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Abstract

Bacterial biofilms create problems in a diverse array of fields, including mechanical and biomedical engineering and human health. It has been proposed that a potential method of destroying biofilms lies in the use of acoustic cavitation. Microbubbles can be injected in a location close to a biofilm and then, upon sonication with ultrasound-frequency waves, will undergo symmetric or asymmetric cavitation, heating the biofilms and causing extreme shear forces in the bacterial membranes. In this project, a simple method is described for growing biofilms in such a way that they can be sonicated in an environment where direct observation with a confocal microscope is possible. Additionally, generational trials involving the use of cavitation to destroy live biofilms were performed. Data is presented that suggests that bacteria do not adapt to cavitation-induced stress, an encouraging result for the future of this technology.
Background Information

Biological Background

**Bacterial Biofilms**

Bacteria are some of the most diverse life forms on Earth and are capable of living in a huge variety of conditions. In order to accomplish this feat, bacteria have developed unique survival techniques and growth patterns. One of these is the ability to form biofilms.

The development of biofilms proceeds similarly across bacterial species. The process is illustrated in Figure 1: 1a shows a schematic depiction while 1b are actual images taken with a fluorescence microscope of a biofilm growing over time. The first step of development is when free-floating individual bacteria – planktonic cells – in a liquid medium transiently associate with the surface along which they are flowing. They then adhere strongly to the surface by the excretion of chemicals that will help them stick to the surface and also bring in more bacteria; they become sessile cells. As more bacteria accumulate, they arrange into microcolonies, characterized by cell differentiation in the community. They adhere together by the excreted exopolysaccharide (EPS), a slimy extracellular matrix. As the biofilm grows, gene expression changes as members communicate and coordinate nutrition uptake and growth. At its height, a biofilm will consist of approximately 10 to 25% cells and 75 to 90% EPS. When a biofilm is destroyed, it breaks open and releases some of its members into the environment and they become planktonic once again.

![Figure 1: Biofilm development](image-url)
More than three billion years ago, biofilms seem to have been a method of bacterial growth, according to the fossil record. Now, they can be found virtually anywhere that an aqueous medium flows by a surface. This diversity includes the slime on rocks in a creek but also on human creations such as household pipes. Perhaps most importantly, biofilms are a problem in human health, as they form on artificial implants in the human body but also play an important role in natural diseases such as Cystic Fibrosis (CF). Today, 70,000 people worldwide are affected by CF, a genetic disorder that results in the growth of *Pseudomonas aeruginosa* biofilms in the lungs and thus greatly increased susceptibility to bacterial lung infections throughout the affected individual’s life.

Usually, antibiotics can be used to treat bacterial infections. There are two problems with this treatment method in the case of the biofilm. First, there is the general problem of bacterial evolution and their ability to adapt to a chemical antibiotic. As bacteria grow and divide, their genes also mutate; sometimes these mutations result in increased biological fitness. If a particular bacterium’s new genes enable it to survive a threat, it has a greater chance of passing on those resistant genes to its progeny, and thus the population evolves. The newer generations have different genes that enable them to survive where some of their forbearers did not. The other problem with antibiotic resistance, one that is specific to biofilms, is that the existence of such a rich and complex community means that even if the outside members are eliminated via a chemical antibiotic, the heart of the biofilm will live on and the effectiveness of the antibiotic is severely reduced.
**Green Fluorescent Protein**

In 2008, the Nobel Prize in Chemistry was awarded to Martin Chalfie, Osamu Shimomura and Roger Y. Tsien for their work with the green fluorescent protein (GFP). Isolated from the jellyfish *Aequorea victoria*, GFP fluoresces green when exposed to blue light. This property, as well as those of GFP mutants with shifted absorption and emission patterns, has been exploited in many ways to observe cellular processes *in situ*. The 27-kD GFP (Figure 2a) has been cloned and expressed as a fusion to many proteins without affecting their functions. Since the light-emitting center of GFP is a chromophore that is natively part of the primary structure of the protein (Figure 2b), no external cofactors or specific enzymes are required for its formation. Thus, tagged proteins can be monitored without disruption of their usual tasks.

![Figure 2: Green Fluorescent Protein](image)

In addition to tagging proteins, GFP can be added to the genomes of organisms in such a way that it is constitutively expressed in the cell cytoplasm. These free-floating proteins will emit green light at 508 nm upon absorbance of blue light with a wavelength of 488 nm, enabling fluorescence imaging of entire organisms. Figure 3 demonstrates its use in *Escherichia coli, Caenorhabditis elegans, Drosophila melanogaster* and *Oryctolagus cuniculus*. 
Figure 3: GFP-expressing organisms: *E. coli, C. elegans, Drosophila* and *O. cuniculus*
Technical Background

Bubble Dynamics

In a situation in which there is an infinite field of incompressible fluid with one gas bubble, the pressure in that bubble ($\varphi_b$) is equal to the atmospheric pressure ($\varphi_o$) plus twice the interfacial tension ($\sigma$) divided by the radius of the bubble (R):

$$\varphi_b = \varphi_o + \frac{2\sigma}{R}$$

The pressure in an ideal gas is also described by the ideal gas law,

$$p_0V = nRT$$

where $p_0$ is the pressure of the gas, $V$ is its volume, $n$ is the number of molecules in that volume, $R$ is the ideal gas constant and temperature is the sample’s absolute temperature. When the diffusion across the bubble wall is negligible, $nR$ is a constant term and thus, in an isothermal situation, bubble internal pressure is inversely proportional to its volume.

Henry’s Law states that the solubility of a gas in a liquid is directly proportional to the partial pressure of the gas above that liquid at a constant temperature. Thus, twice the amount of air can be dissolved in a set volume of water at two atmospheres of pressure than can be dissolved in that same volume of water at one atmosphere. So, in the infinite field and one bubble situation, if the pressure is increased, gas will flow out of the bubble and dissolve in the surrounding liquid because its solubility will have increased. The opposite is true also; if the pressure is decreased, dissolved gas will flow into the bubble through diffusion driven by the concentration gradient of the gas.

When an acoustic field is applied to a bubble, the bubble is forced to oscillate around its equilibrium radius at its resonance frequency. When the bubble contracts, the concentration of gas will increase and thus so will the internal pressure. According to the principles above, gas will then be diffusing into and out of the bubble as it oscillates in size. Since the rate of diffusion is proportional to the bubble surface area, more gas will enter the bubble during an expansion phase than gas will exit during the following contraction.
phase. There is a negligible amount of difference over one cycle of expansion and contraction, but over many cycles the bubble will grow significantly (Figure 4). This process is called rectified diffusion.

![Figure 4: Rectified diffusion](image)

Every bubble has a resonance frequency that is dependent upon the identity of the gas and liquid involved and the radius of the bubble. Driven at this specific frequency, a bubble is at its maximum amplitude of oscillation. A bubble with negligible surface tension in an infinite field will have a resonance frequency given by Minnaert’s 1933 equation:

\[
 f_R = \frac{1}{2\pi R_0} \sqrt[3]{\frac{3\varphi_0 \gamma}{\rho}}
\]

where \( f_R \) is the resonance frequency, \( R_0 \) is the equilibrium radius of the bubble, \( \varphi_0 \) is the atmospheric pressure, \( \gamma \) is the polytropic constant (the ratio of the heat capacities \( C_p/C_v \)), and \( \rho \) is the density of the liquid. For the case of air and water, the resonance frequency is given by:

\[
 f_R = \frac{3.26}{R_0}
\]

This resonance can be damped by a number of factors, including viscosity, thermal damping and acoustic radiation (Figure 5).
Cavitation

If a bubble’s equilibrium radius is below the resonance radius, it will grow through rectified diffusion to resonance size, when it will collapse; this is known as cavitation. It is dependent on damping, the mass of the fluid and the stiffness of the gas. There are several sorts of acoustic cavitation.

Stable cavitation is characterized by small radial excursions during oscillation of less than ten percent of the equilibrium radius. These dynamics are dominated by the stiffness of the gas inside the bubble. Stable cavitation results in the emission of subharmonics at half the frequency of sonication and multiples thereof ($\nu_0/4, \nu_0/8, \text{etc}$).

Inertial cavitation occurs when the excursions are larger, greater than twice the equilibrium radius. Following the rarefactive, or decompressive, portion of acoustic stimulation, the growing bubble will suddenly collapse (Figure 6). The temperature and pressure inside the bubble can reach excesses of 100,000 K and 1000 atm, respectively, light is emitted in the form of sonoluminescence, and hydroxyl free radicals can be produced.
These two previous instances are examples of symmetric cavitation, where the bubble is free-floating and thus spherically symmetric; any point on the surface of the bubble acts like any other. It is, however, possible to have asymmetric cavitation if the bubble is less than ten bubble-diameters away from a surface. In this case, the collapse consists of increased pressure on the side of the bubble away from the surface and results in a high velocity jet of liquid pushed out towards the surface (Figure 7).
Stabilization Agents

In any industrially available collection of microbubbles, there is a wide size distribution. As has been shown, there is an optimal size for the bubbles to be affected by ultrasound of their resonance frequency. Towards the goal of keeping as many bubbles in the optimal range as possible, many stabilizing agents have been developed.

The surface and interfacial tensions of a bubble can be lowered by the addition of surface-active agents, or surfactants. They are generally amphiphilic, meaning they have both a hydrophilic head and a hydrophobic tail. They can be anionic, cationic, non-ionic, or zwitterionic (possessing of both positive and negative charges). Surfactants cover the surface of a bubble with the hydrophilic ends sticking outwards, dissolved in the water surroundings and the hydrophobic ends pointing inwards. The surface tension is thus balanced by the electrostatic repulsion of tail ends with each other and the heads with one another.

In contrast-enhanced ultrasound, visualization is enhanced by injection of microbubbles that have stabilizing shells of albumin (protein), galactose (sugar), lipid or polymer over cores of assorted gases. These bubbles are small enough that they move easily in a patient’s circulation system. Examples include Optison™, a perflutren bubble with a hard shell of human serum albumin, and Definity™, a perflutren bubble with a soft shell of lipids.

Ultrasound

Ultrasound waves are above the frequency of human hearing, so above a frequency of around 20 kHz. Ultrasound can be used to drive microbubbles at their resonance frequency and cause cavitation.
**Previous Work**

This project is generally concerned with the destruction of bacterial biofilms by ultrasound cavitation. This problem has been addressed in various ways for almost twenty years now in the laboratories of Drs. Amy C. Vollmer and E. Carr Everbach. Some challenges that have been encountered throughout the years are presented below, along with the solutions found by different engineers and scientists engaged in the project.

**Effect of Cavitation on Bacteria**

There are two potential ways that cavitation could induce bacterial damage; both can be induced by microbubbles being driven by ultrasonic waves. The first is heating, as symmetrical cavitation of free-floating microbubbles can reach temperatures as high as 5,000 K. This heat is sufficient to result in hydroxyl free radicals that can wreck chemical havoc on bacterial cells. The second is the microjets that result from asymmetric cavitation caused by the collapse of bubbles on a surface.

It has been shown previously by the advisors of this project and past students that acoustic cavitation creates stress in bacteria, indicating bacterial damage (Vollmer et al). These studies were done by applying ultrasound to microbubble-infused *Escherichia coli* cultures and evaluating the resulting damage. The *E. coli* had incorporated into their genomes stress-responsive promoters fused to *lux* genes, resulting in the emission of light if stress-response pathways were activated. In this way, it was determined that most of the bacterial damage was most likely due to the heat from symmetrical cavitation and the microjets from asymmetric cavitation. The results from this experiment are presented below in Figure 8. The bacterial culture that had microbubbles injected and then sonicated is labeled “ultrasound.” The positive control, labeled “2% ethanol,” shows the response of the bacteria to 2% ethanol, a known stress inducer. As one negative control, labeled “sham treatment,” the bioluminescence of otherwise identical cultures without the addition of microbubbles was evaluated; these cultures displayed negligible bioluminescence. The other negative control, “untreated,” shows the luminescence of an *E. coli* culture subjected to neither microbubbles nor sonication and was instead left in the incubator. The bacteria
that were sonicated with microbubbles show a response similar to the bacteria exposed to ethanol, while the other cultures exhibit no substantial response.

Several other studies have been completed that study the effects of ultrasound on bacteria. In one, low-frequency, low-intensity ultrasound was found to actually increase cell growth, probably by disaggregating clumps and increasing the bacterial surface area exposed to nutrients (Pitt and Ross). In another, ultrasound was used to effectively remove biofilms from surfaces (Zips et al). The effects of ultrasound and chemical antibiotics working together as a double stressor on biofilms have also been examined and found to be more effective than either bacteriocide working alone (Qian et al).

Also as an earlier experiment in this project, bacterial adaptation to ultrasound was evaluated. Planktonic bacteria were grown in test tubes and then sonicated. The optical density (OD) was used to monitor cell death. Through multiple rounds of sonication, the drop in OD stayed consistent, suggesting that the bacteria could not adapt to the ultrasound stressor the way it would to a chemical antibiotic. However, this kind of multi-generational adaptation study with ultrasound has never been applied to bacterial biofilms as opposed to free-floating cultures.
**Growing Biofilms**

In order to destroy biofilms, one must first start with living, healthy biofilms. For consistent results, they must be grown in the laboratory. In order to enable visualization of the growing biofilm, they must be grown directly on a microscope slide. Thus, an important component of the project has been designing and implementing an effective system to grow robust biofilms on microscope slides in such a way that ultrasound can then be applied.

Usually, when growing biofilms in a laboratory, a commercial flow cell can be used. These are industrially-available systems in which bacterial medium continually flows by a solid surface on a slide, enabling biofilms to grow up on the slide (Figure 9). These systems involve a medium source, tubing, the actual flow cell and a pump.

![Image of flow cell system](image)

**Figure 9: An example of an industrially-available flow cell system (Stovall, Inc)**

While simply buying a flow cell system would be convenient, it is necessary for the purposes here to design biofilm-growth slides because of the need for ultrasound transducers. In order to deliver ultrasound to the biofilms, there must be built into the slide some transducers in direct contact with the surface upon which the biofilms are growing. Thus, commercial biofilm flow cells are insufficient.
Past Slide Design

Flowthrough System

When setting out to grow biofilms for this project, imitations of commercial flow cells that incorporated transducers were initially considered. These systems incorporated glass slides with two small chambers adhered to the top. On the slide, between the chambers, was a coverslip raised over the slide with transducers, creating a sort of tunnel through which the medium could flow. A peristaltic pump sent bacterial growth medium with growing bacteria through tubes, through one chamber, over the area of the slide surrounded by coverslip and transducers, and out the other side.

A plug input was also glued to the slide that was connected to the transducers. A cable was created with the plug matching that input on one end and a BNC connection on the other; in this way, ultrasound could be delivered to the slide.

Several permutations of this slide were tried, and though they sometimes created wonderfully robust biofilms, all constructed models often leaked growth medium and were thus inconsistent. Below are shown a few prototypes of what were called “flowthrough slides” (Figure 10a, 10b) as well as the setup of the flowthrough system (Figure 10c).

Figure 10: The flowthrough system
**Waterfall System**

Next, what became termed the “waterfall system” was tried. In this system, slides had coverslip-shaped holes cut out of them and then coverslips were attached over the holes with transducers between the slips and the slide surface (see Figure 11a). These slides were put on a rack, which suspended the slides above the bottom of a pyrex dish (Figure 11b). Another dish was inverted over the first, creating a kind of chamber, all of which went inside an incubator. Tubes of growth medium were pumped via a peristaltic pump from the bottom of the chamber, where 100 mL of medium with growing bacteria, through the pump, which was outside the chamber, and back into the top of the chamber, where each slide had a tube suspended over it. In this way, medium was dripped onto the coverslip continuously and biofilms could grow there, next to the transducers.

![Figure 11: Waterfall system](image)

While this system also would sometimes grow robust biofilms, it came with a host of problems. First of all, the slides themselves tended to fall apart, as they were made of delicate materials. In particular, the fragile wires that connected the transducers to the input plug were inclined to break and the input plug itself tended to fall off, as the glue attaching it to the slide was weak. The system was messy to set up and sometimes fell apart as the tubes slipped while the biofilms were growing. The peristaltic pump would also on occasion shred the tubing, resulting in media escape and experiment failure. It was also difficult to keep the system sterile, so that no outside contaminants interfered with biofilm growth. A new biofilms growth system was necessary if any consistent long-term data were to be obtained.
Recent Work

The Shaking System

*Shaking System Development*

While the flowthrough and waterfall systems both produced robust biofilms, neither was a robust enough system to yield consistently good results. Thus, a simpler system was developed. Instead of flowing or dripping growth medium from a large reservoir over a slide continuously, the shaking system just has a small initial aliquot of medium in a slide. Each slide is suspended in a chamber made of Pyrex® dishes and the entire apparatus placed in a shaking incubator. In this way, bacteria is growing in a liquid medium that is moving over a surface; biofilms thus grow on the cover slide. It was initially found that slides tended to dry out due to lack of sufficient medium, so the finalized shaking slides incorporate additional “walls” built up around the central trough so that 1.5 mL of medium can fit comfortably as the slides shake (Figure 12). These walls are strips of thin plastic shim glued upright and sealed.

*Figure 12: The chamber of the shaking system with six shaking slides*
Slide Design Improvements

In order to address the problems with the waterfall slides described above, the basic design of the slides has been improved. Now, instead of attaching the input plug with glue to the surface of the plastic slide, it is attached via a small screw that penetrates through the bottom of the plug and into a tapped hole in the slide (Figure 12). Additionally, instead of using the small fragile wires that are tedious to attach and that tear out easily, the electrical connections between the plug input and the ultrasound transducers are created using conductive paint.

The slides are thus created as follows (see Figure 13). First, a slide-shaped piece of flexible plastic is cut and a rectangular hole milled out of the middle of about 1 by 1.5 cm. A small hole is drilled and tapped on one side. A small screw is screwed partway in the hole and a plug input is slid onto the slide so that the screw is firmly holding it in place. Then, transducers are glued on either side of the hole in the middle of the slide, keeping the space between the two bars consistent with spacers. Conductive paint is applied thickly in two trails. One trail connects the insides of the two transducers to the “hot” end of the input plug. The other connects the outsides of the two transducers and the ground on the input plug. A coverslip is then glued over the transducers and spacers with watertight glue, sealing off the hole in the slide. This creates a shallow trough with the coverslip as the bottom, where biofilms can grow. This slide design is a great improvement over the previous one and enabled further developments in the biofilm growth systems.
Figure 13: Front and back of a shaking slide
Generational Studies

The Concept

One of the potential advantages of using cavitation as a bactericide is that it is possible that bacteria have a harder time adapting to a mechanical stressor as opposed to a chemical stressor. With the antibiotics usually used, bacteria can often quickly evolve resistance. However, it is hypothesized that cavitation damages bacteria primarily via high temperatures and membrane shear stresses. In both cases, bacterial adaptation would likely result in less-viable cells. For example, if a small percentage of the population survives a cavitation event because these cells happen to have particularly strong and inflexible cell walls, the ensuing progeny is probably less likely to survive after ultrasound because of its overly-rigid walls. Similarly, if some bacteria can survive high temperatures, it is likely that they are highly inefficient at regular growing temperatures.

While it has been shown that bacteria and even biofilms specifically can be destroyed by cavitation, there have been no published studies on their ability to adapt. To that end, an experiment has been designed that seeks to measure the ability of bacteria existing in biofilm communities to adapt to stress caused by cavitation. Biofilms are grown for an extended period of time and they are periodically sonicated. The percent destruction of biofilm over the course of the time trial will indicate whether or not there is generational adaptation occurring. If the percent killing goes up over time, it means the bacteria are failing to adapt, whereas if it goes down, the bacteria are finding successful adaptation strategies. Alternatively, the percent destruction over time can have no meaningful trend, indicating that repeated cavitation has no effect on bacterial ability to adapt.

The Experiment

To examine the effects of cavitation on bacterial biofilms over several generations, biofilms were first grown in six slides inside a Pyrex-dish chamber. Tape was stretched over one dish and the slides stuck to the tape, suspended over the dish. About 200 mL of water was placed in the bottom of the Pyrex to keep the environment moist. The chamber was closed with the top dish and the opening between the two dishes was sealed with electrical tape. The chamber, tape, water and slides were autoclaved for 20 minutes on the lowest
temperature setting. When cool, the chamber was opened in a sterile hood and 1500 μL of Luria-Bertani broth (LB) with 25 mg of ampicillin per Liter of medium was pipetted into each slide. Each was then inoculated with 75 μL of stationary-phase GFP-expressing *Pseudomonas aeruginosa* overnight culture. The chamber was resealed, placed in a shaking incubator at 37°C on the lowest shaking velocity, and the bacteria allowed to grow for 24 hours (Figure 14). To counter the drying effects of the convection-based incubator, a small open tub of water was also placed in the incubator for the duration of the experiment.

*Figure 14: Shaking system setup*

After the initial growth period, the following protocol was followed every 24 hours for ten days. The chamber was removed from the shaking incubator and brought to the sterile hood, where it was opened and the slides with growing bacteria were transferred to sterile Petri dishes. They were then brought to the fluorescence confocal microscope, where one by one they underwent the following protocol for sonication. A slide was taken from its Petri dish and placed on the microscope stage. It was then plugged into an amplifier that was attached to a function generator. Using the viewfinder on the microscope, the thickest and strongest-looking biofilm on the slide was located. The microscope focus was centered on the middle of that biofilm and an initial data set was collected.
Each data set consisted of a 100-step Z-stack taken from -245 μm to 245 μm (a 490 μm stack centered on the middle of the biofilm). This means that the microscope begins by focusing 245 μm lower than the middle of the biofilm and taking a picture while shining 488 nm light on the slide, then focuses 49 μm higher and does the same, all the way until it is focused to 245 μm above the middle of the biofilm. The product of each picture is similar to Figure 15a: a field of green. More intense green means there is more GFP, indicating more bacteria. Planktonic bacteria create a more dispersed, less intense green field, while bacteria in a biofilm are much more tightly packed and thus fluoresce much more densely. The Leica software that runs the microscope will automatically quantify the amount of green in one of these pictures, and will graph that quantity over Z-stack distance. Thus, one 2-minute, 12-second Z-stack data set results in one graph, as shown in Figure 15b.

![Figure 15: Example of a typical picture and resulting Z-stack graph with the confocal microscope](image)

After the baseline is established, the biofilms are sonicated. For each of the four trial slides, 200 μL of microbubble solution, made up of 10% Optison microbubbles in 0.8% aqueous sodium chloride (NaCl) solution, was pipetted into the slide and pipetted up and down once. For the control slides, 200 μL of pure 0.8% NaCl solution was added in the same manner. The concentration of salt was chosen to match the salt concentration of the cell to maintain osmotic balance and not burst the cells. The amplifier was then turned on for eight minutes, directly delivering via the transducers on the slides amplified 810 kHz ultrasound to the surface on which the biofilms have grown. This rate and magnitude of
sonication was chosen by an optimization process performed by previous researchers. Directly after sonication, the slide was "washed" with 750 μL of 0.8% NaCl solution by pipetting up and down three times. A second Z-stack was taken afterwards. These Z-stack graphs were given names according to a specific scheme and exported from the confocal software in the form of Excel spreadsheets.

This procedure was repeated every 24 hours for seven days in the case of the first trial and ten days in the case of the second trial.

**Processing the Data**

This procedure proceeded for ten consecutive days. Matlab was then used to process the data. There are three interacting m-files, presented below, that work together to accomplish this. Taken together, the processing begins as the data is first imported from Excel. For one graph coming from one data set at a time, the minimum point is found and subtracted from all data points to eliminate background noise. The area under the data curve is then taken and then that number from each before and after pair is used to calculate percent killing according to the following equation:

\[
\text{% killing} = 1 - \frac{\text{area}_{\text{after}}}{\text{area}_{\text{before}}} \times 100
\]

The percent killing over time for the four trials and the two controls is then graphed. In a separate graph, the four trials are averaged with one another and the error determined by standard deviation; the same process occurs with the controls. Any data points that were major outliers in the negative percent destruction range that resulted from measurements on slides that were completely dried out and thus did not actually contain biofilms were also deleted at this point. The resulting graph is thus simpler and easier to interpret, as it just presents killing over time with microbubbles versus without them and it includes error bars.
for slide=[7 8 9 10 11 12];
i=i+1;
perdestot=[];
    for dat=[416 417 419 420 421 422 423 424 425 426];
        perdestot=descalc(dat, slide);
    end
all(i,:)=perdestot;
end
allr=all;
neg=find(all<0);
all(neg)=0;
allt=all'; %make allr' for raw, all' for all positives

subplot 211
hold on
plot(allt(:,1:4),'b*-
plot(allt(:,5:6),'r*-
title('percent destruction over time, trial #2')
xlabel('day #')
ylabel('% destruction')
xlim([0 11])
ylim([0 100])

[aveexp, stdexp, avectrl, stdctrl]=stats(all);

subplot 212
hold on
errorbar(aveexp',stdexp','b*-
errorbar( avectrl',stdctrl','r*-
title('average percent destruction over time, trial #2')
xlabel('day #')
ylabel('% destruction')
legend('with microbubbles','without microbubbles')
xlim([0 11])
ylim([0 100])

Code 1: Matlab m-detruconAreaFn2.m that brings in the processed data and graphs it
function [perdestot]=descalc(dat,slide)
global perdestot

for w=['b' 'a']
    filename=[num2str(slide),w,num2str(dat),'.csv'];
    ndata=importdata(filename);
    zdist=ndata.data(:,1);
    glow=ndata.data(:,2);
    glowmin=min(glow);
    glow=glow-glowmin;
    spacings=zdist(3)-zdist(2);
    if w=='b'
        areab=spacings*trapz(glow);
    else
        areaa=spacings*trapz(glow);
    end
    perdest=1-(areaa/areab);
    perdestot=[perdestot, perdest];
end

Code 2: Matlab m-file descalc.m that imports raw data and calculates percent destruction for before and after pairs

function [aveexp,stdexp,avectrl,stdctrl]=stats(all)

expe=all(1:4,:);
ctrl=all(5:6,:);

for k=1:length(expe)
    pos=nonzeros(expe(:,k));
    aveexp(1,k)=mean(pos);
    stdexp(1,k)=std(pos);
end

for k=1:length(ctrl)
    pos=nonzeros(ctrl(:,k));
    avectrl(1,k)=mean(pos);
    stdctrl(1,k)=std(pos);
end

Code 3: Matlab m-file stats.m that calculates the averages and standard deviations of sets of data for a given day
Results and Conclusion

The graphs shown in Figures 16 and 17 resulted from the two generational trials conducted and processing with the Matlab codes above.

![Graphs showing percent destruction over time and average percent destruction over time for trial #1.](image)

**Figure 16: Results of the first generational trial**
Data Analysis and Conclusions

In the case of both trials, the overlap of the error bars between the red control lines and the blue lines corresponding to the bacteria exposed to cavitation is great enough to come to the conclusion that there is no significant difference between these two sets of data. Additionally, a linear regression applied to the cavitation-exposed data from the second trial gives a slope of -0.0298 with 95% confidence bounds of -0.0625 and 0.003007. Since these bounds are inclusive of zero, these results suggest that the percent destruction of cavitation-exposed bacteria are neither decreasing nor increasing over time. Both of these facts suggest that bacteria do not adapt to cavitation stress, a hopeful result for the future of this technology.

Figure 17: Results from the second generational trial
**Error Analysis**

Due to the nature of the experiment, large amounts of experimental error are introduced at different points in the procedure. Perhaps the largest error is due to the inability to maintain a sterile system through ten days of bacterial growth, as at least ten minutes per slide is spent outside of any sterile environment and instead on the microscope stage. Every effort is made to keep the slide environment as sterile as possible, including use of a sterile hood and laboratory gloves when handling slides, but the fact remains that the slide with its culture is open to the environment for ten minutes per day.

Another major error is the identification of biofilms on the slides. For each set of data, one biofilm is located and zoomed in on and both the before and after Z-stacks are taken with the slide at this point. Though this method should give an accurate measure of percent destruction, the size of the biofilm and its location on the slide varies wildly between slides and between days.

The temperature of the biofilms was not monitored over the course of cavitation and so some of the destruction may have been due to death by heating rather than cavitation damage.

It is possible that ten days of data-taking is much too small a timescale to see any kind of significant results for bacterial adaptation. However, there is no way to know what a significant timescale for this type of experiment is without actually carrying out the experiment for a longer period of time. Also, a greater number of slides would allow for a better analysis of the results of the experiment, as the inherent nature of biofilm experimentation introduces great variability to any data set, which can be compensated for in part with additional data points.

There is also some question as to the biological relevance of the experimental setup. Generational studies were originally attempted with the flowthrough system, which mimics biofilm growing environments more accurately than the shaking system, but these failed due to the fragile nature of the slides, tubing, and the setup in general. So, the shaking
slides that were eventually used for the data presented here most likely did not produce
the large, advanced-stage biofilms that exist in ideal (for bacteria) conditions in nature.

Finally, an important component of the destruction of biofilms with cavitation is that a
significant way in which the cavitation affects the bacteria is that they do not necessarily
kill them, but rather knock off parts of the biofilm, so that they join the flow of medium
away from the original biofilm site. The “wash” step directly prior to sonication is designed
to consider this, as any biofilm mass that was knocked loose would hopefully be removed
with the salt water during this step. Studies have been undertaken by another student
investigating the susceptibility of the knocked-off biofilms to a double-stressor chemical
antibiotic, but here this experiment is concerned only with removing that knockoff as well
as possible.

Acknowledgements
I would like to thank Amy C. Vollmer and E. Carr Everbach for their advising on this project.
Also a huge thanks go to Omari Faakye, Jeff Santner, Kofi Anguah and Roby Valez for their
previous work on this project. Finally, my colleague Sunjay Barton has my eternal and
heartfelt thanks for his help and guidance in bringing me up to speed on the project and
teaching me the myriad joys of biofilm study.
References


Figures

Figure 1: Fuqua, et al (above)
Figure 2: [my creation]
Figure 4: http://www.npl.co.uk/upload//img_400/ultra_high_intro_1.jpg
Figure 5: Brennan (above)
Figure 6: Kennedy, et al (above)
Figure 7: http://www.noria.com/learning_center/category_article.asp?articleid=380&relatedbookgroup=Hydraulics, http://www.oilanalysis.com/backup/200209/Cavitation-Fig1.jpg
Figure 8: Vollmer, et al (above)
Figure 9: http://www.slscience.com/flowcell.html
Figure 10 : Sunjay Barton, Jeff Santner, Omari Faakye
Figure 11-17: [my photos and data]