



Measurement of primary biofilm formation

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"Measurement of Primary Biofilm Formation"

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ABSTRACT

Biofilm development is a sequence of three events: primary biofilm formation, exponential accumulation, and a quasi-steady-state plateau. Direct and indirect biofilm detection methods used in the latter two stages of development are frequently insensitive to primary film formation.

Methods for detecting primary film formation are presented in relation to their potential use in the power industry. Techniques discussed include the following:

1. Microscopic surveillance
2. Biofilm chemical constituent analysis
3. Viable cell constituent analysis

INTRODUCTION

Biofouling film development in a turbulent flow system progresses in a sigmoidal manner as illustrated in Figure 1. Three phases are evident:

1. Primary Biofilm Formation
2. An Exponential Accumulation
3. Steady State or Plateau Phase

The induction phase terminates when both frictional and heat transfer resistance begin to increase, as shown in Figure 1. The effects of biofilms on both frictional and heat transfer resistance become severe in the latter two phases of development. Naturally, a majority of research has focused on these two biofilm development phases.

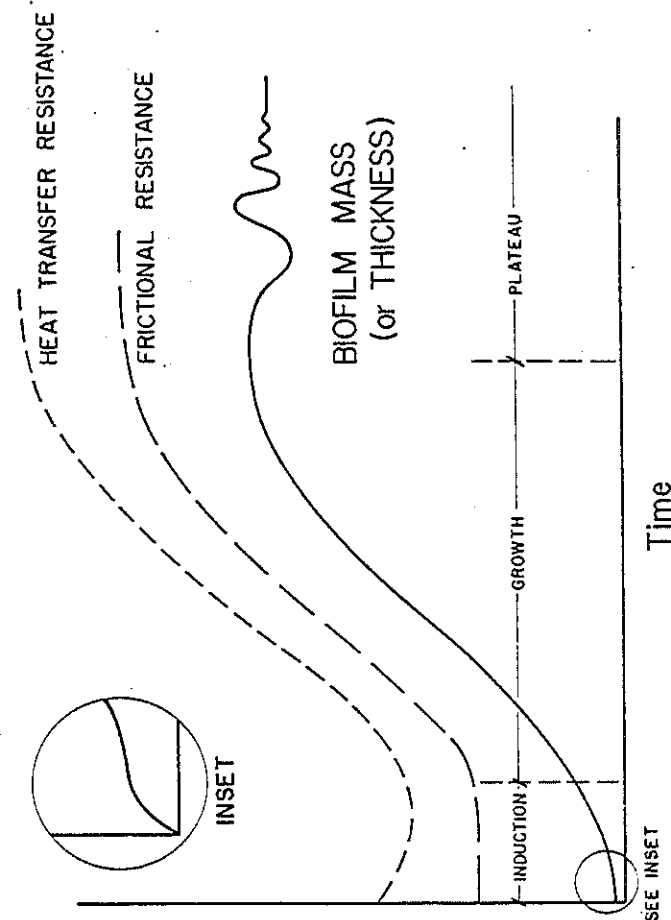


Figure 1. Progression of Biofouling

the percent of polysaccharide within a biofilm increases with time, while the relative amount of viable cells is decreasing. This implies an increasing accumulation of polysaccharides in the biofilm by either cellular production or capture of dispersed materials. Detection of overall biofouling using a viable cell component analysis may seriously underestimate the total amount of biofilm material.

CONCLUSIONS

The feasibility of existing biofilm detection methods to measure primary biofilm formation has been considered. Experimental results of this comparison provide the following conclusions:

1. Direct methods (biofilm thickness, biofilm mass) and indirect methods (frictional and heat transfer resistance increases) are proven reliable, sensitive, indicators of the latter stages of biofilm development.
2. Existing direct and indirect methods do not provide the sensitivity nor the precision to monitor primary biofilm formation.
3. Chemical analysis of either a biofilm or a viable cell constituent provides sensitive, precise detection of early biofilm development. Presently, analysis does require destructive sampling.
4. Responses of biofilm and viable cell component chemical analysis may be different. Biofilm constituents (e.g., mg COD, fiber number, and total polysaccharide attached

per area) increased nonlinearly with time. Viable cell constituents (e.g., nucleic acids and total proteins attached per area) increased linearly. Overall extent of biofouling is underestimated using only viable cell component analysis.

5. Viable cell constituent analysis has two other disadvantages. Use of viable cell components as biofilm indicators after biocide application will overestimate the biocides' biofilm removal efficiency. Viable cell components analysis will also require constant calibration to biomass concentration due to fluctuations in growth conditions.
6. The concept of a uniform biofilm thickness below 10 μm is questionable in light of in situ photomicrographs.

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However, research indicates that the physical, chemical, and biological properties of biofilms may be determined within the primary formation stage.^{1,2,3,4} Previous research regarding primary biofilm formation has generally ignored the effects of parameters of engineering concern. Our work is an attempt to define the stoichiometric and kinetic aspects of primary biofilm formation within a turbulent flow system.

Objectives of our research on primary biofilm formation kinetics are as follows:

1. To develop a biofilm detection method sensitive to early stages of film formation.
2. To develop a kinetic expression for primary biofilm formation as a function of microbial activity and hydrodynamic parameters.
3. To elucidate mechanisms contributing to the overall process of primary biofilm formation.

This paper describes progress on the first objective i.e., measurement of primary biofilm formation. Several existing methods for measuring biofilm quantity are discussed with special attention directed at their suitability for monitoring biofilm formation in the early stages of fouling.

Biofilm Detection Methods

During the growth and plateau phases, biofilm accumulation can be monitored by the several methods listed in Table 1.⁵ Analytical details of these methods are given elsewhere.^{5,6,7,8}

TABLE 1. Measurement of Biofouling

-DIRECT MEASUREMENT OF BIOFILM QUANTITY	
Thickness	
Mass	
-INDIRECT MEASUREMENT: Effects of Biofilm Development on Transport Properties	
Frictional Resistance	
Heat Transfer Resistance	
-MEASUREMENT OF BIOFILM CONSTITUENT	
Polysaccharide	Chemical Oxygen Demand
Nucleic Acid	Organic Carbon
Protein	
-VIABLE CELL CONSTITUENT	
Viable Cell Count	Lipopolysaccharide
ATP	

The direct and indirect biofilm detection methods are excellent measures of biofouling during the latter stages of biofilm development as indicated by other investigators.^{6,7,8} However, lack of both sensitivity and precision restricts use of these methods to the latter two stages of biofilm accumulation. Table 2 lists the sensitivity and precision for the direct detection methods.

TABLE 2. Limits of Direct Biofilm Detection Methods

Method	Sensitivity	Precision	Reference
Biofilm Thickness	9 μm	$\pm 9 \mu\text{m}$	8
	10 μm	$\pm 10 \mu\text{m}$	9
	μm	$\pm 9 \mu\text{m}$	10
Biofilm Mass	2.2 mg/20cm ²	$\pm .01 \text{mg}$	8

Frictional resistance increase is a major detrimental effect of attached biofilms. It is also an important indirect method of biofilm surveillance in the latter stages of biofouling. Evidence indicates that the minimum biofilm detection limit of friction resistance methods is set by the hydrodynamics of the system; specifically, the viscous sublayer thickness. Explanation and experimental observation of the "viscous sublayer" thickness as the lower detection limit to the use of frictional resistance is detailed elsewhere.^{5,8,11}

Schlichting¹² states that pipes with rigid wall roughness appear hydrodynamically smooth unless the roughness protrudes past the viscous sublayer. Norrman⁵ and Zilver⁸ also witness no frictional resistance increases due to biofilms until thicknesses exceed the viscous sublayer. Figures 2 and 3 illustrate this effect as observed by Zilver. A biofilm thickness of 35 μm is observed at 37 hours duration with no effect upon the system friction factor. Above 40 μm , the biofilm creates unexpectedly high increases in frictional resistances. The viscous sublayer thickness for this experiment was 40 μm . Frictional resistance is shown to be unresponsive to biofilm thicknesses less than the viscous sublayer. Therefore, for purposes of this paper, the primary biofilm formation period ceases when the friction factor begins to increase.

Primary Biofilm Detection

Both direct and indirect methods for measuring biofilm quantity were considered insensitive for detection of subtle changes in

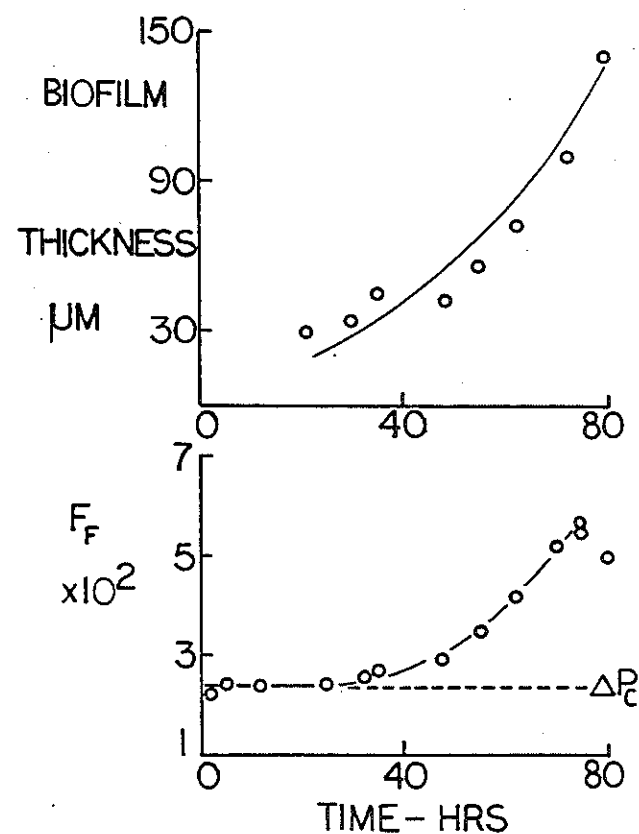


Figure 2. Change in biofilm thickness and friction factor within a circular tube⁸

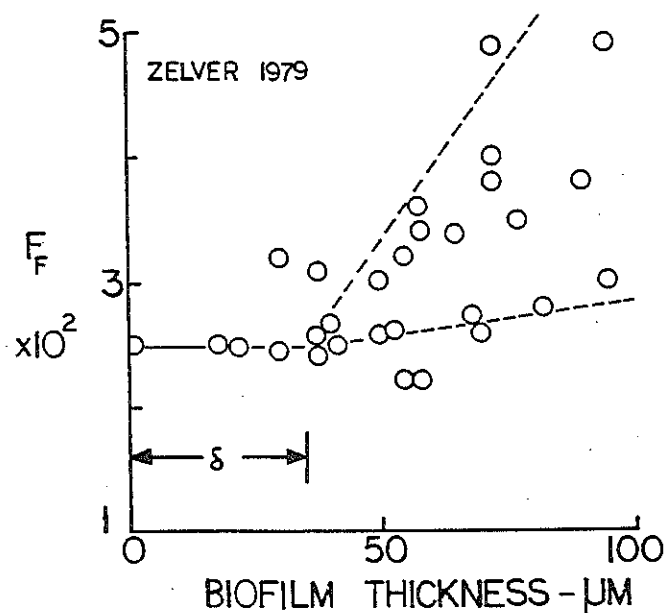


Figure 3. Change in friction factor with increase in biofilm thickness⁸

attached biofilm during the primary formation period. Therefore, biofilm surveillance based upon analysis of either a biofilm or a viable cell constituent was selected. Examples of these methods are given in Table 1.

EXPERIMENTAL PROTOCOL

Apparatus

The Overall System. Microbial fouling experiments were conducted in a 4.75 liter, completely-mixed reactor system. A detailed schematic is presented in Figure 4. Microbial physiological activity (i.e., growth rate) and hydrodynamic conditions can be varied in this system.

The experimental apparatus consists of a nutrient feed system, a 2.0 litre plexiglas mixing vessel, and two internal recycle loops in series.

Nutrient Feed System. A combination of anhydrous dextrose and trypticase soy broth was used as nutrient in all primary biofilm formation experiments. The glucose-to-trypticase soy broth mass ratio entering the reactor was 1:1.

Tap water, used as a diluent for the nutrient feed, was pre-aerated and passed through activated carbon to remove any soluble organics and any chlorine residual.

Sterile nutrients were pumped into the mixing vessel by peristaltic pumps; both nutrients and dilution water were dripped into the reactor fluid to avoid back contamination of the feed lines.

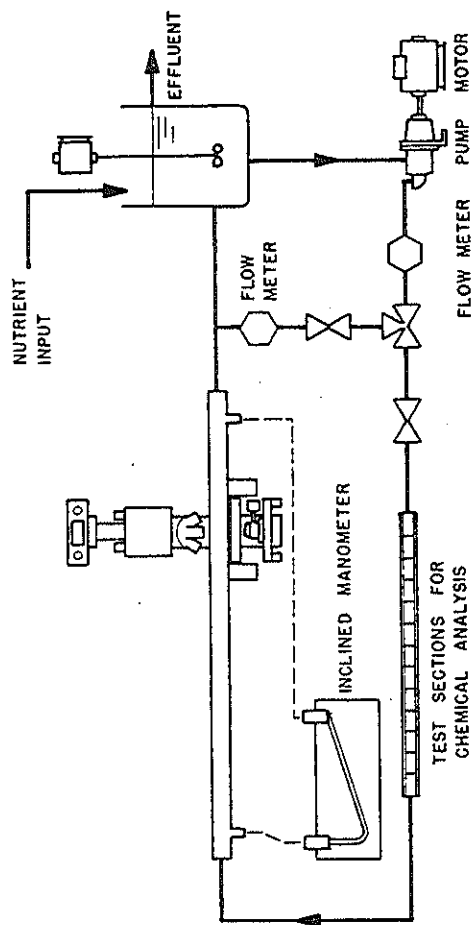


Figure 4. Experimental system for observing primary film formation

Tubular Fouling Reactors. Details of the tubular fouling reactors are shown in Figures 5 and 6.

Reactor One is a 1.27 cm I.D. x 91.4 cm L pyrex glass cylindrical tube equipped with pressure taps 2.50 cm from either end. Pressure drop across the reactor was measured with an inclined mercury manometer (range 15.2 cm Hg). Biofilm development on the inner surface of Reactor One was monitored microscopically.

Reactor Two consists of fifteen 1.27 cm ID x 5.13 cm L pyrex glass test sections within a 1.63 cm ID x 79.6 cm L stainless steel outer tube. Glass sections were periodically withdrawn throughout an experiment to monitor biofilm accumulation.

Both Reactor One and glass test sections of Reactor Two were cleaned using the same procedure prior to each experiment. The procedure is as follows:

1. Soak all glass surfaces in a warm solution of detergent and deionized water.
2. Manually scrub with soft bristle brush.
3. Rinse with alternating solutions of acetone and deionized water.
4. Dry at 288 °C.

Start-Up Procedures. All primary biofouling experiments required the following:

1. All surfaces were clean and unfouled.
2. At zero time, surfaces in the tubular reactors were exposed to a constant biomass concentration of pre-determined growth rate in a fluid flowing at a desired velocity.

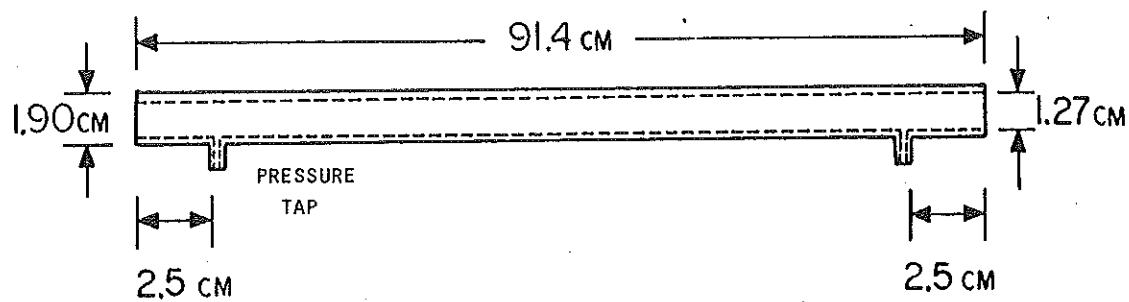


Figure 5. Tubular Reactor One

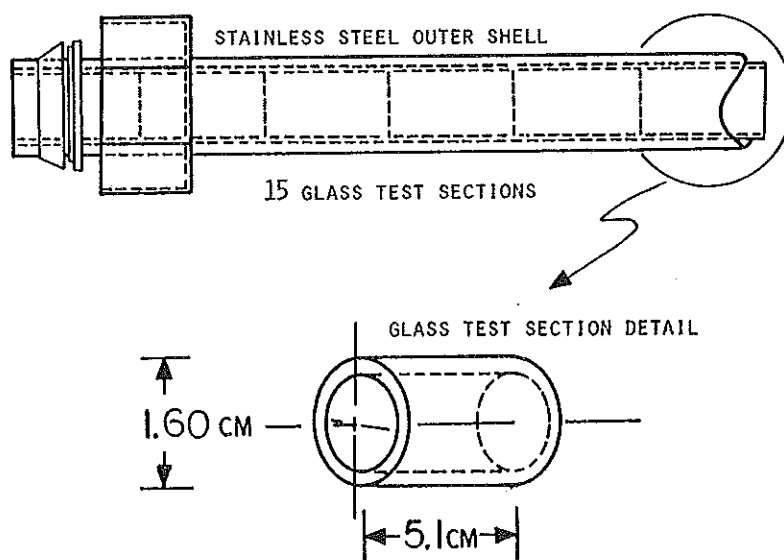


Figure 6. Tubular Reactor Two

These specific requirements dictated the following start-up procedures:

1. All glass reactor surfaces cleaned and in position prior to inoculation.
2. Loop Two containing both tubular fouling reactors was isolated from Loop One by means of two flow control valves.
3. The mixing vessel and Loop One were filled with treated tap water. Recycle flow was started in Loop One.
4. Nutrient was introduced at the desired concentration. Aeration was started.
5. A standard inoculum was introduced into the mixing vessel. Batch growth within the vessel and Loop One was allowed to proceed for 4-5 hours.
6. Continuous reactor operation was started by pumping both nutrients and dilution water into the mixing vessel. Continuous growth conditions were maintained within the vessel and Loop One until the reactor effluent biomass and nutrient concentrations reached a steady-state. This required approximately 2-3 system detention times.
7. Once biomass and nutrient concentrations were steady, recycle flow was set at a predetermined rate in Loop Two. Experimentally, this was considered as zero time.

Analytical Methods

Total amount of oxidizable, attached material is determined using a modified H_2SO_4 - dichromate digestion method.¹³ Concentrations of both the dichromate oxidant and ferrous ammonium sulfate titrant are reduced by one-tenth of the Standard Methods procedure in order to detect the anticipated low levels of attached materials. Replicate samples indicate a minimum detection level of $0.001 \pm .00005$ mg COD/cm².

Total attached polysaccharide is determined colorimetrically by an analysis using concentrated H_2SO_4 for hydrolysis of reducing polysaccharides.¹⁴ The hydrolysis products react with phenol to yield a brownish-yellow color whose intensity is proportional to the polysaccharide concentration. Dubois reports a minimum detection limit of $80 \text{ ng} \pm 1.0\%$.¹⁴ The method is calibrated with reagent grade glucose.

Total attached protein determinations were accomplished by either direct ultraviolet absorbance at 280 nm¹⁵ or by the method of Lowry *et al.*¹⁶ Direct absorbances at 280 nm were corrected for the amount of nucleic acid present. Both protein methods were calibrated with casein dissolved in 3.0% wt aqueous NaOH solution and both analytical methods yielded similar results. Sensitivity was $5.0 \text{ ng} \pm 10\%$.

Total attached nucleic acids (NA) content was determined using ultraviolet absorbance at 260 nm after a cold perchloric acid extraction as detailed by Echlin and Delamater (1962).¹⁷ No relationship between absorbance and concentrations was determined; results are reported as absorbance at 260 nm.

RESULTS AND DISCUSSION

The four types of biofilm detection methods (direct, indirect, biofilm constituent, and viable cell component) are compared for a typical primary biofilm formation experiment conducted under the following conditions:

EXPERIMENTAL CONDITIONS

Recycle Flow Rate	7.95 l/min
Average Recycle Velocity	108.3 cm/sec
Recycle Re	17,200
Inlet Nutrient Concentration	50 mg/l
Dispersed Biomass Concentration	11 mg/l TSS
System Detention Time	60 min
Temperature	32 °C

Figure 7 is photomicrographs taken of the inner surface of Reactor One at elapsed times of 0, 10, 35, and 55 hrs., respectively. During this relatively rapid developing experiment, substantial attached biofilm allowed estimation of an *in situ* biofilm thickness employing the method of Sanders.¹⁸ One fact is obvious from the photographs: a uniform biofilm thickness does not exist until biofilm fibers are of such length or numbers that they entangle and are no longer discrete entities. This lower limit of a uniform biofilm thickness is $9-10 \pm 10 \mu\text{m}$.

Results of both direct and indirect biofilm surveillance for this experiment are presented in Figure 8. A gradual increase in biofilm thickness from $9 \mu\text{m}$ at time = 9 hours to $19 \mu\text{m}$ at time = 34 hours causes no increase in friction factor. However, a rapid increase in biofilm thickness from $19 \mu\text{m}$ to $60 \mu\text{m}$ in an 8-hour

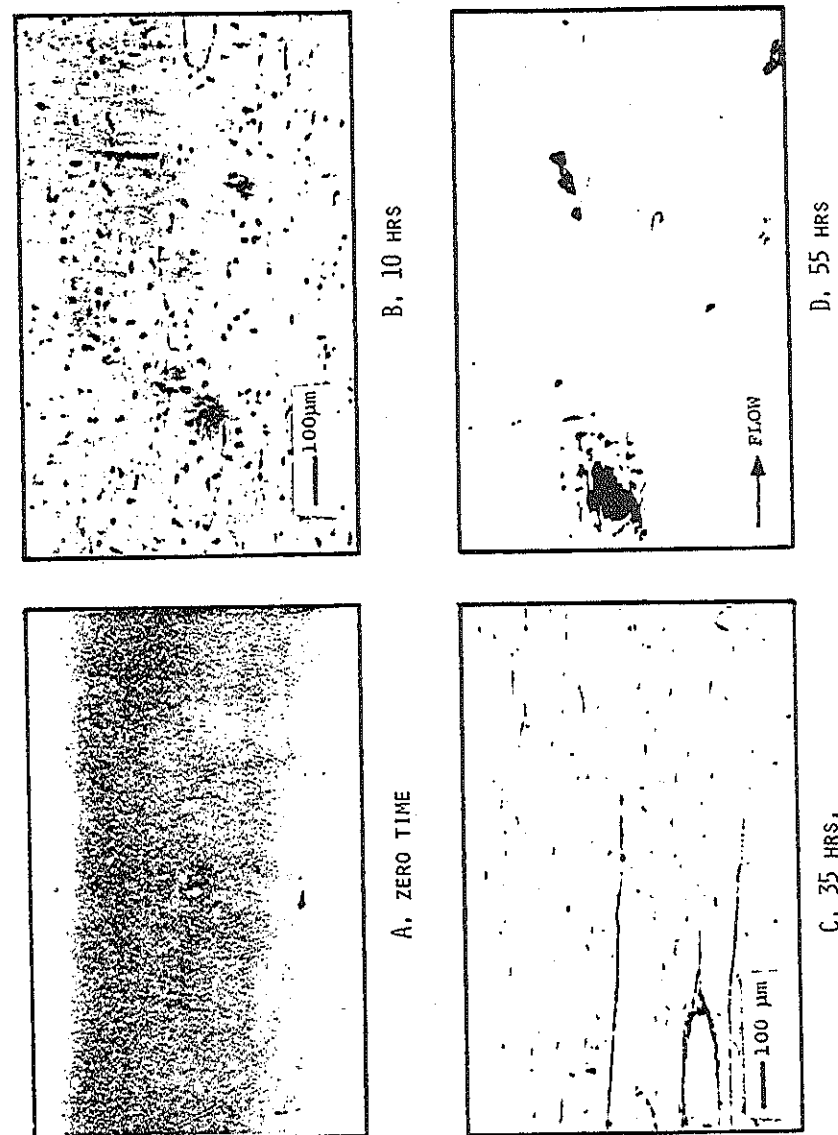


FIGURE 7. PHOTOMICROGRAPHS OF PRIMARY BIOFILM FORMATION

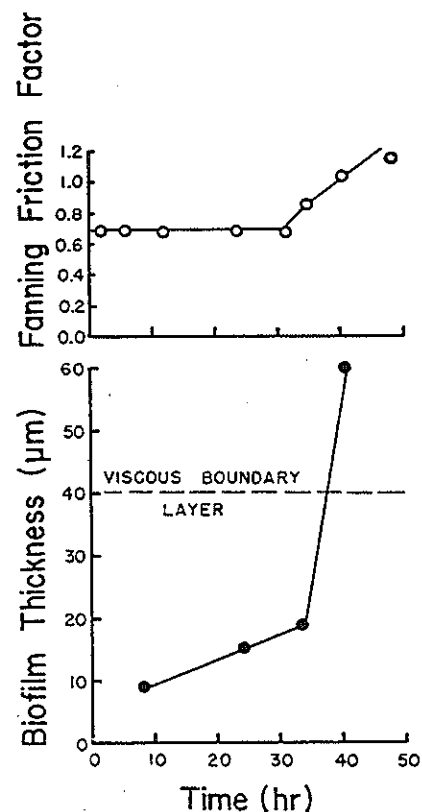


Figure 8. Biofilm development as determined by friction factor and biofilm thickness

period results in a significant increase in observed friction factor. The viscous sublayer thickness for this flow rate is 41 μm , and compares favorably with the estimated biofilm thickness at the onset of increased fluid frictional resistance.

Results of biofilm constituent analysis for the same experiment are presented in Figures 9 and 10. Attached COD, fiber count, and total polysaccharide per unit area exhibit a similar progression; the accumulation increases non-linearly. Results indicate a constant relationship between these three biofilm indicators. Figure 10 infers that a significant polysaccharide adsorption occurs within a very short time period as suggested by the work of Baier *et al.*^{19,20,21} with organic preconditioning films.

Figure 11 summarizes results of two different primary formation experiments at significantly different dispersed biomass concentrations. The amount of attached biofilm is reported as mg COD/cm^2 . The sensitivity of this detection method allows comparison of these two experiments to ascertain their relative rates. Use of either a direct or an indirect method of biofilm detection would not have distinguished a significant difference in these two experiments.

Results of viable cell constituent analysis is depicted in Figure 12. Both total NA and total protein attached increase linearly during the experiment implying a constant rate of viable cell accumulation -- somewhat contradictory to an expected exponential biomass growth rate.

Comparison of Figures 9 and 10 to Figure 12 indicates that

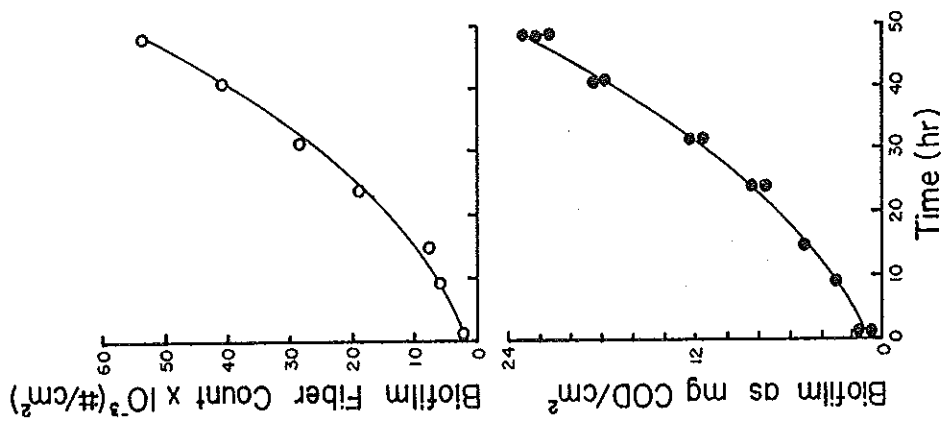


Figure 9. Biofilm development as determined by biofilm fiber count and chemical oxygen demand per unit area

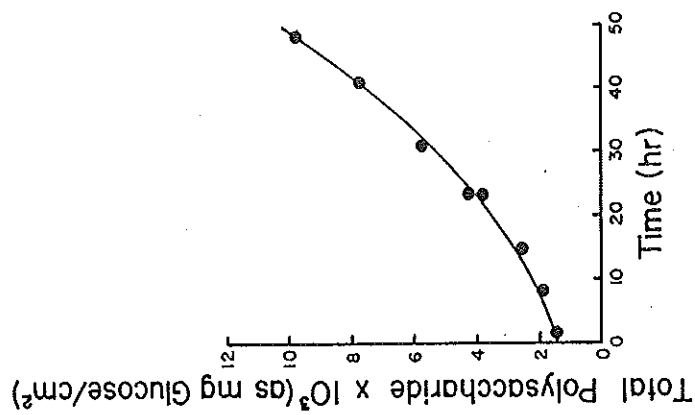


Figure 10. Increase in biofilm polysaccharide

Figure 11. Effect of dispersed biomass concentration in the bulk fluid on biofilm development

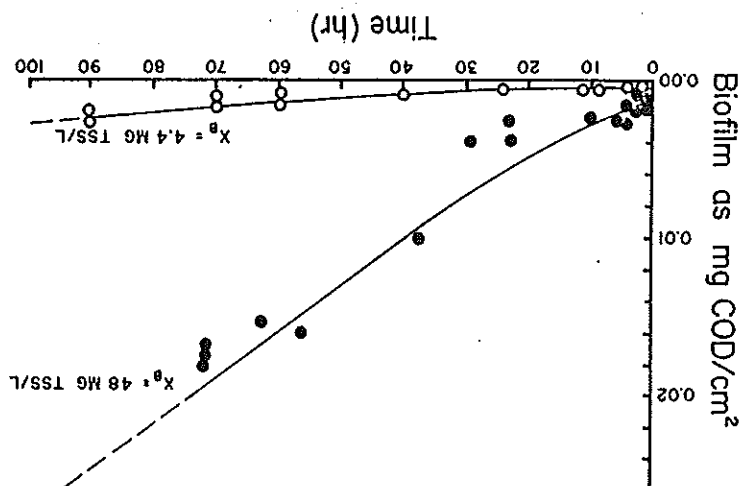
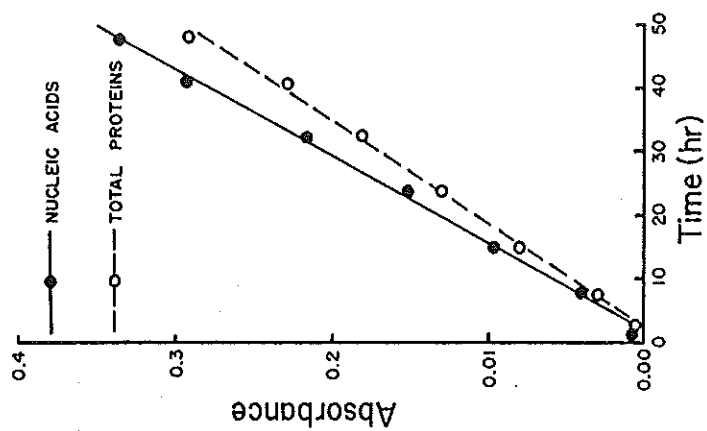


Figure 12. Biofilm development as determined by total attached nucleic acids and total attached proteins

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