

# CHALMERS



**Effect of agitation on antimicrobial activity and  
release  
during *in-vitro* testing of antimicrobial wound care  
dressings**

Master of Science Thesis in Biotechnology

JULIA ÅKERMAN

*Department of Chemical and Biological Engineering*  
CHALMERS UNIVERSITY OF TECHNOLOGY  
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## Abstract

The use of antimicrobial substances to combat microorganisms has been in practice for centuries. Louis Pasteur's germ theory published in 1865 was the first scientific evidence of the link between microorganisms and infectious disease and brought the implementation of antisepsis into hospital routines. The interest in antiseptics diminished with the discovery and introduction of antibiotics in clinical treatment in the 1940s, but the emergence of antibiotic resistance during the past decades has revived the interest in antiseptic agents as treatment of infectious diseases.

The aim of this thesis was to evaluate the antimicrobial effect and release kinetics of two commonly used antimicrobial substances (silver and PHMB) during *in-vitro* testing of antimicrobial wound dressings. Experiments with the active substance in solution as well as experiments with the active substance in the form of antimicrobial wound care dressings were performed. The antimicrobial effect was evaluated using a two-compartment model by determining the number of viable cell counts by standard plate count method. The silver release from wound care dressings was determined by ICP-OES analysis. The PHMB release was assessed with spectrophotometric measurements and a colorimetric method involving the anionic dye Eosin Y. The influence of agitation was evaluated at static conditions and at agitation rates of 50 and 100 rpm respectively.

Experiments with active substance in solution against *P. aeruginosa* showed no influence of agitation on antimicrobial effect. Higher PHMB susceptibility was observed for *S. aureus* compared to *P. aeruginosa*, this could be coupled to the outer cell membrane present in gram-negative bacteria which may act as permeability barrier against the polymeric PHMB. In contrast, experiments involving wound care dressings showed a clear correlation between increased antimicrobial effect and increased agitation rates for *P. aeruginosa*. The contradictory results of the two types of experiments may be explained by the initial mixing of inoculated medium and antimicrobial agent for the experiments with active substance in solution. In the experiments involving wound care dressings no such mixing occurred and hence, the effect of agitation is likely to be greater.

The silver release during incubation was similar in experiments performed at static conditions and agitation at 50 rpm, but a significantly higher silver release was detected when agitation at 100 rpm was employed. As no proper control sample was accessible, the amount PHMB released during incubation was not possible to determine with analytical methods available at MHC. Although the presence of interfering substances was indicated in performed measurements of PHMB concentration it could be concluded that agitation does not seem to greatly influence the amount PHMB released, even at higher agitation rates.

## Abbreviations

cfu	colony forming units
log	logarithmic
MHC	Mölnlycke Health Care
AM	Antimicrobial
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
ppm	parts per million
PW	Peptone Water
SWF	Simulated Wound Fluid
TSB	Tryptic Soy Broth
PBS	Phosphate Buffered Solution
ICP-OES	Inductively Coupled Plasma-Optical Emission Spectroscopy
UV-Vis	UltraViolet-Visible Spectrophotometry

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## 1 Introduction

The use of antimicrobial substances to combat microorganisms has been in practice for centuries. Louis Pasteur's germ theory published in 1865 was the first scientific evidence of the link between microorganisms and infectious disease and brought the implementation of antisepsis into hospital routines (Assadian, 2007). By the turn of the century a significant number of antimicrobial agents were used in hospital settings including substances such as carbolic acid, benzoic acid, sodium hydroxide solution and nitric acid (Assadian, 2007). However, the discovery of penicillin by Alexander Fleming in 1928 and its later introduction in clinical treatment in the 1940s, interest in antiseptics faded. Tremendous faith was put into antibiotics as treatment of infectious disease and many believed in their complete eradication by the turn of the century (Weston, 2008). Yet, by the 1960s the emergence of antibiotic resistance was recognized and today the spread of antibiotic resistance is a serious public health threat in clinical practice, especially considering the slow development of novel antibiotics (Coates, 2012).

The misuse and overuse of antibiotics have resulted in a renewed interest in alternative actions such as treatment with antiseptic agents. The antibacterial effect of silver has been known since antiquity and a wide range of silver preparations were used in the treatment of infectious diseases already in the late 19<sup>th</sup> century (Assadian, 2007). Today an extensive amount of silver containing wound care products are commercially available. Another recognized antimicrobial agent is the organic polymer polyhexamethylene biguanide (PHMB). The compound has low cytotoxicity against human cells and has been shown to exert antimicrobial effect against a wide range of microorganisms (Lachapelle et al, 2013). PHMB is commercially available in various formulations and for different indications including wound antisepsis, rinsing and decolonization of unwanted organisms (Koban, Bender, Assadian, Kramer & Hübner, 2012).

## 1.1 Aim

The aim of this master's thesis is to evaluate the antimicrobial effect (AM-effect) and release kinetics of two commonly used antimicrobial substances during *in-vitro* testing of antimicrobial wound dressings. The aspiration is to determine if and how the AM-effect and release of active substance is influenced by agitation and at what agitation rate this effect might appear. It is desirable to try and sort out if the outcome is a matter of release rate and/or biological effect of antimicrobial substance. The substances in use in this thesis work are silver (Ag) and polyhexamethylene biguanide (PHMB), which may be affected differently by agitation. Therefore, the ambition is also to determine if and why these substances exert different AM-effect and release properties when subjected to agitation. The questions at issue are summarized in the following points:

- How does agitation influence the AM-effect of silver/PHMB in solution?
- How does agitation influence the AM-effect of antimicrobial wound care dressings?
- How does agitation influence the amount of active substance released from antimicrobial wound care dressings?
- How is the antimicrobial effect correlated to release of antimicrobial substance from wound care dressings?
- What may be potential explanations to the observed effect of agitation?

## 2 Background

### 2.1 The skin

The skin is the largest organ in the human body. It constitutes an effective barrier between the organism and its environment and thus prevents the invasion of pathogens and chemicals. Its composition also protects internal organs by decreasing mechanical and physical stress as well as prevents diffusion of ions and nutrients from the body (Ehrhardt & Kim, K.-J. 2008). The skin barrier is essential to skin health and must be preserved in order to prevent disease (Draelos & Pugliese, 2011). Figure 1 displays the structure of the skin, which is usually divided into two layers – the epidermis and the dermis.

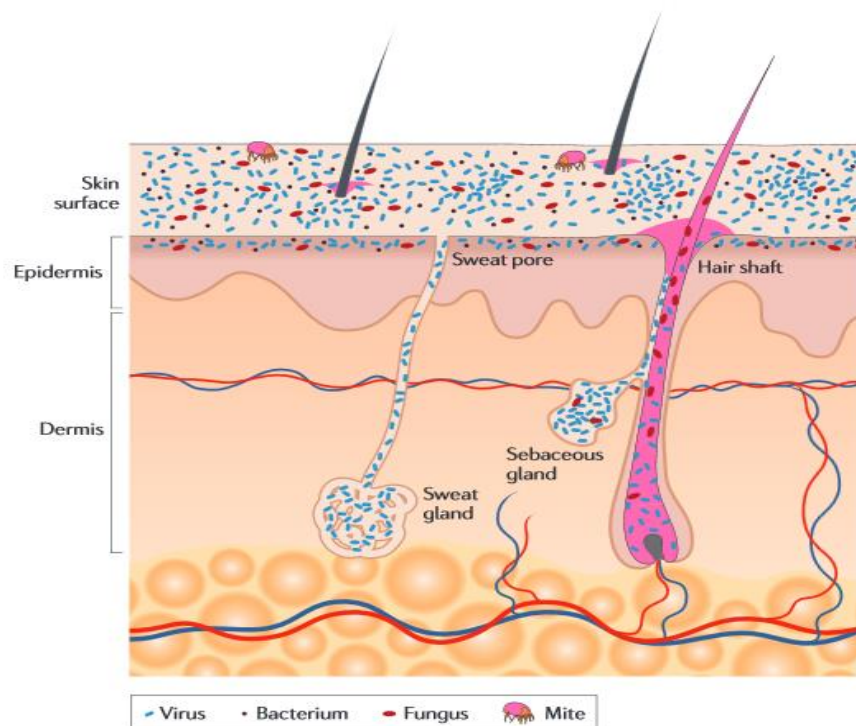


Figure 1. Cross-section of human skin. The dermis is situated underneath the thinner skin layer referred to as the epidermis which constitutes a physical barrier towards the surroundings (Grice & Segre, 2011)

The epidermis is the outer skin layer which acts as a physical barrier, resisting penetration by microorganisms and toxins as well as retaining moisture and nutrients inside the body (Grice & Segre, 2011). The outermost layer of the dermis is referred to as the stratum corneum, also known as the horny layer, and is composed of dead cells. Rapid cell division in the lower regions of the epidermis pushes older cells towards the surface of the skin. During the process cell deformation occurs resulting in destruction of nuclei and other organelles, ultimately leading to cell death. The cell migration continuously builds up the stratum corneum during keratin formation (Bennett, 2008). The high proportion of keratin combined with a complex structure of lipids makes the stratum corneum an effective barrier against the environment even though it holds merely 15-150  $\mu\text{m}$  in thickness (Draelos & Pugliese, 2011).



The dermis, which holds more than 90 % of the skin mass, is situated beneath the epidermis. This layer is in turn divided into the papillary dermis and reticular dermis, respectively (Draelos & Pugliese, 2011). The first mentioned is in direct contact with the epidermis and contains collagen and elastin fibers as well as connective tissue cells, glucosaminoglycans and blood vessels. The constituents of this layer provide nutrients for the epidermis. The reticular dermis lies beneath the papillary dermis and is of similar composition but holds relatively few blood cells and more densely packed collagen and elastin fibers. Instead this layer contains sweat and oil glands along with hair follicles (Draelos & Pugliese, 2011).

## **2.2 Wound healing**

The process of wound healing is a complex sequence of events taking place in a precise and highly programmed manner. The normal stages of healing are commonly divided into four continuous, overlapping phases: hemostasis, inflammation, proliferation, and remodeling. Wounds that undergo these stages are referred to as acute wounds (Guo & DiPetro, 2010). Wounds that are arrested in the late inflammatory or early proliferative phase of normal wound healing are defined as chronic or hard-to-heal wounds (Harding, Morris & Patel, 2002).

### **2.2.1 Acute wounds**

Immediately after tissue injury, platelets in the blood stream will initiate the formation of the fibrin clot and thus reduce bleeding. A network of pro-inflammatory cytokines and growth factors including transforming growth factor (TGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and epidermal growth factor (EGF) are released from both the clot and the surrounding damaged tissue and participate in wound healing activities (Worley, 2004).

The inflammatory phase is entered as leukocytes, mainly neutrophils and macrophages, are guided to the wound site by chemotaxis (Worley, 2004). Neutrophils arrive shortly after the injury, according to Worley (2004) as early as two minutes after injury, and engulf or phagocytose bacteria and foreign material at the wound site. After some time macrophages are activated as well and will continue the process of removing bacteria, dead cells and damaged tissue, i.e. clearing the wound site for further wound healing (Larjava, 2012). Chemical signaling from an increasing number of leukocytes results in an amplified inflammatory response which attracts additional leukocytes and later in time also other cells. Macrophage activity has been shown to be highly significant and of vital importance for the inflammation process whereas the presence of neutrophils seems to have less impact on wound healing (Larjava, 2012). In fact, it has been shown that elimination of neutrophils from wounds with no microbial contamination resulted in accelerated wound closure. A possible explanation for this phenomenon is that microbicidal substances released by neutrophils can also harm healthy host cells (Larjava, 2012).

After two to three days when the wound site has been cleared by leukocytes the proliferative phase is initiated as chemical signaling by mainly platelets, neutrophils and macrophages attracts primarily fibroblasts and endothelial cells to the site (Guo & DiPetro, 2010). Extracellular matrix is then formed as fibroblasts migrate to the site of the injury from surrounding tissues and produce mainly collagen, glycosaminoglycans and proteoglycans. The newly formed collagen presents a supporting structure which enables the migration of endothelial cells into the wound bed, resulting in formation of new epithelium of the wound (Guo & DiPetro, 2010). Re-epithelialization is a crucial step in wound healing as the new epithelial cells constitute a barrier to microbial invasion and help to prevent fluid loss. A moist wound bed facilitates epithelial cell migration and will thus promote the process. After re-epithelialization, wound contraction occurs and the proliferative phase is finalized (Hanna & Giacomelli, 1997).

The final remodeling phase of wound healing is usually entered at around 21 days after injury and may continue for years (Guo & DiPetro, 2010). Collagen bundles produced during the proliferative phase are now reorganized in a parallel arrangement, resulting in a more organized structure. The vascular density is also decreased at this time by regression of many of the newly formed capillaries (Guo & DiPetro, 2010). The result is a tissue similar to the original tissue but the tensile strength is usually comprised to approximately 80% of the original, undamaged tissue strength (Hanna & Giacomelli, 1997).

It should be stated that the much simplified description of the wound healing process presented here does not nearly reflect the true complexity and interaction between various phases of healing. The intricacy of the healing process is a major obstacle in therapeutic treatment of wounds.

### **2.2.2 Hard-to-heal wounds**

Hard-to-heal wounds, also referred to as chronic wounds, are wounds that are arrested in the late inflammatory or early proliferative phase. Both local factors (tissue maceration, presence of foreign bodies and microorganisms, etc.) and systemic factors (age, malnutrition, etc.) may contribute to the development of a hard-to-heal wound (Harding, Morris & Patel, 2002). Reduced levels of active growth factors in the wound environment are also associated with the non-healing condition as well as imbalance between proteinases and their inhibitors where excessive proteinase activity results in abnormal degradation of the extracellular matrix (Harding, Morris & Patel, 2002).

Hard-to-heal wounds frequently enter a state of pathologic inflammation due to the incomplete healing process. Some 5-6 million patients in the United States are affected each year, with persons 65 years and older accounting for 85% of the incidences (Cowan et al, 2013). Consequently, hard-to-heal wounds constitute a major public health concern that results in enormous costs for the health care sector (Guo & DiPetro, 2010).

### 2.2.3 Microorganisms of wounds

Wounds of the skin enable contact between human tissue and the environment and thus present easy access for microorganisms that are normally sequestered on the skin surface. Migration into the underlying tissues commonly results in local infections and impaired wound healing (Kapalschinski et al, 2013). Presence of non-replicating organisms on a wound is referred to as *contamination* whereas *colonized wounds* are defined by the presence of replicating microorganisms yet the absence of tissue damage. Key features of *infected wounds* are the presence of replicating microorganisms and subsequent host injury (Guo & DiPetro, 2010).

Bacteria of infected wounds often occur in the form of biofilms which are constituted by complex communities of aggregated bacteria surrounded by an extracellular polysaccharide matrix (EPS) (Madigan, Martinko & Stahl, 2011). The self-secreted EPS provides a protective barrier towards the surroundings and prevents the access of for example antibiotics. The healing process of many chronic ulcers is probably delayed because of biofilms containing *Pseudomonas aeruginosa* (*P. aeruginosa*) which shield the bacteria from the phagocytic activity of invading neutrophils (Guo & DiPetro, 2010). The development of biofilms on medical devices and surrounding tissues has become one of the major challenges in modern clinical medicine (Salek, 2009). Strains of wound pathogens such as *Staphylococcus aureus* (*S. aureus*), *P. aeruginosa*, and *Candida albicans* (*C. albicans*) commonly express surface molecules with high cell surface hydrophobicity (CSH), such as lipoteichoic acid and surface proteins, which constitute a potential mechanism of initial adhesion and thus biofilm formation (Ljung & Wadström, 1995).

Infected wounds tend to be dominated by gram-positive bacteria in the early phase whereas wounds of greater than one month have gram-negative bacteria present followed by anaerobes (Ousey & McIntosh, 2008). Moreover, delayed wound healing is not dependent on the presence of one specific organism but rather it is the result of multiple species of microorganisms. Common wound pathogens that may cause wound infections include bacteria, fungi, protozoa and viruses. The most common causative wound pathogens include gram-negative bacteria *P. aeruginosa* and *Escherichia coli* (*E. coli*), gram-positive bacteria *S. aureus*/ Meticillin-resistant *staphylococcus aureus* (MRSA) and fungi *C. albicans* (Ousey & McIntosh, 2008). According to Guo & DiPetro (2010), especially *P. aeruginosa* and *S. aureus* appear to play an important role in bacterial infection in wounds. Also Kapalschinski et al (2013) and Hirsch, Seipp & Steintraesser (2010) state that *S. aureus* has been shown to be the most common pathogenic agent in clinical isolates from hard-to-heal wounds. The prevalence of MRSA has progressively increased during the past two decades. From 1995 to 2005 the *S. aureus*-related hospitalizations increased by 62% in the USA and the MRSA-related hospitalizations more than doubled during the same period (Hogea, van Effelterre & Acosta, 2013). The emergence and rapid spread of this organism has created new challenges for infection prevention and control services in hospitals and other health care facilities (Ferreira et al, 2014).

## 2.3 Antimicrobial substances

This section describes general properties of antimicrobial substances, followed by a presentation of the two antiseptic agents used in the experiments of this thesis work.

### 2.3.1 Antibiotics vs. antiseptics

Antibiotics are defined as chemical compounds produced by microorganisms that kill or inhibit other microorganisms (Madigan, Martinko & Stahl, 2011). The antibiotic mode of action is selective, meaning they have a narrow spectrum of destruction. Generally, low concentrations are sufficient to destroy pathogens. Antiseptics on the other hand are non-selective and destroy or inhibit the growth of a broad spectrum of microorganisms in or on living tissue. Disinfectants are similar to antiseptics but generally refer to chemical agents that destroy microorganisms found on non-living objects e.g. surfaces (McDonnell & Russell, 1999).

Antiseptic agents have been in use for centuries but it was first in the middle of the 19<sup>th</sup> century when an increasing number of bacteria were being discovered that it was possible to experimentally verify the effect of a range of antimicrobial substances. This also led to the discovery that antimicrobial agents proved to be effective only when they were in very close contact with different microorganisms and where present in certain concentrations (Assadian, 2007). The discovery of penicillin in 1928 gradually decreased the interest in antiseptics as treatment using antibiotics gained ground. An increasing number of different antibiotics have been discovered over the years and today numerous different types of antibiotics exist on the market. In the late 1960s it was believed by some that the development of antibiotics would lead to complete eradication of infectious diseases by the turn of the century (Assadian, 2007). However, the prediction was mistaken and today bacterial infections continuously constitute a serious threat to public health. In fact, the increasing emergence of antibiotic resistance has become a worldwide issue over the past 20 years (Assadian, 2007). One of the most problematic effects of this is the dramatic increase in the proportion of methicillin-resistant *Staphylococcus aureus* (MRSA) among *S. aureus* during the 1990s in many European countries as well as in America and Asia (Witte, 2013). In this respect antiseptic agents represent an appropriate alternative for the treatment of infected wounds as they have a broader spectrum of activity (particularly against antibiotic-resistant bacteria), and rarely lead to resistance (Ubbink, Vermeulen, Lubbers & Voss, 2009).

Small compounds such as diiodine and silver ions readily penetrate membrane channels of both gram-positive and gram-negative bacteria. Once inside the cell the antimicrobial agent can harm the bacteria by oxidizing proteins present in the cytoplasm. On the contrary, large molecules such as chlorhexidine and polyhexanide are believed to exert antimicrobial activity by adsorbing to the cell membrane of the microbial (Lachapelle et al, 2013). However, less is known on the mechanism of antimicrobial substances compared to antibiotic mode of action

and it is a field that is still being explored.

Moreover, the enhanced usage of antiseptics and disinfectants has raised concerns regarding possible microbial resistance development also for antiseptic agents, a phenomenon that seems to be increasing for some antiseptic agents (Lachapelle et al, 2013). The acquired resistance alters the bacterial susceptibility to antimicrobial agents by for example altering the outer membranes of gram-negative bacteria which thus prevents antiseptic adsorption onto the microbial membrane (Lachapelle et al, 2013). According to Wong, Zhang, O'Donoghue & Boost (2013), plasmid-borne antiseptic resistance (AR) genes, which possess enhanced tolerance to several disinfectants, have been reported in *S. aureus* of human origin. Even though the minimum inhibition concentration MIC and minimum bactericidal concentration MBC of strains harboring these genes remain lower than the recommended working concentrations, the authors set forth concern regarding possible selection for more resistant strains if exposed to lower levels of these antiseptic agents (Wong, Zhang, O'Donoghue & Boost (2013). Also Lepointeur et al (2013) have presented studies that show selection for resistant strains of staphylococci due to exposure to antiseptic agents. One in vitro study showed resistance genes among coagulase-negative staphylococci isolated from bloodstream infections in very preterm neonates after subjected to common antiseptic agents (Lepointeur et al, 2013). In fact, genes that carry resistance to antiseptic agents such as chlorhexidine and quaternary ammonium compounds have been identified in up to 42% of *S. aureus* isolates in Europe and Japan (Lachapelle et al, 2013).

A recent study involving more than 88 000 cases conducted by the Swedish Infection Control Society showed how treatment of impetigo (a highly contagious bacterial infection of the surface layers of the skin commonly caused by staphylococci or streptococci) with antiseptic agent fusidic acid produced an epidemic of fusidic acid-resistant *S. aureus* (Lachapelle et al, 2013). However, the same study also showed that a large reduction of the fusidic acid prescriptions subsequently led to a decrease in resistant strains. This phenomenon confirms the correlation between prescription and the selection of resistance and is an important factor in reducing the risk for any potential antiseptic resistance development. Other important factors include selecting antiseptics with the broadest spectrums of antimicrobial activity and maintaining adequate exposure times and local antiseptic concentrations *in vivo* (Lachapelle et al, 2013). Lepointeur et al (2013) also recognize that the use of topical antimicrobial agents, as well as the use of antibiotics, requires careful monitoring in order to avoid resistance development among microorganisms and argue for the implementation of a stewardship program that controls their use.

Finally, although the antimicrobial effect of antiseptic agents is disambiguated, critics claim that many of the positive effects in terms of *in-vivo* wound healing usually ascribed to antiseptic agents are not clearly established (Ubbink, Vermeulen, Lubbers & Voss, 2009). Despite the widespread use of antiseptics there is currently no consensus regarding the optimal choice of antiseptic agent for each particular clinical setting. Current clinical studies

employ variations in skin prepping, concentration and formulation (e.g. alcoholic or aqueous) of the antiseptic agent etc which disables accurate comparison of antimicrobial efficacy for different antiseptic agents. Even though there is some evidence on clinical benefits for certain antiseptic agents over others there is still an imperative need for well-designed clinical research in the area that enables directly comparison of various antiseptic agents (Lachapelle et al, 2013).

### 2.3.2 Silver

Silver-containing compounds have been used in medicine since antiquity, long before the concept of microorganisms was discovered. From the late 19th century up until the middle of the 20th century a wide range of silver-based products were available on the market in various formulations including oral, injectable and topical agents (Assadian, 2007). Silver in various forms such as silver metal, silver acetate, silver nitrate, and others are currently used to control bacterial growth in a variety of applications. Common medical device applications include orthopedic implants, vascular grafts and wound dressings (Jun Sung et al, 2007). The antimicrobial capability of silver ions against a wide range of bacteria has been well established. The antiseptic activity is dependent on the silver cation  $\text{Ag}^+$ , which binds strongly to electron donor groups of biological molecules containing sulphur, oxygen or nitrogen (Kumar and Munstedt 2005). Consequently, release of  $\text{Ag}^+$  is essential in order for silver containing compounds to exert antimicrobial activity. This is enabled through interaction with water molecules, hence an aqueous or moist environment is required (Lagaron, 2011).

Silver ions have been observed to possess an extreme chemical affinity for sulphur groups, e.g. thiol groups of L-cysteine, resulting in protein denaturation and hence loss of bacterial enzymatic functions. This has several consequences on bacterial viability including loss of proton motive force (leading to release of  $\text{K}^+$ ), disturbance of the respiratory chain and interference with DNA replication (Lagaron et al, 2011). In addition to this, it has also been suggested that the depletion of protective enzymes leads increased levels of reactive oxygen species in the microbial cell which contributes to cell damage (Lagaron et al, 2011). The main bacterial defense mechanism against  $\text{Ag}^+$  mode of action involves up-regulation in production of the destroyed enzymes. As previously mentioned, several genes that hold elevated resistance towards low concentrations of  $\text{Ag}^+$  have been isolated and identified (Lachapelle et al, 2013). Increased efflux rates of silver ions from bacterial cells that are subjected to low concentrations of silver have also been observed (Lagaron et al, 2011).

The use of silver nanoparticles has raised increased interest over the past years. The mode of action is believed to be similar to that described for silver ions and experiments have shown enhanced antibacterial effect compared to the effect of silver ions. Higher chemical stability of the nanoparticles is suggested as a possible explanation since this would enable more efficient penetration of the bacterial cells (Lagaron et al, 2011). Furthermore, the lesser dimensions of nanoparticles result in an increased number of surface silver atoms. The fraction of silver atoms at the surface is of importance since these particles can easily be released in the surrounding medium and hence exerts antibacterial effect (Palomba et al, 2012).

Bacterial susceptibility toward silver ions may vary considerably for different microbial

species and even for different strains within a single species (Ruparelia, 2008). In general, susceptibility appears to be greater for gram-negative bacteria than for gram-positive bacteria (Ruparelia, 2008). A possible explanation to the observed difference in susceptibility is the attraction of the positively charged silver ion towards the negatively charged lipopolysaccharide (LPS) layer present in gram-negative bacteria. A peptidoglycan layer is present in the cell wall of both bacteria types but the much thicker peptidoglycan layer of gram-positives could act as a protective shield and thus prevent access of  $\text{Ag}^+$  to the cell (Lagaron et al, 2011).

One major advantage of silver ions as an antibacterial agent is the unspecific mode of action. However, the strong affinity for sulphides and halides also brings that the presence of molecules containing these compounds may lead to decreased antibacterial activity. A study conducted by Lieu et al showed how the antibacterial activity of silver was diminished when compounds with free thiol groups were present in the test solution (Lagaron et al, 2011). Also Ip et al (2006) stress how compounds with halide and chloride ions present in serum have been observed to alter the antibacterial activity of silver. In an *in-vitro* study performed by Gupta, Maynes & Silver (1998), the presence of chloride ions was shown to have profound effect on silver activity. At low chloride concentrations silver was excluded by precipitating as  $\text{AgCl}$ , but at higher concentrations increased proportions of accessible anionic complexes was observed, resulting in increased antibacterial activity (Gupta, Maynes & Silver, 1998).

### 2.3.3 PHMB

Polyhexamethylene biguanide, also known as polyhexanide or PHMB, is a cationic polymeric compound utilized in a wide variety of antimicrobial applications (Kapalschinski et al, 2013). Due to its positive charge, PHMB is highly adsorptive to anionic surfaces at physiological pH (O'malley, Collins & White, 2006), see Figure 2 for chemical structure. It is currently one of the most commonly used antiseptic agents and several studies have concluded that PHMB-containing solutions are among the most suitable agents for this purpose (Kapalschinski et al, 2013). The polymer binds to the negatively charged phosphate head groups of phospholipids in the bacteria cell membrane which results in increased fluidity and permeability of the membrane. As a consequence, membrane function is lost and intracellular constituents are precipitated, ultimately leading to death of the cell (De Paula, Germano & Mattoso, 2011).

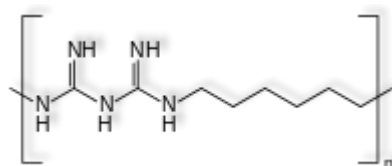


Figure 2. Chemical structure of cationic polymer polyhexamethylene biguanide ([www.worldofchemicals.com](http://www.worldofchemicals.com))

The compound is structurally similar to naturally occurring antimicrobial peptides (AMPs) which enables it to act as an immuno-modulator by enhancing the immune response during infection (Gentile, Gerli & Di Renzo, 2012). AMPs are present in multi-cellular organisms, e.g.



in humans, and kill bacteria by permeating their membranes. Permeation is enabled due to the peptides' ability to arrange in clusters of hydrophobic and cationic amino acids producing an amphiphilic conformation. Due to the non-specific mechanism of action, peptide-based immune defenses have remained an effective weapon of multi-cellular organisms during evolution and it is a general belief that resistance to AMPs seldom occurs (Werthén et al, 2004). However, microbial resistance to AMPs, or deficiencies amongst AMPs, seems to be coupled to various health impaired states, for example enteric infections and chronic leg ulcers (Werthén et al, 2004). In such cases PHMB could be an adequate alternative to the degraded levels of natural immune peptides. This is confirmed in animal studies where PHMB has been shown to stimulate cell proliferation and promote wound healing at low concentrations (Baier et al, 2013). According to Kramer, Hübner, Assadian & Mulder (2010), PHMB is the only currently known wound antiseptic agent that significantly promotes wound healing in the use concentration.

The multiple modes of action of PHMB decreases the risk of associated resistance development and to date there are no reported cases of PHMB resistance (Lachapelle et al, 2013). The compound has a broad spectrum of antibacterial activity against both gram-positive and gram-negative bacteria (Kapalschinski et al, 2013). It has also been shown to have fungicidal effect against organisms such as *Candida spp.* and *Aspergillus spp.* (Lachapelle et al, 2013). Moreover, the ability of PHMB to block microbial attachment to surfaces has been shown both *in-vitro* and *in-vivo* (Hübner et al, 2010). This is a key property in the light of the increased awareness to the significance of biofilms in infected wounds. PHMB has also been shown to effectively remove already formed biofilms in wounds (Hübner et al, 2010). Additional advantageous properties include excellent chemical stability, high availability and reasonable cost (Kapalschinski et al, 2013).

*In-vitro* studies have shown that the antibacterial effect of PHMB is significantly reduced in the presence of albumin, which is the protein that constitutes the major fraction of total protein in wound exudates (Kapalschinski et al, 2013). On the contrary, both Hübner et al (2010) and LaChapelle et al (2013) demonstrate studies where PHMB showed a maintained antibacterial effect in human wound fluid and human tissue. This effect was not impaired even with high loads of blood and albumin. As previously mentioned, the interaction of antiseptic agents in a clinical setting has not been widely investigated. These disputing results regarding the impact of proteins on antiseptic activity further supports the already established high demand for additional *in-vivo* studies in the area.

#### **2.4 *In-vitro* test methods for antimicrobial activity**

The techniques used for determining the *in-vitro* antimicrobial effect of wound care dressings are usually not standardized test methods (MacGregor, 2012). The variety of antimicrobial test methods brings complexity in comparing data derived from different evaluations of wound care products and as a result, confusing differences in results of



different studies may appear (Nadworny & Burrell, 2008). Experimental parameters such as choice and composition of test medium, temperature, type of microorganisms, contact time, sample size etc. will influence the efficacy of the examined antimicrobial wound care product (Tkachenko & Karas, 2012). Commonly used *in-vitro* test methods for determination of the antimicrobial activity of wound care dressings include diffusion based assays and cultured bacterial assays (Nadworny & Burrell, 2008), these are described below.

#### **2.4.1 *In-vitro* diffusion based assays**

Diffusion based assays are similar to antibiotic disk sensitivity assays in which an antimicrobial agent of a specified concentration is allowed to diffuse into an inoculated solid culture medium. The principle is to determine an inhibition zone that is proportional to the microbial susceptibility of the test organism against the antimicrobial agent present in the disk. As the antimicrobial diffuses into the media a gradient is produced and when the concentration of the antimicrobial becomes so diluted that it can no longer inhibit the growth of the test organism, a resulting zone of inhibition (ZOI) is produced (OIE, 2012). When analyzing antimicrobial activity of wound care dressings a piece of dressing is simply placed onto the inoculated solid culture medium, e.g. a Tryptic soy agar (TSA) plate, and the zone around the dressing is measured after incubation. The diameter of the ZOI is related to the MIC of the examined substance, i.e. the larger the diameter the lower the MIC. However, diffusion properties and concentration of the antimicrobial agent will affect the result and for this reason comparison of different types of antimicrobial substances may not be applicable. According to Nadworny & Burrell (2008), diffusion based assays are not appropriate for assessing the activity of silver wound care dressings due to interactions between silver and the test medium. Furthermore, according to Nadworny & Burrell (2008) Rosenkrantz et al (1978) and Spadaro (1985) observed poor correlation between zone size and silver concentration which was attributed to limited diffusion of silver in agar.

#### **2.4.2 *In-vitro* culture based assays**

Cultured based assays are commonly employed in the assessment of microorganism susceptibility against an antimicrobial agent. These types of assays allow for quantitative determination and can also be used for assessment of the antimicrobial activity of wound care dressings. According to Nadworny & Burrell (2008), the most suitable type of assay for determining antimicrobial effect of a wound care dressing is logarithmic reductions assays. This type of test method is very flexible and enables the determination of microbicidal activity of antimicrobial agents in various forms including solutions, creams or devices (e.g. dressings). The antimicrobial agent is exposed to an inoculated test medium and incubated during a specified time. After incubation the antimicrobial activity is stopped by an appropriate neutralizing solution and the logarithmic reduction is determined by subtracting the logarithm of the number of viable cells after incubation compared to the logarithm of the inoculum concentration (Nadworny & Burrell, 2008). The test can be carried out by immersing the antimicrobial dressing in a suspension of inoculated test medium during either static or agitated conditions. Alternatively, the dressing can be subjected to a smaller,

defined amount of inoculated test media (sometimes referred to as a direct contact method). One advantage of culture based assays is that they allow for determination of antimicrobial activity at several time points within the same experiment and hence, both instant and sustained antimicrobial effect can be observed (Nadworny & Burrell, 2008).

### **2.4.3 Influence of agitation on microbial viability and AM-susceptibility during *in-vitro* testing**

The influence of agitation on microbial susceptibility against antimicrobial agents is not a clear cut matter in scientific literature. The positive correlation between agitation and enhanced growth rate of cultivated aerobe microorganisms has however been well established (Najafpour, 2007). The solubility of oxygen in water decreases with increasing temperature and may become a critical parameter for aerobic bacterial growth. Commonly used biological test media contain salts which further decrease oxygen solubility in solution (Sivaprakasam et al, 2007). Transfer of oxygen is a major concern in many bioprocesses that require aerobic growth and the amount dissolved oxygen (DO) is directly related to agitation of the test medium. Apart from increasing the levels of DO, and thus increasing the transport of oxygen to the cell, agitation also increases the transport of nutrients to the microbial cell as well as the transport of waste material away from the cell (Kun, 2006). The growth of aerobic microorganisms decreases during static incubation because transfer of oxygen is limited to the surface of the test medium. The cell cultures will most likely sediment to the bottom and over time become oxygen depleted (Najafpour, 2007). Moreover, too elevated agitation rates may decrease microbial growth by damaging cells or even cause cell lysis, especially if agitation is created using baffles (Beyenal et al, 2002).

Aeration (for example via agitation of test medium) is usually only a major concern for large-scale cultivations whereas aerobic bacteria can be grown in small scale in tubes or flasks under normal atmospheric conditions at static conditions (Najafpour, 2007). Nevertheless, even small-scale cultivations have shown a positive correlation between enhanced agitation and increased bacterial growth. In a study on *P. aeruginosa* incubated for 24 hours during agitation (0-1000 rpm) the bacterial growth was significantly increased for agitation rates up to 200 rpm. For higher agitation rates the growth was decreased, probably due to mechanical damage of cells (Sivaprakasam et al, 2007).

As previously mentioned, *in-vitro* studies are generally not focused on the specific effect of agitation on AM-susceptibility and thus the correlation is unclear. One study performed by Sawyer et al (1997) reported a correlation between enhanced agitation and increased AM-susceptibility for several bacterial species. According to their study, agitation of test medium during incubation nearly always produced lower MICs for bacteria subjected to antimicrobial agent cryptolepine. When cultured in nutrient broth for 24 hours, an eight-fold decrease in MIC was observed for *E. coli* incubated during agitation (150 rpm) compared to the MIC for *E. coli* incubated at static conditions. A similar trend was observed for *P. aeruginosa*, but the MIC was only reduced by a two-fold when incubated during agitation. Increased interactions

between the antimicrobial substance and the bacteria and enhanced aeration of test medium are possible explanations to this phenomenon according to the authors (Sawer et al, 1997). Furthermore, insufficient contact between antimicrobial agent and the test organism during static incubation conditions are often due to adherence of viable microorganisms to the culture vessel surface above the meniscus. This could result in decreased effect of the antimicrobial agent and consequently a higher microbial viability. Several studies have shown decreased adherence of test organisms for cultivations performed at continuous agitation. This effect was particularly observed for cultivation performed with plastic test tubes (National Committee for Clinical Laboratory Standards [NCCLS], 1999).

On the contrary, other studies report unaffected AM-susceptibility for agitated cultivations. A study performed by Klepser et al (1998) involved the assessment of several strains of *C. albicans* subjected to three different antimicrobial agents (fluconazole, amphotericin B and LY303366) at both agitated and static conditions for 48 hours. No difference in AM-effect was detected between agitated and non-agitated samples for either of the test agents (Klepser et al, 1998).

### 3 Materials and Method

In this section the materials and media used in the experiments are presented along with a description of utilized bacterial strains. The test methods employed in this thesis work will also be described in this section. The main test method in use is developed at MHC and is used for determination of both release of active substance and antimicrobial effect of materials and products. Test methods used for determination of silver concentration in protein solution as well as PHMB concentration in saline solution (PBS) are also described.

#### 3.1 Bacteria

Two bacteria species that are commonly found in wounds were evaluated:

- *Pseudomonas aeruginosa* (*P. aeruginosa*); ATCC 15442  
Gram-negative, aerobe bacteria
- *Staphylococcus aureus* (*S. aureus*); ATCC 6538  
Gram-positive, facultive anaerobe bacteria

#### 3.2 Overnight culture conditions

An overnight culture of bacteria for sample inoculation was prepared as follows. One colony of the bacterium was picked from an agar plate and added to a test tube containing 3 ml TSB. The colonies originated from cryopreserved bacteria stored at -70°C. The test tube was vortexed and then incubated overnight at  $35 \pm 2$  °C to produce a bacterial suspension in the stationary phase with an approximate concentration of  $10^9$  cfu/ml. The overnight culture was diluted one tenfold to  $10^8$  cfu/ml by suspending 0.5 ml of the overnight culture in 4.5 ml PW. Aliquots of 1.3 ml bacteria suspension was then dissolved in 45 ml SWF to produce a suspension of approximately  $10^6$  cfu/ml, which was the start concentration in all experiments.

#### 3.3 Test media

Media and chemicals used in the experiments are presented below.

- Deionized, sterile water used for dilution of test solutions
- Simulated Wound Fluid (SWF; Fetal calf serum and Peptone water in proportions 1:1). It contains salts, proteins, carbohydrates, amino acids and vitamins along with other trace elements to simulate a wound like environment and is here used as test media in the 6-well plates.
- Peptone water (PW; 0.85 % NaCl and 0.1 % peptone) used for dilution of overnight culture (O/N culture) and dilution of samples after incubation.
- Tryptic Soy Broth (TSB; 17 g Pancreatic digest of casein, 5 g Sodium chloride, 3 g Papaic digest of soybean meal, 2.5 g Dibastic potassium phosphate, 3.5 g Glucose mixed in 1 L deionized water), used for O/N culturing of bacteria.
- D/E Neutralization Broth (10 g Dextrose, 7g Lecithin, 6g Sodium Thiosulfate , 5g

Enzymatic Digest of Casein, 5 g Polysorbate 80, 2.5 g Yeast extract, 2.5 g Sodium Bisulphite, 1 g Sodium Thioglycollate, 0.02 g Bromocresol Purple). The solution neutralizes a broad spectrum of disinfectants and preservative antimicrobial chemicals, used here for neutralizing silver in inoculated samples after incubation.

- Dextran sulfate, 0.4% (3% asolectin, 0.5% Tween 80 , 0.3% sodiumthioglucolate). Used for inactivation of PHMB in inoculated samples after incubation.
- Petrifilm (3M), aerobic count plate used for determination of cfu in microbiological experiments
- Hydrochloric acid (32% w/w) used for digestion of silver samples prior to ICP-OES analysis.
- Nitric acid (65% w/w) used for digestion of silver samples prior to ICP-OES analysis.
- Silver standard solution ( $\text{AgSO}_4$ ) used for standard solutions in ICP-OES analysis.
- Phosphate buffered saline (PBS; 0.036%  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 0.137%  $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$ , 0.85% NaCl) is a water-based solution that is commonly used in biological research, the osmolarity and ion concentrations match those of the human body. Used as test solution for analysis of PHMB release.
- Mepiseal (Mölnlycke Health Care) is a silicone paste used for sealing edges of punched products.
- Eosin Y (2-(2,4,5,7-tetrabromo-6-oxido-3-oxo-3*H*-xanthen-9-yl) benzoate; C.I Acid Red 87, technical grade) is an anionic red dye used for PHMB detection.

### 3.4 Antimicrobial substances

Two active substances commonly used as antimicrobial agents were evaluated:

- Silver:  $\text{AgNO}_3$  in water (10 795 ppm), diluted in deionized and sterilized water to appropriate concentrations.
- PHMB: Cosmocil PG (20% w/w) diluted in deionized and sterilized water to appropriate concentrations.

### 3.5 Antimicrobial wound care dressings

The following products were included:

**Mepilex Ag:** antimicrobial foam dressing designed for the management of low to moderately exuding wounds. The foam contains silver sulfate (1.2 mg silver/cm<sup>2</sup>) and activated carbon.

**Kendall AMD:** antimicrobial foam dressing designed to provide an ideal moist environment for a wide variety of wounds. It is a highly absorbent and intended for moderate to heavily exuding wounds. The dressing contains PHMB (0.5 %).

**Aquacel Ag:** antimicrobial non-woven absorbent dressing designed for managing high amounts of wound fluid. When in contact with fluid the wound dressing creates a soft, cohesive gel that intimately conforms to the wound surface while maintaining a moist environment. The product is impregnated with ionic silver (1.2 %).

**Suprasorb X + PHMB:** antimicrobial dressing intended for slight to moderately exuding wounds. The product is able to absorb and release moisture as required using “Hydro Balance technology” which keeps the surface of the wound at the optimum moisture level for healing. It contains 4 % cellulose, 96 % water and 0.3 % PHMB.

### 3.6 Test method

The main test method in use is a type of *in-vitro* culture based assay which involves a 6-well plate to which inoculated test media is added. The volume of inoculated test medium (approximate bacteria concentration  $10^6$  cfu/ml) in each well was 2.5 ml for all experiments. Bacteria were subjected to the antimicrobial agent either in the form of active substance in solution or by the presence of test pieces retrieved by punching of antimicrobial wound care dressings. Test pieces were placed onto plastic inserts in the wells which allowed contact between test medium and test piece without complete suspension of the test piece in solution. The 6-well plates were then incubated for 24 hours at  $35\text{ °C} \pm 2\text{ °C}$  during static conditions or continuous agitation at a specific rate. After incubation the solution of each well was mixed by pipetting up and down 5 times and a tenfold dilution was carried out by dissolving aliquots (0.5 ml) from each well in 4.5 ml of appropriate neutralization buffer (D/E for Ag-samples and 0.4% dextrane sulfate for PHMB-samples, respectively). Further tenfold dilution was carried out in PW to reach appropriate concentrations for the determination of viable counts. Finally, 1 ml of appropriate dilutions were pipetted onto petrifilm and incubated at  $35\text{ °C} \pm 2\text{ °C}$  for 1 day (*S. aureus*) or 2 days (*P. aeruginosa*). Then number of viable counts could then be determined by counting the number of cfu on each petrifilm. All experiments were performed using three replicates, performed on two different occasions for verification, and with duplicate replicates on petrifilm.

#### 3.6.1 Determination of appropriate concentrations of active substance in solution

These experiments were performed at static conditions in order to define appropriate test concentrations of active substance. The aim was to define substance concentrations that allowed for the detection of potential alterations of AM-activity due to agitation. For this purpose substance concentrations that produced a reduction in viable counts corresponding to approximately one log reduction (cfu/mL) compared to the inoculum concentration were considered suitable. Initially, a screening experiment was performed using a broad spectrum of active substance concentrations. The experimental setup of the screening experiment is displayed in Table 1.

Table 1. Experimental setup for screening of appropriate test concentrations of silver and PHMB against *P. aeruginosa* and *S. aureus* at static conditions.

Bacteria	Ag concentration (ppm)	PHMB concentration (ppm)
<i>P. aeruginosa</i>	2	20
	20	200
	200	2000
<i>S. aureus</i>	2	20
	20	200
	200	2000

2 ml of inoculated SWF was transferred to each well along with 0.5 ml of appropriate concentrations of either silver or PHMB. In order to validate the growth conditions of the test organism a negative control sample, which consisted of 2 ml inoculated SWF and 0.5 ml deionized and sterilized water instead of the active substance, was included in the experiment as well. The content of each well was mixed by pipetting up and down three times. Incubation and determination of viable counts was then performed as described in section 3.6.

Two additional experimental trials were then performed, each with a more narrow substance concentration range. The experimental set up for these additional experiments are displayed in Table 2 and Table 3.

Table 2. Experimental setup using a less broad concentration range for determination of appropriate test concentrations of silver and PHMB against *P. aeruginosa* and *S. aureus* at static conditions.

Bacteria	Ag concentration (ppm)	PHMB concentration (ppm)
<i>P. aeruginosa</i>	0.5	10
	5	50
	20	100
<i>S. auerus</i>	0.5	10
	5	50
	20	100

Table 3. Experimental setup using a less broad concentration range for determination of appropriate test concentrations of silver and PHMB against *P. aeruginosa* and *S. aureus* at static conditions. Bolded concentrations corresponded to approximately one logarithmic reduction in viable counts and were later used for experiments with applied agitation.

Bacteria	Ag concentration (ppm)	PHMB concentration (ppm)
<i>P. aeruginosa</i>	1	1
	2	5
	<b>4</b>	<b>10</b>
	8	-
<i>S. aureus</i>	1	1
	2	<b>5</b>
	4	10
	<b>6</b>	-
	8	-

### 3.6.2 AM-effect of active substance in solution during agitation

After appropriate test concentrations for each active substance had been established, tests were performed at different agitation rates in order to investigate the influence of agitation on antibacterial effect. Tests were performed as described in section 0 but the 6-well plates were incubated during continuous agitation at 50 or 100 rpm. Concentrations of active substances used in the experiments are displayed in bold in Table 3.

### 3.6.3 Determination of AM-effect for wound care dressings

Test pieces (16 mm in diameter) were punched out from each product under aseptic conditions. The samples were placed onto inserts (pore size 74  $\mu\text{m}$ ) with the wound side facing upwards. A specific volume of non-inoculated SWF (corresponding to the specific maximum absorption capacity of each test piece) was added to the wound side using a pipette. The maximum absorption capacity was determined by subtracting the dry weight of the test piece from the wet weight of the test piece after subjecting it to an excess of SWF for 24 hours (estimating 1 g with 1 ml solution). Test-pieces were left to absorb the added liquid for a few minutes and were then placed onto the inserts with the wound side facing down into the well. Silicone paste (Mepiseal) was added around the samples in order to seal the edges of the punched test pieces. Since the maximum absorption capacity varied for the different wound care dressings, the total concentration of bacteria in the system of each sample varied as well. However, for each experiment the same number of bacteria was added to all wells (each incubation condition was performed within the same experiment, i.e. three experiments were performed in total). The maximum absorption capacities of product test pieces in use are displayed in Table 4.



Table 4. Maximum absorption capacity for examined product test pieces (16 mm in diameter). Results are mean of three replicates.

Test piece ( $\varnothing$ 16 mm)	Maximum absorption capacity (mL)
Mepilex Ag	0.93
Aquacel Ag	1.15
Kendall AMD	0.37
Suprasorb X + PHMB	0.40

Inoculated SWF (2.5 ml) was transferred to each well and the inserts with the pre-wetted test pieces were then replaced into the well, see Figure 3 for a schematic illustration of a well with insert in place. A test liquid volume of 2.5 ml ensures contact between the test medium and the test piece throughout the experiment. The samples were incubated for 24 hours and the number of viable counts was determined as described in section 3.6. In order to reduce the number of experiments the antimicrobial effect of wound care dressings was only evaluated against *P. aeruginosa*.

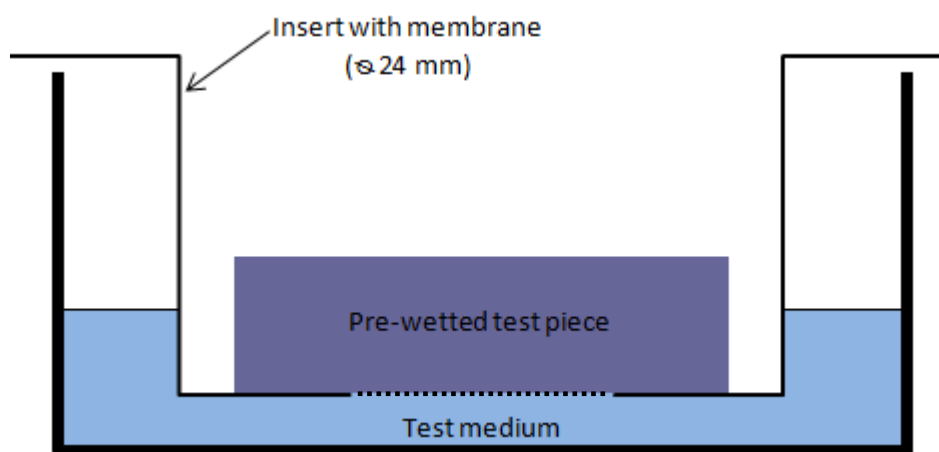


Figure 3. Schematic illustration of one well of a 6-well plate filled with test medium and pre-wetted product placed on insert (membrane diameter 24 mm, pore size 74  $\mu$ m in diameter). The membrane allows contact between product and test medium.

### 3.6.4 Silver release from wound care dressings

After incubation in 6-well plates, an aliquot of test medium was removed from each well (in the same manner as described in section 3.6.3) for further dilution prior to analysis with inductively coupled plasma-optical emission spectroscopy (ICP-OES) which is an analytical technique used for detection of trace metals. Inductively coupled plasma is generated by a flux of argon gas which in turn excites trace metals present in the sample. The excited atoms and ions will emit electromagnetic radiation with intensity proportional to the concentration in the sample. The emitted wavelength is element specific and thus the element of interest can be quantified (Olesik, 1991).

By performing both microbiological and chemical analysis on the same test medium sample, the amount of silver released from each test piece could be coupled to the exerted AM-effect. After removing aliquots for microbiological analysis, the remaining test medium in each well was vortexed and aliquots (1 ml) were transferred to a 50 ml polypropylene tube. In order to digest the samples and dissolve salts present in the solution, hydrochloric acid (15 ml; 32% w/w) and nitric acid (3 ml; 65% w/w) was added to each sample. The tubes were left at room temperature for >4 hours with the caps loose. The solutions were then transferred to 50 ml volumetric flasks by repeated rinsing of the tubes with deionized water. The flask was filled with additional deionized water to a total volume of 50 ml and mixed well before transferring the solution to 15 ml tubes.

Calibration standards with silver concentrations of 1, 7, 50, 300, and 2000 mg/l were prepared in 50 ml tubes by mixing appropriate concentrations of silver standard solution with 2 ml SWF. The purpose of adding SWF, which was the test medium in use in the microbiological experiments, was to produce a standard solution with a composition similar to the composition of the liquid samples. By using solutions with similar composition the accuracy of the analysis is increased. The calibration standards were dissolved in acid in the same manner as previously described for silver samples. Standard 50 mg/l was also used as quality control during analysis with ICP-OES in order to detect potential instrument drift.

Silver was quantified at wavelength 328.068 nm using axial mode in the range 1-2000 mg/l. Three replicate measurements were performed for each sample and a quality control sample was measured each 10<sup>th</sup> injection.

#### **3.6.4.1 Silver content of wound care dressings**

According to the product information sheets, the silver content of examined products was 1.2 mg silver/cm<sup>2</sup> (Mepilex Ag) and 1.2% (Aquacel Ag), respectively. The silver content was verified by ashing and dissolving of samples prior to detection of actual silver concentration using ICP-OES. Appropriate sizes of silver-containing wound care dressings and control sample (Mepilex) were punched out, the weight of each test piece was noted (0.1 – 0.7 g) and the samples were placed in cleaned and dried pots. Silver sulphate (0.010 – 0.035 g) and carboxymethyl cellulose (CMC; 0.02 – 0.07 g) was also added to the pot containing the control sample. A blank sample was also included which consisted of an empty, cleaned and dried pot. All samples were performed in duplicate. Prepared pots were placed in an incinerator and heat-treated over night in an ashing furnace according to a defined temperature program, see Table 5.

Table 5. Temperature program for ashing of samples in an incinerator over night.

Temperature	Time (hours)
Ambient to 300° C	2
Constant at 300° C	2
300° C to 550° C	2
Constant at 550° C	7

Pots were then removed and left to cool for a few minutes until ambient temperature was reached. Nitric acid (5 ml) was transferred to each pot and the ash was gently dispersed in the acid using a glass rod. After 10 minutes, the content of the pot was transferred to a 100 ml E-flask. Pots were rinsed five more times with nitric acid (5ml) and the rinsing solution was also transferred to the E-flask containing the dissolved ash. The E-flasks were covered with watch glasses and placed on a heating plate where the content was boiled gently for > 30 minutes after which the heater was shut off and 50 ml of water was added. The E-flasks were left on the plate for an additional 10 minutes before the content was transferred to a 500 ml volumetric flask using thorough rinsing with water. The volumetric flask was filled with water up to the line and mixed thoroughly.

Standard solutions were prepared in 100 ml plastic volumetric flasks by first adding approximately 50 ml of water to each; thereafter approximately 6 ml of nitric acid is added along with appropriate concentrations of silver sulphate resulting in standard solutions with silver concentrations of 0, 5, 10, 20 and 50 mg/l. Flasks were filled with water up to the line and mixed thoroughly. Standard solution with silver concentration 10 mg/l is also used as QC during analysis with ICP-OES in order to detect potential instrument drift.

Silver was quantified at wavelength 243.779 using axial mode in the range 0.25-50 mg/l. Signal for silver was also measured at 224.874 wavelength nm in order to detect potential interference. Three replicate measurements were performed for each sample and a quality control sample was measured each 6<sup>th</sup> injection.

### 3.6.5 PHMB release from wound care dressings

The potential release of substances other than PHMB into the test medium was a matter of concern in this thesis work. Since no control sample (i.e. a material identical to the product apart from the lack of active substance) was available, the potential interference of other substances present in the analyzed test medium could not be determined. Even though equivalent wound care dressings without antimicrobial agents incorporated exist, it is not certain that the material composition and processing is identical. Hence, it is not certain that such a product would constitute a credible control sample. However, absorption spectroscopy and colorimetric measurements of the two PHMB-containing dressings were performed in an attempt to determine the amount of PHMB released from the test pieces.

### **3.6.5.1 Absorption spectroscopy measurement**

Absorption measurements were carried out in the UV region at a wavelength of 236 nm. Organic molecules such as PHMB undergo electronic transitions on absorbing UV radiation. As defined by Beer's Law, the absorption of light is related to the properties of the material through which the light is traveling. One limitation of absorption spectroscopy analysis is that relatively pure samples are required or alternatively, the level of interference at measured wavelength is known and corrected for (Freeman-Stanfield, 2001).

The presence of proteins in the test liquid prevented determination of PHMB concentration using UV absorption spectroscopy since proteins interfere with the detected signal intensity at the measured wavelength. For this reason absorption measurements were carried out on diluted aliquots collected from samples incubated with phosphate buffer saline (PBS) instead of SWF. As a consequence, AM-effect and PHMB release was evaluated on different samples. Test pieces were incubated in the same manner as described in section 3.6.3 but with uninoculated PBS as test medium, all samples performed in triplicate. After incubation, 50  $\mu$ l from each well of the 6-well plate was transferred to a test tube and 20-fold diluted in distilled water to produce sample concentrations within the measuring range. Aliquots of 300  $\mu$ l of each diluted sample as well as a control sample (PBS) and blank (deionized water) were transferred in duplicate into wells of a microtitration plate. Aliquots of 300  $\mu$ l from each sample spiked with known concentrations of PHMB (25 and 50 ppm) were also transferred to the microtiter plate and the absorption intensity was measured at 236 nm for all wells.

Absorbance spectra were also constructed by measuring absorbance intensity of each sample over a range of wavelengths (200-350 nm, step: 2 nm). An absorbance spectrum for PHMB in distilled water was also produced to be used as a reference spectrum.

### **3.6.5.2 Colorimetric measurements**

The method involves a color reaction between quaternary amines in the PHMB molecule and the anionic Eosin Y. The two molecules form colored ion pairs which results in changes in the Eosin Y spectrum that can be detected by e.g. absorption spectroscopy (Mousavi, Butler & Danaher, 2008). Aliquots of the same test medium used for the absorbance measurements described in section 3.6.5.1 were diluted to appropriate concentrations and allowed to react with Eosin Y prior to absorption analysis at 545 nm.

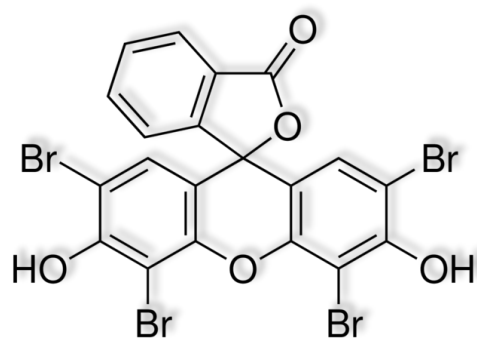


Figure 4. Chemical structure of the anionic dye Eosin Y (Chemspider.com)

A stock solution of 0.6 g of Eosin Y in 250 ml distilled water was prepared which was further diluted by adding 100  $\mu$ l of the stock solution to 1300  $\mu$ l distilled water. A solution of sodium acetate was prepared by adding 10 g of sodium acetate trihydrate to 100 ml distilled water. Next, 10  $\mu$ l of sodium acetate solution and 30  $\mu$ l of diluted Eosin Y solution were transferred to wells of a 96-well microtiter plate. Samples were diluted a 20-fold before 250  $\mu$ l of each sample was added to the wells. For standard addition measurements, 225  $\mu$ l of sample was added to 25  $\mu$ l of either water, a 125 ppm solution of PHMB or a 250ppm solution of PHMB. Also a blank sample consisting of deionized water was prepared. Samples, standard solutions and blank were analyzed with absorption at 545 nm. All measurements were performed in duplicate.

## 4 Results

Results are presented in two sections – microbiological test methods and chemical test methods.

### 4.1 Microbiological test methods

Results are presented in diagrams as logarithmic viable cell counts (log cfu/mL). A negative control sample is also presented for each experiment. The control sample contained the same test medium as the samples but no antimicrobial substance or product and is used to validate the growth condition of the test organisms.

Since there is no equipment for determining the inoculum concentration in real time (for example by using turbidimetric measurements) at MHC, variations in start inoculum concentration is inevitable for microbiological experiments. However, the start inoculum concentration was approximately  $10^6$  cfu/mL for all experiments, the exact concentration for each experiment is presented in the figure captions.

#### 4.1.1 Determination of appropriate test concentrations for silver and PHMB

In this section the results for experiments with active substance in solution performed at static conditions as well as during agitation will be presented. All experiments were performed in triplicate.

##### 4.1.1.1 Experiments at static conditions

Initial experiments indicated that an appropriate test concentration for both *P.aeruginosa* and *S. aureus* was in the range of 2-20 ppm silver, see Figure 5.

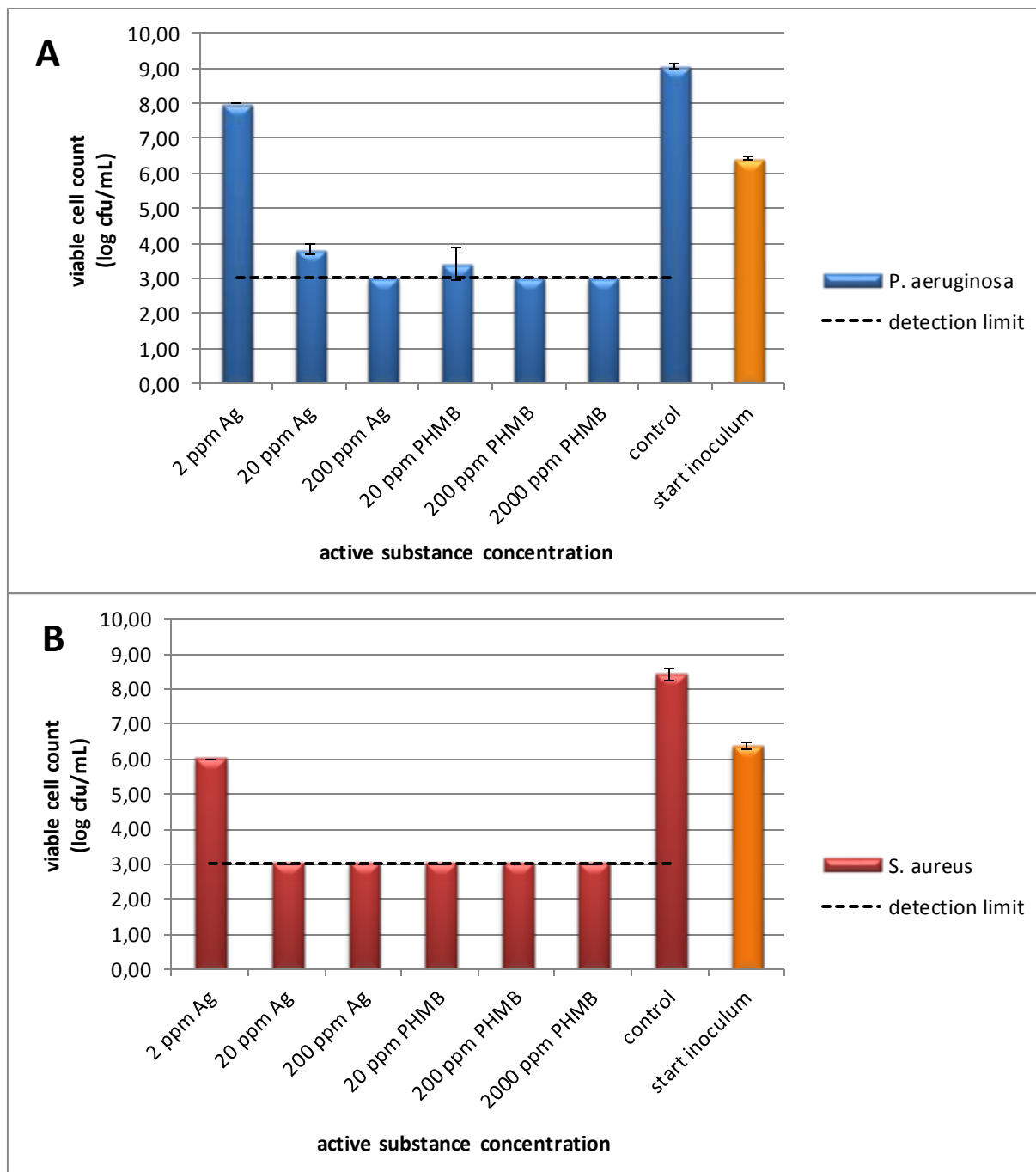


Figure 5. Experiments with a broad concentration range: AM-effect of silver and PHMB after 24 hours incubation at static conditions, presented as viable cell count (log cfu/mL). Results are mean of three replicates; limit of detection was  $10^3$  cfu/mL.

A) AM-effect against *P. aeruginosa*, start inoculum concentration was  $2.5 \times 10^6$  cfu/mL. B) AM-effect against *S. aureus*, start inoculum concentration was  $2.0 \times 10^6$  cfu/mL.

For *P. aeruginosa*, a PHMB concentration of 20 ppm resulted in approximately 3 log reductions. The log reduction for *S. aureus* was below the detection limit, i.e. the viable cell count was reduced more than 3 log units for all examined PHMB concentrations. Results also showed that the appropriate silver concentration was in the range 2-20 ppm for both test organisms. As a consequence, two additional experimental setups with a more narrow concentration range were carried out. Results for experiment with concentrations of 0.5-20

ppm silver and 10-100 ppm PHMB are not shown. Results for experiment with concentrations of 1-8 ppm silver and 1-10 ppm PHMB are presented in Figure 6.

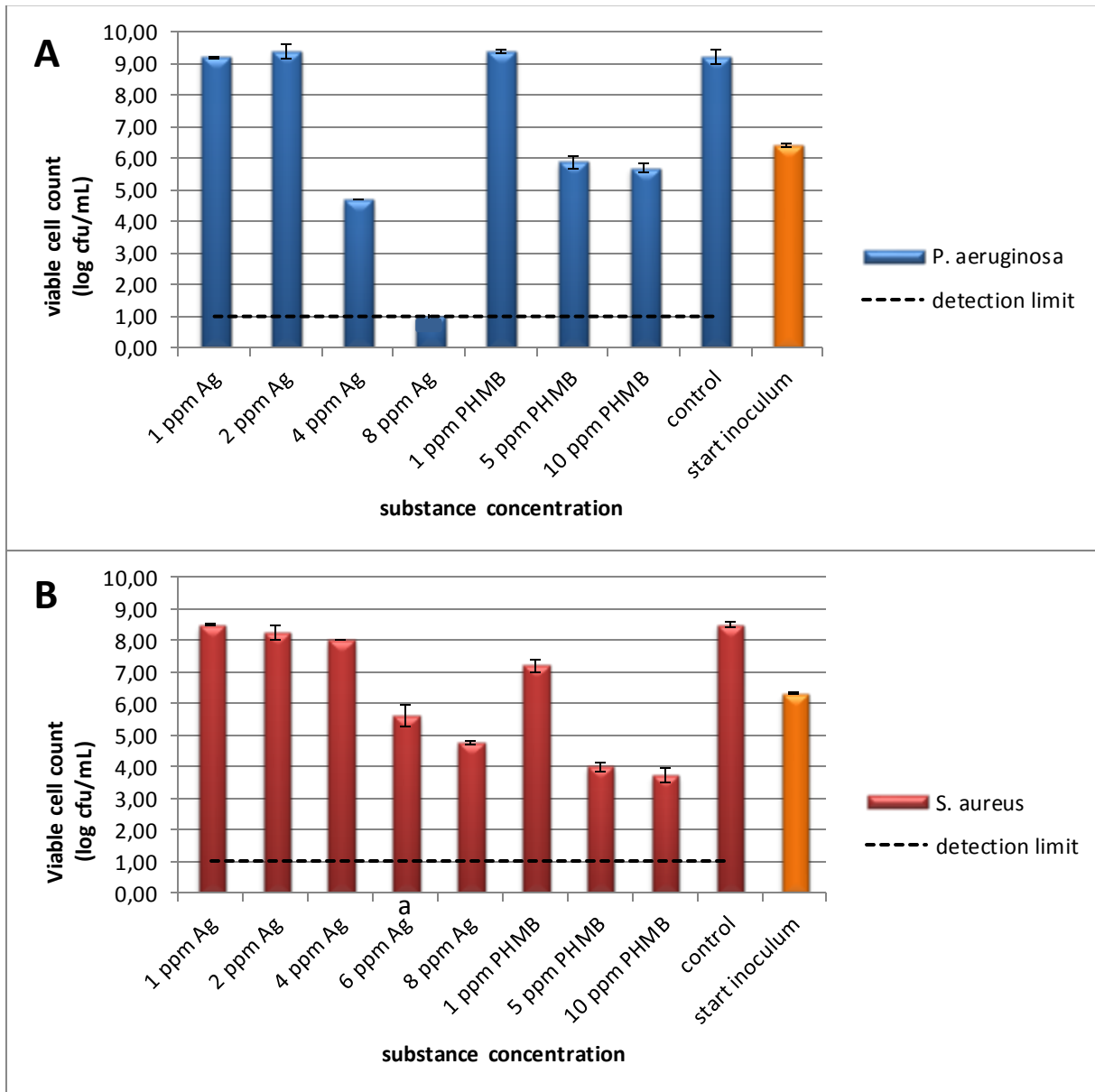


Figure 6. Experiments with a narrower concentration range: AM-effect of silver and PHMB after 24 hours incubation at static conditions, presented as viable cell count (log cfu/mL). Results are mean of three replicates, limit of detection was 10 cfu/mL.

A) AM-effect against *P. aeruginosa*, start inoculum concentration was  $2.5 \times 10^6$  cfu/mL.

B) AM-effect against *S. aureus*, start inoculum concentration was  $2.1 \times 10^6$  cfu/mL. Bar with “a” indicates sample was tested in a separate experiment, start inoculum concentration and viable cell count of control sample for this experiment was  $4.0 \times 10^5$  cfu/mL and  $2.5 \times 10^8$  cfu/mL respectively.

Results show that for *P. aeruginosa*, concentrations of 4 ppm silver and 10 ppm PHMB resulted in log reductions of 1.7 and 0.7 respectively. Corresponding log reductions for *S. aureus* with 6 ppm silver and 5 ppm PHMB were 0.7 and 2.3. The aim of these experiments was to define a specific substance concentration that allowed for the observation of how agitation influences AM-effect. The anticipated result was an increase in AM-effect and for this reason an appropriate concentration was one that produced approximately one log



reduction in viable cell counts. Mentioned concentrations of silver and PHMB were considered adequate for the purpose and thus selected as the concentrations in use for the following.

#### 4.1.1.2 Experiments with agitation

Results for experiments involving incubation during agitation rates of 50 rpm and 100 rpm are presented in Figure 7. Results of experiments performed at static conditions (i.e. at 0 rpm) are also included in the figure in order to display the difference in AM-effect for experiments performed with and without agitation.

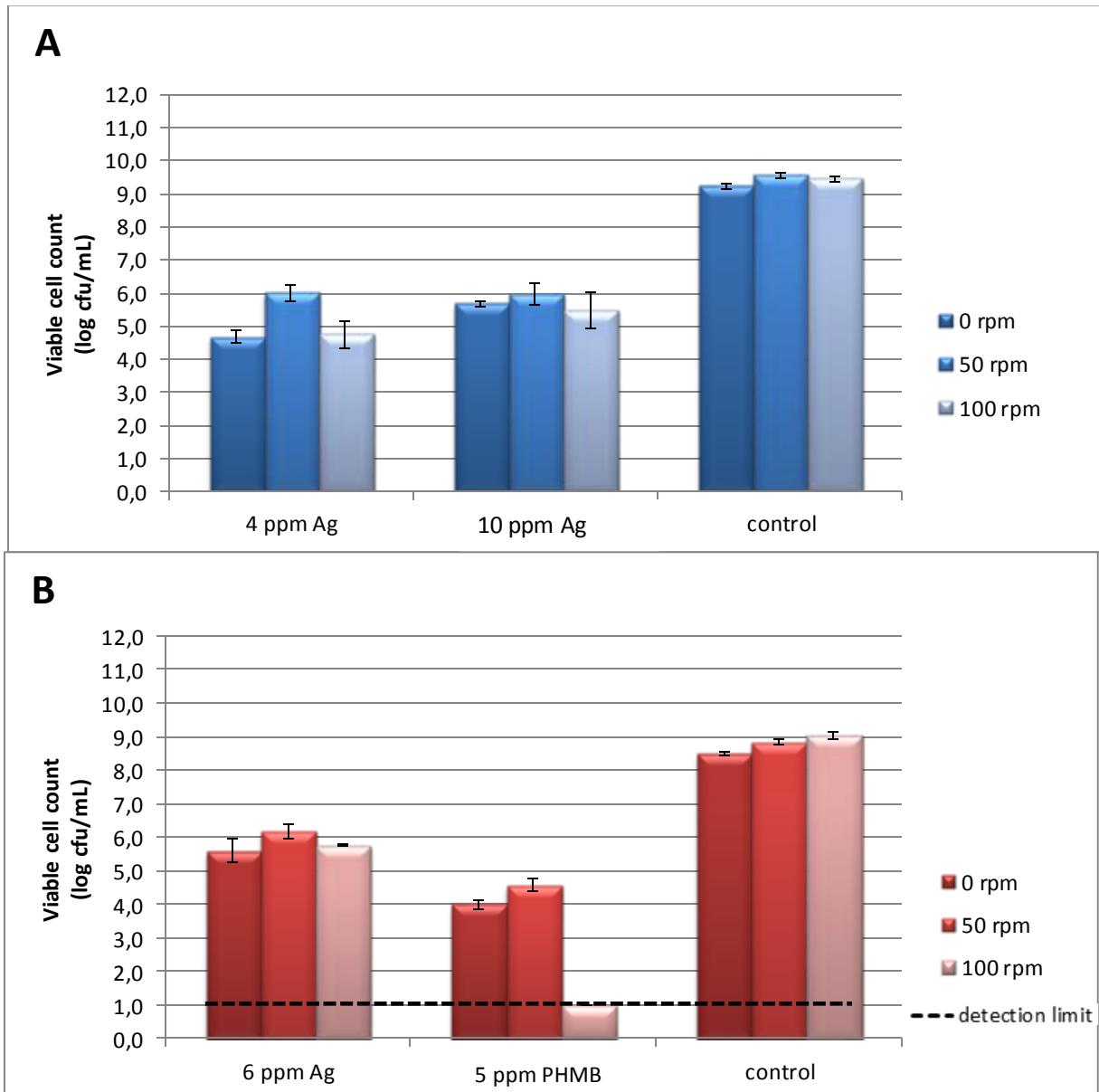


Figure 7. Comparison of AM-effect of silver and PHMB after 24 hours incubation during agitation at 0, 50 and 100 rpm respectively. Data presented as viable counts (log cfu/mL). Results are mean of three replicates.

A) AM-effect against *P. aeruginosa*. Start inoculums concentrations were  $2.5 \times 10^6$  cfu/mL (no agitation),  $1.6 \times 10^7$  cfu/mL (50 rpm) and  $4.0 \times 10^6$  cfu/mL (100 rpm). B) AM-effect against *S. aureus*. Start inoculums concentrations were  $2.0 \times 10^6$  cfu/mL (no agitation),  $4 \times 10^6$  cfu/mL (50 rpm) and  $1.6 \times 10^6$  cfu/mL (100 rpm).

For *P. aeruginosa*, no clear effect of agitation on viable cell count was detected for either silver or PHMB. The same trend was observed for silver solutions tested against *S. aureus*. Conversely, experiments with PHMB against *S. aureus* showed a significant increase in AM-effect when agitation at 100 rpm was employed. The difference in viable cell counts of *S. aureus* between 50 and 100 rpm correlates to >3 log reductions.

#### 4.1.2 AM-effect of wound care dressings

These experiment were only tested against *P. aeruginosa* and showed a correlation between increased agitation and increased AM-activity for all products. The influence of agitation on AM-effect was somewhat greater for the silver-containing products (Mepilex Ag and Aquacel Ag) than the PHMB-products (Suprasorb X+PHMB and Kendall AMD), see Figure 8.

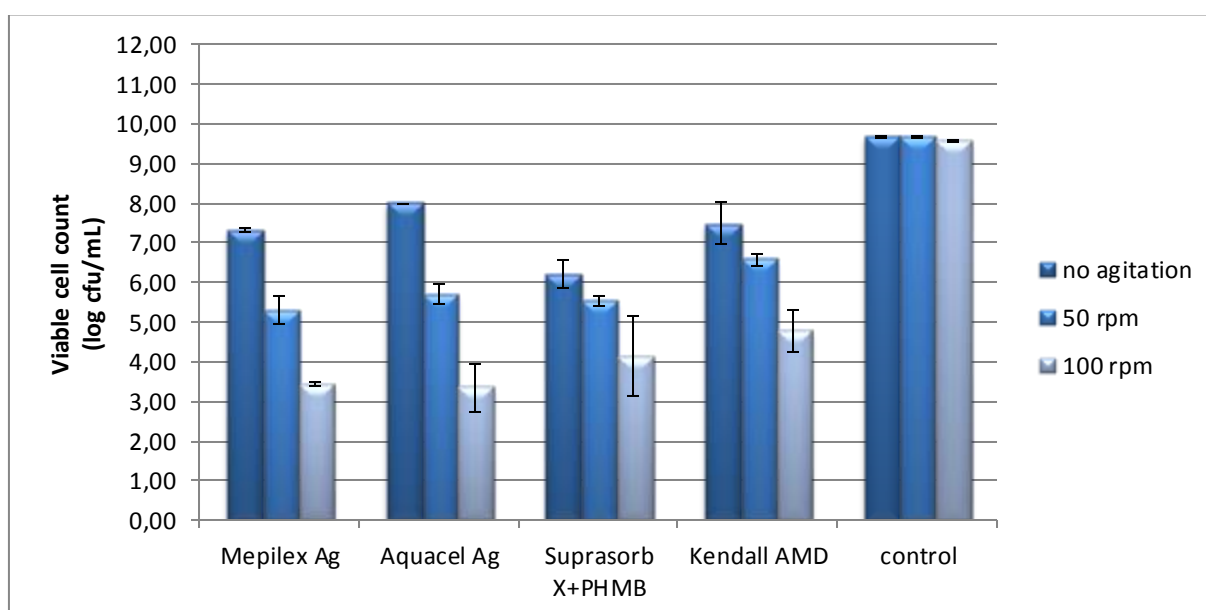


Figure 8. Comparison of AM-effect of products against *P. aeruginosa* after 24 hours incubation during agitation at 0, 50 and 100 rpm respectively. Data presented as viable counts (log cfu/mL). Results are mean of three replicates. Start inoculum concentrations were  $7.9 \times 10^6$  cfu, (no agitation),  $2 \times 10^7$  cfu (50 rpm) and  $2.5 \times 10^7$  cfu (100 rpm).

## 4.2 Release of active substance from products

Results from the determination of silver release using ICP-OES as well as results for determination of PHMB release using absorption measurements are presented in this section.

### 4.2.1 Silver release from products

Results from the detection of silver release from products using ICP-OES are presented in Figure 9. No great difference in the amount silver released from samples incubated at static conditions and agitation at 50 rpm was detected. However, a significantly greater release was detected for samples incubated at 100 rpm, especially for samples of Mepilex Ag.

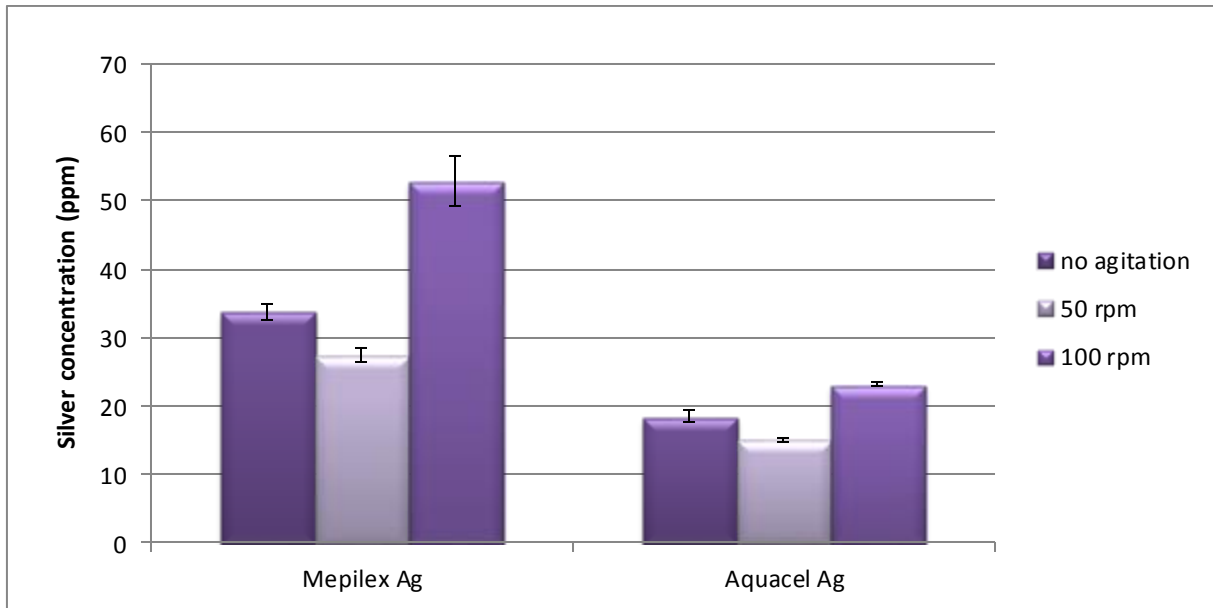


Figure 9. Silver released from products incubated for 24 hours at agitation rates 0, 50 and 100 rpm respectively. Data presented as silver concentration in test medium (mg/L). Results are mean of three replicate samples. Silver content of test pieces correspond to a maximum amount available silver in test medium of 560mg/L (Mepilex Ag; 1.2 mg/cm<sup>2</sup>) and 87mg/L (Aquacel Ag; 0.13 mg/cm<sup>2</sup>) respectively.

#### 4.2.1.1 Analysis of silver content

Results from the experimental verification of reported silver concentrations for the two silver-containing wound care dressings are displayed in Figure 10. The actual yield of silver (calculated from the control samples) in the experiment was approximately 93%.

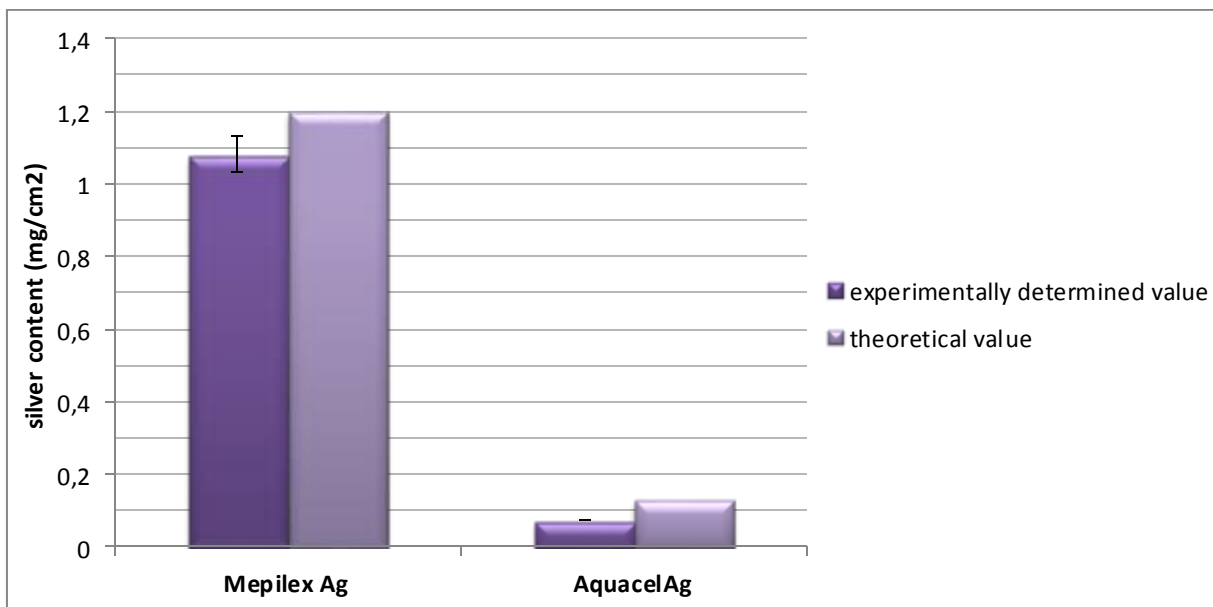


Figure 10. Silver content of products presented in mg/cm<sup>2</sup>. Theoretical value is the reported silver content according to the product data sheet of each product. Experimentally determined value is the silver content obtained by ashing of samples followed by silver analysis by ICP-OES. Results are mean of two replicate samples. Actual yield of silver in the experiment was approximately 93%.

## 4.2.2 PHMB release from products

In this section results for the determination of PHMB release using spectrophotometric (UV absorbance) and colorimetric (anionic red dye Eosin-Y) measurements are presented.

### 4.2.2.1 Spectrophotometric measurements

Standard addition equations were obtained by plotting the detected absorbance signals from each spiked sample (samples spiked with 0, 25 and 50 ppm PHMB), producing a specific standard plot for each product and agitation rate – in total 4 different standard addition plots were constructed. The coefficient of determination ( $R^2$ ) obtained from the regression lines were greater than 0.99 for all samples. Standard addition plots were constructed for all samples, see Figure 11.

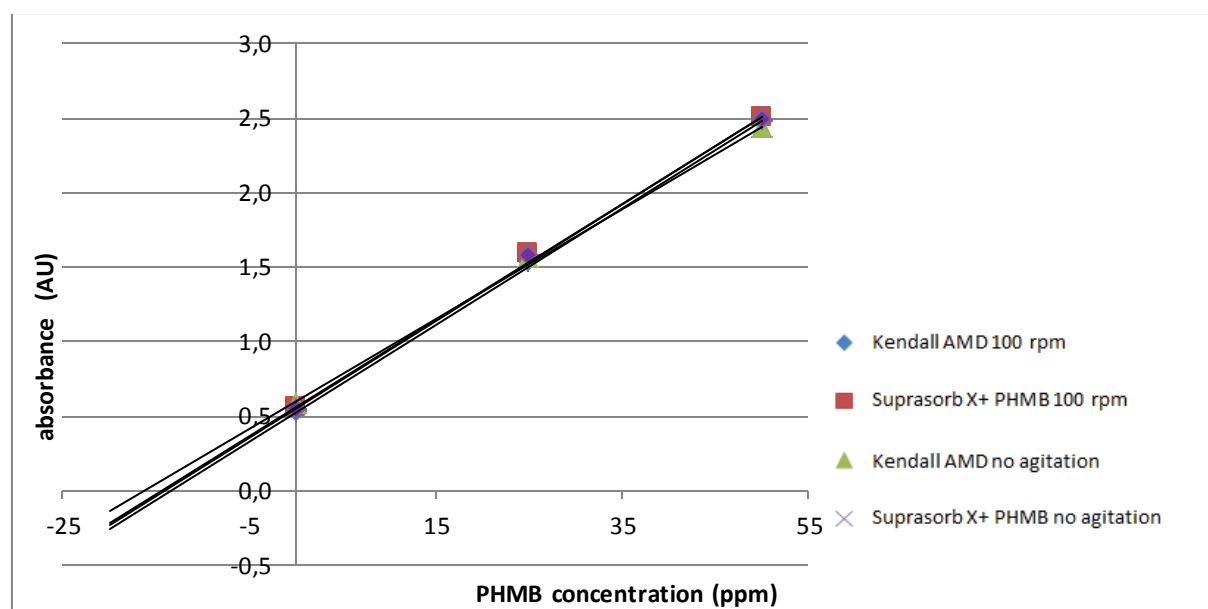


Figure 11. Standard addition plots obtained from absorbance measurements at 236 nm. Data presented in absorbance units (AU). Samples were incubated at 0 and 100 rpm in PBS for 24 hours before 20-fold dilution and spiking of samples with PHMB (0, 25 and 50 ppm). Absorbance values are based on single replicates of each sample, all absorbance measurements performed in duplicate.

According to the data presented in Figure 11, the value of the x-intercept was approximately 15 ppm for all samples which corresponds to a PHMB concentration around 300 ppm (dilution factor 20) for all samples.

Absorbance values for samples incubated at static conditions and during agitation at 100 rpm are presented in Figure 12. Results show that the difference in absorbance between static incubation and incubation at 100 rpm was  $\leq 0.06$  AU for both products. Due to the small difference in absorbance between samples incubated at 0 and 100 rpm, absorbance of samples incubated at 50 ppm was not measured.

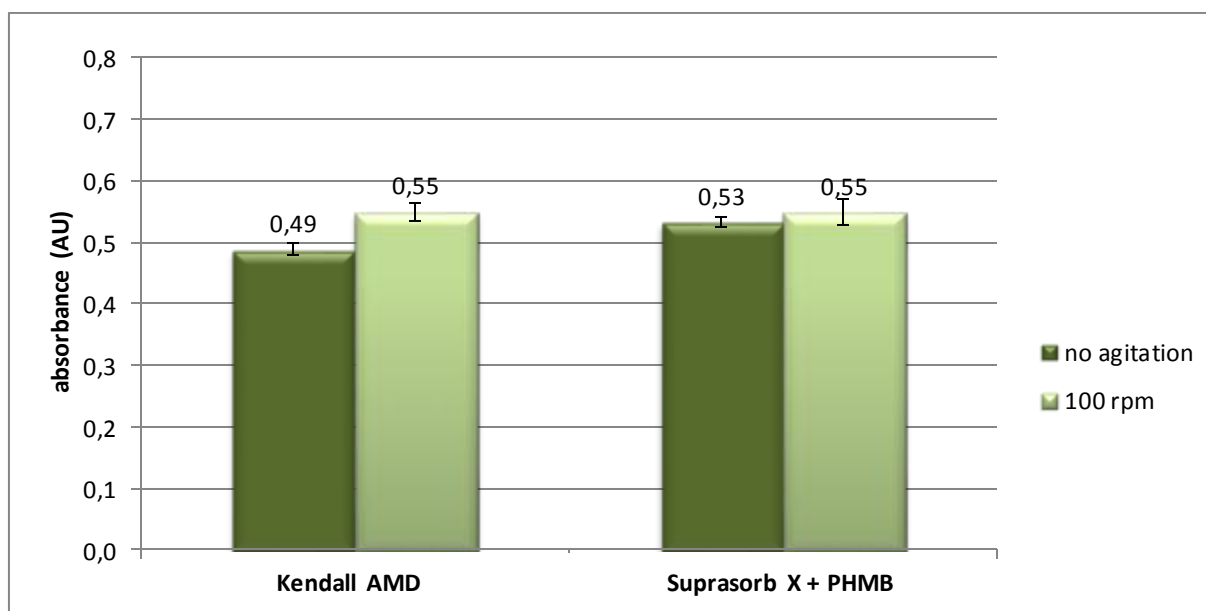


Figure 12. Absorbance values for PHMB-containing samples determined by spectrophotometric measurements at 236 nm. Data presented as absorbance units (AU). Samples were incubated at 0 and 100 rpm in PBS for 24 hours and diluted a 20-fold prior to absorbance measurements. Results are mean of three replicate samples, all spectrophotometric measurements performed in duplicate.

In order to investigate if the detected absorbance was derived from PHMB exclusively, an absorbance spectra was produced for each sample by measuring absorbance over a range of wavelengths. Obtained spectra from the wavelength scan revealed differences in absorbance in comparison with the spectra for PHMB in water, see Figure 13.

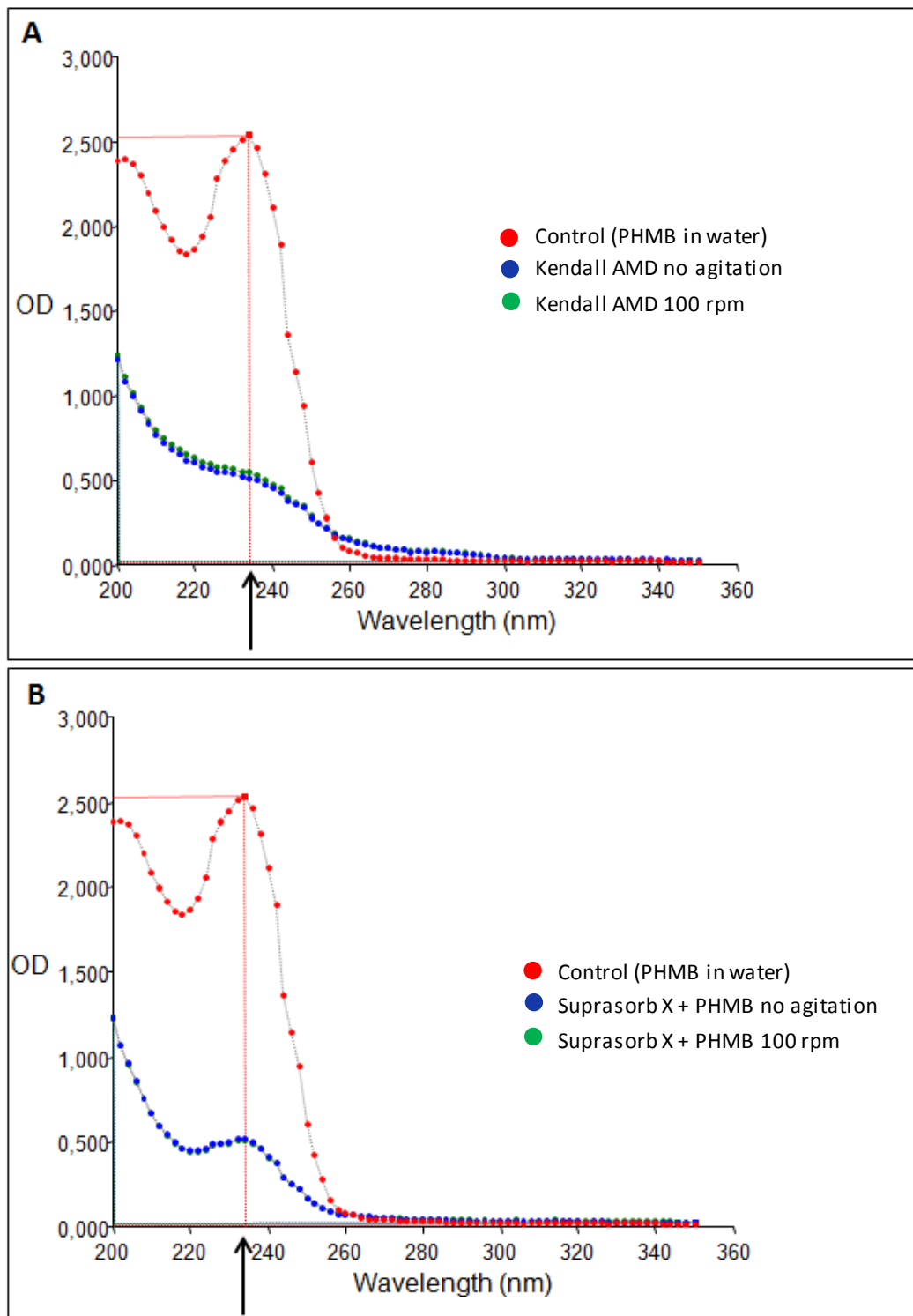


Figure 13. Spectral scan of PHMB concentration over a range of wavelengths (200-350 nm) for products and control sample (PHMB in water). PHMB maximum absorbance wavelength of 236 nm is indicated in the graph by bolded arrow. All spectrophotometric measurements performed in duplicate. A) Absorbance spectrum for Kendall AMD incubated at static conditions and agitation at 100 rpm compared to control sample. B) Absorbance spectrum for Suprasorb X + PHMB incubated at 0 and 100 rpm compared to control sample.

#### 4.2.2.2 Colorimetric measurements

Standard addition plots constructed from absorbance measurements of Eosin Y at 545 nm are displayed in Figure 14. For each product respectively, no clear difference in absorbance between samples incubated at static conditions and during agitation at 100 rpm was possible. Furthermore, Suprasorb X + PHMB samples displayed a greater level of absorbance compared to Kendall AMD samples. According to these data PHMB concentration was around 300 ppm in Suprasorb X + PHMB samples and around 100 ppm in Kendall AMD samples (dilution factor 20).

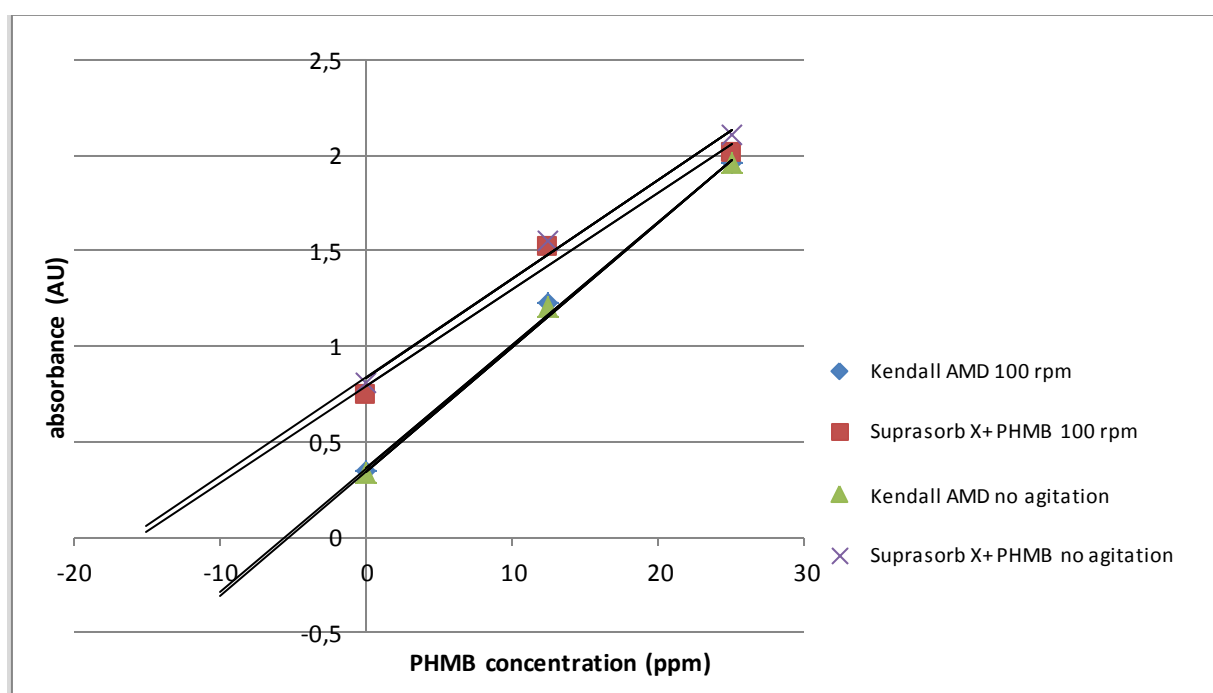


Figure 14. Standard addition plots obtained from absorbance measurements at 545 nm. Data presented as absorbance units (AU). Samples were incubated at 0 and 100 rpm in PBS for 24 hours before 20-fold dilution and reaction with Eosin Y. Absorbance values are based on single replicates of each sample, all absorbance measurements performed in duplicate.

Absorbance values for samples incubated at static conditions and during agitation at 100 rpm are presented in Figure 15. The difference in absorbance between static incubation and incubation at 100 rpm was  $\leq 0.06$  AU for both products.

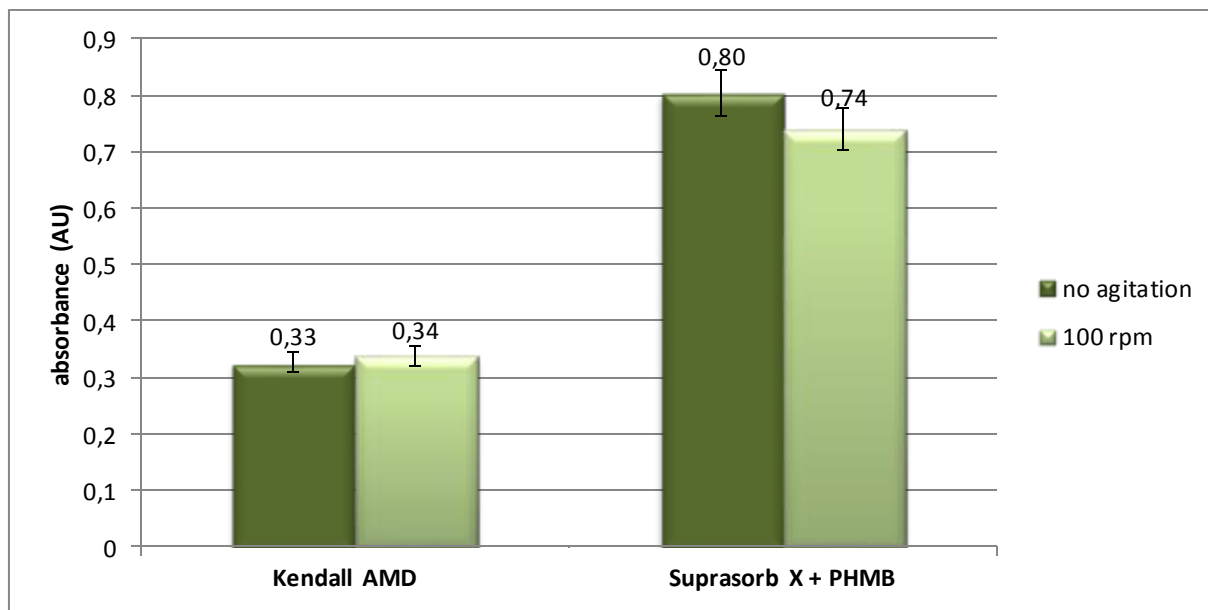


Figure 15. Absorbance values of PHMB-containing samples determined by spectrophotometric measurements at 545 nm after reaction with Eosin Y. Data presented as absorbance units (AU). Samples incubated at 0 and 100 rpm in PBS for 24 hours and 20-fold diluted prior to absorbance measurements. Absorbance values are based on single replicates, all spectrophotometric measurements performed in duplicate.



## 5 Discussion

In this section the results from the experiments of the current study will be discussed and compared to the literature.

### 5.1 Influence of agitation on AM-effect of active substances in solution

According to the *in-vitro* experiments conducted in this thesis work, continuous agitation during incubation seems to have little or no influence on the AM-effect against *P. aeruginosa* when the test organism is directly subjected to active substance in the test solution. This is demonstrated in Figure 7A. The same trend was noted for silver experiments against *S. aureus*. It can also be concluded that silver susceptibility was lower for *S. aureus* compared to *P. aeruginosa*. When subjected to a medium concentration of 4 ppm silver, the number of viable counts for *P. aeruginosa* and *S. aureus* were approximately  $10^5$  and  $10^8$  respectively. This is also in agreement with literature where gram-negative bacteria (e.g. *P. aeruginosa*) have demonstrated higher silver susceptibility compared to gram-positive bacteria (e.g. *S. aureus*). A possible explanation to this phenomenon is the difference in structure of their respective cell wall composition (Tamboli & Lee, 2013). The cell wall of gram-positive bacteria consists of a thick, rigid peptidoglycan layer which can act as a barrier and prevent attachment and penetration of silver ions. In contrast, the cell wall of gram-negative bacteria is comprised of a thin peptidoglycan layer and a negatively charged lipopolysaccharide layer which provides a weak permeability barrier to the positively charged silver ions (Tamboli & Lee, 2013).

Furthermore, PHMB experiments carried out at various agitation rates displayed a clear difference in AM-effect against *S. aureus*. Agitation at 100 rpm resulted in a significant decrease in viable cell counts (>5 log reductions) compared to static conditions and agitation at 50 rpm (approximately 1 log reduction in viable cell counts for both conditions). In contrast to the silver susceptibility of examined test organisms, PHMB susceptibility was greater for *S. aureus* than for *P. aeruginosa*. Antimicrobial activity of PHMB has been proven against both gram-positive and gram-negative bacteria (McDonnell & Russel, 1999). However, lower PHMB susceptibility of some gram-negatives, including *P. aeruginosa*, has been reported in literature, which is in agreement with results of this thesis work. PHMB is a membrane-active agent that may also impair the integrity of the outer membrane of gram-negative bacteria but it has been suggested that the outer membrane may act as a permeability barrier, thus decreasing the antimicrobial effect of PHMB (McDonnell, Russel, 1999). Kramer & Hübner (2010) report PHMB MBCs of 0.1µg/ml for *S. aureus* and 25µg/ml for *P.aeruginosa*. A study performed by Gilbert et al (2001), investigated PHMB susceptibility of *S. aureus* and gram-negative bacteria *E. coli* and also in this study a significantly lower susceptibility was observed for the gram-negative *E. coli* compared to *S. aureus*. Moreover, due to the lack of standardized test methods for *in-vitro* determination of antimicrobial activity it is difficult to compare reported MICs and MBCs of different studies in absolute numbers. Variation in experimental parameters including choice of test medium,

temperature, inoculum concentration etc. prevent accurate comparison of results. In addition to this, the bacterial susceptibility towards AM substances and materials may vary significantly, even within a single bacterial strain (Ruparelia, 2008).

Previous experiments performed at MHC have indicated a correlation between increased agitation rate and enhanced AM-activity of silver in solution (Sandström, 2011). However, these experiments were performed in test tubes whereas the experiments in the present study were performed in 6-well plates with inserts of 24 mm in diameter. As previously mentioned, the meniscus formed in a test tube involves a greater risk of trapping bacteria on the test tube wall which will result in reduced contact between the antimicrobial agent and the test organism. Consequently, agitation will have greater influence on the number of contact points between AM-agent and bacteria in this type of test system. This could partly explain the lack of differentiation between the AM-effect of active substance in solution when incubated at static or agitated conditions observed in the present study. Moreover, the experimental set-up involved initial mixing of test medium in the experiments with active substance in solution by pipetting up and down three times. As a result, enhanced contact between AM agent and bacteria is acquired from start and perhaps the initial mixing provided sufficient contact points and thus the applied agitation during incubation became superfluous. The nonexistent influence of agitation on AM-effect observed in the present study could also be related to the incubation time of the experiments. A study conducted by Sena et al (2006) investigated the influence of agitation on AM-effect by subjecting microorganisms to different AM-agents for a time period of 30 seconds – 60 minutes. Strains of both *S. aureus* and *P.aeruginosa* were evaluated in the study and results showed that mechanical agitation promoted the effectiveness of the antimicrobial agents, i.e. agitation decreased the time required to eliminate the bacteria. A lengthy incubation time of 24 hours would not allow the detection of such an effect.

## 5.2 Pre-wetting of products

In the product experiments, test pieces were pre-wetted with a liquid volume corresponding to the specific maximum liquid absorption capacity of each product. It was desirable to seal the edges of the foam test pieces to ensure that the active substance is released through the wound contact layer and not through the punched edges. Also, highly absorbent test pieces will increase in size as liquid is absorbed by the material and as a consequence, contact between the test piece and the bacteria in the test solution is not maintained during the experiment if edges are sealed with silicone paste. This phenomenon is however avoided by pre-wetting the test pieces. In the present study test pieces that were fully saturated with (non-inoculated) liquid before being placed in the inserts. Even though this procedure involves a difference in total bacteria concentration for different samples the “total challenge” can be considered equivalent since each pre-wetted test piece is subjected to the same volume of inoculated test solution and thus the same number of bacteria. Moreover, alternative experimental procedures could also be considered. For example, it could be argued that the total amount of test liquid should be the same for all test pieces since this

way samples are subjected to the same total concentration of bacteria. This set-up perhaps mimics the situation in a wound more accurately, yet it may impose an unfair challenge considering that the variation in absorption capacity involves a difference in the actual number of bacteria that each sample is subjected to.

### 5.3 Correlation between silver release and AM-effect of wound care dressings

In contrast to the results of experiments performed with active substance in solution, the product experiments conducted in this thesis work showed that agitation seems to have a significant effect on AM-activity.

The correlation between silver release and AM-activity (AM-activity presented as log reductions in viable cell count) is displayed in Figure 16.

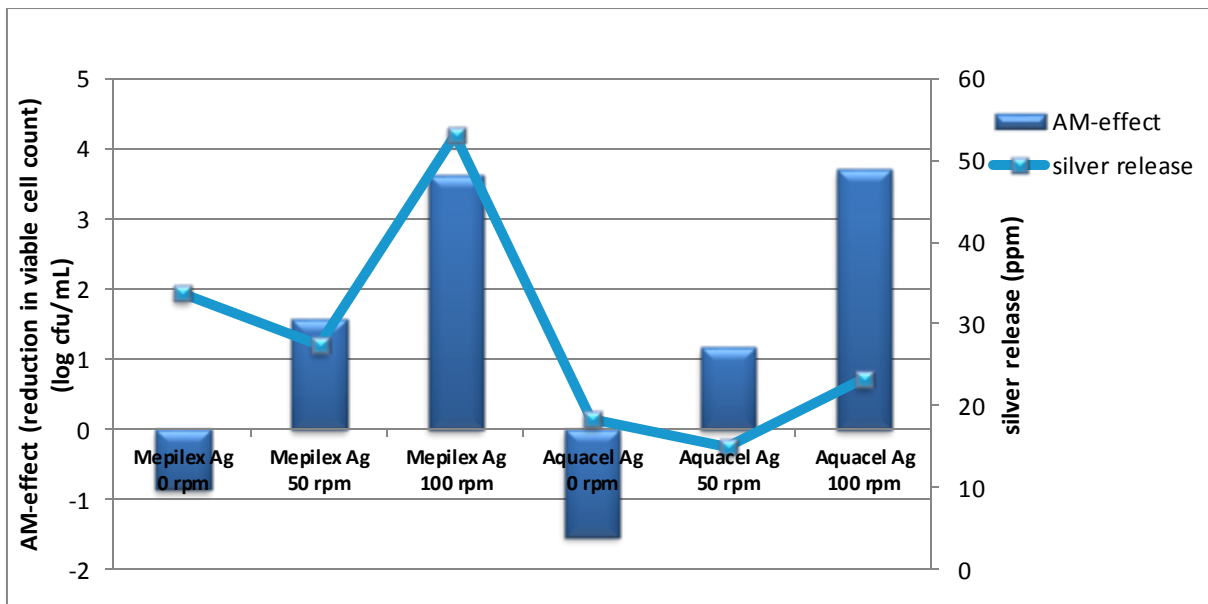


Figure 16. Correlation between AM-effect against *P. aeruginosa* and silver release from products at different agitation rates. Data derived from results presented in section 4. AM-effect presented in viable cell counts (log cfu/mL) on primary axis, silver release presented in ppm on secondary axis.

For both Mepilex Ag and Aquacel Ag, the greatest AM-activity as well as the highest release of silver was observed at 100 rpm. Compared to the result for samples incubated at static conditions, agitation at 50 rpm resulted in a higher AM-effect against *P. aeruginosa*. This was however not reflected in the detected amounts of silver released from the products. A possible explanation to the diverging results of silver release and observed AM-effect is that an agitation rate of 50 rpm results in enhanced contact between bacteria and AM-agent even though the actual amount of silver in the test system is intact. This is also in agreement with the proposed theory that the initial mixing of the active substance and the inoculated test medium will provide sufficient contact between AM-agent and bacteria to increase the AM-effect. In the product tests, no such mixing occurs at static conditions and for this reason it is reasonable to believe that agitation will have a profound effect on the AM-activity.

Furthermore, previous experiments performed at MHC have assessed the AM-effect of different silver release profiles and results showed that a slow release of silver resulted in an increased number of viable cell counts for *P. aeruginosa* compared to when the same amount of silver was added from start (Sandström, 2011). Assuming that static conditions involves a slower release of silver compared to when agitation is employed this may have contributed to the increased AM-effect at 50 rpm compared to static conditions.

### 5.3.1 Analysis of silver content

The total silver content of dressing test-pieces were analyzed using ICP-OES in order to investigate if the experimentally determined values were in accordance with the silver content reported in the product data sheets of each product. The experimentally determined silver content of Mepilex Ag corresponded to 93% of the reported target value. For Aquacel Ag the experimentally determined value was only 57% of the reported value which was surprisingly low. In Mepilex Ag silver is incorporated as silver sulphate which will readily release silver ions when in contact with liquid. According to the manufacturer Aquacel Ag contains ionic silver and carboxymethylcellulose (CMC) and it is possible that additional treatment would have been necessary in order to detect all silver present in the dressing. Silver could for example have reacted with chloride present in the sample resulting in a precipitation of AgCl. By adding chloride ions in excess,  $AgCl_2$  will be formed instead which is soluble and thus possible to detect with ICP-OES. Based on the silver content as presented in the product data sheets the fraction silver released from the silver-containing products during incubation was calculated, see Table 6. The fractional release of silver was higher for Aquacel Ag compared to Mepilex Ag. However, according to the instruction of use, both products can be left in place for up to seven days, which implies that Mepilex Ag has a larger possibility to release silver for the whole wear time.

Product	Agitation rate (rpm)	Silver release (% of total silver content)
Mepilex Ag	0	6
	50	5
	100	10
Aquacel Ag	0	21
	50	18
	100	27

Table 6. Silver released from products during incubation presented as fraction of total silver content in test pieces as stated in product data sheets. Results are mean of six replicate samples.

### 5.4 Determination of PHMB release

Determination of PHMB release proved to be associated with a number of difficulties. The major obstacle was the lack of a representative blank sample which is a necessity in order to detect potential interference from other substances present in the solution which absorb in the same region as PHMB. There are products versions of both Kendall AMD and Suprasorb X + PHMB available on the market which appear to be equivalent in composition apart from the lack of the antimicrobial substance. However, even though the compositions of these

products appear to be identical to their antimicrobial counterparts they could possibly contain substances that interfere with the analysis of PHMB and hence, these products could not serve as proper control samples. The absorbance scan was conducted in an attempt to detect the potential presence of interfering substances. Figure 13 displays deviations between the absorption spectra for PHMB in water (control spectrum) and the absorption spectra for product samples, indicating presence of interfering substances in the samples. If the detected signal from the product samples measurements had been the result of PHMB absorbance exclusively, the appearance of the absorption spectra would have been analogous to that of the control spectrum. Moreover, the appearance of the scanning spectra reveals a greater resemblance between Suprasorb X + PHMB and the control in comparison with spectra of Kendall AMD and the control. This indicates a higher level of interference in the Kendall AMD sample.

Due to the unknown level of interference in the absorption measurements at 236 nm, the actual PHMB content of each sample could not be determined from obtained absorption data. Nevertheless, the detected absorbance corresponds to a PHMB concentration of around 300 ppm and consequently, it could be assumed that this is the *maximum* amount PHMB released from the products. According to the PHMB content stated in the product data sheet of each product this would correspond to a release of approximately 80% of the PHMB content of Kendall AMD and more or less the entire PHMB content of Suprasorb X + PHMB. Even though an unknown fraction of the detected absorbance may be due to interference from other substances these measurements indicate that a substantial amount of the total PHMB content was released during incubation. A high level of release is also in line with the high solubility of PHMB in water (40% w/w). However, even though PHMB solubility is high, the interaction of active substance and the material is also an essential parameter for release properties. It should also be mentioned that the use of PBS instead of SWF as test medium may have affected the release of PHMB. The potential difference in release has not been possible to fully investigate since no proper method for determination of PHMB in SWF is available at MHC. Moreover, detected absorbance values at 236 nm presented in Figure 11 provide an estimation of the difference in PHMB content for the examined samples. According to the standard addition plots, 1 AU corresponds to an approximate concentration difference of 25 ppm for all examined samples. Results for Kendall AMD showed that the difference in absorbance between samples incubated at static conditions (0.49 AU) and during agitation at 100 rpm (0.55 AU) corresponded to an actual concentration difference of around 30 ppm. The concentration difference for Suprasorb X + PHMB samples was around 10 ppm. As earlier discussed, the potential difference in medium composition (and thus potential difference in level of interference) of the two products disables accurate comparison of detected PHMB concentrations for different products.

In contrast to the results of absorbance measurements at 236 nm, measurements of the Eosin Y reaction carried out at 545 nm displayed a distinction between Kendall AMD and Suprasorb X + PHMB samples. The detected amount PHMB in these measurements was

approximately 300 ppm for Suprasorb X + PHMB but only around 100 ppm for Kendall AMD. Furthermore, according to the standard addition plots obtained at 545 nm, the difference in concentration for samples incubated at static conditions and during agitation at 100 rpm was around 20 ppm Suprasorb X + PHMB and only around 5 ppm for Kendall AMD. In conclusion, the two analytical assessments of PHMB release conducted in this thesis work detected different concentrations of PHMB in the analyzed samples. However, both methods displayed small concentration differences between products incubated at static and agitated conditions. It can also be concluded that it seems as though the amount PHMB released is lower than 300 ppm for both products. The considerably lower levels of PHMB detected in the colorimetric measurements of Kendall AMD samples combined with the deviating appearance of the scanning spectra for Kendall AMD compared to the control spectrum indicate a higher level of interference in mentioned product sample in the absorption measurements carried out at 236 nm. A plausible explanation to the diverging results of the two techniques employed in this study is that interfering substances present in the Kendall AMD sample absorb light in the same region as PHMB but do not react with Eosin Y and consequently, these substances will not be detected at 545 nm. According to this hypothesis, the levels of PHMB detected in the colorimetric measurements are to be considered as more accurate. Moreover, even though interference from other substances may have produced falsely high PHMB release concentrations, results for static and agitated conditions of the same products may be still be compared since the level of interference is assumed to be consistent within a single product type. Accordingly, all data collected in the experiments of this thesis work suggest that agitation does not greatly influence the release of PHMB from products.

## 6 Conclusions

According to the experiments conducted in this thesis work agitation seems to have no or little influence on the antimicrobial effect of active substances in solution for the test system in use. The incubation time was 24 hours for all experiments and hence shorter test times could reveal a potential correlation between agitation and antimicrobial effect of active substances in solution. Moreover, bacterial susceptibility varied for different AM-agents in solution. This was most apparent for PHMB for which a significantly greater susceptibility was observed for *S. aureus* compared to *P. aeruginosa*.

As opposed to the results from experiments with bacteria subjected to antimicrobial substances in solution, agitation seems to significantly influence the antimicrobial effect of products for the test system in use. A correlation was observed between silver release and AM-effect at the higher agitation rate (100 rpm), no correlation was observed at the lower agitation rate (50 rpm). A possible explanation to this phenomenon is that agitation at 50 rpm results in enhanced contact between antimicrobial agent and bacteria and as a consequence, an increased AM-effect is attained even though the silver concentration in the test medium is not increased. Another contributing factor to the enhanced AM-effect observed during agitation could be a more rapid release of silver. The release of PHMB from products was difficult to assess due to the lack of a proper blank sample. However, results indicate a greater relative release of PHMB from products compared to the relative amount of silver released. Furthermore, it could be concluded that agitation does not seem to greatly influence the amount PHMB released from products, even at the higher agitation rate.

## 7 Future work

It would be highly desirable to develop a method that enables proper determination of PHMB. Since the composition of the test media may affect the release of PHMB, such a method should preferably enable the analysis of PHMB suspended in proteins-containing solutions, e.g. SWF. This would also enable the determination of both AM-effect and PHMB release on the same test system. Furthermore, it would be interesting to evaluate the influence of agitation on AM-effect and release at shorter time points. This would address the hypothesis of different release kinetics of PHMB and silver. It is also of interest to investigate the influence of agitation on AM-effect against other test organisms, such as *S. aureus*.



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