Removal of Biofilm through Controlled Cavitation

Mathew Chackalackal Mathew

Embry-Riddle Aeronautical University - Daytona Beach

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Removal of Biofilm through Controlled Cavitation

By

Mathew Chackalackal Mathew, B.E.

A Thesis Presented to the Faculty
Emory - Riddle College of Engineering
Department of Aerospace Engineering
In Partial Fulfillment of the Requirements for the
Degree of Master of Science in Aerospace Engineering

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Removal of Bacteria through Controlled Cavitation

Mathew Chackalackal Mathew

This thesis was prepared under the direction of the candidate’s thesis committee chairman, Dr. Sathya Gangadharan, Professor of Mechanical Engineering, and has been approved by the members of his thesis committee. It was submitted to the Aerospace Engineering Department and was accepted in partial fulfillment of the requirements for the degree of Master of Science of Aerospace Engineering.

THESIS COMMITTEE

Dr. Sathya Gangadharan, Chairman
Professor of Mechanical Engineering

Dr. Reda Mankbadi, Co-Chairman
Professor of Aerospace Engineering

Dr. Ram Nayar, Member
Professor of Microbiology and Director of Research, Daytona State College

Dr. Efstratios Nikolaidis, Member
Professor of Mechanical, Industrial and Manufacturing Engineering, University of Toledo

Dr. Chelakara S. Subramaniam, Member
Professor and Program Chair, Mechanical and Aerospace Engineering, Florida Tech

Dr. Sorin Cioc, Member
Assistant Professor of Mechanical Engineering, University of Toledo

Dr. Habib Eslami
Professor and Department Chair, Aerospace Engineering

Dr. James Cunningham
Associate Provost
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Mathew Chackalackal Mathew
Abstract

Author : Mathew Chackalackal Mathew
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Human kind is poised to take the next “giant step” in space exploration, the manned mission to Mars, among other things. But before any plans for such long term habitation on an alien planet, there has to be consideration towards prevention and removal of bacterial contamination. Bacterial contamination or rather degradation of materials was considered of negligible importance. Bacterial degradation can be serious for long-term manned space mission, more so because there is no way to obtain quick replacements. For such conditions, a new innovative way of bacterial removal has been proposed wherein the bacteria is removed with no damage to the underlying material using controlled cavitation. The proposed method involves sonication or bombarding the material surface with cavitation bubbles to remove the bacterial biofilm.

This thesis includes the various experiments done with respect to removal rate for varying parameters (i.e. depth, sonication time, etc). Discussion is presented as to advantages and possible limitations.

The advantages of this procedure include non-invasive surgical procedure to clean prosthetics as well as a cost-effective way of cleaning bacterial growth on a surface. The limitations include the fact that this procedure generates high acoustic waves which causes disturbance to people.
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I. INTRODUCTION

“Space: The final frontier….. To explore strange new worlds, To seek out new life and new civilizations, To boldly go where no man has gone before” – Introductory text at the beginning of the series Star Trek. [1]

As the above text states, Man with his technical sophistication is reaching out towards the stars. This started with the publication of the “Philosophiæ Naturalis Principia Mathematica” by Sir Isaac Newton in 1686, followed by exposition of rocket equations by William Moore and finally the launch of the first liquid fueled rocket by Robert H. Goddard on 16th March 1926. But the first space flight by living organisms was not till the Army Ballistic Missile Agency, USA launched fruit flies into space using a V2 rocket in 1947. This led to the “Great Space Race”, an informal competition between the Unites States and the former Soviet Union. [2]

This friendly competition has pushed human imagination and technical expertise to the limits wherein satellites have been sent to the outer reaches of the solar system (Voyager Mission) and having extended human habitation aboard the International Space Station. The next step in consideration is the Manned Mission to Mars as well as possible human habitation on the planet.

Microorganisms, such as bacteria and fungi, are natural constituents of the Earth’s atmosphere i.e. in the air, water and soil as well as in biotic habitats. Microorganisms can also survive in other planets provided they are under favorable conditions. A characteristic feature of microorganisms is their ability to survive and adapt easily to changes in the environment and nutrient availability.
The deterioration of materials by microorganism, such as bacteria and fungi, was for a long time considered of negligible importance. Experience in long time operation of manned space modules, such as the MIR space station and the ISS, demonstrated that in principle all kinds of constructive and decorative materials, functional units and substances are degraded and/or property altered under the influence of microorganisms. These deteriorations can advance to a status at which the infected materials and compounds become either unsafe or even useless.

There are numerous methods, both biological as well as abiological, of removal of biofilm growth. The most common one involves the use of one or more chlorinated hydantoins, such as dichloro- or monochlorodialkylhydantoin, to the aqueous system. Alternatively, the chlorinated hydantoins can be created by adding a chlorine source and an alkylated hydantoin separately to the aqueous system. [20]

An alternate method to effectively remove this contamination by the bacteria has been proposed here. This method, which will not have any chemicals involved in the process of removal of biofilms, involves the use of a sonotrode to produce ultrasonic waves which in turn remove the biofilm due to the phenomenon of cavitating bubbles. This process is called sonication.

Sonication is defined as the “act of applying sound (usually ultrasound) energy to agitate particles in a sample”. In the laboratory, it is usually applied using an ultrasonic probe, colloquially known as a sonicator.

Cavitation is a phenomenon where a small, high energy and low pressure, fluid bubble forms inside a liquid typically due to vibratory energy, or due to the characteristics of the flow. Cleaning of these bacterial contaminations can be accomplished by use of the acoustic cavitation bubbles. Experiments have been performed with respect to the removal rate due to cavitation bubbles on painted glass surfaces, biofilm covered slides and aluminum foil in an ultrasound standing wave field at 24 KHz. Costerton et al. broadly defined biofilm as communities of microbes associated with a surface, typically
encased in an extracellular matrix. [2] This definition has been expanded to include surfaces as far ranging as steel pipes, soils, medical implants, and epithelial cells.

Many studies have been performed to study the effect of bubbles on the removal of biofilms. Different interactions involved in the removal of biofilm have been studied, ranging from strictly chemical interactions, chemical with limited physical interactions, to solely physical methods of biofilm removal.

The use of bubbles as a means of removing particles from a surface has been explored in recent studies.(3,4) Suarez et al. explored the removal of polystyrene lattices from quartz surfaces as a function of interfacial tension, velocity, and the number of air bubbles passing over the particles. The quartz was placed in a flow cell under a microscope in order to observe the interaction of the air-liquid-polystyrene interface. The polystyrene lattices were added to a potassium nitrate solution which was introduced into the flow cell to allow the lattices to adhere to the surface of the quartz. Flow of potassium nitrate was then introduced into the flow cell to remove any non-adhering lattices. [4]

Bubbles were passed over the surface to remove the particles. In order to manipulate the surface tension of the bubble/liquid interface, various amounts of 1-propanol were added to a potassium nitrate solution. The results of this study showed that the percentage of particles removed was proportional to the interfacial tension and number of bubbles involved in collision, and inversely proportional to the velocity of the moving 3-phase interface. [4]

In addition to removing particles, bubbles have also been used as a means of removing adherent bacteria from a surface. Pitt et al. pumped bacteria through a flow cell containing a glass slide or a polymer substrate. After 1 hour of exposure to the bacterial suspension, the flow cell was rinsed with saline, then with ethanol, and finally with air. During the rinsing process, none of the bacteria adhering to the surface were displaced. This process was repeated using methanol in place of ethanol, and again none of the
bacteria were removed. [5] The process was again repeated, but this time the alcohol rinse step was omitted. During the passage of the air-water interface through the flow cell, all of the bacteria were displaced. In another experiment a static air bubble, surrounded by water, formed on the surface of the flow cell. As the bubble expanded laterally, all the bacteria in its path were displaced. Pitt et al. hypothesized that bacteria were displaced by the air-water interface as a result of the surface tension between air and water, but not by the lower surface tension of the air-alcohol interface. [5]

One common approach to removing biofilm is used by the general public on a daily basis. The use of mouth rinses or pre-brushing solutions are commonly employed in the attempt to remove biofilm, and Landa et al. have created an in vitro model to study the effectiveness of this approach. Biofilm was simulated by allowing Streptococcus sobrinus to adhere to a surface in a parallel plate flow chamber. After this preparation, a mouth rinse (Hibident®, or Scope®) or a pre-brushing solution (Plax®) was passed over the sample. Finally, air was introduced into the chamber so that bubbles with the bacteria created a shear stress at the surface of the bacteria. [6]

The removal of bacteria in the presence of mouth rinses was approximately 6% and 9% for Hibident® and Scope®, respectively, whereas Plax® removed 62% of the bacteria before the bubbles were introduced to the system. After the bubbles were introduced the total percent of bacteria removed for the three rinses were 33%, 89% and 81%, respectively. Control samples which were biofilm not treated with mouth rinse, when exposed to the bubble stream; only 26% of the total bacteria were removed. [6] The partial removal of biofilms from the control experiment indicated that in addition to chemical interactions, the physical interactions also play a role.

Yang et al. performed a study comparing three types of toothbrushes (manual, electric, and sonic) to determine which removes the greatest percentage of biofilm. Sonics and electric toothbrushes are both electrically powered, but the difference between a sonic toothbrush and an electric toothbrush is that the sonic toothbrush operates at speeds
greater than 30,000 brushstrokes a minute whereas an electric toothbrush operates only a few thousand brushstrokes a minute. With respective to biofilm removal, approximately 30% of the biofilm was removed by the manual brush, 60% of the biofilm by the electric, and the sonic toothbrush removed about 90%. [7] From this experiment it appears that the sonic toothbrush is superior to the other toothbrushes at removing biofilm. Other clinical studies also compared manual toothbrushes to sonic toothbrushes. The results of their studies also indicate that sonic toothbrushes are superior to manual toothbrushes at removing supragingival plaque. [8-10]

Carter et al. also performed experiments to compare the difference in toothbrushes by focusing on the ability of the brushes to remove biofilm in a model developed to replicate the interproximal spacing between human teeth, where the bristles cannot reach. In this experiment a slide covered in biofilm of S.mutans was mounted behind two posts that represented two teeth. The toothbrush being tested was partially submerged in water and positioned to operate at the optimal performance angle. The sonic toothbrush removed more than twice the amount of biofilm than the electric toothbrush. An important observation from this study was that the sonic brush created more bubbles than the electric brush. [11]

The fluid dynamics of the sonic toothbrush were also of interest to Stanford et al. A study performed previous to their research by Wu-Yuan et al. reported that the fluid forces and cavitation generated by the sonic toothbrush were able to remove common oral bacteria (S.mutans, Actinomyces viscosus, and Porphyromonas gingivalis) from titanium and hydroxyapatite surfaces at distances of 4mm. [12] Stanford et al. wanted to determine if the fluid forces would be able to remove oral plaque in vivo upon enamel. After the biofilm was grown, the enamel was placed either 2 or 3 mm from the tips of the bristles of the sonic toothbrush and were exposed for 5, 10 or 15 second. After 5 second of exposure at least 56% of the bacteria were removed, and after 15 second at least 65% were removed. Thus the fluid forces generated by sonic toothbrushes are sufficient to remove oral plaque. [13]
Wu-Yuan et al. noted that both cavitation and fluid forces were generated by the toothbrush during the experiments involving biofilm removal. [12] From Stanford et al. it is apparent that the fluid forces are sufficient to remove biofilm. [13] However, the impact of an air-liquid interface present when bubbles are in the solution and the effect of the acoustic waves generated by the toothbrush were not addressed. From the studies of Adams et al. and by Heersink et al., it appears that the air-liquid interface of bubbles is also powerful enough to remove biofilm. [11, 14]

a. Effect of Sound on Bacteria

McInnes et al. have studied the effect of sonic waves on planktonic Actinomyces viscosus and its adherence to hydroxyapatite discs. [15] The culture of A. viscosus was divided into three groups. The first group was exposed to sonic waves before being allowed to attach to the hydroxyapatite discs (pre-exposure group), the second group was first allowed to attach to the discs and were then sonicated (post-exposure group), and the third group was allowed to attach to the discs but were not sonicated (control group). Results from the pre-exposure indicated that the bacteria had to be sonicated at least for 10 second before any significant reduction in the percentage of bacteria binding to the discs was observed. In this same group, an applied acoustic pressure of at least 20 kPa was required to reduce the percentage of binding for solutions of $10^7$ bacteria/mL and a pressure of at least 35 kPa was required to reduce binding in solutions of $10^8$ bacteria/mL.

Results from the study of the post-exposure group indicated that no significant removal occurred after 5 seconds of exposure to acoustic pressures of 50 kPa. After 15 seconds of exposure the difference in percent of bacteria bound to the discs between the post-exposure samples and the controls was statistically insignificant; however, the difference was only 10%. The study did show that the percentage of bacteria that remained bound to the discs reduced with time. After 480 second, the longest reported exposure, only 20% of the bacteria was still bound to the discs. As with the pre-exposure
group, the post-exposure group was tested under various acoustic pressure conditions. It was shown that higher acoustic pressures resulted in greater removal. It was also noted that pressures lower than 30 kPa showed no significant removal of bacteria.

b. Effect of Acoustic Cavitation on Material Surface

Nikolaidis et al. conducted experiments at the University of Toledo to examine the possibility of cleaning medical implants without causing damage to both the implants and the living tissue of a patient. In order to avoid damaging the implants and tissue, the energy and or the force generated by cavitation and its relation to the biofilm removal mechanism was determined by exposing different materials to a Hielscher UP400S ultrasonic processor. [21]

The main objectives of this experiment were (1) determine the approximate pressure exerted on a surface due to the rapid frequent collapses of cavitation bubbles, and (2) to establish the most suitable location of the surface relative to the sonotrode. This location would be the area where the most cavitation bubbles collapse thereby having maximum cleaning effect.

_Experiments with Aluminum Foil using Hielscher UP400S ultrasonic processor_

Several experiments were performed using a Hielscher Ultrasound Technology UP400S ultrasonic processor (Figure 21) and aluminium foil samples, with varying distances between the ultrasonic processor sonotrode and the aluminium sample. The amplitude was set to 50% of the maximum and the cycle duration was set to ½ the total duration of sonication. This duration was 2 minutes in all trials.

Small craters with sizes ranging from 0.2mm to 0.4mm and depth of 25µm to 40µm, were created on the aluminum foil surface which were attributed to collapsing cavitation bubbles. These craters were measured and profiled using a Zygo Profilometer (Figure 1). To measure the pressure exerted on aluminum foil by collapse of cavitating bubbles was estimated by use of the hypothesis: the pressure exerted by cavitation
bubbles is roughly equal to the static pressure that needs to be exerted by the tip of a mechanical pencil in order to create a crater with similar dimensions and shape as those of the crater induced by cavitation.

Figure 1. Image of the Zygo Profilometer
Figure 2 Zygo Profilometer image of a cavitation induced crater. The crater diameter is about 0.3 mm and the depth is 40 μm.

A mechanical weight scale was used to measure the force applied on a foil sample using a mechanical pencil. The Zygo Profilometer was again used to measure the depression caused due to the mechanical pencil and these were compared to the original depressions due to cavitating bubbles.

The bounds of static force that induced these craters (based on the Profilometer images of the foil samples that had different forces applied by a mechanical pencil)

\[ F_{\text{min}} = 4 \text{ g}, \quad F_{\text{max}} = 20 \text{ g} \]

where \( g \) is the acceleration of gravity. The lower bound of the pressure is obtained by using the minimum pressure and the maximum diameter.
The upper bound of pressure is obtained by using the maximum pressure and the minimum diameter.

\[
\text{P}_{\text{min}} = \frac{1}{\min} \left( \frac{D_{\text{min}}^2 \pi}{4} \right)
\]

\[\text{P}_{\text{min}} = 3.122 \times 10^6 \text{ Pa}\]

In conclusion, the static pressure, which induces craters similar to those craters created by cavitation ranges from 0.31 MPa to 6.2 MPa.

![Zygo Profilometer Image](image)

Figure 3. Zygo Profilometer Image of a mechanical pencil crater formed with a 0.2N applied force.
II. Objectives and Significance

a. Objectives

The objectives of the research were (1) to determine the effectiveness of bubbles in removing biofilms from surface within an acoustic field, (2) to determine which parameters would have the greatest influence on the effectiveness of bacterial removal (the parameters of interest are distance from sonicator to the plate and sonication time), and (3) to determine the effect of different acoustic frequencies and amplitudes with respect to biofilm removal by the gas bubbles. In order to accomplish this, the following steps were taken:

1. Setup the experiment using a sonicator.
2. Machine a jig to hold the biofilm covered slides in position while being sonicated.
3. Study the effect of variation in sonication time and distance between sonication tip and slide.
4. The viability of biofilm after impingement by bubbles was studies to determine whether or not the bubbles would kill the bacteria.

b. Significance

The use of bubbles and acoustic waves for bacterial removal could lead to an increase in the bacterial removal for human prosthetics while within the human body. The blood could act as a medium for the ultrasonic waves and can carry the dead bacteria after controlled cavitations. Currently, the practice is to remove biofilm infected prosthetics and replace it with a new one. This involves a lot of recuperation time for the patient as well as money. But controlled cavitations could reduce both these factors.
Also this could be used on manned space missions to remove bacterial contamination on spacecraft surface, in situations where it is not viable to actually replace the bacteria contaminated surface, thereby improving the health and well being of on-board astronauts.
III. Methodology

a. Preparation of Biofilm

i. Media

The bacteria *Pseudomonas aeruginosa* were grown in a solution of Tryptic Soy Broth (TSB). Tryptic Soy Broth is used for the cultivation of a wide variety of microorganisms. It is a general purpose medium and is commonly referred to as Soybean-Casein Digest Medium. This medium was originally developed for use without blood in determining the effectiveness of sulfonamides against Pneumococci and other organisms. (16) *Clostridia* and non-sporulating anaerobes grow luxuriantly in this broth when incubated under anaerobic conditions. In TSB, Enzymatic Digest of Casein and Enzymatic Digest of Soybean Meal are nitrogen sources. Dextrose is the carbon energy source that facilitates organism growth. Sodium Chloride maintains osmotic balance and Dipotassium Phosphate is a buffering agent. [17]

The media solution was prepared by dissolving 24 g of Tryptic Soy Broth instead of the regular 30 g into 1L of purified water. This was done to decrease the nutrients in the medium to provide stressful condition for growth of bacteria which will enable the bacteria to divide more rapidly and when the nutrients are depleted, it will resort to biofilm formation. The media was placed on a hot plate with a magnetic stirrer and the mixture was continuously stirred under low heating until the powder was completely dissolved. This solution was then covered and sterilized at 250 °F (121.11 °C) for 30 minutes at 15psi. The media was removed from the autoclave and placed in the cooler and left to cool overnight.
ii. Storage of Bacteria

The Bacteria (*P. aeruginosa*) was purchased from Carolina Biological Supply Company, NC. These bacteria are lyophilized cultures and they were reconstituted in a nutrient medium and incubated @ 37°C for 24 hours. Then the bacteria were used to make subcultures which were used in experiments. These subcultures were stored at 4°C until use.

iii. Bacteria Growth Phases

In general, a single bacterium takes 23 minutes to divide into two. Bacteria dices by asexual reproduction called *Binary Fission*. The bacterial growth can be divided into 4 phases: lag phase (A), exponential or log phase (B), stationary phase (C), and death phase (D). [18, 19]

1. **During lag phase**, bacteria adapt themselves to growth conditions, it is the period where the individual bacteria are maturing and not yet able to divide. During the lag phase of the bacterial growth cycle, synthesis of RNA, enzymes and other molecules occurs.

2. **Exponential phase** (sometimes called the log phase) is a period characterized by cell doubling. The number of new bacteria appearing per unit time is proportional to the present population. If growth is not limited, doubling will continue at a constant rate so both the number of cells and the rate of population increase doubles with each consecutive time period. For this type of exponential growth, plotting the natural logarithm of cell number against time produces a straight line. The slope of this line is the specific growth rate of the organism, which is a measure of the number of the number of divisions per cell per unit time. The actual rate of this growth (i.e. the slope of the line) depends upon the growth conditions, which affect the frequency of cell division events and the probability of both daughter cells surviving. Exponential growth cannot
continue indefinitely, however, because the medium is soon depleted of nutrients and enriched with wastes.

3. *During* stationary phase, the growth rate slows as a result of nutrient depletion and accumulation of toxic products. This phase is reached as the bacteria begin to exhaust the resources that are available to them. This phase is a constant value as the rate of bacteria growth is equal to the rate of bacterial death.

4. At *death phase*, bacteria run out of nutrients and die except those which form endospores.

![Graph showing the various phases on Bacterial Growth](image)

Growth is shown as $L = \log(\text{numbers})$ where numbers is the number of colony forming units per ml, versus $T(\text{time})$.

**Figure 4.** Graph showing the various phases on Bacterial Growth

*iv. Biofilm Growth*

The biofilm of *P.aeruginosa* was grown on polycarbonate slides in the following manner. 7 gallons of medium was prepared, which was then poured into a large tank. The tank was made up polycarbonate plastic which will not react with the medium. The tank was also fitted with a plastic tube which leads to a beaker to collect overflow of the medium. More medium was prepared and poured into a drip-feed system to enable constant refreshing of the nutrients required for bacterial
growth. This would allow the bacteria to continuously grow without it entering the stationary phase of its growth cycle. Bacteria grow better when there is slow agitation in the medium. For this reason, a magnetic stirrer was also added to the tank to cause motion of the medium.

A polycarbonate sheet of thickness 0.8” was cut into the required design as shown in Appendix A. Laboratory tape was used to cover all no-essential portions of the slides and only the central circular section was left open to enable the bacteria to grow on the slide. The slides were strung up in sets of 12, with the bacteria growth section immersed face down in the medium.

An important part of the biofilm formation was preparation of the bacterial culture. One vial of *P. aeruginosa* was removed from the 40°C freezer and was allowed to thaw just enough to pipette out 0.5mL of suspension and add it to the tank filled with Tryptic Soy Broth medium. The medium containing bacteria was then placed on a base with magnetic stirrer. The bacteria were allowed to grow for two weeks while fresh medium was introduced as and when necessary. This was done so as to prevent the bacteria from entering the stationary phase. This methodology is known as *Continuous Culture Method*.

The bacteria were grown for 2 week to enable a good growth of *P. aeruginosa* bacteria. The growth was further improved by refreshing the nutrients available by replacing the old medium with fresh medium when necessary.
After the two week period, the medium in the tank was drained and stored in flasks for sterilization in the autoclave, so that all the bacteria present in the medium is destroyed and the medium can be safely disposed of. The slides were removed and dipped in water three times. This is done to remove any planktonic bacteria which have not adhered to the slides. The slides are then placed in a slide holder which serves to keep the slides in place while controlled cavitation is being performed. The fixture design is shown in Appendix B.

v. Spectrophotometry

Ultraviolet-visible spectroscopy or ultra-violet spectrophotometry (UV-Vis or UV/Vis) involves the spectroscopy of photons in the UV-visible range, i.e. it uses light in the visible and adjacent (near ultraviolet (UV) and near infrared (NIR)) ranges. The absorption in the visible ranges directly affects the color of the chemicals involved.
This technique is comparable to fluorescence spectroscopy, in that the fluorescence deals with the transitions from the excited state to the ground state, while absorption measures transitions from the ground state to the excited state. [26]

A spectrophotometer works on the basis of Beer-Lambert law, which is given by:

\[ A = -\log_{10}\left(\frac{I}{I_0}\right) = \varepsilon \cdot c \cdot L \]

where \( A \) is the measured absorbance, \( I_0 \) is the intensity of the incident light at a given wavelength, \( I \) is the transmitted intensity, \( L \) the path length through the sample, and \( c \) the concentration of the absorbing species. For each species and wavelength, \( \varepsilon \) is a constant known as the molar absorptivity or extinction coefficient. This constant is a fundamental molecular property in a given solvent, at a particular temperature and pressure, and has units of \( 1 / M \cdot cm \). The ratio \( I/I_0 \) is denoted as transmittance and is usually expressed as a percentage (\%T).

This law states “that the absorbance of solution is directly proportional to the concentration of the absorbing species in the solution and the path length”. Thus, for a fixed length, a spectrophotometer can be used to determine the concentration of the absorber in the solution.

Spectrophotometry involved the use of a device called spectrophotometer, which is a photometer (a device for measuring light intensity) that can measure intensity as a function of the color (or more specifically the wavelength) of light. There are two major classes of devices: single beam and double beam. A double beam spectrophotometer compares the light intensity between two light paths, one containing a reference sample and the other a test sample. A single beam spectrophotometer measures the relative light intensity before and after a test sample is inserted. Although comparison measurement from double beam
instruments are easier and more stable, single beam instruments can have a larger dynamic range and are optically more simpler and compact. [27]

The spectrophotometer quantitively compares the fraction of light that passes through a reference solution and test solution. The sequence of events in a spectrophotometer is as follows:

1. The light source shines into a monochromator
2. A particular output wavelength is selected and beamed at the sample.
3. The sample absorbs the light.
4. Transmitted light is measured using a photo diode or other light sensor.
5. Transmittance value of this wavelength is compared with that of a reference sample.

A UV-2100 Spectrophotometer was used to determine the absorbance values. The UV-2100 is a double-beam, fully automated scanning system capable of a wavelength range of 190-900nm with a photometric accuracy of ±0.3%T (0-100% T) and a wavelength accuracy of ±0.3nm.

Figure 6. UV-2100 Spectrophotometer
Many spectrophotometers must be calibrated by a procedure known as “zeroing.” The absorbency of the reference substance is set as a baseline value, so that the absorbencies of all other substances are recorded relative the initial “zeroed” substance. The spectrophotometer then displays the % absorbency (the amount of light absorbed relative to the initial substance). [27]

To calibrate the UV-2100 spectrophotometer, following steps were performed:

1. Turn on the spectrophotometer 15 min in advance before taking reading.
2. Adjust wavelength to 550nm.
3. Pour 3mL of Ethyl Alcohol into a cuvette and set it in the spectrophotometer slot.
4. Set the spectrophotometer to 100% transmission / 0% absorbance, and pull the knob.
5. The spectrophotometer is calibrated.

After controlled cavitation of the slides, the slides were dipped in a 0.1% solution of crystal violet dye to stain the remaining bacteria on the slides. The slides were then scrapped using a rubber scrapper with 3mL of ethyl alcohol added and then transferred to a cuvette. The samples were then transferred to the spectrophotometer which has been calibrated, and the absorbance reading was taken. This absorbance value is a measure of the amount of bacteria remaining on the slide after controlled cavitation. The greater the absorbance value, greater is the amount of bacterial suspension, and vice-versa.
b. Experimental Sonication

For the sonication experiment a Hielscher UP400S supplied by Heilscher Ultrasonics was used. The UP400S (400W, 24 kHz) is a powerful and reliable ultrasonic device for the sonication of large samples in the laboratory, with a sonotrode number H22 (made of titanium, tip diameter is 22mm, approx. length 100mm, for samples from 100mL up to 2000mL). The amplitude and frequency can be varied from 20 to 100% and from 0 to 100% respectively. This was used to create cavitation which was produced by longitudinal mechanical vibrations through electric excitation. The vibrations occurred at a working frequency of 24 kHz and could be controlled in the range of plus or minus 1 kHz. The power output of the processor could be adjusted to anywhere in the range of 20% to 100% of the total duration of the sonication. The processor output vibrations were amplified by the oscillating sonotrode and transferred by its end face to the medium being sonically irradiated.

Figure 7. Hielscher UP400S sonicator
The biofilm covered slide was placed in the above mentioned fixture and the entire setup was placed inside a wide brimmed beaker filled with 2L of water. The water is added to prevent the sonotrode from heating up during sonication and also as a medium to transmit the high ultrasonic waves generated by the sonotrode.

For the experiment the frequency was set at 50% and amplitude at 100%. The distance (depth) between the base of sonotrode and the biofilm surface, as well as the sonication treatment time was set. The depth was varied from 1” to 0.25” with a decrement of 0.25”, thereby giving us 1”, 0.75”, 0.5”, and 0.25” depth. Two sonication treatment experiments were conducted at times, 30 s and 200 s.

<table>
<thead>
<tr>
<th>Experiment</th>
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</thead>
<tbody>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>3</td>
<td>30</td>
<td>0.5”</td>
</tr>
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</tr>
<tr>
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</tr>
<tr>
<td>7</td>
<td>200</td>
<td>0.5”</td>
</tr>
<tr>
<td>8</td>
<td>200</td>
<td>0.25”</td>
</tr>
</tbody>
</table>

Table 1. Table of experimental conditions for Sonication.
c. Confocal Microscopy Imaging Technique

Confocal microscopy is an imaging technique used to increase the micrograph (photograph or similar image taken through a microscope to show magnified image of the object) contrast and/or to reconstruct three-dimensional images by using spatial pinhole to eliminate out-of-focus light in specimens that are thicker than the focal plane. [28]

The principal of confocal imaging was patented by Marvin Minsky [29] and aims to overcome the limitation of traditional wide-field fluorescence microscopes. In a conventional fluorescence microscope, the entire specimen is flooded in light from the light source thereby exciting all parts of the specimen. Then the resulting fluorescence is detected by the microscope photodetector as a background signal. In contrast, a confocal microscope uses point illumination and a pinhole in an optically conjugate plane in front of the detector to eliminate out-of-focus information. As only light produced by the fluorescence very close to the focal plane can be detected, the image resolution is much better than that of the conventional microscope.

There are three types of confocal microscopes available commercially:

- Confocal laser scanning microscope
- Spinning – disk confocal microscope
- Programmable Array Microscope (PAM)

For imaging of the bacteria slides, a TCS SP5 confocal laser scanning microscope was used. The TCS stands for True Confocal Scanner and the SP5 refers to the 5 SpectroPhotometer channels this system is equipped with. The confocal laser scanning microscope gives images with depth selectivity. In a confocal laser scanning microscope, a laser beam passes through a light source aperture and then is focused by an objective lens into a small focal volume within or onto the surface of a
specimen. Reflected and scattered laser light as well as fluorescent light from the illuminated spot is then re-collected by the objective lens. A beam splitter separates off some portion of the light into the detection apparatus, which will have a filter to selectively pass the fluorescent wavelength while blocking the original excitation wavelength if it is fluorescence confocal microscopy. After passing a pinhole, the light intensity is detected by a photodetection device, transforming the light into an electrical one that is recorded by a computer.

To better visualize the \textit{P.aeruginosa} bacteria, a fluorescent stain called DAPI (4', 6-diamidino-2-phenylindole) was used. DAPI is a fluorescent stain that binds strongly to the DNA and is used to frequently stain both live and dead cells because of its property to pass through intact cell membrane. In confocal microscopy, it is excited by ultraviolet light. \cite{31}

To prevent damage as well as to stop further cell division of the bacteria left on the slides after controlled cavitation, the entire slide was soaked in 10% formaldehyde for 20 minutes. The formaldehyde acts as a fixative by killing and preserving the bacteria / biofilm on the slides. After this, the slides were immersed in distilled water three times, to remove any traces of the formaldehyde. The slides were then soaked in a mixture of DAPI and TBS (Tris Buffered Saline) for 20 minutes. This was repeated two more times. DAPI and TBS are both carcinogenic solutions so gloves are advised. Finally the slides were soaked in distilled water for another 20 minutes to remove any extra traces of the dye. The slides were then removed, and every portion of the slides, except for the area of the biofilm, was wiped down. Add two drops of 60% glycerol with PPD (p-phenyleneamine) on the biofilm and then gently place a cover over the biofilm.

The prepared slides were taken to the confocal laser scanning microscope manufactured by Leica Microsystems and relevant micrographs were taken.
d. Bacterial Growth Count

The number of cells that arise through binary fission can be measured by determining the viable cell number, which equals the number of living organisms/mL culture through either a pour plate or spread plate method. In the pour plate procedure, a diluted bacterial culture is added to melted agar and this mixture is poured into an empty Petri dish. Once the plate cools it solidifies and it is then incubated at optimal temperature to develop colonies. Colonies in this method can develop on the surface and within the agar medium or can be damaged by the melted agar and never develop into colonies. In the spread plate, 0.1mL of diluted bacterial suspension is applied to the center of an agar plate and it is spread out with the use of a curved glass rod. After incubation at the appropriate temperature, the viable colony number is counted. Regardless of the viable cell method used, the countable number of colonies must average 30 to 300 colonies/plate. The number of bacteria present per mL of original suspension is given by the following formulae:

\[
\text{Bacteria/mL of original suspension} = \frac{\text{number of colonies}}{\text{dilution made}}
\]

The principle of spread plate is used to calculate the number of bacterial colony present after sonication at 0.75” and 0.25” depth. The methodology is discussed below.

1. The bacteria from the sonicated plate is scraped and inoculated into a brain-heart infusion broth and it was incubated overnight at 37°C.

2. Serial dilution of the original culture tube was prepared by transferring 1mL of culture into a 9 mL tube of sterile water, mixing and removing from this dilution 1mL to be transferred to another 9mL sterile water blank tube. This is shown in the figure below. Five dilutions (1:10, 1:100, 1:1000, 1:10,000, 1:100,000) of the original bacterial suspension were prepared.
3. Dispense 0.1mL of each dilution onto a plate of T-Soy agar and spread with a bent glass rod. Turn all the plates 45 degrees and spread the diluted suspension on the agar surface in another direction in order to cover the entire surface.

4. Invert all plates and incubate them at 37°C for 24 hours.

5. Count those plates having 30 to 300 colonies each using a Quebec colony counter which is equipped with a magnifying lens and grid. This number refers to the Colony Forming Unit (CFU)

![Figure 8 Quebec Colony Counter with Magnifying Lens and Grid](image)

6. Calculate the average number of bacteria/mL by multiplying the average colony plate number by the reciprocal of the dilution period.
e. Statistical Hypothesis Testing

Science progress in two ways: a) Scientists establish a set of axioms (propositions that are obviously true) and derive models that represent physical phenomena or human behavior on the basis of these axioms. b) Scientists make a hypothesis in order to explain a physical phenomenon, collect observations and test this hypothesis. A hypothesis that gains credibility after passing many tests becomes a theory. [33]

A hypothesis is defined by Webster as “a tentative theory or supposition provisionally adopted to explain certain facts and to guide in the investigation of others.” A statistical hypothesis is a statement about a statistical population and usually is a statement about the values of one or more parameters of the population, or it may be based on empirical evidence, or both. [32] To test the claim about a probabilistic model using a structured, consistent procedure by using data drawn from a model is called statistical hypothesis testing. This is a procedure for determining whether to “accept” or “reject” the hypothesis. The hypothesis to be tested is called null hypothesis. If this hypothesis is false, then the alternative hypothesis is true. The null hypothesis, denoted by $H_0$, usually represents the status quo. It is a specific statement about a probabilistic model. The alternative hypothesis is denoted by $H_1$.

Given below is a methodology to test the claim about a probabilistic model by using data drawn from a model. Take the following example model.

**Example:** A biologist wants to decide if a new cleaning procedure can clean surfaces infected by bacteria. The biologist compares the amounts of bacteria on 11 plates that have been subjected to the procedure to those on 5 control plates. The null hypothesis states that the mean values of amounts of bacteria on the two plates are equal. The alternative hypothesis is that these amounts are different.

Figure 9 compares the conditions of the plates that the biologist tried to clean to those of the control plates. The processed plates seem to have fewer bacteria than the control plates. However, it is not safe to conclude that the cleaning procedure is
effective from the results in Figure 9 because the results could be due to luck. To collect more data from experimentation would be expensive, but statistics provide the biologist with formal procedures to test the hypotheses that the cleaning procedure is effective by using the limited data in Figure 9.

![Figure 9. Cleaned Plates (White Bars) vs. Control Plates (Grey Bars)](image)

The key idea of a statistical hypothesis test is that, if its results are surprising given that the null hypothesis is true, then the hypothesis should be rejected. Thus, a hypothesis test can only falsify a hypothesis -- it cannot prove it.

Example (continued): Suppose that the biologist assumes that the mean absorbance values of the cleaned and control plates are identical. When the biologist examines Figure 1 he/she will be surprised because the absorbance values of the control plates seem to be larger than those of the cleaned plates, on average. In view of these results, he/she will suspect that the mean absorbance values are different.
A decision rule is needed in order to decide if a hypothesis is false. A decision rule tells you whether to reject a hypothesis on the basis of a test statistic. This is a value determined from a sample drawn from the population. The probability distribution of the test statistic, conditioned on the null hypothesis $H_0$ being true is known. The test results suggest that the hypothesis is false if the test statistic assumes an unlikely value.

Example (continued): The test statistic is,

$$T = \frac{\hat{\mu}_A - \hat{\mu}_B}{\sqrt{\frac{\hat{\sigma}_A^2}{n_A} + \frac{\hat{\sigma}_B^2}{n_B}}}$$  \quad (1)

where $\hat{\mu}_A - \hat{\mu}_B$ is the difference of the sample means, and $\hat{\sigma}_A^2$ and $\hat{\sigma}_B^2$ are the sample variances of the two variables. This is a random variable that follows the standard distribution with $\nu - 1$ degrees of freedom.

The confidence interval of the difference of the mean values for unequal sample sizes is [33],

$$\hat{\mu}_A - \hat{\mu}_B - t_{\nu,1-\alpha/2} \sqrt{\frac{\hat{\sigma}_A^2}{n_A} + \frac{\hat{\sigma}_B^2}{n_B}} \leq \mu_A - \mu_B \leq \hat{\mu}_A - \hat{\mu}_B + t_{\nu,1-\alpha/2} \sqrt{\frac{\hat{\sigma}_A^2}{n_A} + \frac{\hat{\sigma}_B^2}{n_B}}$$  \quad (2)

The number of degrees of freedom in Equation (2) is,

$$\nu = \frac{\hat{\sigma}_{AB}^4}{\left(\frac{\hat{\sigma}_A^2}{n_A}\right)^2/(n_A-1) + \left(\frac{\hat{\sigma}_B^2}{n_B}\right)^2/(n_B-1)}$$  \quad (3)

where $\hat{\sigma}_{AB} = \sqrt{\frac{\hat{\sigma}_A^2}{n_A} + \frac{\hat{\sigma}_B^2}{n_B}}$ is the standard deviation of the difference.

In this case, the value of the test statistic is equal to 2.301. Figure 10 shows the Probability Density Function (PDF) of the test statistic, given that the mean absorbance values of the two groups of plates are equal. The test statistic (marked by the dotted
line) lies on the right tail of this distribution, away from the mean. The probability of drawing a value that is located so far from the mean on either side is only 0.061, which is low. This surprising result suggests that the null hypothesis is false, that is, the mean values of the control and cleaned plates are different.

![Test Statistic](image)

**Figure 10.** Probability density function (PDF) of the test statistic conditioned on the hypothesis that the mean absorbance values of control and cleaned plates are equal.

In order to develop a decision rule, the range of the values of the test statistic was divided into rejection regions. The rejection regions are the extreme portions of one or both tails, while the non-rejection region in the main body of the distribution. The value(s) that separate the rejection and non-rejection regions are called critical values. The null hypothesis is rejected if the test statistic falls within the rejection region. The decision rule is expressed in two equivalent forms:

1) For one rejection region, reject the hypothesis if and only if the observed test statistic exceeds the critical value. For two rejection regions, reject the hypothesis if and only if the observed test statistic is smaller than the lower critical value or larger than the upper value.

2) Reject the null hypothesis if and only if the probability of obtaining a more extreme value of the test statistic (called \(p\)-value) is less than the observed
value exceeds a level (called level of significance). The p-value is also called observed level of significance.

Example (continued) Figure 11, shows the rejection region for the test of the hypothesis that the mean values of the absorbance values of the control and cleaned plates are equal. This region has a significance probability of $\alpha = 0.1$. The critical values are $-1.943$ and $1.943$. The biologist should reject the null hypothesis because the observed value of the test statistic (which is $2.301$) lies in the rejection region. The biologist should conclude that the amounts of bacteria in the two plates are different on average, on the basis of this result.

Figure 11. Rejection and non rejection regions for the hypothesis test about the difference of the mean absorbance values.
The probability of the rejection region is $\alpha = 0.1$.

Decision rule: Reject the hypothesis if the test statistic falls in the rejection region.

The $p$-value is equal to $P(T < -t_{observed} \cup T > t_{observed})$, where $T$ is the test statistic and $t_{observed}$ is the observed value of this variable in this particular test. In this example, $p$-value = 0.061. The null hypothesis should be rejected because this value is less than the level of significance: $p$-value < $\alpha$.

There are two types of errors in hypothesis testing: false rejection of a true null hypothesis (Type I error) and failure to reject a false one (Type II error). Type I error is committed if the user is unlucky enough to observe a value of test statistic that lies in the rejection region, despite the fact that the null hypothesis is true. The probability that this can happen is equal to the significance level $\alpha$. To lower this probability it is necessary to reduce the significance level. However, this increases the probability of type II error because it makes it less likely to reject the null hypothesis.

The choice of the probability of type I error depends on the consequences of committing it. Typical values of this probability are 0.01, 0.05 and 0.1.

The probability of type II error is denoted by $\beta$. It is difficult to determine this probability because, usually, one does not know the probability distribution of the test statistic if the null hypothesis is false. One way to reduce $\beta$, without affecting the probability of type I error, is to increase the sample size.

In example 1, there is a probability of 0.1 to conclude that the mean values of the absorbance levels of the control and the cleaned plates are different, while these values are actually equal.

Hypothesis tests can be classified into tests of hypotheses involving one or two populations. Example 1 involves two populations (control and cleaned plates). In addition, hypothesis tests can be classified into one-tail and two-tail tests. The alternative hypothesis in one-tail tests can be expressed in terms of and inequality, e.g.,
In two-tail tests the alternative hypothesis can be expressed in terms of an inequality, $\mu_1 \neq \mu_2$. Example 1 is a two-tail test because the alternative hypothesis is that the mean values of the control and cleaned plates are different.

A hypothesis test involves the following steps

1. State the null and alternative hypotheses.
2. Choose the acceptable probability of type I error $\alpha$ (level of significance) and the sample size(s).
3. Select the test statistic and determine its probability distribution.
4. Determine the critical values that separate the rejection and non rejection regions, or the significance probability.
5. Collect data.
6. Calculate the observed value of the test statistic (or $p$-value).
7. Reject the null hypothesis if the observed value of the test statistic lies in the rejection region (or the $p$-value is smaller than $\alpha$). Do not reject the hypothesis otherwise.
IV. Discussions and Results

a. Confocal Microscopy

The slides were scanned under a confocal microscope manufactured by Leica Microsystems. The model chosen was a TCS SP5 confocal laser scanning microscope. The TCS stands for True Confocal Scanner and the SP5 refers to the 5 SpectroPhotometer channels this system is equipped with. Using a 63x magnification with a zoom of 3.95 photographs was taken of the slides to prove the hypothesis.
The confocal microscopy was conducted at Whitey labs, St. Augustine under the guidance and help of Dr. Paul J Linser.

In the micrograph, the blue picture is one in which the spectrophotometer recognizes the fluorescence dye, DAPI and hence the bacteria DNA. The red and green pictures are the ones which were taken under different wavelengths of light. The grey picture is the actual one on grayscale.
Control Slides

Using the confocal spectrophotometer, localized pictures were taken at various random points on the surface of the control slide as well as the treated slides to better understand biofilm adherence before and after sonication treatment.

Figure 14. Confocal Microscopy picture of control slide
Figure 15: Confocal Microscopy picture at 30 s and 0.25" depth after bacterial removal

No bacteria present
Figure 16: Confocal Microscopy picture at 200 s and 0.5" depth after bacterial removal
The scale in all the micrographs is the same, i.e. 10μm. More confocal microscopy pictures were taken of the other slides for the following treatment conditions:

- 1” depth and 30 seconds of sonication
- 1” depth and 200 seconds of sonication
- 0.75” depth and 30 seconds of sonication
- 0.75” depth and 200 seconds of sonication
- 0.5” depth and 30 seconds of sonication
- 0.5” depth and 200 seconds of sonication
- 0.25” depth and 30 seconds of sonication
- 0.25” depth and 200 seconds of sonication

All the slides after treatment showed similar results as those shown above, namely there was no bacterial growth present.

Is it noticed that the above pictures are multi-colored. This is the basic principle of confocal microscopy wherein reflected light is split into different wavelength so as to offer a wide range of viewing spectrum. The blue-colored pictured is the image due to the light from the fluorescent dye; the red and green colored pictures are the same spot seen under different wavelengths of light, while the grey picture is what is seen under white light.

Comparing the above confocal micrographs of the slides before and after sonication treatment, one can draw a conclusion that there has been effective bacterial removal after controlled cavitation. In Figure 14 (control slide), there is evidence of abundant growth of bacteria on the control slide, seen as a patch growth in the picture. It is also noticed that the growth covers almost the entire picture, thereby giving an idea of the extent of possible growth.
On comparison, to the slides after controlled cavitation (Figures 15 and 16), it is noticed that the amount of bacterial growth remaining on the slides after sonication treatment is almost negligible. After complete scanning of the treated slides, there was very minimal bacterial growth found. This illustrates that controlled cavitation causes effective biofilm removal.

But micrographs cannot be taken as sound empirical data, but rather as a visual confirmation of the data collected from experiments. Thus to further prove that controlled cavitation causes effective biofilm removal, spectrophotometry readings were conducted.
b. Spectrophotometry Absorbance Results

After sonication, the removal rate of bacteria is calculated using a spectrophotometer. Using the absorbance value, a measure of how much bacteria is remaining can be figured out. The results are tabulated below:

**Bacteria Growth Period: 1 week and 3 days**

<table>
<thead>
<tr>
<th>Trial</th>
<th>Time (s)</th>
<th>Depth (inch)</th>
<th>Absorbance Values</th>
<th>AVERAGE ABSORBANCE VALUES</th>
<th>% difference with control</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>-</td>
<td>-</td>
<td>0.056</td>
<td>Control 0.0593</td>
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</tr>
<tr>
<td>C2</td>
<td>-</td>
<td>-</td>
<td>0.066</td>
<td>1&quot; and 30 s 0.0410</td>
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<td>0.5&quot; and 30 s 0.0440</td>
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<td>-</td>
<td>-</td>
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</tr>
<tr>
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<td>30</td>
<td>0.5</td>
<td>0.044</td>
<td>0.5&quot; and 200 s 0.0200</td>
<td>66.29</td>
</tr>
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<tr>
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<td>0.039</td>
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<td>0.028</td>
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</table>

**Table 3: Absorbance results for bacterial growth of 1 week**
Figure 17 Controlled cavitation results for a growth of 1 week
**Bacteria Growth Period:** 2 weeks and 3 days

<table>
<thead>
<tr>
<th>Trial</th>
<th>Time (s)</th>
<th>Depth (inch)</th>
<th>Absorbance Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>-</td>
<td>-</td>
<td>0.056</td>
</tr>
<tr>
<td>C2</td>
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<td>-</td>
<td>0.048</td>
</tr>
<tr>
<td>C3</td>
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<tr>
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<td>1</td>
<td>0.048</td>
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<td>0.034</td>
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**Table 4:** Absorbance results for bacterial growth of 2 weeks

<table>
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<th>Trial ABSORBANCE VALUES</th>
<th>% difference with control</th>
</tr>
</thead>
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<td>Control</td>
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<td>1&quot; and 30 s</td>
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<td>0.5&quot; and 30 s</td>
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<td>1&quot; and 200 s</td>
<td>0.0360</td>
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<tr>
<td>0.5&quot; and 200 s</td>
<td>0.0390</td>
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</tbody>
</table>

**Figure 18.** Controlled cavitation results for a growth of 2 weeks (1" and 0.5" depth)
**Bacteria Growth Period:** 2 weeks

<table>
<thead>
<tr>
<th>Trial</th>
<th>Time (s)</th>
<th>Depth (inch)</th>
<th>Absorbance Value</th>
<th>Control</th>
<th>Average Absorbance Values</th>
<th>% Difference with Control</th>
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<td>-</td>
<td>2.623</td>
<td>2.2595</td>
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<td>-</td>
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<tr>
<td>C2</td>
<td>-</td>
<td>-</td>
<td>1.896</td>
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<tr>
<td>1</td>
<td>200</td>
<td>0.75</td>
<td>1.207</td>
<td>1.500</td>
<td>1.3535</td>
<td>40.10%</td>
</tr>
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<td>200</td>
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<td>1.500</td>
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<td>1.274</td>
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<td></td>
</tr>
</tbody>
</table>

**Table 5:** Absorbance results for bacterial growth of 2 weeks

**Figure 19.** Controlled cavitation results for a growth of 2 weeks (0.75” and 0.25” depth)
The data from spectrophotometry provides conclusive proof of removal of bacterial. From the data, it is seen that greater the sonication time and smaller the distance between the slide surface and the bottom of the sonotrode, greater is the bacterial removal. It was noticed that there is a threshold depth beyond which this rule does not hold. It was noticed that once the depth was reduced to 0.25”, the amount of bacteria removed by means on controlled cavitation is much lesser than when the depth was 0.5”. Hence 0.5” depth can be assumed to be the optimal or threshold depth. To further confirm this tentative conclusion, more experiments and computational modeling needs to be done.

The data collected can be divided into two sets of data. Data set one includes the absorbance values for 1” and 0.5” depth, while set two includes the data for 0.75” and 0.25” depth of sonication. It is noticed that the magnitude of values for data set one is different from data set two. For data set two, the order of the values got was around 2 while for data set one, the order of the values got was much smaller. The reason for this large variation in the values is because during the growth phase, a more concentrated bacterial growth medium was used for the data set two experiment as compared to that for data set one. This caused the bacteria to proliferate rapidly consequently showing a large absorbance reading.
c. Measuring Bacterial Growth

Bacterial growth count methodology was done on three different conditions; on the control slide, a slide sonicated at 0.75” depth for 200 s, as well as a slide sonicated at 0.25” depth for 200 sec. The bacterial counting method described on page 25 was followed for this purpose. The results are tabulated below.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Colonies</th>
<th>Average number of bacteria/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^1$</td>
<td>39</td>
<td>390</td>
</tr>
<tr>
<td>$10^2$</td>
<td>13</td>
<td>1300</td>
</tr>
<tr>
<td>$10^3$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>$10^4$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>$10^5$</td>
<td>2</td>
<td>200,000</td>
</tr>
</tbody>
</table>

**Table 6.** Count of number of bacterial colonies on control slide

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Colonies</th>
<th>Average number of bacteria/mL</th>
<th>Difference between number of colonies between control and treated slides</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^1$</td>
<td>1</td>
<td>10</td>
<td>38</td>
</tr>
<tr>
<td>$10^2$</td>
<td>0</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>$10^3$</td>
<td>TNTC*</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>$10^4$</td>
<td>1</td>
<td>10,000</td>
<td>1</td>
</tr>
<tr>
<td>$10^5$</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

*TNTC – Too Numerous To Count

**Table 7.** Count of number of bacterial colonies on slide sonicated for 200 sec at 0.75” depth
<table>
<thead>
<tr>
<th>Dilution</th>
<th>Colonies</th>
<th>Average number of bacteria/mL</th>
<th>Difference between number of colonies between control and treated slides</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^1$</td>
<td>3</td>
<td>30</td>
<td>36</td>
</tr>
<tr>
<td>$10^2$</td>
<td>229</td>
<td>22,900</td>
<td>216</td>
</tr>
<tr>
<td>$10^3$</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>$10^4$</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>$10^5$</td>
<td>1</td>
<td>100,000</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 8 Count of number of bacterial colonies on slide sonicated for 200sec at 0.25” depth

After conducting the bacterial count, it can be summarized that the numbers of bacteria remaining on the slides have reduced after being treated to sonication. In actuality, the number of colonies should reduce while the dilution increases. For the last two cases, a possible reason for the erroneous number of colonies could be attributed to an error while pipetting out the 0.1mL of bacterial solution. But on the whole, the numbers make sense and thus prove our theory of bacterial removal after sonication.
d. Hypothesis Testing

Statistical Hypothesis testing was done for two different sets of data. The first set was with the absorbance values for 1" and 0.5" depth and the second set was with the absorbance values of 0.75" and 0.25" depth. Hypothesis testing was conducted using an Excel add-on called StatTools.

For all the cases, the null hypothesis considered was, "The mean of the absorbance reading for the control slides as well as the treated slides are the same". The alternative hypothesis states that the values are not equal. Rejection of the null hypothesis, would suggest that controlled cavitation does remove bacterial growth.

First Set: Data from 1" and 0.5" depth

| StatTools (Core Analysis Pack) |
| Analysis: Hypothesis Test |
| Performed By: Mathew C Mathew, |
| Date: Monday, February 22, 2010 |
| Updating: Live |

### Average absorbance value

<table>
<thead>
<tr>
<th>Sample Summaries</th>
<th>Control Slide</th>
<th>Treated Slides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Size</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Sample Mean</td>
<td>0.1900</td>
<td>0.031143</td>
</tr>
<tr>
<td>Sample Std Dev</td>
<td>0.3572</td>
<td>0.008840</td>
</tr>
</tbody>
</table>

### Hypothesis Test (Difference of Means)

<table>
<thead>
<tr>
<th>Hypothesized Mean Difference</th>
<th>Equal Variances</th>
<th>Unequal Variances</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypothesized Mean Difference</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Alternative Hypothesis</td>
<td>&lt;&gt; 0</td>
<td>&lt;&gt; 0</td>
</tr>
<tr>
<td>Sample Mean Difference</td>
<td>0.1589</td>
<td>0.1589</td>
</tr>
<tr>
<td>Standard Error of Difference</td>
<td>0.135060908</td>
<td>0.135060908</td>
</tr>
<tr>
<td>Degrees of Freedom</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>t-Test Statistic</td>
<td>1.1762</td>
<td>1.1762</td>
</tr>
<tr>
<td>p-Value</td>
<td>0.2623</td>
<td>0.2841</td>
</tr>
<tr>
<td>Null Hypoth. at 10% Significance</td>
<td>Don't Reject</td>
<td>Don't Reject</td>
</tr>
<tr>
<td>Null Hypoth. at 5% Significance</td>
<td>Don't Reject</td>
<td>Don't Reject</td>
</tr>
<tr>
<td>Null Hypoth. at 1% Significance</td>
<td>Don't Reject</td>
<td>Don't Reject</td>
</tr>
</tbody>
</table>

### Equality of Variances Test

<table>
<thead>
<tr>
<th>Ratio of Sample Variances</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1633.0603</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>
For this case, the hypothesis test did not reject the null hypothesis. But this is no indication of a failure in the procedure, but rather points to a lack in the number of data points. Usually hypothesis testing is done with large number of data points.

**Second Set: Data from 0.75” and 0.25” depth**

| StatTools (Core Analysis Pack) |
| Analysis: Hypothesis Test |
| Performed By: Mathew C Mathew |
| Date: Monday, February 22, 2010 |
| Updating: Live |

| Average Absorbance Value | 1.07063636363636 | 1.82927727272727 |
| Sample Summaries |
| Sample Size | 5 | 11 |
| Sample Mean | 2.3554 | 1.8293 |
| Sample Std Dev | 0.4475 | 0.3666 |

**Hypothesis Test (Difference of Means)**

| Hypothesized Mean Difference | Equal Variances | Unequal Variances |
| Sample Mean Difference | 0.5261 | 0.5261 |
| Standard Error of Difference | 0.211127407 | 0.228618694 |
| Degrees of Freedom | 14 | 6 |
| t-Test Statistic | 2.4920 | 2.3013 |
| p-Value | 0.0259 | 0.0610 |
| Null Hypoth. at 10% Significance | Reject | Reject |
| Null Hypoth. at 5% Significance | Reject | Don't Reject |
| Null Hypoth. at 1% Significance | Don't Reject | Don't Reject |

**Equality of Variances Test**

| Ratio of Sample Variances | 1.4895 |
| p-Value | 0.5540 |

For this case, hypothesis testing rejected the null hypothesis signifying that sonication treatment will remove bacterial growth on the surface of the slides. For lower levels of significance (1%), the null hypothesis is not rejected stating that there is a lack of data points to draw a more conclusive result.
V. Conclusions

After performing controlled cavitation on the various biofilm covered slides and performing spectrophotometry, confocal micrography and bacterial counting, it is concluded that:

- Controlled cavitation causes bacterial removal.
- Bacterial removal is dependent on the amount of treatment time, *i.e.* greater the time, greater is the bacterial removal.
- Bacterial removal is also dependent on the distance between the sonotrode and the biofilm surface.
- The maximum removal was noted at 0.5" distance between sonotrode and biofilm surface and at 200 s of controlled cavitation, approximately 61% removal.

From the above conclusion, it can be inferred that controlled cavitation does effective remove bacterial growth from the surface. This can be seen by referring to the data in Table 3, 4 and 5. In these tables, the percentage difference is shown, which equates to the amount of bacteria removed after controlled cavitation.

This conclusion was further reinforced by the bacterial counting method, which showed that bacterial colonies reduced after controlled cavitation, as well as from the confocal micrography, which showed that the slides were almost empty of bacterial colonies after controlled cavitation.

This illustrates that controlled cavitation can be used for removal of bacterial growth on a surface. Further experimentation is required to validate the repeatability of these conclusions.
A possible application of this result is onboard manned space missions and habitats. On extended space missions or in extraterrestrial human habitats, there is a high possibility of bacterial growth and consequent contamination and degradation of structural and other materials. In such situations, immediate replacement of materials is not a viable option. Thus to remove the bacterial corrosion, it is suggested to cover the affected area with an air-tight cover filled with water and controlled cavitation is performed on affected area. This would cause the bacteria to break away from the surface and be interspersed in the liquid medium, which can then be safely disposed of to prevent further contamination.

This method can also be used to provide in situ treatment of prosthetic body parts. Overuse of antibiotics by humans can cause a tolerance to the drugs by bacteria and hence they will become resistant to the drugs. In current methods of removal of bacteria from prosthetics using pharmaceuticals, the removal of bacteria is not complete. Therefore, often replacing the old prosthetics with a new one is the norm. This causes the patient to undergo extended recuperation in the hospital and overburden the patient with financial problems. If controlled cavitation can be effectively used, it will provide a remedy that will be quick and easy for prosthetic cleaning. For this, use of a proper isotonic medium without any harmful effects on the body as a result of controlled cavitation has to be developed. Then if controlled cavitation is directed towards the surface of the implants where bacterial growth is seen, there will be complete removal of bacteria.
VI. Future Work

Future researchers can use this data as a basis and build up on this experiment. One possible experiment could be to grow bacterial biofilm on titanium implants, to simulate bacterial growth inside the human body, and then do controlled cavitation to check the bacterial removal rate as well as the effect of cavitation on titanium implants.

Another experiment could be to grow bacteria on fresh meat and do controlled cavitation to document the effect on tissues and cells. This could give a better understanding of possible harmful effects on the human body, if this methodology is to be used for cleaning titanium implants within the human body.

Another experiment would be to check the effect of microgravity and radiation on the growth of biofilm growth and then perform controlled cavitation to measure the amount of biofilm removal. This data would be very valuable if this methodology is going to be used onboard spacecrafts.
Bibliography


19. Documentation from: http://www.ifr.ac.uk/bacanova/project_backg.html


29. United Stated Patents 3013467, Microscopy Apparatus


APPENDICES
Slide Jig Holder Base
Title: Slide Drawing
Scale: 1:2
All dimensions in mm

Slide Drawing
Appendix B:

Confocal Microscopy Pictures of control slides at various positions