Cellular reproduction and extracellular polymer formation in the development of biofilms
by Michael Gerald Trulear

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Civil Engineering
Montana State University
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Abstract:
Bacteria exhibit a tendency for attaching to and colonizing surfaces which are submerged in aquatic environments. Attachment is mediated by extracellular polymer 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 kinetics and stoichiometry of cellular reproduction and extracellular formation in the development of biofilms.

Experiments were conducted using pure cultures of Ps. aeruginosa with glucose and inorganic nutrients providing the necessary requirements for microbial growth. Both attached growth biofilm reactors ' and dispersed growth chemostat reactors were used as experimental systems.

Rate and stoichiometric expressions which describe cellular re-' production and extracellular polymer formation in biofilms are presented. These expressions are compared with corresponding expressions describing the same processes in dispersed growth chemostat reactors.

Results indicate that the rate and extent of cellular reproduction and extracellular polymer formation depend on Ps.. aeruginosa growth rate. At low growth rate, extracellular polymer formation exceeds cellular reproduction, whereas at high growth rate, the rate and extent of cellular reproduction exceed extracellular polymer formation.

CELLULAR REPRODUCTION AND EXTRACELLULAR

POLYMER FORMATION IN THE

DEVELOPMENT OF BIOFILMS

by

Michael Gerald Trulear

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ABSTRACT

Bacteria exhibit a tendency for attaching to and colonizing surfaces which are submerged in aquatic environments. Attachment is mediated by extracellular polymer 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 kinetics and stoichiometry of cellular reproduction and extracellular formation in the development of biofilms.

Experiments were conducted using pure cultures of Ps. aeruginosa with glucose and inorganic nutrients providing the necessary requirements for microbial growth. Both attached growth biofilm reactors and dispersed growth chemostat reactors were used as experimental systems.

Rate and stoichiometric expressions which describe cellular reproduction and extracellular polymer formation in biofilms are presented. These expressions are compared with corresponding expressions describing the same processes in dispersed growth chemostat reactors.

Results indicate that the rate and extent of cellular reproduction and extracellular polymer formation depend on Ps. aeruginosa growth rate. At low growth rate, extracellular polymer formation exceeds cellular reproduction, whereas at high growth rate, the rate and extent of cellular reproduction exceed extracellular polymer formation.

INTRODUCTION

Microorganisms, primarily bacteria, exhibit a dramatic tendency for attaching to and colonizing surfaces which are submerged in aquatic environments. Attachment is mediated by means of extracellular polymer fibers, primarily polysaccharidic in composition, which are produced by the cell and extend from the cell to form a tangled matrix termed a glycocalyx (Costerton et al., 1978). The attached cells reproduce and form additional glycocalyx material. The microorganism-glycocalyx consortium is termed a biofilm.

Biofilms have been used beneficially by engineers for many years as exemplified by fixed-film wastewater treatment processes (e.g., trickling filters and rotating biological contactors; Grady, 1982). Biofilms also play a major positive role in stream purification processes. In fact, microbial activity in natural waters has been found predominantly at surfaces (Marshall, 1976; Geesey et al., 1978; Geesey, 1982). However, biofilms can be quite troublesome in many engineering systems. For example, biofilms in water distribution and heat transfer equipment can cause substantial energy losses resulting from increased fluid frictional resistance and increased heat transfer resistance (Picologlou et al., 1979; Characklis et al., 1981). Biofilms can also play a significant role in the initiation and perpetuation of conditions favorable for corrosion processes (Iverson, 1972).

In the last decade, biofilms have been recognized as major

determinants in various animal and human disease states. Examples of biofilm-associated diseases include cystic fibrosis, pneumonia, intestinal disorders, and dental caries (Costerton et al., 1981).

The Problem

The most common method of controlling biofilm development in engineering systems is through periodic chlorination. In disease states, the method of control commonly involves the application of antibiotics. In both systems, the effectiveness of the control procedures can be significantly reduced by the protective and highly adsorptive nature of the extracellular glycocalyx material (Costerton and Geesey, 1979) which in some biofilm systems can account for up to 90% of the total biofilm volume (Characklis, 1981). To further complicate control methods, the concentration at which chlorine and antibiotics can be applied are usually set at relatively low levels due to environmental and ecological considerations in the former and to human physiological considerations in the latter.

A priori, an understanding of the rate and extent of extracellular polymer formation in biofilm systems appears of fundamental importance. Such an understanding is presently lacking. The rate and extent of biofilm extracellular polymer formation have not been measured. This investigation stems from the apparent need for a fundamental understanding of the rate and stoichiometry of extracellular polymer formation in biofilms.

Research Goal

The goal of this research was to obtain a fundamental understanding of the rate and stoichiometry of polymer formation in biofilms. To accomplish this goal, the following objectives and tasks were established.

Objectives

1. Determine the kinetics and stoichiometry of cellular reproduction and extracellular polymer formation in dispersed growth chemostat reactors and in attached growth biofilm reactors.

2. Determine the applicability of kinetics and stoichiometry determined from a dispersed growth environment for an attached growth environment.

Tasks

1. Determine rate and stoichiometric expressions which describe cellular reproduction and extracellular polymer formation by _Pseudomonas aeruginosa_ in pure culture chemostat and biofilm reactors.

2. Develop and test mathematical models which describe _Pseudomonas aeruginosa_ cellular reproduction and extracellular polymer formation in pure culture chemostat and biofilm reactors.

LITERATURE REVIEW

Cellular Reproduction and Extracellular Polymer Formation

The individual processes of cellular reproduction and extracellular polymer formation have not been quantitatively studied in biofilms. Previous biofilm studies (Kornegay and Andrews, 1967; Lamotta, 1976 a; Zelver, 1979; Trulear and Characklis, 1982) have investigated "total" biofilm production, however these studies did not distinguish between the fundamental processes of reproduction and polymer formation which occur within the biofilm.

This section will discuss literature concerning cellular reproduction and extracellular polymer formation. The literature reviewed is necessarily from dispersed growth studies since results concerning biofilm cellular reproduction and extracellular polymer formation are not available.

Cellular Reproduction

Bacterial cells reproduce by binary fission. For a given set of environmental conditions (e.g., temperature and pH) the rate of reproduction due to binary fission depends on the concentration of nutrients which are available for growth. If all required nutrients are supplied in excess except one, the growth limiting nutrient, the rate of cellular reproduction can be empirically related to the concentration of the limiting nutrient. The equation most widely used to describe this

relation was originally proposed by Monod (1949) to describe the growth

of _Escherichia_ _coli_ on glucose. The Monod equation is written as

follows:

$$\mu = \frac{\mu_{max}\ s}{k_s + s} \tag{1}$$

where

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

μ_{max} = maximum cellular specific growth rate $\qquad$ (t^{-1})

s = concentration of the limiting $\qquad$ $(M_s L^{-3})$
nutrient, commonly referred to as
the substrate

k_s = saturation coefficient, numerically $\qquad$ $(M_s L^{-3})$
equal to the substrate concentration
at $\mu = 1/2\ \mu_{max}$

The maximum specific growth rate, μ_{max}, is a measure of the maximum

rate at which an organism can reproduce under saturating conditions of

the substrate (Monod, 1949; Stanier et al., 1976). The saturation

coefficient, k_s, is a measure of the affinity which an organism's

enzymes exhibit for a particular substrate (Monod, 1949; Shehata and

Marr, 1971). k_s values can also be indicative of extracellular dif-

fusional resistances (Characklis, 1978; Harremoës, 1978). Typical

values of μ_{max} and k_s are given in Table 1.

The hyperbolic form of the Monod equation (Figure 13) is identical

to the Michaelis-Menten equation (1913) for enzyme-catalyzed reactions

and to the Langmuir adsorption isotherm (1918) describing adsorption

kinetics.

TABLE 1

Typical Values for the Maximum Specific Growth Rate, μ_{max}, and
the Saturation Constant, k_s.

Bacterial Species	Substrate	μ_{max} (h^{-1})	k_s (mg/l)	Temperature (°C)	Reference
Escherichia coli	glucose	1.35	4.0	37°C	Monod (1949)
Escherichia coli	glucose	0.78	0.1	30°C	Shehata & Marr (1971)
Escherichia coli	glucose	0.53	–	37°C	Clarke et al. (1968)
Pseudomonas aeruginosa	glucose	0.37	2.8	25°C	Dharmarajan (1981)
Pseudomonas sp.	glucose	0.38	2.0	20°C	Jenkins (1980)
Pseudomonas sp.	lactate	0.55	–	28°C	Matin et al. (1976)

Extracellular Polymer Formation

The formation of extracellular polymer has long been recognized as an important process in the normal metabolism of many bacteria. Traditionally, two types of extracellular polymer have been distinguished depending on the spatial association of the polymer with the cell (Brock, 1978). 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. In recent years with the emergence of an increased level of interest in the development of attached microbial growths (biofilms), the capsule-slime layer component of these deposits has been termed the glycocalyx (Costerton, 1978).

Bacterial extracellular polymers are almost universally composed of polysaccharide subunits, primarily mannans, glucans, and uronic acid (Stainier et al., 1976; Costerton, 1979). The ratio of these subunits and the extent of group substitutions within the subunits can vary widely depending on the type(s) of bacteria present (Geesey, 1981).

Most bacterial extracellular polymers are synthesized at the level of the cell membrane and involve successive transfers of nucleotide-sugar precursors (e.g., UDP-glucose and UDP-galactose) from within the cell via membrane-bound lipid carriers (Stanier et al., 1976; Costerton, 1981). The polysaccharide chain is then assembled by polymerases which are attached to outer portions of the cell membrane.

Extracellular polymer formation is greatest if excess carbon is supplied to cultures which are either nitrogen- or phosphorous-limited.

Several investigators (Tam and Finn, 1977; Williams and Wimpenny, 1977; Mian et al., 1978; Williams, 1978) have used nitrogen limitation to enhance polymer formation in dispersed growth cultures. Characklis and Dydek (1976) found that the amount of biofilm increased with increasing carbon to nitrogen ratio, indicating the importance of extracellular polymer in the overall composition of biofilms.

Numerous equations have been proposed to describe the kinetics of extracellular polymer formation. The majority of these equations are empirical and were originally developed in conjunction with the fermentation industry for the description of various microbial fermentation products. The empirical relation used most widely to describe microbial polymer formation is the equation originally developed by Ludedeking and Piret (1959) to describe the formation of lactic acid by Lactobacillus delbrueckii:

$$R_p = k \mu x + k'x \qquad (2)$$

| rate of polymer formation | rate of growth-associated polymer formation | rate of nongrowth-associated polymer formation |

where

R_p = rate of extracellular polymer formation $(M_p L^{-3} t^{-1})$

k = growth-associated polymer formation rate coefficient $(M_p M_x^{-1})$

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

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

k' = nongrowth-associated polymer formation rate coefficient $(M_p M_x^{-1} t^{-1})$

A review of literature concerning extracellular polymer formation by various bacterial species reveals that, depending on the particular organism, only one of three different forms of the Luedeking-Piret equation are applicable:

case (1) : polymer formation is growth- and nongrowth-associated, i.e., $k \neq 0$, $k' \neq 0$

case (2) : polymer formation is growth-associated, i.e., $k \neq 0$, $k' = 0$

case (3) : polymer formation is nongrowth-associated, i.e., $k = 0$, $k' \neq 0$

Table 2 presents a summary of the above cases for different bacterial species.

Few investigators have presented interpretations of the Luedeking-Piret growth- and nongrowth-associated coefficients and, due to the empirical nature of the Luedeking-Piret equation, it is not clear whether interpretations are warranted. Luedeking and Piret (1959) state that for the case of growth- and nongrowth-association observed in their study (the formation of lactic acid by Lactobacillus delbrueckii), "one can speculate that the cell dissimilates glucose to lactic acid in order to obtain energy required to form new bacterial protoplasm, and at the same time does it as a normal metabolic activity irrespective of growth." Roels and Kossen (1978) have generalized the original interpretation by Luedeking and Piret and conclude that the growth and nongrowth coefficients are indicative of "energy-pathway" associated product formation. The growth-associated coefficient, k, is related to biosynthetic energy requirements, the nongrowth-associated coefficient,

TABLE 2

Growth Association of Luedeking Piret Polymer Formation
Rate Coefficients

Bacterial Species	Polysaccharide Polymer	Growth Association	Reference
Pseudomonas aeruginosa	–	growth and nongrowth	Mian et al. (1978)
Xanthamonas campestris	xanthan gum	growth and nongrowth	Moraine and Rogovin (1971), results reprinted in Weiss and Ollis (1980)
Azotobacter vinelandii	alginic acid	growth	Deavin et al. (1977), results reprinted in Klimek and Ollis (1980)
Pseudomonas sp.	–	nongrowth	Williams (1974), results reprinted in Klimek and Ollis (1980)
Methylomonas mucosa	–	nongrowth	Tam and Finn (1977)

k', is related to maintenance energy requirements.

Biofilm Accumulation

The accumulation of biofilm on a surface exposed to a fluid flow is the net result of several processes including the following:

1. Adsorption of organic molecules to the surface

2. Transport of microbial cells to the surface

3. Microorganism attachment to the surface

4. Microbial metabolism (cellular reproduction and extracellular polymer formation) at the surface resulting in the production of biofilm

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

Biofilm accumulation 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 others.

Adsorption of Organic Molecules to the Surface

Adsorption of an organic monolayer occurs within minutes after exposure of an initially "clean" surface to an aqueous environment containing dissolved organics. 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). The organic molecules involved in this initial adsorption are usually negatively charged (polyanionic) polysaccharides or glyco-proteins (Baier, 1975).

Transport of Microbial Cells to the Surface

The transport of microbial cells from the bulk to the surface

depends on the fluid flow regime (Characklis, 1981). In turbulent flow,

turbulent downsweeps are suspected to be the primary transport mecha-

nism. In laminar flow, chemotaxis and sedimentation may be important.

Microorganism Attachment to the Surface

Observations by Zobell (1943), and later by Marshall et al.

(1971), on the adhesion of bacterial cells to surfaces suggests the

existence of a two-stage adhesion process: 1) reversible adhesion

followed by 2) an irreversible adhesion. Reversible adhesion is charac-

terized by an initially weak adhesion of a cell which can still exhibit

Brownian motion but is readily removed by mild rinsing. The adhesive

forces which hold the cell at the wall during reversible adhesion proba-

bly include the following: a) electrostatic, b) London-van der waals,

c) interfacial tension, and d) covalent bonding (Characklis, 1981).

Conversely, irreversible adhesion is a more permanent bonding to the

surface mediated by the production of extracellular polymers, i.e.,

glycocalyx material (Corpe, 1970; Fletcher and Floodgate, 1973;

Marshall, 1976).

Biofilm Production Due to Microbial Metabolism

The attached microbial cells assimilate nutrients from the bulk

fluid and through processes of microbial metabolism, reproduce and form

additional extracellular polymers. The combined result of these pro-

cesses is the production of biofilm. The rate and extent of biofilm

production depends on the concentration of nutrients in the bulk fluid

and on their subsequent diffusion into the bioflm (Trulear and

Characklis, 1982).

Literature concerning biofilm production (Kornegay and Andrews,

1967; Lamotta, 1976a; Zelver, 1979; Trulear and Characklis, 1982) has

not distinguished between reproduction and polymer formation processes.

Three of these studies (Kornegay and Andrews, 1967; Lamotta, 1976a;

Trulear and Characklis, 1982) have developed models to describe biofilm

production. However, all three models are based on Monod-type satura-

tion kinetics which treat the biofilm as if it were composed entirely

of microorganisms. Kinetic coefficients obtained from these studies are

shown in Table 3. Figure 1 depicts the typical experimental progression

reported.

The references cited above are only a small fraction of the litera-

ture concerning biofilm kinetics. However these references are the only

studies which have explicitly studied biofilm production. Other litera-

ture on biofilm kinetics (Sanders, 1966; Maier, 1966; Tomlinson and

Snaddon, 1966; Sanders et al., 1970; Atkinson and Daoud, 1970; Atkinson

and Davies, 1974; Williamson and McCarty, 1976; Lamotta 1976a and 1976b;

Harris and Hansford, 1976; Harremoës, 1978; Rittmann and McCarty, 1978;

Rittmann and McCarty, 1981) have been primarily concerned with modeling

substrate removal. The majority of these studies have also largely

ignored the internal structure of the biofilm. To this author's know-

ledge, only two groups of investigators (Atkinson and co-workers, and

Rittmann and McCarty, 1981) have included internal structure in their

modeling and only to the extent of recognizing that a certain fraction

TABLE 3

Typical Coefficients from Biofilm Production Models
Utilizing Saturation Kinetics

Influent Glucose Concentration, s_i (mg/1)	Glucose Loading Rate, R_L (mg/m^2 min)	Maximum Specific Production Rate, $r_{p_{max}}$ (t^{-1})	Saturation Constant, k_p (mg/1)	Temperature (°C)	Reference
5.8–130.4	1.7–37.2	0.10–0.52	0.5–69.8	30	Trulear and Characklis (1982)
59.2–577.0	37.6–376.0	0.28	121.0	25	Kornegay and Andrews (1967)
2.2–40.5	1.5–27.0	0.19[a]	7.8[a]	22	Lamotta (1976a)

[a] values based on influent glucose concentration

Induction   Accumulation   Plateau

Glucose Concentration   (mg l⁻¹)

Suspended Biomass Concentration   (mg l⁻¹)

● Glucose Concentration   (mg l⁻¹)

▲ Suspended Biomass Concentration   (mg l⁻¹)

○ Biofilm Thickness   (μm)

Time   (hrs)

32   24   16   8   0

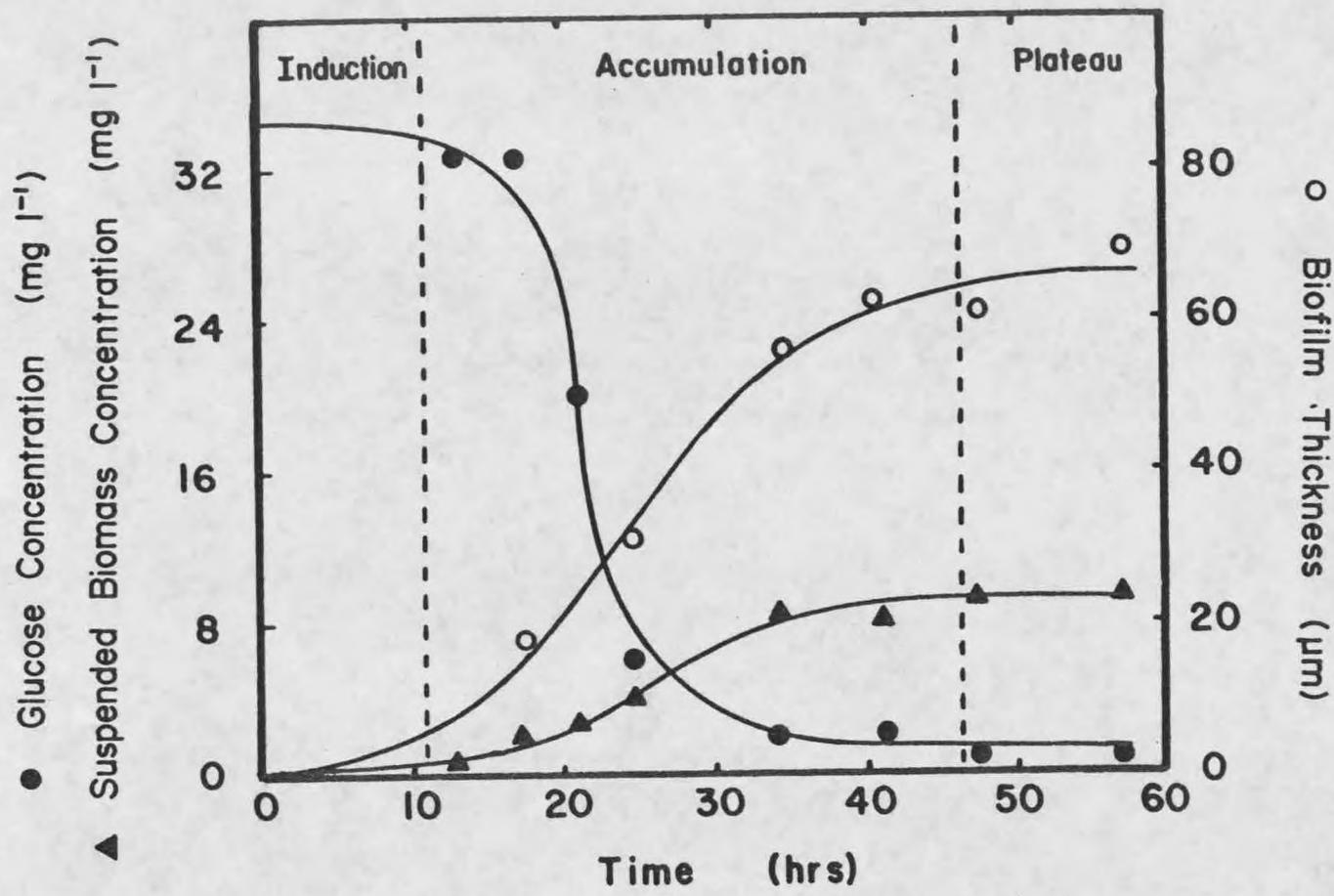0   10   20   30   40   50   60

80   60   40   20   0

Figure 1.   Typical Experimental Progression
(From Trulear and Characklis, 1982).

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of the biofilm, the cellular component, is actually responsible for utilizing substrate.

Biofilm Detachment

At any point in the development of a biofilm, external portions of biofilm are sheared away into the fluid flow. Detachment due to fluid shear is a continuous removal process and is highly dependent on hydrodynamic conditions. Trulear and Characklis (1982) have investigated the rate of biofilm detachment due to fluid shear and report that detachment rate increases with increasing biofilm mass and with increasing fluid velocity.

In addition to shearing, sloughing can also significantly contribute to biofilm detachment. Sloughing refers to a random, massive removal of biofilm attributed to nutrient/oxygen depletion deep within biofilms (Howell and Atkinson, 1976). Sloughing is more frequently witnessed with thicker, less dense biofilms which develop under low fluid shear conditions.

Biofilm Properties

Physical properties of biofilms which have been measured include biofilm thickness and biofilm mass density. In turbulent flow systems, wet thickness (Th) seldom exceeds 1000 μm and is usually considerably less (Zelver, 1979; Trulear and Characklis, 1982). Biofilm mass density (ρ) reflects dry mass per unit wet biofilm volume and measured values in turbulent flow systems range from 10 to 65 mg/cm^3 (Trulear and Characklis, 1982).

Chemical properties of biofilms which have been measured include inorganic composition (reviewed by Characklis, 1981), protein to polysaccharide ratio (Bryers, 1980), and carbon to nitrogen and carbon to phosphorous ratios (reviewed by Characklis, 1981).

Biological properties of biofilms which have been measured indicate that a wide variety of microorganisms, primarily different species of bacteria and algae, are found in naturally occurring biofilms (Haack and McFeters, 1982). There is evidence which suggests organism succession occurs so that at different stages of biofilm development, different groups of microorganisms may predominate (Marshall, 1976; Corpe, 1978).

The available information on biofilm structure suggest that biofilms may be primarily composed of extracellular polymer material (glycocalyx) and that only a small fraction of the biofilm volume is composed of cellular material (Characklis 1981). Unfortunately, the majority of the evidence supporting this assertion is based on electron microscope photomicrographs (Fletcher and Floodgate, 1973; Costerton, 1981). Due to the extremely high degree of biofilm hydration (85-96% water according to Characklis, 1981) interpretations from observations using electron microscopy must be viewed with caution (Geesey, 1982).

Pseudomonas aeruginosa

Ps. aeruginosa is a polymer-forming bacterium which is ubiquitous in nature and is the cause of many infections and disease states (e.g., cystic fibrosis and various bladder infections) in humans (Costerton, 1979). The primary mode of growth of Ps. aeruginosa in nature and

disease is in polymer-enclosed microcolonies attached to a wide variety

of surfaces. The polymer-enclosed, attached mode of growth purportedly

protects Ps. aeruginosa (and other biofilm organisms) from the bac-

tericidal activity of bacteriophages and amoebae which are so 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)

EXPERIMENTAL METHODS

Experimental Systems

Chemostat and annular reactor (AR) systems were used for this research. Components of the chemostat system include four chemostat reactors, sterile substrate feed apparatus, temperature control, and air supply. Components of the annular reactor system include two AR's, sterile dilution water and substrate feed apparatus, temperature control, and air supply. Figures 2 and 3 are schematic diagrams of the chemostat and AR systems.

Chemostat System

Chemostats. The chemostats were 1000 cm^3 Berzelius Pyrex beakers equipped with side arms and rubber stopppers. Figure 4 illustrates details of the chemostats. Stainless steel baffles and magnetic stirring disks provided complete mixing of the liquid solution. A polypropylene scraping disk in each chemostat provided a method of removing attached microorganisms from the inner surfaces of the glass to prevent wall growth. Anti-backflow cylinders on the influent and effluent lines were used to prevent contamination of the substrate feed solution and chemostat due to the backflow of microorganisms. Table 4 presents relevant characteristics and dimensions of the chemostats.
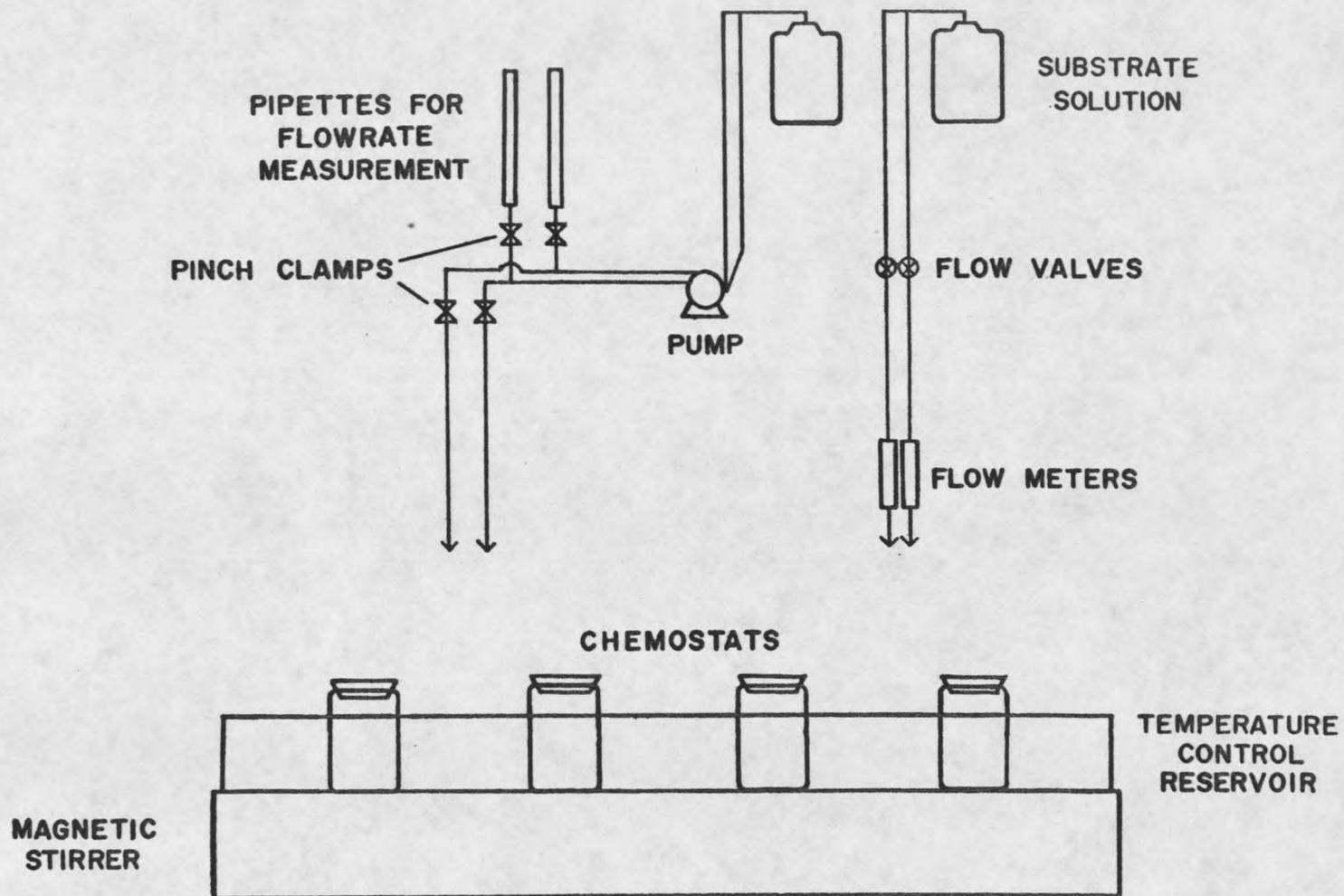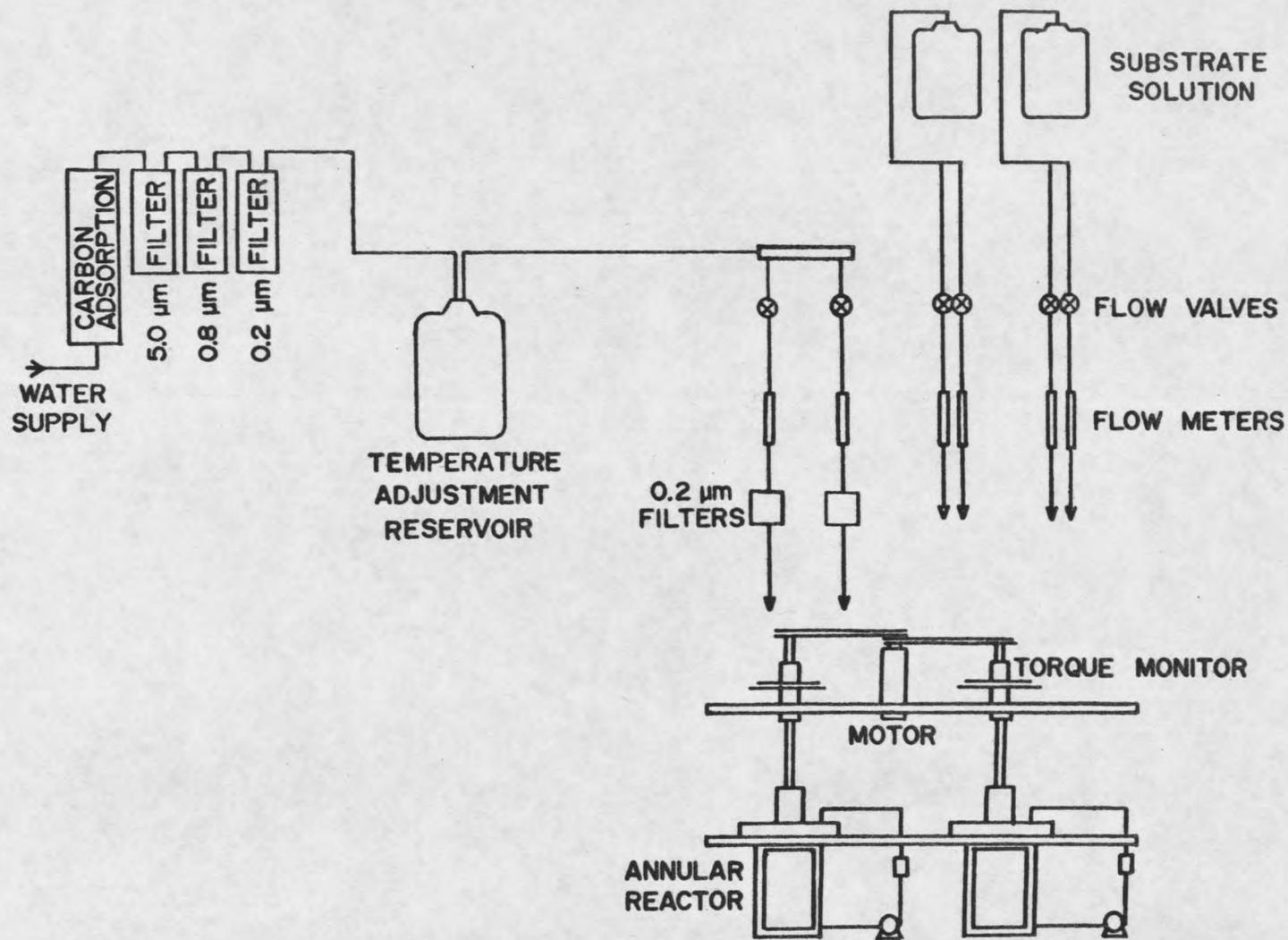
Figure 2.   Schematic Diagram of Chemostat System.

Figure 3.   Schematic Diagram of Annular Reactor System.