Interaction of Detergents and Disinfectants upon Surface Adhered Populations of *Escherichia coli* and *Listeria monocytogenes*

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Abstract

The primary aim of this investigation was to identify and assess the interactions (synergies and antagonisms) that exist between 20 minute detergent and 5 minute disinfectant treatments upon three factory isolated strains of surface adhered (1-hour attached) and surface adapted (24-hour biofilm) populations of *Escherichia coli* and *Listeria monocytogenes*, plus a comparison with vero-toxin producing strains of *E. coli*, when used as part of a cleaning and disinfection regime. The detergents chosen for assessment were two non-ionic (91/4 - Alcohol Ethoxylate and KCL5 - Polyethoxylated Alcohol), two anionic (LX28 - Sodium Lauryl Sulphate and Nec28 - Sodium Laurylether Sulphate) and two novel bismuth thiols (BisEDT - 1:1 Bismuth nitrate 1,2-ethanedithiol and BisTOL - 2:1 Bismuth nitrate 3,4-dimercaptotoluene), developed at Winthrop University Hospital, New York. The disinfectants chosen for assessment were a quaternary ammonium compound (BAC - Benzyl alkonium Chloride) and a chlorine releasing agent (NaDCC - Sodium Dichloroisocyanurate).

The investigation showed that there were no specific cleaning and disinfection regimes that will adequately target both *E. coli* and *L. monocytogenes* strains. It was also concluded that to maximise the removal and disinfection of persistent strains of a given microorganism, it may be necessary to design a regime to specifically target not just the species, but the strain involved and where possible requires mechanical cleaning. The novel bismuth thiols were seen to be promising detergents to aid in the removal of *E. coli* strains and warrant further attention for future studies. Finally, an investigation to identify possible mechanisms of resistance to disinfectant treatments following detergent treatment, showed that different detergents can induce expression of the stress response proteins, HSP60 and HSP70, at differing levels of expression after the same contact time and against different states of adherent populations, i.e. 1-hour attached or 24-hour biofilm populations.

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Abbreviations

The following non-standard abbreviations have been used:

91/4	Alcohol Ethoxylate
BAC	Benzalkonium Chloride
BisEDT	1:1 Bismuth nitrate 1,2-ethanedithiol
BisTOL	2:1 Bismuth nitrate 3,4-dimercaptotoluene
CCFRA	Campden and Chorleywood Food Research Association
KCL5	Polyethoxylated Alcohol
LX28	Sodium Lauryl Sulphate
NaDCC	Sodium Dichloroisocyanurate
Nec28	Sodium Laurylether Sulphate
WoSH	Water of Standard Hardness

1.0 Introduction

1.1 Food Production and Legislation

The food industry is put under considerable pressure to produce large quantities of end product as economically as possible while maintaining very high standards of hygiene. The extensive buying power of multinational supermarket chains combined with the increasing demand by consumers for high quality, low cost foods has forced the food industry to cut production costs without compromising quality. Without stringent legislation, as set down by the UK Government and enforced by the Foods Standard Agency in the UK as well as European wide EC legislature, enforcing strict food safety and hygiene, the cost cutting seen within the industry would have almost certainly led to an increased risk of contamination by microorganisms (Anon, 2006).

During 1999, the legislative Food Standards Act in the UK, empowered the Foods Standards Agency with the ability to control and regulate the food industry by acting upon the consumers interests at any point within the production and supply chain (Anon, 1999). Additional regulations and legislation within Europe came into force on the 1st January 2006 with the Food Hygiene Regulations and extend the 'farm to fork' approach to food safety throughout the supply chain. The legislation makes it a legal requirement for all food businesses (except primary producers) to put in place, implement and maintain a permanent procedure or procedures based on Hazard Analysis Critical Control Point (HACCP) principles (Anon, 2006). By providing a regulatory framework for producers, consumers are better protected against sources of contamination, including microbial, and acts as a guarantee to ensure the quality of food produce is maintained at a high standard.

1.2 Microbial Contamination Risks within the Food Industry

As changes in food production and storage methods have evolved over the last few decades so new challenges have become evident for the food industry to face. The control and elimination of food borne pathogens is the area most relevant to this study, although it should be noted that the control of spoilage microrganisms is concurrent with that of pathogens.

Within the US, the Centers for Disease Control and Prevention have previously identified 14 new pathogens, or pathogens that are newly recognised as predominantly associated with food products, since 1980, of which all but one are bacterial in origin (Tauxe, 1997; Batz, 2005). Of note are entero haemorrhagic *E. coli* and *L. monocytogenes*, both of which have been identified in increasing numbers of clinical cases since 1980. Within the UK, the Health Protection Agency estimates that 20% of the population acquire an infectious intestinal disease each year, with an estimated 1 in 2500 reported cases now being associated with a vero toxin producing entero haemorrhagic strain of *E. coli* (HPA, 2007a), ranging from mild diarrhoea through to severe inflammation of the gut. The incidence of *L. monocytogenes* infections in England and Wales has also been rising over the past 20 years from 25 – 48 cases reported per year in the 1990s to over 60 cases per year since 2003, almost certainly caused by the increase in consumption of more varied dairy and processed meat products (Gillespie *et al.,* 2006; HPA, 2007b).

Entero-haemorrhagic *E. coli* and *L. monocytogenes* are not as common a cause of food borne illness as for example *Campylobacter* spp. and *Salmonella* spp. which are now estimated, based upon laboratory diagnosis, to cause over 46000 and 15000 cases respectively per year in England and Wales (HPA, 2007a). Another common cause of food borne infection within the UK, as reported by the HPA, (2007a) is viral,

including Norovirus and Rotovirus, with over 20000 cases per year. However the severity of the diseases caused by VTEC *E. coli* and *L. monocytogenes* was one of the reasons for inclusion in this study.

1.2.1 Possible Routes of Contamination

There are five primary routes through which food products can become contaminated during processing: raw materials, people, vermin, the air and contact surfaces (Holah & Kearney, 1992; Chmielewski & Frank, 2003). Raw materials entering a factory are prevented from coming into contact with later stages of the production line to avoid contamination. This is achieved by separating the production line into distinct areas to reduce carriage of microorganisms between sections and via limiting movement of the workforce through the production line by only allowing movement from finished product sections towards the raw material entry point (Huggett, 2001).

Enforcing a strict hygiene code of practice for use prior to entering a production line and continuing within the production area, as well as by providing clean coveralls, footwear, hair/beard nets and hats, minimises the risk of contamination from the workforce (Huggett, 2001). Good building maintenance as well as internal and external pest control measures is essential to reduce the risk of vermin originated contamination. Air filtration, to remove particles that might harbour microorganisms, is utilised to minimise this possible route of contamination (Holah & Kearney, 1992). Regular cleaning and disinfection of environmental surfaces is used to remove possible sources of contamination (Poulsen, 1999).

1.2.2 Contact Surfaces and Persistent Biofilms

Possibly the greatest concern and challenge that the food industry faces, with regards to causes of food spoilage and contamination of products with pathogenic

microorganisms, is that posed by the formation and persistence of microbial biofilms within a food processing environment (Poulsen, 1999; Holah *et al.,* 2004). Adherent, persistent populations provide a continual source of microbial contamination via sloughing of cells, this can be a particular problem where a biofilm has formed in a niche with high air or liquid throughput, for example *L. monocytogenes* biofilm populations found within blast (Miettinen *et al*., 2001; Holah *et al*., 2004) or brine (Gailey *et al.*, 2003) chillers were identified as major sources of contamination.

As reviewed by Prakash *et al.,* (2003), an initial reversible attachment occurs rapidly as bacterial cells are surface captured via weak forces of attraction, such as van der Waals forces, electrostatic surface charges and hydrophobic interactions between the bacteria and surface, upon contact. Other than the attachment substrate, few factors influence this stage of adherence, interestingly, it has even been shown that killed microbial cells do not lose this initial adhesive capacity and so can aid living cells with the initial formation of a biofilm (Piette and Idziak, 1992). Zottola (1994) described how bacterial cells readily adhere to most materials and surfaces commonly found within food production lines, particularly strongly to stainless steel food preparation surfaces compared to most plastics. Generally, biofilms in food processing plants that are likely to come into direct contact with products occur on food handling surfaces, food storage areas and food processing surfaces such as equipment or conveyor belts, persistent biofilms are also likely to occur on environmental surfaces such as the wall / floor interface and in and around drains (Kumar and Anand 1998; Wong, 1998). It has also been shown that complex biofilm formation is more likely to occur where nutrient availability is limited but coincides with either static or flowing liquids, which further reinforces the reason for biofilms to

form and persist at the localities detailed above in food processing plants (Hunt *et al.*, 2004).

The transition from reversible to irreversible attachment proceeds via the excretion of extracellular polymeric substances (EPS) and the interaction of pili and fimbrial adhesins with the surface (Dunne, 2002). More recently, Palmer *et al*., (2007) reviewed all of the critical factors affecting initial attachment which was summarised as: surface conditioning, mass transport, surface charge, hydrophobicity, surface roughness and surface micro-topography. Following attachment, a lag phase with regards to cellular multiplication has been noted by Rice *et al*. (2000) by studying a luminescent strain of *Pseudomonas aeruginosa*, during which adaptation from a planktonic state to a sessile state occurs. Biofilm development continues via cell differentiation and the formation of complex matrix architectures, with water channels, pedestal-like structures and pores to trap nutrients. As biofilm maturation progresses, identifiable microcolony structures form and sporadic dispersion of planktonic cells occurs via sloughing (Busscher *et al.*, 1995; Kumar and Anand, 1998; Dunne, 2002). During biofilm formation, the cell differentiation that can occur largely depends upon locality within the matrix, Young (2006) provided a comprehensive review of cell size and shape and how this can change during biofilm maturation.

Microbial EPS are biopolymers, originally defined by Geesey (1982) as "extracellular polymeric substances of biological origin that participate in the formation of microbial aggregates". Both planktonic and adherent populations of bacterial cells synthesise EPS which acts primarily as a protective coating but can also aid with the absorption of nutrients, initialise attachment to surfaces and bind cells into complex biofilm matrices (Mayer *et al*., 1999; Czaczyk & Myszka, 2007). The

primary components of EPS are polysaccharides, nucleic acids, proteins and lipids (Nielsen *et al*., 1997; Dignac *et al*., 1998). EPS content can vary by between 50% and 90% of the total organic carbon present within a biofilm (Donlan, 2002), with the presence of uronic acids or ketal-linked pyruvates conferring an anionic charge to the matrix (Sutherland, 2001) which could potentially interfere with charged cationic disinfectants. It has also been shown that typical biofilms formed by Gram-negative bacteria comprise a greater proportion of proteins within the EPS matrix than seen for Gram-positive bacteria, this is thought to be due to the larger number of protein transport pathways available to Gram-negative bacterial cells (Gerlach & Hensel, 2007). Compared to the fairly simple capsule or slime layer surrounding planktonic cells, the complex structure of EPS in a biofilm acts as a more robust protective barrier against general chemical damage via quenching, chemical mopping and steric hindrance or by simply reducing or preventing diffusion through the matrix which lowers the effective concentration of the chemical in contact with cells (Guiot *et al*., 2002; Vickery *et al*., 2004).

The later stages of biofilms pose a greater risk of persistent contamination to products as cells slough away from the matrix. Cleaning and disinfection of fully formed biofilms is known to be difficult due to the strength of attachment (Eginton *et al.*, 1995; Gibson *et al.*, 1999; Chmielewski and Frank, 2003) and general increases in resistance to environmental stressors for the biofilm as a whole and for individual cells (de Macario and de Macario, 2000; Holah *et al*., 2002), for example raised and lowered temperatures (Perrot *et al.*, 1998 and 2001; Chmielewski and Frank, 2004), damaging chemicals, including detergents and disinfectants (Donlan and Costerton, 2002; Augustin *et al.*, 2004) and changes in pH caused by organic and inorganic acids and alkalis (McNeill and Hamilton, 2004). Many of the changes exhibited by cells

within a biofilm are thought to be due to the increased maintenance of intracellular homeostasis, combined with reduced growth rates and reduced chemical diffusion rates which result in observed enhanced physiological tolerances. An interesting observation by Beyenal and Lewandowski (2002) showed that the internal structure of biofilm micro-channels is largely determined by the environment within which the biofilm forms with regards to velocity and nutrient availability to maximise the diffusivity of available nutrients throughout the biofilm. The increased physiological tolerances of biofilms to multiple, diverse stressors have also been reported to relate specifically to increased tolerance to detergents and biocides used by the food industry (Armon *et al*., 1998; Gibson *et al.,* 1999; Aarnisalo *et al*., 2000), additionally sub lethal concentrations of biocides have actually been shown to enhance biofilm formation (Grant and Bott, 2005) which has serious implications for biofilm control. There are many examples of increased tolerances specific to biocides used by the food industry within the literature although some are more pertinent to this study. Although triclosan was not investigated in this study, it is widely used in sanitation products and impregnated into surfaces, a study by Tabak *et al*. (2007) showed that cells within a biofilm only demonstrated a 1-log reduction in viable count at the same concentration that caused an 8-log reduction in exponentially growing cells of *Salmonella typhimurium*, while planktonic cells derived from a biofilm retained some resistance, with a 3.6-log reduction in viable count observed. The efficacy of benzyl alkonium chloride (BAC) upon *L. monocytogenes* strains as biofilm and planktonic cells was investigated by Romanova *et al.* (2007), where biofilm populations were identified to be up to 1000 times more resistant to treatment than planktonic populations, with the conclusion that concentrations of BAC greater than 10 mg/ml, with a contact time of 30 minutes are required to kill almost all the live cells within a

biofilm, which is a far greater contact time than the 5 minutes suggested for use within the food industry.

Physiologically, populations of cells within a biofilm exhibit very different characteristics to planktonic populations, typically exhibiting reduced growth rates with both up- and down-regulation of many genes (Jain *et al*., 2007). Some studies have even suggested revolutionary ideas concerning the complex systems of cooperation, communication and synchronisation that occur between cells within biofilms relating to social evolutionary theory and how this can apply via quorum sensing to the observation of rapid adaptation to stressors experienced elsewhere within the matrix (Nikolaev & Plakunov, 2007; West *et al*., 2007).

At the moment of surface adhesion, metabolic pathway regulation changes from that seen in planktonic cells, with multiple secretary pathways, including those involved with EPS production, up regulated while cell division is down regulated (Palmer *et al*., 2007). As biofilm formation progresses the observed changes in sensitivity to stressors as described above has been attributed to a number of factors, although specifically with regards to physiological changes, it has been reported that especially at sub-optimal temperatures or during exposure to sub-lethal stressors, like those experienced in a chilled food processing environment, both the strength of adhesion and resistance to general stressors is greatly increased (La Paglia & Hartzell, 1997; Rachid *et al*., 2000). However the precise nature of resistance to such a diverse range of stressors has been summarised by Nikolaev & Plakunov, (2007) as a complex phenomenon which cannot be easily explained by any single or set of mechanisms. They even describe that within a biofilm some "persistor cells" exist which possess extraordinary resistances; these were initially identified by Moyed &

Bertrand (1983) who showed "persistors" are responsible for regenerating biofilms following biocide treatment.

1.2.3 *Escherichia coli* and Entero Haemorrhagic Strains

The ingestion of pathogens poses a significant risk to those people who consume them, especially where the potential infectious dose is very low; within the food industry specific pathogenic bacteria are more commonly associated with certain types of products and therefore the detection and elimination of these organisms is the highest priority. The presence of *E. coli* for example, is indicative of faecal contamination and is a risk factor suggesting the presence of entero haemorrhagic strains. Also studies by Ryu *et al*., (2004), and Wilks *et al*., (2005), have shown VTEC *E. coli* strains can readily form biofilm populations in food processing environments at a range of temperatures, nutrient availability and surface types.

Contamination of foods by vero toxin producing *E. coli* has caused a number of severe outbreaks of entero haemorrhagic disease in recent years; one of the most notorious outbreaks in the United Kingdom was that in November 1996, Lanarkshire - Scotland, when 20 people died and 496 were made seriously ill, all of the deaths were among adults aged from 69 to 93 years (Pennington, 1998). However, not just the elderly are at risk from entero haemorrhagic *E. coli*. In the USA, an outbreak primarily in teenagers was reported during the winter of 1992/1993 following consumption of contaminated hamburgers (Bell *et al*., 1994). During this outbreak a total of 501 cases were reported, with 151 patients requiring hospitalisation and 3 deaths. More recently, in Wales during 2005, an outbreak of *E. coli* O157 was reported primarily amongst school children, where a total of 44 different schools and 157 individuals were infected, with 31 requiring hospitalisation and 1 reported death. The source of contamination for the 2005 outbreak was identified as sliced, cooked

meats distributed to the schools (HPA, 2005). Although food borne transmission routes are most frequently associated with outbreaks of vero toxin producing *E. coli*, in 2004, a single infected individual transmitted the pathogen via a swimming pool in Manchester to 8 other individuals (Verma *et al*., 2007).

Infection with vero toxin producing *E. coli* commonly results in life threatening complications. Typically up to 15% of infected individuals develop complications such as haemolytic uraemic syndrome and thrombotic thrombocytopaenic purpura with mortality rates of up to 30% (Todd and Dundas, 2001). It should be noted that infection with *Campylobacter* spp. and *Shigella* spp. can also lead to the same complications of haemolytic uraemic syndrome and thrombotic thrombocytopaenic purpura, although the rate of complications is considerably lower than that seen for vero toxin producing *E. coli*, estimated to be less than 1% of cases (Cowden, 1992).

The infectious dose for *E. coli* O157 and other entero haemorrhagic strains of *E. coli*, although not specifically quantified, has been estimated to be as few as 10 cells, although not necessarily linked to specific food matrixes as is found with low infective doses of other pathogens (Phillips, 1999; Caprioli *et al.,* 2005). The reported infectious dose is much lower than the infectious doses reported for many other common food borne pathogens, for example the infective dose of *Salmonella* spp. is usually greater than 10^5 cells in most foods, although can be as few as 100 cells in chocolate, where the food matrix increases the effective concentration of quorum signalling molecules surrounding the cells, which leads to expression of virulence determinants at low cell densities (Choi *et al*., 2007). *Campylobacter jejuni* has a fairly low estimated infective dose of just 500 cells, while non vero toxin producing *E. coli* are unlikely to cause disease below 10^5 cells (Kothary and Babu, 2001).

It has been shown that vero toxin producing *E. coli* can persist on stainless steel surfaces as a biofilm in laboratory studies, as used by the food industry, for over 28 days at both refrigeration and room temperatures (Wilks *et al.*, 2005). Within food manufacturing facilities the persistence of *E. coli* O157 and other toxigenic strains of *E. coli* could potentially lead to serious illness, long term complications such as organ failure and even the deaths of consumers. A study by Ueda and Kuwabara (2007) demonstrated that various detergents used within the food industry are unable to remove fully formed biofilm populations of *E. coli* O157 further enforcing the importance of effective sanitisation. Similarly it was shown that as well as detergents failing to remove adherent cells of *E. coli*, the EPS produced during biofilm formation provides protection against removal and against biocides which was increased following detergent application (Ryu and Beuchat, 2004). Regular, effective sanitisation of production lines is used to prevent this situation from occurring. Identifying detergents and disinfectants that positively interact, further enhancing the removal and killing of non toxigenic *E. coli* will help reduce the risk posed to the food industry by vero toxin producing *E. coli* strains.

1.2.4 *Listeria monocytogenes*

Listeria monocytogenes is of great concern to the food industry, most notably due to the risk of foetal damage and miscarriage during pregnancy after consuming contaminated foods (Rocourt, 1996). Prior to the widespread adoption of refrigerators during the late 1940s and early 1950s and due to changes in eating habits, for example wider availability of ready to eat, high risk foods with minimal or no cooking, cases of listeriosis related diseases were rarely seen or possibly rarely looked for (McLauchlin, 2006). As refrigeration as a means of preserving foods became more widespread, listeriosis caused predominantly by *L. monocytogenes* emerged as a new

disease within the developed world. The observed emergence is linked to the ability of *Listeria* spp. to proliferate at low temperatures, with certain food groups, for example unpasteurised dairy produce, posing the greatest risk (Gandhi and Chikindas, 2007). The incidence of listeriosis in the developed world is estimated to be 5 cases per million people per year, but with a very high mortality rate of 20-25% (Hitchins and Whiting, 2001).

Normally found as a soil borne bacterium, *L. monocytogenes* possesses the ability to invasively infect intestinal cells where motility changes from flagella borne to actin polymerisation, enabling secondary cellular infection to occur by passing directly through cellular membranes (Gouin *et al*., 2005). Clinically, besides the invasive form of listeriosis, most commonly associated with immunocompromised patients and during pregnancy, where cells pass across the placental barrier, the disease may present as febrile gastroenteritis or as a cutaneous infection (Swaminathan and Gerner-Smidt, 2007). Although uncommon, *Listeria* spp. outbreaks have been reported, for example in 1997, 1566 students and staff at two primary schools in the USA exhibited febrile gastroenteritis after eating contaminated corn with 292 being hospitalised (Aureli *et al*., 2000). Unusually for listeriosis, the infected individuals were considered to be immunocompetent, whereas normally infection is associated with patients with predisposed conditions, such as the immunocompromised, neonates and pregnant women (Swaminathan and Gerner-Smidt, 2007).

A study by Holah *et al.,* (2004) investigated the persistence and distribution of *Listeria* spp. within food processing environments. Ribotyping, the antibody based bacterial 'fingerprinting' specific to 16s and 23s ribosomal DNA, was used to differentiate members of the same species and to enable, in many cases, the source,

for example of persistent biofilms or raw materials, of contamination to be identified. An important recorded observation was that the majority of isolated *Listeria* spp., including *L. monocytogenes*, were environmentally niche oriented, therefore derived from persistent biofilms, rather than originating on raw material or other external sources of possible contamination. *Listeria* spp. are also capable of multiplication at legally required refrigeration temperatures, 2 - 4°C, as commonly found within food processing environments and used for storage of chilled foods. Møretrø and Landsrud (2004) discuss the implication of *Listeria* spp. biofilm populations, and challenges posed for the food industry, including the high frequency with which biofilm populations are identified as sources of contamination in food production lines and the difficulties encountered with removal and disinfection of biofilm populations. A study by Rodriguez *et al*. (2008) showed that *L. monocytogenes* strains on the smoothest stainless steel surfaces used by the food industry, those that have been electropolished, are able to colonise and form biofilms as readily as on rougher mechanically finished steel surfaces. It has also been shown that some strains of *L. monocytogenes* are able to form highly resistant biofilm populations that can cause a 1000 fold increase in resistance to biocide treatments, including BAC (Romanova *et al*., 2007). These observations highlight the necessity to optimise cleaning and disinfection regimes to reduce the occurrence of environmentally persistent strains that can lead to contamination of food products and therefore human disease.

1.3 Minimising the Microbial Contamination of Food Production Lines

Control of biofilms is currently achieved via strict cleaning and disinfection regimes, for example in the UK, the Food Standards Agency's 'Safer Food Better Business' (SFBB) system is used to implement a five stage advanced food safety management regime which includes rigorous cleaning and disinfection of surfaces.

Elortondo *et al.,* (1999) examined many of the cleaning and disinfection methods employed by the food industry to limit contamination, specifically caused by microbial biofilms. A comparison of the mode of application of detergents, for example in water or as a foam, concluded that the prevention of biofilm formation is a vital strategy for limiting biofilm related contamination due to the inherent difficulty encountered with the removal and disinfection of persistent populations. The development of optimised or alternative cleaning and disinfection regimes against adherent microorganisms could offer the food industry more reliable methods of safely producing foods with regards to microbial contamination. Specifically the food industry requires treatments to be odour and taint free, non-toxic and as effective as possible against a wide ranged of soils and microorganisms that might be encountered within a food processing environment.

1.3.1 Detergents used by the Food Industry

Detergents are primarily used to remove grease and soiling from contact surfaces, floors and walls prior to disinfection, although some detergents are also effective biocides, assisting with the disinfection process. The removal of soiling from surfaces is required to prevent the inactivation of subsequent disinfectant treatments and to remove a source of microbial contamination and nutrients, thereby further enhancing sanitisation. Detergents are usually amphipathic chemicals containing a hydrophilic head group, and a hydrophobic hydrocarbon tail group. This property enables the 'head' of the molecule to be soluble in water while the 'tail' is soluble in organic solvents, for example oils. The interfacial tension between oil and water is reduced after application via absorption at the oil/water interface of the detergent; this enables the removal of hydrophobic soils from surfaces prior to disinfection.

Detergents commonly used by the food industry can be divided into three groups, non-ionic, anionic and cationic, referring to the charge present on the hydrophilic section of the molecule. Cationic detergents generally have poor detergency action and are more commonly used as biocidal disinfectants, for example quaternary ammonium compounds (QACs), fabric softeners or specialist emulsifiers rather than detergents. QACs characteristically cause damage to the outer membrane while promoting their own uptake an entry into cells (Russell, 2002).

Anionic surfactants dissociate in water to give a large negatively charged anion and smaller cation. The anionic portion of the surfactant has a strong detergent mode of action while a weak biocidal action is exhibited by the cation, although at typical 'in use' concentrations the activity is negligible and restricted to Gram-positive bacteria (Pasanen *et al.,* 1997). The use of a charged detergent has potential implications with regards to compatibility with certain disinfectants which may be neutralised by residual detergent if cationic. The anionic detergents, within regular use by the food industry, sodium laurylether sulphate (Nec28) and sodium lauryl sulphate (LX28) were selected for use within this study. Nec28 is a low toxicity, inexpensive and very effective foaming agent found in many products, including soaps, shampoos and toothpastes. LX28 is also a low toxicity surfactant and found in a wide range of products, including bubble bath, shaving foams and some dissolvable aspirin.

Non-ionic surfactants are comprised of uncharged polar groups that form hydrogen bonds with water. The hydrophilic group is usually an ethoxylated chain with no specific biocidal properties, although polysorbates, such as Tween-80, can increase the permeability of the outer envelope of Gram-negative bacteria by acting as an ionophore (Thoman, 1999). The non-ionic detergents, within regular use by the

food industry, alcohol ethoxylate (91/4) and polyethoxylated alcohol (KCL5) were selected for use within this study. 91/4 is readily biodegradable under aerobic and anaerobic conditions and commonly found in a wide range of cleaners and personal care products. KCL5, as with other polyethoxylated surfactants, typically has a greater activity than an etholylated surfactant; it is found within a similar range of products as 91/4 but has reduced biodegradability.

1.3.2 Bismuth Thiol Detergents

A group of novel detergents known as bismuth thiols are currently being investigated for use within clinical situations, the use of these detergents may also lead to improved removal and killing of microorganisms within the food industry. Indeed previous work by Domenico *et al*., (1997, 1999) has suggested that bismuth thiols both aid the removal of biofilms and possess a potent inhibitory effect upon biofilm formation and development due to disrupting the production of EPS, an important component in bacterial attachment and resistance to disinfectants (Kumar and Anand, 1998). Domenico's results that demonstrated the effectiveness of bismuth thiols at removing and inhibiting biofilm populations was the primary consideration for their inclusion within this study.

Bismuth thiol detergents are comprised of a bismuth compound and a thiol moiety, specifically designed to target both EPS *in situ* and inhibit EPS production. To maximise effectiveness, bismuth thiols are likely to require the addition of a conventional detergent for removal of soil from surfaces. Bismuths, like arsenicals, are metabolic poisons with well characterised effects of disrupting ATP production (Dill and McGown, 1994). Thiols are molecules that contain a functional sulfhydryl group and are still frequently referred to as mercaptans. Toxicity studies by Domenico *et al*., (2000) have shown that many bismuth thiol compounds exhibit a

higher LD50 against mice than antimicrobials commonly used by the food industry, such as benzyl alkonium chloride (BAC) and sodium dichloroisocyanurate (NaDCC). Some more recently formulated bismuth thiols combine very low toxicity with no detectable odour or taint, all of which are essential characteristics if a detergent or disinfectant is to be used by the food industry. The bismuth thiols, 1:1 Bismuth nitrate 1,3-propanedithiol (BisEDT) and 2:1 Bismuth nitrate 3,4-dimercaptotoluene (BisTOL) were selected for use within this study by conforming to low toxicity, lack of odour or taint and having been extensively tested against clinically important microbes (Domenico *et al.*, 2000, 2001).

1.3.3 Disinfectants in the Food Industry

Although a wide range of disinfectants exist, only relatively few are considered suitable to use on food production lines where toxicity, odour and taint must be negligible. For information with regards to disinfectants that are not suitable for use within the food industry, a comprehensive review detailing classes of disinfectant (from heavy metals to organics) and mode of action (including oxidising, denaturing and permeablising of cellular membranes) has been written by Fraise (1999). Two types of disinfectant routinely used by the food industry are chlorine releasing agents and quaternary ammonium compounds (QACs), a type of cationic detergent. In general Gram-negative bacteria are seen to be more resistant to biocides than Gram-positive bacteria due to differing penetration of biocides caused by the structural difference with outer membrane and cell wall (Maillard, 2002).

Chlorine releasing agents include hypochlorites and *N*-chloro compounds such as NaDCC; this class of disinfectants are rapidly biocidal and exhibit a broad range of antimicrobial action that is attributed to interference with key metabolic processes via the release of hypochlorous acid, resulting in protein denaturation (McDonnell and

Russell, 1999). Poor activity is seen at low concentrations and under dirty conditions where inactivation occurs; therefore it is important that they are used on clean surfaces (Fraise, 1999). The general mechanisms associated with chlorine releasing agents are oxidative primarily resulting in the oxidation of thiol groups to disulfides, sulfoxides, or disulfoxides and the inhibition of DNA synthesis (McDonnell and Russell, 1999). Chlorine releasing agents are also able to destroy bacterial spores, although activity has been shown to depend upon pH and the concentration of available chlorine (Russell, 1982).

QACs are membrane active biocides where the antimicrobial activity is attributed to inactivation of cell metabolic pathways and the denaturation of proteins, although is has also been reported that QACs alter the hydrophobicity of Gram-negative bacteria (Fitzgerald *et al*., 1992). QACs are also membrane active agents, which are thought to damage the cell wall and outer membrane of Gram-negative bacteria which leads to increased biocide uptake and increased activity against primary target sites at the cytoplasmic membrane and within the cytoplasm (Gilbert *et al*., 1990). The activity of QACs is limited to vegetative bacterial cells with little or no activity against spores and is ineffectual against some bacterial groups such as *Pseudomonas* spp. (Langsrud *et al*., 2003). An early study by Salton (1968), suggested exposure to cationic disinfectants follows the follow sequence: (i) adsorption and penetration of the agent into the cell wall; (ii) reaction with the cytoplasmic membrane (lipid or protein) followed by membrane disorganization; (iii) leakage of intracellular low-molecular-weight material; (iv) degradation of proteins and nucleic acids; and (v) wall lysis caused by autolytic enzymes.

Prior to the application of disinfectants, excess soiling is removed from a surface to eliminate inactivation caused by many organic molecules. Concentrations of

disinfectant used by the food industry are determined by concentration coefficient; typically disinfectants with a low coefficient are preferred to reduce the risk of tainting and disinfectant residues. Other important considerations for the application of disinfectants include the contact time, temperature and pH of the solution which can all affect the efficacy of the disinfectant. The disinfectants that are commonly found within the food industry, sodium dichloroisocyanurate (NaDCC), a chlorine releasing agent and benzyl alkonium chloride (BAC), a QAC, were selected for use within this study. NaDCC is a stable source of chlorine and is widely used as a disinfectant, including in small doses in water purification tablets. BAC is a nitrogenous surface-acting agent and as well as being used as a biocide is frequently used as a microbial corrosion inhibitor, it is found in a wide range of products, including eye and mouth washes.

As with other antimicrobial agents, for example antibiotics, acquired resistance to disinfectants has been reported. A study by Bore *et al*., (2007) has shown that *E. coli* K-12 can readily be induced to tolerate up to 8 times the initial concentration of BAC required for MIC. However other studies have shown that some species do not readily adapt to disinfectants, Aarnisalo *et al*., (2007) showed that 10 species of *L. monocytogenes* when challenged with 8 commercially available disinfectants at most adapted to a 2-fold increase in disinfectant concentration, concluding that with regards to *Listeria* spp. the adaptive response is a minor concern. Additionally, resistances are more likely to develop where partial inactivation of disinfectants by organic particles lowers the effective active concentration to sub lethal levels; this has been demonstrated to occur against *E. coli*, *P. aeruginosa* and *Staphylococcus aureus* when treated with BAC in the presence of dried food elements (Kuda *et al*., 2008).

1.4 Assessment of Cleaning and Disinfection

Research has been conducted to try and assess the effectiveness of current cleaning and disinfection regimes upon biofilm removal and destruction using artificial (Wirtanen *et al.*, 1995) and *in situ* (Gibson *et al.*, 1999) studies. Both studies demonstrated that detergent action typically removed at most 1 log order of microorganisms from surfaces, even though previous studies had suggested over 3 log order is removed following detergent treatment (Dunsmore *et al*., 1981). While environmental factors have been identified that induce a protective response to biocide exposure, for example pre-exposure to HCl at a pH of 5.0 for 4 hours was shown to decrease the sensitivity of 3 strains of *E. coli* O157:H7 to subsequent exposure to high temperatures, 10% NaCl, 15% ethanol and 0.85% bile salt (Cheng *et al.*, 2002); there has been limited investigation of the synergistic opportunities that might exist between detergent and subsequent disinfectant application.

Total viable count (TVC) swabbing can be used to quantify the removal/killing effect of detergents and disinfectants. However, the use of this method is limited in that it will only enable the remaining, viable attached population to be quantified. Other methods are required to study the cells *in situ* and to determine if treatments are removing and/or killing attached microbes. Two additional methods used within this study are that of direct microscopic analysis of treated, attached and biofilm populations and also the use of bioluminescence as a metabolic indicator during and after treatment.

Direct microscopic measurements and observations are frequently used to investigate effects upon adherent populations that would otherwise be missed by viable counting. Some examples where this technique has been successfully used include a study by Stoodley *et al*., (2001), where time lapse microscopy was used to

record the detachment of cell clusters from mature biofilms and a study by Skillman *et al*., (1999), where a mixed population biofilm was examined to determine the proportion and placement of a green fluorescent protein transformed species. A dual staining methodology was developed for use within this study that can be applied to differentiate bacterial and EPS components of biofilm populations using acridine orange and calcofluor white respectively.

Bacteria can be genetically modified to produce light by the addition of a luciferase operon. There are two alternative luciferase gene complexes that are commonly used, the first *lux*AB only encodes the luciferase protein complex and requires the addition of an aldehyde substrate for light production, whereas the complete luciferase operon encodes for both the protein complex and an enzyme required producing the aldehyde substrate (Meighen, 1994).

This study investigated a transformed vero toxin negative, bioluminescent strain of *E. coli* O157:H7 that carried the complete luciferase operon on the plasmid pSB311. Light production requires the presence of a metabolically active cell with freely available ATP, and any influence on free ATP such as non-lethal chemical exposure or stress response may be quantifiable as a reduction in bioluminescence. Possible mechanisms could include the induction of stress response proteins or a sub lethal injury to the cells, both of which should result in reduced bioluminescence as ATP is diverted away from use by the luciferase molecules to counteract the effects of stressors.

1.5 Aims and Objectives

1.5.1 Aims

To quantify, assess and understand the interactions between detergent and disinfectant treatments that are relevant to the food industry, upon 1-hour attached and 24-hour biofilm populations of different strains of *E. coli* and *L. monocytogenes*, leading to the identification of synergies between treatments.

1.5.2 Objectives

1) Development of a reproducible 1-hour surface attachment and 24-hour biofilm model on stainless steel coupons for *E. coli* and *L. monocytogenes* strains.
2) Assessment and refinement of methodology for enumerating and visualising attached and biofilm populations of *E. coli* and *L. monocytogenes* using techniques such as TVC, microscopy and bioluminescence.
3) Identifying the effects that well classified non-ionic and anionic detergents and disinfectants have upon suspended, 1-hour attached and 24-hour biofilm populations.
4) Assessing effects of novel bismuth thiol based detergents upon suspended, 1-hour attached and 24-hour biofilm populations.
5) Investigate potential synergistic effects of detergent and disinfectant treatment regimes on the detachment and loss of viability of 1-hour attached and 24-hour biofilm populations.
6) A comparison of initial attachment and removal of entero haemorrhagic strains of *E. coli* with non-pathogenic strains.
7) Initial investigation into mechanisms involved with resistance to disinfectants after detergent treatments.

2.0 Materials and Methods

2.1 Strains

All strains of *E. coli* and *L. monocytogenes* were maintained in freeze dried ampoules. For recovery, 1ml of TSB was added to resuspend the cells and then spread plated onto TSA and incubated overnight at 37°C. The recovered cells were stored at 4°C for up to 2 weeks before being discarded. The following *E. coli* and *L. monocytogenes* strains were provided by Campden and Chorleywood Food Research Association or from the University of Wolverhampton collection.

E. coli Strains	Information
400	Ribogroup: RIBO1 102-248-S-4. Product isolate from Beef Stew & Dumplings.
453	Ribogroup: RIBO1 102-248-S-7. Environmental isolate from drain in wash down – high / low cook barrier area.
467	Ribogroup: RIBO1 102-575-S-7. Environmental isolate from drain.
CRA 4497 O157:H7	Environmental isolate from Abattoir Survey, Sheffield PHL.
CRA 12628 O157:H-	Product isolate from CPHL, VT 1 & 2.
CRA 13379 O157:H7	Environmental isolate from bovine faeces.
ATCC 43888 O157 WT	VT 1 & 2 negative strain.
O157 WT with pSB311	Transformed ATCC 43888 O157 WT by K. Middleton with plasmid pSB311 containing complete *lux* operon.
K12 W1485	
NCIB 9484	
O128	
HB101 NCIB 11865	
NCIB 12079	

L. monocytogenes Strains	Information
166	Ribogroup: RIBO1 102-195-S-1. Environmental isolate from H/R bund wall.
359	Ribogroup: RIBO1 102-241-S-7. Product isolate from Chicken & White Wine.
367	Ribogroup: RIBO1 102-409-S-3. Environmental isolate from drain by H/R cookline.

Table 2.1: *E coli* and *L. monocytogenes* strain information.

2.2 Growth Media, Stocks and Solutions

Tryptone Soya Agar (TSA) was used as the standard solid growth medium. 37g TSA was added to 1000ml water, sterilisation was carried out at 121°C for 15 minutes. The solution was gently mixed without causing aeration and cooled to 45-50°C prior to pouring 15-20ml into sterile Petri dishes in a laminar airflow cabinet. Agar plates were stored at room temperature with a shelf life of 1 month and were checked for colony growth prior to use.

Tryptone Soya Broth (TSB) was used as the standard liquid growth medium. 30g TSB was added to 1000ml water, sterilisation was carried out at 121°C for 15 minutes. TSB was stored at room temperature with an unopened shelf life of 1 month, unused solution was discarded following experimentation. 1/10 strength, 3g l^{-1}, TSB was prepared to use for biofilm formation.

Tetracycline was used for the maintenance of *E. coli* O157:H7 pSB311. Stock solutions were prepared by dissolving 50 mg tetracycline in 10 ml water. Filter sterilisation was carried out with a maximum effective pore size of 0.45 μm. Stock solutions were wrapped in foil and stored at -20°C. A working concentration of 10 μg ml^{-1} was used; media containing tetracyclione was stored at 4°C for a maximum period of 1 week.

Thymine was used for maintenance of *E. coli* O157:H7 pSB311. Stock solutions were prepared by heating 2g thymine in 500 ml water at 50°C. Filter sterilisation was carried out with a maximum effective pore size of 0.45 μm. Stock solutions were stored at 37°C for up to 1 month to prevent precipitation. A working concentration of 200 μg ml^{-1} was used; media containing thymine was stored at 4°C for a maximum period of 1 week.

Ringer's solution was used as the standard diluent for swabbing, recovery and serial dilution during the study. 1 x Ringer's tablet was added to 500ml water, sterilisation was carried out at 121°C for 15 minutes. Ringer's solution was stored at room temperature with an unopened shelf life of 1 month; unused solution was discarded following experimentation.

2.3 Detergents and Disinfectants

Water of Standard Hardness (WoSH) was used as the diluent for detergent and disinfectant solutions and was prepared as per the Suspension Disinfectant Test - BS EN 1276 : 1997. Two biochemical stock solutions were prepared.

Solution A: 19.84g anhydrous $MgCl_2$ and 46.24g anhydrous $CaCl_2$ were diluted to 1000ml with water. Filter sterilisation was carried out with a maximum effective pore size of 0.45 μm. Storage was achieved at 4°C with a shelf life of 1 month.

Solution B: 35.02g $NaHCO_3$ was diluted to 1000 ml with water. Filter sterilisation was carried out with a maximum effective pore size of 0.45 μm. Storage was achieved at 4°C with a shelf life of 1 month.

In use WoSH was prepared by diluting 6ml of solution A and 8ml of solution B to a total volume of 1000ml with water; pH was adjusted to 7.0 ± 0.2. Filter sterilisation was carried out with a maximum effective pore size of 0.45 μm. WoSH was stored at 4°C with a shelf life of 1 month.

2.3.1 Detergents

91/4 - Alcohol Ethoxylate was provided by Holchem Laboratories Ltd., Rossendale, UK. 91/4 is a non-ionic detergent and used at the recommended in use concentration of 0.1% (v/v). Liquid stock was diluted in WoSH and pH was adjusted to 7.70 prior to use. Shelf life of in use 91/4 was limited to 6 hours.

BisEDT - 1:1 Bismuth nitrate 1,2-ethanedithiol was provided by Winthrop-University Hospital, New York, USA. BisEDT is a bismuth thiol detergent and used at the recommended in use concentration of 10 μg ml^{-1}. Sonication was used for 30 seconds followed by vortexing was used to dissolve 10 mg BisEDT in a solution of 9.9 ml propylene glycol with 100 μl concentrated HCl to produce a stock solution. Stock solutions were stored at room temperature for 1 month. In use concentrations of 10 μg ml^{-1} were prepared by diluting in WoSH. A shelf life of 6 hours was used.

BisTOL - 2:1 Bismuth nitrate 3,4-dimercaptotoluene was provided by Winthrop-University Hospital, New York, USA. BisTOL is a bismuth thiol detergent and used at the recommended in use concentration of 10 μg ml^{-1}. Sonication was used for 30 seconds followed by vortexing was used to dissolve 10 mg BisTOL in a solution of 9.9 ml propylene glycol with 100 μl concentrated HCl to produce a stock solution. Stock solutions were stored at room temperature for 1 month. In use concentrations of 10 μg ml^{-1} were prepared by diluting in WoSH. A shelf life of 6 hours was used.

KCL5 - Polyethoxylated Alcohol was provided by Holchem Laboratories Ltd., Rossendale, UK. KCL5 is a non-ionic detergent and used at the recommended in use concentration of 0.1% (v/v). Liquid stock was diluted in WoSH and pH was adjusted to 7.40 prior to use. Shelf life of in use KCL5 was limited to 6 hours.

LX28 - Sodium Lauryl Sulphate was provided by Holchem Laboratories Ltd., Rossendale, UK. LX28 is an anionic detergent and used at the recommended in use concentration of 0.2% (v/v). Liquid stock was diluted in WoSH and pH was adjusted to 8.65 prior to use. Shelf life of in use LX28 was limited to 6 hours.

Nec28 - Sodium Laurylether Sulphate was provided by Holchem Laboratories Ltd., Rossendale, UK. Nec28 is an anionic detergent and used at the recommended in

use concentration of 0.2% (v/v). Liquid stock was diluted in WoSH and pH was adjusted to 7.75 prior to use. Shelf life of in use Nec28 was limited to 6 hours.

2.3.2 Disinfectants

BAC - Benzyl Alkonium Chloride was provided by Holchem Laboratories Ltd., Rossendale, UK. BAC is a quaternary ammonium compound; a concentration of 0.01% (w/v) was identified to achieve approximately a 4 log kill against *E. coli* and *L. monocytogenes* in suspension. Stock powder was diluted in WoSH and mixed via vortexing prior to use. Shelf life of in use BAC was limited to 6 hours.

NaDCC - Sodium Dichloroisocyanurate was provided by Holchem Laboratories Ltd., Rossendale, UK. NaDCC is a chlorine releasing agent; concentrations of 0.0005% (w/v) for *E. coli* and 0.0008% (w/v) for *L. monocytogenes* were identified to achieve approximately a 4 log kill in suspension. Stock powder was diluted in WoSH and mixed via vortexing prior to use. Shelf life of in use BAC was limited to 6 hours.

2.4 Detergent Activity on Suspended Cells

Detergent activity upon suspended populations of *E. coli* and *L. monocytogenes* was investigated using the following procedure. This method was used for short duration contact of 5 and 20 minutes and also for extended contact to determine MIC and MBC of detergents.

10 ml of a liquid medium, either WoSH for 5 and 20 minute contact time or TSB for MIC/MBC over 24/48 hours, with required concentration of detergent was inoculated with 100µl of overnight culture. The inoculated media were incubated at 20ºC ± 0.5°C.

For all contact times, 100µl of inoculum was removed, serial dilutions carried out and TVC measured using spread plating. For long contact times, presence or absence of turbidity at 24 and 48 hours was also recorded by eye.

Three controls were used. The first was 10 ml medium incubated at 20°C ± 0.5°C and measured as for the procedure. The second control was 10 ml medium plus the required detergent concentration incubated at 20°C ± 0.5°C and measured as for the procedure. The third control was 10 ml medium with 100 µl of O/N culture, incubated at 20°C ± 0.5°C and measured as for the procedure.

2.5 Attachment of Bacterial Cells to Stainless Steel Coupons

Stainless steel coupons for attachment of bacterial cells and biofilm formation (coupons) were provided by Johnson Security Ltd., Gloucester, UK. Coupons were provided measuring 8mm x 8mm x 1.4mm. The grade of steel was type 316 with a 2B finish. Coupons were cleaned in a solution of fairy liquid and rinsed thoroughly before sterilisation was carried out at 121°C for 15 minutes. Unused coupons were discarded following experimentation.

The following methodology was used to obtain reproducible, 1 hour attached populations of both *E. coli* and *L. monocytogenes*. This method was also used prior to biofilm formation. Total viable counts following attachment varied on a day to day basis, the log TVC for *E. coli* strains 400, 453, 467 was 6.56 ± 0.13 and for *L. monocytogenes* strains 166, 359, 367 was 6.76 ± 0.08.

50ml of overnight culture was pelleted via centrifugation at 2000 x G for 30 minutes. The supernatant was removed and the pellet was resuspended in 50ml Ringer's solution, washing was achieved via centrifugation at 2000 x G for 30 minutes. The supernatant was removed and the pellet resuspended in 25ml TSB.

25 coupons were placed in a sterile Petri dish and the cell suspension added. Attachment was achieved after incubating at 20°C for 1 hour. Coupons were gently washed in Ringer's solution to ensure all planktonic cells were removed prior to

experimentation. Attached cells were stored in a covered Petri dish for a maximum of 1 hour before being discarded.

Suspensions of cells in alternative media and at different starting densities were studied to determine the optimum conditions for reproducible attachment. Alternative media tested included different strengths of TSB, overnight supernatant and Ringer's solution.

Three controls were used. The first was a clean coupon. The second control was a clean coupon, rinsed in Ringer's solution. The third control was a clean coupon incubated in sterile attachment medium at 20°C for 1 hour then rinsed in Ringer's solution.

2.6 Biofilm Formation of Bacterial Cells on Stainless Steel Coupons

A reproducible 24 hour biofilm model for *E. coli* and *L. monocytogenes* was achieved using the following methodology. Prior to the steps detailed below, the procedure detailed above for obtaining 1 hour attached populations had been completed. Total viable counts following biofilm formation varied on a day to day basis, the log TVC for *E. coli* strains 400, 453, 467 was 7.23 ± 0.12 and for *L. monocytogenes* strains 166, 359, 367 was 7.51 ± 0.17.

25 coupons with 1 hour attached populations of cells were immersed in 25ml $1/10^{th}$ strength ($3g\ l^{-1}$) TSB. The attached populations were incubated at 20°C for 24 hours to allow biofilm formation. Coupons were gently washed in Ringer's solution to ensure all planktonic cells were removed prior to experimentation. Biofilms were stored in a covered Petri dish for a maximum of 1 hour before being discarded.

Attached cells in alternative media were studied to determine the optimum conditions for reproducible biofilm formation. Alternative media tested included different strengths of TSB, overnight supernatant and Ringer's solution.

Three controls were used. The first was a clean coupon. The second control was a clean coupon, rinsed in Ringer's solution. The third control was a clean coupon incubated in sterile biofilm medium at 20°C for 24 hours then rinsed in Ringer's solution.

2.7 Detergent and Disinfectant Treatments on Attached and Biofilm Populations

The effects of detergent and disinfectant treatments upon attached and biofilm populations of *E. coli* and *L. monocytogenes* were investigated using the following procedure. This method was used to identify individual detergent and disinfectant effects as well as combined detergent followed by disinfectant treatments. The method was designed with controls that can be used to eliminate the variations caused by conducting experiments on different days via regression analysis.

Each experiment to test 3 detergents and 2 disinfectants in triplicate required the preparation of 78 stainless steel coupons with either 1 hour attached or 24 hour biofilm population depending upon the study being conducted. Of the 78 coupons, 72 were used to test the treatment combinations, including controls, 3 were TVC swabbed to determine initial adherent population density and 3 were stained to determine initial total count for 1 hour attached populations or EPS density for 24 hour biofilm populations.

Six washed coupons with adherent populations of cells were placed in a sterile Petri dish. 25ml of a solution of detergent in WoSH was added and incubated at 20°C ± 0.5°C for 20 minutes. The coupons were removed using tweezers and gently washed in 25ml WoSH, this was repeated 3 times to ensure removal of detergent.

The coupons were then placed into new sterile Petri dish and 25ml of a solution of disinfectant was added and incubated at 20°C ± 0.5°C for 5 minutes. The coupons

were removed using tweezers and gently washed in 25ml WoSH, this was repeated 3 times to ensure removal of disinfectant.

Of the six coupons used for each treatment, the upper surface of three of the coupons was vigorously swabbed using a sterile swab stick in Ringer's solution and vortexed in 10ml Ringer's solution to resuspend cells. 3 x 100µl of swabbed, resuspended cells was removed, serial dilutions carried out and TVC measured using spread plating. The remaining 3 coupons were air dried and stained for microscopic analysis as detailed below with acridine orange or calcofluor white.

Three control groups were used. The first was WoSH only, whereby the detergent and disinfectant steps were replaced with 25 ml WoSH. The second control group was detergent only, whereby the detergent steps were replaced with 25 ml WoSH. The third control group was disinfectant only, whereby the disinfectant steps were replaced with 25 ml WoSH.

2.8 Staining of Adherent Populations

2.8.1 Acridine Orange

Acridine orange was used to stain populations of bacteria adhered to stainless steel. Once stained, enumeration of attached populations was achieved via fluorescent microscopy. A total of 30mg acridine orange powder was added to 10ml water, vortexing was used to dissolve the powder. Solutions were wrapped in foil and stored at room temperature with a shelf life of 1 week.

Acridine orange was used to stain populations of *E. coli* and *L. monocytogenes* adhered to stainless steel coupons. The stain binds to DNA within the cells and is viewed using direct epifluorescent microscopy. Combined with calcofluor white, a dual staining method can be used to differentiate between cells and EPS.

Coupons of 1 hour attached and 24 hour biofilm populations of *E. coli* and *L. monocytogenes* were air dried before staining. Immersion in 3mg ml^{-1} acridine orange solution with a contact time of 10 minutes was carried out. Stained coupons were removed from the acridine orange solution and gently washed in Ringer's solution until all excess stain was removed. Coupons were then air dried before being attached, using nail varnish, to microscope slides for viewing under a fluorescent microscope at x 1000 magnification.

Acridine orange stained cells fade rapidly during UV exposure. For dual staining with calcofluor white it is important to complete the calcofluor staining procedure prior to staining with acridine orange.

Three controls were used. The first was a clean coupon, stained with acridine orange. The second control was a clean coupon, rinsed in Ringer's solution and stained with acridine orange. The third control was a clean coupon incubated in sterile attachment medium and stained with acridine orange.

2.8.2 Calcofluor White

Calcofluor white was used to stain the extracellular polysaccharide excreted by adherent populations of bacteria. This enabled measurements of changes to the EPS matrix of biofilm populations following treatment via fluorescent microscopy. A total of 10mg calcofluor white was added to 10ml water, gentle heating was used to dissolve the powder. Solutions were stored at room temperature and used within 24 hours.

Calcofluor white was used to stain populations of *E. coli* and *L. monocytogenes* adhered to stainless steel coupons. The stain binds to the EPS matrix surrounding the cells and is viewed using direct epifluorescent microscopy. Combined with acridine orange, a dual staining method can be used to differentiate between cells and EPS.

Coupons of 1 hour attached and 24 hour biofilm populations of *E. coli* and *L. monocytogenes* were air dried and then immersed in 1mg ml^{-1} calcofluor white solution for 60 seconds. Stained coupons were removed from the calcofluor white solution and gently washed in Ringer's solution until all excess stain was removed. Coupons were then air dried before being attached, using nail varnish, to microscope slides for viewing under a fluorescent microscope at x 1000 magnification.

Calcofluor white stained cells fade rapidly during UV exposure. For dual staining with acridine orange it is important to complete the calcofluor staining procedure prior to staining with acridine orange.

Three controls were used. The first was a clean coupon, stained with calcofluor white. The second control was a clean coupon, rinsed in Ringer's solution and stained with calcofluor white. The third control was a clean coupon incubated in sterile attachment medium and stained with calcofluor white.

2.9 Direct Microscopic Analysis

2.9.1 Microscope Settings for Stained Coupons

Direct epifluorescent microscopy of stained, surface adhered populations was conducted at x1000 magnification using the following settings, equipment and software.

A Spot RT Color camera from Diagnostic Instruments Inc., Michigan, USA was mounted upon a Nikon 'Eclipse' ME600 fluorescent microscope from Nikon Corp., Tokoyo, Japan. Images were gathered using Spot Capture® Version 4.0.2 from Diagnostic Instruments Inc., Michigan, USA and analysed using Image Pro Plus® Version 4.5.1 from Media Cybernetics Inc., Maryland, USA.

Image Pro Plus® Software Settings:

- Calibration > Spatial:
  - Name: 100x
  - Unit: µm
  - Pixels/Unit:
    - X: 13.71450950
    - Y: 13.71450950
  - Aspect Ratio: 1
  - Origin (Pixels):
    - X: 0
    - Y: 0
  - Angle Offset: 0

- Count / Size:
  - All Classes Selected: Automatic Bright
  - Measure Objects: Yes
  - Apply Filter Ranges: Yes
  - Accumulate Count: No
  - Options:
    - 4-Connect: Yes
    - 8-Connect: No
    - Smoothing: 0
    - Pre-Filter: No
    - Fill Holes: No
    - Convex Hull: No
    - Clean Borders: None

- Data Collector:
  - Automatic Collection: Yes
  - Data List: Pooled area (values) data exported to Excel in clump worksheet.
  - Statistics: Sum, area (values) for direct count Excel worksheet.
  - Layout: Count/Size, Area (Values).
  - Export:
    - Data List: Yes
    - Statistics: No
    - With column headers: Yes
    - With row headers: Yes
    - To: Excel (DDE)

Spot Capture® Software Settings:

- Filter Colour: RGB
- Binning: None
- Area: Full Chip
- Gamma: 1.95
- Exposure Times:
  - Red: 240
  - Green: 240
  - Blue: 10
- Gain: 8
- Filter: B-2A

2.9.2 1 Hour Attached Populations

Total counts were obtained via direct microscopic analysis for each treatment combination used against attached *E. coli* and *L. monocytogenes*. Direct total counts of treated populations were recorded using Spot Capture® software and ImagePro Plus® analysis software. Each coupon was imaged in 6 predefined locations, measured in mm from the top left corner of the coupon to the centre of the current image with the following x, y coordinates: 2 x 2.5, 4 x 2.5, 6 x 2.5, 2 x 5.5, 4 x 5.5 and 6 x 5.5. Images were analysed to identify the total area covered by cells, the data was then transformed into cells cm^{-2} by calculating the average size of 10 groups of 5 - 20 individually identifiable cells within each image. This was required because different treatments resulted in different apparent cell sizes after staining.

2.9.3 24 Hour Biofilm Populations

Direct total counts for biofilms of *E. coli* and *L. monocytogenes* were not obtainable due to the dense layering of cells and EPS on the coupons. Instead the density of EPS was assessed via staining with calcofluor white, to assess disruption of the polymeric matrix after detergent and/or disinfectant treatments.

Image intensity of treated populations was recorded to determine density of EPS using Spot Capture® software and ImagePro Plus® analysis software. Additional, untreated controls were measured as a baseline to determine the effects of each treatment, including the WoSH controls. Each coupon was imaged in 6 predefined locations, measured in mm from the top left corner of the coupon to the centre of the current image with the following x, y coordinates: 2 x 2.5, 4 x 2.5, 6 x 2.5, 2 x 5.5, 4 x 5.5 and 6 x 5.5. EPS density was measured using arbitrary brightness values from 1 (black) to 200 (white) for each pixel, 4.1×10^6 per image, allowing a single mean intensity, inferred as EPS density, per image to be calculated.

2.10 Detergent Induced Expression of Stress Response Proteins; HSP60 and HSP70

The expression of the stress response proteins, HSP60 and HSP70, in *E. coli* strain 453 after treatment with water of standard hardness, Nec28 or 91/4 was assessed. Three population types were tested, suspended, 1 hour attached and 24 hour biofilm to determine if the different states had an impact on detergent mediated stress response protein expression. Induction of stress response for the positive control was achieved by heating a population of cells at 50°C for 20 minutes.

Whole cell lysate was prepared to an approximate total protein yield of 10mg ml^{-1}. This was achieved by starting with 10ml of approximately 10^7 cells. The cells were washed in 10 ml of PBS and spun at 400 x g for 10 minutes. The supernatant was discarded and the wash step repeated. Following the second wash step, the supernatant was completely removed and the cell pellet resuspend via vortexing in 1 ml of cold Lysis Buffer (50nM Tris-HCl, pH 8.0, 150mM NaCl, 1% NP-40).

The sample was stored on ice for 30 minutes, with occasional mixing. After 30 minutes the cell lysate was centrifuged at 10,000 x g for 15 minutes at 4°C. Following centrifugation the supernatant was collected without disturbing the pellet and transferred to a clean ependorf tube, the pellet was discarded.

10µl of lysate was transferred to a clean ependorf tube. 10µl of loading buffer and 1µl of beta mercaptoethanol was added. The solution was then heated at 70°C for 2 minutes before being placed on ice.

A standard 12% separation gel, 5% loading gel, was prepared, loaded with lysate and run to separate proteins by molecular weight. The molecular weight markers used were Bio-Rad code: 161-0318, 7.1 kDa - 208 kDa.

A standard Western Blot was conducted using Burnette's (1981) methodology. Rabbit Anti-Hsp60 Polyconal Antibody (SPA-805) and Mouse Anti-HSP70 Monoclonal Antibody (SPA-810) provided by Stressgen Bioreagents, Victoria, Canada, were used to identify the presence and quantity of HSP60 and HSP70 within each sample respectively. A scanning densitometer was used to obtain intensity values for each band.

2.11 Statistical Analysis

GenStat® 9th edition version 9.2.0.152 was used to analyse all data. One-way analysis of variance was used to determine statistical variations for most experiments. The data for the detergent, disinfectant interaction experiments within chapters 4 and 5 (*L. monocytogenes* and *E. coli* respectively) was analysed using a multifactorial regression analyses. This was required to normalise the data and allow for day to day variations that may otherwise have obscured detergent, disinfectant interactions upon 1 hour attached and 24 hour biofilm populations of cells.

3.0 Preliminary Experimentation

3.1 Introduction

The purpose of this chapter is to detail the relevant preliminary experimental work that had a direct bearing on results and data gathering, as described in the *Listeria monocytogenes* and *Escherichia coli* results chapters. This includes essential method development as well as describing promising methods that were rejected after evaluation. Identification, practical assessment and refinement of methodology to measure the interaction between detergents and disinfectants upon suspended, 1-hour attached and 24-hour biofilm populations of bacteria was conducted, including the development of a stable, reproducible attachment and biofilm protocol.

3.2 Bismuth thiol Detergents

The effects of novel bismuth thiol detergents on suspensions of *E. coli* strains 400, 453, 467 and O157:H7 pSB311 and *L. monocytogenes* strains 166, 359 and 367 was assessed. Experimental data was collected for short contact time biocidal effects, minimum inhibitory concentrations and inducible tolerance over time. The detergents BisTOL and BisEDT were identified for use within this study as conforming to the requirements for use within the food industry; little/no odour or taste which might taint food flavours and no toxicological effects as determined in mice studies (Domenico, 1998). Preliminary data to determine any bacteriostatic and biocidal properties of the bismuth thiols was required to develop and understand the interaction of these compounds with *E. coli* and *L. monocytogenes* strains.

3.2.1 Short Contact Time Effects of Bismuth Thiols

Short term exposure to the bismuth thiols, BisTOL and BisEDT was assessed for all *E. coli* and all *L. monocytogenes* strains to determine immediate effects the compounds have upon the different strains. The effect on suspended populations of

contact times of 5 minutes and 20 minutes, for concentrations of 10µg (in use concentration) and 20µg ml^{-1} bismuth thiol, was investigated. Quenching was achieved via serial dilution due to conventional quenching agents being detergents.

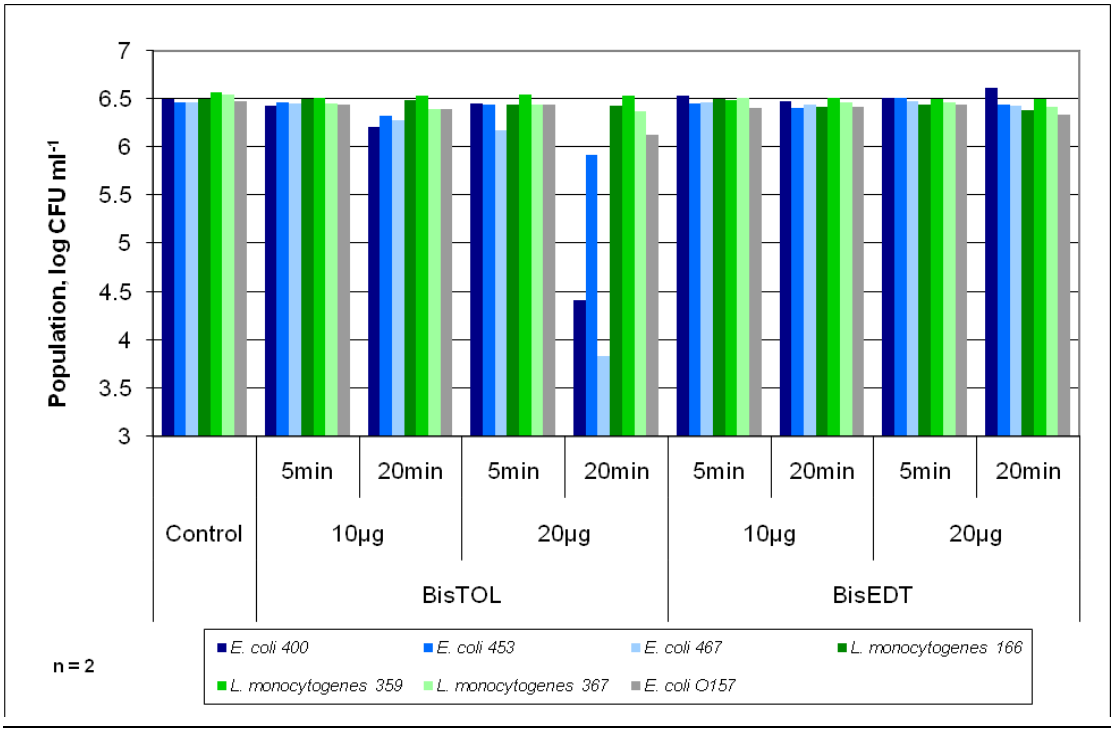

Fig. 3.1: Short contact time biocidal effect of bismuth thiol detergents over 5 and 20 minutes at concentrations of 10µg ml^{-1} and 20µg ml^{-1}.

The results in fig. 3.1 shows that for BisEDT after 5 and 20 minutes contact time, at both 10 and 20 µg ml^{-1} there was no detectable differences in viable population for any strain of *E. coli* or *L. monocytogenes*. No change in viable population was seen at a concentration of 10µg ml^{-1} BisTOL, whereas with a concentration of 20µg ml^{-1} over 20 minutes the viable population of all 4 strains of *E. coli* were observed to fall. Strains 0157:H7 pSB311 and 453 were shown to have a slight reduction in viability of approximately 0.5 log, whereas strains 400 and 467 had a loss of viability greater than 1.5 log and 2 log respectively.

3.2.2 Minimum Inhibitory Concentrations of Bismuth Thiols

Long term exposure to bismuth thiols was investigated to provide minimum inhibitory concentration data. This was required to determine if low level residues of bismuth thiols, for example that may accumulate in drains, has any effects upon viability and growth of *E. coli* and *L. monocytogenes* strains. The effects of long term exposure were recorded as MIC in µg ml^{-1} of bismuth thiol in TSB at 37°C after 24 and 48 hours. Presence or absence of turbidity was used to determine if a concentration inhibited growth after the allotted time.

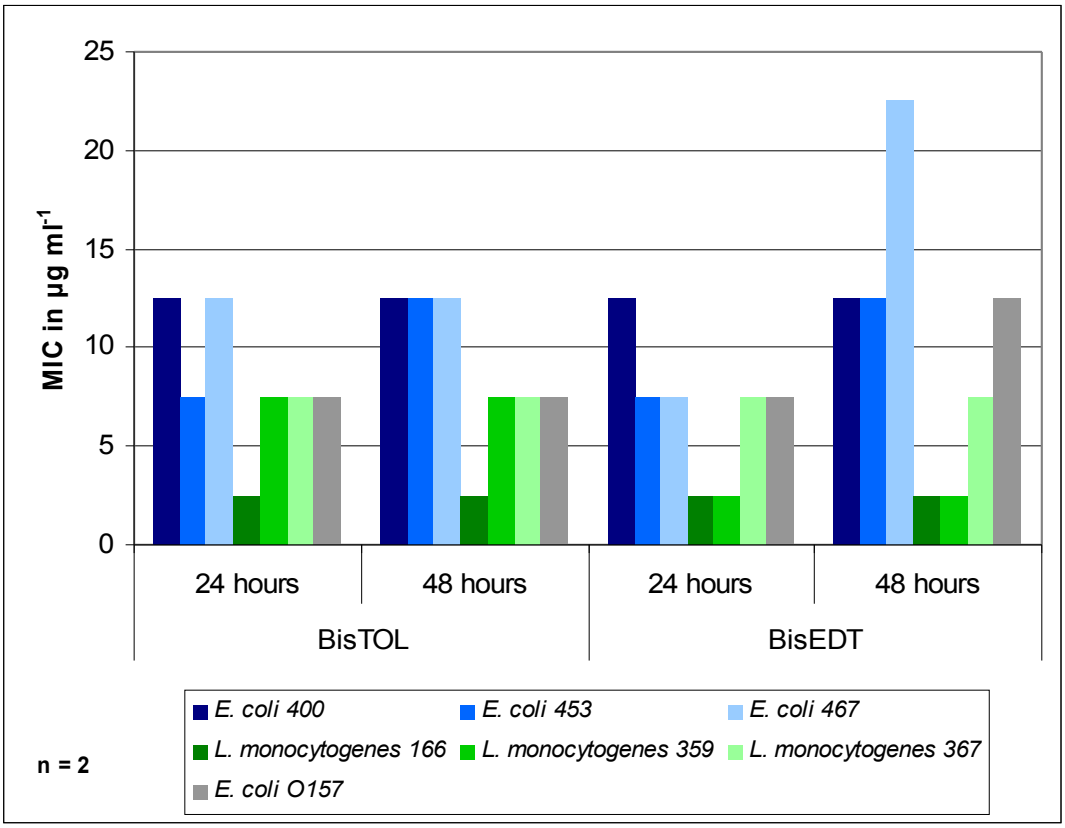

Fig. 3.2: Minimum inhibitory concentrations of bismuth thiol detergents measured over 24 and 48 hours after treatment after an inoculation with 100µl of overnight culture.

Differences in MIC were observed between strains and duration of contact. In fig. 3.2 the results for *E. coli* O157:H7 pSB311 show an MIC of 7.5µg ml^{-1} for BisTOL after 24 and 48 hours contact, for BisEDT an MIC of 7.5µg ml^{-1} was seen after 24 hours but by 48 hours the MIC had risen to 12.5 µg ml^{-1}. The results for *E.*

coli strain 400 show inhibition for BisTOL and BisEDT after 24 and 48 hours to be consistently at 12.5µg ml^{-1}. After 24 hours with both BisTOL and BisEDT an MIC of 7.5µg ml^{-1} was recorded for strain 453, by 48 hours the MIC had risen to 12.5µg ml^{-1}. The MIC of BisTOL against strain 467 was seen to be consistently 12.5µg ml^{-1}, whereas for BisEDT after 24 hours the MIC was 7.5µg ml^{-1} but by 48 hours the MIC was seen to have risen to be 22.5µg ml^{-1}.

No changes in inhibition were seen between 24 and 48 hours for all 3 strains of *L. monocytogenes* in BisTOL or BisEDT. Strain 166 was inhibited by concentrations of BisTOL of 2.5µg ml^{-1}, while strains 359 and 367 were inhibited at 7.5µg ml^{-1}. When treated with BisEDT, strains 166 and 359 were inhibited by 2.5µg ml^{-1} while strain 367 was inhibited at 7.5µg ml^{-1}.

3.2.3 Inducible Tolerance Against Bismuth Thiols

E. coli strain 453 was chosen to identify tolerance/resistance to BisTOL and *E. coli* strain 467 was chosen to identify tolerance/resistance to BisEDT due to previous observations in fig. 3.2 which suggested inducible tolerance might be occurring. 100µl of an overnight culture of each strain was inoculated into a doubling dilution series of the respective bismuth thiol (10ml TSB with 1.25 – 40 µg ml^{-1} bismuth thiol) and incubated at 37°C for 24 hours. Turbidity was noted as present or absent and a new set of doubling dilution bismuth thiols in TSB was inoculated with 100µl from the turbid culture with the highest concentration of bismuth thiol. This was repeated for a total of 8 subsequent passages.

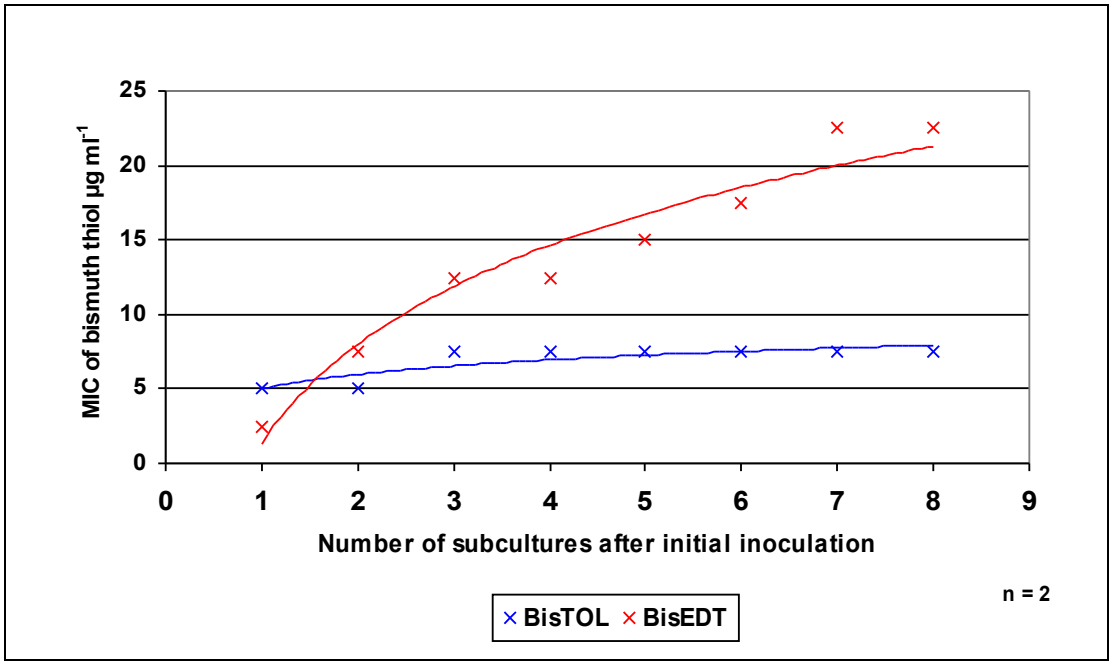

Fig. 3.3: Increased tolerance to bismuth thiol detergents for *E. coli* strain 453 for BisTOL and strain 467 for BisEDT after 7 passages from the previously highest turbid concentration of bismuth thiol.

Fig. 3.3 shows that increasing tolerance to bismuth thiol detergents is possible through successive passage as measured by turbidity after 24 hours at 37°C. The results for BisTOL against strain 453 show that tolerance increased from an initial MIC of 5µg ml^{-1} for the first and second passage to an MIC of 7.5µg ml^{-1}. This showed an overall 1.5 fold increase in tolerance to BisTOL over 8 days continuous exposure.

The results for BisEDT against strain 467 shows that the initial MIC for the first passage was 2.5µg ml^{-1}, the second passage had an MIC of 7.5µg ml^{-1}. The third and fourth subsequent passages had an MIC of 12.5µg ml^{-1}, the fifth and sixth passages had an MIC of 17.5µg ml^{-1} and the final two passages were shown to have an MIC of 22.5µg ml^{-1}. This showed an overall 9 fold increase in tolerance to BisEDT over 8 days continuous exposure.

3.3 Non-ionic and Anionic Detergents

Assessments of the non-ionic detergents 91/4 and KCL5 and the anionic detergents LX28 and Nec28 were conducted on suspended populations of *E. coli* and *L. monocytogenes* strains to determine any biocidal effects over short contact times and biocidal or biostatic effects during long duration contact times. The results for all detergents against all strains of *E. coli* did not show any biocidal effects during short duration contact and no biostatic effects during long term contact with all four detergents.

3.3.1 Short Contact Time Effects of Detergents on *L. monocytogenes* Strains

Recommended in-use concentrations of the non-ionic detergents 91/4 and KCL5 (0.1% v/v) and the anionic detergents LX28 and Nec28 (0.2% v/v) were assessed for biocidal effects after 5 and 20 minutes contact time against *L. monocytogenes* strains 166, 359 and 367.

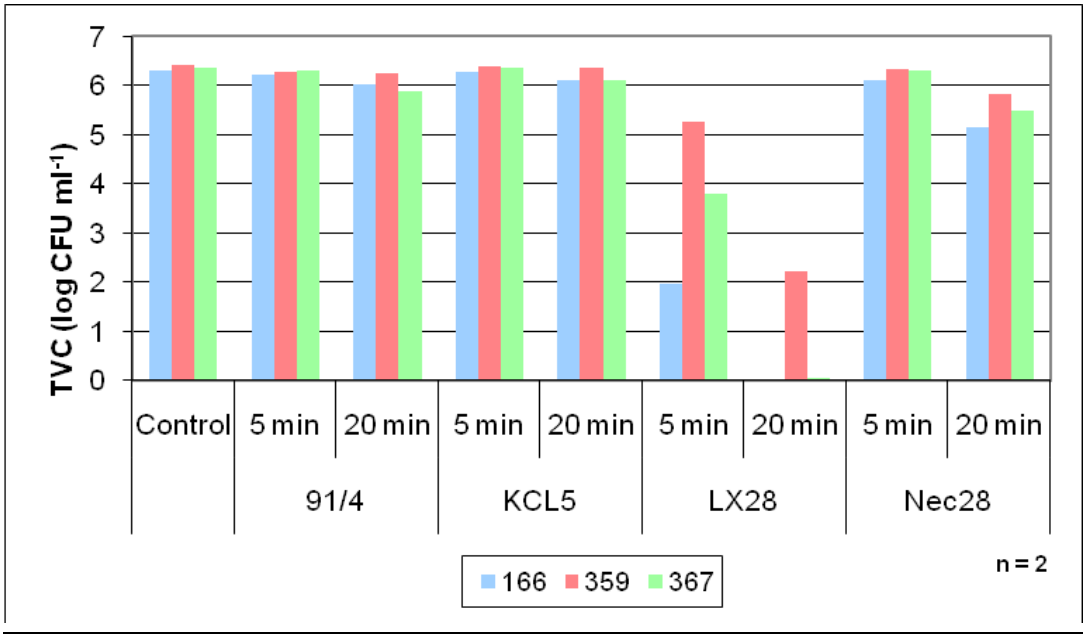

Fig. 3.4: Short contact time biocidal effect of non-ionic and anionic detergents over 5 and 20 minutes contact time at concentrations of 0.1% v/v and 0.2% v/v respectively.

Treatment with the non-ionic detergents 91/4 and KCL5, fig. 3.4, did not noticeably reduce the viable count of strains 166, 359 and 367. Treatment with the

anionic detergent Nec28 of the *L. monocytogenes* strains did not appear to alter viable counts after 5 minutes contact time but did appear to reduce the viable count of strain 166 and 367 after 20 minutes contact time.

The anionic detergent LX28 was seen to reduce the viable count of all 3 strains of *L. monocytogenes* after 5 and 20 minutes contact time. The results for strain 166 show a reduced viable count by over 4.3 log after 5 minutes contact, by 20 minutes the viable counts had been reduced to zero. Strain 359 was seen to have a reduction in viable count by just over 1.1 log after 5 minutes contact and nearly 4.2 log after 20 minutes contact. The results for strain 367 in fig. 3.4 show that a loss of viability greater than 2.5 log was seen after 5 minutes contact with LX28 and by 20 minutes contact a loss of viability greater than 6.2 log was observed, resulting in almost total kill.

3.3.2 24 and 48 Hour Contact Time Effects of Detergents on *L. monocytogenes* Strains

Long term exposure of *L. monocytogenes* to non-ionic and anionic detergents was assessed. 100µl of overnight culture of each strain was inoculated into 100ml TSB containing either in use or 10 x in use concentrations of detergents. The cultures were incubated at 37°C, for 24 and 48 hours. The presence or absence of turbidity and the presence or absence of viable cells was recorded to determine possible bacteriostatic and biocidal activities.

Detergent	Concentration		24 Hours		48 Hours	
		Strain	Viable cells	Turbidity	Viable cells	Turbidity
91/4	0.1%	166	+	-	+	-
		359	+	-	+	+
		367	+	-	+	-
	1%	166	+	-	+	-
		359	+	-	+	+
		367	+	-	+	-
KCL5	0.1%	166	+	+	+	+
		359	+	+	+	+
		367	+	+	+	+
	1%	166	+	+	+	+
		359	+	+	+	+
		367	+	+	+	+
LX28	0.2%	166	-	-	-	-
		359	-	-	-	-
		367	-	-	-	-
	2%	166	-	-	-	-
		359	-	-	-	-
		367	-	-	-	-
Nec28	0.2%	166	+	+	+	+
		359	+	+	+	+
		367	+	+	+	+
	2%	166	+	+	+	+
		359	+	+	+	+
		367	+	+	+	+
Control			+	+	+	+

Table 3.1: Long term effects against *L. monocytogenes* strains 166, 359 and 367 of the non-ionic detergents 91/4 and KCL5 and the anionic detergents LX28 and Nec28 at in use, and 10 x in use concentrations in TSB, after 24 and 48 hours.

The results for the non-ionic detergent 91/4 show that at both concentrations a bacteriostatic effect was observed for all strains after 24 hours. By 48 hours, bacteriostasis was still apparent against strains 166 and 367 while strain 359 was seen to be multiplying in the presence of both 0.1% and 1% 91/4. All strains, when exposed to the non-ionic detergent, KCL5, grew at concentrations of 0.1% and 1% after 24 hours.

After 24 hours, no surviving cells for any strain were recorded after exposure to the anionic detergent LX28. The second anionic detergent Nec28 was not shown to exhibit any inhibitory effects, with all strains seen to be turbid after 24 hours for both 0.2% and 2% detergent concentrations.

3.4 Bioluminescence as a Measure of Stress upon Populations of Cells

Bioluminescence, measured as relative light units (RLU), was investigated as a tool to quantify sub-lethal detergent and disinfectant effects upon metabolism and activity of attached and biofilm cells *in situ*.

Initial data for attached populations of the bioluminescent bacterium, *E. coli* O157:H7 pSB311 resulted in large variations of RLU for the same sample over time. RLU data was collected for suspended populations of cells to identify how RLU varies over time and with different starting population densities.

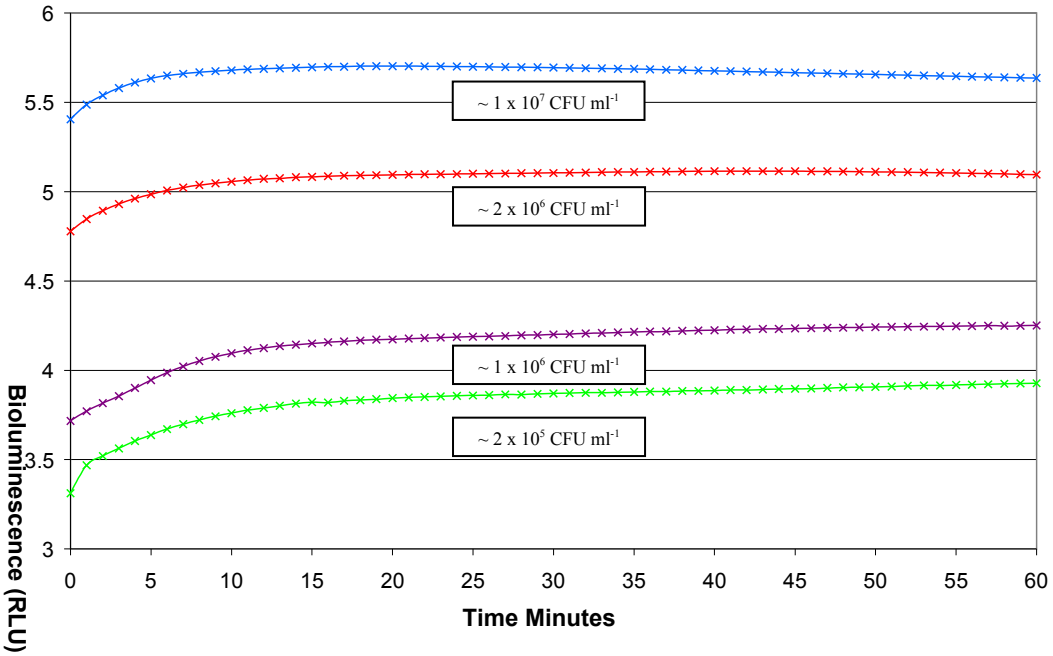

Fig. 3.5: RLU against sampling time after pipetting of 1ml into a Biotrace cuvette of an approximated concentration of suspended *E. coli* O157:H7 pSB311 to demonstrate the changes in RLU as a measure of population size due to variations in RLU measurements for the same population density over time.

Figure 3.5 demonstrates why bioluminescence is unsuitable as a measure of viable population size following treatment. The simple procedure of pipetting a culture of cells into a Biotrace cuvette was shown to result in RLU measurements that vary considerably on a second by second basis based on when a reading was taken. Additional experimental data provided by Betty Ogwaro (personal communication) showed that upon exposure to any environmental stressor, e.g. acid, detergents etc, RLU output ceased with a substantial lag time before RLU was once again detectable.

3.5 Attachment and Biofilm Formation

Reproducible 1-hour attachment and 24-hour biofilm formation for all *E. coli* and *L. monocytogenes* strains at room temperature was required in order to conduct detergent / disinfectant synergy trials upon adherent populations of cells. Experiments using various conditions for attachment and biofilm formation were conducted to determine an optimal, reproducible methodology that was deemed to simulate possible conditions found within food processing plants, i.e. high nutrient availability following a spillage that results in consistent adherent populations of each species and strain followed by more limiting nutrient availability during biofilm formation.

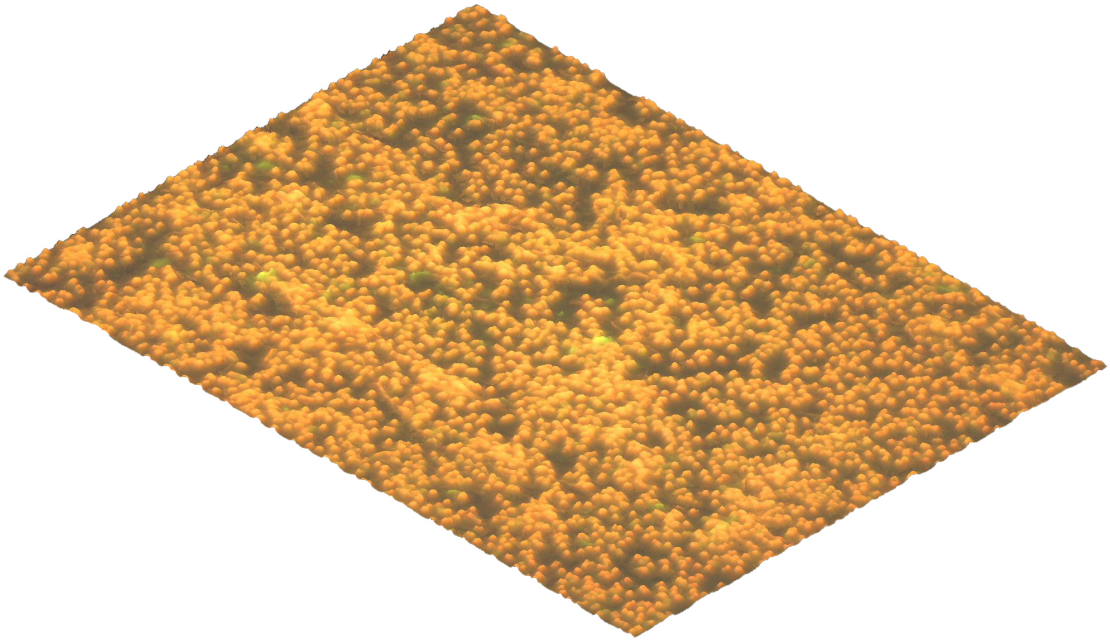

Plate 3.1: 3D topographical epifluorescent rendered image of a typical 1-hour attached population of *E. coli* strain 453 at x 1000 magnification, stained with acridine orange and calcofluor white on stainless steel to differentiate between cells (orange) and EPS (blue) respectively.

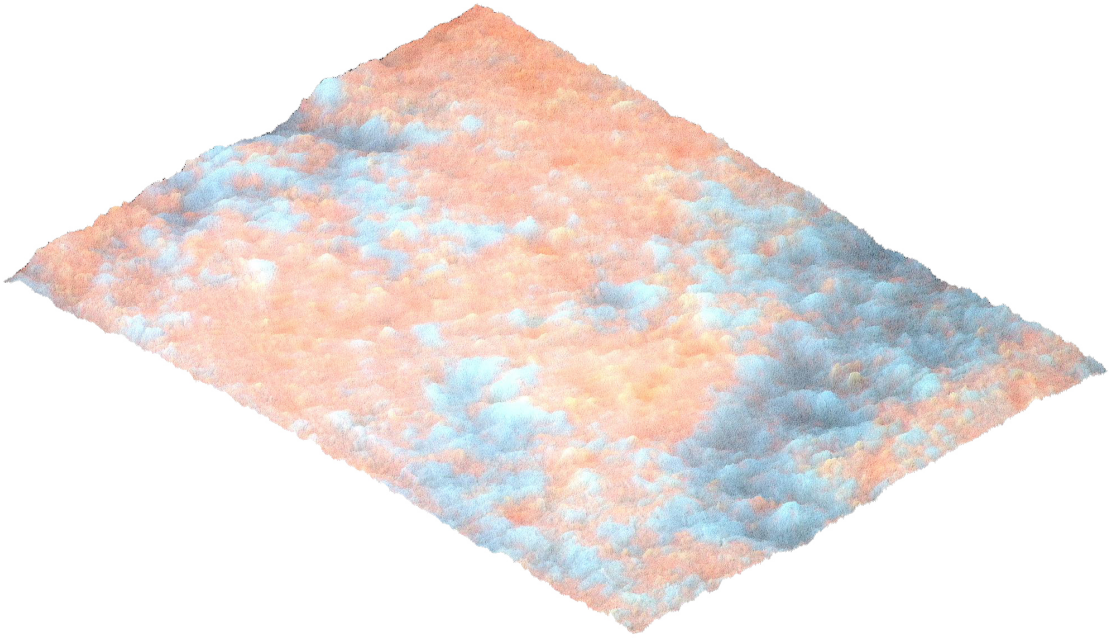

Plate 3.2: 3D topographical epifluorescent rendered image of a typical 24-hour biofilm population of *E. coli* strain 453 at x 1000 magnification, stained with acridine orange and calcofluor white on stainless steel to differentiate between cells (orange) and EPS (blue) respectively.

Plates 3.1 and 3.2 are representative of the typical appearance of 1-hour attached and 24-hour biofilm populations of cells. Generalised morphological differences between *L. monocytogenes* and *E. coli* attached and biofilm populations were not apparent other than cell size, therefore plates of the former have not been reproduced here. Dual staining with acridine orange and calcofluor white was used to differentiate between cells and extra cellular polysaccharide (EPS). Acridine orange binds strongly to DNA rendering the cells visible as orange rods while calcofluor white binds to polymeric matrices, such as the sugars that form EPS, rendering them blue. This provides easy differentiation between cellular and extracellular material present in a biofilm and enables observation of some of the complex multilayering and channels seen within biofilm populations.

3.6 Discussion

The bismuth thiol detergent, BisTOL, at a concentration of 20μg ml^{-1} (twice the in use concentration of 10μg ml^{-1}) was shown to exhibit biocidal properties after 20 minutes contact time when used against *E. coli* strains 400 and 467 and to a lesser extent strains 453 and O157:H7 pSB311. Bismuth thiols are known to disrupt EPS production and *in situ* EPS (Domenico, 1998), although no specific data is available to describe possible biocidal activity, for example if the detergent affects cellular permeability. Identifying the mode of action involved with cell death seen against strains 400 and 467 could provide data that would enable the development of improved bismuth thiol detergents, designed to further enhance cleaning and disinfection regimes via additional killing of bacterial cells. One possible mode of action that can be linked to disruption of EPS surrounding the cells is lysis due to changing osmotic pressure as the EPS is broken down (Domenico, 1998).

Bismuth thiols were shown to possess a secondary desirable mode of action, growth inhibition, which can help reduce colonisation and multiplication of organisms within common risk areas within factories, e.g. drains. Concentrations as low as 5µg ml^{-1} of bismuth thiol inhibited bacterial growth within TSB; the presence of organic material, as found within growth media, has been shown to reduce the effectiveness and inactivate many detergents and disinfectants (Fraise, 1999; Aarnisalo *et al.*, 2000; Augustin *et al.*, 2004). During prolonged treatment, inhibition of growth was initially seen at concentrations below 10µg ml^{-1} for 24 hours for the majority of strains tested. However, by 48 hours contact time, growth was observed within previously inhibitory concentrations of bismuth thiols, this may be due to degradation of the compounds or possibly inducible tolerance. Inducible tolerance to bismuth thiols was then shown to be acquired through prolonged exposure, the results for strain 467 treated with BisEDT showed that tolerance increased by a factor of 9 after 8 day's exposure to gradually increasing concentrations. Locations within factories, e.g. drains, wall/floor joints and cracks, with greater risk of persistent biofouling are possible sites of acquired tolerance to bismuth thiols as suggested by these results. Possible mechanisms involved with inducible tolerance may include selection for increased efflux efficiency or decreased permeability to the agent as reviewed by Prakash *et al.* (2003), although it is also possible that over time the EPS disruption and inhibition may be overcome through adaptation. Although not tested, the stability of the tolerance could be determined by removal of the bismuth thiol challenge via sub-culturing the tolerant population for a number of generations and then re-exposing the population to the bismuth thiol. If different bismuth thiols exhibit differing modes of action then by combining two or more bismuth thiols within a cleaning product, the risk of acquired tolerance would be reduced. Broader spectrum biocidal activity

against a range of bacterial species should also be possible by combining multiple bismuth thiols in a single product (Domenico, 1998).

It was hoped that bioluminescent *E. coli* O157:H7 pSB311 could be used to obtain real time, *in situ* measurements of detergent and disinfectant activity. Previous studies have used bioluminescent strains of both *L. monocytogenes* and *E. coli* O157:H7 as markers to determine survival of inoculum in foods (Hudsen *et al*., 1997; Ramsaran *et al*., 1998). However, it was discovered that RLU measurements were unreliable; with rapidly increasing measurements occurring for up to 17 minutes after a suspension of cells had been simply transferred into a Biotrace cuvette. The comparison of RLU to TVC suggests that the difference seen for RLU could equate to a change in excess of 1 log TVC, with greater inaccuracies for lower population densities, therefore it was decided that the variation observed with RLU measurements was too great to be of use during this study. Betty Ogwaro (personal communication), also demonstrated that after challenge with any chemical, including detergents, disinfectant, acids, alkalis and also environmental challenges such as heating or cooling, light production was halted and did not resume within viable populations for up to 2 hours following the challenge. Within the literature, similar results where luciferase was utilised as a 'real time' marker were observed by Lehtinen *et al*., (2003), following exposure to differing concentrations of ethanol, whereby the first reliable reading obtained was taken 40 minutes following exposure.

The reproducible 1-hour attachment and 24-hour biofilm models shown in plates 3.1 and 3.2 were achieved after considerable refinement of attachment and biofilm formation conditions. The choice of medium for forming a complex biofilm population after initial attachment was seen to be particularly important. Media tested included supernatant, different broth types and different broth concentrations, always

at 20°C. The final choice of 1/10 strength TSB was used because it was discovered that higher concentrations resulted in considerable sloughing of cells into the medium and a much less complex biofilm being formed, while lower concentrations and supernatant did not contain adequate nutrients to allow for sufficient cell multiplication and EPS production.

Acridine orange and calcofluor white were chosen after considering other alternative staining methods such as DAPI. Acridine orange was found to consistently produce good quality stains of attached populations without the complication of extracellular material or attachment surface being stained. During analysis, although the stain degraded, the rate of degradation was slow enough to allow for multiple image cross-sections of attached populations to be sampled. Calcofluor white was also chosen because as with acridine orange, degradation was slow enough during analysis to sample multiple cross-sections. Also during preliminary experimentation, where known concentrations of starch was dried onto coupons, with or without the addition of detergents, it was shown that the image intensity recorded for calcofluor white was directly proportional to the concentration of starch present, which was analogous to the density of EPS present after biofilm formation.

4.0 Effects of Treatments on *Listeria monocytogenes*

4.1 Introduction

The identification of synergistic relationships between specific detergents and disinfectants, with regards to the removal and killing of surface adhered, 1-hour attached and 24-hour biofilm populations of *L. monocytogenes*, will lead to the development of improved cleaning and disinfecting regimes within chilled food processing environments.

All of the TVC results throughout this chapter have been standardised to use the experiments negative control as the zero value for the x-axis on the following graphs. Each graph represents experimentation conducted on multiple days, the standardised data was analysed using multifactorial regression analysis to normalise day to day variation. The inherent variability of adherent populations' meant that no specific population density is stated for each experiment. A treatment value below zero shows a reduction in viable count while a value greater than zero shows an increase in viable count compared to the negative control, the latter does not represent proliferation of the population, merely a greater proportion of viable cells remaining adhered following the treatments compared to the water only negative control treatment.

As detailed in the Materials and Methods chapter, the term 'Attached' refers to a population of *L. monocytogenes* that has been adhered to an 8mm x 8mm stainless steel coupon for 1-hour. The term 'Biofilm' refers to a one hour attached population of *L. monocytogenes* that has then established a biofilm over 24-hours, as described in the Materials and Methods chapter.

4.2 Effect of Detergents on the Viability of Suspensions of *L. monocytogenes*

Preliminary experimentation, as described in 3.3.1, was repeated using controlled experimental procedures to determine statistical significances using ANOVA. Biocidal effects of industrially recommended, in use, concentrations of detergents were determined on 100µl suspensions of *L. monocytogenes* in 9.9ml detergent in WoSH for strains 166, 359 and 367. Detergent activity was compared to a negative control (zero on the x-axis) in which detergent solutions were substituted with WoSH. Two detergent contact times were used, 20 minutes as recommended to the industry and 5 minutes to identify any rapid biocidal effects.

The results in fig. 4.1 show a significant loss of viability ($P < 0.01$) was identified for suspensions of *L. monocytogenes* treated with the anionic detergent LX28, after 5 and 20 minute contact times. Strain 359 was observed to be significantly less sensitive ($P < 0.01$) to LX28 than either strain 166 or 367. A significant ($P < 0.05$) loss of viability was noted for the second anionic detergent, Nec28, for all 3 strains after 20 minutes contact time, however no significant loss was seen after 5 minutes contact time. Strain 359 was observed to be less sensitive ($P < 0.05$) to Nec28 than either strain 166 or 367. The two bismuth thiol and the two non-ionic detergents did not demonstrate any significant effects upon the viability of suspended *L. monocytogenes* strains.

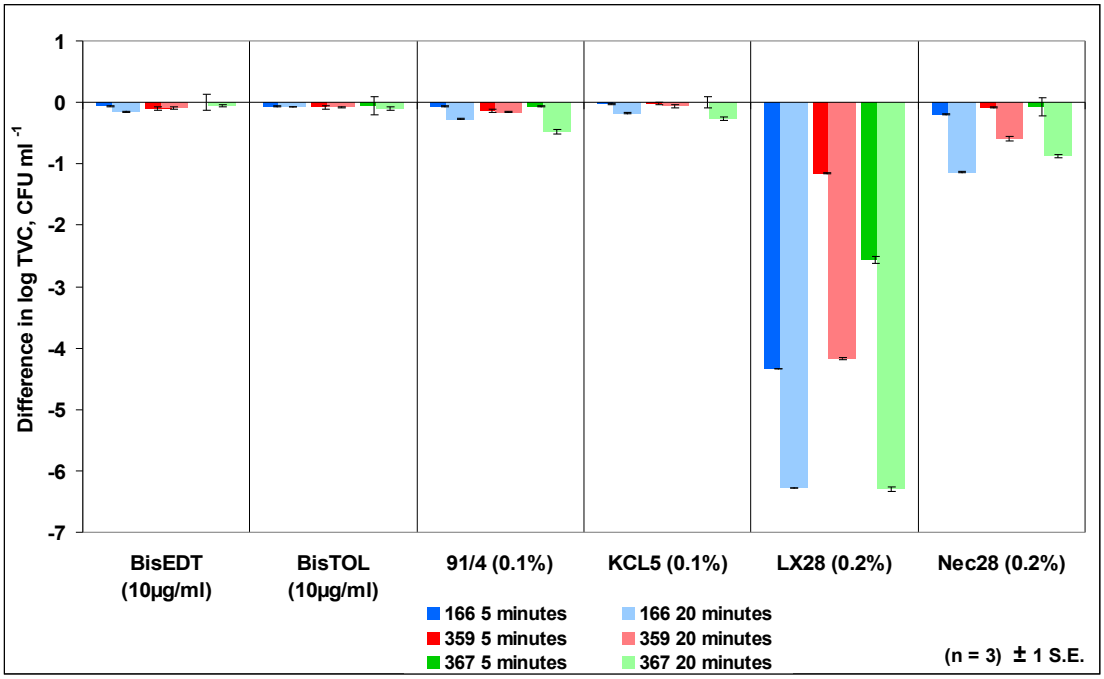

Fig. 4.1: Mean loss of viability after 5 and 20 minute contact times with detergent treatments for approximately 1 x 10^7 *L. monocytogenes* suspended in WoSH at 20°C, shown as log TVC difference to a negative, water only control.

4.3 One Hour Attached Populations – Total Viable Counts

4.3.1 Effect of Detergent Only Treatments

The effect of industrially recommended, in use, concentrations of detergents was determined on one hour attached populations of *L. monocytogenes* strains 166, 359 and 367. This was used to determine the degree of killing and/or removal of the adherent population following treatment. Detergent activity was compared to a negative control (zero on the x-axis) in which detergent solutions were substituted with WoSH.

The results shown in fig. 4.2 for BisEDT and BisTOL do not show any significant change in viable count to the control treatment. Both non-ionic detergents, 91/4 and KCL5, and the anionic detergent Nec28, demonstrated a general trend towards a reduction in viable count against all 3 strains of *L. monocytogenes* with a significant ($P < 0.05$) loss of viability observed for strain 359 when treated with

KCL5 and Nec28. The detergent, LX28, significantly (P <0.01) reduced the viable count of all 3 strains of *L. monocytogenes*.

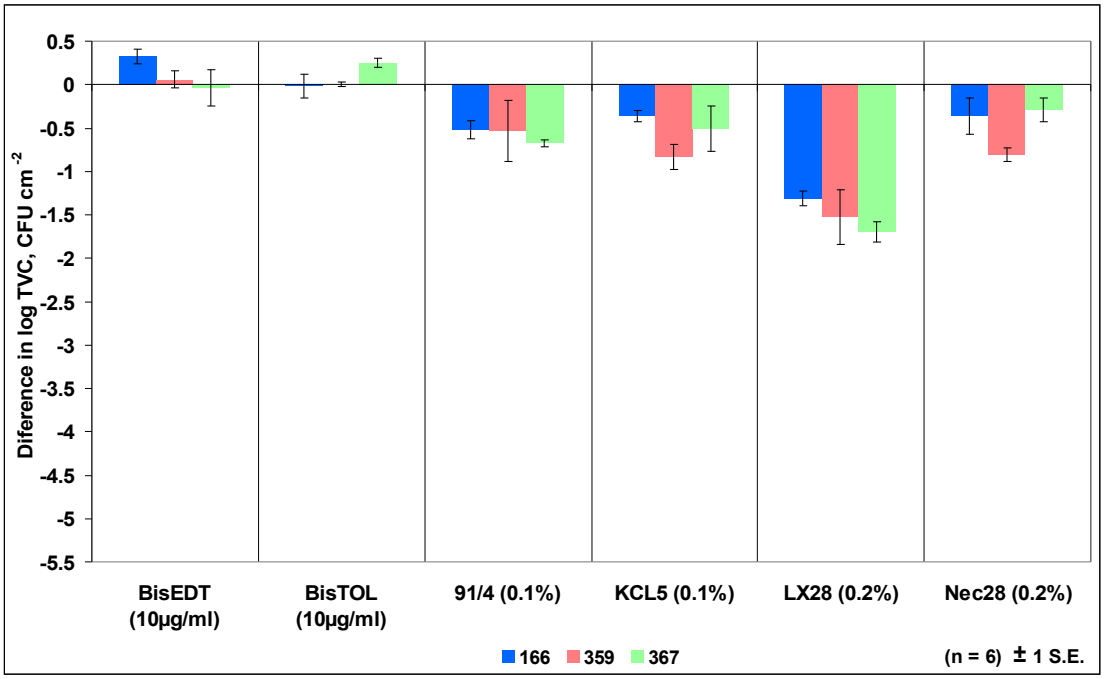

Fig. 4.2: Mean loss of viability after 20 minute contact time with detergent treatments in WoSH for attached *L. monocytogenes* at 20°C, shown as log TVC difference to a negative, water only control.

4.3.2 Effect of Disinfectant Only Treatments

The effect of disinfectants, at concentrations of 0.01% for BAC and 0.0008% for NaDCC, previously identified to give 2 – 4 log kill against *L. monocytogenes* in suspension, was determined against 1-hour attached populations of strains 166, 359 and 367 to measure the biocidal effects against biofilm populations. Disinfectant activity was compared to a negative control (zero on the x-axis) in which disinfectant solutions were substituted with WoSH. The industry recommended contact time of 5 minutes was used. The results are presented in figure 4.3.

Significant differences were observed in disinfectant sensitivity between strains. Strain 367 was significantly less sensitive to BAC (P <0.05) when compared to strains 166 or 359. No significant difference in sensitivity to BAC between strains 166 and

359 was identified. Strain 359 was seen to be significantly more sensitive ($P < 0.01$) to NaDCC when compared to strains 166 and 367.

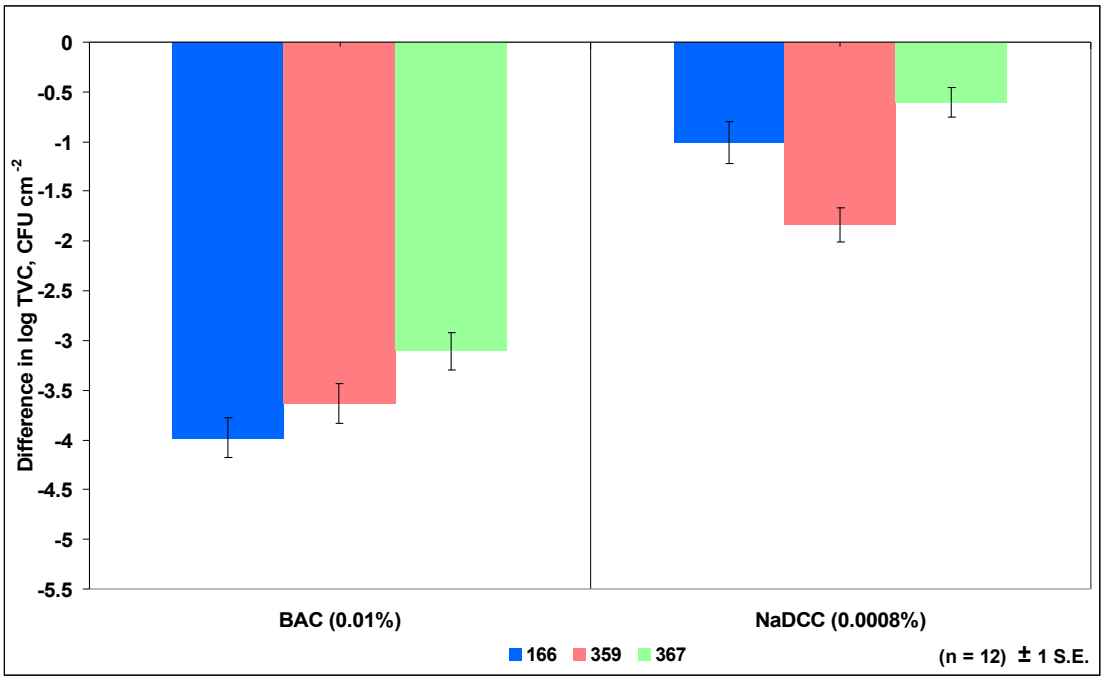

Fig. 4.3: Mean loss of viability after 5 minute contact time with disinfectant treatments in WoSH for attached *L. monocytogenes* at 20°C, shown as log TVC difference to a negative, water only control.

4.3.3 Effect of Detergent Followed by Disinfectant Treatments

The effect of combined detergent (industry recommended, in use, concentrations) and disinfectants, at concentrations of 0.01% for BAC and 0.0008% for NaDCC, previously identified to give 2 – 4 log kill against *L. monocytogenes* in suspension, was determined against 1-hour attached populations of strains 166, 359 and 367. Treatment activity was compared to a negative control (zero on the x-axis) in which detergent and disinfectant solutions were substituted with water of standard hardness. The industry recommended contact times of 20 minutes for detergents and 5 minutes for disinfectants was used. Observed results were compared to expected results, where the expected results were calculated by combining the changes in TVC for individual detergent (fig. 4.2) and disinfectant (fig. 4.3) treatments to determine any interactions between detergent and disinfectant treatments.

The data (fig. 4.4 and table 4.1) shows that significant differences occurred between both strains and treatments when treated with the disinfectant BAC. The only observed synergy (defined as a statistically greater than expected reduction in TVC compared to an additive effect of detergent and disinfectant treatments) with BAC was seen when BisTOL was used against strain 367 ($P < 0.01$). As can be seen with the majority of treatments, all 6 detergents generally exhibited an antagonism (defined as a statistically lower than expected reduction in TVC compared to an additive effect of detergent and disinfectant treatments) against BAC which resulted in a significantly increased rate of survival than expected for all 3 strains of *Listeria*. Treatments against strain 359 were in general seen to result in a greater degree of antagonism than seen against strain 166 and 367.

The two bismuth thiol detergents were seen to have no effect against strain 166, significant (BisEDT: $P < 0.01$, BisTOL: $P < 0.05$) antagonisms against strain 359 and against strain 367, BisEDT demonstrated a significant ($P < 0.05$) antagonism while BisTOL was seen to be significantly ($P < 0.01$) synergistic. Against strains 359 and 367 both of the non-ionic detergents, 91/4 and KCL5, showed significant antagonisms ($P < 0.01$), while against strain 166, 91/4 treatments were not seen to significantly differ from the expected results and KCL5 treatments resulted in a weakly significant antagonism ($P < 0.05$).

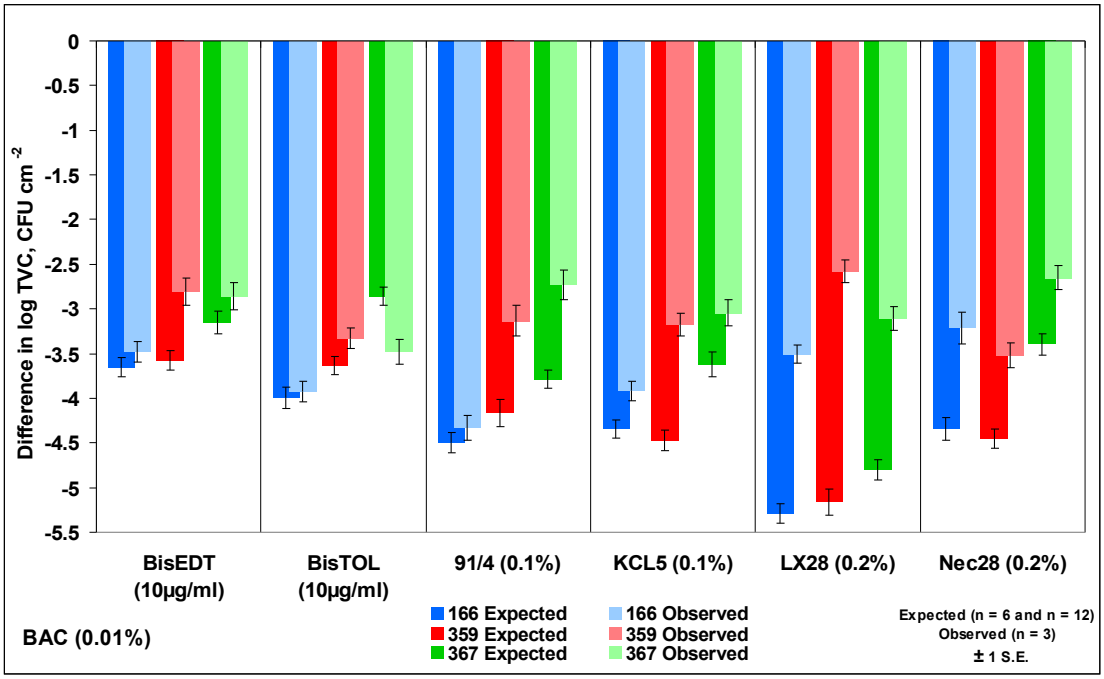

Fig. 4.4: Mean loss of viability after 20 minute detergent treatment followed by 5 minute BAC treatment in WoSH for attached *L. monocytogenes* at 20°C, shown as expected (fig. 4.2 + fig. 4.3) and observed log TVC difference to a negative, water only control.

BAC	166	359	367
BisEDT	-0.177	**-0.770**	-0.291
BisTOL	-0.071	-0.308	**0.625**
91/4	-0.167	**-1.035**	**-1.055**
KCL5	-0.423	**-1.297**	**-0.571**
LX28	**-1.784**	**-2.584**	**-1.693**
Nec28	**-1.132**	**-0.924**	**-0.744**

Table 4.1: Synergies and antagonisms after 20 minute detergent treatment followed by 5 minute BAC treatment in WoSH for attached *L. monocytogenes* at 20°C. Differences to the expected additive results are presented as log TVC with significant synergies (P <0.05), **(P <0.01)** and antagonisms (P <0.05), **(P <0.01)**.

In contrast, for NaDCC treatments many synergies can be observed (fig. 4.5 and table 4.2) in which 12 significant synergies (10: P <0.01, 2: P <0.05) were observed compared to 2 significant antagonisms (1: P <0.01, 1: P <0.05). Only Nec28 and BisTOL treatments were shown to present synergies against all 3 strains.

The summary of synergies and antagonisms in table 4.2 showed there to be large differences (> 2 log) between the 3 strains of *L. monocytogenes*. Treatments against

strain 166 showed 4 significant synergies (KCL5, LX28, Nec28: P <0.01, BisTOL: P <0.05), strain 359 showed 2 significant synergies (BisTOL, Nec28: P <0.01) and 2 significant antagonisms (LX28: P <0.01, BisEDT: P < 0.05) and against strain 367 all treatments were significantly synergistic (P <0.01, BisTOL: P <0.05). In general treatments used against strain 367 were seen to be more synergistic that treatments against strains 166 and 359.

Treatment with BisEDT showed no effect against strain 166, a weak significant (P <0.05) antagonism against strain 359 and a significant (P <0.01) synergy against strain 367. The results for BisTOL showed that significant synergies (166, 357: P <0.05, 359: P< 0.01) were seen against all 3 strains.

The non-ionic detergent, 91/4 showed no significant interaction when used against strains 166 and 359 and a significant (P <0.01) synergy against strain 367. The second non-ionic detergent, KCL5, was seen to be significantly (P <0.01) synergistic against strains 166 and 367, with no interaction observed against strain 359.

Significant synergies (P <0.01) were seen for both anionic detergents, LX28 and Nec28, against all 3 strains with the exception of LX28 when used against strain 359 which was seen to result in a significant (P <0.01) antagonism with NaDCC.

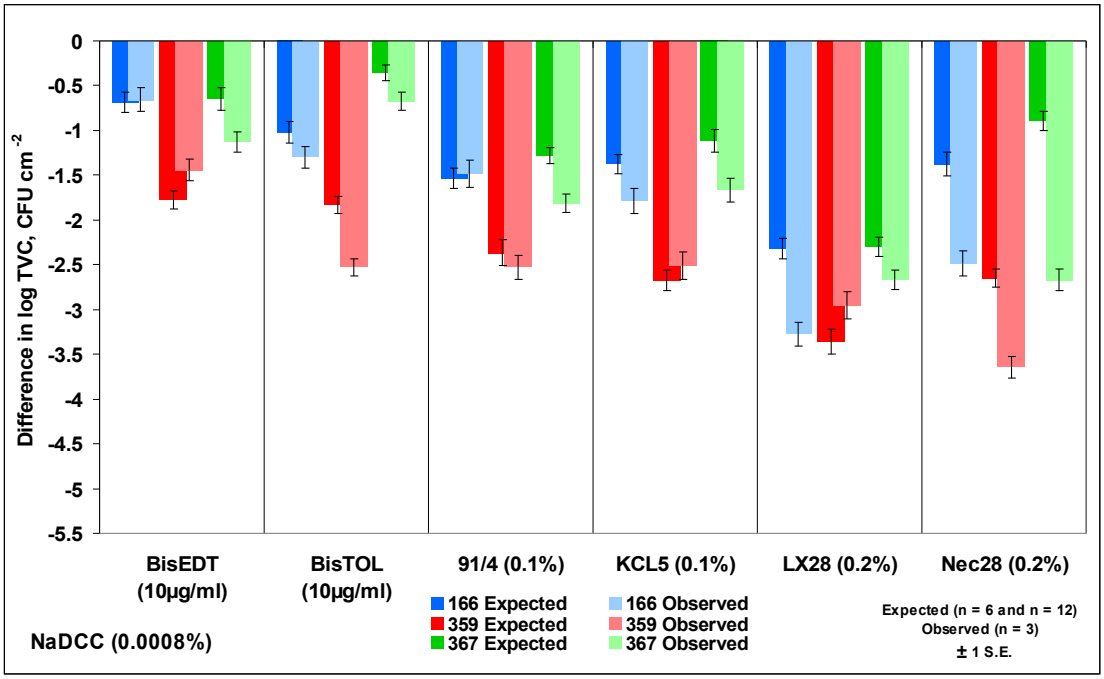

Fig. 4.5: Mean loss of viability after 20 minute detergent treatment followed by 5 minute NaDCC treatment in WoSH for attached *L. monocytogenes* at 20°C, shown as expected (fig. 4.2 + fig. 4.3) and observed log TVC difference to negative, water only control.

NaDCC	166	359	367
BisEDT	-0.028	-0.335	**0.480**
BisTOL	0.274	**0.696**	0.318
91/4	-0.046	0.157	**0.527**
KCL5	**0.416**	-0.158	**0.547**
LX28	**0.952**	**-0.408**	**0.370**
Nec28	**1.103**	**0.997**	**1.776**

Table 4.2: Synergies and antagonisms after 20 minute detergent treatment followed by 5 minute NaDCC treatment in WoSH for attached *L. monocytogenes* at 20°C. Differences to the expected additive results are presented as log TVC with significant synergies (P <0.05), **(P <0.01)** and antagonisms (P <0.05), **(P <0.01)**.

4.3.4 Summary of Treatments for One Hour Attached Populations

The data in fig. 4.6 summarises two general trends with regards to antagonism and synergy. Exposing attached cells of *L. monocytogenes* to detergents prior to treatment with the disinfectant BAC was shown to result in antagonistic effects. Exposing cells to detergents prior to treatment with the disinfectant NaDCC was shown to result in synergistic effects.

Generally treatment with the bismuth thiol detergents, BisEDT and BisTOL, did not demonstrate any significant changes in disinfectant sensitivity to either BAC or NaDCC. Treatment with either of the non-ionic detergents (91/4 and KCL5) showed trends towards antagonisms with BAC and no effect with NaDCC. The anionic detergents, LX28 and Nec28, showed a general trend of antagonistic effects with BAC and synergistic effects with NaDCC.

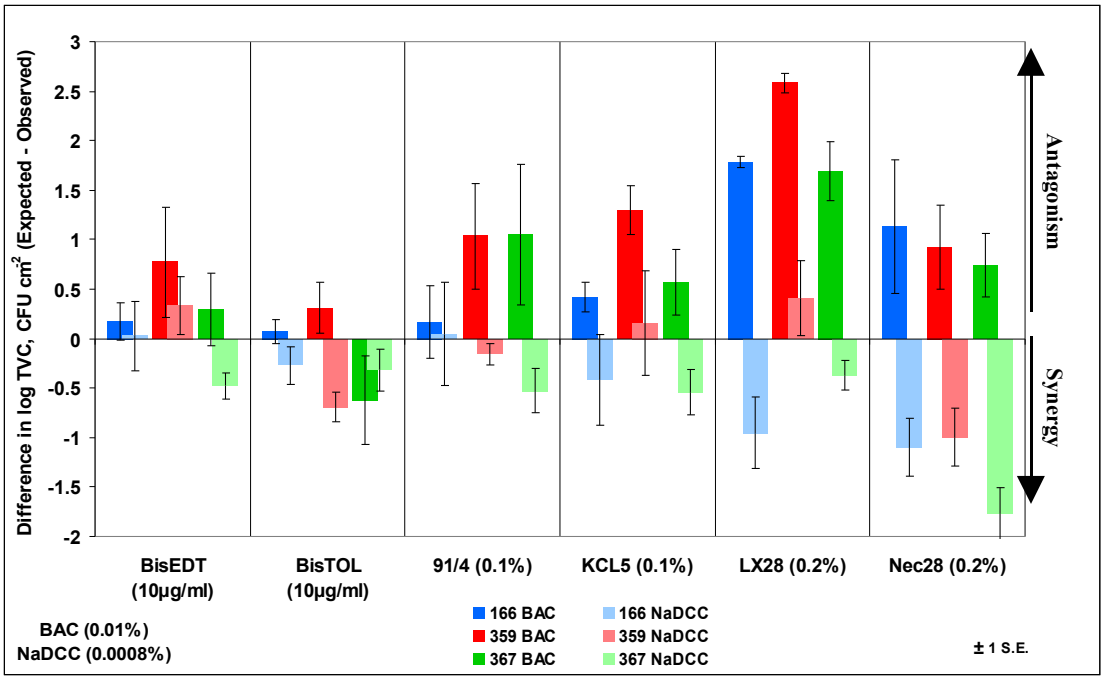

Fig. 4.6: Summary of the effectiveness on attached *L. monocytogenes* of the combined detergent/disinfectant treatments compiled by comparing the difference in viability to the sum of the single detergent and disinfectant treatments, shown as difference in log TVC.

4.4 One Hour Attached Populations – Direct Microscopic Counts

Direct counts were obtained for each treatment combination used against attached *L. monocytogenes* strains. Direct counting was used to determine if the effects seen while measuring TVC were removal and/or killing of bacteria.

Plate 4.1 shows typical slides of each *L. monocytogenes* strain after attaching for 1 hour to stainless steel coupons and staining with acridine orange. Direct counts of treated populations were recorded using Spot® capture software and ImagePro® analysis software. Each image was analysed to identify the area covered by cells, which was then transformed into cells cm^{-2} from calculations of the average size of an individual cell within the image, as described in chapter 2. The direct count for strain 367 was seen to be significantly lower ($P < 0.05$) than that observed for strains 166 and 359.

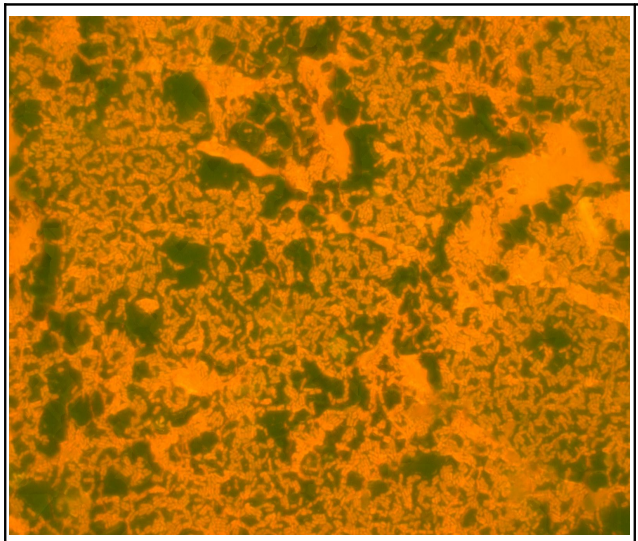	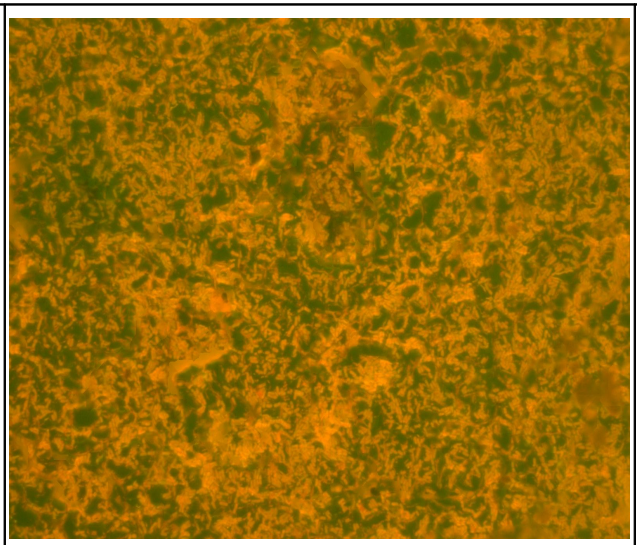	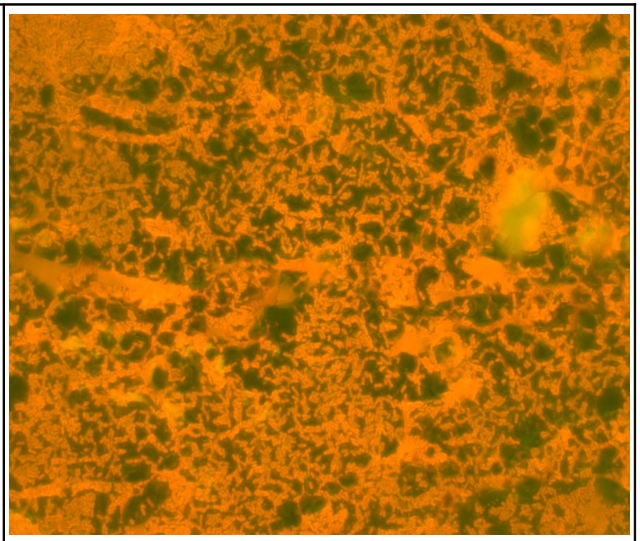
Strain 166 1 hour attachment, n = 12, mean 7.8 log cells cm^{-2}, S.E. 0.3	Strain 359 1 hour attachment, n = 6, mean 7.6 log cells cm^{-2}, S.E. 0.4	Strain 367 1 hour attachment, n = 6, mean 7.1 log cells cm^{-2}, S.E. 0.3

Plate 4.1: Typical 1 hour attachment in TSB for *L. monocytogenes* strains at 20°C on type 316 with 2B finish stainless steel coupons, stained with acridine orange at x 1000 magnification.

4.4.1 Effect of Detergent Only Treatments

Direct counts were obtained for each detergent treatment used against attached *L. monocytogenes* strains. Direct microscopic counting was used to determine if the TVC effects measured were removal and/or killing of bacteria.

The results in fig. 4.7 show that 91/4 and LX28 detergent only treatments had some removal effect against attached populations of all *L. monocytogenes* strains. Significant ($P < 0.01$) loss in total counts was observed with the non-ionic detergent 91/4 (0.45 log – 0.61 log) and the anionic detergent LX28 (0.43 log – 0.81 log). A significant loss ($P < 0.05$) in total count was also seen with BisTOL against strain 367. Significant increases ($P < 0.05$) in total count were observed for strain 359 when treated with BisEDT, KCL5 and Nec28 and also for strain 367 when treated with BisEDT.

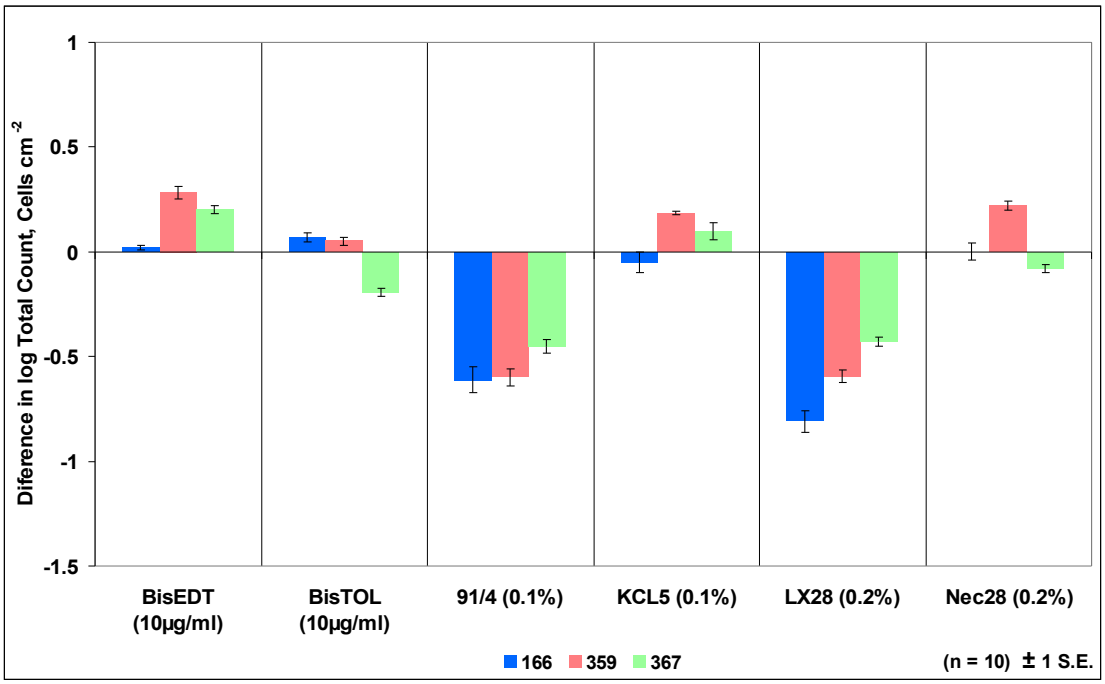

Fig. 4.7: Mean total count after 20 minute contact time with detergent treatments in WoSH for attached *L. monocytogenes* at 20°C, shown as log total count difference to a negative, water only control.

4.4.2 Effect of Disinfectant Only Treatments

Direct counts were obtained for each disinfectant treatment used against attached *L. monocytogenes* strains. Direct microscopic counting was used to determine if the TVC effects measured were removal and/or killing of bacteria.

The results shown in fig. 4.8 demonstrate that disinfectants do not effect removal of attached cells. The disinfectant treatment NaDCC against strain 359 was however significant ($P < 0.05$), with an increase in total count compared to the water only control.

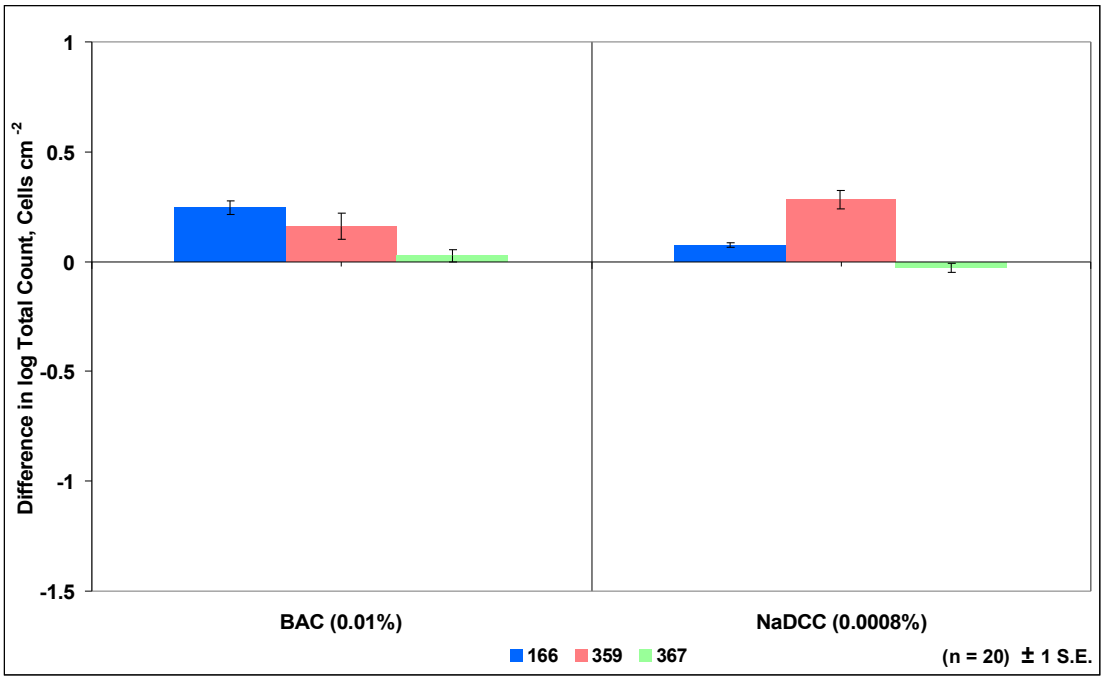

Fig. 4.8: Mean total count after 5 minute contact time with disinfectant in WoSH treatments for attached *L. monocytogenes* at 20°C, shown as log total count difference to a negative, water only control.

4.4.3 Effect of Detergent Followed by Disinfectant Treatments

Direct counts were obtained for each detergent, disinfectant treatment combination used against attached *L. monocytogenes* strains. Direct microscopic counting was used to determine if the TVC effects measured were removal and/or killing of bacteria.

The results presented in fig. 4.9 show that, although not significant for every treatment, in all but one case (LX28:367) each observed treatment resulted in more than the expected number of bacterial cells to remain adhered to the stainless steel surface. Nine of the treatments in fig. 4.9 showed significantly (2: $P < 0.01$, 7: $P < 0.05$) more cells than expected remained adhered to the surface.

With the bismuth thiol treatments, only BisTOL against 367 did not show a significant difference to the water only control. All of the other five bismuth thiol treatments were seen to have significantly ($P < 0.01$) more cells adhered to the surface than for the water only control.

The results for the non-ionic detergents show that observed 91/4 treatments did not significantly differ to the control for strains 359 and 367 but did show a small significant ($P < 0.05$) loss in total count against strain 166. KCL5 was seen to have significantly (166, 367: $P < 0.05$, 359: $P < 0.01$) more cells adhered to the surface than the water only control treatment.

The anionic detergent LX28 was observed to effect significantly ($P < 0.05$) lower total counts against strains 166 and 367 but no significant change in total count against strain 359. The second anionic detergent, Nec28, had significantly ($P < 0.05$) more cells compared to the water only control for strains 166 and 359 and no significant change in total count against strain 367.

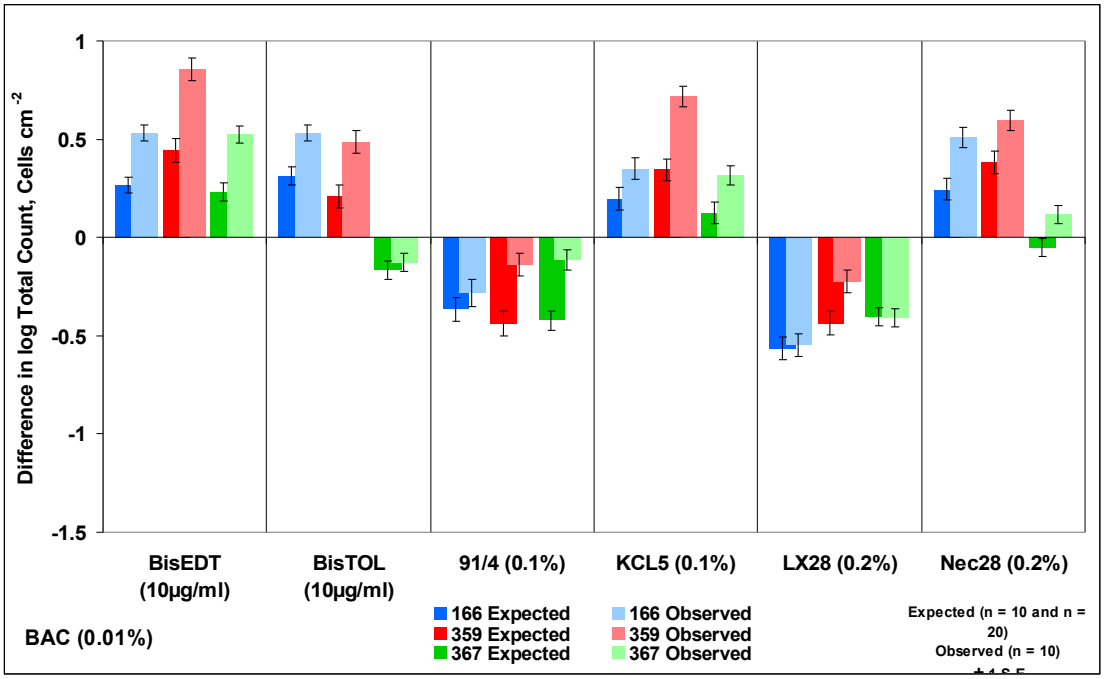

Fig. 4.9: Mean total count after 20 minute detergent treatment followed by 5 minute BAC treatment in WoSH for attached *L. monocytogenes* at 20°C, shown as expected (fig. 4.7 + fig. 4.8) and observed log total count difference to a negative, water only control.

The results for the disinfectant NaDCC are presented in fig. 4.10. Eight of the treatments showed no significant changes in total count to the water only control. The detergent treatment, 91/4, was the only treatment to differ significantly ($P < 0.01$) from the expected counts, with each treatment having fewer than expected cells adhered to the surface. Strain 359 was seen to have the only significantly ($P < 0.01$, BisTOL: $P < 0.05$) increased total counts in comparison to the water only control when treated with BisEDT, BisTOL, KCL5 and Nec28.

The results for the bismuth thiol detergents showed no significant change in total count against strains 166 and 367. Against strain 359 treatment with BisEDT ($P < 0.01$) or BisTOL ($P < 0.05$) were seen to cause significantly more cells to remain adhered to the surface than the control.

The results for the non-ionic detergent 91/4 show that against all 3 strains of *L. monocytogenes* a significantly ($P < 0.01$) reduced total count was observed. The

second non-ionic detergent, KCL5, demonstrated no significant change in total count against strains 166 and 367 and a significant ($P < 0.01$) increase in total count compared to the control against strain 359.

For the anionic detergent LX28, significant reductions in total count were shown against strains 166 ($P < 0.01$) and 367 ($P < 0.05$), while against strain 359 no significant change in total count occurred. Results for the second anionic detergent, Nec28, showed a significant ($P < 0.01$) increase in total count compared to the control for strain 359 and no significant changes in total count against strains 166 and 367.

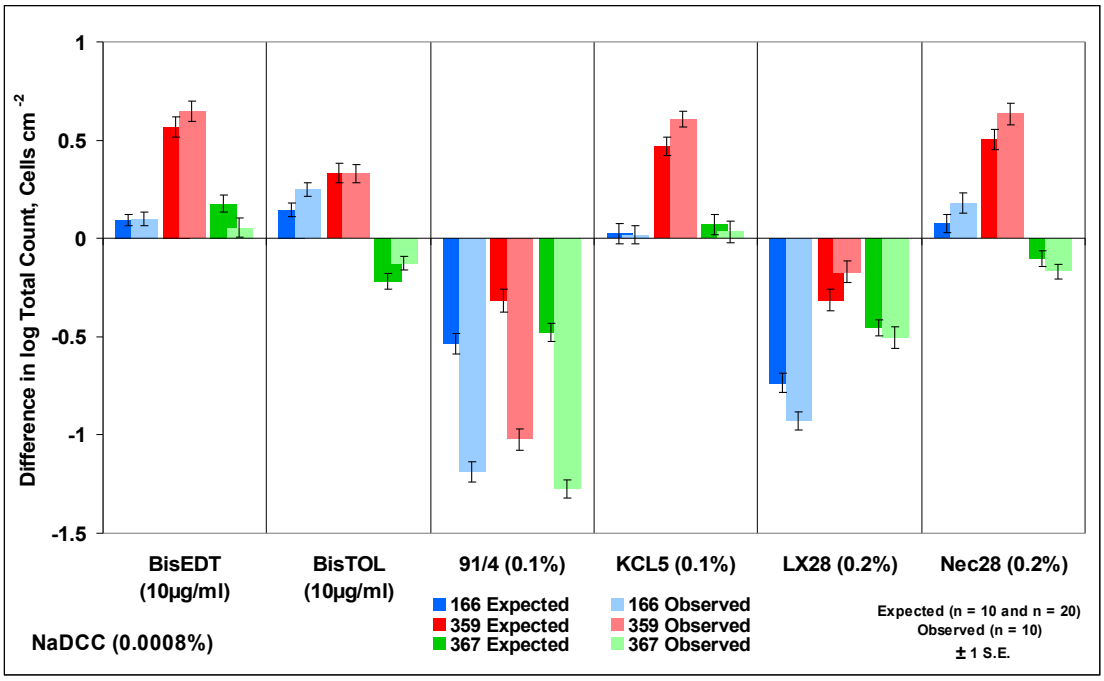

Fig. 4.10: Mean total count after 20 minute detergent treatment followed by 5 minute NaDCC treatment in WoSH for attached *L. monocytogenes* at 20°C, shown as expected (fig. 4.7 + fig. 4.8) and observed log total count difference to a negative, water only control.

4.4.4 Summary of Treatments for One Hour Attached Populations

The data in fig. 4.11 summarises the observed antagonisms and synergies seen for direct count data. Generally, exposing attached cells of *L. monocytogenes* to any detergents prior to treatment with either disinfectant was seen to result in antagonistic effects, resulting in more cells remaining adhered to the surface than expected. The

one notable exception was treatment with 91/4 followed by NaDCC which was seen to significantly (P <0.01) reduce the total cell count remaining on the coupon for all 3 strains.

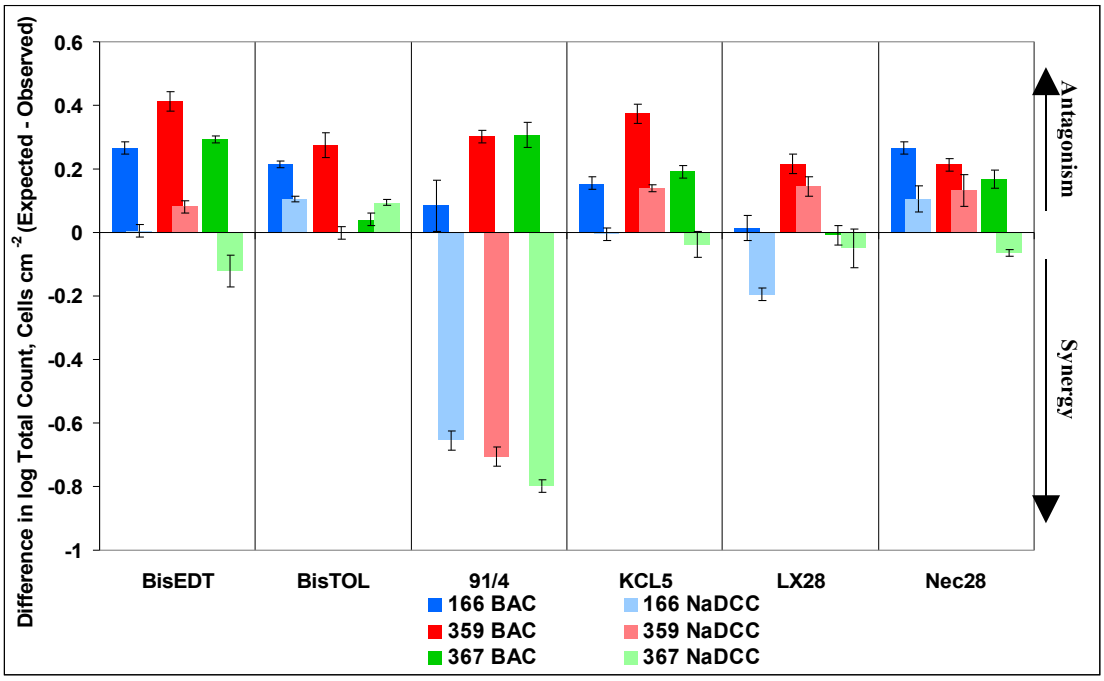

Fig. 4.11: Summary of the effectiveness on attached *L. monocytogenes* of the combined detergent/disinfectant treatments compiled by comparing the difference in total count to the sum of the single detergent and disinfectant treatments, shown as difference in log total count.

4.5 24 Hour Biofilm Populations – Total Viable Counts

4.5.1 Effect of Detergent Only Treatments

The effect of industrially recommended, in use, concentrations of detergents was determined on 24 hour biofilm populations of *L. monocytogenes* strains 166, 359 and 367. This was used to determine the degree of killing and/or removal of the adherent population following treatment. Detergent activity was compared to a negative control (zero on the x-axis) in which detergent solutions were substituted with water of standard hardness.

The results (fig. 4.12) show that both bismuth thiols (BisEDT and BisTOL) demonstrated a statistically higher than expected viable count for strain 359 ($P < 0.01$). The non-ionic detergents (KCL5 and 91/4) did not show any statistical differences to the control. The anionic detergent LX28 demonstrated a significant ($P < 0.01$) reduction in viable count against strains 166 and 367 but not against strain 359, whereas the anionic detergent Nec28, although not statistically significant, can be seen to slightly reduce the viable count of all 3 strains of *Listeria*. For most treatments, strain differences were shown when comparing 359 with 166 and 367, whereby strain 359 had a generally higher viable count.

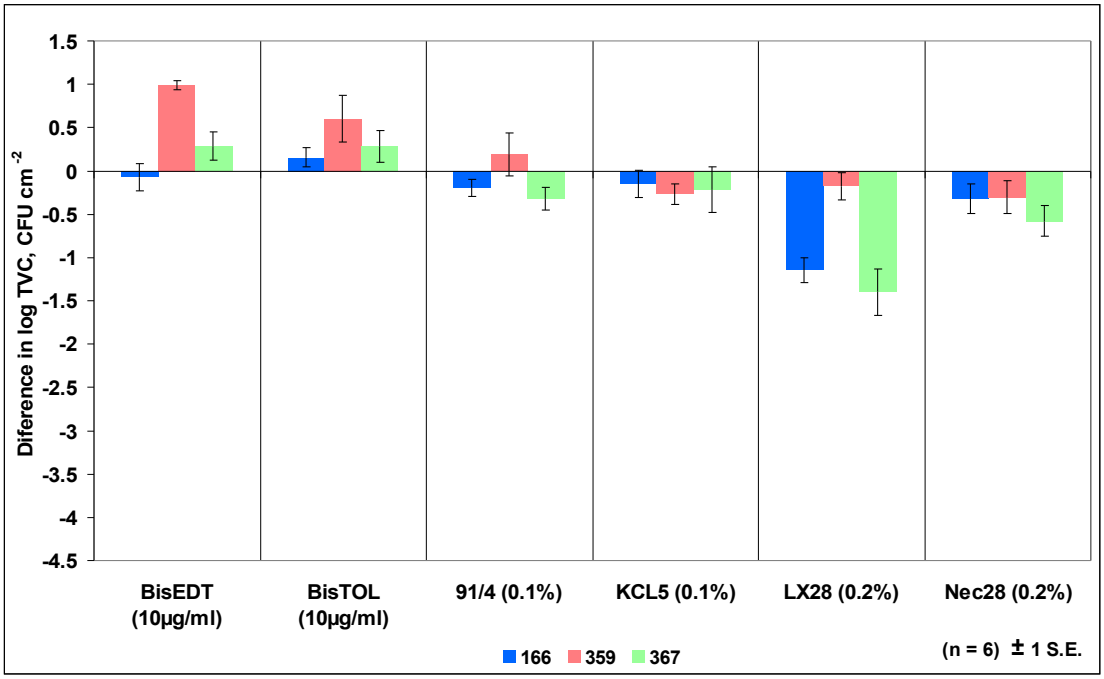

Fig. 4.12: Mean loss of viability after 20 minute contact time with detergent treatments in WoSH for biofilm *L. monocytogenes* at 20°C, shown as log TVC difference to a negative, water only control.

4.5.2 Effect of Disinfectant Only Treatments

The effect of disinfectants, at concentrations of 0.01% for BAC and 0.0008% for NaDCC, previously identified to give 2 – 4 log kill against *L. monocytogenes* in suspension, was determined against biofilm populations of strains 166, 359 and 367 to measure the biocidal effects against biofilm populations. Disinfectant activity was compared to a negative control (zero on the x-axis) in which disinfectant solutions were substituted with WoSH. The industry recommended contact time of 5 minutes was used.

The results (fig. 4.13) show that statistically significant differences were observed in disinfectant sensitivity between strains. Strain 166 was shown to be statistically more sensitive to BAC ($P < 0.05$) when compared to strains 359 and 367. Strain 359 was seen to be significantly less sensitive ($P < 0.05$) to NaDCC when compared to strains 166 and 367.

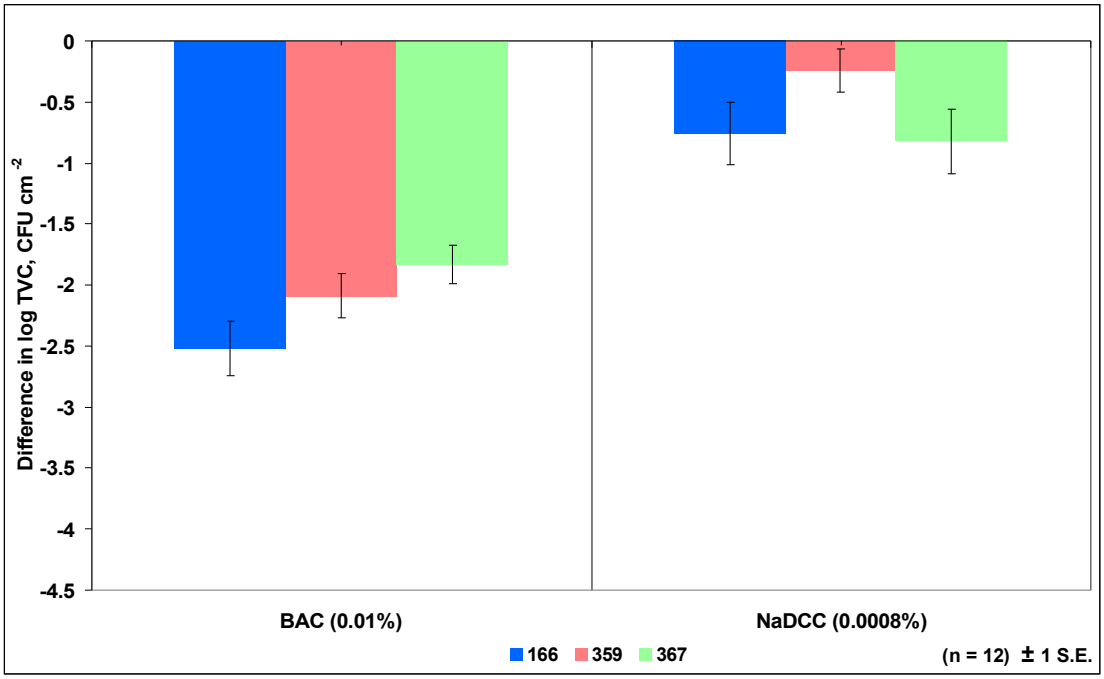

Fig. 4.13: Mean loss of viability after 5 minute contact time with disinfectant treatments in WoSH for biofilm *L. monocytogenes* at 20°C, shown as log TVC difference to a negative, water only control.

4.5.3 Effect of Detergent Followed by Disinfectant Treatments

The effect of combined detergent (industry recommended, in use, concentrations) and disinfectants, at concentrations of 0.01% for BAC and 0.0008% for NaDCC, previously identified to give 2 – 4 log kill against *L. monocytogenes* in suspension, was determined against biofilm populations of strains 166, 359 and 367. Treatment activity was compared to a negative control (zero on the x-axis) in which detergent and disinfectant solutions were substituted with WoSH. The industry recommended contact times of 20 minutes for detergents and 5 minutes for disinfectants was used. Observed results were compared to expected results, where the expected results were calculated by combining the changes in TVC for individual detergent (fig. 4.12) and disinfectant (fig. 4.13) treatments to determine any interaction between detergent and disinfectant treatments.

The results in fig. 4.14 and table 4.3 show a wide range of synergies and antagonisms between treatments and strains with 8 significant (7: $P < 0.01$, 1: $P < 0.05$)

synergies and 4 significant ($P < 0.05$) antagonisms being observed. In general treatments used against strain 367 were seen to result in synergy while those against strains 166 and 359 resulted in treatments with no effect, some synergy and some antagonism.

Both bismuth thiol detergents showed a significant ($P < 0.01$) antagonism with BAC when used against strain 359, while against strain 367 both showed a significant ($P < 0.01$) synergy. Against strain 166 BisTOL exhibited a significant ($P < 0.01$) synergy while BisEDT showed no significant changes.

When using the non-ionic detergent 91/4, significant ($P < 0.01$, 367: $P < 0.05$) synergies with BAC were seen against all 3 strains of *L. monocytogenes*. No significant differences were observed for treatments with the second non-ionic detergent KCL5.

The anionic detergent LX28 showed a significant ($P < 0.01$) antagonism when used against strain 166 while no significant differences were observed against strains 359 and 367. Nec28 also showed a significant ($P < 0.01$) antagonism when used against strain 166 whereas when used against strain 359 and 367, significant ($P < 0.01$) synergies were observed.

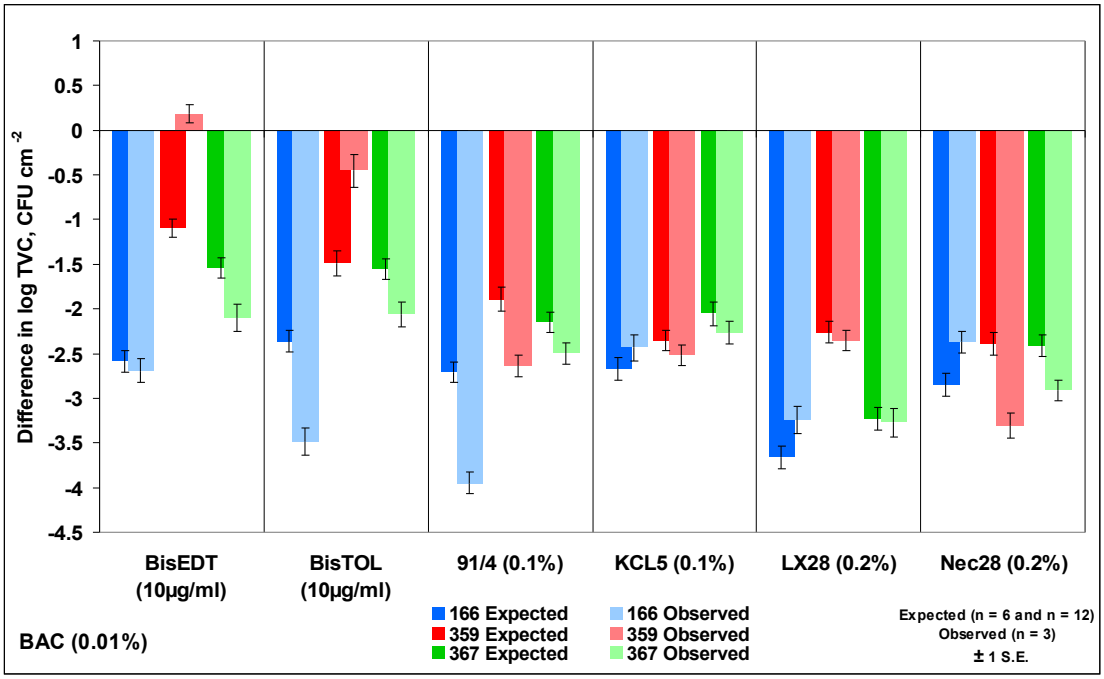

Fig. 4.14: Mean loss of viability after 20 minute detergent treatment followed by 5 minute BAC treatment in WoSH for biofilm *L. monocytogenes* at 20°C, shown as expected (fig. 4.12 + fig. 4.13) and observed log TVC difference to a negative, water only control.

BAC	166	359	367
BisEDT	0.102	**-1.281**	**0.553**
BisTOL	**1.122**	**-1.035**	**0.510**
91/4	**1.238**	**0.744**	0.346
KCL5	-0.236	0.161	0.212
LX28	**-0.421**	0.090	0.042
Nec28	**-0.474**	**0.917**	**0.503**

Table 4.3: Synergies and antagonisms after 20 minute detergent treatment followed by 5 minute BAC treatment in WoSH for biofilm *L. monocytogenes* at 20°C. Differences to the expected additive results are presented as log TVC with significant synergies (P <0.05), **(P <0.01)** and antagonisms (P <0.05), **(P <0.01)**.

Very few antagonisms were observed between detergent treatments and NaDCC as shown in fig. 4.15 and table 4.4; 2 significant antagonisms compared to 13 significant synergies. With the exception of BisEDT against strain 166, treatments against strain 359 were seen to be more synergistic than when used against strains 166 and 367.

The two bismuth thiol treatments used against strain 367 were seen to exhibit significant ($P < 0.01$) antagonistic effects. Against strain 166, treatment with BisEDT resulted in a strong, significant ($P < 0.01$) synergy, whereas against strain 359 no significant interaction was observed. Treatment with the second bismuth thiol, BisTOL, was not seen to result in a significant interaction against strain 166 whereas against strain 359 a strong, significant ($P < 0.01$) synergy was observed.

Of the two non-ionic detergents, 91/4 was seen to have a significant ($P < 0.01$, 166: $P < 0.05$) synergy with NaDCC against all 3 strains of *Listeria*. Significant synergies with NaDCC for KCL5 were seen against strains 359 ($P < 0.01$) and 166 ($P < 0.05$) but no effect was observed against strain 367.

Both of the anionic detergents, LX28 and Nec28, exhibited significant synergies ($P < 0.01$) with NaDCC against all 3 strains of *L. monocytogenes*.

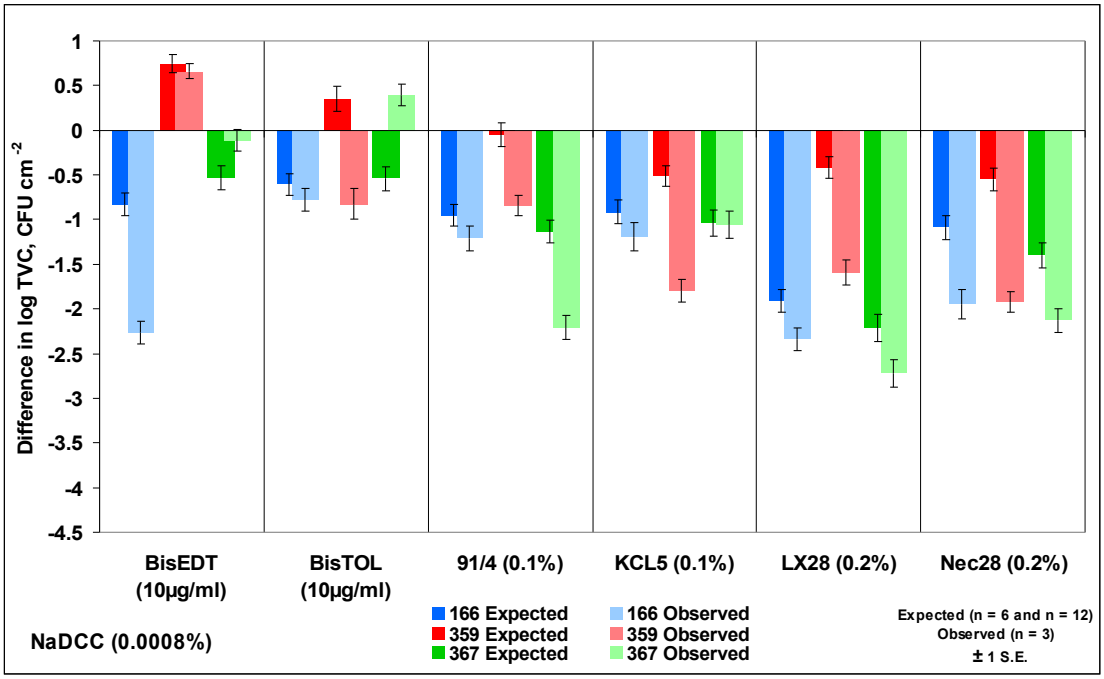

Fig. 4.15: Mean loss of viability after 20 minute detergent treatment followed by 5 minute NaDCC treatment in WoSH for biofilm *L. monocytogenes* at 20°C, shown as expected (fig. 4.12 + fig. 4.13) and observed log TVC difference to negative, water only control.

NaDCC	166	359	367
BisEDT	**1.431**	0.088	**-0.421**
BisTOL	0.176	**1.177**	**-0.934**
91/4	0.255	**0.787**	**1.070**
KCL5	0.281	**1.286**	0.023
LX28	**0.432**	**1.171**	**0.506**
Nec28	**0.858**	**1.372**	**0.732**

Table 4.4: Synergies and antagonisms after 20 minute detergent treatment followed by 5 minute NaDCC treatment in WoSH for biofilm *L. monocytogenes* at 20°C. Differences to the expected additive results are presented as log TVC with significant synergies (P <0.05), **(P <0.01)** and antagonisms (P <0.05), **(P <0.01)**.

4.5.4 Summary of Treatments for 24 Hour Biofilm Populations

The summary in fig. 4.16 shows that in general, exposing biofilm populations of *L. monocytogenes* to a detergent treatment prior to using either BAC or NaDCC results in a synergistic effect. There were very few observed antagonistic effects, fig. 4.15 showed these occurred when using either bismuth thiol with BAC against strain

359 or with NaDCC against strain 367, weak antagonisms were also seen when strain 166 was treated with KCL5, LX28 and Nec28 with BAC.

The only detergent treatment to show entirely synergistic effects with both BAC and NaDCC was 91/4. With the exception of Nec28 with BAC against strain 166, all of the Nec28 detergent treatments were observed to be significantly ($P < 0.01$) synergistic with both disinfectants.

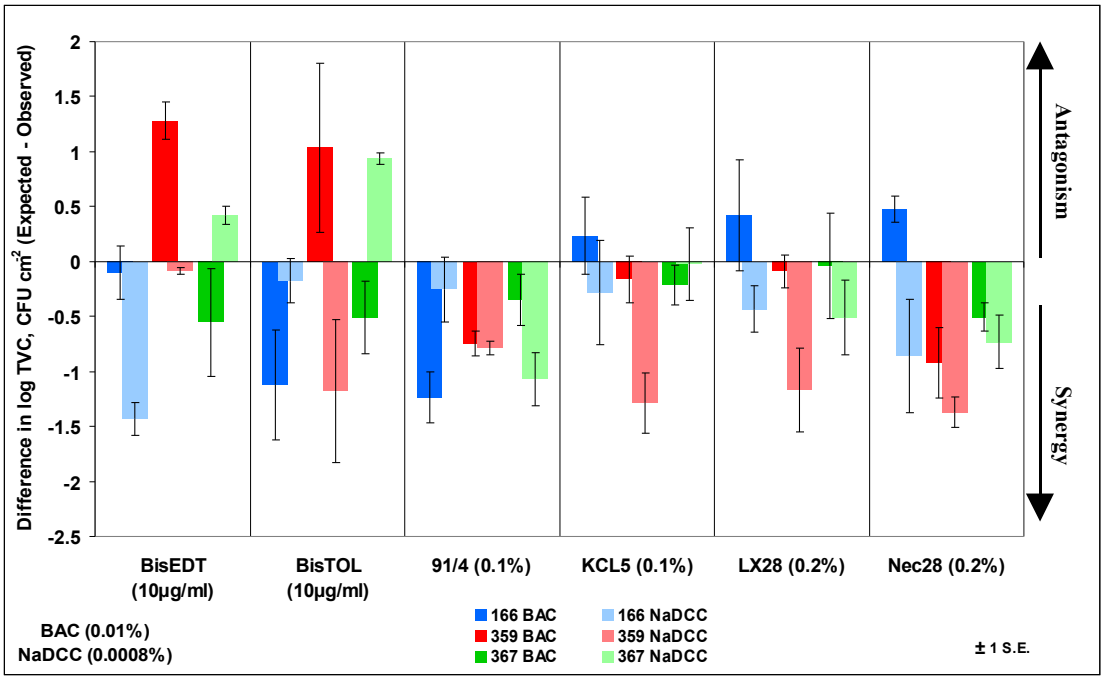

Fig. 4.16: Summary of the effectiveness on biofilm *L. monocytogenes* of the combined detergent/disinfectant treatments compiled by comparing the difference in viability to the sum of the single detergent and disinfectant treatments, shown as difference in log TVC.

4.6 24 Hour Biofilm – Direct EPS Quantification

Direct total counts for biofilms of *L. monocytogenes* were not obtainable due to the dense layering of cells and extra cellular polysaccharide on the coupons. Although measurable, the intensity of cellular material, stained with acridine orange, did not significantly change following treatments, whereas measurements of the intensity of EPS, stained with calcofluor white, were significantly different following treatments.

The density of EPS was assessed via calcofluor staining, to determine if detergent and/or disinfectant treatments caused disruption of the polymeric matrix. Plate 4.2 shows typical slides of each *L. monocytogenes* strain after forming biofilms on stainless steel coupons over 24 hours and stained with calcofluor white. Image intensity of treated populations was recorded to infer the density of EPS using Spot® capture software and ImagePro® analysis software as described in chapter 2. Uneven distribution of EPS was observed for all 3 strains, although this was seen to vary with strain 359 demonstrating the brightest clumping of EPS, indicative of the densest EPS. The brighter areas seen on the plates are denser regions of EPS which when dual stained with acridine orange can also be seen to be the densest regions of cellular material, the darker areas are less dense regions of cells and EPS.

Additional, untreated controls were measured as a baseline to determine the effects of each treatment, including the WoSH controls. EPS was measured using arbitrary brightness values from 1 (black) to 200 (white) for each pixel (4.1×10^6) per image allowing a single mean per image to be calculated.

Strains 166 and 367 appeared, visually, to be most similar, with a smooth layer of EPS, whereas strain 359 consistently produced denser regions of EPS that appeared much brighter than the EPS seen for strain 166 and 367. Statistically strain 359

always produced significantly ($P < 0.01$) brighter EPS than strains 166 or 367 with a mean intensity of 158.4 ± 4.1, indicative of a denser EPS layer, additionally strain 166 was seen to produce significantly ($P < 0.05$) brighter EPS than strain 367 with a mean intensity of 149.3 ± 2.4 compared to a mean intensity of 129.2 ± 7.8.

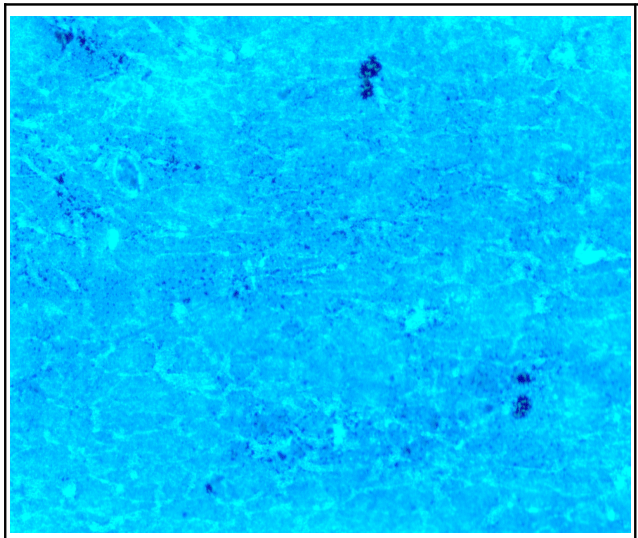	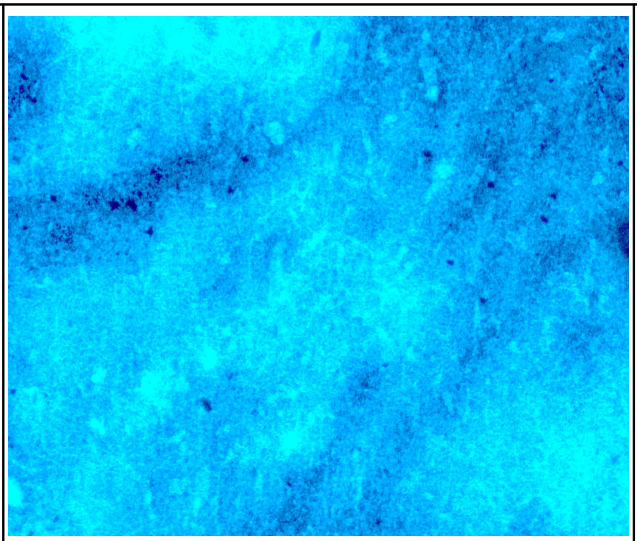	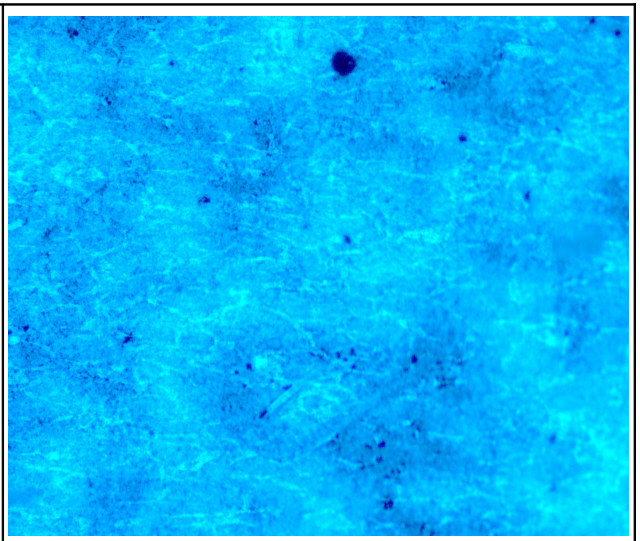
Strain 166 biofilm, n = 6, mean 149.3 intensity, S.E. 2.4	Strain 359 biofilm, n = 6, mean 158.4 intensity, S.E. 4.1	Strain 367 biofilm, n = 6, mean 129.2 intensity, S.E. 7.8

Plate 4.2: Typical 24 hour biofilm in 10% TSB for *L. monocytogenes* strains at 20°C on type 316 with 2B finish stainless steel coupons stained with calcofluor white at x 1000 magnification.

4.6.1 Strain 166 - EPS Intensity after Treatments

As seen in fig. 4.17 the negative, water only control treatment (no detergent/no disinfectant) was observed to have significantly ($P < 0.01$) lower EPS intensity compared to the untreated control (black line) which showed that WoSH alone was sufficient to solubilise some of the EPS. The two disinfectant only treatments significantly ($P < 0.01$) reduced the EPS intensity compared to the water only control by 53.44 for BAC and 37.91 for NaDCC.

The results for the bismuth thiol treatments in fig. 4.17 show there to be no significant differences between BisEDT and the no detergent control. For BisTOL significantly ($P < 0.05$) higher intensities were recorded for the detergent on its own and combined with NaDCC compared to the negative control. No difference to the negative control was identified for BisTOL with BAC.

Of the two non-ionic detergents, 91/4 was shown to significantly ($P < 0.01$) reduce the image intensity substantially when compared to the negative control with and without both disinfectants. The second non-ionic detergent, KCL5 showed no significant difference on its own to the negative control, although significant reductions in image intensity were observed when combined with BAC ($P < 0.05$) and NaDCC ($P < 0.01$).

Both of the anionic detergent treatments caused a significant ([LX28, LX28:NaDCC, Nec28:NaDCC]: $P < 0.01$, [LX28:BAC, Nec28, Nec28:BAC]: $P < 0.05$) reduction in image intensity compared to the negative control with and without both disinfectants.

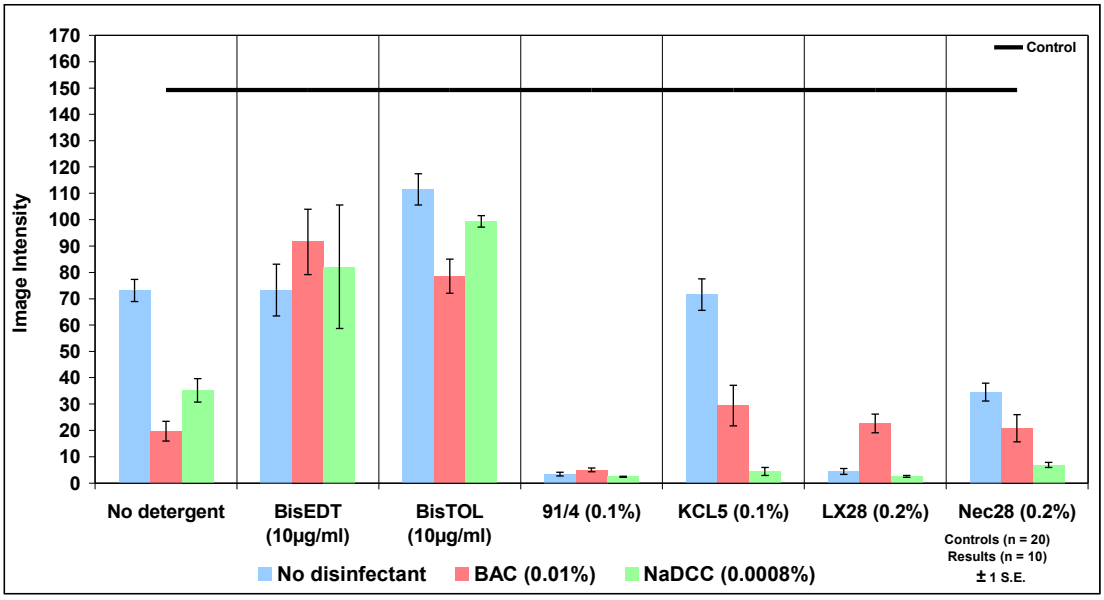

Fig. 4.17: Effect on the density of extra cellular polysaccharide for biofilm *L. monocytogenes* strain 166, for all detergent and disinfectant regimes in WoSH at 20°C, measured as image intensity. The control baseline is an untreated 24 hour biofilm population with no treatments other than gentle washing to remove suspended cells, presented as the lower 5% confidence limits.

4.6.2 Strain 359 - EPS Intensity after Treatments

The negative control, water only treatment, at 39.23, in fig. 4.18 was shown to have significantly ($P < 0.01$) reduced EPS intensity, 119.2 points lower, compared to the untreated control (black line) which showed that WoSH alone was sufficient to solubilise some of the EPS. The disinfectant only treatments were significantly ($P < 0.01$) lower in intensity than the negative control, 1.78 points for BAC and 3.51 points for NaDCC.

When treated with BisEDT on its own, the image intensity was not significantly different to the untreated control and was significantly ($P < 0.01$) more intense than the negative, water only control. When combined with BAC a small significant ($P < 0.05$) reduction in EPS intensity compared to the negative control was observed. When combined with NaDCC there a significant ($P < 0.01$) loss in EPS

intensity compared to BisEDT on its own, although the image intensity was still significantly ($P < 0.01$) higher than for the water only negative control.

BisTOL treatment on its own was not significantly different to the negative control. Combination treatments with either BAC or NaDCC resulted in a significant ($P < 0.01$) reduction in image intensity compared to the negative control.

Both non-ionic detergents were observed to have a significantly ($P < 0.01$, KCL5 and BAC: $P < 0.05$) lower image intensity with and without either disinfectant when compared to the negative control.

The image intensity for the anionic detergent LX28 on its own and also combined with NaDCC was significantly ($P < 0.01$) lower than the negative control, no significant difference was seen when combined with BAC. The second anionic detergent, Nec28 was significantly ($P < 0.01$) lower than the negative control with and without either disinfectant.

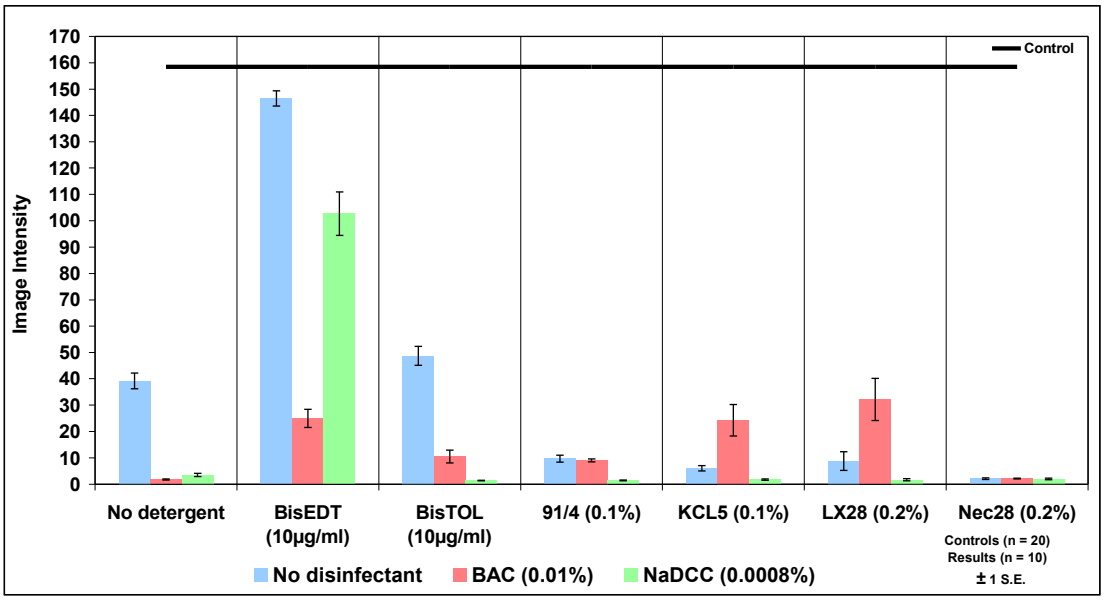

Fig. 4.18: Effect on the density of extra cellular polysaccharide for biofilm *L. monocytogenes* strain 359, for all detergent and disinfectant regimes in WoSH at 20°C, measured as image intensity. The control baseline is an untreated 24 hour biofilm population with no treatments other than gentle washing to remove suspended cells, presented as the lower 5% confidence limits.

4.6.3 Strain 367 - EPS Intensity after Treatments

The data (fig. 4.19) showed that a significant ($P < 0.01$) reduction in image intensity (58.2 lower) was seen when comparing the negative, water only control to the untreated control (black line) showing that WoSH only treatments are able to solubilise some of the EPS. Both of the disinfectant only treatments were significantly ($P < 0.01$) lower in image intensity than the negative control.

The detergent BisEDT showed a small significant ($P < 0.05$) rise in image intensity compared to the negative control on its own, a significant ($P < 0.01$) loss in image intensity when combined with BAC and no significant difference when combined with NaDCC. The second bismuth thiol, BisTOL, had a small significant ($P < 0.05$) rise in image intensity compared to the negative control on its own and with either disinfectant, BAC or NaDCC, the image intensity was significantly ($P < 0.01$) higher than the negative control but not significantly different to the untreated control.

The results in fig. 4.19 for the non-ionic detergents show that significant ($P < 0.01$) reductions in image intensity were observed for 91/4 with and with out both treatments. For KCL5 the image intensity of the detergent on its own and with BAC was significantly ($P < 0.01$) lower than the negative control, while no significant difference was observed when combined with NaDCC.

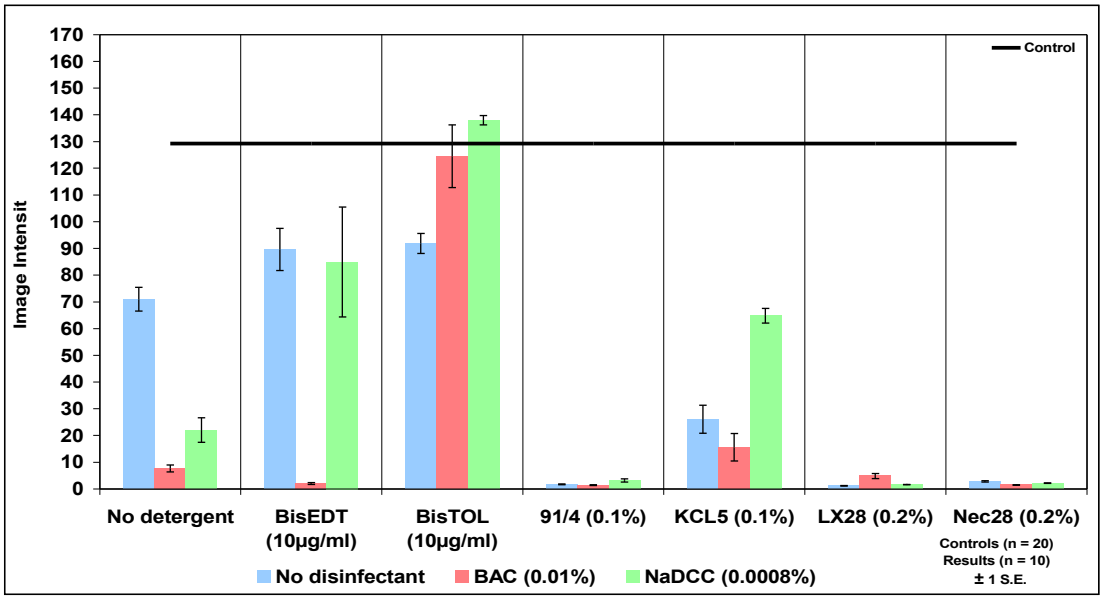

Fig. 4.19: Effect on the density of extra cellular polysaccharide for biofilm *L. monocytogenes* strain 367, for all detergent and disinfectant regimes in WoSH at 20°C, measured as image intensity. The control baseline is an untreated 24 hour biofilm population with no treatments other than gentle washing to remove suspended cells, presented as the lower 5% confidence limits.

4.7 Comparison of Total Viable Counts for Biofilms against Attached Populations

The effectiveness of each treatment, detergent only, disinfectant only and detergent followed by disinfectant, was compared between attached and biofilm populations of *L. monocytogenes*. On the following graphs a value above zero shows the treatment to be more effective against attached than biofilm populations while a value below zero shows the treatment was more effective against biofilms than attached populations.

4.7.1 Effectiveness of Detergent Only Treatments

As seen in fig. 4.20, 2 detergent treatments significantly ($P < 0.05$) reduced the TVC of a biofilm population (BisEDT:166 and Nec28:367) more than the respective attached population. All 16 remaining treatments are seen to have been more

effective against attached populations than biofilms, with 12 significant ($P < 0.05$) differences.

The results for most treatments against strains 166 and 367 are not significantly different to each other; the only exceptions are the results for BisEDT and Nec28. The non-ionic detergents were the only treatments where all 4 results are significantly ($P < 0.05$) more effective against attached cells than biofilms for strains 166 and 367.

Strain 359 was shown to be significantly ($P < 0.05$) different to both strains 166 and 367 for every detergent treatment. All of the treatments used against strain 359 were significantly ($P < 0.01$) more effective against attached populations than against biofilms. The anionic detergent LX28 showed the largest difference, whereby the loss in TVC for an attached population of strain 166 was 1.35 log more than for biofilms of strain 166.

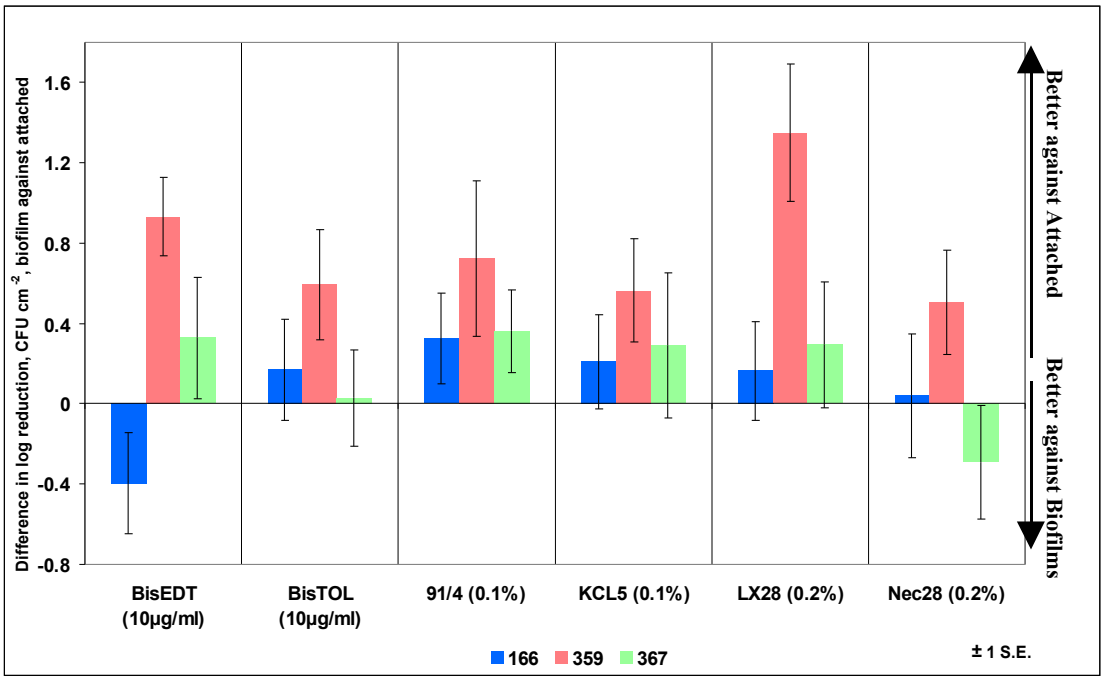

Fig. 4.20: Difference in the effectiveness of detergent treatments against biofilms compared to attached *L. monocytogenes*, shown as difference in log reductions of TVC.

4.7.2 Effectiveness of Disinfectant Only Treatments

The differences in the loss of TVC between biofilms and attached populations can be seen in fig. 4.21. The results for the disinfectant BAC, show that for all three strains of *L. monocytogenes* the loss in TVC was significantly ($P < 0.01$) lower as biofilms than as attached cells, 1.28 log – 1.55 log lower. Against strains 166 and 267, the disinfectant NaDCC shows there were no significant differences between biofilms and attached populations. For strain 359 the loss in TVC caused by NaDCC against biofilms was significantly ($P < 0.01$) lower than that for attached populations, by 1.59 log.

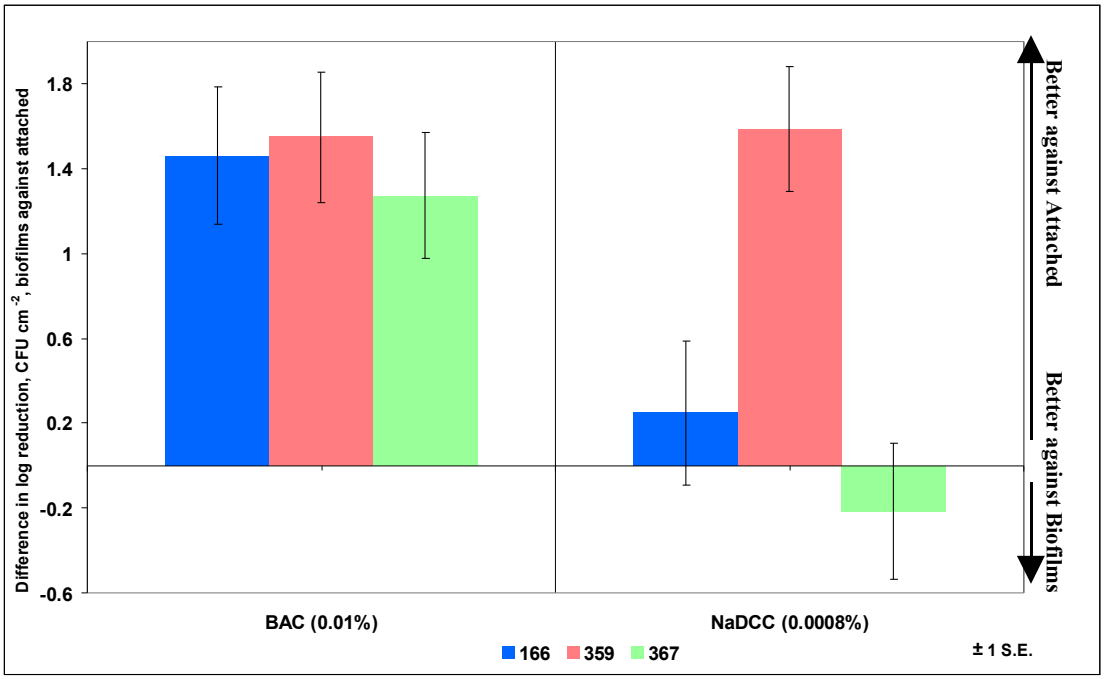

Fig. 4.21: Difference in the effectiveness of disinfectant treatments against biofilms compared to attached *L. monocytogenes*, shown as difference in log reduction of TVC.

4.7.3 Effectiveness of Detergent Followed by Disinfectant Treatments

In fig. 4.22 there are seven significant ($P < 0.05$) instances where the combined detergent/disinfectant treatments were more synergistic against attached populations than against the respective biofilms. There are twenty two significant ($P < 0.05$) examples shown where the combined detergent/disinfectant treatments were more

synergistic against biofilms than against the respective attached populations. This suggests a trend to increasing synergy between detergents and disinfectants when used against biofilm compared to attached populations.

Fig. 4.22 shows that strain 359 was seen to be much more sensitive as an attached population than as a biofilm compared to strains 166 and 367. For most treatment combinations strains 166 and 367 were observed to have similar resistance as biofilm populations, KCL5:BAC and LX28:BAC combinations showed more resistance for strain 166 than 367 for biofilms, BisEDT:NaDCC showed biofilms of strain 166 to be more sensitive than attached populations and Nec28:BAC showed biofilms of strain 367 to did not gain more resistance as seen for strain 166.

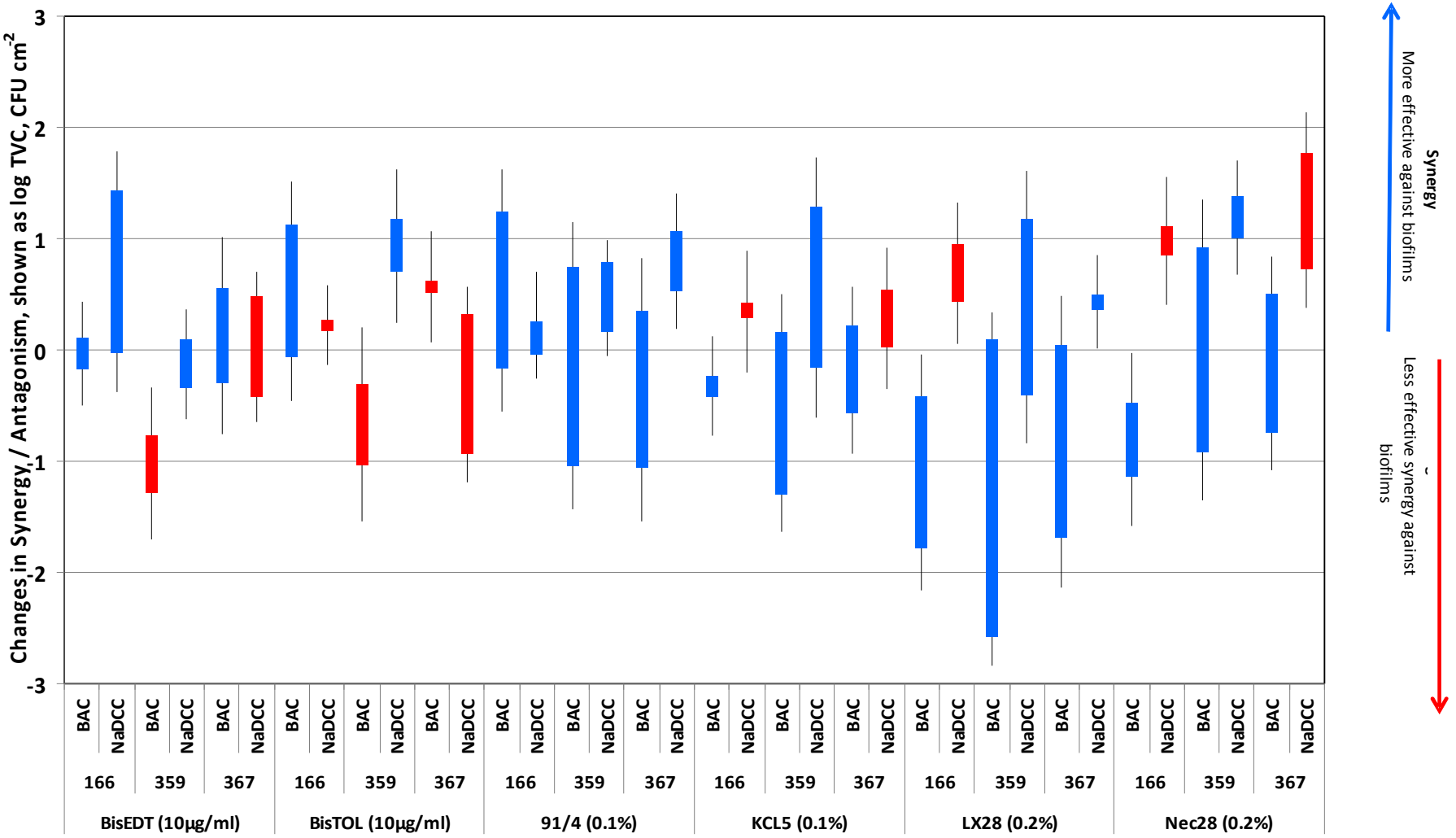

Fig. 4.22: Synergistic/antagonistic effects of combined detergent/disinfectant treatments for biofilm compared to attached *L. monocytogenes*, shown as difference in log TVC synergy also showing the change in synergistic/antagonistic effects of attached compared to biofilm populations. Blue bars show the increase in synergy for biofilms compared to attached while red bars show a decrease, for example strain 166, BisEDT, NaDCC attached synergy of zero increases to biofilm synergy of almost 1.5 log.

4.8 Discussion

The reduction in TVC demonstrated by the anionic detergent LX28 against attached cells of *L. monocytogenes* was expected due to previous observations with suspended populations. Anionic detergents, such as LX28, are known to exhibit some biocidal properties against Gram positive bacteria, whereby the cell membrane is disrupted, although at low concentrations the effect is typically negligible (Pasanen *et al*., 1997). The loss of viability shown for suspended cells during 20 minute contact time with LX28 varied from >4 log (strain 359) to >6 log (strains 166 and 367), whereas for one hour attached populations of *L. monocytogenes* a reduction in TVC from 1.4-1.7 log was observed and for biofilms the loss in TVC varied from 0-1.3 log. This comparison of the results for suspended, attached and biofilm cells shows that the biocidal activity of LX28 was considerably diminished against attached and biofilm populations of all three strains. A reduction in the activity of LX28 was especially seen when comparing biofilm to attached populations for strain 359 where no loss of TVC compared to the control treatment was observed as a biofilm.

The reductions in biocidal activity of LX28 could be attributed to a number of causes. The surface area of attached and biofilm populations as opposed to suspended populations exposed to a biocide will be significantly reduced (Prakash *et al.*, 2003), thus the uptake of biocide into the cells via membrane pores and channels or the damaging effect of biocides against cell membranes as a whole, will also be reduced. Further many biofilms comprise of cells within a thick coating of EPS which may decrease contact with LX28 compared to attached populations. Indeed plate 4.2 (typical biofilm appearance) showed that strain 359 exhibited the densest EPS layer, and it was also shown that LX28 had no biocidal

effect against biofilm populations of 359, yet still had a significant ($P < 0.01$) effect against strains 166 and 367. Therefore *L. monocytogenes* populations are likely to be less sensitive to a given concentration of LX28 when in a biofilm state than attached and less sensitive as an attached population than when suspended. It is also well known that once cells adhere to a surface there are rapid changes in metabolic activity and protein expression as adaptation to a adherent state progresses (Busscher *et al.*, 1995; Kumar and Anand, 1998; Dunne, 2002), these changes may impact the sensitivity of the strains to LX28 by either changing the uptake rate of the detergent, e.g. alteration of ion channels, by altering the active site of the detergent in the cell membrane or within the cells or via expression of stress response proteins e.g. GroEL or HSP60 (Donlan and Costerton, 2002; Augustin *et al.*, 2004; Matin and Lynch, 2005; Bianco *et al.,* 2006).

Against attached populations of cells, both of the non-ionic detergents (91/4 and KCL5) and the anionic detergents (LX28 and Nec28), were seen to show a general trend for a reduction in TVC against all 3 strains. This suggests these four detergents demonstrate some biocidal or removal activity against *L. monocytogenes* as an attached population. Research by Gross and Logan (1995) demonstrated that the non-ionic detergent Tween-20 reduced the viable count of surface attachment *Alcaligenes paradoxus* to glass beads via removal rather than killing. The reductions in TVC seen for attached populations treated with detergents when compared with total count data, indicates that a loss in viability occurred when treated with the non-ionic detergent KCL5 and the anionic detergent Nec28, whereas a removal effect was observed for the non-ionic detergent 91/4, while for the anionic detergent LX28 a mixture of removal and loss in viability was seen. Variations in the composition of the extra cellular

polysaccharide coat produced by each strain may account for observed strain differences, with strain 359 visually seen to produce a more intense EPS layer surrounding individual cells as an attached population. Specific measurements of 1 hour attached population EPS variation would be required to confirm this theory, although research by Korenevsky and Beveridge, (2007), showed that variation in capsule composition and thickness altered the adhesiveness of *Shewanella* strains. With the example of strain 359 this may be the result of increased EPS as the primary mechanism of attachment, resulting in an increase in sloughing of attached cells due to solubilisation of EPS when exposed to either KCL5 or Nec28, whereas strain 166 and 367 initial attachment may be stronger due to a greater dependency of additional surface structures such as pilli and flagella rather than EPS as the primary mechanisms of attachment as suggested by Busscher *et al*., (1995).

Strain differences were observed throughout the attached treatment regimes. The overall trend for each of the strains was that 166 was generally least desensitised, while 359, a food product isolate, was the most desensitised to disinfectant treatments after exposure to detergents. This general pattern was observed for all of the detergent treatments. The strain differences may be explained by differences in the rapidity of stress protein expression following exposure to a detergent (Matin and Lynch, 2005; Bianco *et al.,* 2006), whereby if strain 359 produces stress proteins more rapidly and/or at a higher expression rate than strains 166 and 367, then this strain would be likely to be more resistant to subsequent disinfectant treatments in suspension or as a 1 hour attached population. Conversely if strain 166 is slower at expressing stress response proteins and/or expresses at a lower expression rate then it is likely to be comparatively more sensitive to subsequent disinfectant treatments. Another factor that might influence the expression of

stress proteins is the ease with which detergents can come into contact with adhered cells, e.g. strain 359 produced a denser coating of EPS than strains 166 and 367, therefore detergent treatments may affect cellular protein expression more rapidly against biofilm populations of strain 166 and 367 than against strain 359 due to increased permeability through less dense EPS coatings, differences in permeability of capsules to chemicals based on density and composition has been demonstrated in *Klebsiella pneumoniae* as a key factor in rapidity of stress response (Campos *et al*., 2004).

Surprisingly, strain 359 was identified as a food product isolate rather than an environmental isolate, typically persistent factory isolated strains form the densest biofilms (Gibson *et al*., 1999), which suggests that although product isolated, strain 359 may have been a contaminant from a persistent biofilm within the factory. A study by Holah *et al.* (2004), confirmed most *Listeria* spp. detected within factories were environmentally niche oriented, therefore given the ability of 359 to rapidly form complex, highly resistant biofilms, as typified by environmental, rather than product isolates, it can be surmised that although isolated from a product, strain 359 is likely to have contaminated the product from an environmental source within the production line.

Treatment regimes against attached populations that used the anionic detergents, LX28 and Nec28 combined with BAC, were seen to exhibit strong antagonisms. Given that BAC is cationic, it is possible that sufficient quantities of the anionic LX28 and Nec28 persisted after rinsing to cause an inactivation of the disinfectant, although there are quenching agents available, e.g. lecithin, that could be used to inactivate the detergents, it was decided not to include these to more accurately represent factory treatment regimes. Results showed that individual LX28 and Nec28 treatments

resulted in a loss of TVC, given that anionic detergents are know to bind to and disrupt Gram-positive bacterial cell membranes (Pasanen *et al*., 1997), the likely site of inactivation of BAC would have been at the surface of the bacterial cells, where the anionic detergents were adsorbed and so not removed during rinsing and perhaps therefore not possible to quench either. This suggestion is further enhanced by the findings shown for biofilms where 2 significant ($P < 0.01$) synergies were observed with BAC, in this case the detergents were probably prevented from coming into contact with cell surfaces because of the dense EPS layer and so were fully removed upon rinsing, therefore little or no inactivation occurred.

The total count results for detergent/disinfectant interactions, where 14 treatments resulted in an increase in total count compared to the water only control are comparable to the results shown by Eginton *et al.,* (1998), where an increase in attachment strength was observed, although not explained, following treatment with Tween-80 on stainless steel for *Staph. epidermidis*. Only treatment with the non-ionic detergent, 91/4 plus the disinfectant NaDCC was seen to significantly ($P < 0.01$) reduce the total count for all three strains. Although a removal effect caused by 91/4 had been shown, while NaDCC did not cause a removal effect, a synergy must exist between the detergent and disinfectant treatments because the expected removal effect was significantly ($P < 0.05$) lower than that actually observed. One possible explanation is that the loss in total count was the result of a synergy resulting in enhanced lysis of the population, rather than removal, resulting in the appearance of fewer remaining cells on the surface after acridine orange staining.

The direct counts for detergent only treatments showed two interesting effects. The first effect was that both of the bismuth thiols resulted in an increased population total count compared to the control for strain 359, again this is most likely related to

the findings by Eginton *et al.,* (1998), as mentioned in the previous paragraph. The second effect was that while a loss in TVC was observed for strains 166 and 367 following LX28 treatment, strain 359 did not demonstrate any reduction in TVC as a biofilm population. The results in plate 4.2 showed that strain 359 produced the densest EPS layer compared to strains 166 and 367 and also demonstrated more visible clumping of cells and EPS, which probably prevented LX28 from coming into contact with the cells and so infers a mode of action of reduced sensitivity to chemical treatments due to a denser EPS layer as shown previously by Donlan and Costerton (2002) for clinically important biofilms. The disinfectant only results for NaDCC also suggest that the denser EPS layer produced by strain 359 is responsible for there being no significant loss of TVC after treatment, whereas both strains 166 and 367 showed a significant ($P < 0.01$) loss in TVC.

The summary for biofilm populations showed that compared to the summary for attached populations, there were no generalised trends of effects for all three strains. Overall, synergies were generally observed against biofilm populations, suggesting that physiological differences between 1 hour attached and 24 hour biofilm populations, e.g. the EPS layer surrounding the adherent cells, prevented desensitisation to disinfectants, perhaps by insufficient contact time with the detergent treatments to illicit expression of stress response proteins as shown against *E. coli* in chapter 5. Synergies may also result after detergent treatment due to disruption of the EPS layer, resulting in improved disinfectant contact with the population, indeed the data presented in fig. 4.17-19 (EPS intensity following treatments) suggests this to be the case for the detergents 91/4, LX28 and Nec28 which were observed to reduce the EPS intensity of all three strains typically to below a value of 10. In general, the synergies appearing in the summary coincided with reductions in EPS intensity, while

those treatments with no effect or antagonisms generally coincided with higher EPS intensities following treatments.

The comparison of treatments used against biofilms and against attached cells showed some very interesting effects. Firstly the results for the detergent only comparison showed that in general detergents affect 1 hour attached populations more strongly than 24 hour biofilm populations, physiological differences, e.g. dense EPS layer, protection by surrounding cells or surface adaptation, are likely causes of the differences. One of the most notable effects however, was that strain 359 was much less sensitive to all detergent only treatments as a biofilm, the most likely assessment of this finding can be attributed to the research conducted by Ryu and Beuchat (2005) which showed that strains of *E. coli* with increased EPS production are less sensitive to environmental stressors and have increased adhesive strength. Similar results were seen for the disinfectant NaDCC, whereby strain 359 was seen to be over 1.5 log less sensitive when a biofilm, while both strains 166 and 367 showed no statistical difference between attached and biofilm populations.

The comparison of treatment combination effects against attached and biofilm populations, showed that most treatment combinations were likely to results in a greater degree of synergy against biofilms than against one hour attached populations. This is most likely caused by some disruption of the complex EPS matrix by detergents that surround biofilm populations, indeed Prakash *et al.,* (2003) has suggested that the EPS matrix is the primary factor that increases resistance to a plethora of environmental stresses, therefore a disruption of this matrix is likely to enhance disinfectant or other biocide activity. Conversely to expectation, a comparison of the results showing the observed reduction in TVC for combined treatments against attached populations, with those showing the observed reduction in

TVC for combined treatments against biofilm populations, showed that although synergies were generally seen when biofilms were treated, the actual loss in viable count was still considerably lower than that seen against attached populations. The summation of this information is that although a disruption of the EPS occurred, the protective mechanisms associated with biofilms were still sufficient to prevent much of the loss in TVC associated with attached populations. This has implications for cleaning and disinfection regimes, whereby persistent biofilms, even when treated with a detergent and disinfectant that are known to work in synergy, are still likely to partially survive the treatments unless mechanical cleaning is used in addition to chemicals. Indeed, although synergies are more likely against a biofilm population than an attached population, the observed effects may be due to the failure of the detergent to illicit a protective effect within a biofilm population, possibly due to reduced concentrations of chemical passing through the complex structures found within biofilms. Therefore rather than a true synergy being observed, the actual effects in most cases are likely to be caused by the detergent desensitising the cells in attached populations, resulting in an antagonistic interaction with disinfectant, while against biofilm populations some EPS disruption occurs, although there is insufficient cellular contact time to illicit the same protective response observed for attached populations. This suggests that to optimise treatments against *L. monocytogenes* biofilm populations will require a highly optimised treatment regime, or ideally mechanical cleaning in addition to chemical, which in many situations is simply not practical. Jessen and Lammert, (2003), also highlighted the necessity for mechanical cleaning in meat processing plants after identifying biofilm populations that persisted following chemical sanitisation techniques and concluded that where possible mechanical modes of cleaning should be applied in addition to chemical methods.

5.0 Effects of Treatments on *Escherichia coli*

5.1 Introduction

Identifying synergistic relationships between specific detergents and disinfectants, with regards to the removal and killing of surface adhered, 1 hour attached and 24 hour biofilm populations of *E. coli*, will lead to the development of improved cleaning and disinfecting regimes within food processing environments. Primarily experiments were conducted on factory isolated strains, from product and environment, additional experimentation was undertaken to determine the effects of treatments upon vero toxin producing strains of *E. coli* to assess the similarity with the non pathogenic isolates.

All of the TVC results throughout this chapter have been standardised to use the experiments negative control as the zero value for the x-axis on the following graphs. Each graph represents experimentation conducted on multiple days, the standardised data was analysed using multifactorial regression analysis to normalise day to day variation, the inherent variability of adherent populations' means that no specific population density is stated for each experiment. A treatment value below zero shows a reduction in viable count while a value greater than zero shows an increase in viable count compared to the negative control, the latter does not represent proliferation of the population, merely a greater proportion of viable cells remaining adhered following the treatments compared to the water only negative control treatment.

As detailed in the Materials and Methods chapter, the term 'Attached' refers to a population of *E. coli* that has been allowed to adhere to an 8mm x 8mm stainless steel coupon over one hour. The term 'Biofilm' refers to a one hour attached population of *E. coli* that has then established a biofilm over 24 hours, as described in the Materials and Methods chapter.

5.2 Effect of Detergents on the Viability of Suspensions of *E. coli*

The effect of industrially recommended, in use, concentrations of detergents was determined on suspensions of *E. coli* strains 400, 453 and 467. Detergent activity was compared to a negative control (zero on the x-axis) in which detergent solutions were substituted with WoSH. Two detergent contact times were used, 20 minutes as recommended to the industry and 5 minutes to identify any rapid biocidal effects.

Significant loss of viability ($P < 0.05$) was identified for suspensions of *E. coli* treated with the bismuth thiol detergent BisTOL after 20 minutes contact time and against strain 453 when treated with BisEDT, after 5 and 20 minutes contact time. Strain 400 was observed to be significantly less sensitive ($P < 0.05$) to BisEDT than either strain 400 or 467 after both 5 and 20 minutes contact time.

A significant ($P < 0.01$) loss of viability was seen for the non-ionic detergent, 91/4, against strain 467, signifying a strain specific interaction, however no significant difference was seen between 5 and 20 minute contact time. No significant changes in TVC were observed for the second non-ionic detergent, KCL5, or for either anionic detergent.

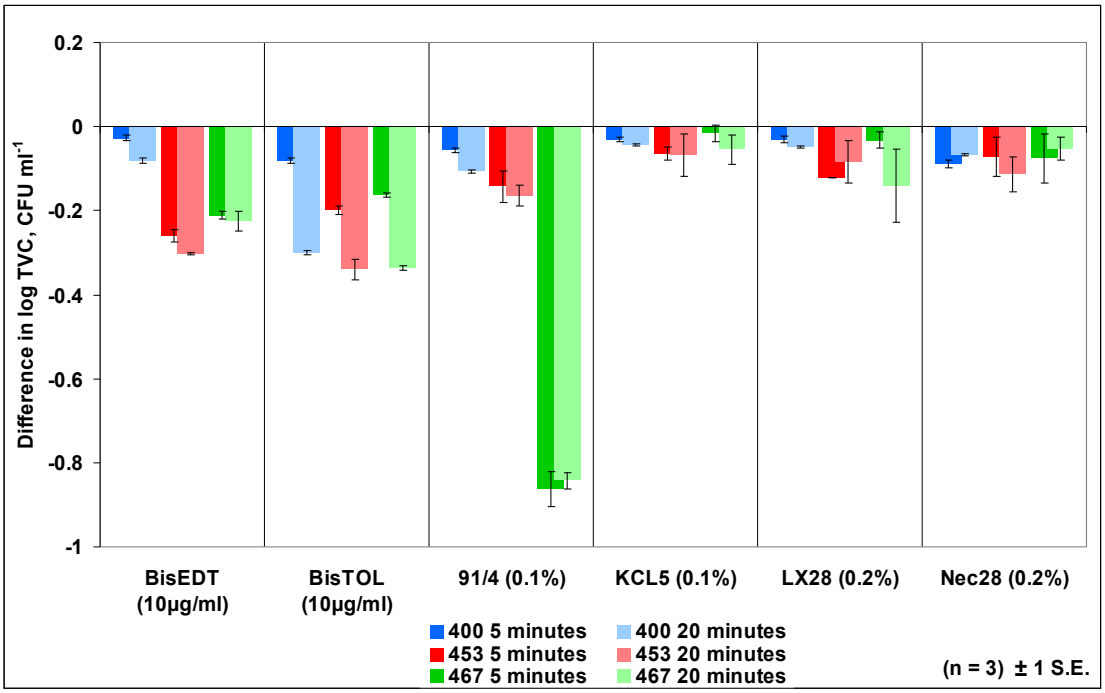

Fig. 5.1: Mean loss of viability after 5 and 20 minute contact times with detergent treatments for approximately 1 x 10^7 *E. coli* suspended in WoSH at 20°C, shown as log TVC difference to a negative, water only control.

5.3 One Hour Attached Populations – Total Viable Counts

5.3.1 Effect of Detergent Only Treatments

The effect of industrially recommended, in use, concentrations of detergents was determined on one hour attached populations of *E. coli* strains 400, 453 and 467. This was used to determine the degree of killing and/or removal of the adherent population following treatment. Detergent activity was compared to a negative control (zero on the x-axis) in which detergent solutions were substituted with WoSH.

The results (fig. 5.2) for BisEDT show a significant reduction ($P < 0.01$) in TVC to the control treatment for strains 400 and 467. A significant ($P < 0.05$) loss in TVC was seen for strain 453 when treated with BisTOL. The non-ionic detergent 91/4 significantly ($P < 0.01$) reduced the viable count of strains 400 and 467, while a significant ($P < 0.05$) increase in viable count compared to the control was seen against strain 453 when treated with KCL5. The anionic detergent LX28

significantly (P <0.05) reduced the viable count of strain 400, while Nec28 showed a significant (P <0.05) increase in TVC against strain 400 and a significant (P <0.05) reduction in TVC against strain 453 when compared to the control.

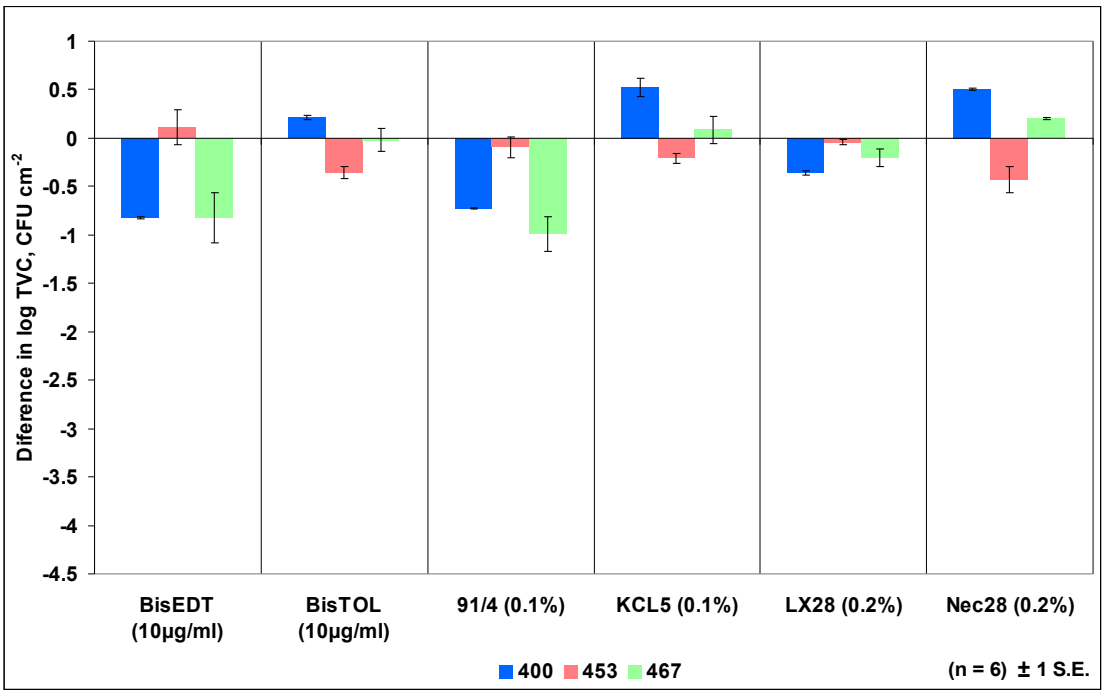

Fig. 5.2: loss of viability after 20 minute contact time with detergent treatments in WoSH for attached *E. coli* at 20°C, shown as log TVC difference to a negative, water only control.

5.3.2 Effect of Disinfectant Only Treatments

The effect of disinfectants, at concentrations of 0.01% for BAC and 0.0005% for NaDCC, previously identified to give 2 – 4 log kill against *E. coli* in suspension, was determined against one hour attached populations of strains 400, 453 and 467. Disinfectant activity was compared to a negative control (zero on the x-axis) in which disinfectant solutions were substituted with WoSH. The industry recommended contact time of 5 minutes was used. The results are presented in figure 5.3.

Strain 400 was shown to be significantly more sensitive to BAC (P <0.05) and NaDCC (P <0.01) compared to strains 453 and 467 to measure the biocidal effects against biofilm populations. No significant differences in sensitivity were observed

between strains 453 and 467. No significant differences were seen between BAC and NaDCC treatments, with the exception detailed above.

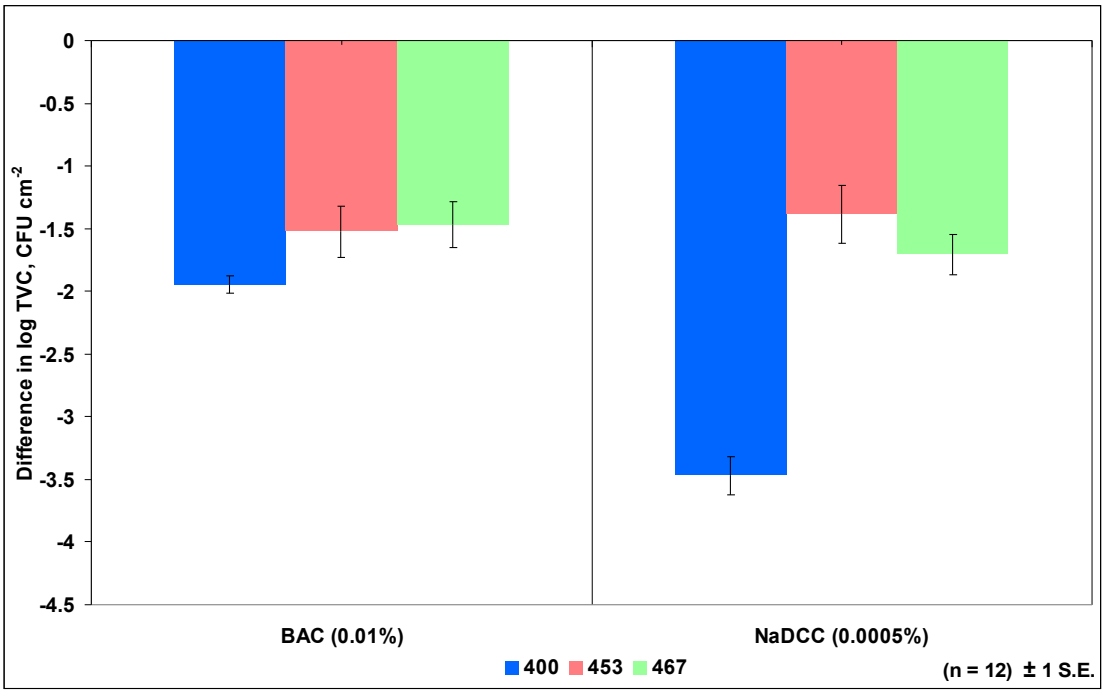

Fig. 5.3: Mean loss of viability after 5 minute contact time with disinfectant treatments in WoSH for attached *E. coli* at 20°C, shown as log TVC difference to a negative, water only control.

5.3.3 Effect of Detergent Followed by Disinfectant Treatments

The effect of detergent treatments (at industry recommended in use concentrations) followed by disinfectants, at concentrations of 0.01% for BAC and 0.0005% for NaDCC, previously identified to give 2 – 4 log kill against *E. coli* in suspension, was determined on one hour attached populations of *E. coli* strains 400, 453 and 467. Treatment activity was compared to a negative control (zero on the x-axis) in which detergent and disinfectant solutions were substituted with water of standard hardness. The industry recommended contact times of 20 minutes for detergents and 5 minutes for disinfectants was used. Observed results were compared to expected results, where the expected results were calculated by combining the changes in TVC for individual detergent (fig. 5.2) and disinfectant (fig. 5.3)

treatments to determine any interactions between detergent and disinfectant treatments.

The results (Fig. 5.4 and table 5.1) showed that significant differences occurred with strain and with treatment when using the disinfectant BAC. Against strain 400 significant ($P<0.01$) synergies were observed with the detergents BisEDT, BisTOL, KCL5 and Nec28, while significant ($P <0.01$) antagonisms were identified with the detergents 91/4 and LX28. For strain 453 only one significant ($P <0.05$) synergy was shown with BisEDT, while significant antagonisms were shown with the detergents BisTOL, KCL5 ($P <0.05$) and Nec28 ($P <0.01$). No synergies were observed against strain 467, but significant ($P <0.01$) antagonisms were shown for 91/4, BisEDT and Nec28.

E. coli strain 400 was observed to be significantly ($P <0.01$) more sensitive to treatment with BisEDT, BisTOL, KCL5 and Nec28 than either strains 453 or 467, whereas when treated with LX28, strain 400 was significantly ($P <0.05$) less sensitive. Strains 453 and 467 were only significantly ($P <0.05$) different when treated with 91/4 whereby strain 467 was more sensitive than 453.

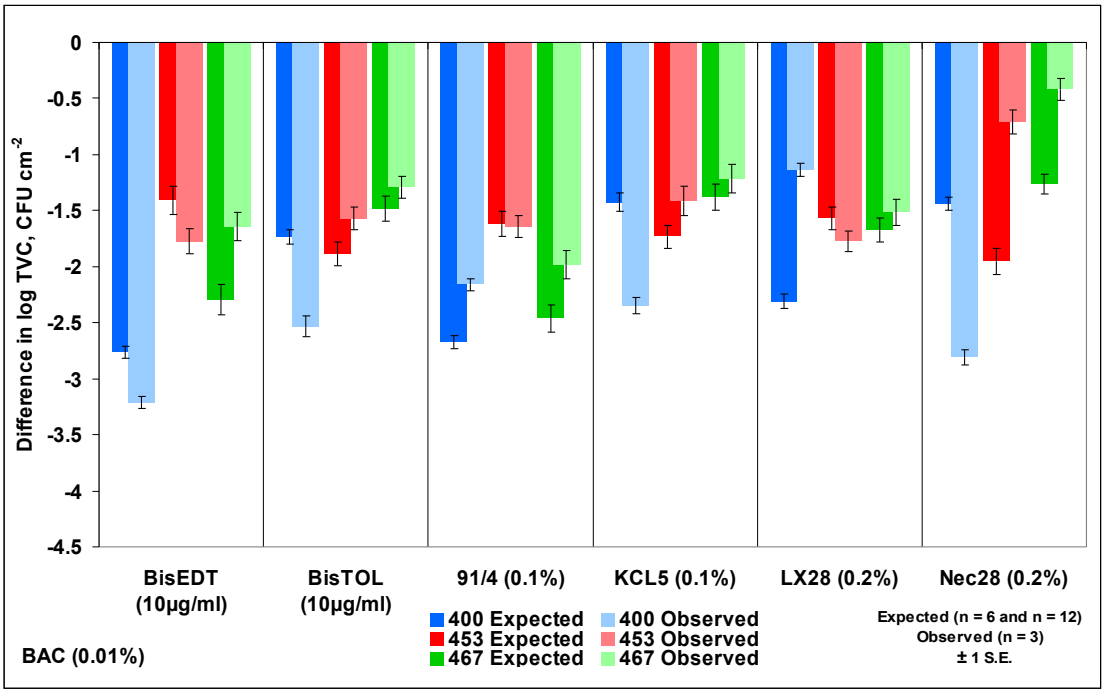

Fig. 5.4: Mean change in viability after 20 minute detergent treatment followed by 5 minute BAC treatment in WoSH for attached *E. coli* at 20°C, shown as expected (fig. 5.2 + fig. 5.3) and observed log TVC difference to a negative, water only control.

BAC	400	453	467
BisEDT	**0.448**	0.361	**-0.646**
BisTOL	**0.797**	-0.309	-0.192
91/4	**-0.514**	0.023	**-0.480**
KCL5	**0.924**	-0.317	-0.165
LX28	**-1.173**	0.205	-0.157
Nec28	**1.368**	**-1.243**	**-0.846**

Table 5.1: Synergies and antagonisms after 20 minute detergent treatment followed by 5 minute BAC treatment in WoSH for attached *E. coli* at 20°C. Differences to the expected additive results are presented as log TVC with significant synergies (P <0.05), **(P <0.01)** and antagonisms (P <0.05), **(P <0.01)**.

Fig. 5.5 and table 5.2 show there to be a total of 5 significant synergies and 6 significant antagonisms with NaDCC. Significant strain differences were also observed. For strain 400, 5 of the 6 treatments resulted in significant antagonisms with NaDCC, although in general, strain 400 was shown to be more sensitive to the treatments than strains 453 and 467. Observations of 3 antagonisms and 1 synergy for strain 453 were made and a single synergy was shown for strain 467.

Significant antagonisms with NaDCC were observed with strain 400 when treated with BisEDT, 91/4, KCL5, LX28 ($P < 0.01$) and Nec28 ($P < 0.05$), with over 1 log higher TVC than expected with detergents BisEDT and 91/4. The results for BisTOL showed there to be no significant interactions with NaDCC.

Treatment with BisEDT, BisTOL and 91/4 were shown to produce significant ($P < 0.01$) synergistic interactions with NaDCC for strain 453. A significant ($P < 0.01$) antagonism with Nec28 was observed, while treatment with detergents KCL5 and LX28 did not result in a significant interaction.

The results for strain 467 show that two significant synergies were observed when treated with the anionic detergents LX28 ($P < 0.01$) and Nec28 ($P < 0.05$). No significant interactions with NaDCC were identified for either the bismuth thiol or non-ionic detergents.

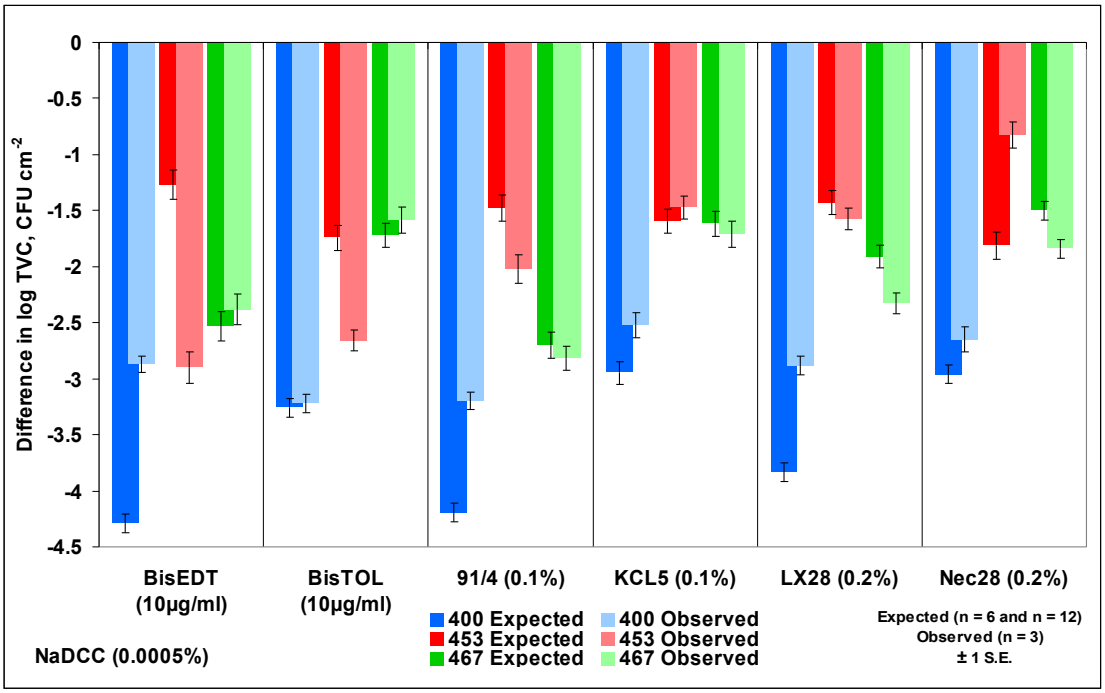

Fig. 5.5: Mean change in viability after 20 minute detergent treatment followed by 5 minute NaDCC treatment in WoSH for attached *E. coli* at 20°C, shown as expected (fig. 5.2 + fig. 5.3) and observed log TVC difference to negative, water only control.

NaDCC	400	453	467
BisEDT	**-1.416**	**1.629**	-0.146
BisTOL	-0.039	**0.917**	-0.140
91/4	**-1.001**	**0.544**	0.120
KCL5	**-0.430**	-0.121	0.092
LX28	**-0.950**	0.146	**0.417**
Nec28	-0.313	**-0.987**	0.340

Table 5.2: Synergies and antagonisms after 20 minute detergent treatment followed by 5 minute NaDCC treatment in WoSH for attached *E. coli* at 20°C. Differences to the expected additive results are presented as log TVC with significant synergies (P <0.05), **(P <0.01)** and antagonisms (P <0.05), **(P <0.01)**.

5.3.4 Summary of Treatments for One Hour Attached Populations

The data in fig. 5.6 shows there to be no general trends for all strains of attached *E. coli* for any combination of detergent and disinfectant, whereas *L. monocytogenes* data had shown trends between strains. A total of 10 significant synergies (8: P <0.01, 2: P <0.05) and 14 significant antagonisms (11: P <0.01, 3: P <0.05) were seen. Differences between strain responses to BAC and NaDCC were observed, for

example strain 400 was seen to be >0.8 log more sensitive to BAC following 4 detergent treatments (BisEDT, BisTOL, KCL5 and Nec28) than to NaDCC.

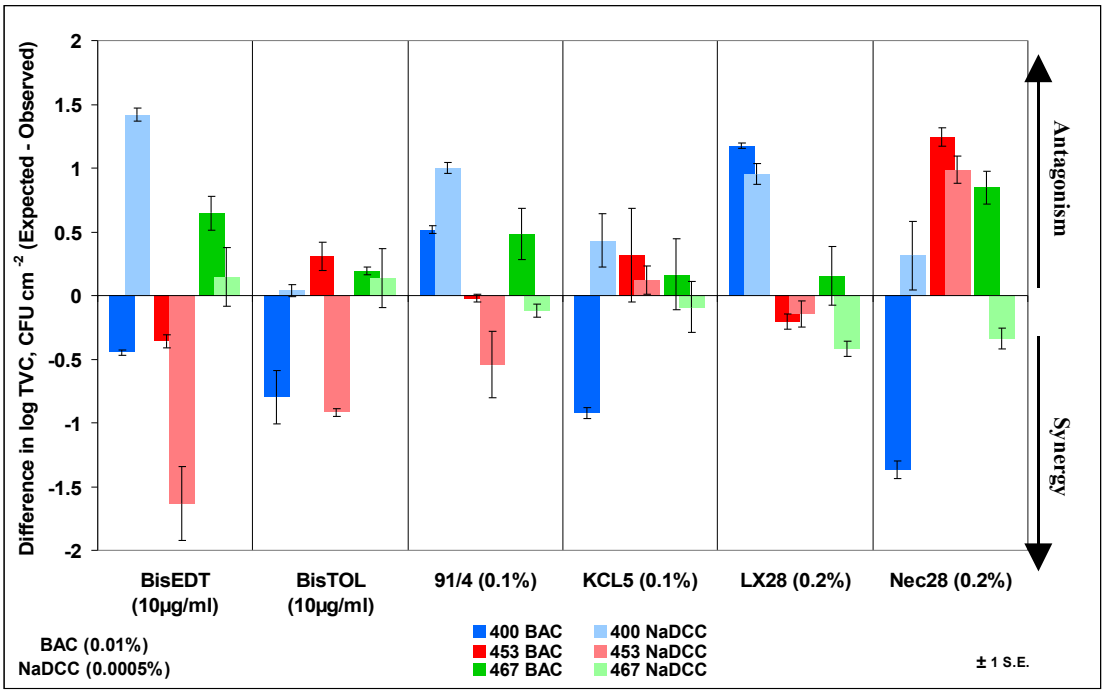

Fig. 5.6: Summary of the effectiveness on attached *E. coli* of the combined detergent/disinfectant treatments compiled by comparing the difference in viability to the sum of the single detergent and disinfectant treatments, shown as difference in log TVC.

5.4 One Hour Attached Populations – Direct Microscopic Counts

Direct counts were obtained for each treatment combination used against attached *E. coli* strains. Direct counting was used to determine if the effects seen while measuring TVC were removal and/or killing of bacteria.

Plate 5.1 shows typical slides of each *E. coli* strain after attaching for 1 hour to stainless steel coupons and staining with acridine orange. Direct counts of treated populations were recorded using Spot® capture software and ImagePro® analysis software. Each image was analysed to identify the area covered by cells, this data was then transformed into cells cm^{-2} from calculations of the average size of an individual cell within the image as described in chapter 2. The direct count for strain 400 was shown to be significantly lower ($P < 0.05$) than for strains 453 and 467.

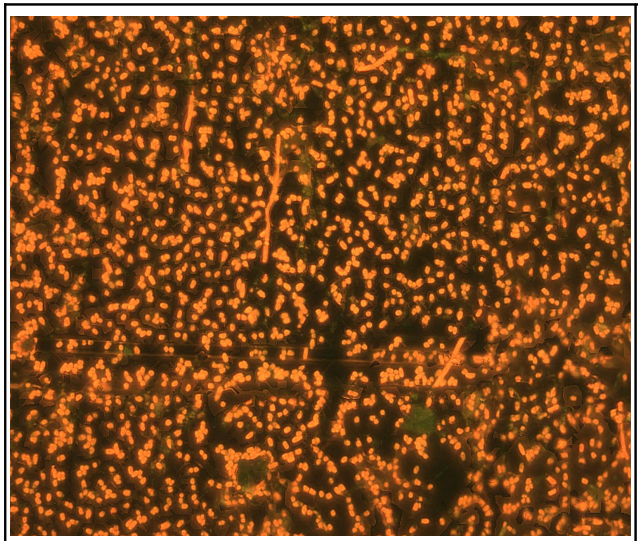	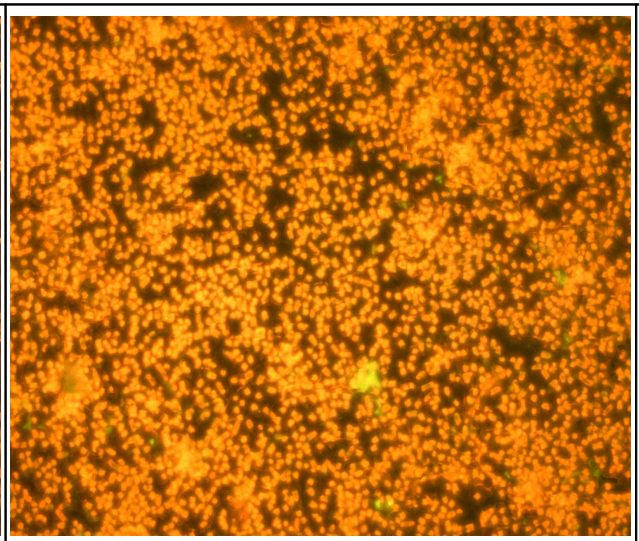	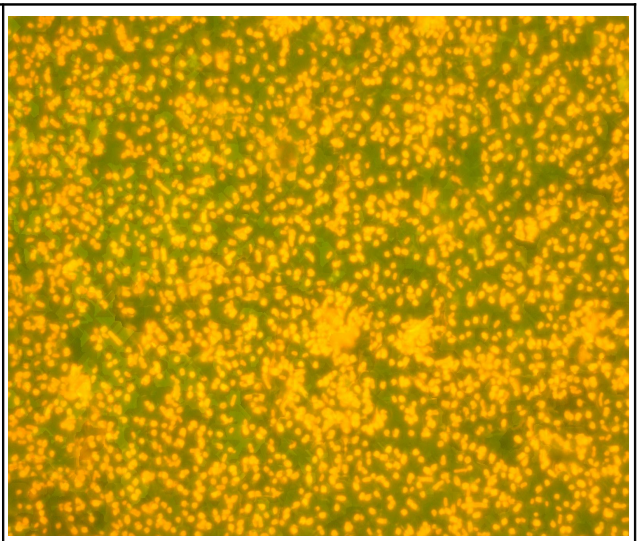
Strain 400 1 hour attachment, n = 6, mean 7.3 log cells cm^{-2}, S.E. 0.03	Strain 453 1 hour attachment, n = 6, mean 7.5 log cells cm^{-2}, S.E. 0.05	Strain 467 1 hour attachment, n = 6, mean 7.4 log cells cm^{-2}, S.E. 0.03

Plate 5.1: Typical 1 hour attachment in TSB for *E. coli* strains at 20°C on type 316 with 2B finish stainless steel coupons, stained with acridine orange at x 1000 magnification.

5.4.1 Effect of Detergent Only Treatments

Direct counts were obtained for each detergent treatment used against attached *E. coli* strains. Direct microscopic counting was used to determine if the TVC effects measured were removal and/or killing of bacteria.

The results in fig. 5.7 show that the detergent only treatments had little or no removal effect against attached populations of *E. coli*. As shown, significant ($P < 0.05$) reductions in total count were seen for BisEDT against strain 453 and 91/4 against strain 467. No other significant changes in total counts observed for any detergent against any strain.

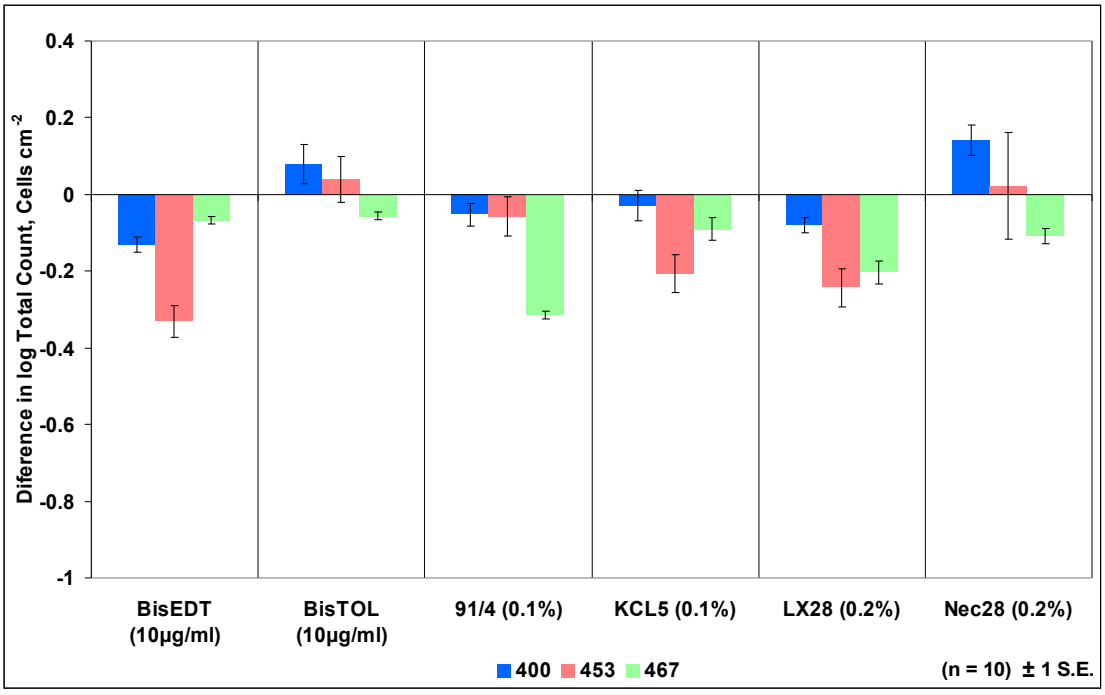

Fig. 5.7: Mean total count after 20 minute contact time with detergent treatments in WoSH for attached *E. coli* at 20°C, shown as log total count difference to a negative, water only control.

5.4.2 Effect of Disinfectant Only Treatments

Direct counts were obtained for each disinfectant treatment used against attached *E. coli* strains. Direct microscopic counting was used to determine if the TVC effects measured were removal and/or killing of bacteria. The results (fig. 5.8)

show there to be no significant difference in total count for any strain/disinfectant combination.

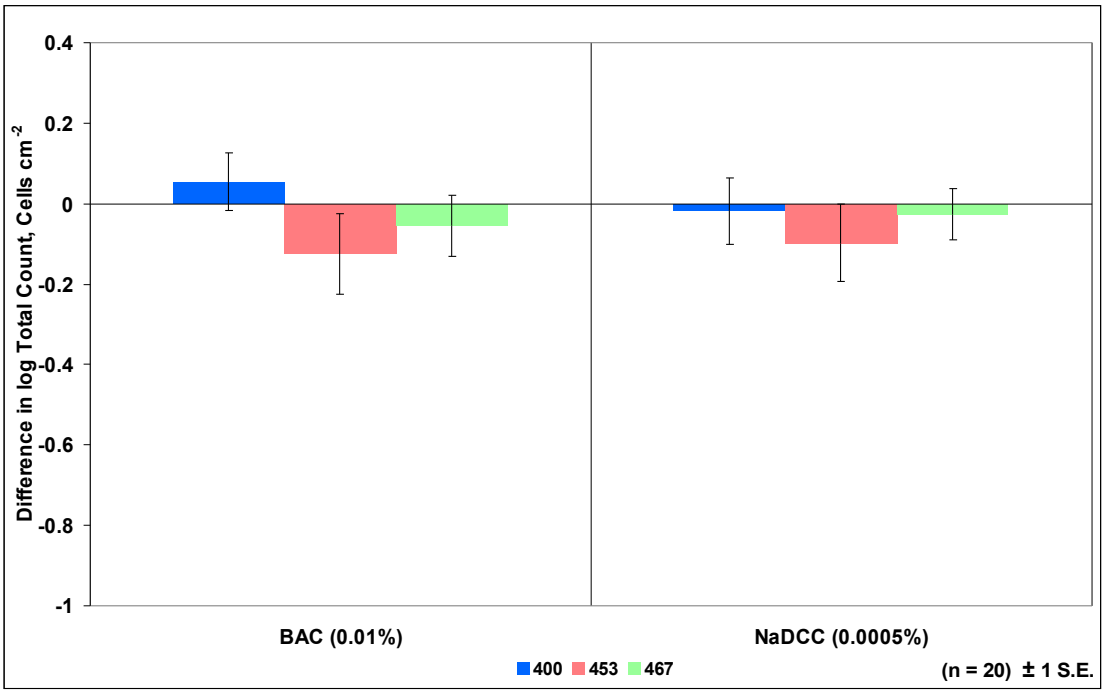

Fig. 5.8: Mean total count after 5 minute contact time with disinfectant treatments in WoSH for attached *E. coli* at 20°C, shown as log total count difference to a negative, water only control.

5.4.3 Effect of Detergent Followed by Disinfectant Treatments

Direct counts were obtained for each detergent, disinfectant treatment combination used against attached *E. coli* strains. Direct microscopic counting was used to determine if the TVC effects measured were removal and/or killing of bacteria.

The results (fig. 5.9) show that significant changes in total count were seen between treatments and between strains. Strain 400 was not seen to have any significant change in total count following any of the treatment combinations. Strain 453 was seen to have significant ($P < 0.05$) reductions in total count when treated with BisEDT, 91/4 and KCL5. Strain 467 was shown to have a significant reduction in total count following treatment with 91/4 and LX28.

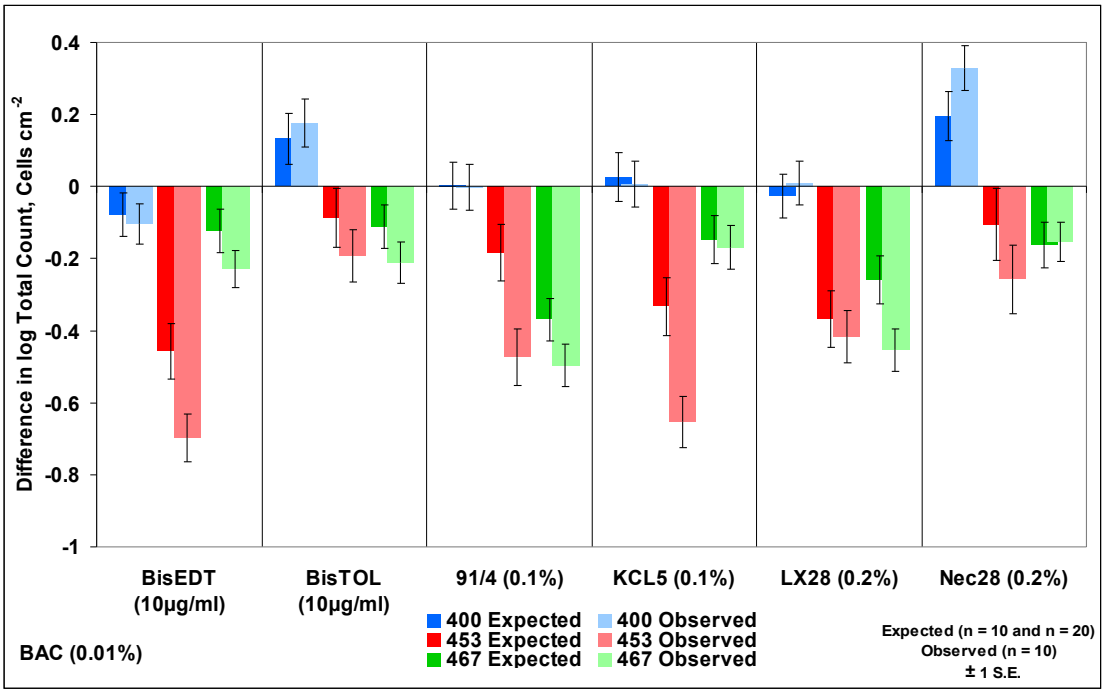

Fig. 5.9: Mean total count after 20 minute detergent treatment followed by 5 minute BAC treatment in WoSH for attached *E. coli* at 20°C, shown as expected (fig. 5.7 + fig. 5.8) and observed log total count difference to a negative, water only control.

In fig. 5.10, no treatments were shown to significantly change the total counts for strain 400. Four NADCC treatments against strain 453 were shown to have significantly reduced ($P < 0.05$) total counts compared to water only controls, BisEDT, BisTOL, KCL5 and LX28, of which treatment with BisTOL, KCL5 and LX28 demonstrated significant ($P < 0.05$) synergy interactions with NaDCC. Against strain 467 significant reductions in total count were observed when treated with 91/4 and LX28.

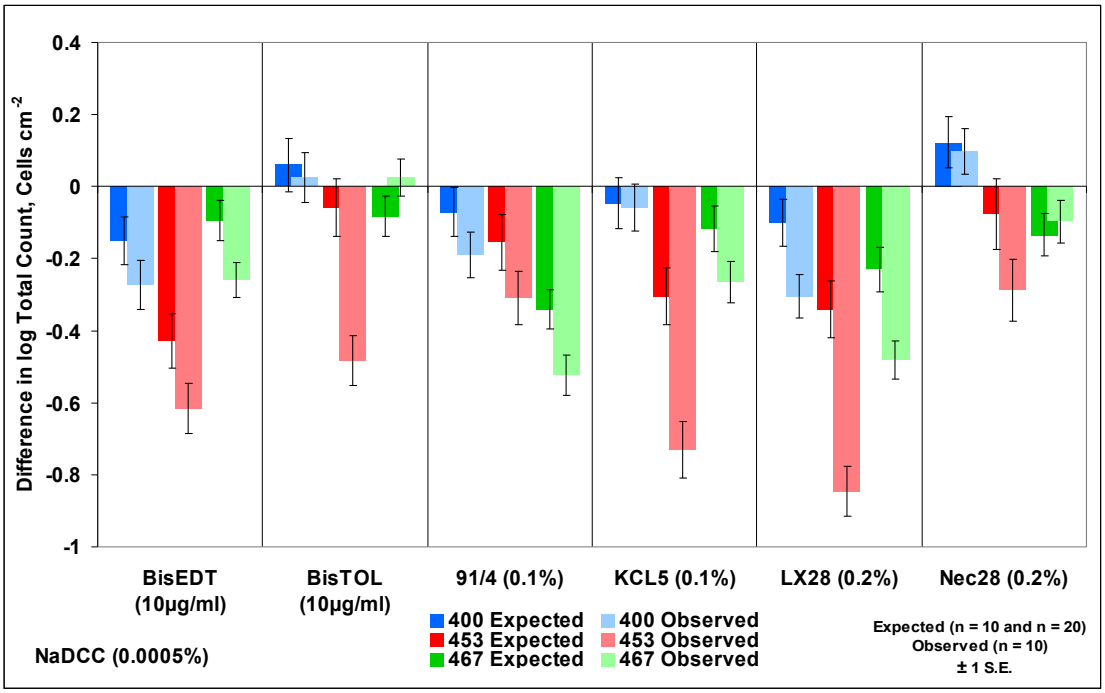

Fig. 5.10: Mean total count after 20 minute detergent treatment followed by 5 minute NaDCC treatment in WoSH for attached *E. coli* at 20°C, shown as expected (fig. 5.7 + fig. 5.8) and observed log total count difference to a negative, water only control.

5.4.4 Summary of Treatments for One Hour Attached Populations

The data in fig. 5.11 summarises the observed antagonisms and synergies seen for direct count data. There were no significant detergent/disinfectant interactions, synergistic or antagonistic, observed against strain 400 or strain 467. Significant ($P < 0.05$) synergistic interactions were observed for strain 453 when treated with BisTOL, KCL5 or LX28 prior to treatment with NaDCC, a general trend towards synergy, although not significant, was seen for many of the other treatment combinations against strain 453.

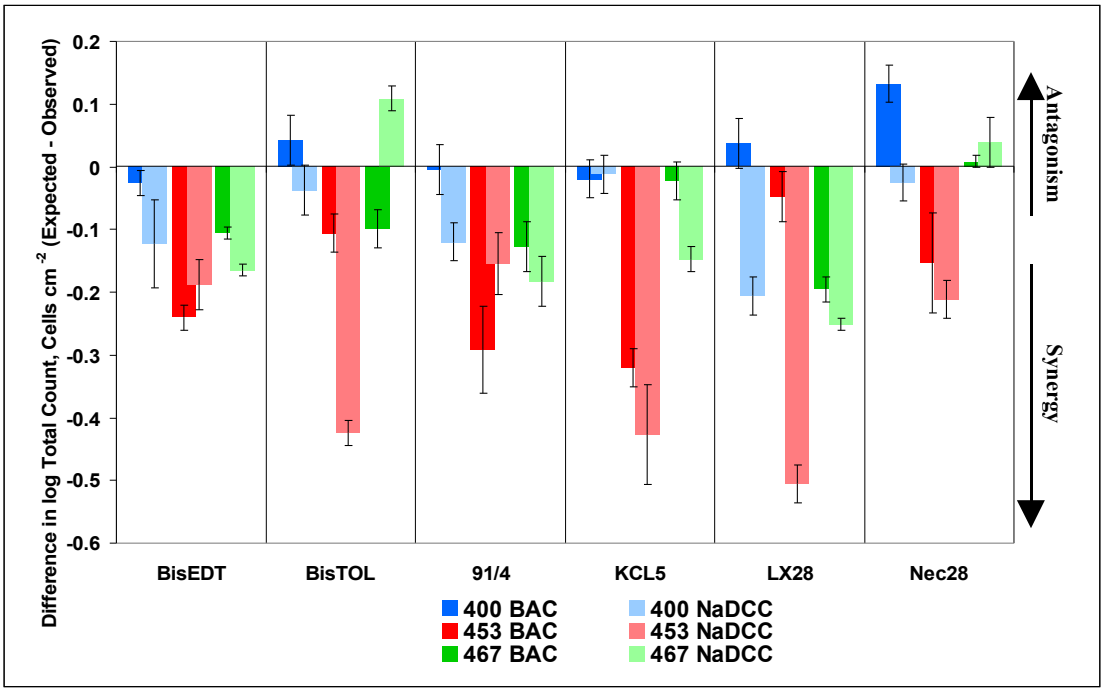

Fig. 5.11: Summary of the effectiveness on attached *E. coli* of the combined detergent/disinfectant treatments compiled by comparing the difference in total count to the sum of the single detergent and disinfectant treatments, shown as difference in log total count.

5.5 24 Hour Biofilm Populations – Total Viable Counts

5.5.1 Effect of Detergent Only Treatments

The effect of industrially recommended, in use, concentrations of detergents was determined on 24 hour biofilm populations of *E. coli* strains 400, 453 and 467. This was used to determine the degree of killing and/or removal of the adherent population following treatment. Detergent activity was compared to a negative control (zero on the x-axis) in which detergent solutions were substituted with water of standard hardness.

The results for the effect of detergents against biofilms in fig. 5.12 show that only 2 statically significant losses ($P < 0.05$) in TVC occurred, strain 467 with BisTOL and strain 400 with Nec28. None of the other detergent only treatments resulted in a significant variation in TVC compared to the controls.

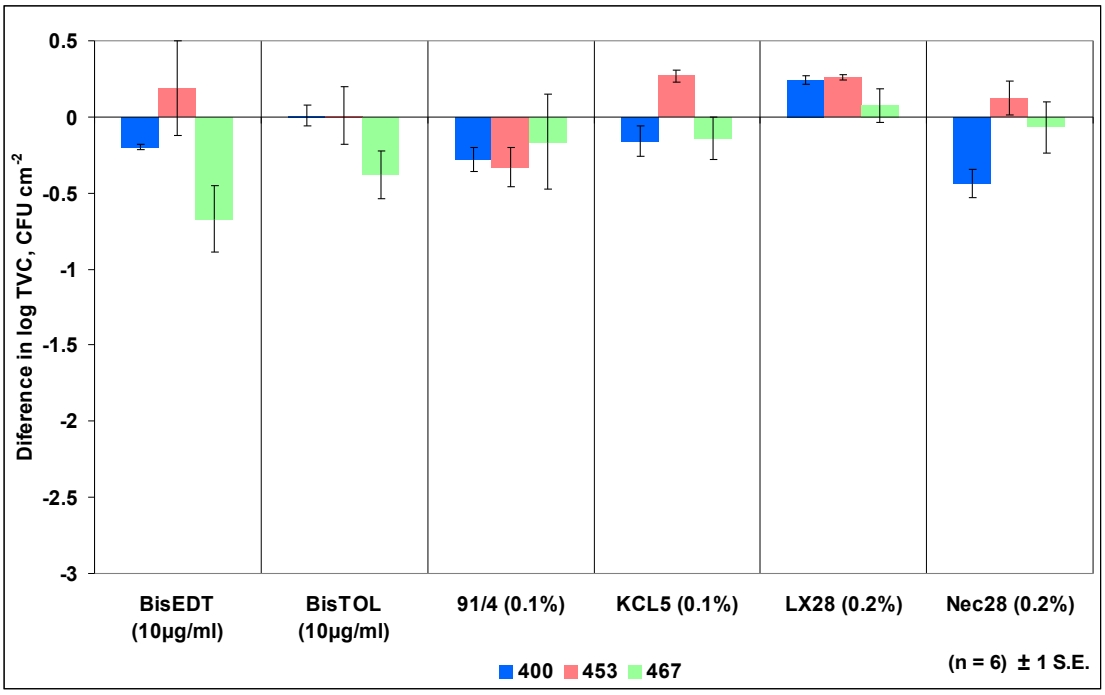

Fig. 5.12: Mean loss of viability after 20 minute contact time with detergent treatments in WoSH for biofilm *E. coli* at 20°C, shown as log TVC difference to a negative, water only control.

5.5.2 Effect of Disinfectant Only Treatments

The effect of disinfectants, at concentrations of 0.01% for BAC and 0.0005% for NaDCC, previously identified to give 2 – 4 log kill against *E. coli* in suspension, was determined against 24 hour biofilm populations of strains 400, 453 and 467 to measure the biocidal effects against biofilm populations. Disinfectant activity was compared to a negative control (zero on the x-axis) in which disinfectant solutions were substituted with water of standard hardness. The industry recommended contact time of 5 minutes was used.

The results presented in fig. 5.13, show that statistically significant differences were observed in disinfectant sensitivity between strains. Strain 400 was shown to be statistically less sensitive to BAC ($P < 0.05$) when compared to strains 453 and 467. Strain 467 was significantly more sensitive ($P < 0.05$) to NaDCC when compared to strains 400 and 453. A trend was also observed, although not statistically significant, suggesting strain 400 was least sensitive to both disinfectants while strain 467 was most sensitive.

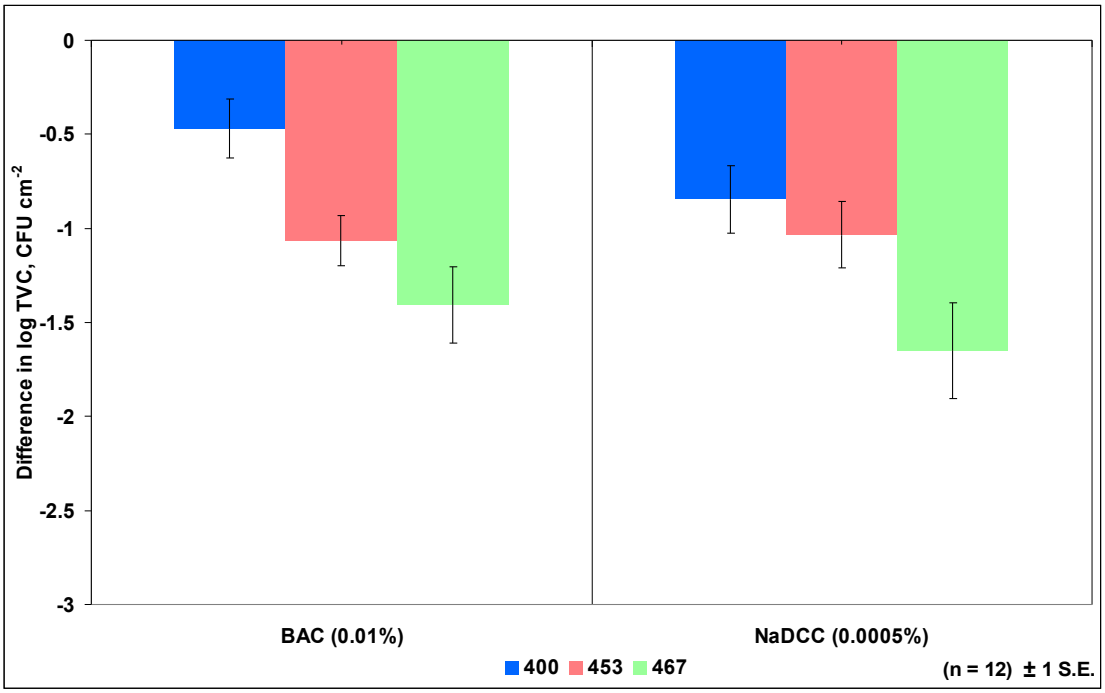

Fig. 5.13: Mean loss of viability after 5 minute contact time with disinfectant treatments in WoSH for biofilm *E. coli* at 20°C, shown as log TVC difference to a negative, water only control.

5.5.3 Effect of Detergent Followed by Disinfectant Treatments

The effect of detergent treatments (at industry recommended in use concentrations) followed by disinfectants, at concentrations of 0.01% for BAC and 0.0005% for NaDCC, previously identified to give 2 – 4 log kill against *E. coli* in suspension, was determined against biofilm populations of *E. coli* strains 400, 453 and 467. Treatment activity was compared to a negative control (zero on the x-axis) in which detergent and disinfectant solutions were substituted with WoSH. The industry recommended contact times of 20 minutes for detergents and 5 minutes for disinfectants was used. Observed results were compared to expected results, where the expected results were calculated by combining the changes in TVC for individual detergent (fig. 5.12) and disinfectant (fig. 5.13) treatments to determine any interaction between detergent and disinfectant treatments.

As seen in fig. 5.14 and table 5.3 both bismuth thiol detergents showed a significant ($P < 0.01$) synergy with BAC against strains 400 and 453, while against

strain 467 a significant ($P < 0.01$) synergy was only observed with BisTOL. Against strain 400 as seen in table 5.3, both bismuth thiols produced a synergistic effect almost 3 fold greater than that observed against strain 453 and 467 (BisTOL only).

The results for the non-ionic and anionic detergents shows there to be very few observed interactions with BAC. A significant ($P < 0.05$) antagonism was seen for 91/4 against strain 453 resulting in 0.336 log greater viable count than expected. The only other observed significant antagonism ($P < 0.01$) was the anionic detergent Nec28 with strain 400, which was shown to increase the expected viable count by 0.67 log. Other than the bismuth thiol detergents only the non-ionic detergent KCL5 was seen to produce a significant synergistic ($P < 0.01$) effect with BAC when used against strain 453.

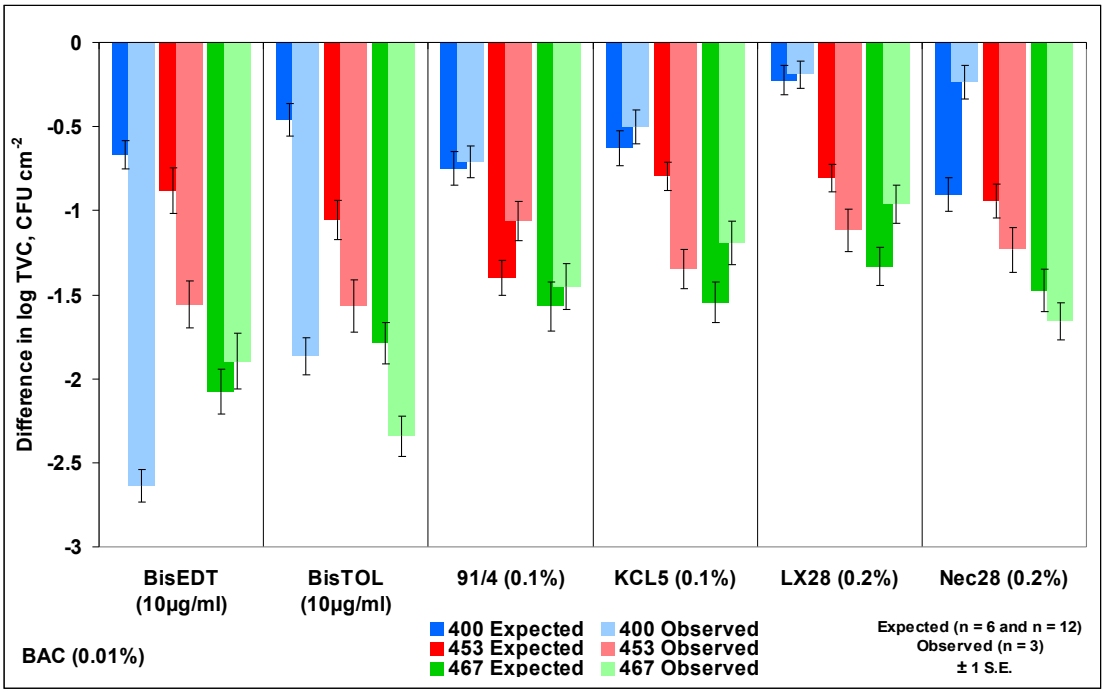

Fig. 5.14: Mean loss of viability after 20 minute detergent treatment followed by 5 minute BAC treatment in WoSH for biofilm *E. coli* at 20°C, shown as expected (fig. 5.12 + fig. 5.13) and observed log TVC difference to a negative, water only control.

BAC	400	453	467
BisEDT	**1.971**	**0.680**	-0.182
BisTOL	**1.41**	**0.512**	**0.554**
91/4	-0.04	-0.336	-0.120
KCL5	-0.126	**0.551**	-0.357
LX28	-0.035	0.311	-0.370
Nec28	**-0.670**	0.291	0.183

Table 5.3: Synergies and antagonisms after 20 minute detergent treatment followed by 5 minute BAC treatment in WoSH for biofilm *E. coli* at 20°C. Differences to the expected additive results are presented as log TVC with significant synergies (P <0.05), **(P <0.01)** and antagonisms (P <0.05), **(P <0.01)**.

A mixture of antagonisms and synergies between detergents, strains and NaDCC were observed (fig. 5.15 and table 5.4). Highly significant synergies (P <0.01) were observed against all 3 strains when treated with the bismuth thiol detergent BisTOL while highly significant antagonisms (P <0.01) were observed against all 3 strains when treated with the non-ionic detergent 91/4. The data in table 5.4 shows that as

observed in table 5.3 for BisTOL, there was a over a 2 fold greater synergy against strains 400 (1.318 log) than strains 453 (0.626 log) and 467 (0.558 log).

The second bismuth thiol, BisEDT, was shown to have a significant synergy ($P < 0.01$) against strain 400 while no effect was seen against strains 453 and 467. The non-ionic detergent KCL5 was observed to result in a significant antagonism ($P < 0.05$) against strain 400 and a significant synergy ($P < 0.05$) against strain 453.

The two anionic detergents LX28 and Nec28 were shown to have markedly different effects. LX28 was seen to produce significant synergies against strains 453 ($P < 0.01$) and 467 ($P < 0.05$), while against strain 400 no significant interaction was observed, although a weakly synergistic interaction can be inferred by the 0.302 log lower viable count than expected. For Nec28 a strong significant antagonism ($P < 0.01$) was seen against strain 400 resulting in nearly a 1 log higher than expected viable count, while a significant synergy ($P < 0.05$) was shown against strain 453.

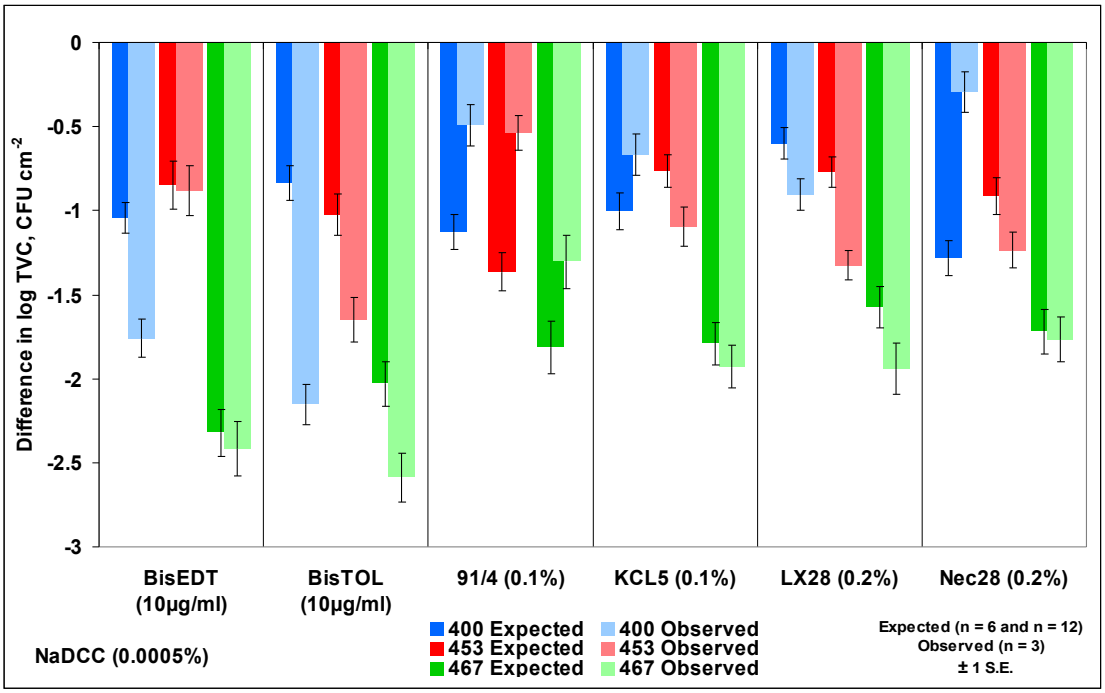

Fig. 5.15: Mean loss of viability after 20 minute detergent treatment followed by 5 minute NaDCC treatment in WoSH for biofilm *E. coli* at 20°C, shown as expected (fig. 5.12 + fig. 5.13) and observed log TVC difference to negative, water only control.

NaDCC	400	453	467
BisEDT	**0.718**	0.036	0.097
BisTOL	**1.318**	**0.626**	**0.558**
91/4	**-0.632**	**-0.829**	**-0.509**
KCL5	-0.338	0.331	0.138
LX28	0.302	**0.552**	0.366
Nec28	**-0.989**	0.326	0.05

Table 5.4: Synergies and antagonisms after 20 minute detergent treatment followed by 5 minute NaDCC treatment in WoSH for biofilm *E. coli* at 20°C. Differences to the expected additive results are presented as log TVC with significant synergies (P <0.05), **(P <0.01)** and antagonisms (P <0.05), **(P <0.01)**.

5.5.4 Summary of Treatments for 24 Hour Biofilm Populations

The results (fig. 5.16) show that in general, exposing biofilms of *E. coli* to a bismuth thiol detergent treatment prior to using either BAC or NaDCC results in a synergistic effect. Exposure to either non-ionic or anionic detergents was shown in general to either result in an antagonistic effect or show no significant interaction.

The only detergent treatment to show entirely synergistic effects against all 3 strains ($P < 0.01$) with both BAC and NaDCC was BisTOL. Conversely the non-ionic detergent 91/4 was observed to result in antagonistic, although not always significant, interactions with both disinfectants for all 3 strains. The bismuth thiol, BisEDT and the second non-ionic detergent, KCL5, as well as both anionic detergents LX28 and Nec28 did not demonstrate any specific trends with regards to synergies or antagonisms, for BisEDT and Nec28 significant antagonisms ($P < 0.01$) were seen for both disinfectants when used against strain 400.

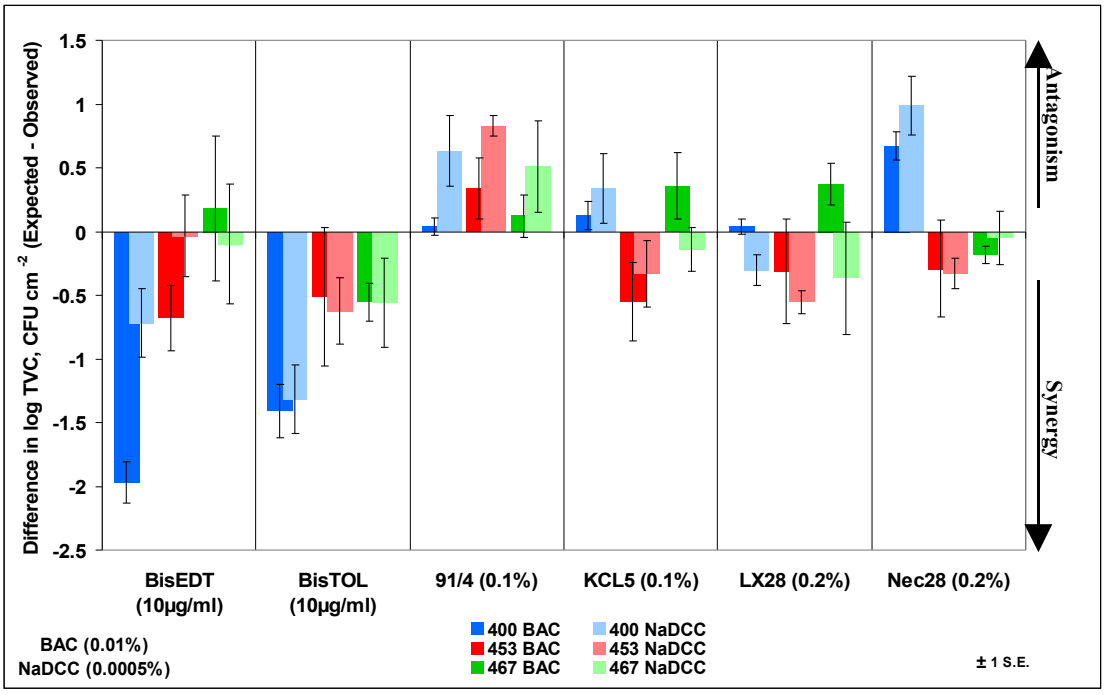

Fig. 5.16: Summary of the effectiveness on biofilm *E. coli* of the combined detergent/disinfectant treatments compiled by comparing the difference in viability to the sum of the single detergent and disinfectant treatments, shown as difference in log TVC.

5.6 24 Hour Biofilm – Direct EPS Quantification

Direct total counts for biofilms of *E. coli* were not obtainable due to the dense layering of cells and extra cellular polysaccharide on the coupons. Although measurable, the intensity of cellular material, stained with acridine orange, did not

significantly change following treatments, whereas measurements of the intensity of EPS, stained with calcofluor white, were significantly different following treatments.

The density of EPS was assessed via calcofluor staining, to determine if detergent and/or disinfectant treatments caused disruption of the polymeric matrix. Plate 5.2 shows typical slides of each *E. coli* strain after forming biofilms on stainless steel coupons over 24 hours and staining with calcofluor white. Image intensity of treated populations was recorded to determine density of EPS using Spot® capture software and ImagePro® analysis software. Uneven distribution of EPS was observed for all 3 strains, although this was seen to vary with strain 400 being seen to produce the densest EPS. The brighter areas seen on the plates are denser regions of EPS which when dual stained with acridine orange can also be seen to be the densest regions of cellular material, the darker areas are less dense regions of cells and EPS.

Additional, untreated controls were measured as a baseline to determine the effects of each treatment, including the WoSH controls. EPS was measured using arbitrary brightness values from 1 (black) to 200 (white) for each pixel (4.1×10^6) per image allowing a single mean per image to be calculated.

Strain 467 was seen to consistently produce approximately half of the calculated amount of EPS that was produced by strains 400 and 453. Strains 400 and 453 did not produce significantly different quantities of EPS after 24 hours, although in appearance strain 453 was seen to produce dense clumps of cells and EPS whereas strain 400 was seen to produce a more even biofilm with fewer channels or clumps observed.

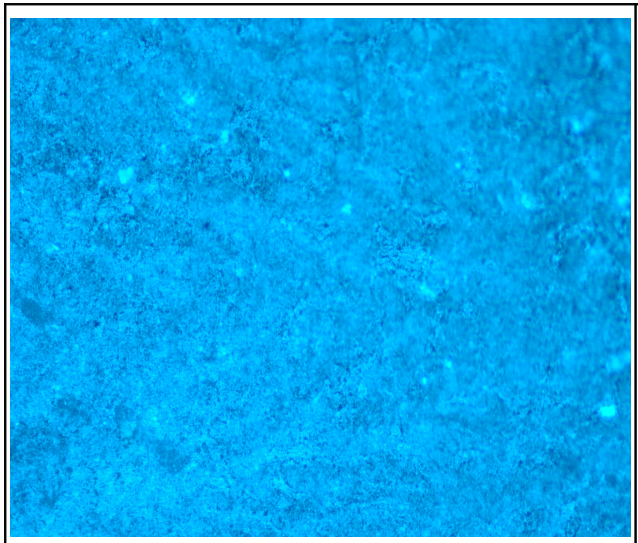	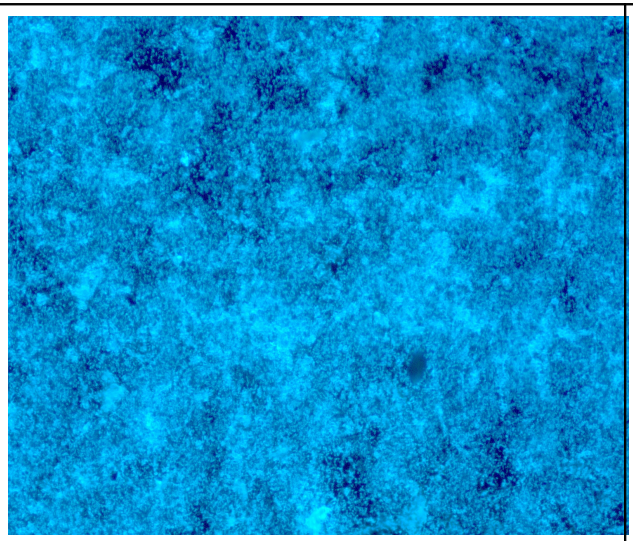	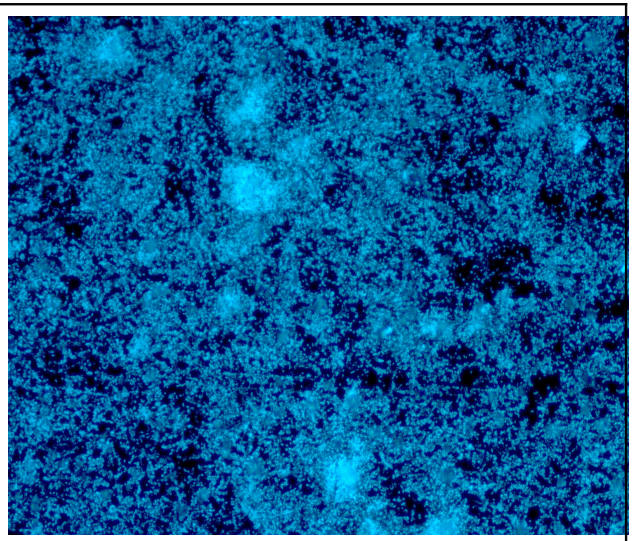
Strain 400 biofilm, n = 6, mean 126.3 intensity, S.E. 6.7	Strain 453 biofilm, n = 6, mean 119.7 intensity, S.E. 4.6	Strain 467 biofilm, n = 6, mean 54.7 intensity, S.E. 3.6

Plate 5.2: Typical 24 hour biofilm in 10% TSB for *E. coli* strains at 20°C on type 316 with 2B finish stainless steel coupons stained with calcofluor white at x 1000 magnification.

5.6.1 Strain 400 - EPS Intensity after Treatments

As seen in fig. 5.17 there were only two treatment combinations the caused a significant reduction (P <0.01) in EPS intensity for strain 400, BisEDT with both BAC and NaDCC, BisEDT only treatments however did not significantly alter the EPS intensity. None of the other treatment combinations were shown to significantly alter the EPS intensity compared to the untreated control (black line). Unlike all of the strains tested, including *L. monocytogenes*, WoSH only treatments did not reduce the EPS intensity of strain 400.

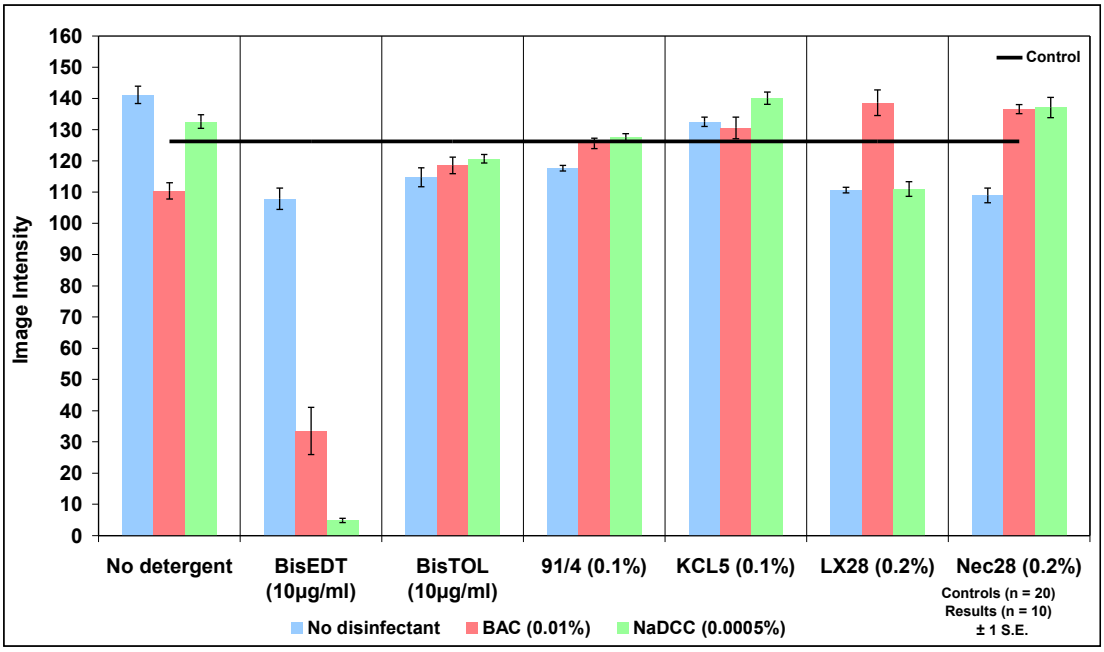

Fig. 5.17: Effect on the density of extra cellular polysaccharide for biofilm *E. coli* strain 400, for all detergent and disinfectant regimes in WoSH at 20°C, measured as image intensity. The control baseline is an untreated 24 hour biofilm population with no treatments other than gentle washing to remove suspended cells, presented as the lower 5% confidence limits.

5.6.2 Strain 453 - EPS Intensity after Treatments

The results (fig. 5.18) for *E. coli* strain 453 shows there to be large variations between treatments. The no detergent, no disinfectant control treatment was seen to significantly reduce (P <0.01) the EPS intensity by almost a factor of 3 compared to the untreated control (black line), showing that WoSH alone was sufficient to

solubilise some of the EPS. The results for the no detergent, with either BAC or NaDCC, treatments also showed a significant reduction ($P < 0.01$) in EPS intensity, although these were not significantly different from the no detergent, no disinfectant control treatment.

The bismuth thiol, BisEDT, was not seen to significantly affect the EPS intensity with or without the presence of a disinfectant treatment. The second bismuth thiol, BisTOL was shown to significantly reduce the EPS intensity when combined with the no disinfectant treatment ($P < 0.01$) and the disinfectant NaDCC ($P < 0.05$), however no significant affect was shown when the disinfectant BAC was used.

The non-ionic detergent, 91/4 was shown to significantly reduce the EPS intensity with the no disinfectant treatment ($P < 0.05$) and with BAC ($P < 0.01$), however there was no significant change in EPS intensity with NaDCC. Treatment with 91/4 and the disinfectant BAC was shown to significantly reduce ($P < 0.01$) the EPS intensity compared to the 91/4 with no disinfectant treatment. All of the treatment combinations with the second non-ionic detergent, KCL5, were seen to significantly reduce ($P < 0.01$) EPS intensity. Significantly higher EPS intensities were shown when KCL5 treatment was followed by either BAC ($P < 0.01$) or NaDCC ($P < 0.05$) compared to the no disinfectant treatment.

The results for the anionic detergent LX28 show a significant ($P < 0.01$), 4 fold reduction in EPS intensity compared to the untreated control for the no disinfectant and NaDCC treatments. When treated with BAC after LX28, the EPS intensity for strain 453 was not significantly different from the untreated control. All 3 of the treatments with Nec28 were seen to have significantly lower ($P < 0.01$) EPS intensity

than the untreated control but with no significant variation between treatments.

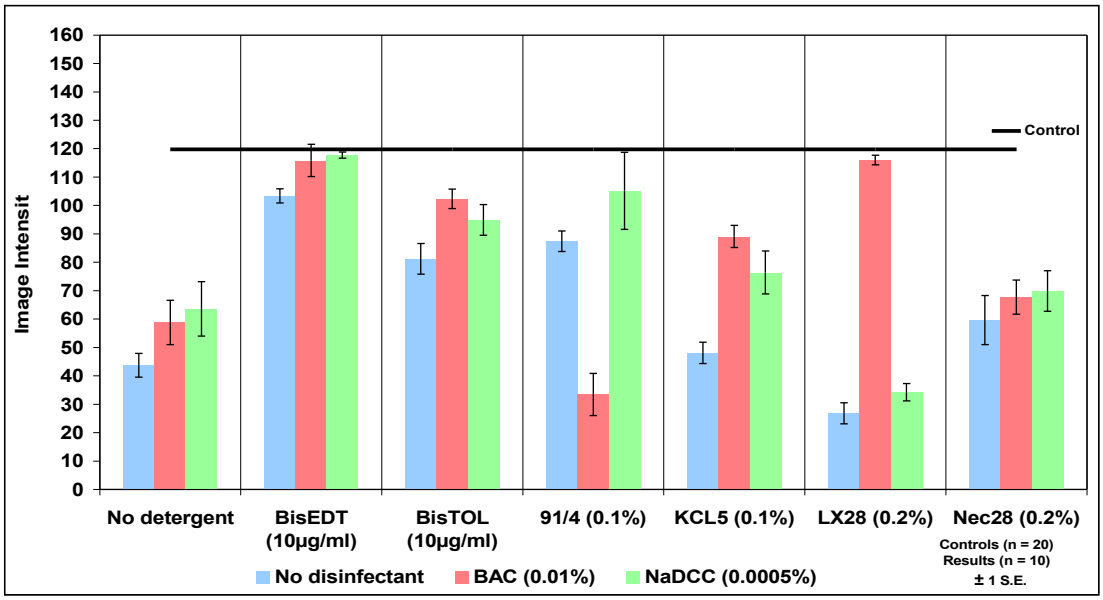

Fig. 5.18: Effect on the density of extra cellular polysaccharide for biofilm *E. coli* strain 453, for all detergent and disinfectant regimes in WoSH at 20°C, measured as image intensity. The control baseline is an untreated 24 hour biofilm population with no treatments other than gentle washing to remove suspended cells, presented as the lower 5% confidence limits.

5.6.3 Strain 467 - EPS Intensity after Treatments

The black control bar for strain 467 (fig. 5.19) was shown to be less than half the value observed for strains 400 (fig. 5.17) and 453 (fig. 5.18), due to consistently producing a much less dense EPS layer. All three, no detergent treatments showed significant reductions ($P < 0.01$) in EPS intensity compared to the untreated control

(black bar) which shows WoSH only treatments solubilise some of the EPS. No significant differences were observed when either disinfectant was present compared to the no disinfectant treatment.

All treatments with the bismuth thiol, BisEDT, were seen to significantly ($P < 0.01$) reduce the EPS intensity of strain 467. There was no observable difference when the disinfectant BAC was used compared to the no disinfectant treatment, although when BisEDT treatment was followed by NaDCC treatment a significant drop ($P < 0.05$) in EPS intensity was seen compared to the other BisEDT treatments. Significant reductions ($P < 0.01$) in EPS intensity were observed for BisTOL with the no disinfectant and BAC treatments, however BisTOL followed by the disinfectant NaDCC did not significantly change the EPS intensity compared to the untreated control. A significant drop ($P < 0.05$) in EPS intensity was shown with the BisTOL, BAC treatment compared to the BisTOL, no disinfectant treatment.

All of the non-ionic detergent treatment combinations for both 91/4 and KCL5 were seen to significantly reduce ($P < 0.01$) the EPS intensity compared to the untreated control. No significant differences between treatments using 91/4 or KCL5 were observed.

Significant reductions ($P < 0.01$) in EPS intensity were seen for both anionic detergents, LX28 and Nec28, with the no disinfectant and NaDCC treatments. There were no significant differences observed between these treatments. When either anionic detergent treatment was followed by treatment with the disinfectant BAC the EPS intensity was not significantly different from that seen for the untreated control.

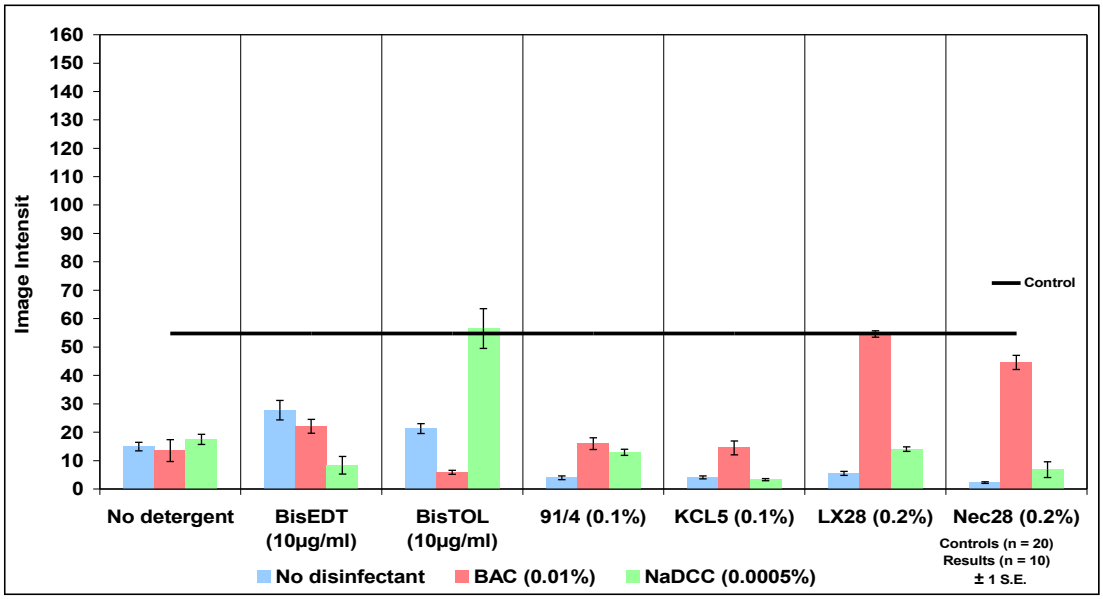

Fig. 5.19: Effect on the density of extra cellular polysaccharide for biofilm *E. coli* strain 467, for all detergent and disinfectant regimes in WoSH at 20°C, measured as image intensity. A The control baseline is an untreated 24 hour biofilm population with no treatments other than gentle washing to remove suspended cells, presented as the lower 5% confidence limits.

5.7 Comparison of Total Viable Counts for Biofilms against Attached Populations

The effectiveness of each treatment, detergent only, disinfectant only and detergent followed by disinfectant, was compared between attached and biofilm populations of *E. coli*. On the following graphs a value above zero shows the treatment to be more effective against attached than biofilm populations while a value below zero shows the treatment was more effective against biofilms than attached populations.

5.7.1 Effectiveness of Detergent Only Treatments

As seen in fig. 5.20, only 2 detergent treatments significantly ($P < 0.05$) reduced the TVC of a biofilm population (KCL5 and Nec28 against strain 400) more than the respective attached population. Three treatments were shown to be significantly more effective ($P < 0.05$) against attached cells than biofilms (BisEDT: 400, 91/4: 467 and

LX28: 400). All of the remaining detergent treatments did not show a significant difference when used against attached cells compared to biofilms.

Significant differences between strains were seen for some treatments. 91/4 was significantly more effective ($P < 0.05$) against attached cells of strain 467 compared to strain 453. KCL5 and Nec28 were significantly more effective ($P < 0.01$) against biofilms of strain 400 than 453.

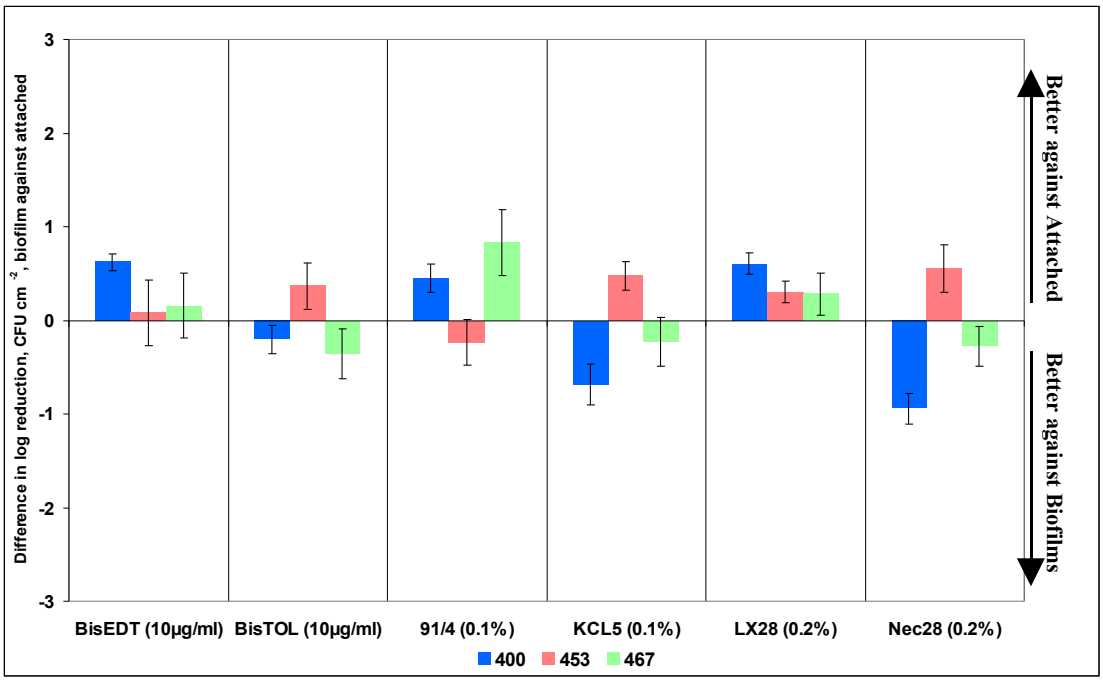

Fig. 5.20: Difference in the effectiveness of detergent treatments against biofilms compared to attached *E. coli*, shown as difference in log reductions of TVC.

5.7.2 Effectiveness of Disinfectant Only Treatments

The differences in the loss of TVC between biofilms and attached populations can be seen in fig. 5.21. Strain differences in sensitivity towards both disinfectants were observed. *E. coli* strain 400 was shown to be significantly more sensitive ($P < 0.01$) to both disinfectants as a 1 hour attached population than as a biofilm. Strain 400 was also seen to gain significantly more tolerance ($P < 0.01$) to BAC and NaDCC compared to strains 453 and 467 after biofilm formation.

Strain 453 showed a small significant increase (P <0.05) in tolerance to disinfectant treatment after biofilm formation against BAC and NaDCC. The tolerance of strain 467 to BAC and NaDCC was not seen to significantly differ between 1 hour attached and 24 hour biofilm populations.

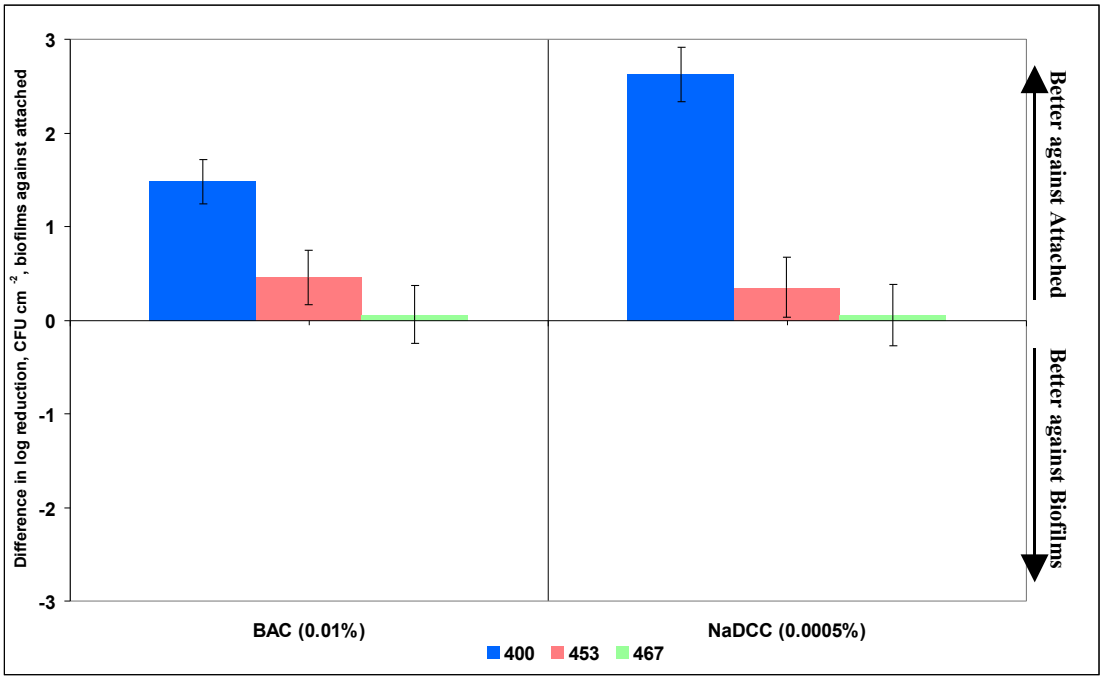

Fig. 5.21: Difference in the effectiveness of disinfectant treatments against biofilms compared to attached *E. coli*, shown as difference in log reduction of TVC.

5.7.3 Effectiveness of Detergent Followed by Disinfectant Treatments

Fig. 5.22 shows there to be a general trend towards increasing synergistic interactions between detergents and disinfectants against 24 hour biofilm populations compared to the interactions seen against 1 hour attached populations. The results show there to be 13 treatments that showed significant increases (8: P <0.01, 5: P <0.05) in synergy against biofilms while only 6 treatments showed a significant increase (4: P <0.01, 2: P <0.05) in antagonism against biofilms compared to attached populations.

Treatment with the detergent BisTOL was seen to results in 5 significant (1: P <0.01, 4: P <0.05) increases in synergy against biofilms compared to attached

populations. BisTOL with NaDCC against strain 453 did not significantly differ in effect against biofilms compared to attached populations. Against strain 400, BisEDT was shown to exhibit a significant increase ($P < 0.01$) in synergy with either disinfectant against biofilms compared to attached populations, however a significantly increased ($P < 0.01$) antagonism was shown for BisEDT with NaDCC against strain 453.

Only a single significant ($P < 0.05$) increase in synergy against biofilms was observed for the two non-ionic detergents (KCL5:BAC:453). Three significantly more antagonistic combinations were shown, 91/4:NaDCC:453 ($P < 0.01$), 91/4:NaDCC:467 ($P < 0.05$) and KCL5:BAC:400 ($P < 0.01$).

Treatments with the anionic detergent LX28 did not differ in effect against biofilms or attached populations of strains 453 and 467. Against strain 400 significant increases ($P < 0.01$) in synergy against biofilm populations were observed. The second anionic detergent, Nec28, was shown to significantly increase ($P < 0.01$) observed synergies against biofilms for strain 453 with BAC and NaDCC and strain 467 with BAC. Significant increases in antagonism were observed against biofilms for strain 400 with BAC ($P < 0.01$) and NaDCC ($P < 0.05$) when treated with Nec28.

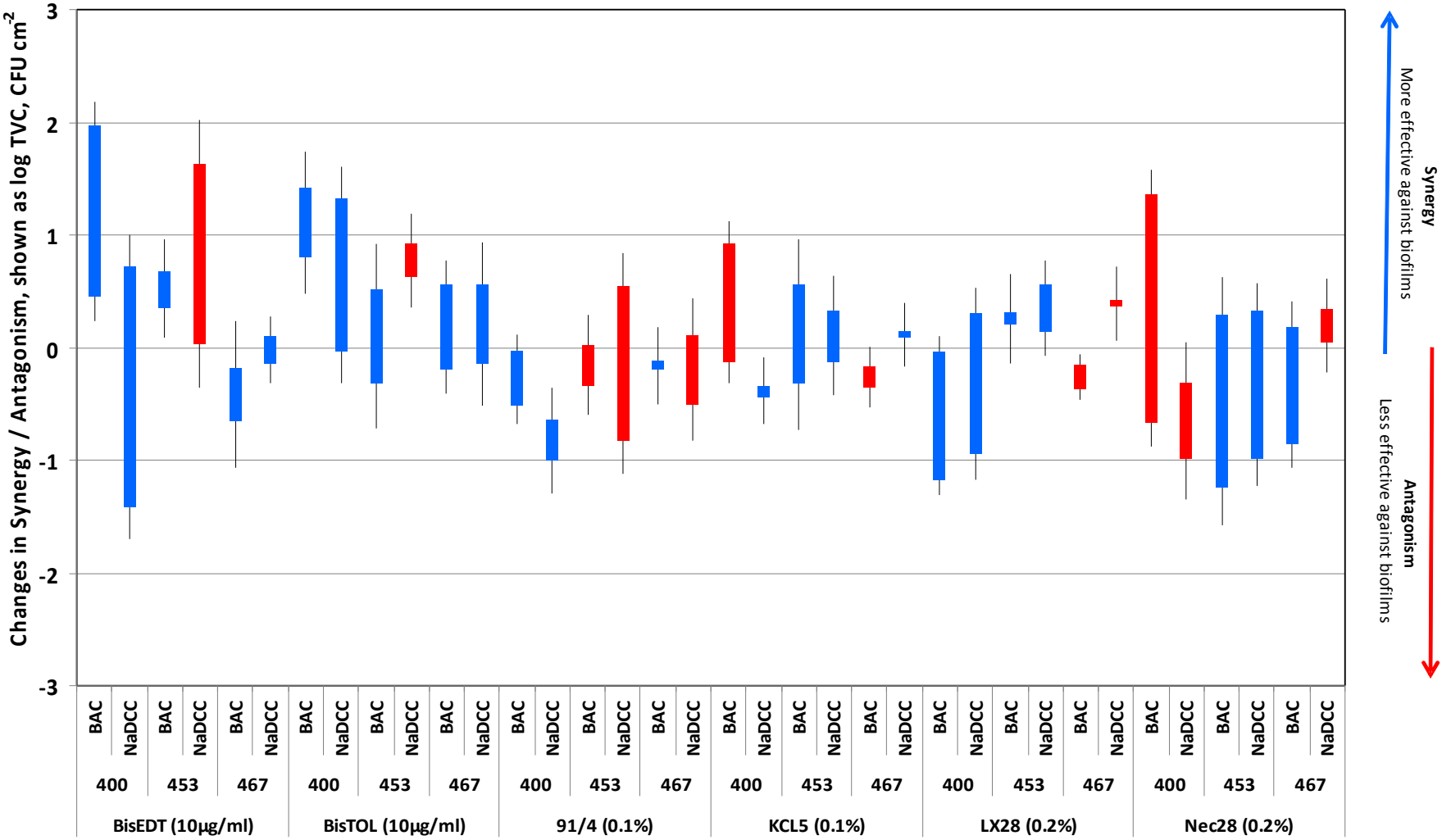

Fig. 5.22: Synergistic/antagonistic effects of combined detergent/disinfectant treatments for biofilm compared to attached *E. coli*, shown as difference in log TVC synergy also showing the change in synergistic/antagonistic effects of attached compared to biofilm populations. Blue bars show the increase in synergy for biofilms compared to attached while red bars show a decrease, for example strain 400, BisEDT, NaDCC attached antagonism of -1.4 log increases to biofilm synergy of almost 0.7 log.

5.8 Attachment and adhesive strength of O157 and other *E. coli* strains

The ability of strains of O157 to adhere to stainless steel and the relative strength of attachment after 1 hour was compared to strains 400, 453 and 467 as well as five typed strains of *E. coli*. Three entero-haemorrhagic ribotyped strains of O157, a wildtype vero toxin negative strain and a luminescent vero toxin negative strain, containing the plasmid pSB311, were chosen for this experiment as representative of O157 strains.

After 1 hour attachment the strains were all treated using the negative control method, where water of standard hardness was used to represent 20 minutes detergent followed by 5 minutes disinfectant treatments. Total viable counts were taken from replicate steel surfaces before and after treatment to determine the initial population density and the population density after treatment.

The results (fig. 5.23) show there to be large differences in initial population density after 1 hour attachment and the remaining population after treatment when comparing the O157 strains with the other *E. coli* strains. Significant reductions ($P < 0.01$) in TVC for all five strains of O157 after treatment were observed. With the exception of O157:H-, 12628, which showed a 0.93 log drop in TVC, the TVC for all strains of O157 was observed to drop by over 1.1 log after treatment.

Significant reductions ($P < 0.05$) in TVC were also seen for some non-O157 strains for strains 453, K12 W1485, HB101 NCIB 11865 and NCIB 12079. For each strain the reduction in total viable count was at most 0.61 log. The TVC for all eight non-O157 strains was not significantly different between strains before or after treatment.

A significantly lower ($P < 0.05$) pre treatment population TVC was observed for all O157 strains compared to the non-O157 strains. After treatment all of the O157

strains showed a significantly lower ($P < 0.01$) TVC than the non-O157 strains that was at least 1.5 log lower than that seen for strain 400 after treatment. The reduction in TVC seen for all strains of O157 was significantly greater ($P < 0.01$) than that seen for any other *E. coli* strain.

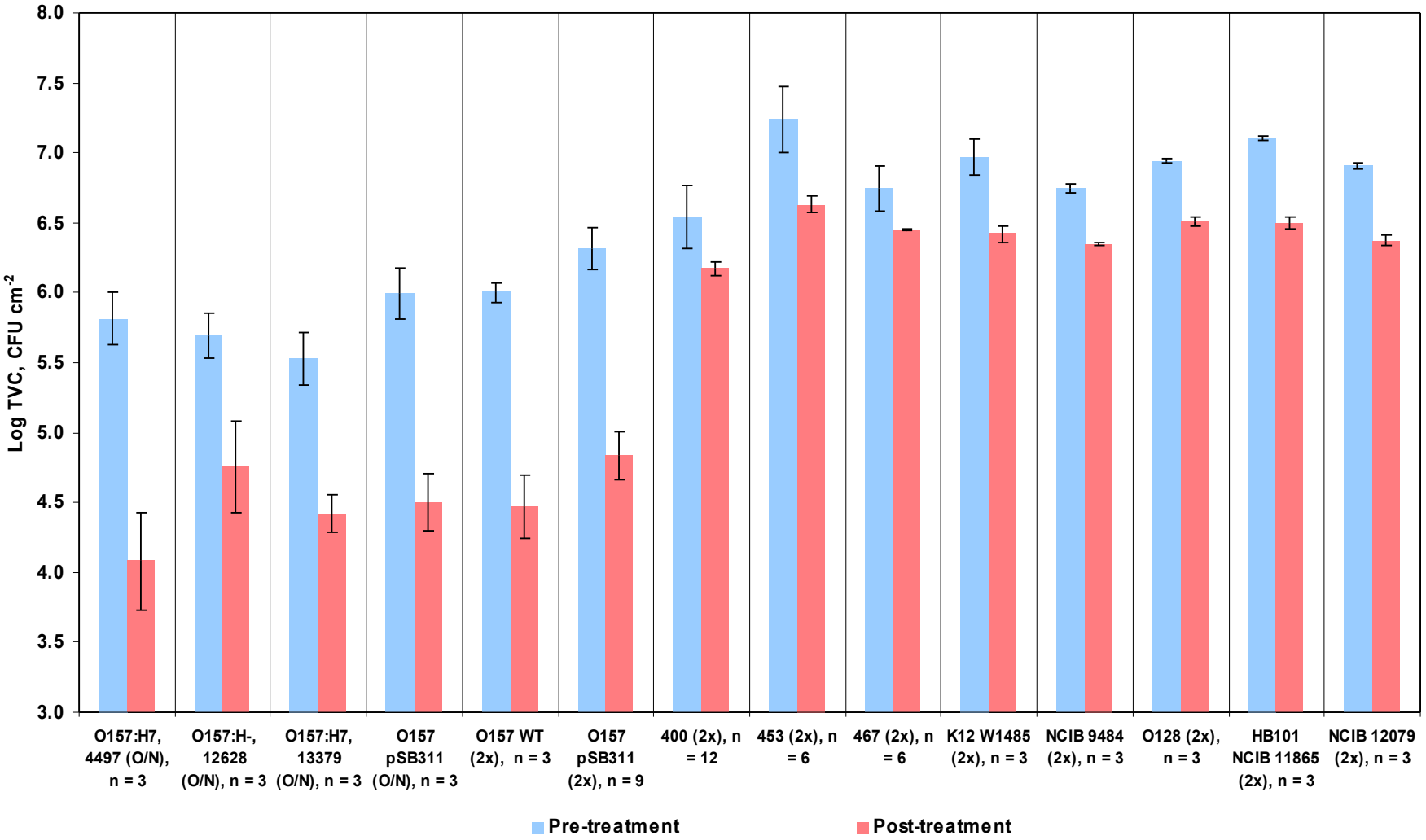

Fig. 5.23: Total viable counts for 1 hour attached populations of six strains of O157, five reference strains of *E. coli* and three factory isolated strains of *E. coli* before and after 20 minutes negative control 'detergent' treatment using a WoSH control followed by 5 minutes negative control 'disinfectant' using a WoSH control treatment.

5.9 *E. coli* O157:H7 pSB311, 1 hour attached and 24 hour biofilm populations

The bioluminescent, vero toxin negative strain, *E. coli* O157 pSB311, was used as a model to determine the effects of treatments on O157 and related vero-toxin producing strains. This was investigated to determine if detergent/disinfectant interactions against O157 strains are likely to be similar or different to those seen for factory isolated non-O157 strains. Identifying changes in interactions will enable more effective sanitisation of factory environments to reduce the risk of vero-toxin producing strains from colonisation, biofilm formation and contamination of food products.

5.9.1 Effect of Detergent Only Treatments

The effect of industrially recommended, in use, concentrations of detergents was determined on one hour attached and 24 hour biofilm populations of *E. coli* O157 pSB311. This was used to determine the degree of killing and/or removal of the adherent population following treatment. Detergent activity was compared to a negative control (zero on the x-axis) in which detergent solutions were substituted with WoSH.

The results (fig. 5.24) show there to be only 3 significant changes in TVC after detergent treatment, all against attached cells, BisEDT, KCL5 ($P < 0.05$) and LX28 ($P < 0.01$), although these were not significantly different to the same treatments when used against biofilms only to the WoSH control. None of the treatments used against biofilms showed any significant change in TVC. No significant differences between attached cells and biofilms were observed. The treatment effects were all comparable to those identified for the three factory isolates of *E. coli*.

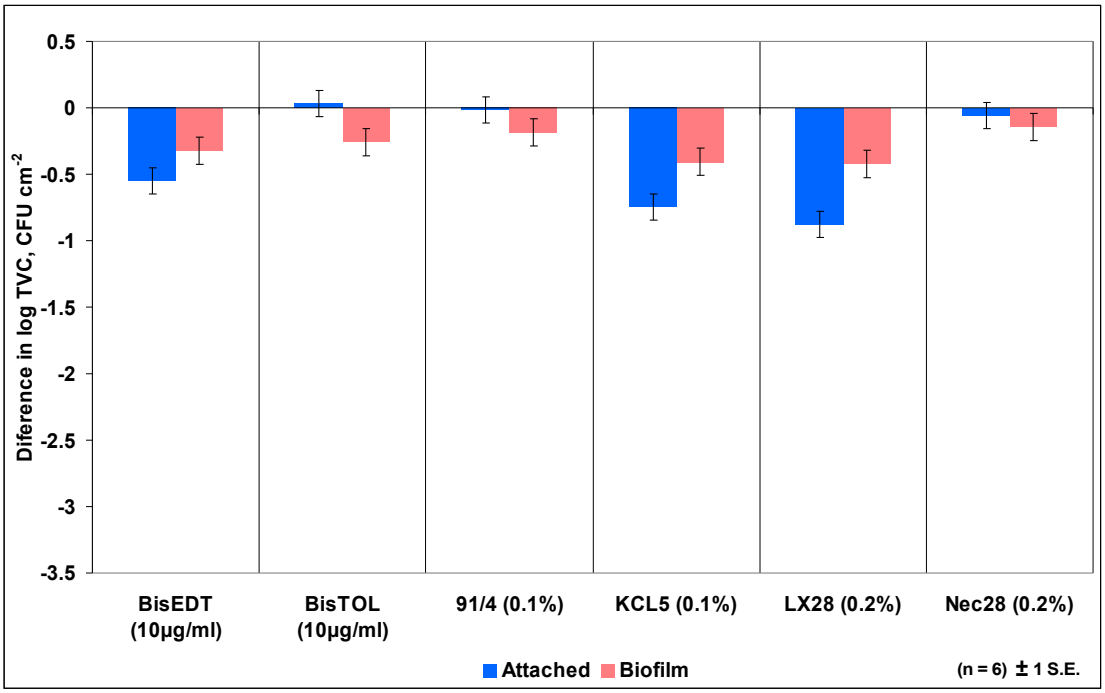

Fig. 5.24: Mean loss of viability after 20 minute contact time with detergent treatments in WoSH for attached and biofilm *E. coli* O157 pSB311 at 20°C, shown as log TVC difference to a negative, water only control.

5.9.2 Effect of Disinfectant Only Treatments

The effect of disinfectants, at concentrations of 0.01% for BAC and 0.0005% for NaDCC, previously identified to give 2 – 4 log kill against *E. coli* in suspension, was determined against one hour attached populations and 24 hour biofilms of the bioluminescent *E. coli* O157 pSB311 to measure biocidal effects against attached and biofilm populations. Disinfectant activity was compared to a negative control (zero on the x-axis) in which disinfectant solutions were substituted with WoSH. The industry recommended contact time of 5 minutes was used.

The data (fig. 5.25) shows there to be no significant differences between disinfectant treatments against attached or biofilm cells of *E. coli* O157 pSB311. The concentration of BAC chosen gave approximately a 1.1 log reduction in TVC for both attached and biofilm cells while approximately a 2 log reduction in TVC was seen when treated with NaDCC.

The loss of TVC identified for NaDCC was comparable to that identified against strains 453 and 467 for one hour attached populations but significantly ($P < 0.05$) increased than observed against all three factory isolates for biofilms. For BAC, a lower, although not significant, reduction in TVC was observed against one hour attached populations than was seen against the three factory isolates, while compared to biofilms a similar loss in TVC was observed with strain 453 and 467 while strain 400 showed a significantly ($P < 0.01$) lower reduction in TVC.

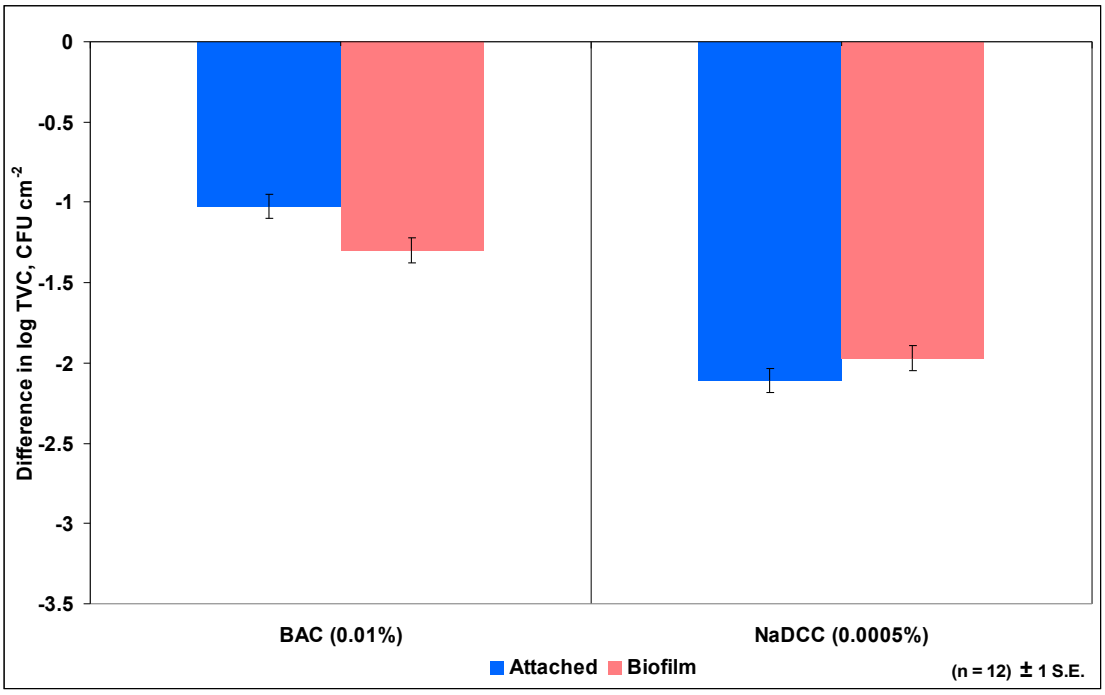

Fig. 5.25: Mean loss of viability after 5 minute contact time with disinfectant treatments in WoSH for attached and biofilm *E. coli* O157 pSB311 at 20°C, shown as log TVC difference to a negative, water only control.

5.9.3 Effect of Detergent Followed by Disinfectant Treatments

The effect of combined detergent (industry recommended in use concentrations) followed by disinfectants, at concentrations of 0.01% for BAC and 0.0005% for NaDCC, previously identified to give 2 – 4 log kill against *E. coli* in suspension, was determined on one hour attached and 24 hour biofilm populations of *E. coli* O157 pSB311. Treatment activity was compared to a negative control (zero on the x-axis)

in which detergent and disinfectant solutions were substituted with water of standard hardness. The industry recommended contact times of 20 minutes for detergents and 5 minutes for disinfectants was used. Observed results were compared to expected results, where the expected results were calculated by combining the changes in TVC for individual detergent (fig. 5.24) and disinfectant (fig. 5.25) treatment to determine any interactions between detergent and disinfectant treatments.

As seen (fig. 5.26) there was only a single significant difference (BisTOL : $P < 0.05$) between the expected effect of detergents and BAC disinfectant treatments against attached and biofilm cells. The observed difference did not show any significant change between attached and biofilm populations. All of the other observed treatments did not show any differences between attached and biofilm cells showing purely additive effects occurred.

Treatment with the bismuth thiol, BisEDT, was shown to be significantly ($P < 0.01$) antagonistic with BAC against attached and biofilm cells. Against attached cells no effect was seen with BisTOL, while against biofilms a significant ($P < 0.01$) antagonism with BAC was observed. Observed treatment effects with both bismuth thiols were lower than those seen against the factory isolates in fig. 5.4 for attached populations and fig. 5.13 for biofilms.

Significant synergies ($P < 0.05$) were observed with the non-ionic detergent 91/4 against attached cells and with KCL5 against biofilms. Compared to the factory isolates, the one hour attached populations were seen to have a similar observed loss in TVC, whereas for biofilms a greater loss in TVC was seen compared to strains 400 and 453 with 91/4 while a greater loss in TVC was observed compared to all three strains when treated with KCL5.

No significant effects were seen between expected and observed results with either of the anionic detergents. Compared to attached populations of the factory isolates a similar observed loss in TVC was seen when treated with LX28, while a greater loss in TVC was observed compared to strains 453 and 467 when treated with Nec28, although strain 400 was seen to have a greater loss in TVC than pSB311. Compared to biofilms of the three factory isolates, treatment with LX28 caused a greater observed loss in TVC for pSB311 while treatment with Nec28 was similar to that seen against strains 453 and 467 but greater than that seen for strain 400.

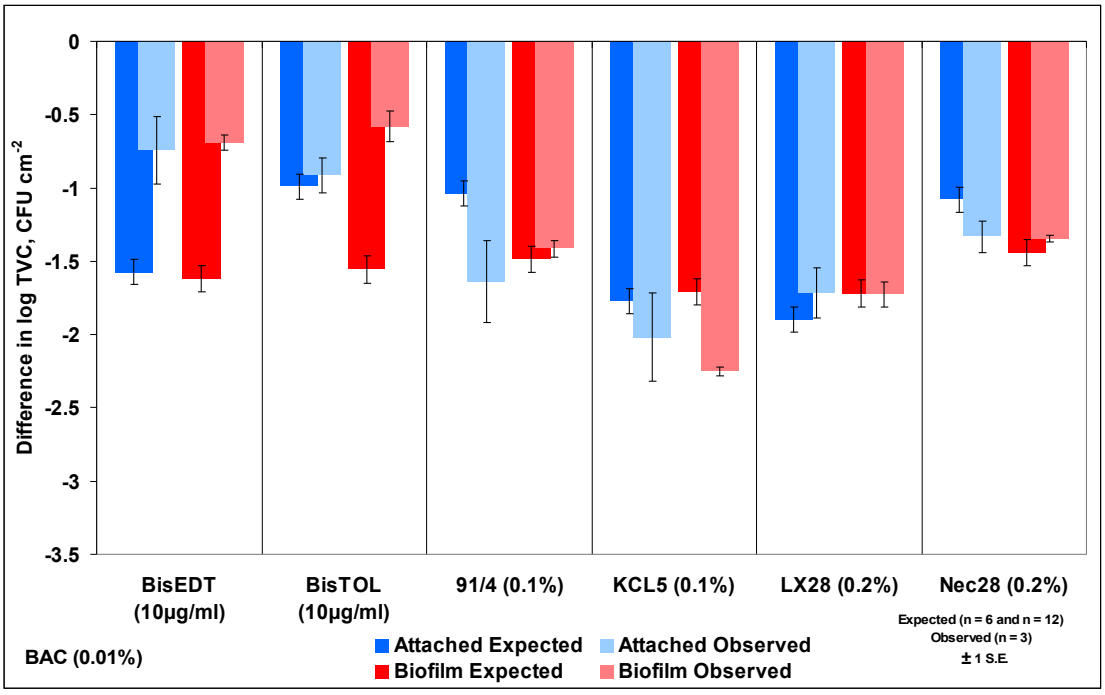

Fig. 5.26: Mean loss of viability after 20 minute detergent treatment followed by 5 minute BAC treatment in WoSH for attached and biofilm *E. coli* O157 pSB311 at 20°C, shown as expected (fig. 5.24 + fig. 5.25) and observed log TVC difference to a negative, water only control.

The results (fig. 5.27), with the exception of the non-ionic detergent KCL5, show there to be no significant differences between observed results for attached and biofilm cells, showing that purely additive affects occurred. The results for KCL5 shows that significantly ($P < 0.05$) less kill was observed for biofilm populations compared to attached cells. Some significant variations in the expected kills for

attached and biofilm cells were seen. For example KCL5 and LX28 were expected to be significantly ($P < 0.05$) less effective against biofilms than attached cells, whereas the observed results showed there to be no difference in loss of TVC.

The bismuth thiol, BisEDT did not show any significant differences between expected and observed effect against attached or biofilm cells. BisTOL was seen to exhibit a significant ($P < 0.01$) antagonism with NaDCC for both attached and biofilm populations. Treatment with BisEDT was seen to reduce TVC against attached populations similarly to the three factory isolates, while against biofilms a greater loss in TVC was seen compared to strains 400 and 453 while strain 467 showed a greater loss in TVC compared to pSB311. For BisTOL, strain 467 was comparable to pSB311 for attached populations while strains 400 and 453 showed greater loss in TVC, when compared to biofilms however strain 453 showed a similar loss in TVC to pSB311 while strains 400 and 467 showed a greater observed loss in TVC.

No significant change was shown for the non-ionic detergent 91/4 against attached cells, while against biofilms a significant ($P < 0.05$) antagonism was observed. Against both attached and biofilm cells a highly significant ($P < 0.01$) antagonism was seen between KCL5 and NaDCC. Compared to the three factory isolates, observed attached results for 91/4 were similar to those seen against strain 453, while strains 400 and 467 showed greater losses in TVC. Observed biofilm results for 91/4 showed that pSB311 was observed to have a greater loss in TVC compared to all three factory isolates. For KCL5 treatments, attached populations of strains 453 and 467 were similar to pSB311 while strain 400 showed a greater loss in TVC, biofilm treatments showed that strains 400 and 453 were similar to pSB311 while strain 467 showed a greater loss in TVC.

Neither anionic detergent were shown to significantly differ from the expected results for attached or biofilm cells. pSB311 was seen to be similar in loss of TVC to strains 400 and 467 for attached populations and show a greater loss in TVC compared to strain 453, for biofilms all three factory isolates showed a lower loss in TVC than pSB311. For attached populations treated with Nec28, pSB311 was similar to strain 467, while strain 453 was seen to have a lower loss in TVC and strain 400 was seen to have a greater loss in TVC. Against biofilms treated with Nec28, pSB311 was similar to strain 467 while both strains 400 and 453 had a lower observed loss in TVC.

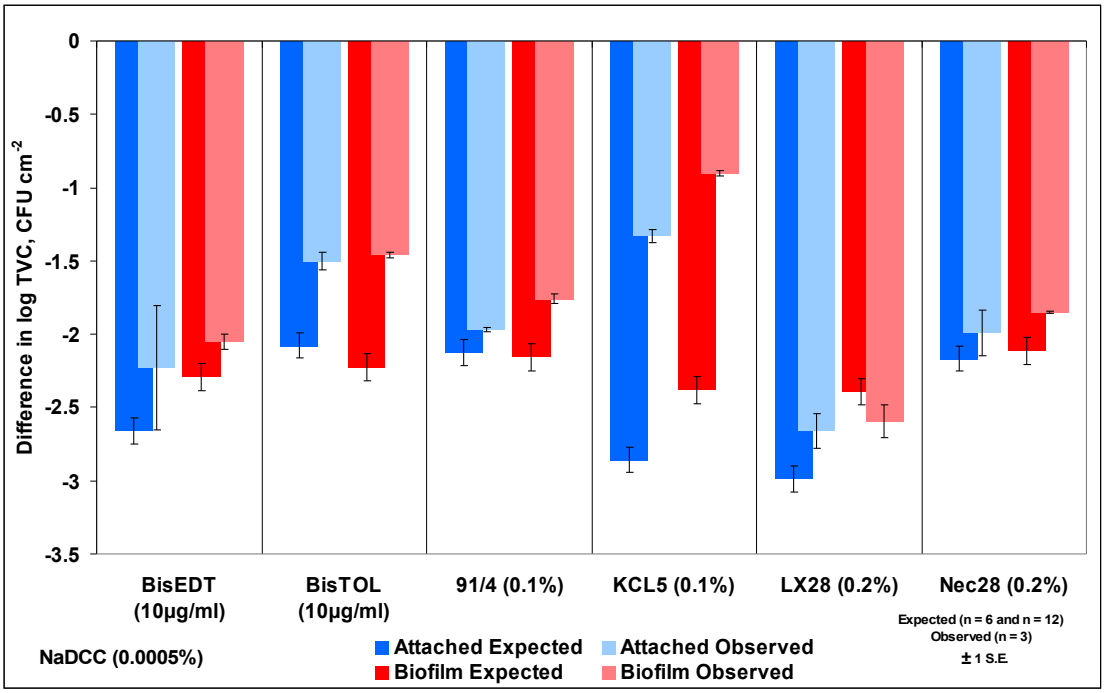

Fig. 5.27: Mean loss of viability after 20 minute detergent treatment followed by 5 minute NaDCC treatment for attached and biofilm *E. coli* O157 pSB311, shown as expected (fig. 5.24 + fig. 5.25) and observed log TVC difference to a negative, water only control.

5.10 Detergent Induced Expression of Stress Response Proteins; HSP60 and HSP70

To identify possible mechanisms involved with disinfectant sensitivity and induced resistance following detergent treatment, the expression of the stress response proteins, HSP60 and HSP70, in *E. coli* strain 453 after treatment with WoSH, Nec28 or 91/4 was assessed. Three population types were tested, suspended, 1 hour attached and 24 hour biofilms to determine if the different states had an impact on detergent mediated stress response protein expression. Whole cell lysate was diluted to a total protein yield of 10mg ml^{-1} before loading onto an SDS-PAGE, after blotting an ELISA was carried out to determine variations in expression of HSP60 and HSP70 between treatments and population states. A scanning densitometer was used to obtain intensity values for each band, the arbitrary values assigned directly correspond to expression rates of HSP60 and HSP70 during each treatment and state compared to the heat shocked positive control sample.

The data in plate 5.3 shows there to be no difference in expression of HSP60 between the positive control, water, Nec28 and 91/4 suspended cell treatments. The results for suspended populations show HSP60 expression at levels similar to the positive control. The results for attached cells show an insignificant expression of HSP60 while those for biofilms do not show any detectable expression of HSP60 when treated with either detergent or the WoSH control.

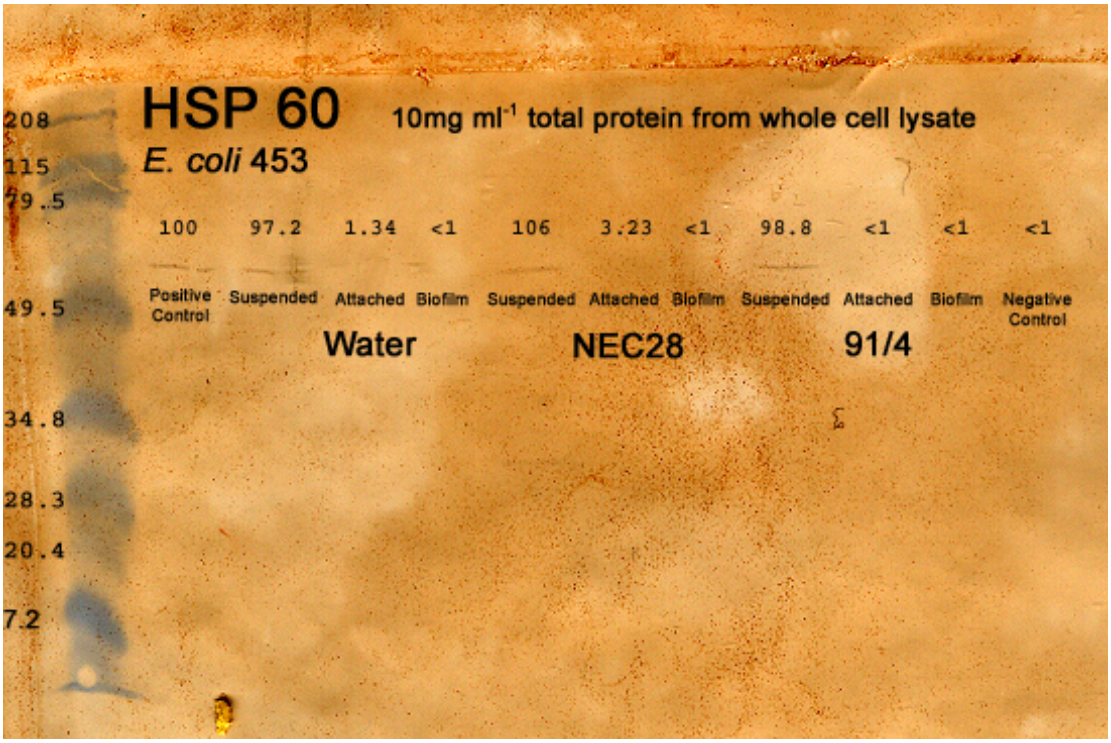

Plate 5.3: The effects of 20 minute detergent contact at 20°C and population state (suspended, attached and biofilm) on the expression of the stress response protein HSP60. HSP60 expression is shown as a percentage of the positive control.

The results in plate 5.4 show that differences in expression of HSP70 occurred between treatments and population states. No detectable HSP70 expression was seen when *E. coli* strain 453 was treated with WoSH as a suspension, attached population or biofilm.

Treatment with the detergent Nec28 was shown to induce 32% more HSP70 in suspension compared to the positive control. The attached population expressed 25.6% and the biofilm only 1.41% of the HSP70 expressed by the positive control. When treated with 91/4 the suspended population of *E. coli* strain 453 expressed 54% more HSP70 than the control, the attached population only expressed 14.5% of the positive control while the biofilm population expressed just 6.45% of the positive control.

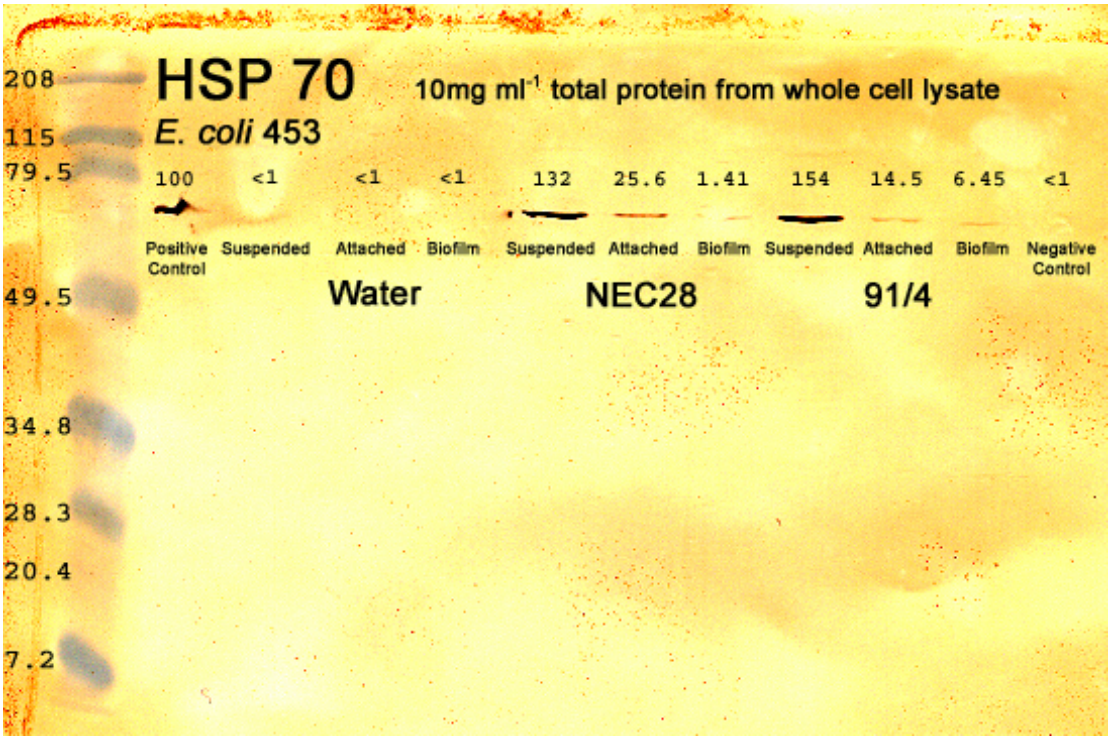

Plate 5.4: The effects of 20 minute detergent contact at 20°C and population state (suspended, attached and biofilm) on the expression of the stress response protein HSP70. HSP70 expression is shown as a percentage of the positive control.

5.11 Discussion

5.11.1 Factory Isolates of *E. coli*

The summary for 1 hour attached populations showed that while there were no specific treatment combination that were effective against all three factory isolated strains, some treatments were seen to be highly effective against specific strains. This suggested that strain differences between *E. coli* will make it very difficult to target a treatment regime to maximise the removal and/or killing of recently adhered strains. This observation suggests that genetic variation between strains of *E. coli* is sufficiently large to result in strain, rather than species, specific interactions with detergents and disinfectants.

The summary for 24 hour biofilm populations identified the detergent BisTOL with either disinfectant as being highly effective at increasing the removal and/or killing of all 3 strains of *E. coli*. Conversely the non-ionic detergent 91/4 was shown to reduce the activity of both disinfectants against all 3 strains of *E. coli*. The summary suggests that it may be possible to target biofilm populations of *E. coli* with specific combinations of detergent and disinfectant to maximise the removal and/or killing of the population.

As expected, detergents which are designed to remove soil prior to disinfection did not exhibit a biocidal effect against strains of *E. coli* in suspension. The one exception appeared to be a strain specific effect, when strain 467 was treated with 91/4, although no supporting literature has been identified that demonstrates a strong biocidal effect of a non-ionic detergent against any bacterial strains. However the loss in TVC recorded after 5 minutes had not increased further by 20 minutes contact time. Combined with the attached population detergent only results for TVC and total direct count, which showed no significant removal, it can be hypothesised that after

exposure to 91/4 a biocidal effect occurred, killing ~ 90% of the population of suspended or attached strain 467, followed by a protective effect, possibly the result of detergent inactivation caused by intracellular material from lysed cells, the expression of stress response pathways or the use of ion channels to actively remove the detergent, which prevented further killing of the remaining population. A comparison of suspended, attached and biofilm detergent only treatments showed that while suspensions and attached populations had a reduction in TVC; there was no loss of TVC observed for biofilm populations. This finding was expected, as suggested by various sources of literature which have shown that cells in biofilms are more resistant than suspensions to external influences such as biocidal chemicals and disinfectant (Aarnisalo *et al*., 2000; Donlan and Costerton, 2002; Prakash *et al*., 2003; Augustin *et al*, 2004) and temperatures (Perrot *et al.*, 1998 and 2001; Chmielewski and Frank, 2004).

The results for strain 400 following treatment with the non-ionic detergent KCL5 and anionic detergent Nec28 showed an apparent significant increase in TVC compared to the control. A possible explanation is that the cells strength of adhesion was increased via a physio-chemical interaction with the detergents, thus resulting in fewer cells sloughing from the surface than were seen with the WoSH control treatment, therefore leading to an apparently higher TVC. Research presented by Eginton *et al*., (1998) also showed an increase in attachment following treatment with the non-ionic detergent Tween-80, supporting this hypothesis, although no explanation was proposed for the mode of action. The comparison between attached and biofilm detergent only treatments showed that against biofilms both the non-ionic KCL5 and the anionic Nec28 detergents reduced the TVC of the population, probably via sloughing of biofilm matrix which was identified as a mechanism of TVC loss by

Vickery *et al*., (2004) following exposure to some detergents, although generally only those with high enzymatic activity.

A comparison of the disinfectant only results for 1 hour attached populations with biofilm populations showed that changes in sensitivity occurred with changes in adherence. Biofilm populations of strain 400 in particular, were seen to be much more resistant to disinfectant treatments compared to attached cells possibly due to a dense EPS matrix that protected the cells by preventing contact with the disinfectants. Previous studies (Poulsen, 1999; Dunne, 2002; Prakash *et al*., 2003) have indeed shown that in general biofilm population's increase in resistance to external stresses, primarily due to the EPS layer and protective effect afforded via complex biofilm structures. A possible explanation for strain 467 showing no significant change in sensitivity to BAC and NaDCC when comparing attached and biofilm populations, is due to reduced EPS intensity compared to strains 400 and 453. The image in plate 5.2 combined with EPS intensity results confirm that this is indeed the case, where less than half of the EPS intensity for strains 400 and 453 was observed for strain 467. The reduced EPS layer observed for strain 467 is unlikely to have been sufficiently thick to prevent contact with the disinfectants, which rendered biofilms of the strain as sensitive to the disinfectant treatments as was seen for attached populations. Observations by Ryu and Beuchat, (2005), identified similar results with regards to chlorine resistance, whereby *E. coli* O157:H7 strains with lower EPS production were seen to be significantly more sensitive to treatment than those with denser EPS layers.

The results described for the disinfectants BAC and NaDCC following detergent treatments, showed there to be statistically significant differences between treatments and also between 1 hour attached and 24 hour biofilm populations of cells. The summaries of synergies and antagonisms showed there to be 7 fewer antagonisms

and 4 more synergies for the biofilm populations than for attached. These observations suggest that against attached populations, in many cases, 20 minutes contact time with detergents resulted in induced resistance to subsequent disinfectant activity, possibly by expression of stress response pathways. Research by Langsrud *et al*., (2004), has demonstrated that exposure to chemical stresses will result in rapid increases in resistance to disinfectants and antibiotics that support the hypothesis. Against biofilm populations, detergent treatments exhibiting a synergy probably disrupted the protective properties of EPS, although without sufficient contact time to desensitise the population to subsequent disinfectant treatments, as was observed as antagonisms for many attached cell treatment combinations. The results for EPS intensity reinforce the notion that a possible mechanism of synergy against biofilm populations is the disruption and/or removal of EPS, Armon *et al*., (1998) previously identified an increase in disinfection activity against biofilms after treatment with the detergent EDTA which was due to EPS disruption. However, although treatment synergies were more frequently observed against biofilm populations, treatments used against biofilm populations were seen to be less effective when total loss of TVC is compared with attached populations, showing that a biofilm population is inherently more resistant to stressors even when treatments are seen to be synergistic.

The two bismuth thiol detergents, BisEDT and BisTOL, which have been previously shown to disrupt EPS production via pleimorphic inhibition of metabolic enzymes (Domenico, 1998; Domenico *et al*., 1999 and 2001), were seen to illicit strong synergies against biofilm populations of strains 400 and to a lesser extent strain 453, however against strain 467, BisEDT treatments were not synergistic with either NaDCC or BAC, although BisTOL treatments were seen to exhibit synergies. In general 24 hour biofilm populations of strain 400 were shown to confer greater

protection against treatments with either anionic or non-ionic detergents than for 1 hour attached populations. It was shown that for these treatments that there was no reduction in the intensity of EPS, therefore it is probable that the EPS layer was unaffected by the detergent treatments and so prevented contact with the disinfectants BAC and NaDCC, hence conferring the observed resistance to the disinfectants. Strain 453 showed an opposite trend than that seen for strain 400, with most non-ionic and anionic treatment combinations being observed as synergistic. The EPS intensity results showed a loss of EPS following detergent treatment, suggesting that these observations are the result of EPS disruption by the detergents prior to disinfectant treatment.

Strain 467 was shown to produce the least intense EPS, therefore inferred as least dense EPS layer, as a biofilm, as was seen in plate 5.2. This observation explains the similarity between TVC loss and antagonisms/synergies seen when comparing 1 hour attached and 24 hour biofilm populations. In general only a slight tendency towards synergy was observed for treatments against biofilm populations of strain 467, further enhancing the hypothesis that a sufficiently dense EPS layer is required to significantly alter the populations response to treatments compared to 1 hour attached populations. Ryu and Beuchat (2005) have shown a similar response with *E. coli* O157:H7 mutants that produce excess EPS, whereby a greater degree of protection was afforded against chlorine than strains producing less EPS.

5.11.2 *E. coli* O157 Strains

The comparison of 1 hour attached O157 strains of *E. coli* with 8 non-O157 strains of *E. coli* showed there to be significant statistical differences between the two groups of strains. All 8 non-O157 strains were seen to produce initial adherent population densities that did not differ statistically between strains. After control

treatments, there were still no significant differences in TVC between all 8 non-O157 strains. Based upon the selection of non-O157 strains it can be suggested that most strains of *E. coli* initially form similarly sized adherent populations and are affected to the same degree by control treatments.

Similarly, each O157 strain produced an adherent population that was not significantly different in size to the other O157 strains, either pre or post treatment. When comparing the two groups of strains however it was immediately apparent that *E. coli* O157 strains produce a statistically ($P < 0.05$) less dense initial surface adhered population and a significantly ($P < 0.01$) greater loss in TVC was seen after control treatments compared to the non-O157 strains. Stoitsova *et al*., (2007) also compared the ability of pathogenic and non-pathogenic strains of *E. coli* to form biofilms, with similar results whereby the non-pathogenic strains were identified as producing the denser biofilms. The two contributing factors of a lower initial adherent population and greater removal after treatment, suggests that persistent populations of O157 are less likely to occur than non-pathogenic strains. One possible explanation is reduced capacity for surface attachment and surface adaptation for O157 strains. Durso *et al*., (2004) have also shown that pathogenic O157 strains have reduced fitness, reduced carbon utilization and compete poorly against non-pathogenic strains. Even though it can be surmised that non-pathogenic strains are likely to dominate and out compete O157 strains, even very low numbers of vero toxin producing strains pose a serious health risk (Phillips, 1999; Caprioli *et al.,* 2005).

As with the three factory isolated strains of *E. coli*, O157 pSB311 was seen to conform to a similar loss in viable count following detergent only treatment against attached and biofilm populations, with fewer cells being lost as a biofilm. For the disinfectant only results a slightly lower loss in viable count was seen compared to

factory isolates for 1 hour attached cells, although over 1.5 log was lost after water only treatments, therefore the total loss in viable count can be estimated as 2.5 - 3.0 log for BAC and 3.5 - 4.0 log for NaDCC, which was considerably greater than that seen for the 3 factory isolates.

There was no observed difference in the loss in TVC for 1 hour attached or 24 hour biofilms of O157 pSB311, suggesting that as with strain 467, during biofilm formation insufficient EPS was excreted to affect treatments (Ryu and Beuchat, 2005), further reducing the likelihood of persistent biofilm formation. This observation was further enhanced when the observed loss in TVC did not differ significantly between 1 hour attached and 24 hour biofilm populations for all but two treatments. Again the loss in TVC appears to be lower than that seen for the factory isolates, 400, 453 and 467, however once the additional 1.5 log loss in TVC, following water only treatments, is taken into account, the results for O157 pSB311 show that overall the treatments had a greater effect at removal and/or killing of the starting population compared to the other strains.

5.11.3 Induction of HSP60 and HSP70 by Detergents.

Stress mediated expression of the heat shock protein; HSP70, occurred after strain 453 was exposed to the detergents Nec28 and 91/4, while the results for HSP60 demonstrate that only some stress response pathways are induced following exposure to the detergents. Indeed the most plausible explanation for the HSP60 expression detected for suspensions of strain 453 is due to osmotic stress as has been identified previously following osmotic stress in *Clostridium difficile* (Hennequin *et al*., 2001). The HSP70 results comparing 1 hour attached and 24 hour biofilm populations also enforces the suggestion that desensitisation to disinfectants occurs after sufficient contact time with detergents has elapsed for mediated expression of stress response

proteins prior to disinfectant treatments. The results for the water only treated populations suggest osmotic stress does not result in the expression of HSP70.

The HSP70 data supports the theory that EPS is likely to prevent or delay contact with detergents during 20 minute treatment time, preventing biofilm populations from expressing sufficient stress response proteins to gain resistance to subsequent disinfectant treatments. *E. coli* exposed to increased radiation, temperature extremes, chemicals, osmotic stresses and microgravity have been shown to be more resistant to biocides coupled with an increased expression of HSP70 (Matin and Lynch, 2005; Bianco *et al.,* 2006), this suggests that mediated stress response protein expression is a common factor from multiple possible stressors for increased resistances to subsequent contact with biocides.

It was observed that for 1 hour attached populations of strain 453, the detergent Nec28 mediated more expression of HSP70 than the detergent 91/4, this suggests that desensitisation to disinfectants can be reduced by selecting detergents that minimise the expression of stress response proteins while still effectively disrupting EPS. Further research would be required to identify detergents that minimally impact stress response protein expression between different species and strains.

6.0 General Discussion

6.1 Bismuth thiols

The bismuth thiol detergents developed by Winthrop University Hospital, New York, were shown to be promising candidates for use within the food industry. Compared to conventional non-ionic and anionic detergents, it was shown both BisEDT and BisTOL performed well and in many instances were seen to be better than 91/4, KCL5, LX28 and Nec28, especially against strains of *E. coli*.

The potential for bismuth thiols to inhibit growth and biofilm formation (Domenico, 1998; Domenico *et al.,* 2001) of *E. coli* and *L. monocytogenes* was investigated. It was discovered that at concentrations at or below those recommended for use (10 μg ml^{-1}), suspensions of all strains tested in TSB were inhibited from growing for 24 hours. By 48 hours only one strain, *E. coli* 467, was able to grow above the in use concentration. This showed that bismuth thiols are not rapidly inactivated by contact with organic material. However, experimentation to induce resistance to bismuth thiols did show that growth in increasing concentrations was possible after prolonged contact.

Against 1 hour attached populations of *E. coli* and *L. monocytogenes* neither bismuth thiol was shown to reduce viable counts. Some synergies with the disinfectants BAC and NaDCC were seen, although in general synergies were stronger against strains of *E. coli* than against *L. monocytogenes*. Given bismuth thiols have been shown to be primarily effective against biofilms rather than simple attached populations of cells (Domenico *et al.,* 1998), the activity shown against attached populations conformed to expectations.

When used against 24 hour biofilms, the bimuth thiols were not shown to reduce viable counts. While none of the treatments were shown to be antagonistic for either

bismuth thiol with disinfectants against *E. coli* strains, a number of antagonisms were observed against *L. monocytogenes* strains. BisTOL treatments were also shown to be synergistic against all strains of *E. coli* with either disinfectant. The results seen for bismuth thiols showed them to be an effective detergent treatment against *E. coli* strains but more variable against *L. monocytogenes*. This enhances the rational for carefully choosing a cleaning and disinfection regime, dependant upon the species or possibly even strain of organism that requires eradication.

6.2 Non-ionic

Neither anionic detergent was seen to reduce the TVC of 1 hour attached or 24 hour biofilm populations of any of the *E. coli* or *L. monocytogenes* strains tested. Given that no biocidal properties have been identified for non-ionic detergents this was expected (Fraise, 1999). However it was predicted that some removal of adherent populations would occur following treatment. Indeed none of the detergents tested demonstrated any significant removal of adherent cells, suggesting that without mechanical cleaning, as demonstrated via TVC swabbing, populations of cells are not dislodged by detergent treatment alone. Therefore to further optimise cleaning and disinfection a mechanical action, for example via foam application of detergent, would be recommended.

Against attached populations of *E. coli*, treatment with either non-ionic detergent resulted in a mixture of effects with the disinfectant BAC. When the same treatments were used against attached populations of *L. monocytogenes* all but one strain (91/4, strain 166) were shown to result in significant antagonisms with BAC. Although the non-ionic detergents did not demonstrate a biocidal effect, it is likely that after detergent treatment, stress response pathways were induced which resulted in the antagonisms observed, as demonstrated in chapter 5 for *E. coli* where HSP70

was induced. However there are other alternative explanations for the observed results which could include a morphological change of the EPS, resulting in decreased permeability to BAC or detergent residue blocking bacterial transport channels that would otherwise internalise the disinfectant. Unlike the anionic detergents it is unlikely that an inactivation of the cationic disinfectant BAC was the cause of the antagonisms.

In general no interaction was observed when treating biofilms of *E. coli* with the non-ionic detergents and BAC. However treatments against strain 453 were seen to result in an antagonism for 91/4 and a strong synergy for KCL5. The synergy may be the result of disruption of the EPS matrix as was shown when measuring the EPS intensity following treatment in chapter 5, leading to increased disinfectant contact and so a greater loss of TVC. Against biofilms of *L. monocytogenes* the non-ionic detergent KCL5 was seen to have no interaction with BAC, while the detergent 91/4 was seen to be synergistic against all 3 strains. For each strain, the EPS density following treatment with 91/4 was seen to be greatly lowered, therefore it can be inferred that the cause of the synergies was a disruption of the EPS matrix and so an increase in the disinfectant contact with the attached population during treatment.

The results for treatments with the non-ionic detergents and NaDCC show a mixture of effects against 1 hour attached *E. coli* strains and a general trend towards synergies against 1 hour attached strains of *L. monocytogenes*. The most plausible explanations for the synergies to occur are detergent induced increase in permeability of the disinfectant or cell membrane damage following detergent treatment which weakened the population, enhancing disinfectant killing.

Non-ionic detergents with NaDCC against biofilms of *E. coli* showed that for KCL5 there was a mixture of effects while for 91/4 strong antagonisms were observed

against all 3 strains. It is possible that the antagonism was a result of induction of a stress response; however given that the data for 1 hour attached populations did not show the same response, it is unlikely to be the cause. An alternative explanation is that the 91/4 treatment altered the properties of the EPS matrix, therefore decreasing the rate at which NaDCC was able to diffuse and so resulted in an antagonistic effect. Against biofilms of *L. monocytogenes*, most of the treatments with non-ionic detergents and NaDCC resulted in synergies. Given that strain 359 was identified as producing the most dense EPS matrix and that it was seen to result in the greatest synergy between detergents and disinfectants in most instances, it can be surmised that the synergies observed for non-ionic detergents with NaDCC were the result of a disruption of the EPS matrix, resulting in an increased rate of diffusion of the disinfectant and so a greater loss in TVC.

6.3 Anionic

During testing of anionic and non-ionic detergents for biocidal effects, the anionic detergent LX28 was found to show activity against all three *L. monocytogenes* strains after 5 and 20 minutes contact time. The identification of detergents that increase the killing of specific species or groups of microorganisms, offers the potential for designing specifically targeted cleaning and disinfection regimes to increase the removal and disinfection of persistent biofilms. *L. monocytogenes* persistent biofilms are frequently identified within chilled food processing plants (Holah *et al.,* 2004), where experimental data such as this can be applied to improve removal and disinfection.

The anionic detergent, LX28, was shown to exhibit biocidal activity against all strains of *L. monocytogenes*, while none was seen against *E. coli* strains. Slight biocidal activity against Gram positive bacteria was expected, whereby anionic

detergents can interfere with the cell membrane (Pasanen *et al.,* 1997), although the strength of activity exhibited by LX28 against *L. monocytogenes* strains was unexpected, suggesting a particular sensitivity to LX28 was the cause. This effect was observed against attached populations of all 3 strains but only against strains 166 and 367 for biofilm populations. Strain 359 was not affected by contact with LX28 as a biofilm; the most likely cause of the observed resistance was due to strain 359 producing a denser EPS matrix than strains 166 and 367, therefore direct cell contact with LX28 was prevented during the 20 minutes detergent treatment, even taking into account possible EPS disruption. No other detergents were seen to reduce the TVC of 1 hour attached or 24 hour biofilm populations of *E. coli* or *L. monocytogenes* strains.

Combining anionic detergents with the disinfectant BAC was shown to produce inconsistent results against attached strains of *E. coli* and had little effect against biofilms. Against all 3 attached strains of *L. monocytogenes*, both anionic detergents were seen to exhibit strong antagonisms. Given that BAC is a cationic disinfectant, this suggests that one possibility is that sufficient residue, adhered to the Gram positive cell membranes, of the anionic detergents LX28 or Nec28 remained after rinsing, which resulted in a partial inactivation of the disinfectant, causing the observed antagonisms. An alternative possibility is that due to the weak biocidal properties of anionic detergents against Gram positive bacteria (Pasanen *et al.,* 1997), desensitisation to BAC occurred after induction of stress response pathways following detergent treatment. Both suggested causes are further enforced when the results against *L. monocytogenes* biofilm populations are taken into account. Both anionic detergents were shown to exhibit mixed effects, suggesting that either the EPS matrix present in biofilms prevented residues of the detergents remaining after rinsing, or that the detergents were prevented from coming into direct contact with the cells for

sufficient time to illicit a stress response and so did not desensitise the population to BAC. As was shown in chapter 5, the production of HSP70 was decreased for biofilm populations supporting the idea that insufficient contact time elapsed for expression of a stress response pathway. Additional experimentation could be undertaken to attempt to determine the porosity of EPS to each detergent by preparing a biofilm on a filter membrane and then comparing diffusion rates of detergent with a control membrane, however given biofilm structure differs depending on substrate (Donlan, 2002) this would be unlikely to be an accurate representation of a biofilm on stainless steel.

Against attached populations of *E. coli*, the anionic detergents showed a mixture of effects with NaDCC. The *L. monocytogenes* attached treatments were all observed to be strong synergies, with the one exception of strain 359 when treated with LX28. Observed antagonisms could be caused by desensitisation of the population to NaDCC following exposure to detergents, causing the expression of stress response proteins. Although it is not clear what caused the synergies, it is possible that this is the result of increasing permeabilisation of the cell membrane, causing Na^+ and K^+ leakage as well as increasing internalisation of the disinfectant resulting in an increase in potency. Given that anionic detergents have a weak biocidal mode of action via the disruption of the cell membrane of Gram positive but not Gram negative bacteria (Pasanen *et al.* 1997); this supports the theory that increased permeability of the cell membrane can cause the observed synergies with NaDCC.

When anionic detergents with NaDCC were used against biofilm populations of *E. coli*, a mixture of effects was seen. Against biofilms of *L. monocytogenes* all of the anionic treatments resulted in strong synergies. It can be surmised that a similar mode of action as observed for the attached populations is the cause of the synergies. Of

note are the results for strain 359, this strain was seen to produce a greater density of EPS than strains 166 and 367 and it was also seen that LX28 treatment did not reduce the TVC of biofilms of this strain but did the other two, although against suspended and attached populations was seen to exhibit strong biocidal properties. The results showed a much greater synergy with either detergent than for strains 166 and 367. Therefore it can be suggested that although detergent was not in contact with the population for sufficient time to illicit a biocidal mode of action, there was sufficient detergent activity to disrupt the membranes or EPS of strain 359, yet not induce stress responses that might otherwise have decreased the synergistic effects observed.

6.4 Conclusions

The investigation showed that there were no specific cleaning and disinfection regimes that will adequately target both *E. coli* and *L. monocytogenes* strains. Indeed there were no specific combinations that were shown to be optimised against attached and biofilm populations of the same species. Direct count results generally showed that detergents do not remove adherent cells from stainless steel and where the EPS density was affected by a treatment; there was rarely a correlation with additional loss of TVC for the affected population. It was also concluded that to maximise the removal and disinfection of persistent strains of a given microorganism, it may be necessary to design a regime to specifically target not just the species, but the strain involved plus combine mechanical with chemical cleaning, which alone was inadequate for removal of adherent populations. As was seen, no detergent treatments, with or without disinfectant treatments, removed adherent populations, so all loss in TVC observed throughout the study was the result of killing rather than removal.

The novel bismuth thiols were seen to be promising detergents to aid in the removal of biofilm *E. coli* strains and warrant further attention for future studies. In general non-ionic and anionic detergents, particularly the non-ionic 91/4 and the anionic LX28, were better optimised for removal and killing of *L. monocytogenes* strains than the bismuth thiols. Against 1 hour attached populations of *E. coli* no specific treatment combination could be selected for optimisation, whereas against 1 hour attached populations of *L. monocytogenes* using the anionic detergent Nec28, with the disinfectant NaDCC, resulted in a highly optimised cleaning and disinfection. Against biofilm populations of *E. coli*, the treatment combination of BisTOL with either BAC or NaDCC were seen to offer optimum cleaning and disinfection, while against biofilms of *L. monocytogenes* the combination of either anionic detergent (LX28 or Nec28) with NaDCC offered the best optimisation for a cleaning and disinfection regime.

Finally, an investigation to identify possible mechanisms of resistance to disinfectant treatments following detergent treatment, showed that different detergents can induce expression of the stress response proteins, HSP60 and HSP70, at differing levels of expression after the same contact time and against different states of adherent populations, i.e. 1 hour attached or 24 hour biofilm populations. This information can be used to identify detergents that result in the lowest expression of stress response proteins possible, yet still have a high efficacy for the removal of soil and other organic material prior to disinfection.

6.5 Further Work

Continued research is required to further establish the interactions between detergents and disinfectants upon additional strains of bacteria that pose a risk to the food industry. Increasing the range of detergents and disinfectants tested will help to

identify additional optimisations of cleaning and disinfection regimes for general use, against specific species and even to target persistent strains within species. Expanding the range of surfaces investigated will enable a greater understanding of how specific surfaces affect the interactions between detergents and disinfectants and so affect the attachment, development and persistency of bacterial populations. The research can be expanded to encompass additional high risk environments where stringent cleaning and disinfection must be maintained, for example by investigating the interaction between detergents and disinfectants as used by the health service upon bacterial species commonly isolated from hospital wards, for example *Staphylococcus aureus*.

The initial investigation into the molecular nature of persistence and acquired resistance to disinfectants following detergent treatment should be expanded. Understanding how surface adhered populations respond to treatments through the expression of metabolic pathways that directly impact the effectiveness of disinfection, will lead to improvements in the application of cleaning agents to minimise induced resistance. Additionally, the identification of chemicals that disrupt or prevent the expression of stress response mechanisms in bacterial cells would lead to improved formulations of detergents that retain the same efficacy for cleaning but do not induce the expression of stress response proteins in treated populations.

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