# Summary

Bacteria are among the most resilient organisms on the planet. It is currently estimated that over 60% of all human infections and 80% of bacterial infections, treated by physicians are due to bacterial biofilms. There is a need to find a method to control biofilms after they have been formed. So far, previous research has been extensively focused on preventing bacterial adhesion using surface coatings antagonistic with bacterial surfaces and developing antimicrobial surface properties for killing adhering bacteria. **Chapter 1** describes some of the properties of biofilms make them special and distinct from their planktonic counterparts. The aim of this thesis is to better understand the visco-elastic properties of mature biofilms.

In order to control biofilms, it is necessary to understand the properties of the initially adhering bacteria which give rise to the biofilms. Therefore, the first experimental study focused on centrifugal damage, which has been known to alter bacterial cell surface properties and interior structures, including DNA. In **Chapter 2,** we provided a simple, versatile method of analysis for describing the compaction of bacteria during centrifugation based on a proposed centrifugation coefficient; *C*. Values of *C* were related to different bacterial cell surface properties. Changing the geometry of the centrifugation container or centrifugation speeds changed the value of *C* significantly. Initial deposition rates of *Staphylococcus aureus* ATCC 12600 to a glass surface decayed exponentially from 4217 to 1478 $cm^{-2}s^{-1}$ with increasing *C*, while the proportion of staphylococci with a zeta potential of around -15 mV, decreased from 97% to 58%. These surface-sensitive parameters were used independently to derive a critical centrifugation coefficient (0.040), above which centrifugation was considered to impact the outcome of

surface-sensitive experiments due to cell surface damage. Moreover, controlling the centrifugation coefficient within narrow limits over a series of experiments yielded 43% smaller standard deviations in initial staphylococcal deposition rates than with centrifugation at fixed speeds for replicate experiments.

Obviously, the next step was to investigate whether the harvesting procedure, along with different growth parameters, could alter properties of resulting biofilms (**Chapter 3**). Therefore, the influence of centrifugal compaction and environmental conditions during growth on visco-elastic properties of oral biofilms was studied. Biofilms were grown out of a layer of initially adhering streptococci, actinomyces or their combination. Different uniaxial deformations were induced on the biofilms and load relaxations measured over time. Linear-Regression-Analysis demonstrated that both centrifugation coefficient for streptococci and induced deformation influenced the percentage of stress relaxation in 100 s. Centrifugal compaction significantly influenced relaxation only upon compression of the outermost 20% of the biofilm ($p<0.05$), whereas biofilm composition became influential upon inducing 50% deformation, invoking re-arrangement of bacteria in deeper biofilm structures. In summary, the effects of centrifugal compaction of initially adhering, centrifuged bacteria extended to visco-elastic properties of biofilms, indicating that the initial bacterial layer influences the structure of the entire biofilm.

Biofilms are often tolerant to antimicrobials, due to a combination of inherent properties of bacteria in their adhering, biofilm mode of growth and poor physical penetration of antimicrobials through

biofilms. Current understanding of biofilm recalcitrance toward antimicrobial penetration is based on qualitative descriptions of biofilms. In **Chapter 4**, we hypothesized that stress relaxation of biofilms would relate with antimicrobial penetration. Stress relaxation analysis of single-species oral biofilms grown *in vitro* identified a fast, intermediate and slow response to an induced deformation, corresponding with outflow of water and extracellular polymeric substances (EPS), and bacterial re-arrangement, respectively. Penetration of chlorhexidine into these biofilms increased with increasing relative importance of the slow and decreasing importance of the fast relaxation element. Involvement of slow relaxation elements suggested that biofilm structures allowing extensive bacterial re-arrangement after deformation are more open, allowing better antimicrobial penetration. Involvement of fast relaxation elements suggested that water dilutes the antimicrobial upon penetration to an ineffective concentration in deeper layers of the biofilm. *Ex situ* chlorhexidine penetration into two-week old *in vivo* formed oral biofilms followed a similar dependence on the importance of the fast and slow relaxation elements as observed for *in vitro* formed biofilms. This study demonstrated that measured biofilm visco-elastic properties could quantitatively explain antimicrobial penetration into a biofilm.

Stress relaxation has been mathematically modeled, but never related back to physical processes. In Chapter 4, we suggested that the speed of stress relaxation for materials was based on their size. In **Chapter 5**, we determined that EPS did in fact relax quicker than bacterial cells after a 20% deformation of *Pseudomonas aeruginosa* biofilms. Using confocal laser scanning microscopy we visualized the

flow of EPS and bacteria that facilitate stress-relaxation. The stain intensity ratio of slime to cells was roughly five times higher for the slime producing parent strain than for the mutant strain. Time-characteristics of the flow coincided with relaxation-time-constants in the mathematical Maxwell model analysis of stress-relaxation, as used in Chapter 4.

Now with visual evidence of the movement of biofilm components combined with the mathematical relationship between antimicrobial penetration and stress relaxation, we closely examined the characteristic time constants of the different Maxwell elements describing stress relaxation of biofilms in an effort to move from arbitrary to established designations. In **Chapter 6**, the role of different matrix constituents on the visco-elastic response of biofilms was identified. Staphylococcal, streptococcal and pseudomonas biofilms were grown under different conditions yielding distinct matrix chemistries. Biofilms were subjected to mechanical deformation and stress-relaxation was monitored over time. A Maxwell model was used to fit the data. Maxwell elements were defined by a relaxation-time constant and their relative importance. Relaxation-time constants varied widely over the 104 biofilms included and were divided into seven ranges. Principal component analysis was carried out to eliminate related time constant ranges, yielding three principal components that could be related with the known matrix chemistries. The fastest relaxation component (< 3 s) was due to the presence of water, combined with the absence of bacteria, i.e. the heaviest masses in a biofilm. An intermediate component (3-70 s) was related to EPS in general, while a distinguishable role was assigned to

eDNA, which possessed a unique principal component with a time constant range (10-25 s) between the one for EPS constituents. This implied that eDNA modulated its interaction with other matrix constituents to control its contribution to visco-elastic relaxation under mechanical stress.

In this thesis, we have provided quantitative relationships between stress relaxation time constants and both biofilm structure-composition and antimicrobial penetration. In **Chapter 7,** an overview of the advances made since the 17$^{th}$ century in the qualitative understanding of the recalcitrance of biofilms toward antimicrobial penetration through advances in microscopic techniques was given. Limitations still associated with these microscopic techniques create the need to find a physical property that can relate to known biofilm structures. We proposed that visco-elastic relaxation under external stress relates to antimicrobial penetration regardless of growth conditions, single or multi-species biofilms, or antimicrobials used. Due to the work described in this thesis, visco-elastic relaxation has become the first physical property capable of describing antimicrobial penetration. Furthermore, key components of the biofilm matrix could be determined utilizing quantitative visco-elastic relaxation. Finally, the possibility of bioacoustics as a method for clinical biofilm control merging our quantitative results to the known enhancement of antimicrobial efficacy was explored.

Summarizing, in this thesis it is demonstrated that there is a connection between the underlying properties of initially adhering bacteria and the resulting properties of biofilms. Stress relaxation of biofilms could be described in three Maxwell elements and was

linked to their structural composition and antimicrobial penetration. Extracellular DNA had a distinguishable role in biofilm stress relaxation, making it a primary target for future research into bioacoustics-associated treatments. Throughout this thesis, relaxation time constants were used to distinguish between Maxwell elements and their associations to biofilm structure and antimicrobial penetration. These time constants could be converted to frequencies for bioacoustics as a useful tool against bacterial biofilms, however we have not tested the efficacy of these frequencies. A potentially successful biofilm treatment would combine antimicrobials with a mechanical frequency that would resonate the bacterial biofilm while leaving neighboring cells and tissues unaffected.