

Bacterial Resistance to Oxidising Biocides: An Assessment of Resistance Mechanisms and Method Development

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Summary

Oxidising biocides are chemical agents grouped together by a similar mechanism of action. They are used in a range of settings to remove unwanted bacterial contamination. Bacterial resistance or decreased susceptibility to biocides was first observed over 70 years ago. The majority of bacteria exist as surface attached communities known as biofilms. Biofilms have a greatly reduced susceptibility to antimicrobials including biocides compared to their planktonic counterparts. For this reason, it is important to study the effect of antimicrobials on biofilms and planktonic cells.

The aim of this project is to understand the mechanisms that allow survival and tolerance of bacteria exposed to oxidising biocides by using *Salmonella* as a representative enteric human pathogen.

A range of methods were used to investigate the response of *Salmonella enterica* to sodium hypochlorite, hydrogen peroxide and peracetic acid. These included culture based methods such as inactivation kinetics and MICs. Flow cytometry, Hoescht assay and GFP reporters were also used to investigate the bacterial response to biocide in the planktonic state. Confocal microscopy was undertaken to investigate the effect of biocides on the structure of biofilms and determine the distribution of survivors within biofilms.

Planktonic *Salmonella* were found to be more susceptible than intact biofilms, but once the biofilms were dispersed this protective effect was reduced. The protective effect was also seen in *Salmonella* Typhimurium SL1344 which is a poor biofilm former. Hydrogen peroxide exposure resulted in a change to cellular permeability, however this was not linked to efflux through the *acrAB* system. Flow cytometry demonstrated differential survival upon exposure to different biocides and identified potentially viable but non-culturable populations. Confocal microscopy demonstrated non-uniform distribution of survival of cells within a biofilm. An additional observation is that bacteria responded differently to each of the three biocides tested; there is not one consistent response to biocides with oxidising activity.

This research shows the importance of using appropriate methods to test biocides. A key recommendation of this study is that all biocides should be tested against biofilms. This would give a better understanding of the efficacy of the biocides in more realistic and challenging conditions.

List of Abbreviations

µg	Micrograms
µL	Microlitres
µM	Micromolar
AMP	Ampicillin
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BOX	Bis (1,3-dibutylbarbituric acid) trimethine oxanol
BS	British Standard
BSAC	British Society for Antimicrobial Chemotherapy
CFU	Colony forming units
CFU/cm ²	Colony forming units per centimetre squared
CFU/mL	Colony forming units per millilitre
CMCC	Colworth Microbiology Culture Collection
cm	Centimetre
diH ₂ O	Deionised water
DNA	Deoxyribonucleic acid
EN	European Standards
FACS	Fluorescence-activated cell sorting
FC	Flow cytometry
<i>g</i>	Gravity
g	Grams
GFP	Green-fluorescent protein
ISA	Iso-sensitest agar
ISB	Iso-sensitest broth
H ₂ O ₂	Hydrogen peroxide
hr(s)	Hour(s)
L	Litre
Log ₁₀	Base 10 logarithm
LPS	Lipopolysaccharide
M	Molar
M9	M9 minimal media
MBC	Minimum bactericidal concentration
mg	Milligrams
min(s)	Minute(s)
MIC	Minimum inhibitory concentration
mL	Millilitre
MOPS	3-(N-Morpholino)propanesulfonic acid
MW	Molecular weight
nm	Nanometre
NaCl	Sodium chloride
NaOCl	Sodium hypochlorite
OD	Optical density
PAA	Peracetic acid
PBS	Phosphate buffered saline
pH	Potentiometric hydrogen ion concentration
PI	Propidium Iodide

ppm	Parts per million
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks
SD	Standard deviation
TSA	Tryptone soya agar
TSB	Tryptone soya broth
TSC	Tryptone sodium chloride
TVC	Total viable count
w/v	Weight for volume

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Chapter One: General Introduction

1.1 Biocides

1.1.1 Definition of a biocide

The EU definition of a biocidal product is a material containing active substances that can inactivate harmful organisms (EU, 2012). It is however, worth noting that biocides are non-specific in their activity and inactivate any organisms present, whether or not they are harmful.

There are three main ways in which biocides are used: preservatives, antiseptics and disinfectants. Preservatives are chemical additives used to prevent premature spoilage of a product. Antiseptics are antimicrobial compounds applied to the skin or tissue to prevent infection. This project focusses on the disinfectant uses of biocides. Disinfectants are used to inactivate microorganisms in the environment such as those found on hard surfaces or in water.

1.1.2 Classes of biocides

There are a wide range of biocides commercially available dependant on the desired application. Some of the most common classes of biocides are listed below in table 1.1 which describes the chemical group they belong to, examples of biocides in that group, the uses of these classes of biocide and the mode of action of the specific examples highlighted.

Table 1.1: Some of the major groups of biocides (Scientific Committee on Emerging and Newly Identified Health Risks (2009); Al-Adham *et al.* (2013)).

Chemical Group	Example biocides	Uses	Mode of action
Alcohols	Ethanol	Cosmetic products, hand sanitizer	Membrane disruption; protein denaturation and therefore metabolic inhibition
	Isopropanol		
Aldehydes	Glutaraldehyde	Previously used for disinfection of reusable medical devices	Cross linking of vital biological macromolecules
	Formaldehyde		
Biguanides	Chlorhexidine	Skin antiseptics; contact lens solutions; endodontic irrigant	At low concentrations- inhibition of membrane enzymes causing cell leakage. At high concentrations- inhibition of ATPase; membrane disruption; coagulation of the cytoplasm
		Water treatment; disinfection of bio-hazardous waste spillages; disinfection of reusable medical devices; environmental disinfection	Oxidises important biomolecules such as key enzymes, structural damage (to proteins, carbohydrates and lipids) and DNA damage
Halogen releasing agents	Sodium hypochlorite	Skin antiseptics; wound cleaning	Oxidises thiol groups in the cytoplasm; disruption of protein and nucleic acid structure and synthesis
	Iodophores	Incorporation into surfaces in hospitals; fabric, wood, paper and paint preservation; water systems; agricultural disinfection	Interaction with thiol groups
Heavy metal derivatives	Copper compounds	Prevention of wound infection; treatment of endodontic infections; water purification	Reacts strongly with thiol groups; induces cytological changes; DNA damage
	Silver compounds	Contact lens disinfection; food industry; disinfection of reusable medical devices	Damage to membrane lipids; DNA damage (both caused by oxidising damage)
Peroxygens	Hydrogen peroxide	Food industry; waste water treatment; disinfection of reusable medical devices	Denaturing proteins; disrupting cell wall permeability; oxidising important biomolecules
	Peracetic acid	Disinfectant; paper and cardboard industry; an additive to paraffin wax for wax paper and bottle cap lining	Membrane disruption; inhibition of cell wall synthesis; coagulation of the cytoplasm
Phenols	2-Phenylphenol	Personal care products; toothpastes; incorporation in plastics and fabrics	At low concentrations triclosan also inhibits lipid synthesis by inhibiting enoyl acyl reductase at low concentration
	Triclosan	Food industry; skin antiseptics; medical eye drops and skin creams; wound cleaning	Membrane disruption
Quaternary ammonium compounds (QACs)	Benzalkonium chloride	Skin antiseptics; wound cleaning	
	Cetrimide		

1.1.3 Use of biocides

Biocides are used in a wide range of environments such as: water treatment; disinfection of a clinical environment; industrial settings for example food, pharmaceuticals and cosmetics; and increasingly in domestic products. The choice of biocide is dependent upon the environment in which it is going to be used; for example in the food industry, where it is important that the by-products are safe, hydrogen peroxide is used extensively (Linley *et al.*, 2012). Another example would be the use of silver alloys for antimicrobial surfaces as they are an alternative to biocides that may be corrosive and are often used for antimicrobial coatings (Silvestry-Rodriguez *et al.*, 2007).

The European market for biocides in 2006 was worth €10-11 billion and was predicted to grow further, having grown 4-5% a year in the preceding 15 years (Scientific Committee on Emerging and Newly Identified Health Risks, 2009). This rate of growth has led to concerns about the safety of the use of biocides with regard to resistance to biocides and antibiotics. For further information see **Chapter One, section 1.4** for bacterial resistance and tolerance to biocides.

1.2 Oxidising biocides

Oxidising biocides are chemical agents grouped together by a similar mechanism of action. The mechanism by which they exert their biocidal activity is through the removal of electrons from susceptible functional groups causing damage to biomolecules (Finnegan *et al.*, 2010). They are usually low molecular weight compounds either readily passing through the membrane of a bacterial cell and causing cell death by damage to internal targets, or they can disrupt the cell wall and membrane and cause cell death by that route (Finnegan, *et al.*, 2010). The targets of these biocides can be the cell surface, cell wall or intracellular components giving these agents a very broad spectrum of activity. There is a wide range of oxidising biocides available but this project focuses on the following three: hydrogen peroxide, peracetic acid and sodium hypochlorite. Sodium hypochlorite is currently the most widely used oxidising biocide for water disinfection and peracetic acid and hydrogen peroxide are being investigated as suitable alternatives. The properties of these three biocides are summarised in table 1.2 and then explored in more detail in below.

Table 1.2: Summary information of hydrogen peroxide, peracetic acid and sodium hypochlorite (Kitis (2003), Fukuzaki (2006), Finnegan, *et al.* (2010), Linley, *et al.* (2012)).

Biocide	Mode of Action	Advantages	Disadvantages
Hydrogen peroxide	<ul style="list-style-type: none"> • Primary mechanism of action is DNA damage • Interaction with intracellular iron to form hydroxyl radicals • Oxidative damage to other biomolecules e.g. lipids and proteins 	<ul style="list-style-type: none"> • Easily degrades into non-toxic by-products • No odour • Can be used on a range of materials • Colourless • Broad spectrum of antimicrobial activity 	<ul style="list-style-type: none"> • High concentration required for antimicrobial activity • Unstable and prone to degradation • Ineffective in the presence of peroxidases and heat
Peracetic acid	<ul style="list-style-type: none"> • Denaturing proteins and lipoproteins disrupting the cell wall and cell membrane • Protein damage of intracellular enzymes • Additionally damages thiol (S-H) and sulphur (S-S) bonds 	<ul style="list-style-type: none"> • Broad spectrum of antimicrobial activity • Degrades into non-toxic by-products • Colourless • Continued activity in the presence of organic material • Greater oxidising potential than either chlorine or hydrogen peroxide 	<ul style="list-style-type: none"> • Strong odour • Unstable and prone to degradation • Acetic acid (one of the by-products of degradation) can be broken down and used as a nutrient source for bacterial regrowth after disinfection
Sodium hypochlorite	<ul style="list-style-type: none"> • Postulated that the primary mechanism of action is DNA damage • Oxidation of C=C bonds, peptide bonds, peptide groups and thiol groups damaging cell structure and respiration 	<ul style="list-style-type: none"> • Broad spectrum of rapid bactericidal activity • Low cost and ease of use • Persistence in water • Use for bleaching in addition to disinfection 	<ul style="list-style-type: none"> • Strong odour • Decreased efficacy when organic material is present • Unstable and prone to degradation • Can result in the formation of toxic by-products

1.2.1 Hydrogen peroxide

Hydrogen peroxide is an oxidising agent used for disinfection, sterilisation and antiseptics. First discovered in 1818 by Louis Thenard (Thenard, 1818) it was first used as a disinfectant in 1891 (Richardson, 1891). Hydrogen peroxide is a colourless liquid that is slightly more viscous than water and is a weak acid with a pK_a of 11.75 (Housecroft & Constable, 2010). Unlike many other biocides (such as sodium hypochlorite which can produce chloramines) hydrogen peroxide degrades into the non-toxic by-products, of water and oxygen (Linley, *et al.*, 2012). Treatment with catalase, peroxidase enzymes or heat degrades hydrogen peroxide leaving no harmful residue.

The effective concentration of hydrogen peroxide varies depending on how it is to be employed; for example, it is used at a concentration range of 3-6% (v/v) in water for wound and surface treatment, 0.4-1% (Linley, *et al.*, 2012) is used as a commercial dental disinfectant and 7.5% is used as a high level disinfectant in a clinical environment (Scientific Committee on Emerging and Newly Identified Health Risks, 2009). Hydrogen peroxide has a number of advantages: no activation is required; it can be used on a wide range of materials; it is safe to dispose of; and there is no odour, unlike chlorine-based alternatives (Rutala & Weber, 1999a).

There have been very few studies describing the precise mechanism of hydrogen peroxide action on bacterial cells at disinfection levels (Linley, *et al.*, 2012). Studies have shown that the biocidal activity of hydrogen peroxide may be due to interactions with intracellular iron forming iron ions and hydroxyl radicals (Finnegan, *et al.*, 2010) according to the Fenton reaction below:

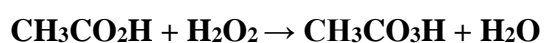


These ions and hydroxyl radicals damage biomolecules which subsequently leads to loss of function and cellular death due to oxidative damage. Several studies have shown hydrogen peroxide to be responsible for damage to DNA (Henle and Linn (1997), (Imlay *et al.*, 1998) proteins and amino acids (Dean *et al.*, 1997), and cell membranes (Baatout *et al.*, 2006; Brandi *et al.*, 1991; Peterson *et al.*, 1995). However, these studies into the biocidal activity of hydrogen peroxide have only used typically low levels of exposure (less than 850 ppm). This means these results may not be applicable to the concentrations of hydrogen peroxide used for disinfection; between 2700 ppm (0.27%) and 60000 ppm (6%) (Linley, *et al.*, 2012), and up to 75000ppm (7.5%) for high level disinfection which is defined as the inactivation of all microorganisms except large numbers of spores (Scientific Committee on Emerging and Newly Identified Health Risks, 2009).

Although bacteria that possess cytochrome systems (aerobes and facultative anaerobes) have catalase as protection against the hydrogen peroxide that is produced due to intracellular metabolism, these defences are completely overwhelmed at the in-use concentrations (6-7.5%) during hydrogen peroxide disinfection (Rutala & Weber, 1999b).

1.2.2 Peracetic acid

Peracetic acid (PAA) is a peroxide of acetic acid produced by reacting acetic acid with hydrogen peroxide in the presence of a sulphuric acid catalyst (Block, 1991). It is soluble in water and other polar solvents and exists in equilibrium between acetic acid (CH₃CO₂H), peracetic acid (CH₃CO₃H), water (H₂O) and hydrogen peroxide (H₂O₂) as shown below (Block (1991); Gehr *et al.* (2002)):



In commercial preparations there is also usually a stabiliser or sequestering agent added to PAA such as dipicolinic acid (Kurschner & Diken, 1997). This is needed because PAA is unstable with a 40% concentration losing 1-2% activity a month (Block, 1991).

PAA is a clear colourless solution with a strong smell similar to that of acetic acid. It is a weak acid with a pK_a of 8.2 (Unis, 2010). It has greater oxidising potential than chlorine or chlorine dioxide and is environmentally safe due to its degradation into non-toxic components (Kitis, 2003). It also has higher antimicrobial activity than hydrogen peroxide, in that lower concentrations of PAA are needed to give the same antimicrobial activity. For example, to reduce the faecal coliform concentration of wastewater to safe levels 106 ppm to 285 ppm of hydrogen peroxide was required compared to 0.6 ppm to 1.6 ppm of peracetic acid (Wagner *et al.*, 2002). These characteristics, along with its continued activity in the presence of peroxidases (unlike hydrogen peroxide) and interfering matter including organic material such as blood (Russell & McDonnell, 1999), makes it an ideal biocide.

PAA is primarily used in wastewater treatment as it is effective at low concentrations and, as previously mentioned, leaves no toxic by-products. It also has a broad spectrum of activity against microorganisms, making it a viable alternative to halogen biocides such as sodium hypochlorite (Kitis, 2003). It finds applications in a medical setting for the disinfection of medical devices, preparation of pharmaceuticals (in the cold sterilisation of emulsions, ointments and powders) and as a hard surface disinfectant (Russell & McDonnell, 1999). Further applications of PAA are in the food, textile and paper industry as a decolouring and antimicrobial agent (Kitis, 2003). However, a possible disadvantage of the use of peracetic acid is that one of its by-products is acetic acid which is readily biodegradable and may be

used as a food source for any remaining organisms present allowing possible bacterial regrowth (Kitis, 2003).

The mechanism of action of peracetic acid has not been fully investigated but it has been hypothesised that it disrupts sulphhydryl (-SH) and sulphur (S-S) bonds in biomolecules (Russell & McDonnell, 1999). It has also been suggested that PAA disrupts the cell wall and cell membrane by oxidising structural lipoproteins and when acting intracellularly may inactivate vital metabolic enzymes and DNA bases (Kitis, 2003).

1.2.3 Sodium hypochlorite

Chlorine was first discovered by the Swedish chemist Scheele in 1774 (Rutala & Weber, 1997) and was later shown to have a wide range of antimicrobial activity. Parisian surgeons experimented in 1825 using sodium hypochlorite in wound treatment by applying bandages containing a dilute solution to carbuncles, ulcers and burns. It was observed that this successfully prevented infection and calcium hypochlorite was also recorded as being a useful environmental disinfectant (Rutala & Weber, 1997).

Although the disinfection of water using sodium hypochlorite was only formally established by Traube in 1894 (Rutala & Weber, 1997), chlorinated lime had been used in the treatment of sewage in London since 1854. After this however, chlorination of water became more widespread with its continual use first being established in the Belgian town of Middlekerke in 1902 with introduction into North America in 1908 (Rutala & Weber, 1997).

In modern manufacture sodium hypochlorite is produced when gaseous chlorine is passed through sodium hydroxide solution as shown in the equation below (Fukuzaki, 2006)-



The sodium chloride is removed from the final product leaving sodium hypochlorite in water with the final commercial product containing between 5 and 12% active chlorine (Fukuzaki, 2006).

Sodium hypochlorite is a salt of the hypochlorite ion dissolved in water which when in solution dissociates as follows-



In water, two different types of available chlorine are present depending on the pH of the solution. This is because hypochlorous acid (HOCl) is a weak acid (pK_a value of 7.5 at 25°C in aqueous solution (Morris, 1966) and will dissociate as shown below-



This dissociation is very important as hypochlorous acid has considerably more antimicrobial activity than the hypochlorite ion and so to maximise the antibacterial efficacy it has been proposed that the optimum pH should be around pH 6 (Rossi-Fedele *et al.*, 2011). In this however, the biocide solution was adjusted to pH 8. This was selected in discussion with collaborators in industry to reflect their worst case scenario testing.

Sodium hypochlorite has a wide range of uses, its primary use being water treatment but it is also employed for the disinfection of medical equipment (such as

haemodialysers, syringes and needles for drug injection and dental prostheses), environmental surfaces, laundry and regulated medical waste (Rutala & Weber, 1997). Even in water treatment it has a variety of applications including potable water, water in medical equipment (flushing through haemodialysers) and even water in vases containing cut flowers in hospital to reduce infection risk. Its use is diverse primarily due to its spectrum of activity, even at low concentration (Rutala & Weber, 1997).

Regardless of the wide range of uses, long history and a great deal of attention from the scientific community the exact mechanism of biocidal action of sodium hypochlorite remains unknown. Hypochlorous acid (HOCl) and the hypochlorite ion (OCl^-) which make up sodium hypochlorite are known to be strong oxidising agents interacting with a range of biological molecules (Fukuzaki, 2006). The chlorine atom in both molecules behaves as Cl^+ which is a strong electrophile, combining with biological molecules with high electron densities. Proteins, peptides, lipids and DNA have all been shown to be oxidised by sodium hypochlorite at physiological pH with C=C double bonds, peptide bonds, peptide groups and thiol groups susceptible to electrophilic damage (Fukuzaki, 2006). This leads to the destruction of the cellular activity of proteins and nucleotide bases as well as disrupting oxidative phosphorylation and other membrane related pathways (Russell & McDonnell, 1999). Notwithstanding this reactivity, it is postulated that the primary action of the biocide is oxidative damage to DNA synthesis since low concentrations of sodium hypochlorite leave protein synthesis far less affected than DNA synthesis (Russell & McDonnell, 1999).

1.3 Factors which affect biocide efficacy

The action of biocides can be affected by a number of factors either inherent to the biocide (such as concentration) or the environment that the biocide is being used in (such as environmental temperature). This is especially important as improper use of biocides has been associated with biocide resistance and antibiotic cross-resistance (Scientific Committee on Emerging and Newly Identified Health Risks, 2009) due to sub-lethal exposure.

1.3.1 Concentration

The concentration of a biocide has a considerable effect on the length of time required to achieve the desired biocidal effect (shown in studies such as Doddamani *et al.* (2011)).

The effect of concentration on activity is represented by the concentration exponent (η value; see equation below) which is likely to be influenced by the mechanism of action of the biocide (Russell & McDonnell, 2000).

$$\eta = \frac{(\text{Log death time at concentration } C_2) - (\text{Log death time at concentration } C_1)}{\text{Log } C_1 - C_2}$$

Broadly speaking, biocides can be classified into one of two categories; those with a chemical action and those with a physical action (Hugo & Denyer, 1987). Biocides with primarily physical mechanisms of action such as phenols are greatly influenced by concentration. This is because their mechanism of action has a cumulative effect; for example phenols are membrane disrupters and so the higher the concentration partitioning into the membrane the greater the likelihood of the membrane bursting. Biocides such as this with a high concentration dependency effect have a high concentration exponent ($\eta > 4$ (Russell & McDonnell, 2000)). This means that the biocidal effect is reduced by the power of the η value on dilution for example a η

value of 4 would mean that two fold dilution reduces the activity by 16 times (Maillard, 2005a).

Biocides that have a chemical action generally have a low concentration exponent ($\eta < 2$) meaning that effect of dilution is not as great. Examples of biocides with a highly chemical action are oxidising agents such hydrogen peroxide (η value of 0.5) and sodium hypochlorite (η value of 0.5) (Tirumalai, 2004).

Biocides with an intermediate concentration exponent such as parabens have a combined chemical and physical action on the target (Russell & McDonnell, 2000).

1.3.2 Temperature

The temperature efficacy relationship is defined by the equation below-

$$Q_{10} = \frac{\text{Time to kill at } T^{\circ}\text{C}}{\text{Time to kill at } (T + 10)^{\circ}\text{C}}$$

Q_{10} is thus a measure of the change in activity associated with a 10°C temperature change. Biocide activity usually increases in proportion to an increase in temperature; some examples of biocides that become more active when the temperature is increased are isoascorbic acid, phenolics and organomercurials (Russell, 2003a). Sodium hypochlorite (Sirtes *et al.*, 2005), peracetic acid (Kitis, 2003) and hydrogen peroxide (Schurman, 2001) have all been shown to have raised antimicrobial efficacy at higher temperatures in accordance with this observation.

1.3.3 pH

The pH of the biocidal formulation can affect its activity in two different ways. Firstly, it can affect the biocide itself and secondly, it can lead to an alteration in the interaction between the biocide and the bacterial cells.

Biocides that are most effective in their un-ionised form such as organic acids and phenols are more effective at lower pH. This is because an increase in pH will

increase the level of dissociation and therefore decrease their biocidal activity (Maillard, 2013a). The Henderson-Haselbach equation can be used to give information about the relationship between pH and the degree of dissociation (see below (Maillard, 2013a)).

$$\text{Log}_{10} \frac{[\text{concentration of ionised molecules}]}{[\text{concentration of unionised molecules}]} = \text{pH} - \text{pKa}$$

The pH of sodium hypochlorite affects its antimicrobial activity, as like phenols and organic acids sodium hypochlorite is most effective when dissociated. In an aqueous solution sodium hypochlorite exists in an equilibrium between hypochlorous acid (HOCl) and the hypochlorite ion (⁻OCl). This equilibrium is dependent on the pH of the solution with the hypochlorite ion predominating at high pH (above pH 9) (Russell & McDonnell, 1999) and hypochlorous acid becoming prevalent at lower pH (see fig. 1.1). If the solution is between pH 6.5 and 8.5, then both hypochlorous acid and hypochlorite ion are present (Harp, 2002).

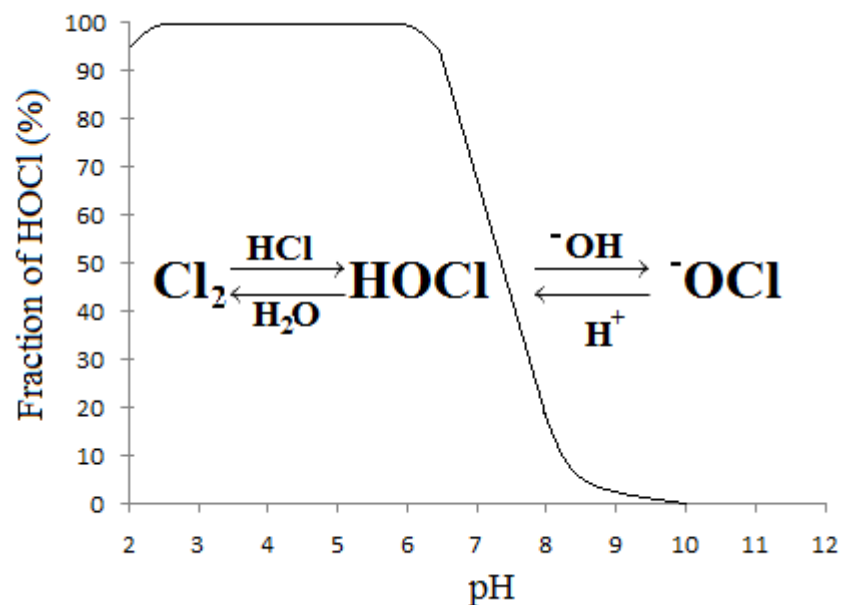


Figure 1.1: Effect of pH on the percentage of hypochlorous acid in sodium hypochlorite (graph adapted from Fukuzaki (2006)).

Hypochlorous acid has considerably more antimicrobial activity than the hypochlorite ion and so to maximise the antibacterial efficacy the pH should be adjusted from its natural range of 8.5 to 10 to a lower range where it would improve efficacy. It has been proposed that the optimum pH should be around pH 6 (Rossi-Fedele, *et al.*, 2011). Hypochlorous acid decomposes at a relatively high rate when undissociated at low pH; Cl₂ which is seen at lower pH has poor solubility in water and is lost from the solution. Hypochlorite ion in alkaline water (above pH 10) is far more stable, with very little change over as long a period as 6 months (Fukuzaki, 2006).

Like sodium hypochlorite, peracetic acid is more active at a lower pH. This is believed to be because the undissociated acid form of peracetic acid is thought to be responsible for its biocidal activity, which predominates at a pH of below 9 (Kitis, 2003). This has been demonstrated by Sanchez-Ruiz *et al.* (1995) who found that peracetic acid reduces total coliforms by 2 to 3 log₁₀ more at pH 7 than pH 10.

Conversely, some biocides are shown to be more active at an alkaline pH. Glutaraldehyde is more active at a higher pH as it has increased interaction with amine groups (Maillard, 2013a). In addition, biocides that are more effective in the ionised form (such as triphenylmethane dyes) have increased activity at higher pH. This is due competition with H⁺ ions causing a greater interaction with target molecules (Maillard, 2013a).

Changes in the interaction between the biocide and bacterial cell surface are due to alterations in the bacterial cell surface. As the pH increases there is an increase in the number of negatively charged groups on the bacterial cell surface. This increase in surface negativity means that positively charged molecules are attracted to a greater

extent to the bacterial cell. This means that biocides such as quaternary ammonium compounds and biguanides are more strongly attracted and are therefore more effective (Russell (2003b);Maillard (2013a)).

1.3.4 Interfering materials

Interfering materials such as soil, food residues and faecal material can interfere with the activity of a biocide. This usually occurs as an unwanted reaction between the antimicrobial and the organic matter which leaves the biocide depleted so it is less available for attacking the target microbes (Ayyildiz *et al.*, 2009).

A study by Pappen *et al.* (2010) into the effect of bovine serum albumin (BSA) on the activity of sodium hypochlorite solution found that the presence of organic matter reduced the efficacy of the biocide against a range of organisms. Even at low concentrations (300 ppm) sodium hypochlorite killed organisms suspended in water within 30 seconds; however, when BSA (a common indicator of organic soil and ‘dirty’ conditions) was added the time taken to kill the organisms was longer. The protection given by BSA was dependant on its concentration, but a concentration above 16700 ppm of BSA gave no kill after 30 mins 300 ppm sodium hypochlorite exposure. It was concluded that BSA was oxidised and has a concentration dependant inhibitory effect on the biocidal action of sodium hypochlorite (Pappen, *et al.*, 2010). Another study by Takehara and Fukuzaki (2006) added extra evidence to this, showing less available chlorine present when 300 ppm of BSA is added to sodium hypochlorite at a pH range of 2-12. This study also found that the effect of organic load was greater at a higher pH range, with the maximum decrease occurring at pH 9.5, leading to the conclusion that hypochlorite ion is more affected by organic matter than hypochlorous acid.

The reactivity of a biocide tends to influence how greatly it is affected by organic matter. Biocides such as sodium hypochlorite which is a reactive oxidising agent are commonly shown to be affected by organic matter as described above; organic matter has been shown to have less of an effect on activity of compounds that are less reactive such as iodine (Russell, 2003b).

1.3.5 Contact time

The contact time between the biocide and the contaminating organisms is also important as it can affect the activity of an agent (Maillard, 2005a). When devising a contact time for a disinfection procedure, a balance needs to be made to ensure that it is long enough to gain the required bactericidal effect, yet reasonable to make sure the disinfection procedure is complied with (Maillard, 2005b).

In healthcare settings, a protocol with a shorter exposure time is more likely to be adhered to. However, a shorter exposure time generally means lower efficacy and less kill than a longer contact time with the same concentration of biocide. Inactivation kinetics studies such as Walsh *et al.* (2003) shows how increased levels of kill are associated with increased contact times when looking at the same concentration of biocide over a time course. Although, a long contact time is more efficacious, it is less likely to be adhered to in an everyday environment (Maillard, 2005a).

There may also have to be an adjustment in exposure time dependant on the bacterial target; for example Gram-negative bacteria will generally require a shorter exposure time than bacterial endospores to achieve the same outcome. This is due to intrinsic differences in bacterial cell structure making some bacteria more susceptible to biocides (Russell, 2003a). For further information on the effect of bacterial structure on biocide susceptibility please see Chapter One, figure 1.2 in section 1.4.

1.4 Bacterial resistance and tolerance to biocides

1.4.1 Definition of biocide resistance and tolerance

There has been a great deal of debate on the topic of defining bacterial resistance to biocides. This is because biocides have a wide range of target sites within a bacterial cell and are used to kill a wide range of organisms. Resistance when discussed in terms of antibiotics is more straightforward to define as it is often associated with a specific change in the bacterial target site (Bloomfield, 2002) which is rarely seen with biocides (with the exception of triclosan Webber, *et al.* (2008)). Cerf *et al.* (2010) therefore recommends the use of the word resistance when discussing concentrations that are associated with bacterial killing.

It is recommended by Cerf, *et al.* (2010) to use the phrase biocide tolerance when there has shown to be bacterial adaptation to concentrations that usually inhibit bacterial growth.

1.4.2 Background to bacterial tolerance and resistance

The first incidence where biocide resistance in *Salmonella Typhi* against chlorine based compounds was identified over 70 years ago by Heathman *et al.* (1936). Over time, in spite of the fact that biocides have a much wider range of targets within a bacterial cell, there has been continued evidence of resistance to biocides after exposure (Braoudaki and Hilton (2004); Randall *et al.* (2007); Whitehead *et al.* (2011)).

Biocide use has become more widespread, especially in a domestic environment with products such as triclosan impregnated wellington boots, paint, toilet seats and chopping boards (Fraise, 2002). There are two perspectives on this increased use of biocides; on the one hand with increasing antibiotic resistance the prevention of

infection by proper cleaning and disinfection is more important than ever. However, it has been postulated that the indiscriminate use of biocides may be linked to increasing antibiotic resistance and therefore their use should be controlled (Bloomfield, 2002).

1.4.3 Mechanisms of bacterial resistance to biocides

Mechanisms of biocide resistance are usually non-specific such as reduced cellular permeability and increased efflux due to the non-specific nature of biocide activity. In Gram-negative bacteria there is evidence that changes in the outer surface of a bacterial cell is part of acquired resistance such as changes in the outer membrane ultrastructure, outer membrane protein and fatty acid composition and surface hydrophobicity (Poole, 2002). Efflux mechanisms allow bacterial cells to remove a range of chemicals with a broad specificity which can lead to cross resistance between the biocide used and a range of other antimicrobial compounds.

A diagrammatic representation of some of the mechanisms of bacterial resistance to biocides is summarised in figure 1.2. This figure shows resistance mechanisms which can be either intrinsic or acquired and protect the cells from a biocide challenge. Examples of biocides which are affected by this change in target site are given.

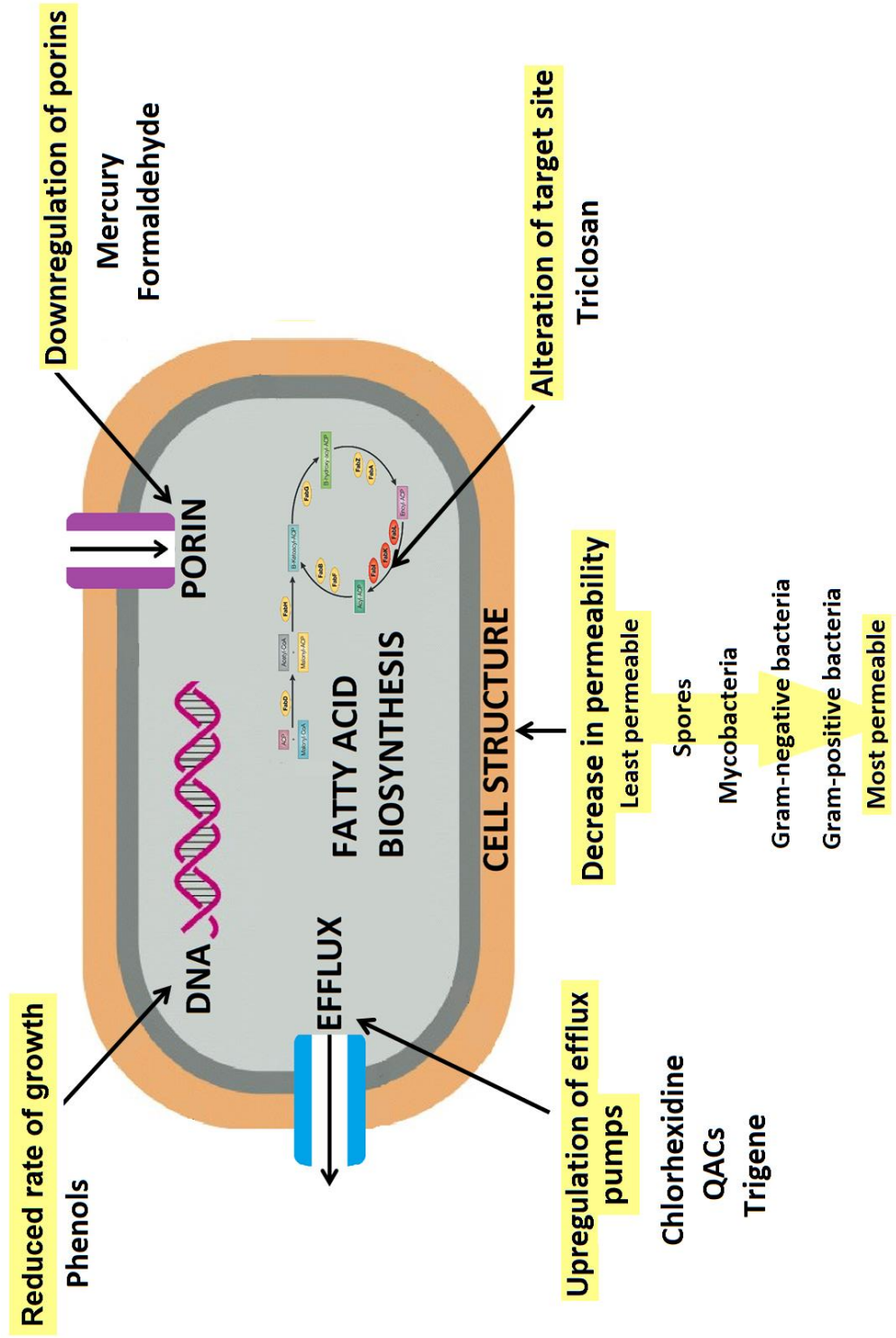


Figure 1.2: Mechanisms of bacterial tolerance to biocides (developed from Hugo and Bloomfield (1971); Heinzel (1998); Tattawasart *et al.* (2000); Levy (2002); Szabo and Minamyer (2014); Kristiansson *et al.* (2011); Webber *et al.* (2008); Bailey *et al.* (2009); Chapman (2003); Maillard (2013b))

1.4.3.1 Cell permeability

Cell permeability can either be an intrinsic mechanism of bacterial resistance to biocides or one that is acquired. Intrinsic bacterial resistance to biocides is associated with factors inherent to the bacteria such as the bacterial cell structure. As shown in figure 1.2 certain types of bacteria are less permeable than others. A decrease in cell permeability is seen in spores, mycobacteria and to a certain degree, Gram-negative bacteria (Scientific Committee on Emerging and Newly Identified Health Risks, 2009). Bacterial spores are protected by the inner and outer spore coat which exists as a barrier and is responsible for resistance to many chemicals, especially oxidising biocides (Wingender & Flemming, 2011). Mycobacterial cell walls also have a protective function due to the waxy mycoylarabinagalactan layer which makes it impermeable to many biocides due to the hydrophobic nature of the cell wall (Ortega Morente *et al.*, 2013).

Gram-negative bacteria possess a lipopolysaccharide (LPS) containing outer membrane (see figure 1.3 for comparison of Gram-positive and Gram-negative cell walls) which acts as a permeability barrier for a number of biocides such as chlorhexidine (Maillard, 2013b). This reduced permeability prevents the biocide from reaching target sites within the cell and therefore acting effectively. Guérin-Méchin *et al.* (1999) found that growing *Pseudomonas* in the presence of quaternary ammonium compounds led to a change in the composition of the fatty acids that make up the lipopolysaccharide layer. In addition, Gram-negative bacteria in the presence of mercury or formaldehyde have also been shown to down regulate porin expression which has been linked to tolerance of these biocides (Heinzel, 1998). There is also a possibility that peptidoglycan may play a role in the intrinsic

resistance of Gram-negative bacteria by supporting the outer membrane lipopolysaccharide layer which reduces permeability (Denyer & Maillard, 2002).

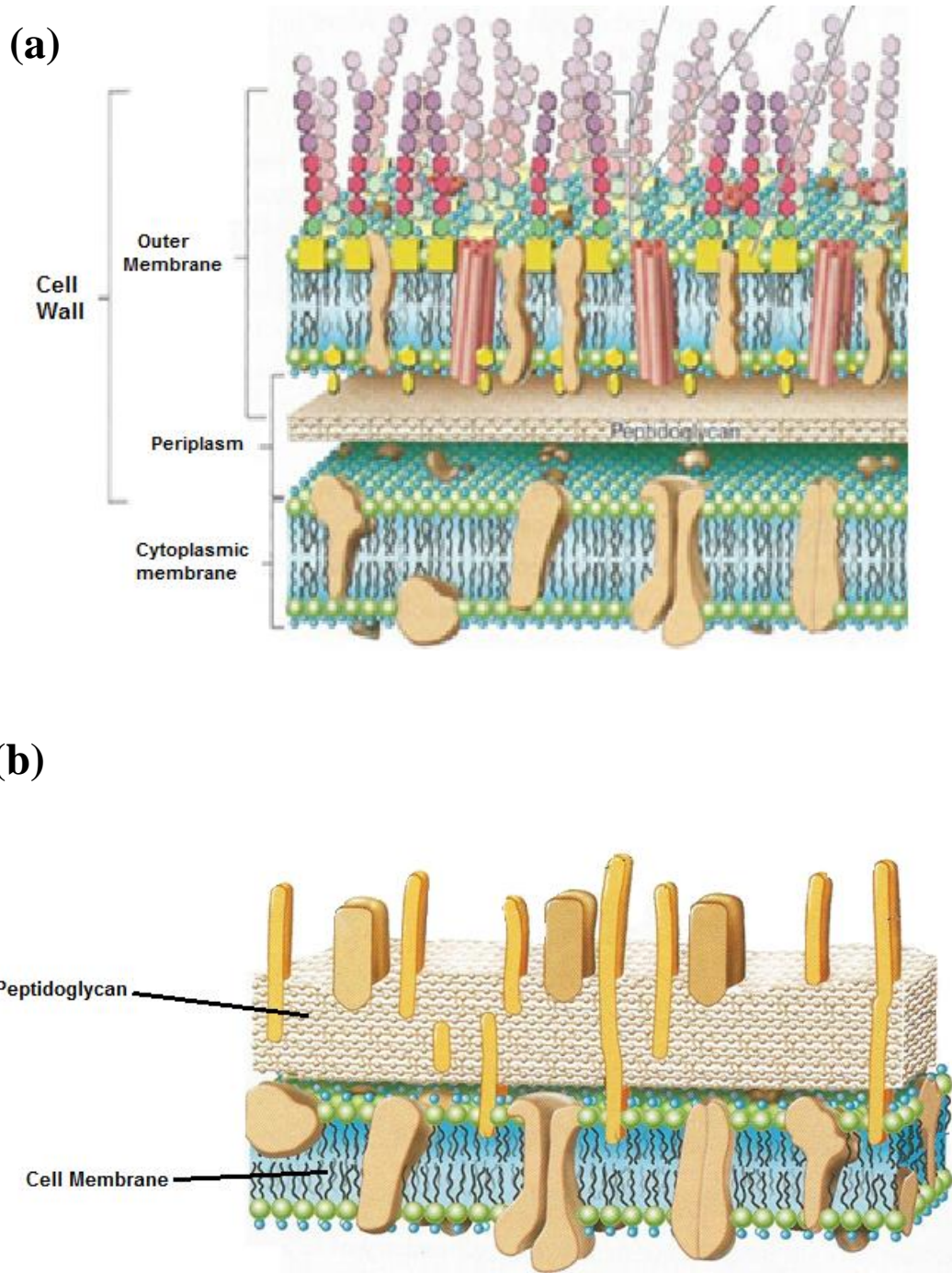


Figure 1.3: Diagrams of the composition of the (A) Gram-negative and (B) Gram-positive bacterial cell wall (adapted from Madigan *et al.* (2009)).

Changes in bacterial cell permeability can also be acquired if bacteria are exposed to a certain set of circumstances that make this favourable. A change in bacterial cell structure has been shown in a number of studies to be a protective mechanism after biocide exposure (Ortega Morente, *et al.*, 2013). These changes have included an alteration in the expression of outer membrane porins (Tattawasart, *et al.*, 2000) and a change in the lipopolysaccharide composition of the cell surface and outer membrane protein composition (Winder *et al.*, 2000).

Karatzas *et al.* (2008b) exposed *Salmonella* Typhimurium SL1344 to a number of widely used farm disinfectants. They found that the response in all resistant mutants produced was to increase the expression porins OmpC and OmpF. There was also an increase in the presence of TolB and ElaB membrane proteins in all mutated isolates; TolB has been linked to the uptake of proteins, however the function of ElaB remains unknown. This study also identified a change in the lipopolysaccharide composition of the cell surface. These findings indicate that the composition of the cell membrane and the permeability of the membrane are important in surviving biocide exposure.

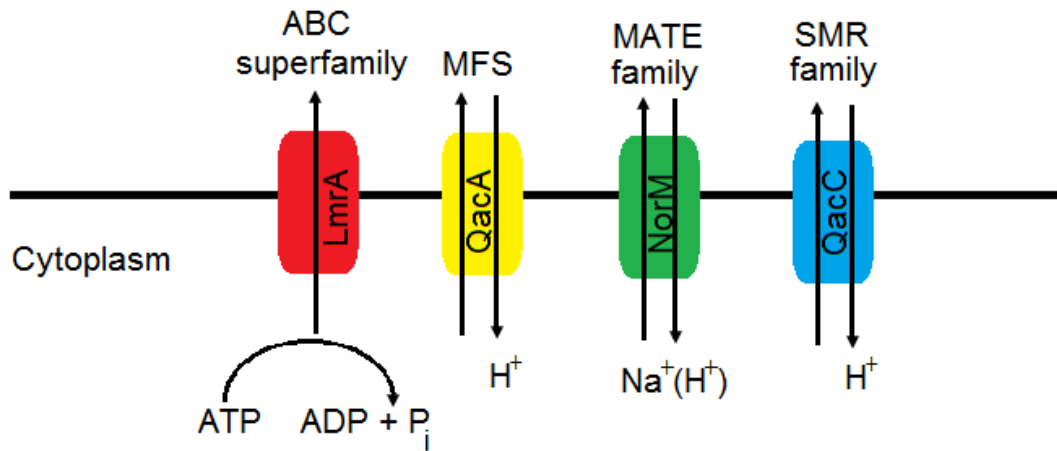
1.4.3.2 Efflux

Efflux pumps are a type of active transport mechanism that remove unwanted or toxic substances from the bacterial cell. The first description of efflux as a resistance mechanism was to tetracycline in *E. coli* in 1980 (McMurray *et al.*, 1980). Efflux pumps can be either an intrinsic or acquired resistance mechanism to antimicrobials. They not only reduce susceptibility to the in-use antimicrobial, but can also be responsible for cross-resistance (Maillard, 2013b). Cross resistance is defined by the Scientific Committee on Emerging and Newly Identified Health Risks (2009) as bacterial strains that can survive biocide challenge due to a resistance mechanism that gives protection against several antimicrobial molecules with related mechanism

of action. A study by Randall, *et al.* (2007) demonstrates cross-resistance between biocides and antibiotics involving efflux.

There are five families of efflux pumps (see fig. 1.4) that have been described in the literature which are: the ATP-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the multidrug and toxic compound extrusion (MATE) family, the small multidrug resistance (SMR) family and the resistance-nodulation division (RND) superfamily (Li & Nikaido, 2009).

Gram Positive



Gram Negative

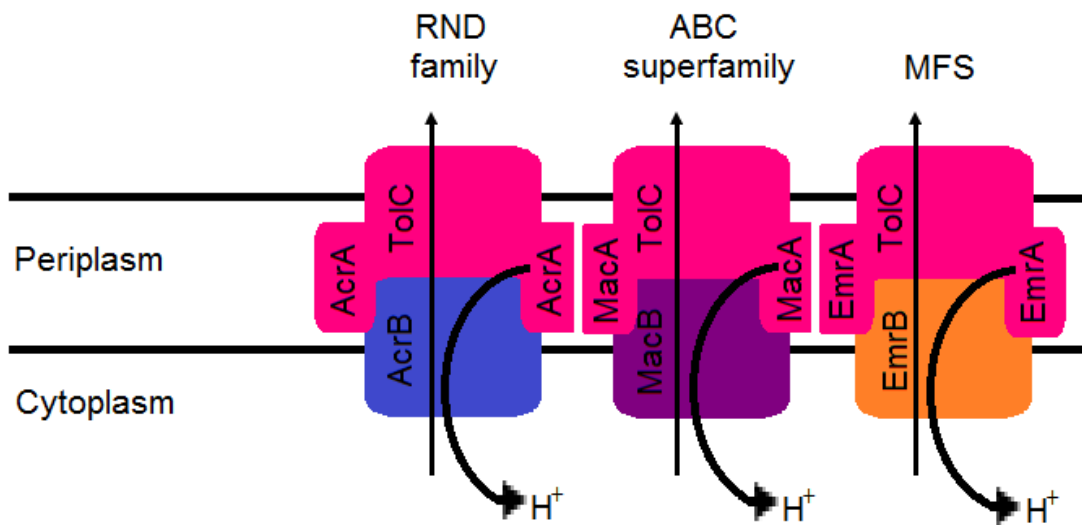


Figure 1.4: Families of efflux pumps in Gram-positive and Gram-negative bacteria adapted from Piddock (2006).

The efflux pumps in Gram-negative bacteria contain more complex machinery which is not seen in Gram-positive bacteria (figure 1.4). This is because Gram-positive bacteria do not have to remove substances from the intermembrane space, with outer membrane channel pumps seen only in Gram-negatives for example the AcrB-TolC

pump. These substrates are pumped out directly from the cytoplasm as opposed to accumulating in the bilayer (Nikaido, 1998).

The RND family of efflux pumps are of particular significance as they contain the AcrB efflux pump (figure 1.5) which has shown to be involved in the efflux of multiple ligands including bile salts (Sun *et al.*, 2014), biocides (Karatzas *et al.*, 2007) and antibiotics causing antibiotic resistance in the clinic (Li & Nikaido, 2009). The AcrB pump cannot function without TolC which is a trimeric outer membrane channel protein and *Salmonella* strains without an active TolC were found to be hypersensitive to biocides (Li & Nikaido, 2009). Levy (2002) also identified AcrB-TolC to be a multidrug resistant efflux pump and showed how the presence of this one efflux pump could offer resistance to a number of different biocides.

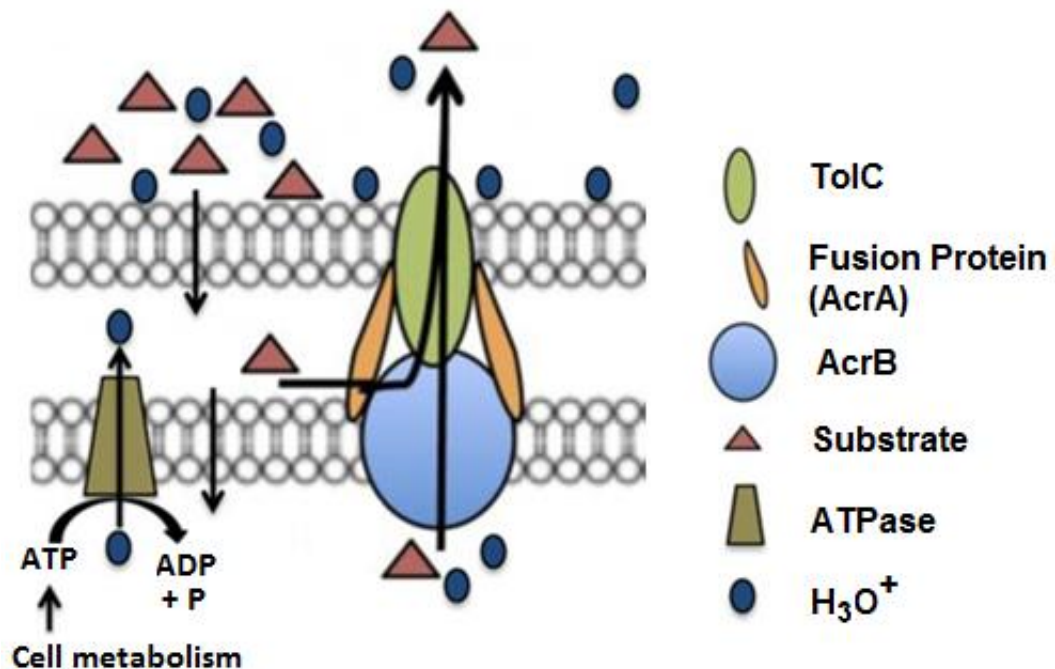


Figure 1.5: A diagrammatic representation of the AcrB-TolC efflux pump adapted from Amaral *et al.* (2013).

The AcrAB-TolC efflux pump described above is regulated by both a local repressor, *AcrR* but is also controlled by a number of different transcriptional activators. Studies have shown that different transcriptional activators are involved in co-ordinating a response to different environmental stresses for example in *E. coli* *MarA* responds to antibiotic stress and *SoxS* responds to superoxide stress (Lawler *et al.*, 2013). The response regulators up-regulate groups of genes involved in defence against the relevant stresses, AcrAB-TolC is part of various stress responses. The dominant regulator of the AcrB-TolC efflux pump in *Salmonella* is the transcriptional activator *RamA* (Lawler, *et al.*, 2013). RamA is a transcriptional activator in the AraC family (van der Straaten *et al.*, 2004), which when over-expressed leads to increased production of the AcrB-TolC efflux pump and when disrupted leads to a decrease in virulence and a decrease in AcrB-TolC production (Bailey *et al.*, 2010). *RamA* itself has a local repressor named *RamR*, the disruption of which results in over-production of *RamA* and consequent multidrug resistance (Abouzeed *et al.*, 2008).

SoxS is an oxidative stress response regulator that controls the *soxR/S* regulon which controls a number of different genes. *SoxR* is again a local repressor of *SoxS* and has a redox-sensing centre within the two homodimeric 17kDa subunits of *SoxR*. Once oxidised, repression of *SoxS* is alleviated which leads to the transcription of *soxS* (van der Straaten, *et al.*, 2004). One of the responses that can be activated by *SoxS* is the upregulation of the efflux pump AcrB-TolC. This may be due to *RamA* dependant (van der Straaten, *et al.*, 2004) or *RamA* independent (Nikaido *et al.*, 2011) mechanisms.

Efflux pump inhibitor (EPI) studies have been conducted to give further information as to the role of efflux pumps in biocide tolerance. Carbonyl cyanide 3-

chlorophenylhydrazone (CCCP) is an efflux inhibitor which acts as a proton uncoupler. CCCP was used as an efflux pump inhibitor in two studies by Rajamohan *et al.* (2010a and 2010b). In the first of these two studies investigated the role of RND transporters in *Acinetobacter baumannii* in response to biocide exposure. Rajamohan *et al.* (2010b) found that the activity of the RND transporters AdeB and AbeJ reduced susceptibility to biocides such as chlorhexidine and benzalkonium chloride in *A. baumannii*. Another study by Rajamohan *et al.* (2010a) also used CCCP to elucidate the role of major facilitator superfamily (MFS) efflux pump AmvA in *A. baumannii*. This study found that AmvA was involved in decreased susceptibility of a number of dyes (e.g. Acridine orange and DAPI) and disinfectants (e.g. chlorhexidine and benzalkonium chloride). A similar effect has also been seen in *Campylobacter* by Mavri and Smole Možina (2012). Mavri and Smole Možina (2012) used two efflux pump inhibitors (1-(1-naphthylmethyl)-piperazine and phenylalanine-arginine β -naphthylamide) to investigate the effect of efflux on susceptibility to a range of different compounds including chlorhexidine and benzalkonium chloride. These studies confirm active efflux as an important factor in antimicrobial susceptibility in a different bacterial species.

1.4.3.3 Target alteration

Biocides usually have multiple cellular targets and so target alternation is not often associated with biocide resistance. An unusually specific bacterial resistance to a biocide is that of *Salmonella* to triclosan. Usually bacteria employ a non-specific mechanism to give resistance (such as a nonspecific efflux pump) due to the many target sites affected by biocidal activity (for example the upregulation of AcrB-TolC observed in response to Triclosan (Webber, *et al.* (2008); Bailey, *et al.* (2009)). Triclosan however, also has an effect on the metabolism of bacterial cells interfering

with *fabI* which encodes the enoyl-acyl carrier protein reductase which is important in fatty acid biosynthesis. Metabolic changes in fatty acid synthesis such as increased pyruvate production or the production of fatty acids using a different pathway give bacteria some protection against triclosan and a combination of different resistance mechanisms (including metabolic changes) can give rise to high level triclosan resistance (Webber, *et al.*, 2008).

1.4.3.4 Bacterial growth conditions

An important bacterial growth condition that affects bacterial susceptibility to biocides is the formation of biofilms. This will be discussed in more detail in Chapter One, section 1.5.

Nutrient levels present in the bacterial environment cause a change in bacterial gene expression. Studies by Hugo and Bloomfield (1971) showed that cells passaged in the presence of glycerol-containing media were less susceptible to benzylpenicillin and certain phenols. Nutrient limitation (Gilbert, 1988) and variable growth rates (Kim *et al.*, 2009) have also been found to impact the susceptibility of bacteria to biocides. Bailey *et al.* (2006) found that the growth media used when growing overnight cultures of *E. coli* affected the production of *acrB*. Minimal medium appeared to induce the expression of *acrB* compared to enriched medium which is a known indicator of increased tolerance to some biocides and antibiotics (see Chapter One, section 1.4.3.2).

Mc Mahon *et al.* (2007) conducted a study on the effect of pre-stress treatment including temperature, osmotic and pH stress on *Salmonella* and *E. coli*. The study found that the presence of stress in bacterial growth conditions of *Salmonella* and *E. coli* led to the transmission of antibiotic resistance plasmids. Although this is not biocide resistance, it demonstrates that bacterial growth conditions and stress can

cause the transmission of antimicrobial resistance genes (see Chapter One, section 1.4.4).

1.4.4 Cross resistance to antibiotics

The rising concern over the excessive use of biocides is not just due to biocide resistance but the cross resistance to clinically relevant antibiotics. Antibiotic resistance has been present since antibiotics have been in use (Plough, 1945) but their over prescribing is believed to be responsible for the widespread resistance that is currently seen in clinical environments (Bloomfield, 2002). In addition however, the widespread use of biocides may be adding to the number of antibiotic resistant strains since cells isolated after biocide exposure have been shown to have increased antibiotic resistance (Walsh, *et al.* (2003) ;Randall, *et al.* (2007); Karatzas *et al.* (2008a); Copitch *et al.* (2010); Soumet *et al.* (2016)). Changes in generic bacterial defence mechanisms which are usually seen in response to biocide exposure as described above may also give bacterial cells protection from antibiotics. For example, changes in the permeability of the bacterial cell caused by biocides such as biguanides could theoretically give protection against aminoglycosides as they gain access to the cell through similar mechanisms (McBain *et al.*, 2002).

Efflux pumps have broad specificities and can be used to expel a wide range of naturally occurring environmental toxicants (McBain, *et al.*, 2002). Changes in multidrug efflux pumps induced by exposure to a biocide could also be used to expel antibiotics from the bacterial cell. An example of this is seen in *Escherichia coli*, a model organism for Gram-negatives, in which the Acr system acts as a transporter for both antibiotics and a range of biocides, for example ciprofloxacin and triclosan, chloroxylenol and quaternary amines (Bloomfield, 2002). *E. coli* when also exposed

to sub-lethal concentrations of chemicals such as pine oil or salicylate can also gain protection from small hydrophilic antibiotics (McBain, *et al.*, 2002).

1.5 Bacterial biofilms

In early research it was assumed that bacteria existed as free living planktonic cells. However, in 1978 the term “biofilm” was defined by Costerton to describe surface-associated microbial agglomerations (Costerton *et al.*, 1978). It is now accepted that the majority of bacteria exist in surface-attached biofilms (Garnett & Matthews, 2012) as it provides them with protection from environmental stress and therefore increases survival chances.

Bacterial biofilms are defined as bacteria that are adhered to a solid surface (either biotic or abiotic) and to each other (Soto, 2013). They can be composed of either one or multiple species of bacteria enclosed in a scaffold of extracellular polymeric substance (EPS) produced by the biofilm itself (Flemming & Wingender, 2010). The cells within a biofilm have also been noted to exhibit an altered phenotype with variation within gene expression (Stewart & Franklin, 2008).

1.5.1 Formation of bacterial biofilm

Bacterial biofilms can form on biotic and abiotic substances and their formation is made up of a number of complex phases. The first phase occurs when the bacteria encounter a surface suitable for colonisation and growth. The surface is usually conditioned with nutrients (such as an indwelling medical device, pipes within a water treatment plant or even a surface in a domestic dwelling) such as water, lipids and protein (Pace *et al.*, 2006). On encountering a suitable surface bacterial cells attach, using non-specific interactions for example van der Waals, electrostatic and hydrophobic forces (Pace, *et al.*, 2006). The cells then switch from planktonic to biofilm growth which in *Salmonella* Typhimurium involves signalling using cyclic dimeric guanosine monophosphate as a secondary messenger which has been linked to the control of exopolysaccharide synthesis Simm *et al.* (2014). Cells then begin to

irreversibly bind to the surface, producing the extracellular matrix and initiating cell division to begin form an established biofilm colony. The absorption of nutrients from the surrounding environment continues this growth and development and the further production of an extracellular matrix until a mature biofilm is produced. After some time an area of the biofilm detaches, which can be due to a number of factors, which include but are not limited to: quorum sensing, shear stress, nitric oxide, a decrease in the concentration of cyclic GMP, depletion of oxygen and sudden increase in carbon source (Petrova & Sauer, 2016). At this point bacteria revert to their planktonic state and possibly move along the surface to form a biofilm in another area, starting the cycle again (Joo & Otto, 2013). Figure 1.6 summarises this cycle of attachment, maturation and detachment.

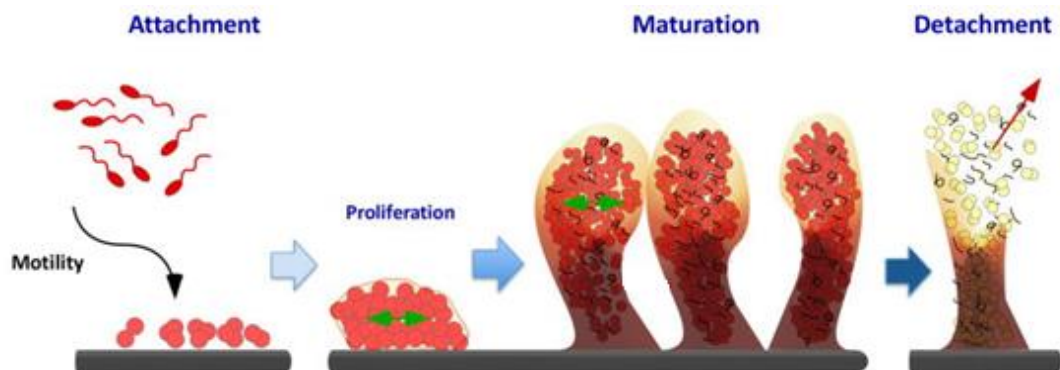


Figure 1.6: Stages of bacterial biofilm development adapted from Joo and Otto (2013). The green arrows refer to adhesive factors within the biofilm.

1.5.2 Genetic heterogeneity in bacterial biofilms

It is well documented in the literature that bacterial physiology in biofilms differs from planktonic cells (Stoodley *et al.* (2002), An and Parsek (2007)). Additionally it has been shown that cells in a biofilm are not homogenous but have physiological and genetic heterogeneity (Stewart & Franklin, 2008). There are three key reasons

for heterogeneity of bacteria in a biofilm; local microenvironment, genetic changes and stochastic gene switching (Stewart & Franklin, 2008).

The first cause of heterogeneity in a biofilm is the chemical microenvironment within the biofilm. This includes the presence or absence of oxygen, metabolic substrates and metabolic product. The concentrations of these substances change throughout the depth of the biofilm with the outermost layers of the biofilm having the most access to oxygen and metabolic substrate and the innermost layers having the least (Rani *et al.*, 2007). Moving from the outermost layer inwards oxygen is often depleted first decreasing growth (Xu *et al.*, 1998) or causing anaerobic respiration to occur. Move deeper into the biofilm structure metabolic substrates will be increasingly depleted leading to slower growth or cell death (Stewart & Franklin, 2008). Metabolic products are being produced by cells in the biofilm constantly and accumulate deeper in the biofilm which would cause changes in pH affecting bacterial physiology (Stewart & Franklin, 2008).

Genetic variation occurs within a biofilm producing regions of cells which are genetically distinct from the biofilm parent strain (Koh *et al.*, 2007). These mutant subpopulations may have a different colony morphology from the parent strain (Kirisits *et al.*, 2005) making them easier to identify. This genetic variation is not caused by the microenvironment of the biofilm but occurs by DNA mutation. However, it is then thought that the mutants which are most suited to their specific biofilm microenvironment thrive and multiply (Koh, *et al.*, 2007).

Stochastic gene expression is variation within a biofilm that is controlled by the presence of a bistable switch. A bistable switch refers to genes that are in a binary

state (either 'on' or 'off') (Stewart & Franklin, 2008). This is operated independently of environmental conditions (McAdams & Arkin, 1997).

Chai *et al.* (2008) investigated the formation of biofilms by *Bacillus subtilis* and found that this was controlled by a bistable switch. The production of the exopolysaccharide matrix and the protein content of the matrix, TasA are controlled by the *epsA-O* operon and the *yqxM-sipW-tasA* operon respectively. These operons are both controlled by the repressor SinR and the anti-repressor SinI. They found that SinI was only expressed in a minority of cells within a population whereas SinR was expressed in almost all of the population. This study therefore indicates that biofilm formation is caused by differential gene expression within a minority of the population (SinI expression), controlled by a bistable switch (Chai, *et al.*, 2008). There is also evidence of the presence of bistable switching in the production of chitin in *Pseudoalteromonas* species (Baty *et al.*, 2000).

Bistable switching is hypothesised to be the mechanism behind the production of persister cells within biofilms (Stewart & Franklin, 2008). Persister cells are not genetically any different from the rest of the bacterial population and experience the same environmental conditions, but they are metabolically dormant and therefore more resistant to antimicrobial challenge. It is not yet fully understood why persister cells form, but it is possible that changes on an individual cellular level caused by a bistable switch could be responsible (Balaban *et al.*, 2004; Fasani & Savageau, 2013).

1.5.3 Biocide insusceptibility in biofilms

It is well established within the literature that bacterial biofilms are far more resistant to biocide challenge than planktonic cells (for example Leung *et al.* (2012); Condell *et al.* (2012); Behnke *et al.* (2011)). Table 1.3 shows a comparison of bacterial susceptibilities to biocides in a planktonic and a biofilm phenotype although it is often difficult to compare between studies due to the different methodologies that have been used (Buckingham-Meyer *et al.*, 2007).

Table 1.3: Comparison of biocide susceptibility in planktonic and biofilm phenotypes for oxidising biocides against Gram-negative bacteria (taken from Bridier, *et al.* (2011)).

Biocide	Bacterial Strain	Killing ratio (Rc) Rc = $\frac{\text{conc. to kill planktonic}}{\text{conc. to kill biofilm}}$	Reference
Chlorine	<i>Pseudomonas aeruginosa</i> ATCC 15442	20	Dubois-Brissonnet <i>et al.</i> 1995
	<i>Pseudomonas aeruginosa</i> ATCC 15442	5	Ntsama-Essomba <i>et al.</i> 1995
Sodium hypochlorite	<i>Escherichia coli</i> ATCC 10536	5	Ntsama-Essomba <i>et al.</i> 1995
Peracetic acid with hydrogen peroxide	<i>Pseudomonas aeruginosa</i> ATCC 15442	40	Dubois-Brissonnet <i>et al.</i> 1995
	<i>Pseudomonas aeruginosa</i> ATCC 15442	4	Ntsama-Essomba <i>et al.</i> 1995
	<i>Escherichia coli</i> ATCC 10536	25	Ntsama-Essomba <i>et al.</i> 1995

The reasons for these significant changes in bacterial susceptibility to biocides have been debated and several theories have been proposed. These theories implicate the structure of the bacterial biofilm and the EPS matrix, an altered biofilm phenotype and an increase in genetic transfer which allows resistance genes to be spread more easily (see table 1.4). It may indeed be one or more likely a combination of these factors that give biofilms the resistance to biocides that have been documented.

Table 1.4: Factors associated with biocide resistance and tolerance in biofilms (adapted from Bridier *et al.* (2011))

Explanation of factors and evidence	
Factors associated with biocide resistance in biofilms	
Limited diffusion into the biofilm structure	The structure of the biofilm gives it protection from biocide penetration and action. The extracellular matrix which surrounds the bacterial cells can prevent the penetration or reduce the effects of biocide by slowing diffusion or by releasing chemicals to react with the biocide to prevent its action. A number of studies have shown that chlorine has limited penetration into biofilms and therefore lower available concentrations of biocide (De Beer <i>et al.</i> 1994 and Jang <i>et al.</i> 2006).
Non-specific interaction with the biofilm matrix	A number of biocides are highly reactive; particularly the oxidising biocides described in section 1.2. This means that the presence of extracellular material as organic matter can interfere with biocidal activity (as described in section 1.3). This has been supported in the specific case of biofilms and biocide activity in a mathematical model by Lambert and Johnston (2001).
Exposure to sub-lethal biocide concentrations	Due to interference of biocide action and penetration described in the above two points some elements of a biofilm can be exposed to sub-lethal concentrations of biocide. <i>Salmonella</i> biofilms exposed to benzalkonium chloride had a better survival outcome than planktonic cells when they were continuously exposed (Mangalappalli-Illathu <i>et al.</i> 2008). This is thought to be linked to a change in fatty acid composition and the up-regulation of specific proteins in biofilms (Mangalappalli-Illathu <i>et al.</i> 2008).
Biofilm phenotype	Biofilm formation begins with cell adhesion, the phenotype of which has in itself been associated with a more resistant phenotype (Chavant <i>et al.</i> 2004). In addition to this, once a biofilm has formed into a three-dimensional structure there is a chemical gradient within the biofilm. The concentration of nutrients and oxygen within a biofilm are higher nearer the periphery and lower in the centre of a cluster of cells. This chemical gradient leads to physiological heterogeneity as the cells with access to oxygen and nutrients are metabolically active whereas those without access have a much lower metabolic rate (Stewart and Franklin 2008). Altered metabolic rates can influence the efficacy of biocides as shown with increased tolerance to hydrogen peroxide and decreased tolerance to chlorine by metabolically dormant cells (Kim <i>et al.</i> 2009).
Genetic transfer and mutation	The environment of a biofilm with its close proximity of a high cell density has been shown to be involved in increased efficacy of gene transfer (Molin and Tolker-Nielsen, 2003) which allows for the spread of resistance genes more easily (as seen with antibiotic resistance by Hannan <i>et al.</i> 2010). There has also been found to be an increase in mutability in (100-fold increase in <i>Pseudomonas aeruginosa</i> (Driffield, <i>et al.</i> 2008)) when cells are grown in a biofilm compared to planktonic growth. The presence of biocide as a selective pressure could lead to clonal expansion of resistant mutants (as has been shown with antibiotic resistance by Driffield, <i>et al.</i> , 2008).
Synergy in a multispecies biofilm	In a natural environment biofilms are formed by a number of different species rather than the single species biofilms created artificially in the laboratory. This combination of different phenotypes can lead to the production of greater biomass (<i>E. coli</i> with <i>Acinetobacter calcoaceticus</i> (Habimana <i>et al.</i> 2010)) which can give protection by limiting diffusion of biocides and increasing non-specific biocide interaction. It has also been shown that multi-species biofilms are generally more resistant to biocide exposure (Simoes <i>et al.</i> 2009; Luppens <i>et al.</i> 2008; van der Veen and Abee 2011) however, the reasons for that are currently unclear. Suggestions include the production of a more viscous matrix produced by the interaction of various polymers produced by different species (Burmolle <i>et al.</i> 2006) or possibly the structure of the biofilm and how species are arranged within it (Leriche <i>et al.</i> 2003).

1.6 Bacterial resistance and tolerance in aqueous environments

Aquatic systems are an important reservoir for antimicrobial resistance and may possibly play a role in the emergence or spread of antimicrobial resistance genes. Bacteria from environments where resistance may be rife (such as a clinical environment with high use of both antimicrobial drugs and biocides) can collect into water courses. The main sources of these high risk resistant bacteria come from sewage, hospital and agricultural waste (Taylor *et al.*, 2011). Sewage and animal faecal matter shed large amounts of resistant bacteria from inappropriate use of biocides or incorrect treatment with antibiotics (Levantesi *et al.*, 2012). These antimicrobials can also remain present at sub-optimal levels in the water sources after treatment leading to the selection of resistant bacteria in the environment where this water ends up. These bacteria in the water supply can then go on to enter the human or animal population leading to the outbreak of multidrug resistant infections ((Levantesi, *et al.*, 2012). It has also been found that the same *Salmonella* isolates have been found in both local water sources and in human infection (Haley *et al.*, 2009). Aquatic systems also mean that horizontal genetic transfer can occur more easily due to the formation of biofilms on hard surfaces in aquatic environments (Shrout & Nerenberg, 2012) which give close proximity and a protected environment for the transfer of genetic material (Molin and Tolker-Nielsen (2003); Taylor, *et al.* (2011)).

1.7 *Salmonella* species

Salmonella species are facultative anaerobes, motile Gram-negative rods from the Enterobacteriaceae family which are associated with both human and animal infections (Madigan, *et al.*, 2009). The genus of *Salmonella* can be divided into two species; *Salmonella enterica* and *Salmonella bongori* (Center for Disease Control, 2011). *Salmonella bongori* has no subspecies whereas *Salmonella enterica* is made up of 6 different subspecies; enterica, salamae, arizonae, diarizonae, houtenae and indica (Sánchez-Vargas *et al.*, 2011). These subspecies can be further divided into serovars with over 2500 serovars known (World Health Organisation, 2005). This means that *Salmonella* Typhimurium is formally named *Salmonella enterica* subspecies *enterica* serotype Typhimurium.

For information on antimicrobial and biocide resistance mechanisms in *Salmonella* please see Chapter One, section 1.4.3.

1.7.1 *Salmonella* infections

Salmonella infections can be split into two main categories based on clinical presentation; typhoid *Salmonella* and non-typhoid *Salmonella*. The organisms causing typhoid or enteric *Salmonella* infections primarily use humans as reservoirs for infection and are predominantly transmitted via contaminated water (Sánchez-Vargas, *et al.*, 2011). Non-typhoidal *Salmonella* strains are predominantly found in animals which is why transmission is generally linked to contaminated food such as poultry (Sánchez-Vargas, *et al.*, 2011). *Salmonella* Typhi and *Salmonella* paratyphi are the causative agents of enteric infections, with all other species of *Salmonella* being responsible for salmonellosis.

The progression of a *Salmonella* infection is shown in figure 1.7. Initially, *Salmonella* enter the intestinal epithelium via the M cells associated with Peyer's patches. Pathogenic *Salmonella* then has the ability to survive inside macrophages and other immune cells (non-pathogenic species cannot (Lahiri *et al.*, 2010)). In non-typhoidal salmonellosis presenting without complications (such as in immune compromised patients) *Salmonella* is contained in the gastrointestinal tract (see figure 1.7)

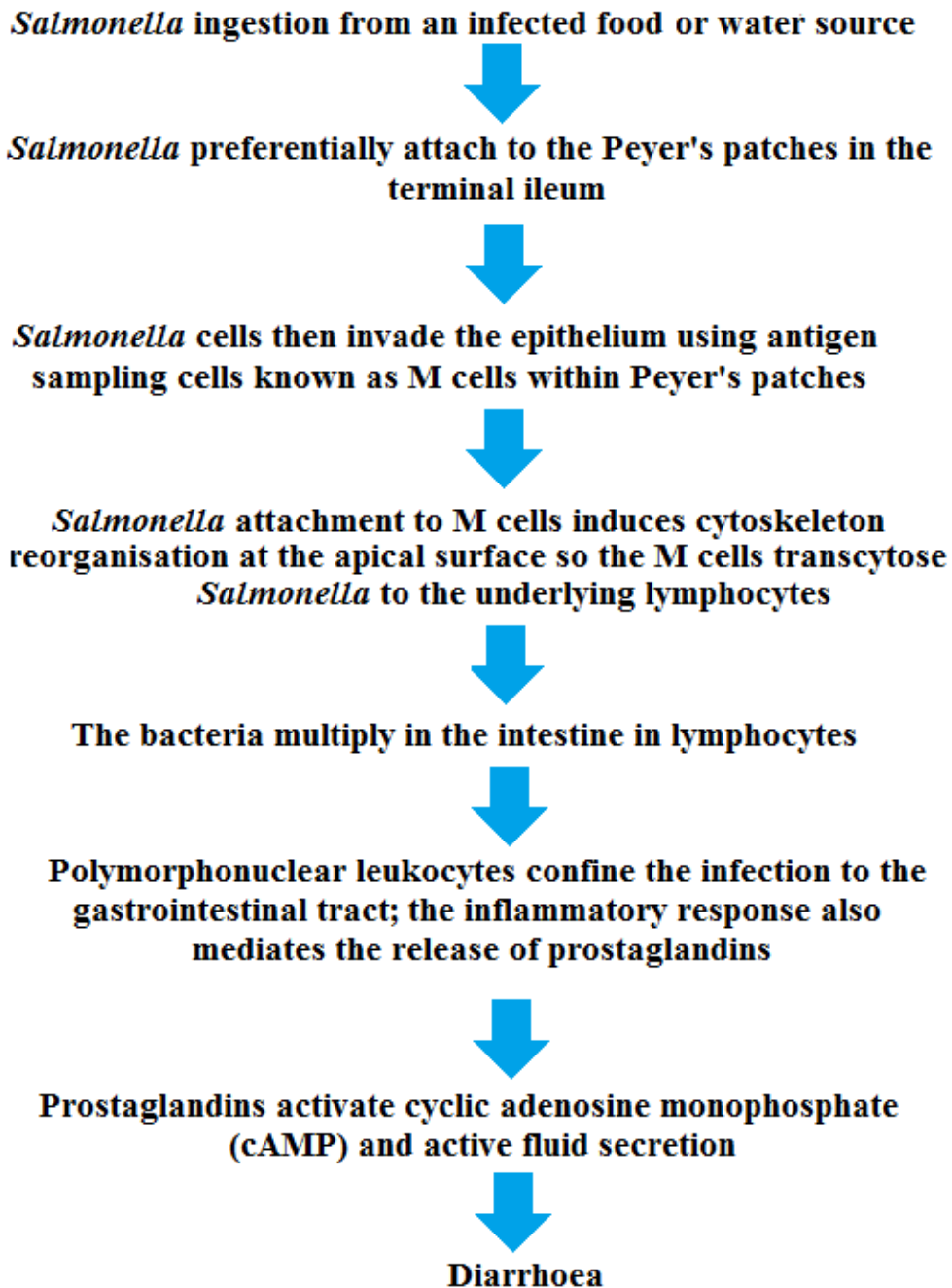


Figure 1.7: Stages of non-typhoidal *Salmonella* infection (Goering *et al.* (2008); Lahiri, *et al.* (2010); Sánchez-Vargas, *et al.* (2011)).

Complications of non-typhoidal salmonellosis can lead to a risk of septicaemia and damage to other organs if the local immune system in the gastrointestinal tract cannot contain the infection so *Salmonella* can reach the bloodstream (Goering, *et al.*, 2008). These can be very serious and potentially fatal if the correct treatment is

not sought. However, complications of this sort are rare and are only seen in patients that are very young or old or immunocompromised (Goering, *et al.*, 2008).

Non-typhoidal salmonellosis is one of the most common foodborne infections in the U.K., responsible for almost 50% of general foodborne outbreaks of infection reported between 1992 and 2009 (Health Protection Agency, 2010). This is a major public health burden, costing millions annually in lost days at work and increased healthcare costs (World Health Organisation, 2005). *Salmonella* can infect anyone who comes into contact with the bacteria through contaminated water, improperly cooked food, household pets and person to person contact with an infected individual (through unwashed hands after using the toilet) (Health Protection Agency, 2011). Symptoms of Salmonellosis include diarrhoea, vomiting and fever with onset occurring 12 to 72 hours after initial contact with the infectious dose of bacterium and may last between 4 and 7 days (Health Protection Agency, 2011). It is unpleasant but nonfatal in the majority of cases and as it is a self-limiting infection is mostly best left untreated. The exception to this is at risk groups such as children and the elderly (Health Protection Agency, 2011). These groups need effective treatment either with antibiotics (either ciprofloxacin or cefotaxime (British National Formulary, 2016)) or fluid and electrolyte replacement therapy to prevent serious illness or even death.

1.7.2 *Salmonella enterica* serovar Typhimurium

Salmonella enterica serovar Typhimurium is a widespread serovar causing a significant burden of morbidity. It has the second highest incidence of a non-typhoidal *Salmonella* serovar to cause human infection (Public Health England, 2014). The number of cases were rising from 1455 in 2004 to 2150 in 2011, at which point the number of infections peaked and began to fall with 1572 recorded as the provisional figure for 2013 (Public Health England, 2014). It also has high incidence with outbreaks in Europe and the United States linked to a wide range of carrier species such as cattle, pigs and poultry (Hauser *et al.*, 2010) making this serovar of interest for further research. There have also been a number of multidrug resistant strains isolated in various different outbreaks (White *et al.* (2001); del Cerro *et al.* (2003); Cabrera *et al.* (2004)) which also makes this serovar of particular interest.

1.7.3 *Salmonella enterica* serovar Agona

Salmonella Agona was first isolated in 1952 from cattle in Ghana (Guinee *et al.*, 1961). *Salmonella* Agona is a human pathogen of some significance in the UK. It was ranked the eighth most common strain of non-typhoidal *Salmonella* serotypes in the most recently produced data. This is an increase in incidence from ninth most common in 2000 to 2012 (Public Health England, 2014). It has been associated with a number of outbreaks from food sources (Brouard *et al.*, 2007; Nicolay *et al.*, 2011) and as such is a public health concern.

1.7.4 *Salmonella enterica* serovar Havana

Salmonella Havana was first isolated from neonatal spinal fluid from a meningitis outbreak (Schiff & Saphra, 1941). It does not feature in the 20 most commonly reported non-typhoidal *Salmonella* serotypes isolated from patients (Public Health England, 2014) so it is relatively uncommon in the UK. *Salmonella* Havana has been

linked to poultry and their feed supplies, human cases are becoming more common (Jafari *et al.*, 1994).

1.7.5 *Salmonella* biofilms

Nair *et al.* (2015) found that of the 40 environmental *Salmonella* isolates it tested 85% were able to produce a biofilm on a polystyrene surface. This ability of *Salmonella* to form biofilms readily on a polystyrene surface is of particular concern as such surfaces are so widely used in the food industry (Corcoran *et al.*, 2014). Russo *et al.* (2013) found that one strain of *Salmonella* Agona was able to persist for 10 years in a food processing facility. This ability to persist in the environment may be related to the ability of *Salmonella* to form a biofilm. Vestby *et al.* (2009) investigated this possibility and found a link between persistence and the ability of a strain of *Salmonella* to form a biofilm. In this study, they found *Salmonella* Agona produced more biofilm and were more likely to persist in the environment than other *Salmonella* strains that produced less biofilm.

Salmonella biofilms can be produced on a number of different surfaces both biotic (e.g. glass, plastic and stainless steel) and abiotic (e.g. gallstones, epithelial cells and sprout seeds) (Steenackers *et al.*, 2012). The biofilm matrix is composed of an exopolysaccharide fraction (cellulose) and a proteinaceous fraction (curli) (Steenackers, *et al.*, 2012). Cellulose is a polysaccharide produced by *Salmonella* (Zogaj *et al.*, 2001) which has an important structural function in the biofilm, helping bind the biofilm together and supporting long-range cell to cell interactions (Steenackers, *et al.*, 2012). Curli are highly aggregative, non-branching, amyloid-like cell surface proteins which have an important role in *Salmonella* biofilm formation as it is involved with initial and subsequent attachments (Barnhart & Chapman, 2006). When bacteria expressing curli are grown on Congo-red agar plates they are

stained red. The rdar (red, dry and rough) which is formed on Congo-red agar is the most commonly studied form of *Salmonella* biofilm morphotype (Steenackers, *et al.*, 2012). However, it has been found that biofilms formed on agar express very different genes (up to a 30% difference) from biofilms grown in liquid media (Wang *et al.*, 2004).

The most important of these genes is CsgD as it is an important controller of the expression of a number of *Salmonella* biofilm matrix compounds (Gerstel & Römling, 2003). It is involved in the expression of both curli and cellulose along with a number of other biofilm regulators such as bapA biosynthesis and O-Ag-Capsule biosynthesis. It has long been recognised as the master regulator of curli production (Grund & Weber, 1988) and as such has been described by Steenackers, *et al.* (2012) as the “biofilm control point” which regulates the switch between planktonic and biofilm behaviour. This can be affected by a range of environmental conditions including ethanol, temperature, nutrient availability and pH (Steenackers, *et al.*, 2012).

1.8 Hypotheses

This project intends to investigate the effect of three oxidising biocides on *Salmonella* in both planktonic and biofilm modes of growth, to do this three hypotheses will be tested:

1. Exposure to oxidising biocides leads to expression changes in efflux and oxidising stress response genes.
2. There is variation in the susceptibility of bacteria to oxidising biocides between bacterial cells in an intact biofilm and cells in a dispersed biofilm.
3. Biocide exposure will have a differential effect depending on the spatial arrangement of the cells in a biofilm.

1.9 Objectives

- Objective 1 of my work was to: Optimise and standardise assays to characterise bacterial survival, efflux, membrane damage and gene expression after exposure to oxidising biocides.
- Objective 2 aimed to investigating the mechanisms enabling survival of bacteria exposed to oxidising biocides.
- As biofilms are intrinsically more tolerant to many stresses, objective number 3 aimed to determine the effect of oxidising biocides on *Salmonella* biofilms.

1.10 Project Aim

To investigate the activity of oxidising biocides on planktonic cells and biofilms to discover the mechanisms that enable survival of biocide challenge and develop new methods for testing biocide efficacy.

Chapter Two: Materials and Methods

2.1 Bacterial strains

2.1.1 Bacterial strains used

The details of the different serovars of *Salmonella enterica* used in this project are outlined in table 2.1. **Unless otherwise stated throughout the thesis *Salmonella Typhimurium* cultures are SL1344.**

All *Salmonella* strains were grown using either tryptone soya agar (Oxoid, Basingstoke, U.K.) or tryptone soya broth (Oxoid, Basingstoke, U.K.) and incubated aerobically at 37°C ($\pm 1^\circ\text{C}$), for 24 hours in an incubator (Memmert INE 600, Schwabach, Germany).

The pMW82 plasmids used in this project were produced by Bumann and Valdivia (2007). The medium copy plasmid pMW82 is composed of a promoterless GFP gene, a transcriptional terminator (*rrnB*) and ampicillin resistance (*AmpR*) cassette. This plasmid fused to the promoter region of a gene of interest produces GFP which can be quantified to indicate gene expression. Strains with the pMW82 plasmids required the broth or agar to be supplemented with ampicillin at a concentration of 50 $\mu\text{g}/\text{mL}$. Ampicillin (Sigma, Dorset, U.K.) was made up to a 10,000 $\mu\text{g}/\text{mL}$ stock in sterile dH_2O which was then added to the growth media to give the correct concentration and mixed.

Table 2.1: Bacterial strains used in this project, their source and the details for each strain.

Bacterial Strain	Laboratory Reference Number	Details	Source
<i>Salmonella enterica</i> serovar Typhimurium SL1344	1344	Sequenced, wild type strain (Wray & Sojka, 1978)	Supplied by the Animal Health and Veterinary Laboratories Agency
<i>Salmonella enterica</i> serovar Typhimurium SL1344	L644	SL1344 lacking a functional <i>AcrB</i>	Department of Infection and Immunity, The Medical School, University of Birmingham, U.K. (Baugh, 2013)
<i>Salmonella enterica</i> serovar Typhimurium SL1344	L1007	SL1344 lacking a functional <i>RamR</i>	Department of Infection and Immunity, The Medical School, University of Birmingham, U.K.
<i>Salmonella enterica</i> serovar Typhimurium SL1344	L1232	SL1344 with a pMW82ramA GFP promoter GFP produced when ramA expressed	Department of Infection and Immunity, The Medical School, University of Birmingham, U.K. (Bumann & Valdivia, 2007; Lawler, <i>et al.</i> , 2013)
<i>Salmonella enterica</i> serovar Typhimurium SL1344	L1361	SL1344 with a pMW82acrAB GFP promoter GFP produced when acrAB expressed	Department of Infection and Immunity, The Medical School, University of Birmingham, U.K.
<i>Salmonella enterica</i> serovar Typhimurium SL1344	L1357	SL1344 with a pMW82soxS GFP promoter GFP produced when soxS expressed	Department of Infection and Immunity, The Medical School, University of Birmingham, U.K.
<i>Salmonella enterica</i> serovar Agona CMCC 3750	3750	Isolated from a contaminated dried vegetable, showing increased tolerance to quaternary ammonium compounds and to an alcohol based disinfectant	Safety and Environmental Assurance Centre, Unilever R&D Colworth, Bedfordshire, U.K.
<i>Salmonella enterica</i> serovar Havana CMCC 3759	3759	Isolated from a contaminated food source, showing an increased tolerance to quaternary ammonium compounds and to an alcohol based disinfectant	Safety and Environmental Assurance Centre, Unilever R&D Colworth, Bedfordshire, U.K.

2.1.2 Recovery of freeze dried strains

One mL of tryptone soya broth (TSB) was added to the freeze dried *Salmonella* which was vortex mixed and 0.45 mL of the resuspended bacterium *Salmonella* in TSB was then added to 10 mL TSB. This was then incubated in a non-shaking incubator at 37°C ($\pm 1^\circ\text{C}$) overnight. A tryptone soya agar (TSA) plate was inoculated from the freeze dried stocks and incubated in a non-shaking incubator at 37°C ($\pm 1^\circ\text{C}$) for 24 hours to check for contamination.

2.1.3 Phenotypic identification of strains

All strains were Gram stained to ensure that they were Gram-negative rods. All three strains were then streaked out on Oxoid Brilliance *Salmonella* agar (Fisher, Leicestershire, U.K.) and incubated in a non-shaking incubator at 37°C ($\pm 1^\circ\text{C}$) overnight. They were identified as *Salmonella* as colonies were purple; other competing bacteria are inhibited (*Escherichia coli* by an Inhibigen™ compound and other bacteria such as *Proteus* spp. and *Pseudomonas* spp. by the presence of novobiocin and cefsulodin) and the purple colour is given by caprylate esterase. Other Enterobacteriaceae spp. also produce caprylate esterase, but these give blue colonies due to the presence of β -glucosidase substrate. These tested were conducted to check for contamination and to confirm the strain at the commencing of the project.

2.1.4 Storage of strains

A number of colonies (approximately) of each *Salmonella* strain were used to inoculate cryogenic vial containing beads (Fisher, Leicestershire, U.K.) coated in a cryopreservative solution (composed of: Beef extract; Peptone; Sodium chloride; Glycerol (20%); De-ionised Water). The beads were mixed thoroughly in the

inoculated solution and the liquid was removed. The inoculated beads were then stored at -80°C ($\pm 1^{\circ}\text{C}$).

All strains were also maintained on a TSA slope and stored at 4°C ($\pm 1^{\circ}\text{C}$). A new slope was inoculated from a freezer stock every 4 weeks. This frequent reversion to the seed culture from the frozen beads was used to keep the genotype as unaltered as possible. Culture purity was checked by inoculating a TSA plate and examining colony morphology. This was used throughout the week for source colonies for experimentation; with a fresh plate being used each week.

2.1.5 Broth cultures

In all experiments requiring a broth culture, a number of colonies (approximately 3 colonies) of the required *Salmonella* strain were picked off a TSA plate using a microbiological loop and used to inoculate 10 mL of TSB. Antibiotics were added as necessary. This was then incubated in a static incubator for 24 hours at 37°C ($\pm 1^{\circ}\text{C}$).

2.1.6 Preparation of a washed bacterial suspension from an overnight culture

A number of experiments require a cell suspension, they were produced this way unless stated otherwise. Overnight cultures (10 mL) were centrifuged (MSE, Mistral 1000, London, U.K.) at $5000 \times g$ for 15 mins at 19°C ($\pm 1^{\circ}\text{C}$). The supernatant liquid was discarded and the pellet was resuspended in 15mM phosphate buffered saline (PBS from Fisher, Leicestershire, U.K.). This was repeated one more time to remove all traces of growth media.

2.1.7 Viable Counts

Viable counts were used to determine the concentration of viable bacteria in a bacterial suspension. *Salmonella* cultures were grown overnight in TSB and washed as described in 2.1.6. Ten μL of bacterial suspension was then serially diluted in 90 μL of sterile PBS eight times in a microtitre plate. Ten microlitre drops were plated

out for each dilution according to the drop counting method in triplicate on TSA (Miles & Misra, 1938). The drops of bacterial suspension were allowed to dry before the plates were incubated overnight at 37°C ($\pm 1^\circ\text{C}$) in a static incubator. Colonies on the plates were then counted, with between 3 and 30 colonies per drop deemed countable. Numbers of CFU/mL in the original cultures were then counted.

2.1.8 Standardisation of cultures

Salmonella cultures were grown overnight in TSB, prepared as described in 2.1.6 and resuspended in 10 mL of PBS. Serial 1 in 5 dilutions were performed and optical density readings were taken on the Ultrospec 3100 pro (Amersham Biosciences, Buckinghamshire, UK) at 600 nm. Each individual dilution was further serially diluted and plated out using the drop counting method (described in 2.1.7). All experiments were repeated in triplicate and incubated for 24 hours at 37°C ($\pm 1^\circ\text{C}$) in a static incubator. This data was then used to create a calibration graph by plotting the optical density against CFU/mL which was used for future reference.

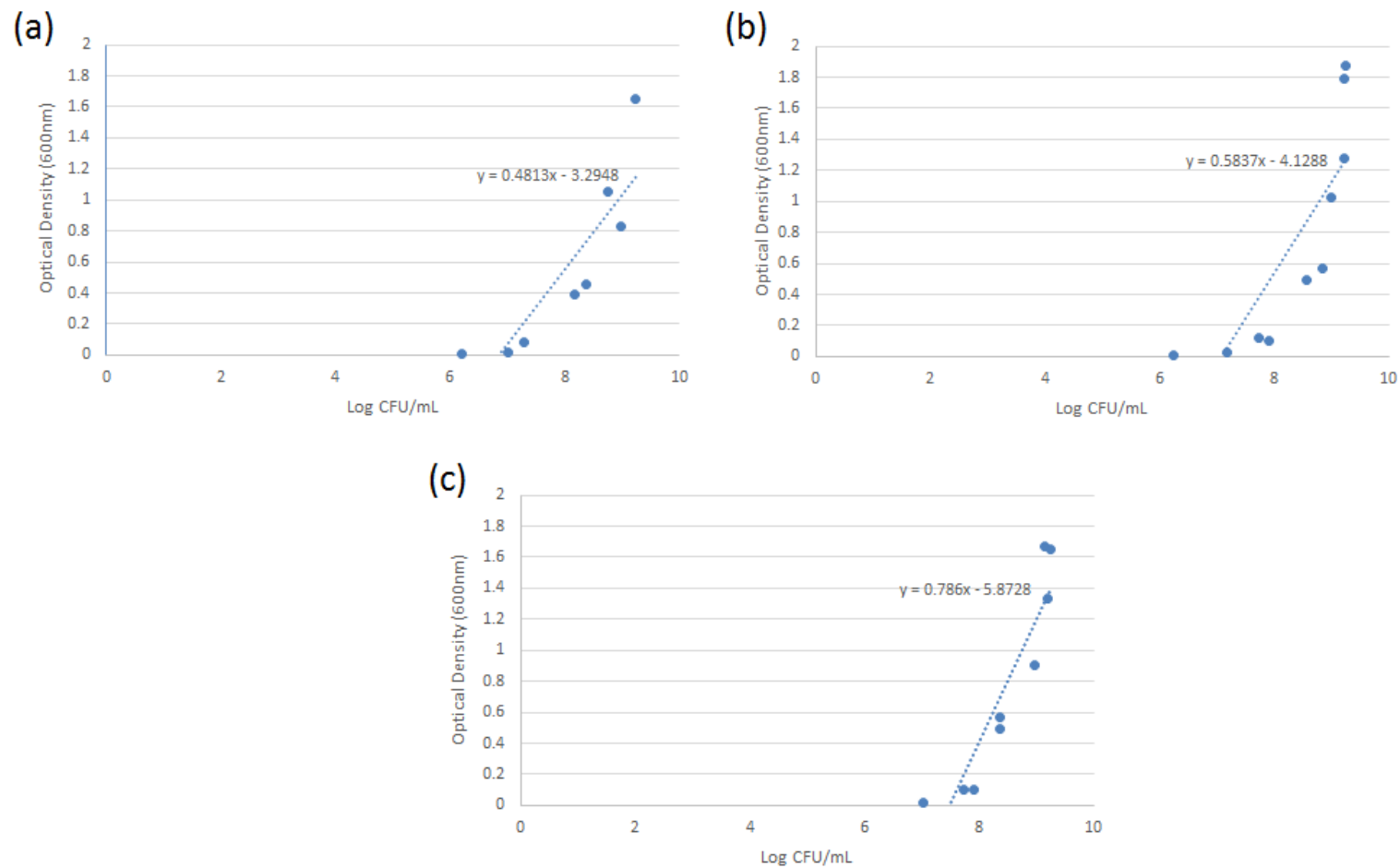


Figure 2.1: Optical density standardisation graphs where the optical density (600nm) is plotted against total viable count (log CFU/mL) for *Salmonella enterica* serovar (a) Typhimurium SL1344 (b) Agona and (c) Havana (n=3).

2.2 Biocides

The biocides used were hydrogen peroxide, peracetic acid and sodium hypochlorite (Sigma, Dorset, U.K.). All three biocides were bought in solution, with the stated concentration of hydrogen peroxide being 30%, peracetic acid 39% and sodium hypochlorite 15% (active chlorine). However, these agents are intrinsically unstable and so the concentration of sodium hypochlorite and hydrogen peroxide were assayed weekly. Peracetic acid was not assayed as it was guaranteed by the manufacturer to remain at that concentration for six months after opening. After this point it was discarded and a new peracetic acid stock was used.

2.2.1 Hydrogen Peroxide Assay

The hydrogen peroxide titration method was taken from the British Pharmacopoeia (2011b). Potassium permanganate (0.02 M) was titrated into 1 M sulphuric acid and a 1:100 dilution of hydrogen peroxide stock until there was a colour change. The volume added was then used to calculate the concentration of hydrogen peroxide present as each mL of 0.02 M potassium permanganate is equivalent to 1.701mg of hydrogen peroxide. The titration was repeated in triplicate.

2.2.2 Sodium Hypochlorite Assay

The sodium hypochlorite titration method was taken from the British Pharmacopoeia (2011a). Sodium thiosulphate (0.1 M) with 1% starch solution as an indicator was titrated into diH₂O, 0.006 M potassium iodide, 2 M acetic acid and a 1:10 dilution of sodium hypochlorite stock until there was a colour change. The volume added was then recorded and used to calculate the percentage of active chlorine as each mL of 0.1 M sodium thiosulphate is equivalent to 3.546 mg of active chlorine. The titration was repeated in triplicate.

2.2.3 Preparation of biocides

All biocides were made up fresh on the day of use in PBS, pH adjusted to 8 using a 9025 microcomputer pH meter (HANNA instruments, Bedfordshire, U.K.) with either a 0.1 M hydrochloric acid or 0.1 M sodium hydroxide as required. The resulting solution was filter sterilised using a 0.2 µm pore size filter attached to a syringe (Fisher, Leicestershire, U.K.).

All experiments were conducted with the biocides prepared to pH 8 as the industry collaborators involved with this project stipulated this pH, as this was the concentration they used for their ‘worst case’ testing.

2.3 Neutralisers

Neutralisers were used to quench the activity of the biocide, when investigating the bactericidal activity of the agent over a time period. The neutralisers used and their components are outlined below (table 2.2 and 2.3).

Table 2.2: Neutralisers used for each biocide and their concentrations.

Biocide	Neutraliser
Hydrogen peroxide	250 ppm catalase (Sigma, Dorset, U.K.)
Peracetic acid	Universal neutraliser (see table 2.3)
Sodium hypochlorite	10,000 ppm sodium thiosulphate (Sigma, Dorset, U.K.)

Table 2.3: The components of universal neutraliser which was prepared and then autoclaved (at 121 °C for approximately 15 mins) to ensure sterility.

Components	Quantity per 1L
Lecithin (Fisher, Leicestershire, U.K.)	3g
Polysorbate 80 (Fisher, Leicestershire, U.K.)	30 mL
Sodium thiosulphate (Sigma, Dorset, U.K.)	5g
L-histidine (Fisher, Leicestershire, U.K.)	1g
Saponin (Fisher, Leicestershire, U.K.)	30g
Phosphate diluent (Potassium dihydrogen phosphate 3.4g/100mL) (Fisher, Leicestershire, U.K.)	10 mL

2.3.1 Neutraliser efficacy test

One mL of biocide (10,000 ppm hydrogen peroxide, 1,000 ppm sodium hypochlorite or 500 ppm peracetic acid) was added to 8 mL of neutraliser (250 ppm catalase solution, 10,000 ppm sodium thiosulphate or universal neutraliser respectively) or PBS (control). This was vortex mixed and then left for 5 min before adding 1 mL of approximately 10^8 CFU/mL *Salmonella* bacterial suspension (all three parent strains were tested). The test suspensions were left for a further 5 min (or 30 min in the case of hydrogen peroxide as it takes longer to give a 5 log₁₀ reduction) before serial dilution in PBS and plating out using the drop counting method (see Chapter Two, section 2.1.7). All experiments were conducted in triplicate, and incubated for 24

hours at 37°C ($\pm 1^\circ\text{C}$) in a static incubator. For results please see appendix section 8.1.

2.3.2 Neutraliser toxicity test

Overnight *Salmonella* cultures grown in TSB and washed as described in 2.1.6, were resuspended in 10 mL of PBS which was then diluted to 1 in 10 in PBS. One mL of this diluted suspension in PBS (10^8 CFU/mL) was added to either 9 mL of neutraliser or 9 mL of PBS as a control. This was left for 5 min before serial dilution and plating out using the drop counting method (see Chapter Two, section 2.1.7). All experiments were in triplicate; and incubated for 24 hours at 37°C ($\pm 1^\circ\text{C}$) in a static incubator. For results please see appendix 8.1.

2.4 Minimal media

Minimal media were used in this study to replicate real world conditions more effectively to simulate the low nutrient environment often associated with formation of biofilms. They were also used to reduce the presence of organics that the oxidising biocides could interact with for MIC tests which required accuracy without interference. The two media selected were M9 minimal medium and a MOPS (3- (N-Morpholino) propanesulfonic acid) based minimal medium, as they are both minimal media used for the growth of enterobacteria. M9 medium composition is outlined in table 2.4; the MOPS based minimal medium was supplied as three solutions (VWR, Lutterworth, U.K.), the composition of which is given in tables 2.5 and 2.6. Both media were supplemented with 400 mg/L histidine (Sigma, Dorset, U.K.) as *Salmonella* Typhimurium SL1344 is an histidine auxotroph.

Table 2.4: The components of M9 minimal medium

Components	Quantity per 1L
Disodium phosphate anhydrous (Sigma, Dorset, U.K.)	6.8g
Monopotassium phosphate (Fisher, Leicestershire, U.K.)	3g
Sodium chloride (Sigma, Dorset, U.K.)	0.5g
Ammonium chloride (Sigma, Dorset, U.K.)	1g
20% D-Glucose solution (Fisher, Leicestershire, U.K.)	20 mL
1 M Magnesium sulphate solution (Sigma, Dorset, U.K.)	2 mL
1 M Calcium chloride (Sigma, Dorset, U.K.)	0.1 mL

Table 2.5: The components of MOPS based minimal media (VWR, Lutterworth, UK)

Components	Quantity per 1L
10 x MOPS mixture	100 mL
0.132 M Dibasic dipotassium phosphate	10 mL
20% D-Glucose solution	10 ml
diH ₂ O	880 mL

Table 2.6: The components of 10 x MOPS mixture (VWR, Lutterworth, UK)

Components	Quantity per 1L
MOPS	83.72g
Tricine	7.17g
0.01 M Ferrous sulphate	10 mL
1.9 M Ammonium chloride	50 mL
0.276 M Potassium sulphate	10 mL
0.02 M Calcium chloride dihydrate	0.25 mL
2.5 M Magnesium chloride	2.1 mL
5 M Sodium chloride	100 mL
Micronutrient stock (composed of- 0.009g ammonium molybdate, 0.062g boric acid, 0.018g cobalt chloride, 0.006g cupric sulphate, 0.04g manganese chloride and 0.007g zinc sulphate to make a 50 mL micronutrient stock)	0.2 mL
Autoclaved diH ₂ O	387 mL

2.5 Antibiotic susceptibility

2.5.1 Antibiotic susceptibility: disc testing

This test was based on the British Society of Antimicrobial Chemotherapy (BSAC) standard method (Howe *et al.*, 2011). All *Salmonella* strains were grown in Iso-Sensitest Broth (ISB, (Oxoid, Basingstoke, U.K.)) and incubated at 37°C overnight. The culture was then centrifuged at 5000 x g for 15 mins at 4°C and the pellet was resuspended in sterile 10 mL diH₂O. This was then diluted to 1 in 1000 in sterile diH₂O (10⁶ cells). This diluted bacterial suspension was then used to inoculate the

whole surface of an Iso-Sensitest agar (ISA) plate using a sterile cotton swab, in three directions, to ensure complete coverage. The plates were then allowed to dry for no more than 15 mins before the antibiotic discs (Oxoid, Basingstoke, U.K.) were applied (a maximum of 6 per plate). The experiment was repeated in triplicate for each organism and incubated overnight (20 hours) at 37°C. The diameter of the zones of inhibition around the antibiotic discs were then measured to the nearest millimetre using a ruler and the averages calculated (see figure 2.2). This was then compared to the clinical breakpoints as outlined in Howe, *et al.* (2011) to determine whether the organism was clinically sensitive.

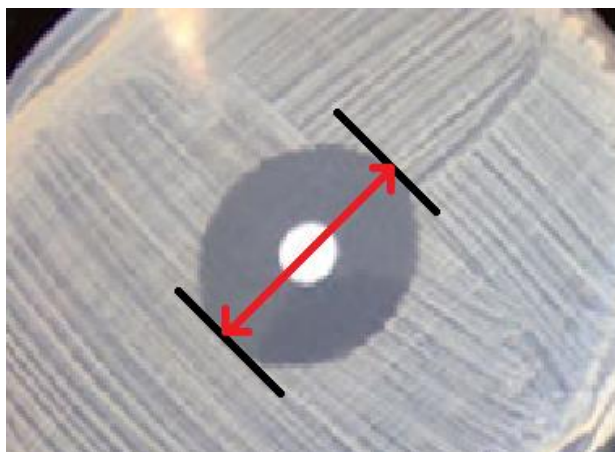


Figure 2.2: A diagram showing how the results of the BSAC method are interpreted: the zone of inhibition is measured as indicated by the red arrow line.

2.5.2 Antibiotic susceptibility: E-strip testing

Cultures of all *Salmonella* strains were prepared and inoculated onto ISA plate described in 2.5.1. When the plates had dried the antibiotic E-test strips (Oxoid, Basingstoke, U.K.) were added. This was repeated in triplicate for each organism and incubated overnight (20 hours), in air, at 37°C. The MIC was then read as the lowest concentration where growth was inhibited on the plate (see fig. 2.3).

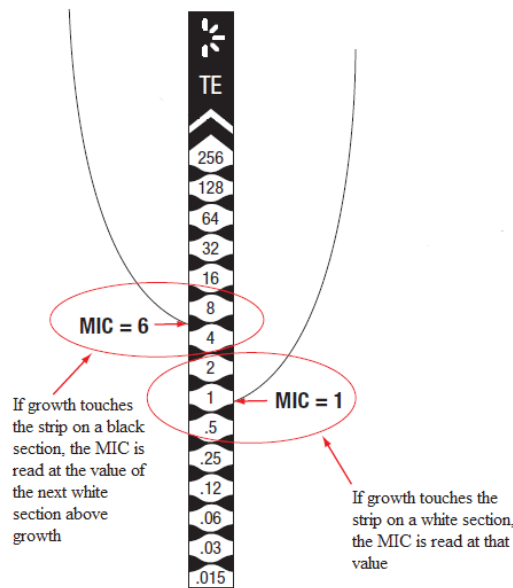


Figure 2.3: A diagram showing how the results of E-test strips are interpreted adapted from (Oxoid, 2008).

2.6 Biocide minimum inhibitory and bactericidal concentration determination

2.6.1 Minimum inhibitory concentration (MIC) testing

MICs were determined using doubling dilutions of biocide to give an indication of the susceptibility of the organism to a specific biocide. For this, 100 μ L of biocide in PBS was added to the first well of a 96 well microtitre plate (Fisher, Leicestershire, U.K.) and 50 μ L PBS was added to wells 2-12. The biocide was double diluted between wells 1-11 with a 50 μ L sample taken from well 1 and added to the second well, mixed thoroughly and then a 50 μ L sample was taken from this well to the third etc. The twelfth well was left containing PBS only; this meant that wells contained 50 μ l of PBS or a biocide in PBS solution. *Salmonella* strains were grown overnight in TSB and prepared as described in 2.1.6 and resuspended in 10 mL PBS. This was then diluted 1 in 10 in double concentrate media to give a cell suspension of 10^8 : either Muller-Hinton broth; M9 minimal medium (see table 2.4) or MOPS

based minimal medium (VWR, Lutterworth, U.K.; see tables 2.5 and 2.6) and 50 μL of the bacterial cell suspension was added to every well making the total volume of each well 100 μl (see fig. 2.4). All samples were completed in triplicate.

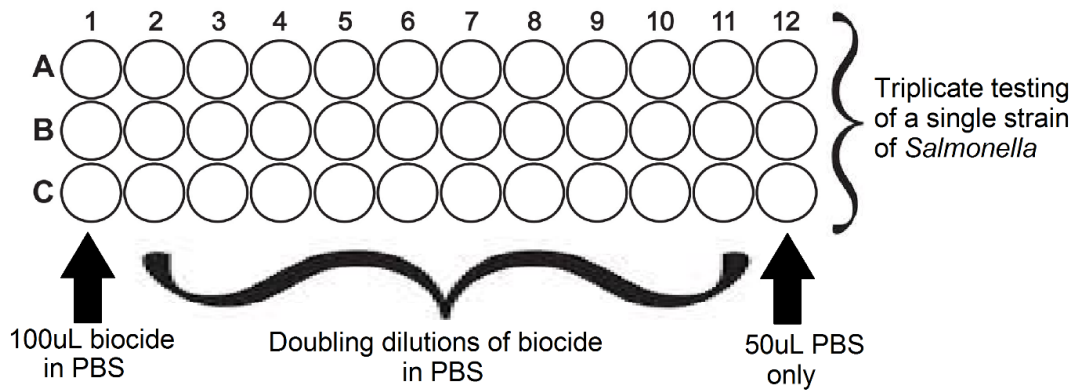


Figure 2.4: Diagram showing the arrangement of the 96 well microtitre plate for doubling dilution MIC testing with one biocide and one *Salmonella* strain in triplicate. Concentrations ranged between 32,000-31 ppm for hydrogen peroxide, 1000-1 ppm for peracetic acid and 1000-1 ppm for sodium hypochlorite.

The microtitre plate was then incubated for 24 hours at 37°C. The minimum inhibitory concentration was the lowest concentration of biocide where bacterial growth was inhibited.

2.6.2 Minimum bactericidal concentration (MBC) testing

20 μL samples were taken from the MIC microtitre wells where no growth was observed by visual examination for turbidity and the two highest concentrations where growth was observed. These samples were added to 180 μL of the appropriate neutraliser for that biocide. This was vortex mixed and left for five mins for the neutraliser to take effect. Twenty five μL of this was then plated out as a single drop on a TSA plate. The lowest concentration where no growth was observed was identified as the minimum bactericidal concentration.

2.7 Bioscreen testing to determine growth rates in different liquid media

Three hundred and fifty microlitres of broth; either tryptone soya broth, Muller-Hinton, MOPS based minimal media or M9 minimal media were added to the bioscreen plate. An overnight culture of *Salmonella* grown in TSB was prepared as described in 2.1.6 and the pellet was resuspended in PBS. The culture was then adjusted to a 10^8 CFU/mL in the appropriate broth and 50 μ L (containing 5×10^6) was added to broth. Negative control samples of broth (used as a blank) contained 400 μ L of broth only. The bioscreen plate was then placed in the Bioscreen C plate reader (Growth Curves, NJ, USA) and incubated at 37°C, for 24 hours with readings taken every 15 min at 420-580nm with samples being gently agitated for 15 seconds before the reading. Each sample had 10 technical repeats which were averaged to give a final reading for each time point.

2.8 Suspension test

Suspension test methods were based on the (BS EN 1276, 2009) method for testing the bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas.

The concentrations of biocide were made up fresh in phosphate buffered saline (PBS) and pH adjusted to 8. This was then sterilised using a 0.2 μ m syringe filter (Fisher, Leicestershire, U.K.). Nine mL of biocide was then added to one mL of a washed overnight culture (approximately 10^7 CFU/mL) and placed in the waterbath to maintain a constant temperature of 25°C. One mL samples were taken at the appropriate time points (either 5, 10, 20, 40 or 60 min) as required and added to 9 mL of neutraliser, vortex mixed, serially diluted and plated out in a drop count

method as described in 2.1.7. This was then incubated for 24 hours at 37°C. All experiments were conducted in triplicate.

2.8.1 Optimisation of suspension test methodology for low concentrations of oxidising biocides and the effect of TSC

Testing for the effects of tryptone sodium chloride (TSC) was only completed with sodium hypochlorite. These experiments were preliminary and when it was discovered that the diluent being used (TSC) was interfering with the activity of the biocide, all future experiments used PBS as a diluent to prevent further interference (see Chapter Three, section 3.2.6).

2.8.1.1 Comparing the effect of TSC and PBS on a range of concentrations of sodium hypochlorite

A stock concentration of biocide was made up fresh in either tryptone sodium chloride (TSC) or phosphate buffered saline (PBS) and pH adjusted to 8-8.3. This was then filter sterilised and diluted into either sterile TSC or PBS to make up the required concentration of biocide (dilution in the reaction vessel was taken into account). Nine hundred μL of biocide was then added to 9 mL of sterile dH_2O . One hundred μL of a washed overnight culture (approximately 10^7 CFU/mL) was added to the biocide solution and placed in the waterbath at 25°C. One mL aliquots were taken at 20, 40 and 60 mins and neutralised in 9mL sodium thiosulphate, serially diluted and plated on TSA using a drop count method (see Chapter Two, section 2.2). This was then incubated for 24 hours at 37°C. All experiments were conducted in triplicate.

2.8.1.2 The effect of the concentration of tryptone in TSC on sodium hypochlorite

A stock concentration of biocide was made up fresh in TSC containing different concentrations of tryptone (between 1000ppm and 10ppm) with the concentration of sodium chloride remaining constant. This was pH adjusted to 8 and then sterilised using 0.2 µm filters attached to the end of a syringe. This was diluted to make up the required concentration of biocide (dilution in the reaction vessel was taken into account). Nine hundred µL of biocide in TSC was then added to 9 mL of sterile diH₂O. One hundred µL of a washed overnight culture (approximately 10⁷ CFU/mL) was added to the biocide solution and placed in the waterbath at 25°C. One mL aliquots were taken at 10 and 20 mins and neutralised in 9 mL sodium thiosulphate, serially diluted and plated out on TSA using the drop count method (see Chapter Two, section 2.2.3). This was then incubated for 24 hours at 37°C. All experiments were conducted in triplicate.

2.8.2 Biocide pulse experiment

The British Standard method (BS EN 1276, 2009) as described above (using PBS instead of TSC) was followed but after 40 mins an additional pulse of biocide (sodium hypochlorite) was added to the reaction vessel. This was to investigate the apparent plateau in the log reduction curve, to see if increased tolerance was observed in the survivors due to either the presence of resistant mutants or persister cells. At the appropriate time points samples were neutralised and enumerated as described in Chapter Two, section 2.1.7.

2.9 Flow cytometry

Overnight broth culture of *Salmonella enterica* Typhimurium 1344 was prepared as described in Chapter Two, sections 2.1.5 and 2.1.6. Bacterial cultures were then exposed to either hydrogen peroxide, peracetic acid or sodium hypochlorite using the suspension testing methodology as described in Chapter Two, section 2.8 (suspension testing). Exposure times were 5 mins and 20 mins for peracetic acid and sodium hypochlorite and 20, 40 and 60 mins for hydrogen peroxide. After neutralisation with the appropriate neutraliser (see Chapter Two, section 2.3) bacteria were centrifuged at 3000 x g for 10 mins at 20°C. The supernatant was discarded and the bacterial pellet was resuspended in 500 µL PBS. An ethanol killed control was prepared by adding 50 µL of the untreated overnight cell suspension to 1 mL of ethanol for 10 mins before pelleting and resuspending in 500 µL PBS.

Frozen and refrigerated aliquots of 10 µL of 10mg/mL Bis (1,3-dibutylbarbituric acid) trimethine oxanol (BOX) (Invitrogen, Paisley, U.K.) and 50 µL of 5mg/mL propidium iodide (PI) (Sigma, Dorset, U.K.) were used to prepare working solutions. Once thawed 10 µL of the BOX was added to 10 mL of diH₂O and 100 µL of 4 mM EDTA (ethylenediaminetetraacetic acid) was added to this. The 50 µL aliquot of PI was added to 10 mL of diH₂O to give a working solution. PI stains the DNA of cells that have lost membrane integrity and is excluded from live cells and BOX is a dye that determines changes in membrane potential. These dyes were used to identify bacterial membrane damage as an indicator of cellular viability in response to biocide challenge as seen in Whitehead, *et al.* (2011).

Fifty µL bacterial cells were added to 1 mL FACSflow buffer (BD, Oxford, U.K.; composition given in table 2.7) in a tube to prepare them for the flow cytometer. The

dyes were then added; 10 μ L of BOX and 50 μ L of PI per 1 mL of bacterial sample in FACSflow buffer. Samples were then analysed using the FACS ARIA II (BD, Oxford, U.K.). One thousand particles were recorded with a 488 nm laser.

Table 2.7: The components of FACSflow buffer (BD, Oxford, U.K.)

Components	Percentage (v/v)
Riboflavin 5'-(sodium hydrogen phosphate)	2.35%
Sodium chloride	0.78%
EDTA Disodium dihydrate	0.36%
Potassium chloride	0.28%
Disodium hydrogen orthophosphate	0.26%
Ethylene glycol	0.21%
Sodium fluoride	0.06%
Water	95.7%

Data were then analysed using FACSDiva (BD, Oxford, U.K.) software. Initially, PBS only and live untreated cells were run and the data were recorded. This was then visualised using the FACSDiva software and gates were applied to remove the particles that were not of the size of normal healthy cells to remove any 'background noise' of other particles. Controls of healthy cells and ethanol killed cells were then run on the FACS; ethanol treated controls were known to be dead and would fluoresce with both PI and BOX, whereas healthy cells would not fluoresce. This was then used to draw the quadrants on the output graphs to show where live and dead cells would be recorded.

2.10 Hoescht assay

The method described below was based on the Hoescht accumulation assay described in Coldham *et al.* (2010) and Webber and Coldham (2010). *Salmonella* strains were prepared as described in Chapter Two, section 2.1.5 and 2.1.6. The washed overnight cultures were diluted to an OD of 0.1 at 600nm in M9 minimal media (see table 2.4) and added to a black flat bottomed 96 well microtitre plate which was covered with a breathable membrane. These were then placed back in the static incubator at 37°C for 3 hours for them to reach late log growth. After 3 hours, a biocide was added to the plate which was then left at room temperature for 1 hour for a change in efflux response to occur. Bis-benzimide (Hoescht 33342) (Sigma, Dorset, U.K.) was dissolved in sterile diH₂O to make a stock of 25 µM H33342, this was diluted to a final concentration of 2.5 µM in the 96 well plate. The bis-benzimide was injected by the FLUOstar Optima (BMG Labtech, Offenberg, Germany) which took fluorescence readings (excitation 355 nm, emission 465 nm, gain 1460) every 2 min for 30 min. A positive control of heat treated cells (5 mins at 99°C) which have no efflux activity was included, as well as L1007 and L644 which have efflux upregulated and downregulated respectively. L1007 lacks a functional RamR which is the RamA repressor, without RamR present RamA is overexpressed which results in the overexpression of the AcrB-TolC efflux pump (see Chapter One, section 1.4.3.2). L644 lacks functional AcrB which results in decreased efflux through the AcrB-TolC pump as it cannot assemble.

2.11 Green fluorescent protein reporter assays

Bacterial cultures of *Salmonella enterica* serovar Typhimurium SL1344, L644, L1007, L1232, L1361 and L1357 (see Chapter Two, table 2.1, section 2.1.1) were grown and prepared as described in Chapter Two, section 2.1.5 and 2.1.6. Ampicillin was added to cultures containing the pMW82 plasmid (L1232, LL1361 and L1357). The resulting washed overnight bacterial suspension was diluted in M9 minimal to an OD of 0.1 at 600 nm. This was added to a 96 well plate with clear well bottoms and black well sides which was then placed inside the FLUOstar Optima. The FLUOstar took fluorescence (excitation 492 nm, emission 520 nm, gain 3010) and growth kinetics readings every every 3 min for 3 hrs. After this time had elapsed and cells had reached late log phase, the plate was removed from the FLUOstar and biocide was added to the wells aseptically. The concentrations of biocide used were as follows: hydrogen peroxide 90 ppm, 67.5 ppm, 45 ppm and 22.5 ppm, peracetic acid 4 ppm, 3 ppm, 2 ppm and 1 ppm, sodium hypochlorite 20 ppm, 15 ppm, 10 ppm, 5 ppm. As soon as possible the plate was returned to the FLUOstar and growth and fluorescence continued to be recorded for every 3 mins for a further 99 mins. Bacteria treated with 70% ethanol for 10 mins and included as a positive control. Chlorpromazine (50 µg/mL) and menadione (200 µM) were also used as controls as chlorpromazine induces *ramA* expression but inhibits *acrB* expression (Bailey *et al.*, 2008) and menadione induces *soxS* (Nunoshiba *et al.*, 1992). This assay was based on the GFP reporter work described in Ricci *et al.* (2012) and Lawler, *et al.* (2013).

2.12 Development of a static *Salmonella* biofilm

2.12.1 Optimising the growth of *Salmonella* biofilm in minimal media

Salmonella cultures were grown overnight in TSB and prepared as described in 2.1.6 and washed once in PBS before being resuspended in PBS. Ten mL of the selected minimal growth medium was then added to each well of a six-well plate (Fisher, Leicestershire, U.K.), then 100 μ L of 10^9 CFU/mL *Salmonella* suspension was added to each well. Stainless steel discs (2 cm diameter, surface area 6.28 cm² (supplied by Goodfellows Cambridge Ltd., Huntingdon, U.K.); prepared by sterilisation in Virkon (Fisher, Leicestershire, U.K.), rinsed, dried and then autoclaved were added. These 6-well plates were then incubated statically for 48 hours at either 30°C or 37°C. After incubation stainless steel discs were washed once in 10 mL PBS to remove any planktonic or loosely adherent cells and then added to a 100 mL Duran bottle (Fisher, Leicestershire, U.K.) containing 5g of glass beads and 10 mL of PBS (Fisher, Leicestershire, U.K.). This was then mixed at 150 rpm for 1 min. The PBS was then serially diluted and plated out according to the drop counting method (see Chapter Two, section 2.1.7).

Some bacteria attached to the polystyrene interior base of the 6-well plate (Fisher, Leicestershire, U.K.) and the growth medium was removed from the well by careful pipetting so as not to disturb the biofilm. The *Salmonella* cells were then washed with Ten mL PBS to remove any non-adherent or loosely adherent cells, which were then pipetted off. 10 mL of PBS was then added to each well and the cells were physically removed using a cell scraper (Fisher, Leicestershire, U.K.). This bacterial suspension was then serially diluted and plated out according to the drop count method (see Chapter Two, section 2.1.7).

TSA plates were then incubated statically overnight at 37°C, in air. The number of colonies was then counted and the CFU/cm² of the surface of the disc or polystyrene well was calculated. This calculation corrects for the difference in the size of surfaces as the area of the bottom of the plastic 6-well plate is 11.34cm² (cells were only removed from the bottom surface, not the sides of the wells in the plate) whereas the area of the stainless steel discs is 6.28cm² (top and bottom).

2.12.2 Quantification of *Salmonella* species biofilms using crystal violet staining

Biofilms were grown from an overnight broth culture prepared as described in Chapter Two, section 2.1.5. This broth culture was then prepared as described in 2.1.6 to give a bacterial suspension in PBS. One hundred µL of *Salmonella* cell suspension was added to 10 mL of MOPS based minimal media in a polystyrene 6-well multidish. This was then incubated statically in aerobic conditions at 30°C for 48, 72 and 96 hours.

After biofilm formation the growth media was removed and the cells were washed twice in sterile diH₂O to remove any planktonic or loosely adherent cells. 10 mL of methanol was added and incubated for 15 mins at room temperature to fix the biofilm. Ten mL of a 0.1% crystal violet solution was added to the wells which were incubated at room temperature for a further 15 mins before being washed off with water. Plates were then dried and photographed for qualitative assays. For quantitative assays 10 mL of 30% acetic acid was added to each well of the multidish and this was incubated at room temperature for a further 15 mins. 1 mL samples of this solubilised crystal violet were then removed (Fisher, Leicestershire, U.K.) and the absorbance was read at 550 nm (Ultraspec 3100 pro, Fisher, Leicestershire, U.K.) using 30% acetic acid as a blank (based on O'Toole (2011)).

2.12.3 Optimised methodology for growing a *Salmonella* biofilm

Biofilms were grown from an overnight broth culture prepared as described in Chapter Two, section 2.1.5. This broth culture was then prepared as described in 2.1.6 to give a bacterial suspension in PBS at a concentration of approximately 10^9 cells per mL. One hundred μL of *Salmonella* cell suspension was added to 3 mL of MOPS based minimal media in a polystyrene 6-well multidish. This was then incubated statically in aerobic conditions at 30°C for 72 hours.

2.13 Inactivation kinetics of a *Salmonella* biofilm

2.13.1 Inactivation kinetics of a dispersed *Salmonella* biofilm

Biofilms were grown as described in Chapter Two, section 2.12.3 using *Salmonella* Typhimurium SL1344 and Agona 3750. After the 72 hours incubation the growth media was removed and the biofilms were washed twice in PBS to remove and planktonic or loosely adherent cells. Ten mL of PBS was then added and the biofilm was removed using a cell scraper (Fisher, Leistershire, U.K.). The suspension was vortex mixed in the presence of 1g glass beads for 1 min (see figure 2.4). After disaggregation the cell suspension is treated the same as planktonic cells and the suspension testing method (see Chapter Two, section 2.8) was followed. Planktonic cells were tested simultaneously with the dispersed biofilm cells as a control.

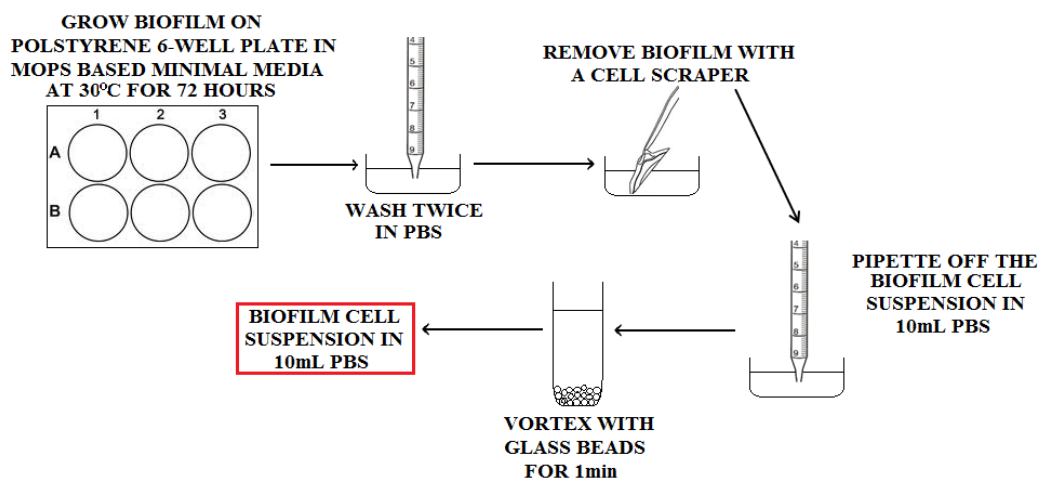


Figure 2.5: A diagram to show how *Salmonella* biofilms were grown and dispersed for dispersed biofilm inactivation kinetics.

2.13.2 Inactivation kinetics of an intact *Salmonella* biofilm

Biofilms were grown as described in Chapter Two, section 2.12.3 using *Salmonella* Typhimurium SL1344 and Agona 3750 on polystyrene 6-well plates. After the 72 hours incubation the growth media was removed and the biofilms were washed twice in PBS to remove planktonic or loosely adherent cells. Biocide was prepared as described in Chapter Two, section 2.2.3 and 3.5 mL of biocide was added directly to the biofilm in situ (see figure 2.6). After the appropriate incubation time had elapsed (20, 40 or 60 mins, incubated at 25°C), 7 mL of neutraliser was added directly to the biofilm-biocide solution. After neutralisation the biofilm was removed from the surface of the polystyrene multidish using a cell scraper and was then disaggregated by vortex mixing for 1 minute with 1g of glass beads. The resulting cell suspension was then enumerated using the drop counting method described in Chapter Two, section 2.1.7.

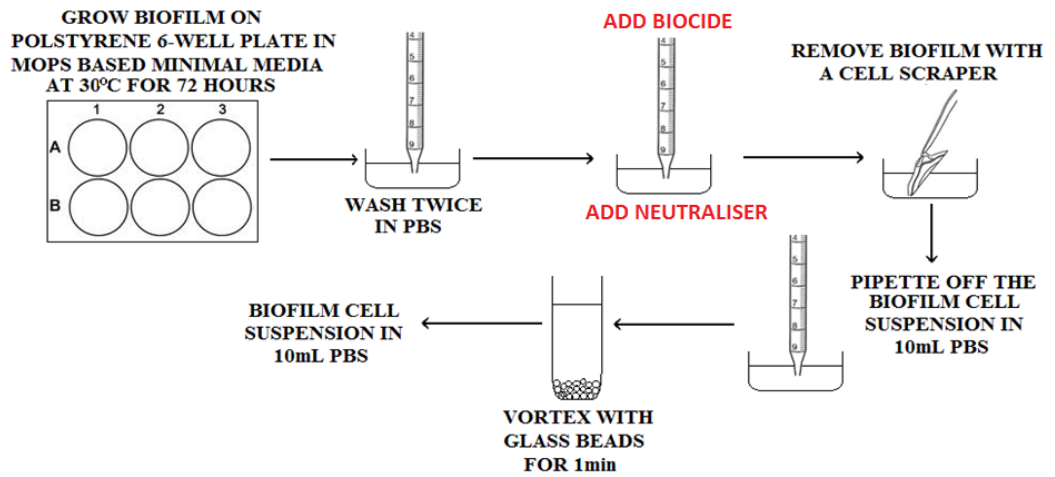


Figure 2.6: A diagram to show how *Salmonella* biofilms were grown, exposed to biocide and dispersed for enumeration for whole biofilm inactivation kinetics.

2.14 Growing a *Salmonella* species biofilm under flow using a microfluidic system

The Bioflux system (Fluxion, U.S.A.) was used to grow a *Salmonella* species biofilm under flow conditions as used by Benoit *et al.* (2010) and Nance *et al.* (2013). Before bacterial cells were added to the Bioflux the channels first needed to be primed. Two hundred microlitres of MOPS based minimal media (see table 2.4 and 2.5) was added to the outlet well (see figure 2.7) and then pressure (5 dyne for 5 mins) was added to the system to push the media through to the inlet well. The excess media was then removed from both the inlet and outlet well, leaving only the media in the inner pockets.

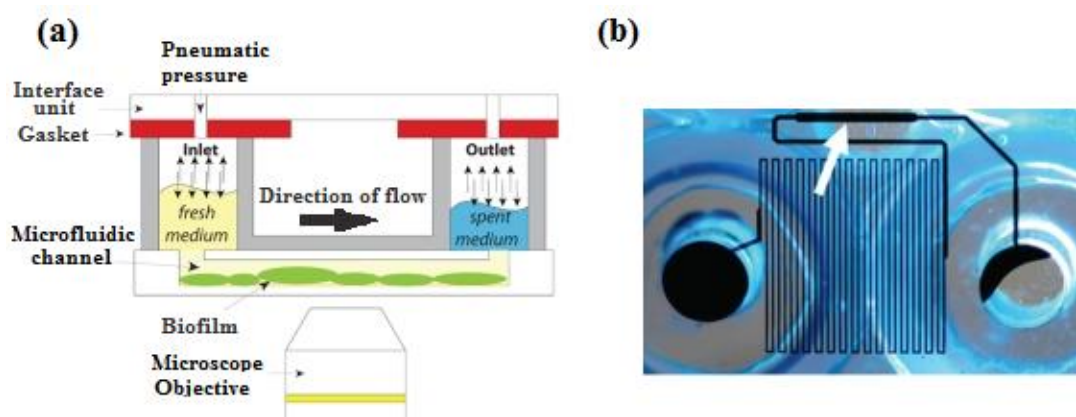


Figure 2.7: Diagram of the Bioflux microfluidic system (a) schematic diagram of one microfluidic channel (b) image of the inlet and outlet wells and the microfluidic channel system that connects them, the white arrow indicates the chamber that is viewed under the microscope. Adapted from Benoit, *et al.* (2010).

Bacterial cell suspensions of *Salmonella* Typhimurium SL1344 and *Agona* 3750 were grown overnight and prepared as described in Chapter Two, sections 2.1.6 and 2.1.7. The bacterial suspensions were then diluted to an OD of 0.1 at 600 nm in MOPS based minimal media. Fifty microlitres of this was then added to the outlet well which was balanced with 50 μ L of growth media in the inlet well. The flow cells were then inoculated by adding 3 dyne of pressure to the outlet well for 5 seconds which forced the *Salmonella* into the microfluidic channels. To confirm that

the channel had been effectively inoculated the viewing chamber was viewed via an LTSi-1000 inverted microscope (Labtech, Massachusetts, U.S.A.). After confirmation that the microfluidic channel had been successfully inoculated, the bacteria were left in static conditions for 1 hour to attach at room temperature ($19 \pm 1^\circ\text{C}$). After the hour had elapsed 1 mL of MOPS based minimal media was added to the inlet well and a flow rate of 0.3 dyne was added to the inlet well (see fig. 2.7). This was left at room temperature ($19 \pm 1^\circ\text{C}$) for either 24 or 48 hours for a biofilm to develop.

2.15 Biocide treatment of a *Salmonella* biofilm grown in a microfluidic flow cell

Salmonella biofilms were grown as described in Chapter Two, section 2.14 for either 24 or 48 hours. After the required period of growth had elapsed the waste was removed from the outlet well and the growth media was removed from the inlet well down to the inner pocket; PBS was then added to the inlet well. The PBS was flowed through the system at a rate of 0.3 dyne for 1 hour. Excess PBS was then removed from the inlet and outlet well and biocide (prepared as described in Chapter Two, section 2.2.3) was added to the inlet well. The biocide was then flowed through the system at a rate of 0.3 dyne for either 20, 40 or 60 mins. Biocide was then removed from the inlet and outlet well; PBS was then added to the inlet well. The microfluidic channel was then washed with PBS at 0.3 dyne for 30 mins. For experiments that had a post-exposure growth condition: PBS was removed, 1 mL of media was then added to the inlet well and pushed through at 0.3 dyne for 24 hours.

2.16 Light microscopy and analysis of a *Salmonella* species biofilm

Biofilms grown in the microfluidic channels of the Bioflux (Chapter Two, section 2.14) were viewed at the x10 and x40 objectives of an LTSi-1000 inverted microscope. Images were taken before biocide exposure (Chapter Two, section 2.15), after 1 hour of biocide exposure and after 24 hours of growth post-biocide exposure. Images were then analysed using Bioflux EZ software (Labtech, Sussex, U.K.) by using their percentage coverage tool and highlighting 3 different fields of view to give an average value for percentage coverage for quantitative comparison.

2.17 Confocal microscopy and analysis of a *Salmonella* species biofilm

Salmonella biofilms were grown in the Bioflux microfluidic system as described in Chapter Two, section 2.14 and treated by biocide as described by Chapter Two, section 2.15. After biocide exposure and 30 mins washing with PBS excess PBS was removed. Two fluorescent stains were added to the outlet well: 748 μM PI (Sigma, Dorset, U.K.) and 10 μM SYTO 9 (Life Technologies, Leicestershire, U.K.) diluted in 1 mL in PBS and flowed to the input well at 2 dyne for 5 mins. After visual confirmation that the dye had travelled through the microfluidic channel the dyes were left to incubate at room temperature ($19 \pm 1^\circ\text{C}$) for 40 mins. After the incubation period any excess dye was removed from the inlet and outlet wells and PBS was run through the outlet to the inlet well for 20 mins at 0.3 dyne to remove any excess dye from the microfluidic channel.

The Bioflux plate was removed from the Bioflux system and any excess PBS was removed from the wells. The plate was then covered and taken to the Zeiss LSM 710 Confocor 3 inverted confocal microscope (Carl Zeiss Ltd., Germany) where it was viewed at x63 in oil. The 488 nm laser was used at 30% power and using the MBS 488/543/633 filter. The range used for SYTO 9 was 500-550 and for PI was 600-650. Images were taken in triplicate across different fields of view. Z-stacks were taken at intervals of 0.5 μm across the depth of the biofilm. Images were taken in triplicate and the colour channels were split with the use of ImageJ (National Institutes of Health, Maryland, U.S.A.). These separate monochrome images were processed with the percentage coverage analysis tool on the Bioflux EZ software. This gave percentage live vs. dead for each image which was then statistically analysed using a Student's T-test in Microsoft Excel.

Chapter Three: Biocide and
Antibiotic Susceptibility
Profile of Planktonic
Salmonella

3.1 Introduction

There are a number of different methodologies that can be used when attempting to determine bacterial susceptibility to a biocide or an antibiotic. The different methodologies are appropriate in different circumstances depending on the application of the antimicrobial being tested and the information required.

Antibiotic testing using the disc method is a very useful tool in assessing antimicrobial susceptibility in a clinical and research environment. It is a very simple, quick method that can be used to test a number of antibiotics and bacterial strains in a very short space of time. It is also very easily standardised with discs supplied at a given concentration and instructions to standardise the bacterial inoculum (Howe, *et al.*, 2011). This is useful for surveillance of antibiotic resistance as the same method is used in more than 175 clinical and research laboratories across the United Kingdom. There are however, limitations on methods that are only standardised nationwide and to address this in January 2016 BSAC are advising that the EUCAST disc diffusion method is used in preference to its own method (British Society for Antimicrobial Chemotherapy, 2015). The EUCAST method is similar to the BSAC method but there are small differences such as Muller-Hinton agar is used instead of ISA, the inoculum preparation is sterile saline as opposed to sterile water or ISA and the bacteria are grown to confluence as opposed to semi-confluence. Changing to using one method across Europe makes it possible for antibiotic susceptibilities to be tracked across the European Union and additionally this method is linked to MIC results gained by using the international standard method for testing antimicrobial susceptibility. Antibiotic disc testing is useful in a clinical environment as it gives information on which drug would be the most appropriate to prescribe. However, from a research perspective what this information gives us is of limited

value; an organism may display changes in susceptibility which can reflect different mechanisms of resistance, the binary nature of susceptibility testing to determine organisms to be sensitive or resistant may not be sensitive to these changes.

Minimum inhibitory concentration (MIC) testing can be used for both antibiotic and biocides. It does not have the direct clinical applications and is more time consuming (in the case of microbroth dilutions) or expensive (in the case of the E-strips) and so the use is primarily in research, although it can be used to assist a prescribing decision. The advantages of MICs are that they give a quantitative as opposed to a semi-quantitative result. This exposes growing bacteria in the presence of a nutrient source to an antimicrobial and records the lowest concentration that inhibits bacterial growth (which does not necessarily mean bacterial cell death). It requires long exposure and incubation times (normally at least overnight) and so is most appropriate to use with antimicrobials with a long exposure time (antibiotics are given in courses that typically last a number of days, whereas biocides have exposure times in mins). The other limitation in terms of using MICs for biocide studies is that the concentrations used in MICs are often much lower than the in-use concentrations of biocides. This means that they are useful in assessing the effect of residual concentrations of biocide (Thomas *et al.*, 2000), but not as useful for the higher concentrations used disinfectant products. As described in the introduction an increase in MIC would fit the definition of an increase in biocide tolerance as they are inhibitory concentrations; whereas true resistance is associated with an ability to survive 'in-use' concentrations (Chapter One, section 1.4.1). This has been confirmed by research showing an increase in MIC does not necessarily mean a failure of a biocide to effectively kill a bacterial target (Lear *et al.*, 2002; Thomas *et al.*, 2005).

Minimum bactericidal concentration (MBC) tests look at the lowest concentration of biocide exposure when bacteria have been exposed to biocide in broth overnight. This test is completed by taking a sample from the well of the MIC plate, neutralising the biocide and plating out onto an enriched media. It does however use long exposure times, potentially unrealistically low concentrations and the presence of organic matter are limitations. However, studies have shown that a combination of MIC and MBC testing may be used to investigate or potentially predict biocide resistance (Knapp *et al.*, 2013).

Inactivation kinetics determined using the suspension testing method (BS EN 1276, 2009) is the British Standard method for testing bacterial susceptibility to biocides. This means that in-use concentrations can be tested in the conditions which would be found in an in-use scenario (with the possibility to add dirty conditions but in a controlled manner). This method can be used to assess bacterial resistance and tolerance and the data can be analysed to give information such as the concentration exponent which gives insight into the biocidal mechanism of action (Russell & McDonnell, 2000). As concentration is key to the activity of many biocides being able to control this and test at suitable concentrations is very important (Russell & McDonnell, 2000).

In summary, there are a range of methods that can be used to investigate the susceptibility of planktonic bacteria to both antibiotics and biocides. They test different responses to antimicrobial challenge and therefore are all useful in building a susceptibility profile. However, despite all the work that has been done it is important to adjust and optimise these methods for testing susceptibility to oxidising biocides.

3.1.1 Aims

- Using existing methodology to identify the most appropriate tools for measuring biocide and antibiotic susceptibility.
- To determine the baseline susceptibility data for planktonic *Salmonella*.
- To determine biocide concentrations which will be appropriate for future work.

3.2 Results

3.2.1 Antibiotic susceptibility testing using the disc method

For the associated method please see **Chapter Two, section 2.5.1.**

Salmonella Typhimurium SL1344, Agona d Havana were first tested for their susceptibility to four clinically relevant antibiotics using the British Society for Antimicrobial Chemotherapy (BSAC) disc method. Two of antibiotics selected were cefotaxime (a ‘third generation’ cephalosporin) and ciprofloxacin (a quinolone) as they were recommended by the British National Formulary (2010) for treatment of non-typhoidal salmonellosis. The other two antibiotics chosen were ampicillin (a beta lactam) and imipenem (a carbapenem) which are both broad spectrum antibiotics used in treating a wide range of infections and so may be used in a clinical environment if the causative organism is unknown. The BSAC disc method is used clinically and gives a semi quantitative result. The zone of inhibition was interpreted according to the British Society for Antimicrobial Chemotherapy (BSAC) guidelines to determine if the *Salmonella* strain was sensitive or resistant to the antibiotic in a clinical setting.

The BSAC disc test showed that all *Salmonella* isolates tested were clinically sensitive to all four antibiotics (table 3.1). The *E. coli* control strain, which was used to confirm that the discs were working appropriately, did so as it was clinically sensitive to all four antibiotics (table 3.1) as would be expected (Howe *et al.* 2011).

Table 3.1: The antibiotic susceptibilities of *E. coli* NCTC 10418 and *Salmonella* Typhimurium SL1344, Agona and Havana tested using the BSAC antibiotic disc method (n=3).

	Antibiotic	Mean zone of inhibition (mm) ± SD	Susceptibility according to BSAC guidelines
Control <i>E. coli</i> NCTC 10418	Ampicillin (10 µg)	26 ± 0	Sensitive
	Cefotaxime (5 µg)	41 ± 0.6	Sensitive
	Ciprofloxacin (5 µg)	36 ± 1.7	Sensitive
	Imipenem (10 µg)	39 ± 1.7	Sensitive
<i>Salmonella enterica</i> serovar Typhimurium SL1344	Ampicillin (10 µg)	30 ± 1.5	Sensitive
	Cefotaxime (5 µg)	41 ± 3.6	Sensitive
	Ciprofloxacin (5 µg)	34 ± 2	Sensitive
	Imipenem (10 µg)	34 ± 1	Sensitive
<i>Salmonella enterica</i> serovar Agona 3750	Ampicillin (10 µg)	32 ± 0.6	Sensitive
	Cefotaxime (5 µg)	39 ± 2.7	Sensitive
	Ciprofloxacin (5 µg)	35 ± 2.3	Sensitive
	Imipenem (10 µg)	37 ± 0.6	Sensitive
<i>Salmonella enterica</i> serovar Havana 3759	Ampicillin (10 µg)	33 ± 1.2	Sensitive
	Cefotaxime (5 µg)	37 ± 1.5	Sensitive
	Ciprofloxacin (5 µg)	35 ± 0.6	Sensitive
	Imipenem (10 µg)	37 ± 0.6	Sensitive

3.2.2 Antibiotic susceptibility testing using E-test strips

For the associated method please see **Chapter Two, section 2.5.2.**

The same four clinically relevant antibiotics were then further tested using the E-strip test method. This is a quantitative method and gives the MIC of the antibiotic for each strain of *Salmonella*. This approach may be used in future experiments to identify any change in antibiotic susceptibility seen in biocide insusceptible mutants rather more effectively than the disc method. Again *E. coli* NCTC 10418 was used as a control to confirm that the strips were working effectively (Andrews 2001).

The E-strip method showed the wild type *Salmonella* Typhimurium SL1344 strain to have the highest MIC against all the clinically relevant antibiotics tested (see table 3.2). *Salmonella* Agona and Havana have identical MICs against cefotaxime, however Agona has a higher tolerance for ciprofloxacin and Agona has a higher tolerance for imipenem (see table 3.2).

Table 3.2: The MIC of *E. coli* NCTC 10418 and *Salmonella* Typhimurium SL1344, Agona and Havana to clinically relevant antibiotics using the E-test strip method (n=3).

	Antibiotic	Mean MIC ($\mu\text{g/mL}$) \pm Standard Deviation
<i>Escherichia coli</i> NCTC 10418	Ampicillin	5.5 \pm 0.7
	Cefotaxime	0.03 \pm 0
	Ciprofloxacin	0.09 \pm 0.1
	Imipenem	0.03 \pm 0
<i>Salmonella enterica</i> serovar Typhimurium SL1344	Ampicillin	6.7 \pm 2.3
	Cefotaxime	0.25 \pm 0.0
	Ciprofloxacin	0.015 \pm 0.0
	Imipenem	0.21 \pm 0.075
<i>Salmonella enterica</i> serovar Agona 3750	Ampicillin	1.0 \pm 0.0
	Cefotaxime	0.12 \pm 0.0
	Ciprofloxacin	0.015 \pm 0.0
	Imipenem	0.12 \pm 0.0
<i>Salmonella enterica</i> serovar Havana 3759	Ampicillin	1.0 \pm 0.0
	Cefotaxime	0.12 \pm 0.0
	Ciprofloxacin	0.005 \pm 0.0
	Imipenem	0.25 \pm 0.0

3.2.3 MIC in different growth media

For the associated method please see **Chapter Two, section 2.6.1.**

MIC results also demonstrated that the composition of media can interfere with biocidal activity. The data shown in table 3.3 compares MIC values given from a doubling dilution MIC in Muller Hinton broth which is an enriched medium, which contains, among other components casein hydrolysates (tryptone). M9 and MOPS based medium however are minimal media which contain only certain salts, buffer and a carbon source (glucose) with no protein hydrolysates present.

The MICs were highest against all bacterial strains when the biocides were used in the presence of MOPS based minimal media; although they were also high in the presence of Muller Hinton Broth (MHB) (table 3.3). This suggests that these broths interfere with biocide activity therefore affecting the MIC. For all biocides the lowest MIC was seen with M9 minimal media. Although some of the intervals between the MICs look large, doubling dilutions were used in MOPS based minimal media and MHB making the concentrations 293 ppm, 586 ppm and 1172 ppm sequential in hydrogen peroxide for example. This means that the gaps are not as great as they would seem due the methodology employed.

Table 3.3: The MIC values of *Salmonella* Typhimurium SL1344, Agona and Havana when exposed to sodium hypochlorite, hydrogen peroxide and peracetic acid in Muller-Hinton broth, MOPS based minimal media and M9 minimal media overnight (n=3).

<i>Salmonella enterica</i> serovar	Biocide	Mean MIC in Muller Hinton Broth (ppm)	Mean MIC in M9 minimal media (ppm)	Mean MIC in MOPS based minimal media (ppm)
Typhimurium SL1344	Hydrogen peroxide	1172 ± 0	90 ± 0	586 ± 0
	Peracetic acid	166.7 ± 72.2	4 ± 0	333 ± 144
	Sodium hypochlorite	500 ± 0	19 ± 4	125 ± 0
Agona 3750	Hydrogen peroxide	293 ± 0	150 ± 0	488 ± 169
	Peracetic acid	125 ± 0	5 ± 1	500 ± 0
	Sodium hypochlorite	500 ± 0	21 ± 0	125 ± 0
Havana 3759	Hydrogen peroxide	293 ± 0	293 ± 0	140 ± 9
	Peracetic acid	166.7 ± 72.2	500 ± 0	5 ± 1
	Sodium hypochlorite	500 ± 0	125 ± 0	21 ± 0

No statistical tests have been performed on this data as most of the standard deviations have a value of 0.

3.2.4 Growth rates in different growth media

For the associated method please see **Chapter Two, section 2.7.**

To confirm that the different MICs seen in table 3.3 were in fact due to the broth interfering with the activity of the biocide, as opposed to lower levels of growth in the different media a growth kinetics of the *Salmonella* strains in the presence of the different media were produced.

Data from the growth kinetics in the Bioscreen C fig. 3.1 show that tryptone soya broth (TSB) gives the highest levels of growth as it is an enriched media. MHB gives a similar final optical density (OD) reading as TSB when growing *Salmonella* Agona, however when growing *Salmonella* Typhimurium SL1344 gave a much lower growth yield. *Salmonella* Havana gave a similar growth kinetics to Agona. Notably however, M9 minimal media gave a higher growth yield than MOPS based minimal media in both strains and higher than MHB with *Salmonella* Typhimurium|. This indicates that the lower MIC with M9 minimal media is not due to lower levels of bacterial growth than MOPS based minimal media or MHB but due to greater biocide interference.

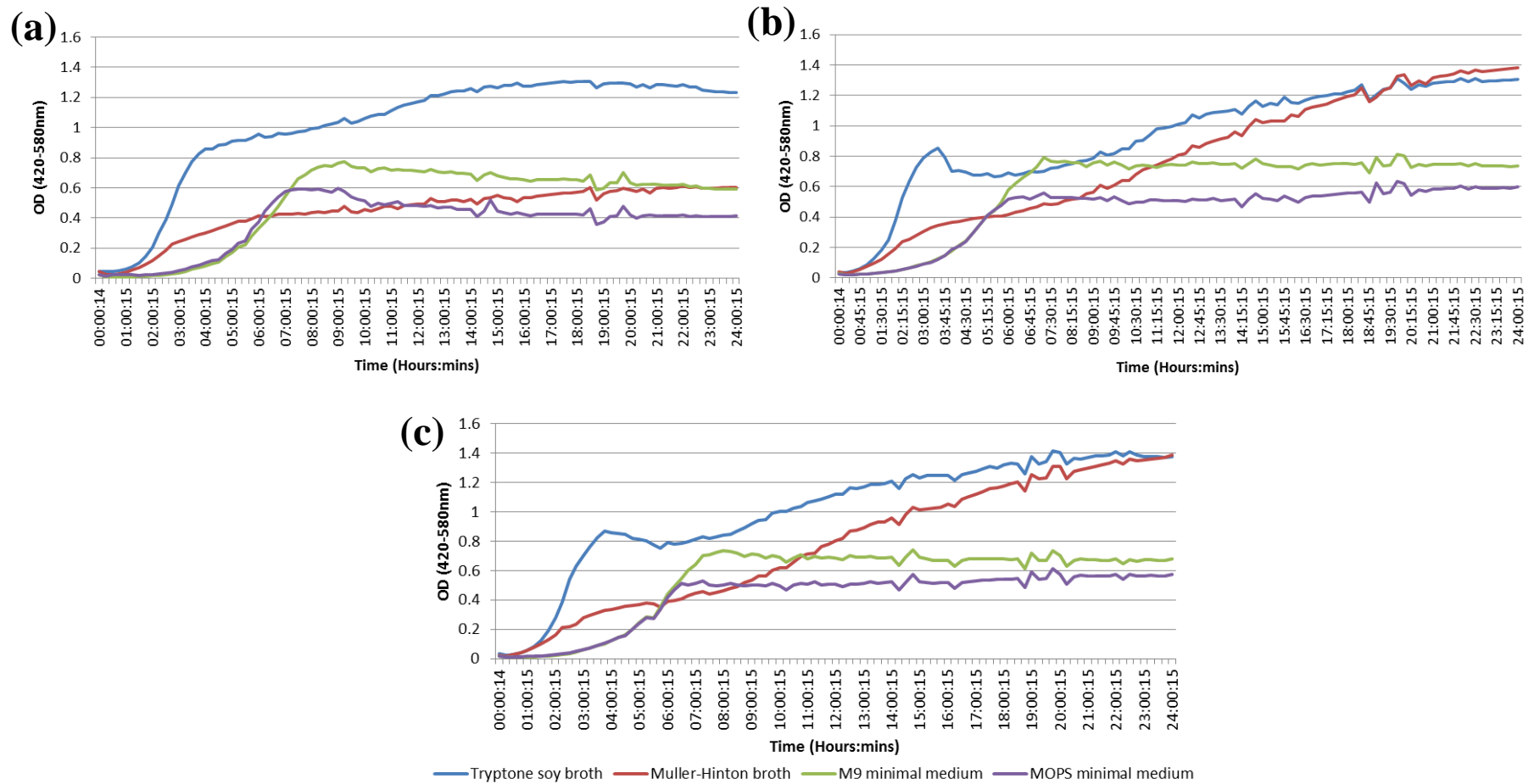


Figure 3.1: Growth curves for different *Salmonella enterica* serovars (a) Typhimurium SL1344 (b) Agona (c) Havana over 24 hours when grown in different liquid growth media (n=10).

3.2.5 MIC and MBC in M9 minimal media

For the associated method please see **Chapter Two, section 2.6.**

After the preliminary studies outlined in Chapter Three, sections 3.3.2 and 3.3.3 M9 minimal media was selected for future MIC and MBC testing as it interferes with the biocide least and yet still gives a high bacterial growth yield.

There was some variation between strains with *Salmonella* Agona having an increased MICs for all three biocides. The differences between the MICs for peracetic acid and sodium hypochlorite are small and within the standard deviations (table 3.4). The differences between hydrogen peroxide MIC against the strains is slightly greater, and so this indicates that *Salmonella* Agona and Havana are more tolerant of growing in the presence of hydrogen peroxide. The MBCs of sodium hypochlorite were higher than the MICs in all strains. Peracetic acid gave the same value for MICs and MBCs in all strains.

Table 3.4: The MIC and MBC values of *Salmonella* Typhimurium SL1344, Agona and Havana exposed to hydrogen peroxide, peracetic acid and sodium hypochlorite.

<i>Salmonella enterica</i> serovar	Biocide	Mean MIC \pm SD (ppm)	Mean MBC \pm SD (ppm)
Typhimurium SL1344	Hydrogen peroxide	90 \pm 0	155 \pm 9
	Peracetic acid	4 \pm 0	4 \pm 0
	Sodium hypochlorite	19 \pm 4	42 \pm 0
Agona 3750	Hydrogen peroxide	150 \pm 0	>165 \pm 0
	Peracetic acid	5 \pm 1	5 \pm 1
	Sodium hypochlorite	21 \pm 0	>42 \pm 0
Havana 3759	Hydrogen peroxide	140 \pm 9	165 \pm 0
	Peracetic acid	5 \pm 1	5 \pm 1
	Sodium hypochlorite	21 \pm 0	>42 \pm 0

3.2.6 Optimisation of suspension test methodology for low concentrations of oxidising biocides and the effect of TSC

For the associated method please see **Chapter Two, section 2.8.1.1.**

TSC is the diluent recommended for inactivation kinetics by the British Standard (BS EN 1276, 2009). However, it was hypothesised that the presence of the tryptone could act as organic load reducing the efficacy of sodium hypochlorite. Figure 3.2 is a comparison graph of the log₁₀ reduction in total viable count of *Salmonella* Typhimurium SL1344 exposed to sodium hypochlorite either prepared in TSC or PBS.

Figure 3.2 shows a very clear difference in the log₁₀ reductions of total viable counts when biocides are prepared in PBS compared to TSC. A 4.3 log₁₀ reduction was seen at only 7 ppm when the biocide was prepared in PBS whereas to get the same result 40 ppm of sodium hypochlorite needs to be present in TSC.

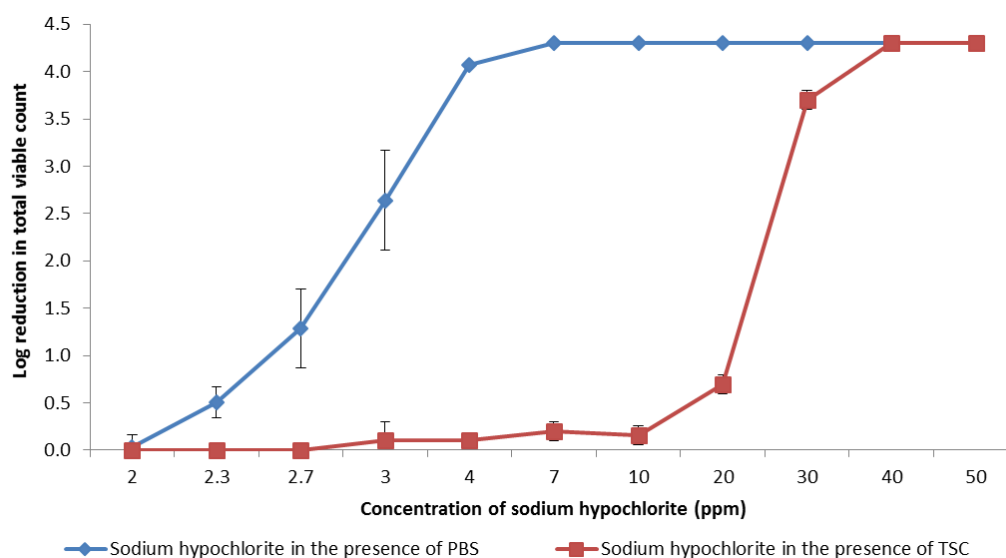


Figure 3.2: Log₁₀ reduction in the total viable count of *Salmonella enterica* serovar Typhimurium SL1344 after 30 mins exposure to a range of concentrations of sodium hypochlorite prepared in either TSC or PBS (n=3). A concentration of 0.01% tryptone was present in the final volume. Based on the limit of detection the maximum log₁₀ reduction which could be determined was a log₁₀ reduction of 4.3. The error bars denote the standard deviation from the mean.

To further investigate the interaction of the tryptone in TSC with sodium hypochlorite a range of concentrations of tryptone in sodium chloride were investigated, with the concentration of sodium chloride remaining constant, (for the associated method please see **Chapter Two, section 2.8.1.2.**). The concentration of tryptone usually found in the suspension test vessel is 100 ppm. Figure 3.3 shows the effect of tryptone concentrations between 1000 ppm and 10 ppm on bacterial survival to 10 ppm of sodium hypochlorite. The positive control containing biocide in sodium chloride showed a 3.8 log₁₀ reduction in 10 mins. The data shown in fig. 3.3 indicate that as the concentration of tryptone present in the TSC increases, the log₁₀ reductions decrease; with no log₁₀ reduction occurring with a concentration of tryptone above 500ppm (0.05%). At concentrations of 0.01% (100 ppm which is BS

EN normal conditions) of tryptone and lower the protective effects begin to disappear with 0.001% (10 ppm) tryptone having no protective effect at all and giving a 3.8 log₁₀ reduction after 10 mins like the sodium chloride control.

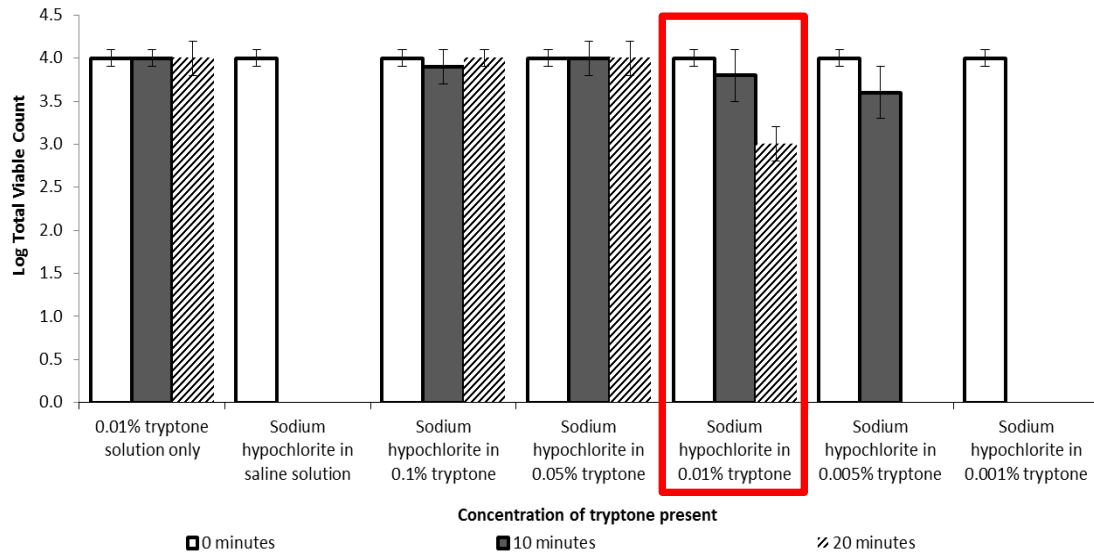


Figure 3.3: Log₁₀ total viable count of *Salmonella enterica* serovar Typhimurium SL1344 when exposed to 10 ppm of sodium hypochlorite prepared in TSC containing various concentrations of tryptone. Based on the limit of detection the maximum log₁₀ reduction which could be determined was a log₁₀ reduction of 3.8 (n=3). The error bars denote the standard deviation from the mean. The red box highlights the concentration of tryptone recommended by the BS EN 1276 standard.

3.2.7 Inactivation kinetics using planktonic *Salmonella* species

3.2.7.1 Hydrogen peroxide exposure

Preliminary suspension testing indicated that the most appropriate concentrations for further testing with hydrogen peroxide were between 800 ppm and 3600 ppm (see appendix 8.2). For the associated method please see **Chapter Two, section 2.8**.

The inactivation kinetics of *Salmonella* Typhimurium SL1344 showed that when exposed to hydrogen peroxide all concentrations under 2400 ppm gave a maximum of a 0.5 log₁₀ reduction after 20 mins (fig 3.4) was observed. Even the higher concentrations of 2800 ppm and 3200 ppm only gave just over a one log₁₀ reduction. After 60 mins there were no detectable survivors at concentrations of above 2400 ppm (fig 3.4a). Concentrations below 2400 ppm the kill reduced in relationship to the decreased concentration down to 1200ppm, with 1200 ppm giving a 0.8 log₁₀ reduction after 60 mins (fig 3.4a). However, the log₁₀ reduction in total viable count at concentrations between 1200ppm and 800ppm hydrogen peroxide exposure are not significantly different (P values of 0.3, 0.26 and 0.93 for 20, 40 and 60 mins respectively).

Salmonella Agona and Havana show similar inactivation kinetics when exposed to hydrogen peroxide (fig 3.4b and 3.4c). This shows the biocide to less than one log₁₀ reduction 20 mins of exposure with the most distinctive separation between the effects of the concentration occurring between 20 and 40 mins. Between 40 and 60 mins this trend continues with further kill occurring. Concentrations of 2800 ppm and higher resulted in a greater than 4 log₁₀ reduction after 60 mins, with concentrations below 1200 ppm having a less than 1 log₁₀ reduction.

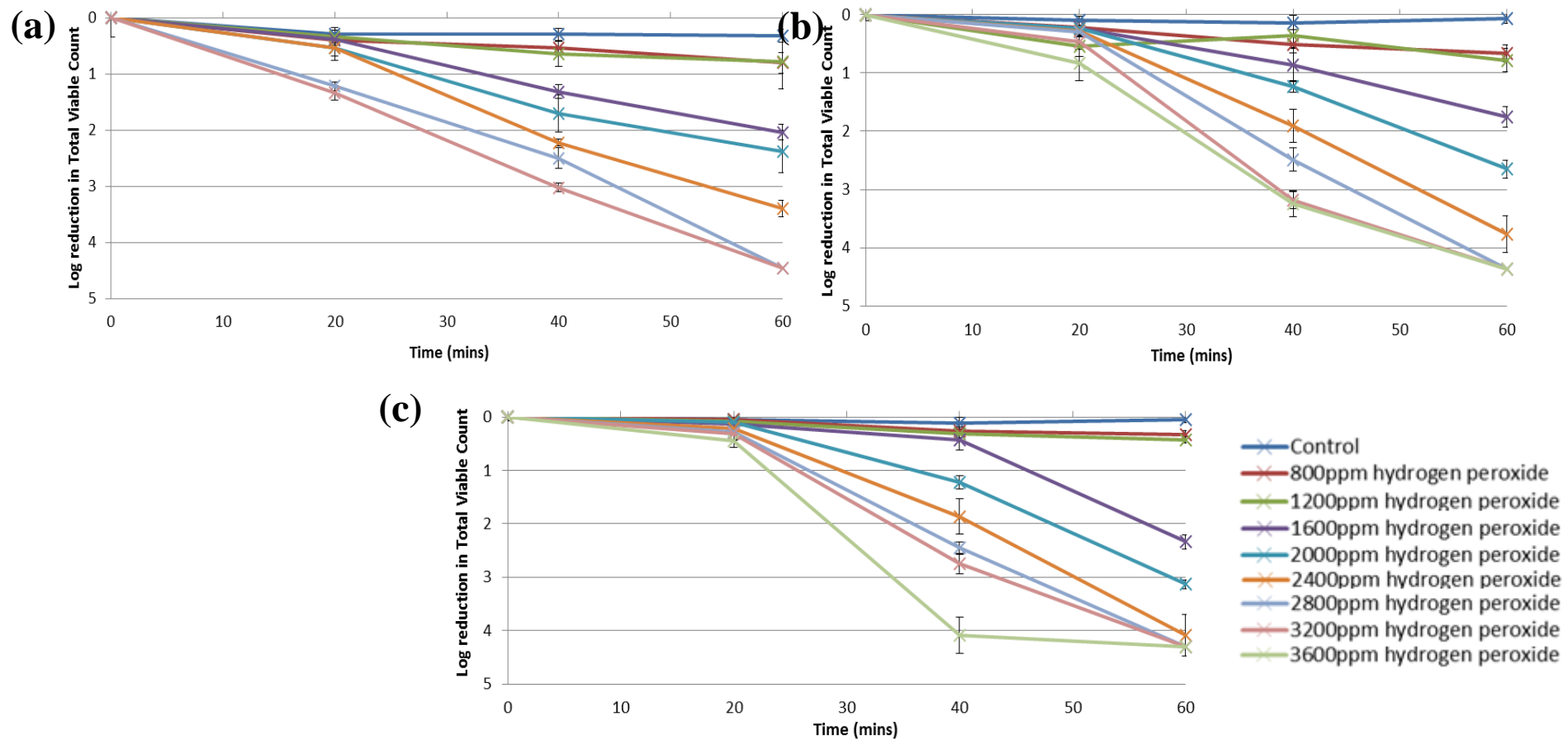


Figure 3.4: Log₁₀ reduction in the total viable count of *Salmonella enterica* serovar (a) Typhimurium SL1344 (b) Agona (c) Havana when exposed to hydrogen peroxide concentrations ranging between 800 ppm to 3600 ppm. Based on the limit of detection the maximum log₁₀ reduction which could be determined was a log₁₀ reduction of between 4.3 and 4.5 (n=3). The error bars denote the standard deviation from the mean.

3.2.7.2 Peracetic acid exposure

Preliminary suspension testing with a concentration range of between 250 ppm and 1 ppm (see appendix 8.2) indicated that the most appropriate concentrations to focus further efforts were between 0.3 ppm and 3 ppm. For the associated method please see **Chapter Two, section 2.8**.

Biocide testing with *Salmonella* Typhimurium SL1344 showed a concentration of 1 ppm of peracetic acid caused a 1.2 log reduction from the control after 60 mins. The concentration of 3 ppm gave a 4 log₁₀ reduction after 20 mins (see fig. 3.5a). As an intermediate concentration of 2 ppm gave a 4.8 log₁₀ reduction after 40 mins. One part per million of peracetic acid was the only concentration to show a lethal effect and yet still have survivors after 40 mins.

Salmonella Agona was exposed to a range of concentrations of peracetic acid between 1 ppm and 3 ppm (fig 3.5b). Concentrations of 3 ppm and above we found to give a greater than 5 log₁₀ reduction after 20 mins, 2 ppm of peracetic acid have the same log₁₀ reduction after 40 mins (a log₁₀ reduction up to the limit of detection). One ppm peracetic acid gave less than half a log₁₀ reduction after 40 mins exposure making it very similar to the unexposed control (fig 3.5b).

The inactivation kinetics of *Salmonella* Havana (fig 3.5c) is more sensitive to peracetic acid than either *Salmonella* Typhimurium SL1344 or Agona. This is shown by concentrations of 2ppm or above having a log₁₀ reduction to the limit of detection after 20 mins whereas Typhimurium SL1344 gives a 3.7 log₁₀ reduction and Agona gives 0.9 log₁₀ reduction at the same concentration and timepoint. When exposed to 1 ppm peracetic acid with approximately a 1 log₁₀ reduction after 20 mins exposure, with a greater than 4 log₁₀ reduction seen after 40 mins exposure. Concentrations of less than 1ppm show less of a log₁₀ reduction with 0.3ppm showing no significant

difference from the control for the first 40 mins and only 0.13 log₁₀ reduction greater than the control at 60 mins. The concentration of 0.7ppm shows no significant difference from the control at 20 mins (p=0.3) and then a 0.8 log₁₀ reduction at 40 mins.

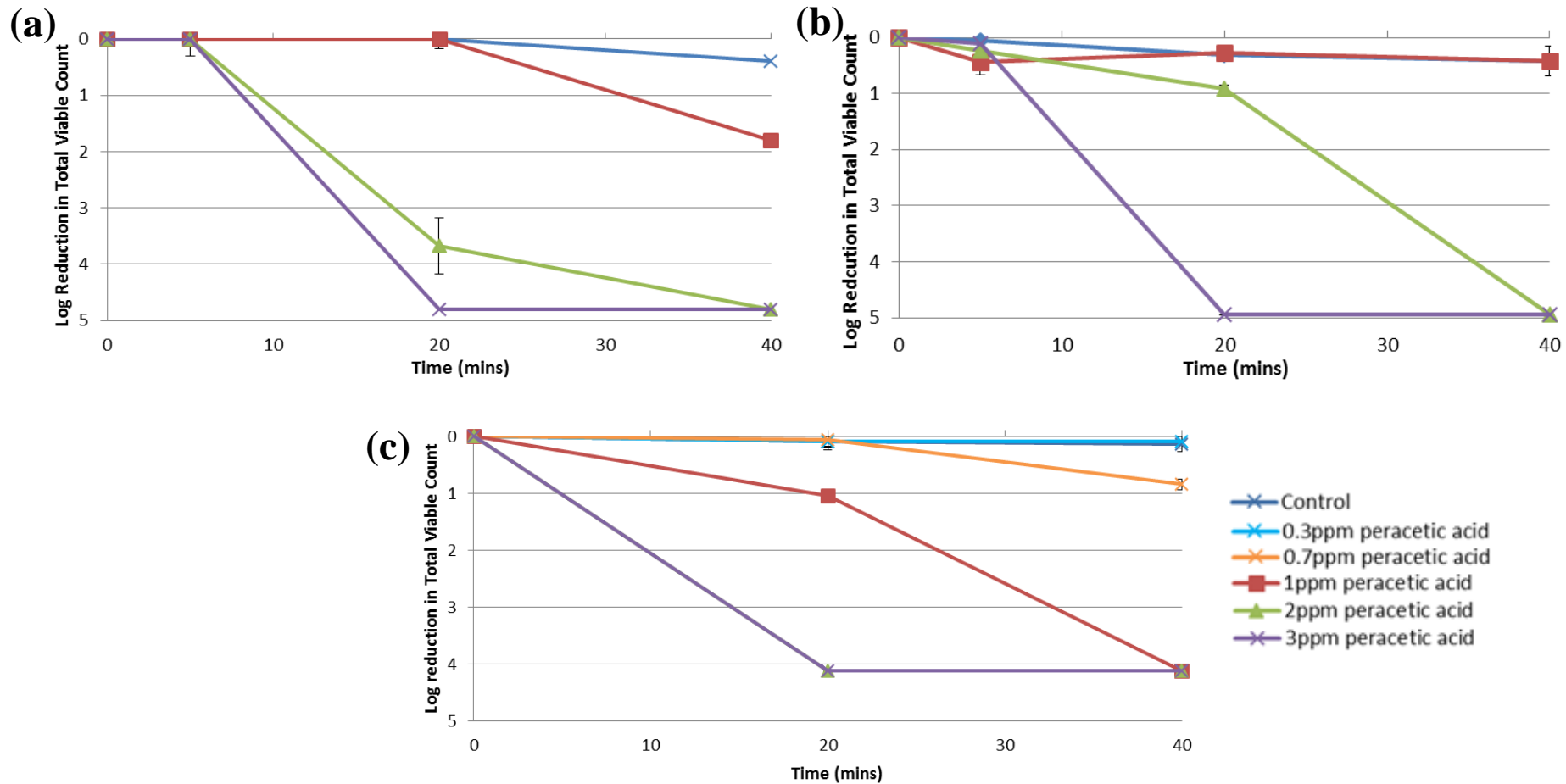


Figure 3.5: Log₁₀ reduction in the total viable count of *Salmonella enterica* serovar (a) Typhimurium SL1344 (b) Agona (c) Havana when exposed to 0.3 ppm to 3 ppm peracetic acid. Based on the limit of detection the maximum log₁₀ reduction which could be determined was a log₁₀ reduction of 4.8, 5 and 4 respectively (n=3). The error bars denote the standard deviation from the mean.

3.2.7.3 Sodium hypochlorite exposure

Preliminary suspension testing was conducted at a wide range of concentrations between 1000 ppm and 1 ppm indicated that the best concentrations to focus on for inactivation kinetics were between 3 ppm and 7 ppm of sodium hypochlorite (see appendix 8.2). For the associated method please see **Chapter Two, section 2.8**.

Salmonella Typhimurium SL1344 was exposed to between 2 ppm and 4 ppm of sodium hypochlorite (fig. 3.6a). Four parts per million of sodium hypochlorite gave a 4 log₁₀ reduction (the limit of detection) within 20 mins. A concentration of 2 ppm gives a maximum log₁₀ reduction of 0.2 at 60 mins which is similar to the decrease seen in the control over the course of the experiment and is therefore only fluctuation within what would be expected. Three parts per million 2.3 log₁₀ reduction after 20 mins increasing to 3.6 log₁₀ reduction after 40 mins with no detectable survivors after 60 mins.

Salmonella Agona (fig. 3.6b) was more tolerant than the wild type (fig. 3.6a) as 7 ppm sodium hypochlorite was the lowest concentration to give a 4 log₁₀ reduction with Agona in 20 mins whereas 4ppm gave the same effect with both Typhimurium SL1344 and Havana. *Salmonella* Agona (fig. 3.6b) also showed survival after 60 mins exposure to both 5 ppm and 4 ppm.

Salmonella Havana shows some similar trend at 2 ppm and 4 ppm to Typhimurium SL1344, with 2 ppm being almost indistinguishable from the negative control (0.1 log₁₀ reduction with a standard deviation of 0.1) and 4 ppm gives no detectable survivors after 20 mins (fig. 3.6). When exposed to 3 ppm of sodium hypochlorite *Salmonella* Havana has more survivors as Typhimurium SL1344 shows no detectable growth (a 4 log₁₀ reduction) whereas Havana only gives a 2.5 log₁₀ reduction (fig. 3.6)..

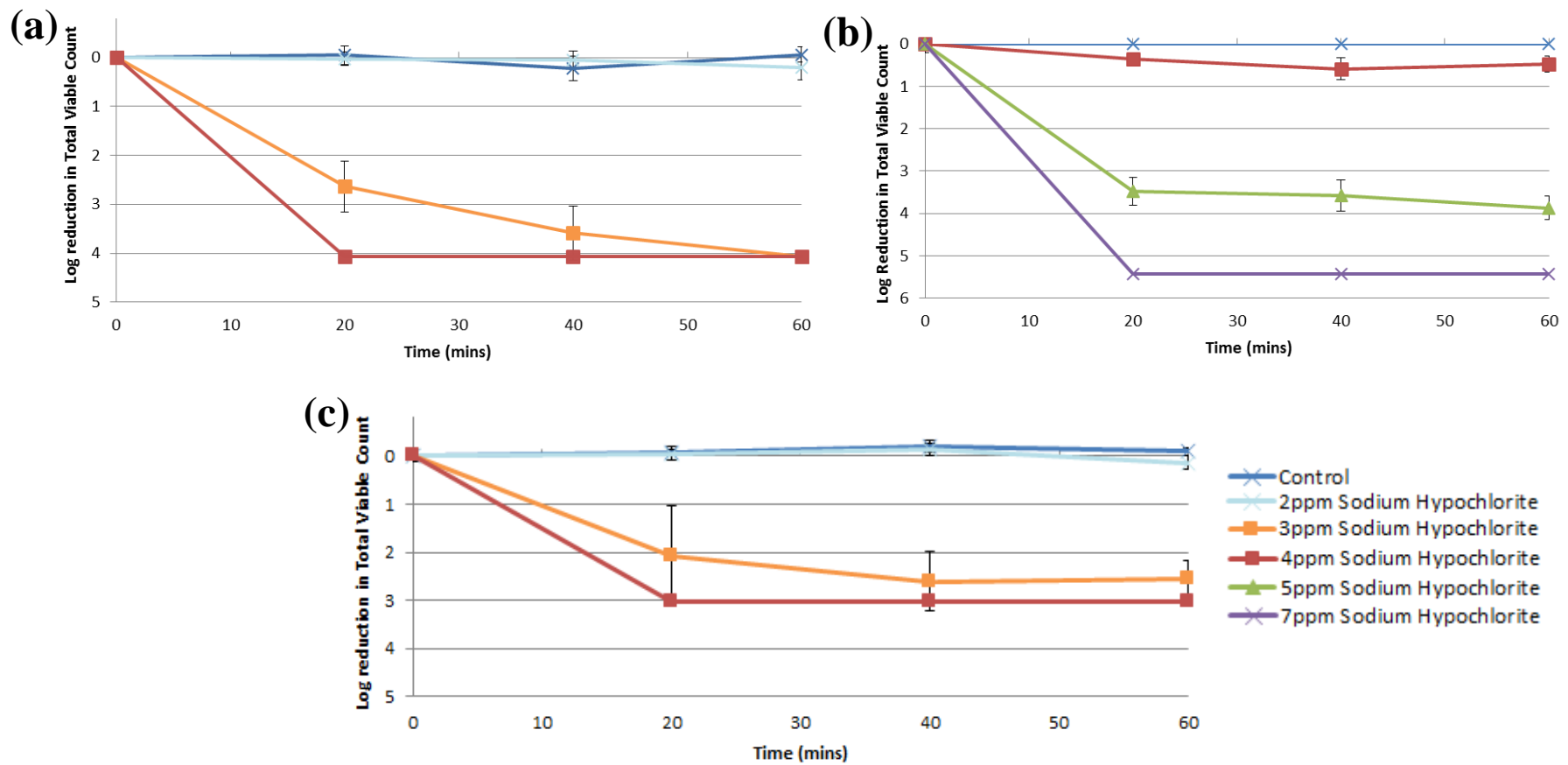


Figure 3.6: Log₁₀ reduction in the total viable count of *Salmonella enterica* serovar (a) Typhimurium SL1344 (b) Agona (c) Havana when exposed to 2 to 7 ppm of sodium hypochlorite prepared in PBS. Based on the limit of detection the maximum log₁₀ reduction which could be determined was a log₁₀ reduction of 4, 5.4 and 3 respectively (n=3). The error bars denote the standard deviation from the mean.

3.2.8 Concentration exponents

Concentration exponents were calculated using the inactivation kinetics graphs shown above, with the time taken to give a 2 log₁₀ reduction being recorded at each concentration. The log₁₀ of the time and concentration were then used to plot a graph. The line of best fit was then added and the slope of the line (η) gave the concentration exponent.

Table 3.5: Concentration exponents calculated from inactivation kinetics data

	<i>Salmonella enterica</i> serovar Typhimurium SL1344	<i>Salmonella enterica</i> serovar Agona 3750	<i>Salmonella enterica</i> serovar Havana 3759
Hydrogen peroxide	1.1	0.8	0.8
Peracetic acid	1.2	1.5	1.5
Sodium hypochlorite	2.6	3.7	1.7

3.2.9 Biocide pulse experiment

The inactivation kinetics were used to determine concentrations of sodium hypochlorite that would give an initial \log_{10} reduction which would then plateau. An additional pulse of biocide was then added to investigate if the surviving population of bacteria were any more or less susceptible to biocide (for the associated method please see **Chapter Two, section 2.8.2.**). This study was only conducted with *Salmonella* Agona because when exposed to sodium hypochlorite this strain reached a plateau after the first 20 minutes of exposure. The other strains were either unaffected or there was a continued decline in the total viable count depending on concentration. *Salmonella* Agona however, appeared to be unaffected after the first 20 minutes.

The concentrations selected were 4 ppm and 5 ppm of sodium hypochlorite which gave a 2.5 and 3.7 \log_{10} reduction respectively within the first 20 mins. The \log_{10} reduction then levelled off between 20 and 40 mins before the second pulse of biocide was added. When the second pulse of biocide was added there were no recoverable survivors at this limit of detection which was a \log_{10} reduction of 5 for both concentrations (fig. 3.7).

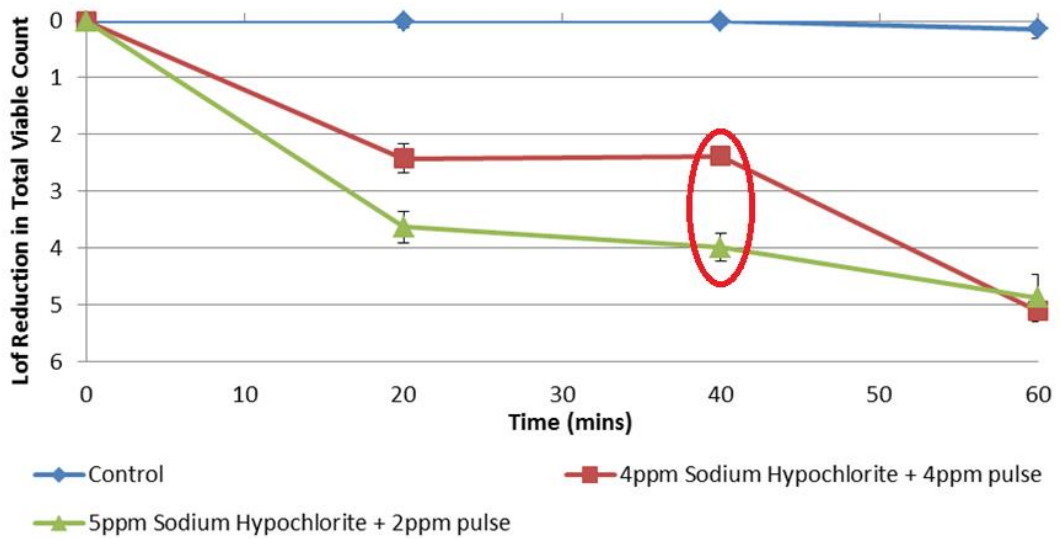


Figure 3.7: Log₁₀ reduction in the total viable count of *Salmonella enterica* serovar Agona when exposed to sodium hypochlorite initially at time 0 and then again at 40 min (circled in red). Based on the limit of detection the maximum log₁₀ reduction which could be determined was a log₁₀ reduction of 5 (n=3). The error bars denote the standard deviation from the mean.

3.3 Discussion

Antibiotic susceptibility testing took two forms: the E-strip testing which used graded concentrations to give the MIC of the antibiotic and the antibiotic disc method which gave the clinical breakpoints. The antibiotics were selected as they were clinically relevant; ciprofloxacin and cefotaxime are recommended treatments in severe cases of *Salmonellosis* or in cases involving vulnerable patients (British National Formulary, 2010) Ampicillin and imipenem were selected as they are broad spectrum antibiotics (British National Formulary, 2010) and so may be used to treat an infection, where the causative bacterium has not been identified.

E-strip test data ranking concurred with the results of the antibiotic disc testing for the antibiotics ampicillin and cefotaxime. The results between the two methods differed slightly for both ciprofloxacin and imipenem. This very small difference between the strains may not be due to any actual difference but that the BSAC disc method was not sensitive enough to detect such a subtle change in susceptibility. This limitation in the methodology is because the BSAC disc method is designed to detect changes in clinical susceptibility and therefore the results it gives are qualitative (e.g. susceptible, intermediate or resistant (Jorgensen & Ferraro, 2009)). The MIC E-strip gives a quantitative result and the results have to be correlated with MICs from broth or agar dilution methods (Jorgensen & Ferraro, 2009). The disc diffusion method is much cheaper than the E-test strip method but as it is designed to only give qualitative results it is less likely than the MIC data to give detailed information about an organisms susceptibility.

All the *Salmonella* strains in this study were susceptible to the clinically relevant antibiotics tested. However, antibiotic resistance is becoming more widespread 2.8%

of non-typhoidal *Salmonella* strains tested show resistance to ceftriaxone (which is rated of critical importance in human medicine (Center for Disease Control, 2010). Studies are showing an increased presence of multidrug resistant *Salmonella* species worldwide (Hur *et al.*, 2012). This makes the monitoring of any change in antibiotic susceptibility after exposure to biocides important as any evidence of cross-resistance is of clinical importance.

The nature of minimum inhibitory concentration (MIC) testing requires the presence of a growth media and therefore organic matter. However, the presence of organic matter affects the activity of biocides and has been well documented in the literature (Ayyildiz, *et al.*, 2009; Casella & Schmidt-Lorenz, 1989; Güzel-Seydim *et al.*, 2004; Otter *et al.*, 2012). This is important when designing reproducible assays such as MICs, because if the presence of interfering material affects the efficacy of the biocide, but the full extent of how this occurs and why are not understood then any data produced may be spurious. If this data goes on to be used as a baseline for further experiments it means that this data will also be flawed. In the environment this is important as organic matter is present in a variety of settings where biocides are in use such as water (Ayyildiz, *et al.*, 2009), the food industry (Güzel-Seydim, *et al.*, 2004) and clinical environments (Casella & Schmidt-Lorenz, 1989). The studies by Otter, *et al.* (2012) and Ayyildiz, *et al.* (2009) are relevant as they evidence that hydrogen peroxide and chlorine based compounds can be less effective in the presence of organics. For these reasons, MICs have limited value when determining biocide efficacy and so a recommendation of this study is to suggest MICs are not appropriate tests for biocides.

There was a difference between the MIC values of sodium hypochlorite and peracetic acid in the two minimal media. It is was not known initially if the

difference between the two minimal media is due to the MOPS based media being more complex than the M9 media or that the M9 media does not support *Salmonella* growth as well as MOPS based media. This would mean that there would be a lower bacterial count to begin with so the rate of inactivation would not need to be as high. Further experimentation found that it was not due to varying growth rates (figures 3.1) and therefore was biocide interaction with the growth media. However, even using a simple minimal growth media like M9 the MIC values for sodium hypochlorite (and to a lesser extent peracetic acid) are elevated above the concentrations that would give a \log_{10} reduction to the limit of detection in the suspension testing seen in Chapter Three, section 3.2.7. Although suspension testing is a very different methodology from MICs the considerable difference (from 7 ppm sodium hypochlorite giving a 5 \log_{10} reduction in suspension testing but with an MIC of 19 ppm to 21 ppm) in bacterial survival is unexpected. It would be expected for the initial bacterial inoculum to be killed before the bacteria have time to reproduce. It is particularly unusual considering metabolically active cells such as those in MICs are often more susceptible to the activity of biocides (Kim, *et al.*, 2009) than those that are not active (such as those in the inactivation kinetics). For this reason, it is hypothesised that the difference in the bacterial susceptibility to oxidising biocides may be due to the presence of interfering materials in the broth and that given the nature of the test this can only be minimised rather than completely eliminated.

The TSC data shown in Chapter Three, section 3.2.6 is another example of the potential for organic material to interfere with the activity of biocides. There is shown to be a considerable difference in biocidal activity when sodium hypochlorite was dissolved in TSC rather than PBS. When sodium hypochlorite was dissolved in

TSC a minimum concentration of 40 ppm was required to achieve a 4.3 log₁₀ reduction, whereas the same log₁₀ reduction was seen at 7 ppm PBS was the diluent. It was postulated that the inhibitory effect of TSC on low concentrations of sodium hypochlorite was caused by the presence of tryptone as this hydrolysates contains peptides which may interact with sodium hypochlorite. Sodium hypochlorite is highly reactive and interacts with all biological molecules and so this may deplete the activity of the biocide (Waters & Hung, 2014). To test this hypothesis, TSC with a range of tryptone concentrations (the concentration of sodium chloride remaining constant) were tested for biocidal activity in the presence of 10 ppm sodium hypochlorite. The data from this (figure 3.3) shows that presence of tryptone in TSC is responsible for the depletion of sodium hypochlorite and that this effect seemed to be dependent on the concentration of tryptone. These findings led to all inactivation kinetics occurring in the presence of PBS as opposed to TSC to prevent interference from organic material affecting the results.

The inactivation kinetics in Chapter Three, section 3.2.7 was produced as baseline susceptibility data for planktonic bacterial cells. The inactivation graphs (figs. 3.4-3.6) show that the responses of the *Salmonella* strains to hydrogen peroxide and peracetic acid are similar. However, there are more recorded survivors of *Salmonella* Agona after exposure to sodium hypochlorite compared to the other two strains. This seems to indicate *Salmonella* serovar Agona is less susceptible to sodium hypochlorite than the other serovars. This data linked with the unusual concentration exponent data may indicate that *Salmonella* serovar Agona interacts differently with sodium hypochlorite than the other *Salmonella* isolates. This requires further investigation into the interaction of *Salmonella* Agona with sodium hypochlorite and perhaps to see if any unique mechanisms may be involved.

The inactivation kinetics data also granted the opportunity to calculate the concentration exponents of sodium hypochlorite, hydrogen peroxide and peracetic acid (table 3.5). Hydrogen peroxide and peracetic acid both had η values lower than 2 which is expected of oxidising agents (Maillard, 2013a; Russell, 2003b). This is because oxidising agents have a chemical mechanism of action. Unusually, sodium hypochlorite has a concentration exponent of between 1.7 and 4. This type of concentration exponent is associated with biocides that have a dual action; with chemical and physical components. The variation between strains is also interesting as concentration exponents often remain consistent between bacterial species (Hugo & Denyer, 1987). Although it is widely accepted that biocides have a range of targets within a cell, this difference in concentration exponents between serovars may indicate different targets within each serovar. *Salmonella* serovar *Agona* has a higher sodium hypochlorite concentration exponent and consequently there may be a quenching reaction with non-critical components of the cell. This may partially neutralise the effect over the concentration range that was used. To investigate this the concentration exponent could be measured over a range of concentrations. Usually the value would remain unchanged if the interaction with the target was not concentration dependent, but this might not be the case if another factor is affecting the effective dose. Alternatively, there could be dual or multiple interactions that are needed to achieve a killing effect and that these are differentially affected by concentration.

The biocide pulse experiment (Chapter Three, section 3.2.9) followed the British Standard (BS EN 1276, 2009) for the beginning of the experiment, with an additional 1 mL of biocide added 40 mins after the commencement of the experiment. This was to investigate the apparent plateau in the \log_{10} reduction curve,

to see if there was any tolerance within the survivors that formed the plateau in the graphs. The results of these experiments indicate that there is no tolerance intrinsic to the survivors that are shown in the plateau in the \log_{10} reduction graphs. It also indicates that the reason for the plateau is not due to depletion of the biocide in the presence of organic material (bacterial cells) as when a sub-lethal concentration is added (2ppm) it gives a killing effect, indicating that is additional to the already present biocide (at 5ppm concentration). Further investigation of this plateau phenomenon is required.

This chapter forms the basis for all future experiments as methods were optimised for working with oxidising biocides. Baseline susceptibilities and conditions were determined and then used in subsequent chapters to examine responses to non-lethal biocide stress. It also forms a useful comparison for the biofilm work in later chapters.

Chapter Four: Phenotypic
responses to biocide
challenge in planktonic
Salmonella

4.1 Introduction

In addition to the culture based methods discussed in Chapter Three, there are a number of rapid methods that were used to elucidate the effects of oxidising biocides on bacterial cells. Rapid methods have an advantage over traditional culture based methods as they gave results within mins or hours and some are high throughput. These methods also gave a greater insight as to what was happening directly after exposure to biocide and allowed the investigation of bacterial mechanisms of resistance.

Flow cytometry is a fluorescence-based method which allows for rapid analysis of whole populations of cells based on the fluorescence of single cells within that population (Brehm-Stecher & Johnson, 2004). This method can detect multiple characteristics based on the use of different molecular probes which are detected at different wavelengths of light and data can be collected simultaneously from multiple probes (Brehm-Stecher & Johnson, 2004). Although flow cytometry was primarily designed for use with mammalian cells, alterations can be made so that particles of a smaller size such as bacteria can be detected (Sincock & Robinson, 2001). Flow cytometry has been increasingly used in a wide range of applications in microbiology: determining efficacy in food pasteurisation (Tamburini *et al.*, 2013), monitoring drinking water quality (Vital *et al.*, 2012) and in medical diagnostics (Qin *et al.*, 2008).

The primary advantage of flow cytometry over traditional culture based approaches is the speed at which results are obtained. Normal culture based methodologies take hours or even days to give information about the survival of a bacterial population; however, flow cytometry can give the same information in minutes (Díaz *et al.*,

2010). Also, traditional culture based methods depend on the bacterial cells present being culturable, however after treatment with a biocide cells present may be viable but non-culturable. This would mean that they would be undetectable using traditional methods, however they may recover and go on to proliferate becoming a risk for infection or contamination. Flow cytometry does not require cells to be culturable and can use alternative measures of cellular viability (Díaz, *et al.*, 2010). Flow cytometry also has the advantage that it can measure a number of dynamic processes in the cell simultaneously.

There are a wide range of fluorescent markers available which can be useful reporters of bacterial viability or physiology depending on the application and the bacterial target being investigated. The fluorescence from two dyes propidium iodide (PI; a dye excluded from live cells which stains the DNA of bacteria that have lost their membrane integrity) and Bis (1,3-dibutylbarbituric acid) trimethine oxanol (BOX; a dye which stains bacteria that can no longer maintain a normal membrane potential) can be used to determine a biocide's effect on a population as seen in Whitehead, *et al.* (2011). This gives information as to the status of individual bacterial cells which combined together give a picture of a bacterial population's response to a biocide challenge, without requiring a recovery/growth step or the bacteria to be culturable.

Important mechanisms of bacterial resistance to antimicrobials include the reduction of access to biocide target sites by reducing cellular permeability (Ortega Morente, *et al.*, 2013) or increasing the number of efflux pumps (Karatzas, *et al.*, 2007). Efflux pumps are membrane bound transport proteins that export a wide range of substances (for further information see Chapter One section 1.4.3.2). Bacterial efflux is a well-documented resistance mechanism to antimicrobial exposure (Fernández Fuentes *et*

al., 2014; Lavilla Lerma *et al.*, 2014; Vikram *et al.*, 2015a). One method that can be used to investigate the effect of cellular permeability and efflux is described by Coldham, *et al.* (2010). This method uses Hoescht 33342 (bis benzimide) which is a membrane permeable fluorophore, which fluoresces when bound to the minor groove of DNA to quantify cellular permeability and efflux. Hoescht 33342 is a substrate for the major efflux systems of Gram negative bacteria and as such, accumulation of Hoescht 33342 can be used as an indicator of efflux activity. Hoescht is also a relatively non-toxic alternative to ethidium bromide and also avoids the self-quenching (Babayan & Nikaido, 2004) and lower quantum yield problems associated with the latter (Coldham, *et al.*, 2010). The main limitation of using Hoescht or related dyes as efflux indicators is that these experiments do not give detailed information as to what is causing any increase or decrease of fluorescence within the cell. This could be due to both cellular permeability or efflux and changes in expression of a range of genes can be involved to give the altered phenotype.

Green fluorescent protein (GFP) is a 238 amino acid long protein that when excited with blue light is fluorescent and emits green light which is stable with minimal photobleaching (Chalfie *et al.*, 1994). GFP was first isolated in 1962 (Shimomura *et al.*, 1962) and sequenced in 1992 (Prasher *et al.*, 1992). GFP can be used as a fluorescent marker of gene expression when fused to a promoter region of a gene of interest and was first used as a reporter gene in 1994 (Chalfie, *et al.*, 1994). Lawler, *et al.* (2013) fused the GFP as a reporter on plasmid pMW82 created by Bumann and Valdivia (2007) to the promoter region of *ramA* to create pMW82-*ramA* (in *S. Typhimurium* SL1344 strain L1232) which can be used to measure expression changes over time in 96 well plate format. Similarly, the pMW82 GFP reporter plasmid has also been fused to *acrAB* and *soxS* promoters which created L1361 and

L1357 strains respectively. These GFP reporter strains were then used in a 96 well plate assay in the Fluostar to investigate the induction of these genes in the presence of a range of biocides.

The GFP reporters for *acrAB*, *ramA* and *soxS* were selected as these genes have a role in the defence of *Salmonella* from antimicrobials: AcrAB is an important part of a key efflux pump involved in antimicrobial resistance (Blair *et al.*, 2015; Randall, *et al.*, 2007; Webber *et al.*, 2015); RamA is involved in the regulation of the production of AcrAB (Bailey, *et al.*, 2010; Bailey, *et al.*, 2008; Baucheron *et al.*, 2014) and SoxS is a global transcriptional regulator which has been linked to the response to oxidative stress (Gil *et al.*, 2009; Spector & Kenyon, 2012) and also has a role in the regulation role of AcrAB-TolC (Lawler, *et al.*, 2013). The key advantage of this method is it can show gene expression in real time, it has high specificity and as it is used in a 96 well plate assay has a very high throughput. The major disadvantage of this method is that it only investigates selected genes of interest and so there is not the breadth of information of the genetic response that would be shown by other methods. GFP reporters are quick, cheap and easy to use which can give very useful information for specific target genes.

It has been shown in the literature (Juzwa *et al.*, 2015; Whitehead, *et al.*, 2011) that population responses to biocides analysed from flow cytometry data can give far more in depth information than culture based studies alone. For this reason, insight into *Salmonella* response to oxidising biocide challenge may be further elucidated by the use of flow cytometry in conjunction with the culture based methods in Chapter Three. In addition to the investigation of population responses independent of culturability this chapter aims to investigate one of the known mechanisms of biocide resistance using a rapid method. Efflux and cellular permeability changes

have been associated with biocide resistance in the literature (Fàbrega *et al.*, 2016; Karatzas, *et al.*, 2008b; Randall, *et al.*, 2007; Winder, *et al.*, 2000). This has yet to be explored in *Salmonella* and the Hoescht assay and GFP reporters provide excellent tools for the investigation of responses associated with efflux.

4.1.1 Aims

- To investigate bacterial responses to biocides that are not dependant on culturable viability.
- To investigate how oxidising biocides can change efflux activity in *Salmonella*.
- To gain insight into the effects of the oxidising biocides being studied on regulatory responses.

4.2 Results

4.2.1 Using flow cytometry to analyse the planktonic bacterial response to oxidising biocides

For the associated methods please see **Chapter Two, section 2.9**. N.B. due to the results on biofilm formation in Chapter Five, section 5.2.1 experiments with *Salmonella enterica* serovar Havana were discontinued. The reason for this decision was it was decided to be more interesting to pursue a strong biofilm former and a weak biofilm former for future experiments.

4.2.1.1 Flow cytometry controls

Controls of ethanol killed bacterial cells and healthy bacterial cells were run through the flow cytometer so that the quadrants could be gated correctly. Q3 contained 'healthy' *Salmonella* cells that had taken up neither dye as the membrane was intact and had membrane potential (figure 4.1a and 4.1b). The graphs from figure 4.1c and 4.1d showed *Salmonella* cells that had been treated with ethanol to give a killed control. The majority of this population should fall within Q2 as the cells should take up both the PI and BOX dyes, however some cells fall into the other quadrants. Q1 contained cells that had taken up BOX showing that they have no membrane potential and Q4 contained cells that had only taken up PI which means they have no membrane integrity. The combination of the untreated and killed controls allowed gating for subsequent experiments.

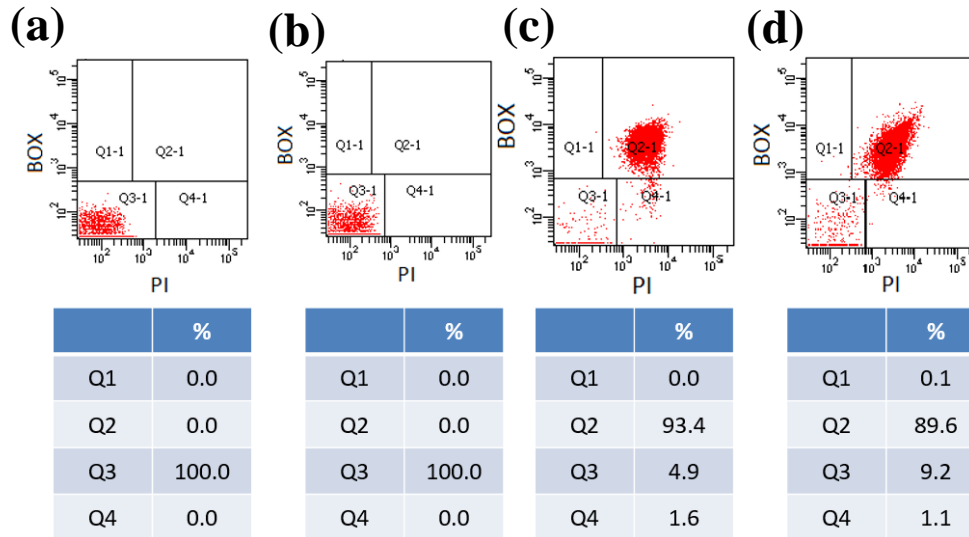


Figure 4.1: The data for the controls used for flow cytometry with PI and BOX added; negative controls containing non-treated *Salmonella enterica* serovar (a) Typhimurium SL1344 (b) Agona cells; *Salmonella enterica* serovar (c) Typhimurium SL1344 (d) Agona cells treated with ethanol. The % symbol denotes the mean percentage of cells found in each quadrant (n=3).

4.2.1.2 Hydrogen peroxide exposure

The FACSdiva graphs produced by *Salmonella* cells exposed to varying concentrations of hydrogen peroxide, at a range of time points showed no uptake of PI or BOX with almost all the events falling in the Q3 ‘healthy’ quadrant (as shown in figure 4.2).

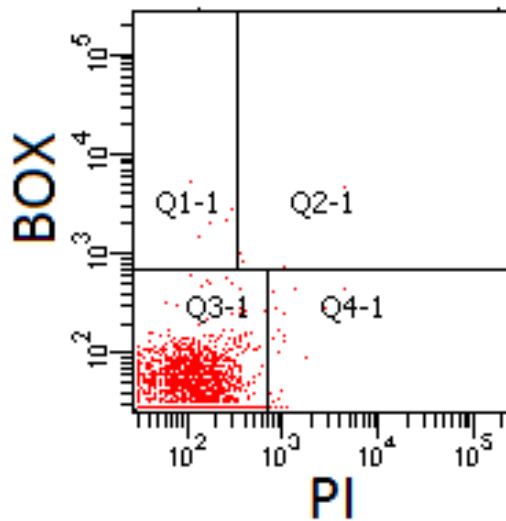


Figure 4.2: A representative graph of the spread of *Salmonella enterica* cells exposed to hydrogen peroxide at all time-points and all concentrations tested (n=3 for each concentration and timepoint tested; 3 concentrations and timepoints were tested).

As the FACSdiva graphs gave little distinction between the samples the data was tabulated. Figure 4.2 shows the average percentages of the events that were recorded in the Q3 quadrant along with the standard deviations. As shown in table 4.1 the averages ranged from 99.5% to 99.8% with a standard deviation of 0.1% or 0.2%. This low standard deviation and consistent results of over 99% show that the majority of the population have intact membranes and maintained membrane potential even after 60 mins exposure of 3000ppm hydrogen peroxide. The culture data (Chapter Three, section 3.2.7.1, figure 3.4) shows approximately a 4.5 log reduction for both strains whereas no membrane damage is detected in either strains from the flow cytometry experiments using PI and BOX dyes.

Table 4.1: The mean percentage viability of *Salmonella enterica* (a) Typhimurium SL1344 and (b) Agona cells (plus or minus standard deviation) that were in Q3 (the “healthy” quadrant) which indicated no damage to the membrane integrity and no loss of membrane potential (as neither BOX nor PI were taken up by the cells) (n=3).

(a)

	1000ppm	2000ppm	3000ppm
20 min	99.7 ± 0.1	99.8 ± 0.1	99.7 ± 0.1
40 min	99.6 ± 0.1	99.8 ± 0.1	99.7 ± 0.1
60 min	99.8 ± 0.1	99.7 ± 0.1	99.8 ± 0.1

(b)

	1000ppm	2000ppm	3000ppm
20 min	99.6 ± 0.1	99.6 ± 0.2	99.7 ± 0.1
40 min	99.6 ± 0.1	99.6 ± 0.2	99.6 ± 0.1
60 min	99.7 ± 0.0	99.6 ± 0.1	99.5 ± 0.1

4.2.1.3 Peracetic acid exposure

Exposure of *Salmonella* strains to peracetic acid at two different concentrations (2 ppm and 3 ppm) at two time points (5 and 20 mins) are shown in figure 4.3 below. These concentrations and time points were selected based on data from culture experiments (see Chapter Three, section 3.2.7.2, figure 3.5).

Salmonella Typhimurium SL1344 exposed to peracetic acid have a very low uptake of the PI dye at all concentrations and exposure times (fig. 4.3a). The uptake of BOX however increases in both concentrations over the time course, indicating that

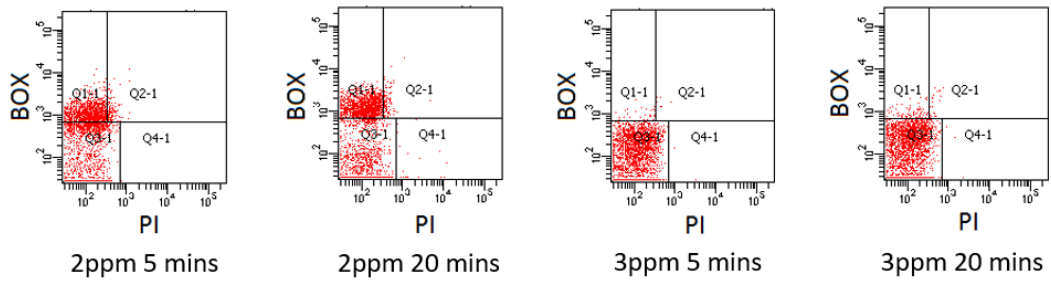
membrane integrity is being maintained but over time membrane potential is being lost in increasingly more cells (an increase of 9.8% at 2 ppm and 2% at 3 ppm).

Similarly figure 4.3b shows *Salmonella* Agona exposed to peracetic acid which also shows low uptake of the PI dye at all concentrations and exposure times. The uptake of BOX however increases at the lower concentration of 2 ppm over the time course, going from 12.6% of the population with lost membrane potential to 15.3%. There is little difference (0.1%) between the uptake of BOX between 5 and 20 mins when exposed to 3 ppm peracetic acid.

In both strains at the higher concentration of peracetic acid (3 ppm) there is little increased uptake of BOX and no increased uptake of PI. However, in the culture data 3 ppm peracetic acid show a 5 log₁₀ reduction but there is no evidence of kill in the flow cytometry data using these specific membrane dyes.

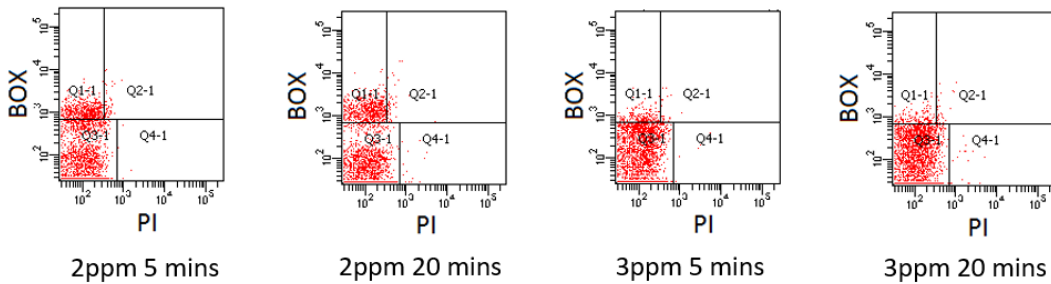
Currently there is no definitive explanation for the differences between the results obtained at 2ppm and 3ppm which show cells to have more membrane damage at a lower concentration which is consistent in both strains of *Salmonella*. However, possible explanations are explored further in Chapter Four, section 4.3.

(a)



	% ± SD		% ± SD		% ± SD		% ± SD
Q1	34.4 ± 3.9	Q1	44.2 ± 7.4	Q1	3.0 ± 2.9	Q1	5.0 ± 3.5
Q2	1.6 ± 0.3	Q2	2.1 ± 0.5	Q2	0.3 ± 0.2	Q2	0.4 ± 0.1
Q3	63.9 ± 4.1	Q3	53.5 ± 7.9	Q3	96.6 ± 3.0	Q3	94.5 ± 3.6
Q4	0.0 ± 0.1	Q4	0.2 ± 0.1	Q4	0.0 ± 0.1	Q4	0.1 ± 1.7

(b)



	% ± SD		% ± SD		% ± SD		% ± SD
Q1	12.6 ± 1.4	Q1	15.3 ± 1.1	Q1	2.3 ± 1.6	Q1	2.4 ± 0.8
Q2	0.7 ± 0.1	Q2	0.7 ± 0.1	Q2	0.2 ± 0.1	Q2	0.3 ± 0.0
Q3	86.7 ± 1.5	Q3	83.9 ± 1.1	Q3	97.3 ± 1.7	Q3	97.2 ± 0.9
Q4	0.1 ± 0.0	Q4	0.2 ± 0.1	Q4	0.1 ± 0.0	Q4	0.2 ± 0.1

Figure 4.3: Flow cytometry data for *Salmonella enterica* (a) Typhimurium SL1344 and (b) Agona exposed to 2 ppm and 3 ppm peracetic acid at 5 and 20 mins (n=3; the figures are of one representative repeat, the percentages are averages of all three repeats). The % symbol denotes the mean percentage of cells found in each quadrant plus or minus the standard deviation.

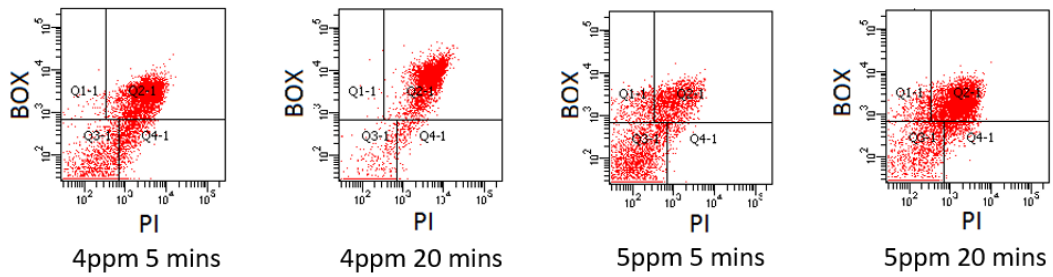
4.2.1.4 Sodium hypochlorite exposure

Exposure of *Salmonella* strains to sodium hypochlorite at two different concentrations (4 ppm and 5 ppm) at two time points (5 and 20 mins) are shown in figure 4.4 on the following page. These concentrations and time-points were selected based on data from culture experiments (Chapter Three, section 3.2.7.3, figure 3.6).

Figure 4.4a shows *Salmonella* Typhimurium SL1344 exposed to sodium hypochlorite. At 4 ppm sodium hypochlorite 23.8% of cells were in Q2 at 5 mins; which goes up to 50% at 20 mins. Q2 contains cells that have taken up both PI and BOX dyes which shows that they have lost membrane integrity and membrane potential and so were 'killed' as far as the dyes showed. The number of cells in the 'healthy' quadrant Q3 decrease from 65.1% to 33.0%. At 5 ppm a similar pattern is observed with an increase in the proportion in Q2 over the time course (38.3% to 86.2%) and a decrease in the proportion of cells in Q3 (34.5% to 9.5%). As would be expected the higher concentration gives a higher proportion of 'killed' cells (in Q2) after 20 mins (50% at 4 ppm and 86.2% at 5 ppm).

Salmonella Agona exposed to sodium hypochlorite and then stained with PI and BOX are shown in figure 4.4b. As seen in the data for *Salmonella* Typhimurium SL1344 the population shifts from Q3 to Q2 over the time course (an increase in the percentage 'killed'); at 4 ppm there are 24.0% cells in Q2 and 66.1% in Q3 at 5 mins which shows an increase to 31.0% cells in Q2 and a corresponding decrease to 41.3% in Q3 at 20 min. At 5 ppm the pattern is similar but with a higher overall percentage of 'killed' cells with more than the half of the population 'killed' at 20 mins; 22.2% cells in Q2 and 54.1% in Q3 at 5 mins which changes to 56.2% cells in Q2 and 31.2% in Q3 after 20 mins.

(a)



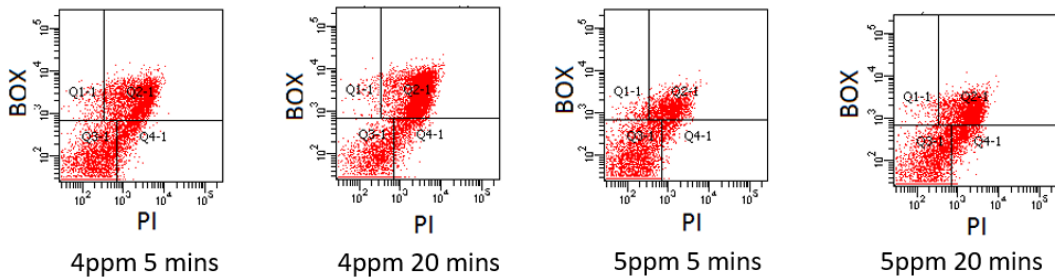
	% ± SD
Q1	9.1 ± 1.5
Q2	23.8 ± 4.0
Q3	65.1 ± 5.5
Q4	2.0 ± 1.4

	% ± SD
Q1	2.9 ± 2.4
Q2	50.0 ± 5.2
Q3	33.0 ± 4.7
Q4	14.1 ± 6.2

	% ± SD
Q1	1.3 ± 0.6
Q2	38.3 ± 34.2
Q3	34.5 ± 11.0
Q4	25.9 ± 31.1

	% ± SD
Q1	0.4 ± 0.1
Q2	86.2 ± 1.6
Q3	9.5 ± 2.3
Q4	3.9 ± 1.9

(b)



	% ± SD
Q1	2.0 ± 0.4
Q2	24.0 ± 13.5
Q3	66.1 ± 15.4
Q4	8.0 ± 2.6

	% ± SD
Q1	1.5 ± 0.6
Q2	31.0 ± 13.7
Q3	41.3 ± 12.5
Q4	26.1 ± 11.9

	% ± SD
Q1	1.8 ± 0.6
Q2	22.2 ± 15.7
Q3	54.9 ± 3.1
Q4	21.1 ± 15.4

	% ± SD
Q1	1.7 ± 0.3
Q2	56.2 ± 8.2
Q3	31.2 ± 4.0
Q4	10.9 ± 4.2

Figure 4.4: Flow cytometry data for *Salmonella enterica* serovar (a) Typhimurium SL1344 and (b) Agona exposed to 4 ppm and 5 ppm sodium hypochlorite at 5 and 20 mins (n=3; the figures are of one representative repeat, the percentages are averages of all three repeats). The % symbol denotes the mean percentage of cells found in each quadrant plus or minus the standard deviation.

4.2.2 Efflux activity in cells after biocide exposure

For the associated methods please see **Chapter Two, section 2.10 and 2.11.**

4.2.2.1 Hoescht assay after biocide exposure

Hoescht 33342 (bis-benzimide) is a permeable dye which enters cells and binds to DNA, causing a change in fluorescence wavelength that can be measured. This method can tell us how much dye is present in the cell which gives indications as to efflux activity and/or the presence of porins.

Salmonella were grown for 3 hrs before biocide was added, this was then incubated at room temperature for 1 hr and then Hoescht dye was added. Readings were taken in the Fluostar every 3 mins for 30 mins and the output was given in the format of an Excel spreadsheet. The raw data was then adjusted for the blank and the averages and standard deviations were calculated. Figure 4.5 shows a representative line graph produced from this data.

Figure 4.5 shows the fluorescence recorded when *Salmonella* Typhimurium SL1344 and *Salmonella* Agona were exposed to peracetic acid (the graphs for hydrogen peroxide and sodium hypochlorite exposure can be found in appendix section 8.3). The *ramR* knockout in figure 4.5 is represented by the purple line at the bottom of the graph, as it has increased efflux due to the AcrB-TolC system not being repressed effectively. This strain has the lowest accumulation of Hoescht 33342 as it is removed by efflux. Conversely, L644 is a *ramA* knockout which reduces the activity of the AcrB-TolC system consequently, this strain shows the highest Hoescht 33342 accumulation (the green line at the top of fig 4.5). The experimental data for SL1344 controls and those exposed to biocide, fall in between L1007 and L644. There is an increase in fluorescence in both strains of *Salmonella* after exposure to 4 ppm and 3 ppm peracetic acid compared to the untreated control (fig.

4.5). The increase in fluorescence indicates that more Hoescht 33342 is bound to DNA inside the cell than the untreated control. However, when both strains of *Salmonella* were exposed to lower concentrations of peracetic acid the levels of fluorescence were similar to the untreated controls (fig. 4.5).

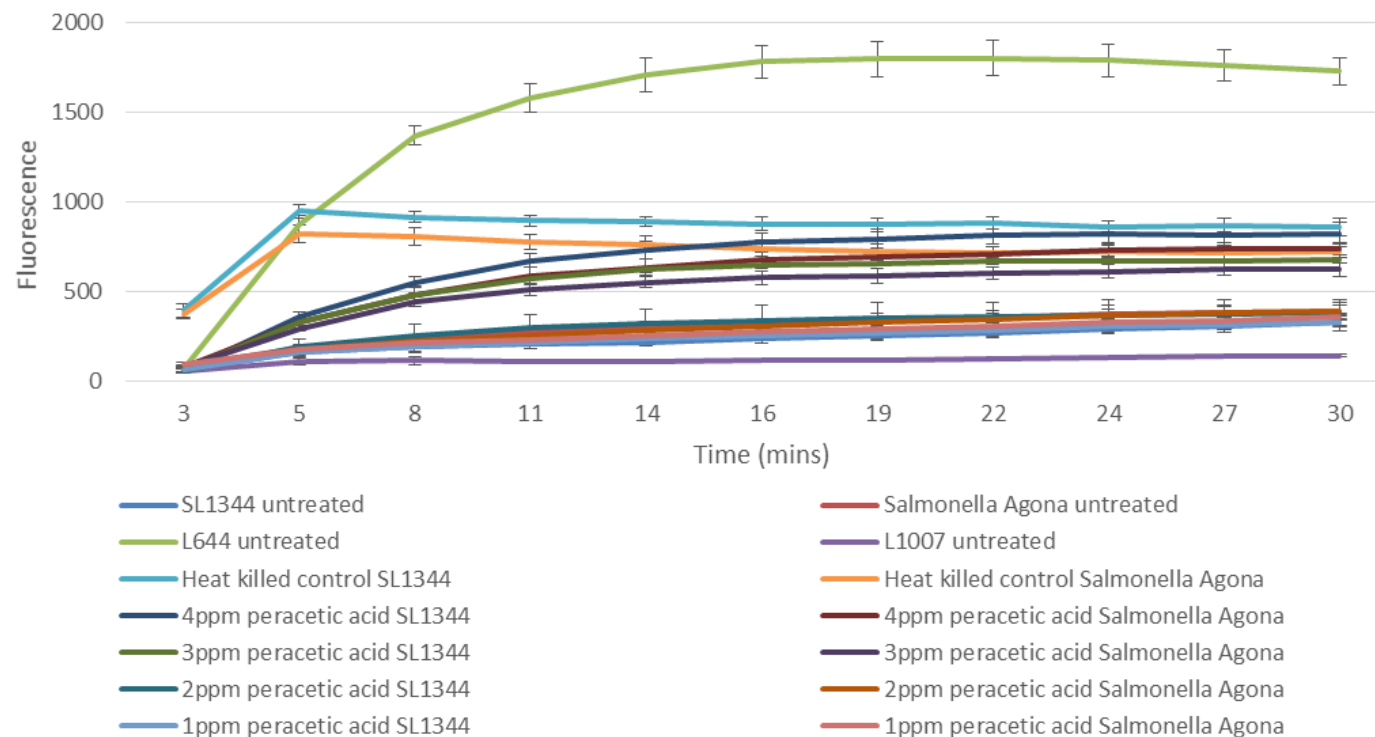


Figure 4.5: The accumulation of Hoescht 33342 (bis-benzimide) in *Salmonella enterica* serovars Typhimurium SL1344 and Agona when treated with peracetic acid. *Salmonella* Typhimurium strains L644 and L1007 were used as controls; L644 is an *acrB* knockout and therefore has decreased efflux and L1007 is a *ramR* knockout which increased *ramA* and therefore increased efflux activity. Heat killed controls give readings of high fluorescence accumulation within the cell as no efflux can occur (n=4). The error bars denote the standard deviation from the mean.

Figure 4.6 is a histogram showing the change in fluorescence from the untreated controls. This was calculated by the fluorescent values of the control unexposed *Salmonella* being subtracted from the final readings of biocide exposed cells to give the difference.

The changes in fluorescence seen in fig. 4.6 seem to be consistent between the two *Salmonella* strains tested. Both *Salmonella* Typhimurium SL1344 and Agona both show a statistically significant decrease in fluorescence after exposure to all concentrations of hydrogen peroxide. There was a significant increase in the fluorescence (fig. 4.6) after exposure to higher concentrations of peracetic acid (3 ppm and 4 ppm).

However, the changes in fluorescence seen in *Salmonella* after sodium hypochlorite exposure were not as clear. Figure 4.6 shows that at higher concentrations (20 ppm and in *Salmonella* Typhimurium SL1344 also at 15 ppm) there is a significant decrease in fluorescence from untreated controls. This appears to reverse at lower concentrations as there is an increase in fluorescence seen in both strains at 5 ppm sodium hypochlorite (fig. 4.6).

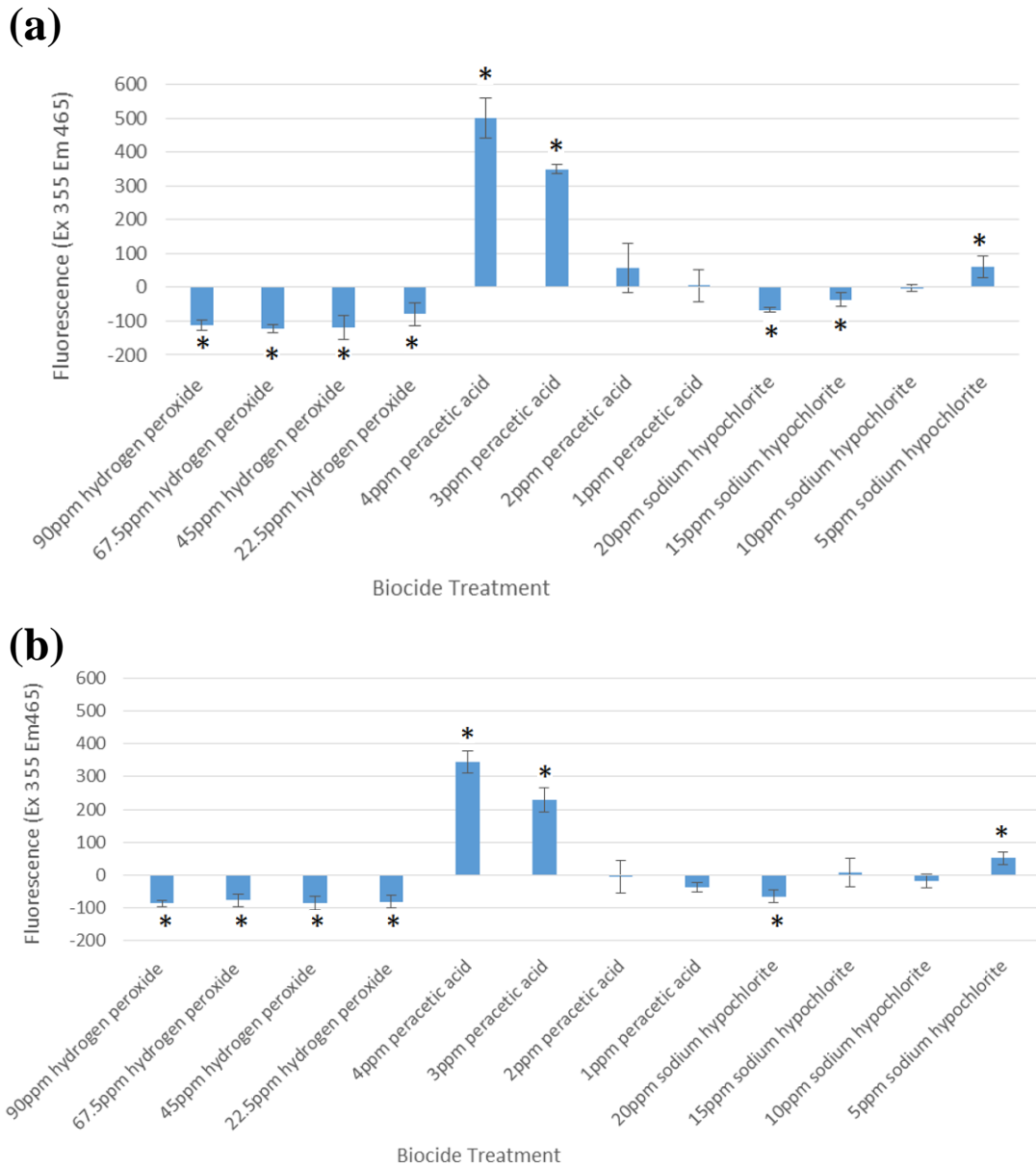


Figure 4.6: The endpoint change in accumulation of Hoescht 33342 (bis-benzimide) between *Salmonella enterica* serovar (a) Typhimurium SL1344 (b) Agona when exposed to oxidising biocides. Asterisks signify statistical significance of *P* values less than 0.05 from the untreated control (n=4). The error bars denote the standard deviation from the mean.

4.2.2.2 *Measuring the activity of acrAB using GFP reporter after biocide exposure*
GFP reporter experiments were conducted using *Salmonella enterica* serovar Typhimurium SL1344 with a fluorescent reporter plasmid inserted. These were created using the method described by Lawler, *et al.* (2013).

Strains were grown in M9 minimal media to late log/early stationary phase in a 96-well plate in a BMG FLUOstar Optima with recordings being taken for OD and fluorescence every 3 min. Biocide was added after approximately 3 hours growth and the OD and fluorescence readings were taken every 3 min.

Figure 4.7 is an example of a line graph showing the change in fluorescence after biocide exposure (in this case hydrogen peroxide) was added to *Salmonella* Typhimurium L1361. All concentrations of hydrogen peroxide showed a decreased in fluorescence and therefore most probably a decrease in *acrAB* expression. Interestingly after approximately 60 mins there seems to be an increase in fluorescence in *Salmonella* exposed to 45 ppm and 22.5 ppm hydrogen peroxide (fig 4.7)

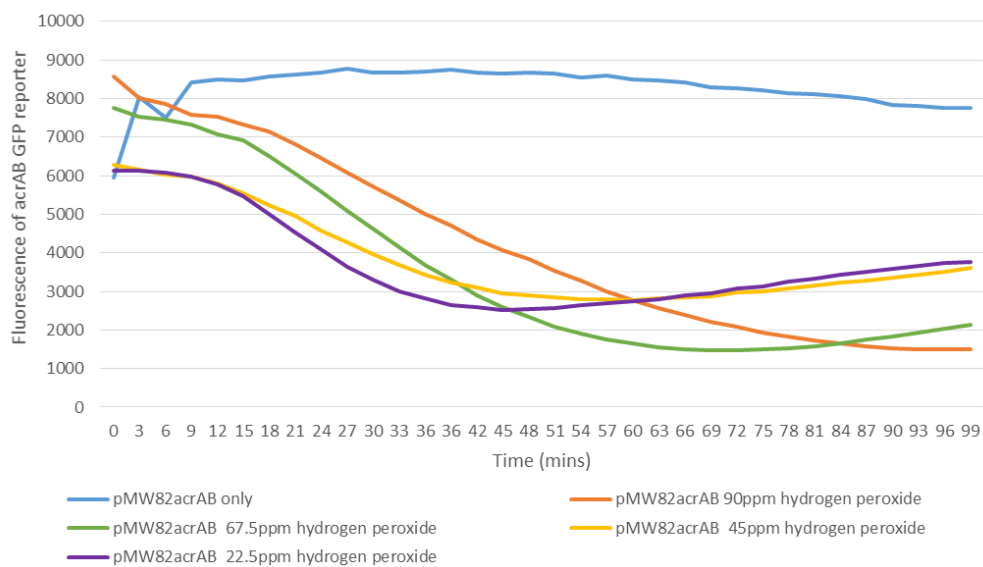


Figure 4.7. The change in fluorescence over time of the *acrAB* GFP reporter in *Salmonella* Typhimurium SL1344 and therefore the expression of *acrAB* when exposed to a range of concentrations of hydrogen peroxide (n=4).

Figure 4.8 collates all of the data from *Salmonella* Typhimurium L1361 exposed to hydrogen peroxide, peracetic acid and sodium hypochlorite at a range of concentrations. To give further information as to the response of *acrAB* in *Salmonella* Typhimurium L1361 the change between the untreated control and the biocide treated samples was calculated and represented in fig 4.8 (the line graphs of the data can be found in appendix section 8.4).

Figure 4.8 indicates there are no statistically significant increases in *acrAB* expression, with the exceptions of 20 ppm sodium hypochlorite, 2 ppm and 1 ppm peracetic acid at 60 mins. All other conditions showed a decrease in expression of *acrAB* after biocide challenge (fig 4.8). Hydrogen peroxide caused the biggest decrease in the presence of *acrAB* and also showed an increase at 99 mins after the decrease at 60 min (as can also be seen in fig 4.7).

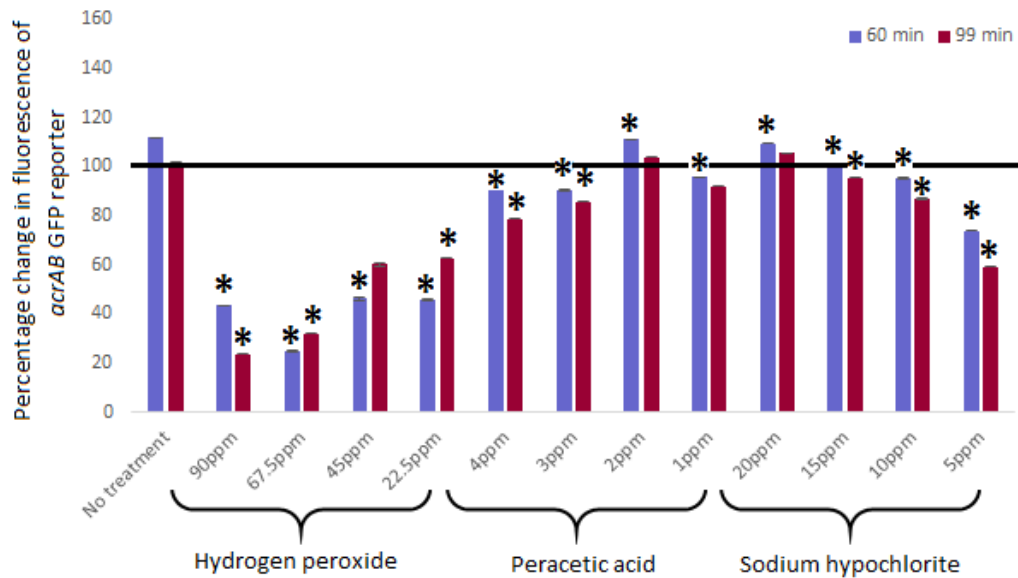


Figure 4.8: The percentage change in expression of *acrAB* GFP reporters quantified by the change in fluorescence from readings taken directly before exposure to biocide treatment (n=4). The black lines show 100% (no change from pre-exposure). Asterisks signify statistical significance of *P* values less than 0.05 from the untreated control (n=4). The error bars denote the standard deviation from the mean. Positive controls were undertaken but have not been shown as they change the scale to an extent it obscures the experimental data.

4.2.3 Regulatory responses of planktonic *Salmonella* to oxidising biocides using GFP reporters

For the associated method please see **Chapter Two, section 2.11.**

4.2.3.1 Measuring the activity of *ramA* using GFP reporter after biocide exposure

These experiments were conducted in the same manner as those for the *acrAB* GFP reporter experiments described above. The line graphs of the change in fluorescence of *ramA* can be found in appendix section 8.4. Figure 4.9 shows the change in *ramA* expression from the untreated controls.

Figure 4.9 showed two conditions (22.5 ppm hydrogen peroxide and 10 ppm sodium hypochlorite) which gave an increase in *ramA* from the pre-treatment condition, all others were less than the 0 min reading. There was no overall trend towards an increase or decrease of *ramA* expression (fig 4.9).

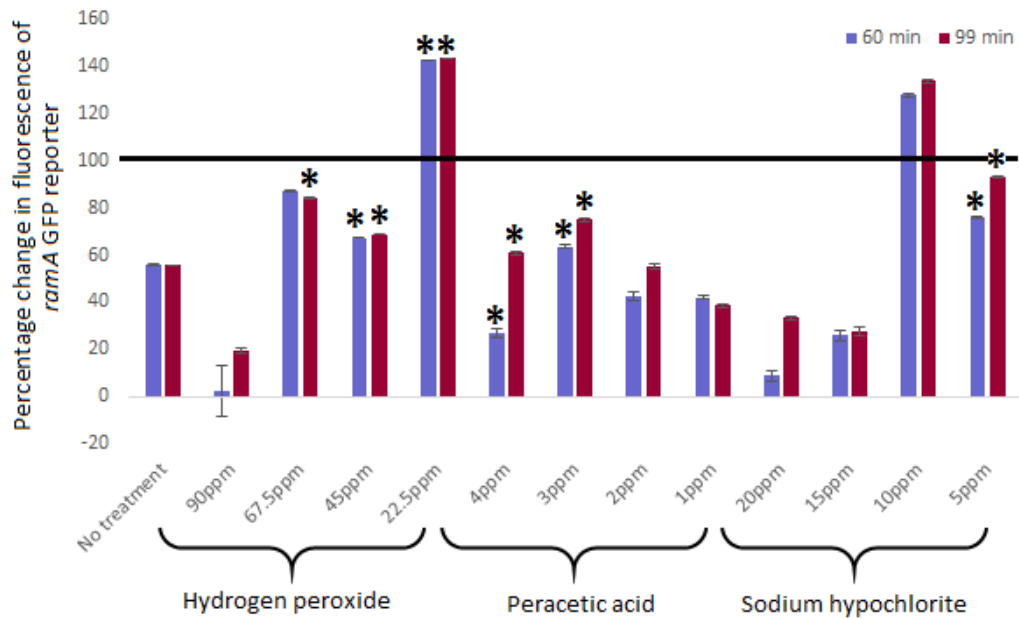


Figure 4.9: The percentage change in expression of *ramA* GFP reporters quantified by the change in fluorescence from readings taken directly before exposure to biocide treatment (n=4). The black lines show 100% (no change from pre-exposure). Asterisks signify statistical significance of *P* values less than 0.05 from the untreated control (n=4). The error bars denote the standard deviation from the mean. Positive controls were undertaken but have not been shown as they change the scale to an extent it obscures the experimental data.

4.2.3.2 Measuring the activity of *soxS* using GFP reporter after biocide exposure

All GFP experiments were conducted in the same manner as described above for *acrAB* reporters. The line graphs of the change in fluorescence of *soxS* can be found in appendix section 8.4. Figure 4.10 shows the variation in *soxS* expression from the untreated controls.

Expression of *soxS* was significantly decreased upon exposure to hydrogen peroxide and sodium hypochlorite (fig 4.10). Peracetic acid exposure did not have a significant effect on the expression of *soxS* at higher concentrations. At 1ppm at 60 and 99mins there was a small but significant increase in *soxS* expression (fig 4.10).

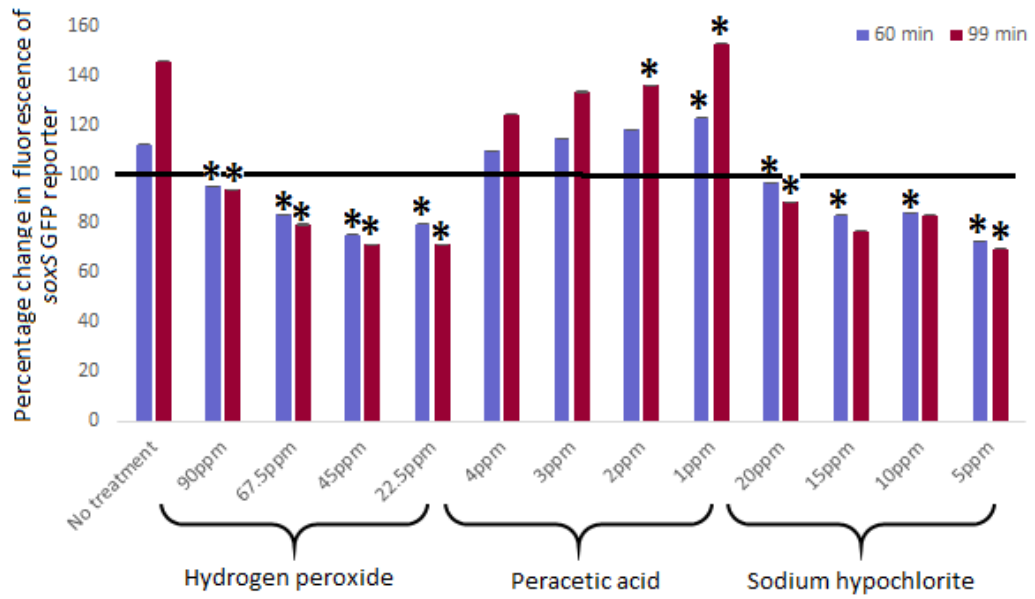


Figure 4.10: The percentage change in expression of *soxS* GFP reporters quantified by the change in fluorescence from readings taken directly before exposure to biocide treatment (n=4). The black lines show 100% (no change from pre-exposure). Asterisks signify statistical significance of *P* values less than 0.05 from the untreated control (n=4). The error bars denote the standard deviation from the mean. Positive controls were undertaken but have not been shown as they change the scale to an extent it obscures the experimental data.

4.3 Discussion

Flow cytometry is a useful tool in examining how *Salmonella* cells respond rapidly after exposure to biocide without having to wait for cells to recover and grow. *Salmonella* cells exposed to hydrogen peroxide showed no damage to cell membrane potential or membrane integrity at all concentrations and time-points tested. This gives an indication that the cells are surviving levels of hydrogen peroxide exposure such as 3000 ppm for 60 mins with no damage to the cell membrane which does not support the culture data shown in Chapter Three, section 3.2.7.1. The culture data shows a 5 log₁₀ reduction at concentrations of 2800 ppm and above after 60 mins. Additionally, peracetic acid showed a large percentage of cells in Q3 (94.5-97.3%) after exposure to a concentration of peracetic acid that gives a 4.8 log₁₀ reduction in culture experiments (3 ppm for 20 mins; Chapter Three, section 3.2.7.2, fig 3.5).

The discrepancy between the flow cytometry data and the culture data may be due to cells exposed to hydrogen peroxide being viable but non-culturable. This would mean that they are not detected by traditional culture methods. This was shown to occur in *Legionella* exposed to lower concentrations of chlorine dioxide by Mustapha *et al.* (2015). In this study, *Legionella* were exposed to a range of concentrations of chlorine dioxide with culturable cells being detected at 4 mg/L of chlorine dioxide but viable but non culturable cells being detected at 4 to 5 mg/L (Mustapha, *et al.*, 2015). However, as the cells were not tested further this cannot be confirmed.

An alternative theory may be that *Salmonella* cells exposed to hydrogen peroxide or peracetic acid may have died due to intracellular damage, such as DNA damage but the membrane has remained intact. The mechanism of action of these biocides on a bacterial cell has yet to be fully understood. However, it is hypothesised that the

effect of hydrogen peroxide on the cell membrane may play an important role in the killing of the cell (Linley, *et al.*, 2012). For this reason the probability of the cell being killed with no membrane damage seems unlikely. Therefore the hypothesis of cells being VBNC seems more likely. This could be investigated further by the use of other dyes to test for cellular viability that are not membrane dependant such as SYTO9.

Salmonella exposed to peracetic acid showed a loss of membrane potential after exposure but no loss of membrane integrity. At the lower concentration tested (2ppm) there seemed to be a separation of the population into two distinct sub-populations; cells which had membrane damage and cells that did not. This could be due to a concentration dependant stress response within the bacterial cell, which is triggered at higher concentrations to give a protective effect but is not activated at 2 ppm.

Cells exposed to sodium hypochlorite displayed a steady shift from live to dead cells with increased exposure time and concentration, which was consistent with the culture data.

The Hoescht 33342 assay showed that when *Salmonella* Typhimurium SL1344 and Agona were exposed to hydrogen peroxide there was a decrease in fluorescence. This means that there was less Hoescht dye bound to the DNA inside the cell. The decrease of Hoescht dye present in *Salmonella* after hydrogen peroxide exposure, could be due to a decrease in cellular permeability (such as a down-regulation of porins) or an increase in efflux activity. The *acrAB* GFP reporter assay showed a decrease in fluorescence in *Salmonella* Typhimurium L1361 exposed to hydrogen peroxide, indicating a decrease in the production of *acrAB* compared to the control.

Therefore, the decrease in accumulation of Hoescht 33342 is not caused by an increase in the AcrAB-TolC efflux pump.

Interestingly, the decrease in *acrAB* expression shown in Chapter Four, section 4.2.2.2 appears to be only temporary, with figure 4.7 showing an increase towards the end of the timescale (at 99 mins). This may mean the adaptations that occur in response to hydrogen peroxide exposure may only be temporary.

There are two hypotheses to explain the decrease of accumulation of Hoescht 33342 in *Salmonella* Agona and Typhimurium SL1344. The first theory is that there could be a decrease in *acrAB* but an increase in the expression of another efflux pump. Whitehead, *et al.* (2011) isolated mutant survivors after in-use concentration exposure to two biocides (an aldehyde and quaternary ammonium formulation and a halogenated tertiary amine compound). These mutant survivors had decreases in Hoescht 33342 accumulation but no increases in *acrAB* expression. Further investigation found that there was an increase in efflux via the AcrEF-TolC system (Whitehead, *et al.*, 2011). It may be that although there is a decrease in *acrAB* expression, another efflux pump may be turned on instead.

An alternate theory for the decrease in Hoescht 33342 accumulation could be a decrease in cellular permeability, due to a decrease in expression of the porin *ompD*. *OmpD* is the most commonly found protein in the outer membrane of *Salmonella* Typhimurium and is known to be linked to the uptake of oxidising agents (Aguayo *et al.*, 2015; Calderón *et al.*, 2011). It has been shown that in the presence of hydrogen peroxide the expression of *ompD* is downregulated as a protection against oxidative damage (Calderón, *et al.*, 2011). Therefore it can be hypothesised, that the decrease in accumulation of Hoescht 33342 dye, could be caused by a decrease in *ompD*,

preventing uptake of the dye. Unfortunately, due to the nature of the Hoescht assay data, it is not possible to determine whether the decrease in accumulation after exposure to hydrogen peroxide is due to an increase in efflux, or a decrease in the number of porins. This could be further investigated by the use of efflux pump inhibitors (as discussed in Chapter One, section 1.4.3.2). Efflux pump inhibitors could be added to the cells and if there is still a decrease in Hoescht accumulation after exposure to biocides then porins are implicated, if not an alternate efflux pump system is responsible for the decreased Hoescht accumulation. An alternative approach would be to investigate the presence and quantify the outer membrane proteins present before and after biocide exposure, to look for an increase in the presence of ompD.

Salmonella Typhimurium SL1344 and Agona exposed to higher concentrations of peracetic acid (3 or 4 ppm) showed an increase in accumulation of Hoescht 33342. Lower concentrations of peracetic acid (1 and 2 ppm) showed no significant change from untreated cells. This shows that the response to peracetic acid is dependent on concentration. There is also a statistically significant decrease in the production of *acrAB* at higher concentrations (3 and 4 ppm) of peracetic acid. There is a small but statistically significant increase in the production of *acrAB* at 2 ppm 60 mins and another similarly small decrease at 1 ppm at 60 mins. Although these changes are statistically significant according to a Student's T-Test the difference is very small. The increase in fluorescence in the Hoescht assay could be due to the decrease in expression of *acrAB*. However, the method is such that this has not yet been proven.

Sodium hypochlorite at the highest concentration tested (20 ppm) showed a slight decrease in fluorescence from the mean in the Hoescht assay in both *Salmonella* Typhimurium SL1344 and Agona and an increase in *acrAB* expression. Intermediate

concentrations gave slight increases and decreases, one of which was statistically significant (15 ppm in *Salmonella* Typhimurium SL1344) but all were very small changes. The lowest concentration of sodium hypochlorite tested (5 ppm) showed a decrease in *acrAB* expression and an increase in Hoescht accumulation. As seen with peracetic acid, these could be linked as the increase of *acrAB* being responsible for the decrease in accumulated Hoescht dye, but this cannot be conclusively proven.

The investigations into the expression of *ramA* using GFP reporters in Chapter Four, section 4.2.2.3 showed no overall trend towards an increase or decrease of *ramA* expression. Also, interestingly the expression of *ramA* did not seem to be linked to the expression of *acrAB* seen in Chapter Four, section 4.2.2.2, which is unusual as *ramA* is an important regulator of *acrAB*.

GFP reporter experiments showed *soxS* expression is decreased in the presence of oxidising biocides in almost all conditions. Hydrogen peroxide and sodium hypochlorite caused a consistent decrease in *soxS* expression. Conversely, lower concentrations of peracetic acid (1 and 2 ppm) showed a statistically significant increase in *soxS* expression. Higher concentrations of peracetic acid showed an increase in *soxS* expression but it was not statistically significant. This was not expected as *soxS* is known to be involved in oxidative stress response (Pomposiello & Dimple, 2000). However, the SoxRS system is not the only system involved with regulating the oxidative stress response, for example the OxyR system is involved with the response to hydrogen peroxide (Spector & Kenyon, 2012). To gain further information as to which oxidative stress responses are induced instead of *soxS*, an experiment, examining the whole transcriptome after exposure to the oxidising biocides used in this study would be preferable.

To summarise, flow cytometry is useful as it allows viable but non culturable cells to be observed and quantified, as may have been observed with hydrogen peroxide exposed cells. It also gives interesting information into the population response to exposure to biocides, which was illustrated when two distinct populations were found using flow cytometry in *Salmonella* cells exposed to peracetic acid. The combination of the Hoescht assay and the *acrAB* GFP reporter experiments gave a useful insight into how cellular permeability and efflux were affected by biocide exposure. Hydrogen peroxide showed a decrease in accumulation of Hoescht dye, but a decrease in the expression of *acrAB*, which may be due to other types of efflux pumps or porins. Peracetic acid and sodium hypochlorite both showed data consistent between the Hoescht assay and *acrAB* study, with a decrease in *acrAB* linked to an increase in Hoescht accumulation. Expression of *ramA* did not show a consistent pattern and *soxS* expression was decreased in most conditions which was unexpected. The combination of these data indicate that the oxidising biocides tested do not induce previously reported stress response pathways.

Chapter Five: Biocide
susceptibility of *Salmonella*
in a static biofilm

5.1 Introduction

Bacterial biofilms are the way that the majority of bacteria exist in nature (Hall-Stoodley *et al.*, 2004). As described in Chapter One, section 1.5, this gives bacteria a survival advantage as it protects them from environmental factors including desiccation, ingestion by protozoa in addition to exposure to biocides (Solano *et al.*, 2014). For this reason it is very important to study bacteria in a biofilm as they present the most common and the most challenging bacterial target.

There are a number of methods to grow biofilms in an in-vitro environment which can be divided into two main types; batch systems which are static and flow systems that allow for the inflow of nutrients and/or the outflow of waste (Coenye & Nelis, 2010). The microtitre plate-based model is used in a wide range of studies (Peeters *et al.*, 2008; Pitts *et al.*, 2003; Shakeri *et al.*, 2007) because of their low price, ease of use and potential for high throughput (Coenye & Nelis, 2010) using multiple different strains and biocide combinations in a single plate (depending on the size of the microtitre plate). An additional advantage of using a microtitre based-plate model is that conditions can be varied easily and without specialist skills or equipment, for instance changing the growth media or the temperature (Coenye & Nelis, 2010). The limitations of this methodology are that it doesn't necessarily reflect real-world conditions and differences in the way that a biofilm is formed can affect the biocide efficacy (Buckingham-Meyer, *et al.*, 2007). However, it is a quick, simple and effective method for comparing planktonic and biofilm phenotypes which is why it was selected for this study.

The environmental conditions can affect the formation of bacterial biofilms. For this reason it was important to identify the optimum growth conditions for the formation

of a static *Salmonella* biofilm. Temperature, attachment surface and media can all affect the growth of a biofilm (De Oliveira *et al.*, 2014; Piras *et al.*, 2015; Ryu *et al.*, 2004); for this reason a range of conditions were tested. In this study minimal media were used for growing biofilms to more accurately reflect environmental conditions seen in a water environment.

Bacterial biofilms are more tolerant to antimicrobials than bacteria growing in a planktonic state (Otter *et al.*, 2015). This is discussed in depth in Chapter One, section 1.5.3 in the General Introduction but to summarise this effect is consistent across a range of different bacterial strains and is seen with many different biocides (Bridier, *et al.*, 2011). The reasons for this have yet to be fully understood; it could be due to the biofilm matrix preventing access to biocide target sites, a result of the expression and exchange of genes that occur in a biofilm, an altered metabolic rate, a heterogenous population or some combination of these factors (Bridier *et al.*, 2015).

Previous studies have used static biofilms as described above to test disinfectant efficacy, such as the American Society for Testing and Materials (2012). However, this has only recently been published and is a qualitative method. A quantitative standard method for determining bacterial susceptibility in a static biofilm has yet to be established. Studies into the susceptibility of biofilms to oxidising biocides requires a reproducible biofilm model for testing which this chapter will establish. This model will then be used for susceptibility testing for both intact and dispersed biofilms against oxidising biocides. The data from these studies will address the remaining problem of whether biofilms are more tolerant of biocides because of their three dimensional structure or due to other factors.

5.1.1 Aims

- To develop a reproducible static biofilm model in minimal media for quantitative biocide testing.
- To determine the effect of oxidising biocides on dispersed biofilm cells which retain the biofilm phenotype but do not have the presence of a protective structure.
- To determine the effect of oxidising biocides on intact biofilms which have structural protection from biocide penetration in addition to the altered biofilm phenotype.

5.2 Results

5.2.1 Developing a *Salmonella* species biofilm

For the associated method please see **Chapter Two, section 2.12.**

5.2.1.1 Identifying the optimum temperature for *Salmonella* biofilm growth

To identify the optimum temperature for growth for a *Salmonella* minimal medium biofilm samples were incubated at two different temperatures: 30°C and 37°C. Samples were grown on polystyrene surfaces in M9 minimal media for 48 hours and after the required incubation time were resuspended in PBS and enumerated (table 5.1). The results of this testing were that growth with *Salmonella* Typhimurium SL1344 ($p < 0.01$) and Havana ($p = 0.03$) were statistically different and showed more growth at 30°C using a Student's T-test. For this reason this was the temperature used for further growth of biofilms (see table 5.1).

Table 5.1: The mean number of colony forming units per cm² of each surface for biofilm growth of *Salmonella* Typhimurium SL1344, Agona and Havana grown in M9 minimal media at either 30°C or 37°C on polystyrene surfaces of 6 well plates for 48 hours (n=3).

	Mean number of colony forming units \pm SD (log CFU/cm ²)	
	30°C	37°C
Typhimurium SL1344	8.1 \pm 0.1	8.3 \pm 0.0
Agona 3750	8.1 \pm 0.1	7.5 \pm 0.7
Havana 3759	8.2 \pm 0.1	7.8 \pm 0.3

5.2.1.2 Identifying the optimum growth medium and attachment surface for *Salmonella* biofilm growth

To identify the optimum growth medium and attachment surface for the biofilm growth of three *Salmonella* strains two different attachment surfaces (polystyrene and stainless steel) and two different minimal media (M9 and MOPS based) were used. Comparison of the stainless steel surface and the polystyrene in M9 minimal media at 30°C (table 5.2) found that there were significant differences for Typhimurium SL1344 ($p=0.04$) and Havana ($p=0.01$) but not Agona ($p=0.97$) when using a Student's T-test. A comparison of the surfaces in MOPS based minimal media (table 5.2) using a Student's T-test gave significant differences ($p<0.01$) for all the strains tested. The results of these tests showed the polystyrene surface gave more growth in the majority of conditions so it was used as the attachment surface for further experiments.

A comparison of growth in the two different media was made using a Student's T-test. First, growth on stainless steel discs in the two media were compared. This gave a significant difference ($p<0.01$) for *Salmonella* Typhimurium SL1344 and Agona but not for Havana ($p=0.96$). The growth on polystyrene was then compared and all three of the strains showed a statistically significant change ($p<0.01$) between growth in M9 minimal media and MOPS based minimal media. As can be seen from table 5.2 there is more growth in MOPS based minimal media which is supported by the statistically significant difference, therefore MOPS based minimal media was used for further experiments.

Table 5.2: The mean number of colony forming units per cm² of each surface for biofilm growth of *Salmonella* Typhimurium SL1344, Agona and Havana grown in either M9 minimal media or MOPS based minimal media at 30°C either on stainless steel discs or polystyrene surfaces of 6 well plates for 48 hours (n=3).

	M9 minimal media ± SD (log CFU/cm ²)		MOPS minimal media ± SD (log CFU/cm ²)	
	Stainless steel disc	Polystyrene plate surface	Stainless steel disc	Polystyrene plate surface
Typhimurium SL1344	7.8 ± 0.3	8.1 ± 0.1	7.4 ± 0.2	9.1 ± 0.1
Agona 3750	8.1 ± 0.2	8.1 ± 0.1	7.6 ± 0.4	9.2 ± 0.1
Havana 3759	7.8 ± 0.3	8.2 ± 0.1	7.8 ± 0.2	9.1 ± 0.1

The results of the preliminary optimisation testing were to grow biofilms for future testing at 30°C in MOPS minimal medium on the polystyrene surfaces of the 6 well multidish.

5.2.1.3 Quantification of *Salmonella* species biofilms using crystal violet staining

Crystal violet staining binds to organic matter and so is therefore a crude measure of the quantity of biofilm that is present. Initially after staining the biofilms in the method described in **Chapter Two, section 2.12.2**; the biofilms were photographed and the image is displayed as figure 5.1. It is easily observed that *Salmonella* Typhimurium SL1344 is a poor biofilm former as there is very little crystal violet staining even after 96 hours of biofilm growth. There also does not seem to be a noticeable difference in the amount of crystal violet staining at 48 72 or 96 hours. *Salmonella* Agona however shows very dark staining after only 48 hours of biofilm growth and this remains visually consistent over the time course.

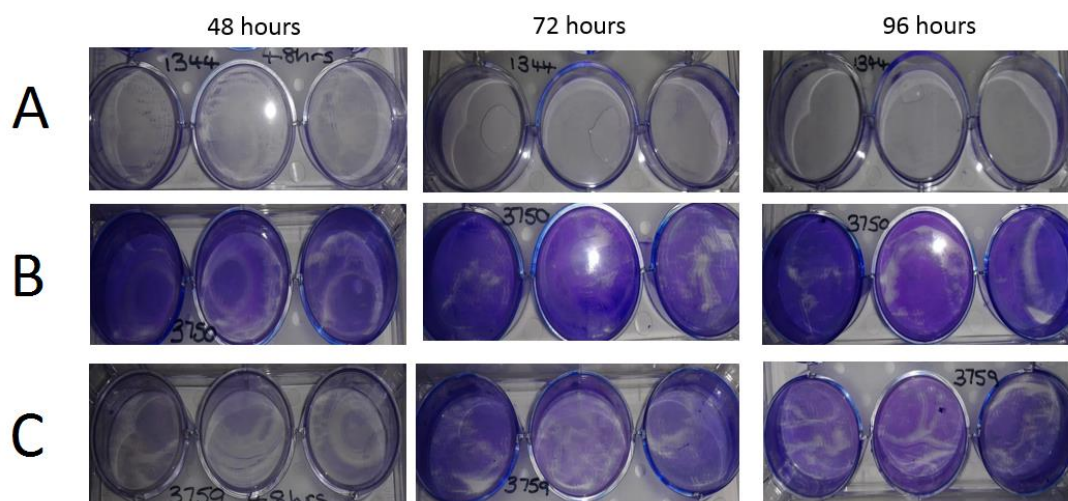


Figure 5.1: Crystal violet staining of *Salmonella* species (A) *Salmonella* Typhimurium SL1344 (B) *Salmonella* Agona 3750 (C) *Salmonella* Havana biofilms after growth over 48, 72 and 96 hours (n=3).

To quantify the results from the crystal violet staining, the crystal violet was solubilised in acetic acid and then the optical density was read at 550 nm (table 5.3). It is obvious that *Salmonella* Agona is a much better biofilm former than *Salmonella* Typhimurium SL1344. It can also be seen from table 5.3 how the quantity of the biofilm has increased over the time period with both strains showing an increase in the optical density every 24 hours.

Table 5.3: Optical density readings of solubilised crystal violet biofilm assay of *Salmonella* species over 96 hours (n=3).

<i>Salmonella enterica</i> serovar	Optical density at 550nm \pm SD		
	48 hours	72 hours	96 hours
Typhimurium SL1344	0.190 \pm 0.02	0.309 \pm 0.04	0.869 \pm 0.3
Agona 3750	1.723 \pm 0.3	2.230 \pm 0.3	2.621 \pm 0.2
Havana 3759	1.044 \pm 0.2	1.122 \pm 0.3	1.285 \pm 0.1

The data shown in figure 5.1 and table 5.3 confirm that *Salmonella* Typhimurium is a poor biofilm former. This is consistent with what would be expected as it has been

reported in the literature that it does not produce cellulose properly (García *et al.*, 2004). This is consistent with the viable count data obtained in Chapter Five, section 5.2.1.2 as crystal violet stains biomass and so the decreased EPS seen with *Salmonella* Typhimurium SL1344 is responsible for the lack of staining, rather than the lack of viable cells. It may also be possible that the wash step to remove excess crystal violet which is required for the result to be accurate, may have caused some of the *Salmonella* Typhimurium SL1344 biofilm to detach as it not as strongly attached due to the lack of biofilm matrix.

After identifying *Salmonella* Typhimurium SL1344 as a poor biofilm former and *Salmonella* Agona as a strong biofilm former, further experiments continued with only these two strains.

5.2.2 Inactivation kinetics using *Salmonella* in dispersed biofilms

For the associated method please see **Chapter Two, section 2.13.1.**

5.2.2.1 Hydrogen peroxide exposure

The inactivation kinetics of *Salmonella* Typhimurium SL1344 when exposed to hydrogen peroxide showed that all concentrations gave a 3.3 log₁₀ reduction (which was the limit of detection) after 60 mins (fig. 5.2a). The highest concentration of 4000ppm gave a 3.3 log₁₀ reduction (the limit of detection) after 20 mins. Both 2000ppm and 3000ppm showed a similar trend log₁₀ reduction line on the graph; 1 and 1.7 log₁₀ reductions at 20 mins and 2.8 and 3.3 log₁₀ reduction at 40 mins respectively (fig. 5.2a).

The inactivation kinetics of *Salmonella* Agona when exposed to hydrogen peroxide showed that all concentrations gave a 4.2 log₁₀ reduction (which was the limit of detection) after 60 mins (fig. 5.2b). The highest concentration of 4000ppm gave a log₁₀ reduction to the limit of detection after 40 mins. All three concentrations (2000 ppm, 3000 ppm and 4000 ppm) gave a similar trend log₁₀ reduction line on the graph; 0.5, 1 and 1.9 log₁₀ reduction at 20 mins and 2.6, 4 and 4.2 log₁₀ reduction at 40 mins respectively (fig. 5.2b).

Comparison of the planktonic and dispersed biofilm data for both strains of *Salmonella* when exposed to hydrogen peroxide shows the dispersed biofilm cells to be slightly more susceptible to biocide exposure than planktonic cells.

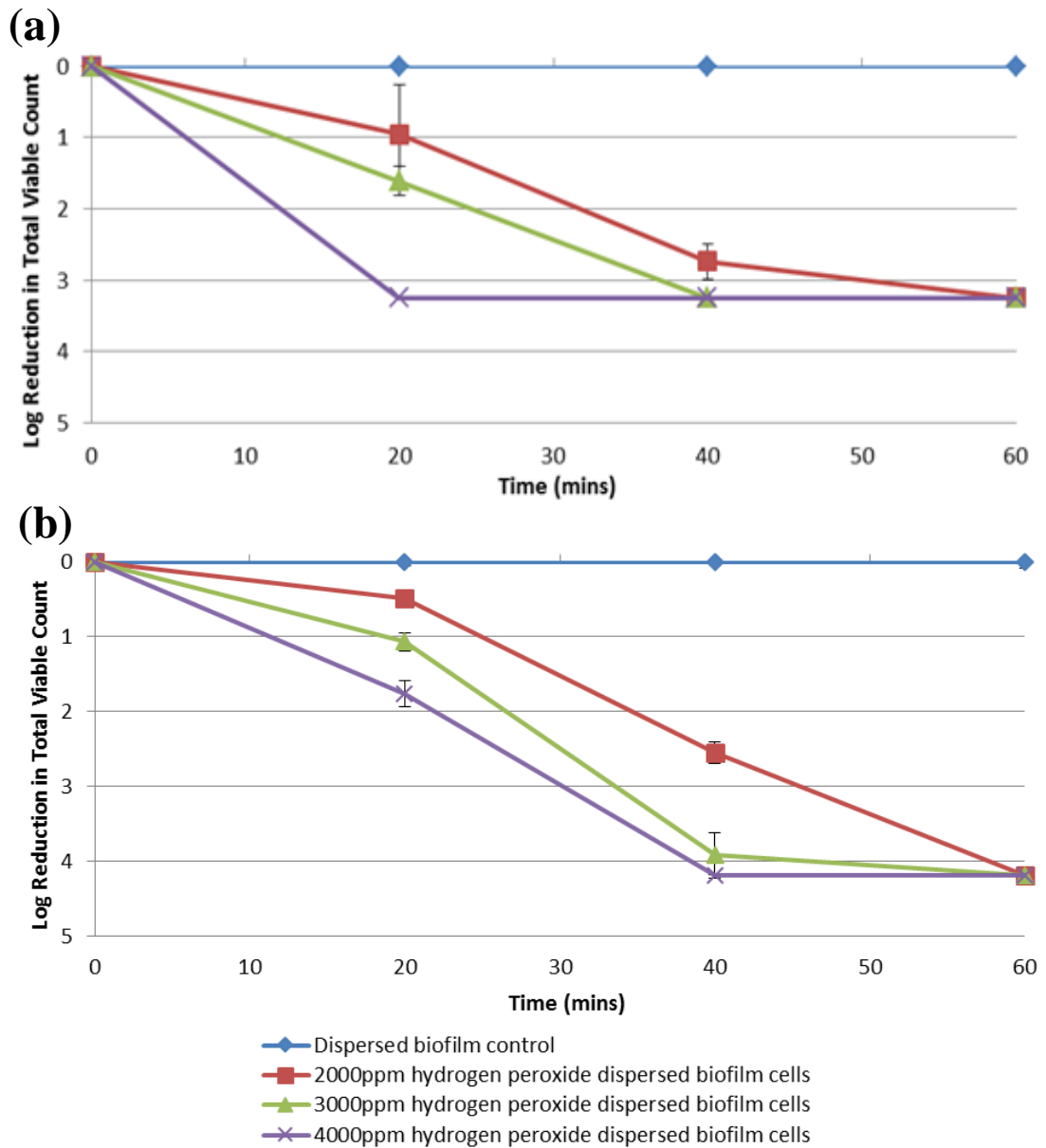


Figure 5.2: Log₁₀ reduction in the total viable count of dispersed biofilm cells of (a) *Salmonella enterica* serovar Typhimurium SL1344 (b) *Salmonella enterica* serovar Agona when exposed to 2000 ppm to 4000 ppm hydrogen peroxide. Based on the limit of detection the maximum log₁₀ reduction which could be determined was a log₁₀ reduction of (a) 3.3 and (b) 4.2 (n=3). The error bars denote the standard deviation from the mean.

5.2.2.2 Peracetic acid exposure

The inactivation kinetics of *Salmonella* Typhimurium SL1344 when exposed to peracetic acid is shown in figure 5.3a. At 3 ppm there was a 1.2 log₁₀ reduction after 5 mins and a 3.3 log₁₀ reduction after 20 mins. At 1 ppm and 2 ppm there no log₁₀ reduction and 0.3 log₁₀ reduction at 5 mins respectively. At 20 mins 1 ppm of peracetic acid still shows no log₁₀ reduction which increases to a log₁₀ reduction at the limit of detection at 3.3. Two parts per million of peracetic acid gives a 3.3 log₁₀ reduction after 20 mins.

The inactivation kinetics of *Salmonella* Agona when exposed to peracetic acid is shown in figure 5.3b. The concentrations of 2 ppm and 3 ppm follow a very similar shape with no log₁₀ reduction after 5 mins and a 4.1 log₁₀ reduction (which was at the limit of detection) at 40 mins. At 20 mins the lines separate out with 2 ppm giving a 1.5 log₁₀ reduction at 20 mins and 3 ppm giving a 2.1 log₁₀ reduction at the same time point. The pattern of 1 ppm of peracetic acid exposure seems to be different with the line remaining level for the first 20 mins at 0.2 log₁₀ reduction, then dropping to 2.7 log₁₀ reduction at 40 mins exposure.

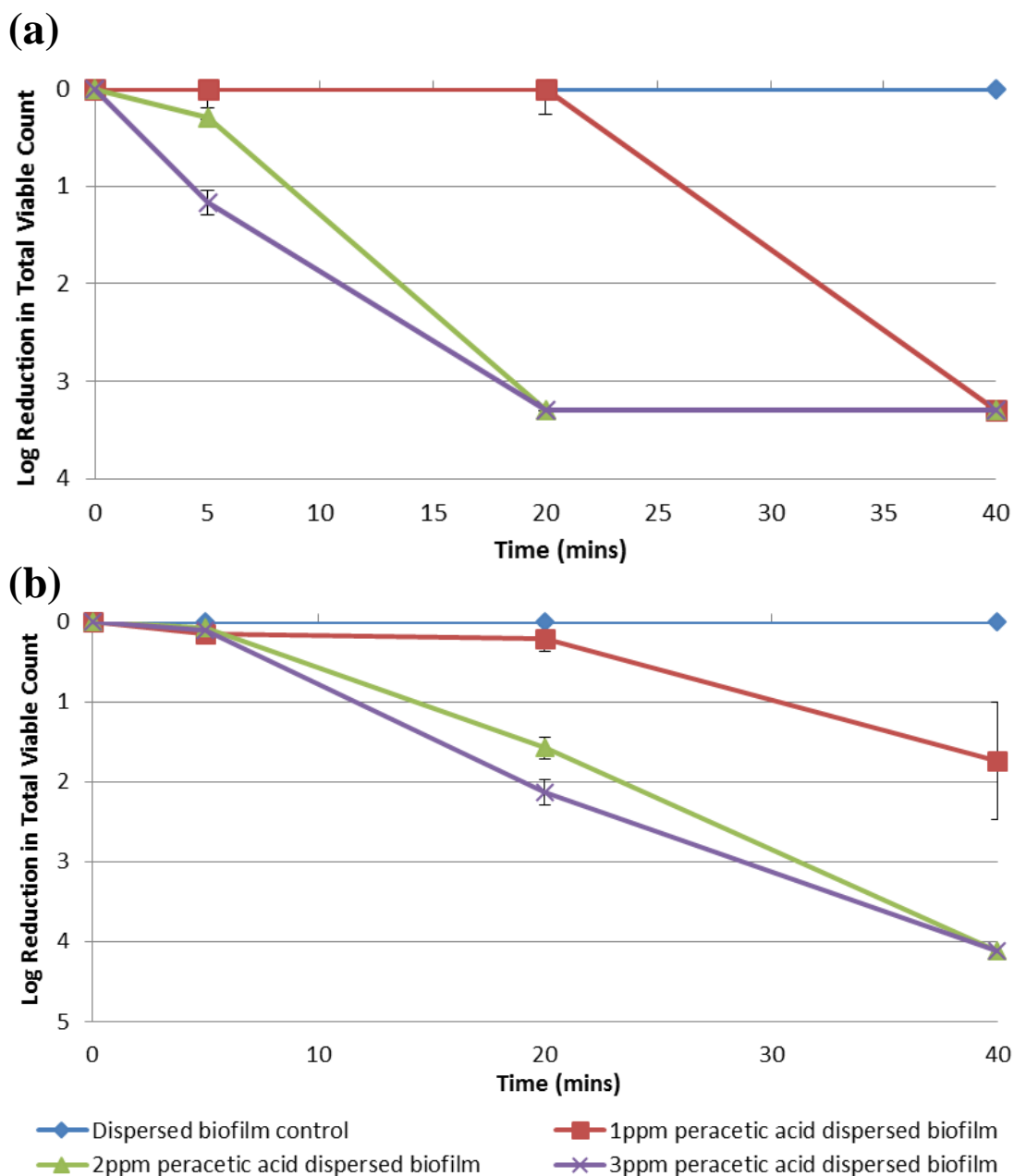


Figure 5.3: Log₁₀ reduction in the total viable count of dispersed biofilm cells of (a) *Salmonella enterica* serovar Typhimurium SL1344 and (b) *Salmonella enterica* serovar Agona when exposed to 1 ppm to 3 ppm peracetic acid. Based on the limit of detection the maximum log₁₀ reduction which could be determined was a log₁₀ reduction of (a) 3.3 and (b) 4.1 (n=3). The error bars denote the standard deviation from the mean.

The dispersed biofilm cells seemed more susceptible to peracetic acid exposure than planktonic cells; a pattern which was also seen in hydrogen peroxide exposed cells.

5.2.2.3 Sodium hypochlorite exposure

The inactivation kinetics of *Salmonella* Typhimurium SL1344 when exposed to sodium hypochlorite is shown in figure 5.4a. At 3 ppm and 4 ppm show similar levels of \log_{10} reduction (between 0 and 0.3) to the untreated control over the 40 mins time course. The *Salmonella* cells exposed to 5 ppm sodium hypochlorite showed a 3.7 \log_{10} reduction in the first five mins.

The inactivation kinetics of *Salmonella* Agona when exposed to sodium hypochlorite is shown in figure 5.4b. All concentrations exposed to sodium hypochlorite show a similar pattern with approximately one log difference between each concentration. The concentrations tested were 4 ppm, 5 ppm and 7 ppm of sodium hypochlorite at 20 mins were 0.9 \log_{10} reduction, 2 \log_{10} reduction and 3 \log_{10} reduction respectively. At 40 mins the \log_{10} reductions were 1.1, 2.4 and 3.2 \log_{10} and at 60 mins 1.3 \log_{10} reduction, 2.4 \log_{10} reduction and 3.7 \log_{10} reduction.

Dispersed biofilm cells exposed to sodium hypochlorite were less susceptible to biocide exposure, unlike those exposed to peracetic acid and hydrogen peroxide which more susceptible.

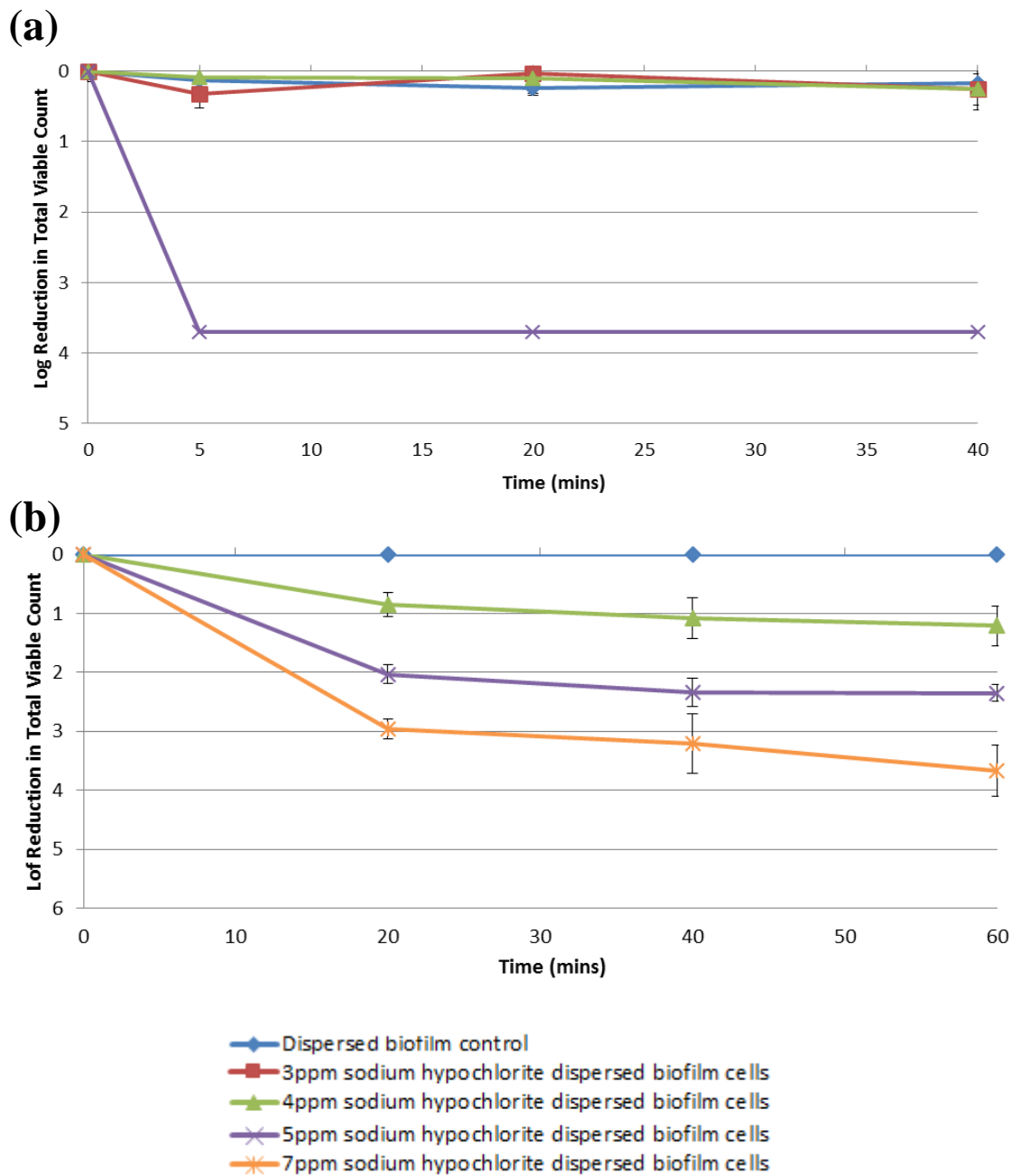


Figure 5.4: Log₁₀ reduction in the total viable count of dispersed biofilm cells of (a) *Salmonella enterica* serovar Typhimurium SL1344 (b) *Salmonella enterica* serovar Agona when exposed to 3 to 7 ppm of sodium hypochlorite. Based on the limit of detection the maximum log₁₀ reduction which could be determined was (a) 3.7 (b) 5 (n=3). The error bars denote the standard deviation from the mean.

5.2.3 Inactivation kinetics using *Salmonella* in intact biofilms

For the associated method please see **Chapter Two, section 2.13.2.**

5.2.3.1 Hydrogen peroxide exposure

Preliminary biocide testing indicated that the most appropriate concentrations for further testing with hydrogen peroxide were between 2000 ppm and 25,000 ppm (data not shown).

The inactivation kinetics of *Salmonella* Typhimurium SL1344 when exposed to hydrogen peroxide showed that none of the concentrations tested gave a \log_{10} reduction of greater than 2 even after 60 mins exposure to 25000 ppm (fig. 5.5a). All concentrations show a similar trend with the initial \log_{10} reduction occurring in the first 20 mins and then remaining in a plateau over the remainder over the time course (with less than 0.7 \log_{10} reduction variation over the remaining 40 mins). All concentrations ranging from 5000 ppm to 25000 ppm gave between a one and two \log_{10} reduction over the time course (fig. 5.5a).

The inactivation kinetics of *Salmonella* Agona when exposed to hydrogen peroxide showed a very similar response to that of *Salmonella* Typhimurium SL1344. The maximum \log_{10} reduction was slightly greater than 2 (2.16) even after 60 mins exposure to 25000 ppm. All concentrations show a similar trend with the initial \log_{10} reduction occurring in the first 40 mins and then forming a plateau for the next 20 mins (figure 5.5b). This differs from the Typhimurium SL1344 data as the initial reduction only took 20 mins with that strain. Similarly, all concentrations ranging from 5000 ppm to 25000 ppm gave between a 1 and 2.2 \log_{10} reduction over the time course, showing a similar level of kill when it had reached the plateau phase (figure 5.5).

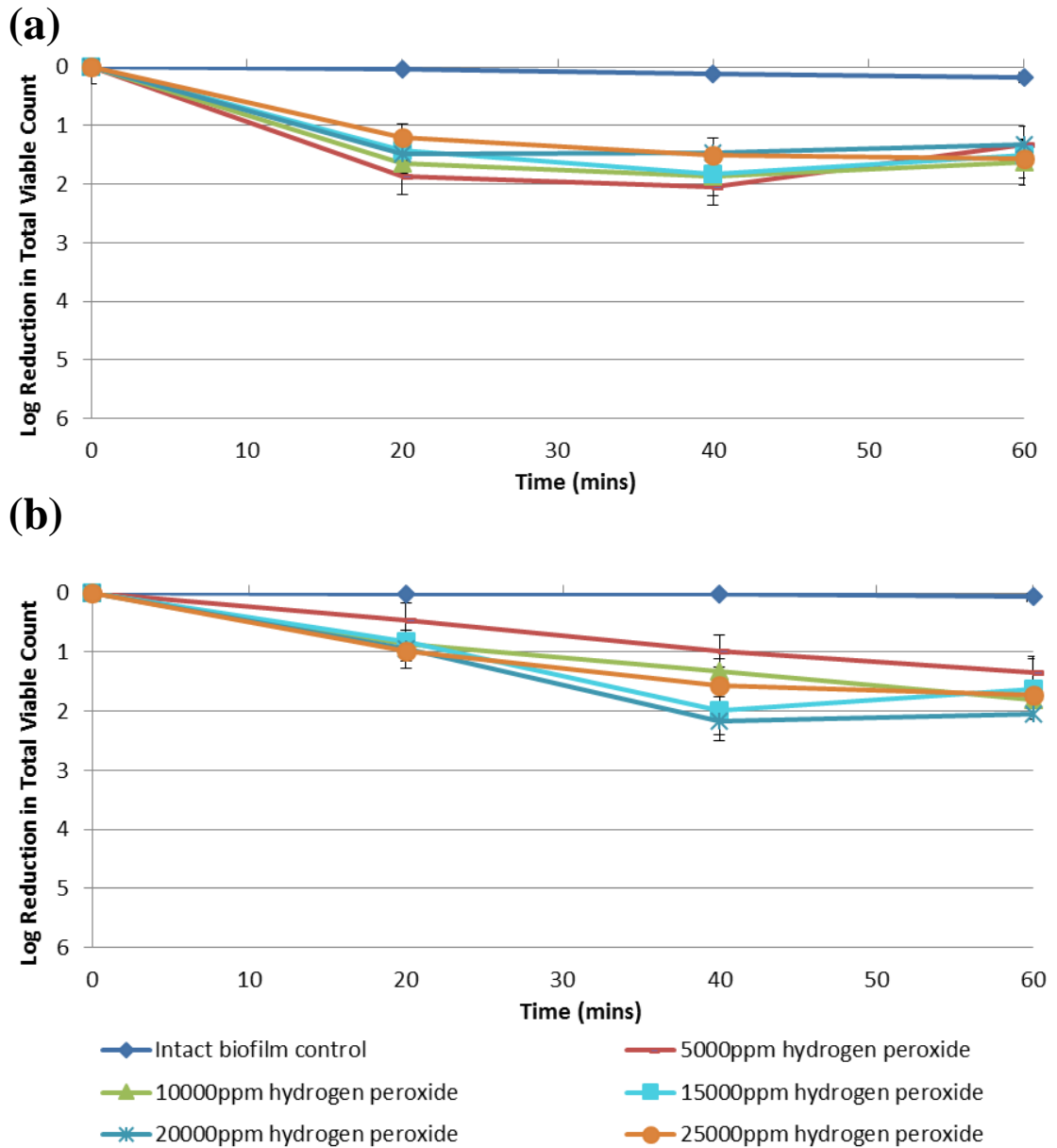


Figure 5.5: \log_{10} reduction in the total viable count of intact biofilm cells of (a) *Salmonella enterica* serovar Typhimurium SL1344 (b) *Salmonella enterica* serovar Agona when exposed to 5,000 ppm to 25,000 ppm hydrogen peroxide. Based on the limit of detection the maximum \log_{10} reduction which could be determined was a \log_{10} reduction of (a) 5.7 (b) 6 ($n=3$). The error bars denote the standard deviation from the mean.

As would be expected both strains of *Salmonella* are less susceptible to hydrogen peroxide when in the biofilm form or growth that in planktonic growth or in a disrupted biofilm.

5.2.3.2 Peracetic acid exposure

Preliminary biocide testing indicated that the most appropriate concentrations for further testing with peracetic acid were between 5 ppm and 40 ppm (data not shown).

The inactivation kinetics of *Salmonella* Typhimurium SL1344 when exposed to peracetic acid is unusual when compared to sodium hypochlorite and hydrogen peroxide. There is a great variation in responses as indicated by the large standard deviations and although some of the lines would be as expected in an inactivation kinetics (the response to 10 ppm and 20 ppm shows a continuous \log_{10} reduction 2.2, 3.7, 4.2 and 3.1, 5, 5.1 respectively over the time course) not all concentrations show a similar response (figure 5.6a). In the concentrations of 5 ppm, 30 ppm and 40 ppm there are increases at 40 or 60 mins (figure 5.6a). This may be due to incomplete disaggregation of the samples or interaction of the biocide in such a way that interferes with disaggregation so that counting is inaccurate or it could be due to some kind of recovery process.

Similarly the inactivation kinetics of *Salmonella* Agona when exposed to peracetic acid is also unusual. Again there are large standard deviations indicating great variation between responses. At the lower concentrations of biocide exposure (5 ppm and 20 ppm) there is an initial decline in the first 20 mins exposure that plateaus in the case of 5 ppm for the remainder of the time course or for 20 ppm for 20 mins until there is a final decrease in the number of survivors between 40 and 60 mins (figure 5.6b). In the higher concentrations of peracetic acid (35 ppm, 50 ppm and 60 ppm) there is a very noticeable increase in the number of detectable survivors between 40 mins and 60 mins with an increase of over one \log_{10} in the number of

survivors (figure 5.6b). This may be due to incomplete disaggregation of the samples or interaction of the biocide in such a way that interferes with disaggregation so that counting is inaccurate.

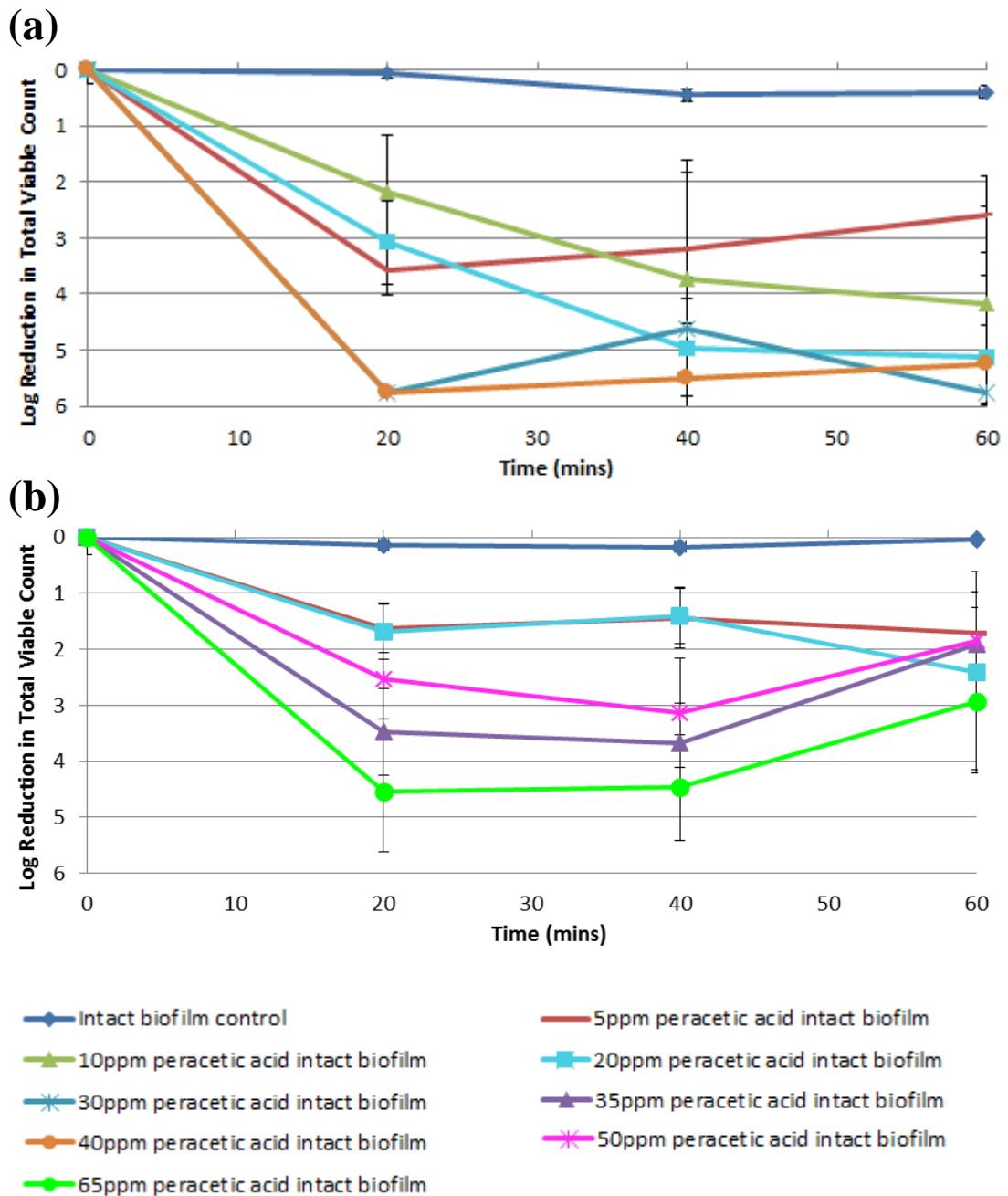


Figure 5.6: Log₁₀ reduction in the total viable count of intact biofilm cells of (a) *Salmonella enterica* serovar Typhimurium SL1344 (b) *Salmonella enterica* serovar Agona when exposed to 5 ppm to 65 ppm peracetic acid. Based on the limit of detection the maximum log₁₀ reduction which could be determined was a log₁₀ reduction of 6 (n=3). The error bars denote the standard deviation from the mean.

Both strains of *Salmonella* are less susceptible to peracetic acid when in the biofilm form or growth than in planktonic growth or in a disrupted biofilm.

5.2.3.3 Sodium hypochlorite exposure

Preliminary biocide testing indicated that the most appropriate concentrations for further testing with sodium hypochlorite were between 10 ppm and 100 ppm (data not shown).

The inactivation kinetics of *Salmonella* Typhimurium SL1344 when exposed to sodium hypochlorite showed similar patterns of \log_{10} reduction over the time course. The first 20 mins is where the greatest \log_{10} reduction was observed and after that there is a steadier decline to the limit of detection for concentrations above 20 ppm (figure 5.7a). Ten parts per million of sodium hypochlorite gives an initial 2.4 \log_{10} reduction at 20 mins which increases to 3.4 at 40 mins and 3.8 \log_{10} reduction at 60 mins (figure 5.7a). Concentrations of 20 ppm and above gave a \log_{10} reduction to the limit of detection after 40 mins exposure. Twenty parts per million of sodium hypochlorite then gives a decrease in the \log_{10} reduction at 60 mins of 1 \log_{10} . This is unusual as in all other concentrations a steady increase or a plateau is seen over the time course. However, as the standard deviation of this data point is 1 \log_{10} this may not be a true result and may be an experimental artefact.

The inactivation kinetics of *Salmonella* Agona when exposed to sodium hypochlorite showed similar patterns of \log_{10} reduction over the time course. All concentrations show an initial increase in the \log_{10} reduction in the first 20 mins of biocide exposure and then plateau after that (figure 5.7b). Twenty parts per million of sodium hypochlorite showed a 1.6 \log_{10} reduction in the first 20 mins which then plateaued

for the remainder of the time course. The concentrations of 40 ppm and 60 ppm remained between a \log_{10} reduction of 2.6 and 3.3 with a standard deviation of 0.4. Similarly, concentrations of 80 ppm and 100 ppm remained steadily in the region of a \log_{10} reduction of between 4.0 and 4.5 with a standard deviation of approximately 0.5.

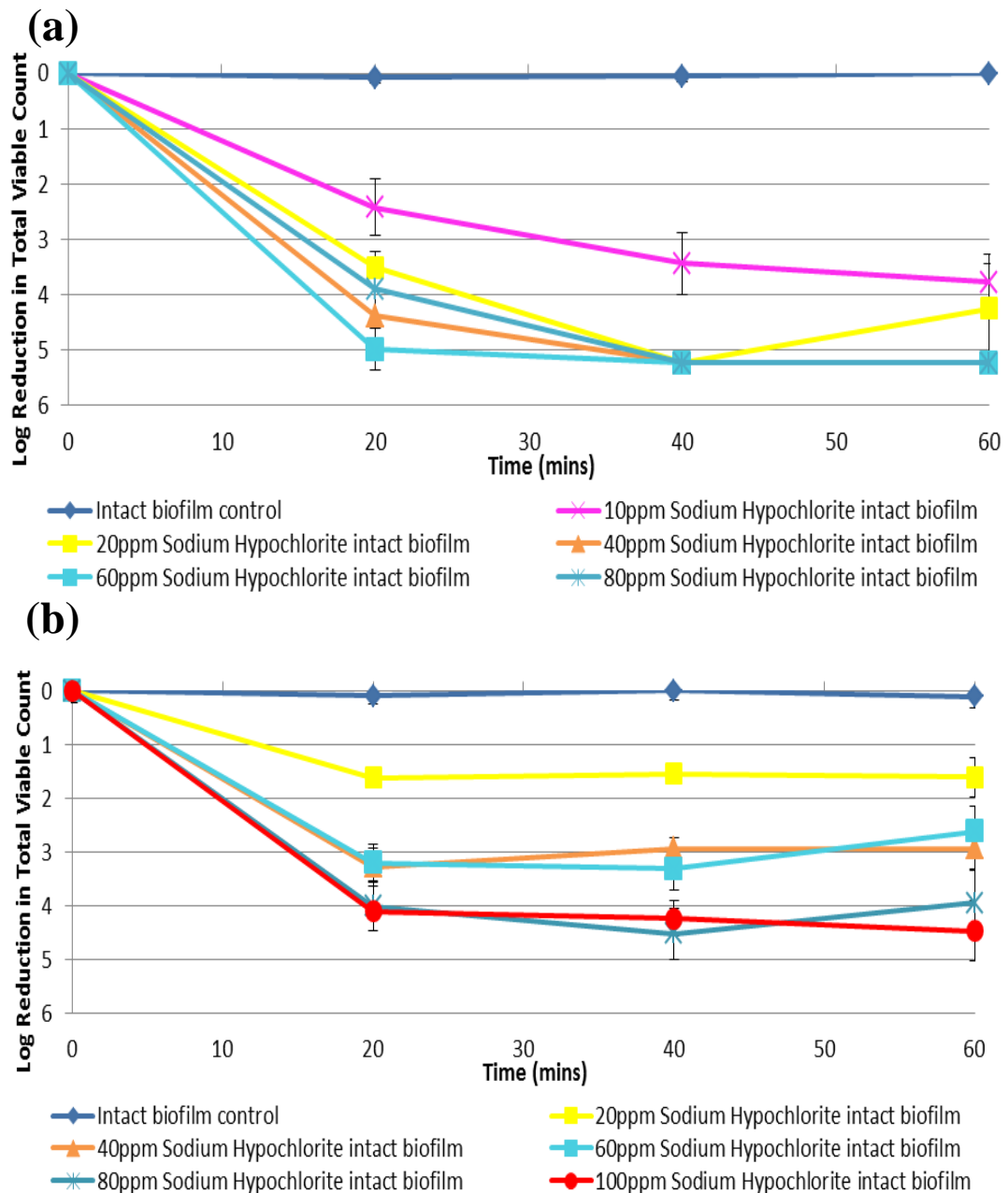


Figure 5.7: Log₁₀ reduction in the total viable count of intact biofilm cells of (a) *Salmonella enterica* serovar Typhimurium SL1344 (b) *Salmonella enterica* serovar Agona when exposed to 10 to 100 ppm of sodium hypochlorite. Based on the limit of detection the maximum log₁₀ reduction which could be determined was (a) 5.2 (b) 5 (n=3). The error bars denote the standard deviation from the mean.

Both strains of *Salmonella* are less susceptible to sodium hypochlorite when in the biofilm form or growth that in planktonic growth or in a disrupted biofilm. This effect is observed with both strains in response to all biocides.

5.3 Discussion

A reproducible biofilm model was developed using *Salmonella* cells grown in an MOPS based minimal media on a polystyrene surface at 30°C. The literature shows that bacterial cells adhere more strongly to hydrophobic surfaces than to hydrophilic surfaces (Kim *et al.*, 2012). This would be consistent with the findings that biofilms formed better on a plastic rather than a stainless steel surface as plastic is hydrophobic whereas stainless steel is hydrophilic (Pagedar *et al.*, 2010). It has also been shown that lower temperatures (Giaouris *et al.*, 2005) and low-nutrient environments act as a stressor promoting biofilm growth (Pagedar, *et al.*, 2010; Ryu, *et al.*, 2004; Stepanović *et al.*, 2004; Wong *et al.*, 2010a). Thus the combination of a hydrophobic surface, a low nutrient medium and a lower than optimum temperature produced high yield biofilms.

In the crystal violet experiments *Salmonella* Agona produced the highest yield of biofilm of the three strains tested, with Typhimurium SL1344 producing the lowest yield and Havana being an intermediate biofilm producer (fig 5.1 and table 5.3). *Salmonella* Typhimurium SL1344 is a poor biofilm former as it does not produce cellulose (García, *et al.*, 2004) which is an important part of the biofilm matrix (Hobley *et al.*, 2015). As a consequence of these results, it was decided to use an example of both a poor biofilm former and a strong biofilm former. Therefore, further studies have used *Salmonella* Typhimurium SL1344 as the poor biofilm former and *Salmonella* Agona as the strong biofilm former.

Inactivation kinetics have allowed a simple method for comparison of the efficacy of the biocides used against planktonic data presented in Chapter 3. Both dispersed biofilm cells and intact biofilms were exposed to hydrogen peroxide, peracetic acid

and sodium hypochlorite. Dispersed biofilm cells appear to be as sensitive as planktonic cells with concentrations well below those in-use for disinfection giving log₁₀ reductions up to the limit of detection. Intact biofilms however, are far less susceptible to biocides. This is what would be expected given the literature on biofilm recalcitrance (Chen *et al.*, 1993; Griebe *et al.*, 1993; Tachikawa *et al.*, 2005). Behnke, *et al.* (2011) found that the size of the clusters and the structure of the biofilm were crucial to the efficacy of disinfection, as the larger the cluster of cells the more difficult it was to disinfect.

The mechanisms that give *Salmonella* biofilms protection against oxidising biocides have yet to be fully investigated and there are several mechanisms that have been proposed that may be involved in biofilm recalcitrance. A full list of all the potential mechanisms that are involved can be found in Chapter One, section 1.5.3 (table 1.4). The most common reasons suggested are that the structure of the biofilm has a protective role and that the varying metabolic states and the presence of metabolically dormant ‘persister’ cells survive when the actively respiring cells are killed (Bridier, *et al.*, 2011). The presence of dormant ‘persister’ cells giving added protection seems unlikely given that dispersed biofilms were very similar in their susceptibility to planktonic cells. However, an inactivation kinetics only shows the ‘average’ response of the total biofilm which is heterogeneous and does not give information about individual cells. The lack of sensitivity of the test means that there may be dormant responses that cannot be detected by culture methods alone.

Bacterial cells in intact biofilms may be offered some protection by the presence of the extra-polymeric matrix. This may explain why *Salmonella* Typhimurium SL1344 is less tolerant to biocide exposure than *Agona* as it does not produce cellulose (García, *et al.*, 2004) and produces less EPS (as can be seen in the crystal violet

assays in figure 5.1 and table 5.3). However, even with poor EPS production *Salmonella* Typhimurium SL1344 was still considerably more tolerant of biocide in a biofilm structure than in planktonic form which indicated that biofilm structure may not be the only factor.

In summary, the work completed in this chapter has two key findings; the first is that intact biofilms are more tolerant of biocides than planktonic cells. The second is that dispersed biofilms lose much of the protective effect of an intact biofilm and respond in a similar way to planktonic cells. These two findings were applicable for both good and poor biofilm formers. Therefore, new biocides need to be tested against intact biofilms to give accurate data as to their efficacy in real world environments; as the data showed the concentration required to give the same level of \log_{10} reduction was much higher for intact biofilms than for planktonic cells. Comparison of the intact biofilm and dispersed biofilm data also indicates that the structure of the biofilm may have had a protective role for the bacteria. However, biofilm recalcitrance is a complex phenomenon and needs further investigation to be fully understood.

Chapter Six: Responses of
Salmonella grown in a
microfluidic flow biofilm
model to biocide challenge

6.1 Introduction

Bacterial biofilms can form under static conditions (see Chapter Five), or as is more likely in real-world environments they can form under flow conditions (Gomes *et al.*, 2014). There are a number of different systems that replicate flow conditions found in the environment such as a CDC biofilm reactor, the Robbins device and microfluidic flow systems (Gomes, *et al.*, 2014).

The Bioflux system was first described for the use of the investigation of the behaviour of biofilms by Benoit, *et al.* (2010). It is comprised of a Bioflux plate, a pressurised cover and a pneumatic pump (for the diagram see Chapter Two, section 2.14, figure 2.7). It is a continuous flow system; fresh media added to the inlet well, then flows through the serpentine tubes into the viewing channel where the biofilm grows and then flows out to the outlet well. The serpentine tubes help to keep the inlet well sterile so the media reaches the biofilm without contamination.

This Bioflux method of flow biofilm investigation was selected for a number of reasons; firstly the growth conditions were very easy to control using a computer attached to the pneumatic pump and were easily standardised by using the same computer settings (Benoit, *et al.*, 2010). Secondly, as the Bioflux plates were delivered sterile and do not require any assembly meaning there is a decreased risk of contamination. Thirdly, there is a built in coverslip on the viewing channel of the Bioflux plate meaning that cells can easily be microscopically examined without disturbing the system. Finally, the advantage of the microfluidic system is that the small volumes involved conserves reagents. The limitations of the system are the high cost of the Bioflux plates, the lack of access to the biofilms and lack of biofilm surface area meaning that the flow cells can become blocked if a good biofilm forms.

Confocal scanning laser microscopy uses an optical microscope that utilises lasers to give a high definition image when studying dense samples such as biofilms (Pantanella *et al.*, 2013). It allows for non-invasive optical sectioning, which can be processed to produce 3D images of the sample structure (Ross *et al.*, 2014). Confocal microscopy requires fluorescent staining of samples; this can be done in a number of different ways: either induction of the expression of a fluorescent gene such as a constituent GFP gene, or a GFP reporter on a gene of interest, or by staining using fluorescent dyes (Pamp *et al.*, 2009). Staining with a combination of SYTO9 and PI can give information as to the viability of cells within a biofilm as SYTO9 stains live cells and PI only stains cells that are dead (due to loss of membrane integrity). Staining with this combination of dyes is available in a commercial kit known as BacLight and is used effectively to study biofilm behaviour as seen in Mah *et al.* (2003).

The key advantage of using confocal scanning laser microscopy is that it is non-invasive and can be used to image live samples with minimal preparation. Another advantage is that depending on the fluorescent stains or GFP reporters used, important information as to the structure and behaviour of a biofilm can be discovered (Ross, *et al.*, 2014). In biocide studies this is useful as it provides a non-culture dependant method of investigating biofilm response to biocides. Confocal microscopy also allows for investigation of spatial analysis of the biofilm and to see if structural changes occur after biocide exposure. As images are taken in sections a view of the entire biofilm can be observed so spatial patterns of cell death can be investigated. A limitation of this methodology is that it gives a semi-quantitative result and only a limited number of fluorescent dyes can be used simultaneously which limits the amount of data that can be obtained (Pantanella, *et al.*, 2013).

The combination of microfluidic biofilm formation and the analysis of the resulting biofilms have already been used. Nance, *et al.* (2013) used the Bioflux system to grow multispecies biofilms in human saliva as a model for dental plaque. The mixed species biofilms were then exposed to a biocide challenge from cetylpyridinium chloride which is found in over the counter oral healthcare products. Biofilms were then stained using LIVE/DEAD from the BacLight kit to investigate the most effective concentration of biocide for biofilm removal. This study shows how the Bioflux microfluidic system can be effectively paired with confocal microscopy to investigate biocide efficacy.

In summary, the Bioflux system is an effective method of forming reproducible biofilms under flow conditions. It has been effectively paired with confocal microscopy to gain insight into the effect of biocides on flow biofilms. It would be valuable to use this system to further investigate the effect of oxidising biocides on *Salmonella enterica* biofilms.

6.1.1 Aims

- To examine how treatment with oxidising biocides can affect the percentage coverage of a biofilm in a microfluidic system and to determine if there is recovery after treatment.
- To investigate bacterial survival within a biofilm formed in a microfluidic system after a biocide challenge using membrane dyes (LIVE/DEAD).
- To explore the activity of oxidising biocides on different areas within the biofilm.

6.2 Results

6.2.1 Light microscopy and percentage coverage of different ages of biofilms

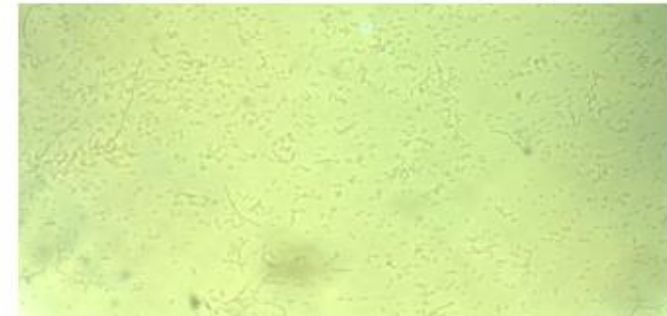
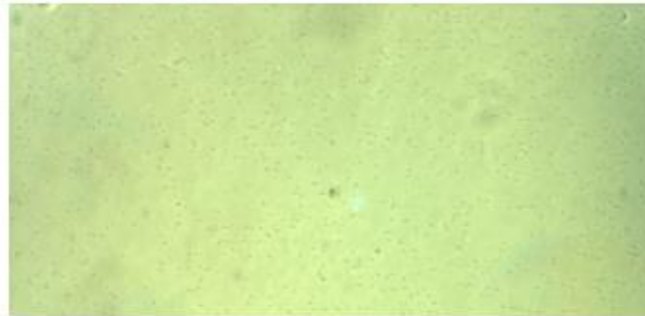
Overnight cultures of *Salmonella* were grown in TSB, centrifuged, washed and resuspended in MOPS based minimal media at an OD of 0.1 at 600 nm. The resulting cell suspension was used to inoculate the microfluidic channels of the Bioflux plates. After a 1 hr attachment period, fresh media was added to the inlet well and a flow rate of 0.3 dyne was set (for full details of the associated methods please see **Chapter Two, section 2.14 2.16**). After 24 hrs and 48 hrs images of the growth of *Salmonella* Typhimurium SL1344 and *Agona* were taken as shown in figure 6.1.

Figure 6.1 shows a comparison of the growth of *Salmonella* Typhimurium SL1344 and *Salmonella* *Agona* under flow conditions at 24 and 48 hrs. Clearly there was more coverage of the microfluidic viewing channel at 48 hours than 24 hours for both strains; but at both timepoints there was greater coverage when the channel was inoculated by *Salmonella* *Agona* (fig 6.1).

24 hours growth

48 hours growth

Salmonella
Typhimurium



Salmonella
Agona

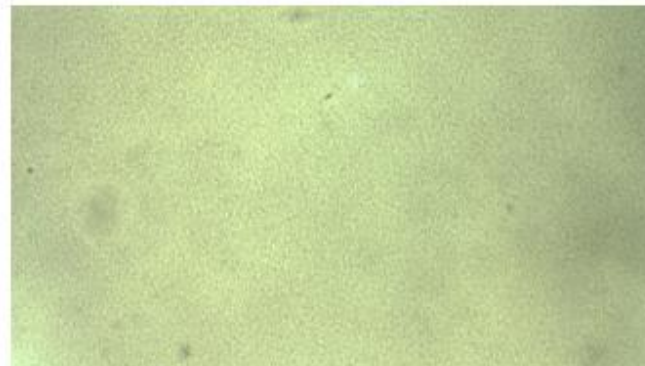


Figure 6.1: Light microscopy images taken of *Salmonella enterica* serovar Typhimurium SL1344 and Agona grown in the Bioflux microfluidic system.

The images shown in figure 6.1 were used with other replicates and an additional timepoint of 72 hrs, to generate the percentage coverage data shown in figure 6.2 below. The percentage coverage data was generated by the analysis of 3 different fields of view with the analyse function in the Bioflux software. The average was then taken and the standard deviation was calculated. This was consistent with the data gathered in Chapter Five (section 5.2.1.3, figure 5.1 and table 5.3) that showed *Salmonella* Agona was a better biofilm former in static conditions. Figure 6.2 showed that *Salmonella* Agona was a better biofilm former under flow conditions. There was an increase in percentage coverage over time for both *Salmonella* Typhimurium SL1344 and Agona as would be expected with a growing biofilm.

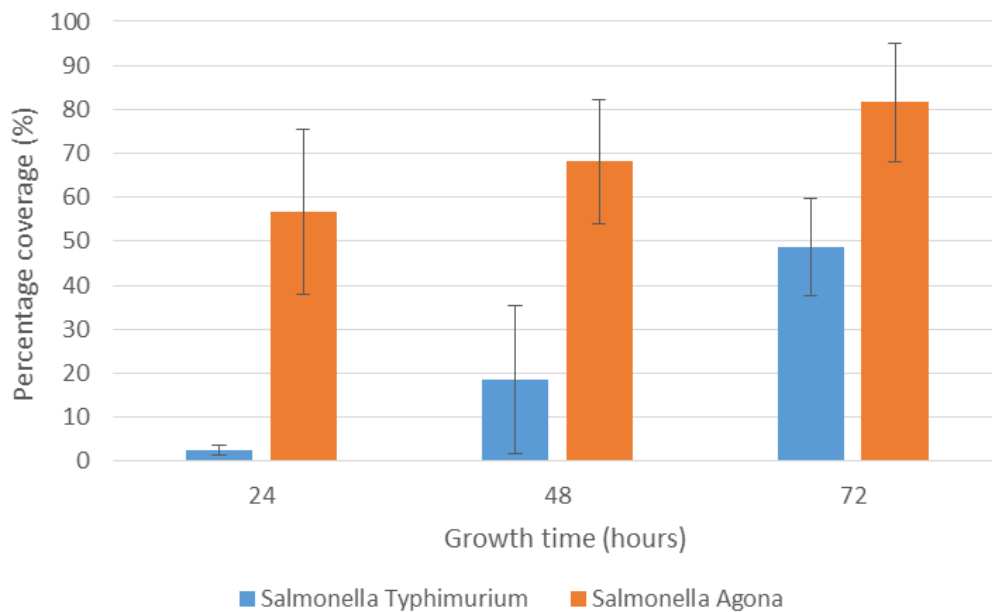


Figure 6.2: Growth of *Salmonella enterica* serovar Typhimurium SL1344 and Agona measured as percentage coverage of the surface of the microscope viewing chamber on Bioflux plate (n=12). Grown in MOPS based minimal media at 20°C (room temperature) for either 24 hrs, 48 hrs and 72 hrs. The error bars denote the standard deviation from the mean.

6.2.2 Light microscopy and percentage coverage of biofilms exposed to oxidising biocides

Salmonella biofilms were grown in the Bioflux plates as described in **Chapter Two, section 2.14** for either 24 or 48 hrs. The biofilms were then exposed to biocide for 60 min and then washed and fresh medium was added (please see **Chapter Two, section 2.15**). The biocide concentrations used were: 25,000 ppm hydrogen peroxide, 30 ppm peracetic acid and 40 ppm sodium hypochlorite. Images of the biofilms were taken before and after biocide exposure and 24 hrs post-biocide exposure to investigate the bacterial recovery. These images were then analysed with the Bioflux software which gave values for percentage coverage; the average of three fields of view were used to give the percentage coverage values (please see **Chapter Two, section 2.16**). The percentage coverage values of the biofilms before exposure to biocide were then subtracted from the percentage coverage values after biocide exposure, to give the change in percentage coverage.

Figure 6.3 showed the change in percentage coverage of biocide exposed biofilms compared to the pre-treatment control. There was a statistically significant increase in biofilm percentage coverage in a number of biocide treated biofilms, predominantly seen in *Salmonella* Typhimurium SL1344. The increase in biofilm percentage coverage was seen in *Salmonella* Typhimurium SL1344 biofilms after 24 hrs growth with 1hr exposure to hydrogen peroxide ($p=0.01$), after 48 hrs growth, 1hr exposure hydrogen peroxide and 24 hrs recovery ($p=0.02$) and after 48 hrs growth, 1 hr exposure to peracetic acid followed by 24 hrs recovery ($p=0.01$). Figure 6.3 also showed an increase in the biofilm percentage coverage in *Salmonella* Agona after 48 hrs growth and 1 hr exposure to peracetic acid ($p=0.01$).

Figure 6.3 also showed a decrease in the biofilm percentage coverage as seen in *Salmonella* Typhimurium SL1344 after 24 hrs growth, 1hr exposure to sodium

hypochlorite ($p=0.02$) and in the same condition after 24 hrs recovery ($p=0.03$). In *Salmonella* Agona a decrease in biofilm percentage coverage was seen after 24 hrs growth, 1 hr exposure to peracetic acid and 24 hrs recovery ($p=0.04$) and 48 hrs growth, 1 hr exposure to sodium hypochlorite and 24 hrs recovery ($p=0.01$).

There are a number of trends that are apparent on fig 6.3. Firstly, as mentioned previously there seem to be more statistically significant increases in the percentage coverage in *Salmonella* Typhimurium SL1344 biofilms exposed to biocides. Secondly, exposure to sodium hypochlorite seems to have a consistent effect across both strains and ages of biofilm in decreasing the percentage coverage. Hydrogen peroxide exposure gave a consistent increase in the percentage coverage of *Salmonella* Typhimurium SL1344 biofilms and also gave a less consistent (and not statistically significant) increase in *Salmonella* Agona biofilms. Finally, biofilms exposed to peracetic acid did not show a consistent pattern of increased or decreased percentage coverage.

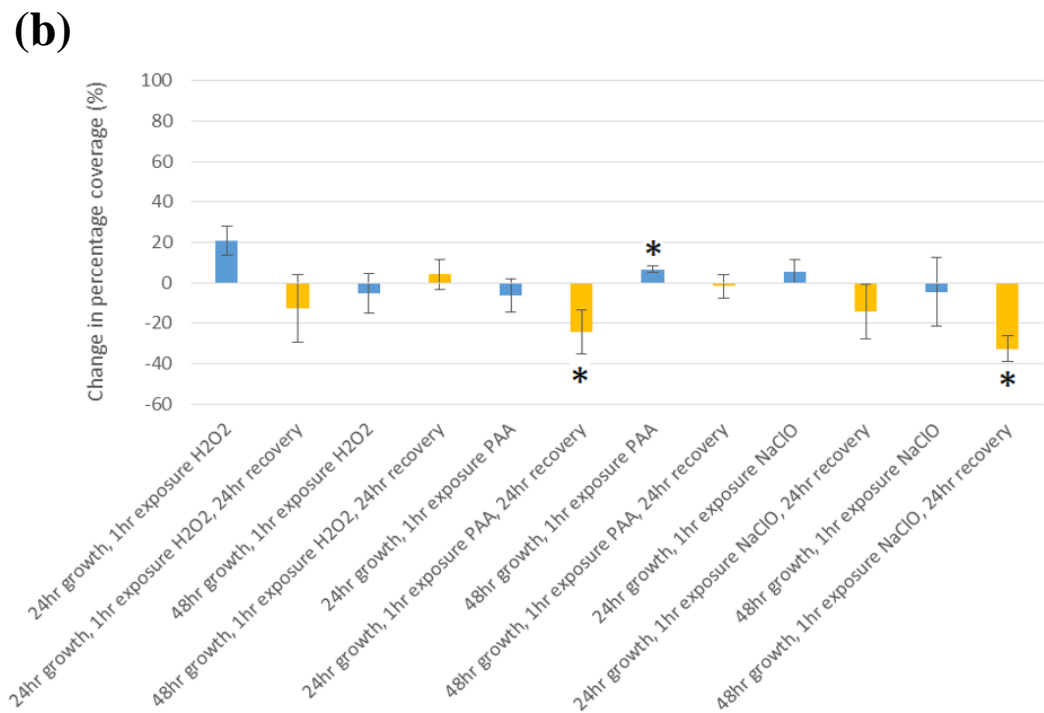
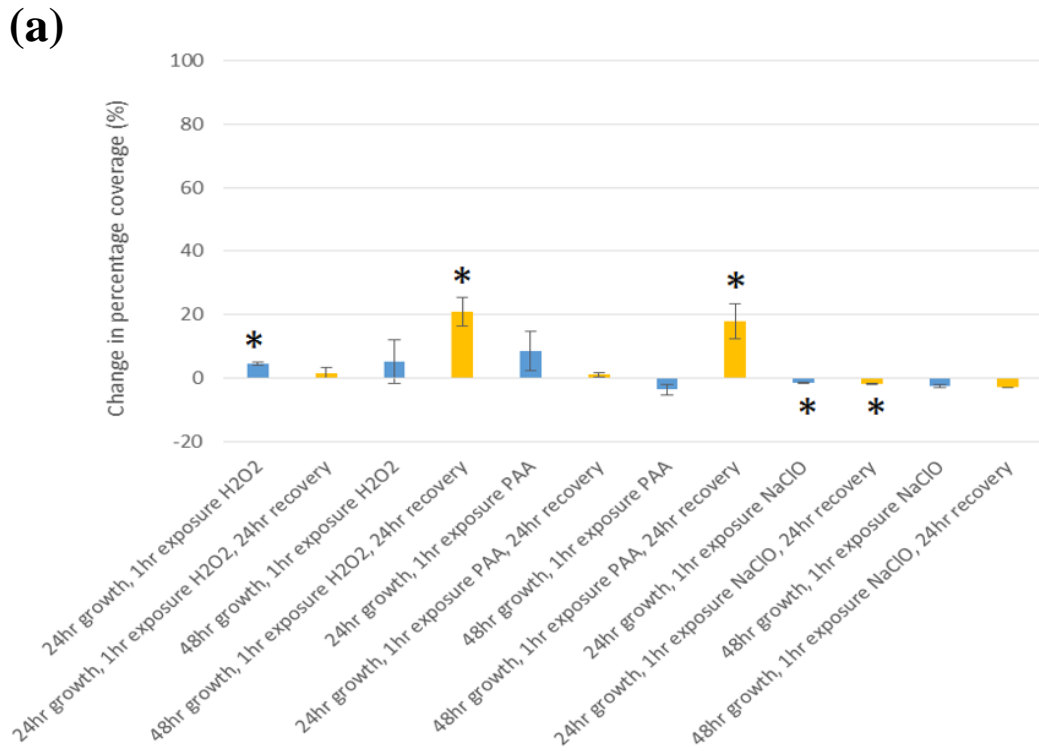


Figure 6.3: Change in the percentage coverage of *Salmonella enterica* serovar (a) Typhimurium SL1344 (b) *Agona* biofilms grown for either 24 hrs or 48 hrs and then exposed to oxidising biocides. Photographs were taken of the same biofilm at the following stages: pre-exposure, after 1 hour exposure and with 24 hrs recovery (n=3). The asterisks denote a statistically significant ($p \leq 0.05$) change in percentage coverage from biofilm pre-exposure according to a Student's T-test. The error bars denote the standard deviation from the mean.

6.2.3 Confocal microscopy with LIVE/DEAD staining images

Microfluidic channels in the Bioflux plate were inoculated with *Salmonella* grown overnight in TSB, washed and resuspended in MOPS based minimal media at an OD of 0.1 at 600 nm. The *Salmonella* cells were left in static conditions to attach for one hour and then 0.3 dyne of flow was added (see **Chapter Two, sections 2.14, 2.15 and 2.17**). All confocal images can be found on the CD in the appendix.

Figure 6.4a is an image of *Salmonella* Typhimurium SL1344 biofilm grown for 24 hrs that was untreated. After 24 hours there were only small clusters of attached cells which were mostly stained by STYO9 indicating they were alive. Figure 6.4b is an image of a *Salmonella* Typhimurium SL1344 exposed to 70% ethanol for 1 hr, most of the cells are stained red with PI which indicates that they are dead.

Figure 6.4c and fig 6.4d show the untreated control and the ethanol treated control after 48 hours of *Salmonella* Typhimurium biofilm growth. It can be observed that after 48 hours there is considerably more growth of the *Salmonella* Typhimurium SL1344 biofilm (fig 6.4c and 6.4d), however it still only forms a monolayer of cells. There is also considerably less cell death seen as PI fluorescence after exposure to one hour of ethanol when the biofilm was grown for 48 hrs (fig 6.4b and 6.4d).

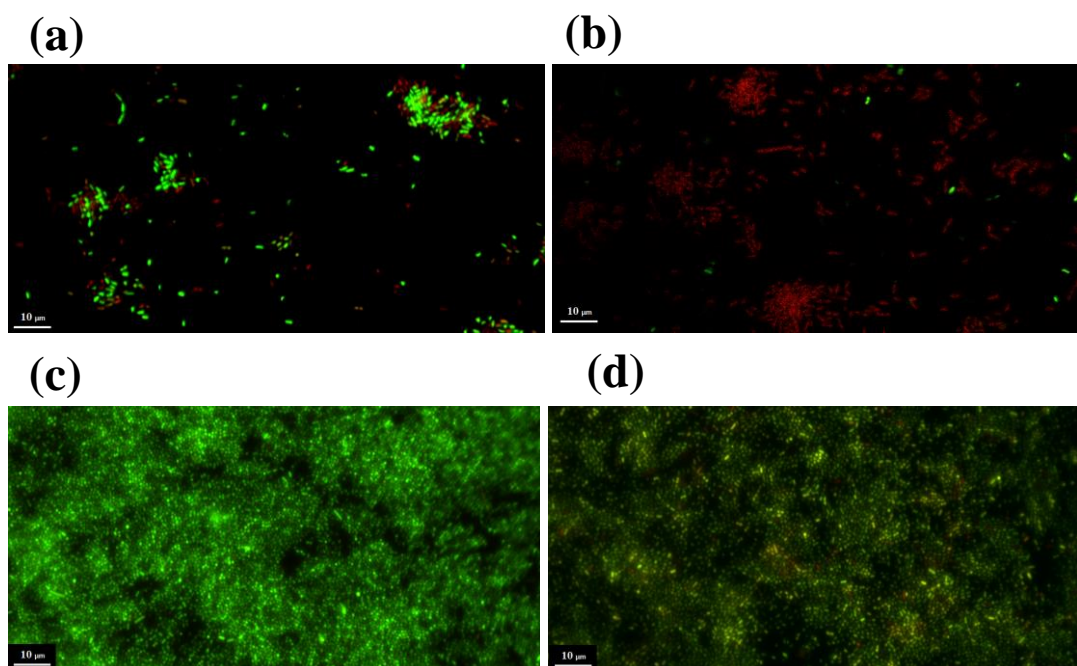


Figure 6.4: Representative confocal microscopy controls for *Salmonella enterica* serovar Typhimurium SL1344 grown in a microfluidic flow system for (a and b) 24 hrs (c and d) 48 hrs at 20°C (room temperature); live untreated cells and cells exposed to 70% ethanol for 60 mins. Cells were stained with PI and STYO9; live cells were shown in green and dead cells were shown in red. Imaged using a x63 magnification in oil.

Figure 6.5 and 6.6 show *Salmonella* Typhimurium SL1344 biofilms grown for 24 hours and 48 hours respectively in the Bioflux microfluidic system. The biofilms were then exposed to oxidising biocides for either 20, 40 or 60 mins, washed and stained with SYTO9 and PI.

The first column of figure 6.5 shows representative images of 24 hour *Salmonella* Typhimurium SL1344 biofilms exposed to hydrogen peroxide. After 20 and 40 mins exposure the vast majority of observable cells were alive (stained green by SYTO9). However, after 60 mins exposure all observable cells are stained red with PI indicating that they are dead. The second column of fig 6.5 shows peracetic acid exposure to 24 hour *Salmonella* Typhimurium SL1344 biofilms throughout the time course there are a mixture of live and dead cells present, however more PI stained cells can be seen after 60 mins peracetic acid exposure. The third column of figure

6.5 showed images of *Salmonella* Typhimurium SL1344 biofilms exposed to sodium hypochlorite. Sodium hypochlorite exposed biofilms show a similar pattern to peracetic acid exposed biofilms in that more cells are stained with SYTO9 after 20 and 40 mins and more cells are stained with PI after 60 mins.

In figure 6.6 *Salmonella* Typhimurium SL1344 biofilms were grown for 48 hours under flow conditions in the Bioflux before exposure to hydrogen peroxide, peracetic acid and sodium hypochlorite. The majority of cells within the biofilms fluoresce green which indicated they had survived all the biocide challenges at all timepoints. Hydrogen peroxide shows a reduction in the biofilm as less cells appear to be attached after 40 and 60 mins biocide exposure. After exposure to hydrogen peroxide it could be observed using both the light and confocal microscope that there was an increase in unattached live cells moving in the PBS in the microfluidic channel. This may be linked to the decrease in attachment seen in the confocal microscopy images. There also appeared to be a decrease in the mass of the biofilm after 60 mins exposure to peracetic acid and sodium hypochlorite, but the increase in planktonic live cells seen with hydrogen peroxide was not seen in these cases.

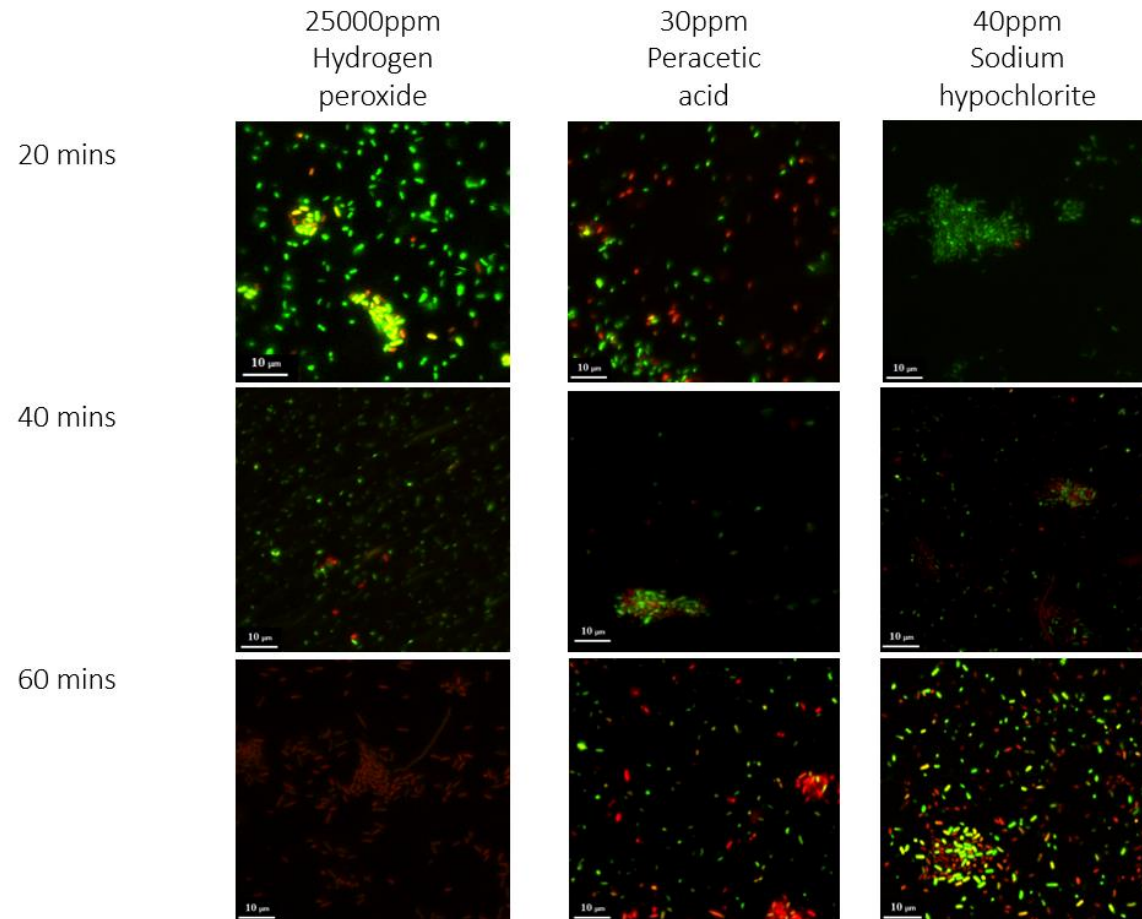


Figure 6.5.: Representative confocal microscopy images of *Salmonella enterica* serovar Typhimurium SL1344 exposed to hydrogen peroxide, peracetic acid and sodium hypochlorite for 20, 40 or 60 mins. *Salmonella* biofilms were grown in a microfluidic flow system for 24 hrs at 20°C (room temperature). Cells were stained with PI and STYO9; live cells were shown in green and dead cells were shown in red. Imaged using a x63 magnification in oil.

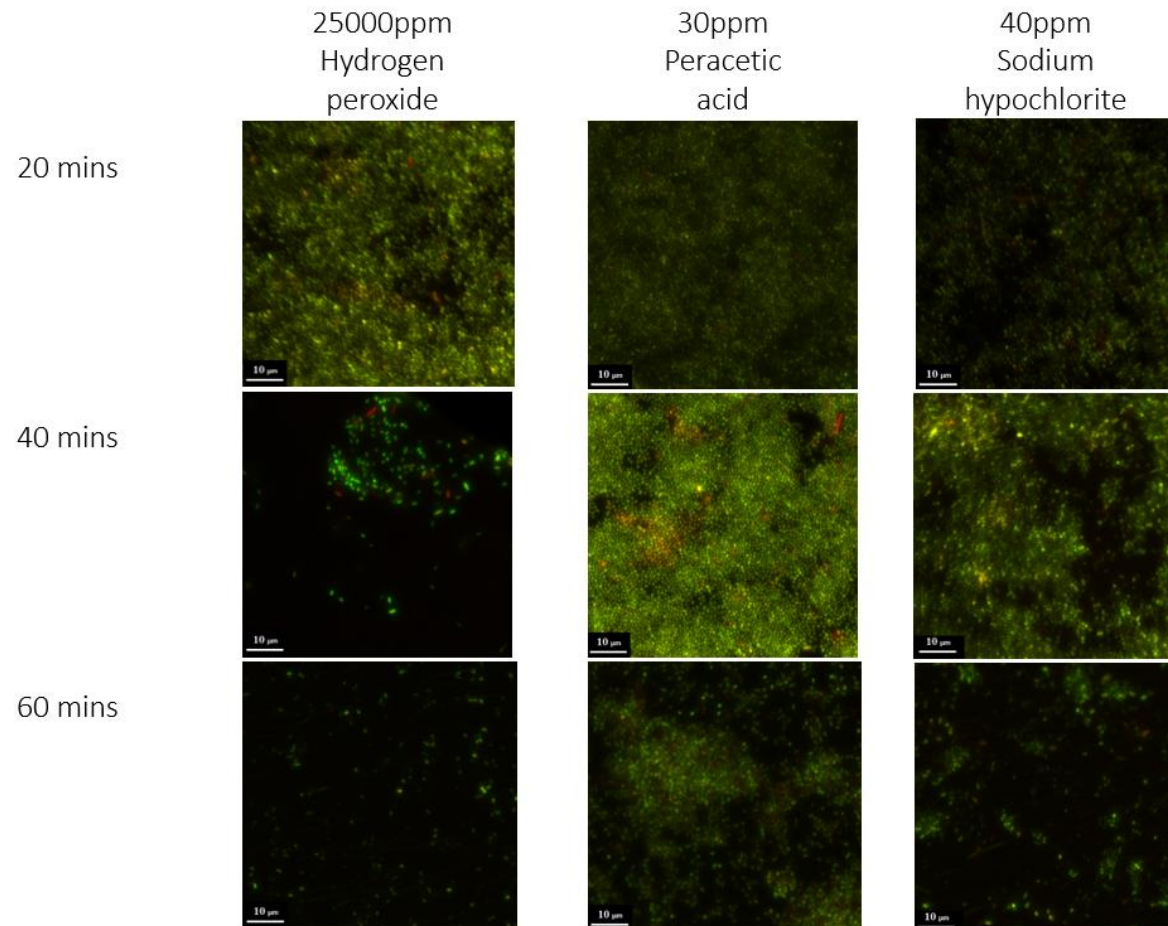


Figure 6.6: Representative confocal microscopy images of *Salmonella enterica* serovar Typhimurium SL1344 exposed to hydrogen peroxide, peracetic acid and sodium hypochlorite for 20, 40 or 60 mins. *Salmonella* biofilms were grown in a microfluidic flow system for 48 hrs at 20°C (room temperature). Cells were stained with PI and STYO9; live cells were shown in green and dead cells were shown in red. Imaged using a x63 magnification in oil.

Figure 6.7 shows the confocal microscopy controls for *Salmonella* Agona. *Salmonella* Agona is a much better biofilm former than Typhimurium SL1344 as was discussed in Chapter Five (section 5.3). The confocal microscopy data is consistent with this finding. Figure 6.7a shows an untreated *Salmonella* Agona biofilm grown for 24 hours, most of the cells are stained with SYTO9 with a few stained by PI; figure 6.7c has a similar image. In figure 6.7b and d which have been treated for 1 hour with 70% ethanol all cells are stained with PI indicating that they were all dead. Figure 6.7d differs slightly from figure 6.7b in that the coverage is less consistent but there was greater development of the biofilm structure which led to greater masses in certain areas.

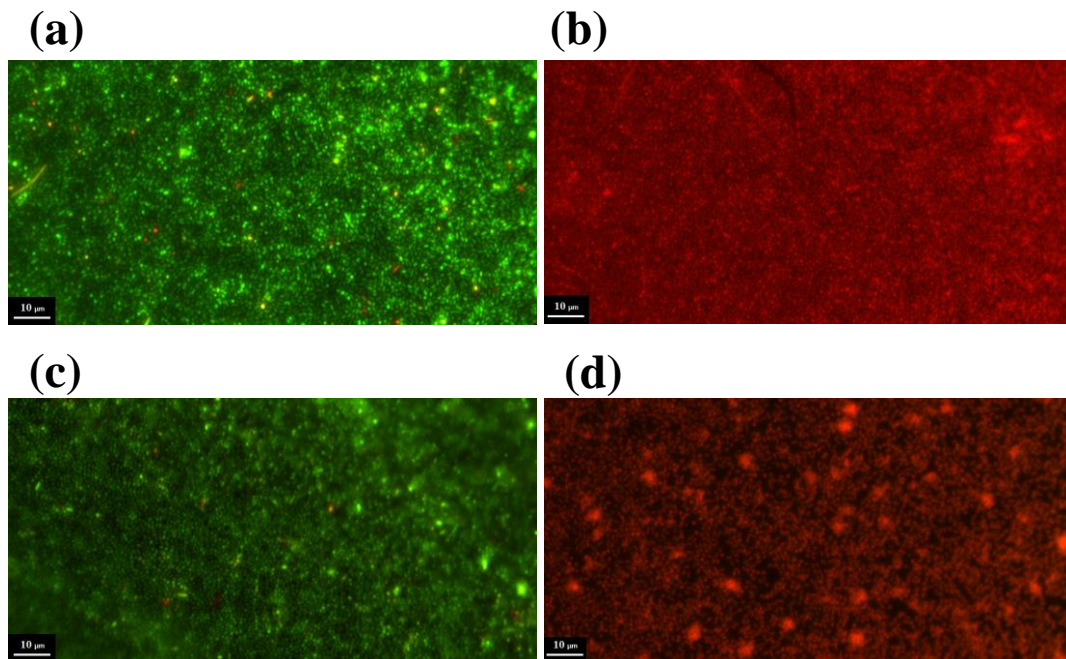


Figure 6.7: Representative confocal microscopy controls for *Salmonella enterica* serovar Agona grown in a microfluidic flow system for (a and b) 24 hrs (c and d) 48 hrs at 20°C (room temperature); live untreated cells and cells exposed to 70% ethanol for 60 mins. Cells were stained with PI and STYO9; live cells were shown in green and dead cells were shown in red. Imaged using a x63 magnification in oil.

The images in figure 6.8 were taken after a *Salmonella* Agona biofilm was grown for 24 hours and then exposed to oxidising biocides for 20, 40 and 60 mins. When the biofilm was exposed to hydrogen peroxide for 20 mins and 40 mins there was a mix between live and dead cells (shown as green and red respectively). After 60 mins however, all observable cells were stained with PI indicating that they are dead due to loss of membrane integrity (fig 6.8). The *Salmonella* Agona biofilms grown for 24 hours and treated with peracetic acid or sodium hypochlorite (fig 6.8) show a similar pattern; the vast majority of observable cells within the biofilm are dead with a small number of live cells randomly distributed across the field of view.

Figure 6.9 is composed of images taken from *Salmonella* Agona biofilms grown for 48 hours and then exposed to oxidising biocides. They presented a very different set of results from those shown in figure 6.8. The great majority of cells with the 48 hour biofilms fluoresced green (due to the staining of SYTO9) indicating they were alive across all biocide treatments and all timepoints. There were some observable red cells in the images but so few, it is difficult to visually distinguish images of treated cells from untreated controls. The only observable effect seen is a decrease in cell density in *Salmonella* Agona biofilms treated with hydrogen peroxide for 40 mins and 60 mins. However, the cells that remain appeared to be alive as they emitted a green fluorescence (fig 6.9).

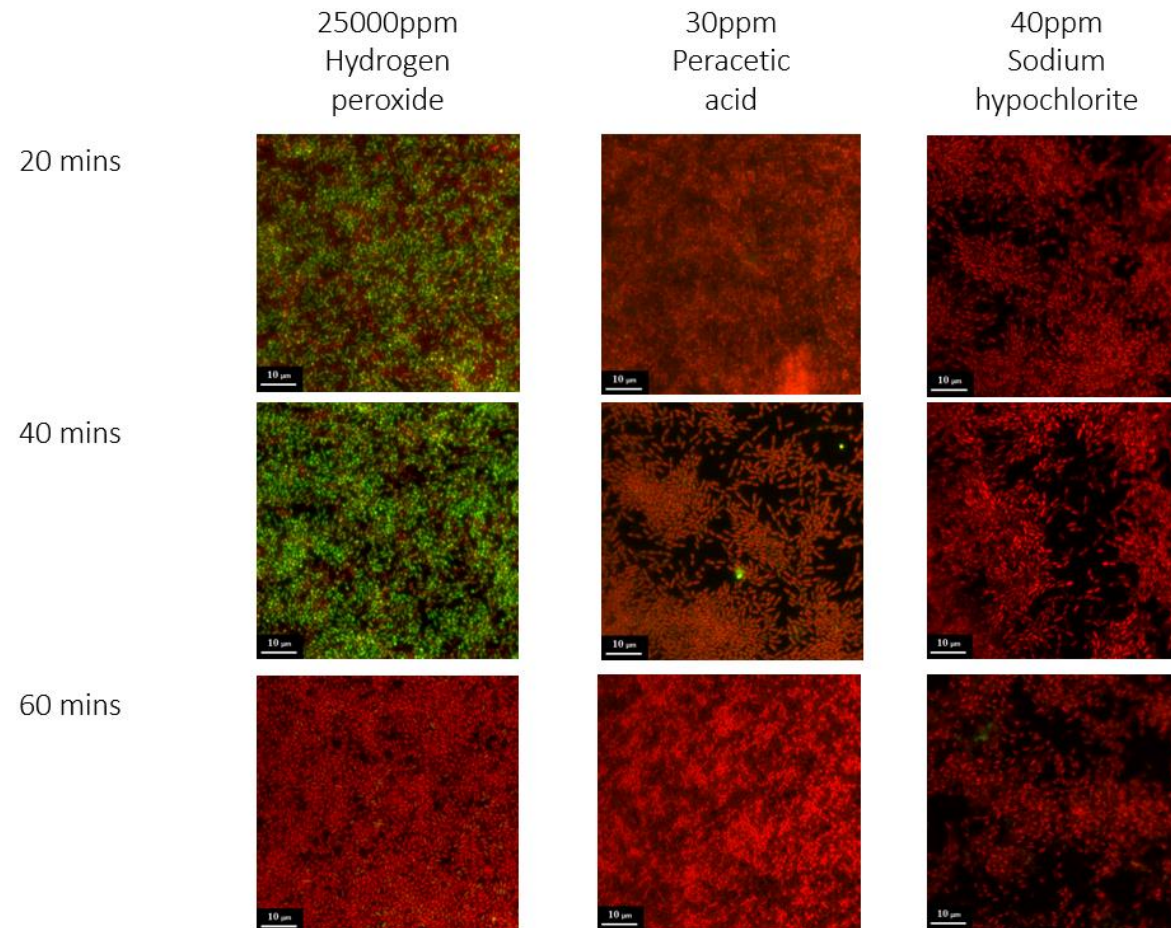


Figure 6.8: Representative confocal microscopy images of *Salmonella enterica* serovar Agona exposed to hydrogen peroxide, peracetic acid and sodium hypochlorite for 20, 40 or 60 mins. *Salmonella* biofilms were grown in a microfluidic flow system for 24 hrs at 20°C (room temperature). Cells were stained with PI and STYO9; live cells were shown in green and dead cells were shown in red. Imaged using a x63 magnification in oil.

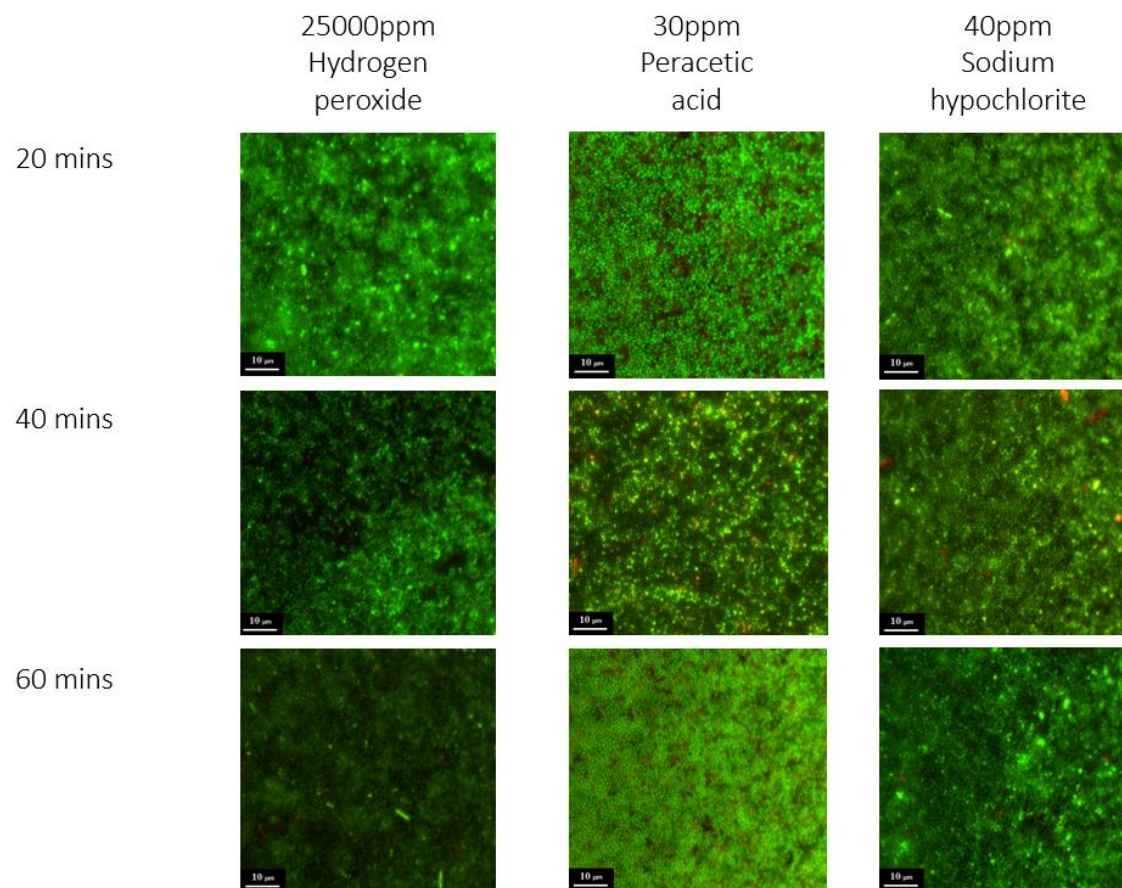


Figure 6.9: Representative confocal microscopy images of *Salmonella enterica* serovar Agona exposed to hydrogen peroxide, peracetic acid and sodium hypochlorite for 20, 40 or 60 mins. *Salmonella* biofilms were grown in a microfluidic flow system for 48 hrs at 20°C (room temperature). Cells were stained with PI and STYO9; live cells were shown in green and dead cells were shown in red. Imaged using a x63 magnification in oil.

6.2.4 *Salmonella enterica* serovar Agona Confocal Z-stacks

Salmonella Agona produced good biofilms that formed a 3D structure quickly, which meant that in addition to the 2D images shown in section 6.2.3, 3D images were produced. These were produced when a series of images taken at 0.5 μm intervals (a Z stack) were combined. These Z stacks were used to gain a further insight as to the areas in which the biofilm structure was affected by biocide exposure. For full methods see **Chapter Two, sections 2.14, 2.15 and 2.17**, all confocal Z stack images can be found on the CD in the appendix.

The Z-stack images show the 3D structure of the biofilms. They are not flat structures as they have 'peaks' where the biofilm has developed in some areas differently to others. The advantage of using Z-stacks is that these structural variations can be seen clearly and investigated.

Figure 6.10 is composed of assembled Z stacks taken of control biofilms that were either untreated or treated with 70% ethanol. Figure 6.10a and fig 6.10b show the 3D structure of *Salmonella* Agona biofilms grown in a microfluidic flow cell; fig 6.10a in the untreated control, almost all cells are alive and in 6.10b all cells are dead. In figure 6.10c there cells are predominately alive but there are some dead cells present. Figure 6.10d shows complete cell death and it can be seen that this is consistent across the entire 3D structure including raised masses present on the surface down to the very bottom.

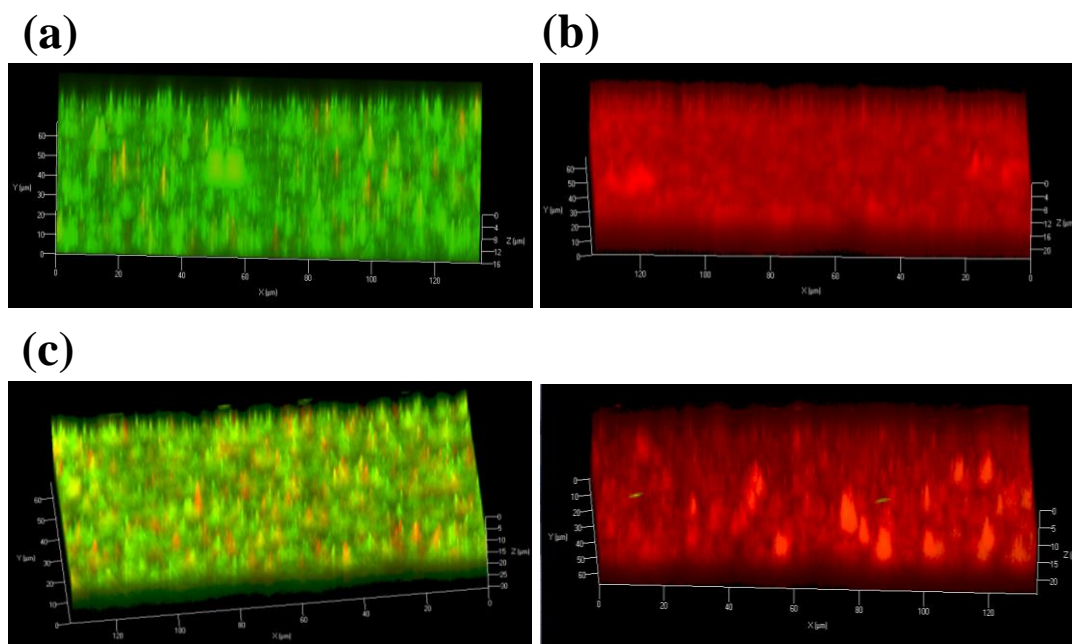


Figure 6.10: Confocal microscopy controls for *Salmonella enterica* serovar Agona grown in a microfluidic flow system for (a and b) 24 hrs and (c and d) 48 hrs at 20°C (room temperature); live untreated cells and cells exposed to 70% ethanol for 60 mins. Cells were stained with PI and STYO9; live cells were shown in green and dead cells were shown in red. Imaged using a x63 magnification in oil.

The most obvious difference between the 2D images shown in fig 6.8 and 6.9 are the voids that can be seen in the biofilm structure after exposure to biocide. This can be seen in figure 6.11 after only 24 hours of biofilm growth, voids can be seen appearing in the structure after exposure to all of the biocides. This is most obvious when observing hydrogen peroxide at 40 mins exposure and at all timepoints of sodium hypochlorite exposure (fig 6.11). The other advantage of 3D images over their 2D counterparts was that the areas where biofilm cell death occurred was clearly visible. For instance, in fig 6.11, after 20 mins exposure to hydrogen peroxide, sporadic cell death was seen throughout the biofilm.

Figure 6.12 was made up of Z stacks of *Salmonella* Agona biofilms grown for 48 hours and then exposed to oxidising biocides. There was less cell death observed in the more mature biofilms compared to the younger biofilms seen in figure 6.11. However, figure 6.12 further confirms that when cell death does occur in a biofilm as

a result of biocide exposure, the areas of cell death are clumped together in a random pattern. This response can be seen in the biofilms exposed to peracetic acid and sodium hypochlorite. The biofilms exposed to hydrogen peroxide have shown sporadic areas of cell death after 20 mins exposure, but after 40 mins and 60 mins very little cell death was observed. Instead, the dispersal of the biofilm was visible. Large voids appeared in *Salmonella Agona* biofilms that were grown for 48 hours (fig 6.12) after 40 mins and 60 mins of hydrogen peroxide exposure.

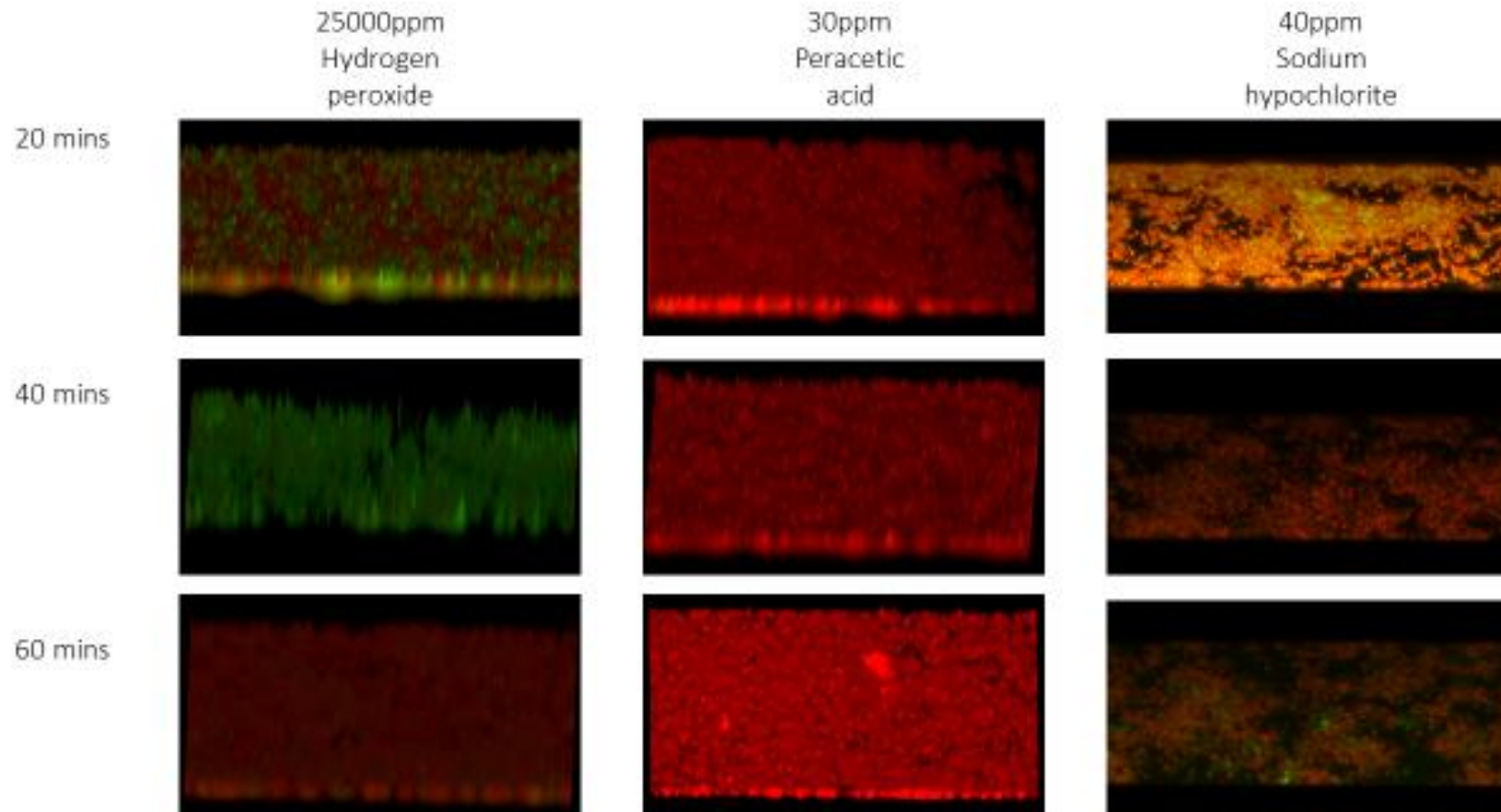


Figure 6.11: Confocal microscopy images of *Salmonella enterica* serovar Agona exposed to hydrogen peroxide, peracetic acid and sodium hypochlorite for 20, 40 or 60 mins. *Salmonella* biofilms were grown in a microfluidic flow system for 24 hrs at 20°C (room temperature). Cells were stained with PI and STYO9; live cells were shown in green and dead cells were shown in red. A series of images were taken at 0.5 μm intervals and combined in Zen or ImageJ software to form 3D images. Imaged using a x63 magnification in oil.

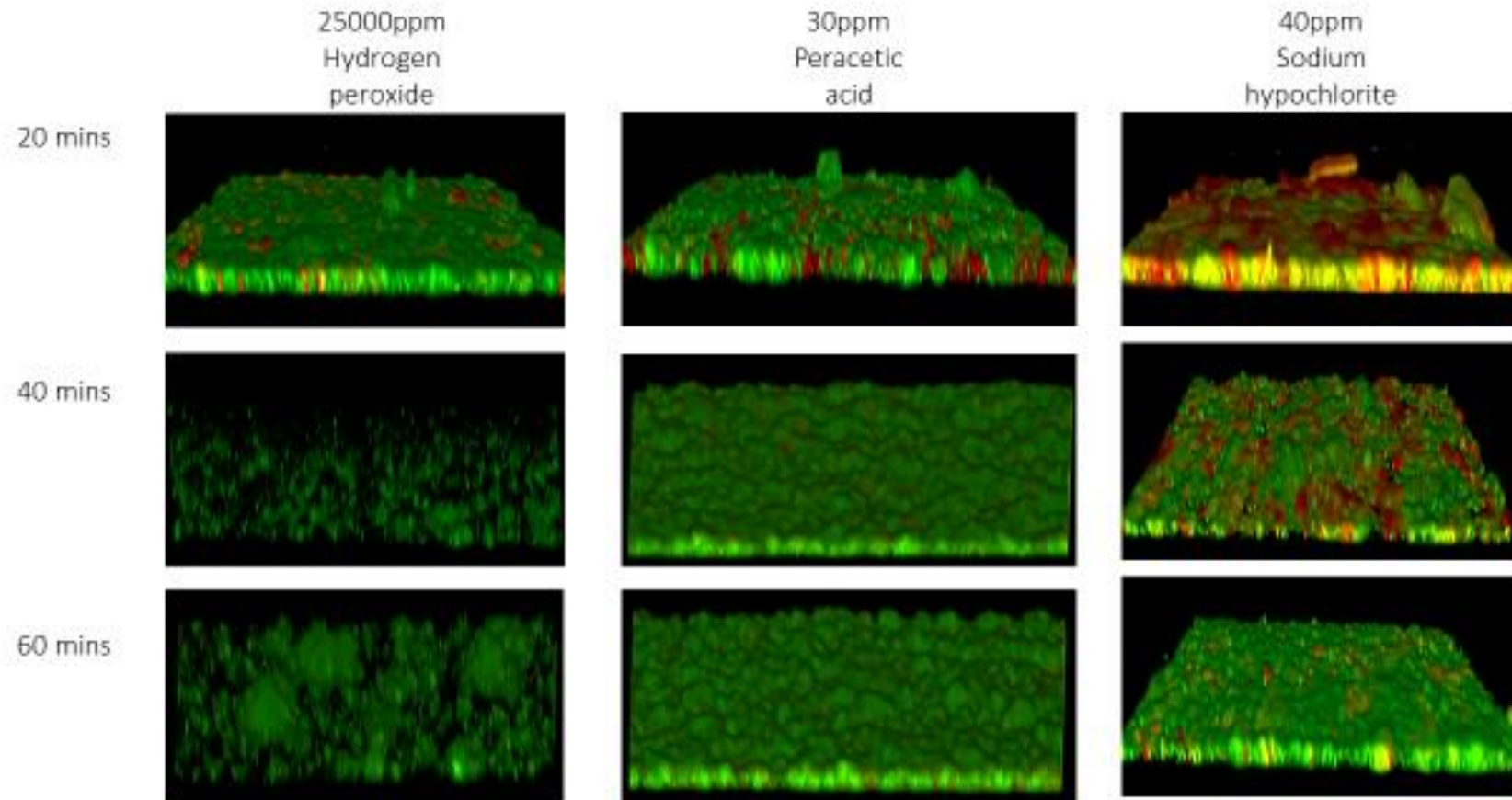


Figure 6.12: Confocal microscopy images of *Salmonella enterica* serovar Agona exposed to hydrogen peroxide, peracetic acid and sodium hypochlorite for 20, 40 or 60 mins. *Salmonella* biofilms were grown in a microfluidic flow system for 48 hrs at 20°C (room temperature). Cells were stained with PI and STYO9; live cells were shown in green and dead cells were shown in red. A series of images were taken at 0.5 μm intervals and combined in Zen software to form 3D images. Imaged using a x63 magnification in oil.

6.2.5 Quantitative analysis of confocal microscopy images

For some experiments a complex pattern of live and dead cells was seen, spread in a non-uniform way. However, on some occasions numbers of live and dead cells were spread relatively equally across the flow cell making these amenable to quantitative analysis. To quantify the number of live and dead cells in a confocal image, the image was split into red and green channels using ImageJ to give a monochrome output image. The single channel images were then analysed using the Bioflux EZ percentage coverage tool which gave a percentage for the coverage of cells stained red and green.

Salmonella Agona exposed to hydrogen peroxide was selected as the confocal images shown in section 6.2.3 and 6.2.4 seemed to indicate a different response to biocide exposure than that seen in cells exposed to peracetic acid and sodium hypochlorite. Biofilm cells exposed to peracetic acid and sodium hypochlorite seemed to fluoresce either red or green, however cells exposed to hydrogen peroxide also seemed to disperse from the biofilm. The method of quantification selected allowed for coverage to be quantified as well as percentage red or green.

There were a greater percentage of PI stained cells in the untreated control at 48 hours (53%) than 24 hours (6%) indicating there is more cell death as the biofilm matures (fig 6.13). There was also an increase in the percentage of cells stained by PI in the ethanol treated control after 48 hours (81%) compared to 24 hours (58%) which could be due to increased surface coverage of the biofilm and more cells being present (fig 6.13).

The hydrogen peroxide treated biofilms showed very different responses between the biofilms grown for 24 hours and those grown for 48 hours. In figure 6.13a there were more cells stained with PI and at all time points of exposure to hydrogen peroxide

than stained with SYTO9. This indicates there are more dead cells than living cells. This increase in the fluorescence of PI was statistically significant from the untreated control at all time points ($p < 0.01$). There is also a statistically significant decrease in the percentage of SYTO9 fluorescent cells at 20 mins ($p = 0.01$) and 40 mins ($p = 0.05$).

In figure 6.13b there was no statistically significant difference between the percentages of cells that fluoresced SYTO9 in the untreated control than those in the hydrogen peroxide treated biofilm cells. There was a statistically significant decrease in the percentage of cells that fluoresced PI from the untreated control at 20 mins ($p = 0.05$) and 40 mins ($p = 0.05$) hydrogen peroxide exposure (fig 6.13). There was also a decrease in the overall percentage coverage of the 48 hour biofilm after hydrogen peroxide exposure (fig 6.13).

Although it was useful to have quantitative data as a tool for investigating the response of biofilms to biocide challenge, the graphs shown in figure 6.13 do not give the depth of information that can be obtained from examining the confocal images directly. In this instance, from the confocal images (fig 6.9 and 6.12) and from observation microscopically it was very suggestive that hydrogen peroxide was involved with biofilm dispersal in more mature biofilms. However, it was very difficult to see this effect using quantitative analysis shown below in fig 6.13. For this reason, figure 6.13 was the only quantitative analysis performed, as although quantitative analysis can be a useful tool for investigating bacterial responses, it did not seem appropriate in this case.

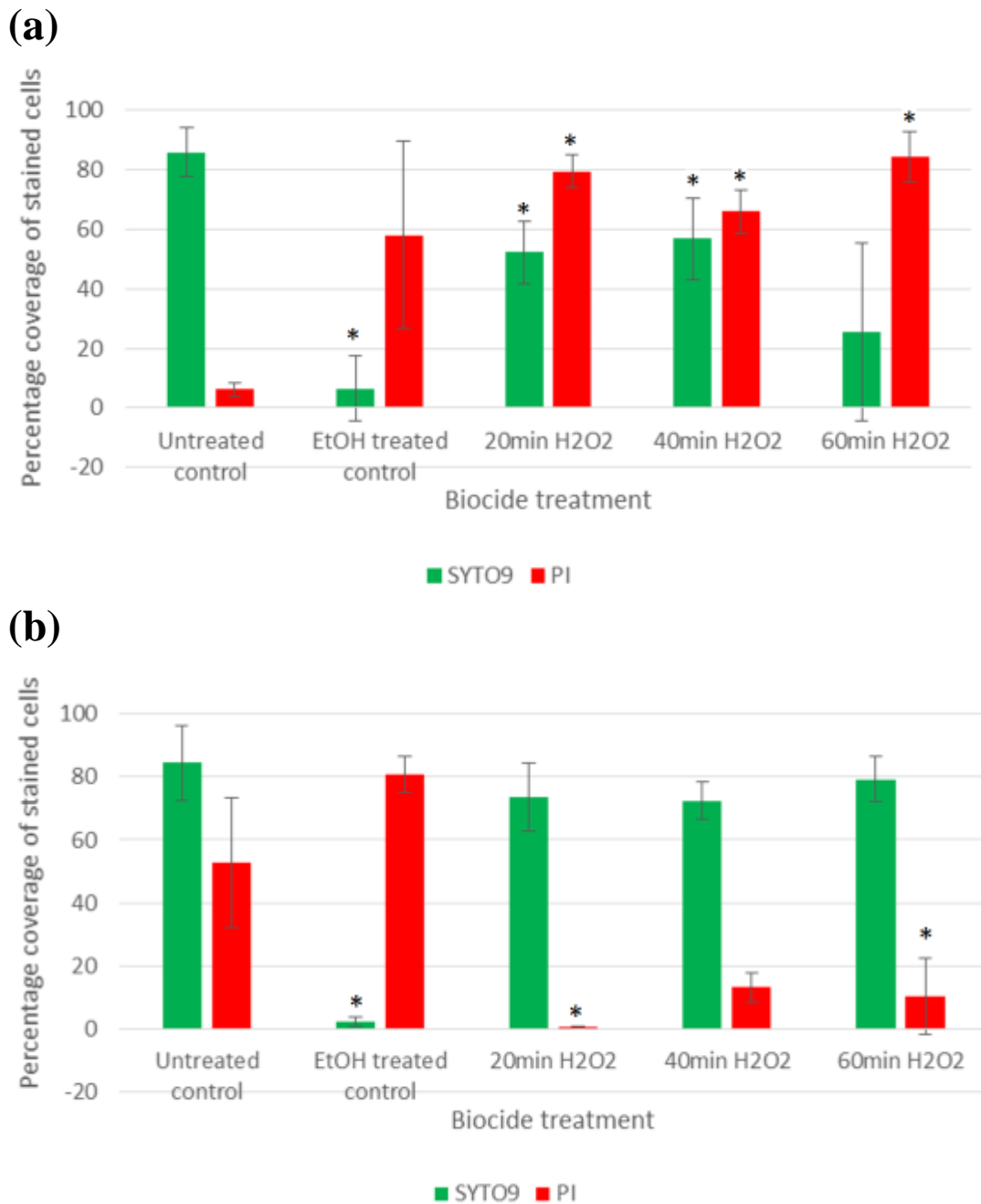


Figure 6.13: *Salmonella enterica* serovar Agona biofilms after (a) 24 hrs and (b) 48 hrs growth in a microfluidic flow system after exposure to hydrogen peroxide. Images were taken in triplicate and the colour channels were split with the use of ImageJ. These separate monochrome images were processed with the percentage coverage analysis tool on the Bioflux EZ software. The error bars denote the standard deviation from the mean. The asterisks denote a statistically significant ($p \leq 0.05$) change in percentage coverage of either SYTO9 or PI stained cells from the control untreated biofilm according to a Student's T-test.

6.3 Discussion

Biofilm experiments conducted under flow with a microfluidic system, built on the data presented in Chapter Five. In both chapters it can clearly be seen that *Salmonella* Agona was a more effective biofilm former than *Salmonella* Typhimurium SL1344. This was supported in both the light microscopy images and the confocal images. This was due to the inability of *Salmonella* Typhimurium SL1344 to make cellulose (García, *et al.*, 2004). It was considered important to use these as comparisons of how effective biocides were with both good and poor biofilm formers.

The light microscopy data presented in section 6.2.2 showed the effect of biocide exposure on percentage coverage of the microfluidic viewing channel of the Bioflux plate. There is a statistically significant increase in the percentage coverage of biofilms grown for 24 hours and then exposed to hydrogen peroxide for 1 hour and biofilms grown for 48 hours and exposed to hydrogen peroxide and peracetic acid for 1 hour and allowed to recover for 24 hours for *Salmonella* Typhimurium SL1344. There was only one condition with a statistically significant increase for *Salmonella* Agona which was 48 hours of biofilm growth followed by 1 hour exposure to peracetic acid. There was however a statistically significant decrease in biofilm percentage coverage in *Salmonella* Agona after 24 hours of biofilm growth, 1 hour exposure to peracetic acid followed by 24 hours recovery.

The data described above indicated that hydrogen peroxide and peracetic acid were ineffective at biofilm removal after 48 hours growth. After hydrogen peroxide exposure, it would seem that cells in both the 24 hours and 48 hour growth biofilms survive and multiply to give the increase percentage coverage. Lakretz *et al.* (2011)

found that higher concentrations of hydrogen peroxide managed to limit biofilm regrowth but only for short time periods, lower concentrations of hydrogen peroxide did not prevent biofilm regrowth at all, which supports the light microscopy data. Peracetic acid seem to be effective at biofilm removal after only 24 hours growth as there was a decrease in percentage coverage. However, once the biofilms have matured it was no longer effective as additional growth was seen 24 hours after biocide exposure. Based this data it could be speculated that ineffective biocide treatment could actually increase biofilm formation.

Light microscopy data showed a consistent decrease in the percentage coverage of *Salmonella* biofilms after exposure to sodium hypochlorite. This decrease was statistically significant in *Salmonella* Typhimurium SL1344 after 24 hours of biofilm growth and 1 hour of biocide exposure, there was a another decrease observed after 24 hours recovery. *Salmonella* Agona grown for 48 hours, exposed to sodium hypochlorite for 1 hour and then allowed 24 hours for recovery also shows a statistically significant decrease in the percentage coverage of the biofilm. This evidence shows that sodium hypochlorite was good at preventing biofilm regrowth even after considerable recovery time. A study by del Carpio-Perochena *et al.* (2015) found that sodium hypochlorite was only associated with recovery at 1% after 5 mins exposure and at higher concentrations and longer time points no recovery was seen. Although far lower concentrations of sodium hypochlorite were used in this study, they were used for a much longer exposure time (60 mins).

Another consideration to be made in regards to the light microscopy data was that although biocide killing may occur, the biofilm may not be removed (Simões *et al.*, 2011). This biofilm may either be made up of dead cells that remain attached or may contain injured cells that given the right opportunity may regrow. Farrell *et al.*

(1998) found that culturable cells may not be recovered from a biofilm, however if the biofilm itself was enriched viable cells may be recovered. This was seen to be the case in those hydrogen peroxide treated biofilms that showed regrowth, however it would be useful to know the viability of the attached cells in the biofilm.

Confocal microscopy was completed with similar biocide treatments to the light microscopy, however this method has the advantage of giving viability data of cells within the biofilm. Biofilms grown in the microfluidic system were exposed to oxidising biocides for 20, 40 and 60 mins, washed with PBS and then stained with SYTO9 and PI (LIVE/DEAD stained). Confocal images of *Salmonella* Typhimurium SL1344 showed a majority of bacterial cell survival after biocide exposure; after 20 mins and 40 mins biocide treatment the majority of cells showed SYTO9 fluorescence. After 60 mins exposure to biocide, hydrogen peroxide exposed cells were dead. There were both live and dead cells present after 60 mins exposure to peracetic acid and sodium hypochlorite. The images did indicate that the majority of cells exposed to peracetic acid were dead as opposed to sodium hypochlorite where the majority of cells were alive. *Salmonella* Typhimurium SL1344 biofilms grown for 48 hours in the Bioflux system appeared to survive all biocide challenges at all time points with minimal cell death.

Confocal images of *Salmonella* Agona taken after 24 hours of growth and exposure to hydrogen peroxide showed an interesting pattern. After 20 mins exposure to hydrogen peroxide there was an even mix of a live and dead cells. However, after 40 mins exposure there were less dead cells present but also voids forming within the biofilm which can be seen on both the 2D and 3D images. Hydrogen peroxide exposure for 60 mins showed the great majority of the cells as dead, with a very small number of live cells present in a non-uniform distribution.

Peracetic acid and sodium hypochlorite treatment of *Salmonella* Agona biofilm grown for 24 hours displayed predominantly dead cells after all contact times. There were some live cells present after 20 mins and 40 mins peracetic acid exposure and after 60 mins sodium hypochlorite exposure. The live cells present were a very small minority but were again distributed in a non-uniform pattern.

Salmonella Agona biofilms grown for 48 hours showed high levels of bacterial cell survival after exposure to all biocides. There were a minority of dead cells present in all conditions (most notably after 20 mins sodium hypochlorite exposure) and again these were found to be distributed in a non-uniform pattern. Hydrogen peroxide exposure did not seem to cause cell death but, there was a clear decrease in the biomass of the biofilm. However, the cells that remained in the biofilm after hydrogen peroxide exposure were almost all alive. The high levels of bacterial survival can also be seen in other studies which show that sodium hypochlorite and hydrogen peroxide respectively could not remove the biofilms (Flach *et al.*, 2015; Lin *et al.*, 2011).

The ability of hydrogen peroxide to disperse bacterial biofilms can be seen in *Salmonella* Agona at both 24 hours and 48 hours growth and after 48 hours growth of *Salmonella* Typhimurium SL1344. This was also seen by Ferris *et al.* (2015) who found that exposure to 1% hydrogen peroxide could give a decrease in *E.coli* biofilm biomass and CFUs. This reduction in biofilm biomass was also seen by Arias-Moliz *et al.* (2015) who found that sodium hypochlorite and peracetic acid caused cell death and a reduction in biomass of *Enterococcus faecalis* biofilms. In this project these biocides showed greater cell death than hydrogen peroxide but not the same the loss of biomass. Peracetic acid did not appear to show any loss of biomass and sodium hypochlorite did show a loss of biomass in *Salmonella* Typhimurium

SL1344 biofilms grown for 48 hours and *Salmonella* Agona biofilms grown for 48 hours. This discrepancy may be due to a number of methodological differences such as the difference in test bacterium used or that the biofilms were of a different age (5 days old).

A key observation from the confocal microscopy data was that an increase in the age of the biofilm was associated with a decrease in biocide efficacy. This was also seen by Stojicic *et al.* (2013) who found that biofilms that were over three weeks old were less susceptible to biocide challenge. Comparatively, the biofilms tested in this study were very young, however, there was still a marked difference in the susceptibility of biofilms grown for 24 hours compared to 48 hours. Other studies also found that biofilms were more tolerant of biocide the older they were (Fraud *et al.*, 2005; Wang *et al.*, 2014). Conversely, Wong *et al.* (2010b) found that there were no viable cells isolated after 1 min sodium hypochlorite exposure to 5 and 7 day old biofilms, but there were recoverable cells from the 3 day old biofilms. This difference may be due to differences in growth conditions as Wong, *et al.* (2010b) used a static biofilm model and LB growth media as opposed to MOPS based minimal media.

Another interesting result from the confocal microscopy work was that after 24 hours growth *Salmonella* Typhimurium SL1344 biofilms appeared to survive biocide exposure better than *Salmonella* Agona cells. This was an anomalous result as it would be expected that the strain that forms the strong biofilm (Agona) would survive biocide challenge better due to the protection of the extracellular matrix. However, this was not the case and further investigation would be required to understand this phenomenon.

The non-uniform distribution of dead cells within biocide treated biofilms was unexpected as it was hypothesized in the literature that one of the defence mechanisms of a biofilm was that there was ineffective biocide penetration (Bridier, *et al.*, 2011). Cochran *et al.* (2000) and De Beer, *et al.* (1994) found that there was a decreased penetration of hydrogen peroxide and chlorine into biofilms. This would lead to the hypothesis that the majority of cell death within a biofilm exposed to biocides would be seen at the most exposed surface. This was seen in a study by Takenaka *et al.* (2008) who found that cell death, as seen by the loss of green fluorescence, was first seen at the periphery of cell clusters which then moved inward over time. However, cell death in biocide exposed biofilms seen in this study does not follow this pattern. This could be due to the heterogeneity within the biofilms (Stewart & Franklin, 2008); either genetic variation as each area of death could be grown from a more susceptible parent bacterium or due to bistable switching of gene expression affecting biocide susceptibility.

In summary, the light microscopy data indicated recovery in some biofilms exposed to hydrogen peroxide and peracetic acid and a consistent decrease in biofilm percentage coverage after sodium hypochlorite exposure. Confocal microscopy experiments showed that *Salmonella* Typhimurium SL1344 was more tolerant of biocide after 24 hours of biofilm growth compared to a 24 hour *Salmonella* Agona biofilm. In addition it was also found that biofilms were less susceptible to biocide exposure with age. Hydrogen peroxide had a dispersal effect rather than a killing effect, on both *Salmonella* Agona and Typhimurium SL1344 biofilms. Moreover, the cell death that did occur within biofilms was non-uniformly distributed as opposed to occurring at the surface exposed to biocide.

Chapter Seven: General Discussion

7.1 General Discussion

7.1.1 Optimisation of methods was required to investigate bacterial responses to biocides

A number of different methods were used in this project to investigate the bacterial response to biocides. Some of these methods were adapted or optimised so that they were appropriate and gave accurate results. The most important change that had to be made was the alteration of the diluent used for the inactivation kinetics testing throughout the project. This was necessary, as it was found that TSC which was the diluent recommended by the BS EN 1276 (2009) interfered with the activity of sodium hypochlorite (Chapter Three, section 3.2.6, figs 3.2 and 3.3). On a related note, the media used for MIC and all other testing conditions for bacterial growth (Hoescht assay and the GFP reporter assays) needed to be changed from an enriched media to M9 minimal media (Chapter Three, section 3.2.3, table 3.3). This again was due to the presence of organic materials in the growth media interacting with the biocides. This was important as diluent and media interference with biocidal activity would have rendered the resulting data inaccurate.

7.1.2 Flow cytometry data gives information on cellular response to biocide that cannot be obtained using culture methods

Flow cytometry was used alongside culture data to give information about not only cell viability but also to look for differences within populations in response to biocide exposure. Concentrations of 2 ppm of peracetic acid give a split population at 5 mins and 20 mins exposure with some of the cells taking up BOX indicating that they have lost membrane potential (Chapter Four, section 4.2.1, fig 4.3). The explanation for this split population has yet to be understood, especially as it decreases at 3 ppm exposure. Another interesting feature of the peracetic acid flow cytometry data is that at 3 ppm after 20 mins the majority of the population is in Q3 (94.5-97.3%) which means they do not take up either PI or BOX, indicating the

membranes of the cells are intact. This concentration gives a 4.8 log₁₀ reduction in culture experiments (Chapter Three, section 3.2.7.2, fig 3.5). This pattern is also seen in hydrogen peroxide exposed cells analysed using flow cytometry (Chapter Four, section 4.2.1, fig 4.2 and table 4.1). At all concentrations and all time points including conditions that give a 4.5 log₁₀ reduction in culture conditions (3000 ppm for 60 mins; Chapter Three, section 3.2.7.1, fig 3.4) the majority of cells (99%) are found in Q3. This could be due to the mechanism of action of the biocides not damaging the bacterial cell wall so the dyes were not taken up by the cells. Alternatively, this could be due to the cells entering a viable but non-culturable state, which require a certain set of conditions for resuscitation that are not met in culture conditions.

7.1.3 Exposure to hydrogen peroxide leads to changes in efflux or porin expression

In Chapter Four, section 4.2.2.1 it was shown that when *Salmonella* Typhimurium SL1344 and Agona were exposed to hydrogen peroxide there was a decrease in the accumulation in Hoescht dye in the cell. This finding was consistent across both strains and all concentrations of hydrogen peroxide tested, indicating hydrogen peroxide exposure induced a specific response in *Salmonella*.

A commonly identified efflux pump involved in bacterial resistance to biocides is AcrAB-TolC (Levy, 2002; Webber, *et al.*, 2015). For this reason, it was investigated if AcrAB was involved in the decrease in accumulation of Hoescht dye in Chapter Four section 4.2.2.2 using a GFP reporter attached to the *acrAB* promoter. This showed that the decrease in accumulation of Hoescht dye was not linked to an increase in *acrAB* expression, in fact *acrAB* was downregulated.

Changes in the expression of both efflux pumps (Bogomolnaya *et al.*, 2013) and porins (Aguayo, *et al.*, 2015; Calderón, *et al.*, 2011) have been associated with hydrogen peroxide exposure in *Salmonella* species. Although, this study has not identified which mechanism was being used, it does confirm that cellular permeability is involved in *Salmonella* response to hydrogen peroxide.

The role of cellular permeability could be further investigated by the use of efflux pump inhibitors (as discussed in Chapter One, section 1.4.3.2). Overnight cultures of *Salmonella* could be treated with an efflux pump inhibitor and then exposed to hydrogen peroxide. A decrease in Hoescht accumulation after exposure to hydrogen peroxide would implicate porins, as opposed to an alternate efflux pump system to AcrB-TolC. Alternatively, the presence and quantity of the outer membrane proteins present before and after biocide exposure could be investigated, to look for an increase in the presence of porins.

7.1.4 Adherent cells are more tolerant of biocide stress than planktonic cells

In Chapter Five and Six it can be observed that biofilms are more tolerant of biocide exposure than planktonic cells. This can be observed in both *Salmonella* Typhimurium SL1344 and *Agona* even though *Salmonella* Typhimurium SL1344 is a poor biofilm former. It is unsurprising that this study has found that biofilms are more tolerant of biocide exposure than planktonic cells as this has been seen in numerous studies across the literature (Behnke, *et al.*, 2011; Bridier, *et al.*, 2011; Condell, *et al.*, 2012; Leung, *et al.*, 2012). What is interesting however, is that *Salmonella* Typhimurium SL1344 is known to be a poor biofilm former as it lacks the ability to produce cellulose (García, *et al.*, 2004) which is an important part of the biofilm matrix. This means that although *Salmonella* Typhimurium SL1344 could attach they could not form a 3D dimensional biofilm structure when tested in

Chapter Six (sections 6.2.1 and 6.2.3, figs 6.1 and 6.4-6.6). After 24 hours of biofilm growth, there actually appears to be more cell death in *Salmonella* Agona (Chapter Six, section 6.2.3, fig 6.8) than Typhimurium SL1344 (Chapter Six, section 6.2.3, fig 6.5). After 48 hours the majority of cells in the biofilms of both strains appear to survive biocide exposure at all time points as seen in the confocal microscopy images (Chapter Six, section 6.2.3, figs 6.6 and 6.9). In Chapter Five (section 5.2.3, figs 5.5-5.7) static biofilms grown for 72 hours were tested using culturable viability methods after exposure to biocides. *Salmonella* Typhimurium SL1344 biofilms survived far higher concentrations than planktonic cells as can be seen in table 7.1.

Table 7.1: A comparison of biocide susceptibility of *Salmonella enterica* serovar Typhimurium SL1344 in planktonic and biofilm growth states after exposure to biocides. The biocide concentration required to give a 4.5 log₁₀ reduction after 60 mins biocide exposure is listed for both planktonic cells and static biofilms. Full results for planktonic cells in Chapter Three (section 3.2.7, figs 3.4-3.6) and static biofilms in Chapter Five (section 5.2.3, figs 5.5-5.7).

	Planktonic	Static biofilm
Hydrogen peroxide	2800ppm	>25000 ppm
Peracetic acid	2 ppm	30 ppm
Sodium hypochlorite	3 ppm	40 ppm

In table 7.1, it can be seen that although *Salmonella* Typhimurium SL1344 does not have the ability to form a 3D biofilm structure, some physiological changes have clearly occurred to allow the attached cells to survive higher concentrations of all three biocides. Further study would be required to investigate changes in gene transcription to find the underlying cause of this phenomenon.

7.1.5 *Salmonella* cells from a dispersed biofilm have a similar susceptibility to biocides as planktonic cells

In Chapter Five (section 5.2.2) the inactivation kinetics of dispersed biofilms were tested alongside those of intact biofilm cells. It was expected that intact biofilms would be less susceptible to biocide challenge than planktonic cells as described above and it was expected that dispersed biofilm cells would retain some of the features that were protective within the biofilm. However, it was found that this was not the case and that dispersed biofilm cells had very similar susceptibilities to planktonic cells. It has been reported that detached biofilms have a different phenotype for the first few hours after detachment (Rollet *et al.*, 2009), however this may not be relevant as this refers to cells that have broken away naturally as opposed to being dispersed artificially. This loss of biocide tolerance may be associated with the loss of the protective biofilm structure or a rapid change in gene expression after detachment from the biofilm structure.

7.1.6 Biocides cause cell death within a biofilm in a non-uniform pattern

The confocal images in Chapter Six (section 6.2.3) show a surprising pattern. The biofilms exposed to a biocide challenge do show cell death in some instances (shown by the uptake of red PI dye), however this pattern of death is random across the biofilm. It might have been expected that the uppermost layers of the biofilm would show the greatest cell death whereas the lower layers closest to the attachment surface would show the least. The lower layers would have had the protection of; reduced biocide penetration into the biofilm structure, non-specific biocide interactions with the biofilm matrix and additionally the possibility of dormancy of nutrient starved cells at the bottom of the biofilm which have all been associated with tolerance (Jang, *et al.*, 2006; Kim, *et al.*, 2009; Lambert & Johnston, 2001). However, this was not the case as cell death occurred within biofilms seemingly at

random. It may be that this is due to heterogeneity within the biofilm (Stewart & Franklin, 2008) as if some cell clusters are expressing different genes which may be more or less favourable to surviving biocide exposure.

7.1.7 Hydrogen peroxide is consistently the least effective biocide

Responses to oxidising biocides were not consistent across the oxidising biocides tested. Hydrogen peroxide was consistently the least effective biocide that was tested. In the initial planktonic culture data (Chapter Three, section 3.2.7.1, fig 3.4), much higher concentrations of hydrogen peroxide were required to give a 4 log₁₀ reduction (2800 ppm compared to 2 ppm peracetic acid and between 4 ppm and 7 ppm of sodium hypochlorite). In Chapter Four, flow cytometry data conflicted with this culture based data as the hydrogen peroxide exposed cells did not take up either PI or BOX indicating that the membranes of the cells were intact (Chapter Four, section 4.2.1, fig 4.2 and table 4.1). This may have meant that they could have recovered in the right conditions and regrown.

Static biofilm studies showed that the maximum concentration tested (25000 ppm) only gave a maximum of a 2 log₁₀ reduction (Chapter Five, section 5.2.3.1, fig 5.5). In microfluidic studies confocal microscopy after 24 hours *Salmonella Agona* biofilm growth, followed by 20 mins and 40 mins exposure to hydrogen peroxide showed there were still survivors observed (Chapter Six, section 6.2.3, fig 6.5 and 6.8). Peracetic acid and sodium hypochlorite showed no survivors at any time point in *Salmonella Agona*. After 48 hours of *Salmonella Agona* biofilm growth it was observed that there was a decrease in the percentage coverage as there were no cells stained by PI present (Chapter Six, sections 6.2.3-6.2.5, figs 6.9, 6.12-6.13). However, the light microscopy data showed an increase in the percentage coverage of microfluidic channels containing *Salmonella* biofilms grown for 48 hours, treated

with hydrogen peroxide for 1 hour and having 24 hours recovery. This increase was present in both strains but only statistically significant for *Salmonella* Typhimurium SL1344 (Chapter Six, section 6.2.2, fig 6.3). The increase in percentage coverage indicates that biofilms may lose cell density after hydrogen peroxide treatment, but they recover and start re-growing within 24 hours.

Hydrogen peroxide showed a decrease in cellular permeability in the Hoescht assay (Chapter Four, section 4.2.2.1, fig 4.6) which could have consequences as the phenotypic change may make those cells more likely to survive challenges from other antimicrobials. This is obviously a very poor outcome and is not a quality that would be found in an effective biocide.

7.2 Future work

7.2.1 Analysis of bacterial survival and recovery using flow cytometry

The hydrogen peroxide and peracetic acid flow cytometry results in Chapter Four (section 4.2.1.2, figure 4.2 and table 4.1) showed that *Salmonella* cells did not take up either PI or BOX. This meant that there was no loss of membrane potential and no loss of membrane integrity. To investigate if this was due to the mechanism of action of hydrogen peroxide causing a lack of membrane damage, or due to the cells becoming viable but non-culturable. The use of a dye that measures respiratory activity such as 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) could give additional information as to the viability of hydrogen peroxide exposed cells. CTC fluoresces in actively respiring bacterial cells (cells with active electron transport) and was used in conjunction with SYTO9 to analyse bacterial response to a biocide challenge in Warnes and Keevil (2011). This would be useful in investigating if hydrogen peroxide exposure interfered with respiratory pathways, in addition to being an indicator of viability.

Another method of investigating the response of *Salmonella* to hydrogen peroxide exposure is to investigate bacterial recovery. After biocide exposure, cells could be treated with either glucose or pyruvate. This can give insight as to whether given the right environmental conditions, the bacterial cells can recover from the damage inflicted by the biocide. Different recovery media could also be used to see if this affects survival and susceptibility to discover if bacterial cells are damaged but in a reversible way that may not be detected in normal culture techniques, as the media used can have a considerable impact on the number of bacteria recovered (Stephens *et al.*, 2000; Wesche *et al.*, 2009).

7.2.2 Isolating biocide surviving planktonic cells using fluorescence activated cell sorting

The peracetic acid data as shown in Chapter Four (section 4.2.1.3, figure 4.3) displayed a distinct separation between Q3 and Q1 when *Salmonella* was exposed to a two parts per million concentration. This is as yet unexplained and it is important to investigate the cells surviving biocide exposure, to see if there are potentially resistant subpopulations present. This would involve exposing the *Salmonella* cells to peracetic acid and then analysing the results using flow cytometry. If a surviving sub-population can be seen in Q3, these cells can then be sorted and collected using fluorescence activated cell sorting (FACS). These cells would then be regrown overnight and tested to see if they have a different response when re-exposed to biocide. If so, then it may be caused by a genetic change which would need to be investigated further using a method such as by RNA-sequencing (Casey *et al.*, 2014; Vikram *et al.*, 2015b). Alternatively, a phenotypic change may be responsible and investigation of factors such as the up-regulation of efflux or porins may be worthy of investigation by performing a Hoescht assay (Webber, *et al.*, 2015; Whitehead, *et al.*, 2011).

7.2.3 Recovery of biocide treated cells from the Bioflux to test for viability and other characteristics

In Chapter Six, microscopy of *Salmonella* in a biofilm was undertaken to gain a better understanding of the response of a biofilm to a biocide challenge. To investigate this further it would be useful to remove the cells from the microfluidic cell chamber for further analysis. Cells could be removed by flushing through the channels with PBS at the maximum flow rate, these cells could then be harvested from the output well for further testing. It would be interesting to undertake a viable count and to test the antibiotic and biocide susceptibility of these dispersed biofilm cells using the antibiotic disc diffusion and a biocide MIC as described in Chapters

Two and Three (section 2.1.7, 3.2.2 and 3.2.5). Further experiments could be completed with these cells, such as RNA sequencing, however this would only be possible if there were sufficient numbers of cells present.

Other studies have implicated the AcrEF efflux pump in response to biocide exposure in *Salmonella* Typhimurium SL1344 (Whitehead, *et al.*, 2011). An alternative method of investigation would be to use efflux pump inhibitors (as discussed in Chapter One, section 1.4.3.2). Efflux pump inhibitors could be added to the experiment and if there is still a decrease in Hoescht accumulation after exposure to biocides then porins are implicated, if not an alternate efflux pump system is responsible for the decreased Hoescht accumulation.

To investigate the role of porins in response to hydrogen peroxide exposure a Western blot could be used. This would quantify the outer membrane proteins present before and after biocide exposure and so it would be possible to look for an increase in the presence of ompD.

7.2.4 RNA sequencing of *Salmonella* exposed to biocide

Chapter Four (section 4.2.2.2 and 4.2.3) used GFP reporters to analyse the responses of three specific genes (*acrAB*, *ramA* and *soxS*) after biocide challenge. This targeted approach provided interesting data, however it revealed there was more activity occurring in response to biocide exposure than those captured using the GFP reporter assays. RNA sequencing allows for the whole transcriptome to be analysed and does not require any previous knowledge of the sequence (Wang *et al.*, 2009). This could be used to investigate the changes in gene expression that occur after biocide exposure to the three different oxidising biocides used. RNA sequencing would give a comprehensive view of the transcriptome and capture a wide range of changes in

response to biocide challenge. This method has been used by Vikram, *et al.* (2015b) to investigate the changes in the transcriptome that occur in *Pseudomonas* after exposure to glutaraldehyde. This shows that RNA sequencing could be used for *Salmonella* and the range of oxidising biocides described in this thesis to investigate the transcriptional changes of both planktonic cells and biofilms.

7.3 Concluding remarks

Various methods have been used to investigate the mechanisms of bacterial survival to a range of oxidising biocides. It has been identified that an alteration of cellular permeability is important in the bacterial response to oxidising biocides. In addition, it has been discovered that some cells may enter a viable but non culturable state in response to biocide challenge and there may be a split into different subpopulations after biocide challenge. Biofilms play an important role in the recalcitrance of bacteria and this was confirmed even in poor biofilm formers such as *Salmonella* Typhimurium SL1344. Dispersed biofilm cells however, lost the protection associated with the biofilm when they were exposed to a biocide challenge. Confocal microscopy elucidated that this may not be due to a structural protection as cell death occurred sporadically throughout the biofilm, not just the cells on the top of the surface exposed to biocide. Further study is required to investigate these mechanisms and the use of RNA-sequencing to explore the changes in the transcriptome would be especially useful.

References

- Abouzeed, Y.M., Baucheron, S. & Cloeckert, A. (2008) ramR Mutations Involved in Efflux-Mediated Multidrug Resistance in *Salmonella enterica* Serovar Typhimurium. *Antimicrob Agents Chemother* **52**: 2428-2434.
- Aguayo, D., Pacheco, N., Morales, E.H., Collao, B., Luraschi, R., Cabezas, C., Calderón, P., González-Nilo, F., Gil, F., Calderón, I.L. & Saavedra, C.P. (2015) Hydrogen Peroxide and Hypochlorous Acid Influx Through the Major S. Typhimurium Porin OmpD is Affected by Substitution of Key Residues of the Channel. *Archives of Biochemistry and Biophysics* **568**: 38-45.
- Al-Adham, I., Haddadin, R. & Collier, P. (2013) Types of Microbicidal and Microbistatic Agents. 5-70. John Wiley and Sons, Chichester, U.K.
- Amaral, L., Martins, A., Spengler, G. & Molnar, J. (2013) Efflux Pumps of Gram-negative Bacteria: What They Do, How They Do It, With What and How To Deal With Them. *Frontiers in Pharmacology* **4**: 1-11.
- American Society for Testing and Materials (2012) ASTM E2799 - 12: Standard Test Method for Testing Disinfectant Efficacy against *Pseudomonas aeruginosa* Biofilm using the MBEC Assay. ASTM International, West Conshohocken, Pennsylvania, USA.
- An, D. & Parsek, M.R. (2007) The Promise and Peril of Transcriptional Profiling in Biofilm Communities. *Current Opinion in Microbiology* **10**: 292-296.
- Arias-Moliz, M.T., Ordinola-Zapata, R., Baca, P., Ruiz-Linares, M., García García, E., Hungaro Duarte, M.A., Monteiro Bramante, C. & Ferrer-Luque, C.M. (2015) Antimicrobial Activity of Chlorhexidine, Peracetic Acid and Sodium Hypochlorite/Etidronate Irrigant Solutions Against *Enterococcus faecalis* Biofilms. *International Endodontic Journal* **48**: 1188-1193.
- Ayyildiz, O., Ileri, B. & Sanik, S. (2009) Impacts of Water Organic Load on Chlorine Dioxide Disinfection Efficacy. *Journal of Hazardous Materials* **168**: 1092-1097.
- Baatout, S., De Boever, P. & Mergeay, M. (2006) Physiological Changes Induced in Four Bacterial Strains Following Oxidative Stress. *Prikl Biokhim Mikrobiol* **4**: 418-427.
- Babayán, A. & Nikaido, H. (2004) In *Pseudomonas aeruginosa* ethidium bromide does not induce its own degradation or the assembly of pumps involved in its efflux. *Biochemical and Biophysical Research Communications* **324**: 1065-1068.
- Bailey, A.M., Webber, M.A. & Piddock, L.J. (2006) Medium Plays a Role in Determining Expression of *acrB*, *marA*, and *soxS* in *Escherichia coli*. *Antimicrobial Agents and Chemotherapy* **50**: 1071-1074.

- Bailey, A.M., Paulsen, I.T. & Piddock, L.J.V. (2008) RamA Confers Multidrug Resistance in *Salmonella enterica* via Increased Expression of *acrB*, Which Is Inhibited by Chlorpromazine. *Antimicrobial Agents and Chemotherapy* **52**: 3604-3611.
- Bailey, A.M., Ivens, A., Kingsley, R., Cottell, J.L., Wain, J. & Piddock, L.J.V. (2010) RamA, A Member of the AraC/XylS Family, Influences Both Virulence and Efflux in *Salmonella enterica* Serovar Typhimurium. *Journal of Bacteriology* **192**: 1607-1616.
- Bailey, A.M., Constantinidou, C., Ivens, A., Garvey, M.I., Webber, M.A., Coldham, N., Hobman, J.L., Wain, J., Woodward, M.J. & Piddock, L.J. (2009) Exposure of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium to Triclosan Induces a Species-specific Response, Including Drug Detoxification. *Journal of Antimicrobial Chemotherapy* **64**: 973-985.
- Balaban, N.Q., Merrin, J., Chait, R., Kowalik, L. & Leibler, S. (2004) Bacterial Persistence as a Phenotypic Switch. *Science* **305**: 1622-1625.
- Barnhart, M.M. & Chapman, M.R. (2006) Curli Biogenesis and Function. *Annual review of microbiology* **60**: 131-147.
- Baty, A.M., Eastburn, C.C., Diwu, Z., Techkarnjanaruk, S., Goodman, A.E. & Geesey, G.G. (2000) Differentiation of Chitinase-Active and Non-Chitinase-Active Subpopulations of a Marine Bacterium during Chitin Degradation. *Appl Environ Microbiol* **66**: 3566-3573.
- Baucheron, S., Nishino, K., Monchaux, I., Canepa, S., Maurel, M.-C., Coste, F., Roussel, A., Cloeckert, A. & Giraud, E. (2014) Bile-mediated Activation of the *acrAB* and *tolC* Multidrug Efflux Genes Occurs Mainly Through Transcriptional Derepression of *ramA* in *Salmonella enterica* serovar Typhimurium. *Journal of Antimicrobial Chemotherapy* **69**: 2400-2406.
- Baugh, S. (2013) The Role of Multidrug Efflux Pumps in Biofilm Formation of *Salmonella enterica* serovar Typhimurium. Thesis, University of Birmingham, Birmingham, UK.
- Behnke, S., Parker, A.E., Woodall, D. & Camper, A.K. (2011) Comparing the Chlorine Disinfection of Detached Biofilm Clusters with Those of Sessile Biofilms and Planktonic Cells in Single- and Dual-species Cultures. *Applied and Environmental Microbiology* **77**: 7176-7184.
- Benoit, M.R., Conant, C.G., Ionescu-Zanetti, C., Schwartz, M. & Matin, A. (2010) New Device for High-Throughput Viability Screening of Flow Biofilms. *Applied Environmental Microbiology* **76**: 4136-4142.

- Blair, J.M.A., Bavro, V.N., Ricci, V., Modi, N., Cacciotto, P., Kleinekathöfer, U., Ruggerone, P., Vargiu, A.V., Baylay, A.J., Smith, H.E., Brandon, Y., Galloway, D. & Piddock, L.J.V. (2015) AcrB Drug-Binding Pocket Substitution Confers Clinically Relevant Resistance and Altered Substrate Specificity. *Proceedings of the National Academy of Sciences of the United States of America* **112**: 3511-3516.
- Block, S.S. (1991) *Disinfection, Sterilization and Preservation*. Lea & Febiger Pubs, Philadelphia.
- Bloomfield, S.F. (2002) Significance of Biocide Usage and Antimicrobial Resistance in Domiciliary Environments. *Journal of Applied Microbiology Symposium Supplement* **92**: 144S-157S.
- Bogomolnaya, L.M., Andrews, K.D., Talamantes, M., Maple, A., Ragoza, Y., Vazquez-Torres, A. & Andrews-Polymenis, H. (2013) The ABC-Type Efflux Pump MacAB Protects *Salmonella enterica* serovar Typhimurium from Oxidative Stress. *mBio* **4**: e00630-00613.
- Brandi, G., Salvaggio, L., Cattabeni, F. & Cantoni, O. (1991) Cytocidal and Filamentous Response of *Escherichia coli* Cells Exposed to Low Concentrations of Hydrogen Peroxide and Hydroxyl Radical Scavengers. *Environmental and Molecular Mutagenesis* **18**: 22-27.
- Braoudaki, M. & Hilton, A.C. (2004) Adaptive Resistance to Biocides in *Salmonella enterica* and *Escherichia coli* O157 and Cross-Resistance to Antimicrobial Agents. *Journal of Clinical Microbiology* **42**: 73-78.
- Brehm-Stecher, B.F. & Johnson, E.A. (2004) Single-Cell Microbiology: Tools, Technologies, and Applications. *Microbiology and Molecular Biology Reviews* **68**: 538–559.
- Bridier, A., Briandet, R., Thomas, V. & Dubois-Brissonnet, F. (2011) Resistance of Bacterial Biofilms to Disinfectants: A Review. *Biofouling* **27**: 1017-1032.
- Bridier, A., Sanchez-Vizueté, P., Guilbaud, M., Piard, J.C., Naïtali, M. & Briandet, R. (2015) Biofilm-associated Persistence of Food-borne Pathogens. *Food Microbiology* **45, Part B**: 167-178.
- British National Formulary (2010) *British National Formulary 60*. BMJ Group, London, UK.
- British National Formulary (2016) *British National Formulary* (online). BMJ Group and Pharmaceutical Press, London.
- British Pharmacopoeia (2011a) Sodium Hypochlorite Solution. The Stationary Office, British Pharmacopoeia Online.

British Pharmacopoeia (2011b) Hydrogen Peroxide Solution (30 per cent). The Stationary Office, British Pharmacopoeia Online.

British Society for Antimicrobial Chemotherapy (2015) BSAC Susceptibility Testing Method: Message to users of BSAC disc diffusion method.

Brouard, C., Espié, E., Weill, F.-X., Kérouanton, A., Brisabois, A., Forgue, A.-M., Vaillant, V. & de Valk, H. (2007) Two Consecutive Large Outbreaks of *Salmonella enterica* Serotype Agona Infections in Infants Linked to the Consumption of Powdered Infant Formula. *The Pediatric Infectious Disease Journal* **26**: 148-152.

BS EN 1276 (2009) Chemical Disinfectants and Antiseptics — Quantitative Suspension Test for the Evaluation of Bactericidal Activity of Chemical Disinfectants and Antiseptics Used in Food, Industrial, Domestic and Institutional Areas — Test Method and Requirements. British Standard.

Buckingham-Meyer, K., Goeres, D.M. & Hamilton, M.A. (2007) Comparative Evaluation of Biofilm Disinfectant Efficacy Tests. *Journal of Microbiological Methods* **70**: 236–244.

Bumann, D. & Valdivia, R.H. (2007) Identification of Host-induced Pathogen Genes by Differential Fluorescence Induction Reporter Systems. *Nature Protocols* **2**: 770-777.

Burmolle, M., Webb, J.S., Rao, D., Hansen, L.H., Sorensen, S.J. & Kjelleberg, S. (2006) Enhanced Biofilm Formation and Increased Resistance to Antimicrobial Agents and Bacterial Invasion are Caused by Synergistic Interactions in Multispecies Biofilms. *Applied Environmental Microbiology* **72**: 3916-3923.

Cabrera, R., Ruiz, J., Marco, F., Oliveira, I., Arroyo, M., Aladuena, A., Usera, M.A., Jimenez De Anta, M.T., Gascon, J. & Vila, J. (2004) Mechanism of Resistance to Several Antimicrobial Agents in *Salmonella* Clinical Isolates Causing Traveler's Diarrhea. *Antimicrobial Agents and Chemotherapy* **48**: 3934-3939.

Calderón, I.L., Morales, E., Caro, N.J., Chahúan, C.A., Collao, B., Gil, F., Villarreal, J.M., Ipinza, F., Mora, G.C. & Saavedra, C.P. (2011) Response Regulator ArcA of *Salmonella enterica* serovar Typhimurium Downregulates Expression of OmpD, a Porin Facilitating Uptake of Hydrogen Peroxide. *Research in Microbiology* **162**: 214-222.

Casella, M.L. & Schmidt-Lorenz, W. (1989) Disinfection with Gaseous Formaldehyde. Fifth Part: Influence of Albumin, Mucin and Blood on the Bactericidal and Sporocidal Effectiveness. *International journal of hygiene and environmental medicine* **189**: 37-49.

Casey, A., Fox, E.M., Schmitz-Esser, S., Coffey, A., McAuliffe, O. & Jordan, K. (2014) Transcriptome Analysis of *Listeria monocytogenes* Exposed to Biocide Stress

Reveals a Multi-System Response Involving Cell Wall Synthesis, Sugar Uptake, and Motility. *Frontiers in Microbiology* **5**: 68.

Center for Disease Control (2010) National Antimicrobial Resistance Monitoring System: Enteric Bacteria. Center for Disease Control (CDC).

Center for Disease Control (2011) National *Salmonella* Surveillance Overview. CDC, Atlanta, Georgia.

Cerf, O., Carpentier, B. & Sanders, P. (2010) Tests for Determining In-use Concentrations of Antibiotics and Disinfectants are Based on Entirely Different Concepts: “Resistance” has Different Meanings. *International Journal of Food Microbiology* **136**: 247-254.

Chai, Y., Chu, F., Kolter, R. & Losick, R. (2008) Bistability and Biofilm Formation in *Bacillus subtilis*. *Molecular Microbiology* **67**: 254-263.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. & Prasher, D. (1994) Green Fluorescent Protein as a Marker for Gene Expression. *Science* **263**: 802-805.

Chapman, J.S. (2003) Disinfectant resistance mechanisms, cross-resistance, and co-resistance. *International Biodeterioration & Biodegradation* **51**: 271-276.

Chavant, P., Gaillard-Martinie, B. & Hébraud, M. (2004) Antimicrobial Effects of Sanitizers Against Planktonic and Sessile *Listeria monocytogenes* Cells According to the Growth Phase. *FEMS Microbiology Letters* **236**: 241-248.

Chen, C.-I., Griebe, T. & WG, C. (1993) Biocide Action of Monochloramine on Biofilm Systems of *Pseudomonas aeruginosa*. *Biofouling* **7**: 1-17.

Cochran, W.L., McFeters, G.A. & Stewart, P.S. (2000) Reduced Susceptibility of Thin *Pseudomonas aeruginosa* Biofilms to Hydrogen Peroxide and Monochloramine. *Journal of Applied Microbiology* **88**: 22-30.

Coenye, T. & Nelis, H.J. (2010) In vitro and In vivo Model Systems to Study Microbial Biofilm Formation. *Journal of Microbiological Methods* **83**: 89-105.

Coldham, N.G., Webber, M., Woodward, M.J. & Piddock, L.J.V. (2010) A 96-well Plate Fluorescence Assay for Assessment of Cellular Permeability and Active Efflux in *Salmonella enterica* serovar Typhimurium and *Escherichia coli*. *Journal of Antimicrobial Chemotherapy* **65**: 1655-1663.

Condell, O., Iversen, C., Cooney, S., Power, K.A., Walsh, C., Burgess, C. & Fanning, S. (2012) Efficacy of Biocides Used in the Modern Food Industry to Control *Salmonella enterica*, and Links Between Biocide Tolerance and Resistance to Clinically Relevant Antimicrobial Compounds. *Applied Environmental Microbiology* **78**: 3087-3097.

- Copitch, J.L., Whitehead, R.N. & Webber, M.A. (2010) Prevalence of Decreased Susceptibility to Triclosan in *Salmonella enterica* Isolates from Animals and Humans and Association with Multiple Drug Resistance. *International Journal of Antimicrobial Agents* **36**: 247-251.
- Corcoran, M., Morris, D., De Lappe, N., O'Connor, J., Lalor, P., Dockery, P. & Cormican, M. (2014) Commonly Used Disinfectants Fail To Eradicate *Salmonella enterica* Biofilms from Food Contact Surface Materials. *Applied Environmental Microbiology* **80**: 1507-1514.
- Costerton, J.W., Geesey, G.G. & Cheng, K.J. (1978) How Bacteria Stick. *Scientific American* **238**: 86-95.
- De Beer, D., Srinivasan, R. & Stewart, P.S. (1994) Direct Measurement of Chlorine Penetration into Biofilms During Disinfection. *Applied and Environmental Microbiology* **60**: 4339-4344.
- De Oliveira, D., Fernandes Júnior, A., Kaneno, R., Silva, M., Araújo Júnior, J., Silva, N. & Rall, V. (2014) Ability of *Salmonella* spp. to Produce Biofilm Is Dependent on Temperature and Surface Material. *Foodborne Pathogens and Disease* **11**: 478-483.
- Dean, R.T., Fu, S., Stocker, R. & Davies, M.J. (1997) Biochemistry and Pathology of Radical-mediated Protein Oxidation. *Biochemical Journal* **324**: 1-18.
- del Carpio-Perochena, A., Monteiro Bramante, C., Hungaro Duarte, M., Bombarda de Andrade, F., Zardin Graeff, M., Marciano da Silva, M., Cavalini Cavenago, B. & Lucas Fernandes, S. (2015) Effect of Temperature, Concentration and Contact Time of Sodium Hypochlorite on the Treatment and Revitalization of Oral Biofilms. *Journal of Dental Research, Dental Clinics, Dental Prospects* **9**: 209-215.
- del Cerro, A., Soto, S.M. & Mendoza, M.C. (2003) Virulence and Antimicrobial-Resistance Gene Profiles Determined by PCR-based Procedures for *Salmonella* Isolated from Samples of Animal Origin. *Food Microbiology* **20**: 431-438.
- Denyer, S.P. & Maillard, J.Y. (2002) Cellular Impermeability and Uptake of Biocide and Antibiotics in Gram-negative Bacteria. *Journal of Applied Microbiology Symposium Supplement* **92**: S35-S45.
- Díaz, M., Herrero, M., García, L.A. & Quirós, C. (2010) Application of Flow Cytometry to Industrial Microbial Bioprocesses. *Biochemical Engineering Journal* **48**: 385-407.
- Doddamani, S., Patil, R.A. & Gangadhar, S.A. (2011) Efficacy of Various Spray Disinfectants on Irreversible Hydrocolloid Impression Materials: An In Vitro Study. *Indian Journal of Dental Research* **22**: 764-769.

- Driffield, K., Miller, K., Bostock, J.M., O'Neill, A.J. & Chopra, I. (2008) Increased Mutability of *Pseudomonas aeruginosa* in Biofilms. *Journal of Antimicrobial Chemotherapy* **61**: 1053-1056.
- EU (2012) Regulation (EU) Number 528/2012 of the European Parliament and of the Council. Official Journal of the European Communities.
- Fàbrega, A., Ballesté-Delpierre, C. & Vila, J. (2016) Differential Impact of *ramRA* Mutations on Both *ramA* Transcription and Decreased Antimicrobial Susceptibility in *Salmonella* Typhimurium. *Journal of Antimicrobial Chemotherapy* **71**: 617-624.
- Farrell, B.L., Ronner, A.B. & Lee Wong, A.C. (1998) Attachment of *Escherichia coli* O157:H7 in Ground Beef to Meat Grinders and Survival after Sanitation with Chlorine and Peroxyacetic Acid. *Journal of Food Protection* **61**: 817-822.
- Fasani, R.A. & Savageau, M.A. (2013) Molecular Mechanisms of Multiple Toxin–antitoxin Systems are Coordinated to Govern the Persister Phenotype. *Proceedings of the National Academy of Sciences of the United States of America* **110**: E2528-E2537.
- Fernández Fuentes, M.Á., Ortega Morente, E., Abriouel, H., Pérez Pulido, R. & Gálvez, A. (2014) Antimicrobial Resistance Determinants in Antibiotic and Biocide-resistant Gram-negative Bacteria From Organic Foods. *Food Control* **37**: 9-14.
- Ferris, R.A., McCue, P.M., Borlee, G.I., Loncar, K.D., Henet, M.L. & Borlee, B.R. (2015) In Vitro Efficacy of Non-Antibiotic Treatments on Biofilm Disruption of Gram-Negative Pathogens and an In Vivo Model of Infectious Endometritis Utilizing Isolates from the Equine Uterus. *Journal of Clinical Microbiology*.
- Finnegan, M., Linley, E., Denyer, S.P., McDonnell, G., Simons, C. & Maillard, J.Y. (2010) Mode of Action of Hydrogen Peroxide and Other Oxidizing Agents: Differences Between Liquid and Gas Forms. *Journal of Antimicrobial Chemotherapy* **65**: 2108-2115.
- Flach, N., Böttcher, D.E., Parolo, C.C.F., Firmino, L.B., Malt, M., Lammers, M.L. & Grecca, F.S. (2015) Confocal Microscopy Evaluation of the Effect of Irrigants on *Enterococcus faecalis* Biofilm: An In Vitro Study. *Scanning* n/a-n/a.
- Flemming, H.-C. & Wingender, J. (2010) The Biofilm Matrix. *Nature Reviews Microbiology* **8**: 623-633.
- Fraise, A.P. (2002) Biocide Abuse and Antimicrobial Resistance- A Cause for Concern? *Journal of Antimicrobial Chemotherapy* **49**: 11-12.
- Fraud, S., Maillard, J.-Y., Kaminski, M.A. & Hanlon, G.W. (2005) Activity of amine oxide against biofilms of *Streptococcus mutans*: a potential biocide for oral care formulations. *Journal of Antimicrobial Chemotherapy* **56**: 672-677.

- Fukuzaki, S. (2006) Mechanisms of Action of Sodium Hypochlorite in Cleaning and Disinfection Processes. *Biocontrol Science* **11**: 147-157.
- García, B., Latasa, C., Solano, C., Portillo, F.G.-d., Gamazo, C. & Lasa, I. (2004) Role of the GGDEF Protein Family in *Salmonella* Cellulose Biosynthesis and Biofilm Formation. *Molecular Microbiology* **54**: 264-277.
- Garnett, J.A. & Matthews, S. (2012) Interactions in Bacterial Biofilm Development: A Structural Perspective. *Current Protein and Peptide Science* **13**: 739–755.
- Gehr, R., Cochrane, D. & French, M. (2002) Peracetic Acid as a Disinfectant for Municipal Wastewaters: Encouraging Performance Results from Physicochemical As Well As Biological Effluents.
- Gerstel, U. & Römling, U. (2003) The csgD Promoter, A Control Unit for Biofilm Formation in *Salmonella* Typhimurium. *Research in Microbiology* **154**: 659-667.
- Giaouris, E., Chorianopoulos, N. & Nychas, G.J.E. (2005) Effect of Temperature, pH, and Water Activity on Biofilm Formation by *Salmonella enterica* Enteritidis PT4 on Stainless Steel Surfaces as Indicated by the Bead Vortexing Method and Conductance Measurements. *Journal of Food Protection* **68**: 2149-2154.
- Gil, F., Hernandez-Lucas, I., Polanco, R., Pacheco, N., Collao, B., Villarreal, J.M., Nardocci, G., Calva, E. & Saavedra, C.P. (2009) SoxS Regulates the Expression of the *Salmonella enterica* serovar Typhimurium ompW Gene. *Microbiology* **155**: 2490-2497.
- Gilbert, P. (1988) Microbial Resistance to Preservative Systems. 171-194. Chicester, U.K., Ellis Horwood.
- Goering, R.V., Dockrell, H.M., Zuckerman, M., Wakelin, D., Roitt, I.M., Mims, C. & Chiodini, P.L. (2008) *Mims' Medical Microbiology*. Mosby Elsevier, Philadelphia.
- Gomes, I.B., Simões, M. & Simões, L.C. (2014) An Overview on the Reactors to Study Drinking Water Biofilms. *Water Research* **62**: 63-87.
- Griebe, T., Chen, C.-I., Srinivasan, R. & Stewart, P. (1993) Analysis of Biofilm Disinfection by Monochloramine and Free Chlorine. *Biofouling/Biocorrosion in Industrial Systems*, (Geesey GG, Lewandowski Z & Flemming H-C, ed.^eds.), p.^pp. 151-161. Lewis Publications Inc., Chelsea, Michigan.
- Grund, S. & Weber, A. (1988) A New Type of Fimbriae on *Salmonella* Typhimurium. *Journal of Veterinary Medicine, Series B* **35**: 779-782.
- Guérin-Méchin, L., Dubois-Brissonnet, F., Heyd, B. & Leveau, J.Y. (1999) Specific Variations of Fatty Acid Composition of *Pseudomonas aeruginosa* ATCC 15442 Induced by Quaternary Ammonium Compounds and Relation with Resistance to Bactericidal Activity. *Journal of Applied Microbiology* **87**: 735-742.

- Guinee, P., Kampelmacher, E. & Willems, H. (1961) Six new *Salmonella* types, isolated in Ghana (*S. volta*, *S. agona*, *S. wa*, *S. techimani*, *S. mampong* and *S. tafo*). *Antonie Van Leeuwenhoek* **27**: 469-472.
- Güzel-Seydim, Z., Bever Jr, P.I. & Greene, A.K. (2004) Efficacy of Ozone to Reduce Bacterial Populations in the Presence of Food Components. *Food Microbiology* **21**: 475-479.
- Habimana, O., Heir, E., Langsrud, S., Åsli, A.W. & Møretrø, T. (2010) Enhanced Surface Colonization by *Escherichia coli* O157:H7 in Biofilms Formed by an *Acinetobacter calcoaceticus* Isolate from Meat-Processing Environments. *Applied Environmental Microbiology* **76**: 4557-4559.
- Haley, B.J., Cole, D.J. & Lipp, E.K. (2009) Distribution, Diversity, and Seasonality of Waterborne *Salmonellae* in a Rural Watershed. *Applied and Environmental Microbiology* **75**: 1248-1255.
- Hall-Stoodley, L., Costerton, J.W. & Stoodley, P. (2004) Bacterial Biofilms: From the Natural Environment to Infectious Diseases. *Nature Reviews Microbiology* **2**: 95-108.
- Hannan, S., Ready, D., Jasni, A.S., Rogers, M., Pratten, J. & Roberts, A.P. (2010) Transfer of Antibiotic Resistance by Transformation with eDNA within Oral Biofilms. *FEMS Immunology & Medical Microbiology* **59**: 345-349.
- Harp, D.L. (2002) Current Technology of Chlorine Analysis for Water and Wastewater. Hach Company, U.S.A.
- Hauser, E., Tietze, E., Helmuth, R., Junker, E., Blank, K., Prager, R., Rabsch, W., Appel, B., Fruth, A. & Malorny, B. (2010) Pork Contaminated with *Salmonella enterica* serovar 4,[5],12:i:-, an Emerging Health Risk for Humans. *Applied and Environmental Microbiology* **76**: 4601-4610.
- Health Protection Agency (2010) Foodborne General Outbreaks of Infection Caused by Foodborne Pathogens/toxins in England and Wales in Relation to Implicated Food Vehicle (1992-2009). Health Protection Agency, HPA Website.
- Health Protection Agency (2011) *Salmonella*. Health Protection Agency, HPA Website.
- Heathman, L.S., Pierce, G.O. & Kabler, P. (1936) Resistance of Various Strains of *E. typhi* and *coli* Aerogenes to Chlorine and Chloramine. *Public Health Reports* **51**: 1367-1387.
- Heinzel, M. (1998) Phenomena of Biocide Resistance in Microorganisms. *International Biodeterioration & Biodegradation* **41**: 225-234.

- Henle, E.S. & Linn, S. (1997) Formation, Prevention, and Repair of DNA Damage by Iron/Hydrogen Peroxide. *Journal of Biological Chemistry* **272**: 19095-19098.
- Hobley, L., Harkins, C., MacPhee, C.E. & Stanley-Wall, N.R. (2015) Giving Structure to the Biofilm Matrix: An Overview of Individual Strategies and Emerging Common Themes. *FEMS Microbiol Reviews* **39**: 649-669.
- Housecroft, C.E. & Constable, E.C. (2010) *Chemistry*. Pearson Education Limited, Essex, England.
- Howe, R., Andrews, J., MacGowan, A., Livermore, D., Brown, N., Winstanley, T., Brown, D., Glynn, G., Perry, J., MacKenzie, F., Kahlmeter, G., Teale, C., Morrissey, I., Bowker, K. & Wootton, M. (2011) Methods for Antimicrobial Susceptibility Testing. British Society for Antimicrobial Chemotherapy.
- Hugo, W.B. & Bloomfield, S.F. (1971) Studies on the Mode of Action of the Phenolic Antibacterial Agent Fentichlor Against *Staphylococcus aureus* and *Escherichia coli* II. The Effects of Fentichlor on the Bacterial Membrane and the Cytoplasmic Constituents of the Cell. *Journal of Applied Bacteriology* **34**: 569-578.
- Hugo, W.B. & Denyer, S.P. (1987) The Concentration Exponent of Disinfectants and Preservatives (biocides). 281-291. Blackwell Scientific Publications, Oxford, UK.
- Hur, J., Jawale, C. & Lee, J.H. (2012) Antimicrobial Resistance of *Salmonella* Isolated From Food Animals: A Review. *Food Research International* **45**: 819-830.
- Imlay, J.A., Chin, S.M. & Linn, S. (1998) Toxic DNA Damage by Hydrogen Peroxide Through the Fenton Reaction in vivo and in vitro. *Science* **240**: 640-642.
- Jafari, A., Bouzari, S., Farhoudi-Moghaddam, A.A., Parsi, M. & Shokouhi, F. (1994) In Vitro Adhesion and Invasion of *Salmonella enterica* serovar Havana. *Microbial Pathogenesis* **16**: 65-70.
- Jang, A., Szabo, J., Hosni, A., Coughlin, M. & Bishop, P. (2006) Measurement of Chlorine Dioxide Penetration in Dairy Process Pipe Biofilms During Disinfection. *Applied Microbiology and Biotechnology* **72**: 368-376.
- Joo, H.-S. & Otto, M. (2013) Molecular Basis of in-vivo Biofilm Formation by Bacterial Pathogens. *Chemistry and Biology* **19**: 1053-1513.
- Jorgensen, J.H. & Ferraro, M.J. (2009) Antimicrobial Susceptibility Testing: A Review of General Principles and Contemporary Practices. *Clinical Infectious Diseases* **49**: 1749–1755.
- Juzwa, W., Myszka, K., Białas, W., Dobrucka, R., Konieczny, P. & Czaczyk, K. (2015) Investigation of the Effectiveness of Disinfectants Against Planktonic and

Biofilm Forms of *P. aeruginosa* and *E. faecalis* Cells Using a Compilation of Cultivation, Microscopic and Flow Cytometric Techniques. *Biofouling* **31**: 587-597.

Karatzas, K.A., Webber, M.A., Jorgensen, F., Woodward, M.J., Piddock, L.J. & Humphrey, T.J. (2007) Prolonged Treatment of *Salmonella enterica* serovar Typhimurium with Commercial Disinfectants Selects for Multiple Antibiotic Resistance, Increased Efflux and Reduced Invasiveness. *Journal of Antimicrobial Chemotherapy* **60**: 947-955.

Karatzas, K.A., Hocking, P.M., Jorgensen, F., Mattick, K., Leach, S. & Humphrey, T.J. (2008a) Effects of Repeated Cycles of Acid Challenge and Growth on the Phenotype and Virulence of *Salmonella enterica*. *Journal of Applied Microbiology* **105**: 1640-1648.

Karatzas, K.A., Randall, L.P., Webber, M., Piddock, L.J., Humphrey, T.J., Woodward, M.J. & Coldham, N.G. (2008b) Phenotypic and Proteomic Characterization of Multiply Antibiotic-Resistant Variants of *Salmonella enterica* serovar Typhimurium Selected Following Exposure to Disinfectants. *Applied Environmental Microbiology* **74**: 1508-1516.

Kim, J., Park, H.-D. & Chung, S. (2012) Microfluidic Approaches to Bacterial Biofilm Formation. *Molecules* **17**: 9818-9834.

Kim, J., Hahn, J.-S., Franklin, M.J., Stewart, P.S. & Yoon, J. (2009) Tolerance of Dormant and Active Cells in *Pseudomonas aeruginosa* PA01 Biofilm to Antimicrobial Agents. *Journal of Antimicrobial Chemotherapy* **63**: 129-135.

Kirisits, M.J., Prost, L., Starkey, M. & Parsek, M.R. (2005) Characterization of Colony Morphology Variants Isolated from *Pseudomonas aeruginosa* Biofilms. *Applied Environmental Microbiology* **71**: 4809-4821.

Kitis, M. (2003) Disinfection of Wastewater with Peracetic Acid: A Review. *Environment International* **30**: 47-55.

Knapp, L., Rushton, L., Stapleton, H., Sass, A., Stewart, S., Amazquita, A., McClure, P., Mahenthiralingham, E. & Maillard, J.Y. (2013) The Effect of Cationic Microbicide Exposure against *Burkholderia cepacia* complex (Bcc); The Use of *Burkholderia lata* Strain 383 as a Model Bacterium. *Journal of Applied Microbiology* **115**: 1117-1126.

Koh, K.S., Lam, K.W., Alhede, M., Queck, S.Y., Labbate, M., Kjelleberg, S. & Rice, S.A. (2007) Phenotypic Diversification and Adaptation of *Serratia marcescens* MG1 Biofilm-Derived Morphotypes. *Journal of Bacteriology* **189**: 119-130.

Kristiansson, E., Fick, J., Janzon, A., Grabic, R., Rutgersson, C., Weijdegård, B., Söderström, H. & Larsson, J.D.G. (2011) Pyrosequencing of Antibiotic-

Contaminated River Sediments Reveals High Levels of Resistance and Gene Transfer Elements. *PLoS One* **6**.

Kurschner, L.M. & Diken, G.M. (1997) Use of peracetic acid to sanitize processed fowl. Google Patents.

Lahiri, A., Lahiri, A., Iyer, N., Das, P. & Chakravortty, D. (2010) Visiting the Cell Biology of *Salmonella* Infection. *Microbes and Infection* **12**: 809-818.

Lakretz, A., Ron, E.Z. & Mamane, H. (2011) Biofilm Control in Water by a UV-based Advanced Oxidation Process. *Biofouling* **27**: 295-307.

Lambert, R.J.W. & Johnston, M.D. (2001) The Effect of Interfering Substances on the Disinfection Process: A Mathematical Model. *Journal of Applied Microbiology* **91**: 548-555.

Lavilla Lerma, L., Benomar, N., Sánchez Valenzuela, A., Casado Muñoz, M.d.C., Gálvez, A. & Abriouel, H. (2014) Role of EfrAB Efflux Pump in Biocide Tolerance and Antibiotic Resistance of *Enterococcus faecalis* and *Enterococcus faecium* Isolated from Traditional Fermented Foods and the Effect of EDTA as EfrAB Inhibitor. *Food Microbiology* **44**: 249-257.

Lawler, A.J., Ricci, V., Busby, S.J.W. & Piddock, L.J.V. (2013) Genetic Inactivation of *acrAB* or Inhibition of Efflux Induces Expression of *ramA*. *Journal of Antimicrobial Chemotherapy* **68**: 1551-1557.

Lear, J.C., Maillard, J.Y., Dettmar, P.W., Goddard, P.A. & Russell, A.D. (2002) Chloroxylenol- and Triclosan-Tolerant Bacteria from Industrial Sources. *Journal of Industrial Microbiology and Biotechnology* **29**: 238-242.

Leriche, V., Briandet, R. & Carpentier, B. (2003) Ecology of Mixed Biofilms Subjected Daily to a Chlorinated Alkaline Solution: Spatial Distribution of Bacterial Species Suggests a Protective Effect of One Species to Another. *Environmental Microbiology* **5**: 64-71.

Leung, C.Y., Chan, Y.C., Samaranyake, L.P. & Seneviratne, C.J. (2012) Biocide Resistance of *Candida* and *Escherichia coli* Biofilms is Associated with Higher Antioxidative Capacities. *Journal of Hospital Infection* **81**: 79-86.

Levantesi, C., Bonadonna, L., Briancesco, R., Grohmann, E., Toze, S. & Tandoi, V. (2012) *Salmonella* in Surface and Drinking Water: Occurrence and Water-mediated Transmission. *Food Research International* **45**: 587-602.

Levy, S.B. (2002) Active Efflux, A Common Mechanism for Biocide and Antibiotic Resistance. *Journal of Applied Microbiology Symposium Supplement* **92**: S65-S71.

Li, X.Z. & Nikaido, H. (2009) Efflux-Mediated Drug Resistance in Bacteria: An Update. *Drugs* **69**: 1555-1623.

- Lin, S.-M., Svoboda, K.K.H., Giletto, A., Seibert, J. & Puttaiah, R. (2011) Effects of Hydrogen Peroxide on Dental Unit Biofilms and Treatment Water Contamination. *European Journal of Dentistry* **5**: 47-59.
- Linley, E., Denyer, S.P., McDonnell, G., Simons, C. & Maillard, J.-Y. (2012) Use of Hydrogen Peroxide as a Biocide: New Consideration of its Mechanisms of Biocidal Action. *Journal of Antimicrobial Chemotherapy* **67**: 1589-1596.
- Luppens, S.B.I., Kara, D., Bandounas, L., Jonker, M.J., Wittink, F.R.A., Bruning, O., Breit, T.M., Ten Cate, J.M. & Crielaard, W. (2008) Effect of *Veillonella parvula* on the Antimicrobial Resistance and Gene Expression of *Streptococcus mutans* Grown in a Dual-species Biofilm. *Oral Microbiology and Immunology* **23**: 183-189.
- Madigan, M.T., Martinko, J.M., Dunlap, P.V. & Clark, D.P. (2009) *Brock Biology of Microorganisms*. Pearson Benjamin Cummings, San Francisco, USA.
- Mah, T.-F., Pitts, B., Pellock, B., Walker, G.C., Stewart, P.S. & O'Toole, G.A. (2003) A Genetic Basis for *Pseudomonas aeruginosa* Biofilm Antibiotic Resistance. *Nature* **426**: 306-310.
- Maillard, J.Y. (2005a) Biocides in the Healthcare Environment. Webber Training, www.webbertraining.com.
- Maillard, J.Y. (2005b) Antimicrobial Biocides in the Healthcare Environment: Efficacy, Usage, Policies and Perceived Problems. *Journal of Therapeutics and Clinical Risk Management* **1**: 307-320.
- Maillard, J.Y. (2013a) Factors Affecting the Activities of Microbicides. John Wiley and Sons, Oxford, UK.
- Maillard, J.Y. (2013b) Mechanisms of Bacterial Resistance to Microbicides. 108-130. John Wiley and Sons, Oxford, UK.
- Mangalappalli-Illathu, A.K., Vidovic, S. & Korber, D.R. (2008) Differential Adaptive Response and Survival of *Salmonella enterica* Serovar Enteritidis Planktonic and Biofilm Cells Exposed to Benzalkonium Chloride. *Antimicrobial Agents and Chemotherapy* **52**: 3669-3680.
- Mavri, A. & Smole Možina, S. (2012) Involvement of Efflux Mechanisms in Biocide Resistance of *Campylobacter jejuni* and *Campylobacter coli*. *Journal of Medical Microbiology* **61**: 800-808.
- Mc Mahon, M.A.S., Blair, I.S., Moore, J.E. & Mc Dowell, D.A. (2007) The Rate of Horizontal Transmission of Antibiotic Resistance Plasmids is Increased in Food Preservation-stressed Bacteria. *Journal of Applied Microbiology* **103**: 1883-1888.

- McAdams, H.H. & Arkin, A. (1997) Stochastic Mechanisms in Gene Expression. *Proceedings of the National Academy of Sciences of the United States of America* **94**: 814-819.
- McBain, A.J., Rickard, A.H. & Gilbert, P. (2002) Possible Implications of Biocide Accumulation in the Environment on the Prevalence of Bacterial Antibiotic Resistance. *Journal of Industrial Microbiology and Biotechnology* **29**: 326-330.
- McMurray, L.M., Petrucci, R. & Levy, S.B. (1980) Active Efflux of Tetracycline Encoded by Four Genetically Different Tetracycline Resistance Determinants in *E. coli*. *Proceedings of the National Academy of Sciences of the United States of America* **77**: 3974-3977.
- Miles, A.A. & Misra, S.S. (1938) The Estimation of the Bactericidal Power of Blood. *Journal of Hygiene* **38**: 732-749.
- Molin, S. & Tolker-Nielsen, T. (2003) Gene Transfer Occurs with Enhanced Efficiency in Biofilms and Induces Enhanced Stabilisation of the Biofilm Structure. *Current Opinion in Biotechnology* **14**: 255-261.
- Morris, J.C. (1966) The Acid Ionization Constant of HOCl from 5 to 35^o. *Journal of Physical Chemistry* **70**: 3798-3805.
- Mustapha, P., Epalle, T., Allegra, S., Girardot, F., Garraud, O. & Riffard, S. (2015) Monitoring of *Legionella pneumophila* viability after chlorine dioxide treatment using flow cytometry. *Research in Microbiology* **166**: 215-219.
- Nair, A., Rawool, D.B., Doijad, S., Poharkar, K., Mohan, V., Barbuddhe, S.B., Kolhe, R., Kurkure, N.V., Kumar, A., Malik, S.V.S. & Balasaravanan, T. (2015) Biofilm Formation and Genetic Diversity of *Salmonella* Isolates Recovered from Clinical, Food, Poultry and Environmental Sources. *Infection, Genetics and Evolution* **36**: 424-433.
- Nance, W.C., Dowd, S.E., Samarian, D., Chludzinski, J., Delli, J., Battista, J. & Rickard, A.H. (2013) A High-Throughput Microfluidic Dental Plaque Biofilm System to Visualize and Quantify the Effect of Antimicrobials. *Journal of Antimicrobial Chemotherapy* **68**: 2550-2560.
- Nicolay, N., Thornton, L., Cotter, S., Garvey, P., Bannon, O., McKeown, P., Cormican, M., Fisher, I., Little, C., Boxall, N., De Pinna, E., Peters, T.M., Cowden, J., Salmon, R., Mason, B., Irvine, N., Rooney, P. & O'Flanagan, D. (2011) *Salmonella enterica* serovar Agona European Outbreak Associated With a Food Company. *Epidemiology & Infection* **139**: 1272-1280.
- Nikaido, E., Shirosaka, I., Yamaguchi, A. & Nishino, K. (2011) Regulation of the AcrAB multidrug efflux pump in *Salmonella enterica* serovar Typhimurium in response to indole and paraquat. *Microbiology* **157**: 648-655.

- Nikaido, H. (1998) The Role of Outer Membrane and Efflux Pumps in the Resistance of Gram-negative Bacteria. Can We Improve Drug Access? *Drug Resistance Updates* **1**: 93-98.
- Nunoshiba, T., Hidalgo, E., Amábile Cuevas, C.F. & Demple, B. (1992) Two-stage control of an oxidative stress regulon: the Escherichia coli SoxR protein triggers redox-inducible expression of the soxS regulatory gene. *J Bacteriol* **174**: 6054-6060.
- O'Toole, G.A. (2011) Microtiter Dish Biofilm Formation Assay. *Journal of Visualized Experiments : JoVE* 2437.
- Ortega Morente, E., Fernández-Fuentes, M.A., Grande Burgos, M.J., Abriouel, H., Pérez Pulido, R. & Gálvez, A. (2013) Biocide Tolerance in Bacteria. *International Journal of Food Microbiology* **162**: 13-25.
- Otter, J.A., Yezli, S. & French, G.L. (2012) Impact of the Suspending Medium on Susceptibility of Methicillin-resistant *Staphylococcus aureus* to Hydrogen Peroxide Vapour Decontamination. *Journal of Hospital Infection*.
- Otter, J.A., Vickery, K., Walker, J.T., deLancey Pulcini, E., Stoodley, P., Goldenberg, S.D., Salkeld, J.A.G., Chewins, J., Yezli, S. & Edgeworth, J.D. (2015) Surface-attached Cells, Biofilms and Biocide Susceptibility: Implications for Hospital Cleaning and Disinfection. *Journal of Hospital Infection*.
- Oxoid (2008) M.I.C. Evaluator Multilanguage Instructions For Use. 1-6.
- Pace, J.L., Rupp, M.E. & Finch, R.G. (2006) *Biofilms, Infection and Antimicrobial Therapy*. CRC Press, Florida, USA.
- Pagedar, A., Singh, J. & Batish, V.K. (2010) Surface Hydrophobicity, Nutritional Contents Affect *Staphylococcus aureus* Biofilms and Temperature Influences its Survival in Preformed Biofilms. *Journal of Basic Microbiology* **50**: S98-S106.
- Pamp, S.J., Sternberg, C. & Tolker-Nielsen, T. (2009) Insight into the Microbial Multicellular Lifestyle via Flow-Cell Technology and Confocal Microscopy. *Cytometry Part A* **75A**: 90-103.
- Pantanella, F., Valenti, P., Natalizi, T., Passeri, D. & Berlutti, F. (2013) Analytical Techniques to Study Microbial Biofilm on Abiotic surfaces: Pros and Cons of the Main Techniques Currently In Use. *Annali di Igiene* **25**: 31-42.
- Pappen, F.G., Qian, W., Aleksejuniene, J., Leonardo Rde, T., Leonardo, M.R. & Haapasalo, M. (2010) Inhibition of Sodium Hypochlorite Antimicrobial Activity in the Presence of Bovine Serum Albumin. *Journal of Endodontics* **36**: 268-271.
- Peeters, E., Nelis, H.J. & Coenye, T. (2008) Evaluation of the Efficacy of Disinfection Procedures Against *Burkholderia cenocepacia* Biofilms. *Journal of Hospital Infection* **70**: 361-368.

- Peterson, H.G., Hrudey, S.E., Cantin, I.A., Perley, T.R. & Kenefick, S.L. (1995) Physiological Toxicity, Cell Membrane Damage and the Release of Dissolved Organic Carbon and Geosmin by *Aphanizomenon flos-aquae* after Exposure to Water Treatment Chemicals. *Water Research* **29**: 1515-1523.
- Petrova, O.E. & Sauer, K. (2016) Escaping the biofilm in more than one way: desorption, detachment or dispersion. *Current Opinion in Microbiology* **30**: 67-78.
- Piddock, L.J.V. (2006) Multidrug-Resistance Efflux Pumps- Not Just for Resistance. *Nature Reviews Microbiology* **4**: 629-636.
- Piras, F., Fois, F., Consolati, S.G., Mazza, R. & Mazzette, R. (2015) Influence of Temperature, Source, and Serotype on Biofilm Formation of *Salmonella enterica* Isolates from Pig Slaughterhouses. *Journal of Food Protection* **78**: 1875-1878.
- Pitts, B., Hamilton, M.A., Zelter, N. & Stewart, P.S. (2003) A Microtiter-Plate Screening Method for Biofilm Disinfection and Removal. *Journal of Microbiological Methods* **54**: 269-276.
- Plough, H.H. (1945) Penicillin Resistance of *Staphylococcus Aureus* and its Clinical Implications. *American Journal of Clinical Pathology* **15**: 446-451.
- Pomposiello, P.J. & Demple, B. (2000) Identification of SoxS-Regulated Genes in *Salmonella enterica* Serovar Typhimurium. *J Bacteriol* **182**: 23-29.
- Poole, K. (2002) Mechanisms of Bacterial Biocide and Antibiotic Resistance. *Journal of Applied Microbiology Symposium Supplement* **92**: S55-S64.
- Prasher, D.C., Eckenrode, V.K., Ward, W.W., Prendergast, F.G. & Cormier, M.J. (1992) Primary structure of the *Aequorea victoria* green-fluorescent protein. *Gene* **111**: 229-233.
- Public Health England (2014) PHE Gastrointestinal Infections Data: Summary of *Salmonella* Surveillance, 2013. Public Health England.
- Qin, D., He, X., Wang, K. & Tan, W. (2008) Using Fluorescent Nanoparticles and SYBR Green I Based Two-Color Flow Cytometry to Determine *Mycobacterium tuberculosis* Avoiding False Positives. *Biosensors and Bioelectronics* **24**: 626–631.
- Rajamohan, G., Srinivasan, V.B. & Gebreyes, W.A. (2010a) Molecular and functional characterization of a novel efflux pump, AmvA, mediating antimicrobial and disinfectant resistance in *Acinetobacter baumannii*. *Journal of Antimicrobial Chemotherapy* **65**: 1919-1925.
- Rajamohan, G., Srinivasan, V.B. & Gebreyes, W.A. (2010b) Novel role of *Acinetobacter baumannii* RND efflux transporters in mediating decreased susceptibility to biocides. *Journal of Antimicrobial Chemotherapy* **65**: 228-232.

- Randall, L.P., Cooles, S.W., Coldham, N.G., Penuela, E.G., Mott, A.C., Woodward, M.J., Piddock, L.J. & Webber, M.A. (2007) Commonly used Farm Disinfectants Can Select for Mutant *Salmonella enterica* serovar Typhimurium with Decreased Susceptibility to Biocides and Antibiotics Without Compromising Virulence. *Journal of Antimicrobial Chemotherapy* **60**: 1273-1280.
- Rani, S.A., Pitts, B., Beyenal, H., Veluchamy, R.A., Lewandowski, Z., Davison, W.M., Buckingham-Meyer, K. & Stewart, P.S. (2007) Spatial Patterns of DNA Replication, Protein Synthesis, and Oxygen Concentration within Bacterial Biofilms Reveal Diverse Physiological States. *Journal of Bacteriology* **189**: 4223-4233.
- Ricci, V., Busby, S.J.W. & Piddock, L.J.V. (2012) Regulation of RamA by RamR in *Salmonella enterica* Serovar Typhimurium: Isolation of a RamR Superrepressor. *Antimicrobial Agents and Chemotherapy* **56**: 6037-6040.
- Richardson, B.W. (1891) On Peroxide of Hydrogen or Ozone Water as a Remedy. *The Lancet* **i**: 707-709.
- Rollet, C., Gal, L. & Guzzo, J. (2009) Biofilm-Detached Cells, a Transition From a Sessile to a Planktonic Phenotype: A Comparative Study of Adhesion and Physiological Characteristics in *Pseudomonas aeruginosa*. *FEMS Microbiology Letters* **290**: 135-142.
- Ross, S.S., Tu, M.H., Falsetta, M.L., Ketterer, M.R., Kiedrowski, M.R., Horswill, A.R., Apicella, M.A., Reinhardt, J.M. & Fiegel, J. (2014) Quantification of Confocal Images of Biofilms Grown on Irregular Surfaces. *Journal of Microbiological Methods* **100**: 111-120.
- Rossi-Fedele, G., Guastalli, A.R., Dogramaci, E.J., Steier, L. & De Figueiredo, J.A. (2011) Influence of pH Changes on Chlorine-containing Endodontic Irrigating Solutions. *International Endodontic Journal* **44**: 792-799.
- Russell, A.D. (2003a) Similarities and Differences in the Responses of Microorganisms to Biocides. *Journal of Antimicrobial Chemotherapy* **52**: 750-763.
- Russell, A.D. (2003b) Factors Influencing the Efficacy of Antimicrobial Agents 98-118. Blackwell Publishing Ltd, Oxford.
- Russell, A.D. & McDonnell, G. (1999) Antiseptics and Disinfectants: Activity, Action and Resistance. *Clinical Microbiology Reviews* **12**: 147-179.
- Russell, A.D. & McDonnell, G. (2000) Concentration: A Major Factor in Studying Biocidal Action. *Journal of Hospital Infection* **44**: 1-3.
- Russo, E.T., Biggerstaff, G., Hoekstra, R.M., Meyer, S., Patel, N., Miller, B. & Quick, R. (2013) A Recurrent, Multistate Outbreak of *Salmonella* Serotype Agona Infections Associated with Dry, Unsweetened Cereal Consumption, United States, 2008. *Journal of Food Protection* **76**: 227-230.

- Rutala, W.A. & Weber, D.J. (1997) Uses of Inorganic Hypochlorite (bleach) in Health-care Facilities. *Clinical Microbiology Reviews* **10**.
- Rutala, W.A. & Weber, D.J. (1999a) Disinfection of Endoscopes: Review of New Chemical Sterilants Used for High-Level Disinfection. *Infection Control and Hospital Epidemiology* **20**: 69-76.
- Rutala, W.A. & Weber, D.J. (1999b) Infection Control: The Role of Disinfection and Sterilization. *Journal of Hospital Infection* **43**: S43-S55.
- Ryu, J.-H., Kim, H. & Beuchat, L.R. (2004) Attachment and Biofilm Formation by *Escherichia coli* O157:H7 on Stainless Steel as Influenced by Exopolysaccharide Production, Nutrient Availability, and Temperature. *Journal of Food Protection* **67**: 2123-2131.
- Sanchez-Ruiz, C., Martinez-Royano, S. & Tejero-Monzon, I. (1995) An Evaluation of the Efficiency and Impact of Raw Wastewater Disinfection with Peracetic Acid Prior to Ocean Discharge. *Water Science and Technology* **32**: 159–166.
- Sánchez-Vargas, F.M., Abu-El-Haija, M.A. & Gómez-Duarte, O.G. (2011) *Salmonella* infections: An Update on Epidemiology, Management and Prevention. *Travel Medicine and Infectious Disease* **9**: 263-277.
- Schiff, F. & Saphra, I. (1941) A New Salmonella Type: Salmonella Havana. *The Journal of Infectious Diseases* **68**: 125-127.
- Schurman, J.J. (2001) Antibacterial Activity of Hydrogen Peroxide Against *Escherichia coli* and *Salmonella* spp. in Fruit Juices, Both Alone and In Combination with Organic Acid. Thesis, Faculty of the Virginia Polytechnic Institute and State University, Virginia.
- Scientific Committee on Emerging and Newly Identified Health Risks (2009) Assessment of the Antibiotic Resistance Effects of Biocides. Scientific Committee on Emerging and Newly Identified Health Risks.
- Shakeri, S., Kermanshahi, R.K., Moghaddam, M.M. & Emtiazi, G. (2007) Assessment of Biofilm Cell Removal and Killing and Biocide Efficacy Using the Microtiter Plate Test. *Biofouling* **23**: 79-86.
- Shimomura, O., Johnson, F. & Saiga, Y. (1962) Extraction, Purification and Properties of Aequorin, a Bioluminescent Protein from Luminous Hydromedusan, *Aequorea*. *Journal of Cellular and Comparative Physiology* **59**: 223-239.
- Shrout, J.D. & Nerenberg, R. (2012) Monitoring Bacterial Twitter: Does Quorum Sensing Determine the Behavior of Water and Wastewater Treatment Biofilms? *Environmental Science and Technology* **46**: 1995-2005.

- Silvestry-Rodriguez, N., Sicairos-Ruelas, E.E., Gerba, C.P. & Bright, K.R. (2007) Silver as a Disinfectant. *Reviews of Environmental Contamination and Toxicology* **191**: 23-45.
- Simm, R., Ahmad, I., Rhen, M., Le Guyon, S. & Römling, U. (2014) Regulation of Biofilm Formation in *Salmonella enterica* serovar Typhimurium. *Future Microbiology* **9**: 1261-1282.
- Simões, L.C., Lemos, M., Araújo, P., Pereira, A.M. & Simões, M. (2011) The effects of glutaraldehyde on the control of single and dual biofilms of *Bacillus cereus* and *Pseudomonas fluorescens*. *Biofouling* **27**: 337-346.
- Simões, M., Simões, L.C. & Vieira, M.J. (2008) Species Association Increases Biofilm Resistance to Chemical and Mechanical Treatments. *Water Research* **43**: 229-237.
- Sincock, S.A. & Robinson, J.P. (2001) Flow Cytometric Analysis of Microorganisms. *Methods in Cell Biology* **64**: 511-537.
- Sirtes, G., Waltimo, T., Schaetzle, M. & Zehnder, M. (2005) The Effects of Temperature on Sodium Hypochlorite Short-term Stability, Pulp Dissolution Capacity, and Antimicrobial Efficacy. *Journal of Endodontics* **31**: 669-671.
- Solano, C., Echeverz, M. & Lasa, I. (2014) Biofilm Dispersion and Quorum Sensing. *Current Opinion in Microbiology* **18**: 96-104.
- Soto, S.M. (2013) Role of Efflux Pumps in the Antibiotic Resistance of Bacteria Embedded in a Biofilm. *Virulence* **4**: 223-229.
- Soumet, C., Méheust, D., Pissavin, C., Le Grandois, P., Frémaux, B., Feurer, C., Le Roux, A., Denis, M. & Maris, P. (2016) Reduced Susceptibilities to Biocides and Resistance to Antibiotics in Food-associated Bacteria Following Exposure to Quaternary Ammonium Compounds. *Journal of Applied Microbiology* n/a-n/a.
- Spector, M.P. & Kenyon, W.J. (2012) Resistance and Survival Strategies of *Salmonella enterica* to Environmental Stresses. *Food Research International* **45**: 455-481.
- Steenackers, H., Hermans, K., Vanderleyden, J. & De Keersmaecker, S.C.J. (2012) *Salmonella* Biofilms: An Overview on Occurrence, Structure, Regulation and Eradication. *Food Research International* **45**: 502-531.
- Stepanović, S., Ćirković, I., Ranin, L. & Svabić-Vlahović, M. (2004) Biofilm Formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. *Letters in Applied Microbiology* **38**: 428-432.
- Stephens, P.J., Druggan, P. & Nebe-von Caron, G. (2000) Stressed *Salmonella* are exposed to reactive oxygen species from two independent sources during recovery in

conventional culture media. *International Journal of Food Microbiology* **60**: 269-285.

Stewart, P.S. & Franklin, M.J. (2008) Physiological Heterogeneity in Biofilms. *Nature Reviews Microbiology* **6**: 199-210.

Stojicic, S., Shen, Y. & Haapasalo, M. (2013) Effect of the Source of Biofilm Bacteria, Level of Biofilm Maturation, and Type of Disinfecting Agent on the Susceptibility of Biofilm Bacteria to Antibacterial Agents. *Journal of Endodontics* **39**: 473-477.

Stoodley, P., Sauer, K., Davies, D.G. & Costerton, J.W. (2002) Biofilms as Complex Differentiated Communities. *Annual Review of Microbiology* **56**: 187-209.

Sun, J., Deng, Z. & Yan, A. (2014) Bacterial Multidrug Efflux Pumps: Mechanisms, Physiology and Pharmacological Exploitations. *Biochemical and Biophysical Research Communications* **453**: 254-267.

Szabo, J. & Minamyer, S. (2014) Decontamination of Biological Agents from Drinking Water Infrastructure: A Literature Review and Summary. *Environment International* **72**: 124-128.

Tachikawa, M., Tezuka, M., Morita, M., Isogai, K. & Okada, S. (2005) Evaluation of Some Halogen Biocides Using a Microbial Biofilm System. *Water Research* **39**: 4126-4132.

Takehara, A. & Fukuzaki, S. (2006) Relationship Between the pH and Decrease in Available Chlorine of Sodium Hypochlorite in the Presence of Organic Compounds or During Freezing Treatment. *Report of Industrial Technology Centre of Okayama Prefecture* **32**: 10-13.

Takenaka, S., Trivedi, H.M., Corbin, A., Pitts, B. & Stewart, P.S. (2008) Direct Visualization of Spatial and Temporal Patterns of Antimicrobial Action within Model Oral Biofilms. *Applied Environmental Microbiology* **74**: 1869-1875.

Tamburini, S., Ballarini, A., Ferrentino, G., Moro, A., Foladori, P., Spilimbergo, S. & Jousson, O. (2013) Comparison of Quantitative PCR and Flow Cytometry as Cellular Viability Methods to Study Bacterial Membrane Permeabilization Following Supercritical CO₂ Treatment. *Microbiology* **159**: 1056-1066.

Tattawasart, U., Maillard, J.Y., Furr, J.R. & Russell, A.D. (2000) Outer Membrane Changes in *Pseudomonas stutzeri* Resistant to Chlorhexidine Diacetate and Cetylpyridinium Chloride. *International Journal of Antimicrobial Agents* **16**: 233-238.

Taylor, N.G., Verner-Jeffreys, D.W. & Baker-Austin, C. (2011) Aquatic Systems: Maintaining, Mixing and Mobilising Antimicrobial Resistance? *Trends in Ecology and Evolution* **26**: 278-284.

- Thenard, L.J. (1818) Observations sur des nouvelles combinaisons entre l'oxygène et divers acides. *Annales de chimie et de physique* **8**: 306-312.
- Thomas, L., Russell, A.D. & Maillard, J.Y. (2005) Antimicrobial Activity of Chlorhexidine Diacetate and Benzalkonium Chloride Against *Pseudomonas aeruginosa* and its Response to Biocide Residues. *Journal of Applied Microbiology* **98**: 533-543.
- Thomas, L., Maillard, J.Y., Lambert, R.J. & Russell, A.D. (2000) Development of Resistance to Chlorhexidine Diacetate in *Pseudomonas aeruginosa* and The Effect of a "Residual" Concentration. *Journal of Hospital Infection* **46**: 297-303.
- Tirumalai, R.S. (2004) Disinfectants and Antiseptics. Pharmacopeial Forum, Pharmacopeial Forum Online.
- Unis, M.M.A. (2010) Peroxide Reactions of Environmental Relevance in Aqueous Solution. Thesis, Northumbria University, Newcastle.
- van der Straaten, T., Zulianello, L., van Diepen, A., Granger, D.L., Janssen, R. & van Dissel, J.T. (2004) Salmonella enterica Serovar Typhimurium RamA, Intracellular Oxidative Stress Response, and Bacterial Virulence. *Infection and Immunity* **72**: 996-1003.
- van der Veen, S. & Abee, T. (2011) Mixed Species Biofilms of *Listeria monocytogenes* and *Lactobacillus plantarum* Show Enhanced Resistance to Benzalkonium Chloride and Peracetic Acid. *International Journal of Food Microbiology* **144**: 421-431.
- Vestby, L.K., Møretrø, T., Langsrud, S., Heir, E. & Nesse, L.L. (2009) Biofilm Forming Abilities of *Salmonella* are Correlated with Persistence in Fish Meal- and Feed Factories. *BMC Veterinary Research* **5**: 20-20.
- Vikram, A., Bomberger, J.M. & Bibby, K.J. (2015a) Efflux as a Glutaraldehyde Resistance Mechanism in *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* Biofilms. *Antimicrobial Agents and Chemotherapy* **59**: 3433-3440.
- Vikram, A., Bomberger, J. & Bibby, K.J. (2015b) Efflux as a Glutaraldehyde Resistance Mechanism in *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* Biofilms. *Antimicrobial Agents and Chemotherapy*.
- Vital, M., Dignum, M., Magic-Knezev, A., Ross, P., Rietveld, L. & Hammes, F. (2012) Flow Cytometry and Adenosine Tri-phosphate Analysis: Alternative Possibilities to Evaluate Major Bacteriological Changes in Drinking Water Treatment and Distribution Systems. *Water Research* **46**: 4665-4676.
- Wagner, M., Brumelis, D. & Gehr, R. (2002) Disinfection of Wastewater by Hydrogen Peroxide or Peracetic Acid: Development of Procedures for Measurement

of Residual Disinfectant and Application to a Physicochemically Treated Municipal Effluent. *Water Environment Research* **74**: 33-50.

Walsh, S.E., Maillard, J.Y., Russell, A.D., Catrenich, C.E., Charbonneau, D.L. & Bartolo, R.G. (2003) Development of Bacterial Resistance to Several Biocides and Effects on Antibiotic Susceptibility. *Journal of Hospital Infection* **55**: 98-107.

Wang, Q., Frye, J.G., McClelland, M. & Harshey, R.M. (2004) Gene Expression Patterns During Swarming in *Salmonella* Typhimurium: Genes Specific to Surface Growth and Putative New Motility and Pathogenicity Genes. *Molecular Microbiology* **52**: 169-187.

Wang, R., Kalchayanand, N., King, D.A., Luedtke, B.E., Bosilevac, J.M. & Arthur, T.M. (2014) Biofilm Formation and Sanitizer Resistance of *Escherichia coli* O157:H7 Strains Isolated from High Event Period Meat Contamination. *Journal of Food Protection* **77**: 1982-1987.

Wang, Z., Gerstein, M. & Snyder, M. (2009) RNA-Seq: A Revolutionary Tool for Transcriptomics. *Nature reviews. Genetics* **10**: 57-63.

Warnes, S.L. & Keevil, C.W. (2011) Mechanism of Copper Surface Toxicity in Vancomycin-Resistant *Enterococci* following Wet or Dry Surface Contact. *Applied Environmental Microbiology* **77**: 6049-6059.

Waters, B.W. & Hung, Y.-C. (2014) The Effect of Organic Loads on Stability of Various Chlorine-based Sanitisers. *International Journal of Food Science & Technology* **49**: 867-875.

Webber, M. & Coldham, N. (2010) Measuring the Activity of Active Efflux in Gram-Negative Bacteria. *Antibiotic Resistance Protocols*, Vol. 642 (Gillespie SH & McHugh TD, ed. eds.), p. pp. 173-180. Humana Press.

Webber, M.A., Randall, L.P., Cooles, S., Woodward, M.J. & Piddock, L.J. (2008) Triclosan resistance in *Salmonella enterica* serovar Typhimurium. *Journal of Antimicrobial Chemotherapy* **62**: 83-91.

Webber, M.A., Whitehead, R.N., Mount, M., Loman, N.J., Pallen, M.J. & Piddock, L.J.V. (2015) Parallel Evolutionary Pathways to Antibiotic Resistance Selected by Biocide Exposure. *Journal of Antimicrobial Chemotherapy* **70**: 2241-2248.

Wesche, A.M., Gurtler, J.B., Marks, B.P. & Ryser, E.T. (2009) Stress, Sublethal Injury, Resuscitation, and Virulence of Bacterial Foodborne Pathogens. *Journal of Food Protection* **72**: 1121-1138.

White, D.G., Zhao, S., Sudler, R., Ayers, S., Friedman, S., Chen, S., McDermott, P.F., McDermott, S., Wagner, D.D. & Meng, J. (2001) The Isolation of Antibiotic-Resistant *Salmonella* from Retail Ground Meats. *New England Journal of Medicine* **345**: 1147-1154.

- Whitehead, R.N., Overton, T.W., Kemp, C.L. & Webber, M.A. (2011) Exposure of *Salmonella enterica* serovar Typhimurium to High Level Biocide Challenge Can Select Multidrug Resistant Mutants in a Single Step. *PLoS One* **6**: e22833.
- Winder, C.L., Al Adham, I.S., Abdel Malek, S.M., Buultjens, T.E., Horrocks, A.J. & Collier, P.J. (2000) Outer Membrane Protein Shifts in Biocide-Resistance *Pseudomonas aeruginosa* PAO1. *Journal of Applied Microbiology* **89**: 289-295.
- Wingender, J. & Flemming, H.-C. (2011) Biofilms in Drinking Water and Their Role as Reservoir for Pathogens. *International Journal of Hygiene Environmental Health* **214**: 417-423.
- Wong, H.S., Townsend, K.M., Fenwick, S.G., Trengove, R.D. & O'Handley, R.M. (2010a) Comparative Susceptibility of Planktonic and 3-day-old *Salmonella* Typhimurium Biofilms to Disinfectants. *Journal of Applied Microbiology* **108**: 2222-2228.
- Wong, H.S., Townsend, K.M., Fenwick, S.G., Maker, G., Trengove, R.D. & O'Handley, R.M. (2010b) Comparative Susceptibility of *Salmonella Typhimurium* Biofilms of Different Ages to Disinfectants. *Biofouling* **26**: 859-864.
- World Health Organisation (2005) Drug-resistant *Salmonella*. World Health Organisation, World Health Organisation website.
- Wray, C. & Sojka, W. (1978) Experimental *Salmonella* Typhimurium Infection in Calves. *Research in Veterinary Science* **25**: 139-143.
- Xu, K.D., Stewart, P.S., Xia, F., Huang, C.-T. & McFeters, G.A. (1998) Spatial Physiological Heterogeneity in *Pseudomonas aeruginosa* Biofilm Is Determined by Oxygen Availability. *Applied Environmental Microbiology* **64**: 4035-4039.
- Zogaj, X., Nimtz, M., Rohde, M., Bokranz, W. & Römling, U. (2001) The Multicellular Morphotypes of *Salmonella* Typhimurium and *Escherichia coli* Produce Cellulose as the Second Component of the Extracellular Matrix. *Mol Microbiol* **39**: 1452-1463.

Appendices

8.1 Neutraliser efficacy and toxicity data

Neutralisers were judged to be efficacious and nontoxic if a less than one log₁₀ reduction was seen after the exposure time.

8.1.1 Neutraliser efficacy and toxicity data for catalase

Catalase was tested to see if it effectively neutralised 10,000 ppm of hydrogen peroxide. This concentration was selected as this gave the appropriate level of kill in preliminary experiments.

The total viable count for a control exposed only to PBS was compared to samples exposed to hydrogen peroxide neutralised with catalase and there was less than a one log₁₀ reduction observed in both strains (table 8.1) demonstrating that the biocide was effectively neutralised.

The neutraliser toxicity test showed there was less than a one log₁₀ reduction between *Salmonella* exposed to PBS compared to those exposed to 250 ppm catalase (table 8.1). This shows that 250 ppm of catalase was not toxic to the *Salmonella* serovars tested.

Table 8.1: Neutraliser efficacy and toxicity data for 250 ppm catalase which is used to neutralise 10000 ppm hydrogen peroxide (n=3). Based on the limit of detection the maximum log₁₀ reduction which could be determined was a log₁₀ reduction of 5.17 for *Salmonella* Typhimurium, 5.25 for *Salmonella* Agona and 5.23 for *Salmonella* Havana.

<i>Salmonella enterica</i> serovar	Exposure	Mean log ₁₀ reduction ± SD
Typhimurium SL1344	10000 ppm hydrogen peroxide	5.17 ± 0.0
	Neutralised 10000 ppm hydrogen peroxide	-0.05 ± 0.12
	Neutraliser only	0.32 ± 0.29
Agona 3750	10000 ppm hydrogen peroxide	5.25 ± 0.00
	Neutralised 10000 ppm hydrogen peroxide	0.04 ± 0.11
	Neutraliser only	0.61 ± 0.08
Havana 3759	10000 ppm hydrogen peroxide	5.23 ± 0.00
	Neutralised 10000 ppm hydrogen peroxide	-0.06 ± 0.15
	Neutraliser only	0.10 ± 0.08

8.1.2 Neutraliser efficacy and toxicity data for universal neutraliser

The total viable counts of *Salmonella* serovars when exposed to universal neutraliser and PBS indicates that universal neutraliser is not toxic to the *Salmonella* strains tested as less (table 8.2). There is also less than a one log₁₀ reduction (table 8.2) when the *Salmonella* are exposed to neutralised peracetic acid showing the neutraliser to be effective. The concentration of 500 ppm peracetic acid was selected as this was the highest concentration this gave the appropriate level of kill in preliminary experiments.

Table 8.2: Neutraliser efficacy and toxicity data for universal neutraliser which is used to neutralise 500 ppm peracetic acid (n=3). Based on the limit of detection the maximum log₁₀ reduction which could be determined was a log₁₀ reduction of 5.30 for *Salmonella* Typhimurium, 5.28 for *Salmonella* Agona and 5.21 for *Salmonella* Havana.

<i>Salmonella enterica</i> serovar	Exposure	Mean log ₁₀ reduction ± SD
Typhimurium SL1344	500 ppm peracetic acid	5.30 ± 0.00
	Neutralised 500 ppm peracetic acid	0.21 ± 0.21
	Neutraliser only	0.20 ± 0.16
Agona 3750	500 ppm peracetic acid	5.28 ± 0.00
	Neutralised 500 ppm peracetic acid	0.15 ± 0.23
	Neutraliser only	0.03 ± 0.13
Havana 3759	500 ppm peracetic acid	5.21 ± 0.00
	Neutralised 500 ppm peracetic acid	-0.08 ± 0.08
	Neutraliser only	0.00 ± 0.12

8.1.3 Neutraliser efficacy and toxicity data for sodium thiosulphate

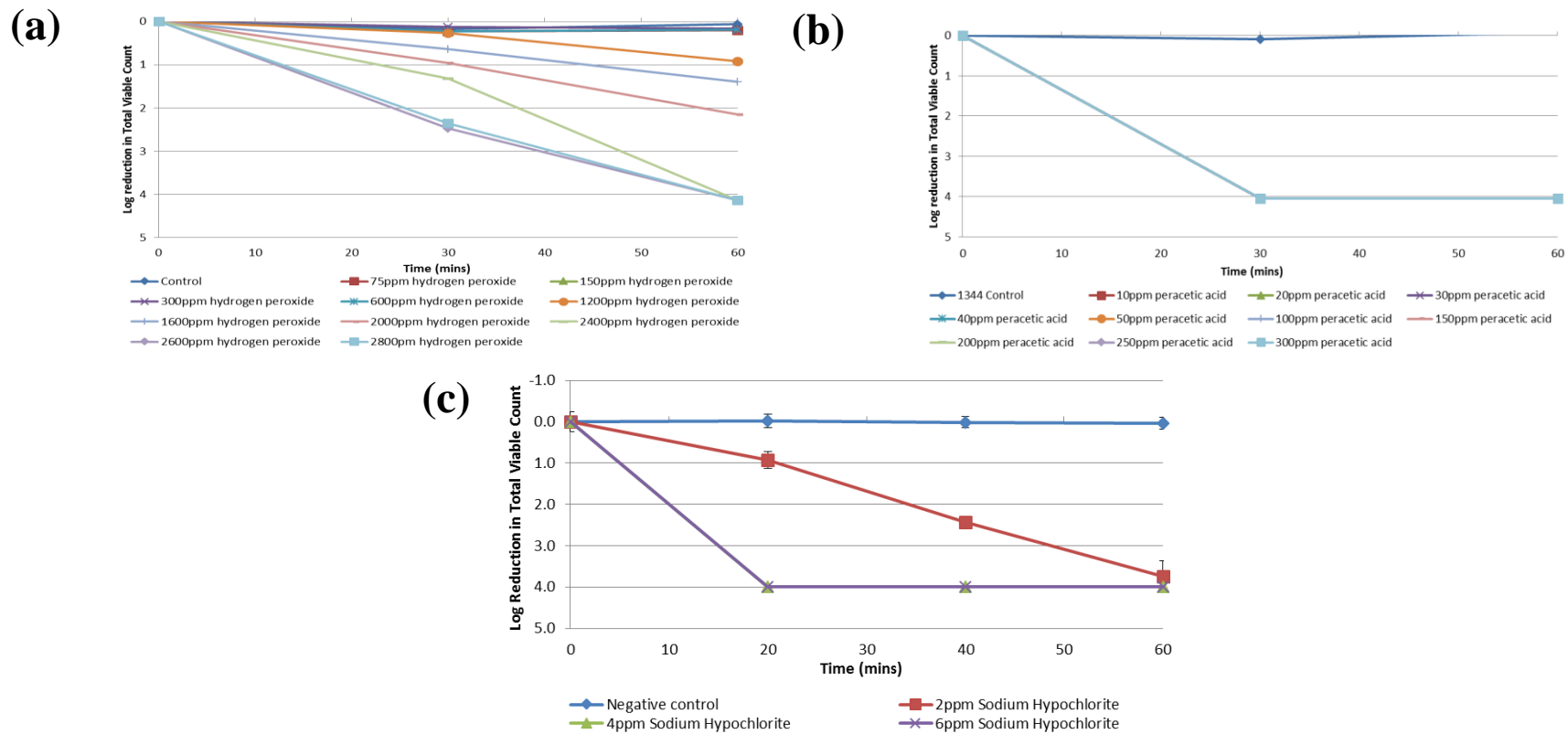
Total viable counts of *Salmonella* strains were taken using the drop counting method after exposure to a PBS negative control, a positive control of 1000 ppm of sodium hypochlorite and a test sample of 1000 ppm of sodium hypochlorite neutralised by 1000 ppm of sodium thiosulphate. The concentration of 1000 ppm of sodium hypochlorite was selected as this was the highest concentration this gave the appropriate level of kill in preliminary experiments. The positive control showed that 1000 ppm of sodium hypochlorite caused a 5 log₁₀ reduction in all three *Salmonella* serovars (table 8.3). The neutraliser efficacy test showed that there was less than a 1 log₁₀ reduction from the PBS control indicating that the neutraliser was effective (table 3.3).

Table 8.3: Neutraliser efficacy and toxicity data for 1000 ppm sodium thiosulphate which is used to neutralise 1000 ppm sodium hypochlorite (n=3). Based on the limit of detection the maximum log₁₀ reduction which could be determined was a log₁₀ reduction of 5.28 for *Salmonella* Typhimurium and 5.23 for *Salmonella* Agona and Havana.

<i>Salmonella enterica</i> serovar	Exposure	Mean log ₁₀ reduction ± SD
Typhimurium SL1344	1000 ppm sodium hypochlorite	5.28 ± 0.00
	Neutralised 1000 ppm sodium hypochlorite	0.11 ± 0.10
	Neutraliser only	0.11 ± 0.14
Agona 3750	1000 ppm sodium hypochlorite	5.23 ± 0.00
	Neutralised 1000 ppm sodium hypochlorite	0.83 ± 0.24
	Neutraliser only	0.09 ± 0.11
Havana 3759	1000 ppm sodium hypochlorite	5.23 ± 0.00
	Neutralised 1000 ppm sodium hypochlorite	0.37 ± 0.09
	Neutraliser only	0.13 ± 0.16

8.2 Preliminary inactivation kinetics

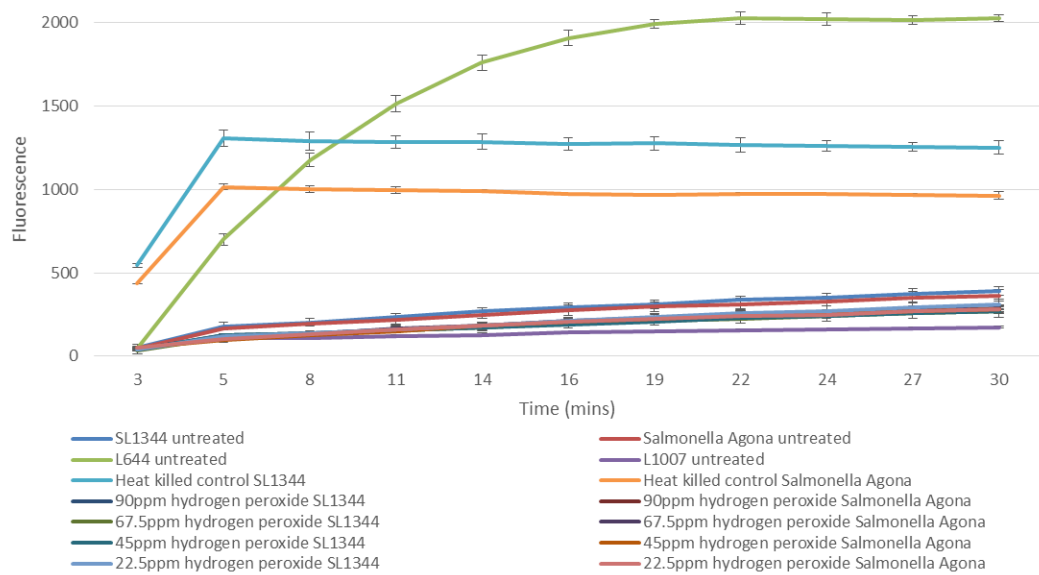
Log₁₀ reduction in the total viable count of *Salmonella enterica* serovar Typhimurium SL1344 when exposed to (a) hydrogen peroxide (b) peracetic acid (c) sodium hypochlorite prepared in PBS. Based on the limit of detection the maximum log₁₀ reduction which could be determined was a log₁₀ reduction of 4.1, 4 and 4 respectively (n=3).



8.3 Hoescht assay additional data

The accumulation of Hoescht 33342 (bis-benzimide) in *Salmonella enterica* serovars Typhimurium SL1344 and Agona when treated with (a) hydrogen peroxide (b) sodium hypochlorite. *Salmonella* Typhimurium strains L644 and L1007 were used as controls; L644 is an *acrB* knockout and therefore has decreased efflux and L1007 is a *ramR* knockout which increased *ramA* and therefore increased efflux activity. Heat killed controls give readings of high fluorescence accumulation within the cell as no efflux can occur (n=4). The error bars denote the standard deviation from the mean.

(a)



(b)

