**Effects of biofilm heterogeneity on the apparent mechanical properties obtained by shear rheometry**

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**Abstract**

Rheometry is an experimental technique widely used to determine the mechanical properties of biofilms. However, it characterizes the bulk mechanical behavior of the whole biofilm. The effects of biofilm mechanical heterogeneity on rheometry measurements are not known. We used laboratory experiments and computer modeling to explore the effects of biofilm mechanical heterogeneity on the results obtained by rheometry. A synthetic biofilm with layered mechanical properties was studied, and a viscoelastic biofilm theory was employed using the Kelvin-Voigt model. Agar gels with different concentrations were used to prepare the layered, heterogenous biofilm, which was characterized for mechanical properties in shear mode with a rheometer. Both experiments and simulations indicated that the biofilm properties from rheometry were strongly biased by the weakest portion of the biofilm. The simulation results using linearly stratified mechanical properties from a previous study also showed that the weaker portions of the biofilm dominated the mechanical properties in creep tests. We note that the model can be used as a predictive tool to explore the mechanical behavior of complex biofilm structures beyond those accessible to experiments. Since most biofilms display some degree of mechanical heterogeneity, our results suggest caution should be used in the interpretation of rheometry data. It does not necessarily provide the “average” of the mechanical properties of the entire biofilm if the sample is vertically stratified.

**Keywords:** Biofilm, heterogeneous mechanical properties, rheometer, Kelvin-Voigt model, biofilm modeling

1. **Introduction**

Rheometry is commonly used to determine the mechanical properties of viscoelastic materials, such as biofilms. The simplicity of rheometry, and the ability to quickly obtain mechanical parameters for a range of biofilm samples, make it a popular choice among researchers. Biofilm samples are typically placed between two flat or conical plates, and the changes of strain and stress are measured. In biofilm research, rheometry has been used for a range of different types of studies, including creep tests (Gloag et al., 2018; Houari et al., 2008; Jones et al., 2011; Shaw et al., 2004; Towler et al., 2003), relaxation tests (Li et al., 2020; Vinogradov et al., 2004), and oscillatory tests (Brugnoni et al., 2014; Gloag et al., 2018; Lieleg et al., 2011; Rühs et al., 2014).

Rheometry is typically used for homogeneous materials, as it captures bulk rather than spatially varying properties. However, biofilms are known to have high spatial variability of mechanical properties (Boudarel et al., 2018; Li et al., 2021). For example, using microscale techniques, biofilms have been shown to be more compliant near the surface and more rigid near the base (Birjiniuk et al., 2014; Galy et al., 2012; Pavissich et al., 2021; Safari et al., 2015). The elastic compliance values in the same biofilm can spatially vary by up to three orders of magnitude (Galy et al., 2012). Consolidation occurs in the deeper biofilm, leading to a denser and stiffer biofilm interior (Laspidou & Rittmann, 2004). Chemical gradients of oxygen and nutrients can further stratify the local mechanical heterogeneity in the biofilm (Pavissich et al., 2021; P. S. Stewart & Franklin, 2008). In some studies, biofilms were found to have a stiffer center due to hydrodynamic effects (Karampatzakis et al., 2017; Pavissich et al., 2021). The internal voids and channels that are filled with fluid (Birjiniuk et al., 2014) increase the mass transport within the biofilm (P. S. Stewart, 2012), alter mechanical heterogeneity (Laspidou & Aravas, 2007) and potentially lead to detachment (Kim et al., 2020). Due to the heterogeneous nature of biofilms, rheometry results can vary dramatically, potentially leading to incorrect interpretations of biofilm properties and mechanical behavior.

Even though average mechanical properties are desired in some studies, the effect of the heterogeneous mechanical properties of biofilms on their assessment by shear rheometry is not clear. Such heterogeneity may result in the average mechanical properties in some cases, but not in others. Considering large variations of local mechanical properties, and assuming that these variations are often depth dependent (i.e., parallel to the attachment surface), we hypothesized that the mechanical properties determined by a shear rheometer of a stratified biofilm are dominated by the weakest portion of the sample, instead of providing the thickness-averaged properties of the entire biofilm.

The goals of this research were to explore the effects of biofilm mechanical heterogeneity on the results of rheometry analyses. To experimentally tests our hypothesis, we used synthetic stratified biofilms made from agar. Layered hydrogels using agar or alginate have been widely used to mimic heterogeneous living tissues such as biofilms (Fuentes-Caparrós, Ana M., et al., 2021; (Liou et al., 2021). Synthetic biofilms were used instead of real biofilms, because of the complexity of assessing a biofilm’s spatial distribution of mechanical properties, then assessing the same biofilm by rheometry. Synthetic biofilms offer homogenous and easily characterized mechanical properties and known property distributions. A two-dimensional (2D) Kelvin-Voigt model was also employed to simulate creep responses in rheometer measurements. The shear modulus and viscosity were obtained by calibrating Kelvin-Voigt model to experimental data. The results of this study can help biofilm researchers better understand the uncertainties and limitations of rheometry.

1. **Materials and Methods**

To explore the effects of mechanical heterogeneity on the results obtained by rheometry, a layered synthetic biofilm was prepared using agar solutions with defined concentrations. The layered synthetic biofilm was composed of two layers of equal thickness: a stiffer bottom layer and a softer top layer, using higher agar concentration and lower agar concentration, respectively. The mechanical parameters of each layer (stiff and soft agars) were independently determined using each pure (non-layered) agar biofilm using creep tests in a shear rheometer, followed by calibration of the Kelvin-Voigt model. The mechanical parameters of layered synthetic biofilm were determined following the same protocol.

**2.1 Preparation of synthetic biofilms**

Three agar synthetic biofilms were prepared: (1) a stiff biofilm with 1.5% weight-to-volume (w/v) of agar concentration, (2) a soft biofilm with 0.75% w/v of agar concentration, and (3) a layered biofilm with the stiff biofilm on the bottom and the soft biofilm on the top.

The following procedure was adapted from (Liou et al., 2021). To prepare the agar solution, agar powder was mixed with deionized water (DI) and heated in a microwave oven for 30-60 seconds until fully dissolved. Next, 4 mL of warm agar solution (1.5% w/v and 0.75% w/v) was added to a petri dish (diameter = 60mm) for the preparation of homogeneous agar biofilm. Meanwhile, 2 mL of warm agar solution with a concentration of 1.5% w/v was added in a separate petri dish for the layered biofilm preparation. The petri dishes were stored horizontally in a refrigerator at 4 °C for gelation. After gelation, 2 mL of warm agar solution with a concentration of 0.75% w/v was added on the top of the 1.5% w/v agar gel. All the petri dishes were placed horizontally position in a refrigerator until rheometer tests were carried out. The agar thickness was 4 mm for all synthetic biofilms. A schematic of agar gel preparation can be found in Fig. S1 in Supplementary Information (SI).

**2.2 Rheometry analysis**

After the agar gel fully solidified, the petri dish with the agar gel was transferred directly to a rheometer (Discovery HR-2 Hybrid; TA Instruments, IL). Each petri dish was fixed to the lower plate of the rheometer using modeling clay (see Fig. S2 in SI). The rheometer was used with a flat 25-mm diameter upper plate. The gap was set in the position where the normal force on the agar biofilm was around 0.4 N (Vinogradov et al., 2004).

Two types of rheological measurements were performed during this study. Stress sweep tests in shear were performed first to determine the linear viscoelastic region of the agar sample. The storage modulus (G’) and loss modulus (G”) were plotted as a function of stress. Fig. S3 shows the storage and loss moduli as functions of shear stress for all three types of agar biofilms. Triplicate measurements were performed using different locations of the agar sample. The linear viscoelastic region (LVR) for all three agar samples were determined and 10 Pa of stress was selected within this region for the next step of rheological measurement (Fig. S3).

To characterize the mechanical properties of the layered biofilm, creep tests were performed. A constant stress of 10 Pa was set during the creep test, based on the previous sweep tests. Five replicated measurements were performed on five agar samples for each condition.

**2.3** **Kelvin-Voigt model**

To quantitatively compare the effects of biofilm mechanical heterogeneity on the results obtained by rheometer a standard Kelvin-Voigt model was used to determine the viscoelastic properties of the biofilm, including shear modulus () and viscosity (). Kelvin-Voigt model is a commonly used model to describe the creep response of viscoelastic materials (Ferry, 1980). In this study, Kelvin-Voigt model was used to (1) calibrate the mechanical parameters using experiments, and (2) in simulations to model the viscoelastic behavior.

The Kelvin-Voigt model describes the viscoelastic behavior as a combination of a spring and a dashpot in parallel, resulting in the following stress/strain relationship:

where is strain [-], is shear stress [Pa], is shear modulus [Pa], and is viscosity [Pa·s].

Model calibration was performed with a trust-region refractive algorithm (Coleman & Li, 1996) using MATLAB (Mathworks, Natick, Massachusetts, USA, [www.mathworks.com](http://www.mathworks.com)).

**2.4 Finite element modeling**

The biofilm was modeled as a viscoelastic material to further validate our hypothesis and study the impact of mechanical heterogeneity. A 2D plane-strain continuum model was implemented using COMSOL Multiphysics (COMSOL v5.6, Comsol Inc., Burlington, MA) using a finite element method. The built-in solid mechanics module was used. The experimentally calibrated Kelvin-Voigt model was used as the constitutive equation for biofilm viscoelastic behavior (see Section 2.3).

2.4.1 Layered biofilm model and linearly stratified biofilm model

Two types of heterogeneous biofilms were simulated. First, we simulated the layered biofilm using the experimental data from Section 2.1 for the stiff, soft, and layered biofilm as modeling inputs with a simplified geometry. Second, we used the model to explore the impact of having a continuous linear variation in the mechanical properties, rather than two discrete layers. The stratified mechanical parameters measured from our previous paper (Pavissich et al., 2021) were used as modeling inputs with a simplified geometry, as explained below.

2.4.2 Model geometry

As shown in Fig. 1a, the synthetic biofilm was simplified as a rectangular 2D geometry, with a dimension of 8 mm × 2 mm (length × depth) for the layered synthetic biofilm case (Fig. 1a) and a dimension of 0.8 mm × 0.075 mm (length × depth) for the continuous linear variation biofilm case. Although the rheometer used in this study has circular plates that rotate in their plane, the deformation is greatest in the outer portions of the plates, furthest from the center. This can be approximated to a rectangle deforming linearly, as shown in Fig. 1a.

2.4.3 Model implementation



For simulations with the experimental data from this study, the simulated biofilm consisted of two layers. The upper layer had a smaller shear modulus and viscosity (1 mm < Y < 2 mm); the lower layer had a larger shear modulus and viscosity (0< Y<1 mm) (Fig. 1b and Table 1). Shear moduli and viscosities for both layers were obtained from the experiments described in Section 2.1 and 2.2. For simulations with the linearly stratified mechanical properties, we used the data from a previous study (Pavissich et al., 2021). In that study, magnetic tweezers were used to determine the spatial distribution of the elastic modulus in *Pseudomonas aeruginosa* biofilms. The elastic modulus was found to vary with depth in the biofilm. The experimental data for the growth under 0.1 mL/h flow rate, DO saturation, and no Ca2+ addition was considered. Since elastic modulus was the only parameter measured in that study, we assumed that the viscosity distribution was the same as elastic modulus in our model. A linear equation was used to fit the experimental data in this simulation (Fig. S4 &1b).

The Poisson ratio (υ) was set as 0.45, since the value between 0.4-0.5 is usually applied in biofilm studies (Böl et al., 2009; Picioreanu et al., 2018; Safari et al., 2015; Taherzadeh et al., 2010). For both shear modulus () and viscosity (), a same slope-intercept form was used in our model, where the slope is -1.02×10-3 and the y-intercept is 2.08. The y value in this form is the biofilm depth [m]. A detailed list of modeling parameter values is provided in Table 1.

For comparison, the layered mechanical distribution was also obtained from the same study by fitting the previous data (Pavissich et al., 2021) with a stepwise function. The fitted functions used for biofilm mechanical properties can be found in Fig. S4 in SI.

2.4.4 Boundary conditions and numerical implementations

Interactions between the rheometer upper plate and biofilm top layer are complex and uncertain. In our model, we assumed that the upper boundary was a “loading boundary” with a constant applied shear stress (=10 Pa for synthetic layered biofilm and =10-3 Pa for linearly stratified biofilm) in the x direction to mimic the applied shear stress in rheometer tests. For the synthetic layered biofilm simulations, the loading was set as the same value in the rheometer test. Free boundary conditions were used for the left and right boundaries. To avoid computational errors, the loading on the upper wall was ramped up from 0 to within 0.01s. A visco-elastic simulation was performed for 5 s. The inertia forces in the finite element model were neglected resulting in the quasi-steady approximation (i.e., the time dependence is a result of material viscosity). A maximum mesh size of 1×10-4 m was applied in this study using quadratic elements. The number of elements is 6250 for agar biofilm simulations and 2428 for stratified biofilm simulations.

1. **Results and Discussion**

In this study, we first used a layered synthetic (agar) biofilm to quantitatively explore the impacts of mechanical heterogeneity on mechanical property measurements using a rheometer. The mechanical properties of biofilm in each layer were experimentally determined using a rheometer. The mechanical response of layered agar biofilm was then measured using a rheometer and simulated using a 2D finite element model. Second, we used the material data from our previous experimental study (Pavissich et al., 2021) to develop a linearly stratified biofilm system to predict the mechanical behavior.

**3.1 Experimental measurements**

Stress sweep tests were performed to identify the LVR for all three types of agar biofilms, and the rheometer analyses used in this study were all within the LVR. While large deformations may capture the nonlinear region of viscoelasticity (Charlton et al., 2019; Jana et al., 2020; Li et al., 2020, 2021; Xia et al., 2022), we chose to avoid this mechanical behavior to simplify our analyses.

The viscoelastic moduli from stress sweep test (Fig. S5) were compared between the layered agar biofilm and the pure agar biofilms (stiff and soft). For viscoelastic materials, the storage modulus (G’) represents the elastic behavior while loss modulus (G”) represents viscous behavior. All three types of agar biofilms exhibited higher G’ compared to G”, indicating a more elastic behavior of the agar biofilms than viscous behavior. The small value of standard deviation shows that synthetic biofilms made with agar are mechanically more homogeneous than typical real biofilms. Both G’ and G” for layered biofilm were closer to the values of soft biofilm, which were one order of magnitude smaller than stiff biofilm. The results demonstrated that the layered biofilm had apparent mechanical properties, as measured by rheometry, more similar to the soft biofilm.

To be consistent with the model set-up in the following study, we also performed creep tests using three types of agar biofilms (stiff, soft, and layered) and use those to calibrate Kelvin model. Five replicated measurements were performed for each type of agar biofilms. Under a constant stress of 10 Pa, shear strain over time was plotted. Figure 2 shows the results. The data showed viscous behavior after 3s, i.e., the rate of change of strain over time did not change. All creep curves indicated typical viscoelastic behavior. Similar to the results of sweep tests, the creep curve of layered agar biofilm was closer to the curve of soft agar biofilm.

The Kelvin-Voigt model was used to calibrate averaged creep curves. After calibration with Kelvin-Voigt model, the elastic modulus (G) and viscosity () were obtained. For the stiff agar biofilm, the G and were 1.8×104 ± 0.5×104 Pa and 1.5×104 ± 0.6×104 Pa·s, respectively. For the soft agar biofilm, the G and were 4.4×103 ± 0.7×103 Pa and 3.4×103 ± 1.1×103 Pa·s, respectively. The values obtained for the layered agar biofilm were 5.7×103 ± 0.2×103 Pa and 5.6×103 ± 0.7×103 Pa·s, similar to those for the soft agar biofilm.

Agar has been widely used to prepare homogeneous artificial biofilms (Dalsgaard et al., 1995; Jouenne et al., 1994; Phoenix et al., 2008). The values of G and from previous rheometry tests have a wide range of values, e.g., G=2×10-2 – 2×105 Pa, =1×101 – 5×108 Pa·s (Böl et al., 2012; Shaw et al., 2004). The parameters from our study were within the range of reported data.

The layered agar biofilm was made of stiff agar on the bottom half (2 mm thickness) and soft agar on the top half (2 mm thickness). However, the measured properties of the layered biofilm were much closer to that of the soft agar. This indicates that the mechanical behavior of a heterogeneous stratified biofilm, with variation stratified parallel to the attachment surface, is strongly biased towards the mechanically weaker material. Since the spatial distribution of the mechanical properties are usually unknown, the rheometry results would suggest the entire biofilm is soft, while in reality it is half soft and half stiff. While the measured mechanical properties would be suitable to predict mechanical behavior under conditions similar to the test (a shear test parallel to the mechanical property stratification), it would not be accurate for other types of conditions, such as deformation perpendicular to the stratification. Also, mechanical properties are often related to other biofilm properties, such as diffusivity and permeability (Kovach et al., 2020; Picioreanu et al., 2018; Wagner et al., 2017). These properties are highly impacted by a dense layer. Therefore, the mechanical property determined from rheometry would give a mistaken indication of other behaviors.

**3.2 Simulated creep responses of the layered biofilm**

To further explore the effects of mechanical heterogeneity on mechanical property measurement, we developed a 2D finite element model to simulate the deformation of layered biofilm in rheometer creep test. Fig. 1b shows the distribution of elastic modulus over biofilm depth. The average experimental elastic modulus G and viscosity of individual layers from Section 3.1 were used as the input for the model. The 2D geometry of the layered biofilm was a simplification of the biofilm sample in rheometer test. We simplified the 3D biofilm tested under a rotating disk to a 2D rectangular biofilm with a shear stress on the top surface.

The deformation in the x direction was plotted in Fig. 3. From Fig. 3b, we can see that the weaker, upper part of the biofilm contributed most to the biofilm deformation.

Averaged strain over the upper boundary of the biofilm (Fig. 1a) was exported and the strain over time relationship was plotted in Fig. 4. For comparison, we also simulated the biofilm deformation with the homogeneous soft biofilm (G=4.4×103 Pa, =3.4×103 Pa·s) and the homogenous stiff biofilm (G=1.8×104 Pa, =1.5×104 Pa·s) (Fig. 4).

In Fig. 4, the creep curve of the layered biofilm is much closer to that of the soft biofilm, rather than that of the stiff one. Thus, the simulated layered biofilm exhibited a mechanical behavior similar to the soft biofilm. The simulated results are consistent with the experimental results, indicating that the biofilm mechanical properties are dominated by the weaker part of the stratified biofilms. The modeling results in Fig. 4. also show a better prediction for the stiffer biofilm compared to the results of the softer one. This can be expected as the soft biofilm exhibits larger strains and more nonlinear behavior. A nonlinear model as in Li et al., 2021 could improve the response. As discussed in the previous section, the mechanical behavior of a layered biofilm was mainly contributed by the weaker area of the biofilm, in both experimental and modeling results.

**3.3 Simulated creep response of a linearly stratified biofilm and layered biofilm**

To explore the effects of mechanical heterogeneity for more realistic conditions, we used modeling to assess a hypothetical biofilm with linearly stratified mechanical properties. A layered biofilm with the same averaged properties were also modeled for comparison as the experimental data indicate a layered like profile (see Fig. S4 in SI). The parameters from this model were compared to a homogeneous biofilm with the mean values of the linear distribution. The linear distribution of shear modulus G was based on experimental data from a previous study (Pavissich et al., 2021). The viscosity was assumed to have the same relationship with biofilm depth as shear modulus G. The input shear modulus G had a range of 0.04 - 2.04 Pa. The averaged G of 1.06 Pa was computed over the biofilm domain and then implemented into the homogeneous model for comparison. Similarly, the averaged of 1.06 Pa·s was used in the homogeneous model.

We compared the strain over time for the linearly stratified biofilm, the layered biofilm and homogeneous biofilm with the averaged properties (Fig. 5). It is noted that, compared to the previous agar biofilm, the real biofilm was much softer. However, it still was in a reasonable range, considering previously reported biofilm data (G=2×10-2 - 2×105 Pa, =1×101 - 5×108 Pa·s from (Böl et al., 2012; Shaw et al., 2004). The strain for all three types of biofilms increased for the first 3 s and then stabilized. The stabilized strain in layered biofilm (ε = 2×10-3) was three times higher than the homogeneous biofilm (ε = 7×10-4), but smaller than the linearly stratified biofilm (ε = 0.01). The significantly higher strain in the linear stratified biofilm compared to the layered and homogeneous biofilms indicates importance and sensitivity of material data on response of a biofilm. It also shows the importance of simulations in case the experimental data is missing. From the synthetic biofilm study, it is reasonable to assume that the linear stratified result, or the layered model are more realistic given the input material data (see Fig. S4 in SI). Moreover, it is reasonable to infer that material properties and resulting behavior of stiff portion of the stratified biofilm cannot be captured properly in a standard rheometry test.

The model was also shown to be a useful tool for mechanical parameter prediction for heterogeneous biofilms. The differences between average mechanical properties in theory and the mechanical properties in practice indicate that the rheometer results should be used with caution. The results may not reflect the mechanical properties of each layer of the biofilm. Rather, they may be biased of the weakest part of the biofilm.

**3.4 Implications**

Rheometry is a standard technique that is widely used to determine biofilm mechanical properties. It is a convenient tool for understanding of the biofilm response under mechanical changes. The data from rheometry are usually assumed to reflect the properties of the entire biofilm, and treated as an intrinsic bulk biofilm property, independent of the amount of biofilm (Billings et al., 2015; Tallawi et al., 2017). However, in previous studies, biofilms have been shown to be heterogeneous materials with high spatial variations in mechanical properties (Galy et al., 2012; Pavissich et al., 2021). Given the mechanical heterogeneity of biofilms, our goal was to understand whether mechanical properties determined using rheometry can be assumed to reflect the properties of all layers. This can be important, as sometimes mechanical properties are used as a surrogate for other properties, such as biofilm diffusivity or permeability. For these properties, a thin dense layer could have a significant impact.

We used both experimental and modeling methods to study the mechanical properties of the heterogeneous biofilms and compare to the data from homogeneous biofilms. A heterogeneous biofilm with a weak top and a stiff base was considered. Both experimental and modeling results demonstrated that mechanical responses of a heterogeneous biofilm are dominated by the weaker part. Then we simulated a heterogeneous biofilm with linear and stepwise stratification using experimental data from a previous study. The strain and displacement were compared with the results of homogeneous biofilm. A weaker response was shown in the linearly stratified biofilm, indicating that the properties of the stiff basal layer was not fully reflected in the result. Thus, rheometry may provide misleading results for biofilms with horizontally stratified mechanical properties, when tested under shear.

Previous studies have revealed that biofilms are often composed of a stiffer base layer and a weaker outer layer (Galy et al., 2012; Paramonova et al., 2009; Pavissich et al., 2021). Biofilms increase their stiffness when the porosity decreases at higher flow velocities (Blauert et al., 2015; Laspidou & Aravas, 2007; Picioreanu et al., 2018). Biofilm consolidation is also a possible reason that biofilms stratify mechanically (Laspidou & Rittmann, 2004). Hydrodynamic conditions can promote biofilm mechanical heterogeneity (Paramonova et al., 2009), or, conversely, reduce biofilm mechanical heterogeneity (Pavissich et al., 2021). With a similar mechanical property distribution over biofilm depth, our results indicated that the data from rheometry measurements do not reflect the aggregate biofilm properties. For stratified systems, the measured properties are more similar to the weakest part of the biofilm. The impact of the stiff basal layer on biofilm properties can thus be underestimated. Of course, if the properties of the weakest portion of the biofilm are of interest, then rheometry would be suitable. This could be the case where rheometry is used to assess the viscoelastic response of the top biofilm, which is affected the biofilm’s interactions with fluid flows.

Although not studied here, a related concern exists with the gap between the upper rheometer plate and the biofilm. This gap is often selected based on the biofilms average thickness. However, for rough biofilms this can result in the upper rheometer plate only partially contacting the biofilm, with a fluid layer in areas where the biofilm is thinner than the average thickness. Based on our results, such a layer could also have a significant impact on the extant mechanical properties. This suggests that shear rheometry may also not be suitable for very rough biofilms.

Biofilm mechanical properties are increasingly studied as a critical indicator for the physical-chemical-biological interactions (Kim et al., 2020; Kundukad et al., 2016; Paramonova et al., 2009; Pavlovsky et al., 2013; E. J. Stewart et al., 2015; Tallawi et al., 2017). The underestimation of stiff layers in the biofilm can lead to misinterpretation in other aspects of the biofilm studies. However, the heterogenous nature of biofilms over microscopic spatial scales present challenges in terms of characterization of many other properties of biofilms including, chemistry, structure, microorganism distribution and physiology. Heterogeneity in itself is difficult to quantify and a horizontally stratified biofilm will behave differently than a biofilm that has an equal distribution of variability in terms of volume, but distributed as vertical “pockets" throughout the biofilm.

For mechanical properties, a particular pattern of heterogeneity may manifest itself differently in terms of measurements depending on the nature of the test. For example, an axial compression test may be less influenced by layers forming slip-planes but be more reflective of the properties of the surface layer of the biofilm that interact with the probe. Therefore, rheometry measurements in biofilm studies should be interpreted with caution for biofilms with large spatial variation of mechanical properties.

The experimentally calibrated model we developed can be used as a tool to study the biofilm heterogeneity with complex structures. The model can also be used to predict biofilm deformation in situations where the distribution of biofilm mechanical properties is known, but measurement of biofilm deformation is not possible.

**4. Conclusions**

In this study, we used both experimental and modeling method to test our hypothesis: that the measured mechanical properties using rheometry is not the average for the entire biofilm, especially for highly heterogeneous biofilms where the properties vary with depth. The measured mechanical properties are closer to the property of the weakest region of biofilm. The contribution of biofilm stiff basal layer can be underestimated using rheometry.

Rheometry is likely to yield a more representative result when heterogeneity is not significant, when the stratification is perpendicular to the shear, or when weak layers are very thin. Significant errors can be generated otherwise. Biofilm heterogeneity should be considered when using shear rheometry to assess the mechanical properties. If there is a stratification parallel to the plates, other methods, such as in-situ microrheology, may more accurately characterize the mechanical properties.

In cases where biofilm axial heterogeneity is not significant, rheometry can still a suitable method, given the low cost and short required time. Also, rheometers are increasingly used for biofilm axial tests, and these can complement shear tests by inferring the significance of the axial variability in mechanical properties.

**Acknowledgement**

The rheology tests were performed with instruments at the Notre Dame’s ND Energy Materials Characterization Facility. The computational resources to run COMSOL Multiphysics were provided by the Notre Dame Center for Research Computing. Partial funding was provided by NSF project #1805406 (RN) and the CEST Bayer Fellowship (ML).

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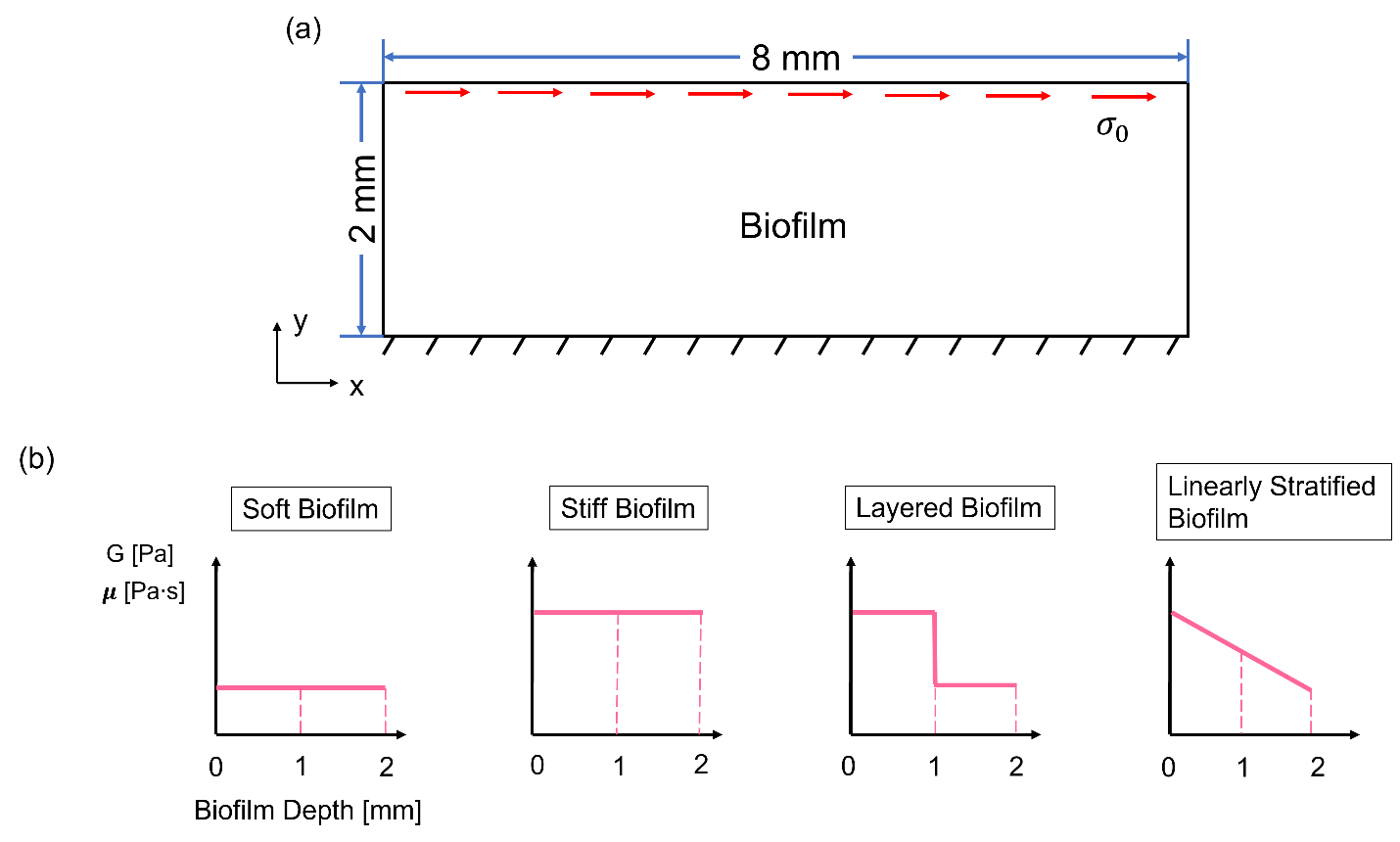
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**Table 1.** Model Parameters

|  |  |  |  |
| --- | --- | --- | --- |
| **Name** | **Value** | **Unit** | **Description** |
| h | 2 | mm | Biofilm height |
| w | 8 | mm | Biofilm width |
| υ | 0.45 | **-** | Poisson’s ratio |
|  | 10 | Pa | Applied shear stress |
|  | 1.8×104 (0<Y<1mm) | Pa | Top shear modulus for Layered Biofilm |
| 4.4×103 (1mm<Y<2mm) | Pa | Bottom shear modulus for Layered Biofilm |
|  | 1.5×104 (0<Y<1mm) | Pa·s | Top viscosity for Layered Biofilm |
| 3.4×103 (1mm<Y<2mm) | Pa·s | Bottom viscosity for Layered Biofilm |



**Figure 1.** Model geometry and viscoelastic properties used in the layered synthetic biofilm simulations. a) Two-dimensional biofilm geometry description. The red arrows indicate the location of the applied stress . (b) Schematic showing the viscoelastic properties distribution of three synthetic biofilms - soft, stiff, and layered, and linearly stratified biofilm. Y-axis value is the biofilm depth of 2 mm in (a).



**Figure 2**. Rheometer creep curves for soft agar biofilm (blue square), layered agar biofilm (green circle), and stiff biofilm (pink diamond). The creep tests were under performed a constant stress of 10 Pa. The error bars represent standard deviations from five measurements.

Chart

Description automatically generated

**Figure 3**. Simulated results of layered biofilm for t=5 s. (a) Figure showing deformation u (mm) in the x direction for entire biofilm, and (b) deformation u (mm) as a function of biofilm depth at the center of the biofilm top, as indicated by the red arrow in (a).



**Figure 4**. Creep curves (strain over time) of biofilm from modeling. The applied stress for both the experiment and model was 10 Pa. The markers indicate averaged strain of the upper biofilm boundary over time in rheometer tests. The solid lines indicated simulated strain over time for soft, stiff, and layered biofilms. The error bars represent standard deviations from five measurements.

Chart, diagram

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**Figure 5**. Simulated results for linear stratified biofilm, layered biofilm, and homogeneous biofilm. Strain over time for the biofilms under the shear stress of 10-3 Pa.