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Technical Report

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Executive Summary

Attachment and Early Biofilm Development of Methane Forming Anaerobic Microbial Cultures

One of the disadvantages of anaerobic methane-forming biofilm reactors is their long start-up time. Improvement and optimization of initial bacterial attachment and biofilm development would help to further implement the use of anaerobic wastewater treatment processes. The purpose of this study, which was conducted from 1983-1986, was to obtain basic information on bacterial attachment and biofilm development for methane-forming anaerobic mixed cultures.

An anaerobic attachment vessel was designed, constructed, and used to quantify and visualize the initial attachment of chemostat grown anaerobic bacteria and the development of anaerobic biofilms. Bacteria from methane-forming anaerobic chemostat cultures attached rapidly to washed/autoclaved glass slides in the attachment vessel. Within one to three hours, the number of irreversibly attached bacteria increased by approximately two orders of magnitude from 0 bacteria per 10,000 square micrometers to 100 to 250 bacteria per 10,000 square micrometers. Only a slow increase in the number of irreversibly attached cells was measured after the initial rapid increase. The counts of total bacteria after one week of inoculation were in a range of 250 to 450 bacteria per 10,000 square micrometers. No statistically significant difference was noted in the pattern of attachment of the eight day solids retention time culture and a twenty day solids retention time culture.

Two mathematical models were developed to describe early attachment and growth. Each model contained three coefficients. One coefficient represented the maximum number of bacterial cells that could initially attach to the surface. One coefficient was a rate coefficient describing the initial rate of attachment. One coefficient described the specific growth rate of the bacteria after they had attached to the surface.

A significant fraction of the bacteria counted on the washed/autoclaved slides were methanogens. The counts of methanogenic bacteria, which were counted by epifluorescent microscopy, followed a similar pattern of attachment over time when compared to the counts of total bacteria over time. The counts of methanogenic bacteria were generally 25% to 75% as high as the counts of total bacteria. This is a conservative estimate since one type of acetate utilizing methanogen is not readily observed to fluoresced.

Autoclaving as a final step in the wash procedure had a statistically significant effect on attachment. The counts of irreversibly attached bacteria on washed/unautoclaved slides over time were one half to one and one half orders of magnitude lower than the corresponding counts for washed/autoclaved slides.

Scanning electron microscopy showed some cells possess conspicuous appendages or extracellular fibers which appear to be used for attachment while other cells do not have such conspicuous structures. At long inoculation times, more extensive development of extracellular fibers was sometimes observed and more amorphous extracellular material was present. At short and long inoculation times, cells were attached as individuals and in clumps. The clumps were covered and/or interspersed with the amorphous, extracellular, gluelike material. Some clumps and individual cells also appear to have a ring around them. It was speculated this ring is either from the secretion of extracellular polymers or enzymes.

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CHAPTER I. INTRODUCTION

In recent years, there has been a renewed interest in the use of methane generating anaerobic fermentation processes to degrade organic wastes. In 1964, McCarty (25) summarized the advantages and disadvantages of methane generating anaerobic waste treatment with microorganisms as compared to aerobic treatment with microorganisms. At the time of McCarty's paper, methane generating anaerobic waste treatment systems were based on the suspended growth of bacteria. The advantages he listed were as follows:

1) a high degree of waste stabilization is possible,
2) low microbial yields result in low production of sludge,
3) low nutrient requirements,
4) no oxygen requirement, and
5) methane gas production from degraded organic matter.

The disadvantages he discussed were:

1) optimum process temperature requires heating the waste,
2) poor process stability due to slow growth rates,
3) lack of knowledge about nutritional requirements,
4) difficulty in treating low strength wastes, and
5) long start-up times.

Since the mid 1960's, several new designs for the methane generating anaerobic fermentation process, based on the used of fixed microbial films (or biofilms) have been developed. Some of the most significant new designs include the anaerobic filter, the anaerobic upflow sludge blanket reactor, the anaerobic attached film expanded bed reactor, and the anaerobic baffled reactor. A complete description of these new designs may be found elsewhere

(Speece (39) and Switzenbaum (41)). Also since the mid-1960's, there has been a substantial increase in the knowledge about the nutritional requirements and basic microbiology of methane generating anaerobic cultures.

There are three important advantages of the anaerobic biofilm reactors when they are compared to suspended growth systems.

1) They achieve substantial substrate removal with much shorter hydraulic detention times than suspended growth systems.
2) They are more stable to shock loads and toxic substances than complete mix systems.
3) Some operate effectively at less than optimum temperatures and their performance is less effected by changes in temperature.

The advantages of methane generating anaerobic biofilm reactors listed above, coupled with the new basic knowledge on nutrition and microbiology, have addressed many of the disadvantages listed by McCarty (25). One new advantage, discovered in recent work, is that methanogenic anaerobic cultures are capable of degrading aromatic compounds (20) and halogenated aliphatic compounds (3,4). The former group was previously considered nonbiodegradable anaerobically (27). The latter group is generally believed to be refractory under aerobic conditions (27). The long start-up time and difficulty in treating low strength wastes remain as persistent problems.

The goal of this project was to obtain basic knowledge about attachment of methane forming microbial cultures and early biofilm development. Such information is important in understanding biofilm development and thus reducing start-up time. In particular, this study conducted from 1983-1986,

examined the influence of three parameters on bacterial attachment and early biofilm development of methane forming microbial cultures. They are:

1. growth rate of the culture of microorganisms,
2. cleaning preparation of the glass surface used for attachment, and
3. inoculation time - the amount of time bacteria were exposed to the attachement surface.

CHAPTER II. BACKGROUND

A. *Methanogenesis From Complex Organic Substrates*

Before considering the attachment of bacteria to surfaces, it is necessary to review how microorganisms convert complex organic molecules to methane and carbon dioxide. There are five groups of organisms involved in methanogenesis (Figure 1). A consortium of microorganisms from these five groups (numbers are shown in Figure 1) are necessary to bring about methanogenesis from complex organic compounds. Group 1 represents a wide range of fermentative bacteria that take complex organic polymers, convert them to monomers and oligomers, and convert monomers and oligomers to H_2, CO_2, acetate, and longer chain fatty acids. Group 2 are the hydrogen producing acetogenic bacteria. These bacteria convert fatty acids, longer than acetate, to acetate, carbon dioxide, and hydrogen. Group 3 are the hydrogen consuming acetogenic bacteria. These bacteria reduce carbon dioxide to acetate. Groups 4 and 5 are the methanogens. Almost all known methanogens are capable of converting H_2 and CO_2 to methane. Only two methanogenic genera, Methanothrix (filaments composed of rods) and Methanosarcina, are known to be capable of converting acetate to methane and carbon dioxide.

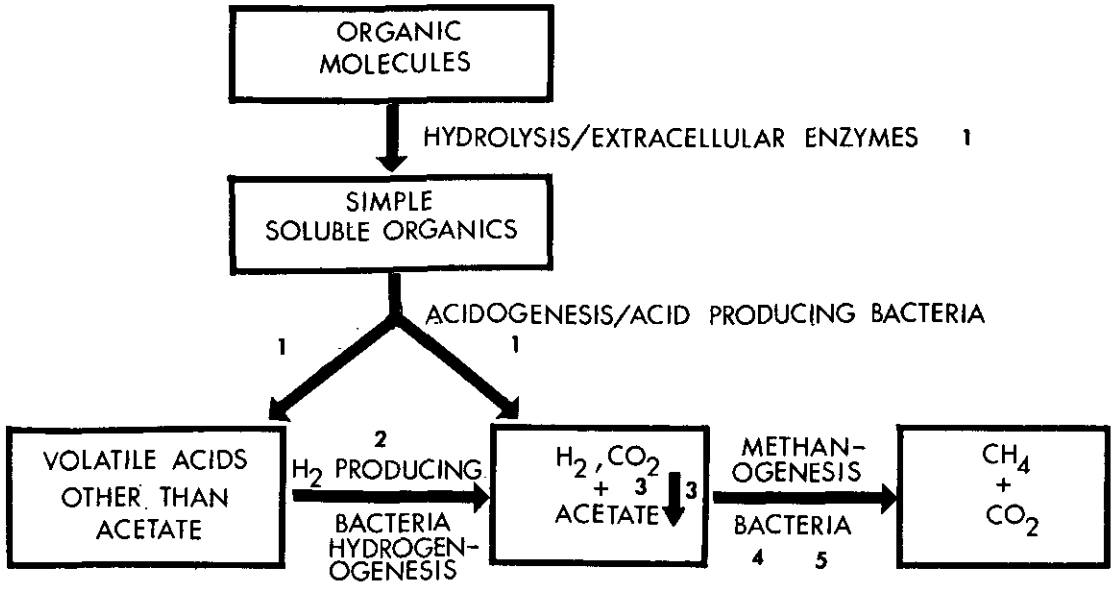


Figure 1

Anaerobic Degradation of Organic Matter

The organisms of a methanogenic consortium are closely interdependent on one another for survival. For example, the conversion of proprionate to hydrogen gas, carbon dioxide, and acetate is only thermodynamically favorable if the hydrogen partial pressure is between 10^{-4} and 10^{-6} atmospheres (26). The methanogens keep the concentration of hydrogen low for the proprionate oxidizing bacteria by utilizing the hydrogen as an electron donor. So these two groups of organisms have a syntrophic relationship.

The acetate utilizing methanogens play an important role in methanogenesis. It was pointed out earlier that only two methanogens are capable of converting acetate to methane and CO_2. It has also been found that approximately two-thirds of the methane formed in wastewater treatment reactors comes from acetate via these microorganisms (22). de Vocht et al. (11) performed experiments that indicated reactors which selected for sedimentation of organisms favored Methanothrix, while reactors selecting for organisms which attach to surfaces favored Methanosarcina. In our laboratory an electron microscopy study comparing biofilm development in three reactor types found relatively more sarcina in the high shear anaerobic fluidized bed than the low shear anaerobic filter and anaerobic upflow sludge blanket reactor. In the latter two reactors, rod type organisms were more numerous than sarcina. In Robinson's (35) electron microscopy study of eight methanogenic, anaerobic fixed film reactors, Methanothrix spp. was found in high numbers at film surfaces whereas Methanosarcina spp. was commonly embedded in the lower regions of the film.

There are some kinetic data for Methanosarcina spp. and Methanothrix spp. Methanothrix spp. have a doubling time of between four and nine days

(21), uses only acetate as a substrate (26), and have a K_m value of less than one millimolar (21). Doubling times for Methanosarcina spp. grown on acetate have been reported as short as one day (38). The K_m values are from three to five millimolar for growth on acetate (38).

One final item of importance when comparing Methanothrix spp. with Methanosarcina spp. is that species of Methanosarcina autofluoresce whereas Methanothrix spp. do not (21,45). The autofluorescence is due to the presence of Factor 420, a compound methanogens use to accept electrons from hydrogen. The reduced form of Factor 420 then donates its electrons to NAD to give the cell reducing power (5). Factor 420 absorbs light at 420 nm and fluoresces blue-green light when placed in an oxidized environment.

B. How and Why Bacteria Stick to Surfaces

It is generally accepted that there are two classification of attachment of bacteria to surfaces, "reversible attachment" and "irreversible attachment" (24). Reversible attachment is defined as an instantaneous attraction to a surface where the cell still exhibits Brownian motion but can be removed by washing. Irreversible attachment results when bacteria are firmly adsorbed to a surface. They no longer exhibit Brownian motion and are not removed by washing.

The most widely accepted explanation of reversible attachment involves the Vervey and Overbeek; and Derjaguin and Landau (VODL) theory. VODL theory predicts a general pattern of attractions and repulsions between colloids and surfaces. The repulsive energy is due to the electrostatic interaction between the like charges of the colloid and the surface. The attractive energy is due to van der Waals attractive forces. The sum of these two forces results in a total energy such that a repulsive energy

barrier exists as the colloid and surface approach each other. At a slightly greater distance apart from the energy barrier, the colloid and surface actually attract one another at a region called the secondary minimum. It is proposed that bacteria can be attracted to the secondary minimum because of the energy pattern described in the VODL theory.

As the radius of a sphere approaching a surface is reduced, the VODL repulsive energy barrier is reduced. Thus if a cell produces a small diameter probe, the probe would have a much smaller energy barrier to surmount. Such a probe might then be capable of forming a bond to the surface. Rogers (36) states that sufficient energy required for such a probe to overcome the energy barrier could be provided by the forces of locomotion developed by a bacterial cell or by molecular bombardment.

Marshall (24) obtained data that supported the VODL theory for a marine bacterium. He compared reversible sorption of bacteria and the theoretical double layer thickness with the log of the electrolyte concentration. He also compared the energy of interaction between glass and bacterial surfaces with the particle separation (a VODL type plot) for different electrolyte concentrations. Data showed that there was only slight reversible attachment when the electrolyte concentration was low and the double layer thickness large. However at high electrolyte concentrations and small double layer thicknesses, the reversible attachment of microorganisms was high.

Meadow's (29) experiments showed that freshwater bacteria and marine bacteria responded very differently when attachment was related to ionic strength. Attachment of freshwater bacteria was optimum at low ionic strengths as compared to marine bacteria which attached optimally at the

normal ionic strength of seawater. It is unclear from the experimental procedure whether this experiment actually measured reversible or irreversible attachment. Slides were grown for three hours in an appropriate broth, counted and transferred to various test solutions at different ionic strengths for 1.5 hours, then counted again. There was no washing step but one would imagine that reversibly attached organisms would detach if the test solution was not at an appropriate ionic strength.

Bacteria use their glycocalyx to irreversibly attach to surfaces. A glycacalyx is a mass of tangled polymer fibers which extend from the surface of a cell. The exact composition of the polymers is not known but they are thought to be primarily polysaccharides and glycoprotein type molecules (12). The existence of the glycocalyx has only been known since the late 1960's (7). The reason for its recent discovery is that the glycocalyx typically does not form in pure laboratory cultures, the major investigative system used by microbiologists (7). Apparently the production and maintenance of a glycocalyx requires a substantial metabolic expenditure. Cells which are not burdened with this metabolic expense are selected for in pure laboratory culture (7). In naturally occurring environments, however, selection favors microorganisms that produce a glycocalyx. The glycocalyx had been found to be a universal structure in bacteria and is thought to be essential to the biological success of most bacteria (7).

It is interesting to note that most of the cells from the other kingdoms of organisms also possess an external polymer coating. Plant cells have an outer layer containing cellulose, hemicelluloses, pectin and lignin (34). Fungal cells have an outer layer of chitin (34). Animal cells have a variety of polysaccharides in their glycocalyxes (7). At least some of the

Protista (i.e., algae) contain a variety of polymers in their cell walls (34).

The attachment of bacteria to a surface can be nonspecific or specific (7). The exact mechanism of a nonspecific bond to an inert surface is unknown (7). Specific bonds are usually formed between bacteria and other higher organisms. Higher organisms have their own chemically defined glycocalyx. The glycocalyx of the bacteria and the higher organism can either be held together by polar attraction (i.e. two negatively charged polymers joined by a divalent cation) or be joined by a lectin molecule. Lectins are molecules found primarily in highly developed organisms which can form a bond bridge between two specific sugar molecules. If the sugar molecules happen to be at the ends of two polysaccharide chains, then the lectin molecule can bind the two chains together. Thus, lectins are able to bind a higher organism to chemically specific polysaccharide chains, sometimes belonging to bacteria. If a particular bacterium cannot form a bridge via a lectin molecule to a higher organism, or if the bacterium polysaccharide coat cannot bind directly to the polysaccharide of the glycocalyx of the higher organism, then no adherence will occur. Specificity will be achieved. Some examples of bacterially produced lectins are known (8,40).

In an electron microscopy study, Fletcher and Floodgate (15) determined that the glycocalyx contains a primary and secondary acidic polysaccharide. The primary polysaccharide was composed of an inner thin dense line on the cell wall surface and an outer fringe region. The fringe region was about 15-25 nanometers thick. The secondary polysaccharide was associated primarily with groups of organisms. It was a fibrous, netlike substance

that stretched from bacteria to bacteria and from bacteria to the surface. They later proposed (16) the primary polysaccharide was responsible for initial adhesion while the secondary polysaccharide strengthened the cells attachment to the surface. Calcium and magnesium were demonstrated to be important for the maintenance of the secondary polysaccharide intercellular matrix.

There are several reasons why it is advantageous for a bacterium to adhere to a surface. Attachment may be specific or nonspecific. In a specific interaction with another organism the reasons are obvious. The ability of the bacterium to attach to the higher organism allows the bacterium to infect the host. Once the infection occurs, relationships ranging from pathogenic to symbiotic may develop. In general, the bacterium has found an environment where it can obtain the substances which are necessary to grow and reproduce.

The explanation for nonspecific attachment to nonliving surfaces is more subtle. Costerton (7) speculated the following reasons.

1) Because of the hydrophobic nature of portions of many organic molecules, the molecules tend to accumulate at surfaces providing a food source.
2) A microorganism attached to a surface with a fluid passing by would experience a continuous supply of substrate and nutrients.
3) A microorganism attached to a surface with a fluid passing by would continually have its wastes removed.
4) The presence of the surface and attached glycocalyx impedes the movement of exoenzymes away from the cell.

5) The presence of the glycocalyx, and being attached with other microorganisms to a surface, provides the cell with some physical protection (i.e. from drying, toxic substances, etc.).

6) The polymer molecules of the glycocalyx possess negatively charged sites to which free cations (nutrients) may bond. Thus the glycocalyx may act like an ion exchange resin and collect nutrient cations.

7) Attachment of cells to a surface may allow the establishment of a specific geometric orientation of the cells at a surface. Such an orientation might be important for some processes such as interspecies hydrogen transfer.

C. The Pattern of Biofilm Development

The following steps in biofilm development have been postulated by Trulear and Characklis (42):

1. Transport and adsorption of organic molecules to the surface.
2. Transport of microbial cells to the surface.
3. Microorganism attachment to the surface.
4. Microbial transformations (growth and exopolymer production) at the surface resulting in the production of biofilm.
5. Partial detachment of biofilm.

The formation of a biofilm begins with the initial adsorption of a layer of biological macromolecules to the surface (1). The macromolecules are primarily glycoproteins, proteoglycans, or their end product humic residues (28). Microorganisms are transported to the surface either by turbulent flow conditions, diffusion, or chemotaxis (42). Once in close proximity to the surface, the organism will experience a net attractive

force at a particular distance due to forces theorized in the VODL theory. The attractive force will tend to hold the organism close to the surface. In this location, the organism can then use its smaller diameter appendages, pili, flagella, fibriae, and most likely the glycocalyx polymers, to stick to the surface. Once the bacteria have attached successfully, they enter the growth phase and also produce additional exopolymers to strengthen their attachment and begin to reproduce (42).

Finally, partial detachment of the biofilm occurs as segments periodically break off (42). The breaking off most likely has three causes; shear stress, nutrient or oxygen (in the case of aerobic systems) depletion, or cell death. A change in the hydraulic regime, or the increased frictional resistance of the growing biofilm, could increase the shear forces. Depletion of nutrients could cause cell death in the deepest attached portions of the biofilm. Likewise cell death due to aging could also cause the detachment of biofilm.

Trulear and Characklis (42) conducted an extensive series of experiments on overall growth of biofilms. They used an annular reactor composed of two concentric cylinders. The inner cylinder rotated and its speed was controlled. A removeable slide, which formed an integral fit with the inside wall of the outer cylinder, was used to monitor biofilm development. Trulear and Characklis summarized their findings as follows.

1. Biofilm accumulation is the net result of substrate removal, biofilm production (results from metabolic growth) and biofilm detachment (caused by fluid shear).

2. Glucose removal is directly proportional to biofilm thickness up to an active thickness that corresponds to the depth of glucose penetration into the biofilm.

3. The depth of glucose penetration increases with increasing reactor glucose concentration.

4. Glucose removal is limited by the transfer of glucose from the bulk fluid to the fluid-biofilm interface at low velocities.

5. The rate and extent of biofilm accumulation increase with glucose loading rate.

6. The rate and extent of biofilm accumulation increase with fluid velocity at low velocities and decrease with increasing fluid velocities at high velocities.

7. Biofilm detachment increases with fluid velocity and the mass of attached biofilm.

8. Biofilm density increases with glucose loading rate.

9. Biofilm density and morphology are related. Low density biofilms exhibit a filamentous structure. High density biofilms exhibit a non-filamentous structure characterized by dense patches of microbial colonies.

10. Biofilm accumulation increases fluid frictional resistance. Once a critical biofilm thickness is reached, frictional resistance increases in proportion to biofilm thickness. For a given biofilm thickness, frictional resistance increases with filamentous structure.

Bryers and Characklis (6) have postulated that the overall progression of biofilm development can be represented in three stages for a turbulent flow system (induction, growth and plateau). During the induction period, initial biofilm formation takes place. The growth period is a time of exponential accumulation of the biofilm. Frictional resistance increases and becomes more severe as growth continues. Finally, at the plateau stage, the biofilm reaches steady state thickness as growth and detachment are balanced.

D. Factors Affecting Biofilm Development

Daniels (10) listed the following as significant parameters affecting the adsorption of microorganisms to solid surfaces.

1. Character of microorganism

   a) Species
   b) Culture Medium
   c) Culture Age

d) Concentration

2. Character of adsorbent

a) Type
b) Ionic Form (ion exchange resin)
c) Particle Size
d) Cross-linkage (ion exchange resin)
e) Concentration

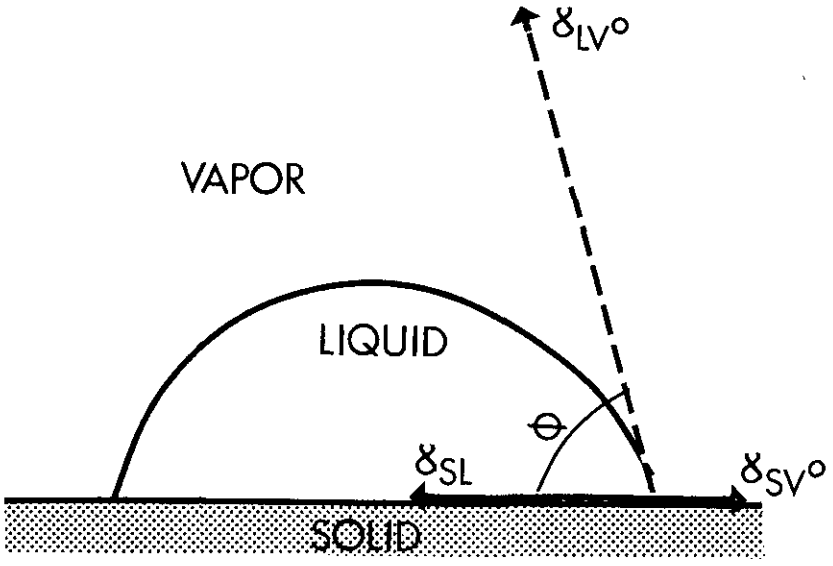
3. Character of the environment

a) Hydrogen Ion Concentration
b) Inorganic Salt Concentration
c) Organic Compounds
d) Agitation
e) Time of Contact
f) Temperature

Several of these parameters will be discussed below.

Dexter (13) proposed that two parameters, critical surface tension and the "interaction parameter" between the inert solid surface and the organic layer of molecules forming on the inert surface, determine the number of bacteria attached per unit area. Dexter's procedure also included a rinsing step in order to measure irreversible attachment.

Critical surface tension, γ_c, is an empirical parameter to measure the wettability of a surface. It is obtained by measuring the contact angle, θ, between a liquid droplet and a solid surface (for a series of droplets from fluids with known surface tensions), and plotting the surface tensions of the liquids tested against the cosine of angles formed by the droplets (Figure 2, after (2)). The critical surface tension for wetting of the substrate is defined as the intercept of the best straight line through the data with the $\cos \theta = 1$ axis. Physically, the critical surface tension separates liquids which form contact angles with the substrate of less than about 1° (in other words spontaneous spreading) from those forming higher contact angles and not spreading.



γ_c-critical surface tension

γ_{LV}-interfacial tension between the liquid and the vapor phase

γ_{SL}-interfacial tension between the solid and the liquid phase

γ_{SV}-interfacial tension between the solid and the vapor phase

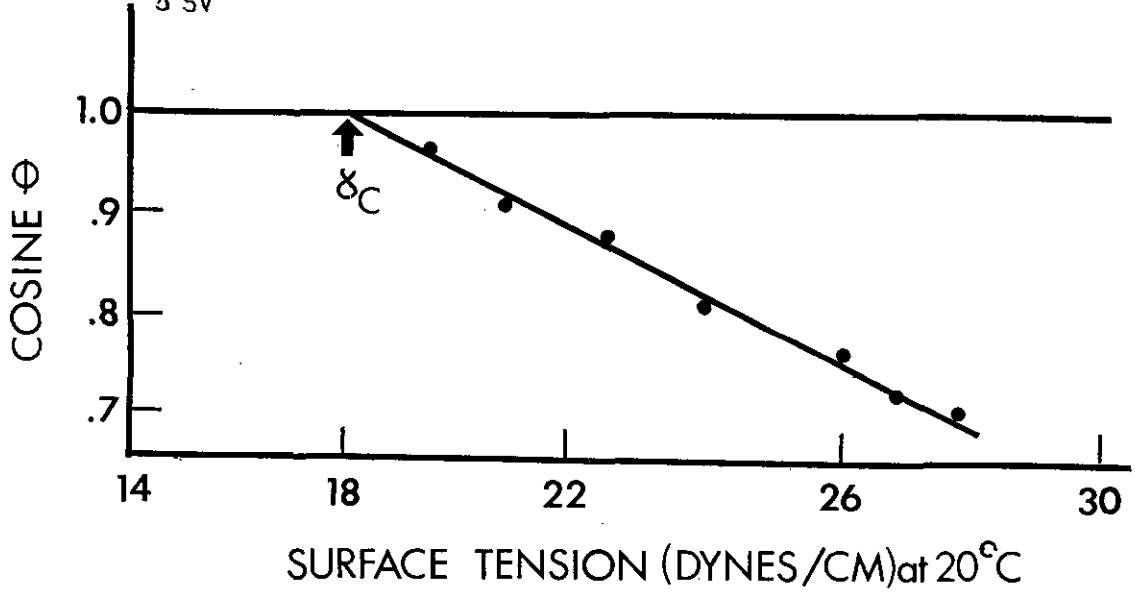


Figure 2

Determining Critical Surface Tension

Dexter observed that studies comparing attachment of bacteria to critical surface tension obtained different results when the studies were done in situ versus in vitro. He proposed a two-step model to account for the observed discrepancy in the data (12). In step one, the wettability of the surface influences the rate of formation or the composition of the initial film of organic molecules on the surface, what he calls the conditioning film. In step two, the rate of formation on composition of the conditioning film influences bacterial attachment. The driving force for the adsorption of the conditioning film is the Helmholtz Free Energy, which is determined by the interfacial tensions as described in the equation below:

$$\Delta F = \gamma_{SO} + \gamma_{OW} - \gamma_{SW} \qquad (1)$$

ΔF = change in the Helmholtz Free Energy

γ_{SO} = interfacial tension between the solid support surface and the adsorbed organic layer

γ_{OW} = interfacial tension between the adsorbed organic layer and water

γ_{SW} = interfacial tension between the solid support surface and water

The interfacial tension between the solid support surface and water, γ_{SW}, is the most significant parameter in determining the Helmholtz Free Energy, ΔF, for adsorption of the organic layer to the surface.

Dexter (13) used the work of Girifalco and Good (18) and Good (19) to explain that the interfacial tension between the solid and water is a function of the interaction parameter. The interaction parameter, ϕ_{SL}, is a constant which is dependent upon the molecular properties of the solid and the organic compound adsorbing to the solid. Dexter postulated that adsorption of the conditioning film is a function of the critical surface tension, γ_c, of the solid and the interaction parameter, ϕ_{SL}, between the solid and the organic molecules adsorbing to the surface. He proposed that the relationship of critical surface tension and bacterial attachment is determined by the interfacial surface tension between the solid and the water, which is closely related to the tendency of organics to adsorb to the solid surface.

Fletcher (14), Bryers and Characklis (6), and Shapiro and Switzenbaum (37) each found that organism concentration affected irreversible attachment. In general, an increase in organism concentration resulted in an increase in the number of bacteria attaching to a surface. Fletcher's and Shapiro and Switzenbaum's data imply there is a maximum amount of bacteria that can attach in a given area.

Fletcher (14) found her data fit a modified Langmuir type adsorption plot (the usual Langmuir assumption of an equilibrium between adsorption and desorption was not included) but did not fit a Freundlich or BET type of plot. She suggested the fit of the data to a Langmuir isotherm may mean that irreversible bacterial attachment conforms to the assumptions and principles of the model.

The equations she used to develop the model are as follows:

(1) $R = k[X]_s\ (1-\theta)$ (2)

R = rate of irreversible attachment

k = constant indicating the intensity of adsorption

$[X]_s$ = organism concentration in the bulk of fluid

θ = fraction of surface covered with bacteria

$$[X]_{ad} = k' \theta \qquad (3)$$

k' = a limiting constant depending on the adsorption capacity of the surface

$[X]_{ads}$ = the number of bacteria adsorbed to the surface

$$[X]_s = \frac{[X]_{ads}[X]_s}{k'} + \frac{R}{k} \qquad (4)$$

If the model were true, there are several implications. The rate of irreversible attachment is dependent upon the bulk organism concentration, the extent which bacteria cover the surface, and the "intensity of adsorption" of the microbes in question. There is a maximum number (k') of cells that can attach to a given surface in a layer one cell thick (recall Langmuir isotherms assume monolayer adsorption). The irreversible attachment process can be described mathematically by equations 2, 3 and 4.

The integrated form of equations 2, 3 and 4 (assuming X_s constant) results in the following relationship:

$$(4) \quad -K^1 \left[\exp\left(\frac{-K \; X_s t}{K^1}\right)-1\right]^{X_{st}} = Xads \qquad (5)$$

The data of Fletcher (14), Marshall (24) and Dexter (13) show that as inoculation time increases, the number of irreversibly attached cells increases. Fletcher's model implies that as inoculation time increases and surface coverage increases, the rate of attachment would decrease. As inoculation time approaches infinity, the rate of attachment would be zero. The integrated form of her equations relates time of inoculation to number

of bacteria adsorbed, bulk fluid organism concentration, the intensity of adsorption, and the maximum adsorptive capacity of the surface.

The data existing on the effect of growth rate on irreversible attachment are somewhat contradictory. Several studies are summarized in Table 1. General observations from these studies imply that log phase organisms attach faster than stationary phase organisms, which attach faster than death phase organisms. For example, Bryers and Characklis (6) observed that attachment rate was directly proportional to growth rate in a mixed culture system when feeding the biofilm reactor from a chemostat. Shapiro and Switzenbaum (37) found in their methane forming anaerobic mixed culture that the slow growing culture attached at about the same rate as the fast growing culture. Nelson et al. (32) however observed a decrease in attachment rate with increasing specific growth rate for a Pseudomonas species (pure culture) in a similar experimental system to Bryers and Characklis (6). Marshall (24) found that providing 7 mg/L of glucose to Pseudomonas R3 stimulated irreversible adsorption but glucose additions of 30 mg/L and 70 mg/L completely inhibited irreversible adsorption. The limiting substrate concentration determines the growth rate of microorganisms. One would expect equal or faster growth rates at higher glucose concentrations (f glucose is limiting). Given the generalization above that faster growth rates result in quicker attachment, Marshall's data

Table 1. Effect of Phase of Growth and Growth Rate on the Rate of Irreversible Attachment or Biofilm Development

Ref. # Study	Aerobic or Anaerobic	Type of Culture	Rate of Irreversible Attachment or Biofilm Development
			High > Low
DIFFERENT PHASES OF GROWTH			
15	Aerobic	Pure	log phase > stationary phase > death phase
32	Aerobic	Pure	log phase > "older bacteria"
WITHIN LOG PHASE GROWTH			
39	Aerobic	Pure	fast growth rate > slow growth rate
6	Aerobic	Mixed	fast growth rate > slow growth rate
44	Anaerobic	Mixed	fast growth rate = slow growth rate
32	Aerobic	Pure	slow growth rate > fast growth rate

is contradictory. Pavoni (33) found that bacteria do not flocculate until they have entered the endogenous growth phase. He also found a dramatic increase in the presence of exocellular polymers at this stage. He did not determine whether the origin of the polymer at this stage was from autolysis of bacteria or from living bacteria. From these findings, one would expect organisms in the stationary phase and in the death phase to attach faster than organisms in the log phase. Thus this study also provides contradictory information.

Shear has an important effect on attachment and biofilm development. Trulear and Characklis (42) noted that it was important to operate the mixed aerobic culture in their annular reactor in the batch mode for about eight hours before beginning to rotate the inner cylinder and allowing shear forces into their experimental regime. This technique minimized the induction period. Their experiments on shear showed that there was an optimum speed for the fluid to pass by the biofilm to achieve the highest biofilm accumulation rate. They concluded the peak probably represented an optimum balance between enhancing biofilm development, by increasing the availability of substrate at high velocities, and hindering biofilm development, by increasing shear stress at high velocities.

Shapiro and Switzenbaum (37) obtained different results for the effect of shear on the development of a mixed anaerobic biofilm. Higher accumulations of biofilm were observed at the lower and higher bulk liquid flow velocities with lower accumulations in the middle range. They felt there were two possible explanations for their results. Either the competing phenomena of fluid shear and mass transport caused the shape of the curve or different

shear conditions selected for different species which had different growth patterns.

The influence of ionic strength on reversible attachment was discussed earlier and considered the data of Marshall (24) and Meadows (29). Meadows showed that marine bacteria attach optimally in a high ionic strength environment whereas freshwater bacteria attach optimally in a low ionic strength environment. Marshall showed that reversible attachment in a marine bacteria follows the principles of VODL theory and double layer thickness with respect to ionic strength.

Marshall (24) and Fletcher and Floodgate (16) found that the presence of calcium and magnesium were important for irreversible attachment to take place and be maintained. Marshall found that either calcium or magnesium must be present for irreversible attachment to take place and attachment was highest when both were present. Fletcher noted complete disruption of the secondary polysaccharide when calcium and magnesium concentrations in the growth media were reduced.

There have not been any truly in-depth studies of the effects of temperature and pH on attachment to this writer's knowledge. Fletcher obtained some data on the attachment of a stationary phase marine pseudomonad. Cells suspended in filtered seawater at $3^{\circ}C$ did not attach as rapidly as those suspended in filtered seawater at $20^{\circ}C$. Fletcher and Floodgate (15) observed a high pH in the growth medium prevented the appearance of primary polysaccharide in preparations of naturally attached bacteria. Adhesion was not impaired.

CHAPTER III. METHODS AND MATERIAL

A. General Experimental Approach

One of the persistent disadvantages of methane generating anaerobic biofilm reactors is their long start-up time. Improvement or optimization of initial biofilm development would help make the methane forming anaerobic digestion process more acceptable to potential users. Understanding of how microbes attach and form biofilms is in its infancy. To date, most research has been done on aerobic cultures and only recently has work begun on mixed anaerobic cultures. The contradictory data for aerobic systems together with the dearth of data for anaerobic systems create a need for more information specific to methane forming anaerobic cultures. Accordingly, the experiment described below investigated the effect of three pertinent parameters on the attachment of methane forming anaerobic bacterial cultures to a glass surface. Also, some refinements in the techniques of studying methane forming anaerobic biofilms were developed and utilized.

The three parameters which were varied in these experiments were culture growth rate, inoculation time (the time that bacteria were exposed to the surface) and surface preparation. The experimental set-up is depicted schematically in Figure 3. It included a completely mixed anaerobic chemostat, in which the culture growth rate was controlled, and an anaerobic attachment vessel in which irreversible attachment was measured. The anaerobic attachment vessel was designed and constructed for this experiment and used microscope slides as the surface on which irreversible attachment was observed both quantitatively and qualitatively. Bacterial attachment was measured at progressing inoculation times by removing the slides at different time intervals and counting the bacteria which attached to the slides.

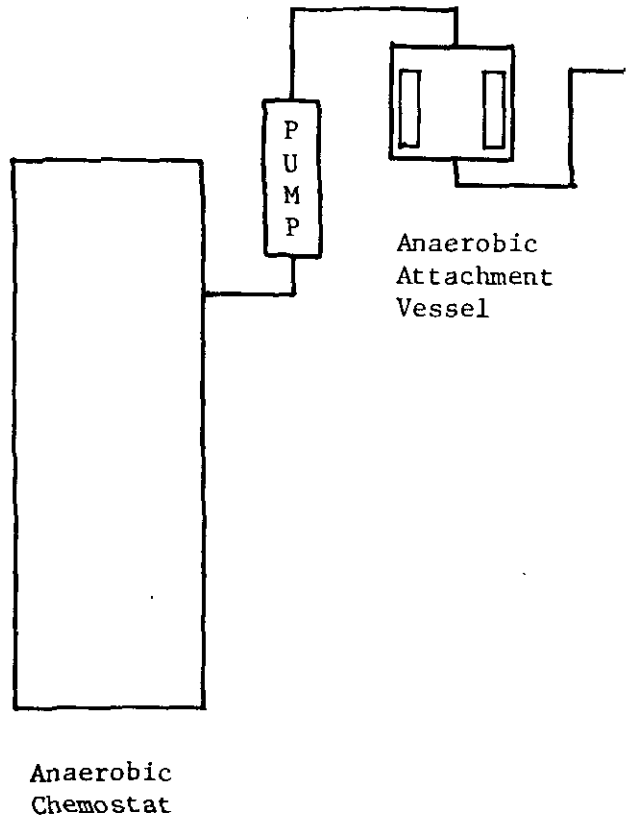


Figure 3

General Experimental Setup

The inoculation time discussed in the experiments was the amount of time a slide was left in the flow thru attachment vessel and exposed to the culture of bacteria before it was removed to be counted or photographed. Irreversible attachment was thus measured after the inoculation period was completed. The hydraulic detention time of the attachment vessel was approximately 1.3 to 1.4 days. Slide preparation could be varied by simply using different preparation procedures before inserting slides into the attachment vessel and starting an experiment. While other surfaces might be better for bacterial attachment, the glass slides were convenient for qualitative and quantitative measurement.

Finally, the use of microscope slides as the attachment surface allowed qualitative observations to be made and photographs taken under the phase contrast and scanning electron microscopes.

A number of parameters were kept constant in this experiment. They include:

1. -glass attachment surface -- Attachment took place on glass microscope slides provided from the same supplier (VWR Scientific Precleaned Plain Microscope Slides, No. 48300-25).

2. -the organism concentration (measured as bacteria per 100 mls) -- The organism concentration in the chemostat was adjusted to keep it constant at different growth rates by altering the substrate concentration in the feed to the chemostat.

3. -the overall environment in which attachment was measured or observed -- Slides were placed in a radially symmetrical fashion in an acrylic cylinder (the attachment vessel) so that each slide experienced the same environment (with respect to fluid mechanics, shear, proximity to wall, etc.).

4. -the temperature of the chemostat effluent/attachment vessel influent -- The temperature of the chemostat and the attachment vessel was maintained at 36°C $\pm$ 2°C.

5. -the pH of the chemostat effluent/attachment vessel influent -- The pH of the chemostat effluent was held constant for a given growth rate and between the two growth rates by adding a constant, sufficient amount of alkalinity to each feed such that the pH's of the effluent were stable and approximately equal (pH 7.1).

Table 2. Overall Experimental Procedure

1. wash glass slides (chromic acid wash, distilled water rinse/ferrour amoniumsulfate wash/distilled water rinse/deionized water rinse)

2. place glass slides in attachment vessel

3. remove slides from attachment vessel and rinse with wash buffer after varied inoculation times

4. count microorganisms

   a) total count of all bacteria per area (counts at cocci > 0.6 micrometers, cocci < 0.6 micrometers and noncocci)

   b) count methanogens with fluorescence scope per area

parameters varied - culture growth rate - (8 day solids retention time/0.5 volumes per day dilution rate)

- (20 day solids retention time/0.125 volumes per day dilution rate)

inoculation time - (0 to 165 hours)

slide preparation - (chromic acid wash - autoclave)
- (chromic acid wash - no autoclave)

parameters constant - organism concentration
- salinity
- pH
- surface for attachment
- temperature
- fluid shear

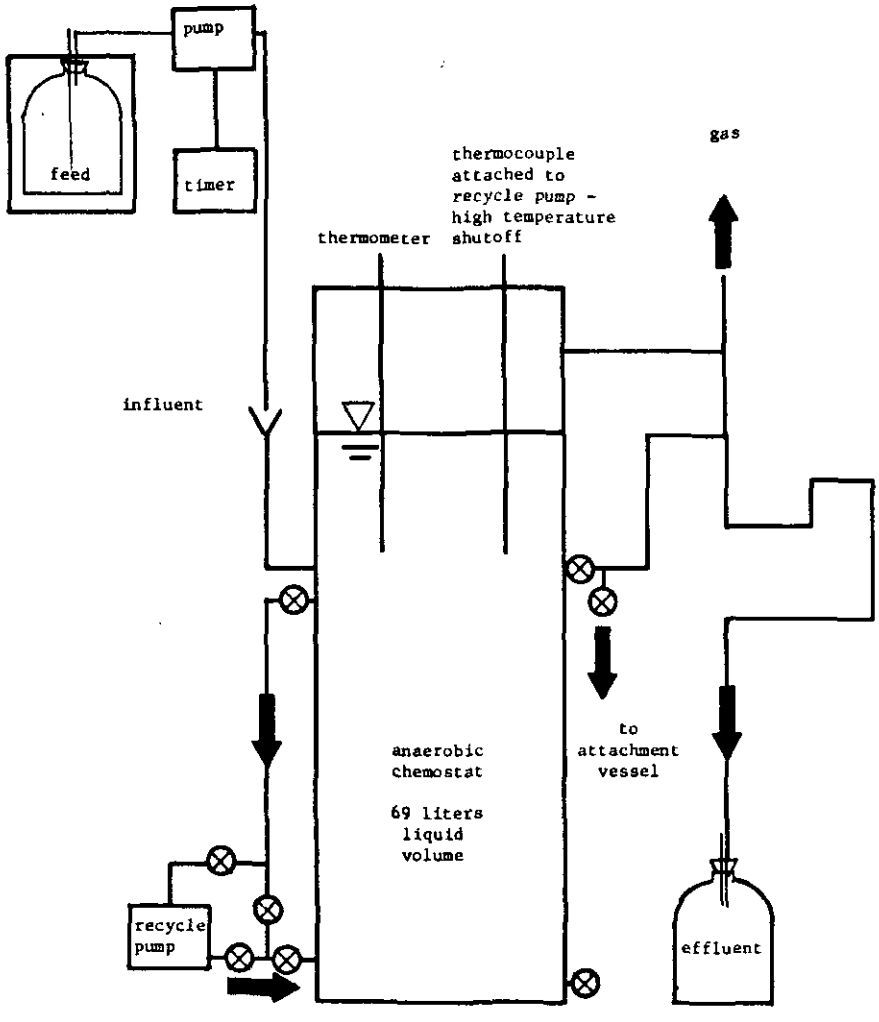


Figure 4

Anaerobic Chemostat

anaerobic chemostats between 10 and 15 day SRT's (23). The two growth rates also offered an opportunity to compare attachment of fast and slow growing cultures.

Two 15 liter working volume inoculating reactors were maintained in addition to the experimental reactor. One operated at an eight day SRT and the other operated at a 20 day SRT. The two inoculating reactors and the experimental reactor were all seeded simultaneously from a seed prepared from a mixture of sources (Table 3). The inoculating reactors served three primary functions. They provided a source of inoculum in case of an accident with the experimental reactor. They provided a controlled source of inoculum when the experimental reactor was switched from one growth rate to another. Having the two experimental growth rates operating simultaneously allowed experimentation with feed to obtain similar environmental conditions in the reactors.

The feeds used in the reactors are summarized in Table 4. The feed can be broken down into five major components. Organic carbon was primarily supplied as sucrose. Alkalinity was primarily supplied as sodium bicarbonate. A variety of inorganic salts were added as nutrients. L-cysteine was provided as a sulfur source (sulfate might have acted as a competing electron acceptor and allow sulfate reducers to out-compete methanogens). Yeast extract was added to supply trace nutrients. The sucrose feed concentrations for the two reactors differed so that the organism concentration would be the same in the two reactors. The salt concentrations were based on two concepts. First, the amount of a particular salt necessary for a culture with a 67 percent cell yield and carbon as the limiting nutrient was

Table 3. Inoculum

SOURCE	COMMENTS
Dairy Manure Digester	plug flow
Sewage Digester	complete mix
Research Fluidized Bed Reactor	fed lactose/salts
Research Upflow Sludge Blanket Reactor	fed lactose/salts
Research Complete Mix	fed lactose/nutrient broth/salts
Rumen Fluid	

Table 4. Reactor Feeds

	0.125 vol/day Dilution Rate 8 day SRT, mg/L	0.05 vol/day Dilution Rate 20 day SRT, mg/L
Sucrose	8,500	10,000
Nitrogen as N	657	657
Phosphorus as P	292	292
Potassium as K	735	735
Magnesium as Mg	139	139
Iron as Fe	155	155
Chloride as Cl	2,425	2,425
Sodium as Na	2,300	2,300
Cobalt as Co	8.4	8.4
Nickel as Ni	4.2	4.2
Calcium as Ca	33	33
L-Cysteine as S	67	67
Yeast Extract	200	200
Alkalinity as $CaCo_3$	5,000	5,000

determined for the 20 day SRT reactor. Second, the concentrations of influent salt used in other successfully operated research reactors were reviewed. The higher of these two concentrations was used. The salts concentration for the two reactor feeds was kept the same to keep the effluent salts concentration approximately the same. Feed was delivered to the reactor by a timer activated peristaltic pump once each hour.

The overall stoichiometric reactions predicted to occur at the two growth rates were as follows.

20 Day SRT

$$0.25\ CH_2O + 0.0097\ HCO_3^- = 0.110\ CO_2 + 0.101\ CH_4 + 0.0097\ NH_4^+ + 0.039\ H_2O + 0.0097\ C_5H_7O_2N \quad (6)$$

8 Day SRT

$$0.25\ CH_2O + 0.0117\ HCO_3^- = 0.107\ CO_2 + .\ 0.0958\ CH_4 + 0.0117\ NH_4 + 0.0117\ C_5H_7O_2N + 0.047\ H_2O \quad (7)$$

The reactors were brought to steady state before experiments were carried out. Reactors were operated for at least three times longer than the experimental SRT to achieve steady state. pH, temperature, effluent volume, gas composition, gas quantity, volatile suspended solids (organism concentration), suspended bacteria concentration and soluble chemical oxygen demand removal were monitored regularly to insure steady state conditions.

2. Attachment Vessel

Each attachment vessel was a four-inch inside diameter by six-inch high working dimensions sealed acrylic cylinder which was capable of holding 36 glass slides (Figure 5). The slides were radially arranged so that each slide experienced the same environment. The hydraulic detention time was approximately 1.3 to 1.4 days.

Before each experiment was started, the attachment vessel was thoroughly cleaned and disinfected. It received a soap and water wash and was rinsed with distilled water until all suds were removed. It was then disinfected overnight with 100 mg/l as Cl_2, NaOCl solution. Finally, the attachment vessel was then rinsed four times with distilled water.

The slides were thoroughly cleaned before placing them in the attachment vessel. The wash procedure was as follows:

1) soap and water wash using a sponge,
2) rinse with tap water to remove suds,
3) rinse with distilled water,
4) soak in chromic acid for at least 1 hour,
5) rinse 10 times with distilled water,
6) soak in 0.25 M ferrous ammonium sulfate for at least 1 hour,
7) rinse 10 times in distilled water, and
8) rinse 4 times in deionized water.

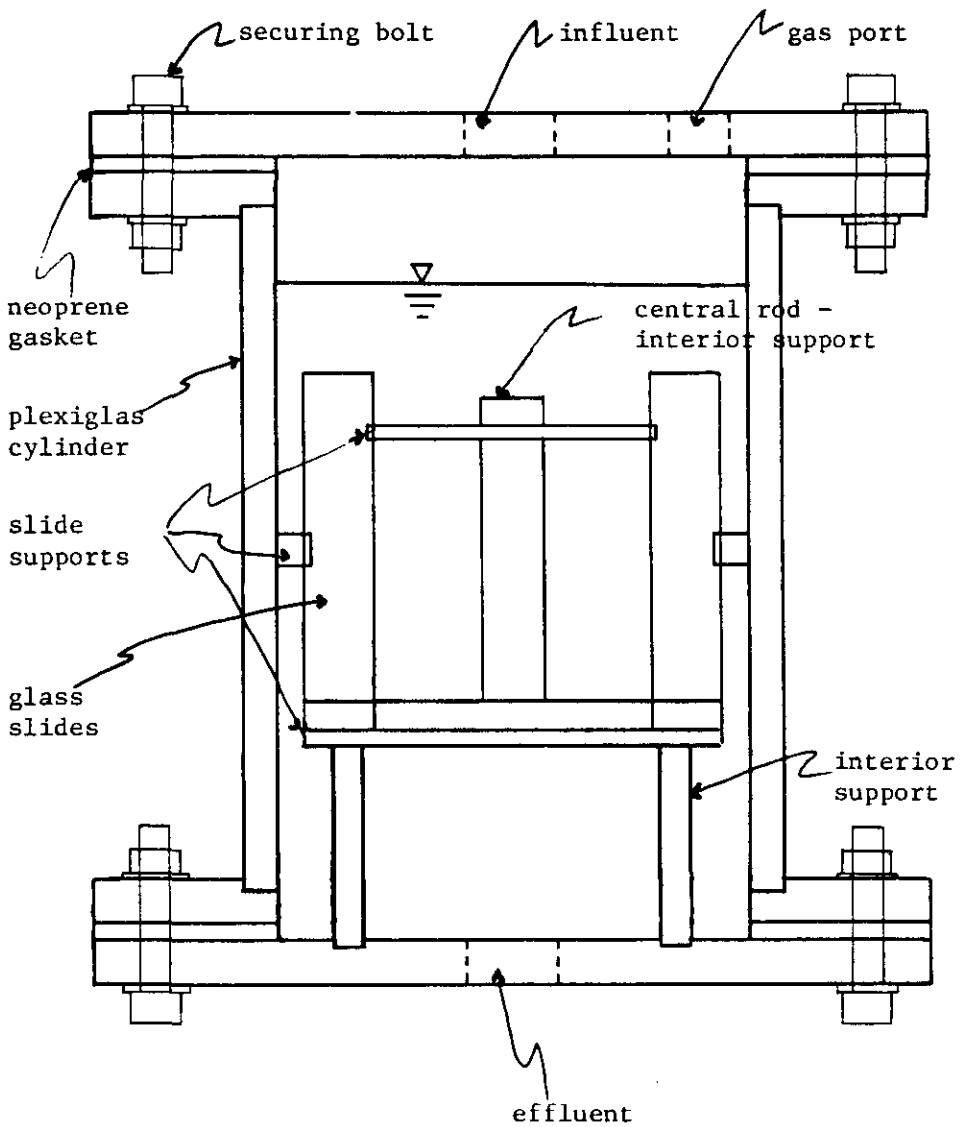


Figure 5

Anaerobic Attachment Vessel - Cross Section

The slides were stored in the dark, submerged in deionized water at room temperature in a similarly cleaned, parafilm covered beaker. The slides that were not autoclaved in the experiment were simply removed from these beakers the day of an experiment, loaded into the attachment vessel. For slides which were autoclaved, the procedure was as follows.

The beaker containing the slides was set on a stainless steel tray with holes in the bottom. The parafilm was removed from the top of the beaker. A larger beaker (which had been through the same chronic acid wash procedure as the slides) was inverted and placed over the top of the smaller beaker containing the slides. The tray and the beakers were loaded into the autoclave and autoclaved for 30 minutes at 270°F. The tray and the beakers were then removed and the beaker with submerged slides was allowed to cool. When the slides were cool they were loaded into the attachment vessel.

When these preparations were completed for the attachment vessel and the slides, an experimental run commenced. The recycle pump for the chemostat was operated continuously for the 30-minutes prior to filling the attachment vessel. The effluent valve on the chemostat was closed. Five hundred milliliters of mixed liquor was flushed through the effluent sample tap and returned to chemostat through the feed port. Then the attachment vessel was filled. The effluent valve of the chemostat was reopened and it was thus returned to its initial state. The attachment vessel was then hooked up to a timer activated pump which pumped approximately 30 milliliters once each hour. Slides were removed

from the attachment vessel after being submerged in the attachment vessel for a period of time, the inoculation time. Once the attachment vessel was filled, the inoculation time clock started running. Slides were removed at the appropriate times, rinsed to remove reversibly attached cells, and counted.

3. Slide Removal and Rinse Technique

At a given inoculation time, the liquid effluent valve, the feed influent valve, and the gas port valve of the attachment vessel were all closed. The attachment vessel was removed from the 35°C room and carried to the lab bench. At the lab bench, the head space of the attachment vessel was gassed with nitrogen (O_2 < 3ppm) while the top of the attachment vessel was being removed and after it was removed. Slides were grabbed by the top with a tweezers and carefully removed. The slides were immediately rinsed with a wash bottle (see Table 5) whose magnesium and calcium concentrations, pH, and ionic strength were designed to be approximately equal to the mixed liquor of the chemostat.

The buffer was prepared within a month of the time for an experimental run, with deionized water. After mixing, it was filter sterilized through a 0.2 µm filter into an autoclaved flask (15 minutes @ 230°C). It was then transferred to an autoclaved culture bottle and stored at 4°C in the dark.

An attempt was made to make the rinse procedure as uniform as possible. Slides were held next to a stand which had a 45° angle (see Figure 6). The rinse buffer was dispensed from a 25 ml Fisher brand

Table 5 Wash Buffer

	g/l
$CaCl_2$	0.092
$MgCl_2 \cdot 6H_2O$	1.178
K_2HPO_4	0.696
KH_2PO_4	0.136
$NaCl_2$	4.62
KCl	5.89

pH measured = 7.1 to 7.2

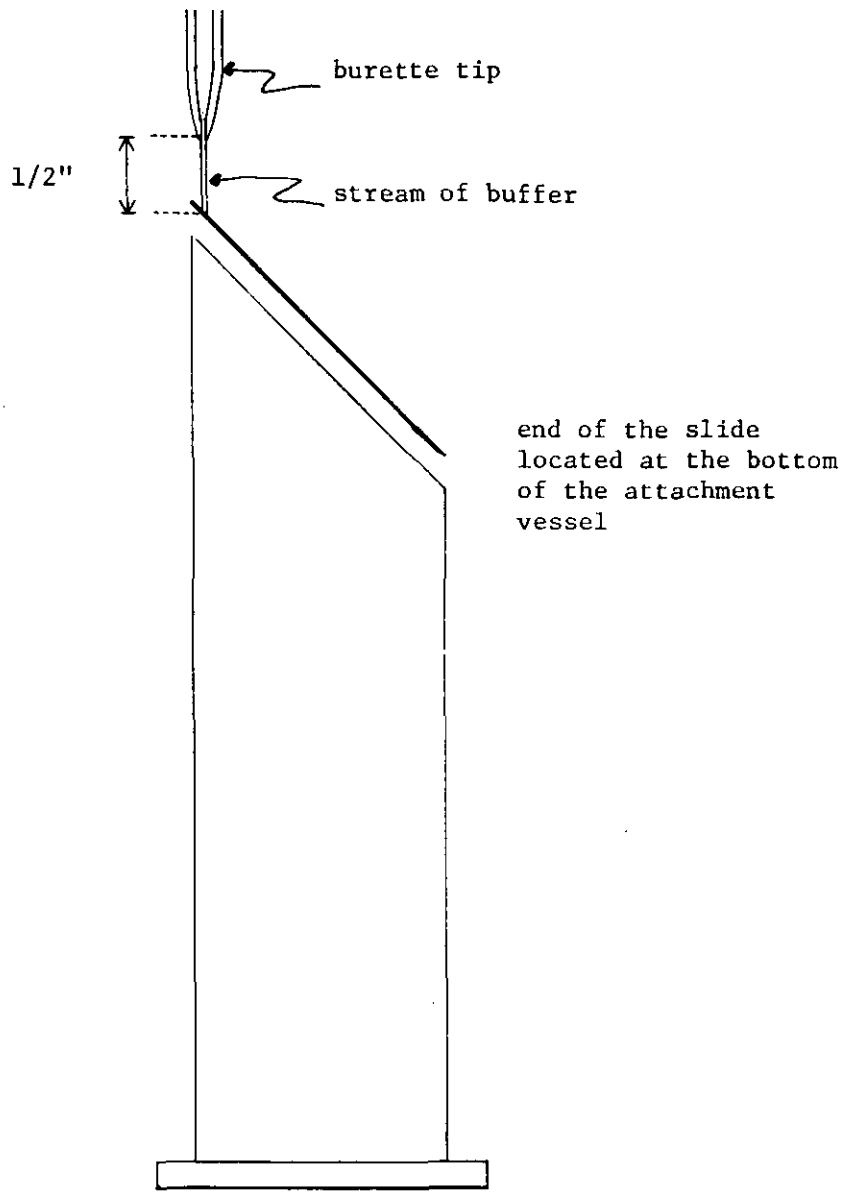


Figure 6

Slide Rinsing Technique

Schellbach burret with a 3-way stopcock and automatic zeroing. The tip of the burrette was held approximately one half inch from the elevated edge of the slide. The stream of buffer from the burrette was directed on the top quarter inch of the elevated portion of the slide. The fluid flowed down the slide and off the end. The stream was shifted from side to side on the slide so that the entire slide was rinsed. When a slide was rinsed, the side to be counted was rinsed initially with 25 ml of buffer. Keeping the elevated end elevated, the slide was flipped over and the bottom side of the slide was rinsed with 5 ml of buffer. Finally, the slide was flipped over again keeping the elevated side elevated, and the side to be counted was rinsed again with 5 more milliliters of buffer.

The final preparation of the slide was the fixing of a cover-slip. After rinsing with rinse-buffer, the small residual of buffer on the slide was used to make a wet mount by placing a coverslip on the slide. The coverslip was placed so that its end closest to the edge of the slide was 17mm from the edge of the slide (see Figure 7). This end had been at the bottom of the attachment vessel. The edges of the coverslip were then sealed with nail polish to prevent evaporation. Three coats of nail polish were applied to the coverslip edges. A few minutes were allowed for drying after each application. The bacteria were then counted as soon as possible. After four or five days, the slide would begin to dry out.

4. Cell Counts

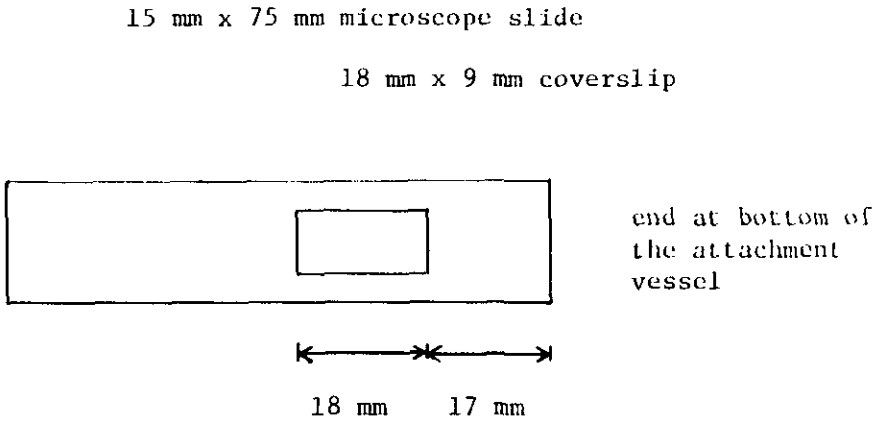


Figure 7

Microscope Slide And Coverslip Mounting Location
Used For Bacterial Attachment Counts

The irreversibly attached bacteria on the prepared slides were next counted. An Ernst Leitz Wetzlar SM Phase Contrast Microscope was used for the April 1, 1986 and May 21, 1986 experimental runs. A Zeiss GFL Phase Contrast Microscope was used for the July 15, 1986 and August 19, 1986 experimental runs. Photographs were taken using a Zeiss D-7082 Standard Phase Contrast Microscope. Counts were carried out on the Leitz microscope using the 100x oil immersion phase objective, a 10x eye piece, and Cargille Type A immersion oil. On the Zeiss GFL microscope, counts were done under the 100x oil immersion phase objective and a 12.5x eye piece, and Cargille Type A immersion oil.

An attempt was made to count approximately the same locations for each slide. The slides were placed on the microscope mechanical stage with the same orientation each time. The mechanical stage was adjusted so the same coordinates of microscope fields were brought into view each time. For phase contrast counts of a particular slide, usually twenty-four fields were counted. For fluorescence microscopy, twenty-four to seventy-two fields were counted. For phase contrast counts, the fields that were counted were in two rows of twelve fields (see Figure 8). Within each row, the fields that were counted were 0.5 mm apart. The two rows were 1 mm apart. The field closest to the bottom edge of the slide is 20 mm from the edge of the slide. The rows are located approximately 7 mm from either edge and are 1 mm apart.

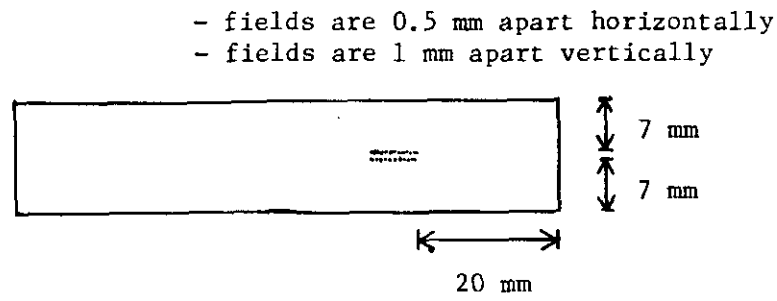


Figure 8

Location Of Phase Contrast Microscope Fields Used
For Bacterial Attachment Counts

The counting technique as follows: In most cases, only bacteria in one quarter of the oil immersion field, the upper right quadrant, were counted. Each slide was counted three times under the phase contrast microscope. The first time noncocci were counted. The second time cocci greater than 0.6 micrometers in diameter were counted. The third time, cocci less than 0.6 micrometers in diameter were counted. After counting on the phase contrast microscope, the oil was not wiped off the slide. It was found that wiping the oil off would damage the biofilm and cause attached bacteria to become unattached. So the slides were stored with the oil remaining on them until the fluorescence counts were done.

Fluorescence microscopy was carried out using an Olympus BHS-2 microscope with a reflected light fluorescence attachment. The filter mode used was with the exciter filter BP-490 (B) (allows light with a wavelength of 490 nm and less to reach the specimen) and the barrier filter O-515 (allows light with a wavelengh of greater than 515 nm to be seen through the eyepiece). Counts were done using the 100x oil immersion objective and, a 10x eyepiece, using Cargille Type A immersion oil.

The method of counting under the fluorescence scope differed from the phase counts slightly. Due to the rapid fading (a few seconds) of many of the fluorescent bacteria, the area of the field counted was reduced and the number of fields counted increased. Field sizes counted were either 5030 square micrometers, 745 square micrometers, 331 square

micrometers, depending on the density of attachment. From 24 to 72 field were counted.

The fluorescence counts were carried out in a fashion similar to the phase contract counts except that up to six rows of twelve fields were counted (72 fields total) instead of just two rows (see Figure 9). The outer rows were 6.5 mm from the edge of the slide. Three of the spaces between the six rows is 0.5 mm. Two of the spaces between the six rows is 0.25mm.

The timing of the counts is summarized in Appendix A. In all cases, the phase-contrast counts of autoclaved slides were done within 26 hours of the time the slides were sampled. The majority of these slides were counted within 5 hours of being sampled. The autoclaved/florescence microscope counts and the unautoclaved/phase contrast microscope counts were completed within 5 days of sampling. The majority of these slides were counted within 3 days of sampling.

5. Scanning Electron Microscopy

The techniques for counting bacteria were modified so that scanning electron microscopy could be performed. Microscope slides were cut into small rectangular pieces with a glass saw approximately five to ten millimeters by five to ten millimeters, washed (in the same manner as microscope slides for counting), autoclaved, and oven-dried. These pieces were then glued with nail polish to 15 mm x 75 mm microscope slides used for the attachment study in the same location that counts were done (see Figure 10). The slides fit into the

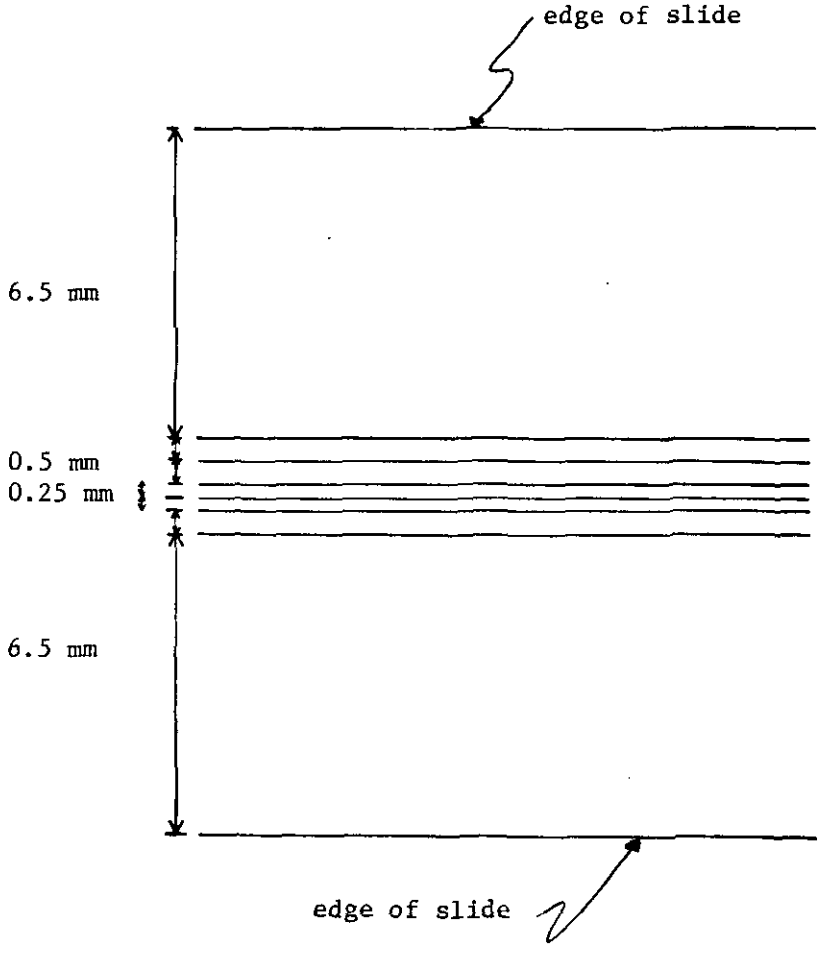


Figure 9

Fluorescence Microscope Counting Technique - Location Of Rows Counted

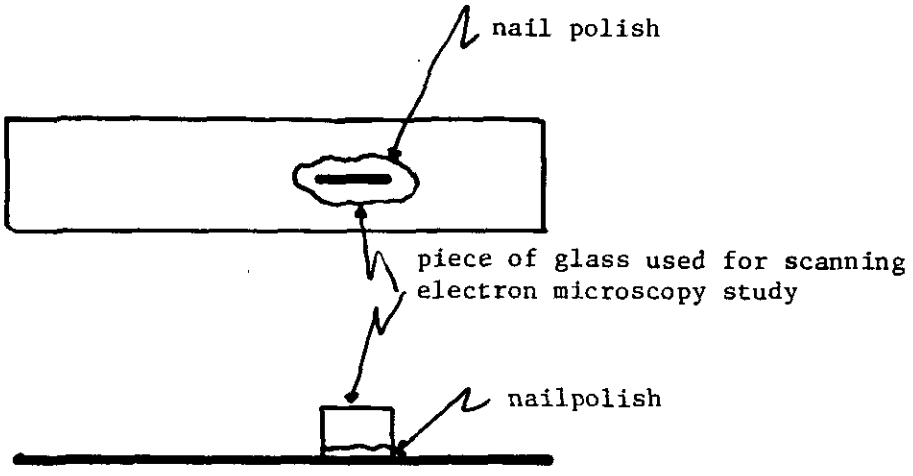


Figure 10

Slide Apparatus For Scanning Electron Microscopy

attachment vessel just as the other slides fit. However, the attachment vessel could only hold 18 slides at a time of electron microscope slides, compared to the normal 36, due to interference caused by the extra glued piece.

The procedure used to sample and prepare the electron microscope samples was as follows. Slides were placed in the attachment vessel at staggered times so they could be removed the day before sample preparations were carried out for the SEM. When slides were removed, they were immediately placed in a Petri plate containing the slide rinse buffer. Here the sample piece of glass was removed using an ethanol flame sterilized razor knife to cut at the nail polish. When the sample of glass was cut, it usually fell on one side, this side was thereafter treated as the "up" side and kept up for the rest of the preparations. Excess nail polish was trimmed off the sample piece of glass using the razor knife. The sample was then carefully removed from the Petri plate with a forceps and placed in a 25 milliliter beaker containing enough rinse buffer to cover the sample. The buffer was then removed by suction with a Pasteur pipette and the beaker refilled with buffer four times to remove reversibly attached cells. Care was taken not to hit the sample piece of glass with a direct stream of fluid when refilling the beaker to prevent irreversibly attached bacteria from being knocked off. After the buffer was removed for the fourth time, the beaker containing the glass sample was refilled with 2% glutaraldehyde in Millonig's buffer. Samples were stored overnight at 4°C in 2%

glutaraldehyde/Millonig's buffer solution. The shape of glass samples were then physically sketched so "up" side could be recognized in case the glass samples were jossled or flipped during the drying process. The following day, the samples were first washed twice with Millonig's buffer. Next the samples were initially dried by submerging them in a series of increasing strengths of ethanol for five minutes each; twenty percent, fifty percent, seventy percent, ninety-five percent, and one hundred percent ethanol solutions were used. The sample was submerged twice in the 100% ethanol. Critical point drying was next carried out under CO_2 atmosphere with a Polaron Equipment Ltd. E3000 Critical Point Dryer. The samples were mounted on aluminum pegs used for the SEM and sputter coated with a layer of gold 500-735A° thick. Finally, the samples were examined on a JEOL Model JSM 255 Scanning-Electron Microscope.

6. Monitoring of Anaerobic Chemostat

A number of parameters were measured in order to monitor the condition of the anaerobic chemostat and to insure it was at steady state. They included daily effluent volume, mixed liquor temperature, mixed liquor pH, daily gas volume produced, gas composition, mixed liquor volatile suspended solids, mixed liquor total bacteria count, and feed total, effluent total, and effluent soluble chemical oxygen demand. The methods are summarized in Table 6.

A. Effluent Volume -- Effluent was collected in a plastic carboy and the volume was measured each day. The volume

Table 6. Monitoring of Chemostat

PARAMETER	TEST PROCEDURE
Effluent Volume	Graduated Cylinder
pH	Fisher Accumet pH Meter, Model 600
Gas Volume	Wet Tip Gas Meter
Gas Composition	Gow-Mac Gas Chromatograph Series 550 Thermal Conductivity Detector
Volatile Suspended Solids	Dried at 103-105°C/Combusted at 550°C
Total Suspended Bacteria Count	Petroff-Hausser Counting Chamber
Chemical Oxygen Demand	Closed Reflux, Colorimetric Method
Temperature	Taylor Dial Thermometer

measurement was done after any leftover feed in the feed bottle was emptied into the reactor.

B. pH -- pH was measured by removing a 25 ml sample from the effluent sample tap. The sample was allowed to a sit for 3 to 5 minutes before the pH reading was taken on a Fisher Accumet Model 600 pH meter.

C. Gas Production -- Gas production readings were recorded each day at the time the reactor was fed. The meter was calibrated every three to four weeks. A wet tip meter manufactured by Wet Tip Gas Meter Company (472 Sharon Drive, Wayne, Pennsylvania, 19087) was used.

D. Gas Composition -- Gas composition was measured on a Gow-Mac Gas Chromatograph with a Series 350 Thermal Conductivity Detector which was interfaced with a Hewlett Packard 3390 Integrator. Samples for the experimental reactor were done in triplicate and averaged. Samples from the inoculating reactors were done in duplicate. The gas chromatograph was calibrated prior to measurement on a given day with the exception of some measurements during the start-up of the eight day SRT reactor. Gas measurements were made almost daily during this period (start up of the 8 day SRT reactor) but the gas chromatograph had not always calibrated before making the measurements. These data points are noted. It should be pointed out that other researchers in the lab calibrated the gas chromatograph on a

daily basis and the gas chromatograph was probably calibrated almost every day.

E. Volatile Suspended Solids and Chemical Oxygen Demand -- Volatile suspended solids (VSS) and chemical oxygen demand (COD) were performed according to the procedures set forth in the sixteenth edition of Standard Methods for the Examination of Water and Wastewater (17) The sampling technique was as follows: The recycle pump for the reactor was turned on for thirty minutes of continuous operation. At the end of the thirty minutes, with the recycle pump still on, the effluent valve to the reactor was closed. Five hundred ml of mixed liquor was flushed through the effluent sample tap and poured back into the reactor feed port. Then another 500 ml was removed from the effluent sample port and this sample was used for VSS and COD measurements. The sample was then mixed with a magnetic stir bar. Aliquots for measurements were removed using pipettes which had sawed off ends or were open ended so that a representative sample of particulate matter would be obtained. Solids were captured on and soluble COD samples were filtered through an eleven centimeters in diameter Whatman 934-AH-filter (pose size 1.5 um). All solids samples were done in triplicate. COD samples were done in duplicate. The feed total COD sample was diluted 20 fold, the effluent total COD was diluted 5 fold, the effluent soluble COD was diluted 2 fold to carry out the COD

measurements. A standard curve was performed each time a COD analysis was done. For COD, the spectrophometric method was used.

F. Bacterial Counts -- Total bacteria counts for the suspended growth of the mixed liquor were done using a Petroff-Hausser bacterial counting chamber. The sampling technique from the chemostat was the same as described above for COD and solids analysis. The sample was diluted by a factor of twenty. The counts were done on the Ernst Leitz Wetzlar-SM Phase Contrast microscope using the 40x objective and a 10x eyepiece.

CHAPTER IV. RESULTS AND DISCUSSION

A. Steady State Data

Before beginning experiments at each particular growth rate, it was necessary to bring the chemostat to a steady state condition. A listing of steady state variables which were monitored and an explanation of these parameters along with the actual time trends can be found in Appendix B.

B. Initial Attachment Data

Five experimental runs were carried out to count attached bacteria or take scanning electron microscope photographs. These runs are summarized in Table 7.

The data for each of the bacterial counts are summarized in Figures 11 to 15. The graphs contain a set of data points for cocci < 0.6 micrometers in diameter/slides washed and autoclaved; cocci > 0.6 micrometers in diameter/slides washed and autoclaved; noncocci/slides washed and autoclaved; blue-green fluorescing (methanogenic) bacteria/slides washed and autoclaved; cocci < 0.6 μm + cocci > 0.6 μm + noncocci/slides washed and autoclaved; cocci > 0.6 μm + noncocci/slides washed and autoclaved; and cocci > 0.6 μm + noncocci/slides washed and unautoclaved.

In addition, on each graph a weighted regression curve was added for the cocci < 0.6 μm + noncocci/slides washed and autoclaved data set.

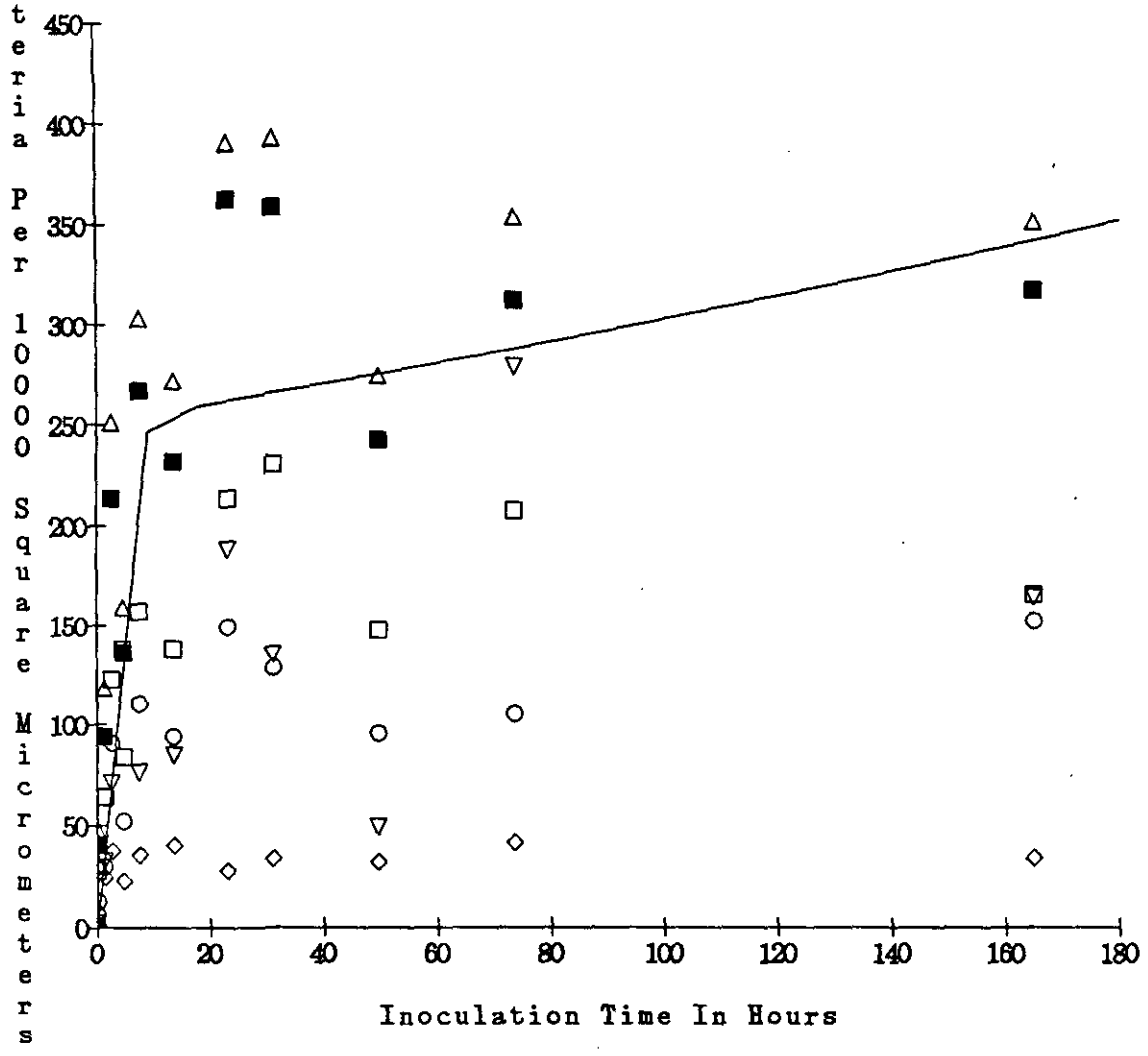
Table 7. Experimental Runs
Dates, Data Obtained, Slide Preparation

Experimental Run No.	Dates	SRT	Data Obtained BA = bacterial attach. SEM = Scanning Electron Micrographs	Slide Preperation W = Washed A = Autoclaved U = Unautoclaved
1	4/1/86-4/8/86	20	BA	Slides W, A
2	5/2/86-5/28/86	20	BA	Slides W, A
		20	BA	Slides W, U
3	6/1/86-6/8/86	20	SEM	Slides W, A
		20	BA	Slides W, U
4	7/15/86-7/22/86	8	BA	Slides W, A
		8	BA	Slides W, U
5	8/19/86-8/26/86	8	BA	Slides W, A
		8	BA	Slides W, U
		8	SEM	Slides W, A

Figure 11

Inoculation Time
Versus
Number Of Bacteria Irreversibly Attached
Per 10000 Square Micrometers

20 Day SRT - 0.05 Volumes Per Day Dilution Rate
April 1, 1986 Experimental Run



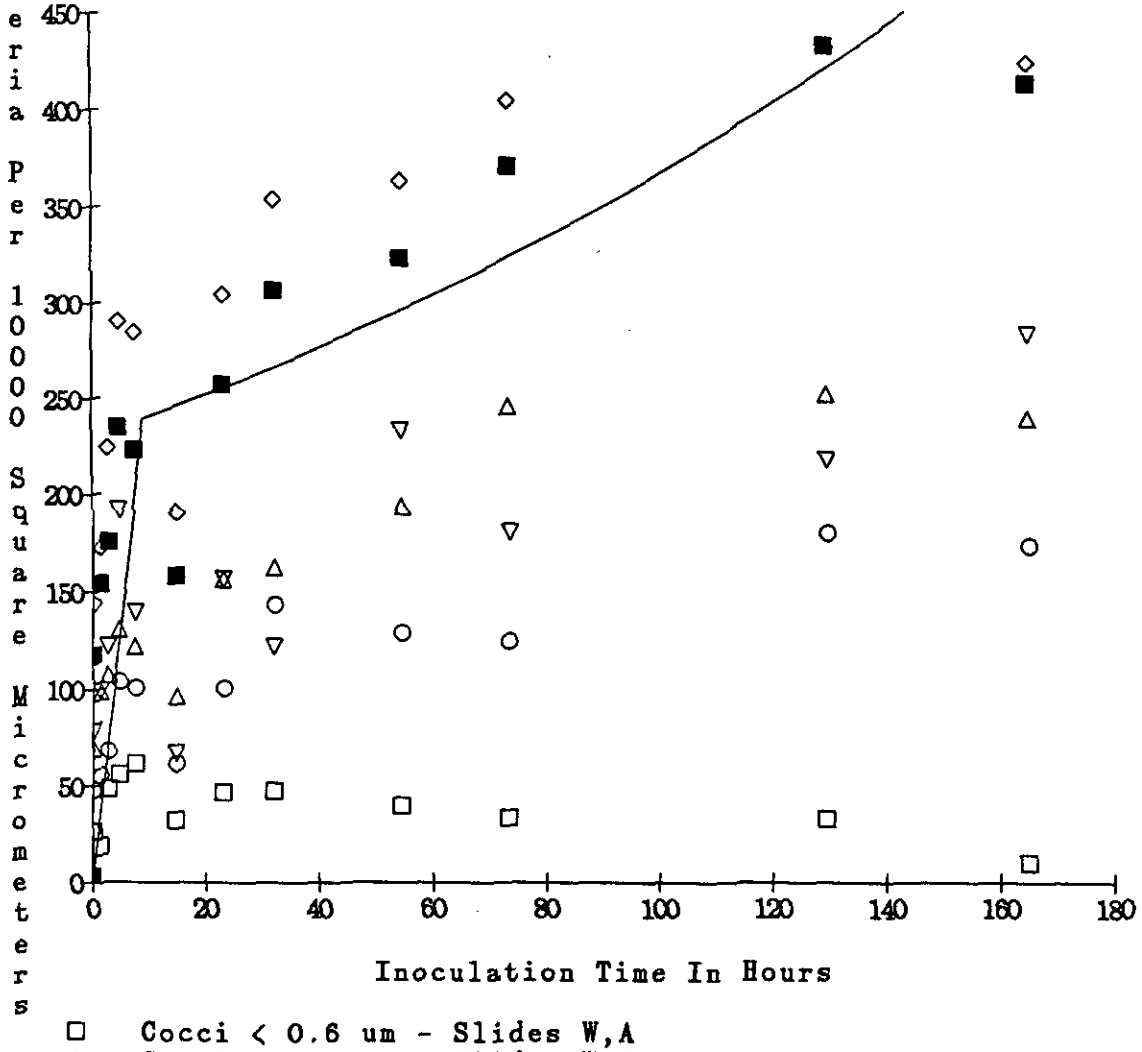
◇ Cocci < 0.6 um - Slides W,A
○ Cocci > 0.6 um - Slides W,A
□ Noncocci - Slides W,A
▽ Blue-green Fluorescing Bacteria (Methanogens) - Slides W,A
△ Cocci < 0.6 um + Cocci > 0.6 um + Noncocci - Slides W,A
■ Cocci > 0.6 um + Noncocci - Slides W,A
——— Weighted Regression - Cocci > 0.6 um + Noncocci
$Y = A*(1-\exp(k*t)) + A*(\exp(u*t)-1)$

Flow Rate Through Attachment Vessel = 29 ml/hr
A = Autoclaved W = Washed U = Unautoclaved

Figure 12

Inoculation Time
Versus
Number Of Bacteria Irreversibly Attached
Per 10000 Square Micrometers

20 Day SRT - 0.05 Volumes Per Day Dilution Rate
May 21, 1986 Experimental Run



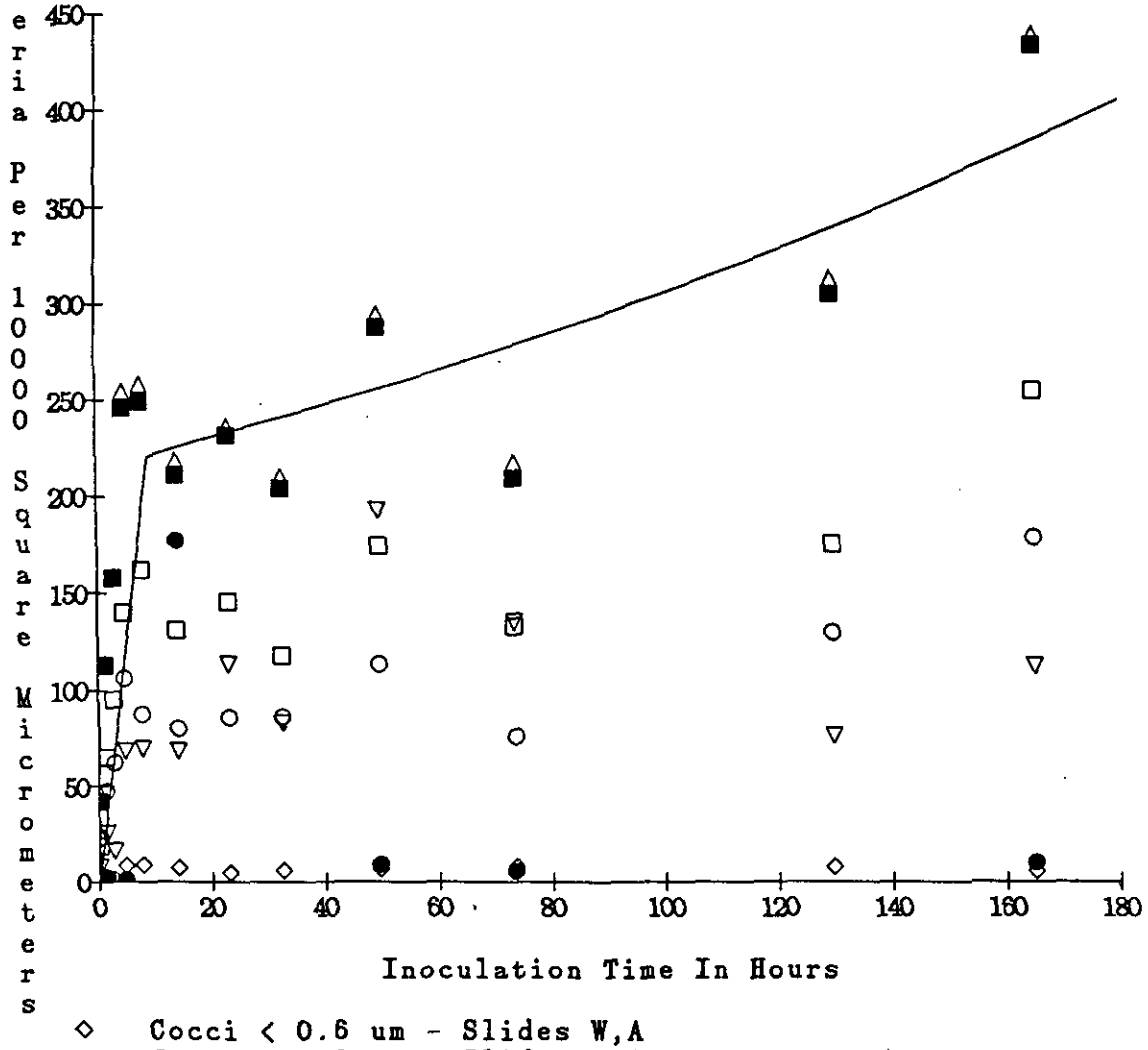
□ Cocci < 0.6 um - Slides W,A
○ Cocci > 0.6 um - Slides W,A
△ Noncocci - Slides W,A
▽ Blue-green Fluorescing Bacteria (Methanogens) - Slides W,A
◇ Cocci < 0.6 um + Cocci > 0.6 um + Noncocci - Slides W,A
■ Cocci > 0.6 um + Noncocci - Slides W,A
● Cocci > 0.6 um + Noncocci - Slides W,U
—— Weighted Regression - Cocci > 0.6 um + Noncocci
Y = A*(1-exp(k*t)) + A*(exp(u*t)-1)

Flow Rate Through Attachment Vessel = 27 ml/hr
A = Autoclaved W = Washed U = Unautoclaved

Figure 14

Inoculation Time
Versus
Number Of Bacteria Irreversibly Attached
Per 10000 Square Micrometers

8 Day SRT - 0.125 Volumes Per Day Dilution Rate
July 15, 1986 Experimental Run



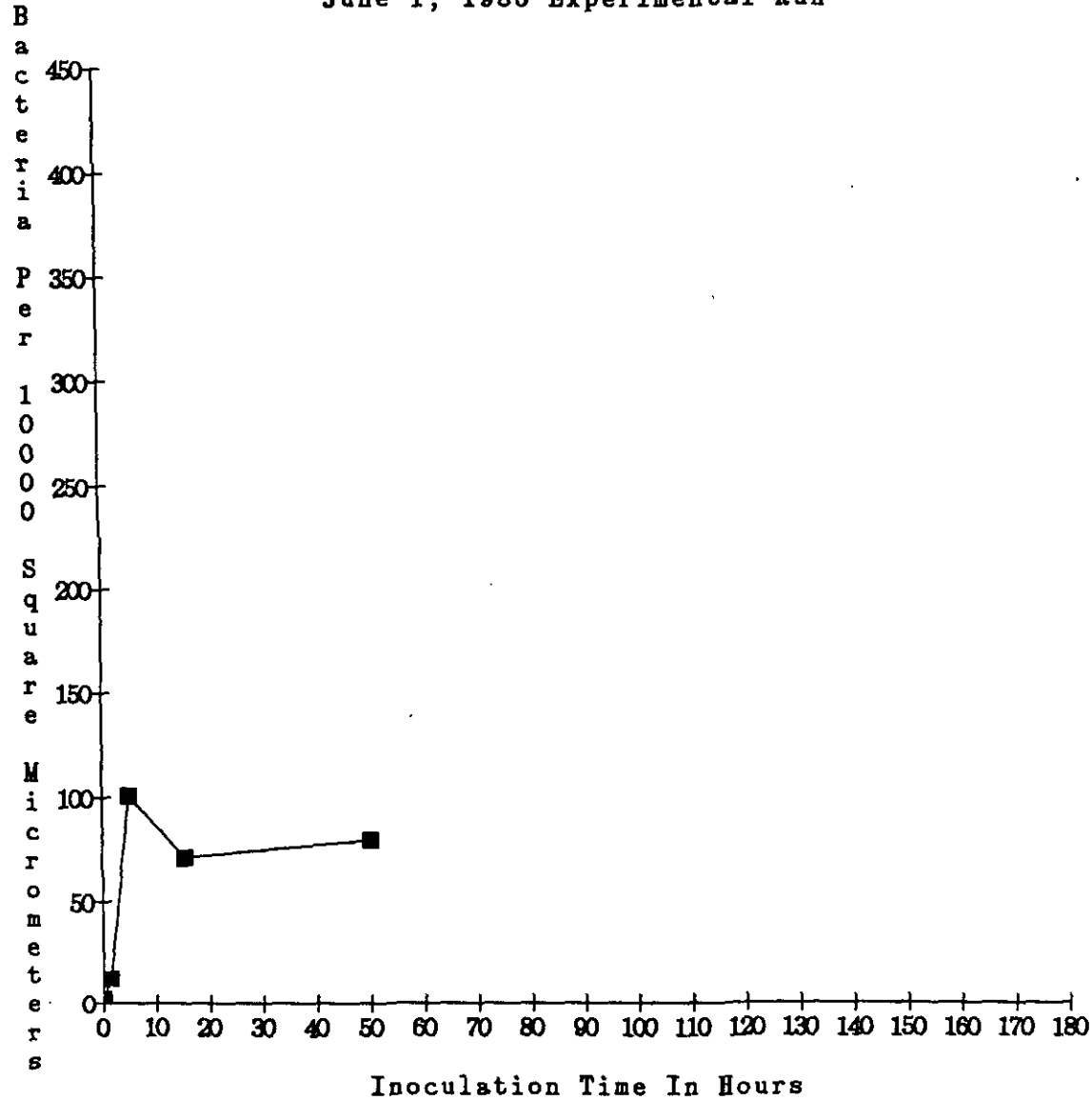
◇ Cocci < 0.6 um - Slides W,A
○ Cocci > 0.6 um - Slides W,A
□ Noncocci - Slides W,A
▽ Blue-green Fluorescing Bacteria (Methanogens) - Slides W,A
△ Cocci > 0.6 um + Cocci > 0.6 um + Noncocci - Slides W,A
■ Cocci > 0.6 um + Noncocci - Slides W,A
● Cocci > 0.6 um + Noncocci - Slides W,U
—— Weighted Regression - Cocci > 0.6 um + Noncocci
Y = A*(1-exp(k*t)) + A*(exp(u*t)-1)

Flow Rate Through Attachment Vessel = 31 ml/hr
A = Autoclaved W = Washed U = Unautoclaved

Figure 13

Inoculation Time
Versus
Number Of Bacteria Irreversibly Attached
Per 10000 Square Micrometers

20 Day SRT - 0.05 Volumes Per Day Dilution Rate
June 1, 1986 Experimental Run

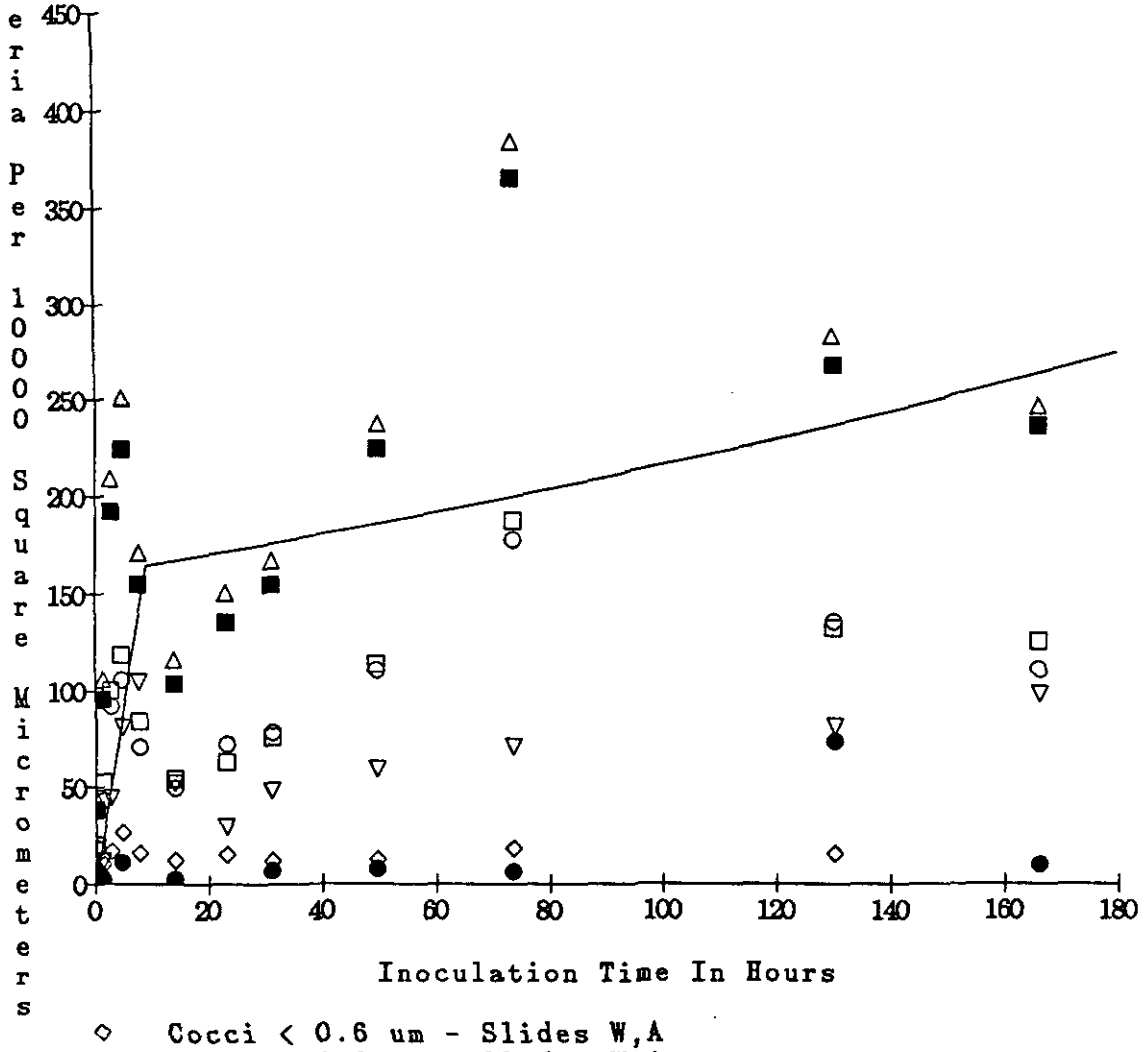


Flow Rate Through Attachment Vessel = 29 ml/hr
A = Autoclaved W = Washed U = Unautoclaved

Figure 15

Inoculation Time
Versus
Number Of Bacteria Irreversibly Attached
Per 10000 Square Micrometers

8 Day SRT - 0.125 Volumes Per Day Dilution Rate
August 19, 1986 Experimental Run



◇ Cocci < 0.6 um - Slides W,A
○ Cocci > 0.6 um - Slides W,A
□ Noncocci - Slides W,A
▽ Blue-green Fluorescing Bacteria - Slides - W,A
△ Cocci < 0.6 um + Cocci > 0.6 um + Noncocci - Slides W,A
■ Cocci > 0.6 um + Noncocci - Slides W,A
● Cocci > 0.6 um + Noncocci - Slides W,U
——— Weighted Regression - Cocci > 0.6 um + Noncocci
Y = A*(1-exp(k*t)) + A*(exp(u*t)-1)

Flow Rate Through Attachment Vessel = 30 ml/hr
A = Autoclaved W = Washed U = Unautoclaved

The model function for the regression curve was

$$Y = A(1-e^{kx}) + A(e^{\mu x}-1) \qquad (8)$$

where: Y = number of bacteria attached per 10,000 square micrometers

A = maximum number of bacteria that can initially attach per 10,000 square micrometers

K = rate coefficient - $\frac{\text{number of attachment sites/time disappearing}}{\text{number of attachment sites remaining}}$

μ = specific growth rate $\frac{\text{number of bacteria/time produced}}{\text{number of bacteria present}}$

x = inoculation time

The model will be discussed in more detail later in this chapter. The curve was included here to show the general pattern of attachment over time.

C. Data Analysis

1. Deciding Which Bacteria Counts to Analyze

At the beginning of the data analysis, a few decisions were made that deserve discussion. First, it was decided that the most pertinent parameter to consider with respect to the bacteria counts was the sum of cocci > 0.6 μm in diameter + noncocci. The counts for cocci < 0.6 μm in diameter were generally low and remained fairly constant through time. This category was created because it was difficult to be sure whether these small items were indeed bacteria or whether they were just dust or other particulate matter.

It was also decided to consider the sum of cocci > 0.6 μm diameter and noncocci rather than to break these two categories up. The categories were initially created during the early attempts to arrive at

the best way to count bacteria on slides. In these early attempts, a fluorescent stain, acridine orange, was used to stain the cells and the counts were carried out on a fluorescence microscope. Slides which were stained in this manner often contained many tiny circular droplets (0.2 - 3 μm in diameter) of stain which were difficult to differentiate from bacteria. Hence, it seemed important at that time to create separate categories in the counts for noncocci which were definitely bacteria, and cocci > 0.6 μm, for which there was less certainty that one was counting bacteria as opposed to droplets of stain. Eventually, the use of the stain was given up but the procedure of counting categories was retained. In general, the average counts for each, the cocci > 0.6 μm and the noncocci at a particular time, were similar. Also, there was always some uncertainty when making the counts where to categorize a short, stubby rod with rounded edges. Was it a cocci or a rod? Despite attempts to be consistent in counting, inevitably sometimes such an organism would be counted as a cocci, sometimes a noncocci. Thus, the sum of the two categories seemed to provide the most relevant information.

The counts using the fluorescence microscope were pertinent but contained some limitations. As was discussed in the literature review, methanogens are the only known bacteria which fluoresce blue-green when illuminated with light of 420 nm wavelength. However, one of the most important methanogens, Methanothrix soehngenii, does not noticeably fluoresce (Zehnder et al (44). Methanothrix soehngenii is important because it is an acetate utilizing methanogen. Acetate is known to be the major intermediate in methanogenesis in digestors. Only two methanogens are known to be acetate utilizers, Methanosarcina barkeri

and Methanothrix soehngenii. So the inability to count one of the most important methanogens was a significant drawback for this data set and meant caution was required in the analysis.

Also, some bacteria fluoresce brightly while others are dim. A field containing brightly fluorescing bacteria can make it difficult to see the dimly fluorescing ones. Further, the fluorescence of the bacteria tended to fade in about one second. This problem was negotiated by counting many small areas quickly taken together, these drawbacks restricted the value of completing a formal statistical analysis on these data.

2. Statistical Design

The data analysis was carried out using inferential statistics and descriptive statistics. The experimental design, for the purpose of statistics, can be represented as shown in Figure 16. The initial desire was to compare bacterial attachment at the two different growth rates. Replicate runs of each growth rate were done to allow the use of inferential statistics. During the second run, it was noticed that slide preparation apparently dramatically influenced bacterial attachment. So an extra experimental run on attachment to unautoclaved slides was carried out so that data for replicate runs on unautoclaved slides would also be available. This run was done with the run performed on June 1, 1986 for scanning electron microscopy. The fact that run #1 and #3 comparing autoclaved and unautoclaved slides were not conducted simultaneously, while for the other runs, autoclaved and unautoclaved experiments were conducted simultaneously, posed a problem

Figure 16. Experimental Design -- Statistical Perspective

	Growth Rate		Slide	Inoculation Time	Points - Hours								
	SRT	Run #	Preparation	0.0	0.08	1.25	2.67	4.67	7.5	14	23	31	49.5	73	165
paired observations	20	1	W, A												
	20	2	W, A												
	20	2	W, U												
	20	3	W, U												
paired observations	8	4	W, A												
	8	4	W, U												
paired observations	8	5	W, A												
	8	5	W, U												

for the inferential statistical analysis. The problem was addressed by using a different methods of analysis of each growth rate for autoclaved versus unautoclaved data.

3. Inferential Statistics

For the entire duration of this study, from the 1984 to 1987, intermitent consultation was obtained from the Statistical Consulting Center at the University of Massachusetts/Amherst. Professional statisticians were consulted about the experimental design and formal statistical analysis using inferential statistics. A more complete discussion of inferential statistics used in this study can be found in Appendix C. A brief synopsis of the analysis follows.

There were two questions addressed by formal inferential statistics in this study.

1. Is there a statistically significant difference in the pattern of bacterial attachment and initial biofilm development at the two growth rates tested (8 day SRT vs 20 day SRT)? and,
2. Is there a statistically significant difference in the pattern of bacterial attachment and initial biofilm development for the two slide preparations used (washed/autoclaved vs. washed/unautoclaved)?

The answer to the first question is no. The answer to second question is yes.

To answer the first question, three different methods of analysis were employed. They were a repeated measures growth curve analysis, a "t" test comparison of growth curve coefficients using an unweight regression analysis to determine the coefficients, and a "t" test comparison of growth curve coefficients using a weighted regression analysis to determine the coefficients. The repeated measures growth

curve analysis used the individual data points in the statistical analysis. The "t" test used coefficients, derived from a mathematical model to describe the pattern of attachment, for the statistical analysis. The analyses are summarized at the end of this section.

To answer the second question, three different methods of analysis also were employed. They were a randomized complete block analysis for the 8 day SRT data, a repeated measures growth curve analysis for the 20 day SRT data, and a "t" test comparison of the means at each inoculation time point for the data at both growth rates. The analyses are summarized below.

Comparison of Growth Rates

1) Repeated Measures Growth Curve Analysis	The two curves describing attachment at different growth rates were essentially the same curve. The attachment values changed over time.
2) "t" test Comparison of Growth Curve Coefficients	
unweighted	Hypothesis that the coefficients were equal between growth rates were accepted when hypotheses were considered simultaneously or independently.
weighted	Hypotheses that the coefficients were equal between growth rates were accepted when the hypotheses were considered simultaneously or independently.

Comparison of Slide Preparation	Autoclaved vs Unautoclaved
1) Randomized Complete Block Analysis (8 day SRT only)	The null hypothesis tests if the mean difference (between autoclaved and unautoclaved) were equal at each time point. It was known that the mean differences was approximately 0 at time zero. Thus the null hypothesis tested if the two curves were the same curve. The null hypothesis was rejected for the 8 day SRT data.

2) Repeated Measures Growth Curve Analysis (20 day SRT only)	The analysis concluded that the curves for autoclaved versus unautoclaved slides were very different. They were not parallel. Their overall mean values were different, and their values changed over time.
3) t Test Comparison of the Means at Each Time Point	The "t" test compared the mean attachment at each inoculation time point. The null hypothesis was that the means of attachment number at a certain inoculation time were equal for autoclaved and unautoclaved slides. For the 8 day SRT, the null hypothesis was rejected at 5 out of the 6 non-zero inoculation times. For the 20 day SRT, the descriptive level of the test was less than 0.085 in 3 out of the 4 non-zero inoculation times.

4. Descriptive Statistics

The attachment data was also considered using descriptive statistics. The most important data for each growth rate can be shown on a single graph. Figure 17 is a graph of inoculation time versus number of bacteria attached per 10,000 square micrometers for the 20 day SRT. Figure 18 is for the 8 day SRT data. Each data point on these graphs represents the average number of bacteria attached at the particular inoculation time for the replicate experimental runs. Only three categories of organism type/slide preparation technique are shown in these graphs. They are cocci > 0.6 μm + noncocci/slides washed and autoclaved; cocci > 0.6 μm + noncocci/slides washed and unautoclaved; and blue-green fluorescing bacteria. Also included on these graphs for washed/autoclaved slide preparations, and for the blue-green fluorescing bacteria, are the least squares regression curves for the first order

Figure 17. 20 Day SRT Data

1st Order Model

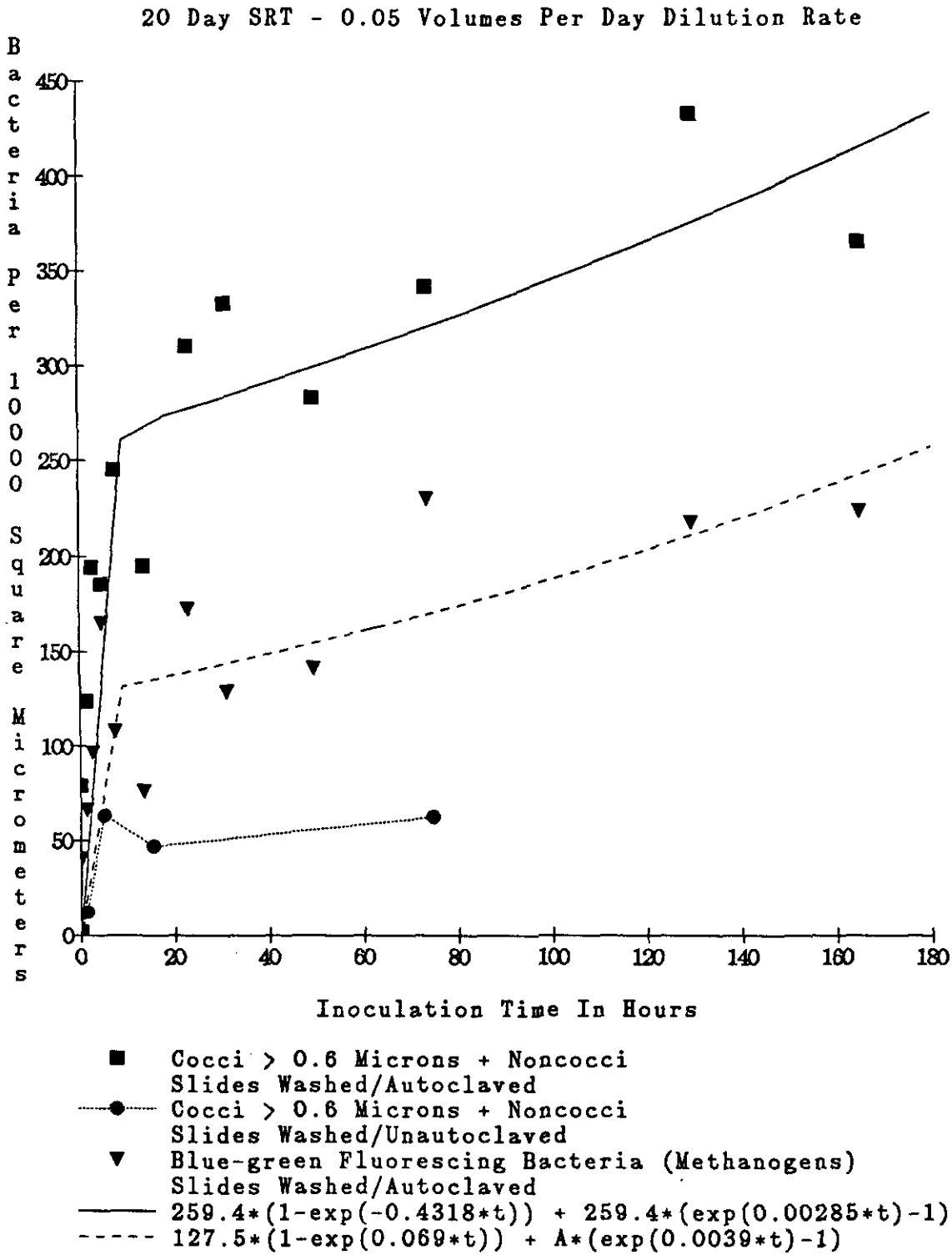
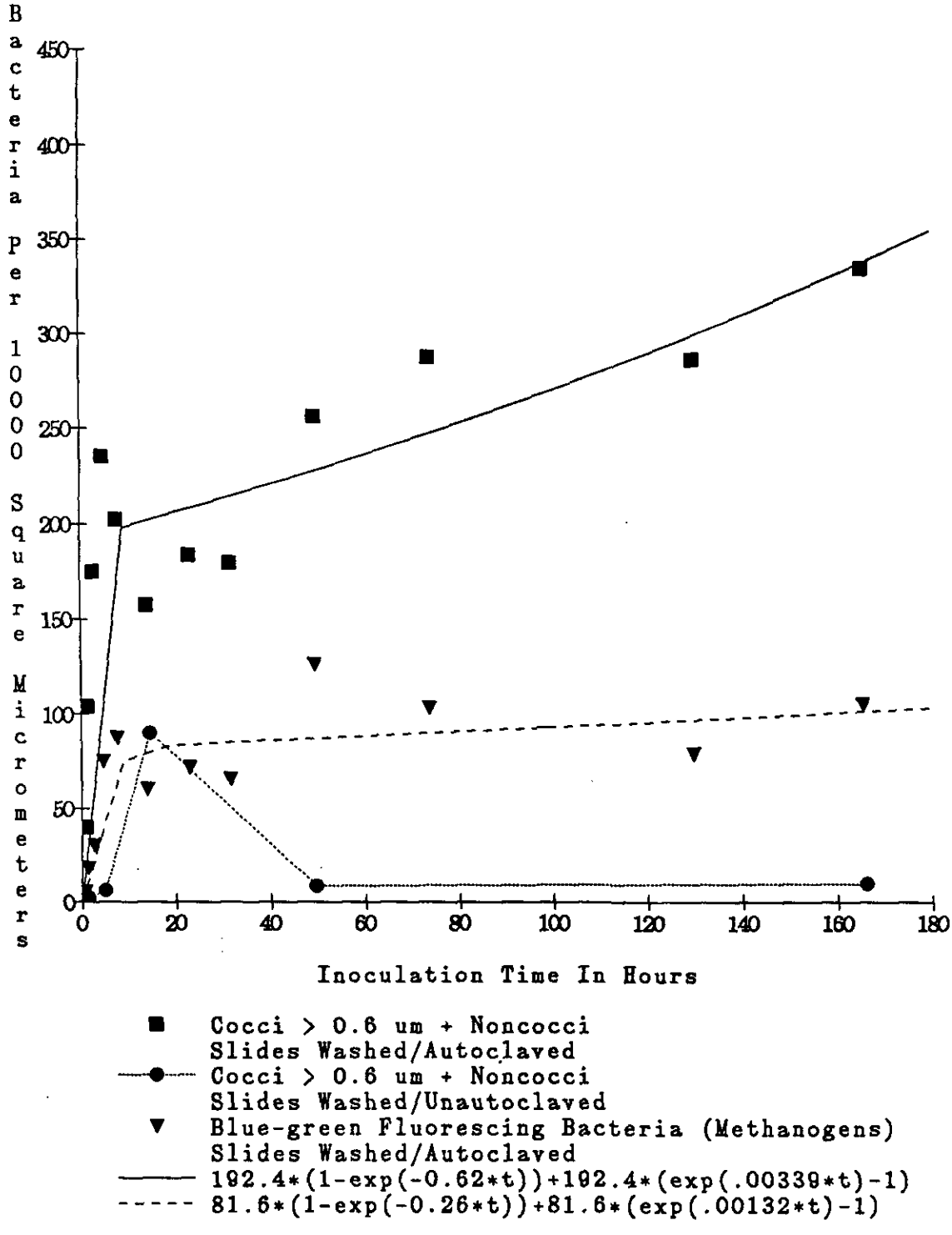


Figure 18. 8 Day SRT Data

1st Order Model

Inoculation Time
Versus
Number Of Bacteria Irreversibly Attached
Per 10000 Square Micrometers

8 Day SRT - 0.125 Volumes Per Day Dilution Rate



model described earlier. In Figures 19 and 20 the same graphs are shown with the Michaelis-Menten type model. Figure 21 shows that the curves obtained by the two models are very similar.

The following observations and conclusions can be made from inspection of Figures 17 to 21. Bacteria from methane-forming anaerobic chemostat cultures attached rapidly to washed/autoclaved glass slides in the attachment vessel. Within one to three hours, the number of irreversibly attached bacteria increased by two orders of magnitude from 0 bacteria per 10,000 square micrometers to 100 to 250 bacteria per 10,000 square micrometers. Initial attachment plateaued between 3 hours and 2 days inoculation time in the range of 200 to 350 bacteria per 10,000 square micrometers. Only a slow increase in the number of irreversibly attached was measured after the initial rapid increase. The counts of total bacteria after one week of inoculation were in the range of 250 to 450 bacteria per 10,000. From the results of the inferential statistics analysis no appreciable differences can be noted in the pattern of attachment on washed/autoclaved glass slides for the cocci > 0.6 μm and noncocci from inoculation cultures at the 8 day SRT versus the 20 day SRT. Bacteria which have been illuminated with light of 420 nm and fluoresce blue-green (methanogens) also attached rapidly to washed/autoclaved glass slides. The counts of methanogenic bacteria were generally 25% to 75% as high as the counts of total bacteria.

Autoclaving as a final step in the wash procedure had a dramatic effect on attachment. The counts of irreversibly attached bacteria on washed/unautoclaved slides over time were one half to one and one half

Figure 19. 20 Day SRT Data

Michaelis-Menten Type Model

Inoculation Time
Versus
Number of Bacteria Irreversibly Attached
Per 10000 Square Micrometers

20 Day SRT - 0.05 Volumes Per Day Dilution Rate

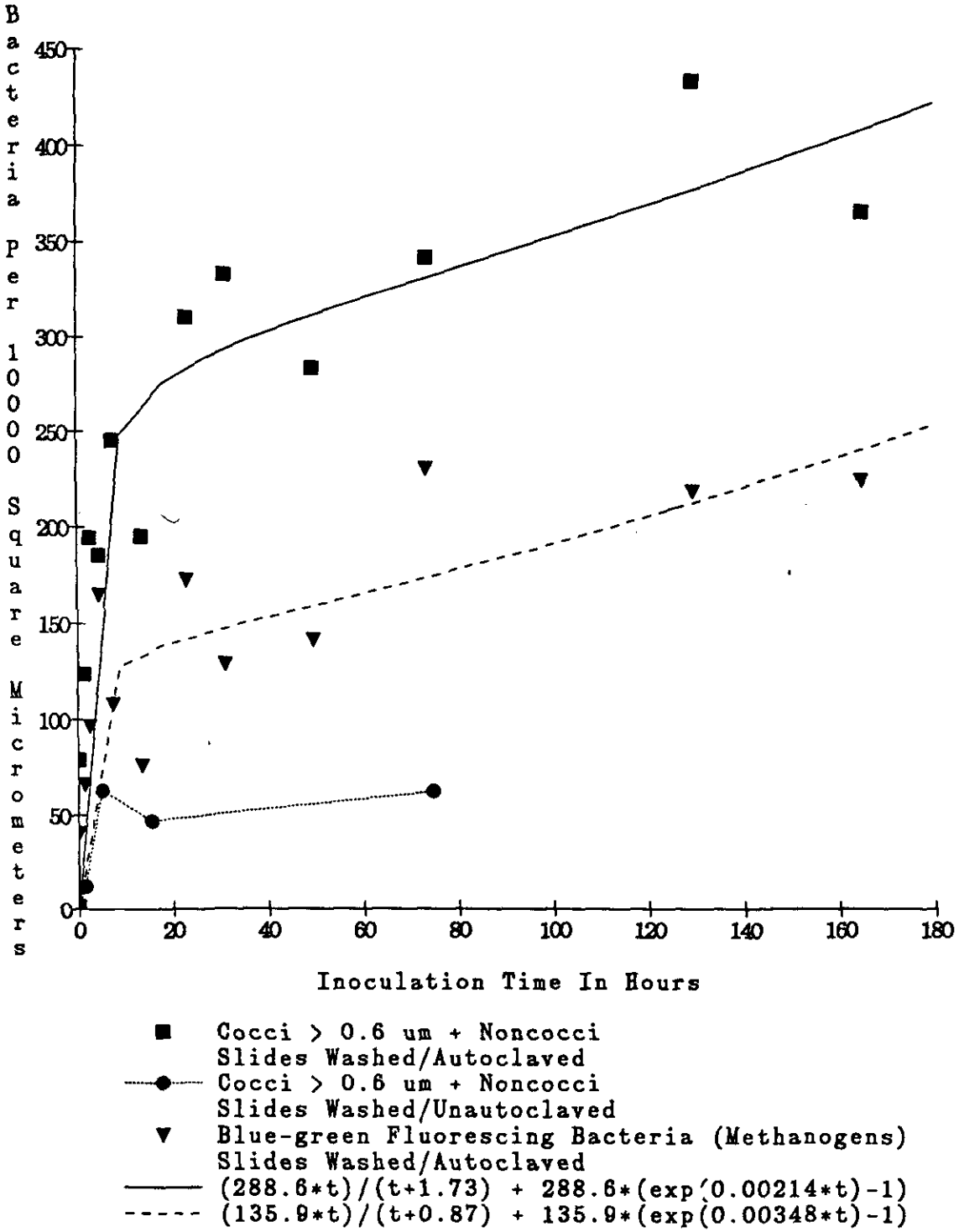


Figure 20. 8 Day SRT Data

Michaelis-Menten Type Model

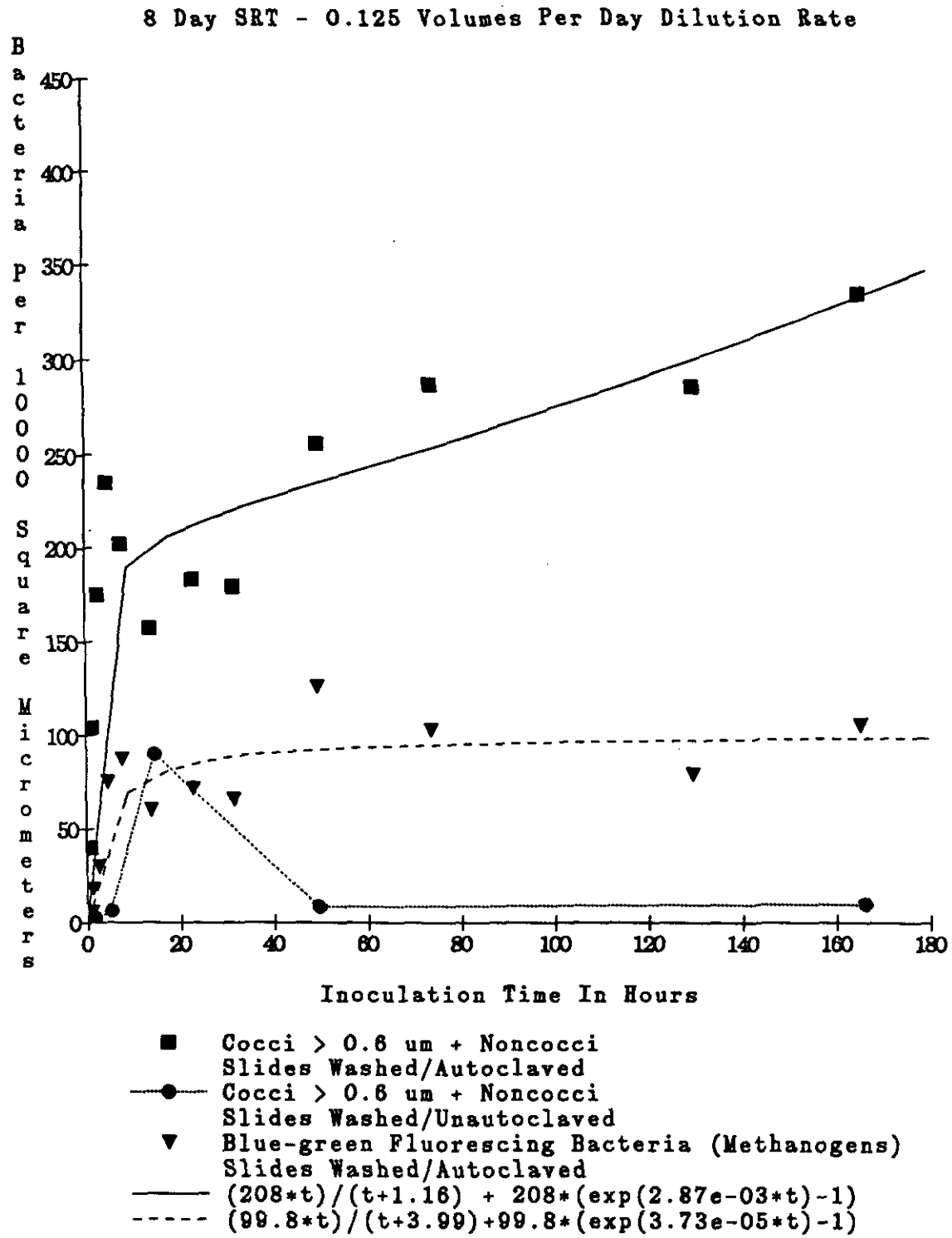
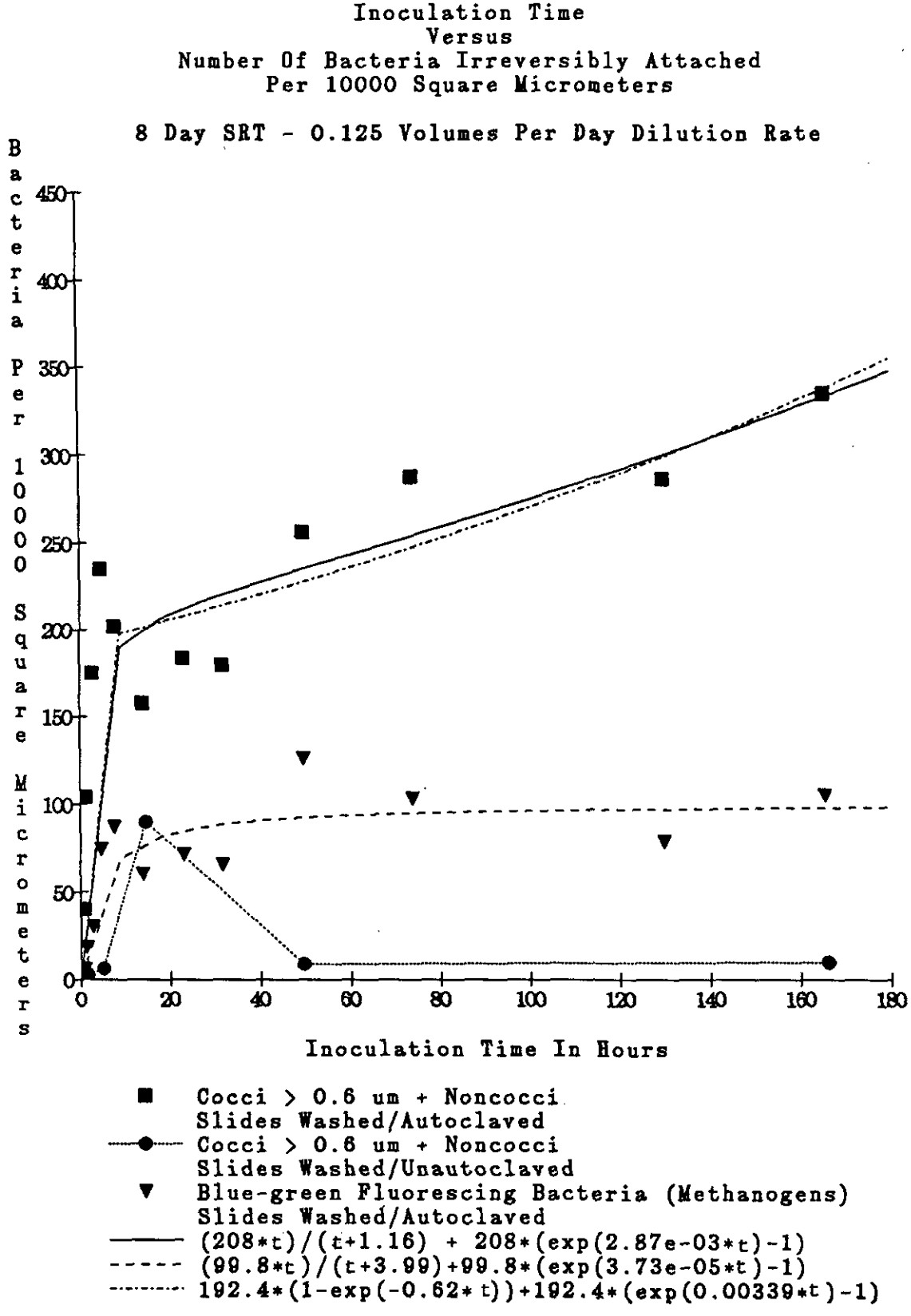


Figure 21. 8 Day SRT Data

Comparison of Models



orders at magnitude lower than the corresponding counts for washed, autoclaved slides. The differences between data for autoclaved and unautoclaved slides was confirmed using inferential statistics.

For washed/unautoclaved slides higher numbers of irreversibly attached bacteria were found on slides which were exposed to the 20 day SRT culture when compared to the 8 day SRT culture.

Table 8 summarizes the values that were obtained for the growth rate, μ, in the two models. Both the values that were obtained in the inferential statistical analysis and the values determined from the model to fit data points representing averages of the replicate runs are included. In all cases, the growth rate value were of a similar order of magnitude as the chemostat that was feeding the attachment vessel. The bacteria from the 8 day SRT chemostat appear to have a slightly higher growth rate.

D. Phase Contrast Microscope Photographs

Figure 22 is an inoculation time sequence of attachment photographs of the 8 day SRT culture/autoclaved slide preparation for inoculation times ranging from 0 to 166 hours. Photographs provide a realistic presentation of what was seen under the phase contrast microscope when the bacteria counts were done. Note that within minutes, significant concentrations of cells can be found irreversibly attached to the microscope slide (b). Bacteria are attached as single cells and in clumps. Mixed clumps, single cell type clumps, and single cell chains were attached to the surface within minutes and the first few hours. It is not possible to see in the still photographs, but many rods were attached on one of their short diameter ends while the rest of the

Table 8. Summary of Values Obtained for Growth Rates From Descriptive and Inferential Statistics Growth Rates - Cocci > 0.6 um + Noncocci

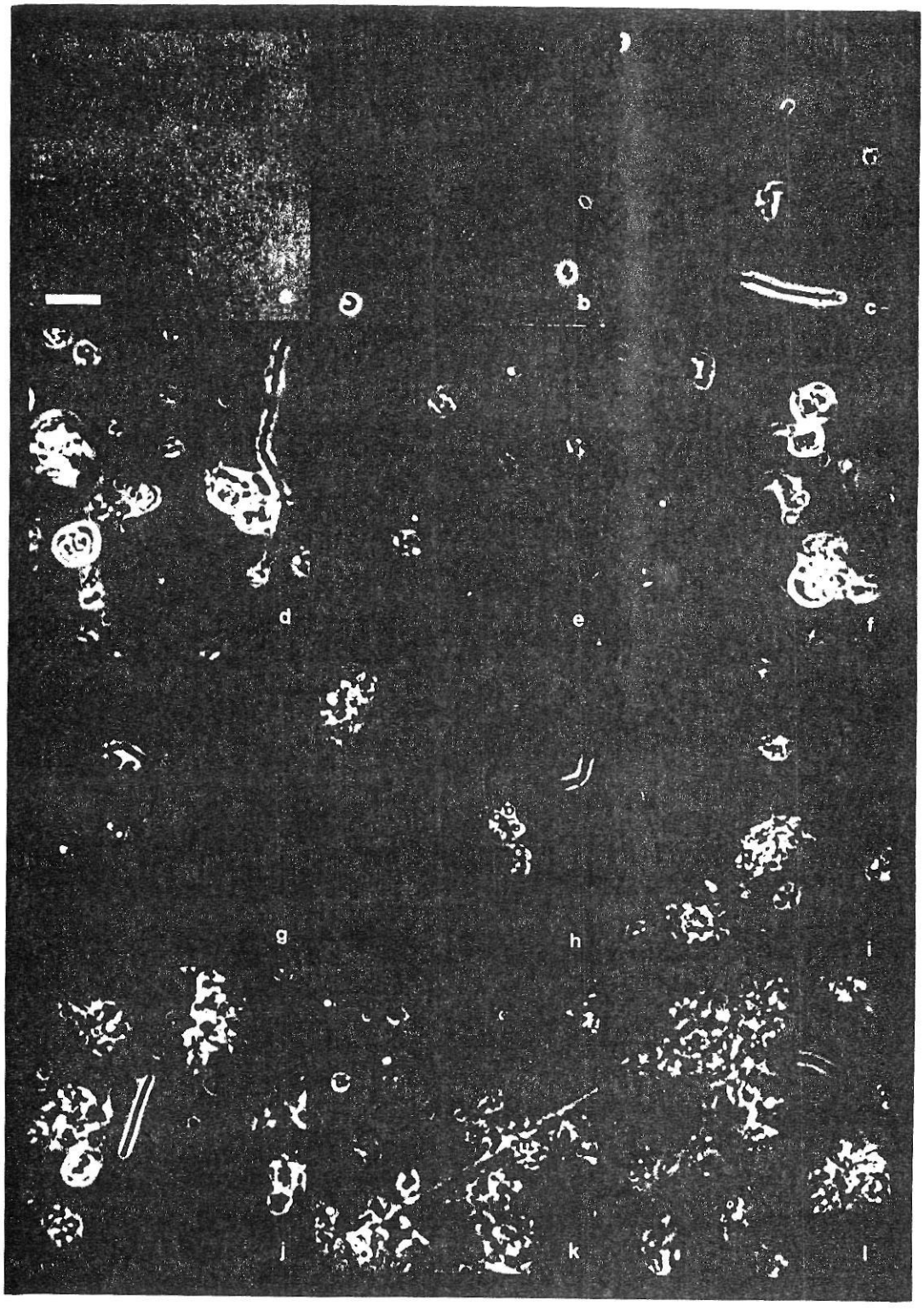
Descriptive Statistics Values		μ doublings per hour	SRT days per doubling
20 Day SRT	First Order Model	.00285	14.6
	Michaelis-Menten Type Model	.00714	19.5
8 Day SRT	First Order Model	.00339	12.3
	Michaelis-Menten Type Model	.00287	14.5
Inferential Statistics Values			
20 Day SRT	Unweighted-Average of Coefficients for Individual Curves	.002764	15.07
	Weighted-Average of Coefficients for Individual Curves	.003284	12.69
8 Day SRT	Unweighted-Average of Coefficients for Individual Runs	.00341	12.2
	Weighted-Average of Coefficients for Individual Runs	.003259	12.8

Figure 22. Attachment Sequence - Phase Contrast - 8 Day SRT

Slides Washed and Autoclaved

Bar = 10 micrometers

Inoculation Times in Hours: (a) 0, (b) 0.083, (c) 1.25, (d) 2.75, (e) 4.67, (f) 7.5, (g) 14.0, (h) 23.0, (i) 31.0, (j) 49.5, (k) 73.5, (l) 166.0.


a
b
c
d
e
f
g
h
i
j
k
l

bacterium moved vigorously. Other rods appeared to be attached by long thin, threadlike, appendages. One of these can be seen in (k) three quarters of the way up the photo in the center. The clumps grew larger at longer inoculation times and it became more difficult to differentiate individual bacteria at the longer inoculation times.

Figure 23 is an inoculation time sequence of attachment photographs of the 8 day SRT culture/unautoclaved slide preparation for inoculation times ranging from 0 to 166 hours. The photographs show very little attachment. A dramatic difference can be seen when these photographs are compared with photographs of washed/autoclaved slides (Figure 22).

E. Scanning Electron Microscopy

A selection of scanning electron microscope photographs are shown in Figures 24 to 28. Included are an inoculation time sequence of photographs for both the 20 day SRT (Figure 24) and the 8 day SRT (Figure 25), a plate of conspicuous attachment structures and small attached clumps (Figure 26) and two plates of large clumps and other photographs (Figures 27 and 28).

Figure 24 shows a selection of scanning electron microscope (SEM) photographs over a range of inoculation times from 0 hours to 134 hours for the 20 day SRT culture. It can be seen that within minutes, significant concentrations of cells were found on the surface (b). Mixed clumps, single cell type clumps, and single cell chains were attached to the surface within minutes and the first few hours (b, c, d, e, f). Single bacteria also attached to the surface initially (b, c, d, e, f). Some cells appeared to be attached by conspicuous fibers or appendages (c, d, e, f, g, h, i). Some cells did not appear to be

Figure 23. Attachment Sequence - Phase Contrast - 8 Day SRT

0.125 Volumes/Day Dilution Rate

Slides Washed and Unautoclaved

Bar = 10 micrometers

Inoculation Times in Hours: (a) 0, (b) 5, (c) 14.5, (d) 31, (e) 130, (f) 166.

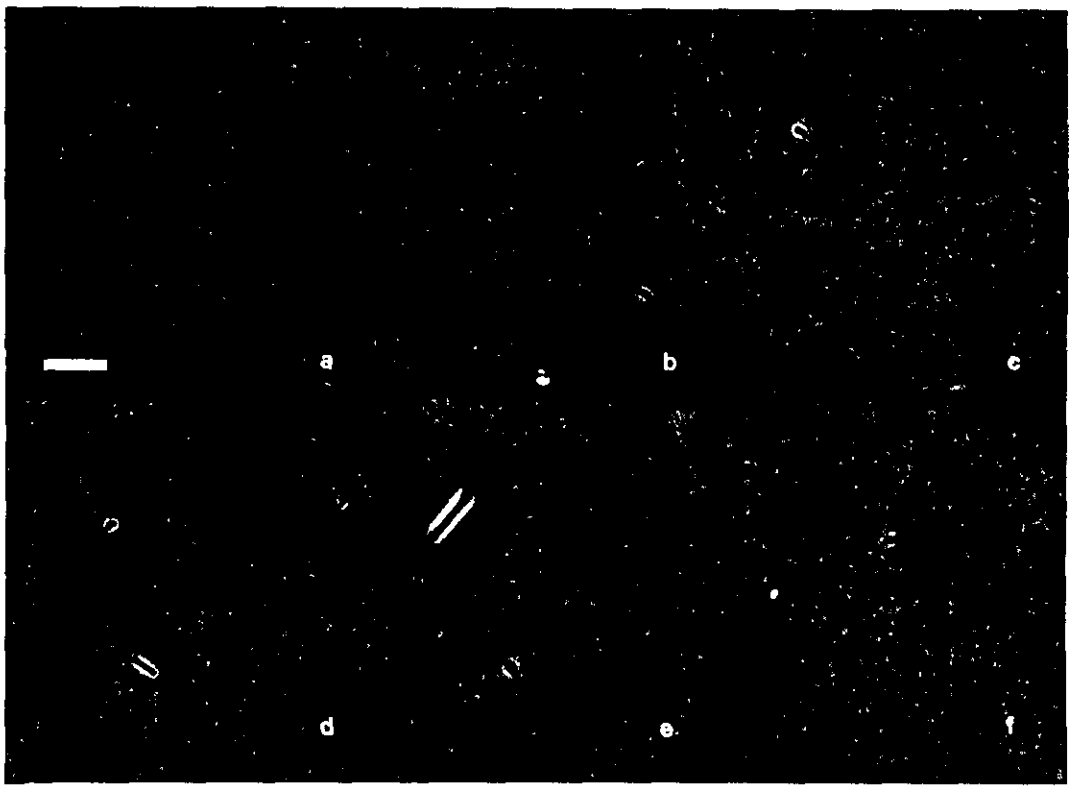

a
b
c
d
e
f

Figure 24. Attachment Sequence - Scanning Electron Microscope

20 Day SRT - 0.05 Volumes/Day Dilution Rate

Slides Washed and Autoclaved

Specimen Stage Angle = 45° (c & h are 0°)

Bar = 10 micrometers

Inoculation Time in Hours: (a) 0.0, (b) 0.25, (c) 1.25, (d) 4.67, (e) 7.5, (f) 16.5, (g) 49, (h) 76.5, (i) 134.

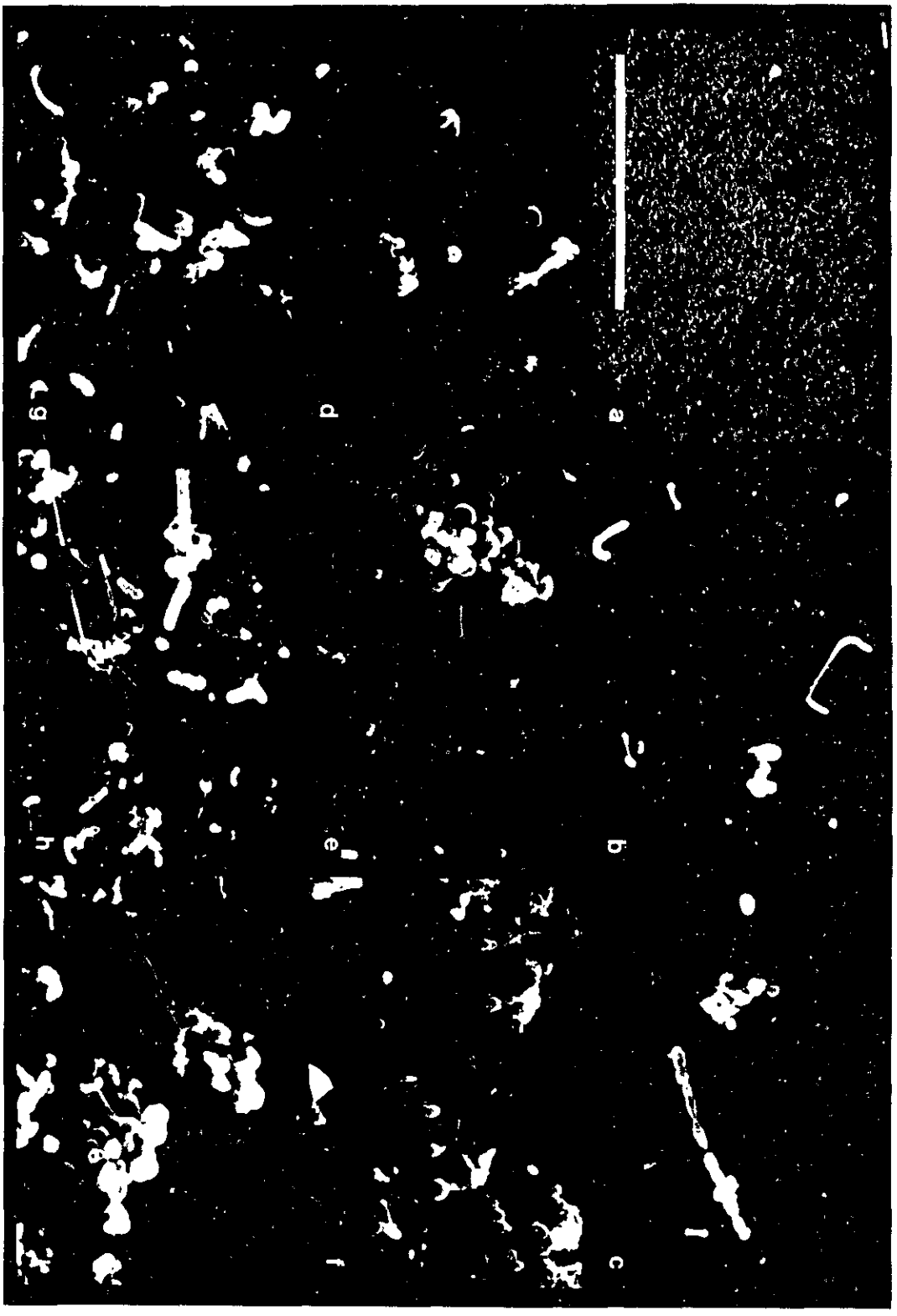

a
b
c
d
e
g

Figure 25. Attachment Sequence - Scanning Electron Microscope

8 Day SRT - 0.125 Volumes/Day Dilution Rate

Slides Washed and Autoclaved - Specimen Stage Angle = 45°

Bar = 10 micrometers

Inoculation Time In Hours: (a) 0.0, (b) 0.25, (c) 1.25, (d) 2.67, (e) 4.83, (f) 16, (g) 50, (h) 76.5, (i) 120.


a
b
c
d
e
f
g
h
i

Figure 26. Conspicuous Attachment Structures - Scanning Electron Microscope Slides Washed and Autoclaved (h - unautoclaved)

Bar = 1 micrometer

Information listed below for each photo is inoculation time in hours, specimen stage angle, and solids retention time:

(a) 1.25, 45°, 20; (b) 4.67, 0°, 20; (c) 0.25, 0° 20; (d) 0.25, 45°, 8; (e) 76.5, 45°, 20; (f) 2.67, 45°, 20; (g) 4.67, 45°, 20; (h) 5.0, 0°, 20; (i), 134, 0°, 20; (j, k) 1.25, 45°, 8; (l) 4.83, 45° 8; (m) 4.67, 0°, 20; (n) 0.25, 45°, 8; (o) 0.25, 45°, 8; (p) 1.25, 45°, 20.


a
b
c
d
e
f
g
h
i
k
l
m
n
o
p

Figure 27. Other Scanning Electron Microscope Photographs

Information listed below for each photograph is inoculation time in days, solids retention time, specimen stage angle, bar length in micrometers.

(a) 16, 8, 0°, 10 -- Note difference in tone surrounding clumps of bacteria - possibly due to extracellurlar materials or secretions by the cells.

(b) 16, 8, 0°, 10 -- Note difference in tone surrounding clumps of bacteria - possibly due to extracellular materials or secretions by the cells.

(c) 16, 8, 0°, 10 -- Note diversity of morphological cell types, extracellular material, and clumped attachment and growth.

(d) 12, 8, 0°, 10 -- Note diversity of morphological cell types, extracellular material, and clumped attachment and growth.

(e) 12, 8, 0°, 10 -- Note diversity of morphological cell types, extracullar material and clumped attachment and growth.

(f) 16, 8, 0°, 10 -- Note diversity of morphological cell types, extracellular maerial, and clumped attachment and growth.

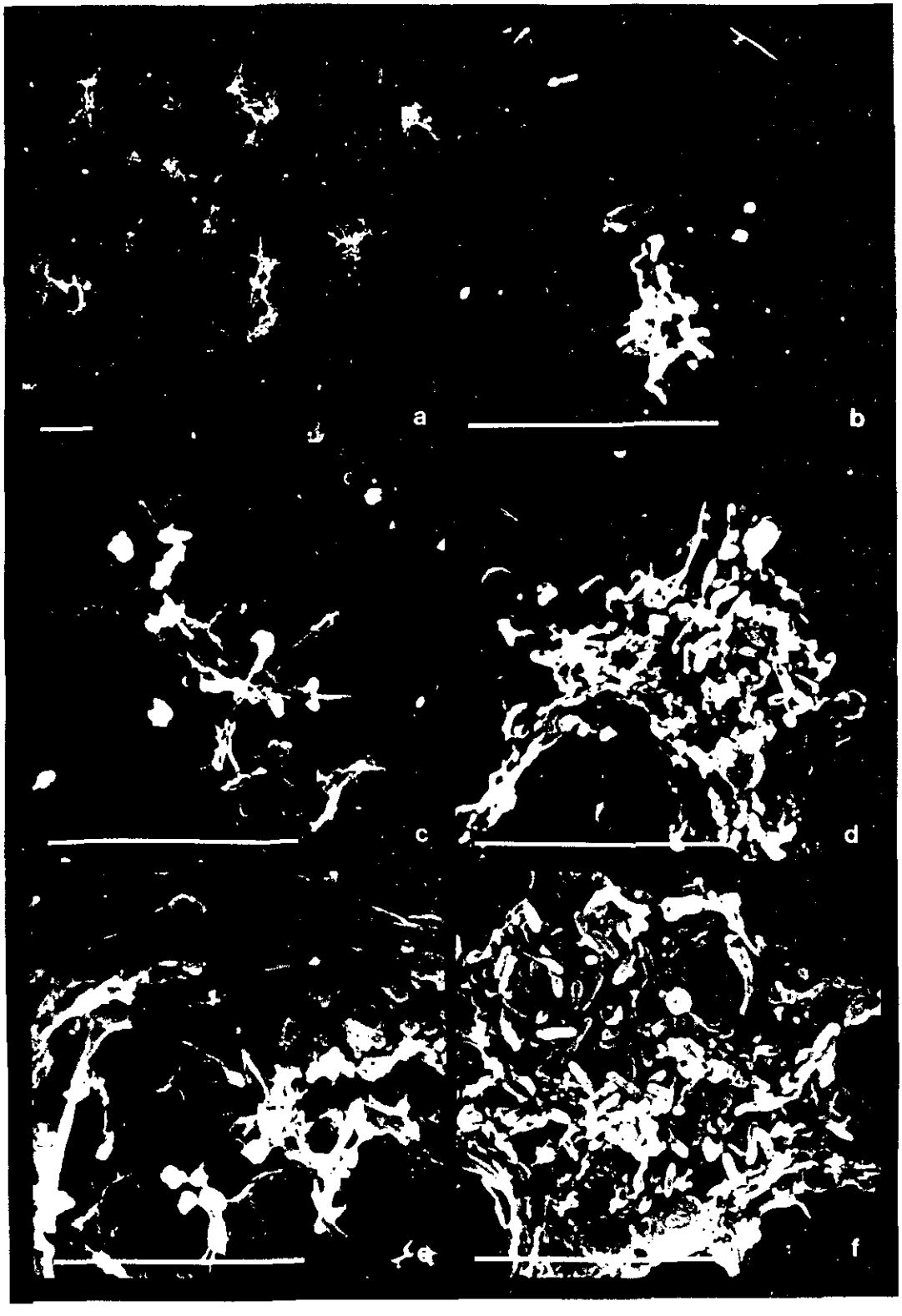

a
b
c
d
e
f

Figure 28. Other Scanning Electron Microscope Photographs

Information listed below for each photograph is inoculation time, solids retention time in days, specimen stage angle, bar length in micrometers.

(a) 12 days, 8, 45°, 20 -- Note extracellular material and clumped attachment and growth.

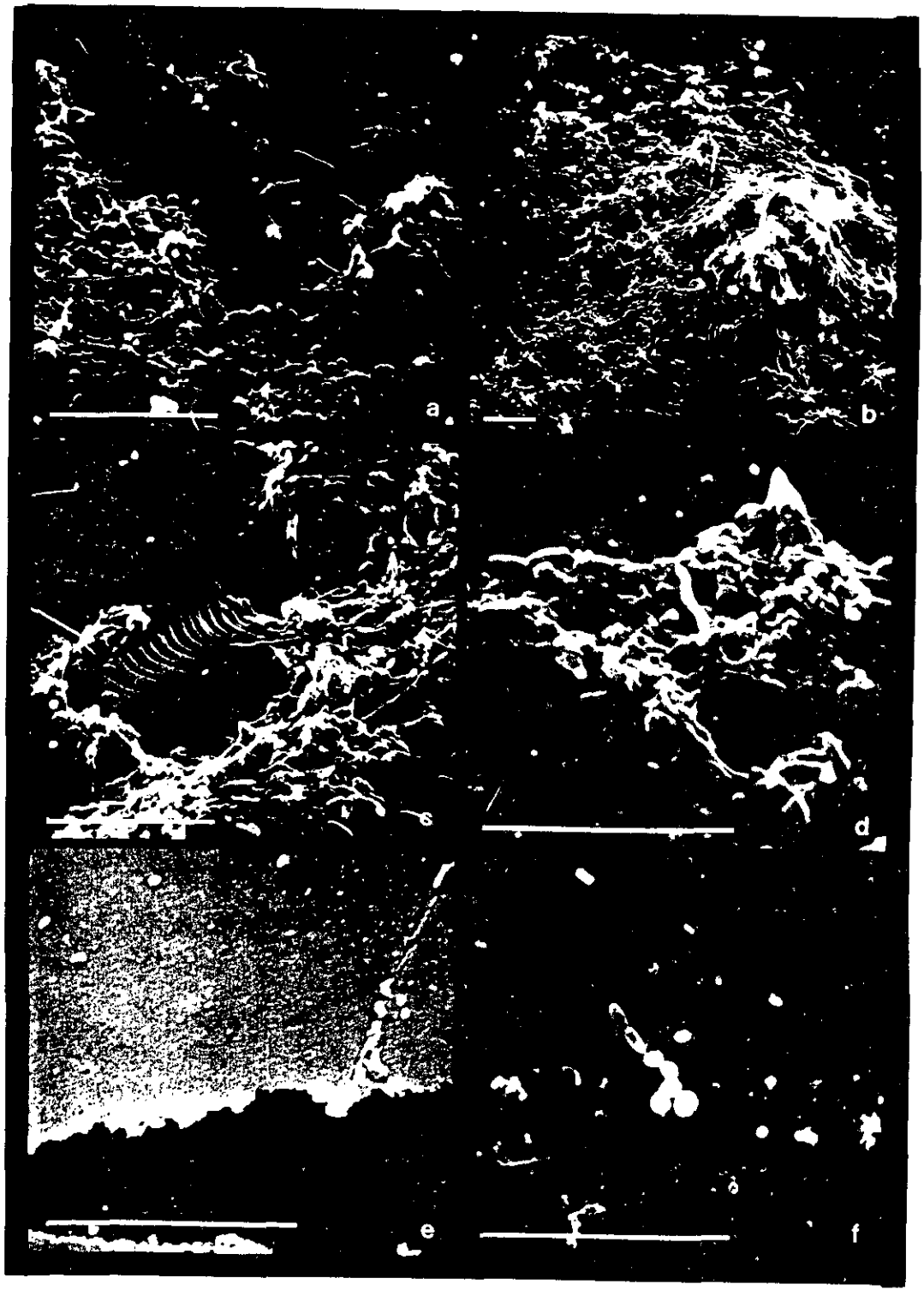
(b) 12 days, 8, 45°, 10 -- Note extracellular material and clumped attachment and growth.

(c) 12 days, 8, 45°, 10 -- Note extracellular material and clumped attachment and growth.

(d) 16 days, 8, 45°, 10 -- Note extracellular material and clumped attachment and growth.

(e) 15 min, 20, 45°, 10 -- Note large scratch - possibly an example of initial organic film.

(f) 76.5 hrs, 20, 0°, 10 - Note extracellular filamentous material.


a
b
d
e
f

attached by conspicuous fibers or appendages (b, c, d, e f, g , h, i). At the longer inoculation times, more extracellular, fiber-like material was seen (f, g, h). Branching or distinct angular sections of the extracellular fiber-like material can be seen at longer inoculation times (h). At the longest inoculation time, amorphus extracellular material can be seen (i).

Figure 25 shows a selection of SEM photographs over a range of inoculation times from 0 hours to 120 hours for the 8 day SRT culture. Many of the comments about attachment of the 20 day SRT culture apply to the 8 day SRT culture but there are a few differences. Once again, it can be seen that within minutes, significant concentrations of cells can be found on the surface (b). Mixed clumps, single cell type clumps (b, c, d, e, f) and single cell chains (see Figure 26, n) are attached within minutes and the first hours of inoculation. Single bacteria are also attached to surface initially (b, c, d, e, f, g). Some cells appear to be attached by conspicuous fibers or appendages (b, c, d, e, f, g) but these are less evident than those found in the 20 day SRT photographs. Some cells do not appear to be attached by conspicuous fibers or appendages (b, c, d, e, f, g, h). Unlike the 20 day SRT, there was not a lot more extracellular fiber-like material visualized at the later inoculation times. Extracellular amorphous material is not shown in this figure for the 8 day SRT culture but it was seen in other long inoculation time 8 day SRT cultures (see Figure 27 and 28). Finally, some of the morphological types of bacteria seen in the 8 day SRT cultures are similar to the 20 day SRT culture and some are different.

Figure 26 shows conspicuous attachment structures that were seen in the SEM study (a-m) and clumps of bacteria attached at very short inoculation times (n-p). Extracellular straight, fiber-like material apparently used for bacterial attachment was seen (a,c, f, g, m) that seemed to fuse and flatten where it contacted the surface. Rods were seen with a square or rectangular "foot" apparently used for attachment either at the end of the rod (b), or at the end of a long slender appendage extending from the main body of the rod (j, k). Curved filament-like appendages with distinct, slightly thicker ends at the attachment sight were seen (d, h). On one occasion a ring-like structure was observed (e). Very short appendages or extracellular material was seen (1). A fuzzy border surrounding an entire cell was also seen (i).

Photographs 26, n, o, and p were included to show that clumps and chains of cells were also attached at very early times (1.25 hours or less).

Figure 27 shows more SEM photographs of the attached 8 day SRT culture at much longer inoculation times, twelve and sixteen days. All the photographs show the dramatic development of mixed cell clumps and extracellular gluelike material. Photo (a) was included to show a low magnification perspective of the bacteria attached to the surface and the tone shading difference that was noticeable around the clumps of bacteria and single bacteria. The cause of these rings is unknown but one can speculate they are the result of either extracellular production of polymers or extracellular secretion of enzymes breaking down organic molecules attached to the surface. Photo (b) is a higher magnification photograph of a clump surrounded by one of these rings. Photos (c),

(d), (e), and (f) are included to show high magnification photographs of the extensive development of the clumps of bacteria. Note the diversity of morphological cell types, extracellular amorphus and fiber-like material, and the large diameter of the clumps.

Figure 28 shows more long inoculation time clumps (a-d), one photograph possibly showing an initial layer or organic molecules on the glass surface, and one photograph showing more extracellular fiber-like material. Photos (a), (b), and (c) are relatively low magnification photographs of extremely large, clumped growth. There is also extensive presence of the extracellular glue-like material. In (c), the glue-like material seems to have moved far away from the cells or clumps. The curved parallel lines which would be bisected by an axis running from the lower left to the upper right of the photograph might be some sort of scratch caused during the cleaning process. The glue-like material appears to be draped over the gap caused by the scratches; (d) also shows the extensive presence of extracellular material. Many of the bacteria appear to have lost their distinct shapes and appear as if covered with snow. Nevertheless, there are a few bacteria on top of the others which still have a distinct shape. The theory of bacterial attachment supposes that a layer of organic molecules forms very rapidly on a surface before the bacteria attach. Photo (e) was included to show what may be an example of that film of organic molecules. This is a fifteen minute inoculation time photograph. It appears the section was scratched during the SEM fixing or drying procedure revealing the initial organic film. Photo (f) was included as another example of extracellular fiber development. This was a 76.5 hour inoculation time from the 20 day SRT culture.

CHAPTER V. SUMMARY AND CONCLUSIONS

Based on the results of this study, the following conclusions can be made:

1. Bacteria from methane-forming chemostat cultures attached rapidly to chromic acid washed/autoclaved glass slides in a quiescent environment. Within one to three hours, the number of irreversibly attached bacteria increased by two orders of magnitude from 0 bacteria per 10,000 square micrometers to 100 to 250 bacteria per 10,000 square micrometers. Initial attachment plateaued between 3 hours and 2 days inoculation time in the range of 200 to 350 bacteria per 10,000 square micrometers.
2. After initial attachment, only a slow increase in the number of irreversibly attached cells was observed. The growth rate was of the same order of magnitude as the growth rate for the bacterial cultures from the chemostat. The counts of total bacteria after one week of inoculation were in the range of 250 to 450 bacteria per 10,000 square micrometers.
3. Two mathematical models were developed to describe early attachment and growth. Each model contained three coefficients to describe the pattern of early attachment and growth. In the first order attachment model the following coefficients were used:

   A_0 = maximum number of cells that can initially attach

   k = rate coefficient indicating the rate that initial attachment sites are disappearing

   μ = rate coefficient indicating the rate cells reproduce once they are attached

   The first order attachment model is:

$$Y = A_o (1 - e^{kt}) + A_o (e^{ut} - 1)$$

Y = number of bacteria irreversibly attached

t = inoculation time

In the Michaelis-Menten type model the following coefficients were used:

A_o = maximum number of cells that can initially attache

Km = inoculation time when the number of attached cells is one half the maximum number of initially attached cells ($A_o/2$)

μ = rate coefficient indicating the rate cells reproduce once they are attached

The Michaelis-Menten type model is

$$Y = \frac{(A_o\ t)}{A_o + Km)} + A_o (e^{\mu t} - 1)$$

Y = number of bacteria irreversibly attached

t = inoculation time

4. No statistical difference could be noted in the pattern of attachment on chromic acid washed/autoclaved glass slides for the cocci > 0.6 μm + noncocci from inoculum cultures growing at an 8 day SRT and a 20 day SRT. However, the small number of replications and the large variance in the attachment counts makes the probability of a Type II error (failing to statistically note a true difference in the curves) high. For future experiments, the only way to reduce the probability of a Type II error are to increase the number of times the experiment is carried out or reduce the variance in the bacteria counts.

5. Bacteria which have been illuminated with light at 420 nm and fluoresce blue-green (methanogens) also attach rapidly to chronic acid washed/autoclaved glass slides. The counts of methanogenic bacteria were generally 25% to 75% as high as the counts of total bacteria.

6. Autoclaving as a final step in slide washing procedure had a dramatic effect on attachment. The counts of irreversibly attached bacteria on chromic acid washed/unautoclaved slides over time were one half to one and one half orders of magnitude lower than the corresponding counts for chromic acid washed, unautoclaved slides. The difference between the data for autoclaved and unautoclaved slides was statistically significant.
7. Scanning electron microscopy revealed five noteworthy items.
   a. Some bacteria possess conspicuous attachment structures. After 1 to 2 days inoculation, one begins to notice the production of extracellular fiber like material. These fibers have been observed in dental studies also.
   b. Between 2 days and 2 weeks inoculation time, there begins to be an extensive production of extracellular material that looks like glue or snow and is spread everywhere.
   c. Bacteria are found singly but also found in large clumps or colonies.
   d. The colonies are often covered or interspersed with the glue-like material.
   e. Some colonies appear to have a ring around them. One must suppose this is either extracellular material the cells have secreted or the result of the secretion of extracellular enzymes.
8. An anaerobic attachment vessel was developed which allows the systematic investigation of the attachment of anaerobic bacteria to microscope slides or other surfaces.

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APPENDIX A. TIMING OF PHASE CONTRAST AUTOCLAVED COUNTS

Table A-1. Time Sequence -- Sampling and Bacteria Counts
April Experimental Run
Start Time 2:20 p.m. -- April 1, 1986

Autoclaved (A) or Unautoclaved (U)	Inoculation Time Hours	Sampling Time	Time Date	Phase Contrast Count Time	Phase Contrast Count Date	Elapsed Time Between Sampling and Counting	Flourescence Count Time	Flourescence Count Date	Elapsed Time Between Sampling and Counting	Comment
A	.08	2:25 pm	4/1/86	3:05 pm	4/1/86	40 min.	DNR	4/4/86	3 days	
A	1.25	3:35 pm	4/1/86	4:14 pm	4/1/86	40 min.	DNR	4/4/86	3 days	
A	2.58	4:55 pm	4/1/86	5:55 pm	4/1/86	1 hrs.	12:10 pm	4/4/86	67.25 hrs.	
A	4.67	7:00 pm	4/1/86	8:05 pm	4/1/86	1.08 hrs.	11:50 am	4/4/86	64.83 hrs.	
A	7.5	9:50 pm	4/1/86	11:30 pm	4/1/86	1.67 hrs.	2:40 pm	4/4/86	64.83 hrs.	
A	13.5	3:50 am	4/2/86	11:20 am	4/2/86	7.5 hrs.	6:15 pm	4/4/86	62.42 hrs.	
A	23	1:20 pm	4/2/86	3:35 pm	4/2/86	2.25 hrs.	DNR	4/4/86	2 days	
A	31	9:20 pm	4/2/86	10:00 pm	4/2/86	40 min.	11:00 am	4/4/86	37.67 hrs.	
A	49.5	3:50 pm	4/3/86	4:35 pm	4/3/86	45 min.	10:35 am	4/7/86	90.75 hrs.	
A	73.5	3:50 pm	4/4/86	4:40 pm	4/4/86	50 min.	DNR	DNR		
A	165	11:20 am	4/8/86	1:20 pm	4/8/86	2 hrs.	11:40 am	4/8/86	20 min.	

Table A-2. Time Sequence -- Sampling and Bacteria Counts
May Experimental Run
Start Time 2:20 p.m. -- May 21, 1986

Autoclaved (A) or Unautoclaved (U)	Inoculation Time Hours	Sampling Time	Time Date	Phase Contrast Count Time	Date	Elapsed Time Between Sampling and Counting	Flourescence Count Time	Date	Elapsed Time Between Sampling and Counting	Comment
A	0	11:00 am	5/21/86	11:30 am	5/21/86	30 min.	11:30 am	5/21/86	30 min.	
A	.083	2:25 pm	5/21/86	2:55 pm	5/21/86	30 min.	2:05 pm	5/26/86	119.67 hrs.	
A	1.25	3:35 pm	5/21/86	5:30 pm	5/21/86	1.92 hrs.	DNR	5/26/86	5 days	
A	2.67	5:00 pm	5/21/86	8:00 pm	5/21/86	3 hrs.	DNR	5/26/86	5 days	
A	4.67	7:00 pm	5/21/86	12:00 noon	5/22/86	17 hrs.	DNR	5/25/86	4 days	
A	7.5	9:50 pm	5/21/86	2:25 pm	5/22/86	16.58 hrs.	DNR	5/25/86	4 days	
A	14.75	5:05 am	5/22/86	5:20 pm	6/22/86	12.25 hrs.	10:30 pm	5/25/86	89.42 days	
A	23	1:20 pm	5/22/86	6:55 pm	5/22/86	5.58 hrs.	4:15 pm	5/25/86	74.92 hrs.	
A	31	9:20 pm	5/22/86	DNR	5/23/86	1 day	10:00 pm	5/25/86	72.67 hrs.	
A	54.5	8:50 pm	5/23/86	9:45 pm	5/23/86	55 min.	3:30 pm	5/25/86	43.53 hrs.	
A	73.5	3:50 pm	5/23/86	5:00 pm	5/24/86	25.16 hrs.	2:45 pm	5/25/86	46.92 hrs.	
A	129.75	12:05 am	5/27/86	11:20 am	5/27/86	11:25 hrs.	11:30 am	5/29/86	59.42 hrs.	
A	165	11:20 am	5/28/86	10:15 pm	5/28/86	10.92 hrs.	DNR	5/29/86	1 day	
U	0	11:05 am	5/27/86	11:10 am	5/27/86	5 min.				
U	1.417	3:45 pm	5/21/86	3:25 pm	5/23/86	47.67 hrs.				
U	5	7:20 pm	5/21/86	1:35 pm	5/24/86	66.25 hrs.				
U	15	5:20 am	5/22/86	2:30 pm	5/24/86	57.16 hrs.				
U	74	4:20 pm	5/24/86	12:05 am	6/25/86	7.75 hrs.				

Table A-3. Time Sequence -- Sampling and Bacteria Counts
June Experimental Run
Start Time 6:05 p.m. --
DNR did not record -- Fluorescence count not recorded

Autoclaved (A) or Unautoclaved (U)	Inoculation Time Hours	Sampling Time	Time Date	Phase Contrast Count Time	Phase Contrast Count Date	Elapsed Time Between Sampling and Counting	Flourescence Count Time	Flourescence Count Date	Elapsed Time Between Sampling and Counting	Comment
U	0	1:40 pm	6/1/86	1:45 pm	6/1/86	5 min.				
U	1.42	7:30 pm	6/1/86	9:50 pm	6/1/86	2.33 min.				
U	5	11:05 pm	6/1/86	11:55 pm	6/1/86	50 min.				
U	15.5	9:35 am	6/2/86	9:40 am	6/2/86	5 min.				
U	50	8:05 pm	6/3/86	2:20 pm	6/7/86	90.25 hrs.				
U	77	11:05 pm	6/4/86	DNR	6/5/86	1 day				

Table A-4. Time Sequence -- Sampling and Bacteria Counts
July Experimental Run
Start Time: 3:05 p.m.
DNR = did not record

Autoclaved (A) or Unautoclaved (U)	Inoculation Time Hours	Sampling Time	Time Date	Phase Contrast Count Time	Phase Contrast Count Date	Elapsed Time Between Sampling and Counting	Flourescence Count Time	Flourescence Count Date	Elapsed Time Between Sampling and Counting	Comment
A	0	12:55 pm	7/15/86	1:00 pm	7/15/86	5 min.		7/23/86	5 min.	blank sample taken on 7/23
A	.083	3:10 pm	7/15/86	3:35 pm	7/15/86	25 min.	9:30 pm	7/17/86	54.33 hrs.	
A	1.33	4:25 pm	7/15/86	5:00 pm	7/15/86	35 min.	9:50 pm	7/17/86	53.417 hrs.	
A	2.75	5:50 pm	7/15/86	6:40 pm	7/15/86	50 min.	10:15 pm	7/17/86	52.417 hrs.	
A	4.67	7:45 pm	7/15/86	8:40 pm	7/15/86	55 min.	10:35 pm	7/17/86	50.83 hrs.	
A	7.67	10:45 pm	7/15/86	11:20 pm	7/15/86	35 min.	DNR	7/17/86	2 days	
A	14.0	5:05 am	7/16/86	11:55 am	7/16/86	6.83 hrs.	11:30 pm	7/17/86	42.417 hrs.	
A	23.0	2:05 pm	7/16/87	3:35 pm	7/16/87	1.5 hrs.	DNR	DNR	DNR	
A	31.417	10:30 pm	7/16/87	2:00 pm	7/17/86	15.5 hrs.	4:10 pm	7/18/86	41.67 hrs.	
A	49.5	4:35 pm	7/17/86	8:15 pm	7/17/86	3.67 hrs.	2:20 pm	7/20/86	71.75 hrs.	
A	73.5	4:35 pm	7/18/86	1:25 pm	7/19/86	20.83 hrs.	3:10 pm	7/20/86	46.583 hrs.	
A	129.5	DNR	7/21/86	DNR	7/21/86	< 1 day	11:05 pm	7/21/86	< 1 day	
A	165	12:05 pm	7/22/86	4:50 pm	7/22/86	475 hrs.	12:05 am	7/23/86	12 hrs.	
U	0	1:55 pm	7/15/86	2:00 pm	7/15/86	5 mins.				
U	1.5	4:35 pm	7/15/86	12:55 pm	7/16/86	20.33 hrs.				
U	5	8:05 pm	7/15/86	1:15 pm	7/16/86	17.16 hrs.				
U	14.67	5:45 am	7/16/86	5:05 pm	7/16/86	11.33 hrs.				
U	49.5	4:35 pm	7/17/86	1:40 pm	7/18/86	21.08 hrs.				
U	74	5:05 pm	7/18/86	1:35 pm	7/20/86	44.5 hrs.				
U	165.5	12:30 pm	7/22/86	6:10 pm	7/22/86	5:07 hrs.				

Table A-5. Time Sequence -- Sampling and Bacteria Counts
August Experimental Run
Start Time: 2:55 p.m. (8/19/86)
DNR = did not record

Autoclaved (A) or Unautoclaved (U)	Inoculation Time Hours	Sampling Time	Sampling Date	Phase Contrast Count Time	Phase Contrast Count Date	Elapsed Time Between Sampling and Counting	Flourescence Count Time	Flourescence Count Date	Elapsed Time Between Sampling and Counting	Comment
A	0	1:35 pm	8/19/86	1:40 pm	8/19/86	5 min.	10:50 am	8/22/86	5 min.	Flourescence blank sample taken on 8/2
A	0.083	3:00 pm	8/19/86	3:30 pm	8/19/86	30 min.	DNR	DNR	DNR	
A	1.73	4:15 pm	8/19/86	5:00 pm	8/19/86	45 min.	9:35 am	8/22/86	65.33 hrs.	
A	2.75	5:40 pm	8/19/86	6:55 pm	8/19/86	1:25 hrs.	3:25 pm	8/21/86	45.75 hrs.	
A	4.75	7:40 pm	8/19/86	9:00 pm	8/19/86	1:33 hrs.	4:40 pm	8/21/86	45 hrs.	
A	7.67	10:35 pm	8/19/86	10:55 am	8/20/86	12.33 hrs.	11:10 am	8/22/86	60.58 hrs.	
A	14	4:55 am	8/20/86	12:05 pm	8/20/86	7.16 hrs.	11:35 am	8/22/86	54.67 hrs.	
A	23	1:55 pm	8/20/86	3:05 pm	8/20/86	1.16 hrs.	12:10 pm	8/22/86	46.25 hrs.	
A	31	9:55 pm	8/20/86	9:30 am	8/21/86	11.58 hrs.	12:35 pm	8/23/86	62.67 hrs.	
A	49.5	4:25 pm	8/21/86	DNR	8/22/86	1 day	DNR	DNR	DNR	
A	73.5	4:25 pm	8/22/86	9:00 pm	8/22/86	4.58 hrs.	DNR	DNR	DNR	
A	130	12:55 am	8/25/86	3:25 pm	8/25/86	14.5 hrs.	10:55 am	8/26/86	34 hrs.	
A	166	12:55 pm	8/26/86	9:30 pm	8/26/86	8:58 hrs.	3:55 pm	8/27/86	27 hrs.	
U	0	1:45 pm	8/19/86	1:50 pm	8/19/86	5 min.				
U	1.5	4:25 pm	8/19/86	4:15 pm	8/20/86	23.83 hrs.				
U	5	7:55 pm	8/19/86	DNR	8/20/86	1 day				
U	14.5	5:25 am	8/20/86	4:40 pm	8/20/86	11.25 hrs.				
U	31	9:55 pm	8/20/86	2:45 pm	8/21/86	16.83 hrs.				
U	49.5	4:25 pm	8/21/86	DNR	8/22/86	1 day				
U	73.5	4:25 pm	8/22/86	5:55 pm	8/23/86	25.5 hrs.				
U	130	12:55 am	8/25/86	DNR	8/25/86	1 day				
U	166	3:55 pm	8/26/86	10:55 pm	8/26/86	7 hrs.				

APPENDIX B. STEADY STATE CHEMOSTAT DATA

Prior to beginning experiments at a particular growth rate, it was necessary to bring the chemostat to a steady state condition. One rule of thumb often used is that a chemostat must be operated at least three times as long as the solids retention time to achieve stedy state operation. The timing of the experimental runs in relation to the days after start-up is shown in Table B-1. There are also a number of parameters which, taken together, give one a good idea of the condition of a methane-forming, anaerobic chemostat culture. The steady variables monitored in this study are listed below. They include:

a) effluent volume

b) temperature

c) bacteria concentration

d) volatile suspended solids

e) pH

f) feed total, effluent total, and effluent soluble chemical oxygen demand

g) gas composition, and

h) gas production rate

A brief discussion of these parameters is included below.

a) Effluent Volume - An experimenter determines the growth rate of chemostat by the rate that the mixed liquor of the chemostat (including the cells) are washed out of the reactor vessel. Thus the effluent volume measurement shows what volume of the mixed liquor is being washed through each day. A constant effluent volume lets the researcher know that a constant growth rae is being maintained.

Table B-1. Timing of Experimental Runs in Relation to Start-Up of Chemostat at a Particular Growth Rate.

Run #	Days	Dates	Day of Operation	Days After Start-Up at the Growth Rate Tested
1	20	4/1/86 - 4/8/86	482 - 489	482
2	20	5/21/86 - 5/28/86	532 - 539	532
3	20	6/1/86 - 6/8/86	543 - 550	543
4	8	7/15/86 - 7/22/86	587 - 594	24
5	8	8/19/86 - 8/26/86	623 - 630	60

b) Temperature - Temperature is an important factor influencing the metabolic rates of microorganisms. It should be kept as constant as possible.

c) Bacteria Concentration - Other workers have shown bacteria concentration in the bulk fluid has an influence on attachemnt. Bacteria concentration in a chemostat is influenced by feed strength and growth rate. In the set of experiments described in this study, the feed strength was altered to compensate for the different growth rates and to try to obtain the same organism concentration at the two growth raes. A chemostat operating at steady state has a constant bacteria concentration.

d) Volatile Suspended Solids - Volatile suspended solids is also a crude measure of the bacteria concentration.

e) pH - Properly operating methane-forming anaerobic digesters usually operate at a stable, neutral pH. Upsets usually result in a drop in the pH. If the pH drops below 6.5, the methane-forming consortium is in danger of being inhibited.

f) Chemical Oxygen Demand - Chemical oxygen demand (COD) is a measure of oxidizeable organic matter. Feed total COD gives a measure of organic strength of the feed. Effluent soluble COD gives a measure of the concentration of the limiting nutrient for growth (carbon) in the chemostat. Thus effluent soluble COD concentration determines the growth rate of the microorganisms. The feed total, effluent total, and effluent soluble COD should all be constant for a chemostat at steady state.

g) Gas Composition - The microorganisms in a methane-forming anaerobic chemostat produce large amounts of the gases methane and carbon

dioxide (see stoichiometry in the Methods and Materials section). The relative amounts of these gases (percentage in head space atmosphere) should remain fairly constant in a chemostat operating at steady state.

h) Gas Production Rate - The microorganisms in a chemostat operating at steady state should produce gas at a constant rate.

20 Day SRT - 0.05 Volumes Per Day (cell population doublings per day) Dilution Rate:

The experimental runs for the 20 day SRT/0.05 volumes per day dilution rate were carried out beginning on day 482 and day 532 of operation. The time frame of the experiments for the 20 day SRT in relation to monitoring of the steady state parameters is shown in Figures B-1 and B-2. All the parameters monitored were virtually constant for the 60 days (three times the SRT) prior to the beginning of the first experimental run. They stayed fairly constant once the experiments began also. Volatile suspended solids did show a slow gradual increase over the period from day 420 to day 560. Volatile suspended slides (VSS) were measured to provide a crude measure of the organism concentration. The direct count of microorganisms did not confirm this VSS increase. The difference between effluent total COD and effluent soluble COD, another crude measure of bacteria concentration, also did not confirm the VSS increase.

8 Day SRT - 0.125 Volumes Per Day (cell population doublings per day) Dilution Rate:

The operation of the 70 liter chemostat at a 20 day SRT was terminated after 559 days of operation. The reactor was drained and thoroughly cleaned and rinsed.

Figure B-1

Day Of Operation
Versus
Steady State Parameters
20 Day SRT - 0.05 Volumes/Day

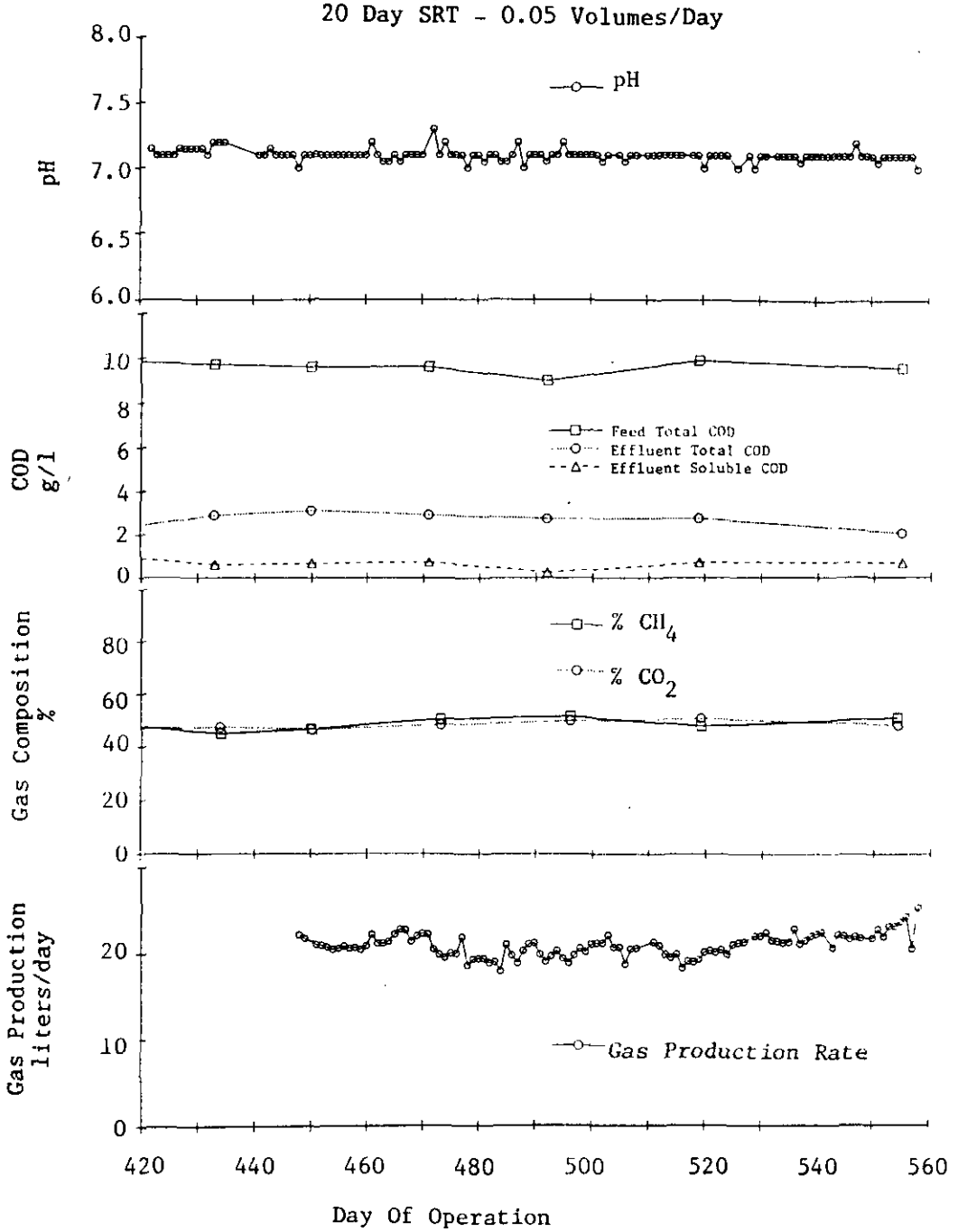
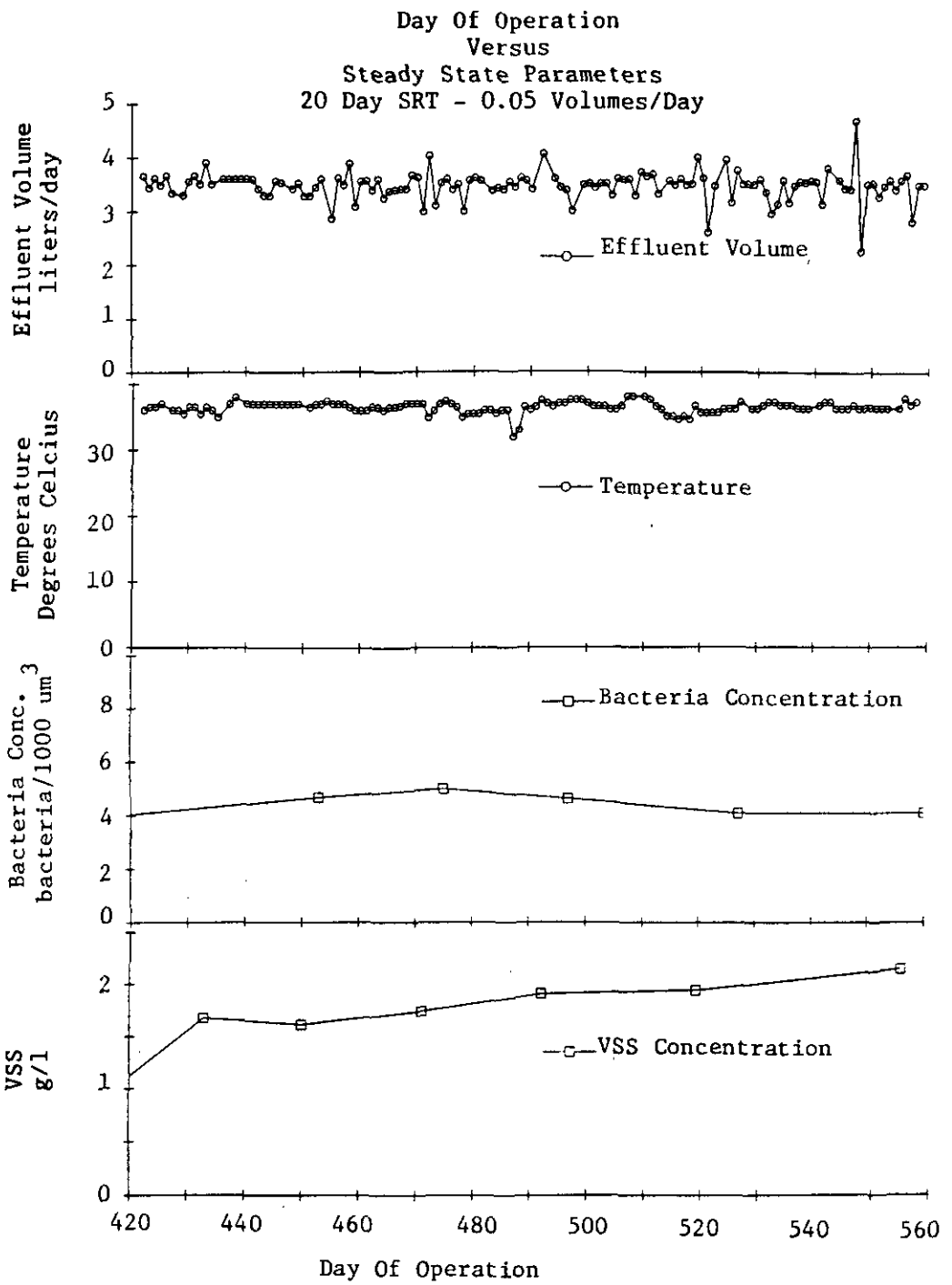


Figure B-2


Day Of Operation
Versus
Steady State Parameters
20 Day SRT - 0.05 Volumes/Day
Effluent Volume liters/day
Effluent Volume
Temperature Degrees Celcius
Temperature
Bacteria Conc. bacteria/1000 um 3
Bacteria Concentration
VSS g/l
VSS Concentration
Day Of Operation

On day 562 the 70 liter experimental reactor was restarted at the 8 day SRT. The inoculum used to restart the reactor was approximately 20 to 30 liters of effluent from the 15 liter, 8 day SRT inoculating reactor that had been saved from the previous two to three weeks. The remainder of the liquid added at the time of inoculation was the normal feed with sucrose omitted. Thus the ionic strength and nutrient concentrations of mixed liquor in the reactor vessel would be approximately equal to what was experienced in the inoculating reactor.

Two actions were taken during the first eleven days of operation to ease stress during the start-up. First, on the first two days after start-up, part of the influent volume to the experimental included the 1.875 liters of effluent from the 8 day SRT inculating reactor. This was done to help build up the cell population in the experimental reactor and to provide fresh organisms in case the ones in the reactor were under stress. Second, on days 566, 570, and 573, no sucrose was added to the feed. On each of these days the pH had dropped slightly and it was felt the population of acid formers might be growing faster and producing more acids than the methanogens could metabolize.

The steady state parameters for the 70 liter experimental reactor operating at an 8 day SRT are summarized in Figures B-3 and B-4. In the week prior to the start of the July experimental run on day 587, the reactor appeared to have achieved a steady state condition. Day 587 was 24 days (3 times the 8 day solids retention time) after the start-up of the reactor. The running seven previous day average gas production rate stabilized on day 582 after climbing progressively before that time. The COD and VSS levels measured on days 574, 582, and 585 were stable. VSS were somewhat lower

Figure B-3

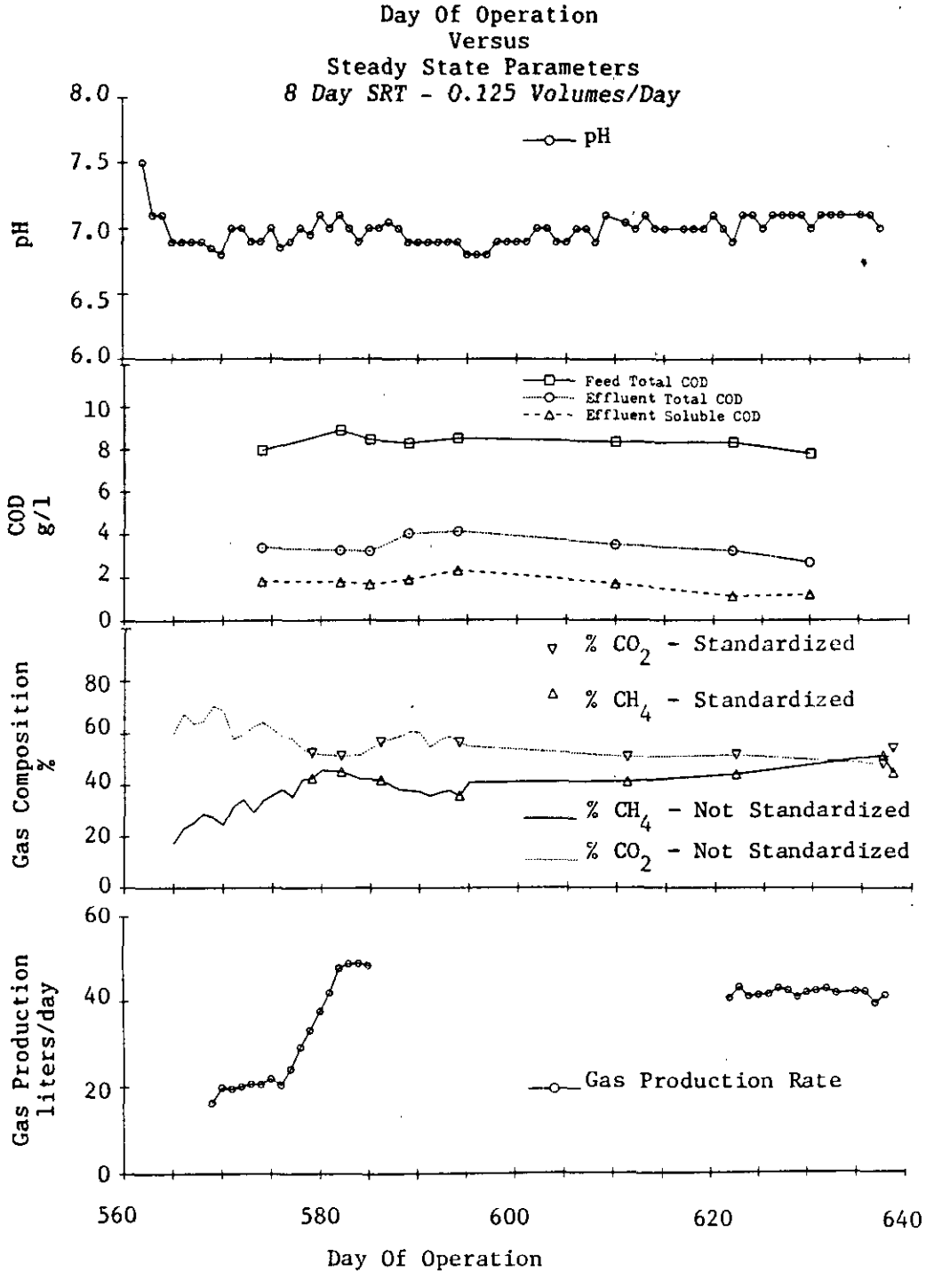
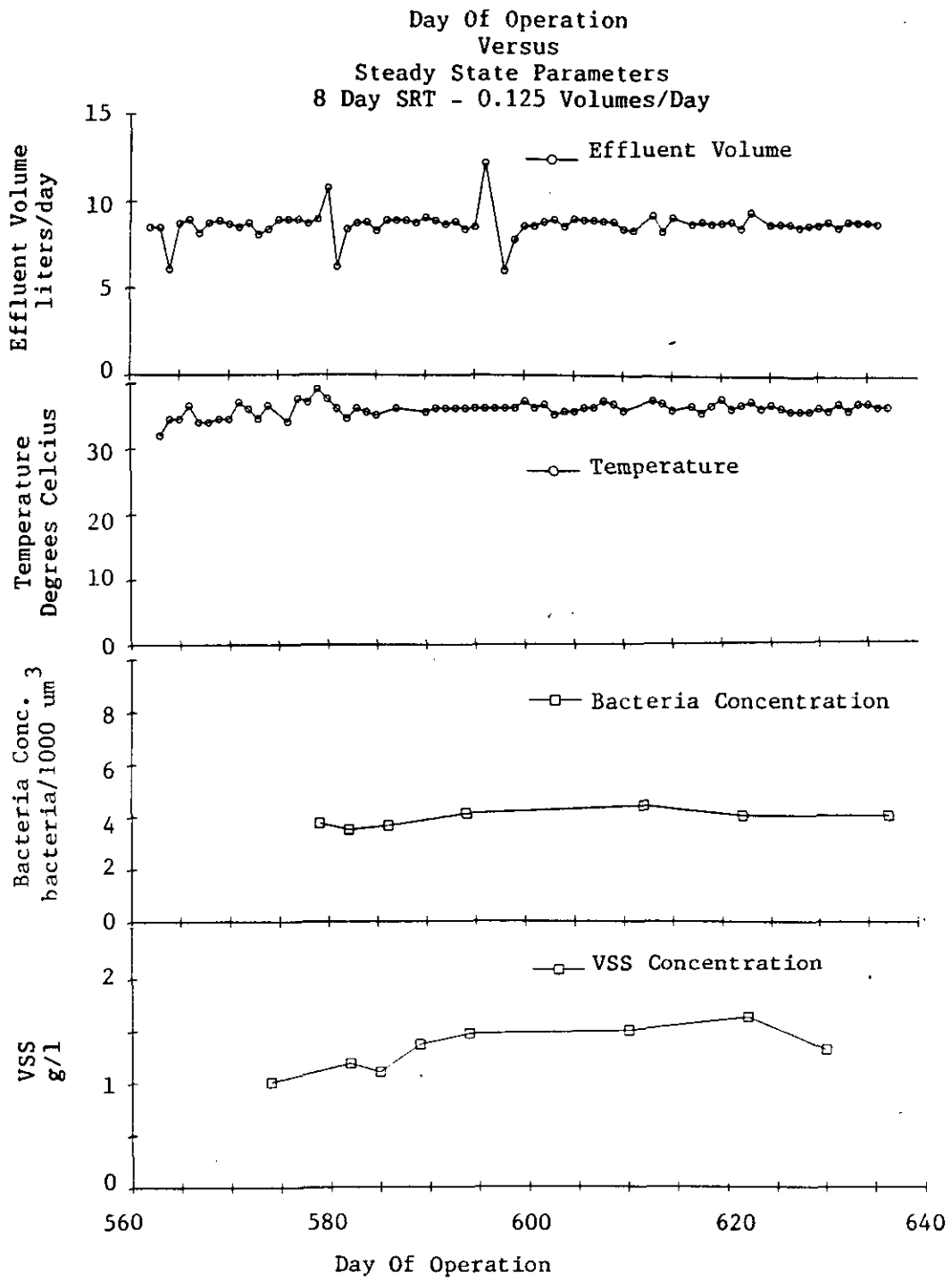

Day Of Operation
Versus
Steady State Parameters
8 Day SRT - 0.125 Volumes/Day
pH
8.0
7.5
7.0
6.5
6.0
pH
Feed Total COD
Effluent Total COD
Effluent Soluble COD
COD g/l
10
8
6
4
2
0
% CO2 - Standardized
% CH4 - Standardized
% CH4 - Not Standardized
% CO2 - Not Standardized
Gas Composition %
80
60
40
20
0
Gas Production liters/day
60
40
20
0
Gas Production Rate
560
580
600
620
640
Day Of Operation

Figure B-4


Day Of Operation
Versus
Steady State Parameters
8 Day SRT - 0.125 Volumes/Day
Effluent Volume
liters/day
Effluent Volume
Temperature
Degrees Celcius
Temperature
Bacteria Conc.
bacteria/1000 um 3
Bacteria Concentration
VSS
g/1
VSS Concentration
0
5
10
15
0
10
20
30
0
2
4
6
8
0
1
2
560
580
600
620
640
Day Of Operation

than the 20 day SRT steady state levels which implied the bacteria concentration might be lower for the 8 day SRT. However, direct counts of the bacteria concentration in the mixed liquor on days 579, 582, and 586 were stable and approximately equal to the counts for the 20 day SRT culture. It was felt the direct counts were a more reliable indicator of the bacteria concentration than the VSS measurement. The pH values of the mixed liquor were stable and ranged from 6.9 to 7.1, which was approximately the same as the 20 day SRT culture. The gas composition was measured on days 579, 582, and 586 and similar values were obtained. On days 579 and 582, the recycle pump was inadvertently left on for a few hours and the reactor temperature rose to 40 degrees celcius each time, but this did not seem to have a noticeable effect on the reactor. Also for an unexplained reason, the effluent volume was high on day 580 and low on day 581. Taken together, the effluent volume for the two days was normal. On day 586, a clamp was left on effluent gas line. This apparently caused a pressure buildup and a leak to occur becasue the gas production rate dropped from close to 50 liters/day to approximately 10 liters per day almost instantaneously. This mistake had been made before (leaving a gas clamp on a line causing a leak). On the earlier occasions it had not seemed to effect the reactor. It was expected that gas produced by the reactor would keep gas flowing out of the reactor system rather than letting oxygen in. Thus, it was decided to begin the 8 day SRT experiments. The first experimental run for the 8 day SRT took place on July 15, 1986, day 587.

During and after the July experimental run, there were some indications the reactor was slightly stressed. Beginning day 589, the pH dropped to 6.9 where it stayed until day 593 when it dropped further to 6.8. In the 3 measurements of gas composition prior to the experimental runs the percent

methane had ranged from 42 to 46% CH_4, and 51 to 57% CO_2. The measurement taken at the end of the experimental run (day 594) was 36% CH_4 and 57% CO_2. The three measurement of soluble COD before the run ranged between 1644-1793 mg/l. On day 589 the reading was 1898 mg/l. On day 594 the reading was 2283 mg/l. It was decided that if the August replicate run showed a large difference from the July run, the July run would have to be thrown out or thrown out and repeated. However, the data for the August experimental run was very similar to the July experimental run.

The final experimental run was carried out on day 623 to 630. By this time, all parameters indicated the reactors had rstabilized (Figures B-3 and B-4). The pH ranged fom 6.9 to 7.1. One day 622, the gas composition was measured at 44% CH_4. 52% CO_2. Effluent soluble COD was 1680 mg/l on day 608 and 1119 mg/l on day 620. The gas production rate was stable. The bacteria concentration was 4 per 1000 μm^3 or 4 x 10^9 per ml. On day 636, the feed pump was accidently not turned on. This probably effected the second to last gas composition reading on day 637. On day 637, before the feed pump was turned back on, the methane level showed a slight increase and the carbon dioxide level showed a slight drop. The unadded feed was then added in a batch and feed pump and timer hooked up with the next day's feed. On day 638, the gas composition was measured again and the methane and carbon dioxide levels had reurned to their previous values.

APPENDIX C. INFERENTIAL STATISTICS

1. Comparison of Attachment Curves at Two Different Growth Rates

Two methods of inferential statistics were used to compare bacterial attachment at the two growth rates. The first method was a repeated measures growth curve analysis. In this analysis the individual data points were compared with one another. The second method was a comparison of mean coefficients for a mathematical model that was used to describe the attachment curves. Each set of attachment data was described by the same mathematical model using three coefficients. The means of these coefficients for each growth rate were compared using a "t" test.

Repeated measures growth curves analyses are discussed in detail by Winer (43). The data summary for such an analysis is presented in Table C-1. The analysis of variance table is presented in Table C-2.

Such an analysis tested three hypotheses (see Figure C-1). The first hypothesis tests, as the hull hypothesis, whether the means of all the data points for a particular growth rate were equal to the means of all the data points for another growth rate. The alternative is they are not equal. The second hypothesis tested whether the sums of data points at each inoculation time point were equal. The alternative was they were not all equal. The third hypothesis tests whether the differences of the data points at each inoculation time were equal. In other words, the third hypothesis tests whether the two curves are parallel. The alternative was they were not parallel.

Table C-1. Repeated Measures Growth Curve Analysis
Comparison of Bacterial Attachment/Growth at Different Growth Rate
Computational Set-Up

p = # of SRT's = 2
q = # of inoculation times = 12
n = # of experimental runs/SRT = 2

Growth Rate	Run	Inoculation Time in Hours												
		0.0	0.08	1.33	2.75	4.67	7.67	14	23	32	49.5	73.5	165	
9 Day	July	0.25	42.03	112.71	157.65	245.91	249.27	211.1	231.59	204.24	287.6	209.12	433.26	2384.72
SRT	August	1.98	38.42	95.83	192.35	224.61	155.21	104.21	135.88	155.09	224.84	365.96	236.25	1930.6
20 Day	April	0.38	40.37	93.94	213.53	136.28	267.21	232.01	362.73	359.11	242.76	312.84	317.55	2578.71
SRT	May	3.4	117.61	153.82	175.61	234.93	223.24	158.35	257.66	306.8	323.59	370.93	413.75	2739.69
		6.01	238.43	456.3	739.14	841.73	894.9	705.67	987.86	1025.23	1078.79	1258.85	1400.81	G=9633.72

Summary

9 Day														
SRT		2.23	80.45	208.54	350	470.52	404.48	315.31	367.47	359.32	512.44	575.08	669.51	4315.37
20 Day														
SRT		3.78	157.98	247.76	389.14	371.21	490.45	390.35	620.39	665.9	566.35	683.77	731.3	5318.38
		6.01	238.43	456.3	739.14	841.73	894.9	705.67	987.86	1025.23	1078.79	1258.85	1400.81	G=9633.75

(1) $\frac{G^2}{npq} = \frac{(9633.72)^2}{(2)(12)(2)} = 1933511.7$

(2) $\Sigma x^2 = 2522342.6$

(3) $\frac{\Sigma A_i^2}{nq} = \frac{(4315.37)^2 + (5318.38)^2}{2\ (12)} = 1954482.7$

(4) $\frac{\Sigma B_j^2}{np} = \frac{9556471.9}{(2)(2)} = 2389120$

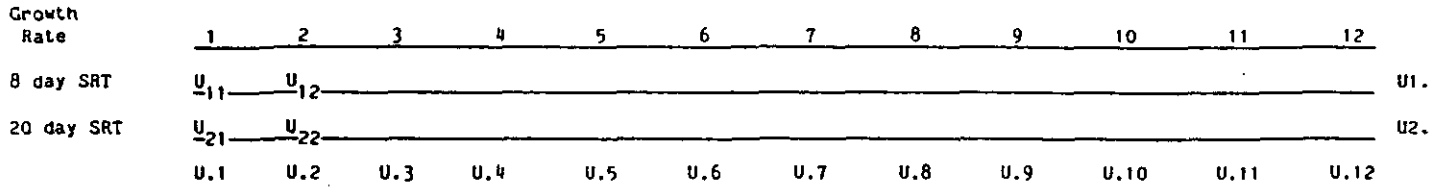
(5) $\frac{[\Sigma\ (^{AB}_{ij})^2]}{n} = \frac{4882478}{2} = 2441238$

(6) $\frac{(\Sigma Pk)^2}{q} = \frac{23569752.4}{12} = 1964146$

Table C-2. Repeated Measures Growth Curve Analysis
Comparison of Bacterial Attachment at Different Growth Rates
Analysis at Variance

Source of Variation	Computational Formula	SS	DF	MS	F.
Between Runs	(6)-(1)	30634.3	3		
A (growth rate)	(3)-(1)	20970.3	1	20970.3	4.34
Runs within growth rates	(6)-(3)	9664	2	4832	
Within Runs	(2)-(6)	558196.6	44		
B (inoculation time)	(4)-(1)	455608.3	11	41418.9	12.75
AB	(5)-(3)-(4)+(1)	31147.7	11	2831.6	0.87
Bx runs within growth rate	(2)-(5)-(6)+(3)	71440.6	22	3247.3	

Figure C-1. Schematic Representation of Hypothesis Testing of Repeated Measures Growth Curve Analysis Comparison of Bacterial Attachment at Different Growth Rates



Hypothesis 1 tested if U1. = U2. - Alternative U1. ≠ U2.

Hypothesis 2 tested if U.1 = U.2 = U.3 = U.4 . . . = U.12 - Alternative U.1 ≠ U.2 ≠ U.3 ≠ . . . U.12

Hypothesis 3 tested if $(U_{21} - U_{11}) = (U_{22} - U_{12}) = (U_{23} - U_{13}) \ldots = (U_{212} - U_{112})$

The hypothesis testing results for the repeated measures analysis are summarized in Table C-3 and Figure C-2. The hypothesis that the means of all he data points for each growth rate were equal was accepted. They hypothesis that the curves were parallel was accepted. They hypothesis that the means of the sums of all the data points at each inoculation time were equal was rejected. Thus, this would lead one to conclude that the curves for each growth rate are parallel, have the same mean value, but the means of their values for each time point change over time. In other words, the curves are the same curve and the value of the function changes over time.

Next, the "t" test analysis to compare attachment for the two microbial cultures growing at different growth rates was carried out by comparison of mathematical model coefficients. The first step was to attempt to find a meaningful mathematical model to describe the data. Two models were investigated. Both models included a sum of two values. One value of the sum described initial attachment and had a maximum value. The second value described the population growth after the cells have attached. Both models included a coefficient, A, which gives a plateau number of initially attached cells. Both models included a rate coefficient which gave one an idea how rapidly bacteria initially attach to the surface. Both models included a specific growth rate term, μ, which described exponential growth of the attached microbial population after attachment.

The mathematics of bacterial attachment in the first model is based on first order decay models and is analagous to the mathematics used in the development of the concepts of biochemical oxygen demand. For the attachment term:

Let A = the number of attachment sites available or remaining.

Then:

Table C-3. Summary of the Hypothesis Testing Results for the Repeated Measures Growth Curve Analysis - Comparison of Growth Rates

Null Hypothesis	α	Experimental F Value	Critical F Value	Accept or Reject Null Hypothesis
1	.05	4.33	18.51	Accept
2	.05	12.76	2.26	Reject
3	.05	0.87	2.26	Accept

Figure C-2 Schematic Summary Of Hypothesis Testing Results For The Repeated Measures Growth Curve Analysis - Comparison of Growth Rates.

		Inoculation Time Data Points					
		1	2	3		12	
Growth Rate	8 day SRT	U_{11}	U_{12}	U_{13}		U_{112}	U1.
	20 day SRT	U_{21}	U_{22}	U_{23}		U_{212}	U2.
		U.1	U.2	U.3		U.12	

Hypothesis 1 - U1. = U2. Accepted

Hypothesis 2 - U.1 = U.2 = U.3 ... U.12 Rejected

Hypothesis 3 - $U_{21} - U_{11} = U_{22} - U_{12} \cdots = U_{212} - U_{112}$ Accepted

$$\frac{dA}{dt} = kA \qquad \text{(C-1)}$$

that is, the rate that attachment sites disappear is directly proportional to the number of attachment sites remaining where

k = constant of proportionality -

$$\left\{\frac{\text{number of attachment sites disappearing/time}}{\text{number of attachment sites remaining}}\right\}$$

The equation can then be integrated:

$$\frac{1}{A}dA = kdt \qquad \text{(C-2)}$$

$$\int_{A_o}^{At} \frac{1}{A}\, dA = \int_{o}^{t} kdt \qquad \text{(C-3)}$$

$$\ln \frac{A_t}{A_o} = kt \qquad \text{(C-4)}$$

$$e^{kt} = \frac{A_t}{A_o} \qquad \text{(C-5)}$$

$$A_o\, e^{kt} = A_t \qquad \text{(C-6)}$$

where, A_o = total number of attachment sites, and

A_t = the number of attachment sites remaining at time t

$$Y = A_o - A_t \qquad \text{(C-7)}$$

$$A_t = A_o - Y \qquad \text{(C-8)}$$

$$A_o e^{kt} = A_o - Y \qquad \text{(C-9)}$$

substituting equation 6 into equation 7 yields equation 10

$$Y = A_o - A_o e^{kt} \qquad \text{(C-10)}$$

$$Y = A_o\,(1-e^{kt}) \qquad \text{(C-11)}$$

where, Y = number of bacteria attached at time t

Schematically, equations (1), (6), (7) and (11) can be represented as shown in Figure C-3.

For the growth term:

$$\frac{dY}{dt} = \mu Y \qquad \text{(C-12)}$$

where, μ = specific growth rate $\left\{\frac{\text{cells produced/time}}{\text{cells present}}\right\}$

$$\frac{1}{Y}\, dY = \mu dt \qquad \text{(C-13)}$$

$$\int_{Y_o}^{Y_t} \frac{1}{Y}\, dY = \int_{o}^{t} \mu dt \qquad \text{(C-14)}$$

$$\Big]_{Y_o}^{Y} \ln Y = \Big]_{o}^{t} \mu t \qquad \text{(C-15)}$$

$$\ln Y - \ln Y_o = \mu t \qquad \text{(C-16)}$$

$$\ln \frac{Y}{Y_o} = \mu t \qquad \text{(C-17)}$$

$$e^{\mu t} = \frac{Y}{Y_o} \qquad \text{(C-18)}$$

$$Y_o e^{\mu t} = Y \qquad \text{(C-19)}$$

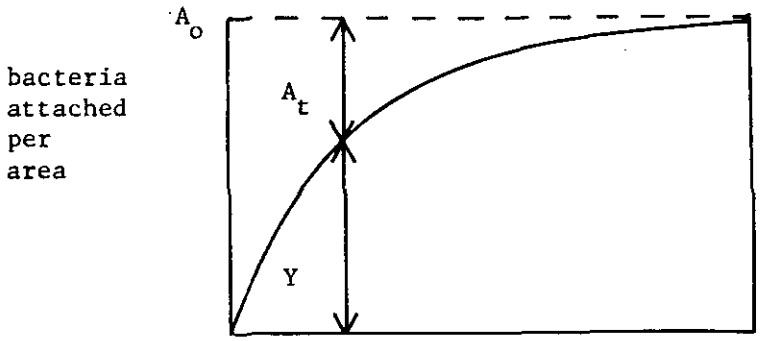
where, $Y = Y_t$ = number of bacteria attached at time t per area, and

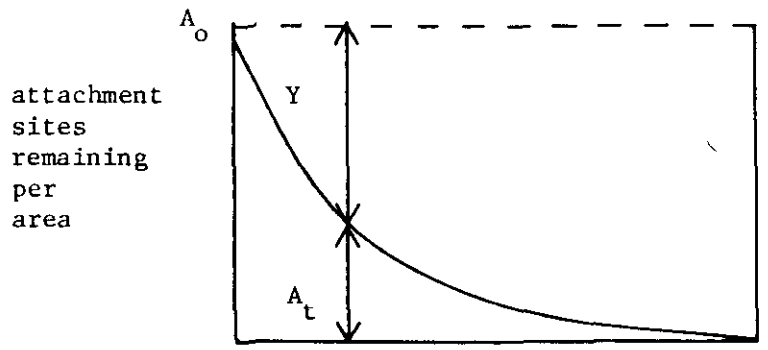
Y_o = number of bacteria attached at time 0 per area

Thus when the growth and attachment terms are combined (with slight modification) the following equation is obtained:

$$Y = \underset{\text{attachment}}{A_o\,(1 - e^{kt})} + \underset{\text{growth}}{A_o\,(e^{\mu t} - 1)} \qquad \text{(C-20)}$$

One noticeable simplifying assumption is made here. It is that the maximum number of cells very rapidly attach to the surface. The growth term assumes that from time 0, the maximum number of cells have attached





Y = number of bacteria attached at time t
A_t = number of attachment sites remaining at time t
A_o = total attachment sites or maximun number of bacteria initially attached
$A_o - A_t = Y$

Figure C-3
Schematic - First Order Attachment Model

their growth is beginning at time = 0. This is not exactly the case. It takes a few hours at least for the concentration of cells on the surface to reach its maximum. However, the growth rate of the bacteria is so slow, it seemed a reasonable simplifying assumption to make. Also, the clarify terminology, Y_o of equation (19) becomes A_o in equation (20). A° is subtracted from the growth term because the initially attached cells are accounted for in the attachment term.

The mathematics of bacterial attachment in the second model is similar to the equation used in Michaelis-Menten enzyme kinetics, Monod bacterial growth, and Langmuir adsorption isotherms.

Let:

A_o = the maximum number of bacteria that can initially attach to the surface per area

K_m = time it takes for bacterial concentration on the surface to reach, $A_o/2$ one half the maximum concentration

Y = the number of bacteria attached per area at time t, and

t = inoculation time

Then:

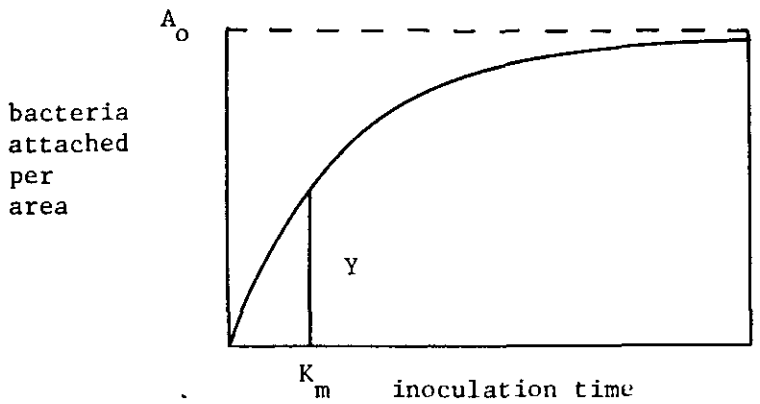
$$Y = \frac{A_o\, t}{t + Km} \qquad \text{(C-21)}$$

Schematically, equation (21) can be represented as shown in Figure C-4.

When the growth term from equation (19) is combined with the attachment term of equation (21), equation (22) is obtained.

$$Y = \underbrace{\frac{A_o\, t}{t + Km}}_{\text{attachment}} + \underbrace{A_o\,(e^{\mu t} - 1)}_{\text{growth}} \qquad \text{(C-22)}$$

The same simplifying assumption that the maximum number of cells is attached at time = 0 is made. Again to clarify terminology Y_o at equation (19)



Y = number of bacteria attached at time t
A_o = total attachment sites or maximum number of bacteria initially attached
K_m = inoculation time when $Y = A_o/2$

Figure C-4

Schematic - Michaelis-Menten Type Attachment Model

becomes A_0 in equation (22). Also, A° is subtracted from the growth term because initially attached cells are accounted for in the attachment term.

It was decided to pursue the inferential statistical analysis using the first order rate model to model the attachment curves. The other model is very similar as is shown in the descriptive statistics section. The data set that was used for the first order model was for cocci > 0.6 μm + noncocci versus inoculation time. A regression analysis was performed, the best fit (minimum residual sum of squares) was obtained, and the three parameters, A_0, K, and μ were used to describe the curves.

In addition, because the variance of the attachment counts increased with time and the number of bacteria attached to surface increased, a "weighted" fit to the data was also carried out. For a "weighted" fit, each component of each sum of squares term is multiplied by a "weighting" factor when computing the sum of squares. The weighting factor, W_i, equals

$$W_i = \frac{1}{\delta^2} \qquad (C\text{-}23)$$

the inverse of the variance. Thus, data points which have a high variance get a low weight when computing the sum of squares. Conversely data points with a low variance receive a high weight when computing the sum of squares. The implications for this study are that data points at the earlier inoculation times would receive a higher weight in determining the regression curve. The parameters that were determined for the "weighted" and "unweighted" fits are summarized in Table C-4.

On a theoretical basis, the weighting was not done in a completely justifiable way. The weights that were used were determined using the measured variance of the bacteria counts on a particular slide. The variance, in truth, was contributed to by four sources.

Table C-4. Least Squares Regression Curve Coefficients
First Order Attachment Model Including Growth Term
Unweighted and Weighted Analyses

	Growth Rate	Run #	A	K	μ	R^2	Std Dev of Reg	F	Significance Level
Unweighted	20 Day SRT	1	303.09	-0.24	0.000214	.964	53.6	80.43	.0001
	20 Day SRT	2	233.32	-0.80	0.004123	.970	53.9	106.9	.0001
	Means		268.2	-0.52	0.002169				
	8 Day SRT	1	206.71	-0.666	0.00389	.980	38.11	159.4	.0001
	8 Day SRT	2	175.70	-0.913	0.00292	.917	63.72	36.71	.001
	Means		191.2	-0.79	0.00341				
Weighted	20 Day SRT	1	251.12	-0.37	0.001882	.896	4.59	25.71	.001
	20 Day SRT	2	229.43	-0.885	0.004685	.962	18.95	84.32	.0001
	Means		240.28	-0.628	0.003284				
	8 Day SRT	1	214.8	-0.56	0.003523	.977	1.31	143.5	.0001
	8 Day SRT	2	160.26	-1.040	0.002994	.926	11.98	41.48	.0001
	Means		187.53	-0.8	0.003259				

$$\sigma_T^2 = \underset{\substack{\text{physical act of counting differences}}}{\sigma^2} + \underset{\substack{\text{slide to slide differences}}}{\sigma^2} + \underset{\substack{\text{field to field differences on a slide}}}{\sigma^2} + \underset{\substack{\text{random error differences}}}{\sigma^2} \qquad \text{(C-24)}$$

The sources were the physical act of counting, the use of different slides for each count, the use of different fields on a slide, and random error. Only the variance associated with the use of different fields on a slide, the physical act of counting, and random error can be estimated given the data that was collected. No attempt was made to estimate the other source of variance, slide to slide differences. The weights were determined using only the variance associated with field to field differences, the physical act of counting differences, and random error. If these sources of variance were the major sources, then the weights were a valid concept to use. Given the high field to field differences, it may indeed be true that these were the largest contributor to the variance.

The coefficients were compared using a "t" test. The "t" test was carried out two ways. One test considered all three parameters simultaneously. The second test considered the parameters independently. The test which considers the three coefficients simultaneously tests if the two curves are equal. In statistical terminology:

H_0: $\overline{A_{20}} = \overline{A_8}$ A: $\overline{A_{20}} \neq A_8$

$\overline{K_{20}} = \overline{K_8}$ $\overline{K_{20}} \neq \overline{K_8}$

$\overline{U_{20}} = \overline{U_8}$ $\overline{U_{20}} \neq \overline{U_8}$

All three hypotheses must be accepted in order for the null hypothesis to be accepted. If any one of the subhypotheses was rejected, the entire hypothesis would have to be rejected. In determining the critical value of the test statistic, the α values (significance levels) using the terminology

of Montgomery (31) were divided by 2 to account for the two-sided nature of the alternative hypotheses, and also divided by 3 to account for the three simultaneous hypotheses being tested. Thus, the α value was divided by 6 to determine the critical "t" value.

The second method of testing the coefficients is to simply test them independently. Here each null hypothesis is considered as its own separate test with a two sided alternative. Accepting or rejecting a particular hypothesis has no bearing on whether one accepts or rejects the other hypotheses. In statistical notation

$$H_0: \quad \overline{A_{20}} = \overline{A_8} \qquad\qquad A: \quad \overline{A_{20}} \neq \overline{A_8}$$
$$\overline{K_{20}} = \overline{K_8} \qquad\qquad \overline{K_{20}} \neq \overline{K_8}$$
$$\overline{U_{20}} = \overline{U_8} \qquad\qquad \overline{U_{20}} \neq \overline{U_8}$$

The α value used to determine the critical value of the test statistic was $\alpha/_2$.

The results of the simultaneous t test for the parameters determined in the unweighted analysis are summarized in the top portion of Table C-5. All three hypotheses were accepted. For two of the parameters, k and u, the descriptive level of the test (the probability of obtaining a result as extreme as the one that was obtained) was high. For A, the descriptive level of the test was lower but still reasonable for $\overline{A_{20}} = \overline{A_8}$. Thus the hypothesis that the two curves for the two different growth rates were essentially equal was accepted.

The hypotheses testing the equality of the coefficients were also carried out considering the coefficients independently (Table C-5). In such an analysis, the coefficients were determined in the same way as the simultaneous analysis using a regression analysis. However, each hypothesis was then considered separately. Because the hypotheses were considered

Table C-5 Summary Of t Test Comparison Of Least Squares Regression Curve Coefficients- Hypotheses Considered Simultaneously And Independently. Unweighted Analysis.

	H_o	α	t calculated	t critical	accept or reject	descriptive level	94% confidence interval	Probability of a Type II Error $\alpha = 0.05$
Hypotheses considered simultaneously	$\bar{A}_{20} = \bar{A}_8$	.06	2.02	6.965	accept	.187	$-188.9 \leq \bar{A}_{20} - \bar{A}_8 \leq 342.9$	
	$\bar{k}_{20} = \bar{k}_8$	.06	0.882	6.965	accept	.482	$-1.86 \leq \bar{k}_{20} - \bar{k}_8 \leq 2.40$	
	$\bar{\mu}_{20} = \bar{\mu}_8$	.06	-0.61	6.965	accept	.62	$-0.0123 \leq \mu_{20} - \mu_8 \leq 0.0927$	
							95% confidence interval	
Hypotheses considered independently	$\bar{A}_{20} = \bar{A}_8$	.05	2.02	4.303	accept	0.187	$-87.29 \leq \bar{A}_{20} \quad \bar{A}_8 \leq 241.2$	0.90
	$\bar{k}_{20} = \bar{k}_8$	.05	0.882	4.303	accept	0.482	$-1.047 \leq \bar{k}_{20} - \bar{k}_8 \leq 1.587$	0.95
	$\bar{\mu}_{20} = \bar{\mu}_8$	.05	-0.61	4.303	accept	.62	$-0.0099 \leq \mu_{20} - \mu_8 \leq .007441$	0.90

separately, critical value of the "t" statistic for rejecting the null hypothesis was the $\alpha/_2$ "t" value instead of the $\alpha/_6$ "t" value. Each hypothesis was still accepted.

The probability of a Type II error (failing to reject the null hypothesis that the coefficients are equal when they truly are not equal) is high. The values were estimated for the independent hypothesis cases and are included in Table C-5. The values were estimated from Montgomery (31) (p. 25). The values ranged from 0.9 to 0.95 which means there is a 70% to 95% probability and one would fail to reject the null hypothesis that the coefficients are equal if they are truly different. There are three ways the probability of a Type II error can be reduced. The probability of a Type II error would be reduced if: (1) the number of experimental runs was increased, (2) the standard deviation of the coefficient values determined for the different runs was reduced, or (3) if the difference between the coefficient means was increased. For this experiment, the researcher only can easily control the first of these parameters. But just to increase the number of runs would require a substantial effort (beyond the scope of this study).

As was stated earlier, because the variance of the bacteria counts increased at long inoculation times and high bacteria counts, the curve coefficients were also determined using a weighted analysis. The coefficients determined by the weighted and unweighted analyses are available in Table C-6. The weighted coefficients for the two growth rates were compared using a "t" test in the same methods the unweighted coefficients were compared. No dramatic changes were observed in the weighted analysis compared to the unweighted analysis. However, it should be noted that the descriptive level of the "t" test comparison of mean

Table C-6 **Summary Of t Test Comparison Of Least Squares Regression Curve Coefficients[4] Hypotheses Considered Simultaneously and Independently. Weighted Analyses.**

	H_o	α	t calculated	t critical	accept or reject	descriptive level	94% confidence interval	probability of a type II error α = 0.05
Hypotheses	$\tilde{A}_{20} = \tilde{A}_8$	.06	1.79	6.965	accept	.2252	$-151.6 \leq \tilde{A}_{20} - \tilde{A}_8 \leq 257.1$	
considered	$\tilde{k}_{20} = \tilde{k}_8$	.06	0.49	6.965	accept	.69	$-2.266 \leq \tilde{k}_{20} - \tilde{k}_8 \leq 2.61$	
simultaneously	$\mu_{20} = \mu_8$	.06	0.0175	6.965	accept	> 0.8	$-0.009907 \leq \tilde{\mu}_{20} - \tilde{\mu}_8 \leq .009957$	
							95% confidence interval	
Hypotheses	$\tilde{A}_{20} = \tilde{A}_8$	.05	1.79	4.303	accept	.2252	$-73.5 \leq \tilde{A}_{20} - \tilde{A}_8 \leq 179.0$	0.90
considered	$\tilde{k}_{20} = \tilde{k}_8$	.05	0.49	4.303	accept	0.69	$-1.334 \leq \tilde{k}_{20} - \tilde{k}_8 \leq 1.678$	0.95
independently	$\tilde{\mu}_{20} = \tilde{\mu}_8$	.05	0.0175	4.303	accept	> 0.8	$-0.006111 \leq \tilde{\mu}_{20} - \tilde{\mu}_8 \leq .00616$	0.95

coefficients was higher for all three coefficients in the weighted analysis. Thus the weighted analysis indicated it was more likely the mean coefficient values were equal than the unweighted analysis. The same results were obtained in the hypothesis testing when comparing the weighted and unweighted analyses. The hypothesis that the two attachment curves at the two different growth rates were the same has accepted. The hypotheses that each individual coefficient were the same across the two growth rates were also accepted.

2. Comparison of Attachment on Autoclaved vs Unautoclaved Slides

This was not an experiment that was originally planned. Between the April and May (1986) experimental runs, the scanning electron microscopy (SEM) work associated with this experiment was commencing. It was noticed while making some preparations for SEM work that there was very little attachment on glass that was not autoclaved. At this time, it was recalled that during the intial preliminary attachment test runs, there was also very little attachment on unautoclaved slides. So some unautoclaved slides were installed in the attachment vessel for the May experimental run. During the June experimental run for SEM work, unautoclaved slides were again installed to obtain replicate run for the 20 day SRT. For the July and August (1986) experimental runs, unautoclaved slides were included in the attachment vessel simultaneously with the autoclaved slides.

The ad-hoc experimental set-up for autoclaved versus unautoclaved slides posed some problems for the use of inferential statistics. The experimental design is shown in the schematic Figure C-5. For the May, July, and August (1986) experimental runs, paired observations were obtained. The slides were in the same attachment vessel at the same time and were removed as close as was physically possible by one person. Hence, these observations were not independent of each other. However, it could be argued that for the April and June experimental runs, the observations were not carried out simultaneously and thus are independent. This line of reasoning would continue that for the 20 day SRT culture, there was not a true paired replicate experiment carried out. Accordingly, a different method of analysis was used to compare attachment on autoclaved and unautoclaved slides for each growth rate. For the 20 day SRT growth rate (the April, May, and June experimental runs), a repeated measures growth curve analysis was used. For the 8 day SRT (the July and August experimental runs), a randomized complete block design as described by Montgomery (31) was used. For both growth rates, a "t" test was used to compare the means of the differences between autoclaved and unautoclaved slides.

In the randomized complete block design for the 8 day SRT runs, the analytical set-up is shown in Figure C-6. In order to evaluate the data, for each of the July and August runs, the differences between the number of attached bacteria for autoclaved and unautoclaved slides were calculated (Table C-7). The differences were then used to carry out the statistical analysis (Table C-8 and C-9). The differences for the July

Figure C-5. Experimental Design
Statistical Perspective Comparison of the Effect of Slide Preparation on Bacterial Attachment

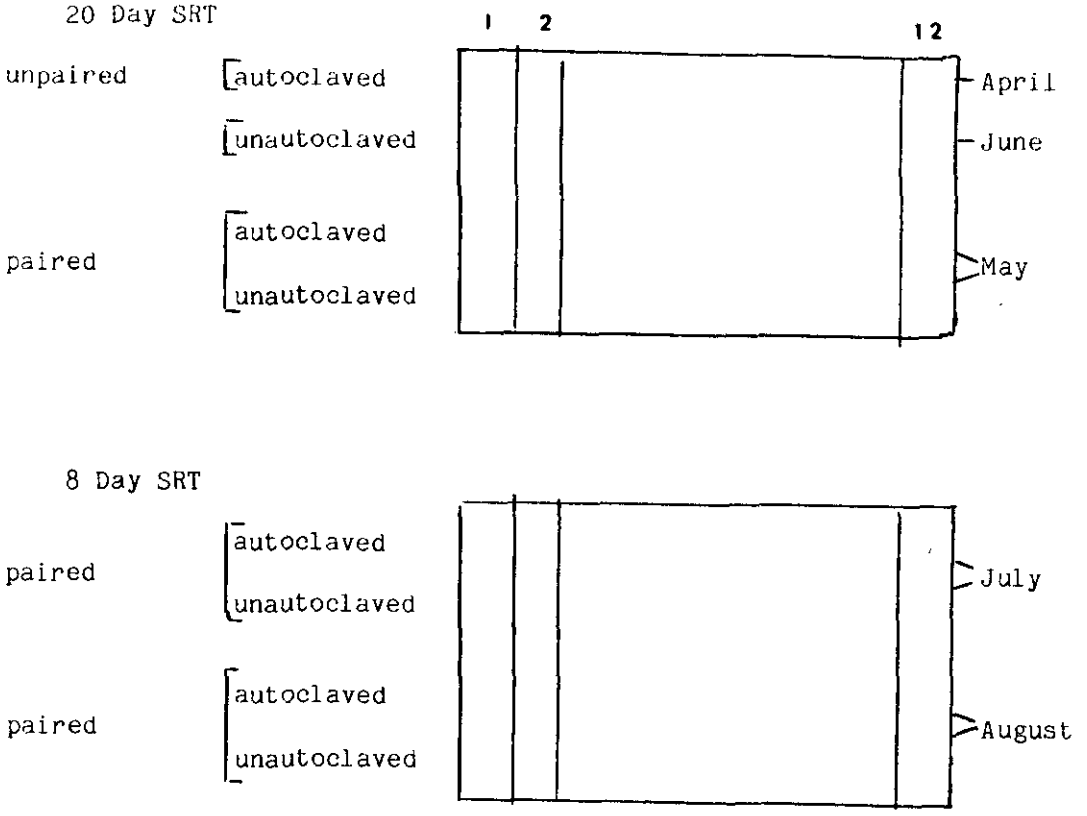


Figure C-6. Randomized Complete Block Design to Compare the Effect of Slide Preparation on Bacterial Attachment - 8 Day SRT

N = ab = total number of differences = 14

July (autoclaved minus unautoclaved)

August (autoclaved minus unautoclaved)

Blocks (b = 2)

Treatments (a=7) Inoculation Times	inoculation times-hours	Block 1	Block 2	Yi.	$\bar{\bar{Y}}i.$
	0	Y11	Y12	Y1.	$\bar{\bar{Y}}1.$
	1.33	Y21	Y22	Y2.	$\bar{\bar{Y}}2.$
	4.67	Y31	Y32	Y3.	$\bar{\bar{Y}}3.$
	14	Y41	Y42	Y4.	$\bar{\bar{Y}}4.$
	49.5	Y51	Y52	Y5.	$\bar{\bar{Y}}5.$
	73	Y61	Y62	Y6.	$\bar{\bar{Y}}6.$
	165	Y71	Y72	Y7.	$\bar{\bar{Y}}7.$
	Y.j	Y.1	Y.2	Y..	$\bar{\bar{Y}}..$

Yi, j = autoclaved - unautoclaved for block i, treatment j

$Yi. = \sum_{j=1}^{2} Yi, j$ - sum of the differences at each inoculation time

$Y.j = \sum_{i=1}^{7} Yi, j$ - sum of the difference for each entire block

$Y.. = \sum_{i=1}^{7}\sum_{j=1}^{2} Yi, j = \sum_{i=i}^{a} Yi. = \sum_{j=1}^{b} Y.j$ - sum of all the differences

$Y.. = \frac{Y..}{N} = \frac{Y..}{14}$ = average of all the difference

$\bar{\bar{Y}}i. = \frac{Yi.}{2}$ - average difference at each inoculation time

Table C-7. Calculations of Differences in Bacterial Attachment/Growth from Slide Preparation at Each Inoculation Time - 8 Day SRT

July

inoculation time-hours	cocci > o.6 um + noncocci Slides W, A (A)	cocci > 0.6 um + noncocci Slides W, U (U)	differences A - U
0	0.25	0.79	-0.54
1.33	112.71	2.56	110.15
4.67	245.91	1.28	244.63
14	211.10	177.22	33.88
49.5	287.6	9.31	278.29
73	209.12	5.71	203.41
165	433.26	10.13	423.13

August

inoculation time-hours	cocci > o.6 um + noncocci Slides W, A (A)	cocci > 0.6 um + noncocci Slides W, U (U)	differences A - U
0	1.98	7.57	-5.59
1.33	95.83	2.56	93.27
4.67	224.61	11.18	213.43
14.67	164.21	2.79	101.42
54.5	224.84	7.92	216.89
73.5	365.96	6.64	359.32
165	236.25	10.01	226.24

Table C-8. Randomized Complete Block Analysis Calculations - 8 Day SRT

a = 7
b = 2
N = 14

		July A-U	August A-U		
		Blocks b = 2			
	Inoculation Times-Hours	Block 1	Block 2	Yi.	$\bar{Y}i.$
Treatments	0	-0.54	-5.59	-6.13	-3.065
a = 7	1.33	110.15	93.27	203.42	101.71
	4.67	244.63	213.43	458.06	229.03
Inoculation	14.33	33.88	101.42	135.30	67.65
Times	52	278.29	216.89	495.18	247.59
	73.25	203.41	359.32	562.73	281.37
	165	423.13	226.24	649.37	324.69
	Y.j	1292.95	1204.98	2497.9	178.42
				Y..	$\bar{Y}$..

Table C-9. Randomized Complete Block Analysis at Variance - 8 Day SRT

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F_o	Descriptive Level
Treatments	180867	6	30144.5	5.05	.04265
Blocks	563.4	1	563.4		
Error	35781.1	6	5963.5		
Total	217211.5	13			

Critical Value

$F_{.05,\ 6,\ 6} = 4.28$

Reject null hypothsis - The differences, A - U, are not constant over time. This implies the two curves representing autoclaved and unautoclaved slides are different.

run composed Block 1. The differences for the August run made up Block 2. The counts at different inoculation times were considered the treatments. In this statistical set-up, the null hypothesis was that the means of the differences of all the inoculation times were equal

H_o: $\overline{Y1.} = \overline{Y2.} = \overline{Y3.} = \overline{Y4.} = \overline{Y5.} = \overline{Y6.} = \overline{Y7.}$

A_o: they are not all equal

Seeing both curves start at the same place, where the difference between autoclaved and unautoclaved is zero, the null hypothesis really asked if the two curves were the same curve. The alternative to the null hypothesis would be that the curves were different.

The analysis at variance table is summarized in Table C-9. The calculated F value is 5.05. The critical F value at a significance level $\alpha = .05$ is 4.28. This would lead one to reject the null hypothesis that all the differences are equal. As was discussed in the preceding paragraph, the null hypothesis implies that the two curves are identical. Rejecting the null hypothesis implies the curves are not identical and the mean differences between autoclaved and unautoclaved slides are not all equal. Thus the bacteria attach in a different pattern on autoclaved versus unautoclaved. By inspection of the graphs, it is clear the more bacteria attach overall and they attach at a faster initial or autoclaved slides versus unautoclaved slides.

The same repeated measures growth analysis method that was used to compared attachment at different growth rates was used to compare the unpaired data for autoclaved versus unautoclaved slides at the 20 day SRT. The data summary for the analysis is presented in Table C-10. The analysis of variance table is presented in Table C-11.

Table C-10. Repeated Measures Growth Curve Analysis to Compare Autoclaved Versus Unautoclaved Slides

p = slide preps = 2 $\quad q = t = 5 \quad n = \frac{\#\text{ of runs}}{\text{slide prep}} = 2$

Slide Prep	Run	Inoculation Times 0	1.25	4.67	14.0	73.5	Total
Autoclaved	May	3.40	153.82	234.93	158.35	370.93	921.43
$\Sigma x^2 = 241528$							
Autoclaved	April	0.38	93.94	136.28	232.01	312.84	775.45
$\Sigma x^2 = 179094.6$							
Unautoclaved	May	1.79	12.83	24.62	22.64	58.76	120.64
$\Sigma x^2 = 4739.3$							
Unautoclaved	June	2.17	12.07	101.1	71.1	65.83	252.27
$\Sigma x^2 = 19760.4$							

SUMMARY

Autoclaved	3.78	247.76	371.21	390.36	683.77	1696.88
$\Sigma x^2 = 819118.5$						
Unautoclaved	3.96	24.9	125.72	93.74	124.59	372.91
$\Sigma x^2 = 43342.87$	7.74	272.66	496.93	484.10	808.36	2069.79 = G

(1) $\frac{G^2}{npq} = \frac{(2069.79)^2}{(2)(5)(2)} = 214201.53$

(2) $\Sigma x^2 = \quad = 445122.3$

(3) $\frac{\Sigma A_i^2}{nq} = \frac{(1696.88)^2 + (372.91)^2}{(2)(5)} = 301846.36$

(4) $\frac{\Sigma B_j^2}{np} = \frac{1209141.5}{(2)(2)} = 302285.4$

(5) $\frac{\Sigma (AB_{ij})^2}{n} = \frac{862461.4}{2} = 431230.7$

(6) $\frac{(\Sigma P_k)^2}{q} = \frac{1528550.1}{5} = 305710$

Table C-11. Repeated Measures Growth Curve Analysis to Compare Autoclaved Versus Unautoclaved Slides - Analysis of Variance

Source of Variation	Computational Formula	SS	df	MS	F
Between runs	(6)-(1)	91508.47	3		
A (slide prep)	(3)-(1)	87644.83	1	87644.83	45.34
runs within slide prep	(6)-(3)	3863.64	2	1931.82	
Within runs	(2)-(6)	139412.3	16		
B (inoc. time)	(4)-(1)	88083.87	4	22020.97	17.57
AB	(5)-(3)-(4)+(1)	41300.5	4	10325.1	8.24
B x runs within slide prep	(2)-(5)-(6)+(3)	10027.96	8	1253.5	

The analysis tests three hypotheses (Figure C-7). The first hypothesis tests, as the null hypothesis, that the means of all the data points for a particular slide preparation are equal to the mean of all the data points for the other slide preparation. The alternative is they are not equal. The second hypothesis tests whether the sum of the data points at each inoculation time point are equal. The alternative is they are not all equal. The third hypothesis tests whether the differences of the data points at each inoculation time are equal. In other words, the third hypothesis tests whether the two curves are parallel. The alternative is they are not.

The hypothesis testing results for the repeated measures analysis are summarized in Figure C-8 and Table C-12. The hypothesis that the means of all the data points for each slide preparation were equal was rejected. The hypothesis that the means of the sums of all the data points at each inoculation time were equal was rejected. The hypothesis that the curves were parallel was rejected. These results lead one to conclude that the two sets of data are very different. The curves are not parallel, their overall mean values are different, and their values change over time.

The comparison of attachment of cocci > 0.6 μm + noncocci on autoclaved versus unautoclaved slides was also carried out using a "t" test. The mean attachment counts for autoclaved and unautoclaved slides were calculated at each inoculation time. The 8 day SRT data is included in Table C-13. The 20 day SRT data is included in Table C-14. The mean number of attached cells at each inoculation time was then compared for autoclaved versus unautoclaved slides using a "t" test. The results for the 8 day SRT are shown in Table C-15. The results of the 20 day SRT are shown in Table C-16.

Figure C-7 Schematic Representation Of Hypothesis Testing For The Effect Of Slide Preparation On Bacterial Attachment - Repeated Measures Growth Curve Analysis - 20 Day SRT.

slide treatment	inoculation time 1	2	3	4	5	
autoclaved	U_{11}	U_{12}	U_{13}	U_{14}	U_{15}	U1.
unautoclaved	U_{21}	U_{22}	U_{23}	U_{24}	U_{25}	U2.
	U.1	U.2	U.3	U.4	U.5	

Hypothesis 1 tests if U1. = U2. alternative U1. ≠ U2.

Hypothesis 2 tests if U.1 = U.2 = U.3 = U.4 = U.5

Hypothesis 3 tests if $(U_{21} - U_{11}) = (U_{22} - U_{12}) = (U_{23} - U_{13})...$ $(U_{25} - U_{15})$

alternative $(U_{21} - U_{11}) \neq (U_{22} - U_{12}) ... \neq (U_{25} - U_{15})$

Figure C-8 Schematic Summary Of Hypothesis Testing Results For The Repeated Measures Growth Curve Analysis - Comparison Of The Effect Of Slide Preparation On Bacterial Attachment - 20 Day SRT.

Slide Preparation	Inoculation Time Data Points 1	2	3	4	5	
Autoclaved	U_{11}	U_{12}	U_{13}	U_{14}	U_{15}	U1.
Unautoclaved	U_{21}	U_{22}	U_{23}	U_{24}	U_{25}	U2.
	U.1	U.2	U.3	U.4	U.5	

Hypothesis 1 - U1. = U2. rejected

Hypothesis 2 - U.1 = U.2 = U.3 = U.4 = U.5 rejected

Hypothesis 3 - $(U_{21}-U_{11}) = (U_{22}-U_{12}) \ldots (U_{25}-U_{15})$ accepted

Table C-12. Summary of the Hypothesis Testing Results for the Repeated Measures Growth Curve Analysis -- Comparison of Slide Preparation Techniques

Null Hypothesis	α	Experimental F Value	Critical F Value	Accept or Reject Null Hypothesis
1	.05	45.34	18.51	Reject
2	.05	17.57	3.84	Reject
3	.05	8.24	3.84	Reject

Table C-13. Computation of Mean Attachment Values for Washed/Autoclaved and Washed/Unautoclaved Slides - 8 Day SRT

Slides Washed Autoclaved	July Cocci > 0.6 um + noncocci	August Cocci > 0.6 um + noncocci	
inoculation time-hours	Slides W, A bacteria/10000 μm^2	Slides W, A bacteria/10000 μm^2	ave bacteria/10000 μm^2
0	0.25	1.98	1.115
1.33	112.71	95.83	104.27
4.67	245.91	224.61	235.26
14.33	211.10	164.21	187.66
52	287.6	224.84	256.22
73.25	209.12	365.96	287.54
165	433.26	236.25	334.76

Slides Washed Unautoclaved	July Cocci > 0.6 um + noncocci	August Cocci > 0.6 um + noncocci	
inoculation time-hours	Slides W, A bacteria/10000 μm^2	Slides W, A bacteria/10000 μm^2	ave bacteria/10000 μm^2
0	0.79	7.57	4.18
1.33	2.56	2.56	2.56
4.67	1.28	11.18	6.23
14.33	177.22	2.79	90.0
52	9.31	7.92	8.62
73.25	5.71	6.64	6.18
165	10.13	10.01	10.07

Table C-14. Computation of Mean Attachment Values for Washed/Autoclaved and Washed/Unautoclaved Slides - 20 Day SRT

(All values as bacteria per 1000 square micrometers)

inoculation time-hours	May cocci > 0.6 um + noncocci Slides W, A	April cocci > 0.6 um + noncocci Slides W, A	ave
0	3.40	0.38	1.89
1.25	153.82	93.94	123.88
4.67	234.93	136.28	185.6
14.0	158.35	232.01	195.18
73.5	370.93	312.84	341.89

inoculation time-hours	May cocci > 0.6 um + noncocci Slides W, U	June cocci > 0.6 um + noncocci Slides W, U	ave
0	1.79	2.17	1.98
1.25	12.83	12.07	12.45
4.67	24.62	101.1	62.86
14.0	22.64	71.1	46.87
73.5	58.76	65.83	62.30

Table C-15. 8 Day SRT t Test Comparison of Means Autoclaved versus Unautoclaved

Inoculation Time	Mean Autoclaved μ_1	Mean Unautoclaved μ_2	Ho	α	t Calculated	t Critical	Accept or Reject	Descriptive Level	95% Confidence Interval
0	1.115	4.18	$\mu_1=\mu_2$	.05	-0.878	4.303	accept	.482	$-18.08 \leq \mu_1 - \mu_2 \leq 11.95$
1.33	104.27	2.56	$\mu_1=\mu_2$	.05	12.05	4.303	reject	.0074	$65.39 \leq \mu_1 - \mu_2 \leq 138.03$
4.67	235.26	6.23	$\mu_1=\mu_2$	.05	19.51	4.303	reject	.0032	$178.85 \leq \mu_1 - \mu_2 \leq 279.89$
14.33	187.66	90.0	$\mu_1=\mu_2$	.05	1.08	4.303	accept	.426	$-290.94 \leq \mu_1 - \mu_2 \leq 486.26$
52	256.22	8.62	$\mu_1=\mu_2$	.05	7.88	4.303	reject	.017	$112.49 \leq \mu_1 - \mu_2 \leq 382.71$
73.25	287.54	6.18	$\mu_1=\mu_2$	.05	3.58	4.303	accept	.076	$-56.08 \leq \mu_1 - \mu_2 \leq 618.8$
165	334.16	10.07	$\mu_1=\mu_2$	.05	3.30	4.303	accept	.086	$-99.16 \leq \mu_1 - \mu_2 \leq 748.54$

Table C-16 20 Day SRT t Test Comparison Of Means-Autoclaved Versus Unautoclaved.

Inoculation Time	Mean Autoclaved μ_1	Mean Unautoclaved μ_2	Ho	α	t Calculated	t Critical	Accept or Reject	Descriptive Level	95% Confidence Interval
0	1.89	1.98	$\mu_1=\mu_2$	.05	-0.059	4.303	accept	>.8	$-6.63 \leq \mu_1 - \mu_2 \leq 6.45$
1.25	123.88	12.45	$\mu_1=\mu_2$	.05	3.73	4.303	accept	.071	$-17.23 \leq \mu_1 - \mu_2 \leq 240.09$
4.67	185.6	62.86	$\mu_1=\mu_2$	.05	1.97	4.303	accept	.192	$-145.94 \leq \mu_1 - \mu_2 \leq 391.4$
14.0	195.18	46.87	$\mu_1=\mu_2$	.05	3.36	4.303	accept	.084	$-41.4 \leq \mu_1 - \mu_2 \leq 337.98$
73.5	341.89	62.30	$\mu_1=\mu_2$	.05	9.34	4.303	reject	.012	$153.7 \leq \mu_1 - \mu_2 \leq 405.45$

The results of the comparisons of the means at each inoculation time by a "t" test tended to confirm the results of the randomized block analysis and the repeated measures analysis. For the 8 day SRT data the null hypothesis, that attachment number for autoclaved and unautoclaved slides was equal at a given inoculation time, was rejected at 5 of the 7 data points. The null hypothesis was only accepted at the zero inoculation time, which one would expect, and one other data point. For the 20 day SRT data, the null hypothesis was only rejected at 1 of the 5 data points. However, if one excluded the zero inoculation time, the descriptive level of the tests was less than 0.085 for three of the four remaining data points. The descriptive level of the test gives the probability that such an extreme result would occur. In this case three of four points obtained an extreme result that had only a very low probability of occurring randomly.