Microfluidic Device for the Evaluation of Biofilm Removal under Shear Stress

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ABSTRACT

Biofilms are a relevant problem in the medical field and many other industries. Biofilms can lead to proliferation of pathogens, loss of life, equipment failure, and loss in productivity. Biofilms also have increased resistance to antibiotics and are harder to remove than planktonic bacteria. Motile bacteria behave differently than the immotile bacteria that compose biofilms, and so studies that focus specifically on biofilms are necessary.

The experiments consisted of growing biofilms *Pseudomonas aeruginosa*, subjecting them to stress in the presence of a removal agent, and capturing images of the biofilms. A microfluidic device allowed for the use of existing microscope setups to image the biofilms, which allows for broad application of the device. The microfluidic device also mimicked the scale at which bacteria operate. Pressure was applied at increasing magnitude to find the threshold at which the biofilm began to be removed. The images were then analyzed to determine the amount of biofilm removal that occurred.

Polydimethylsiloxane (PDMS) microfluidic devices were used to evaluate treatment methods in conjunction with shear stress. The experiments that were conducted show the differences in the effect of shear stress with two removal agents on a biofilm. The pressures at which phosphate buffered saline (PBS) and sodium dodecyl sulfate (SDS) removed biofilms were statistically different. SDS required a lower pressure threshold to remove the biofilm than PBS. The percentage of biofilm that was removed was also statistically different between the PBS and SDS. At their respective removal pressures and in the same time span, SDS removed more of the biofilm than PBS.
The biofilms were characterized using confocal imaging, which creates a 3D reconstruction of the biofilm. The biofilms were 2.5 μm thick and had one to two layers of bacteria. The biofilms in the microfluidic device have an average radius of 28.3±11.5 μm after overnight growth.

The shear stress applied to the biofilm was modeled using COMSOL Multiphysics® software. The model includes a profile of the shear stress in the fluid as a function of position in the working fluid. Pressure-driven flow rates and biofilm size were varied in the model. The location of the maximum shear correlates with the portion of the biofilm that faces and extends into the fluid flow. The model shows, as expected, that increasing biofilm size decreases shear stress and increasing flow rate increases the shear stress.

The described device has been used to successfully compare chemical treatment methods while applying a shear stress to the biofilms.
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1.0 INTRODUCTION

Biofilms are a collection of adherent bacterial cells inside of a complex extracellular matrix, which improves the proliferation of the pathogens by modifying the local environment [1]. The characteristics of a mature biofilm make the pathogens more difficult to remove and the infections that result more serious. The extracellular matrix inhibits the transport of chemicals in the biofilm, which causes a higher antibiotic resistance in biofilms than planktonic bacteria [2,3]. Planktonic bacteria are individual bacterial cells that are mobile and can swim in their environment. Furthermore, the extracellular matrix acts as a protective barrier for the cells, which reduces the effect of physical stresses on the bacteria. Bacteria in biofilms behave differently than their planktonic counterparts, which creates the need to study how antibiotics and other removal agents affect mature biofilms.

In the medical field, biofilms that grow on medical devices lead to hospital associated infections [4]. In 2002, it is estimated that there were 1.7 million hospital-associated infections, which led to 99,000 deaths [5], many of which are thought to be caused by bacteria biofilms [6]. These infections cause an estimated $37.5-$45 billion in direct costs for inpatient services [7]. The costs stem from the prevalence and severity of the infections that are associated with biofilms. The cost of life and the billions spent in care make prevention of infectious biofilms imperative.

*Pseudomonas aeruginosa* (*P. aeruginosa*) is a medically relevant bacteria that causes many healthcare associated infections. The Center for Disease Control (CDC) and Prevention reports that 51,000 health care associated infections are caused by *P.
*P. aeruginosa* and that 13% of them are multidrug-resistant [8]. Furthermore, *P. aeruginosa* is known to form strong biofilms in patients with compromised immune systems [9]. The extracellular matrices in these strong biofilms increase drug resistance and proliferation of *P. aeruginosa*. These biofilms are tough to remove and cause chronic illnesses. *P. aeruginosa* is therefore a strong candidate for the study of biofilm removal.

Outside of the medical field, in industries like food processing and paper production, biofilms can form on various equipment. These biofilms can lead to persistence of pathogens, biofouling, and inoperability of the equipment [10,11]. The contamination with pathogens can lead to dangerous disease transmission through poor food handling [10]. The proliferation of pathogens can directly lead to increased problems for healthcare. Furthermore, biofilms can also clog and damage equipment by forming thick and corrosive slimes on equipment [11]. The clogging and damages lead to losses in productivity and a waste of resources. Therefore, the removal of biofilms from equipment has broad implications across many fields.

Many industries require the removal of biofilms, but there are few commercially available devices and no government guidelines for evaluating the removal of established biofilms from medical devices, which are hard to image [12]. Susceptibility testing of treatment methods on planktonic bacteria is often not a direct indicator of the treatment’s efficacy on a biofilm [13]. Planktonic bacteria behave differently than immobilized bacteria in biofilms. Therefore, there is a need for methods of evaluating the efficacy of antibiotic susceptibility and bacterial removal agents on mature biofilms.

The objective of this study is to create a means to evaluate the effectiveness of biofilm removal methods combined with an applied shear stress. The microfluidic device
needs to be able to grow biofilms, have a means to remove the mature biofilm, and accurately assess the efficacy of the removal of the biofilm. These functional specifications can be validated through fluorescence and confocal microscopy. The device should show the difference between chemical treatment methods as a means to compare the treatments. The microfluidic device has the added benefit of operating at the scale of bacteria. A microfluidic device is chosen because it is easily integrated into existing setups to allow for broad application in a variety of studies while minimizing costs.
2.0 CRITICAL LITERATURE REVIEW

There are many studies on the properties of biofilms, including their formation, proliferation, and removal. The effects of chemical treatment of biofilms have been explored, as well as the effects of mechanical forces from fluid shear on biofilms. However, there are no studies that explore the effects of chemical treatment in conjunction with dynamic conditions in the removal of a mature biofilm.

2.1 STUDIES ON BIOFILM ADHESION

Figure 1: Photograph of a 1 liter glass CDC Biofilm Reactor [17].
The Center for Biofilm Engineering (CBE) at Montana State University created the CDC Biofilm Reactor, which grows and quantifies biofilms of various species [14]. The CDC Biofilm Reactor was an improvement on the rotating disk reactor [15]. The American Society for Testing and Materials has approved E2562, which was a method for the quantification of *P. aeruginosa* biofilm growth using the CDC Biofilm Reactor [16]. The reactor was a vessel with rods that held the biofilms extended into the fluid (Figure 1). Shear was applied to the biofilm by a stir bar. The reactor was able to grow multiple biofilms under high shear stress, and individually harvest biofilms for testing. The CDC Biofilm Reactor was also used to grow biofilms for *in vivo* implantation in animal studies [17].

*Figure 2: Schematic of a Drip Flow Reactor [18]*
Like the CDC Biofilm Reactor the Drip Flow Reactor (DFR) was also made by the CBE. Unlike the CBC, the DFR grew *P. aeruginosa* biofilms under low shear stress conditions by dripping bacteria onto a slide while the device was held at an angle to cause gravimetric flow [18]. The methods that were used for the DFR were accepted by the ATSM with the designation E2647.

Park *et al.* (2011) used a polydimethylsiloxane (PDMS) based microfluidic device to grow biofilms and assess the biofilm formation under a fluid shear. In the device, biofilms were formed by flowing bacteria into the device at varying shear stresses. Biofilm growth in microfluidic devices was affected by the shear stress applied by the fluid containing the bacteria being deposited on the surface [19]. The study concluded that the time required to form a biofilm decreased as the velocity increased, until there was a sufficient shear such that the dissociation of the bacteria from the surface outweighed the increase in number of bacteria that reach the surface. They found that any shear stress applied in the device above 0.17 dyn/cm² (0.00017 mbar) would remove more bacteria than the flow added. This study was successful in establishing the mechanics of biofilm adhesion in fluid flow.

An interesting study was conducted by Wang and Libera (2013) which showed that biofilm formation can be inhibited by patterning the surface of a device. They took scanning electron micrographs (SEM) of patterned surfaces, which showed that when the size of the patches was smaller than 1 µm the bacteria had trouble adhering to the surface [20]. They argued that when the *Staphylococcus aureus* tried to adhere to the surface, they were bent and curved by the pattern, which created a morphology that was
energetically unfavorable for adhesion [20]. These SEM images showed that the patterned surfaces prevented the adhesions and proliferations by showing groups of bacteria in biofilms on unpattern surfaces while there was a noticeable decrease in bacteria on the patterned surfaces. Their result indicated that biofilm formation can be reduced simply by affecting the morphology of the bacteria.

In a biofilm reactor, Liu et al. (2001) observed that biofilms were able to respond metabolically and physically change under shear stress [21]. In the reactor, the changes in shear stress affected the anabolism and catabolism rate as well as the density and size of the biofilm. However, this setup only showed a macroscopic effect on biofilms because the reactor volume was 4 L, which was much larger than the scale of bacteria. They were able to show that biofilm characteristics changed depending on the shear stress applied. As shear stress increased, the biofilms became smoother and denser [21]. The denser biofilm correlates to the finding of the DFR biofilm reactor, where biofilms grown under lower shear stress were less tolerant to treatment methods [18]. Shear stress not only affects the rate of formation, but also the properties of the formed biofilm.

Foncesa et al. used a microtiter plate assay to evaluate biofilm adhesion under antibiotic treatment [22]. Orbital shaking applied a shear force to create dynamic conditions for the assessment of biofilm adhesion. The shear stress mimics the conditions that biofilms form under. Their experiments treated the planktonic bacteria and observed the adhesion and formation of the biofilm. They showed that under dynamic conditions, the antibiotic was more effective at preventing biofilm formation. While, this study successfully indicates that the combination of shear stress and a chemical treatment could
affect biofilm formation more than each one does individually, they do not explore biofilm removal.

The studies that focus on biofilm adhesion all neglected to address the resistance to chemical and physical stress that a mature biofilm has. These studies focused on the characteristics of planktonic bacteria as they are forming a biofilm, which may not be indicative of an established biofilm. Furthermore, chemical treatment in conjunction with shear stress could provide greater effects than just a chemical treatment.

2.2 STUDIES ON BIOFILM REMOVAL
Figure 3: A) The tilt table that created shear during biofilm formation. B) Cutaway view of the device showing the pins sitting in the channels of the incubation tray C) Top plate of the device [23].

Ceri et al. (1999) used the Calgary Biofilm Device (CBD) to test the susceptibility of biofilms to antibiotics (Figure 3). This device grows biofilms in a 96 well plate, which are then tested and evaluated through microbiology and quantitative scanning electron microscopy [23]. The CBD can further be combined with Phenotypic MicroArray to assess metabolic activity through the use of a dye [13]. They observed that antibiotic concentrations necessary to remove biofilms were 100 to 1,000 times higher than concentrations to remove their planktonic counterparts [23]. However, the CBD only grows biofilms in a monolayer and cannot evaluate biofilms grown in multiple layers. Furthermore, the CBD does not combine chemical treatment with application of a shear. The CBD was successful in demonstrating the differences between the removal of bacteria in biofilms and planktonic bacteria.

Annular reactors are another method of evaluating biofilm removal, which can be monitored by using laser-based Focused Beam Reflectance Measurements [24]. Choi et al. (2003) attempted to establish which detachment process dominates the removal of a biofilm subjected to fluid flow. The use of the reactors successfully determined that most of the biofilm removal due to shear stress was done through erosion, which is the transfer of small particles from the biofilm into the bulk fluid [24]. This result indicates that at steady state, gradual removal of the biofilm should be expected. While the annular reactors evaluated biofilm removal under shear stress, they were not used to evaluate the efficacy of a chemical treatment.
The biofilms grown by the CBD and DRF were used to evaluate biofilm removal [16,18]. Once the biofilms were grown in both devices, they were removed from the device and tested. The biofilms that were formed under higher shear stress were more resilient to treatment methods [18]. These results may be explained by the work done by Liu et al. (2001), where biofilms formed under higher shear stress were denser than biofilms formed under low shear stress [21].

The studies conducted by Ceri et al. and Choi et al. addressed chemical treatment and shear stress, respectively. By first growing a biofilm, and then testing the properties of the biofilm, they ensured that they were testing the biofilm and not planktonic bacteria. Moreover, the studies indicated that there is a difference between planktonic bacteria and bacteria in biofilms. However, their studies did not combine chemical and mechanical treatment.

2.3 IMPORTANT CONCEPTS AND TECHNIQUES

2.3.1 Chemotaxis as a Means to Guide Biofilm Formation

Chemotaxis can be used to target biofilm formation at controlled locations. Chemotaxis is the ability for bacteria to move in response to chemical stimuli. Motile bacteria have been known to travel towards organic nutrients since a series of studies conducted in the 19th century [25]. Creating a point source of nutrients guides the bacteria through chemotaxis towards the nutrients. Upon reaching the nutrients, the bacteria aggregate and proliferate around the source. Thus, by creating point sources of nutrients, the growth of biofilms can be targeted to only occur at those sources.
2.3.2 Fluorescent Microscopes

A fluorescent microscope uses an ultraviolet light to induce fluorescence of a labeled specimen. Bacteria that are induced to produce a fluorescent protein emit a specific wavelength of light that can be filtered. The filtered light creates an image that shows the proliferation of the bacterial species of interest. The fluorescence can also be an indicator of bacterial health, as bacterial species can ‘kick out’ the gene coding for the fluorescent protein when their survival is in question. The intensity of the light also correlates to the amount of bacteria in an area. The images have good contrast and clarity, which is useful in the quantification of bacterial coverage.

Most fluorescent microscopes are also able to take brightfield images. Brightfield images are taken using visible light that is transmitted through the specimen. It is a simple microscopy technique that offers high contrast between specimens with a bright background.

2.3.3 Confocal Microscopes

A confocal microscope is an excellent tool for the characterization of cells on a plane. Unlike traditional fluorescent microscopes, confocal microscopes improve the quality of the image by eliminating out-of-focus light through the use of spatial filtering techniques. Serial images at varying heights are made possible by reducing the background, which normally blurs the image. The serial images can be combined to create a 3D recreation of the subject. Therefore confocal microscopy is becoming an increasingly popular technique to produce high quality images of biological specimens [26]. The produced images accurately show the depth of the sample.
2.3.4 COMSOL Multiphysics®

COMSOL Multiphysics® (COMSOL) is a modeling software that can analyze many physical phenomena. In fluidics, it can calculate pressure, velocity, and shear in a model. To calculate those parameters the model is given a geometry, boundary conditions, initial conditions, and a set of guiding equations. For example, the laminar flow module can take the properties of water and the geometry of a pipe to find the numerical solutions to the Navier-Stokes equations. This solution provides insight to the fluid dynamics in that pipe. The modules in COMSOL can model heat, mass, and momentum transfer. The models can also provide information about the effects of varying parameters.

2.3.5 Welch’s t-test as a Means for Significance Testing

The Welch’s t-test, also known as the unequal variances t-test, tests the hypothesis that two samples have the same means. On the other hand, the Student’s t-test tests the same hypothesis, but assumes the variances are equal. When the null hypothesis is rejected by the result of the Welch’s t-test, the means are statistically different. The Welch’s t-test uses the normalized variance of the two samples instead of an assumed variance of the overall population for the statistical analysis. The Welch’s t-test therefore better represents the sample data. The Welch’s t-test performs as well as the Student’s t-test when the variances of the two samples are the same, and does better when the variances are different.
3.0 EXPERIMENTAL

3.1 FABRICATION OF THE MICROFLUIDIC DEVICE

3.1.1 Master Wafer Design and Fabrication

The master wafer was fabricated by Nil Tandogan through a previously defined lithography process [27]. First, poly (methyl methacrylate) (PMMA) resist was spun on a silicon wafer and baked. The thickness is confirmed with an optical profilometer. The constrictions were marked in the PMMA with an electron-beam lithography. The wafer was then developed in isopropanol and methyl isobutyl ketone. Chromium metal was sputtered onto the wafer and hot acetone lift-off left only the constrictions remaining on the wafer. The positive photoresist, S1827, was then spun on the wafer and baked. The food chamber and main channel mask were aligned and exposed to UV light. The features were then developed in deionized water and AZ400K developer, which completes the wafer. The steps of the wafer fabrication are shown in Figure 4(a-g).
The design of the wafer consists of a main channel, seven food chambers, and constrictions that connect the main channel to the food chambers (Figure 5). The food chambers and main channel were 100 µm wide and 2.5 µm high. The height of the main channel restricts the biofilm height. The main channel and the food chambers had the same height to reduce the number of steps needed for the fabrication of the wafer. The main channel is 4 mm long and the food chamber is 1 mm long. The constrictions are 700 nm high, 20 µm long, and vary in width from 1 to 2 µm. The separation of the constrictions and therefore biofilms allows the flow in the main channel to return to...
normal before reaching the next biofilm. Each wafer was designed to make eight PDMS devices.

![3D rendering of the PDMS device, including the main channel, food chamber, and the constrictions before the access holes are drilled.](image)

**Figure 5: 3D rendering of the PDMS device, including the main channel, food chamber, and the constrictions before the access holes are drilled.**

### 3.1.2 PDMS Device Fabrication

PDMS devices were made from the master wafer. The wafer was surrounded by an aluminum foil ‘boat’, and cleaned with an airbrush before pouring uncured PDMS into the wafer. The base and curing agent for the PDMS was mixed in a 5:1 (w/w) ratio (Sylgard 184 Silicone Elastomer Kit, Dow Corning) and poured so that it formed a 1 cm thick layer on the wafer. The wafer with PDMS was then placed in a vacuum chamber to degas, removing the bubbles from the PDMS. The PDMS was then cured in an oven at 70 °C for one hour. After curing, the PDMS was separated from the wafer and cut into individual devices. The access holes to the main channel and the food chambers were then drilled with a sharpened blunt tip 14 gauge needle (Small Parts). The holes drilled with the 14 gauge needle fit the 15 gauge needles (Small Parts) that were used later.
Oxygen plasma (Anatech SP-100 Plasma System) at 100 W, 0.4 Torr for 5 sec permanently bonded the imprinted side of the PDMS to a microscope cover slip (Fisher Scientific, microscope cover glass, 0.17–0.25 mm thick). The fabrication steps are outlined in Figure 4(h-i). The coverslip allows for imaging under a microscope while acting as the wall for the microfluidic channels. The bonding step completes the fabrication of the PDMS microfluidic device (Figure 6).

Figure 6: Photo of PDMS Device

3.2 EXPERIMENTAL PROCEDURE

3.2.1 Growth of the Biofilm

A day prior to preparing the device, *P. aeruginosa* was inoculated in 6 mL of Lysogeny broth (LB) and incubated overnight at 37 °C. The strain of *P. aeruginosa* used was PAO1. LB is a nutrient rich medium for bacterial growth. The access holes to the food channels of the device were filled with LB using a 1 mL syringe and a 25 gauge needle. The LB was then forced into food channels using a 15 gauge needle and 3 mL syringe. The LB was pushed until it reached the main channel, which was validated visually. Once all of the food channels were filled, the main channel was filled with
Phosphate Buffered Saline (PBS, 100 mM), which was a buffered solution that has no nutrients. Like filling the food chambers, the access holes to the main channel were filled with PBS and a syringe pushes the PBS from one access hole to the other. Once the main channel was filled, the main channel was inoculated with the previously cultured *P. aeruginosa*, which was diluted at 1:1 (v/v) ratio with LB. The inoculation consisted of dripping in a drop of the diluted *P. aeruginosa* in both main channel access holes with a syringe. The device was then placed in a culture plate with some wet laboratory wipes. The plate was sealed with laboratory film and incubated overnight at 37 oC. Figure 7a and Figure 7b show how the biofilms form in the main channel.

### 3.2.2 Biofilm Removal Experiments

![Flow diagram of the experiment](image)

**Figure 7**: Flow diagram of the experiment. a) Inoculated bacteria moved from the access hole towards the food chambers by chemotaxis, but were trapped by the constriction. b) The bacteria proliferated and formed a biofilm around the constriction. c) Fluid was flowed through the main channel. d) Eventually, the biofilm broke apart due to the fluid flow and chemical treatment.
After the biofilms formed in the device during overnight incubation, 40x brightfield and fluorescence images were taken of the biofilms that formed in the device. A pressure driven microfluidic flow control system (Fluigent MFCS) connected to an in-house air line, provided constant pressure. The main channel access hole was filled with the test fluid using a 25 gauge needle and syringe. A 15 gauge needle with some tubing was used to connect the main channel to the Fluigent. Pressure driven flow was then applied to the device, as shown in Figure 7c. After 10 minutes, the flow was stopped, and images were taken of the biofilms. The pressure was then adjusted and applied for another 10 minutes. This process of taking images and adjusting the applied pressure was repeated until biofilms showed signs of removal (Figure 7d). Biofilm removal was indicated by a change in the biofilm observed in the brightfield image and a similar change in fluorescence of the biofilm. The change was a decrease in the amount of bacteria in the biofilm after a flow was applied, as shown in Figure 7d. Further analysis on the biofilm removal was completed later as outlined in Section 3.3.3. The applied pressure was varied from 50 to 700 mbar.

The experiments compared the removal capabilities of PBS and Sodium dodecyl sulfate (SDS). SDS was chosen because it is an anionic surfactant. SDS is used in soaps and is therefore a good candidate for biofilm removal.

3.3 CHARACTERIZATION OF THE BIOFILM

3.3.1 Fluorescence Microscopy Procedure

The microscope used for imaging was a Zeiss Axiovision A2. Zeiss Axiovision (version 4.8.2.0) imaging software was the interface for an AxioCam MRm CCD camera.
The camera used took black and white images. Fluorescent images were therefore white and grey for anything that fluoresced and black for areas that did not. The 40x images centered around the biofilm near each constriction. To remain consistent, the image was adjusted until the constriction was in focus.

3.3.2 Confocal Microscopy of Mature Biofilms Specifications

Confocal microscopy was conducted on the biofilms in the devices. The microscope used was Zeiss LSM 700. The devices are bonded to thin glass cover slips, which were well suited for confocal microscopy. The cyan fluorescent protein labeled *P. aeruginosa* emitted blue light that was well detected in the 485 nm region using the filter set for DAPI [28]. Unlike nuclei stains, the cyan fluorescent protein is located in the cytoplasm of the cells. Thus, the whole cell is illuminated by the fluorescence. Images were taken at 63x magnification. Z-stacks were taken to produce a 3D image with 100 nm between each slice. The Z-Stack spans include the entire biofilm depth and a buffer area above and below the biofilm. The microscope was adjusted so that it was just out of focus below the biofilm and the images were taken until the microscope was out of focus above the biofilm. The height of the stack was between 4 and 5 µm, which includes the biofilm and the buffer depth. The 3D images were then reconstructed in ImageJ.

3.3.3 ImageJ Image Analysis

The removal of the biofilm was evaluated on ImageJ. The fluorescence image of when the biofilm showed signs of removal was compared to the fluorescence image of the biofilm at the start. The boundary of the biofilm was adjusted until the biofilm area included the bacteria up to the grey that outlined the outmost bacteria. Any dark areas around the outline were not considered part of the biofilm. The colors of the resulting
image were inverted because ImageJ naturally analyzes for the area that is not selected. Inverting the colors of the image allows ImageJ to analyze for the area of the selected biofilm. A space around the biofilm was selected and measured for total area (including biofilm and empty space) and area fraction that was the biofilm. The area of the biofilm was then found from the total area of the selection and the fraction that was biofilm. The area of the biofilm that showed signs of removal was divided by the original biofilm area to find the area of the biofilm that remained.

The confocal Z-stacks were reconstructed as a 3D projection in ImageJ. The contrast was adjusted to remove the background. The 3D projection was made using the mean brightness setting. The 3D model can be rotated to view the biofilm at multiple angles. Orthogonal views were also made to show cross sectional slices of the biofilm.

3.3.4 COMSOL Multiphysics® Model Construction

The COMSOL version used was COMSOL Multiphysics® 4.4. The model was created with the laminar flow module. The model was specified for static conditions, which means that the model was not time dependent. The model was created as a 2D model where the height of the device was neglected. The model focused on the main channel because there should not have been flow into the constriction and the food chambers. The main channel was modeled as 100 µm wide and 15,000 µm long. Two assumptions made were that the fluid is water and the biofilm was a solid semicircle with a radius of 30 µm. The biofilms were placed where the constrictions are, which was 3,000 µm from the wall for the first constriction and 3,000 µm between each constriction with each constriction on alternating sides of the main channel. The left wall was set to a pressure of 278 mbar and the right wall was set as the outlet at 0 mbar. The flow was
pressure driven. The mesh size for the model was normal. A shear stress plot was created by plotting the shear rate multiplied by the viscosity of the fluid. To find the shear stress dependence, the inlet pressure and biofilm radius were varied. Biofilm radii tested ranged from 10 \( \mu \text{m} \) to 70 \( \mu \text{m} \) in increments of 10 \( \mu \text{m} \). The inlet pressures tested ranged from 100 mbar to 700 mbar in increments of 100 mbar and included 278 mbar.
4.0 RESULTS AND DISCUSSION

4.1 CHARACTERIZATION OF THE BIOFILM RESULTS
4.1.1 Biofilm Size and Distribution

![Image](image.png)

Figure 8: a) fluorescence image of biofilm before PBS treatment, b) fluorescence image of biofilm after PBS treatment, c) fluorescence image of biofilm before SDS treatment, d) fluorescence image of biofilm after SDS treatment, e) threshold adjusted image of biofilm before PBS treatment, f) threshold adjusted image of biofilm after PBS treatment, g) threshold adjusted image of biofilm before SDS treatment, and h) threshold adjusted image of biofilm after SDS treatment.

The image analysis included only the area inside the red box, which selects only the area around the biofilm and excludes planktonic bacteria and the bacteria in the food channels (Figure 8). Figure 8(a-d) the threshold was adjusted, which created in Figure 8(e-h) respectively. In threshold-adjusted images, the black area included only the area that is bright enough be considered a component of the biofilm. Only the selections that are outlined in the images were included, which excludes areas that are clearly not a part of the biofilm. The biofilm area was calculated for each of the threshold-adjusted images.

The average initial radius of each biofilm was 28.3±11.5 μm. The calculation was conducted by assuming the biofilm was a semicircle and using the area measured in the
ImageJ analysis. The measurement justifies the assumption in the COMSOL model that the radius of the biofilm was 30 μm during the pressure variation calculations.
Figure 9: 40x brightfield and fluorescence images of each biofilm from left to right
In the devices, the biofilm size was not dependent on the size of the position of the biofilm in the main channel Figure 9. Furthermore, the biofilm size within one device was consistent.

4.1.2 3D Confocal Model Shows Biofilms are one to two Layers Thick

Figure 10: Confocal images: a) 3D Projection, b) yz cross section and c) xy cross section. The cross sections were taken at the respective yellow lines. The white arrows indicate areas where bacteria are on top of each other.
The images in Figure 10 show that the biofilms were one to two bacteria thick. When observing the 3D stack from a 45° angle (not shown), the bacteria were clearly on top of each other in some sections and monolayer in others. Another indication of two layers was to notice that there are areas of brighter bacteria that covered the darker bacteria beneath them. The cross section Figure 10b and Figure 10c further validated that the biofilm is one to two bacteria thick. The biofilm thickness was about 2.5 µm, which included the bacteria and their extracellular matrix. The thickness was limited by the height of the channel, which is also 2.5 µm.

4.2 COMSOL Multiphysics® MODEL RESULTS

4.2.1 Stability of the Fluid Flow

The shear stress plots from the COMSOL model showed the shear profile in the device as a function of position. The flow was pressure driven, where the pressure was applied from the left. In both Figure 11e and Figure 13e, after the flow is interrupted by a biofilm, the flow returned to fully developed flow before reaching the next biofilm. Both Figure 11e and Figure 13e had the same biofilm geometry. The difference between Figure 11e and Figure 13e was the pressure applied (700 mbar vs. 300 mbar), but both have fully developed flow before each biofilm. Therefore, the model showed that there was sufficient distance between the biofilms for the flow to return to steady flow after the disturbance from the previous biofilm at the operating pressure of the device. The shear applied to each biofilm in the device was the same regardless of the position of the biofilm.
4.2.2 Effect of Inlet Pressure on Shear Stress at Constant Biofilm Size

The effect of various inlet pressures was predicted with the assumption of biofilms with 30 µm radii. Figure 11(a-d), which was an expanded image plot around the central biofilm, shows that the maximum shear was achieved at the boundary that extends into the fluid flow in the direction of the fluid flow. Also, the shear stress on the biofilm near the wall of the channel and on the portion of the biofilm facing away from the direction of flow was much less than the forward facing portion of the biofilm. The removal of the biofilms was, therefore expected near the areas of higher shear stress.

![Figure 11: Shear stress in the main channel with biofilm radii of 30. a) Center biofilm with 100 mbar inlet pressure. b) Center biofilm with 300 mbar inlet pressure. c) Center biofilm with 500 mbar inlet pressure. d) Center biofilm with 700 mbar inlet pressure. e) Main channel with 700 mbar inlet pressure. The main channel contains seven locations where biofilms can form in a single experiment. The color scale is set such that the upper end is maximum shear applied by the 300 mbar inlet pressure.](image)

Notably, Figure 11 had a scale that caped at around the maximum shear applied to the biofilm with an inlet pressure of 300 mbar, which meant the differences in colors are exaggerated in Figure 11(c&d). The shear in the bulk fluid was mostly on the lower end of the scale (green to blue). The shear only approached the maximum in the boundary
layer near the biofilm, which would have been difficult to visualize with a scale that spans the whole range. Therefore, high shear forces were localized only to the boundary that extends into the fluid flow in the direction of the fluid flow.

The maximum shear stress applied to the biofilm with respect to the pressure applied is plotted in Figure 12. The equation of the best fit linear trend line is given by Eqn. 1. The trend line in figure 12 had a $R^2$ value of 0.999.

$$\tau = 0.0212 \cdot P - 0.801$$ \hspace{1cm} (Eqn. 1)

Where:

$\tau = $ Maximum shear stress in the main channel (mbar)

$P = $ Pressure applied to the main channel (mbar)

![Figure 12: Plot of maximum shear stress observed in the main channel as a function of pressure applied to the device. The trend line was the linear trend of maximum shear stress on the biofilm as function of the applied pressure given by $\tau = 0.0212 \cdot P - 0.801$. The trend line had a $R^2$ value of 0.999.](image)
4.2.3 Effect of Biofilm Size on Shear Stress at Constant Inlet Pressure

The relative location of the shear stress applied to the biofilm remains the same regardless of the size of the biofilm at 278 mbar. As seen in Figure 13, the biofilm always received the maximum stress at the boundary that faces and extends into the fluid flow. Therefore, the region where the biofilm began to be removed was expected to be the same regardless of the size of the biofilm.

Figure 13: Plots of shear stress in the main channel around the central biofilm at an applied pressure of 278 mbar and biofilm radii of a) 10 µm, b) 30 µm, c) 50 µm and d) 70 µm. e) flow between two biofilms with a radius of 30 µm at 278 mbar.

Figure 14 shows that there was a linear relationship between shear and biofilm radius size at 278 mbar. The trend line equation is given by Eqn. 2.

\[ \tau = -0.00625 \cdot r + 5.25 \]  \hspace{1cm} \text{(Eqn. 2)}

Where:

\( \tau \) = Maximum shear stress in the main channel (mbar)

\( r \) = Radius of the biofilm (µm)
The linear relationship between the points tested was not strong. The trend line in Figure 14 had an $R^2$ value of 0.714. Testing more points or with a finer mesh size could improve the model and the trend. However, the change in biofilm radius had a lower effect than inlet pressure on the shear stress, which is made apparent by comparing Eqn. 1 and Eqn. 2. Therefore, the shear was assumed to be constant despite the changes in biofilm size because the effect of the inlet pressure on the maximum shear stress would outweigh the effect of changing biofilm size.

Figure 14: Plot of maximum shear stress observed in the main channel as a function of the biofilm radii. The trend line was the linear trend of maximum shear stress on the biofilm as function of the biofilm radius given by $\tau = -0.00625 \cdot r + 5.25$. The trend line had a $R^2$ value of 0.741.

With an inlet pressure of 278 mbar and a biofilm radius of 30 \( \mu \)m, the maximum shear stress in the main channel was 5.02 mbar (Eqn 1 & Eqn. 2).
4.3 BIOFILM REMOVAL COMPARISON BETWEEN PBS AND SDS

4.3.1 Biofilm Growth and Characterization of Biofilm Size

![Comparison of biofilm growth](image)

**Figure 15:** 40x a) brightfield and b) fluorescence images of the main channel at the start of a PBS experiment.

![Comparison of biofilm growth](image)

**Figure 16:** 40x a) brightfield and b) fluorescence images of the main channel from PBS experiment after 100 mbar of pressure for ten minutes.

As seen in Figures 15 and Figure 16, biofilm removal was observed in the portion of the biofilm that faced the flow and extended into the fluid, in agreement with the COMSOL model. The removal is indicated by a decreased density of bacteria in the biofilm surrounding the constriction in both the brightfield and fluorescence images. The experiment was stopped when the biofilm began to be removed because the biofilm has
already been weakened at that point, and further experiments on that biofilm would not be comparable. Of the biofilms tested, signs of removal were observed in 21 biofilms exposed to PBS and 19 biofilms exposed to SDS. Biofilms that did not show significant removal were not included in the data analysis. The areas before and after treatment were then compared to find the percentage of biofilm remaining after treatment.

4.3.2 Pressure Required for Biofilm Removal

The Welch's t-test revealed that the difference in pressure that was required to remove the biofilm between PBS and SDS was statistically significant (p=0.011). The average pressure of removal under PBS and SDS were 278±175 mbar and 167±60 mbar, respectively. Therefore, SDS allowed for the biofilm to be removed at a significantly lower pressure than PBS did. SDS performed better as a removal agent by requiring a lower pressure threshold to begin the removal of biofilms.

Figure 17: Average pressure required for removal of biofilm exposed to PBS and SDS.

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4.3.3 Percent Area Remaining After Achieving Removal Threshold

The Welch's t-test revealed that the difference in the amount of biofilm that was removed between PBS and SDS was statistically significant (p<0.001). The average percentage of biofilm remaining after removal under PBS and SDS were 74.0±16.7 percent and 38.1±25.6 percent, respectively. Therefore, SDS removed more of the biofilms after reaching the minimum threshold required for biofilm removal than PBS. SDS again was a better removal agent because it removed more biofilm in the same time span at the minimal pressure requirement.
5.0 CONCLUSION

In these experiments, chemical treatment is combined with application of a stress on the biofilm. The device is capable of forming biofilms at specific locations. Biofilms formed around the entrance to the food channels from bacteria that were drawn to the constriction by chemotaxis. The biofilms that form are about 2.5 μm, corresponding to one to two layers of bacteria. The average initial radius of each biofilm is 28.3±11.5 μm. The shear stress applied to the biofilm is focused on the leading edge that faces into the fluid flow, which is where the biofilm removal begins. The COMSOL model is confirmed by the experimental data. The shear stress applied varies from 7.5 to 40 mbar depending on the applied pressure. The microfluidic device is able to consistently form biofilms and evaluate their removal.

Biofilm removal is observed through a fluorescence microscope. The brightfield and fluorescence images are compared before and after flow was applied to the film. The images provided a threshold for biofilm removal as well as the area of biofilm removed. The device designed shows that SDS is indeed a better removal agent than PBS, which is expected. SDS performs better by having a lower required pressure for removal and increased removal at that pressure.

This result validates that the device can be used to compare treatment options for the removal of mature biofilms. Tests on biofilm removal are different than the tests of biofilm formation. Notably, the shear force required to remove a 30 μm biofilm with PBS is 5.02 mbar. This is over four orders of magnitude larger than 0.00017 mbar, which is the critical shear stress where biofilm adhesion decreases found by Park et al. [19].
The device can explore the effects of shear stress in combination with a chemical treatment. It has the advantages of being on the scale of bacterial and is easily incorporated into existing microscope setups to provide a simple analysis of chemical treatments on biofilms. Furthermore, this microfluidic device is much smaller, thus requiring less resources. This microfluidic device has broad applications in the study of biofilm removal.
6.0 RECOMMENDATIONS

Future experiments can include testing different strains and species of bacteria. The difference in removal of *P. aeruginosa* between PBS and SDS was distinct, but a weaker biofilm forming bacteria may have a less distinguishable result. Such experiments could validate the broad applicability of this evaluation technique.

Other studies could also look to evaluate new treatment methods on biofilms. Once a baseline treatment is evaluated, other treatment methods can be tested and compared to the baseline. Various treatment methods could also be compared to one another. The device can test the removal of biofilms from different bacteria and treatment methods under the application of a shear stress.

The COMSOL model could also be expanded upon. Currently the model only looks at the effects of biofilm size and pressure applied individually. A coupled model could provide more insight to the flow dynamics inside the main channel at more extreme conditions. The model can also be used to improve the design of the device to increase throughput and stability. For example, as long as the flow in the main channel is fully developed, the constrictions can be moved closer together, which would allow for more constrictions in each device.
REFERENCES


16. ASTM E2562 - 12 Standard Test Method for Quantification of Pseudomonas aeruginosa Biofilm Grown with High Shear and Continuous Flow using CDC


20. wang, y., libera, m. (2013). length-scale effects on the differential adhesion of bacteria and mammalian cells. proquest llc.


23. ceri, h., olson m.e., stremick, c. read, r.r., morck, d., buret, a. (1999). the calgary biofilm device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. journal of clinical microbiology, 37(6), 1771-1776.


