Effects of ultrasonic exposure on growth and attachment rates of a capsule-forming bacterium
by Teresa Ann Campbell

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemical Engineering
Montana State University

Abstract:
The initial goal of this project was to study changes in the growth rate of a capsule-forming bacteria (Pseudomonas aeruginosa) with exposure of a culture to low intensity ultrasonic waves. Preliminary experiments had earlier indicated that the possibility existed for increasing the growth rate by "scraping" the capsule layer from the cell. This might reduce the resistance to mass transport, resulting in a higher growth rate. Because the bacterial cell also uses this capsule layer to attach to surfaces, attachment ability was also studied.

Growth rates of cultures were measured by viable cell counts. Cell concentration measurements of sonicated samples were compared to concentration measurements of control cultures exposed to identical environmental conditions with the single exception of the absence of ultrasonic exposure. Although some experimental runs indicated a beneficial effect of ultrasound on the growth rate of the culture, the majority of the data showed that sonication was actually decreasing the growth rate. In addition, these "positive" experimental runs could not be duplicated, even under identical conditions. Varying such parameters as temperature, exposure intensity, exposure duration, or nutrient concentration also did not produce evidence of growth rate enhancement.

Surface attachment of cells after exposure to ultrasound was studied using a specialized flow system. Cell attachment from sonicated cultures was compared to controls at identical environmental conditions. The flow system used in this series of experiments was still in the development phase, so precise quantitative results were not attainable. However, even after taking experimental error into account, results do indicate a marked decrease in attachment after exposure to ultrasound. Additional experimentation was conducted which indicated that this decrease in ability to attach might be passed on to daughter cells, even without additional exposure.

Although precise determination could not be made as to whether or not ultrasonics could be used to decrease the capsule layer of bacteria, some key conclusions can be drawn. First, bacteria can grow and reproduce in a low intensity ultrasonic field, although at a slightly retarded rate. Second, ultrasonic exposure seems to diminish the ability of cells to attach to surfaces, a characteristic which might be passed on to daughter cells.

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by

Teresa Ann Campbell

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APPROVAL

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Teresa Ann Campbell

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style and consistency, and is ready for submission to the College of Graduate Studies.

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Date May 28, 1991

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ABSTRACT

The initial goal of this project was to study changes in the growth rate of a capsule-forming bacteria (Pseudomonas aeruginosa) with exposure of a culture to low intensity ultrasonic waves. Preliminary experiments had earlier indicated that the possibility existed for increasing the growth rate by "scraping" the capsule layer from the cell. This might reduce the resistance to mass transport, resulting in a higher growth rate. Because the bacterial cell also uses this capsule layer to attach to surfaces, attachment ability was also studied.

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INTRODUCTION AND BACKGROUND

The first American Institute of Chemical Engineers symposium on ultrasonics was held in Boston, Massachusetts in May of 1950 and was soon followed by another in Columbus, Ohio in December of that same year (1). The Boston symposium was "developed with three specific objectives in mind: 1) to point up the potentialities of sonic and ultrasonic energy, 2) to emphasize known limitations, and 3) to stimulate investigations and reports of further work in this field." Topics discussed in this symposium included application to aerosol collection problems, colloidal effects of ultrasonics, and testing and gaging of materials. The Columbus symposium included topics such as biological effects, power requirements and applications to industrial processing.

As interest in ultrasonics grew, so did the range of applications. By 1971, the term "sonochemical engineering" had been coined to describe the "application of sonic and ultrasonic waves to chemical transport and processing operations" (2). In that year, H. Scott Fogler edited a symposium by the American Institute of Chemical Engineers to report on some of the "unusual phenomena" that had been discovered to be associated with ultrasonic waves. For the

most part, topics in this symposium dealt with mixing, drying, viscosity control, and increases in catalytic reaction rates, all common processes in industrial chemical engineering. The general consensus of this symposium was that ultrasonic energy can greatly affect, often beneficially, typical processes within the field of chemical engineering.

Ultrasonic waves are sound waves above the audible frequency range. The accepted "breaking point" between sonic and ultrasonic waves can range anywhere from a frequency of 16 kHz to a frequency of 20 kHz (1, 2), but since there are no fundamental differences between ultrasonic and sonic energies, the "dividing line" is immaterial.

Sound waves are longitudinal waves, and thus vibration of a particle in the medium is in the direction of wave propagation. The word 'particle' can refer to an actual particle suspended in the medium (most often a liquid), or a fluid particle. The accepted definition of a fluid particle is a volume of fluid in which the pressure, temperature, density, and velocity are considered equal for each molecule.

As sound waves propagate through a medium, they create zones of low pressure and temperature and zones of high pressure and temperature. When passed through liquids or gases, sound waves have produced some interesting effects,

including cavitation, acoustic streaming and interfacial instabilities.

Cavitation is the most studied of these effects, perhaps because it is the most easily observed. Cavitation is the formation, growth, and collapse of tiny bubbles in liquids. When cavitation occurs, the rarefaction portion of the ultrasonic wave is lower in pressure than the vapor pressure of the liquid, causing tiny bubbles to be formed. However, when the high pressure zone propagates through the medium, many of these bubbles will collapse, generally quite violently.

Three types of bubbles have been observed, stable resonant, unstable resonant, and collapsing (3). Resonant bubbles are seen to expand and contract with the sound waves in an oscillatory fashion for an indefinite number of cycles. Resonant bubbles are stable if the average size of the bubble is independent of time. Resonant bubbles are unstable if they continue to grow with each successive wave until they reach an unstable size and collapse. Collapsing bubbles are seen to grow very large and collapse in one cycle. The most fascinating aspect of cavitation is the energy generated by the collapse of cavitation bubbles. While measurements are impossible to obtain directly, the pressure may reach twenty-thousand atmospheres and the temperature may reach 10,000^{o}C (4). These regions of high temperature and pressure may be responsible for the

acceleration of chemical reaction rates as well as heat and mass transfer rates.

Acoustic streaming has been less studied, and thus is less well understood. Basically, acoustic streaming refers to an observed pattern of time independent circulations in sonicated fluid, resulting in a "gentle" mixing pattern at all but the highest sonication intensities (2). What exact effects, beneficial or detrimental, acoustic streaming may have on liquid systems are not completely known or understood at this time.

The third major effect of ultrasonics is interfacial instabilities. This effect is due to the fact that particles at the interface of two liquids are subject to oscillations in and out of the two liquids. One result of these oscillations is the emulsification of "immiscible" liquids. While it could be argued that the collapse of cavitation bubbles could also cause emulsification, emulsions of mercury and water have been produced by sonication without the occurrence of cavitation (5).

One of the earliest studies of ultrasonics on biological cell systems was performed by T.F. Hueter (6) as reported in the 1950 Columbus symposium. However, the viability of the cells after ultrasonic irradiation was not a consideration. In most of Hueter's experiments, cells were killed with ultrasonics and then studied. This is typical of the history of ultrasonics and biological cell systems. In

fact, today, ultrasonic exposure is one of the most common methods of cell destruction. In order to study the internal components in cells, the membrane can be disrupted with high intensity ultrasound and then the various constituents can be centrifuged for separation and analysis. Thus, it is known that the effects of ultrasound can produce cell death. However, very little is known about the effects that ultrasound can have at low intensities on living cells.

As previously mentioned, one of the effects of sonication can be an increase in chemical reaction rate. This is illustrated in the work of Kenneth S. Suslick of the University of Illinois at Urbana-Champaign (7). The majority of work in this study dealt with high intensity sound waves and the benefits that cavitation can have on inorganic systems. Although some effects were observed at low intensity, all of the dramatic effects observed came from the fact that as the cavities collapse, the temperature and pressure reach short lived, but extremely high levels. Thus, it only makes sense that many reaction rates will increase as it has been known for quite some time that nearly all chemical reaction rates are temperature dependent. It might seem from these results that since the metabolism of a cell can be thought of as simply a series of chemical reactions, the growth rate could be significantly increased with this higher intensity sonication.

Unfortunately, there are several other aspects to take into consideration before applying these ideas to living systems.

A living cell is a very delicate system, composed of very large and complex organic molecules, a situation not explored by Suslick. According to Heat Systems, one manufacturer of ultrasonic machines, the larger the molecule, the less likely that it will be able to withstand sonication (8). Therefore, it is only reasonable that larger organic molecules and bacterial cells would be more likely to be disrupted by sonication. However, the fact that some inorganic reactions can be accelerated is reason enough to believe that there may be a possibility of accelerating biochemical organic reactions, and thus cell metabolism, under certain controlled conditions.

Perhaps the most important consideration when dealing with living cells and potentially hazardous conditions is the strength and durability of the cell membrane. A diagram of a typical bacterial cell membrane is given in Figure 1. The internal components of the cell are contained in a fluid called the cytoplasm, which is contained within the cell membrane. The cell wall gives structure to the cell and determines the shape. The majority of the molecules in the membrane are phospholipids which are held together by hydrophobic interactions, not actual covalent bonds. Other molecules contained within the membrane

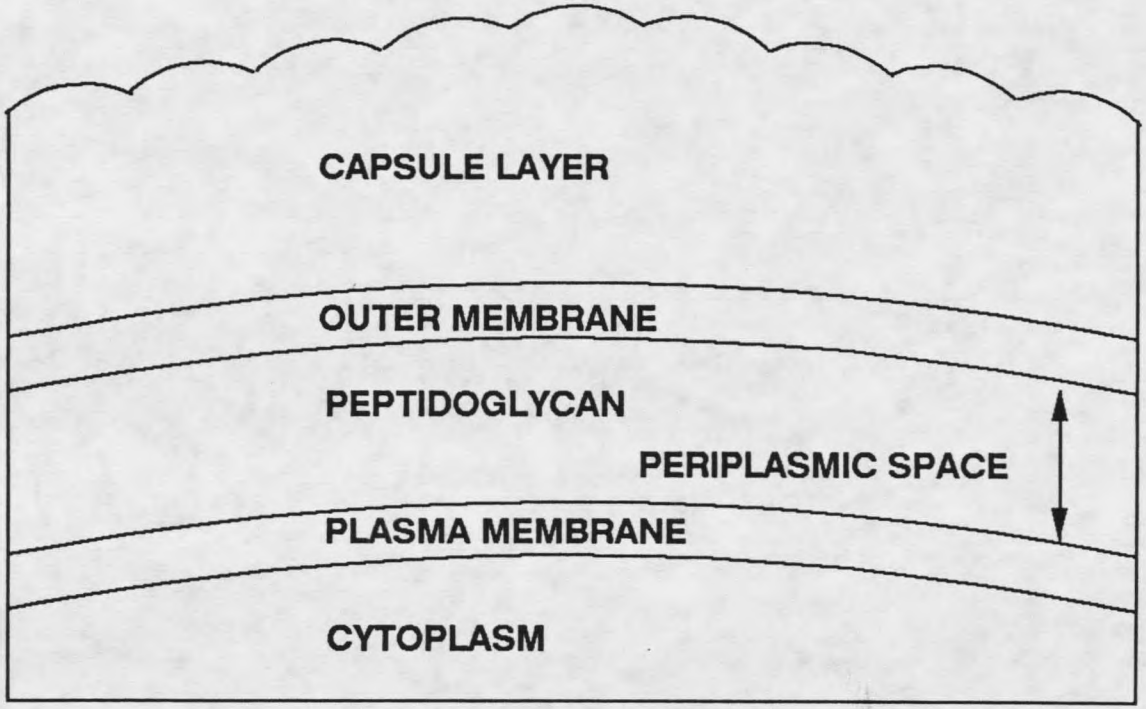

Figure 1. Outer Cellular Structure

include transport proteins, which are responsible for taking in nutrients and disposing of waste products. However, these molecules are not actually bonded to the membrane, but instead are held in place in a sort of a fluid mosaic pattern. It can easily be seen that the membrane of the cell would be one of the weaker parts of the cell and thus likely to be disrupted with ultrasound.

As shown in Figure 1, the exterior of a bacterial cell can be covered with a substance known as a slime or capsule layer. This layer can vary in thickness up to three times the width of the cell itself. The capsule layer is generally composed of lipopolysaccharides and does not have a rigid structure. While this layer serves no known purpose for cell metabolism, it does allow the bacterial cells to adhere to surfaces and to grow together in colonies, thus serving a beneficial purpose by allowing the bacteria to remain stationary. However, it has been proposed that if the capsule layer is thick enough, it may prove to be a hindrance to cell metabolism in that necessary nutrients would have to diffuse through this excessively thick layer in order to get to the cell wall and membrane.

As previously mentioned, sound waves produce vibratory motion in the direction of wave propagation. Due to differences in particle characteristics, the amplitude of vibration is not constant for all particles in the system. These differences can lead to significant shear stresses

between particles and the surrounding media. These differences in amplitude occur at all intensities as do the shear stresses that they produce.

This research project was designed to take into account the properties of the cell as well as the effects of ultrasound. As the effects of ultrasound at a low intensity should be less damaging than those at high intensity, it was speculated that low intensity ultrasound might produce the same vibratory particle movement as high intensity, but result in a gentle scraping of the capsule layer instead of cell destruction. This scraping could either reduce the thickness of the capsule layer, or remove it all together, which would then reduce the resistance to mass transport. This lower resistance should then manifest itself as a higher metabolism which could be observed as an increase in the culture growth rate. By carefully controlling other environmental parameters such as temperature and nutrient availability, any difference between the concentration of a sonicated culture and the concentration of a control culture should be attributable to the effects of the sonication. In this way, growth enhancement due to sonication could be both observable and measurable.

MICROBIOLOGY

The living microbial cell can be thought of as an extremely sophisticated chemical reactor, involving over 1000 reactions in an open operating system (9). Each of these reactions fulfills some purpose of cellular life. Due to the complexity of this system, there is no feasible way to look at the detailed effects of a change in any one parameter on the entire system. One would have to choose between either looking at the effects on the system as a whole, or on each individual reaction system within the cell. Therefore, in the present study, the effects of ultrasound on cells are analyzed for the impacts on cells as whole systems and not for the distinct internal processes of microbial life. Thus, the detailed knowledge of microbiology necessary for the current project is limited and highly specific towards the particular bacterium and its particular growth needs and characteristics.

Pseudomonas Aeruginosa

The bacteria chosen for this study is a strain of Pseudomonas aeruginosa. P. aeruginosa are straight rods, approximately 1 micron in diameter and 1.5 to 2.0 microns in length. They are motile bacteria, the motility being

provided by the one flagellum, or "tail", on one end of each cell. They are very adaptable, and they tend to grow well on all usual laboratory culture media. Like many microorganisms, they require water, oxygen, and a temperature-controlled environment for significant growth to occur.

As mentioned, P. aeruginosa are a rod-shaped bacteria. Since the major thrust of the current project is aimed at enhanced transport through the capsule layer and cell wall, the shape of the cell is very important. The more surface area per unit volume a cell has, the more likely that a transport benefit would be seen. However, it is known that the effects of ultrasound can be violent and that the smaller, more compact cells can generally withstand it much better (10). This would mean that cocci, or spherical bacteria might be best for this study. Unfortunately, these have the least surface area per unit volume of any shape. Thus, the rod-shaped cells are somewhat of a compromise between the two extremes. They provide the added surface area while still retaining a compact shape.

Also, P. aeruginosa are Gram-negative. This test distinguishes between the two common types of cell walls. The Gram-negative wall was depicted in Figure 1. In Gram-negative bacteria, there is a second membrane around the cell wall. This has various effects on the cell, one of which is to give the cell added protection, again perhaps

adding to the ability of the cell to withstand ultrasonic exposure.

Finally, P. aeruginosa are known capsule producers. The main thrust of this project is to increase cell growth rates by increasing mass transfer through the slime layer. Thus, this attribute is of prime importance to the current study.

Growth and Metabolism

Microorganisms require a variety of conditions and nutrients in order to grow and reproduce. One important condition is temperature. While a wide range of viable temperatures exists for most microorganisms, there is a relatively narrow band which will result in the maximum growth rate. The optimum temperature is the temperature at which the microorganism will grow the fastest. Figure 2 illustrates how a change in temperature can alter the growth rate (11). P. aeruginosa, the bacteria used for this study, are able to grow in temperatures from 15 - 50 C with optimum growth occurring at 37 - 42 C (12).

The growth of a bacterial culture can be defined as an increase in the number of cells, and the growth rate is the change in cell number per unit time. The growth rate is not constant, but will change with concentration and environmental conditions. A typical growth curve of a batch culture is shown in Figure 3. As shown, the culture will go

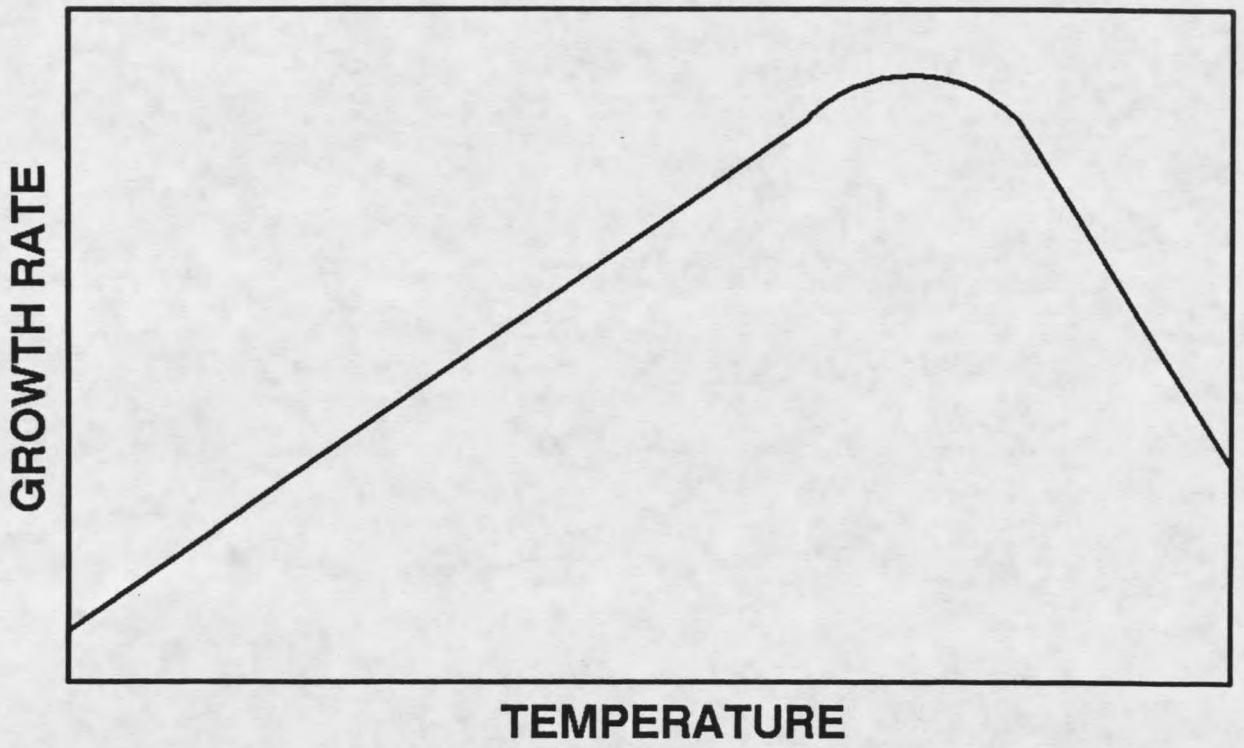

Figure 2. Bacterial Growth Rate versus Temperature

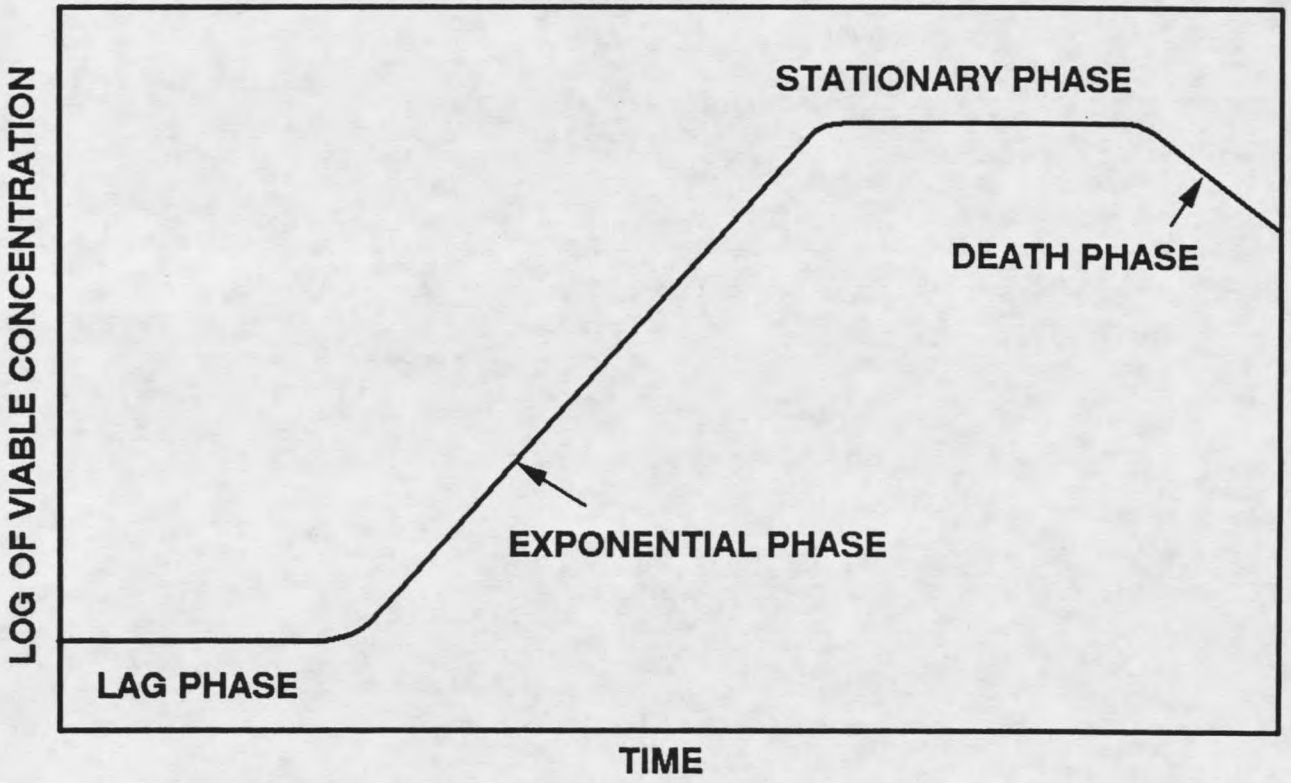

Figure 3. Typical Bacterial Growth Curve (Base 10 Logarithm of the Viable Cell Concentration versus Time

through a lag phase, an exponential phase, a stationary phase, and finally, a death phase. This type of curve is typical of a batch culture that has been freshly inoculated from a parent culture itself in a stationary phase. If the parent culture had been in an exponential phase and then inoculated into a fresh batch of the same media, an exponential phase would have continued. The reason for the lag phase from a stationary parent is due to the condition of the culture in stationary phase, as explained below.

Growth of bacteria in an adequate media is characteristically exponential for a period of time (11). However, it is obvious that this cannot continue forever. The generation time is the time required for the number of viable cells in a culture to double, with twenty minutes being common for many bacterial strains. If exponential growth could continue indefinitely, a typical laboratory culture with a generation time of twenty minutes would produce a population weighing about 4000 times the weight of the earth in just forty-eight hours (13). As the concentration of the culture increases, either a necessary nutrient is depleted or a waste product is accumulated to a level that inhibits further growth, and exponential growth ceases. When this happens, the constituents of the cell that are used for reproduction deplete in concentration within the cell. The bacterial cell is extremely conservative in that it only produces what it actually needs. Therefore, when the environmental conditions are

such that the rate of metabolism of nutrients is decreased, the production of enzymes necessary for synthesis is decreased. When cells in this condition are inoculated into fresh media, a lag time is required to resynthesize enzymes. A lag time also occurs for damaged or injured cells where the time is required to repair the cell. In addition, cells transferred from a rich medium to a poorer one also undergo a lag phase as the balance of the various enzymes must be adjusted to the nutrient concentrations in the new medium.

Attachment

As mentioned previously, P. aeruginosa are motile bacteria, meaning that they have the ability to move themselves through a medium. This gives them the ability to adapt to their environment to the extent that they can move short distances to a more desirable location. Unfortunately, in a liquid medium, a cell in suspension is at the mercy of the currents and any bulk flow that may be occurring. Thus, even though it can propel itself, to some extent, to desirable locations, the cell requires a means of staying there. This is accomplished through attachment. Attachment is the process by which the cells adhere to surfaces, generally by means of either the flagella, the capsule layer, or both. Once attached, the cell can acquire nutrients from the liquid media, allowing for growth,

reproduction and the production of additional capsular lippopolysaccahride. This is the beginning of a "biofilm" and results in "biofouling". Biofouling can result in decreased efficiency and significant corrosion in industrial processes and thus can be a very serious problem.

LITERATURE SURVEY

To date, nearly all of the work relating ultrasonics to biological systems has dealt with the destructive effects of cavitation. In cavitation, tiny bubbles are formed by the rarefaction portion of the wave where the pressure is low and are collapsed by the high pressure crests. The violence of the collapse causes extremely detrimental effects on biological systems, such as cell lysis, and thus cavitation was avoided whenever possible for the current project.

Cavitation is enhanced by several factors, including dissolved gases and particles in solution (10). However, it has also been found that these factors which enhance cavitation also tend to reduce the severity of effects if cavitation does occur (10). Therefore, for this study, if some cavitation did occur due to dissolved gases and the presence of the bacteria themselves, there is some reason to believe that the cells would be able to survive anyway, provided the intensity of exposure was not too strong.

There have been many misconceptions regarding ultrasonics in the past. One of these is that ultrasonics could cause DNA depolymerization (14). This was found to be incorrect by several investigators (14-19). The reason for

wrong conclusions in the literature was due to errors in experimental interpretation. Sound waves are a form of energy and will dissipate through a medium in the form of heat. Heat can and will cause depolymerization, so the damage observed was due to the temperature increase and not the sonication itself. One opponent of damage due to ultrasonics stated that "neither pulsed nor continuous wave application of ultrasonics cause chromosome damage" (20).

It was also thought that enzymes could be inactivated by ultrasonics (21). However, it has since been found that the observed deactivation was again due to the temperature increase and not the sonication itself (12-25).

There are also other effects of sonication that could have beneficial results on microbial systems. One such effect is micro-streaming. When the bubbles of cavitation form in liquid systems, they do not always collapse immediately but will often build in size until they reach a critical size and then collapse. When this occurs, the fluid is seen to eddy about the bubbles and suspended particles are seen to rotate about an equilibrium axis without damage to the particles themselves (26). This could indicate that if cavitation did occur, but at a low intensity, the bacterial cells could withstand the treatment.

Another effect of sonication on biological cells is that in some instances, the cell walls of bacteria have appeared

thinner (10), indicating a significant effect on the outer surface of the cell. One investigator even suggested that "it may be possible ... to strip a polysaccharide outer coating of a bacteria cell wall, with very low intensities and without injury to the cell itself." (10).

Lastly, it has been shown that shear stress may increase mass transfer in systems of biological cells. The hemoglobin in blood is normally found only within the red blood cells and not in the plasma. In fact, hemolysis, or the release of hemoglobin into the blood system can be very dangerous to human beings. However, hemoglobin has been found in solutions of blood after exposure to shear stresses without breaking of the cell walls (27), indicating a transfer through the cell membrane. Admittedly, the external structure of mammalian cells is much thinner and much less rigid than bacterial cells, but the fact that mass transfer was accomplished without cell destruction shows some indication that mass transfer may be enhanced by ultrasound without destruction to cell membranes.

All of this information indicates that very little is actually known about the effects of low intensity ultrasonic energy on living systems. However, if ultrasonic energy could be applied at a level which would not kill bacterial cells, but would still create vibratory motion of the medium, it could be possible to enhance growth through sonication by increasing the metabolism of the culture.

RESEARCH OBJECTIVES

This research was undertaken to explore the possibilities of enhancing bacterial growth through the ultrasonic removal of the cell capsule layer. Due to the exploratory nature of this project, other objectives were developed as additional information was obtained. These included inhibiting attachment of cells to surfaces by the removal of the capsule layer.

Initially, the primary goal of this work was to show a increase in the growth rate of the bacteria after exposure to ultrasound. Based on initial results, other parameters, such as temperature and nutrient concentration, were to be evaluated for their effect on growth enhancement.

The primary goal at the end of this work was to show a decrease in the attachment of bacteria after exposure to ultrasound, which could indicate a partial removal of the capsule layer. These results can be used to identify a suitable course for future research.

EXPERIMENTAL APPARATUS

The Sonicator

All sonication in this study was done using a Heat Systems Ultrasonic Sonicator with a one-half inch titanium horn. While the frequency of this machine is not variable, being set on 20 kHz, there is some room for exposure regulation by controlling the output intensity, the percent duty cycle, and the cycle time.

The output control regulates how much of the power of the system is actually put out through the horn, thus controlling the sonication intensity. Settings are from zero to ten, which proportionally represent intensities from zero to 450 watts through the half-inch horn tip. Control from zero to one was not judged as precise. Since the amplitude of vibration and cavitation effects are directly related to the intensity, this control was always set relatively low, usually on one, or roughly 50 watts.

The machine is also designed so that sonication can occur for intermittent cycles. This is done using the cycle time and the percent duty cycle. The cycle time indicates how long the complete cycle of sonication and silence will

last. The choices on this system are one, two, or five seconds, or continuous. "Continuous" is the setting used if cycles are not desired. The percent duty cycle determines the fraction of the total cycle time during which sonication will occur. The range for this is from ten to ninety percent. Thus, a cycle time of five seconds and a percent duty cycle of ten percent would result in sonication for 0.5 seconds and silence for 4.5 seconds.

To ensure even exposure of all of the bacterial cells to the sonication, a continuous flow cell was used. The majority of the effects of ultrasound are felt within approximately one-quarter of an inch from the probe itself, making intimate contact with the probe by all the cells very important. By using the flow cell, as shown in Figure 4, this can be accomplished. For these experiments, flow entered the chamber of the cell using the bottom port and exited the cell using the higher side port, ensuring that all liquid, and thus all cells, came into close contact with the probe, giving the most even exposure possible.

The Flow System

The experimental setup for these experiments is shown in Figure 5. A constant temperature water bath was used to

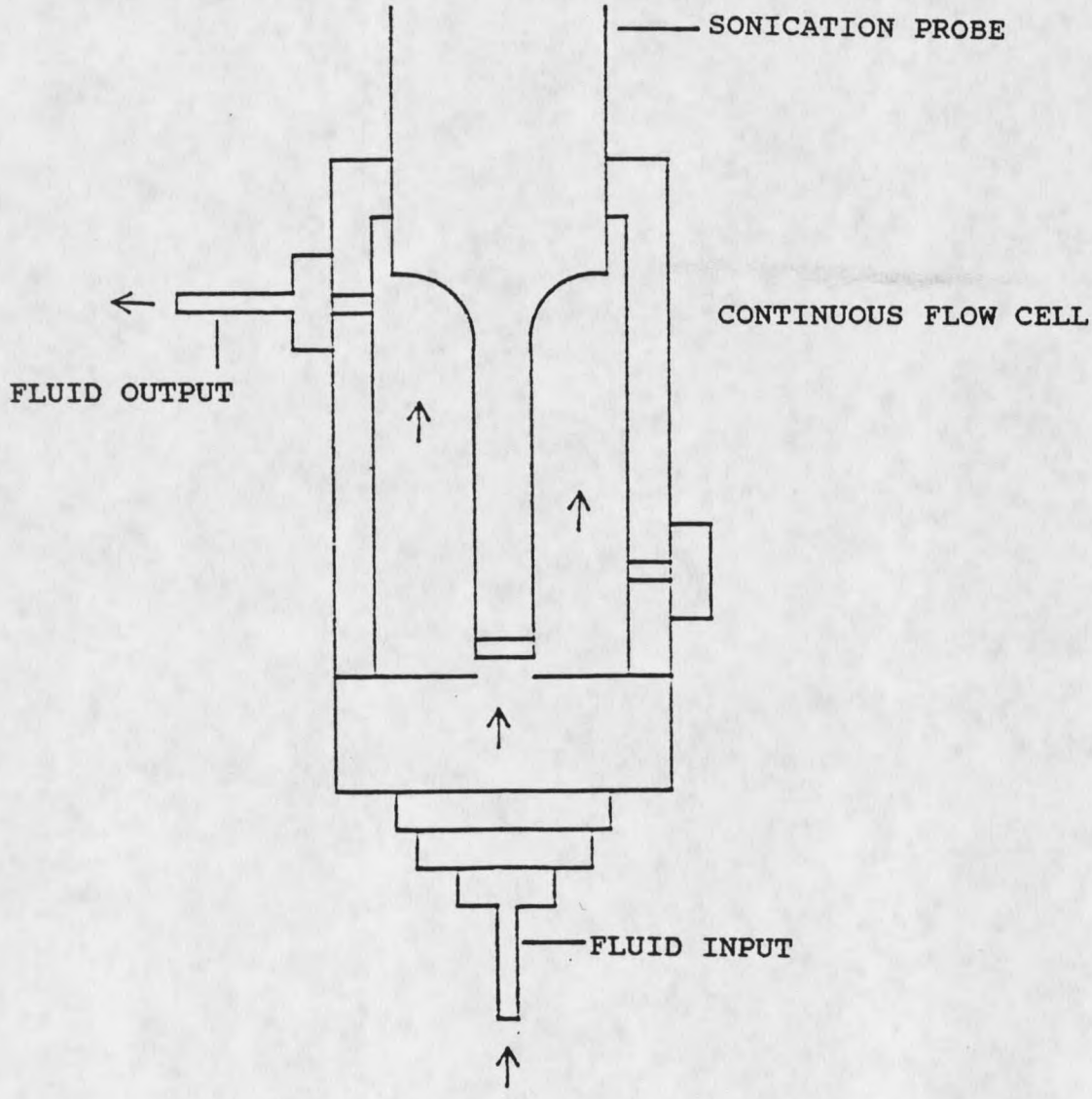

Figure 4. Sonication Probe with Continuous Flow Cell

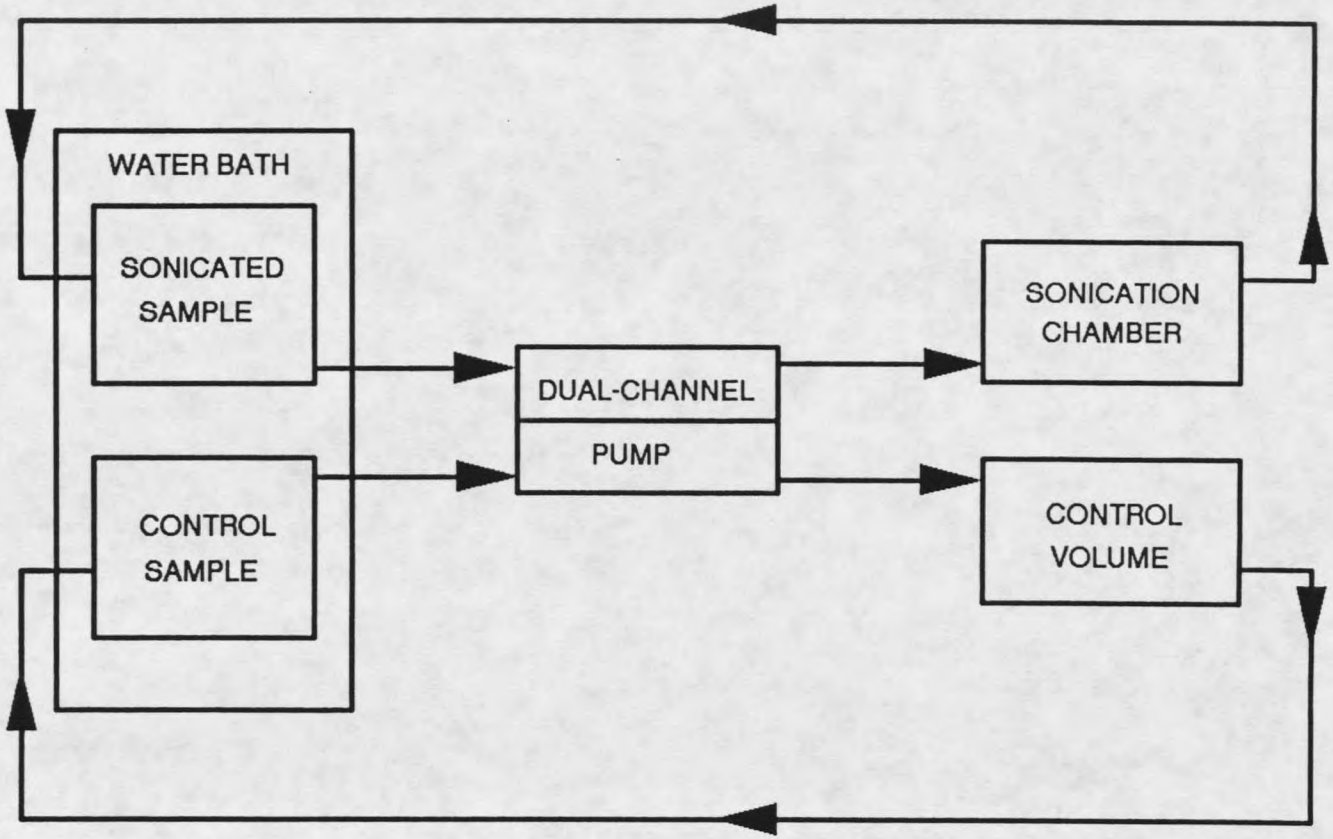

Figure 5. Experimental Apparatus

maintain and regulate temperature for the system. A Masterflex (trademark) peristaltic pump model N-06241-20 was used to force flow through the sonication cell and to provide mixing in the system. The 1 to 100 rpm model was chosen because it was thought that the 6 to 600 rpm model might cause excessive shear stresses in the liquid within the pump head. Since the basis of the experiments was to alter the growth characteristics of the cells through shear stress provided by the sonication, additional stresses were not desired, and the 1 to 100 rpm model was chosen.

For the sonicated sample, fluid was drawn out of the flask, through the pump, through the sonication cell, and back to the flask. The flow line for the control sample was identical with the exception of the sonication cell. Since there was not a sonication cell for the control line, the cell was replaced by a control volume, a stoppered test tube that had a volume as close to the volume of the sonication cell as possible. Volume was still a small amount less than the sonication cell, so the additional volume needed to give both lines the same total volume was obtained by using additional tubing. Since the flow configuration for both lines was nearly identical, any differences in growth would have to be due to the sonication itself.

Wide-mouth 500-ml Erlenmeyer flasks with specially designed stoppers were used for these experiments. The stoppers and entry/exit tubes were designed to maximize

mixing of both the liquid and gas portions in the flasks. This is shown in Figure 6. The fluid was drawn from the bottom of the flask on one side and returned near the top of the liquid level on the other. As for the gas line, pure oxygen was put in near the gas/liquid interface and allowed to escape near the top of the flask. As the gas outlet was the portal in the system open to the surroundings, a short piece of tubing was attached with the open end facing down to prevent air-borne contamination from entering the system.

The flasks could contain a maximum of about 550 ml, including both the gas and liquid phases. This gave an operating volume in the flasks of about 350 ml liquid and 200 ml gas. The sonication cell contained 60.5 ml of fluid, while the entire flow loop contained approximately 100 ml of liquid when size 17 (0.322 cm^2 cross sectional area) silicon tubing was used. The silicon tubing was chosen because it is compatible with biological systems and because it has a relatively long "tubing life" within the pump head (825 hours at 100 rpm). A high flow rate with a low flow loop volume was desired, so size 17 tubing was chosen. Size 16 tubing has a maximum flow rate of 167 ml/min at 100 rpm (as opposed to about 250 ml/min with size 17) while size 18 would have resulted in an increase in flow loop volume of approximately 30 percent. Thus, the size 17 tubing was judged to provide the best compromise for operation of the present system.

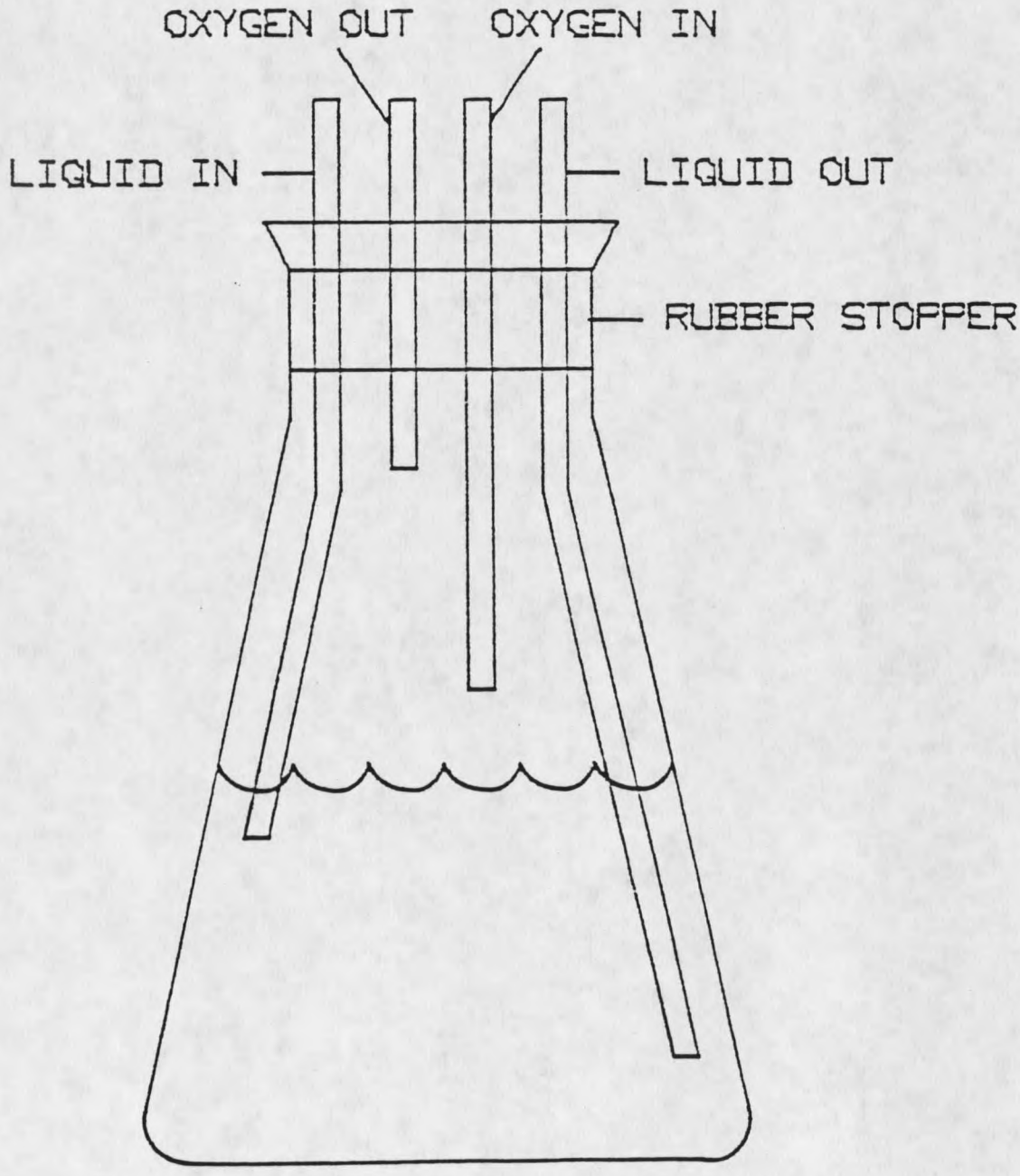

Figure 6. Reaction Flask

EXPERIMENTAL PROCEDURES

Preparation

Before any experimentation could be preformed, a substantial amount of preparation was required, including sterilization of the system, pouring of petri plates, and preparation of dilution blanks, nutrient broth, and bacterial culture solution.

Before each run, the entire system had to be dismantled, washed, partially reassembled, and heat sterilized. After washing, each section of the system was assembled as much as possible before sterilization in the autoclave. This was done to minimize manipulation of sterile components thus minimizing the chance for contamination during assembly. Next, each piece was wrapped in aluminum foil and then sterilized in the autoclave for at least 20 minutes. In addition to the overall wrapping, each end of each section of hose and all of the exposed fittings were also wrapped in foil. This was done to keep each section of the system sterile while being assembled. Sterilization had to be done to each piece of the system that came into contact with the

bacterial solution. The only exception to this was the sonication probe itself, which was cleaned with 95% ethanol.

The petri plates were poured as described in the Laboratory Techniques section of this report. However, in the planning stage of each experimental run, it had to be decided how many plates would be needed. Since data was to be taken from both the sonicated and control lines, and each data point needed three plates of three different dilutions, a total of 18 uncontaminated plates were needed for each data point to be taken. To allow for contamination and errors in plating, 20 plates were poured for each data point.

The dilution blanks were also prepared prior to each run. However, due to the exceptionally high number of samples and plates needed in a single run, additional blanks had to be made during the course of the run.

Schussners Mineral Salts Media, a common nutrient source growth media for many types of bacteria, was prepared and used for the bacterial nutrient broth. This broth is made by mixing the following ingredients in one liter of distilled water:

7.0 g dibasic potassium phosphate

3.0 g monobasic potassium phosphate

1.0 g ammonium nitrate

0.1 g magnesium sulfate

0.1 g glucose

After mixing, this solution was covered and sterilized in the autoclave.

The preparation of the bacterial culture began two days before the experiment was to take place. On this day, a cryovial of P. aeruginosa was obtained from Cooley Laboratory, thawed, and inoculated into approximately 300 ml of sterile nutrient broth using a sterile syringe. This flask was placed either in the incubator or the water bath at 35 C. After approximately 24 hours, the flask was placed in the refrigerator until use. Depending on the scheduled starting time the next day, the duration of refrigeration was from 10 to 24 hours. Since the purpose of refrigeration was to slow growth, the exact time had little effect on the outcome of the experiments as long as it was not too extensive.

Running the Experiment

The day of the experiment, a volume of approximately 460 ml of sterile nutrient broth was placed into each of the two sterile wide mouth Erlenmeyer flasks. The system was then assembled in the water bath and flow was started. A specified amount of the bacterial solution (usually 5.00 ml) was then added to each flask. This was done using a pipetor to ensure that each flask received exactly the same amount.

Also, prior to dispensing, the bacterial solution to be used for inoculation was mixed to ensure homogeneity within the solution so that the reaction flasks would have as close to identical concentrations as possible. Five minutes after the bacteria were added to the reaction system, the initial sample was taken and sonication began. This five-minute time interval allowed for mixing within the flow system, and since the flow rate was approximately 250 ml/min, five minutes allowed for the entire system to circulate two and a half times, and thus was considered adequate.

Sample times varied from experiment to experiment and are recorded in the raw data accordingly. All times recorded were rounded off to the nearest 5-minute interval. Initially, sampling was done using a long-needle syringe. However, it was discovered after the first few runs that samples could be taken using a pipetor with equal sterility and more accuracy. Therefore, after the third run, all samples were taken using a pipetor.

Oxygen flow to the gas line was started immediately after the initial sample was taken. Since a pure oxygen atmosphere was not necessary, the flow was kept as low as possible and was meant merely to replenish the oxygen consumed by the bacteria. The flow rate was controlled at approximately 10 ml per minute.

LABORATORY TECHNIQUES

Certain laboratory techniques were necessary for the acquisition and subsequent analysis of experimental data. These included methods for sterile manipulation of cultures, preparation of agar plates, maintaining stock cultures, culture identification, and viable cell counts.

Sterilization

Sterilization was a primary consideration for every phase of this project. For sterilization, an autoclave was used. This is an oven-type apparatus that uses moderate temperature (250 C) and pressure (15 psig) steam to kill any micro-organisms that may be present. The sterilization cycle is identical for both liquids and solid apparatus. Both raise the temperature and pressure and maintain them at the elevated levels for a specified period of time, such as twenty minutes as was used for the current study, however, the cool-down cycle is different. For liquids, the pressure must be lowered slowly such that the elevated temperatures will not vaporize any liquid present. However, if the temperature is allowed to decrease with the pressure, this

problem can be avoided. Therefore, the machine is set with the option of sterilizing liquids or apparatus. Everything that came into contact with the bacterial culture was first sterilized in the autoclave.

Preparation of Agar Plates

Nutrient agar on petri plates was used extensively throughout this study, both for cell isolation and for viable cell counts. Agar, which is derived from seaweed, is used as the solidifying agent for solid media cultures. Agar is similar to gelatin in that it can be mixed with water at elevated temperatures and then causes the solution to solidify as it cools. However, some bacteria have the ability to metabolize gelatin while very few can metabolize agar. For this reason, agar has replaced gelatin as the solidifying agent in solid growth media. When the cells are grown on this solid surface, they cannot move around and thus they grow and replicate in one spot, forming distinct colonies.

Solid agar media on plates generally contains the agar plus whatever nutrients are necessary. The composition of the nutrients can be made to enhance the growth of certain organisms while harming or even killing others. While these characteristics could be beneficial for impure cultures,

they are not as necessary when working with pure cultures. For this reason a commercially prepared agar, specifically Difco Standard Methods Plate Count Agar (trademark), was used.

The agar solution was prepared according to a fixed standard procedure. The agar powder was suspended in an appropriate amount of distilled water in a flask that would hold roughly twice the volume as was actually placed in the flask. The additional volume was to insure that the solution would not boil over during autoclaving. For sterilization, each flask was loosely sealed with a styrofoam stopper and covered with aluminum foil. The foam stoppers help to keep the solution from boiling over while allowing steam to escape, and the foil aids in keeping the solution sterile. After autoclaving, the solution was allowed to cool to 40°C and poured into disposable petri dishes, each plate being about 1/3 full. The flask was swirled gently prior to pouring to mix the solution, but not so violently that bubbles would form. Five hundred milliliters of the agar solution would pour approximately twenty plates. After solidifying, these plates were inverted for storage until use.

There are possibilities for contamination everywhere, so much care was taken to ensure that as little contamination as possible was allowed to occur. All work was done close to a flame. This creates air currents that help prevent

air-borne bacteria from landing on sterile materials. Secondly, the lip of the flask was flamed prior to pouring to kill any microorganisms there. Thirdly, the lids of the plates were moved only as far as necessary to pour the plates, returned promptly, and not removed again until the plate was ready to be used. As an added precaution, the plates were prepared one or two days in advance so that if contamination did occur, a colony would grow to the point of visibility, and any contaminated plate was discarded before it was used to acquire experimental data. Even with this extreme care, approximately three to five percent of the plates were discarded before use.

Identification

The bacteria used in this study were obtained in pure culture form. However, as an added check, colony isolation and identification were performed to confirm that only P. aeruginosa bacteria were present.

The first step to identification is isolation. This is to ensure that only one type of cell is used in the identification process and is done by using cells that are all descendants of one cell. An isolation streak was preformed on an agar plate as illustrated in Figure 7. As shown, the concentrated culture was streaked across section

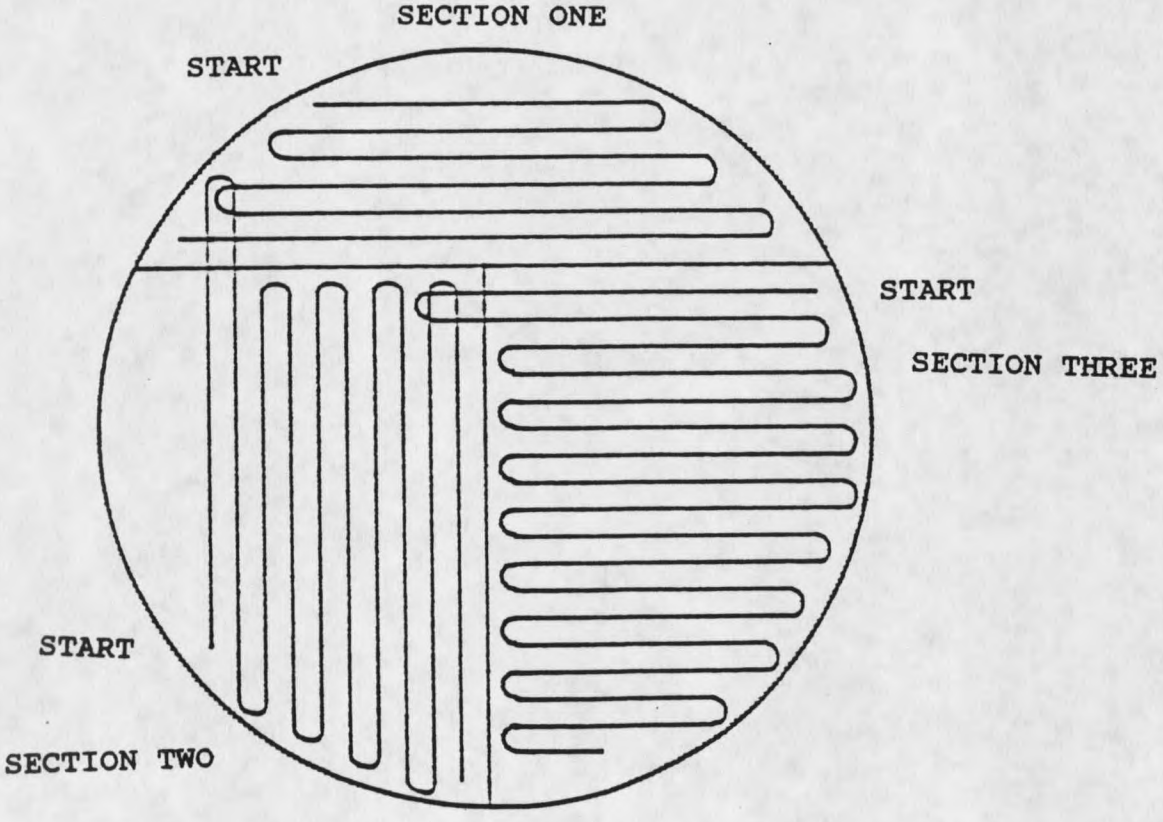

Figure 7. Streak for Isolation

one using an inoculating loop. The loop was then sterilized in the flame and cooled. To streak the second section, the loop was dragged across section one once or twice and then through section two. This same procedure was used to transfer the cells from section two to section three. The result of this streaking from section to section is a dilution of concentration and acquisition of single colonies in section three. One colony from section 3 was removed using the inoculating loop and another streak for isolation was performed using these cells.

After these cells had grown into colonies, identification was performed using a Non-Fermenters Test (NFT). This test is actually a commercially prepared series of microbial tests by Inalatab Products, Incorporated, which can be used to determine the identity of a culture. While these tests are quite simple to perform, they do require specific training to interpret. Therefore, the details of the tests will not be included with this study with the exception of the result: they did show the culture to be P. aeruginosa.

Stock Cultures

Stock cultures are used in microbial work because they ensure that exactly the same organism is used for each

experiment and provide availability of pure cultures. For this study, preservation was achieved by freezing.

The frozen cultures for this study were obtained from the second isolation streak used for identification. One or two colonies were removed from that plate and spread over an agar plate. This was repeated for each of the frozen cultures to be made. When the cells had grown to nearly their maximum concentration, or late logarithmic/early stationary phase (about 2 days), they were harvested. This was done using two milliliters of sterilized glycerol peptone solution which were dispensed onto the plate using a pipetor with sterile disposable tips. The glycerol peptone solution was composed of 2% peptone, 40% glycerol, and 58% water. The cells were then loosened with an inoculating loop and this solution was then placed into a cryovial, again using the pipetor, one cryovial per plate. Once this was done for each plate, the vials were labeled with the name of the person who prepared them, the date, and the name of the organism. They were then taken to Cooley Laboratory to be frozen at -70 C.

Viable Cell Counts

There are several methods that can be used to determine cell concentration. These include particle counts, viable

plate counts, the "most probable number" method, and spectrophotometry. In this study, only the cells that were able to withstand the ultrasonic treatment were to be considered, so the method of viable plate counts was used.

In this method, a known volume of bacterial solution is spread onto an agar plate and colonies are counted after they grow to countable size. This method assumes that each cell in the original solution forms one colony on the plate and the concentrations are reported as colony forming units (CFU's) per volume and not cells per volume. If the cells tended to grow in groups, this might not have been a good choice for these experiments as, theoretically, the sonication could have affected the ability of the cells to "clump" together without affecting the cells themselves. For example, if the cells tended to grow in groups of four (a tetrad) and both the sonicated and non-sonicated samples contained 160 cells, the non-sonicated sample might contain 40 CFU's while the sonicated sample would contain 160. It is evident that this would lead to significant errors. However, the bacteria used in this study, Pseudomonas aeruginosa, do not have a tendency to "clump" together in liquid media, and the viable plate count method seemed to be the most valid method of enumeration.

Fluid media cell concentrations can reach 10^9 cells/ml. Thus, even 0.1 ml of the solution could contain 10^8 cells, far too many to count on a single agar plate. Thus the

culture must be diluted so that enumeration can be done. This was done using dilution blanks.

Dilution blanks were made by autoclaving distilled water, clean test tubes with caps, and a syringe-type dispenser. Using the dispenser, exactly 9.0 ml of the sterile water was placed into each of the test tubes. The lip of each test tube was flamed before and after filling and promptly capped.

For each sample to be counted, exactly 1.0 ml of the sample was added to a dilution blank and mixed. Then, exactly 1.0 ml of this solution was transferred to another blank and mixed. In this fashion, the concentration of the culture was reduced one order of magnitude with each successive dilution. The dilution to be plated (0.1 ml spread onto an agar plate) is the one that results in between 30 and 300 colonies on the plate. Unfortunately, this is never known in advance, so it was guessed and then three dilutions were plated: the guess, one dilution greater, and one dilution less. This increased the chance that at least one of these dilutions would contain a countable number of colonies. This method worked extremely well as in only 5 of over 180 samples did none of the dilutions contain the proper number of colonies. However, in each sample, only the dilution that contained 30 to 300 colonies per plate was recorded and used as data.

PRELIMINARY EXPERIMENTATION

Four preliminary experimental runs were done in the Spring of 1987 by Jim Duffy and Chris Allen, B.S. thesis students, at Montana State University. Although the main purpose of those experiments was also to attempt to increase the growth rate of Pseudomonas aeruginosa, the experimental system used differed greatly from the system eventually developed for the current study.

While both sets of experiments were done using the Heat Systems Ultrasonic Sonicator, the system used by the B.S. students was strictly a batch system. Also, sonication time was much shorter, being one hour for three runs and two hours for a fourth and final run.

The preparation of the bacteria was also different in that for these preliminary runs the culture was inoculated from a pure parent culture and grown at 37 C for 48 hours. This solution was then divided equally between the two samples (control and sonicated). The sonicated sample was exposed at an intensity setting of two (roughly 100 watts) with a five-second cycle time and a ten percent duty cycle while the control sample was simply allowed to grow in a quiescent pool for the first two runs and was stirred with a magnetic stirrer for the last two experimental runs.

Results were measured by two methods, viable plate counts and the biological oxygen demand (BOD) method. The BOD measures the amount of oxygen required by a culture in a sealed container. The waste carbon dioxide generated by the culture is adsorbed by lithium hydroxide placed in the cap of the container and thus, as oxygen is used by the cells, a partial vacuum is created and can be measured as a drop in pressure. Plate counts were done at the end of the run and BOD measurements were taken for several hours after sonication had ceased. The results obtained can be found in Table 1 and in Figures 8-11, taken from the cited B.S. thesis. It would appear from this data that there is a distinct likelihood that the bacteria can indeed be benefitted by ultrasonic exposure. However, there are several discrepancies that would tend to affect the interpretation of these results.

The first place where there is room for significant error in the B.S. thesis work is in the lack of control over some of the parameters in the system, particularly the temperature. A small difference in temperature can result in a large difference in growth rate. Therefore, in the initial stages of the current study, two experiments were done to study temperature effects in the system.

In the first of these current experiments, the heat input from the sonicator was analyzed to see which operating parameter ranges could be used without significant

	Control	Sonicated
Run 1	42	53
	32	66
Run 2, #1	18	32
	14	34
	18	54
	18	54
Run 2, #2	137	275
	151	238
	155	346
	193	274

Table 1. Viable Plate Count Results, Duffy and Allen.

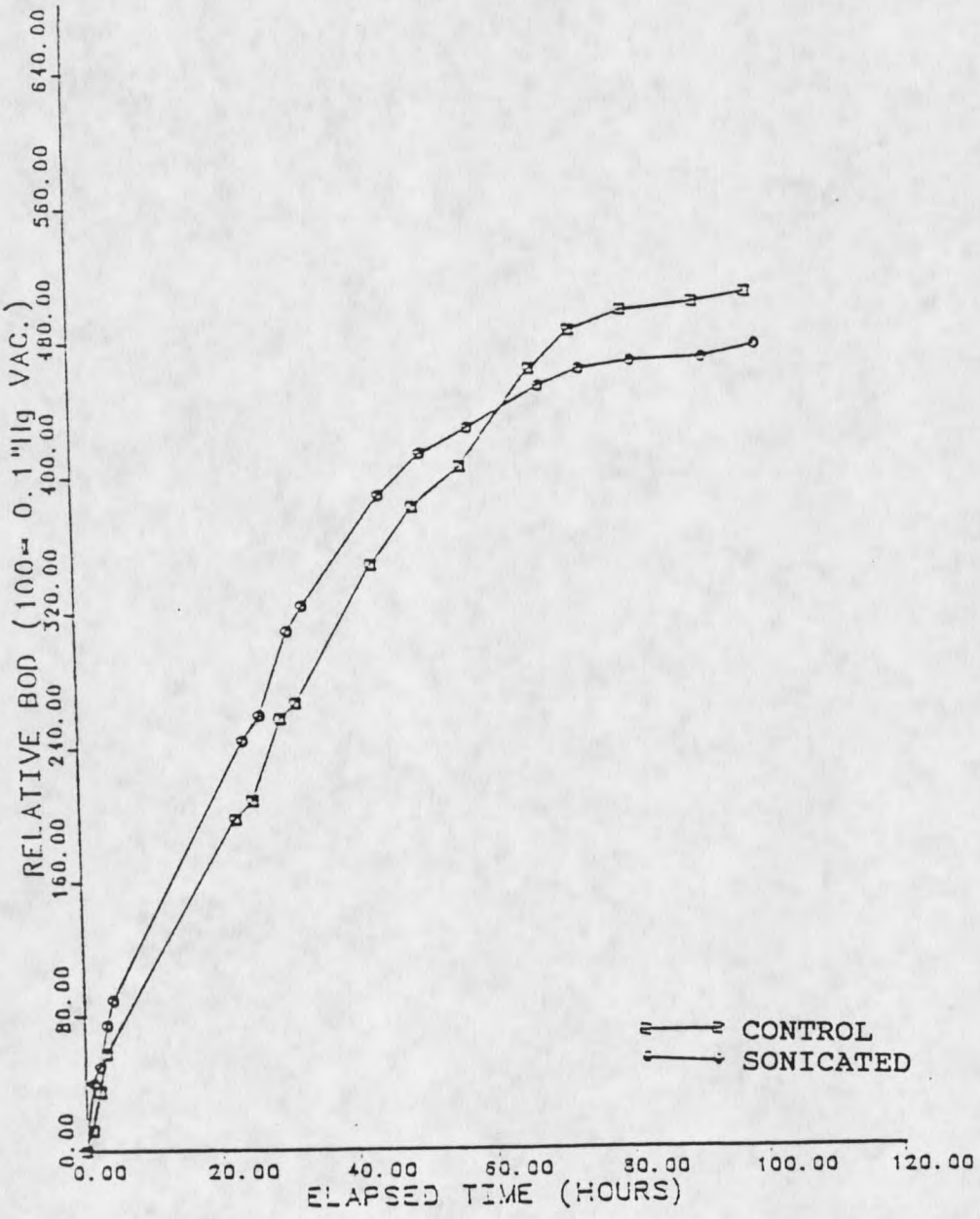

Figure 8. Relative Biological Oxygen Demand versus Time, B. S. Thesis, First Run

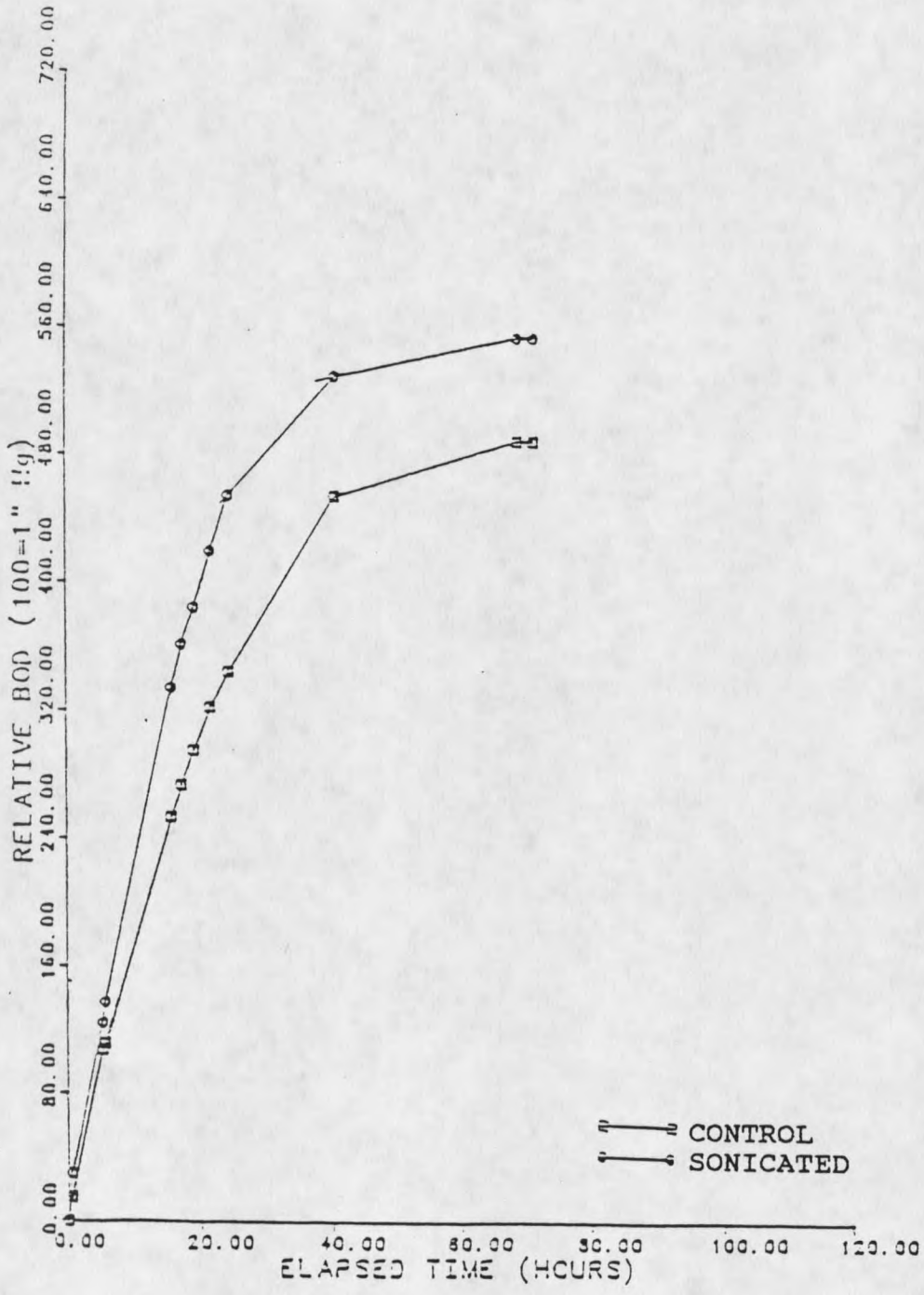

Figure 9. Relative Biological Oxygen Demand versus Time, B. S. Thesis, Second Run

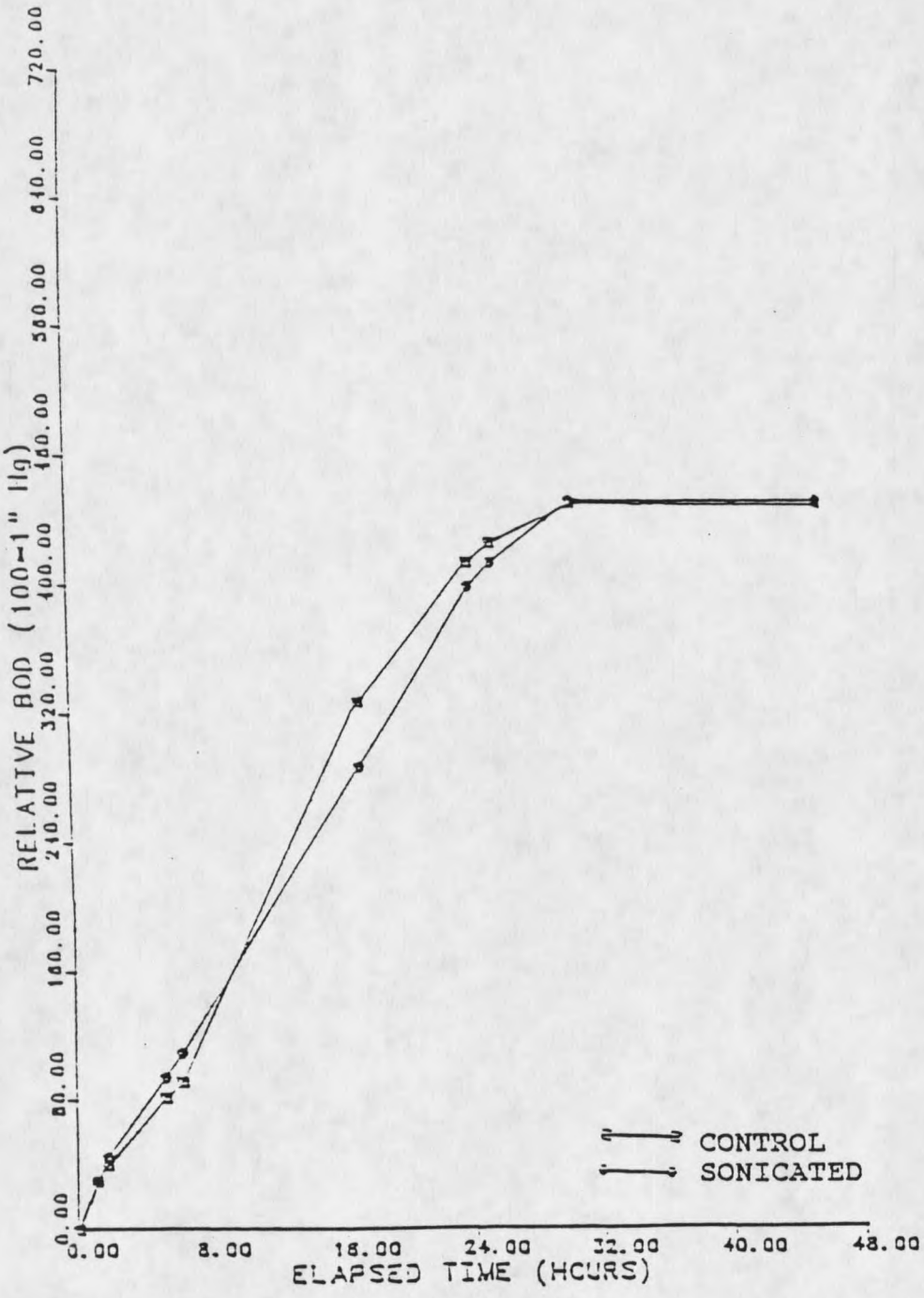

Figure 10. Relative Biological Oxygen Demand versus Time, B. S. Thesis, Third Run

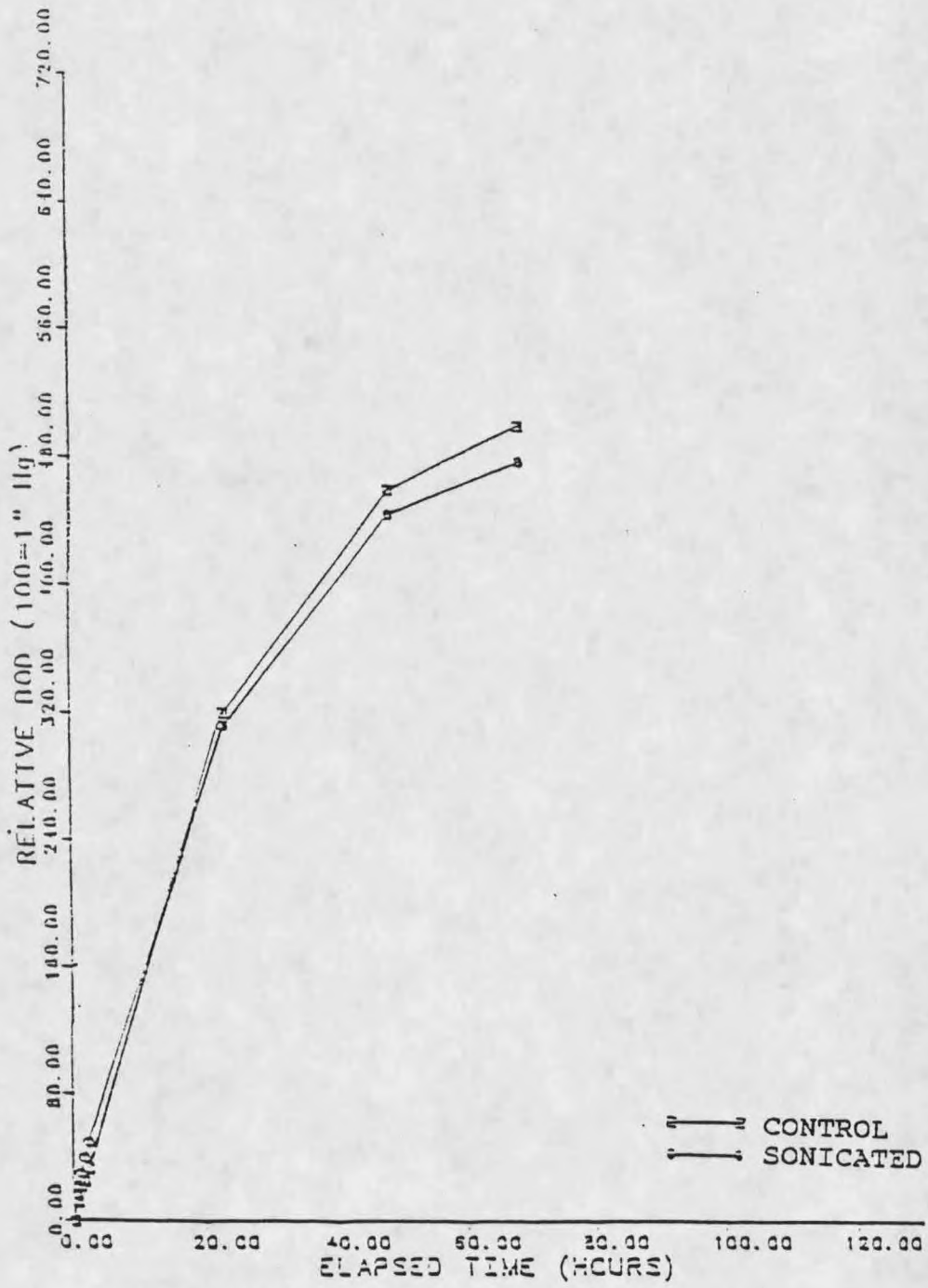

Figure 11. Relative Biological Oxygen Demand versus Time, B. S. Thesis, Fourth Run

temperature increase. To accomplish this, the flow system was set up using tap water with a thermocouple inserted into the tubing immediately after exiting the flow cell. If significant heat were being added to the system, it would be most evident at this point. Since the thermocouple was accurate only to 0.2 C, if the temperature at this point were less than 0.3 C above the steady state value with the sonicator off, the parameters used were determined to be in the allowable operating range. The cycle time was not found to affect the temperature at all, but this was expected as the cycle time does not affect the amount of energy output. The percent duty cycle and the intensity did strongly affect the system, so the allowable operating range was determined based on these two parameters. The results of this experiment are shown in Figure 12 and in Tables 2 and 3. As can be seen, a wide range of operating parameters is possible. As expected, as the intensity increases, the allowable percent duty cycle decreases.

The second of the two current experiments was done in order to determine the amount of heat added to the system from a magnetic stirrer. Approximately 100 ml of tap water in a small beaker was exposed to ultrasound with a magnetic stirrer present. The water used for this experiment was poured and left standing for several hours to ensure that it was initially at room temperature. Temperatures were

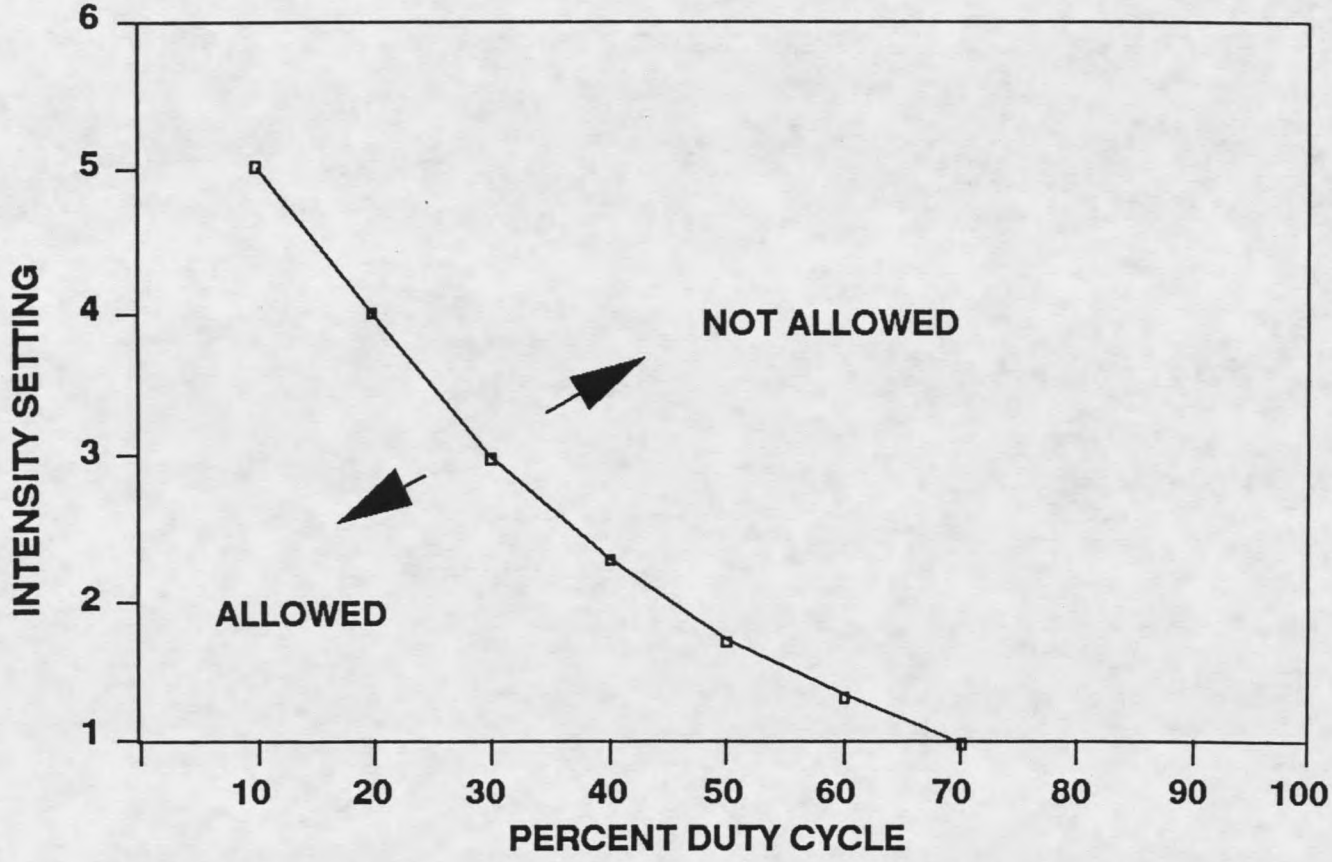

Figure 12. Maximum Sonicator Intensity versus Percent Duty Cycle for Negligible Culture Temperature Increase

CYCLE TIME	%DUTY CYCLE	OUTPUT CONTROL	THERMOCOUPLE READING C	$T_{exit}-T_{ss}$ C
CONTINUOUS	100	OFF	28.8	0.0
CONTINUOUS	100	5.0	34.8	6.0
CONTINUOUS	100	4.0	32.6	3.8
CONTINUOUS	100	3.0	31.4	2.6
CONTINUOUS	100	2.0	30.1	1.3
2 SECONDS	70	5.0	32.9	4.1
2 SECONDS	70	4.0	31.5	2.7
2 SECONDS	70	3.0	30.6	1.8
2 SECONDS	70	2.0	29.8	1.0
2 SECONDS	70	1.5	29.4	0.6
2 SECONDS	50	5.0	31.6	2.8
2 SECONDS	50	4.0	30.7	1.9
2 SECONDS	50	3.0	30.1	1.3
2 SECONDS	50	2.0	29.5	0.7
2 SECONDS	50	1.5	29.2	0.4
2 SECONDS	30	5.0	30.1	1.3
2 SECONDS	30	4.0	29.6	0.8
2 SECONDS	30	3.0	29.2	0.4
2 SECONDS	30	2.0	28.8	0.0
2 SECONDS	30	1.5	28.8	0.0
2 SECONDS	30	5.0	29.4	0.6
2 SECONDS	30	4.0	29.2	0.4
2 SECONDS	30	3.0	29.1	0.3
2 SECONDS	30	2.0	28.8	0.0
2 SECONDS	30	1.5	28.8	0.0
2 SECONDS	10	5.0	29.2	0.4
2 SECONDS	10	4.0	29.1	0.3
2 SECONDS	10	3.0	29.0	0.2
2 SECONDS	10	2.0	28.8	0.0
2 SECONDS	10	1.5	28.8	0.0

Table 2. Flow System Temperature Analysis.

CYCLE TIME	%DUTY CYCLE	OUTPUT CONTROL	THERMOCOUPLE READING C	$T_{exit}-T_{ss}$ C
5 SECONDS	50	5.0	31.7	2.9
2 SECONDS	50	5.0	31.7	2.9
1 SECOND	50	5.0	31.7	2.9
5 SECONDS	50	3.0	30.0	1.2
2 SECONDS	50	3.0	30.0	1.2
1 SECOND	50	3.0	30.0	1.2
5 SECONDS	20	5.0	29.5	0.7
2 SECONDS	20	5.0	29.5	0.7
1 SECOND	20	5.0	29.5	0.7
5 SECONDS	20	3.0	28.9	0.1
2 SECONDS	20	3.0	28.8	0.0
1 SECOND	20	3.0	28.8	0.0
5 SECONDS	70	5.0	33.5	4.7
2 SECONDS	70	5.0	33.4	4.6
1 SECOND	70	5.0	33.5	4.7
5 SECONDS	70	3.0	30.8	2.0
2 SECONDS	70	3.0	30.8	2.0
1 SECOND	70	3.0	30.8	2.0
5 SECONDS	70	2.0	29.5	0.7
2 SECONDS	70	2.0	29.5	0.7
1 SECOND	70	2.0	29.5	0.7

Table 3. Flow System Temperature Analysis, Continued.

recorded periodically until a steady state was reached. These temperatures are listed in Table 4. Sonication was done at an intensity of two and a ten percent duty cycle.

Time (minutes)	Temperature (C)
0	22.0
15	24.0
25	25.0
40	30.0
60	30.5
80	30.8
90	31.0
105	31.0

Table 4. Batch System Temperature Analysis

Since a temperature elevation of even a few degrees can significantly affect the growth rate of bacteria, a change of 10 C can have dramatic effects, and the conditions for the control and sonicated samples cannot be assumed to be equal. Since differences other than just sonication existed, the differences in growth rate cannot be attributed solely to sonication.

A second place where there is significant room for error is in the interpretation of BOD measurements. The growth of the organism depends on far more than just the amount of

oxygen utilized. The pH, concentration of nutrients in the solution, cell concentration, and particular growth phase of the culture will all affect a cell's ability to replicate. Also, if either the control or sonicated sample had grown significantly faster during the sonication period, when oxygen consumption was not recorded, it could have used up more of its nutrients and thus had less available for consumption in the BOD container. Without knowing definitely what all the conditions of a culture are for each sample, a comparison based strictly on the oxygen consumed is not likely to be a good indication of the effects of sonication on a culture.

The third major potential error in the earlier experimental methodology was the lack of plate counts before sonication. The plate counts done after the sonication indicate the number of viable cells after exposure, but without knowing initial viable cells counts, this number has little value. The bacterial cells used for this study are motile and they have a tendency to settle out and concentrate at the bottom of the flask. Thus, even with the utmost of care, initial differences in concentration can occur, especially in an unmixed batch experiment.

For these reasons, no definite conclusions could be drawn from these earlier results, except that even after exposure to sonication, the cells were able to replicate, indicating survival. Since the most common use of

ultrasonics in biology is for cell disruption, the fact that cells can survive at these lower exposure intensities was encouraging.

RESULTS AND DISCUSSION

Flow System Development

Initially, use of experimental apparatus similar to that used by the B.S. thesis students cited earlier was planned. However, after careful consideration it was decided that an entirely new system would be needed to solve the previously discussed problems inherent in that approach.

The first consideration was to maintain the system at a designated, controlled temperature for the duration of each experiment. This is done using a constant temperature water bath, but this solution created another problem. It had been previously decided that stirring was required for both the sonicated and control samples. However, with the flasks in a water bath, it would be difficult to use magnetic stirrers. While submersible magnetic stirrers are commercially available, the cost for these units was prohibitive for this project.

This problem was solved by changing from a non-flow batch system to a flow system. By designing the entrance and exit ports for the flasks to maximize mixing, the magnetic stirrers were not needed. Admittedly, it is doubtful that this design provided mixing equal to that provided by a mechanical stirrer, but since mixing problems were never observed, it was assumed that the mixing was at least adequate. The flow past the sonication probe tip was easily accomplished using the continuous flow cell.

The last major new feature in the system was the addition of a gas line to provide additional oxygen. To avoid the possibility of contamination from the gas supply bottle, a filter was placed in each gas line prior to sterilization in the autoclave.

The experiments performed for this project can be divided into four categories: Scouting (Experimental Runs 1-4), First Series (Runs 5-9), Second Series (Runs 10-14, 18), and Third Series (Runs 15-17, 19).

Scouting

In preparation for the first experimental run, 400 ml of mineral salts medium (MSM) was inoculated with a frozen stock culture of Pseudomonas aeruginosa and grown at 27 C in

a water bath for 21 hours. At this time, it was divided by 25-ml increments into the two reaction flasks. An additional 300 ml of MSM was added to each flask, the solution was mixed, and plate counts were done to determine the initial concentrations. Sonication was employed at an intensity setting of two (roughly 90 watts) with a ten percent duty cycle and a one-second cycle time. An experimental temperature of 34 C, slightly below the temperature for the maximum growth rate, was chosen for this experiment. Flow rates for both the gas and the liquid were adjusted and measured at the beginning and conclusion of the experiment, with the flow rate being 250 ml/min for the liquid and 40 ml/min for the gas. Samples were taken after 1.4 hours. The next sample was to be taken at 3.5 hours, but between these last two samples, the tubing split in the pump head and the experiment was stopped.

Although little data was obtained through this initial experiment, a better understanding of the entire system was gained. The plans for this experiment had included taking samples from the flasks using a long-needle syringe through the gas outlet tube. In addition, the volume of the sample itself was to also be measured using the syringe. However, the pipetor used for the plate counts is much more reliable for obtaining consistent and nearly exact sample volumes, so it was decided that samples of two to three milliliters would continue to be taken using the long-needle syringe,

but this sample would be remeasured, using the pipetor, for accuracy of sample size for determining concentration. Also, the plates from all three dilutions were counted for this experimental run. This was discovered to be not only unnecessary, but inaccurate for data acquisition. Therefore, starting with the second run, only the dilution where the counts were from 30 to 300 colonies per plate were recorded. The results of the plate counts that were done for this run are given in Table 5. From this data it can be seen that the initial counts were significantly different from each other. This can be attributed to the possibility that sample sizes may not have been the same, leading to observed differences, or to the possibility that mixing was not adequate while dividing the culture, leading to actual differences. To avoid these problems in future experiments, the culture was thoroughly mixed prior to division and sample sizes were carefully measured using a pipetor. Also, the concentration is seen to drop from the initial reading to the first sample. While this was observed on several runs, it could not be definitely attributed to any one cause at this time in the experimental program.

For the second run, 200 ml of MSM was inoculated with the frozen stock culture and grown at 28 C for 19 hours. The bacterial culture was added as in Run 1, using 25-ml increments, to 375 ml MSM in each of the two reaction

TIME	TYPE	DILUTION	COUNTS			CONCENTRATION CELLS/ML
INITIAL	CONTROL	10^{-6}	62	87	80	7.63×10^7
	SONICATED	10^{-6}	68	67	71	6.87×10^7
1.4 HOURS	CONTROL	10^{-6}	65	73	69	6.90×10^7
	SONICATED	10^{-6}	37	46	50	4.43×10^7

(Sometime after 1.4 hours, the line of tubing inside the pump head split, and thus the run was aborted.)

Table 5. Plate Count Results, Run 1.

flasks. However, for this experimental run, the flask containing the concentrated bacterial culture was mixed while being divided between the two flasks. As in the first experiment, sonication was conducted at an intensity setting of two with a cycle time of one second and a ten percent duty cycle, while the water bath was maintained at 35 C. Samples were taken at 2.00 hours, 4.08 hours, 5.92 hours, and 8.25 hours. These results are given in Figure 13. Also, after the end of the run, the system was left running to see what longer term effects sonication may have, and samples were taken after 22.25 hours. The graph including these points is given in Figure 14. It is important to note that the starting concentrations of the two flasks were quite close. This would indicate that mixing the culture during division helped to avoid the extreme differences seen in the starting concentrations in the two flasks in the first experimental run.

The results from the first eight hours of this experiment seemed to indicate that sonication significantly slowed the growth of the bacteria without causing severe detrimental effects on the viability of the cells. However, the data points at twenty-two hours indicated that the bacteria might develop an ability to withstand the treatment and might eventually begin to grow and replicate in an ultrasonic field. This possibility was temporarily shelved

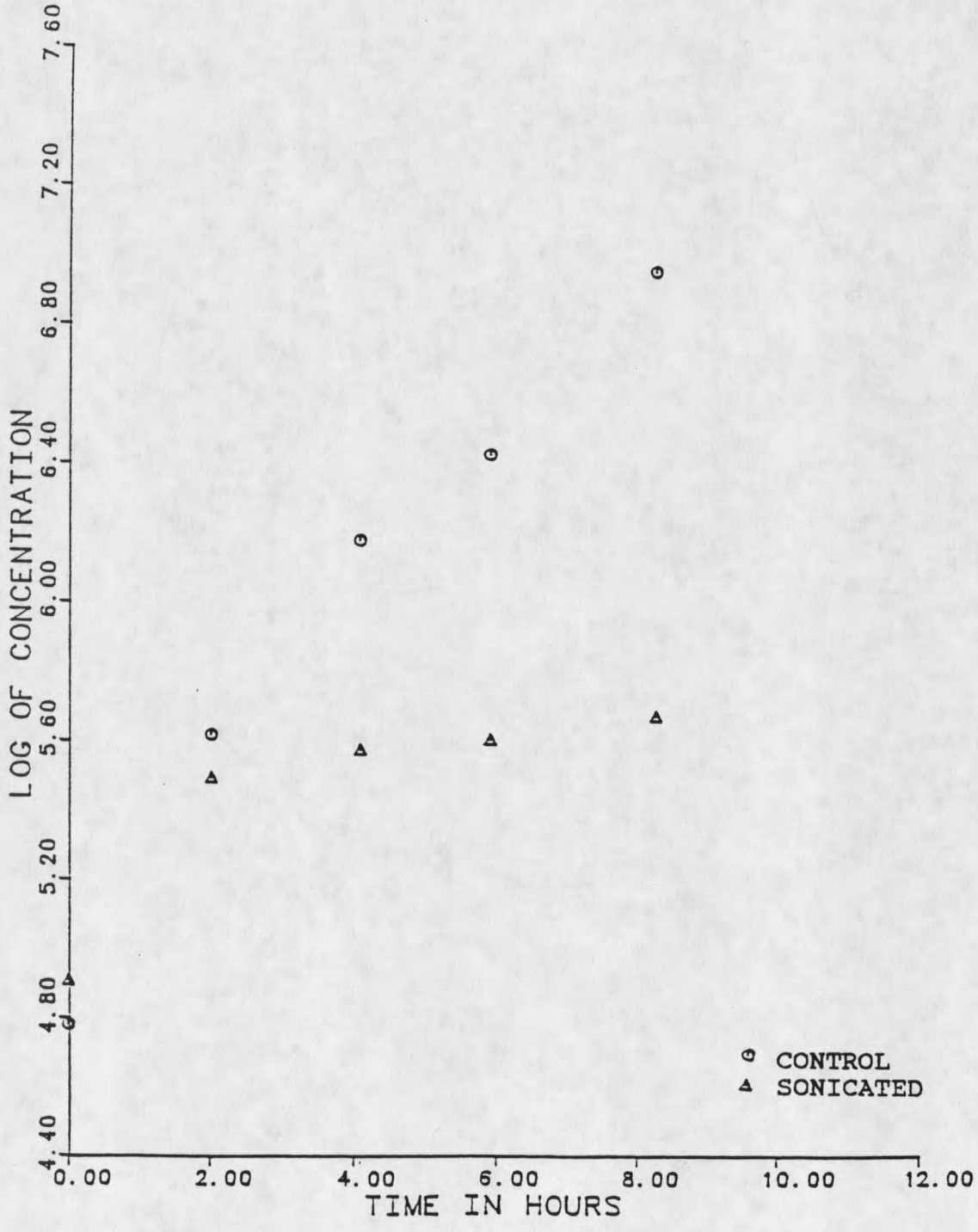

Figure 13. Base 10 Logarithm of the Culture Concentration (Cells per Milliliter) versus Time (Hours) Run 2

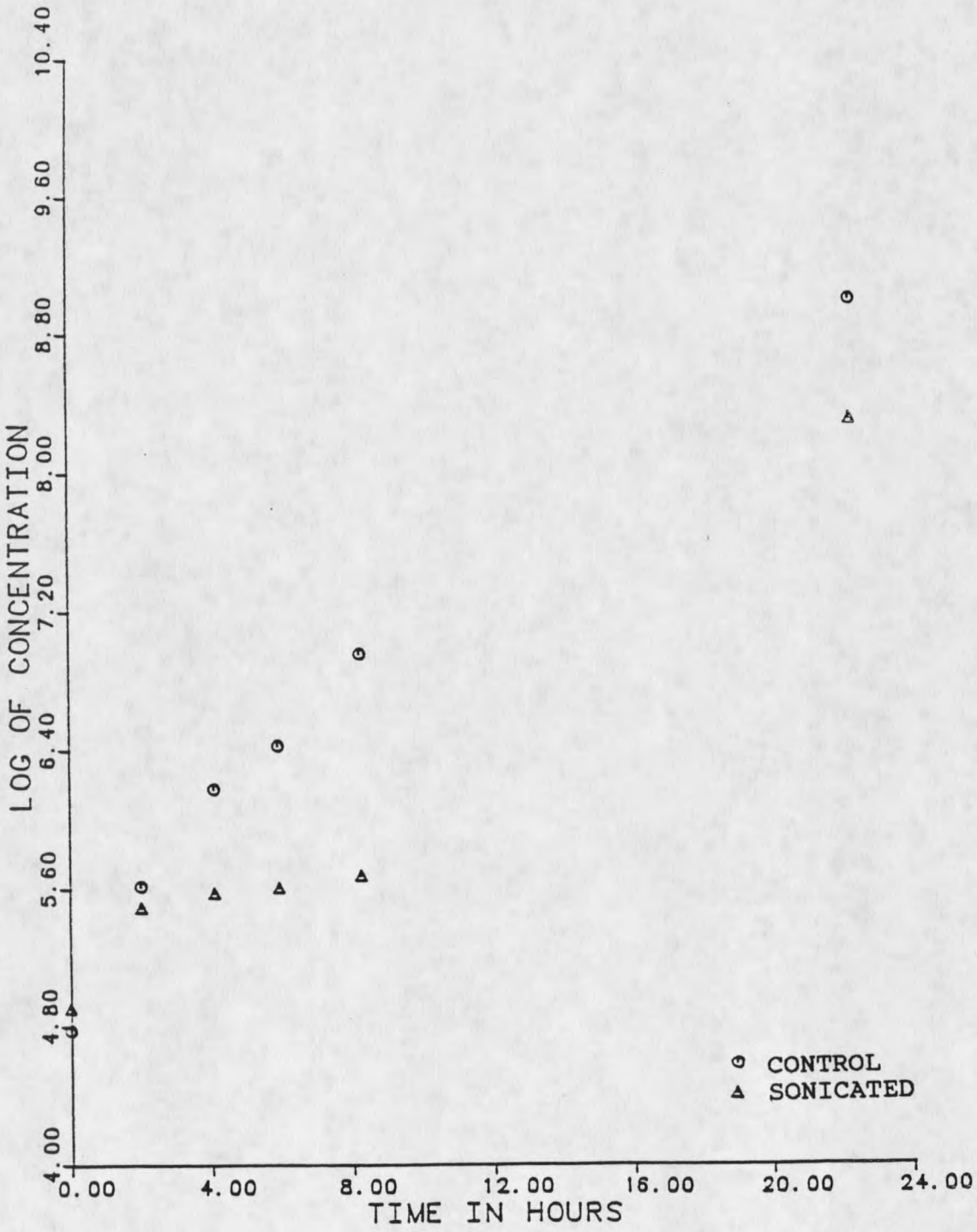

Figure 14. Base 10 Logarithm of the Culture Concentration (Cells per Milliliter) versus Time (Hours), Run 2, Extended Graph

and the effects at lower intensities were scouted, beginning with the third experiment.

As for the second experiment, the nutrient source to be used for this experiment was sterilized and then divided into volumes of 350 ml in each of the reaction flasks and 300 ml to be inoculated with the bacteria culture. Incubation was done at 30 C for 16.5 hours, after which the culture was divided by 25-ml increments and added to the nutrient broth in the reaction flasks. Sonication was conducted at an output setting of one (roughly 45 watts), a cycle time of one second and a ten percent duty cycle with the water bath temperature maintained at 32 C. This temperature was slightly below the temperatures used for the first experimental runs. However, since the purpose of the water bath was to maintain the temperatures within both flasks at equal levels for the duration of sonic exposure, the exact value of the temperature is not important as long as it is consistent for both flasks, as well as being near the range for maximum growth rate. Temperatures generally stayed within ± 1 C for the duration of any run. However, temperatures had to be monitored and adjusted manually as the water bath did not have a reliable temperature controller. Thus, slight deviations did occur within some of the experiments, but these were never observed to be

greater than one degree. For these reasons, on all experiments, any temperature within the range of 32-35 C was accepted. Samples were taken at two-hour intervals for a total of twelve hours.

The plates were to be counted after two days as had been done previously. However, the counting was halted after the first sample because the plates were not countable. Instead of growing into small distinct colonies, bacteria completely covered the plates. It was assumed that, for some reason, the plates contained too much moisture. While it is still unknown why this might have happened, a couple of theories were proposed. The first was that the agar solution was not mixed properly prior to sterilization. The second was that the agar settled out while pouring and thus some of the plates were deficient in solidifying agent. Even while warm, agar is not completely soluble in water. In the event that the second theory was correct, the agar used for all plates used subsequent to this experiment was mixed gently while being poured to help to alleviate this possibility.

The culture preparation for the forth run was identical to the preparation used for the third experimental run. Run conditions were also the same, using an intensity setting of one, with the exception that the water bath was kept at 35 C as opposed to 32 C for Run 3. Samples were taken at two-

hour intervals for six hours with a final sample at nine hours. The results from this run are given in Figure 15.

In this experiment, both samples grew to approximately the same concentration during the nine-hour experiment. However, while the control sample seemed to begin to grow immediately, the sonicated sample seemed to go through somewhat of a lag period. As discussed in the Microbiology section of this paper, when bacteria are exposed to a new environment, they often go through a period of adaptation before they begin to divide. It was not initially thought that the sonication would produce this effect, but the results of this experiment seemed to indicate that sonic energy might yield a lag effect. This can be seen in Figure 15 by the fact that after two hours, the concentration of the sonicated sample had not quite doubled while the concentration of the control sample had nearly quadrupled.

It was then decided that, if the sonicated sample would have to go through a lag period, it would be ideal if both the sonicated and the control samples could be forced to go through a lag phase to ease the subsequent comparison. Up until this time, it was desired that the culture be in the exponential phase just prior to the start of each experiment so that growth would continue in this mode. However, with the possibility of a sonically-induced lag phase, it was thought that if the culture were in stationary phase instead, both samples would have to go through a lag phase.

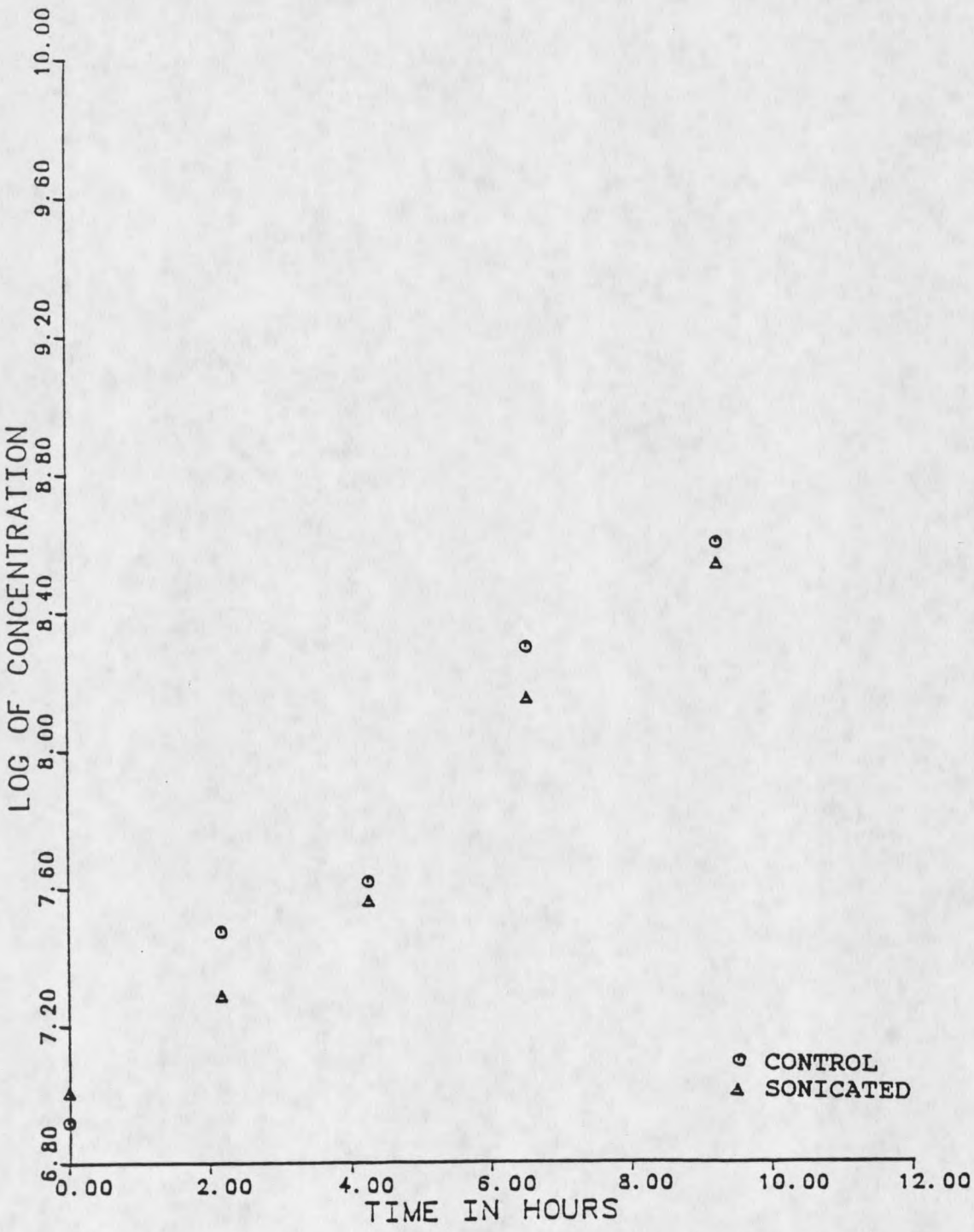

Figure 15. Base 10 Logarithm of the Culture Concentration (Cells per Milliliter) versus Time (Hours) Run 4

If the sonicated sample could adapt to the sonication at the same time as the new environment, the lag time could potentially be the same for both samples and the growth curves could be more easily compared. This idea was incorporated into the experimental procedure starting with Run 5.

First Series

At this point, the majority of the techniques that were to be used hereafter in this project had been developed. While operating parameters continued to be changed, the basic methods of data acquisition had been developed and changed very little until much later in this project. Therefore, Run 5 marks the beginning of the first series of standardized experiments.

The fifth experimental run was done as a repeat of Run 4 for two reasons: to extend the data beyond ten hours to eighteen hours and to attempt to force both the sonicated and the control samples to go through a similar lag phase. To achieve the second of these goals, the sterile growth media was inoculated from the pure frozen parent culture, grown at room temperature for two days and then refrigerated

for three days. It was hoped that this would result in a culture in the stationary phase of growth.

At the beginning of the experiment, approximately 50 ml of this concentrated and refrigerated culture was inoculated into 400 ml sterile nutrient broth in each of the reaction flasks, and then the experiment proceeded as in Run 4. All experimental conditions were identical to those used in Run 4, including temperature and flow rates. Samples were taken at two, five, eight, eleven, fourteen, and eighteen hours. The results are given in Figure 16.

It can be seen that the attempt at inducing a lag phase in both samples was apparently successful. However, after the lag phase, both samples grew at approximately the same rate, with the exception of the sample point at eleven hours. Because the results of the plate counts cannot be known until the colonies are large enough to count, if an error is made in the plate count procedure, it is not apparent until much later and steps cannot be made to correct it. It is believed that this was the case for the eleven hour sample. Due to the fact that, with the exception of that one data point, the curve for the sonicated sample is quite smooth, and that both the sonicated and control samples were exceptionally low, it is very possible that incorrect volumes were used for that sample. As the pipetors used for the determination of sample volumes are adjustable over a range of volumes,

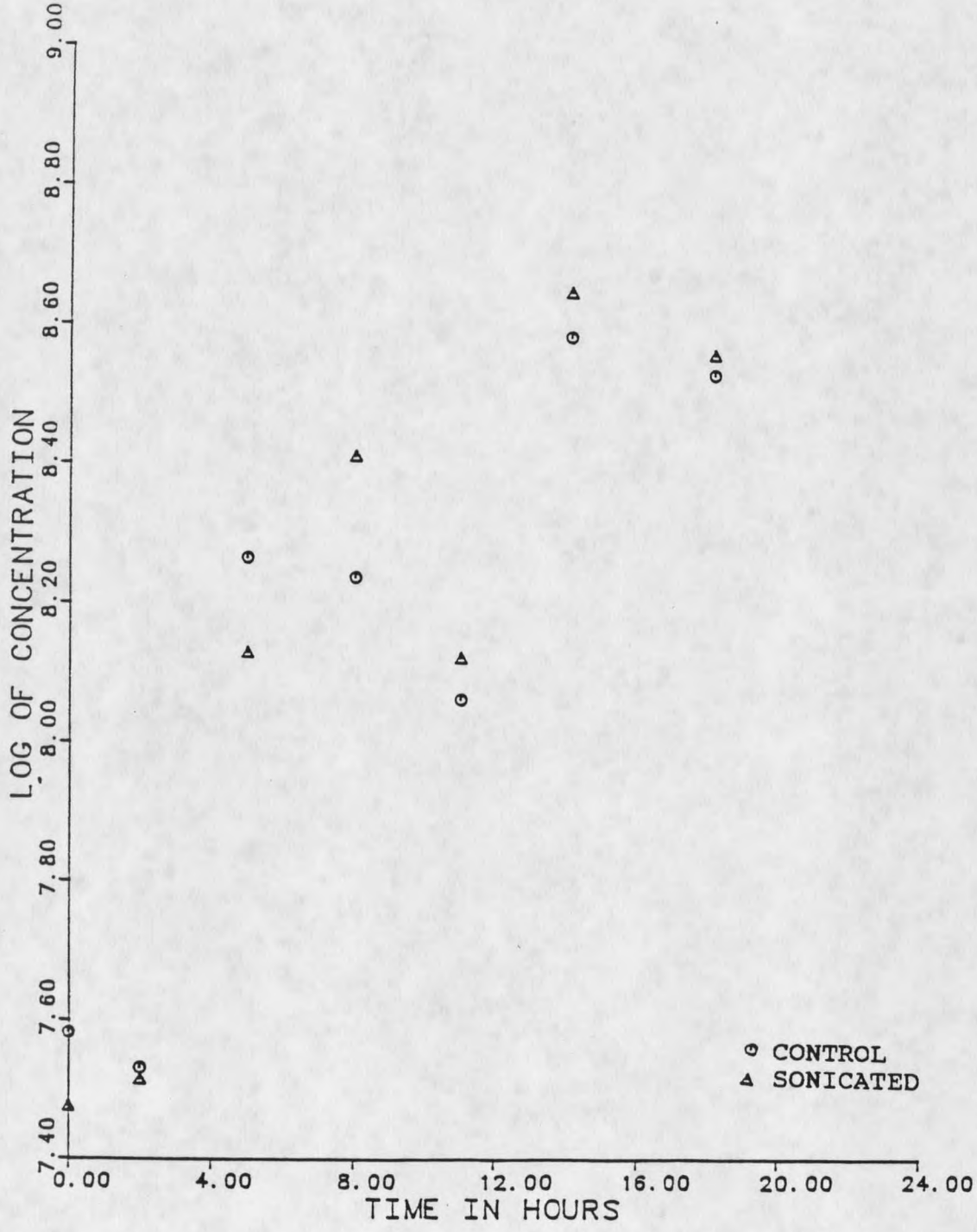

Figure 16. Base 10 Logarithm of the Culture Concentration (Cells per Milliliter) versus Time (Hours) Run 5

extreme care was taken hereafter to assure that the volumes being used were the volumes desired.

Figure 16 shows that the viable cell concentration of the sonicated sample was consistently greater than that of the control sample after five hours. However, the differences did not seem great enough to validly attribute them to anything other than either experimental error or the lack of tight precision in viable cell counts on petri plates. If these results could be found to be consistent for several runs, then it might be possible to come to a conclusion, but with only one weak positive result, no firm conclusions could be made at this time. For these reasons, the next few experiments were conducted as attempts to repeat these results.

The culture preparation and run conditions for Run 6 were the same as those used for Run 5, including temperatures, flow rates, incubation times, and refrigeration times. The only exception to this was that each reaction flask contained 450 ml of sterile nutrient broth with only 10 ml of the concentrated bacterial solution. The reason for this change was that it would result in lower initial concentrations, thus allowing for a longer exponential phase and increasing the likelihood that any actual differences could be discernible Also, it was

decided to record the data more often at the beginning so that a more accurate description of the growth curve could be obtained. In addition, it was hoped that it would be easier to compare the lengths of the lag phases in both samples with more data points. For these reasons, samples were taken every hour for the first five hours and then every two hours after that for a total of thirteen hours. The results obtained from this experiment are given in Figure 17.

This figure shows the viable cell concentrations in both samples dropped more than an entire order of magnitude after being stable for an hour near the initial concentrations. While an error in plating could account for one data point being so far off, the fact that both samples for two data point pairs were off would suggest that the concentrations were actually falling. The reason for this dramatic change is as of yet unknown. Sample sizes were meticulously measured and plated, and contamination was unlikely as all of the colonies on every plate had essentially the same size, shape, and color. Thus, while it is uncertain as to why the concentration in both samples experienced this drop in concentration, it is apparent from this data that there was no significant difference in either growth rates or the shape of the growth curve itself during latter stages of this experiment.

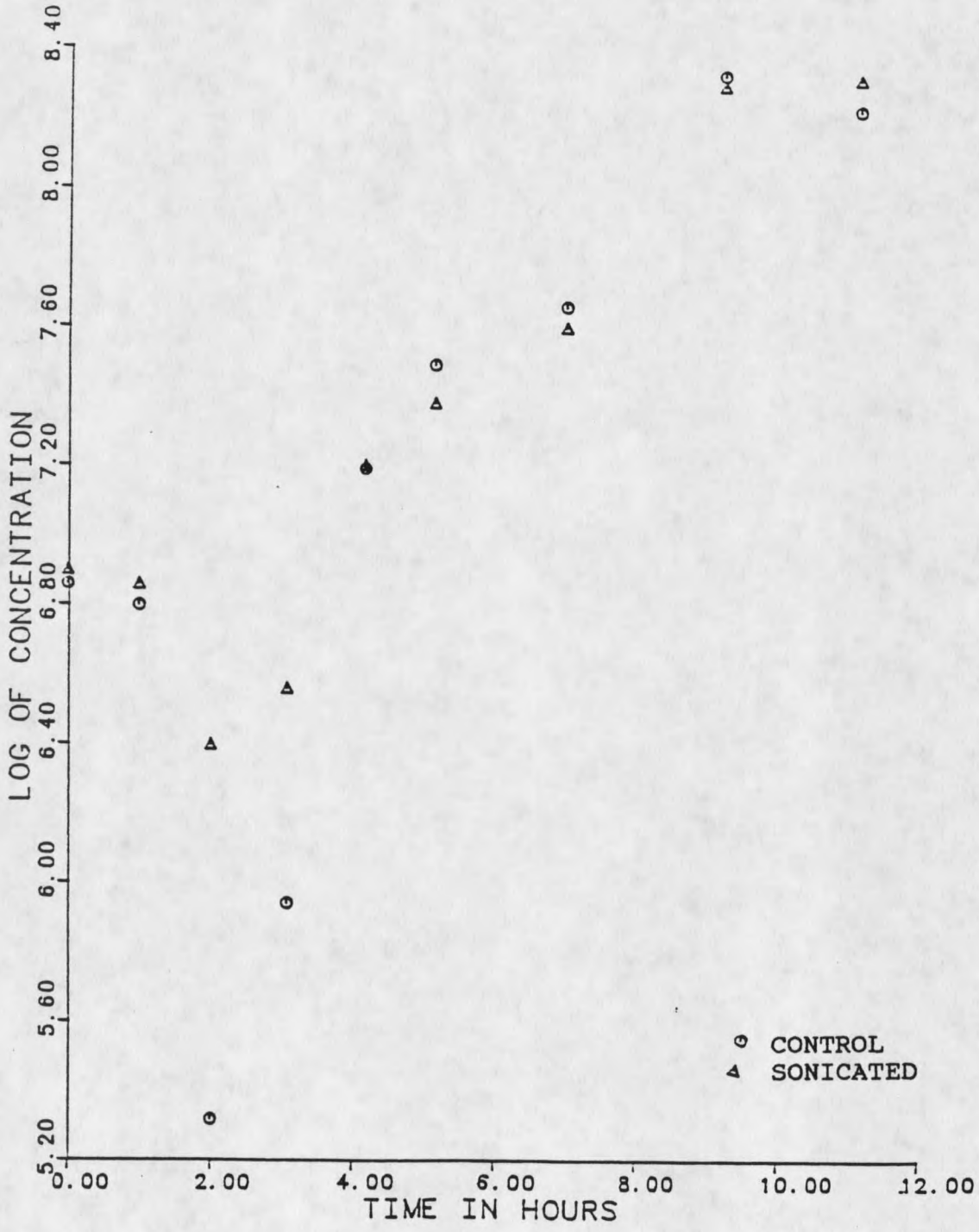

Figure 17. Base 10 Logarithm of the Culture Concentration (Cells per Milliliter) versus Time (Hours) Run 6

The cause of the drop in the concentration is unexplainable at this time. The smallest change in environmental conditions can have a large effect on the growth of a bacterial culture. Also, bacterial viruses and phages will affect the culture and can be quite difficult to discover without elaborate testing. Thus, the cause for the drop in concentration remains unknown. However, it is important to note that whatever was causing the drop in concentration affected the control sample far more than the sonicated sample. One possible reason for this is that the sonication was somehow "protecting" the cells. However with only one experimental run experiencing this initial drop in concentration, no firm conclusions can be reached at this time.

Run 7 was conducted, once again, as an attempt to repeat Run 5 and perhaps shed more light on the unexpected curve shape of Figure 17. The run conditions and culture preparation were the same as in Run 6. Data are shown in Figure 18. The reason for the unusual shape and scatter of the data is unknown. However, while no thorough identification tests were run, there did appear to be a significant amount of contamination. This was evident by the morphology of the colonies on the plates. Plates from both the sonicated and control flasks resulted in two different kinds of colonies. The normal Pseudomonas

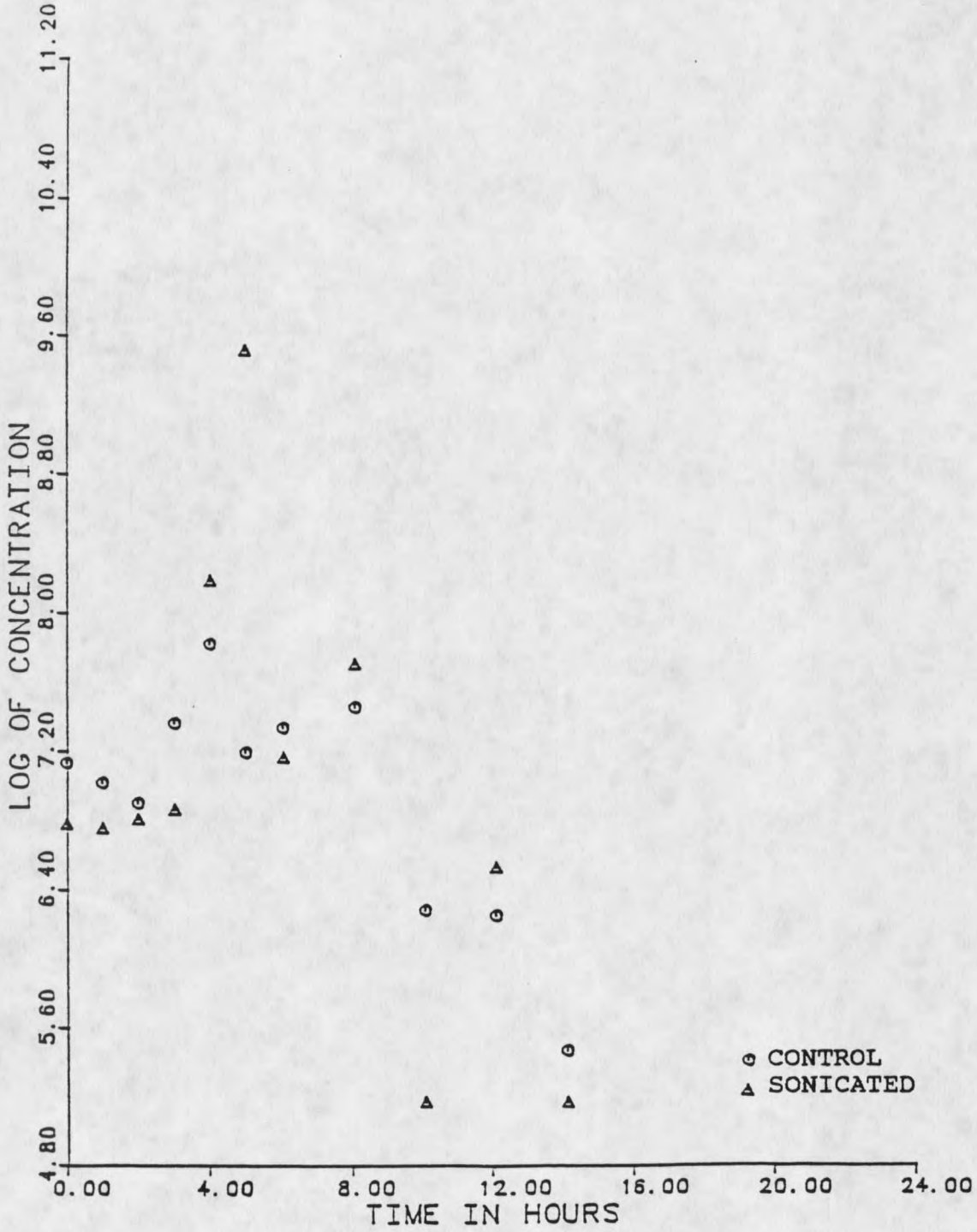

Figure 18. Base 10 Logarithm of the Culture Concentration (Cells per Milliliter) versus Time (Hours) Run 7

aeruginosa colonies accounted for about 50% of the colonies while the remainder were smaller in size and lighter in color, indicating a different type of bacteria, and thus, contamination.

It is unknown how or when the contamination of the culture took place. However, since both the control and sonicated samples were contaminated, it probably existed before the reaction flasks were inoculated, or as a result of subjecting both samples to a common source of contamination. Since everything that came into contact with either sample was sterilized, and no bacteria were ever transferred between cultures, the chance of exact and simultaneous contamination occurring is extremely rare, thus the assumption that contamination took place before the culture was inoculated into the reaction flasks.

There are several ways that the culture could have become contaminated, and while there are methods that can be used to reduce the chance of these, there are no guarantees that contamination cannot occur. Since the culture was inoculated from a frozen parent culture, there is a chance that contamination occurred in the preparation of that parent culture. Or, if the Mineral Salts Medium became contaminated, the contaminating bacteria would have grown along with the Pseudomonas. Regardless of how contamination occurs, it is a very serious threat to research of this

type, as generally it cannot be detected until it is too late and valuable time and material have been wasted.

Since the data obtained from this experiment included significant contamination, the results could not be assumed to be valid or to represent the uncontaminated behavior in any respect.

Run 8 was the third attempt to duplicate the results of Run 5. Culture preparation and run conditions were the same as used before. In this case, each reaction flask contained 475 ml of sterile nutrient broth with six milliliters of the concentrated culture solution. This was done to slightly lower the starting concentration in the flasks to allow for a longer exponential growth phase. The bath was maintained at 33 C for the duration of the run and samples were again to be taken every hour for the first five hours and every two hours after that for a total of eighteen hours. However, this run resulted in no solid results because, after the third sample was taken, the rubber stopper in the test tube used for the control volume came loose. When an attempt was made to secure it, the tube itself broke, contaminating the culture and putting an end to this run. The results of the plate counts that were done for this experiment are given in Table 6, and in graph form in Figure 19. This graph is included to illustrate that, as in Run 6,

TIME	TYPE	DILUTION	COUNTS			CONCENTRATION CELLS/ML
INITIAL	CONTROL	10^{-5}	70	79	80	7.63×10^6
	SONICATED	10^{-5}	64	73	80	7.23×10^6
1 HOUR	CONTROL	10^{-5}	68	68	63	6.57×10^6
	SONICATED	10^{-5}	55	79	71	6.83×10^6
2 HOURS	CONTROL	10^{-5}	73	80	63	7.20×10^6
	SONICATED	10^{-5}	73	56	90	7.30×10^6
3 HOURS	CONTROL	10^{-5}	64	73	78	7.17×10^6
	SONICATED	10^{-5}	52	88	65	6.83×10^6

The test tube contained in the apparatus broke after the 3 hour reading and thus the run had to be aborted.

Table 6. Plate Count Results, Run 8.

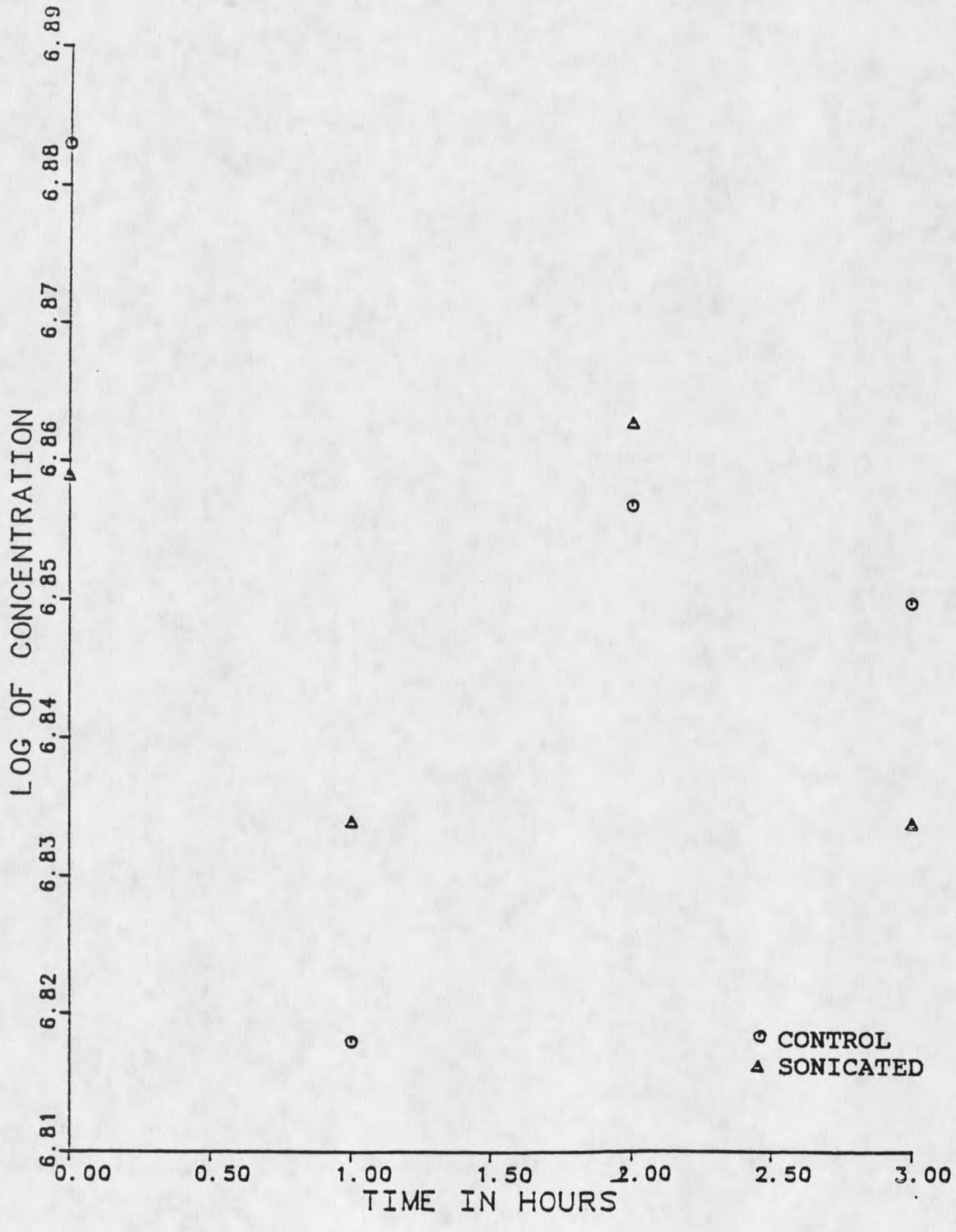

Figure 19. Base 10 Logarithm of the Culture Concentration (Cells per Milliliter) versus Time (Hours) Run 8

the concentration of both samples dropped significantly in the first few hours of the experiment, and then began to increase again. Also, as in Run 6, the control sample seemed to be affected more than the sonicated sample. Unfortunately, this experiment did not last long enough to give the full shape of the growth curves, thus, no firm conclusions can be reached.

Run 9 was the fourth attempt to duplicate Run 5. For this run, a sample of the bacteria prepared for Run 8 was used, as this culture had been kept refrigerated after the required 12 ml had been removed. Therefore, a small sample (approximately six milliliters) was inoculated into fresh, sterile nutrient broth. This was grown as a new batch at 33 C in the water bath for eighteen hours and then used as the concentrated culture for Run 9. For this run, each reaction flask contained 460 ml of sterile nutrient broth with nine milliliters of the concentrated culture. Although the preparation of the concentrated culture was the same as that used previously, this culture did not appear to be as concentrated (less turbid) as the cultures for previous runs, thus a few extra milliliters were added to account for this. While adding "a few extra milliliters" may seem unscientific, with scouting experimentation of this sort, it is often the best route to choose. Because the concentration and condition of the culture are not known

until the experiment itself is over, it is often up to the researcher to determine if small changes such as this will be necessary for the success of the individual runs. This proved to be the case in this instance, as even with the extra volume of concentrated culture, the initial concentrations were significantly lower than they had been in the last few runs.

The water bath was maintained at 34 C, and as usual, the sonication probe was set with an intensity setting of one, cycle time of one second, and a ten percent duty cycle. Samples were taken every hour for the first five hours and every two hours after that for a total of approximately eighteen hours. The results from this run are given in Figure 20.

Once again, the differences between the two curves were not large enough to attribute to differences due to sonication. In any case, the control culture samples are more concentrated throughout this experiment while the sonicated microbes seemed to do better in Run 5. For these reasons, it was concluded that no significant effect of sonication could be obtained under these conditions, and new variables, such as exposure intensity and duration, were now considered. Therefore, Run 9 was the last run of the First Series.

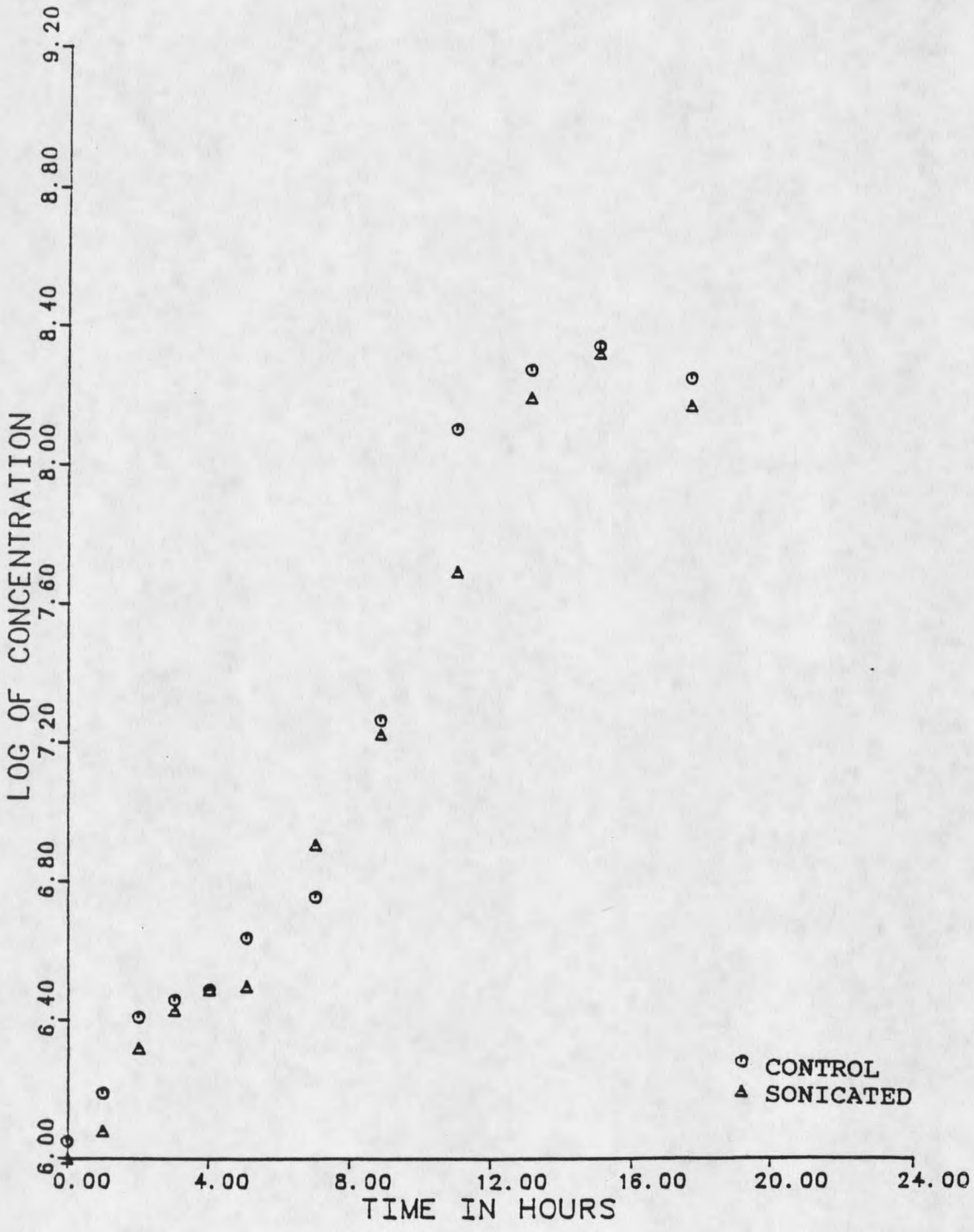

Figure 20. Base 10 Logarithm of the Culture Concentration (Cells per Milliliter) versus Time (Hours) Run 9

Second Series

Run 10, the first run of the Second Series, was conducted to determine if more exposure at the same intensity could result in increased cell concentrations. The data from the First Series gave few conclusive results and showed a great deal of randomness. Thus, it could have been true that the sonication was, indeed, improving growth, but since the exposure was low, evidence of improvement was becoming "lost" in experimental scatter. Run 10 was a scouting trial to see if this could be the case.

The frozen stock culture was inoculated into approximately 200 ml of sterile nutrient broth and grown for twenty-six hours at 35 C in an incubator, after which it was refrigerated for sixteen hours. Five milliliters of this culture were added to 460 ml of sterile nutrient broth in each of the reaction flasks. While the cycle time and output control both remained at the settings used for the first nine experiments, five seconds and an intensity setting of one, respectively, the percent duty cycle was changed to forty percent versus the previous ten percent. As the percent duty cycle time determines how much exposure the medium will receive for a given intensity, increasing the duty cycle to forty percent resulted in four times the

exposure as at ten percent. The results of this run are given in Figure 21.

This run was the first that resulted in distinct and rather consistent differences in plate count results and was also the first time that these differences were observable throughout the course of the run. After six hours, the sonicated sample appeared to be far less concentrated as it was much less turbid than the control sample. The difference in the turbidity of the two samples seemed to decrease as the experiment progressed, but the sonicated sample never appeared to be as turbid as the control sample. These observations were confirmed by the results of the plate counts, as shown in Figure 21. Thus, it was not difficult to see that while the increased sonication did have an effect, it was not a beneficial one, and this line of experimentation was halted.

At this point, the possibility of "pretreating" the culture was proposed. It was thought that perhaps the sonicated sample needs to adapt to the sonic energy exposure and that it never is able to "catch up" and thus always remains less concentrated than the control sample. One proposed method of pretreating the culture was to suspend the sonication probe in a small beaker containing a culture in solution and sonicate at an intensity setting of two,

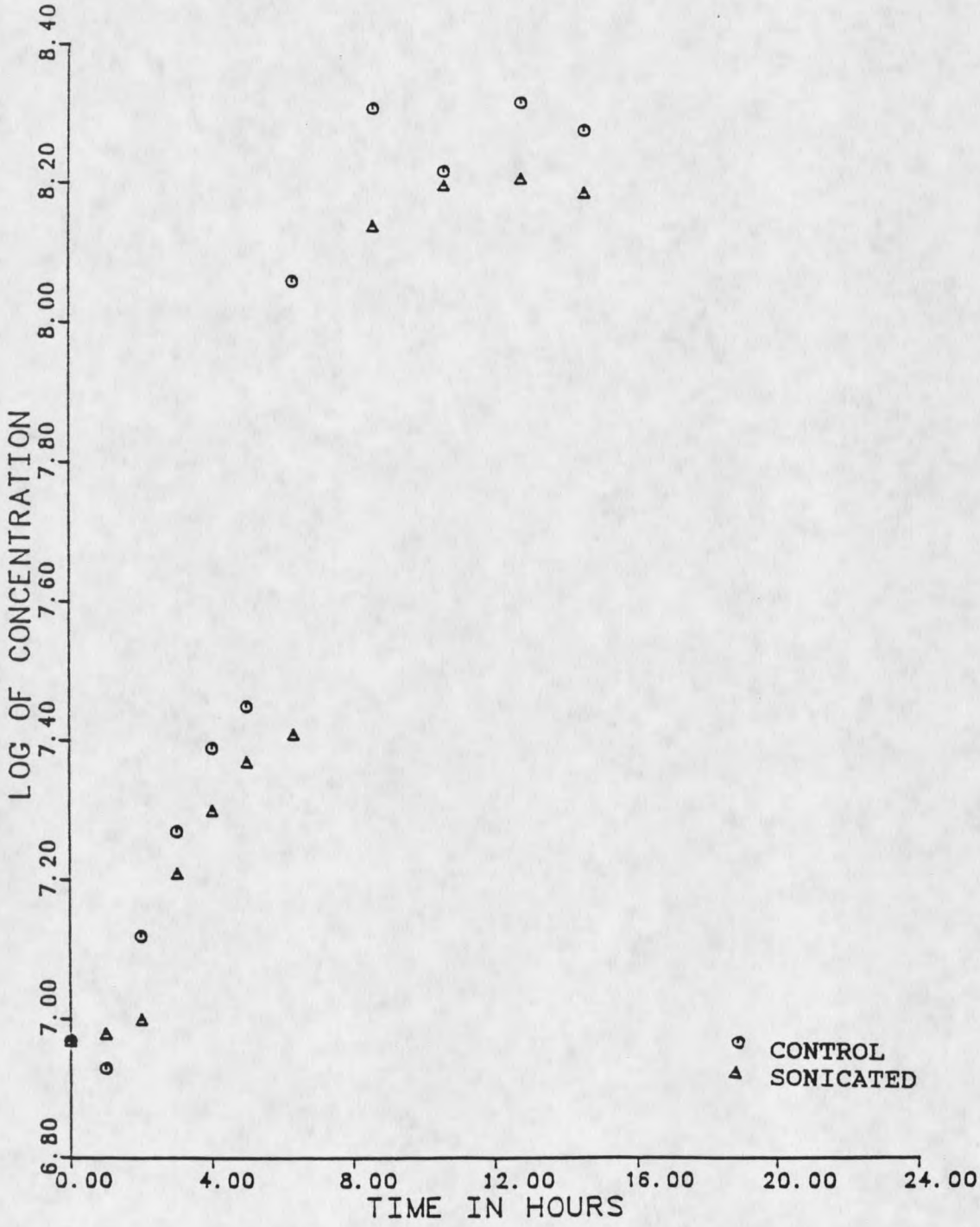

Figure 21. Base 10 Logarithm of the Culture Concentration (Cells per Milliliter) versus Time (Hours) Run 10

roughly 90 watts. Due to the relatively small volume in the proposed beaker, a short preliminary experiment was performed to determine if temperature increases would be a concern. To test this issue, a 140 ml beaker with approximately 100 ml of water was sonicated and magnetically stirred, with the temperature being taken periodically until a steady state temperature was reached. The water reached 31 C after ninety minutes, but the majority of the heat increase was attributed to the heat output from the motor of the stirrer, based on the findings of the temperature experiments done previously.

The next step was to determine if these operating parameters would adversely affect the viability of the cells. This was accomplished by doing the same experiment as described above with a bacteria solution instead of water. Also, a sheet of aluminum foil was formed into a "tent" above the beaker to prevent air-borne microorganisms from falling into the beaker and contaminating the culture. The culture consisted of one milliliter of concentrated culture in 100 ml of sterile nutrient broth. This experiment was run for over eleven hours and during that entire time, no visible sign of bacterial growth was observed. The solution remained completely clear.

Samples were taken as rapidly as possible for the first ninety minutes and then less frequently after that for a total exposure time of 11.5 hours. Plate counts were

performed and the results of these can be found in Figure 22. From these results it was determined that if this method were to be used for sample preparation, sonication for more than five hours would have strongly detrimental effects on the culture. For this reason, pretreatment using the flow system was thought to be the more effective and perhaps a better way to acclimate the bacteria to ultrasonic exposure.

The purpose of Run 11 was to attempt to both roughly duplicate the sonicated curve of Run 2, and to obtain some "pretreated" bacteria for use in Run 12. Since the shape of the growth curve for the sonicated sample was the desired information, no control curve was run for this experiment. The water bath was maintained at 36 C to achieve the maximum growth rate, and the sonication probe was set with the intensity setting at two, a one-second cycle time, and a ten percent duty cycle. The results of this run are given in Figure 23.

While the preparation and sonication procedures for this run were nearly the same as those used for the sonicated sample of Run 2, it is quite evident in comparing Figures 13, 14, and 23 that the results from the two experiments were very different. One possibility for this might be that the cultures were not in the same phase of growth at the beginning of the experiments, and thus acted differently

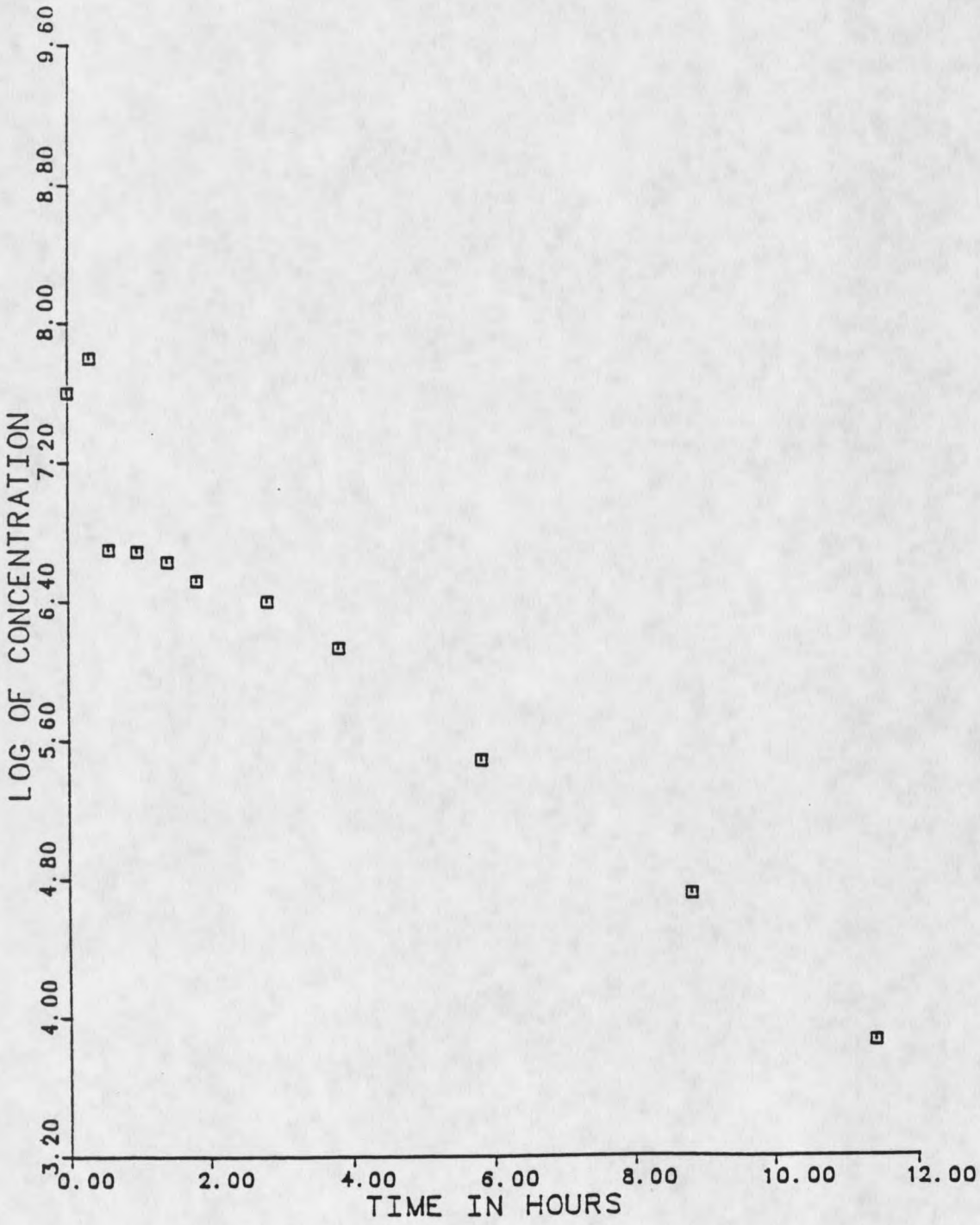

Figure 22. Pretreatment Experiment (Base 10 Logarithm of the Cell Concentration versus Time)

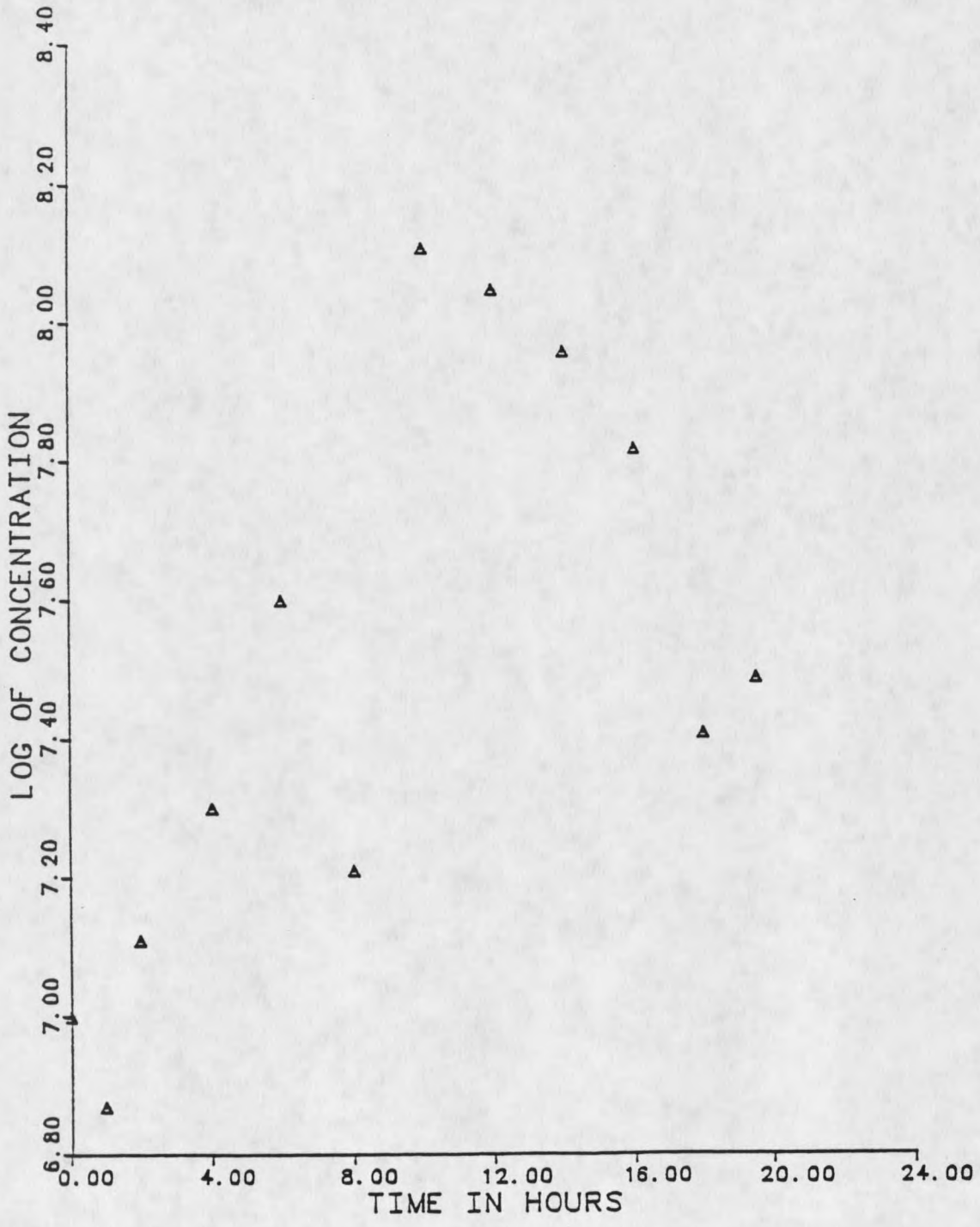

Figure 23. Base 10 Logarithm of the Culture Concentration (Cells per Milliliter) versus Time (Hours) Run 11

when inoculated into the fresh broth and exposed to the ultrasound. Differences might also be attributed to some unknown source of experimental error as the plot from Run 11 contained a fair amount of scatter. Regardless of the cause, the differences between the results of the two experiments lead to no definite conclusions.

This attempt to "duplicate" previous results illustrates one of the major difficulties in dealing with living systems; that even under seemingly identical conditions, growth patterns do not always remain the same. For this reason, attempts to compare results run-to-run with different starting cultures were abandoned, and only the two growth curves within each individual run were compared.

Run 12 was performed as a second attempt to determine if the culture could adapt to the ultrasonic field. It was thought that if the culture were acclimated to this field, it may grow faster if left in the field. This test was accomplished by inoculating the sonicated bacteria from Run 11 into fresh, sterile nutrient broth and placing it in an incubator for thirty-six hours. After this, the culture was refrigerated for seventeen hours. At this time, exactly five milliliters was added to 460 ml of sterile nutrient broth in each of the two reaction flasks. The bath temperature was maintained at 34 C and sonication was done at an intensity setting of one with a ten percent duty cycle

and a one-second cycle time. Samples were taken every hour for three hours and every two hours after that for a total of seventeen hours. At thirteen hours the control sample was observed to be far more cloudy than the sonicated sample. The results of Run 12 are given in Figure 24. Once again, the control sample was generally more concentrated than the sonicated sample for the duration of the run. This would seem to indicate that bacteria will grow faster outside of a ultrasonic field at the conditions of this run, regardless of the history of the culture and whether or not it had previously been exposed to ultrasound.

At this point, it was speculated that the concentration of nutrients in the broth was so high that diffusion through the capsule layer was not a limiting resistance for cell metabolism. Therefore, Run 13 was designed to determine if limiting the sugar, or carbon source, in the nutrient broth could force diffusion to be the limiting factor, resulting in increased growth and replication with sonication.

For this experimental run, 250 ml of sterile MSM was inoculated from the pure frozen parent culture and grown in the incubator at 35 C for 24 hours. Under these conditions, the culture should be in the stationary phase of growth, and should have metabolized nearly all of the glucose present in the MSM. After incubation, the culture was refrigerated for

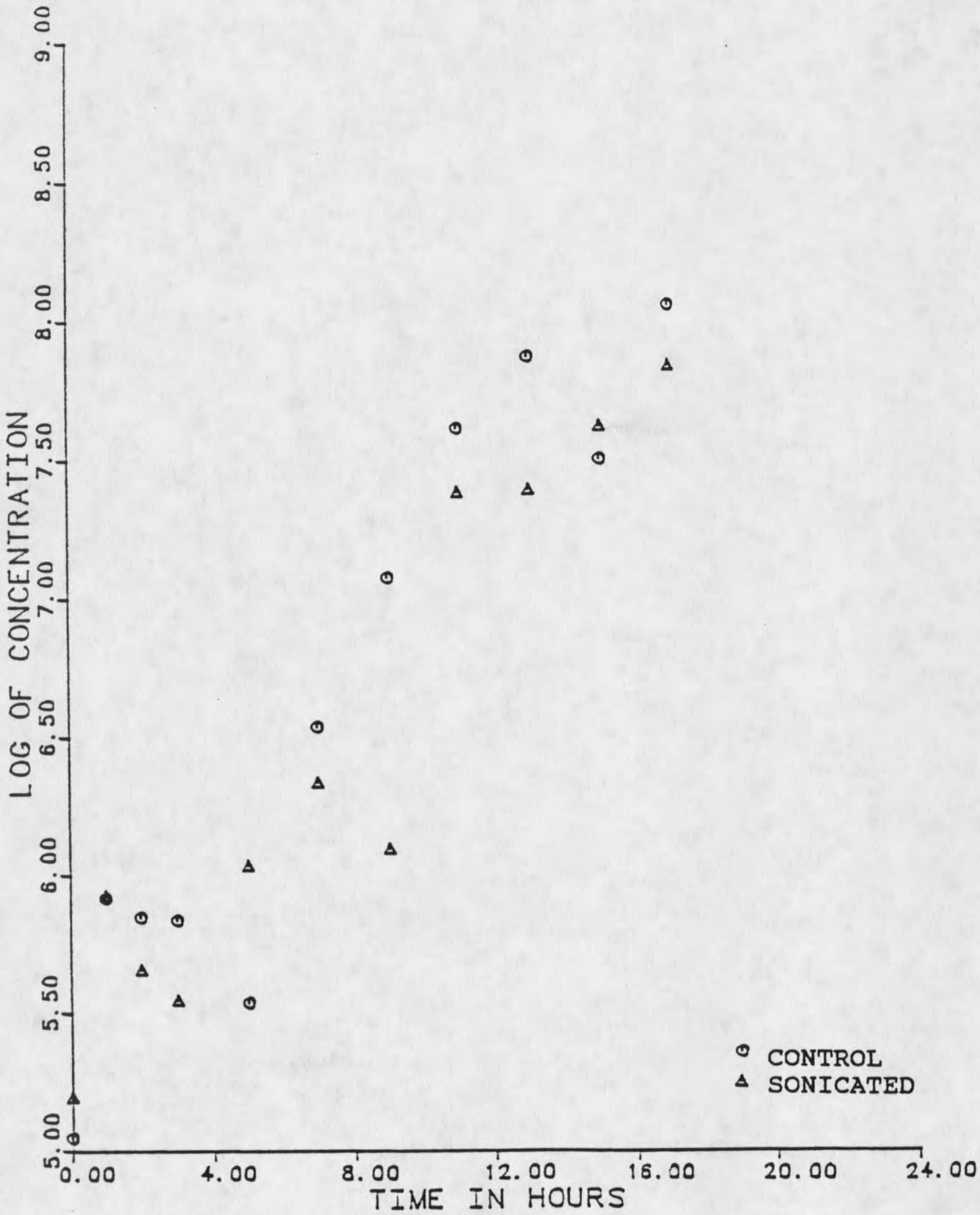

Figure 24. Base 10 Logarithm of the Culture Concentration (Cells per Milliliter) versus Time (Hours) Run 12

18 hours to help induce the lag phase for both the sonicated and control samples. Immediately prior to the run, three milliliters of this concentrated culture was added to each of the reaction flasks.

Preparation for this run was completed as had been done previously, with one major exception: the MSM that was to be used in the reaction flasks was prepared without any sugar source. Instead, a sugar source was prepared by sterilizing the glucose normally present (0.1 g in one liter) in 50 ml of demineralized water. This sugar source would then be fed to both samples at a rate of 1.00 ml/hr for the duration of the run. It was hoped that this would make the medium sugar-deficient, and growth of the cells would depend on their ability to obtain sugar. If this were true, then the transport of the sugar through the capsule layer might be the limiting step for metabolism and cell growth.

As usual, the sonicated culture was exposed at an intensity setting of one with a cycle time of one second and a ten percent duty cycle at a temperature of 34 C. Samples were taken every hour for three hours, and then every two hours for a total of seventeen hours. The data obtained from this experiment are given in Figure 25. Once again, it is not known why the concentration in both samples dropped so far early in the run, but it is evident that restricting

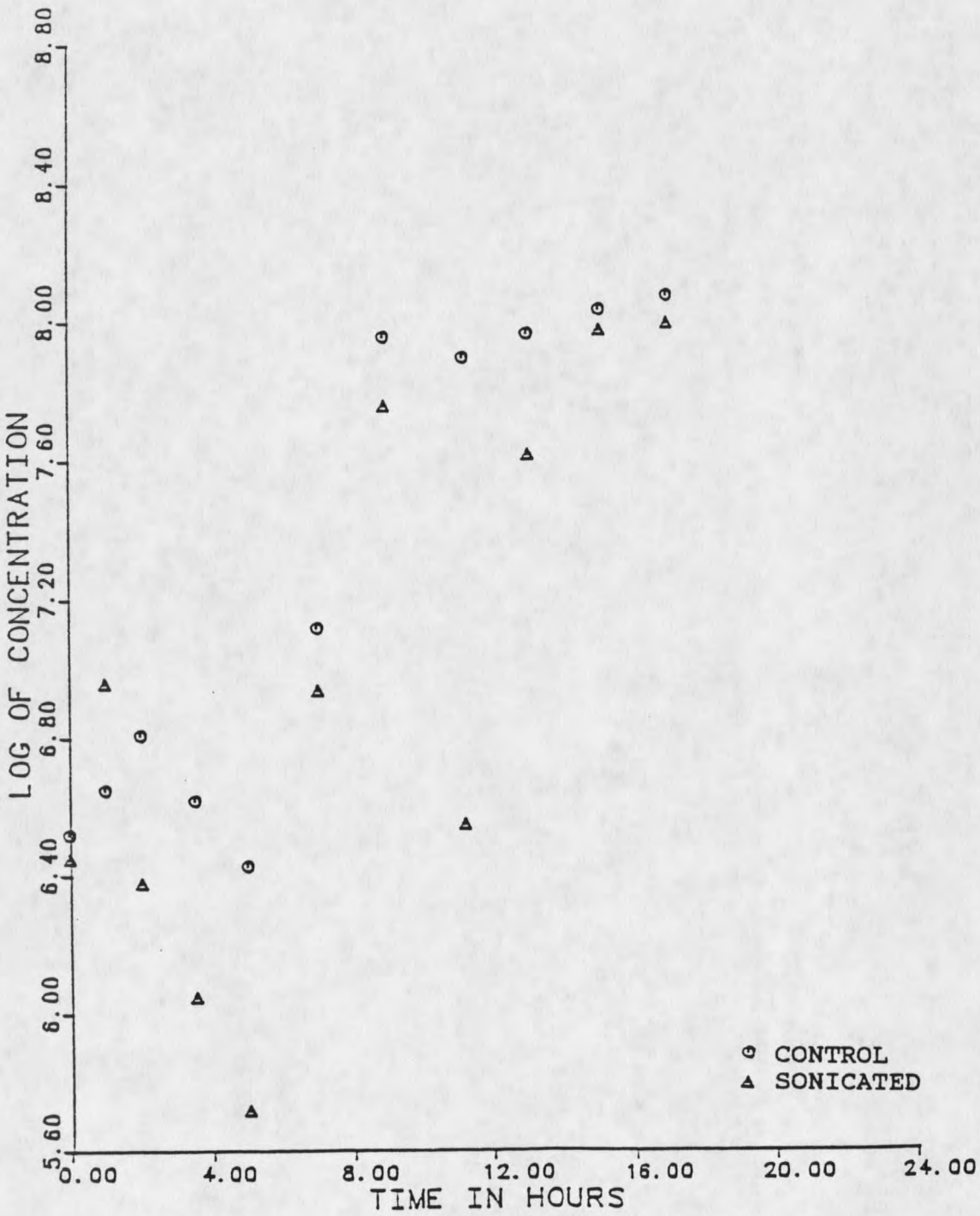

Figure 25. Base 10 Logarithm of the Culture Concentration (Cells per Milliliter) versus Time (Hours) Run 13

the carbon source did not show beneficial growth results for the sonicated sample.

It is important to note that in this case, the sonicated sample seemed be more greatly affected early in the run than the control sample. This is contrary to observations from the other two runs where a sharp initial drop in concentration was observed. Because the cause of this drop is unknown, it is impossible to tell whether or not the change from the sonicated to the control sample as the "protected" culture was due to the concentration of the glucose, or if all of the differences were due simply to unknown factors.

The idea of a nutrient-restricted environment was tried again in Run 18. In this case, the initial MSM contained only the potassium phosphates while the remainder of the nutrients, ammonium sulfate, magnesium sulfate, and glucose were sterilized separately in 20 ml of water. While they do provide the culture with necessary nutrients, the main purpose of the potassium phosphates is to buffer the solution so that the culture does not experience osmotic shock in an environment of pure water.

Run and exposure conditions for Run 18 were identical to those used in Run 13, with the exception that all of the nutrients except the phosphates were fed on an hourly basis instead of just the sugar. Samples were taken every hour

for the first four hours and then every two hours for a total of 14 hours. The results of this experiment are given in Figure 26.

As seen in Figure 26, the data from this experiment is very scattered, even more so than was previously observed. While the precise reasons for this are unknown, it could be that the concentration of the nutrients could have affected the growth of the cultures by limiting the nutrients available to the culture at certain times in the experiment. The fact that the nutrients were fed in small amounts at distinct time intervals and not continuously would lead to sudden changes in nutrient concentration at each feeding. This, in turn, could lead to sporadic culture growth. Also, the same amount of nutrients was added at each "feeding". Since the concentration of the culture increased by two to three orders of magnitude during each experimental run, the culture would naturally utilize less nutrients at the beginning of the experiment and more toward the end. Thus, feeding the culture regular quantities at regular times could theoretically lead to the culture having a surplus of nutrients at the beginning of the experiment and not enough at the end.

Ideally, nutrients should be fed as they are needed. Unfortunately, this depends on current concentration of both the culture and the nutrient broth. These, in turn, depend on the initial stage of the culture, the initial

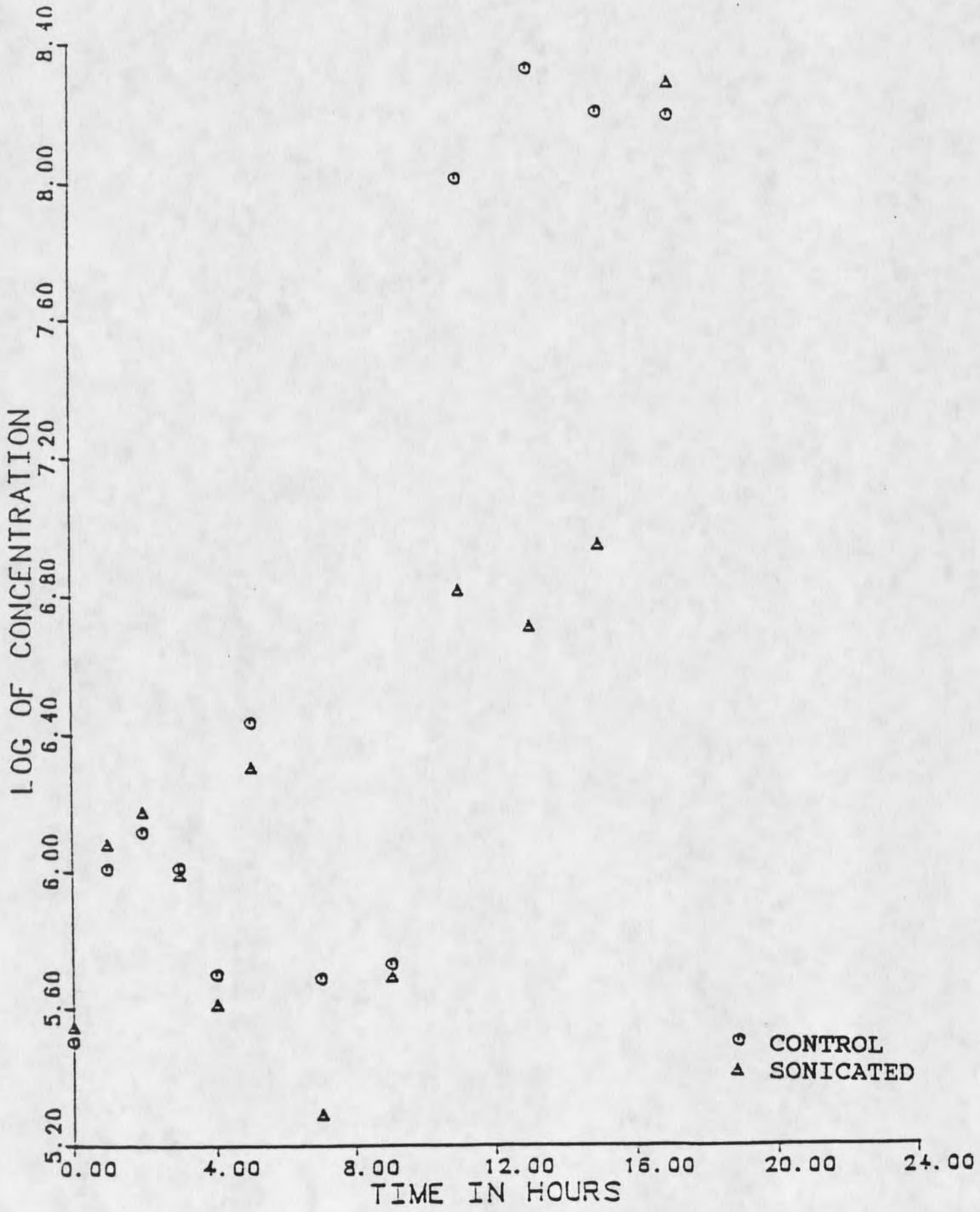

Figure 26. Base 10 Logarithm of the Culture Concentration (Cells per Milliliter) versus Time (Hours) Run 18

concentration of the culture, and nutrients utilized from previous feedings, none of which are available at the time that the experiment is executed.

At this time, it was concluded that if Pseudomonas aeruginosa could be benefitted by exposure to ultrasound, the conditions investigated and methods of analysis used for the current study were not sensitive enough to discover this fact. Thus, as several obvious parameters had been explored with no discernible beneficial results, the current approach to enhancing growth of this particular strain of bacteria with sonication was suspended, and a new avenue of research was pursued.

Third Series

At the conclusion of the Second Series of experimental runs, it was still inconclusive whether one of the major objectives of the current project had actually been accomplished: the shearing of the capsule layer by sonication. The fact that no metabolic evidence of shearing was observed is not definite proof that no capsule shearing had occurred. Therefore, the possibility of observing evidence of this shearing by some other method was explored.

Among microbiologists, it is believed that the capsule layer contains the products of metabolism of the cell, and

because of its sticky nature, allows adherence of the cells to each other and to surfaces within their environment. It is not thought to be directly necessary for cell life. In fact, "rough" strains of bacteria are nearly identical to "smooth" strains with the exception that the "rough" strains do not possess a capsule layer. Also, due to the nature of cell metabolism, the limiting metabolic step could be a number of things and would not necessarily have to be the transport of the nutrients through the capsule layer. Thus, if diffusion through the capsule layer were not a limiting step in cell metabolism, even if the layer were to be sheared, this may not be evident by viable cell concentration counts. However, it could possibly be observed through other methods, such as attachment ability. Developing concurrently with the present study at MSU was a separate project designed to examine the attachment of bacteria to various metals in a flow situation. Unfortunately, this project had not progressed to the point of being able to reliably quantify results, however, observation of the individual bacteria was possible. For this reason, the results obtained in the next series of experiments, especially the numbers generated, cannot be taken to be absolute, but should be thought of as a basis for possible future research.

The experimental apparatus used in this section of the current project consisted of a new flow cell, a microscope,

and a computer. The computer and microscope used are components of "Image Analysis", a method of computer observation and enhancement operated at MSU by the Institute of Biological and Chemical Process Analysis (IPA). In this system, the microscope "observes" the image and sends information about it to the computer. The computer then analyzes the image and uses various contrast and shading options in order to display the clearest picture possible on a computer monitor. In addition, a program for computerized counting of attached bacterial cells called "Bug Count" was used to some degree.

The developing IPA project dealt with attachment of bacteria on the surface of various metals, and the majority of the work had been done using stainless steel. Therefore, stainless steel was also chosen as the surface to be used in this series of experiments. The surface of even a small object is usually far too large to count all cells attached. Therefore, certain locations on the surface are analyzed and a comparison of overall attachment can be done based on attachment at these locations. The IPA project had designated a 3 x 4 grid of locations on the surface, so these were also used for the current project. These positions correspond to location settings on the stage of the microscope. One very important benefit of this system is that it will not allow researcher bias. That is, the locations for analysis are predetermined and, thus, the

researcher cannot simply pick locations based on the apparent attachment at that point. Using this methodology, the computer can be used to actually count attached cells using the "Bug Count" program. However, this computer program had not yet been completely adapted to the IPA flow cell and resulted in significant problems, and thus, it was seldom used in the current study.

The flow cell used in this series of experiments differed greatly from the flow cell used in the sonication system. Therefore, for the remainder of this report, to avoid any confusion between the two, the flow cell used in the Image Analysis system will be referred to as the IA flow cell, while the flow cell used in the sonication system will continue to be called the flow cell.

The IA flow cell is given in Figure 27. The fluid lines shown were "built in" to the cell and flow was drawn through the cell from tygon tubing attached to these lines. For cleaning and preparation purposes, the glass slide, and then the stainless steel surface were removable. The IA flow cell was attached to the microscope platform using the screws shown.

The preparation of the IA flow cell was significantly different from the preparation of any equipment used up until this point. Previously, it was sufficient to simply sterilize the equipment used for experimentation. This was not the case with the IA flow cell. In addition to

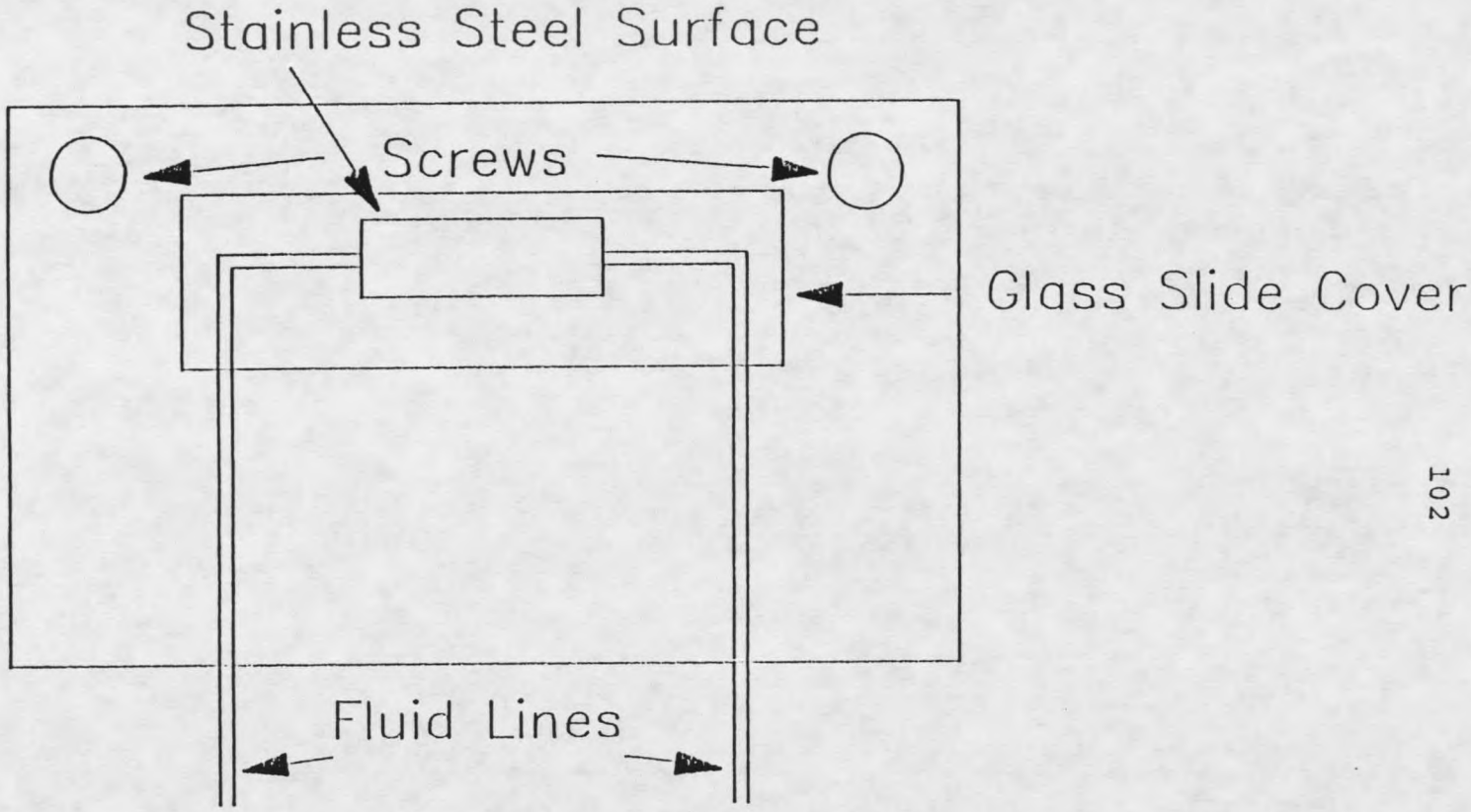

Figure 27. Image Analysis flow Cell

sterilization, the surface to be used had to be polished before each sample to be analyzed. Other than this, preparation took very little time. For each sample, the culture was placed in a 250 ml Erlenmeyer flask with a sterilized magnetic stirrer. The surface, stainless steel, was polished and the IA flow cell assembled. Tubing to be used for the transport of the culture solution was washed in ethanol. Then, the flow cell was mounted in place on the microscope and the tubing lines were attached. The tubing forming the outlet from the cell was attached to a small pump. In this way, the bacterial solution was pulled through the system, allowing the pump to be used without requiring sterilization, and avoiding additional shear. Before the culture solution was passed through the system, the system was flushed with sterile water to remove any residual ethanol. The system was then ready for use.

A series of experiments (Runs 15 - 17, and 19) was designed to examine and compare the attachment abilities of bacteria that had been exposed to ultrasound to those that had not. If differences were found, a test of the daughter cells was planned to determine whether differences would be passed on to subsequent generations. In order to have a quantitative comparison, all samples tested should be of exactly the same concentration and exactly the same flow through the IA flow cell. Due to the fact that viable cell concentrations cannot be determined without significant time

elapsing, this was never entirely possible. However, concentrations were generally very close, making some comparison possible.

The sonication system used to prepare the bacteria for this series of experiments was the same as that described in the Experimental Apparatus section of this report. In the IA flow cell apparatus, the culture solution left the stirred flask, flowed across the metal surface within the IA flow cell and then exited the cell into the graduated cylinder to determine flow rates. As mentioned before, this system was not fully developed, and one significant fault was that the method used to determine flow rates was to collect and measure the solution volume for a designated period of time and then compute the flow rate from this information. This inability to control flow would not be a problem with small differences in flow rate. However, at times the flow rate would be two or three times as great for one run as for another run at the same setting on the pump. Since flow controlled the number of bacterium that passed through the IA flow cell as well as the volumetric flow rate and velocity of the fluid, changing this one variable actually affected three variables within the experimental system. Thus, this problem was by far the biggest obstacle in this series of experiments and contributed heavily to the inability to precisely quantify results.

Run 15, the first of the attachment experiments, was conducted without using the IA flow cell. It was thought that if attachment differences existed, they might be observed by simply exposing a surface, such as glass, to the culture and observing attachment after a specified period of time. The plan for this experiment was to expose a sample of the bacteria to sonic energy, after which one glass slide would be vertically submerged in each of the sonicated and control samples in separate beakers. A diagram of how this was done is given in Figure 28. After one hour, the slides were to be removed from the culture, rinsed in sterile water, and observed under the microscope. This experiment was not intended to result in quantitative results, but was meant as a "quick and dirty" trial run to see if further experimentation was warranted. If results could be seen with this crude experimental method, then the IA flow cell would be used to obtain further experimental data.

In preparation for this run, the inoculation bacteria were taken from a pure, frozen parent culture and put into 250 ml of sterile nutrient broth, grown at 35 C for 24 hours, and then refrigerated for about 1.5 hours to temporarily slow growth. After this, 5.0 ml of this concentrated solution was put into each of the reaction flasks along with 460.0 ml of sterile nutrient broth. Sonication was conducted as in the past: the cycle time was

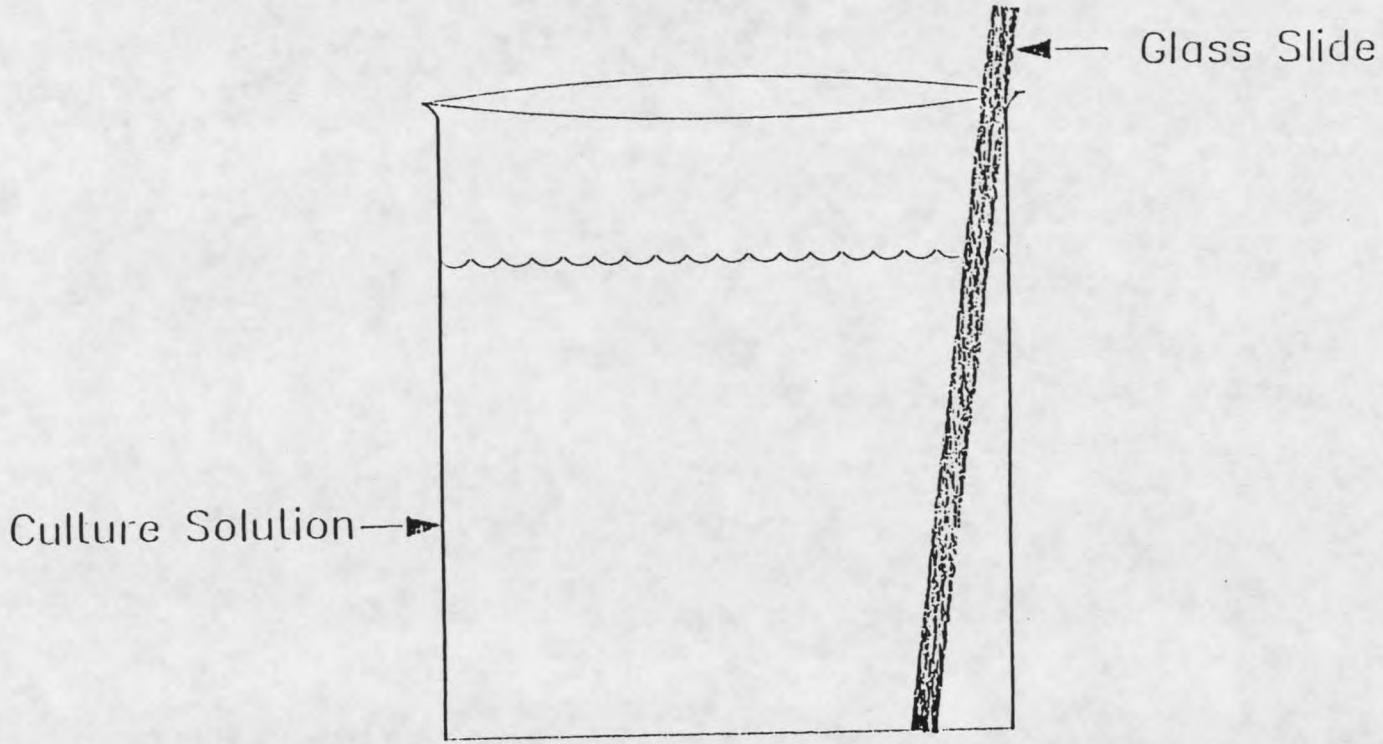

Figure 28. Glass Slide Experimental Apparatus

1.0 second, the percent duty cycle was 10 percent, the intensity setting was at one for 18.5 hours, and the temperature was 34 C. After exposure, the cultures were placed in the beakers and the glass slides were immersed. No samples for viable plate count analysis were taken until the end of the sonic exposure, at which time viable plate counts were complete. The concentrations (in cells/ml) were within one order of magnitude, being 8.87×10^8 for the control and 1.19×10^8 for the sonicated sample. Far more cells appeared to be attached to the control slide than the sonicated slide. Although these results were tentative, it was decided that there was enough evidence to justify a more detailed experimental approach using the IA flow cell.

Run 16 was designed to analyze the ability of the bacteria to attach in a flow situation. In this case, the IA flow cell was used along with the Image Analysis computer system. The cells were prepared for the IA flow cell in the same manner as they were prepared for the glass slides in Run 15.

After exposure to the sonication, both the sonicated and control samples were removed from the water bath and refrigerated. As only one IA flow cell existed, only one of the samples could be tested at any one time. Since it was desirable to have both samples experience identical conditions, and it took approximately two to three hours to

test each sample on the Image Analysis system, it was decided that each test would begin with the culture at refrigerated temperatures. This would result in nearly identical temperatures for each sample at the beginning of each test as well as slowing any growth in the sample that was forced to wait.

Under normal conditions, this particular strain of bacteria has a high affinity for attaching to surfaces. Therefore, a concentration of 10^8 cells/ml, typical of concentrations at this point in the research, was thought to be too high to give readable results as the cells might attach far too quickly. Therefore, this culture was diluted by about two orders of magnitude for use in the IA flow cell. The diluent used was water buffered with potassium phosphate. This dilution was done for both the control and sonicated samples. Exact measurement of culture broth and diluent were not necessary in this instance. This was due to the fact that the concentrations between the control and sonicated samples were never the same at this point in the experiment. Since nearly identical concentrations were desired for comparisons in the IA flow cell, the exact measurements used in the dilutions were based on the estimated concentration due to the visual appearance of the culture, and on previously sonicated samples under similar conditions. In this case, 4.0 ml of sonicated sample was added to 250 ml of buffer while 2.0 ml of control solution

was added to 250 ml of buffer. Since it was speculated that the sonicated sample would not be able to attach as well as the control sample, it was decided that if either sample were to be higher in concentration than the other, it would be best if it were the sonicated sample, thus weighting the results somewhat against the expected outcome.

As in all of the experiments of this series, the only heat added to the IA flow cell system was that provided by the magnetic stirrer. From the heating experiments performed earlier in this project, it was known that the magnetic stirrer would keep the solution at approximately 31 C. Flow was measured using the graduated cylinder and was found to be 120 ml/hour for the control sample, and 90 ml/hour and 60 ml/hour for the sonicated culture. Attachment of daughter cells was also studied by innoculating fresh broth with a small amount of the sonicated sample, allowing this culture to grow overnight, and then testing it in the IA flow cell. The concentration as measured by viable cell counts on petri plates was 4.5 x 10^5 cells/ml for the control sample and 1.4×10^6 cells/ml for the sonicated sample.

For both the control and sonicated samples, viable plate counts were done both immediately before and immediately after testing on the Image Analysis system. These counts, which are given in Table 7, show that the sonicated sample was significantly more concentrated both before and after

Microscope Setting	Control Sample	Sonicated Sample	Daughter Cell Sample
1	12	7	7
2	10	4	5
3	13	2	10
4	11	1	1
5	10	3	5
6	15	2	10
7	11	0	2
8	8	6	9
9	15	0	8
10	10	4	5
11	10	3	8
12	11	1	7
Average	11.3	2.8	6.4
Flow Rate	2.0 ml/min	1.5 ml/min	1.0 ml/min
Concentration			
Initial	2.4×10^5	1.0×10^6	1.4×10^6
Final	6.6×10^5	1.4×10^6	2.9×10^6

Table 7. Bacterial Attachment, Run 16

the test. However, when the attached cells were counted at the end of one hour, the sonicated sample had an average of less than 3 cells per screen while the control had an average of over 11 cells per screen, or nearly four times the attachment of the sonicated sample. Considering that the sonicated sample was higher in concentration, this result seems very significant.

In addition to the immediate effect, a daughter cell analysis was prepared by inoculating approximately 250 ml of sterile nutrient broth with the sonicated culture and growing this new culture at 35 C for 24 hours. This new culture was again decreased in concentration by dilution with buffered water and tested on the IA system. This resulted in an average of five to six cells per screen, still only about half of the average for the control sample. Once again, concentrations were measured by viable plate counts before and after the experiment. While the counts done at the completion of the experiment were unreadable, the counts before the test indicated a concentration of 1.4×10^6, well above the concentration for the control sample.

The results of this experiment seemed to indicate that sonication actually does affect the ability of P. aeruginosa to attach and that this ability seems to be passed on to future generations.

Run 17 was performed in an attempt to duplicate the results of Run 16. All system parameters and culture preparation steps were conducted as in Run 16. Once again, an attempt was made to achieve concentrations that would be nearly the same. The results of this experiment are given in Table 8. In this case, the concentrations were very close. Nevertheless, when the attached cells were counted, the control sample averaged 10 cells per screen while the sonicated sample averaged about 1.5 cells per screen, or about one-seventh the attachment level for the control sample. This experiment again seemed to indicate that sonic energy can be employed to disrupt the ability of the cells to form biofilms.

Run 19, was a final attempt to show that attachment of viable cells could, in fact, be adversely affected by sonication. The culture preparation for this experiment was identical to that used for Runs 16 and 17. However, it is doubtful that the cultures were at the same point in the growth curve as had previously been obtained. This was because of the apparent condition of the cells, especially the cells of the control sample, as viewed through the microscope. In all other experiments in this series, the individual bacterium were easily visualized. This was not the case for Run 19. The bacterium here were very small and difficult to see. This made comparisons between the

Microscope Setting	Control Sample	Sonicated Sample
1	10	3
2	16	0
3	10	0
4	13	1
5	12	3
6	13	4
7	7	0
8	9	3
9	11	1
10	4	0
11	8	2
12	6	0
Average	9.9	1.4
Flow Rate	1.5 ml/min	1.7 ml/min
Concentration		
Initial	2.0×10^5	1.6×10^6
Final	2.5×10^6	2.1×10^6

Table 8. Bacterial Attachment, Run 17

sonicated and control samples very difficult within this experiment, as well as impairing the comparison of Run 19 with Runs 16 and 17. The results from this experiment are given in Table 9.

In Run 19, as in Runs 16 and 17, the bacteria that had been exposed to ultrasound yielded fewer cells attached to the stainless steel surface than attached with the control sample. However, as stated above, cells in the control sample appeared different than those observed in other samples in this series of experiments. While it is unknown whether this was an actual difference or simply an artifact of the experimental method, the general results again pointed to sonication having an adverse effect on bacterial attachment rate. Admittedly, the results here were not nearly as dramatic as had been obtained previously, but they still indicated less attachment. For the sonicated sample, the concentration was lower, the flow rate higher, and the number of attached cells lower than that of the control sample. However, the difference between the two numbers, 3.5 cells per screen for the control sample and 2 for the sonicated sample, was so slight that it could easily be attributed to experimental error. These facts alone would make any comparison difficult. In addition, the concentration of the control sample was higher than that of the sonicated sample, while the flow rate of the sonicated sample was higher than that of the control sample. These

Microscope Setting	Control Sample	Sonicated Sample	Daughter Cell Sample (1 Day)	Daughter Cell Sample (2 Days)
1	2	2	63	1
2	2	3	86	1
3	4	0	73	1
4	2	2	3	0
5	4	0	19	2
6	3	0	34	2
7	1	3	16	0
8	4	1	39	0
9	9	4	39	1
10	4	6	24	1
11	5	0	20	0
12	3	5	42	0
Average	3.6	2.2	38	0.8
Flow Rate (ml/min)	1.9	2.0	1.5	1.3
Concentration				
Initial	$5.1x10^6$	$7.9x10^6$	$4.9X10^6$	$5.1X10^5$
Final	$5.7x10^6$	$6.7x10^6$	$9.0x10^6$	$6.1x10^5$

Table 9. Bacterial Attachment, Run 19

differences between the two samples make any definite conclusions risky in a system of such uncertainty as the IA flow cell.

In addition to the problems experienced with the direct comparison between sonicated and non-sonicated samples, the cultures grown from the sonicated sample for one and two days also resulted in some unexpected attachment findings. While the plate counts from both samples indicate that the cultures were still increasing in number, the numbers of attached cells did not correlate with any previous results. The concentration of the culture grown for one day was only slightly higher than concentrations used in previous runs and very similar to the concentrations of the initial control and sonicated samples. However, the attachment was much higher than experienced before. In fact, there were too many cells attached to count by eye (average of 38 per screen), and the Bug Count program had to be used. However, the culture that had been growing for two days gave results that were back to normal, averaging less than one attached cell per screen.

It is unknown why the culture grown for one day gave such drastically higher attachment results, but one possibility is that the surface became contaminated after polishing and before insertion into the IA Flow Cell. A second possibility is that one or more large clusters of cells were suspended in the sample and became attached when

they were pulled through the IA flow cell. This latter possibility became even more likely when further examination of the metal surface revealed large groups of cells that could not have grown to such large populations within the one hour duration of the experiment. For these reasons, the results obtained from the first day generation of sonicated cells cannot be accepted as being valid.

CONCLUSIONS

The original theory to be tested in this project was that growth rates of bacterial cell populations can be enhanced by the total or partial removal of the surrounding capsule layer by low intensity ultrasonic shearing. Resolution of this theory remains somewhat in question, largely due to complexities encountered in processing "living" systems. However, a number of tentative conclusions related to this theory can be drawn from results of the present work.

1. At the low intensity ultrasonic exposures of this work Pseudomonas aeruginosa are able to adapt and remain generally viable in a flow system. However, there is no definite evidence that a beneficial metabolic effect occurs due to ultrasonic exposure compared with non-sonicated control samples.

2. The bulk of evidence suggests that the low intensity ultrasonic exposures of this study retard metabolic growth of Pseudomonas aeruginosa, although only slightly.

3. Low intensity ultrasonic exposures seem to diminish the ability of Pseudomonas aeruginosa to attach to a

stainless steel surface. This result implies an impact of ultrasound on capsule layer thickness.

4. The diminished attachment ability apparently due to low intensity ultrasonic exposure seems to be passed on, at least to some degree, to daughter Pseudomonas aeruginosa cells.

RECOMENDATIONS FOR FUTURE WORK

While the original theory to be tested in this project was neither proven nor disproven, it still presents various other possibilities that can be explored using bacteria and sound waves.

1. The attachment results obtained from the third series of experiments were, for the most part, quite solid. These experiments would provide a follow-up experimenter with an excellent place to start. However, until the IA flow cell system is better developed, precise results from this system would still be difficult to obtain. Fortunately, there are some fundamental improvements that could easily be made to improve the system, although not perfecting it.

    a. A metering pump would eliminate the differences in the flow rates and allow the experimenter to set distinct values that could be kept consistent for samples within each experiment, as well as from experiment to experiment.

    b. Duplicate cells could be built so that the time required to change from one sample to the next could be significantly shortened to the one hour required for the experimental run itself.

Unfortunately, one of the major problems with these experiments, the inability to predetermine the concentration, would still exist. However, as was discovered in these experiments, this should not be a significant problem as long as the concentration of the sonicated sample is higher than that of the control sample. Exact quantified results may still not be possible to obtain, but qualified results should be very possible, as long as any differences are biased against the expected results. Based on what is already known in this area, this seems to be the most promising experimental direction to take at this point in time.

2. A second possibility for future work would be to use an electron microscope and capsule stains to attempt to directly determine if the capsule layer were affected at all by sonication. This approach would most likely require a large amount of assistance from a microbiologist and some technique development for P. Aeruginosa.

3. There are other possibilities for microbial growth enhancement due to ultrasonic exposure that were not explored in this project, but in light of the general lack of success so far, this area seems to hold little promise. These possibilities could include such things as growth at either very high or very low temperatures, growth in broth

starved for nutrients other than carbon or nitrogen, or using a different type of capsule-forming bacteria. As stated before, due to the lack of success in this area during the current project, it is questionable, although not impossible, that positive results could be found in this area.

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