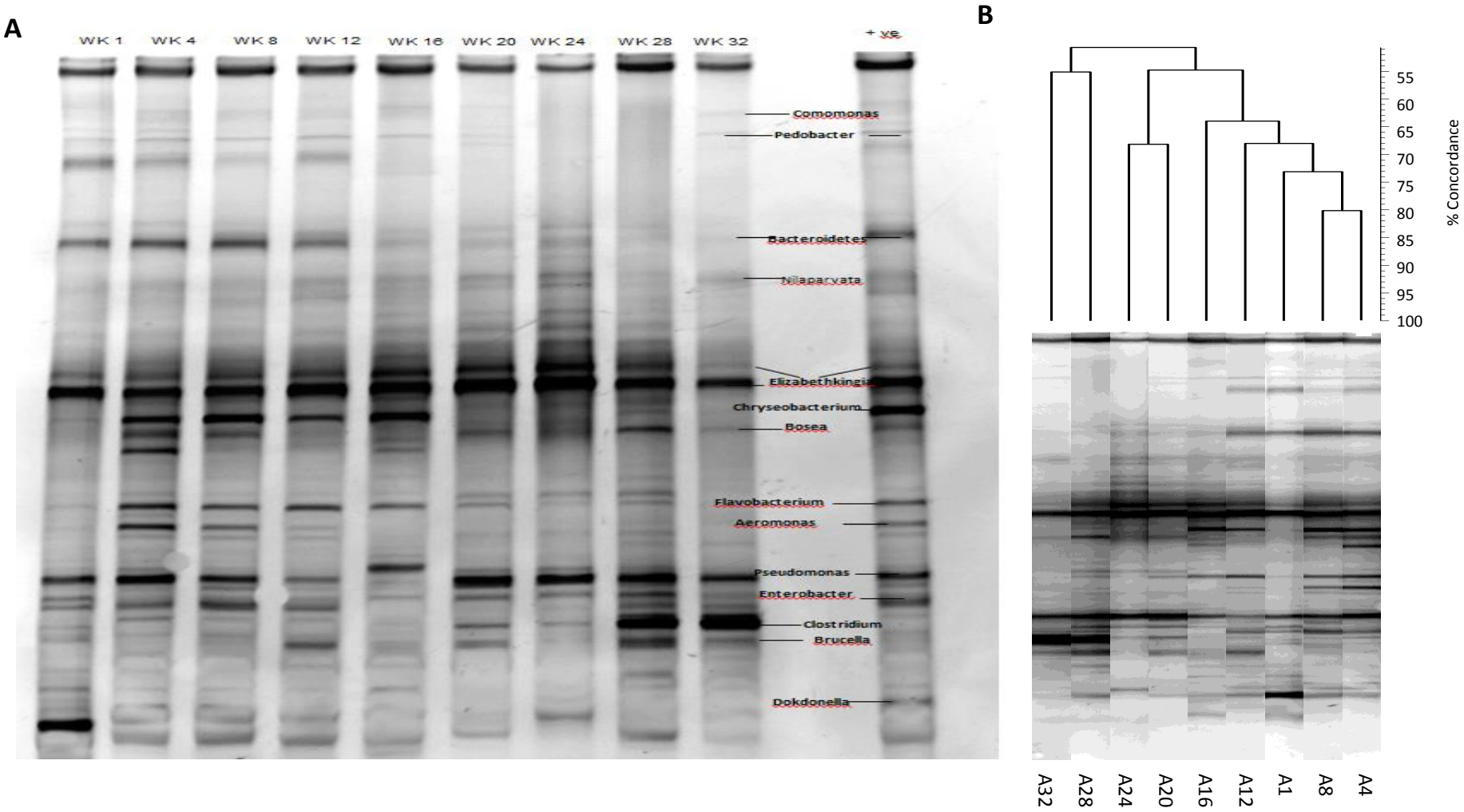

Figure 5.20 A) DGGE dendrogram from system A (formulation), showing separation of the various different bacterial species identified. Lane 1, week 1; lane 2, week 4; lane 3, week 8; lane 4, week 12; lane 5, week 16; lane 6, week 20; lane 7, week 24; lane 8, week 28; lane 9, week 32; lane 10, positive control. B. shows clustering of weeks deemed to be similar based on their phylogenetic profile.

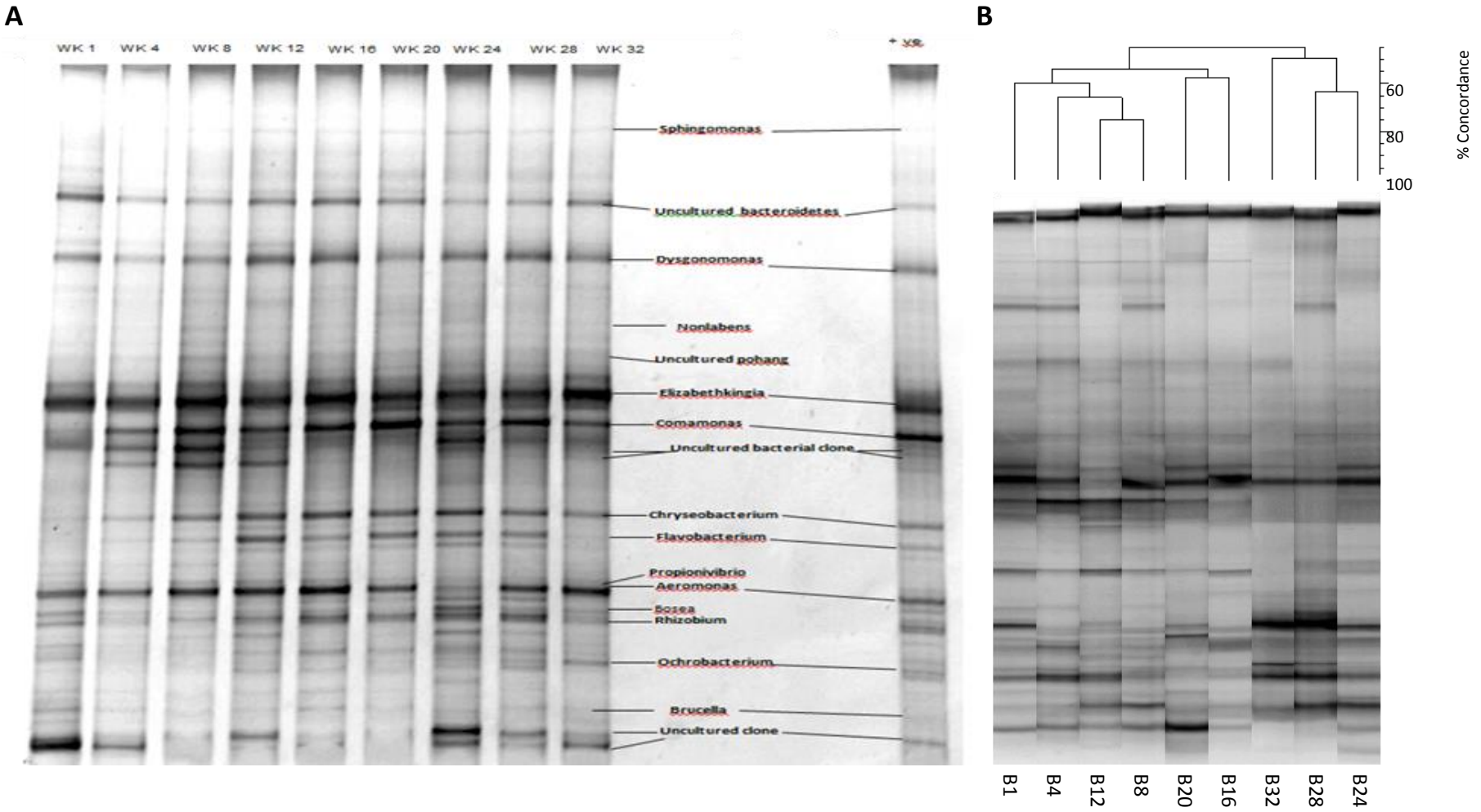

Figure 5.21 A) DGGE dendrogram from system B (active), showing separation of the various different bacterial species identified. Lane 1, week 1; lane 2, week 4; lane 3, week 8; lane 4, week 12; lane 5, week 16; lane 6, week 20; lane 7, week 24; lane 8, week 28; lane 9, week 32; lane 10, positive control. B. shows clustering of weeks deemed to be similar based on their phylogenetic profile.

5.3.3 Next Generation Sequencing analysis

These data show results from NGS analysis processed using QIIME. Charts showing abundance allow a visual guide to loss or gain of abundant genus for the BAC formulation system (Figure 5.9) and BAC active system (Figure 5.10). The BAC formulation system showed a high relative abundance of *Elizabethkingia, Klebsiella, Aeromonadaceae, Hydrogenophaga* initially (Figure 5.9); with *Elizabethkingia* remaining the most abundant genus even after exposure to 0.01% and 0.1% BAC formulation (Figure 5.9). Shifts in relative abundance are seen with each subsequent increase in BAC concentration, with *Elizabethkingia, Aeromonadaceae* and *Pedobacter* remaining at high levels (≥1%) throughout. After 1% BAC formulation exposure the dominant genus of *Elizabethkingia* was reduced from 54% relative abundance (Figure 5.9) to 15% (Figure 5.9); *Achromobacter* increased in relative abundance from 3% after 0.1% exposure (Figure 5.9) to 42% after 1% exposure (Figure 5.9) making it the dominant genus at 1% exposure levels. The BAC system showed a high relative abundance of *Elizabethkingia, Bacteriodes, Comamonas, Dygonamonas* initially (Figure 5.10); with *Elizabethkingia* remaining the most abundant genus when the system was subjected to 0.01% and 0.1% BAC (Figure 5.10). Shifts in relative abundance are seen with each increase in BAC concentration, with *Elizabethkingia, Comamonadaceae, Comamonas, Rhizobiales* and *Xanthomonadaceae* remaining at high levels (≥1%) throughout.

After 1% exposure to BAC in aqueous solution, the dominant genus of *Elizabethkingia* was reduced from 58% relative abundance after 0.1% exposure (Figure 5.10) to 29% after 1% exposure (Figure 5.10); *Achromobacter* increased in relative abundance from 2% after 0.1% exposure (Figure 5.10) to 12% after 1% exposure (Figure 5.10); *Aeromonadaceae* increased in relative abundance from 2% after 0.1% exposure (Figure 5.10) to 32% after 1% exposure (Figure 5.10) making it the most dominant genus. Shannon-Wiener diversity index demonstrated little difference in the taxa diversity between treatments in each system; formulation 2.65 and active 2.81, using a paired *t*-test; ($P = 0.278$); and concentrations; formulation 0% 2.94, 0.01% 2.55, 0.1% 2.67, 1% 2.44; active 0% 2.72, 0.01% 3.42, 0.1% 2.78, 1% 2.33. Clustering of similar samples, in terms of detected genus are seen in the PCoA plots (Figure 5.11.A; 5.11.B). Weekly samples (Figure 5.11.A) show clustering with weeks 8 and 12, showing phylogenetic similarities; other weeks show further distance from initial weeks, with

week 32 being the most distance phylogenetically to all other samples. Figure 5.12 shows both systems to overlap and cluster between samples showing similarities in taxonomy for both systems and comparable samples.

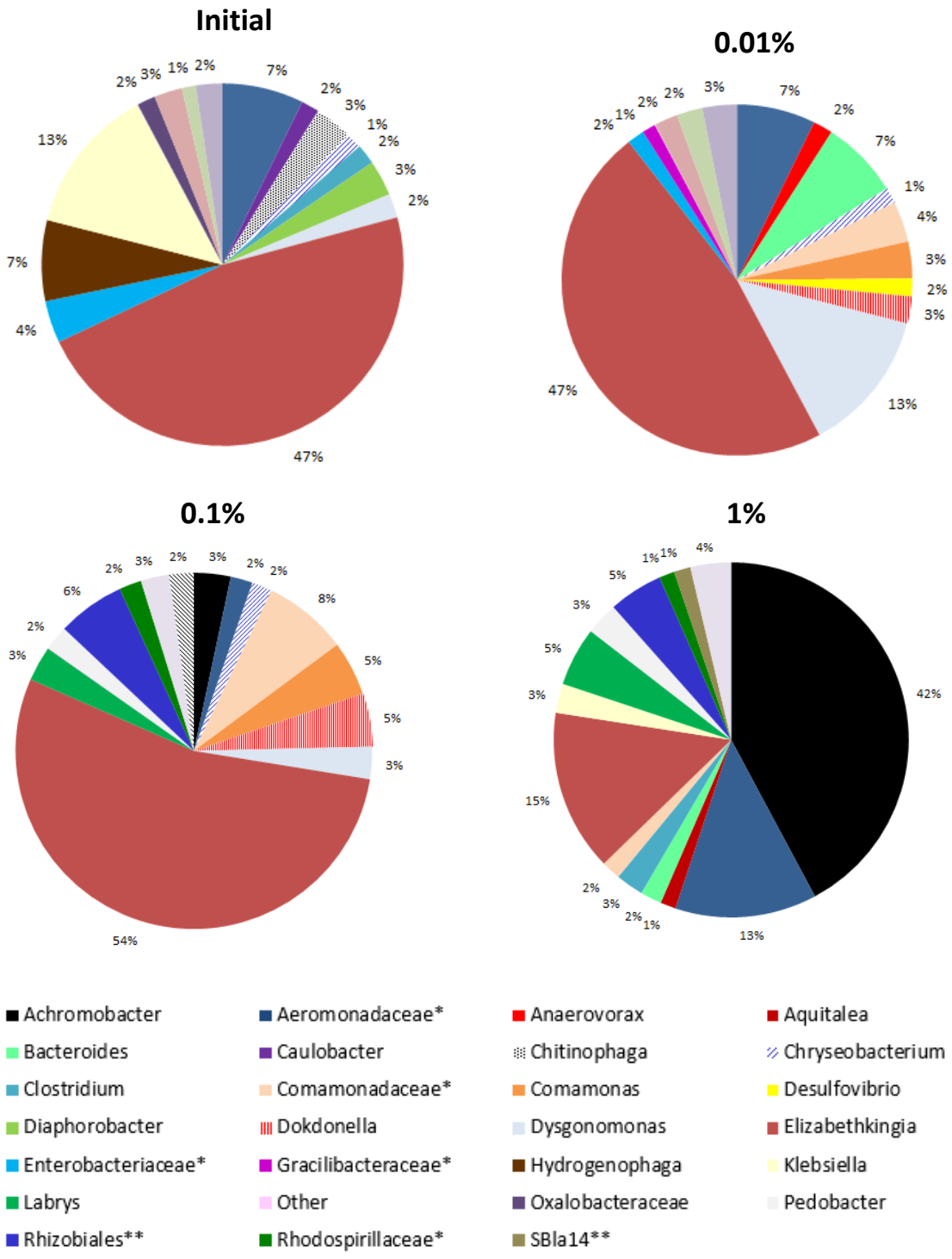

Figure 5.22 Pie charts showing relative abundance of bacteria for initial; 0.01%; 0.1% and 1% exposure in the BAC formulation system. Minority genus - bacteria found at lower than 1% relative abundance levels. * classification under bacterial family, ** classification under bacterial order.

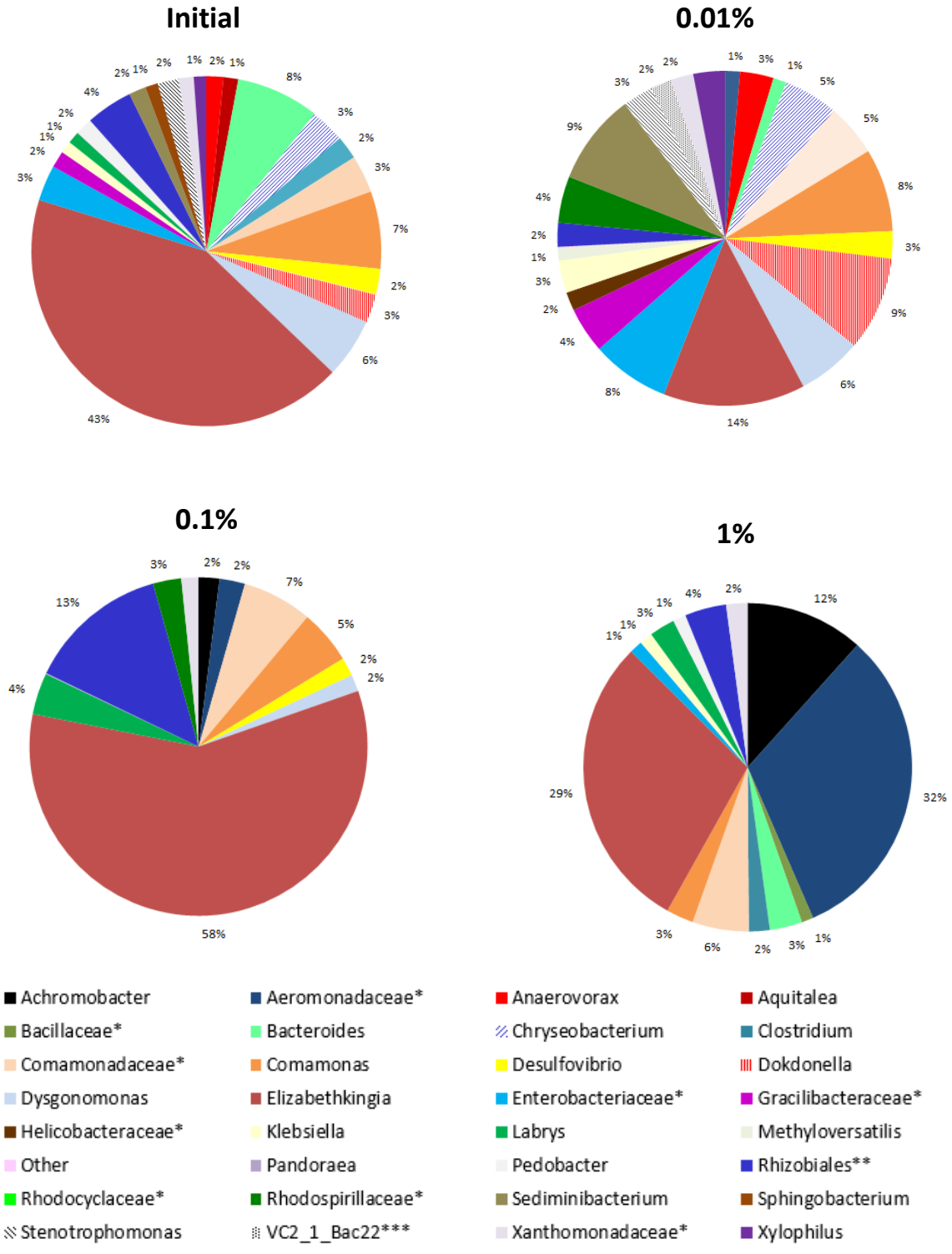

Figure 5.23 Pie charts showing relative abundance of bacteria for initial; 0.01%; 0.1% and 1% exposure in the BAC system. Minority genus - bacteria found at lower than 1% relative abundance levels. * classified under bacterial family, ** classified under bacterial order, *** classified under bacterial class.

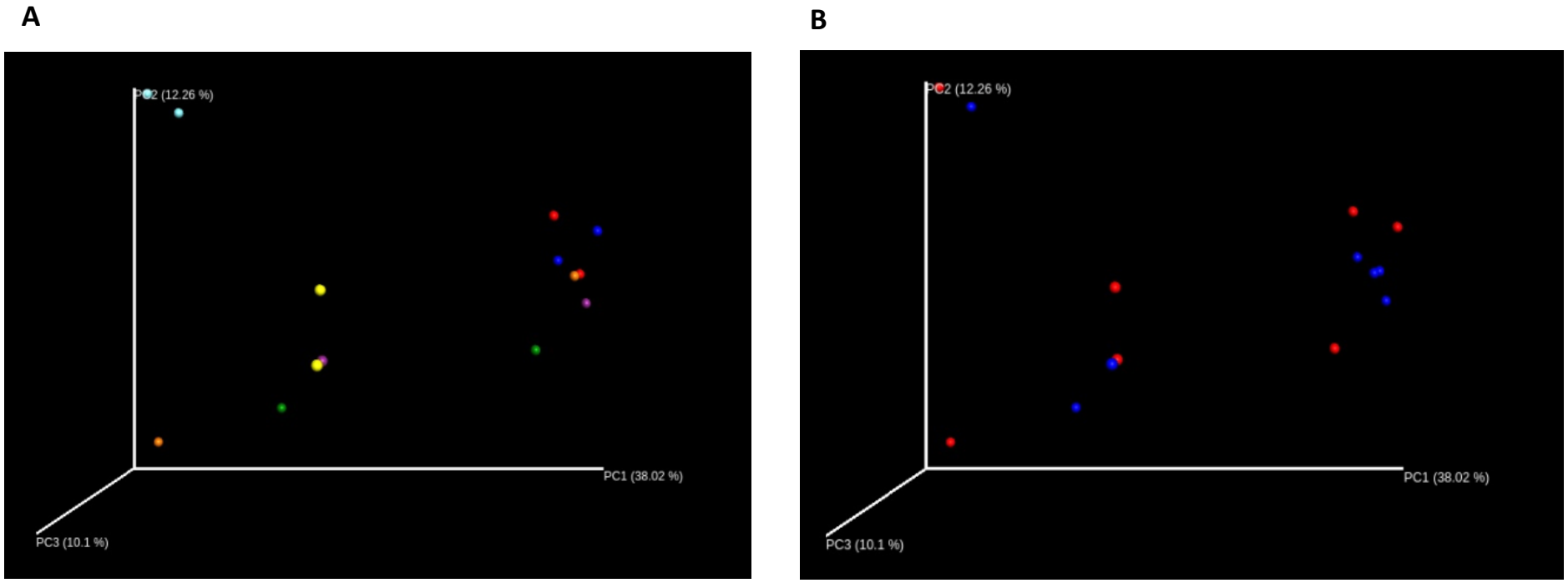

Figure 5.24 Principle coordinate analysis (PCoA) plot showing clustering of similar phylogenetic samples in both systems. A. shows PCoA plot based on weekly samples that were taken; 8 (red); 12 (blue); 16 (orange); 20 (green); 24 (purple); 28 (yellow); 32 (light blue). B. shows PCoA plot based on treatment; active (red) formulation (blue). Plots were created using Qiime.

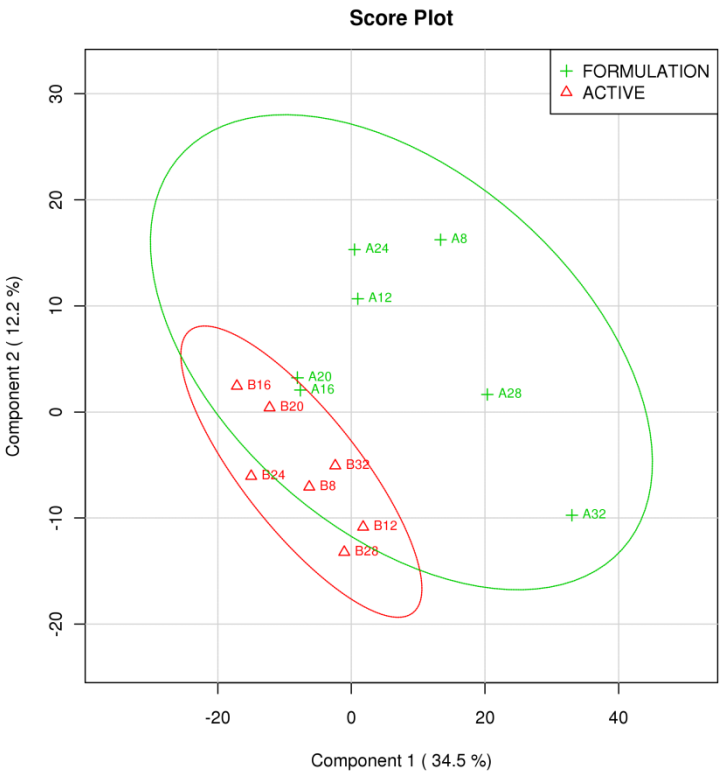

Figure 5.25 2D PLS-DA score plot of samples subjected to active (red) and formulation (green). The data illustrates a 95% confidence ellipse for the samples given. The score plot was created in MetagenAssist.

5.4 Discussion

In the past 60 years the increasing use of microbicides has been well documented (Maillard et al., 2013). Though knowledge of microbicide mode of action and mechanisms of induced cross-resistance are not always well-understood, there has been little evidence to suggest that microbicide in formulation exposure could lead to the development microbicide or antibiotic resistance, as defined by in-use or therapeutic concentrations, with studies examining effects of aqueous microbicides (Keren et al., 2004, Walsh et al., 2003b). BAC is a commonly used microbicide found in a wide-array of personal care products. Exposure of bacterial communities to sub-lethal concentrations of BAC has been previously seen to reduce bacterial diversity and promote the clonal expansion of intrinsically insusceptible bacterial species altering community antimicrobial susceptibility profiles (Tandukar et al., 2013).

5.4.1 Use of Domestic Drain Sampling and Constant Depth-Film Fermenter

As the basis of the project is to obtain an understanding of selective antimicrobial pressures that bacteria may experience in a domestic environment, the use of domestic drain biofilm as an inoculant for CDFF microcosm systems provides an effective means to recreate the interactions that may occur between microbicides and bacteria in the home (McBain et al., 2003b, McBain et al., 2003c, McBain et al., 2004). The CDFFs allow even bacterial biofilm growth on individual pegs at a set depth, while allowing the biofilms to be uniformly exposed to varying treatments. Each pan contained 5 pegs, which enabled technical replicates to be examined and samples to be stored for further analysis (DGGE, NGS). Though 2 CDFFs were established, both gave good similarities in terms of pre-treatment results. Marshall *et al.* (2012) used established household bacterial biofilms residing in drains for analysis; however as mentioned in the paper, it is more difficult to dictate product use within households than in a CDFF.

5.4.2 Use of Metagenomics for Data Analysis

16S rRNA gene sequencing allows bacterial identification based upon variable regions of the 16S region of bacteria rRNA genes (Stackebrandt and Goebel, 1994). Variable region V3 - V4 was amplified in this experiment. These regions are considerably more conserved and undergo less changes or mutations, allowing partial sequence analysis using specific primers complementary to the conserved sequence areas (Neefs et al., 1990). Amplification of genetic material using PCR is a well-established method, implementing the use of heat to disrupt hydrogen bonds holding helical structures in place. The cooling process allows annealing of the primers to the specific region of the RNA, due to the high abundance of primers, single stranded RNA annealing with the primers will occur; with the final step involving primer extension using DNA polymerase enzyme (Schochetman et al., 1988). Analysis of PCR products using DGGE is a useful tool for large community examination, as it allows a visual understanding of changes occurring within various bacteria. DGGE involves separation of RNA fragments down a urea

gradient by denaturation creating melting domains (Muyzer and Smalla, 1998). The denatured RNA leaves genetic fingerprints across the polyacrylamide gel which, when in combination with a fluorescence marker, becomes visible under UV light (Muyzer and Smalla, 1998).

DGGE has been previously used to examine community changes and dynamics successfully (Muyzer et al., 1993). Limitations in DGGE is evident; with potential of co-migration of DNA from different species occurring (Gafan and Spratt, 2005); also accuracy in identification past genus of bacteria not being reliable due to the short fragmented DNA being used. NGS is a useful analysing tool, which enables multiple bacterial partial sequences to take place at one time. Using the Illumina enables sequencing by synthesis by using fluorescently labelled nucleotides to be added to clustered lawns of anchored RNA after bridge amplification (Mardis, 2008). A laser emitted allows excitation of the fluorescently labelled nucleotides, which are then process and compared with a reference database to reveal the bacterial identification (Morozova and Marra, 2008). The technique is useful for examining large communities of bacteria, though large quantities of data are given and can become difficult to interpret (Reis-Filho, 2009).

5.4.3 The Effects of Microbicide Formulation of Anti-biofilm Potency

BAC has been seen to inhibit biofilm formation in numerous bacteria when used at below in-use concentrations (Houari and Di Martino, 2007). With previous studies (Chawner and Gilbert, 1989, Salton, 1968), explaining the loss of fluidity and cytoplasmic leakage after in-use concentration of QACs; Houari and Di Martino (2007) theorised the decrease in membrane fluidity at low concentration of BAC, may cause the bacteria to be unable to form biofilms, due to membrane vesicles unable to be released (Kadurugamuwa and Beveridge, 1995). If BAC was inhibiting biofilm formation in microcosm systems, due to decreases in membrane fluidity (Houari and Di Martino, 2007) then this may lead to disruption of the biofilm as shedded cells would be unable to attach to pegs and the biofilm would be unable to remain maintained. Due to the complex structure of biofilms, there have been studies which demonstrate lower susceptibility to

antimicrobials, due to failure to penetrate the biofilm (De Beer et al., 1994) or the slow growth caused by stress induced responses (Tuomanen et al., 1986).

5.4.4 The Effects of Microbicide Formulation on Preventing the Development of Microbicide and Antibiotic Insusceptibility in the Bacterial Biofilm

Decreases in BAC and antibiotic susceptibility were seen in both CDFF systems, with the BAC system showing a higher percentage of population able to grow on the higher concentrations of BAC or antibiotics. Loughlin *et al.* (2002) demonstrated that *P. aeruginosa* became less susceptible to BAC after prolonged sub-lethal exposure; however, cross-resistance to selected antibiotics did not occur, nor was the bacteria less susceptible to other antimicrobials. *P. aeruginosa* has been shown to be less susceptible to BAC (Loughlin et al., 2002), and has the ability to upregulate the production of efflux pumps readily (Chuanchuen et al., 2001). The study shows in both the culture method and NGS that Pseudomonads were prominent throughout both systems, which may explain the susceptibility changes, which occur. As previously mentioned, *Pseudomonas sp*. are well documented to have a higher tolerance towards many antimicrobials including, BAC (Adair et al., 1969), tetracycline (Li et al., 1994), ampicillin (Sivanmaliappan and Sevanan, 2011) and kanamycin (Poole, 2005). *S. pasteuri* has been known to be intrinsically less susceptible towards QACs, as well as, numerous antibiotics including tetracycline (Savini et al., 2009). Due to the mechanism of action of disruption of the cellular membrane, BAC is ineffective against spores as it is unable to penetrate the spore coating (Russell et al., 1985), and does not prevent the germination process (McDonnell and Russell, 1999). As *Bacillus* sp. are spore forming bacteria, it is likely the spores have survived in the CDFF vessel and germinated onto the cultured plates. *Alcaligenes* sp*.* are reportedly less susceptible towards quaternary ammonium compounds such as BAC (Meade et al., 2001), with changes to cellular membrane or the upregulation of efflux pumps reportedly being the mechanism for reduced susceptibility (Nagai and Ogase, 1990). Studies have shown *Micrococcus* sp. susceptibility to BAC to demonstrate (after exposure) a decrease of susceptibility (Hattori et al., 2003), with one study showing an

approximate 10 times decrease in susceptibility after exposure (Moore et al., 2008) and with another study showing *Micrococcus* sp. to be less susceptible to a number of antibiotics (Corse and Williams, 1968).

5.4.5 Analysis of Diversity and Susceptibility of CDFF

The data indicate BAC in formulation has higher anti-biofilm efficacy in comparison to the non-formulated counterpart which supports observations reported in Chapter 3.3, where microbicides in formulation enhanced antimicrobial potency. Formulations that contain non-ionic surfactants and sequestrants have been shown to have a bacteriostatic effect (Moore et al., 2006); as well as increasing bioavailability (Guha and Jaffe, 1996). The increase in bioavailability, as well as the further disruption to the cellular membrane (Moore et al., 2006), may enable the microbicide to penetrate the biofilm more readily than is the case in aqueous solution. This could explain the mitigation of the development of BAC and antibiotic insusceptibility, since the microbicide in a formulation has increased potency which decreases the likelihood bacterial viability following exposure as the potential for a formation to interact with multiple pharmacological sites in the bacterium (Moore et al., 2006). The bacteria identified from cultured methods are seen to be intrinsically less susceptible to BAC in aqueous solution, demonstrating susceptible bacteria had been eradicated after BAC exposure, whilst less susceptible species remained in the system; initially at low copy numbers which may not have been detectable in the culture methods but are seen in the NGS methods (*Alcaligenes* sp. are seen at low levels in NGS). It has been previously shown that *Achromobacter* sp. in biofilms are less susceptible to BAC exposure (Chang et al., 2015), which correlates with the increases seen in the systems. Intrinsically resistant species of bacteria to BAC have previously been researched, to find an increase in cellular fatty acids and phospholipids decreases absorption of BAC into the bacteria cell (Sakagami et al., 1989). The results suggest compositional similarities with exposure of BAC and BAC formulation, with variations between concentrations.

5.5 Conclusion

Domestic biofilm microcosms subjected to BAC in aqueous solution or within a formulation representative of realistic deployment underwent changes in bacterial community diversity and BAC and antibiotic susceptibility profiles. Decreases in numbers of viable bacteria appear to be more pronounced in the system treated with BAC alone rather than BAC formulation. Although there were varying degrees of difference of reduction in viable bacteria in each system, in terms of detected genus, both systems showed a high degree of phylogenetic similarity. Culture-dependent and DNA-based analyses suggest that changes in community susceptibility profiles may, in part, be due to the clonal expansion of intrinsically antimicrobial insusceptible species such as Pseudomonads and Bacillus. This provides further evidence of the increased antimicrobial efficacy of microbicides in formulation and provides evidence on their potential to mitigate the development of antimicrobial insusceptibility in a mixed species biofilm.

Chapter 6

General Discussion

6.1 Study Overview

The widespread use of microbicides in consumer products over the past decade has led to increased interest and concern about potential effects on bacterial susceptibility. There is however, limited evidence that acquired bacterial resistance towards microbicides occurs in the environment. Many *in vitro* studies have however demonstrated that susceptibility changes can occur mainly in experiments that have utilised single bacterial species, after repeated exposure to sub-inhibitory concentrations of microbicides (Walsh et al., 2003b). This type of experiment, although a useful tool to evaluate potential for susceptibility changes to occur does not reproduce the conditions in which bacteria are likely to be exposed to microbicides in the real world, which is in multispecies communities, often in sessile form (biofilms). The common use of microbicides in aqueous solution is another potential confounder since microbicides are almost always used in formulated products.

The majority of reports in the literature that have focussed on the effects microbicide exposure on bacterial susceptibility have not investigated other phenotypic alterations that may occur as a consequence of microbicide adaptation including changes in pathogenicity, and processes that may determine the likelihood of an adapted bacterium persisting in the environment (broadly termed “fitness”). The overall aim of Chapter 3 therefore was to evaluate the consequences of microbicide adaptation in bacteria. This thesis had three distinct foci; i) to assess the comparative antibacterial activity of test microbicides as aqueous solutions versus the same microbicides incorporated into formulations (BAC, BIT, CHX, DDAC, DMDM hydantoin, PHMB, thymol and triclosan) were evaluated against a panel of bacteria which had been repeatedly exposed to microbicides often used in home and personal care products both as pure actives and in complex formulations that mimicked their real world use. The stability of any observed susceptibility changes were then determined by subsequently passaging the antimicrobial exposed bacteria in the absence of microbicide/ formulation and re-evaluating susceptibility. The intention of this was to i) assess whether alterations in susceptibility were likely to be due to short-term phenotypic adaptation of more stable genetic changes and also to better understand the potential of an adapted bacterium to persist in the environment without reverting

to its original susceptibility profile. Following this, Chapter 4 examined further phenotypic changes in the antimicrobial exposed bacteria were examined through analysis of the relative pathogenesis using a wax worm model, biofilm formation, antibiotic susceptibility and competitive fitness of the exposed bacteria compared to the unexposed isolates. Finally, using a constant depth film fermenter microcosm system Chapter 5 investigated the effects of exposure over *n* days or *n* months of a mixed species bacterial biofilm community to benzalkonium chloride (BAC) in aqueous solution and benzalkonium chloride (BAC) in formulation examining community diversity and susceptibility.

6.2 The Formulation of Microbicides Increases Antibacterial Potency and Mitigates the Development of Insusceptibility

Initial susceptibility testing showed the majority of bacteria to exhibit susceptibility changes in response to the selected microbicides, with increases in potency observed when microbicides were used in formulation (average of 11-fold lower MIC and MBC for formulated microbicides), likely the addition of surfactants and sequestrants which are known to exhibit biocidal properties (Guha and Jaffe, 1996). Both biguanides, CHX and PHMB, were effective at inhibiting the growth of test bacteria at low concentrations, with strong bactericidal activity evident at higher concentrations. Previous investigations have documented the strong antimicrobial and anti-biofilm potency of CHX and PHMB (Nascimento et al., 2008). Equally, Chapter 3 demonstrates both QACs (BAC and DDAC) as well as the bisphenol (triclosan) to be effective against the test bacteria, with low concentrations showing both bacteriostatic and bactericidal potential, with the exception of the test *Pseudomonas* sp. Pseudomonads are known to be relatively insusceptible towards triclosan and QACs such as BAC (Chuanchuen et al., 2001, Chuanchuen et al., 2003). Triclosan insusceptibility has been widely documented in *P. aeruginosa,* and has been attributed to expression of the multidrug efflux pump MexCD-OprJ.

Microbicide-containing formulation initially demonstrated increased inhibitory and bactericidal activity compared with the non-formulated microbicides. Microbicides are frequently

in deployed in formulation that contain surfactants and sequestrants, which have been seen to increase the activity of microbicides (Guha and Jaffe, 1996). This could potentially be due to their effects on the bacterial cell envelope increasing cell permeability and exposure to intracellular microbicide target sites. Additionally, various excipients such as non-ionic surfactants and sequestrants can interact with different cellular targets to the accompanying microbicide (Rosen and Kunjappu, 2012, White, 1993), therefore possibly producing a cumulative antimicrobial effect that increases antibacterial potency as well as resulting in the bacteria requiring multiple physiological adaptations in order to become insusceptible. Data presented in Chapter 3 support the relevance of this phenomenon because fewer, and generally lower in extent, susceptibility changes being observed in bacteria after exposure to microbicides in formulation, compared to microbicide in aqueous solution.

Of the microbicides tested, triclosan induced the highest frequency of susceptibility changes in the test bacteria, probably due to the acquisition of mutations in the specific intracellular target enzyme, fabI (McMurry et al., 1998). There was a high frequency of susceptibility changes in the test bacteria to both quaternary ammonium compounds (QACs); BAC and DDAC. The induction of insusceptibility towards QAC has largely been previously attributed to the increased expression of efflux pumps (Okusu et al., 1996, Poole, 2005, Romanova et al., 2006). Efflux pumps are transport proteins which move harmful agents out of the cell more rapidly than the influx process (Nikaido, 1996) and this may explain the decreases in susceptibility after long term exposure of sub inhibitory concentrations of DDAC and BAC, which were observed in many of the bacterial isolates. Whilst the overexpression of efflux pumps may explain many of the changes in susceptibility which occur in the bacterial isolates, other factors may be involved, such as; changes to the lipid composition of the cytoplasmic membrane (Arias et al., 2011), or changes in cell envelope permeability due to LPS modifications as well as target site adaptation (McMurry et al., 1998). After recovery in an antimicrobial-free environment, 72% of the tested bacteria reverted back to pre-exposure susceptibility which suggests that phenotypic adaptation were responsible, such as the temporary increased expression of efflux pumps, which revert once the stress-induced response has been removed (Kaatz and Seo, 1995). Importantly, although

adaptations to susceptibility of the bacteria were observed, adapted bacteria have been shown to be mostly susceptible to in-use concentrations of the microbicides (Kawamura-Sato et al., 2008) after adaptations have occurred.

In terms of minimum biofilm eradication concentration (MBEC), the microbicides were more effective when in formulation than when in aqueous solution, with the most notable difference seen between pure triclosan and triclosan-formulation. As mentioned above, surfactants and sequestrants are compounds with the ability to preserve products and increase bioavailability. Surfactants, such as, alcohol ethoxylates are known to disrupt bacterial cell membranes, increasing permeability of the cells, leading to leakage of potassium ions causing bacterial inhibition (Moore et al., 2006). Surfactants can therefore increase potency of microbicides when they are combined and can affect a bacterial cells ability to form biofilms by inhibiting bacterial growth. Sequestrants, such as, STP-P and EDTA, are also have antimicrobial activity (Vareltzis et al., 1997). STP-P and EDTA are chelating agents, which cause disruption of the bacterial membrane through the binding and removal of cations, which destabilises the outer membrane (Haque and Russell, 1974). EDTA causes reductions in bacterial biofilms due to this membrane instability effect, with studies showing substantial reduction in biofilms incidence in hemodialysis catheters (Kite et al., 2004, Banin et al., 2006). Banin *et al.* (2006) showed detachment and dispersal of *P. aeruginosa* cells from biofilms, as well as complete killing of planktonic species caused by chelating of divalent cations from lipopolysaccharide (LPS), causing LPS to be released from the cell. Sequestrants have previously been shown to bind cations, such as Zn^{2+} in *S. aureus*, which are essential for biofilm formation (Geoghegan et al., 2013, Barbu et al., 2014). Previous studies have shown reductions bacterial growth and in bacterial biofilm formation after exposure to sequestrants due to the binding of divalent cations (Humphreys et al., 2011, Maier et al., 1999). Maier *et al.* (1999) reported reductions in septum formation of dividing cells when a sequestrant is added, due to the binding of divalent cations which are needed for GTPase activity of FtsZ which is a protein required for septum formation of the cell.

The majority of previous studies in this area have analysed sub inhibitory concentrations of microbicides upon single bacterial species (Cookson et al., 1991, Walsh et al., 2003b), with

little focus on microbicide in formulation and whether susceptibility changes are sustained or transient after removal of the microbicide selective pressure. Data presented in this doctoral thesis are in agreement with previous reports that have indicated that changes in susceptibility can occur after prolonged exposure to microbicides or formulations; however, data in this thesis suggest that in majority of cases, these susceptibility changes revert back to pre-exposed values once the antimicrobial was removed. This is in agreement with the limited number of available previous studies (Tattawasart et al., 1999) which have shown similar reversions in susceptibility in bacteria after prolonged antimicrobial exposure. Tattawasart *et al.* (1999) demonstrated this by exposing *P. stutzeri* to increasing concentrations of chlorhexidine diacetate (CHX) resulted in a reduction in CHX susceptibility, with the strain reverting back to pre-exposure values. Sustained changes after removal of the microbicide can indicate inherently stable mutations occurring, causing continued decreases in susceptibility.

6.3 Microbicide-Induced Phenotypic Adaptations in Bacteria

As mentioned in Chapter 4, previous studies have examined the effects of microbicide adaptation in bacteria in terms of susceptibility changes, with few studies being conducted into the resulting phenotypic adaptations which may occur after prolonged microbicide exposure (Knobloch et al., 2002, Latimer et al., 2012). Phenotypic adaptations can result in changes in the ability of bacteria to form biofilms (Knobloch et al., 2002a) or changes in planktonic growth rate; reductions in competitive fitness (Bouma and Lenski, 1988); relative pathogenicity (Li et al., 1998) and changes to antibiotic susceptibility (Walsh et al., 2003b). In Chapter 4, the data indicates that there were significant changes in planktonic growth rates in multiple bacterial isolates after microbicide/formulation exposure. Decreases in growth of bacteria may directly correlate to changes, for instance in antibiotic susceptibility, in the case of antibiotics such as ciprofloxacin that target actively growing cells (Tomasz, 1979). This provides a possible mechanism by which microbicide adaptation may lead to the induction of antibiotic-cross resistance for certain bacteria.

Changes in biofilm formation were also evident in many of the adapted bacterial isolates. *P. aeruginosa* demonstrated a marked decrease in biofilm formation after exposure to both QACs (BAC and DDAC), with DDAC exposure demonstrating a sustained adaptation. *P. aeruginosa* is known to cause persistent infections in many immunocompromised patients, especially those suffering from cystic fibrosis due to the ability of the bacteria to readily form biofilms (Costerton, 2001). A decrease in biofilm formation could potentially lead to decreases of *P. aeruginosa* infections and contaminations in hospitals, if they have an inability to attach to surfaces. Changes to the lipopolysaccharide of the bacteria can lead to reductions in attachment and potentially decreases in biofilm formation (Makin and Beveridge, 1996). Increases in biofilm formation were also seen, with *E. coli* exposed to BAC showing a notable increase. Hoffman *et al.* (2005) show antibiotic exposure to bacteria, *P. aeruginosa* and *E. coli*, can induce biofilm formation as a specific defensive reaction. Exposure of microbicide in formulation to bacteria demonstrates fewer changes to biofilm formation than exposure of microbicide. Formulations containing surfactants and sequestrants are known to reduce biofilm formation in bacterial species via chelation (Moore et al., 2006).

A reduction in competitive fitness may result in the potential decrease of the bacteria to persist or establish in the environment and failure to compete with their congeners (Besier et al., 2005). After exposure to microbicides, the majority of bacteria were shown to exhibit a decrease in competitive fitness, with the most marked changes being observed in *E. coli* and *S. aureus* when exposed to triclosan. Therefore, it is apparent that bacterial adaptations to microbicide exposure can result in costs in bacterial fitness. Similar changes in bacterial fitness have been frequently demonstrated with antibiotic resistant bacteria, with previous investigations suggesting reversion of the fitness cost once the selective pressure is removed (Björkman and Andersson, 2000, Levin et al., 2000). Exposure of bacteria to microbicides in formulation resulted in a reduction in competitive fitness, with the most notable decrease occurring in *S. aureus* after exposure to BAC. There were increased fitness costs seen with microbicide exposed bacteria, showing adaptations to susceptibility leading to fitness costs.

Examining the relative pathogenesis of bacteria can provide insight into their ability to cause infection, with decreases in relative pathogenicity potentially being of clinical importance (Wilson et al., 1995). In the thesis, the *G. mellonella* model was used to determine the relative pathogenicity of the bacteria isolates. Data indicate that the majority of exposed bacteria underwent decreases in relative pathogenicity, with the most marked decreases observed in *E. coli* exposed to BAC and triclosan and the *E. coli* drain isolate exposed to DDAC, DDAC formulation and triclosan. Induced changes in antibiotic susceptibility in bacteria after exposure to microbicide can also have a substantial impact on the clinical environment. This type of induced cross-resistance has been seen previously shown in bacteria to quinolones, beta-lactams or aminoglycosides (Sanders et al., 1984). Cross-resistance to microbicides is less well documented, although some studies have shown decreases in third party microbicides after microbicide exposure (Chuanchuen et al., 2001, Walsh et al., 2003b). In this thesis, statistically significant changes in antibiotic susceptibility were observed in 29% of the selected isolates exposed to microbicides, with 14% demonstrating decreases and 15% demonstrating increases in overall antibiotic susceptibility. Of the bacteria exposed to microbicides in formulation, 16% demonstrated changes to antibiotic susceptibility, with 8% showing increases and 8% showing decreases. Overall, the data in this thesis shows no evidence of cross-resistance to occur with any combination of microbicides/antibiotics.

Out of all the test actives, triclosan exposure resulted in the highest frequency of changes in antibiotic susceptibility in the test bacteria, with marked decreases in ampicillin susceptibility seen in *S. aureus* and *C. sakazakii*. Since ampicillin causes cellular damage by disrupting cell wall synthesis, it is possible that decreases in ampicillin susceptibility may be due to an already slow growth rate in bacteria that occurs as a result of triclosan exposure, possible due to the inhibitory effects on fatty acid synthesis. Previous studies have shown potential cross-resistance occurring in triclosan exposed organisms due to an increase in efflux pumps (Chuanchuen et al., 2001), which may explain the lowering of susceptibility in some of the test bacteria. However, the majority of bacteria exposed to triclosan showed an increase in antibiotic susceptibility, demonstrating a potential cost in fitness. Overall, the data represent the array of phenotypic

changes that may occur in bacteria after exposure to microbicides and microbicide formulations. In the majority of cases these phenotypic defects, for instance in competitive fitness and relative pathogenicity, appear to be more pronounced after exposure to the formulations rather than the pure microbicides. This may be due to the fact that formulations have a multi-targeted site mode of action on the bacterial cell, therefore may potentially induce more considerable levels of adaptation in the bacteria.

6.4 Changes in Community Composition and Antimicrobial Susceptibility Profile of a Mixed Species Biofilm after Exposure to Benzalkonium Chloride and a Benzalkonium Chloride Containing Formulation.

In this thesis, the adaptations exhibited by single species of bacteria after exposure to microbicides in aqueous solution and microbicides containing formulation were initially examined. To enable a deeper understanding of the effects of antimicrobials in the domestic environment, the final parts of the study focused upon assessing changes in the community composition and antimicrobial susceptibility profile of a mixed species bacterial community after long-term exposure to benzalkonium chloride or a benzalkonium chloride containing formulation. Previous similar investigations into the impact microbicide exposure to bacteria in a domestic setting have used the biofilm models based upon the bacterial composition that you may find in a kitchen drain biofilm community (McBain et al., 2003c), as well as examining the effects that microbicides have upon the community (McBain et al., 2004, McBain et al., 2003b). However, there has been no previous study which has evaluated the effects that exposure to microbicides containing formulation has upon the bacterial community in comparison to exposure to microbicide in aqueous solution. Examining the effects that microbicides have upon domestic drain microorganisms will help provide a clearer understanding of the impact that the continuous use of household cleaning products may have upon domestic bacterial communities, and potentially allow us to develop more realistic risk assessments of the potential induction of bacterial insusceptibility in the environment.

In Chapter 5, the overall taxonomic diversity of the bacterial community was significantly reduced after exposure to BAC or BAC in formulation after exposure to 1% Diversity of bacterial communities may alter due to changes in nutrient availability (Lebaron et al., 1999), alterations of oxygen concentrations (Tunney et al., 2013) and the introduction of harmful substances, such as antibiotics or microbicides (Moore et al., 2008). In addition to a progressively more pronounced decreases in total bacterial viability, changes in the relative abundance of detected genus was observed after each increase in BAC dosing concentration, through QIIME analysis. Decreases in the abundance of initially prominent genera were evident such as, *Bacteriodes, Elizabethkingia, Comamonas* and *Dygonamonas*, after dosing with BAC in aqueous solution (section 5.3.3), with many innately susceptible species *Elizabethkingia, Klebsiella, Aermonadaceae* and *Hydrogenophaga* rapidly decreasing in relative abundance after BAC in formulation addition (section 5.3.3). CDFF bacterial microcosm communities exposed to BAC in aqueous solution or BAC in formulation, showed similarity in detected genus throughout the BAC exposure period. This demonstrates that both aqueous microbicides and microbicides in complex formulations cause similar shifts in the bacterial community composition. However, after determining the viability of the treated biofilm it was evident that BAC in formulation had substantially enhanced greater antibacterial potency when compared to the BAC in aqueous solution.

The thesis shows that reductions in BAC and antibiotic susceptibility occur within a bacterial community after exposure to BAC. Although, identification of isolated bacteria at the end of the exposure period from both microcosms indicated that this was probably due to the clonal expansion of innately insusceptible bacterial species such as *Pseudomonas*, *Bacillus* and *Clostridium* through QIIME analysis. Decreases in BAC and antibiotic susceptibility were observed during exposure to both BAC in aqueous solution and BAC in formulation. The clonal expansion of insusceptible bacteria with the community, after more susceptible species were eradicated, may explain the continuously shifting community profiles observed within the microcosm. Equally the induction of change in susceptibility may also have occurred; however, further testing would be needed to determine this point.

6.5 Future Work

The current study examined the exposure of antimicrobial compounds to bacterial communities in realistic conditions, examining aspects of the development of microbicide insusceptibility with single species of bacteria and within mixed species communities. The research has enabled a further understanding into microbicide-containing formulations in realistic approaches to antimicrobial susceptibility changes by examining effects and changes occurring with domestic drain biofilms, as well as, providing analysis of phenotypic adaptations. Further analysis into the impact of other microbicides (biguanides, bisphenols, essential oils, etc.) and formulations on domestic drain bacterial communities, would allow a more detailed risk assessment to be developed of the potential risk of using microbicide-containing products in the home. Chapter 5 examined the impact of BAC and BAC in formulation on the bacterial drain community; however, this could be expanded to include all other microbicides/formulations mentioned in the study.

Future work could examine the effects residual concentrations of microbicides that reside in waste water have upon bacterial communities. Previous studies have suggested that concentrations up to 50ng/L and 100ng/L for triclosan and BAC respectively, may be found in rivers or waste water systems (Martínez-Carballo et al., 2007, Lawrence et al., 2015). Examining the effects residual concentration may enable further understanding of the impact that microbicides have on wider-environment, not just within the domestic setting. Transcriptomic and proteomic analysis of microbicide/formulation exposed bacteria would aid in the understanding of the mechanisms which allow the bacteria to become less susceptible toward certain antimicrobial. RNA-seq is a technique which could be used to provide comparisons in the transcriptomes of the selected bacteria, enabling an understanding of the alterations in gene transcripts in response to a particular antimicrobial stress (Ozsolak and Milos, 2011). Understanding how bacteria adapt towards microbicides on a molecular level may provide insight into further therapeutic target sites within the bacteria that could be exploited in the production of future antimicrobial agents.

6.6 Conclusion

As discussed through this thesis, evaluating susceptibility changes in bacteria exposed to microbicides via realism-based approaches, may enhance understanding of the potential effects of microbicides in real world deployment. Chapter 3 describes studies in which bacteria were exposed to microbicide in aqueous solution or microbicide-containing formulation. The study demonstrated that susceptibility changes may occur but generally fewer changes were observed for bacteria following exposure to microbicide-containing formulations. In Chapter 4, phenotypic adaptations were detected for antimicrobial exposed bacteria with reduced susceptibility, although there were fewer changes occurring with bacteria exposed to microbicide-containing formulations, with no evidence of cross-resistance occurring. Chapter 5 describes studies in which long-term exposure of domestic biofilm communities to antimicrobial were investigated. Microbicide exposure of the test biofilms resulted in decreases in viable bacteria and shifts in community composition, potentially caused by the increase of the occurrence of intrinsically insusceptible bacterial species; an effect that was more marked in microbicide in aqueous solution exposed biofilms. Throughout the doctoral project, the formulation of microbicides with surfactants and sequestrants was observed to increase potency and mitigate the development of insusceptibility. In conclusion, the studies demonstrated throughout the doctoral thesis considered not only any induced microbicide susceptibility changes in bacteria but also the stability of such changes as well further consequential phenotypic alterations. This enabled further analysis of drain biofilm community shifts after long term use of microbicide, as well as examining all aspects of microbicide deployment as a formulation. The considerable analysis led to an overall conclusion that changes to bacterial susceptibility, phenotypic alterations and community shifts that occur after long term selective exposure to microbicide in aqueous solutions, are not reflective of real world deployment, with formulations being seen to show fewer and less sustained changes throughout the studies.

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