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# The antibacterial effect of silver with different release kinetics

Master of Science Thesis in Biotechnology

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# Abstract

Hard to heal wounds are painful and the patient has to stand with them for a long time, also these wounds are an economical burden for health care systems. One important factor that may interrupt the healing of wounds is contaminating bacteria and they can also cause infections. Today, there are numerous of silver releasing dressings on the market that have effect against a wide range of bacteria over multiple days. The available silver dressings contain different amounts of silver and have different release kinetics.

The aim with this Master thesis is to find out how the release profile of silver influences the antibacterial effect, and if one silver release profile is more preferable to another and if it is valid for bacteria both in logarithmically and stationary growth phase.

Two wound pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, in either log- (~$10^6$ CFU/mL) or stationary- growth phase ($10^8$-$10^9$ CFU/mL) were cultured in simulated wound fluid. Silver sulfate solutions were added to the bacterial suspension, the suspensions were incubated for 24 h and viable count were determined by plate counts method. For log-phase bacteria, the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were 8 ppm and 32 ppm for *S. aureus* and, 6.5 ppm and about 7 ppm of silver for *P. aeruginosa*, respectively. For stationary-phase bacteria MBC's were found to be above 987 ppm and above 64 ppm of silver for *S. aureus* and *P. aeruginosa*, respectively. The MIC and MBC values were determined to find appropriate silver concentrations to simulate silver release profiles. It was also found that vortex of the bacterial suspensions after addition of silver increased the antibacterial effect significantly.

Three silver release profiles (corresponding MIC, boost, linear) were simulated by adding different amounts of silver every second hour (0, 2, 4 and 6 h) for six hours and then incubated further for 24 h in total. There was no significant antibacterial difference between the three release profiles when *S. aureus* was exposed. In contrast, the linear release profile for *P. aeruginosa* in log- and stationary-phase had 3 and 3.5 log reductions less antibacterial effect, respectively, compared to the profile corresponding to MIC measurements.

**Keywords:** silver, release, antibacterial, wound, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, dressing

# Abbreviations

| | |
|---|---|
| CFU | colony forming units |
| log | logarithmic |
| MHC | Mölnlycke Health Care |
| MIC | Minimum Inhibitory Concentration |
| MBC | Minimum Bactericidal Concentration |
| ppm | parts per million |
| PW | Peptone Water |
| STS-1 | Sodium Thioglycolate Solution 1 |
| STS-2 | Sodium Thioglycolate Solution 2 |
| SWF | Simulated Wound Fluid |
| TSA | Tryptic Soy Agar |
| TSB | Tryptic Soy Broth |

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# 1. Introduction

Hard to heal wounds are of great concern since they cause great pain and suffer to patients (Lo et al., 2009). Also, hard to heal wounds are a great economic burden for health care systems. Venous leg ulcers, which are loss of epidermis and parts of dermis, are one common type of hard to heal wound (Sibbald et al., 2007). These kind of wounds are caused by chronic venous insuffiency, venous hypertension and calf-muscle pump insuffiency; where only the chronic venous insuffiency is estimated to cost 0.75-1 billion per year in the United States (Sibbald et al., 2007). One important factor that interrupts the healing is bacterial burden in the wound. When bacteria proliferate a wound above a manageable level for the host, the healing may be interrupted (Lo et al., 2009). The disturbance caused by bacteria often involves prolonged inflammatory response, changes in the normally molecular and cellular environment of the wound bed and breakdown of granulation tissue (Lo et al., 2009).

Today it exists a lot of silver containing dressings on the market and most of them promise antibacterial effect against a broad range of bacteria over multiple days (Cavanagh et al., 2010). There is an ongoing debate whether which type of silver dressing that has the most efficient antibacterial properties, regarding the silver release kinetics and amounts of silver released over a certain time. Recently, it has been shown that two different silver containing dressings had various antibacterial effects after 24 hours, although the silver concentration at this time was the same (Jakobsen, 2010). It turned out that the release kinetics of the two different wound care products was different and this could be the reason of the various antibacterial effects.

## 1.1. Aim

The aim with this Master thesis is to find out whether it is best to give a quick boost of silver, in a wound like environment, to achieve the best antibacterial effect, or if another silver release profile is more preferable. Normally these kinds of experiments are performed with growing log-phase bacteria. But in this project it will also be investigated if the release profiles also apply to bacteria that have established into stationary-phase.

# 2. Background

The area of wound healing and infection with its microbiology will be explained in this chapter. First the skin and acute wound healing are described. Then, the disturbance of the healing by bacterial infection is explained. Last, topical treatment with silver and its antibacterial properties are mentioned.

## 2.1. The skin

The skin is the largest organ of the body and it has several of essential functions (Tortora & Derrickson, 2010). The most important functions are: regulation of body temperature, storage of blood, detection of cutaneous sensations, excretion and absorption of substances, production of vitamin D and last protection from external threats. Bennett (2008) mentions that the skin also is vital because it functions as a barrier against diffusing nutrients and ions in the body, additionally the skin prevents us from desiccation and death by its water proof properties. The structure of the skin is composed of mainly two parts: the superficial and thinnest part is called epidermis and the deeper and thicker part is called dermis (Tortora & Derrickson, 2010), both illustrated in Figure 1. Below dermis the subcutaneous layer is located, although this layer is not a part of the skin organ.

The thickness of the epidermis layer ranges from 0.1 mm up to 1 mm at regions that are exposed to more friction, such as, soles and palms (Bennett, 2008). Thick skin consists of five layers of keratinocytes: stratum basale, stratum spinosum, stratum granulosum, stratum lucidum and the most superficial stratum corneum, while thin skin also contains these except for the stratum lucidum (Tortora & Derrickson, 2010). In the deepest layer, stratum basale, which is composed of one single layer of columnar keratinocytes, some of them are stem cells.

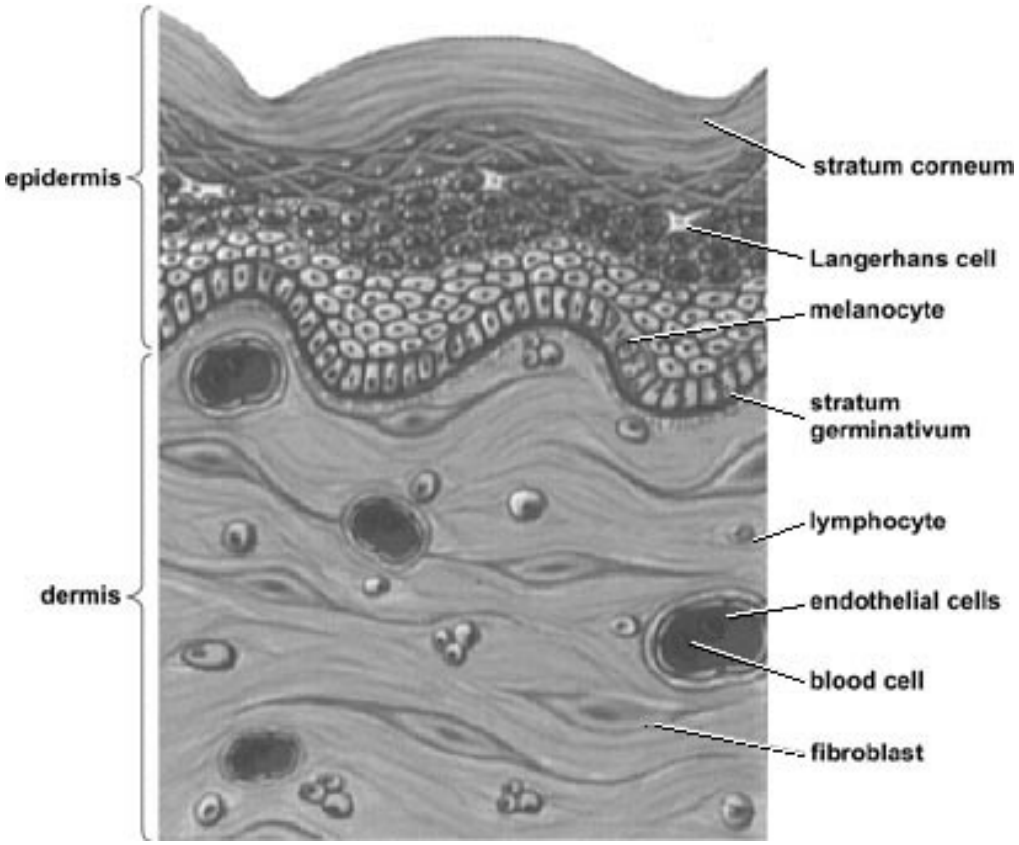


**Figure 1.** A cross section of the human skin. (Bennett, 2008)

New forming keratinocytes, originating from the stem cells, are pushed toward the surface. In the superficial part of stratum spinosum the cells become somewhat flattened and this phenomena proceeds towards the surface of the skin. Subsequently stratum spinosum comes stratum granulosum, where the keratinocytes undergo apoptosis; hence the more superficial layers consist of dead keratinocytes. During the traverse through the epidermis to the surface the keratinocytes produce and accumulate more and more keratin, this is known as keratinization (Tortora & Derrickson, 2010). It takes about one month for a keratinocyte to reach the surface and be sloughed off. Keratin plays a major role to the barrier function and in the stratum corneum. Filaggrin, a matrix protein, aggregates with keratin filaments into a highly ordered structure that promotes keratinocytes to collapse and flattened (Proksch, Brandner & Jensen, 2008). Filaggrin together with keratin constitute 80-90 % of the protein mass in the epidermis of mammalians.

Epidermis consists of four different cell types: keratinocytes, melanocytes, Langerhans cells and Merkel cells (Tortora & Derrickson, 2010). About 90 % of epidermal cells are keratinocytes. As earlier mentioned, keratinocytes produce the tough and fibrous protein keratin, which contributes to the skin's great barrier function against e.g. microbes, heat and chemicals. Also produced are lamellar granules, which release a water proof sealant and therefore prevent desiccation. Melanocytes, about 8 % of the epidermal cells, enclose and protect keratinocytes and other cells via release of the pigment melanin, which absorbs ultraviolet light. Melanin also gives the skin its color; exposure to ultraviolet light stimulates melanin secretion that leads to a darker skin (Bennett, 2008). Langerhans cells detect antigens from e.g. bacteria so the immune system is able to recognize threats (Tortora & Derrickson, 2010). Merkel cells are involved in touch sensation, via connection to a tactile disc that further is connected to a sensory neuron.

Dermis is the deeper layer below the epidermis, see Figure 1, and it is thicker than epidermis, 1-3 mm thick (Bennett, 2008). This region of the skin is composed of fewer cells but more of elastic and collagenous fibers that give strength, cohesion and elasticity to the skin. According to Lindholm (2007), 70-80 % (dry weight) of dermis consists of collagen. Throughout dermis there are capillaries that also supplies epidermis, which lack blood vessels, with nutrients and oxygen (Bennett, 2008). To increase the surface area of dermis to epidermis, finger-like projections, called dermal papillae, are located into the under-surface of epidermis. According to Tortora & Derrickson (2010), some dermal papillae also contain free nerve endings, which are sensitive to warmth, coolness, itching, tickling and pain, and touch sensitive tactile receptors. In the deeper dermis, between fibers of collagen and some elastic fibers; nerves, adipose cells, hair follicles, oil glands and sweat glands are located (Tortora & Derrickson, 2010). Collagen and elastic fibers provide strength and elasticity, respectively, to the skin. The deeper part of dermis is attached to the subcutaneous layer, which contains large blood vessels that supplies the skin. It is also the fat storage depot and fibers that extend through the skin are anchored in the sub-cutaneous layer (Tortora & Derrickson, 2010).

### 2.1.1. Human microbiota

Normally, a human being has around $10^{12}$ bacteria on their approximately 2 $m^2$ skin surface (Harley, 2008). In addition, bacteria are present on other surface tissues as the mucous membranes. Microorganisms that frequently are found on us are called the normal microbiota. Bacteria that originate from the surrounding environment and come in contact to us are called the transient microbiota, though they normally do not establish and multiply. According to Harley (2008), bacteria on the skin live on superficial dead cells or close to oil and sweat glands where the secretions provide them with nutrients, such as water, amino acids, urea electrolytes and also for some bacteria fatty acids. An evidence of their existence is the odor of sweat (apocrine glands) from the underarm pits that comes from the activity of microorganisms, since the

apocrine secretion is odorless (Madigan & Martinko, 2006).

Normally the microbiota of the skin consists of Gram-positive bacteria as the genera *Staphylococcus*, where its barrier properties against dehydration are favored due to the skin's relatively dry environment (Madigan & Martinko, 2006). Gram-negative bacteria as *Escherichia coli* do not have the same barrier against dehyd-ration as the Gram-positive bac-teria. Therefore *E. coli* bacteria prefer more moist environments as in the intestine (Madigan & Martinko, 2006).

## 2.2. Acute wound healing

Wounds are often divided into two types: acute and chronic, also known as hard to heal, wounds (Bowler, Duerden & Armstrong, 2001). Acute wounds follow after an external damage to the skin, such as bites, burns, cuts, abrasions and also after surgical operation. Acute wounds normally heal in a predicted time depending on site, depth and type of wound. Severe acute wounds caused by burns or gunshots have more problematic issues with devitalized tissue and often contaminated with bacteria and foreign materials (Bowler, Duerden & Armstrong, 2001), therefore e.g. some kind of antibacterial therapy could be preferable to aid in the healing of the wound. The source of an infection normally origin from the trauma that cause the wound, the own normal microbiota, health care providers or from sur-gical operation and as a result the healing of the wound will be disturbed and prolonged (Lindholm, 2007). The healing of an acute wound is described in this chapter while the complexity of bacterial disturbance in hard to heal wounds will be explained in the chapter *Infection and Wound microbiol-ogy*.

As mentioned above, wound healing normally is a rapid process and takes around a few weeks (Martin, 1979; Metcalfe & Ferguson, 2011). Though, complication as connective tissue scar most often remains in the collagen matrix within dermis after the wound has healed (Martin, 1979). Alternatively the tissue is completely regenerated and heals without a scar as a result (Metcalfe & Ferguson, 2011). The healing process of mammalian tissue is often divided into four overlapping phases as shown in Figure 2: injury and haemostasis, inflammation, proliferation and remodeling (Metcalfe & Ferguson, 2011). Wound healing is a complicated process and all the phases are controlled of different signal substances, such as growth factors and cytokines (Metcalfe & Ferguson, 2011).

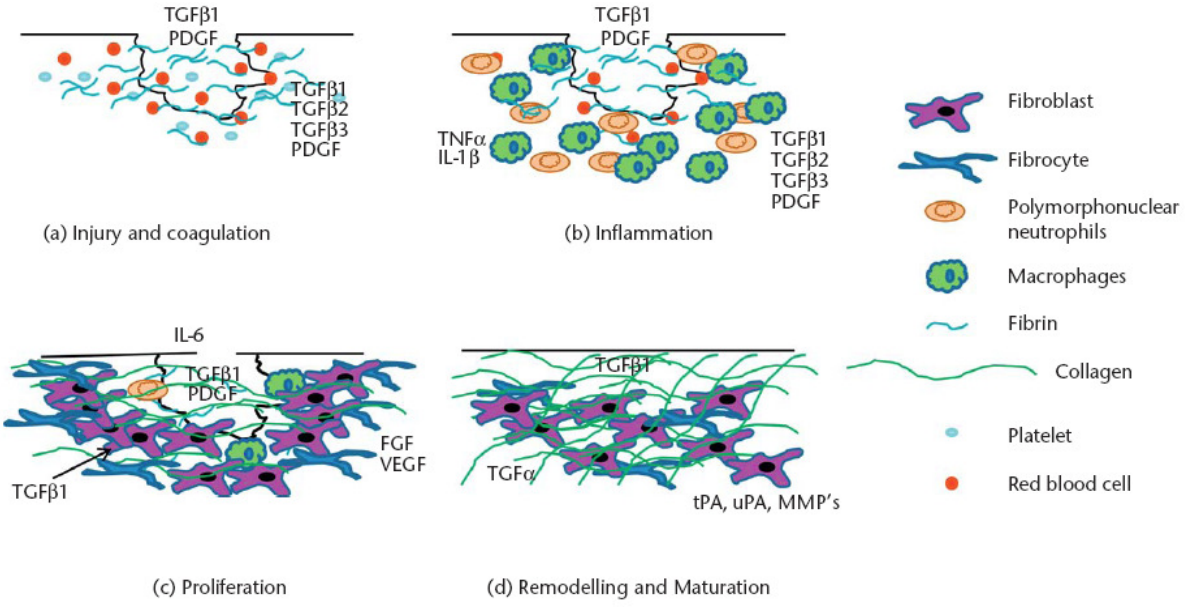


**Figure 2.** Wound healing divided in four phases: a) Injury and Coagulation (Haemostasis), b) Inflammation, c) Prolif-eration and d) Remodeling and Maturation. (Metcalfe & Ferguson, 2011)

After an injury the blood vessels around the wound are contracting (vasoconstriction) and the capillaries are widening to reduce the blood loss and provide the haemostasis (Lindholm, 2007). The haemostasis phase, through clot formation, starts directly to prevent hemorrhage from damaged blood vessels (Martin, 1979). The clot mainly consists of aggregated platelets and a mesh of cross linked fibrin fibers, which are produced through cleavage of fibrinogen by the enzyme thrombin. The platelets also release cytokines and growth factors, such as: platelet-derived growth factor (PDGF), transforming growth factors (TGF-α and TGF-β), epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1) and fibroblast growth factors (FGF's) (Metcalfe & Ferguson, 2011). These molecules function as inflammatory mediators and they play a crucial role in the wound repair progression. Simultaneously as clot formation proceeds another process counteracting clot formation via the proteolytic enzyme plasmin. Plasmin becomes activated when the skin is damaged and then degrades fibrin (Lindholm, 2007). This process is called fibrinolysis and its purpose is to prevent clot formation at places where new blood vessels are being formed and to ease cell migration. Clot formation and fibrinolysis are perfectly balanced in wounds (Lindholm, 2007).

The inflammatory phase is often described to start when polymorph nuclear neutrophils arrive to the wound site (Metcalfe & Ferguson, 2011). Neutrophils degrade and remove damaged and denatured extracellular matrix components through release of proteases, such as, collagenase and elastase. In addition, neutrophils fight the infection by killing bacteria (Lindholm, 2007). Metcalfe & Ferguson (2011) say that the maximum number of neutrophils in the wound has been reached after 24-48 hours from the trauma. Further the neutrophils decline and instead monocytes from the blood stream enter the wound and differentiate to macrophages (Lindholm, 2007). Macrophages engulf bacteria through a process called phagocytosis and therefore prevent infection. Furthermore, macrophages are believed to play the most important role in the release of growth factors at the wound site due to their duration for several days in the wound (Metcalfe & Ferguson, 2011). According to Lindholm (2007), macrophages attract fibroblasts to the wound and also stimulate the fibroblasts proliferation through release of the growth factors TGF-β and PDGF. Throughout the epithelium another type of defensive cells, Langerhans cells, also mentioned in chapter *The Skin*, are located (Metcalfe & Ferguson, 2011). Langerhans cells are dendritic cells and their main function is to present foreign protein antigens as e.g. bacteria to T-lymphocytes in the immune system. Langerhans cells also engulf and degrade bacteria in their lysosomes (Metcalfe & Ferguson, 2011).

During the proliferation that lasts for 3-4 weeks new tissue and blood vessels are being formed (Lindholm, 2007). Macrophages decline and keratinocytes, fibroblasts and epithelial cells take over the production of growth factors and secretion (Metcalfe & Ferguson, 2011). Fibroblasts arise 48 hours after the wound appeared (Lindholm, 2007) and become the leading cell type with secretion of collagen to the extracellular matrix (Metcalfe & Ferguson, 2011). In addition to collagen secretion, Lindholm (2007) mentions that fibroblasts main functions are those also mentioned before: growth factor production to stimulate cell growth and angiogenic factors production for regulation of new blood vessels to form. Formation of new blood vessels is crucial, because the fibroblasts require nutrients from the blood to survive and proliferate. If the formation of new blood vessels are disrupted the consequence will be stopped wound healing. The arrangement of small blood vessels and connective tissue with different kind of cells and collagen, which replaces the fibrin clot through fibrinolysis and cell migration, is called granulation tissue (Singer & Clark, 1999). Reepithelialization of the wound starts from wound margins, detached cells from stratum basale and possibly from remnants of hair follicles (Martin, 1979). Keratinocytes migrate in the wound and dissolve the fibrin clot through the enzyme plasmin, which becomes activated of tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA) and fibrinolysis. When two or more keratinocytes meet, epithelialization stops, a

phenomenon called contact inhibition (Lindholm, 2007). In the end of the epithelialization when the wound is covered of new keratinocytes the stratum basale is recovered. At approximately the fifth day post wounding the wound starts to contract. Fibroblasts and myofibroblasts contract the wound margins with bundles of actin-filaments (Singer & Clark, 1999).

The remodeling phase last for months to years and aims to remodel collagen, in the skin, into a structure with more tensile strength (Metcalfe & Ferguson, 2011). Matrix metalloproteinases, produced by fibroblasts and macrophages, remodel collagen and strengthen the skin by forming larger collagen bundles with an increased number of cross-links (Singer & Clark, 1999). However, the result will most often be a scar and the total tensile strength, compared to normal skin, will be at maximum 70 %.

## 2.3. Infection & Wound microbiology

All wounds contain some bacteria more or less, yet it is not said that all wounds are infected (Percival & Cutting, 2010). A chronic wound is by definition "a wound that does not heal in an orderly set of stages". Chronic wounds may appear due to a lot of reasons, such as diabetes, circulatory impairment, inflammation, infection, reduced ability to move and malnutrition (Bowler et al., 2001; Sussman & Bates-Jensen, 2007; Bryant & Nix, 2007). According to Percival & Cutting (2010), chronic wounds that show signs of erythema, swelling, heat and pain probably are infected with bacteria. Still, chronic wounds with a lack of these signs may be infected. Some other infection signs are: lack of granulation tissue, friable granulation tissue, unhealthy color, degraded wound bed and excessive of exudate in the wound (Percival & Cutting, 2010).

Whether a wound is of acute or chronic character it offers a moist, warm and nutrient environment (Bowler, Duerden & Armstrong, 2001), hence an infection of a wound by bacteria is not unexpected. Most often, the infection is caused by transient bacteria, which then might multiply in the beneficial wound environment. In some cases, when the normal microbiota bacteria are moved from their normal environment, they also can cause an infection of a wound (Lindholm, 2007). Factors that may predispose for an infection are a decreased host immune response, insuffiency of nutrients, trauma and implantation of foreign materials (Lindholm, 2007).

By definition, a clinical infection can be described as an invasion of pathogenic microorganisms in viable tissue. The quantitative measure of pathogenicity in a host is called virulence (Madigan & Martinko, 2006). To be called an infection a wound should contain microorganisms with a density of $10^5$ CFU/g tissue or mL tissue fluid (Lindholm, 2007; Percival & Cutting, 2010). Bowler et al. (2001) claim that the majority of the bacteria causing infections or disruptions of the healing process in both acute and chronic wounds are aerobic or facultative pathogens. The most common ones are beta-hemolytic streptococci, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. However, Bowler (1998) reviewed studies and in 33 of them, which all were about wound infection, he found that anaerobic bacteria, such as *Bacteroides* species and *Peptostreptococcus* species, were among the most frequently microbial isolates. The reason to why anaerobic bacteria often not are mentioned could be cause of the difficulties in isolating and identifying them. At last, it should be mentioned that wound infections, chronic or not, are polymicrobial (Percival & Cutting, 2010).

### 2.3.1. Pathogenic bacteria

*Staphylococcus aureus* is an aerobic Gram-positive bacterium (Madigan & Martinko, 2006). It tolerates reduced water potential fairly well, as mentioned in chapter *Human microbiota*, and also high salt concentrations. *S. aureus* grow in clusters and are facultative aerobes, further they produce acids from glucose both aerobically and anaerobically. To spread from an initial location *Staphylococcus* bacteria are able to produce hyaluronidase, which is an enzyme that breaks down hyaluronic acid, an intercellular polymer that works as cementing substance (Madigan &

Martinko, 2006). In addition, these bacteria produce proteases, lipases and nucleases that break down proteins, lipids and nucleic acids, respectively. One example is coagulase that is a fibrin-clotting enzyme, which promotes formation of fibrin clots that often appear at sites of wound infections. Hence, the enzyme coagulase protects the bacteria from attack by host cells by causing coating of the bacterial cells with the fibrin. Madigan & Martinko (2006) describe that pathogen produced extracellular proteins are called virulence factors if they help in establishment and maintenance of disease. Virulence factors most often are enzymes that aid in the colonization and growth of a pathogens, for example above mentioned coagulase.

*S. aureus* can cause serious infections in humans and is the most predominant bacteria found in chronic wounds (Percival & Cutting, 2010). Moreover, it is the most problematic bacterium in burn and surgical wound infections. *S. aureus* can be recognized by its yellow pigment and it can be found in pathological conditions as boils, pimples, arthritis and meningitis (Madigan & Martinko, 2006). Studies have shown that *S. aureus* has been found in wounds together with other bacteria, such as *P. aeruginosa* (Percival & Cutting, 2010)

*Pseudomonas aeruginosa* is an aerobic Gram-negative bacterium, which usually has a much thinner cell wall compared to Gram-positive bacteria (Madigan & Martinko, 2006). They are rods with polar flagella, which offer them mobility. Primarily, *P. aeruginosa* is a soil bacterium and has simple nutritional requirements; hence it can grow in many different conditions (Madigan & Martinko, 2006). *P. aeruginosa* is known to produce a wide range of virulence factors, such as adhesions, flagella, pili, alginate, elastases, proteases and pyocyanin, some of these are a part of the intrinsically resistance against antimicrobial agents (Percival & Cutting, 2010; Lyzak, Cannon and Pier, 2000; Van Delden and Iglewski, 1998).

Since *P. aeruginosa* is an opportunistic pathogen, it only infects hosts with reduced resistance, such as patients having a wound or a disease (Madigan & Martinko, 2006). According to Percival & Cutting (2010), *P. aeruginosa* is the primary cause of infection in chronic wounds. Moreover, infections of the urinary and respiratory tract of humans are frequently caused by *P. aeruginosa*; also the bacteria can be problematic in severe burns and cystic fibrosis patients (Madigan & Martinko, 2006).

### 2.3.2. Microbial progression of wound infections

Concerning the microbiology of infections, the progression of an infection and establishment of microorganisms can be divided in six overlapping stages (Percival & Cutting, 2010): *Contamination*, *Colonization with reversible adhesion*, *Colonization with irreversible adhesion*, *Critical colonization*, *Local infection* and *Systemic infection*.

Biofilm has been observed and found for many years in chronic wounds (Percival & Cutting, 2010). Since chronic wounds heal poorly biofilm is thought to be one disrupting factor in the healing process of wounds. Furthermore, *critical colonization* is in good correlation to the biofilm model, which therefore may be a good model and aid in describing the complexity of the microbiology of a wound infection. In this chapter the microbiology of infections is described in parallel with the biofilm formation theory.

According to Percival & Cutting (2010), all wounds are or have been in the *contamination* stage, which means that all wounds contain microorganisms, though, necessarily not harmful ones or harmful concentrations. Microorganisms in wounds originate from the skin, mucosa membranes or the surroundings (transient). They are most often not harmful for the host, since the host defense system prevents the microorganisms from multiply into a dangerous number. A wound is a new environment for contaminating microorganisms; therefore the microorganisms have to assess the surroundings, which represent the adaptive- or lag-phase in their growth cycle. Environmental factors, such as pH, temperature, oxygen content and certainly the availability of nutrients, which is of excess in the wound, will

influence the growth of micro-organisms (Madigan & Martinko, 2006).

As mentioned, bacteria in a new environment will start with adaption and assessment of the surroundings. This includes that chemical receptors on the cell wall of the bacteria will assess the environment and if it is beneficial the bacteria will attach to a surface (Percival & Cutting, 2010). This also is between bacterial cells (Dunne, 2002). However, bacteria are not believed to directly attach to an abiotic or a biotic surface, instead bacteria interact with a conditioning film that coats all surfaces within milliseconds (Percival & Cutting, 2010). The conditioning film in humans consists of collagen, fibrinogen, water, serum albumin, lipids and extracellular polymers, which originate from tears, blood, urine, saliva and other organic body fluids. Dunne (2002) explains that for an attachment between a surface and the surface of a planktonic (free swimming) bacterium to occur the distance has to be around 1 nm and the net sum of the physiochemical interactions (e.g. hydrophobic and electrostatic forces; steric hindrance) has to be attractive. Planktonic bacteria often have flagella, which via chemotaxis enhance the probability to find a surface. In addition, an environment that benefits attachment for bacteria will stimulate the production of adhesins, such as pili (Dunne, 2002). Adhesins are stereo specific molecules located on the surface of bacteria, which help bacteria interacting with surfaces (Percival & Cutting, 2010). This stage where single planktonic bacteria assemble to the surface is called *colonization with reversible adhesion* and is included in Figure 3. If attachment between bacteria and surface is favorable, reversible attachment will proceed, and as result, a cluster of cells will proliferate on the exposed surface. Since microorganisms in the *contamination* and *colonization with reversible adhesion* stages are considered to be planktonic and reversibly attached, respectively, they can easily be washed away with low levels of shear forces, for example a saline solution (Percival & Cutting, 2010).

As bacteria attach to surfaces and go from a planktonic phenotype to a more biofilm-like phenotype they will resist the host defense and antibacterial agents better (Percival & Cutting, 2010). This can be explained by that bacteria begin to produce intracellular and extracellular substances that will aid in the formation of biofilm, such as extra-cellular polymeric substances (EPS), which are composed of polysaccharides, nucleic acids, proteins and glycoproteins. EPS act as a cementing matrix that also, complementary to adhesins, enhance the attachment for bacteria to surfaces (Percival & Cutting, 2010).

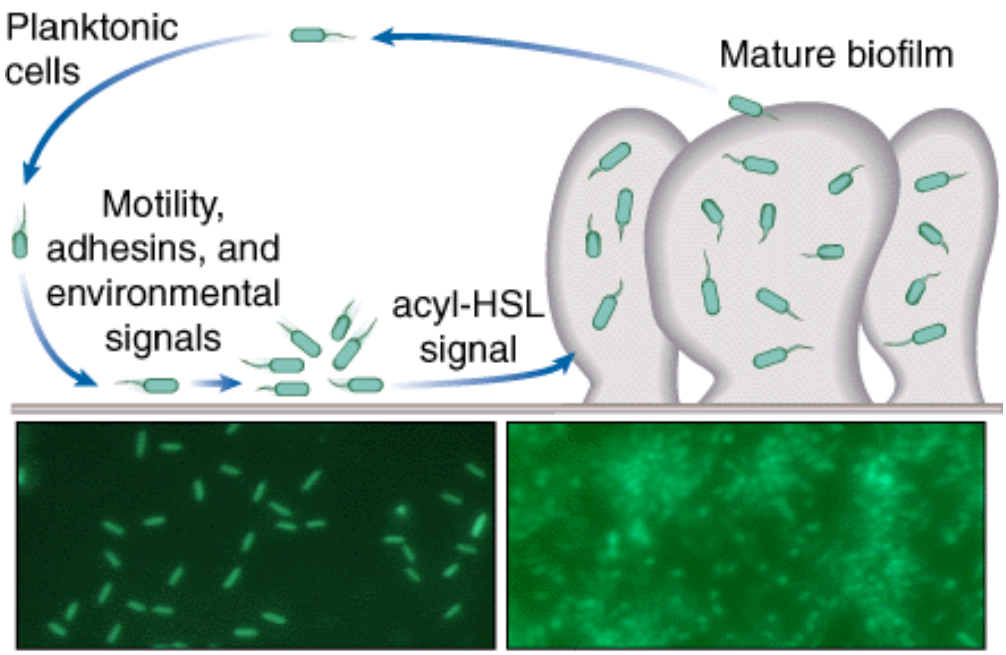


**Figure 3.** Formation of a biofilm, which is common in chronic wound infections. Single planktonic *P. aeruginosa* bacteria assemble to the surface and proliferate. Further cell-to-cell communication (QS) induces formation of a biofilm, through acyl homoserine lactones (acyl-HSL) signal molecules, that mainly is composed of the protective and viscous EPS. The lower left and lower right images show free floating planktonic bacteria and bacteria in biofilm, respectively. (Kolter & Losick, 1998)

In the *colonization with irreversible adhesion*, bacteria are favored of the environment and the net interaction between bacteria and surface is stronger compared to the net interaction in the *colonization with reversible adhesion* stage (Percival & Cutting, 2010). EPS production proceeds and bacteria become encased of EPS, which strengthen the resistance against removal from the wound bed. Further, bacteria multiply and form aggregates, inheritance of antibacterial resistance develops and as a result of the assessment of the environment the genetic profiles are influenced. Dunne (2002) claims that adhesion between bacterial cells can occur, not only within species, but also between different kinds of species, hence one kind of species is able to promote establishment for another type of specie in a wound. Along with proliferation of bacteria in a wound the nutritional and surrounding conditions change, which may favor other kinds of micro-organisms (Percival & Cutting, 2010).

One example of dramatic change of the surrounding is when massive proliferation of aerobes in parts of a biofilm will result in hypoxia (Percival & Cutting, 2010). Bowler et al. (2001) mention that local $pO_2$-levels of 5 to 20 mmHg have been found in non-healing wounds. In comparison, reports tell that well perfused periwound tissue has between 60-90 mmHg $pO_2$ while dead tissue can have total lack of oxygen (Bowler et al., 2001). Moreover, infected tissues have been studied and levels below 30 mmHg were measured. These low oxygen levels in comparison to the process of active cell division, which requires around 30 mmHg (Bowler et al., 2001), may explain the difficulties in healing of an infected wound. Additionally, at the 30 mmHg $pO_2$, polymorphonuclear leukocytes killing capacity was reduced, significantly (Bowler et al., 2001). Hence the host immune system with its antibacterial properties will be depressed. The low oxygen levels in a wound will probably result in establishment of anaerobic bacteria, which will increase the diversity of microbes in the wound (Bowler et al., 2001; Percival & Cutting, 2010). All these facts indicate that poorly perfused wound tissues have a much greater probability of getting a prolonged healing and infected than well perfused wound tissues.

*Critical colonization* is often referred to when the number of bacteria has reached $10^5$ CFU/g

tissue, or mL wound fluid (Percival & Cutting, 2010). However, since *critical colonization*, for a microbiologist, means the critical level of bacteria in a wound the number differs depending on patient, wound type and bacterial species. According to Siddiqui and Bernstein (2010), colonization of a wound sometimes may aid and hasten the healing, due to that a local infection stimulates inflammation that further increase perfusion, which is an important factor in the healing process. Nevertheless, in the *critical colonization* stage bacteria have colonized and proliferated to a critical number where the healing of wounds is delayed (Percival & Cutting, 2010). Although in *critical colonization* there are no clinically signs of infection and no visible host reaction can be observed. Moreover, microorganisms continue to multiply and the diversity of the wound (biofilm) microbiota increases cause of the many interaction and synergistic effects that occur between species. In the wound microbiota many niches develops that leads to persistence and a greater chance for survival for the microorganisms. Siddiqui & Bernstein (2010) claim that the wound in the *critical colonization* stage might go into a non-healing chronic inflammatory state.

The wound is considered to enter the *local infection* stage when bacteria grow exponentially into a fully mature biofilm (Percival & Cutting, 2010). Also, in this stage bacteria master the host's immune response and host injury is observed (clinical signs mentioned earlier) (Siddiqui & Bernstein, 2010). The efficacy of the host immune system can be reduced by a lot of reasons, such as, malnutrition, obesity, diabetes and age. Therefore the risk of getting an infection differs among people and time in life.

If the local infection not is treated and bacteria proliferate further the local infection may spread in the body and turn into a severe *systemic infection* (Percival & Cutting, 2010). Also, in mature biofilms some bacteria detach from the biofilm and become free swimming planktonic bacteria. The way for bacteria to enter new tissues can be through the blood (bacteremia). In more severe cases bacteria may proliferate in the blood and start toxin production (sepsis) (Percival & Cutting, 2010). In worst case scenario the outcome can be failure of organs and even death of the host.

### 2.3.3. Does infection depend on the bacterial phenotype?

Interestingly, The National Institutes of Health (NIH) states that as much as 80 % of human infected wounds, for example chronic wounds and medical device infections, are caused of biofilm phenotype bacteria (Percival & Cutting, 2010). The last 20 % of human infections, typically acute infections, as sepsis, are instead caused by planktonic phenotype bacteria. So, why is the biofilm phenotype that successful in proliferating wounds compared to the planktonic phenotype?

According to Siddiqui & Bernstein (2010) and also earlier described, a biofilm is a community of bacteria living at a liquid interface. Bacterial colonization and biofilm formation occur on almost any surface, such as, ship hulls, petroleum and dairy pipelines, contact lenses, biomedical devises and wounds (Dunne 2002). The plaque on teeth is composed of biofilm, though we prevent the maturation of biofilm by brushing our teeth daily (Percival & Cutting, 2010). Mature biofilms are composed of microcolonies encased of the main matrix substance EPS (Siddiqui & Bernstein, 2010), see Figure 4. As seen in Figure 3 the mature biofilm is composed of a special structure called mushrooms and pillars (Serralta et al., 2001). The microcolonies within this structure are supplied with nutrients and are able to remove their metabolic waste products in a very efficient way via water channels, which act as a circulation system throughout structure. Additionally, the biofilm give shelter against the often hostile surrounding environment and communication opportunities, called quorum sensing, that may lead to increased virulence and risk for an infection (Siddiqui & Bernstein, 2010).

Recently, it has been shown that several unicellular bacteria have the ability to communicate with each other and therefore act as multicellular bacteria (Hooshangi & Bentley, 2008). This is known as quorum sensing that often is connected to biofilm formation, bacterial

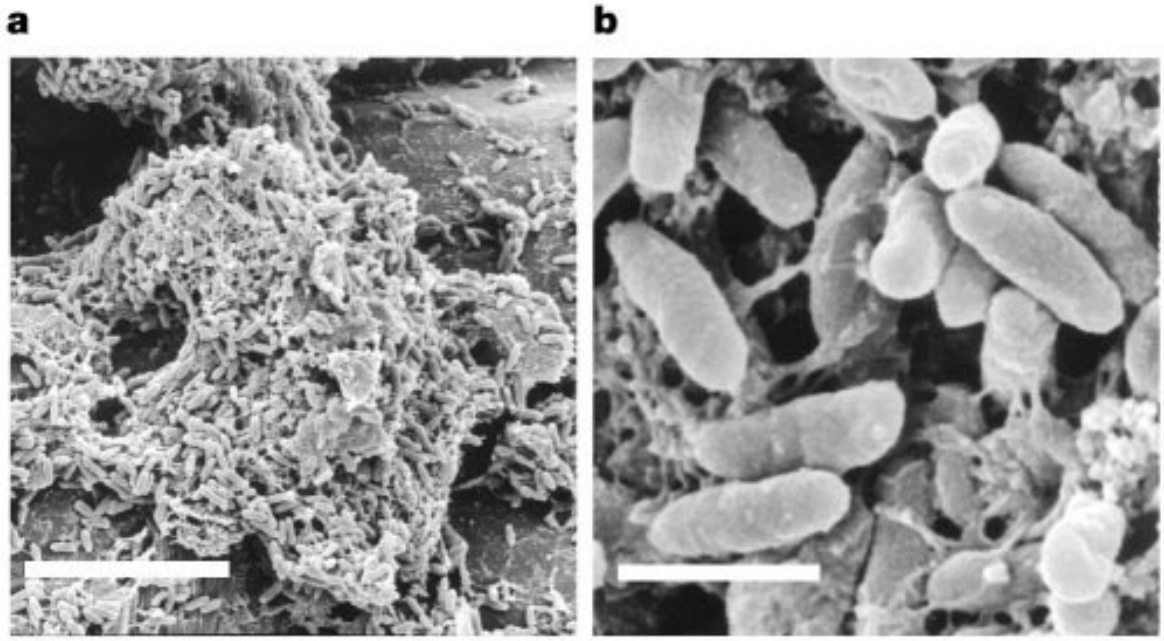


**Figure 4.** Scanning electron microscopy images of *P. aeruginosa* in biofilm. a) Biofilm on the surface of a pebble, scale bar: 10 mm. b) Rod shaped *P. aeruginosa* in a biofilm, also EPS between the bacterial cells is shown, scale bar: 1 mm. (Whiteley et al., 2001)

pathogenicity and virulence (Hooshangi & Bentley, 2008). In the communication process bacteria produce and release signal-molecules, which can be detected by other bacteria. Further the signal-molecules regulate gene expression in the bacterial receiver cells. According to Hooshangi & Bentley (2008), there exist three types of signaling-molecule classes: oligopeptides, acyl homoserine lactones (acyl-HSL) and autoinducer-2. As shown in Figure 3 acyl-HSL is used as a communication molecule by *P. aeruginosa* but acyl-HSL is also commonly used by other Gram-negative bacteria. The oligopeptide class is used by Gram-positive bacteria while the autoinducer-2 molecule has been found to be a universal signal molecule, which means that it is used by many Gram-negative and Gram-positive bacteria. Quorum sensing is critical for multicellular communities, such as biofilms, to be able to establish (Kolter & Losick, 1998).

Fux at al. (2005) mean that bacteria in biofilm have a lot of similarities to stationary-phase bacteria. Both these phenotypes live in a high cell density environment and as a consequence nutrient limitations follows. Moreover, studies of antibiotics have shown that antibiotics with a good effect against stationary-phase bacteria *in vitro* also cure biofilm infections *in vivo* more successful (Fux at al., 2005). The fact that bacteria in biofilm grow slower than the free swimming planktonic phenotype may affect the efficacy of antibacterial substances, due to decreased uptake of substance and physiologic changes of bacteria in biofilm (Siddiqui & Bernstein, 2010). Studies tell that bacteria in biofilm resist antibacterial agents up to 500 times better than planktonic bacteria. Percival & Cutting (2010) confirm this fact by reporting a 1000 times better resistance against biocides and antibiotics than planktonic bacteria, also they report that biofilm bacteria are 1000 times less affected of the host immune system.

The reasons for the significant increased resistance for the biofilm community may be explained by that more than 800 proteins showed a six fold or greater change in expression level between planktonic and matured biofilm cells (Sauer et al., 2002). The changed protein production patterns in mature biofilm phenotype cells surely contribute to the sustained host inflammation that appears in biofilm infections (Percival & Cutting, 2010). Studies have shown that bacteria in biofilm manipulate the host innate immune response to produce more of proinflammatory cytokines and also to enhance the proteolytic activity in the environ-

ment. Inflammation in the wound provides bacteria with nutrients and remains the beneficial environment of an open wound. Since biofilm communities have an increased resistance against the host immune system a sustained inflammation will be a beneficial strategy for the biofilm bacteria.

The importance of the biofilm matrix for bacterial survival is confirmed in a study of Fux et al. (2005), where they studied bacterial mutants with a knockout of polysaccharide intercellular adhesin (PIA). In staphylococcal biofilms PIA is one of the major biofilm matrix components. Consequently, as expected, the bacteria with the PIA mutation were killed easier by the host immune defense, through macrophages, for example. In addition, antibacterial peptides that naturally are present on the human skin had an increased killing effect on the PIA mutants.

In comparison, planktonic bacteria, as mentioned, most often are found in acute wound infections (Percival & Cutting, 2010). The planktonic phenotype bacteria are besides to antibacterial agents also easier affected of environmental changes, ultraviolet light and host immune defense compared to the biofilm phenotype bacteria. Percival & Cutting (2010) tell that the planktonic phenotype tend to behave more predatory by attacking its host by destroying and degrading. Subsequently, the bacteria feed on the dead cells and other material. Also, planktonic bacteria secrete virulence factors and proteins that cause invasion of the host immunity, destruction of the host cells and further feeding on the dead material (Percival & Cutting, 2010). The predatory behavior leads to relative quick responses in the host. This because planktonic bacteria lack the advantage of a colony defense with the protecting EPS, thus they are more susceptible to antibiotics, which results in that an acute infection normally is cured after a few days or weeks. On the other hand, if the antibiotics fail the host may die relative rapidly (Percival & Cutting, 2010).

### 2.3.4. Topical treatment of wounds

Bacteria colonizing wounds are one of all factors that may disturb wound healing (Dowsett, 2004). Vermeulen et al. (2009) tell that topical agents, such as antibiotics, antiseptics and disinfectants, which may kill microorganisms or prevent their growth, can be used together with a dressing to prevent or treat a wound infection. Today, there are several dressings with topical agents on the market designed to prevent and treat burn, surgical and chronic infections (Dowsett, 2004). One group of topical agents includes ointments, creams and impregnated dressings, which are designed to stay in contact with the wound surface for a longer time (Vermeulen et al., 2009). Silver dressings are one of the most used impregnated dressings. First of all, silver dressings function, as also non-impregnated dressings do, by acting as a barrier against exogenous bacteria and therefore prevent infection or wound contamination. Additionally, silver containing dressings release silver to the wound, alternatively bacteria are absorbed into the silver dressing and thereafter killed by silver within the dressing (Lansdown, 2004). The speed of silver release can be controlled by incorporate silver as complex silver molecules into foam dressings but also into ointments, creams and hydrogels (Vermeulen et al., 2009).

Today, there exist a lot of different kinds of silver dressings with variety in composition, mechanism of action and silver release rate (Lansdown, 2006). The different kinds of silver dressings are produced and adapted for treating burns, acute surgical wounds and hard to heal wounds, which are related to severe patient discomfort with exudations and unpleasant odors.

## 2.4. Silver

Humans have used silver as an antimicrobial agent since ancient times (Lansdown, 2004). Monarchs of dynasties in the ancient Middle East and South America put silver coins in their drinking water for purification. Furthermore, when settlers came to western America they put silver dollars in their water barrels to preserve the water (Burrell, 2003). So the effect of silver has been known for a very long time, and present silver is being used in surgical needles, catheters, dentistry and in wound care (Lansdown, 2004). In some of these

products silver is incorporated in polymeric substances, such as artificial skin, bone cement or nylon (Schierholz et al., 1998).

Silver is a precious metal, which means that it is chemically stable, and it is not toxic against human tissue in its non-ionized form (Ag) (Lansdown, 2004). Nevertheless, ionized silver has antimicrobial effect and occurs predominantly as $Ag^{+}$, but also as $Ag^{2+}$ and $Ag^{3+}$, though these last two forms are unstable and therefore very rare (Lansdown, 2004). Silver becomes ionized when it comes in contact with moisture and fluids, such as wound fluids. Since it is generally accepted that it is the silver ion ($Ag^{+}$) that has antimicrobial properties, different silver salts and complexes are being used as carriers in order to release $Ag^{+}$ when the salt or complex are dissolved in fluid (Schierholz et al., 1998). One historically used silver salt is silver nitrate, which has been successful in treatment of infectious diseases and especially in burn wounds (Lansdown, 2004). In 1884 Crede used a 1% solution of silver nitrate to prevent eye infections in newborn babies (Burrell, 2003). *In vitro* tests have shown that silver nitrate affects fibroblasts, lymphocytes and hepatocytes negatively. Atiyeh et al. (2007) also claim that silver is toxic, but mean that silver containing dressings has a definite positive effect on wound healing by reducing the microbial burden that disturbs the healing.

Silver is effective against wide range of aerobic, anaerobic, Gram-negative and Gram-positive bacteria, filamentous fungi, yeast and viruses (Burrell, 2003). The reason why the silver ion ($Ag^{+}$) has antimicrobial properties is because it is highly reactive and binds strongly to electron donor groups containing sulfur, oxygen and nitrogen (Schierholz et al., 1998). Such electron donor groups are for example thio-, amino- phosphate groups, which all are common biological molecules. According to Lansdown (2004), Jung et al. (2008) and Pal et al. (2007), silver ions absorbed by sensitive strains:

- Impair bacterial cell wall and/or cell membrane structure
- Bind and disrupt subcellular components
- Inactivate bacterial DNA and RNA
- Impair essential enzymes and metabolic enzymes modulated by sodium, magnesium, phosphate etcetera

Since silver ions are very reactive the choice of medium in *in vitro* testing is crucial and will affect the antimicrobial effect (Hermans, 2006). In a wound environment fluids, such as exudates, may be present and exudates contains a high percentage of chloride ions ($Cl^{-}$) that together with silver ions ($Ag^{+}$) precipitate to silver chloride (AgCl), which has a very low solubility. The common opinion is that silver chloride has not any antimicrobial properties, thus the antimicrobial effect in a fluid or medium with chloride will be reduced (Hermans, 2006). Burrell (2003) means that the concentration of silver in an aqueous system may be as low as 0.01 ppm to control bacteria. In contrast the MIC in a complex media may be around 20-40 ppm of silver. Another study reports that the MIC for five clinically relevant bacteria in Mueller-Hinton broth, a complex media, was found between 5-12.5 ppm of silver (Yin et al., 1999).

The increased use of silver in wound care has formed a debate whether silver resistance is a problem or not (Percival et al., 2005). There are studies that have reported bacterial resistance to silver, but since the exact mechanism of action is unknown and little evidence exist, more studies have to be done. The fact that antibiotic resistance is a problem is because antibiotics often target a specific site on the cell wall or within the cell. In contrast, silver and other biocides normally have several modes of actions and target multiple sites. Therefore the probability of resistance against the silver ion is less than for antibiotics (Percival et al., 2005). Nevertheless, silver resistance genes have been found sporadically in certain bacteria, so more studies are necessary and they will probably tell more about the mechanism of resistance.

# 3. Materials and Methods

In this chapter, the media, other substances and the two bacterial strains that were used in the experiments to resemble a wound like environment are presented. Also, the preparation of silver solution that was used to simulate silver release profiles is presented. Last, the performance and experimental plan for the different kinds of experiments are presented.

## 3.1. Media and chemicals

- Simulated Wound Fluid (SWF), used to simulate a wound like environment, consists of fetal calf serum mixed with an equal amount of peptone water. The SWF contains of salts, proteins, carbohydrates, amino acids, vitamins and other trace elements.
- Peptone water (PW), contains of 0.85% NaCl and 0.1% peptone
- STS-1 (STS-1: 0.9% NaCl, 1% Tween 20, 0.1% sodium thioglycolate), inactivation solution for silver used for *P. aeruginosa*
- STS-2 (STS-2: 6.0% NaCl, 1% Tween 20, 0.1% sodium thioglycolate), inactivation solution for silver used for *S. aureus*
- Tryptic Soy Broth (TSB: 17 g Pancreatic digest of casein, 5 g NaCl, 3 g Papaic digest of soybean meal, 2.5 g Dibasic potassium phosphate, 2.5 g Glucose mixed in 1 L of deionised water)
- Tryptic Soy Agar (TSA: 15 g Tryptone, 15 g Agar, 5 g Soya peptone, 5 g NaCl mixed in deionised water)

## 3.2. Bacteria

To simulate wound like environment two pathogenic bacteria, which are frequently appearing in wounds, were used:

*Pseudomonas aeruginosa*, PAO1
(*P. aeruginosa*)

*Staphylococcus aureus*, ATCC 6538
(*S. aureus*)

## 3.3. Silver solutions

Silver sulfate ($Ag_2SO_4$) was used, since it is one of the silver substances used in MHC's silver containing wound care products. A stock silver sulfate solution was prepared by dissolving 0.57 g of silver sulfate (Merck KGaA, EG-nr 2336537) in 100 mL of milli-Q water, to reach the concentration of approximately 3950 ppm silver.

## 3.4. Culturing of bacteria

The experiments started with inoculation of bacteria, either *S. aureus* or *P. aeruginosa*, into a test tube with 3 mL TSB. The colonies were picked from an agar plate, where the colonies originated from cryopreserved bacteria, which have been kept in a -70°C freezer. The test tubes were then incubated at 35°C over night.

The over night cultures had an approximate concentration of $10^9$ CFU/mL. In the next step, the over night culture was transferred to SWF and diluted to approximately $10^6$ CFU/mL in two steps. Firstly, ten fold diluted in PW. Secondly, the diluted bacteria in PW were transferred to SWF, such as the volume ended up as one hundredth of the desired SWF volume.

### 3.4.1. Log-phase experiments

The above mentioned bacterial suspension with SWF had an approximate concentration of $10^6$ CFU/mL and will be in logarithmic phase when it starts to duplicate. These kinds of experiments will further be called *Log-phase experiments*. Aliquots of 2.7 mL of log-phase bacterial suspension were transferred to test tubes for *Log-phase experiments* before exposure to silver.

### 3.4.2. Stationary-phase experiments

For *Stationary-phase experiments* aliquots of 2.25 mL of the bacterial suspension with bacteria in log-phase were transferred to test tubes. The test tubes were incubated further at 35°C for 24 h to reach the stationary-phase with an approximate concentration of $10^8$-$10^9$ CFU/mL before exposure to silver. The bacterial suspensions with SWF and bacteria in sta-

tionary-phase will further be called *Stationary-phase experiments*.

## 3.5. Exposure to silver

To find appropriate silver concentration for silver release experiments, screening and *MIC & MBC* (see chapter *MIC & MBC* for definitions) for *Log-phase experiments* and MBC for *Stationary-phase experiments* were performed. The added silver should reduce the viable count but still be above level of detection.

Silver sulfate was added, at time point 0 h, to the test tube filled with a bacterial suspension, with either ~$10^6$ CFU/mL (*Log-phase experiments*) or $10^8$-$10^9$ CFU/mL (*Stationary-phase experiments*). Subsequently, an initial vortex for each test tube was done for approximately 10 s to mix the silver with the bacterial suspension. All experiments included a negative control (0 ppm of silver) where milli-Q water was added instead of silver solution.

The experimental setup for the screening of the *Log-phase experiments*, presented in Table 1, included both experiments with and without initial vortex after silver had been added. The ambition was to do experiments without initial vortex, since that would resemble the real situation better. However, the results of the screening experiments without initial vortex showed that the highest silver concentration had a very little reducing effect. Better effect and higher silver concentration were required, but could not be achieved due to limited solubility of silver sulfate or too diluted bacterial suspension in the test tube. Thus, it was decided that the further experiments should be performed with initial vortex for approximately 10 s.

**Table 1.** The experimental setup for the screening of bacteria in log-phase (*Log-phase experiments*). These experiments were performed both with and without initial vortex.

| Bacteria | Silver concentration (ppm) | | | |
|---|---|---|---|---|
| *S. aureus* | 0 | 9.87 | 98.7 | 987 |
| *P. aeruginosa* | 0 | 9.87 | 98.7 | 987 |

Each experiment throughout this present study was performed in triplicate and repeated on two different days to verify the results.

### 3.5.1. MIC & MBC (finding appropriate silver concentrations)

The *MIC & MBC* experiments were performed to determine the MIC and MBC for silver sulfate on *S. aureus* and *P. aeruginosa* in log-phase. MIC is the lowest concentration of a substance that completely inhibits growth of bacteria (Madigan & Martinko, 2006). To achieve MIC, the substance is in general, as in this study, added to the test system at the same time as the bacteria and then incubated for 24 h (Nadworny and Burrell, 2008). MBC is in this Master thesis defined as the concentration of a substance that reduces the number of bacteria with at least three log units.

The experimental setup for the *MIC & MBC* experiments is shown in Table 2. By doubling the silver concentration for each new sample the MIC and MBC could be found. Since the step between no inhibition and total reduction (below the detection level) for *P. aeruginosa* was narrow further experiments were done to achieve a more adequate silver concentration with an appropriate antibacterial effect.

**Table 2.** The experimental setup for *MIC & MBC, Log-phase* experiments. The bolded concentrations were later used in *Simulation of silver release profiles experiments*.

| Bacteria | Silver concentration (ppm) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *S. aureus* | 0 | 2 | 4 | **8** | **16** | **32** | 64 | 128 | |
| | | | | | | | | | |
| *P. aeruginosa* | 0 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | |
| | 0 | | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 |
| | 0 | 4 | 5 | 6 | | | | 7 | |
| | 0 | | | **6** | 6.25 | 6.50 | **6.75** | 7 | **8** |

For the *Stationary-phase experiments* only *MBC* experiments were done, since bacteria have stopped grow in stationary phase and therefore MIC could not be determined. In Table 3 the experimental setup is presented.

**Table 3.** The experimental setup for *MBC, Stationary-phase experiments*. The bolded concentrations were later used in *Simulation of silver release profiles experiments*.

| Bacteria | Silver concentration (ppm) | | | | | |
|---|---|---|---|---|---|---|
| *S. aureus* | 0 | 64 | 128 | 256 | 512 | **987** |
| | | | | | | |
| *P. aeruginosa* | 0 | 64 | 128 | 256 | 512 | 987 |
| | 0 | 4 | 8 | 16 | 32 | **64** |

## 3.6. Simulation of silver release profiles

Three types of silver release profiles were simulated by adding silver to the test system every second hour during a six hour period. An example of the silver release profiles is shown in Figure 5.

The amount and volume of the actual silver solution added to the different samples were equal after six hours for all the silver release profiles. Here follows a description of the silver release profiles:

Release profile 1 - Corresponding MIC, corresponds to the MIC & MBC determination where the total amount of silver is added at the start (0 h)

Release profile 2 - also called boost, starts with a boost of silver solution followed with a declining addition of silver solution

Release profile 3 - also called linear, the addition of silver solution is distributed equally over the 6 hours period

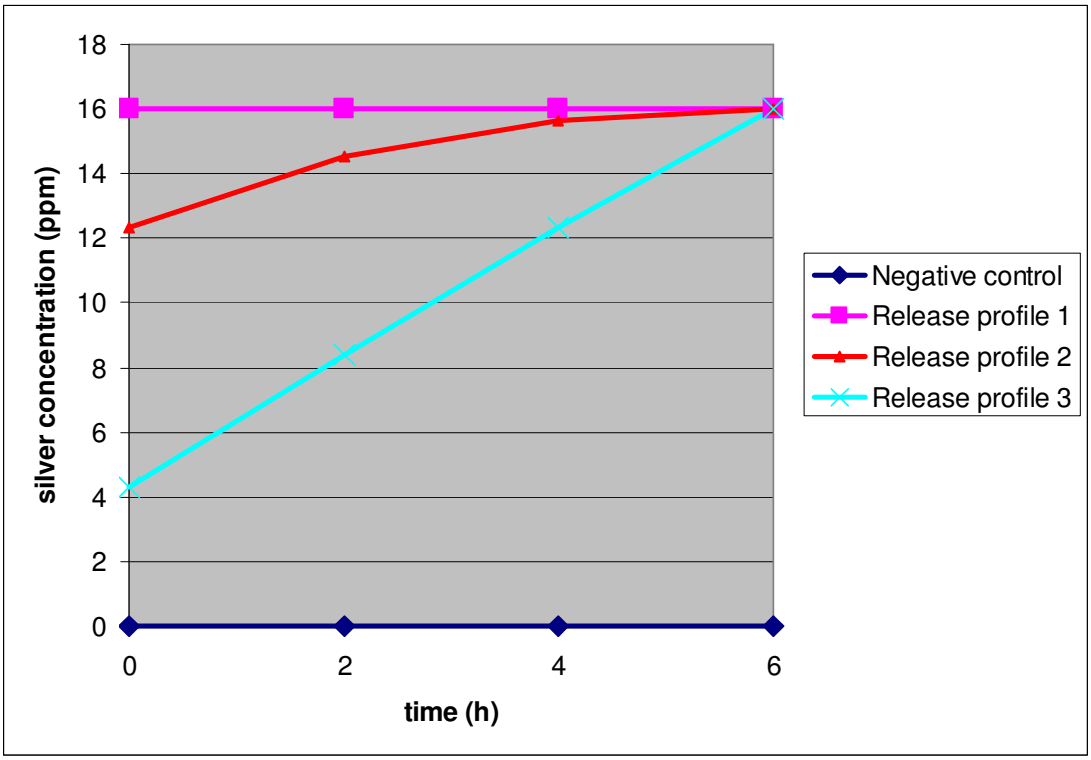


**Figure 5.** The simulation of silver release profiles for *S. aureus* in log-phase with a final concentration of 16 ppm is shown. Silver solution is added at time point 0, 2, 4 and 6 h for Release profiles 2 and 3. Though, release profiles were vortexed at 0, 2, 4 and 6 h, even if there were no addition of silver solution. This figure is one example, all the other *Simulation of silver release profiles* experiments have a corresponding figure (see *Appendix*).

After each addition of silver solution, the test tubes were incubated at 35°C. The Negative control and the Release profile 1 only got addition of milli-Q water and silver solution, respectively, in the start, though all samples were vortexed at 0, 2, 4 and 6 h to be treated equally. After the first addition of silver solution, the experiments were incubated at 35°C in total for 24 h.

## 3.7. Determination of viable count

After incubation, the experiments were stopped by transferring 0.1 mL of the bacterial suspension from each test tube to test tubes filled with either 0.9 mL STS-1 or STS-2 for *P. aeruginosa* or *S. aureus*, respectively. Thereafter, STS was allowed to react, for at least 10 minutes at room temperature, to inactivate silver.

The antibacterial effect in all experiments was determined by viable count and the spread-plate method (Madigan & Martinko, 2006). The test tubes with STS and bacteria were ten-fold serial diluted in PW, and 0.1 mL of appropriate dilutions was spread on duplicate TSA-plates. The same procedure was done on the bacterial suspensions at time point 0 h to get a start concentration of bacteria. Subsequently, the TSA-plates were incubated at 35°C and after night/nights each formed colony represented one single viable bacterial cell. Plates with a range of approximately 30-300 colonies per plate were counted and the number of CFU was recorded.

The detection level for the viable count method used above is $10^2$ CFU/mL.

# 4. Results

The results are presented in diagrams, where the viable cell count (log CFU/mL) for each experiment is shown. First the *Log-phase experiments* are presented thereafter the *Stationary-phase experiments*.

## 4.1. Log-phase experiments

The screening of the *Log-phase experiments* on *S. aureus*, see Figure 6, showed that initial vortex reduced the viable count to a lower level with increasing silver concentration. At 98.7 ppm and 987 ppm, the viable count was reduced by almost 4 log units compared to the inoculation (start culture). Without initial vortex the viable count were reduced by 1-1.2 log units for all three concentrations tested.

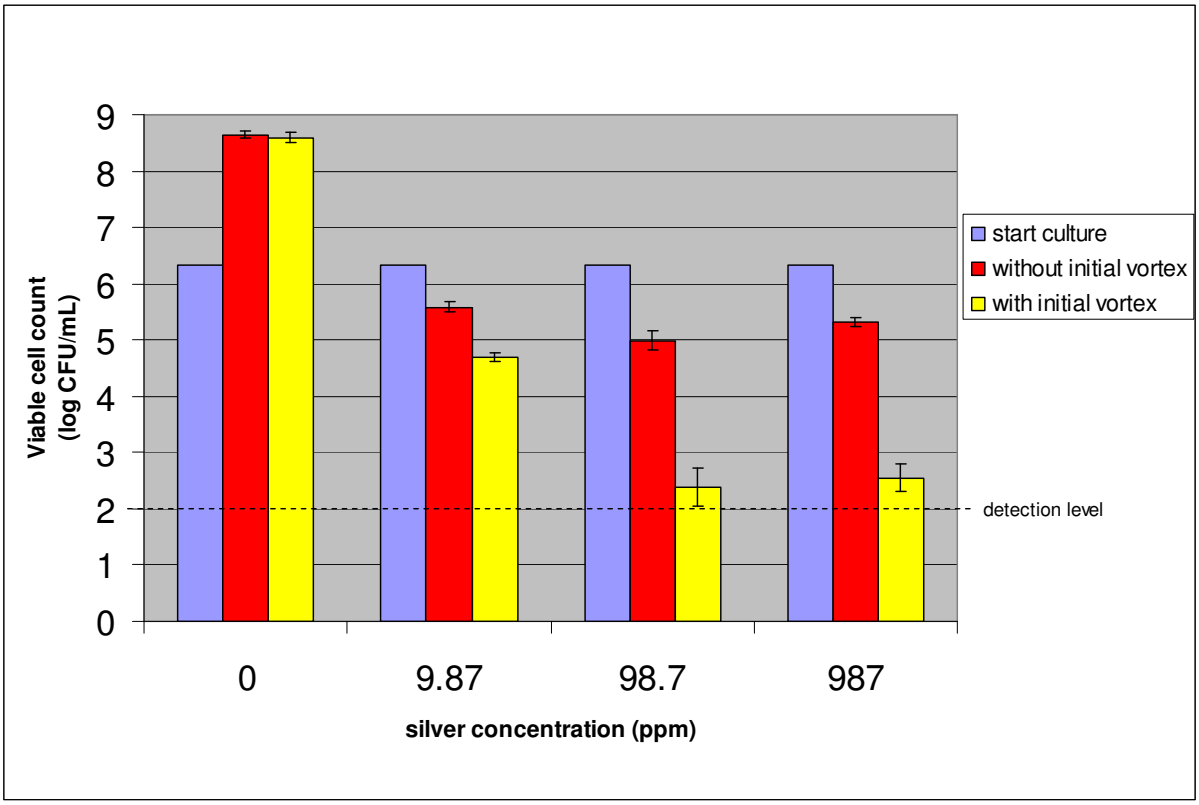


**Figure 6.** Screen of the effect of silver on *S. aureus* in log-phase, with and without initial vortex, with 24 h incubation time. Results are mean of three replicates and standard deviation is indicated by error bars.

The screening of the *Log-phase experiments* on *P. aeruginosa*, see Figure 7, showed that initial vortex reduced the viable count by >4 log units (below detection level) for all three concentrations. Without initial vortex the viable count maintained at the level of inoculation (start culture).

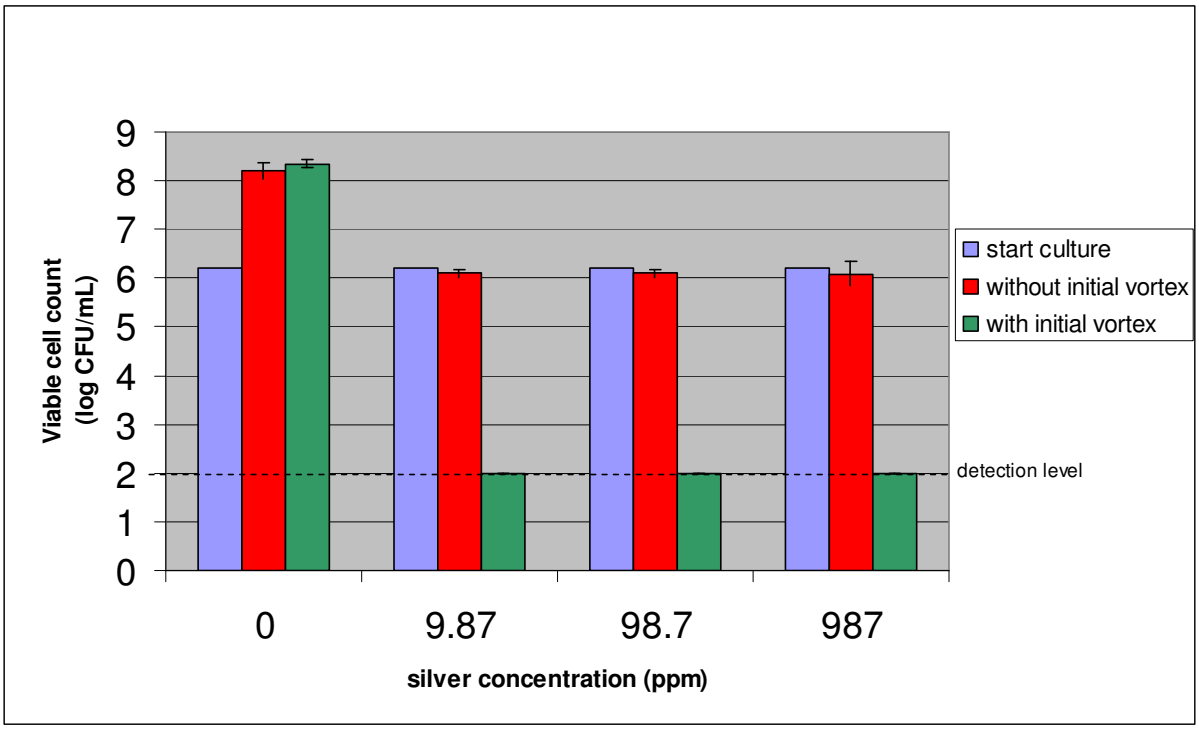


**Figure 7.** Screen of the effect of silver on *P. aeruginosa* in log-phase, with and without initial vortex, with 24 h incubation time. Results are mean of three replicates and standard deviation is indicated by error bars.

### 4.1.1. MIC & MBC

The *MIC & MBC* experiments on *S. aureus* are shown in Figure 8. The MIC for *S. aureus* was 8 ppm and the MBC was 32 ppm of silver.

The silver concentrations above 32 ppm did not reduce the viable count any further.

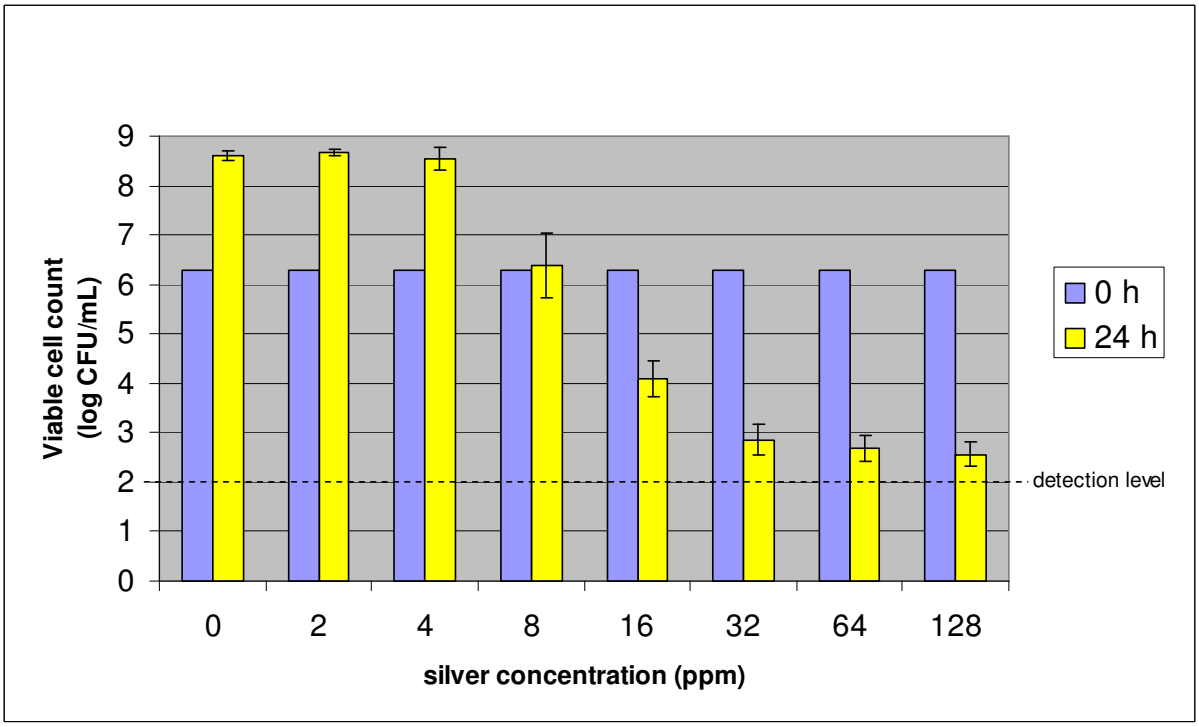


**Figure 8.** *MIC & MBC* experiments on *S. aureus* in log-phase, with initial vortex, with 24 h incubation time. Results are mean of six replicates and standard deviation is indicated by error bars.

The *MIC & MBC* experiments on *P. aeruginosa*, see Figure 9, included more than one experimental setup. Because the step between no inhibition and total reduction for *P. aeruginosa* was narrow the experimental setup had to be repeated with a smaller range to find an appropriate silver concentration for the simulation of the silver release profiles. Therefore, additional experiments were done with silver concentrations between 4-8 ppm (data not presented, see *Appendix*) and 6-7 ppm (see Figure 9B). MIC for *P. aeruginosa* was 6.5 ppm and the MBC was about 7 ppm of silver.

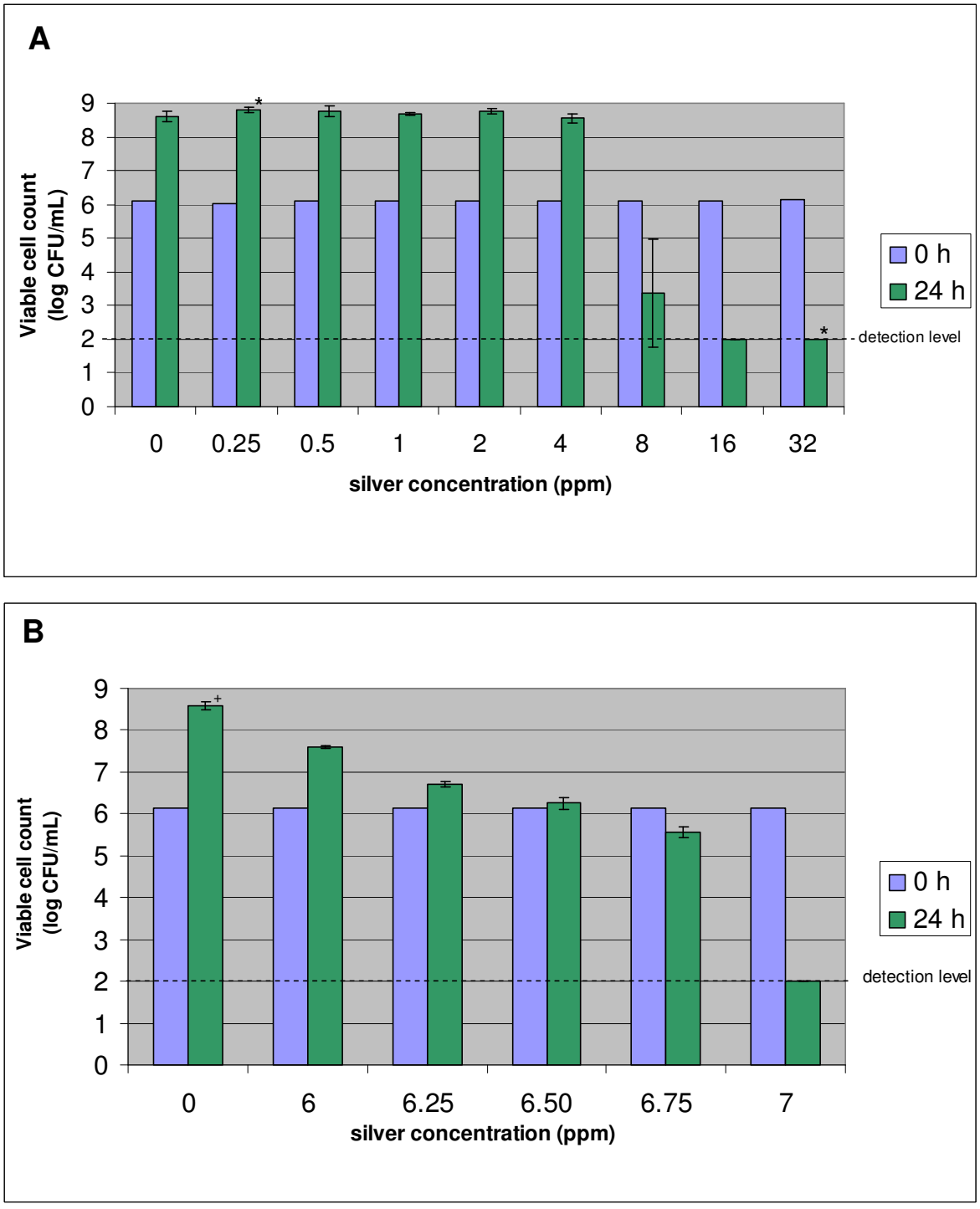


**Figure 9.** *MIC & MBC* experiments on *P. aeruginosa* in log-phase, with initial vortex, with 24 h incubation time. **A)** Broad range: 0-32 ppm of silver. Results are mean of six replicates and standard deviation is indicated by error bars, except for bars indicated by *, which only includes three replicates. **B)** Smaller range: 6-7 ppm of silver. Results are mean of three replicates and standard deviation is indicated by error bars, except for bars indicated by $^{+}$, which only includes two replicates.

### 4.1.2. Simulation of silver release profiles

The three different silver release profiles with 16 ppm reduced the viable count of *S. aureus* to approximately the same level, i.e. 3.3-3.7 log CFU/mL, see Figure 10. When the experiment was repeated with 8 and 32 ppm, the three silver release profiles were reduced to 4.1-5 log CFU/mL and 3-3.7 log CFU/mL, respectively (data not shown) and no difference between the three release profiles could be observed.

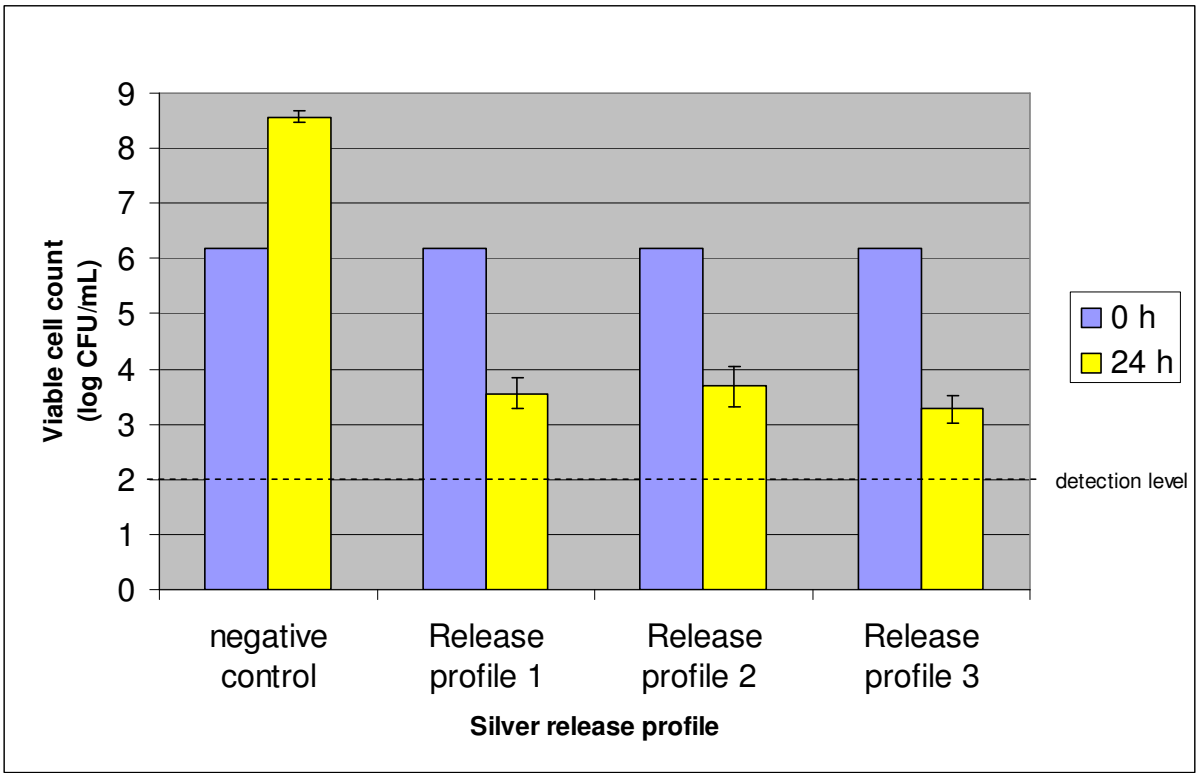


**Figure 10.** Simulation of silver release profiles on *S. aureus* in log-phase, with initial vortex, with 24 h incubation time. The concentration of silver was 16 ppm after 6 h, except for the negative control (0 ppm). Release profile 1, 2 and 3 were simulated as a corresponding MIC, boost and linear silver release profile, respectively (see chapter *Materials and Methods*). Results are mean of six replicates and standard deviation is indicated by error bars.

The concentration used to simulate the silver release profiles on *P. aeruginosa* in log-phase was 3 ppm (see why in chapter *The effect of vortex*), see Figure 11. Release profile 1 (Corresponding MIC) and Release profile 2 (boost) reduced the viable count to 4.1 log CFU/mL and 4.8 log CFU/mL, respectively. For Release profile 3 (linear), an increase of viable count to 6.8 log CFU/mL was observed. When the experiment was repeated with 6, 6.75 and 8 ppm, the viable count were in most cases reduced to below the level of detection for all three release profiles and no difference could therefore be observed (data not shown).

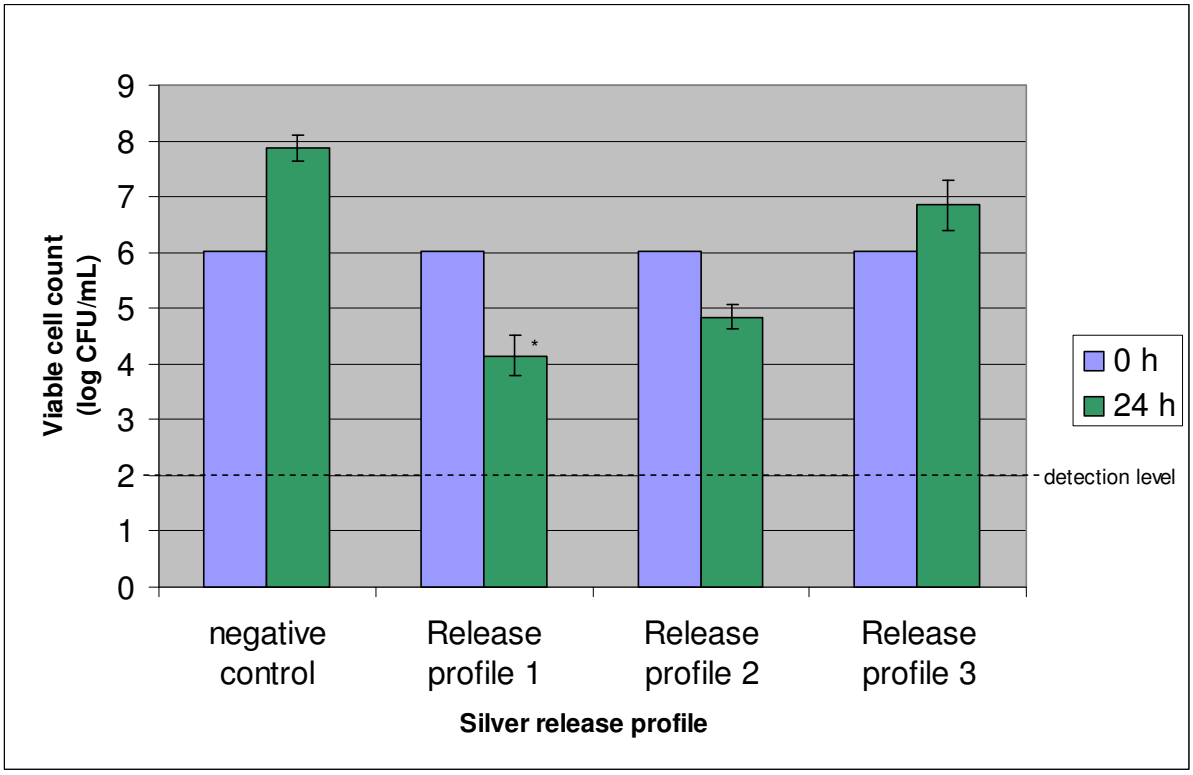


**Figure 11.** Simulation of silver release profiles on *P. aeruginosa* in log-phase, with initial vortex, with 24 h incubation time. The concentration of silver was 3 ppm after 6 h, except for the negative control (0 ppm). Release profile 1, 2 and 3 were simulated as a corresponding MIC, boost and linear silver release profile, respectively (see *Appendix*). Results are mean of six replicates and standard deviation is indicated by error bars, except for bars indicated by *, which only includes five replicates.

## 4.2. Stationary-phase experiments

In the *Stationary-phase experiments*, *MBC* experiments were done to screen for the silver release profiles. Time point 0 h corresponds to the time point when addition of silver started.

### 4.2.1. MBC

In the *MBC* experiments on *S. aureus* in stationary-phase, see Figure 12, 987 ppm of silver showed approximately 1.8 log reductions in viable count. Since no higher silver concentration was used, the MBC was not determined.

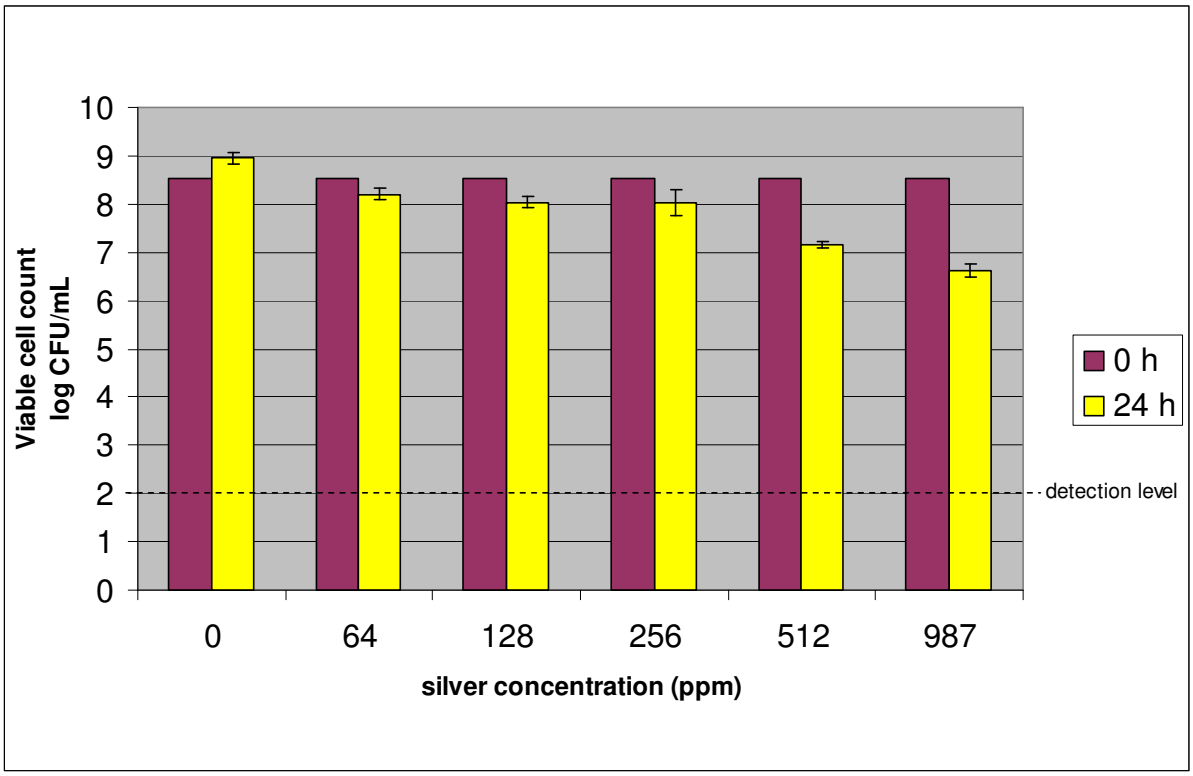


**Figure 12.** *MBC* experiments on *S. aureus* in stationary-phase, with initial vortex, with 24 h incubation time. Results are mean of three replicates and standard deviation is indicated by error bars.

In the *MBC* experiments on *P. aeruginosa* in stationary-phase, see Figure 13, 64 ppm of silver showed approximately 2 log reductions. Though, the MBC was not determined since it is above 64 ppm of silver.

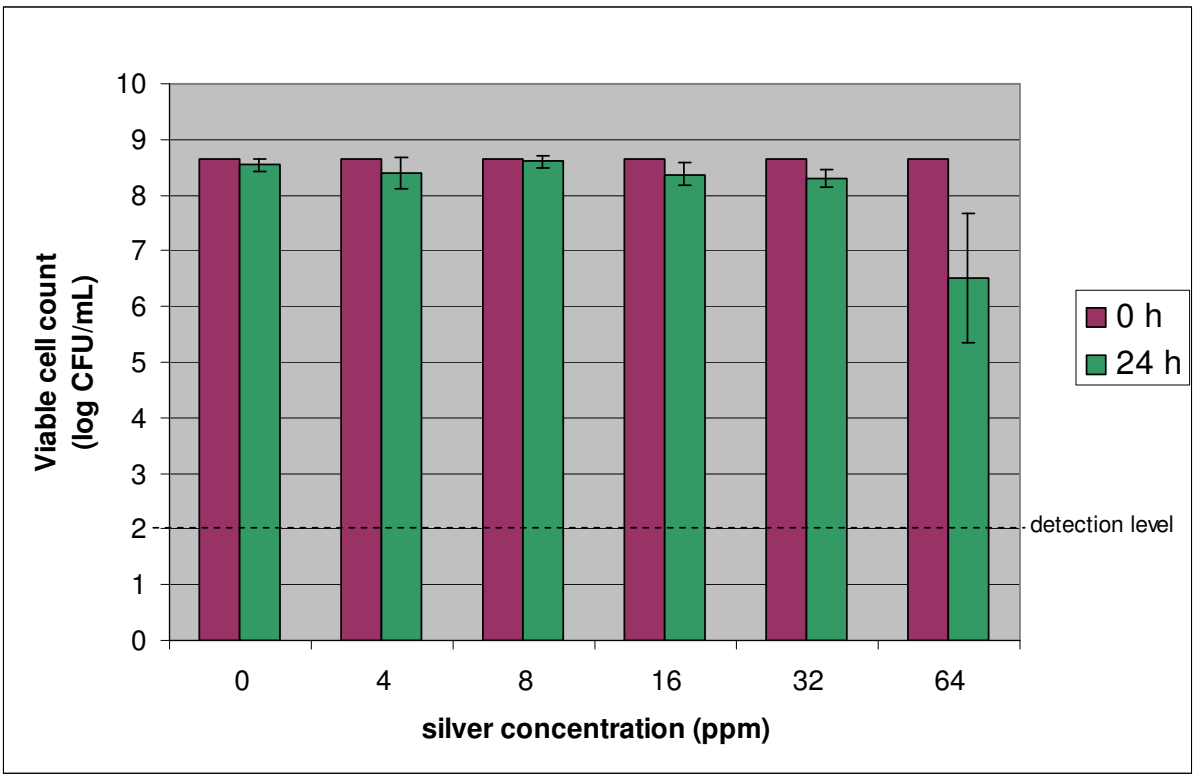


**Figure 13.** *MBC* experiments on *P. aeruginosa* in stationary-phase, with initial vortex, with 24 h incubation time. Results are mean of six replicates and standard deviation is indicated by error bars.

### 4.2.2. Simulation of silver release profiles

The three different silver release profiles with 987 ppm of silver reduced the viable count to the same level, i.e. 7.1 log CFU/mL, see Figure 14.

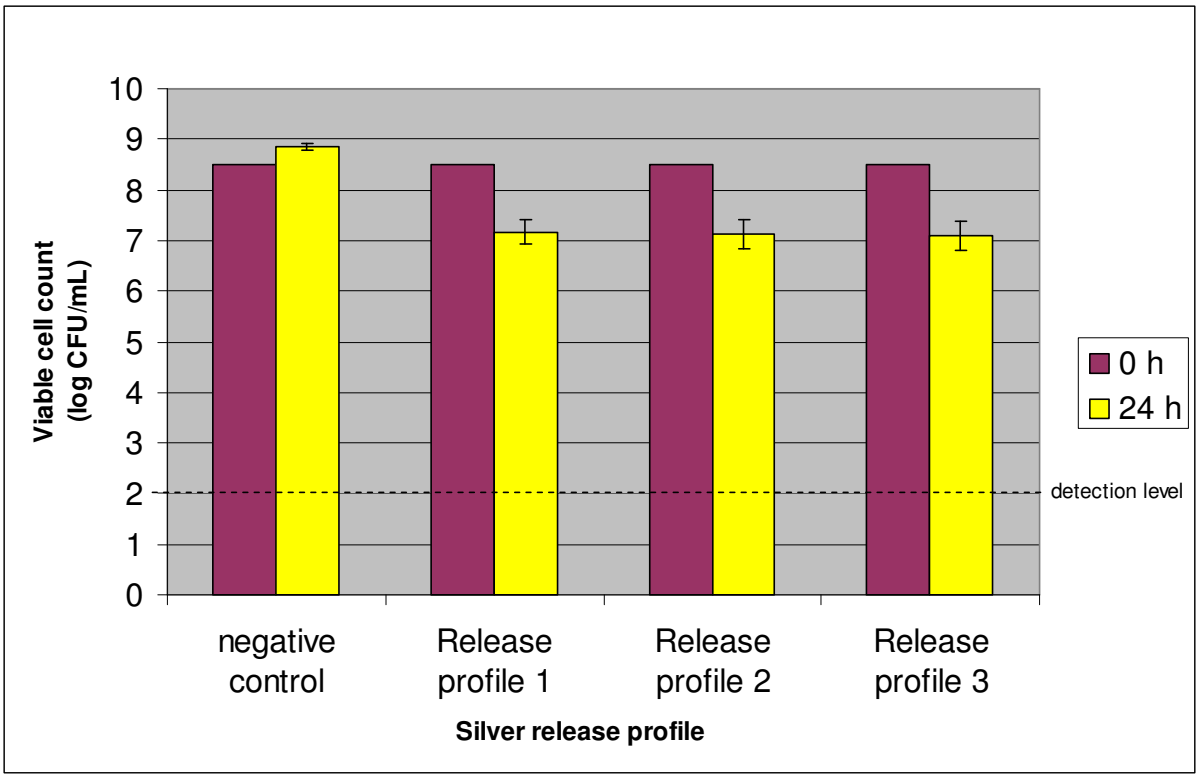


**Figure 14.** Simulation of silver release profiles on *S. aureus* in stationary-phase, with initial vortex, with 24 h incubation time. The concentration of silver was 987 ppm after 6 h, except for the negative control (0 ppm). Release profile 1, 2 and 3 were simulated as a corresponding MIC, boost and linear silver release profile, respectively (see *Appendix*). Results are mean of six replicates and standard deviation is indicated by error bars.

In the *Simulation of silver release profiles* on *P. aeruginosa* in stationary-phase with 64 ppm after 6 h, see Figure 14, Release profile 1 (Corresponding MIC) reduced the viable count to below detection level, which was at least 6.7 log reductions. Release profile 2 (boost) and Release profile 3 (linear) reduced the viable count to 2.8 and 5.7 log CFU/mL respectively.

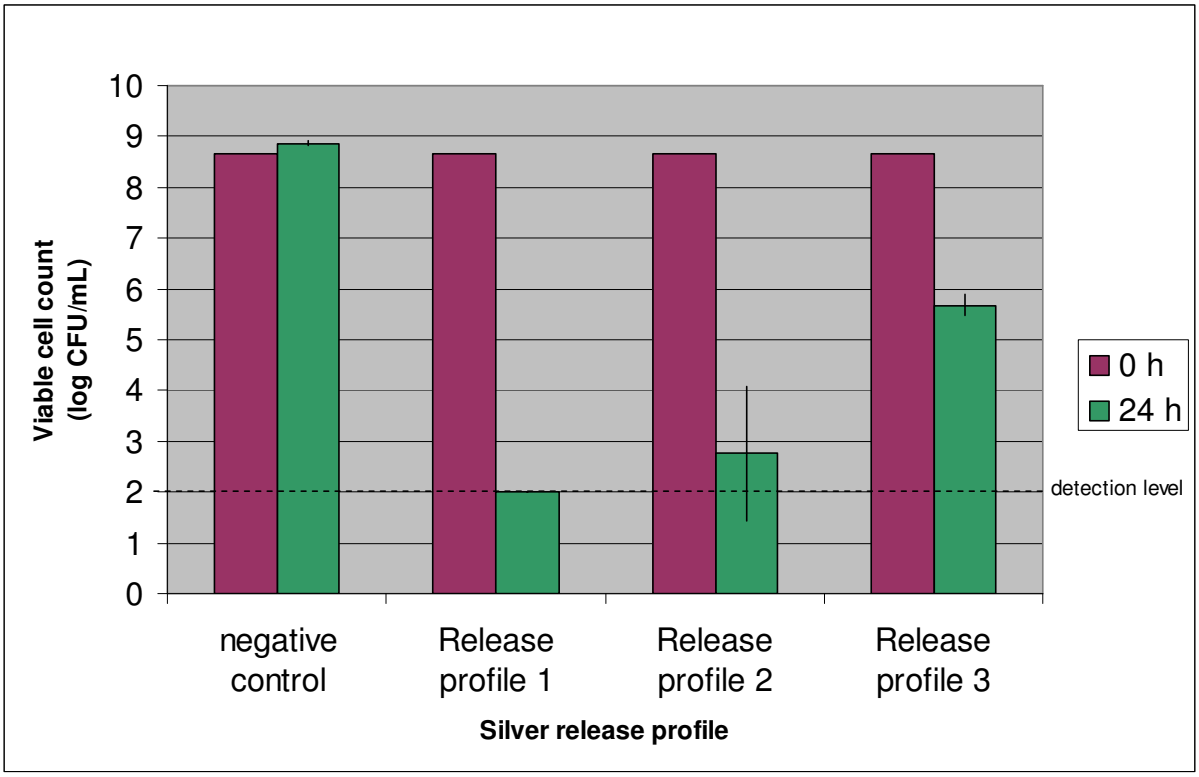


**Figure 15.** Simulation of silver release profiles on *P. aeruginosa* in stationary-phase, with initial vortex, with 24 h incubation time. The concentration of silver was 64 ppm after 6 h, except for the negative control (0 ppm). Release profile 1, 2 and 3 were simulated as a corresponding MIC, boost and linear silver release profile, respectively (see *Appendix*). Results are mean of three replicates and standard deviation is indicated by error bars.

# 5. Discussion

In this chapter the results of the *Log-* and *Stationary-phase experiments* including *MIC & MBC*, *MBC* and *Simulation of silver release profiles* experiments will be discussed and compared to the literature. Additionally, the differences between bacteria in log- and stationary phase to tolerate silver and the contribution of vortexing will be handled.

## 5.1. Log-phase experiments

Silver has antibacterial properties, which clearly can be seen when looking on the *MIC & MBC* experiments in log-phase. *P. aeruginosa* had a MIC of 6.5 ppm and a MBC about 7 ppm of silver when incubated in SWF for 24 h, while S. *aureus* had a MIC of 8 ppm and a MBC of 32 ppm. These data go in hand with other studies (Jung et al., 2008; Schierholz et al., 1998) where Gram-negative (*P. aeruginosa*) are more sensitive to silver than Gram-positive (*S. aureus*) bacteria. An explanation to the differences in antibacterial effect between the two bacteria may be due to the thickness of the peptidoglycan layer (cell wall); the much thicker wall of Gram-positive bacteria may prevent the diffusion of silver ions through the cell wall better (Jung et al., 2008).

When comparing MIC's to the literature, the choice of media and strain used in the studies will be crucial (Nadworny and Burrell, 2008). Ricketts et al. (1970) reported that the MIC for *P. aeruginosa* ranged from 20-40 ppm of silver in nutrient broth, and that the amount of silver required when adding organics to the media could be increased up to 80 times. Regarding *S. aureus*, Spadaro et al. (1974) determined the MIC for *S. aureus* to 7 ppm of silver in unknown media. Also, Yin et al. (1999) reported that MIC for five clinically relevant bacteria ranged from 5 to 12.5 ppm of silver with a Mueller-Hinton broth (a complex organic growth medium). To demonstrate that the MIC depends on the media used, Spacciapoli et al. (2001) determined the MIC for *Porphyromonas gingivalis* in a buffer to 0.003 ppm and when serum was added to the buffer the MIC increased 2500 times to 8 ppm. The MIC's in this study, 8 and 6.5 ppm of silver for *S. aureus* and *P. aeruginosa*, respectively, were determined in SWF as media, which contains fetal calf serum and therefore a lot of proteins etcetera that gives a favorable environment for bacterial growth. To get as relevant results as possible in *in vitro* models, for wounds containing microorganisms, the media should be mimicking a wound like environment. Else, in for example water or buffers, the resulting MIC may be much lower than it really is compared to the reality.

The *Simulation of silver release profiles* on *S. aureus* showed no differences in antibacterial effect between the three release profiles. The same result was found when the same experiments were performed with 8 and 32 ppm of silver.

In contrast to *S. aureus*, *Simulation of silver release profiles* on *P. aeruginosa* showed that Release profile 1 (corresponding MIC) had the most efficient antibacterial effect, 1.9 log reductions, as expected. Further, Release profile 3 (linear) did not even inhibit growth and instead showed an increase with 0.8 log units, 2.7 log units more growth compared to Release profile 1. Release profile 2 (boost) had the second best antibacterial effect, with ~0.7 log reduction less than Release profile 1. The reason to that release kinetics matter for *P. aeruginosa* and not for *S. aureus* may be that *P. aeruginosa* almost had the same value on the MIC and MBC, i.e. only a small increase in silver concentration had a large impact on the antibacterial effect. In other words, the lowest concentration of silver with an inhibitory effect on *P. aeruginosa* (see 8 ppm in Figure 9A) also showed killing effect. Since *P. aeruginosa* seems to be vulnerable to small changes in silver concentration, Release profile 3 with the least antibacterial effect may be explained by that only 25 % of the total amount of silver was available at time point 0 h, which gave a concentration in the test system that had no effect on bacteria. Therefore, bacteria in Release profile 3 experiments had more time to grow before the whole amount of silver had been added at time point 6 h.

Even more interesting is that the results for the *MIC & MBC* experiments for *P. aeruginosa* were not transferable to the *Simulation of silver release* experiments. In the *MIC & MBC* experiments with *P. aeruginosa* 4 ppm of silver showed no antibacterial effect (see Figure 9A), whereas 3 ppm in the *Simulation of silver release profiles* showed 1.9 log reductions (see Figure 11). This will be discussed below in chapter *The effect of vortex*.

## 5.2. Stationary-phase experiments

The MBC was not determined in none of *MBC* experiments on *S. aureus* and *P. aeruginosa* in stationary-phase. Though, silver concentrations with an appropriate antibacterial effect were found, 987 ppm and 64 ppm for *S. aureus* and *P. aeruginosa*, respectively, and these concentrations were further used in the *Simulation of silver release profiles*.

The *Simulation of silver release profiles* in stationary-phase showed a similar pattern for both bacteria as the same type of experiments in log-phase. Regarding *P. aeruginosa*, Release profile 3 (linear) was reduced at least 3.7 log units less than Release profile 1 (corresponding MIC). Release profile 2 (boost) was reduced around 0.8 log units less than Release profile 1. Since there is no growth in stationary-phase, bacteria in Release profile 3 experiments had not any advantage in growing before the whole amount of silver had been added. Therefore, it can be speculated that bacteria may be able to adapt to silver when it is at concentrations that have no effect during the first six hours, compared to the other two release profiles with more instant silver release. Thereby bacteria in Release profile 3 experiments may tolerate silver better when it later on is bactericidal concentrations. This theory may be valid for bacteria in log-phase as well.

## 5.3. Log- versus Stationary-phase bacteria

Bacteria in stationary-phase tolerated silver better compared to when they were in log-phase. This was probably due to that the bacterial concentration for bacteria in stationary-phase approximately was 1000 times higher. Moreover, as for *S. aureus* that normally grows in clusters of cells, the inner cells of a cluster would probably be better protected against the surroundings than the outer cells of a cluster. In stationary-phase, it most likely exists more of larger clusters of bacterial cells. Therefore a larger amount of cells may be protected against silver, compared to bacteria in log-phase.

Another aspect is the fact that log-phase bacteria grow exponentially while stationary-phase bacteria basically have stopped in growth. This may be one reason why bacteria in log-phase were more vulnerable to silver, since the respiratory chain, DNA and RNA etcetera, which are affected of silver (Lansdown, 2004), are important factors in cell growth.

## 5.4. The effect of vortex

When the screening of the *Log-phase experiments* was performed two types of experiments were included: with initial vortex at time point 0 h (addition of silver) and without initial vortex. The latter type was performed to mimic a silver dressing on a wound. In the experiments on *S. aureus* with 9.87, 98.7 and 987 ppm of silver the experiments without initial vortex were reduced approximately 1, 2.5 and 2.5 log units, respectively, less than the experiments with initial vortex (see Figure 6). Remarkably, the experiments without initial vortex on *P. aeruginosa* were reduced at least 4 log units less for all silver concentrations (see Figure 7). Thus, an initial vortex of the sample to mix bacteria, media and silver increases the effect of silver significantly on *P. aeruginosa* compared to not vortex the sample. Concerning *S. aureus* it seems that the effect of an initial vortex becomes fairly greater if the silver concentration is increased, at least from 9.87 to 98.7 ppm.

The results from the *MIC & MBC* experiments on *P. aeruginosa* were not transferable to the *Simulation of silver release profiles*, therefore other silver concentration had to be tested in order to achieve appropriate reduction in the *Simulation of silver release profiles*. However, the reason for this was a small change in methodology, where one initial vortex in the *MIC & MBC* experiments became four vortexes, spread over six hours (with vortex every

time silver was added), in the *Simulation of silver release profiles*. In Figure 16 the effect of one versus four vortexes (corresponding MIC) is illustrated.

The experiments with four vortexes did not enhance the antibacterial effect of silver more than the experiments with one vortex for *S. aureus*. On the other hand, the viability for *P. aeruginosa* was reduced with at least 5.6 and 4.5 log units in the experiments with four vortexes in the log- and stationary-phase, respectively, compared to the experiments with one vortex. The reason to the significant better effect with four vortexes spread over the first six hours over a total incubation time of 24 hours is unknown. Though, when a sample with silver and bacteria is mixed the outcome will be that the probability for bacteria to come in contact with the silver will increase compared to if the sample was not mixed. Concerning the different effects between the two studied bacteria, *P. aeruginosa* has a thinner cell wall than *S. aureus*, therefore silver ions may penetrate *P. aeruginosa* to a larger extent since the probability for a silver ion to hit and penetrates a cell wall increases when vortexing is applied on bacteria with a relative thin cell wall. In other words, every silver ion that hit a cell wall has a higher probability to penetrate the cell wall if it is thin compared to if it is thick. Thus, silver ions may affect intracellular components, such as DNA, RNA and enzymes more likely in *P. aeruginosa*, and maybe also in other Gram-negative bacteria, than in *S. aureus*.

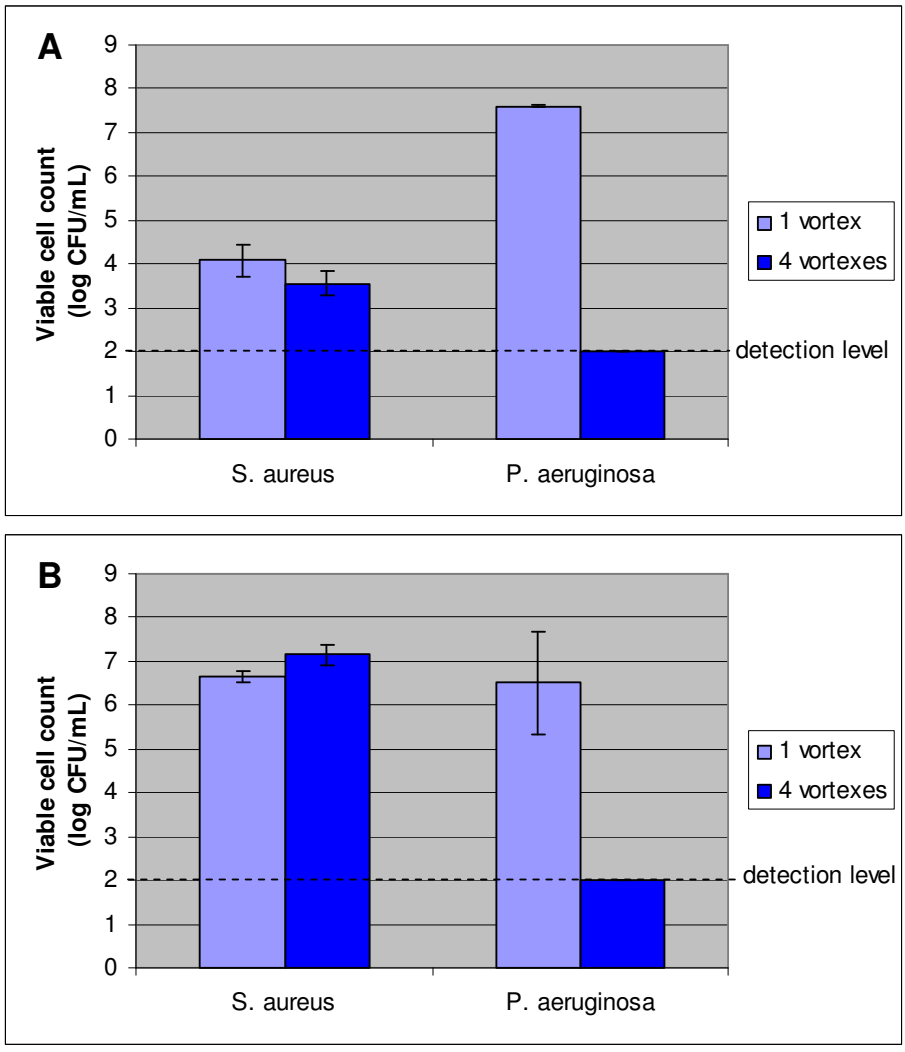


**Figure 16.** The *MIC & MBC* and *MBC* experiments are compared to the corresponding MIC (Release profile 1) in the *Simulation of silver release profiles*. **A)** Log-phase, *S. aureus*: 16 ppm of silver, *P. aeruginosa*: 6 ppm of silver. **B)** Stationary-phase, *S. aureus*: 987 ppm of silver, *P. aeruginosa*: 64 ppm of silver.

Another explanation to the great effect of vortex may be that insoluble particles are formed with the silver ions. Then, when the suspen-

sions are vortexed the particles may destroy the cell wall and membrane.

It is interesting that the *in vitro* methods to determine effect of antimicrobial substances in the field of wound care contain the step of vortexing the sample in order to mix the active substance with bacteria. In studies, it is not very often stated weather vortex is used or not. Even worse, the test system can be agitated during the whole incubation time, which certainly not mimics the real situation of e.g. a silver dressing, but then it is most often stated. Most likely the mixing effect in a cell based model system enhances the effect of other active substances, such as antibiotics and other drugs, too. Therefore several methodologies that determine the effect of active substances should be considered.

## 5.5. The relation of the silver release profiles to silver dressings

Recently, a study done by Jakobsen (2010) compared the release of silver between different silver containing dressings and their antibacterial efficacy over 24 h in SWF. In the study, increased release of silver after 24 h showed a relation to increased antibacterial effect of the silver dressings. This relation was true up to 126 ppm and 91 ppm of silver for *P. aeruginosa* and *S. aureus*, respectively, where 91 ppm corresponded to the dressing with highest release for *S. aureus*. It should be mentioned that it was only the dressing with highest release, which reduced the initial bacterial concentration (~$10^6$ CFU/mL); most of the other dressings were only able to prevent growth (Jakobsen, 2010). These amounts of silver released from the dressings (after 24 h) were higher than the MBC in the present Master thesis for *P. aeruginosa* and sometimes for *S. aureus*. This could be due to that no mixing was done at all in the study done of Jakobsen (2010) and then higher silver concentrations are required, which was discussed in chapter *The effect of vortex*.

The results from present Master thesis defend the fact that higher silver concentrations gives better antibacterial effect up to a certain concentration, and thereafter a further increase of the silver concentration does not increase the antibacterial effect significantly. This implies that a high silver release is preferable to achieve an appropriate antibacterial effect that contributes to healing up to a certain concentration. Probably, more complex *in vivo* situations require more and higher concentration of silver compared to the simpler *in vitro* models.

Moreover, in the study of Jakobsen (2010), the silver release profiles, in the range of 0-4 or 0-8 h, for three different dressings corresponded most to Release profile 2 (boost) in the present Master thesis, where a high amount, ~75 %, of the silver released over 6 h was released instantly. The silver release profile of another dressing corresponded more to Release profile 3 (linear) in the present Master thesis. According to the results in the present Master thesis, there was a difference between these two profiles for *P. aeruginosa*, where Release profile 2 (boost) was reduced 2.1 and 2.9 log units more in log- and stationary-phase, respectively, compared to Release profile 3 (linear). Therefore, the dressings with the silver release profile that released a boost of silver instantly (after one hour) and delivered an appropriate silver concentration would be preferable if *P. aeruginosa* is present in the wound. Of course, Release profile 1 with the best antibacterial effect would be best, though it would be hard to design a dressing that releases the whole amount of silver into a wound instantly.

Present results also show that traditionally MIC-measurement may not be sufficient to predict the antibacterial properties of a silver containing dressing, since the silver release kinetics has a great effect on at least *P. aeruginosa*, which is one of the most problematic wound pathogens.

# 6. Conclusion

Silver had an increased antibacterial effect with increasing concentration on two wound pathogens, *S. aureus* and *P. aeruginosa*, in log- and stationary-phase when the samples were vortexed after addition of silver to the bacterial suspension. The test media used was SWF, which mimics a wound like environment. S. *aureus* tolerated silver better than *P. aeruginosa*, probably because *S. aureus* is a Gram-positive bacterium, which has a much thicker cell wall compared to a Gram-negative bacterium (*P. aeruginosa*). Also, both bacteria in stationary-phase tolerated silver better compared to when they were in log-phase.

The three different release profiles had the same antibacterial effect on *S. aureus* in both phases. Though for *P. aeruginosa* the release profiles had different antibacterial efficacy. Release profile 3 (linear) had the least antibacterial effect, while Release profile 1 (Corresponding MIC) had the most efficient antibacterial effect since it released all silver instantly. This concerned *P. aeruginosa* in both growth phases.

Silver experiments with, compared to without, initial vortex after addition of silver had much better antibacterial efficacy on *P. aeruginosa* and *S. aureus*, respectively, in log-phase. Also, the increased antibacterial effect of silver when vortexing a sample was confirmed when experiments with one vortex (*MIC & MBC*) and corresponding experiments with four vortexes spread over six hours (*Simulation of silver release profiles*) were compared.

# Further work

Included in this Master thesis were two common wound pathogens, further studies could be done on other bacterial species to validate the silver release profiles efficacy on Gram-negative bacteria and maybe also on Gram-positive bacteria.

To mimic the conditions of a wound containing biofilm it would be interesting to do the same kind of experiments in a biofilm model. Then the correlation between the stationary-phase and biofilm model also could be determined.

## Acknowledgements

First of all I would like to acknowledge MHC and all employees that have been contributing to a nice atmosphere for me to work in. I also would like to thank my supervisor Kristina Hamberg for all help and support I have got with my Master thesis.

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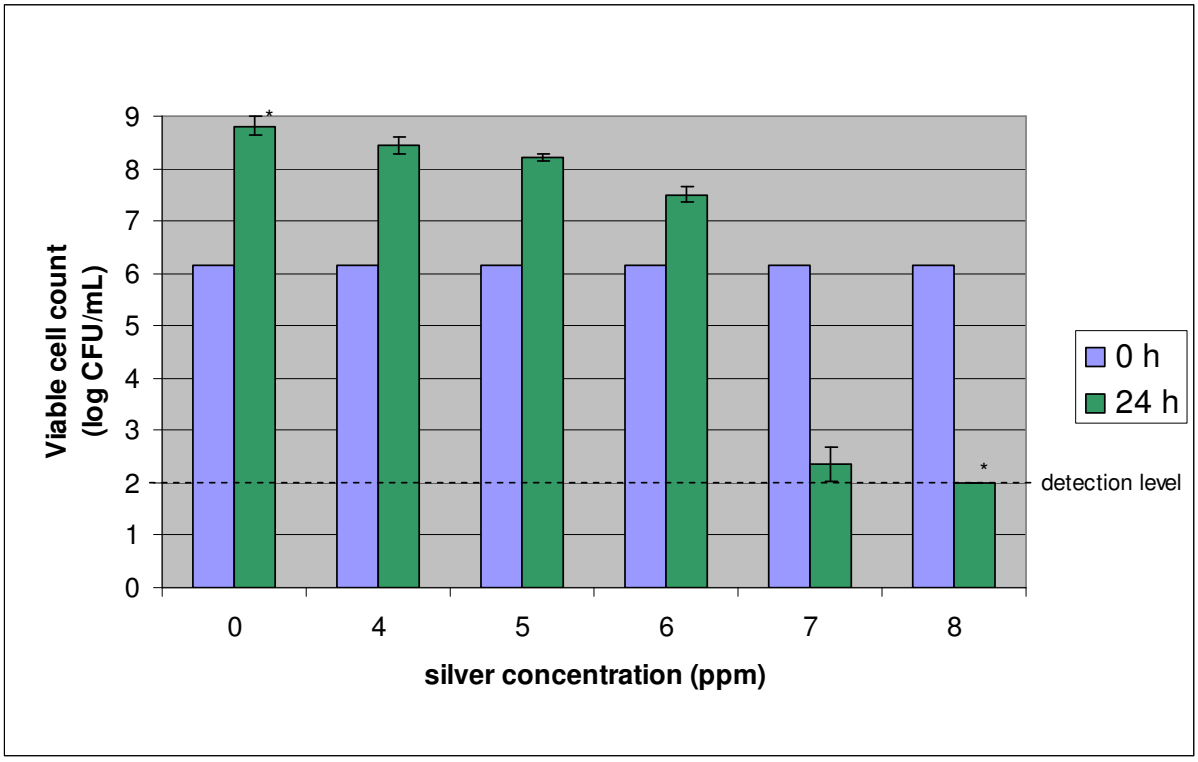
# Appendix

Here are supplementary data and the three simulated silver release profiles that were not shown in chapter *Material and Methods* presented.

## MIC & MBC

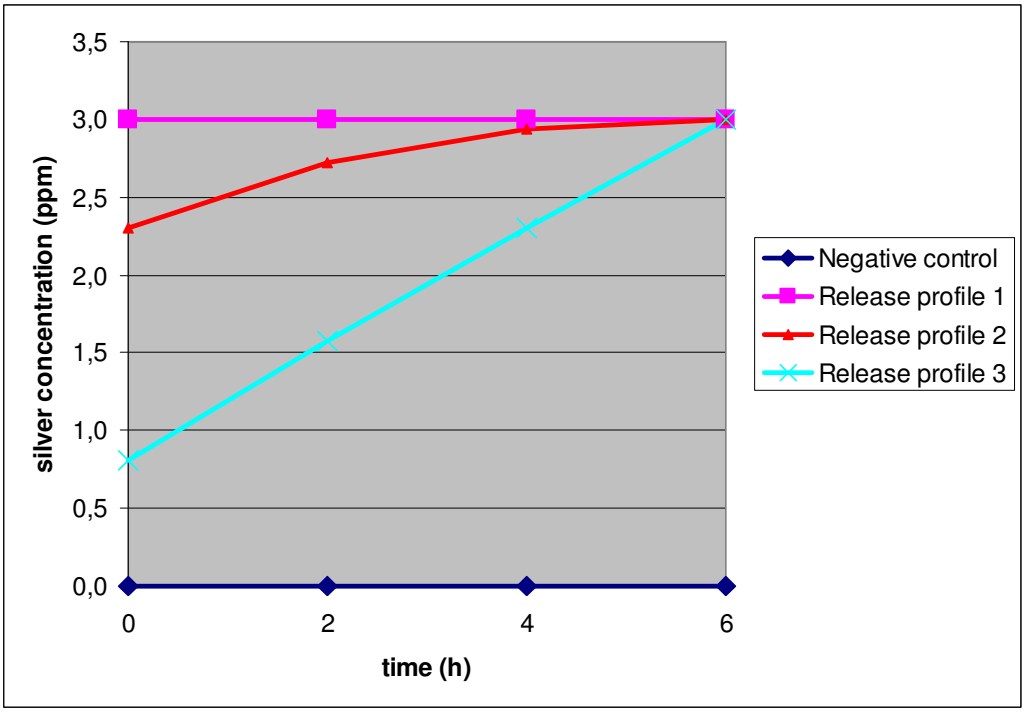
*MIC & MBC* experiments on *P. aeruginosa* in log-phase, with initial vortex, with 24 h incubation time. The experiments included silver concentrations with 4-8 ppm, i.e. these experiments were done in between the *MIC & MBC* experiments on *P. aeruginosa* in log-phase. From the results below the experimental plan with 6-7 ppm of silver were done, which are presented in the report.




*MIC & MBC* experiments on *P. aeruginosa* in log-phase, with initial vortex, with 24 h incubation time. Range: 4-8 ppm of silver. Results are mean of three replicates and standard deviation is indicated by error bars, except for bars indicated by *, which only includes two replicates.

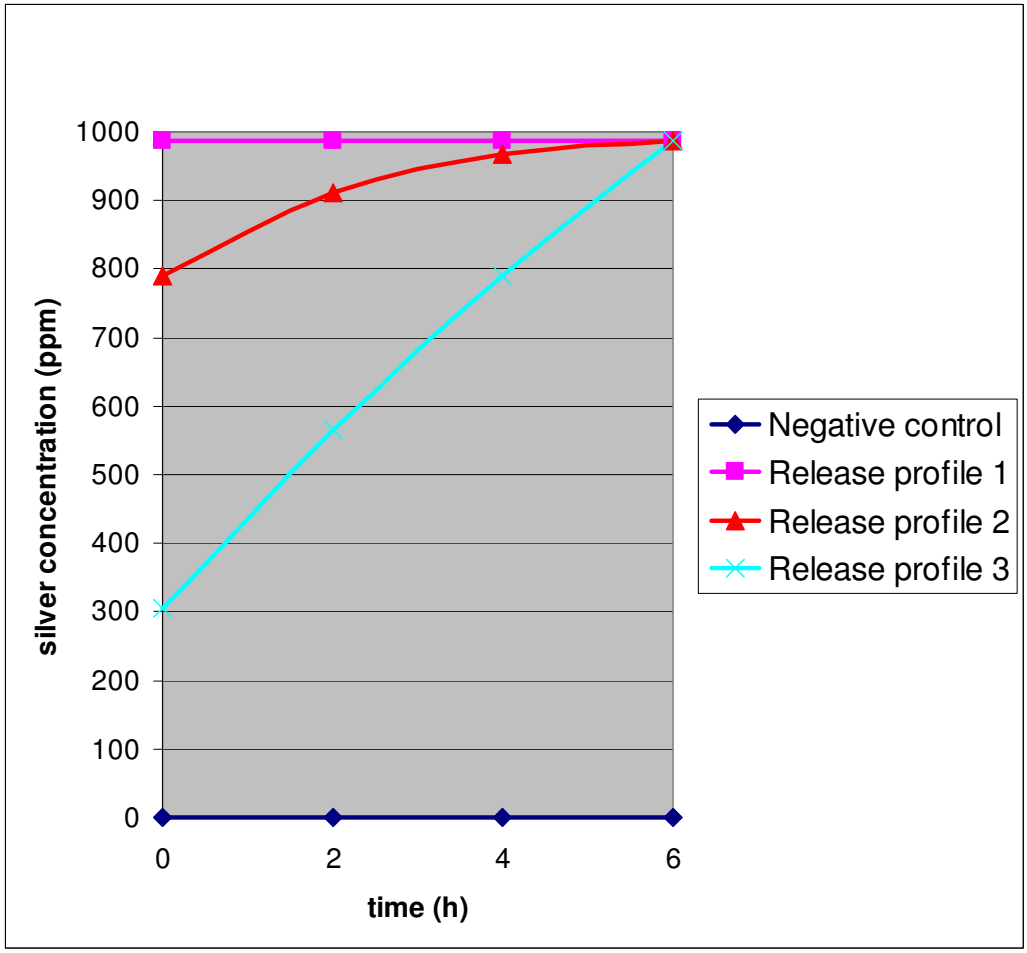
## Simulation of silver release profiles

Silver release profiles for *P. aeruginosa* in log-phase with a final concentration of 3 ppm are shown below.



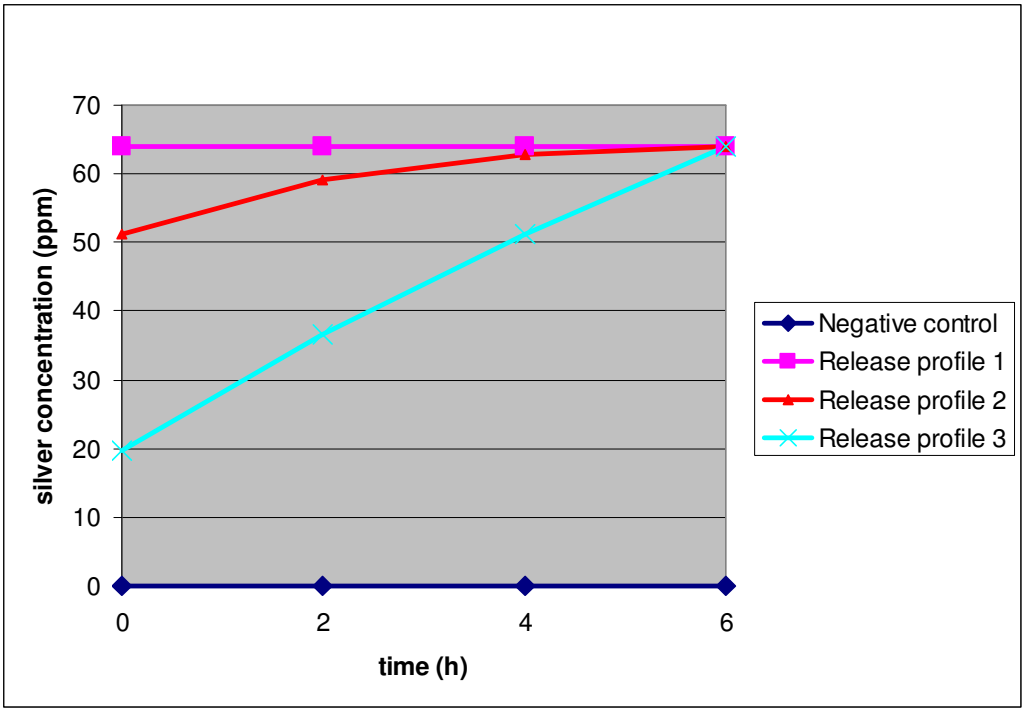

The simulation of silver release profiles for *P. aeruginosa* in log-phase with a final concentration of 3 ppm is shown. Silver solution is added at time point 0, 2, 4 and 6 h for Release profiles 2 and 3. Though, release profiles were vortexed at 0, 2, 4 and 6 h, even if there were no addition of silver solution.

Silver release profiles for *S. aureus* in stationary-phase with a final concentration of 987 ppm are shown below.




The simulation of silver release profiles for *S. aureus* in stationary-phase with a final concentration of 987 ppm is shown. Silver solution is added at time point 0, 2, 4 and 6 h for Release profiles 2 and 3. Though, release profiles were vortexed at 0, 2, 4 and 6 h, even if there were no addition of silver solution.

Silver release profiles for *P. aeruginosa* in stationary-phase with a final concentration of 64 ppm are shown below.




The simulation of silver release profiles for *P. aeruginosa* in stationary-phase with a final concentration of 64 ppm is shown. Silver solution is added at time point 0, 2, 4 and 6 h for Release profiles 2 and 3. Though, release profiles were vortexed at 0, 2, 4 and 6 h, even if there were no addition of silver solution.