Understanding the Resilience of Dry Surface Biofilms to Disinfection

Isabella Centeleghe

Cardiff School of Pharmacy and Pharmaceutical Sciences

Cardiff University

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Dedication

To my Mum and Dad, Hilary and Domenico Centeleghe.

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Thesis Summary

Dry surface biofilms (DSB) are present on healthcare surfaces throughout the world. They have been found to harbour pathogenic organisms, yet still the infection risk DSB pose remains unknown. Previous work has alluded to the resistance of DSB to disinfectants but there remains a gap in the literature. This thesis sought to understand the resistance of DSB with new strains of bacteria, including environmental isolates and explore the idea of how to combat DSB in the future. We combined laboratory experiments with surveys and interviews to gain a full understanding on the current knowledge and opinions of infection control measures in the working environment.

We investigated DSB resistance to key disinfectant components readily used in the healthcare environment and identified the efficacy of commercially available wipe products on DSB. Of the species tested, *Bacillus* spp. were much less susceptible to disinfectants. Resistance of DSB over time was also investigated, which highlighted the resistance of environmental isolates from initial DSB formation. Although wipe products were successful in the removal of some DSB from surfaces, they failed to inhibit direct transfer of DSB. *Staphylococcus aureus* was most susceptible to both disinfectant treatment and wiping. The success in the formation of a dual species DSB has impact on future work into the investigation of multispecies biofilms, which should be considered as they are better representative of what occurs in the natural environment.

Healthcare professionals provided essential insights into cleaning and disinfection practice. There is still a gap in knowledge of DSB amongst healthcare workers. It is clear that a greater deal of education is required to overcome current barriers. Our work has concluded that manufacturers should start focusing on combatting DSB alongside education into correct product usage, as this remains an issue.

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Annex 2. List of Abbreviations

AEWD: Automated endoscope washer disinfector

AGP: Aerosol generating procedures

ASTM: American society for testing and materials

ATCC: American type culture collection

ATP: Adenosine Triphosphate

BSA: Bovine serum albumin

BZK: Benzalkonium Chloride

CDC: Centre for disease control and prevention

CFU: Colony forming units

DE: Dey-engley neutralising agar

DSB: Dry surface biofilm

EN: European standard

EPS: Exopolysaccharide substance

HAI: Hospital acquired infection

HCAI: Healthcare associated infection

HCAI: Healthcare associated infection

HPV: Vaporised hydrogen peroxide

MDRO: multi-drug resistant organism

NaDCC: Sodium dichloroisocyanurate

NCTC: National collection of type cultures

PAA: Peracetic acid

PPE: Personal protective equipment

SEM: Scanning electron microscopy

TSA: Tryptone soya agar

TSB: Tryptone soya broth

TSC: Tryptone sodium chloride

UV: Ultraviolet

Annex 3. Glossary

Term	Definition
Disinfectant	A chemical agent used to kill or inhibit the growth of microorganisms
Surfactant	Amphiphilic molecules which, when added to liquid, decrease surface tension
Detergent	Water soluble cleaning agent containing surfactants, used to remove dirt from objects, including fabrics
Wipe	Piece of fabric used remove grime from an object or surface, usually pre-impregnated with cleaning product
Biocide	A chemical substance or microorganism used to destroy, deter, have a harmful effect on any harmful organism by chemical or biological means
Culturability	The ability of bacteria to grow and form colonies on specific nutrient agar that are detectable in the laboratory setting
Viable but non culturable (VBNC)	Bacteria that are considered living (i.e metabolically active) but cannot be detected on liquid or solid media
Dry Surface Biofilm	Aggregation of microorganisms housed in exopolymeric matrix that has been exposed to periodical wetting and drying phases, found in the healthcare environment on dry surfaces
Multi-drug resistant organism (MDRO)	Organisms which are resistant to more than one type of antibiotic

Biological repeat	Biologically distinct samples, using the same organism under the same conditions from a different starting inoculum
Virulence	A microorganism’s ability to cause infection within a host
Pathogenicity	The absolute ability to cause disease, infectious agents are classed as pathogenic or not
Healthcare associated infection (HCAI)	Any infection that develops as a result of being treated, or coming into contact with a healthcare facility including nursing homes, GP practice, hospitals
Hospital acquired infection (HAI)	Any infection that has resulted from being treated or being in contact with a hospital facility

Annex 4. List of Suppliers

Supplier name	Address
Acros Organics	Loughborough, UK
Biosystems Technology	Devon, UK
Corning Incorporated	New York, USA
Decon Laboratories	Hove, UK
Diversey Solutions	Amsterdam, Netherlands
E & O Laboratories	Bonnybridge, UK
FisherBrand	Loughborough, UK
Fisher Scientific	Loughborough, UK
GraphPad Software	San Diego (CA), USA
Goodfellow Cambridge Ltd.	Huntington, UK
Hamilton	Birmingham, UK
IBM SPSS Software	Chicago, USA
Invitrogen	Loughborough, UK
Labnet International	Edison, USA
Life Sciences (Tecan Gorup)	Reading, UK
Memmert	Schwabach, USA
Merck	Dorset, UK
Microspec	Bromborough, UK
Sartorius	Stonehouse, UK
Terumo	Shibuya, Japan
ThermoFisher Scientific	Loughborough, UK
Wiperator FitaFlex Ltd.	Ontario, Canada

Chapter 1. General Introduction

1.1 The Global Burden of Healthcare-Associated Infections

The concern of healthcare-associated infections (HCAI) throughout the world is ever increasing as healthcare professionals and scientists have still not been able to resolve this global problem. Although it is not possible to completely eradicate HCAI, adherence to strict infection control measure, such as hand hygiene and use of personal protective equipment (PPE), can help to reduce the risk (Flodgren et al., 2013; Greene and Wilson, 2022). The World Health Organisation (WHO) describes HCAI as those infections which occur within a patient during their time spent in a hospital or other healthcare facility, which was not present upon initial admission (WHO, 2011). These infections typically occur within 48 hours of admission or, within a period of 30 days after receiving care (Haque et al., 2018). HCAI can arise endogenously through patients own microflora (Collins, 2008), or most commonly, through exogenous contamination from patient-to-patient contact, transmission from surfaces, equipment or even healthcare professionals themselves (Daud-Gallotti, 2012). HCAI are also spread through aerosol generation, not only from patients or other people in the hospital but also medical procedures carried out in hospitals. Considering the current climate and aerosol transmission of SARS-CoV-2, aerosol generating procedures (AGP) set out by the UK government include protection procedures which is not limited to the use of FFP3 respirator mask, PPE including gowns and gloves, and facial protection. Spread can be exacerbated in buildings with poor ventilation and high relative humidity levels (D'Alessandro and Fara, 2017). Improvements in ventilation within a hospital environment can improve air quality, in turn, reducing microbial contamination in the air (Ilić et al., 2022). High humidity levels lead to a greater amount of moisture in the air and on surfaces, lending themselves to microbial colonisation. In addition, the greater number of beds occupied, leads to higher bioburden levels as a greater number of microorganisms are shed into the environment (Beggs, 2003). The term HCAI itself encompasses a wide range of infections. Mandatory reporting within the NHS is required for the most common causative organisms; methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-sensitive *Staphylococcus aureus* (MSSA), *Clostridium difficile* and *Escherichia coli* (NICE, 2016). In 2011, an estimated 300,000 patients acquired a HCAI after being treated within the NHS in England, bringing the total prevalence of HCAI to 6.4% (NICE, 2014). Whereas in 2020, an estimated 653,000 HCAI cases were recorded, this shows an increase in number of HCAI infections throughout the

years. In Wales, 5.5% patients in the acute sector of hospitals contracted HCAI, and 6% in non-acute sectors (Public Health Wales, 2017). Of these, the most common infections (in both England and Wales) are outlined in Table 1.1. Although those described are most common in healthcare facilities, the emergence of pathogens continues to put strain on healthcare such as *Candida auris* (Eyre, 2022) and methicillin-resistant *Staphylococcus capitis* (Carter et al., 2018).

Table 1.1. Most common HCAI. HCAI accounting for more than 50% of infection within hospitals, their causative organism, and the prevalence of each infection within NHS England in 2011.

Hospital-associated infection	Causative organisms	Prevalence of Infection
Respiratory infections	*Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*	22.8%
Urinary tract infections	*Escherichia coli*, *Candida albicans*, *Enterococcus faecalis*, *P. aeruginosa*	17.2%
Surgical site infections	*Staphylococcus aureus*	15.7%

There has been a considerable amount of work to identify the important role of environmental contamination in nosocomial (hospital) transmission of not only multi-drug resistant organisms, but mycobacteria, viruses, and fungi (Dancer, 2014). This is pertinent now considering the emergence of SARS-CoV-2, which has spread rapidly throughout the world and led to a global pandemic in 2020. The current socioeconomic, environmental, and ecological impact of SARS-CoV-2 pandemic has had detrimental effects on the global population, whilst we are all currently still, and will continue to battle recurrent diseases and antimicrobial resistance (Haque et al., 2020).

Increased morbidity and mortality are amongst the major concerns associated with HCAI (Cassini et al., 2016). HCAI can lead to severe health issues such as

bloodstream infections and often death (Wenzel and Edmond, 2001). HCAI are responsible for a huge economic burden globally, the NHS (England) spent £2.1 billion for the year 2016-17, and NHS (Wales) around £50 million per year (Guest et al. 2020). Over half of this sum is predicted to have been spent on infections after patients have been discharged from hospital (Mantle, 2015). The discrepancies between the two nations may have arisen from the population size, but also patient management. Worldwide, for every 100 patients that are admitted to hospital, 7% from high-income economies and 10% from low-income economies will acquire at least one type of HCAI upon hospital admission (WHO, 2016).

Many have argued that a significant number of HCAI are in fact preventable, the total numbers are considered a measure of the lack of patient care given in healthcare facilities (Umscheid et al., 2011). Unfortunately, due to the rapid spread of pathogenic, drug-resistant bacteria, the problem of HCAI will only worsen without appropriate avoidable measures being put in place. HCAI are a key priority for the NHS, which has led to the implementation of a range of policies and infection prevention and control measures such as hand hygiene and PPE. Ultimately infection prevention control (IPC) policy should prevent the spread of antimicrobial resistance (AMR) and HCAI. The government also intends to prioritise AMR through the UKs current 5-year AMR strategy from 2019 – 2024 (Department of Health, 2019). The strategy includes three main factors to work towards combatting AMR, they are as follows; reducing the need for unintended exposure to antimicrobials, optimising the current use of antimicrobials, and investing in innovation, supply and access to tackle the current problems with AMR. The term ‘antimicrobial’ encompasses all drug agents which are active in killing all microbes, including bacteria, viruses and fungi.

1.2 Infection Prevention and Control

Infection prevention and control (IPC) is an essential component to healthcare services around the world. According to the World Health Organisation, implementation of appropriate infection control measures can reduce HCAI by 30% (WHO, 2016). IPC incorporates both a scientific and practical approach designed to mitigate HCAI, transmission and spread of infection to patients and healthcare workers (WHO, 2019). Policy and guidance are constantly evolving and aim to inform IPC staff and help shape IPC nursing practices. In the UK, specific guidelines

for IPC for each major sector in healthcare services such as GP surgery, nursing homes and hospitals, are set out. These include hand hygiene, aseptic technique and environmental cleaning and decontamination. Within each guideline are specific measures to follow depending on the situation. The implementation of IPC is vital to stop outbreaks of pathogenic diseases and infection. The Global IPC network aims to bring major IPC organisations together from around the globe to strengthen IPC systems and programmes and support outbreak preparedness and response (Allegranzi et al., 2017). It is evident that IPC is at the core of healthcare systems globally, however these guidelines are constantly evolving with improvements being made to ensure the safest environments for all. Globally, there are numerous challenges faced by IPC. The World Health Organisation (WHO) outlined those challenges to IPC including IPC is not considered a priority in some facilities, the leadership for IPC is missing, implementation expertise is not available and a crucial point, financial resources are limited.

1.2.1 Cleaning and Disinfection

It is important to establish the difference between cleaning and disinfection. Cleaning is classed as the physical removal of dirt or organic matter with the use of a soap and/or detergent. Often soap or cloths are used, which increase the transmission of microbes from one site to another (Dancer, 2011). Disinfection involves killing a substantial number of microbes present on a surface, rendering them incapable of reproducing using products such as chlorine, bleach and peracetic acid-based product (Rutala and Webber, 1999). The cleanliness of a hospital has a direct impact on patient recovery from an illness. Although there remains much debate over cleaning practices within hospitals (Doll et al., 2018). Much of the time, hospitals rely on areas looking visually clean rather than a reliable assessment of infection risk (Dancer, 2009). Mulvey et al. (2011) proved that visual inspection of cleanliness did not reflect the levels of bacterial species contamination in the environment when testing with adenosine triphosphate (ATP) bioluminescent assays. Other studies have revealed that a lack of thoroughness and insufficient cleaning practices are often inadequate in the removal of microbial contamination from surfaces (Carling et al., 2010). The type and frequency of cleaning and disinfection protocols depends on numerous factors including clinical risk, patient turnover and surface characteristics (Dancer, 2014; Siani and Maillard, 2015).

Surfaces can fall into two main categories, low risk and high risk/touch areas (**Figure 1.1**).

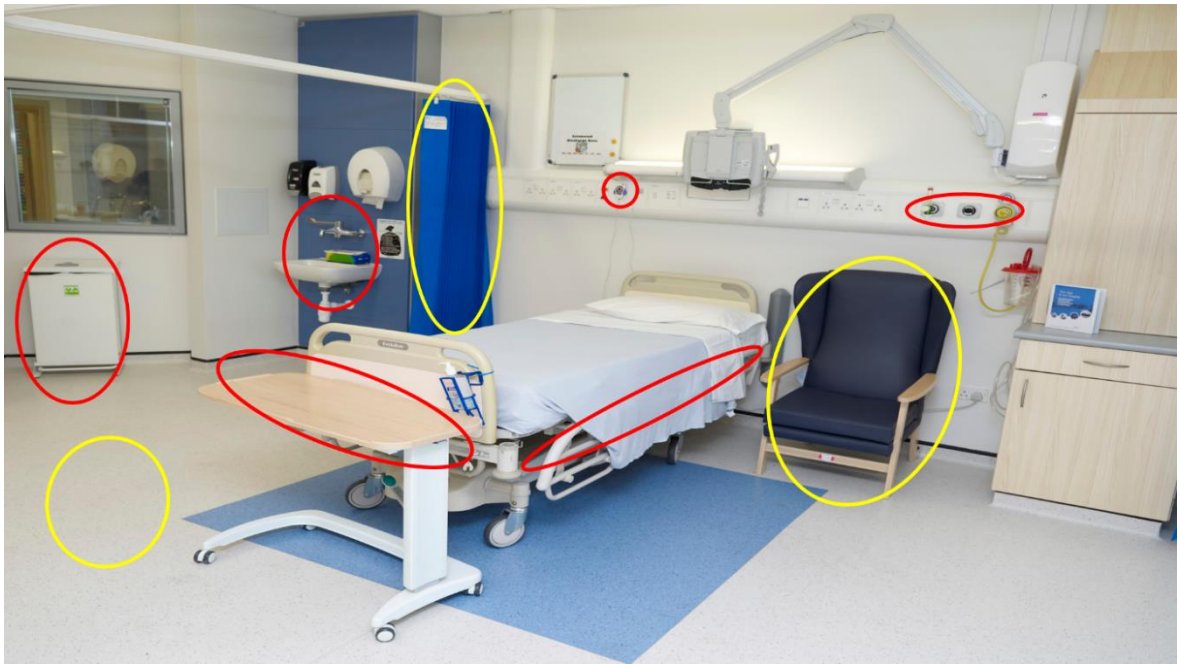


Figure 1.1. Surfaces and items within hospitals fall under two categories, non-critical (yellow**) or critical (**red**).** Circles encompass both low risk (yellow) and high risk/touch (red) surfaces and equipment within a patient room.

Low risk surfaces are usually cleaned with detergent and water. However, water needs to be changed regularly as it can easily become contaminated when continually used. Sifuentes et al. (2013) highlighted the problem of microbial growth on cloths used for cleaning hospital rooms, where hospital laundering services were insufficient to thoroughly remove contaminants. Pre-impregnated detergent and disinfectant wipes are commonly used due to ease of use (Sattar and Maillard, 2013). Wiping has been proven to be more effective due to the physical action of removal of dirt and debris from a surface (Dancer and Kramer, 2019; Song et al., 2019) (**Figure 1.2**). Using this S-shape motion with a wipe transference of pathogens is avoided and the product does not dry out, which is essential for surface cleaning (Boyce, 2021).

In addition to wiping, automated devices, such as vaporised hydrogen peroxide machines, are also frequently used and are most effective in terms of capacity for high-level disinfection, with longer term beneficial effects but they come at a cost and can be very labour and time intensive (White et al., 2007). Currently, systems are used to complement cleaning rather than replace it altogether (Diab-El Schahawi et al., 2021). However, automated systems, eliminate the reliance on an operator which ensures an adequate distribution and contact time of a disinfectant which may not be true for human controlled measures (Otter et al., 2014).

Figure 1.2. Appropriate way to clean a surface. The S-shaped method should be used to maximise contact with the surface and minimise the amount of hand contact. *Copyright from Dancer and Kramer (2019).*

Critical surfaces require disinfection and are occasionally checked with environmental screening such as fluorescent markers (Smith et al., 2012), however, most often surfaces are only checked for microbial contamination following an outbreak (Rawlinson et al., 2019). It is thought daily routine cleaning with disinfectant wipes may be enough to reduce and control infection in the localised area (Bogusz et al., 2013). Intensive care units (ICU) may require more attention

due to susceptible patients that are most vulnerable to infection, more care tends to be taken when cleaning and disinfecting these areas.

If one looks at the cleaning and disinfection of devices, the classification of what remains a high risk to the patient is slightly different. The Spaulding classification, originally proposed in 1957 explains the risk of three main groups: critical, semi-critical and non-critical devices (McDonnell and Burke, 2011). Critical pose the highest risk, as these are categorised as those devices which enter the body. Critical devices require sterilisation to ensure the device is completely free of any viable bacteria. Semi-critical pose a lower risk as they often only encounter mucosal barriers so high-level disinfection is adequate. Cleaning must always follow sterilisation and high-level disinfection to ensure all pathogens capable of causing infection are eliminated (Rutala and Weber, 2019). Finally, non-critical devices pose the lowest risk to patients, which only come into contact with the skin but still require low level of disinfection. Although guidelines are in place for decontamination of equipment, there are discrepancies over protocols within the NHS, specifically the person liable for decontamination of those objects that are considered low risk is unclear (Castelli et al., 2021).

1.2.2 Surfaces at Risk

High touch surfaces, such as door handles, keyboards and bed rails, are often the most contaminated areas within hospitals (Vickery et al., 2012). It has been highlighted that even after cleaning and disinfection, surfaces can remain contaminated with pathogens (Perry-Dow et al., 2022). An added problem, is microbes including bacterial spores, vegetative bacteria, fungi and viruses can survive on these dry surfaces for extended periods of time, creating a reservoir of micro-organisms (Otter et al., 2015). The longer a pathogen survives in the environment on a dry surface, the greater the risk of an HCAI (Querido et al., 2019). Hospitals implement various methods to test/sample surfaces for microbial contamination (**Table 1.2**).

1.2.3 Deep Clean

Deep cleans are usually performed following patient discharge (Dancer, 2014). When a patient has had a known infection, protocols for decontamination can be augmented to use disinfectant at a specific strength for specific targets. Methods can vary between hospitals, especially when we consider the SARS-CoV-2 pandemic, whereby strength and frequency of cleaning not only varied between

country but also between regions and even areas of the hospital (Kanamouri et al., 2021). Generally, all detachable items, including curtains and bedding are removed. The area and items are then cleaned downwards towards floor level. Housekeeping staff usually conduct terminal cleans of the room, with nursing staff taking care of the beds and clinical equipment. This separation has caused much confusion over who cleans what and can lead to items not being cleaned (Anderson et al., 2011). Generally, in the UK, the room is viewed before admission of another patient and deemed clean through visual inspection rather than other more reliable methods to check for cleanliness, such as the ATP assay or culture of microorganisms (**Table 1.2**) (Dancer, 2004).

Table 1.2. Methods to evaluate and quantify cleanliness within hospital environments. (*Information taken from Mitchell et al. (2013)).

Method	How to perform	Advantages	Problems	Extra information
Visual Inspection	Primary method using own eyesight.	Quick and easy Economical to implement Surveys the whole area	· Biased upon individual observing area · Cannot detect bacterial soiling	17-93% more surfaces are passed as clean using this method
Fluorescent gel marker	Transparent gel left on surface and visualised under UV light.	Easy to implement Highlights lack/good cleaning on near patient areas	· Associated ongoing costs · Highest rate of false results · Only measure how well a surface is wiped	Enables immediate response directly following cleaning
ATP assay	Surface is swabbed, then placed into a solution containing active enzymes (luciferase and luciferin) to catalyse reaction with ATP. Luminometer reads light output.	Easy to implement Although includes all bacteria (inc. non-viable) and organic debris on surfaces, you can remove this data through enzymatic removal before the assay	· Associated ongoing costs · Also includes information on bacteria in a non-viable state and other organic debris · Low sensitivity and specificity · Measurements can be compromised by disinfectant products	Sensitivity and specificity have been calculated as low as 57%
Microbial culturing	Swabs are taken of the surface and sent off to a lab for culture and identification of species present, settle plates are also used directly on surfaces.	High sensitivity Directly identifies species Suggests environmental reservoirs of organisms around the area	· Takes a long to time to receive results · Very costly to outsource to a lab	Generally, only used for outbreak situations rather than everyday

1.3 Biofilms

Bacteria are often thought of as planktonic cultures, however in the natural environment, most microorganisms exist as self-sufficient, complex communities, called biofilms. These aggregates of microorganisms can adhere to both abiotic and biotic surfaces (Flemming et al., 2016). Bacterial cells are embedded within a matrix of extracellular polymeric substances (EPS), which contains polysaccharides (1-2%), DNA (<1%), RNA (<1%) and water (Jamal et al., 2015). EPS are responsible for both the structural and functional integrity of the biofilm and can vary from 50% to 90% of the total biofilm mass (Wingender et al., 1999). The EPS matrix confers a particular mode of life for bacteria ensuring they can survive and interact with their natural environment (Flemming et al., 2007). Importantly, it provides a strong barrier against the penetration of antimicrobials, acting as both a chemical and physical diffusion barrier, owing to their high resilience within the natural environment (Percival et al., 2015; Singh et al., 2021). The 'housing' matrix of EPS and vital proteins, DNA, and other organic material, produced by the species in the latter stages of development encases all bacteria together, however if some is left behind on a surface, this leaves a structure for another biofilm to be established rapidly. Approximately 99.9% of all known bacteria possess the ability to form a biofilm (Costerton, 1995). Most biofilms are multi-species, which are structurally and spatially defined communities in response to interspecific interactions (Lui et al., 2016). These interactions, tend to be competition over nutrients and synergistic, meaning the activity of two or more bacterial species working together is greater than that when they are individuals. These factors influence overall evolutionary fitness of species within biofilms (Sadiq et al., 2021), including promotion of biofilm formation, increased antibiotic resistance or metabolic cooperation (Elias and Banin, 2012).

1.3.1 Biofilm Formation

Biofilm formation comprises of 5 main stages: reversal attachment, irreversible attachment, colonisation, maturation and finally dispersal (**Figure 1.3**).

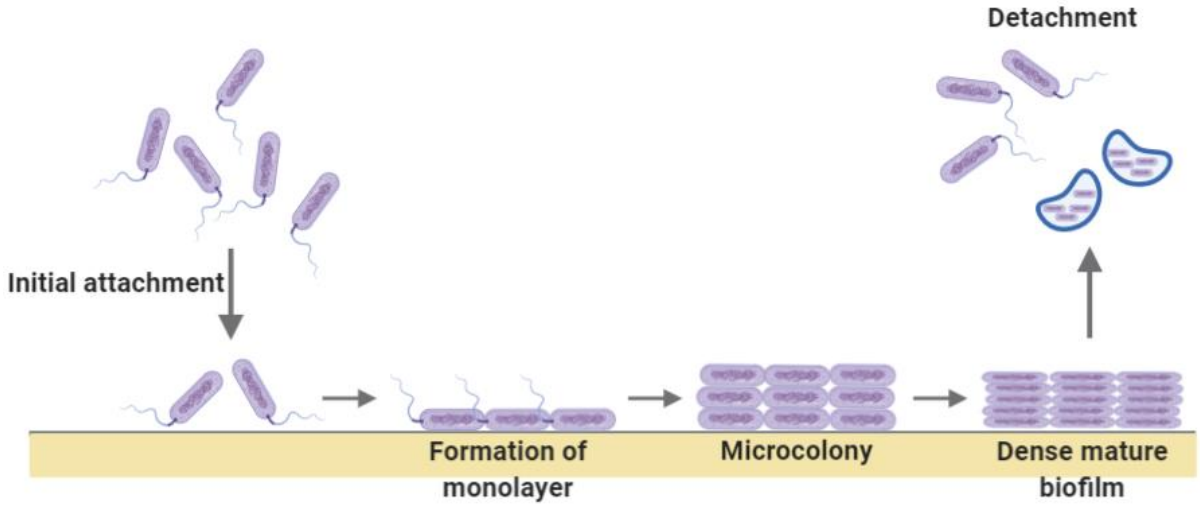


Figure 1.3. Schematic representation of a biofilm. The 5 stages of biofilm formation from the initial attachment to the surface (1), leading to the formation of a monolayer of bacterial cells housed in the EPS matrix (2). This small monolayer results in colonisation of more cells through quorum sensing to enhance the biofilm (3). At the later stages of development, a mature biofilm has now formed and become very dense (4), detachment occurs, mediated by secondary signal molecules, resulting in dispersal from cells and 'rafts' before the process starts again (5).

Specific mechanisms to allow initial surface attachment, development of the microbial biofilm community and subsequent detachment from the biofilm have arisen in microorganisms living within a biofilm (Percival et al., 2015). The initial attachment is the most important aspect of biofilm formation and can be dictated by increased shear forces, various electrostatic and physicochemical interactions between microorganisms and the appropriate surface, such as covalent bonds or

van der Waal forces (Donlan, 2001; Jamal et al., 2018). Depending on the nature of attachment it can be classed as either transient or permanent. Following the initial reversal attachment, irreversible attachment is facilitated by either fimbrial (pili) or non-fimbrial adhesins (Berne et al., 2015). Fimbrial adhesins tend to be multifaceted and are mainly involved in the transition between motility and irreversible attachment. The non-fimbrial adhesins are widespread throughout bacterial cells and are used when attaching to an abiotic surface or host cell, they aid in linking the bacterial cells to the housing matrix of the biofilm (Berne et al., 2015).

The cell population density of a biofilm is constantly fluctuating throughout development. Quorum sensing (QS) is used to regulate gene expression (Miller and Bassler, 2001), allow control of specific processes including biofilm formation and stress adaption mechanisms (Pena et al., 2019) and cause microbial physiological changes and virulence potential (Warrier et al., 2021). QS is a form of communication between bacterial cells to convey their presence to one another (Li and Tian, 2012). Eberhard et al. (1981) first described quorum sensing in *Vibrio fischeri*, a marine bioluminescent bacterium, QS uses diffusible chemical signal molecules called autoinducers. Alterations in gene expression occur when autoinducers reach a critical threshold within the biofilm, leading to changes in motility, sporulation and the release of virulence factors (Mangwani et al., 2012). Gram-negative and Gram-positive bacteria release different QS molecules which may implicate different pathological processes within the biofilm (Percival et al., 2015). For example, biofilms are common in patients with cystic fibrosis. *Pseudomonas aeruginosa* and *Burkoholderia cepacian* are two common causative organisms. *P. aeruginosa* produces C_4 and 3-oxo-C_{12} homoserine lactones which are recognised by *B. cepacia* and activate its *cep* quorum sensing systems which contributes to the development of a multi-species biofilm and has implications for the pathogenesis of the disease (Jayaraman and Wood, 2008).

1.3.2 Biofilm Dispersal

Biofilm dispersal constitutes the last stage of the biofilm cycle and can be either passive or active, whereby matrix-encased cells are released from the biofilm and revert back to planktonic mode of growth (Rumbaugh and Sauer, 2020). It is considered the most important point in biofilm development as it allows bacteria to leave the biofilm and spread to new niches, especially when nutrients and other resources are limited (Guilhen et al., 2017). Passive dispersal involves sloughing,

in which many cells are stripped from the surface causing radical loss to the biofilm mass, or erosion, where a small portion of the biofilm is gradually and continually separated from the biofilm matrix (Kim and Lee, 2015; Fleming and Rumbaugh, 2017). Active dispersal using specific molecules, enzymes, and proteins to aid the dispersal of cells from the biofilm. Cyclic-di-GMP, a secondary messenger molecule, has been identified as one of the main intracellular mechanisms responsible for dispersal of bacteria from a biofilm (Karatan and Watnick, 2009). Biofilm dispersal is characterised by an active phenotypic switch involving, several environmental factors including temperature, oxygen and nutrients, which allows the release of cells and/or aggregates from the biofilm (Guilhen et al., 2017). These environmental cues can induce the production of exoenzymes and surfactants that degrade the EPS matrix, identified in *Staphylococcus aureus* biofilms (Lister and Horswill, 2014). The dispersal cells are highly specialised compared to those bacterial cells that are cast off the biofilm during development. Dispersed cells have specific properties that enable them to colonise a new surface quickly, including greater motility, and an increased expression of adhesion and virulence factors (McDougald et al., 2012; Guilhen et al., 2017). Once dispersal occurs, dissemination of bacteria can be extremely widespread, which is of concern in the healthcare environment, especially those biofilms associated with medical devices.

1.4 Biofilms within Healthcare Environments

Biofilms have been at the forefront of medical microbiology research due to their implication in the development of chronic wounds, catheter-associated infections and even pneumonia in cystic fibrosis patients (Bjarnsholt, 2013) (**Table 1.3**).

Table 1.3. Common biofilms associated with the healthcare environment and their most common causative microorganism.

Biofilm type	Most common causative species	Reference
Catheter-associated urinary tract infections (CAUTI)	*Escherichia coli* most common, *Enterobacter cloacae* highest biofilm production	Sabir et al. (2017)
Central line associated bloodstream infections	Gram-positive organisms most common (coagulase negative Staphylococci, Enterococci, *S. aureus*) *Candida albicans*	Haddadin and Regunath (2019)
Cystic fibrosis patients	*Pseudomonas aeruginosa* (mucoid strains)	Høiby et al. (2010)
Surgical site	*S. aureus*, *S. epidermis*, MRSA	Percival et al. (2015)

Several factors enhance tolerance of biofilms to antimicrobial agents. Many biofilms within the healthcare environment possess resistance to antimicrobial agents as the EPS matrix housing bacterial cells causes slow or incomplete penetration of these products (Francolini and Donelli, 2010). It has been reported that extracellular DNA (eDNA) in the biofilm matrix induce additional resistance mechanisms (Li et al., 2020). Bacteria within a biofilm have reduced metabolic activity, as antibiotics depend upon active bacterial metabolism to work, this has been one reason for their failure in the treatment of biofilm infections (Koo et al., 2017). Biofilms also contain a diverse range of bacterial species in different physiological states, which can readily adapt to changing environmental and chemical gradients (Stewart and Franklin, 2008). The existence of persister bacterial cells, those dormant cells created spontaneously upon biofilm formation are accountable for approximately 1% of the stationary state of biofilms and arise due to metabolic inactivity (Wood et al., 2013). As they are in a dormant state, they are somewhat responsible for the

tolerance to antibiotic treatment as most classes of antibiotics rely on active metabolism to work (Kim et al., 2009).

1.4.1 Medical Devices and Implant Biofilms

Medical devices and implants have revolutionised medicine but come with an increased risk of foreign body infection (Stewart and Bjarnsholt, 2020). In the USA alone, device associated infections account for 25.6% of HCAI (Magill et al., 2014). Most medical devices and implants are susceptible to microbial colonisation due to the type of materials used. The most common include heart valves, catheters, orthopaedic implants and intrauterine devices (Costerton et al., 1999; Von Eiff et al., 2012).

When a prosthetic implant is put inside the body, our natural immune response causes the production of a film around the foreign object. This contains proteins which serve as binding ligands for bacteria and subsequent colonisation (Bryers, 2008). Once bacteria have attached to the implant, the process of biofilm formation begins allowing bacteria to survive in hostile environments. Most often, infections will occur after 3 months of surgery/implantation of a device, however, sometimes infections take up to 24 months to arise (Zimmerli et al., 2014). This delay can lead to dissemination of bacteria within a biofilm to other sites in the body (Arciola et al., 2018).

Biofilms on implant surfaces are mostly difficult to treat effectively, in the past implants harbouring biofilms are removed and the infection treated with antibiotics before replacing the implant. However, this has proven to be both costly and stressful for the patient (Carmen et al., 2005). Other methods aim to directly treat the device/implant through hydrophilic coatings and impregnation of antimicrobial agents, such as peptides, on the surface of the device or implant (Francolini and Donelli, 2010; Yao et al., 2022). Whilst work on surface coatings and antimicrobial surfaces has been proven effective only in laboratory settings, more works needs to be done to investigate the “kill-first” or “repel-first” mechanisms of action for the long-term prevention of medical device HCAI (Zander and Becker, 2018). This process looks at either killing microbes as they settle on a surface with underlying repellent mechanism or repelling them before they reach the surface with an underlying killing mechanism.

To date new methods are taking a different approach (**Table 1.4**). It is still apparent that keeping general hygienic conditions and practices is most important in hospitals.

Table 1.4. New approaches to combatting biofilm colonisation on medical devices and implants.

Preventative method	Mechanism of action	Reference
Antibody-based approaches	Antibodies can interfere with biofilm formation and cause dispersal of cells from the biofilm through; opsonophagocytosis, binding to bacterial surface proteins, activation of the complement pathway and target matrix component	Raafat et al. (2019)
Antibiotic loaded calcium beads	For orthopaedic periprosthetic joint infections, calcium beads dissolve when implanted in soft tissue and release antibiotics	Knecht et al. (2018)
Antimicrobial coatings with surface-bound biocides	Coatings contain cationic compounds that target microorganisms after adherence to a surface through alterations in membrane permeability and other disruption	Krishnan (2015)
Antimicrobial peptides	AMPs display broad spectrum biocidal activity where they bind to bacteria causing them to become permeated	Bayramov and Neff (2017)

1.4.2. Multispecies Biofilms

Many different environments, including ocean deep sub-surfaces (Flemming and Wuertz, 2019), medical devices (Ramstedt and Burmølle, 2022) and even the digestive system of animals (Arias and Brito, 2021) support life of bacteria in

multispecies biofilms (Sadiq et al., 2021). The structure of multispecies biofilm differs from monospecies due to the spatial organisation of the species which are well organised in layers, or clusters. Spatial organisation contributes to ecological interactions between different populations in the biofilm, thus, prompting the overall community functions (Costa et al., 2017). Coaggregation of multispecies biofilms is partly governed by the production of specific cell surface structures of early colonisers, enabling other microorganisms to adhere to the biofilm through cell receptors for these structures (Simões et al., 2008).

In recent years, research has alluded to the increased resistance of multispecies biofilms to the action of disinfectants, compared to their monospecies counterparts (Bridier et al., 2011a). Also evidenced in real world scenarios, resident surface flora of the oral biofilm, normally non-virulent, have been shown to protect more pathogenic species (Luppens et al., 2008). Although it is not definite, as research is still ongoing, there are numerous factors thought to be involved in this process of protection. Firstly, the EPS matrix plays a vital role in the structure of biofilms. A more viscous matrix is formed in multispecies biofilms from the production of polymers by each species, in turn decreasing the ability of biocidal products to penetrate the deepest layers of the active biofilm (Burmølle et al., 2006). Metabolic cross feeding between cells within a biofilm can enhance the growth of dwelling cells within the matrix, allowing for longer survival when exposed to biocidal products (Stacy et al., 2014).

The worrying issue with the possible protection of species within a multispecies biofilm is the protection of those pathogenic strains, specifically within the healthcare environment.

1.4.3 Dry Surface Biofilms

With regards to biofilm resistance and appropriate cleaning/disinfection within healthcare environments, dry surface biofilms (DSB) have had limited attention compared to their counterpart, the hydrated biofilm. DSB are biofilms which have been exposed to lowered water potential, reduced nutrient resources and periodic disinfection on clinical surfaces (Almatroudi et al., 2015; Ledwoch et al., 2019a&b). The presence of DSB with a thicker EPS than hydrated biofilms was confirmed by Hu et al. (2015). This thick EPS layer creates a barrier for the dry biofilm allowing it to resist desiccation and tolerate standard cleaning and disinfection methods,

prolonging their survival on hospital surfaces. The complexity of environmental DSB is hard to replicate in the laboratory, Almatroudi et al. (2015) created one of the first DSB models similar, in composition and architecture, to those found in the clinical setting. Although they used a CDC Biofilm Reactor and different media concentrations, the DSB model in this project is based around their findings.

1.4.3.1 Environmental DSB

There are now a handful of studies identifying the presence of dry biofilms on hospital surfaces. Much of which is a concern due to the levels of multi-drug resistant organisms (MDRO) found within the biofilms. Vickery et al. (2012) were one of the first groups to identify reservoirs of MDRO existing in the environment as dry biofilms. Following a two-step terminal cleaning protocol of an intensive care unit (ICU) in Australia, equipment and furniture was removed from the unit and subject to both culture of any microorganisms presents on sampled surfaces and scanning electron microscopy (SEM) imaging. They found the pathogenic species, MRSA within the samples, as a pathogen, MRSA is responsible for 20% of hospital mortalities globally (Stefani et al., 2012). A similar study was carried out by Hu et al. (2015) in an ICU, to determine the prevalence of DSB in the immediate vicinity of a patient within the unit. MRSA, VRE- and ESBL- positive multi-drug resistant organisms were mostly found within the immediate patient vicinity. Interestingly, the average number of species per 1% of the biofilms detected in this area was 23, which seems extremely high. This study not only highlights the ability of DSB to remain in the environment for long periods, but also the interaction between both pathogenic and non-pathogenic organisms within the biofilm, which could play a vital role in DSB resistance. Since the release of these publications, DSB, containing MDRO have now been detected on hospital surfaces in Australia, Saudi Arabia (Johani et al., 2016), and the UK (Ledwoch et al., 2018; Ledwoch et al., 2021a).

1.4.3.2 Biofilm Tolerance to Cleaning and Disinfection

With regards to infection control, the importance of studying DSB lies within their significant tolerance to destruction by cleaning agents or disinfectants (Smith and Hunter, 2008). A DSB model of the pathogenic species, *S. aureus*, commonly found in healthcare environments, was produced by Almatroudi et al. (2016). DSB were exposed to several concentrations of sodium hypochlorite solution, a common disinfectant used in terminal cleaning. Following exposure to solutions of 1,000ppm,

5,000ppm, 10,000ppm and 20,000ppm for 10 minutes, bacterial counts were reduced by 7 log_{10}. However, even after exposure to 20,000ppm of sodium hypochlorite viable cells released as planktonic cells from DSB were still evident through confocal microscopy. Although the methods used to quantify bacterial cells were not as accurate as direct quantification through serial dilution, this paper highlights the resistance of at least 10% of the biofilm following exposure to extreme concentrations of disinfectant. This is further proven by data on recovery of bacteria from DSB, after exposure to 1,000ppm, DSB were able to regrow in < 6 days at 25ºC. Similarly, Ledwoch et al. (2021b) investigated the efficacy of several commonly used disinfectants and found that mechanical removal was essential to reduce bacterial concentration in DSB, whilst exposure to disinfectant, including vaporised H_2O_2 failed to control DSB. In addition, they explored regrowth of *S. aureus* DSB following treatments and reported regrowth time as low as 1 day. This is of great concern to healthcare environments, as without routine cleaning and disinfection procedures, DSB are able to continue colonising surfaces even after treatment with biocidal products.

Not only is there a concern over ineffective disinfectant measures to control DSB growth but transfer of DSB throughout the healthcare environment must also be considered, which has not yet been tested in the healthcare environment. The hospital environment is frequently touched by hands of both patients and healthcare workers, providing ample opportunity for the spread of pathogenic bacteria (Chowdhury et al., 2018), together with the risk of object-to-object transmission (Cheeseman et al., 2009). Tahir et al. (2019) investigated transfer of *S. aureus* DSB by three types of glove materials (nitrile, latex and surgical). Only one touch of DSB was all it took to transfer *S. aureus* in significant quantities to cause infection. However, more interestingly, after treatment with 5% neutral detergent, the rate of DSB transfer increased 10-fold, attributed to the surfactants in the detergent which change the physiochemical property of DSB to a hydrated type increasing the cell detachment from the biofilm and subsequently increasing transference. Wipes are also commonly used in the hospital environment for ease of use and convenience (Ramm et al., 2015). However, work has shown that wipes are a vessel for transfer of DSB from surfaces. DSB isolated from keyboard samples were transferred by wipes loaded with either sterile water or 1,000ppm sodium hypochlorite solution (> 50% transfer of samples) (Ledwoch et al., 2021a).

It is apparent that DSB are widespread throughout healthcare environments and are tolerant to standard cleaning and disinfection procedures. There is a need for new methods to help reduce the threat of DSB globally.

1.5 Overall project aims and workplan

The overarching aim of this project is to investigate dry surface biofilms (DSB) and understand their resilience to common disinfectants. Such an understanding will impact on the process of developing new products to eradicate DSB in healthcare settings.

The project can be broken down into four main areas surrounding the interaction of disinfectants and DSB with associated research questions (**Figure 1.4**):

i) Knowledge of IPC on biofilms is unclear. Hence the first objective will be to explore knowledge of IPC on infection control measures and biofilms. This will be done in the form of a survey and interviews.

ii) Although the efficacy of disinfectants against DSB has been reported, the second of objective of this thesis is to identify resilience of species that are commonly found in DSB in the healthcare environment to disinfectant products that are routinely used in disinfectant products.

iii) Environmental DSB contain several bacterial species, yet current DSB laboratory models concern single bacterial species. The third objective of this work will be to explore dual bacterial species biofilm and the role of environmental species commonly found in DSB to protect bacterial pathogens from disinfection.

iv) DSB has been shown to be difficult to eliminate from surfaces. The fourth objective of this work will be to identify current methods to decontaminate surfaces and determine how long DSB can survive in the environment to help progress in combatting DSB in the future.

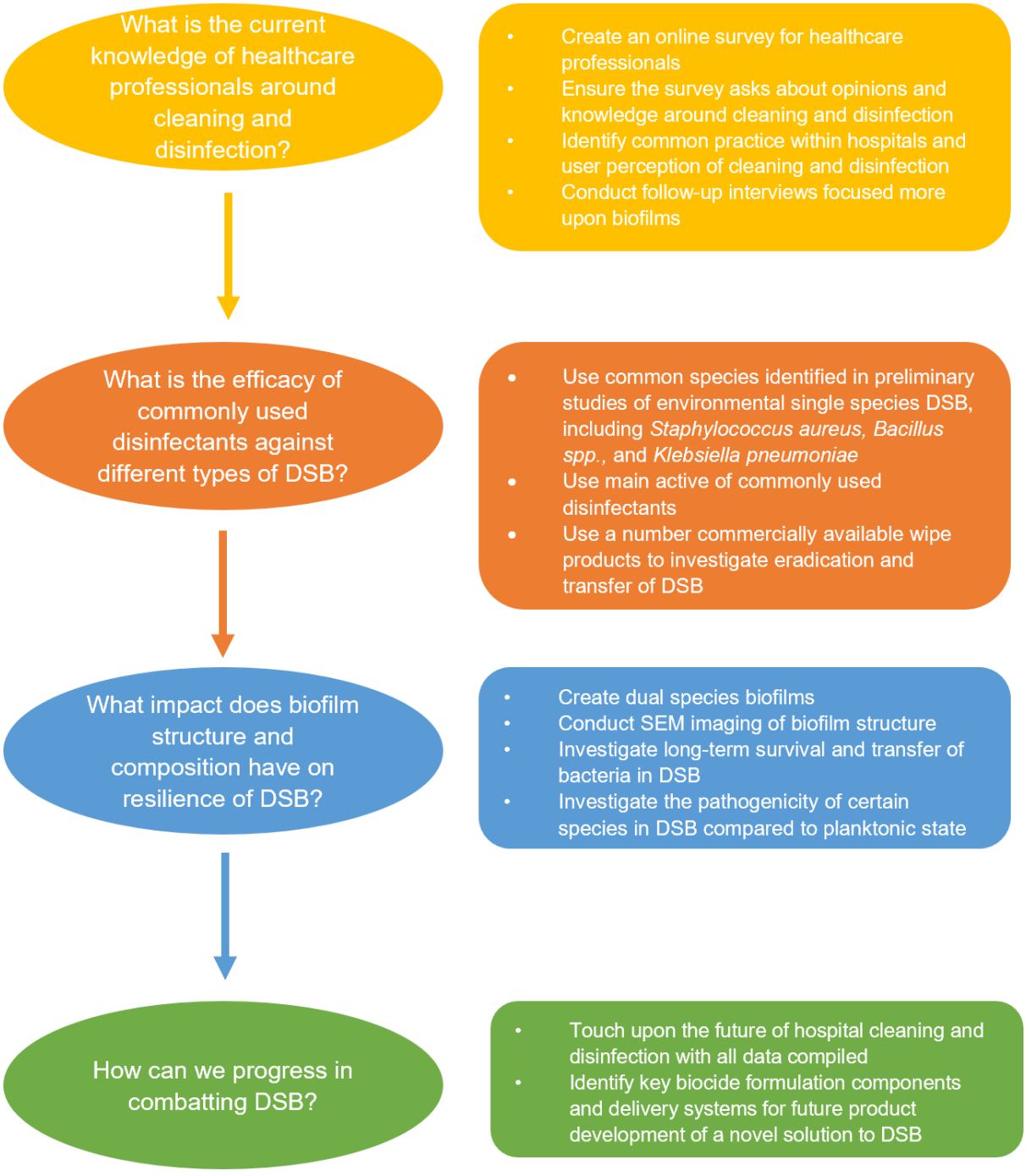


Figure 1.4. Project work plan for the PhD project.

Chapter 2
Healthcare Professional's Opinions and Knowledge of Microbes and Cleaning/Disinfection Routine in the Workplace

2.1 Introduction

Whilst laboratory experiments and testing are crucial in understanding the efficacy of disinfectant products, as will be shown in the following chapters, it is also important to focus upon the cleaning/disinfection protocols used in healthcare settings and the general knowledge of healthcare professionals on microbial environmental contamination. Nonetheless, hospital cleaning also comes with many challenges, including the cleaning process, which products should be used and how to effectively use them (Boyce et al., 2010). This is all encompassed in infection, prevention and control (IPC), which can be defined as the practice involved with reducing healthcare associated infections (HCAI).

Contaminated surfaces allow for transmission of HCAI throughout the healthcare environment, including pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA), norovirus and *Clostridioides difficile* (Weber et al., 2010). Approximately 30 – 50% of all HCAIs are linked to environmental contamination (Peters et al., 2018). Ensuring patient safety is crucial in the hospital environment, one of the biggest factors contributing to this is the cleaning and disinfection protocols used. Although national guidelines for cleaning and disinfection do exist, these can be complex and differ greatly between healthcare facilities and there remains a gap in the European and International market (Assadian et al., 2021).

Studies have previously shown that, after thorough cleaning by healthcare workers, the areas around a patient bed remain contaminated as they have been inadequately cleaned (Carling et al. 2008; Sitzlar et al. 2013). Disinfectant products may kill a species, but, if compliance with products and effective cleaning methods are not followed and workers do not understand the general theory behind disinfection, the potential for spread of infection is not as greatly reduced.

The Research Effective Approaches to Cleaning in Hospitals (REACH) training study has had a great impact on cleaning practices and reducing hospital acquired infections (HAIs) throughout Australia. Mitchell et al. (2018) used pre and post questionnaires to test staff members involved in hospital cleaning, on their knowledge and reported practice following implementation of their REACH cleaning bundle, including information on appropriate product use and techniques. They identified an increase in knowledge of environmental service staff members following REACH training audits, leading to an overall increase in understanding of

their role and knowledge relating to general cleaning practices. After implementing the REACH training bundle Mitchell et al. (2019) went back to look at the effectiveness of the trial. They observed improvements in cleaning thoroughness and reduction of bacterial infections, including *Staphylococcus aureus* and *Clostridioides difficile*. This meant the implementation of their training reduced infection rates within the hospitals. In addition, work by Bernstein et al. (2016) has also shown that by assessing the knowledge, attitudes, and practices of environmental cleaning alone, environmental service workers from 5 hospitals in New York, USA, could benefit from additional education in order to enhance cleaning practice. Similarly, both these studies have indicated the importance of teaching and implementation of good working practice in hospitals throughout the world.

Researchers have highlighted two major gaps within hospital disinfection; healthcare staff may not know how to clean effectively or forgotten what they have been taught without recurrent training courses to keep their existing knowledge updated. There is a requirement for an intervention to improve knowledge of both cleaning protocols and microbial contamination (as described above). Similarly, this area of research means to address healthcare staff knowledge of cleaning and disinfection, but also assess their understanding of biofilms, specifically dry surface biofilm (DSB), and the spread of infection, which has had limited attention in the literature.

To our knowledge, there is no current published research looking at healthcare professionals' knowledge of biofilms within healthcare environments. It is important to emphasise that even if we come up with an effective solution to eradicate DSB and pathogens from healthcare surfaces, if users are not compliant, or they do not understand the reason for good practice, these solutions will be redundant.

2.2 Aims and Objectives

The main aim of this chapter is to identify user perception of both cleaning and disinfection, including protocols and equipment, used in healthcare. This will be achieved through the following objectives:

- To survey relevant healthcare staff, using an online questionnaire in order to:

  - Assess reported disinfectant product usage within healthcare environments
  - Identify current cleaning and disinfection routine by healthcare professionals
  - Map knowledge of relevant healthcare staff with regards to microorganisms, transmission of infection and disinfection solutions within healthcare settings
- To undertake semi-structured interviews with a purposive sample of healthcare staff to:
  - Gain an in-depth insight into healthcare cleaning and disinfection by further exploration of the topics covered in the survey
  - Allow healthcare professionals to express all their opinions on the topic freely and without judgment
  - Allow for more open questioning on biofilms, specifically dry surface in order to explore the topic in depth and identify any gaps in understanding
  - This work is intended to help in the future by making recommendations to industrial sponsor and influence future training workshops

2.3 Methodology

The study consists of two main aspects: a survey and interviews. A qualitative approach was chosen. Qualitative research seeks to identify behavioral characteristics and understand a phenomenon at greater depth using observational methods such as interviews (Ritchie et al., 2013). Whereas quantitative research employs numbers and very large sample sizes using statistical models to describe phenomena (Stockemer, 2019). A qualitative approach was used as the chapter intended to observe how participants thought, their opinions and perceptions on aspects of cleaning and disinfection to identify and understand the knowledge of healthcare professionals. Qualitative research methods include interviews, participant observation and focus groups. Participant observation and focus groups were not used as they were not deemed appropriate for the data. Participant observation would show current practices but would not allow for the direct analysis of specific areas within cleaning and disinfection and the attitudes and opinions of

healthcare professionals towards this. Focus groups were not chosen as I wanted participants to be able to speak freely and not feel pressured, as they might in a group setting especially as some questions related to knowledge or practices within workplaces which they may not wish to express in front of those from other settings/workplaces. Also, the group dynamics were not needed to uncover the data that was sought. Interviews conducted 1-2-1 work just as well and so the added time and additional complications of setting up focus groups did not feel justified to add more to the research data.

A mixed methods approach was chosen to gain as much information as possible, both broad and more in depth on the topic of cleaning and disinfection which in turn, allows us to pinpoint areas of the research which we felt were important (Almaki, 2016). A survey was used as it is the quickest and most efficient way to gain lots of information over a short period during the first stages of the project (Babbie, 2020). Semi-structured interviews were commenced once the survey had been out for a few months to gain more thorough knowledge from the target audience.

Two approaches were used to complement one another. Whilst the online survey benefits from gaining a lot of data from a lot of people over a wider geographic area, it can lack detailed understanding or exploration into the reasons behind their responses. The interviews therefore provide a follow-up opportunity as they allow for a more in depth look into findings from the survey and gain a richer understanding which has been used to interpret the survey results.

2.3.1 Survey

This section of the project employed the use of a self-complete survey aimed at healthcare workers, particularly those with a role/background in infection prevention and control, in order to assess the opinions and perceptions of those working in healthcare with regards to cleaning and disinfection.

2.3.1.1 Population and Recruitment

The survey was aimed at healthcare professionals, specifically those involved in infection prevention and control. There was no complete list of all people fitting the inclusion criteria (healthcare professionals from all healthcare facilities, only needed to have worked in healthcare at some point), which meant that sample size calculations and probability sampling could not be completed prior to the launch of the survey. As such, the survey was reliant on non-probability convenience and

purposive sampling (Babbie, 2020). Although this did mean that it would be difficult to know when there were enough responses to the survey, it was the only feasible option for this type of open population. In order to target healthcare professionals working in infection, prevention and control (IPC), it was important to use a range of approaches for recruitment. Using social media (Twitter) focussed on specialism groups, retweeting was effectively a form of snowball sampling (Naderifar et al., 2017) and enabled sharing within the IPC community. In addition, IPC conferences and workshops where healthcare professionals were likely to attend provided another route. The processes for recruitment are covered in more detail in 2.3.1.3.

2.3.1.2 Questionnaire Design

A survey was developed based on the research question and relevant literature (refer to appendix 2.1). The questions that were chosen provided a platform to receive as much information on cleaning and disinfection protocol and general knowledge in the workplace. The questionnaire was ensured as fit for purpose due to the nature of the questions and the information that could be gained, it was also checked with the supervisory team. In section A, non-identifiable personal data was requested including job role, demographics and most importantly completed training courses relating to infection prevention and control. Sections B and C focused on daily routine and cleaning and disinfection prevention methods. Information regarding the best intervention methods for spread of infection, the most high-risk areas for transmission of infection and the current methods for measuring cleanliness were asked. Participants were asked questions that would distinguish cleaning from disinfection, which was key to the survey. The final section, D, touched upon the scientific aspect of the survey. It was designed to gain a primary insight into healthcare professionals and their current knowledge of microbes in the healthcare environment.

Closed answer questions were used in all sections for ease of response although the option of 'other' was also used for where an individual's response did not fit the provided options. Open option questions were also added, mainly in section D, to allow people to expand upon their answers. The option of 'not applicable' was also included where necessary as not to alienate participants.

An online survey was chosen as it is easily accessible, as most people today have access to the internet on multiple devices, it is available to all without having to

identify personal details for a postal survey and, it allows for a quick response rate (Dillman et al., 2014). Ease of access and completion is especially important when considering the target audience, healthcare professionals, have extremely busy workdays. It also meant that the survey could easily be accessed worldwide through social media (Twitter®). Online surveys are also easy to fill out and will not get lost. The survey was made anonymous as personal details would not have enhanced the outcome and so this was appropriate from an ethical approval perspective. The survey was formatted using Online Surveys® due to the method for launching the survey and to maximise response.

The survey was reviewed by members of the research team and refined, although not formally piloted as, given the challenges of recruiting, it was important not to use people from the study population who then could not be involved in the final survey.

2.3.1.3 Data Collection

As per 2.3.1.2, two methods were used for recruitment. A tweet was written:

"I am PhD student at Cardiff Uni, conducting a survey to assess attitudes & perceptions to cleaning and disinfection in healthcare. This survey is anonymous and only takes 7 mins to complete! It can be found at: https://cardiff.onlinesurveys.ac.uk/infection-control".

Initially, a gatekeeper (healthcare company) was used to share the tweet, from their primary twitter account in order to maximise the number of relevant people the survey would reach. Anyone who fit the criteria was firstly taken to an information sheet (see below) which led to the online survey. The gatekeeper was needed and chosen as someone who worked in the field of IPC and had many links with colleagues and societies, working in this area.

Conferences identified as relevant, where GAMA Healthcare had a stand were targeted. The information sheet was available in printed form and iPads were available with the survey pre-loaded so participants could quickly and easily fill out the survey. In all cases, potential participants had the opportunity to ask questions before choosing to participate. Implied consent was assumed upon submission of a completed questionnaire, which has proven to work well with online surveys by healthcare professionals (McGowan et al., 2018).

The information sheet (refer to appendix 2.2) was created in order to fully inform participants before completing the survey. This allowed implied informed consent, as they had a choice of whether to complete the survey or not.

The survey was left open for 24 months to allow maximum opportunity for responses, especially as there was no opportunity for follow-ups to non-responders as the population was unknown and submission of the survey was anonymous. Responses were encouraged approximately every 6 months. After 11 months of the survey being open, the SARS-CoV-2 pandemic occurred. As there may be new rules for disinfection procedures and more awareness/knowledge of disease within the IPC community due to the pandemic, it was decided to leave the survey open but to separate pre and post SARS-CoV-2* responses, using the stamp date of the survey prior to analysis.

2.3.1.4 Data Analysis

The data was extracted from the completed surveys (online) into Microsoft Excel® and from there, imported into statistical analysing software (IBM SPSS Statistics v.27®). Appropriate descriptive statistical analyses, which included Chi squared test and frequency analysis were conducted once data had been acquired. Comparative analysis of pre and post SARS-CoV-2* responses based on the sector of work were also conducted on some occasions where it was deemed useful and added value to the results. Post-pandemic was classified as time after and including March 2020, when the UK national lockdown occurred.

2.3.1.5 Ethical consideration

The study was approved by the Cardiff University School of Pharmacy and Pharmaceutical Sciences Research Ethics Committee (application number: 1819-14). Ethical approval was required due to the use of human participants in the study, although there was no risk to participants. No other approvals were required as this survey was not conducted and targeted to the NHS. Data from the survey data will remain anonymous, confidential and no identifiable data will be required.

2.3.2 Interviews

This section of the project employed the use of interviews aimed at healthcare workers, as previously described in the survey section 2.3.1. Interviews were conducted to allow participants to speak freely and answer in more depth than achieved by the survey (Aksu, 2009). The interviews focused on the knowledge of

biofilms, specifically dry surface biofilms within healthcare environments, more so than the survey.

2.3.2.1 Development of Interview Schedule

Interview questions were developed based on the research question and the survey. The semi-structured nature of the interviews allowed flexibility in the interviews, meaning that questions could be asked in a different order from the interview schedule and focus could be put on those responses which were deemed important in relation to the research aims (Brinkmann, 2014). The schedule was used to guide through the interview but not planned to adhere to it rigidly (refer to appendix 2.3). This also meant that particular answers, if appropriate for the research question, could be probed further to explore the participant's answers. Having a schedule was important to facilitate a more natural conversation and enable the interview to be driven more so by the participant so that there was less prompting and leading by the interviewer, whilst covering what mattered to the participant being questioned. The other types of interviews; structured and unstructured were not used as structured interviews provide too much of a rigid schedule without allowing for freedom to further explore responses from participants, and unstructured interviews do not allow for any predetermined questions which means there would be a lack of reliability between interviews and there would be a chance topic areas, important to the research question, might not be covered.

The opening section of the interview schedule gathered non-identifiable information on the participant: their current and previous workplace, and years within the role. The second section of the interview focused on cleaning and disinfection. Questions were asked in more detail than in the survey, gaining information on the situation in the participants workplace, their involvement in cleaning and outbreak situations. The third section of the interview focused on pathogens and their survival in the environment. This was to gain information on whether healthcare professionals know about the surrounding pathogenic threats to their patients. The final section was solely dedicated to biofilms. This included biofilm formation, survival, and dry surface biofilms. The interview questions allowed open-ended answers and unlike the survey, the interviews leaned more towards the scientific aspect of the research. The schedule was reviewed via the research team. No formal pilot was conducted, instead the interview schedule was used with on the first participant and time was

then spent reflecting on how the interview went. The level of detail obtained, the flow of the interview and the understanding gained from asking the participant questions was reflected on to ensure the interview was fit for purpose. This was necessary to highlight anything that could have been missed in the interview that would be pertinent to the research questions and ensured smooth running of following interviews. As a novice interviewer, interviews were reviewed, initially a few cues were missed in the first interview and so this was noted and changed for future interviews. No significant changes were needed so the data from this interview has been included.

2.3.2.2 Population and Recruitment

The interview was aimed at the same population as the survey (section 2.3.1.1). It was important to purposively recruit healthcare professionals with an IPC background to further explore topics covered in the survey. Convenience sampling, a form of non-probability sampling was used as it is quick, effective, and easy to implement. Although sampling was from within a defined sample based on inclusion criteria, participants volunteered themselves to be involved in the study after the interviews were announced, unlike in purposive sampling whereby participants are directly selected by the researcher (Stratton, 2021). Due to the nature of this qualitative research, which focuses more on data than on numbers (Babbie, 2020), it was not necessary to calculate a planned sample size. Typically, analysis is carried out cyclically and data collection continues until no new data appears and theoretical saturation has occurred. Theoretical saturation is found when a researcher has reached the point where no additional data can be added to your research topic and data is considered adequate in relation to the purpose and goal of the research (Faulkner and Trotter, 2017). In this project, theoretical saturation was found when interviews were not revealing any more data to the researcher from what had been mentioned in previous interviews, one more interview was conducted when this was thought to identify if theoretical saturation had been reached. Recruitment was mainly conducted by word of mouth at conferences, however prior to each conference a tweet (below) was sent out by me to make people aware of the interviews. Leaflets were also placed on the GAMA Healthcare stand at such conferences to gauge interest.

"Going to the … conference in … this weekend? I'll be conducting interviews for my PhD regarding cleaning, disinfection and pathogens in healthcare facilities. If you would like to know more, please find me at the GAMA stand! For more info email centeleghei@cardiff.ac.uk"

Unfortunately, due to the COVID-19 pandemic, there were several conference cancellations, meaning it was more difficult to recruit for interviews. Due to this (and following amendment to the Ethics application), a tweet (below) was sent out by February 2021 to invite those who were willing to take part in interviews over Zoom by contacting the researcher directly. The participant information sheet was attached to the tweet, and it was explained that the camera did not have to be on for the duration of the interview.

"Happy New Year! I will be conducting short <30 min interviews over zoom due to the continued cancellation of conferences. If you are interested in cleaning, disinfection & pathogens, or know anyone that might be please contact centeleghei@cardiff.ac.uk for more details"

2.3.2.3 Data Collection

Pre-covid, the researcher attended conferences (IPS Liverpool 2019 and 2021) and had a stand with GAMA Healthcare with leaflets and information about the interviews, including why they were being conducted and the available time slots for interviews. Potential participants who expressed interest in the study were given both an information sheet and consent form prior to the interview (refer to appendix 2.3). There was the opportunity to ask questions before providing informed consent. Consent forms were collected and stored in a locked file cabinet. Interviews were carried out in a private room or a quiet space during conferences to minimise disruption and maintain confidentiality. The interviews were carried out face-to-face either individually or in a group depending on the participant's preference and the time frame in which to conduct the interview. Both single and group interviews were offered to maximise the number of participants. Single interviews can allow for more detail and accuracy whereas group interviews take less time and are considered more of a conversation between participants and the interviewer. One implication

for group interviews is that participants speak less freely in front of others or are influenced by what others say. On the other hand, in single interviews participants may be more nervous in the interview setting. However, interviews conducted were driven by interview preference. After obtaining consent, interviews were guided by the interview schedule and were audio recorded using a digital recorder. The interviews were then transcribed by the interviewer (myself) using intelligent verbatim and the transcriptions were reviewed to remove any identifiable data.

For online interviews during the pandemic and associated restrictions consent forms were sent via email to the participant after they had expressed interest in completing an interview through initial email to myself. As not all have access to scanners, an online signature was acceptable for consent forms. Data was collected via Zoom or telephone call in a private room so that participants could be recorded on loudspeaker through the digital recorder or audio stream. If conducted via Zoom, participants were told prior to the interview that they could either have their camera on or off.

2.3.2.4 Data Analysis

Research is often categorised into four main research paradigms: positivism, realism, interpretivism and pragmatism. Positivism is used in quantitative research, which relies on scientific evidence including controlled experiments. It works only on what can be observed and considers pure data (Alharahsheh and Pius, 2020), which was why it was discounted. Realism accepts the existence of social facts are as real as physical facts, and they are known through a mix of observations and measurements, but knowledge of these facts is objective (Bunge, 2001). Realism also relies little on experimental data and was not deemed appropriate. Although interpretivism and pragmatism share some commonalities, interpretivism was not used for this research. Interpretivism, on the other hand, looks at the beliefs and reasons of individuals in a particular situation to describe social interactions (Nickerson, 2022). Research through an interpretivist manner is shaped widely by the researcher and so, usually the researchers own view, based on personal experience, is brought into play. In interpretivism understanding is seen as a value on its own, whereas pragmatism is concerned with the link between knowledge and action/change (Goldkuhl, 2012), making it appropriate for the research question here and the potential for intervention into new guidance for IPC.

In this thesis the research paradigm pragmatism, was used. Using this standpoint, appropriate qualitative analyses, including open coding, was conducted once data was acquired. Pragmatism is a paradigm that focuses on the premise of what will work best for the research (Morgan, 2014). Pragmatism states that a researcher should use the methodological approach that directly connects to the problem being investigated, where there is an inherent focus on consequences of the research rather than the methods chosen (Kaushik and Walsh, 2019). Using a pragmatist approach, the research conducted could be innovative and dynamic to find answers to the research problems which included frequency of cleaning/disinfecting, who should be cleaning and what, and how much was known around biofilms.

Thematic analysis was used to analyse interview data which allowed the researcher to understand what phenomena are mentioned frequently and in depth to allow connections throughout the themes. Thematic analysis was based upon the process outlined by Braun and Clarke (2006). Initially the researcher familiarised themselves with the data, allowing the generation of initial codes before identifying and reviewing themes. Once these had been found key themes were produced and named. Transcripts were reviewed, coded, and developed into themes deductively. A deductive approach was used as this allowed the researcher to start off with generalisations, then produce more specifics observations from the research (Hyde, 2000). Included in this was the use of pragmatism to outline key themes throughout the interviews. Two approaches were used throughout analysis. Both conventional and summative content analyses were used to directly derive codes from the interview text, identify keywords/content and count these in order to interpret the overall themes (Hsieh and Shannon, 2005). Constant comparison was also used to review codes between interviews throughout analysis allowing for comparison with existing codes that have arisen throughout analysis (Lewis-Beck et al. 2004). Once codes had been generated, themes were generated that encompassed codes of a similar standpoint. To assure quality analysis, one interview was coded by both the main researcher and a supervisor to sense check the coding and ensure both were in agreement. Where appropriate, illustrative quotes were used to enhance the theme and point of view from the interviewees.

Trustworthiness was also considered to establish the credibility, transferability, dependability and confirmability of the research as described by Lincoln and Guba

(1985). Trustworthiness has been shown through the detailed description and full disclosure of the methods used in interview analysis, the precise and consistent analysis through recording and thematic analysis used. Analysis was also confirmed by a supervisor to ensure credibility and enhance trustworthiness.

2.3.2.5 Ethical consideration

Ethical approval was sought as described in section 2.3.1.5. The study was approved by the Cardiff University School of Pharmacy and Pharmaceutical Sciences Research Ethics Committee (application number: 1819-27). Although due to the nature of the method the data for the could not be completely anonymous confidentiality was maintained throughout the process. Recorders used for interviews were stored in a locked drawer and any recording was destroyed after the transcripts were edited to remove any identifiable data. Audio streams from any Zoom interviews were saved to a password protected computer then deleted after transcription. Data was stored on a password protected computer and consent forms were kept in a safe, locked place.

2.4 Research Findings

2.4.1 Survey

2.4.1.1 Response rate and demographic data

A total of 137 healthcare professionals completed the online survey, of these 120 were completed pre-pandemic and 17 were completed post-pandemic. Out of a total of 137 participants, the majority (70.6%) worked in a hospital (**Figure 2.1**). Of these, a large proportion of participants post covid (n = 12) worked in a hospital.

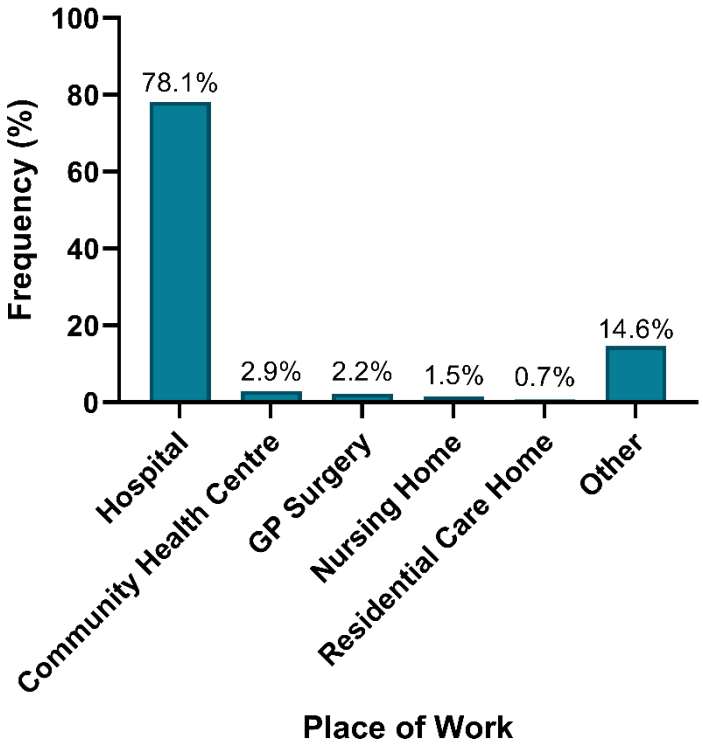


Figure 2.1. Participant response to place of work when completing the survey, including the frequency of each choice (n = 137).

When asked of their job title, there were multiple different responses. To analyse the data, each title was added into a broader job role. For example, if the response was IPC nurse this was added into the job role "nurse". All responses were grouped into 8 categories: doctor, nurse, midwife, consultant, clinical scientist, pharmacist, academic and other. Out of the 137 responses, one person did not respond to job title or country of residence, so they have been taken out of these responses. The number of respondents in each category is outlined in Table 2.1. Just under half (47.1%) of participants following the pandemic were nurses, and just over half pre pandemic (52.5%).

Table 2.1. Job role of each participant from the survey given as number both pre- and post-pandemic (n = 136).

Job Role	Number Pre-Pandemic (n = 119)	Number Post Pandemic (n = 17)
Nurse	63	8
Doctor	11	-
Midwife	1	-
Consultant	6	1
Clinical Scientist	5	-
Pharmacist	2	-
Academic	2	-
Other*	25	12

*Other comprises microbiologist, virologist, managers (risk and deputy), theatre co-ordinator, care co-ordinator, housekeeping supervisor, medical advisor, and operating department practitioner

When asked of their country of residence, the majority (66.4%) participants were from the UK, this included Scotland, Northern Ireland, England, and Wales. Responses came from all over the world, with representation from 5 continents: Europe, North America, Africa, Australia and Asia (**Figure 2.2**). One person provided a location that wasn't recognisable and so has been excluded from this response, as was the individual who did not provide an answer to the question. Most participants were from Europe, this included: UK, Spain, Finland, Denmark and France.

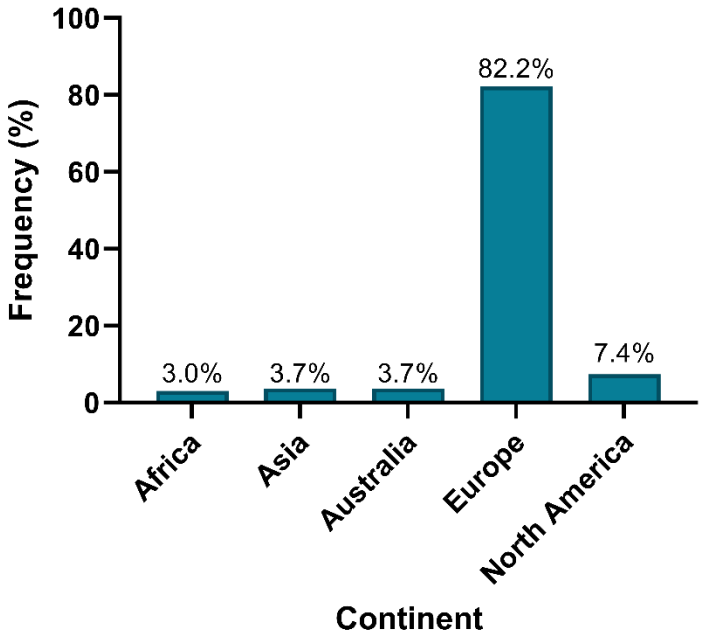


Figure 2.2. Location of each respondent grouped into continent location (n = 135).

The two final "about you" questions looked to identify what the participants main area of practice is and for how long each participant had been working in their field. From 137 participants, 84 of these (61.3%) work in infection prevention and control (IPC) (**Figure 2.3**). A total of 70/84 responses were pre-pandemic. A total of 82.4% of participants post-pandemic work in IPC. However, this figure is most likely due to the target audience and conference attendances. Following those that responded "other" (19%), the most common area of healthcare from all responses was surgery (4.4%). Those responding with other included areas such as palliative end of care, microbiology and radiology. Every area that was depicted in the survey had at least 1 response.

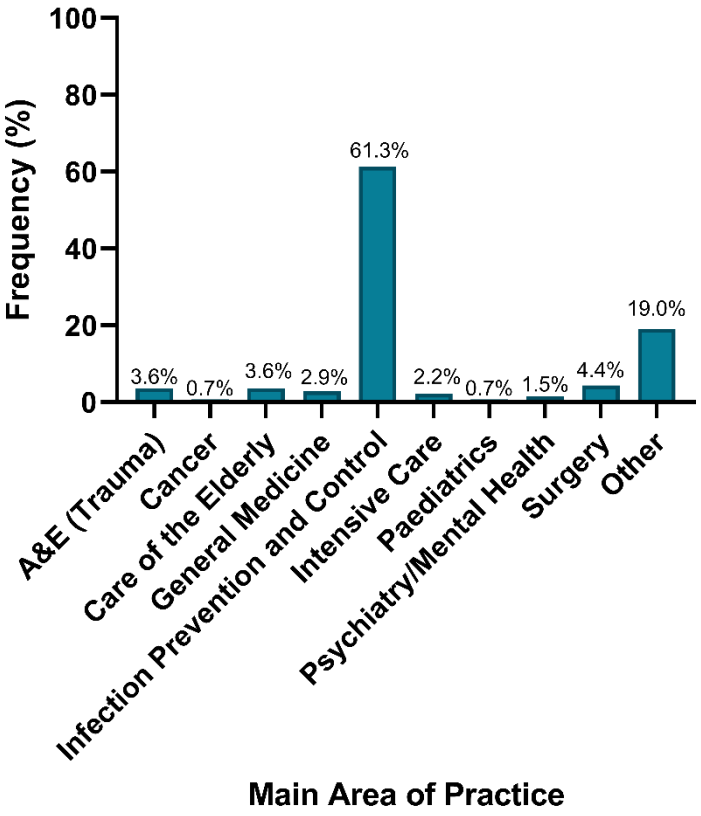


Figure 2.3. Frequency of each area of practice that was mentioned in the survey (n = 137).

Length of service resulted in the most even spread of data from all demographic data (**Figure 2.4**). There was not much variation between the categories, the most frequently chosen answer saw participants that had only been working in their subsequent field for 1-5 years (30.7%). The second most common was the complete opposite, 21+ years (24.8%). Of the 137 responses, 94 said they have received specific training qualifications relating to IPC, of these 56.4% were higher education certificates.

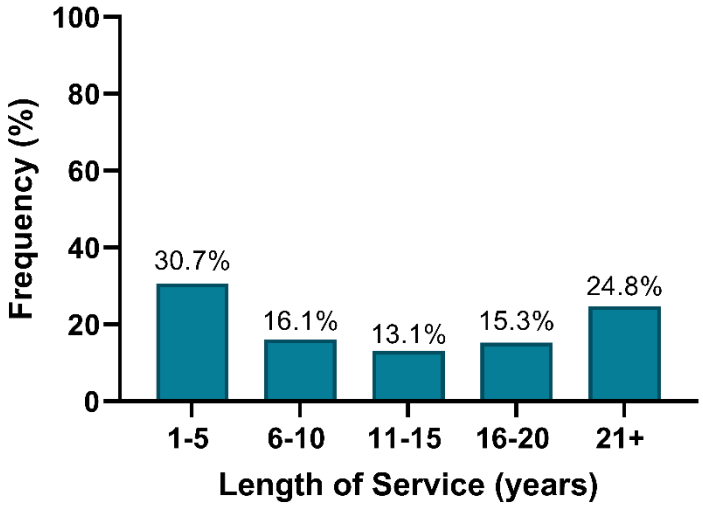


Figure 2.4. Length of service of each respondent in their respective field, displayed in years (n = 137).

2.4.1.2 Cleaning and Disinfection

To understand cleaning and disinfection routine, participants were asked how many times a day they felt hospital surfaces (including- desks, bedside tables etc) should be cleaned or disinfected. Most participants pre-pandemic believed that hospital surfaces should be disinfected between every patient, however post-pandemic the highest response was twice a day (**Figure 2.5a&b**). Pre-pandemic data showed a much more even spread over the four categories in response to cleaning every day. Both between once a day and twice a day were the most popular answers (**Figure 2.5a**), however post pandemic there was much more variation in responses. As different healthcare environments have different guidelines and different severities of patients with infectious diseases, we wanted to consider the relationship between the area of work and how often a surface should be cleaned/disinfected. Overall, considering all 137 participants' responses there was no statistically significant difference between the area of work (i.e., hospital etc) and the number of times a surface should be cleaned, X^2 (15, N = 137) = 23.284, $p > 0.05$, or disinfected X^2 (15, N = 137) = 10.436, $p > 0.05$. The National Standards of Cleanliness (NHS, 2021) state that the frequency of cleaning does depend on the healthcare

organisation in order to meet each facility's needs, however the baseline for cleaning surfaces when they are being used falls into the daily cleaning category, especially those touchpoint areas.

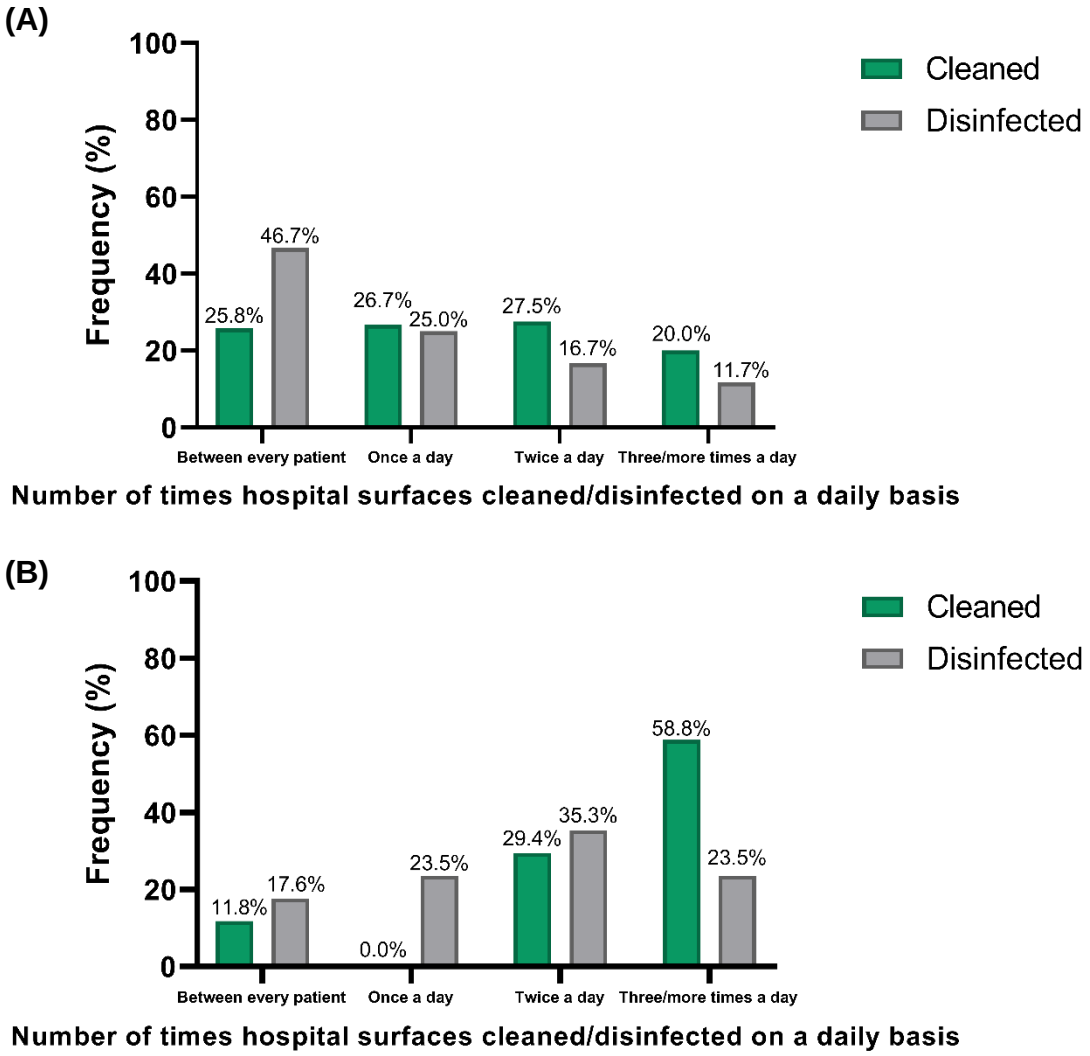


Figure 2.5. Response data pre- and post-pandemic to how often healthcare professionals believe hospitals surfaces should be cleaned or disinfected on a daily basis, pre-pandemic (A) (n = 120) and post-pandemic (B) (n = 17).

Participants were given the option of varying methods to deliver the best infection prevention to a contaminated area. These contained a mix of cleaning and disinfection, and methods to deliver these such as automated machinery, single use

wipes and liquid-based products (**Table 2.2**). Interestingly, both pre- and post-pandemic highest responses were: "Cleaning followed by automated disinfection" and "Cleaning followed by liquid-based disinfection". Post-pandemic there were fewer participants in favour of "Automated disinfection methods only and cleaning only", which saw no responses. There were a higher percentage of participants that thought "Single use loaded wipes only" were the best method for IPC. Those few who responded with "Other" (n = 2) mentioned it depended on what the equipment and environment was, and community deep clean.

Table 2.2. Percentage of both pre- and post-pandemic responses to what participants believe to be the best method for IPC in a contaminated environment.

Best method	Frequency (%)	
	Pre-pandemic (n = 120)	Post-pandemic (n = 17)
Automated disinfectant methods only (e.g., UV light, hydrogen peroxide)	12.5	5.9
Cleaning followed by automated disinfection	37.5	35.3
Cleaning followed by liquid-based disinfection	33.3	35.3
Cleaning only (detergent)	1.7	0
Liquid based disinfectants only	0	5.9
Single use loaded wipes only (antibacterial, sporicidal)	14.2	11.8
Other*	0.8	5.9

*Other included community deep clean and best method is dependent on the environment and where the contamination is located

Survival time of microorganisms is essential for companies to consider when creating products for cleaning/disinfection in healthcare environments. It is also necessary to understand survival times so that outbreaks can be dealt with effectively and help everyday cleaning and disinfection protocols. When asked

about survival of the 4 groups of microorganisms (bacteria, viruses, fungi and spores) there were only a couple of respondents who answered, "Don't know". Response to spore survival was much more conclusive than the other three microorganisms, where most believe that spores can survive on surfaces for long periods of time (months and years) (**Figure 2.6, Table 2.3**). Response to bacterial and viral survival was much more spread, but it was evident that very few respondents believe they can survive for extended periods (years). Overall, participants believe that spores can survive the longest in the environment, followed by fungi, bacteria and finally viruses (**Figure 2.6, Table 2.3**). When looking at both pre- and post-pandemic data it is interesting to see that over 50% of respondent's post-pandemic think that viruses can survive on surfaces for days, whereas this was lower pre-pandemic, at 30%. However, there was no statistically significant difference between pre or post pandemic responses and survival time of viruses in the environment, X^2 (6, N = 137) = 5.604, $p > 0.05$.

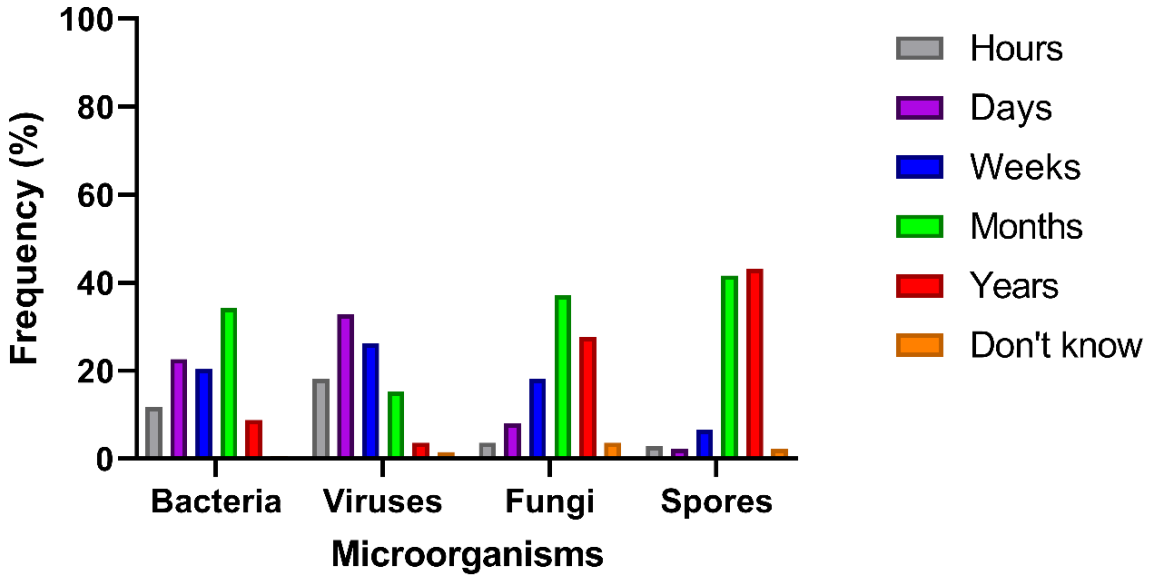


Figure 2.6. Frequency (%) of all participants response to survival time of microorganisms on environmental surfaces (n = 137).

Table 2.3. Frequency (%) of all participants response to survival of microorganisms in the environment (n = 137).

Length of survival	Microorganism (frequency of choice %)			
	Bacteria	Viruses	Fungi	Spores
Hours	11.7	18.2	3.6	2.9
Days	22.6	32.8	8.0	2.2
Weeks	20.4	26.3	18.2	6.6
Months	34.3	15.3	37.2	41.6
Years	8.8	3.6	27.7	43.1
Don't know	0.7	1.5	3.6	2.2

2.4.1.3 Daily Routine

Methods for prevention of colonisation of microorganisms are important in healthcare settings. Interventions can be implemented in order to prevent microbial contamination. Participants were given a choice of four different intervention methods and asked which they felt had the greatest impact on IPC (**Figure 2.7**). It was unanimous that hand hygiene is believed to be the best intervention method, with 88.3% of all 137 participants selecting this (**Figure 2.7**). Vaccinations were least popular with only 1.5% response rate. Pre-pandemic data showed that a large 89.2% of respondents thought hand hygiene had the greatest impact. This percentage decreased slightly to 82.4% post-pandemic and we saw an increase in surface cleaning and disinfection, where response rate almost quadrupled from pre- to post-pandemic. Those who selected "Other" mentioned that there is not just one intervention method, it is a combination of all (n = 2) and care of invasive devices following by cleaning/disinfection (n = 1).

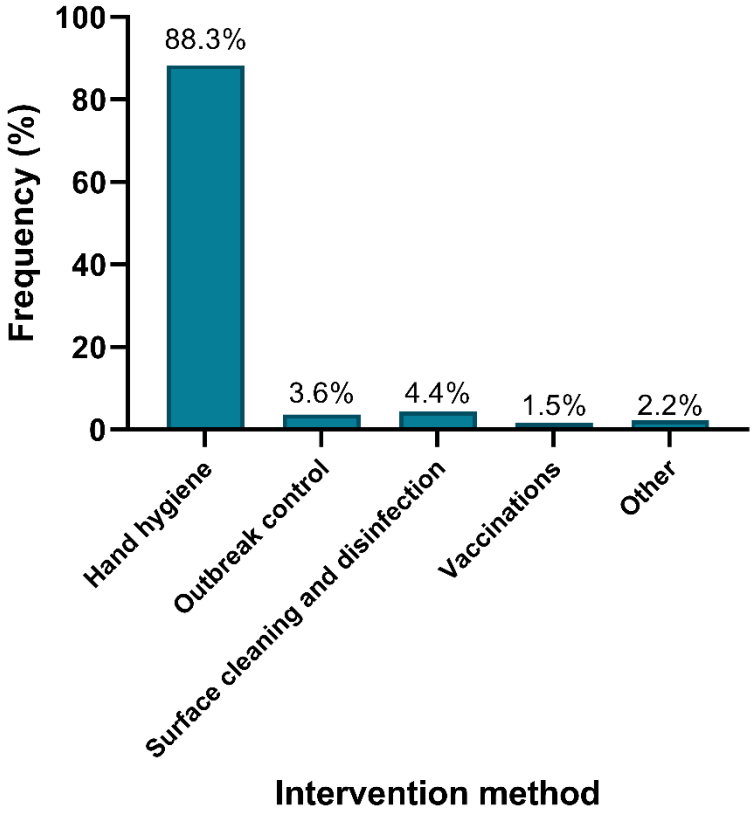


Figure 2.7. Frequency (%) of participants response to which intervention method they believe has the greatest impact on IPC (n = 137).

Measuring microbial contamination of surfaces is important to use alongside cleaning/disinfectant methods to ensure an area is clean and ready. The main choices for measuring cleanliness are Adenosine Triphosphate (ATP) markers, Ultraviolet (UV) light, culture swabs of microorganisms present on surfaces, indicator products (stickers, tapes) or visibly an area looks clean. These were given as options to participants alongside "Other", and all were asked to choose all that they thought applied to the question of which methods are best for measuring cleanliness. A total of 48.1% of 135 participants chose multiple methods. Out of the 51.9% that only chose a single method, culture swab was the most common answer (20.4%). The two participants that chose "other" were excluded from this as they one individual believes you cannot measure cleanliness, and the other said it varies on the task.

When asked specifically which single method is best for measuring cleanliness, culture swab was the most popular answer, 38.3% of all 133 participants chose this (**Figure 2.8**). The answer with the lowest response was indicator products (5.3%) (**Figure 2.8**). Again, the same two individuals who chose "Other" were excluded as they gave the same reasons as previously mentioned. Interestingly, there were two more participants who chose "Other" as an option, they stated that chlorine-based disinfectant and rates of infection were the single most important measures of cleanliness. They have also been excluded from whole responses.

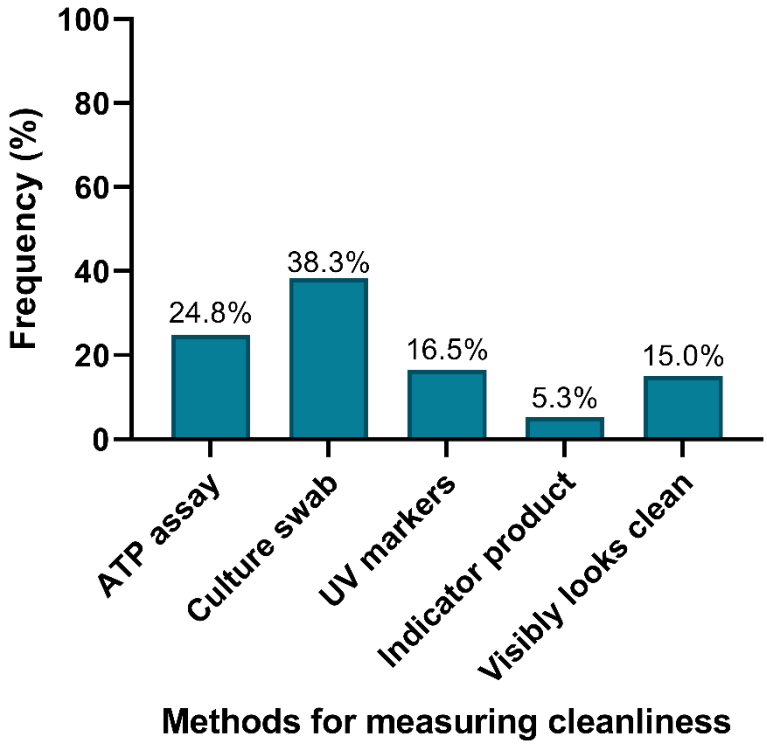


Figure 2.8. Frequency (%) of participants who chose each option for what they believe was the single most important method for measuring cleanliness (n = 137).

There are multiple areas in a hospital where there is a risk for transmission and spread of infectious diseases. Using low (green), medium (orange) and high (red) risk, participants were asked to put each area into a category as to what they believe the risk of transmission of pathogens in these areas is (**Table 2.4**). Most areas were categorised as high risk by the majority of participants (> 55%), with the exception of nurse station which received 45.2% medium risk and 45.9% high risk. Only café, clean utility, floors and television were categorised as low risk areas (**Table 2.4**). Clean utility is categorised as the area for storage of clean supplies like disposable bedpans. Although much like nurse station, clean utility received 45.1% of votes for low risk and 43.6% for medium risk. Outpatient area and curtains were seen as the only medium risk areas, receiving 43.7% and 50.7% of responses respectively. Patient area was the only environment that did not receive any votes for low risk and saw majority (76.5%) high risk. Although door handle was seen the most high-risk area with 77.6% of participants classifying door handles as this.

Table 2.4. Areas of a hospital and their risk of transmission as rated by participants. Green indicates low risk; orange indicates medium risk and red indicates high risk.

Area	Modal level for risk of transmission	Response for risk of transmission (%)		
		Low	Medium	High
Café		42.1	25.6	32.3
Clean Utility		45.1	43.6	12.0
Television		50.0	35.1	17.2
Floor		54.1	26.3	21.1
Outpatient Area		21.5	43.7	35.6
Curtains		21.6	50.7	28.4
Nurse Station		8.9	45.2	45.9
Patient Side Room		9.6	33.8	56.6
Patient Table		6.0	35.3	59.4
Light Switch		8.0	30.1	63.9
Keyboard		8.1	27.4	64.4
Mattress		9.0	26.1	65.7
Sink		5.2	28.4	66.4
Sluice Rooms		6.0	26.7	71.1
Bed Rails		3.0	26.5	71.3
Call Button		4.5	23.1	73.1
Patient Bathroom		5.0	21.5	76.3
Patient Area		0.0	32.0	76.5
Door Handle		2.2	20.9	77.6

2.4.1.4 Knowledge of Biofilms

The last section of the survey looked to identify current knowledge on biofilms. A total of 87.6% of all participants had heard of the term biofilm, and of these, 83.9% knew what the term biofilm means. Of these, a greater number of participants post-pandemic (94.1%) had heard the term biofilm compared to that pre-pandemic (86.7%). The same can be said for knowledge of the meaning of the term biofilm, where 88.2% of participants post-pandemic understood the term, whereas 83.3% did pre-pandemic. There was very little uncertainty over the term biofilm as 4.4% of all participants are not sure if they had heard the term before and 6.6% were not sure they know what a biofilm is.

Pearsons Chi-Square Test was used to identify if there was any relationship between years of experience (length of time working as a healthcare professional) and knowledge of the term biofilm. There was no statistical significance, X^2 (8, N = 137) = 9.066, $p > 0.05$, between years of experience and having heard the term biofilm, suggesting that these two factors are not associated with one another.

We also wanted to investigate the relationship between higher education and knowledge/understanding of the term biofilm. There was a statistically significant difference between higher education and knowledge of the term biofilm, X^2 (6, N = 137) = 148.864, $p < 0.001$. Post-hoc z-test and Bonferroni correction with adjusted p value of 0.05 outlined the statistically significant difference arose between those that hold a higher education certificate and those that don't in response to both questions and each answer choice ($p < 0.05$). 100% of those that hold a higher education certificate answered yes to hearing the term biofilm, whereas 79.8% of those who do not hold one answered yes. When we compare this to knowledge of the term biofilm, results are similar, 96.2% of those that hold a higher education certificate know the term and 76.2% of those who don't hold a higher education certificate know the term biofilm.

Conference/study days were the main source where all participants have found out about biofilms (60%) (**Figure 2.9**). This was followed by scientific journals (42.6%) and talking to other colleagues (36.5%). Talking to a company rep was the place from where the least amount of information is gained (6.1%). A total of 10/13 participants who said "Other" attained their information from a university degree, both bachelor's and Postgraduate were mentioned. Only one participant mentioned

that their own research was how they gained information on biofilms. Those participants who had answered the previous question with "No" or "Not sure" did not respond to this question. Although conference/study days was the most frequently answered, 55.7% of participants chose more than one option.

There are multiple types of biofilms in the healthcare environment both relating to invasive devices (Kavanagh et al., 2021) and both surfaces and general equipment (Vickery, 2019). Here, we asked participants which types of biofilms they were aware of in relation to health and asked them to indicate each biofilm they have heard of. Dry surface biofilms were the least well known of all mentioned, less than half (39.1%) of participants had heard the term. Medical device (86.1%) and drain biofilm (76.5%) were unsurprisingly, the most common answers. The vast majority of participants, 90.4% mentioned more than one biofilm in relation to those that they had come across before. Those who chose "Other" indicated biofilms on implants, and one participant said that biofilms will form anywhere and so did not make a choice. Again, as previously, those participants who did not know or had not heard the term biofilm or were unsure did not respond to this question.

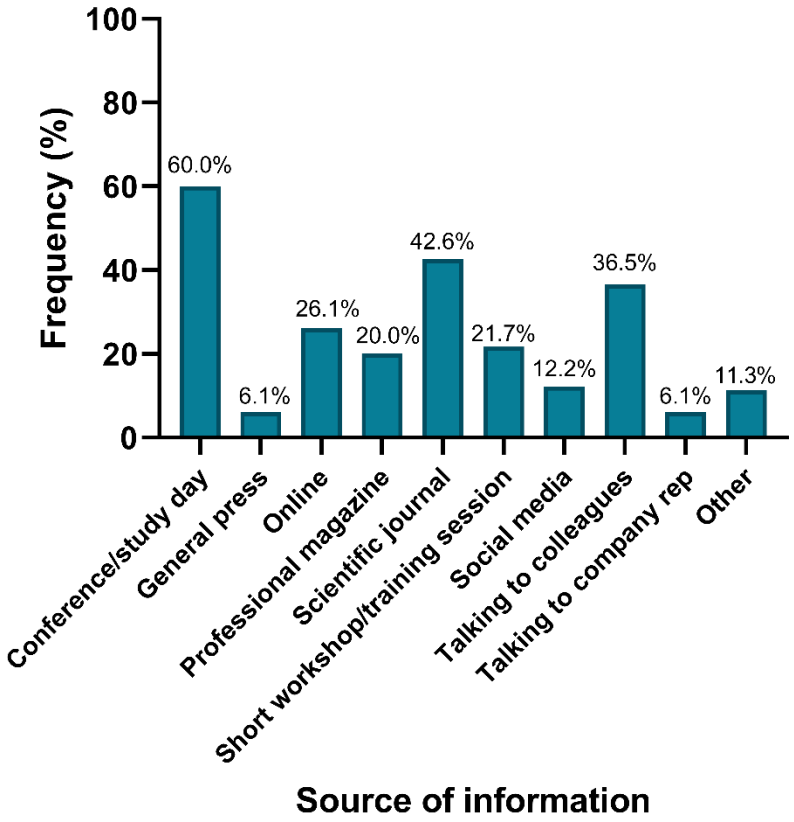


Figure 2.9. Percentage of participants that chose each information source for their knowledge of microbial biofilms. Respondents could select > 1 option, so % add up to more than 100%.

2.4.2 Interviews

2.4.2.1 Demographic data

In total, 10 interviews were conducted over two conferences. All interviews were face-to-face as there was no response to invitations to be interviewed over Zoom. Participants were asked of their current and previous job roles, and the healthcare setting in which they currently and previously worked in (**Table 2.5**). Most (n=8) participants had worked in a hospital previously, but 3 of these no longer worked in a hospital setting.

Table 2.5. Demographic of Interviewees, indicating current and previous job roles and also current and previous place of work.

Interview no.	Pre/Post Pandemic	Previous Job Role	Current Job Role	Previous Place of Work	Current Place of Work
1	Pre	Senior IPC nurse	CQC Manager	Community and Acute Trusts	Nursing Home
2	Pre	Senior IPC Nurse	Manager	Acute Trust	Community Trust
3	Pre	(Same as current)	Lead Nurse	(Same as current)	Hospital
4	Pre	(Same as current)	IPC Nurse	(Same as current)	Hospital
5	Pre	Doctor (Medical Microbiologist)	Manager	Hospital	Office
6	Post	Haematology Nurse	Clinical Educator	Hospital	Hospital
7	Post	Clinical trainer and Nurse	Clinical Educator	Hospital and Community Trust	Hospital
8	Post	IPC Nurse	Head of IPC	Hospital	Community and Acute Trust
9	Post	(Same as current)	IPC practitioner	(Same as current)	Hospital and Community Trust
10	Post	Clinical Director and IPC Nurse	Consultant Practitioner (IPC)	Hospitals and Combined Facilities	Office

2.4.2.2 Content analysis

Before considering themes of the interviews, general questions based around surface cleaning and disinfection were asked to gain a better understanding of the practices going on in the healthcare environment. The first question sought to ask about pathogen surface tests and if any were used in their place of work. Interestingly, when considering current procedures, most (n=7) participants mentioned that they either do not use any form of surface test, or, they have trialled but do not routine used surface tests (**Table 2.6**). Of the surface tests mentioned,

ATP occurred the most (7/10). Interestingly, one point that arose from interviews 7 and 8 is that sampling the surface for pathogens tends to be used as a teaching tool. By using sampling methods, they can assure an area is clean and teach staff the importance of cleaning an area correctly.

Table 2.6. Usage and type of surface tests in healthcare environments.

Interview no.	Usage of Sampling Methods	Type of Test Used
1	Yes	UV and ATP
2	None currently but have done in the past	ATP
3	None	None
4	None	None
5	Occasionally	ATP
6	None	None
7	None	None
8	Yes but circumstantial	UV and ATP
9	Occasionally	N/A
10	None currently but have done in the past	ATP

Interviewees were asked how they would wipe a surface if required to clean, all 10 participants mentioned the "S Shape" method as described by Dancer and Kramer (2019). All participants, except one, also highlighted the fact that they did have knowledge of the length of survival of different types of microorganisms and that survival is dependent on the type of pathogen. Although this was frequently mentioned, only 6/10 could give examples of pathogens and survival times. Specifically, *Clostridioides difficile* was mentioned most often (5 participants) followed by spores as long-term survivors.

Interviewees were asked if they participated in cleaning of their work area, 3/10 stated that they do not and have never done cleaning (interviews 3,4 and 5). Often mentioned was the fact that if something either looked dirty, or there was an outbreak situation, they would intervene and help with cleaning. It was mentioned

that nurses often have specific areas to clean and would always take care of their own equipment before and after use on a patient. When asked what their opinion on the most important areas to be cleaned and disinfected and the best intervention methods to stop the spread of bacteria in hospitals, high touch points and hand hygiene came up most frequently (**Figures 2.10. & 2.11.**). No participant mentioned more than one intervention method, however two participants mentioned everywhere.

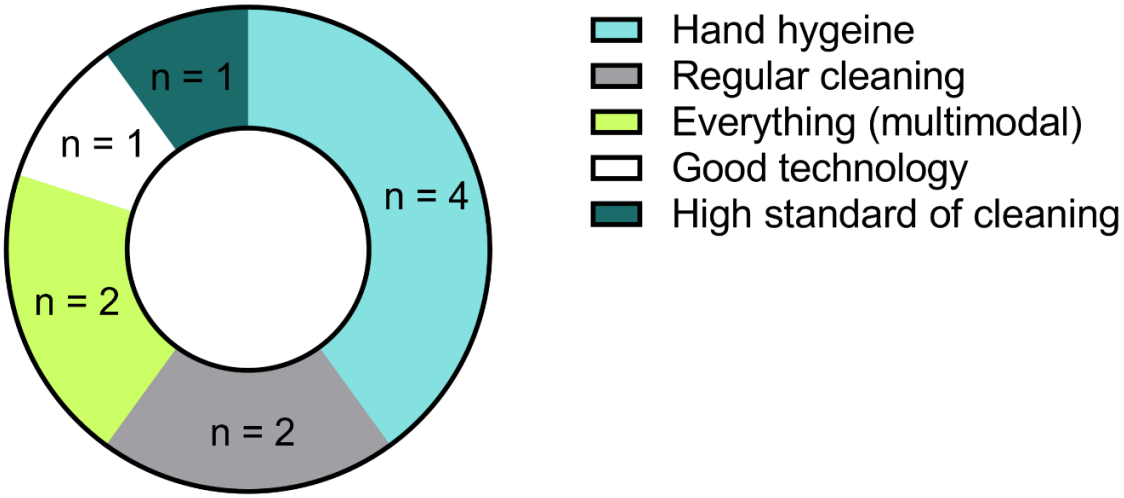


Figure 2.10. Frequency chart of each intervention method that arose during interviews (n = 10).

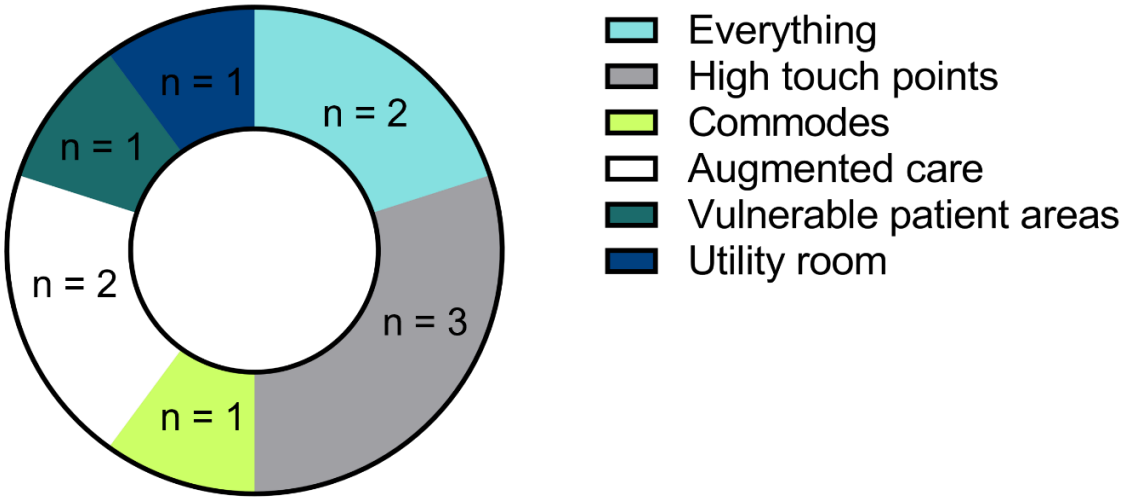


Figure 2.11. Frequency chart of each area that participants believe to be the most important areas to be cleaned and disinfected most regularly (n = 10).

Every participant questioned confirmed they knew what a biofilm was. Those who have been or are still currently nurses first heard the term when on training courses for wound care or water safety. It appears that most participants have been taught about biofilms either through their degree or their job role. When asked about DSB specifically, 9/10 participants had heard the term before. A total of 6 participants had heard the term through conference talks or published material. Most participants were very vague on their knowledge of DSB and had only heard the term briefly quite recently, mentioning that ***"when we think of biofilms we think of areas that are moist not dry surfaces* (interviewee 2)*"***. Participants mentioned that although they had heard the term, other colleagues in their workplace would not know what DSB are.

2.4.2.3 Themes identified from healthcare professional interviews

This study sought to investigate the opinions of healthcare professionals on cleaning and disinfection routine in the hospital environment, as well as general knowledge of pathogens and their risk to patients. Six themes and 18 sub-themes were identified, written in Table 2.7 with illustrative quotes to expand on each sub-theme.

Table 2.7. Thematic analysis of interview data, including a total of 6 themes and 18 sub-themes.

No.	Theme	Sub-themes
1	Reactive judgement	Pandemic has led to compliance and more attention to detail
		Risk leads to vigilance
2	Time limitations	Staff have other work to do before cleaning
		Patient environment is distracting
		Sampling surfaces is time consuming for labs
		Delivery of courses in small time frames
3	Cost limitations	Sampling surfaces is costly
		Wages not sufficient
4	Barriers that can be controlled	Language and terminology
		Access to courses and policy documentation
5	Barriers that cannot be controlled	Poor infrastructure and upkeep of estates
		Outside company providing domestic services
		Lack of interest and engagement
6	Understanding and knowledge	Access to journals limited to certain individuals
		Lack of training
		Subject interest leads to finding out more on your own
		Importance of cleaning is overlooked

Theme 1: Reactive Judgement

Interviews explored views on how outbreak situation were handled and general participation of cleaning in their area. It was evident that outbreaks called for a different judgement and different response from workers. Many participants mentioned that workers become more vigilant and compliant in outbreak situations and must react quickly to solve the problem as fast as possible. Vigilance comes when **"*there is suddenly some sort of threat and they and see what the risk is to them* (interviewee 2)"**. However, this has led to the argument of reactive versus

proactive cleaning; when an outbreak is over, do people then resort back to old habits.

The pandemic was mentioned in 4 out of the 5 interviews conducted after 2020. This highlighted the fact that since then, not only have staff taken more care with cleaning and have focused on areas that may lead to transmission of COVID-19, mainly high touchpoints, **"*through the pandemic there was a huge push on all the touchpoint cleaning which should have been a priority beforehand anyway and it wasn't* (interviewee 6)"**. Also mentioned was the fact that companies have started providing more detail on the correct use of their products ensuring appropriate use of cleaning/disinfectants. It is clear the pandemic has highlighted problems that may have been frequently occurring in hospitals, but went relatively unnoticed, **"*…the pandemic brought this to the forefront (not touching things unnecessarily) staff often let this slip* (interviewee 6)".**

Theme 2: Time Limitations

When talking about daily routine and the cleaning situation in their area, one common theme that came up was time limitations. People working on the ward are often very busy, some participants mentioned that domestic areas that are meant to be cleaned by nurses were skipped due to high workflow, **"*it was hit and miss, some people cleaned well but some people were very busy so it wasn't always done as well as it could have been* (interviewee 7)"**. When you are time limited, cleaning can be a rushed job. This leads to either areas getting missed, or healthcare assistants/students cleaning these areas who may not be fully trained. Interestingly, it was mentioned that if you did have time to clean this showed compassion and reflected patient care. Most participants mentioned that in their place of work, domestic housekeeping staff were hired to clean certain areas. One time constraint of this was they worked limited hours, and so if you needed them after this time you would have to wait till the following day in order to resolve a situation.

When discussing sampling the surface for pathogens, as previously mentioned, no-one produces environmental samples to check an area is free of microorganisms. There were two main reasons for this, one which is connected to the next theme, these are 1) labs at hospitals do not have the time to process environmental samples

as they are so busy already and 2) processing is costly and the money is required elsewhere… **"*the problem is it takes a while to get the results* (interviewee 9)"** and, **"*our lab doesn't process environmental swabs because they just don't have the time to do it* (interviewee 4)"**.

Theme 3: Cost Limitations

Cost limitations have some overlap with theme 2 (time limitations). As mentioned above, environmental sampling the surface for pathogens is needed but comes at a cost **"*…we do believe ATP would be a good thing but the cost is an issue* (interviewee 5)"**. Although this shows that staff do believe sampling the surface would be beneficial. As well as this, domestic housekeeping staff are often quite poorly paid and so the job is not appealing for many, **"*…there is always a shortage of staff as they come and go. It's a very poorly paid job* (interviewee 3)"**. This in turn, leads to staff shortages having a detrimental effect on cleaning of healthcare facilities. One way of overcoming these problems is providing new technologies which are self-sufficient and can alleviate the problems of time and staff shortages. However, these are also expensive and require upkeep and maintenance with added costs.

Theme 4: Barriers that can be Controlled

Throughout all interviews it was apparent that there is a general sense of confusion throughout hospital environments over who is cleaning what, **"*…looking back there was a lack of appreciation of the importance as a clinical member of staff around cleaning. I think there were grey zones of areas of responsibility* (interviewee 8)"**. As domestic staff from external companies are used for cleaning there are guidelines to what they are required to clean. However, it was stated that in some cases this is not quite clear, especially when one piece of equipment is cleaned by two types of staff, **"*…we have a split role, so the mattress and the bed rails would be the responsibility of the nurses to clean but underneath of the beds and the mechanisms would be down to the domestic services to clean* (interviewee 2)"**.

Language and terminology are another barrier that arose. Even the term "clean" is defined differently in separate hospitals within the same trust, **"*there are different***

levels of cleaning, there is a lot of confusion over terminology **(interviewee 9)"**. Not only our definitions different, but within national standards there are several words used for one term, creating further confusion. Language and terminology can be improved through communication, more detailed policy documentation and educational courses as there are evidently gaps in knowledge, **"*I think there is also in both community and acute in healthcare there is a lack of training and knowledge on why they are doing what they are doing*** **(interviewee 1)"**. By giving staff the opportunity to attend teaching courses, they will have an up to date understanding of the current terminologies. By keeping policy documentation updated and easy to access for all, confusion over language used and general protocols for cleaning can be reduced.

Theme 5: Barriers that cannot be Controlled

The poor upkeep of estates falls both under this theme and theme 3. Generally poor infrastructure is a cost limitation and not within the control of nurses and other staff members. It is essential that moving forward to the future, expert input is required when building new facilities so that surfaces can be easily cleaned but this is often missed. If surfaces are not in good condition, they cannot be cleaned effectively, here the estate is contributing to infection spread throughout hospitals**, *"… with the infrastructure starting to crumble which gives the organisms opportunities and even if you are cleaning you aren't able to clean as well*** **(interviewee 5)"**. No matter how much cleaning you do to a high standard, the environment does not lend itself to be clean.

By having an external company providing cleaning services, there is a barrier in communication. Unfortunately, this cannot be controlled unless an internal company is used but this is not always possible. This leads to confusion over cleaning protocols, and means should a problem arise it is hard for it to be sorted efficiently and effectively.

Although to some extent lack of interest can be increased to actively engage staff members, it is difficult. It seems it is even difficult to engage those who are commenting on policies and documentation in the workplace, **"*I think the whole science of cleaning needs to be addressed… there's a lack of interest***

(interviewee 10)". If workers are not interested then how, in the future, are we meant to progress engaging staff who are busy with everyday problems.

Theme 6: Understanding and knowledge

Access to publications, although not required, provides direct knowledge from experts on the current climate and science around cleaning and disinfection. Often this means that only those at senior level have a greater understanding of what is happening in the environment. Not having access to publications means that there is a lack of understanding of pathogen survival in the environment, which is essential to identify if an area is clean and understand why you are doing what you are doing, **"*when nurses get to a senior level and more so in an IP role, they know about how long pathogens will last in the environment* (interviewee 1)"**. Also, the point was made that a lack of effort from staff leads to gaps in knowledge. If you are interested in a subject, you will seek to find out more in-depth information gaining a greater knowledge and understanding, **"*…I think that's because of my interest. When it comes to the cleanliness in the environment because for any IPCA it is about that level of cleanliness…* (interviewee 1)"**. Training courses also provide important information on how to use products. Although all participants understood the "S-shape" motion when cleaning, many mentioned the fact that staff will simply scrunch a bunch of wipes together to clean a surface as they think the soap suds mean they will be more effective. It was also apparent that staff do not know what is in each product and what each one should be used for, **"*lots of misunderstanding, some people think Clinell is a detergent wipe* (interviewee 10)"**. Without proper understanding of how and why you should be using products a certain way, staff will continue with bad habits.

Understanding and knowledge became apparent when questioned on biofilms, specifically DSB. Most participants were senior in their field and had knowledge of biofilms, their risk to patients and DSB. The majority found had this information from conferences, again like publications only a select few have access to conference attendances, so only those with access have a greater understanding and knowledge.

The importance of cleaning is often overlooked in healthcare environments. Frequently, participants mentioned that sampling should be a requirement as it is

an excellent training tool for staff members to fully understand whether a surface is clean or not. **"*Nothing matters unless its personal* (interview 9)"** … many interviewees stated that through visual aids such as sampling, staff would learn how to effectively clean in the long term and the risk this puts on patients if cleaning is not followed correctly. Linked to theme 1, a reaction can also cause an increased knowledge of cleaning in the environment. One participant mentioned an outbreak of *Pseudomonas* spp. in a neonatal unit they were working on. In order to understand where the infection risk was coming from, they ended up learning more about biofilms and cross contamination.

2.5 Discussion and Conclusions

Healthcare professionals provided an essential insight into the daily routine and knowledge of those in hospital environments around the UK. This study highlighted the importance of those in a healthcare role, to understand and learn about why and how they should be cleaning the environment on a daily basis.

Survey data provided information on daily routine, cleaning and disinfection and touched upon biofilm knowledge. Most participants work in the field of IPC, in a hospital and are nurses which were exactly the criteria the survey was aiming to target. Data came from all over the world, most responses coming from Europe. Within each country there are different measures for cleaning and disinfection practices regardless of location.

When asked how often a surface should be cleaned or disinfected, pre-pandemic data showed that between every patient and once a day were the most popular responses. This is interesting to note as these responses are opposing. However, post-pandemic data showed a lean to the three/more times a day, especially when considering cleaning. We know from the SARS-CoV-2 pandemic, the virus has been found to survive on surfaces from between 4 – 6 days (Goldman, 2020). This data may have influenced choices of both cleaning/disinfection frequency and survival of microorganisms in the environment. When we consider responses to how long microorganisms survive in the environment, most participants responded with answers > days and very little stated hours. Thus, cleaning or disinfecting only once a day does not correlate to their current knowledge of survival.

When asked what the best methods to control IPC in a contaminated area are, the choices that mentioned use of a single product or machine were the least popular. This coincides with results from the survey, where it was obvious that cleaning and disinfecting of an area is multimodal and not one method will suffice. Both pre- and post-pandemic respondents were unanimous in thinking that either cleaning and automated disinfection or cleaning and liquid-based disinfection were best for decontamination. It is well known that both cleaning and disinfection is essential for reducing the threat of HCAIs and reduce transmission of pathogens (Weber et al. 2013). As also seen with the REACH training programme by Mitchell et al. (2018 & 2019) a multimodal cleaning approach reduced the occurrence of hospital pathogens.

Most survey participants appeared to have a good idea about the survival of four different microorganisms on hospital surfaces. It was clear that after the pandemic, there was an increase in knowledge of viral survival periods. This indicates that the pandemic, in a way, has been beneficial as it taught healthcare professionals new information that they may not have either had access to before or had the time to learn about. This premise fits in well with the interview themes sought, specifically reactive judgement and the thought that "nothing matters unless its personal". As mentioned in interviews, the pandemic made people think, it educated those who did not know previously and highlighted the importance of IPC in healthcare. This is especially pertinent as we know that bacteria, especially DSB, are readily spread by contaminated gloves of healthcare professionals, even following treatment with a cleaning detergent (Tahir et al., 2018).

Hand hygiene was unanimously chosen as the best method for helping prevent the spread of pathogens in the healthcare environment, not dissimilar to interview data. It is apparent that there is much confusion within the sector, especially when most hospitals employ external contractors to clean certain areas and pieces of equipment. This is a worry as it may lead to areas being missed out, and as we know, pathogens residing in DSB can survive for prolonged periods in the environment and go unnoticed (Vickery et al., 2012). Most areas either near patient, where the patient and healthcare staff touch frequently, or with high footfall of patients/healthcare staff were flagged as high-risk areas of transmission. The only low risk areas were those we would consider as not frequently used or touched by

either patients or healthcare staff. However, the floor was chosen as a low-risk area. Floors have been found to be one area that is a reservoir for the transmission of pathogens throughout the hospital environment but readily goes unnoticed (Deshpande et al., 2017). Linking with interview data on knowledge, it appears this is an area where healthcare professionals require more education so that they understand the risks associated with areas they believe to be "safe". Supporting this argument, Houghton et al. (2020) reviewed IPC guidelines and adherence of healthcare workers worldwide during the SARS-CoV-2 pandemic. They found that when knowledge of IPC was limited, those workers did not adhere to IPC guidelines set out by hospitals, subsequently increasing infection risk.

It is clear that there is a requirement for more teaching tools and workshops to help staff understand the science around cleaning. Frequently mentioned was the use of visual aids and without a known threat, staff will not recognise that bacteria are always present in the environment and cleaning protocols need to be followed correctly in order to reduce the risks associated. Some survey participants did choose "visibly looks clean" as one of the best methods for measuring cleanliness. This is really important to understand, as they may be relying on other staff members to have completed their job of cleaning/disinfecting an area appropriately, or as mentioned above in interviews, if they cannot see contamination, they do not think it is hidden on a surface. This leads on to the risk of the pandemic. We can conclude from interviews the overall standpoint was that the COVID-19 pandemic was in some way positive for the infection control community. It meant that there was more attention to detail in terms of cleaning and hygiene. When the threat is there and real, people will react accordingly.

All but one of those interviewed knew what DSB are. All those interviewed were in senior positions or had been in their line of work for a long time and were quite specialised. However, there were very few (%) survey participants who had heard the term DSB. Whilst we had a wide range of length of service, there was no correlation between having heard the term biofilm previously and length of service. This highlights the view that DSB are still relatively unknown throughout the healthcare profession. Interviewees also all touched upon the point that they only knew about DSB from conferences, publications or collaborations, again highlighting the importance of teaching and access for all. Similarly,

conference/study days and scientific journals were also the two most popular answers from the survey in relation hearing the term biofilm. Again, this raises questions as to the accessibility of these sources of information, however it is important to note that most survey participants are IPC nurses.

Talking to colleagues was also amongst the most popular. Talking to others about current issues and educating through social/professional networks has always been at the forefront of the healthcare profession and shows that word of mouth is essential as an unofficial education tool. Healthcare professionals use virtual communities of practice as a tool for continued learning, support, and education (McLoughlin et al., 2017).

It is worth noting that it appears there is no current requirement microbial method for sampling surfaces for pathogens to check cleanliness. Only ATP kits are occasionally used but not employed as part of regular routine. Although ATP is useful for determining bioburden on a surface, it produces quite variable results and has low sensitivity compared to microbial culturing, which can be used on a wide range of surfaces and produces accurate data on species of bacteria (Mitchell et al., 2013). Understandably this takes longer to get results, but currently no methods are being used in hospitals to determine whether a surface has been cleaned properly. Interestingly, the culture swab of microorganisms was chosen by most participants as effective in measuring cleanliness of an area. Following interviews, it appears that the cost associated is too great for this to be done routinely, even if healthcare staff believe it is worthwhile.

There are some important implications that arose from interviews. When questioned about other staff under their supervision, interviewees were reluctant to say that they knew what they were doing when it comes to correct protocol. This was apparent when questioned about how to wipe a surface. The "S shape" wipe action was designed as it is an easy method to follow, but it appears even simple instructions are not followed in the workplace. The importance of the "S shape" means that clean surfaces are not wiped again with a dirty wipe, potentially spreading microbial contamination back over the clean surface. As this is not being followed, are the surfaces really clean in healthcare environments?

2.6 Limitations

One main limitation of the study was that it was not possible to go back for follow-up interviews to go more in depth and understand some points that were made by participants which weren't quite clear. Ideally building this opportunity into the consent procedures may have been helpful but there would be practical considerations given the way the data was collected as it would have required follow-up via another means.

The COVID-19 pandemic occurred from March 2020, with conference attendance not starting again until September 2021. Unfortunately, due to the nature of this work, the planned participants were infection prevention control professionals for both the survey and interviews. These people were extremely busy on the frontlines, fighting the pandemic and therefore participation has been lower than we would have hoped.

There were limitations in relation to responder bias. As convenience sampling was used, participants were self-selected. However, this did not mean that those participants were selected based on their knowledge. For example, we could have selected those that we assumed knew a lot about dry surface biofilms, which could have been an issue, this was clearly not the case, given that a lack of knowledge of the term dry surface biofilm was evident in survey responses.

Questions related to the survival of microorganisms on surfaces in the survey required honesty from participants regarding their knowledge, although we can assume participants genuinely do know how long, they may have been inclined just to submit an answer rather than indicating that they do not know.

2.7 Recommendations and Conclusions

Both interview and survey analysis have shown that education is really key to tackling problems in the healthcare environment that surround IPC. Without knowledge an understanding, healthcare professionals cannot fully appreciate the risks in the surrounding environment and understand how to tackle them appropriately and effectively. There is also a large gap in knowledge of DSB even though they present a threat to the near patient environment. Based on data compiled from both surveys and interviews, this would be best provided through initial education courses, such as nursing degree, or, through conference and study

days where the information could spread via word of mouth, where attendees go back to work and tell their colleagues what they have learnt. Future work should look to test these methods and uncover the best approach.

One main issue with effective cleaning and disinfection is confusion. This can only be overcome through strict rules and regulations. The input of healthcare workers in developing policies could help overcome problems of ineffective cleaning. Also, those who are required to look and understand trust policies need more encouragement and enforcement to do so. Although it is mandatory now, there may be a need for some stricter measurements so that those in senior roles can check that their workers are reading policy documents and keeping up to date. Perhaps policies require some change to help with this problem and those who are enrolled to clean understand exactly what they are supposed to clean and make it simpler for them to follow, especially when workers are pushed for time. This links to the problem of money, which will inevitably always be around if funding is not provided. Money links to so many issues raised including infrastructure, sampling surfaces and jobs. Not enough provision goes into checking if a surface is clean, perhaps a new inexpensive, effective sampling method for measurement of cleanliness, which is both visible and quick needs to be addressed. Funding should be provided by the NHS, if they can free up money through savings, which is enabled by better procurement of effective cleaning equipment and thus, less wastage.

It is clear that although there are many issues within the healthcare environment surrounding IPC and work must be done to improve standards of cleanliness, the healthcare professionals in this study understand the environment and are keen to move forward in tackling these issues to ensure patient safety.

This work clearly illustrates the need for education, training, and stricter compliance to IPC control measures in the healthcare environment. It highlights the gaps in knowledge and cleaning/disinfection protocols. The results will be revisited in relation to the lab work in the final general discussion chapter at the end of the thesis.

Chapter 3
General Methods and Materials

3.1 Bacterial Strains

Several bacterial species were chosen for the PhD project (**Table 3.1**).

Table 3.1. Bacterial species used in this project.

Species	Reason for Use	Place of Purchase	Reference
Staphylococcus aureus NCTC 10788	Clinically relevant and previous use in dry biofilm formation	PHE[1]	Almatroudi et al. (2015) Ledwoch et al. (2018)
Bacillus subtilis NCTC 1400	Laboratory reference strain for biocidal testing	PHE[1]	BS EN standard tests
Bacillus subtilis AEWD	Resilience to disinfectant and ability to adapt to the surrounding environment, identified as heavy biofilm producer	Environmental isolate (Automated Endoscope Washer-Disinfector)	Martin et al. (2008)
Bacillus licheniformis ATCC 14580	Main component species of dry biofilms in the hospital environment	LGC[2]	Ledwoch et al. (2018)
Klebsiella pneumoniae ATCC 13883	Pathogenic Gram-negative species, prevalent throughout hospitals and associated with HCAIs	LGC[2]	Anderson et al. (2014)

[1]Public Health England Culture Collections (Salisbury, UK); [2]LGC Standards (Middlesex, UK)

3.2 Sterilisation of Equipment

All growth media, glassware and materials were sterilised at 121 ºC for 15 minutes following standard protocol (British Pharmacoepia, 2019), and allowed to cool down before use. All media was stored at 4 ºC and materials were stored in a cupboard in the dark. Tryptone Soya Broth (TSB) growth media used in biofilm formation was sterilised and heat liable nutrients were added aseptically using a 0.2 µm membrane filter.

3.3 Bacterial Stocks and Storage

Long-term freezer stocks were prepared for storage at -80 ºC, using an 20-30% sterile glycerol solution. A cryovial (Fisher Scientific, Loughborough, UK) was used to store a 1 mL bacterial suspension containing 500 µL of bacterial culture (approx. 10^9 CFU/mL) and 500 µL of 20-30% glycerol solution. For shorter term storage, working stocks were maintained on tryptone soya agar (TSA) plates and kept at 4ºC. Stocks were replaced every two months to ensure viability. Purity of cultures was checked under a light microscope (Leica DM IL LED Inverted Laboratory Microscope) using Gram-staining developed by Hans Christian Gram in 1884 (Coico, 2005).

3.4 Culture conditions

Overnight cultures were made using 20 mL of TSB. Two colonies were picked aseptically using an isolation loop from working TSA stocks and suspended in 20 mL of TSB. All cultures were left to grow aerobically for 18 hours at 37 ºC in a shaking incubator (Sanyo Orbital Incubator) at 120 rpm.

3.5 Dry Surface Biofilm (DSB) Formation

Bovine serum albumin (BSA) 3 g/L was added to 15 mL of full-strength TSB and left to dissolve completely so no BSA crystals were visible. The TSB + BSA growth media was further sterilised by passing he solution aseptically through a 0.2 µm membrane filter. Working overnight cultures (section 3.4) were centrifuged at 5000 g (Beckman Coulter Avanti J-20 XP centrifuge) for 10 minutes at room temperature (21 ºC). The supernatant was discarded, and the pellet resuspended in 20 mL of TSB. All *Bacillus* spp. were vortexed with glass beads in order to disaggregate the bacterial clumps which are naturally formed due to the nature of the species growth

when grown in liquid culture. Using an Ultrospec 3100 pro UV/Vis spectrophotometer (Amersham Biosciences, UK) to measure absorbance at 600 nm, cultures were diluted with the TSB + BSA solution to a density of 1 x 10^6 CFU/mL. Bacterial attained concentration was verified by comparing the A_{600} value to growth curves created previously for all species (appendix 1.1).

Before use, stainless steel discs (0.7 ± 0.07 mm thickness, 10 ± 0.5 mm diameter; 2B finish) were placed in 3-5% Decon 90 solution for 30 minutes. Discs were washed with distilled water and submerged 70% ethanol. Discs were incubated at 50 ºC for the ethanol to evaporate and then sterilised 121 ºC in a glass container. This process ensured all organic load debris was removed from the disc. Sterile clean discs were placed in a Corning™ Costar™ flat bottom 24 well cell culture plate ready for biofilm growth. The Dry biofilm takes a period of 12 days to grow, alternating 48-hour hydration and 48-hour dehydration “dry” phases (**Figure 3.1**) (Ledwoch et al., 2019).

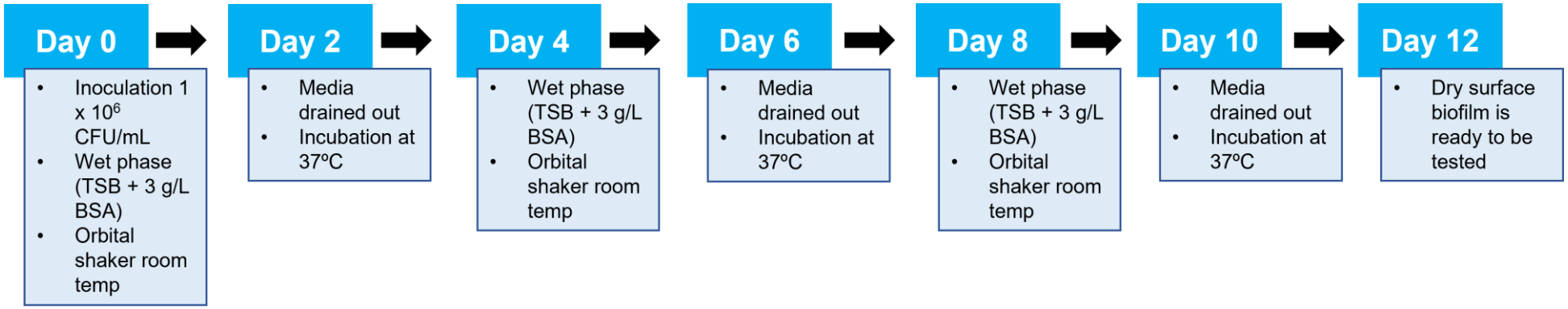


Figure 3.1. Schematic representation of biofilm formation and growth. For the first hydration phase, 1 mL of inoculum, (BSA 3 g/L in full strength TSB, plus 1 x 10^6 CFU/mL), was added into each well containing a disc. The 24 well plate was incubated at room temperature (20 – 23 ºC) for 48 hours in an Orbital shaker (Labnet International) at 200 rpm. Following hydration, all media was drained from the well containing the biofilm and left for a further 48 hours at 37 ºC. This process was repeated until a mature biofilm was produced at 12 days following methods outlined by Ledwoch et al. (2019).

3.6 Carrier Test

The efficacy of different disinfectants was tested against single species DSB (described in section 3.5). The test disinfectants were based on current commercially available wipe formulations. Four key components of known disinfectant products were identified, including benzalkonium chloride (BZK), peracetic acid (PAA) and chlorine (sodium dichloroisocyanurate/NaDCC). NaDCC was prepared by in accordance with manufacturer's instructions, by allowing one tablet (Titan chlor tablets, Netherlands) to dissolve in 1 L of sterile deionised water to make a final concentration of 1,000 ppm (pH 5.8), or 1 tablet in 100 mL diluent for 10,000 ppm (pH 5.8). Both BZK (Sigma, Dorset, UK) and PAA (Acros Organics, Geel, Belgium) were prepared by adding the agents in sterile deionised water at room temperature. BZK (pH 4.8) was added in powder form and vortexed to fully dissolve all BZK, PAA was added in liquid form to the diluent. Both BZK and PAA are currently used in GAMA Healthcare disinfectant wipes and final concentrations cannot be disclosed due to confidentiality issue. BZK was measured as % weight and the concentration of PAA was calculated using the Pocket Colorimeter™ (HACH®, Manchester, UK), following the N, N-diethyl-p-phenylenediamine (DPD) method. Where the amount of available chlorine is measured and PAA subsequently calculated using equations outlined by Domínguez-Henao et al. (2018). PAA was used in two concentrations, further referred to as low concentration (pH 3.0) and high concentration (pH 2.5) hereafter.

Each DSB was exposed to 1 mL of disinfectant for 5 min. Control consisted of untreated DSB. DSB of *Bacillus subtilis* AEWD and *B. licheniformis* were also exposed to the disinfectants for a 60 min treatment time due to the lack of activity of the disinfectant after 5 min contact time. Following exposure to the disinfectant, discs were taken out of the well, using sterile tweezers, and added into 1 mL of universal neutraliser for 10 min. The universal neutraliser solution used to quench the activity of the disinfectants contained 8.5 g/L sodium chloride, 1 g/L tryptone, 1 g/L L-Histidine, 3 g/L lecithin, 5 g/L sodium thiosulphate, 30 g/L polysorbate 80 and 30 g/L saponin and was prepared in distilled deionised water. Bacteria were recovered from the disc and revived as described in section 3.8.

3.7 Wiperator experiment

The antibiofilm effectiveness of five commercial wipes and a water control was also investigated. All wipes were pre-packaged excluding the water control wipe (Table 3.2).

Table 3.2. Commercial wipe products used and control water wipe.

Product	Formulation/Active ingredients
Control (water)	Water on Rubbermaid® HYGEN™ disposable microfiber cloth 284
A	>5% non-ionic surfactants + preservatives
B	DDAC (6.4g/Kg) + N-(3-aminopropyl)-N-dodecylpropane-1,3-diamine (5.31g/Kg)
C	DDAC 0.450% w/w
D	<1% cationic biocides + additional surfactants
E	Peracetic acid

Wipe efficacy was tested using the Wiperator (FitaFlex Ltd., Ontario, Canada) following a modified version of ASTM E2967-15 standard (2015). Wipes were cut into 4 x 4 cm squares and loaded into the Wiperator using the boss. Discs were mounted onto the metal stage and pushed into the Wiperator so that wiping action could start (**Figure 3.2**). Once finished, a fresh clean wipe was loaded, and the disc was turned over to be wiped on the other side.

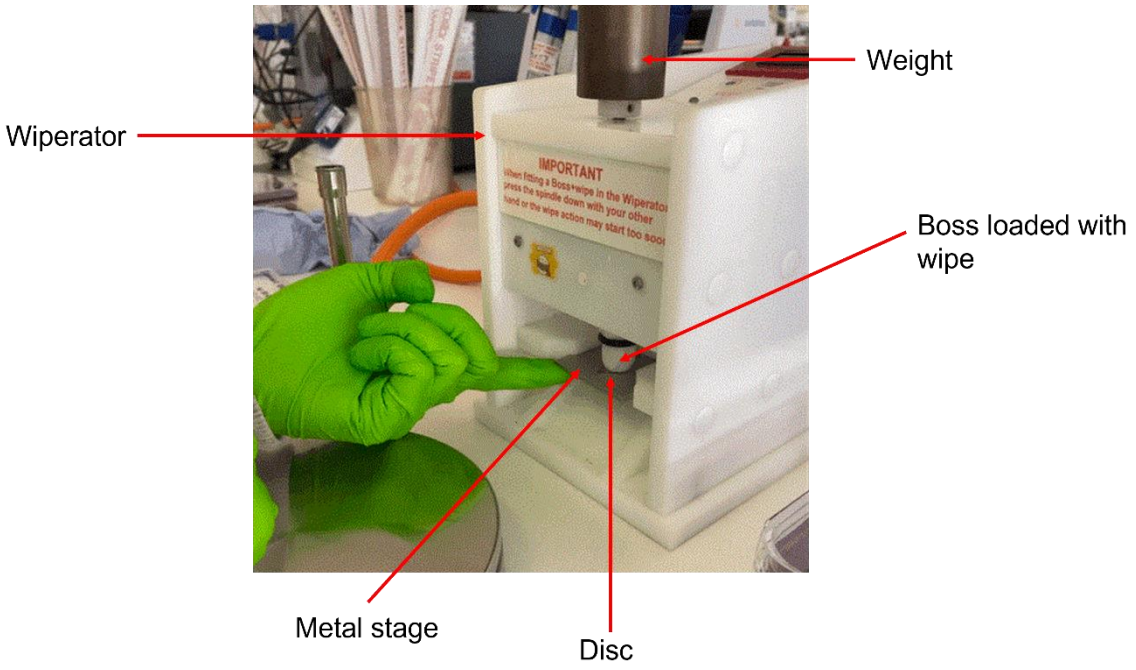


Figure 3.2. Schematic presentation of the operation of the Wiperator. Discs are placed on the metal stage and a wipe loaded onto the boss. When ready the metal stage is pushed upwards to start the wiping action.

DSB discs were wiped on both sides for 10 seconds each, under 500 g of pressure. To determine log_{10} reduction in bacterial viability, wiped discs were then left dry at room temperature for 30 or 60 seconds before being placed in 1 mL of universal neutraliser for 10 min. Discs were then placed in TSB for 2 hours and bacteria recovered from disc revived and enumerated as described in sections 3.8 and 3.9. Log_{10} reduction was calculated as the difference in bacterial number between wiped samples and unwiped DSB.

The direct transfer of bacteria to a new surface was also investigated, mimicking what may occur within hospitals following routine cleaning and disinfection protocols. Original disc containing DSB was wiped as described above and a sterile disc was wiped immediately using the same wipe as described in the ASTM2967-15 protocol. This “clean” disc then went through the same neutralisation and bacterial enumeration process as described in sections 3.8 and 3.9.

Another test was conducted to measure bacterial transferability directly post wiping from the wiped disc. Following wiping, DSB disc were pressed 36 times onto Dey-Engley neutralising agar (DE agar) at a pressure of 100 g (Ledwoch et al., 2019a&b). 36 adpressions was the maximum amount of recordable adpressions possible for

the size of the plate (Gosselin™ square petri dish, 12.05 cm diameter). DE agar plates were inverted and incubated at 37ºC overnight. Positive growth was recorded, and transferability was calculated as the number of positive contacts out of the 36 adpressions and expressed as percentage transfer.

3.8 Revival of Bacteria from the Dry Biofilm

Following exposure to disinfectants, DSB discs were placed in a McCartney bottle with 2 mL of sterile TSB and 1 g of glass beads for 2 hours at 37 ºC. Preliminary results showed poor bacteria culturability without a revival step (data not shown). 2 hours was chosen as a suitable time to allow biofilm recovery without promoting bacterial growth. Bacterial growth curves showed active exponential growth phase was starting after 5 hours of incubation (appendix 1.2). Following incubation, the bottle containing the disc and glass beads was vortexed for 4 minutes using the multitube vortexer (FisherBrand) to disrupt the biofilm. Bacterial suspension was then serial diluted using phosphate buffered saline (PBS) and plated on TSA, either by drop count (DCM; Miles and Misra method, 1938) or spread plate technique (Sanders, 2012) depending on the species (section 3.9). Plates were placed in the incubator overnight (18 hours) at 37 ºC and colonies counted after incubation.

3.9 Quantification of Bacteria

All revived suspensions (section 3.8) were serially diluted by adding 100 µL of sample to 900 µL of diluent, PBS, from a range of dilution factors starting at 10^0 (neat sample) to 10^6. Dilutions were carried out in 1.5 mL Eppendorf tubes and vortexed thoroughly to ensure mixing of the samples. Viability counts (CFU/mL) using the Miles and Misra method were used with *S. aureus.* Three 10 µL drops of each dilution were dropped immediately using a 100 µL pipette, onto corresponding sections on a TSA plate.

Due to the formation of swarming colonies with all *Bacillus* spp. and *K. pneumoniae* 200 µL of each dilution was spread onto a TSA plate using a sterile plastic spreader – DCM and spread plates were then incubated at 37ºC for 18 hours. Colonies were then counted, and the average number was calculated. To calculate total bacteria viability (CFU/mL), average colony count was multiplied by the dilution factor and either 50 (20 µL drops) or 5 (200 µL). If growth was absent in the 10^0 dilution the lower limit of detection (LLD) was applied. For DCM plate as 20 µL drops were used,

the LLD was 50 CFU/mL. When using the 200 µL spread plate, the LLD was 5 CFU/mL. Difference in bacterial viability counts amongst control and test groups were calculated as log-reductions using the formula $Log_{10}(A/B)$, where A represents the viability of the control group and B the viability of tested group.

3.10 Neutraliser Toxicity validation

The neutraliser used was validated against all bacterial species used in this study. Since planktonic cultures are considered more susceptible to the action of disinfectants than dry surface biofilms (Nkemngong et al., 2020)., the validation tests were performed using planktonic bacteria. To determine neutraliser efficacy and toxicity, a modified version of the EN16615 test was performed. To evaluate the neutraliser efficacy a sterile stainless-steel disc was placed in 1 mL of disinfectant or TSC control for 5 min then placed in 9 mL of universal neutraliser for 10 min. After the allotted time, 1 mL of a bacterial suspension (10^7 CFU/mL) was placed into the solution for 10 min. For neutraliser toxicity, 1 mL of bacteria (10^7 CFU/mL) was added to 9 mL of universal neutraliser for 10 min. As an untreated control equivalent, bacteria were also added to TSC for 10 min. After serial dilution and, all suspensions were plated on TSA using DCM or spread plate (section 3.9) to enumerate bacterial colonies.

For the experiments which involved the wiperator, an uninoculated stainless steel disc was wiped on each side for 10 seconds at 500 g to follow experimental procedures described in section 3.7. The disc was left for 60 seconds and then added to universal neutralising solution for 10 min. A 1 mL of x 10^7 CFU/mL bacterial suspension was then added after 10 min, the suspension was serially diluted and plated on TSA, and colonies enumerated as described in section 3.9.

A validation count excel spreadsheet based on the EN16615 was created to determine the validation of neutraliser efficacy and toxicity, the neutraliser was determined as non-toxic as there than a 50% reduction in viability after treatment compared with the untreated control and also for the effect of neutraliser to quench residual biocidal activity of both disinfectants and wipe products.

3.11 Statistical Analyses

Statistical analyses were carried out using GraphPad Prism9 software (version 9.3.1) when a minimum of three biological replicates have been achieved in

experiments. Throughout this thesis, all error bars on graphs define standard deviation around man values. In order to compare multiple means, one-way and two-way analysis of variance (ANOVA) were mainly used to determine any statistically significant difference within the datasets. A *p* value of 0.05 was always used as the cut off for statistical significance. One-way ANOVA was used to determine statistical significance between one treatment over different timepoints. Whereas two-way ANOVA were used to determine statistical significance between different treatments and different time points. T-tests were also used where appropriate to identify any statistically significant difference between an individual disinfectant treatment and its respective control.

Post-Hoc tests were performed to allow for multiple comparisons between individual means, these were Tukey, Dunnetts and Šidák. Post hoc tests were chosen over t-tests to see differences between defined groups as t-tests do not control the inflated type 1 error rate, also referred to as a “false-positive” error of rejecting the null hypothesis when it is true (Kao and Green, 2008). Each Post-Hoc test was chosen dependent on the comparisons to be made (**Table 3.3**).

Table 3.3. Post-Hoc tests used for multiple comparisons of statistical analyses (Motulsky, 2016).

Post-Hoc test	Test comparison
Tukey	Compares the means of all treatment groups to the mean of every other treatment group after one-way ANOVA
Dunnetts	Compares the means of the treatment group to that of the control group
Šidák	Compares the means of all treatment groups to every other treatment group at each time point and species after two-way ANOVA

Chapter 4
Structure and Longevity of Dry Surface Biofilms

4.1 Introduction

In 1975 the term “biofilm” was first used by Mack, Mack and Ackerson after using transmission and scanning electron microscopes to view biofilm development (Mack et al., 1975). then, scientists have focused on the structure of the biofilm and it became clear that bacterial cells were embedded in some sort of self-produced matrix, which we now know as exopolymeric substance (EPS) (Schilcher and Horswill, 2020). A wealth of research has been conducted on the composition and structure of the EPS matrix housing the residing bacterial cells. The EPS is commonly comprised of lipids, eDNA, proteins and other biomolecules (Hobley et al., 2015). Predominantly, approximately 85% of the biofilm is matrix material (Kokare et al., 2009). The EPS plays vital roles in protecting biofilms from outside stressors and maintaining biofilm structure (Dufour et al., 2012). There are several beneficial functions of the EPS matrix (**Table 4.1**), which allow bacteria to thrive in otherwise extreme environments.

Table 4.1. Main crucial functions of the components within EPS matrix which provide advantages to life in a biofilm (Fleming and Wingender, 2016).

Beneficial function	Key role
Initial adhesion of bacterial cells	First steps for colonisation of biofilm by planktonic cells, allowing for attachment of biofilms to surfaces for extended periods
Aggregation of other bacterial cells	Allows for dense biofilm formation
Water retention	Prevent desiccation in extreme environments, especially those deficient in water
Protective barrier	Increased tolerance to antimicrobial agents such as disinfectants and antibiotics
Maintain gradients (oxygen, pH, nutrients)	For metabolic heterogeneity, provide habitat diversity and aid development of subpopulations and persister cells

The ability of some species to produce a thick layer of EPS matrix to which the bacteria are heavily embedded is highlighted in Table 4.2.

Table 4.2. Examples of environmental isolates of species identified as heavy producers of biofilms.

Species	Source	Reference
Bacillus subtilis	Endoscope washer-disinfector	Martin et al., (2008 & 2015); Bridier et al. (2012)
Micrococcus luteus	Endoscope washer-disinfector	Martin et al. (2008)
Pseudomonas aeruginosa	Various clinical and food isolates	Bridier et al. (2010)

However, the structure and characteristics of dry surface biofilms (DSB) have not been investigated in such detail. There is supporting evidence for the idea that the active production of EPS protects bacteria from desiccation (Fleming and Windgender, 2010). The EPS provides a microenvironment in which water is retained, enabling survival of bacteria on abiotic/biotic surfaces that include desiccation stressors (Roberson and Firestone, 1992).

Ledwoch et al. (2018) recently observed an interesting phenomenon in dry biofilm growth and composition. When dry surface samples were rinsed, bacteria did not grow in selected media which suggested that there were no planktonic bacteria present in samples. However, following immersion of dry samples in nutrient broth, multiple species were identified following appropriate incubation. It was suggested that the rinsing step contributed to accidental removal/dislodging of DSB from the surface. The Vickery group have also identified DSB growth on healthcare surfaces and commented on the impact of biocides and hospital surface decontamination procedures (Vickery et al., 2012; Almatroudi et al., 2015 & 2016). The group has shown that after current cleaning practices in Australian hospitals, biofilms were still able to persist on clinical surfaces in an intensive care unit. These studies identify the complexity of DSBs in hospital environments but have not looked at longevity of the dry surface biofilm in a laboratory setting.

5.2 Aims and Hypotheses

To monitor key biofilm structure and bacterial growth within the DSB, including establishing the characteristic morphology of DSBs. As previous work with *S. aureus* DSBs has shown dense layers and aggregations of bacteria within the DSB (Ledwoch et al., 2019), it seems reasonable to hypothesise a similar structure to the DSBs tested at this stage. Survival and longevity of DSB will also be investigated, it is anticipated that those species that produce a dense biofilm will persist on surfaces for longer periods of time.

4.3 Methods

Single species DSB were used in this chapter at three stages of development: 4, 8 and 12 days. Biofilm formation includes 4 and 8-day DSB, whilst post formation/mature DSB includes only 12-day DSB.

4.3.1 Scanning Electron Microscopy

Scanning electron microscopy (SEM) was employed to allow identification of the structure and characteristics of the dry surface biofilm at each stage of formation.

4.3.1.1 Sample Preparation

DSB samples were prepared by overnight incubation of discs in 2.5% glutaraldehyde solution. Following this, discs were placed, through a series of ethanol washes; 5%, 25%, 50%, 75%, 90% and 100% ethanol. Each disc was submerged in each ethanol concentration for 10 minutes to ensure DSB were fixed to the surface (Ledwoch et al., 2018). The DSB discs were then coated with a thin layer (20 nm) of gold-palladium for high resolution images, in a vacuum chamber, using a Bio-Rad Sputter Coater SC500. Argon gas was used to purge the sputter chamber before coating.

4.3.1.2 SEM Imaging

The DSB discs were using the secondary electron image (SEI) setting on a Philips XL30 field emission gun-scanning electron microscope (FEG-SEM) at x10000 and x2000 magnification and 5-7 mm working distance.

4.3.2 Long-term survival

Growth of DSB was recorded over extended periods of drying to understand survival of bacteria on dry surfaces. DSB were formed over a 12-day period as described in Chapter 3, section 3.5. Following maturation of the biofilm, DSB were stored in well plates in a sealed box at room temperature (21 ºC) and relative humidity (55 ± 5%).

Humidity was regulated using Prosorb humidity control cassettes. After 2 weeks, 2 months 4 months and 6 months DSB were tested for culturability and transferability. Culturability, was measured as the number of bacteria that were able to be cultured onto agar followed incubation and was tested as described in Chapter 3, section 3.8 and 3.9. Transferability of bacteria embedded in DSB was measured by direct adpressions onto DE agar. Each disc was pressed 36 times in total and percentage transfer from the dried disc was calculated number of positive growth contacts /36 x 100. The presence of spores of *Bacillus* spp. longevity was also investigated. DSB were vortexed for 4 min and 1 mL of DSB suspension was pipetted into an Eppendorf and placed in a dry bath for 30 min at 80 ºC to kill vegetative cells within the sample (Chapter 5, section 5.3.3). The samples were subsequently diluted and plated as described (Chapter 3, section 3.9) previously to enumerate spores within the sample.

4.3.3 Statistical analyses

Statistical analysis was carried out, where appropriate, using GraphPad Prism 9 software (version 9.3.1). One-way and Two-way ANOVA were used to determine statistically significant differences within long-term survival datasets (Chapter 3, section 3.11). Post-hoc tests were used for multiple comparisons.

4.4 Results

4.4.1 SEM Imaging of 4-, 8- and 12-day DSB

Dry surface biofilms were imaged at 4 and 8 days (x5000 and x10,000 magnification and at 12 days when the mature biofilm had formed (x2000, x5000 and x10,000 magnification) to evaluate development of the DSB over time. SEM imaging confirmed the overall DSB structure.

4.4.1.1 *Bacillus subtilis* AEWD

From 4 days, *B. subtilis* AEWD DSB had formed clusters of cells across the disc surface (**Figure 4.1**). There was a significant change in density and development of the DSB from 8 days to the mature biofilm at 12 days (**Figure 4.1 & 4.2**). At all stages of growth, *B. subtilis* formed large clusters of bacterial cells. At 12 days, a covering layer of EPS at x2000 was evident (**Figure 4.2a**), however this concentration of EPS is not evident at x10000 (**Figure 4.2c**). There was a high concentration of bacteria within the biofilm, and they appeared deeply embedded into the matrix at x2000. SEM imaging only allows the surface of the biofilm to be

viewed, however it was apparent that there are multiple layers to the biofilm structure (**Figure 4.2**). At 12 days, the biofilm was much more homogenous and covers the whole disc surface, which was not as evident at 4 days. DSB seem to become denser from 8 days onwards. There was no evidence of spores, from either bulging of the end of rod-shaped vegetative cells, or small circles within SEM images.

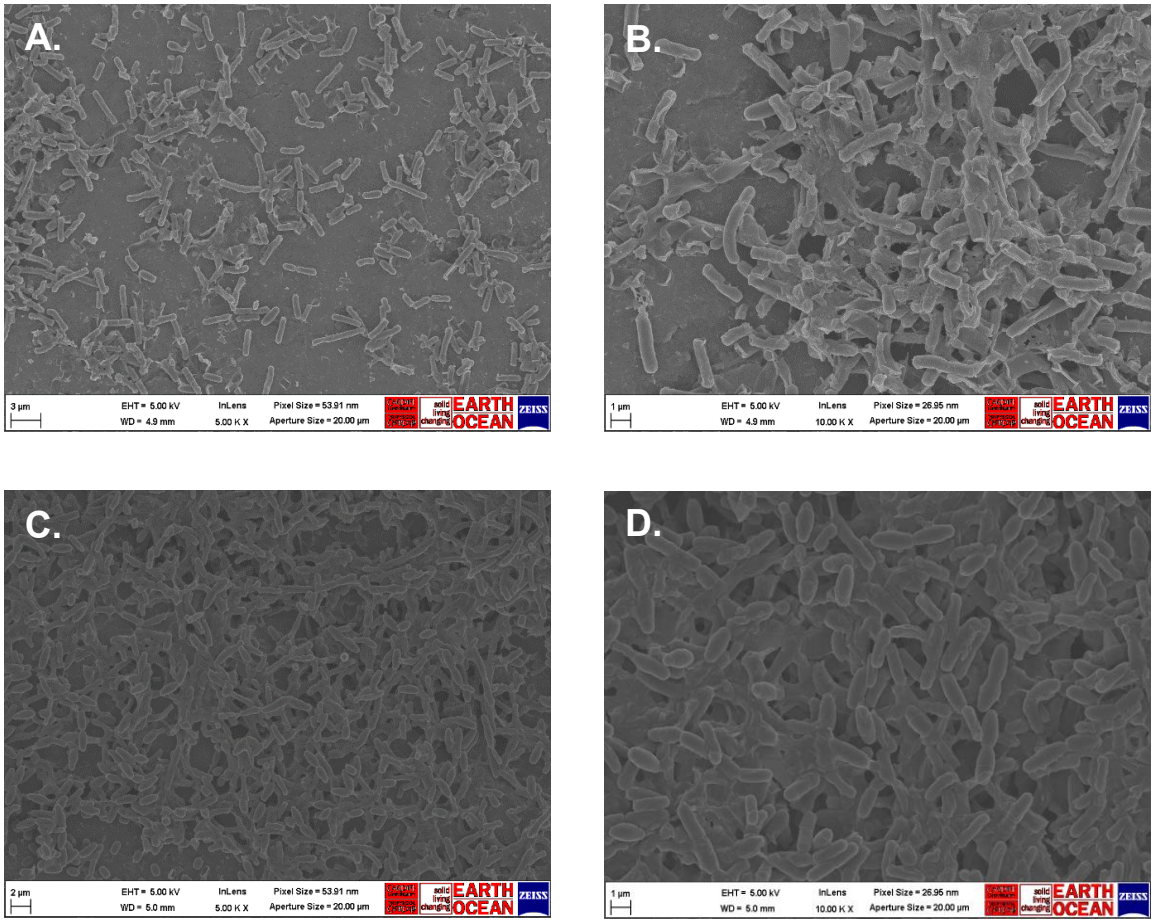


Figure 4.1. SEM images of *B. subtilis* AEWD 4- and 8-day DSB. Images presented are representative of the whole disc surface. Images taken at x5000 and x10000 magnification. (A & B) 4-day DSB, (C & D) 8-day DSB. There was no evidence of spores within DSB.

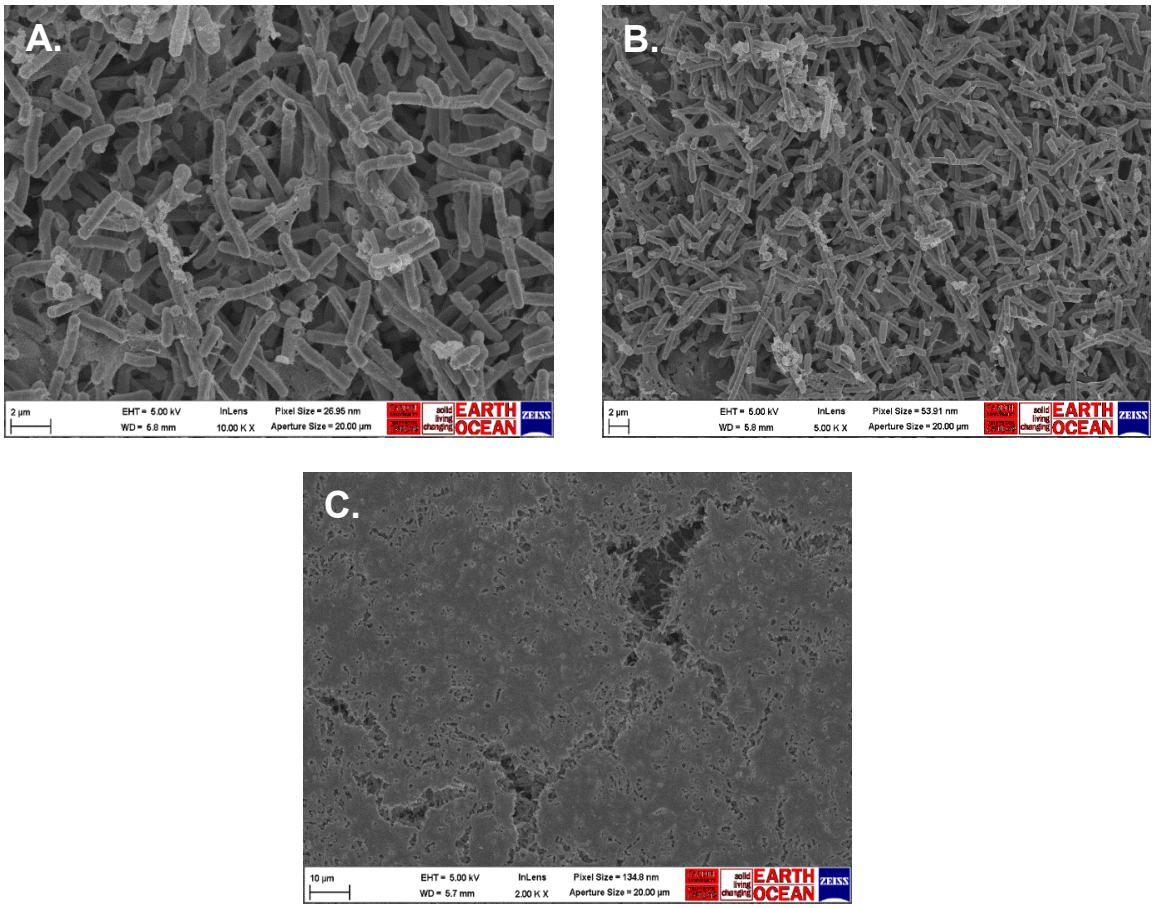


Figure 4.2. SEM images of *B. subtilis* AEWD 12-day mature DSB. Images presented are representative of the whole disc surface. DSB at (A) x 10000, (B) x5000 and (C) x2000 magnifications. At 12 days the biofilm is very dense which high cell population covering the disc surface. There was no evidence of spores within DSB.

4.4.1.2 *Bacillus licheniformis*

Bacillus licheniformis DSB formed a sparser biofilm at all stages of development than the *B. subtilis* AEWD (**Figure 4.3 & 4.4**). At both 4 and 8 days it appears that the biofilm was not fully matured as there was not a homogenous full covering of bacteria over the disc surface (**Figure 4.3**). Bacteria formed multiple aggregates within the biofilm, but a lack of EPS was evident at 4 and 8 days. At x2000 magnification of 12-day DSB, large aggregations of bacteria were visible, with evidence of the EPS matrix, which appeared to be holding the cells together (**Figure 4.4c**). Similarly, to *B. subtilis* AEWD, there was no evidence of spores.

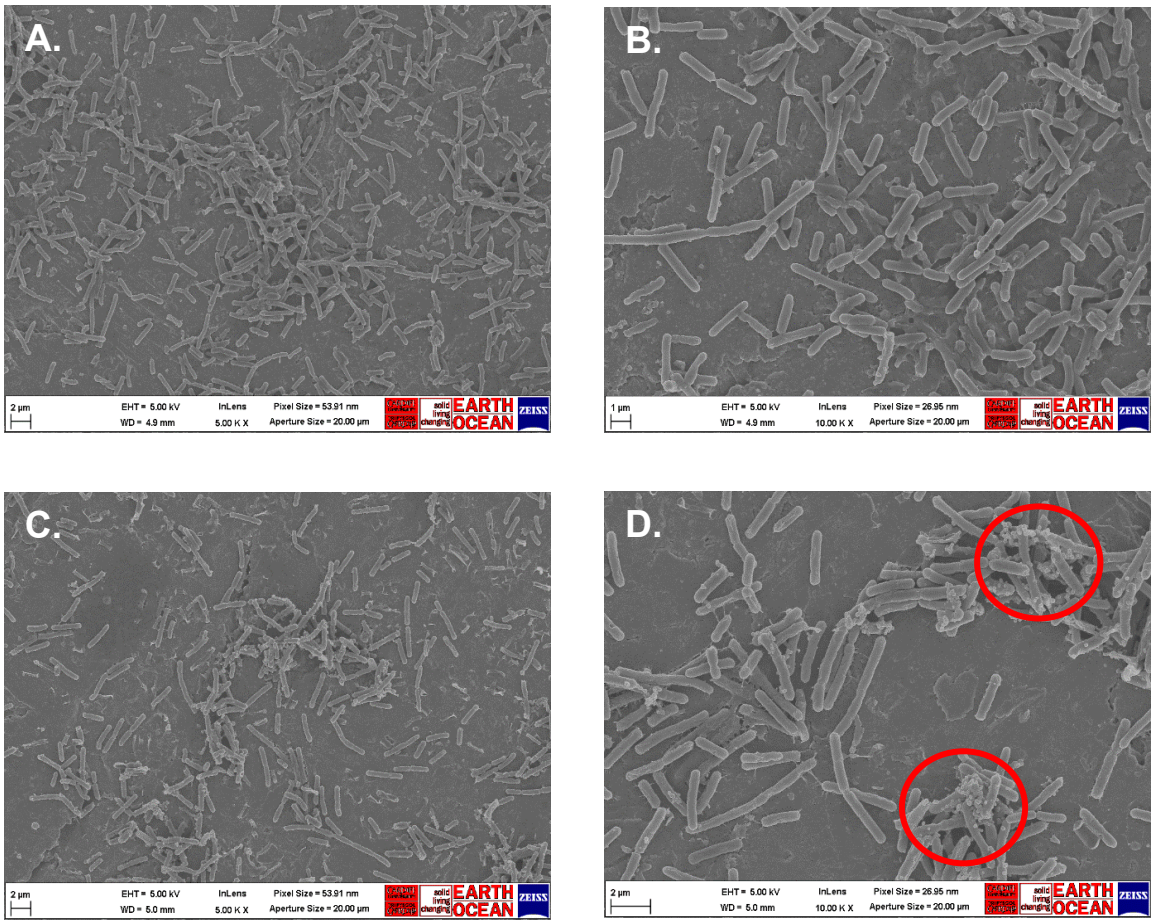


Figure 4.3. SEM images of *B. licheniformis* 4- and 8-day DSB. Images presented are representative of the whole disc surface. Images taken at x5000 and x10000 magnification. (A & B) 4-day DSB, (C & D) 8-day DSB. Red circles indicate EPS and clumping of bacterial cells in DSB. There was no evidence of spores within DSB.

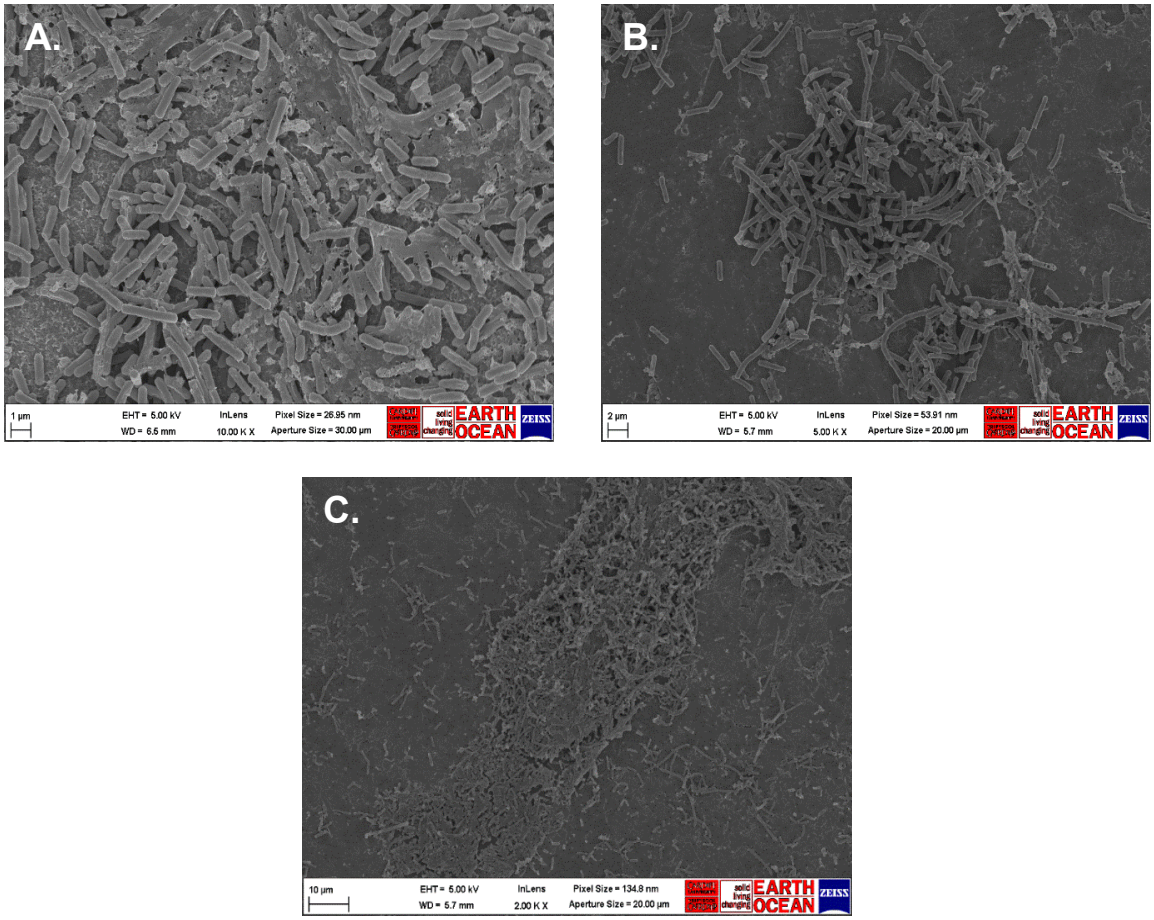


Figure 4.4. SEM images of *B. licheniformis* 12-day mature DSB. Images presented are representative of the whole disc surface. DSB at (A) x 10000, (B) x5000 and (C) x2000 magnifications. Large aggregates are evident over the disc surface with high cell density.

4.4.1.3 *Bacillus subtilis* NCTC 10400

Bacillus subtilis NCTC 10400 had a lower cell density than the previous *Bacillus* spp. At 4 days, there were very few bacterial cells on the surface of the disc and no matrix was evident (**Figure 4.5a&b**). After 8 days of development, DSB appeared to gain more structural integrity and house clusters of cells, evident at x10000 magnification (**Figure 4.5d**). However, there was still a large proportion of the disc surface that was not covered in biofilm, with single rods sparsely scattered. There was no evidence of DSB covering the whole disc surface (**Figure 4.6**). However, from 4 and 8 days there was visible progression of DSB formation. There was evidence of increased organic matter in the biofilm structure, but cells were not well

embedded into any matrix, instead rods appeared to connect to form long chains (**Figure 4.6**). Instead of a smooth surface, the cell rods appeared to have a rough membrane. In comparison to the two environmental isolates of *Bacillus* spp., NCTC 10400 formed a less dense DSB over the disc surface. Again, we observed no evidence of spores from SEM images.

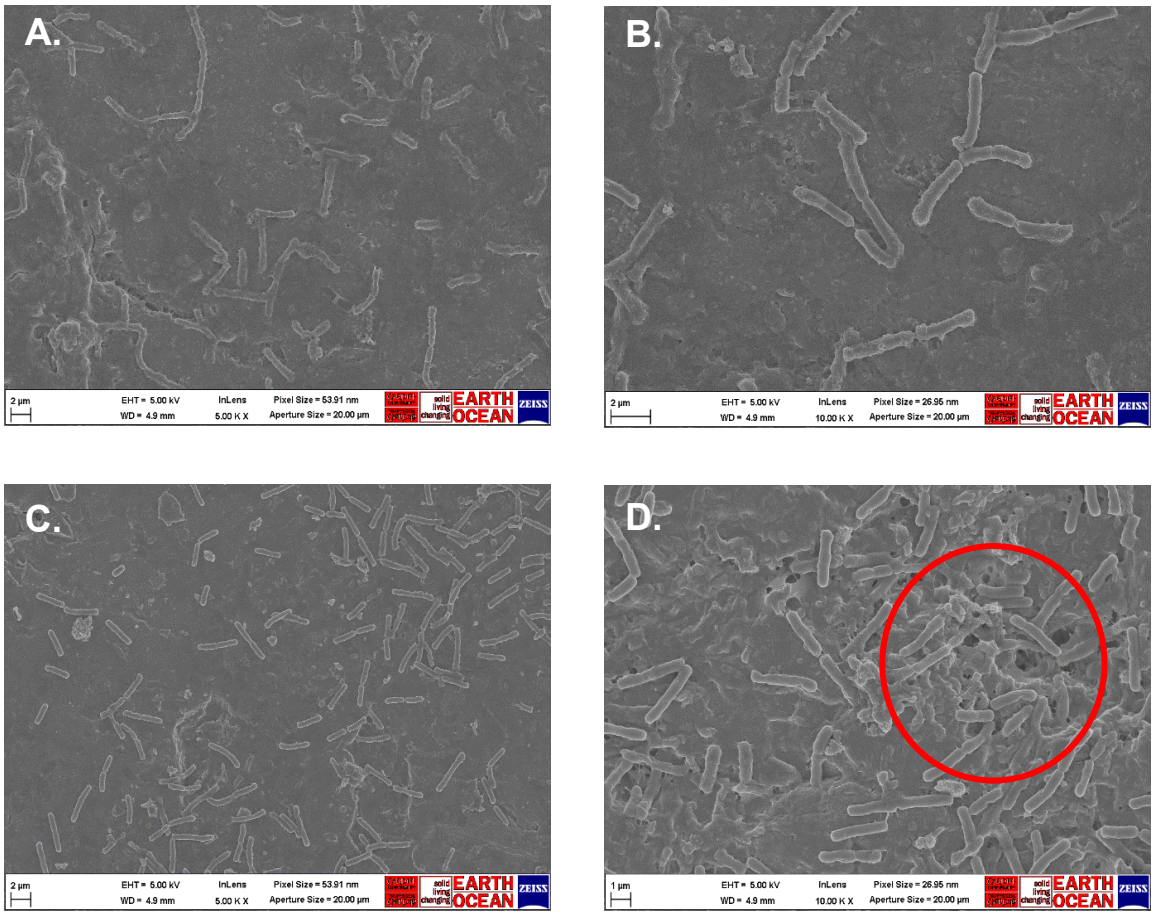


Figure 4.5. SEM images of *B. subtilis* NCTC 10400 4- and 8-day DSB. Images presented are representative of the whole disc surface. Images taken at x5000 and x10000 magnification. (A & B) 4-day DSB, (C & D) 8-day DSB. Red circles indicate clusters of cells developed at 8-day DSB. There was no evidence of spores within DSB.

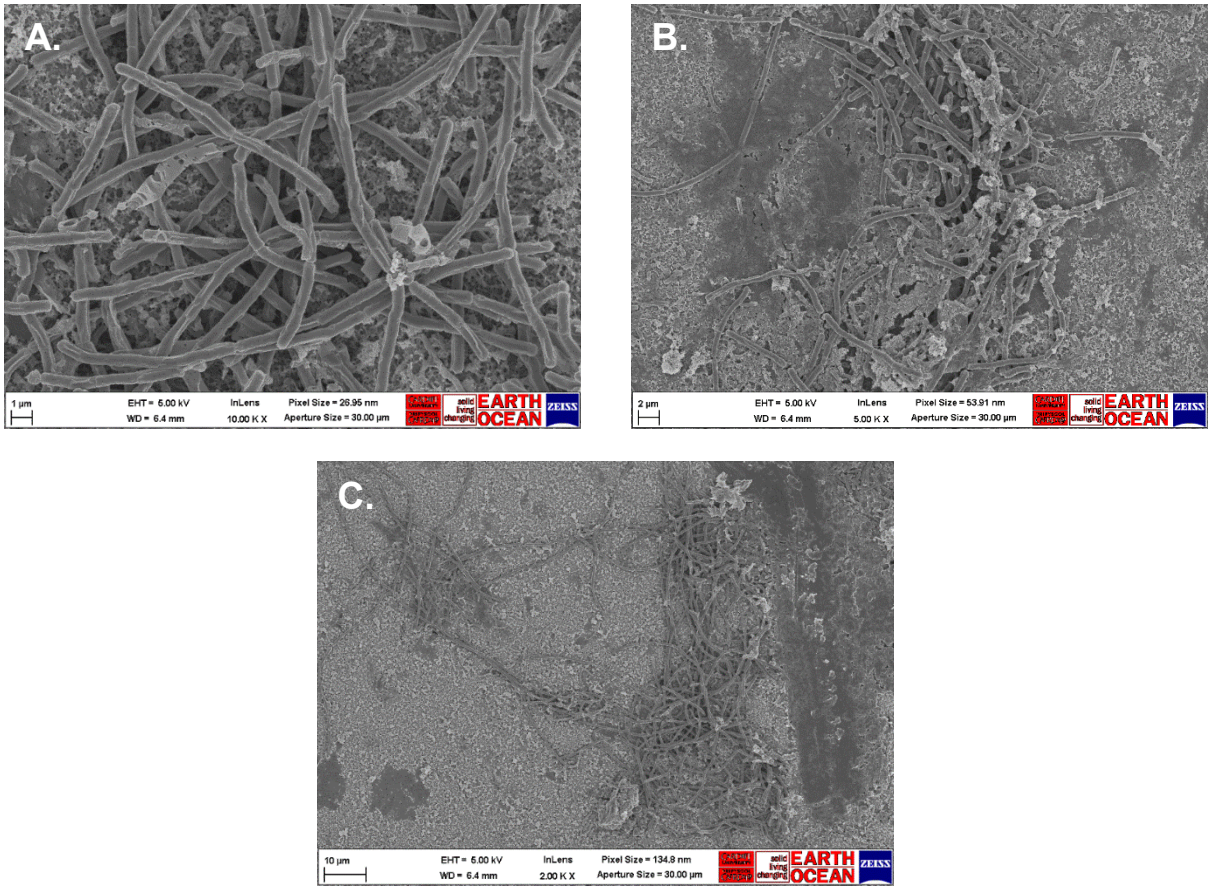


Figure 4.6. SEM images of *B. subtilis* NCTC 10400 12-day mature DSB. Images presented are representative of the whole disc surface. DSB at (A) x 10000, (B) x5000 and (C) x2000 magnifications. DSB are much sparser than what we have previously seen with environmental isolates. Bacterial rods are connected to form what looks like long chains. There was no evidence of spores within DSB.

4.4.1.4 *Staphylococcus aureus*

S. aureus started to form a densely packed biofilm from 4 days (**Figure 4.7a&b**). Across the surface of the disc, large clumps of cocci cells embedded in an EPS matrix can be seen at DSB ages 4 and 8 days (**Figure 4.7**). High cell density was apparent from 4 days and appears to increase till the mature DSB is 12 days old (**Figure 4.8**). At 12 days, *S. aureus* cells covered the whole surface of the disc, with evidence of EPS matrix and multiple layers to the DSB (**Figure 4.8**). In comparison to the *Bacillus* spp. presented here, *S. aureus* formed a dense biofilm much like *B. subtilis* AEWD.

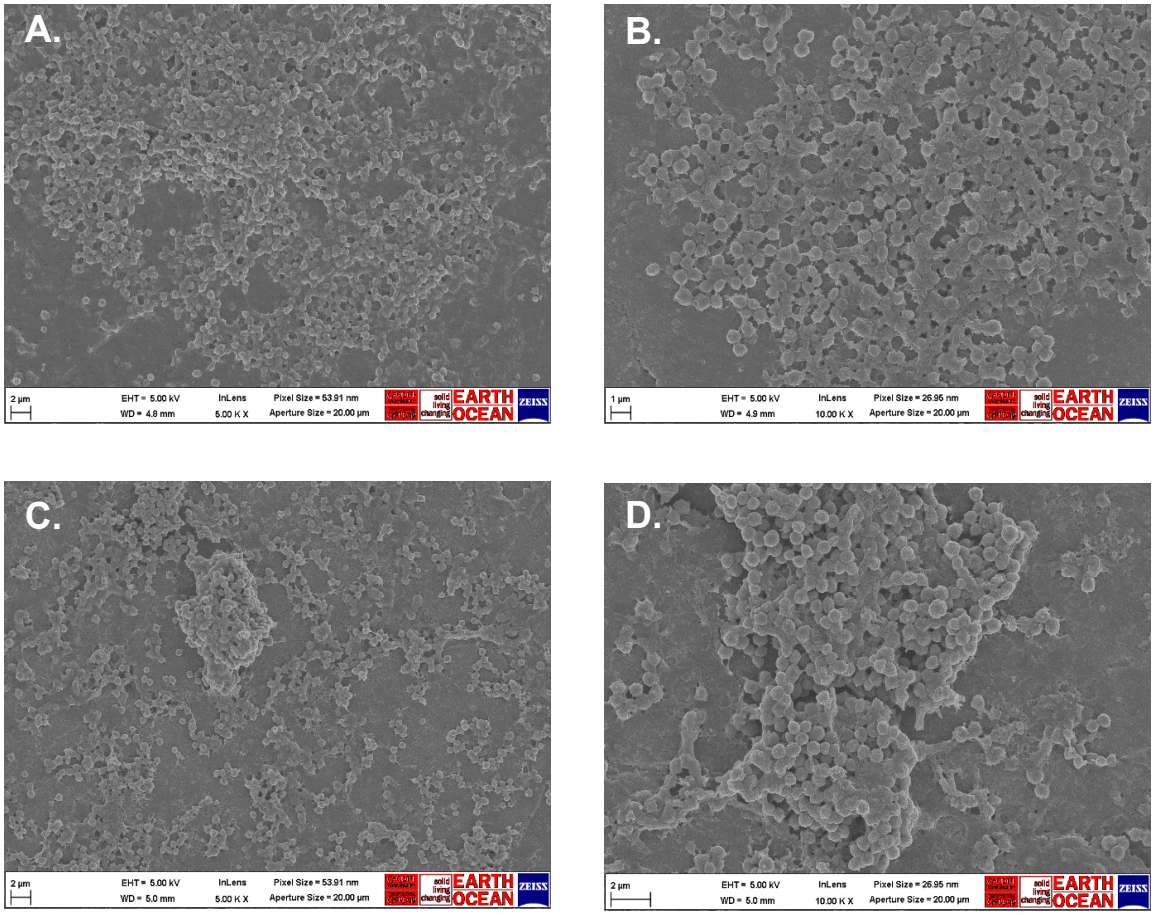


Figure 4.7. SEM images of *S. aureus* 4- and 8-day DSB. Images presented are representative of the whole disc surface. Images taken at x5000 and x10000 magnification. (A & B) 4-day DSB, (C & D) 8-day DSB.

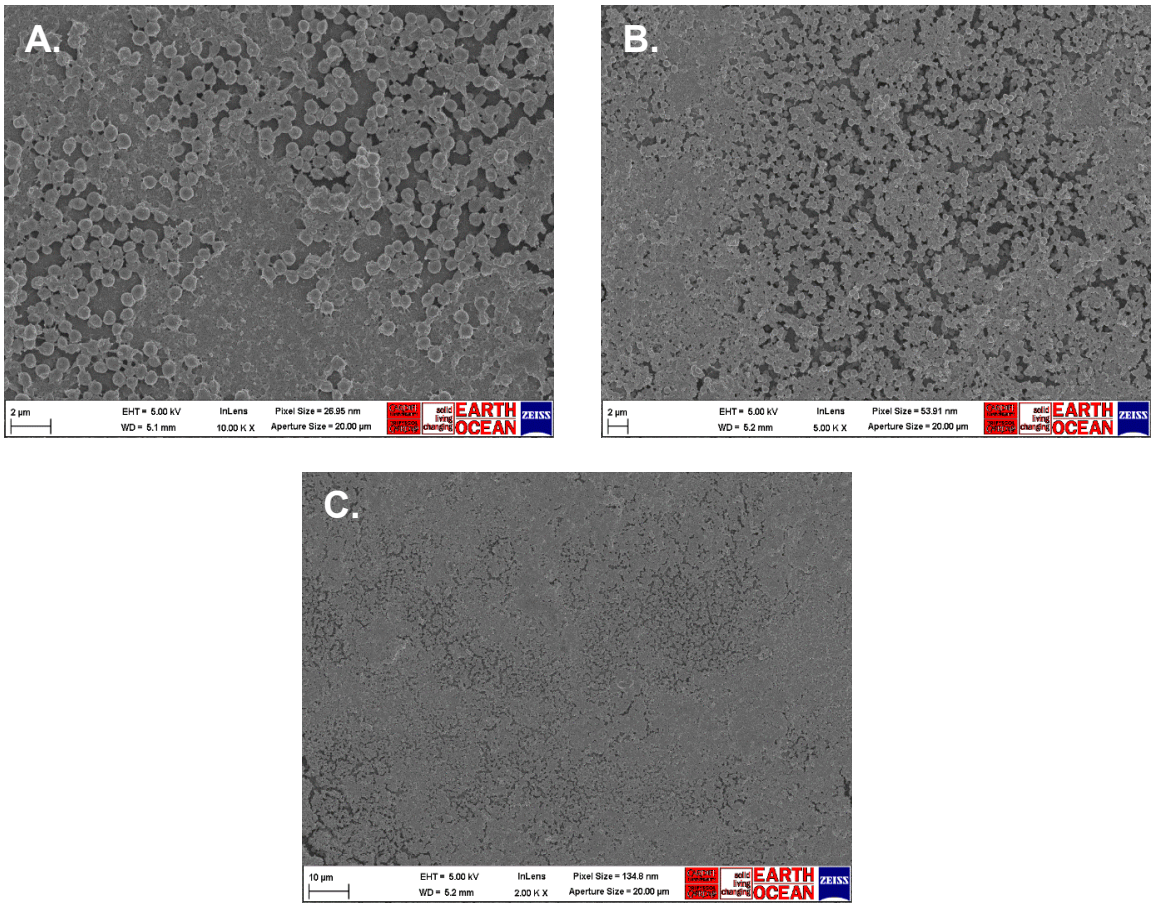


Figure 4.8. SEM images of *S. aureus* 12-day mature DSB. Images presented are representative of the whole disc surface. DSB at (A) x 10000, (B) x5000 and (C) x2000 magnifications. DSB has formed a dense covering over the surface of the disc, with evidence of EPS matrix housing cells.

4.4.2 Culturability of long-term DSB

DSB of *Bacillus* spp. and *S. aureus* were tested for culturability after 2 weeks and 2, 4 and 6 months of drying in a controlled environment to evaluate survival on surfaces over extended periods of time. Bacteria recovered remained circa 6 $\log_{10}$ CFU/mL with both environmental isolates; *B. subtilis* AEWD and *B. licheniformis* (**Figure 4.9**). Over the course of 6 months $\log_{10}$ CFU/mL changed by less than 0.2 $\log_{10}$ for both species, culturability remained consistent and there was little variation between biological replicates. There was no statistically significant difference between culturability counts over 6 months for either *B. subtilis* AEWD or *B. licheniformis* (One-way ANOVA, $p > 0.05$).

Log_{10} CFU/mL recovered from *B. subtilis* NCTC 10400 and *S. aureus* DSB were much more variable than with the other *Bacillus* spp. (**Figure 4.9**). Initially *B. subtilis* 10400 had the lowest CFU/mL counts of all species After 2 weeks and 2 months of drying, *B. subtilis* 10400 were approx. 4 $\log_{10}$ CFU/mL and decreased to 2.8 $\log_{10}$ CFU/mL at 4 months, there was no statistically significant difference between bacteria recovered from all ages (One-way ANOVA, $p > 0.05$). Unusually, after 6 months of drying the number of bacteria recovered increased slightly by 0.5 $\log_{10}$, but remained 3 $\log_{10}$ lower than the other *Bacillus* spp. It is worth noting that variation in $\log_{10}$ CFU/mL recovered amongst biological replicates of *B. subtilis* 10400 was much higher than other species throughout the long-term experiment.

S. aureus on the other hand had a high starting CFU/mL (6 $\log_{10}$) consistent with environmental isolates of *Bacillus*. However, after 2 months of drying this had already dropped by 2.3 $\log_{10}$ (**Figure 4.9**). From 2 weeks to 4 months there was an exponential decrease of 73% (rate of decline) in bacteria recovered from *S. aureus* DSB, which plateaued from 4 to 6 months as no bacteria were recovered from DSB (limit of detection was reached). There was a statistically significant difference between 2-week culturability counts and all other incubation ages (ANOVA, Tukey's $p < 0.05$). *S. aureus* was the only species in which this happened.

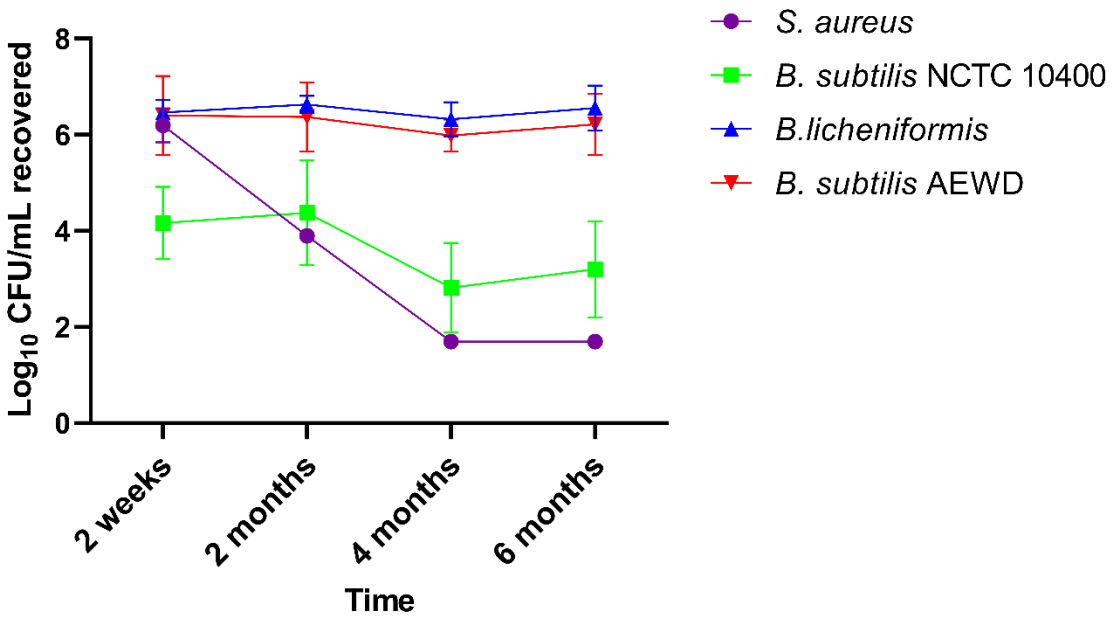


Figure 4.9. Log_{10} CFU/mL recovered of single species DSB after extended periods of drying. DSB were left in a controlled environment and tested for culturability at 2 weeks and 2-, 4- and 6-months following formation. Both *B subtilis* AEWD and *B. licheniformis* log_{10} recovery counts remained high (circa 6 log_{10}), whereas *B subtilis* 10400, although bacteria were still recovered at 6 months, was much lower. Log_{10} CFU/mL recovered from *S. aureus* DSB exponentially decreased from 2 weeks to 4 months. At 4 and 6 months no bacteria were culturable.

4.4.3 Transferability of long-term DSB

The transferability of bacteria embedded in DSB was also investigated at 2 weeks and 2, 4 and 6 months. Much like culturability data, transferability of DSB of both *B. licheniformis* and *B. subtilis* AEWD isolate remained at high, a 100% transfer rate was observed throughout the 6-month period, there was no statistically significant difference between % transfer over 6 months (One-way ANOVA, $p > 0.05$) (**Figure 4.10**). Transferability of *B. subtilis* 10400 akin to culturability data, there was no statistically significant difference between % transfer from 2 weeks to 6 months (One-way ANOVA, $p > 0.05$). Although transferability fluctuated over 6 months, there was always direct transfer of bacteria from DSB. Transferability was lowest at

4 months (avg. 70.4%), where variability between biological replicates was also highest (**Figure 4.10**). Similarly, to *B. subtilis* 10400, transferability of *S. aureus* DSB followed that of culturability data (**Figure 4.9**). After 2 weeks of drying, a 100% transfer was found, soon after there was an exponential statistically significant decrease (ANOVA, Tukey's, $p < 0.05$) in direct transfer from DSB, of 97.2%, from 2 weeks to 4 months (**Figure 4.10**). Unsurprisingly, after 6 months of drying, there was no direct transfer of *S. aureus* from DSB to agar.

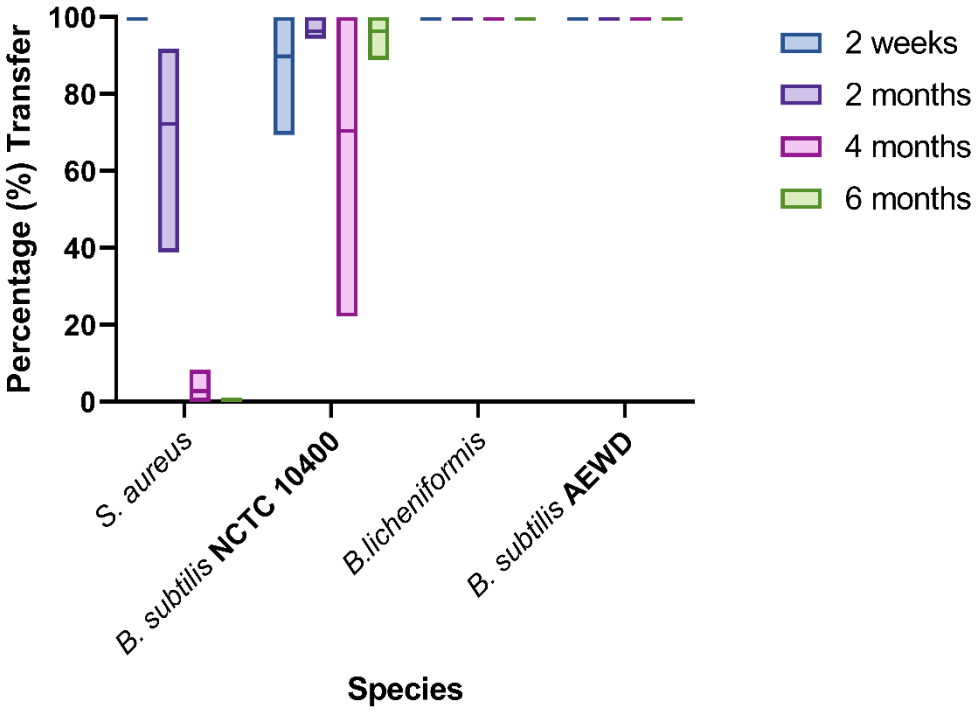


Figure 4.10. Percentage (%) transfer of single species DSB directly to new surface following long-term incubation. Transfer tested after 2 weeks and 2, 4 and 6 months of drying in a controlled environment. *B. licheniformis* and *B. subtilis* AEWD displayed 100% transfer from DSB over 6-month period. *B. subtilis* 10400 fluctuated over 6 months, with lowest % transfer at months. *S. aureus* decreased exponentially from 2 weeks, where transfer was 100%, to 4 months. At 6 months there was no transfer from DSB. Percentage transfer calculated as number of squares with positive growth, divided by 36, multiplied by 100.

4.4.4 Survival of spores in *Bacillus* spp. DSB

All three *Bacillus* spp. were investigated for the presence of spores within DSB over the 6-month period. Even after 2 weeks, no spores were recovered on agar following heat treatment of *B. subtilis* NCTC 10400 (**Figure 4.11**). There was a statistically significant difference between Log_{10} CFU/mL recovered *vs.* Log_{10} spores/mL recovered between each respective incubation time from 2 weeks to 6 months (ANOVA, Šidáks, $p < 0.05$). On the other hand, *B. subtilis* AEWD had extremely consistent spore counts of 4 log_{10} throughout each time point of 6-month period, no overall statistically significant difference in spores/mL recovered was identified from 2 weeks to 6 months (One-way ANOVA, $p < 0.05$) (**Figure 4.11**). *B. licheniformis* spore counts were much more variable that what was previously seen with whole DSB counts. A statistically significant difference between log_{10} CFU/mL recovered and spores/mL recovered was identified at 2 weeks and 2 months (ANOVA, Šidáks, $p < 0.05$). At 2 months, spore counts dropped, due to the wide range of values from biological replicates (**Figure 4.11**). At 4 months, spore counts increased by 3.6 log_{10} and remained high at 5 log_{10} by 6 months (**Figure 4.11**). At 2 weeks, 4 and 6 months, spore counts are more consistent than what was identified at 2 months, and the highest of all three *Bacillus* spp.

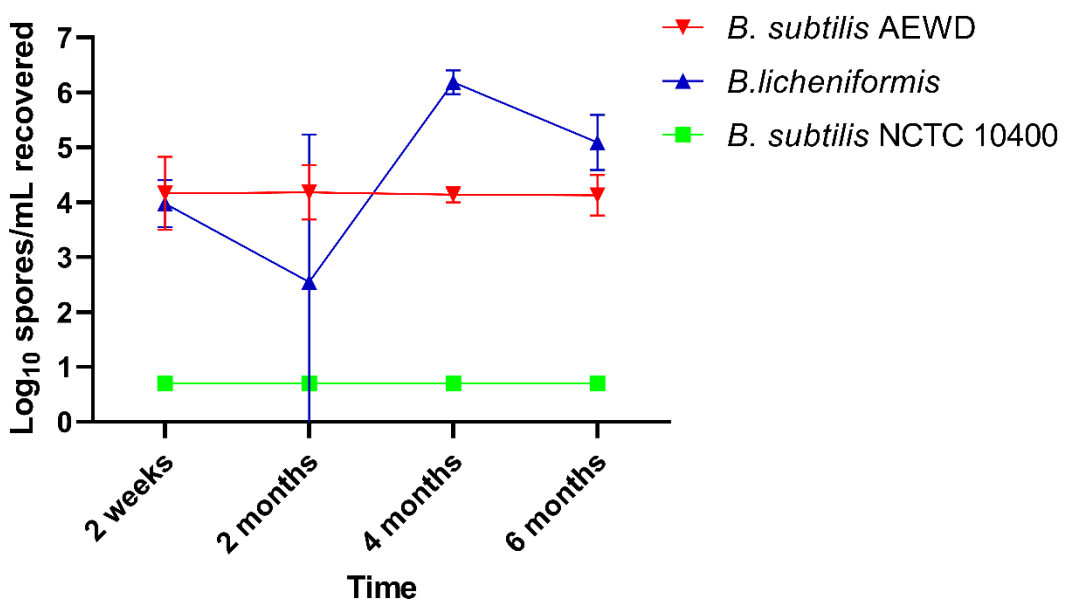


Figure 4.11. Spore counts expressed as Log_{10} spores/mL recovered from DSB of *Bacillus* spp. over 6 months. Nothing was recovered from *B. subtilis* NCTC 10400 after 2 weeks. *B. subtilis* AEWD was very consistent, and spores recovered remained at 4 log_{10} over 6 months. *B. licheniformis* spore counts fluctuated, decreasing at 2 months where there was a lot of variation between biological repeats. At 4- and 6-months counts were the highest from all species.

4.4.5 SEM images of long-term DSB

4.4.5.1 *Bacillus subtilis* AEWD

There is evidence of the presence of high organic load at all ages of DSB over 6months (**Figure 4.12**). DSB appear to be densely packed with cells covering the full disc surface. When comparing all ages of DSB, the structure of the biofilm looks very similar, coinciding with consistencies identified in culturability and transferability data.

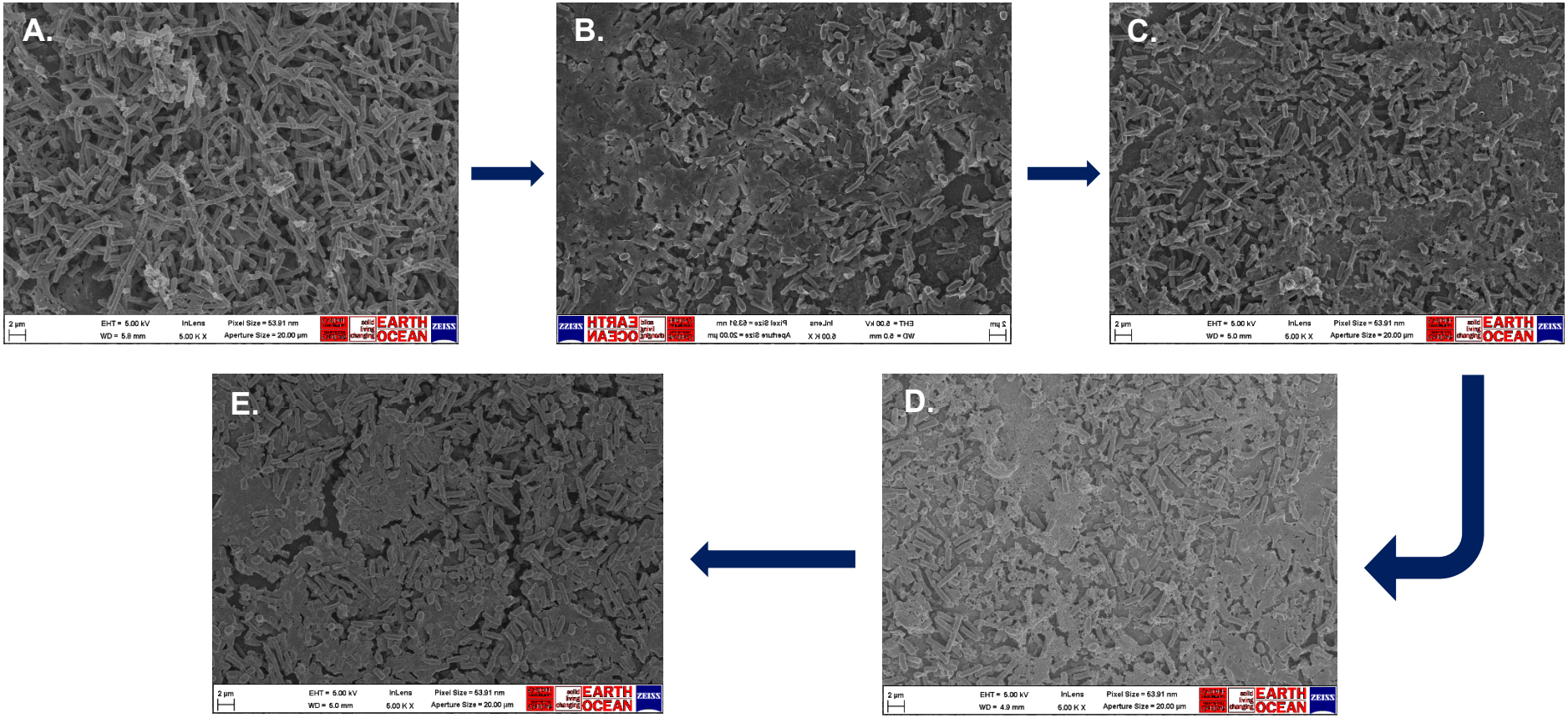


Figure 4.12. SEM images of *B. subtilis* AEWD DSB after long-term incubation periods. SEM images taken at x5000 magnification and representative of whole disc surface. DSB at (A) 12 days, (B) 2 weeks, (C) 2 months, (D) 4 months and (E) 6 months.

4.4.5.2 *Bacillus licheniformis*

Similarly, to what we have observed previously with *B. subtilis* AEWD, DSB of *B. licheniformis* did not change structurally over 6 months (**Figure 4.13**). Evidence of a matrix is apparent at all DSB ages. At 6 months, cell density is still extremely high, and cells form large aggregates over the disc surface. DSB are homogenous at all ages.

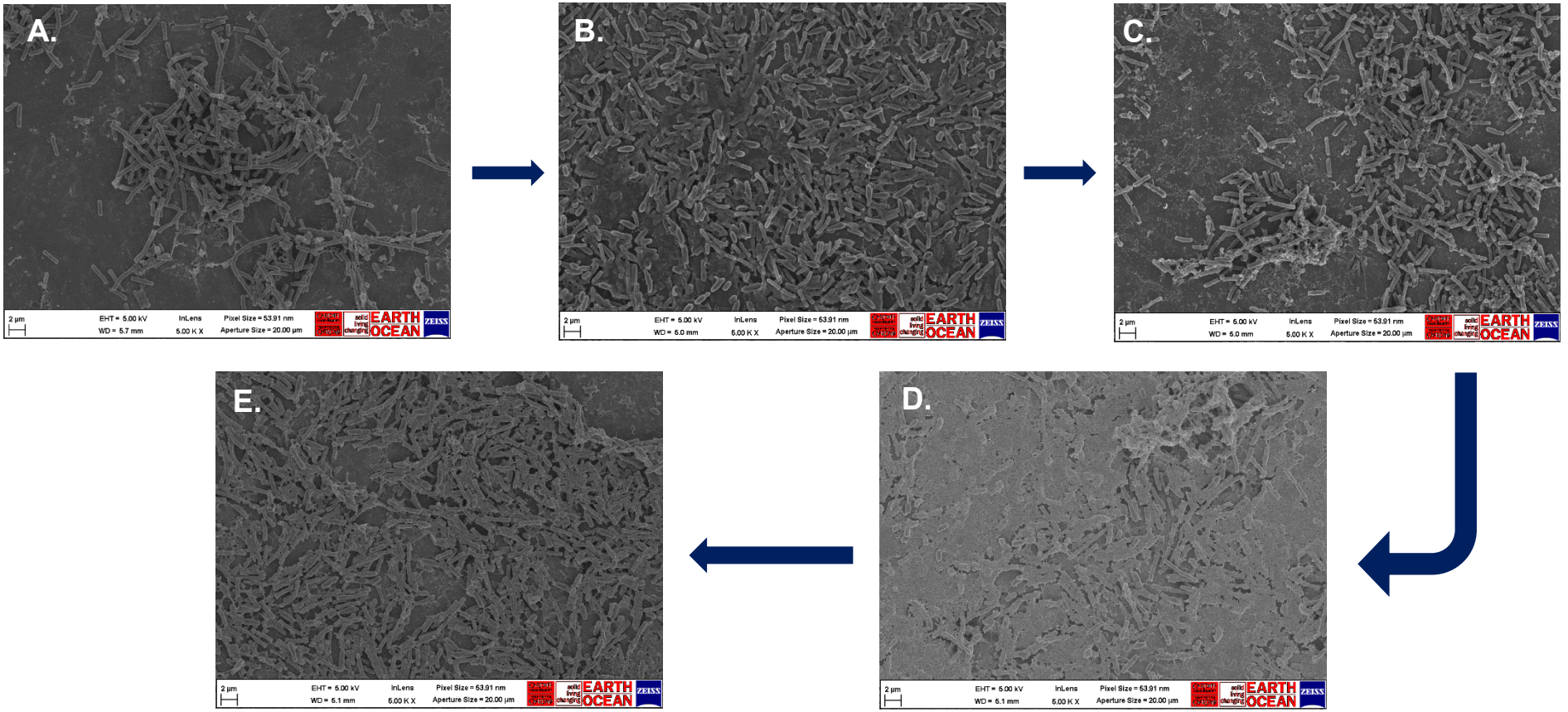


Figure 4.13. SEM images of *B. licheniformis* DSB after long-term incubation periods. SEM images taken at x5000 magnification and representative of whole disc surface. DSB at (A) 12 days, (B) 2 weeks, (C) 2 months, (D) 4 months and (E) 6 months.

4.4.5.3 *Bacillus subtilis* NCTC 10400

DSB of *B. subtilis* 10400 are sparser than those of the other two *Bacillus* spp. However, there was evidence of cells embedded in a matrix at all four ages of DSB (**Figure 4.14**). At 6 months, there are large aggregates of bacterial cells which is not as prominent at both 2 and 4 months. EPS is evident at all periods of time. At 4 months, cells are more scattered around the surface of the disc.

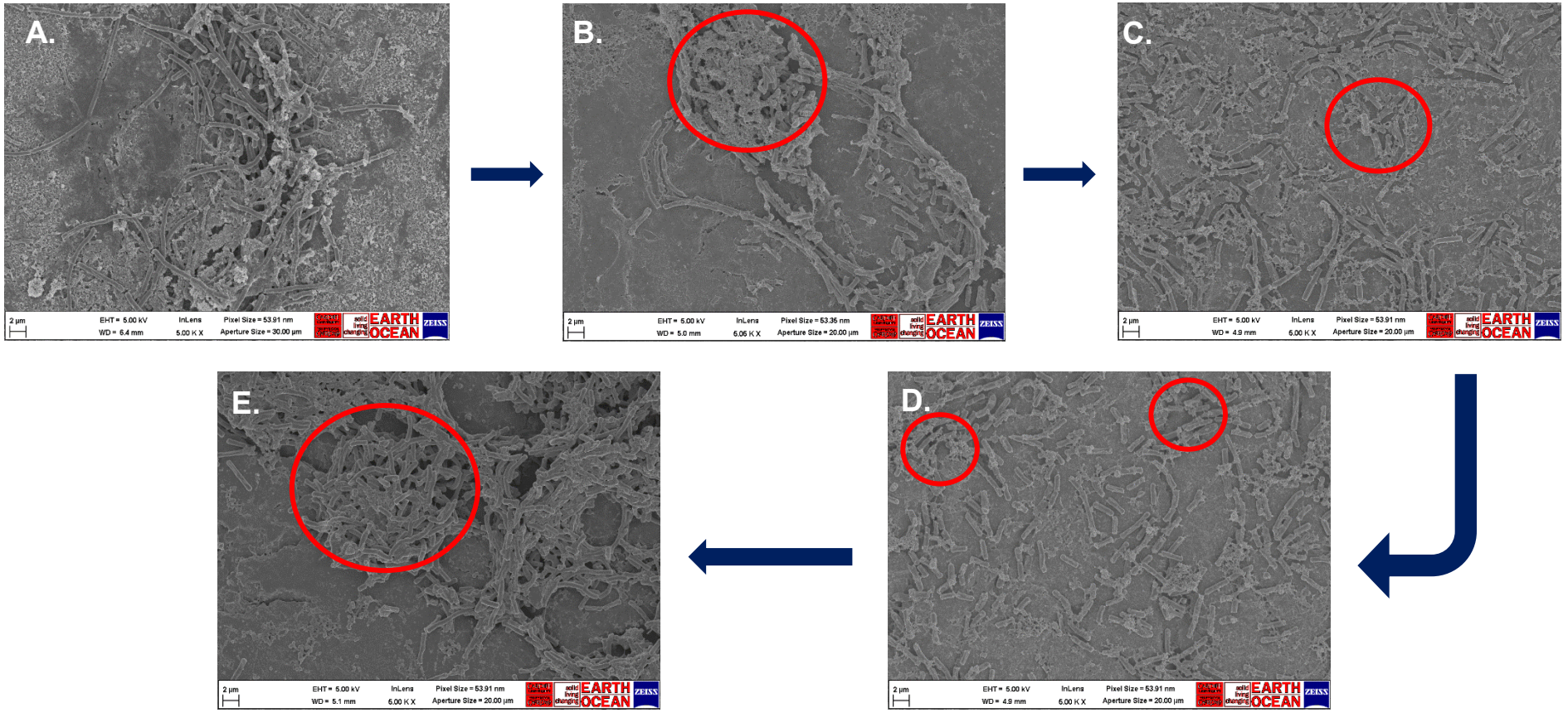


Figure 4.14. SEM images of *B. subtilis* NCTC 10400 DSB after long-term incubation periods. SEM images taken at x5000 magnification and representative of whole disc surface. DSB at (A) 12 days, (B) 2 weeks, (C) 2 months, (D) 4 months and (E) 6 months. Red circles indicate presence of matrix.

4.4.5.4 *Staphylococcus aureus*

Unlike *Bacillus* spp., DSB of *S. aureus* appeared structurally different throughout the ages. At 2 weeks, it was apparent that a dense biofilm had formed, cells were tightly packed and embedded in a matrix which we could see from the layers of the biofilm (**Figure 4.15b**). The biofilm was homogenous and covered the whole disc surface. Whereas at 2 months DSB appeared to have dense clumps of bacterial cells and EPS, but some areas of the disc surface only had a single layer of cells (**Figure 4.15c**). At both 4 and 6 months, the biofilm had changed structurally and appeared dense, it is important to note that here, although culturability and transferability data were null, *S. aureus* cells are still present on the surface of the disc (**Figure 4.15d&e**).

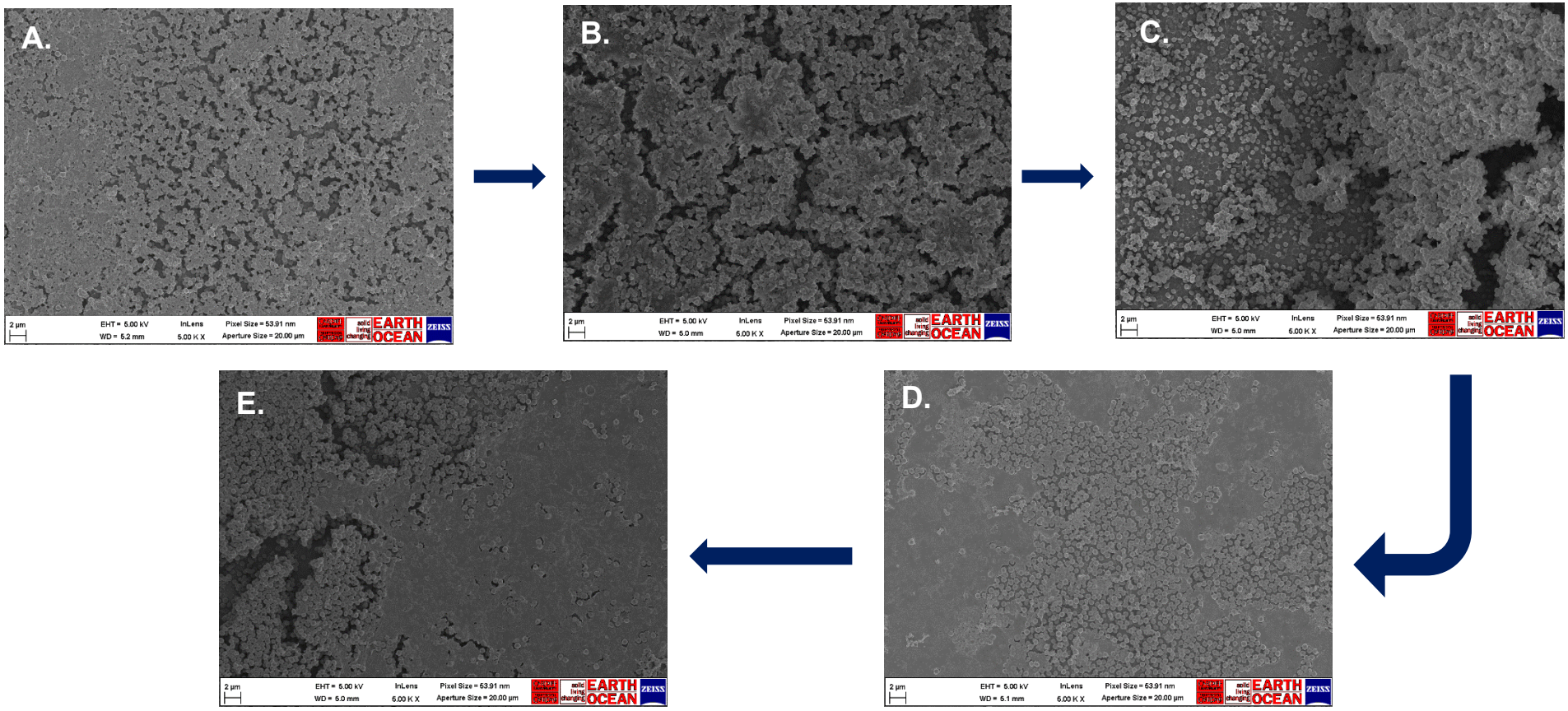


Figure 4.15. SEM images of *S. aureus* DSB after long-term incubation periods. SEM images taken at x5000 magnification and representative of whole disc surface. DSB at (A) 12 days, (B) 2 weeks, (C) 2 months, (D) 4 months and (E) 6 months.

4.5 Discussion

This is the first study investigating the structure of DSB over extended periods of time and DSB changes in structure and complexity whilst in a laboratory setting. SEM images showed the uniformity of DSB at different developmental stages. The unevenly scattered clusters of bacteria, especially within the *B. licheniformis* and *B. subtilis* NCTC 10400 biofilms, were akin to DSB found in healthcare environments (Almatroudi et al., 2015). However, environmental DSB tend to be more heterogenous, most likely due to the multiple species making up the biofilm (Hu et al., 2015). SEM imaging confirmed full coverage of EPS in DSB of environmental isolate of *B. subtilis* compared to the wildtype strain *B. subtilis* NCTC 10400. Rods of *B. subtilis* NCTC 10400 embedded in DSB showed evidence of elongated rods and formed long chains which has been evidenced at the initial stages of biofilm formation in *B. subtilis* strains previously (Branda et al., 2006). The large quantity of EPS in *B. subtilis* AEWD DSB after 12 days was expected, as reported by Martin et al. (2015 & 2008). However interestingly, at 4 days, *B. subtilis* AEWD DSB was less dense and displayed a lack of biofilm coverage across the surface of the disc, highlighting the importance of a longer development time in order to produce a thick DSB. Martin et al. (2008) showed the *B. subtilis* AEWD isolate heavily embedded in thick EPS when in hydrated biofilm, after viewing under SEM at x10,000 magnification, which was similar to what was seen in the 12-day DSB. These results importantly tell us that no matter what state *B. subtilis* AEWD, whether that be wet or dry biofilm, EPS remains thick. it appears that species which do not produce as robust DSB at the end of formation (12 days) did not change much structurally over this period, as evidenced by *B subtilis* NCTC 10400. All other species including *S. aureus*, appeared to have an increase in cell density and overall structural integrity of DSB over time. SEM imaging of the same *S. aureus* strain DSB by Ledwoch et al. (2019) showed similar cell density and proportion of cell clusters within the biofilm after 12 days. Although *S. aureus* was not culturable at 4 and 6 months, the evolution of the structural integrity of *S. aureus* over the 6-month period has shown that the species is visible on dry surfaces over long periods of time.

Environmental DSB have been shown to survive up to 12 months on healthcare surfaces (Hu et al., 2015). *Bacillus* spp. have been identified within these DSB (Ledwoch et al., 2018). After long-term incubation from 2 weeks up to 6 months, we have demonstrated the culturability, transferability and structural integrity of *B.*

subtilis AEWD and *B. licheniformis* DSB. This has highlighted the importance of non-pathogens for long term survival on surfaces, as we have shown culturability and transferability of DSB of *S. aureus* decrease after just 2 months. *B. subtilis* NCTC 10400 showed a much more varied response to long-term incubation. Although SEM images show bacteria covering the surface of the disc, embedded in what appears to be a matrix of EPS, CFU counts were not as high as other *Bacillus* spp. With this in mind, spore counts were also extremely low as none were recovered. Whereas *B. subtilis* AEWD had a 4 $\log_{10}$ recovery of spores from DSB over 6 months, which remained stable much like whole DSB counts. It is well known that spores are resistant to desiccation (Setlow, 2014), which will impact on the survival of *B. subtilis* AEWD and *B. licheniformis* over long-term incubation and desiccation. Interestingly at 2 months, variation amongst replicates of *B. licheniformis* spore counts was inconsistent with data from 2 weeks, 4 and 6 months and whole DSB CFU counts. This variability may be attributed to natural variation within population production of spores.

All *Bacillus* spp. were recoverable and transferable after 6 months. *B. subtilis* AEWD and *B. licheniformis* were again, consistent with whole CFU counts and displayed a 100% transfer from DSB throughout the drying periods. Although *B. subtilis* NCTC 10400 fluctuated, similarly to culturability data, bacteria were still transferred from DSB. Transfer of bacteria from surfaces to near patient areas plays a crucial role in the spread of disease throughout hospital environments, which has been evidenced by DSB of *S. aureus* (Chowdhury et al., 2018). As *Bacillus* is a common genus found in the environment, this data suggests that DSB can be readily transferred to new sites, all it requires is the touch of a worker's hands to spread bacteria from one surface to another.

S. aureus however displayed a different pattern that was consistent between culturability and transferability data (**Figure 4.16**). After 2 weeks of drying, high CFU counts and % transfer was observed. Following this an exponential decrease leading to no recoverable bacteria either from culture or transfer, was identified (**Figure 4.16**). SEM images presented displayed *S. aureus* embedded in DSB up to 6 months, but the transferability and culturability of bacteria were low at this point. This work was limited as we only tested for culturable bacteria and did not examine viability of bacteria embedded in DSB through live/dead staining. *S. aureus* follows a similar pattern with both transferability and culturability, whereby DSB were not

culturable at the same time they were not transferred to a new surface. Although not in a biofilm, *S. aureus* has been shown to survive on stainless steel surfaces for only 96 hours (Fuster-Valls et al., 2008). Here, we have shown, when embedded in a DSB, *S. aureus* does survive for prolonged periods, both culturable and transferable up to 2 months, which may present an advantage to the pathogenic nature of the species and increase infection risk from healthcare surfaces.

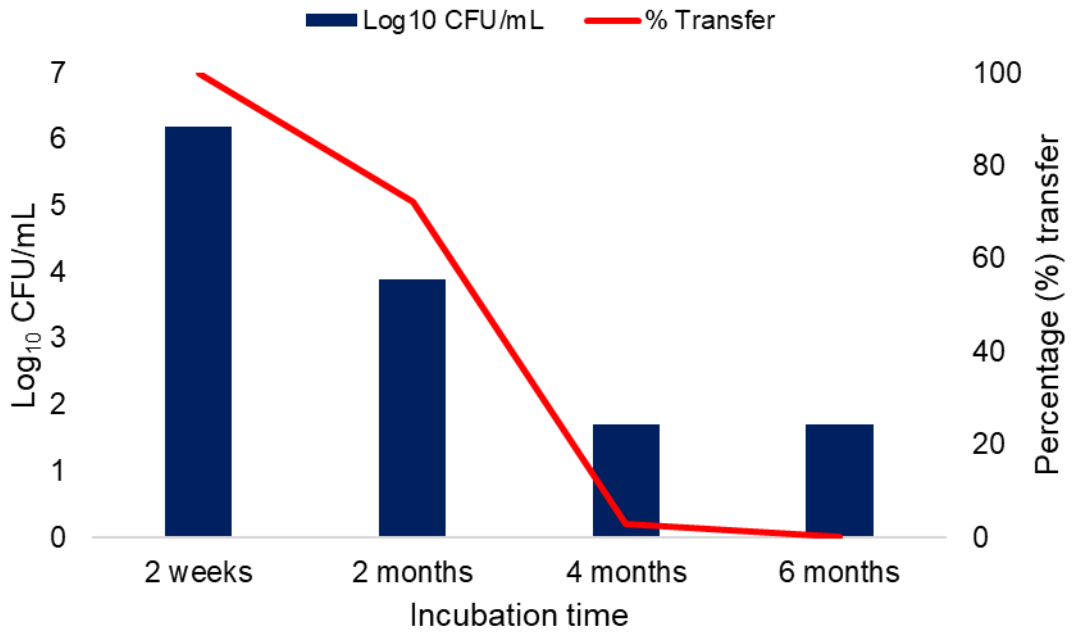


Figure 4.16. *S. aureus* transferability (%) and culturability (Log_{10} CFU/mL) over 6 months of incubation. Transferability and culturability decreased to below detectable limits after 4 and 6 months of incubation.

4.6 Chapter conclusions

We have shown that over 12 days of formation, DSB did change structurally and develop to form the mature biofilm. Environmental isolates of *Bacillus* spp. can survive for extended periods of time on surfaces and be readily transferred which, in part, may be due to the presence of spores. *S. aureus* is much less resistant to desiccation than the other species tested and, at a certain point in time, is not culturable or transferable but the presence of a biofilm on the disc surface is still noted. If species are not transferable, they may not present a risk to the healthcare environment. The addition of viability staining would be an important factor to assess

for VBNC cells within DSB in the future. The results presented here have implications on healthcare and environmental decontamination, if a surface is not cleaned/disinfected properly, bacteria are able to survive as DSB for extended periods of time.

Chapter 5
Resistance of Single Species Dry Surface Biofilms to Disinfectant Products

5.1 Introduction

Healthcare-associated infections (HCAI) affects approximately 20% of NHS patients in the UK. With appropriate intervention methods, such as improved hand and environmental hygiene, use of personal protective equipment and screening, 20-30% of HCAIs could be prevented (National Audit Office, 2009). Bacteria can persist on environmental surfaces for days, months and even years if surfaces have not been decontaminated effectively (**Table 5.1**) (Kramer et al., 2006). Survival on surfaces, coupled with prior occupation of a room with a patient infected with an MDRO has proven to increase risk of acquisition (Mitchell et al., 2015). Detergents are being used mostly for routine cleaning, although cleaning methods vary between healthcare facilities. Chlorine-based high-level disinfectants are required for terminal or specialised cleaning of areas exposed to multi-drug resistant bacteria (Dancer, 2016).

Table 5.1. Range of persistence on dry surfaces of common bacterial species responsible for infection within hospitals (Adapted from Kramer et al., 2006).

Species	Persistence range
Acinetobacter spp.	3 days to 5 months
Escherichia coli	1.5 hours to 16 months
Klebsiella spp.	2 hours to > 30 months
Pseudomonas aeruginosa	6 hours to 16 months
Staphylococcus aureus (inc. MRSA)	7 days to 7 months

Hydrated biofilms, a consortium of organisms housed in an exopolymeric matrix in an environment with high relative humidity, account for 68% of total HCAI and are commonly found on medical devices such as urinary catheters and endoscopes (Percival et al., 2014), ventilators (Dewi et al., 2021), sink, shower, pipes, water storage and tanks and generally drain systems in healthcare settings (Hayward et

al., 2020; Ledwoch et al., 2020). Studies have demonstrated the high tolerance of wet biofilms to antimicrobials compared to their planktonic equivalents including peracetic acid (Akinbobola et al., 2017), benzalkonium chloride and chlorhexidine gluconate (Smith and Hunter, 2008). However, evidence for the eradication and removal of dry biofilms remains limited within the literature. Although there is some controversy on a clear definition of DSB (Nkemngong et al., 2020) commonly, DSB are described as biofilms which have been exposed to desiccation, reduced nutrient resources and periodic disinfection on clinical surfaces (Almatroudi et al., 2015; Ledwoch et al., 2019a). There is currently no standard method for detecting DSB in the environment, or in a laboratory setting.

DSB are prevalent within healthcare environments all around the world, they have been identified in hospitals in Australia and Saudi Arabia (Johani et al., 2017) and in the UK (Ledwoch et al., 2018). But DSB seem to have enhanced resilience to the common detergents and disinfectants used within hospitals (Almatroudi et al., 2015; Chowdhury et al., 2018). DSB can harbour pathogenic bacteria, some of which are associated with outbreaks or HCAI, which compel testing the impact or efficacy of cleaning regime and disinfection practices such as hydrogen peroxide vapour (HPV) fumigation and disinfectant products, such as chlorine, against DSB. Chowdhury et al. (2018) found that dry biofilms disperse bacterial cells of pathogen *Staphylococcus aureus*, even though they are dehydrated. Although bacterial numbers were less than that released from hydrated biofilms, it still impacts on bacterial dissemination and potential outbreaks. This is especially pertinent when regarding multi-species biofilms as many harbour different pathogenic MDRO species which can contribute to infection risk within healthcare environments.

The Wiperator (FitaFlex) was developed to enable surface testing of wipe products at a constant pressure and time (Sattar et a., 2015). Ledwoch et al. (2021a) investigated the reduction in viability of *S. aureus* DSB when treated with commercially available disinfectants in wipe form. They concluded that several of the disinfectant products were effective in killing/removing bacteria embedded in DSB, but this test alone was not sufficient in determining product performance. Indeed, both reduction in microbial bioburden combined with measuring bacterial transfer post-wiping were recommended to be a better indicator of product performance (Ledwoch et al., 2021a).

Overall, the number of studies that have looked at the resistance of DSB to disinfectant products and commercially available products is still limited (**Table 5.2**).

Table 5.2. Studies conducted on DSB and their resilience to disinfectants/commonly used products or detergents. Outlined are the test protocols followed, what species was used and what the outcome of the study was.

Reference	Testing procedure	Products used	Species tested	Results
Almatroudi et al. (2016)	Exposure to liquid disinfectant to measure eradication of DSB and regrowth	Sodium hypochlorite (1,000-20,000ppm)	*S. aureus*	Reduced DSB counts by 7 log_{10} but regrew after incubation
Almatroudi et al. (2018)	DSB exposed to hot-air oven, water bath or autoclave	Heat treatment	*S. aureus*	DSB remained culturable after exposure to dry heat (100ºC) for 60 min
Chowdhury et al. (2018)	Transfer by glove	5% neutral detergent	*S. aureus*	Enough bacteria to cause infection were transferred after one touch
Ledwoch and Maillard (2018)	Wiping to measure eradication of DSB, transfer and regrowth	Commercially available wipe disinfectant products	*Candida auris*	50% of products failed to decrease viability, 58% did not prevent transfer from DSB and 75% did not stall regrowth
Ledwoch et al. (2019b)	Wiping to measure eradication of DSB, transfer and regrowth	Sodium hypochlorite (1,000ppm) on microfibre cloth	*S. aureus*	Circa. 4 log_{10} reduction in viability, high transfer rate of DSB (68% avg.) and regrowth quick (2.8 days avg.)
Ledwoch et al. (2021a)	Wiping to identify transfer	Sodium hypochlorite (1,000ppm)	Multi-species DSB (environmental sample from keyboard)	54% of isolates transferred post-wiping
Ledwoch et al. (2021b)	Wiping to identify reduction in viability, direct transfer, cross contamination and regrowth	Commercially available disinfectant and detergent products	*S. aureus*	9/11 products effective in eradicating DSB, recovery in less than 2 days after from 8/11 products and only 2 products reduced direct and cross contamination transfer
Tahir et al. (2018)	Transfer by latex, nitrile and surgical gloves	5% neutral detergent	*S. aureus*	Bacteria transmitted by all types of gloves

5.2 Aims and Hypotheses

The first aim of this chapter was to determine the susceptibility of single species DSB to key disinfectants of in biocidal products. Two environmental *Bacillus* spp. isolates, one standard culture collection strain of *Bacillus subtilis* and *S. aureus* were used. It was hypothesised that different disinfectants would not be able to eradicate DSB, based on the literature on DSB resilience and high transfer rate post-intervention.

The second aim was to identify commonly used disinfectant wipe products in healthcare settings in the UK, and test these against single species DSB, monitoring not only kill/reduction in viability, but also transfer to a new surface with a wipe and direct transfer of DSB post-wiping. It was hypothesised that i) disinfectant wipe products would prevent direct transfer and surface transfer of bacteria embedded in DSB and ii) products would cause a limited reduction in viability, failing to completely eradicate DSB.

5.3 Methods

5.3.1 Carrier Test Experiments with Single Species Dry Biofilms

DSB were prepared in accordance with Chapter 3., section 3.5. Neutraliser toxicity tests were conducted before testing (please refer to Chapter 3, section 3.10). To test for susceptibility to different disinfectants, single species dry biofilms were used in a carrier test experiment, adapted from EN 13697:2015 carrier test (EN 13697, 2015). Carrier tests were chosen as they are the easiest way to measure the efficacy of disinfectant products of DSB without adding another factor such as mechanical action. Methods for the carrier tests are outlined in Chapter 3 section 3.6. Four key disinfectants of known disinfectant wipes were investigated, including benzalkonium chloride (BZK), peracetic acid (PAA) and chlorine. The amount of BZK and PAA were based on concentrations used in current commercially available disinfectant wipes (GAMA Healthcare, UK) (Chapter 3, section 3.6). However, due to proprietary reasons the disinfectant concentrations cannot be disclosed.

DSB of *Staphylococcus aureus* NCTC 10788, *Bacillus licheniformis* ATCC 14580, *Bacillus subtilis* NCTC 10400 and *Bacillus subtilis* automated washer endoscope isolate (AEWD) were prepared as described in Chapter 3, section 3.5. DSB were tested at each of the dehydration phases during biofilm development: 4 , 8 and 12 days. Testing at each timepoint meant that we could use the disinfectants as a

marker of emerging biocidal resistance during biofilm formation *in vitro*. Each DSB was exposed to 1 mL of the disinfectant, enough to completely submerge the disc, for 5 min, the control was left dry without treatment. Two environmental species, *Bacillus subtilis* AEWD and *B. licheniformis* ATCC 14580 were also exposed to the disinfectants for a 60 min treatment time due to their high resistance to 5 min treatment. As *Bacillus* spp. is a known spore former, all three *Bacillus* spp. were also tested for the potential contribution of spores on the efficacy of NaDCC, BZK and PAA against single species DSB. Following exposure to the disinfectant, discs were taken out and added into a neutralising solution. Bacteria that remained in DSB were revived in accordance with Chapter 3, section 3.8 and quantified on TSA plates as described in Chapter 3, section 3.9.

5.3.2 Wet Biofilm Experiments

Wet biofilms of each species were tested to provide a comparison of bactericidal efficacy with 12-day mature DSB, biofilms being more tolerant to antimicrobial treatment than planktonic bacteria (Stewart, 2015).

Planktonic cultures for all species were prepared in 20 mL of sterile TSB and left to grow overnight for 18 hours at 37 ºC in a shaking incubator (Sanyo orbital incubator) at 120 rpm. After incubation, cultures were centrifuged, resuspended, and diluted to 1 x 10^7 CFU/mL. Bovine serum albumin (BSA) representative of soiling on hospital surfaces, was added into cultures before testing. A 1 mL culture suspension (1 x 10^7 CFU/mL) was placed onto a stainless-steel disc in a 24 well plate and left on an orbital shaker at 200 rpm for 48 hours, the same time as the first wet phase in DSB formation. A modified carrier test experiment, without desiccation onto the surface of the disc, was then performed as outlined in section 5.3.1.

5.3.3 Quantification of spores from *Bacillus* biofilms

To determine if there was any impact of *Bacillus spp.* spores formed within the DSB on the efficacy of biocidal products (as outlined in section 5.3.1), 1mL of BZK, PAA and NaDCC solution was added to DSB and a contact time of 5 min was used. DSB were vortexed for 4 min and 1 mL of DSB suspension was pipetted into an Eppendorf and placed in a dry bath for 30 min at 80 ºC to kill any vegetative cells within the sample. The samples were subsequently diluted and plated as described previously to enumerate spores within the sample. No treatment controls were performed in the same manner without exposure to disinfectants to determine the quantity of spores within the DSB. Untreated DSB samples were viewed using Leica

DM IL LED light microscope, using phase contrast to highlight any refringent spores within samples.

5.3.4 Statistical analysis

Statistical analysis was carried out, where appropriate, using GraphPad Prism 9 software (version 9.3.1). Two-way ANOVA and t-test were used to determine statistically significant differences within datasets (Chapter 3, section 3.11). Post-hoc tests were used for multiple comparisons.

5.4 Results

5.4.1 DSB Growth

DSB were tested at 4, 8 and 12 days, indicating the three stages of desiccation during DSB development. *S. aureus*, *B. licheniformis* and *B. subtilis* AEWD all displayed similar $\log_{10}$ recovery of bacteria embedded within DSB after 12-days (circa. 6.5 $\log_{10}$) (**Figure 5.1**). At 4-, 8- and 12-days bacterial viability was also consistent and remained within the range of 6 – 7 $\log_{10}$ for all three species independently of one another. On the other hand, *B. subtilis* NCTC 10400 displayed much lower bacterial viability, an average of 5.1 $\log_{10}$ was observed between 4, 8 and 12 days. Viability of *B. subtilis* NCTC 10400 was between 1.6 – 2.2 $\log_{10}$ less than the other three species at 8- and 12-days, bacterial counts were not consistent over the three desiccation phases (**Figure 5.1**). After 4 days of growth avg. $\log_{10}$ reduction was 5.9, 0.6 $\log_{10}$ more than at 12 days. Overall, quantity of bacteria embedded in DSB of all species did not change much from 4 to 12 days. There was no statistically significant difference between DSB counts at 4-, 8- and 12 days for each species respectively (One-way ANOVA, $p > 0.05$).

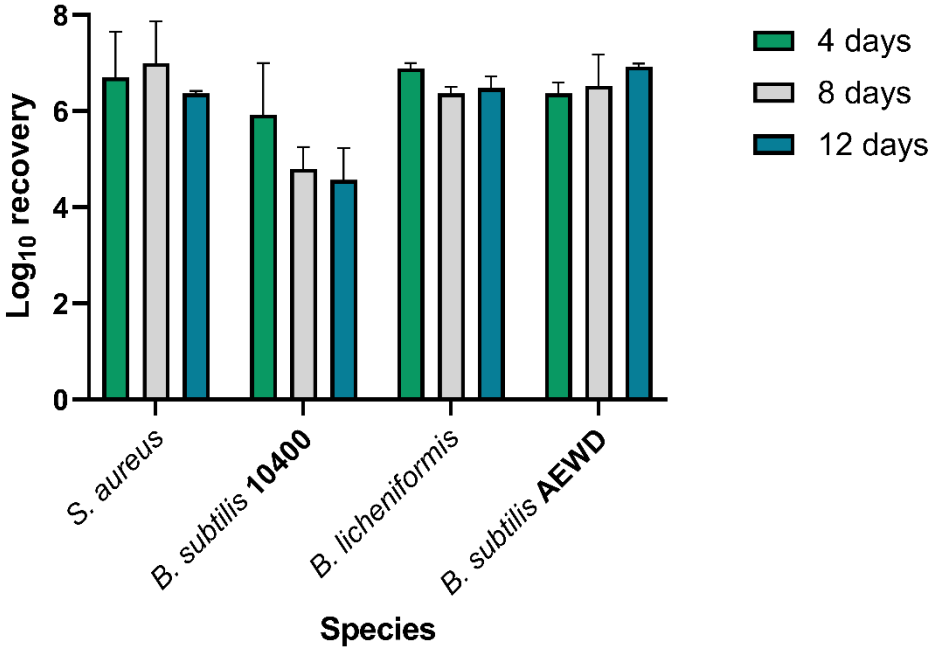


Figure 5.1. Log_{10} recovery of all species after 4-, 8- and 12-days growth. The quantity of bacteria embedded in DSB remained consistent between all three DSB ages for all species. Whilst *S. aureus*, *B. licheniformis* and *B. subtilis* AEWD displayed log_{10} recovery values circa 6.5 log_{10}. Whereas *B. subtilis* NCTC 10400 remained lower at 5.1 log_{10}.

5.4.2 Single species DSB Susceptibility to Disinfectants

The following section presents the susceptibility of four bacteria: *S. aureus*, *B. subtilis* AEWD, *B. subtilis* NCTC 10400 and *B. licheniformis* to commonly used disinfectants in biocidal products. Presented data outlines the difference between efficacy of disinfectants on DSB survival versus the untreated control, and the impact of DSB maturation on susceptibility to disinfectants. Results in figures are stated as % reduction. This value means % reduction of bacteria embedded in DSB calculated as the quantity of recovered bacteria from untreated control minus the quantity of recovered bacteria from disinfectant treated samples. Log_{10} CFU/mL was taken before and after treatment and % reduction is calculated from these two values. By doing so, the data is better visualised and represented in the figure.

Each DSB was exposed to NaDCC (1,000 ppm), BZK and two concentrations of PAA for 5 min at room temperature (21ºC) and relative humidity (40±5%). The PAA concentrations are described as “high” and “low”, both within the range of 500-4,000 ppm, low being 500 ppm and high being 4000 ppm for reference. BZK concentration used was < 1% w/v. Results presented include a minimum of three biological replicates. Following initial experiments, it was identified that NaDCC at the original concentration (1,000 ppm) had little effect on biofilm kill in comparison to the other disinfectants used, < 2 $\log_{10}$ reduction was reported for all species, and so NaDCC at a concentration of 10,000 ppm was also added to this set of experiments.

5.4.2.1 *Staphylococcus aureus* NCTC 10788

Overall, there was a significant difference between each disinfectant and their respective untreated controls at all 3 stages of biofilm development (ANOVA, Šidák, $p < 0.05$) (**Figure 5.2, Table 5.2**). PAA (high conc) and NaDCC (10,000 ppm) had the greatest bactericidal effect against *S. aureus* DSB at 4, 8 and 12 days (**Figure 5.2**). When exposed to the lower concentration of PAA, reduction in viability decreased from 5.5 $\log_{10}$ at 8 days to 2.0 $\log_{10}$ at 12 days. As DSB developed over the 12-day period, generally the efficacy of disinfectants decreased, particularly with NaDCC (1,000 ppm) and BZK (**Figure 5.2**). The higher concentration of NaDCC used had a lethal effect against DSB from 4 days, 71% reduction of DSB was observed.

No viable bacteria were recovered from 4-day DSB following exposure to all treatments; a statistically significant difference between treated samples and their respective untreated control was observed (ANOVA, Šidák, $p < 0.0001$). When 8-day DSB was exposed to both high and low concentrations of PAA, a $\log_{10}$ reduction of > 4.4 was observed (> 70% reduction in DSB) (**Table 5.2**).

When comparing 4 *vs.* 8 *vs.* 12-day DSB, exposure to BZK, PAA (at both high and low concentrations) and NaDCC (1,000 ppm) resulted in a statistically significant difference amongst all 3 biofilm ages (One-way ANOVA, $p < 0.05$). When exposed to NaDCC (10,000 ppm), no statistically significant difference between all ages of DSB was observed (One-way ANOVA, $p > 0.05$). A post hoc Tukey test revealed no significant difference between 4- and 8-day DSB when exposed to NaDCC (1,000 ppm), BZK and PAA (low conc) (ANOVA, Tukey test, $p > 0.05$). Following exposure to the high concentration of PAA there was no statistical difference between 8- and 12-day old DSB (ANOVA, Tukey test, $p > 0.05$).

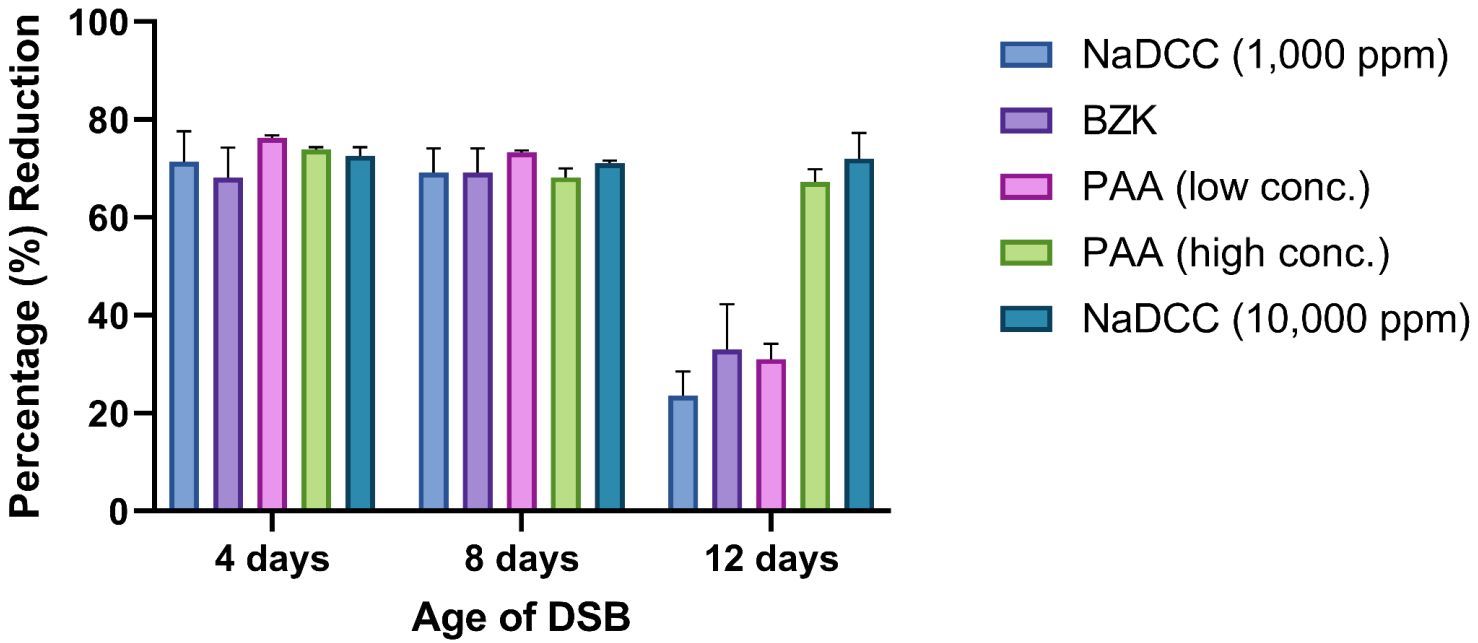


Figure 5.2. *S. aureus* DSB 5 min exposure to three disinfectants using modified carrier test. DSB exposure to disinfectants at three drying phases during biofilm formation. Reported values represent percentage (%) reduction, calculated from $\log_{10}$ values and standard deviation. The efficacy of NaDCC (1,000 ppm), BZK and PAA (low conc.) decreased over the 12-day formation, whereas both PAA (high conc.) and NaDCC (10,000 ppm) expressed high bactericidal activity (> 70% reduction in DSB survival) throughout biofilm development. (% reduction of bacteria embedded in DSB calculated as the quantity of recovered bacteria from untreated control minus the quantity of recovered bacteria from disinfectant treated samples).

Table 5.2. Statistical significance and average Log_{10} reduction values of each *S. aureus* DSB age when comparing treatment to respective untreated controls. Statistical significance calculated from Šidák's multiple comparison test (ANOVA). Stars indicative of statistical significance.

Disinfectant	Age of DSB (days)	Avg. Log_{10} reduction (± SD)	Statistical Significance^
NaDCC (1,000ppm)	4	4.7 (± 1.0)	****
	8	4.6 (± 1.0)	****
	12	1.5 (± 0.3)	*
BZK	4	4.5 (± 0.7)	****
	8	4.6 (± 1.0)	****
	12	2.1 (± 0.6)	***
PAA (low conc.)	4	5.5 (± 0.0)	****
	8	5.5 (± 0.0)	****
	12	2.0 (± 0.4)	****
PAA (high conc.)	4	5.7 (± 0.0)	****
	8	4.4 (± 0.0)	****
	12	4.4 (± 0.4)	****
NaDCC (10,000ppm)	4	4.5 (± 0.4)	****
	8	4.2 (± 0.1)	****
	12	4.9 (± 0.4)	****

^statistical significance values correspond to $p \leq 0.05$*, $p \leq 0.01$**, $p \leq 0.001$*** and $p \leq 0.0001$

5.4.2.2 *Bacillus licheniformis* ATCC 14580

Both BZK and NaDCC (1,000 ppm) decreased DSB counts by < 1 log_{10} respectively (≤ 6% reduction in total DSB) (**Figure 5.3**). Interestingly, both disinfectants had little effect on DSB, even at 4 days. There was no statistically significant difference between treated and untreated controls for NaDCC and BZK at each time point (ANOVA, Šidák, $p > 0.05$).

At 4 days, both PAA (high concentration) and NaDCC (10,000 ppm) produce ≥ 4.9 log_{10} reduction, > 75% reduction, of bacteria embedded in DSB (**Figure 5.3**). These numbers decrease too circa. 2.5 log_{10} reduction (< 40% reduction) at 8 days and remain low when DSB is 12 days old. The lower concentration of PAA appeared to decrease DSB survival at 8 and 12 days than 4 (**Figure 5.3, Table 5.3**). There was a significant difference between the high concentration of PAA treated samples and the untreated control at 4 and 12 days (ANOVA, Šidák, $p < 0.05$) (**Table 5.3**). The overall highest log_{10} reduction value recorded was at 4 days with the higher concentration of PAA, > 5 log_{10} or 80% reduction in total DSB as shown by Figure 5.2. However, PAA did have the largest log_{10} reduction values. There was a significant difference between the untreated control and NaDCC (10,000 ppm) over all three DSB drying phases (ANOVA, Šidák, $p < 0.05$). At both 8 and 12 days, NaDCC (10,000 ppm) had the largest impact on DSB survival out of all disinfectants used (**Figure 5.3**, **Table 5.3**).

When considering efficacy of disinfectants over DSB maturation, there was no significant difference between 4, 8 and 12 days when *B. licheniformis* DSB was exposed to NaDCC (1,000 ppm) or PAA (low concentration) (One-way ANOVA, $p > 0.05$). There was a significant difference between all DSB ages with both BZK and PAA (high concentration) (One-way ANOVA, $p < 0.05$), specifically this arose from 4 *vs.* 12-day DSB and 4 *vs*. 8-day DSB respectively (ANOVA, Tukey test, $p < 0.05$). DSB were least resistant to effects of NaDCC (10,000 ppm), there was an overall significant difference between log_{10} reduction values for all DSB ages (One-way ANOVA, $p < 0.05$), a post-hoc Tukey test showed this significance arose from 4-day DSB and the other two biofilm ages ($p < 0.05$).

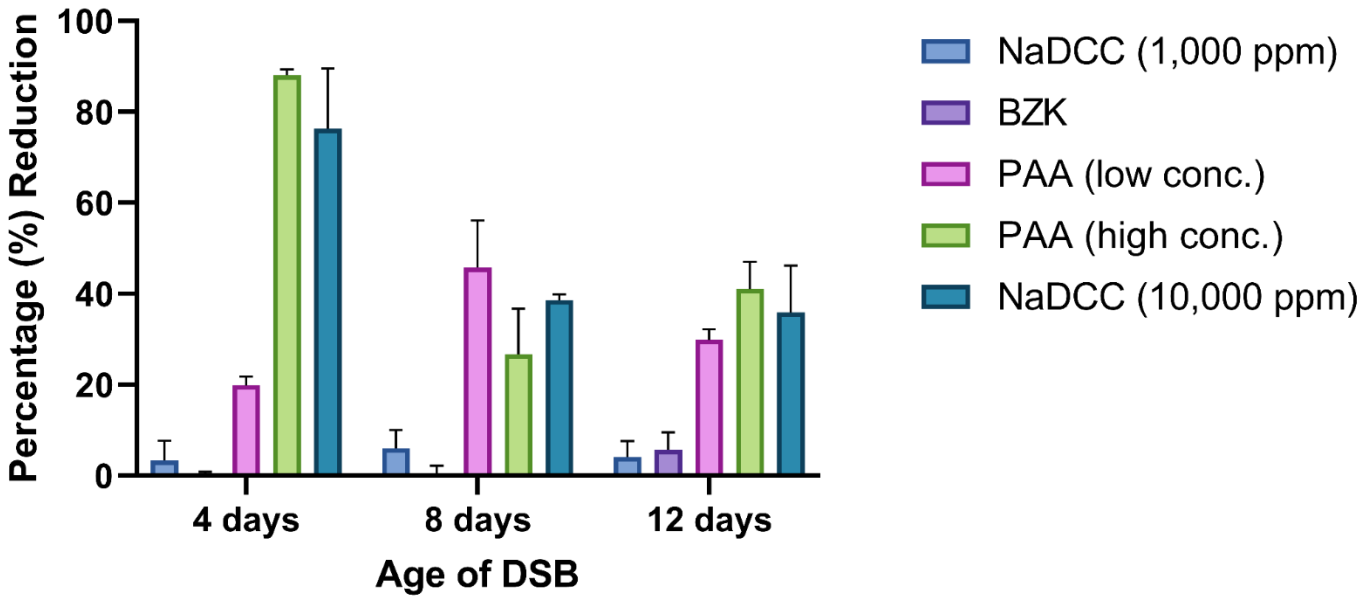


Figure 5.3. *B. licheniformis* DSB 5 min exposure to three disinfectants using carrier test. DSB exposure to disinfectants at the three drying phases during biofilm formation. Reported values represent percentage (%) reduction, calculated from $\log_{10}$ values and standard deviation. After 4 days of DSB development, PAA (high conc.) and NaDCC (10,000 ppm) were effective in reducing bacteria embedded in DSB by > 70%. Whereas, at all stages of DSB development both NaDCC (1,000 ppm) and BZK did not possess bactericidal activity on *B. licheniformis* DSB. (% reduction of bacteria embedded in DSB calculated as the quantity of recovered bacteria from untreated control minus the quantity of recovered bacteria from disinfectant treated samples).

Table 5.3: Statistical significance and average Log_{10} reduction values of each *B. licheniformis* DSB age when comparing treatment to respective untreated controls. Statistical significance calculated from Šidák's multiple comparison test (ANOVA). Stars indicative of statistical significance, 'NS' indicates no statistically significant difference.

Disinfectant	Age of DSB (days)	Avg. Log_{10} reduction (± SD)	Statistical Significance^
NaDCC (1,000ppm)	4	0.2 (± 0.2)	NS
	8	0.4 (± 0.3)	NS
	12	0.3 (± 0.1)	NS
BZK	4	-0.1 (± 0.2)	NS
	8	0.0 (± 0.2)	NS
	12	0.4 (± 0.2)	NS
PAA (low conc.)	4	1.2 (± 0.4)	*
	8	2.4 (± 0.9)	****
	12	1.7 (± 0.4)	**
PAA (high conc.)	4	5.4 (± 0.0)	****
	8	1.7 (± 0.5)	NS
	12	2.0 (± 1.0)	***
NaDCC (10,000ppm)	4	4.9 (± 1.1)	****
	8	2.4 (± 0.1)	****
	12	2.3 (± 0.9)	***

^statistical significance values correspond to $p \leq 0.05$*, $p \leq 0.01$**, $p \leq 0.001$*** and $p \leq 0.0001$

5.4.2.3 *Bacillus subtilis* NCTC 10400

Out of all species tested, there was no evidence of increased resistance to disinfectants as DSB of *B. subtilis* NCTC 10400 developed over the 12-day period (**Figure 5.4**). Overall PAA displayed the greatest bactericidal effect on *B. subtilis* DSB (**Figure 5.4**). Although PAA (both concentrations) had the greatest bactericidal effect against *B. subtilis* DSB, there was not much difference in $\log_{10}$ reduction value between the two concentrations used, the greatest reduction observed being 0.5 $\log_{10}$ (**Table 5.4**). There was a statistically significant difference between treated DSB and respective untreated controls at all three DSB ages (ANOVA, Šidák, $p < 0.05$).

Overall, we observe a lack of activity of NaDCC (1,000 ppm) and BZK over the 12-day period, there was no statistically significant difference between NaDCC treatment and the untreated control at 4 and 12 days (ANOVA, Šidák, $p > 0.05$). Whereas for BZK an increased efficacy over the 12 days was observed (**Figure 5.4**) There was a statistically significant difference between 8- and 12-day untreated controls *vs.* BZK treatment (ANOVA, Šidák, $p < 0.05$). Log_{10} reduction remained low (circa. 2.5 $\log_{10}$) when DSB were exposed to NaDCC (10,000 ppm) (**Table 5.4**). A statistically significant difference was identified between the untreated control and treated samples at all stages of biofilm development respectively (ANOVA, Šidák, $p < 0.05$).

It is worth noting that the bacterial numbers in untreated controls of *B. subtilis* NCTC 10400 were lower (circa. 3-4 $\log_{10}$) than that of the other 3 species tested (circa. 6-7 $\log_{10}$). Over the biofilm development of 4 to 12 days, there was no significant difference in $\log_{10}$ reduction values when DSB were exposed to both concentrations of PAA or NaDCC (10,000 ppm) (One-way ANOVA, $p > 0.05$). However, there was a significant difference between stages of DSB development after exposure to BZK or NaDCC (1,000 ppm). A post-hoc Tukey test showed this statistically significant stemmed from 8 and 12 days when treated with NaDCC (1,000ppm) (ANOVA, Tukey test, $p < 0.05$) and 4 *vs.* both 8 and 12 individually when exposed to BZK (ANOVA, Tukey test, $p < 0.05$).

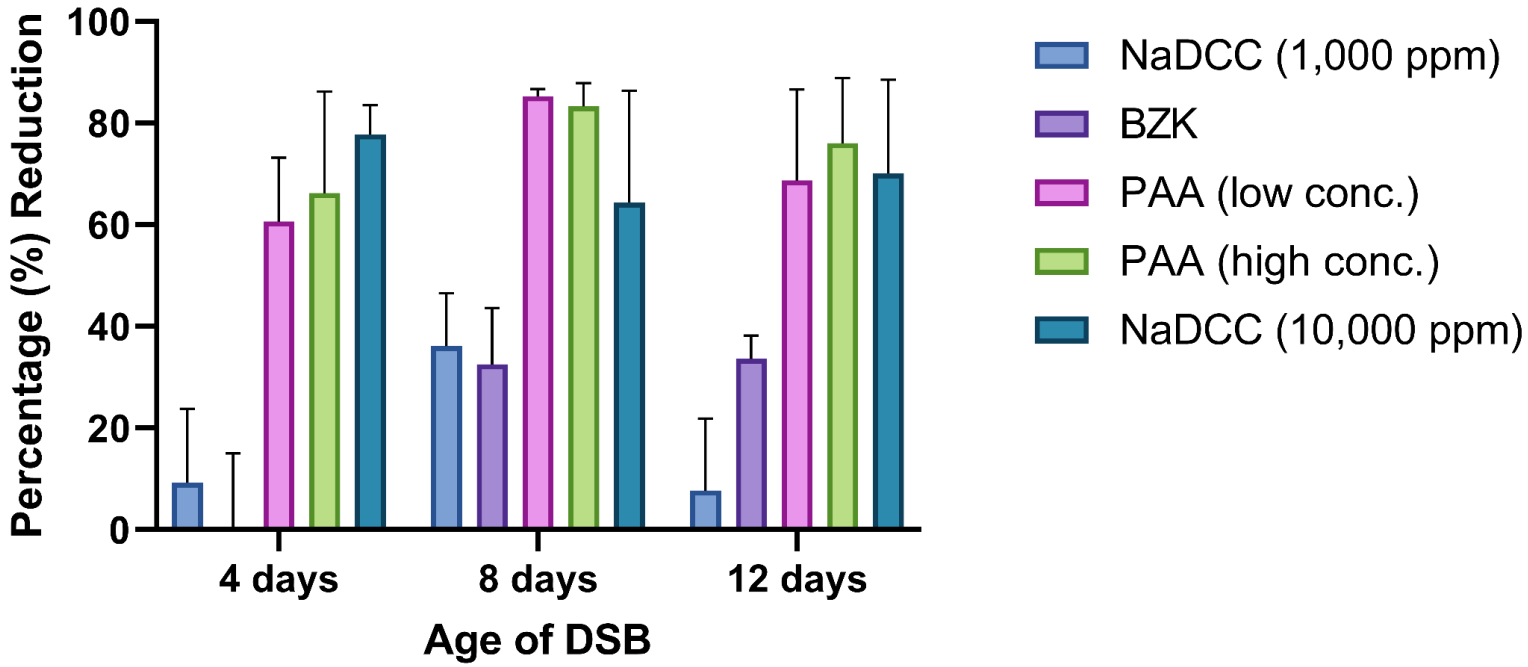


Figure 5.4. *B. subtilis* NCTC 10400 DSB 5 min exposure to three disinfectants using carrier test. DSB exposure to disinfectants at the three drying phases during biofilm formation. Reported values represent percentage (%) reduction, calculated from $\log_{10}$ values and standard deviation. Efficacy of PAA (low and high conc.) and NaDCC (10,000 ppm) was greatest out of all disinfectants used, all caused a > 50% reduction in bacteria survival in DSB from 4 days. There was no pattern of resistance observed over the 12-day DSB formation. (% reduction of bacteria embedded in DSB calculated as the quantity of recovered bacteria from untreated control minus the quantity of recovered bacteria from disinfectant treated samples).

Table 5.4: Statistical significance and average Log_{10} reduction values of each *B. subtilis* NCTC 10400 DSB age when comparing treatment to respective untreated controls. Statistical significance calculated from Šidák's multiple comparison test (ANOVA). Stars indicative of statistical significance, 'NS' indicates no statistically significant difference.

Disinfectants	Age of DSB (days)	Avg. Log_{10} reduction (± SD)	Statistical Significance^
NaDCC (1,000 ppm)	4	1.1 (± 1.1)	NS
	8	1.8 (± 0.7)	**
	12	0.6 (± 0.6)	NS
BZK	4	0.5 (± 0.4)	NS
	8	1.6 (± 0.4)	**
	12	1.7 (± 0.5)	**
PAA (low conc.)	4	3.4 (± 0.9)	**
	8	4.2 (± 0.0)	***
	12	3.5 (± 0.8)	****
PAA (high conc.)	4	3.2 (± 1.1)	**
	8	3.7 (± 1.1)	****
	12	3.7 (± 0.6)	****
NaDCC (10,000 ppm)	4	2.6 (± 0.7)	**
	8	2.1 (± 0.5)	**
	12	2.6 (± 0.4)	**

^statistical significance values correspond to $p \leq 0.05$*, $p \leq 0.01$**, $p \leq 0.001$*** and $p \leq 0.0001$

5.4.2.4 *Bacillus subtilis* AEWD

Alongside *B. licheniformis* this strain showed the greatest overall resistance to the disinfectants tested. DSB of *B. subtilis* AEWD were resistant to disinfectants from 4 days of development. Throughout formation of the mature DSB, < 50% of the biofilm was eradicated by all disinfectants and concentrations used, leaving over half of the biofilm remaining on the surface of the disk.

BZK and NaDCC (1,000 ppm) displayed the lowest bactericidal activity throughout all dry phases (≤ 1.6 $\log_{10}$ reduction, or < 27% reduction) (**Figure 5.5**). Similarly, to *S. aureus*, efficacy of NaDCC (1,000 ppm), BZK and PAA (low concentration) decreased with the ageing of the biofilm. Both PAA (high concentration) and NaDCC (10,000 ppm) effectively eradicated circa. 3 $\log_{10}$ DSB at 4, 8 and 12 days (**Figure 5.5, Table 5.5**). The resilience of DSB to these disinfectants did not change much from 4 to 12 days. Both PAA (high concentration) and NaDCC (10,000 ppm) were significantly different to respective untreated controls throughout biofilm ages (ANOVA, Šidák, $p < 0.05$). There was no statistical difference between untreated controls of 8- and 12-day DSB when exposed to BZK or NaDCC (1,000 ppm) (ANOVA, Šidák, $p > 0.05$). It is worth noting the resilience of *B. subtilis* AEWD DSB to the lower concentration of PAA at 12 days (0.4 $\log_{10}$ reduction) is much greater than 4 and 8 days (2.8 and 2.4 $\log_{10}$ respectively) (**Table 5.5**). PAA is the only disinfectant used that showed a stark difference between the young DSB at 4 days and mature 12-day DSB. There was a statistically significant difference between untreated controls of 4- and 8-day DSB when exposed to low concentration of PAA (ANOVA, Šidák, $p < 0.05$), although the same was not identified for 12-day DSB (ANOVA, Šidák, $p > 0.05$). There was no significant difference between $\log_{10}$ reduction values over biofilm development when *B. subtilis* AEWD was exposed to the high concentrations of PAA or NaDCC (One-way ANOVA, $p < 0.05$). Overall, NaDCC (1,000 ppm), BZK and PAA (low concentration) resulted in a significant difference between 4-, 8- and 12-day DSB (One-way ANOVA, $p > 0.05$). A post-hoc Tukey test revealed that significance came from 4 *vs*. 8- and 12-day DSB with NaDCC (1,000 ppm), 4 *vs*. 12-day DSB with BZK and 12 *vs*. 4- and 8- day DSB with low concentration of PAA (ANOVA, Tukey test, $p > 0.05$).

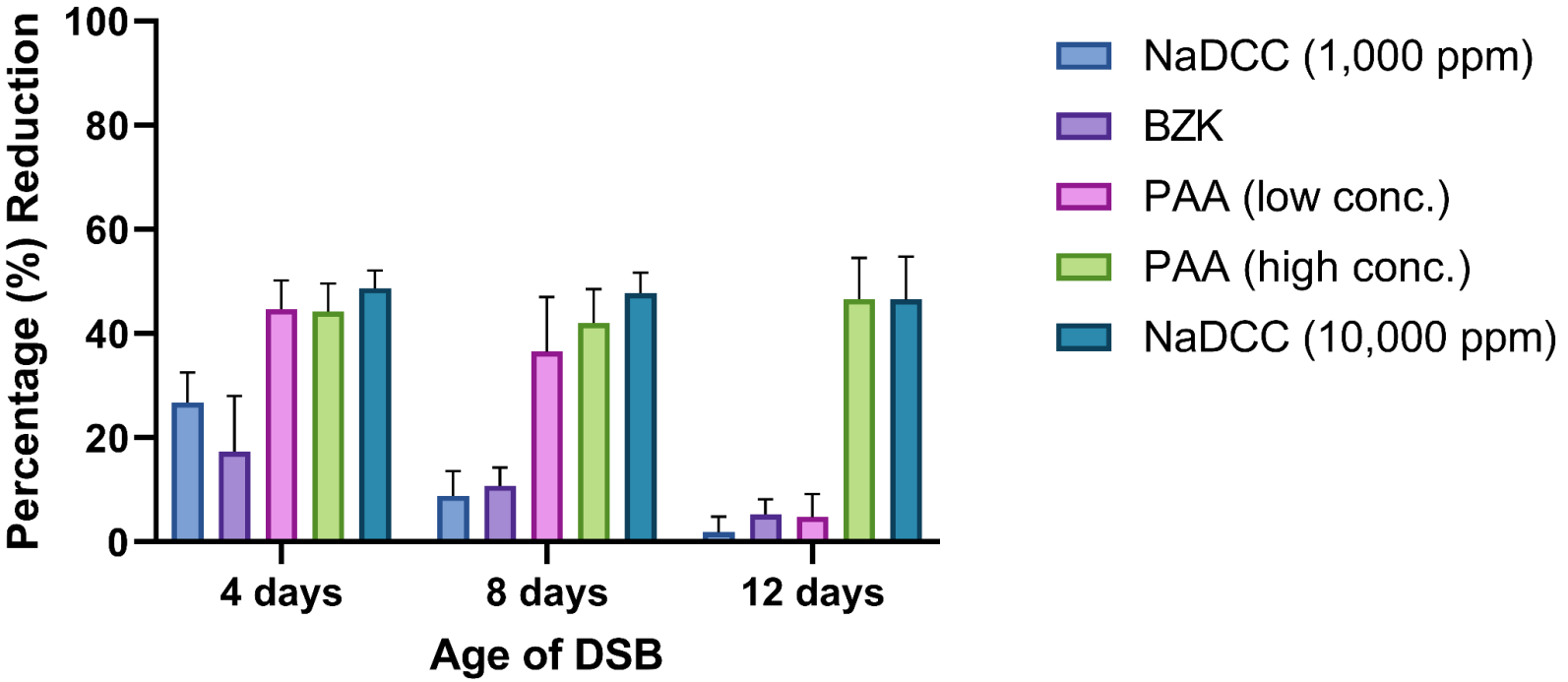


Figure 5.5. *B. subtilis* AEWD DSB 5 min exposure to three disinfectants using carrier test. DSB exposure to disinfectants at the three drying phases during biofilm formation. Reported values represent percentage (%) reduction, calculated from $\log_{10}$ values and standard deviation. Overall, % reduction did not exceed 50%. At 12 days, efficacy of NaDCC (1,000 ppm), BZK and PAA (low conc.) is reduced to < 5% reduction of bacteria surviving within DSB. (% reduction of bacteria embedded in DSB calculated as the quantity of recovered bacteria from untreated control minus the quantity of recovered bacteria from disinfectant treated samples).

Table 5.5. Statistical significance and average Log_{10} reduction values of each *B. subtilis* AEWD DSB age when comparing treatment to respective untreated controls. Statistical significance calculated from Šidák's multiple comparison test (ANOVA). Stars indicative of statistical significance, 'NS' indicates no statistically significant difference.

Disinfectants	Age of DSB (days)	Avg. Log_{10} reduction (± SD)	Statistical Significance^
NaDCC (1,000 ppm)	4	1.6 (± 0.6)	***
	8	0.6 (± 0.3)	NS
	12	0.1 (± 0.2)	NS
BZK	4	1.1 (± 0.4)	**
	8	0.8 (± 0.1)	NS
	12	0.4 (± 0.2)	NS
PAA (low conc.)	4	2.8 (± 0.3)	****
	8	2.4 (± 0.8)	***
	12	0.4 (± 0.3)	NS
PAA (high conc.)	4	2.9 (± 0.1)	****
	8	2.9 (± 0.5)	****
	12	3.3 (± 0.5)	****
NaDCC (10,000 ppm)	4	3.2 (± 0.3)	NS
	8	3.2 (± 0.4)	NS
	12	3.1 (± 0.6)	****

^statistical significance values correspond to $p \leq 0.05$*, $p \leq 0.01$**, $p \leq 0.001$*** and $p \leq 0.0001$

5.4.3 Long exposure to disinfectants

As described in section 5.3.1, *B. licheniformis* ATCC 14580 and *B. subtilis* AEWD were exposed to disinfectants NaDCC (1,000ppm), BZK, and two concentrations of PAA for 60 min due to the species' previously identified resilience to disinfectants tested when DSB had reached maturity at 12 days. Disinfectants did not prove effective in eradication of either *Bacillus* spp. DSB (< 2 $\log_{10}$ reduction reported) after exposure to disinfectants: NaDCC (1,000 ppm), BZK and PAA (low concentration) after 60 min contact time (**Figure 5.6 & 5.7**). In both species DSB, the disinfectant with the greatest efficacy in eradicating DSB was PAA (higher concentration).

Much like the 5 min exposure (**Figure 5.3**), NaDCC and BZK were least effective in eradicating DSB of *B. licheniformis* (**Figure 5.6**). There was no statistically significant difference between 60 min exposure and their respective 5 min exposure from DSB of *B. licheniformis* for each disinfectant tested (t-test, $p < 0.05$). Both BZK and PAA (low concentration) had slightly greater effects on biofilm eradication when used for 60 min, although both were less than 1 $\log_{10}$ difference (**Figure 5.6**). Although the higher concentration of PAA appeared to have a greater effect after 5 min, both 5- and 60-min treatment types resulted in ≥ 4.5 $\log_{10}$ reduction of *B. licheniformis*.

After 60 min of exposure to disinfectants, *B. subtilis* AEWD was more susceptible to treatment than the *B. licheniformis* DSB. Figure 5.6 shows that the high concentration of PAA had the greatest effect, recording a 4.9_{10} log reduction after 60 min contact time, unlike *B. licheniformis* (**Figure 5.7**). As seen previously, BZK had very little biocidal effect on *B. subtilis* AEWD DSB, only a 0.2 $\log_{10}$ reduction was obtained after 60 min contact and 0.4 $\log_{10}$ after 5 min (**Figure 5.7**). BZK was the only disinfectant that did not show a statistical difference between the 5 min and 60 min exposure (t-test, $p < 0.05$). When exposed to NaDCC (1,000 ppm) and PAA (both concentrations), there was a significant difference between 5 min and 60 min contact times upon treatment of *B. subtilis* AEWD (t-test, $p < 0.05$) (**Figure 5.7**).

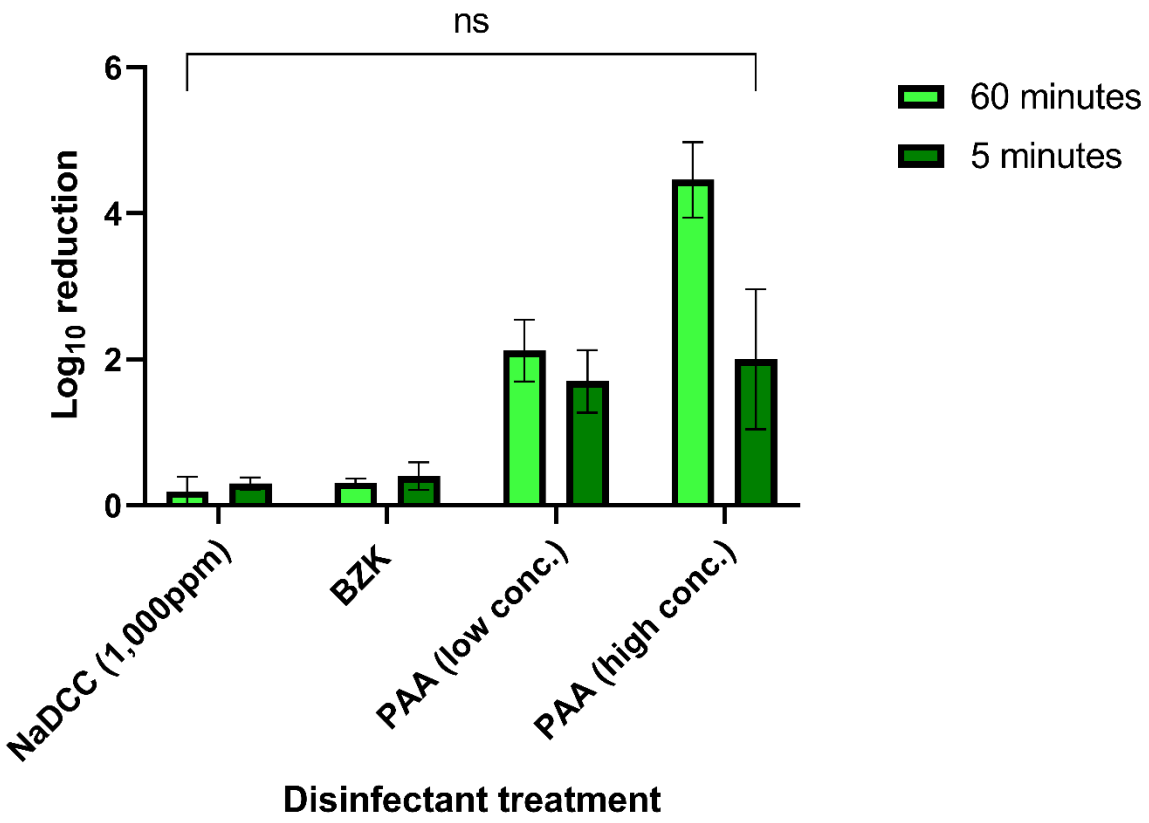


Figure 5.6. *B. licheniformis* DSB exposure to three disinfectants of differing concentration, using carrier test. Mature 12-day DSB exposure to disinfectants, 60 min treatment time. Reported values as $\log_{10}$ reduction and standard deviation. NS indicates no statistically significant difference between 5- and 60-min contact times (t-test, $p < 0.05$).

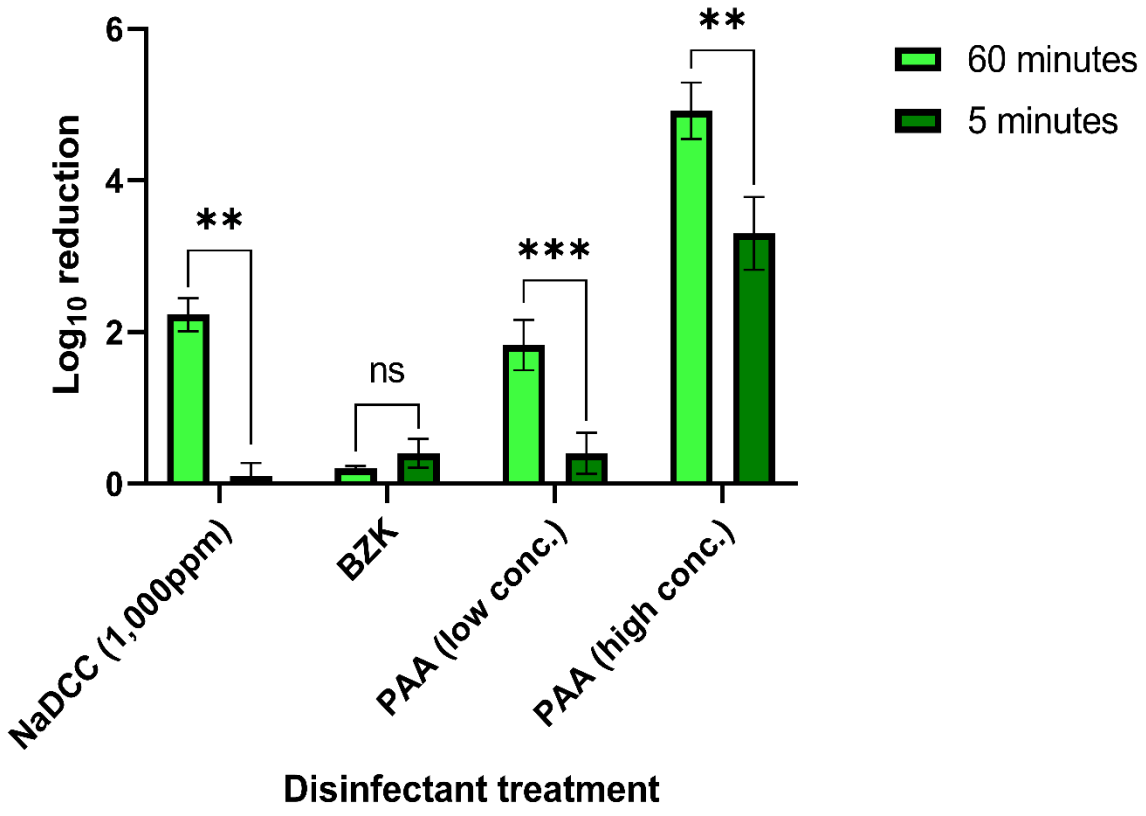


Figure 5.7. *B. subtilis* AEWD DSB exposure to three disinfectants of differing concentration, using carrier test. Mature 12-day DSB exposure to disinfectants, 60 min treatment time. Reported values as $\log_{10}$ reduction and standard deviation. NS indicates no statistically significant difference between 5- and 60-min contact times. Stars indicate a statistically significant difference between contact times, as seen here for NaDCC and both PAA concentrations (t-test, $p > 0.05$).

5.4.4 Effect of disinfectants on spores within DSB of *Bacillus* spp.

Bacillus spp. are spore forming bacteria. To determine the quantity and contribution of spores to the resilience of dry surface biofilms from each *Bacillus* spp. to disinfectants, carrier tests were conducted with NaDCC (1,000 ppm), BZK and both high and low concentrations of PAA.

5.4.4.1 *Bacillus subtilis* NCTC 10400

This species showed the highest susceptibility out of all three *Bacillus* strains tested. Spores were only recovered (after 80 ºC treatment) following exposure to NaDCC and BZK at 4 days and BZK at 8 days. There was no visual evidence of spores under phase-contrast microscopy at any data points. There was no evidence of the presence of spores in the prepared DSB (**Figure 5.8**), or with the untreated control following heat treatment revealed no detectable germinated spores on agar plates. There was a statistically significant difference between spore count and total DSB counts for NaDCC, BZK and the untreated controls at 4 days (ANOVA, Šidák, $p < 0.05$) (**Figure 5.8a**). There was no statistically significant difference between $\log_{10}$ recovery of spores from DSB vs total DSB count at both 8 and 12 days (ANOVA, Šidák, $p > 0.05$). This was due to both concentrations of PAA reducing DSB counts to below detectable limits (**Figure 5.8**).

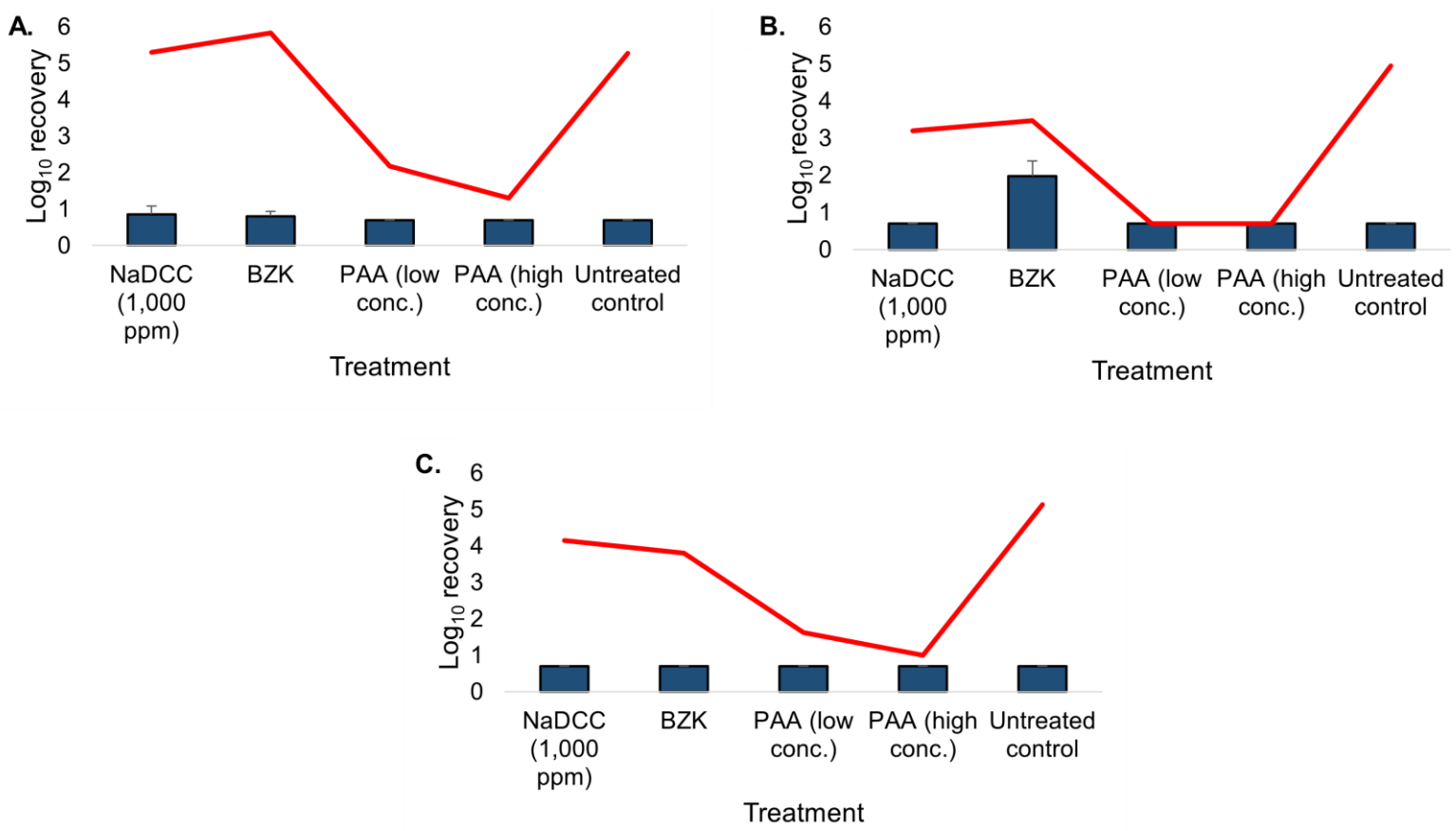


Figure 5.8. *B. subtilis* NCTC 10400 spore and total DSB counts following exposure to disinfectants. DSB exposure to disinfectants, using a 5 min treatment time at 4 (a), 8 (b) and 12 (c) days. Spore counts are represented by blue bar, reported values as log_{10} reduction. Total DSB recovery counts are reported by the red trendline.

5.4.4.3 *Bacillus licheniformis* ATCC 14580

B. licheniformis displayed the overall highest number of recovered spores out of all three *Bacillus* species. Spores were visible under phase-contrast microscopy, evidenced by the bright refringence in 12-day DSB (**Figure 5.9**). Spore numbers were significantly lower than whole DSB counts at 8 and 12 days (ANOVA, Šidák, $p < 0.05$) (**Figure 5.10b&c**) At 4 days, spore counts (> 5 $\log_{10}$ recovered) were relatively equal to the DSB as a whole (**Figure 5.10a**). Only a significant difference between the high concentration of PAA between spores and total DSB was identified (ANOVA, Šidák, $p < 0.05$) (**Figure 5.10a**). There was much more variation between the PAA (high conc) samples where a significant difference was identified at 4 and 8 days (ANOVA, Šidák, $p < 0.05$).

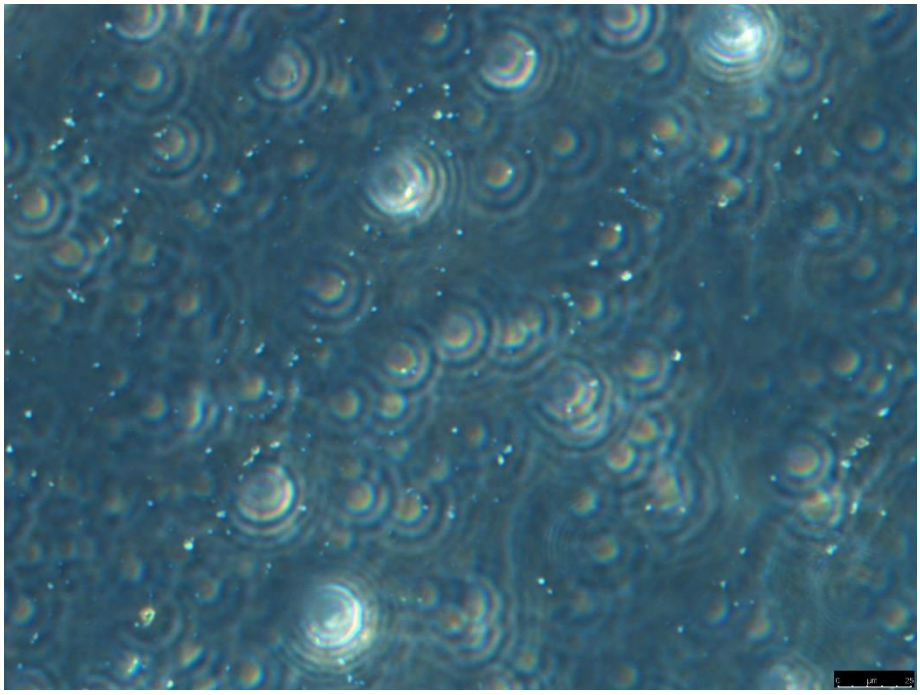


Figure 5.9. Phase-contrast microscope image of spores within 12-day DSB of *B. licheniformis*.

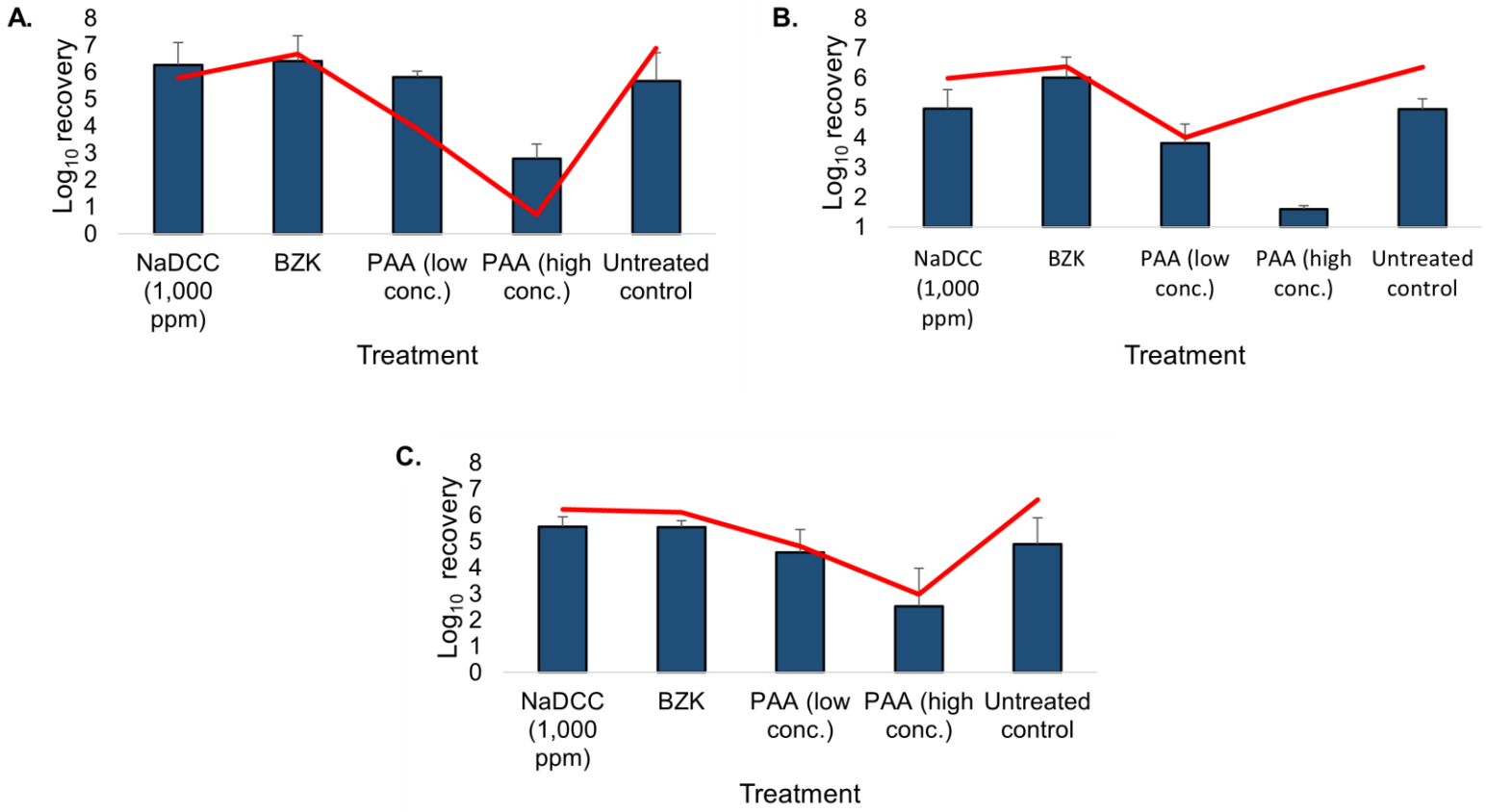


Figure 5.10. *B. licheniformis* spore and total DSB counts following exposure to disinfectants. DSB exposure to disinfectants, using a 5 min treatment time at 4 (a), 8 (b) and 12 (c) days. Spore counts are represented by blue bar, reported values as log_{10} reduction. Total DSB recovery counts are reported by the red trendline.

5.4.4.2 *Bacillus subtilis* AEWD

Spores were visible under phase-contrast microscopy of *B. subtilis* AEWD 12-day DSB, although other debris can be identified there are round refringent spores within the sample (**Figure 5.11**). There was a statistically significant higher concentration of vegetative cells within the DSB compared to spores at both 4 and 12 days (ANOVA, Šidák, $p < 0.05$) (**Figure 5.12a&c**) At 12 days, spore counts were statistically significantly lower than total DSB counts (ANOVA, Šidák, $p < 0.05$) (**Figure 5.12c**). However, spore counts were still high after treatment with NaDCC and BZK (circa. 4.2 $\log_{10}$ recovered). There was no significant difference amongst spore count and total DSB count at any treatment or untreated control with 8-day biofilm (ANOVA, Šidák, $p > 0.05$), with the exception of PAA (high conc) (ANOVA, Šidák, $p < 0.05$) (**Figure 5.12b**). At 4 days, only the high concentration of PAA and the untreated control resulted in significantly higher total DSB counts compared to spore counts and total DSB (ANOVA, Šidák, $p < 0.05$) (**Figure 5.12a**). PAA had the greatest effect on reducing spore counts as presented in Figure 5.9.

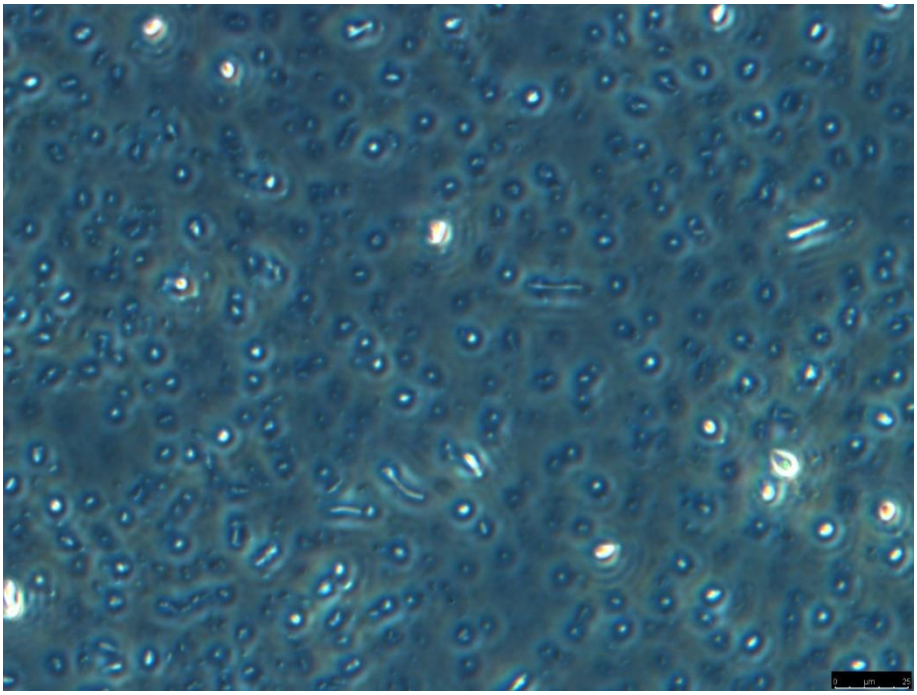


Figure 5.11. Phase-contrast microscope image of spores within 12-day DSB of *B. subtilis* AEWD.

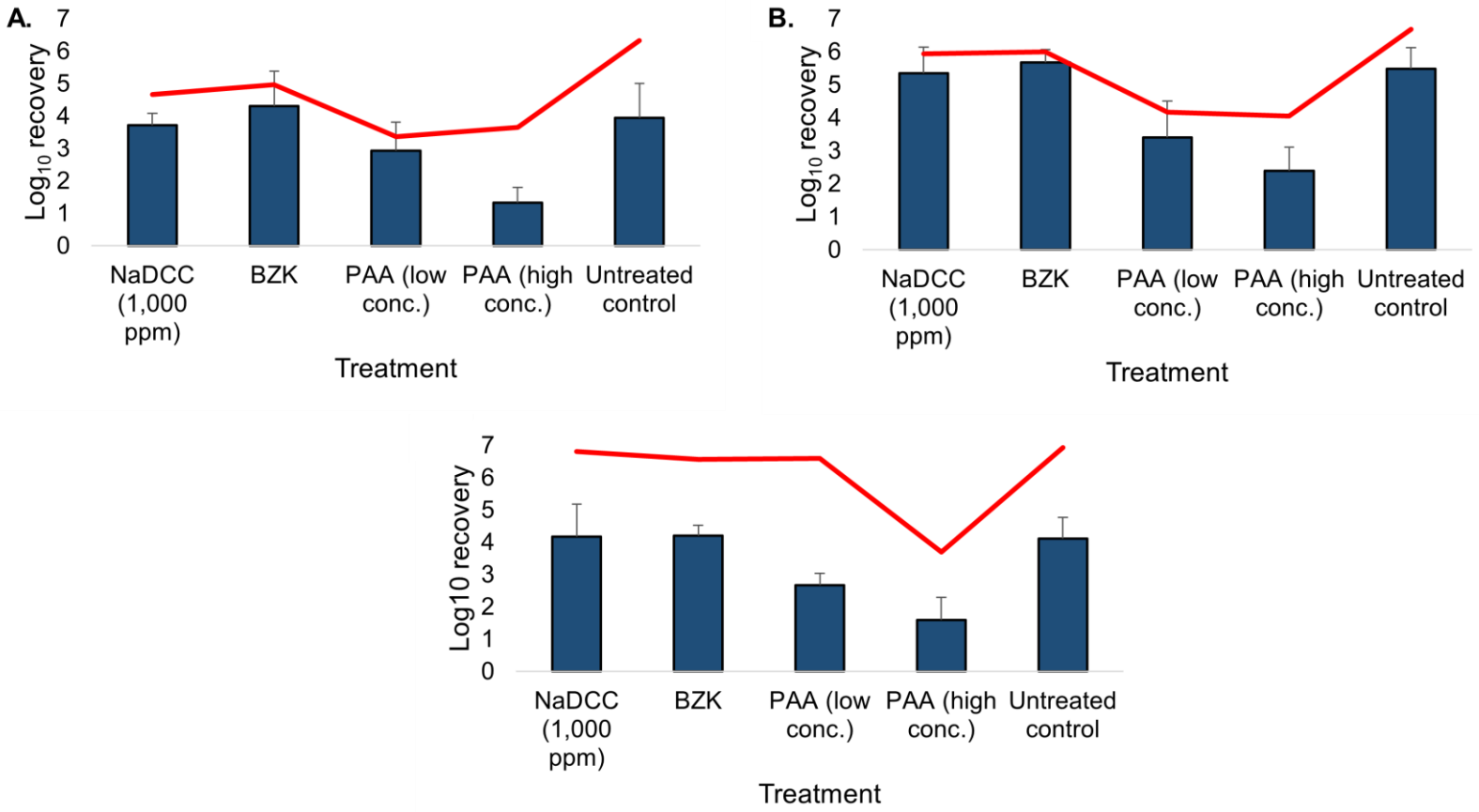


Figure 5.12. *B. subtilis* AEWD spore and total DSB counts following exposure to disinfectants. DSB exposure to disinfectants, using a 5 min treatment time at 4 (a), 8 (b) and 12 (c) days. Spore counts are represented by blue bar, reported values as log_{10} reduction. Total DSB recovery counts are reported by the red trendline.

5.4.5 Wiperator experiments

Three tests were conducted with wipe products (**Chapter 3, Table 3.2**), measuring removal of bacteria from the surface by wipe, direct surface to surface transfer of bacteria from DSB after wiping, and transfer of DSB by the wipe itself. Post-wiping contact times of 30 and 60 sec were used after the 10 sec wiping (500 g). Only *B.subtilis* NCTC 10400 and *B. subtilis* AEWD are presented here as both *S. aureus* and *B. licheniformis* results are presented in chapter 6 (6.4.2, 6.4.3 and 6.4.4).

5.4.5.1 *Bacillus subtilis* NCTC 10400

After both 30 and 60 sec contact times, there was no observed statistically significant difference between $\log_{10}$ reduction when DSB of *B. subtilis* was treated with each individual wipe product respectively (One-way ANOVA, $p > 0.05$) (**Figure 5.13**).

Following 30 sec contact time, when comparing wipes A – E (refer to Table 3.2) to the control water wipe, only wipe B showed a significant difference in reducing bacterial viability (ANOVA, Dunnett's, $p < 0.05$). Reduction in viability for all other products at 30 or 60 sec were not significantly different from the water control (ANOVA, Dunnett's, $p > 0.05$). Negative values recorded for $\log_{10}$ reduction at 30 seconds (mainly wipes A and C) arose due to lower untreated control counts (average 4.6 and 4.8 $\log_{10}$ respectively) than what was recovered from wiped DSB (average 4.1 $\log_{10}$) (**Figure 5.13**).

Overall, wipe B proved least effective when compared to the untreated control at both 30 and 60 seconds with average $\log_{10}$ reductions of 0.1 and -0.1 respectively.

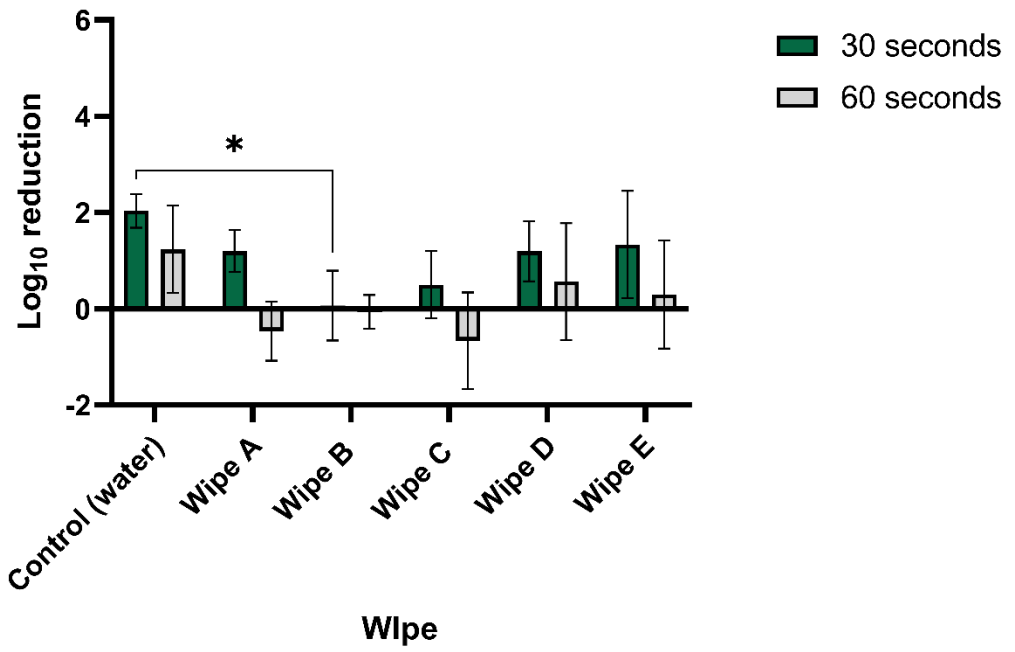


Figure 5.13. Log_{10} reduction of *B. subtilis* NCTC 10400 after 30 and 60 second contact time with wipe products. Values expressed as mean with standard deviation from three biological replicates. There was no significant difference between 30 and 60 second contact for each corresponding wipe (ANOVA, Šidák, $p > 0.05$).

When considering transfer of bacteria by the wipe itself, there was less transfer of bacteria embedded in DSB after 60 sec contact time with wipes B, D and E (**Figure 5.14**). Of these, log_{10} transfer was significantly different to untreated controls between 30 and 60 sec with wipes B and D (ANOVA, Šidák, $p < 0.05$). There was no significant difference between any wipe and the control water wipe after both 30 and 60 sec contact times (ANOVA, Dunnett's, $p > 0.05$). Wipe D transferred significantly less bacteria than wipe A (ANOVA, Tukey, $p < 0.05$) after 60 sec. Wipe A transferred the greatest number of bacteria after 60 sec (average 3.3 log_{10}), whereas wipe B transferred the largest quantity of bacteria embedded in DSB after 30 sec contact time (average 3.0 log_{10}) (**Figure 5.14**). All wipes including water control, with the exception of A and C, transferred more bacteria after 30 second contact time than 60 seconds.

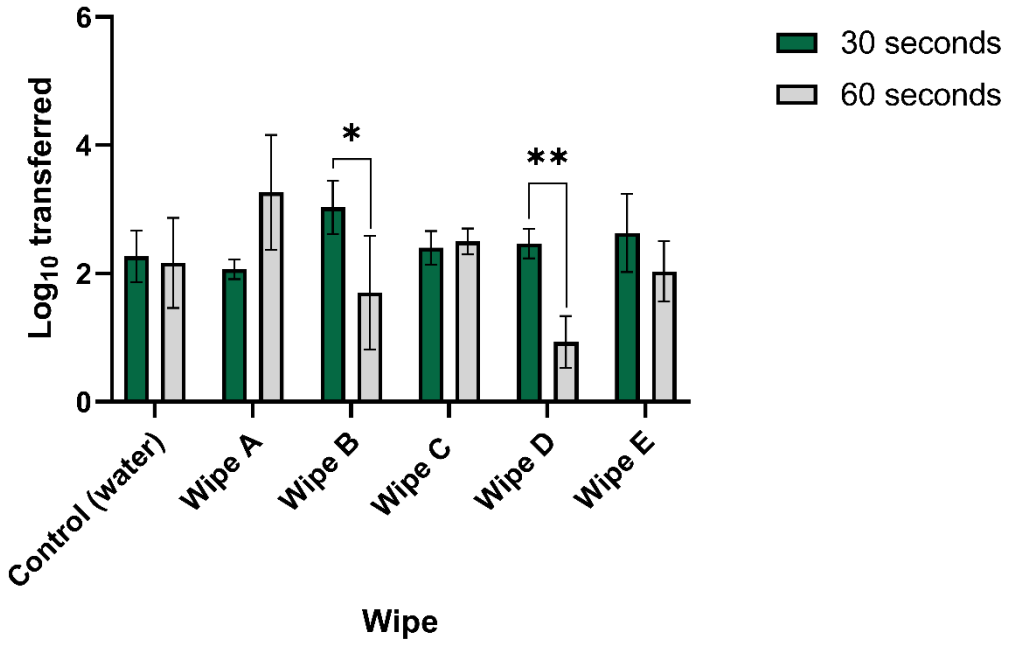


Figure 5.14. Log_{10} transfer of *B. subtilis* NCTC 10400 by wipe after 30 and 60 second contact time. Graph representative of log_{10} transfer of bacteria embedded in DSB by wipe products. Values expressed as mean with standard deviation from three biological replicates. There was a significant difference between 30 and 60 second contact for wipes B and D (ANOVA, Šidák, $p < 0.05$).

Finally, we looked at direct transfer of bacteria to DE agar surface following wiping. There was no significant difference between either wipe products when comparing 30 and 60 sec % transfer (ANOVA, Šidák, $p > 0.05$). There was also no significant difference between any wipe products and the water control transfer after 30 or 60 sec contact (ANOVA, Dunnett's, $p > 0.05$). We observed > 80% average transfer of *B. subtilis* NCTC 10400 direct transfer after treatment with all wipes at both 30 and 60 sec (**Figure 5.15**). Wipes A, B, C and E all displayed a 100% transfer rate, whereas the water control and wipe D transferred average 84% and 99% respectively (**Figure 5.15**). It is evident that direct % transfer of bacteria by the water control was more variable than any of the commercially available wipe products.

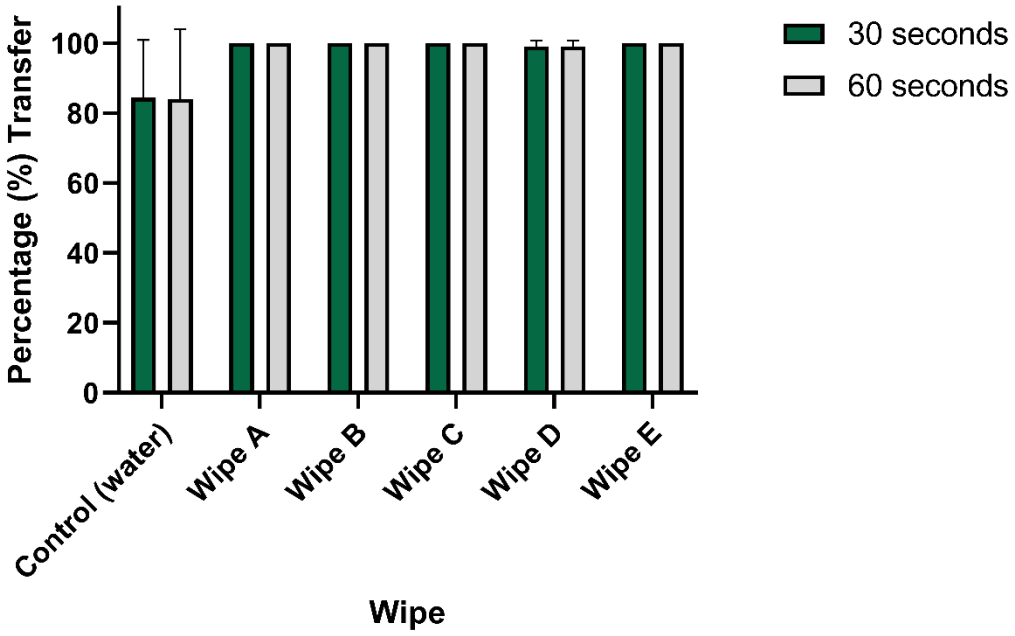


Figure 5.15. Direct percentage (%) transfer of *B. subtilis* NCTC 10400 after wiping for both 30 and 60 second contact time. Graph representative of % transfer of bacteria embedded in DSB following wiping. Values expressed as mean with standard deviation from three biological replicates. All wipes transferred > 80% bacteria. There was no significant difference between 30 and 60 second contact for all wipes including water control (ANOVA, Šidák, $p > 0.05$).

5.4.5.2 *Bacillus subtilis* AEWD

As seen previously with *B. subtilis* NCTC 10400, generally $\log_{10}$ reduction values were relatively low (**Figure 5.16**). No significant difference was identified between 30 and 60 sec contact times for each individual wipe (ANOVA, Šidák, $p > 0.05$). Wipe E performed best overall with average $\log_{10}$ reductions of 2.5 (30 second) and 2.2 (60 second) respectively. Negative values correspond to lower untreated control value of 4.4 $\log_{10}$. Wipe E also had the greatest range in $\log_{10}$ reduction values after 60 sec contact time, as it had both the highest and lowest values of all. When

comparing wipes A – E to the water control, only wipe E was significantly different after 30 sec contact time (ANOVA, Dunnett's, $p < 0.05$) (**Figure 5.16**).

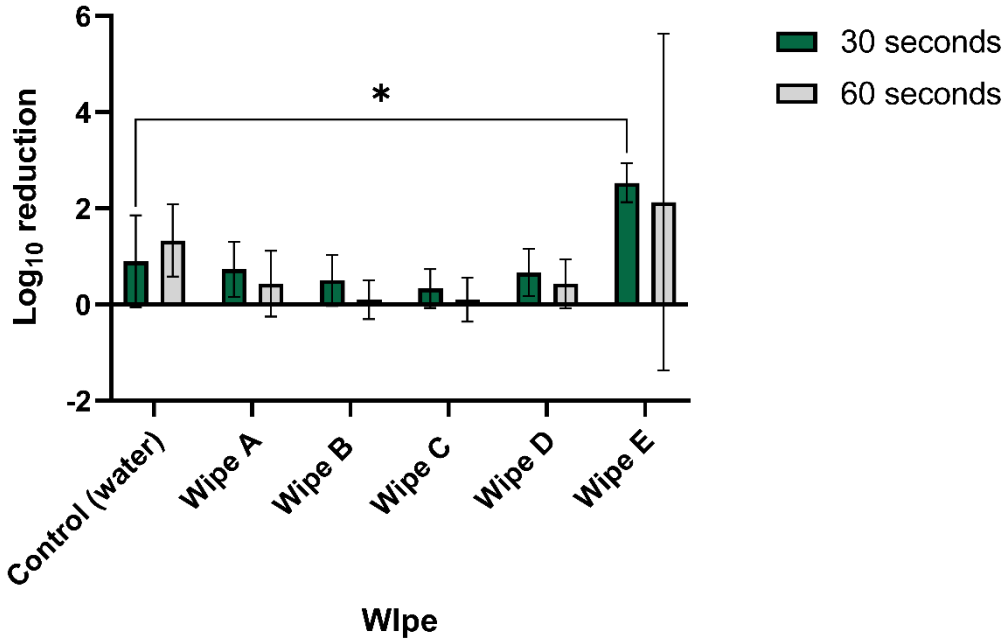


Figure 5.16. Log_{10} reduction of *B. subtilis* AEWD after 30 and 60 second contact time with wipe products. Values expressed as mean with standard deviation from three biological replicates. There was no significant difference between 30 and 60 second contact for each corresponding wipe (ANOVA, Šidák, $p > 0.05$).

Similarly, we found no significant difference between 30 and 60 sec contact times when considering quantity of bacteria transferred via the wipe itself (ANOVA, Šidák, $p > 0.05$). Wipe B transferred the highest number of bacteria, average 4.4 log_{10} at 60 sec, and wipes B and C transferred the highest after 30 sec contact time, average 4.5 log_{10} (**Figure 5.17**). Overall, there was a statistically significant difference between all wipe products when considering 30 sec contact time used (One-way

ANOVA, $p < 0.05$). Specifically, wipe E was significantly different to wipe B and C (ANOVA, Tukey, $p < 0.05$). Overall, wipe products transferred > 2.5 log_{10} after 60 second contact and > 3 log_{10} after 30 sec contact time (**Figure 5.17**). If we exclude wipe E, these values change to ≥ 3.7 log_{10}.

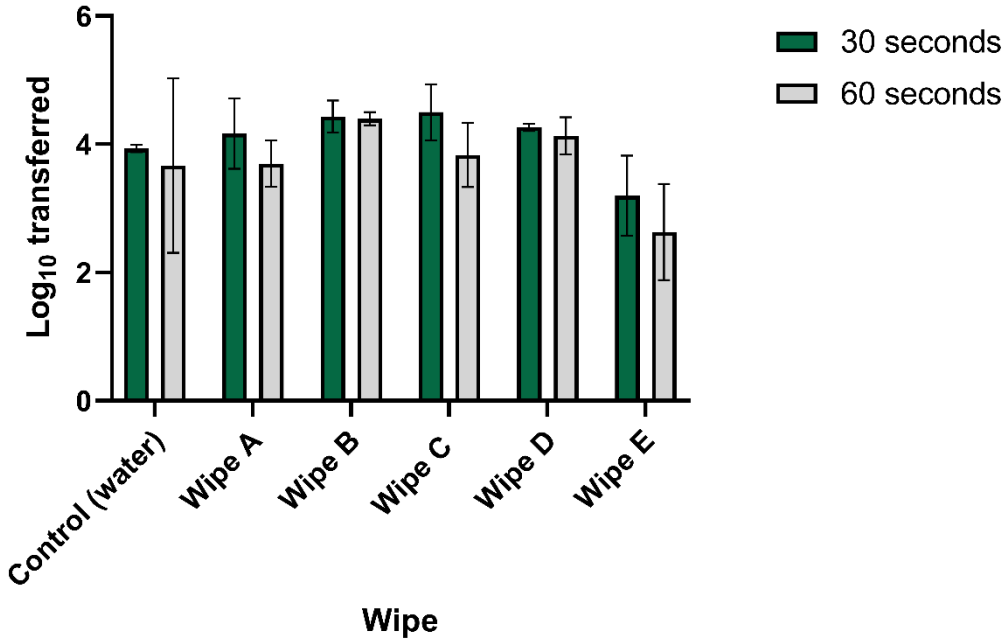


Figure 5.17. Log_{10} transfer of *B. subtilis* AEWD by wipe after 30 and 60 second contact time. Graph representative of log_{10} transfer of bacteria embedded in DSB by wipe products. Values expressed as mean with standard deviation from three biological replicates. There was no significant difference between 30 and 60 second contact times between each individual wipe product (ANOVA, Šidák, $p > 0.05$), nor the wipe product *vs.* the control (ANOVA, Dunnett's, $p > 0.05$).

Lastly, for the direct transfer of bacteria following wiping, we observed a 100% transfer of *B. subtilis* AEWD after wiping by each product, including water wipe (**Figure 5.18**). Statistical analyses cannot be performed as all biological replicates produced a 100% transfer and so no variation was observed between treatments.

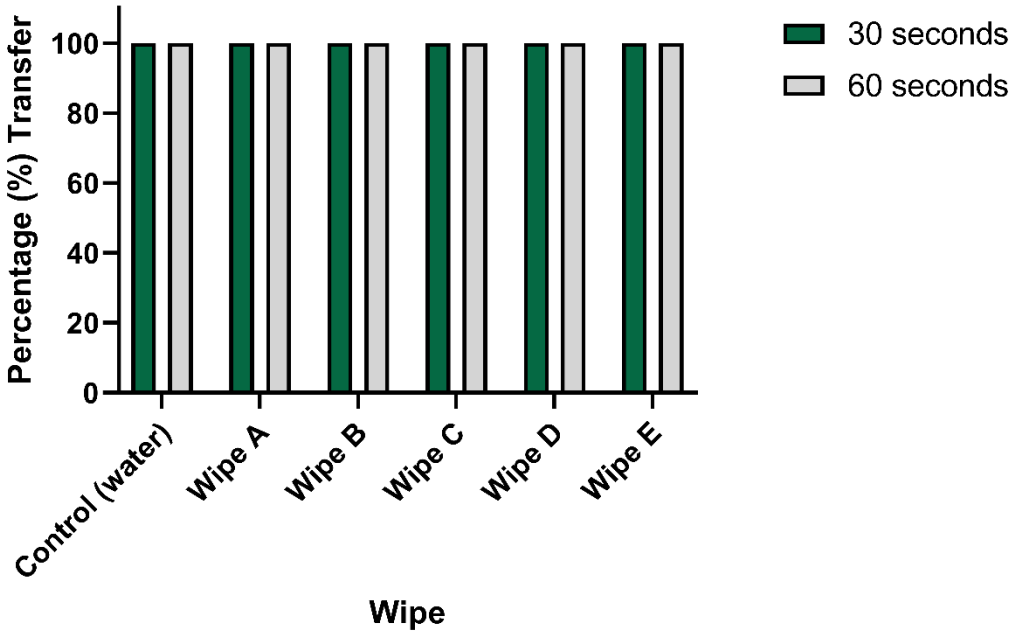


Figure 5.18. Direct percentage (%) transfer of *B. subtilis* AEWD after wiping for both 30 and 60 second contact time. Graph representative of % transfer of bacteria embedded in DSB following wiping. Values expressed as mean with standard deviation from three biological replicates. All treatments produced 100% transfer from all three biological replicates.

5.4.6 Wet Biofilm Comparison

No viable bacteria were recovered following 5 min treatment of all disinfectant products and concentrations. Viable untreated control counts of *S. aureus* and *B. licheniformis* circa 7 $\log_{10}$ (**Figure 5.19**) were similar to DSB counts of circa 6.5 $\log_{10}$ (**Figure 5.1**). *B. subtilis* AEWD displayed slightly lower viability between wet biofilm (avg. 5.8 $\log_{10}$) and 12-day DSB (avg. 6.8 $\log_{10}$). *B. subtilis* NCTC 10400 counts

were higher in wet biofilm, circa 5.5 log_{10}, (**Figure 5.19**), compared to DSB (**Figure 5.1**).

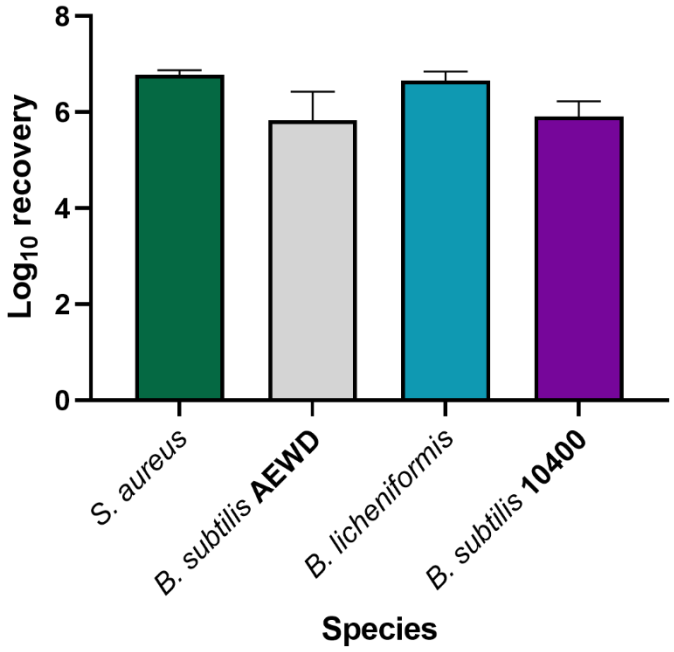


Figure 5.19. Log_{10} recovery of bacteria in wet biofilm of all four species. Bacterial numbers recovered from wet biofilm were fairly consistent between species, both *S. aureus* and *B. licheniformis* exhibited similar counts of circa 7 log_{10}. Both *B. subtilis* remained slightly lower circa 5.5 log_{10}.

5.5 Discussion

Dry surface biofilms are prevalent within healthcare environments (Vickery et al., 2012; Almatroudi et al., 2015; Ledwoch et al., 2019a). Both *Bacillus* spp. and *S. aureus* have been identified as two common species found in DSB from hospital surfaces (Ledwoch et al., 2018). Even with effective cleaning protocols, DSB are not removed from the surfaces and have been shown to withstand terminal cleaning followed by 500 ppm chlorine disinfection (Vickery et al., 2012).

Here, we report findings from three *Bacillus* spp. and *S. aureus* DSB exposure to common disinfectants. This chapter presents the susceptibility of *Bacillus* spp. in a DSB to disinfectants, which has not been reported previously in the literature. We also present work on the development of DSB and use disinfectants as a marker for resistance over DSB development.

S. aureus DSB was susceptible, meaning all disinfectants displayed a bactericidal effect on the DSB and showed the greatest reduction in bacterial counts compared to the untreated control. Previous work has presented similar susceptibility results of *S. aureus* DSB to sodium hypochlorite (Almatroudi et al., 2016; Ledwoch et al. 2019b).

Testing of DSB of *Bacillus* spp. is novel and so cannot be compared to previous cited literature. The action of the disinfectants decreased throughout DSB formation, from 4 to 12 days, indicating an increased resilience of the mature biofilm compared to the early stages of development. At 12 days the biofilm is fully formed which will decrease its susceptibility, we know from previous work that the biofilm is mainly made up of protein (96%) and carbohydrates, with little DNA (Ledwoch et al., 2019b). The disinfectants had the least bactericidal effect on against both *B. subtilis* AEWD and *B. licheniformis* DSB. Previous work has shown the resistance of *B. subtilis* AEWD vegetative cells to chlorine dioxide and hydrogen peroxide treatment (Martin et al., 2008). Bridier et al. (2011a) then confirmed the production of a voluminous biofilm by this strain and associated it with large quantities of EPS. EPS has been found to inhibit the penetration of biocides (Maillard, 2007) and play a vital role in protection of the bacterial cells (Martin et al., 2015). Further work by Martin et al. (2015) identified three key resistance mechanisms of *B. subtilis* AEWD vegetative cells including: bacterial cell aggregation, presence of a large quantity of EPS and expression of detoxification enzymes. These characteristics may have contributed to the resistance of *B. subtilis* AEWD to disinfection and not the

presence of spores in DSB, especially at 12 days when the biofilm has developed, and a large quantity of EPS is present (4.4.1, 4). Similarly, *B. licheniformis* ATCC 14580 has been identified as a heavy producer of EPS (Malick et al., 2017). Here, marked differences in DSB susceptibility was observed between the *Bacillus* spp. With *B. subtilis* AEWD being the least susceptible. Such difference in efficacy could be attributed to EPS but we did not quantify EPS in the mature 12-day biofilm. Future work is required to confirm the quantity of EPS production when in DSB to gain a better understanding of EPS involvement in the resistance mechanisms of each strain.

The wildtype strain used, *B. subtilis* NCTC 10400 displayed a greater susceptibility to the disinfectants compared to the other *Bacillus* spp. Unlike the other species, the effect of PAA on the biofilm remained consistent during the development of DSB to the mature 12-day biofilm, but the efficacy of BZK and NaDCC on DSB survival varied between the three drying phases tested (**Figure 5.3**). It appeared there was no correlation of increased resistance of *B. subtilis* NCTC 10400 to each disinfectant and concentration tested with biofilm maturity.

In all species, the effect of PAA on DSB was apparent. This disinfectant appeared to have the best bactericidal activity, which could be due to its high redox potential causing high sporicidal and bactericidal efficacy (Finnegan et al., 2010). PAA has been reported to react strongly with bacterial cell membranes and cause the denaturing of proteins, ultimately ending in bacterial cell death (Kitis et al., 2004). The effects of PAA were greater than the other two disinfectants, $\log_{10}$ reduction of < 5.5 in all *Bacillus* spp. and < 5.8 in *S. aureus* DSB. Compared to results with other disinfectants, Almatroudi et al. (2016) achieved a 7 $\log_{10}$ reduction of *S. aureus* removed/killed from DSB by treatment with 1,000 ppm of sodium hypochlorite. Here our results showed that *Bacillus* spp. DSB were tolerant of the same concentration as Almatroudi used in their study (1,000 ppm) of sodium dichloroisocyanurate (NaDCC). This is of particular interest as chlorine at a concentration of 1,000 ppm is required for routine cleaning in NHS hospitals (NHS England, 2022). As stated by Dancer (2011), the use of chlorinated products within hospitals only offers a limited control measure for removing bacterial contamination on a surface, physical action is required for enhanced use. Coinciding with results presented here, whereby treatment with NaDCC (1,000ppm) in solution, with no mechanical removal, was not enough to have a damaging effect on the DSB. Furthermore, chlorine is inactivated

by disinfectant by organic matter (Gallandat et al., 2021), which may contribute to the resistance seen here as we add bovine serum albumin to the DSB. The addition of BSA has been shown to reduce activity of sodium hypochlorite when added at the same concentration as used here (Clayton et al., 2020).

We also used NaDCC at 10,000 ppm, the requirement for blood spillages, and although $\log_{10}$ recovery in general was high, still resistance to such a high concentration of disinfectant was observed with both *Bacillus* environmental isolates (**Figure 5.2 & 5.4**). NaDCC certainly had the greatest activity against 4-day DSB of all species. This indicates that although products may work in the early stages of biofilm development, they may not be as effective on mature biofilms. DSB may remain on surfaces for extended periods of time in hospitals (Otter et al., 2015). This in turn could create a problem for general cleaning protocol as on a daily basis standard cleaning routines utilise lower concentrations of products, which appear less effective in eradication of DSB and may leave some bacteria remaining on surfaces in near patient areas from biofilm, which has been shown by Costa et al. (2019), where MDRO were present on surfaces following sub-optimal cleaning measures.

Upon testing of environmental isolates *B. licheniformis* and *B. subtilis* AEWD, it became clear that $\log_{10}$ reduction did not exceed 1.5, with the exception of PAA at high concentration (**Figure 5.5 & 5.6**). Increasing contact time correlated to enhanced performance of NaDCC (1,000 ppm) and both PAA concentrations when exposed to *B. subtilis* 12-day DSB. We can conclude from these results that increased contact times are required for a greater kill rate of DSB. Time constraints do not allow for 60 min contact times, as nurses are already under pressure and find it difficult to clean surfaces adequately (Curryer et al., 2021). It is extremely difficult to keep a surface submerged even for 5 min. Although contact time may have had a significant difference on *B. subtilis* AEWD, this cannot be said for *B. licheniformis* 12-day DSB. Generally, *B. licheniformis* possesses the greatest resistance to disinfectant treatment through suspension testing. From the results presented here, it is clear that some *Bacillus* spp. can persist on surfaces following disinfectant treatment.

The spore experiments were intended to understand whether DSB survival to disinfection was entirely attributed to spores. Under stressful conditions, *B. subtilis* sporulates (Tan and Ramamurthi, 2014). The process of DSB development lends

itself to a stressful environment as bacteria undergo several periods of desiccation. It should be noted that not all spores within DSB samples may have germinated at the same time, as dormant and super dormant spores may have also been missed (Setlow, 2003) and so the results presented may be a slight underestimation of the total number of spores. Spores are considered more resistant to disinfectant treatment than vegetative cells due to their morphology. Spores are encased in impermeable outer layers and possess a low water content (Leggett et al., 2016). From the results presented, it appears that *B. subtilis* NCTC 10400 does not display evidence of spore production in the DSB, evidenced by both heat treatment and light microscopy.

Overall, treatment with PAA lead to the lowest spore counts from the DSB. PAA is a sporicidal product and used commercially in wipes branded as sporicidal, this indicates that a wipe product is sufficient in eradicating spores from the surface environment (Leggett et al., 2016). However, the low efficacy of NaDCC, also a sporicidal product, is surprising. There is an overall lack of efficacy of NaDCC against *B. licheniformis* and *B. subtilis* AEWD throughout all carrier tests, 5- and 60-min exposure, and spore tests. This is important to contribute to hospital guidance on infection prevention and control.

Results suggest that much of *B. licheniformis* resistance and *B. subtilis* AEWD, particularly at 12 days, is due to total DSB cells. This suggests the reason for the high resistance of *B. licheniformis* DSB to disinfectant products from the beginning. Chapter 4 illustrates images of all single species DSB through SEM imaging. There was no evidence of spores within these images, further suggesting that *B. licheniformis* and *B. subtilis* AEWD resistance is due to vegetative cells and their ability to form robust DSB. *B. subtilis* NCTC 10400 presented no evidence of spore production in DSB. *B. subtilis* NCTC 10400 was much less resistant to disinfection treatment than the other two *Bacillus* spp., suggesting that spore production may be a contributing factor to DSB susceptibility.

Pre-impregnated wipes are routinely used throughout hospitals on equipment and the near patient environment (Dural-Erem et al., 2019). Efficacy is based upon multiple factors. Sattar and Maillard (2013) describe the key factors in decontaminating a surface effectively: (i) the towelette, including size and material, (ii) the wiping action, (iii) the surface to be wiped, (iiii) contact time, and (iv) disinfectant used.

Although log_{10} reduction is a primary method of assessing the ability of a products to eradicate either planktonic cells or bacteria, it does not accurately demonstrate product efficacy (Ledwoch et al., 2019a). Healthcare workers gloves contribute to high transferability of environmental contamination throughout hospitals (Morgan et al., 2012), thus it is important to investigate all aspects of a wipes ability to reduce and transfer bacteria. Here, we observed that commercially available wipe products (**Table 3.2,** Chapter 3) were not that effective in eradicating *Bacillus* spp. when embedded in DSB. Generally, the product containing PAA (wipe E) had the greatest overall effect on bacterial viability. A similar observation was made by Ledwoch and Maillard (2018) who observed a successful log_{10} reduction of DSB of *Candida auris* when wiped with 3,500 ppm PAA and 1,000 ppm of sodium hypochlorite. Although they found these to be successful, of the 12 commercial wipe products they tested, 50% were not deemed effective in reducing bacterial viability and 58% failed to prevent transfer of bacteria from DSB surface. The PAA based wipe E yielded an average log_{10} reduction of 2.4 for both 30 and 60 sec contact (*B. subtilis* AEWD) and 0.8 (*B. subtilis* NCTC 10400). Both these values are lower than previously reported studies, Chowdhury et al (2019) used commercially available PAA based wipes, with concentration of 2,200 ppm active PAA, and found a 6.3log_{10} reduction of *S. aureus* DSB. It appears that the commercial product used here are not as effective against spore-formers. Calculations of available PAA from wipes ranged from 1,000 – 3,400 ppm (appendix 2.4).

We observed no significant difference between 30 and 60 sec contact time when regarding log_{10} reduction and direct surface transfer post wiping, indicating that either contact time is not as pertinent to DSB removal from a surface as we would expect, or a greater contact time than 60 sec is required to see significant effects. As we observed with increasing carrier test contact time to 60 min, DSB survival decreased (**Figure 5.5&6**). One issue with increasing contact time is the actual time that the surface remains wet for, increasing this can prove difficult as even after 30 seconds wiped discs appeared dry. All wipes displayed a 100% transfer rate of *B. subtilis* AEWD post wiping, this shows that even with mechanical action, bacteria are not fully removed when embedded in DSB. Interestingly, we have observed a robust DSB of *B. subtilis* AEWD which has proven difficult to eradicate via suspension tests and wiping.

The added mechanical action of a wipe-based product enhances cleaning performance (Sattar and Maillard, 2013; Ledwoch et al., 2021b; Pascoe et al., 2022). However, the high transfer of *B. subtilis* AEWD by the wipe itself was identified for all wipes. Previous work has alluded to the importance of transferability, especially when considering the transfer from hospital workers. DSB of *S. aureus* were readily transferred after treatment with a neutral detergent (Chowdhury et al., 2018). The wipe materials dislodge bacteria embedded in DSB and have inevitably transferred a proportion of total DSB to a new surface. This also indicates the contact time or active ingredient has not been sufficient to kill any viable bacteria within DSB. However, when considering *B. subtilis* NCTC 10400 wildtype strain, transfer by wipe was much reduced. Wipes B and D performed much better after a 60 second contact time compared to the others investigated. Although retention of bacteria and spores by the wipe was not looked at, we can conclude that these wipes did effectively stop the transmission of DSB from one surface to another.

5.6 Chapter conclusion

We have demonstrated that environmental isolates, *B. subtilis* AEWD and *B. licheniformis* are resistant to disinfectant treatment. Even after extended contact times of up to 60 min they able to remain on surfaces. *S. aureus* and *B. subtilis* NCTC 10400 are much more susceptible to disinfectant protocol but do increase in resistance to disinfectants over time of biofilm formation. Summary Figure 5.20 shows the development of resistance of species in DSB to each disinfectant tested. Both *S. aureus* and *B. subtilis* AEWD exhibit increased resistance over development of DSB to NaDCC (1,000 ppm) and BZK indicating that the longer time taken for DSB to develop, the decrease in susceptibility of DSB to products treatment. Although *B. licheniformis* did not display a similar trend to those disinfectants, as they were not efficient in reducing *B. licheniformis* DSB from the initial starting phase of DSB development. An increase in concentration of both PAA and NaDCC do not have increased effects on the resistance of all DSB over 12 days to all species with the exception of a high concentration of PAA against *B.licheniformis*, whereas the other biocidal products follow a different pattern. The use of a wipe product to remove DSB from a surface has proven relatively ineffective and has confirmed that DSB do remain on surfaces post wiping. Companies should take into consideration testing transference by wipe as we have shown that many commercially available

products are able to spread bacteria from one surface to another despite 60 second contact times.

Chapter 6
Dual Species DSB; the impact of *Bacillus* species on *Staphylococcus aureus*

6.1 Introduction

Biofilms are considered the most prevalent form of microbial existence in natural ecosystems (Nozhevnikova et al. 2015), where bacteria mostly reside in multispecies biofilms. There is a concern regarding the synergies, such as metabolic cooperation, resistance and quorum sensing systems, that can arise from species within a multispecies biofilm. These synergies can lead to the development of a mild infection becoming severe or life threatening (Lopes et al., 2018). Multispecies biofilms have a different spatial organisation compared to monospecies, which contributes to the fitness of the whole population within the biofilm (Elias and Banin 2012; Liu et al., 2016).

There is a plethora of research conducted on monospecies biofilms, predominantly due to the experimental limitations that come with complex communities of microorganisms (Hall-Stoodley et al., 2004; Sanchez-Vizuete et al., 2015). This does not concur with what is happening in real life scenarios, where it is widely evidenced that biofilms reside on ships hulls, in wastewater treatment, oral cavities, medical devices and wounds (Vishwakarma, 2020).

In recent years however, research has started to move towards multi-species biofilms; their complexity and interactions (Burmølle et al., 2014). Coaggregation, a process where genetically distinct bacterial species become attached to one another, is thought to aid multi-species biofilm formation in the natural environment (Yang et al., 2011). Interactions between the different species within a biofilm can change the structural and functional dynamics, influencing pathogenicity of the biofilm and promoting antimicrobial resistance (Harriott and Noverr, 2010). Not only do these interactions provide strength to an ever-changing environment, but competition is also common within the biofilm.

Several studies investigating the tolerance of multispecies biofilms to biocides, including chlorine (Schwering et al. 2013) and benzalkonium chloride (Ibusquiza et al. 2011) observed a decrease in biocide susceptibility of multispecies biofilms compared to monospecies biofilms (Sanchez-Vizuete et al. 2015). A worrying fact shows that resident flora, for example non-pathogenic bacteria found within the oral cavity, can protect pathogenic species from disinfection (Luppens et al. 2008). This was shown by Bridier et al. (2012) who evidenced the protection *Staphylococcus aureus* when embedded in a hydrated biofilm with *Bacillus subtilis* AEWD (isolate used in this thesis) from peracetic acid disinfection. Other studies on the oral

multispecies biofilm, identified a decreased in cell population upon exposure to chlorhexidine gluconate, however after time the cells within the biofilm began to recover and the number of viable bacteria within the biofilm increased back to where it began pretreatment (Shen et al., 2016). The results of this study were attributed to the presence of persister cells within the biofilm.

Environmentally isolated DSB are polymicrobial, containing both pathogenic and non-pathogenic species (Ledwoch et al., 2018). Although research has begun on these multispecies biofilms, there is still much to learn about these complex communities, especially their role in the hospital environment and implications for cleaning and disinfection protocols.

6.2 Aims and hypotheses

It was hypothesised that certain bacterial species, especially those with high resistance to disinfectants as a single species dry biofilm (Martin et al., 2015; Almatroudi et al., 2016), are able to protect other bacterial species that may be more susceptible to desiccation and subsequently, protect from disinfectant/biocidal products.

The main aim of the initial dry biofilm experiments was to formulate a dual species DSB that can be used for subsequent testing. Once established, the study aims to explore the potential impact dual species DSB have on the efficacy of commonly used disinfectant products and investigate virulence of a pathogen in DSB.

6.3 Methods

6.3.1 Bacterial growth and DSB formation

Bacillus subtilis washer-disinfector isolate (AEWD) (Martin et al., 2008), *Bacillus licheniformis* ATCC 14580 and *Staphylococcus aureus* NCTC 10788 were used (**Table 3.1**). Overnight cultures of each species were prepared as described in Chapter 3, section 3.4. Single species DSB of *B. licheniformis* and *S. aureus* were prepared through sequential dehydration and hydration phases as mentioned in Chapter 3, section 3.5. For dual species DSB (*B. subtilis* + *S. aureus*, *B. licheniformis* + *S. aureus*) were prepared in the same manner however bacterial inoculum consisting of 10^6 CFU mL of either *B. licheniformis* or *B. subtilis* and 10^{6-7} CFU mL of *S. aureus* with 3 g/L BSA was used as the start-up inoculum. The ratio (0.5:1) was found to produce the most consistent dual species DSB. The overnight

washed broth culture of *Bacillus* spp. used as an inoculum was not checked for the presence of endospores, since the overnight broth culture conditions were optimal for bacterial growth and not conductive for sporulation.

6.3.2 Wipe product testing

Three methods were used to test the overall effectiveness of six commercially available wipe products, including the water control wipe, as described in Chapter 3, section 3.7. These were determination of $\log_{10}$ reduction within DSB for each species, direct wipe transfer of bacteria and, transfer of bacteria following wiping. Specific selective media to distinguish between *Bacillus* spp. and *S. aureus* were not used to avoid introduction of potential additional stressors post wiping. Instead, TSA plates were used since *S. aureus* was easily distinguishable against *Bacillus* spp. due to the characteristic phenotypes of the colonies. Whilst *S. aureus* produces small round colonies that are golden yellow in colour, *Bacillus* spp. produce larger colonies that are whitish in colour. Single species DSB were also investigated according to Chapter 3, section 3.7.

6.3.3 Scanning Electron Microscope (SEM) Imaging

High vacuum SEM imaging of dual species DSB was performed using a Philips XL30 field emission gun-scanning electron microscope. Samples were prepared by submerging overnight in 2.5% glutaraldehyde solution, followed by a series of ethanol washes in increasing concentration (Chapter 4, section 4.3.1.1). Samples were coated with gold palladium before viewing under the microscope (Chapter 4, section 4.3.1.1).

Low vacuum SEM imaging was used to identify whether or not bacteria from the DSB were retained within the wipe following treatment. Only commercially available wipes A – E (detergent/disinfectant wipes) were analysed. Discs containing dual DSB were wiped on the same day as imaging took place. Used wipes were cut to 10x10mm squares and immediately attached to 12.5 mm aluminium SEM stubs (TAAB Laboratories Equipment Ltd) without any treatment and imaged with a Tescan MAIA3 FEG-SEM at a working distance of 5 mm. Samples were analysed at -5 ºC at 50 or 80 Pa.

6.3.4 Virulence assay using *Galleria mellonella*

A virulence assay using the wax moth larvae, *Galleria mellonella* was performed to assess pathogenicity of *S. aureus* within DSB to aid understanding of multispecies biofilms within hospitals and their risk to patients.

6.3.4.1 Preparation of planktonic suspension and DSB for injection

Cultures of *S. aureus* were prepared overnight in TSB as described in section 3.4 (chapter 3). Bacterial suspensions were centrifuged for at 5000 g for 10 mins at 21ºC. Pellets were resuspended in TSC and cultures were adjusted to approx. 10^8 CFU/mL at OD_{625nm} and subsequently diluted down to 10^6 CFU/mL. Suspensions were plated to ensure the correct concentration was used. Single species DSB of *S. aureus* were vortexed in TSC with 1 g of glass beads for 4 minutes. DSB were not diluted but plated and counted to confirm CFU/mL.

6.3.4.2 Storage and injection of *Galleria mellonella*

Larvae of the greater wax moth, *G. mellonella* are widely recognised as an alternative to mammalian models in the study of bacterial pathogenesis (Ramarao et al., 2012). *G. mellonella* larvae (Biosystems Technology, Exeter, UK) weighing between 0.18 – 0.35 g were stored at 4ºC and used within 2 weeks of delivery. Prior to experiments, groups of 10 individual larvae were placed in 9 cm petri dishes and left to acclimatise at room temperature (21ºC).

G. mellonella larvae were injected with 10 µL of bacterial or DSB suspension containing 10^7 and 10^6 CFU/mL, corresponding to an injection of 10^5 and 10^4 CFU/mL. Larvae were injected into the hemocoel via the last left proleg using a 50 µL Hamilton syringe with a 22-gauge needle (Sheehan et al., 2019). Needles were decontaminated with 70 % (v/v) ethanol before use and then rinsed with the relevant suspension before injection. Each needle was only used for a total of 5 injections. Controls were used to ensure injection accuracy these included a toxicity control of 100 % DMSO and an injection of TSC. An untreated control was also used to confirm death was not due to larval health. Injected larvae were incubated at 37ºC and survival was monitored every 24 hours for 5 days. Larvae were considered dead when no movement was observed in response to the light touch of a sterile pipette tip (Peleg et al., 2009). Ten larvae were used for each condition, which was performed in triplicate (**Figure 6.1)**.

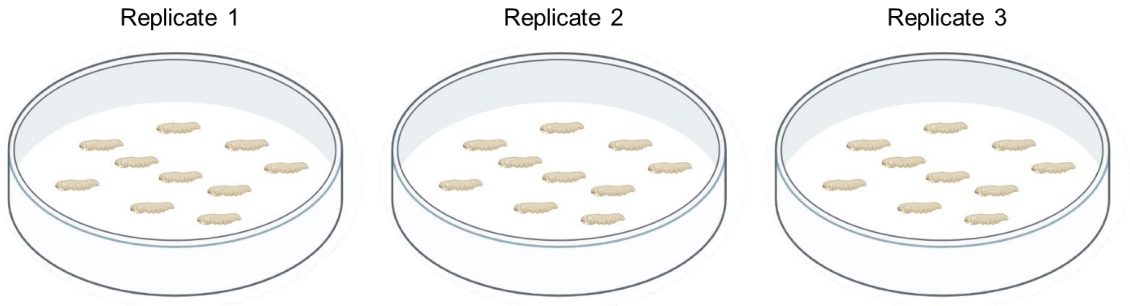


Figure 6.1: Schematic of *G. mellonella* virulence assay. Each petri dish contains 10 larvae. Three repeats were performed for each test condition to give a total sample size of 30 larvae (Image created with BioRender.com).

6.3.5. Statistical analyses

Statistical analysis was carried out, where appropriate, using GraphPad Prism 9 software (version 9.3.1). ANOVA (One-way and Two-way) were used to determine statistically significant differences within datasets (Chapter 3, section 3.11). Post-hoc tests were used for multiple comparisons.

6.4 Results

6.4.1 Growth of dual DSB

The environmental isolate, *B. subtilis* AEWD inhibited the growth of *S. aureus* when grown as a dual DSB (**Table 6.1**). Observations taken from SEM images of *B. subtilis*/*S. aureus* confirm the absence of *S. aureus* cells when embedded in a dual DSB with *B. subtilis* (**Figure 6.1**). *B. subtilis* formed a dense biofilm covering the disc surface. This does, however, make it harder to identify any *S. aureus* cells that could be hidden under the top layer of *B. subtilis* cells. The formation of a dried dual *S.* aureus/*B. licheniformis* species biofilm was confirmed with SEM imaging (**Figure 6.3.**). Whilst both bacterial species can be clearly identified, *S. aureus* culturability when the DSB was plated on TSA was not always consistent as *S. aureus* only grew in 50% of samples following DSB formation (**Table 6.1**).

Table 6.1. Culturability of both bacterial species from DSB recovered on TSA plates. Starting inocula: *Bacillus licheniformis*: 6 Log_{10} cfu/mL; *Bacillus subtilis*: 6 Log_{10} cfu/mL, *Staphylococcus aureus*: 6-7 Log_{10} cfu/mL.

Biological repeats	**Log_{10} cfu/mL recovered after DSB formation**			
	***B. licheniformis* – *S. aureus* dual DSB**		***B. subtilis* – *S. aureus* dual DSB**	
	B. licheniformis	*S. aureus*	*B. subtilis*	*S. aureus*
1	5.6	5.04	5.5	No growth
2	5.8	5.84	5.5	No growth
3	6.0	No growth	5.9	No growth
4	5.2	No growth	6.2	No growth
5	5.4	4.0	6.3	No growth
6	5.6	No growth	-	-

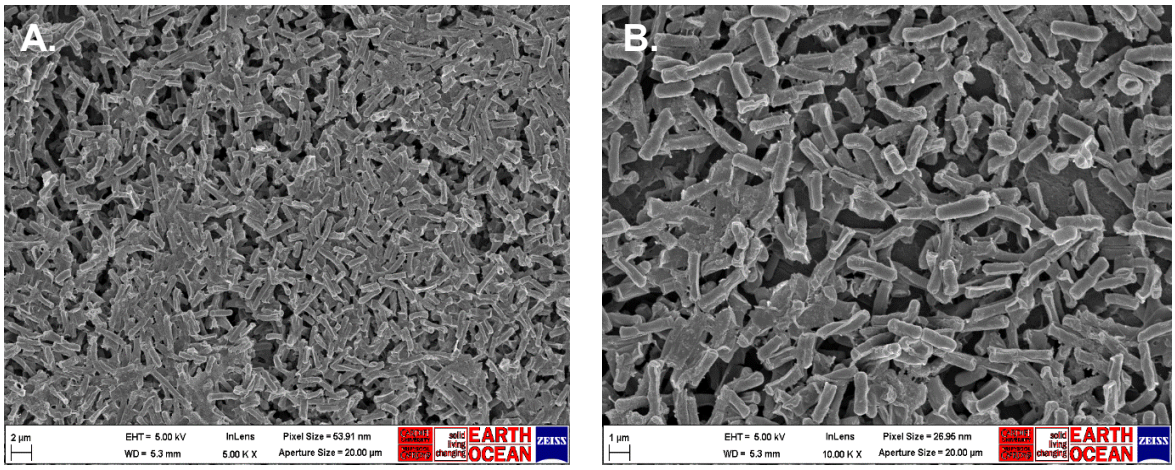


Figure 6.2. SEM imaging of dual DSB (*Bacillus subtilis* and *Staphylococcus aureus*). Images taken at (A) x 5000 magnification and (B) x 10,000 magnification. Only *B. subtilis* can be identified in the images. Images presented are representative of the whole disc surface. Observations were made on three independent repeats.

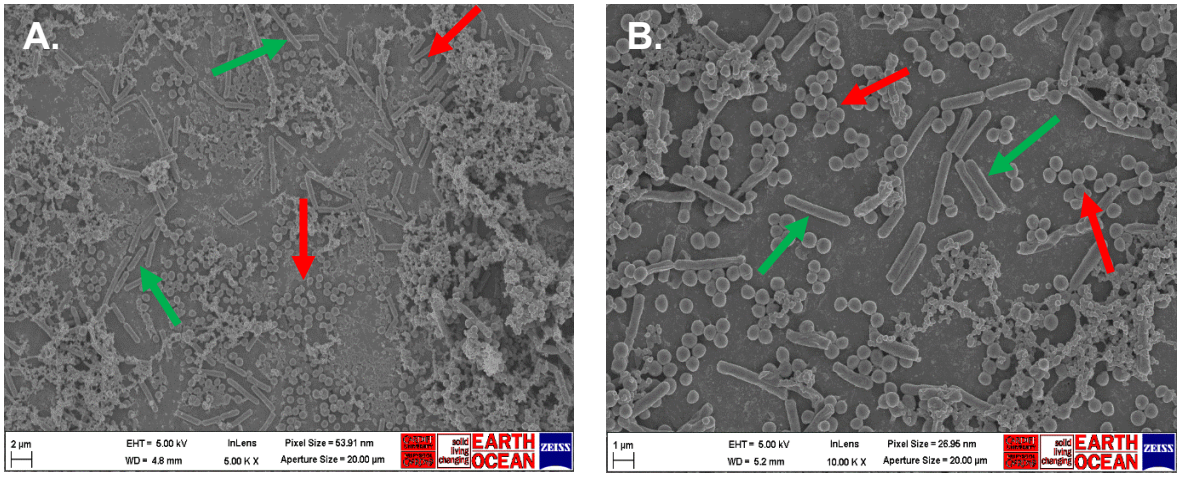


Figure 6.3. SEM imaging of dual DSB (*Bacillus licheniformis* and *Staphylococcus aureus*). Images taken at (A) x 5000 magnification and (B) x 10,000 magnification. Both bacterial species can be identified (green arrows: *Bacillus licheniformis*; red arrows: *Staphylococcus aureus*). To note, there was no evidence of bacterial endospores. We would expect bulging at the very end of rod cells if spores were present, or very small circular spores in the sample. Images presented are representative of the whole disc surface. Observations were made on three independent repeats.

6.4.2 Log_{10} reduction of bacteria embedded in DSB

6.4.2.1 *Staphylococcus aureus*

Although inconsistencies in culturability of *S. aureus* within the dual DSB were identified, repeats where there was positive growth of *S. aureus* in the untreated control were selected to allow for a better understanding of the protection of *S. aureus* by *B. licheniformis*. Variability in results observed with some products with the dual DSB originated with some low *S. aureus* count in dual DSB.

When single biofilm is considered, only 2 products (A & E) produced a > 4 log_{10} reduction in *S. aureus* after 30 sec contact (**Figure 6.4a**). Wipes A, C and D all performed better against single species DSB than dual species DSB after 60 sec (**Figure 6.4b**). There was no overall statistically significant difference between single and dual species DSB at either 30 or 60 sec contact time when comparing all wipe

products used (Two-way ANOVA, $p > 0.05$). When comparing individual wipes only wipe C after 60 sec contact time was significantly different between the two types of DSB (ANOVA, Šídák, $p = 0.04$) (**Figure 6.4**). Interestingly, wipe B had little activity against *S. aureus* single species DSB (avg. 1.6 $\log_{10}$ reduction) compared to dual species DSB after 60 sec (avg. 3.4 $\log_{10}$ reduction). Whereas the opposite can be seen for wipe C, whereby an avg. 1.2 $\log_{10}$ reduction was observed when *S. aureus* was in a dual DSB, but avg. 3.7 $\log_{10}$ reduction in single DSB (**Figure 6.4b**). It is important to note that *S. aureus* results from the single DSB were consistent with what has been previously reported in other studies (Almatroudi et al., 2018; Ledwoch et al., 2019). There was no statistically significant difference (One-way ANOVA, $p > 0.05$) between the water control and wipe products A – E when *S. aureus* was in a dual DSB at either 30 or 60 sec (**Figure 6.4**). However, in a single species DSB both 30 and 60 sec showed overall statistically significant differences between all wipe products (One-way ANOVA, $p < 0.05$). When compared to the water control, only wipe B was significantly different at 60 sec (ANOVA, Dunnett, $p = 0.02$) (**Figure 6.4b**). When comparing individual wipe products at 30 vs 60 sec contact time, there was no statistically significant difference with dual DSB (ANOVA, Šídák, $p > 0.05$). Only wipe C was significantly different between 30 and 60 sec contact time when *S. aureus* was in a single DSB (ANOVA, Šídák, $p < 0.05$).

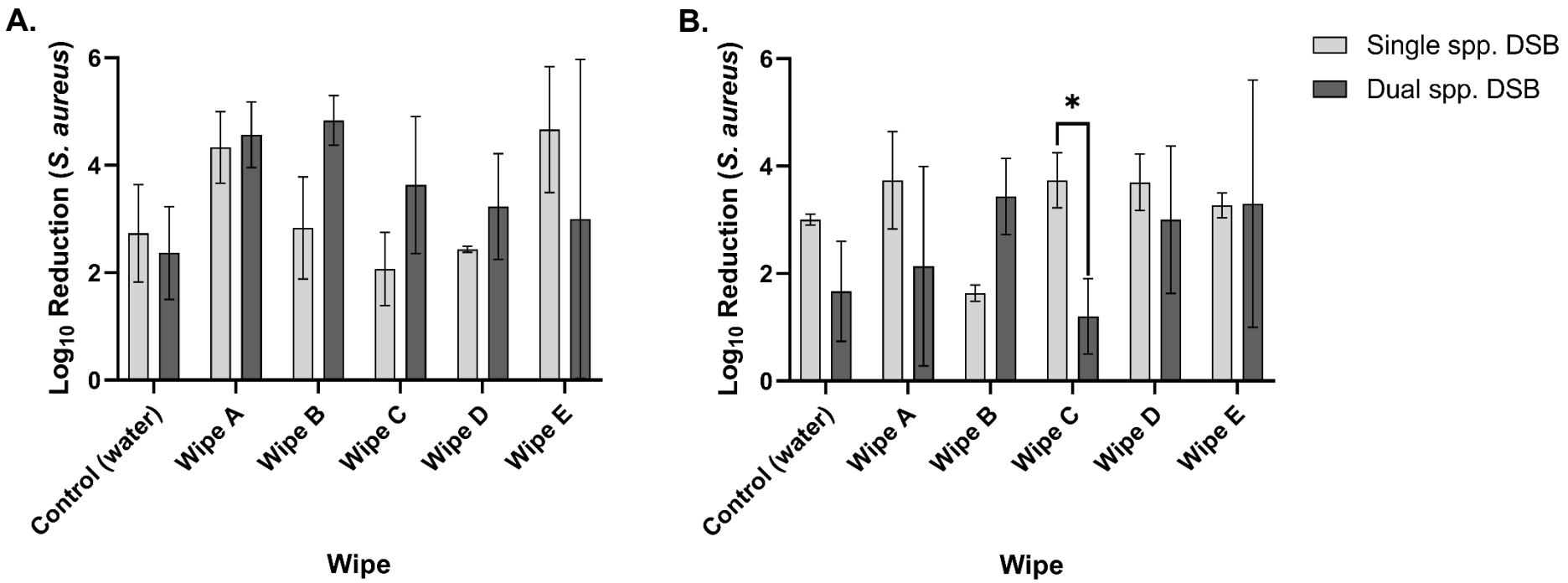


Figure 6.4. Product efficacy in reduction of *S. aureus* in a single and dual spp. DSB after 30 and 60 sec contact time with wipe. (A) Product efficacy after 30 sec contact time, (B) Product efficacy after 60 sec contact time. There was no overall statistically significant difference between single and dual species DSB at 30 or 60 sec contact time when comparing all wipe products (two-way ANOVA, $p > 0.05$). Individually, only $\log_{10}$ reduction values from wipe C after 60 sec were significantly different between single and dual DSB (ANOVA, Šídák, $p = 0.04$). Wipe B was significantly different to the water control wipe after 60 sec contact time in a single DSB (ANOVA, Dunnett, $p < 0.05$).

6.4.2.2 *Bacillus licheniformis*

The majority of commercially available disinfectant and detergent wipes used here, did not effectively eradicate *B. licheniformis* in DSB (**Figure 6.5**). There was no statistically significant difference in activity between any of the products tested after 60 sec contact time (one-way ANOVA, $p < 0.05$) against *B. licheniformis* dual DSB separately (**Figure 6.5b**). The same can be said for 30 sec contact time with single species DSB (one-way ANOVA, $p < 0.05$). However, when *B. licheniformis* was embedded in a dual species DSB, there was a significant difference between all wipes (one-way ANOVA, $p > 0.05$) (**Figure 6.5a**). Log_{10} reduction values from wipes C and E were significantly different to the water control after 30 sec in dual species DSB (ANOVA, Dunnett, $p > 0.05$) (**Figure 6.5a**), however, bacterial reduction with all other wipe products when compared with the water control, when both 30 and 60 sec contact times were used, were not statistically significant (ANOVA, Dunnett, $p < 0.05$). With the dual species DSB at 60 and 30 sec, wipe E performed the best overall in reduction of *B. licheniformis* from the biofilm, demonstrating average log_{10} reductions of 3.5 and 3.9 respectively (**Figure 6.5**). Interestingly, wipe E, performed much better in eradicating *B. licheniformis* from the dual species DSB compared to a single species DSB (**Figure 6.5**). The negative log_{10} reduction values from treatment of dual DSB with wipe D (30 sec) likely impacted the statistical difference significance, as untreated control counts of *B. licheniformis* were low (**Figure 6.5a**). The maximum reduction in bacteria from a single species DSB was circa 2 log_{10} at both 30 and 60 sec. With the dual species DSB, there was no bacterial reduction with the water control and 2 of the products (A & D) after 30 sec contact time (**Figure 6.5a**). A two-way ANOVA showed a significant difference in log_{10} reduction from *B. licheniformis* in dual DSB between 30 and 60 sec contact times ($p > 0.05$). A post hoc test showed specifically the control water wipe and wipe A were statistically significant (ANOVA, Šídák, $p < 0.05$). With regards to single species DSB, there was a statistically significant difference between 30 and 60 sec contact time (two-way ANOVA, $p < 0.05$), but no statistically significant difference between wipe products (two-way ANOVA, $p > 0.05$). Differences in log_{10} reduction were statistically significant between single and dual DSB at 30 sec contact time for water control and wipes A, D and E (ANOVA, Šídák, $p < 0.05$).

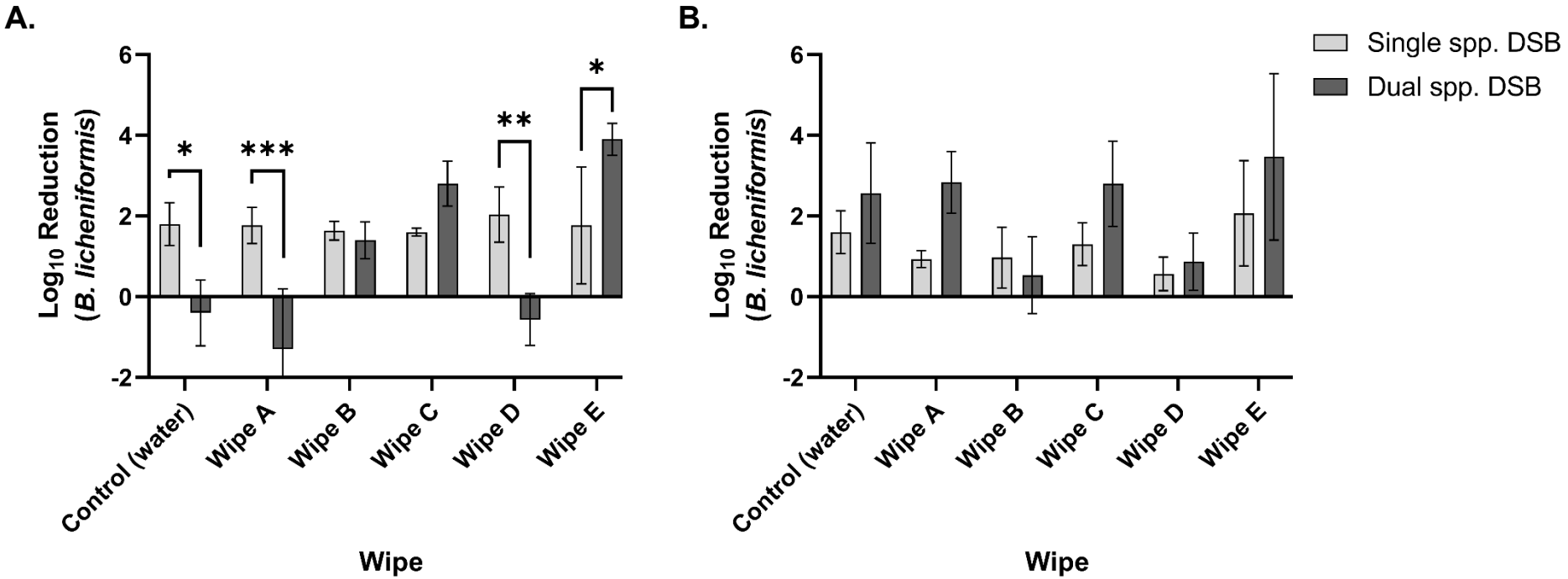


Figure 6.5. Product efficacy in reduction of *B. licheniformis* in a single and dual spp. DSB after 30 and 60 sec contact time with wipe. (A) Product efficacy after 30 sec contact time, (B) Product efficacy after 60 sec contact time. There was no statistically significant difference in efficacy of wipe products after 60 sec contact time (one-way ANOVA, $p < 0.05$). At 30 sec, there was no statistical significance between all wipe products when *B. licheniformis* was embedded in a single species DSB (one-way ANOVA, $p < 0.05$). There was a statistically significant difference between $\log_{10}$ reduction of bacteria embedded in a single vs dual DSB after 30 sec contact time for the water control and wipes A, D and E (ANOVA, Šídák, $p < 0.05$).

6.4.3 Direct wipe transfer of bacteria to a new surface

The efficacy of antimicrobial wipes is measured by the number of bacteria removed/killed on surfaces but also by the ability of the wipe not to transfer microorganisms to other surfaces (Wesgate et al. 2019).

Wipe products (B – E) did not transfer any *S. aureus*, regardless of being in a single or dual species DSB (**Table 6.2**). The water control wipes transferred significantly more (two-way ANOVA, $p < 0.05$) *S. aureus* (> 4 $\log_{10}$) in a single species DSB, than any of the wipe products (**Table 6.2**). More *S. aureus* were transferred from the single species biofilms (circa 3-4 $\log_{10}$) than from the dual biofilm by the water control and wipe A (circa 1-2 $\log_{10}$) (**Table 6.2**). There was no difference in *S. aureus* transfer from the single species DSB between 30 and 60 second contact time (two-way ANOVA, $p > 0.05$). There was a statistically significant difference between $\log_{10}$ transfer of *S. aureus* in a single DSB versus dual DSB after 30 and 60 sec contact time for both control water and wipe A (ANOVA, Šídák, $p < 0.05$).

Table 6.2. Direct wipe transfer of *S. aureus* from a single and dual species DSB at both 30 and 60 second contact time following wiping. Number represents $\log_{10}$ transfer of bacteria with standard deviation.

	Log10 transfer of bacteria (± SD)			
	Single species DSB		**Dual species DSB**	
Wipe	**30 seconds**	**60 seconds**	**30 seconds**	**60 seconds**
Control (water)	4.6 (± 0.2)	4.1 (± 0.3)	2.7 (± 0.7)	2.0 (± 1.7)
A	3.5 (± 0.5)	3.2 (± 0.2)	0.9 (± 0.4)	0.9 (± 0.3)
B	No transfer	No transfer	No transfer	No transfer
C	No transfer	No transfer	No transfer	No transfer
D	No transfer	No transfer	No transfer	No transfer
E	No transfer	No transfer	No transfer	No transfer

Overall, wipe products including water control transferred a high amount of *B. licheniformis* (2-4.6 log_{10} transfer) following wiping of DSB both in a single and dual species DSB at 30 and 60 sec contact times (**Table 6.3**). Noticeably, more *B. licheniformis* was transferred when embedded in a single species DSB, all products transferred ≥ 3.5 log_{10} bacteria. Whereas, when in a dual DSB, log_{10} transfer was ≥ 1.7, a 1.8 log_{10} difference between the lowest transfer values (**Table 6.3**). There was a statistically significant difference in wipe transfer of *B. licheniformis* between the dual and single DSB for all products after 60 sec contact time (two-way ANOVA, $p < 0.05$) (**Table 4**). ANOVA, Šídák revealed wipe E had the greatest significant difference in *B. licheniformis* transfer from wipes between dual and single DSB ($p = 0.0005$). After 30 second contact time with *B. licheniformis*, there was no difference in transfer between the dual and single DSB for all products (two-way ANOVA, $p > 0.05$).

Table 6.3. Direct wipe transfer of *B. licheniformis* from a single and dual species DSB at both 30 and 60 second contact time following wiping. Number represents log_{10} transfer of bacteria with standard deviation.

	Log10 transfer of bacteria (± SD)			
	Single species DSB		**Dual species DSB**	
Wipe	**30 seconds**	**60 seconds**	**30 seconds**	**60 seconds**
Control (water)	3.5 (± 0.2)	3.9 (± 0.6)	2.1 (± 0.8)	2.3 (± 0.5)
A	4.1 (± 0.6)	4.1 (± 0.5)	3.8 (± 0.5)	2.8 (± 0.6)
B	4.3 (± 0.5)	4.6 (± 0.4)	3.9 (± 0.4)	3.1 (± 1.0)
C	3.7 (± 0.1)	4.6 (± 0.2)	3.3 (± 0.8)	3.4 (± 0.1)
D	4.3 (± 0.4)	4.6 (± 0.1)	4.0 (± 0.3)	3.2 (± 0.2)
E	4.0 (± 1.3)	3.6 (± 0.3)	3.2 (± 1.9)	1.7 (± 0.5)

6.4.4 Transfer of bacteria from wiped discs

One pertinent question about infection prevention and control is whether a surface is safe following cleaning and disinfection. Here, we investigated whether any viable bacteria in DSB remaining on surfaces post-wiping could be transferred. Overall, *B. licheniformis* remaining on surfaces was easily transferred by direct adpression of the surface to DE agar following 30 and 60 sec contact time post wiping (**Figure 6.6**). All wipes transferred > 90% of *B. licheniformis* in a dual species DSB except for wipe D at 30 sec. In a single species DSB, all wipes transferred 100% of *B. licheniformis*. With *S. aureus*, the results are more variable. After 30 sec contact time post wiping A, B, C and E did not transfer any *S. aureus* (**Figure 6.7a**) whilst the water control and wipe D did not prevent the direct transfer of *S. aureus* from dual DSB (**Figure 6.7a**). *S. aureus* direct transfer was much reduced (< 10%) from dual species DSB following wiping at a contact time of 60 sec with products D and E (**Figure 6.7b**). Variation was highest with wipes B – E, when considering % transfer of *S. aureus* from dual DSB, **Figure 6.7** shows the range of data from 0 – 100%.

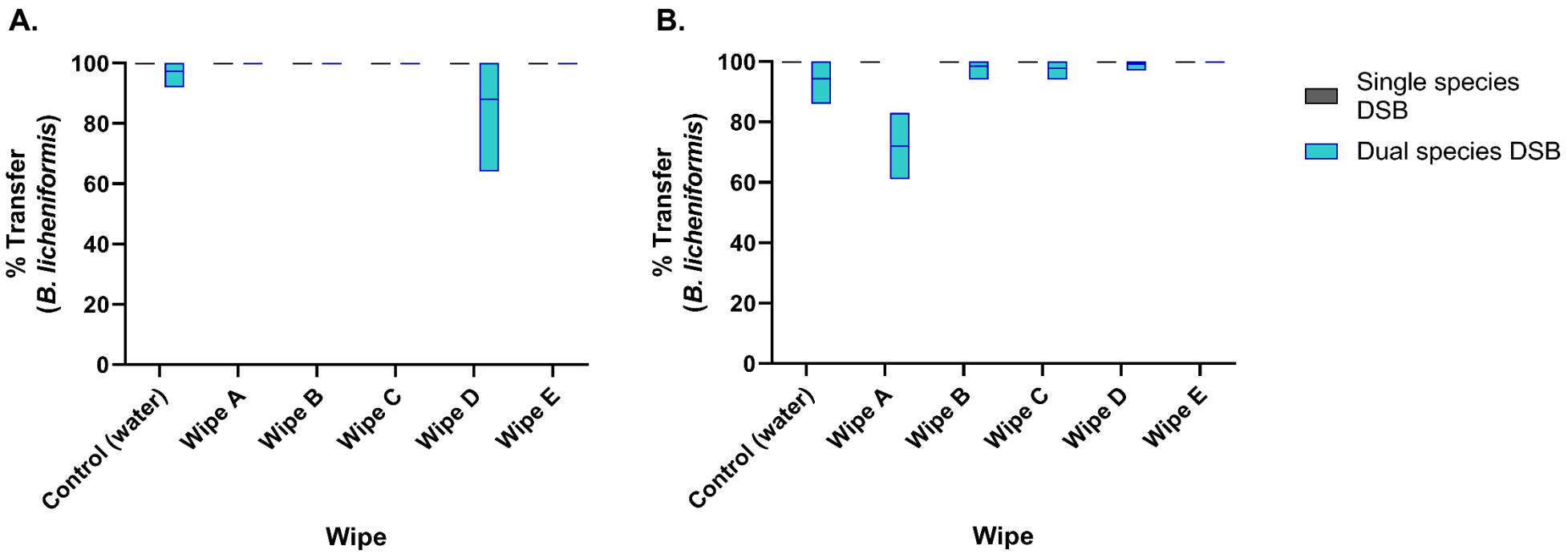


Figure 6.6. Percentage of *B. licheniformis* transferred from a dual or single DSB post wiping. (A) 30 second contact time, (B) 60 second contact time following wiping. Direct transfer was measured after pressing the disc directly onto DE agar following wiping. Percentage (%) transfer was calculated as the number of squares containing growth divided by the total squares (36) multiplied by 100. Box plots show the full spread of data including mean.

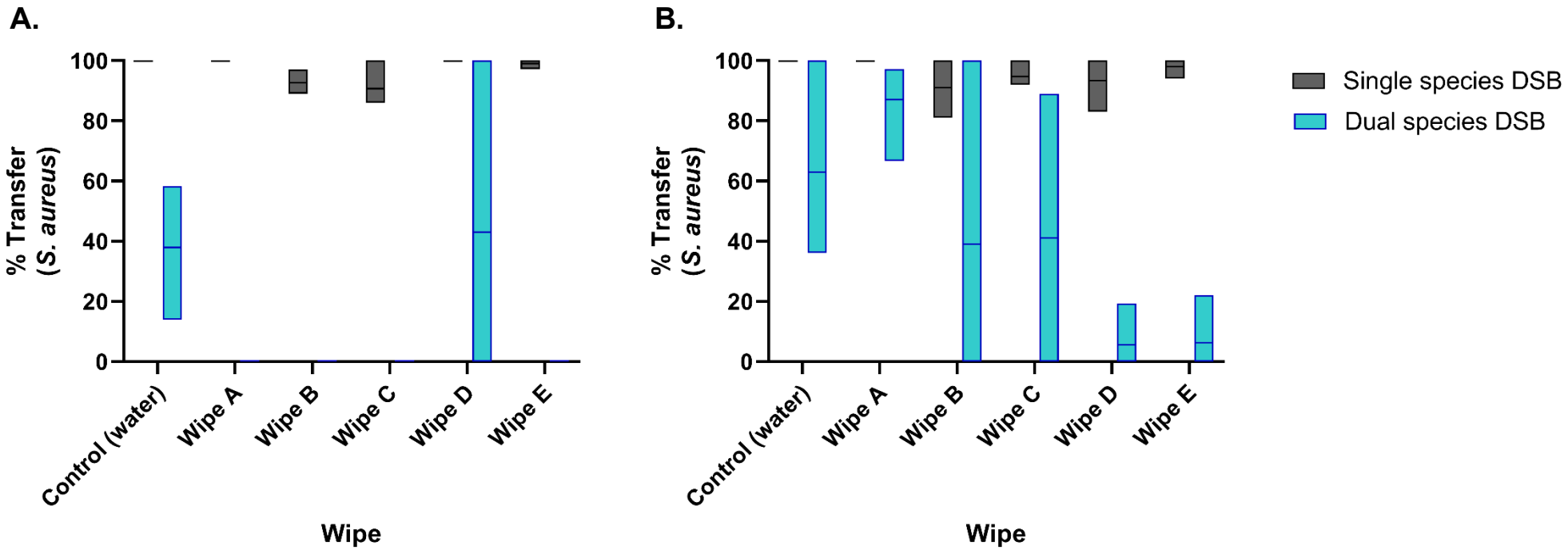


Figure 6.7. Percentage of *S. aureus* transferred from a dual or single DSB post wiping. (A) 30 second contact time, (B) 60 second contact time following wiping. Direct transfer was measured after pressing the disc directly onto DE agar following wiping. Percentage (%) transfer was calculated as the number of squares containing growth divided by the total squares (36) multiplied by 100. Box plots show the full spread of data including mean.

6.4.5 DSB embedded in wipes

Following wiping of dual species DSB disc, commercially available wipes were imaged under low vacuum SEM to identify if DSB rafts were embedded into the wipe fibres. The presence of DSB within all wipes is evident from imaging, with bacterial clusters present in wipe fibres (**Figure 6.8**). Both *S. aureus* and *B. licheniformis* cell morphologies are clear, especially within wipe D. Aggregates of DSB are mainly seen in wipes A – D, however images of wipes E show a homogenous spread of bacteria all over the wipe material. Wipe E has a much rougher texture than the other wipes, where the fibres are smooth (data not shown).

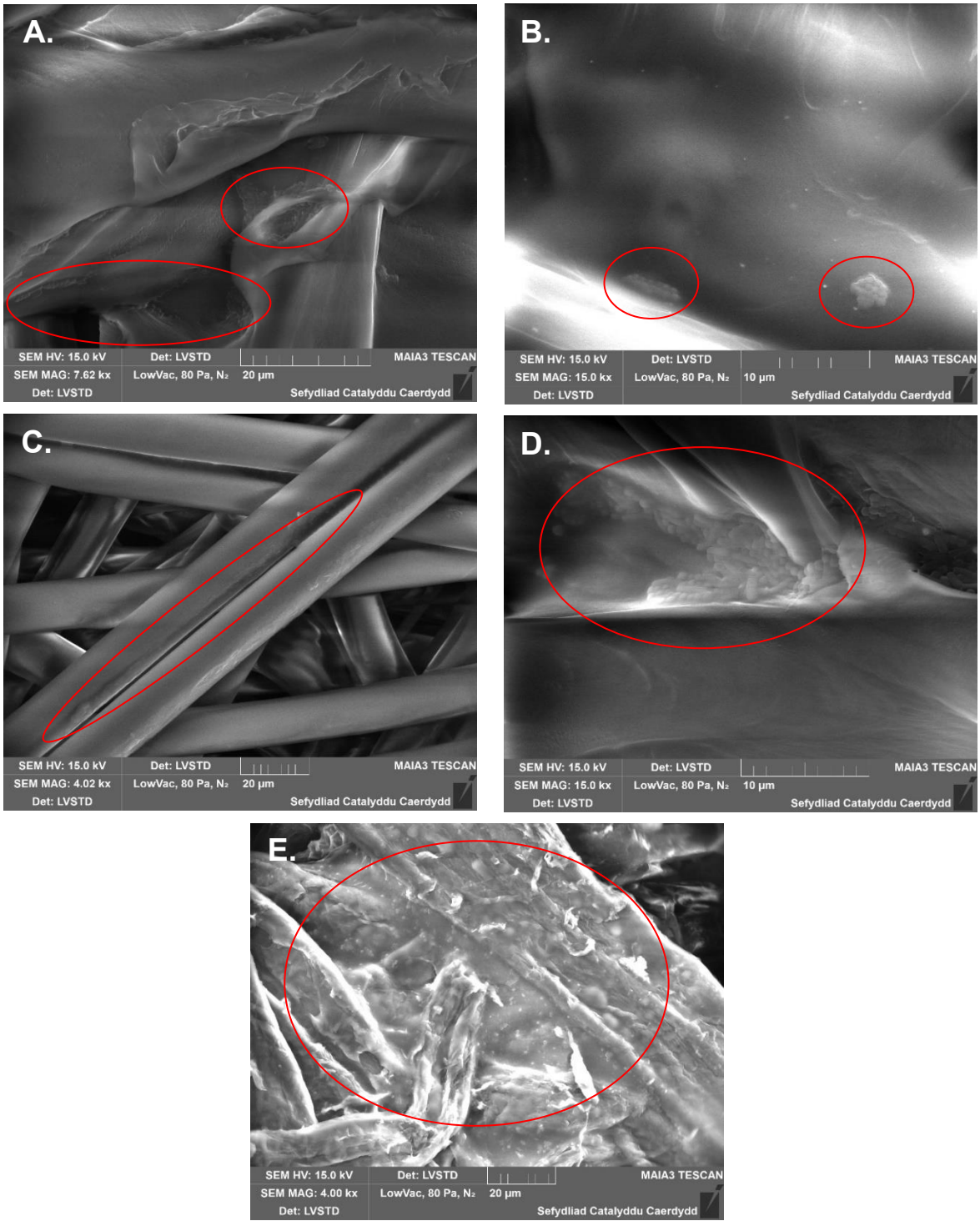


Figure 6.8. Low vacuum SEM imaging of wipe materials A – E post-wiping of dual DSB (*Bacillus licheniformis* and *Staphylococcus aureus*). Red circles indicate presence of bacteria and DSB rafts. (A) wipe A, (B) wipe B, (C) wipe C, (D) wipe D and (E) wipe E. Images taken between x4,000 and x15,000 magnification.

6.4.6 *G. mellonella* virulence assay

Single species *S. aureus* DSB was assessed for any changes to virulence of the species when compared to a planktonic suspension. After 5 days of incubation, 100 % survival of *G. mellonella* larvae was recorded for both untreated controls and TSC controls (**Figure 6.8**). After 48 hours, all larvae injected with 100% DMSO solution had died, only 10% survival rate was recorded after 24 hours. *S. aureus* DSB grew to 10^6 CFU/mL and so was compared to the equivalent bacterial concentration in planktonic suspension. There was no significant difference between survival of *G. mellonella* larvae when injected with either *S. aureus* DSB or planktonic suspension (two-way ANOVA, $p > 0.05$) (**Figure 6.8**). After 5 days of incubation, average survival rate was 83% and 73% for planktonic suspension and DSB respectively. After 24 hours, all larvae remained alive after injection with *S. aureus* DSB, but this dropped to 87% survival after 48 hours (**Figure 6.8**).

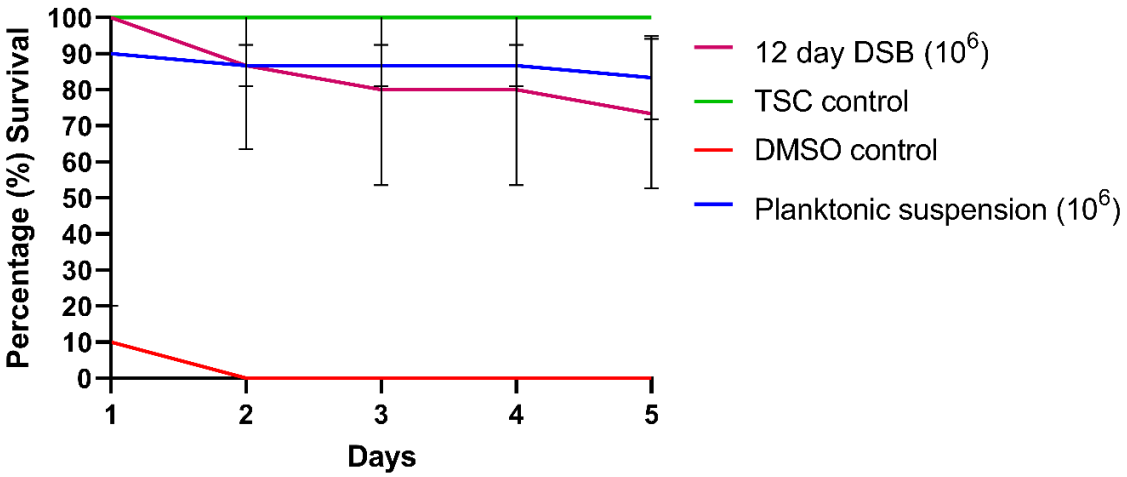


Figure 6.8. Percentage survival of *G. mellonella* larvae after injection with *S. aureus* DSB or planktonic suspension. There was no significant difference between the planktonic suspension and DSB survival rate (two-way ANOVA, $p > 0.05$). After 48 hours all larvae in the DMSO control were confirmed dead, whereas both TSC and untreated controls saw 100% survival after 5 days.

6.5 Discussion

Aapproximately 40 – 80% of all living bacteria on earth reside in the form of a biofilm (Hall-Stoodley et al. 2004). The complexity and distinct spatial organisation of multispecies biofilms presents advantages in natural habits compared to those housed in a single species biofilm (Røder et al. 2020), this includes an increased tolerance to disinfectants like chlorine (Schwering et al. 2013).

DSB colonise various dry surfaces throughout hospitals (Hu et al. 2015; Ledwoch et al. 2018) and contribute to the contamination of near patient environments, providing an ideal substrate for microbial contamination over prolonged periods of time (Weber et al. 2013). Cleaning and disinfection of these dry surfaces is essential for patient recovery and reduction in HCAIs (Doll et al. 2018).

To our knowledge, this is the first study to produce a dual species dry surface biofilm for testing against commercially available wipes. We measured the efficacy of commercially available wipe products using three different parameters: reduction in bacterial viability, direct surface to surface transfer post wiping and transfer of bacteria by the wipe itself.

Here, we present culturability data, which can be defined as the detectable replication of bacteria, i.e. growth on agar (Barer and Harwood 1999). We observed a large variation in culturability of *S. aureus* on agar when in a dual DSB with *B. licheniformis*. Behaviour between species in a biofilm can be cooperative, competitive or neutral (Nadell et al. 2016; Alonso et al. 2020; Li et al. 2021). Cooperation can include protection from biocides whereas competition may be the result of lack of space, nutrients and other resources (Alonso et al., 2020). Results inconsistency could be attributed to competition between the two species; *B. licheniformis* becomes the dominant species within the dual DSB and thus *S. aureus* is hard to identify on agar when in laboratory conditions. Although some studies have previously shown that *B. licheniformis* prevents the initial adhesion of *S. aureus* cells in a biofilm by producing a biosurfactant (Sayem et al., 2011), this is unlikely to be the case here as *S. aureus* cells were evident under SEM and light microscope (data not shown) in samples where *S. aureus* did not grow on agar, indicating *S. aureus* did adhere to the surface in a dry biofilm as they could not be present in a planktonic state.

Gause's law states that two species in competition cannot remain at stable levels when exposed to limited resources over time, so one must become dominant

(Rescigno and Richardson, 1965). The same principle is observed with *B. subtilis* and *S. aureus*. When we observed both SEM images taken and culturability data there is no evidence of *S. aureus* within the DSB. The observed inhibitory effect of *B. subtilis* against *S. aureus* supports evidence of its use as a probiotic cleaner, as it has been shown to counteract growth of pathogens on dry hospital surfaces (Caselli et al. 2016). Microbial cleaning with *B. subtilis* on hospital surfaces has been shown to be effective in reducing HCAIs over prolonged periods compared to more conventional disinfectant cleaning methods (Vandini et al. 2014). *B. subtilis* has also been found to produce exogenous D-amino acids which can interfere with other species in a biofilm, specifically preventing biofilms of *S. aureus* from reaching maturity (Kolodkin-Gal et al., 2010).

Our original hypothesis was the ability of a non-pathogenic environmental species, *Bacillus* species to protect a pathogenic species, *S. aureus* from disinfection. Bridier et al. (2012) demonstrated the impact of *B. subtilis* hydrated biofilm to protect *S. aureus* against exposure to 3500 ppm peracetic acid. It should also be noted that although they did observe a lower log reduction value in the mixed biofilm, it was still very high (5.9 $\log_{10}$). Such a strong protective effect was not observed in our study. Instead, *B. subtilis* AEWD (the same isolate used in Bridier's study) inhibited the growth of *S.* aureus. When *B. licheniformis* was used, no protective effect was observed (**Figure 6.4 & 6.6, Table 6.2**), apart from wipe C after 60 sec contact time (**Figure 6.4b**). A noticeable difference between hydrated biofilm and DSB is the amount of EPS observed. Although SEM images indicated presence of EPS in the DSB, which is consistent with other studies (Almatroudi et al. 2015), the extensive EPS network described in hydrated biofilms was not observed as identified by Bridier et al. (2012). The impact of EPS in protecting bacteria from disinfection has been well reported (Almatroudi et al. 2018; Ledwoch et al. 2019b; Nkemngong et al. 2020) and is one of the major mechanisms responsible for the decreased biofilm susceptibility to disinfection (Vickery et al. 2012; Xue et al. 2012).

The majority of commercially available disinfectant and detergent wipes used here, do not effectively eradicate *B. licheniformis* from dry surfaces. In a single species DSB *B. licheniformis* is more resistant to cleaning and disinfection than in a dual species DSB with *S. aureus*. Wipe E was most effective at removing *B. licheniformis* from DSB, indicating a requirement for sporicidal products on hospital surfaces.

The efficacy of a surface disinfectant should not only be measured as log reduction in viability, but also as both decreasing bacteria transfer directly or indirectly through cloth/wipe and time taken for biofilm to regrow (Ledwoch et al. 2021b). Indeed, accepted efficacy product test standards such as the ASTM 2967-15 and the EN 16615-15 (ASTM E2967 2015; EN 16615 2015) measure both reduction in viability and transfer. Our results confirmed the direct transfer (> 80%) of *B. licheniformis* and *S. aureus* from single species DSB when treated with all wipes, in agreement with previous studies (Ledwoch et al. 2018, 2019b & 2021b). This indicates that not all microorganisms and their organic load is removed from a surface when wiping. When dual species were evaluated, a decreased transfer in *S. aureus* was observed (**Figure 6.6**). Although there was a reduction in percentage transfer when in a dual species DSB, *B. licheniformis* transfer remained high (**Figure 6.5**). Single species DSB of both *S. aureus* and *B. licheniformis* appeared to be denser and colonised more of the disc surface (Chapter 4, sections 4.4.1.2 and 4.4.1.4) than the dual DSB, therefore harder to remove from the disc surface by wipes. Preventing bacterial transfer from a contaminated wipe to another surface overall makes a product safer to use. Again, we observed high variability within the dual dataset for direct surface transfer when considering *S. aureus*, hence statistics were not performed due to different populations within the dataset. This observation has previously been mentioned when considering dual species DSB culturability.

There was no transfer of *S. aureus* from either a single or dual species DSB when wipes B – E were used. These products are highly effective and should be used on hospital surfaces to stop the spread of pathogenic species. However, a high transfer of *B. licheniformis* from wipes to a new surface was observed with the water control, a microfibre cloth. The effectiveness of microfibre cloths has been previously shown to be not better than that of non-woven conventional cloths, with the added risk of recontamination of surfaces with microorganisms (Moore and Griffith 2006). Although the removal of bacterial from surfaces using water and cloth was similar to cleaning/disinfectant wipes (**Figure 6.3 & 6.4**), the ability of the water control wipe to transfer bacteria was not. The effectiveness of water on microfibre cloth has previously been investigated by Robertson et al. (2019), who similarly showed that water alone is much less effective at reducing transfer of microorganisms between surfaces and should not be used as a replacement to disinfectant wipes. Our results show that wipe material alone may result in the removal of microorganisms, but

disinfectants are key to also reducing transfer, rendering the product safer, and the surface safe post-wiping. The spread of bacteria from one surface to another by microfibre cloth has also been described previously and questions the use of this material for environmental cleaning (Bergen et al. 2009). Parvin et al. (2019) investigated the ability of cloth moistened with water to remove *S. aureus* DSB and observed a 1.48 $\log_{10}$ reduction, even with wiping the DSB surface up to 50 times. This reduction is much lower than the results presented here (≤ 3 $\log_{10}$ reduction with water control), which may be explained with differences in methodology and wipe materials. In our study, DSB are formed by sedimentation in well plates, whilst Parvin et al. (2019) used the CDC Biofilm Reactor. In their study a viscose/polyester blend material was also used to wipe surfaces. It has been reported that, different wipe materials vary greatly in their ability to remove bacteria from surfaces (Boyce 2021). The presence of dual DSB aggregates on wipe products identified through low vacuum SEM and low transfer rate of bacteria to a new surface suggests that the wipes do not release bacteria to new surfaces assuming correct practice use, one wipe one direction (Williams et al. 2007), is followed.

Although boxplots might not be recommended for use with small data samples (Krzywinski and Altman 2014), they are used in this study as they are a much better figure for presentation of the data collected to evidence variability of dual DSB.

The greater wax moth larvae, *G. mellonella*, have been widely used to investigate virulence of microorganisms (Tsai et al., 2016) due to their ease of use, low cost, and high throughput (Ménard et al., 2021). Over 5 days of recording larval survival, there was no statistical difference between *S. aureus* planktonic control suspension and DSB. Virulence of *S. aureus* does not change when embedded in a DSB indicating the species is still a threat to patients when colonising dry hospital surfaces. Larval survival steadily decreased over 5 days but did not reach below 70%, this does remain high, but the concentration of injected bacteria decreased by 2 $\log_{10}$ from initial inoculum due to the quantity injected.

When we consider contact times used, there was little difference between 30 and 60 sec contact times. With the exception of *B. licheniformis* when in a dual DSB. This most likely arose from the low untreated control counts. This indicates that the products do not perform better with an increased contact time with DSB and have started to effectively eradicate bacteria even when in contact for just 30 sec. However, the poor performance of all wipes at both 30 and 60 sec with single

species DSB when considering direct transfer of bacteria indicates the need stronger wipe products regardless of contact time.

6.6 Chapter conclusions

Here, it has been demonstrated that environmental isolates can survive on healthcare surfaces even after cleaning and disinfection protocols. Overall, *S. aureus* was more susceptible to wipe products than *B. licheniformis*. There was no evidence to suggest that *B. licheniformis* protected *S. aureus* from the action of wipe products, indicating that environmental species might not contribute to pathogen protection in a DSB state. When we consider the natural environment, complex DSB found on hospital surfaces have been found to contain up to 18 different bacterial species dominated by Staphylococci and *Bacillus* spp. (Ledwoch et al., 2018). From the results presented here, with two different species of *Bacillus*, the study of dual species DSB may not be sufficient to answer whether a complex multispecies DSB protect pathogens from cleaning and disinfection or not. It is also clear that dual species DSB behave differently from a wet biofilm. *S. aureus* maintains virulence within a DSB, providing evidence that DSB found naturally in the hospital environment may house species that are still, harmful to health even if in a reduced metabolic state. This study outlines environmental species in DSB can easily be transferred from wipe or directly following wiping. In future, it would be of manufacturers interest to consider testing products against DSB.

Chapter 7
Survival and Longevity of a Gram-negative bacterium in Dry Surface Biofilm

7.1 Introduction

Healthcare-associated infections (HCAIs) refer to infections linked with admission to hospital, or those that have been developed in any healthcare facility, either from 48 hours after admission or up to 30 days after release (Haque et al., 2018). The Centre for Disease Control and Prevention (CDC) has outlined 10 microorganisms that are responsible for more than 80% of HCAIs, including *Klebsiella pnuemoniae* (Hidron et al., 2008; Sievert et al., 2013; Weiner et al., 2016) (Table 7.1).

Table 7.1. Microorganisms responsible for > 80% HCAIs worldwide.

Causative organism	Information	Reference
Staphylococcus aureus	Pathogenic Gram-positive cocci bacterium residing as part of natural human flora on the skin	Humphreys (2012)
Coagulase-negative Staphylococci	Staphylococcal species that do not produce coagulase enzyme, part of inherent microbiome	Piette and Verschraegen (2009)
Enterococcus spp.	Gram-positive facultative cocci residing in gastrointestinal tract	Fisher and Phillips (2009)
Escherichia coli	Gram-negative rod-shaped bacterium colonises the gastrointestinal tract	Kaper et al. (2004)
Pseudomonas aeruginosa	Gram-negative Bacillus, opportunistic pathogen associated with infections in Cystic Fibrosis patients	Wu et al. (2015)
Klebsiella pneumoniae	Gram-negative Bacillus, associated with hospital-acquired pneumonia	Paczosa and Mecsas (2016)

Enterobacter spp.	Gram-negative rod-shaped facultative anaerobe, found in gut microbiota	Regli et al. (2019)
Acinetobacter baumannii	Gram-negative coccobacilli, opportunistic pathogen found predominantly in hospitals	Antunes et al. (2014)
Klebsiella oxytoca	Gram-negative, emerging pathogen with multiple drug resistance to common antibiotics	Singh et al. (2016)
Candida spp.	Yeast species that reside naturally on skin and inside the body, commonly causing fungal infection of the mouth, throat or genital area	Papon et al. (2013)

The rapid spread of MDROs continues to put pressure on healthcare environments, associated with increased mortality and prolonged hospital stays leading to increased cost of care per patient (Giske et al., 2008). Vickery et al. (2012) confirmed reservoirs of pathogenic MDROs in hospitals residing in a dry biofilm state. Since then, DSB containing MDROs have now been reported in healthcare settings across the world (Ledwoch et al., 2018; Johani et al., 2016). Bacteria residing in a biofilm have proven difficult to culture, which is also a concern for DSB, limiting detection techniques (Hu et al., 2015).

Klebsiella pneumoniae is often classed as an MDRO, due to its ever-increasing widespread carbapenemase resistance (Moradigaravand et al., 2017). A common opportunistic bacterium, *K. pneumoniae* is found in the environment, where it resides in soils and surface waters (Paczosa and Mecsas, 2016) and in humans, where it colonises intestines and faeces. *K. pneumoniae* is an opportunistic pathogenic, non-motile bacterium, associated with pneumonia, septicaemia and surgical site infections and is the second most common Gram-negative bacterium, after *Escherichia coli*, causing invasive infections (Anderson et al., 2014; Vading et al., 2018). Patients on ventilators, receiving antibiotics or with catheters are at a

much greater risk of becoming infected with *K. pneumoniae* through person to person contact or environmental contamination (CDC, 2009). The rise of carbapenemase producing *Klebsiella* species has resulted in more deaths and fewer treatment options, due to this increasing threat, hospitals worldwide, are starting to implement stricter infection control measures, such as screening and early intervention, to prevent any further spread of the organism (Samra et al., 2007; Nordmann et al., 2009).

The survival of microorganisms on environmental surfaces is dependent on multiple factors including pH, ambient temperature, nutrient availability, presence of other species and biofilm formation (Katzenberger et al., 2021). It has been reported that *K. pneumoniae* readily form biofilms on catheters and other surgical equipment (Percival et al., 2015). This is related to the expression of type 1 and 3 fimbrial adhesins during biofilm formation (Schroll et al., 2010; Chatterjee et al., 2014). One study concluded that the majority of medical device infections (> 90%) from an intensive care unit in India were caused by biofilm producing *K. pneumoniae* (Singhai et al., 2012). *K. pneumoniae* pose a greater threat due to the increased resistance to antibiotic treatment when in the form of a biofilm compared to planktonic state of growth, meaning there are less treatment options if a patient were to become infected (Vuotto et al., 2014).

It is known that the longer microorganisms can survive in the environment, the greater the risk of infection to the patient (Otter et al., 2015). Microorganisms in a dry state can survive on surfaces for extended periods compared to those residing in a planktonic form, increasing the chance of patient infection (Kramer and Assadain, 2014). To date, the literature available on the survival of *K. pneumoniae* in a dry state is limited and contradictory. Hirai (1991) reported the absence of detectable viable *K. pneumoniae* following desiccation on a range of surface materials. However, Kramer et al. (2006) reported *K. pneumoniae* surviving from two hours to up to 30 months on inanimate surfaces. Both of these studies were performed with planktonic bacteria and not with bacteria embedded in a biofilm.

7.2 Aims and hypotheses

The main aim of this chapter was to produce a dry surface biofilm of *Klebsiella pneumoniae* and investigate its tolerance to desiccation and the implications DSB may have on bacterial transfer and virulence. This is especially pertinent as *K.*

pneumoniae has been previously previously identified in environmental samples of dry surface biofilms, but little is known about its virulence and longevity when in this state (Costa et al., 2019). If the species can survive alone in a DSB this could have major implications for the spread of disease within hospitals.

7.3 Methods

7.3.1 Bacterial growth and DSB formation

K. pneumoniae ATCC 13883 was suspended in tryptone soya broth (TSB), and a culture prepared as previously described (Chapter 3, section 3.4). DSB of *K. pneumoniae* were formed over a 12-day period with sequential hydration and dehydration phases as described in Chapter 3, section 3.5. Identification of *K. pneumoniae* was confirmed with the API 20E test kit (bioMérieux, USA).

7.3.2 Culturability of bacteria embedded in DSB

Bacterial culturability was investigated with 12-day DSB (referred to from now on as DSB_0). Bacteria from DSB were revived as outlined in Chapter 3, section 3.8, followed by enumeration onto TSA plates as described in Chapter 3, section 3.9. Other 12 days-DSB were incubated for a further two-weeks and one-month in relative humidity 55 ± 5% at room temperature (21ºC) in a sealed box. Humidity was regulated with Prosorb humidity control cassettes. Following specific incubation periods, bacteria remaining on discs were processed as described in Chapter 3, section 3.8 and 3.9. Minimal recovery agar, R2A, was also used alongside TSA to facilitate growth of stressed bacteria following long periods of desiccation, once disrupted from the DSB.

To compare survival of *K. pneumoniae* in DSB with a *K. pneumoniae* planktonic suspension dried on surfaces, a control was performed following a modified version of ASTM2967-15 (ASTM2967-15, 2015) inoculum preparation. Cultures of *K. pneumoniae* were prepared overnight in 10 mL TSB at 37 ºC in a shaking incubator at 120 rpm. Pellets were centrifuged at 5000 g for 10 mins at 21 ºC and resuspended in 5 mL TSC to produce a bacterial suspension of 10^8 CFU/mL. A 10 µL drop was placed onto a sterile stainless steel disc and left to completely dry completely at room temperature (21 ºC) for 30 min. Discs were left for a total of 12 days and culturability was tested every 2 days as described in Chapter 3, sections 3.8 and 3.9.

7.3.3 Viability of bacteria embedded in DSB

Bacterial viability was investigated by live/dead staining using a BD LSR Fortessa flow cytometer. DSB were vortexed for 2 min in phosphate buffer saline (PBS) with 1 g of glass beads to ensure biofilm removal from the disc surface and disaggregation of bacterial clumps. The resulting suspension (approx. 10^5 bacterial cells) was used neat and stained using the LIVE/DEAD® BacLight™ bacterial viability kit (Invitrogen, Thermo Fisher Scientific) with Syto 9 and Propidium Iodide in a 1:1 ratio. Live (washed overnight bacterial suspension in TSB; Chapter 3, section 3.4) and dead (washed overnight bacterial suspension exposed to 85ºC for 10 min in a dry heat bath) planktonic cultures were used as a control. The FITC-A (green fluorescence) and PE-Texas Red-A (red fluorescence) channels were used to detect live and dead bacteria. The flow cytometer was initially adjusted using a population of unstained cells. For each sample, 10,000 events were recorded to yield mean values for green and red fluorescence. Data obtained by flow cytometry were analysed using FlowJo™ flow cytometry analysis software (version 10.8.1).

7.3.4 Transferability of bacteria from DSB

Dry Transfer: Discs containing DSB_0, two-weeks or one-month old DSB were pressed 36 times on Dey-Engley Neutralising (DE) agar at a pressure of 100 g. Following transfer, each plate was incubated at 37ºC overnight. Positive growth was recorded, and transferability was calculated as the number of positive contacts out of the 36 adpressions and expressed as percentage transfer (Chapter 3, section 3.7).

Wet Transfer: Discs containing DSB_0, two-weeks or one-month old DSB were wiped with a detergent wipe (TRICLEAN™) or a wipe (Rubbermaid microfibre cloth) containing sterile water. Each disc was wiped for ten seconds using the Wiperator (Fitaflex Ltd.) at 500 g pressure, left to air dry for 30 seconds before being pressed 36 times onto DE agar at 100 g pressure (3.7). Plates were then incubated and positive growth recorded as described above.

7.3.5 Scanning Electron Microscope (SEM) Imaging

DSB_0, two-weeks and one-month old DSB samples were imaged using a Philips XL30 field emission gun-scanning electron microscope as outlined in Chapter 4, section 4.3.1.1). Discs containing DSB were submerged overnight in a 2.5% glutaraldehyde solution. To ensure samples were fixed, discs were then put through a series of ethanol concentrations before coating with gold palladium.

7.3.6 Capsule staining

A negative capsule stain was used to identify the presence of a capsule around *K. pneumoniae* cells both from a planktonic suspension and from DSB_0. Capsules provide protection from desiccation and have been categorised as virulence determinants (Schembri et al., 2004). A 10 µL loop of *K. pneumoniae* from planktonic washed overnight culture (Chapter 3, section 3.4) or DSB resuspended cells (section 7.3.3) was mixed with 10 µL of 10% nigrosine on a glass slide as demonstrated by Struve and Krogfelt (2003). A thin film of this mixture was left to dry at room temperature so that the capsule was not affected as it would be with heat fixation. Once completely dry, the slide was flooded with a 1% solution of crystal violet for 1 minutes and again left to dry at room temperature at an angle so any excess stain could flood off the slide. Slides were then viewed with a Leica DM IL LED microscope under x100 oil immersion lens.

7.3.7 Virulence assay using *Galleria mellonella*

A virulence assay was used to explore the effects of *K. pneumoniae* pathogenicity in DSB compared to planktonic suspension. Only DSB_0 was investigated as culturability was low and remained inconsistent with two-week and one-month DSB (section 7.4.1) Cultures of *K. pneumoniae* were prepared overnight in TSB (Chapter 3, section 3.4) or from DSB_0. Planktonic bacterial suspensions were centrifuged at 5000 g for 10 min at 21ºC. Pellets were resuspended in TSC and cultures were adjusted to approx. 10^8 CFU/mL at OD_{625nm} and subsequently diluted down to 10^5 CFU/mL. Suspensions were plated to ensure the correct concentration was used. DSB_0 suspension were vortexed in TSC with 1 g of glass beads for 4 min. DSB_0 were not diluted but plated and counted to confirm CFU/mL.

G. mellonella larvae (Biosystems, UK) were kept and stored in the fridge for a maximum of 2 weeks as described in Chapter 6, section 6.3.4.2. *G. mellonella* larvae were injected with 10 µL of planktonic or DSB suspension containing 10^6 and 10^5 CFU/mL, corresponding to an injection of 10^4 and 10^3 CFU/mL. Larvae were injected and determined “dead” or “alive” (Chapter 6, section 6.4.3.2). Controls included untreated larvae and larvae injected with 10 µL of TSC or 10 µL of 100% DMSO.

7.3.8 Statistical Analyses

Statistical analysis was carried out, where appropriate, using GraphPad Prism 9 software (version 9.3.1). One-way and Two-way ANOVA were used to determine

statistically significant differences within datasets (Chapter 3, section 3.11). Post-hoc tests were used for multiple comparisons.

7.4 Results

7.4.1 Culturability of *K. pneumoniae* from DSB

Bacterial culturability varied between the different desiccation periods (**Figure 7.1**). There was a statistically significant difference between Log_{10} CFU/mL recovered from all three ages of DSB: DSB_0, two-week and one-month (One-way ANOVA, $p < 0.0001$). However, a post hoc Tukey test showed no significant difference ($p > 0.05$) between two-week and one-month DSB. An average of $5{\cdot}15 \pm 0.60$ log_{10} CFU/mL was recovered from DSB_0. After one-month of incubation at 21 ºC and 55 ± 5% RH, 4.01 ± 1.64 log_{10} CFU/mL were recovered, but this number dropped to 1.58 ± 0.66 log_{10} CFU/mL after two-weeks of incubation. Results from DSB of two-weeks and one-month varied between batches of biofilms, not just between biological repeats. No viable bacteria were detected from the dried planktonic suspensions of *K. pneumoniae* on stainless steel discs after 2 days incubation. Similarly, minimal media, R2A had no impact on recovery of *K. pneumoniae* after two-weeks or one-month in DSB as no viable bacteria were detected on agar plates.

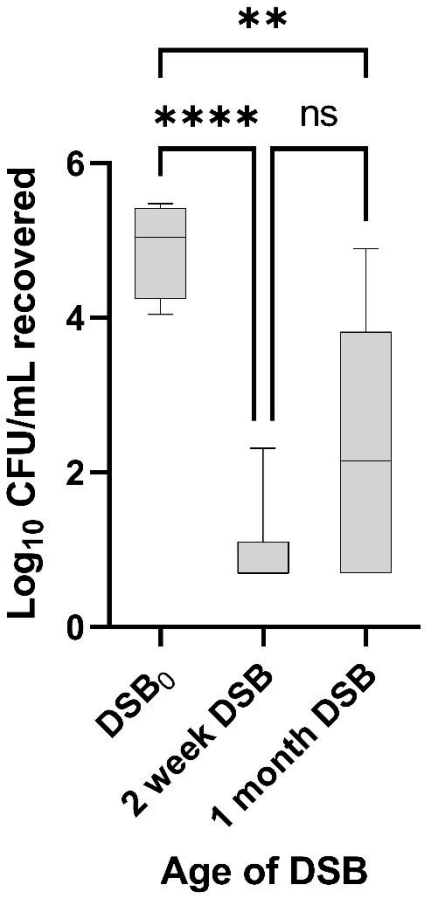


Figure 7.1. Culturability of *K. pneumoniae* at 12 days (DSB_0), two-weeks and one-month old DSB. Represented by Log_{10} CFU/mL. Data based on ≥ 6 biological replicates. Culturability of DSB_0 was more consistent than two-weeks and one-month.

7.4.2 Viability of bacteria embedded in DSB

Viability of *K. pneumoniae* embedded in a DSB was measured by live/dead staining using flow cytometry. The population of live/dead cells in DSB at different incubation times; 12 day (DSB_0), two-week and one-month is presented in Figure 7.2. By performing manual gating, as shown by the black box/circles in Figure 7.2, mean fluorescence values were calculated for live (FITC-A channel) and dead cells (PE-Texas Red-A channel) (**Table 7.1**). In all three ages of DSB both live and dead cells were identified within the samples. With DSB_0 mean fluorescence values of 3953 (FITC-A) and 1351 (PE-Texas Red-A) were determined. Whereas two-weeks and one-month DSB had lower mean fluorescence values of dead cells of 1026 and 765

respectively. Figure 7.3 shows histograms of both FITC-A channel (**Figure 7.3a**) mean fluorescence values and PE-Texas Red-A channel (**Figure 7.3b**). The presence of live cells in all two week and one-month DSB samples is indicative of a VBNC (viable but non culturable) state, as culturability was extremely low at both ages.

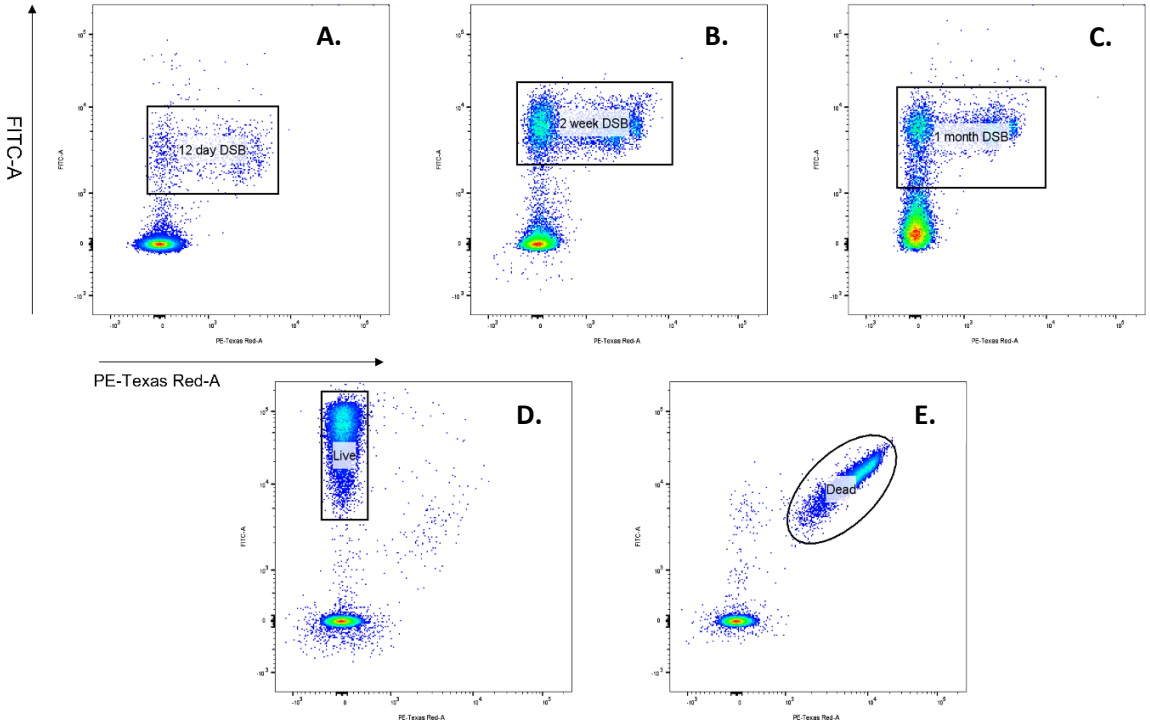


Figure 7.2. Dotplots from flow cytometric analysis of 12 day, two-week and one-month DSB. Areas gated by black rectangles/squares show the population analysed for live/dead cells with the elimination of any background noise. FITC-A (green channel) is presented on the y axis, and PE-Texas Red-A (red channel) is presented on the x axis. (A) DSB_0, (B) two-week DSB, (C) one-month DSB, (D) planktonic live control, (E) planktonic dead control. All DSB ages showed presence of both live and dead cells in all samples (n = 3).

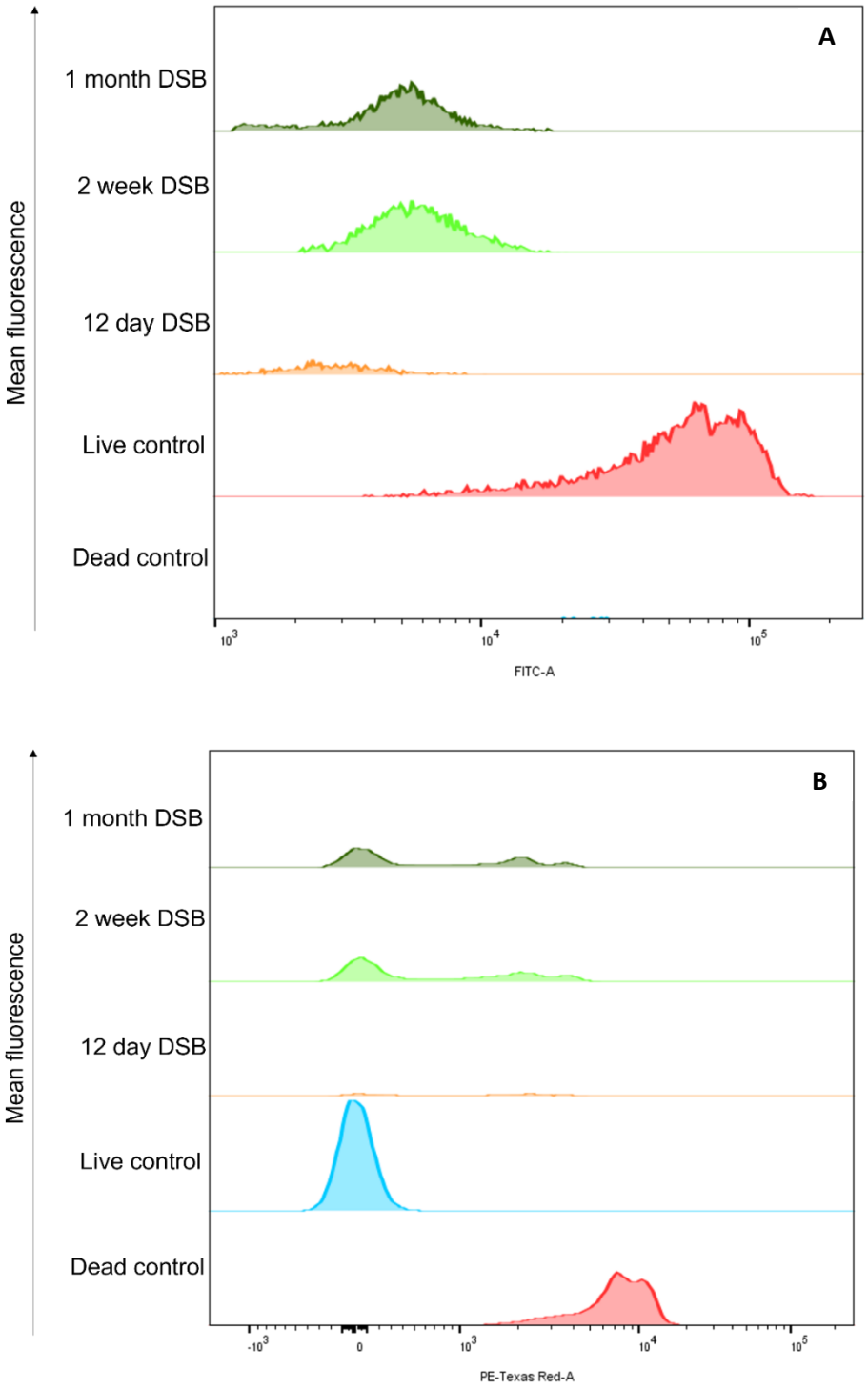


Figure 7.3. Histograms of mean fluorescence peaks of FITC-A (Green) and PE Texas Red-A (Red) channels following live/dead staining. Histograms of FITC-A channel (A) show the live staining of cells within all samples, with the exception of the dead control, histograms of PE Texas Red-A (B) show dead staining of cells within all samples. There is a reduced mean fluorescence of all DSB samples when observing the PE-Texas Red A channel. The shift of histograms to the right indicates

a positive dead stain, as shown by the dead control sample. The peaks displayed to the left of the graph indicate background staining which have not included in the dead stain.

Table 7.1. Average mean fluorescence values of FITC-A (green channel) and PE-Texas Red-A (red channel) for *K. pneumoniae* DSB. Total mean fluorescence values calculated for 12 day, two-week and one-month old DSB using live/dead bacterial viability staining kit. Live and dead planktonic controls are included. Higher values indicate greater cell numbers have been stained, lower values indicate less cells with the stain.

	Average mean fluorescence value				
Live/Dead stain	**12-day DSB**	**Two-week DSB**	**One-month DSB**	**Live control**	**Dead control**
LIVE	3953	6079	5645	60906	13607
DEAD	1351	1026	765	23	8020

7.4.3 Transferability of DSB

Percentage transfer was calculated from the number of positive adpression (positive colony growth regardless of number) out of 36 total adpressions. Dry transfer yielded the highest percentage transfer compared to wet transfer following wiping with a detergent or water (**Figure 7.4**). There was a significant difference (one-way ANOVA, $p < 0.05$) between all dry, water and detergent transfer for both DSB_0 and one-month DSB respectively. The greatest percentage transfer was from the DSB_0, with one-month old the lowest (**Figure 7.4**). One-month DSB was directly transferred when dry but variability between biological replicates remained high as 50% of the time no bacteria were transferred. This is similar to what was observed with the culturability experiments. However, after wiping with water or detergent,

there was no bacterial transfer from all replicates represented by dots in Figure 7.4. *K. pneumoniae* embedded in two-week DSB was directly transferred to agar following wiping with either detergent or water. There was no overall statistical significance in percentage transfer between dry transfer and wet transfer for two-week DSB (one-way ANOVA, $p > 0.05$). A significant difference was identified between dry or for water transfers between DSB_0 and one-month DSB (ANOVA, Tukey, $p < 0.05$).

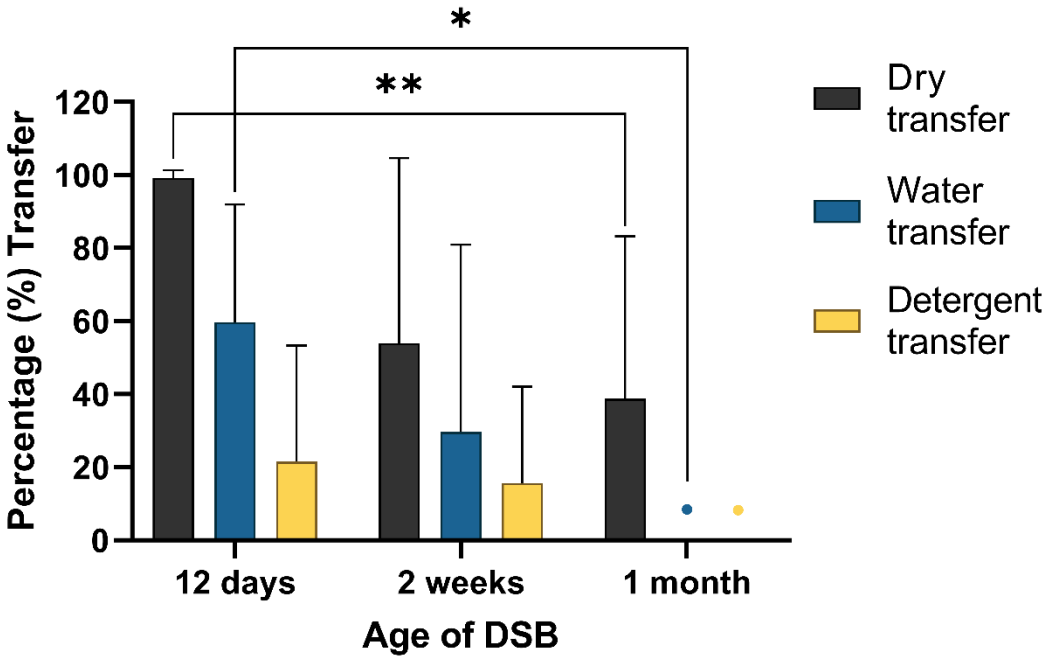


Figure 7.4. Percentage transfer of bacteria from 12 days, two-week and one-month old DSB following dry transfer and wet transfer (water and detergent wipes). Dots (•) indicates no transfer of bacteria from DSB after wiping with water or detergent. There was a significant difference between dry and water transfers of 12 day and one-month DSB (ANOVA, Tukey, $p < 0.05$). (n > 3)

7.4.4 Scanning Electron Microscope Analysis

DSB of *K. pneumoniae* were homogenous and uniform within the same biofilm age based on multiple pictures (n > 6) taken of three replicates of each biofilm. There were however differences in the structure of the biofilm matrix between DSB_0, two-weeks and one-month old DSB. DSB_0 presented an uneven covering of bacteria, over the surface of the disc and a higher concentration of cells compared to that observed in the two-week old DSB (**Figure 7.5a,b**). Perhaps not surprisingly owing the regular addition of BSA, two-week old DSB showed more organic matter, and less cells present within the biofilm matrix (**Figure 7.5d)**. However, those cells present appear to be well embedded into the matrix (**Figure 7.5c,d**). Spatial separation of bacterial cells within the one-month DSB can be observed, with little EPS identifiable (**Figure 7.5e,f**), and number of observable bacteria is lower than the DSB_0 and two-week old DSB.

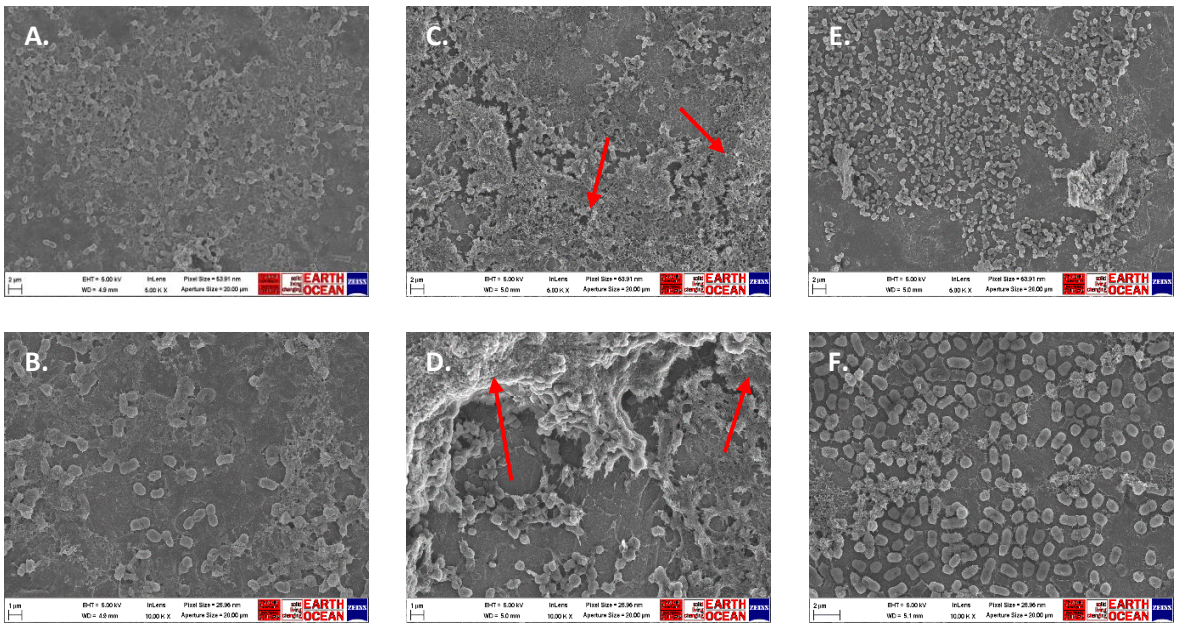


Figure 7.5. Scanning electron microscope images of *K. pneumoniae* dry surface biofilm. Images taken at x5,000 and x10,000 magnifications. Images presented are representative of the whole disc surface. 12-day DSB x5,000 (A) and x10,000 (B), two-weeks DSB x5,000 (C) and x10,000 (D), one-month DSB x5,000 (E) and x10,000 (F). Arrows indicate large presence of organic load.

7.4.5 Confirmation of capsule using nigrosine stain

The presence of a capsule was confirmed through negative nigrosine staining. Where the area around the cells is clear, this indicates the presence of a capsule (**Figure 7.6**). The images taken of the *K. pneumoniae* planktonic suspension, which is used as the starting inoculum for DSB formation, clearly shows the presence of a capsule around the individual cells. Once the mature DSB has been developed, the capsule is not as prominent compared to the planktonic inoculum, as shown in Figure 7.6.

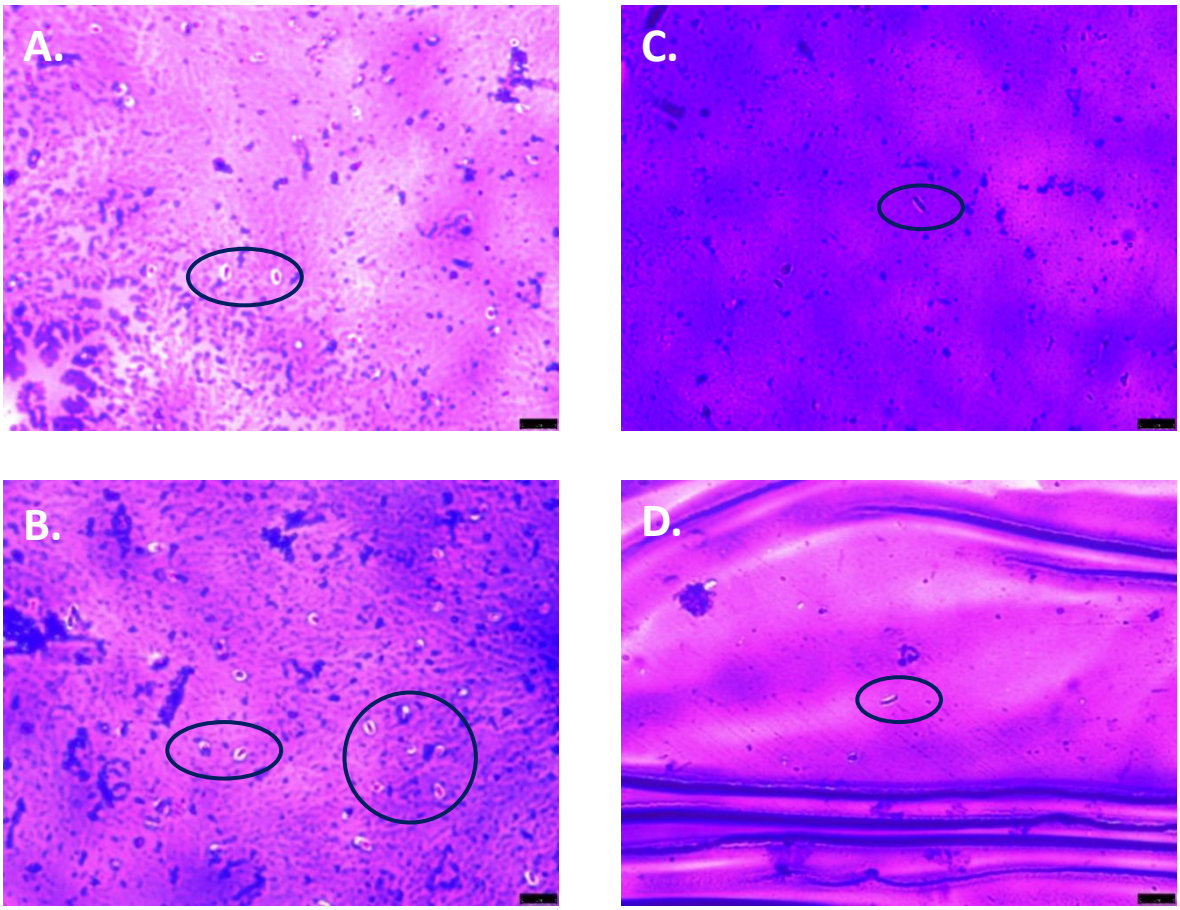


Figure 7.6. Capsule staining of *K. pneumoniae* planktonic suspension and DSB_0. The presence of a capsule is indicated by the white ring around the individual cells, highlighted by black circles. (A) and (B) *K. pneumoniae* planktonic suspension, (C) and (D) *K. pneumoniae* mature DSB_0. Images representative of 3 biological replicates and whole sample surface.

7.4.6 *K. pneumoniae* DSB effect on virulence

The untreated and TSC control groups had no effect on *G. mellonella* larvae, showing a 100% survival rate after 5 days (**Figure 7.7b**). All larvae injected with 100% DMSO (positive death control) died within 48 hours of injection; after 24 hours only 10% survival was recorded (**Figure 7.7b**). Counts for DSB_0 corresponded to 10^6 CFU/mL. After 3 days of incubation, there was still 100% survival of larvae after injection with DSB_0 (**Figure 7.7a**), the same % survival was also observed when larvae were injected with 10^6 planktonic suspension (**Figure 7.7b**). There was no significant difference (two-way ANOVA, $p > 0.05$) of overall larval survival over the 5 day period between the control planktonic suspension and DSB_0. After 5 days of incubation, larval survival rates were 97% for both DSB_0 and the planktonic suspension (**Figure 7.7**). Over the course of 5 days, % survival did not differ between DSB_0 and planktonic suspension.

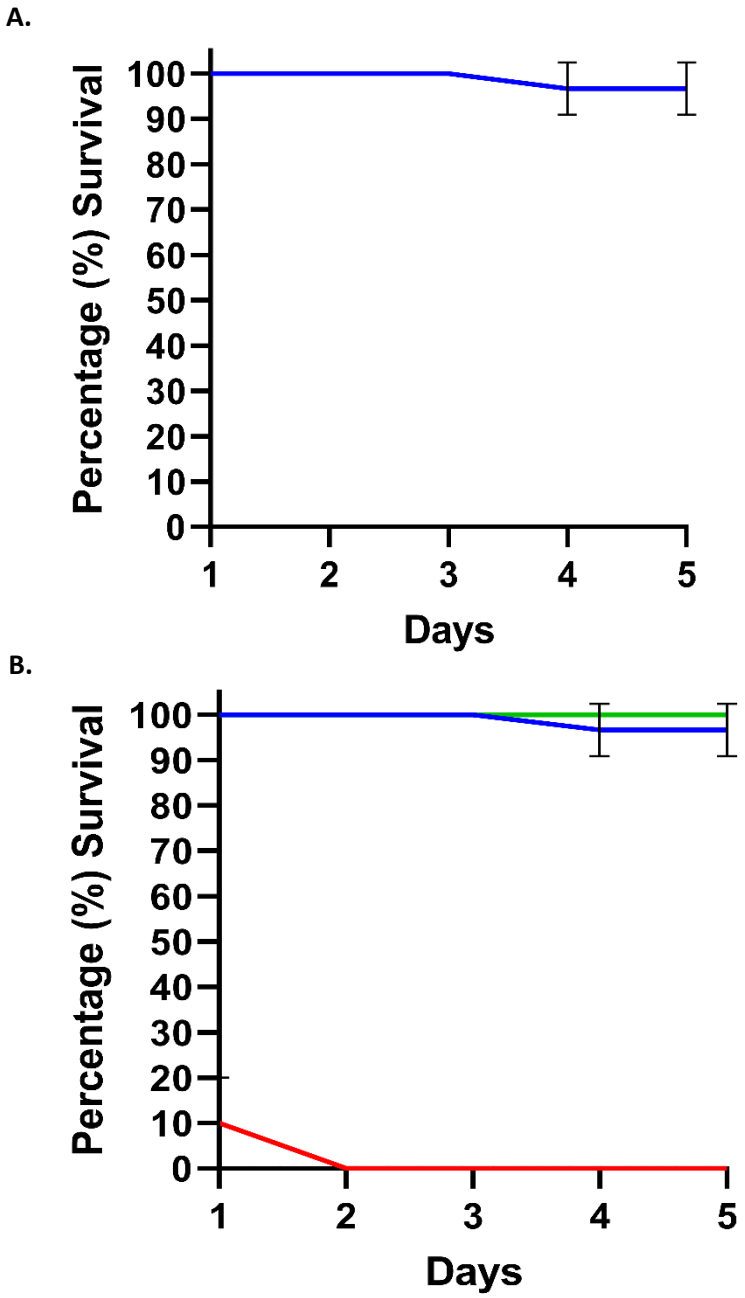


Figure 7.7. Percentage survival of *G. mellonella* larvae over 5 days when injected with DSB_0 and controls (planktonic suspension, TSC and DMSO). (A) % survival of larvae when injected with DSB_0 (avg. 10^6 CFU/mL). (B) % survival of control groups when injected with TSB (green), DMSO (red) and 10^6 CFU/mL planktonic suspension (blue). There was no statistically significant difference between all DSB_0 and the control planktonic suspension (two-way ANOVA, $p > 0.05$).

7.5 Discussion

K. pneumoniae is associated with a large proportion of hospital acquired pneumonia and ventilator acquired pneumonia, specifically amongst vulnerable patients in intensive care units (Podschun and Ullman, 1998; Kalanuria et al., 2014). Several sources of transmission within hospitals have been identified, including direct person to person contact and contaminated surfaces and instrumentation (Martin et al., 2018). The production of a *K. pneumoniae* DSB and survival in a desiccated state over a one-month period was successful. Owing to pathogen transmission from surfaces, the question was whether bacteria in DSB were transferable or not. To respond to this question, both direct 'dry' transfer and 'wet' transfer were evaluated following wiping with water or a detergent wipe. Perhaps not surprisingly, dry transfer test resulted in the highest percentage of transferred *K. pneumoniae* from DSB, since the mechanical action of wiping likely removed some bacteria from the disc surface resulting in lower percentage transfer (Williams et al., 2007). There was less direct transfer from surface to surface when all DSB were wiped with both water and detergent materials. The effect of wiping DSB_0 resulted in lower % of bacterial transfer post-treatment (**Figure 7.4**). Wet transfer of both two-week (< 30%) and one-month (0%) was extremely low, comparable to the culturability data reported in **Figure 7.1**.

K. pneumoniae has been identified as one of 85 known species, mostly comprised of Gram-negatives, to enter the VBNC state supporting long term survival under stressful conditions (Li et al., 2014). The dormant state of bacterial cells has been proven to last several months in Gram-negative species, the species also remained infectious after resuscitation from dormancy (Baffone et al., 2006). Our flow cytometry analysis shows that *K. pneumoniae* remains in a VBNC state following two weeks in stable dry conditions, up to one-month. At this point, there were little bacteria grown following transfer and viability tests. In hospitals cells in a VBNC state create a great problem as swabs taken from the environment will not identify these bacteria residing on surfaces and other methods are considered costly and time consuming. It is evident, that there is a gap in literature available on the presence of VBNC biofilms within healthcare environments and the threat they may pose. One of the main limitations around analysis of cells in a VBNC state, is the lack of methods to quantify the live/dead presence within a sample. Here, staining is used as it was considered most appropriate for biofilms.

Single species DSB models, with *K. pneumoniae* (this study), *S. aureus* (Ledwoch et al 2018; Almatroudi et al., 2015) or *Candida auris* (Ledwoch et al., 2018) provide robust, reproducible platforms for testing culturability and viability over period of time or following cleaning or/and disinfection. These simple models do however not represent DSB *in situ* where more complex multi species DSB have been identified (Vickery et al. 2012, Hu et al. 2015, Ledwoch et al. 2018). In addition, environmental DSB isolated from healthcare settings seemed to be associated with more expolymeric substance (Vickery et al., 2012) than our single species *K. pneumoniae* DSB despite the regular addition of BSA (**Figure 7.5**). Although *K. pneumoniae* DSB may not produce a biofilm containing large quantities of cells and EPS as previously shown for species like *S. aureus* (Ledwoch et al., 2019b; Nkemngong et al., 2020), we have shown the increased survival of *K pneumoniae* cells when housed in a DSB, compared to those just dried onto a surface. This has great implications for the healthcare environment, where DSB reside even after cleaning and disinfection routines (Hu et al., 2015).

The effect of DSB on virulence of a bacterial species is novel data. Percentage survival of *G. mellonella* did not differ when injected with either 12 day (DSB_0) or the equivalent concentration of planktonic suspension indicating no changes in virulence of the bacteria. Globally, *K. pneumoniae* has gained interest as an opportunistic pathogen due to its resistance to numerous antibiotics and thus, the use of models to assess virulence has become increasingly popular (Wand et al., 2013). Although there was no significant difference between the death rate of larvae injected with planktonic suspension versus DSB_0, overall death rate was generally low. After 4 days, the larvae survival rate remained high at 97%, which did not change to 5 days. Interestingly, the presence of a capsule, which is expressed by the *K. pneumonaie* used in this study, is considered an important virulence factor of the species (Struve and Krogflet, 2003), which may contribute to the initial survival and development of *K. pneumoniae* DSB as presented here.

The large values of standard deviation recorded are due to biofilm growth, over extended formation period there is a higher capacity for variation amongst biofilm replicates. Variation in biofilms is largely due to the biofilm architecture and development (Haney et al., 2018). An additional factor to high variation amongst replicates is the DSB model utilised. Using equipment such as the CDC biofilm reactor creates constant shear stress and controls biofilm development in a reliable

and reproducible manner (Goeres et al., 2005). However, it is important to note that variability in results did not just occur between repeats, but within batches that used the same original inoculum. A similar concept was identified by Ledwoch et al. (2019b), after treatment with sodium hypochlorite, transferability results showed DSB created with organic load showed greater standard deviation values amongst replicates compared to those without.

7.6 Chapter Conclusions

This chapter successfully showed that *K. pneumoniae* can persist in a desiccated stage as DSB on surfaces for at least one-month (55% RH, 21ºC) unlike planktonic bacteria dried onto surfaces. *K. pneumoniae* in DSB could be transferred to another surface directly or post wiping after a month. However, using a wipe did reduce the quantity of bacteria transferred from surface to surface. In addition, *K. pneumoniae* persistence at a VBNC stage was observed. This alone might be cause for concern as this pathogen might not be detectable on surface in a VNBC state.

Chapter 8
General Discussion and Conclusions

8.1 Summary of project findings

This thesis sought to understand the resilience of DSB in the healthcare environment, by using species that have been frequently found in hospital DSB (Vickery et al., 2012; Ledwoch et al., 2018). Resilience to disinfectants and commercially available products, long-term survival of DSB, and the formation of a dual species biofilm has added invaluable information to current knowledge on DSB. Alongside laboratory experiments, surveys and interviews were conducted on healthcare professionals (HCP). This section of the thesis aims to tie all aspects of laboratory testing together, which will be reviewed in this chapter. Table 8.1 summarises findings from key objectives within the thesis.

Table 8.1. Short summary of findings related to key aims addressed throughout the thesis.

Key Aims	Short Summary of Findings
Understanding of HCP opinion and knowledge on cleaning and disinfection protocols	There remains a lack of education and knowledge of HCP in healthcare. Although generally they have an idea of cleaning/disinfection protocols, this is not always adhered to. There is a lot of confusion over who cleans what.
Understanding of HCP knowledge of biofilms	In depth knowledge of biofilms appears to be directly correlated to higher degree level certification. Understanding of DSB remains very limited.
Efficacy of key disinfectant components of biocidal products as markers of resistance in DSB development	As *S. aureus* DSB develops, it becomes less susceptible to NaDCC and BZK. Both PAA and high concentrations of NaDCC have lethal effects from 4 days.
	B. licheniformis is resistant to chlorine-based disinfectants and BZK treatment from 4 days but remains susceptible to PAA. Extending contact times to 60 min did not significantly lower bacterial numbers in DSB.
	B. subtilis NCTC 10400 was more susceptible to disinfectant treatment than the other two *Bacillus* spp. No pattern of resistance with DSB development was identified.
	B. subtilis AEWD was resistant from 4-day DSB. Increasing contact time to 60 min did significantly increase efficacy of NaDCC and PAA.
Efficacy of wipe products to effectively eradicate and stop transfer of DSB from surfaces	Transfer of *S. aureus* DSB was inhibited by wipe products. Although *S. aureus* remained on surfaces post-wiping, wipes were much more effective than carrier tests.

	B. subtilis NCTC 10400 was transferred by wipe and remained on surfaces post wiping.
	B. licheniformis was not effectively removed by wipes, neither did wipes prevent transfer of *B. licheniformis* from DSB to new surfaces, or by the wipe itself.
	B. subtilis AEWD was readily transferred to new surfaces by all wipes tested and large quantity of DSB remained on the disc surface after wiping.
Develop dual species DSB for testing protocols	Dual DSB was formed and tested. Although inconsistencies were found, a basis for multispecies testing has been developed.
Investigate protection of species within dual DSB from current commercially available products	There was no evidence of protection of *S. aureus* by *B. licheniformis* in dual DSB.
Long-term survival and transfer of DSB from surfaces	*B. licheniformis* and *B. subtilis* AEWD survive up to 6 months on surfaces with no additional nutrients. *B. subtilis* NCTC 10400 is also viable up to 6 months at lower densities. No bacteria from *S. aureus* DSB were recovered ≥ 4 months.
Visualise overall structure and architecture of DSB	Thick, homogenous DSB of *B. subtilis* AEWD and *S. aureus* were identified. *B. licheniformis* was densely packed with clumps of bacteria rather than covering hole disc surface. *B. subtilis* NCTC 10400 was generally sparser and did not cover whole DSB surface as the other species.
Determine if species residing in DSB remain as pathogenic as planktonic cultures	Both *K. pneumoniae* and *S. aureus* remained as pathogenic as planktonic counterparts when recovered from DSB.

8.1.1 Efficacy of disinfectant products against DSB

Chapter 5 sought to test single species DSB to disinfectants, carrier tests were chosen to test disinfectants in solution, and the Wiperator (ASTM2967) was used to test commercially available wipe products. Results from this work has highlighted the increased resistance of *S. aureus* DSB over development to disinfectant treatment (NaDCC low conc, PAA and BZK), with the exception of NaDCC at high concentrations (**Figure 8.1**). Similarly, *B. subtilis* AEWD also displayed an increase in resistance with increasing DSB age to the majority of disinfectant products (**Figure 8.1**).

If we consider DSB architecture, at 12 days *B. subtilis* AEWD has formed a thick DSB with high cell density (**Figure 4.2**, Chapter 4), and EPS is evident throughout DSB structure, potentially owing to its increasing resistance with development. These observations of EPS was similar to Bridier et al. (2012) work on 24-hour wet biofilms of *B. subtilis*. *B. subtilis* NCTC 10400 was the only species that did not show any pattern of resistance over 12 days (**Figure 8.1**). Low $\log_{10}$ reduction values with *B. subtilis* NCTC 10400 were partly due to low levels of growth within the untreated control DSB. SEM images of *B. subtilis* NCTC 10400 showed that the biofilm if very sparse compared to the other species, especially at 4 and 8 days which coincided with low levels of $\log_{10}$ CFU/mL recovered. Although this is true, *B. subtilis* NCTC 10400 remained recoverable at reduced levels for up to 6 months on stainless steel surfaces. *B. licheniformis*, much like *B. subtilis* AEWD remained on surfaces following disinfectant treatment (**Figure 8.1**). However, when we consider DSB structure, *B. licheniformis* DSB are densely packed with clumping of cells rather than a full homogenous covering over the disc surface, and much less EPS is evident compared to *B. subtilis* AEWD.

8.1.2 Practicality of carrier tests and use of environmental isolates for DSB testing

Suspension tests are the most common form of standard testing procedure for disinfectants. Here we have used carrier tests as they were deemed most appropriate for DSB research using stainless steel disc carriers. Although suspension tests are useful, there is a consideration to be made of the application of the research. Not only is there no standard for DSB testing, but all protocols look to test standard strains, but do they really inform product efficacy in practice? There are both arguments for and against the use of environmental isolates.

Environmental isolates are physiologically representative of real-life, they have not been cultured numerous times and they are harder to recover on media (Sandle, 2020). However, there is still an argument to be made that culture collection strains are standardised and so researchers around the world are able to use them (Sandle, 2020). We have clearly shown that environmental isolates are more resistant to disinfection protocol, whether that be against key components of wipe formulations, or against pre-impregnated disinfectant wipes. In the future, we suggest looking to test environmental isolates taken from hospital samples, which has been shown to be beneficial for water treatment testing previously (Bailey et al., 2021). This could create more realistic testing conditions and allow for better practice of product testing (Sandle, 2021).

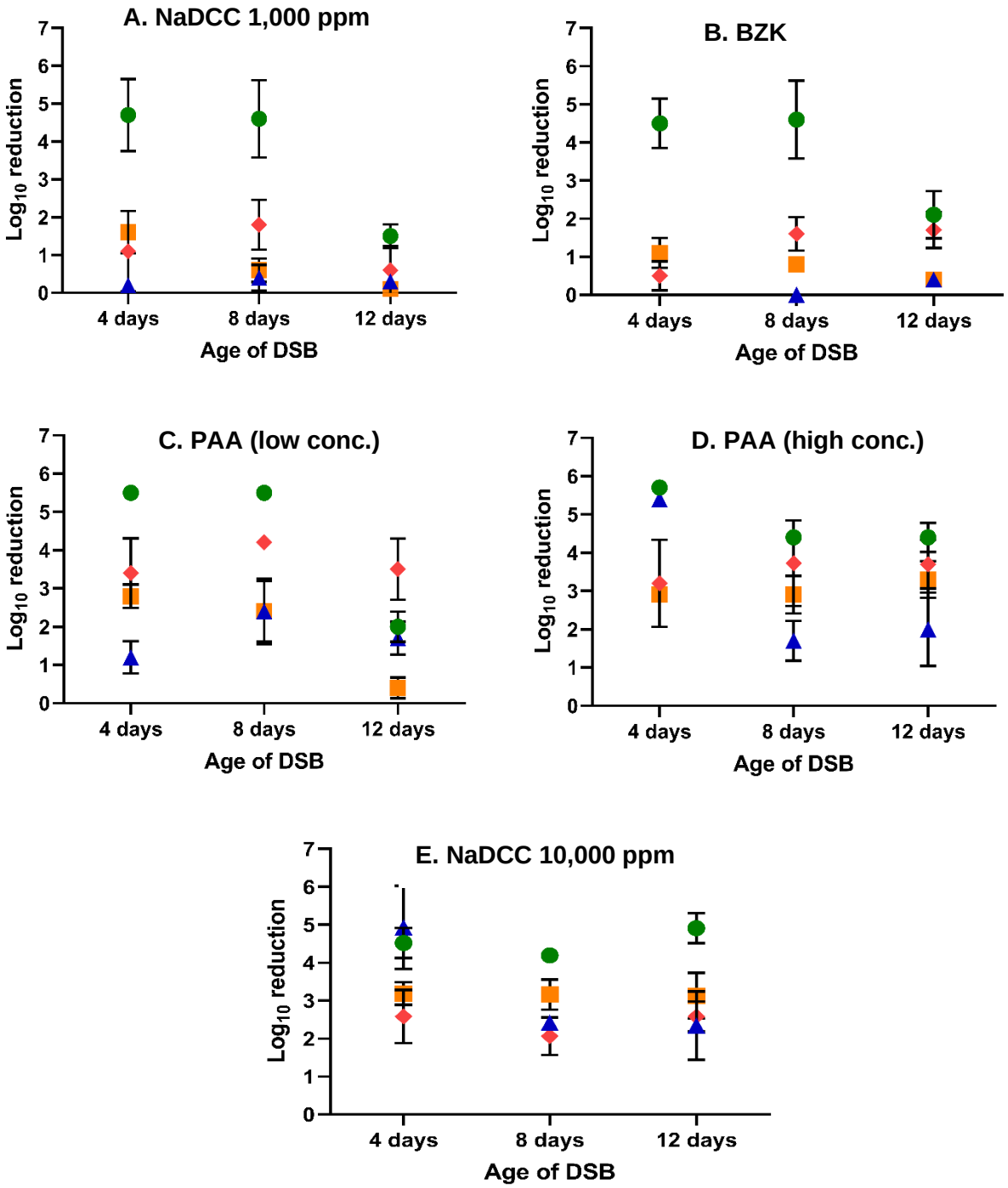


Figure 8.1. Summary of development of resistance to disinfectants over time of DSB development for all four species. (A) NaDCC 1,000 ppm, (B) BZK, (C) PAA low conc., (D) PAA high conc., (E) NaDCC 10,000 ppm. Species as follows; *S. aureus* (**green**), *B. licheniformis* (**blue**), *B. subtilis* NCTC 10400 (**red**) and *B. subtilis* AEWD (**orange**).

8.1.3 Factors effecting wipe performance

Generally, increasing contact time with wipes from 30 to 60 seconds did not greatly increase efficacy of the wipe against all single species DSB and all testing procedures examined (log_{10} reduction of bacteria embedded in DSB, transfer of DSB post-wiping and transfer between surfaces). Akin to wiperator testing, increasing contact time in carrier test experiments with *B. licheniformis* DSB to 60 min, did not significantly decrease bacterial reduction from 5 min contact time. Similar observations have been previously made, concluding that disinfectant mode of action, rather than contact time, significantly increases bactericidal efficacy of a wipe product (West et al., 2019). Pascoe et al. (2022) also mentioned that the use of longer contact times, may become less apparent when comparing performance of different wipes.

An important observation of wipe efficacy was the poor performance of wipes following transfer tests. Both tests conducted, direct transfer post-wiping and transfer by wipe between surfaces, showed products did not inhibit *Bacillus* spp. transfer. *S. aureus*, although more susceptible to transfer by the wipe itself, remained on surfaces post wiping, much like results presented by Ledwoch et al. (2021b), whereby certain biocidal products failed to inhibit transfer of *S. aureus* DSB. We would assume the mechanical action of the wipe would add an additional impact to reduce DSB but when compared to carrier tests, as mechanical wiping has shown to increase efficacy of disinfectants previously (Song et al., 2019; Sloan et al., 2022), however efficacy of wipes was not considerably greater. Transfer tests, both through surfaces and healthcare personnel (although we do not present results for this in this thesis), are an important addition to any testing procedure on wipe products supported by numerous studies (Ledwoch et al., 2021b; Chowdhury et al., 2018; Tahir et al., 2018) and through amendments to EN16615:2015 protocol highlighting the importance of understanding transference of bacteria by wipes. As we have shown here, DSB persist on surfaces between 4 and 6 months and can be readily transferred in this time frame. If a wipe is unable to inhibit transfer of bacteria embedded in DSB, this causes implications for HCAI and a greater infection risk from surfaces.

Wipes A – D (**Table 3.2,** Chapter 3) did not differ greatly in product efficacy, but there were differences in liquid weight (**Table 8.2**) and overall surfactant release from wipes onto disc surfaces (**Table 8.2**). Wipe D (cationic biocide based)

displayed the highest content of total surfactant, and amount released onto disc surface but contained the lowest water weight out of all wipes. This suggests that manufacturers may not need high liquid content of their wipes but an effective release of surfactant onto a surface; other authors have noted the effective adsorption of surfactants when used in mop materials (Bloß et al., 2010).

Wipe E (PAA based) displayed the best product performance from all wipes, most likely due to its PAA content. PAA has been shown as an effective disinfectant for microbial decontamination against biofilms previously (Vásquez-Sánchez et al., 2014; Chowdbury et al., 2019), including those grown on stainless steel (Lee et al., 2016) and as an anti-biofilm agent (Farjami et al., 2022). As with carrier tests, PAA had overall greater biocidal properties than lower concentration of NaDCC and BZK.

Table 8.2. Liquid content, total surfactant content and surfactant release onto disc surface from wipes A – D. Wipe A did not contain any anionic surfactants which this calculation is based off (DDAC and BZK).

Wipe	Liquid weight (g)	Total surfactant content (g/L)	Surfactant release per mass/cm^2
A	3.3	N/A	N/A
B	3.7	2.6	0.2
C	4.5	3.1	0.3
D	2.9	4.0	0.5

8.1.3 Culturability and survival of DSB

The formation of a dual species DSB was successful, although inconsistencies remain. We found inhibition of *S. aureus* by *B. subtilis* AEWD when embedded in DSB together, confirmed through culturability counts and imaging. However, variation in culturability counts of *S. aureus* when in dual DSB with *B. licheniformis* were not explained through SEM imaging as we could see both bacterial species in dual DSB matrix. Although our results did not confer with that of Bridier et al. (2012), we have highlighted the importance of testing multispecies biofilms against disinfectant products.

Although culturability of *K. pneumoniae* DSB was reduced over time, we could clearly see *K. pneumoniae* cells on the disc surface and viable cells through live/dead staining. As found from survey data, the majority of participants believed that culture swabs were the most effective way to measure cleanliness of the surface environment. But as we have shown here, with *K. pneumoniae* DSB, pathogenic species may enter VBNC state when in DSB and so culture swabs would not be appropriate. These results enforce the current view that DSB do go unnoticed on surfaces and if correct cleaning and disinfection measures are not in place, have the potential to be a source of infection for patients in the healthcare environment.
HCP knowledge of survival of different microorganisms in the environment was generally good, especially those who were interviewed and able to give specific examples of species. It was noticeable that since the SARS-CoV-2 pandemic many individuals had more accurate knowledge on the survival of viruses on environmental surfaces. This fits in with a common theme found in interviews, that in order for someone to learn, a reaction is required from a problematic situation. When we consider the long-term survival of *Bacillus* spp. presented in this thesis, up to 6 months on surfaces without the addition of a nutrient source, unless presented at conferences and subsequently spread by word of mouth would not reach HCPs. This further confirms the importance of collaboration between NHS trusts, universities, and industrial partners.

8.2 Recommendations and Future Work

Several recommendations are proposed following the overall outcomes of the thesis. Combining both the knowledge and opinions of healthcare professionals (HCPs) and experimentation has broadened our understanding of DSB and the present challenges of cleaning and disinfection.

- There is clearly, a gap in knowledge of DSB and other biofilms in the healthcare sector. Although literature is widely available on biofilms, there remains a lack of research on DSB. Increased teaching/educational workshops is needed to enhance HCP understanding of DSB so that the limited research is available to those who need it. A suggestion would be to target higher education, integrating the problem of DSB and ineffective cleaning and disinfection measures in the workplace. Inductions in IPC and

workshops on the importance of cleaning and disinfection would also benefit HCP when they enter the working environment.

- The work in this thesis has highlighted the resilience of environmental isolates to commonly used disinfectant products, especially chlorine, (NaDCC) and disinfectant-based wipes, and DSB persistence to remain and become transferred from surfaces following treatment. Healthcare companies should start testing their products against DSB, which will also have subsequent effects on learning of HCPs. If products are more explicit in their testing against DSB, hopefully this will improve general knowledge of DSB.
    - Currently there is no standard testing protocol (BS, EN or ASTM standard) for DSB against biocidal treatment. By implementing a standard testing procedure, companies will be more likely to investigate efficacy against DSB. A DSB standard is becoming more attainable in the near future as some companies are already starting to mention effectiveness of their products against DSB on packaging.
- Although we have not looked in depth at the relationship between the NHS and industry, interview data suggests a lack of co-operation between the two parties. Given that new data is constantly being generated, a recommendation would be to increase communication between the NHS, industry, and universities through collaborative projects, so they can work towards the most effective ways to combat microorganisms in the environment, considering both nurses time and efficacy data on current products.
- DSB can go unnoticed on healthcare surfaces as they are difficult to detect through swabbing (Ledwoch et al., 2021a). We have found that HCP have made it explicit that there is a severe lack of routine testing of surface contamination in healthcare environments. Alongside longevity data where we have observed persistence of up to 6 months on surfaces of *Bacillus* spp., there is a need for new technology to sample surfaces quickly and easily for microbial contamination. Simpler sampling methods are required for detection of DSB on surfaces, perhaps a visual technique that will allow the user to detect the location of DSB. Furthermore, this should be implemented

in healthcare facilities and become a priority. Surface sampling will also help IPC protocols and assure a safe patient environment.

- We have already begun work on detection of DSB on surfaces (appendix 8.1a). Using Raman Spectroscopy, we can differentiate between different microorganisms in a culture (Stöckel et al., 2015). We were successful in identifying species from a dual DSB, and also differentiating between vegetative cells and spores in *Bacillus* spp. DSB (appendix 8.1a). Our results are promising and although we have some detection issues to overcome, we still believe that in the future, will be able to engineer the principle of Raman Spectroscopy for the detection of DSB into a device that can be used in healthcare environments.

There remains a lot to understand about DSB. It is well known that multispecies communities are the dominant form of microbial life in the natural environment (Tan et al., 2016). One main problem within laboratory experiments is the lack of multispecies biofilm testing. Although this can come with difficulties in reproducibility of data, there is a requirement for testing multispecies DSB and other biofilms to better represent the natural environment. Not only this, but laboratory strains are not sufficient to mimic those present on surfaces in healthcare facilities, throughout this thesis it has been shown that environmental isolates are much more resistant to disinfection protocol than standard strains. To overcome issues associated with multispecies testing, experimental conditions should be modified to mimic the “real-life” environment. Imaging techniques are also essential when looking at multiple species in a sample, as with SEM imaging used here on dual species DSB, we could clearly see the structure and the arrangement of species.

Almatroudi et al. (2015) and Ledwoch et al. (2019) outlined composition and architecture of DSB from clinical surfaces and those models they made in laboratory environment, they highlighted similarities between the two different DSB types. As this has been well investigated, future work should look into gene expression within DSB. An insight into genetic expression of stress responses and biocidal resistance of DSB would help further understanding of their resilience in the environment and help combat potential infection risks by developing new solutions or modifying existing products.

One focal point from interview data was the lack of funding provided by the NHS for lower paid jobs, infrastructure, and cleaning apparatus. Future work should be done to help outline potential new funding sources for the NHS and potentially change the allocation of money supplied to these areas so that we are ensured everything is being done to reduce infection risk and help cleaning/disinfection protocols in NHS facilities.

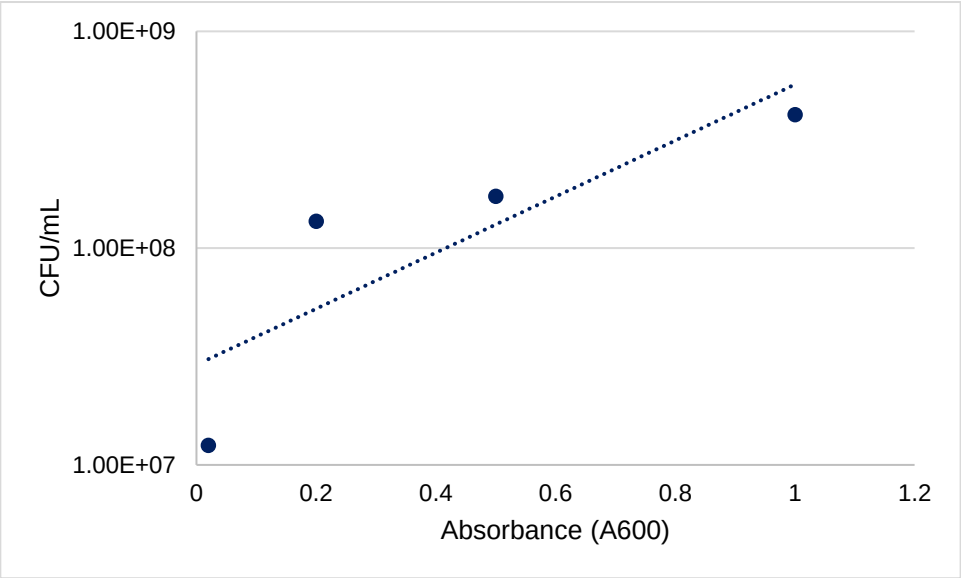
It is understood that disinfectant wipes are frequently being chosen for cleaning of hospital surfaces (Sattar and Maillard, 2013). However, as we have found, wipe products are still ineffective in the removal and inhibition of transfer of DSB from surfaces. Further investigation into potential other methods of disinfectant application (i.e., sprays, disinfectant then automated machinery) and type of disinfectant is required to combat the threat of DSB.

8.3 Final Conclusions

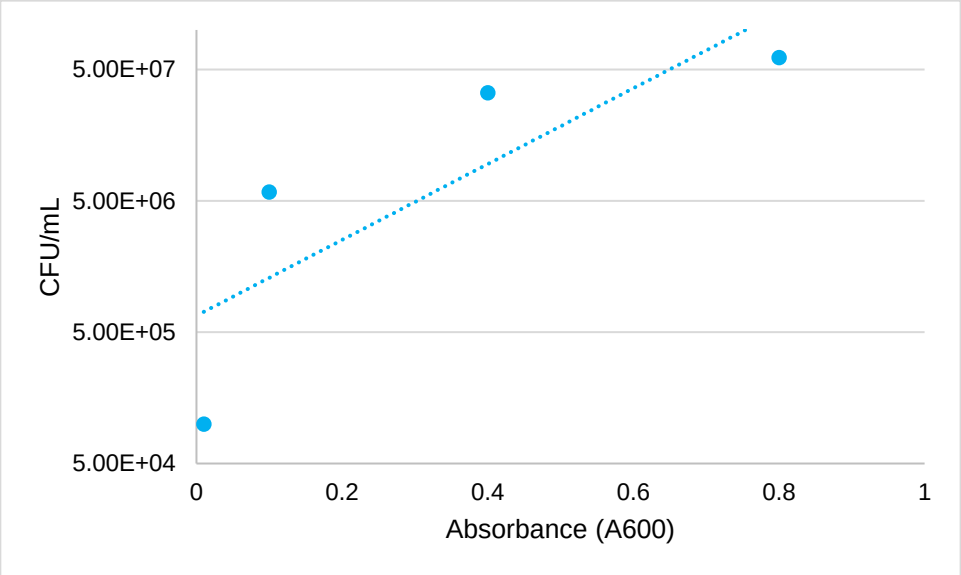
Our results have shown the resistance of DSB to disinfectant products, especially those species considered non-pathogenic (*Bacillus* spp.). Environmental isolates can persist on surfaces for extended periods and can remain culturable up to 6 months of incubation. We have highlighted the importance for new measures to include DSB in standard testing procedures and for companies to consider DSB when creating their products. Still there remains a lack of knowledge around DSB, especially with those working in healthcare which must be addressed in order to further work in combatting DSB from healthcare environments. An important highlight from this thesis is the benefit of social research alongside product testing. As found in surveys and interviews, HCP working with commercially available products that we are testing in the laboratory, are not using them appropriately. Without the correct usage, product efficacy is reduced and so the time taken to produce the highest quality product with the best efficacy against target microorganisms becomes somewhat redundant.

Appendices

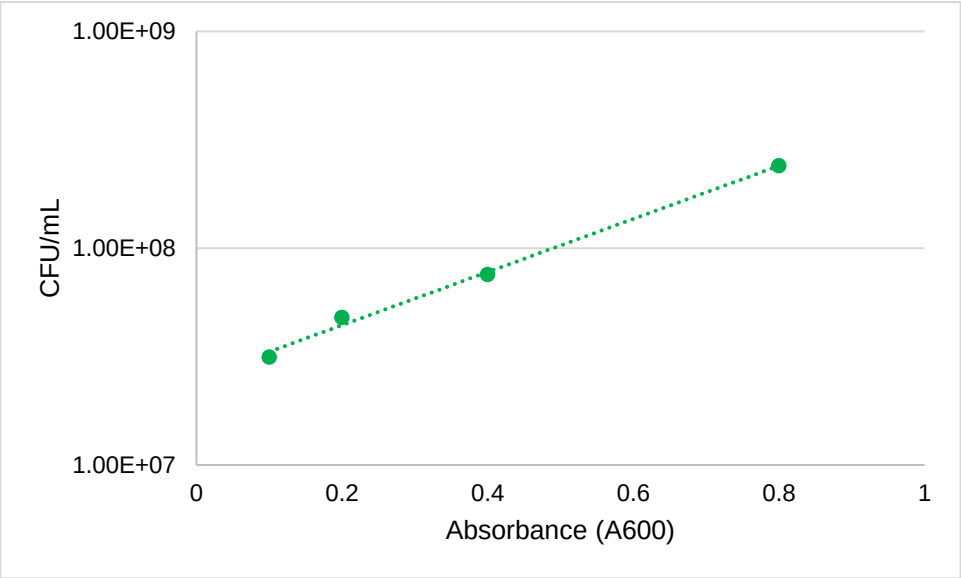
Appendix 1.1. Species Growth Curves



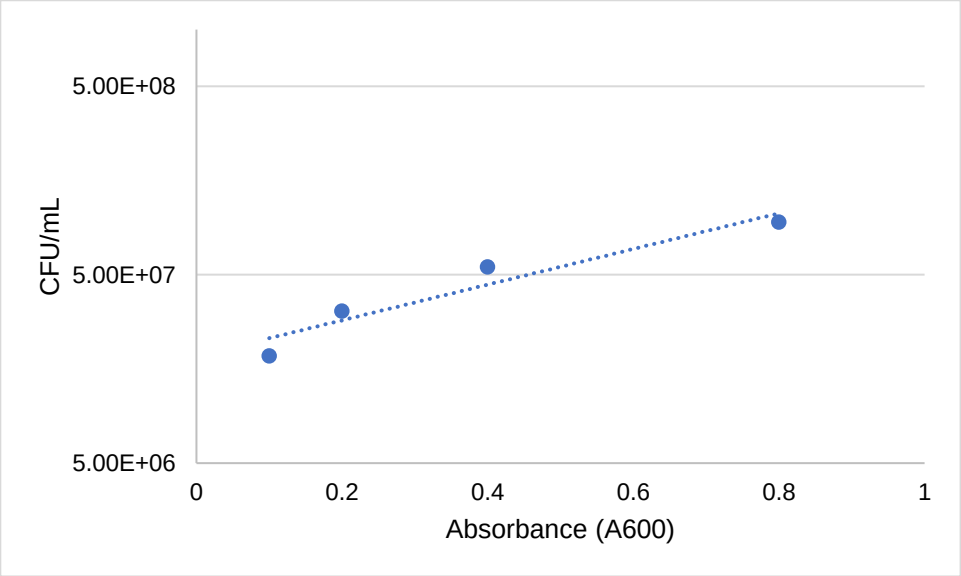
S. aureus standard growth curve.



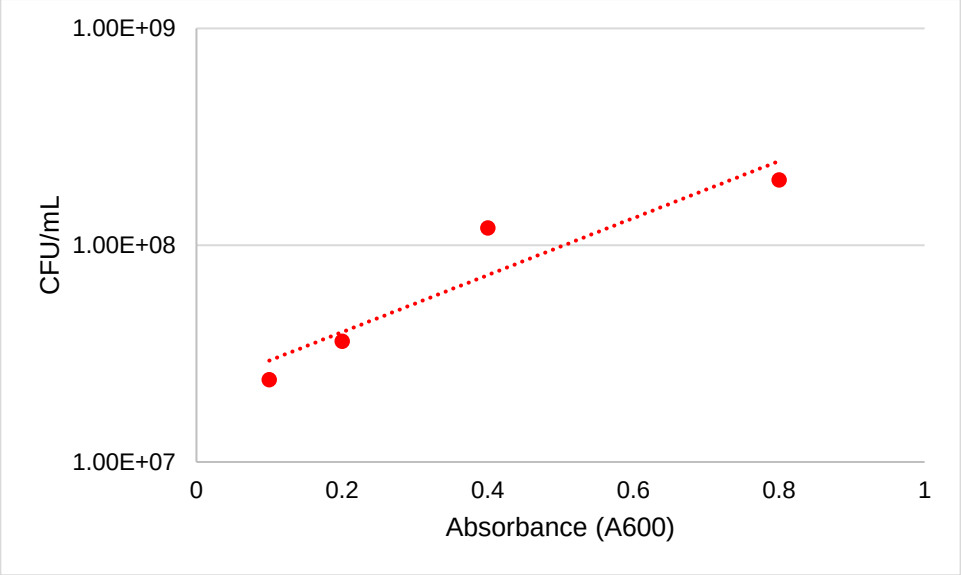
B. subtilis AEWD standard growth curve.



B. licheniformis standard growth curve.

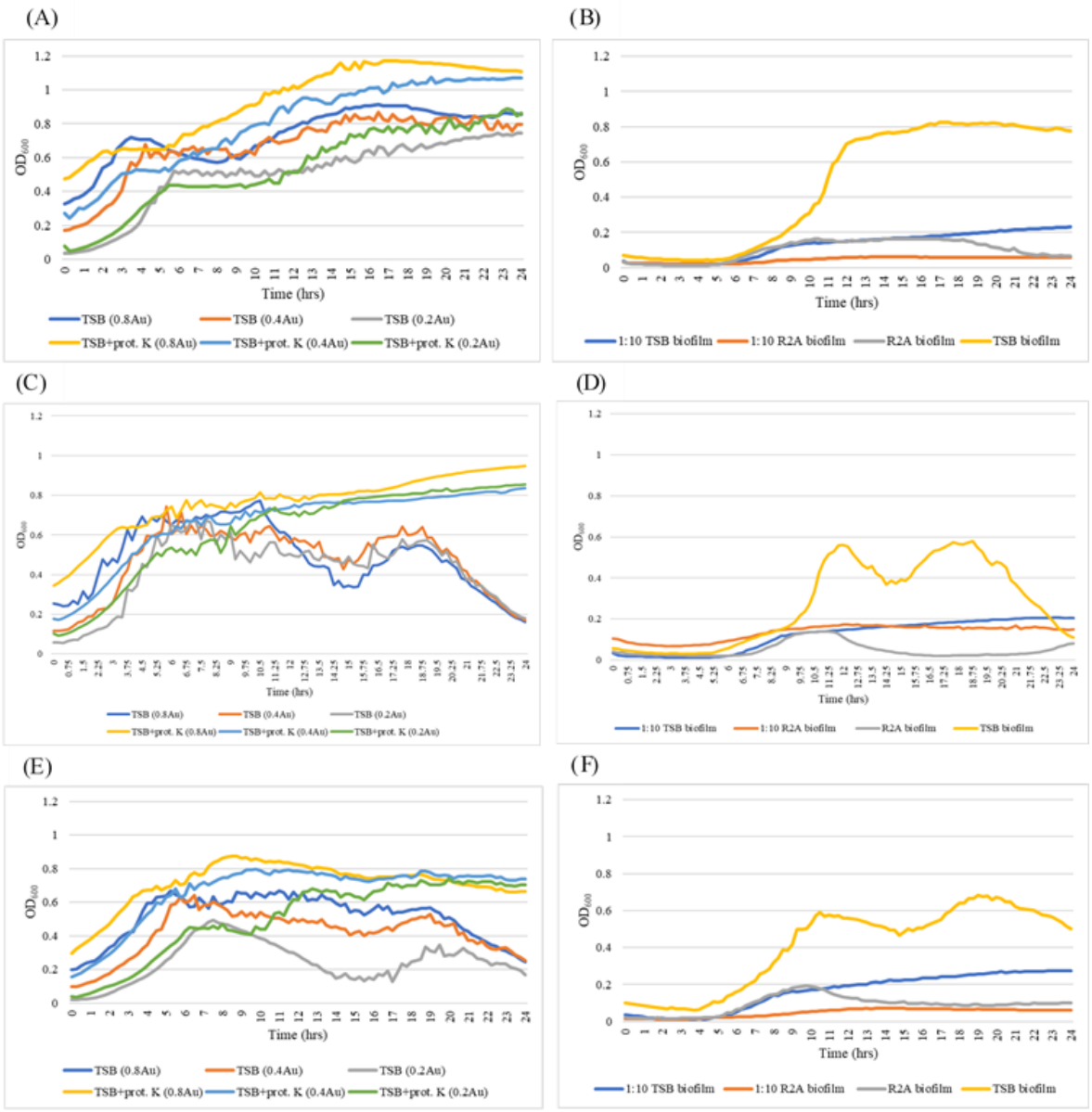


B. subtilis NCTC 10400 standard growth curve.

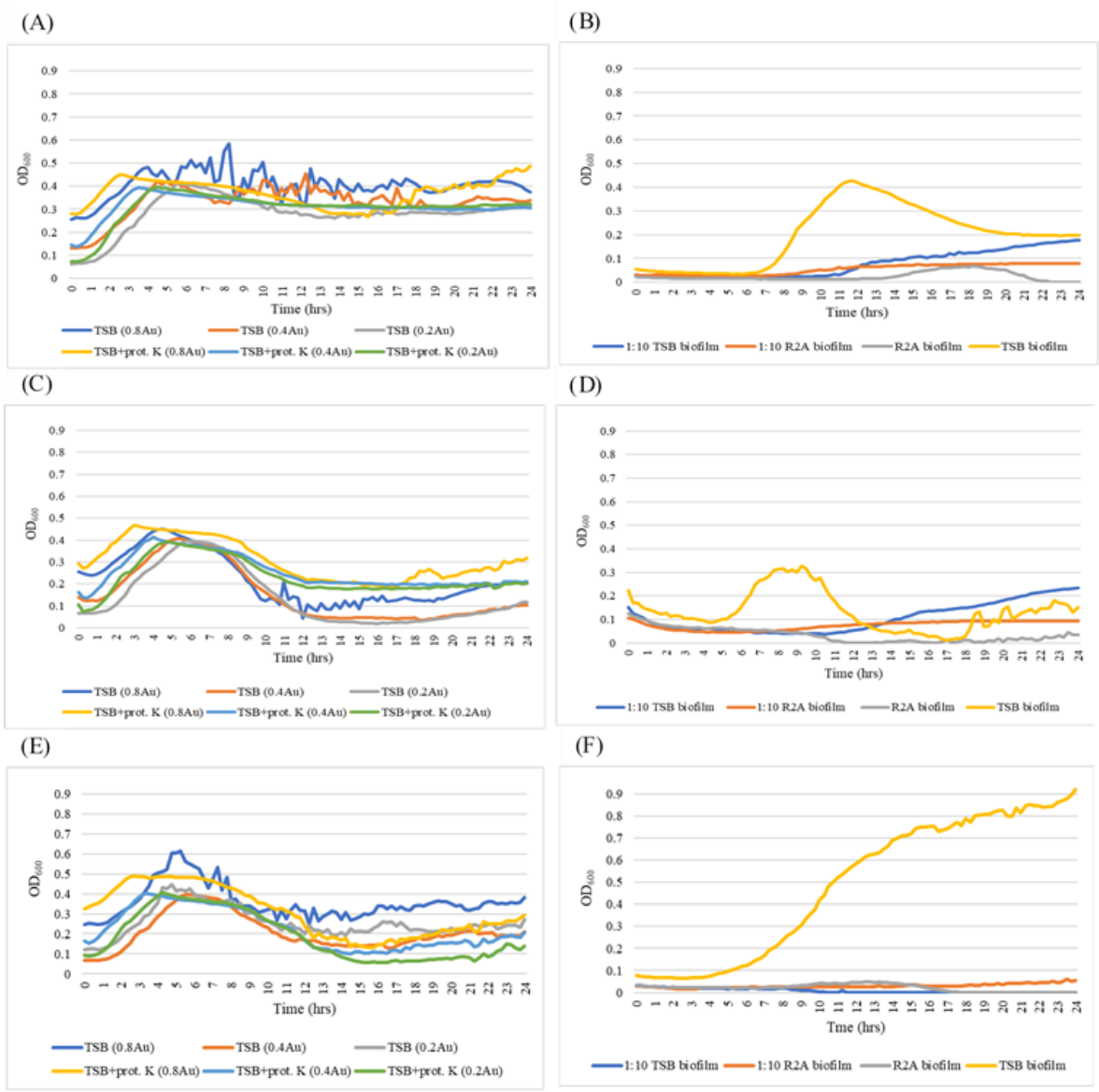


K. Pneumoniae standard growth curve.

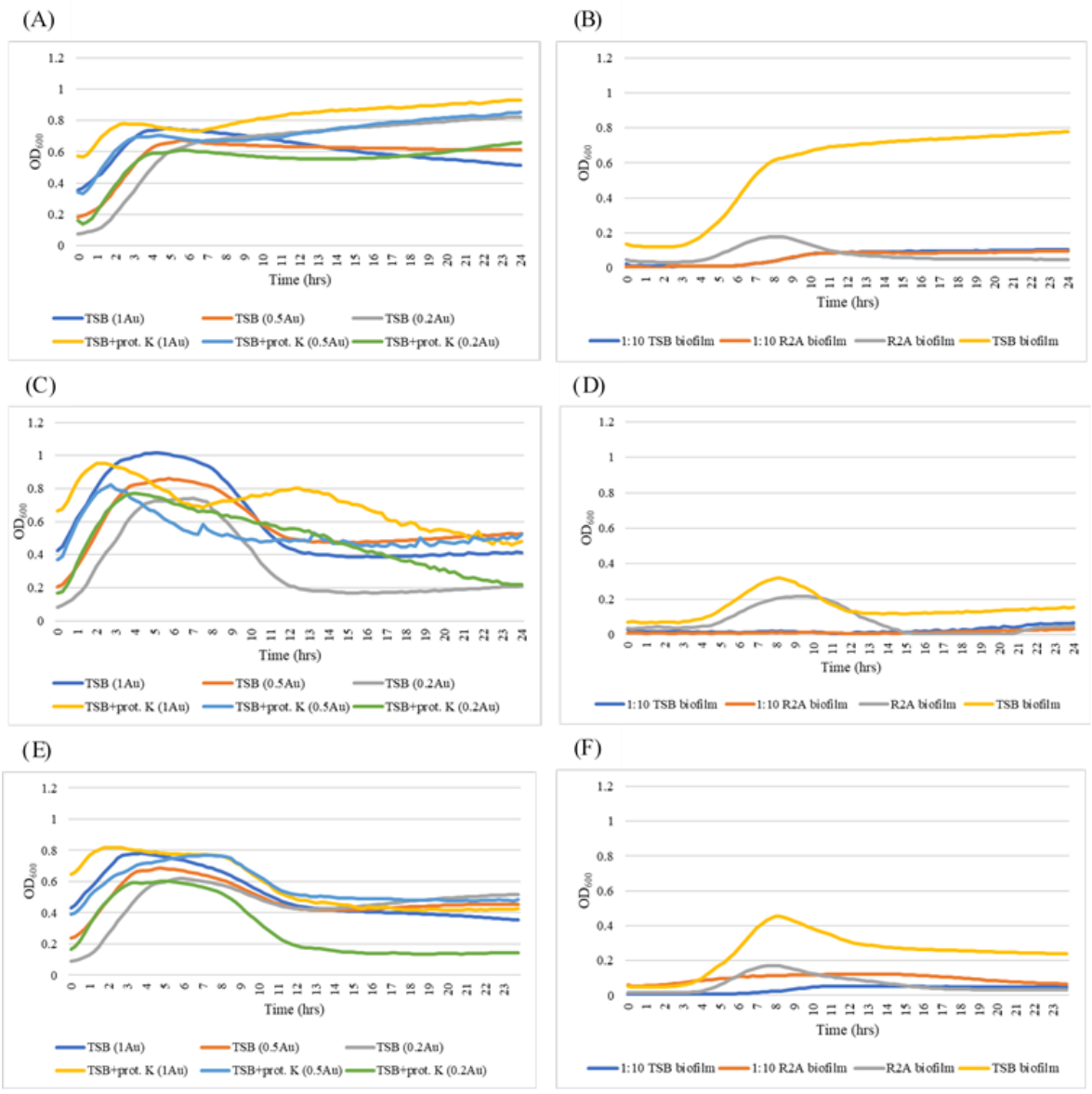
Appendix 1.2 Growth Curves of DSB *vs.* planktonic culture



Growth curves of planktonic cultures and DSB of *B. licheniformis*. (A) planktonic growth at 4 days, (B) DSB growth at 4 days, (C) planktonic growth at 8 days, (D) DSB growth at 8 days, (E) planktonic growth at 12 days, (F) DSB growth at 12 days.



Growth curves of planktonic cultures and DSB of *B. subtilis* NCTC 10400. (A) planktonic growth at 4 days, (B) DSB growth at 4 days, (C) planktonic growth at 8 days, (D) DSB growth at 8 days, (E) planktonic growth at 12 days, (F) DSB growth at 12 days.



Growth curves of planktonic cultures and DSB of *S. aureus*. (A) planktonic growth at 4 days, (B) DSB growth at 4 days, (C) planktonic growth at 8 days, (D) DSB growth at 8 days, (E) planktonic growth at 12 days, (F) DSB growth at 12 days.

Appendix 2.1. Online survey for Healthcare Professionals

SECTION A: ABOUT YOU

1. Which healthcare setting do you work in?
   a.) Hospital
   b.) Residential care home
   c.) Nursing home
   d.) GP surgery
   e.) Community health centre
   f.) Other please state …….

2. What is your job title?
   Please state……

3. What is your country of residence?
   Please state……

4. What is your main area of practice?
   a.) A&E (Trauma)
   b.) Cancer
   c.) Care of the elderly
   d.) General medicine
   e.) Infection prevention and control
   f.) Intensive Care
   g.) Paediatrics
   h.) Psychiatry/mental health
   i.) Surgery
   j.) Other please state …….

5. How long have you been working in your healthcare role?
   a.) 1-5 years
   b.) 6-10 years
   c.) 11-15 years
   d.) 16-20 years
   e.) 21+ years

6. Have you attained any specific training/qualifications relation to infection control?
   Please state……..

SECTION B: CLEANING AND DISINFECTION

1. How often do you think hospital surfaces (e.g. desks, bedside tables) should be **cleaned** on a routine basis?
   a.) Once a day
   b.) Twice a day
   c.) Three/more times a day
   d.) Between every patient

2. How often do you think hospital surfaces (e.g. desks, bedside tables) should be **disinfected** on a routinely basis?
   a.) Between every patient
   b.) Once a day
   c.) Three/more times a day
   d.) Twice a day

3. Which one of the methods below do you believe delivers the **best** infection prevention in a contaminated area?
   a.) Automated disinfectant methods only (e.g. UV light, hydrogen peroxide)
   b.) Cleaning followed by automated disinfection
   c.) Cleaning followed by liquid based disinfection
   d.) Cleaning only (detergent)
   e.) Liquid based disinfectants only
   f.) Single use loaded wipes only (antibacterial, sporicidal)
   g.) Other please state …….

4. From the following options, typically, how long do you think these microorganisms can survive in the healthcare environment? (Please tick <u>one</u> option for each microorganism)

	Microorganisms			
Survival	Bacteria	Viruses	Fungi	Spores
Hours				
Days				
Weeks				
Months				
Years				
Don’t know				

SECTION C: DAILY ROUTINE

1. In your opinion, which intervention has the greatest impact on infection prevention and control within the healthcare setting?
   a.) Hand hygiene
   b.) Outbreak control
   c.) Surface cleaning
   d.) Surface cleaning and disinfection
   e.) Vaccinations
   f.) Other please state …….

2. Out of these methods, which methods are most effective when measuring cleanliness? (Please tick all that apply)
   a.) Adenosine Triphosphate (ATP) assay
   b.) Culture of microorganisms from a swab taken
   c.) Indicator products (stickers/tape)
   d.) Ultraviolet (UV) markers
   e.) Visibly it looks clean
   f.) Other please state …….

3. From the above, which do you believe is the **single most important** method?
   a.) Adenosine Triphosphate (ATP) assay
   b.) Culture of microorganisms from a swab taken
   c.) Indicator products (stickers/tape)
   d.) Ultraviolet (UV) markers
   e.) Visibly it looks clean
   f.) Other please state …….

4. Please tick one out of the three risk factor options for each area/room in the grid, with regards to the transmission and spread of infection in the healthcare setting. For example, high would mean that you believe there is a high chance of spread and transmission of infection in this area/room.

	Risk of transmission		
Area/room	Low	Medium	High
Café			
Clean utility			
Nurse station			
Outpatient area			

Patient area			
Patient bathroom			
Patient side room			
Sluice rooms			

5. Please tick one out of the three risk factor options for each item in the grid, with regards to the transmission and spread of infection in the healthcare setting. For example, high would mean that you believe that this item definitely enables the spread and transmission of infection.

	Risk of transmission		
Item	**Low**	**Medium**	**High**
Bed rails			
Call button			
Curtains			
Door handle			
Floor			
Keyboard			
Light switches			
Mattress			
Patient table			
Sink			
Television			

SECTION D:

1. Have you **heard** the term “microbial biofilms”?
   a.) Yes
   b.) No
   c.) Not sure

2. Do you know what a microbial biofilm is?
   a.) Yes
   b.) No
   c.) Not sure

If YES, please continue to question 3. If NO or NOT SURE, thank you for your time in completing the survey.

3. Where did you gain this information on microbial biofilms? (Please tick all that apply)
   a.) Conference/study day
   b.) General press (e.g. Newspapers)
   c.) Online
   d.) Professional magazine (e.g. Nursing Times)
   e.) Scientific journal (e.g. Journal of Clinical Microbiology, Nature)
   f.) Short workshop/training session
   g.) Social media (e.g. Twitter)
   h.) Talking to colleagues
   i.) Talking to a company rep
   j.) Other please state …….

4. What type of biofilms are you aware of? (Please tick all which apply)
   a.) Dental
   b.) Drain
   c.) Dry surface
   d.) Medical device
   e.) Wet
   f.) Other please state …….

Thank you for taking the time to complete the survey. If you have anything else you want to mention on the areas touched upon, please could you state below.

Appendix 2.2. Information Sheet given to Participants before Completion of the Online Survey.

Thank you for your interest in this survey regarding the perception of cleaning and disinfection in the healthcare environment.

The survey is part of a PhD project being undertaken at Cardiff University, with support from GAMA Healthcare. The survey will ask for your views and responses on infection prevention and control, focusing on cleaning and disinfection processes. Your contribution will enhance our understanding of the importance of cleaning and disinfection within the healthcare environment.

Participation is completely voluntary, and your response will not be included in the study until you click the final submit button. You can withdraw at any point up to this time and the research team will not know. (Please note that due to the anonymous nature of the survey, it is not possible to remove an individual response after submission).

This survey should only take 7 minutes to complete. All responses will remain **anonymous:** you will not be required to enter your name or personal information.

Please tick **one answer to all questions unless stated otherwise.**

If you have any concerns or complaints during this survey, please contact Dr Louise Hughes, HughesML@cardiff.ac.uk, who will address the issue. If you remain unhappy and wish to complain formally, you can do this by contacting the Director of Research, at Cardiff School of Pharmacy and Pharmaceutical Sciences on phrmyresoffice@cardiff.ac.uk.

If you should have any further questions in relation to the purpose of the study please do not hesitate to contact myself, Isabella Centeleghe, PhD student, on CenteleghеI@cardiff.ac.uk.

Appendix 2.3. Semi-structured Interview Schedule.

Introduction to participants

- Thanks for agreeing to participate
- Explanation/reminder of the aims of the study: finding out opinions and knowledge of healthcare professionals with regards to cleaning and disinfection in the hospital environment, pathogen survival and risk in healthcare facilities
- Check consent for interview/audio recording – sign all relevant sheets
- Housekeeping (use normal language and terminology/no right or wrong answers/speak freely: confidentiality etc.)

About Them – Opening Questions

- What is your job? *Tell me about what this entails*
- How long have you been in this role?
- In which healthcare facility do you work?
- In which department (i.e. A&E, Pediatrics)

Main Body of Interview

- I wanted to start off with cleaning and disinfection: *As a healthcare professional, how much do you participate in cleaning of your area? Do you ever join in? (i.e. if you think something looks dirty)*

What do you currently think about the cleaning situation in your area of work?

How would you wipe surfaces? (i.e. do they use one wipe one surface one direction without saying this)

Which areas do you think are most important to be cleaned & disinfected daily? How often? And why is that?

Do you sample surfaces for pathogens? If so, how? Wet/dry any difference?

- Looking at Trust policies of cleaning & disinfection, do you have specific policy in your work place? i.e. do you employ the red/yellow/green system for cleanliness or?

What do the different levels of clean involve (if have)?

Do you do things differently in outbreak situations?

- Next, I wanted to talk about the transmission of pathogens through hospitals…

Where do you think the highest risk of transmission of pathogens are in hospitals?

What do you believe is the best intervention method to prevent such spread?

- Do you know how long pathogens survive on surfaces in healthcare environments? *Expand please/can you give me an example/tell me more about that/is that the case or are there any exceptions?*

What surfaces do you believe are ideal for pathogen survival? Why is that?

- Biofilm related questions, start off with … do they know what a biofilm is/can they give examples/where did they first hear about biofilms?

If yes to knowing what a biofilm is – do they know how they form/develop? Look out for knowledge of medical device biofilms.

Do they know how long they can survive in the environment for?

Do you know the risk of infections from biofilms to patients in hospitals?

Have you heard the term 'dry surface biofilms' – expand.

<u>Closing</u>

- Thank you for answering my questions
- Is there anything related to the topics covered (cleaning & disinfection, biofilms etc.) that you wanted to say but haven't had the chance?
- If yes, explore and check if anything else to add after this
- Explanation of what will happen to data
- Thanks and bye

Appendix 2.4. Participant Information Sheet and Consent Form for Interviews.

Participant Information Sheet

Thank you for your interest in this study regarding pathogens and their risk in the healthcare environment.

The interviews are part of a PhD project being undertaken at Cardiff University, with support from GAMA Healthcare. The interview will consist of questions around the main topics of cleaning and disinfection, pathogenic bacteria, specifically biofilms and their associated risks. The purpose of this study is to enhance our understanding of the importance of cleaning and disinfection within the healthcare environment.

Why have I been asked to participate?

You have been asked to take part in this study as you are a healthcare professional. Your participation is voluntary and your consent is needed to participate in this project. If you do not wish to take part, you do not have to do anything in response to this request. If you decide to take part and then change your mind you can withdraw from the study at any time without giving reasons, and the audio recorder can be stopped at any time at your request.

What am I being asked to do?

The study will involve you taking part in a one-to-one interview. The interview will be held on a date and time that suits you, via Zoom or telephone if you prefer. The interview will consist of you giving your opinions and knowledge in relation to the project topic. You do not have to answer any questions you do not wish to answer. The interview is expected to last approximately 20-30 minutes. The interview will be audio recorded with your consent via a Dictaphone, even over Zoom. Should the interview be conducted over Zoom, you may choose whether to have your camera on or off for the duration. The participant consent form will be sent to you via email, which you will need to complete by hand. This can either be scanned or a picture of the form can be taken by a mobile phone/camera and emailed. Or if you do not have access to a scanner, an electronic completion, including e-signature, will be sufficient and return via email prior to the interview.

Will I be paid for taking part?

No, you should understand that any information you give will be as a gift.

What are the benefits of taking part?

There will be no direct advantages or benefits to you from taking part, but your contribution and others, will go on to help and advise on cleaning regulations within hospitals.

Will my taking part in this research be kept confidential?

Whilst you will be asked to answer questions on your views and perceptions, all the information you provide will be kept confidential and any personal information you provide will be managed in accordance with data protection legislation. The audio recordings will be typed up and any information that could identify you will be removed – audio recordings will then be deleted. Although the findings of this project may be reported (including direct quotes from the interviews), all information will be made anonymous i.e. no personal details which could identify you will be reported, and quotes will not be attributable to any specific identifiable individual.

What will happen to my personal data?

Cardiff University is the Data Controller and is committed to respecting and protecting your personal data in accordance with your expectations and Data Protection legislation. Further information about Data Protection, including:

- Your rights
- The legal basis under which Cardiff University processes your personal data for research
- Cardiff University's Data Protection Policy
- How to contact the Cardiff University Data Protection Officer
- How to contact the Information Commissioner's Office

may be found at https://www.cardiff.ac.uk/public-information/policies-and-procedures/data-protection

After data collection, the research team will anonymise all the personal data it has collected from, or about, you in connection with this research project, except for your consent form. Your consent form will be retained for one year and may be accessed by members of the research team and, where necessary, by members of the University's governance and audit teams or by regulatory authorities. Anonymised information will be kept for a minimum of 3 years but may be published in support of the research project and/or retained indefinitely, where it is likely to have continuing value for research purposes.

If you wish to withdraw from the project at any time, personal data collected up until this point will be destroyed. It will not be possible to withdraw any anonymised data that has already been published.

What will happen to the data at the end of the research project?

The data collected during the research project will may be used in publications or presented at relevant conferences. However, no identifiable information will be included.

What if there is a problem?

If you have any concerns or complaints about the interview, please contact Dr Louise Hughes, HughesML@cardiff.ac.uk, who will address the issue. If you remain unhappy and wish to complain formally, you can do this by contacting the Director of Research, at Cardiff School of Pharmacy and Pharmaceutical Sciences on phrmyresoffice@cardiff.ac.uk.

Who has reviewed this research?

This study has been reviewed by Cardiff University School of Pharmacy Research Ethics Committee.

What to do next?

If you would like to take part please let myself know through the email address CenteleghеI@cardiff.ac.uk, to arrange an interview. If you should have any further questions in relation to the purpose of the study please do not hesitate to contact myself, Isabella Centeleghe, using the above email.

Thank you for reading this information sheet and for your consideration in taking part in this research.

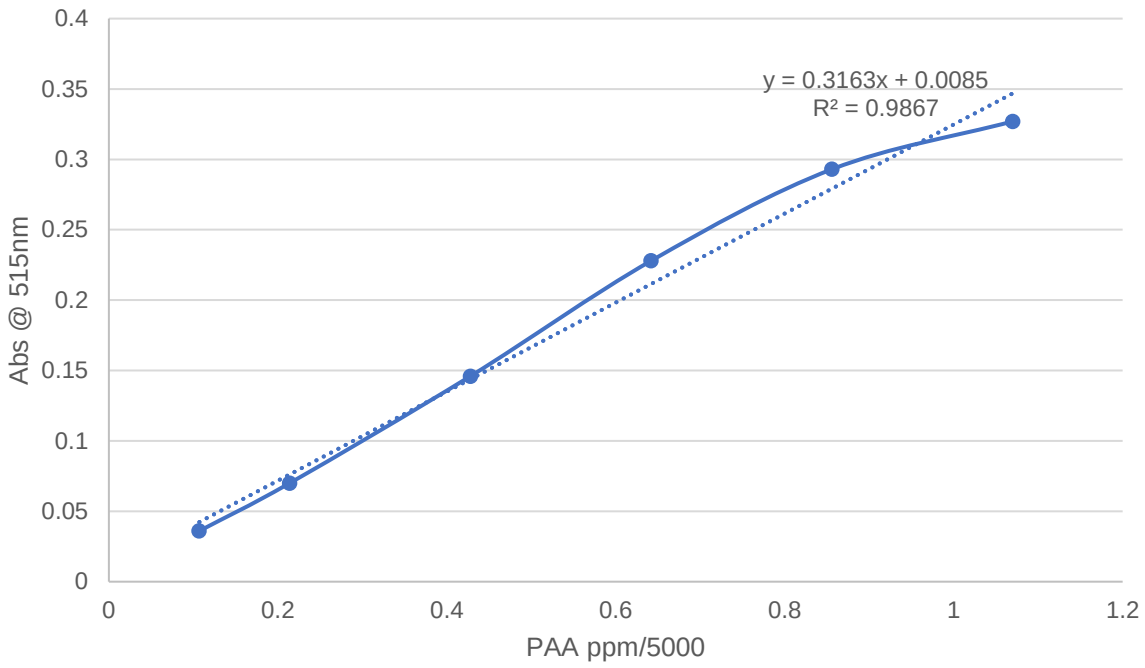
PARTICIPANT CONSENT FORM

Title of research project: Pathogens and their risk to the healthcare environment
SREC reference and committee:
Name of Researcher: Isabella Centeleghe

	Please initial box
I confirm that I have read the information sheet dated January 2021 (version 1.1a) for the above study.	
I confirm that I have understood the information sheet dated January 2021 (version 1.1a) for the above research project and that I have had the opportunity to ask questions and that these have been answered satisfactorily.	
I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my legal rights being affected.	
I consent to the processing of my personal information (name, contact details) for the purposes explained to me. I understand that such information will be held in accordance with all applicable data protection legislation and in strict confidence unless disclosure is required by law or professional obligation.	
I understand who will have access to personal information provided, how the data will be stored and what will happen to the data at the end of the research project.	
I consent to being audio recorded for the purposes of the research project and I understand how it will be used in the research.	
I understand that anonymised excerpts and/or verbatim quotes from my interview may be used as part of the research publication.	
I understand that I have the choice to keep my camera on or off, should the interview be conducted via Zoom.	
I understand how the findings and results of the research project will be written up and published.	
I agree to take part in this research project.	

Name of participant (print) Date Signature

Appendix 3.1. Peracetic acid calibration curve and corresponding calculation chart done in UV spectrophotometer.



PAA (ppm)	Absorbance @ 515 nm
0	0
535	0.036
1070	0.07
2140	0.146
3210	0.228
4280	0.293
5350	0.327

Appendix 8.1a. Raman microscopic analysis of dry-surface biofilms on clinically relevant materials (SUBMITTED).

Raman microscopic analysis of dry-surface biofilms on clinically relevant materials

Thomas J. Tewes[1], Isabella Centeleghe[2], Jean-Yves Maillard[2], Frank Platte[1] and Dirk P. Bockmühl[1]

[1] Faculty of Life Sciences, Rhine-Waal University of Applied Sciences, Marie-Curie-Straße 1, 47533 Kleve, Germany; thomasjohann.tewes@hsrw.eu (T.J.T.); frank.platte@hsrw.eu (F.P.)

[2] School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Cardiff CF10 3NB, Wales, UK; CenteleghеI@cardiff.ac.uk (I.C.); maillardj@cardiff.ac.uk (J.-Y.M.)

***** Correspondence: dirk.bockmuehl@hochschule-rhein-waal.de; Tel.: +49-2821-806-73208

Abstract: Moist/hydrated biofilms have been well studied in the medical area, and their association with infections is widely recognized. In contrast, dry surface biofilms (DSB) on relevant surfaces in healthcare settings have received less attention. DSB have been shown to be widespread on commonly used items in hospitals and to harbor bacterial pathogens which could lead to healthcare acquired infections (HAI). DSB cannot be detected by routine surface swabbing or contact plate and studies have shown DSB to be less susceptible to cleaning/disinfection products. As DSB are increasingly reported in the medical filed, and it highly probable they also occur in food production areas, there is a growing for the rapid in situ detection of DSB and the identification of pathogens within DSB. Raman microspectroscopy allows both to obtain spatially resolved information about the chemical composition of biofilms, and to identify microbial species. In this study, we investigated Staphylococcus aureus mono species DSB on polyvinylchloride blanks and on stainless steel coupons, and dual species (S. aureus/B. licheniformis) DSB on steel coupons. We demonstrated that Raman microspectroscopy is not only suitable to identify specific species, but also enables differentiation of vegetative cells from their sporulated form. Our findings provide the first step towards the rapid identification, and characterization of the distribution and composition of DSB on different surface areas.

Keywords: Dry-surface biofilms, Raman microspectroscopy, *Staphylococcus aureus*, *Bacillus licheniformis*, support vector machine, stainless steel, PVC

Appendix 8.1b. Dual species dry surface biofilms; *Bacillus* species impact on *Staphylococcus* aureus survival and surface disinfection (PUBLISHED)

Dual species dry surface biofilms; *Bacillus* species impact on *Staphylococcus aureus* survival and surface disinfection

Running title; Survival of dual species biofilms

Isabella Centeleghe[1], Phillip Norville[2], Louise Hughes[1], Jean-Yves Maillard[1]*

[1]School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Cardiff, Wales

[2]GAMA Healthcare Ltd., Hemel Hempstead, Hertfordshire, United Kingdom

*Correspondence: Jean-Yves Maillard, MaillardJ@cardiff.ac.uk

School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Redwood Building, King Edward VII avenue, CF10 3NB, Cardiff, Wales

Key words: Bacillus, Staphylococci, Biofilms, Disinfection, Biocides

Abstract

Aims

Dry surface biofilms (DSB) survive on environmental surfaces throughout hospitals, able to resist cleaning and disinfection interventions. This study aimed to produce a dual species DSB and explore the ability of commercially available wipe products to eliminate pathogens within a dual species DSB and prevent their transfer.

Methods and Results

Staphylococcus aureus was grown with two different species of *Bacillus* on stainless steel discs, over 12 days using sequential hydration and dehydration phases. A modified version of ASTM 2967-15 was used to test six wipe products including one water control with the Fitaflex Wiperator. *S. aureus* growth was inhibited when combined with *Bacillus subtilis*. Recovery of *S. aureus* on agar from a dual DSB was not always consistent. Our results did not provide evidence that *Bacillus licheniformis* protected *S. aureus* from wipe action. There was no significant difference of *S. aureus* elimination by antimicrobial wipes between single and dual species DSB. *B. licheniformis* was easily transferred by the wipe itself and to new surfaces both in a single and dual species DSB, whilst several wipe products inhibited the transfer of *S. aureus* from wipe. However, *S. aureus* direct transfer to new surfaces was not inhibited post-wiping.

Conclusions

Although we observed that the dual DSB did not confer protection of *S. aureus*, we demonstrated that environmental species can persist on surfaces after disinfection treatment. Industry should test DSB against future products and hospitals should consider carefully the products they choose.

Significance and Impact of the Study

To our knowledge, this is the first study reporting on the production of a dual species DSB. Multispecies DSB have been identified throughout the world on hospital surfaces, but many studies focus on single species biofilms. This study has shown that DSB behave differently to hydrated biofilms.

Appendix 8.2. Conference Acceptance

Centeleghe, I., Norville, P., Hughes, L., Maillard, J-Y. Dual species dry surface biofilm: are pathogens being protected from disinfection? **Poster Presentation**, ECCMID annual conference 2022.

Centeleghe, I., Norville, P., Hughes, L., Maillard, J-Y. Efficacy of commercial wipes on a dual species biofilm. **Oral presentation**, PGR Research Day 2021.

Centeleghe, I., Norville, P., Hughes, L., Maillard, J-Y. The hidden threat of *Klebsiella pneumoniae* in a dry surface biofilm. **Poster presentation**, IPS annual conference 2021.

Centeleghe, I. Dry surface biofilms in the healthcare environment. **Oral presentation,** Southwest Branch meeting and IPS MHLD SIG meeting 2021.

Centeleghe, I., Norville, P., Hughes, L., Maillard, J-Y. *Klebsiella pneumoniae* survives on surfaces as dry biofilm. **Poster presentation**, ECCMID annual conference 2020.

Centeleghe, I., Norville, P., Hughes, L., Maillard, J-Y. The secret infection risk. **Poster presentation**, L'AIPI annual conference 2020.

Centeleghe, I., Ledwoch, K., Norville, P., Hughes, L., Maillard, J-Y. The secret infection risk: Dry surface biofilms resisting disinfection within healthcare settings. **Poster presentation**, IPS annual conference 2019.

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