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ATTACHMENT AND EARLY BIOFILM DEVELOPMENT

OF

METHANE-FORMING ANAEROBIC MICROBIAL CULTURES

A Dissertation Presented

by

Jeffrey P. Robins

Submitted to the Graduate School of the
University of Massachusetts in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Department of Civil Engineering

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Dedication

To the ten million, billion bacteria
that devoted their lives to this study.

ABSTRACT

ATTACHMENT AND EARLY BIOFILM DEVELOPMENT

OF

METHANE-FORMING ANAEROBIC MICROBIAL CULTURES

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This study investigated the influence of growth rate and glass slide preparation on bacterial attachment and biofilm development over time for methane-forming, anaerobic, mixed, microbial cultures. Photomicrographs and microscopic observations were also recorded.

An anaerobic attachment vessel was designed, constructed, and used to quantify and visualize the initial attachment and biofilm development of chemostat grown bacterial cultures. The bacteria attached rapidly to washed/autoclaved glass slides. Within one to three hours, the number of irreversibly attached bacteria increased by approximately two orders of magnitude from 0 to 100 - 250 bacteria per 10,000 square micrometers. Only a slow increase in the number of attached bacteria 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 for 8 day solids retention time (SRT) and 20 day SRT cultures. Two mathematical models were developed to describe the results. A significant percentage, usually 25% - 75%, of the bacteria counted on the washed/autoclaved slides were methanogens. Final step autoclaving in the slide wash procedure had a statistically significant effect on attachment. Irreversibly attached bacteria counts 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 do, and some do not, possess conspicuous appendages or extracellular fibers which appear to be used for attachment. At long inoculation times, more extensive development of extracellular fibers was observed sometimes and more amorphous, extracellular, gluelike material was present. Occasionally, extracellular fibers were observed to branch at longer inoculation times. Tip growth was proposed to account for this observation. At short and long inoculation times, cells attached as individuals and in clumps. The clumps were covered and/or interspersed with the gluelike material. Some clumps and individual cells appeared to have a ring around them, perhaps the secretion of extracellular polymers or enzymes. Higher concentrations of attached bacteria were sometimes observed on surface irregularities.

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CHAPTER I

INTRODUCTION

In the last twenty-five years, there has been a renewed interest in the use of methane generating anaerobic fermentation processes to degrade organic wastes. In 1964, McCarty 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
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
5. long start-up times
6. many parameters must be monitored to maintain the stability of the complex microbial ecosystem (process control)

7. competition between sulfate-reducing bacteria and methanogens results in the production of hydrogen sulfide
8. odorous end products are sometimes produced
9. the systems generally have high buffer requirements.

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, 1983 and Switzenbaum, 1983). 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 engineering 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 (1964). The long start-up time, difficulty in treating low strength wastes, competition with sulfate reducing bacteria, and odorous end-products remain as persistent problems. One new advantage, discovered in recent work, is that methanogenic anaerobic cultures are capable of degrading aromatic compounds (Healy and Young, 1979) and halogenated aliphatic compounds (Bouwer and McCarty, 1981; Bouwer and McCarty, 1983). The former group was previously considered nonbiodegradable anaerobically (McCarty, 1982).

The goal of this dissertation 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 examines 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
3. inoculation time - the amount of time bacteria were exposed to the attachment surface.

CHAPTER II

BACKGROUND

Methanogenesis From Complex Organic Substrates

Before considering the attachment of bacteria to surfaces, it is necessary to review how anaerobic microorganisms convert complex organic molecules to methane and carbon dioxide. There are five groups of organisms involved in methanogenesis (Figure 2.1; Zinder, 1984).). A consortium of microorganisms from these five groups 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 (Zinder, 1984).

The organisms of a methanogenic consortium are closely interdependent on one another for survival. For example, the

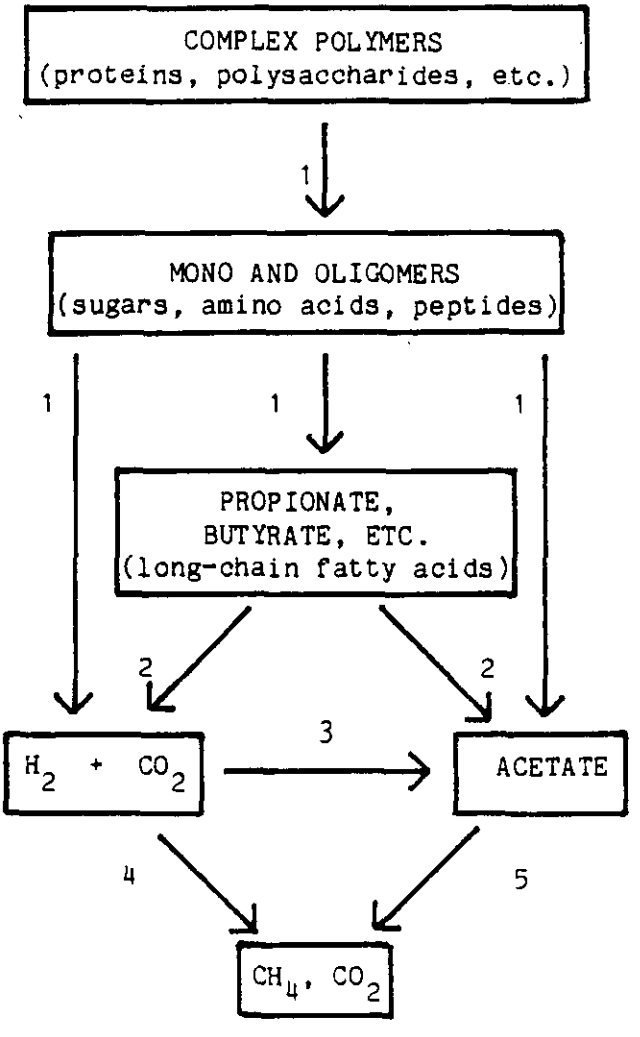


Figure 2.1 Anaerobic Degradation Of Organic Matter To Methane (after Zinder, 1984). Group 1, fermentative bacteria; group 2, hydrogen-producing acetogenic bacteria; group 3, hydrogen-consuming acetogenic bacteria; group 4, carbon dioxide-reducing methanogens; group 5, aceticlastic methanogens.

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 (McCarty, 1981). 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 biological processes reactors comes from acetate via these microorganisms (Jeris & McCarty, 1965; de Vocht et al. 1983) performed experiments which indicated reactors which selected for sedimentation of organisms favored Methanothrix, while reactors selecting for organisms which attach to surfaces favored Methanosarcina. Switzenbaum's (1986) 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 (1984) electron microscopy study of eight methanogenic, anaerobic fixed film reactors, Methanothrix spp. was found in high numbers at film surfaces whereas Methanosarcina was commonly embedded in the lower regions of the film.

There is some kinetic data for Methanosarcina spp. and Methanothrix spp. Methanothrix spp. have a doubling time of between four and nine days, only use acetate as substrate, and have a substrate concentration at which they reach one-half their maximum growth rate, K_m, of less than one millimolar (Huser et al., 1982). Doubling times for Methanosarcina spp. grown on acetate have been reported as short as one day (Smith et al., 1980) The K_m values are from three to five millimolar for growth on acetate (Smith et al., 1980).

One final item of importance when comparing Methanothrix spp. with Methanosarcina spp. is that Methanosarcina species autofluoresce whereas Methanothrix spp. do not (Huser et al., 1982; Zinder, 1984). 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 (Brock et al., 1984). Factor 420 absorbs light at 420 nm and fluoresces blue-green light when placed in an oxidized environment (Brock et al., 1984).

How and Why Bacteria Stick to Surfaces

Reversible Attachment, Irreversible Attachment, And The Glycocalyx

It is generally accepted that there are two classifications of attachment of bacteria to surfaces, "reversible attachment" and

"irreversible attachment" (Marshall et al., 1971). 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 (Marshall, 1985). 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. The colloid and the surface are also attracted to one another if the energy barrier is surmounted and their separating distance is less than the distance to the barrier. It is proposed that bacteria can be attracted to the region known as the secondary minimum simply 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 (Weiss and Harlos, 1977).

Thus if a cell produces a small diameter probe, this would have a much smaller energy barrier to surmount. Such a probe might then be capable of forming a bond to the surface. Rogers (1979) 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 (1971) obtained data that supported the application of VODL theory to explain reversible attachment 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 electyrolyte concentrations and small double layer thicknesses, the reversible attachment of micro-organisms was high.

Bacteria use their glycocalyx to irreversibly attach to surfaces. The glycocalyx was defined as "the polysaccharide containing structures of bacterial origin that lie outside the integral elements of the outer membrane of Gram-negative cells and the peptidoglycan of Gram-positive cells" (Costerton, 1985). In the past, the glycocalyx was often referred to as the slime layer, capsule, or microcapsule. The term glycocalyx implies its composition is essentially polysaccharide in nature (Wicken, 1985). Wicken (1985) suggested the

term glycocalyx is not the best term because the outer layer of polymers contains molecules other than polysaccharides. Glycoprotein molecules are another important polymer found in glycocalyces. Some genera, notably Bacillus, are capable of forming glycocalyces composed primarily of polypeptides under growth conditions with excess nitrogen (Wicken, 1985). Nevertheless, glycocalyces are most commonly composed of polysaccharide materials (Wicken, 1985). The glycocalyx forms a mass of tangled polymer fibers which extend from the surface of the cell (Costerton, 1978). In has been found in virtually all in situ observations of bacteria living in natural ecosystems and is involved in bacterial attachment to surfaces and each other (Costerton, 1984). It is also usually relatively thick, greater than 0.1 micrometers (Costerton, 1984).

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 (Raven et al., 1981). Fungal cells have an outer layer of chitin (Raven et al., 1981). Animal cells have a variety of polysaccharides in their glycocalyxes (Costerton et al., 1978). At least some of the Protista (e.g., algae) contain a variety of polymers in their cell walls.

The existence of the glycocalyx has only been known since the late 1960's (Costerton et al., 1978). There are two reasons why its existence has only recently been recognized. First, the glycocalyx typically does not form in pure laboratory cultures, the major

investigative system used by microbiologists (Costerton et al., 1978). Apparently, the production and maintenance of the glycocalyx requires a substantial metabolic expenditure (Costerton et al., 1978). Cells which are not burdened with this metabolic expense are selected for in pure laboratory cultures. In naturally occurring environments, however, natural selection favors microorganisms that produce glycocalyx. Second, the glycocalyx was not detected in early electron microscopy work (Costerton, 1985). The polysaccharides of the bacterial glycocalyx did not attract the heavy metal stains used at that time. With the development and use of polyanion-specific stains (i.e. ruthenium red & alcian blue), the glycocalyx was first seen but distorted due to dehydration. The glycocalyx is ninety-nine percent water and the dehydration involved in the preparation of specimens for electron microscopy collapsed the overall structure. In the mid 1970's and early 1980's, methods were developed to stabilize the glycocalyx using lectin (Birdsell, et al., 1975) and specific antibodies (Mackie et al., 1979; Chan, et al., 1982). These techniques allowed visualization of the glycocalyx in its naturally occuring, uncollapsed form.

Structure Of The Cell Membrane And The Cell Wall In Bacteria

Before examining bacterial attachment, it is useful to review the structure of what has been traditionally considered the outer surface of the bacterial cell, the plasma membrane and the cell wall (Wicken,

1985). It is from these structures that the glycocalyx emerges. Bacteria or procaryotes are classified into two groups, the eubacteria and the archaebacteria (Stanier, 1986). Archaebacteria are thought to be the most primitive organisms on earth in an evolutionary sense (Woese, 1981). They were recently classified as a separate group from eubacteria based on 16S ribosomal RNA sequencing studies, the structure of their menbrane lipids, the lack of peptidoglycan in their cell walls, their spectrum of antibiotic sensitivity, and certain details of their protein synthesizing machinery (Brock, 1984). Bacteria which are not archaebacteria are eubacteria. The eubacteria are further broken down into three groups, the Gram-positive bacteria, Gram-negative bacteria, and the mollicutes. These three groups are classified based on the structure of their cell walls. The archaebacteria are also divided into three groups; the methanogens, the halophiles, and the acidophiles. There are differences in the structure and composition of the cell wall of three groups of archaebacteria compared to the eubacteria, and compared to each other. The archaebacteria are not as well studied as the eubacteria.

The general structure of a gram-positive plasma membrane and cell wall, with an emphasis on polymeric substances, is summarized below (Wicken, 1985). Gram-positive bacteria have an inner plasma membrane which is surrounded by a relatively thick (compared to gram-negative bacteria) layer of peptidoglycan. There are some secondary polymers which are either covalently bound or noncovalently associated with cell wall or plasma membrane. Teichoic acids, teichuronic acids, and

polysaccharides are covalently bound to the peptidoglycan and in some cases extend from the surface of the cell. Teichoic acids are polymers of either ribitol phosphate or glycerophosphate joined by phosphodiester bonds to alcohol groups of the polyol residues. Teichuronic acids are a group of acidic polysaccharides that can replace teichoic acids, in some gram-positive bacteria, when the bacteria are grown under a limiting phosphate conditions. The polysaccharides are generally heteropolymers of two to four different neutral or amino sugars. The relative amount of peptidoglycan to secondary wall polymers is fairly constant under different growth conditions.

Proteins are associated and sometimes covalently bound to the cell wall (Wicken, 1985). Noncovalently bound proteins are found within the peptidoglycan and the glycocalyx regions. Covalently associate proteins may exist as globular or fibrillar proteins at the outer surface of thee cell wall. The basal bodies of flagella are composed of protein, beginning at the plasma membrane, and extended through the peptidoglycan outside the cell wall. The filament of the flagella is also composed of a protein, flagellin. Gram-positive cells excrete a wide range of hydrolytic enzymes. Some cells excrete enzymes related to polymer synthesis. Some cells have surface arrays of glycoprotein molecules which are electrostatically associated to the cell surface.

Lipoteichoic acids are molecules similar to teichoic acids except they are covalently linked to a glycolipid or a phosphatidylglycolopid

molecule; thus they have a hydrophobic region which can be associated with the plasma membrane while the hydrophilic portion of the molecule has been detected at the surface of the cell wall (Wicken, 1985). Lipoteichoic acids are excreted by the cell. They can from micellar aggregates when excreted from the cell. They also interact ionicly and hydrophobically with proteins and form complexes with polysaccharides.

In some organisms, there is turnover of peptidoglycan and secondary polymers (Wicken, 1985). In other organisms, the covalently linked cell wall polymers are conserved.

The general structure of the plasma membrane and cell wall of a gram-negative bacterium, with an emphasis on polymeric substances, is summarized below (Wicken, 1985). Gram-negative bacteria have an inner plasma membrane, an outer membrane and a relatively thin (compared to gram-positive bacteria) layer of peptidoglycan sandwiched in between the two membranes. The space between the inner cell membrane and the cell wall is known as the periplasmic space. At least two types of proteins are associated with the inner face of the outer membrane and serve to chemically stabilize the membrane and the peptidoglycan as a single complex. The inner and outer membrane are connected to each other in places. The outer membrane is asymmetric. The inner face of the outer membrane bilayer is composed primarily of phospholipids and protein. The outer face of the outer membrane bilayer is composed of lipopolysaccharides and protein.

The lipopolysaccharides molecules have three distinct regions, the lipid component, the core polysaccharide, and O-antigen polysaccharide side chain (Wicken, 1985). The lipid component associates with the hydrophobic portion of the outer cell membrane. The core polysaccharide is relatively constant series of polysaccharides. The O-polysaccharides are polymers containing repeating sequences of two to four monocaccharide units. These polysaccharide polymers are generally more complex than the polysaccharides which emerge from Gram-positive bacteria. Some bacterial strains do not possess the O-antigen polysaccharide (called "rough stains") and sometimes there is variability of composition within the same preparation for bacteria that do possess the O-antigen polymer. In addition to the polysaccharides associated with the lipopolysaccharide molecules, there are other polysaccharides emerging from the membrane whose hydrophobic regions are not fully elucidated. The outer membrane also contains divalent metal cations.

The glycocalyx region also contains glycoprotein S layers, extra cellular polysaccharides, extracellular proteins, excreted outer membrane fragments (Wicken, 1985). Turnover of cell wall components has been observed in some strains and not observed in other strains. The basal body structure for flagella spans the inner cell membrane and the cell wall. Both the filaments of flagella and pili are composed of protein. Fimbriae are short filaments composed of protein which extend from the cell surface.

The secondary polymers associated with the Gram-negative peptidoglycan are different than those associated with the Gram-positive peptidoglycan (Wicken, 1985). In Gram-positive bacteria, the cell wall peptidoglycan is associated primarily with the carbohydrate-type polymers (polysaccharides, teichoic acids, and teichuronic acids) and to a lesser extent with proteins (Wicken, 1985). In Gram-negative bacteria the primary component of the outer cell membrane associated with the peptidoglycan is lipoprotein, which provides a covalent linkage between the outer membrane and the peptidoglycan (Wicken, 1985).

Mollicutes, the third type of eubacteria, lack a defined cell wall outside the plasma membrane (Stanier, 1986). However, they have substantial amounts of hexose, hexosamine, and N-acetylglycosamine containing polysaccharides associated with their membrane. They are all parasites on eucaryotic organisms which implies they are successful in attaching to the tissue of host organisms.

The plasma membrane and cell wall of the archaebacteria is different than the plasma membrane and cell wall of the eubacteria (Stanier, 1986). Their membrane lipids contain ether-linked isoprenoid side chains in contrast to the ester-linked hydrocarbons found in the eubacteria (and all other biological systems). They lack muramic acid as a constituent of the cell wall peptidoglycan in contrast to its nearly universal presence in the walled eubacteria. The cell walls of methanogens have a composition and structure which varies between species. Pseudomurein, protein, and

heteropolysaccharides are the compounds that make up their cell walls. The composition of the cell walls of halophiles is either an acidic glycoprotein (Halobacterium) or similar to the Gram-positive eubacterial cell wall (Halococcus). Among the thermoacidophiles there are three groups, Sulfolobus, Thermoplasma, and Thermoproteus. The cell wall of Sulfolobus forms a distinct layer outside the cell membrane and is composed of lipoprotein and carbohydrate. Thermoplasma lacks a cell wall but its cell membrane contains large amounts of lipopolysaccharide and glycoprotein, both of which contain mannose as their principal sugar monomer. The cell wall of Thermoproteus appears to be composed of glycoprotein.

Bacterial Cell Appendages

There are a few types of appendages emerging from the bacterial cell wall, other than the glycocalyx material, some of which are known or implicated to be involved in bacterial attachment. Flagella, pili, frimbriae and prosthecae are discussed briefly below.

Flagella are relatively thin (12-18 nm. in diameter) helical, proteinaceous filaments which rotate (Brock, 1984). Their primary function is to provide cells with locomotion. The average length of an E. coli flagella is five micrometers.

Fimbriae are straight, proteinaceous filaments which also extend from the surface of cells (Wicken, 1985). They are shorter than

flagella but more numerous on the surface of the cell. There is some evidence they are involved in bacteria attachment (Brock, 1984).

Pili are also filamentous structures emerging from the cell wall of bacteria (Stanier, 1986). They are composed primarily of protein. Some cells possess only one or a few pili, other cells possess many per cell (i.e. hundreds per cell). Pili are thought to be involved with the adhesion of bacterial cells to surfaces. Some pili are also involved with conjugation between bacterial cells. Gram-negative cells which donate their genetic material to other bacteria must have a sex pilus which they use to attach to the other bacterium and transport the genetic material.

Prosthecae are cytoplasmic extrusions from cells such as stalks, buds, or hyphae (Brock, 1984). Prosthecae are still bounded by the plasma membrane and cell wall. Stalks in some cases (i.e. Caulobacter) are involved with attachment.

The Glycocalyx

Finally, the greater than 0.1 micrometer in cross section outer bacterial coat in nature is the glycocalyx region (Costerton, 1984). It is composed primarily of a matrix of polysaccharide material . The outer surface is composed mainly of these polysaccharides and protruding pili. The polysaccharides possess many negatively charged sites at neutral pH's. These available negative charges are important in holding positively charged nutrients in the glycocalyx. They also

may be involved in the mechanism of attachment by forming bond bridges, via a cation, to negative charges on another bacterium or a surface. They also are important in determining the charge on the surface on cell and whether the cell surface is hydrophobic or hydrophilic.

In an electron microscopy study, Fletcher and Floodgate (1973) determined that the glycocalyx contains a primary and secondary acidic polysaccharide. The primary polysaccharide was composed of an inner thin dense line on cell wall surface and an outer fringe region. The dense line was about 5 nanometers thick and 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 (Fletcher & Floodgate, 1973) later proposed the primary polysaccharide was responsible for initial adhesion while the secondary polysaccharide strengthened the cells attachment to the surface. Finally, in an experiment where bacteria attached to Millipore filters suspended in broth, calcium and magnesium were demonstrated to be important for the maintenance of the secondary polysaccharide intercellular matrix (Fletcher & Floodgate, 1976). Within five minutes of being transferred into a calcium and magnesium deficient media, the secondary polysaccharide was disrupted.A schematic diagram of the glycocalyx structure they described is shown in Figure 2.2.

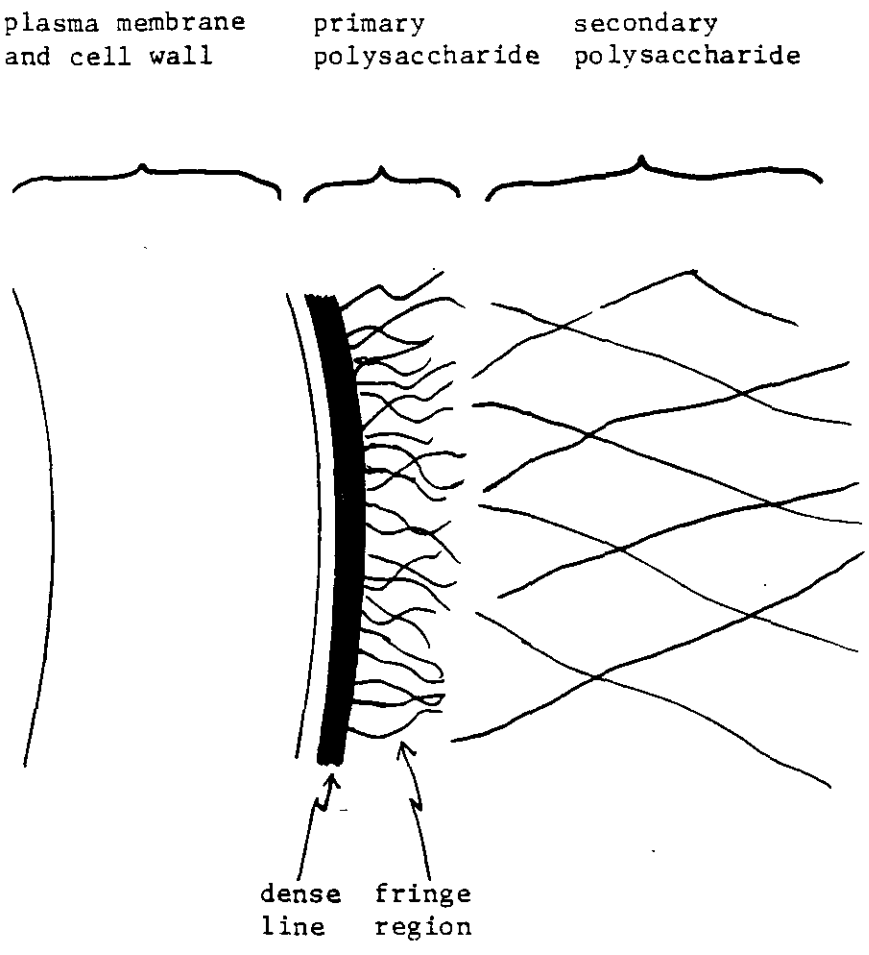


Figure 2.2 Schematic Diagram Of Glycocalyx Structure.
Based on the work of Fletcher and Floodgate, 1973;
and Fletcher and Floodgate, 1976.

Specific And Nonspecific Attachment

The attachment of bacteria to a surface can be nonspecific or specific (Costerton et al., 1978). The exact mechanism of a nonspecific bond to an inert surface is unknown. 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 (Cumsky & Zusman, 1981).

Sizes Of Cell Structures

Table 2.1 shows a comparison of the sizes of various cell structures including the VODL secondary minimum distance, the theoretical location of a reversibly attached bacterial cell about to irreversibly attach. The range of distances given for the secondary minimum are from an approximate numbers given by Wicken (1985), from reading off the graphs of Marshall (1972), and from reading off the graphs of Weiss and Harlos (1977). The distance to the secondary minimum, 10-100 angstroms, is small in comparison to many other structures of the cell. The cell has many small diameter probes (polysaccharide polymers, pili, flagella, etc.) which would reduce the energy barrier that must be overcome for the cell to contact the surface. Fletcher and Floodgate (1976) proposed that the primary polysaccharide region appears to be involved in initial, irreversible attachment. It is of the correct size range to span the secondary minimum distance.

Advantages To A Bacterium Of Living On A Surface

There are several reasons why it is advantageous for a bacterium to adhere to a surface. In a specific interaction with another organism the reasons are obvious.

1. Attachment to other organisms allows relationships ranging from pathogenic to symbiotic to develop.

Table 2.1 Size Ranges Of Various Bacterial Cell Structures And The Secondary Minimum Distance.

angstroms	
1 - 2	radius of most atoms (Dickerson, Gray and Haight, 1974)
30 - 100	distance of the VODL secondary minimum between bacteria and a surface (Marshall, 1972; Wicken, 1985)
40 - 350	diameter of pili (Stanier, 1986)
50	width of the dense line (measured off photograph) of the primary polysaccharide (Fletcher and Floodgate, 1973)
70 - 80	width of cell membranes (Stanier, 1986)
120 - 180	diameter of flagella (Stanier, 1986)
150 - 250	width of the outer fridge region (measured off photograph) of the primary polysaccharide (Fletcher and Floodgate, 1973)
1000	minimum width of the glycocalyx of most bacteria in vivo (Costerton, 1984)
10000	typical diameter of a bacterial cell
50000	typical length of an E.coli flagellum

2. Attachment to other organisms facilitates the exchange of genetic material.
3. Attachment to other organisms facilitates the development of a habitat that may be more favorable for survival (i.e. granules or a biofilm).
4. Attachment to other organisms may help conserve heat.
5. Attachment to other organisms may provide protection from predators.

The explanation for nonspecific attachment to nonbiological surfaces is more subtle. Costerton et al. (1978; and 1985) proposed the following reasons.

1. Because of the hydrophobic nature of portions of many organic molecules, they 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 some physical protection (i.e. from drying, toxic substances, surfactants, antibodies, 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 a process like interspecies hydrogen transfer or other transfers of chemicals from one cell to another.
8. The glycocalyx polymers which organisms produce have a carbohydrate storage function in some bacteria (Dudman, 1977). These bacteria can use their own exocellular polysaccharides, that they themselves produced, to support growth.

Further, it has been proposed that the attached mode of growth represents a distinct physiological state of bacteria (Costerton, 1985; Wicken, 1985; Whittenbury and Dow, 1977). They suggest that many bacterial species exist in two physiologically distinct forms, sessile microcolonies surrounded by an extensive glycocalyx and mobile "swarmer" cells that are dispatched to colonize new environments. The "swarmer" cells are the glycocalyx lacking cells that are usually selected for in laboratory cultures where the competitive challenges and hazards of the natural environment demand the presence of a glycocalyx.

Other Observations Of Bacterial Attachment From The Electron Microscope

A number of electron microscope photographs have already been discussed in relation to bacterial attachment. The primary and secondary polysaccharides of the glycocalyx have been shown. The importance of stabilizing the glycocalyx using monoclonal antibodies or lectins has been reviewed. The importance of calcium and magnesium in maintaining the integrity of the secondary polysaccharide has been considered.

There have been a few other observations about bacterial attachment from electron microscopy that are worthy of note. Wardell et al. (1984) observed that the initial biofilm development of a pure culture of Pseudomonas sp. occurred in small microcolonies or clumps on the surface. Several researchers (Wardell, et al., 1984; Lie, 1977; Beeftink and Staugaard, 1986) have recorded the presence of fibrils reaching from attached bacteria to the surface and other bacteria. Wardell et al. (1984) noted that the fibrils branch. The branching can be seen also in Beeftink and Staugaard's (1986) photographs.

The Pattern of Biofilm Development

Descriptive Models Of Attachment And Early Biofilm Development

The following sequential steps in the development of a biofilm have been postulated by Trulear and Characklis (1982).

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 (Baier, 1980). The macromolecules are primarily glycoproteins, proteoglycans or their end product humic residues (Baier, 1980). Microorganisms are transported to the surface either by turbulent flow conditions, diffusion, or chemotaxis (Trulear and Characklis, 1982). 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, fimbriae, and most likely the glycocalyx polymers, to stick to the surface. Once the bacteria have attached successfully, they enter the growth phase. They produce

additional exopolymers to strengthen their attachment and reproduce (Trulear and Characklis, 1982).

Finally, partial detachment of the biofilm occurs as segments periodically break off (Trulear and Characklis, 1982). 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 (1982) 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 could be 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 (1982) summarized their findings as follows.

1. Biofilm accumulation is the net result of substrate removal, biofilm production resulting 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 (1981) 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.

Mathematical Models of Attachment and Early Biofilm Development

There have not been many mathematical models to quantify the bacterial attachment and early biofilm development over time. A few are discussed below.

Fletcher (1977) 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 were as follows:

$$R = k[X]_s\ (1-\theta) \qquad (2.1)$$

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

$$[X]_{ad} = k'\theta \qquad (2.2)$$

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

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

$$[X]_s = \frac{[X]_{ads}[X]_s}{k'} + \frac{R}{k} \qquad (2.3)$$

Fletcher did not provide an integrated form of her equations. If she had, she might have developed the following:

$$R = \frac{dX_{ads}}{dt} = k\ X_S\ (\theta) \qquad (2.4)$$

$$\theta = \frac{X_{ads}}{k'} \qquad (2.5)$$

$$\frac{dX_{ads}}{dt} = k\, X_S \left(1 - \frac{X_{ads}}{k'}\right) \text{ assume } X_S = \text{constant} \tag{2.6}$$

$$\left(1 - \frac{X_{ads}}{k'}\right)^{-1} dX_{ads} = k\, X_S\, dt \tag{2.7}$$

$$\int_o^{X_{ads}} \left(1 - \frac{X_{ads}}{k'}\right)^{-1} dX_{ads} = \int_o^t kX_S dt \tag{2.8}$$

$$-k' \ln \left(1 - \frac{X_{ads}}{k'}\right) = kX_S t \tag{2.9}$$

$$\ln \left(1 - \frac{X_{ads}}{k'}\right) = \frac{-k}{k'} X_S t \tag{2.10}$$

$$\exp\left(-kX_s t/k'\right) = 1 - \frac{X_{ads}}{k'} \tag{2.11}$$

$$-k' \left(\exp\left(-kX_s t/k'\right) - 1\right) = X_{ads} \tag{2.12}$$

$$k' \left(1 - \exp\left(-kX_s t/k'\right)\right) = X_{ads} \tag{2.13}$$

If the models are 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). As inoculation time increases, surface coverage increases, and the rate of attachment decreases. As inoculation time approaches infinity, the fraction of the surface covered approaches one, and the rate of attachment approaches 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.

Verrier (1984) obtained data for attachment of volatile fatty acid fed methanogenic cultures on to polyvinyl chloride over time. He carried out his experiments at three pH's. He observed rapid attachment over the first few hours of incubation followed by a plateauing of the cell numbers attached to the surface after the first few hours. Verrier used the following mathematical equation to model his attachment curve:

$$X_{ads} = k_1\sqrt{t} + k_2 t \text{ or} \qquad (2.14)$$

$$\frac{X_{ads}}{t} = \frac{k_1}{\sqrt{t}} + k_2 \qquad (2.15)$$

where X_{ads} = concentration of bacteria on the surface

t = incubation time

k_1 = constant representing the initial adsorption rate

k_2 = constant influencing the variation in the adsorption rate over time

Caldwell et al. (1981) proposed a model to take into account bacterial attachment and subsequent growth after attachment. Their model was based on the following relationships.

$$\frac{dN}{dt} = \mu N + A \qquad (2.16)$$

where:

N = number of cells on the surface (cells/field)

t = incubation time (hours)

μ = specific growth rate (1/hours)

A = attachment rate (cells/(field x hours))

They assumed the attachment rate was constant and the microbial growth rate was exponential. After integrating their equation they obtained the following:

$$N = (A/\mu)e^{\mu t} - A/\mu \qquad (2.17)$$

Brannan and Caldwell (1982) experimentally tested the model in equation 2.17. They found the attachment rate was not constant but increased with time. Their empirical data for the time course of colonization fell in the 95% confidence interval of the predicted values. Their experiment tested bacterial attachment over a 6.5 hour period.

Caldwell et al. (1983) proposed another mathematical model to quantify microbial growth. The purpose of this model was to improve

the earlier model (equation 2.17). The first model required a computer to solve for μ and involved counting bacterial cells, which they felt was awkward. The newer model also attempted to account for attachment and growth. They again assumed the attachment rate was constant. The derivation they presented was as follows. The rate one-celled microcolonies form is equal to the attachment rate.

$$\frac{dC_1}{dt} = A \tag{2.18}$$

where:

C_1 = number of microcolonies

t = incubation time (hours)

A = attachment rate (cells/hour)

The rate that one-celled microcolonies become two-celled colonies (and the rate one-celled colonies disappear) is:

$$\frac{dC_2}{dt} = \mu C_1 \tag{2.19}$$

where:

μ = specific growth rate

Thus the net rate of formation of one-celled colonies is:

$$\frac{dC_1}{dt} = A - \mu C_1 \tag{2.20}$$

At some point an equilibrium will be reached such that:

$$\frac{dC_1}{dt} = 0 \tag{2.21}$$

Then:

$$0 = A - \mu C_1 \tag{2.22}$$

They propose that this equation would actually apply to any size colony because the rate of formation of each colony size would eventually come into equilibrium.

So:

$$0 = A - \mu C_i \tag{2.23}$$

where:

C_i = number of microcolonies with i cells

This equation simplifies to:

$$\mu = \frac{A}{C_i} \quad \text{or} \tag{2.24}$$

$$C_i = \frac{A}{\mu} \tag{2.25}$$

From these equations the growth rate on the surface can be determined by the empirically derived quantities, the attachment rate and the number of colonies of a particular size. When equation 2.25 was substituted into equation 2.17 of their earlier study, they obtained the following expression:

$$\mu = \frac{\ln \left[(N/C_i) + 1\right]}{t} \tag{2.26}$$

This equation relates growth rate to the total number of cells on the surface, the concentration of a particular colony size and the incubation time.

Factors Affecting Biofilm Development

Overview

Daniels (1980) 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.

Surfaces

Dexter (1979) 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 included a rinsing step so he was measuring irreversible attachment.

Critical surface tension, γ_{crit}, 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 (from 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. 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^{o} (in other words spontaneous spreading) from those forming higher contact angles and not spreading.

Dexter (1979) observed that studies comparing attachment of bacteria to critical surface tension obtained different results when the studies were done <u>in situ</u> versus <u>in vitro</u>. He proposed a two-step model to account for the observed discrepancy in the data. 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:

$$\Delta F = \gamma_{SO} + \gamma_{OW} - \gamma_{SW} \qquad (2.27)$$

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 (1979) used the work of Girifalco and Good (1957) and Good (1964) to explain that the interfacial tension between the solid and water, γ_{SW}, 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 graphed the interfacial tension of the solid-water interface as a function of the critical surface tension of the solid and the interaction parameter between the solid surface and a

hydrocarbon liquid which would be adsorbing to the solid. When ϕ_{sl} = 1.0, the curve resembled the bacterial attachment curve for in vitro studies. When ϕ_{sl} = 0.5, the curve resembles the bacterial attachment curve for in situ studies. Dexter thus postulated that adsorption of the conditioning film is a function of the critical surface tension, γ_{crit}, 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 dependent on the tendency of organics to adsorb to the solid surface.

Pringle and Fletcher (1983) investigated the influence of the work of adhesion (by varying the solid surface for attachment) on bacterial attachment. Their interpretation of work of adhesion theory for bacterial attachment was based on the following:

$$W_{A \cdot BS} = \gamma_{SL} + \gamma_{BL} - \gamma_{SB} \qquad (2.28)$$

where:

$W_{A \cdot BS}$ = work of adhesion between the bacteria and the solid

γ_{SL} = interfacial free energy for the solid-liquid interface

γ_{BL} = interfacial free energy for the bacterium-liquid interface

γ_{SB} = interfacial free energy for the solid-bacterium interface.

They tested a number of pure strains of freshwater bacteria that were isolated by submersing slides in the River Sowe Coventry, England.

For each isolate they investigated the influence of the work of adhesion on bacterial attachment. They found that each strain had its own pattern of attachment for a range of surfaces. Each strain had a range of work of adhesion values where its attachment values reached a maximum. Maximum attachment occurred at work of adhesion values between 75 and 105 millijoules per square meter (high energy or hydrophilic surfaces > 100 millijoules per square meter; low energy or hydrophobic surfaces < 100 millijoules per square meter - Loeb, 1985).

Absolom et al. (1983) tested a thermodynamic model to explain attachment of bacteria to a surface. They proposed, in theory;

$$\Delta F^{adh} = \gamma_{BS} - \gamma_{BL} - \gamma_{SL} \qquad (2.29)$$

where:

ΔF^{adh} = free energy of adhesion/surface area

γ_{BS} = bacterium-substratum interfacial tension

γ_{BL} = bacterium-liquid interfacial tension

γ_{SL} = substratum-liquid interfacial tension

also

$$\gamma_{SV} - \gamma_{SL} = \gamma_{VL}\cos\theta \qquad (2.30)$$

where:

γ_{SV} = solid-vapor interfacial tension

γ_{SL} = solid-liquid interfacial tension

γ_{LV} = liquid-vapor interfacial tension

θ = contact angle of the liquid on the solid.

γ_{LV} and cos θ are easily measured. γ_{LV} is the surface tension of the liquid. θ is the contact angle between the liquid and the solid. γ_{SL} and γ_{SV} can be determined by mathematical relationships (not shown).

They then tested the theory by investigating the influence of γ_{LV}, γ_{SV}, and γ_{BV} (the interfacial tension of the bacterium-vapor interface) on bacterial attachment. When $\gamma_{LV} > \gamma_{BV}$, ΔF^{adh} became more positive (less energetically favorable for attachment) with increasing γ_{SV}. When $\gamma_{LV} < \gamma_{BV}$, ΔF^{adh} became more negative (more energetically favorable for attachment) with increasing γ_{SV}. They obtained experimental data that supported this model. Further, when $\gamma_{LV} = \gamma_{BV}$, the model predicts changes in γ_{SV} should not effect bacterial attachment. This was also confirmed experimentally.

van Loosdrecht et al. (1987a) obtained data showing that the characteristics of the cell surface are important in determining bacterial attachment. They measured the contact angle of a drop of 0.1M NaCl solution on a layer of cells for twenty-three strains of bacteria. They found contact angles ranging from 15°-70°. As the contact angle increased (increased hydrophobicity of the cell), irreversible attachment increased.

In another study, van Loosdrecht et al. (1987b) found that contact angle (hydrophobicity of the cell surface), and electrophoretic mobility (surface charge of the cell surface) were important parameters in determining irreversible attachment. Surface hydrophobicity was the dominant characteristic. At high contact angles (high hydrophobicity of the cell surface) almost complete

adhesion was found irrespective of the electrophoretic mobility (surface charge). At more hydrophilic contact angles, complete adhesion was not found and electrophoretic mobility influenced adhesion as well. Finally, they obtained some evidence that growth rate influenced hydrophobicity. Cells with higher growth rates had higher contact angles (increased hydrophobicity).

Mahoney et al. (1984) had several findings concerning surface phenomena and bacterial attachment. Contact angles of anaerobic sewage cells increased (the cells became more hydrophobic) when they were exposed to metal ions. They proposed the positively charged metal ions were neutralising the cell surface charges making the cells more hydrophobic. Flocculation increased with increased concentration of metal ions in the medium. An increase in ionic strength of cell suspension medium resulted in a reduction of negative cell surface charge. At constant ionic strength, if the ions in the bulk medium were varied, the surface charge of cells changed. Surface charge of cells changed with surface pH of the cells.

Verrier (1984) compared early biofilm development of methanogenic cultures grown on a mixture of volatile fatty acids for glass and polyvinychloride surfaces. He found much greater biofilm development on the polyvinylchloride than the glass.

Switzenbaum et al. (1985) studied biofilm development of a methane-forming anaerobic mixed culture on four surfaces. The surfaces they investigated were stainless steel, polyvinylchloride, teflon, and aluminum. They found a more rapid biofilm development on

stainless steel and teflon compared to polyvinylchloride and aluminum. They noted that the biofilm development occurred most rapidly on the solid surfaces with high critical surface tension (teflon) or low critical surface tension (stainless steel) while the lowest rates of initial biofilm development occurred on the surfaces with intermediate values of critical surface tension (polyvinyl chloride and aluminum).

Organism Concentration

Fletcher (1977), Bryers and Characklis (1981), Shapiro and Switzenbaum (1984), Wardell et al. (1984), and Verrier (1984) 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 (1977), Shapiro and Switzenbaum's (1984), Wardell et al. (1984) and Verrier's (1984) data imply there is a maximum amount of bacteria that can attach in a given area. Fletcher's (1977) mathematical model (equation 2.1) assumed that the rate of bacterial attachment was directly proportional to the organism concentration and the fraction of the surface covered with bacteria.

Inoculation Time

The data of Fletcher (1977), Marshall (1971) Dexter (1979) and Verrier (1984) show that as inoculation time increases, the number of

irreversible attached cells increases. To repeat, Fletcher's (1977) mathematical models of bacterial attachment (equations 2.1, 2.2, 2.3, and 2.4) suggest the following influences of inoculation time. As inoculation time increases, surface coverage increases, and the rate of attachment decreases. As inoculation time approaches infinity, the fraction of the surface covered approaches one, and the rate of attachment approaches 0. The integrated form of her equation (equation 2.4) relates time of inoculation to the number of bacteria adsorbed, bulk fluid organism concentration, the intensity of adsorbtion, and the maximum adsorptive capacity of the surface.

Growth Rate

The data existing on the effect of growth rate on irreversible attachment are somewhat contradictory. Several studies are summarized in Table 2.2. General observations from these studies imply that log phase organisms attach faster than stationary phase organisms, which attach faster than death phase organisms. And fast growing cells within log phase attach more rapidly than slow growing log phase organisms. However conflicting data does exist. Shapiro and Switzenbaum (1984) found, in their methane-forming anaerobic mixed culture, that the slow growing log phase culture attached about the same rate as the fast growing log phase culture. Nelson et al. (1985) reported that attachment decreased linearly with an increase in specific growth rate history (the log phase growth rate of organisms

Table 2.2 Effect Of Phase Of Growth And Growth Rate On The Rate Of Irreversible Attachment Or Biofilm Development.

Reference	Aerobic or Anaerobic	Type of Culture	Rate of Irreversible Attachment or Biofilm Development High > Low
DIFFERENT PHASES OF GROWTH			
Fletcher, 1977	Aerobic	Pure	log phase > stationary phase > death phase
Marshall et al., 1971	Aerobic	Pure	log phase > "older bacteria"
WITHIN LOG PHASE GROWTH			
Molin, et al., 1983	Aerobic	Pure	fast growth rate > slow growth rate
Bryers & Characklis, 1981	Aerobic	Mixed	fast growth rate > slow growth rate
Shapiro & Switzenbaum, 1984	Anaerobic	Mixed	fast growth rate = slow growth rate
Nelson, et al., 1985	Aerobic	Pure	slow growth rate > fast growth rate
Fletcher and McEldowney, 1984	Aerobic	Pure	fast growth rate > slow growth rate
Fletcher and McEldowney, 1984	Aerobic	Pure	fast growth rate > slow growth rate
Fletcher and McEldowney, 1984	Aerobic	Pure	fast growth rate > slow growth rate
Fletcher and McEldowney, 1984	Aerobic	Pure	slow growth rate > fast growth rate

before they are pumped into their bacterial attachment reactor) for Pseudomonas sp. 224S. In a study of Pseudomonas aeruginosa, Robinson et al. (1984) noted a decrease in extracellular polymer carbon with increasing growth rate. More extracellular polymer production is generally associated with better irreversible attachment. So this study may provide some evidence that increased growth rate of the microorganisms does not result in quicker or better irreversible attachment. Marshall 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. Given the generalization above that faster growth rates result in quicker attachment, Marshall's data is contradictory. Pavoni et al.(1972) 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.

The explanation of these contradictory results may ultimately be that different species behave differently. Fletcher and McEldowney

(1985) reported the pattern of bacterial attachment versus growth rate differed for the 4 species they tested. Flexibacter sp. and Chromobacterium sp. showed increased attachment with increased growth rate. Pseudomonas fluorescens showed little change in attachment at different growth rates. Enterobacter cloacae showed a small decrease in attachment with increased growth rate. One final study that concerned growth rate and was mentioned earlier, van Loosdrecht et al. (1987b) found that cells with faster growth rates were more hydrophobic.

Species

There is some data on the influence of species on bacterial attachment. As mentioned previously, Pringle and Fletcher (1983) found each species of bacteria displayed its own pattern of attachment over a range of surfaces. Fletcher and McEldowney (1985) report the pattern of bacterial attachment versus growth rate differed for the four species of bacteria they tested Hulshoff Pol et al. (1984) investigated the effect of growth substrate composition on the formation of granules. They found the composition of substrates (mainly volatile fatty acids versus mainly sucrose) influenced the species composition and the character of the granules that were formed.

Shear

Shear has an important effect on attachment and biofilm development. Trulear and Characklis (1982) 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 (1984) obtained different results for the effect of shear on the development of a mixed anaerobic biofilm. Their experiments on shear showed there were intermediate liquid flow velocities where 5 day biofilm development was at a minimum. At relatively lower and higher liquid flow velocities, 5 day biofilm development was higher. 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.

Several researchers have noted that initial bacterial attachment and colonization of surfaces occurs in low shear environments. Beeftink and Staugaard (1986) studied the formation of bacterial aggregates. They noted that the aggregates (bacterial clumps or granules) only formed when their reactor possessed sand grains. Bacteral attachment and microcolony formation preferentially occurred in depressions or crevices in the surface of the sand grains. They proposed these regions were initially colonized because of the low shear forces in the protected regions. Oakley et al. (1985) found that initial colonization of keiselguhr particles occurred in crevices of the solid surface and spaces between solid surfaces (low shear environments). Lie (1977) observed that the colonization of hydroxyapatite splint segments attached to the surface of teeth began in grooves and pits on the surface. Saxton (1973) noted the presence of organic materials accumulating in the cracks of artificial teeth despite brushing.

Powell and Slater (1982) studied the removal of bacteria, already attached to glass, by shear. They obtained the following results. The rate of removal of bacteria from glass could be modeled by the following first order of relationship.

$$\frac{dN}{dt} = [\mu_s - \psi(\tau_o)]N \qquad (2.31)$$

where:

N = number of bacteria on the glass surface per area

$\frac{dN}{dt}$ = rate of change of the number of bacteria on the glass surface

μ_s = specific growth rate of bacteria on the surface

$\psi(\tau_o)$ = removal rate constant - which is a function of τ_o, the shear stress

Data was obtained which supported their model. A shear stress that is so high that all bacteria will be removed from the surface was noted. They termed this the critical shear stress value. The initial time period that bacteria were allowed to attach under no shear influenced the critical shear stress value. They tested four species and found that, in general, as the initial attachment period was increased, up to one hour, the critical stress increased. Increasing the initial attachment period beyond one hour did not influence the critical shear stress. There was actually variation between species on the actual boundary time at which initial attachment influenced critical shear stress. In one species initial attachment time greater than 15 to 30 minutes did not effect critical shear stress.

Mahoney et al. (1984) found that when cells were subjected to shear stress, their surface charge was reduced with increased length of time that they were subjected to the shear stress. They hypothesized that surface polymer, with its negatively charged sites, is removed from the cells by shear stress.

Ionic Strength

There have been a few studies investigating the influence of ionic strength on bacterial attachment. Meadows (1965) showed that marine bacteria attach optimally in a high ionic strength environment whereas freshwater bacteria attach optimally in a low ionic strength environment. Marshall (1971) demonstrated that reversible attachment in a marine bacteria followed the principles of VODL theory and double layer thickness with respect to ionic strength. Mahoney et al. (1984) noted that an increase in ionic strength resulted in a reduction of negative cell surface charge.

Calcium and Magnesium

Calcium and magnesium have been implicated as important ions, whose presence is required, for irreversible attachment to occur. Marshall (1971) 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. Mahoney et al. (1984) found that the extracellular polymeric substances of granules from an upflow anaerobic sludge blanket reactor contained 55% more calcium than whole granules. The percentage of sodium was also higher while the percentage of iron was reduced. Only small differences were observed

for magnesium and potassium. They also found that addition of EDTA, a chelating agent which would bind metal ions such as calcium, reduced the rate of flocculation.

Temperature and pH

There have only been a few studies of effects of temperature and pH on bacterial attachment to this writer's knowledge. Fletcher (1977) obtained some data on the attachment of stationary phase marine pseudomonad with respect to temperature. Cells suspended in filtered seawater at $3^{o}C$ did not attach as rapidly as those suspended in filtered seawater at $20^{o}C$. Fletcher and Floodgate (1973) observed a high pH in the growth medium prevented the appearance of primary polysaccharide in preparations of naturally attached bacteria. Adhesion was not impaired. Verrier (1984) compared bacterial attachment of methanogenic cultures, after a four hour incubation time, for a range of pH values between pH 6.5 and pH 8.0. The cultures had been fed either volatile fatty acids or sucrose and attachment was on to a polyvinylchloride surface. For the volatile fatty acid fed cultures, there was peak attachment at pH 7.4 at about 100 bacteria per 10^{-3} mm^2 (1000 bacteria per 10000 square micrometers). For sucrose fed cultures, there was peak attachment at pH 7.2 at about 300 bacteria per 10^{-3} mm^2 (3000 bacteria per 10000 square micrometers). As was mentioned earlier, Mahoney et al. (1984) noted that the surface pH of cells influence the cells' surface charge.

CHAPTER III

METHODS AND MATERIAL

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 3.1. It included a completely mixed anaerobic chemostat in which the culture growth rate was controlled, and an anaerobic attachment vessel in which

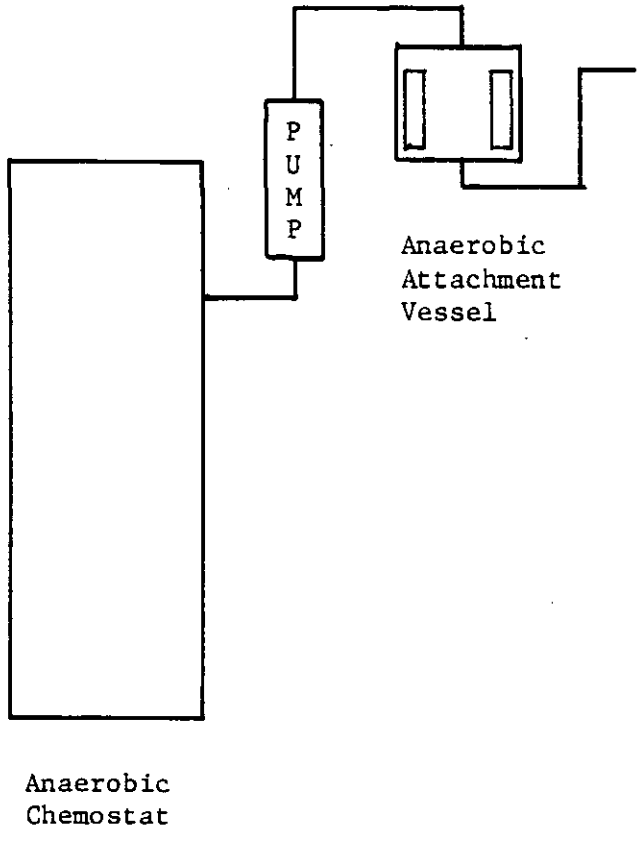


Figure 3.1 General Experimental Set-Up.

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 slides at time intervals and counting the bacteria which attached to the slides.

The inoculation time discussed in the experiments was the amount of time a slide was left in the 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.

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 included:

1. -glass attachment surface -- Attachment took place on glass microscope slides all provided from the same supplier (VWR Scientific Precleaned Plain Microscope Slides, No. 48300-25). The slides were cut to the size of 15 mm x 75 mm. They all received the same thorough

cleaning regime (described later in the methods section).

2. -the organism concentration (measured as bacteria per 100 mL) -- The organism concentration in the chemostat was adjusted to be kept 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 (7.1 0.2) 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.

6. -the salinity of chemostat effluent/attachment vessel influent -- The salinity was held constant for a given growth rate and between the two growth rates by adding a constant, large sufficient amount of dissolved solids to the feed such that the salinity of the effluents was approximately stable and equal.

The overall experimental procedure is outlined in Table 3.1.

Methods

Chemostat

The methane-forming anaerobic cultures used in the experiments described above were taken from a 69 liter working volume anaerobic chemostat (Figure 3.2) There are two reasons such a large chemostat was used. First, in the design stages of this experiment, it was believed that three attachment vessels would be hooked up simultaneously and it would have been necessary to supply all three mixed liquor on a daily basis. Second, other researchers (e.g. Molin et al., 1982) have observed significant attachment when their chemostat was operated at washout. They attributed this phenomenon to the attachment of sloughing bacteria from the walls of their chemostat. Thus a large reactor was used to minimize this problem.

Table 3.1 Overall Experimental Procedure

1. wash glass slides (chromic acid wash, distilled water rinse/ferrous ammonium sulfate wash/distilled water rinse/deionized water rinse)

2. place glass slides in attachment vessel

3. remove slides from attachment vessel and rinse 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

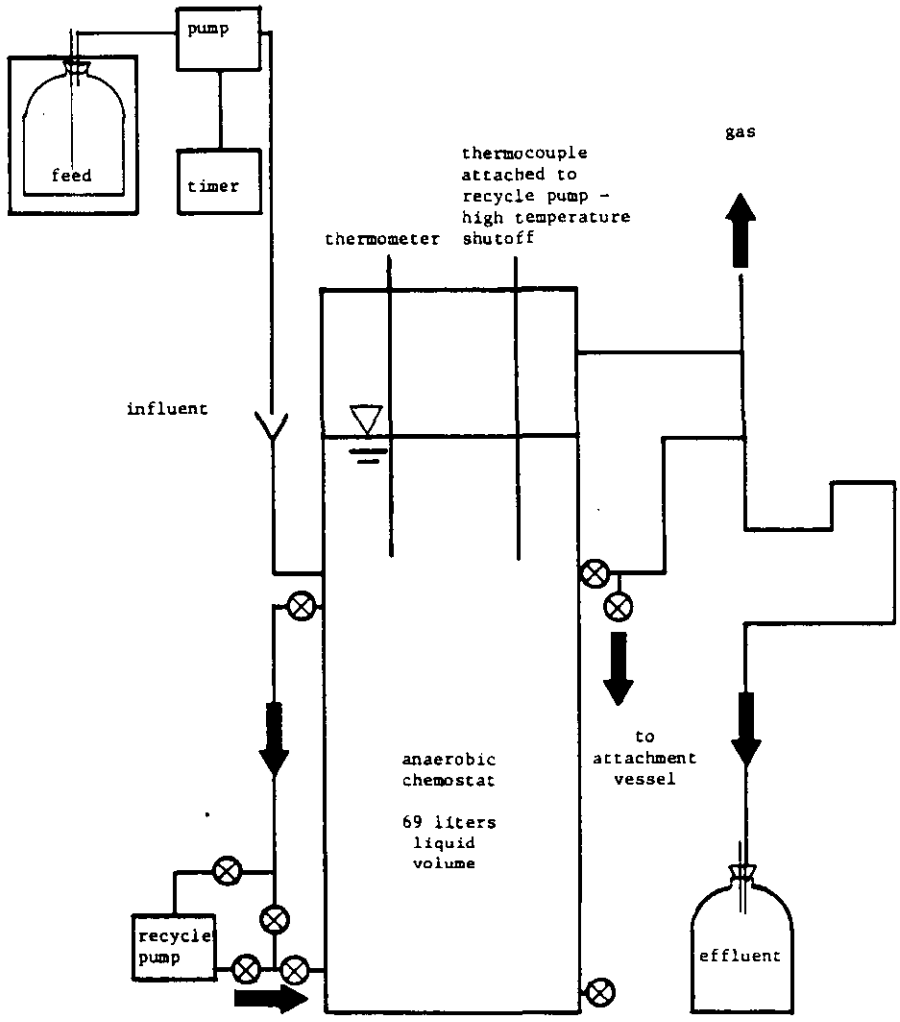


Figure 3.2 Anaerobic Chemostat.

The reactor was mixed by a recycle pump which took mixed liquor from the top of the reactor and pumped it into the bottom of the reactor. The recycle pump was only operated for one 5-minute interval at each half hour to prevent heat build-up in the reactor.

The chemostat was operated at two growth rates during the experiments, a eight day solids retention time (SRT) (0.125 per day dilution rate) and a 20 day SRT (0.05 per day dilution rate). These two growth rates were chosen because there is believed to be a population shift in the methanogen population of methane-forming anaerobic chemostats between 10 and 15 day SRT's (Lawrence and McCarty, 1969). Another, more recent, study concluded that a population shift occurs between a 6.5 and 9.6 day SRT (Noike et al., 1985). 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 8 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 variety of sources (Table 3.2). 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.

Table 3.2 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	

The feeds used in the reactors are summarized in Table 3.3, 3.4, and 3.5. 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 growth rates differed so that the organism concentration would be the same at the two growth rates. 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 determined for the 20 day SRT reactor. Second, the concentrations of influent salts used in other successfully operated research reactors was 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

$$0.25\ CH_2O + 0.0097\ HCO_3^- = 0.110\ CO_2 + 0.101\ CH_4 \qquad (3.1)$$
$$+\ 0.0097\ NH_4^+ \qquad\qquad + 0.039\ H_2O + 0.0097\ C_5H_7O_2N$$

Table 3.3 Stock Solutions And Daily Mixing Proportions - Day 124 To Day 559 - 20 Day SRT Experimental Reactor

	grams/L	
Stock #1	25.1	NH_4Cl
	16.4	K_2HPO_4
Stock #2	29.45	$MgCl_2.6H_2O$
	13.8	$FeCl_2.4H_2O$
	2.3	$CaCl_2$
	0.85	$CoCl_2.6H_2O$
	0.42	$NiCl_2.6H_2O$
Stock #3	17.8	L-cysteine-hydrochloride monohydrate
Stock #4	10	Yeast Extract

$NaHCO_3$	51.2 g	
Stock #1	609 mL	
Stock #2	244 mL	
Stock #3	122 mL	Basal Medium
Stock #4	122 mL	
Distilled Water	4.993 L	

Feed A - add 42.15 g sucrose to 4.215 liters of Basal Medium
Feed B - add 15.94 g sucrose to 1.875 liters of Basal Medium

-use 3.465 liters/day of Feed A for the 70 liter reactor (20 day SRT)
-use 1.875 liters/day of Feed B for the 15 liter reactor (8 day SRT)
-use 0.75 liters/day of Feed A for the 15 liter reactor (20 day SRT)

Table 3.4 Stock Solutions And Daily Mixing Proportions - Day 562 To Day 638 - 8 Day SRT Experimental Reactor

	grams/L	
Stock #1	50.2	NH_4Cl
	32.8	K_2HPO_4
Stock #2	58.9	$MgCl_2.6H_2O$
	27.6	$FeCl_2.4H_2O$
	4.6	$CaCl_2$
	1.7	$CoCl_2.6H_2O$
	0.85	$NiCl_2.6H_2O$
Stock #3	35.6	L-cysteine-hydrochloride monohydrate
Stock #4	20	Yeast Extract

$NaHCO_3$	94.8 g	
Stock #1	565 mL	
Stock #2	226 mL	
Stock #3	113 mL	Basal Medium
Stock #4	113 mL	
Distilled Water	10.273	

Feed A - add 89.57 g sucrose to 10.538 L Basal Medium
Feed B - add 7.5 g sucrose to 0.75 L Basal Medium

-use 8.663 L/day of Feed A for the 70 liter reactor (8 Day SRT)
-use 1.875 L/day of Feed A for the 15 liter reactor (8 Day SRT)
-use 0.75 L/day of Feed B for the 15 liter reactor (20 Day SRT)

Table 3.5 Reactor Feeds Final Concentrations

	0.125 volumes/day Dilution Rate 8 day SRT, mg/L	0.05 volumes/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

8 Day SRT

$$0.25\ CH_2O + 0.0117\ HCO_3^- = 0.107\ CO_2 + .\ 0.0958\ CH_4 \qquad (3.2)$$
$$+\ 0.0117\ NH_4 \qquad +\ 0.0117\ C_5H_7O_2N + 0.047\ H_2O$$

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.

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 3.3). 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

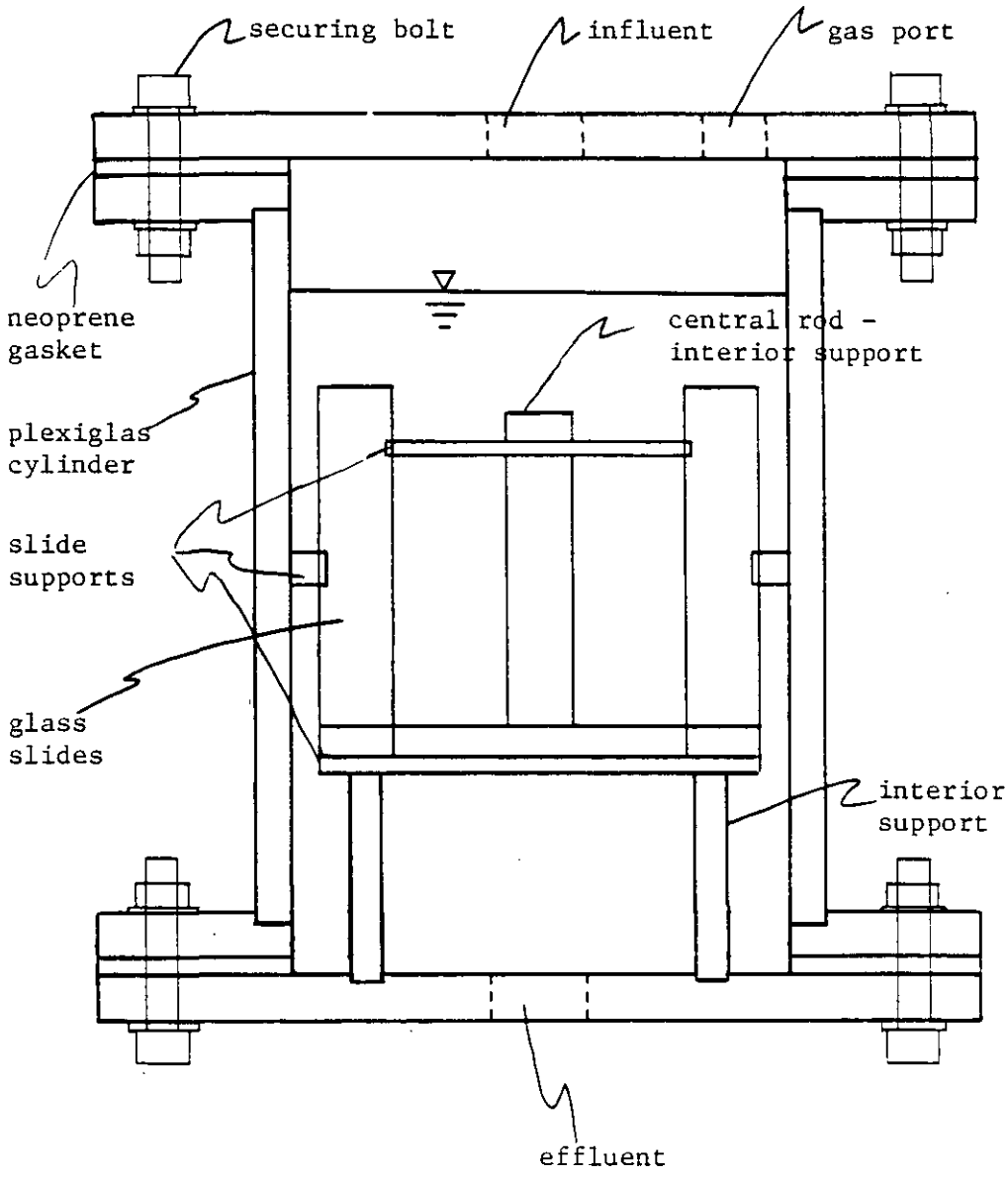


Figure 3.3 Anaerobic Attachment Vessel-Cross Section.

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
8. rinse 4 times in deionized water.

The components of the chromic acid and ferrous ammonium sulfate solutions are listed in Table 3.6.

Note that all references to "deionized water" in this dissertation describe water that has been treated by the Super Q treatment system (Millipore Corporation; Bedford, Massachusetts). Super Q water has been treated with reverse osmosis, carbon adsorption, ion exchange, and filtration (0.22 micrometer pore size).

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.

Table 3.6 Recipes For Chromic Acid Washing Solution And Ferrous Ammonium Sulfate Washing Solution.

Chromic Acid

-$NaCr_2O_7.2H_2O$	120 grams
-Distilled H_2O	1 liter
-Concentrated H_2SO_4	870 mL

Ferrous Ammonium Sulfate

-$Fe(NH_4)_2(SO_4).6H_2O$	98 grams
-dissolved in distilled H_2O	
-add 20 mL concentrated H_2SO_4	
-cool	
-dilute to 1 liter	

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 a Castle Sybron Corporation 1250 Labclave 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. 500 mL of mixed liquor were 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 mL 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.

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 < 3 ppm) 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 buffer (see Table 3.7) 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 of 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 3.4). The rinse buffer was dispensed from a 25 ml Fisher brand Schellbach burrets with 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 one quarter inch of the elevated portion of the slide. The fluid flowed down the slide and off the end. The

Table 3.7 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

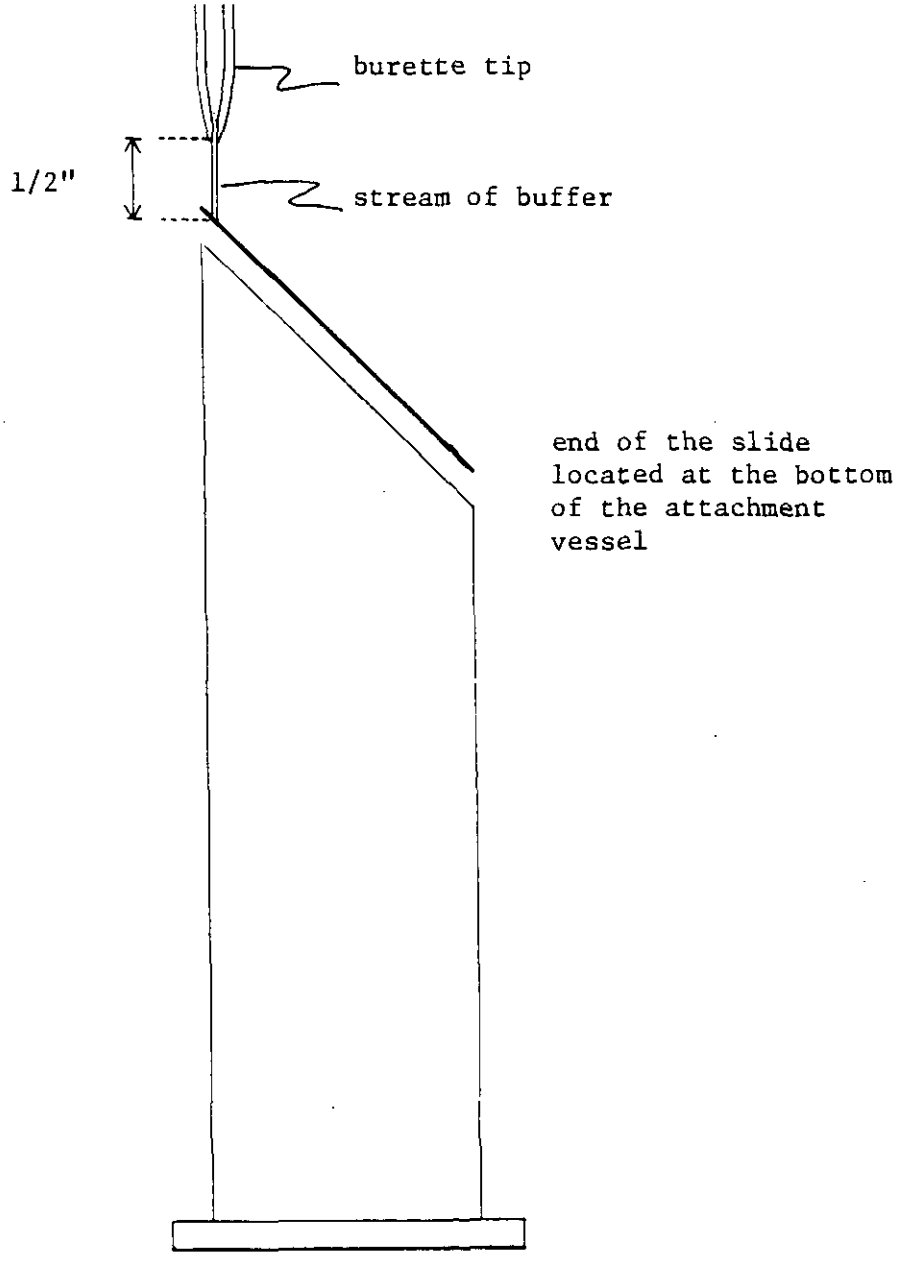


Figure 3.4 Slide Rinsing Technique.

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 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 17 mm from the edge of the slide (Figure 3.5). 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.

Cell 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

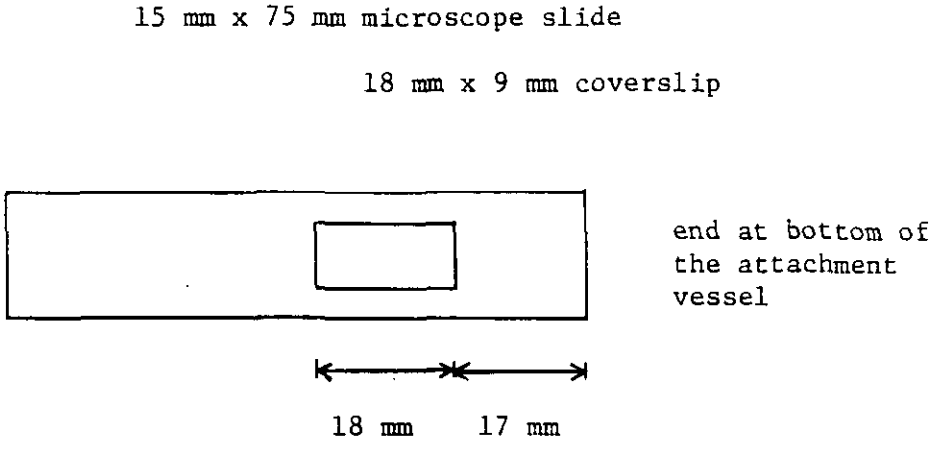


Figure 3.5 Microscope Slide And Coverslip Mounting Location Used For Bacterial Attachment Counts.

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 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 flourescence 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 3.6). 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.

The counting technique was 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

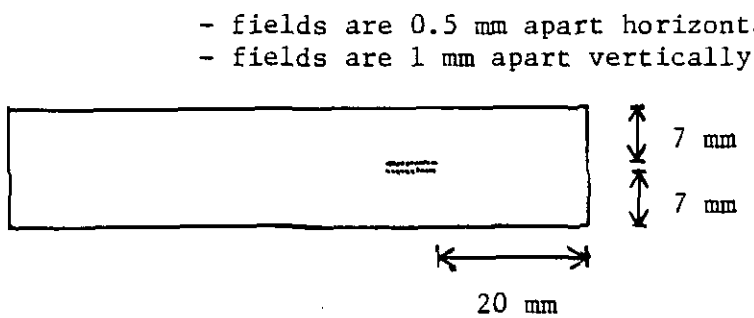


Figure 3.6 **Location Of Phase Contrast Microscope Fields Used For Bacterial Attachment Counts.**

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 wavelength 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. Do 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 5030 square micrometers, 745 square micrometers, 331 square micrometers, depending on the density of attachment. From 24 to 72 fields 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 3.7). 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 0.25 mm.

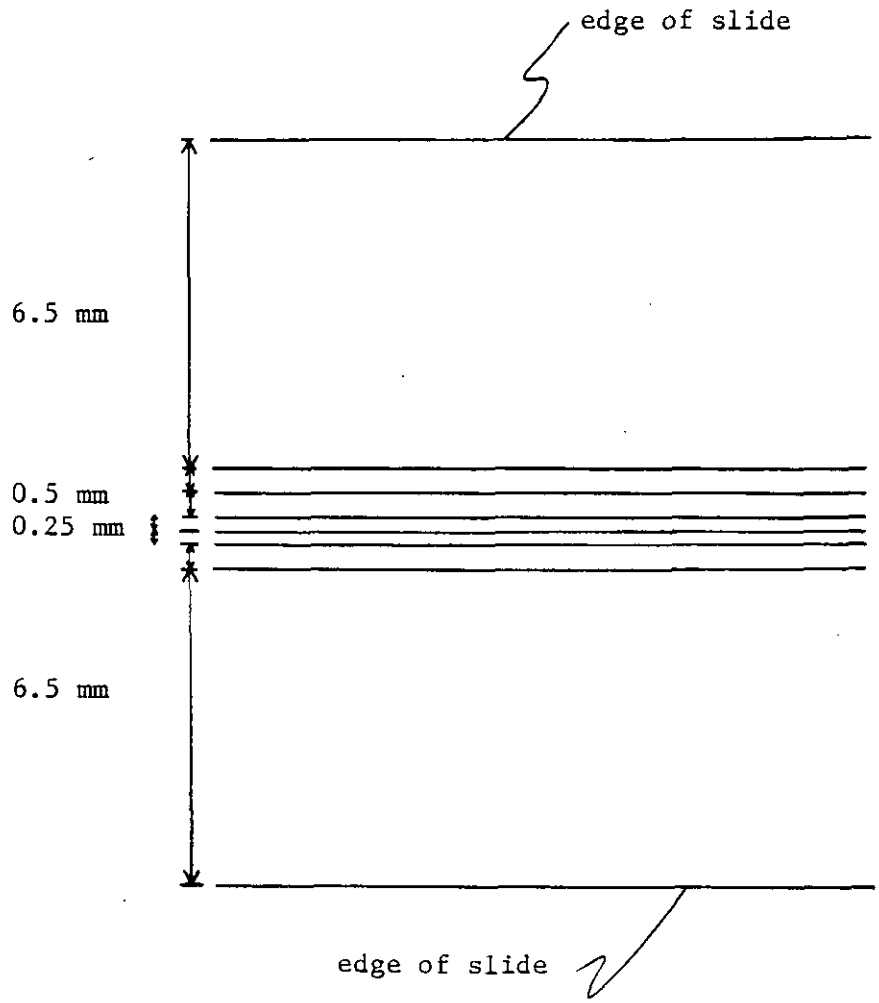


Figure 3.7 Fluorescence Microscope Counting Technique. Location Of Rows Counted.

The timing of the counts is summarized in Appendix B. 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.

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 5 - 10 mm by 5 - 10 mm, washed (in the same manner as microscope slides for counting), autoclaved, and over-dried at 100^{o}C. 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 3.8). The slides fit into the 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

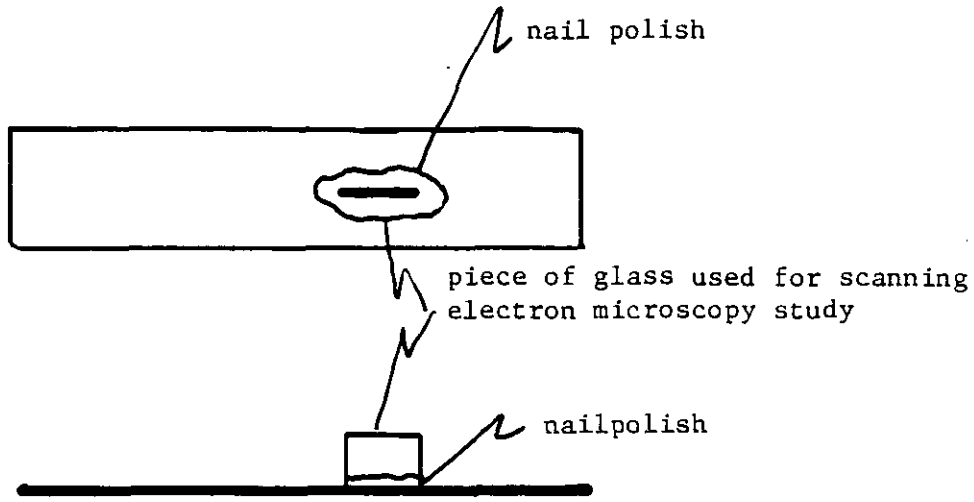


Figure 3.8 Slide Apparatus for Scanning Electron Microscopy.

rinse buffer. Here the sample piece of glass was removed using a ethanol flame sterilized razor knife to cut at the nail polish. When the nail polish holding the sample of glass was cut, the glass usually fell on one side. The side facing up 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, by grabbing it on the edges, and placed in a 25 mL 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, by hand, so the "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; 20%, 50%, 70%, 95%, and 100% 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.

Monitoring

A number of parameters were measured to monitor the condition of the anaerobic chemostat 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; feed total, effluent total, and effluent soluble chemical oxygen demand. The methods are summarized in Table 3.8.

Effluent Volume

Effluent was collected in a plastic carboy and the volume was measured each day. The volume measurement was done after any leftover feed in the feed bottle was emptied into the reactor.

pH

pH was measured by removing a 25 ml sample from the effluent sample tap and placing the pH probe in the sample. The sample was

Table 3.8 Monitoring of Chemostat

PARAMETER	TEST PROCEDURE	STANDARD DEVIATION	REFERENCE
Effluent Volume	Graduated Cylinder	± 40 mL	estimated
pH	Fisher Accumet pH Meter, Model 600	± 0.1 pH units	Standard Methods
Gas Volume	Wet Tip Gas Meter	± 10 %	estimated
Gas Composition	Gow-Mac Gas Chromatograph Series 550 Thermal Conductivity Detector	± 2 %	Standard Methods
Volatile Suspended Solids	Dried at 103-105°C/Combusted at 550°C	± 200 mg/L	data from this study
Total Suspended Bacteria Count	Petroff-Hausser Counting Chamber	± 0.5 bacteria/1000 μm^3	data from this study
Chemical Oxygen Demand	*Closed Reflux, Colorimetric Method*	± 10 % (coef. of variation)	Standard Methods
Temperature	Taylor Dial Thermometer	± 1 %	manufacturer's claim

allowed to a sit for 3 to 5 minutes before the pH reading was taken on a Fisher Accumet Model 600 pH meter.

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.

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 a set of measurements 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 start-up period but the gas chromatograph had not always been 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.

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 (Franson, 1985) 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 those thirty minutes, with the recycle pump still on, the effluent valve to the reactor was closed. 500 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(effective retention, 1.5 micrometers). 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.

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 20. The counts were done on the Ernst Leitz Wetzlar-SM Phase Contrast microscope using the 40X objective and a 10X eyepiece.

Organic Deposition/Removal Experiment

An experiment was done to see if the autoclaving caused the deposition or removal of organic compounds on the surface of the microscope slide during their preparation for the attachment experiments. All glassware and microscope slides for this experiment received the chromic acid/ferrous ammonium sulfate/distilled water/deionized water wash and rinse procedure described earlier for microscope slides. Caps for culture tubes received a soap and water wash, rinse with tap water to visibly remove soap bubbles, three rinses with distilled water, submerge in 20% H_2SO_4 for fifteen minutes, ten rinses with distilled water, and four rinses with deionized water. Microscope slides either received or did not receive a final autoclaving before an analysis was done for organic film deposition. The slides to be tested were placed, three at one time, in 30 mL of 1 Normal H_2SO_4 (made up using deionized water). The 30 mL of 1 Normal H_2SO_4 was contained in a Kimax Borosilicate Glass, 25

mm O.D x 150 mm long Reusable Culture Tube sealed with a teflon lined cap. For each group of three slides, the tube was suspended in a boiling water bath for fifteen minutes. The slides were removed and three more slides added. This procedure was followed until a total of twelve slides had been treated. The concentration of total organic carbon was then measured. This procedure was carried out for autoclaved slides and unautoclaved slides. A control in which no slides was used was also carried out. Each of the preparations described above (autoclaved, unautoclaved and control) was done in triplicate. Total organic carbon analyses were carried out using a Dohrmann DC-80 Total Organic Carbon Analyzer. The procedures descibed in Standard Methods For The Examination Of Water And Wastewater (Franson, M. et al., 1985), Section 505 B were followed.

CHAPTER IV

RESULTS

Steady State Data

Overview

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 steady state operation. The timing of the experimental runs in relation to the days after start-up is shown in Table 4.1. There are also a number of parameters which, taken together, give one a good idea of the condition of the methane-forming, anaerobic chemostat culture. The steady state variables monitored in this study are listed below. They include:

1. effluent volume
2. temperature
3. bacteria concentration
4. volatile suspended solids
5. pH
6. feed total, effluent total, and effluent soluble chemical oxygen demand
7. gas composition, and
8. gas production rate.

Table 4.1. Timing Of Experimental Runs In Relation To Start-Up Of Chemostat At A Particular Growth Rate.

Run #	SRT	Dates	Day of Operation	Days After Start-Up 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

A brief discussion of these parameters is included below.

Effluent Volume

An experimenter determines the growth rate of a chemostat by the rate that the mixed liquor of the chemostat (including the cells) is 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 rate is being maintained.

Temperature

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

Bacteria Concentration

Other workers have shown bacteria concentration in the bulk fluid has an influence on attachment (Chapter II). 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 rates. A chemostat operating at steady state has a constant bacteria concentration.

Volatile Suspended Solids

Volatile suspended solids is also a crude measure of the bacteria concentration.

pH

Properly operating methane-forming anaerobic digestors 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.

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.

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.

Gas Production Rate

The microorganisms in a chemostat operating at steady state should produce gas at a constant rate.

20 Day Solids Retention Time

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 Figure 4.1 and 4.2. 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 solids (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 bacteria concentration, also did not confirm the VSS increase.

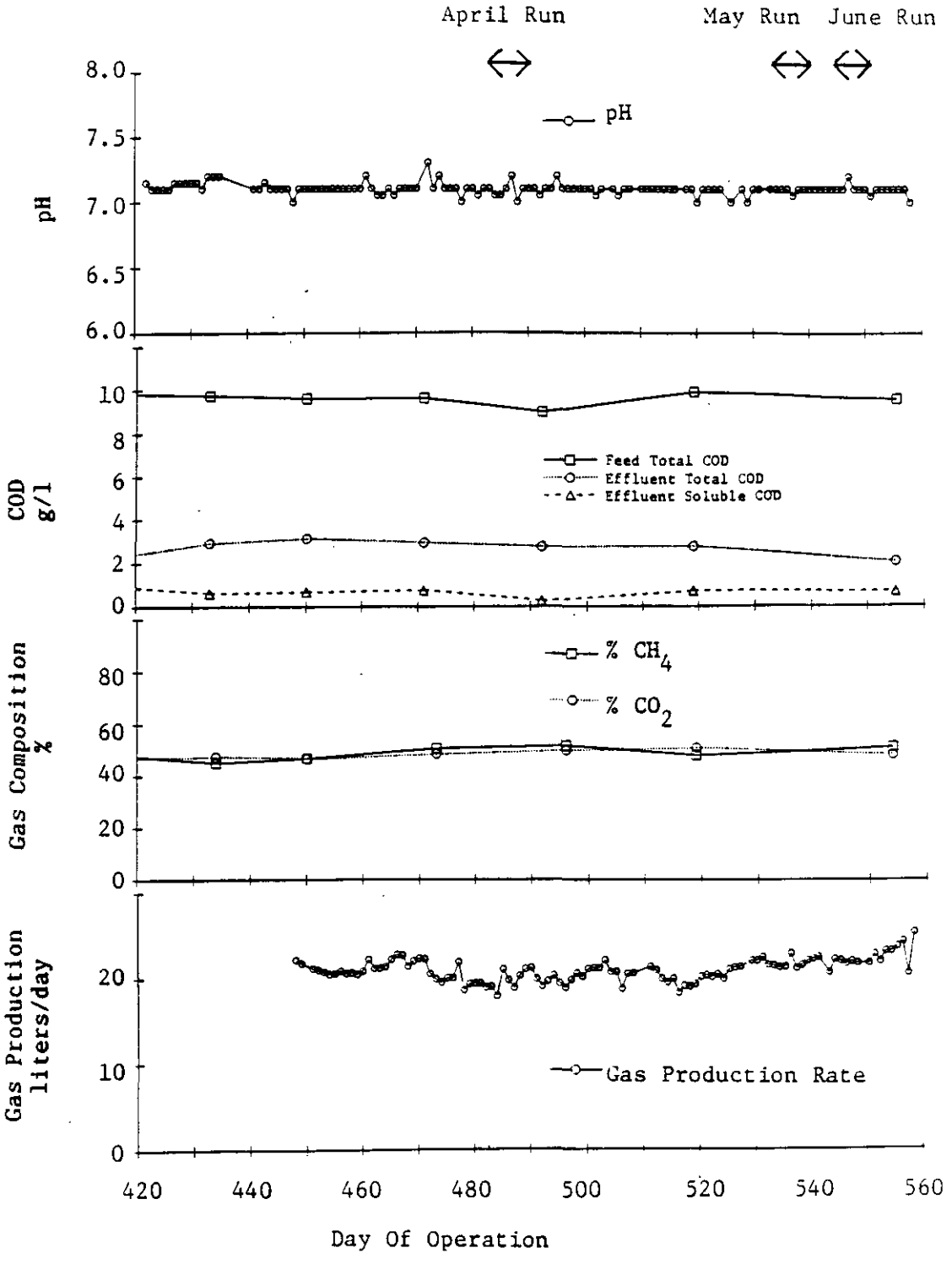


Figure 4.1 Day Of Operation Versus Steady State Parameters - 20 Day SRT - 0.05 Volumes/Day.

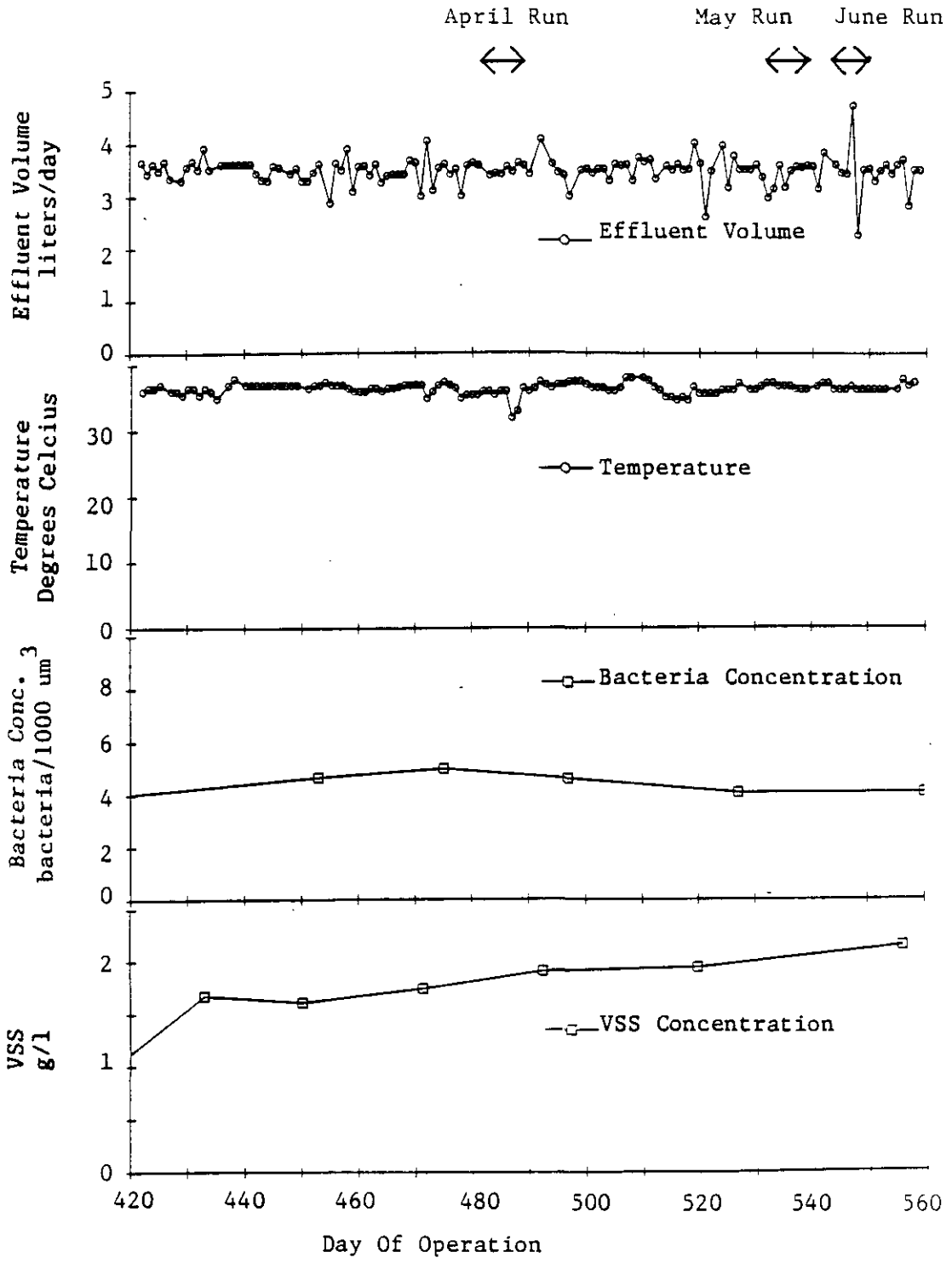


Figure 4.2 Day Of Operation Versus Steady State Parameters - 20 Day SRT - 0.05 Volumes/Day.

8 Day Solids Retention Time

The operation of the 69 liter chemostat at a 20 day SRT was terminated after 559 days of operation. The reactor was drained, thoroughly cleaned and rinsed.

On day 562 the 69 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 reactor 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 69 liter experimental reactor operating at an 8 day SRT are summarized in Figures 4.3 and 4.4. 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 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

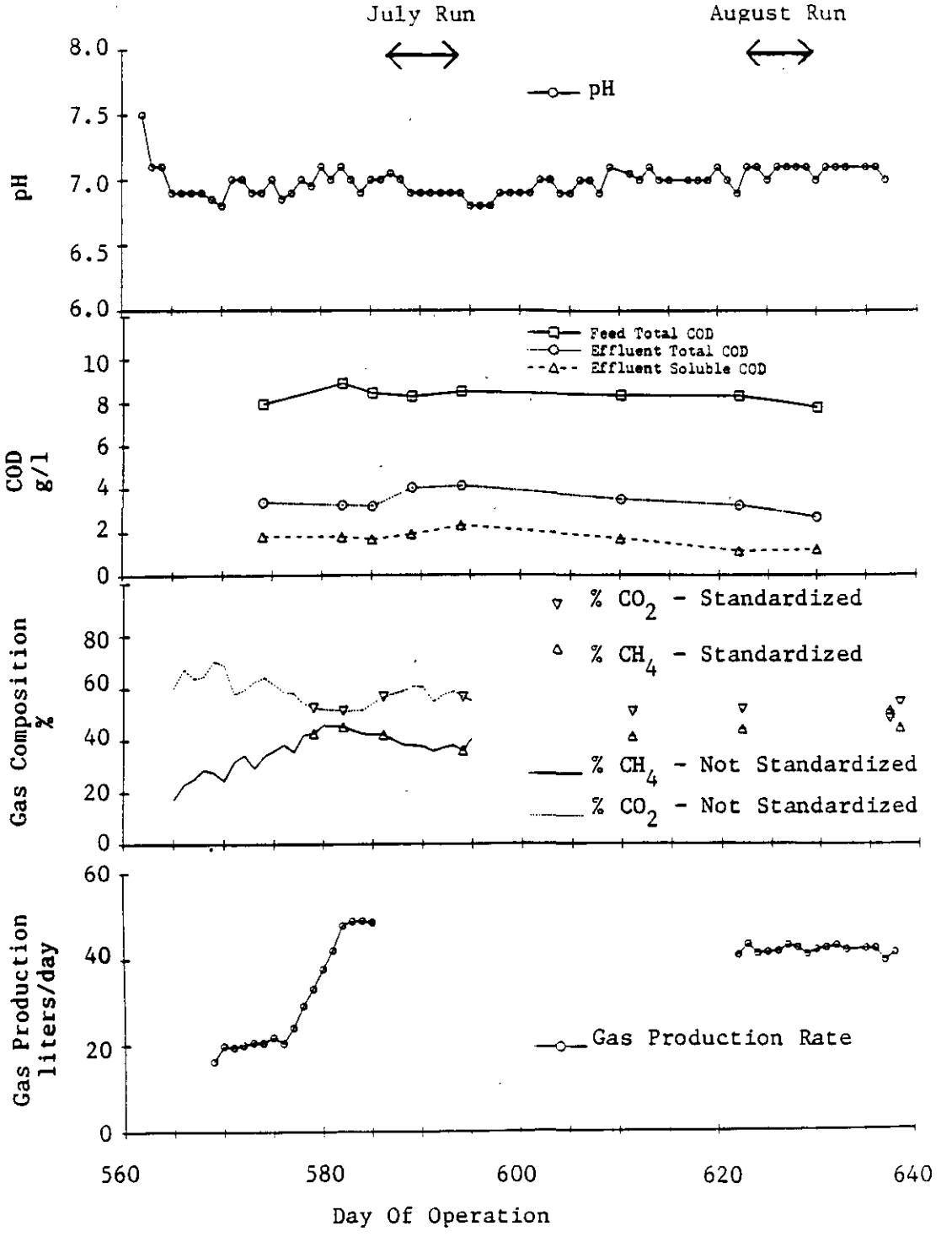


Figure 4.3 Day Of Operation Versus Steady State Parameters - 8 Day SRT - 0.125 Volumes/Day.

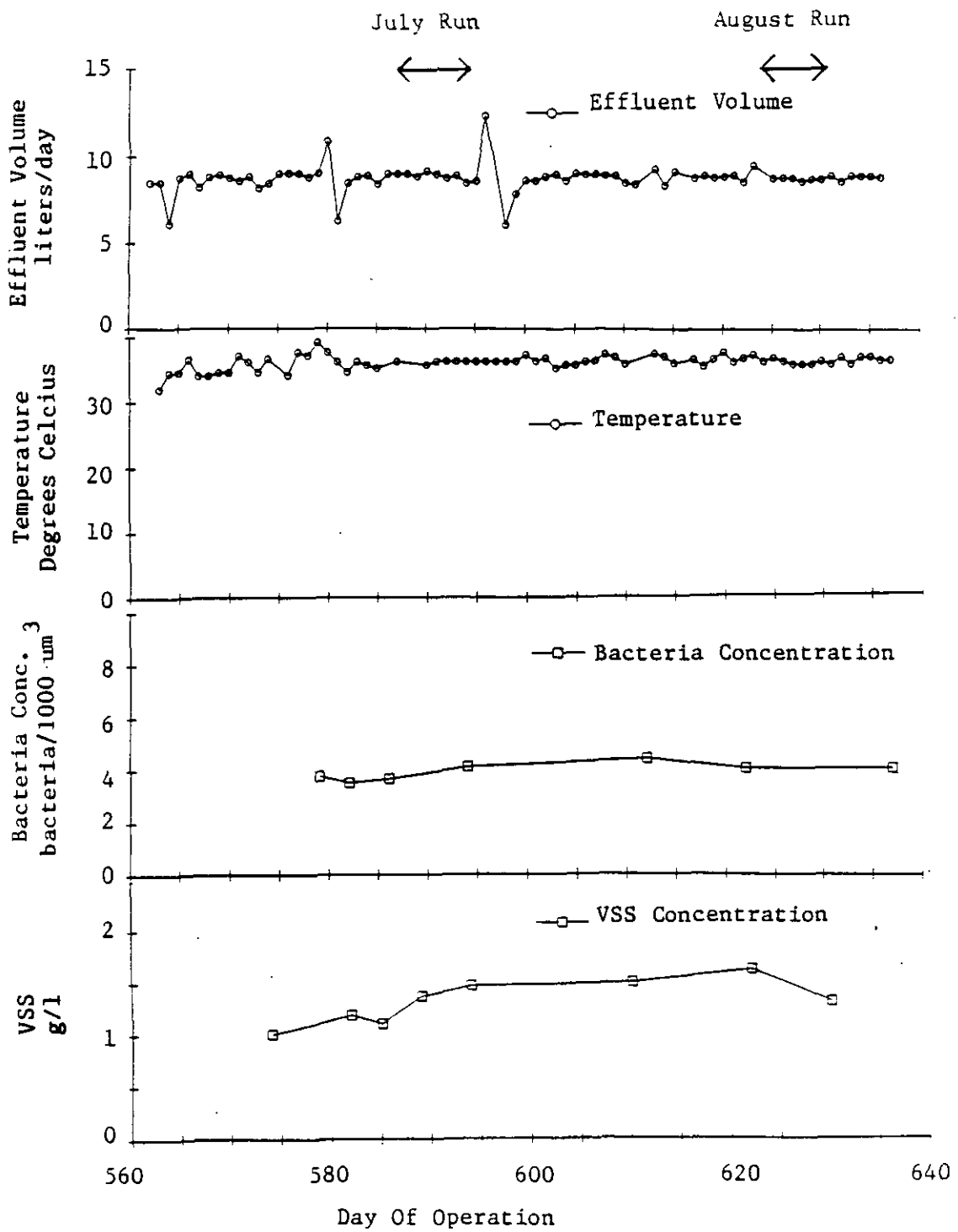


Figure 4.4 Day Of Operation Versus Steady State Parameters - 8 Day SRT - 0.125 Volumes/Day.

effluent gas line. This apparently caused a pressure buildup and a leak to occur because 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 measurements of soluble COD before the run ranged between 1644-1793 mg/l. Day 589's reading was 1898 mg/l. Day 594's 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 restabilized

(Figures 4.3 and 4.4). 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 1120 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 affected 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 returned to their previous values.

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 4.2

The data for each of the bacterial counts are summarized in Figures 4.5 to 4.9. The data used to construct these graphs are included in tabular form in Appendix C. 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

Table 4.2 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	5/21/86-5/28/86	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

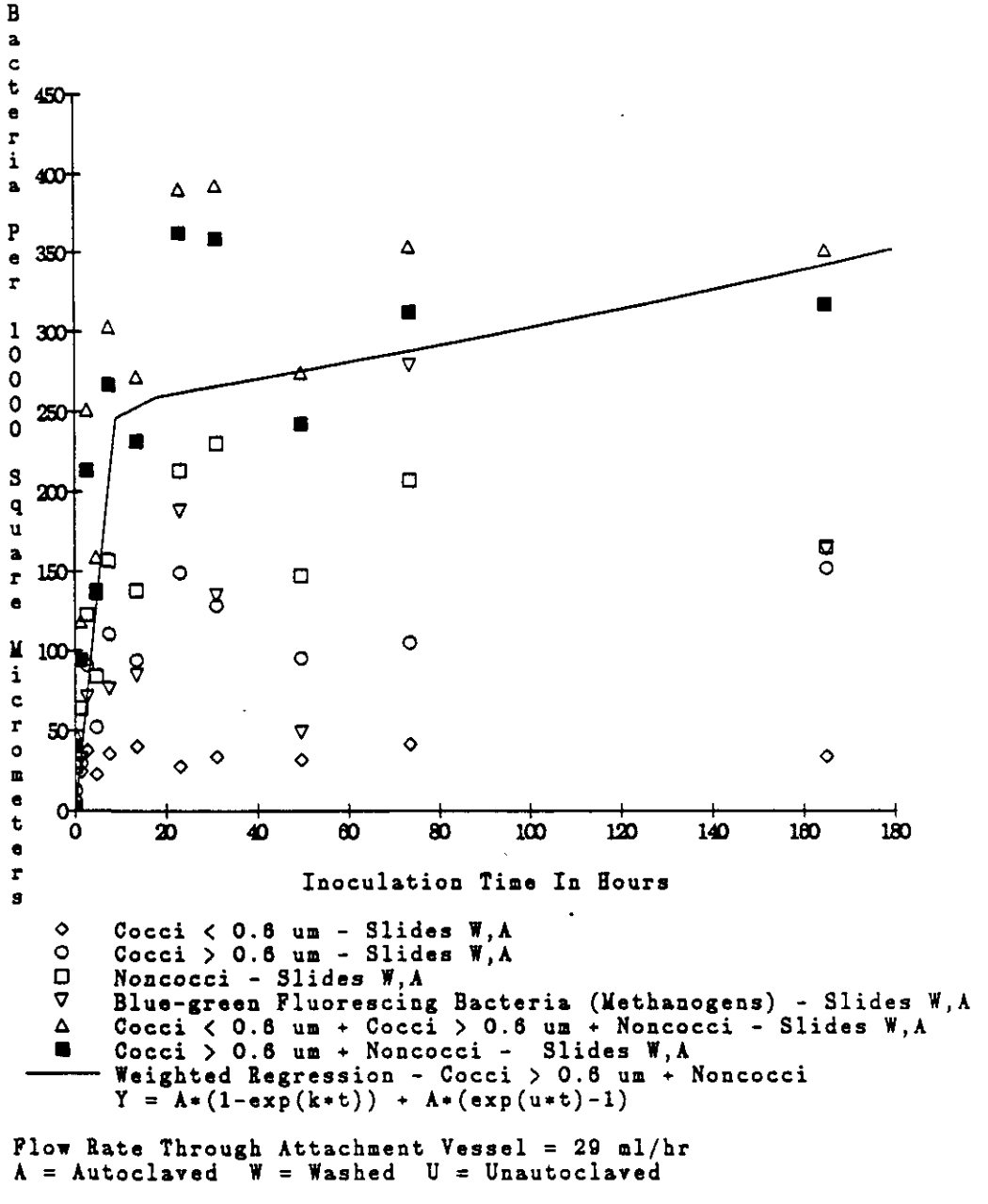


Figure 4.5 Inoculation Time Versus Number of Bacteria Irreversibly Attached Per 10000 Square Micrometers. 20 Day SRT - 0.05 Volumes Per Day Dilution Rate. April 1, 1986 Experimental Run.

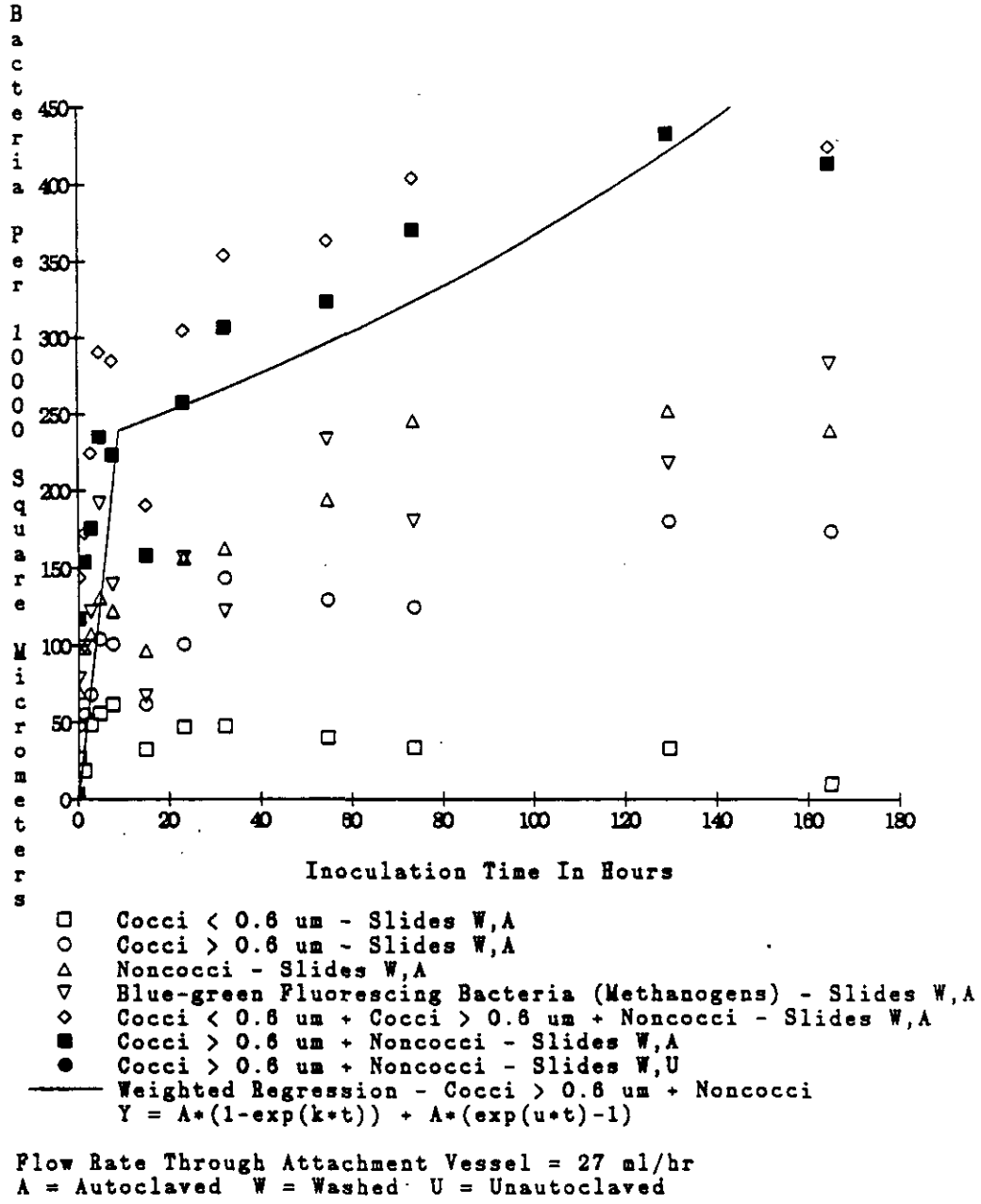


Figure 4.6 Inoculation Time Versus Number of Bacteria Irreversibly Attached Per 10000 Square Micrometers. 20 Day SRT - 0.05 Volumes Per Day Dilution Rate. May 21, 1986 Experimental Run.

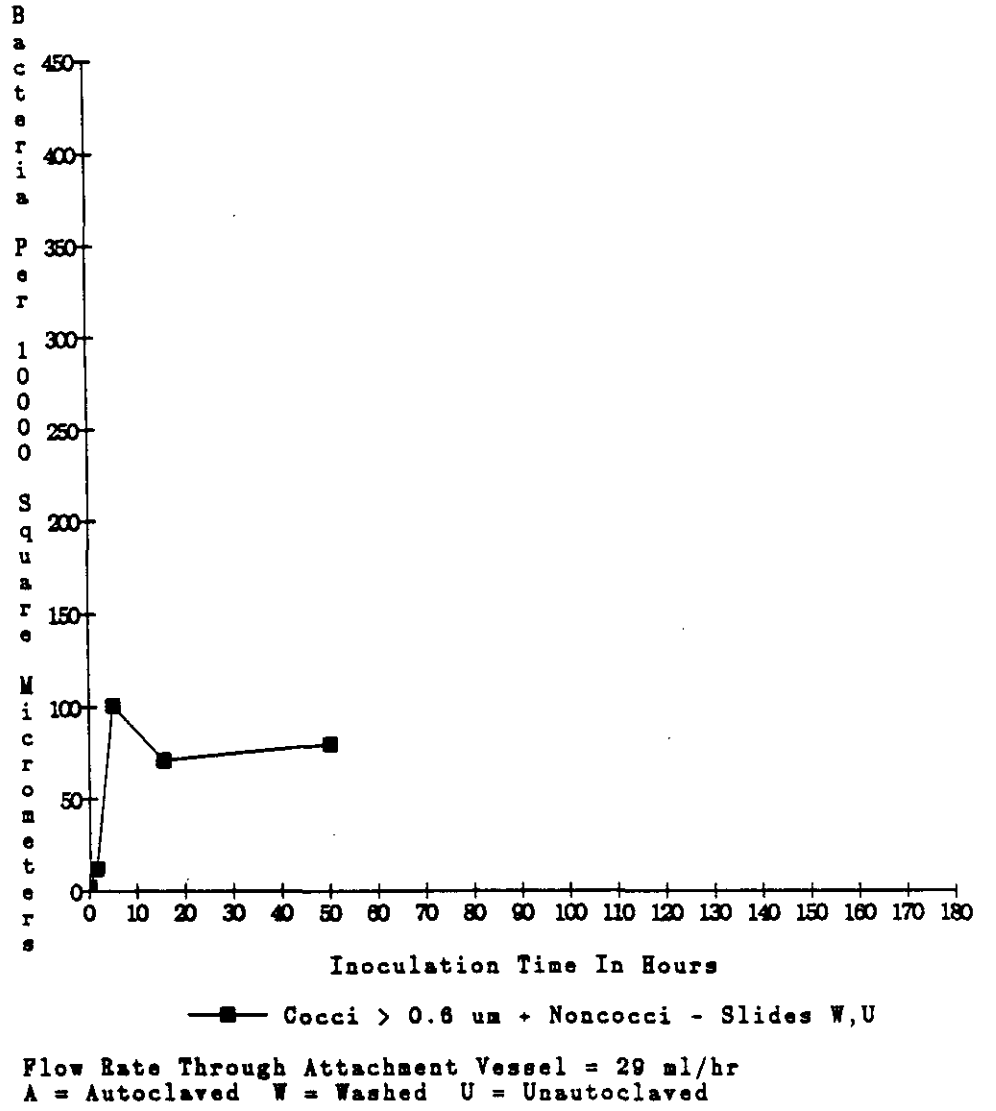


Figure 4.7 Inoculation Time Versus Number of Bacteria Irreversibly Attached Per 10000 Square Micrometers. 20 Day SRT - 0.05 Volumes Per Day Dilution Rate. June 1, 1986 Experimental Run.

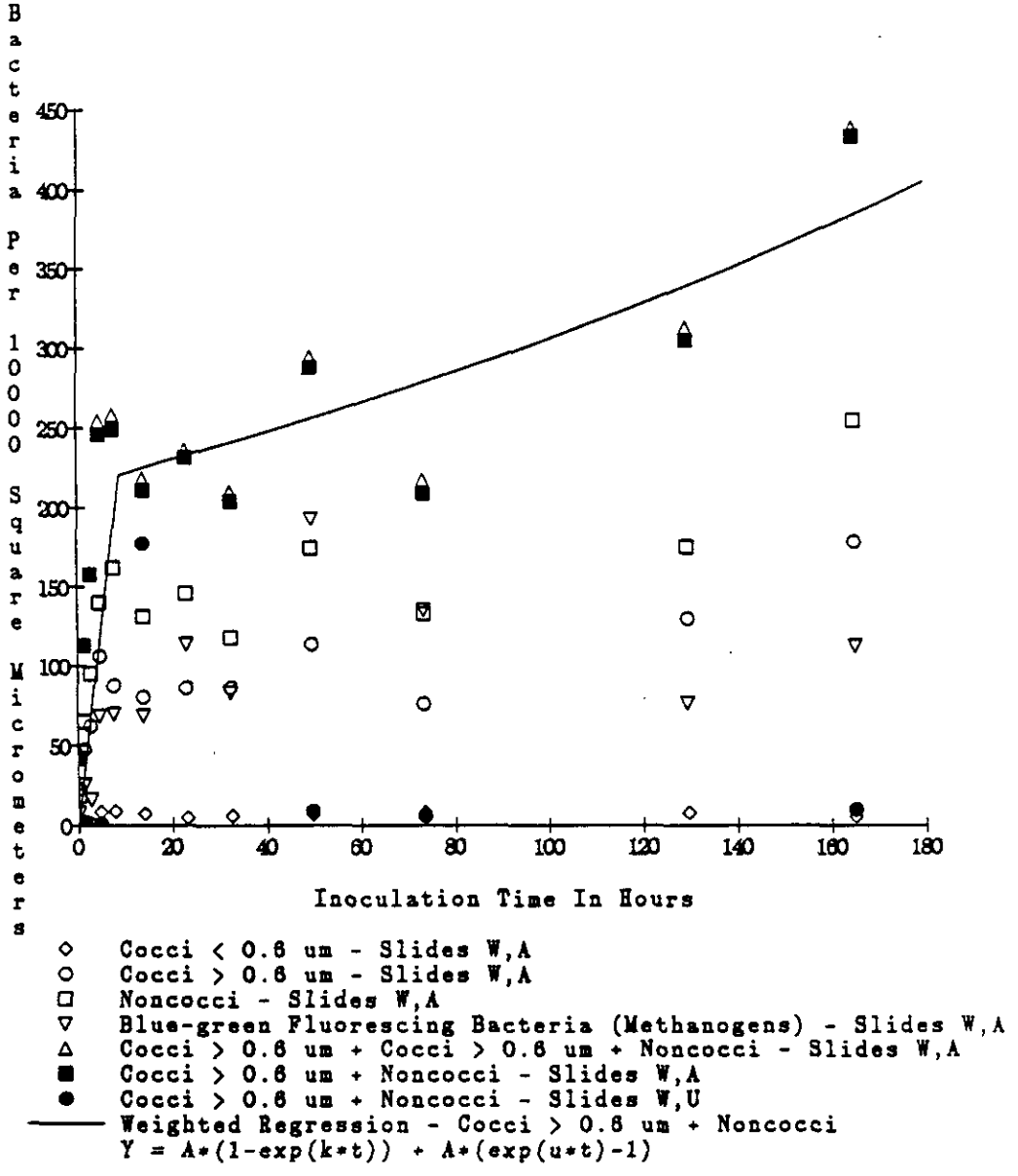


Figure 4.8 Inoculation Time Versus Number of Bacteria Irreversibly Attached Per 10000 Square Micrometers. 8 Day SRT - 0.125 Volumes Per Day Dilution Rate. July 15, 1986 Experimental Run.

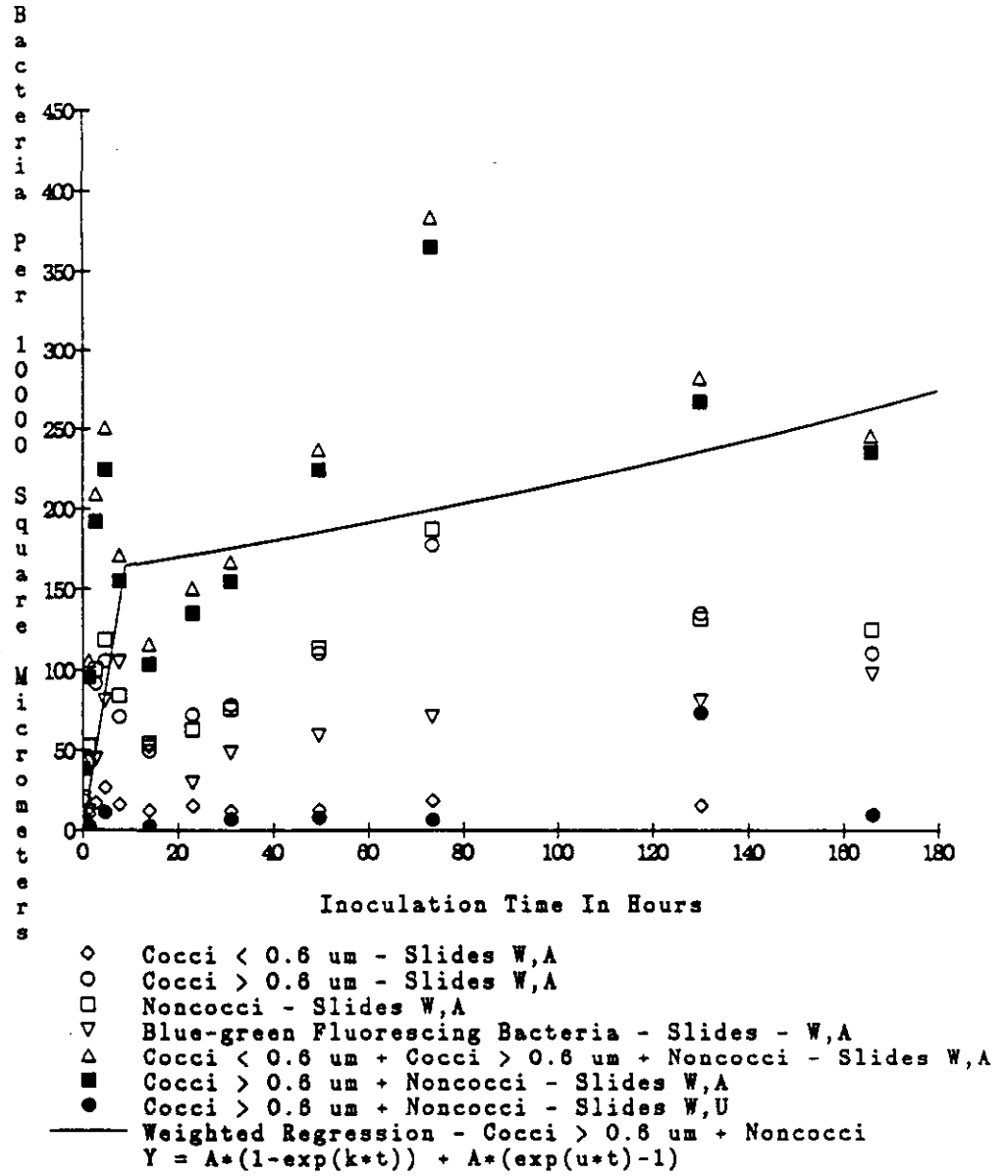


Figure 4.9 Inoculation Time Versus Number of Bacteria Irreversibly Attached Per 10000 Square Micrometers. 8 Day SRT - 0.125 Volumes Per Day Dilution Rate. August 19, 1986 Experimental Run.

fluorescing bacteria (methanogens)/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. The model function for the regression curve was

$$Y = A(1-e^{kx}) + A(e^{\mu x}-1) \qquad (4.1)$$

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.

Data Analysis

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 preliminary 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 decipher 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 coccus 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., 1980). 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. Thus, just as a full moon can make dim stars difficult to see, 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.

Finally, it appeared that when slides were stored for extremely long periods of time, greater than approximately 5 days, the fluorescent material inside the cells began to leak out (perhaps due to cell lysis or death) and coat the outside of the cells. This phenomenom may also have been caused by the fact that the wet mount of the slides tended to dry out at long storage times. The nail polish, used to seal the outer edges of the coverslip for the wet mount, also fluoresced under the fluorescence microscope set-up. Thus the nail polish might have a role in this phenomenom. It should be pointed out again at this point that all the fluorescence counts were done in 5 days or less and the majority of the fluorescence counts were completed in 3 days or less. Taken together, all these drawbacks listed above restrict the value of completing a formal statistical analysis on these data.

Statistical Design

The experimental design, for the purpose of inferential statistics, can be represented as shown in Figure 4.10. 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 6/1/86 run for scanning electron microscopy. The fact that run #1

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

Figure 4.10 Experimental Design -- Statistical Perspective

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 for the inferential statistical analysis. The problem was addressed by using a different method of analysis for comparing autoclaved versus unautoclaved data at the two growth rates.

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. Professional statisticians were consulted about the experimental design and formal statistical analysis using inferential statistics.

There were three 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 versus 20 day SRT)?
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 versus washed/unautoclaved)?
3. Is there a statistically significant change in content of total organic carbon on the surface of the microscope slides,

used to study bacterial attachment, caused by autoclaving the slides?

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 unweighted 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 chapter.

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.

To answer the third question, three different methods were also employed. A One-Way Classification Fixed Effects Model was used to assess whether the mean Total Organic Carbon values for each experimental slide treatment (autoclaved, unautoclaved, and control) was the same. When it was determined that all the means were not equal, Tukey's Test and the Newman-Keuls test were used to carry out pairwise comparison of each possible pair.

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 was compared using a t test.

Repeated measures growth curves analyses are discussed in detail by Winer (1971). The data summary for such an analysis is presented in Table 4.3. The analysis of variance table is presented in Table 4.4.

Such an analysis tested three hypotheses (Figure 4.11). The first hypothesis tests, as the null 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 sum 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 4.3 Repeated Measures Growth Curve Analysis.
Comparison Of Bacterial Attachment/Growth
At Different Growth Rates. 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	
8 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

bacteria/10000 μm^2

Summary

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

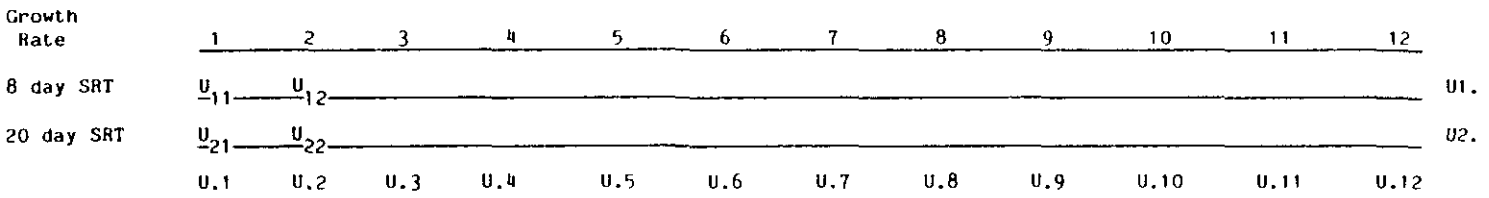
(4) $\frac{\Sigma B_j^2}{np} = \frac{9556471.9}{(2)(2)} = 2389120$

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

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

Table 4.4 Repeated Measures Growth Curve Analysis.
Comparison Of Bacterial Attachment At Different Growth Rates.
Analysis Of 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	



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

Figure 4.11 Schematic Representation Of Hypothesis Testing Of Repeated Measures Growth Curve Analysis. Comparison Of Bacterial Attachment At Different Growth Rates.

The hypothesis testing results for the repeated measures analysis are summarized in Table 4.5 and Figure 4.12. The hypothesis that the means of all the data points for each growth rate were equal was accepted. The hypothesis that the curves were parallel was accepted. The 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

Table 4.5 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 4.12 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 - $U_{21} - U_{11} = U_{22} - U_{12} \ldots = U_{212} - U_{112}$ Accepted

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:

$$\frac{dA}{dt} = kA \qquad (4.2)$$

that is, the rate that attachment sites disappear is directly

proportional to the number of attachment sites remaining where:

k = constant of proportionality -

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

The equation can then be integrated:

$$\frac{1}{A}dA = kdt \qquad (4.3)$$

$$\int_{A_o}^{At} \frac{1}{A}\, dA = \int_{o}^{t} kdt \qquad (4.4)$$

$$\ln \frac{A_t}{A_o} = kt \qquad (4.5)$$

$$e^{kt} = \frac{A_t}{A_o} \qquad (4.6)$$

$$A_o\, e^{kt} = A_t \qquad (4.7)$$

where:

A_o = total number of attachment sites, and

A_t = the number of attachment sites remaining at time t

$$Y = A_o - A_t \qquad (4.8)$$

$$A_t = A_o - Y \qquad (4.9)$$

$$A_o e^{kt} = A_o - Y \qquad (4.10)$$

substituting equation 4.7 into
equation 4.8 yields equation 4.12

$$Y = A_o - A_o e^{kt} \qquad (4.11)$$

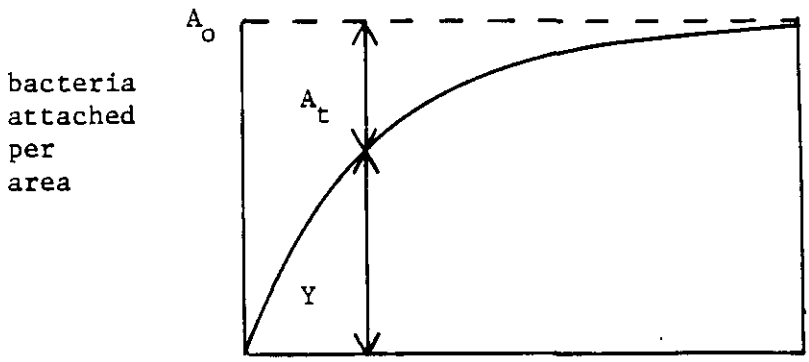
$$Y = A_o\,(1-e^{kt}) \qquad (4.12)$$

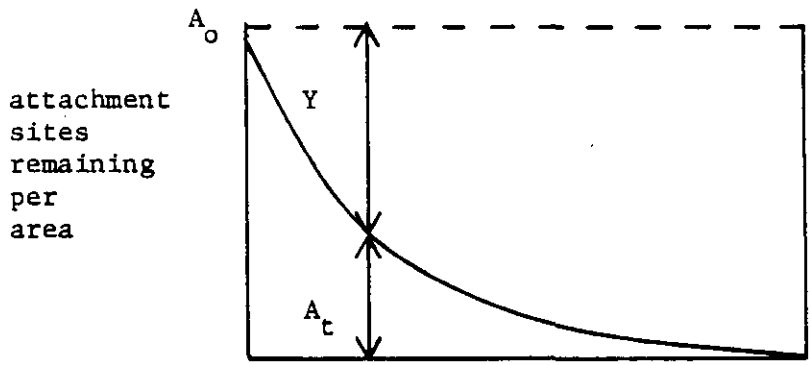
where:

Y = number of bacteria attached at time t

Schematically, equations (4.2), (4.7), (4.8) and (4.12) can be represented as shown in Figure 4.13.

For the growth term:





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 4.13 Schematic-First Order Attachment Model.

$$\frac{dY}{dt} = \mu Y \qquad (4.13)$$

where:

μ = specific growth rate $\quad \frac{\{\text{cells produced/time}\}}{\text{cells present}}$

$$\frac{1}{Y}\, dY = \mu dt \qquad (4.14)$$

$$\int_{Y_o}^{Y_t} \frac{1}{Y}\, dY = \int_{o}^{t} \mu dt \qquad (4.15)$$

$$\Big]_{Y_o}^{Y} \ln Y = \Big]_{o}^{t} \mu t \qquad (4.16)$$

$$\ln Y - \ln Y_o = \mu t \qquad (4.17)$$

$$\ln \frac{Y}{Y_o} = \mu t \qquad (4.18)$$

$$e^{\mu t} = \frac{Y}{Y_o} \qquad (4.19)$$

$$Y_o e^{\mu t} = Y \qquad (4.20)$$

where:

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

Y_0 = 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:

$$Y = \underset{\text{attachment}}{A_0 (1 - e^{kt})} + \underset{\text{growth}}{A_0 (e^{\mu t} - 1)} \qquad (4.21)$$

One noticeable simplifying assumption is made here. It is that the maximum number of cells attach very rapidly to the surface. The growth term assumes that from time 0, the maximum number of cells have attached and 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, to clarify terminology, Y_0 of equation (4.20) becomes A_0 in equation (4.21). A_0 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_0 = 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, $A_0/2$, one half the maximum concentration

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

t = inoculation time

Then:

$$Y = \frac{A_0\ t}{t + Km} \qquad (4.22)$$

Schematically, equation (4.22) can be represented as shown in Figure 4.14.

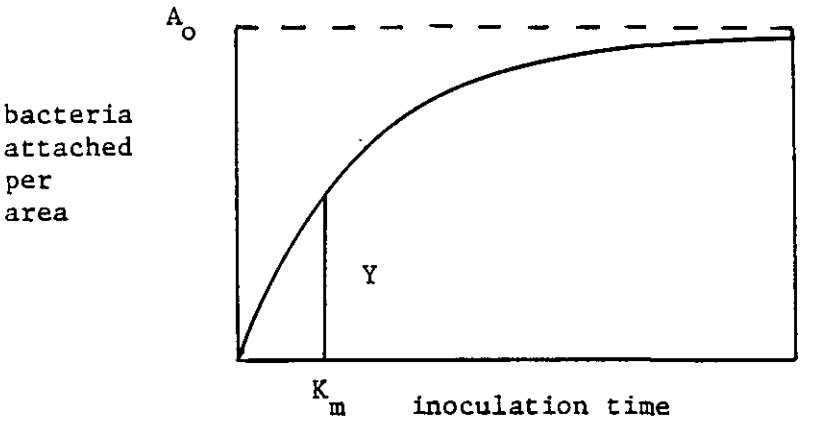
When the growth term from equation (4.20) is combined with the attachment term of equation (4.22), equation (4.23) is obtained.

$$Y = \underbrace{\frac{A_0\ t}{t + Km}}_{\text{attachment}} + \underbrace{A_0\ (e^{\mu t} - 1)}_{\text{growth}} \qquad (4.23)$$

The same simplifying assumption that the maximum number of cells is attached at time = 0 is made. Again, to clarify terminology, Y_0 in equation (4.20) becomes A_0 in equation (4.22). Also, A_0 is subtracted from the growth term in equation (4.23) 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 (Chapter V). 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



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 4.14 Schematic-Michaelis-Menten Type Attachment Model.

"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

$$W_i = \frac{1}{\sigma^2} \qquad (4.24)$$

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 inoculations 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 4.6.

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.

$$\sigma_T^2 = \sigma^2 + \sigma^2 + \sigma^2 + \sigma^2 \qquad (4.25)$$

total variance	physical act of counting differences	slide to slide differences	field to field differences on a slide	random error differences

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

Table 4.6 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				

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: $\overline{A_{20}} = \overline{A_s}$ A: $\overline{A_{20}} \neq \overline{A_s}$

$\overline{k_{20}} = \overline{k_s}$ $\overline{k_{20}} \neq \overline{k_s}$

$\overline{\mu_{20}} = \overline{\mu_s}$ $\overline{\mu_{20}} \neq \overline{\mu_s}$

All three hypotheses must be accepted in order of 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 (1984) 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: $A_{20} = A_8$ $\quad$ A: $A_{20} \neq A_8$

H_0: $K_{20} = K_8$ $\quad$ A: $K_{20} \neq K_8$

H_0: $U_{20} = U_8$ $\quad$ A: $U_{20} \neq U_8$

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 4.7. All three hypotheses were accepted. For two of the parameters, k and μ, 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 $A_{20} = A_8$. 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 4.7). In such an analysis, the coefficients were determined in the same way as the simultaneous analysis using a regression analysis. However,

Table 4.7 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 α = 0.05
Hypotheses	$\bar{A}_{20} = \bar{A}_8$	.06	2.02	6.965	accept	.187	$-188.9 \leq \bar{A}_{20} - \bar{A}_8 \leq 342.9$	
considered	$\bar{k}_{20} = \bar{k}_8$	.06	0.882	6.965	accept	.482	$-1.86 \leq \bar{k}_{20} - \bar{k}_8 \leq 2.40$	
simultaneously	$\bar{\mu}_{20} = \bar{\mu}_8$	.06	-0.61	6.965	accept	.62	$-0.0123 \leq \mu_{20} - \mu_8 \leq 0.0927$	
							95% confidence interval	
Hypotheses	$\bar{A}_{20} = \bar{A}_8$	.05	2.02	4.303	accept	0.187	$-87.29 \leq \bar{A}_{20} - \bar{A}_8 \leq 241.2$	0.90
considered	$\bar{k}_{20} = \bar{k}_8$	.05	0.882	4.303	accept	0.482	$-1.047 \leq k_{20} - k_8 \leq 1.587$	0.95
independently	$\mu_{20} = \mu_8$	.05	-0.61	4.303	accept	.62	$-0.0099 \leq \mu_{20} - \mu_8 \leq .007441$	0.90

each hypothesis was then considered separately. Because the hypotheses were considered separately, the 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 4.7. The values were estimated from Montgomery (1984, p. 25). The values ranged from 0.9 to 0.95 which means there is a 90% 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. If the number of experimental runs was increased, if the standard deviation of the coefficient values determined for the different runs was reduced, or if the difference between the coefficient means was increased, the probability of a Type II error would be reduced. For this experiment, the researcher can only 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 4.8. The weighted coefficients for the two growth rates were compared using a t test in the same methods the

Table 4.8 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	$\bar{A}_{20} = \bar{A}_8$	.06	1.79	6.965	accept	.2252	$-151.6 \leq \bar{A}_{20} - \bar{A}_8 \leq 257.1$	
considered	$\bar{k}_{20} = \bar{k}_8$	.06	0.49	6.965	accept	.69	$-2.266 \leq \bar{k}_{20} - \bar{k}_8 \leq 2.61$	
simultaneously	$\mu_{20} = \mu_8$	.06	0.0175	6.965	accept	> 0.8	$-0.009907 \leq \bar{\mu}_{20} - \bar{\mu}_8 \leq .009957$	
							95% confidence interval	
Hypotheses	$\bar{A}_{20} = \bar{A}_8$	.05	1.79	4.303	accept	.2252	$-73.5 \leq \bar{A}_{20} - \bar{A}_8 \leq 179.0$	0.90
considered	$\bar{k}_{20} = \bar{k}_8$	.05	0.49	4.303	accept	0.69	$-1.334 \leq \bar{k}_{20} - \bar{k}_8 \leq 1.678$	0.95
independently	$\bar{\mu}_{20} = \bar{\mu}_8$	.05	0.0175	4.303	accept	> 0.8	$-0.006111 \leq \bar{\mu}_{20} - \bar{\mu}_8 \leq .00616$	0.95

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 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 was accepted. The hypotheses that each individual coefficient was the same across the two growth rates were also accepted.

Comparison Of Attachment On Autoclaved Versus Unautoclaved Slides

This was not an experiment that was originally planned. Between the April and May experimental runs, the scanning electron microscopy (SEM) work associated with this experiment began. 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 initial, 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 a replicate run for the 20 day SRT. For the July and August 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 4.15. For the May, July, and August 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 (1984) 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 4.16. 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 4.9). The differences were

Figure 4.15 Experimental Design
Statistical Perspective Comparison Of The Effect
Of Slide Preparation On Bacterial Attachment

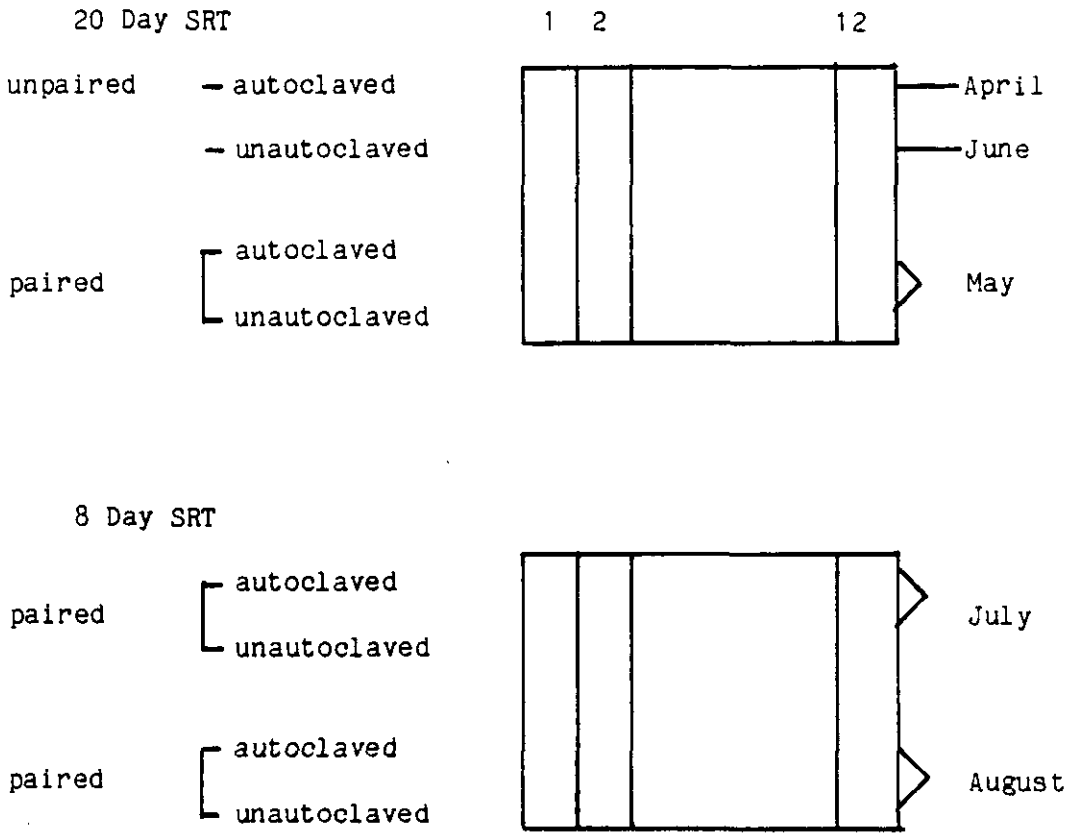


Figure 4.16 Randomized Complete Block Design To Compare The Effect Of Slide Preparation On Bacterial Attachment - 8 Day SRT

N = ab = total number of differences = 14

July (autoclaved minus unautoclaved)

August (autoclaved minus unautoclaved)

Blocks (b = 2)

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

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

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

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

Table 4.9 Calculations Of Differences In Bacterial Attachment/Growth From Slide Preparation At Each Inoculation Time - 8 Day SRT.

July
bacteria/10000 μm^2

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
bacteria/10000 μm^2

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

then used to carry out the statistical analysis (Table 4.10 and 4.11). The differences for the July 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: $Y_{1.} = Y_{2.} = Y_{3.} = Y_{4.} = Y_{5.} = Y_{6.} = Y_{7.}$

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 of variance table is summarized in Table 4.11. 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 that more bacteria attach overall and they attach at a faster initial rate on autoclaved slides versus unautoclaved slides.

The same repeated measures growth analysis method that was used to compare attachment at different growth rates was used to compare the

Table 4.10 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 4.11 Randomized Complete Block Analysis Of 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.

unpaired data for autoclaved versus unautoclaved slides at the 20 day SRT. The data summary for the analysis is presented in Table 4.12. The analysis of variance table is presented in Table 4.13.

The analysis tests three hypotheses (Figure 4.17). 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 4.18 and Table 4.14. 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

Table 4.12 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 = t 0	1.25	4.67	14.0	73.5	Total
Autoclaved	May	3.40	153.82	234.93	158.35	370.93	921.43
Σx^2 = 241528							
Autoclaved	April	0.38	93.94	136.28	232.01	312.84	775.45
Σx^2 = 179094.6							
Unautoclaved	May	1.79	12.83	24.62	22.64	58.76	120.64
Σx^2 = 4739.3							
Unautoclaved	June	2.17	12.07	101.1	71.1	65.83	252.27
Σx^2 = 19760.4							
SUMMARY							
Autoclaved		3.78	247.76	371.21	390.36	683.77	1696.88
Σx^2 = 819118.5							
Unautoclaved		3.96	24.9	125.72	93.74	124.59	372.91
Σx^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) $\frac{\Sigma A_i^2}{nq_2} = \frac{(1696.88)^2 + (372.91)^2}{(2)(5)} = 301846.36$

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

(5) $\frac{\Sigma(AB_{ij})^2}{n_2} = \frac{862461.4}{2} = 431230.7$

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

Table 4.13 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	

Figure 4.17 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 4.18 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 4.14 Summary Of The Hypothesis Testing Results For The Repeated Measures Growth Curve Analysis - Comparison Of Slide Preparation Techniques- 20 Day SRT.

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

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 4.15. The 20 day SRT data is included in Table 4.16. 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 4.17. The results of th 20 day SRT are shown in Table 4.18.

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.

Table 4.15 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, U bacteria/10000 μm^2	Slides W, U 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 4.16 Computation Of Mean Attachment Values For Washed/Autoclaved And Washed/Unautoclaved Slides - 20 Day SRT,

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 4.17 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 4.18 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	$153.7 \leq \mu_1 - \mu_2 \leq 405.45$

Testing Of Total Organic Carbon On the Surface Of Glass Slides Used For Attachment

The data for the testing of total organic carbon on the surface of glass slides is summarized in Table 4.19. The statistical analysis of this data is described below.

One-Way Classification Analysis of Variance. The data and its statistical set-up for the surface organics experiment are summarized in Table 4.20. The analysis of variance table for the one-way classification fixed-effects model (Montgomery, 1984) is summarized in Table 4.21. There were three different treatments for the slides before the procedure to measure total organic carbon on the surface was carried out. Slides were washed and autoclaved, washed and unautoclaved, or no slides were included in the extraction procedure (total organic carbon was measured of the blank sample). The experiment was repeated three times so there are three measurements for each treatment. The null hypothesis is that the means for all three treatments are equal, versus the alternative, that all three means are not equal. In statistical terminology

$$H_0: \mu_1 = \mu_2 = \mu_3$$

$$H_1: \mu_i \neq \mu_j \text{ (for at least one i, j)}$$

The null hypothesis was rejected for this analysis indicating at least one of the means was not equal to the others. The hypothesis testing results are summarized in Table 4.22.

Table 4.19. Results Of Total Organic Carbon (TOC) Analysis From Digestion Solution Of Microscope Slide Surface.

Slide Preparation	mg TOC/l for 12, 15 mm x 75 mm x 1 mm slides digested in 30 ml 1 N H_2SO_4				TOC On Slide Surface
Concentration	experiment replication mg TOC/l			ave.	ug TOC/cm^2
	#1	#2	#3		
autoclaved	1.1	1.0	1.0	1.033	0.11
Unautoclaved	1.1	1.7	1.8	1.533	0.16
No slides-control	0.75	0.72	0.83	0.767	0.079

Table 4.20 Data For One-Way Classification Fixed Effects Model - Analysis Of Total Organic Carbon On Slide Surface

Total Organic Carbon Measurement
(mg TOC/l for 12, 15 mm x 75 mm x 1 mm slides digested in 30 ml 1 N H_2SO_4)

		1	2	3	Y_i	$\bar{Y}_i$
Treatments	autoclaved	1.1	1.0	1.0	3.1	1.033
	unautoclaved	1.1	1.7	1.8	4.6	1.533
	no slides	0.75	0.72	0.83	2.3	0.767
					10.0	1.11

H_0: $\mu_1 = \mu_2 = \mu_3$

H_1: $\mu_i \neq \mu_j$ for at least one i, j

Table 4.21 Analysis of Variance For One-Way Classification Fixed Effects Model - Analysis Of Total Organic Carbon On Slide Surfaces.

Source of Variation	Sum of Squares	Degree of Freedom	Mean Square	F_0
Treatments	0.9089	2	.4545	9.095
Error	0.2998	6	.04997	
Total	1.2087	8		

$F_{(.05,2,6)} = F_{critical} = 5.14$

$F_0 > F_{critical}$ ---> *reject null hypothesis*

Table 4.22 One-Way Classification Fixed Effects Model-Summary.

$$H_0: \ \mu_1 = \mu_2 = \mu_3$$

$$H_1: \ \mu_i \neq \mu_j \ \text{(for at least one i,j)}$$

Null hypothesis rejected - At least one average value of total organic carbon on the slides is different from the others.

Tukey's Test And Newman-Keuls Test. In order to try to assess statistically which mean(s) differs from the others, two other analyses were carried out which compare pairs of treatment means. These two analyses are discussed by Montgomery (1984). They are Tukey's Test (Table 4.23) and the Newman-Keuls Test (Table 4.24). The statistical tests show:

1. there is a statistically significant difference between the control and the washed/unautoclaved sample
2. there is no statistically significant difference between the control and the washed/autoclaved sample.
3. it is a borderline case if there is a statistically significant difference between the total organic carbon measurements for washed/autoclaved and washed/unautoclaved slides (Tukey's Test - no difference, Newman-Keuls - significant difference.

While the statistical results are somewhat inconclusive, it is important to consider the actual concentrations of total organic carbon that were found on the slide surfaces. They are shown in Table 4.19. It should be pointed out that all the values are extremely low. It appears doubtful that anything of significance with respect to organic compounds is happening on the surface of the glass slides during the autoclaving process.

Table 4.23 Summary Of Tukey's Test Comparing Pairs Of Treatment Means - Analysis Of Total Organic Carbon On Slide Surfaces.

for each mean

$H_0: \mu_1 = \mu_2$

$H_1: \mu_1 \neq \mu_2$

$T_\alpha = q_\alpha (a,f) S_{\bar{y}_i}$

$S_{\bar{y}_i} = \sqrt{\frac{MS_E}{n}}$

$f = n - a$

$T_{(.05)} = [q_{(.05)} (3,6)] \quad (\sqrt{\frac{.04997}{3}})$

$N - a = 9 - 3 = 6$

$T_{(.05)} = (4.34)(.129)$

$T_{(.05)} = 0.56 =$ critical value

autoclaved vs. unautoclaved	1.533 - 1.033 = 0.5	no significant difference
autoclaved vs. control	1.033 - 0.767	no significant difference
unautoclaved vs. control	1.533 - 0.767	significant difference

Table 4.24. Summary Of Newman - Keuls Test Comparing Pairs Of Treatment Means - Analysis Of Total Organic Carbon On Slide Surfaces.

$\bar{y}_{3.} = 0.767$

$\bar{y}_{1.} = 1.033$

$\bar{y}_{2.} = 1.533$

$K_p = q_\alpha (p,f) S_{\bar{y}_i} \qquad p = 2,3$

$K_2 = q_{.05} (2,6) S_{\bar{y}_i} = (3.46) (0.12906) = 0.4465$

$K_3 = q_{.05} (3,6) S_{\bar{y}_i} = (4.34) (0.12906) = 0.56$

unautoclaved vs. control	$1.533 - 0.767 = 0.766 > 0.56$	reject
unautoclaved vs autoclaved	$1.533 - 1.033 = 0.50 > 0.4465$	reject
autoclaved vs. control	$1.033 - 0.767 = 0.266 < 0.4465$	accept

Chapter V

DISCUSSION

Summary Of Inferential Statistics

The results of the inferential statistical analysis are summarized below.

Comparison of Bacterial Attachment at Two 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 Bacterial Attachment For Two Slide Preparations

1) Randomized Complete Block Analysis (8 day SRT only)	The null hypothesis tested if the mean differences (between autoclaved and unautoclaved) were equal at each time point. It was known that the mean difference 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.

Comparison of Total Organic Carbon on the Slide Surface

For Two Slide Preparations

1) One-Way Classification Values Fixed Effects Model	Hypothesis that all three for TOC on slide surfaces (autoclaved, unautoclaved, control) were equal was rejected
2) Tukey's Test	Pairwise comparisons of TOC on slide surfaces yielded the following results. autoclaved vs. unautoclaved - no significant difference autoclaved vs. control - no significant difference unautoclaved vs. control - significant difference
3) Newman-Keuls Test	Pairwise comparisons of TOC on slide surfaces yielded the following results

autoclaved vs. unautoclaved -
significant difference

autoclaved vs. control -
no significant difference

unautoclaved vs. control -
significant difference

Descriptive Statistics

The attachment data were also considered using descriptive statistics. The most important data for each growth rate can be shown on a single graph. Figure 5.1 is a graph of inoculation time versus number of bacteria attached per 10,000 square micrometers for the 20 day SRT. Figure 5.2 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 techniques 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

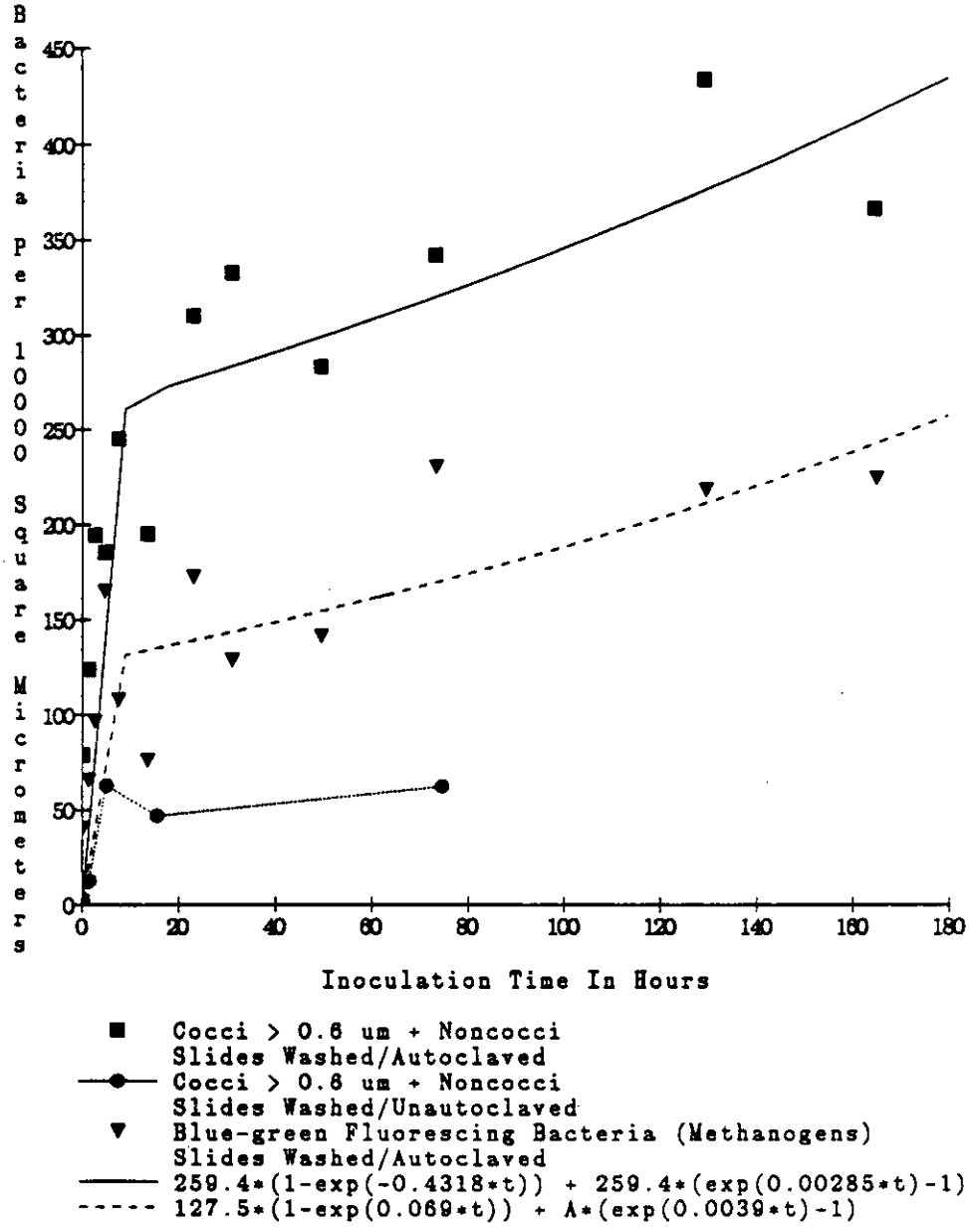


Figure 5.1 20 Day SRT Data-First Order Model. Inoculation Time Versus Number Of Bacteria Irreversibly Attached Per 10000 Square Micrometers.

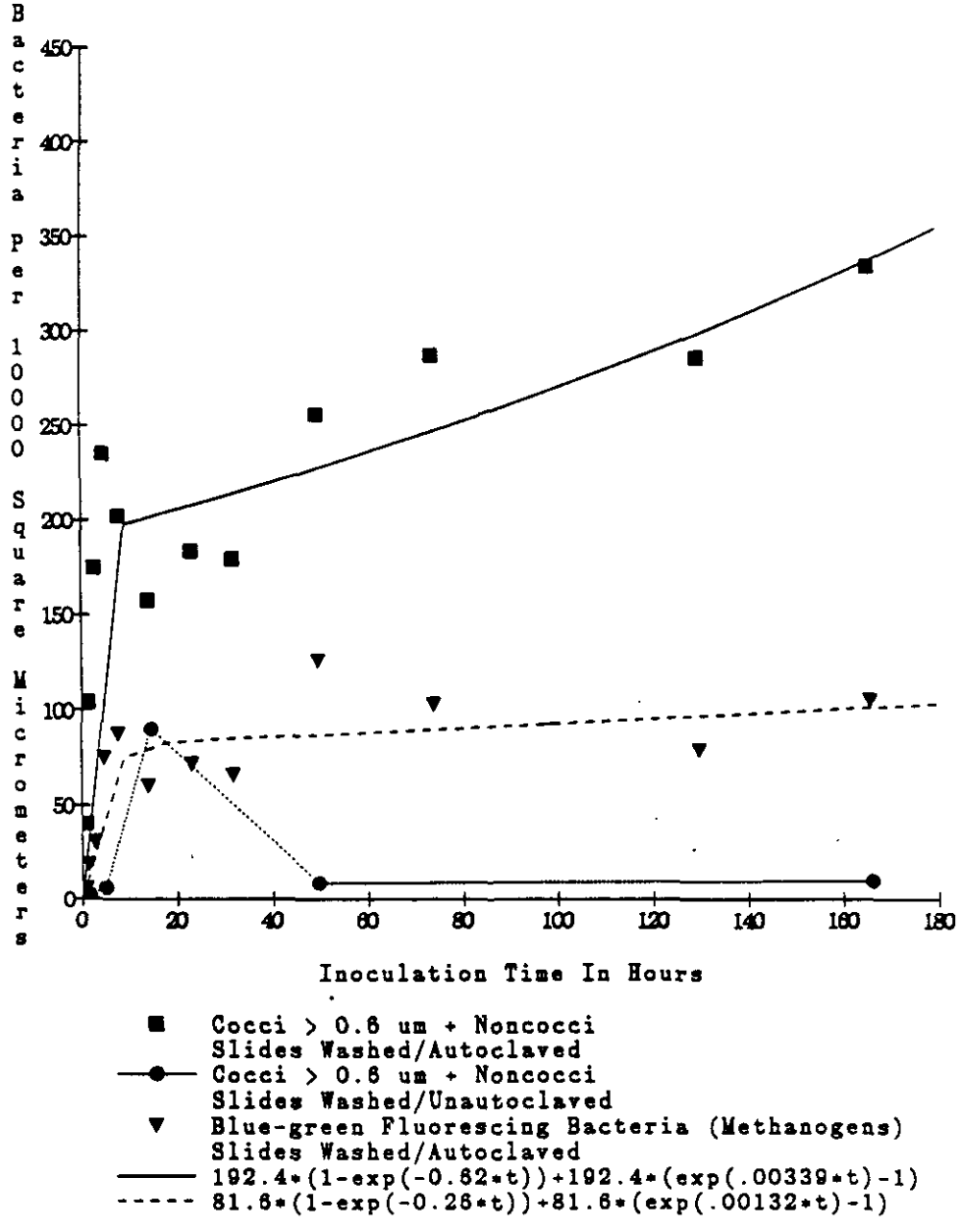


Figure 5.2 8 Day SRT Data-First Order Model.
Inoculation Time Versus Number Of Bacteria
Irreversibly Attached Per 10000 Square Micrometers.

curves for the first order model described earlier. In Figures 5.3 and 5.4 the same graphs are shown with the Michaelis-Menten type model. Figure 5.5 shows that the curves obtained by the two models are very similar.

The following observations and conclusions can be made from inspection of Figures 5.1 to 5.5. 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 difference can be noted in the pattern of attachment on washed/autoclaved glass slides for the cocci > 0.6 μm plus 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 (most 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

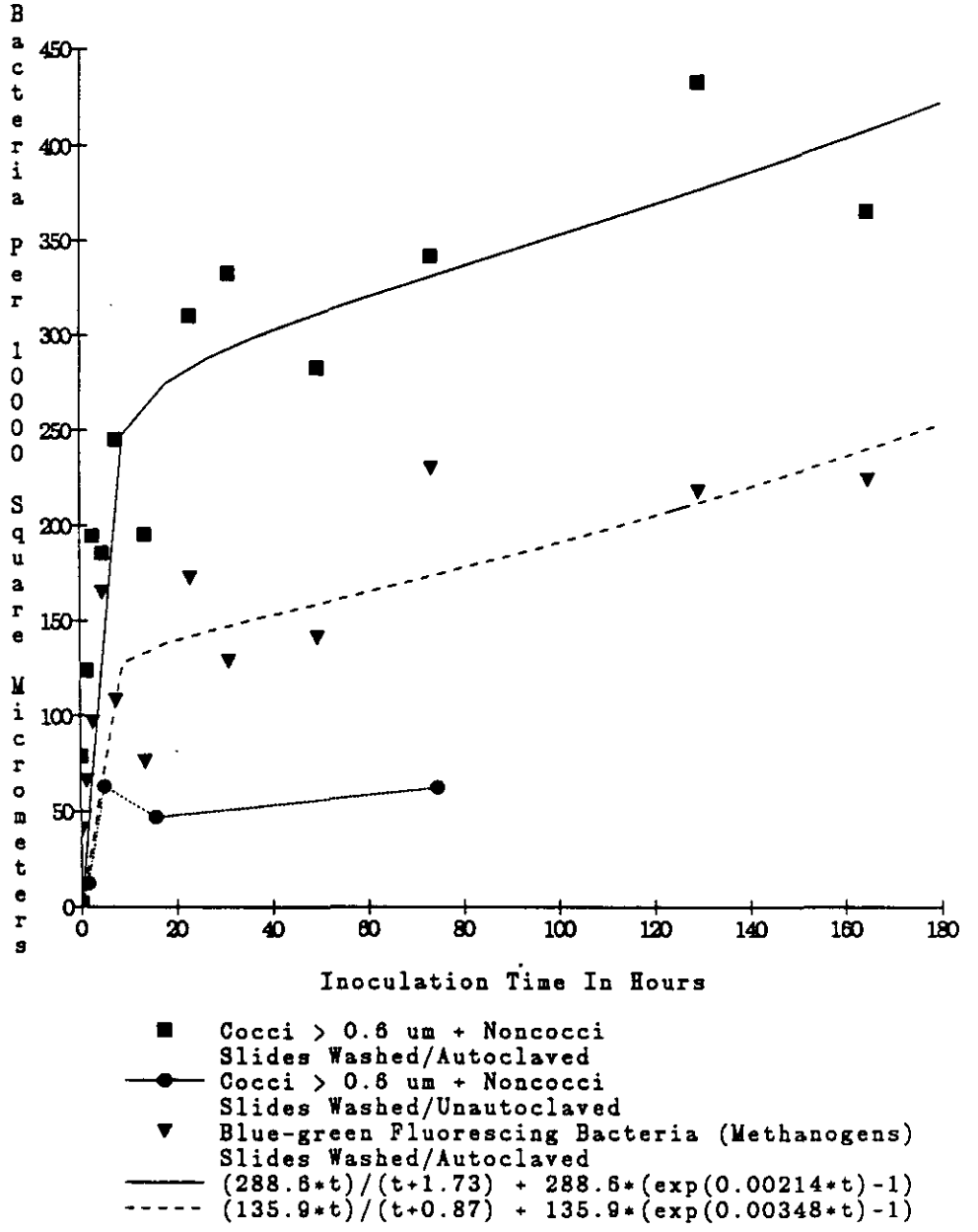


Figure 5.3 20 Day SRT Data-Michaelis-Menten Type Model. Inoculation Time Versus Number Of Bacteria Irreversibly Attached Per 10000 Square Micrometers.

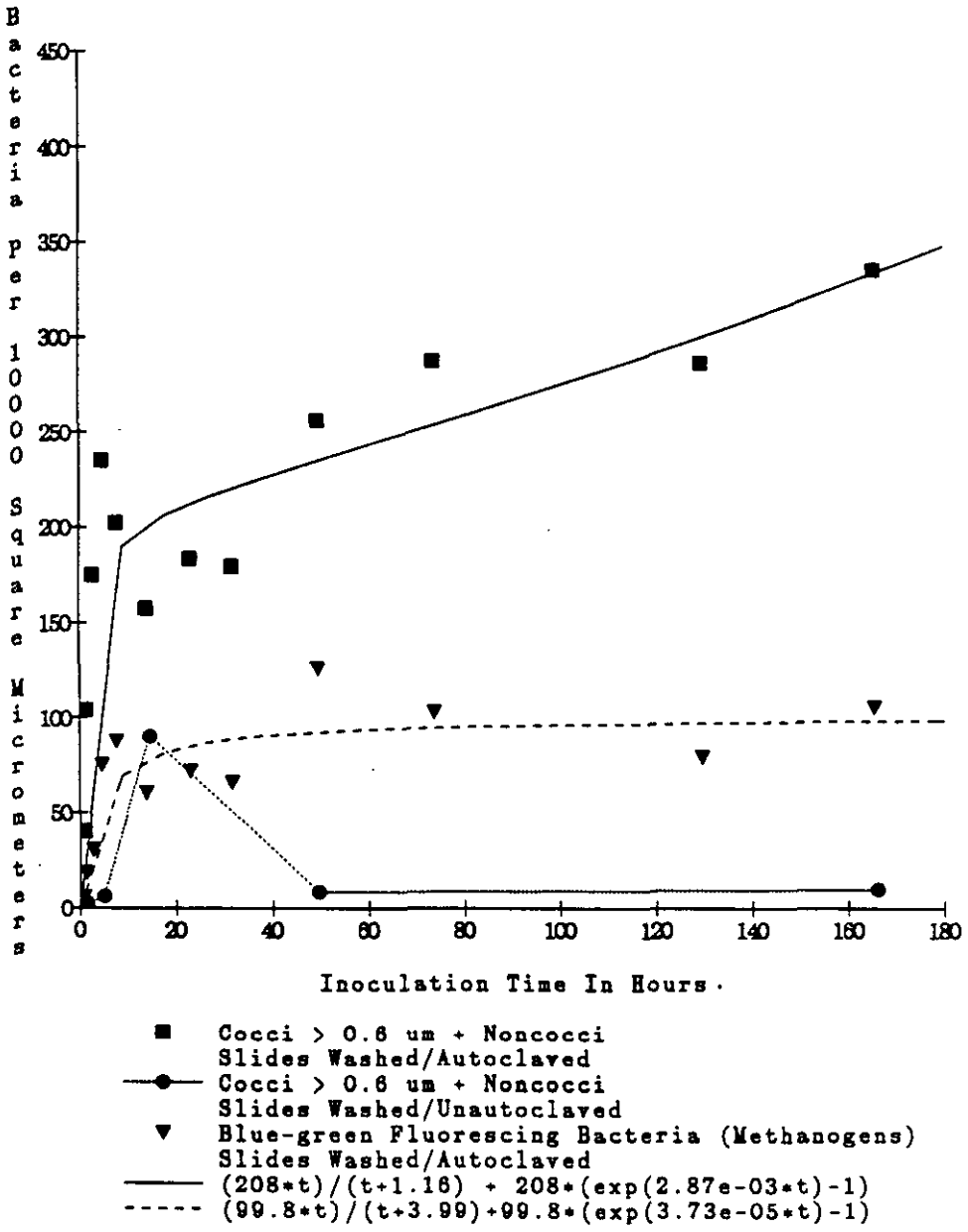


Figure 5.4 8 Day SRT Data-Michaelis-Menten Type Model. Inoculation Time Versus Number Of Bacteria Irreversibly Attached Per 10000 Square Micrometers.

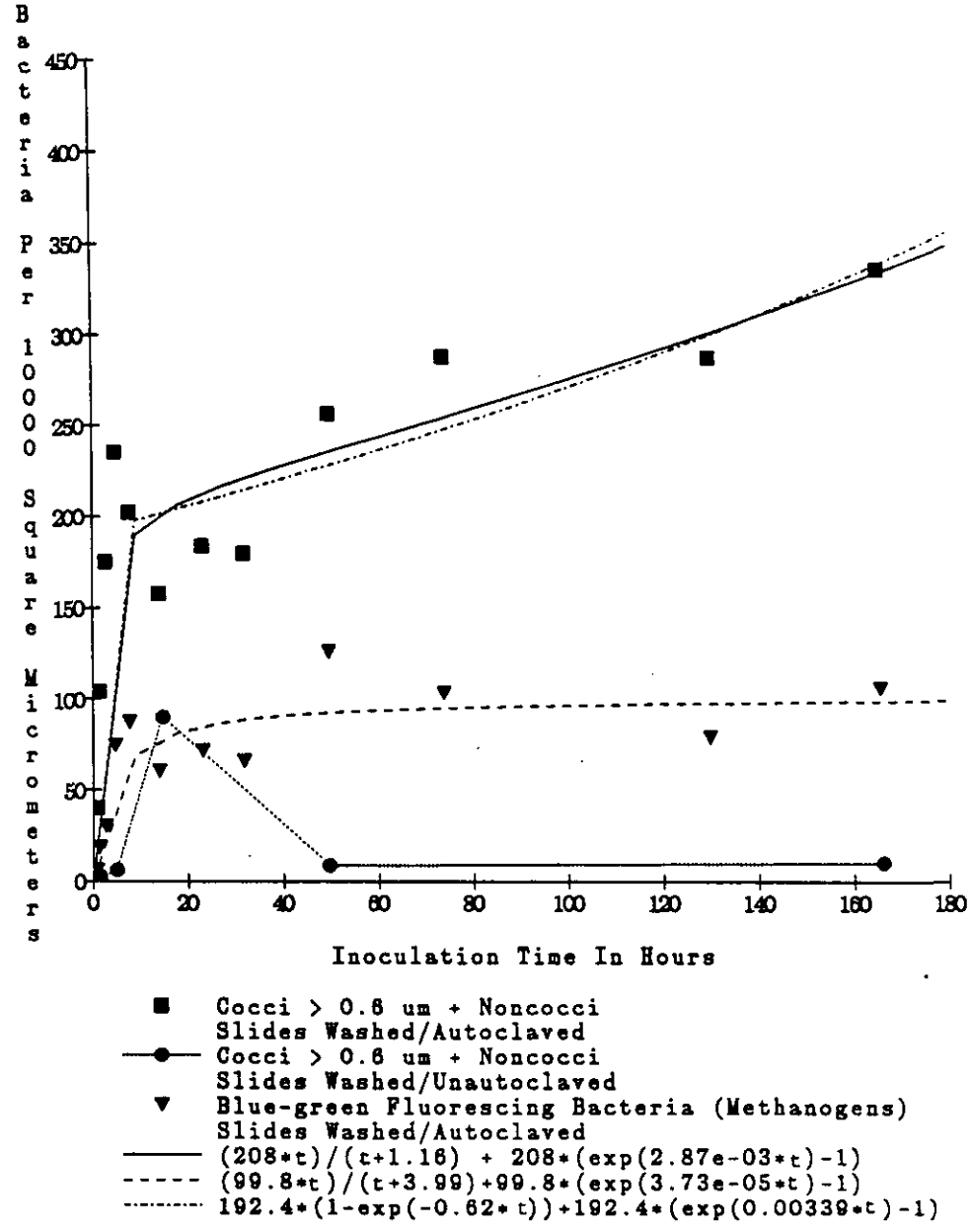


Figure 5.5 8 Day SRT Data-Comparison of Models. Inoculation Time Versus Number Of Bacteria Irreversibly Attached Per 10000 Square Micrometers.

washed/unautoclaved slides over time were one half to one and one half 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 5.1 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 values were of a similar order of magnitude as the growth rate in the chemostat that was feeding the attachment vessel. The bacteria from the 8 day SRT chemostat appear to have a slightly higher growth rate.

Phase Contrast Microscope Photographs

Figure 5.6 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

Table 5.1 Summary Of Values Obtained For Growth Rates Of Attached Cocci > 0.6 Micrometers + Noncocci From Descriptive And Inferential Statistics.

Descriptive Statistics Values		μ doublings per hour	μ doublings per day	SRT days per doubling
20 Day SRT	First Order Model	.00285	.0684	14.6
	Michaelis-Menten Type Model	.00214	.0514	19.5
8 Day SRT	First Order Model	.00339	.08136	12.3
	Michaelis-Menten Type Model	.00287	.06888	14.5
Inferential Statistics Values				
20 Day SRT	Unweighted-Average of Coefficients for Individual Curves	.002764	.0663	15.07
	Weighted-Average of Coefficients for Individual Curves	.003284	.0788	12.69
8 Day SRT	Unweighted-Average of Coefficients for Individual Runs	.00341	.0818	12.2
	Weighted-Average of Coeffients for Individual Runs	.003259	.0782	12.8

Figure 5.6 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

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 bacterium moved vigorously. Other rods appeared to be attached by long thin, threadlike, appendages. One of these can be seen in (k) three quarters 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 5.7 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 5.6).

Scanning Electron Microscopy

A selection of scanning electron microscope photographs are shown in Figures 5.8 to 5.12. Included are an inoculation time sequence of photographs for both the 20 day SRT (Figure 5.8) and the 8 day SRT (Figure 5.9), a plate of conspicuous attachment structures and small attached clumps (Figure 5.10) and two plates of large clumps and other photographs (Figures 5.11 and 5.12).

Figure 5.8 shows a selection of scanning electron microscope (SEM) photographs over a range of inoculation times from 0 hours to 134

Figure 5.7 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.

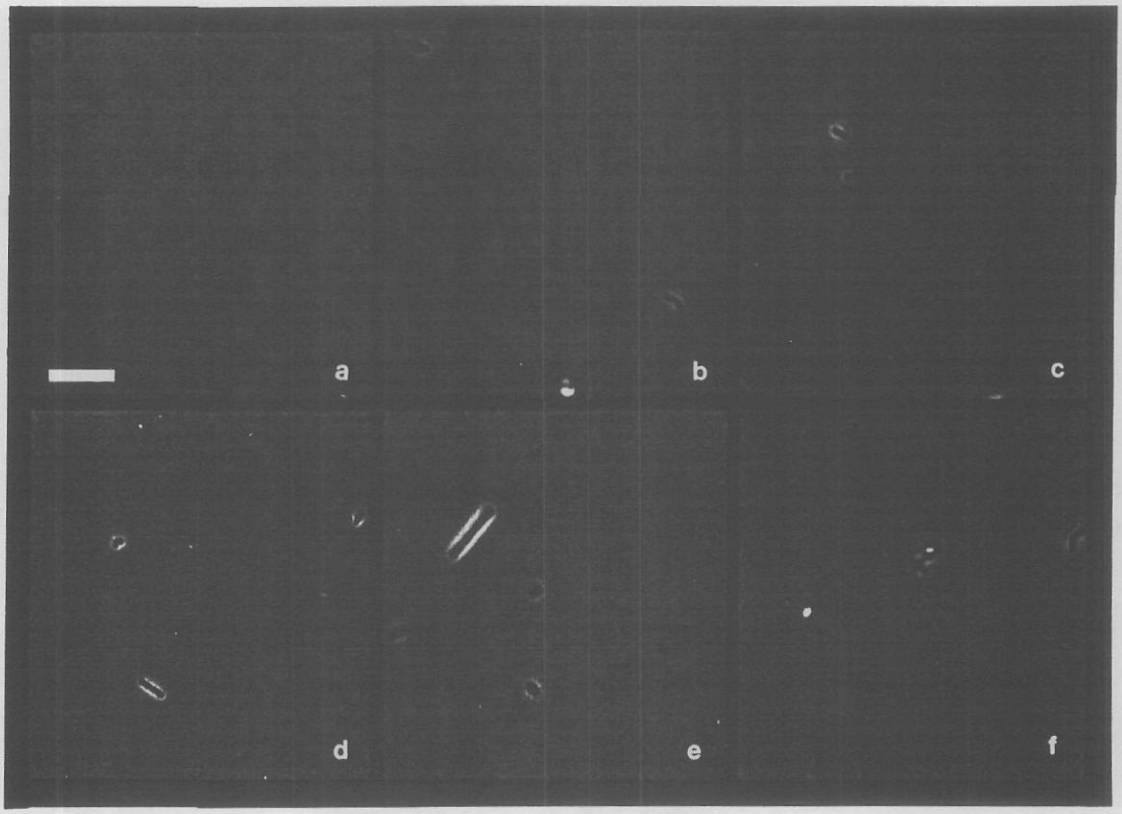

a
b
c
d
e
f

Figure 5.8 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.

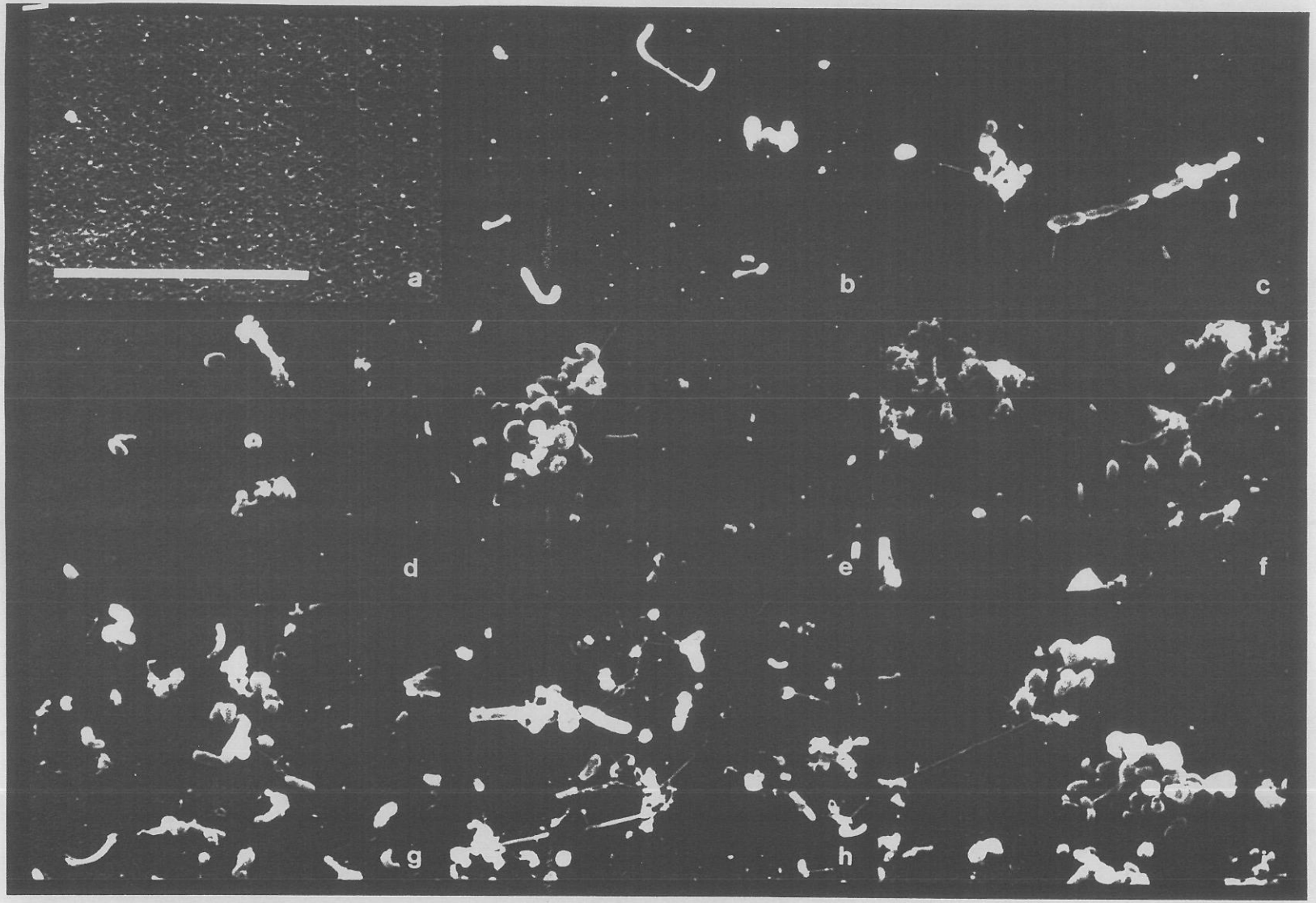

a
b
c
d
e
f
g
h

Figure 5.9 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.

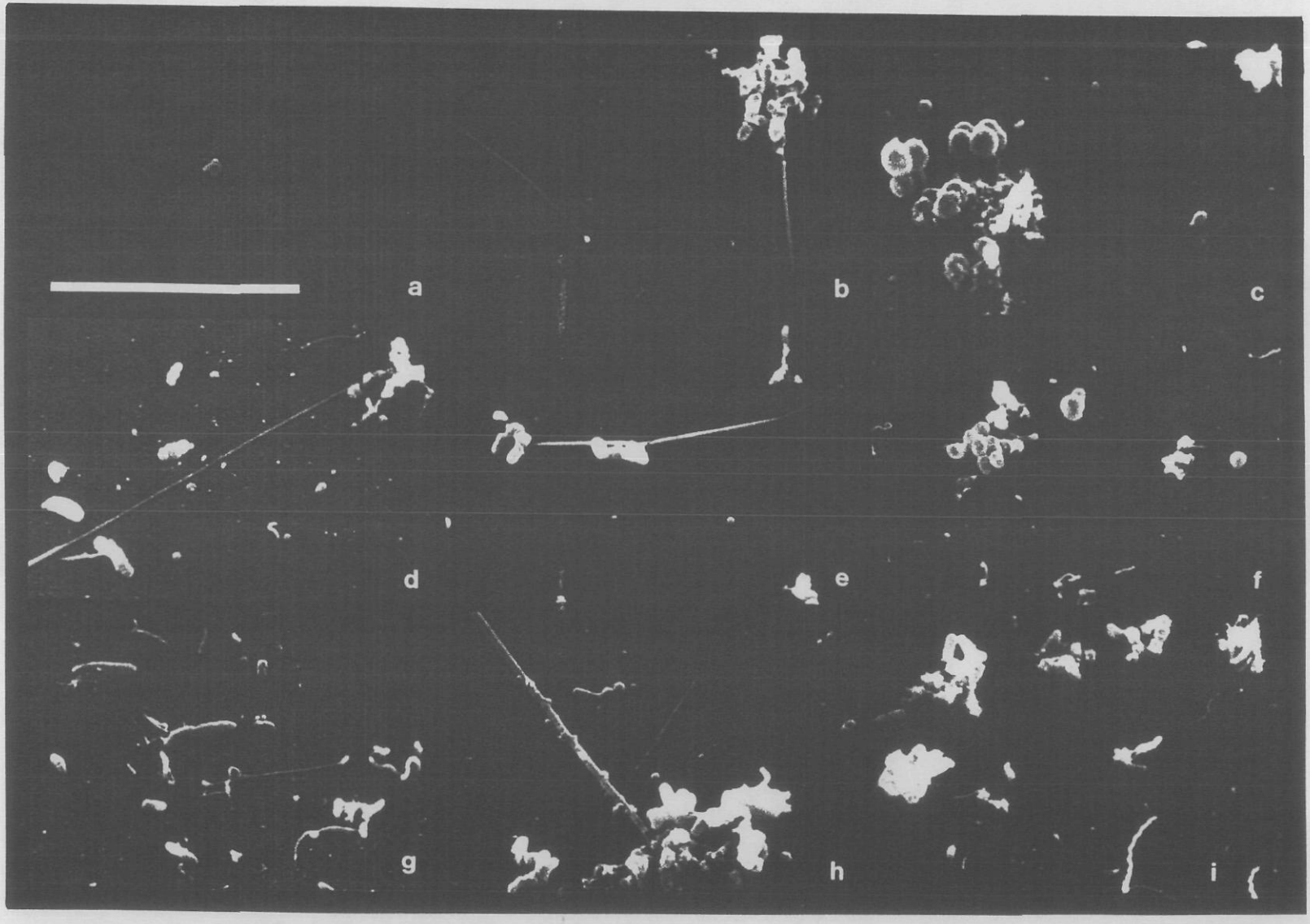

a
b
c
d
e
f
g
h
i

Figure 5.10 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
j
k
l
m
n
o
p

Figure 5.11 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 color surrounding clumps of bacteria - possibly due to extracellular materials or secretions by the cells.

(b) 16, 8, 0°, 10 -- Note difference in color 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, extracellular material and clumped attachment and growth.

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

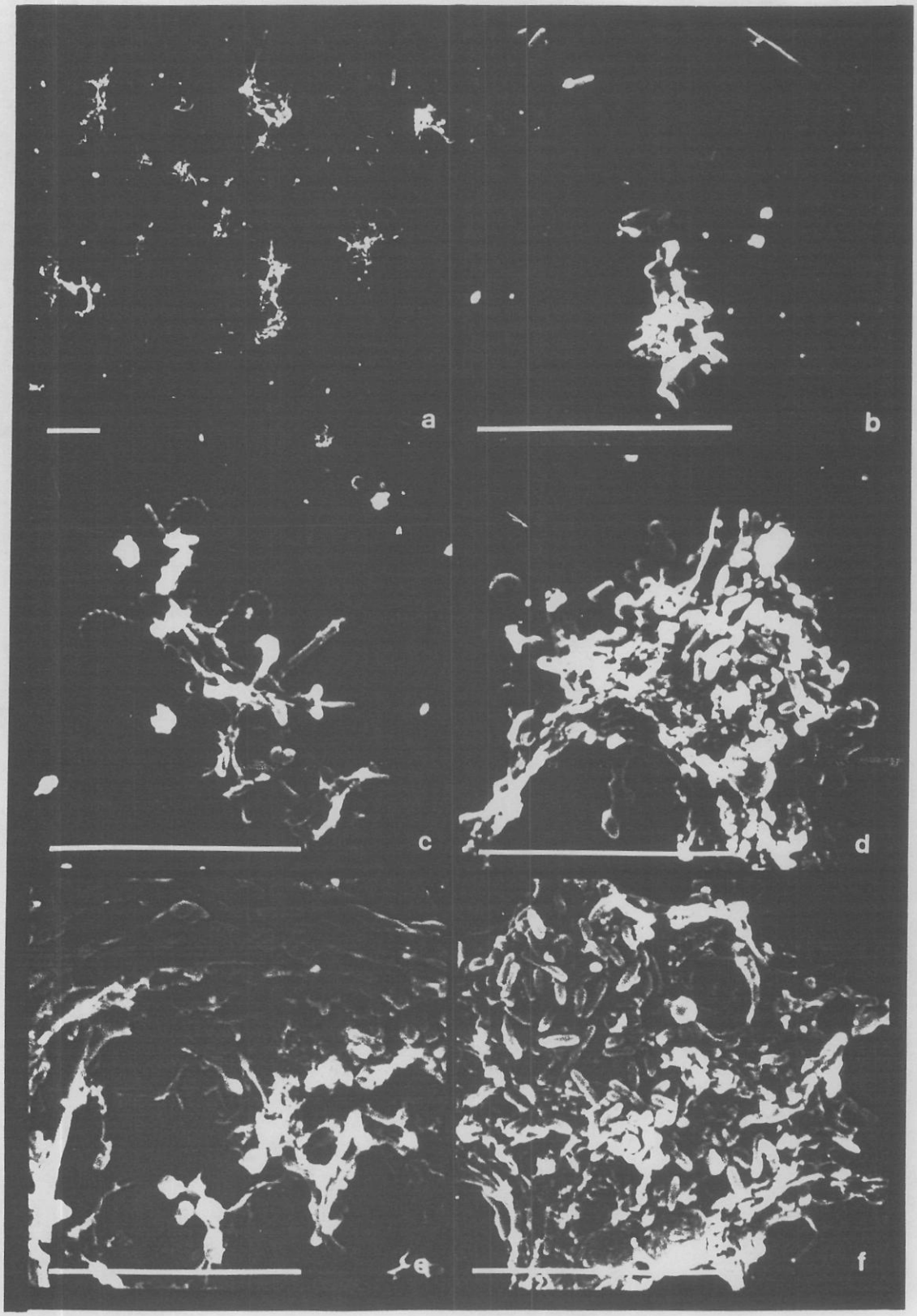

a
b
c
d
e
f

Figure 5.12 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.

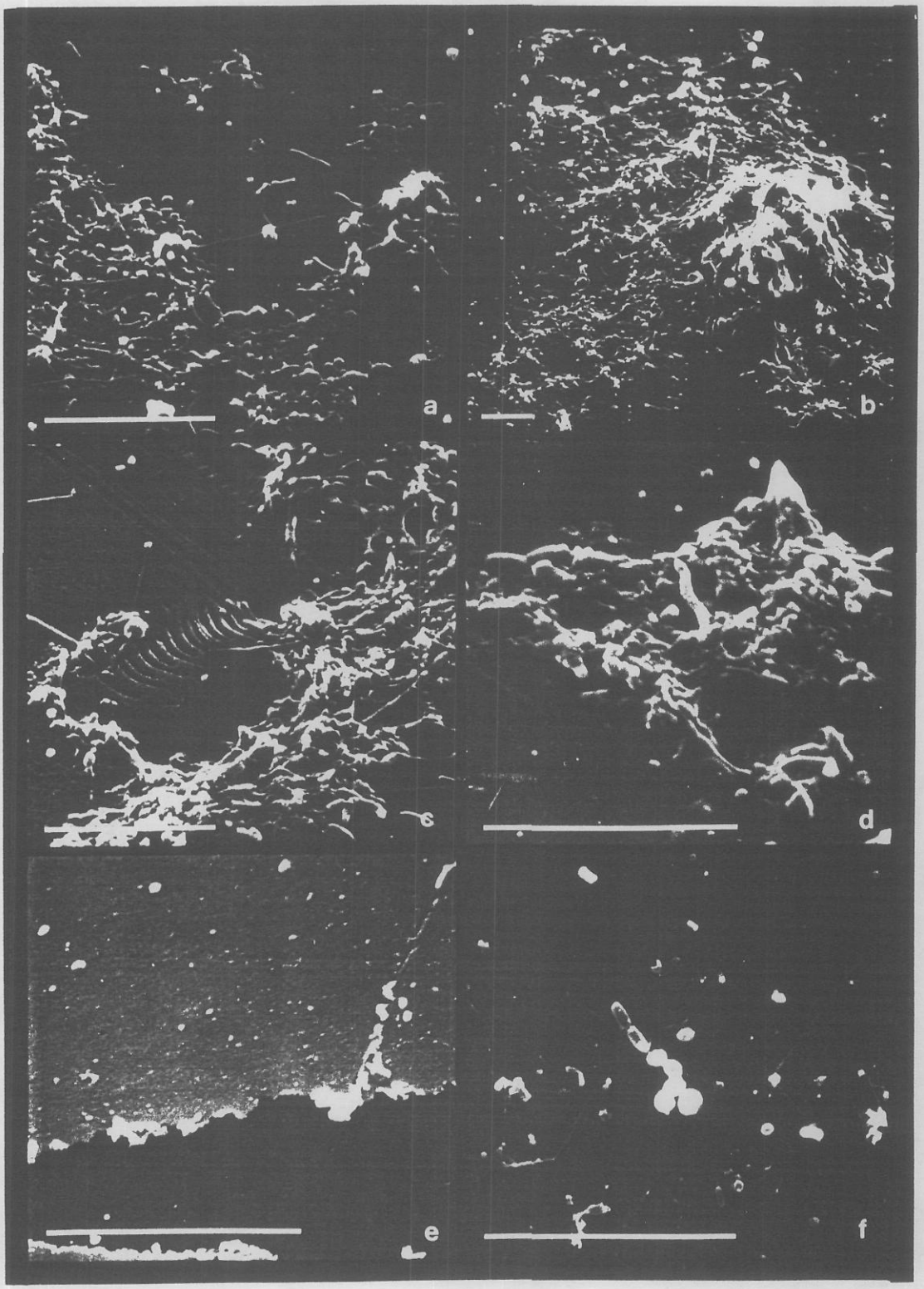
(b) 12 days, 8, 45°, 10 -- Note extracellular material and clumped attachment and growth.

(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
c
d
e
f

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 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, amorphous extracellular material can be seen (i).

Figure 5.9 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 seem 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 5.10 n) are attached within minutes and the first hours of inoculation. Single bacteria area 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 (Figure 5.11 and 5.12). 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 5.10 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 site were seen (d, h). On one occasion a ring-like structure was observed (e). Very short appendages or extracellular material was seen (l). A fuzzy border surrounding an entire cell was also seen (i).Photographs 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 5.11 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. (a) was included to show a low

magnification perspective of the bacteria attached to the surface and the color 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. (b) is a higher magnification photograph of a clump surrounded by one of these rings. (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 amorphous and fiber-like material, and the large diameter of the clumps.

Figure 5.12 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. (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 extracellular material. 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. (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. (f) was included as another example of extracellular fiber development. This was a 76.5 hour inoculation time from the 20 day SRT culture.

There was one other observation for the scanning electron microscope photographs for which no photographs were included. It was occasionaly observed that the rough, cracked, glass sawn edges of the pieces of glass used to observe bacterial attachment sometimes had much higher concentrations of bacteria than smooth, flat plane areas of the glass. Several other researchers (Beeftink and Staugaard, 1986; Oakley et al., 1985; Lie, 1977; and Saxton, 1973) have noted early bacterial attachment in cracks, crevices, and other irregularities of a surface. The same phenomenon seemed to have occurred in this study.

Other Comments

The successful development and application of a mathematical model which accounts for growth and attachment is a significant finding. It has a number of implications. One set of phenomena (most likely

physical and chemical) are involved in the attachment process. A second set of phenomena (biological) are involved in early development of the biofilm. Attachment occurs at a much faster rate (hours) than the reproduction of the slow growing cells of this study. There is a maximum number of cells that can initially irreversibly attach to the surface. Once attached, though, the maximum is easily surpassed by the growing cells. The growth rate of an organism in the bulk fluid is similar to its growth rate growing attached on a surface.

The attachment term of the first order attachment equation is of the same form as the attachment term derived in equation 2.4 from Fletcher's (1977) work. Fletcher's k^1 is A in this work. Her $\frac{-k}{k^1} X_s$ is k in this work. Fletcher's (1977) model assumed bacteria attach in a monolayer. From the photographs taken in this study, and the successful application of the model, that assumption is supported to a large degree. However, following initial attachment, the monolayer is not valid as the cells reproduce and form multilayer mixed colonies.

In cases where bacterial attachment occurs at the maximum rate (100 to 250 bacteria attached per the first 1-3 hours), the slow growth rate of the methanogenic consortium limits early development of the biofilm. From an engineering viewpoint to maximize the growth rate of organisms attached to the surface, a relatively high substrate concentration (high enough to obtain the close to the maximum growth rate of the methanogens) would be the logical choice.

The fact that slide preparation had a dramatic effect on bacterial attachment is significant finding. It is not apparent why autoclaving

slides should have such an important effect. However in the case where the surface preparation was not done to allow optional attachment, bacterial attachment could be a important hindrance to building up a biomass in a methanogenic fixed film reactor.

It was noted in this study and other studies that cracks, crevices, other imperfections on the glass surface were important locations where bacteria attach and biofilms began to form. Simply from reviewing the SEM photographs, it appeared that a surface with bacteria sized depressions or crevices, 1-3 micrometers in depth, would probably allow more rapid bacterial attachment than a smooth surface.

The anaerobic attachment vessel which was developed for this study worked well. It provided a quiescent enviromment in which bacteria could adhere to a surface. If counting techniques were developed for a different material, the attachment vessel could be used to test attachment on different kinds of materials. The attachment vessel could also possibly be used to monitor long term growth under quiescent conditions. Initially, it had been intended that this study would also monitor long term biofilm development by assessing total protein and chemical oxygen demand accumulation of the biofilm accumulating on the surface over time. The attachment vessel was designed to hold 15 mm x 75 mm x 1 mm slides so that these slides could be inserted into heavy duty screw top culture tubes. It was intended that the slides would be inserted into heavy duty screw cap culture tubes for an acid or alkaline digestion followed by the chemical determination of biofilm accumulation.

The biofilm was very delicate. At the early stages of the experiments, when techniques were still being worked out, slides received their initial rinse to remove reversibly attached cells by applying the rinse buffer from the burette directly onto the area of the slide to be counted. It became apparent though that this procedure damaged the biofilm. Observing the biofilm under the phase-contrast microscope it looked like pieces of ripped wax paper, with bacteria embedded, and was torn off the surface. Likewise, if the immersion oil was wiped off a slide and the slide was looked at again under the microscope, the biofilm was similarly damaged. Unfortunately, no pictures were taken of this phenomenom.

There are a couple of implications to these observations concerning damage to the biofilm.. First, future researchers in this area should make an effort to standardize their rinsing procedure. Most researchers indicate they "gently rinse" their samples. It seems that a more consistent method should be found, preferably one in which the shear stress could be quantified. A repeatable method is described in this paper but it does not allow the easy measurement of shear stress.

Second, there is much discussion in the literature of attachment and early biofilm formation about the stages of biofilm formation including the deposition of an initial conditioning film of organic molecules on the surface (i.e. Dexter, 1979). These damaged biofilms gave further support for those proposals and provided evidence that the bacteria are more firmly attached to the conditioning film than the conditioning film is bound to the glass.

In the process of working out a counting technique, staining the attached cells and the biofilm with acridine orange, and counting under an epifluorescence microscope, was tried at the early phases of this research several times. This technique has been used by many researchers and is considered to be an excellent method. However, for the set experiments described in this dissertation, it did not work well. There often appeared to be many (sometimes thousands per microscope field under high power-oil immersion), small fluorescent dots. Their size range was from one micrometer in diameter down to the resolving power limit of the microscope. It was difficult to tell if these dots were artifacts of the staining process or tiny bacteria. Recently, other researchers have also described problems with the acridine orange staining technique. Bergstrom et al. (1986) reported that acridine orange precipitated with dissolved humic material in highly humic water. Perhaps a similar occurence caused the fluorescent dots in this study. Finally, the actual manual manipulation of the microscope slide during the staining process (staining, rinsing, wicking off excess water or buffer with lens paper) resulted in some damage to the biofilm.

CHAPTER VI

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

Summary

The results of this study are summarized below.

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_o = 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:

$$Y = A_o (1 - e^{kt}) + A_o (e^{\mu t} - 1) \qquad (6.1)$$

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 attach

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

$$Y = \frac{(A_o\ t)}{A_o + Km} + A_o (e^{\mu t} - 1) \qquad (6.2)$$

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 ways 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 were illuminated with light at 420 nm and fluoresce blue-green (most methanogens), also attached rapidly to chromic 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. No explanation was proposed to account for this phenomenon.

7. Scanning electron microscopy revealed six noteworthy items.
   a. Some bacteria possess conspicuous attachment structures. Some of these structures appear to be appendages and some appear to be extracellular fibers. The appendages were seen at all

inoculation times and their appearance did not change appreciably over time. The extracellular fiber material was also present at the early inoculation times (within the first few hours), but the character of the extracellular material did change over time. These fibers have been observed in dental studies also. Some extracellular fibers branch at longer inoculation times. These extracellular branching fibers were also noted by Wardell et al. (1984). The presence of the branches at longer inoculation times leads to the hypothesis that fibers grow from the tip.

b. Between 2 days and 2 weeks inoculation time, there begins to be an extensive production of extracellular material. Some extracellular fibers appear longer and/or branched at inoculation times of approximately 2 days and longer. Beginning at approximately 5 days an amorphous, gluelike, extracellular material starts to form and it ultimately spreads extensively.

c. Attached 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.

f. Higher concentrations of attached bacteria were sometimes observed in the crevices and surface irregularities at the edge of glass cut with a glass saw.

8. An anaerobic attachment vessel was developed which allows the systematic investigation for the attachment of anaerobic bacteria to microscope slides.

Conclusions

The practical implications of the findings for this study are listed below.

1. The slow growth rate of the methanogenic consortium is a more significant, ultimate limiting factor in the start-up of a methane-forming biofilm reactor than the rate of bacterial attachment. Under optimum conditions, bacterial attachment can occur rapidly (within a few hours) whereas the maximum doubling time of a methanogenic consortium is on the order of days.

2. Achieving initial bacterial attachment in starting up a biofilm reactor is still s significant concern. If a surface is not prepared properly, bacterial attachment can be extremely slow. More research is needed into understanding how surfaces affect bacterial attachment.

3. Bacteria seem to require a low shear environment for initial attachment. The testing and development of rough media, with bacteria sized cracks, crevices, and depressions would be a worthwhile enterprise. From qualitative review of the SEM photographs, it appears that depressions on the order of 1-5 micrometers in depth would be best. Also low shear and quiescent periods for a reactor starting up would seem appropriate.

Recommendations

With the results of this study in mind, suggestions for future research are listed below.

1. The attachment vessel described in this study could be used for other research. Some possibilities include:
   a. more long term quantitative study of biofilm development using other parameters (e.g. chemical oxygen demand or protein) to quantify bacterial attachment
   b. more qualitative study of biofilm development using the electron microscope including other electron microscope preparation techniques and longer inoculation times
   c. testing the influence of other attachment surfaces (if a suitable counting technique is developed) or other slide preparations
   d. testing the influence of other principal carbon sources on attachment and biofilm development (Certain bacteria, e.g. Leuconostoc mesenteroides, are known to produce large amounts of extracellular polysaccharides when growing on sucrose, which was the principal carbon source in this study. It would be interesting to see if the pattern of production of extracellular material was similar for a different principal carbon source.).

2. The phenomenon of autoclaving the slide surface resulting in a striking influence on bacterial attachment should be investigated.

3. The importance of surface irregularties for bacterial attachment should be investigated.

4. A standardized method to rinse a slide under a measureable shear stress and leave irreversibly attached cells intact needs to be developed.

5. It would be worthwhile to examine why the acridine orange/fluorescense microscopy counting technique for bacterial cells did not work well.

6. Topics with a microbiological emphasis might include:
   a. isolating and identifying attached microorganisms
   b. detailing more extensively how extracellular fibers grow
   c. examining the exact mechanism(s) of bacterial attachment
   d. investigating the genetic basis and regulation of bacterial attachment.

Appendix A Sizing Of Experimental Reactor

Table A.1 Comparison Of Percentage Volatile Solids On Reactor Walls Versus The Size Of The Reactor - Predicted Values.

height of reactor	volume of reactor	surface area of reactor	surface area of tubing	volatile solids in suspension	volatile solids on walls	percentage volatile solids on walls
inches	liters	cm^2	cm^2	g	g	
6	10.7	1430	798	10.7	2.04	16
12	21.3	2860	798	21.3	3.35	13.6
18	32.0	4290	798	32.0	4.66	12.7
24	42.6	5720	798	42.6	5.96	12.3
30	53.3	7140	798	53.3	7.26	12.0
36	64.0	8570	798	64.0	8.57	11.8
42	74.6	10000	798	74.6	9.88	11.7
48	85.3	11400	798	85.3	11.1	11.5
54	96.0	12900	798	96.0	12.5	11.5
60	107	14300	798	107	13.8	11.4
66	117	15700	798	117	15.1	11.4
72	128	17100	798	128	16.4	11.4
78	139	18600	798	139	17.7	11.3
84	149	20000	798	149	19.0	11.3

Appendix B Times For Sampling And Bacteria Counts.

Table B.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 B.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 B.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	Date	Elapsed Time Between Sampling and Counting	Flourescence Count Time	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 B.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	Date	Elapsed Time Between Sampling and Counting	Flourescence Count Time	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	12:35 am	7/21/86	DNR	7/21/86	< 1 day	11:05 pm	7/21/86	10.5 hrs.	
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 B.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	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	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	1.33	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 C Attachment Data

Table C.1 Attachment Data - April Experimental Run.

Inoculation Time Hours	Slides Autoclaved Or Unautoclaved	Average- Cocci > 0.6µm Plus Noncocci Per 10000 µm^2	Standard Deviation	Number Of Fields Counted	Field Size µm^2	1/Variance	Blue-green Fluorescing Bacteria (Methanogens) Per 10000 µm^2	Standard Deviation	Number Of Fields Counted	Field Size µm^2
0.0	A	0.38	0.86	24	4418	1.35	0.17	0.81	24	5027
0.08	A	40.37	11.30	24	4418	0.0078	2.16	1.75	24	5027
1.25	A	93.94	23.97	24	4418	0.0017	33.82	18.36	48	745
2.58	A	213.53	36.64	24	4418	0.00075	71.0	33.44	48	745
4.67	A	136.28	30.02	24	4418	0.0011	137.93	67.17	44	745
7.5	A	267.21	57.00	20	4418	0.00031	76.11	45.76	48	331
13.5	A	232.01	59.63	24	4418	0.00028	84.92	42.08	48	331
23.0	A	362.73	70.02	24	4418	0.00020	188.08	72.16	48	331
31.0	A	359.11	71.26	24	4418	0.00020	135.24	70.19	48	331
49.5	A	242.76	66.58	24	4418	0.00023	49.06	42.07	48	331
73.5	A	312.84	98.99	24	4418	0.00010	279.29	98.18	48	331
165.0	A	317.55	95.17	24	4418	0.00011	164.18	89.37	48	331

Table C.2 Attachment Data - May Experimental Run.

Inoculation Time Hours	Slides Autoclaved Or Unautoclaved	Average- Cocci > 0.6µm Plus Noncocci Per 10000 µm^2	Standard Deviation	Number Of Fields Counted	Field Size µm^2	1/Variance	Blue-green Fluorescing Bacteria (Methanogens) Per 10000 µm^2	Standard Deviation	Number Of Fields Counted	Field Size µm^2
0.0	A	3.4	3.89	24	4418	0.066	0.33	1.27	24	5027
0.08	A	117.61	26.3	24	4418	0.0014	78.42	48.52	72	331
1.25	A	153.82	25.63	24	4418	0.0015	99.39	47.39	72	331
2.67	A	175.61	28.82	24	4418	0.0012	122.03	48.72	72	331
4.67	A	234.93	30.75	24	4418	0.0011	192.06	67.48	72	331
7.5	A	223.24	53.9	24	4418	0.00034	139.64	83.95	72	331
14.67	A	158.35	99.0	24	4418	0.00010	67.10	59.15	72	331
23.0	A	257.66	32.98	24	4418	0.00092	156.84	70.52	72	331
32	A	306.8	44.6	24	4418	0.00050	122.45	61.21	72	331
54.5	A	323.59	70.19	24	4418	0.00020	233.58	162.24	72	331
73.5	A	370.93	69.08	24	4418	0.00021	180.73	70.64	72	331
129.5	A	433.09	31.84	6	4418	0.00099	218.06	95.51	36	331
165	A	413.75	78.59	24	4418	0.00016	283.9	133.08	72	331
0.0	U	1.79	3.83	24	4418					
1.42	U	12.83	7.94	24	4418					
5.0	U	24.62	8.89	24	4418					
15.0	U	22.64	10.58	16	4418					
74.0	U	58.76	21.94	24	4418					

Table C.3 Attachment Data - June Experimental Run.

Inoculation Time Hours	Slides Autoclaved Or Unautoclaved	Average- Cocci > 0.6um Plus Noncocci Per 10000 μm^2	Standard Deviation	Number Of Fields Counted	Field Size μm^2
0.0	U	2.17	1.94	24	4418
1.42	U	12.07	8.00	24	4418
5.0	U	101.10	30.29	24	4418
15.5	U	71.11	40.5	24	4418
50	U	79.69	50.46	24	4418
77	U	65.83	15.15	24	4418

Table C.4 Attachment Data - July Experimental Run.

Inoculation Time Hours	Slides Autoclaved Or Unautoclaved	Average- Cocci > 0.6µm Plus Noncocci Per 10000 μm^2	Standard Deviation	Number Of Fields Counted	Field Size μm^2	1/Variance	Blue-green Fluorescing Bacteria (Methanogens) Per 10000 μm^2	Standard Deviation	Number Of Fields Counted	Field Size μm^2
0.0	A	0.25	0.46	22	14314	4.73	0.17	0.56	24	5027
0.08	A	43.2	26.69	24	3579	0.0014	7.87	6.88	24	5027
1.33	A	112.71	25.73	24	3579	0.0015	25.63	20.52	48	1325
2.75	A	157.65	25.8	24	3579	0.0015	16.19	14.10	48	1325
4.67	A	245.91	42.52	24	3579	0.00055	68.21	42.59	48	745
7.67	A	249.27	43.46	20	3579	0.00053	69.6	55.19	48	745
14.0	A	211.10	52.98	24	3579	0.00036	68.21	37.83	48	745
23	A	231.59	39.26	24	3579	0.00065	113.22	90.76	72	331
32.4	A	204.23	63.05	24	3579	0.00025	83.03	57.95	72	331
49.5	A	287.6	62.05	24	3579	0.00026	192.48	121.46	72	331
73.5	A	209.12	60.43	24	3579	0.00027	135.03	96.01	72	331
129.5	A	304.93	71.41	25	3579	0.00020	76.32	58.22	72	331
165	A	433.26	70.57	24	3579	0.00020	112.39	68.03	72	331
0.0	U	0.79	1.24	24	14314					
1.33	U	2.56	3.68	24	3579					
4.67	U	1.28	2.33	24	3579					
14.0	U	177.22	41.21	24	3579					
49.5	U	9.31	15.05	24	3579					
73.5	U	5.71	5.24	24	3579					
165	U	10.13	7.17	24	3579					

Table C.5 Attachment Data - August Experimental Run.

Inoculation Time Hours	Slides Autoclaved Or Unautoclaved	Average- Cocci > 0.6μm Plus Noncocci Per 10000 μm^2	Standard Deviation	Number Of Fields Counted	Field Size μm^2	1/Variance	Blue-green Fluorescing Bacteria (Methanogens) Per 10000 μm^2	Standard Deviation	Number Of Fields Counted	Field Size μm^2
0.0	A	1.98	2.91	24	3579	0.12	0.91	1.31	24	5027
0.08	A	38.42	29.67	24	3579	0.0011	3.70	4.88	36	5027
1.33	A	95.83	27.25	24	3579	0.0014	11.76	12.21	60	2070
2.75	A	192.35	29.62	24	3579	0.0011	44.35	46.13	72	745
4.67	A	224.61	33.78	24	3579	0.00088	81.25	49.54	72	745
7.67	A	155.21	40.75	24	3579	0.00060	104.84	90.65	72	331
14.0	A	105.37	38.00	24	3579	0.00069	52.42	62.06	72	331
23.0	A	135.88	33.00	24	3579	0.00092	29.77	40.06	72	331
31.0	A	155.09	45.37	24	3579	0.00049	48.45	55.32	72	745
49.5	A	224.84	143.03	24	3579	0.000049	59.55	93.57	72	331
73.5	A	365.96	118.44	24	3579	0.000071	71.29	91.12	72	331
130	A	268.15	68.16	24	3579	0.00022	81.35	79.91	72	331
166	A	236.25	85.44	24	3579	0.00014	98.13	110.18	72	331
0.0	U	7.57	8.58	24	3579					
1.33	U	2.56	4.28	24	3579					
4.67	U	11.18	9.21	24	3579					
14	U	2.79	3.40	24	3579					
31	U	6.99	6.85	24	3579					
49.5	U	7.92	7.93	24	3579					
73.5	U	6.64	5.58	24	3579					
130	U	73.82	24.58	24	3579					
166	U	10.01	6.16	24	3579					

REFERENCES

Absolom, D. R., Lamberti, F. W., Policova, Z., Zingg, W., van Oss, C. J. and Neumann, A. W. 1983. "Surface Thermodynamics Of Bacterial Adhesion." Applied And Environmental Microbiology. 46:90-97.

Aveyard, R. and Haydon, D. A. 1973. An Introduction To The Principles Of Surface Chemistry. Cambridge University Press. London, England.

Baier, R. E. 1980. "Substrata Influences On Adhesion Of Microorganisms And Their Resultant New Surface Properties." In Adsorption of Microorganisms To Surfaces. Eds. Bitton, G. and Marshall, K. John Wiley and Sons. pp. 59-104.

Baier, R. E., Shafrin, E. G., Zisman, W. A. 1968. "Adhesion: Mechanisms That Assist Or Impede It." Science. 162:1360-1368.

Beeftink, H. H., and Staugaard, P. 1986. "Structure And Dynamics Of Anaerobic Aggregates In A Gas-Lift Reactor." Applied And Environmental Microbiology. 52:1139-1146.

Birdsell, D. C., Doyle, R. J. and Morgenstern, M. 1975. "Organization Of Teichoic Acid In The Cell Wall Of Bacillus subtilis." Journal of Bacteriology. 121:726-734.

Bouwer, E. J., and McCarty, P. L. 1983. "Transformations Of 1- and 2-Carbon Halogenated Aliphatic Organic Compounds Under Methanogenic Conditions." Applied and Environmental Microbiology. 45:1286-1294.

Bouwer, E. J., McCarty, P. L. 1981. "Anaerobic Degradation of Halogenated 2- and 2-Carbon Organic Compounds." Environmental Science and Technology. 15:596-599.

Brannan, D. K. and Caldwell, D. E.. 1982. "Evaluation Of A Proposed Surface Colonization Equation Using Thermothrix thiopara As A Model Organism." Microbial Ecology. 8:15-21.

Brock, T. D., Smith, D. W. and Madigan, M. T. 1984. Biology of Microorganisms, Fourth Edition. Prentice-Hall, Inc. Englewood Cliffs, N.J.

Bryers, J. and Characklis, W. 1981. "Early Fouling Formation In A Turbulent Flow System: Overall Kinetics." Water Research. 15:483-491.

Caldwell, D. E., Brannan, D. K., Morris, M. E., and Betlach, M. R. 1982. "Quantitation of Microbial Growth on Surfaces." Microbial Ecology. 7:1-11.

Caldwell, D. E., Malone, J. A., Kieft, T. L. 1983. "Derivation of a Growth Equation Describing Microbial Surface Colonization." Microbial Ecology. 9:1-6.

Chan, R., Acres, S. D. and Costerton, J. W. 1982. "The Use Of Specific Antibody To Demonstrate Glycocalyx, K99 Pili, And The Spatial Relationships Of K99+ Enterotoxigenic E. coli In The Ileum Of Colostrum-Fed Calves."Infection And Immunology. 37:1170-1180.

Chang, R. 1981. Physical Chemistry With Applications to Biological Systems. MacMillian Publishing Co., Inc.

Costerton, J. W. 1984. "Mechanisms of Microbial Adhesion to Surfaces - Direct Ultrastructural Examination of Adherent Bacterial Populations in Natural and Pathogenic Ecosystems." In Current Perspectives In Microbial Ecology - Proceedings of the Third International Symposium on Microbial Ecology. Eds. Klug, M. J. and Reddy, C. A. American Society for Microbiology. Washington, D.C. pp. 115-123.

Costerton, J. W., Gessey, G. C. and Cheng, K. J. 1978. "How Bacteria Stick." Scientific American. pp. 86-95.

Costerton, J. W., Marrie, T. J. and Cheng, K. J. 1985. "Phenomena of Bacterial Adhesion." In Bacterial Adhesion. Eds. Savage, D.C. and Fletcher, M. Plenum Press.

Cumsky, M. G. and Zusman, D. R. 1981. "Purification and Characterization of Myxobacterial Hemagglutin, A Development-Specific Lectin of Myxococcus xanthus." Journal of Biological Chemistry. 256:12512587.

Daley, R. J. and Hobbie, J. E. 1975. "Direct Counts of Aquatic Bacteria By a Modified Epi-fluorescent Technique." Limnol. Oceanogr. 20:875-882.

Daniels, S. L. 1980. "Mechanisms Involved In Sorption of Micro-organisms To Solid Surfaces." In Adsorption of Micro-organism To Surfaces. Eds., Bitton, G. and Marshall, K. John Wiley and Sons. pp. 7-58.

de Vocht, M., van Meenen, P., van Assche, P., and Verstraete, W. 1983. "Influence of Sedimentation and Adhesion on Selection of Methanogenic Associations." Process Biochemistry. pp. 31-34.

Dexter, S. C., Sullivan, J. D., Williams, J., and Watson, S. W. 1975. "Influence of Substrate Wettability on the Attachment of Marine Bacteria to Various Surfaces." Applied Microbiology. 30:298-308.

Dexter, S. C. 1979. "Influence of Substratum Critical Surface Tension On Bacterial Adhesion - In Situ Studies." Journal of Colloid and Interface Science. 70:346-354.

Dickerson, R. E., Gray, H. B., and Haight, G. P. Jr. 1974. Chemical Principles, Second Edition. W. A. Benjamin, Inc. Menlo Park, California.

Dudman, W. F. 1977. "The Role of Surface Polysaccharides in Natural Environments." In Surface Carbohydrates of the Procaryotic Cell. Ed. I. Sutherland. Academic Press Inc. New York.

Fletcher, M. 1977. "The Effects of Culture Concentration and Age, Time, and Temperature on Bacterial Attachment to Polystyrene." Canadian Journal of Microbiology. 23:1-6.

Fletcher, M., and Floodgate, G. D. 1973. "An Electron-microscopic Demonstration of An Acidic Polysaccharide Involved in the Adhesion of a Marine Bacterium to Solid Surfaces." Journal of General Microbiology. 74:325-334.

Fletcher, M., and Floodgate, G. D. 1976. "The Adhesion of Bacteria to Solid Surfaces." In Microbial Ultrastructure. Eds. Fuller, R. .and Lovelock, D. W. Academic Press. London. pp. 101-17.

Fletcher, M. and McEldowney, S. 1984. "Microbial Attachment to Nonbiological Surfaces." In Current Perspectives In Microbial Ecology - Proceedings of the Third International Symposium on Microbial Ecology. Eds. Klug, M. J. and Reddy, C. A. American Society for Microbiology. Washington D.C. pp. 124-129.

Francisco, D.E., Mah, R.A., and Rabin, C.A. 1973. "Acridine Orange-Epifluorescence Technique For Counting Bacteria In Natural Waters." Transactions Of The American Microscopical Society. 92:416-421.

Franson, M. A. H. Managing Editor. 1985. Standard Methods for the Examination of Water and Wastewater. Sixteenth Edition.

Girifalco, L. A. and Good, R. J. 1957. Journal of Physical Chemistry. 61:904.

Good, R. J. 1964. Contact Angle, Wettability and Adhesion-Advances In Chemistry Series 43. American Chemical Society. Washington, D. C. pp. 74-87.

Healy, J. B., Jr., and Young, L. Y., 1979. "Anaerobic Biodegradation of Eleven Aromatic Compounds to Methane." Applied and Environmental Microbiology. 38:84-89.

Hulshaft Pol, L. W., Dolfing, J., van Straten, K., de Zeeuw, and Lettinga, G. 1984. "Pelletization of Anaerobic Sludge in Upflow Anaerobic Sludge Bed Reactors on Sucrose-Containing Substrates." In Current Perspectives in Microbial Ecology - Proceedings of the Third International Symposium on Microbial Ecology. Eds. Klug, M. J. and Reddy, C. A. American Society for Microbiology. Washington, D.C., pp. 636-642.

Huser, B. A., Wuhrman, K. and Zehnder, A. J. B. 1982. "Methanothrix soehngenii gen. nov. sp. nov., New Acetotrophic Non-hydrogen-oxidizing Methane Bacterium." Archives of Microbiology. 132:1-9.

Jeris, J. S. and McCarty, P. L. 1965. "The Biochemistry of Methane Fermentation Using C_{14} Tracers." Journal of the Water Pollution Control Federation. 37:2:178-192.

Lawrence, A. W. and McCarty, P. L. 1969. "Kinetics of Methane Fermentation in Anaerobic Treatment." Journal of the Water Pollution Control Federation. 41:2:R1-R17.

Lie, T. 1977. "Early Dental Plaque Morphogoresis." Journal of Periodontal Research. 12:73-89.

Loeb, G. I. 1985. "The Properties of Nonbiological Surfaces and Their Characterization." In Bacterial Adhesion. Eds. Savage, D.C. and Fletcher, M. Plenum Press.

Mackie, E. B., Brown, K. N., Lam, J. and Costerton, J. W. 1979. "Morphological Stabilization of Capsiles of Group B Streptococci, Type Ia, Ib, II and III, with Specific Antibody." Journal of Bacteriology. 138:609-617.

Mahoney, E. M., Varangu, L. K., Cairns, W. L. and Kosaric, N. 1984. "Cell Surface and Aggregation Studies of Microbes From Anaerobic Systems." Presented at 57th Conference of the Water Pollution Control Federation, New Orleans, LA, October 1984.

Marshall, K. 1985. "Mechanisms of Bacterial Adhesion At Solid-Water Interfaces." In Bacterial Adhesion. Eds. Savage, D.C. and Fletcher, M. Plenum Press.

Marshall, K. C., Stout, R., and Mitchell, R. 1971. "Mechanism of the Initial Events in the Sorption of Marine Bacteria to Surfaces." Journal of General Microbiology. 68:337-348.

McCarty, P. L. 1964. "Anaerobic Waste Treatment Fundamentals." Public Works, 95: Sept. 107-112, Oct. 123-126, Nov. 91-94, Dec. 95-99.

McCarty, P. L. 1981. "One Hundred Years of Anaerobic Treatment." In Anaerobic Digestion. Eds., Hughes, et al. pp. 3-22.

McCarty, P. L. 1982. "Impact of Fundamental Research on Anaerobic Wastewater Treatment." In Proceedings of: Fundamental Research Needs for Water and Wastewater Systems. Ed. Switzenbaum, M. S. pp. 33-38.

McCarty, P. L. 1975. "Stoichiometry of Biological Reactions." Progress in Water Technology. 7:157:172.

McCarty, P. L. 1985. "Historical Trends in the Anaerobic Treatment of Dilute Wastewaters," In Proceedings of the Seminar/Workshop Anaerobic Treatment of Sewage. Ed. Switzenbaum, M. National Science Foundation.

Meadows, P. S. 1965. "Attachment of Marine and Freshwater Bacteria to Solid Surfaces." Nature 207:1108.

Mink, R. W. and Dugan, P. R. 1977. "Tentative Identification of Methanogenic Bacteria By Fluorescence Microscopy." Applied and Environmental Microbiology. 33:713-717.

Molin, G., Nilson, I. and Stenson-Holst, L. 1982. "Biofilm Build-Up of Pseudomonas putida in a Chemostat at Different Dilution Rates." European Journal of Applied Microbiology and Biotechnology. 15:218-222.

Montgomery, D. C. 1984. Design and Analysis of Experiments, 2nd Edition. Wiley.

Nelson, C. H., Robinson, J. A., and Characklis, W. G. 1985. "Bacterial Adsorption to Smooth Surfaces: Rate, Extent, and Special Pattern." Biotechnology and Bioengineering. 27:1662-1667.

Noike, T., Endo, G., Chang, J., Yaguchi, J., and Matsumoto, J. 1985. "Charcteristics Of Carbohydrate Degradation And The Rate-Limiting Step In Anaerobic Digestion." Biotechnology And Bioengineering. 27:1482-1489.

Oakley, D. L., Wase, D. A. J., and Forster, C. F. "Development of a Biofilm in an Expanded Bed Reactor." Paper presented at Trans. Inst. Chem. Engineers Conference. London.

Pavoni, J. L., Tenney, M. W., and Echelberger, W. F., Jr. 1972. "Bacterial Polymers and Biological Flocculation." Journal of the Water Pollution Control Federation. 44:414-431.

Powell, M. S. and Slater, N. K. H. 1982. "Removal Rates of Bacterial Cells From Glass Surfaces by Fluid Shear." Biotechnology and Bioengineering. pp. 2527-2537.

Pringle, J. H. and Fletcher, M. 1983. "Influence of Substratum Wetability on Attachment of Freshwater Bacteria to Solid Surfaces." Applied and Environmental Microbiology. 45:811-817.

Rao, S. R. 1972. Surface Phenomena. London, England.

Raven, P., Evert, R. and Curtis, H. 1981. Biology of Plants, Third Edition. Worth Publishers.

Robinson, R. W., Akin, D. E., Nordstedt, R. A., Thomas, M. V., and Aldrich, H. C. 1984. "Light and Electron Microscopic Examinations of Methane Producing Biofilms from Anaerobic Fixed Bed Reactors." Applied and Environmental Microbiology. 48:127-136.

Robinson, J. A., Trulear, M. G. and Characklis, W. G. 1984. "Cellular Reproduction and Extracellular Polymer Formation By Pseudomonas aeruginosa In Continuous Culture." Biotechnology and Bioengineering. 26:1409-1417.

Rogers, H. J. 1979. "Adhesion of Microorganisms to Surfaces: Some General Consideration of the Cell Envelope." In Adhesion of Microorganisms to Surfaces. Eds. Ellwood, D. C. and Melling, J. Academic Press.

Sawyer, C. N. and McCarty, P. L. 1978. Chemistry for Environmental Engineering. McGraw-Hill Book Company.

Saxton, C. A. 1973. "Scanning Electron Microscope Study of the Formation of Dental Plaque." Caries Research. 7:102-119.

Shapiro, M. and Switzenbaum, M. S. 1984. "Initial Anaerobic Biofilm Development." Biotechnology Letters. 6:729-734.

Smith, M. R., Zinder, S. H., and Mah, R. A. 1980. "Microbial Methanogenesis from Acetate." Process Biochemistry. 15:34-39.

Speece, R. E. 1983. "Anaerobic Biotechnology for Industrial Wastewater Treatment." Environmental Science and Technology. 17, 416A-427A.

Stanier, R. Y., Ingraham, J. L., Wheelis, M. L. and Painter, R. R. 1986. The Microbial World. Prentice-Hall. Englewood Cliffs, New Jersey.

Stumm, W. and Morgan, J. J. 1981. Aquatic Chemistry. John Wiley & Sons.

Sutherland, I. W. 1983. "Microbial Exopolysaccharides - Their Role in Microbial Adhesion in Aqueous Systems." CRC Critical Reviews in Microbiology. 10:173-201.

Switzenbaum, M. S. 1983. "Anaerobic Treatment of Wastewater: Recent Developments." American Society of Microbiology News. 49:532-536.

Switzenbaum, M. S. 1986. "Comparison of Attached Films In Several Anaerobic Reactor Configurations." Final Report For Argonne National Laboratory. Report No. Env. E. 90-86-1. Department of Civil Engineering. University of Massachusetts At Amherst.

Switzenbaum, M. S., Scheuer, K. C. and Kallmeyer, K. E. 1985. "Influence of Materials and Precoating on Initial Anaerobic Biofilm Development." Biotechnology Letters. 7:585-588.

Trulear, M. and Characklis, W. 1982. "Dynamics of Biofilm Processes." Journal of the Water Pollution Control Federation. 54:1288-1301.

van Loosdrecht, M. C. M., Lyklema, J., Norde, W., Schraa, G., and Zehnder, A. J. B. 1987a. "The Role of Bacterial Cell Wall Hydrobicity in Adhesion." Applied and Environmental Microbiology. 53:1893-97.

van Loosdrecht, M. C. M., Lyklema, J., Norde, W., Schraa, G. and Zehnder, A. J. B. 1987b. "Electrophoretic Mobility and Hydrophobicity as a Measure to Predict the Initial Steps of Bacterial Adhesion." Applied and Environmental Microbiology. 53:1898-1901.

Verrier, D. 1984. "Methanisation D'Effluents Agro-alimentaires En Reacteurs A Cellules Fixees." Ph.D. Dissertation at L'ecole Nationale Superieure Des Industries Agricoles Et Alimentaires.

Wardell, J. N., Brown, C. M., Ellwood, D. C., and Williams, A. E. Continuous Culture 8. 1984. "Bacterial Growth On Inert Surfaces." In Continuous Culture 8, Continuous Culture, Biotechnical, Environmental, And Medical Applications #8. Ed. Dean, A.C. et al. Halsted Press.

Weiss, L. and Harlos, J. P. 1977. "Cell Contact Phenomena And Their Implicatich In Cell Communication." In Intercellular Communications. Ed. by de Mello, W. C. Plenum Publishing Corporation. New York. pp. 33-59.

Whittenbury, R. and Dow, C. S. 1977. "Morphogenesis And Differentiation Of Rhodomicrobium vanielii And Other Budding And Prosthecate Bacteria." Bacteriological Reviews. 41:754-808.

Wicken, A. J. 1985. "Bacterial Cell Walls And Surfaces." In Bacterial Adhesion. Eds. Savage, D.C. and Fletcher, M. Plenum Press.

Winer, B. 1971. Statistical Principles in Experimental Design, 2nd Edition. McGraw-Hill.

Woese, C. R. 1981. "Archaebacteria." Scientific American. 244:98-122.

Zehnder, A. J. B., Huser, B. A., Brock, T. D., and Wuhrmann, K. 1980. "Characterization Of An Acetate-decarboxylating Non-hydrogen-oxidizing Methane Bacterium. Archives of Microbiology. 124:1-11.

Zinder, S. H. 1984. "Microbiology Of Anaerobic Conversion Of Organic Wastes To Methane: Recent Developments." American Society of Microbiology News. 50:294-298.