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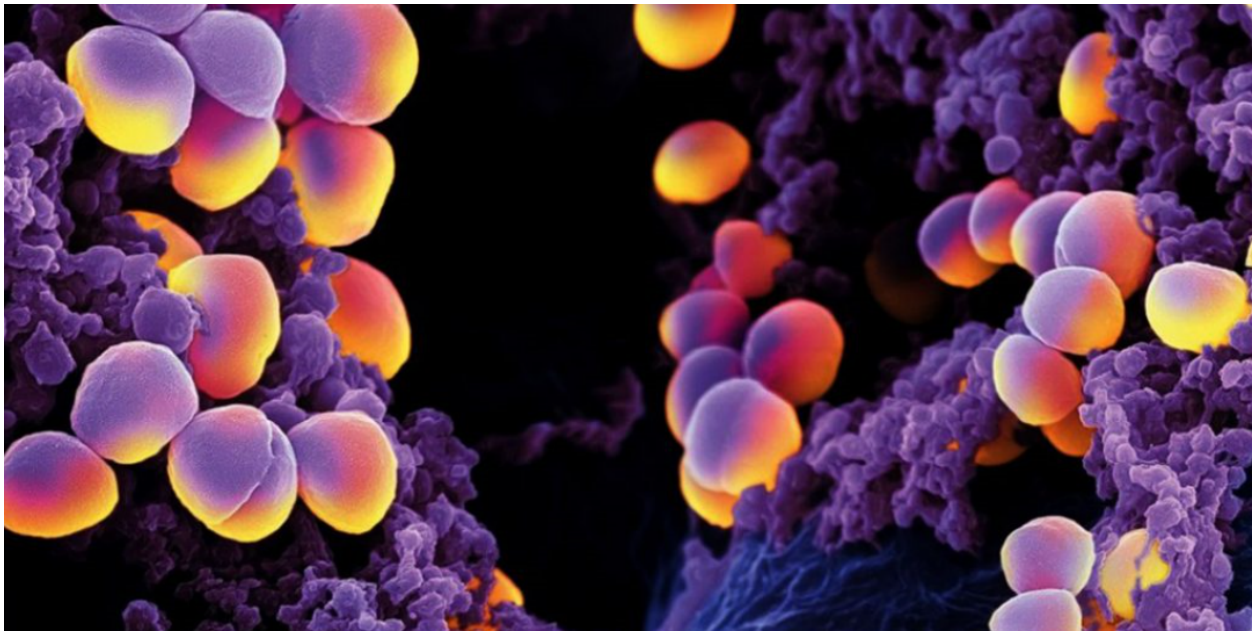

Antimicrobial Effect on Bacteria in Biofilm and Planktonic state

Time-kill studies of Chlorhexidine digluconate against *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilms during static and dynamic conditions

Master's thesis in Biotechnology

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Gothenburg, Sweden 2018

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Cover: Picture of *S. aureus* bacteria growing in biofilm [1]

Typeset in LaTeX
Printed by Reproservice, Chalmers University of Technology
Gothenburg, Sweden 2018

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Abstract

The elimination of contaminating bacteria is essential in order to achieve proper wound healing. Bacteria can occur in two different states, as free-living (planktonic) cells or in aggregates adhered to a surface (biofilm). The bacteria behaves differently depending on in which state they occur and their differences and their sensitivity against Chlorhexidine digluconate have been of key interest during this thesis work. To investigate this, time-kill studies have been performed on the two common wound bacteria *P. aeruginosa* and *S. aureus* under static and dynamic conditions. The studies were performed on bacteria in both biofilm and in planktonic state. The minimum inhibitory concentration (MIC), the minimum biocidal concentration (MBC) and the minimum biofilm eradication concentration (MBEC) for the antimicrobial compound Chlorhexidine digluconate against the bacteria were used in time-kill studies under static and dynamic conditions. In the static time-kill studies the bacterial were treated with different concentrations of Chlorhexidine digluconate in the range from below the MIC to above the MBC. In the dynamic time-kill studies Chlorhexidine digluconate was added continuously to the bacteria to reach the determined MBC in different times. Results from the experiments showed that *P. aeruginosa* was less sensitive against treatment of Chlorhexidine digluconate than *S. aureus* was. There seemed to be persister cells within the *P. aeruginosa* population during all experiments since they managed to recover from antimicrobial treatment even if the bacterial concentration at a point was below the limit of detection. The experiments also showed that killing of *P. aeruginosa* seemed to be C_{max}-driven, which means that a high concentration Chlorhexidine was needed to be delivered fast to the bacteria to achieve the best rate of killing. For *S. aureus* the killing seemed to be AUC-driven which means that a lower amount of the antimicrobial could be used but the bacteria needed to be exposed for a longer time. When the two bacterial species were in biofilm, both were less sensitive to Chlorhexidine digluconate and a higher concentration was needed in order to achieve the same rate of killing as for bacteria in planktonic state.

Keywords: *Pseudomonas aeruginosa*, *Staphylococcus aureus*, biofilm, time-kill, bacteria, Chlorhexidine digluconate, chronic wounds, antimicrobial

Acknowledgements

We would like to thank Mölnlycke for having us and letting us perform this thesis work with them. Thanks to all the members in the preclincial laboratory group for helping us during our time at the company. A special thanks will be given to Eric Wellner and Aida Bibic for their guidance, help and useful discussions. Also, a special thanks to Christin Karlsson for teaching us new methods, always answering our questions and helping us in the laboratory.

Anna Larsson & Julia Linse, Gothenburg, May 2018

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List of Abbreviations

AUC	Area Under the Curve
CFU	Colony Forming Unit
C_{max}	Maximum serum concentration
DNA	Deoxyribonucleic Acid
ECM	Extracellular matrix
EPS	Extracellular Polymeric Substances
LPS	Lipopolysaccharide
MBC	Minimal Biocidal Concentration
MBEC	Minimal Biofilm Eradication Concentration
MDK	Minimal Duration of Killing
MIC	Minimal Inhibitory Concentration
MRSA	Methicillin Resistant *Staphylococcus aureus*
MSSA	Methicillin Sensitive *Staphylococcus aureus*
P.a	*Pseudomonas aeruginosa*
PBP	Penicillin Binding Protein
PD	Pharmacodynamic
PK	Pharmacokinetic
PMN	Polymorphonuclear leukocytes
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
S.a	*Staphylococcus aureus*
SWF	Simulated Wound Fluid
$T_{1/2}$	Elimination half life
T_{max}	Time to maximum serum concentration
T>MIC	Time above minimal inhibitory concentration
TSB	Tryptic Soy Broth
TSS	Toxic Shock Syndrome
TSST	Toxic Shock Syndrome Toxin

1
Introduction

A life essential process in which many cell types in the body collaborates is the wound healing process. In the event of a tissue lesion the regeneration and repair process immediately starts [2]. The wound healing process is often divided into four main phases; hemostasis, inflammation, proliferation and remodelling. These processes are overlapping one another and they are precisely programmed [3].

Improper and impaired healing and remodelling of the tissue can occur if the events in these phases are somehow disturbed. Many factors can contribute to this by interfering with the tissue remodelling processes, causing them to exceed their time frame and by that not occur in their proper sequence. A normal part in the wound healing process is inflammation but this process can be prolonged if removal of contaminating microorganisms is improper. Bacteria can cause elongation of the inflammatory phase by extending the elevation of pro-inflammatory cytokines. The consequences of this continuing may cause the wound to enter a chronic state and fail to heal [3].

Chronic wounds constitute a great economic burden to health care systems and a significant reduction in life quality for those who are affected [4]. Chronic wounds are an increasing problem worldwide, 1-2 % of the population in developed countries have chronic wounds [5]. It may require several years to heal a chronic wound, which is associated with great suffering for the patients, emotional and physical stress, reduced life quality and high cost to the health care system [5, 6]. The search for a solution to this problem is a key priority for the company Mölnlycke, which is a world leading supplier of disposable products in surgery and wound care for health care and patients. One approach they have is to incorporate antimicrobial agents in the dressing of their wound care products in order to reduce the number of harmful pathogens within wounds [7]. In this thesis work this has been approached by studying an antimicrobial agent, that potentially could be used in wound dressings, called Chlorhexidine digluconate and its effect on two types of common wound bacteria, *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) [3]. The bacterial behavior over time when treated with Chlorhexidine digluconate have been studied in this thesis work. The minimum concentration of the antimicrobial that is needed to reach inhibition of growth, the minimum concentration needed to achieve a biocidal effect of the bacteria and the time-dependency of when these concentrations are reached have been investigated. In infected wounds, the bacteria tend to form biofilms, which are clusters of bacteria which are embedded in self-secreted extracellular matrix. Within the biofim the bacteria collaborates metabolically and

the extracellular matrix are protecting the bacteria from the body's phagocytic activity. This can cause chronic ulcers with great difficulties to heal [3, 8]. The formation of biofilm could affect how the bacteria reacts to treatment with antimicrobial agents and therefore, investigations done in this thesis have included both bacteria in planktonic state as well as bacteria in biofilm. A particular interest during this thesis work has been to investigate whether the bacteria develop tolerance or persistence against the antimicrobial agent and under which circumstances this occur.

1.1 Aim

The aim of this thesis work is to investigate the effect of the antimicrobial agent Chlorhexidine digluconate against the bacteria *Staphylococcus aureus (S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*). The investigations comprise examination of potential differences in effect when being treated with the antimicrobial compound when the bacteria exist in biofilm or in planktonic state. Examination of pharmacodynamic properties, such as the minimum inhibitory concentration (MIC), minimum biocidal concentration (MBC) and the minimum biofilm eradication concentration (MBEC) for the antimicrobial compound against the bacteria will be determined. Further studies involving how the bacteria behaves over time will be performed to investigate whether the time to reach a certain concentration of an antimicrobial drug affects the behaviour of the bacteria and whether any indications that the bacteria develops tolerance or persistence can be seen.

2

Background

2.1 Bacteria

Bacteria is a group of unicellular microorganisms with the absence of a nuclei and membrane bound organelles which make bacteria a large domain of prokaryotic microorganisms [9]. The genetic material of bacterial cells exist as a single, circular, tightly packed chromosome and therefore bacteria is often classified as relatively simple cells. Bacteria occur in different shapes and sizes but are approximately within the range of $0.5 - 5\ \mu m$ in diameter, which is about ten times smaller than an average eukaryotic cell [10, 9, 11]. The main morphological categories in which bacteria may occur in are spherical cells (cocci), rods (bacilli) and spirals (spirillum/spirochaete). These types of bacterial cells can be found singly arranged but bacilli and cocci can also be arranged in pairs, chains and irregular clusters [10].

Bacteria are often categorized as Gram-positive or Gram-negative. The main difference between Gram-positive and Gram-negative bacteria is their cell wall composition which can be seen in Figure 2.1. Gram-positive bacteria has relatively thick cell walls, about 20-80 nm, which mainly consists of a peptidoglycan network [12, 9]. The peptidoglycan network is a unique structure among prokaryotic cells and its main function is to confer strength and shape to the bacteria. It consists of a polymer of dissacharide repeating units which are cross-linked with short peptide chains. Certain Gram-positive bacteria, Staphylococci included, have teichoic acids located on the surface of the peptidoglycan network [13]. These teichoic acids are highly negatively charged polymers which are covalently bound to the cell wall of the bacteria [14]. Gram-negative bacteria has two cell membranes but their cell walls are much thinner than the cell walls of Gram-positive bacteria, less than 10 nm thick. Gram-negative bacteria has a thin peptidoglycan layer and between the two membranes of the bacteria it has a periplasmatic space. The periplasmatic space facilitates the nutrition transport and cell wall maintenance and it consists of proteins and polysaccharides [15]. The outer membrane of Gram-negative bacteria contains lipopolysaccharides (LPS) and proteins, in particular porins [16, 17, 15]. The porins in the membrane are hydrophillic channels which the bacteria uses to regulate membrane permeability [15].

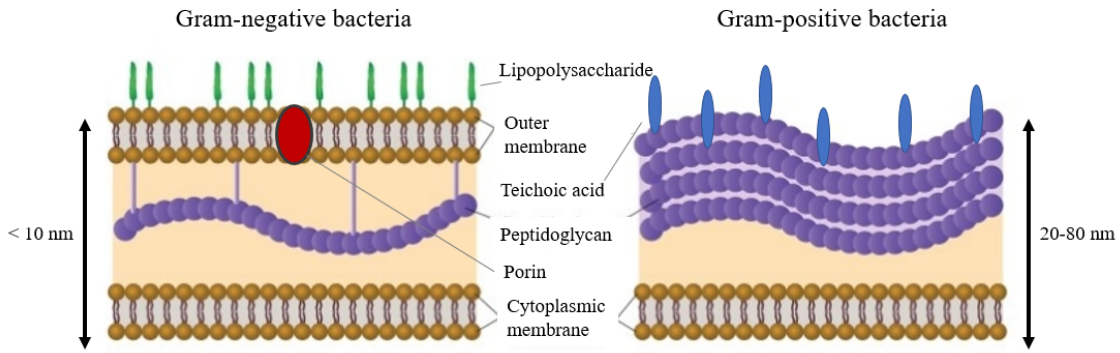


Figure 2.1: Picture of the differences in cell wall composition of Gram-positive (e.g *S. aureus*) and Gram-negative (e.g *P. aeruginosa*) bacteria. Modified from [17]

These different types of bacteria also differ from each other when it comes to their resistance against lysozyme which is an enzyme present in different secretions from animals and humans such as in tears, saliva and other mucous [18, 12, 16]. This enzyme also occurs in monocytes, macrophages and polymorphonuclear neutrophils (PMNs) [19]. Gram-positive bacteria are relatively easy digested by lysozyme while Gram-negative bacteria are resistant to the digestion [12, 16]. The performance of a Gram stain test allow bacteria to be distinguished as Gram-positive or Gram-negative, where the method utilizes the differences in cell wall composition of the bacteria [10, 20].

2.1.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative rod shaped bacteria with a width of 0.5-0.8 µm and a length of 1.5-3.0 µm. The bacteria also have a single polar flagellum which makes it motile. A picture of the bacteria can be seen in Figure 2.2. *P. aeruginosa* normally inhabits water, soil and vegetation but it can also be found on the skin of humans [21]. The bacteria is considered a opportunistic pathogen which means that it seldom affects healthy individuals and is favoured by a weakened immune system [22, 23]. *P. aeruginosa* has a broad range of growth substrates, has minimal nutrient requirements, is capable of growing under anaerobic conditions and is tolerant against temperatures up to 50°C. It also produces many virulence factors, is resistant to a large number of antibiotics and is capable of forming biofilms. This makes *P. aeruginosa* a challenging pathogen. Since *P. aeruginosa* is an opportunistic pathogen it never causes diseases in an immunocompetent host because their immune system effectively prevents the infection [24]. The bacteria displays high resistance towards a wide range of antibiotics and the main reason for this is the limitation of penetration rate of antibiotic molecules into the cells due to the low permeability of the bacterial outer membrane [25].

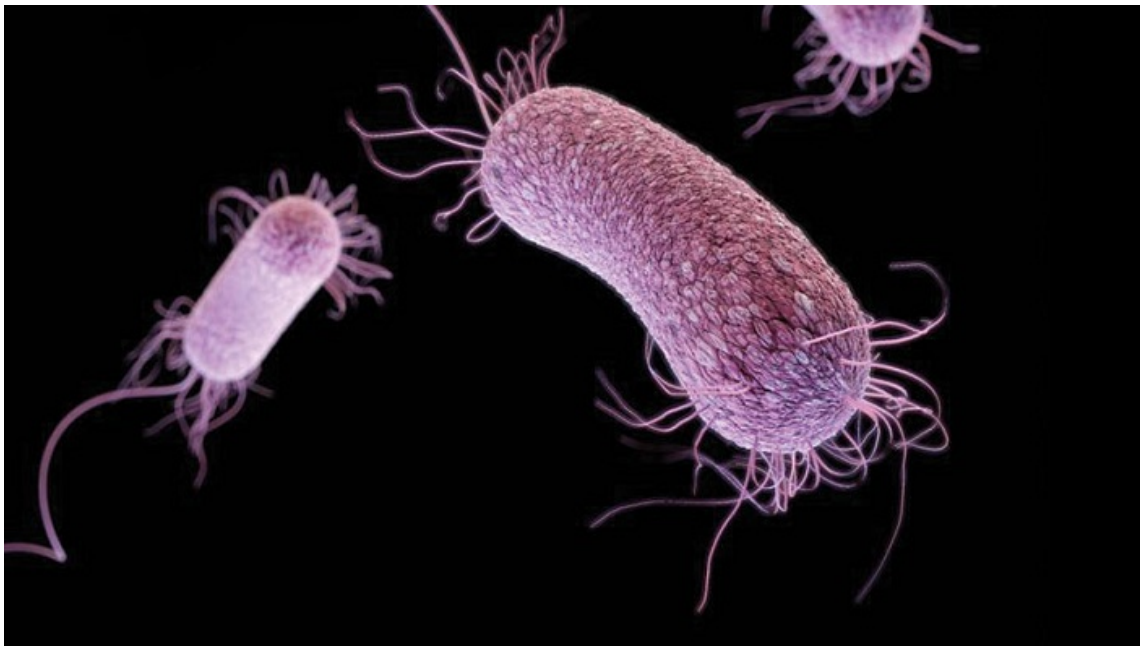


Figure 2.2: Picture of the Gram-negative bacteria *P. aeruginosa* [26]

2.1.1.1 Prolonged wound healing by *P. aeruginosa*

P.aeruginosa produces many factors that may contribute to its virulence [21]. The virulence factors can disrupt the host cells signalling pathways while targeting the extracellular matrix. They also play an initial role in motility and adhesion to the epithelium. *P. aeruginosa* is an unique organism because it is capable of causing severe invasive diseases and evading the immune system and by that cause persisting infections that are very hard to eliminate. The many virulence factors produced by the bacteria are likely to contribute to tissue damage, invasion of the tissue and dissemination of *P. aeruginosa* [24].

Below are a few examples of virulence factors produced by *P. aeruginosa* that can interfere with the wound healing process. One virulence factor is LPS which is a component of the outer membrane of the bacteria. LPS plays a prominent role in activation of the immune response of the host and eventually causes dysregulation of inflammation responses that contribute to mortality and morbidity [26]. Flagellum is another virulence factor that is responsible for the motility of *P. aeruginosa*. It has been shown to have a critical role in attachment, invasion, biofilm formation and mediation of inflammatory responses [24]. Another virulence factor called type IV pili is involved in adhesion to several cell types and is important in the process of attachment to particular tissue as well as initiation of biofilm formation [27]. A variety of secretion systems that interfere with wound healing are also present in *P. aeruginosa* [24]. *P. aeruginosa* also produce several different proteases that destroy host tissue and by that play a significant role in wound infections [24]. Moreover, a cell to cell communication mechanism of bacterial cells that work through diffusible chemical compounds called quorum sensing is another virulence factor of *P. aeruginosa*. This mechanism controls more than 300 genes in *P. aeruginosa*.

The most common signaling molecule used by Gram-negative bacteria produced by this mechanism trigger the formation of a complex that activates genes involved in biofilm formation and coding of virulence factors. Example of these virulence factors are extracellular enzymes and cellular lysins that act as a protective shield against phagocytes which makes them important for the pathogenesis of infections [24].

As mentioned above, *P. aeruginosa* is capable of forming biofilm. The most important feature of such infections is its resistance to antimicrobial agents. The process of biofilm development by *P. aeruginosa* is complex and controlled by a variety of components as well as quorum sensing signals. Type IV pili and flagella are just two examples of components involved in the initial attachment of cells to the surface and biofilm matrix development. *P. aeruginosa* has been shown to form biofilms that are hard to remove, particularly in the site of burn wounds [24].

2.1.2 *Staphylococcus aureus*

Staphylococcus aureus is a 1 µm in diameter Gram-positive cocci bacterium which is a major human pathogen that causes a wide range of clinical infections such as skin infections and post-operative wound infections [28, 29, 11]. A picture of *S. aureus* bacteria can be seen in Figure 2.3. *S. aureus* bacteria is adjustable to survive both aerobic and anaerobic environments and it has relatively low demands for humidity and nutrition and can therefore survive a certain period of dehydration, where it survives as dust particles [9]. *S. aureus* is a normally occurring bacteria among humans. It colonizes on the tissue of humans in some cases without causing infections and it also behaves pathogenic and causes a variety of different infection types [9]. Most people are periodically carriers of this bacteria, normally in the nose but also in other mucous and on the skin. The risk for skin contamination increases upon injury of the skin, for example in eczema and in wounds. The bacteria can also be involved in respiratory tract infections, but these are more dominant in nosocomial patients, which are patients already under medical care at a hospital, with weakened immune defence [30]. This bacteria is also one of the most common reasons for infectious wounds and ulcers. The risk for such an infection is greater in health care environments, for example after surgery since such patients often are immune-compromised and can be the carriers of viral infections [30, 31]. Carriership of the bacteria is more common among health care personal, injection abusers, diabetics and dialysis patients [31]. Among nosocomial pathogens the risk for morbidity and mortality is highest with *S. aureus* present. *S. aureus* strains can also produce a variety of enterotoxins which are toxins that interfere with intestine function which can cause emesis and diarrhea. The toxic shock syndrome toxin (TSST) is one of the most famous toxins of *S. aureus* and is what causes toxic shock syndrome (TSS) by stimulating the release of a variety of cytokines. *S. aureus* also work as a pathogen by secretion of enzymes, mostly proteases, that degrades host molecules, interfere with host signalling pathways or disturbing the host's metabolic network [30].

Nowadays, around 90% of all *S. aureus* strains can produce the enzyme Penicillinase and are therefore resistant to penicillin [9]. Methicillin was therefore introduced as treatment for *S. aureus* but not long after the introduction various methicillin-resistant clones of *Staphylococcus aureus* was discovered worldwide. A penicillin-binding protein (PBP) in *S. aureus* is what causes resistance towards β-lactam antibiotics, where methicillin is included. In the strains of *S. aureus* that are sensitive towards methicillin (MSSA) the methicillin can perform its intended purpose. The methicillin will cause the death of *S. aureus* by disrupting the synthesis of its peptidoglycan layer caused by the binding of the antibiotic to the native penicillin-binding protein present in the cell wall of the bacteria. In the methicillin-resistant strains of *S. aureus* (MRSA) a foreign penicillin-binding protein is present which makes the methicillin unable to bind and no disruption of the peptidoglycan layer occurs [28, 31].

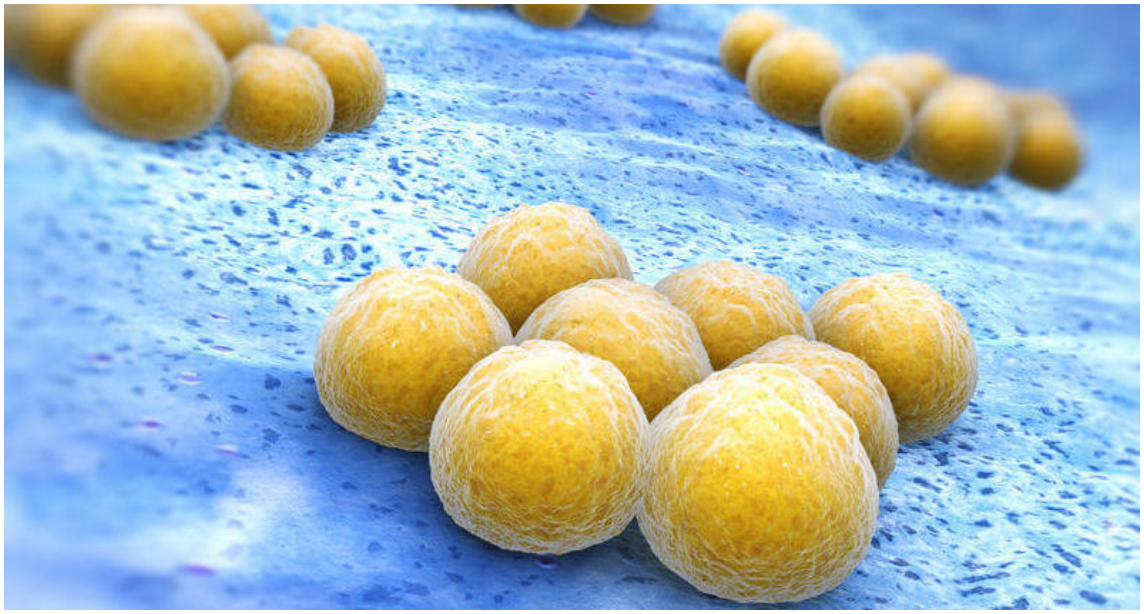


Figure 2.3: Picture of the Gram-positive bacteria *S. aureus* [32]

2.1.2.1 Prolonged wound healing by *S. aureus*

There are several factors that make the bacteria *S. aureus* a dangerous pathogen. The bacteria has several virulence factors, which are molecules that increases the capacity of the bacteria to work as a pathogen which increases its potential of causing diseases. *S. aureus* can decrease the neutrophil function and immune responses of the host [32, 33]. It produces and secretes surface-located proteins which enables the bacteria to attach to damaged host tissue [30]. The bacteria can also decrease the neutrophil function and immune responses of the host [32, 33].

S. aureus produces coagulase which converts the protein fibrinogen to insoluble fibres of fibrin and effectively encloses the invading bacteria to a lump which prevents the defence of the host to get to it [34, 35]. Many bacterial toxins often target the membrane of the host cell. These toxins are cytolytic since they form pores

in the membrane which activates the efflux pump in the cells leading to increased removal of vital molecules.

S. aureus is known to mostly target red and/or white blood cells and it produces cytolytic toxins which causes these cells to lyse. Many of the cytolytic toxins of *S. aureus* have been shown to work through specific receptor interaction. The most studied toxin of *S. aureus* is Alpha-toxin. Alpha-toxin forms a pore in the membrane of the affected host cell which causes efflux of ions out of the cell.

2.2 Biofilm

The formation of a biofilm can occur when individual organisms adhere to a surface. A biofilm is an aggregation of bacteria adhered to a surface and held together by a self-secreted extracellular matrix consisting of polysaccharides [36]. This is often referred to as extacellular polymeric substances (EPS) and are mainly composed of polysaccharides, but also other components such as proteins, nucleic acids and lipids. EPS makes up the intracellular space of the bacterial aggregates and form the structure of the biofilm matrix. The main function of EPS are to protect the bacteria against environmental stress [37]. Biofilm formation can occur on surfaces of liquids, solids and on living tissue. The involved organisms can be from the same or from different species. A period after they have adhered to the surface and have had time to grow and reproduce they start to form the extracellular matrix. The formed matrix is mucous-like and has the purpose to bind the bacteria to the surface and to hold them together. The properties of bacteria existing in a biofilm can differ a lot from the same type of bacteria existing in a planktonic state [36].

Bacteria in biofilm collaborate metabolically and can communicate with each other through signalling in a process known as quorum sensing [36]. In this process bacteria in the biofilm-clusters can regulate gene expression by using signalling molecules and it is a key mechanism for bacterial behaviour-coordination [38]. The biofilm helps the bacteria to be shielded from its surroundings and within chronic wounds it shields the bacteria from the body's phagocytic activities which makes the wounds difficult to heal [3, 8]. Biofilms are nutrition-rich environments for bacteria to grow in and an other advantage for bacteria to grow in this formation is that it confers resistance to antimicrobial agents [36].

2.2.1 Biofilm formation

In the process of biofilm formation the bacteria switch phenotype from planktonic mode of growth to attached mode of growth [39]. This process proceeds through a series of different steps; attachment to a surface, formation of microcolony, three dimensional structure formation, biofilm formation, maturation and detachment [40].

Biofilm formation starts with that a freely floating bacteria adhere to a surface [41]. Structures such as flagella, pili or polysaccharides on the cell surfaces may possibly

provide an advantage for adherence during biofilm formation [42]. Microcoloni formation occurs after the adherence between the bacteria and surface have become a stable binding. Chemical signals between the bacteria result in division of the bacteria. When the intensity of this signaling reach a certain threshold the genetic mechanisms of the production of exopolysaccharides starts [40]. Bacterial cell division within this exopolysaccharide matrix finally results in microcolony formation [43]. After the micro-colony is formed, expression of certain genes that are related to biofilm takes place. The gene products of these genes are needed for the EPS. After formation of the matrix, formation of water-filled channels occurs. The purpose of these channels is to transport nutrients and remove waste products within the biofilm [43]. After formation of the biofilm, bacteria leave the biofilm and can by that undergo rapid division and dispersal. This detachment of the planktonic cells from the biofilm is programmed and have a natural pattern [40]. The detachment occurs either by cells that are newly formed from growing cells or as an effect of quorum-sensing. Dispersed cells from the biofilm can retain some properties of biofilm, such as antibiotic in-sensitivity [44]. The life-cycle of a biofilm can be seen in Figure 2.4 below.

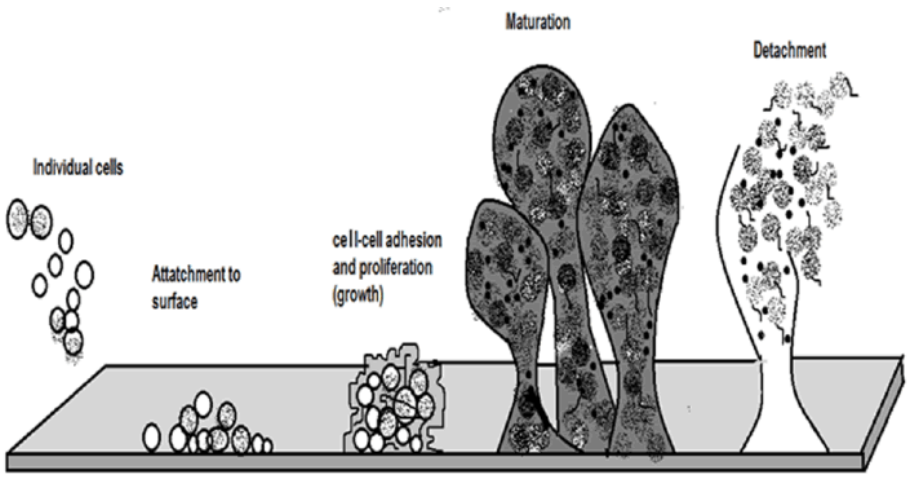


Figure 2.4: Picture of the life-cycle of a biofilm [41]

2.3 Antimicrobial substances

For thousands of years antimicrobial substances have been used in the field of wound care. Continued search for novel substances has been essential in the field due to the emergence of resistant strains [10].

The use of disinfectants and antiseptics is essential in health care settings and hospitals to reduce risk of contamination, control infections and to prevent nosocomial infections. Antiseptic and disinfectant products can contain a variety of different an-

timicrobial chemical agents, called biocides, which many of them have been widely used to achieve antisepsis, disinfection and preservation. The difference between them are basically the concentrations. Disinfectants occur in high concentrations that can be toxic to humans whereas the antiseptics occur in considerably lower concentrations since they are frequently used for treatment of external parts of the body, such as in wounds [34]. One example of a commonly used disinfectant is Ethanol and an example of a normally used antiseptic is Chlorhexidine [45, 46]. Even though these biocides have been in use for hundreds of years there is still little known about their antimicrobial mode of action compared to antibiotics. The main differences between the different types of antimicrobial agents is that antibiotics tend to have more specific targets than biocides, which tend to have multiple targets and a broader spectrum of activity. Antibiotics are often defined as chemical substances that can inhibit or kill selective bacteria or other microorganisms, this is often achieved at low concentrations. An example of an antibiotic is Penicillin [28]. Products containing biocides that destroy or inhibit growth of microorganisms in or on living tissue is often referred to as antiseptics. Disinfectants are similar to antiseptics but differ in the way that they are generally used on surfaces and non-living objects [47, 34]. Antiseptics are generally better to control Gram-positive bacteria than Gram-negative. It is more difficult for an antiseptic to penetrate the double membrane of a Gram-negative bacteria than to incapacitate Gram-positive bacteria [34].

2.3.1 Chlorhexidine against bacteria

Chlorhexidine is a topical antimicrobial agent that is bactericidal [48]. It is a cationic surfactant with broad antibacterial activity and less pronounced antifungal activity [49]. The positive charge of Chlorhexidine causes it to have a high binding affinity to the negatively charged cell wall of bacteria. On Gram-positive bacteria the negative charges comes from the teichoic acids and polysaccaride components located on their cell walls and from the cytoplasmic membrane itself. On Gram-negative bacteria it is their LPS layer and the cytoplasmic membrane that gives them their negative charge [13]. Chlorhexidine is effective against both Gram-positive and Gram-negative bacteria but have shown to be more effective against Gram-positive bacteria [50, 13]. Chlorhexidine binds to the negative cites of the bacterial cell wall [51]. Since the cell wall of Gram-positive bacteria is composed essentially of peptidoglycans and teichoic acids, and neither of them appears to act as an effective barrier for the entry of Chrorhexidine, the molecule can easily attack the cell membrane [47]. The outer membrane of Gram-negative bacteria limits the entry of Chlorhexidine by acting as a barrier. It is the outer membrane of *P. aeruginosa* that is responsible for its low sensitivity to Chlorhexidine [47]. The reason for this is that *P. aeruginosa*, compared to other organisms, have a different LPS composition and a different cation content of the outer membrane [52]. Production of strong links between the LPS is aided by high Mg^{2+} contents, and small size of the porins may prevent general diffusion through them. The major target site for Chlorhexidine against *P. aeruginosa* is its inner membrane [47]. Chlorhexidine have shown to have better efficacy for bacterial infections, especially Staphylococcus than for fungus or

yeast infections [49]. Its action against viruses is uncertain [50].

Chlorhexidine has been applied in several medical devices allowing killing of organisms and protection against microbial colonization and biofilm formation [53]. Chlorhexidine has shown the ability to bind to the proteins present in human tissues, such as skin, with limited bodily or systemic absorption [54]. This phenomenon provides prolonged activity since the protein bound Chlorhexidine releases slowly and allows for longer duration of antimicrobial action [53]. The antimicrobial activity has been documented to last at least 48 hours on the skin [55].

In Figure 2.5 below, the chemical structure of Chlorhexidine is shown. The molecule contain two positively charged biguanide-groups, which are marked with red circles in the figure. Marked with a blue circle are the hydrophobic hexamethylene groups. What also is shown in the figure below, marked with a blue circle, is that the hydrophobic region of the Chlorhexidine molecule is relatively short, six carbons long. This makes the molecule relatively inflexible and incapable of folding which makes it unable to penetrate the cell membrane of the bacteria. The hydrophobic region of Chlorhexidine can instead be seen as a bridge between the two biguanide binding sites. This structure of the Chlorhexidine molecule is what makes the binding of it to the bacteria so successful. The distance between the two positively charged biguanide sites on the molecule is approximately the same as the distance between the negatively charged phospholipids on the membrane of bacteria [13].

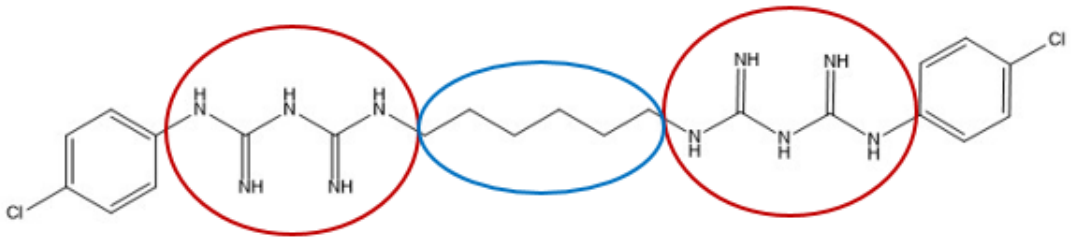


Figure 2.5: Chemical structure of Chlorhexidine

2.3.1.1 Mechanism of action

The progressive mechanism of action of Chlorhexidine can be seen in Figure 2.6. In this figure the intact, untreated bacterial membrane can be seen in 1). It can be seen in 2) that the positively charged biguanide-groups of the Chlorhexidine molecule react with the negatively charged phospholipids on the cell membrane of the bacteria [53, 13]. In 3) the progressive action of more Chlorhexidine molecules binding to the membrane can be seen and the binding of the molecules disrupt the electrolyte layer of the bacteria through stabilization of the net-negative charge of the membrane. After binding has occurred, divalent cations such as Mg^{2+} and Ca^{2+} are pushed aside by the Chlorhexidine and can by that no longer bind to the membrane of the bacteria. It can be seen in 4) in the figure that this causes destabilization of the cell membrane [48, 53, 13]. When low concentrations of Chlorhexidine are used, the fluidity of the membrane will decrease and metabolic and osmoregulatory functions

of the bacterial membrane will be influenced by the binding of the molecule. At high concentrations, "in-use"-concentrations, the binding of the Chlorhexidine molecule leads to a greater damage for the bacteria where the bacterial membrane changes to a liquid crystalline state. This will cause the intracellular components of the bacteria to leak due to the loss of membrane integrity, causing cell death [13, 53].

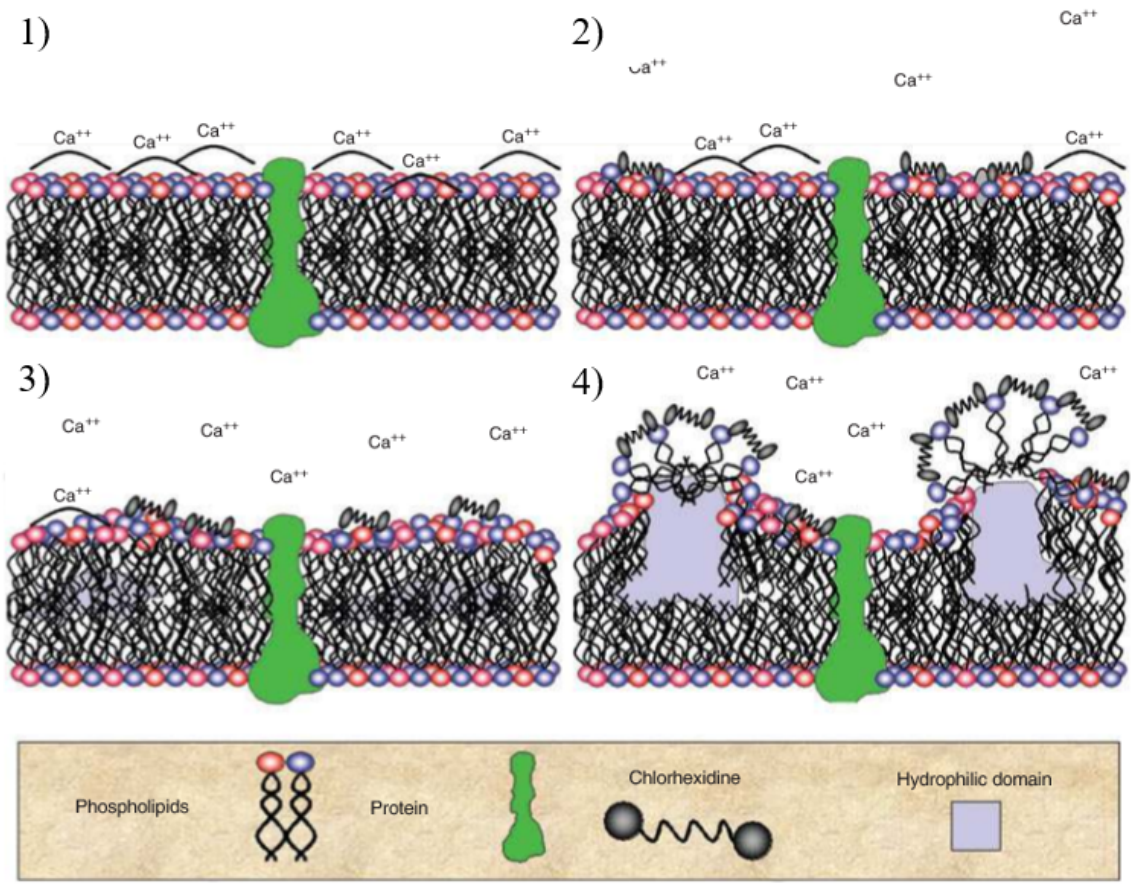


Figure 2.6: The progressive action of Chlorhexidine on the bacterial membrane [13]

The uptake of the Chlorhexidine by the bacteria is very rapid, and is typically occurring within 20 seconds. The mechanism of action for Chlorhexidine makes development of bacterial resistance highly unlikely [48]. Chlorhexidine is not solubilized in the membrane core of the bacteria and therefore efflux pumps have no effect on the substance [13].

2.4 Chronic wounds

A chronic wound is defined as a wound that has proceeded through the repair process without establishing anatomic and functional integrity within a period of 3 months [56, 57]. This definition is not yet agreed, since sometimes these wounds are referred to as difficult or hard to heal wounds, as well as the time span is defined in the range from 4 weeks up to more than 3 months [57, 58, 59]. Chronic wounds are an

increasing problem worldwide, 1-2 % of the population in developed countries have chronic wounds [5]. It may require several years to heal a chronic wound, which is associated with great suffering for the patients, emotional and physical stress, reduced life quality and high cost to the health care system [5, 6].

2.4.1 Causes of chronic wounds

The wound healing process involves four main phases: hemoestasis, inflammation, proliferation and remodeling. Multiple signalling pathways and cascades traverse through the various stages of wound healing. The first event of wound healing is hemostasis and is, compared to the whole wound healing process, very short. Thus, in most cases this phase is not considered as a separate phase and is instead included in the inflammatory phase. In hemostasis the blood vessels are opened and blood clots are formed around the opened vessel walls to prevent excessive bleeding. Bacteria, foreign bodies and dead skin formed when the skin was injured that can interfere with the wound healing process are removed from the wound during the inflammatory phase. This is mainly done by the inflammatory cells in the bloodstream. This phase normally lasts about 3-5 days. The proliferation phase includes an overall process where the defects in the wounded skin are filled up through tissue regeneration. This is done by cells in the skin such as keratinocytes and fibroblasts that produce collagen, proteoglycans and other extracellular matrices to fill up the wounded skin. This phase normally takes about 3 weeks and by the end of this phase the wound is filled up and covered with the epidermis and by that protected from invasion of external bacteria and viruses. In the remodelling phase which is the last phase, the wound defect that was temporarily filled up in the proliferative phase, is replaced by proper tissue to resemble the original tissue as good as possible. This phase continuous for more than a year [60]. The normal signalling pathways which prevent the normal progression of healing is in some way disrupted in chronic wounds [61]. Chronic wounds are believed to be captured in the inflammatory phase [62].

Age as well as diseases linked to lifestyle such as diabetes and obesity can contribute to that a wound turn into a chronic state [4]. The majority of the chronic wounds fall into the main categories: venous ulcers, pressure ulcers, and diabetic ulcers [63, 64].

2.4.1.1 Venous ulcers

Venous ulcers will affect 1-2% of the adult population and represent more than half of all the chronic wounds in the lower limb. Venous hypertension and congestion due to venous thrombosis or valvular incompetence makes these kind of wounds to arise. Macromolecules and red blood cells act as chemottractants for leukocyte infiltration and they have leaked into the perivascular space due to backpreassure that increases blood vessel permeability [65].

2.4.1.2 Pressure Ulcers

Pressure ulcers are common in patients that are either unconscious and by that do not respond to the need for repositioning or patients that in some way have reduced mobility. Unrelieved and prolonged pressure causes the tissue compression to exceed the capillary pressure which leads to ischaemia [66]. Some especially venerable areas are the sacrum, hips and malleoli where the skin is close to the bone [67].

2.4.1.3 Diabetic Ulcers

Diabetic foot ulcers are a common complication of diabetes [68]. Repeated mechanical stress from peripheral neuropathy, which is associated with diabetes, together with disrupted perfusion increase the risk of ulceration. Diabetes also causes metabolic derangements that directly disrupt wound healing by inducing oxidative stress, impairs skin and inflammatory cell function and increases ECM stiffness [69].

2.4.1.4 Bacterial colonization

Bacterial colonization within a wound can cause delayed wound healing [70]. The bacteria damage the host tissue and attract leukocytes which leads to an incerase in inflammatory cytokines, reactive oxygen species (ROS) and proteases which maintain the inflammatory cascades [71]. The closure of the wound is inhibited by proteases derived from bacteria which degrades the ECM, and also by growth factors which disrupt the cell migration. Colonization by bacteria in chronic wounds is often in form of biofilms [72]. The most common bacteria isolated in chronic leg ulcers are *S. aureus* and *P. aeruginosa*. They are both expressing surface proteins and virulence factors that are affecting wound healing, which is described in section 2.1.1.1 and 2.1.2.1. Since co-infection of *S. aureus* and *P. aeruginosa* is more virulent than single infection and both bacteria exhibit intrinsic and acquired antibiotic resistance, clinical management of such infections is a real challenge [73, 74].

2.4.2 Current treatment of chronic wounds

There are many approaches against treatment and prevention of infected wounds [75]. Numerous of topical formulations of antibiotics have been developed to be applied to wound sites [76]. There are also many topical antiseptic products available for treatment of chronic wounds. Some examples of these aniseptics are Acetic acid, Cadexomer idine, Cetrimide, Chlorhexidine gluconate, Hexachlorophene, Iodine compounds, Sodium hypochlorite, Hydrogen peroxide and Silver [76]. Removal of non-viable tissue material is an important concept in wound care. This can be done in different ways but the goal is to expose healthy, well-perfused tissue that is able to proliferate and populate the wound bed via epithelial cell migration [77]. There are many wound dressings on the market that have the purpose to protect the healing wound from infection but also help promote the wound healing process itself. There are also wound dressings with integrated antimicrobial substances [78]. Skin substitutes has been used for a long time for replacement of large surface areas of tissue. These consists of biologically derived substances combined with a material that is placed on the wound [79]. Native pressure wound therapy also called

vacuum-assisted closure is another method used to try to heal chronic wounds. The blood flow is optimized, exudates are removed, a moist environment is maintained and pressure is applied to promote wound closure. The reduced rates of infections in wounds have also been shown to be associated with these devices [80]. Another approach that have been applied the last few decades is growth factors in wound healing, but the results from treatment with this kind of therapy have been rather modest [81]. Hyperbaric oxygen is also a method that have been used to try to heal chronic wounds but the effect of other treatments could be of more benefit [78].

2.5 Pharmacokinetics and Pharmacodynmics

To optimize the use of an antimicrobial agent, pharmacokinetic (PK) and pharmacodynamic (PD) principles can be used as tools. PK is often referred to as "what the body does to the drug" while PD is referred to as "what the drug does to the body". Studies of these parameters provide knowledge of factors that are important for optimization and determination of in what way an antimicrobial drug should be administered. The PK profile of an antimicrobial agent describes its absorption, metabolism and elimination. PK describes how the concentration of a drug change over time after dosage. There are several parameters involved including bioavailability, peak serum concentration (C_{max}), time to peak serum concentration (T_{max}), volume of distribution, area under the serum concentration-time curve (AUC), elimination half-life ($T_{1/2}$) and amount of time serum concentration above the minimum inhibitory concentration ($T > MIC$). The PD profile describes the antimicrobial agents effect on the pathogen. The knowledge in PD is limited relative to the knowledge in PK since these parameters are more difficult to determine. Similar to PK there are several parameters to describe PD including the time the concentration of a drug is above the MIC ($T > MIC$), ratio of the maximum serum concentration to MIC (C_{max}/MIC) and the area of the concentration time curve (AUC) divided by the MIC (AUC/MIC) [82]. These parameters can be seen in the concentration-time curve in Figure 2.7. As pathogens become more resistant to antimicrobial substances it becomes more important that the efficacy is optimized to enable reduction in the dosing [83]. Combination of PK and PD parameters enables optimization of an effective use of an antimicrobial agent and they provide knowledge and understanding of the impact of the drug against the specific pathogen [82].

When it comes to PK and PD principles, the bioavailability of a drug in the host is often considered, which is the degree of absorption of the drug to the treated host [84]. This concentration increases dependent on e.g the solubility and permeability of the drug and the absorption of the host. The bioavailable concentration of the drug decreases depending mainly on the volume of distribution which is the apparent volume of which a drug is distributed and this is based on the administrated amount of the substance and the measured concentrations in the blood of the host. The administrated drug can be distributed to e.g. surrounding tissue in the host or through clearance and elimination mechanisms which lowers the concentration of the drug in the blood [85]. The knowledge about PK and PD enables prediction of the relationship between drug exposure and effect and this relationship is expected to work in a similar manner in different disease models, including humans [86]. Therefore, a similar PK profile can also be determined for bacteria treated with an antimicrobial agent. Binding of the antimicrobial to the bacteria would cause the antimicrobial concentration in the bacterial solution to decrease and a similar concentration-time-curve can be seen for the bacterial solution treated with an antimicrobial as for an affected host treated with a drug.

There are two major patterns of antimicrobial killing: time dependent killing and concentration dependent killing [83]. In time dependent killing the degree of killing

of a pathogen is determined by how long the bacteria is exposed to the drug rather than how high concentration of the drug the bacteria is exposed to [82]. The goal in time dependent killing is to optimize the duration of exposure to the drug. The risk of development of persistence to the antimicrobial substance is minimal with time dependent killing. For time dependent killing the time above MIC ($T > MIC$) is an important parameter to determine the efficacy [83]. In concentration dependent killing the goal is to maximize the concentration of the antimicrobial agent and reach the highest concentration possible at the infection site. The PD parameters that enables prediction of the bactericidal efficacy of a concentration dependent killing compound is correlated with the AUC/MIC ratio or the C_{max}/MIC ratio where the efficacy depends upon the ratio values of those parameters [82, 83].

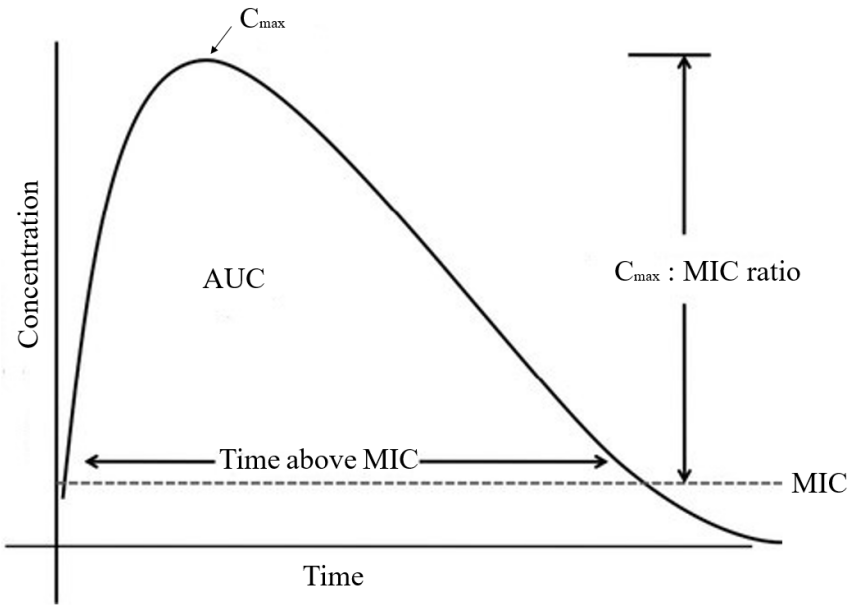


Figure 2.7: Example of a concentration curve showing how the concentration of a drug increases and decreases over time when given in a single dose at time 0 hours [81]

2.5.1 Minimum Inhibitory Concentration

Minimum inhibitory concentration (MIC) is a key indicator of an antimicrobial agents potency and is defined as the lowest concentration of an antimicrobial substance that will prevent visible growth of a microorganism under defined growth conditions [87, 88, 89, 90]. The MIC-values are mainly used to define the sensitivity of a microorganism to an antimicrobial compound and to confirm resistance.

Resazurin can be used as a color indicator to determine the lowest concentration of antimicrobial substance needed to inhibit growth of bacteria. Active bacterial cells reduce the blue/purple, non- fluorescent dye resazurin to the fluorescent dye resorufin that appears pink, see Figure 2.8 below. The color shift will occur when the metabolic activity in the bacterial samples increases, which changes the pH in

the solution. Since a bacterial amount below the pH-shift limit is added from the beginning a direct quantifiable measure of the increase of metabolic activity is given through visualization of the color shift and the minimum concentration needed to inhibit the growth can be determined [91, 89].

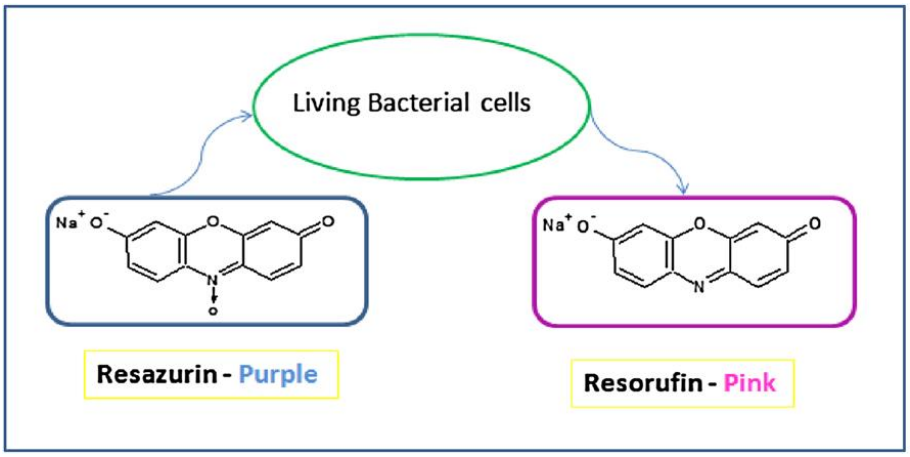


Figure 2.8: Reduction of Resazurin [88]

2.5.2 Minimum Biocidal Concentration

The lowest concentration of an antimicrobial drug that is sufficient to kill the examined test organism in planktonic state is considered as the Minimum Biocidal Concentration (MBC). Tests to investigate the MBC-value of an antimicrobial compound are usually performed after the Minimum Inhibitory Concentration (MIC) has been settled [92]. The MBC-testing can be useful for ranking a large number of antimicrobial compounds after their potency against different types of organisms. It is a relatively inexpensive method that can be used for screening purposes [93, 92].

A common way to perform a MBC-test is to culture samples of the examined bacteria together with a growth media for the bacteria and the examined antimicrobial substance in different concentrations. The bacterial samples are then re-cultured on an agar plate to see in which of the used antimicrobial concentrations the bacteria manage to grow and form colonies in on the agar plate. The lowest used concentration of the antimicrobial in which the bacteria are prevented to form colonies on the agar plate is then considered as the MBC of the antimicrobial substance against the bacteria [94].

2.5.3 Minimum Biofilm Eradication Concentration

Bacteria in biofilms are different from planktonic bacteria in many ways, for example in sensitivity against antimicrobial substances, as described more in detail in section 2.2. Therefore the MBC-value for planktonic bacteria may not be applicable for biofilms and MBEC is being used in the context of biofilms. The Minimum Biofilm Eradication Concentration (MBEC) is defined as the minimum concentration of antimicrobial that is sufficient to eradicate the examined biofilm. There are several

ways to determine the eradication of biofilm which is done after the incubation of the biofilm together with an antibiotic or biocide [95].

A common way to perform a MBEC-test is to culture biofilms by incubating the examined bacteria in growth media together with a structure that can serve as a surface for the bacteria to adhere to. The biofilm formed on the surface is then removed from the bacterial suspension and planktonic bacteria are gently removed from the biofilm. The biofilms are then treated with different concentrations of the examined antimicrobial and incubated further. To be able to see which concentration of the used antimicrobial substance that manged to eradicate the biofilm, the biofilm needs to be destroyed and dissolved into a suspension. That suspension can then be re-cultured on e.g. an agar-plate or a Petrifilm, to see if there are any viable bacteria within the suspension that can form colonies on the plate or the film. The lowest used concentration in which there are no formed colonies is determined as the MBEC for the antimicrobial against the bacteria [96].

2.5.4 Area Under the Curve

The term Area Under the Curve (AUC) represents the integral of the plasma concentration against a defined time interval which is sometimes referred as the concentration-time profile [97, 98]. The number of concentration measurements taken during the given time interval determine the precision of the AUC. In clinical pharmacology the AUC is often determined since it can be interpreted as the total uptake of an administrated drug. In this way, the bioavailability of different drugs can be compared [99]. This kind of concentation-time curves can be used for *in vitro* systems as well. The curves are simulated by addition of antimicrobial compound or by addition of growth medium to a bacterial suspension, to either increase or decrease the concentration of the antimicrobial substance [100].

2.6 Resistance, tolerance and persistence

Failure of antibiotic treatment and relapse of several bacterial infections is the outcome of bacteria that has developed resistance, tolerance or persistence. It is important to distinguish them from one another to avoid miss-classification of bacterial strains that could result in ineffective treatment [101]. A simplified description of the differences of resistance, tolerance and persistence can be seen in Figure 2.9.

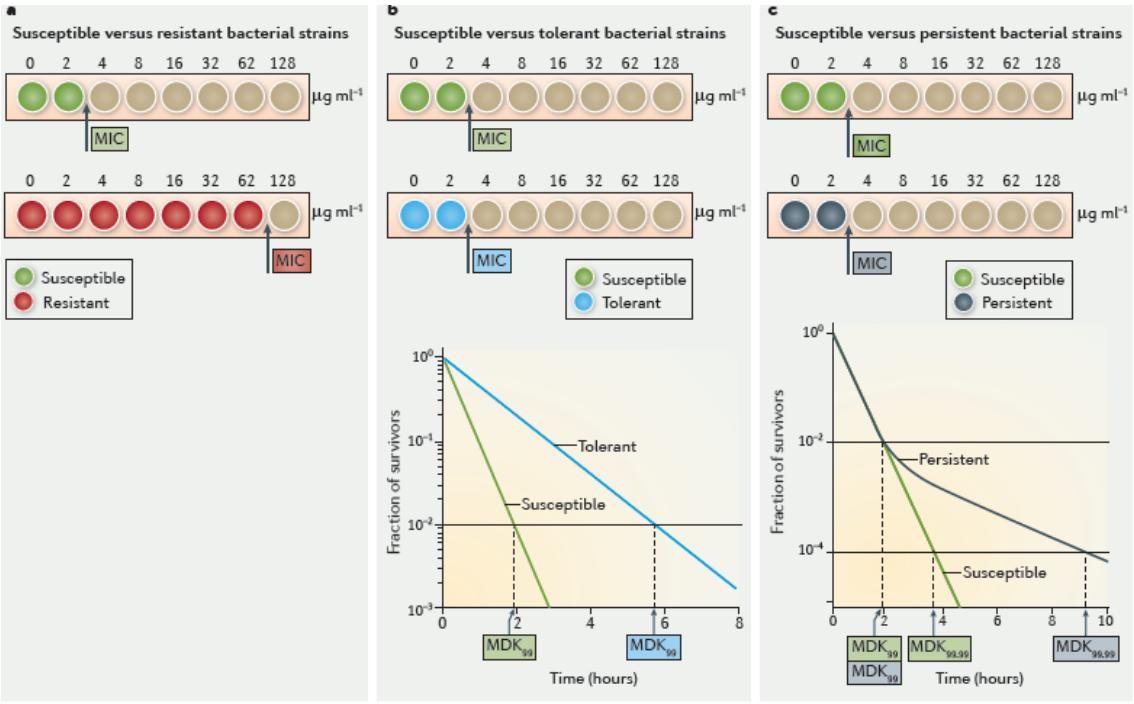


Figure 2.9: Simplified figure describing the characteristic drug responses of strains expressing resistance, tolerance and persistence. It can be seen in a) that a resistant bacterial strain has a higher MIC than a susceptible strain. In b) the behaviour of a tolerant strain versus a susceptible stain can be seen where they differ from each other in MDK (minimal duration of killing). It takes a longer time for an antimicrobial to lower the bacterial amount in a tolerant strain to the same bacterial amount as in a susceptible strain. It can be seen in c) that a susceptible strain and a persistant strain first behave similar in response to antimicrobial treatment but in the persistant strain, a subpopulation manage to change in phenotype to survive the antimicrobial treatment, and in order to kill that subpopulation a longer exposure time is needed and therefore that subpopulation has a higher MDK than the susceptible strain [100]

2.6.1 Resistance

The phenomenon when a system is being exposed to an external disturbing factor but has the ability to withstand it is known as resistance [102]. When it comes to resistance to antimicrobial substances it is typically caused by inherited mutations in the microorganism which affect numerous of molecular mechanisms. Important

resistance mechanisms can be enzyme production, reduction of expression of external membrane proteins and efflux systems [103]. When a bacterial strain has started to develop resistance against an antimicrobial compound much larger doses are needed in order to get an antimicrobial effect, compared to when the compound is used against a susceptible strain. A resistant bacterial strains has the ability to grow at high concentration of the antimicrobial compound, higher than a non-resistant strain, independent of the duration of the treatment. The higher MIC-value a bacterial strain has, the more resistant it is to a certain antimicrobial drug. In cases where the bacterial strain show total insusceptibility against the antimicrobial it can be viewed as an extreme case of resistance [101].

2.6.2 Tolerance

When a bacteria is tolerant to an antimicrobial substance it has the ability to survive a transient exposure to a high concentration, higher than for a non-tolerant strain, without a change in the MIC, but needs to be exposed during a longer period of time to achieve the same rate of killing [104]. This is often achieved by slowing down an essential bacterial process [101]. To enable the same level of killing in a tolerant strain compared to a susceptible strain of bacteria, a longer exposure to the antimicrobial agent is required rather than a high concentration. The time needed to kill a bacterial strain is known as the Minimal Duration of Killing (MDK) [101].

A tolerant and a non-tolerant strain of bacteria can have the same MIC, therefore, other measurements should be used when evaluating tolerance in bacteria. One proposed approach is measurement of time-kill curves at different concentrations of antimicrobial substances [105]. These time kill-curves can be used to study and compare the MDK of bacterial strains treated with an antimicrobial compound, as can be seen in Figure 2.9.

2.6.3 Persistence

There are many mechanisms in bacteria that are thought to be adaptions for changes in the environment, where one of them are the bacterial persistence phenotype [106]. Persistence is the ability of a subpopulation of a bacterial culture to survive when exposed to high concentration of an antimicrobial agent, high enough concentration to kill the majority of the population. This phenomenon is called "bacterial persistance" and the surviving bacterial cells are referred to as persisters [101].

When bacteria cultures are exposed to an antimicrobial treatment, the whole culture is not completely killed, there is a small fraction of bacterial cells that "persist". This insensitivity in the persister cells to the drug is not inheritable, if these persisters cells are regrown the culture will be as sensitive to the drug as the parent culture from which the persiters were derived. The persisters do not remain in the persistent state indefinitely, they spontaneously switch back to the nonpersitent state where they regain the sensitivity to antimicrobial compounds [106].

Bacteria can, by suspending their growth, protect them self from different kind of stress, including antimicrobial compounds. When bacteria are in a nutrient-rich environment they have two strategies to choose between. Either they proliferate and risk death if stress conditions are encountered, or they suppress growth and by that have protected them selves from stress. The risk reducing strategy is referred to as persistence, where the majority of the population proliferate quickly but a small fraction significantly suppresses growth. The slow growing persiter cells could save the population from extinction when they are exposed to stress [106].

3
Materials and Methods

A complete list of the used materials including instruments as well as chemicals is presented in Appendix section A1 and A2.

3.1 Test organisms

All of the performed methods described below were limited to the use of the test organisms *P. aeruginosa* and *S. aureus*, both commonly found in wounds. *P. aeruginosa* ATCC® 15442 originally isolated from water and soil was used in the experiments involving planktonic cells, *P. aeruginosa* ATCC® 15692 originally isolated from wounds was used during the experiments involving biofilm. *S. aureus* ATCC® 6538 originally isolated from human lesion was used in the experiments on planktonic cells as well as experiments on biofilm.

3.2 Cultivation of bacteria

For preparation of the inoculums, 1-2 colonies of wanted bacterial strain, grown on a refrigerated agar plate were transferred to a 15 ml Falcon® tube containing 3 ml of Tryptic Soy Broth (TSB). The tube was then incubated at 35°C overnight (18-20 hours). This bacterial suspension is later referred to as the overnight culture.

3.3 Minimum Inhibitory Concentration

When the Minimum Inhibitory Concentration (MIC) of the antimicrobial compound against the bacteria was determined the following method was used.

The overnight culture was diluted in test media, which is a mixture of Mueller Hinton broth and Resazurin, to a concentration of 1-3 x 10^6 CFU/ml. For quantification of the bacterial suspension it was diluted to 10^2 and 10^1 CFU/mL and plated on Petrifilms™ which were incubated in 35°C for 24 hours (*S. aureus*) or 48 hours (*P. aeruginosa*) followed by enumeration. The test compound (the antimicrobial substance) was prepared by dilution in peptone water to a concentration of 200 mM. The dissolved compound was then diluted in test media to a concentration that was two times higher than the highest concentration that was tested. The test compound was diluted by twofold dilution, according to Table 3.1 using a 96- well plate and 8 replicates of each concentration were made. 200 µl of the test compound

was transferred to the wells in column 1 and 100 µl of test media was transferred to the wells in column 2-12. A multi-pipette was used to transfer 100 µl from column 1 to 2, the pipette tips were then refreshed and the solution was mixed by flushing up and down five times before 100 µl was transferred to the next column. The same procedure was repeated all the way to column 12 where the redundant 100 µl of the antimicrobial solution was removed. This resulted in 100 µl of antimicrobial solution in each well with a two-fold decrease in concentration from column 1 to 12. This procedure was followed by addition of 100 µl of the suspension of the bacteria (1-3 x 10^6 CFU/ml) to each well. Three replicates of both positive and negative control were used during this test. In the positive control 100 µl of test media and 100 µl of the bacterial suspension were used and in the negative control 100 µl of test media and 100 µl of the test compound were used. The plate was mixed for 30 seconds at 500 rpm and then incubated at 35°C for 24 hours. The concentration was considered as the Minimal Inhibitory Concentration (MIC) if 67%, which is the requirement-level that Mölnlycke uses in their tests, of the replicates affected by a specific concentration showed no growth (purple color) on the well plates after incubation.

Table 3.1: Concentrations of Chlorhexidine digluconate used in the MIC-tests

Bacteria	*P. aeruginosa*	*S. aureus*
Column 1	1.400 mM	0.175 mM
Column 2	0.700 mM	0.088 mM
Column 3	0.350 mM	0.044 mM
Column 4	0.175 mM	0.022 mM
Column 5	0.088 mM	0.011 mM
Column 6	0.044 mM	0.005 mM
Column 7	0.022 mM	0.002 mM
Column 8	0.011 mM	0.001 mM
Column 9	0.005 mM	0.0005 mM
Column 10	0.002 mM	0.00025 mM
Column 11	0.001 mM	0.000125 mM
Column 12	0.0005 mM	0.0000625 mM

3.4 Minimum Biocidal Concentration

The Minimal Biocidal Concentration (MBC) of the antimicrobial compound against the bacteria was determined by the following method.

The overnight culture was diluted in simulated wound fluid (SWF) to 4 x 10^6 CFU/ml. For quantification of the bacterial suspension the culture was diluted to 10^2 and 10^1 CFU/mL and then cultured on Petrifilm™ for 24 hours for enumeration. The test compound was prepared by dilution in peptone water to a concentration of 200 mM. The dissolved compound was then diluted in SWF to a concentration that was two times higher than the highest concentration that was

tested. The test compound was diluted by twofold dilution using a 96- well plate and 8 replicates of each concentration were made. The concentrations can be seen in table 3.2. 200 µl of the test compound was transferred to the wells in column 1 and 100 µl of SWF was transferred to the wells in column 2-12. A multi-pipette was used to transfer 100 µl from column 1 to 2, after that the pipette tips were refreshed and the solution in the wells was mixed by flushing up and down five times before 100 µl was transferred to the the wells in the next column. The same procedure was repeated all the way to column 12 where the redundant 100 µl of the antimicrobial solution was removed. This resulted in 100 µl of antimicrobial solution in each well with decreasing concentration from column 1 to 12. This followed by addition of 100 µl of the bacterial suspension to each well. The plate was mixed for one minute at 500 rpm and then incubated at 35°C for 24 hours together with the controls. Three replicates of both positive control and negative control were used. For the positive control 100 µl of SWF and 100 µl of the bacterial suspension was used and for the negative control 100 µl of SWF and 100 µl of the test compound was used. After the incubation, 5 µl from each well was cultured at Tryptic soy agar plates. The plates were dried in a laminar air flow cabinet and then incubated at 35°C for 24 hours (*P. aeruginosa*) or 48 hours (*S. aureus*). The concentration was considered as the Minimal Biocidal Concentration (MBC) if 67% of the replicates affected by a specific concentration showed no growth on the Tryptic soy agar plates after incubation.

Table 3.2: Concentrations of Chlorhexidine digluconate used in the MBC-tests

Bacteria	*P. aeruginosa*	*S. aureus*
Column 1	1.400 mM	1.400 mM
Column 2	0.700 mM	0.700 mM
Column 3	0.350 mM	0.350 mM
Column 4	0.175 mM	0.175 mM
Column 5	0.088 mM	0.088 mM
Column 6	0.044 mM	0.044 mM
Column 7	0.022 mM	0.022 mM
Column 8	0.011 mM	0.011 mM
Column 9	0.005 mM	0.005 mM
Column 10	0.002 mM	0.002 mM
Column 11	0.001 mM	0.001 mM
Column 12	0.0005 mM	0.0005 mM

3.5 Creation of biofilms

The overnight culture was diluted in SWF to a bacterial concentration of 10^6 CFU/ml. 1.5 ml of the bacterial suspension was added into each well of a 24 well plate. A piece of Mesoft®, which is a non-woven swab, with a diameter of 12 mm was transferred into each well containing bacteria suspension. The plate was then incubated at 35°C with an agitation of 100 rpm for 24 hours (*P. aeruginosa*) or 48 hours (*S. aureus*).

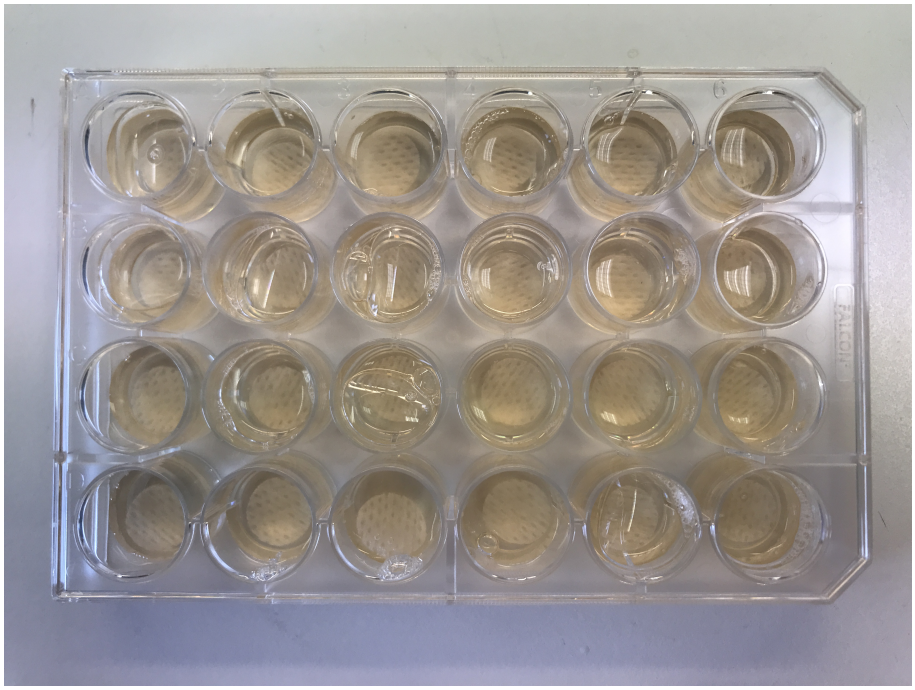


Figure 3.1: Creation of biofilms. A 24 well plate where each well contain 1 piece of Mesoft® (12 mm Ø) and 1.5 ml of bacterial suspension

3.6 Minimal Biofilm Eradication Concentration

A piece of biofilm crated as described in section 3.5 was transferred into each well of a 24 well plate. 1.5 ml of the antimicrobial substance diluted in SWF to different concentration was added to each well. The concentrations used can be seen in Table 3.3. The plate was then incubated at 35°C with an agitation of 100 rpm for 24 hours. Each biofilm was then transferred to a 50 ml Falcon® tube containing 10 ml of Dextran sulphate. The tubes were shaken for 10 minutes at 950 rpm. 1 ml from each tube was cultured on a Petrifilm™ to distinguish growth. The films were incubated for 24 hours (*S. aureus*) or 48 hours (*P. aeruginosa*). A number of six replicates per concentration were used in this test. The concentration was considered as the Minimal Biofilm Eradication Concentration (MBEC) if 67% of the replicates affected by a specific concentration showed no growth on the Petrifilms™.

Table 3.3: Concentrations of Chlorhexidine digluconate used in the MBEC-tests

Bacteria	*P. aeruginosa*	*S. aureus*
Concentration 1	1.792 mM	1.792 mM
Concentration 2	0.896 mM	0.896 mM
Concentration 3	0.448 mM	0.448 mM
Concentration 4	0.224 mM	0.224 mM
Concentration 5	0.112 mM	0.112 mM
Concentration 6	0.056 mM	0.056 mM
Concentration 7	0.028 mM	0.028 mM

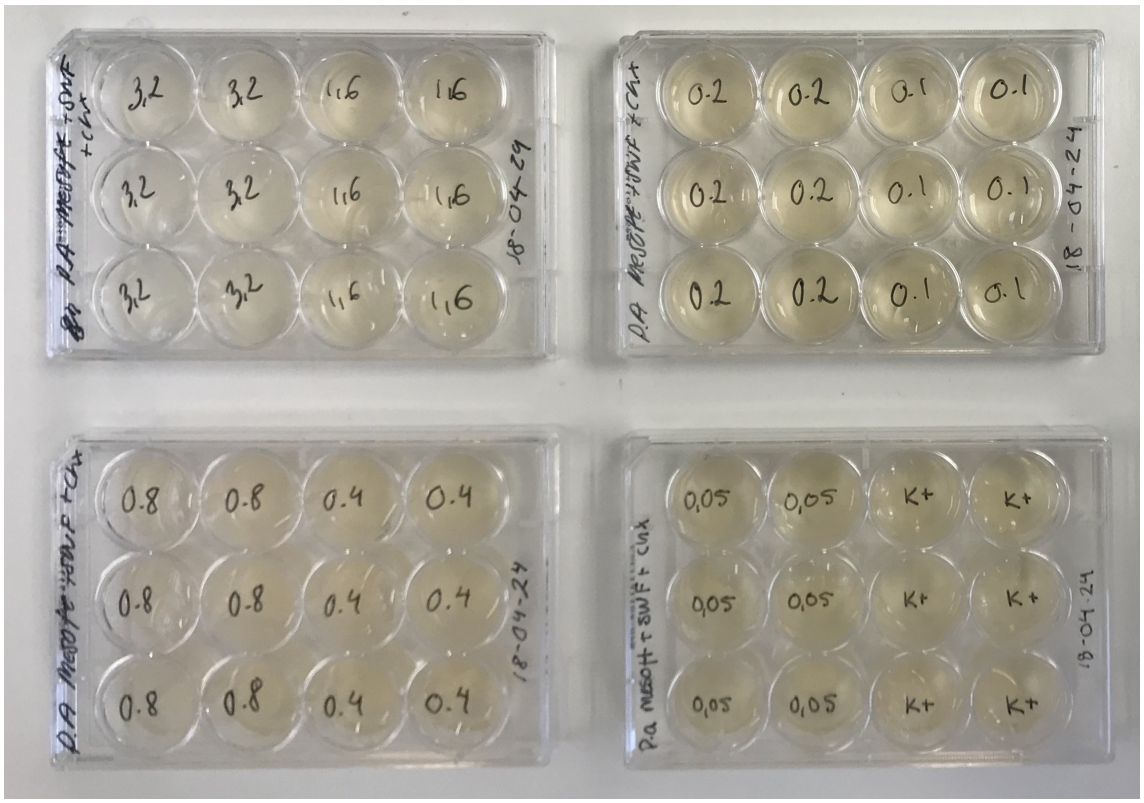


Figure 3.2: MBEC-test: A 24 well plate where each well contain 1 piece of Mesoft® (12 mm Ø) that has been incubated over night in bacterial suspension and have then been placed in 1.5 ml of antimicrobial solution with various concentrations

3.7 Time-kill study on planktonic cells

Time-kill studies of planktonic bacteria in both a static and a dynamic system were performed. These systems will be described in more detail below.

3.7.1 Static system

In the static system, four antimicrobial concentrations derived from the previous MIC/MBC studies for *P. aeruginosa* and five concentrations derived from the studies with *S. aureus* were used. The lowest concentration used was below the established MIC-values and the highest a bit above the established MBC-values, which later on is referred to as the superMBC-value. Exact values can be seen in Table 3.4. The antimicrobial compound where diluted in SWF to the different concentrations and 10 ml where added to an E-flask. Bacterial suspension where then added to each flask to achieve a concentration of 10^6 CFU/ml of the test organisms *P. aeruginosa* or *S. aureus*. The suspensions were then placed in an incubator at 35°C an at an agitation of 100 rpm for 48 hours. Samples of a volume of 0.125 ml were taken from each flask at 1, 2, 4, 6, 24 and 48 hours after addition of the antimicrobial substance, and added into 1.125 ml of Dextran sulphate, which deactivated the Chlorhexidine digluconate. The samples were then tenfold diluted in 0.1% pepton water up to eight times using Microlab STAR pipetting system. 1 ml of samples at different dilutions were then grown on Petrifilms™ for 24 hours (*S. aureus*) or 48 hours (*P. aeruginosa*) in an incubator at 35°C. After incubation the colonies grown on the Petrifilms™ were counted to investigate how the bacteria was affected by the used antimicrobial substance. All concentrations were tested in three replicates and a control group which wasn't exposed any antimicrobial substance was also included.

Table 3.4: Concentrations of Chlorhexidine digluconate used in the static time-kill studies on planktonic bacteria

Bacteria	S1	S2	S3	S4	S5
P. aeruginosa	0.022 mM	0.044 mM	0.088 mM	0.112 mM	-
S. aureus	0.001 mM	0.002 mM	0.022 mM	0.044 mM	0.056 mM

3.7.2 Dynamic system

The dynamic time-kill studies were performed using four different groups where different concentrations of antimicrobial substance were added to continuously with different gradients to reach the superMBC-value, which is a bit above the determined MBC-value, in 1, 3, 6 and 8 hours. This was done by using four different start concentrations, calculated according to Equation 3.1. These concentrations were pumped into different E-flask, each from start containing 10 ml SWF and a concentration of 10^6 CFU/ml of the examined bacteria. The setup for this system can be seen in Figure 3.3 and in Figure 3.4. The pump operated with a flow rate of 0.7 µl/min, the start concentrations of the used antimicrobial solutions can be seen in Table 3.5.

$$C_{start} = \frac{(V + (Q \times t \times 60)) \times MBC}{(Q \times t \times 60)} \tag{3.1}$$

Equation 3.1: Equation for calculation of start concentrations [mM] of antimicrobial substance to be used to reach the MBC-value in the bacteria solutions at different times. V = Sample volume [ml], Q = flow rate [ml/min], t = in which time the MBC-value should be reached [h], MBC = determined MBC-value for the test organism toward the used antimicrobial [mM]

The concentration profiles of the added antimicrobial substance can be seen in Figure 3.5. This concentration-time curves have also been used to calculate the AUC of Chlorhexidine for the PK and PD profiles. Once the bacteria-containing reservoirs had reached the MBC-value, the tubes pumping in the substance were removed and the antimicrobial concentration in the E-flasks were thereby held constant at the MBC-concentration throughout the experiment. In the same way as in the static system, samples in the volume of 0.125 ml were taken at 1, 2, 4, 6, 24 and 48 hours after start and added to 1.125 ml of Dextran sulphate, which was used to deactivate the Chlorhexidine digluconate. The samples were then tenfold diluted in 0.1% peptone water up to eight times using Microlab STAR pipetting system. 1 ml of samples at different dilutions were then grown on Petrifilms^{TM} and incubated at 35°C for 24 hours (*S. aureus*) or 48 hours (*P. aeruginosa*) for later quantification of the bacterial concentration. All concentrations were tested in three replicates and a control group which wasn't exposed any antimicrobial substance was also included.

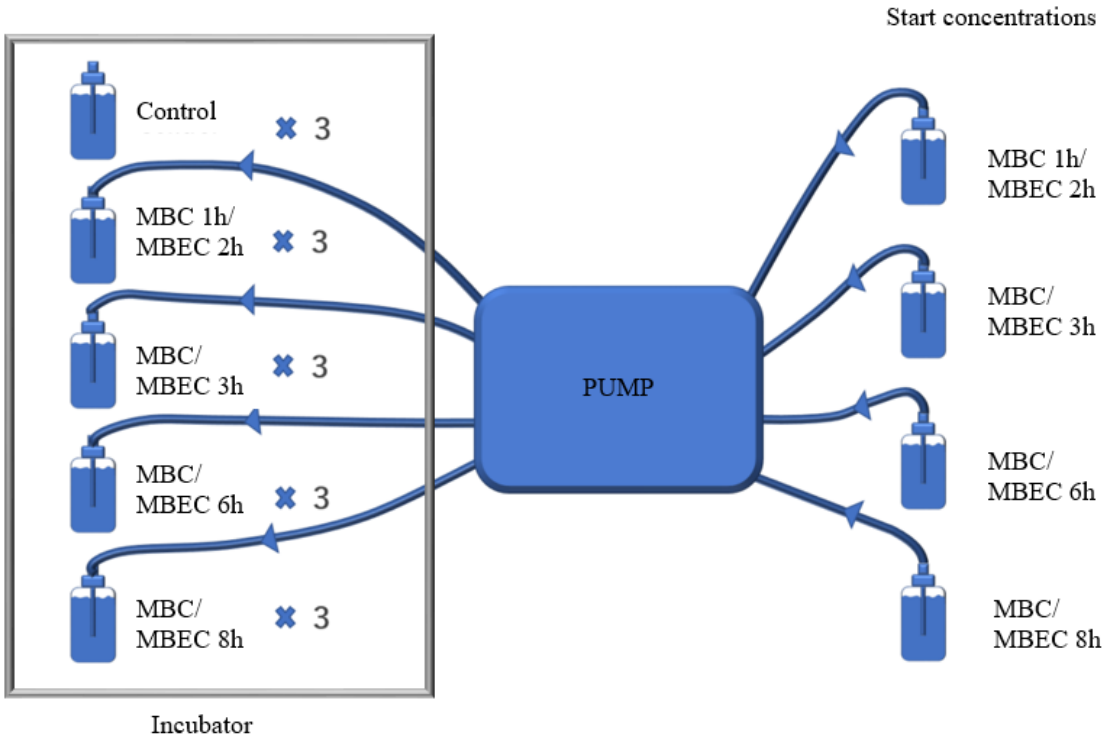


Figure 3.3: Set up for dynamic time-kill studies

Figure 3.4: Picture of the set up used in the dynamic time-kill studies

Table 3.5: Start concentrations of Chlorhexidine digluconate used in the dynamic time-kill studies on planktonic bacteria, calculated using Equation 3.1

Bacteria	MBC 1h (D1)	MBC 3h (D2)	MBC 6h (D3)	MBC 8h (D4)
P. aeruginosa	26.78 mM	9.00 mM	4.56 mM	3.45 mM
S. aureus	13.39 mM	4.50 mM	2.28 mM	1.72 mM

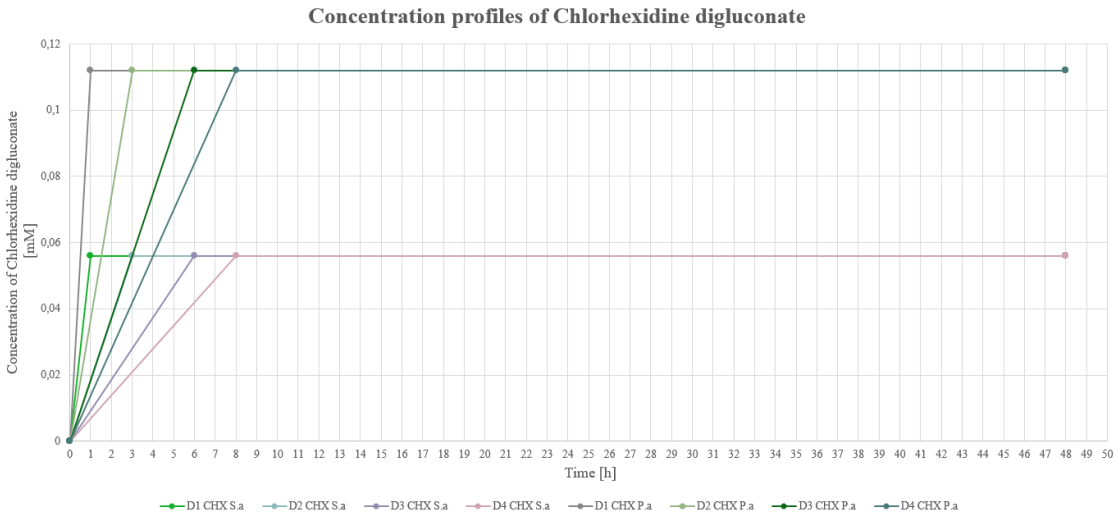


Figure 3.5: Concentration profiles of Chlorhexidine digluconate when being used against planktonic *P. aeruginosa* and *S. aureus*

3.8 Time-kill study on biofilms

Time-kill studies on biofilms were performed using both a static and a dynamic system which are described below.

3.8.1 Static system

Five biofilms created as described in section 3.5 were transferred to a E-flask containing 15 ml of antimicrobial substance diluted in SWF. Each E-flask contained different concentration of the used antimicrobial substance. The concentrations used can be seen in Table 3.6. The flasks were incubated at 35°C with an agitation of 100 rpm. Samples were taken after 2, 4, 6, 24 and 48 hours. A number of three replicates were used for all concentrations. When a test was performed, a biofilm was taken from each flask and transferred to a 50 ml Falcon® tube containing 10 ml of Dextran sulphate. The biofilm was then shaken in the Dextran sulphate for 10 minutes at 950 rpm. 0.125 ml of the suspension was transferred from each tube and added to 1.125 ml of 0.1% peptone water in a 96 well plate. The bacterial suspension was then diluted tenfold up to eight times using a Microlab STAR pipetting system. 1 ml of different dilutions were then cultured on Petrifilms™ which were incubated at 35°C for 24 hours (*S. aureus*) or 48 hours (*P. aeruginosa*) for later quantification of the bacterial concentration. All concentrations were tested in three replicates and a control group which wasn't exposed to any antimicrobial substance was also included.

Table 3.6: Concentrations of Chlorhexidine digluconate used in the static time-kill studies with bacteria in biofilm

Bacteria	S1	S2	S3	S4
P. aeruginosa	0.280 mM	0.560 mM	0.896 mM	1.120 mM
S. aureus	0.112 mM	0.224 mM	0.448 mM	0.560 mM

3.8.2 Dynamic system

Dynamic time-kill studies were performed on biofilms where the established MBEC-value were reached at four different times, after 2, 3, 6 and 8 hours. The concentration profiles can be seen in Figure 3.6. Similar to the static system five biofilms were added to each E-flask, all containing 15 ml of SWF. The flasks were then incubated at 35°C with an agitation of 100 rpm. Samples were taken after 2, 4, 6, 24 and 48 hours in the same way as described in section 3.8.1. To reach the MBEC-value at the different times four different concentrations of antimicrobial substance were added continuously to the E-flask containing the biofilms with a flow rate of 0.7 µl/min. These concentrations were calculated according to Equation 3.2. The calculated start concentrations can be seen in Table 3.7. The setup is the same as the one used for planktonic cells and can be seen in Figure 3.3.

$$C_{start} = \frac{(V + (Q \times t \times 60)) \times MBEC}{(Q \times t \times 60)} \tag{3.2}$$

Equation 3.2: Equation for calculation of start concentrations [mM] of antimicrobial substance to be used to reach the MBEC-value in the bacteria solutions at different times. V = Sample volume [ml], Q = flow rate [ml/min], t = in which time the MBEC-value should be reached [h], MBEC = determined MBEC-value for the test organism toward the used antimicrobial [mM]

Table 3.7: Start concentrations of Chlorhexidine digluconate used in the dynamic time-kill studies on bacteria in biofilm, calculated using Equation 3.2

Bacteria	MBEC 2h (D1)	MBEC 3h (D2)	MBEC 6h (D3)	MBEC 8h (D4)
P. aeruginosa	201.04 mM	134.40 mM	67.76 mM	50.96 mM
S. aureus	133.84 mM	89.60 mM	44.80 mM	44.80 mM

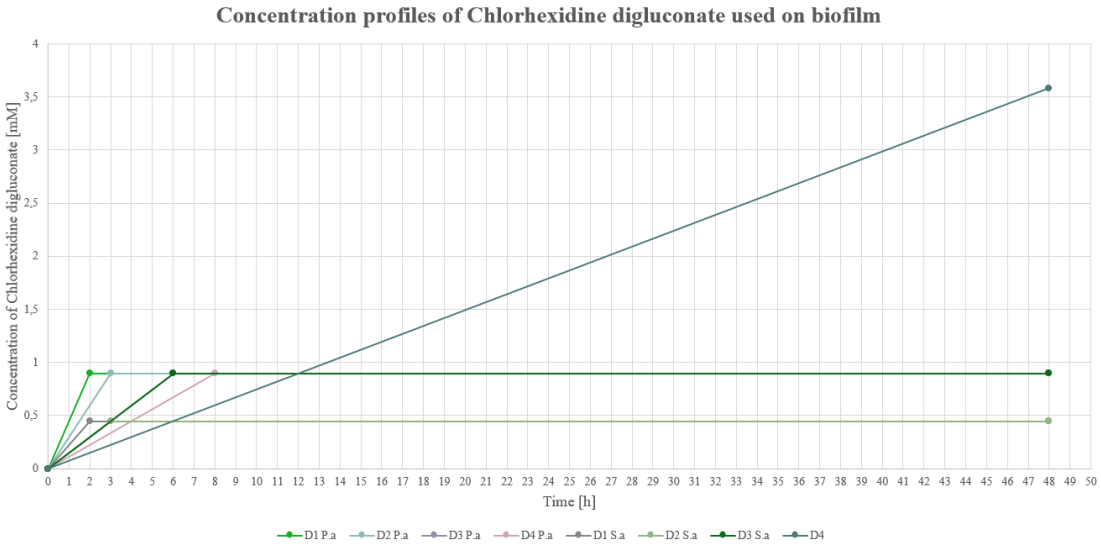


Figure 3.6: Concentration profiles of Chlorhexidine digluconate when being used against *P. aeruginosa* and *S. aureus* biofilms

4

Results

The established results from the performed experiments described in chapter 3 can be seen in the following sections below.

4.1 Minimum Inhibitory Concentration

The established results from the performed MIC-tests described in section 3.3 are listed in Table 4.1 below. The concentrations of antimicrobial substance used in the different tests can be seen in table 3.1 where column 1 is referred to as the column to the left in Figure 4.1 a) and b), followed by column 2, 3 etc. Resazurin was used as the color indicator to visualize the MIC. The pink color indicates growth of bacteria whereas the blue/purple colour indicates that growth is inhibited. Seen below in Figure 4.1 a) the MIC of Chlorhexidine digluconate against *S. aureus* is column 7 which is a concentration of 0.002 mM. In Figure 4.1 b) the MIC of Chlorhexidine digluconate against *P. aeruginosa* is column 6 which is a concentration of 0.044 mM.

The established MIC-values are valid for the bacterial concentration established from the counted CFU/ml that were cultured on Petrifilms^{TM} when the MIC-tests were performed. The bacterial concentration calculated in CFU/ml established from bacterial growth on Petrifilms^{TM} can be seen in Table 4.2 below. These bacterial concentrations were then multiplied by a dilution factor of 0.5 resulting in the concentrations for which the calculated MIC-values seen in Table 4.1 are valid.

Table 4.1: Determined MIC-values from the performed experiments with planktonic *P. aeruginosa* and *S. aureus* treated with Chlorhexidine digluconate

Bacteria	Conc. of Chlorhexidine digluconate
P. aeruginosa	0.044 mM
S. aureus	0.002 mM

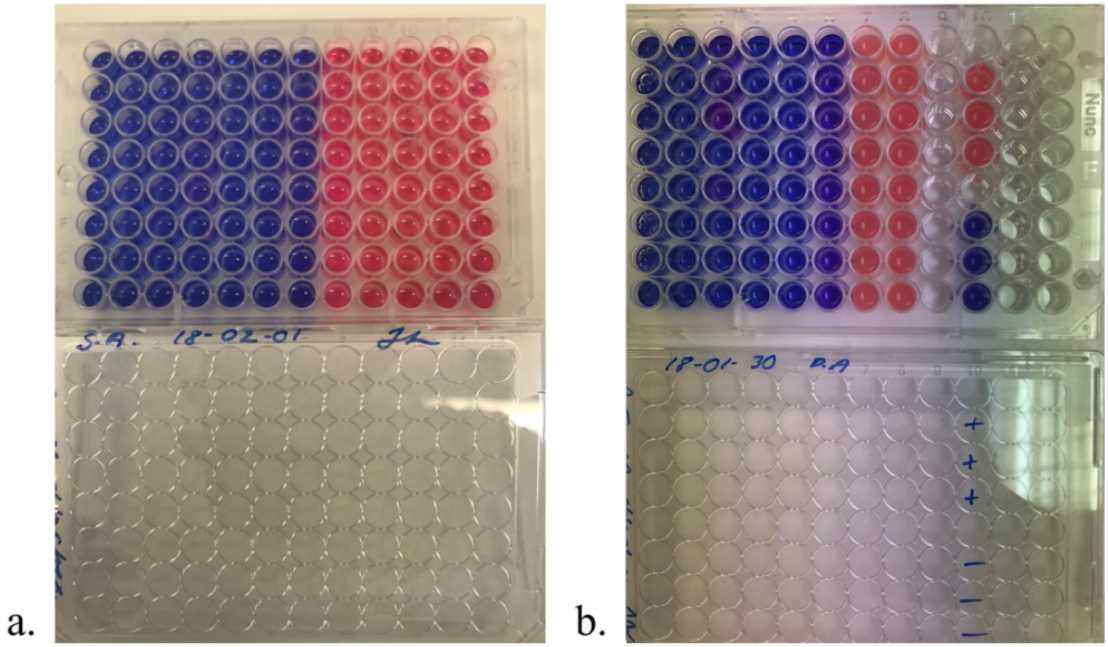


Figure 4.1: Pictures from the performed MIC-tests where in a) Chlorhexidine digluconate was tested against *S. aureus* and in b) Chlorhexidine digluconate was tested against *P. aeruginosa*

Table 4.2: CFU/ml of planktonic *P. aeruginosa* and *S. aureus* in bacterial suspension used in the MIC-tests

Bacteria	Bacterial concentration
P. aeruginosa	2.49 $\times 10^6$ CFU/ ml
S. aureus	2.59 $\times 10^6$ CFU/ ml

4.2 Minimal Biocidal Concentration

The established results from the performed MBC-tests described in section 3.4 can be seen in Table 4.3 below. The concentrations of antimicrobial substance, seen in Table 3.2, were used against *P. aeruginosa* and *S. aureus*. Cultivation of *S. aureus* on agar plate can be seen in Figure 4.2 b) where the 8 replicates where grown vertical with decreasing concentration from the left to right on the agar plate, where the highest concentration is referred to as column 1 in Table 3.2. There are no growth when using concentration 1-6 making concentration 6 the MBC. At concentration 7 there are no growth in 4 out of the 8 replicates which is below 67% of the replicates which is considered as the limit for a concentration to be considered as the MBC.

The established MBC-values are valid for the bacterial concentration established from the counted CFU/ml that were cultured on Petrifilms^{TM} when the MBC-tests were performed. The bacterial concentration calculated in CFU/ml established from

bacterial growth on Petrifilms^{TM} can be seen in Table 4.4 below. These bacterial concentrations were then multiplied by a dilution factor of 0.5 resulting in the concentrations for which the calculated MBC-values seen in Table 4.3 are valid.

Table 4.3: Determined MBC-values from the performed experiments with planktonic *P. aeruginosa* and *S. aureus* treated with Chlorhexidine digluconate

Bacteria	Conc. of Chlorhexidine digluconate
P. aeruginosa	0.088 mM
S. aureus	0.044 mM

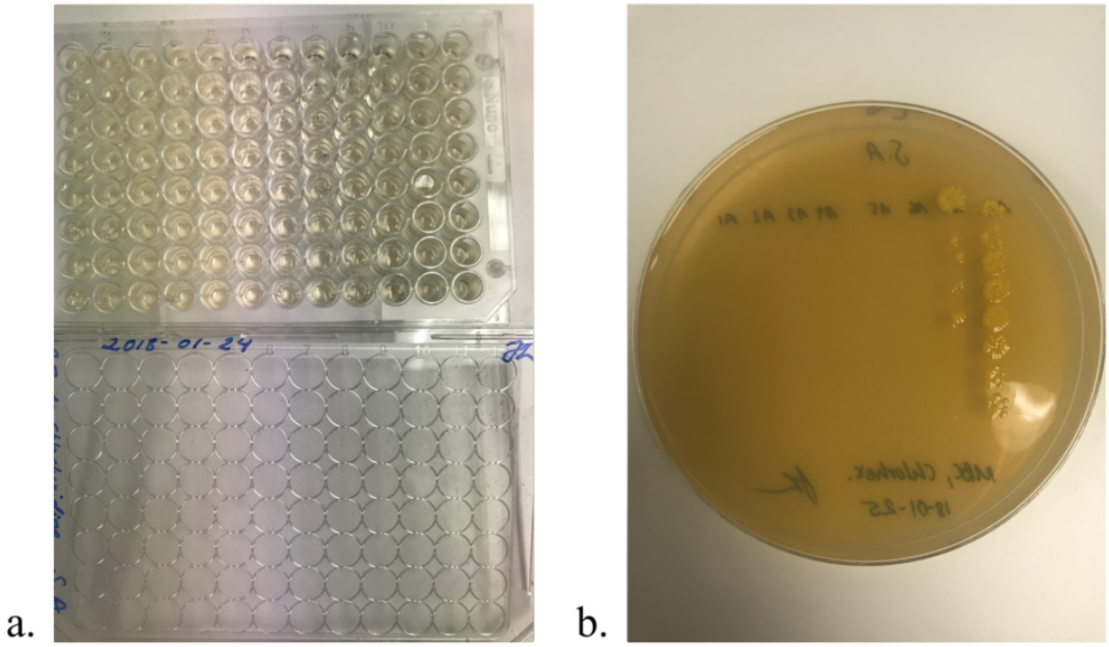


Figure 4.2: Pictures from the performed MBC-tests where Chlorhexidine digluconate was tested against *S. aureus*. It can be seen in a) the cultivation of the bacteria in different concentrations of the antimicrobial in a 96 well plate and in b) the bacteria-antimicrobial solutions can be seen cultivated on an agar plate

Table 4.4: CFU/ml of planktonic *P. aeruginosa* and *S. aureus* in bacterial suspension used in the MBC-tests

Bacteria	Bacterial concentration
P. aeruginosa	3.9 $\times 10^6$ CFU/ ml
S. aureus	4.6 $\times 10^6$ CFU/ ml

4.3 Minimal Biofilm Eradication Concentration

The established results from the performed MBEC-tests described in section 3.6 can be seen listed in Table 4.6 below. The concentrations of antimicrobial substance, seen in Table 3.3, were used against *P. aeruginosa* and *S. aureus*.

The established MBEC-values are valid for the bacterial concentration established from the counted CFU/ml that were cultured on Petrifilm™ when the MBEC-tests were performed. The bacterial concentration calculated in CFU/ml established from bacterial growth on Petrifilm™ can be seen in Table 4.5 below.

Table 4.5: CFU/ml of bacterial suspension from biofilms of *P. aeruginosa* and *S. aureus* used in the MBEC-tests

Bacteria	Bacterial concentration
P. aeruginosa	1.47 $\times 10^9$ CFU/ ml
S. aureus	3.4 $\times 10^8$ CFU/ ml

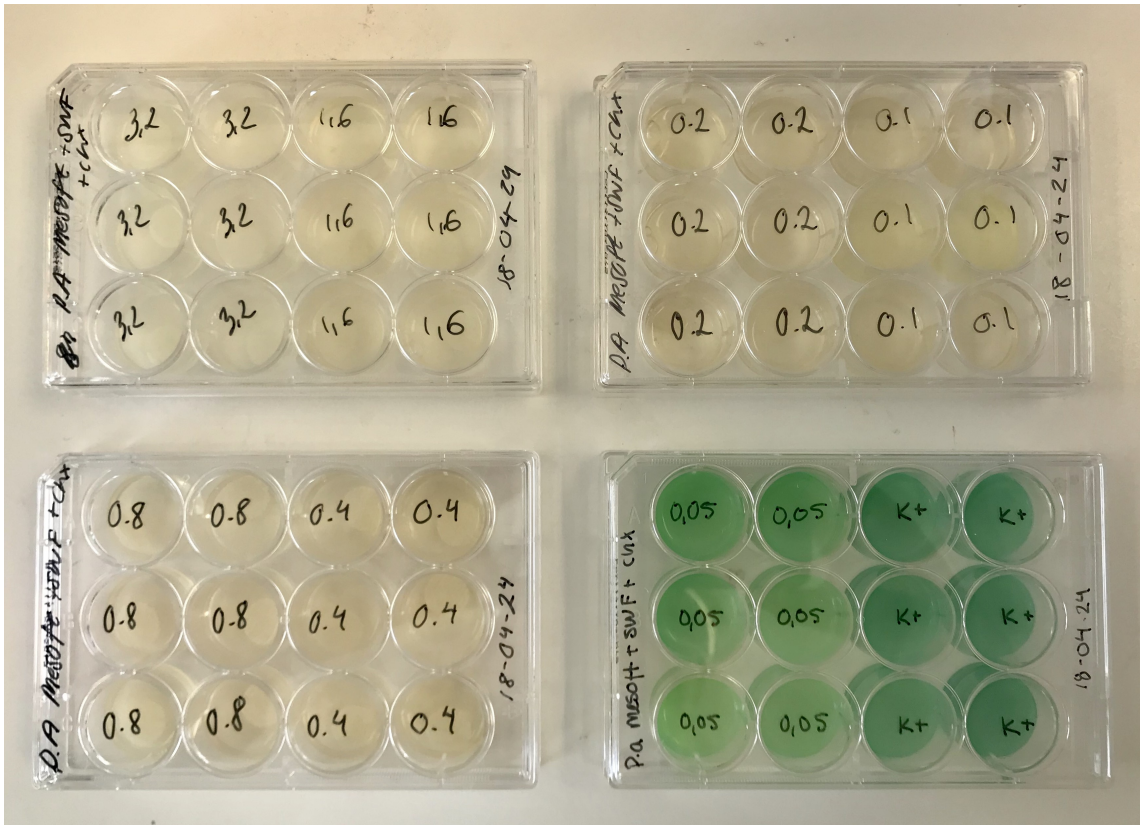


Figure 4.3: Picture of a MBEC-test where biofilms of *P. aeruginosa* are exposed to various concentrations of Chlorhexidine digluconate before the biofilm suspension were cultured on Petrifilms™

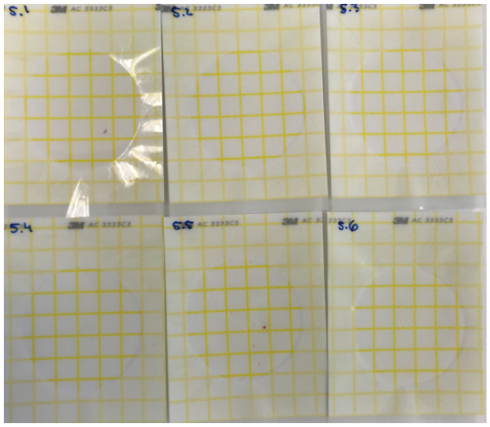
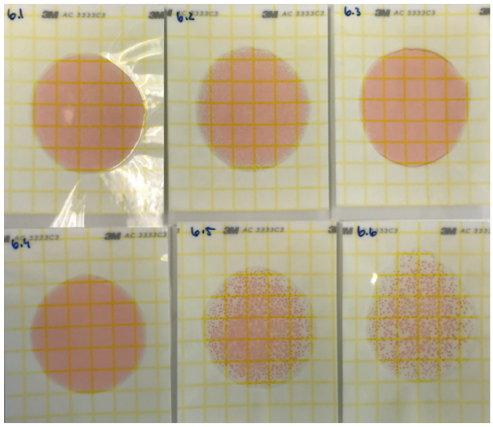
a.  b. 

Figure 4.4: Pictures of bacterial suspension from biofilms of *P. aeruginosa* cultured on Petrifilms^{TM}. It can be seen in a) No growth on four replicates and little growth on two replicates when being cultured in concentration 5 of Chlorhexidine digluconate, which can be seen in Table 3.3. This makes concentration 5 the MBEC-value for biofilms of *P. aeruginosa*. It can be seen in b) that there is much growth on the Petrifilms^{TM} when being exposed to a lower concentration of Chlorhexidine digluconate, concentration 6, seen in Table 3.3

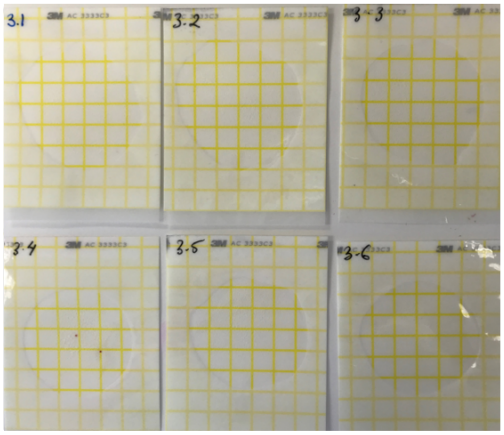
a.  b. 

Figure 4.5: Pictures of bacterial suspension from biofilms of *S. aureus* cultured on Petrifilms^{TM}. It can be seen in a) No growth on five replicates and little growth on one replicate when being cultured in concentration 3 of Chlorhexidine digluconate, which can be seen in Table 3.3. This makes concentration 3 the MBEC-value for biofilms of *S. aureus*. It can be seen in b) that there is growth on three of the Petrifilms^{TM} when being exposed to a lower concentration of Chlorhexidine digluconate, concentration 4, seen in Table 3.3

Table 4.6: Determined MBEC-values from the performed experiments with biofilms of *P. aeruginosa* and *S. aureus* treated with Chlorhexidine digluconate

Bacteria	Conc. of Chlorhexidine digluconate
P. aeruginosa	0.896 mM
S. aureus	0.448 mM

4.4 PK and PD ratios

In Table 4.7, Table 4.8 and Table 4.9 below the calculated ratios of C_{max}/MIC and AUC/MIC can be seen for Chlorhexidine digluconate against the two different bacterial species. The C_{max}-values are the ones earlier referred to as superMBC for each bacteria and the MIC values are the values seen in Table 4.1 in section 4.1. The AUC values are calculated from the curves in the concentration profiles in Figure 3.5.

Table 4.7: C_{max}/MIC ratios calculated for *P. aeruginosa* and *S. aureus* treated with Chlorhexidine digluconate

Bacteria	C_{max}/MIC
P. aeruginosa	2.56
S. aureus	20.41

Table 4.8: AUC/MIC ratios calculated for *P. aeruginosa* and *S. aureus* treated with Chlorhexidine digluconate in dynamic studies

Bacteria	$\frac{AUC(D1)}{MIC}$	$\frac{AUC(D2)}{MIC}$	$\frac{AUC(D3)}{MIC}$	$\frac{AUC(D4)}{MIC}$
P. aeruginosa	121.79	119.23	115.38	112.82
S. aureus	969.39	958.98	918.37	897.96

Table 4.9: AUC/MIC ratios calculated for *P. aeruginosa* and *S. aureus* treated with Chlorhexidine digluconate in static studies

Bacteria	$\frac{AUC(S1)}{MIC}$	$\frac{AUC(S2)}{MIC}$	$\frac{AUC(S3)}{MIC}$	$\frac{AUC(S4)}{MIC}$	$\frac{AUC(S5)}{MIC}$
P. aeruginosa	24.62	49.23	98.46	123.08	-
S. aureus	24.49	48.98	391.84	685.71	979.59

In Figure 4.6 and Figure 4.7 below, the concentration-time curves of Clorhexidine digluconate when used against the two bacteria are shown. The dotted line in each graph represents the MIC value. It can be seen that the T>MIC are much longer in the experiments with *S. aureus* compared to the experimets with *P. aeruginosa*. In the experiments with *S. aureus* the MIC is reached within the first hour for all of the groups, even the one that reached MBC in 8 hours. In the experiments with

P. aeruginosa it is only the group that reached MBC in 1 hour that also reached the MIC within the first hour, and the group that reached MBC in 8 hours hadn't reached it until after three hours.

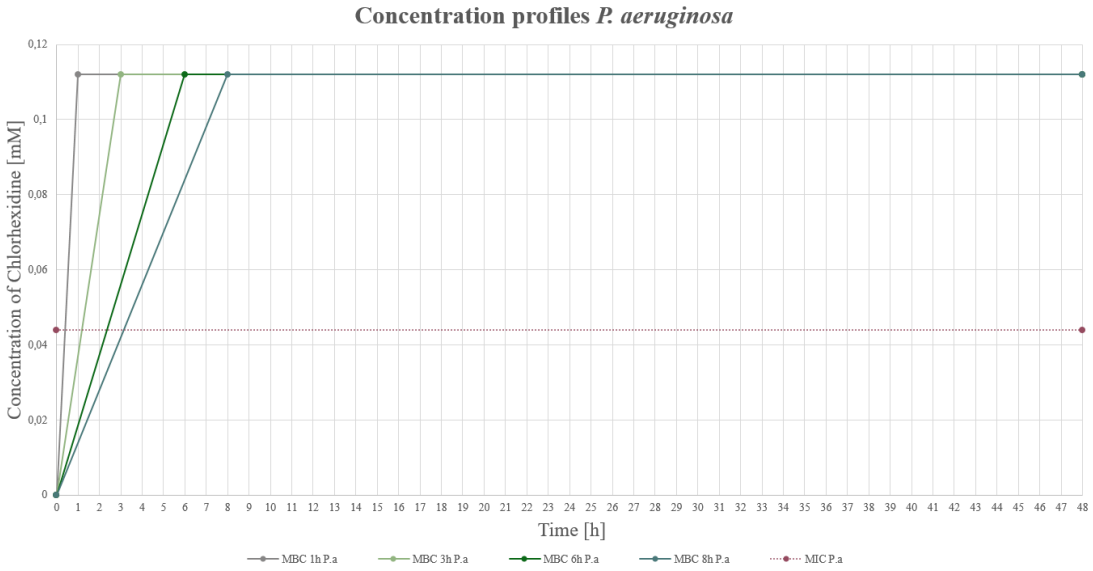


Figure 4.6: Concentration-time curves for dynamic time-kill studies with *P. aeruginosa* where T>MIC is indicated

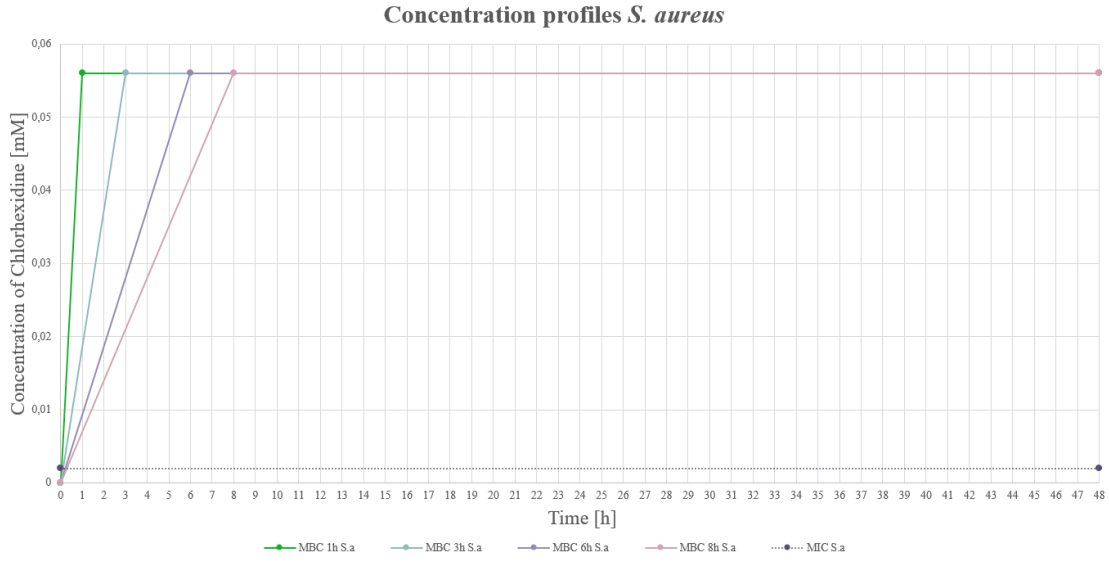


Figure 4.7: Concentration-time curves for dynamic time-kill studies with *S. aureus* where T>MIC is indicated

4.5 Dynamic time-kill studies on planktonic cells

The established results from the performed dynamic time-kill studies on planktonic *P. aeruginosa* and *S. aureus* described in section 3.7.2 can be seen in Figure 4.8 and Figure 4.9. The concentration of the used antimicrobial substance was increased over time to reach the superMBC-concentration at different times, the concentration profile of Chlorhexidine digluconate can be seen in Figure 3.5. This was performed using a set-up that can be seen in Figure 3.3. All concenrtaions were tested in three replicates and the graphs presenting the results below represents a mean value of those. The graphs are showing the log_{10} value of the counted CFU/ml cultured on Petrifilms^{TM} as a function of time. The limit of detection was 10^1. All groups started with a bacterial concentration of approximately 10^6 CFU/ml and the error bars at each test point are showing a confidence interval of 95%. Changes in volume due to taking samples and inflow of antimicrobial substance were neglected.

4.5.1 Chlorhexidine digluconate against *P. aeruginosa*

Results from the dynamic time-kill studies testing Chlorhexidine digluconate against *P. aeruginosa* are shown in Figure 4.8 below. The calculated superMBC of 0.112 mM were roughly reached in 1, 3, 6 and 8 hours and are referred to as in the graph D1, D2, D3 and D4 respectively. The control group was not exposed to any antimicrobial substance and the MIC-group was exposed to the established minimum inhibitory concentration of 0.044 mM, presented in section 4.1.

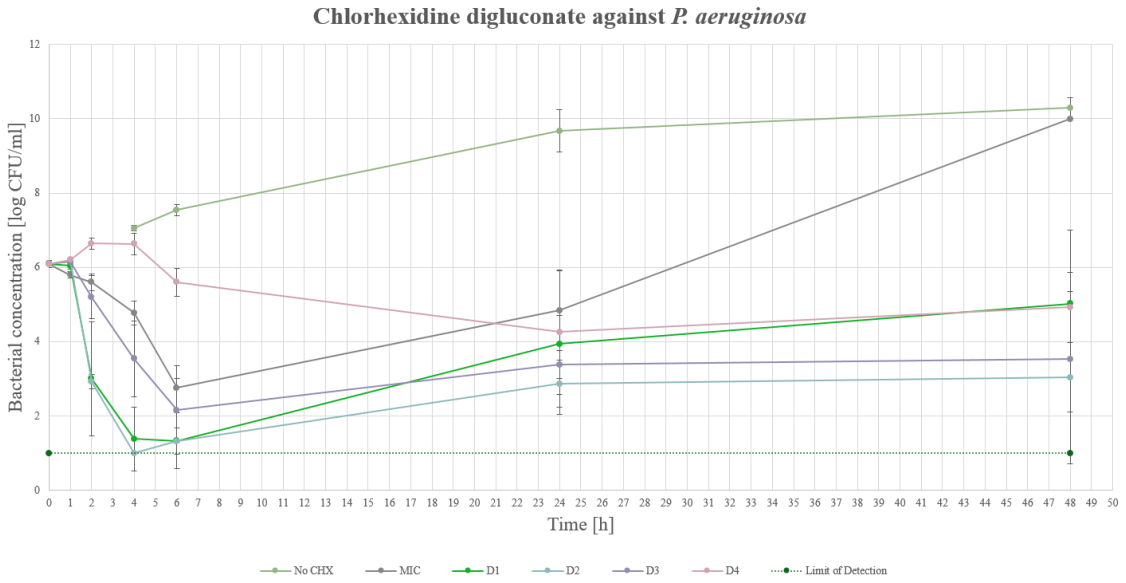


Figure 4.8: CFU counts of *P. aeruginosa* over 48 hours when increasing the concentration of Chlorhexidine digluconate over time and reaching the superMBC-value of 0.112 mM after 1, 3, 6 and 8 hours

A decrease in bacterial concentration can be seen in the three groups D1, D2 and D3 up to 6 hours after start. In the group D4 an increase of bacterial concentration

can be seen up to 4 hours followed by a decrease in the bacterial concentration up to 24 hours. The D4 group grew approximately in the same speed as the control group up to 4 hours but started to decrease at that time. In the two groups D1 and D2 the change in bacterial concentration followed approximately the same pattern with a reduction of approximately 5 log_{10}-units within the first 6 hours. The D3 group showed a lower rate of killing within this time and the reduction of bacteria was approximately the same as for the MIC group. In the three groups where the amount of bacteria decreased in the first hours an increase can be seen after 6 hours. The final reduction of bacteria in the four different groups is 1-3 log_{10}-units after 48 hours. The control group and the MIC group had increased to approximately 10^{10} CFU/ml after 48 hours.

4.5.2 Chlorhexidine digluconate against *S. aureus*

Results from the dynamic time-kill studies testing Chlorhexidine digluconate against *S. aureus* are shown in Figure 4.9 below. The calculated superMBC of 0.056 mM were roughly reached in 1, 3, 6 and 8 hours and are referred to as in the graph D1, D2, D3 and D4 respectively. The control group was not exposed to any antimicrobial substance and the MIC-group was exposed to the established minimum inhibitory concentration of 0.002 mM, presented in section 4.1.

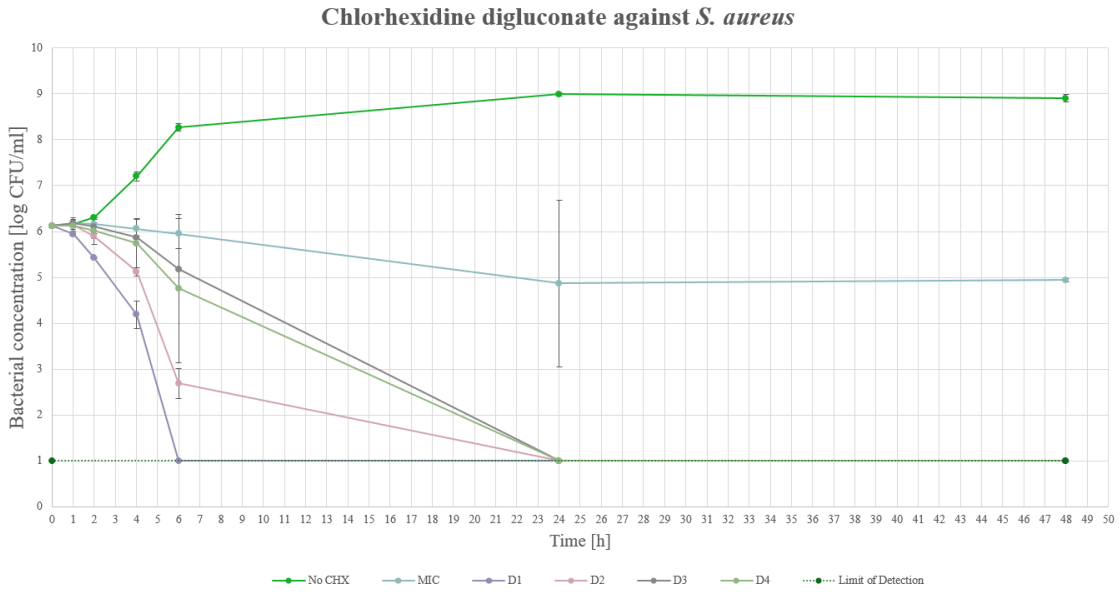


Figure 4.9: CFU counts of *S. aureus* over 48 hours when increasing the concentration of Chlorhexidine digluconate over time and reaching the superMBC-value of 0.056 mM after 1, 3, 6 and 8 hours

From figure 4.9 it can be seen that the used superMBC-concentration of Chlorhexidine digluconate of 0.056 mM against *S. aureus* managed to lower the amount of bacteria below the detection limit of 1 log_{10}-unit independent of the time the superMBC-concentration was reached. At the test point at 6 hours the samples with bacteria that had reached the superMBC-concentration in 1 hour were below

the limit of detection. After 24 hours the samples that had reached the superMBC-value after 3, 6 and 8 hours were also below the limit of detection. None of the samples showed any indication of growing back after 48 hours.

4.6 Static time-kill studies on planktonic cells

The results from the performed static time-kill studies on planktonic *P. aeruginosa* and *S. aureus* described in section 3.7.1 can be seen below. All concentrations were tested in three replicates and the graphs presenting the results below represents a mean value of those. The graphs are showing the log_{10} value of the counted CFU/ml cultured on Petrifilms^{TM} as a function of time. The limit of detection was 10^1. All groups started with a bacterial concentration of approximately 10^6 CFU/ml and the error bars at each test point are showing a confidence interval of 95%. Changes in volume due to taking samples were neglected.

4.6.1 Chlorhexidine digluconate against *P. aeruginosa*

Results from the static time-kill studies testing Chlorhexidine digluconate against *P. aeruginosa* are shown in Figure 4.10 below. The used concentrations of Chlorhexidine digluconate can be seen in Table 3.4, which can be seen in section 3.7.1, where the concentration referred to as S2, is approximately the determined MIC-value of 0.044 mM and the highest used concentration is the superMBC-value of 0.112 mM, referred to as S4. The control group was not exposed to any antimicrobial substance.

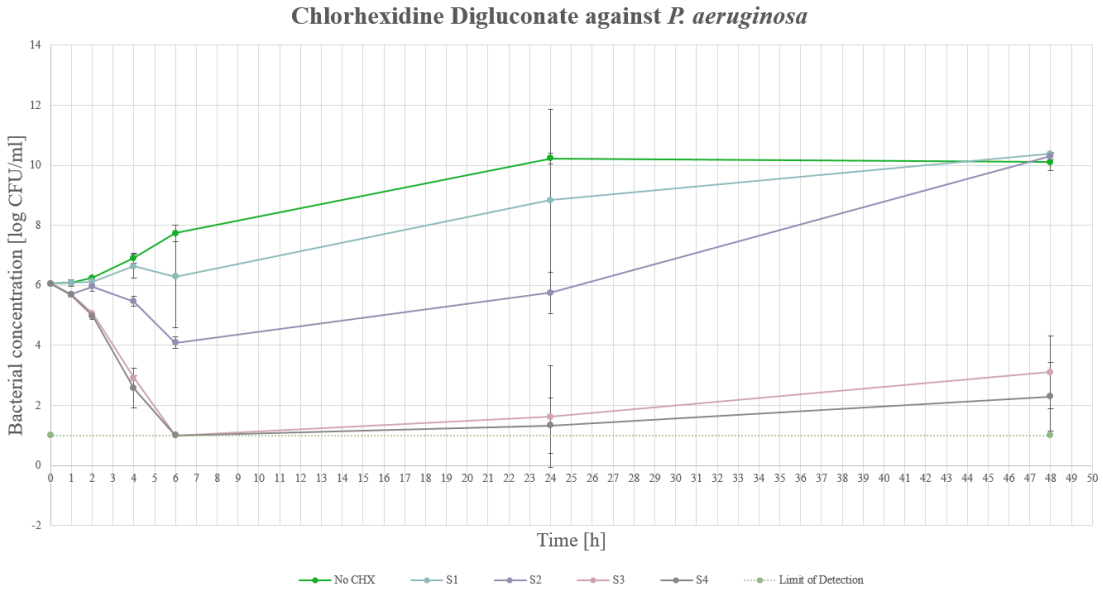


Figure 4.10: CFU counts of *P. aeruginosa* over 48 hours where the concentration of Chlorhexidine digluconate was static and added to the bacteria samples at time 0 hours of the experiment

In Figure 4.10 it can be seen that the two highest concentrations that were used, seen in Table 3.4, referred to as S3 (0.088 mM) and S4 (0.112 mM), managed to lower the amount of bacteria from 10^6 CFU/ml to below 10^1 CFU/ml, which was the detection limit, after 6 hours. The samples taken after 24 hours indicated that the test groups affected by these concentration had managed to grow back to a bit above 10^1 CFU/ml. After 48 hours the amount of bacteria in the samples affected by S3 had grown back to reach the bacterial amount of about 10^3 CFU/ml and the ones affected by S4, the superMBC, just slightly lower. The group referred to as S2 (0.044 mM) had approximately half of the bacterial amount compared to the control group after 6 hours up to 24 hours. The group affected by S1 (0.022 mM) had roughly 1 log_{10}-unit lower bacterial amount after 6 hours up to 24 hours. After 48 hours, both the group containing S1 as well as S2 had increased in bacterial amount to around 10^9 CFU/ml, which was approximately the same as the control group.

4.6.2 Chlorhexidine digluconate against *S. aureus*

Results from the static time-kill studies testing Chlorhexidine digluconate against *S. aureus* are shown in Figure 4.11 below. The used concentrations of Chlorhexidine digluconate can be seen in Table 3.4, which can be seen in section 3.7.1 where the concentration referred to as S2 is approximately the determined MIC-value of 0.002 mM and the highest used concentration is the superMBC-value of 0.056 mM referred to as S5. The control group was not exposed to any antimicrobial substance.

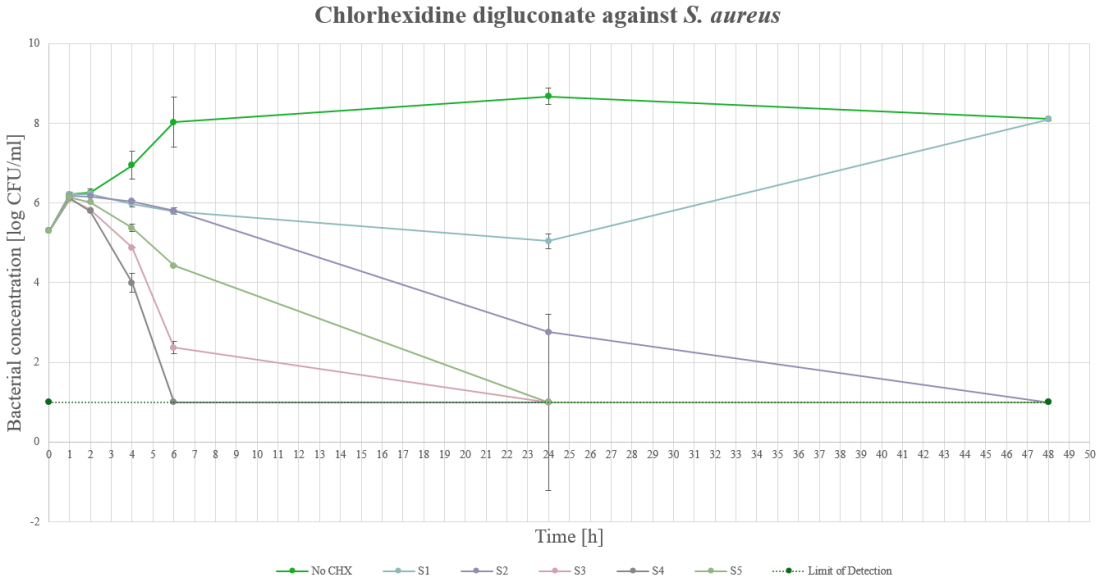


Figure 4.11: CFU counts of *S. aureus* over 48 hours where the concentration of Chlorhexidine digluconate was static and added to the bacteria samples at time 0 hours of the experiment

The graph in Figure 4.11 is showing the bacterial decrease from 10^6 CFU/ml to below 10^1 CFU/ml after 48 hours for all groups of the used concentrations, seen in Table 3.4, except S1 (0.001 mM). The group of samples containing concentration 1 had decreased to about 10^5 CFU/ml after 24 hours but at the next test point at 48 hours, the bacterial amount had increased to reach the same value as the control group of about 10^8 CFU/ml. After 24 hours the samples from the groups treated with S2 (0.002 mM) had decreased in bacterial amount to about 10^3 CFU/ml and the ones treated with S3 (0.022 mM) had decreased to about 10^1 CFU/ml. After 48 hours both these groups were below the detection limit of 10^1 CFU/ml. The samples treated with S4 (0.044 mM) showed bacterial growth of about 10^4 CFU/ml after 4 hours and after 6 hours these samples had decreased to a bacterial amount below detection limit. After 6 hours the samples treated with the highest concentration of Chlorhexidine digluconate, S5 (0.056 mM) showed a decrease in bacterial amount of about 2 log_{10}-units and after 24 hours the bacterial amount in these samples was below detection limit.

4.7 Dynamic time-kill studies on biofilm

The established results from the performed dynamic time-kill studies of *P. aeruginosa* and *S. aureus* biofilms described in section 3.8.2 can be seen in Figure 4.12 and Figure 4.13 below. The concentration of Chlorhexidine digluconate was increased over time to reach the MBEC, as can be seen in Figure 3.6. The set-up used can be seen in Figure 3.3. All concentrations were tested in three replicates and the graphs presenting the results below represents a mean value of those. The graphs are showing the log_{10} value of the counted CFU/ml cultured on Petrifilms^{TM} as a function of time. The limit of detection was 10^1. All biofilms started at a bacterial concentration of approximately 10^9 CFU/ml and the error bars at each test point are showing a confidence interval of 95%. Changes in volume due to inflow of antimicrobial substance were neglected.

4.7.1 Chlorhexidine digluconate against *P. aeruginosa*

Results from the dynamic time-kill studiy testing Chlorhexidine digluconate against *P. aeruginosa* biofilm are shown in Figure4.12 below. The determined MBEC-value for *P. aeruginosa* was estimated to be 0.896 mM. This concentration was roughly reached in 2, 3, 6 and 8 hours and is referred to as in the graph D1, D2, D3 and D4 respectively.

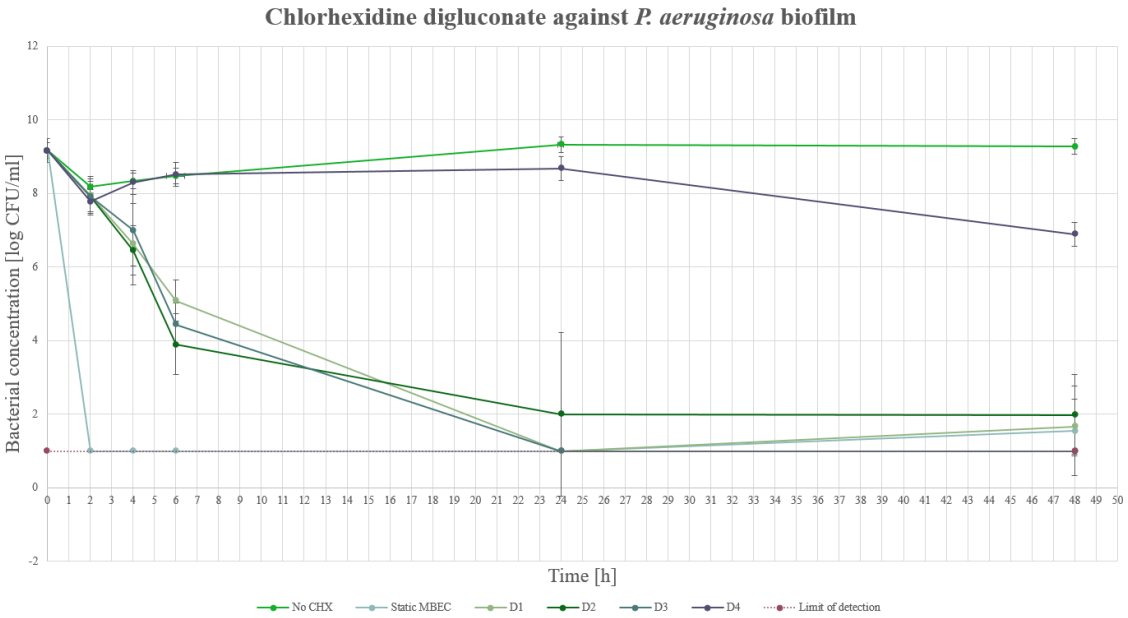


Figure 4.12: CFU counts of *P. aeruginosa* biofilm over 48 hours using the dynamic system

The result from the test with the dynamic system can be seen in Figure 4.12. The group that reached the MBEC-value in 2 hours referred to as D1, the bacterial concentration is just at the limit of detection after 24 hours and grows back slightly after 48 hours to a concentration of 10^1 CFU/ml. The group that reach MBEC in 3 hours referred to as D2 shows a 5 log_{10} reduction after 6 hours and continues to decrease in bacterial concentration to approximately 10^1 CFU/ml after 24 hours and after that it further decreases in bacterial concentration to a concentration just at the limit of detection after 48 hours. The group that reaches MBEC in 6 hours referred to as D3 decreases in bacterial concentration to approximately 10^5 CFU/ml after 6 hours from start, and are after 24 hours it is below the limit of detection and have after 48 hours not recovered and are considered as dead. The group that reaches MBEC in 8 hours referred as D4 followed the control group, which wasn't exposed to any Chlorhexidine digluconate, until 24 hours after start, but is after that decreasing in bacterial concentration to approximately 10^6 CFU/ml.

4.7.2 Chlorhexidine digluconate against *S. aureus*

Results from the dynamic time-kill studiy testing Chlorhexidine digluconate against *S. aureus* biofilms are shown in Figure 4.13 below. The calculated MBEC of 0.448 mM were roughly reached in 2, 3 and 6 hours. There was also one group that kept adding Chlorehexidine digluconate for 48 hours to a final concentration of 3.58 mM. These are referred to as D1, D2, D3 and D4 respectively in the graph.

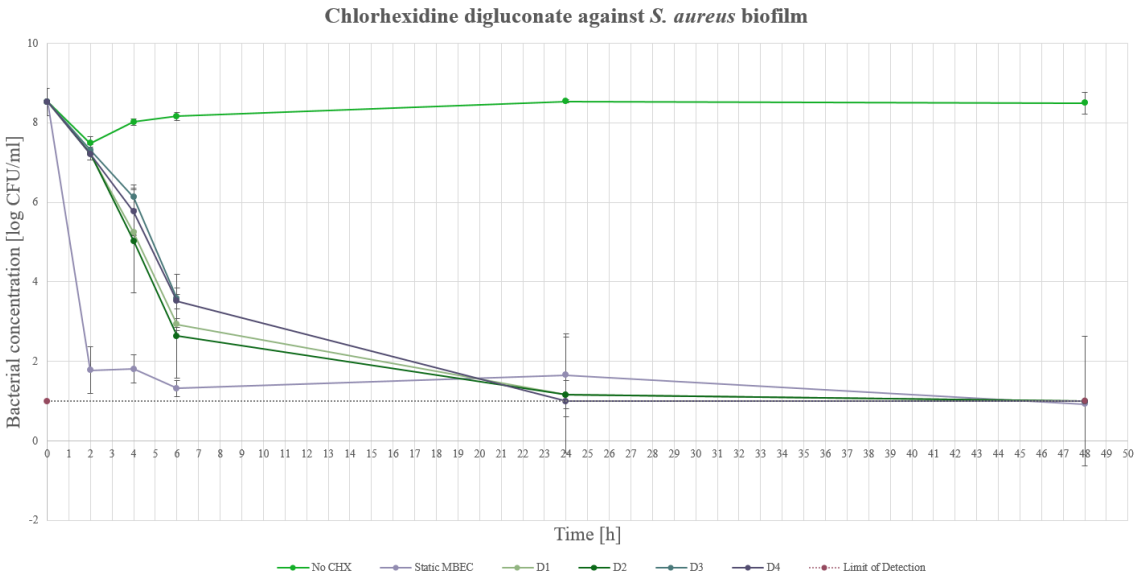


Figure 4.13: CFU counts of *S. aureus* over 48 hours using the dynamic system

The first group in the dynamic system which reached the MBEC in 2 hours, in Figure 4.13 referred to as D1 show a 7 log_{10} unit reduction within the first 6 hours and decreases further until 24 hours to about the limit of detection. After that there was a slight further decrease in the bacterial concentration to under 10^1 CFU/ml after 48 hours. The second group referred to as D2, reached MBEC in 3 hours, showed a 6 log_{10} unit reduction in the bacterial concentration within the first 6 hours to about 10^3 CFU/ml. After 24 hours the bacterial concentration had decreased further to under 10^1 CFU/ml and after 48 hours the amount of bacteria was below the limit of detection. The third group in the dynamic system that reached MBEC in 6 hours, in the graph referred to as D3, showed a reduction of 5 log_{10} units within the first 6 hours, to approximately $10^{3.5}$ CFU/ml. The bacterial concentration in this group had decreased to under the limit of detection after 24 hours and the same was seen after 48 hours. The fourth group in the dynamic system, in the graph referred to as D4 had the same gradient of increase in Chlorhexidine digluconate concentration as D3, but the inflow of antimicrobial compound was not stopped after 6 hours as for D3, it was continued to ass Chlorhexidine for 48 hours, see concentraion profile in Figure 3.6, resulting in a final concentration of 3.58 mM of Chlorhexidine digluconate. D4 shows the same reduction in bacterial concentration as D3 until 6 hours after start, but are after 24 hours just at the limit of detection, and after 48 hours the bacterial concentration is under the limit of detection.

4.8 Static time-kill studies on biofilm

The results from the performed static time-kill studies of *P. aeruginosa* and *S. aureus* biofilms described in section 3.8.1 can be seen in Figure 4.14 and Figure 4.15. The different concentrations of Chlorhexidine digluconate used can be seen in table 3.6 presented in section 3.8.1. All concenrtaions were tested in three replicates and the graphs presenting the results below represents a mean value of those. The graphs

are showing the log_{10} value of the counted CFU/ml cultured on Petrifilms^{TM} as a function of time. The limit of detection was 10^1. All biofilms started at a bacterial concentration of approximately 10^9 CFU/ml and the error bars at each test point are showing a confidence interval of 95%.

4.8.1 Chlorhexidine digluconate against *P. aeruginosa*

Results from the static time-kill studies testing Chlorhexidine digluconate against *P. aeruginosa* biofilm are shown in Figure 4.14 below.

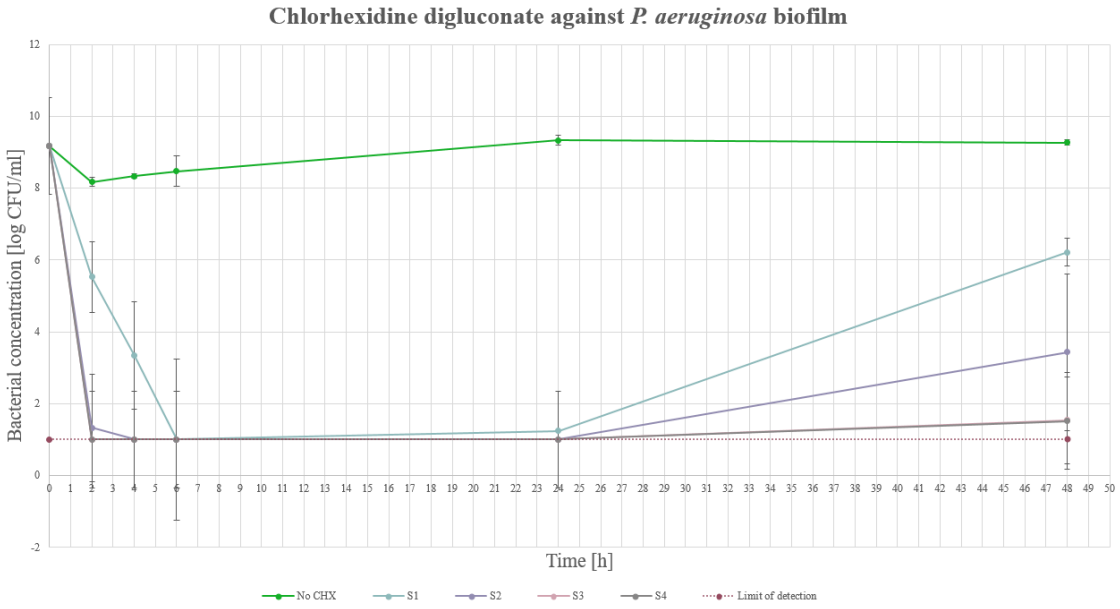


Figure 4.14: CFU counts of *P. aeruginosa* biofilm over 48 hours using the static system

The results form the test using the static system can be seen in Figure 4.14. The three groups exposed to the highest concentration, referred to as S2, S3 and S4, have decreased to a bacterial concentration that is under the limit of detection after 24 hours, independent of which of the used concentrations of Chlorhexidine digluconate that were added. In the groups S3 and S4 the bacterial concentrations were under the limit of detection already after 2 hours from start. Both of these groups have after 48 hours grown back slightly, to approximately 10^1 CFU/ml. In the group S2 the bacterial concentration is under the limit of detection after 4 hours and in the group S1 the bacterial concentration approximatley at the bacterial concentration of 10^1 CFU/ml after 24 hours. Both of these groups have also recovered after 48 hours and the bacterial concentration have increased to approximately 10^4 CFU/ml and 10^6 CFU/ml respectively.

4.8.2 Chlorhexidine digluconate against *S. aureus*

Results from the static time-kill studies testing Chlorhexidine digluconate against *S. aureus* biofilm are shown in Figure 4.15 below.

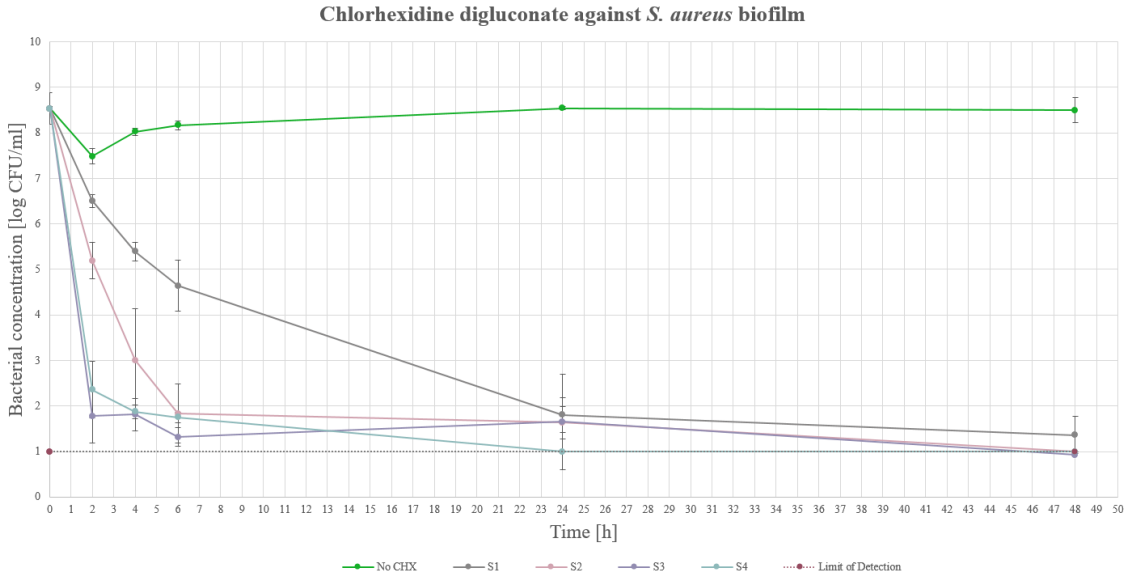


Figure 4.15: CFU counts of *S. aureus* over 48 hours using the static system

Seen in Figure 4.15 is that the group that had the lowest amount of Chlorhexidine digluconate added from start in the static system, in the graph referred to as S1 decreases to approximately 10^2 CFU/ml in 24 hours from start, which is a 7 log_{10} unit reduction of the bacterial concentration after that it decreases further to a bacterial concentration a bit above 10^1 CFU/ml. The group with twice as high concentration of Chlorhexidine digluconate added from the start, in the graph referred to as S2 show a reduction in bacterial concentration to about 10^2 CFU/ml within the first 6 hours. That concentration is then constant until 24 hours, but have after 48 hours decreased to under the limit of detection. The group referred to as S3 in the graph, which was added twice as high concentration of Chlorhexidine digluconate compared to the S2 group, is the group affected by the concentration of the established MBEC (seen in section 4.3). The S3-group decreases to under 10^2 CFU/ml in 2 hours from start, which is a 7 log_{10} unit reduction. A further reduction in the bacterial concentration to a bit above 10^1 CFU/ml is seen after 6 hours. This concentration slightly increases until 24 hours, but after that decreases to just around the limit of detection. The group referred to as S4 show a 6 log_{10} unit reduction within the first 2 hours and then a further reduction in bacterial concentration to approximately 10^2 CFU/ml after 6 hours. After 24 hours the bacterial concentration have decreased to just at the limit of detection and after 48 hours the bacterial concentration is below the limit of detection.

5

Discussion

5.1 Bacterial susceptibility to Chlorhexidine

From the performed experiments involving *P. aeruginosa* and *S. aureus*, a clear pattern can be seen. What is shown is that the Gram-positive bacteria *S. aureus* achieved a lower MIC-, MBC-, and MBEC-value compared to the Gram-negative bacteria *P. aeruginosa* when being treated with the antimicrobial compound Chlorhexidine digluconate, which indicates that *P. aeruginosa* is less sensitive to the antimicrobial. The MIC-value for planktonic *S. aureus* was estimated to be 0.002 mM and for planktonic *P. aeruginosa* it was found to be 0.044 mM. The determined MBC-value for planktonic *S. aureus* was 0.044 mM and for planktonic *P. aeruginosa* it was found to be 0.088 mM. The determined MBEC-value for *S. aureus* biofilm was 0.448 mM and for *P. aeruginosa* it was 0.896 mM when being treated with Chlorhexidine digluconate.

It is shown in all of the performed time-kill studies done on planktonic cells that *S. aureus* do not recover from the treatment with Chlorhexidine digluconate in the same way as *P. aeruginosa* does. The dynamic time-kill study on planktonic cells showed that the bacterial samples containing *S. aureus* eventually died independently in which time the MBC-concentration was reached. In the bacterial samples containing *P. aeruginosa*, the bacterial concentrations were almost back to the initial concentrations in all samples after 48 hours, even the ones reaching the MBC within 1 hour. The same trend was seen in the static experiments performed on the two types of bacteria in planktonic state. All of the used concentrations, except the lowest one, managed to lower the amount of *S. aureus* bacteria to under the detection limit within 48 hours, with no sign of them growing back. The static time-kill study performed on *P. aeruginosa* showed that the two lowest concentrations did not have any effect on the bacterial concentration after 48 hours and the two highest concentrations seemed to have killed the bacteria within 6 hours, or at least lower the concentration to below the limit of detection, but after 48 hours a clear up going curve of the bacterial concentration was seen.

All of these results indicates that *P. aeruginosa* is less sensitive to Chlorhexidine digluconate than *S. aureus* is and these results also goes in line with previous studies of the two bacteria where it has been shown that Chlorhexidine is more effective against Gram-positive bacteria [13]. *S. aureus* is a Gram-positive bacteria and *P. aeruginosa* is a Gram-negative bacteria which means that the two types of bacteria

differ from each other in cell wall composition. The main difference between these types of bacteria is that the Gram-negative bacteria *P. aeruginosa* has two cell membranes which the Chlorhexidine molecule needs to bind to and to disrupt [10, 20]. The main mode of action of Chlorhexidine is to bind to the cell membrane of bacteria, causing it to lose its integrity, leading to leakage of intracellular components leading to cell death. Since the Gram-negative bacteria has two cell membranes it is then two borders for the Chlorhexidine to bind to and break before the antimicrobial has performed its action. It has previously also been shown that antimicrobial agents can in some cases disturb the outer membrane of Gram-negative bacteria without causing cell death of the bacteria [107] and in this case this could be one reason for why *P. aeruginosa* is less sensitive to treatment with Chlorhexidine, since it might manage to survive with only one intact membrane. *S. aureus* only has one membrane and the destruction of the membrane will cause cell death of the bacteria. It has also been reported that *P. aeruginosa* can to some extent degrade Chlorhexidine [108] which further would make it less sensitive to treatment since that would lead to a decrease in Chlorhexidine concentration within the bacterial solution. The cell wall of the Gram-positive bacteria *S. aureus* is also more negatively charged than the cell wall of the Gram-negative bacteria *P. aeruginosa* due to the teichoic acids on the cell wall of *S. aureus* [14]. This would make binding of the positively charged Chlorhexidine molecule to the bacterial cell wall of *S. aureus* more easy than binding to the cell wall of *P. aeruginosa*. This could also be a reason for why lower concentration of Chlorhexidine digluconate is needed in order to kill *S. aureus* compared to *P. aeruginosa*.

5.2 Patterns for antimicrobial killing

Pharmacodynamic and pharmacokinetic parameters are important to evaluate for the correct treatment with an antimicrobial agent and this is further described in section 2.5. When considering the pharmacokinetic profile of an examined bacteria to the used antimicrobial it can be seen in some cases, as for Chlorhexidine, that the antimicrobial acts through an irreversible process in which the main mode of action is to bind to the bacterial membrane, causing it to loose its integrity and eventually break. The binding of the Chlorhexidine to the bacterial membrane would then cause the antimicrobial concentration in the bacterial solution to decrease, but the concentration on the target, in this case the bacterial membrane, is unchanged [109]. Therefore, considerations regarding the dosing of Chlorhexidine towards the two different bacteria have been made.

From the determined MIC- and MBC-values, calculations of the C_{max}/MIC were made. From these calculations it could be seen that the ratio for *S. aureus* was determined to be 20.41 which was about ten times higher than the ratio for *P. aeruginosa* which was determined to be 2.56. A C_{max}/MIC ratio of 8-10 has previously shown to achieve maximum bactericidal effect against Gram-negative bacteria [110]. In concentration dependent killing with quinolones the AUC/MIC ratio needed to kill a Gram-positive bacteria effectively have shown to be greater or equal to 30, and for *P. aeruginosa* the value has shown to be 125 [110, 111]. The goal in general

is to reach a C_{max}/MIC of 10-12 or a total AUC/MIC > 150 to achieve the best effect [112]. This is not achieved in the performed time-kill studies performed on *P. aeruginosa* since the reached C_{max} for the used bacteria was the determined MBC. This could indicate that *P. aeruginosa* is less sensitive to Chlorhexidine digluconate than *S. aureus* since it seems to be necessary to reach a higher concentration than the determined MBC for *P. aeruginosa* in order to kill the whole population of the bacteria without risk of regrowth. Calculations of the AUC/MIC for the bacteria species were made and the results from this can be seen in Table 4.8 and 4.9 where it e.g. can be seen that *P. aeruginosa* reaching MBC in 1 hour was determined to be 121.79 and for *S. aureus* 969.39.

It is shown in the performed time-kill studies on planktonic cells that between time 0-24 hours in the samples that weren't treated with any Chlorhexidine, *P. aeruginosa* seems to replicate faster than *S. aureus*. This could be a further reason to why the treatment with Chlorhexidine on *P. aeruginosa* is not as efficient as on *S. aureus*. On the *P. aeruginosa* bacteria the binding of Chlorhexidine to the membrane and thereby the lowering of the bacterial amount, the elimination of bacteria, is not enough in relation to its replication rate and therefore an up-going curve can be seen for *P. aeruginosa* concentration in the performed experiments. This would mean that the more bacteria in the samples, the more of the Chlorhexidine in the solution will be bound to the bacteria, which in its turn lower the amount of Chlorhexidine in the bacterial solution, which then could be a reason for regrowth of the bacteria. From the performed time-kill experiments it seems like the maximum concentration (C_{max}) is a driving factor for killing the bacteria *P. aeruginosa*. All of the performed time-kill studies on *P. aeruginosa*, both on planktonic cells as well as on biofilm show that the bacteria has the potential of growing back. It seems like if not a complete killing has been reached, it doesn't matter if the bacteria is exposed during longer periods by smaller doses of the antimicrobial, it has the potential to grow back anyway. This indicates that the bacteria could be C_{max}-driven. In the experiments performed on *P. aeruginosa* biofilm the same trend was seen. Even if the the bacterial concentration was under the limit of detection at one time point, later measurements of the bacterial concentration showed regrowth. From these considerations it seems like a high concentration of Chlorhexidine is needed to be reached in a short time, before the bacteria have had time to grow to a too large extent that outnumbers the Chlorhexidine concentration.

When it comes to *S. aureus* it is shown in the performed experiments that a high concentration of the used antimicrobial lowers the bacterial amount faster than a lower concentration of the antimicrobial. But, when a lower concentration of Chlorhexidine is used but during a longer time, it can still be seen that the bacterial concentration is decreasing. This could also be connected to the replication rate of the bacteria. Since *S. aureus* replicates slower than *P. aeruginosa*, the amount of Chlorhexidine bound to *S. aureus* bacteria in the bacterial samples won't be as much as for the amount to *P. aeruginosa* in the samples, which would mean that the bacteria still existing in the bacterial samples with *S. aureus* are treated with a higher concentration of Chlorhexidine than the bacteria in the *P. aeruginosa* sam-

ples. Therefore, it might be in that case that there will be enough Chlorhexidine to sufficiently kill the newly replicated *S. aureus* bacteria in the solution and the bacteria don not manage to regrowth during these conditions. This behaviour of the bacteria indicates that the killing of the bacteria might be AUC-driven.

The concentration needed to inhibit growth of *S. aureus* is much lower than the concentration needed to inhibit growth of *P. aeruginosa*. It is a much greater difference between the MIC- and MBC-values for *S. aureus* compared to the values for *P. aeruginosa*. This can also be seen in the picture from the performed MBEC-tests, when comparing Figure 4.4 to Figure 4.5, where *S. aureus* is inhibited at a much lower concentration than the MBEC, compared to *P. aeruginosa* where the inhibitory concentration seems to be about the same as the MBEC. This would make killing of *S. aureus* easier since the growth of *S. aureus* is inhibited faster than the growth of *P. aeruginosa*. This would result in a lower concentration of the bacteria within the suspension that needs to be killed by the Chlorhexidine. This means that in the performed dynamic time-kill studies the T>MIC, which is an important parameter in time-dependent killing, is higher for *S. aureus* than for for *P. aeruginosa*. This can be seen in Figure 4.7, where T>MIC can be seen for *S. aureus* compared to Figure 4.6, where it can be seen for *P. aeruginosa*. It is suggested that the T>MIC should be at 40-70% for a compound that act time-dependent [112]. In the performed experiments the T>MIC for both of the bacteria is much higher than that, even for *P. aeruginosa*.

These different killing-patterns for the two bacteria exposed to the antimicrobial Chlorhexidine digluconate affects how the dosing of the antimicrobial for treatment of the bacteria types should be optimized. When it comes to treatment of *P. aeruginosa* it seems like, from the performed experiments, that treatment with a high concentration of Chlorhexidine delivered fast to the target would probably be the only way to eliminate the bacteria. Fast delivery is necessary to avoid that the bacteria will have time to replicate to a greater extent than the Chlorhexidine is capable of binding to and eliminate. This might not be the optimal solution when being applied in a wound dressing since high concentrations of Chlorhexidine might be cytotoxic. Further investigations of this is needed for proper treatment of wounds infected with *P. aeruginosa*. When it comes to treatment of *S. aureus*, it seems like, from the performed experiments, that treatment with a high concentration of Chlorhexidine manage to lower the bacterial amount fast but the treatment with a lower concentration of Chlorhexidine but for a longer time also seems to have this effect on the bacteria. Therefore, it is not necessary to reach to high concentrations of Chlorhexidine to eliminate the bacteria *S. aureus*. If the concentration of Chlorhexidine is just above the MIC for the bacteria it seems to be sufficient to kill the bacteria after a longer exposure. This could then be applied in a wound dressing where the Chlorhexidine concentration is delivered from the wound dressing in doses resulting in a concentration of Chlorhexidine just above the MIC. This could be achieved either by the product itself where the product releases small doses of Chlorhexidine, slowly over a longer time, to have a constant concentration of Chlorhexidine in the

wound just above the MIC, or it could be achieved by changing the dressing regularly.

5.3 Survival mechanism

In all of the time-kill studies performed on *P. aeruginosa* it could be seen that the *P. aeruginosa* recover even if the bacterial concentration had decreased to below the limit of detection. This was seen in the experiments with *P. aeruginosa* biofilms as well as planktonic *P. aeruginosa* treated with static antimicrobial concentration. In all of the performed time-kill studies on *S. aureus* it could be seen that once the bacterial concentration was below the limit of detection there was no sign of the bacteria growing back. Also, in the cases where the bacteria was treated with antimicrobial compound, even if the bacteria was not killed by the treatment there were no indications of that the amount of bacteria was increasing, which can be seen in section 4.7.1.

In section 2.6 a definitions of resistant, tolerant and persistent bacteria are described. On this basis there is likely to be persister cells in the inoculum of *P. aeruginosa*. This conclusion can be made since it can be seen that a small part of the population survives the antimicrobial treatment and thereby causing survival and regrowth of the whole population. The exact mechanism for how the bacteria adapts to this kind of stress full environments is still under debate but previous studies have shown that small parts of the population changes in phenotype and suppress growth to adapt to external environmental changes and has the ability to restart growth after stress. Further research has to be done to fully understand how persister cells manage to survive extreme environmental conditions in order to be able to design better therapeutic strategies [113, 106].

5.4 Biofilm

Clear differences could be seen between the studies done on planktonic cells compared to biofilm for both of the types of bacteria. Much higher concentration of Chlorhexidine digluconate was needed to kill the bacteria in biofilm compared to the bacteria in planktonic state, which can be seen in the established MBC- and MBEC-values. The established MBEC-value for *S. aureus* was determined to be 0.448 mM which is about 10 times higher than the MBC for the bacteria in planktonic state which was 0.044 mM. The established MBEC-value for *P. aeruginosa* was determined to be 0.896 mM which also is about 10 times higher than the MBC for the bacteria which was 0.088 mM. The potential explanation for this can be found in section 2.2 where it is described that bacteria in biofilm can collaborate metabolically and can be shielded from external factors, such as antimicrobial com-

pounds which leads to less sensitivity to the used antimicrobial [36]. What also should be taken into account here is that in the studies performed on biofilms in this project, the initial bacterial concentration was higher than in the tests performed on planktonic cells, 10^9 CFU/ml compared to 10^6 CFU/ml, which probably also has an impact on the concentration of Chlorhexidine needed to kill the bacteria.

6
Conclusions

6.1 Conclusions

From the performed experiments and after analyzing the results it can be concluded that *P. aeruginosa* is less sensitive than *S. aureus* to treatment with Chlorhexidine digluconate. When *S. aureus* was decreasing in bacterial concentration due to addition of antimicrobial compound there were no signs of regrowth in any of the experiments performed. There seemed to be persister cells in the *P. aeruginosa* population during all experiments since they managed to recover after antimicrobial treatment even if the bacterial concentration was at a point below the limit of detection.

It can also be concluded that it seems like the killing of *P. aeruginosa* is C_{max}-driven. *P. aeruginosa* does not die over time during treatment. The only powerful treatment is probably therefore to quickly increase to a high concentration of the used antimicrobial agent. *S. aureus* die fast when treated with high concentration of Chlorhexidine digluconate but the bacteria also decrease in bacterial concentration when being treated with a lower concentration, just above the determined MIC, but for a longer time. This indicates that the killing of *S. aureus* seems to be time-dependent (AUC-driven).

It can also be concluded that bacteria in biofilm is harder to kill and a higher concentration of Chlorhexidine digluconate is needed in order to achieve the same rate of killing as for bacteria in planktonic state.

6.2 Further research

Further research that can be done within this area is to examine the volume of distribution of the used antimicrobial substance. This would be tested to see if the added concentration of the antimicrobial compound to the bacteria actually is the concentration that affects the bacteria within the samples, or if some of it could have reacted with something in the suspension. This could be for example, reaction with proteins in the growth media, or that the antimicrobial have bound to bacteria within the bacterial suspension which can lead to that the antimicrobial concentration in the solution decreases.

One thing to examine could be a combination of bacteria, not only looking at one type at a time, since this would better simulate the actual condition of bacteria existing in a chronic wound. Co-cultures of *S. aureus* and *P. aeruginosa* have been shown to be more difficult to treat with antibiotics [74]. Therefore, dynamic time-kill studies, as the ones performed in this project, on co-cultures of these two bacterial species using Chlorhexidine as the antimicrobial would be of great interest.

One factor that would be interesting to further investigate is treatment of the bacteria with a combination of different antimicrobial compounds. The use of two different types of antimicrobial agents might create a synergistic or an additive effect that would be even more effective for killing of the bacteria. One possible option is to use silver in combination with Chlorhexidine digluconate. This is suggested since the company Mölnlycke already uses silver in some of their wound care products [7], and the mode of action of silver differs from how Chlorhexidine acts [114]. This combination might lead to a higher degree of killing of bacteria. Also, since Chlorhexidine showed to be more effective against *S. aureus* compared to *P. aeruginosa* in the performed studies, it would be interesting to treat the bacteria with a mixture of Chlorhexidine and an other substance that is shown to be more effective against Gram-negative bacteria in order to see if that might yield a higher rate of killing, compared to if Chlorhexidine is used individually. Previous studies performed on these two bacteria, using silver as the antimicrobial, have shown that a lower concentration of silver was needed to inhibit the growth of *P. aeruginosa* compared to *S. aureus* [115].

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A

Appendix

A.1 Materials

12 channel IPC-N tubing pump - Acquired from ISMATEC®

Beakers - Range 20 ml to 500 ml acquired from VWR International

Clicking press - acquired from ATOM S.p.A designs

Colony counter - acuired from GERBER INSTRUMENTS AB

Erlenmeyer flasks - Range 20 ml to 500 ml acquired from VWR International

Falcon® tubes - 15 and 50 ml acquired from VWR International

GRANT DEN-1B densitometer - acquired from Grant Instruments

IKA MTS 2/4 shaker - Digital microtiter plate shaker acquired from IKA®-Werke GmbH Co. KG

Mesoft ® - Nonwoven swabs produced by Mölnlycke Health Care®

Microlab STAR - Technology system for pipetting acquired from HAMILTON®

Multi-well plates - 96, 48, 24 and 12 wells. Acquired from VWR International

Multi-well plates - 96 wells. Acquired from Thermo Scientific™

Multi Vortex Genie orbital shaker - Multi-sample digital shaker acquired from Scientific Industries™

Petrifilm™ - Aerobic Count Plates acquired from 3M™

Petrifilm™ plate reader - acquired from 3M™

Pipetboy acu 2 - Pipette aid acquired from INTEGRA Biosciences AG

Polystyrene pipettes - Range 10 ml to 100 ml acquired from VWR International

Shaking humidified incubators - acquired from Thermo Scientific

Shaking non-humidified incubator - acquired from Infors AG

Single Channel Autopipettes - Range 1 µl to 5 ml acquired from Thermo Scientific™

Stainless steel tube connectors - ISM580 with inner diameter 0.3 mm, length 0.63 mm. Acquired from ISMATEC®

Static humidified incubators - acquired from Termaks AS

Tygon® extension tubing - 19 mm inner diameter, length 10 m, cut to pieces of 1 m or 40 cm. Acquired from ISMATEC®

Tygon® pump tubing - 19 mm inner diameter, length 40 cm. Acquired from ISMATEC®

Vortex-Genie 2 ® Mixer - Multi-tube holder mixer acquired from Scientific Industries™

A.2 Chemicals

Chlorhexidine digluconate - 20% in H_2O acquired from Sigma-Aldrich® Life Science

D/E Deactivation-broth - Acquired from Sahlgrenska Universitetssjukhuset Bakteriologiska Laboratoriet

Dextran sulphate - Acquired from Sigma-Aldrich® Life Science

Mueller Hinton broth - Acquired from Sahlgrenska Universitetssjukhuset Bakteriologiska Laboratoriet

Peptone water (0.5% Peptone; 0.5% NaCl; 0.5% Tween 80) - Acquired from Sahlgrenska Universitetssjukhuset Bakteriologiska Laboratoriet

Peptone water (0.85% NaCl with 0,1% Peptone) - Acquired from Sahlgrenska Universitetssjukhuset Bakteriologiska Laboratoriet

Resazurin - Acquired from Sigma-Aldrich® Life Science

Simulated Wound Fluid (SWF) - Acquired from Sahlgrenska Universitetssjukhuset Bakteriologiska Laboratoriet

Tryptic Soy Broth (TSB) - Acquired from Sahlgrenska Universitetssjukhuset Bakteriologiska Laboratoriet

Tryptic soy agar plates - Acquired from Sahlgrenska Universitetssjukhuset Bakteriologiska Laboratoriet