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The influence of physical stress on biofilm susceptibility to antimicrobials

Master thesis

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Declaration

I hereby declare that I have completed this thesis having the topic *The influence of physical stress on biofilm susceptibility to antimicrobials* independently and I have included a full list of used references.

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Abstraktní

Většina bakterií žije v komunitách nazývaných biofilmy, které se vyvíjejí na vlhkých površích. Tyto biofilmy jsou v matrici nazvané EPS, která chrání bakterie před chemickým a mechanickým odstraněním. Biofilmy se vyvíjejí na různých površích, včetně lodních trupů, průmyslových potrubí a zdravotnických implantátů. Tyto bakterie mohou způsobit několik onemocnění. Biofilmy jsou zkoumány a zobrazovány mnoha způsoby, aby se zabránilo nebo odstranilo. Současné procesy stály spoustu peněz pro průmysl. Stresová relaxace je typickou odezvou biofilmu v určité míře deformace a je účinným způsobem studia viskoelastických vlastností biofilmu. Pro popis relaxace stresu biofilmu byl použit tříčlenný Maxwellův model a prvky byly hypotéz, že souvisejí se třemi hlavními složkami biofilmu: vodou, extracelulárními polymerními látkami (EPS) a bakteriemi. Maxwellův model však pouze zhruba popisuje relativní význam každé hlavní složky během uvolnění napětí v biofilmu, ale detailní proces přeuspořádání hlavních složek biofilmu zůstává nejasný. V tomto projektu budeme sledovat strukturální přeskupení celého biofilmu a vizualizovat proudění vody a pohyb bakterií při relaxaci stresu biofilmu pomocí optické koherentní tomografie (OKT).

Klícová slova

Biofilmy, viskoelastické, infekce, OKT, CFU

Abstract

Most bacteria live in communities called biofilms, which develop in wetted surfaces. These biofilms are in a matrix called EPS, which protects the bacteria from chemical and mechanical removal. The biofilms develop on various surfaces including ship hulls, industrial pipelines and medical implants. These bacteria can cause several diseases. Biofilms are studied and imaged by many methods, in an attempt to prevent it or remove it. The current processes cost a lot of money for the industry. Stress relaxation is a typical response of a biofilm under certain extent of deformation and it is an efficient way to study viscoelastic properties of a biofilm. A three-element Maxwell's model has been used to describe biofilm stress relaxation and the elements have been hypothesized to be related with three major components of a biofilm: water, extracellular polymeric substances (EPS) and bacteria. However, the Maxwell's model only roughly describes the relative importance of each major component during biofilm stress relaxation, but the detailed process of the rearrangement of the biofilm major components remains unclear. In this project we will observe the structural rearrangement of the whole biofilm and visualize the water flow and bacteria movement during biofilm stress relaxation by optical coherence tomography (OCT).

Keywords

Biofilms, viscoelastic, infection, OCT, CFU

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Abbreviations

CCD	Charge-coupled device
CFU	Colony-forming unit
CMOS	Complementary metal-oxide-semiconductor
EPS	Extracellular Polymeric Substance
OCT	Optical coherence tomography
PBS	Phosphate Buffered Saline
RPM	Revolutions per minute
TSB	Tryptone Soya Broth

Objective

Compare slime and non-slime producing bacteria in two different growing conditions in order to study their viscoelastic behaviour by compressing the biofilm formed around them. The results are measured by optical coherence tomography.

Analyse the penetration of antimicrobials in biofilm in order to understand the importance of new methods of preventing and treating biofilm contaminations.

1. Introduction

1.1. Bacteria

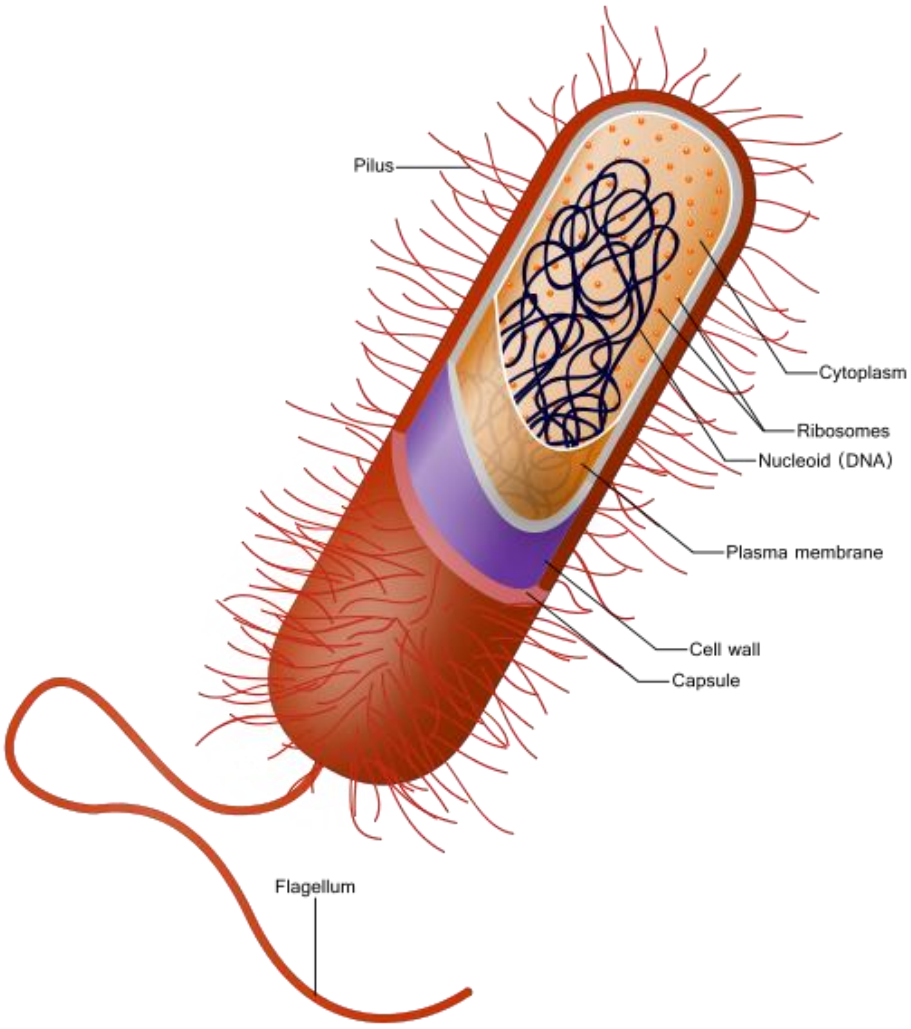


Figure 1: Prokaryote cell[1]

Bacteria might appear on the first study as a very simple form of life. They are single celled, are metabolically active, divide by binary fission, and lack a nuclear membrane. This is true for all bacteria. Upon further study, it is possible to see that they are actually a very sophisticated and highly adaptable. Different species of bacteria may multiply at rapid rates, can make use of various hydrocarbons (including phenol, rubber, and petroleum), and may exist as both parasitic life

forms and free-living forms. They are extremely small, and are usually measured in microns (equivalent to 10-6 meters). Bacteria can be divided into species by analysing and classifying their environmental habitat, effect it has on the environment, among others.[2]

Dues to the ubiquitous nature of bacteria and their high degree of adaptability, they are studied extensively, in order to prevent the damage they might cause in various areas, like medicine and food industries. This has led to the discovery of specific vaccines to diseased caused by bacteria (like diphtheria, tetanus and cholera, among many others), but most importantly the development of antibiotics. The vaccines and antibiotics have not eradicated the harmful effects of bacteria, but their use has proven to be a powerful tool against it. [2]

The advances in the study of bacteria have led to the development of means to reduce their damage, in the form of vaccines, antibiotics (generally antimicrobial substances). Although these methods are a powerful tool against harmful bacteria, they have not removed their risk completely. [2]

Bacteria usually have specific shapes and many times occur in specific types of aggregates characteristic to them, both characteristics that are typical for their biological classification and can aid in removing them. [2]

The most common shapes found are cocci, which are round or ellipsoidal cells, rods, which are long, filamentous branched cells, comma-shaped and spiral cells. The most typical arrangements of aggregates are chains, clusters of spherical cells, and cubic packets. [2]

Another important bacteria characteristic is their specific surface structure. They may be *flagella*, *Pili* or *Capsules*. *Flagella* is a basal body attached to the membrane, which will form a cylindrical protein filament. The movement is created by rotating the long axis. *Pili*, which can also be referred to as *fimbriae*, are appendages on the surface of many bacteria. These are slender, hairlike and proteinaceous, and are important in adhesion to host surfaces. *Capsules* are a

thick outer layer that may be composed of high-molecular-weight, viscous polysaccharide gel or amorphous slime layers. [2]

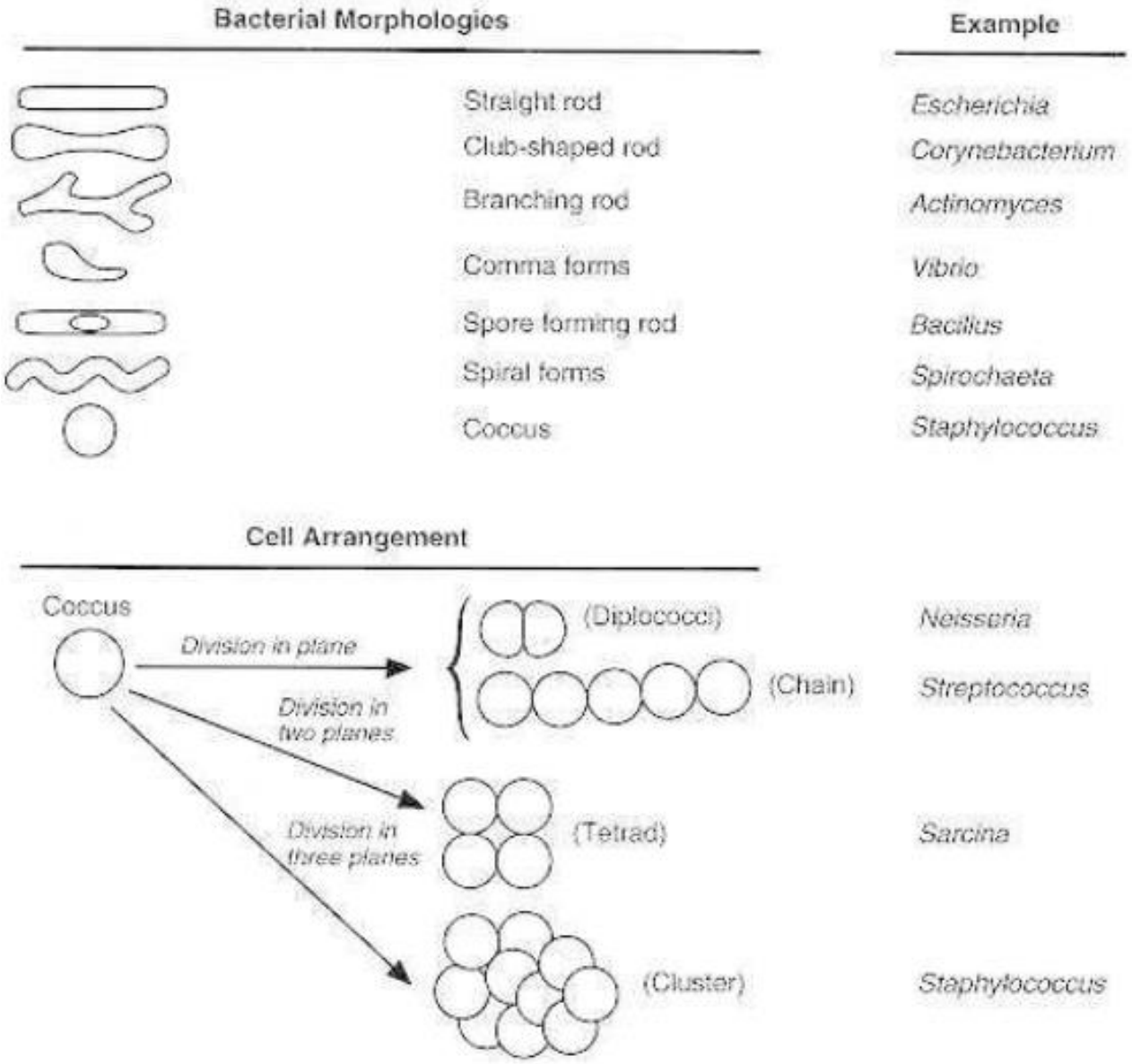


Figure 2: Cell morphologies and arrangements[2]

1.2. Biofilm

The study of biofilms and evaluation of biofilms is a technical area that involves the development of new methods and tools for analysis and computer science techniques. For that reason, it involves the collaboration of scientists from multidisciplinary fields. For these studies, it is necessary to use a variety of microscopy and physical methods associated with the biology and chemical approaches.[3]

Biofilms were first discovered in the 17th century by Antonie van Leeuwenhoek. He observed the accumulation of matter on his teeth. He reported to the Royal Society of London: "The number of these animalcules in the scurf of a man's teeth are so many that I believe they exceed the number of men in a kingdom". He also observed that the vinegar with which he washed his teeth could kill the 'Animals' around the scurf, but could not penetrate the film entirely. This has shown for the first time the difficulties of antibacterial substances to penetrate the biofilm.[4]

The formation of biofilms was also observed by a marine biologist called Claude Zobell in 1943. He observed that if fresh sea water was confined in a glass bottle, the microorganisms on the bottle's walls increased, while the free microorganisms in the water decreased.[5]

Further studies into biofilms have shown that they aggregate more in an environment with a lack of nutrients, compared environments that are rich in nutrients. This suggests that the adhesion and biofilm formation is a survival strategy for survival in hostile environments.[6]

In 1978, it was accepted by the work of Costertan and collaborators that bacteria (and most microorganisms) live attached to a surface within a structured biofilm, and not as single cells.[5]

Due to the flexibility in their gene expressions, bacteria are able to survive in environments with rapidly changing conditions. One particularly important and

clinically relevant example of bacterial adaptation through systematized gene expression is the ability to grow as part of a sessile, exopolymer-enshrouded community referred to as a biofilm.[7]

It is now appreciated that the formation and maintenance of structured multicellular communities critically depends upon the production of extracellular substances that, in conglomerate, constitute an extracellular matrix.8

The major matrix components are microbial cells, polysaccharides and water, together with excreted cellular products. EPS is produced from cell lysis and adsorbed organic matter from wastewater, generating a complex high molecular weight mixture of polymers.[9,10]

Biofilms are defined as consortia of microorganisms that are attached to a biotic or abiotic surface. Biofilm formation is a multi-stage process in which microbial cells adhere to the surface (initial reversible attachment), while the subsequent production of an extracellular matrix (containing polysaccharides, proteins and DNA) results in a firmer attachment. Sessile (biofilm-associated) cells are phenotypically and physiologically different from non-adhered (planktonic) cells and one of the typical properties of sessile cells is their increased resistance to antimicrobial agents.[8,9]

Individuals in bacterial communities such as biofilms experience increased resistance to antibiotics, thermal stress, and predation. These communities also allow bacteria to stay in favourable environments without being swept away. However, because doubling rates of individuals in a community are generally lower than those living outside. The majority of the bacteria live in biofilms. That is because, although they reproduce in a lower rate, their environment is more favourable for survival.[10,11]

There are multiple mechanisms by which biofilms protect themselves from antimicrobials. The first is the failure of the antimicrobial to penetrate the biofilm. Chlorine, one of the most commonly used disinfectant, does not reach more that

20% of the bacteria in a culture. The second is the slowing of the growth when the culture becomes starved for a particular nutrient.[12]

Perfect cooperation in biofilms is predicted when they contain a single strain (high genetic relatedness). Genetically identical cells, as occurs in most multicellular organisms, do not have evolutionary conflicts of interest, and they are predicted to behave simply as is optimal for the group. [13]

Biofilms might have different appearances in different environments. However, their formation will always follow a specific sequence of events. In Figure 3, it is possible this sequence is shown. Part *a,* shows the first layer of molecules adsorbed. This happens very quickly, in seconds after the exposure of a surface to a biofluid. The first molecules to arrive are the proteins with higher mobility, since they are transported through the substratum a lot faster and have a much higher concentration, and therefore arrive well before the cells do. The once pristine surface is now basically an adsorbed protein layer, which is what the cells will encounter once they arrive.[14]

The protein, once adsorbed, will most likely have its structure altered by the adhesion. The type and extent of alteration will be defined by the characteristics of the surface (charge density, hydrophobicity and steric effects). In systems containing various proteins, the type of sorbent surface will determine the composition of the adsorbed protein layer. [14]

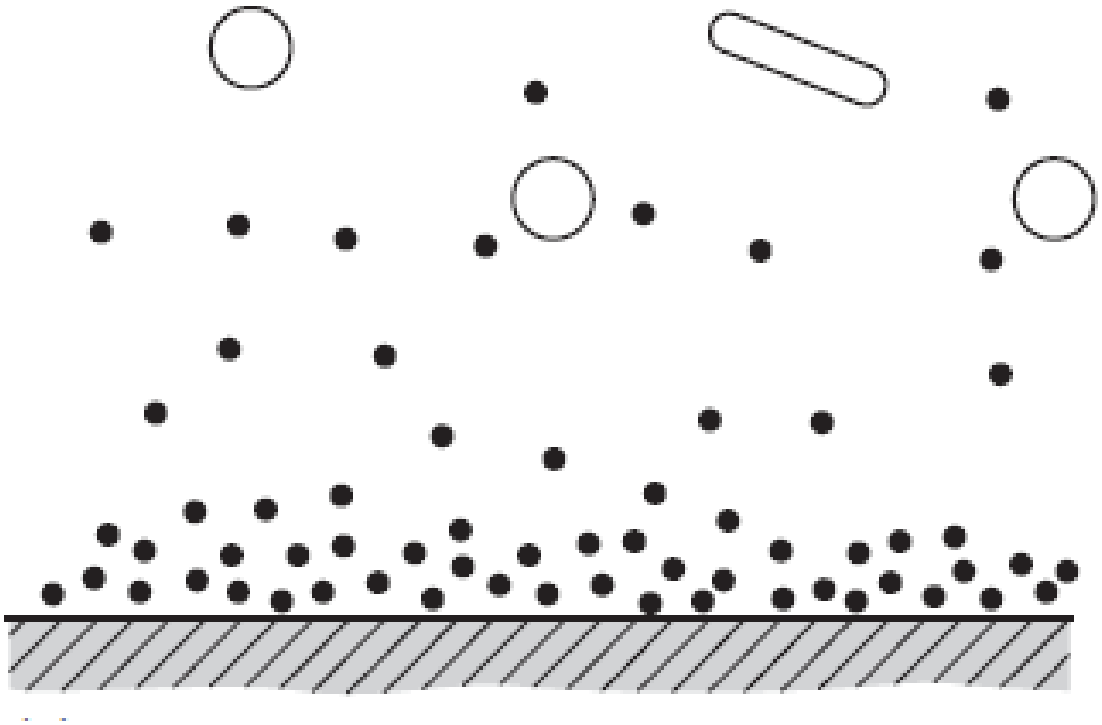


Figure 3: Biofilm formation phase 1

The cells use various transport mechanisms to reach the surface. These may be diffusion, convection, sedimentation, active transport (locomotion), or a combination of those. Once the cell reaches the protein layer, two stages can be noticed. In the first, generic physical-chemical interactions operating over a relatively long separation distance can be determined. After that short range interactions take over, acting on a subnanometer scale. These show range interactions are also of physical chemical nature, but are called ‘specific’. That is because they originate in specific surface architectures of the cell and the substratum, in strongly localized groups. The cell and substratum are stereochemically complementary, making specific recognition possible. (Figure 4).[14]

After the attachment, the cells might form pdeudopods and extrusions, and the microorganism may excrete substances that will help it anchor at the surface. (Figure 5)[14]

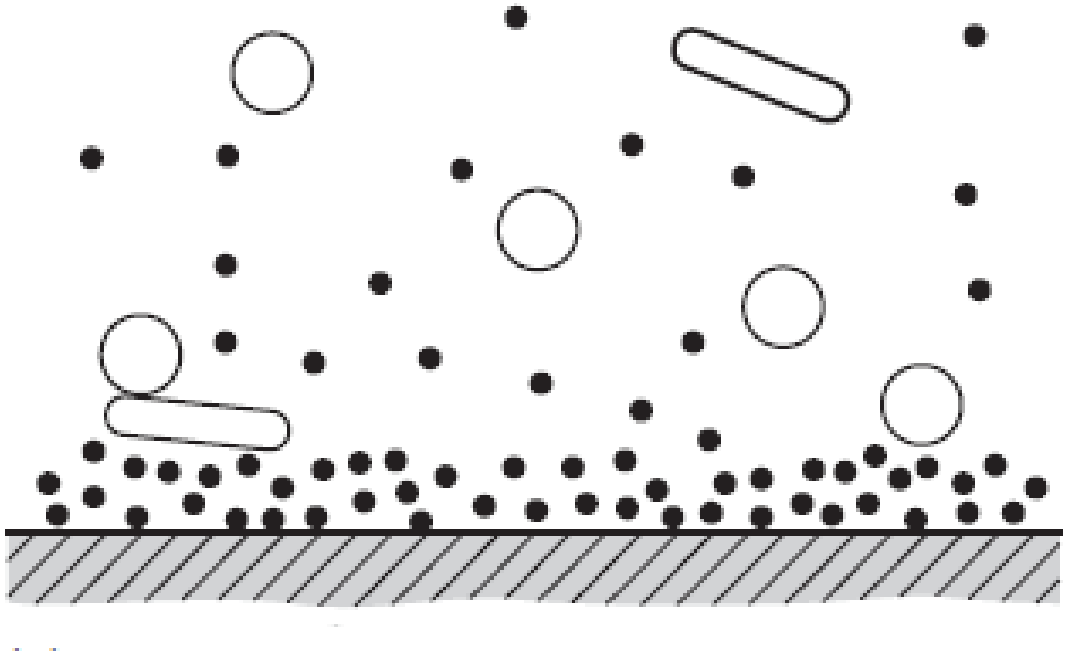


Figure 4: Biofilm formation phase 2

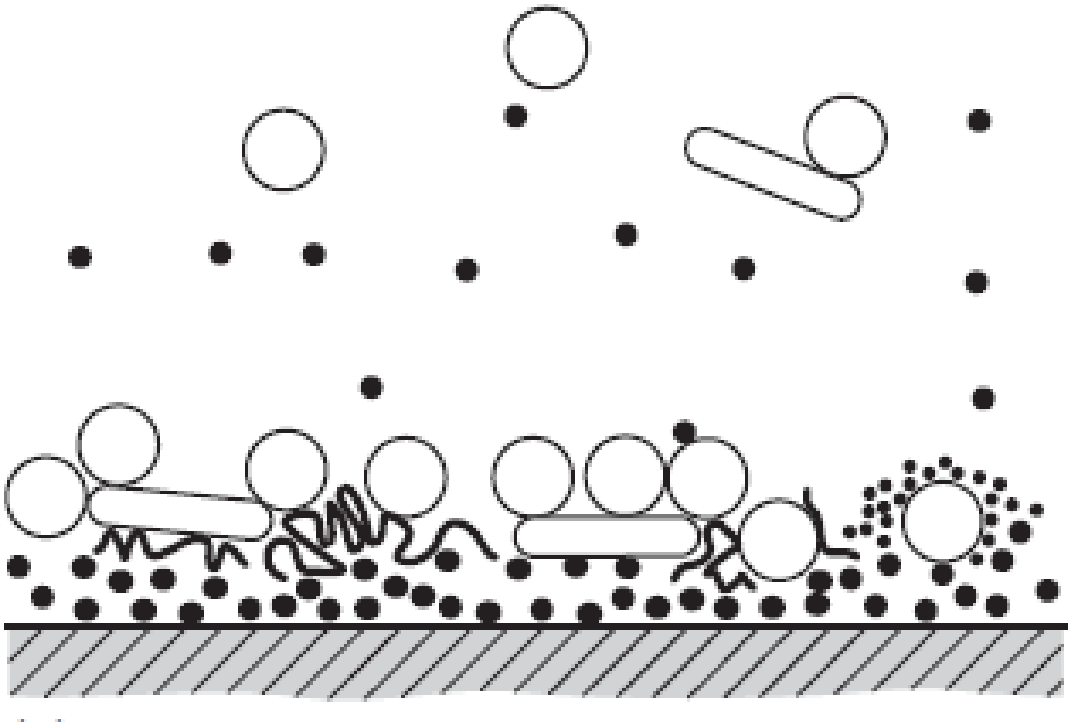


Figure 5: Biofilm formation phase 3

The adhered cells will then start to from and proliferate in order to colonize the substratum. That is the formation process of the biofilm. (Figure 6)[14]

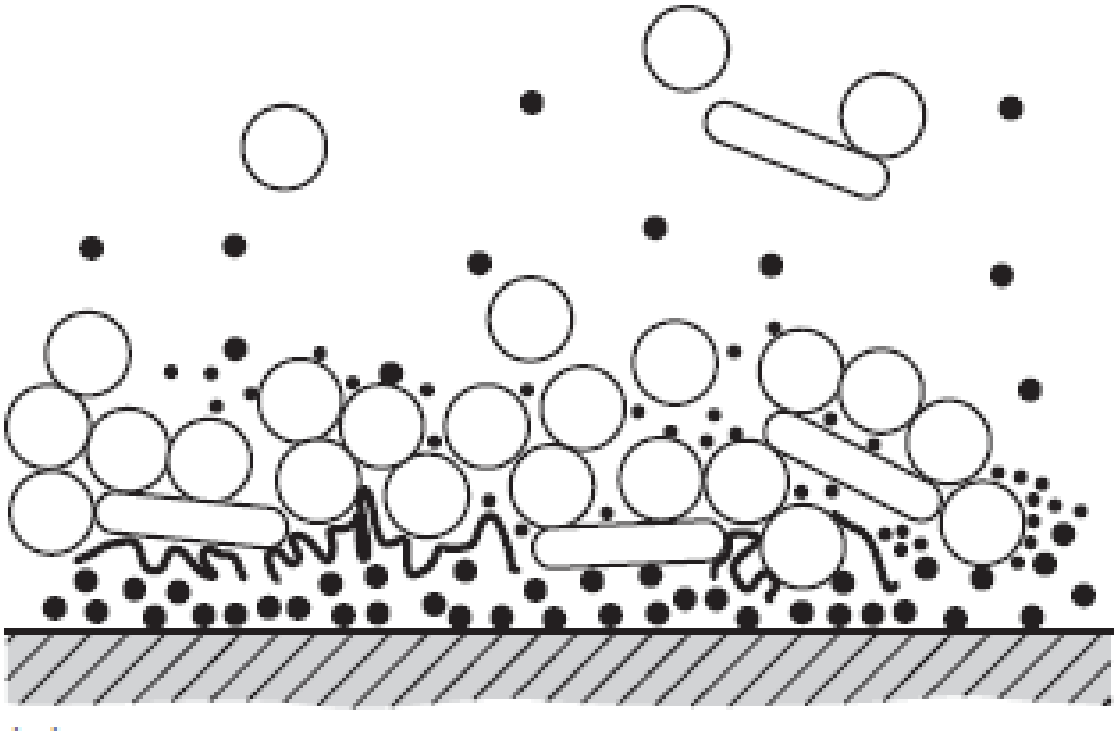


Figure 6: Biofilm formation phase 4

In biofilms, microorganisms from different species or single-species heterogeneous structure organized in communities. In order to transport nutrients, oxygen, genes and antimicrobial agents, the colonies microcolonies are connected by water channels and voids responsible for this transport. [15]

Initially, the cells adhered to the surface start growing relatively quickly. This will make the colony grow in volume. The cells closer to the surface will have difficulty reaching nutrients, while at the same time the cells closer to the extrernal environment will have access to these nutrients. Those closer to the external environment and more access to nutrient will be able to multiply continuously.[16]

In the majority of biofilms, the extracellular material, called EPS, represents 90% of the dry mass. The microorganisms represent only 10 % of that mass. The EPS will enhance the adhesion to the surface and cohesion of the biofilm, and form the platform for the three dimensional architecture of the biofilm structure.[10]

The main functions of the EPS are listed in the table below, as well as their relevance for biofilms and the EPS components involved:

Table 2: Function, relevance and components of EPS[17]

Function	**Relevance for biofilms**	**EPS components involved**
Adhesion	Allows the initial steps in the colonization of surfaces by planktonic cells and enhances the attachment of the whole biofilm to surfaces	Polysaccharides, proteins, DNA and amphiphilic molecules
Aggregation of bacterial cells	Enables bridging between cells, the development of high cell densities and cell-cell recognition	Polysaccharides, proteins and DNA
Cohesion of biofilms	Forms a hydrated polymer network, mediating the mechanical stability of biofilms, determining biofilm architecture and cell-cell communication	Neutral and charged polysaccharides, proteins, and DNA
Retention of water	Maintains a highly hydrated microenvironment around biofilm organisms, leading to their tolerance of desiccation in water-deficient environments	Hydrophilic polysaccharides and possibly, proteins

Protective barrier	Confers resistance to nonspecific and specific host defences during infection and confers tolerance to various antimicrobial agents	Polysaccharides and proteins
Sorption of organic compounds	Allows the accumulation of nutrients from the environment and the sorption of xenobiotics (thus contributing to environmental detoxification)	Charged or hydrophobic polysaccharides and proteins
Sorption of inorganic ions	Promotes polysaccharide gel formation, ion exchange, mineral formation and the accumulation of toxic metal ions (thus contributing to environmental detoxification)	Charged polysaccharides and proteins
Enzymatic activity	Enables the digestion of exogenous macromolecules for nutrient acquisition and the degradation of structural EPS, allowing the release of cells from biofilms	Proteins
Nutrient source	Provides a source of carbon, nitrogen and phosphorus containing compounds for utilization by the biofilm community	Potentially all EPS components
Exchange of genetic information	Facilitates horizontal gene transfer between biofilm cells	DNA
Export of cell components	Releases cellular material as a result of metabolic turnover	Membrane vesicles containing nucleic acids, enzymes, lipopolysaccharides and phospholipids

Studies have shown that the content of the EPS of a biofilm can change according various environmental cues, such as the levels of oxygen, The availability of nitrogen, the desiccation, the temperature, the pH and the nutrient availability.[18]

The EPS surrounds and immobilizes the bacteria in then biofilm, permitting them to be close enough for communication and synergistic microconsortia. Extracellular enzymes present in the EPS also generate something similar to an external digestive system. The enzymes degrade dissolved nutrients present in the water phase, and the organisms in the biofilm can then use them as energy sources. The biofilm architecture can also be influenced by the EPS (depending on the concentration, cohesion, charge, sorption capacity, specificity and nature of the individual components of the EPS) and the three-dimensional architecture of the matrix. It is possible to assert that the EPS is much of the reason why the biofilms are such a complex and dynamic system.[19,20,21]

1.3. Mechanical properties

Two main relations might be made between the deformation and relaxation once a force is applied. One is that the material deforms at a constant rate while the force is being imposed, and this deformation is maintained once the force is released. The materials that behave this way are called viscous (gases and most liquids are examples). If a material behaves fully viscous, the force applied will disrupt all bonds. Viscous materials can be represented by the dashpot.

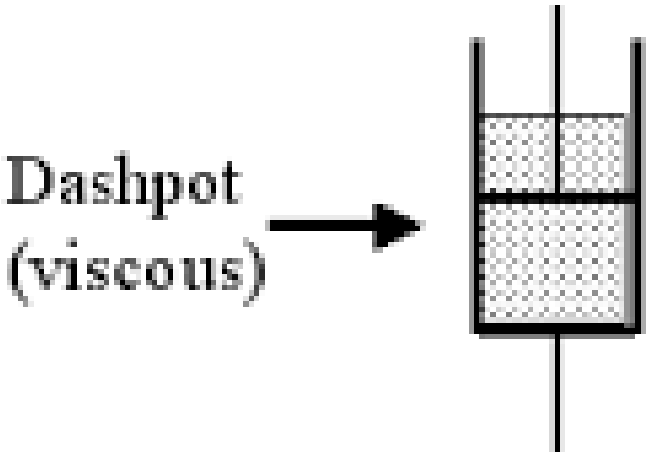


Figure 7: Graphical representation of viscous material

The other is that the material will deform as long as the force is applied and, once the form is released, the material will return to its original state. The deformation is always directly proportional to the imposed force. The materials that behave this way are called elastic. These materials not suffer any breaking of their bonds when the force is applied. They are represented by the spring.

The main difference between those materials is the properties of their intermolecular bonds (most notably their strength in relation to the strength of the applied force).

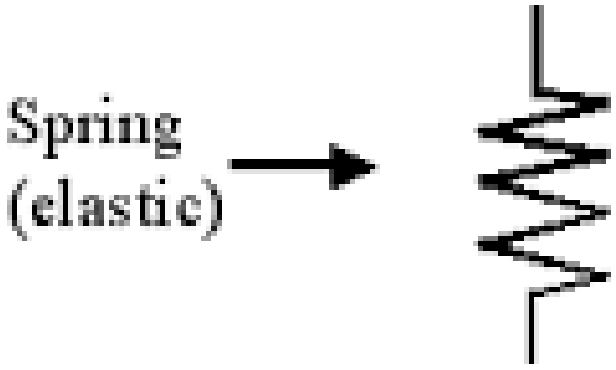


Figure 8: Graphical representation of elastic material

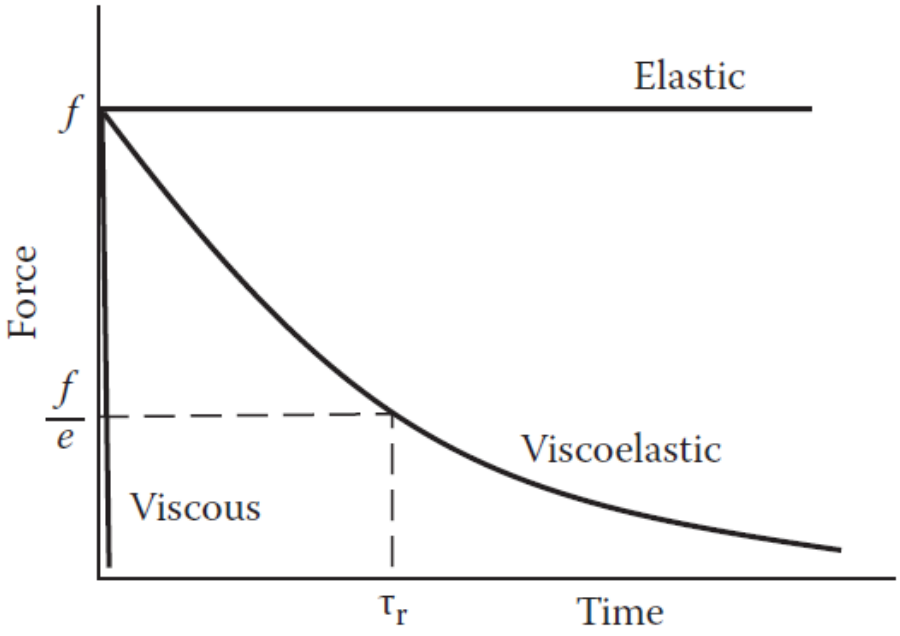


Figure 9: Force relation to time

The difference of behaviour of the viscous and elastic materials can be represented in the figure x. Elastic materials present a long relaxation time (τ r), and viscous materials present a very short τ r. Relaxation time is defined by Willem Norde as “time in which the required force f has decayed to 1/e of its original value”. The ratio of relaxation time per observation time (τ r /t_{obs}) ia called Deborah number (*De*). This number can be used as base to classify if a material is viscous or elastic. If *De* is above one, the material behaviour is elastic, and if *De* is below one, the behaviour is vicous.

Many materials behave partly elastic and partly viscous. Those are called viscoelastic. It is related to the ease of which the bonds are broken. The measurement of deformation for viscoelastic materials will depend on the timescale of the measurement. The bonds in this case are progressively disrupted in a way

that the force decreases gradually during the time of observation. This material can be represented by the spring and dashpot together, either in series or in parallel.

The representation in series is also called Maxwell element.

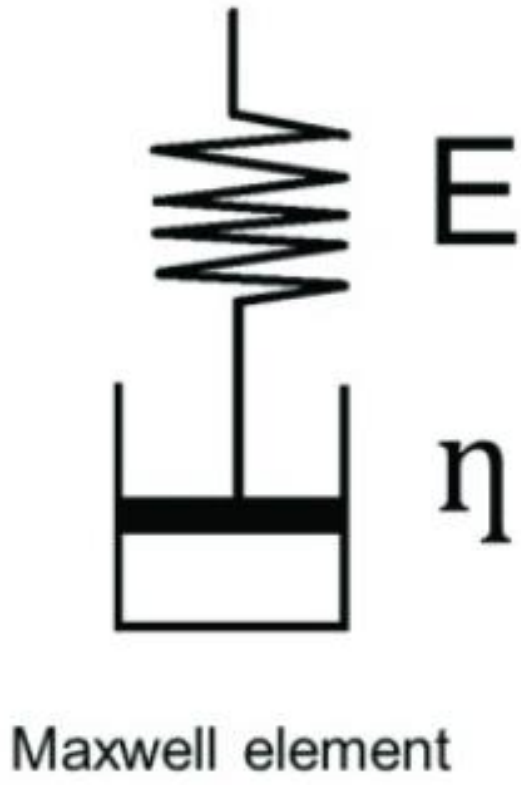


Figure 10: Maxwell element

The system represented in parallel is also called Kelvin-Voigt element.

Klapper et al (2002) found the prediction of an elastic relaxation time on the order of a few minutes—biofilm disturbances on shorter time scales produce an elastic response, biofilm disturbances on longer time scales result in viscous flow, i.e., nonreversible biofilm deformation. [22]

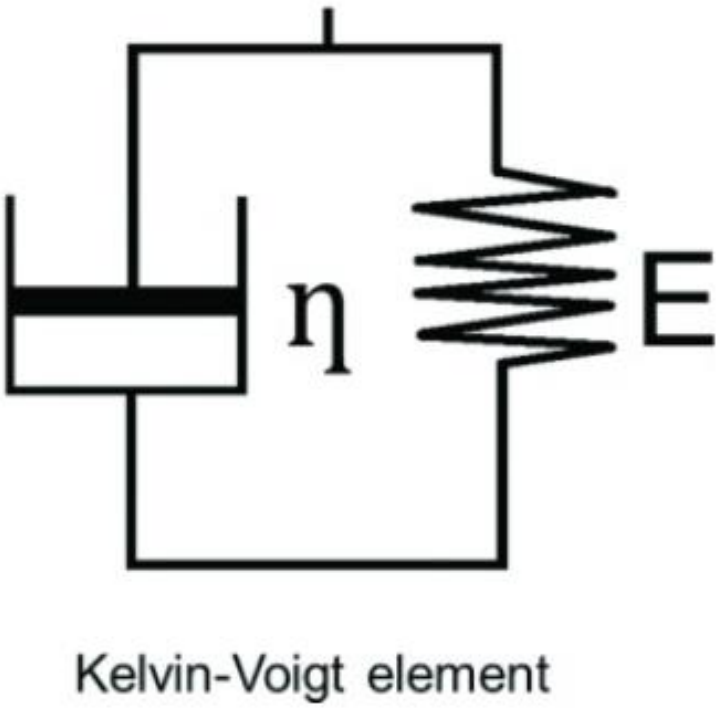


Figure 11: Kelvin-Voigt element

Although Maxwell analysis of stress-relaxation to derive the characteristic time-constants of the various relaxation processes that occur in a biofilm under external loading has been done before, results have been regarded mainly from a mathematical perspective and the details of the relaxation-structure-composition relation in biofilms and the physical processes associated with the different time-constants, are mostly neglected. Stress relaxation may involve a number of processes, like the outflow of water and EPS from the biofilm and re-arrangement of the bacteria in the biofilm. Since penetration of an antimicrobial into a biofilm depends on diffusion and therewith on its structural and compositional features, like the presence of water-filled channels in the biofilm or EPS-containing spaces, we here hypothesize that the penetration of an antimicrobial into a biofilm may relate with stress relaxation and its underlying processes.[23]

Biofilms can be mechanically challenged during growth, for instance by water pressure in marine environments, industrial pipelines or membrane filtration, in the

oral cavity during fluid flow arising from powered tooth brushing and tongue movement, from pulsatile blood flow in intravascular catheters or from the movement of tissues, fluid and biomaterial components in an orthopaedic joint prosthesis. When mechanical challenges occur and detachment forces acting on a biofilm exceed the forces acting between different organisms in a biofilm, the biofilm is overloaded and failure occurs in the biofilm ('cohesive failure'). Alternatively, when detachment forces operate exceeding the forces by which the initially adhering organisms connect with a substratum surface, the entire biofilm dislodges from the substratum surface ('adhesive failure'). Often biofilms go through cycles of fluctuating mechanical challenges and cohesive or adhesive failure along with growth occur accordingly.[24]

A number of studies suggest that biofilms behave as viscoelastic liquids.[24]

1.4. Antimicrobials and biofilm

It has been suggested that the EPS surrounding the bacteria in biofilms, among other functions, prevents the access of antibiotics to the bacterial cells embedded in the community. Although mathematical models suggest that, for many antibiotics, there should be no barrier to their diffusion into a biofilm, some studies have shown an apparent failure of certain antimicrobial agents to penetrate the biofilm.

When a bacterial cell culture becomes starved for a particular nutrient, it slows its growth. Transition from exponential to slow or no growth is generally accompanied by an increase in resistance to antibiotics. Because cells growing in biofilms are expected to experience some form of nutrient limitation, it has been suggested that this physiological change can account for the resistance of biofilms to antimicrobial agents.[25]

Many factors may have an influence biofilm tolerance to antimicrobials in a particular biofilm. Regarding antimicrobial chemistry, there is no way to predict its efficacy against a biofilm based on its chemistry.

Studies made with different substratum materials achieved different results at antimicrobial penetration. However, some results may have been different because of different methodologies.

The density of the biofilm also plays a role in the penetration of antimicrobials, as well as the extent of biofilm accumulation. Older, thicker biofilms are invariably less susceptible than younger, less dense biofilms. [12]

Regarding the species composition, tolerance is not specific to any particular subgroup of microorganisms. In fact, reduced biofilm susceptibility appears to be a broadly distributed capability across the microbial world. However, Antimicrobial susceptibility can be very sensitive to the composition of the medium used in the assay.

It can be said that there is there is no discernible generalized role of antimicrobial size, antimicrobial chemistry, substratum material, or microbial species composition on the quantitative level of tolerance established during biofilm formation. Only areal cell density and biofilm age partially correlate with antimicrobial tolerance. Case study results also point to an important role for medium composition, and hence physiology, in biofilm tolerance. [12]

One simple mechanism of biofilm protection is depletion of the antimicrobial agent in the fluid bathing the biofilm. The antimicrobial could be depleted either by reaction in the fluid phase, by reaction with the biofilm or attachment substratum, or by sorption to constituents of the biofilm or substratum material. [12]

The extent of antimicrobial penetration into a biofilm is expected to depend on biofilm thickness, effective diffusivity of the agent in the biofilm, reactivity of the agent in the biofilm, the sorptive capacity of the biofilm for the agent, the dose concentration and dose duration, and external mass transfer properties.[12]

1.5. Impact of biofilms on the industry and medicine

Human technological development has had an enormous impact in nature and living conditions. That has given new opportunities for microorganisms to colonize and grow in new places, creating new challenges related to biofilms.[26]

A spectrum of indwelling medical devices or other devices used in the health-care environment have been shown to harbour biofilms, resulting in measurable rates of device-associated infections. Biofilms of potable water distribution systems have the potential to harbour enteric pathogens, *L. pneumophila*, nontuberculous mycobacteria, and possibly *Helicobacter pylori*.[11]

The accumulation of microorganisms in industrial water systems happens when surfaces in contact with water are colonized by microorganisms. However, the economic implications of accumulation of microorganisms in industrial water systems are much greater than many people realize. In a survey conducted by the National Association of Corrosion Engineers of the United States, it was found that many corrosion engineers did not accept the role of bacteria in corrosion, and many of them that did, could not recognize and mitigate the problem.[27]

Some of the major developments in recent years have been a redefinition of biofilm architecture and the realization that microbiologically influenced corrosion of metals can be best understood as biomineralization.27

Biofilm control in drinking water distribution systems is crucial, as biofilms are known to reduce flow efficiency, impair taste and quality of drinking water and have been implicated in the transmission of harmful pathogens.[38]

Biofilms are also a problem in places that depend on desalinated water, due to the attachment of microorganisms on the desalinization plants, leading to the formation of biofilms.[39]

Industrial heat exchangers also suffer from deposition problems, which generate an extremely high cost of cleaning for the industry.[40]

Heat exchangers have also been implicated in outbreaks of diseases related to bacteria. They also suffer damage through corrosion and decreasing energy efficiency by clogging hydraulic systems and increasing heat transfer resistance across fouled surfaces.[41]

The detachment of bacterial cells from biofilms is of fundamental importance to the dissemination of infection and to contamination in both clinical and public health settings. However, detachment is the least-studied biofilm process and remains poorly understood.[42]

1.6. Prevention and control of biofilm contamination

Many techniques are applied in order to try to prevent or, if that fails, control biofilm contamination.

High throughput screening of small molecule libraries has been one of the major approaches to search for drug leads.

Disrupting or degrading the extracellular polymeric matrix of biofilms can weaken and disperse biofilms. There have been a number of studies done to degrade matrix components such as polysaccharide, eDNA and proteins.

The material surface can also be coated with bactericidal/bacteriostatic substances, generally antibiotics, which are supposed to prevent the bacteria from adhering in the first place. The surface can also be coated with silver, which is a strong antibacterial agent.[44]

1.7. Optical Coherence tomography

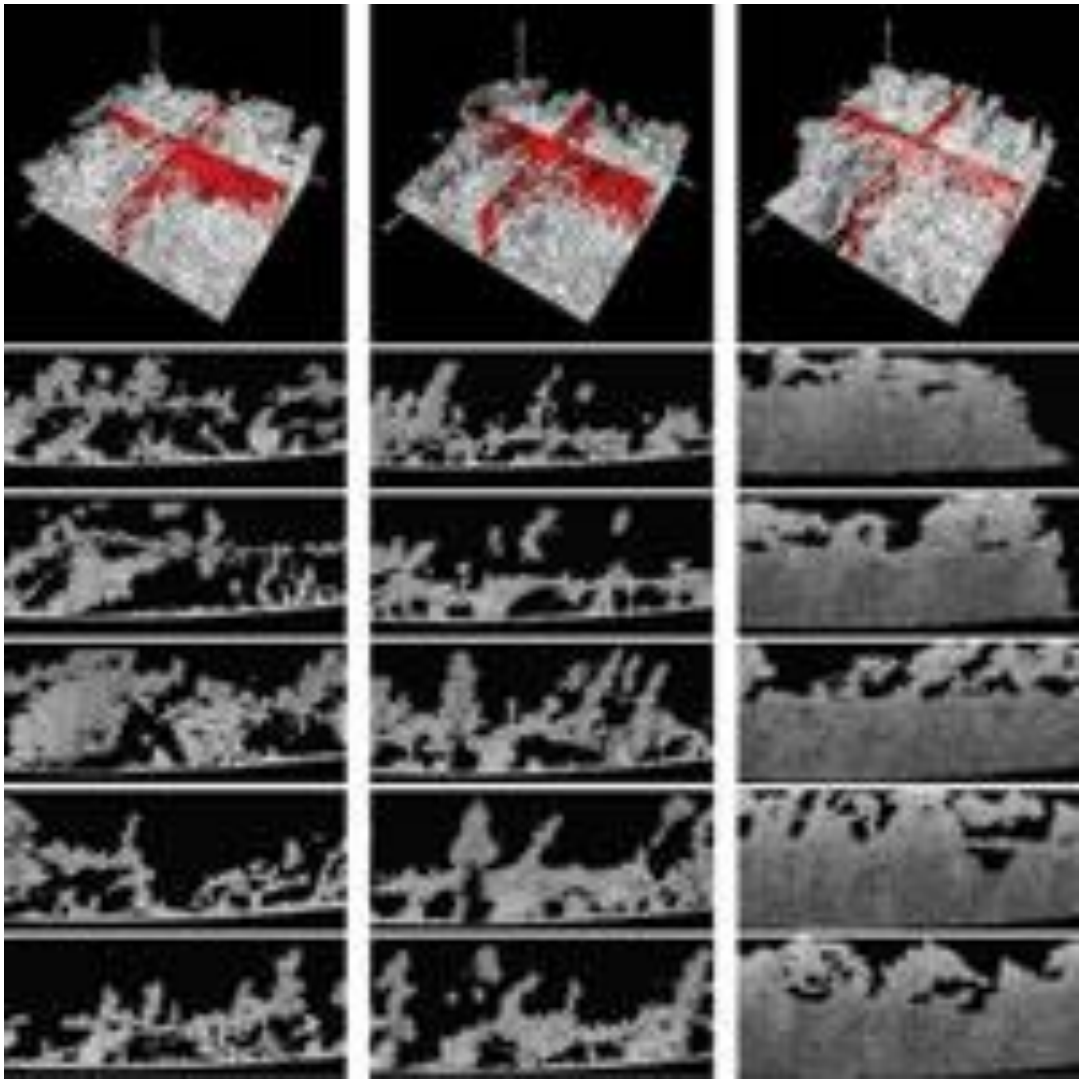


Figure 12: Representation of biofilms in OCT from Thorlabs

Optical coherence tomography (OCT) is a non-invasive imaging methods used for obtaining cross-sectional images of internal structures in biological tissues by measuring their optical reflections.[45]

Optical coherence tomography performs high resolution cross sectional imaging. It is similar to ultrasound imaging, using light instead of sound. The technology uses backscattered light. Its widest use is in ophthalmology. Various

other methods are used for imaging biofilms at the microscopic level, however, few are the ones that measure biofilms on the mesoscopic level, that is, between microscopic and macroscopic, providing information about the sample's global structure. Magnetic resonance imaging (MRI) can be used for that purpose; however, it is costly and complex. OCT also allows *in situ* and in real time imaging to be made.[47]

OCT synthesises cross-sectional images from a series of laterally adjacent depth-scans. At present OCT is used in three different fields of optical imaging, in macroscopic imaging of structures which can be seen by the naked eye or using weak magnifications, in microscopic imaging using magnifications up to the classical limit of microscopic resolution and in endoscopic imaging, using low and medium magnification. First, OCT techniques, like the reflectometry technique and the dual beam technique were based on time-domain low coherence interferometry depth-scans. Later, Fourier-domain techniques have been developed and led to new imaging schemes. Recently developed parallel OCT schemes eliminate the need for lateral scanning and, therefore, dramatically increase the imaging rate. These schemes use CCD cameras and CMOS detector arrays as photodetectors. Video-rate three-dimensional OCT pictures have been obtained. Modifying interference microscopy techniques has led to high-resolution optical coherence microscopy that achieved sub-micrometre resolution.[48]

OCT is increasingly applied in medical diagnostics. It reveals photon-reflecting structures in tissue with lateral and axial resolution in the range of 10 μm. It can monitor biofilm structures and their detachment. OCT is able to reveal spatially resolved structural information on biofilm without staining, and monitor transient processes with temporal resolution in a second to minute scale.[49]

Although the light scattering properties of biological tissues typically limit light penetration to less than 2 mm, this imaging depth has proven sufficient to provide valuable information about tissue pathology in a number of biomedical fields.[50]

OCT has been increasingly a common tool when analysing biofilms and creating three dimensional images of the structures. These three-dimensional OCT images are visually similar to images obtained with confocal laser scanning microscopy, but are obtained at greater depths.[51, 52, 7]

1.8. Colony forming unit counting (CFU)

Colony forming counting unit is a method of estimating the count of living and growing bacteria or other biological cells in a sample. The process involves creating a colony, diluting the cell solution until a concentration that is practical to count annually is reached. Another colony is made with that concentration, and the number of viable colonies are counted.

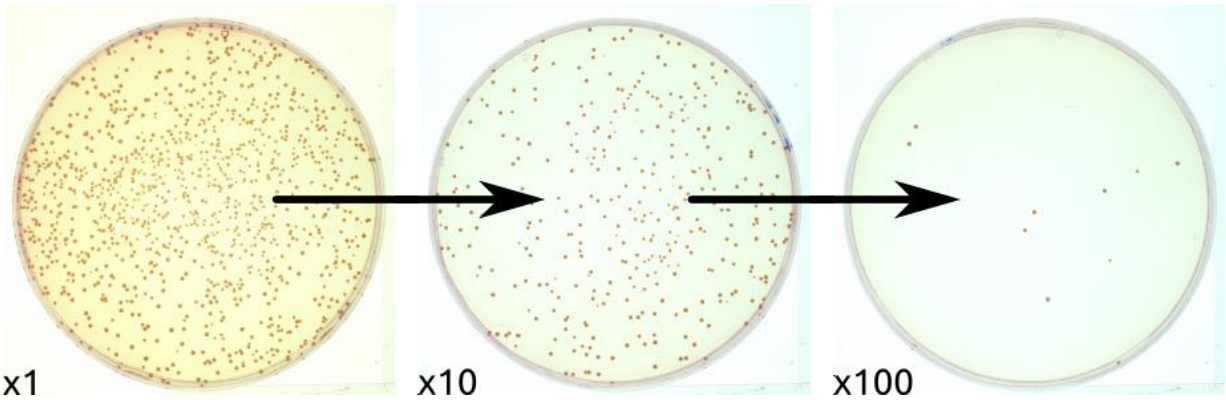


Figure 13: Dilution in CFU

The bacteria solution with is made with a $1x10^{8}$ concentration. This is diluted until $1x10^{3-5}$. This dilution is spread on agar plates by the spread plate technique. It consists of adding the diluted aliquot to a previously prepared and solid agar plate and spread it on the surface. This is left in the incubator to grow for 24 hours. After that, the colonies are counted.[8]

2. Materials and Methods

2.1. Bacterial strains

Staphylococcus aureus was chosen because it is a very common type of bacteria. The strains *Staphylococcus aureus* ATCC 12600 and *Staphylococcus aureus* 5298 were chosen to be compared. The ATCC 12600 strain produces EPS matrix, while the 5298 strain does not.[8, 10, 11]

2.1.1. Bacterial culture

A blood agar plate kept at 4º C was used for the bacterial culture. The bacterial strains was kept at -80º C in 7% dimethylsulphoxide before being used. A solution was made with 30-40 µl of bacteria. The bacteria solution is spread on the blood agar plate with an inoculation loop. The agar plate is placed in the incubator at 37º C for 24 hours. In that period the bacteria grew into individual colonies, which were then used for the pre-culture of the biofilm.

2.1.2. Biofilm formation

The process used to make a biofilm was doing a pre-culture, a main culture, using that to form biofilms in 12 well plates

For the medium, tryptophan soy broth was diluted in water, 30 grams for every litre, and the solution was autoclaved.

The pre culture was made by using the inoculation loop to pick up a single colony from the blood agar plate and stirring the loop in a tryptone soya broth (TSB) medium. That was left for 24 hours in the incubator at 37º C.

For the main culture, the pre culture was mixed into 200 ml of TSB medium. The main culture is then placed in the incubator at 37º C for another 24 hours.

The main culture was then centrifuged at 6500 RPM for 5 minutes at 10º C. The medium was thrown away and the bacteria are re-suspended in 5 ml of autoclaved demi water, and centrifuged again with the same settings. That process needed to be made three times. After the last time, the solution was sonicated in order to remove eventual clusters of bacteria.

A solution was made with 5 ml of normal tap water and 100 µl of the bacteria solution. That is used to count the bacteria with the Bürker Türk.

After calculating the concentration of each strain, the solution was pipetted into the well plates in an amount according to its concentration. That was completed until 2 ml with the attachment buffer and NaCl, which is known to enhance the formation of biofilm. The attachment buffer was made with a 50 times dilution of a Potassium Phosphate buffer in autoclaved water.

The well plates were placed in the shaker at 37º C and 60 RPM for 2 hours. After those 2 hours, the attachment buffer was removed from the well plates and 2 ml of TSB medium was added. Then, the well plates were placed either in the incubator at 60 rpm or in the shaker, both for 48 hours and at 37º C. The medium needed to be changed after 24 hours and returned to the incubator or shaker.[5]

2.2. CFU counting

After the biofilm is ready, one of the well plates put in the shaker and one of the plates left in the incubator were used for the CFU counting. The antimicrobial, chlorhexidine, was placed in half of the well for 30 seconds, then removed and replaced with buffer. The biofilm was re-suspended and diluted enough to be counted after growth. Since the concentration in the well plates were initially around 10^9, the bacterial suspension needed to be diluted until 10^{3-5}. The diluted bacteria solution was spread in agar plates and left in the incubator for 24 hours. After that, the colonies that were formed were counted.

2.3. OCT compression

The equipment used for compression was designed in RUG Biomedical Engineering department. The device would not reach the bottom of the biofilm initially, so it had to be extended with parallel glass plates.

The other plates left in the incubator and shaker, were used in the OCT. The TSB was removed and 2 ml of buffer was added in order for the biofilm to appear in the OCT imaging.

The biofilms were measured. The compressor was placed between the lens and the biofilm, as shown below.

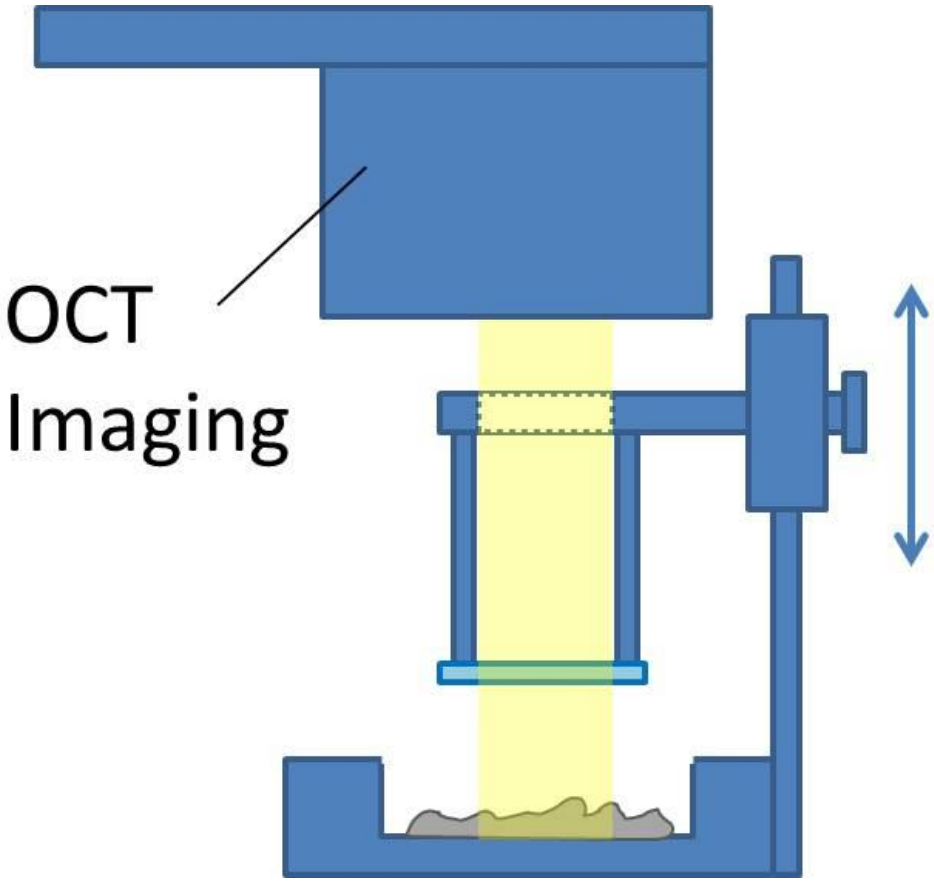


Figure 14: Compression model

Images were captured at six stages, which will from now be called frames. The first is without any compression, and the compressor not touching the biofilm. The second was with the compressor just above the biofilm, touching it but not compressing. The third was with compression to half of the original size of the biofilm (around 100 µm, since all biofilms were around 200 µm). One minute was counted until the next frame, without moving the plunger. The fourth frame was with the compressor back to where it was in the second frame, around 200 µm, which was also left for one minute before the sixth and final frame.

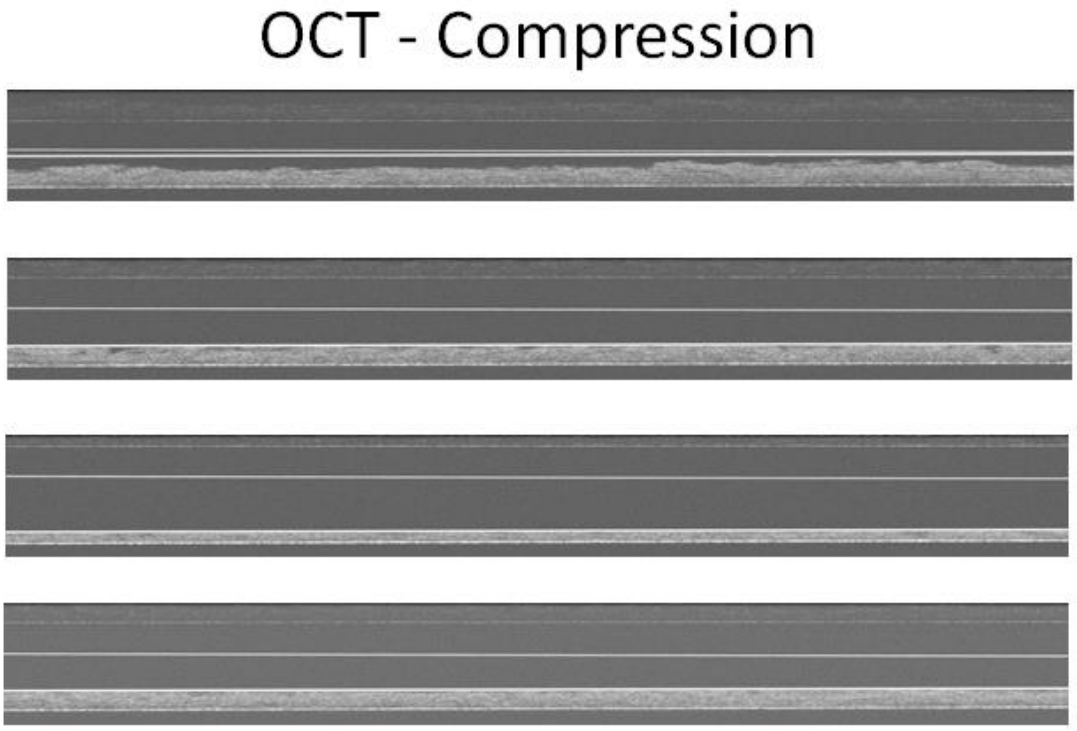


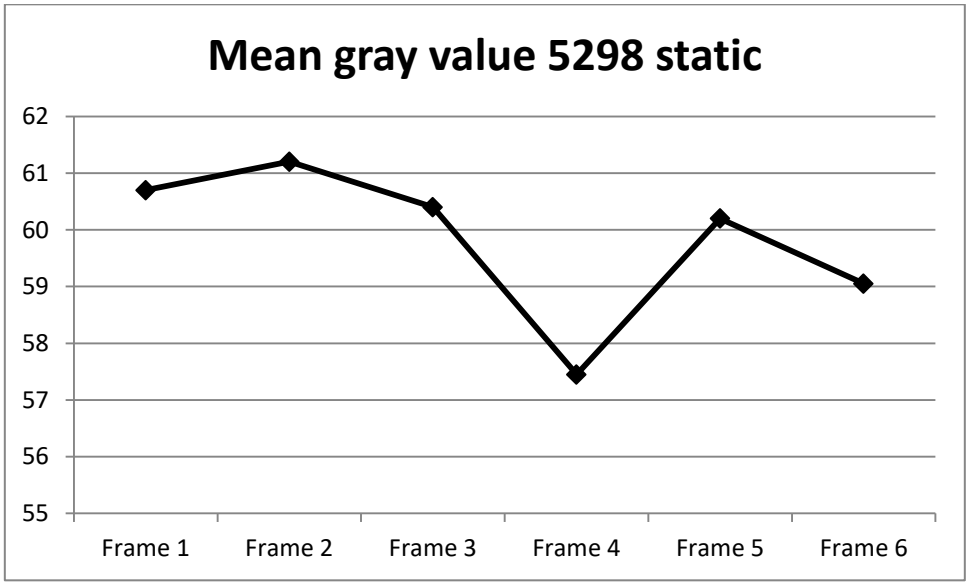
Figure 15: Biofilms in all stages of compression

The images were exported to a software designed by the University of Groningen based on LabView. This software counted the grey level of all the biofilms, which were transformed in graphics and analysed.

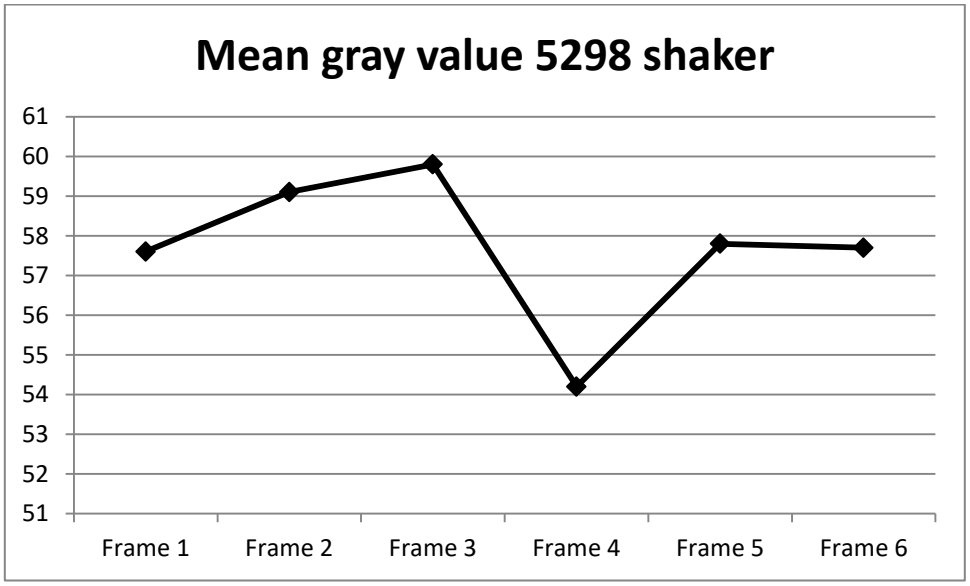
This was made with three colonies from each strain and each condition of growth.

3. *Results*

3.1. OCT compression

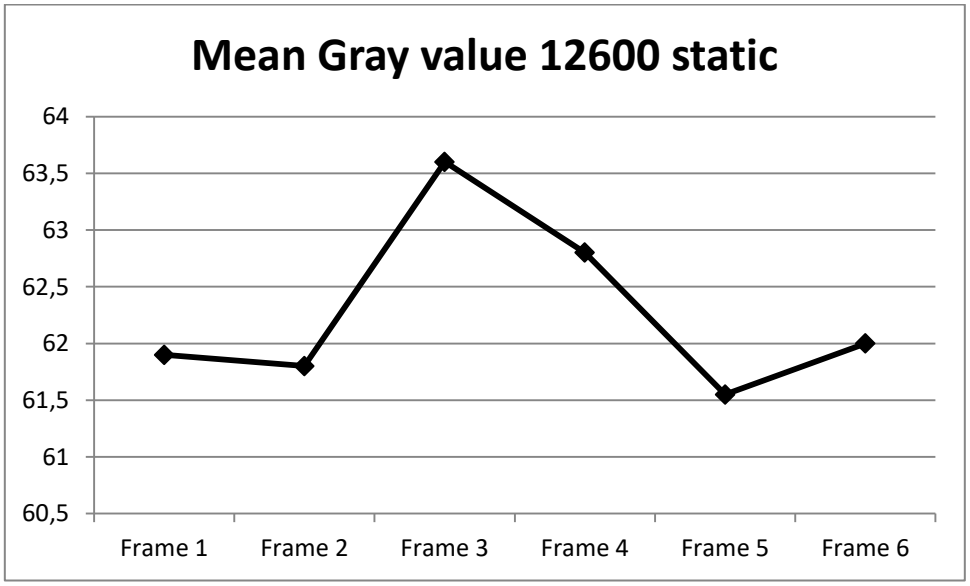


Graphic 1: Mean grey value of S. aureus 5298 grown on static condition.



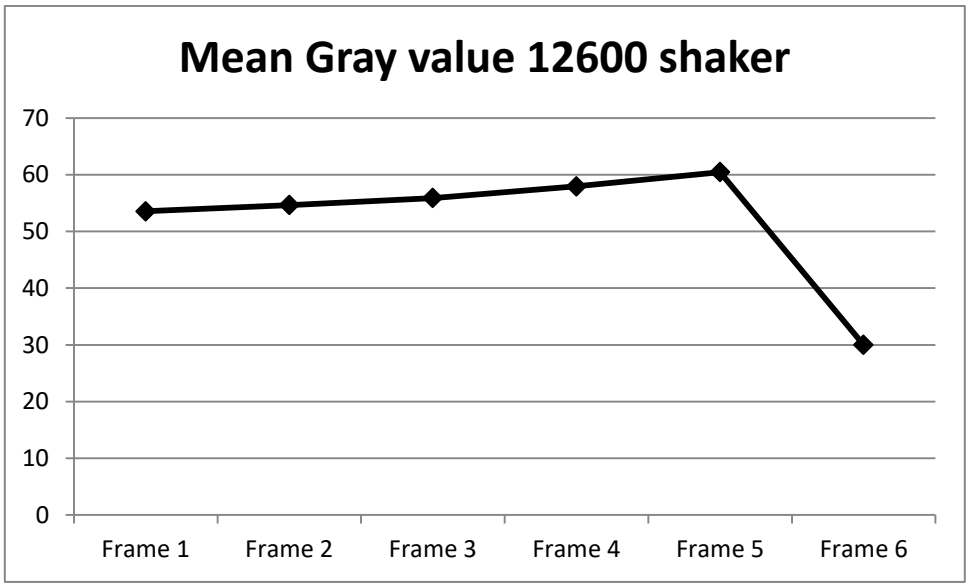
Graphic 2: Mean grey value of S. aureus 5298 grown on shaker.

It is possible to see, comparing the mean grey value of the *S. aureus* 5298 grown in the two different conditions, that growing in an incubator has a lower grey level than the one grown in the shaker, which means it has more water content. The two graphics show a similar pattern, the biggest difference being that when the biofilm is compressed to half of its size, while the biofilm grown in the shaker has a decrease of water level, the one grown in the incubator has an increase. This shows that there is a pattern to the behaviour of the 5298 strain.



Graphic 3: Mean grey value of S. aureus 12600 grown on static conditions.

The *S. aureus* ATCC did not present a pattern in both conditions. While the biofilm grown in the incubator presented similar patterns to the 5298 strain, the biofilm grown in the shaker presented a very different curve. That may be because one of the biofilms were destroyed and could not be tested by the OCT, generating less reliable results.



Graphic 4: Mean grey value of S. aureus ATCC 12600 grown on shaker.

The behaviour expected from the biofilm is that, when being compressed, the water content would decrease, because water is the most dynamic component of the biofilm. That is proven true for the ATCC 12600 strain grown in an incubator, and for the 5298 strain grown in the shaker.**Erro! Fonte de referência não encontrada.**

When the biofilm is left for a minute, with no compression or compressing the biofilm to half of its size, the expected behaviour is that the biofilm resettles, and the water that was pushed before also does.

After the plunger was retracted to the original biofilm thickness, the water was supposed to be pulled back into the surface. That behaviour can only be observed in the 12600 grown under static conditions.

When the biofilm is left without compression again, it is supposed to imitate the previous behaviour, in a smaller level. This is also only true for the 5298 strains.

3.2. CFU counting

All the plates from the 5298 strain (non-slime producing) were contaminated.

Table 2: Colonies counted by CFU method

	Colony 1	Colony 2	Colony 3
12600 static non-treated 10^3	312	320	204
12600 static non-treated 10^4	>300	>300	>300
12600 static treated 10^3	17	5	4
12600 static treated 10^4	51	20	<4
12600 shaker non-treated 10^3	Contaminated	38	98
12600 shaker non-treated 10^4	Contaminated	>300	>300
12600 shaker treated 10^3	63	6	5
12600 shaker treated 10^4	>300	95	Contaminated

It can be seen in the table above that the chlorhexidine, even with a high killing efficiency, cannot completely kill all the bacteria. The surviving bacteria will form other communities, and the surface will be contaminated again.

4. Discussion

When this study was designed, the method of growing biofilms was going to be a flow chamber. This equipment consists of two glass plates with the bacteria chosen between them, surrounded by two metal plates held together by screws. Plastic pipes attached to both sides of these metal plates would push water through. As discussed previously, Biofilm tends to form when the conditions are not favourable for growth. Many studies of biofilms had been done using this method.

However, unlike other studies, not only the image had to be made, but the biofilm had to be compressed. For that, the glass plates had to be separated. At doing that, the biofilm would not be intact enough in any of the two glass slides to be compressed. So other method had to be developed.

The biofilm grown in the shaker was intended to try to emulate the harder conditions of the flow chamber or an environment with harsh conditions.

5. Conclusion

It can be concluded that, barring some mistakes, the biofilm has behaved as expected. That means that in the compression, at least one of the conditions or strains of the biofilms had the rise or fall in grey level that was expected.

The CFU showed, even if in just one of the strains, that even if a antimicrobial has a high level of killing efficiency, it still needs some aid from anoother method, because even just a few bacteria are enough to cause an infection or contamination.

The method used can be developed further for testing of mechanical properties of biofilms. Future works should be to test growing the biofilm in a petri dish instead of well plates, since those interfere with each other both in biological contamination and in mechanical failures.

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