The influence of growth rate and cell concentration on bacterial attachment to surfaces in a continuous flow system
by Christopher Henry Nelson

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Environmental Engineering
Montana State University
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Abstract:
Many factors influence the rate of bacterial attachment to surfaces. Two factors of interest in this study were the growth rate and concentration of cells in the bulk water. A pure culture of Pseudomonas 224S was used as the test organism. The experimental system consisted of smooth, hydrophilic (glass) surfaces placed in a well-mixed continuous flow system. The results indicate attachment rate was greatest with cells growing at approximately 1/2 their maximum growth rate and the surfaces became saturated with cells at approximately 0.1% coverage. The cells tended toward a uniform distribution when surface saturation occurred. The results of this study suggest that bacterial colonization of surfaces occurs in two phases. Initially, cells are transported to a surface where attachment occurs until the surface becomes saturated with cells. After the surface is saturated, the primary mechanism for cell accumulation is growth of attached cells.

THE INFLUENCE OF GROWTH RATE AND CELL CONCENTRATION ON BACTERIAL ATTACHMENT TO SURFACES IN A CONTINUOUS FLOW SYSTEM

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A thesis submitted in partial fulfillment
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Date Dec. 8, 1983

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ABSTRACT

Many factors influence the rate of bacterial attachment to surfaces. Two factors of interest in this study were the growth rate and concentration of cells in the bulk water. A pure culture of *Pseudomonas 224S* was used as the test organism. The experimental system consisted of smooth, hydrophilic (glass) surfaces placed in a well-mixed continuous flow system. The results indicate attachment rate was greatest with cells growing at approximately 1/2 their maximum growth rate and the surfaces became saturated with cells at approximately 0.1% coverage. The cells tended toward a uniform distribution when surface saturation occurred. The results of this study suggest that bacterial colonization of surfaces occurs in two phases. Initially, cells are transported to a surface where attachment occurs until the surface becomes saturated with cells. After the surface is saturated, the primary mechanism for cell accumulation is growth of attached cells.

INTRODUCTION

Bacterial attachment to surfaces occurs in many diverse environments. Subsequent growth of attached bacterial cells results in the formation of a biofilm. Biofilms have many beneficial uses. For example, they are used in wastewater treatment (e.g., rotating biological contactors). Biofilms also cause problems in many engineering systems. For example, they increase heat transfer resistance in heat exchangers.

The goal of this study was to conduct fundamental research on the attachment process. Specifically, the influence of growth rate and concentration of cells in the bulk water on attachment rates was investigated.

The results of this study should increase a fundamental understanding of the attachment process which may be extrapolated to either control or promote biofilm growth.

LITERATURE REVIEW

Bacterial attachment to surfaces is a common occurrence in aquatic environments. This review will focus on attachment at solid/liquid interfaces, on clean and smooth surfaces, and in a turbulent flow regime.

Adsorption of Conditioning Film

Studies have shown that when a clean surface is immersed in water, an organic conditioning film is adsorbed on the surface. The rate and extent of adsorption is influenced by many factors including the organic content and relative turbulence of the bulk water and the available free surface energy (Fletcher, 1980). The rate of adsorption of the conditioning film is, in general, faster than bacterial attachment rates so most surfaces will have conditioning films present when attachment occurs.

Transport to Surface

Many natural and industrial environments are open, turbulent flow systems. Yet, most laboratory studies of bacterial attachment have been conducted under quiescent, batch conditions. Fluid flow conditions must be taken into account because they will influence bacterial cell transport from the bulk water to the surface. For example, gravity may play an important role in transport under quiescent conditions but convective transport (turbulent bursts) may be more important under turbulent flow conditions.

The concept of a viscous sublayer is important in the discussion of a turbulent flow system. The viscous sublayer is a thin film of water which is in contact with a surface during turbulent bulk water flow. For example, water flowing through a pipe under turbulent conditions will produce a viscous sublayer which is in contact with the inner pipe wall

and is typically measured in microns. Fluid forces under turbulent flow conditions transport cells to the viscous sublayer but the sublayer acts as a barrier to transport and it causes the cells to lose their momentum as they approach the surface. How, then, are cells transported to the surface? Observations indicate that velocity fluctuations in the bulk water (turbulent bursts) disrupt the viscous sublayer and penetrate to the wetted surface (Campbell and Hanratty, 1983). These velocity fluctuations appear to provide a mode of transport for the cells to contact the surface.

Reversible/Irreversible Attachment

Once the bacterial cells have been transported to the wetted surface, two types of attachment are possible; reversible and irreversible. Reversible attachment is the initial step in the attachment process. In this phase, cells exhibit random motion and can be removed by gently rinsing the surface with water. Irreversible attachment is firm adhesion to the surface at which point cells no longer exhibit random motion and cannot be removed by gentle rinsing (Marshall et al., 1971). Irreversible attachment is usually associated with the production of extracellular polymeric substances (EPS) (Fletcher, 1980). Regardless of mechanism, after a cell has attached to a surface, subsequent growth and transport processes lead to the formation of microcolonies and eventually to the formation of a mature biofilm.

Factors Influencing Attachment

There are many factors which may influence attachment including the relative roughness and free energy of the surface, the fluid dynamics, nutrient concentration, cation concentration, pH, and temperature of the bulk water, and bacterial species present and their physiological state. Two factors tested in this study are the growth rate and the bulk water concentration of the cells.

The quantity typically measured in most attachment studies is cell accumulation rate on the surface. Cell accumulation is the result of several processes; transport of cells to the surface, attachment of cells to the surface, detachment of cells from the surface, and growth of attached cells. The attachment and detachment processes can be combined as net attachment. In this study, net attachment will be referred to as simply attachment. Attachment is the dominant mechanism for cell accumulation in most bacterial attachment studies because the relatively short experimental times (e.g., 2-6 hours) limit growth of attached cells. Growth becomes more important as the experimental time is increased (e.g., > 6 hours) as typically occurs in biofilm studies.

Growth Rate

The growth rate of bacterial cells used in attachment studies can influence the rate of attachment. Fletcher (1977) reports that in batch studies, the rate of cell accumulation on a surface is greatest with cells taken from log phase cultures after a 2 hour exposure time. Molin et al. (1982) found that the rate of microcolony accumulation on a surface increases as the growth rate of the cells in the bulk water is increased with maximum accumulation occurring near the maximum specific growth rate. Trulear (1983) observed in chemostat studies that the extent of EPS production decreases as the growth rate of the cells is increased. All three studies used a pure culture of *Pseudomonas* as the test organism.

The results suggest that growth rate, which is a measure of physiological activity, influences the attachment process. The rate of attachment appears to increase as growth rate increases. These results also suggest the rate of attachment is increased when minimal amounts of EPS are associated with the cells, i.e., EPS does not appear to have a direct role in initial attachment. Another possibility is that the structure of the EPS produced by the cells varies as the physiological state of the cell changes. Changes in the structure of EPS may influence its relative adhesiveness.

Concentration

The concentration of cells in the bulk water and on the surface can influence attachment rates. Bryers and Characklis (1982) found cell accumulation rates on the surface to be proportional to the suspended biomass concentration in the bulk water. Fletcher (1977) observed that surfaces become saturated with cells as the concentration of cells in the bulk water is increased for a given exposure time. Brannan and Caldwell (1982) found cell accumulation rates on the surface to increase continuously with time.

These observations can be interpreted as follows. The transport rate of the cells to the surface is proportional to the cell concentration in the bulk water. Attachment rate is proportional to transport rate until the surface becomes saturated with cells. After saturation, accumulation rates continue to increase due mainly to growth of attached cells rather than attachment of cells from the bulk water. In other words, surface saturation will not be observed if growth contributes significantly to observed cell accumulation rates or if the cell concentration in the bulk water (and therefore transport rate) is not sufficient for saturation to occur.

Fletcher observed saturation because her relatively short exposure time (2 hours) minimized attached cell growth and her relatively high concentration of cells in the bulk water ($\sim 10^9$ cells ml^{-1}) resulted in a relatively high cell transport rate to the surface. Bryers and Characklis did not observe saturation because their relatively long exposure time ($\sim$ 50 hours) and the addition of substrate into their system allowed attached cell growth to be significant. Brannan and Caldwell did not observe saturation because their relatively low concentration of cells in the bulk water (a natural hot springs) resulted in a relatively small cell transport rate to the surface and nutrients in their natural system allowed for attached cell growth.

EXPERIMENTAL APPARATUS AND METHODS

Experimental System

Figure 1 is a schematic drawing of the experimental system used in this study. The reactor is enlarged to show detail. The reactor consisted of a glass beaker, 9 cm in diameter by 18.5 cm tall. The capacity of the reactor was 450 ml. The reactor was a continuous flow system with cells and dilution water as the influent. Four glass microscope slides were suspended in the reactor by a fixture consisting of silicon tubing and plastic support structures. The chemostat is identical to the reactor except it does not have removable glass microscope slides. The variables of interest in this study were the bulk water cell concentration in the reactor, X, and the growth rate of these cells, μ. Growth rate was varied by varying the flow of nutrients through the chemostat after steady state was reached. Cell concentration was varied by varying the flow of dilution water through the reactor. Cells were pumped from the chemostat to the reactor at a constant flow rate (0.3 ml min^{-1}) for all experiments. The relative turbulence of the reactor bulk water was kept constant in all experiments by maintaining the same stirring rate setting on the magnetic stirrer. Dye tests indicated that this stirring rate was adequate to assume complete mixing of fluids inside the reactor. The temperature, T, of the chemostat was controlled by a water bath (T = 20°C). A single species of bacteria, *Pseudomonas 224S*, was used in this study. *224S* had a maximum growth rate, $\mu_m = 0.45\ hr^{-1}$ (J. A. Robinson, personal communication). Nutrient and dilution water compositions are listed in Appendix 1. Growth was glucose limited. Nutrient and dilution water solutions were autoclaved prior to use.

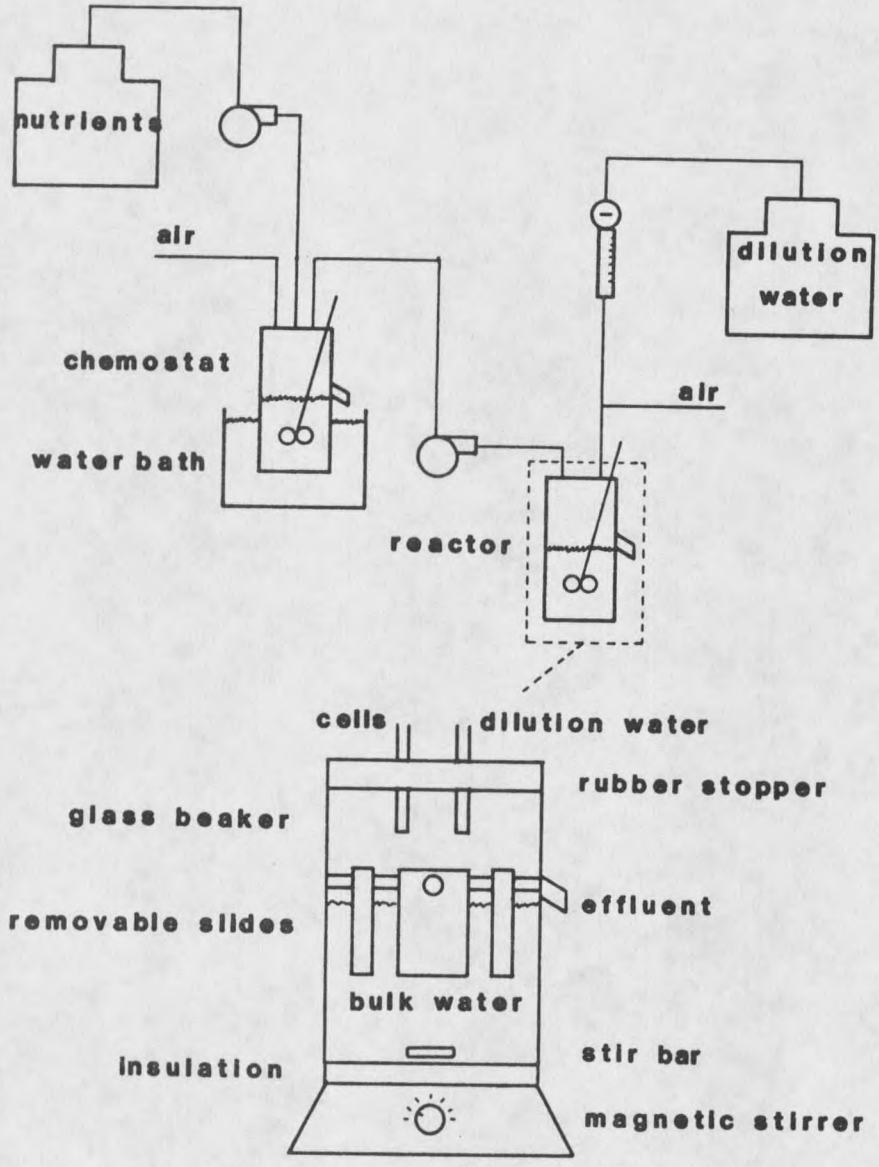

Figure 1. Experimental design.

Cleaning Procedures

The glass microscope slides used in the reactor were cleaned in a consistent manner in order to insure relatively uniform surfaces were used in all experiments. First, the slides were immersed in tetra chlorethylene (TCE) for 2 minutes. Next, the slides were immersed in 10% hydrochloric acid (HCl) for 2 minutes and rinsed with distilled water.

The reactor was washed with 10% HCl and then rinsed with distilled water. Clean slides were mounted inside the reactor and the reactor was autoclaved.

The pipets used for staining were washed with 10% HCl and then rinsed with distilled water. The pipets were autoclaved with the reactor.

The chemostat and all associated tubing were autoclaved prior to the start of the experiments.

Experimental Procedure

The chemostat was innoculated with *Pseudomonas 224S* and allowed to run in batch mode for 12 hours. Then, the dilution rate was adjusted for the desired growth rate, μ. The chemostat was allowed to run for 6 detention times to reach steady state. Experiments were begun after steady state was reached.

The dilution water flow rate was adjusted to give the desired cell concentration in the reactor, X. The duration of exposure in each experiment was 6 hours. After 6 hours, the slides were removed from the reactor and stained.

Staining Procedure

The reagents used in staining the slides from the reactor were as follows:

1. distilled water
2. 70% ethanol
3. acridine orange solution

The acridine orange solution consisted of 1 mg acridine orange per 1 ml of 2% formaldehyde. All three reagents were filtered through 0.22 μm filters. The same glass pipets were used for staining in each experiment, in order to minimize potential differences in delivery velocities between pipets. Ten ml pipets were used to deliver the distilled water and the 70% ethanol, and a 5 ml pipet was used to deliver the acridine orange solution.

A staining procedure was developed in order to observe cells attached to the glass slides with minimum surface alteration. After the slides were removed from the reactor, they were rinsed with 10 ml of distilled water per slide in a reproducible pattern. Next, the slides were stained with 1 ml of acridine orange solution per slide for 15 minutes. Next, the slides were rinsed with 10 ml of 70% ethanol per slide in a reproducible pattern and allowed to dry for approximately 10 minutes. Finally, approximately 0.1 μl of immersion oil was applied to each slide and a cover slip was placed on top of the oil in preparation for cell counting.

Counting Procedure

Attached cells were counted using epifluorescence microscopy. Ten fields of 1×10^4 μm^2 size were counted per slide. Additional counts were made if the initial counts appeared to be erratic in order to minimize the standard deviation of the data. The counts were made in the same relative location on each slide. Both cell numbers and potential colony forming units (PCFU) were counted. PCFU were defined as any group of cells in physical contact with each other, a cell in the process of division, or a single cell attached to the microscope slide surface after the 6 hour exposure time. The differentiation between cell numbers and PCFU was deemed necessary in order to minimize the effect of surface cell growth on the determination of attachment rates. This differentiation was especially important since cell growth rate in the bulk water, μ, was one of the variables tested. A PCFU is assumed to originate from a single cell (possibly in the process of division) which

attaches to a surface and has the potential for subsequent growth. The data were analyzed in terms of PCFU although most PCFU consisted of either 1 or 2 cells (refer to Appendix 2). An average PCFU value per 1×10^4 μm^2 was determined for each experiment by taking an average of counts made from approximately 40 fields from 4 slides.

Chemostat cell counts were determined by epifluorescence microscopy according to the procedure proposed by Hobbie et al. (1977). The results are documented in Appendix 3.

RESULTS

Experimental Results

Experiments were statistically designed according to Hunter (1960). Twelve experiments were arranged in an octagon design (Figure 2). The limits on each variable were determined by preliminary experiments and the capability of the experimental system. A second order polynomial was proposed as a model to approximate the results as suggested by Hunter (1960) although other models could have been proposed. The second order polynomial model is shown in Table 1. The model was tested to determine if the approximation was reasonable. The resulting analysis of variance table (Table 1) indicates the lack of fit of the model to be insignificant at the 5% rejection level and the second-order terms in the model to be significant at the 5% rejection level. In other words, the response surface generated by the proposed second order polynomial model is a statistically valid description of the observed results. Figure 3 compares the response surface generated by the model and the experimentally determined points. The response surface can be described as a "rising ridge" and the experimental points are in good agreement with the response surface.

Response Surface Analysis

In order to observe the response of each variable more closely, cross sections of the response surface were taken at the midpoint of each axis (μ = 0.17 hr^{-1}, F_D = 16 ml min^{-1}). The resultant graphs (Figures 4 and 5) illustrate the response of one variable over the experimental range while the other variable is held constant. In both figures, the lines

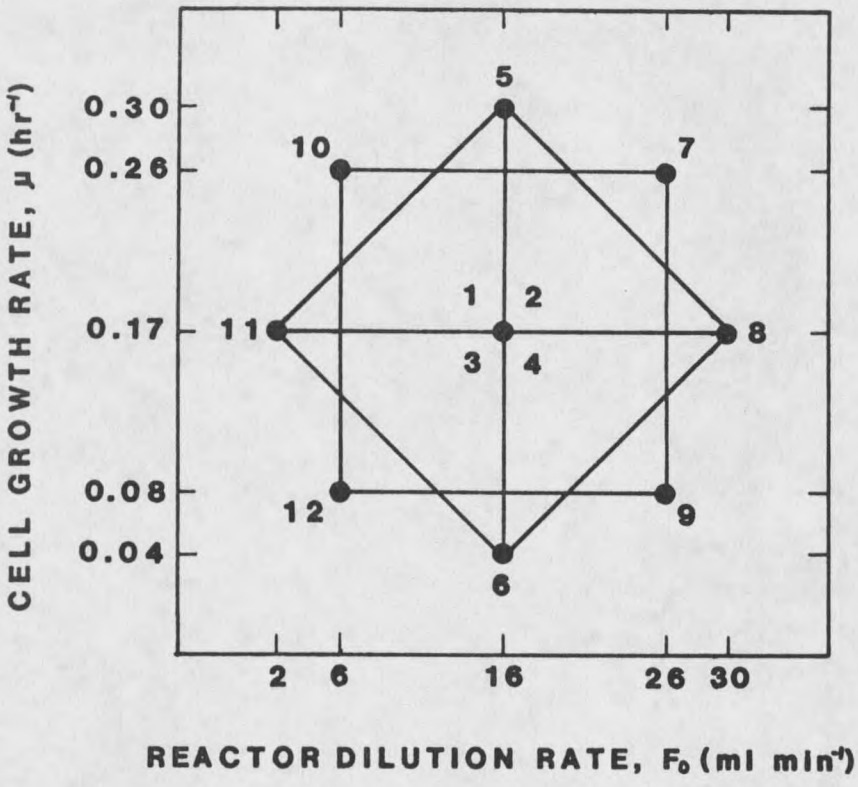

Figure 2. Octagon design.

Table 1. Model and ANOVA Table.

Proposed Model

$$\hat{y} = 2.35 + 0.43\, x_1 - 0.77\, x_2 - 0.53\, x_1^2 - 0.004\, x_2^2 - 0.24\, x_1\, x_2$$

where: $\hat{y}$ = predicted response
x_1 = relative values $(-\sqrt{2}, -1, 0, 1, \sqrt{2})$ on vertical scale of octagon design
x_2 = relative values $(-\sqrt{2}, -1, 0, 1, \sqrt{2})$ on horizontal scale of octagon design

Analysis of Variance Table
(2nd Order Model, K = 2)

Sum of Squares			d.o.f.	Mean Squares	F Ratios
Crude	S	56.95	12		
b_0	S_0	47.60	1		
b_1 b_2	$S_{1.0}$	6.24	2		
b_{11}, b_{22} b_{12}	$S_{2.10}$	2.11	3	0.70	$F_{3,3} = 11.11$ ($F_{.05} = 9.28$) (significant)
residual = $\Sigma (y - \hat{y})^2$	S_R	1.00	6		
Lack of fit	$S_R - S_E$	0.81	3	0.27	$F_{3,3} = 4.28$ ($F_{.05} = 9.28$) (nonsignificant)
Error	S_E	0.19	3	0.063	

were generated by the model and the points and error bars were determined experimentally.

Figure 4 shows the influence of cell concentration in the bulk water, X, on PCFU on the surface. The growth rate of the cells used for this figure is $\mu = 0.17\ hr^{-1}$. Figure 5 shows the influence of specific growth rate of cells in the bulk water, μ, on PCFU on the surface. The cell concentration used in this figure is $X = 1.3 \times 10^5$ cells ml^{-1}.

Variance/Mean Ratio

To help in the analysis of Figure 5, a variance/mean ratio analysis was conducted. The variance/mean ratio provides a test of a population distribution on a surface. If the ratio equals one, the population has a random distribution. If the ratio is greater than one, the

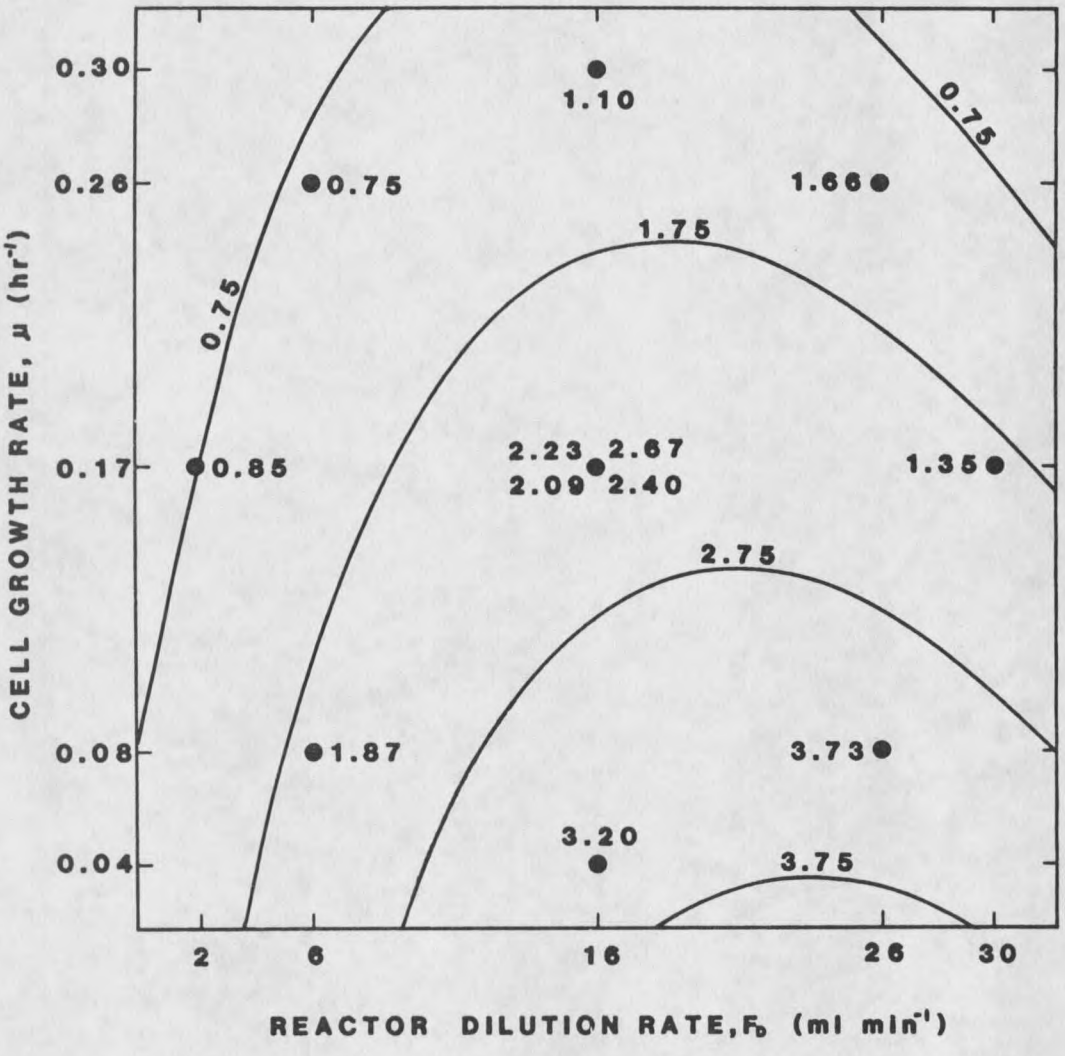

Figure 3. Response surface.

population has a contagious or clustered distribution. If the ratio is less than one, the population has a more uniform distribution (Zar, 1974). The details and results of the variance/mean analysis as applied to the experimental data from this study are presented in Appendix 4.

A variance/mean ratio was calculated from the mean PCFU values from each experiment. The value of the variance/mean ratio was calculated to be less than one, suggesting a relatively uniform distribution. The null hypothesis tested was that the calculated variance/mean ratio is not significantly different from 1.00. The results of the test indicated that the null hypothesis could be rejected at the 7.5% rejection level. In other words, the probability of the calculated variance/mean ratio being less than 1.00 is 92.5%. This suggests the PCFU are more uniformly distributed rather than randomly distributed or contagiously distributed (clustered).

DISCUSSION

Interpretations of the experimental results are presented in this section with emphasis on interpetations of the observed responses in Figures 4 and 5. The response in Figure 5 will be interpreted in terms of surface saturation, zones of inhibition, and surface thermodynamics. A model is also proposed which describes bacterial accumulation on surfaces.

Growth Rate

Growth rate is a measure of the physiological state of the cell. Figure 4 shows the relationship between cell growth rate in the reactor bulk water, μ, and potential colony forming units, PCFU. The resulting curve is "concave down" in shape with the maximum number of PCFU occurring at approximately $\mu = 0.2\ hr^{-1}$. These results are consistent with those of Fletcher and McEldowney (1983) who found maximum attachment rates for *Pseudomonas fluorescens* to hydrophilic surfaces at $\mu = 0.15\ hr^{-1}$ in quiescent bulk water conditions. In this study the maximum number of PCFU occurred at a growth rate which is approximately 1/2 the maximum growth rate, μ_m, of *Pseudomonas 224S*. It is difficult to speculate about the mechanism(s) responsible for promoting attachment from Figure 4. In general, it can be concluded that the physiological state of the cell does influence its attachment properties.

Surface Saturation

Initially clean surfaces appear to allow only a limited number of cells to attach. This phenomenon is termed surface saturation. Figure 5 is a conceptual representation of surface saturation at different cell concentrations in the bulk water. The surface is shown to

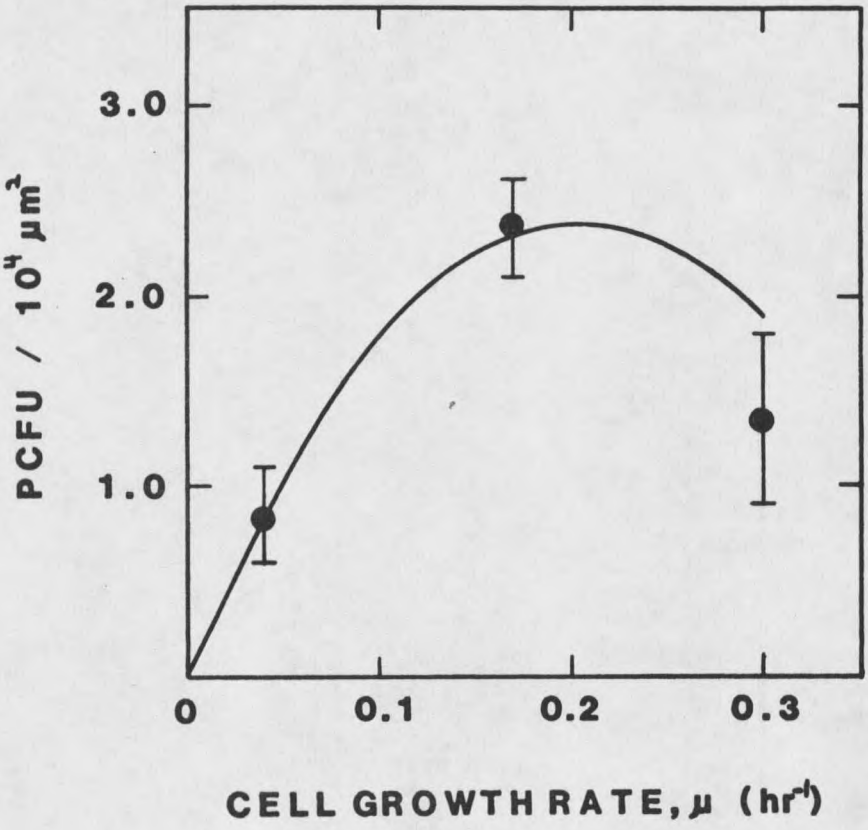

Figure 4. Influence of bulk water cell growth rate on potential colony forming units.

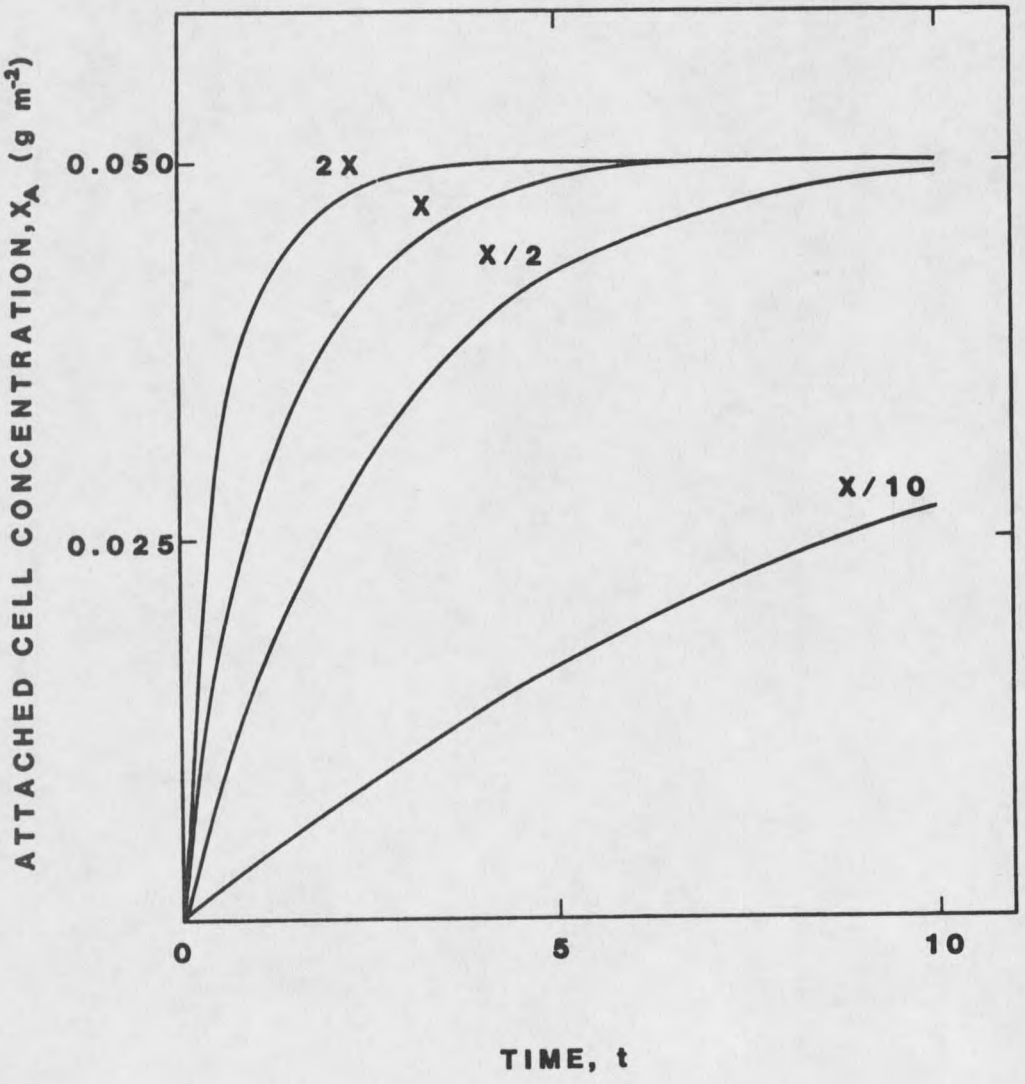

Figure 5. Predicted influence of bulk water cell concentration, X, on attached cell concentration (X = 0.13 gm^{-3}).

become saturated with cells as the time of exposure is increased and the cell concentration in the bulk water is held constant.

Figure 6 shows the relationship between cell concentration in the bulk water, X, and potential colony forming units, PCFU. In this case, the surface appears to approach saturation as the cell concentration in the bulk water is increased and the time of exposure is held constant.

Surface saturation has been observed by other investigators. Fletcher (1977) observed surface saturation to occur when approximately 40% of the surface was covered with cells. Powell and Slater (1983) observed surface saturation to occur when approximately 1% or 5% of the surface was covered with cells depending on the experimental surface. In this study, surface saturation occurred at approximately 0.1% coverage. The saturation coverage from each study and calculated transport and accumulation rates are included in Table 2. The reported saturation coverages decrease as the relative turbulence of the bulk water increases. This response can be attributed to an increase in the detachment rate of cells from the surface. The detachment rate can be approximated by subtracting the rate of accumulation from the rate of transport. It is evident from this calculation that detachment rate increases as the relative turbulence of the bulk water increases.

Table 2. Literature Comparisons.

Flow Regime	% Coverage at Saturation	Rate of Transport (cells $m^{-2} s^{-1} \times 10^{-4}$)	Rate of Accumulation (cells $m^{-2} s^{-1} \times 10^{-4}$)	Presumed Transport Mechanism	Reference
quiescent	40	5000	4170	sedimentation	Fletcher (1977)
laminar	5	167	31	diffusion	Powell and
	1	472	3	diffusion	Slater (1982)
turbulent	0.1	—	—	—	this study

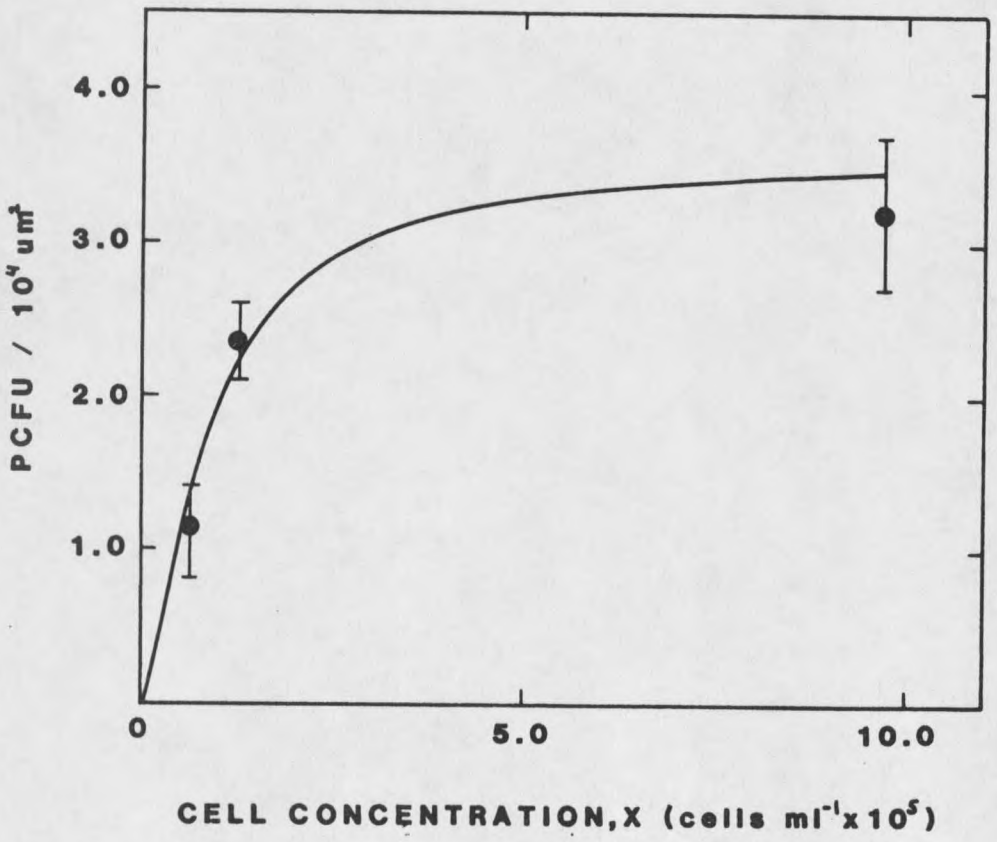

Figure 6. Influence of bulk water cell concentration on potential colony forming units.

Zones of Inhibition

In order to investigate the surface saturation phenomenon further, a variance/mean ratio analysis was performed on the experimental data. The details of this analysis are presented in the Results section and in Appendix 4. The analysis indicates that the distribution of cells on the surface approaches a uniform distribution. The relatively small percent surface coverage of cells at saturation (0.1%) and the relatively uniform distribution of these cells suggest "zones of inhibition" around each attached cell where subsequent attachment of cells from the bulk water is prevented as long as the attached cell remains at the surface. A conceptual representation of the "zones of inhibition" is shown in Figure 7.

Microscopic observations made with a continuous flow system similar to the system used by Powell and Slater (1983) support the "zones of inhibition" concept. Observations of the surface revealed a very dynamic situation with cells constantly attaching and detaching. However, cells preferentially attached to relatively unpopulated areas even if they were initially transported to relatively colonized areas first.

Surface Thermodynamics

Surface thermodynamics provides a reasonable explanation of the "zones of inhibition." Surfaces can be characterized by the concept of surface free energy. Measurements of surface free energy are made by immersing a solid in water and determining the surface tension at the solid/liquid interface. Marshall (1976) observed a "marked lowering" of the surface tension of germanium prisims exposed to pure cultures of bacteria suspended in an artificial seawater medium. He suggests this response is due to the adsorption of bacterial protein; presumably cells and extracellular polymeric substances (EPS). The decrease in surface tension corresponds to a decrease in surface free energy. According to the first law of thermodynamics, the energy within a defined system, must be conserved. Thus, the decrease in surface free energy must be accounted for by a trans-

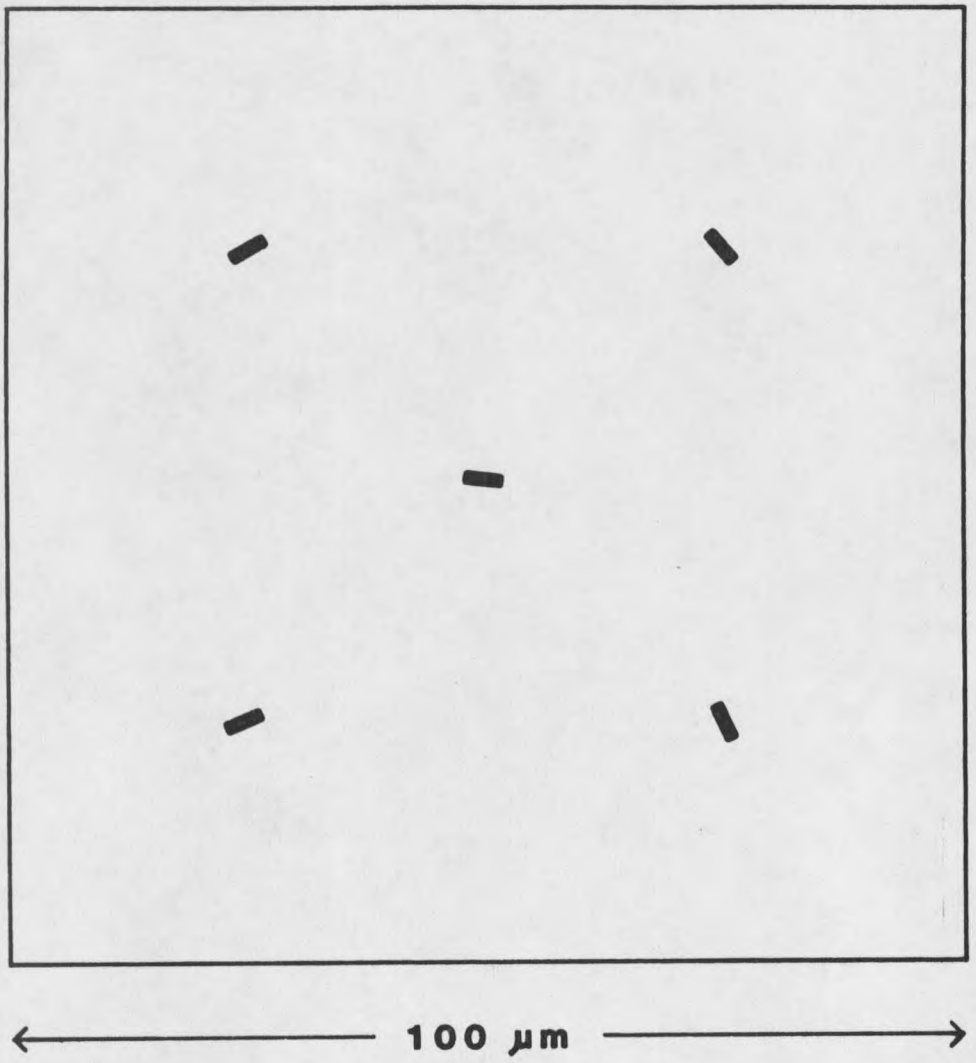

Figure 7. Scale drawing of attached cells covering 0.1% of surface area with a uniform distribution, suggesting "zones of inhibition" concept.

fer of energy somewhere on the surface. It is proposed that the decrease in surface energy is accounted for by the formation of adhesive bonds between the cell (and possibly its EPS) and the surface during the attachment process. Once the available bonding energy of a localized area on the surface is utilized, energy required for attachment is no longer available and the "zone of inhibition" is formed. The size of the "zone of inhibition" is proportional to the bonding energy required for attachment to occur, the available bonding energy per unit area of the surface and of the cell, and localized conditions at the surface/bulk water interface such as the relative turbulence of the bulk water. In these experiments, the "zones of inhibition" were relatively large since approximately 99.9% of the surface remained free of cells after saturation was reached. Regardless of the mechanism of formation, the "zone of inhibition" could be advantageous to cells which attach to surfaces because it would decrease competition for nutrients from other cells in the surrounding micro-environment.

Assumptions

The following assumptions were made in the interpretation of the experimental results:

1. The effect of attached cell growth on the determination of attachment rates was assumed to be negligible because of the following reasons:
   a. Nutrients were not added to the reactor.
   b. Experiments were limited to 6 hours.
   c. PCFU were used in the data analysis instead of cell numbers.
2. The cell concentration in the chemostat was assumed to remain constant in all experiments as indicated by chemostat cell counts (Appendix 3).