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Centrifugal compaction causes changes in the surface properties of bacterial cells. It has been shown previously that the surface properties of planktonic cells change with increasing centrifugal compaction. This study aimed to analyze the influences of centrifugal compaction and environmental conditions on the visco-elastic properties of oral biofilms. Biofilms were grown out of a layer of initially adhering streptococci, actinomyces or a combination of these. Different uni-axial deformations were induced on the biofilms and the load relaxations were measured over time. Linear-Regression-Analysis demonstrated that both the centrifugation coefficient for streptococci and induced deformation influenced the percentage relaxation. Centrifugal compaction significantly influenced relaxation only upon compression of the outermost 20% of the biofilm ($p < 0.05$), whereas biofilm composition became influential when 50% deformation was induced, invoking re-arrangement of the bacteria in deeper biofilm structures. In summary, the effects of centrifugal compaction of initially adhering, centrifuged bacteria extend to the visco-elastic properties of biofilms, indicating that the initial bacterial layer influences the structure of the entire biofilm.

Keywords: centrifugation; visco-elasticity; mechanical properties; dental plaque; biofilm structure

Introduction

Bacteria as single cells are vulnerable to minute changes in their natural environment and therefore group themselves into protective structures, known as biofilms. Biofilms were initially thought to be structurally homogenous, but it is now universally accepted that biofilms have a 3-dimensional structure with site-specific cohesive strengths, diffusion rates, and growth dynamics (Rochex et al. 2009). Dental plaque is an example of a complex biofilm consisting of over 600 bacterial species of both symbiotic and pathogenic bacteria (Marsh 2006). From its earliest point in development, dental plaque is a multi-species biofilm that promotes a metabolic and growth advantage for the participating organisms (Palmer et al. 2003). Multiple species coexist with each other and physically interact with selected other strains and species *via* specific adhesion molecules to cause co-aggregation (Ruhl et al. 2004; Riihinen et al. 2011). The co-aggregating bacteria form microcolonies and excrete extracellular polymeric substances (EPS) that provide structure, mechanical strength and resistance against chemotherapeutics to the biofilm (De Beer et al. 1994; Flemming and Wingender 2010). Effective protection against external removal forces, frequently occurring in the oral cavity (Shaw et al. 2004), requires both viscous and elastic properties of the biofilm (Klapper et al. 2002). The EPS matrix and the 3-dimensional structure have both been implicated in the visco-elastic properties of a biofilm and may vary significantly depending on the environmental conditions during growth (Williams and Bloebaum 2010).

There are currently different models for growing biofilms, although they focus on reproducing the environmental growth conditions rather than on the resulting properties of the biofilm. These models include, but are not limited to, cycles of feast and famine (Giertsen et al. 2011), use of a chemostat (Herles et al. 1994) and flow (Dunsmore et al. 2002). Zero (1995) best describes the complications in model development, saying that it is not possible to have a completely 'natural' model. Thus, whereas a completely natural *in vitro* model may be out of reach, it remains important to identify the environmental conditions that influence the *in vitro* properties of a biofilm.

An important parameter in this respect is possible bacterial cell surface damage due to centrifugal compaction. Most *in vitro* models involve harvesting of bacteria *via* centrifugation, while bacteria in their

natural environment have never experienced centrifugation. Centrifugal damage has been known to alter the cell surface properties of bacteria (Allan and Pearce 1979; Pembrey et al. 1999). The compaction of bacteria during centrifugation causes shear forces acting on bacterial surface structures that can be described by the so-called centrifugation coefficient, C (see Equation 1 below, Peterson et al. 2012). Initial deposition rates of *Staphylococcus aureus* ATCC 12600 to a glass surface decayed exponentially from 4217 to 1478 cm^{-2} s^{-1} with increasing centrifugation coefficients, while the proportions of staphylococci with a more negative zeta potential decreased. Moreover, controlling the centrifugation coefficient within narrow limits over a series of experiments yielded 43% smaller standard deviations in initial staphylococcal deposition rates than when using fixed centrifugation speeds (Peterson et al. 2012), in essence because the centrifugation coefficient accounts for compaction due to both the centrifugation speed and the shape of the centrifugation containers.

The aim of this study was to demonstrate and analyze the influence of environmental conditions such as induced deformation, the type and number of bacterial strains, the shear rate, and the influence of sedimentation as well as of centrifugal compaction on the visco-elastic properties of oral biofilms *in vitro*. An influence of centrifugation on the properties of a biofilm in which generations of bacteria have never experienced centrifugation, would severely impact the way microbiologists should regard *in vitro* experiments with biofilms.

Materials and methods

Bacterial strain, culturing and harvesting by centrifugation

S. oralis J22 and *A. naeslundii* T14V-J1 were precultured from blood agar plates into 10 ml of enriched Brain Heart Infusion medium (eBHI, 37.0 g l^{-1} BHI, 5.0 g l^{-1} yeast extract (OXOID, Basingstoke, England), 0.725 g l^{-1} L-cystiene-HCl, 0.0025 g l^{-1} hemin (Sigma, St Louis, MO, USA), and 0.002 g l^{-1} vitamin K_1 (Alfa Aesar GmbH, Karlsruhe, Germany)) and grown (24 h, 37°C) in aerobic and anaerobic (85% N_2, 10% H_2, 5% CO_2,) conditions, respectively. *S. oralis* (20 μl) or *A. naeslundii* (200 μl) were then passed into eBHI (200 ml) and allowed to grow for 17 h at 37°C. The bacterial cells were harvested by centrifugation at 1,380, 2,460 or 15,300g and washed (30 ml) before final suspension in 10 ml of buffer (50mM KCl, 2mM KH_2PO_4/K_2HPO_4, 1mM $CaCl_2$ [Merck, Darmstadt, Germany]). After each centrifugation cycle, the pellet dimensions and the number of bacteria in a pellet were measured for calculation of the centrifugation coefficient, C according to Equation 1 (Peterson et al. 2012)

$$C = 0.63 \frac{n_{bacteria} V_{bacterium}}{V_{pellet}} \qquad (1)$$

where $n_{bacteria}$ is the number of bacteria harvested in the pellet, $V_{bacterium}$ is the volume of a single bacterium and V_{pellet} is the volume of the pellet. The total volume centrifuged (200 $\pm$ 10 ml) and the temperature of the centrifuge during operation both affected the pellet dimensions, and therewith the value of C, impeding full control over the resulting C values by adjusting the centrifugation speed. The final suspension was sonicated (on ice, 10 s, 30 W; Vibra cell model 375, Sonics and Materials Inc., Newtown, CT, USA) to break up bacterial chains and aggregates after which the bacteria were diluted in buffer to a density of 3×10^8 ml^{-1} with the aid of a Bürker-Türk counting chamber.

Bacterial adhesion and biofilm growth in a parallel plate flow chamber

Salivary conditioning films were formed on cleaned microscope glass slides (76 $\times$ 26 mm, Braunschweig, Germany) from reconstituted human whole saliva prepared by dissolving lyophilized saliva from a pool of donors in buffer to a concentration of 1.5 mg ml^{-1} (Van der Mei et al. 2008). Glass slides, constituting the top and bottom of the parallel plate flow chamber (see below) were exposed to reconstituted saliva at 4°C for 17 h in a sterile Petri dish (10 ml per slide) in order to form a salivary conditioning film.

Next, a bacterial suspension was allowed to flow through a parallel plate flow chamber with dimensions 17.5 $\times$ 1.6 $\times$ 0.075 cm (Busscher and Van der Mei 2006) at one of the three different wall shear rates (7, 15 or 50 s^{-1}), for up to 150 min at 21°C. Adhesion took place on both the top and bottom plate of the flow chamber and sedimentation contributed to the bacterial mass transport on the bottom plate, but not on the top plate. Adhesion was monitored only on the bottom plate using phase contrast microscopy. Images were taken every minute, and adhering bacteria were enumerated using software based on MATLAB developed in-house. For single species biofilms, bacterial adhesion was stopped at a surface density of 2×10^6 cm^{-2}. When mixed species biofilms were to be obtained, *S. oralis* J22 was deposited first, followed by deposition of *A. naeslundii* T14V-J1 until equal surface densities (1×10^6 cm^{-2}) of both strains were obtained. Before perfusion of the flow chamber with *A. naeslundii*, a 15 min wash-out was performed at the experimental shear rate to remove non-adhering streptococci from the tubes and flow chamber. After initial deposition, the flow cell was washed with

sterile buffer (30 min) at the experimental shear rate and subsequently biofilms were allowed to grow at 37°C in a flow with 20% eBHI in buffer for 48 h. After incubation, the biofilms were submerged in sterile buffer to remove any non-adhered bacteria and to keep the biofilm hydrated prior to measurement. All experiments were carried out seven-fold with different bacterial cultures.

Low load compression testing

Biofilm thickness and compressive strength were measured using uni-axial compression on a low-load compression tester (LLCT) (Korstgens et al. 2001). During all LLCT measurements, biofilms were kept hydrated with buffer. Biofilm thickness was measured by first moving the plunger of the LLCT (diameter 2.5 mm) towards a clean, uncultured region of the glass slide until a touch load of 0.01 g and the plunger position was registered. Next, this procedure was repeated for a biofilm covered region of the glass slide and the difference in positions of the plunger in both cases taken as the thickness of the biofilm. For compression, the biofilms were then subjected to a quasi-instantaneous deformation of 10, 20, and 50% for 1 s and the induced deformation was subsequently held constant for 100 s by keeping the plunger in place, (Noble et al. 1990). For further calculations, deformation was expressed in terms of strain, ε, using

$$\varepsilon = \ln\left(1 + \frac{\Delta h}{h}\right) \qquad (2)$$

where Δh is the decrease in height and h is the undeformed height of the biofilm.

The stiffness of the biofilm was quantified as the slope of the stress *vs* the strain plot during initial deformation of the biofilm (Figure 1a). The deformation induced load in the biofilm and its relaxation were monitored over time (see also Figure 1a) and normalized over the cross-sectional area of the plunger to calculate the induced stress (σ). The percentage change in induced stress occurring within 100 s from its initial value was termed the percentage stress relaxation. At each induced deformation, three measurements were performed at three different locations within the same biofilm, yielding nine measurements per biofilm. In order to avoid mechanical interference from neighboring indentions, a space of 2.5 mm was taken in between two indentations.

Visco-elastic properties

Measured relaxation curves for each biofilm were modeled with a generalized Maxwell model
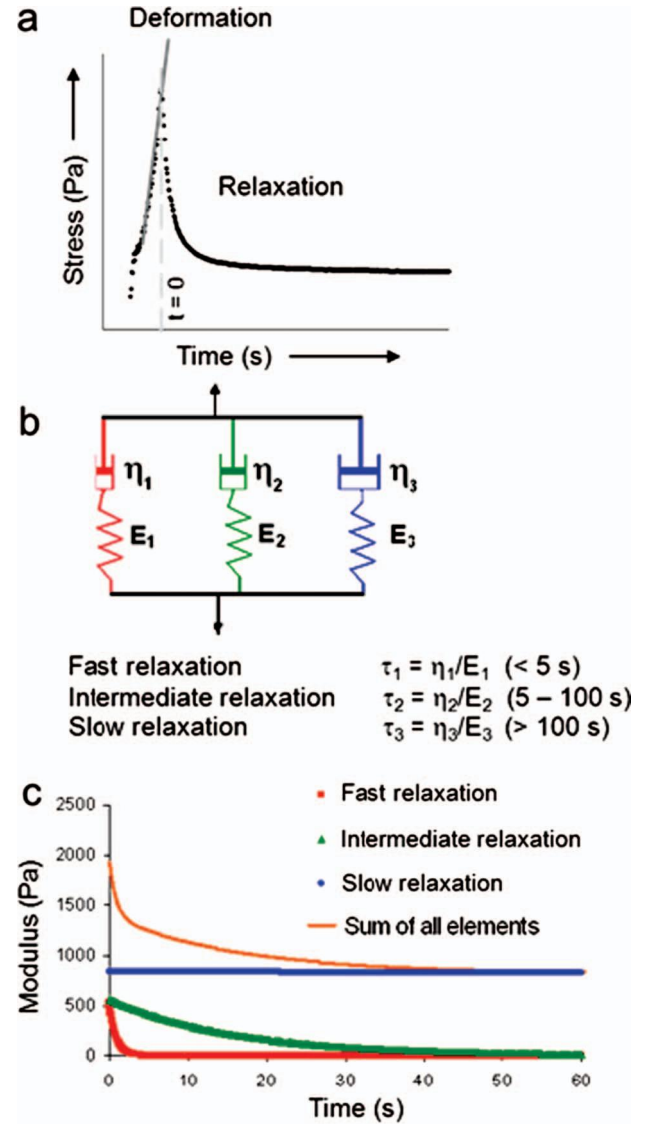
Figure 1. Data acquisition and analysis from Low Load Compression Testing. (a) Applied stress as a function of time. Biofilm stiffness can be derived from the linear increase in stress up to t = 0. Relaxation is initiated at t = 0. (b) Schematic presentation of a visco-elastic model, comprising three generalized Maxwell elements with a spring constant E_i, viscosity η_i, and characteristic decay time τ_i. (c) Separation of measured biofilm relaxation as a function of time, into three different Maxwell elements according to Figure 1b and Equation 3. The fit of the sum of the modeled elements fully corresponded with the measured data.

containing at most three elements (see Figure 1b) according to

$$E(t) = E_1 e^{-t/\tau_1} + E_2 e^{-t/\tau_2} + E_3 e^{-t/\tau_3} \qquad (3)$$

in which E(t) is the total stress exerted by the biofilm divided by the strain imposed (ie 0.1, 0.2 or 0.5). E(t)

was expressed as the sum of three Maxwell elements with a spring constant E_i, and characteristic decay time, τ_i (see also Figure 1b). The model fitting for E(t) and τ values of the three elements was done by minimizing the chi-squared value (Figure 1c) using the Solver tool in Microsoft Excel 2010. Fitting to three Maxwell elements yielded the lowest chi-squared values and increasing the number of Maxwell elements only yielded minor decreases in chi-squared values of <3%. Subsequently, the Maxwell elements were somewhat arbitrarily named fast, intermediate or slow based on their τ values, ie $\tau < 5$, $\tau = 5$–100, and $\tau > 100$ s respectively (see also Figure 1b). The relative importance of each element, based on the value of its spring constant E_i, was expressed as the proportion of its spring constant to the sum of all spring constants.

Statistical analysis

Data were compiled, and the visco-elastic properties of the biofilms were analyzed for significant influences of induced deformation, centrifugal compaction, type of bacteria used, sedimentation, and shear rate using a Mixed Linear Regression Analysis (MLRA, SPSS 16.0, see also Altman 1991–1999). The MLRA creates a linear regression analysis for each environmental condition, and normalizes the influences of each environmental condition based on the linear regression curves established. This eliminates the influences of co-factors in the analysis, and yields the relative importance of each environmental condition with respect to relaxation. Significant influences at p < 0.05 were isolated into cross-tabs, while non-significant influences were combined in the data representation. The significance between groups formed after separation of significant influences was calculated using a Student t-test (unequal number, equal variance), where the t variable and df (degrees of freedom) were calculated and referred to the Student distribution.

Results

During harvesting of bacteria, the centrifugation coefficient varied between 0.008 and 0.065 for *S. oralis* J22 and between 0.022 and 0.155 for *A. naeslundii* T14V-J1. The overall variation in biofilm thickness, stiffness and the percentage relaxation ranged from 25 to 327 μm, 24 to 228850 Pa and 6 to 100% respectively, irrespective of the environmental conditions: the type and number of bacterial strains present in the biofilm, shear rate, and the influence of sedimentation, as well as of centrifugal compaction. MLRA demonstrated that both the centrifugation coefficient for *S. oralis* and the induced deformation had a significant influence on the percentage relaxation after biofilm deformation (see Table 1).

Accordingly, the percentage stress relaxation for *S. oralis, A. naeslundii* and dual species biofilms was presented for the different induced deformations of the biofilms, separated for cultures centrifuged above and below the median centrifugation coefficient (0.038 for *S. oralis* J22 and 0.059 for *A. naeslundii* T14V-J1) in Table 2. Separation of the data above and below the median centrifugation coefficient could not be done for dual species biofilms since both strains demonstrated different centrifugal compaction. The percentage stress relaxation decreased in a similar way with increasing induced deformation for all biofilms, while it was significantly higher for the dual species biofilms at the highest induced deformation than for *S. oralis* biofilms (p < 0.05). Furthermore, centrifugation above the median centrifugation coefficient yielded a higher percentage stress relaxation for *S. oralis* biofilms than centrifugation below the median centrifugation coefficient at induced deformations of 10% and 20%.

Breakdown of the relaxation curve (see Figure 1a) into its three Maxwell elements (Figure 1b) yielded the contribution of each element as a function of time (Figure 1c). The relative importance of each Maxwell element at t = 0 was subsequently presented for the different induced deformations and (combinations of)

Table 1. Significant influences of the environmental conditions affecting the visco-elastic properties obtained by MLRA*.

Environmental condition	Percentage stress relaxation	Fast relaxation	Intermediate relaxation	Slow relaxation	Biofilm stiffness	Biofilm thickness
Induced deformation	**<0.001**	0.420	**<0.001**	**<0.001**	**<0.001**	0.123
Centrifugal compaction for *S. oralis* J22	**0.002**	**0.011**	0.777	**0.015**	**0.011**	0.496
Centrifugal compaction for *A. naeslundii* T14V-J1	0.297	0.580	**0.044**	0.241	0.254	0.240
Strain(s) used	0.133	0.993	0.154	0.234	0.065	0.411
Sedimentation	0.083	0.487	0.739	0.294	**0.035**	0.472
Shear rate	0.558	0.600	0.695	0.955	0.154	0.107

Note: *Biofilm stiffness and biofilm thickness were both co-factors in the analysis. Biofilm stiffness significantly altered the percentage relaxation *via* its fast element (p < 0.001), while biofilm thickness significantly influenced the fast and slow elements (p < 0.001). Conditions were considered significant for p < 0.05 (data in bold).

Table 2. Percentage stress relaxation for *S. oralis*, *A. naeslundii* and dual species biofilms as a function of induced deformation on the biofilms.

Induced deformation	*S. oralis* J22	*A. naeslundii* T14V-J1	Dual species
Average of all data			
10%	83.5 ± 3.6	82.4 ± 3.3	88.2 ± 2.6
20%	72.5 ± 4.0	75.2 ± 3.5	77.1 ± 3.7
50%	50.6 ± 4.7	55.5 ± 3.8	64.3 ± 4.2†
Centrifugal compaction above the median centrifugation coefficient			
10%	92.5 ± 2.2*	87.1 ± 2.9	–
20%	81.2 ± 3.7*	81.1 ± 3.7	–
50%	60.2 ± 4.7	60.1 ± 4.0	–
Centrifugal compaction below the median centrifugation coefficient			
10%	79.0 ± 3.6	84.0 ± 3.0	–
20%	68.5 ± 3.7	71.7 ± 3.5	–
50%	55.0 ± 4.5	60.3 ± 4.3	–

Note: *indicates significant ($p < 0.05$) differences of data above and below the median centrifugation coefficient, while † indicates significance between dual species and *S. oralis* biofilms. Data are presented regardless of centrifugal compaction and separated for cultures centrifuged above and below the median centrifugation coefficient (0.038 for *S. oralis* J22 and 0.059 for *A. naeslundii* T14V-J1). Separation of the data above and below the median centrifugation coefficient could not be done for dual species biofilms since both strains suffered different centrifugal compaction.
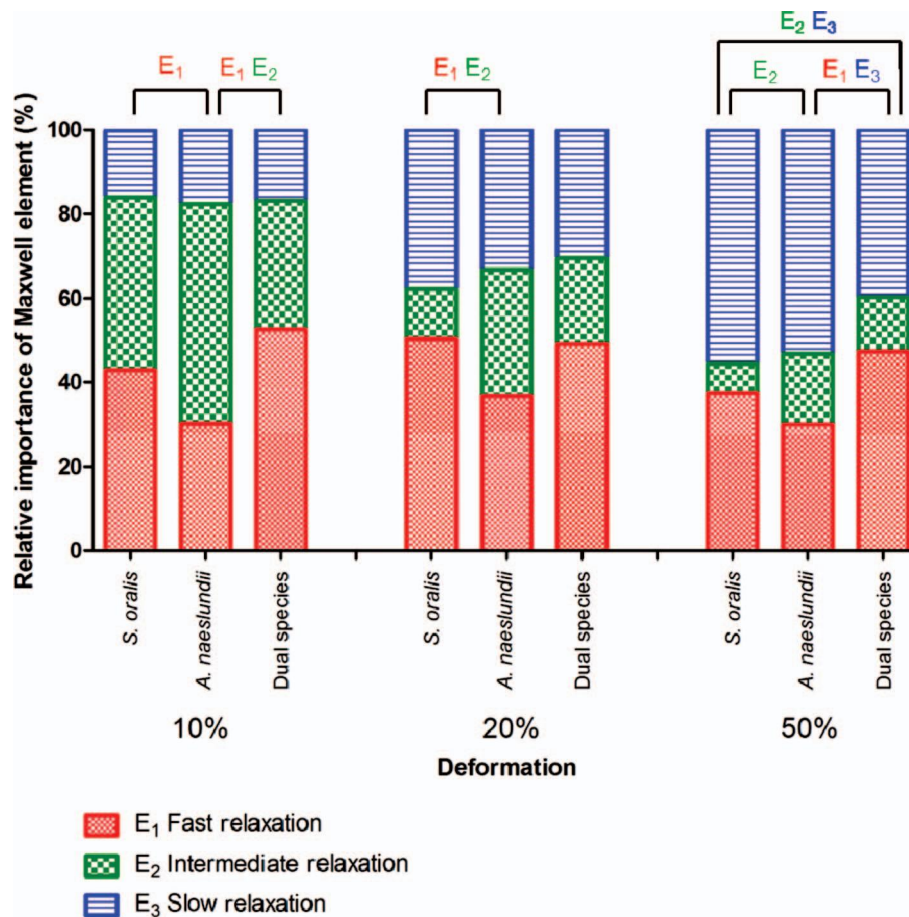

Figure 2. Dependence of the relative importance of the three Maxwell elements on deformation for the two single species and the dual species biofilm used in this study, neglecting possible centrifugation effects. Significant comparisons (Student t-test, $p < 0.05$) are indicated by the connecting bars with names of the Maxwell element(s) involved.

bacterial strain(s) (Figure 2) and separated in cases where the centrifugation coefficient was below or above the median (Figure 3). The relative importance

of fast relaxation was significantly ($p < 0.05$) smaller for *A. naeslundii* biofilms than for *S. oralis* biofilms at 10 and 20% induced deformations and for dual species
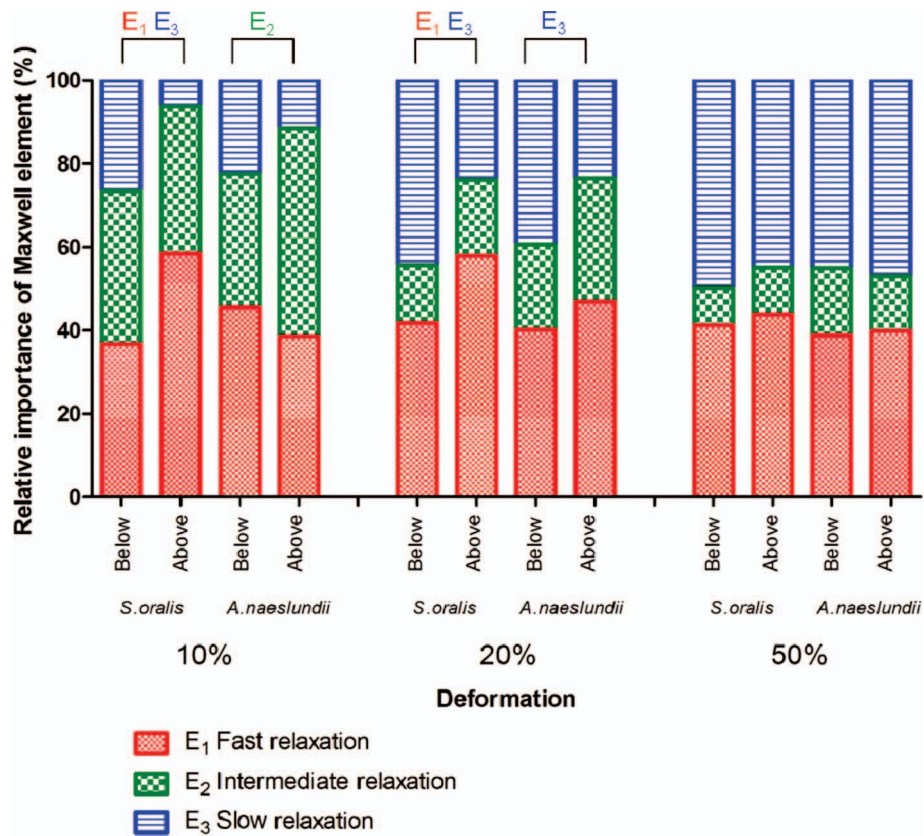
Figure 3.   Dependence of the relative importance of the three Maxwell elements on deformation for the two single species and the dual species biofilm used in this study, separated according to the centrifugation coefficient being below or above median. Significant comparisons (Student t-test, $p < 0.05$) are indicated by the connecting bars with the names of the Maxwell element(s) involved.

biofilms at 10 and 50% induced deformations. The relative importance of slow relaxation was smallest ($p < 0.05$) at 50% induced deformation for dual species biofilms as compared to both single species biofilms (Figure 2).

Centrifugal compaction had a significant influence on the relative importance of Maxwell elements at induced deformations of 20% or less, as demonstrated by the differences between the relative importance of Maxwell elements of biofilms formed from bacteria centrifuged above and below the median centrifugation coefficient (Figure 3). At 50% induced deformation, differences in the relative importance of the Maxwell elements of biofilms formed from bacteria centrifuged above and below median centrifugation coefficient disappeared.

Discussion

Bacteria in their natural environment have never been exposed to centrifugal compaction, and the potential effects of centrifugal damage are generally ignored.

Previously, it has been showed that centrifugal damage can alter the surface charge and initial adhesion of a staphylococcal strain (Peterson et al. 2012). Here it is demonstrated that centrifugal compaction influenced relaxation only upon compression of the outermost 20% of an oral biofilm, whereas the composition of the biofilm became influential with 50% deformation, invoking deeper biofilm structures. Thus centrifugal damage may extend to the visco-elastic properties of biofilms after growth, despite the fact that many generations of bacteria in a biofilm have never experienced centrifugation.

Biofilms have been described as visco-elastic fluids (Klapper et al. 2002), and accordingly the part of a deformed biofilm that flows out of the loaded region is expected to participate less in the relaxation process than the part of the biofilm directly underneath the plunger. Hence, a constant cross-sectional area is assumed in calculating the stress (σ) and generalized Maxwell model to fit the stress relaxation process. However, it is not entirely proven whether this approach holds, and whether a large strain model as

described for hyperviscoelastic materials (Holzapfel 1996) should not have been used. However, application of a large strain is presently impossible for deformed biofilms, as how much the biofilm bulges outwards as a function of relaxation time cannot be estimated. Nevertheless, the results of the current study remain valid on a qualitative, comparative level and allow meaningful conclusions with respect to centrifugal damage and their influence on biofilm structure and therewith on the visco-elastic properties of a biofilm.

Maxwell analysis of the visco-elastic response of biofilms upon induced deformations is usually confined to a mathematical presentation of results, but the processes underlying relaxation as occurring in a biofilm are unknown and seldom alluded to. Mathematical analysis allowed identification of three Maxwell elements in biofilm relaxation that were more or less arbitrarily named fast, intermediate or slow, based on their characteristic time constants. In a biofilm, the bacterial cells have the largest mass, which implies that their re-arrangement after an induced deformation of the biofilm may be associated with the slow relaxation element, ie τ_3. In contrast, water may be anticipated to flow out relatively fast through pores and channels in a deformed biofilm, and it was associated with the fast relaxation element, ie τ_1. The EPS produced by the organisms is still highly hydrated (Flemming 2011) but is more viscous than water and the outflow after an induced deformation is associated with the intermediate relaxation element τ_2 (see Figure 1b). Bacterial re-arrangement plays a larger role after 50% induced deformation (see Figures 2 and 3) than after deformations of 10% and 20%, while the role of a possible outflow of EPS decreases, likely due to extensive narrowing of the pores and channels in biofilms after 50% deformation. Narrowing of the pores and channels will affect possible outflow of water less than EPS, because water is less viscous than EPS.

It is difficult to understand at first glance how effects of centrifugation can extend beyond the level of the initially adhering organisms and influence the properties of a 48 h old biofilms. Biofilms grow from adhering single bacteria into microcolonies within 16 h (Guggenheim et al. 2001). These microcolonies form a foundation that supports the entire biofilm and therewith may profoundly impact its properties. Thus, any change to the foundation of a biofilm through centrifugal damage to the initially adhering bacteria will influence the properties of the biofilm structure, as demonstrated here for the visco-elasticity of different oral biofilms.

Bacteria in direct contact with the substratum surface were the only ones to experience centrifugation, but yet the effects of centrifugal compaction on stress relaxation were only observed for induced deformations of 10% and 20%, which mostly relate to the outermost part of the biofilm, while at 50% induced deformation, the effects of centrifugal compaction had disappeared (Figure 3). Previously, it has been shown that deformation of oral biofilms beyond 50% required an exponentially increasing loading force, probably because 50% deformation invokes deeper, more compact layers of bacteria (Paramonova et al. 2009). As a consequence, relaxation of these layers will involve bacterial re-arrangement much more than outflow of water or EPS. This is confirmed by the present data, since relaxation following 50% induced deformation showed a bacterial strain dependent response, ie different for spherical cocci than for rod-shaped actinomyces, including a combination of both.

In summary, the effects of bacterial cell surface damage due to centrifugal compaction can extend to the visco-elastic properties of biofilms after growth, despite the fact that many generations of bacteria in a biofilm have never experienced centrifugation. Centrifugal compaction influenced relaxation only upon compression of the outermost 20% of the biofilm, whereas the composition of the biofilm became influential upon inducing 50% deformation, invoking deeper biofilm structures. In conclusion, the structure of a biofilm is determined to a large extent by the layer of initially adhering bacteria that form a foundation that supports the biofilm growing on top and therewith influence the structure of the entire biofilm.

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