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Development and Validation of a Biofilm Model to Establish the Effect of Chemical and Physical Treatments on Cellular Viability

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Abstract

Biofilms are multicellular aggregates that can form in a multitude of environments and affect many different areas such as healthcare, domestic and industrial setting causing a huge economic burden. Removing these biofilms from the environment is normally done by chemical cleaning products using mechanical action in domestic, health care and industrial settings. The antibacterial efficacy of these chemical cleaning products is tested using standardised methods to determine their efficacy in a range of settings and conditions and is used to provide antimicrobial claims for the products. Currently, there is a lack of standardised methods for testing antimicrobial efficacy against biofilms, and the aim of this research was to generate a reproducible and relatively cheap method for growing single species and polymicrobial biofilms suitable for testing against chemical and physical disinfectants. *Pseudomonas aeruginosa* and *Staphylococcus aureus* were grown on polycarbonate membrane filters at 36.5°C for 48-72 hours to generate single species and polymicrobial biofilms. Minimum Biofilm Eradication Concentrations (MBECs), of two chemical disinfectants; hydrogen peroxide and a quaternary ammonium compound (QAC), were determined against these biofilms. Combinations of these disinfectants with UV-C exposure were carried out to establish their potential synergistic effect against biofilms. The QAC disinfectant was more efficacious against both biofilm species when compared to hydrogen peroxide, however both disinfectants were less effective against the polymicrobial biofilms. This is due to the two organisms having a synergistic effect, especially *S. aureus* which can revert to small colony variants and in this reduced state can become much more tolerant to antimicrobials. There was a synergistic effect using hydrogen peroxide and UV-C treatment on all of the biofilms tested possibly be due to the production of hydroxyl free radicals, although hydrogen peroxide was less effective in eradicating the biofilms than the QAC. The QAC in combination with UV-C also showed synergism, although the addition of the UV-C after the QAC seemed to be time dependent and was not as effective after a 1-hour contact time. This research supports previous studies in demonstrating that the combination of UV-C with the disinfectant is more

efficacious than the chemical disinfectant by itself. This could be further analysed in future projects by optimising the minimum contact needed for biofilm eradication.

Abbreviations and Definitions

AOP	Advanced oxidation process
ASTM	American society for testing and materials
ATP	Adenosine triphosphate
CBD	Calgary biofilm device
CDC	Center for disease control
CF	Cystic fibrosis
CFU	Colony forming units
CLSM	Confocal laser scanning microscope
CVCs	Central venous catheter
DI	Deionised water
DSB	Dry surface biofilm
DWDS	Drinking water distribution system
e-DNA	Environmental deoxyribonucleic acid
EPS	Extracellular polymeric substances
e-RNA	Environmental ribonucleic acid
EU	European Union
GBNs	Graphene-based nanomaterials
H_2O_2	Hydrogen peroxide
HQNO	2-heptyl-4-hydroxyquinoline *N*-oxide
HW	Synthetic hard water
LEDs	Light emitting diodes
MBEC	Minimum biofilm eradication concentration
MRSA	Methicillin-resistant *S. aureus*
•OH	Hydroxyl free radicals
PBS	Phosphate buffered saline
PC	Polycarbonate
QAC	Quaternary ammonium compound disinfectant
Synergism	The interactions of two or more substances to produce a combined effect greater than the sum of their separate effects.
TSA	Tryptone soy agar

TSB	Tryptone soy broth
UV	Ultraviolet light

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1. Introduction

1.1 The biofilm mode of growth

Bacteria and their mode of growth has been categorised over the years by microbiologists into two different forms; single planktonic cells and multicellular aggregates known as a biofilm (Høiby et al., 2015). Biofilms occur in an environment where the planktonic cells are sessile (Kolpen et al., 2022) and from here they are known to form aggregates and produce a polymer extra-cellular matrix. Biofilm production is an integral process in the prokaryotic life cycle and is a decisive component for survival in diverse environments (Hall-Stoodley, Costerton and Stoodley, 2004). *Pseudomonas aeruginosa* was one of the first biofilm forming bacteria studied to categorise distinct and observable changes during the formation of a biofilm. It was originally found that the formation of a biofilm takes place in five stages, based on the requirement of a surface for the microbes to attach to: (i) reversible attachment, (ii) irreversible attachment, (iii) maturation-1/proliferation, (iv) maturation-2 and (v) dispersion (Sauer et al., 2002). These stages are outlined in **Figure 1**.

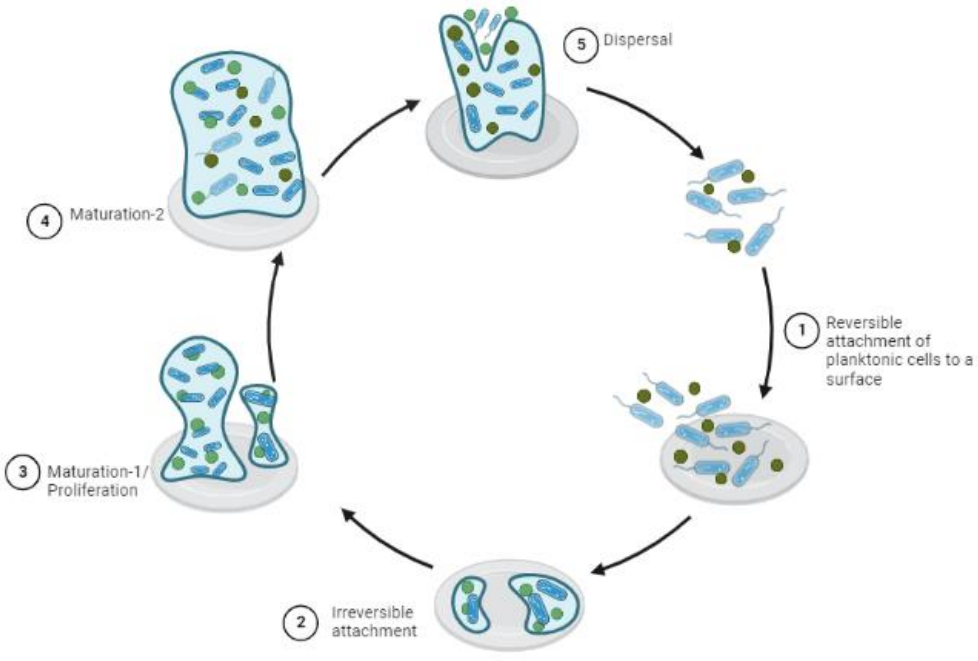

Figure 1.1. The five-stage biofilm growth model. Adapted from Sauer et al (2002), the five stages: (1) Reversible attachment of planktonic cells to a surface, (2) Irreversible attachment, (3) Maturation-1/proliferation, (4) Maturation-2 and (5) Dispersal of *P. aeruginosa.* Adapted from "Circular Pathway with Patient" by BioRender.com (2022) Blue rods indicate *Bacilli* bacteria with and without flagella. The light blue cells are planktonic cells and the darker blue rods are biofilm cells. The green circles indicate cocci bacteria with the darker green cocci being planktonic cells and the lighter green cocci being biofilm cells.

However, this model has been recently criticised due to the limitations surrounding the consideration of different habitats, growth conditions and microenvironments (Sauer et al., 2022). The previous model (**Figure 1.1.**) considers a biofilm to be reliant on a surface to be able to colonise, however a proposed new model (**Figure 1.2**) acknowledges that aggregates may also form in fluids and that aggregation and co-aggregation may be induced by bacterial extracellular polymeric substances (EPS) or host fluids (Sauer et al., 2022). Aggregates not associated with a surface have recently been recognised in clinical settings and can be prevalent in infections of the respiratory tract or soft tissue (Lebeaux, et al., 2013). Instead, this new model proposes just three main events in the formation of a biofilm, independently of surfaces and initiation from single cell planktonic bacteria (**Figure 1.2**). The updated model accounts for the multiple processes of biofilm growth being different and not occurring sequentially and can be used to depict the different scenarios for growth *in vivo*, *in situ* or *in vitro*.

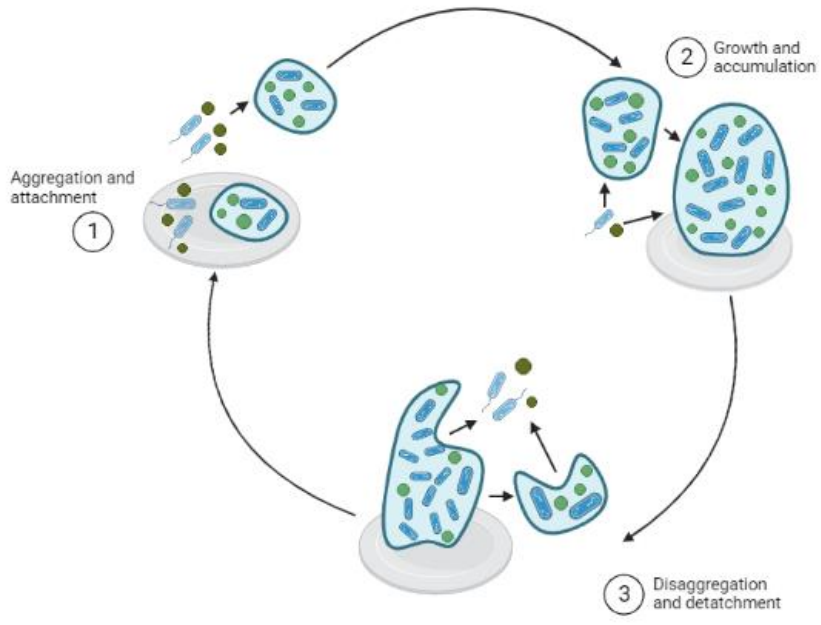

Figure 1.2 The proposed updated three-stage biofilm growth model. Adapted from Sauer et al. (2022), the proposed new three stages: (1) Aggregation and attachment, (2) Growth and accumulation and (3) Disaggregation and detachment. Made on BioRender.com (2022). Blue rods indicate *Bacilli* bacteria with and without flagella. The light blue cells are planktonic cells and the darker blue rods are biofilm cells. The green circles indicate cocci bacteria with the darker green cocci being planktonic cells and the lighter green cocci being biofilm cells.

1.1.2 Significance of biofilm production

Although biofilms may have advantages in some areas such as the potential application of a bio fertiliser in the agricultural setting (Pandit et al., 2020), it is the detrimental effects of biofilms to human and animal health that has demanded more attention. The economic burden of biofilms is estimated to be in excess of $5000bn a year, which is due to the prevalence in areas such as healthcare, the built environment, food and agriculture and water systems (Cámara et al., 2022).

Biofilms in the healthcare industry are of great interest and importance as 78.2% of chronic wounds are associated with biofilms (Malone et al., 2017). Biofilm infections from medical devices such as central venous catheters (CVCs), pacemakers and endotracheal tubes are one of the largest financial burdens and have been associated with an increase in patient mortality and morbidity (Donlan, 2008).

The spread of bacteria, and the potential of biofilm formation, can be through person-to-person interactions or through indirect contact with surfaces or air-borne contaminants. Biofilms cannot only survive and contaminate surfaces but also drinking water distribution systems (DWDS), which can cause illness to humans through drinking water, showering and swimming pools (Speight, et al. 2019). The negative impact of biofilms can be aggravated in areas with a high volume of people where their control is critical. These include places such as hospitals, schools, work places and establishments that work with food where biofilm treatment is normally done by manual cleaning using specialised cleaning products which have been estimated to cost $41.5bn annually (Cámara et al., 2022).

1.1.3 Biofilms components

It is well recognised that bacteria in most environments produce multicellular aggregates held together by a self-produced extra-cellular matrix, called biofilms. This matrix is formed of extra-cellular polymeric substances (EPS) such as nucleic acids (e-DNA and e-RNA), exopolysaccharides, proteins, lipids and polysaccharides (**Table 1.1**), the production of which is triggered by

environmental signals (Costa, Raaijmakers and Kuramae, 2018; Flemming et al., 2016; Karygianni et al., 2020). The EPS is predominant in most stages of the biofilm formation as it embraces the organisms, facilitates their attachment to a surface, forms a central part of the EPS matrix and protects them against stressors (Decho and Gutierrez, 2017). This matrix component can often account for over 90% of the biofilm and its integrity is vital in the survival of the biofilm (Flemming and Windenger, 2010) as it can provide stable and complex microenvironments that are fundamental for the biofilm lifestyle (Koo and Yamada, 2016). The matrix can also be stabilised by extra-cellular bacterial structures such as flagella, fimbriae and pili (Zogaj et al., 2001).

Table 1.1 Biofilm matrix components and their role in the biofilm. The components included are exopolysaccharides (Skillman, Sutherland and Jones, 1998), enzymes (Flemming and Wingender, 2010), non-enzymatic proteins (Lasa and Penadés, 2006), e-DNA (Whitchurch et al, 2002.) and surfactants and lipids (Flemming and Wingender, 2010).

Biofilm Matrix Component	Role in the Biofilm
Exopolysaccharides	Associated with adhesion to surfaces and maintenance of the structural integrity
Enzymes	Involved in the degradation of biopolymers that are assimilated and used as sources of carbon and energy
Non-Enzymatic Proteins	Involved in the formation and stabilisation of the matrix. They are a bond between extracellular EPS and the bacterial surface and aid in adhesion to inanimate surfaces and host cells
e-DNA	Can act as an intercellular connector. Can also act as an adhesive or an antimicrobial agent which can cause cell lysis
Surfactants and Lipids	Essential in aiding with adhesion to hydrophobic surfaces and can promote initial formation of microcolonies and contributing to biofilm dispersal

1.2 Impact of biofilms in the clinic

Biofilm formation has a considerable impact on humans due to their formation in natural, industrial and medical settings, especially in hospital acquired infections, which have recently been increasing (Assefa and Amare, 2022). *P. aeruginosa* is a very common pathogen associated with hospital-acquired illnesses and can affect patients suffering with cystic fibrosis by forming biofilms

in the lungs (Moreau-Marquis, Stanton and O'Toole, 2008). In addition to this, *P. aeruginosa* biofilms can form in chronic wounds and cause long-term infections in patients (Kirketerp-Møller et al., 2008). These biofilms are often the cause of unsuccessful antibiotic treatment and usually require surgical removal of afflicted areas. *Staphylococcus aureus* biofilms are also highly resistant to antibiotics and are therefore the cause of many recurrent infections (Jones et al., 2001). These recurring biofilm-meditated infections include infections of the bones such as osteomyelitis (Lew and Waldvogel, 2004), which can be caused by surgery, trauma or an underlying infection (Ziran, 2007). Other *S. aureus* biofilm infections come mostly from implanted medical devices and orthopaedic implants such as plates, screws, wires, pins, prosthetic joints, stents, central venous cathetors (CVCs), ventilators and pacemakers (Costerton, Montanaro and Arciola, 2005). Infections of this kind often result in the removal of the device to treat the infection effectively, and in some cases, this can cause further complications for patients.

1.2.1 Impact of polymicrobial biofilm production

In nature, microbes rarely exist in isolation but instead are often found in polymicrobial biofilms, which can alter the behaviour of individual bacteria compared to when grown *in vitro* as single species biofilms. Polymicrobial biofilms are the cause of persistent infections in humans in places such as ears, lungs, urinary tract and wounds (Orazi and O'Toole, 2019) however, in some settings, polymicrobial infections can lead to worse outcomes such as the requirements for surgical treatment and a higher rate of mortality (Pammi et al., 2014; Jorge et al., 2018). This is due to the interspecies interactions that occur in a polymicrobial community, which can alter biofilm formation and cause changes in the production of virulence factors leading to resilience to treatment (Korgaonkar et al., 2013).

P. aeruginosa and *S. aureus* are two of the most common bacteria known to cause chronic infections in humans and are frequently found together in locations such as the lungs of cystic fibrosis (CF) patients (Hogan et al., 2016) and in wounds (Trivedi et al., 2014). Polymicrobial biofilms containing *P. aeruginosa* and

S. aureus have been specifically documented for their interactions *in vitro.* Some of these interactions have had a synergistic effect which is making the strains more virulent and resistant to certain antibiotics when compared to their monocultured counterparts (DeLeon et al., 2014). When grown in co-culture with *S. aureus*, the physiology of *P. aeruginosa* has been found to change with regard to iron availability, which changes due to the lysis of the *S. aureus* releasing iron molecules (Mashburn et al., 2005). Other interactions shown by Filkins et al. (2015) include *P. aeruginosa* switching *S. aureus* from aerobic respiration to fermentation due to the production of 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) and siderophores by the former. Michelsen et al. (2014) also demonstrated an increase in antibiotic resistance in *S. aureus* in the presence of HQNO. The interactions between these two species when co-cultured support the idea that, when developing antimicrobial treatments, polymicrobial interactions should be taken into consideration.

1.2.2 Biofilm growth on surfaces

Surfaces in multiple environments such as the home, healthcare settings, workplaces and food manufacturing establishments can be contaminated with biofilms. The biofilms found on these surfaces are likely to be dry surface biofilms (DSB) rather than the highly researched hydrated biofilm, which have been shown to be even more resistant when it comes to treatment, resisting conditions of 100°C dry heat (Almatroudi et al., 2018). These types of biofilms are especially prevalent in healthcare settings amongst commonly used areas and equipment, with a study showing polymicrobial biofilms being recovered from 95% out of 61 sample points in a hospital (Ledwoch et al., 2018). However, *P. aeruginosa* is rarely isolated from these dry biofilms (Hu et al., 2015). To remove these biofilms, specific cleaning protocols must be followed which often include using effective biocides and mechanical action e.g. wiping. However studies such as Hu et al. (2015) identify that current cleaning methods are not effective in eradicating DSBs.

1.3 Recalcitrance of biofilms to antimicrobial agents

Treatment of biofilms is often unsuccessful due to cells exhibiting distinctly different properties under this mode of growth compared to planktonic cells. When in a biofilm, the growth of most of the bacterial population slows down significantly or stops altogether which has been proposed to promote tolerance to antimicrobials. These changes confer antimicrobial resistance, which are genetically coded mechanisms that allow the organism to grow in the presence of an agent, or tolerance, which is the ability to survive transient exposure of an agent by genetically encoded or phenotypic mechanisms (Mah and O'Toole, 2001). The expression of both tolerance and resistance to antimicrobials is referred to as 'recalcitrance', which is exhibited by biofilms (Orazi and O'Toole, 2019). Recalcitrance can occur in biofilms due to the failure of the antimicrobial to penetrate the biofilm. Exchanging genetic material between cells or spontaneous mutations allows recalcitrance due to slow growth in mature biofilms (Brown et al., 1988), because of oxygen depletion, nutrient limitation and an altered stress response (Orazi and O'Toole, 2019; Mah and O'Toole, 2001).

1.3.1 Recalcitrance of biofilms to antibiotics

Monospecies and multispecies biofilms are of great economic significance due to their ability to cause persistent infections, for which healthcare professionals would prescribe antibiotics to treat. Biofilm infections require antibiotics administered at higher concentrations and for a longer period of time than when treating planktonic cells (Hoiby et al., 2015). This is because the penetration of antibiotics is delayed due to the presence of the biofilm matrix, the poor availability of bacterial targets and the presence of enzymes such as β-lactamases, which inactivate antibiotics before they target the bacterial cells (Ciofu et al., 2022).

1.3.2 Recalcitrance of biofilms to disinfectants

The term 'biocide' refers to the chemical agent, which inhibits or kills microbes (White and McDermott, 2001) and cleaning using different types of biocide agents is the main way to control biofilm growth on dry surfaces in

environments such as the home, hospitals, schools and workplaces. However, numerous reports have been published outlining the risks of resistance to biocidal agents, which often can render the biocide ineffective. Bacterial populations can acquire resistance to biocides through cellular gene mutations, acquiring foreign resistance genes or a combination of the two (Davies, 2007). These methods of acquiring resistance alongside the slow-growing state of the bacterial cells that are encapsulated within the matrix can increase the recalcitrance to biocides. Studies have demonstrated that in some instances, biofilms have up to 1,000 fold increased resistance compared to their planktonic counterparts (Hoyle and Costerton, 1991). This has resulted in organisms such as *Pseudomonas putida,* being found to grow in high concentrations of disinfectants where a lower concentration was previously found to be effective in eradicating the organism in a planktonic state (Inoue and Horikoshi, 1989).

1.4 Efficacy of disinfectants

Biocidal efficacy varies depending on types of organisms, concentrations of each component and synergism among components (White and McDermott, 2001). Biocides usually interact with bacteria at the cell surface, which confers resistance from the cell surface structure and chemical composition (Tattawasart et al., 2000). This means the efficacy of biocides against Gram-positive and Gram-negative is often different, with Gram-positive organisms being generally more susceptible due to their thick cell wall, which is permeated by the biocidal agents (Nikaido, 1994). However, in some instances this can change depending on the specific mechanism of action of the disinfectant. Other types of bacteria such as *Mycobacteria* and bacterial spores are less susceptible to biocides than normal bacteria as their cell surfaces are protected by waxy envelopes and spores, respectively (White and McDermott, 2001). There are many different types of active chemical components incorporated into commercially used disinfectants (**Table 1.2**.), each of which has a different mechanism of action and therefore a different efficacy to different organisms.

The efficacy of biocides is very much dependent upon the conditions in which they are used. These include: the contact time required for their activity,

temperature conditions of the test, the compatibility with a given surface, the practical use of the product (e.g. on a wipe or as a surface spray liquid), soiling of the intended surface and the stability of the product (Maillard and McDonnell, 2012; Prescott and Dunn, 1949). These are all considerations to address when undergoing efficacy tests before consumer use.

Table 1.2. Commercially used disinfectants and their main chemical component. Information adapted from Maillard and McDonnell (2012).

Chemical Disinfectant	Main Chemical Component
Aldehydes	Glutaraldehyde Formaldehyde
Quaternary Ammonium Compounds	Benzalkonium Chloride Cetrimide
Peroxygens	Hydrogen Peroxide Peracetic Acid Chlorine Dioxide
Phenolics	Triclosan
Alcohol	Ethanol Isopropanol
Biguanides	Chlorhexidine Polyhexamethylene

1.4.1 Disinfectants mode of action

As the main active chemical components in disinfectants are different, this means that their mode of action to eradicate the bacteria also differs. Previous studies examining the mechanism of action of biocides have used techniques such as examination of biocide uptake within the cell (Russell and Chopra, 1996), disruption of cell homeostasis (Russell et al., 1988) and interaction with macromolecules (Russell, Morris and Allwood, 1973). This means that they can differ in effectiveness between Gram-positive and Gram-negative organisms due to the different cell walls. The main known mechanisms of action of currently used biocides and their efficacy are outlined in **Table 1.3**.

Table 1.3. The mechanism of action of widely used disinfectants and the scope of organisms they are effective against. Information adapted from McDonnell and Russell (1999).

Disinfectant	Organisms Effective Against	Organisms Not Effective Against	Mechanism of Action
Ethanol	Vegetative bacteria, viruses, fungi	Bacterial Spores	Causes membrane damage and rapid denaturation of proteins and can also interfere with metabolism and cell lysis
Isopropanol	Vegetative bacteria, viruses, fungi	Bacterial Spores Less active against hydrophilic viruses e.g. poliovirus	Causes membrane damage and rapid denaturation of proteins and can also interfere with metabolism and cell lysis
Glutaraldehyde	Bacterial spores (at high concentrations), vegetative bacteria, viruses, fungi and mycobacteria	Bacterial spores (at low concentrations) although can be sporostatic	Binds to outer layers of bacteria and inhibits dehydrogenase activity, and periplasmic enzymes. In Gram-negative bacteria it inhibits transport and prevents lysostaphin-induced lysis in *S. aureus*
Formaldehyde	Vegetative bacteria, bacterial spores (at high concentrations), fungi and viruses but works slower than glutaraldehyde	Bacterial spores (at low concentrations) although can be sporostatic	Interacts with protein, DNA (by inhibiting DNA synthesis) and RNA and can penetrate into bacterial spores
Chlorhexidine	Vegetative bacteria, yeasts, some activity against lipid enveloped	Bacterial spores although can be sporostatic Mycobacteria although can be	Up taken rapidly by bacteria and yeast. Causes damage to outer cell layers and then crosses the

	viruses	mycobacteristatic Low activity against many viruses	membrane and attacks the cytoplasm. High concentrations cause coagulation of intracellular components. High concentrations can inhibit membrane bound and soluble ATPase
Hydrogen Peroxide	Vegetative bacteria, Bacterial spores, viruses, yeasts, fungal spores (at high concentrations)	Fungal spores (at low concentrations), less activity against catalase producing organisms	Produce hydroxyl free radicals (⍰OH) which attacks essential cell components including lipids, proteins and DNA
Peracetic Acid	Vegetative bacteria, bacterial spores, fungi, viruses	N/A	Denatures proteins and enzymes and increases cell wall permeability
Quaternary Ammonium Compounds	Vegetative bacteria, yeasts, lipid viruses, enveloped viruses	Bacterial spores although can be sporostatic Mycobacteria although can be mycobacteristatic Non-enveloped viruses	Absorbs and penetrates the cells wall and reacts with the lipids and proteins in the cytoplasmic membrane and causes leakage of intracellular components. It degrades proteins and nucleic acids, eventually causing the cell to lyse. They damage the outer membrane of Gram-negative bacteria, promoting their own uptake

1.4.2 Efficacy of disinfectants against biofilms

Chemical disinfectants are currently the most widely used biocides due to factors such as their low cost and ease of use, and recent intensified use of these disinfectants have been shown to reduce microbial contamination levels of hand contact surfaces (Dancer et al., 2009). Unlike antibiotics, biocides are non-specific and often have a broad-spectrum, which can have both positive and negative impacts. Cells encapsulated in the biofilm matrix undergo phenotypic changes that can make them resilient to biocidal treatment (Nett et al., 2008). This results in issues such as low penetration of biocides through the EPS matrix and the emergence of persister cells (Buckingham-Meyer et al., 2007). To be effective against biofilms, biocides are required to reach one or more of the following targets: stopping growth (bacteriostatic), mechanical removal, killing (bactericidal), stopping attachment or promoting detachment (Stewart, McFeters and Huang, 2000). It has been found that the firmly attached cells which are usually on the base of the biofilm where it interacts with a surface or where the initial irreversible attachment occurred, are the least susceptible to biocidal treatment whereas loosely attached cells, which are usually are on the surface of the biofilm, are more susceptible (Eginton et al., 1998). Usually, a ≥3 log reduction shows bactericidal efficacy of a product, however these efficacy limits have not been established for use against biofilms.

1.5 Current non-biofilm biocidal test methods used

Currently, there are hundreds of standards that test the efficacy of biocidal agents worldwide. These have to line up with regulatory requirements for specific countries or regions such as the European Union (EU) where different regulatory bodies accept these standards based on the setting in which they will be used. For example, there are different standards for the same test depending on whether the product being tested will be used in a general, medical or agricultural setting. In the UK and EU, biocidal efficacy tests are split into three chronological phases, which companies manufacturing the biocide are advised to follow (**Table 1.4**).

Table 1.4. The three phases of testing to produce a biocidal claim. Information adapted from www.MelbecMicrobiology.co.uk (2022).

Phase	What the Tests Within This Phase Show
1	Used in the early development stages of a biocidal product where the main active substance(s) can be assessed for biocidal activity. Tests within this phase do not account for soiling and they do not consider specific applications and cannot be used to make a biocidal claim.
2/1	Phase 2/Step 1 includes quantitative suspension tests that establish whether a product has biocidal activity within a liquid suspension. Tests within this phase consider appropriate simulated soiling and when used alongside Phase 2/step 2 tests, they can be used to make a biocidal claim.
2/2	Phase 2/Step 2 refers to quantitative non-suspension test that establish whether a product has biocidal activity when applied to a surface or skin. Tests within this phase consider appropriate simulated soiling and when used alongside Phase 2/Step 1 tests, they can be used to make a biocidal claim.
3	Includes tests that are field-tests or on-site tests which are tested under real life conditions. These are often bespoke tests that are created to aid in the biocidal claim of a product.

The standards that are used in these phase steps are British (BS)/European (EN) standards that have been regulated for selling products in the UK and the EU. These tests evaluate efficacy against different planktonic bacteria (*P. aeruginosa, S. aureus, Escherichia coli, Enterococcus hirae)*, yeast (*Candida albicans*), fungal spores (*Aspergillus brasiliensis),* bacterial spores (*Clostridium difficile, Bacillus cereus, Bacillus subtilis)* and mycobacterium (*Mycobacterium avium, Mycobacterium terrae).* They also vary by the level of soiling and test temperature to make them specific to certain areas such as medical or agriculture. Current standards used for making biocidal claims in the UK and EU are outlined in **Table 1.5**, there are currently no available standards for phase 3 testing (British Standards Institute, 2019a; 2019b; 2015+2019; 2018; 2005).

Currently, many of these standards in both phase one and two are tested *in vitro* and do not replicate the use of the biocidal agent in real life conditions. The phase one suspension tests are mostly used as screening tests to assess the biocidal activity of the main active component of a product but as they do not involve any simulated soiling, they cannot be used to make a biocidal claim. Phase two suspension tests do include simulated soiling which changes

depending on the test standard and area of product intended use, and the soiling level can include the addition of erythrocytes or milk powder. Even with the addition of this soiling, the suspension test does not replicate using the product in a real-life scenario. The phase two surface tests are more realistic, as bacteria is dried onto a surface, which is more representative of how it would be found in the environment, and the product is applied onto the organisms instead of within a suspension. These tests also include the addition of simulated soiling but the tests are not carried out on different surfaces types, mimicking a real life setting. Currently, there are standards, which use surfaces such as stainless steel (BSI 2015+2019), glass (BSI, 2006) or plastic (BSI, 2011+2019). The aims of these tests are reproducibility and accuracy of results as well as taking into consideration ease and cost of testing. These aims are mostly achieved with these standards as all elements are standardised, making the process quick and reproducible. However, the lack of application to a real-life scenario pushes the focus onto phase three testing for which there are currently no standards available (British Standards Institute, 2019a; 2019b; 2015+2019; 2018; 2005).

Table 1.5. An outline of the current in-use standards within the UK and EU for making a biocidal claim. The standard number, the scope of the standard, the required organisms used for testing, the type of type and the phase that the standard belongs to is all presented. Information adapted from www.MelbecMicrobiology.co.uk (2022).

Standard	Scope	Organisms Tested	Phase	Test Type
EN 1040	Basic bactericidal activity of chemical disinfectants or antiseptics	*P. aeruginosa* and *S. aureus*	1	Suspension
EN 1275	Basic fungicidal/yeasticidal activity of chemical disinfectants or antiseptics	*C. albicans* and *A. brasiliensis*	1	Suspension
EN 14347	Basic sporicidal activity of chemical disinfectants or antiseptics	*Bacillus cereus* and *Bacillus subtillis*	1	Suspension
EN 1276	Bactericidal activity in food, industrial, domestic and institutional areas	*P. aeruginosa, S. aureus, E. coli* and *E . hirae*	2/1	Suspension
EN 1650	Yeasticidal/fungicidal activity in food, industrial, domestic and institutional areas	*C. albicans* and *A. brasiliensis*	2/1	Suspension
EN 13727	Bactericidal activity in a medical area	*P. aeruginosa, S. aureus, E. coli* (if the product is for hand hygiene) and *E. hirae*	2/1	Suspension
EN 13624	Yeasticidal/fungicidal activity in a medical area	*C. albicans, A. brasiliensis*	2/1	Suspension
EN 14348	Mycobactericidal activity in a medical setting	*Mycobacterium terrae, Mycobacterium avium*	2/1	Suspension
EN 17126	Sporicidal activity in a medical setting	*C. difficile, B. cereus, B. subtilis*	2/1	Suspension
EN 1656	Bactericidal activity in an agriculture setting	*P. aeruginosa, S. aureus, E. hirae* and *Proteus hauseri* for surface testing. *S. aureus, E. coli*	2/1	Suspension

		and *Streptococcus uberis* for teat disinfectant		
EN 1657	Yeasticidal/fungicidal activity in an agriculture setting	*C. albicans, A. brasiliensis*	2/1	Suspension
EN 13623	Bactericidal activity in aqueous systems	*Legionella pneumophila*	2/1	Suspension
EN 13704	Sporicidal activity in food, industrial, domestic and institutional areas	*C. difficile, B. cereus, B. subtilis*	2/1	Suspension
EN 14349	Bactericidal activity in a veterinary setting	*P. aeruginosa, S. aureus, E. hirae* and *Proteus vulgaris*	2/2	Surface
EN 16438	Yeasticidal/fungicidal activity in a veterinary setting	*C. albicans, A. brasiliensis*	2/2	Surface
EN13697	Bactericidal/yeasticidal/fungicidal activity in food, industrial, domestic and institutional areas	*P. aeruginosa, S. aureus, E. coli, E. hirae, C. albicans* and *A. brasiliensis*	2/2	Surface
EN 17387	Bactericidal/yeasticidal/fungicidal activity in a medical setting	*P. aeruginosa, S. aureus, E. hirae, C. albicans, A. brasiliensis*	2/2	Surface
EN 16437	Yeasticidal/fungicidal activity in a veterinary setting	*C. albicans, A. brasiliensis*	2/2	Porous Surface
EN 16615	Bactericidal/yeasticidal wipes activity in a medical setting	*P. aeruginosa, S. aureus, E. hirae, C. albicans*	2/2	Wipes Surface
EN 14561	Bactericidal activity in a medical setting	*P. aeruginosa, S.aureus, E. hirae*	2/2	Surface Glass Slides
EN 14562	Yeasticidal/fungicidal activity in a medical setting	*C. albicans, A. brasiliensis*	2/2	Surface Glass Slides
EN	Mycobactericidal activity in a medical setting	*M. terrae, M. avium*	2/2	Surface

14563				Glass Slides
EN 17272	Bactericidal/yeasticidal/fungicidal/mycobactericidal/sporicidal/ virucidal activity of airborne room disinfection by automated process	*P. aeruginosa, S. aureus, E. coli, E. hirae, Acinetobacter baumanii, P. hauseri, C. albicans, A. brasiliensis, M. terrae, M. avium, B. subtilis*	2/2	Surface Fogging
EN 16616	Bactericidal/yeasticidal/ fungicidal/mycobactericidal of chemical thermal textile disinfection	*P. aeruginosa, S. aureus, E. coli, E. hirae, E. faecium, C. albicans, A. brasiliensis, M. terrae, M. avium*	2/2	Textile

1.5.1 Novel surface disinfection methods

In recent years, there have been several novel surface disinfectant tests arising such as fogging (EN 17272), surface coatings, blue light activated disinfectants and UV-C disinfection. Antimicrobials derived from plants such as phytols from the desert plant *Leptadenia pyrotechnica* have also been evaluated for their antimicrobial efficacy (Ghaneian et al., 2015). Surface coatings such as silver-silica (Varghese et al., 2013) and graphene-based nanomaterials (GBNs) (Ayub et al., 2021) have been tested and found to have potential applications in healthcare by maintaining a background antimicrobial activity in addition to the cleaning procedures. Disinfecting methods that are increasingly being tested are the effects of UV-C, which have been found to be as effective as biocides when tested against different surface materials (Guridi et al., 2019) and can be a potential application in disinfection protocols in healthcare settings. In addition to UV-C as a solo treatment, it has now successfully been used in combination treatment with agents such as chlorine (Oppenheimer et al., 1997), peracetic acid (González et al., 2012), ozone (Wu et al., 2016) and hydrogen peroxide (Guan, Fan and Yan, 2013), which have been shown to increase performance as combination therapy. Visible light is also being explored as a combination therapy as disinfectants mixed with light-activated photosensitizers to eradicate microbes (Wylie, et al., 2021) and has also been applied to surface coatings such as cellulose acetate films containing photosensitizers (Wilson, 2003). This approach has been very successful in eradicating contamination.

1.5.2 Current biocidal efficacy test methods used against biofilms

Currently, there are few standards or test methods, which involve the novel ideas such as UV-C disinfection widely used to test against biofilms. However, there are several methods available which describe either a protocol to grow or to test against a biofilm. These that specialise in growing biofilms for testing include the CDC reactor, 96-well microtiter plates, Calgary biofilm device (CBD), drip flow biofilm reactor and the Robbins device, all of which are successful in producing a biofilm for biocidal testing. However, most of these methods are low-throughput, require the purchase of specialised equipment and the end-product biofilm cannot often be accessed for efficacy testing or examination (McBain, 2009).

Biofilm growth methods that do not require the purchase of special equipment and are accessible to high-throughput screening such as the microtiter plate biofilm method and the CBD are usually only successful in producing a loosely attached biofilm (Azeredo et al., 2017). All these methods can easily introduce variability in the biofilm structure and thickness.

As the current biocidal standards available are used against planktonic cells, they can't be translated to biofilms. There are however several American standards for testing biocidal efficacy against biofilms such as ASTM E2871-19 which is a single tube method based on testing biofilms grown using the CDC reactor or the MBEC Assay® test using specific pegged MBEC Assay® 96 well microtiter plates. However, these current standards have limitations such as low-throughput, variability in biofilm production and the need to purchase specialised equipment due to the biofilm growth method used within the standards. Currently, there is a shortage of accepted methods for measuring biocide efficacy against biofilms. Specific bespoke methods have been designed to evaluate biocidal efficacy in environments such as toilet bowls, the human mouth and cooling water systems (Pitts et al., 2001, Bradshaw et al., 1996, Green and Pirrie, 1993.) but there is still no high-throughput reproducible general-purpose biocidal biofilm efficacy model for use.

1.6 Physical methods to treat biofilms

There are lots of new methods being explored to treat biofilms that move away from chemical disinfection and focuses more on physical disinfection. These methodologies don't always focus on the biocidal removal of biofilms, but also assess the removal of biofilms as a preventative measure by stopping them from attaching to a surface and to minimise their formation (Liu et al., 2022). These methods include things such as UV, ultrasound and electric currents. Ultrasound is a newly emerged chemical energy technology, which has been shown to be effective in destroying biofilm structure in a low frequency, high intensity dose and when paired with chemical disinfectants it shows a higher percentage of biofilm cell eradication than from one method alone (Yu et al., 2020). However, when used in a low intensity, low frequency dose, the ultrasound is effective in

stimulating bacterial metabolism and can increase the resistance and the surface adherence of the biofilm (Erriu et al., 2014). Electric fields and currents are another physical treatment that has been found effective in eradicating biofilm formation by interacting with biological membranes and metabolic processes and cell responses (Ravikumar, Basu and Dubey, 2019). The application of these electric currents can increase repulsive forces, which facilitate the surface detachment of biofilms and have also been found to act synergistically when used in combination with a chemical disinfectant (Van der Borden, Van der Mei and Busscher, 2005).

1.6.1 UV-C testing against biofilms

Ultraviolet light (UV) is the most studied physical form of disinfection. It has three wavelengths; UV-A, UV-B and UV-C with UV-C being the wavelength that achieves biocidal effects when tested against bacteria. Mercury vapour lamps, excimer lamps, xenon pulse lamps and light emitting diodes (LEDs) can all artificially produce UV light. UV-C wavelengths of 200-280nm (**Figure 1.3**) are becoming more commonly used as a method of disinfection, especially in wastewater treatment. This is because it targets nucleic acid molecules and can damage cell membranes and disrupt proteins by photo oxidation (Würtele et al., 2011) as DNA can absorb UV-C light in these wavelengths. However, the biocidal effects of UV-C have been mostly focused on planktonic bacteria and have been found to be successful in eradicating these cells (Chen, Craik and Bolton, 2009) but the current knowledge against biofilms is limited. Although UV-C is not able to eradicate biofilms, as it can planktonic cells, UV-C has been found to slow biofilm formation (Wenjun and Wenjun, 2009).

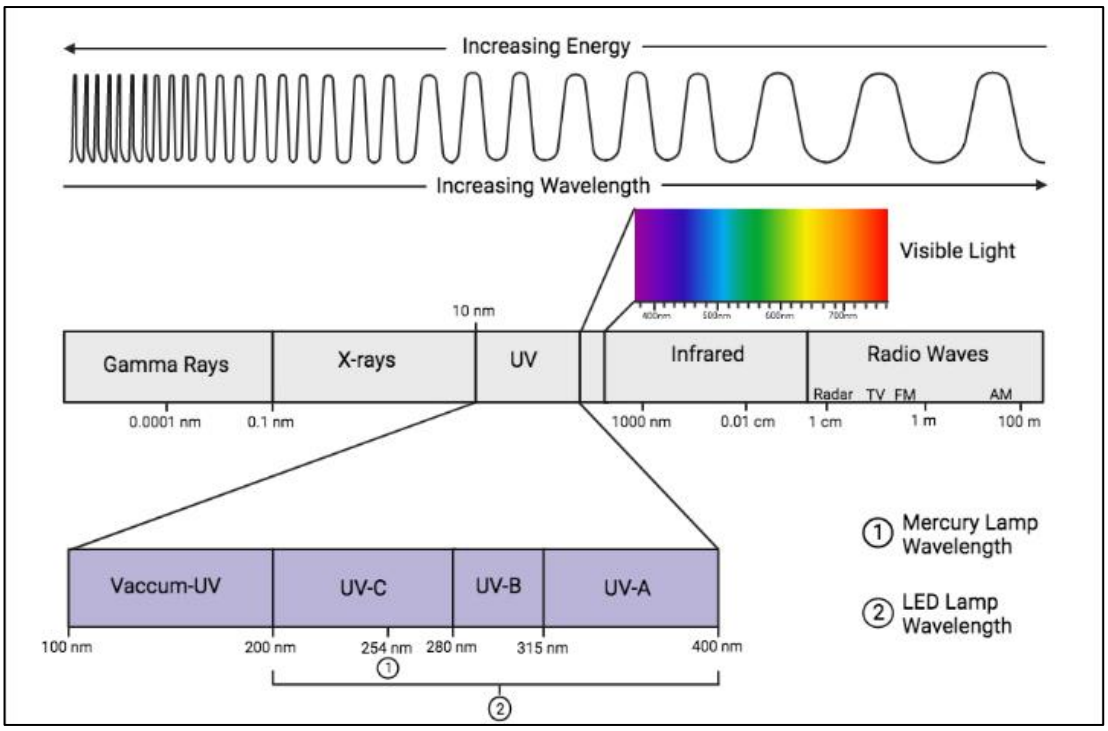

Figure 1.3. The light spectrum from 0.0001 nm to 100 m. This figure illustrates UV-C and the different wavelengths in this UV category used in disinfection. The specific wavelengths of the two most widely used types of UV disinfection lamps; mercury and LED, are highlighted. Made on BioRender.com (2022).

1.7 Aims of the study

Currently, there are very few standardised methods available for biocidal efficacy testing against biofilms. The non-standard tests are mostly low throughput and involve the need for purchasing specialised equipment, which can be costly. The aim of this study was to produce a biocidal efficacy testing model, which is reproducible, cheap and has a high throughput. This model should be sufficient in providing accurate results against both chemical disinfectants and UV-C. Another main aim of this study was to use two of the most common biofilm producing pathogens; *P. aeruginosa* and *S. aureus*, to assess this method for both the growth of biofilms and efficacy testing. These organisms are often found in biofilms in most aspects of everyday such as healthcare, agriculture, domestic and the workplace and are often found growing in a co-culture. Due to the change in bacterial behaviour and the synergistic effects caused by the two species surviving in the same environment, using this multi-species biofilm against the method would show whether this method could be used against multi-species biofilms. I would also show whether a combination

of chemical and physical disinfection treatment is successful in eradicating the biofilm.

2. Materials and Methods

2.1 Bacterial strains

Bacterial strains were purchased in lyophilised form from NCIMB (Aberdeen, Scotland). One strain of *S. aureus* (NCTC 10788) and two strains of *P. aeruginosa* (NCTC 12924/13359) were purchased for testing. All organisms are mandatory test organisms used for biocidal efficacy tests such as BS EN 1276 and BS EN 13697 (BSI 2019a; 2015+2019). *P. aeruginosa* NCTC 12924 and *P. aeruginosa* NCTC 13359 are pyocyanin and non-pyocyanin producing, respectively. All isolates were maintained on tryptone soy agar (TSA; Oxoid, Basingstoke, UK) and were incubated under aerobic conditions at 37°C for 18-24 hours. All strains were stored in 80% (v/v) glycerol (Vickers Laboratories, Yorkshire, UK) at −80°C and freshly sub-cultured onto tryptone soy agar (TSA) before each experiment. These strains were maintained and preserved as stated in BS EN 12353 (BSI, 2021), which is the standard that determines the preservation of test organisms used for the determination of bactericidal activity. This standard states that lyophilised purchased organisms are re-constituted in tryptone soy broth (TSB) and grown up on TSA for 18-24 hours. Once grown up, these are transferred to cryoprotectant fluid with or without beads and frozen at –80 for at least 24 hours. Once frozen, a culture can be made on TSA from the frozen stock which becomes a 'stock plate' which can be kept at 2-5°C for 1 month. This stock plate can be used to passage twice more for use in biocidal testing.

2.2 Biocidal methods

Industrial biocides were obtained in commercial preparations. These were: a product containing a mixture of the Quaternary Ammonium Compounds (QACs) (n-Alkyl dimethyl benzyl ammonium chloride <2.3% and didecyl dimethyl ammonium chloride <3.4%) manufactured by an undisclosed manufacturer and a commercially available product containing 7.9% (w/w) hydrogen peroxide manufactured by Endo Enterprises (Cheshire, UK). All products were supplied by Melbec Microbiology Ltd (Haslingden, UK). The ingredients are shown in **Table 2.1**.

Table 2.1. Ingredients in the disinfectants used for biofilm testing.
Hydrogen Peroxide and QAC ingredients that are displayed on the packaging.

Ingredients	
Hydrogen Peroxide	**QAC**
Hydrogen Peroxide (CA 7722-84-1) 7.9% w/w	Didecyl dimethyl ammonium chloride <3.4%
92.1% Water	n-Alkyl dimethyl benzyl ammonium chloride <2.3%
	Inert ingredients 94.3%

The portable UV disinfection machine was an undisclosed UV machine manufactured by an undisclosed manufacturer. The UV machine emitted UV-C at a wavelength of 254nm with 660 W and 600 V.

2.3 Biofilm growth method

Frozen bacterial cultures were defrosted, cultured onto TSA and incubated for 18-24 hours at 36.5°C. This culture was then used to inoculate 5 mL tryptone soy broth (TSB; Neogen, Lansing, Michigan, USA) containing 1% w/v glucose (Vickers Laboratories, Yorkshire, UK) which was then incubated in an orbital incubator (GallenKamp, Cambridge, UK) for 18-24 hours at 36.5°C. The resulting culture was diluted 1/1000 to achieve $1x10^5$ CFU/mL using phosphate buffer saline (PBS; Sigma Aldrich, Dorset, UK). The optimal density of these suspensions pre and post dilution were measured using a spectrophotometer (Cecil 2041, Cecil Instrumentational Services, Cambridge, UK) at a wavelength of 620nm to ensure the same starting inoculum level. Data was gathered and calibration curves made for future testing. The cell suspension containing $1x10^5$ CFU/ml was then centrifuged at 4,000 x *g* for 10 minutes and re-suspended in the same volume of PBS to wash the culture. This was repeated twice more. Pre-sterilised 13mm polycarbonate (PC) membrane filtration discs (Sigma Alridch, Dorset, UK) were placed on pre-poured 5 mL set TSA in 6 well plates. From the washed suspension, 10 µL was pipetted onto the centre of the disc without spreading and incubated at 36.5°C for 48 hours. When making a polymicrobial biofilm, *S. aureus* was added to the filter first and grown for 24 hours at 36.5°C before adding

P. aeruginosa and growing for 48 hours at the same temperature (3.1 Biofilm Growth) which meant *S. aureus* grew for 72 hours in total. To determine the CFUs of the biofilm, the disc was placed in 10 mL PBS and mixed for 30 seconds using a vortex mixer (Vortex Genie 2, Scientific Industries, Bohemia, New York, USA) and then transferred to an ultrasonic bath (Allendale Ultrasonics, Hertfordshire, UK) for an exposure time of 15 minutes. This was then serially diluted in 96 well plates and plated out using the Miles Misra technique (Miles, Misra and Irwin, 1938) onto organism specific agar; mannitol salt (Neogen, Lansing, Michigan, USA) for *S. aureus* and agar base with an additional CN supplement (Neogen, Lansing, Michican, USA) for *P. aeruginosa.* The full dilution series was plated out from the neat suspension to 10^{-7}. Plates were then incubated at 36.5°C for 24-48 hours (**Figure 2.1**.)

2.4 Disinfectant efficacy testing method for QAC disinfectants

QAC dilutions to achieve desired concentration for testing were made in synthetic hard water (HW). The instructions on how to prepare this are outlined below.

Preparation Instructions for Synthetic Hard water

Solution A 50 mL: 0.992g magnesium chloride (Vickers Laboratories, Yorkshire, UK), 2.312g calcium chloride (Sigma Alridch, Dorset, UK), 46.696 mL sterile deionised water (DI) mixed and sterilised at 121°C for 15 minutes.

Solution B 50 mL: 1.751g sodium bicarbonate (Vickers Laboratories, Yorkshire, UK), 48.249 mL sterile DI mixed and filter sterilised.

Add 6 mL of solution A and 8 mL of solution B into 600 mL of sterile DI and dilute to 1000 mL. pH adjustments can be done by adding a sterile amount of 40g/L sodium hydroxide or 36.5g/L of hydrochloric acid (1% w/v citric acid can be used if hydrochloric acid unavailable).

The PC filter containing the grown biofilm was placed on a sterile surface, which was small enough to fit into the final vessel such as a sterile stainless steel disc

used in the BS EN 13697 surface test was used in this experiment. Once in place, 200 µL of disinfectant product was then added onto the surface of the filter, directly onto the biofilm for *'t'* (*'t'* refers to the contact time decided upon before testing. The contact time used in this test was 60 minutes, based on MBEC Assay®; Innovotech, Canada). After *'t'* the sterile surface containing the PC filter and the 200 µL of disinfectant was transferred to a sterile universal containing 10 mL of neutralising broth (Southern Group Laboratories, Northamptonshire, UK). This was mixed using a vortex mixer for 30 seconds and then transferred to an ultrasonic bath at a frequency of 40kHz for an exposure time of 15 minutes. This was then enumerated using 96 well plates and plated out using the Miles Misra technique (Miles, Misra and Irwin, 1938) onto organism specific agar, mannitol salt (Neogen, Lansing, Michigan, USA) for S. *aureus* or *Pseudomonas* agar base with an additional 5 mL CN supplement (Neogen, Lansing, Michigan, USA) for *P. aeruginosa*. The full dilution series was plated out from the neat suspension to 10^{-7}. Plates were then incubated at 36.5°C for 24-48 hours (**Figure 2.2**.). The test was undergone at room temperature (19-21°C). All tests using the QAC consisted of 3 technical and 3 biological replicates (n=9) and the test conditions were as follows:

1. 200 µL QAC applied to the disc
2. 200 µL Synthetic hard water applied to the disc
3. Untreated biofilm disc recovered in 10 mL PBS

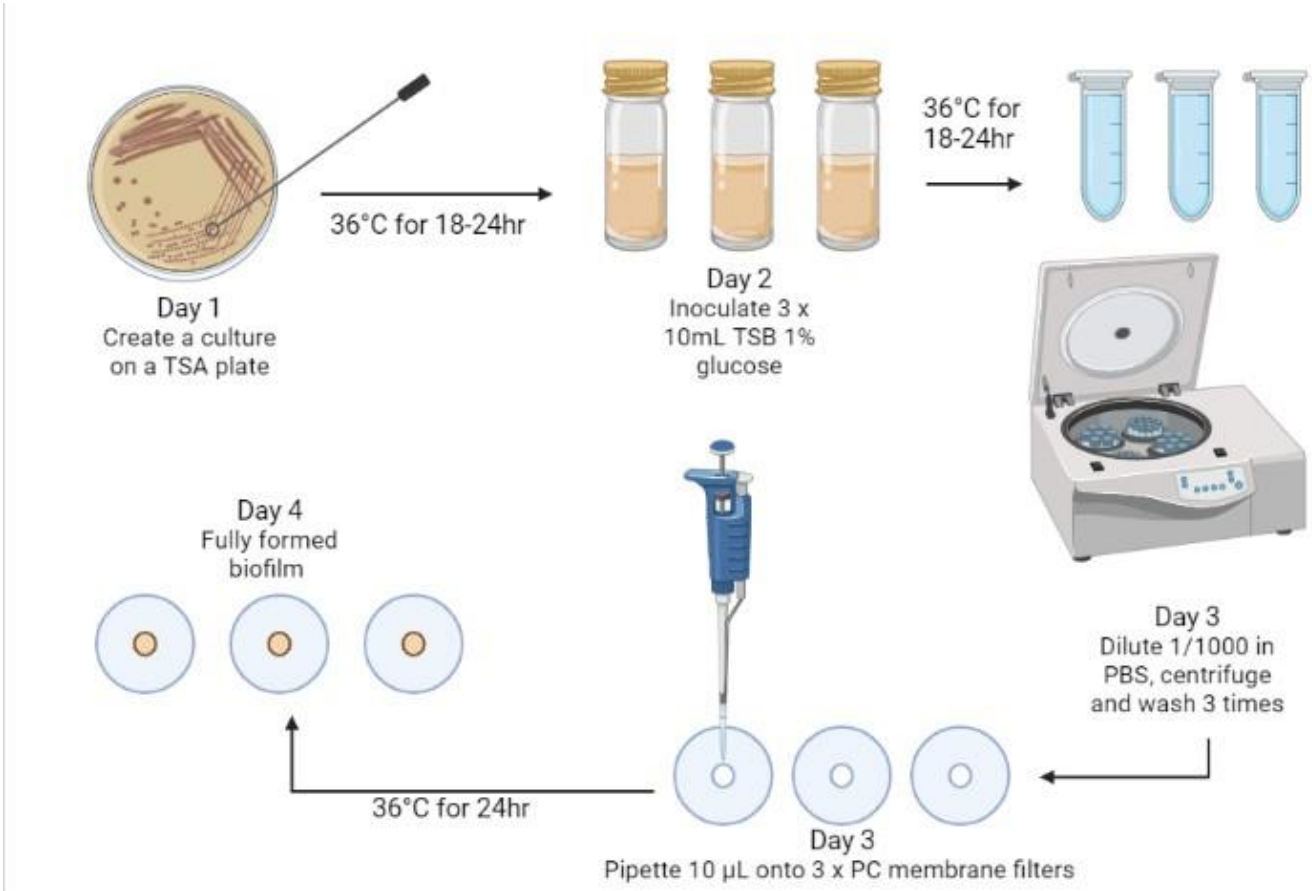

Figure 2.1. Biofilm growth method flowchart. The process of growing the biofilms using the colony biofilm method outlined in 2.3 Biofilm Growth method outlined. Made on by BioRender.com (2022).

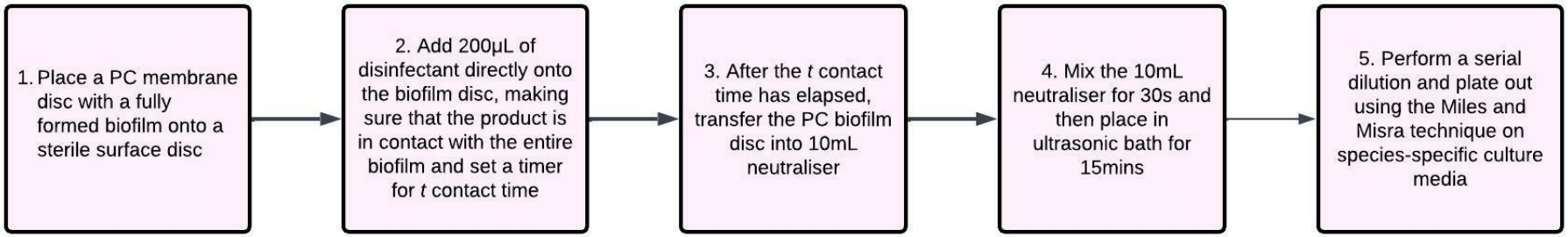

Figure 2.2. Disinfectant efficacy testing method for QACs flowchart. The process of testing the biofilms against a QAC chemical disinfectant outlined in 2.4 Disinfectant Efficacy Testing Method for QAC Disinfectants.

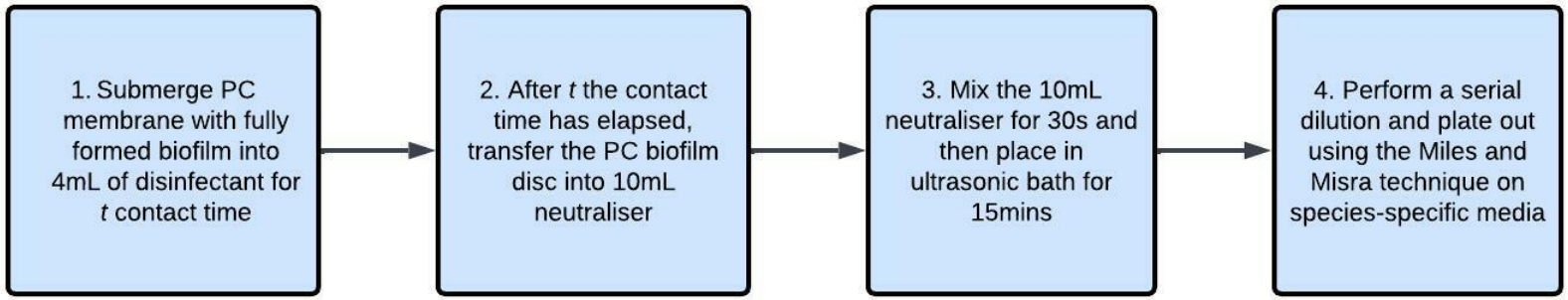

Figure 2.3 Disinfectant efficacy testing method for hydrogen peroxide flowchart. The process of testing the biofilms against a hydrogen peroxide chemical disinfectant outlined in 2.5 Disinfectant Efficacy Testing Method for Hydrogen Peroxide Disinfectants.

2.5 Disinfectant efficacy testing method for hydrogen peroxide disinfectants

Product dilutions to achieve desired concentration for testing were made in synthetic hard water. The PC filter containing the grown biofilm was submerged into 4 mL of the product to mimic the similar method used in Lineback et al (2018). In essence, the biofilm disc was submerged for *'t'* (*'t'* refers to the contact time). The contact time used in this test was 30 minutes. After *'t'* the disc was removed from the disinfectant and submerged in 10 mL neutralising broth. This was mixed using a vortex mixer for 30 seconds and then transferred to an ultrasonic bath for an exposure time of 15 minutes. To determine the CFUs, serial dilutions were carried out in 96 well plates followed by plating out, using the Miles Misra technique (Miles, Misra and Irwin, 1938), onto organism specific agar mannitol salt (Neogen, Lansing, Michigan, USA) for S. *aureus* or *Pseudomonas* agar base with an additional 5 mL CN supplement (Neogen, Lansing, Michigan, USA) for *P. aeruginosa*. The full dilution series was plated out from the neat suspension to 10^{-7}. The plates were then incubated at 36.5°C for 24-48 hours (**Figure 2.3**). The test was undertaken at room temperature (19-21◦C). All tests using the hydrogen peroxide consisted of 3 technical and 3 biological replicates (n=9) and the test conditions were as follows:

1. Biofilm disc submerged ('dipped') into 4 mL hydrogen peroxide
2. Biofilm disc submerged into 4 mL of synthetic hard water
3. Untreated biofilm disc recovered in 10 mL PBS

2.6 UV-C efficacy testing method

Prior to testing, the UV disinfection system was 'warmed up' by running for 5 minutes. The grown biofilm on the PC disc was placed on a sterile surface, which was small enough to fit into the final vessel such as a sterile stainless steel disc, the one used in the BS EN 13697 surface test were used in this experiment. The disc was placed on a sterile surface at a flat angle and 18cm from the UV-C machine, with care taken to remove any potential shadowing. The test was run with the biofilm directly underneath the arm of the UV machine, with the direction of the UV travelling downwards (**Figure 2.4**). The UV-C machine was run for *t* contact time and after this time has elapsed, the disc was placed into 10 mL saline. Note: if doing a combined disinfectant and UV-C treatment, the disc

must be placed in 10 mL neutralising broth. This suspension was mixed for 30 seconds using a vortex mixer and was then placed in an ultra-sonic bath for an exposure time of 15 minutes. This was then serially diluted using 96 well plates and plated out using the Miles Misra technique (Miles, Misra and Irwin, 1938) onto organism specific agar mannitol salt (Neogen, Lansing, Michigan, USA) for *S. aureus* or *Pseudomonas* agar base with an additional 5 mL CN supplement (Neogen, Lansing, Michigan, USA) for *P. aeruginosa*. The full dilution series was plated out from the neat suspension to 10^{-7}. The plates were then incubated at 36.5°C for 24-48 hours (**Figure 2.5**). The test was carried out at room temperature (19-21°C). All tests using the UV-C consisted of 3 technical and 3 biological replicates (n=9) and the test conditions were as follows:

1. Biofilm disc in direct contact with UV-C light
2. 200 µL of synthetic hard water applied to biofilm disc for 50 minutes and then the disc in direct contact with UV-C light for 10 minutes
3. 200 µL of synthetic hard water applied to biofilm disc for 1 hour
4. Untreated biofilm disc recovered in 10 mL PBS

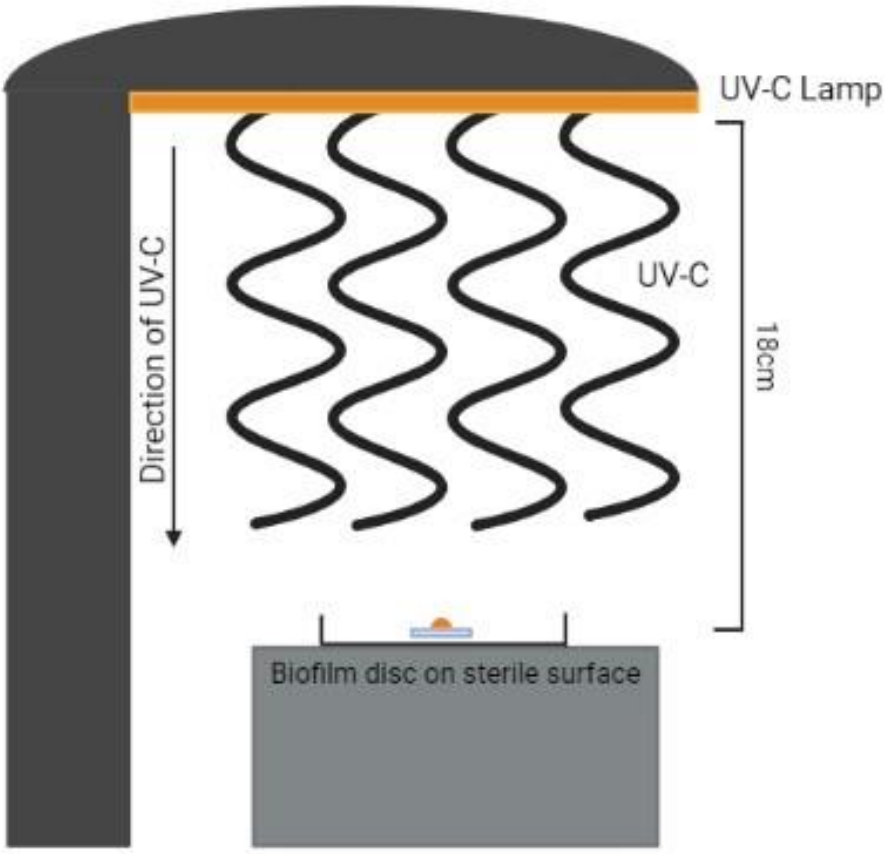

Figure 2.4. Visual representation of the set-up of UV-C testing. The image shows the set-up of the UV-C machine with the arms containing the bulbs coming out directly over the biofilm disc, at 18cm. The biofilm disc is flat underneath the UV bulb, with the direction of UV-C travelling downwards onto the biofilm disc. Made on by BioRender.com (2022).

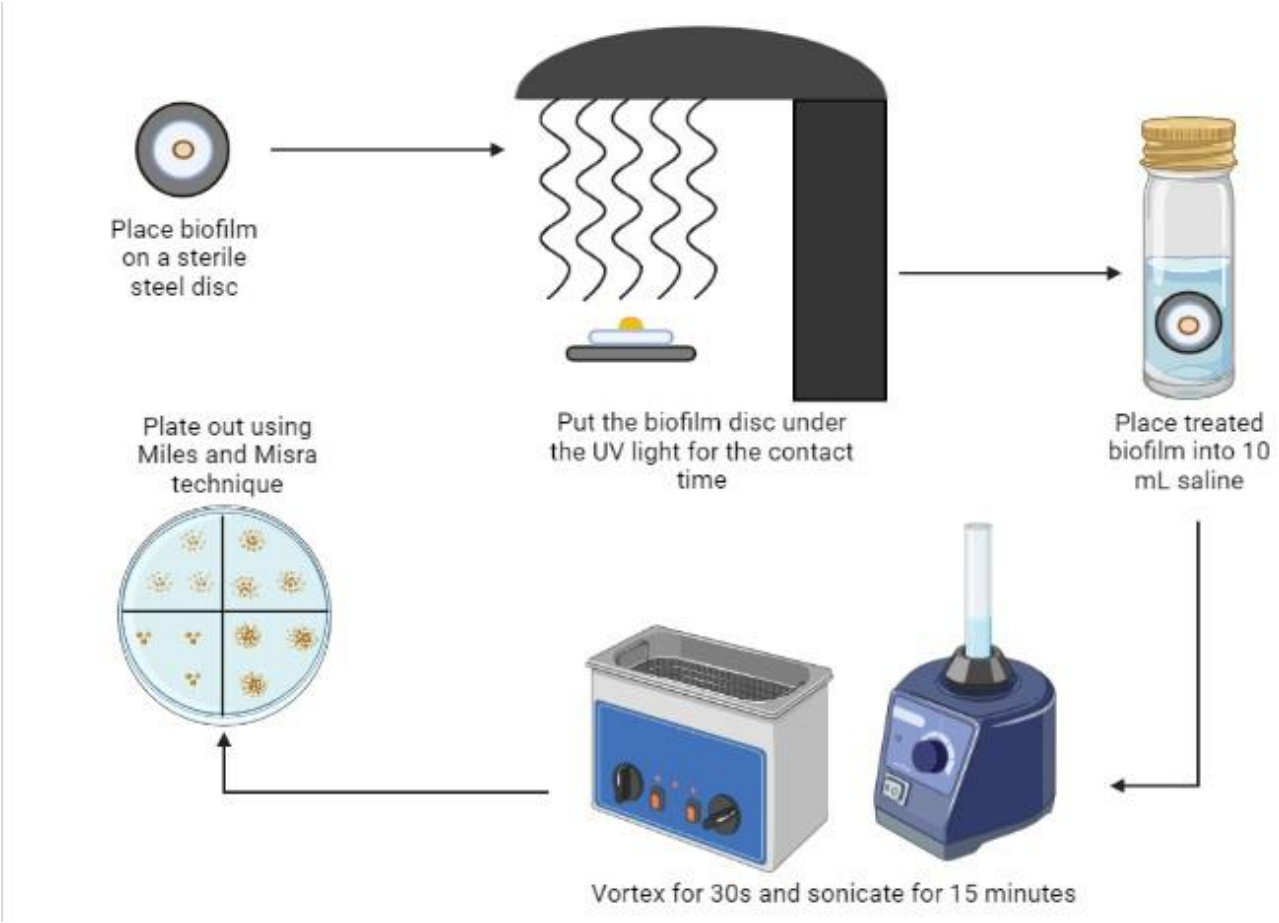

Figure 2.5 UV-C disinfectant efficacy test method flowchart. The process of testing the biofilms against a UV-C physical disinfection outlined in 2.6 UV-C Efficacy Testing Method. Made on by BioRender.com (2022).

2.7 Combined disinfectant and UV-C method

When combining the two methods, the disinfectant was applied first and prior to testing and the UV machine was 'warmed up' by running for 5 minutes prior to testing. When using the test method for QAC disinfectants, the UV-C was applied 10 minutes before the end of *'t'* in the disinfectant test. This way, the two tests can run in parallel. When using the test method for hydrogen peroxide disinfectants, the UV-C application must be done after the disc has been removed from the disinfectant. The biofilm disc was placed on a sterile surface at the desired angle and distance from the UV-C, with care taken to remove any potential shadowing. The UV-C was then activated for *'t'* (t=10 minutes) and once *'t'* had elapsed, the disc was placed in the neutralising broth. This suspension was mixed for 30 seconds using a vortex mixer and was then placed in an ultra-sonic bath for an exposure time of 15 minutes. This was then serially diluted using 96 well plates and plated out using the Miles Misra technique (Miles, Misra and Irwin, 1938) onto organism specific agar mannitol salt (Neogen, Lansing, Michigan, USA) for S. *aureus* or *Pseudomonas* agar base with an additional 5mL CN supplement (Neogen, Lansing, Michigan, USA) for *P. aeruginosa*. The full dilution series was plated out from the neat suspension to 10^{-7}. The plates were then incubated at 36.5°C for 24-48 hours. All tests using the combined method consisted of 3 technical and 3 biological replicates (n=9) and the test conditions were as follows:

1. QAC method
    i. 200 µL of disinfectant applied to biofilm disc for 50 minutes and then the disc in direct contact with UV-C light for 10 minutes
    ii. 200 µL of synthetic hard water applied to biofilm disc for 50 minutes and then the disc in direct contact with UV-C light for 10 minutes
    iii. 200 µL of disinfectant applied to biofilm disc for 1 hour
    iv. 200 µL of synthetic hard water applied to biofilm disc for 1 hour
    v. Untreated biofilm disc recovered in 10 mL PBS

2. Hydrogen peroxide method
    i. Biofilm disc submerged ('dipped') into 4 mL disinfectant for 50 minutes and then the disc removed and put in direct contact with UV-C light for 10 minutes
    ii. Biofilm disc submerged ('dipped') in synthetic hard water for 50 minutes and then the disc removed and put in direct contact with UV-C light for 10 minutes
    iii. Biofilm disc submerged ('dipped') into 4 mL disinfectant for 1 hour
    iv. Biofilm disc submerged ('dipped') into 4 mL synthetic hard water for 1 hour
    v. Untreated biofilm disc recovered in 10 mL PBS

2.8 Microscopy

The biofilms exposed to each treatment were stained with 100 µL of a 1:1 ratio of SYTO9 and propidium iodide in the LIVE/DEAD™ BacLight™ bacterial viability kit (Invitrogen, ThermoFisher Scientific, Massachusetts, USA) diluted to 6% concentration using DI. This was used to evaluate the viability of attached cells by fluorescent staining. When undergoing the microscopy, it was found that diluting the stain in DI water disrupted the biofilm too much and lifted the biofilm colony from the PC disc, which meant there was only a monolayer of cells left for imaging. Diluting the 1:1 stain with 60% glycerol concentration diluted with DI water rectified this, as the stain being more viscous did not disrupt the biofilm enabling the transfer of the biofilm to the glass slide for imaging the entire colony. The addition of glycerol was only required for the microscopy, not the testing. Images of all the biofilms tested were taken using the ZEISS LSM 700 AxioObserver (Carl Zeiss, Germany) confocal laser-scanning microscope (CLSM) using an EC Plan-Neofluar 10×/0.30 M27 objective. Viable and non-viable biofilm biomass quantification from image stacks of biofilms was done with the open-source software Fiji-ImageJ v2.1.0/1.53c. Live/dead ratios were established for each treatment and compared to untreated controls. The images taken using the confocal microscope were analysed using ZEN 3.6 (ZEN lite). Z-stack images (n=9) were taken of each test condition. Due to time constraints regarding

training, the viable and non-viable biofilm mass quantification was done by Dr Manuel Romero at The University of Nottingham.

2.9 Statistical analysis

Data and statistical analyses were undertaken on GraphPad Prism 9 unless otherwise stated. Graphs presenting the data were made using the average CFU/mL recovery of 3 technical replicates per biological replicate (n=9), unless otherwise stated. One-way and two-way ANOVA statistical tests were used to study the interaction between independent variables that influence the value of dependent variables. Dunnett's, Šídák's and Tukey's multiple comparisons test were applied to assess significant interaction between specific variables against the controls. For the microscopy quantification of the live/dead cells, one-way ANOVA tests were applied to determine whether viability differed significantly between treatment conditions ($p < 0.05$) when compared with the variations within the replicates using Prism 8.0 (GraphPad Software).

3 Results

In order to test the efficacy of biocide combinations against biofilms, the first step was to develop methodology to reproducibly grow representative biofilms.

3.1 Optimisation of biofilm growth

The colony biofilm method procedure was followed to grow single and polymicrobial biofilms using both *P. aeruginosa* and *S. aureus*. Initially both organisms were added onto the disc simultaneously when growing a polymicrobial biofilm, however, this caused an inconsistency in recovery of CFU/mL between the two organisms (data not shown). This affected the CFU/mL of *S. aureus* by decreasing the recovery compared to the CFU/mL recovered from parallel single organism biofilm. An alteration was made to the method for growing polymicrobial biofilms in which *S. aureus* was inoculated onto the PC disc 24 hours before *P. aeruginosa* and incubated. This increased the CFU/mL of *S. aureus* by up to 2-log so the bacterial count of both organisms within the same biofilm community were comparable (**Figure 3.1**). When testing this method alteration, *P. aeruginosa* NCTC 13359 was used.

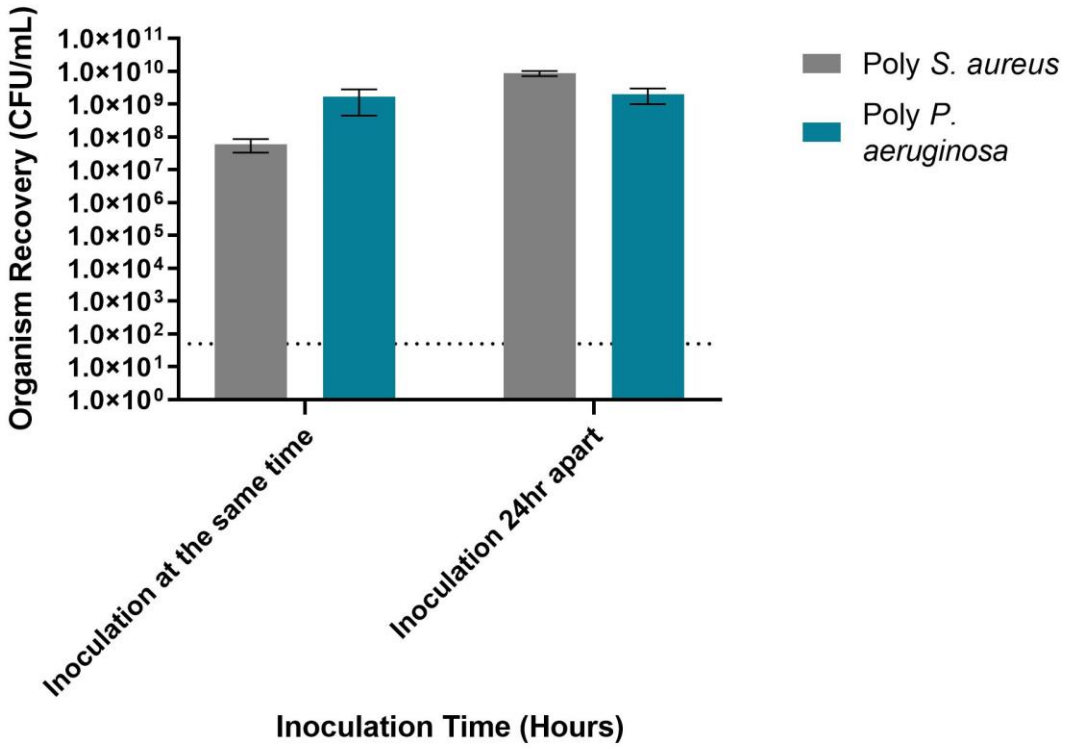

Figure 3.1. Comparison of inoculating PC discs with *S. aureus* and *P. aeruginosa* simultaneously or 24 hours apart to create polymicrobial biofilms. The 'inoculation at the same time' refers to both the *S. aureus* and *P. aeruginosa* being inoculated at the same time. The 'inoculation 24hr apart' refers to the *S. aureus* being inoculated, incubated for 24 hours, and then the *P. aeruginosa* being inoculated on top of the already established *S. aureus* biofilm. Once grown, these were then recovered by placing in 10 mL PBS. All recovery is in CFU/mL. The dotted line depicts the minimum detection value is 50 CFU/mL. Data shown are mean ± SD. Technical replicates n=3, biological replicates n=1 (n=3).

The colony biofilm growth method was optimised to achieve a bacterial count of between 10^9 and 10^{10} CFU/mL in the biofilm. Initially, the method used tryptone soy broth (TSB) to grow up the overnight inoculum, however, this only achieved an average CFU/mL of 10^8 for both *P. aeruginosa* and *S. aureus* as single organism and polymicrobial biofilms. Imitating multiple studies that use the addition of glucose within a TSB culture to increase CFU/mL (Mathur et al., 2006; Deka, 2014), 1% glucose was added to the TSB to try and achieve an increase in recovery. The addition of the 1% glucose was efficient in increasing the CFU/mL in both *P. aeruginosa* and *S. aureus* (**Figure 3.2**). The CFU/mL increased up to 10-fold and 100-fold for *P. aeruginosa* and *S. aureus* respectively. When testing this method alteration, *P. aeruginosa* NCTC 13359 was used.

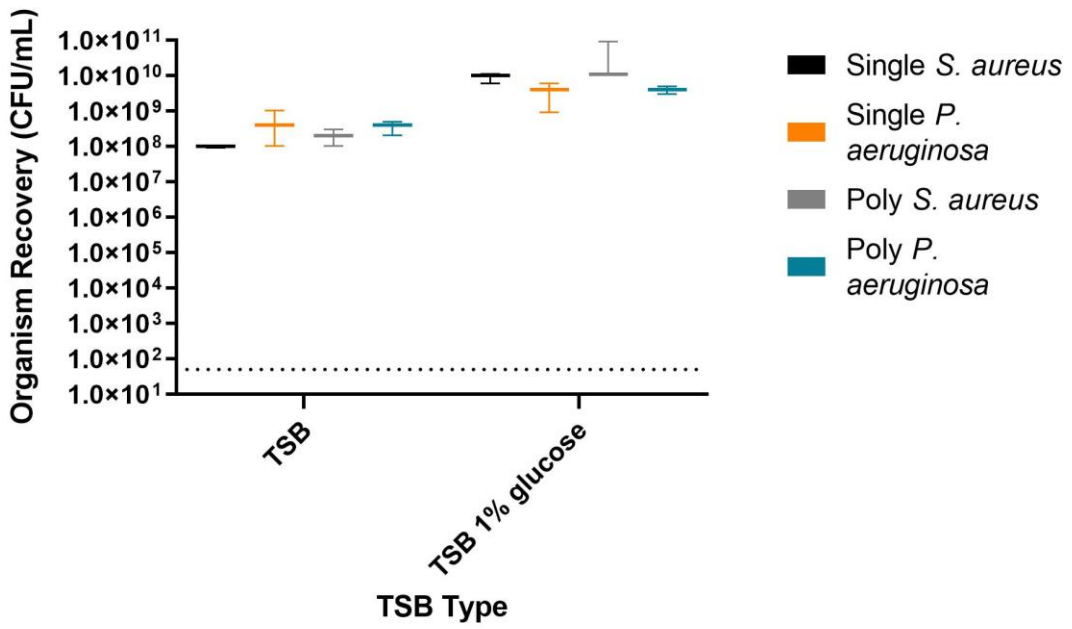

Figure 3.2. Comparison of supplementing TSB with 1% glucose on organism recovery of the single and multi-organism biofilms of *S. aureus* and *P. aeruginosa*. Overnight cultures of the bacteria were grown in TSB or TSB supplemented with 1% glucose and grown up using the standard biofilm growth method with the two bacterial species inoculated sequentially as indicated in **Figure 3.1**. These were then recovered by placing in 10 mL PBS. All recovery is in CFU/mL. The dotted line depicts the minimum detection value is 50 CFU/mL. Data shown are mean ± SD. Technical replicates n=3, biological replicates n=1 (n=3).

The incubation time of the biofilm to optimise CFU/mL was also investigated, with the difference between 24, 48 and 72 hours of incubation at 36◦C tested. It was found that 48 hours was the optimum incubation time for *P. aeruginosa* and *S. aureus* within both single and multi-organism biofilms (**Figure 3.3**). The CFU/mL recovery decreased up to 1-log when incubated for 72 hours compared to 48 hours. However, when grown in a polymicrobial biofilm, the *P. aeruginosa* had 72 hours incubation, but this longer incubation time did not affect the recovery as much as *S. aureus* incubated for this time period. When testing this method alteration, *P. aeruginosa* NCTC 13359 was used.

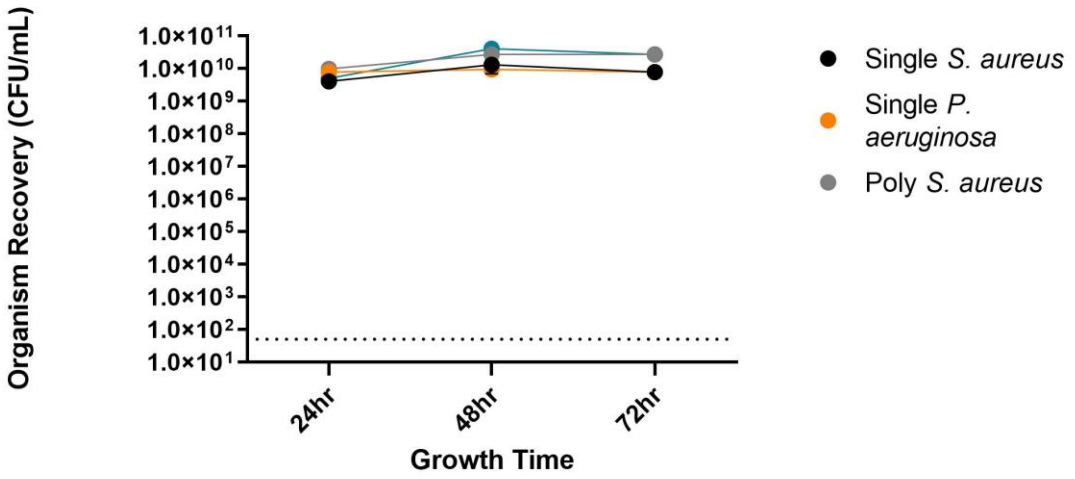

Figure 3.3. The effect of incubation time on organism recovery of the single and multi-organism biofilms of *S. aureus* and *P. aeruginosa*. Single and multi-organism species biofilms were grown up using the standard biofilm growth method but incubated for 24, 48 or 72 hours at 36.5°C. These were then recovered by placing in 10 mL PBS. All recovery is in CFU/mL. The dotted line depicts the minimum detection value is 50 CFU/mL. Data shown are mean. Technical replicates n=1, biological replicates n=1 (n=1).

During biocidal efficacy tests such as BS EN 1276 and BS EN 13697 (BSI 2019; BSI 2015+2019a), the required incubation temperature is 36±1 °C and this was replicated in the biofilm growth method. However, this temperature was compared to 32.5°C, which achieved the highest CFU/mL for *S. aureus* within single and multi-organism biofilms but was shown not to affect the CFU/mL of *P. aeruginosa.* Even though the CFU/mL of *S. aureus* is increased at the lower temperature, the higher temperature provided more consistent results between the two organisms. At 32.5°C the CFU/mL fluctuates between 10^9 and 10^{11} for *S. aureus*, but *P. aeruginosa* only achieves 10^8 to 10^9 when grown at 32.5°C. When grown at 36 degrees, the CFU/mL between both organisms is between $1.22x10^9$ and $5.33x10^9$ for both single and multi-organism biofilms (**Figure 3.4**). When testing this method alteration, *P. aeruginosa* NCTC 13359 was used.

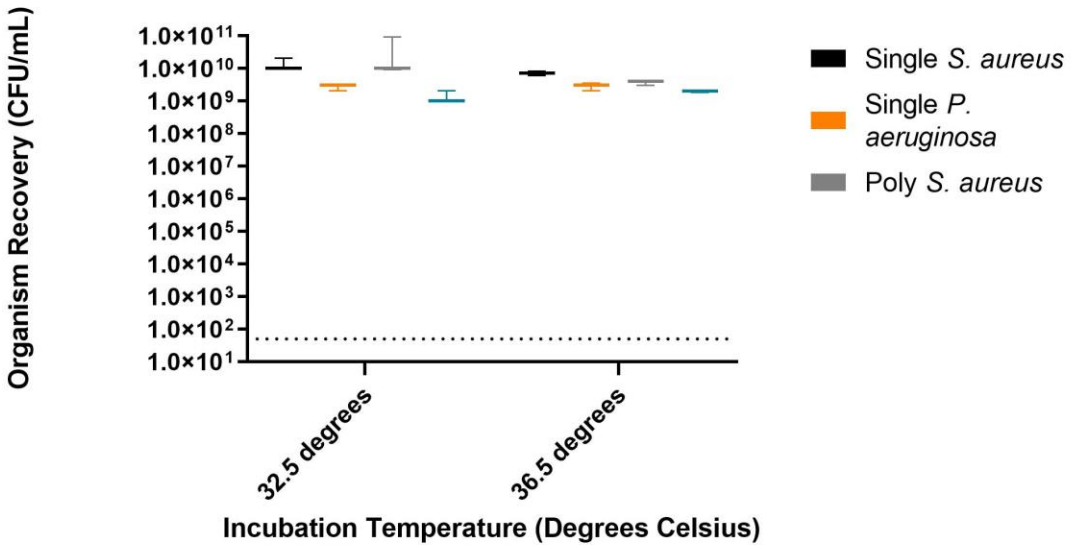

Figure 3.4. The effect of incubation temperature on organism recovery of the single and multi-organism biofilms of *S. aureus* and *P. aeruginosa*. Single and multi-organism species biofilms were grown up using the standard biofilm growth method but incubated at 32.5°C or 36.5°C for 48 hours. These were then recovered by placing in 10 mL PBS. All recovery is in CFU/mL. The dotted line depicts the minimum detection value is 50 CFU/mL. Data shown are mean ± SD. Technical replicates n=3, biological replicates n=1 (n=3).

After the above modifications to the method to optimise the CFU/mL recovery, the method was altered to include the addition of growing the overnight inoculum in TSB supplemented with 1% glucose, a 48-hour incubation time at 36.5°C. When producing a polymicrobial biofilm, it was altered to inoculate the PC disc with *S. aureus* 24 hours before inoculating with *P. aeruginosa.* All work undertaken to optimise the biofilm growth method has used *P. aeruginosa* NCTC 13359, which is a strain that doesn't produce pyocyanin. This strain is required for British Standard efficacy tests such as BS EN 1276 (BSI, 2019a), hence the focus on this strain in the beginning. *P. aeruginosa* NCTC 12924 is a strain that does produce pyocyanin and was introduced to assess the differences when introducing a biocidal product onto the biofilm. The biocidal testing involving the hydrogen peroxide and QAC disinfectant use against both strains of *P. aeruginosa* to see if there was any differences that may be attributed to the pyocyanin production.

3.2 Minimum biofilm eradication concentration (MBEC) for QAC disinfectants

Before testing the application of the QAC disinfectant in combination with the UV-C, the Minimum Biofilm Eradication Concentration (MBEC) had to be established for both single and multi-species biofilms.

The single organism *S. aureus* biofilm had an MBEC for the QAC of 0.24% w/w, which achieved a significant 9-log reduction (**Figure 3.5**) from the control ($P<0.0001$). This shows that the desirable concentration in which to combine the UV-C would be between 0.12% and 0.18%. For the single organism *P. aeruginosa* biofilms (NCTC 12924, NCTC 13359), the MBEC was higher than for the *S. aureus* biofilm at 5.31% w/w (**Figure 3.6, Figure 3.7**) for both strains. This concentration achieved a significant 9-log reduction for NCTC 12924 ($P<0.0001$) and NCTC 13359 ($P<0.0002$) and using this information, a concentration of between 4.15% or 4.8% should give the desired concentration to be used in combination with UV-C to establish a synergistic effect for both strains of *P. aeruginosa*.

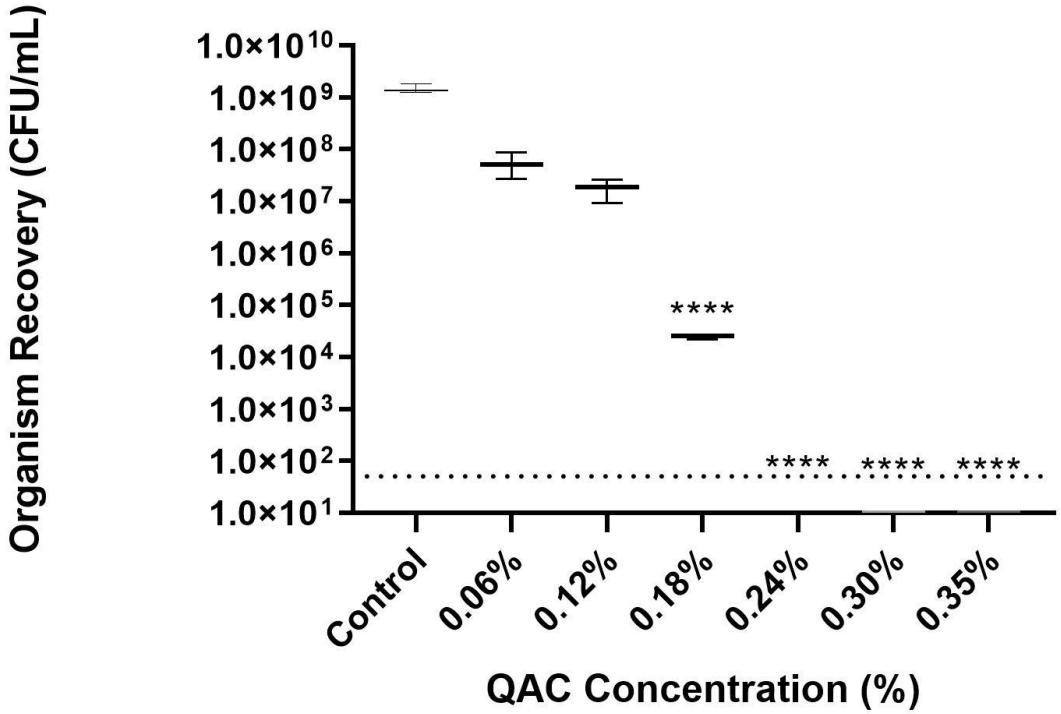

Figure 3.5. MBEC of the single organism *S. aureus* biofilm against QAC disinfectant. Results after *t* contact time (*t*=1 hour) with 200 µL QAC disinfectant at 6 concentrations compared to a control of synthetic hard water for *t*. All recovery is in CFU/mL. The dotted line depicts minimum detection value is 50 CFU/mL. Data shown are mean ± SD. **** Refers to statistical significance of data when compared to a control, calculated using a one-way ANOVA and Dunnett's multiple comparisons test (P<0.0001). Technical replicates n=3, biological replicates n=3 (n=9).

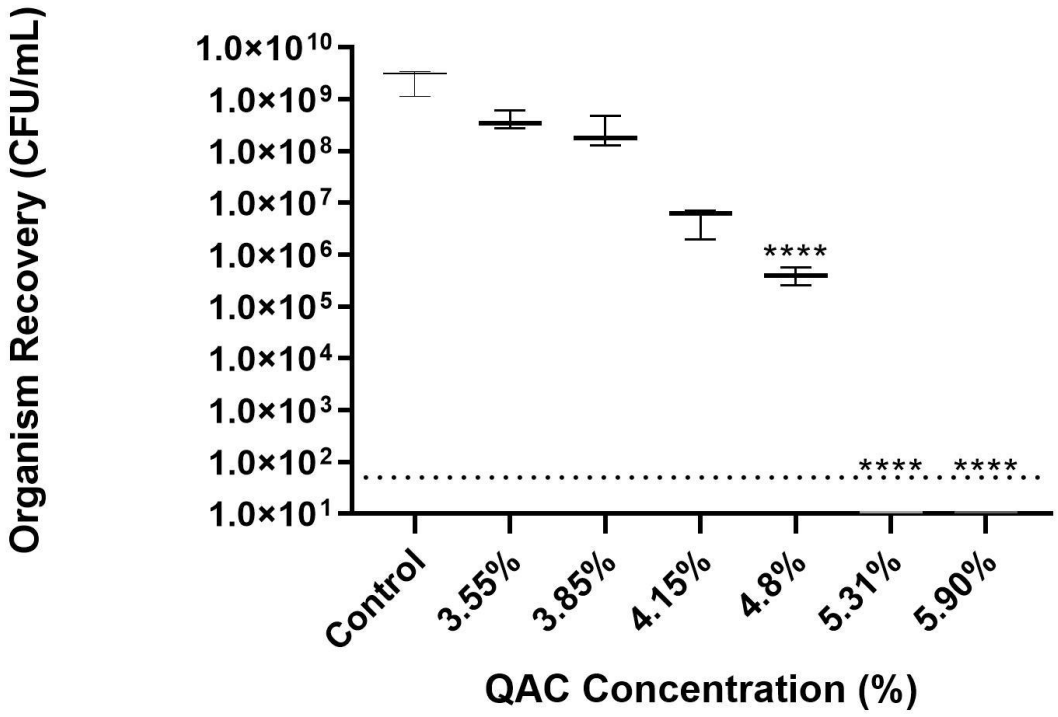

Figure 3.6. MBEC of the single organism *P. aeruginosa* (NCTC 12924) biofilm against QAC disinfectant. Results after *t* contact time (*t*=1 hour) with 200 µL QAC disinfectant at 6 concentrations compared to a control of synthetic hard water for *t*. All recovery is in CFU/mL. The dotted line depicts minimum detection value is 50 CFU/mL. Data shown are mean ± SD. **** Refers to statistical significance of data when compared to a control, calculated using a one-way ANOVA and Dunnett's multiple comparisons test (P<0.0001). Technical replicates n=3, biological replicates n=3 (n=9).

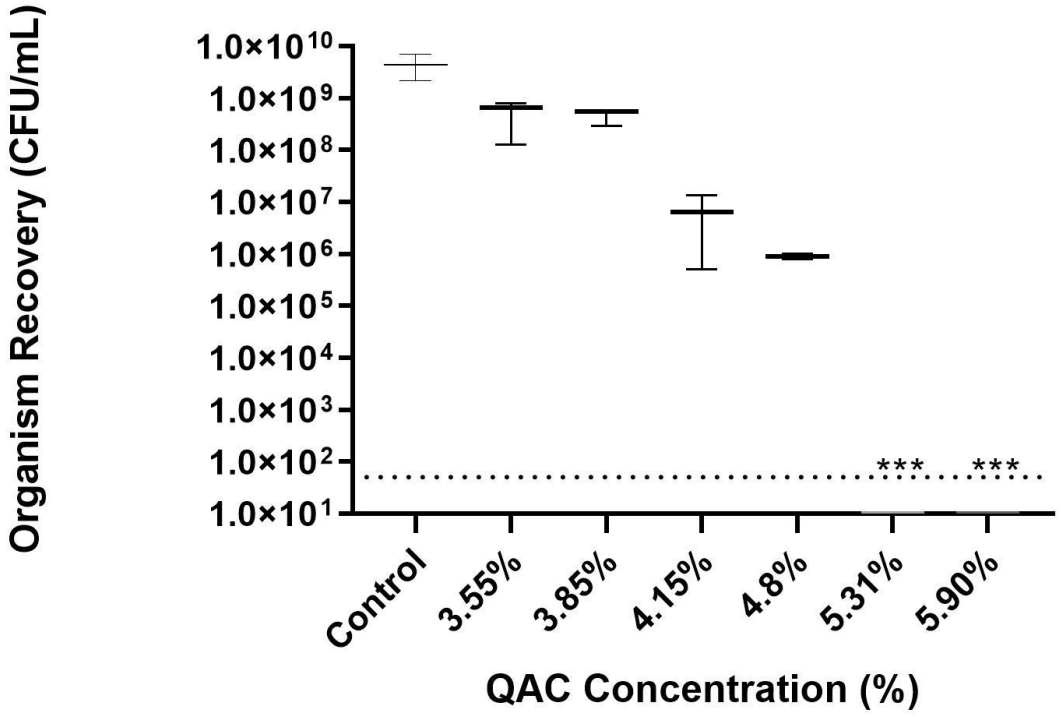

Figure 3.7. MBEC of the single organism *P. aeruginosa* (NCTC 13359) biofilm against QAC disinfectant. Results after *t* contact time (*t*=1 hour) with 200 µL QAC disinfectant at 6 concentrations compared to a control of synthetic hard water for *t*. All recovery is in CFU/mL. The dotted line depicts minimum detection value is 50 CFU/mL. Data shown are mean ± SD. *** Refers to statistical significance of data when compared to a control, calculated using a one-way ANOVA and Dunnett's multiple comparisons test (P<0.0002). Technical replicates n=3, biological replicates n=3 (n=9).

When both organisms were grown in the same multi-species biofilm, the MBEC changed due to the synergistic effects of the organisms within the biofilm. The concentration achieving a complete MBEC of both organisms simultaneously, for the biofilm containing *P. aeruginosa* (NCTC 13359) was 5.31% w/w. However, 4.80% was effective in achieving an MBEC against *S. aureus* but only achieved a 4-log reduction for *P. aeruginosa.* For *S. aureus,* this showed a 182.70% increase of the product concentration, which was needed to achieve a significant log reduction when in a polymicrobial biofilm compared to a single species biofilm (**Figure 3.8**). However, the same changes were not observed in *P. aeruginosa* (NCTC 13359), as the log reductions achieved by the QAC disinfectant were comparable when testing against the single species and multi-species biofilms.

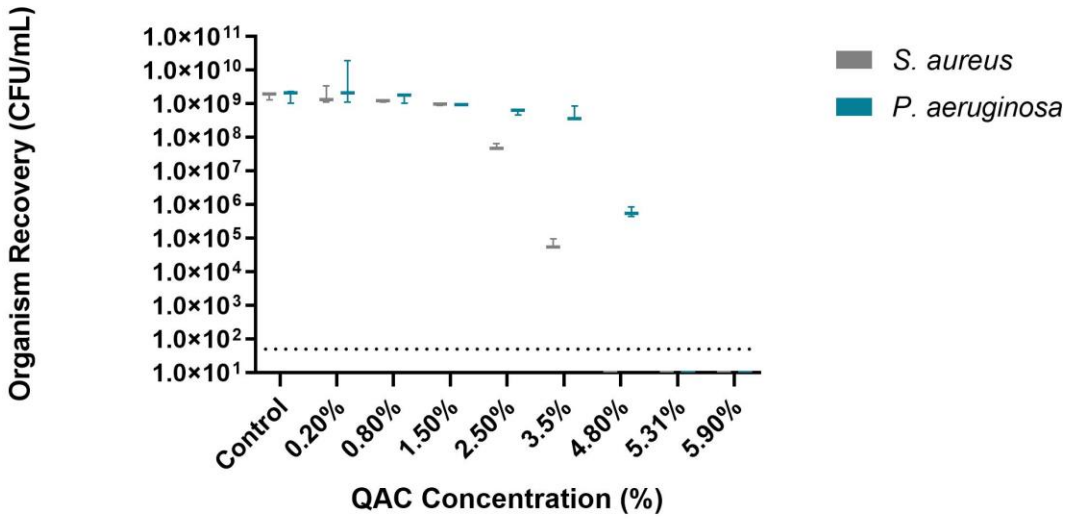

Figure 3.8. MBEC of the multi organism biofilm of *S. aureus* and *P. aeruginosa* (NCTC 13359) against QAC disinfectant. Results after *t* contact time (*t*=1 hour) with 200 µL QAC disinfectant at 8 concentrations compared to a control of synthetic hard water for *t*. All recovery is in CFU/mL. The dotted line depicts minimum detection value is 50 CFU/mL. Data shown are mean ± SD. No statistical significance of data when compared to a control, calculated using a two-way ANOVA and Dunnett's multiple comparisons test. Technical replicates n=3, biological replicates n=3 (n=9).

Similar results were shown for the polymicrobial biofilm containing *P. aeruginosa* (NCTC 12924) with the complete MBEC for both organisms being the same concentration of 5.31% w/w. However, unlike for the other *P. aeruginosa* strain, the 4.80% concentration only achieved a 7-log reduction for the *S. aureus,* rather than the 8-log reduction achieved on the multi-species biofilm containing *P. aeruginosa* (NCTC 13359). *P. aeruginosa* (NCTC 12924), within a multi-species biofilm was not affected like *S. aureus* as the log reductions achieved by the QAC disinfectant and comparable when testing against the single species and multi-species biofilms (**Figure 3.9**).

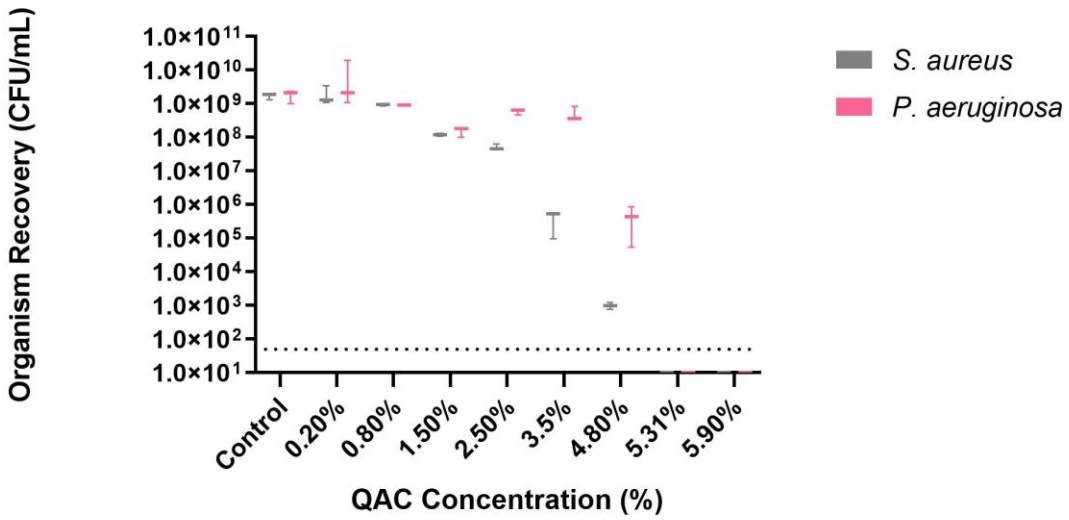

Figure 3.9. MBEC of the multi organism biofilm of *S. aureus* and *P. aeruginosa* (NCTC 12924) against QAC disinfectant. Results after *t* contact time (*t*=1 hour) with 200 µL QAC disinfectant at 8 concentrations compared to a control of synthetic hard water for *t*. All recovery is in CFU/mL. The dotted line depicts minimum detection value is 50 CFU/mL. Data shown are mean ± SD. No statistical significance of data when compared to a control, calculated using a two-way ANOVA and Dunnett's multiple comparisons test. Technical replicates n=3, biological replicates n=3 (n=9).

3.3 Optimising the disinfectant efficacy test method for hydrogen peroxide

When establishing the MBEC for the hydrogen peroxide disinfectant, the method used for the QAC disinfectant was not viable, as it was not achieving an end point concentration. Due to this, the method was altered (2.5 Disinfectant efficacy testing method for hydrogen peroxide disinfectants) to mimic the disinfectant volume from a study by Lineback et al (2018). The contact time used throughout the method was 1 hour, however for the hydrogen peroxide, this was lowered due to the disinfectant activating the catalases and potentially causing resistance over the long contact time.

The contact time using the initial method used for the QACs was re-tested using a shortened contact time of 1 minute and 30 minutes where 200 µL of 7.9% w/w (supplied concentration) hydrogen peroxide was applied to the biofilm discs and was tested against a control of the same procedure but using sterile DI, but no significant reduction was found (**Figure 3.10**).

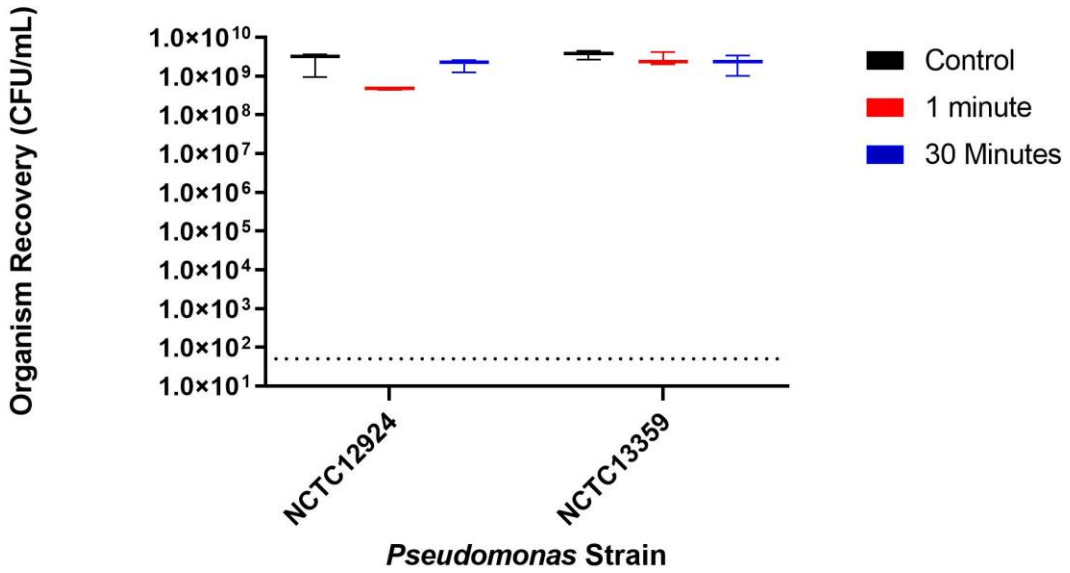

Figure 3.10. The single species biofilm of *P. aeruginosa* (NCTC 12924 and NCTC 13359) against hydrogen peroxide disinfectant against two contact times using the QAC disinfectant method. Results after *t* contact time (*t*=1 minute or 30 seconds) with 200 µL hydrogen peroxide disinfectant at 1 concentration (7.9% w/w) compared to a control of sterile DI for *t*. All recovery is in CFU/mL. The dotted line depicts minimum detection value is 50 CFU/mL. Data shown are mean ± SD. No statistical significance of data calculated using a two-way ANOVA and Dunnett's multiple comparisons test. Technical replicates n=3, biological replicates n=1 (n=3).

After the reduced contact time using the method viable for the QAC disinfectant proved to be ineffective, the method was altered to replicate Lineback et al. (2018) by increasing the disinfectant volume to 4 mL. The new method 'dipped' the biofilm disc into 4 mL of hydrogen peroxide disinfectant for the contact time '*t*' and then after this time had elapsed, transferred the biofilm disc into 10 mL of neutralising broth. This method showed increased log reductions compared to the previous method (**Figure 3.11**) as 7.9% w/w hydrogen peroxide tested against *P. aeruginosa* (NCTC 12924) achieved a significant 8-log reduction ($P<0.0003$) when compared to a control of sterile DI water. This can also be compared to no reduction for *P. aeruginosa* (NCTC 12924) when using the previous method. The same disinfectant concentration also achieved up to a 3-log reduction when tested against *P. aeruginosa* (NCTC 13359) compared to no log reduction when using the previous method. The results for *P. aeruginosa* (NCTC 13359) were not significant as they would not provide a substantial log reduction to comply with BS EN 13697 or BS EN 1276 tests which require a 4 and 5-log reduction, respectively (BSI, 2015; 2019a). Due to *P. aeruginosa* (NCTC

12924) being the strain that was most affected by the 'dipping' testing method, this is the strain that is focused on going forward in the testing due to time constraints.

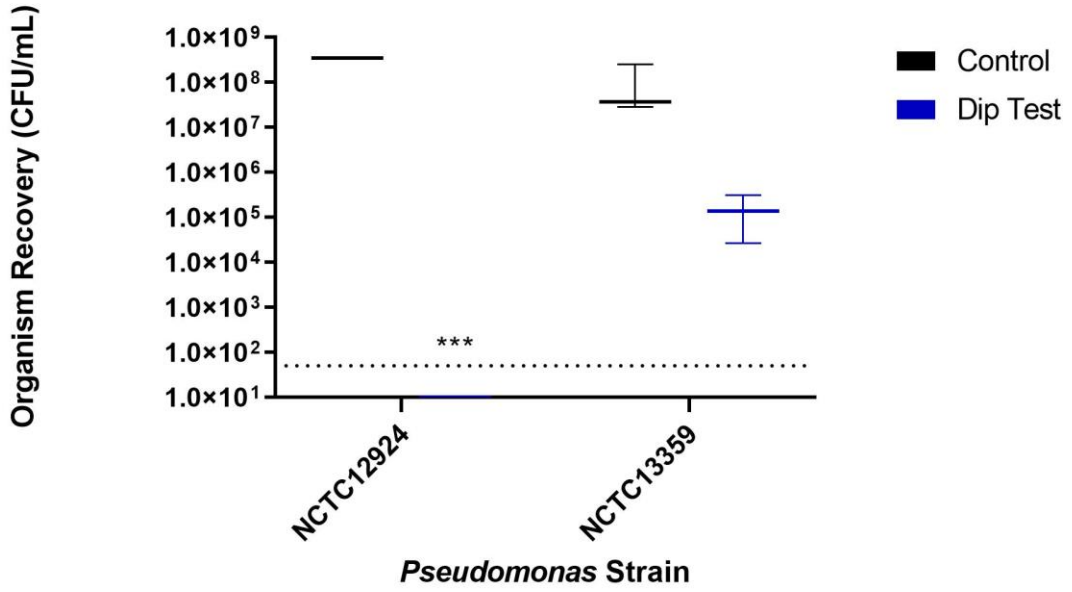

Figure 3.11. The single species biofilm of *P. aeruginosa* (NCTC 12924 and NCTC 13359) against hydrogen peroxide disinfectant using the modified disinfectant method. Results after *t* contact time (*t*=30 minutes) with 4 mL hydrogen peroxide disinfectant at 1 concentration (7.9% w/v) compared to a control of sterile DI for *t*. All recovery is in CFU/mL. The dotted line depicts minimum detection value is 50 CFU/mL. One data set is below the limit of detection. Data shown are mean ± SD. *** refers to statistical significance of data calculated using a two-way ANOVA and Sidak's multiple comparisons test ($P<0.0003$). Technical replicates n=3, biological replicates n=1 (n=3).

Using this modified method for the hydrogen peroxide disinfectant introduced the concern that the biofilm would be disrupted and that cells would detach when in contact with the disinfectant/water. The lost cells would then not be transferred to the neutralising broth. A comparison was done to see how many cells detached from each biofilm type when tested against 4 mL 7.9% w/w hydrogen peroxide (**Figure 3.12 A**) and 4 mL sterile DI (**Figure 3.12 B**). After the disc was removed from the test product and put into the neutralising broth, the test product was centrifuged to remove any of the active ingredients and re-suspended in 4 mL DI water. This was then diluted and plated out using Miles Misra technique. The CFU/mL recovered from the detached cells in the DI water are between 10^8 and 10^9 but when tested in hydrogen peroxide, both *P. aeruginosa* strains had no viable detached cells recovered. The *S. aureus* had a

high number of viable cells detach during the test but the hydrogen peroxide did not achieve a significant log reduction. There is a difference in the test results between the two strains of *P. aeruginosa* with the pyocyanin producing strain (NCTC 12924) being more susceptible to the hydrogen peroxide than the non-pyocyanin producing strain (NCTC13359).

Using the recovery of detached cells in the water that the biofilm disc had been 'dipped' in (**Figure 3.12 B**), the average CFU/mL percentage (%) decrease was determined using the following equation where:

N_1 The average CFU/mL of viable detached cells from the 4 mL DI water that had been used to dip the biofilm disc

N_2 The average CFU/mL of viable detached cells from the 10 mL neutralising broth used in the test

$$\text{Percentage Decrease} = \frac{|N_1 - N_2|}{N_1} \times 100$$

For *S. aureus* using the results gathered:

$$\text{Percentage Decrease} = \frac{|800000000 - 75000000|}{800000000} \times 100 = 90.625\%$$

For *P. aeruginosa* (NCTC 12924) using the results gathered:

$$\text{Percentage Decrease} = \frac{|3000000000 - 50000000|}{3000000000} \times 100 = 98.333\%$$

The percentage decrease for *P. aeruginosa* (NCTC 13359) was not calculated due to the lack of significant difference in CFU/mL between both variables.

Going forward in the study, when using the 'dipping' method for the hydrogen peroxide disinfectant, this average percentage decrease will be accounted for when displaying recovered CFU/mL.

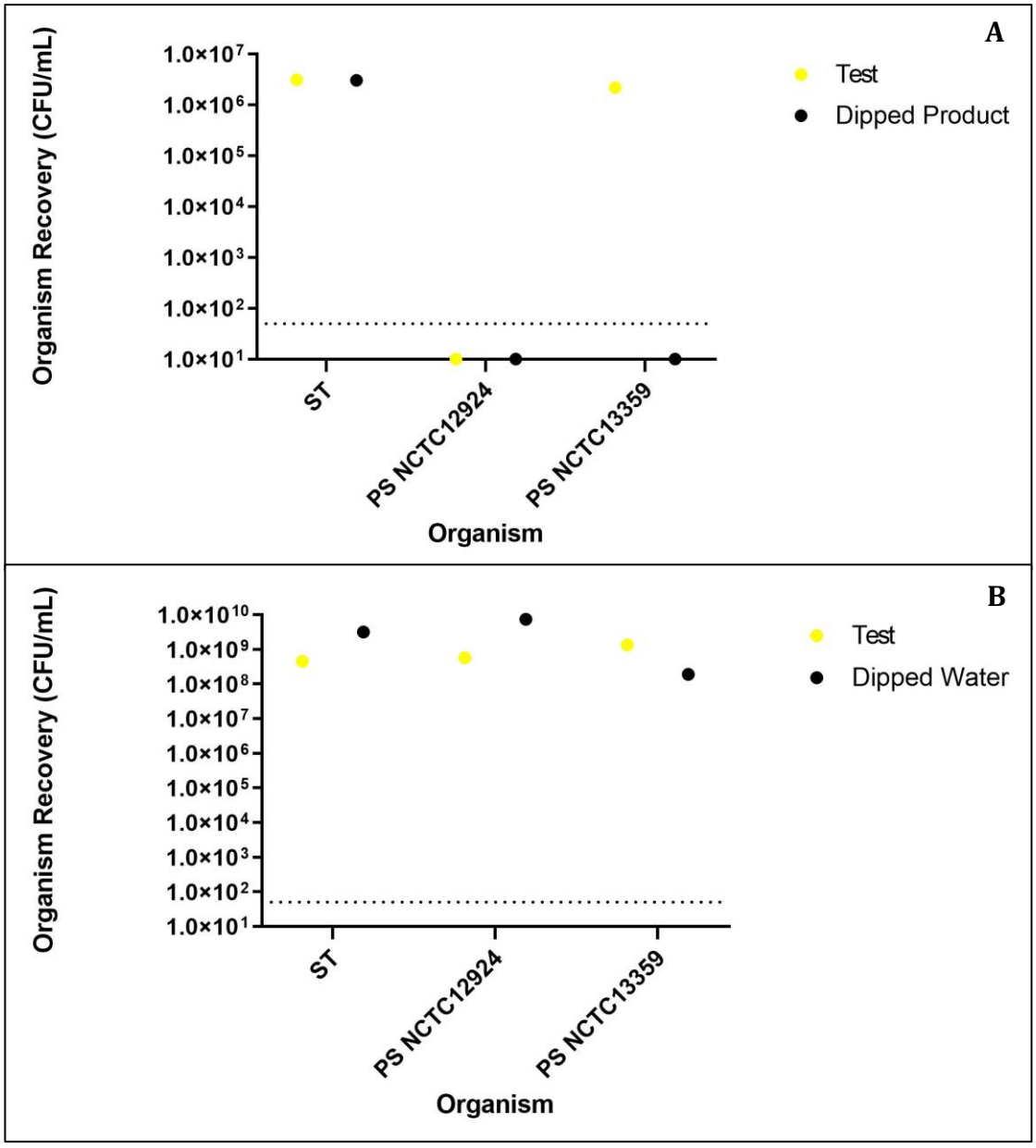

Figure 3.12. The viable cell detachment of *P. aeruginosa* (NCTC 12924 and NCTC 13359) and *S. aureus* in single species biofilms against hydrogen peroxide disinfectant and sterile DI using the modified disinfectant method. Results after *t* contact time (*t*=30 minutes) with 4 mL hydrogen peroxide disinfectant at 1 concentration (7.9% w/v) compared to a control of sterile DI water for *t*. 'Dipped product' or 'dipped water' is the diluent or product used for dipping that was recovered, 'test' is the neutralising broth that was recovered from the same disc. ST (*S. aureus*); PS (*P. aeruginosa*). All recovery is in CFU/mL. The dotted line depicts minimum detection value is 50 CFU/mL. Technical replicates n=1, biological replicates n=1 (n=1). Figure A is the recovery of viable detached cells when dipped into hydrogen peroxide; Figure B is the recovery of viable detached cells when dipped into sterile DI.

3.4 Minimum biofilm eradication concentration (MBEC) for hydrogen peroxide (H_2O_2)

Before testing the application of the hydrogen peroxide disinfectant in combination with the UV-C, the Minimum Biofilm Eradication Concentration had to be established for both single and multi-species biofilms. This was achieved by using the modified 'dipping' method outlined in 3.3 Optimising the disinfectant efficacy test method for hydrogen peroxide. An MBEC could not be achieved for the single organism *S. aureus* biofilm (**Figure 3.13**). However, since the combination of hydrogen peroxide and UV-C was tested using a disinfectant concentration achieving a partial-kill, reaching an MBEC was not necessary for the continuation of the study.

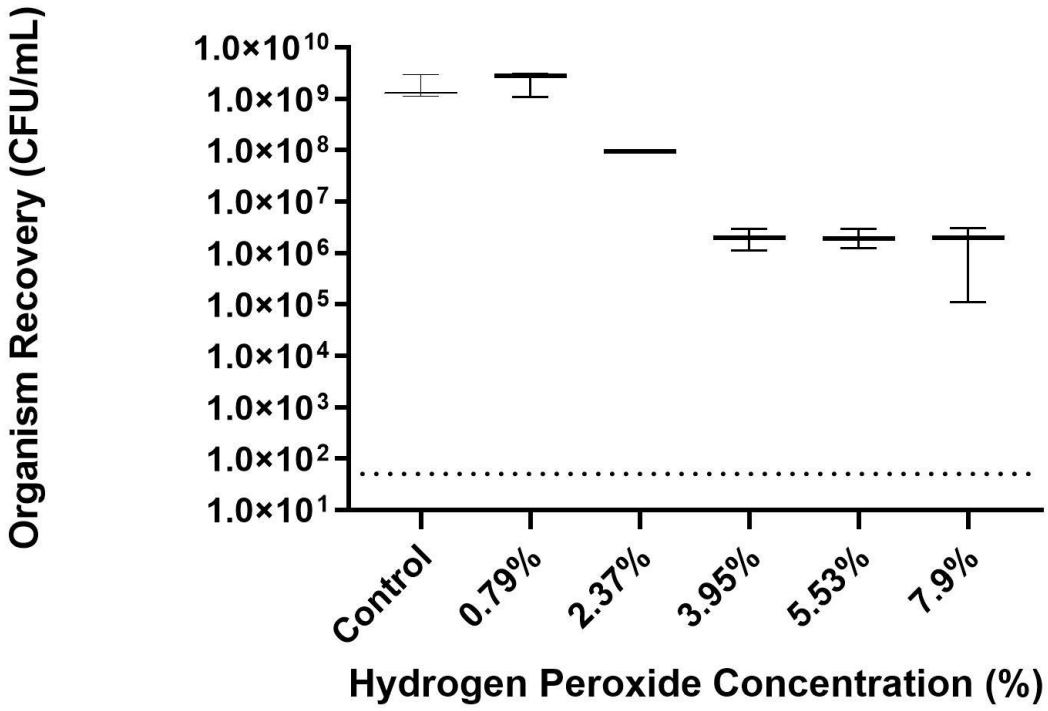

Figure 3.13. MBEC of the single organism *S. aureus* biofilm against hydrogen peroxide disinfectant. Results after *t* contact time (*t*=30 minutes) with 4 mL hydrogen peroxide disinfectant at 5 concentrations compared to a control of synthetic hard water for *t*. All recovery is in CFU/mL. The dotted line depicts minimum detection value is 50 CFU/mL. Data shown are mean ± SD. No statistical significance of data when compared to a control, calculated using a one-way ANOVA and Dunnett's multiple comparisons test. Technical replicates n=3, biological replicates n=3 (n=9).

For the single organism *P. aeruginosa* biofilms (NCTC 12924), the MBEC was at 5.53% w/w (**Figure 3.14**). This concentration achieved a significant 8-log

reduction for NCTC 12924 (P<0.0001) and using this information, a hydrogen peroxide concentration of between 0.79% and 3.95% should give the desired concentration to be used in combination with UV-C to establish a synergistic effect. The MBEC was not completed on the *P. aeruginosa* (NCTC 13359) due to time constraints.

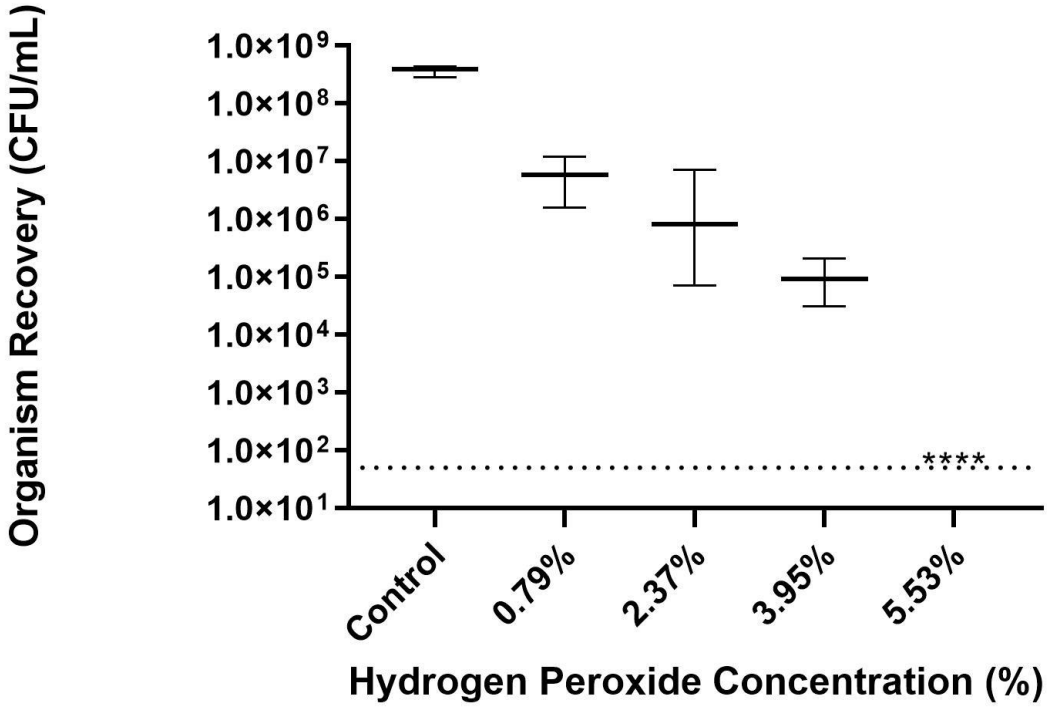

Figure 3.14. MBEC of the single organism biofilm of *P. aeruginosa* (NCTC 12924) against hydrogen peroxide disinfectant. Results after *t* contact time (*t*=30 minutes) with 4 mL hydrogen peroxide disinfectant at 4 concentrations compared to a control of synthetic hard water for *t*. All recovery is in CFU/mL. The dotted line depicts minimum detection value is 50 CFU/mL. Data shown are mean ± SD. **** refers to statistical significance of data when compared to a control, calculated using a one-way ANOVA and Dunnett's multiple comparisons test (P<0.0001). Technical replicates n=3, biological replicates n=3 (n=9).

When both *S. aureus* and *P. aeruginosa* (NCTC 12924) were grown in a polymicrobial biofilm, the MBEC of hydrogen peroxide increased. The MBEC for the polymicrobial biofilm was 7.9%, which gave a complete kill for *P. aeruginosa* but only achieved just over a 4-log reduction for *S. aureus* (**Figure 3.15**). When assessed in a single organism biofilm, *P. aeruginosa* was eradicated by 5.53% hydrogen peroxide which achieved a log reduction of >8, however that same concentration achieved a 5-log reduction against *P. aeruginosa* within the polymicrobial biofilm. A suitable concentration to be used in combination with UV-C would be between 0.79% and 3.95% or above. The MBEC was not

completed on the multi-species biofilm containing *P. aeruginosa* (NCTC 13359) due to time constraints.

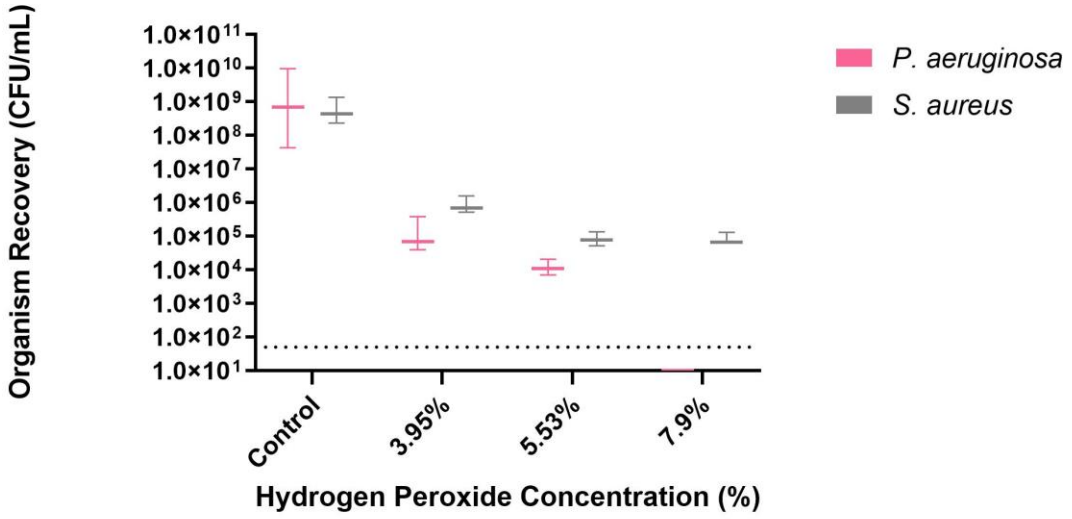

Figure 3.15. MBEC of the multi organism biofilm of *S. aureus* and *P. aeruginosa* (NCTC 12924) against hydrogen peroxide disinfectant. Results after *t* contact time (*t*=30 minutes) with 4 mL hydrogen peroxide disinfectant at 3 concentrations compared to a control of synthetic hard water for *t*. All recovery is in CFU/mL. The dotted line depicts minimum detection value is 50 CFU/mL. Data shown are mean ± SD. No statistical significance of data when compared to a control, calculated using a two-way ANOVA and Dunnett's multiple comparisons test. Technical replicates n=3, biological replicates n=3 (n=9).

3.5 UV-C treatment

The single species biofilms were assessed against UV-C treatment without disinfectant. The longest time point (*t*=10 minutes) of UV-C exposure achieved no significant log reduction (**Figure 3.16**) for *S. aureus, P. aeruginosa* (NCTC 13359) and *P. aeruginosa* (NCTC 12924) when compared to a control of discs without exposure to UV-C light. The average UV-C dose for 1 minute, 5 minutes and 10 minutes exposure was 23.22 mJ/cm^2, 71.88 mJ/cm^2 and 104.10mJ/cm^2 respectively.

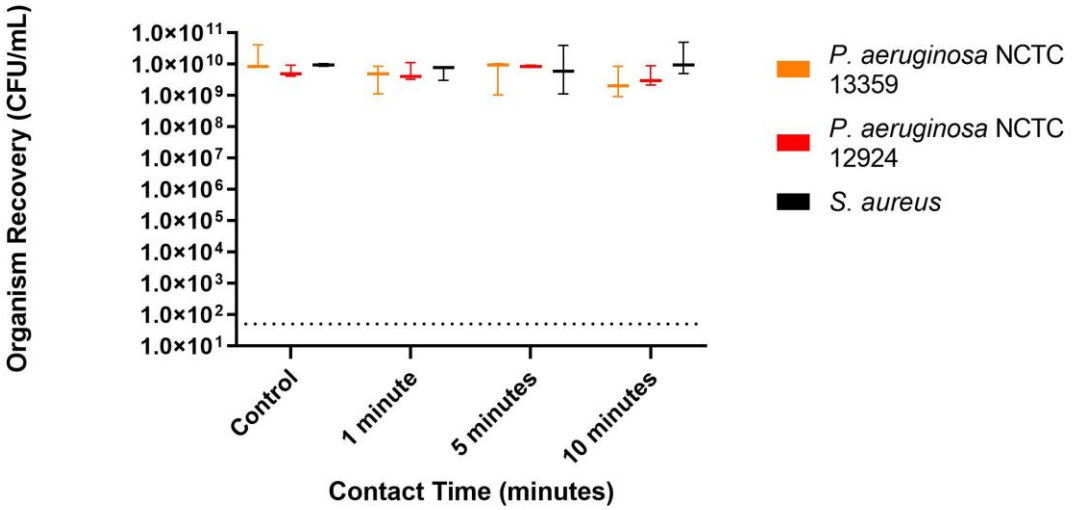

Figure 3.16. Organism recovery of single organism biofilm of *S. aureus* and *P. aeruginosa* (NCTC 12924) and *P. aeruginosa* (NCTC 13359) after exposure to UV-C. Results after *t* contact time (*t*=10, 5 or 1 minute) of exposure to direct UV-C light 18cm away from the light source, against a control of no exposure for *t*. All recovery is in CFU/mL. The dotted line depicts minimum detection value is 50 CFU/mL. Data shown are mean ± SD. No statistical significance of data when compared to a control, calculated using a two-way ANOVA and Dunnett's multiple comparisons test. Technical replicates n=3, biological replicates n=3 (n=9).

3.6 Combined UV-C disinfectant treatment

After the disinfectant concentration that gave a mid-range kill was established using the MBEC, this was used in combination with UV-C exposure. To determine the effect of UV-C application contact time on efficacy, *S. aureus* single species biofilms were used. The UV-C was added either prior to the disinfectant added or whilst the disinfectant was in contact with the biofilm, in the last 10 minutes of the contact time.

3.6.1 UV-C administered post or prior to chemical disinfectant

When comparing the log reductions achieved between the *S. aureus* single species biofilm being exposed to UV-C at the beginning or the end of the contact with hydrogen peroxide, the results differed, but not significantly (**Figure 3.17**). When the UV-C was administered before any hydrogen peroxide, there was a lower rate of log reduction compared to when it was administered after the hydrogen peroxide. However, this was completed before the addition of the 'dipping' method, so these results were not comparable to the end test. It would have been beneficial to do this test using the QAC instead of the hydrogen

peroxide as they use two different methods, but this was not done due to time constraints.

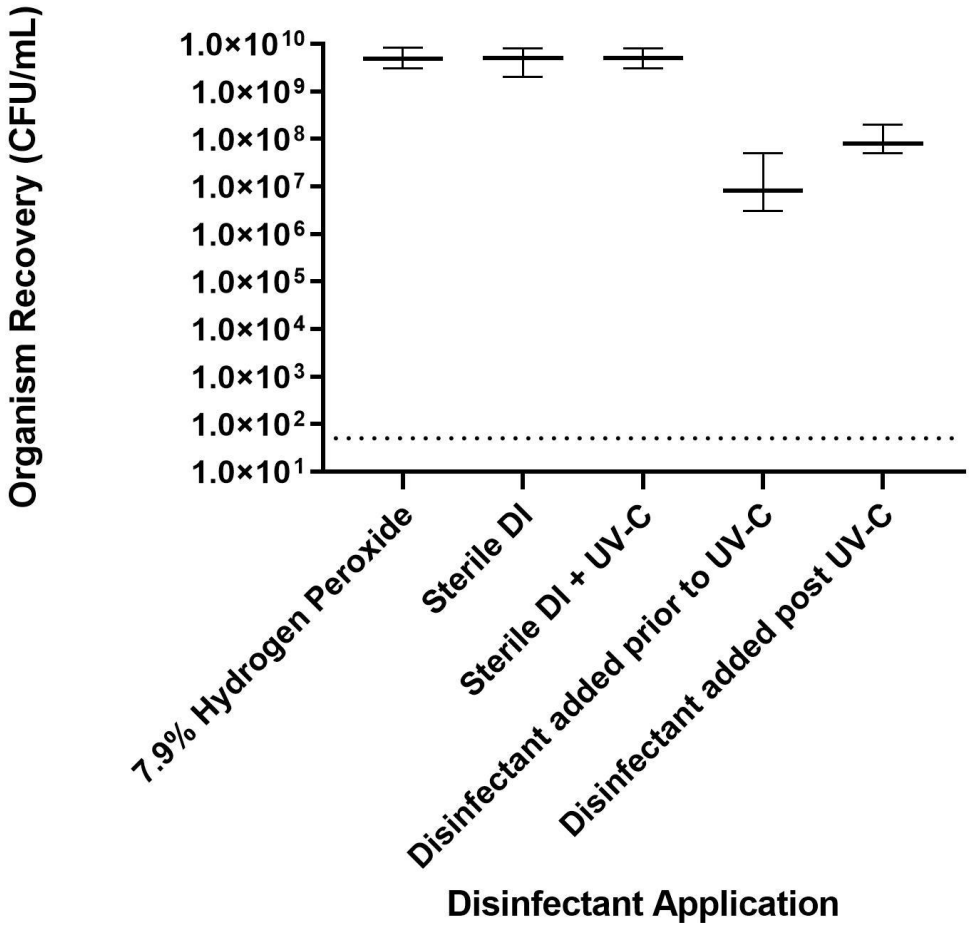

Figure 3.17. Organism recovery of single organism biofilm of *S. aureus* after exposure to UV-C and hydrogen peroxide. Results after *t* contact time (*t*=60 minutes made up of 50 minutes disinfectant with 10 minutes of UV-C and 7.9% w/w hydrogen peroxide or 70 minutes made up of 10 minutes UV-C then 60 minutes hydrogen peroxide). Exposure to UV-C is direct light 18cm away from the light source, against a control of no exposure, 7.9% w/w hydrogen peroxide without UV-C exposure, sterile DI without UV-C exposure and sterile DI with UV-C exposure for *t*. All recovery is in CFU/mL. The dotted line depicts minimum detection value is 50 CFU/mL. Data shown are mean ± SD. No statistical significance of data calculated using a one-way ANOVA and Tukey's multiple comparisons test. Technical replicates n=3, biological replicates n=3 (n=9).

For this test, the UV-C treatment was applied for 10 minutes before the disinfectant was added or 10 minutes before the contact time of the disinfectant ended. The UV-C dose was between 526.5MJ/CM^2 and 831.1MJ/CM^2. As the UV-C being applied after 50 minutes of the disinfectant being in contact with the biofilm, achieved a better log reduction, this method was used going forward.

3.6.2 Combined UV-C and QAC disinfectant treatment

For the single organism *S. aureus* biofilm tested with a combination of 0.18% QAC and 10 minutes of UV-C exposure, there was an increase in log reduction compared to just the QAC alone (**Figure 3.18**). The QAC achieved almost a 4-log reduction (P<0.0170) whilst the addition of UV-C alongside the disinfectant achieved an 8-log reduction.

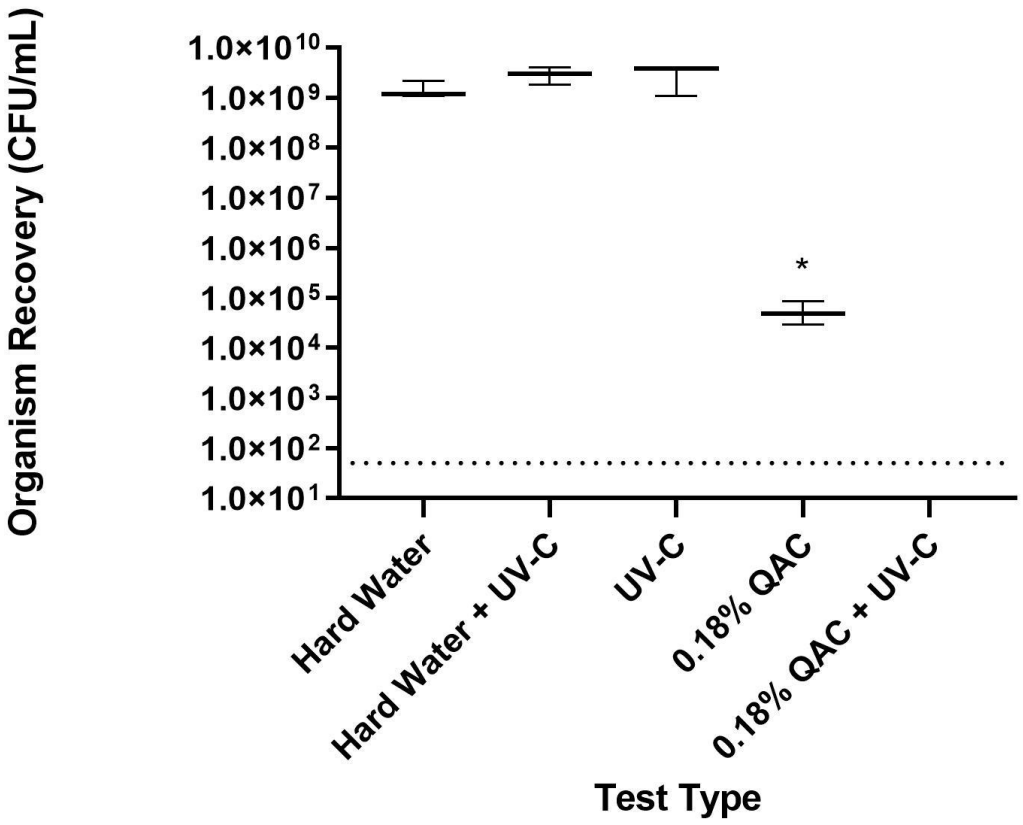

Figure 3.18. Combined treatment of the single organism biofilm of *S. aureus* against QAC disinfectant. Results after *t* contact time (*t*=60 minutes) with 200 µL QAC disinfectant at 1 concentration for 50 minutes and UV-C combined for a further 10 minutes (*t*=60 minutes) compared to controls of synthetic hard water (*t*= 60 minutes), QAC at 1 concentration (*t*= 60 minutes) and synthetic hard water for 50 minutes and UV-C combined for a further 10 minutes. All recovery is in CFU/mL. The dotted line depicts minimum detection value is 50 CFU/mL. Data shown are mean ± SD. * refers to statistical significance of data calculated using a one-way ANOVA and Tukey's multiple comparisons test (P<0.0170). Technical replicates n=3, biological replicates n=3 (n=9).

The log reduction of *P. aeruginosa* (NCTC 12924) single organism biofilm increased by the addition of UV-C used in combination with the QAC (**Figure 3.19**). The 4.15% QAC achieved a 3-log reduction, with the addition of UV-C increasing this to 5-log.

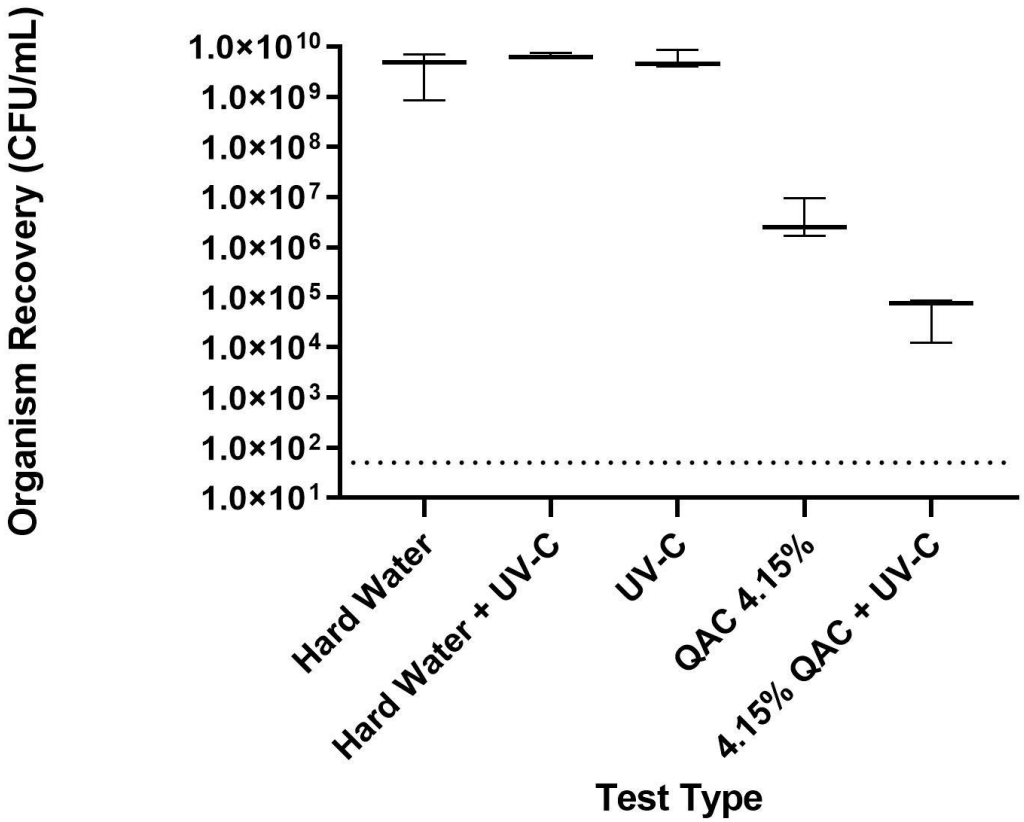

Figure 3.19. Combined treatment of the single organism biofilm of *P. aeruginosa* (NCTC 12924) against QAC disinfectant. Results after *t* contact time (*t*=60 minutes) with 200 µL QAC disinfectant at 1 concentration for 50 minutes and UV-C combined for a further 10 minutes (*t*=60 minutes) compared to controls of synthetic hard water (*t*= 60 minutes), QAC at 1 concentration (*t*= 60 minutes) and synthetic hard water for 50 minutes and UV-C combined for a further 10 minutes. All recovery is in CFU/mL. The dotted line depicts minimum detection value is 50 CFU/mL. Data shown are mean ± SD. No statistical significance of data calculated using a one-way ANOVA and Tukey's multiple comparisons test. Technical replicates n=3, biological replicates n=3 (n=9).

For the poly organism biofilm of *S. aureus* and *P. aeruginosa* (NCTC 12924), the addition of UV-C alongside the QAC increased the log reduction for both organisms (**Figure 3.20**) when compared to the QAC treatment alone. The QAC disinfectant as a solo treatment achieved a 2-log reduction for the *P. aeruginosa* and a 4-log reduction for the *S. aureus.* However, with the combination of the disinfectant and the UV-C, this increased to 5-log and 8-log respectively ($P<0.0001$). The combination treatment using QAC and UV-C was not explored using *P. aeruginosa* (NCTC 13359) due to time constraints.

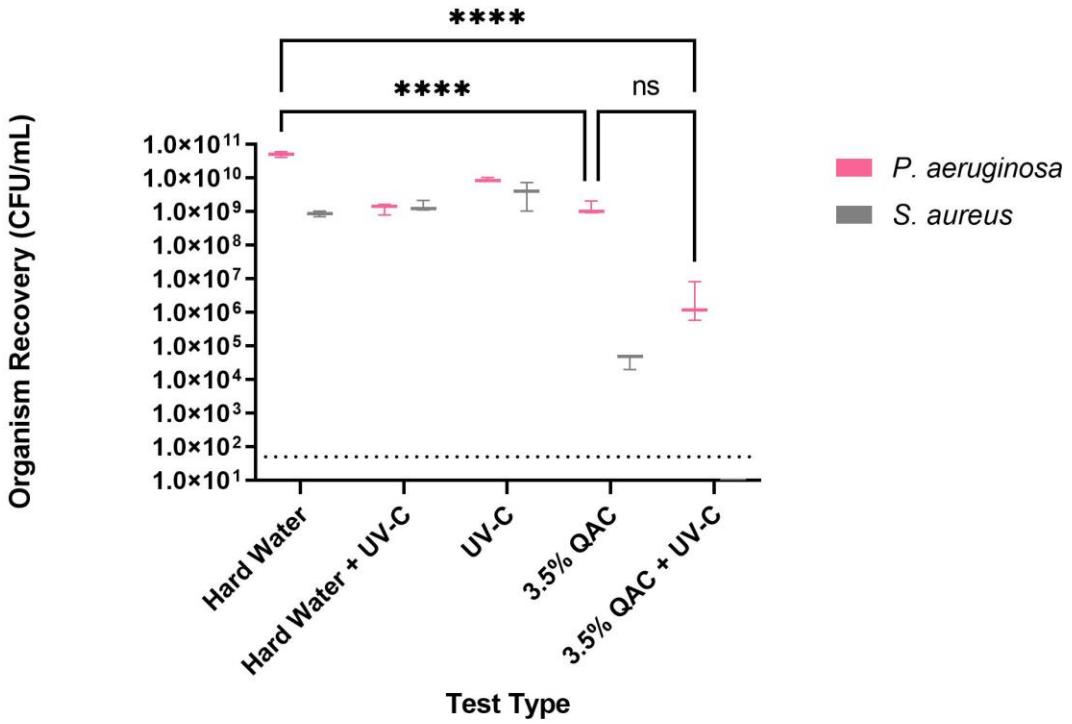

Figure 3.20. Combined treatment of the multi organism biofilm of *S. aureus* and *P. aeruginosa* (NCTC 12924) against QAC disinfectant and UV-C. Results after *t* contact time (*t*=60 minutes) with 200 µL QAC disinfectant at 2 concentrations for 50 minutes and UV-C combined for a further 10 minutes (*t*=60 minutes) compared to controls of synthetic hard water (*t*= 60 minutes), QAC at 2 concentrations (*t*= 60 minutes) and synthetic hard water for 50 minutes and UV-C combined for a further 10 minutes. All recovery is in CFU/mL. The dotted line depicts minimum detection value is 50 CFU/mL. Data shown are mean ± SD. **** refers to statistical significance of data calculated using a two-way ANOVA and Sidak's multiple comparisons test (P<0.0001). Technical replicates n=3, biological replicates n=3 (n=9).

3.6.3 Combined UV-C and hydrogen peroxide treatment

For the poly organism biofilm of *S. aureus* and *P. aeruginosa* (NCTC 12924), the addition of UV-C alongside the hydrogen peroxide increased the log reduction for both organisms (**Figure 3.21**). The hydrogen peroxide disinfectant as a solo treatment achieved a 5-log reduction for the *P. aeruginosa* and a 4-log reduction for the *S. aureus.* However, with the combination of the disinfectant and the UV-C, this increased to 7-log for both organisms. The combination treatment using hydrogen peroxide and UV-C was not explored using *P. aeruginosa* (NCTC 13359) due to time constraints.

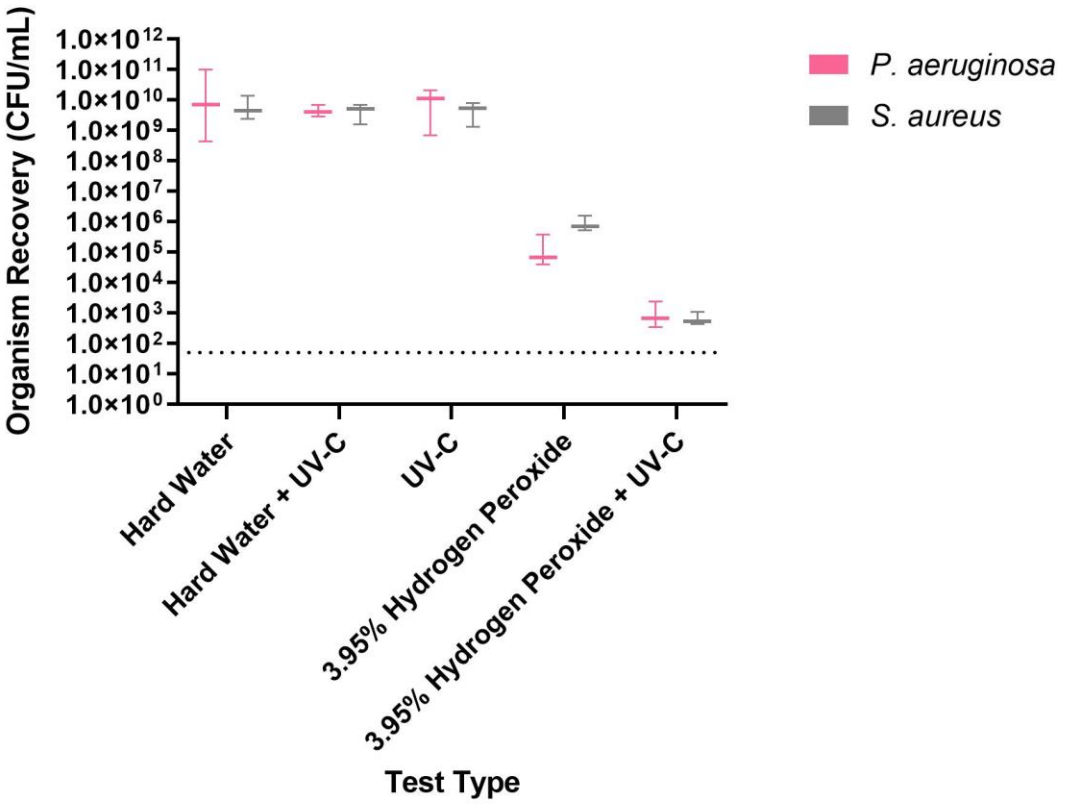

Figure 3.21. Combined treatment of the multi organism biofilm of *S. aureus* and *P. aeruginosa* (NCTC 12924) against hydrogen peroxide disinfectant and UV-C. Results after *t* contact time (*t*=30 minutes) with 4 mL hydrogen peroxide disinfectant at 3.95% w/v for 20 minutes and UV-C combined for a further 10 minutes (*t*=30 minutes) compared to controls of synthetic hard water (*t*= 30 minutes), hydrogen peroxide 3.95% w/v (*t*= 30 minutes) and synthetic hard water for 20 minutes and UV-C combined for a further 10 minutes. All recovery is in CFU/mL. The dotted line depicts minimum detection value is 50 CFU/mL. Data shown are mean ± SD. No statistical significance of data calculated using a two-way ANOVA and Tukey's multiple comparisons test. Technical replicates n=3, biological replicates n=3 (n=9).

3.7 Microscopy

Microscopy was undertaken at The University of Nottingham on the combined UV-C and disinfectant treatment of both QAC and hydrogen peroxide against the multi-species containing *S. aureus* and *P. aeruginosa* (NCTC 12924). Microscopy of multi-species biofilms containing *P. aeruginosa* (NCTC 13359) was not undertaken due to time constraints.

The quantification of the live/dead attached cells from the microscopy showed a significant difference ($p < 0.0001$) between the control and the QAC and the QAC combined with UV-C. However, there was no statistical significance between the QAC and the QAC combined with UV-C. This was similar for the hydrogen

peroxide, with there being a significant difference between the control and both the hydrogen peroxide and the hydrogen peroxide combined with UV-C but no significant difference between the hydrogen peroxide and the hydrogen peroxide combined with UV-C (**Figure 3.22**).

This quantification was taken from different images of the biofilm in contact with sterile deionised water (DI), sterile DI in combination with UV-C, disinfectant, and disinfectant in combination with UV-C. The red colouring indicates dead cells and the green colouring indicates live cells. The controls are imaged in **Figure 3.23** where the colour is mostly green, indicating that there was little or no dead cells within the biofilm and this was also shown in the CFU/mL of viable cells recovered from the biofilm. The imagines of the biofilm after being exposed to the QAC and the QAC combined with UV-C are displayed in **Figure 3.24**. Compared to the controls, these biofilms display a much higher percentage of dead cells, which was expected and in line with the CFU/mL of viable cells recovered from the biofilm. There is very little difference between live and dead cells from the two conditions which is also expressed in the CFU/mL data. The imaged of the biofilm after being exposed to the hydrogen peroxide and the hydrogen peroxide combined with UV-C is displayed in **Figure 3.25**. The biofilms imaged were heavily disrupted by the 'dipping' process so the biofilm that was imaged was thinner than the ones imaged for the other conditions. As it was a monolayer of cells remaining, the imaging was not as accurate. The images show dead cells, which was expected but compared to the images taken of the biofilm after exposure with the QAC and QAC combined with UV-C, there was an increase in dead cells after the UV-C is introduced with the hydrogen peroxide.

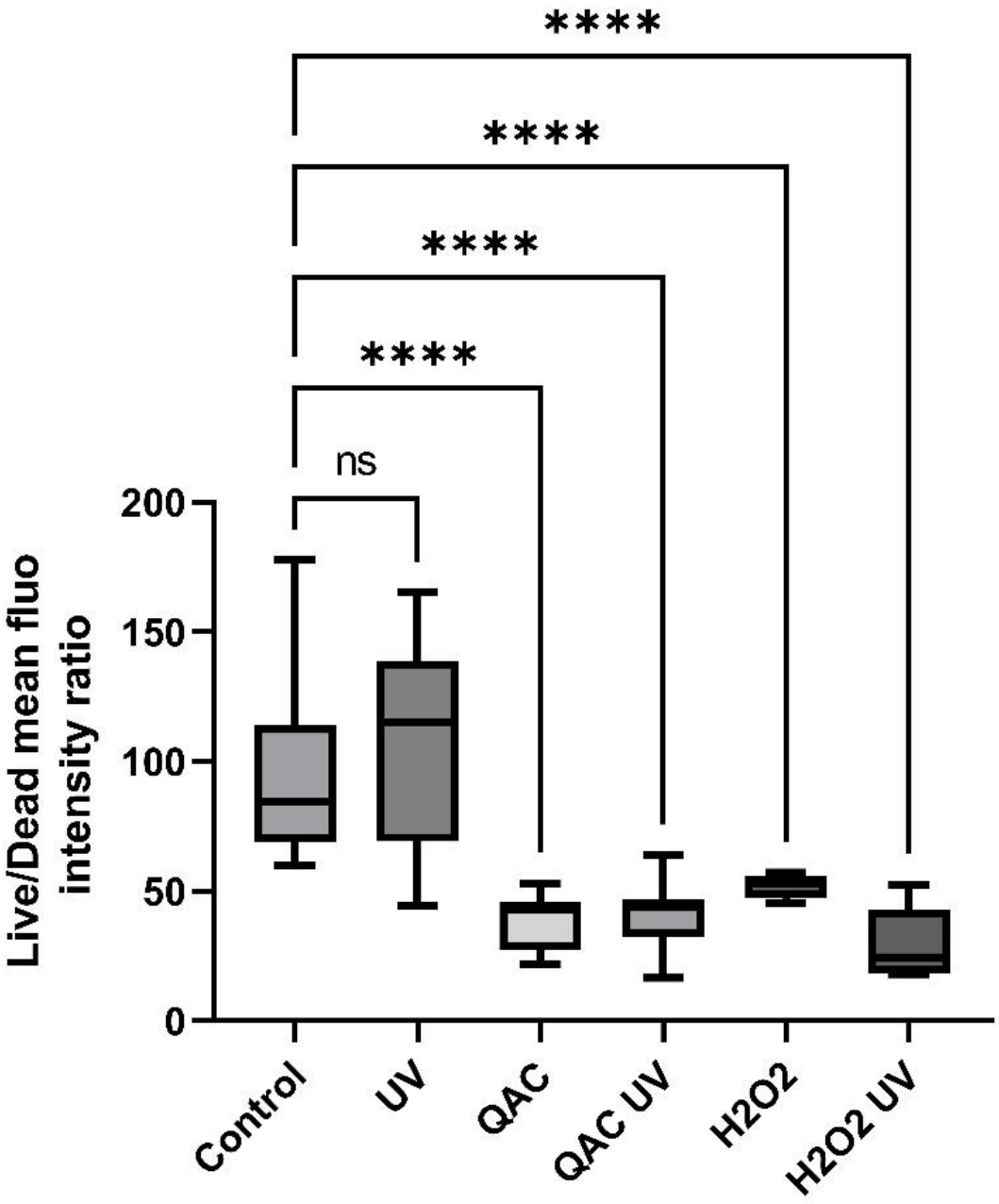

Figure 3.22. Biofilm viability of *S. aureus* and *P. aeruginosa* (NCTC 12924) after exposure to QAC combined with UV-C and hydrogen peroxide combined with UV-C. 72-hour old (72 hour growth of *S. aureus* and 48 hour growth of *P. aeruginosa*) biofilm viability after 60 minutes exposure to different treatments quantified as live/dead mean fluorescent ratios and normalized to untreated controls. Data shown are mean ± SD. Statistical significance was determined with One-way ANOVA test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

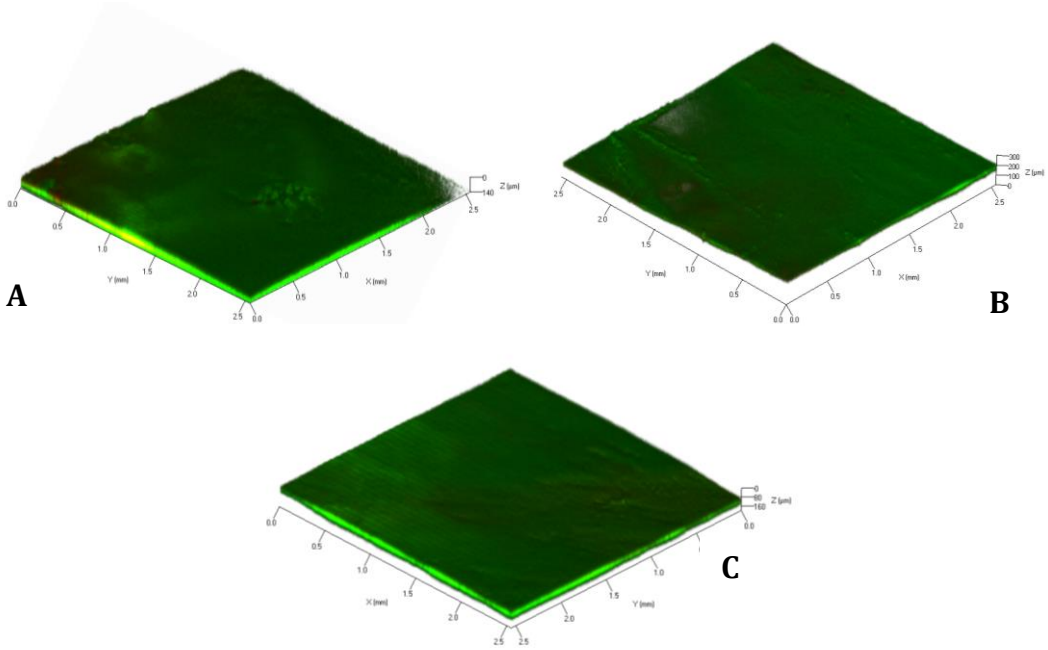

Figure 3.23. CLSM images of multi-species biofilms of *S. aureus* and *P. aeruginosa* (NCTC 12924) after exposure to DI, 60% glycerol and UV-C. A biofilm after exposure to DI for 60 minutes; B after exposure to 60% glycerol for 60 minutes and C after exposure to UV-C for 10 minutes. Green colouring indicates live cells and red colouring indicates dead cells.

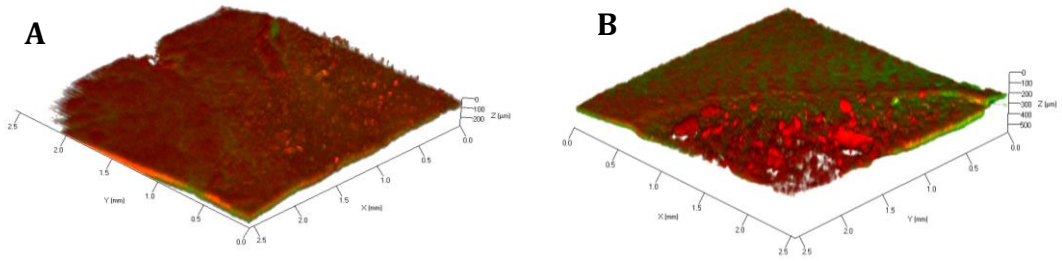

Figure 3.24. CLSM images of multi-species biofilms of *S. aureus* and *P. aeruginosa* (NCTC 12924) after exposure to QAC and QAC combined with UV-C. A biofilm after exposure to QAC for 60 minutes; B after exposure to QAC for 50 minutes and the addition of UV-C for a further 10 minutes. Green colouring indicates live cells and red colouring indicates dead cells.

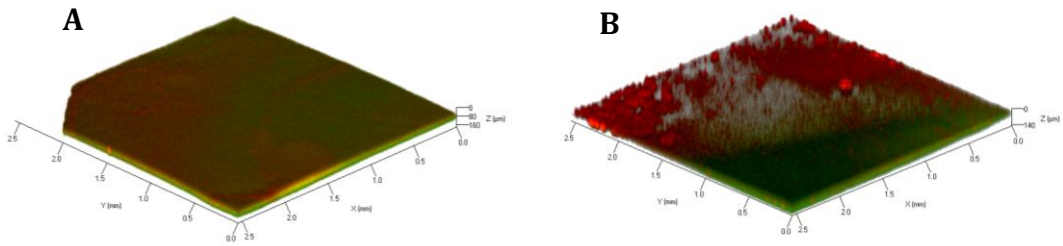

Figure 3.25. CLSM images of multi-species biofilms of *S. aureus* and *P. aeruginosa* (NCTC 12924) after exposure to hydrogen peroxide and hydrogen peroxide combined with UV-C. A biofilm after exposure to hydrogen peroxide for 30 minutes; B after exposure to hydrogen peroxide for 20 minutes and the addition of UV-C for a further 10 minutes. Green colouring indicates live cells and red colouring indicates dead cells.

Alongside the microscopy, enumeration of the viable cells in the biofilm was determined (**Figure 3.26**) for one of the three biological replicates. This however, was not in line with the CFU/mL that was recovered from the same conditions previously. This was most likely due to the increased contact time of the disinfectant with the biofilm whilst the staining and microscopy was taking place, meaning 3.5% QAC that originally achieved a 5-log reduction achieved an 8-log with the extended contact time.

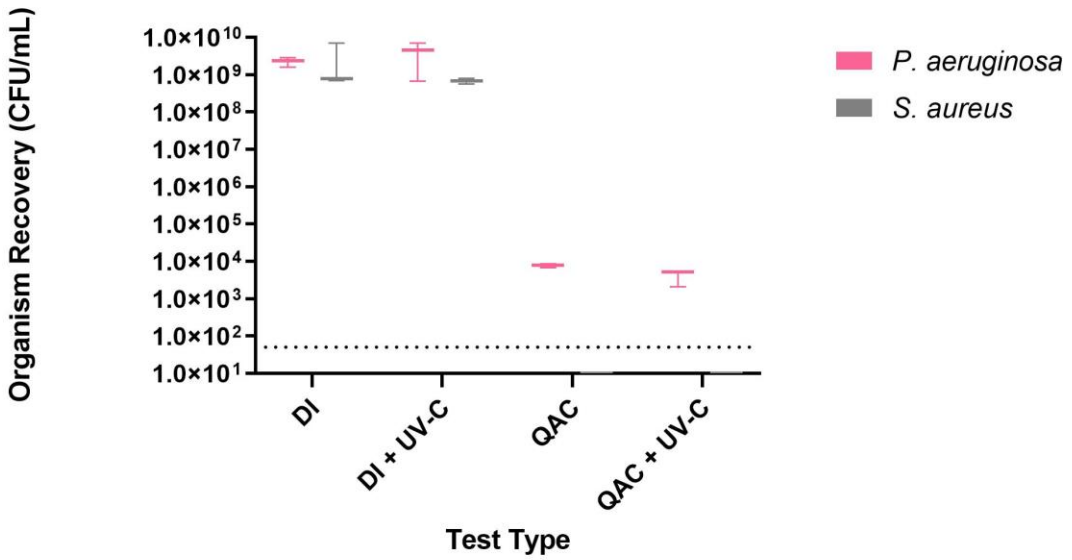

Figure 3.26. Combined treatment of the multi organism biofilm of *S. aureus* and *P. aeruginosa* (NCTC 12924) against QAC disinfectant and UV-C. Results after *t* contact time (*t*=60 minutes) with 200 µL QAC disinfectant at 3.50% w/v for 50 minutes and UV-C combined for a further 10 minutes (*t*=60 minutes) compared to controls of synthetic hard water (*t*= 60 minutes), QAC 3.50% w/v (*t*= 60 minutes) and synthetic hard water for 50 minutes and UV-C combined for a further 10 minutes. All recovery is in CFU/mL. The dotted line depicts minimum detection value is 50 CFU/mL. Data shown are mean ± SD. No statistical significance of data calculated using a two-way ANOVA and Sidak's multiple comparisons test. Technical replicates n=3, biological replicates n=1 (n=3).

To establish that the increase in log reduction of the recovered cells from the microscopy was due to the increased contact time, a repeat of the test combining the UV-C and QAC was taken with contact times of 1 (**Figure 3.27 A**), 2 (**Figure 3.27 B**) and 3 hours (**Figure 3.27 C**). The 2 hour and 3 hour contact time achieved a 5-log reduction for *P. aeruginosa* and an 7-log reduction for *S. aureus* and this was comparable to the results shown in **Figure 3.26** which can be used to demonstrate that the increase in contact time caused the increase in log reduction and that the microscopy can still be used as a comparison for the CFU/mL. It is also noted that the largest synergistic effect between the combined treatment was seen at a contact time of 1 hour but then did not increase at the 2 or 3-hour contact times.

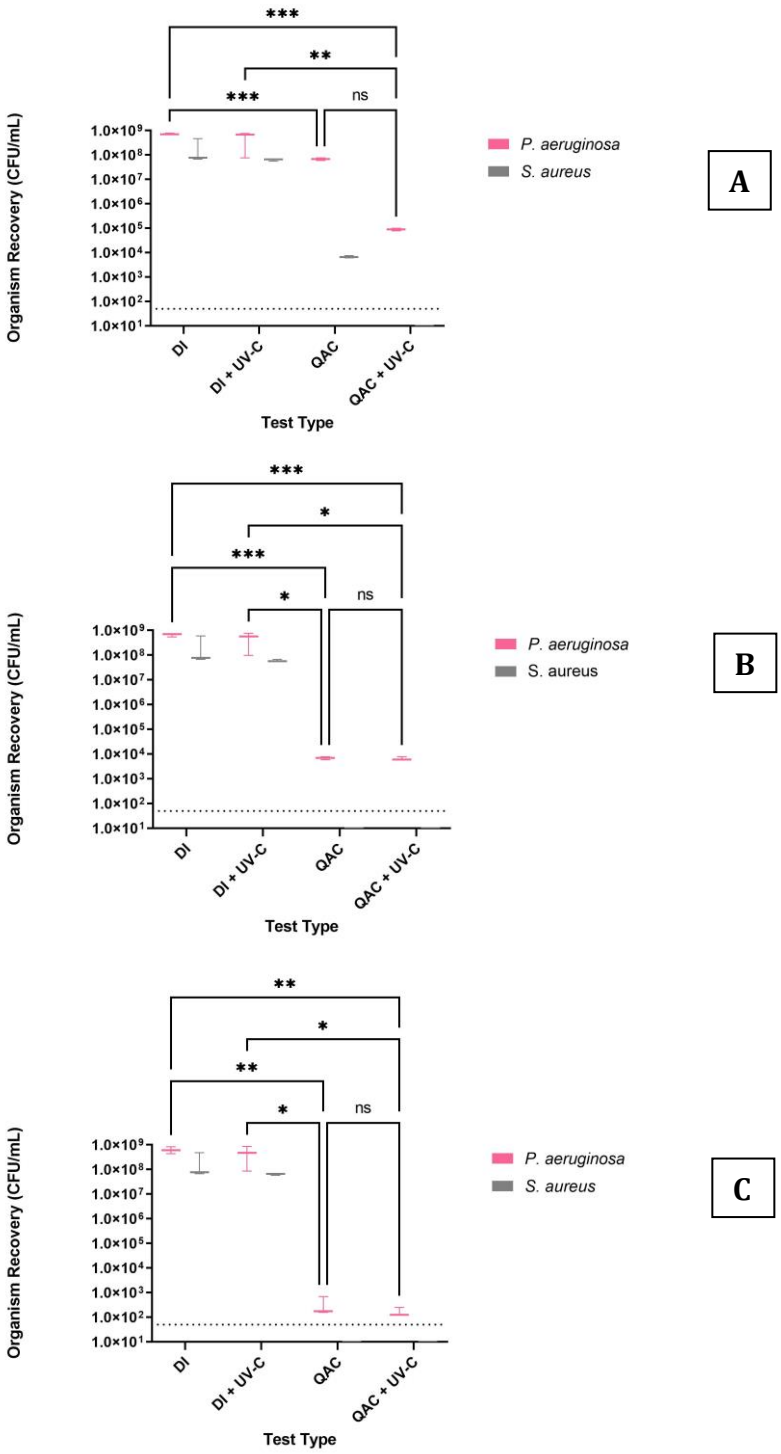

Figure 3.27 Combined treatment of the multi organism biofilm of *S. aureus* and *P. aeruginosa* (NCTC 12924) against QAC disinfectant and UV-C. A= Results after *t* contact time (*t*=60 minutes) with 200 µL QAC disinfectant at 3.50% w/v for 50 minutes and UV-C combined for a further 10 minutes (*t*=60 minutes) compared to controls of synthetic hard water (*t*= 60 minutes), QAC 3.50% w/v (*t*= 60 minutes) and synthetic hard water for 50 minutes and UV-C combined for a further 10 minutes. B= Results after *t* contact time (*t*=120 minutes) with 200 µL QAC disinfectant at 3.50% w/v for 110 minutes and UV-C combined for a further 10 minutes (*t*=120 minutes) compared to controls of synthetic hard water (*t*= 120 minutes), QAC 3.50% w/v (*t*= 120 minutes) and synthetic hard water for 110 minutes and UV-C combined for a further 10 minutes. C= Results after *t* contact time (*t*=180 minutes)

with 200 µL QAC disinfectant at 3.50% w/v for 170 minutes and UV-C combined for a further 10 minutes (*t*=180 minutes) compared to controls of synthetic hard water (*t*= 180 minutes), QAC 3.50% w/v (*t*= 180 minutes) and synthetic hard water for 170 minutes and UV-C combined for a further 10 minutes. All recovery is in CFU/mL. The dotted line depicts minimum detection value is 50 CFU/mL. Data shown are mean ± SD. ***/** refers to statistical significance of data calculated using a two-way ANOVA and Sidak's multiple comparisons test (**P<0.0066; ***P<0.0002). Technical replicates n=3, biological replicates n=3 (n=9).

4 Discussion

4.1 Optimisation of biofilm growth method

The biofilm viability test method described within this study was designed to be a relatively quick and easy method that can be used to grow single and multi-species biofilms for efficacy testing against chemical and physical disinfectants. This method aimed to be cheap and reproducible whilst delivering antimicrobial efficacy results for use alongside antibacterial claims of biocidal products marketed for domestic, healthcare, and industrial use. To be able to achieve this aim using this method, the quantification method used to establish biofilm cell viability after contact with a biocide was viable cell enumeration. Viable cell enumeration is a determination of viable cell numbers by plate count (CFU/mL) and is a standard quantification method used throughout biocidal efficacy testing, including on biofilms (Adetunji and Odetokun, 2012). This is a way to quantify live cells without the need for dyes or instrumentation. With this being the main focus of quantification during this study and for the use of the method going forward, all results used the viable cell enumeration quantification method, and all the method alterations were used to optimise the viable cell count (CFU/mL).

When creating a method to grow biofilms, there were multiple elements that were optimised to achieve the highest recovery of CFU/mL. These optimisations included the addition of 1% glucose to tryptone soy broth (TSB), the incubation time and temperature and the necessary delay when inoculating two organisms within the same biofilm.

When growing polymicrobial biofilms using the colony biofilm method, the addition of both *S. aureus* and *P. aeruginosa* to the PC disc at the same time resulted in the *S. aureus* being inhibited by the dominant *P. aeruginosa* and the recovery (CFU/mL) decreasing. This suggests an antagonistic relationship between the two organisms, which has been supported in many instances. Bacteria can excrete antimicrobial components to eliminate other bacterial competitors, which are often regulated by quorum sensing (Li and Tian, 2012). *P. aeruginosa* is frequently the dominant pathogen because of the large range of mechanisms it uses to adapt to and survive changing environments. When

P. aeruginosa encounters *S. aureus,* it can use mechanisms such as quorum sensing which increases the virulence of the organism, assisting in taking over the biofilm (Hotterbeekx et al., 2017). *P. aeruginosa* can also produce molecules such as 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNOs), which are controlled by quorum sensing through the formation of small colony variants in *S. aureus* which in turn causes aminoglycoside resistance, especially in Cystic Fibrosis (CF) patients (Hoffman et al., 2006). The production of HQNOs has been said to be an adaptive response to nutritional stress, which increases the level of nutrient availability for *P. aeruginosa* due to *S. aureus* consuming nutrients at a reduced rate (Chirathanamettu and Pawar, 2020). In sputum samples taken from CF patients, *P. aeruginosa* is often found in the absence of *S. aureus,* even though *S. aureus* is a common organism recovered from CF patients. This suggests that *P. aeruginosa* can kill or inhibit the growth of *S. aureus* when they're in a co-culture where they are both growing and competing for nutrients in the same environment (Machan et el., 1991). *P. aeruginosa* can lyse the cells of gram-positive organisms including *S. aureus* by producing extracellular antimicrobial molecules so the lysed cells can be used as an iron source (Déziel et al., 2004; Mashburn et al., 2005). These molecules produced by *P. aeruginosa* include rhamnolipids, which are surfactant molecules that increase cell permeability by interacting with the plasma membrane (Sotirova et al., 2008). In polymicrobial communities, it is suggested that *P. aeruginosa* can detect peptidocylcan, which may be shedded by Gram-positive organisms such as *S. aureus,* and this can enhance the production of potent antimicrobials that may kill the Gram-positive bacteria (Korgaonkar and Whiteley, 2011). When isolated together in chronic would infections, both *P. aeruginosa* and *S. aureus* have been found in separate aggregates (Rudkjøbing et al., 2012) and this has been supported *in vitro* (Barraza and Whiteley, 2021).

When adding 1% glucose to the TSB used for the overnight growth stage of the biofilm formation, it increased the CFU/mL of the biofilm by up to 1-log. This has been supported by previous studies growing biofilms (Mathur et al., 2006; Deka, 2014). However, it has also been found that the addition of glucose can inhibit biofilm growth at low concentrations from 0.25%. Jahid et al. (2013) found this

using *Aeromonas hydrophilia* biofilms. Regarding *S. aureus,* Waldrop et al. (2014) found that the addition of glucose increased biofilm mass with a certain level of glucose providing a threshold for growth. This has also been shown by Adetunji and Odetokun (2012) who demonstrated that the quantification of biofilms increased when supplemented with additional nutrients, however a concentration of 0.5% glucose provided a threshold for optimum recovery of cells. This can be explained by sequestration to a nutrient-rich area, which is one of the motivations for bacteria to produce biofilms (Jefferson, 2004). In glucose rich conditions, biofilm growth can be prolonged which can lead to increased EPS production (Flemming and Wingender, 2001). This increased EPS production is correlated with glucose levels in the initial media that the culture was grown in (Dewanti and Wong, 1995), which is supported in this study, as the glucose-supplemented TSB is the initial media where the biofilm is developed. It has also been found that in the presence of glucose, *S. aureus* can breakdown glucose and this glycolysis produces substances such as acetoin and acetic acid that can eradicate *P. aeruginosa* (Kvich et al., 2022). Lactic acid is also produced from the glycolysis and this production from a high glucose concentration can also induce aggregation of *S. aureus*, and therefore potentially increasing the biofilm density (Luo et al., 2019). The same study also found that the increased glucose also significantly increased the extracellular polysaccharide on the bacterial surface which can aid in biofilm formation as the polysaccharides promote aggregation to each other and to surfaces (Limoli, Jones and Wozniak, 2015).

When assessing the influence of incubation time on optimum viable cell recovery, 24-, 48- and 72-hours of growth time at 36°C were evaluated. The most favourable growth time was 48 hours, which achieved the highest viable cell count of $8x10^9$-$5x10^{10}$ CFU/mL whereas a 24-hour and 72-hour growth time achieved lower cell counts of $3x10^9$-$9x10^9$ and $7x10^9$-$3x10^{10}$ CFU/mL respectively. This has also been found in other studies where a biofilm grown for 24-hours had up to 1-log less CFU/mL when determined by CFU counts (Chen et al., 2020). When the tolerance was compared, the 24-hour biofilm was a lot more susceptible to antibiotics compared to the 72-hour biofilm. This is expected

when taking into account the bacterial growth curve, which defines the different stages of planktonic growth; lag, log, stationary and death. During the log phase, the bacteria are more tolerant to antimicrobials, but in the lag phase as the metabolism slows, they become more tolerant before then becoming more susceptible again in the death phase. However, there is not a biofilm growth curve that can be applied to this. The parameters that determine planktonic bacterial growth are very different to bacterial cells grown within a biofilm as the biofilm growth is significantly reduced. The susceptibility of biofilms of different ages to antimicrobials also differs and this may be due to the current phase of the growth of the biofilm. Biomass and thickness of the biofilm increases over time which can affect bacteria on the outer edge compared to the bacteria at the bottom of the biofilm attached to a surface as oxygen and nutrients are more readily available in the outer layers (Crabbé et al., 2019). In *P. aeruginosa* biofilms, subpopulations that have been found to be tolerant to antibiotics are normally located internally within the biofilm and have low growth rates and low metabolic activity (Sønderholm et al., 2017). To reproduce this, it is important to establish the optimum growth time for a biofilm to be used in biocidal tests, which will provide a mature biofilm with thickness enough to confer the slow metabolic activity to a percentage of the internal bacterial population. In current biocidal test standards such as EN 1276 (BSI, 2019a), the planktonic growth time is 18-24 hours, which aims for the organism to be used for testing when in the stationary phase. In biofilm growth, the ideal biofilm growth stage (**Figure 1.1**) would be stage 4, which depicts a mature biofilm before dispersal.

The optimum incubation temperature for biofilm formation varies between bacterial strains and can range from 20°C to 65°C for organisms that are prevalent within the dairy industry such as *Geobacillus stearothermophilus* (Kumar et al., 2021). For bacterial species *P. aeruginosa* and *S. aureus,* often the incubation temperature is between 30-37°C, with 36±1°C being the specific incubation time for biocidal tests such as EN 1276 (BSI, 2019a). The effect of growth at 32.5°C and 36.5°C on cell enumeration of biofilms of these organisms was evaluated. *S. aureus* in both single and polymicrobial biofilms and

P. aeruginosa in single species biofilm had an increased CFU/mL at 32.5°C. This has been supported by other studies assessing the effects of temperature on biofilm growth such as Hoštacká, Čižnár and Štefkovičová (2010) who found an increase in biofilm production at 30°C compared to 37°C. However, in this study 36.5°C achieved a more consistent CFU/mL amongst both species in both single and polymicrobial biofilms. The consistent recovery of both species was preferred as the similar recovery means *P. aeruginosa* and *S. aureus* should be in equal proportions to each other which means the results of the test are more valid.

All these modifications were added to the biofilm growth methods as they increased the viable recovery (CFU/mL) from the biofilms, which is the quantification method used in this study.

4.2 Quaternary ammonium compounds (QAC) disinfection treatment

In this study, the MBEC for the QAC disinfectant being used was established for single species biofilms of *S. aureus, P. aeruginosa* (NCTC 13359) and *P. aeruginosa* (NCTC 12924). These concentrations were 0.24% and 5.31% respectively. When challenged against a QAC disinfectant, specifically n-alkyl dimethyl benzyl ammonium chloride and didecyl dimethyl ammonium chloride as used in this study, the planktonic cells of these species can be eradicated by much lower concentrations. Studies have found that benzalkonium chloride concentrations as low as 0.900mM (1.62%) are proven effective for *P. aeruginosa* planktonic cells (Machado et al., 2012) and 0.12% for *S. aureus* planktonic cells (Bondurant et al., 2020). These effective concentrations are significantly lower than the concentrations required to eradicate a biofilm of the same species as it is well known that biofilms are more recalcitrant to biocidal agents (Percival et al., 2016).

It is widely known that QACs are less effective on Gram-negative bacteria and *Pseudomonas spp.* in particular have high level of intrinsic resistance when compared to other Gram-negative organisms (Russell and Chopra, 1996). This is supported by the results from this thesis, where nearly a 23 times increase in the

MBEC was observed for the *P. aeruginosa* compared to the *S. aureus.* The main mechanism of action of QACs such as benzalkonium chloride is absorption, through penetration of the bacterial cell wall. This causes cell lysis by damaging the membrane structural integrity (McDonnell and Russell, 1999). This is due to the positively charged quaternary nitrogen in the QAC, which associates with the negatively charged head of the acidic phospholipids within the bacterial membrane and can cause solubilisation of the hydrophobic cell membrane components (Gilbert and Moore, 2005). The EPS matrix of the biofilm generally has a negative charge (Gordon, Hodges and Marriott, 1988) which could serve to isolate positively charged compounds such as QACs, preventing the exposure of these compounds to the cells embedded within the biofilm (Costerton et al., 1987). The mechanism of action of QACs when faced with a biofilm differs and has been suggested to be the polycationic compounds interacting with the biofilm EPS which can lead to dispersion enabling the QAC to kill the planktonic cells, or the QAC could agitate the biofilm through electrostatic interactions and then kill the cells using the same lysing mechanism used on planktonic cells (Ganewatta, et al., 2014). This could be seen in the microscopy images of the *P. aeruginosa* (NCTC 12924) and *S. aureus* polymicrobial biofilm after contact with the QAC disinfectant as the cells on the surface were dead but further into the matrix of the biofilm and near the bottom where the initial cell attachment formed, the cells were still live, which suggests that the QAC had not fully penetrated the biofilm. When compared to a control of sterile DI, there was a statistically significant difference between the ratio of live/dead cells after 60 minutes treatment with the QAC.

Resistance mechanisms in bacteria that have been in contact with a QAC disinfectant have been correlated with changes in fatty acid composition, which could play a major role in resistance along with other mechanisms such as efflux, slime formation and the degradation of the disinfectant (Méchin et al., 1999). For *P. aeruginosa,* intrinsic resistance to QACs have been found to be due to changes in the cell wall and cell membrane. The outer cell membranes express lower levels of permeability, making it more difficult for antimicrobials to reach their site of action (Nikaido, 1994). It has also been found that intrinsically tolerant organisms are likely to exhibit cross-tolerance to other types of QACs (Voumard et al., 2020). The

increase of efflux pumps causes an increased level of efflux of antimicrobial agent (Li, Nikaido and Poole, 1995). Other phenotypic changes expressed in strains of *P. aeruginosa* that convey resistance to QACs include changes in lipids (Loughlin, Jones and Lambert, 2002), lipopolysaccharides (Tattawasart et al., 2000) and cell surface hydrophobicity (Tabata et al., 2002). Whilst much of the focus is on the resistance posed in *P. aeruginosa, S. aureus* has also been found to express resistance to QACs, especially methicillin-resistant *S. aureus* (MRSA; Jennings et al., 2015). Community strains of MRSA have been found to possess *qacA* and *qacR* resistance genes, and the percentage of isolates with these genes increased from 10.2% in 1992 (Buffet-Bataillon, 2012) to up to 83% in 2012 and 2015 (Shamsudin et al., 2012; Liu et al., 2015). *qacA* codes for a multidrug exporter protein which mediates resistance to antimicrobial compounds (Brown and Skurray, 2001) and *qacR* codes for a repressor protein which can regulate the expression of *qacA* (Grkovic et al., 1998).

When the QAC was tested against a multi-species biofilm, the MBEC values increased from that of a single species biofilm. *S. aureus* seemed to become more tolerant to the QAC disinfectant when in a polymicrobial biofilm. This is shown with the MBEC of *S. aureus* when grown with *P. aeruginosa* (NCTC13359) in a polymicrobial biofilm being 4.8% compared to the previous 0.24% for the single *S. aureus* biofilm. This difference between the single and multi-species biofilm increased further for *S. aureus* when grown with *P. aeruginosa* (NCTC12924) with an MBEC of 5.31%. Even though *P. aeruginosa* is known to inhibit *S. aureus* within a co-culture, it has been found that co-isolation of these organisms from a patient sample correlates with worse patient outcomes compared to when isolated separately (Limoli et al., 2016), and this has been supported in this study. *P. aeruginosa* has demonstrated abilities to alter the susceptibility of *S. aureus* to antibacterial agents *in vitro* through lysis facilitated by LasA endopeptidase, rhamnolipids facilitating antimicrobial uptake and HQNO inducing changes in respiration (Radlinski et al., 2017). HQNO inhibits respiration, which is the most efficient ATP generation mechanism in *S. aureus* and this has been supported by Radlinksi et al. (2017) who found that *P. aeruginosa* induces significant depletion of intracellular ATP in *S. aureus* when

assessing ciprofloxacin tolerance. Alongside this, *P. aeruginosa* has been found to become less aggressive towards *S. aureus* by quorum sensing factors such as the production of alkyl-quinolones, which reduces its inhibitory effect against *S. aureus* and this is often presented in the lungs of cystic fibrosis (CF) patients (Baldan et al., 2014).

One notable difference between *P. aeruginosa* (NCTC 13359) and *P. aeruginosa* (NCTC 12924) is that NCTC 13359 produces the virulence factor pyocyanin and NCTC 12924 does not. Pyocyanin is a redox-active phenazine compound, which acts through the regeneration of reactive oxygen species (Hassett et al., 1992), and is produced by over 95% of *Pseudomonas* isolates (Gonçalves and Vasconcelos, 2021). Pyocyanin inhibits *S. aureus* respiration, which forces it to acquire energy from fermentation and adopt the phenotypic expression of a small colony variant (SCV; Noto et al., 2017). This could increase the tolerance of *S. aureus* to antimicrobials due to a potential more dormant state, with the *S. aureus* expressing behaviour similar to persister cells. However, in this study the growth alongside the pyocyanin producing *P. aeruginosa* (NCTC 13359) increased the susceptibility of *S. aureus* to the QAC compared to when grown with the non-pyocyanin producing *P. aeruginosa* (NCTC 12924) which shows that the pyocyanin may not have had an effect of the respiration of *S. aureus. P. aeruginosa* can also detect peptidoglycan which has been shedded from Gram-positive organisms and this facilitates the production of pyocyanin (Korgaonkar and Whiteley, 2011), growing more virulent biofilms.

4.3 Hydrogen peroxide method optimisation

An MBEC could not be established for hydrogen peroxide for the single and multi-species *P. aeruginosa* and *S. aureus* biofilms using the same method as the QAC. Therefore, a new method was optimised based on Lineback et al (2018) which increased the product applied to 4 mL compared to 200 µL used FOR the QAC. A number of studies have found hydrogen peroxide to be effective against *P. aeruginosa* and *S. aureus* biofilms. However, the results in this study have shown it to be less effective than the QAC disinfectant. This could be due to biofilm penetration failure because of a neutralising reaction between the

antimicrobial agent and a constituent of the biofilm (Stewart and Raquepas, 1995). There may be an interaction between the antimicrobial agent and the biofilm that neutralises the antimicrobial activity due to a chemical modification that prevents it from penetrating the biofilm. This causes the rate of deactivation of the hydrogen peroxide to exceed the rate of diffusive penetration. This is supported by de Beer et al (1994), Chen and Stewart (1996), Xu et al (1996) and is important for highly reactive oxidants such as ozone and hydrogen peroxide. Biofilms with catalase producing species such as *P. aeruginosa* and *S. aureus* may prove more tolerant to hydrogen peroxide due to the catalases degrading it and hence protecting the bacteria (Ma and Eaton, 1992).

The main method optimisation was the increase of the volume of the product tested from 200 µL to 4 mL. This was achieved by submerging the full biofilm in a suspension of product as opposed to the previous method where a smaller volume of product was added to the surface of the biofilm to replicate real-life surface/product interactions during a cleaning process. The aim of this was to try and replicate a phase 2/step 2 test such as a BS EN 13697. However, many previous biofilm efficacy studies use 4 mL of product by fully submerging the biofilm with no mixing (Goeres et al., 2019; Charaf, Bakich and Falbo, 1999; Lineback et al., 2018). There are other studies that even increase this volume to 10 mL such as Buckingham-Meyer, Goeres, and Hamilton (2007). This improves the efficacy of the hydrogen peroxide due to increased contact of the product with the biofilm, but also gives the treatment two sides of penetration into the biofilm due to the porous PC membrane it grows on. There are few studies which use <1mL of treatment in the biofilm efficacy test such as the MBEC™ test (Parker et al., 2014), but these are often used on biofilms grown in microtiter plates which are thinner biofilms than those grown on the PC membrane. The 200 µL of product was originally chosen because of the 100 µL treatment volume in the EN 13697, as well as this being the treatment amount in standardised biofilm tests such as the MBEC® assay. With these method alterations, an MBEC was achieved for all the biofilms tested using hydrogen peroxide disinfectant.

As this method detaches a high number of the biofilm cells through the 'dipping' process, this needed to be incorporated into the log reduction calculation to normalise to values obtained. The number of detached cells was calculated for all species (**Figure 3.12**) but due to time constraints, this was not completed for *P. aeruginosa* (NCTC 13359) in this study, as the dipping method did not reach an MBEC. For this strain, the results show that there were no viable cells detached from the biofilm during the 'dipping' process. However, it was confirmed that cells had detached by looking under a light microscope, concluding that the detached cells had been eradicated by the hydrogen peroxide. This shows that the 7.9% w/w hydrogen peroxide could penetrate and eradicate the biofilm of a non-pyocyanin producing *P. aeruginosa* (NCTC 12924) but was less effective against the pyocyanin producing strain (NCTC 13359). It has been demonstrated that the presence of pyocyanin can increase the prevalence of persister cells in a biofilm, causing higher resistance to antimicrobial agents and this has been seen in *Acinetobacter baumannii* (Bhargava, Sharma and Capalash, 2014). Pyocyanin can be oxidised by hydrogen peroxide, which inactivates it, whilst also being a substrate for peroxidases, which can contribute to the removal of hydrogen peroxide (Reszka et al., 2004). This might provide an explanation for the increased tolerance of the pyocyanin producing *P. aeruginosa* to hydrogen peroxide.

4.4 Hydrogen peroxide disinfectant treatment

In this study, the MBEC for the hydrogen peroxide disinfectant being used was established for single species biofilms of *P. aeruginosa* (NCTC 12924), but not for *S. aureus.* The MBEC concentration of hydrogen peroxide for *P. aeruginosa* (NCTC 12924) was 5.53% but the supplied concentration of 7.9% w/w did not achieve a significant log reduction for *S. aureus.* The MBEC was not determined for *P. aeruginosa* (NCTC 13359) due to time constraints and the inability to achieve an end-point concentration on this strain when undertaking the model optimisation.

One potential reason for the hydrogen peroxide not eradicating *S. aureus* biofilms could be the presence of persister cells. These are a small number of

free-floating planktonic cells within the biofilm that are in a passive, non-dividing state with reduced metabolism that can aid survival when exposed to extensive antimicrobial treatment (Fisher, Gollan and Helaine, 2017). Spoering and Lewis (2001) found that during elevated concentrations or prolonged exposure of antimicrobial agents that kill most cells within a biofilm, there is a subpopulation of persisters that could reseed the biofilm. QACs have been found to rapidly kill persister cells (Basak et al., 2017) but so have reactive oxygen species such as hydrogen peroxide (Kawano et al., 2020). This suggests that hydrogen peroxide has the ability to eradicate biofilm cells, including persister cells, but this relies on the ability to penetrate the membrane.

The MBEC for the single species *P. aeruginosa* (NCTC 12924) biofilm for hydrogen peroxide was higher at 5.53% than for the QAC at 5.31%. This may be due to the catalases being produced by *P. aeruginosa* and could also explain the lack of susceptibility of the *S. aureus* biofilm. Stewart et al. (2000) found that hydrogen peroxide fails to fully penetrate a *P. aeruginosa* biofilm due to a reaction-diffusion interaction. This has also been supported by Perumal et al. (2014) who found hydrogen peroxide ineffective in eradicating *P. aeruginosa* biofilms. Furthermore, they also demonstrated that within a *P. aeruginosa* biofilm, the highest hydrogen peroxide activity on the biofilm was between 2 and 4 minutes, with very little eradication continuing after this. They proposed multiple potential biofilm eradication mechanisms such as an initial oxidation of exposed cells and penetration of hydrogen peroxide in the membrane with oxidation of the matrix and the cells. As well as catalases, alginate or other free radical scavengers secreted by the bacterial cells can break down hydrogen peroxide before it reaches the cell to protect the biofilm from the antimicrobial (Yun et al., 2012).

When assessing the *S. aureus* and *P. aeruginosa* (NCTC 12924) polymicrobial biofilm against hydrogen peroxide, the MBEC increased compared to the single species biofilms. The MBEC for *P. aeruginosa* in the single species biofilm was 5.53% but this increased to 7.9% when in the polymicrobial biofilm. The MBEC was never established for *S. aureus* but the results across the single and multi-

species biofilms are very similar. This suggests that when growing within a polymicrobial biofilm, *P. aeruginosa* is more resistant to hydrogen peroxide due to the presence of *S. aureus*. This has been supported by the microscopy images taken, which shows there is a higher ratio of live cells after being in contact with the hydrogen peroxide compared to after exposure to the QAC. It has been demonstrated that *S. aureus* can secrete products, which interact with *P. aeruginosa* and these interactions can enhance the formation of aggregates and induce antibiotic resistance (Beaudoin et al., 2017). *Staphylococcus aureus* protein A (SpA) is an extracellular adhesin produced by *S. aureus*, which impacts the resistance related behaviour of *P. aeruginosa* by binding to certain cell surface targets (Armbruster et al., 2016). Furthermore, Armbruster et al. (2016) identified two of the *P. aeruginosa* cell structures as the Psl polysaccharide and the PilA protein component of type IV pili, both of which are integral factors in *P. aeruginosa* biofilm formation. Psl has been known to increase the elasticity and facilitate effective cross-linking within the matrix (Chew et al., 2014), which could increase tolerance to antimicrobials by inhibiting the penetration into the matrix. Specifically, Chew et al. (2014) also found that Psl was important for forming *P. aeruginosa* biofilms on top of already establish *S. aureus* biofilms, which is the method adopted in this study. The ability of Psl to stabilise the cell wall could in turn, reduce the production of molecules such as HQNO and pyocyanin that have been known to eradicate *S. aureus.*

4.5 UV-C treatment

When assessing the efficacy of UV-C against *S. aureus, P. aeruginosa* (NCTC 12924) and *P. aeruginosa* (NCTC 13359) in single species biofilms, there was very little effect on the biofilm viability. This is mostly due to the UV-C not being able to penetrate the biofilm due to the thickness increasing the irradiation path length (Baqué et al., 2013). This is supported by the microscopy images taken of the *S. aureus* and *P. aeruginosa* (NCTC 12924) biofilm after 10 minutes contact time with UV-C where there are almost only live cells in the biofilm. Alginate within the biofilm has also been suggested to play a part in protecting the biofilm from UV-C exposure, with secreted exopolymers attenuating the UV-C (Elasri and Miller, 1999). Studies have demonstrated that UV irradiation can slow biofilm

formation, even when it is ineffective in reducing the biofilm density or destroying cells (Wenjun and Wejnun, 2009). The EPS has also been found to scavenge reactive oxygen species molecules, which can protect the cell structure and the matrix components from oxidative damage. The EPS also often uses photosynthetic pigments and enzymes such as amino acids, carotenoid pigments and catalases to absorb UV or degrade reactive oxygen species, which protects the cells (Hu et al., 2019).

UV-C disinfection is widely used in the treatment of wastewater and drinking water and is seen as a more advantageous treatment when compared to chemical disinfection due to the lack of toxic chemical by-products and the broad-spectrum biocidal activity (Luo et al., 2022). When tested against planktonic bacteria, UV-C has demonstrated biocidal efficacy in many different areas such as wastewater and drinking water disinfection (Masschelein and Rice, 2016). This is due to the DNA absorbing the UV-C, inducing production of oxygen reactive species, which causes photo oxidation that can disrupt proteins and damage cell membranes (Chevremont et al., 2012). For common planktonic bacteria such as *P. aeruginosa* and *E. coli,* UV LED wavelengths between 255 and 280nm have proved to be effective in eradicating the bacterial cells, with 280nm proving the be the most efficacious (Rattanakul and Oguma, 2018). The 280nm wavelength has also been shown to cause irreversible protein damage, which represses the photoreactivation of the organisms (Nyangaresi et al., 2018). Even though the efficacy of UV-C against multi-species biofilms was not assessed within this research, multiple studies (Yuan et al., 2021; Bak et al., 2009) have shown that multi-species biofilms show more tolerance to UV-C than their single-species counterparts and this mirrors the findings of this study when looking at tolerance of chemical disinfectants.

Some studies have alternative findings where UV-C has been found effective in eradicating biofilms (Li et al., 2010), however this may be due to using a 96-well microtiter plate method to grow the biofilms. This method produces much thinner biofilms than the biofilm growth method outlined in this study, meaning that they may be penetrated by UV-C easier. Differences are also seen between

the different types of UV emitter (mercury or LED lamps) and the effect this has on biofilms. Mercury lamps, like the UV system used in this research, are used more frequently then LED due to having a higher radiance because of having a higher voltage. However, LED UV lamps that emits 280nm UV-C are known to cause irreversible protein damage and this may enhance the penetration into a biofilm matrix because protein is the main composition of the EPS (Luo et al., 2022).

Studies have also assessed the efficacy differences between continuous and pulsed UV-C against bacteria in planktonic and biofilm states. Fine and Gervais (2004) found that pulsed UV-C light is 4-6 times more effective than continuous UV-C light due to additional inactivation mechanisms produced by pulsed UV-C when tested against planktonic bacteria. Additionally, Li et al. (2010) found that pulsed UV-A LED irradiation had a stronger biocidal effect on *E.coli* and *Candida* biofilms. This has also been confirmed by Chen et al (2020a) who found that pulsed UV lamps have enhanced biocidal effects on biofilms through photothermal and photophysical effects. These additional mechanisms of pulsed UV-C have been suggested to work through photothermal reactions and photophysical high intensity pulses on bacterial structure (Krishnamurthy, Demirci and Irudayaraj, 2007). Whereas the inactivation mechanisms of continuous UV-C light are through photochemical reactions within the DNA which leads to cell death (Rowan et al., 1999). Another suggestion amongst research to increase the UV-C penetration in biofilms is to use a synergistic combination of both UV-A and UV-C. UV-A is capable of penetrating microbial membranes more than UV-C, which increases the sensitivity of the organism and this has been supported by Elasri and Miller (1999) who found that 33% of UV-A was transmitted in the biofilm compared to 13% of UV-C. Using UV-A followed by UV-C against biofilms could increase the efficacy of UV against biofilms (Chevremont, et al. 2012).

4.6 Quaternary ammonium compounds (QAC) disinfection in combination with UV-C

When looking at the synergistic effect of QAC disinfectant in combination with UV-C against single species biofilms of *S. aureus* and *P. aeruginosa* (NCTC 12924), the treatment was more effective against *S. aureus.* When compared to the QAC treatment alone, the addition of UV-C increased the log reduction by 4-log for *S. aureus* but only by 2-log for *P. aeruginosa* (NCTC 12924). There are very few studies that have investigated the possible synergistic effects of QACs and UV-C but this has been supported by a small number of studies that found that combining the use of a QAC disinfectant such as benzalkonium chloride with UV-C has a greater ability to kill bacteria than just using the QAC alone (Zeber et al., 2019). There is very little information on the QACs and UV irradiation interacting with each other but separately, UV irradiation has been shown to induce typical caspase-dependent cell death and QACs induce caspase-dependent and independent cell death (Buron et al., 2006). Caspases are protease enzymes that are involved in programmed mammalian cell death. The independent cell death might be the reason that the combination of the two treatments causes a larger log reduction of cellular viability. The efficacy of the combined treatment follows the same pattern of the QAC only treatment, where it is less effective against *P. aeruginosa* than *S. aureus* so it could be proposed that the effect the addition of the UV-C is having is equally active against both species.

When applied to the *S. aureus* and *P. aeruginosa* (NCTC 12924) polymicrobial biofilm, this followed the same pattern as in the single species biofilms with *S. aureus* being more susceptible but the addition of the UV-C increasing the log reduction of both species when compared to the QAC as a solo treatment. This was not supported by the microscopy images but it may be explained as the biofilm used in this treatment condition may have been thicker and affecting the effectiveness of the treatment or it could have been disturbed through the imaging process. When in a polymicrobial biofilm, the same tolerance-inducing organism interactions are not seen with the addition of UV-C as with the other treatments. With the QAC treatment alone, the *S. aureus* single species biofilm achieved a 5-log reduction at 0.18% w/v but no log reduction at 0.20% w/v

when in a poly microbial biofilm. However, when this is replicated with the UV-C in combination, the log reduction of the single species and multi-species biofilm is the same. This would suggest that there are interactions happening throughout the treatment that makes the combination treatment a more successful application for polymicrobial biofilms that often show more resistance compared to single species counterparts.

When undergoing the microscopy, the enumeration recovery (CFU/mL) was done alongside the imaging. However, due to the length of time it took to stain and image the samples, the biofilm was in contact with the disinfectant for longer than the stated contact time and because of this, a higher log reduction was achieved than the previous work that was undertaken. To confirm this, the experiment was repeated with 1, 2 and 3 hour contact times to imitate the real-life timings of the microscopy. The results mirrored the results originally found whilst doing the microscopy, however as the contact time of the disinfectant increased, the log reduction remained the same. This shows that after 1 hour, the QAC disinfectant did not seem to have any further impact on killing the *P. aeruginosa* (NCTC 12924). This suggests that there is a time limit for the efficacy of the QAC, which may be mediated by the intrinsic resistance of *P. aeruginosa* when in contact with sub-lethal levels of QAC disinfectant. This activity could not be assessed against *S. aureus,* as 2 hours contact time achieved no cellular viability.

4.7 Hydrogen peroxide disinfection in combination with UV-C

In this study, the combination of hydrogen peroxide and UV-C as a treatment was effective in increasing the efficacy against the *P. aeruginosa* (NCTC 12924) and *S. aureus* polymicrobial biofilms when compared to hydrogen peroxide as a solo treatment when looking at cellular viability. The combination of these physical and chemical treatments together causes an advanced oxidation process (AOP), which is known to degrade trace organic compounds, resulting in bacterial cell death. This AOP produces hydroxyl radicals, which are strong oxidisers that are extremely reactive with organic molecules (Davis and Cornwell, 2008). The hydroxyl radicals eradicate bacteria by oxidising vital bonds and attacking the

thiol group on proteins, which can weaken structures such as the cell wall, eventually causing osmotic lysis. Furthermore, the hydroxyl radicals can inhibit enzyme activity by attacking the ribosomes (Armistead, 2003). The increased biocidal efficacy of this combined method has been supported by multiple studies in both a treatment and a preventative measure in biofilm removal (Lakretz et al., 2018; Bounty, Rodriguez and Linden, 2012; Vankerckhoven et al., 2011). When assessing the effectiveness of treatment by microscopy, there was an increase in dead cells from the biofilm exposed to the combined treatment compared to the biofilm exposed to hydrogen peroxide only, however, the increase was not statistically significant along with the enumeration (CFU/mL) recovery even though an increase of up to 3-log was observed. When undergoing the hydrogen peroxide treatment via the 'dipping' method, the biofilms were detached and the visual colony formation removed very easily. This resulted in 90.625% and 98.333% of the cells removed from the biofilm in *S. aureus* and *P. aeruginosa,* respectively. When imaging the biofilms post-treatment, this caused issues as only a monolayer of cells were left still attached to the membrane, therefore the live/dead cell analysis was not based on the fully formed biofilm being exposed to the treatment and this may be why there were such significant differences in the visual cellular viability.

Although, the combination of the two treatments offered a decrease in cellular viability of the biofilm, it did not fully eradicate the biofilm. This may be seen when using a higher concentration of hydrogen peroxide that provided a larger initial log reduction in the cellular viability. An increase or decrease in contact time may also increase the log reduction as when the optimisation of the hydrogen peroxide method was being established, it was observed that in *P. aeruginosa* (NCTC 12924) the 1-minute contact time performed slightly better in eradicating bacterial cells. However, the effect on contact times with the combined method was not fully established and could increase the efficacy of the treatment. Increasing the specific UV-C contact time element, which was consistently 10 minutes in all of these experiments, could increase the killing effect as it can be hypothesised that more hydroxyl radicals could be formed over a longer period of time, which could result in the lysis of a higher number of cells.

An increase of the UV-C contact time paired with a decrease in the pre-UV hydrogen peroxide contact time may also be beneficial the 20 minutes contact between the hydrogen peroxide and the biofilm may be inducing tolerance. This could be caused by the catalases present in both the *S. aureus* and the *P. aeruginosa,* which can protect the bacterial cells in the presence of hydrogen peroxide. *KatB* catalase induction was found to occur in biofilms after a 20-minute exposure with hydrogen peroxide, which suggests that biofilms of catalase producing bacteria are capable of a rapid adaptive response to the treatment, especially when disinfectant levels are sub-lethal (Elkins et al., 1999). The same study also found that *KatA* catalase is also produced in high concentrations of hydrogen peroxide and is responsible for resistance to hydrogen peroxide. The combination of the two treatments applied together could be suggested to be more effective as opposed to one after the other as this may perform quicker than the production of the *KatB* so the hydroxyl radicals are able to penetrate and lyse the cells more quickly.

4.8 Limitations

Within this study there were multiple limitations, which affected the results or the amount of research undertaken. The first limitation was the time constraints that are mentioned throughout this thesis and these constraints resulted in *P. aeruginosa* (NCTC 13359) not being studied past the QAC MBEC stage of the study. The second limitation is the use of enumeration (CFU/mL) as a quantification method and an important consideration for this method is that only live cells, capable of forming a colony will be counted and used as data. This technique may not be preferable in all situations as it is labour and time intensive, sometimes requiring multiple days to perform enough replicates to obtain reproducible results. However, this study, unless stated otherwise, used 3 biological and 3 technical replicates (n=9), despite the time needed to complete this. Also, since the biofilm requires the generation of a suspension for the establishment of CFUs, some errors can occur due to bacterial clumping. This technique is also vulnerable to counting error and user bias when the given number of colonies is too high and the count is done manually. As the CFU/mL counts were plated as ‘spot plates’ using the Miles Misra method, a limit of 50

colonies was set to try and avoid the inaccuracy of counting. This method also reduced the sensitivity of the test, with the minimum detection at 50 CFU/mL. The third limitation was when undergoing the hydrogen peroxide treatment via the 'dipping' method, as the biofilms were detached and the visual colony formation removed very easily. This resulted in 90.625% and 98.333% of the cells removed from the biofilm in *S. aureus* and *P. aeruginosa,* respectively. When imaging the biofilms post-treatment, this caused issues as only a monolayer of cells were left still attached to the membrane, therefore the live/dead cell analysis was not based on the fully formed biofilm being exposed to the treatment and this may be why there has been so significant difference in the visual cellular viability. This is also applicable to the controls as most of the biofilm was disrupted and left detached in the diluent it was 'dipped' into. However, the equation used to account for detached cells was accounted for in all the controls used. The disruption of the biofilm during the microscopy was also present when imaging the control for the QAC as the sterile DI used lifted the biofilm off the membrane so it was unable to be imaged and the live/dead cells accurately quantified. This was rectified by adding glycerol to both the sterile DI and the cell staining used to achieve 60% glycerol to minimise biofilm disruption for imaging.

4.9 Conclusion and further research

Methods that are validated, often by standard setting organisations, enable decisions to be made on the acceptability of products being manufactured for consumers in a domestic, industrial, veterinary, or healthcare setting. This is because standard methods are often reproducible when performed by different operators in different laboratories. The development of biofilm standard testing methods results in the creation of laboratory protocols for the purpose of comparison with a single laboratory or between multiple laboratories. These methods, once internally validated, are reproducible enough to be used as a method to grow and test the biocidal efficacy of single and polymicrobial bacterial biofilms. QAC disinfectants are effective in eradicating single and polymicrobial biofilms and have shown increased efficacy when used in combination therapy with UV-C. However, sub-lethal doses of this disinfectant

have been shown to be time sensitive in their effectiveness against *P. aeruginosa* (NCTC 12924) biofilms, possibly due to the intrinsic resistance factors getting activated by the QAC. Hydrogen peroxide is less effective in eradicating both single species and polymicrobial biofilms but also has increased efficacy when in combination with UV-C presumably due to the production of hydroxyl radicals causing cell lysis.

The research from this thesis alongside the supporting research mentioned has raised multiple questions, which could be addressed through continued research on this subject. This method could be continued to assess the optimum timings needed for increased efficacy of the combination therapy of both QACs and hydrogen peroxide with UV-C. The administering of UV-A prior to UV-C may be an interesting direction as previous studies have found that UV-A makes bacterial cells more sensitive to UV-C and this may also work well in a combination therapy with chemical disinfectants. Pyocyanin production is also another thought-provoking area that could be studied with production of the virulence factor being associated with tolerance to chemical disinfectants.

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