Dynamics of biofilm processes : substrate load variations
by Rune Bakke

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

Abstract:
A biofilm is defined as biomass, consisting of bacteria and extracellular polymer substances (EPS), growing on submerged surfaces. The purpose of this study was to investigate the effects of substrate load changes on biofilms.

Variations in soluble and suspended organic concentrations and biofilm mass in a fixed film continuous stirred tank reactor were monitored. Two analyses were conducted on the filtered effluent to measure soluble organics: 1) substrate concentrations were measured to deter- mine substrate uptake rates and 2) soluble organic carbon concentration was measured to determine variations in product formation. Biomass, suspended and attached, was divided into two parts: 1) cellular, de- termined through cell counts, and 2) non-cellular, determined by subtracting the cellular from the total biomass.

An overshoot in reactor substrate and product concentrations, compared to the steady state concentrations before and after the transition, was observed. An immediate increase in substrate flux into the biofilm, termed a biofilm reaction potential, was observed when the inlet substrate concentration was increased.

The initial effects on the biofilm populations from substrate load changes varied depending on the type of substrate used. Dextrose had no significant effect. Lactose and especially lactate load changes resulted in a significant release of EPS as the initial response. A temporary increase in bulk liquid biomass concentration was observed in all experiments following the change in substrate loading.

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Abstract

A biofilm is defined as biomass, consisting of bacteria and extracellular polymer substances (EPS), growing on submerged surfaces. The purpose of this study was to investigate the effects of substrate load changes on biofilms.

Variations in soluble and suspended organic concentrations and biofilm mass in a fixed film continuous stirred tank reactor were monitored. Two analyses were conducted on the filtered effluent to measure soluble organics: 1) substrate concentrations were measured to determine substrate uptake rates and 2) soluble organic carbon concentration was measured to determine variations in product formation. Biomass, suspended and attached, was divided into two parts: 1) cellular, determined through cell counts, and 2) non-cellular, determined by subtracting the cellular from the total biomass.

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The initial effects on the biofilm populations from substrate load changes varied depending on the type of substrate used. Dextrose had no significant effect. Lactose and especially lactate load changes resulted in a significant release of EPS as the initial response. A temporary increase in bulk liquid biomass concentration was observed in all experiments following the change in substrate loading.

CHAPTER 1

INTRODUCTION

Biomass, consisting of bacteria and extracellular polymer substances (EPS), growing on submerged surfaces is termed a biofilm. Biofilms have been used beneficially by engineers as illustrated by fixed-film wastewater treatment systems such as rotating biological contactors. Biofilms also play a major positive role in stream purification (1). Negative effects caused by biofilms in engineering systems include energy losses in water distribution systems and heat transfer equipment resulting from fluid frictional resistance and heat transfer resistance (2). Correlation between corrosion processes and biofilms has been documented (3). Biofilms have also been recognized as major determinants in various human and animal disease states. Biofilm-associated diseases include dental caries, intestinal disorders, pneumonia and cystic fibrosis.

The Problem

Most studies on biofilms have been conducted under steady state or pseudo-steady state conditions, while in many natural systems as well as human-controlled systems, rapid and frequent changes in the biofilm environment may occur. The effects of such changes on the biofilm system are important, and models to predict transitional behavior in

biofilm systems are desirable. Studying transitional behavior may also aid in the understanding of biofilm structure.

In this study, major emphasis was on bulk liquid concentrations: 1) biomass and 2) soluble organics. Two analyses were conducted on the filtered effluent to measure the soluble organics: 1) substrate concentrations were measured to determine substrate uptake rates and 2) total soluble organic carbon concentration was measured to determine variations in product formation.

Biomass was divided into two types: cellular and non-cellular biomass. Cellular biomass consists of bacterial cells. Non-cellular biomass consists of EPS and is determined as the difference between cellular biomass and total biomass. The rationale for studying variations in EPS is as follows:

1. The physiological importance of EPS to the microbial population is not well understood (5).
2. EPS is, on a carbon basis, the major constituent of many biofilms (6).
3. Certain EPS are of importance from an industrial standpoint (e.g., Xanthan gum from Xanthamonas campesteris).
4. Shock loads causing release of biomass with low settleability in wastewater treatment systems, has been observed. The release of non-cellular biomass may be the reason since EPS have lower mass density than bacteria (discussed further in Chapter 4).

Research Goal

The goal of this study is to model transient behavior in biofilm systems. To accomplish this goal the following objectives were established:

Objectives

1. Monitor variations of substrate uptake by biofilms during transients caused by substrate load increases.
2. Monitor variations in product formation by biofilms during transients caused by substrate load increases.
3. Determine variations in microbial cell numbers in the biofilm as a result of substrate load increases.
4. Determine the effect of substrate load increases on biofilm EPS.

CHAPTER 2

METHODS

Experimental Systems

All experiments were conducted in an annular reactor (AR) system consisting of the AR, sterile dilution water and substrate feed apparatus including temperature control as shown in Figure 1.

Annular Reactor

The AR is constructed of acrylic plastic and consist of two concentric cylinders, a stationary outer cylinder and a rotating inner cylinder. Figure 2 illustrates details of the reactor. Rotational velocity was controlled by a fractional horsepower gear motor (Model No. NSH11D3 with Series 200 Speed Controller, Bodine Electric Co., Chicago, IL) and continuously monitored by a tachometer/torque transducer unit (submitted for United States Patent, Patent Application Serial No. 388,972) mounted on the shaft between the rotating cylinder and the motor drive pulley assembly. The tachometer/torque transducer unit also continuously monitored changes in fluid frictional resistance caused by biofilm development. Rotational velocity and torque were continuously displayed and recorded by an Apple Computer-CRT monitor system (Apple Computer Inc., Cupertino, CA, Model II Plus, and Montgomery Ward, Chicago, IL, Model No. GGY 12310-A). Each AR contained 4 thin removable slides which were used for biofilm sampling and thick-

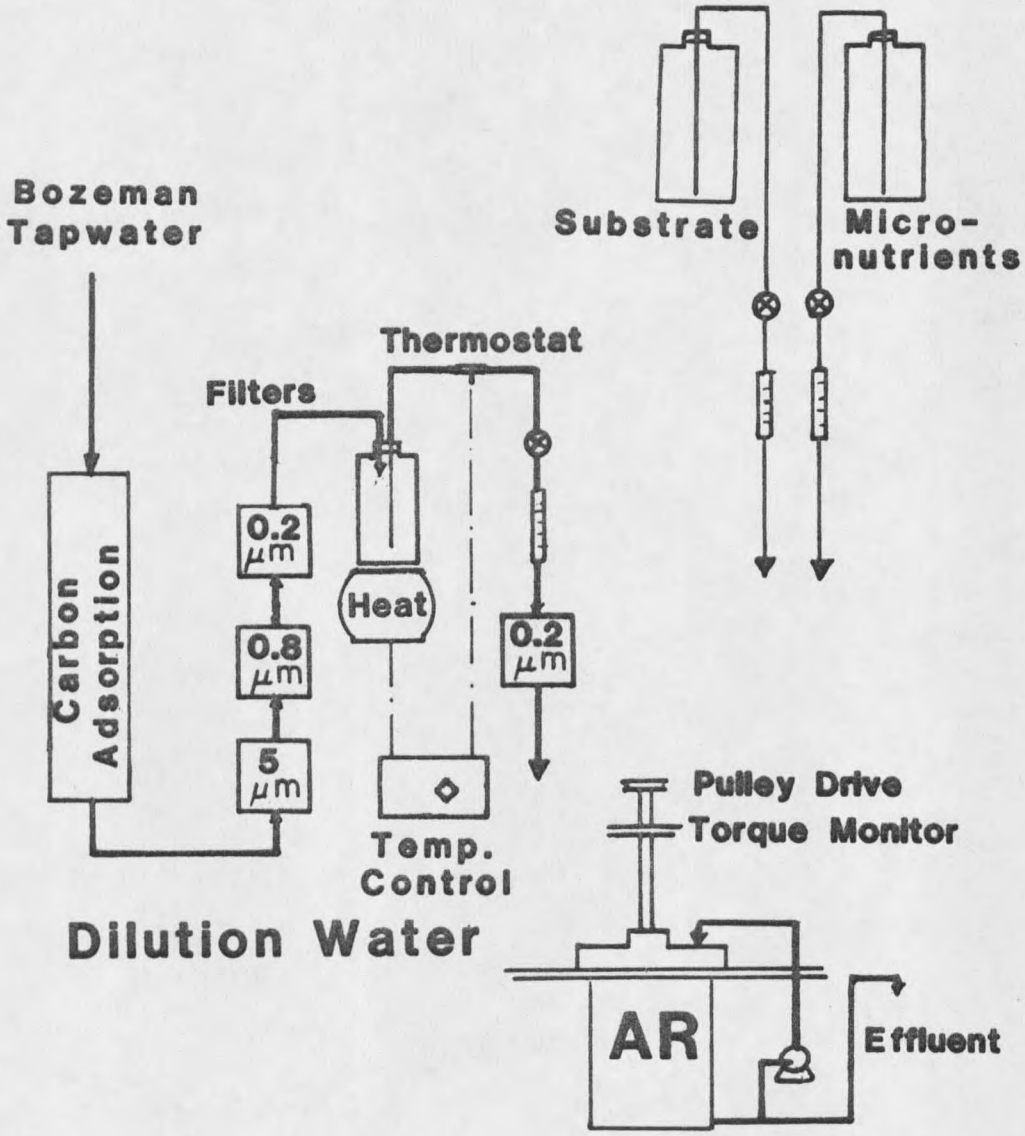

Figure 1 Annular Reactor (AR) System.

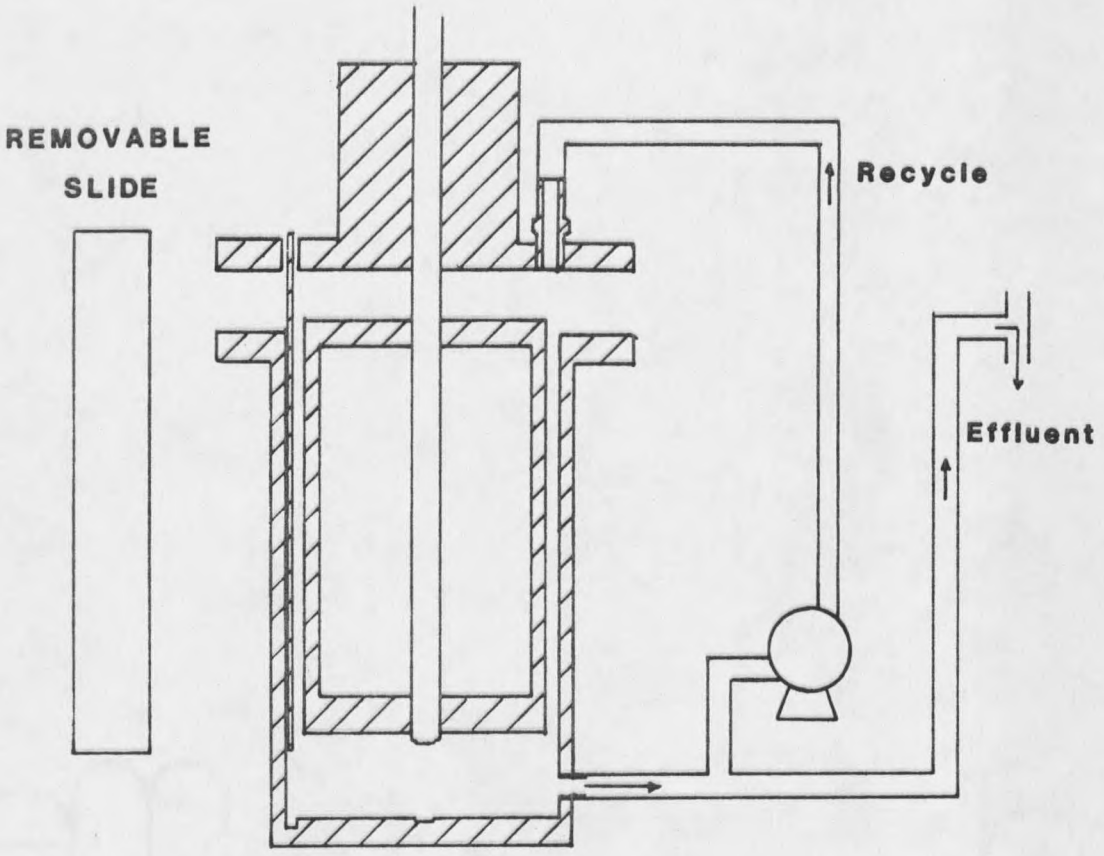

Figure 2 Annular Reactor.

ness measurements. The slides fit next to the inside walls of the outer cylinders.

The AR's were completely mixed (6) by virtue of the recirculating action of a peristaltic pump (Cole-Parmer Instrument Co., Chicago, IL, Model No. WZ1R057) which was used to pump AR liquid solution from the bottom to the top of each AR at volumetric flow rates approximately 10 times greater than the overall volumetric flowrate through each AR. Table 1 presents relevant characteristics and dimensions of the AR's. Advantages of the AR configuration include the following:

1. No concentration gradients exist in the bulk fluid due to complete mixing. This simplifies mathematical description and sampling.
2. Fluid shear stress at the wall can be varied independent of mean residence time.
3. High surface area to volume ratio.

Dilution Water and Substrate Feed

Treated dilution water was continuously fed into the AR's using reduced tap water line pressure (AW Cash Valve Mfg. Corp., Decatur, IL, Type A-315 Pressure Regulator). Dilution water flowrates were controlled at 66.5 ± 2.5 cm^3/min using needle valves (Whitey Co., Oakland, CA, Model No. ORM2) and monitored with in-line flow meters (Gilmont Instruments Inc., Great Neck, NY, Size No. 13).

Sterile substrate solution was continuously fed to the AR's by gravity flow. Flowrates were controlled with Dial-A-Flo® valves (Sorenson Research Co., Salt Lake City, Utah, Cat. No. DAF-30) and

Table 1. Relevant Characteristics and Dimensions of the Annular Reactor

Reactor	
Liquid Volume	675 cm^3
Total Wetted Surface Area (including recycle tubing)	1860 cm^2
Inner Cylinder Wetted Surface Area	734 cm
Outer Cylinder Wetted Surface Area	920 cm^2
Diameter of Inner Cylinder	10.2 cm^2
Width of Annular Gap	0.6 cm
Wetted Height of Inner Cylinder	17.8 cm
Wetted Height of Outer Cylinder	20.0 cm
Volumetric Flowrate	67.5 cm^3/min
Mean residence Time	10 min
Removable Slide	
Wetted Surface Area	28.7 cm^2
Height	24.5 cm
Width	1.9 cm

monitored with in-line flow meters (Gilmont Instruments Inc., Great Neck, NY, Size No. 11).

Substrate Solution Preparation

The substrate solution used in each experiment was the sole energy and carbon source with micronutrients added as shown in Table 2. The micronutrients solution and the substrate solution were prepared separately with distilled water and sterilized by autoclaving.

Table 2. Micronutrients Solution Composition Relative to the Substrate Carbon Concentration

Constituent	Relative Concentration [g per g substrate carbon]
NH_4Cl	0.90
$MgSO_4$ $7H_2O$	0.25
$CaCl_2$	0.025
$FeCl_3$ $6H_2O$	0.005
K_2HPO_4	0.0045
KH_2PO_4	0.75

Dilution Water Treatment

Montana State University tap water was the source of the dilution water. Dilution water treatment consisted of passage through a carbon adsorption column for the removal of residual chlorine and soluble organics followed by filtration through a four filter cascade (5.0 µm, 0.8 µm, 0.2 µm, 0.2 µm; Gelman Sciences, Inc., Ann Arbor, MI, Product Nos., 12585, 12623, 12580, and 12112, respectively) for the removal of particulate and suspended cellular material.

Temperature

Dilution water passed through a Pyrex glass temperature adjustment reservoir (volume 9500 cm^3) before entering the AR's. Reactor temperatures were controlled at 25 ± 1°C by a temperature controller (Yellow Springs Instruments Co., Yellow Springs, OH, Model No. 74) activating a hot plate (VWR Scientific Inc., Dyla-Dual Hot Plate-Stirrer). The hot plate was located under the temperature adjustment reservoir and the temperature controller thermister was located in the dilution water distribution manifold directly upstream of the AR's.

Experimental Procedures

Established biofilms were used for all experiments. Stable conditions, were maintained for a minimum of 100 detention times before the transition was induced.

Sampling

Solution samples were collected directly from the AR effluent lines and stored as follows:

a) substrate - 10 cm^3 samples were filtered (Nuclepore Corp., Pleasanton, CA. No., 111107, average pore size 0.45 μm) and frozen until analysis.

b) suspended solids - 50 cm^3 samples were filtered (Nuclepore Corp., Pleasanton, CA, No., 111107, average pore size 0.45 μm), dried, and weighed.

c) acridine orange direct count (AODC) - 10 cm^3 samples were fixed in 2% formalin solution (7) and stored at 2°C.

d) organic carbon - 20 cm^3 samples were filtered (Nuclepore Corp., Pleasanton, CA. No., 111107, average pore size 0.45 μm) and frozen until analysis.

Biofilm samples were obtained from the AR removable slides by scraping the biofilm into 35 cm^3 of filtered (Millipore Corp., Bedford, MA, 0.22 μm, Type GS filter), carbon-free (Barnstead Co., Boston, MA, Combination Exchange Cartridge, Cat., No. 08922), deionized water. The resulting solution was homogenized (DuPont Co., Instrument Products, Newtown, CN, Sowall Omni-Mixer) and subsamples were prepared and stored as follows:

a) mass density - 10 cm^3 samples were filtered (Nuclepore Corp., Pleasanton, CA. No., 111107, average pore size 0.45 μm), dried, and weighed.

b) organic carbon - 10 cm^3 samples were frozen until analysis.

c) acridine orange direct count - 5 cm^3 were fixed in 2% formalin solution (7) and stored at 2°C.

Analytical Methods

Suspended Solids

Suspended solids concentration was determined by filtering 50 cm^3 of reactor effluent through predried (103°C for 1 hour), preweighed, Nuclepore filters (Nuclepore Corp., Pleasanton, CA, No. 111107, average pore size 0.45 μm). After filtration, the filters were dried at 103°C

for 1 hour and weighed (Mettler Instruments Corp., Hightstown, NJ, Type H6 Digital Balance).

Glucose

Glucose concentration was measured using a modified version (6) of the Sigma 510 Glucose Analysis Procedure (Sigma Chemical Co., St. Louis, MO). The measured glucose concentration was multipled by 0.4 g glucose carbon/g glucose to determine glucose carbon concentration.

Lactate

Lactate concentration was measured using a modified version (8) of the Sigma 500 Lactate Analysis Procedure (Sigma Chemical Co., St. Louis, MO).

Acridine Orange Direct Count

Total cell number concentration was determined by enumerating cells stained with acridine orange using epifluorescence microscopy (Leitz Wetzlar, Rochleigh, NJ, Ortholux II Universal Microscope) according to the methods of Hobbie et al., (7).

Biofilm Mass

Total biomass was measured as carbon using the ampule analysis module of an Oceanography International Carbon Analyzer (Oceanography International Corp., College Station, TX, Total Carbon System, Cat. No. 0524B).

CHAPTER 3

RESULTS

Comprehensive listings of raw data for all experiments are found in Appendix B.

Four transient experiments in biofilm reactors were conducted with lactate, lactose, lactose and glucose as substrates in Experiments 1, 2, 3, and 4, respectively. Experimental conditions are listed in Table 3.

Variations in substrate and SOC concentrations in the bulk liquid are presented first, followed by effects on the biological population measured as cellular and non-cellular biomass.

Experimental progressions are presented in which time zero is defined as the time at which the substrate load was changed.

Substrate Removal

Figures 3 and 4 indicate the response of bulk substrate and SOC concentrations to a doubling of the influent substrate concentrations in Experiments 1 and 4. Substrate was not measured in Experiments 2 and 3 because no method for measuring lactose concentrations was available.

An overshoot in substrate concentration during the transition compared to the steady state concentrations before and after the transition was observed. A similar overshoot in SOC was also observed, but

Table 3. Experimental Conditions

	Experiment #			
	1	2	3	4
Substrate	Lactate	Lactose	Lactose	Dextrose
Dilution Rate, D, hr^{-1}	6	6	6	6
Temperature, °C	25	25	25	25
Aerobic/Anaerobic Biofilm	no/yes	yes/no	yes/no	yes/yes
Biofilm Thickness, μm	60 - 600	22 - 75	22 - 75	22 - 75
Microbial Population	mixed culture	mixed culture	*P. aeruginosa*	mixed culture
Inlet Substrate Concentration S_i [mg C/ℓ]	4 → 8*	4 → 8*	4 → 8*	8 → 16*

*before and after initiation of transient experiment.

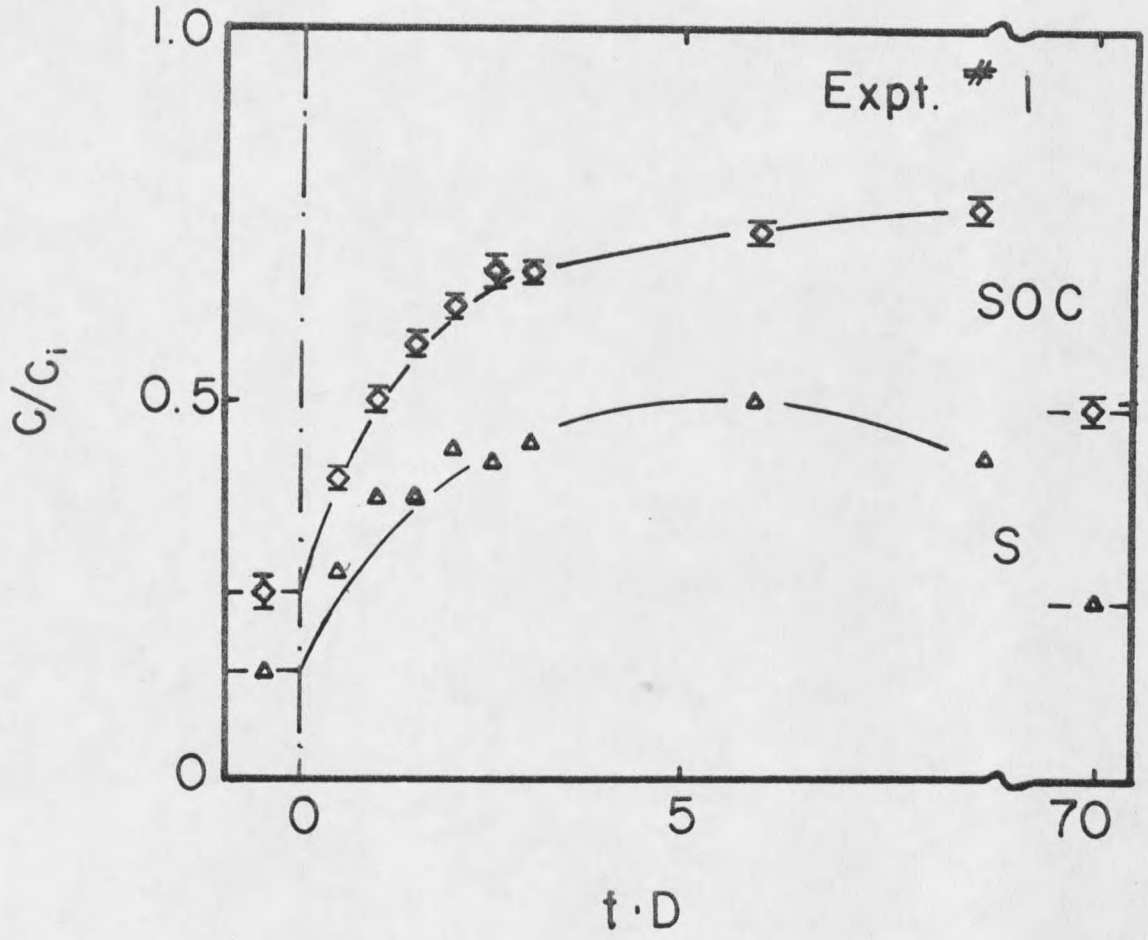

Figure 3 Progression of dimensionless substrate, S, and soluble organic carbon, SOC, concentrations before and after an increase in inlet lactate concentration, Si. Lines drawn by observation. Error bars represent standard deviation of two measurements of the same sample.

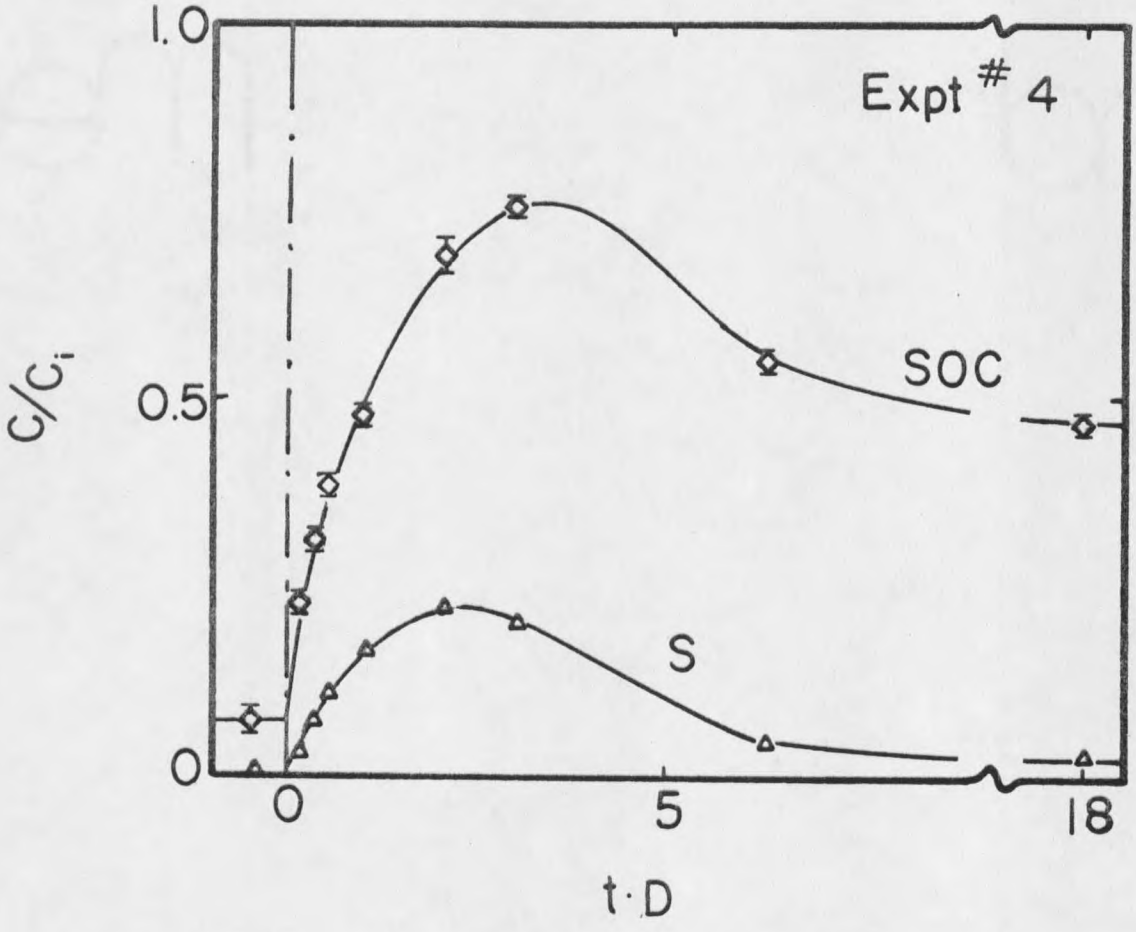

Figure 4 Progression of dimensionless substrate, S, and soluble organic carbon, SOC, concentrations before and after an increase in inlet dextrose concentration, Si. Lines drawn by observation. Error bars represent standard deviation of two measurements of the same sample.

with the peak occurring later. These results suggest that formation of soluble organic products reached a maximum after the substrate overshoot peak had been reached.

Biomass Variations

Changes in the biofilms during the transients are illustrated in Figures 5 through 9.

Experiment 1

Figure 5 indicates no significant changes in biofilm cell numbers or in effluent cell numbers until after approximately 1.5 residence times. Total effluent biomass concentration was, however, increased by an order of magnitude within 5 minutes, and the biofilm mass dropped to less than half of its previous mass within 30 minutes. Total biomass can be considered the sum of organisms and EPS (6). Since there were no significant initial effects on the population numbers, the drastic effects on the total biomass concentration is attributed to EPS detachment.

Only the effluent was sampled in Experiments 2, 3, and 4. To eliminate the possiblity that the effluent mass concentration increase, as observed in Experiment 1 was due to physical disturbance of the biofilm from biofilm sampling. No changes, other than the substrate increase, were induced on the reactors within three detention times before and after the substrate load change.

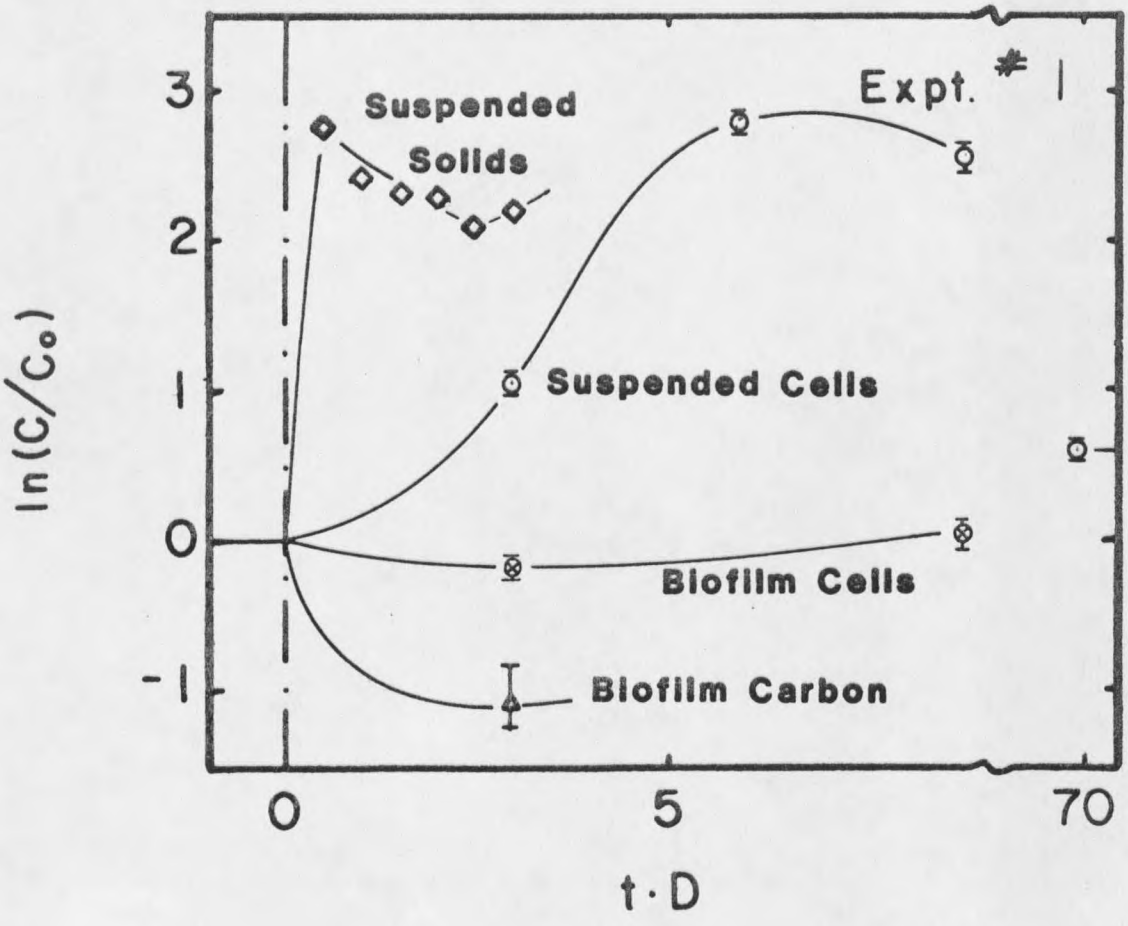

Figure 5 Progression of biomass concentrations relative to the pre-change concentration, C_o, after an increase in inlet lactate concentration. Lines drawn by observation. Error bars represent standard deviation of measurements of the same sample.

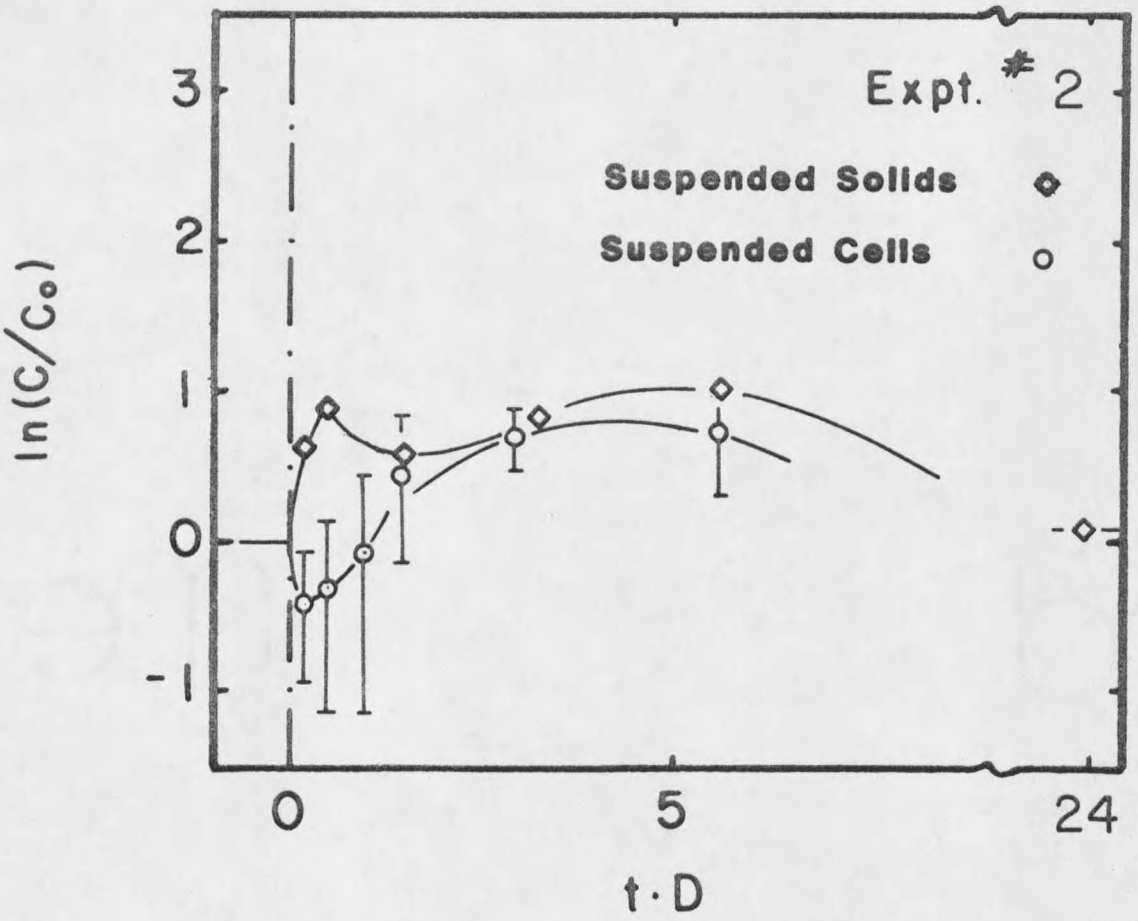

Figure 6 Progression of biomass concentrations relative to the prechange concentrations, C_o, after an increase in inlet lactose concentration. Lines drawn by observation. Error bars represent standard deviation of measurements of the same sample.