The influence of calcium on biofilm processes
by Mukesh Harilal Turakhia

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemical Engineering
Montana State University
© Copyright by Mukesh Harilal Turakhia (1986)

Abstract:
Bacteria exhibit a tendency for adsorbing to and colonizing surfaces which are submerged in aquatic environments. Adsorption is mediated by extracellular polymeric material which is formed by the bacteria and extends from the cell to the attachment surface. The attached cells reproduce and form additional extracellular polymer increasing the mass of the deposit. The cellular-extracellular matrix is termed a bipfilm.

The purpose of this study was to investigate the effect of calcium on cellular reproduction and extracellular polymer formation by Pseudomonas aeruginosa in a biofilm.

Experiments were conducted with a pure culture of Ps. aeruginosa using fixed film bioreactors with glucose serving as the limiting nutrient.

Results indicate calcium increases the rate and extent of cellular carbon accumulation at the surface. However, there was no effect of calcium on the amount of polymer carbon accumulated on the surface. Results also suggest that free calcium (or calcium-assisted ligands) is essential to the structural integrity of the biofilm. The energy required for biochemical conversion of glucose into biomass by suspended or immobilized culture of Ps. aeruginosa was constant and was independent of time, biomass concentration, specific cellular growth rate, and calcium concentration in the medium.

THE INFLUENCE OF CALCIUM ON BIOFILM PROCESSES

by

Mukesh Harilal Turakhia

A thesis submitted in partial fulfillment,
of the requirements for the degree

of

Doctor of Philosophy

in

Chemical Engineering

MONTANA STATE UNIVERSITY
Bozeman, Montana

March 1986

ii

APPROVAL

of a thesis submitted by

Mukesh Harilal Turakhia

This thesis has been read by each member of the thesis committee
and been found to be satisfactory regarding content, English usage,
format, citations, bibliographic style, and consistency, and is ready
for submission to the College of Graduate Studies.

5 March 1986
Date

W. G. Charachlis
Chairperson, Graduate Committee

Approved for Major Department

5 March 1986
Date

John T. Sears
Head, Major Department

Approved for College of Graduate Studies

March 10, 1986
Date

Henry L. Parsons
Graduate Dean

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the require-
ments for a doctoral degree at Montana State University, I agree that
the Library shall make it available to borrowers under rules of the
Library. I further agree that copying of this thesis is allowable only
for scholarly purposes, consistent with "fair use" as prescribed in the
U.S. Copyright Law. Requests for extensive copying or production of
this thesis should be referred to University Microfilms International,
300 North Zeeb Road, Ann Arbor, Michigan 48106, to whom I have granted
"the exclusive right to reproduce and distribute copies of the disser-
tation in and from microfilm and the right to reproduce and distribute
by abstract in any format."

Signature _Mukesh Turakhia_

Date ___S March 1986___

ACKNOWLEDGMENTS

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

ABSTRACT

Bacteria exhibit a tendency for adsorbing to and colonizing surfaces which are submerged in aquatic environments. Adsorption is mediated by extracellular polymeric material which is formed by the bacteria and extends from the cell to the attachment surface. The attached cells reproduce and form additional extracellular polymer increasing the mass of the deposit. The cellular-extracellular matrix is termed a biofilm.

The purpose of this study was to investigate the effect of calcium on cellular reproduction and extracellular polymer formation by Pseudomonas aeruginosa in a biofilm.

Experiments were conducted with a pure culture of Ps. aeruginosa using fixed film bioreactors with glucose serving as the limiting nutrient.

Results indicate calcium increases the rate and extent of cellular carbon accumulation at the surface. However, there was no effect of calcium on the amount of polymer carbon accumulated on the surface. Results also suggest that free calcium (or calcium-assisted ligands) is essential to the structural integrity of the biofilm. The energy required for biochemical conversion of glucose into biomass by suspended or immobilized culture of Ps. aeruginosa was constant and was independent of time, biomass concentration, specific cellular growth rate, and calcium concentration in the medium.

INTRODUCTION

Microorganisms, primarily bacteria, exhibit a tendency for adsorbing to and colonizing surfaces which are submerged in aquatic environments. The immobilized cells grow, reproduce, and produce extracellular polymeric substances (EPS) which frequently extend from the cell, forming a tangled mass of fibers lending structure to the entire assemblage which shall be termed a biofilm.

Biofilms serve beneficial purposes in natural environments and in some modulated systems. For example, biofilms are responsible for removing organic or inorganic "contaminants" from natural streams and in wastewater treatment processes (e.g., trickling filters and rotating biological contactors). Biofilms can, however, impair the performance of process equipment. They can impede the flow of heat across the surface, increase fluid frictional resistance at the surface, and increase the corrosion rate at the surface. Fouling of heat exchange equipment was estimated to cost the United States billions of dollars annually (Lund and Sandu, 1981).

Most studies on the effect of dissolved constituents on biofilm formation have been limited to the effect of organic constituents. There is little or no information on the effect of dissolved inorganic constituents. There are a number of inorganic components which can have an effect. However, the scope of this study was limited to the effect of calcium on biofilm formation.

The presence of calcium in the microbial growth medium has been shown to (1) influence microbial adsorption to the solid substratum, (2) be required for cellular growth and reproduction, (3) influence the composition of EPS. Published reports on the influence of calcium on the above processes is contradictory and the majority of the studies were conducted in quiescent conditions where the cells are not subject to shear stress. Understanding the role of calcium in biofilm processes may be useful in ecosystem analysis, control of biofouling in heat exchangers and/or pipelines, operation of fixed film biological wastewater treatment processes and increasing the rate or strength of cell immobilization in a biofilm reactor for biotechnology applications.

The major emphasis of this study was to determine the influence of calcium on (1) cellular reproduction and polymer formation in a biofilm, and (2) the cohesive strength of biofilm. Pseudomonas aeruginosa was used as a test organism because this organism has been extensively studied both in continuous flow stirred tank reactors, i.e., chemostats, and in biofilm reactors. Specific cellular growth rates and EPS formation rates are known under defined experimental conditions in chemostat and biofilm environments (Robinson et al., 1984; Bakke et al., 1984).

Research Goal and Objectives

The goal of this research was to determine the role of calcium in the formation and maintenance of a biofilm. To accomplish this goal, the following objectives were established:

1. Determine the influence of calcium on cellular reproduction and extracellular polymer formation by <u>Pseudomonas</u> <u>aeruginosa</u> in a biofilm.

2. Determine the influence of calcium on biofilm cohesiveness.

3. Determine the influence of calcium on the net accumulation rate of biofilm resulting from processes and characteristics in Objectives (1) and (2).

LITERATURE REVIEW

Biofilm Formation: A Process Analysis

The adsorption of bacteria is a general phenomenon encountered in natural environments with important ecological implications. Bacterial adsorption to surface offers advantages in terms of nutrient availability, particularly in fast flowing and nutrient deficient habitats. The adsorbed cells reproduce and form extracellular polymers leading to the formation of a biofilm. Accumulation of biofilm at the surface is the net result of the following fundamental processes (Characklis, 1981):

1. Adsorption of organic molecules to the surface forming a conditioned surface.

2. Transport of microbial cells to the conditioned surface.

3. Microbial adsorption to the conditioned surface.

4. Microbial transformation (growth, reproduction, etc.) at the surface resulting in the formation of biofilm.

5. Partial detachment of biofilm due to fluid shear stress.

Biofilm formation is not a sequence of the above rate processes occurring individually but rather the net result of these processes occurring simultaneously. At specific times in the overall development, certain rate processes contribute more than the others. This literature review will focus on the influence of calcium on the processes involved in the formation of biofilm.

Organic Adsorption

Adsorption of an organic monolayer occurs within minutes of exposure of an initially "clean" surface to an aqueous environment containing dissolved organics, microorganisms, and nutrients. This adsorption changes the properties of the wetted surface and actually conditions the surface for subsequent attachment and colonization (Loeb and Neihof, 1975; Baier and Depalma, 1977). These conditioning films have been investigated by various means. Baier and various co-workers have characterized these acquired films as negatively charged (poly-anionic) polysaccharides or glycoproteins (Baier, 1980, Baier and Weiss, 1975; Marshall, 1979).

There appears to be no evidence, however, that microorganisms can only attach to conditioned surfaces. Also, little is known regarding the influence of calcium on organic adsorption.

Transport of Microbial Cells

Microbial cells (0.5 - 10.0 μm) can be transported from the bulk fluid to the wetted surface by several mechanisms, including the following: diffusion (Brownian), gravity, thermophoresis, taxis, and fluid dynamic forces (inertia, drag, drainage, and downsweeps).

In general, the transport of microbial cells from the bulk fluid to the wetted surface depends on fluid flow conditions and is not known to be influenced by the presence of calcium in the bulk fluid.

Microbial Adsorption

Once bacterial cells have been transported to the wetted surface, two types of adsorption are possible; reversible and irreversible (Marshall et al., 1971; Zobell, 1943). Reversible adsorption is characterized by an initially weak adsorption of a cell which still exhibits Brownian motion and is readily removed by mild rinsing. Conversely, irreversible adsorption is a permanent bonding to the surface, usually aided by the production of EPS (Fletcher, 1980). Cells attached in this way can only be removed by rather severe mechanical or chemical treatment.

Calcium and Microbial Adsorption

The role of cations in the adsorption of a cell to the substratum is presently unknown. Roux (1894) reported the necessity for divalent cations, notably Ca^{2+}, in cellular adsorption. Calcium has been shown to be necessary for adsorption of aquatic bacteria (Marshall et al., 1971; Fletcher and Floodgate, 1973; Stanley, 1983) and marine diatoms (Cooksey, 1981). For example, a marine pseudomonad would not irreversibly adsorb in the absence of Ca^{2+} and Mg^{2+}, but would adsorb when either of the cations were present (Marshall et al., 1971). Stanley (1983) observed that Ps. aeruginosa adsorbed poorly in distilled water with adsorption increasing as calcium chloride concentration was increased to 10 mM.

Fletcher (1980) observed the influence of cations on the adsorption process by "chemically treating" free-living cells and

observing any influence on their subsequent adsorption to surfaces. The adsorption of a marine pseudomonad (Fletcher, 1980) was inhibited by the presence of EDTA (ethylenediaminetetra-acetic acid) suggesting that the chelant removed surface-bound divalent cations conceivably involved in intercellular-ionic bridging. Fletcher (1980) noted that lanthanum decreased bacterial adsorption and postulated that lanthanum prevented adsorption through interaction with and subsequent denaturation of EPS. Lanthanum is known to inhibit calcium transport into cells, and to displace calcium from cellular membranes (Weiss, 1974), so that the effect observed by Fletcher (1980) may have been related to the diminution of the flux of calcium to the intracellular space.

Divalent cations, calcium in particular, have been shown to influence microbial adsorption. However, the role of cations in the adsorption process is much disputed. It has been suggested that cations influence adsorption (1) by directly influencing cell physiology or membrane permeability (Drapeau and MacLeod, 1965), and (2) directly by their accumulation at the cell surface where they mediate the formation of the electric double layer (Shaw, 1970). Moscona (1968) suggested that the removal and/or absence of divalent cations inhibits adsorption via calcium sensitive ligands. It has also been suggested that divalent cations, especially calcium, can form bridges between negatively charged substrata and microorganisms, can stabilize the structure of EPS (Fletcher and Floodgate, 1973), or cause precipitation of EPS in the space between a cell and a substratum (Rutter, 1980). Further evidence for the involvement of calcium in the adsorption process comes from the use of complexing agents EDTA

(Fletcher, 1980) and EGTA (Appendix H). The chelant did not remove cells irreversibly adsorbed (ethylene glycol-bis(β-aminoethyl ether)-N, N-tetraacetic acid) to the surface (Fletcher, 1980; Appendix H) suggesting that calcium was not involved in the adsorption of cells to the substratum.

Microbial Transformation

The attached microorganisms assimilate nutrients, reproduce, and form extracellular polymers. The combined result of these processes is the formation of biofilm. The characteristic of the biofilm accumulated will depend on the microbial species, the polymers produced, and the environmental conditions.

Biofilm studies thus far (Kornegay and Andrews, 1967; Lamotta, 1967; Zelver, 1979; Trulear and Characklis, 1982) relied on a relatively unstructured approach to the analysis of biomass components. The biotic component was generally characterized only in terms of cell number and cell mass with little attention to the physiological state of the organisms, although there have been some limited attempts at distinguishing between reproduction and polymer formation (Trulear, 1983; Bakke et al., 1984). Trulear (1983) and Bakke et al. (1984) have used process analysis techniques in experimental biofilm reactors to quantify the fundamental rate processes within a biofilm at steady state. Their results suggest the following:

1. Ps. aeruginosa does not behave differently in biofilms than in suspension at steady state. The biofilm activity measurements in

this study were made in situ, and without significant diffusional resistance.

2. Intrinsic rate and stoichiometric coefficients derived in the chemostat can, therefore, be used to describe steady state biofilm processes.

Process analysis techniques can be useful in determining the effect of calcium on cellular reproduction and polymer formation. The effect of calcium on microbial transformation within a biofilm can also be inferred from more easily observed rate processes such as substrate consumption and oxygen consumption. However, the observed rate processes are not a sufficient criterion for comparing microbial activity under different experimental conditions because they are the net result of several fundamental processes.

Calcium and Microbial Growth

Calcium plays a vital function inside the cell. The concept of calcium as intracellular messenger and/or regulator was proposed 30-40 years ago (Campbell, 1983). For example, Hojeberg and Rydstrom (1977) suggested that calcium is a potent positive effector of nicotinamide nucleotide transhydrogenase in Ps. aeruginosa. Several extracellular degradative enzymes in both eucaryotes and procaryotes require calcium for stability and/or maximal activity (Campbell, 1983).

Calcium is required for growth and function of many bacterial species (Campbell, 1983, Weinberg, 1977). Marshall et al. (1971) reported that omission of calcium and magnesium from artificial sea water prevented growth and polymer production of Pseudomonas R3.

Shooter and Wyatt (1955) investigated the mineral requirements of Staphylococcus pyogenes and found that calcium and magnesium were needed for growth. Kenward et al. (1979) reported that inclusion of calcium and/or magnesium in the media had no effect on the exponential growth rate (0.66 h^{-1}) of Ps. aeruginosa. Calcium was shown to be necessary for the growth of marine Bdellovibrio sp. (Huang and Staff, 1973; Bell and Lantham, 1975).

Calcium appears to be exclusively extracellular and is not accumulated by a normal growing cell (Silver, 1977; Belliveau and Lanyi, 1978; Wacker and William, 1968). A special situation of calcium accumulation occurs during unusual conditions such as bacterial sporulation. Similarly, a number of different major ions (e.g., magnesium, iron, sodium, and potassium) were shown to be required for growth (Shankar and Bard, 1952; Weinberg, 1977; Shooter and Wyatt, 1955).

Very little is known regarding the effect of calcium on specific cellular growth rate and/or polymer production. A clear-cut requirement of calcium for growth in microorganisms has rarely been demonstrated (Wyatt, 1961, 1964; Wyatt et al., 1962; Hunter, 1972). This is due to the fact that it is very difficult to reduce the concentration of free calcium below 1 μM. The concentration of the free calcium can be lowered by the addition of calcium-specific chelant (EGTA). Turakhia (1984) was able to grow Ps. aeruginosa in the presence of 0.006 M EGTA (free calcium in the media was approximately 10^{-10} M). His results (Appendix G) showed that either EGTA or free calcium affected the maximum specific growth rate of Ps. aeruginosa.

Bacterial EPS

The formation of extracellular polymer has long been recognized as an important process in the metabolism of many dispersed and immobilized bacteria. Traditionally, two types of extracellular polymer have been distinguished depending on the spatial association of the polymer with the cell (Brock, 1979). Extracellular polymer which remains in a rather compact layer attached to the cell is referred to as a capsule. Conversely, extracellular polymer which does not exhibit a close association with the cell and can exist as a rather dispersed accumulation is referred to as a slime layer. The capsule-slime component of biofilms is termed extracellular polymeric substances (EPS) because little is known about its composition.

EPS Characterization. Numerous microorganisms produce exopoly-saccharides, i.e. polysaccharide found outside the cell wall, either attached to the cell in the form of capsules or secreted into the extracellular environment in the form of slime. Such polymers vary considerably in their chemical structures. There are many qualitative analyses of bacterial EPS, usually considered to be carbohydrate with acidic groups (Corpe et al., 1976; Fletcher and Floodgate, 1973), amino groups (Baier, 1975), and sometimes associated with proteins (Corpe et al., 1976). A variety of chemical structures is represented in the polysaccharides synthesized by bacteria (Sutherland, 1982). Some components such as D-glucose, D-mannose, D-galactose, and D-glucuronic acid occur very frequently; others such as L-rhamnose, L-fucose, D-mannuronic acid, and D-guluronic acid are slightly less common.

Ps. aeruginosa EPS. Pseudomonas aeruginosa was the test organism for this experimental program. The literature on the composition of slime produced by dispersed culture of Ps. aeruginosa is contradictory. Eagon (1956) reported that Ps. aeruginosa produces slime, which consists largely of mannans, but no uronic acid or amino sugars were detected. Later, Eagon (1962) showed that, in addition to mannose, which accounted for 50% of the material, the slime contained appreciable amounts of nucleic acid (mostly DNA), and small amounts of proteins. Linker and Jones (1964) showed the production, by a pathogenic Pseudomonas organism, of a polysaccharide very similar to alginic acid, a polyuronide usually obtained from sea weed. Both mannuronic and small amounts of guluronic acid appear to be present. Carlson and Mathews (1966) have reported that Ps. aeruginosa slime is a polymer composed of uronic acids. Brown et al. (1969) reported the slime produced by eight strains of Ps. aeruginosa (stationary phase) to be qualitatively the same. The slime was shown to be predominantly polysaccharide (mainly glucose with smaller amount of mannose) with some nucleic acids material and a small amount of protein.

The extracellular polymers have been shown to be involved in the selective accumulation of ions in many gram negative bacteria (Galanos et al., 1977; Leive, 1974). Buckmire (1983) observed preferential adsorption of Ca, K, P, and S (2-4 times greater than in growth medium) on individual cell and extracellular components, indicating the role of EPS (and possibly associated macromolecules) in the adsorption of ions. The lipopolysaccharide of gram-negative bacteria contains a number of potential cation-binding sites (Schindler and Osborn, 1979; Galanos et

al., 1977) having a high affinity for calcium and magnesium. Outside the cell, calcium can bind to carboxylate and sulfate groups of many polysaccharides, many of which are also linked to proteins (Levine and William, 1984).

Calcium and EPS Formation

Wilkinson and Stark (1956) observed that calcium, magnesium, and potassium stimulated polysaccharide production by Enterobacter aerogenes. Linker and Evans (1976) observed that the composition of Pseudomonas aeruginosa alginate was not influenced by different calcium levels in the medium. However, the composition of Az. vinelandii (Larsen and Haug, 1971) was affected by the calcium concentration in the medium and postulated that mannuronic acid residues were epimerised to guluronic residue by an extracellular enzyme dependent on calcium ion. Couperwhite and McCallum (1974) observed that the addition of EDTA to batch culture media affected the ratio of D-guluronic acid to D-mannuronic acid in the alginate produced by Az. vinelandii. Corpe (1964) observed increased polysaccharide production by Chromobacterium violaceum in the presence of calcium.

Calcium and Biofilms

Calcium has been implicated in direct or indirect bridging between adjacent cell surfaces. Fletcher (1980) considers that calcium may act as a cross-linking or charge screening agent for the ionic groups in the EPS. Turakhia et al. (1983) were able to detach a mixed microbial film in a turbulent flow system with EGTA (a calcium-specific chelant)

and postulated that calcium was essential to the structural integrity of the biofilm.

EDTA chelates divalent cations in the following preferential sequence:

$$Ca^{2+} > Mg^{2+} > Sr^{2+} > Ba^{2+}$$
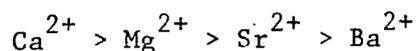
However, disaggregation in EDTA might be connected with chelation of less strongly bound cations, as well as Ca^{2+}. In recent years, however, EDTA has been superseded in many studies involving calcium (Cooksey and Cooksey, 1980; Turakhia et al., 1983) by EGTA, which can bind calcium over 10^5 times (Reed and Bygrave, 1975) more effectively than it binds magnesium.

It is likely that calcium plays a more important role in adsorption than any other cation. Calcium has a higher coordination number (7 or 8) and the coordination geometry is irregular in both bond angle and bond length. In both regards, calcium is quite different from magnesium, which maintains six coordination in a closely regular octahedron. Because magnesium requires a certain specific geometry, it is weakened in its ability to bind irregular geometries of coordination sites of biological molecules. Magnesium does not cross-link structures readily, for cross-linking usually demands a high coordination number and irregular geometries, which are characteristics of calcium.

Biofilm Detachment

At any point in the development of a biofilm under turbulent flow conditions, external portions of biofilm are sheared away into the fluid flow.

Detachment phenomenon can be arbitrarily categorized as erosion or sloughing. Erosion refers to continuous removal of small portions of biofilm, which is highly dependent on fluid dynamic conditions. Under these circumstances, rate of detachment increases with increasing biofilm thickness and fluid shear stress at the biofilm-fluid interface (Trulear and Characklis, 1982). Sloughing refers to a random, massive removal of biofilm attributed to nutrient or oxygen depletion deep within the biofilm (Howell and Atkinson, 1976). Sloughing is more frequently witnessed with thicker, less dense film which develops under low shear conditions.

Detachment can also occur for reasons other than hydrodynamic forces. Bakke (1983) has observed a massive detachment when substrate (lactate) loading to the biofilm was instantaneously doubled. Turakhia et al. (1983) and Characklis (1980) have observed increased detachment (of mixed microbial film) upon addition of chelants (EGTA and EDTA respectively) suggesting the importance of calcium to the cohesiveness of the biofilm. Many other chemicals (e.g., chlorine, bromine chloride, bromo-chloro-dimethyhydantoin, surfactants) have also been used for detachment with varying success.

Most of the detachment work with chemicals has been monitored directly by measuring the changes in frictional resistance and/or heat transfer resistance. No significant work has quantified the amount of material remaining on the surface, identified the detached material, or quantified the amount of material detached.

Detachment of biofilm is the major objective of many anti-fouling additives. Very little is known regarding the kinetics and the extent

of detachment. Such kinetic expressions would be useful for modelling purposes and as a comparative criterion for evaluating anti-fouling treatments.

Organism

Pseudomonas aeruginosa

This organism has been studied extensively both in continuous flow stirred tank reactors (CFSTR), i.e., chemostats, and in annular biofilm reactors. Information on growth rate and EPS formation rates are known under defined experimental conditions in chemostat and biofilm environments (Robinson et al., 1984; Bakke et al., 1984). Pseudomonas aeruginosa is a common waterborne polymer-forming bacteria capable of causing severe infections in a compromised host (Woods et al., 1980; Costerton, 1979). The primary mode of growth of Ps. aeruginosa in nature and disease is in polymer-enclosed microcolonies attached to a variety of surfaces. The polymer-enclosed, attached mode of growth purportedly protects Ps. aeruginosa (and other biofilm organisms) from the bactericidal activity of bacteriophages and amoebae which are numerous in natural systems and from antibiotics and host defense mechanisms in diseased systems (Costerton, 1979). Ps. aeruginosa can be considered a classic biofilm organism and for this reason is the bacterial species used in this study.

Relevant characteristics describing Ps. aeruginosa are as follows.

a) gram stain: negative (Buchanan et al., 1974)

b) morphology: rod shaped, typically 0.5 - 0.8 µm

by 1.5 - 3.0 μm (Buchanan et al., 1974)

c) metabolism: chemoorganotroph (Buchanan et al., 1974)

d) respiration: strict aerobe (Buchanan et al., 1974)

e) motility: polar monotrichous flagellation (Buchanan et al., 1974)

f) polymer composition: primarily mannuronic and glucuronic acids (Evans and Linker, 1973; Mian et al., 1978)

MATHEMATICAL DESCRIPTION OF THE SYSTEM

Cellular production and polymer formation by Ps. aeruginosa can be described mathematically using a mass balance approach. This section contains a mathematical model which describes biofilm processes, including accumulation and activity of bacteria immobilized in a biofilm and dispersed in the bulk phase. In this model the substrate carbon is partitioned into extracellular product and biomass carbon. For both of these processes, some substrate carbon is oxidized to carbon dioxide providing energy for synthesis of cells and EPS (Trulear, 1983).

The annular reactor (Figure 1) and the chemostat (Figure 2) were operated as continuous flow stirred tank reactors (CFSTR) in which bulk fluid concentration gradients do not exist. Accumulation of compounds in the bulk fluid can be described by a material balance of the general form:

$$
\begin{array}{ccccc}
\text{net} & & \text{net} & & \text{net} \hspace{2cm} (1)\\
\text{rate of} & = & \text{rate of} & + & \text{rate of}\\
\text{accumulation} & & \text{transport} & & \text{transformation}
\end{array}
$$

Accumulation of attached biofilm components can be described by a constitutive equation of the same general form as Equation 1, but with no transport term.
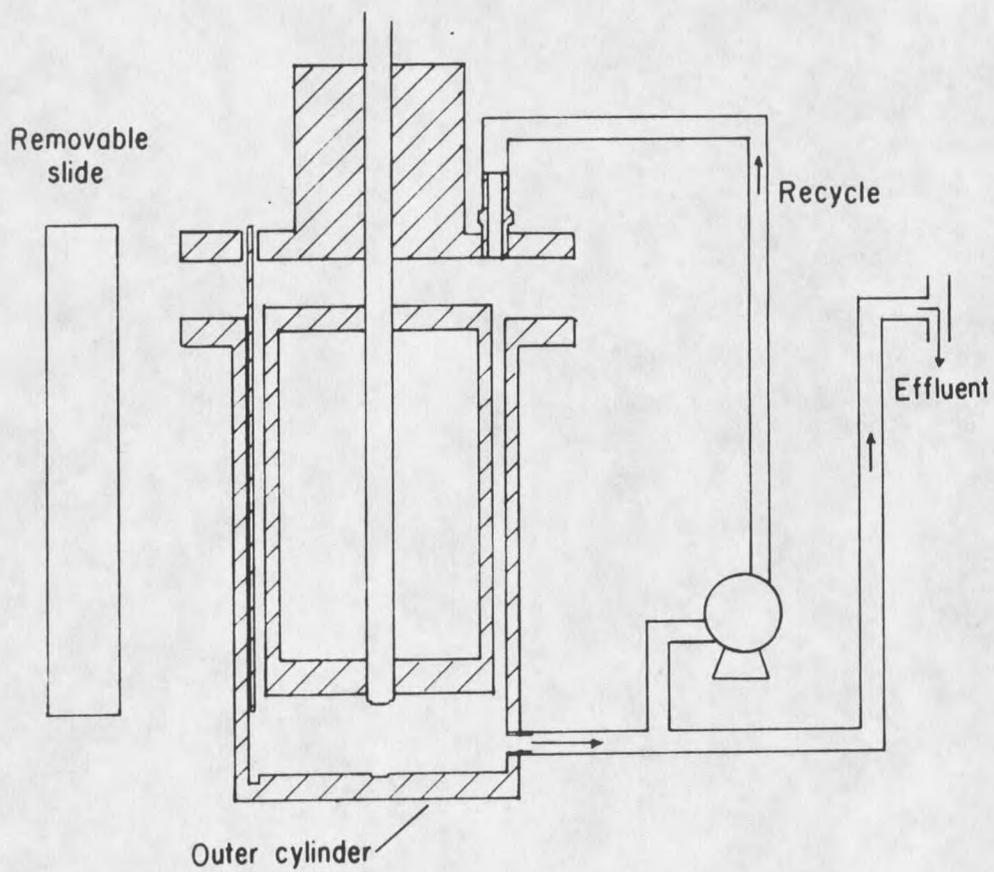
Figure 1. A simplified diagram of the annular reactor.
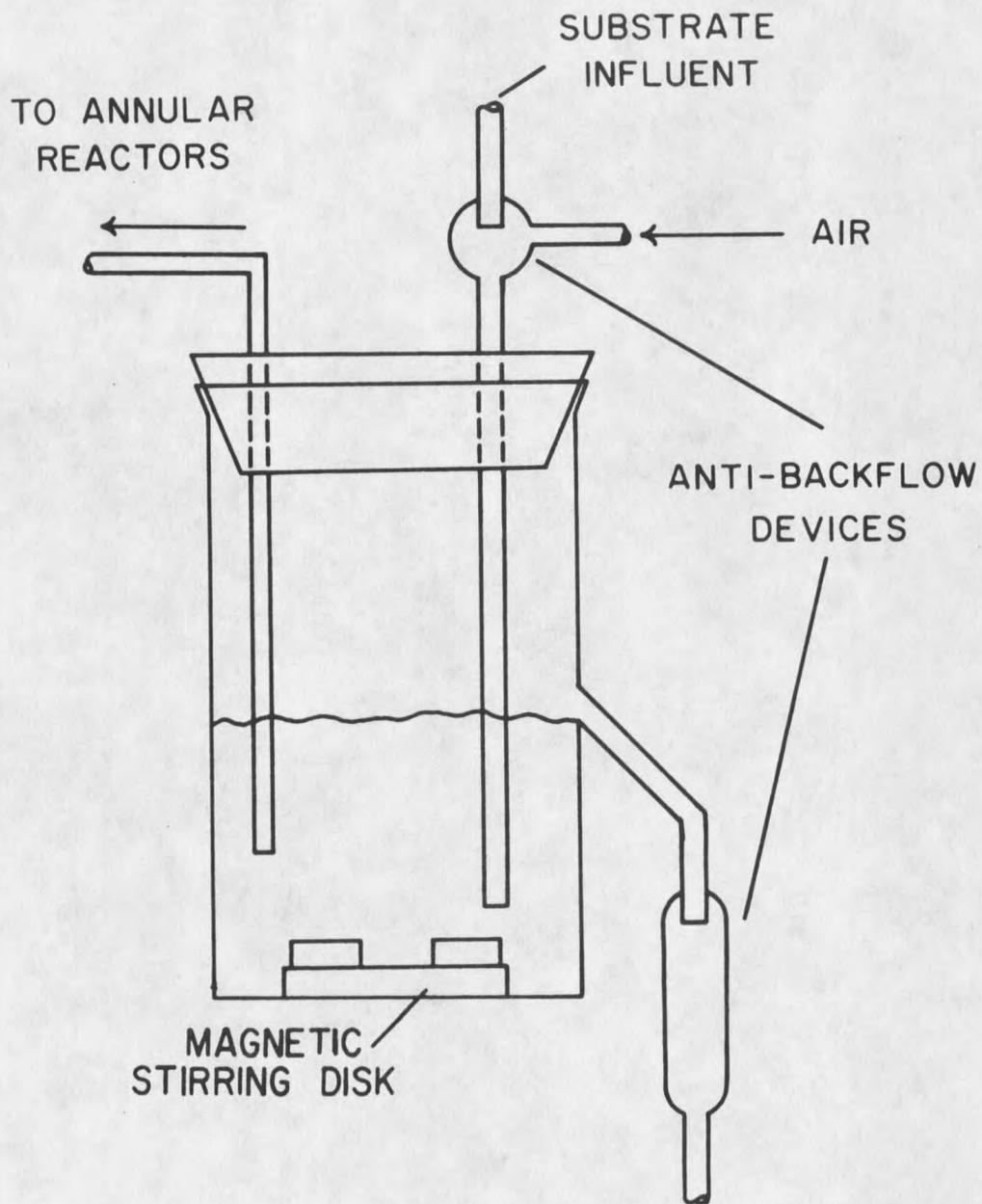
Figure 2. A simplified diagram of the chemostat.

Chemostat Equation

Cellular carbon. A mass balance across the chemostat for cellular carbon can be written as follows:

$$V \, dx/dt \quad = \quad F \, (x_i - x) \quad + \quad \mu \, x \, V \qquad (2)$$

net rate   net rate   rate of
of cellular   of cellular   cellular
accumulation   input by flow   production

where,

V = volume of the system (L^3)

x = cellular carbon concentration $(M_x L^{-3})$

t = time (t)

F = volumetric flow rate of the reactor feed $(L^3 t^{-1})$

x_i = influent cellular carbon concentration $(M_x L^{-3})$

μ = specific cellular growth rate (t^{-1})

The chemostat was operated at steady state and the influent feed was sterile ($x_i = 0$). Incorporating these conditions, Equation 2 can be simplified as follows:

$$F \, x \quad = \quad \mu \cdot x \, V \qquad (3)$$

Dividing both sides of equation by V and noting that F/V is equal to dilution rate, D, Equation 3 can be simplified to:

$$D \quad = \quad \mu \qquad (4)$$

According to Equation 4, the growth rate of the suspended cells in the chemostat can be maintained constant by controlling the dilution rate.

Annular Reactor Equations

The effect of calcium on cellular, polymer, and glucose carbon in the annular reactor (Figure 3) can be meaningfully analyzed using a mass balance approach.

Biofilm cellular carbon. A mass balance for the accumulation of cellular carbon in the biofilm can be written as follows:

$$A \, dx_b/dt \quad = \quad R_{xb} \, A \quad + \quad R_{dx} \, A \qquad (5)$$

| net rate of cellular carbon accumulation in the biofilm | rate of cellular reproduction in the biofilm | rate of cellular detachment from the biofilm |

where,

A = surface area $\qquad (L^2)$

x_b = cellular carbon areal density in the biofilm $\qquad (M_x L^{-2})$

R_{xb} = cellular carbon reproduction rate in the biofilm $\qquad (M_x L^{-2} t^{-1})$

R_{dx} = cellular carbon detachment rate from the biofilm $\qquad (M_x L^{-2} t^{-1})$

Defining biofilm specific cellular growth rate, μ_b, as:

$$R_{xb} \quad = \quad \mu_b \, x_b \qquad (6)$$

where,

μ_b = specific cellular growth rate in the biofilm $\qquad (t^{-1})$

The biofilm cellular carbon balance (Equation 5) can be written as:

$$A \, dx_b/dt \quad = \quad \mu_b \, x_b \, A \quad - \quad R_{dx} \, A \qquad (7)$$
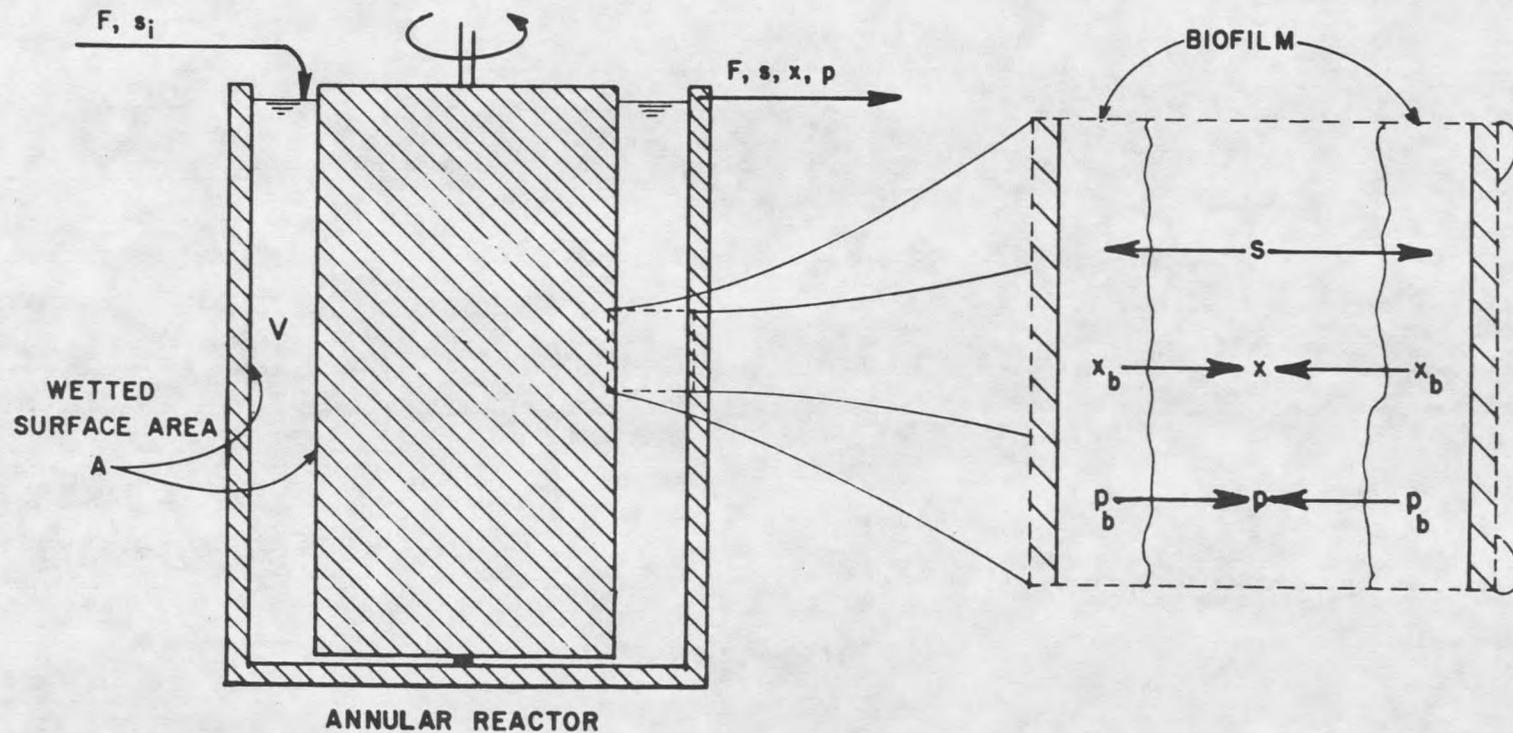
Figure 3. The annular reactor (AR) was operated as a continuous flow stirred tank reactor (CFSTR). The insert describes the local environment in the reactor. The mathematical model was based on this conceptual model.

Biofilm polymer carbon. A mass balance for the accumulation of polymer carbon can be written as follows:

$$A \, dp_b \, / \, dt \quad = \quad R_{pb} \, A \quad - \quad R_{dp} \, A \qquad (8)$$

net rate of polymer carbon accumulation in the biofilm 　　rate of polymer formation in the biofilm 　　rate of polymer detachment from the biofilm

where,

p_b = polymer carbon density in the biofilm $\quad (M_p L^{-2})$

R_{pb} = polymer carbon formation rate in the biofilm $\quad (M_p L^{-2} t^{-1})$

R_{dp} = polymer carbon detachment rate from the biofilm $\quad (M_p L^{-2} t^{-1})$

Polymer formation may be related to organism growth rate and population density according to the Luedeking and Piret equation for product formation (Luedeking and Piret, 1959; Robinson et al., 1984; Bakke et al., 1984).

$$R_{pb} \quad = \quad k_b \, \mu_b \, x_b \quad + \quad k'_b \, x_b \qquad (9)$$

where,

k_b = growth-associated polymer formation rate coefficient in the biofilm $\quad (M_p M_x^{-1})$

k'_b = nongrowth-associated polymer formation rate coefficient in the biofilm $\quad (M_p M_x^{-1} t^{-1})$

The biofilm polymer carbon balance (Equation 8) can be written:

$$A \frac{dp_b}{dt} \quad = \quad (k_b \, \mu_b \, x_b \quad + \quad k'_b \, x_b) \, A - R_{dp} \, A \qquad (10)$$

Liquid cellular carbon. A mass balance across an AR on liquid cellular carbon can be written as follows:

$$V \frac{dx}{dt} \quad = \quad F (x_i - x) \quad + \quad R_{dx} A \quad + \quad \mu x V \qquad (11)$$

net rate of cellular carbon accumulation in the liquid	net rate of cellular input by flow	rate of cellular detachment from the biofilm	rate of cellular reproduction in the liquid

Influent cellular carbon concentration in this study was equal to zero. Furthermore, the AR was operated at a high dilution rate, D = 6 h^{-1}, which is an order of magnitude greater than the maximum specific cellular growth rate. Cellular reproduction in the liquid phase is therefore negligible. Also, accumulation rate in the liquid phase is negligible (Trulear, 1983). Incorporating these conditions and dividing both sides of Equation (11) by V, the AR liquid cellular carbon balance can be written as:

$$R_{dx} \quad = \quad D x V / A \qquad (12)$$

Liquid polymer carbon. A mass balance across the AR on the liquid polymer carbon can be written as follows:

$$V \frac{dp}{dt} \quad = \quad F (p_i - p) \quad + \quad R_{dp} A \quad + \quad r_p x V \qquad (13)$$

net rate of polymer carbon accumulation in the liquid	net rate of polymer input by flow	rate of polymer detachment from the biofilm	rate of polymer formation in the liquid

Influent polymer carbon concentration in this study was equal to zero. Furthermore, due to the 10 minute hydraulic retention time the liquid phase polymer carbon formation and accumulation can be assumed to be negligible (Bakke et al., 1984). Incorporating these conditions and dividing both sides of Equation 13 by V, the AR liquid polymer carbon balance can be written as:

$$R_{dp} = D p V / A \qquad (14)$$

Glucose carbon. A mass balance across an AR on glucose carbon can be written as follows:

$$V \frac{ds}{dt} = F (s_i - s) - \frac{R_{xb} A}{Y_{xb/s}} - \frac{R_{pb} A}{Y_{pb/s}} \qquad (15)$$

| net rate of glucose carbon accumulation in the liquid | net rate of glucose input by flow | rate of glucose removal for cellular reproduction in the biofilm | rate of glucose removal for polymer formation in the biofilm |

where,

$Y_{xb/s}$ = yield coefficient of cellular carbon from substrate $\qquad (M_x M_s^{-1})$

$Y_{pb/s}$ = yield coefficient of polymer carbon from substrate $\qquad (M_p M_s^{-1})$.

Note that the glucose removal term for liquid phase reproduction and polymer formation have not been included in the substrate balance since they were assumed to be negligible in the preceding sections.