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Study of *Pseudomonas aeruginosa* and different wound dressing products

Master of Science Thesis

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Abstract

An injury to the skin resulting in a wound starts the complex healing machinery of the body, normally leading to rapid wound closure. In some cases the normal wound healing process is disturbed and prolonged; which can lead to chronic, non-healing wounds such as venous leg ulcers. Chronic wounds in general are estimated to affect 1 – 2 % of the world's population, and about 1 % is suffering from venous leg ulcers.

The persistency of chronic wounds is often caused by bacterial infections, where the invading microorganisms cause significantly impaired wound healing. There is now increasing knowledge that infections in humans often involve bacterial biofilms, where bacteria form microcolonies embedded in a protective extracellular polysaccharide matrix. Bacteria growing in mature biofilm are very hard to eradicate due to development of several resistance mechanisms. One of the most common pathogens in chronic wounds is *Pseudomonas aeruginosa*, a very problematic microbe due to its ability to form resistant biofilms. The bacteria produce several virulence factors that allows for establishment in host tissue, where one important substance is the blue pigment pyocyanin. Pyocyanin has several toxic effects on host cells, including depletion of NADH, glutathione and other antioxidants in the host cell, an alteration of the redox status, and generation of oxygen radicals.

Chronic wounds, especially venous leg ulcers, are often treated with foam dressings capable of absorbing high amounts of wound exudates. This project is an attempt to elucidate if polyurethane foam dressings can promote wound infection by *P. aeruginosa.* This was investigated by in vitro studies of bacterial growth, biofilm formation and production of pyocyanin in the absence and presence of wound dressing samples.

P. aeruginosa PAO1 was cultured together with wound dressing pieces. Viable cell counts showed that growth of *P. aeruginosa* was not affected by the presence of any wound dressing product included in the study. The presence of polyurethane foam dressings in *P. aeruginosa* cultures caused an altered environment, which was observed as a change in color from green to yellow due to reduction of pyocyanin. Measurement by absorbance spectrophotometry also showed that the presence of wound dressing products resulted in lower concentration of pyocyanin in the solution, compared to the control. The amount of pyocyanin in *P. aeruginosa* cultures was found to increase over time, where the control culture showed the largest increase. In cultures containing polyurethane foam dressings the pyocyanin content increased in the solutions only between day 1 and day 3. Between day 3 and day 7 the increase in pyocyanin content instead occurred inside the dressing products. Dressings were also placed in a cell-free pyocyanin solution derived from stationary phase culture. Absorbance measurements of the pyocyanin solutions showed pyocyanin adherence to the dressing material polyurethane, even in the absence of bacteria.

The polyurethane foam dressing products included in this study all show similar results in the experiments. The wound dressings do not seem to influence the number of viable cells in the bacterial suspensions. The presence of dressings in cultures seems to have a decreasing effect on the level of the virulence factor pyocyanin in the bacterial suspensions, due to accumulation and adherence inside the wound dressing.

Keywords: biofilm, chronic wounds, infection, polyurethane foam dressings, *Pseudomonas aeruginosa*, pyocyanin, wound dressings

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1. Introduction

The skin is one of the largest organs of the body and functions as a protective barrier from the outside environment with temperature differences, radiation and pathogenic microorganisms. An injury to the skin immediately starts the complex wound healing mechanism, involving blood cells, epithelial and connective tissue cells, extracellular matrix and a variety of cell mediators usually resulting in wound closure within days or weeks. In a non-healing, chronic wound the healing process has been disturbed and is often detained in an inflammatory phase, and is unable to progress into the next stage of cell proliferation. The most common examples of non-healing wounds are venous leg ulcers, diabetic foot ulcers and pressure ulcers.

The awareness is increasing of the persistency of non-healing wounds often being caused by bacterial infections (Bjarnsholt et al 2006, Gjødsbøl et al 2006, Kirketerp-Møller et al 2008, and Power et al 2001). All wounds are contaminated by several strains of bacteria, derived from endogenous sources such as the gastrointestinal tract, the surrounding skin, the environment or from the healthcare provider. A wound is said to be infected when the invading microorganisms cause notably impaired wound healing. When infectious bacteria are invading a host, toxic substances are produced by the microorganisms that cause damage to the host tissues. These substances are called virulence factors and allow the bacteria to establish in the host. The host responds to the bacterial invasion with attack of inflammatory cells such as neutrophils which release cytotoxic enzymes, oxygen radicals and inflammatory mediators which cause further damage to host tissue. This host response mechanism is also contributing to the non-healing stage of the infected wound. (Bjarnsholt et al 2006)

It is believed that about 80 % of human infections involve bacterial biofilms (Bjarnsholt et al 2006). Biofilms form when bacteria attach to a surface or aggregates and form microcolonies, embedded in a protective extracellular polysaccharide matrix. Bacteria growing in mature biofilm are very hard to eradicate from the host due to development of several resistance mechanisms. One of the most common pathogens in chronic wounds is *Pseudomonas aeruginosa*, a very problematic microbe due to its ability to form resistant biofilms (Thomsen et al 2010). *P. aeruginosa* is an opportunistic pathogen, often acquired in hospital environments and is often associated with respiratory and urinary infections, in burn damage wounds, and in chronic wounds (Gjødsbøl et al 2006, Madigan & Martinko 2006). Once established in a wound, the bacterium is almost impossible to eliminate with antibiotics due to biofilm formation (Kirketerp-Møller et al 2008). Since bacterial infections of *P. aeruginosa* in chronic wounds are so common, it is important that the wound dressing used for treatment is not promoting or facilitating bacterial growth.

1.2 Objective

The objective of this master thesis project was to investigate if polyurethane foam dressings can promote wound infection by *P. aeruginosa.* This was examined by culturing *P. aeruginosa* in the presence and absence of wound dressing samples. The effect on bacterial growth was studied by performing viable cell counts, and production of biofilm and the virulence factor pyocyanin were measured by absorbance spectrophotometry. The experiment included different commercially available dressing products from Mölnlycke Health Care as well as from competitors.

2. Background

The background section will provide a short description of acute wound healing as well as what will happen if the wound healing process fails, resulting in a non-healing wound. The resistance mechanisms of the immune system are explained as well as how a bacterial infection is affecting the wound healing. Special focus is placed on the pathogenic bacterium *Pseudomonas aeruginosa* which is commonly found in chronic wounds, and how this microorganism is growing and causing injury to the host.

2.1 Wound healing

Cutaneous wound healing is a dynamic and complex process that involves blood cells, epithelial and connective tissue cells, extracellular matrix and a variety of cell mediators. The process is in general consisting of three phases – inflammation, granulation tissue formation, and tissue remodeling. (Singer & Clark 1999) In the first phase, platelets and blood proteins cause the blood to coagulate into a fibrin clot which quickly stops blood loss and also serves as a provisional extracellular matrix. This allows for first line immune system cells such as neutrophils, monocytes and macrophages to migrate into the blood clot directed by different chemoattractants. They remove bacteria, cell debris and foreign particles by phagocytosis but also release several growth factors that influence surrounding tissue cells to initiate production of granulation tissue, which in the second phase replaces the blood clot. Fibroblasts which are attracted into the wound area produce new extracellular matrix that provide a scaffold for further cell proliferation and ingrowth, and new blood vessels grow into the wound in order to supply the cells with oxygen and nutrients. Epidermal cells start to proliferate and migrate into the wound area in a process called reepithelialization. In the last phase, wound contraction occurs by the action of fibroblasts which are also responsible for remodeling of the collagen matrix into mature tissue. (Singer & Clark 1999)

2.2 Non-healing wounds

There are many cases where the normal wound healing process is disturbed and prolonged; this can lead to the development of a chronic, non-healing wound. The most common examples are pressure ulcers, diabetic ulcers, and venous leg ulcers. (Rickard et al 2009) Venous leg ulcers are believed to be caused by inadequate function of the valves in lower extremity veins, leading to increased hydrostatic pressure and edema in subcutaneous tissues which increases the risk for ulcer formation (Thomsen et al 2010). The treatment is compression therapy and wound fluid handling in order to restore the venous pressure. Diabetic foot ulcers are resulting from repetitive loading on the neurophatic foot where the tactile sense is impaired. In combination with an inadequate blood perfusion and weakened immune system with low motility of neutrophils, ulcers are prone to occur. In extreme cases non-healing diabetic foot ulcers lead to amputation of the foot or the leg. Treatment consists of off-loading of the foot and improvement of circulation (James et al 2008, Sibbald et al 2003). Pressure ulcers arise from long-term loading on sensitive areas such as shoulders, sacral region and heels on the immobilized patient and are treated with off-loading and movement. Chronic wounds in general are estimated to affect 1 – 2 % of the world's population (Gottrup 2004), and about 1 % is

suffering from venous leg ulcers (Gottrup 2004, Trent et al 2005). Apart from contributing to increased cost in the health care sector, chronic wounds cause pain, decreased quality of life and the risk for further, more dangerous complications in patients. Along with the worldwide increase in diseases like obesity, diabetes and cardiovascular disease, it can be assumed that the number of patients with chronic wounds will continue to grow. Other life style factors that can affect the ability of wound healing are smoking, malnutrition and alcoholism (Bjarnsholt et al 2008).

The general characteristic of a chronic non-healing wound is a detention in the inflammatory phase of healing and an inability of the wound to progress into the proliferative phase. This features a continuous influx of neutrophils into the wound area, which release free radicals, cytolytic enzymes and inflammatory mediators that cause damage to invading pathogens as well as to host tissue (Sibbald et al 2003). Other leukocytes present produce inflammatory mediators that affect inflammatory cells as well as fibroblasts, epithelial cells and vascular epithelial cells, hence contributing to the detention in the inflammation phase (Sibbald et al 2003). Another feature is elevated amounts of matrix metalloproteinases (MMP), enzymes produced mainly by neutrophils. These enzymes play a key role in normal wound healing for removing dead tissue and later for enabling migration of fibroblasts, keratinocytes and other cells. An excess amount of MMPs is however believed to impair wound healing (Bjarnsholt et al 2008). The balance between pro-inflammatory substances and their inhibitors that exist in acute wounds has been altered in chronic wounds; resulting in degradation of extra-cellular matrix, impaired cell migration and reduced fibroblast proliferation and collagen synthesis (Stojadinovic et al 2008).

Progression towards healing is further inhibited by inadequate tissue perfusion caused by arterial or venous insufficiency, blood loss, trauma or edema. Poor blood supply results in hypoxic conditions in tissues which can lead to cell death and tissue necrosis. This provides good growing conditions for pathogenic microorganisms leading to establishment and colonization of bacteria in the host tissue (Sibbald et al 2003).

2.2.1 Infection in wounds

During the last years there has been growing knowledge and evidence for that the persistency of chronic wounds is in many cases caused by bacterial infections (Gjødsbøl et al 2006, Kirketerp-Møller et al 2008, Bjarnsholt et al 2008, and Power et al 2001). All wounds are *contaminated* by bacteria, meaning that microorganisms are present but not replicating. Wounds become contaminated from endogenous sources such as the gastrointestinal tract, the surrounding skin, the environment or from the healthcare provider (Sibbald et al 2003). The wound is said to be *colonized* when bacteria are replicating to a certain number, but are still not impairing the healing process and do not cause host response. If the established bacteria are allowed to continue to grow, the microbial load will eventually initiate a host response, thus an inflammatory reaction. *Infection* is when the invading microorganisms are present in high amounts that cause significantly impaired wound healing and toxicity (Stojadinovic et al 2008, Sibbald et al 2003). Previous attempts have been made in order to find a threshold value for bacterial load, suggesting that a concentration above 10^6 colony forming units (CFU)/mL of wound fluid (Sibbald et al 2003) or above 10^5 bacteria/g tissue (Bjarnsholt et al 2008) would be a sign of infection. However, the perspective has been altered to that no clear limit in amount of bacteria exists between a colonized wound and an infected wound. Instead, the relationship between the bacterial load and the host resistance determines

the outcome (Stojadinovic et al 2008, Bjarnsholt et al 2008, and Sibbald et al 2003). This implies that a patient with strong host response can handle a higher bacterial load without resulting in disease compared to a patient with compromised host resistance. Consideration must also be taken into what bacteria that is invading. Bacteria have different capabilities to cause injury to the host, where some species can be present in relatively low amounts and still cause significant harm, while other bacteria can exist in greater number without affecting the wound healing process (Schultz et al 2003). Pathogenic bacteria invading a host are producing several toxic substances that allow them to establish in the host tissue. These substances are called virulence factors and are described in more details below. When the immune system of the host responds to the bacterial invasion, inflammatory cells like neutrophils release cytotoxic enzymes, oxygen radicals and inflammatory mediators which cause further damage to host tissue. This mechanism is also contributing to the non-healing stage of the infected wound (Bjarnsholt et al 2008).

The classic signs of infection in wounds include swelling, redness, pain, heat, purulence, and impaired function. Chronic wounds can also show symptoms such as low transcutaneous oxygen tension, development of necrotic tissue, foul odor and wound breakdown (Gardner et al 2001), as well as deterioration and discoloration of granulation tissue and increased friability (Sibbald et al 2003). As some of the above signs may be absent in chronic wounds even if the healing process is disturbed, the most important symptom of infection is failure to heal (Schultz 2003, Sibbald et al 2003).

2.2.2 Biofilm formation

The classic view of bacterial growth in a culture is as freely dispersed bacterial cells in a culture medium, just like in a beaker in the laboratory. This perspective is however not a realistic description of the microbial world, and ongoing research is continuously increasing our understanding about bacterial growth. A great majority, 99.9 %, of the total microbial biomass on earth is instead growing in biofilms (Bjarnsholt et al 2008). Biofilm is commonly encountered as the plaque forming on teeth, the gunk that has to be removed from household pipes, or the slippery green coatings on stones in a creek. Biofilm forms when bacteria adhere to a surface where they start to proliferate into microcolonies. It is important to note that the surface can be biological e.g. living or dead tissue in wounds, non-biological material e.g. medical implants, as well as bacterial aggregates. The bacteria produce and embed themselves in an exopolymer matrix, which functions as a shield allowing for attachment and protection from the host responses. The matrix consists mainly of polysaccharides and proteins, and to a lesser extent DNA (Donlan & Costerton 2002, Wolcott & Rhoads 2008). Mature biofilm has a complex three-dimensional structure consisting of dense regions with bacterial microcolonies, interspersed with channels carrying water, nutrients and waste products (Lindsay & von Holy 2006). The EPS matrix constitutes a physical barrier and slows down diffusion of antibiotics. It also prevents penetration of cells of the immune system and the antimicrobial oxygen species which they produce. The antibiotic resistance of bacteria in biofilm is believed to rely on other causes as well, e.g. a steady state growth mode caused by limitations of oxygen and nutrients. (Drenkard 2003, Costerton et al 1999) Many antibiotics only inhibit growth of cells, and do not affect existing ones. Yet another protection mechanism is that bacterial cells in biofilms develop a specific phenotype with altered expression patterns of genes and proteins, which could be linked to a higher resistance to antimicrobials. (Drenkard 2003, Costerton et al 1999). In 2003

showed Harrison-Balestra in an in vitro study that *P. aeruginosa* forms biofilm in just ten hours, but hypothesized that the formation in wounds probably take longer time.

More than 80 % of persistent infections involve bacterial biofilms (Bjarnsholt et al 2008). The strong resistance of bacteria in chronic infections, example given in endocarditis, osteomyelitis and periodontitis, is most likely due to the formation of biofilm (Costerton et al 1999, Parsek & Singh 2003, Drenkard 2003). Therefore, an expected conclusion is that the healing process of chronic wounds is inhibited by the same reason (Thomsen et al 2010, Ammons et al 2009, Kirketerp-Møller et al 2008, James et al 2008). The wound bed provides a good location for bacterial cell attachment due to the fibrin network and the nutrient levels in the environment promotes encapsulation in biofilm, resulting in great difficulties for treating the infection with antibiotics (Harrison-Balestra 2003, Costerton et al 1999).

2.2.3 Pseudomonas aeruginosa

One of the most common pathogens in chronic wounds is *P. aeruginosa*, a very problematic microbe due to its ability to form resistant biofilms (Thomsen et al 2010, Ammons et al 2009, Kirketerp-Møller et al 2008, Bjarnsholt et al 2008). The presence of these bacteria in wounds seems to impair or even stop the healing process (Bjarnsholt et al 2008). Gjødsbøl *et al* found 2006 that 52.2 % of venous leg ulcers were infected with *P. aeruginosa*, and also that these wounds tended to be larger. *P. aeruginosa* is a gram-negative bacteria belonging to the proteobacteria phylum (Madigan & Martinko 2006), originally found in soil and water but is common everywhere in the environment including hospitals. The metabolism is aerobic and very versatile due to many inducible operons in its genome, allowing for growth on a large variety of carbon compounds. It is an opportunistic pathogen, often acquired in hospital environments and is often associated with infections in the respiratory and urinary tracts, in burn damage wounds and in lungs of patients with cystic fibrosis. (Madigan & Martinko 2006) It has been estimated that about ten percent of all hospital-acquired infections are caused by *P. aeruginosa,* and for immune-compromised patients the mortality rate range from 20 to 70 % (Parsons et al 2007). It has a natural resistance mechanism to many antibiotics because of a *resistance transfer plasmid,* extra genetic material carried in the cells with genes that code for proteins that destroy antibiotic substances. (Madigan & Martinko 2006) The strong persistency of *P. aeruginosa* in chronic wounds due to biofilm production makes the bacteria almost impossible to eliminate from a wound with antibiotics, once established. Kirketerp-Møller et al (2008) demonstrated that colonies of *P. aeruginosa* were located deeper inside, underneath the wound bed surface, and were imbedded in a matrix consisting mainly of alginate. This was in contrast to the also very common wound pathogen *Staphylococcus aureus,* which was found to colonize the wound surface. The different locations probably contribute to the risk of misinterpretation of culture wound swabs, taken only from the wound surface. (Kirketerp-Møller et al 2008) The exopolysaccharide alginate produced by *P. aeruginosa* contributes to the antibiotic resistance, since the substance has been shown to inhibit diffusion of, and reduce the antimicrobial activity of several antibiotics (Ammons et al 2009). The non-healing wound is sustained in the healing process by *P. aeruginosa* through the release of toxic substances called virulence factors, explained further in the following paragraph.

2.2.4 Virulence factors

When pathogenic bacteria are entering host tissue, different substances called virulence factors are released from the bacteria. Virulence factors cause damage to tissues through toxicity and invasiveness and allow for establishment of bacteria. *P. aeruginosa* produce a variety of virulence factors, both extracellular and cell-associated products. (Kipnis et al 2006) The secreted virulence factors maintain the chronic wound in a persistent inflammatory phase (Kipnis et al 2006). Exotoxins are secreted which bind to specific sites on the ribosomes in host cells, resulting in inhibited protein synthesis and cell death (Madigan & Martinko 2006). Several enzymes are produced e.g. proteases and phospholipases that disrupt host cell structures, and adhesins that allow for binding to epithelial cell receptors (Bjarnsholt et al 2008). Bjarnsholt et al (2008) suggest that the virulence factor rhamnolipid plays an important role in chronic *P. aeruginosa* infections. Rhamnolipids destroy neutrophils, resulting in leakage of several different substances with harmful effects on both tissue cells and bacteria. On the outer surface of the bacterial cell are lipopolysaccharides deposited, which contribute to attachment to host cells but also stimulate inflammatory responses. (Kipnis et al 2006) Other cell surface structures such as flagella also allow for adherence to endothelial cells (Kipnis et al 2006). Another important virulence factor for *P. aeruginosa* is the toxic blue pigment pyocyanin, which is further described below.

2.2.5 Pyocyanin

Pyocyanin is a blue pigment belonging to the group of phenazine compounds. Phenazines are low-molecular weight, heterocyclic compounds with redox-active properties, produced by *Pseudomonas* species and some other Pseudomonads. (Parsons et al 2007) Pyocyanin is the major phenazine pigment produced by *P. aeruginosa* and has been shown to contribute to its pathogenicity (Parsons et al 2007). The presence of pyocyanin is easy to detect due to its blue color that turns stationary phase cultures of *P. aeruginosa* green, and is commonly found to stain infected tissues, pus, or dressings (Cox 1986). Muller et al (2009) examined wound dressings from burn patients infected with *P. aeruginosa* and found that four of seven dressings contained pyocyanin. One important function of the pigment is to assist in iron uptake from the environment, i.e. host tissue. (Cox 1986) Due to its redox-active properties, pyocyanin can cause reduction and release of iron from transferrin, a protein that transports iron in our bodies. Hence this crucial mineral can be accumulated by the bacteria (Cox 1986).

The redox-active properties also play an important part for the toxicity of the substance. Pyocyanin can exist in an oxidized, blue form or in a reduced, colorless form, and can easily cross cell membranes. When entering a host cell, pyocyanin reacts with NADH and NADPH, leading to reduction of pyocyanin (O'Malley et al 2004). The reduced form can react with molecular oxygen and give rise to reactive oxygen species like superoxide ($O_2^{\bar{}\cdot}$) and hydrogen peroxide (H_2O_2) as was first showed by Hassan and Fridovich in 1980. The created oxygen species impose oxidative stress on host cells which cause cellular damage. One important defense mechanism in host cells to oxygen species is the glutathione redox cycle, where e.g. hydrogen peroxide is reduced to water. (O'Malley et al 2004) Studies have shown that pyocyanin decreases glutathione levels in host cells resulting in modulation of the defense cycle. (Lau et al 2004) The result is a depletion of NADH, glutathione and other antioxidants in the host cell and an alteration of the redox status, together with generation of oxygen radicals (Muller 2002, Lau et al 2004, Rada et al 2008).

Figure 2.1 *Pyocyanin in water solution*

Several effects of the toxin have been observed on human cells, including inhibited cell respiration, ciliary function and epidermal cell growth (Hasset 1992, Lau 2004). Pyocyanin has also been shown to inactivate catalase, an enzyme responsible for decomposing hydrogen peroxide, and induce apoptosis in neutrophils (Lau 2004). Muller et al showed in a study that pyocyanin inhibits proliferation of human dermal fibroblasts and induced premature senescence, even at low concentrations. These are typical characteristics for fibroblasts in chronic non-healing wounds; therefore the authors speculated that this partly explains how *P. aeruginosa* hinders wound healing. (Muller et al 2009)

2.3 Wound dressings

Chronic wounds are commonly treated with a highly absorbing wound dressing, in order to remove the large volumes of exudate coming from the wound. Examples of appropriate dressings for exudating wounds are made of foams, hydrofibers or crystalline sodium chloride gauze. (Schultz et al 2003) Other non-healing wounds may be dry or necrotic, in these cases instead dressings made of hydrogels or hydrocolloids are more suitable to promote healing (Schultz et al 2003). Venous leg ulcers are often treated with an absorbing foam dressing together with compression therapy in order to decrease subcutaneous pressure (Milic et al 2009). Foam dressings provide high absorption capacity, thermal insulation, permeability to air and a moist wound environment. The wound should be maintained moist in order to promote migration of epidermal and inflammatory cells into the wound, preventing crust formation and keeping an electrical gradient. However, since chronic wound fluid contains substances which impairs cell proliferation and leads to breakdown of extracellular matrix, excess wound fluid should be removed by the dressing (Schultz et al 2003).

The dressing products included in this study consists of polyurethane foam which absorbs wound exudate, a wound contact layer that prevents adherence to the wound bed but instead only adheres to the dry skin, and a polyurethane film backing to prevent leakage of fluids.

3. Materials and Methods

3.1 Cell culturing

The *Pseudomonas aeruginosa* strain PAO1 was used for all experiments. The bacterium is stored in microbank containing beads, in -70°C. One bead was taken out and spread on a tryptic soy agar (TSA) plate and incubated in 35°C over night. An overnight culture was prepared by removing one colony from the TSA plate and transferring it to 3 mL tryptic soy broth (TSB) medium in a test tube, which was incubated at 35°C for 24 hours. In the experiments, the bacteria were cultured in several different media including Simulated Wound Fluid (SWF), Peptone Water (PW) and physiological NaCl (All media and agar plates from department of Bacteriology, Sahlgrenska Hospital, Gothenburg).

> *TSB* – general bacterial growth medium containing enzymatic digests of soybean and casein
> *SWF* – 50 % fetal calf serum and 50 % PW
> *PW* – physiological (0.9 %) NaCl with 0.1 % Peptone

Cell culture plates of polystyrene were used for the experiments (BD Falcon, USA). The cultures were incubated at 35°C. In all performed experiments a start inoculation size of 10^4 CFU/mL was used.

3.2 Wound dressing products

Small pieces of the dressing materials with a diameter of 12 mm were prepared for the experiments by punching under aseptic conditions. The wound dressing products included in the project are:

Product A: Polyurethane foam, soft silicone wound contact layer, polyurethane film backing.

Product B: Polyurethane foam, super-absorbent fiber mix of viscose, polyester, polyacrylate, cotton fibers and binding fibers, soft silicon wound contact layer, polyurethane film backing.

Product C: Soft silicone wound contact layer, polyamide net.

Product D: Hydrocellular polyurethane foam, soft gel adhesive wound contact layer, polyurethane film backing.

Product E: Hydrocellular polyurethane foam, soft silicon wound contact layer, polyurethane film backing.

Product F: Hydrocellular polyurethane foam, polyurethane film backing.

3.3 Viable cell count

Viable cell counts were measured with the spread plate method. *P. aeruginosa* were cultured in 24-wells cell culture plates in presence of dressings, in 1.5 mL SWF. The dressing pieces were completely submerged in the bacterial cultures. After 21 h incubation, samples from cultures were diluted in PW to appropriate concentrations, and spread on TSA agar plates (Department of Bacteriology, Sahlgrenska Hospital, Gothenburg). The plates were incubated at 35°C overnight, and colonies were counted the next day. The result is presented as $\log_{10}$ colony forming units per mL test medium (CFU/mL).

3.4 Biofilm formation

The formation of biofilm was quantified by cell staining with crystal violet and absorbance measurement. *P. aeruginosa* were cultured in 12-wells cell culture plates in presence of dressings, in 3 mL SWF. The dressing pieces were completely submerged in the bacterial cultures. Wound dressing pieces (12 mm) were taken from *P. aeruginosa* cultures in SWF on day 2, they were squeezed to remove excess suspension and washed three times in 4 mL milli-Q water. The dressing pieces were squeezed 15 times between each wash with a tweezer. This washing step was performed in order to remove excess bacteria, biofilm and cell medium components from the samples. Then, 0.5 mL 0.5 % crystal violet solution (Department of Bacteriology, Sahlgrenska Hospital, Gothenburg) was added to each sample and incubated for 20 minutes. The dressing samples were then washed 13 times in 30 mL milli-Q water, and once in 40 mL milli-Q water in a falcon tube with vortexing in order to remove excess crystal violet dye. All samples were squeezed 15 times with a tweezer between each step. After the washing steps, 4 mL 95 % ethanol was added to each sample in order to solubilize crystal violet and extract the dye. After 40 minutes the pieces were once again squeezed 15 times to ensure a homogenous crystal violet solution. As a negative control also clean dressing samples were stained with crystal violet using the same procedure. The clean dressing samples were soaked in SWF before the quantification for the same time period as the cultured samples. 200 µL of the crystal violet ethanol solutions were sampled for absorbance measurement at 570 nm with an ELISA reader (VERSAmax microplate reader, Molecular Devices, USA). Polystyrene 96-wells plates were used for the measurements (Maxisorb U-96, Nunc, Denmark).

3.5 Spectroscopic analysis of pyocyanin

Since pyocyanin absorbs light in the visible light spectrum, the levels can be quantified by absorbance spectrophotometry. A wavelength spectrum was measured which was in accordance to literature (Price-Whelan et al 2007, Parsons et al 2008, Wang & Newman 2008, Watson et al 1986). Quantifications of pyocyanin were measured as absorbance at 690 nm, as described in literature (Parsons et al 2007, Rada et al 2008, Reszka et al 2004). *P. aeruginosa* were cultured in 3 mL SWF in 12-wells cell culture plates in presence of wound dressings, and incubated at 35°C. Also here the dressing pieces were completely submerged in the bacterial cultures. At the time points of 1 day, 3 days and 7 days, solution samples of 1 mL were taken from the bacterial suspensions, centrifugated in 4000 rpm for 10 minutes (3-18K, Sigma, Germany) and filtrated (0.20µm pore size, SartoriusSedium, Germany). The treatment of the samples consisting of repeatedly

pipetting up and down, ensured that yellow suspensions were properly aerated to green hence the present pyocyanin were oxidized to the maximal extent. The wound dressing pieces present in the bacterial cultures were soaked in 3 mL milli-Q in order to extract pyocyanin. The blue liquid was then centrifugated and filtrated as above.

For the wavelength scans, 1 mL of filtrated suspensions were transferred to plastic kyvettes and measured in a spectrophotometer (U-2900, Hitachi, Japan). Samples for pyocyanin quantifications were transferred to polystyrene 96-wells plates (Maxisorb U-96, Nunc, Denmark), and absorbance was measured at 690 nm in an ELISA reader (VERSAmax microplate reader, Molecular Devices, USA).

4. Results

This section provides the results from the experiments carried out in the project. This includes examinations of how polyurethane foam wound dressings influence growth of *Pseudomonas aeruginosa* and production of the bacterial virulence factor pyocyanin.

4.1 The effect of wound dressings on bacterial growth

Wound dressing samples were placed in *P. aeruginosa* cultures in order to study effects on bacterial cell number. Bacterial growth was measured with the spread plate method, to determine if presence of wound dressings would influence how *P. aeruginosa* grow compared to the control not containing dressing sample. The result from the spread plate count is presented in figure 4.1 below. The viable cell count showed that the control culture as well as all cultures containing dressing pieces grew to the maximal extent, between 10^8 and 10^9 CFU/mL. This implies that none of the included wound dressings influenced the viable cell count in cultures of *P. aeruginosa.* The dressings included in this experiment were: product A, product B, product D, product E and product F.

The experiments were performed in medium containing 50 % serum. Also included in the chart are the results from *P. aeruginosa* cultures grown in media containing no serum, and with 5 % serum. Even in such low amount of serum as 5 % the bacteria grew maximally, showing how low the nutritional needs are for this microorganism. The sample cultured completely without serum also managed to grow, but this sample had the lowest increase in cell count.

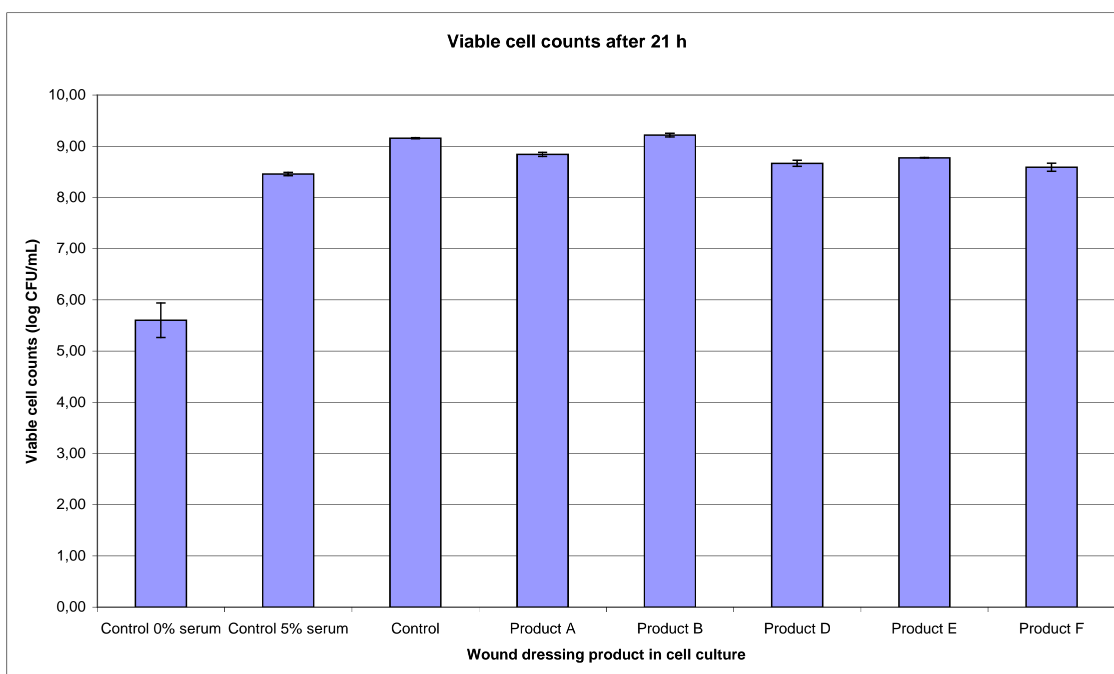

Figure 4.1 *Viable cell counts in cultures of P. aeruginosa PA01 incubated in SWF with wound dressing pieces for 21 h. Control sample was incubated without dressing material. Also included are control samples cultured in media containing 0 % serum and 5 % serum. The start inoculation size was* 10^4 *CFU/mL. N = 3.*

4.2 Quantification of biofilm in wound dressings

The formation of biofilm inside the foam in the wound dressings was assayed by cell staining with crystal violet and absorbance measurement. Wound dressing samples were positioned in *P. aeruginosa* cultures and incubated for two days. The dressing samples were then stained with crystal violet. As a negative control, clean dressing pieces were also treated with crystal violet, in order for measuring unspecific binding of the dye.

Included products in this analysis were product A and product D, and the result is accessible in figure 4.2. For both products, there was a high degree of unspecific binding of crystal violet to the dressing materials. Even so, a clear difference exists between the negative control and the cultured dressing samples, for both product A and product D. Product D shows a larger difference in absorbance between negative control and cultured sample than product A, suggesting that more biofilm has been formed in product D.

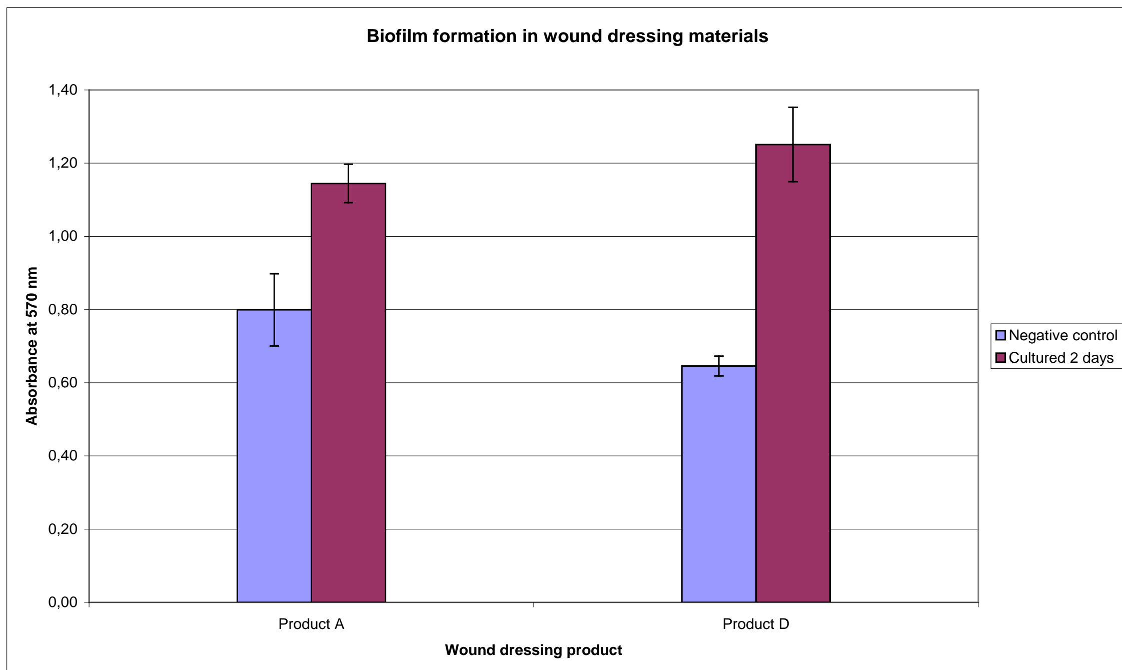

Figure 4.2 *Assessment of biofilm formation of P. aeruginosa in wound dressings, measured as absorbance of crystal violet. P. aeruginosa was cultured in SWF together with dressing pieces, for 48 h at 35°C. The negative control samples represent unspecific binding of crystal violet to the dressing materials. N = 3.*

4.3 The colors of Pseudomonas aeruginosa cultures

In a series of experiments, *Pseudomonas aeruginosa* was cultured in Peptone Water with varying serum levels. At high serum levels the production of biofilm was high in the cultures, resulting in dark green cultures with a slimy texture. The dark green color means a high production of the virulence factor pyocyanin which was also confirmed with spectroscopic analysis. The experiments described and presented hereafter in this report were carried out at this high serum level. At low serum levels a clearer appearance could be observed, caused by smaller quantities of biofilm produced by *P. aeruginosa*. Pyocyanin was produced, but in lower amounts observed as a light blue color of the cultures. The different colors obtained in *P. aeruginosa* cultures are visualized in figure 4.3. When comparing cultures with low serum level with high serum level, no simultaneous increase was found between bacterial cell number and production of pyocyanin and biofilm. At low serum levels the production of pyocyanin and biofilm was low, but *P. aeruginosa* were even so found to grow maximally except for in serum-free medium, see figure 4.1.

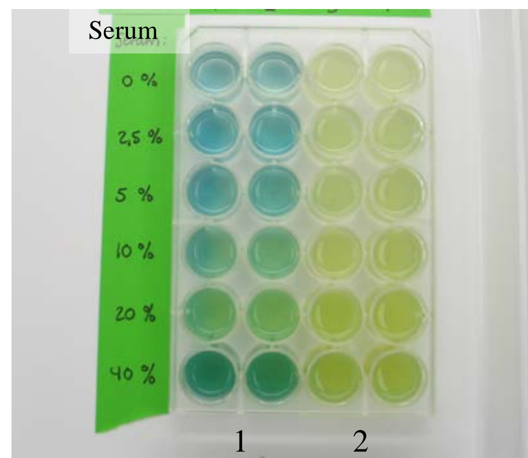

Figure 4.3 *P. aeruginosa cultured for three days in varying serum levels. 1: The two columns to the left are control cultures. Observe the clearer, blue appearance of the cultures with lower serum level, and the denser, green cultures in high serum level. 2: The two columns to the right have been incubated with wound dressing samples, yielding the yellow color of the culture.*

The presence of wound dressings in the *P. aeruginosa* culture usually resulted in yellow color of the culture medium, while the control culture with no wound dressing usually had a dark green color, see figure 4.3. However, when a yellow wound dressing piece was lifted up from the culture into the air, it quickly turned green as can be seen in figure 4.4. This is consistent with a previous study describing how the reduced, colorless form of pyocyanin is oxidized in air to a green color (Price-Whelan et al 2007). If a wound dressing piece was added to a green control culture in its stationary phase, it resulted in a slow color change of the culture medium from green to yellow, overnight.

Figure 4.4 *Wound dressing pieces from stationary phase P. aeruginosa culture. 1: The yellow dressing has very recently been removed from the culture. 2: Dressing left in air for ~5 min, the color has transitioned to dark green.*

The yellow suspension resulting from culturing *P. aeruginosa* together with wound dressings could be turned green, by flushing the liquid with air using a pipette. In a few cases the control culture did also turn yellow; the suspension could then be turned green by the same method. This is illustrated in figure 4.5, showing two samples from the same *P. aeruginosa* culture. The test tube to the right contains the yellow, non-treated culture sample, where a thin green layer can be observed on the surface signifying spontaneous oxidation of pyocyanin. The sample to the left has been flushed with air, thus oxidizing pyocyanin and turning the suspension green.

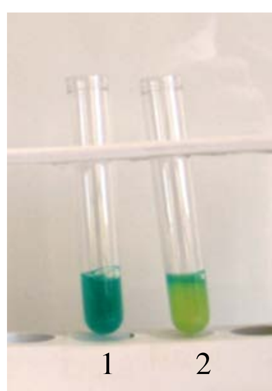

Figure 4.5 *1: The sample has been flushed with air which oxidizes pyocyanin, turning the culture green. 2: The sample contains pyocyanin in its reduced, colorless form.*

4.4 Quantification of pyocyanin

4.4.1 WAVELENGTH SPECTRUM OF PSEUDOMONAS AERUGINOSA CULTURE

A wavelength scan was performed on cell-free filtrate from *P. aeruginosa* culture, in order to find an absorbance maximum for pyocyanin. The presence of pyocyanin was easily observed in the culture due to the green color. The resulting spectrum is presented in figure 4.6, and correlates very well with spectra found in the literature. Three distinct peaks are visible at 311, 378 and at 691 nm which also correlates with the expected result. (Price-Whelan et al 2007, Parsons et al 2008, Wang & Newman 2009, Watson et al 1986)

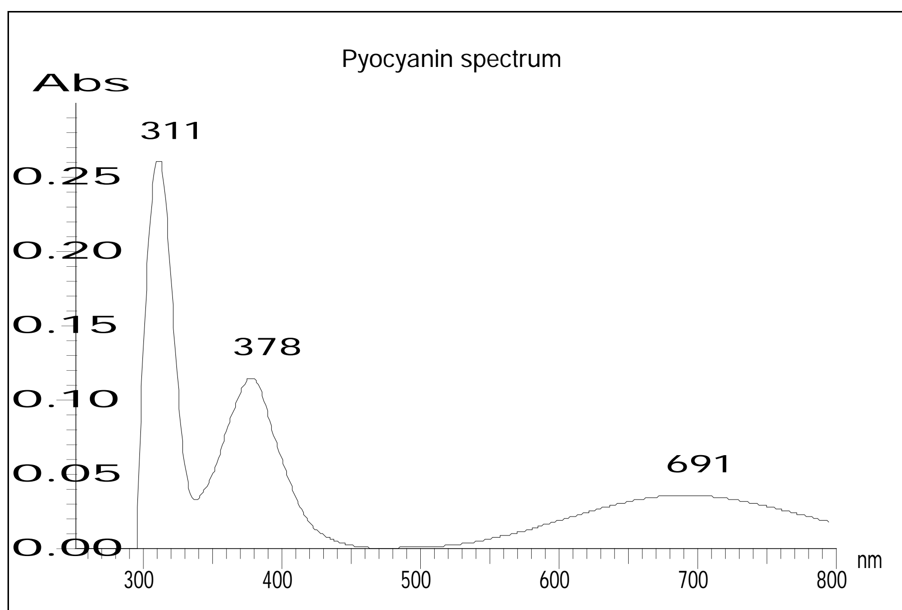

Figure 4.6 *Wavelength spectrum measured in filtrated P. aeruginosa culture grown in SWF, after 72 h incubation at 35°C. The three characteristic absorbance peaks of pyocyanin at 311, 378 and 691 nm are depicted in the figure.*

The absorbance maximum at 691 nm was chosen for measurement of pyocyanin in the following quantifications. This was because of a low level of noise at this wavelength, and also consistency with quantification methods found in literature (Parsons et al 2007, Rada et al 2008, Reszka et al 2004). In other wavelength scans performed in the project, additional peaks could arise in the spectra at shorter wavelengths, which was probably due to other sorts of pigments being produced in the bacterial culture.

4.4.2 PYOCYANIN PRODUCTION IN PRESENCE OF WOUND DRESSINGS

4.4.2.1 Analysis of pyocyanin in cultures

In this analysis, the amount of pyocyanin was assessed in *P. aeruginosa* cultures grown in the presence of wound dressings. The control sample was grown with no dressing piece. Samples from the cultures were taken on day 1, day 3 and on day 7 for measurement by absorbance spectroscopy. The result which is presented in figure 4.7 shows a considerable difference in absorbance between the control and the four cultures with wound dressings. The higher absorbance of the control culture suggests a higher content of pyocyanin, compared to the cultures with dressing materials. This also suggests an increasing production of pyocyanin in the control culture over time, with the highest content measured on day 7. The control culture shows higher absorbance than the wound dressing cultures at all three time points. The culture grown with product C wound dressing showed higher absorbance than the three other dressings. However, since product C consists only of a silicon wound contact layer, lacking the polymer foam, the absorbance values are not comparable. The results indicate that the polyurethane foam has a role for the lower amounts of pyocyanin in those cultures. The wound dressings containing a polyurethane foam layer, product A, product D and product E, displayed very similar results. The chart shows that the absorbance values of these three cultures reached the same levels at all three time points, suggesting similar amounts of pyocyanin being produced by *P. aeruginosa* in cultures containing product A, product D or product E. An interesting feature of the cultures containing foam dressings is that the amount of pyocyanin does not seem to increase from day 3 to day 7, in contrast to the control culture.

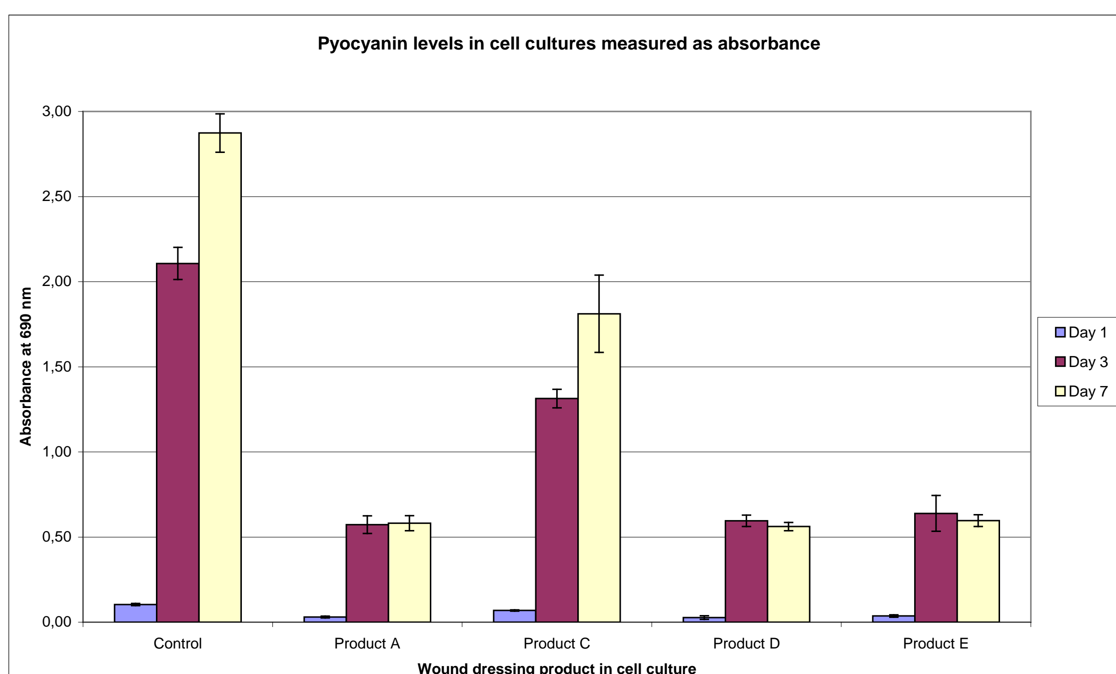

Figure 4.7 *Levels of pyocyanin in P. aeruginosa cultures, measured as absorbance at 690 nm. Measurements were made on day 1, day 3 and day 7 of incubation. The bacterial cultures were grown in SWF medium at 35°C. N = 3.*

4.4.2.2 Quantity of pyocyanin in wound dressings

The amounts of pyocyanin were also measured inside wound dressings. This analysis was performed in order to investigate if the wound dressings could provide an environment that enhanced pyocyanin production. Wound dressing pieces were taken from *P. aeruginosa* cultures on day 3 and on day 7, and soaked in water. This rinse water was colored blue due to pyocyanin being washed out from the dressings. The absorbance values of the rinse water from all dressings are presented in figure 4.8. It is important to note that the assay does not provide values for the total amounts of pyocyanin inside a dressing sample, but serves as estimations for comparing the included dressing products to each other. The dressing sample product C showed the lowest absorbance value, indicating the lowest amount of pyocyanin being washed out from this dressing. But as described above, wound dressing C does not contain a foam layer, thus a lower amount of pyocyanin is expected. The three dressings with polyurethane foam layers, product A, product D and product E, presented similar absorbance values on day 3. This indicates similar amounts of pyocyanin in these wound dressings after three days incubation in bacterial culture.

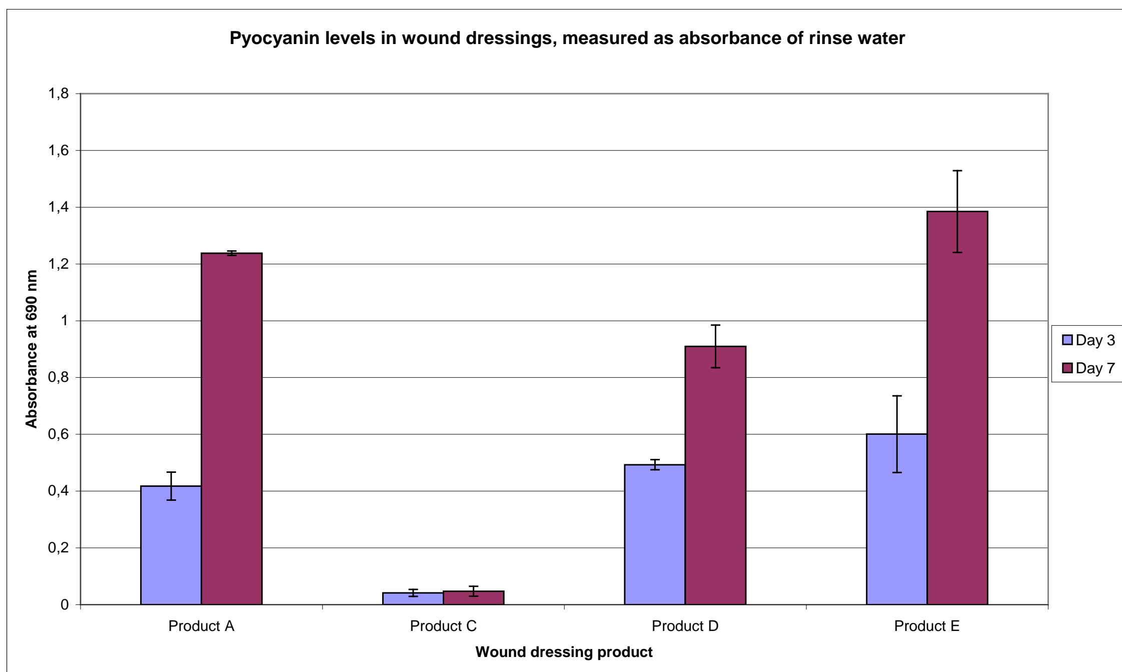

Figure 4.8 *Assessment of pyocyanin levels inside wound dressings, measured as absorbance of rinse water. Dressing samples were taken from P. aeruginosa cultures on day 3 and day 7, and soaked in water. Pyocyanin present in wound dressing pieces was washed out into the rinse water. N = 3.*

After seven days incubation, differences in absorbance arise between the foam dressings product A, product D and product E. Product D shows lower absorbance at this time point than the other two, suggesting a lower amount of pyocyanin in this dressing. Product A and product E shows rather similar absorbance values at day 7, where product E has a slight tendency of a higher value.

A common feature of the polyurethane foam dressings, product A, product D and product E, is that the absorbance values increase significantly from day 3 to day 7. The

result indicates that the amounts of pyocyanin inside the wound dressings are increasing with time. This is in contrast to the levels of pyocyanin in cultures outside the wound dressings, which does not seem to increase from day 3 to day 7, see figure 4.7.

4.4.2.3 Effects of adding wound dressings to stationary phase culture

In this experiment, *P. aeruginosa* was cultured for four days. Wound dressing samples from product A, product B and product D were added from start on day 0 to some cultures, and on day 3 to other cultures. The idea was to measure possible differences in pyocyanin content in cultures when the wound dressing was added in the lag phase or in the late stationary phase of *P.aeruginosa* growth cycle. The control cultures were cultured without wound dressings, and were measured both on day 3 before adding of the dressing samples, and on day 4.

In the result which is presented in figure 4.9, higher absorbance values were measured in the control compared to the wound dressing cultures. This is consistent with earlier results, see figure 4.7. For the cultures incubated with dressing products, very similar absorbance values were measured for each wound dressing. The result indicates that for each wound dressing, similar amount of pyocyanin was present in the cultures where the dressing samples were added on day 0, and in the cultures where the samples were added on day 3. This suggests that it does not make a difference for the pyocyanin production if the wound dressing is added in the lag phase (before growth starts) or in the stationary phase to a *P. aeruginosa* culture. It also suggests that there is no difference in pyocyanin levels after four days incubation when a wound dressing has been present in bacterial cultures from the start or only during the last 24 hours of culturing.

An important observation in this experiment is that the addition of wound dressing samples to the cultures causes a decrease in absorbance, indicating a decrease in pyocyanin concentrations. This can be seen in figure 4.9, where the column *control d 3* represents absorbance value on day 3 before addition of wound dressings. When measuring again 24 hours later, the absorbance values have diminished, represented by the columns for *Wound dressing added on day 3*.

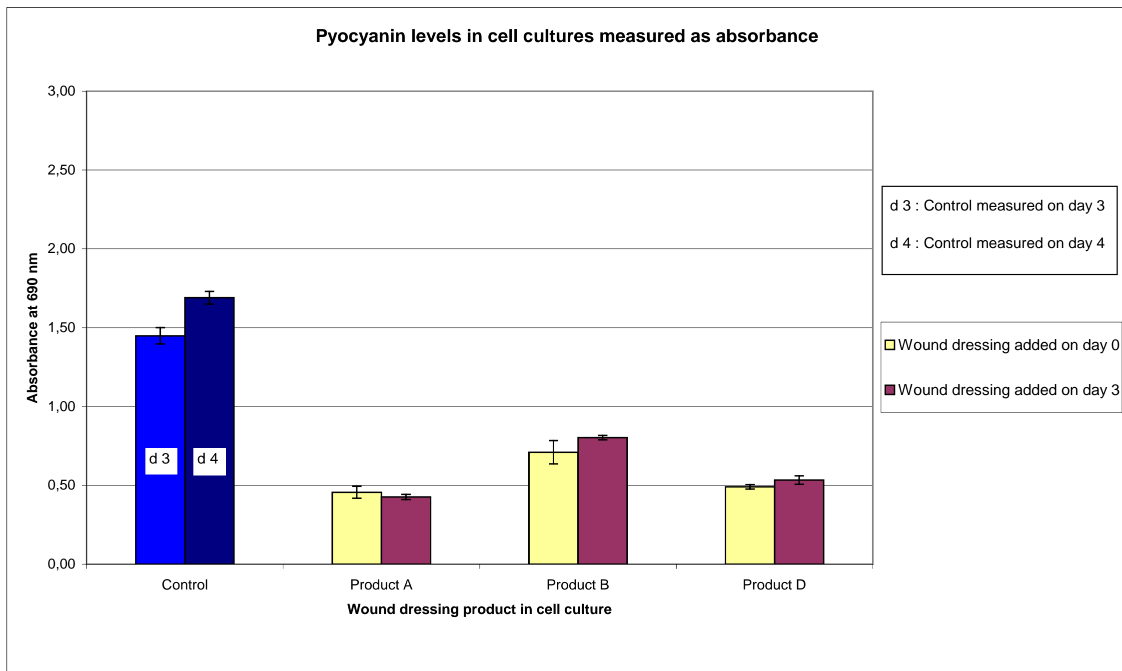

Figure 4.9 *Quantification of pyocyanin in bacterial cultures, measured as absorbance. P. aeruginosa was cultured in SWF and wound dressing samples were added on day 0 and on day 3, with measurement on day 4. The absorbance of pyocyanin on day 3, before adding of dressing samples, was also measured and is represented by the* d 3 *column. N = 3.*

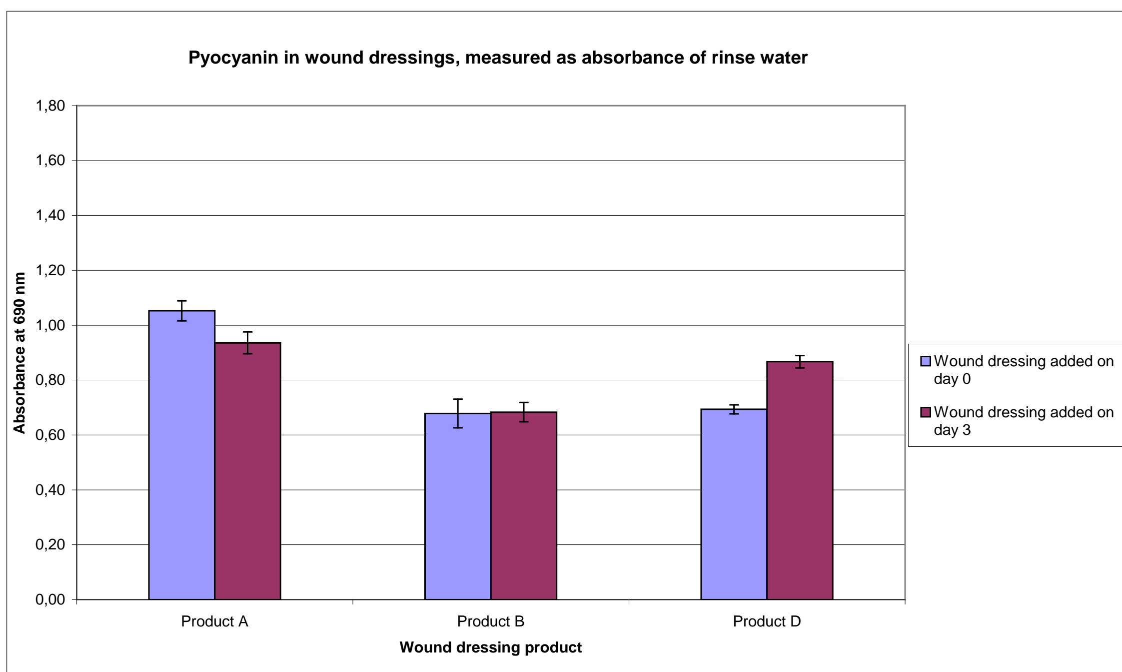

Figure 4.10 *Assessment of pyocyanin inside wound dressings, measured as absorbance of the rinse water that the dressing samples were soaked in after incubation. P. aeruginosa was grown in SWF and dressing samples were added on day 0 and on day 3. On day 4 the dressings were removed from the cultures and soaked in distilled water. N = 3.*

In the same experiment, the levels of pyocyanin were also measured inside the wound dressings. As described above, the wound dressings had been added to *P. aeruginosa* cultures on day 0, and on day 3. The outcome of the measurement is presented in figure 4.10. A common trait for each of the included wound dressings is that the absorbance values do not differ much when the dressings were added on day 0 compared to when added on day 3. This result suggests that the pyocyanin content inside the wound dressings added on day 0 is rather similar to the pyocyanin content in the dressings added on day 3.

When comparing the three included wound dressings to each other, the differences in measured absorbance between them are minor. However, some observations can be done when comparing figure 4.9 and figure 4.10. One feature is that the dressing product B which has the highest absorbance of the three dressing products in the bacterial culture, also shows the lowest absorbance value of the included products for the extracted pyocyanin from inside the dressing. This reverse relationship indicates that a higher content of pyocyanin in the cell culture corresponds to lower pyocyanin content in wound dressings. For product A the opposite pattern is obtained, this product shows the lowest absorbance value of the cell culture compared to products B and D, and the highest absorbance value of extracted pyocyanin from dressings, indicating a lower pyocyanin content in the culture combined with a higher pyocyanin level in the wound dressing.

4.4.2 ADHERENCE OF PYOCYANIN TO WOUND DRESSINGS

The ability of pyocyanin to adhere to wound dressings in a non-biological manner was investigated in this experiment. Pieces of the dressing products A, B and D were soaked in a cell-free pyocyanin solution in water, see figure 2.1. The absorbance of the solution was measured before, depicted as control in figure 4.11, and after 24 hours. The result showed that the absorbance of the pyocyanin solution had decreased for all three dressing products, indicating that the concentration of pyocyanin had also decreased in the solutions. A lowered concentration is in turn indicating that pyocyanin had adhered to the wound dressings. The two polyurethane foam dressing products A and D showed similar result, whereas a lower amount of pyocyanin adhered to product B.

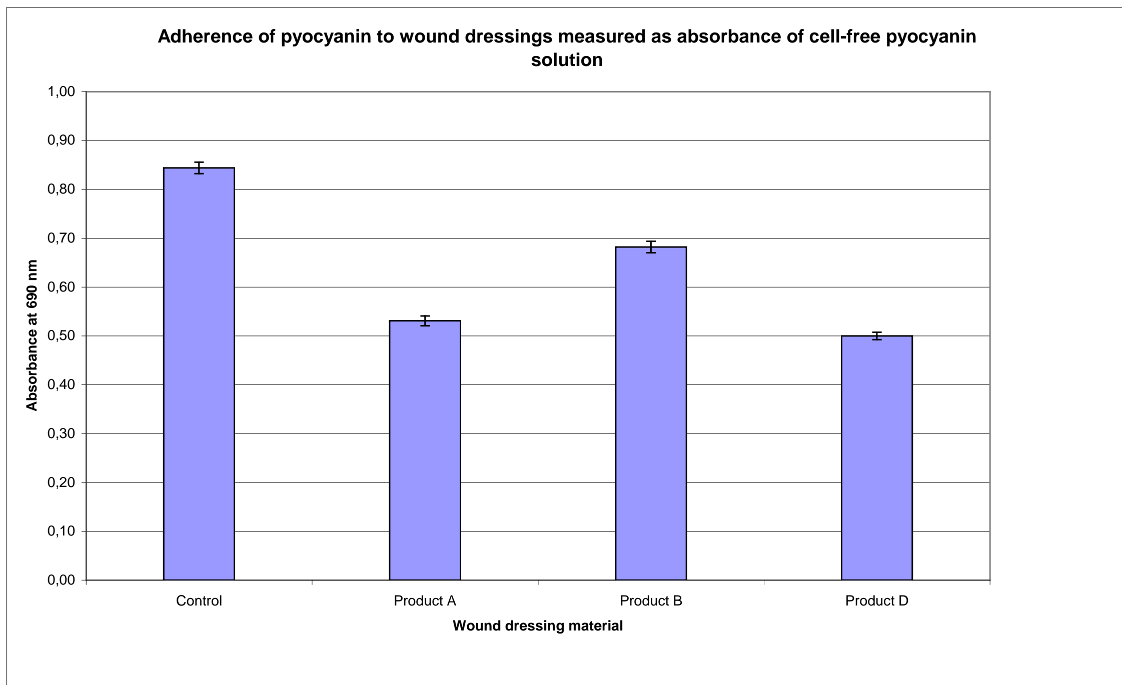

Figure 4.11 *Diagram showing the cell-independent adherence of pyocyanin to wound dressing products A, B and D. Dressing samples were placed in a cell-free pyocyanin solution, and absorbance of the solution was measured after 24 hours. A decreased absorbance compared to the control indicates lower concentration of pyocyanin in the solution, meaning that some pyocyanin has adhered to the dressings. N = 2.*

5. Discussion

In this project attempts were made to investigate if polyurethane foam dressings can promote wound infection. This was investigated by studying bacterial growth, biofilm formation and production of the virulence factor pyocyanin in absence and presence of wound dressing samples. Several different experiments were performed in this project constituting of culturing the pathogenic bacteria *Pseudomonas aeruginosa* together with different wound dressing products. The first part of the experimental process consisted of determination if wound dressings could influence the bacterial growth. The results of this measurement showed that cultures containing wound dressings grew to the same extent as the control, which was between 10^8 and 10^9 CFU/mL. Hence, none of the included dressing products influenced the viable cell count of *P. aeruginosa* in cultures. The result was not unexpected since the included dressings do not have any content or release of any antibacterial substance. A difficulty with the analysis is the possibility that wound dressing cultures grow better than the control, which cannot be measured since all cultures grow to the maximal extent. The bacteria *P. aeruginosa* have low nutritional requirements and are capable of a very versatile metabolism with growth on many different carbon sources, which obstructs this kind of analysis. The aim of the test was to find a nutritional mode where wound dressings could be found to influence bacterial cell number. Such nutritional level could not be found, except for a completely serum-free medium. Since wounds contain relatively high values of nutrients and the experiments should be relevant for their applications, further attempts were rejected.

The amounts of biofilm formed inside wound dressings were analyzed by cell staining with crystal violet and absorbance measurement. The dressings included in the assay were product A and product D. Although there was a high degree of unspecific binding of crystal violet to both dressing products, there were apparent differences between the negative control and the cultured samples for both products. The results indicate that the assay was a good method for measuring biofilm formed inside the foam of the dressings. Dressing product D showed a larger difference between the negative control and the cultured sample than product A, indicating that more biofilm has formed inside product D after two days incubation. A factor that could contradict this result is that product D is slightly thicker than product A. This could lead to more crystal violet stain being washed out from the dressing sample A before measurement, making the procedure in favor of product A.. However, since the unspecific binding of crystal violet is higher in product A, this factor does not seem to inflict on the result.

The project also focused on an analysis of the production of pyocyanin, a bacterial virulence factor generated by *P. aeruginosa.* The bacteria were cultured together with wound dressing pieces and at different time points the amounts of pyocyanin were measured by a spectrophotometric method. In the first part of this analysis, dressing pieces from product A, product C, product D and product E were added to *P. aeruginosa* cultures on day 0 and incubated. Measurements were made on day 1, day 3 and on day 7. The results show that the absorbances of the bacterial cultures were low on day 1 for all samples, indicating that the production of pyocyanin is very low during the log phase growth of *P. aeruginosa* and starts when the culture is approaching the stationary phase. This observation is analogue with results from a study by Rada et al in 2008, who showed that production of pyocyanin by *P. aeruginosa* started in late exponential phase and thereafter was increasing with time. On day 3 the absorbance values had increased significantly in all cultures, where the control sample showed the highest value. The control also presented the highest value on day 7, indicating that the

culture with no wound dressing had the highest concentration of pyocyanin. Of the included wound dressings, product C resulted in the largest amount of pyocyanin in the culture, at both time points. However, since product C is a dressing consisting of only soft silicon and polyamide layers without the polyurethane foam, the result cannot be compared to the other included products. It is yet interesting that addition of product C to a *P. aeruginosa* culture leads to lower amount of pyocyanin in the culture. The three foam dressings product A, product D and product E resulted in similar amounts of pyocyanin in the cultures. The quantities of pyocyanin were also measured inside the dressing products after water extraction. The results indicate that the amounts of pyocyanin are increasing with time inside the foam dressing products A, D and E. Some conclusions can be drawn from this first part of the analysis. The total quantity of pyocyanin in *P. aeruginosa* cultures is increasing with time, but in cultures containing polyurethane foam dressings the increase mainly occurs inside the dressing product. Also, the addition of a polyurethane foam dressing leads to lower concentration of pyocyanin in the culture compared to the control. The results suggest that both the presence of a wound dressing and the polyurethane foam have significant roles for lower amounts of pyocyanin in the bacterial cultures.

Some factors should be considered regarding the conclusions made above. The first aspect is the redox-active properties of pyocyanin, as described in the section 2.2.5. As is visualized in figure 4.3, culturing *P. aeruginosa* together with a dressing sample causes a change in color from green to yellow due to reduction of pyocyanin. Since pyocyanin in reduced form does not absorb light at 690 nm, there would be a risk that the measurement does not provide correct information about pyocyanin levels in these cultures. However, the yellow culture suspensions were always flushed with air several times before measurement, leading to reoxidation of pyocyanin and regaining of the green color. This procedure is described by Price-Whelan et al in a study 2007, where yellow *P. aeruginosa* suspensions were turned green by aeration and fully regained the absorbance maximum at 690 nm. Therefore, an assumption is made that all pyocyanin present in cultures was completely oxidized by aeration, thus the assay gives correct levels. Another factor would be a potential abiotic degradation of pyocyanin. In order to study this in the project, filtrated suspensions from *P. aeruginosa* cultures were measured spectrophotometrically at 690 nm, left standing in an incubator for 24 hours and then measured again. Since the absorbance values did not decrease, a conclusion was made that pyocyanin is not degraded abiotically.

An important question is if the large increase in pyocyanin concentration over time inside foam dressings is coupled to a higher bacterial density. A theory could be that the wound dressing provides an environment where higher cell density is possible compared to the solution outside the dressing. The high cell density, together with limited oxygen diffusion through the dressing materials causes oxygen limitations for the bacteria. The limitations of oxygen result in electron transfer to pyocyanin instead of oxygen, in order to oxidize NADH and restore the redox balance (Price-Whelan et al 2007). Pyocyanin thus is reduced, which explains the change in color to yellow of the cultures containing wound dressings. The production of pyocyanin as other phenazines increases with high cell density (Price-Whelan et al 2007), and reduction of pyocyanin occurs faster in high cell densities due to oxygen limitations (Cox 1985). However, the viable cell count showed a maximum cell density of about 10^9 CFU/mL in all cultures. Therefore, there are no indications for a potential higher cell density in cultures containing foam dressings. Also, the results from the biofilm formation assay and quantifications of pyocyanin in this project contradict the suggestion that higher cell

density is coupled to higher production of pyocyanin. The biofilm formation assay suggested higher content of biofilm, measured as amounts of cells, in product D compared to product A. However, cultures with product A contained slightly higher amounts of pyocyanin than product D. Therefore, the number of cells was not found to correlate to the production of pyocyanin.

The quantities of produced pyocyanin were also measured in cultures where wound dressing pieces were added either from start, or on day 3 with all measurements on day 4. The included wound dressings were product A, product B and product D. The results showed that for each included dressing product, the two different time intervals for presence of dressing samples resulted in similar absorbance values on day 4. As performed in the previous experiment, absorbance values were measured both in cultures and inside wound dressing samples. The results indicate that after four days incubation in the presence of wound dressing, the total amount of pyocyanin, i.e. in culture and in dressing, was almost identical to the amount in the culture in presence of dressing only during the last 24 hours. An interesting feature is that the concentration of pyocyanin in the culture is decreasing when dressing pieces are added on day 3. Instead, this amount of pyocyanin has been transferred to the inside of the wound dressings. The same pattern was seen for all three included dressing products.

Three different theories can be possible explanations to this observation that presence of dressing from day 0 and from day 3 results in the same pyocyanin amount. The first theory states that the total amount of pyocyanin is identical in these two cultures, as well as the control without wound dressing, throughout the experiment. If the pyocyanin contents are the same on day 4, they are also the same on previous days. This would imply that the presence of wound dressing in the culture does not influence the production of pyocyanin. The second possibility would be that the culture with no dressing had higher concentration of pyocyanin on day 3, but the addition of a dressing caused the concentration to diminish to the same level as in the culture with dressing from start. Pyocyanin content could decrease either by degradation, or by intracellular uptake by *P. aeruginosa* cells. As mentioned previously, pyocyanin was not found to be degraded in a cell-free system, making this explanation unlikely. The third possibility would be that the culture with no wound dressing contained lower concentration of pyocyanin on day 3, but the addition of dressings caused the pyocyanin production to increase, resulting in the same concentration on day 4 as in the culture with dressing piece from day 0. An increased production of pyocyanin due to addition of wound dressing could be triggered by oxygen limitations as described above. However, an increased production of pyocyanin inside wound dressings would indicate that bacterial ingrowth occurs in wound dressings added on day 3. This would require reorganization of stationary phase bacteria and start of new biofilm production.

A certain conclusion is that the addition of wound dressings to a stationary phase culture of *P. aeruginosa* causes a large alteration in the living environment for the bacteria. This can be visualized not the least by the change in color from green to yellow, signalling reduction of pyocyanin possibly due to oxygen limitations in the whole culture, inside dressing and in solution, caused by obstructed diffusion of oxygen.

In order to elucidate if a wound dressing triggers production of pyocyanin, the total amounts of pyocyanin in cultures and inside wound dressings must be determined. The measured absorbance values in cultures and inside wound dressings, see figure 4.7 and 4.8, may hypothetically be added together to yield the total amount of pyocyanin for

each dressing culture. When comparing the total absorbance values of each dressing culture to the control, the control culture still contains the highest value. Therefore, a suggestion is that presence of a wound dressing in a *P. aeruginosa* culture results in lower production of the virulence factor pyocyanin.

An additional explanation to the lower pyocyanin concentration in presence of wound dressings lies in that pyocyanin adheres to the dressing materials. In an experiment in this project fresh dressing samples were soaked in a cell-free water solution of pyocyanin. The absorbance of the solution was measured before and after 24 hours. The result showed a decreased absorbance value in all samples, indicating that pyocyanin had adhered to the dressing resulting in a lower concentration in the solution. It could also be observed visually that pyocyanin adhered to the polyurethane foam in all dressings. In product B which consists of two different absorbent layers, pyocyanin did not adhere significantly to the super-absorbent fibre layer. Although pyocyanin was found to adhere to the dressing products, this does not completely explain the differences between the control and the dressing cultures. The change in color to yellow in dressing cultures signals reduction of pyocyanin. Since reduction is performed by bacteria, it indicates that the wound dressing contributes to an altered environment for the microorganisms.

6. Conclusions

The experiments performed in this project have resulted in some conclusions about growth of *Pseudomonas aeruginosa* and production of the virulence factor pyocyanin in the absence and the presence of polyurethane foam dressings.

- Bacterial growth of *P. aeruginosa* was found to be maximal in all culture media containing any degree of serum. Inhibition of growth was only found in completely serum-free medium.

- Bacterial growth of *P. aeruginosa* was found not to be affected by the presence of any wound dressing product included in the project.

- The production of biofilm was low in nutrient-poor medium seen in cultures as a clear appearance, and high in nutrient-rich medium observed as a thick, green appearance with viscous texture.

- The presence of polyurethane foam dressings in *P. aeruginosa* cultures caused an altered environment for the bacteria, visualized as a change in color from green to yellow due to reduction of pyocyanin.

- The presence of wound dressing products in *P. aeruginosa* cultures resulted in lower concentration of pyocyanin in the culture, compared to the control. The tested polyurethane foam dressings presented similar results.

- The amount of pyocyanin in *P. aeruginosa* cultures was found to increase over time, where the control culture showed the largest increase. In cultures containing polyurethane foam dressings the pyocyanin content increased in the cultures only between day 1 and day 3. Between day 3 and day 7 the increase in pyocyanin content instead occurred inside the dressing products.

- Pyocyanin was found to adhere to the dressing material polyurethane, leading to lowered concentration of pyocyanin in the solution outside the dressing.

7. Acknowledgements

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Staff at the Preclinical Lab at Mölnlycke Health Care

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9. Appendix

A.

A green colored suspension from a stationary phase *P. aeruginosa* culture was filtrated, and diluted with SWF to obtain a dilution serie. The absorbance was measured for each dilution and the result is presented in figure 9.1. The diagram shows a very good correlation between the measured absorbance and the dilution factor. Therefore, it was assumed that bacterial filtrates could be diluted 50 % before absorbance measurements, and the absorbance value obtained could be doubled.

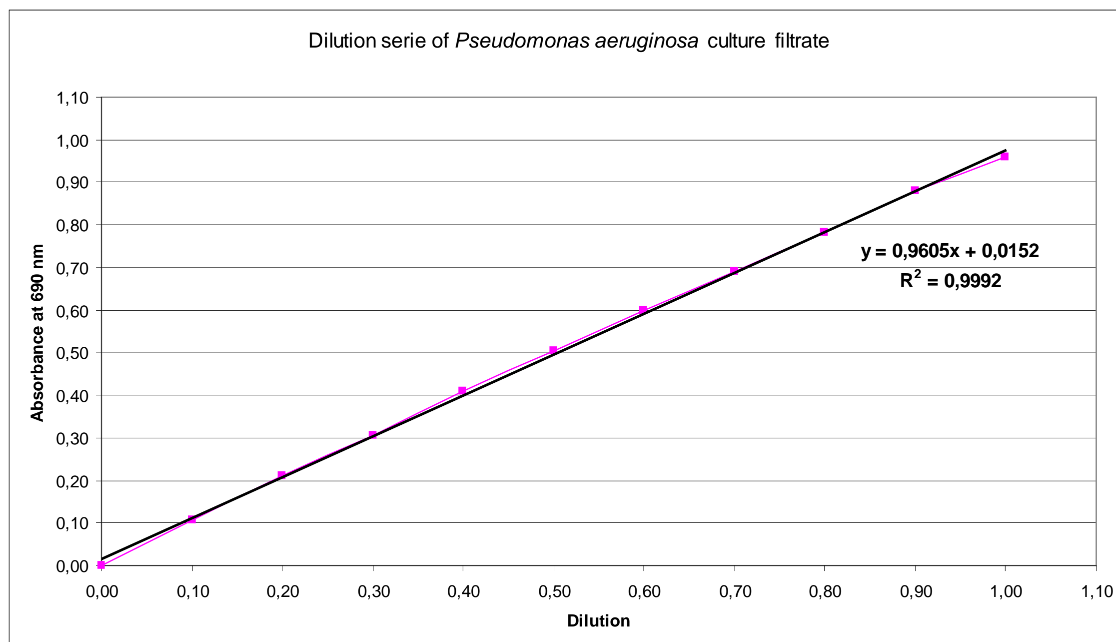

Figure 9.1 *Cell culture filtrate from P. aeruginosa cultures was diluted in a series and the absorbance was measured. The result shows a very good correlation between the absorbance and the dilution factor.*

B.

The result from an additional experiment is presented here, where an in vitro wound model was constructed with nutrient-free agar in inserts and SWF culture medium in the wells of a six-well plate. Wound dressing pieces were placed on the agar, and on top of the dressings small weights were placed giving a pressure of approximately 5.8 mm Hg. *Pseudomonas aeruginosa* suspension was added to the agar plates and the cultures were incubated for three days in 35°C. On day three pyocyanin levels were measured in the bacterial suspensions as well as inside the wound dressings, as described previously in the report.

Some general observations can be made when comparing these results to the experiment where dressing samples were completely soaked in SWF medium, see section 4.4.2.1 and 4.4.2.2. In the in vitro wound model lower levels of pyocyanin were measured in the cultures, except for the culture with Product A without a weight. The presence of a weight seems to decrease the production of pyocyanin, possibly due to reduced oxygen availability. The pyocyanin levels inside wound dressings were significantly lower in this experiment, compared to the experiment where dressing pieces were soaked. A suggestion is that the experiments described in sections 4.4.2.1 and 4.4.2.2 provide a more beneficial environment for *P. aeruginosa,* resulting in a higher production of pyocyanin. This approach with an in vitro wound model also seems to be a more insecure and precarious method, which can be observed as higher standard deviations for all measurements.

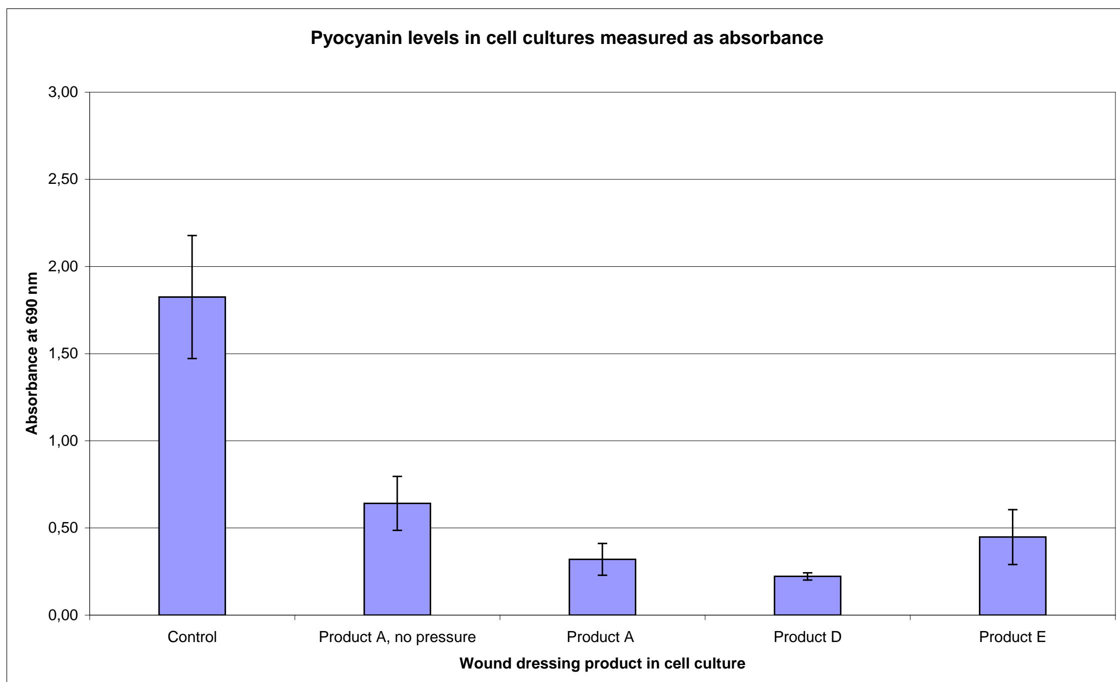

Figure 9.2 *Diagram showing absorbance values of bacterial suspensions, describing levels of pyocyanin. P. aeruginosa was cultured in an in vitro wound model consisting of nutrient-free agar and SWF medium, where the bacteria were added to the agar. The agar were located in inserts with filters, inhibiting presence of bacteria in the medium underneath. Product A was incubated both with a weight and without. N = 3. Product B was excluded from this measurement due to total absorption of the cell medium by the dressing, unabling sampling of the suspension.*

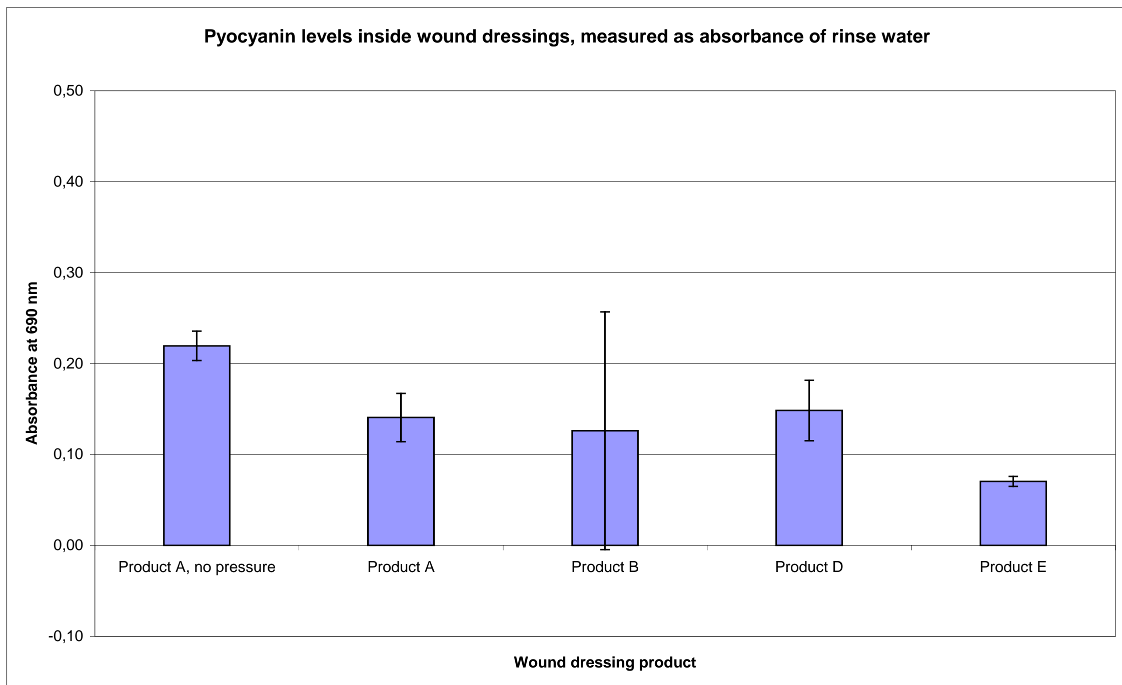

Figure 9.3 *Pyocyanin levels inside wound dressings, measured as absorbance of the rinse water they were soaked in. As described above, the cultures consisted of constructed wound models made of nutrient-free agar and SWF medium. The cultures were incubated for three days. N = 3. Observe the change in scale compared to previous charts.*