MIXED POPULATION BIOFILMS

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1. Introduction

Multiple species biofilms comprise mixed bacterial populations within an extracellular polymeric matrix bound to a substratum. These attached mixed cultures are subject to interactions such as symbiosis or competition for either space or a common substrate; such interactions are directly or indirectly influenced by a myriad of variables associated with the surrounding environment. Spatial distributions of microbial populations are everchanging under the selection pressures exerted by processes such as: *(a)* exchange of bacterial species with the bulk liquid phase; *(b)* the relative efficiency of each species to metabolize their limiting substrate(s) into viable cell mass and non-viable extracellular polymers; *(c)* transport of limiting substrates and essential nutrients by molecular and convective transfer mechanisms; and *(d)* biofilm removal processes brought on by either physiological mechanisms (sloughing) or as a result of prevailing hydrodynamics (shear-related detachment). Evolution of spatial distributions of species within a biofilm can affect the biofilm's overall performance in specific situations (*e.g.,* natural purification of contaminated surface- or ground-waters, *in situ* bioremediation of xenobiotics, fate of recombinant DNA sequences and host microbes in the open environment, specific biofilm wastewater treatment systems, and specialty bioconversions). Consequently, it is critical to know how the ever-changing attached population distributions can affect overall system performance in order to better design, interpret, and operate biofilm systems.

2. Models of Biofilm Formation

Models which describe microbial growth either as an increase in total biomass or which consider only simple, single microbial conversion reactions are called *unstructured models.* Such lumped parameters approaches do not detail, for example, mixed culure population dynamics or changes in cellular composition in response to changes in environmental conditions. A structured model based on total cellular mass can not actually distinguish between pure and mixed cultures. Although relatively simple, these models have been applied throughout the industrial fermentation industry and the biological treatment sector verify experimental observations and provide initial process design parameters. Since such models are based simple biological conversion of substrate to cell mass, they are best suited for "balanced growth" steady-state systems.

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Conversely, *structured models* do consider the additional detail of mixed culture population dynamics, cell molecular composition changes, and multiple reaction sequences. Consequently, a structured model is inherently better suited for simulating microbial physiology or mixed population dynamics under transient, unsteady conditions. While structured models provide more detail about a system, this necessitates a more phenomenological picture of the complex process interactions involved and quantitative information regarding process rate parameters. Historically, models of biofilm performance were initially unstructured, inductive "black-box" approaches that did not consider the *formation history* of a biofilm but rather simply the biofilm's efficiency in metabolizing a single limiting substrate. Evolution of our need to predict pure then mixed populations dynamics within an developing biofilm as led to a number of successive derivations that span the spectrum of unstructured to structured models as summarized by Wanner & Gujer (1986).

In the following, both modeling philosophies will be applied to biofilm formation beginning with a simplistic unstructured biofilm formation model followed by three different structured models; the first two structured models presume the biofilm is a well mixed continuum with no spatial distribution of species possible and the third, more complex model that accounts for spatial distributions of different species can arise in a developing biofilm.

2.1 AN UNSTRUCTURE MODEL OF BIOFILM FORMATION

2.1.1. *Basic Model Assumptions.* Unstructured versus structured modeling concepts are discussed in some detail in this volume's chapter on process analysis (Bryers and Characklis). Biofilm formation is assumed to occur on the exposed surface areas of a CSTR; thus, tacitly neglecting any spatial heterogeneity in the bulk liquid. Suspended biomass in the bulk liquid arises due to either cell growth and replication or due to shear-related erosion of biofilm material. Planktonic cells leave the liquid phase by either the effluent liquid leaving the reactor or by cell deposition onto the reactor surfaces. A single sterile growth limiting substrate, S, enters the reactor where it is consumed by either the suspended or biofilm-bound cells. Any reasonable measure of both biofilm and suspended cell concentrations is acceptable (cell number, biomass dry weight, biomass organic carbon) but note in the unstructured approach, no distinction between species or between cell carbon and EP carbon is made or required. However, one most know the stoichiometric relationship between changes in the suspended and attached biomass concentrations due to growth and the limiting substrate utilized.

2.1.2. *A General Unstructured Model.* Based on these assumptions and the scenario of biofilm formation detailed by Bryers and Characklis (this volume; process analysis), material balances for both the planktonic cell biomass and single growth limiting substrate can be written as Eqns. (1) and (2),

Suspended biomass balance:

$$VdX/dt = -FX + r_{gs}V + r_{ero}A - r_{dep}A \quad (1)$$

Limiting substrate balance:

$$VdS/dt = F(S_{in} - S) - [r_{gs}V/Y_{X/S}] - [r_{gb}A/Y_{B/S}] \quad (2)$$

where X = suspended cell biomass, S = limiting substrate concentration, V = reactor volume, $Y_{X/G}$ = yield coefficient for suspended cells, $Y_{B/S}$ = yield coefficient for biofilm mass, and A =

reactor area. Rate expressions for the remaining process rates (r_{gs}, r_{ero}, r_{dep}, r_{gb}) have been defined in the chapter on process analysis (Bryers & Characklis; this volume). with several depending directly on changing biofilm amount, limiting substrate and suspended biomass concentration. Should the reactor be operated at a residence time shorter than the generation time of the cells, then one can assume the term $r_{gs}V$ is negligible; however, the corresponding term in Eqn. (2) respresenting suspended cell substrate metabolism $[r_{gs}V/Y_{X/S}]$ can not be disregarded unless in the specific system it is determined small compared to the other terms.

Biofilm net accumulation can be described by Eqn. (3),

Biofilm net accumulation:

$$A\,[dm_f/dt = r_{dep} + r_{gb} - r_{ero}] \qquad (3)$$

Since rate expressions for both planktonic and biofilm growth are non-linear saturation kinetic functions of the instantaneous substrate concentration and first order functions of X and m_f, respectively, Eqns. (1) - (3) are coupled, non-linear ordinary differential equations that require either simultaneous numerical integration (for the transient situation) or simultaneous algebraic solution under conditions of steady-state (*i.e.*, dS/dt, dX/dt, and $dm_f/dt = 0$). Trulear (1983) determined, by independent experiments on a mixed culture biofilm, values for the various process rate kinetic constants which were then used to simulate changes in X, S, and biofilm thickness, y_f, in a rotating annular reactor operated at a residence time well in excess of the generation time for the culture (Figure 1). In this study, the biofilm thickness y_f was determined as the ratio of the biofilm area mass concentration to the biofilm density (m_f/ρ_f).

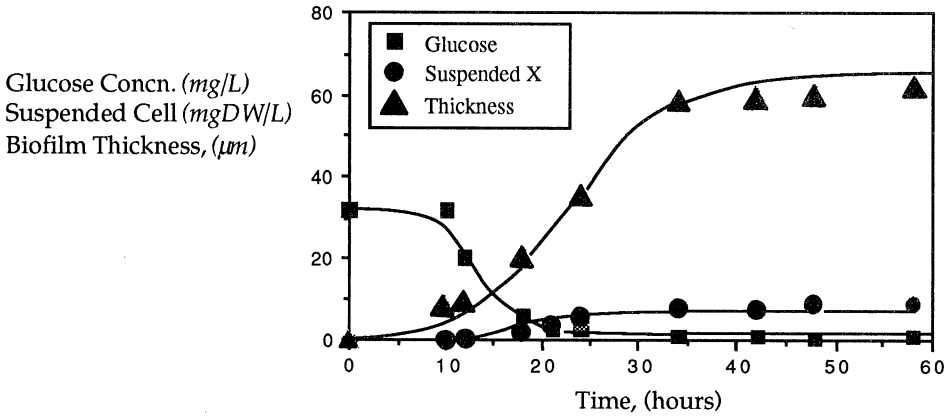

Figure 1. Progression of both liquid phase and surface parameters during mixed culture biofilm formation in a completely mixed rotating annular reactor operated at a dilution rate well in excess of the culture maximum growth rate. (Trulear, 1983). Lines represent predictions of a computer simulation of an unstructured model.

2.2 STRUCTURED MODELS OF BIOFILM FORMATION

A number of different structured models of biofilm formation exist that vary depending on how "structure" is applied to fulfill the needs of the research. Three will be detailed below; two models add structure to the biofilm composition yet treat it spatially as a well mixed continuum while a third model accounts for spatial distributions of both substrates and species within the biofilm.

2.2.1. *A Structured Model of Biofilm Composition.* Bakke *et al* (1984) presents a model of a pure culture biofilm system similar to that detailed in Section 2.1 above but in their model "structure" is added by considering the total biofilm amount per area, m_f, (measured as total organic carbon/area) as the sum of attached cell carbon/area, B, plus extracellular polymer carbon/area, EP_b, (*i.e.*, $m_f = B + EP_b$). A similar distinction is made for planktonic biomass (*i.e.*, $X = Xc + EP_s$). As in the unstructured model in Section 2.1.2 above, this model treats the biofilm as a well mixed reactor in that the components B and EP_b are assumed uniformily distributed throughout the depth of the biofilm; no spatial gradients in the various biofilm components are considered. By necessity, this structured model requires material balances for Xc, EP_s, and S in the liquid phase and B and EP_b in the biofilm phase. Similar equations to Eqns. (1) - (3) can be written that now require new process rate details regarding *(a)* the rate of EP production by both suspended and attached cells, *(b)* separate erosion rates for biofilm cells and EP_b, and *(c)* additional stoichiometric coefficients relating substrate conversion to both cell and EP production. The series of ordinary differential equations forming the structured model of a pure culture biofilm cell and EP components are summarized below:

Substrate Carbon Balance:

$$dS/dt\ [M_{sc}L^{-3}t^{-1}] = D(S_i - S) - \{\mu_b/Y_{b/s} + q_{pb}/Y_{ep/s}\}B - \{\mu_c/Y_{x/s} + q_{pc}/Y_{ep/s}\}X_c \quad (4)$$

Suspended Cell Balance:

$$dX_c/dt\ [M_{cc}L^{-3}t^{-1}] = -DX_c + r_{er/c}A - r_{dep/c}A + \mu_c X_c \quad (5)$$

Suspended Polymer Carbon Balance:

$$dEP_s/dt\ [M_{pc}L^{-3}t^{-1}] = -DEP_s + r_{er/p}A - r_{dep/p}A + q_p X_c \quad (6)$$

Biofilm Cellular Carbon:

$$dB/dt\ [M_{cc}L^{-2}t^{-1}] = \mu_b B + r_{dep/c} - r_{er/c} \quad (7)$$

Biofilm Extracellular Polymer Carbon:

$$dEP_b/dt\ [M_{pc}L^{-2}t^{-1}] = q_{pb}B - r_{er/p} + r_{dep/p} \quad (8)$$

where D = system dilution rate = flow rate F/reactor volume V; q_{pb} = specific polymer production rate for biofilm cells = $k\mu_b + k'$ where k is a growth related polymer production rate constant and k' is a non-growth related polymer production rate constant; a similar term, q_{pc}, exists for suspended cell polymer production rate; $Y_{x/s}$ and $Y_{b/s}$ are stoichoimetric coefficients for substrate carbon conversion to, respectively, suspended and biofilm cell carbon; $Y_{ep/s}$ = the stoichiometric coefficient for conversion of substrate to polymer; $r_{er/c}$ and $r_{er/p}$ are, respectively, the erosion rates for cells and polymer per area biofilm, $r_{dep/c}$ and $r_{dep/p}$

are, respectively, the net deposition rates of cells and polymer from the liquid onto the biofilm; and μ_b and μ_c are, respectively, the cell growth rates for biofilm and suspended cells. If the reactor in question is operated as a CSTR at a residence time less than the generation time of the cells, the last term in Eqns. (5) can be assumed negligible. Bakke *et al* (1984) used results from pure *Pseudomonas aeruginosa* biofilm experiments and steady-state versions of Eqns. (4) - (8), to determine the individual stoichiometric and kinetic parameters associated with the rate processes above. The reader should consult Bakke *et al* (1984 and 1990) for exact details of their experimental study and the different functions assumed for each rate expression above. Predictions of the above structured model compare well with experimental results of Trulear (1983) for a *Pseudomonas aeruginosa* biofilm cultivated in an annular rotating reactor (Figure 2).

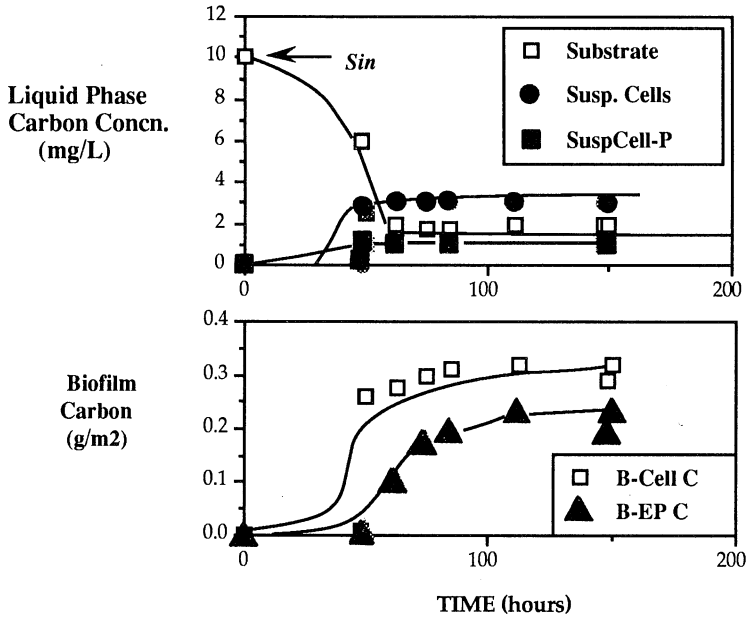

Figure 2. Progression of liquid and biofilm parameters for *Pseudomonas aeruginosa* biofilom formation in a rotating annular reactor. Lines represent predictions of a structured biofilm model.

2.2.2. *A Structured Model of a Mixed Culture Biofilm.* Bryers (1984) proposed a multiple species structured biofilm model that was a simple extension of the unstructured model in Section 2.1 that incorporated additional material balances per each species in both the biofilm and the fluid phase. Eqns. (4) - (8) can be repeated for each bacterial species identified, provided the individual kinetic and stoichiometric parameters for the new process rates are known. Either a separate growth limiting substrate can be assumed for each species necessitating additional material balances or, should the bacterial species compete for the same substrate, all that is required are additional substrate depletion terms per bacterial species in Eqn. (4). Models of this type are essentially the same as the two previous versions in that no consideration is given to any spatial segregation of cells or substrates within the biofilm. Siebel *et al* (1987) report on the use of just such a structured model of a two bacterial

species, *Pseudomonas aeruginosa* and *Klebseilla pneumoniae* , each competing for the same electron donor (glucose) and acceptor (oxygen) within a rotating annular biofilm reactor. Values for the individual rate constants for the processes of deposition, cell growth, and biofilm erosion were determined experimentally from pure culture biofilm formation studies similar to those depicted in Figure 2. Using the parameters from the pure culture studies, a binary population model was employed to simulate the formation of the mixed culture biofilm; a comparison of predictions and observations are provided in Siebel *et al.* (1987).

3. Modeling Spatial Distributions in Multiple Species Biofilms

A number of existing complex models can predict the development of spatial distributions of different bacterial species within a developing biofilm competing for multiple growth limiting substrates (Wanner & Gujer, 1986; Kissel *et al.* , 1984). The version of Wanner & Gujer (1984) will be used here; for details regarding the derivation of their original model and subsequent modifications the reader is directed elsewhere (Gujer and Wanner, 1990; Gujer, 1987).

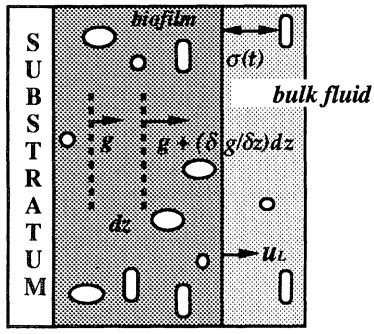

Figure 3. Flux of species biomass in an expanding biofilm.

A number of fundamental assumptions are necessary in order to model a mixed culture biofilm developing in a direction perpendicular to the plane of the substratum (Figure 3). The model is based on assumptions that *(a)* properties of the biofilm - *i.e.*, bacterial species and substrate concentrations - change only in the direction perpendicular to the substratum and *(b)* that the biofilm is a continuum. Biomass of each species "moves" due to convective motion of that species' mass at a velocity dependent not only on the cells growth rate at a point in the biofilm but also due to displacement by neighboring cells. Dissolved components in the liquid phase are assumed to diffuse within the biofilm by molecular diffusion assuming a constant diffusion coefficient; (*i.e.*, Fick's law prevails within the biofilm). *(c)* The biofilm overall volumetric density is assumed to remain constant. *(d)* The model is capable of accounting for both extracellular polymers and dead cell mass if desired. *(e)* Growth rate dependencies of each species on its limiting substrate(s) can be any type of functional dependency (*e.g.*, Monod, dual-Monod, Blackman kinetics) and will vary locally with time as the biofilm develops. Finally, *(f)* exchange of cells and dissolved components in the fluid phase with the biofilm can be linked with a material balance over the reactor volume by assuming the biofilm develops within a well-mixed CSTR.

A mass balance on microbial species i set up over a differential element of the biofilm can be written as,

$$\partial f_i/\partial t = \mu^o{}_i f_i - (1/\rho_i)\, \partial g_i/\partial z \qquad (9)$$

where ρ_i = the constant density, f_i = the volume fraction, $\mu^o{}_i$ = the net specific growth rate, and g_i = the mass of bacterial species i that is displaced per unit time and area relative to the

substratum. The mass flux g_i of species i can also be expressed as the product of the velocity u at which microbial mass is displaced and its mass concentration,

$$g_i\,(t,z) = u(t,z)\rho_i f_i(t,z) \tag{10}$$

The velocity$u$ is unknown but can be determined by relating the biofilm volume expansion to biomass production. Summarizing the results of Wanner and Gujer (1986), the mean observed growth rate of biomass, μ_o, is

$$\mu_o(t,z) = \partial u(t,z)/\partial z \tag{11}$$

Integrating to solve for the velocity yields,

$$u(t,z) = \int_0^z \overline{\mu_0}(t,z')\ dz' \tag{12}$$

If the biofilm grows or shrinks, the thickness L changes and the biofilm-fluid interface moves at a velocity u_L ,

$$u_L = dL(t)/dt \tag{13}$$

Defining $\sigma(t)$ as the rate at which biomass is exchanged between the biofilm and the fluid interface, then the velocity of the biofilm-fluid interface can also be expressed as,

$$u_L(t) = \int_0^L \overline{\mu_o}(t,z')dz' + \sigma(t) \tag{14}$$

In a similar fashion, a mass balance for any substrate *i*, can be written as,

$$\partial S_i(t,z)/\partial t = r_i(t,z) - \partial j_i(t,z)/\partial z \tag{15}$$

where S_i = the local, instantaneous concentration, j_i = the mass flux, and r_i = the observed biological conversion rate of substrate i. The mass flux of substrate can be related to the concentration gradient by Fick's first law,

$$j_i(t,z) = - \mathcal{D}_i\, \partial S_i(t,z)/\partial z \tag{16}$$

The reaction rate expressions depend upon the microbial conversion and bacterial species in question. Reaction rate expressions take the form of dual substrate limited saturation kinetics minus an oxygen dependent endogenous decay rate,

$$r_i\,(t,z) = \mu^*_i\rho_i f_i[S_{O2}/(K_{O2} + S_{O2})]\,[S_{EDi}/(K_{EDi} + S_{EDi})] - k_e\rho_i f_i[S_{O2}/(K_{O2} + S_{O2})] \tag{17}$$

where μ^*_i = the maximum growth rate constant for species i ; S_{O2} = the local instantaneous concentration of oxygen (which is the common terminal electron acceptor for the ecology considered in this study); K_{O2} = the saturation rate constant; k_e = the endogenous decay rate constant for species i; and S_{EDi} and K_{EDi} = the local, instantaneous concentration and the saturation rate constant for the specific electron donor used by species i.

In one study, Gujer and Wanner (1990) considered populations of heterotrophic carbon-oxidizing bacteria competing with autotrophic nitrogen-oxidizing species. In the case of the heterotrophs, the electron donor is organic carbon, for *Nitrosomonas spp.* the electron donor is ammonia nitrogen (represented here as NH_4^+-N), and Nitrite nitrogen (NO_2^--N) for *Nitrobacter spp.* Note: the oxygen dependent endogenous decay rate expression is identical to that in the growth rate term which, should the oxygen at some depth in the biofilm go to zero, renders the overall reaction zero at that (t,z). While this computationally prevents negative reaction rates, it also tacitly ignores the potential for denitrification by facultative heterotrophs; the biofilm model of Kissel *et al.* (1984) does account for possible denitrification. Bryers and Banks (1990) used the Gujer-Wanner model to simulate biofilm development of two heterotrophs, a *Hyphomicrobium spp.* growing exclusing on methanol and a *Pseudomonas putida* metabolizing exclusively glucose, both competing for oxygen.

Maximum overall uptake of any substrate varies with time as the biofilm increases (or decreases) and as the bacterial populations change; this maximum observed uptake rate per unit area of biofilm can be calculated as the flux of substrate evaluated at $z = L$. Bacterial species and substrate profiles in the developing biofilm can be numerically determined using the *methods of lines* as detailed in Wanner and Gujer (1986).

3.1 COMBINED NITRIFICATION:CARBON OXIDATION BIOFILMS

Wanner and Gujer (1986) present an illustration of the utility of the multiple species biofilm above for the competition between autotrophic nitrifying bacteria oxidizing ammonia and nitrite nitrogen and heterotrophic carbon-oxidizing bacteria. An inert fraction of the biofilm was also modeled to represent polymer production and inert dead cell material. Dissolved components considered were organic carbon (as COD), NH_4^+-N, and O_2. In the following example, reactor inlet (and initial) conditions in the fluid phase were 3 $(gCOD)m^{-3}$, 13 $(gNH_4^+\text{-}N)m^{-3}$, and 8 $(gO_2)m^{-3}$. Biofilm thickness was initially set at 5 μm and the various biofilm components set at 0.0 inert fraction, 0.65 heterotrophs, and 0.35 autotrophs; expressed as fractions of the total biofilm density here assumed to be 35 g cm^{-3}. Figure 4 illustrates predictions of the Wanner & Gujer model for the progression of biofilm thickness and species distrubution with depth for the first ten days of the development.

Bryers and Banks (1990) employed the above model under slightly different conditions to simulate experimental observations a nitrifyier:heterotroph biofilm. Parameters employed for the computer simulations are provided in Table 1. Predicted fractions of biofilm species were compared to the concentrations determined for both HET and NTF using dual radiolabelling of the biofilm bacteria. Heterotrophs were radiolabelled with ^{14}C-acetate (0.5 μCi/mL) while autotrophic nitrifiers were labelled with $NaH^{14}CO_3$ (58μCI/mmol) followed by microtome

sectioning of the biofilm at 20μm depth intervals parallel to the plane of the substratum. For details regarding these experiments the reader is directed to Bryers and Banks (1990).

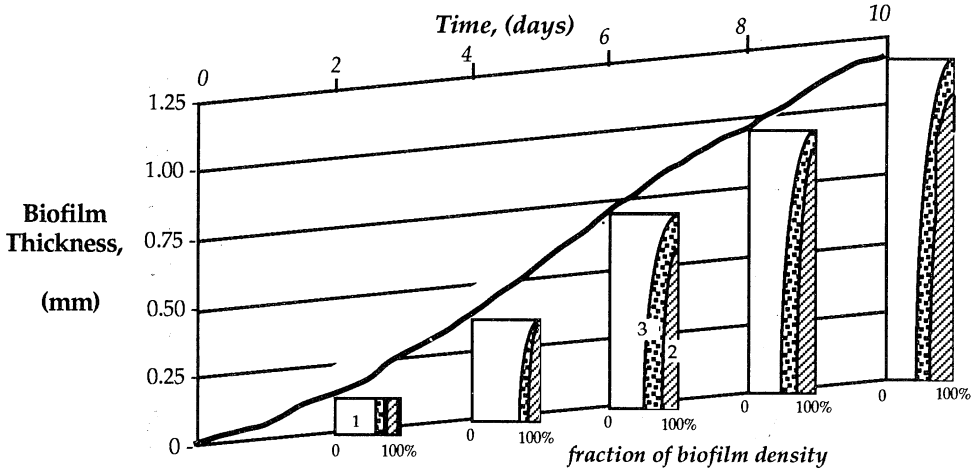

Figure 4. Model predictions of hetrotrophic:nitrifier biofilm species profiles. Fraction of biofilm density of each species is based on a total biofilm density of 35 g cm^{-3}. Feed conditions are stated in the text above. Fractions considered a part of the biofilm mass are: (1) heterotrophs, (2) nitrifiers, and (3) exopolmyers. Wanner and Gujer (1986).

Experimental disintegrations per minute were converted to cell numbers then to cell carbon by calibration curves for each species. Predicted species concentrations in the Wanner-Gujer model were converted from fractions of the biofilm density to mass-carbon areal concentrations using the experimentally determined biofilm density. A comparison of predicted and observed biofilm species distributions are illustrated in Figure 5A&B for two different experimental conditions: a situation to promote nitrifyier dominance (NH_4^+-N = 5.0; O_2 = 8.5; and Acetate = 1.0 ppm) and one to promote heterotroph dominance (NH_4^+-N = 5.0; O_2 = 8.5; and Acetate = 3.0 ppm).

Table 1. Parameters Employed in Computer Simulations of Multiple Species Biofilms

		Ecosystem I		Ecosystem II	
PARAMETERS		Heterotrophs	Nitrifiers	Ps. putida	Hyphomicro.
μ*	(hr^{-1})	0.2	0.039	0.54	0.24
K_S	(mgS/L)	5 (acetate)	1 (ammonia)	10.0 (C6)	0.01 (C1)
K_{O2}	(mgO_2/L)	0.1	0.1	1.0	0.10
$Y_{x/s}$	(mgX/mgS)	0.45	0.10	0.42	0.30
k_e	(h^{-1})	0.008	0.004	0.005	0.002
D_{eff}	(cm^2/sec)	acetate:	9.6E-06	methanol:	15.0E-06
		ammonia:	17.2E-06	glucose:	5.0E-06
		oxygen:	20.2E-06	oxygen:	20.2E-06

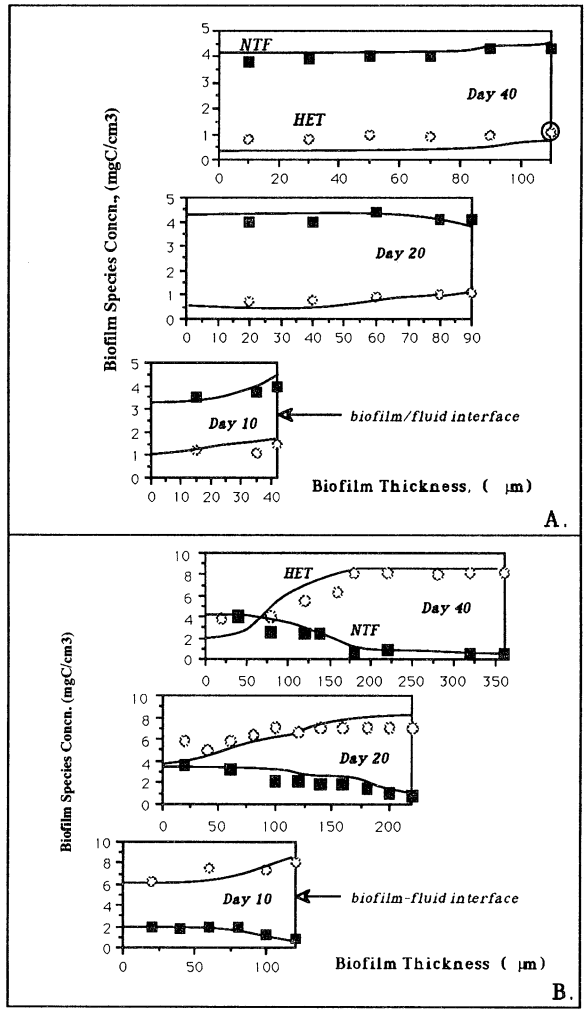

Figure 5. Comparison of computer simulation using Gujer-Wanner model to mixed heterotrophic-autotrophic culture biofilm experiments. (**A.**) NH_4^+-N = 5.0; O_2 = 8.5; and Acetate = 1.0 ppm. (**B.**) NH_4^+-N = 5.0; O_2 = 8.5; and Acetate = 3.0 ppm.

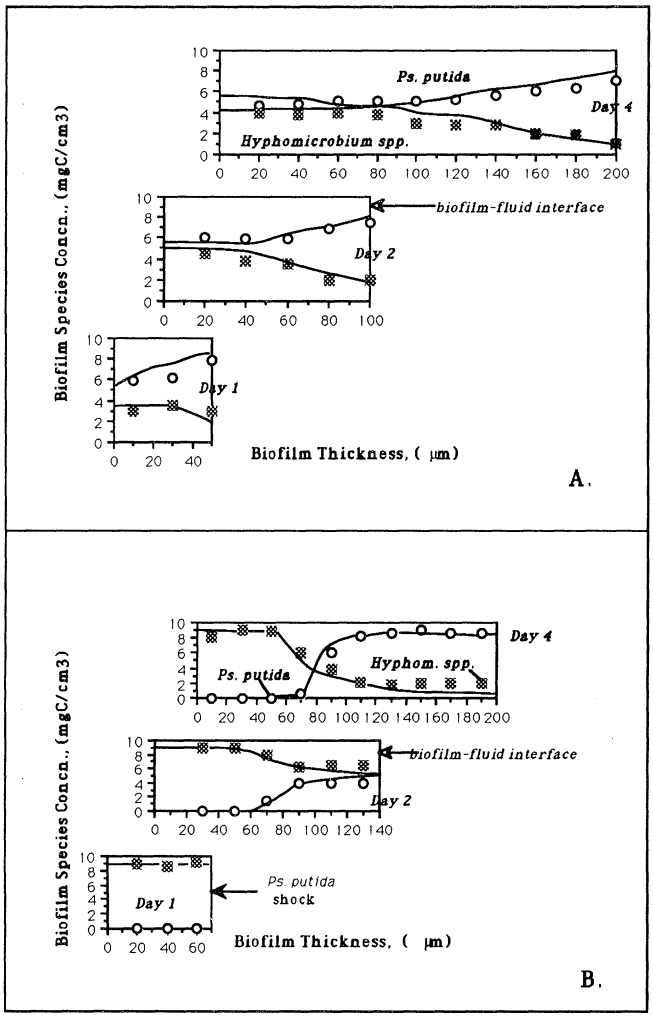

Figure 6. Comparison of computer simulation using Gujer-Wanner model to a binary heterotrophic mixed culture biofilm experiment. (**A.**) Both *Pseudomonas putida* and *Hyphomicrobium spp.* seeded on surface at same time; nutrient conditions detailed in text. (**B.**) *Hyphomicrobium spp.* pure culture biofilm grown for one day, exposed to *Pseudomonas putida* suspension for 2 hours, then biofilm fed as in A. An artificial bi-layer biofilm is created.

Bryers and Banks (1990) also simulated a second system, also summarized in Table 1, of two chemoheterotrophs metabolizing separate electron donors but both competing for oxygen. *Pseudomonas putida* ATCC 11172 metabolized exclusively glucose while *Hyphomicrobium spp. ZV620* grew only on methanol. Subsamples of the same biofilm sample could thus be exposed to either ^{14}C-glucose or ^{14}C-methanol to label the separate species. Samples were fixed, embedded, and sectioned in 20 μm thick increments parallel to the plane of the substratum as describe in Bryers and Banks (1990). A comparison of predicted and observed biofilm speciesdistributions are illustrated in Figure 6A&B for two different experimental conditions: *(1)* an artificially constructed bi-layer biofilm where a pure culture of *Hyphomicrobium spp.* is overgrown with *Pseudomonas putida* and then both electron donors suppled and *(2)* where both *Pseudomonas putida* and *Hyphomicrobium spp.* are inoculated at time equal zero and grown on equal concentrations of glucose and methanol; the biofilms were oxygen limited.

4. Summary

Biofilm systems are very complex. Often times out of experimental expediency, research simplifies these complexities by dealing with *in vitro*, well-controlled laboratory systems that use pure bacterial cultures. Such environmentally controlled systems have in the past provided much information on the dynamics of biofilm formation and activity. However, only in rare situations are the results of such pure culture work additive; mixed population biofilms pose additional nuances on the structure and function of biofilms that can not be garnered from pure culture systems. This lecture will exemplify a number mixed population bacterial biofilm systems. Effects of mixed population biofilms on our understanding of biofilm structure, mass transport properties of the biofilm matrix, and morphological characteristics of the biofilm will be outlined. Mathematical modeling of population distributions within developing biofilms will be presented and experimentally verified. Models presented could be extended to other mixed cell biofilms including multiple species or strains, mixed procaryotic-eucaryotic biofilms, and mixed cell line (bacteria, platelets, endothelial cell) biofilms.

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