Binary population biofilms
by Maarten Alexander Siebel

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Civil Engineering
Montana State University

Abstract:
Biofilm research has been restricted to studies of undefined mixed microbial populations and to investigations of (defined) mono microbial populations. In the first case, the organisms are considered as a homogeneous mass, biomass, ignoring the properties of individual species, the sum of which determines the observed phenomena. The second case concentrates on the properties and processes of one microbial species ignoring the influence of the broader environment, eg. other microbial species, on this species.

Goal of this research was to study a defined mixed microbial biofilm and to determine possible interactive effects between the species comprising the biofilm. Biofilm experiments were conducted with mono populations of Klebsiella pneumoniae and Pseudomonas aeruginosa and with binary populations of K. pneumoniae and P aeruginosa . Process rates and stoichiometric coefficients, determined for the mono population biofilms were compared with those found in the binary population biofilm.

Results indicate that the specific cellular product formation rate of K. pneumoniae or P. aeruginosa in the binary biofilm is not affected by the presence of the other species. Similarly, the glucose-oxygen stoichiometric ratio of K. pneumoniae or P. aeruginosa in the binary biofilm is not affected by the presence of the other species. Many processes at the cellular level are performed faster by K. pneumoniae than by P. aeruginosa: eg. biofilm specific product formation rate and the maximum specific growth rate of K. pneumoniae are 5 times that rate of P. aeruginosa. Nevertheless, K. pneumoniae cell mass does not dominate the biofilm, possibly because of its non-motility and its product formation properties.

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Sept 18, 1987 — Date

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ABSTRACT

Biofilm research has been restricted to studies of undefined mixed microbial populations and to investigations of (defined) mono microbial populations. In the first case, the organisms are considered as a homogeneous mass, biomass, ignoring the properties of individual species, the sum of which determines the observed phenomena. The second case concentrates on the properties and processes of one microbial species ignoring the influence of the broader environment, eg. other microbial species, on this species.

Goal of this research was to study a defined mixed microbial biofilm and to determine possible interactive effects between the species comprising the biofilm. Biofilm experiments were conducted with mono populations of Klebsiella pneumoniae and Pseudomonas aeruginosa and with binary populations of K. pneumoniae and P. aeruginosa. Process rates and stoichiometric coefficients, determined for the mono population biofilms were compared with those found in the binary population biofilm.

Results indicate that the specific cellular product formation rate of K. pneumoniae or P. aeruginosa in the binary biofilm is not affected by the presence of the other species. Similarly, the glucose-oxygen stoichiometric ratio of K. pneumoniae or P. aeruginosa in the binary biofilm is not affected by the presence of the other species. Many processes at the cellular level are performed faster by K. pneumoniae than by P. aeruginosa: eg. biofilm specific product formation rate and the maximum specific growth rate of K. pneumoniae are 5 times that rate of P. aeruginosa. Nevertheless, K. pneumoniae cell mass does not dominate the biofilm, possibly because of its non-motility and its product formation properties.

INTRODUCTION

A biofilm is a layer of microbial cells and inorganic debris held together in a polymeric matrix and firmly attached to a substratum. Accumulation of biofilm is encountered in many natural environments. Natural purification of surface waters depends to a large extent on the activity of adsorbed microorganisms to remove pollutants from the bulk liquid. In certain engineered environments, adsorption of organisms is fundamental to the processes anticipated eg., fixed-film biological wastewater treatment. In other environments, adsorption of microorganisms is considered a nuisance resulting in energy losses, sudden deterioration of water quality and possible destruction of long-term exposed surfaces.

A major dilemma in the study of biofilm processes is the extreme complexity of natural biofilms which makes distinguishing individual processes and obtaining process oriented information virtually impossible. This is in contrast with study of biofilms of one single microbial species, mono population biofilms. As far as we know, however, these films do not exist in natural systems and information exposed serves mainly to improve theoretical understanding.

The focus of this research is accumulation of binary population biofilms, biofilms resulting from accumulation of two identified microbial species. The behaviors of different microbial species respiring in close proximity does not necessarily equal the sum of the behavior of the individual microbial species. Different interactions between organisms have been distinguished. However, most studies in this area have been conducted in suspended growth systems and results may not be directly applicable to systems with attached microbial growth. The scope of

this study was to elucidate processes describing accumulation of a binary population biofilm by comparison with processes describing accumulation of mono population biofilms.

Goal and Objective

The goal of this research was to determine the effect of a mixed microbial population on biofilm accumulation processes and biofilm properties.

The objective was to describe biofilm accumulation of a binary population of Klebsiella pneumoniae and Pseudomonas aeruginosa in terms of the accumulation of the mono population biofilms of K. pneumoniae and P. aeruginosa. Process variables were monitored to determine the process kinetics and stoichiometry for mono population biofilms of each species and for the two species combined.

LITERATURE REVIEW

Biofilm Processes

Biofilm accumulation is the result of microbial, chemical and physical processes occurring in the liquid phase, both within the biofilm and at the substratum:

Transport and adsorption of organic macromolecules and nutrients from the liquid phase to the substratum: Research indicates that adsorbed macromolecules may both enhance and inhibit microbial adsorption. Characklis and Cooksey (1983) conclude that the role of conditioning film in microbial adsorption to surfaces is not yet clear.

Transport and adsorption of microorganisms from the liquid phase to the substratum: Microbial cells (0.5-10 μm effective diameter) can be transported from the bulk fluid to the wetted substratum by (Brownian) diffusion, gravity, thermophoresis, taxis and fluid dynamic forces like inertia, lift, drag, drainage and downsweep. The relative contribution of each of those forces depends on the size of the organism, its density, etc. Motility can contribute significantly to the rate of transport of a microbial cell to the substratum. Adsorption of microorganisms may be reversible or irreversible, closely related to the bonding energy between the substratum and the macromolecular conditioning film (Mitchell and Kirchman, 1981). Irreversible adsorption occurs following the production of extracellular fibers that allow the bacteria to overcome repulsive forces (Marshall et al. 1971).

Reactions within the biofilm: Once organisms are adsorbed energy is expended for growth or replication, for the formation of extracellular products (eg. polysaccharides, proteins, small molecules, peptides,

antimicrobial agents), for cell maintenance, and for death or lysis of the cell.

Detachment of biofilm and associated products: Parts of the biofilm may separate from the biofilm and become reentrained in the bulk liquid. Erosion occurs when detachment concerns individual cells. Detachment generally refers to separation of pieces of biofilm (cells and products). Desorption refers to the reentrainment of cells from the substratum.

Reactions between the biofilm and the substratum: Reactions may occur between microbial products and the substratum and reactions may occur between locations of the substratum, covered with biofilm and locations not covered. The adsorbed cells grow and reproduce forming colonies which constitute physical anomalies on a surface. Nonuniform or "patchy" colonization by microorganisms results in the formation of differential aeration cells where areas under respiring colonies are depleted of oxygen relative to surrounding, non-colonized areas, forming differential surface chemistries.

Biofilm Properties

Biofilm properties can be distinguished in physical, chemical and biological properties. Relevant thermodynamic properties are volume (thickness seldom over 1000 μm) and mass (dry mass density varies from 10 - 50 $kg.m^{-3}$ in fluid flow systems) (Characklis and Cooksey, 1983). Both values are highly dependent on hydraulic conditions and on the chemical regime to which the film is exposed. Transport properties of biofilms determine mass, heat and momentum transfer. Diffusion coefficients in biofilms are most probably related to biofilm density (Characklis and Cooksey, 1983); thermal conductivity is not significantly

different from that of water (Characklis et al., 1981).

Chemical properties of the biofilm vary with the chemical composition of the bulk liquid and probably affect the physical and biological structure of the film, eg., interspecies bonding strength is probably affected by calcium (Turakhia, 1986). In addition, inert suspended solids and corrosion products, when substratum is ferro-metallic, may accumulate in the biofilm matrix.

Organic composition of biofilms is closely related to the energy, carbon and nutrient sources available for metabolism. For example, nitrogen limitation can result in a relatively large amount of carbon being devoted to production of extracellular microbial polysaccharide (EPS). In terms of macromolecular composition, Bryers (1979) has measured protein-to-polysaccharide mass ratios from 0 - 10 (the former in terms of casein equivalents, the latter in terms of glucose equivalents). In addition, the physiological state of organisms is of importance in determining the composition of a biofilm: stationary phase cells are generally EPS producers rather than log phase cells (Characklis and Cooksey, 1983).

Biological properties of a biofilm strongly depend on the species colonizing the substratum in addition to the physical and chemical properties of the environment in which the biofilm accumulates. Initial microbial activity on the substratum results in small colonies of cells distributed randomly. In time, colonies may grow together forming a relatively smooth biofilm or grow as isolated colonies with bare substratum in between, forming a patchy biofilm.

Viable cell numbers are relatively low in relation to the biofilm volume ($10^{10} - 10^{14}.m^{-3}$), occupying only from 1 to 10% of the biofilm volume in dilute nutrient solutions (Characklis, 1980; Trulear, 1983). Cell densities are in

the order of 10^{10}-10^{13} cells.m^{-2}.

Microbial Species

Klebsiella pneumoniae

K. pneumoniae, also described as Klebsiella type I (Erbing et al., 1976), is an organism in the coliform group. It is commonly found in soil and water and is present in 30 - 40 % of all warm-blooded animals, including humans. Individual densities range up to 10^8.(gram of feces)$^{-1}$. K. pneumoniae is frequently implicated in hospital infections. Approximately 60 - 85% of all Klebsiella from feces and clinical specimens are K. pneumoniae. Strains that are positive by the fecal coliform test (ferment lactose with gas production at 44.5°C) are considered K. pneumoniae (Geldreich and Rice, 1987). This organism is the rare cause of pneumonia in humans (Brock, 1979). Several investigations (Knittel, 1975; Brown and Seidler, 1973) suggest that the origin of K. pneumoniae in surface waters could be human, animal or mixed sources (Campbell et al., 1976). When growing anaerobically, Klebsiella strains fix N_2, a property not found among other enteric bacteria (Brock, 1979). K. pneumoniae is generally a non-motile, gram negative, rod shaped bacterium. Relevant characteristics of K. pneumoniae are summarized in Table 1.

NB. K. pneumoniae appears to be referred to by a variety of names including, Aerobacter aerogenes, Klebsiella aerogenes, K. eduardii, and K. oxytocum (Brown and Seidler, 1973).

Pseudomonas aeruginosa

P. aeruginosa is a polymer-forming bacterium, ubiquitous in nature and the cause of many infections and disease. The primary mode of growth is in polymer-enclosed

microcolonies (Buchanan and Gibbons, 1977), attached to a wide variety of substrata. The polymer capsule is assumed to act as a protective layer. This polymer layer is relatively diffuse, and is easily dispersed in the liquid phase.

P. aeruginosa has been studied extensively in both suspension and in biofilms (Trulear, 1983; Mian et al, 1978; Robinson et al., 1984; Bakke et al., 1984; Turakhia, 1986). Kinetic and stoichiometric coefficients for this organisms have been determined. Relevant characteristics of P. aeruginosa are summarized in Table 1.

Table 1. Relevant characteristics of K. pneumoniae and P. aeruginosa.

	K. pneumoniae	P. aeruginosa
shape	rod shaped [1]	rod shaped [1]
breadth (μm)	0.3 - 1.5 [1]	0.5 - 0.8 [1]
length (μm)	0.6 - 6.0 [1]	1.5 - 4.0 [1]
EPS formation	+	+
EPS composition	glucose, fucose, glucuronic acid, and pyruvic acid [4]	primarily mannuronic and guluronic acid [5]
motility	non motile	polar flagella [1]
respiration	facultative anaerobic [3]	obligate aerobe except when nitrate present [3]
denitrification	+ [3]	
metabolism	chemoorganotroph [2]	chemoorganotroph
gram stain	negative [1]	negative [1]
optimal temperature	35-37°C [1]	35-37°C [3]
optimal pH	7.2 [1]	6.8 [3]

[1] (Holt, 1977)
[2] (Sutherland, 1977)
[3] (Buchanan and Gibbons, 1974)
[4] (Erbing et al., 1976)
[5] (Mian et al., 1978)

Extracellular Products

Extracellular Polymeric Substances (EPS) are by-products of microbial metabolism produced mostly in the cytoplasm and transported through the cell wall. These, generally large chain macromolecules may either take the form of a discrete capsule located around the cell perimeter or may be present as extracellular slime apparently unattached to the bacterial surface, depending on the species (Sutherland, 1977). Capsules are generally quite stable but stability depends on cell age, chemical composition of surrounding liquid, etc. Consequently, capsule layer thickness will vary from hardly discernible to extending 0.1 to 10 μm beyond the exterior of the cell wall (Sutherland, 1977). It should be noted that observations of capsular material very much depend on the sample preparation procedure. Several methods and resulting observations on K. pneumoniae, discussed by Roth (1977), show that capsule appearance can range from a clearly visible, layered structure with a well-defined edge to not visible.

Cell growth rate and the nature of the compound limiting cell growth rate, eg. nitrogen or carbon, strongly influence the rate of product formation and the product yield. Under nitrogen limitation in chemostat growth, specific polysaccharide synthesis by P. aeruginosa increased from 0.27 g.(g of cell.h)$^{-1}$ at a dilution rate of 0.05.h^{-1} to 0.44 g.(g of cell.h)$^{-1}$ at a dilution rate of 0.1.h^{-1}. Polysaccharide yields, based on glucose used, ranged from 56 to 64% (Mian, 1978).

Polysaccharides were also produced under carbon limitation at a rate of 0.19 g.(g of cell.h)$^{-1}$ at D=0.05.h^{-1} at a yield of 19%. (Mian et al., 1978). Similar results were obtained with Klebsiella aerogenes. Product formation

increased with nitrogen relative to carbon as limiting nutrient (Sutherland, 1977). It was even observed that Klebsiella continued to produce considerable amounts of polysaccharide after cessation of cell growth despite the presence of residual carbohydrate in the growth medium (Dudman, 1960). Neijssel and Tempest (1975), however, report that K. aerogenes, growing at very high substrate concentrations going into the chemostat (10 g $C.l^{-1}$ when carbon limited, 30 g $C.l^{-1}$ when nitrogen, phosphate or sulphate limited) and at low dilution rate, 0.17.h^{-1}, did not produce any polysaccharide or other metabolic products when growing under carbon limitations. In that case, cell yields were reportedly 0.45 (g cell-C.(g glucose-C)$^{-1}$) for growth on glucose carbon; glucose-oxygen stoichiometric ratio was 2.83 (g O_2.(g glucose-C)$^{-1}$).

Oxygen has also been implicated in production of polymeric material. Dudman (1960) found that increased aeration led to decreased product yields in a variety of microorganisms but resulted in an increased polysaccharide production in E. coli and Klebsiella species. It seems possible that in bacteria, which are facultative anaerobes utilizing the Embden-Meyerhof pathway, optimal conditions of polysaccharide production include aeration, whereas in obligate aerobes this leads to reduced yield of extracellular polysaccharide (Sutherland, 1977).

Microbial Interactions

Many examples of microbial interactions are described in the context of biodegradation, microbial communities degrading compounds. Slater and Lovatt (1984) give an overview of the significance of microbial communities in biodegradation and discuss why techniques for the isolation of strains might fail to reveal community interactions.

Batch culturing of an originally adsorbed community, plating and nutrient excess are examples of conditions that are liable to disturb and not reveal species interactions.

Traditionally microbial interaction studies have been conducted in laboratory type chemostats (Frederickson, 1977) which allow the definition of the various types of interactions between microbial organisms. Generally interaction has a strong tendency to result in exclusion of one of the competitors. But often mitigating circumstances are present, resulting in 'balanced coexistence' (Van Gemerden, 1974).

Extrapolation of results from suspended growth interaction studies to attached growth cultures is virtually impossible. Homogeneity of chemostat conditions is not available in the attached growth system while characteristic aspects of the latter (transport through laminar sublayer, adsorption, growth on substratum, shear force, gliding, desorption, detachment) are not accounted for in the suspended growth system. Interestingly, discrepancy between the mathematical description of a predator - prey relation and observations could be removed by addition of a wall growth term in the equation, and Frederickson (1977) concludes that 'variation of the ratio of chemostat wetted surface area to culture volume should be an important part of future studies on microbial predator - prey relations'.

If interorganism distance is a variable of importance in microbial interaction (Crisp, 1981), attached growth seems more liable to result in interaction than suspended growth. Based on data from biofilm experiments in Rototorque reactors (Trulear, 1983), cell density is approximately 4 orders of magnitude higher in biofilms than in suspension (cell number per volume of biofilm or volume of suspension).

Interactions between algal and bacterial species in a variety of environments have been documented (Schiefer and Caldwell, 1982; Haack and McFeters, 1982; Escher and Characklis, 1982; Sandbeck and Ward, 1981). Few have been described to occur on solid substrata. Holmes (1986) studied the colonization of vinyl by 2 algal species on open air swimming pool walls. Rate and extent of algal colonization drastically increased in the presence of bacteria. Bacterial extracellular products (EPS) seemed to play a role in initial bacterial and in subsequent algal colonization. In a laboratory assembled (no growth took place during biofilm formation) algal bacterial biofilm (Murray et al., 1986), it was shown that algal exudates served as a sole carbon source for attached bacteria.

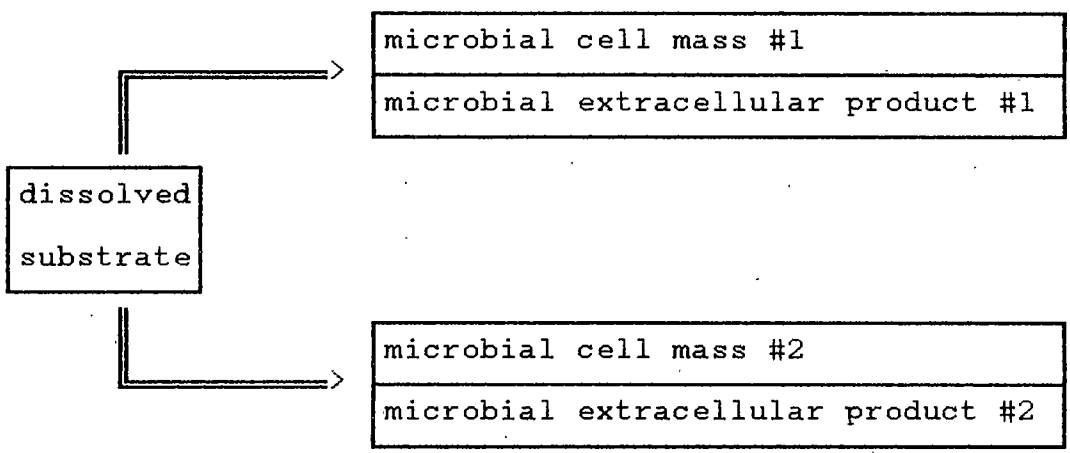
Microbial interaction is mathematically approached by Wanner and Gujer (1985-a) describing biofilm formation by a heterotrophic and autotrophic bacteria. Frederickson (1977) is the first to denote a microbial system consisting of 2 identified species by a binary population.

MATHEMATICAL DESCRIPTION

Microbial conversion of substrate into cell mass can be mathematically described using a mass balance approach. The equation describing a mass balance is of the following general form:

net rate of accumulation	=	net rate of transport	+	net rate of transformation

Mass accumulation equals the sum of rates of mass transformation and rates of mass flow in or out of a control volume.
For two microbial species in the system, the following sequence of events is considered:



Substrate carbon is used to provide microbial cell carbon and extracellular product carbon. CO_2 is produced as a respiration product. Thus, microbial cell carbon is used for cell synthesis (cell mass) and respiration (energy).

Mass Balance Equations for the Biofilm Reactor

Liquid phase substrate carbon (1):

$$\frac{dS_{Sl}}{dt} = D(S_{Si}-S_{Sl}) - X_{Mf}.\frac{A}{V}.r_S - X_{Ml}.\left[\frac{\mu}{Y_{MS}} + \frac{r_{Pl}}{Y_{PS}}\right]$$

net rate of substrate accumulation = net rate of substrate inflow − rate of substrate uptake by biofilm − rate of substrate uptake in liquid phase

where:

S_{Sl} = substrate carbon concentration in liquid phase $[M_S L^{-3}]$

t = time $[t]$

D = dilution rate $[t^{-1}]$ (= flow rate into divided by volume of reactor)

S_{Si} = substrate carbon concentration in influent $[M_S L^{-3}]$

X_{Mf} = cell carbon concentration in biofilm $[M_M L^{-2}]$

A = substratum area $[L^{-2}]$

V = reactor volume $[L^{-3}]$

r_S = biofilm specific substrate uptake rate $[M_S M_M^{-1} t^{-1}]$

X_{Ml} = cell carbon concentration in liquid phase $[M_M L^{-3}]$

μ = specific cellular growth rate $[t^{-1}]$

Y_{MS} = yield of cell carbon from substrate carbon $[M_M M_S^{-1}]$

r_{Pl} = specific product formation rate in liquid phase $[M_P M_M^{-1} t^{-1}]$

Y_{PS} = yield of product carbon from substrate carbon $[M_P M_S^{-1}]$

Liquid phase cell carbon (2):

$$\frac{dX_{Ml}}{dt} = D(X_{Mi}-X_{Ml}) + X_{Mf}.\frac{A}{V}.r_{Md} + X_{Ml}.\mu$$

net rate of cell mass accumulation = net rate of cell mass inflow + rate of cell mass detachment + rate of cell growth in liquid phase

where:

X_{Mi} = cell carbon concentration in influent $[M_M L^{-3}]$

r_{Md} = specific cell detachment rate $[t^{-1}]$

Liquid phase product carbon (3):

$$\frac{dS_{Pl}}{dt} = D(S_{Pi}-S_{Pl}) + S_{Pf}.\frac{A}{V}.r_{Pd} + XM_l.r_{Pl}$$

net rate of product accumulation = net rate of product inflow + rate of product detachment + rate of product formation in liquid phase

where:

S_{Pl} = product carbon concentration in liquid phase $[M_P L^{-3}]$

S_{Pi} = product carbon concentration in influent $[M_P L^{-3}]$

S_{Pf} = product carbon concentration in biofilm $[M_P L^{-2}]$

r_{Pd} = specific rate of product detachment $[t^{-1}]$

Biofilm substrate carbon (4):

$$\frac{dS_{Sf}}{dt} = X_{Mf}.r_S - X_{Mf}.f_D.\left[\frac{\mu}{Y_{MS}} + \frac{r_{Pl}}{Y_{PS}}\right]$$

net rate of substrate accumulation = net rate of substrate uptake − rate of substrate consumption for cell growth − rate of substrate consumption for product formation

where:

S_{Sf} = substrate carbon concentration in biofilm $[M_S L^{-3}]$

f_D = effectiveness factor for substrate diffusion [-]

Biofilm cell carbon (5):

$$\frac{dX_{Mf}}{dt} = - X_{Mf}.r_{Md} + X_{Mf}.f_D.\mu$$

net rate of cell mass accumulation = net rate of cell mass detachment + rate of cellular growth

Biofilm product carbon (6):

$$\frac{dS_{Pf}}{dt} = - S_{Pf}.r_{Pd} + X_{Mf}.r_{Pf}$$

net rate of product accumulation = net rate of product detachment + rate of product formation

where:

r_{Pf} = specific rate of product formation in biofilm $[M_P M_M^{-1} t^{-1}]$

Oxygen (7):

$$\frac{dS_{Ol}}{dt} = D(S_{Os}-S_{Ol}) + N_O.\frac{A}{V} +$$

net rate of oxygen accumulation = net rate of oxygen inflow in dilution water + rate of oxygen inflow by diffusion

$$- X_{Mf}.f_D.\left[\frac{\mu}{Y_{MO}} + \frac{r_P}{Y_{PO}}\right].\frac{A}{V} - X_{Ml}\left[\frac{\mu}{Y_{MO}} + \frac{r_P}{Y_{PO}}\right]$$

- rate of oxygen uptake for cell growth and product formation in biofilm
- rate of oxygen uptake for cell growth and product formation in liquid phase

where:

S_{Ol} = concentration of oxygen in liquid phase $[M_O L^{-3}]$

S_{Os} = oxygen saturation concentration in influent $[M_O L^{-3}]$

N_O = flux of dissolved oxygen into reactor system $[M_O L^{-2} t^{-1}]$

Y_{MO} = yield of cell carbon from oxygen $[M_M M_O^{-1}]$

Y_{PO} = yield of product carbon from oxygen $[M_P M_O^{-1}]$

The following simplifying assumptions are made:

1. With long biofilm detention times, biofilm substrate concentration (S_{Sf}) is zero.
2. Glucose is the only carbon and energy source in the influent.
3. Specific product formation rate is (Luedeking & Piret, 1959) growth associated and non-growth associated:

$$r_{Pl} = k_{Pg}.\mu + k_{Pn} \qquad (8)$$

where:

k_{Pg} = growth associated product formation coefficient $[M_P M_M^{-1}]$

k_{Pn} = non-growth associated product formation coefficient $[M_P M_M^{-1} t^{-1}]$

4. Specific microbial growth rate is dependent on substrate concentration according to Monod (1949):

$$\mu = \frac{\mu_m . S_{Sl}}{K_S + S_{Sl}} \qquad (9)$$

where:

μ_m = maximum specific cellular growth rate $[t^{-1}]$

K_S = half saturation concentration $[M_S L^{-3}]$

5. The flux of oxygen into the reactor can be estimated as (Appendix A):

$$N_O = k_c.(S_{Os}-S_{Ol}).\frac{V}{A} \qquad (A\text{-}2)$$

where:

k_c = dissolved oxygen specific mass transfer rate $[t^{-1}]$

With dilution water at saturation concentration, the oxygen diffusion component in Eq. 17 can be substituted by an expression similar to transport of oxygen by dilution water:

$$N_O.\frac{V}{A} = k_c.(C_{Os}-C_{Ol}) \qquad (10)$$

Mass Balance Equations for the Chemostat

Liquid phase substrate carbon (11):

$$\frac{dS_{Sl}}{dt} = D(S_{Si}-S_{Sl}) - X_{Ml}\cdot\left[\frac{\mu}{Y_{MS}} + \frac{r_P}{Y_{PS}}\right]$$

net rate of substrate accumulation = net rate of substrate inflow − rate of substrate uptake in liquid phase

Liquid phase cell carbon (12):

$$\frac{dX_{Ml}}{dt} = D(X_{Mi}-X_{Ml}) + X_{Ml}.\mu$$

net rate of cellular accumulation = net rate of cellular inflow + rate of cell growth in liquid phase

Liquid phase product carbon (13):

$$\frac{dS_{Pl}}{dt} = D(S_{Pi}-S_{Pl}) + XM_l.r_{Pl}$$

net rate of product accumulation = net rate of product inflow + rate of product formation in liquid phase

Mass Balance Equations for the Batch Reactor

Liquid phase substrate carbon (14):

$$\frac{dS_{S1}}{dt} = - X_{M1} \cdot \left[\frac{\mu}{Y_{MS}} + \frac{r_P}{Y_{PS}} \right]$$

net rate of substrate accumulation = rate of substrate uptake

Liquid phase cell carbon (15):

$$\frac{dX_{M1}}{dt} = X_{M1} \cdot \mu$$

net rate of cell mass accumulation = rate of cell growth

Liquid phase product carbon (16):

$$\frac{dS_{P1}}{dt} = XM_1 \cdot r_{P1}$$

net rate of product accumulation = rate of product formation

Oxygen (17):

$$\frac{dS_{O1}}{dt} = - X_{M1} \left[\frac{\mu}{Y_{MO}} + \frac{r_P}{Y_{PO}} \right] + R_O$$

net rate of oxygen accumulation = − rate of oxygen uptake for cell growth and product formation + rate of oxygen generation

EXPERIMENTAL APPROACH

The experimental work can be divided in three parts according to the reactor used. Experiments conducted in the Rototorque resulted in stoichiometric and kinetic coefficients for biofilm processes for both the mono and binary populations. Chemostat experiments provided kinetic and stoichiometric coefficients for mono population processes in suspension. Batch experiments provided stoichiometric data on the consumption of oxygen.

Rototorque

The Rototorque, essentially a Couette vessel, consists of two concentric cylinders, a stationary outer cylinder and a rotating inner cylinder (Figure 1). Removable slides (4-12) which form an integral part of the inside wall of the outer cylinder permit sampling of the biofilm so that thickness, mass, and/or biofilm chemical composition can be determined. The reactor liquid phase is completely mixed by virtue of draft tubes bored through the solid inner cylinder (Figure 1). The draft tubes are positioned at angles so that the rotation of the inner cylinder pumps the fluid through the tubes. By virtue of the complete mixing, effluent liquid samples represent the reactor liquid composition.

The Rototorque is a continuous flow stirred tank reactor (CFSTR), an open reactor (i.e., there are flows in and out) in which concentration gradients within the liquid volume are minimized. The CFSTR provides significant advantages for observing, separating and evaluating the kinetics and stoichiometry of each biofilm process:

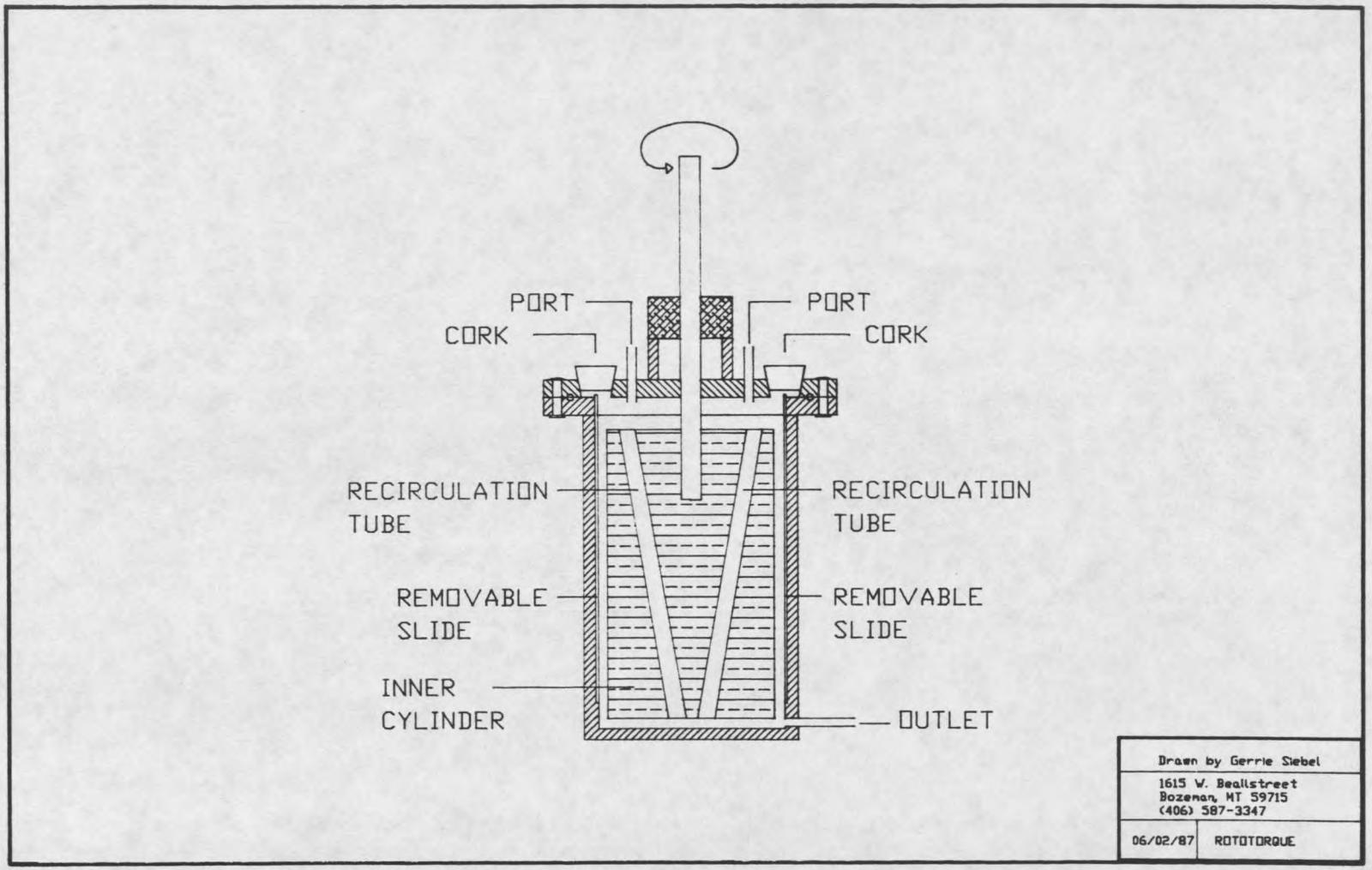


Figure 1. Schematic of a Rototorque reactor.

1. The liquid phase is homogeneous which simplifies sampling, chemical analysis, and mathematical modelling.
2. The mass transfer rate and shear stress in the annular geometry at different rotational speeds has been described mathematically (Mizushina, 1971).
3. The annular geometry has been used in numerous experimental observations related to biofilm processes (Trulear & Characklis, 1982; Bakke et al. 1984; Turakhia, 1986).

Material balance calculations for carbon permit a measure of biofilm activity. Oxygen balances can also be performed. Fluid shear stress at the wall is a function of rotational speed. Mean liquid residence time depends on dilution flow rate through the reactor. Thus fluid shear stress and residence time can be varied independently. Reactor residence time was maintained at approximately 10 minutes so that suspended growth was negligible and all reactor activity can be attributed to the biofilm. The reactor has a high surface area-to-volume ratio and most of its surface area is exposed to a uniform shear stress. A summary of characteristics and dimensions of the Rototorque is presented in Appendix B.

Preparation of Rototorque

Biofilm experiments were prepared by initiating the standard cleaning procedure for reactor and tubing components. This consisted of brushing all reactor components in a solution of 2% active chlorine followed by rinsing with distilled water. After drying, the slides were inserted and the intake ports on top of the reactor were filled with loosely packed cotton plugs. The air vent was attached, the effluent line connected and closed with a

screw clamp. Parts of the slide were provided with a thin layer of resin for the preparation of biofilm Transmission Electron Micrographs (TEM). The entire assembly was then autoclaved for 15 minutes at 121°C.
Tubing and flow meters between feed stock solutions and the reactor were flushed with distilled water and tube ends were sealed with aluminum foil and clamped. Tubing was autoclaved for 15 minutes at 121°C.

Preparation of Chemostat

A 500 ml solution of nutrient medium was made up, added to the clean chemostat and autoclaved for 15 minutes at 121°C. After cooling to room temperature, the chemostat was inoculated with 1 ml of the appropriate microbial suspension, previously grown in batch culture. The chemostat was operated in batch mode for 12 to 16 hours to obtain a cell density between 10^{12} and $10^{13}.m^{-3}$. Then a continuous flow of nutrients and substrate (40 g $C.m^{-3}$) was started. Steady state conditions were assumed to have been reached after 6 residence times.

Start-up and Operating Conditions

The Rototorque setup was assembled. The reactor was then filled with dilution water and continuous flows started (dilution water $3.6*10^{-3}$ $m^3.h^{-1}$) giving final concentrations of calcium 25 g $Ca.m^{-3}$, buffer 1.5 mMol of mono- and dibasic phosphate and substrate 8 or 20 g $C.m^{-3}$. The rotational speed was set at 200 rpm. After a minimum of 15 minutes, the Rototorques were inoculated by starting a flow of microorganisms ($0.06*10^{-3}$ $m^3.h^{-1}$, cell density 10^{12}-$10^{14}.m^{-3}$) from the chemostat at time zero. The inoculation period was 12 hours.
A schematic of the Rototorque experimental setup is presented in Figure 2.