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ASSESSMENT OF BIOFILM ECODYNAMICS

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SUMMARY

Processes governing the population dynamics of multiple species biofilms are reviewed. Existing structured models of multiple species biofilms are used to describe the spatial and time-dependent changes in species concentration and extracellular polysaccharide for two different mixed culture systems. An experimental protocol is presented that allows verification of the model predictions for the two systems considered.

1. INTRODUCTION

Almost any material, exposed to an aqueous environment provides a substratum for microbial cell attachment. Attached cells grow, replicate, and produce extracellular polymers forming a gel matrix which collectively is called a **BIOFILM.**

A biofilm community begins initially when a "clean" surface is exposed to an aqueous environment and becomes conditioned by chemical constituents prevailing therein. Inevitably, microorganisms become associated with the surface, adhere, then attach tenaciously. Once these primary colonizers are attached, the activity of the biofilm community is dependent upon cell metabolism and growth as dictated by local surface conditions. These metabolic activities include nutrient consumption, cellular growth and replication, and the synthesis of extracellular polymers. As the biofilm gel matrix accumulates on a surface, it acts to trap elusive nutrients and if in a mixed species system, to attract other microbial participants into the biofilm community. Thus, the microbial diversity of the biofilm community is everchanging, both spatially (with depth or from one location to another) and with time.

Most laboratory studies on bacterial biofilm formation and

persistence have been carried out using a single growth limiting nutrient to cultivate pure culture biofilms. Mixed culture biofilm studies have simplified analysis by using an "unstructured" approach to population diversity -- i.e., viable cells are lumped into single groups; either all "heterotrophs" or all "autotrophs". Consequently, little knowledge exists on how various environmental selection pressures affect biofilm population dynamics. One goal of our research group is to evolve the means to understand those processes controlling population dynamics during biofilm formation. This paper presents both the mathematical and experimental tools to determine the spatial distribution of multiple bacterial species within a developing biofilm.

2. BIOFILM MODEL SYNOPSIS

An unsteady-state model for multiple biofilm components (microbial species, extracellular polymers) accumulating within a biofilm growing on a number of possible limiting substrates has been derived by Kissel et al (1) and Wanner and Gujer (2). Details and complete discussion of the original model are presented in Wanner and Gujer (2).

2.1 Model Equations

A balance for the mass flux of the i-th microbial species, g_i, in the differential element shown in Fig. 1 can be written

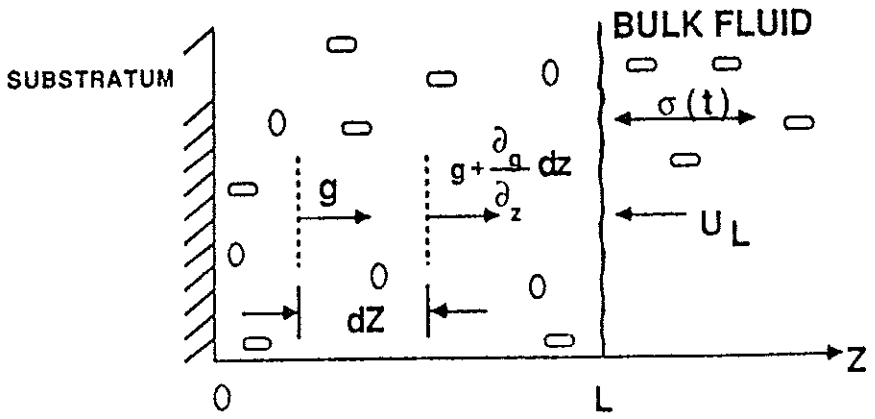

FIGURE 1. FLUX OF BIOMASS THROUGH AN EXPANDING BIOFILM

as,

$$\rho_i \partial f_i/\partial t = \mu_i \rho_i f_i - \partial g_i/\partial z \qquad [1]$$

where

$$g_i(t,z) = u(t,z)\rho_i f_i(t,z) \qquad [2]$$

Substituting [2] into [1] yields,

$$\partial f_i(t,z)/\partial t = (\mu_i - \partial u/\partial z) f_i - u\partial f_i/\partial z \qquad [3]$$

where f_i = volume fraction of biofilm that is microbe i, μ_i = observed or net specific growth rate of microbe i, ρ_i = biofilm species density and u = velocity at which microbial mass for species i is displaced relative to the inert support. Summation of this equation from i = 1 → nx yields the expression for the mean observed specific growth rate of biofilm mass.

$$\mu^*(t,z) = \partial u(t,z)/\partial z \qquad [4]$$

Integrating [4] yields,

$$u(t,z) = \int_0^z \mu^*(t,z)dz \qquad [5]$$

The velocity of the biofilm thickness increase (or decrease) can be defined simply as,

$$U_L(t) = dL/dt \qquad [6]$$

The biofilm interfacial velocity U_L can also be expressed as

$$U_L(t) = \int_0^L \mu^*(t,z)dz + \sigma(t) \qquad [7]$$

where $\sigma(t)$ is the net rate (velocity) at which biomass is exchanged between the biofilm and the bulk liquid, $\sigma(t)$ thus can represent the rate of biofilm detachment, the rate of cellular deposition, or the net combination of both processes.

Similarly mass balances for i = 1 → ns substrates take the form,

$$\partial S_i(t,z)/\partial t = r_i(t,z) - \partial j_i(t,z)/\partial z \qquad [8]$$

where S_i is the concentration of substrate i, j_i = substrate flux, and r_i the conversion rate of substrate i. Using Fick's Law, this becomes,

$$\partial S_i(t,z)/\partial t = r_i + \partial z\{D_i \partial S_i(t,z)/\partial z\}\partial/\partial z \qquad [9]$$

Specific boundary conditions and solution techniques to the multispecies biofilm model can be found elsewhere (2). An updated version of this model is now available in a personal computer software package called BIOSYM (3).

This model was employed to simulate the two different bacterial ecosystems investigated experimentally below. One system consists of (1) a mixed culture of heterotrophs oxidizing reduced organic carbon (acetate) and (2) a mixed autotrophic culture (Nitrosomonas and Nitrobacter spp.) oxidizing reduced NH_4^+-N to NO_2^--N then NO_3^--N. The second ecosystem consists of two chemoheterotrophic species, Pseudomonas putida and Hyphomicrobium spp. that independently metabolize two separate electron donors while both competing for dissolved oxygen.

3. METHODS

3.1 Microbial Cultures

3.1.1 Experimental System I. One biofilm ecosystem consists of two enriched mixed cultures. One culture, the chemoheterotrophs, are grown, aerobically from a municipal wastewater treatment inoculum, in a separate inoculum reactor fed continuously a defined basal nutrient media with organic carbon (as acetate) the carbon energy limiting substrate. A second culture of autotrophic nitrifiers are grown aerobically in a separate inoculum system (a fluidized bed of biomass coated quartz sand) fed exclusively a bicarbonate buffered medium with NH_4^+-N the growth limiting substrate. The inlet NH_4^+-N concentration of 100 mg/ℓ was completely oxidized to NO_3^--N indicating the presence of both Nitrosomonas and Nitrobacter spp. Cell biomass from both inoculum reactors was used to inoculate surfaces of the biofilm study reactors with desired initial concentrations of each culture.

3.1.2 Experimental System II. The second ecosystem studied consisted of two pure culture bacteria. Pseudomonas putida ATCC 11172 was maintained on agar slants stored at 4°C with new slants reinoculated every six weeks. Every three months a new freeze dried culture of Pseudomonas putida was obtained from the

American Tissue Culture Collection. Cells were grown to late exponential stage prior to inoculating suspended culture chemostats. P. putida was grown at 28°C, pH = 7.0 in 2 liter (1.76 liter working volume) using sterile media (4) with glucose (3g/ℓ) as the carbon source. Cells from the steady state chemostat at a dilution rate of 0.20 h^{-1} served to inoculate surfaces of biofilm study reactors.

Hyphomicrobium ZV620, reported in Grazer-Lambert et al (5), was supplied courtesy of Professor G. Hamer, Institute of Aquatic Sciences, ETH-Zurich and maintained on slants as per P. putida. Hyphomicrobium spp. was grown at 28°C and pH = 7.0 in a separate 2-litre bioreactor on a basal medium described elsewhere (4). Methanol (1 g/ℓ) served as the carbon and energy limiting substrate. Cells from the steady state chemostat, at a dilution rate of o.09 h^{-1}, served to inoculate surfaces of the biofilm study reactor. These two heterotrophs were selected because (1) both are common residents in biofilm systems, (2) both compete for oxygen substrate, and (3) each species grows exclusively on its own individual carbon source. Repeated chemostat experiments confirmed P. putida did not metabolize methanol and Hyphomicrobium spp. did not utilize glucose.

3.2 Reactor Systems

Biofilm formation studies were carried out in the reactor system, illustrated in Fig. 1a and b, which consisted of the rectangular duct biofilm reactor situated within a liquid phase recycle loop. During surface inoculation (Fig. 2a), suspended cells of either pure culture or combinations of both cultures (heterotrophs:autotrophs in exp. series I or P. putida: Hyphomicrobium spp. in exp. series II) are recirculated through the biofilm reactor for six hours. During biofilm formation, the chemostats were removed, a mixing/aeration chamber inserted, and nutrient addition to the recycle loop initiated (Fig. 2b). In all experiments, recycle flow rate was much greater than the influent nutrient feed rate essentially insuring completely mixed liquid phase conditions.

The biofilm study reactor itself (Fig. 3) was a rectangular duct fabricated of optically clear plexiglass. One half of the reactor chamber was constructed with a recessed trough that accommodated three (2.5 cm (W) x 7.6 cm (L) x 0.1 cm (D) glass microscope slides. Once assembled, the dimensions of the actual

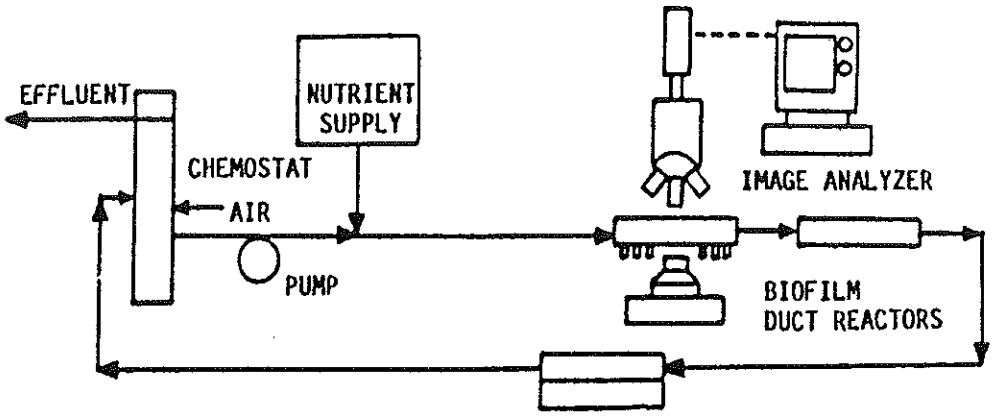

Fig. 2A. Biofilm reactor system during surface inoculation.

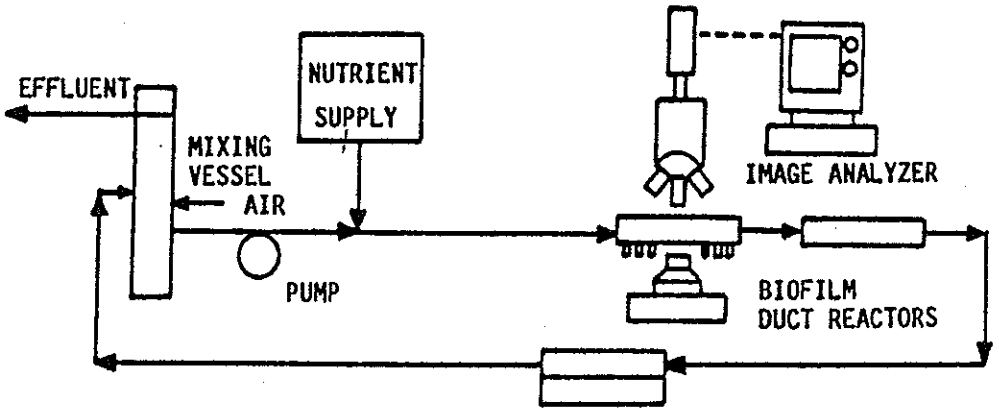

Fig. 2B. Biofilm reactor system during biofilm formation

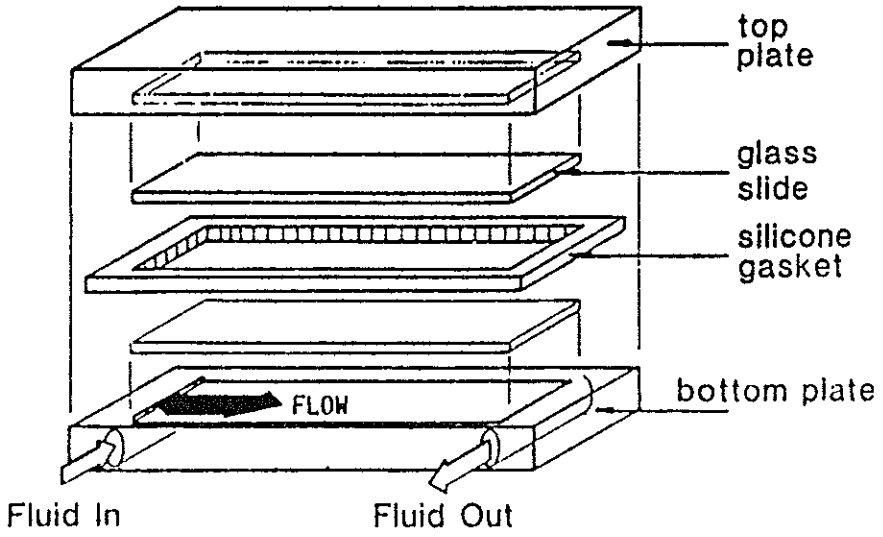

Fig. 3. Rectangular duct biofilm study reactor.

rectangular flow chamber were 2.5 cm (w) x 25.0 cm (L) x 0.5 cm (D).

3.3 Experimental Conditions

3.3.1 Experimental Series I. Two experiments investigating the population dynamics of a combined heterotrophic:autotrophic biofilm were carried out in the recycle system above. In both cases, the initial surface composition of heterotrophs, autotrophs, and exopolymer were the same at 0.63, 0.32, and 0.05 respectively. One experiment was carried out with an inlet feed composition of 3.0 g/m^3 acetate, 5.0 g/m^3 NH_4^+-N, and 8.5 g/m^3 O_2. The second experiment maintained an influent feed composition of only 1.0 g/m^3 acetate, 5 g/m^3 NH_4^+-N, and 8.5 g/m^3.

3.3.2. Experimental Series II.

Two different inoculation patterns were carried out in order to assess the ability of the analytical techniques to discern spatial location of different species. In one experiment, a pure culture biofilm of Hyphomicrobium spp. is cultivated to a biofilm thickness of ~ 70 μm. The biofilm is then exposed for six hours to a bulk fluid suspension containing both methanol and glucose substrate and P. putida cells at 1.5 x 10^8 cells/mℓ. After the six hour exposure, only glucose and methanol substrate are delivered to the system.

In the second series, both P. putida and Hyphomicrobium spp. are exposed to the biofilm reactor surfaces in equal mass concentrations for six hours. After inoculation, attached cells were continuously supplied an aerated basal medium containing both glucose (0.5 gm/ℓ) and methanol (0.5 gm/ℓ).

3.4 Analytical Methods

Glucose concentrations were determined enzymatically using a modified glucose oxidase assay (Kit No. 510) from Sigma Biochemicals. Methanol concentrations were determined using a Shimadzu GCA-9 gas chromatograph equipped with an 80/100 Carbopack C - 0.1% SP 1000 column and flume ionization detector. Acetate concentrations were also determined by gas chromatography. NH_4^+-N concentrations were determined using a gas sensitive electrode (Phillips IS 570-NH_3) after adding 5 mℓ of reagent (1M NaOH and 0.1 M Na_2EDTA) to 50 mℓ sample. Nitrate was determined by ion selective electrode (Phillips IS 561-NO_3; ref. electrode R 44/2-SD/1) after adding 5 mℓ reagent (0.5 M

H_2SO_4 and 1 M KH_2 $_4$) to 50 mℓ sample. Nitrate was determined as per Standard Methods (6). Total bacterial cell counts in the liquid phase or in resuspended biofilm samples (4) were determined by acridine orange staining followed by epifluorescence microscopy as per Hobbie et al (7). Suspended cell mass per volume or biofilm mass per area were determined gravimetrically after drying for 1 hour at 100°C the biomass deposited on a 0.2 μm pore diameter membrane. Biofilm thickness was determined using the micrometer focus adjustment of a Reichert-Jung microscope at 10x magnification; thickness was the distance measured between focusing on the biofilm upper layer and the biofilm-glass interface.

3.4.1. Radiolabelling Techniques. In experimental series I, heterotrophic bacteria were labelled with ^{14}C-acetate (0.5 μCi/mℓ) for one hour. Autotrophic nitrifier cells were labelled using $NaH^{14}CO_3$ (58 mCi/mmoℓ) for 2 hours. In experimental series II, P. putida bacteria were labelled using ^{14}C-glucose (0.5μCi/mmol for 1 hour) while Hyphomicrobium spp. cells were labelled using ^{14}C-methanol (0.5 μCi/mℓ for 1 hour).

All calibrations between radiolabeled cell counts (DPM/mℓ) and either cell numbers (epifluorescent cell count/mℓ) or cell biomass (dry weight/ℓ) were carried out using suspended cell samples from the chemostats. Overall concentration of a particular species within the biofilm was determined by removing the entire depth of biofilm from a 2 cm^2 section of glass slide, resuspended the biofilm mass in 5 mℓ of phosphate buffer, then radiolabelling as above. After incubation, the cell suspension is filtered through a 0.2 μm membrane, and the biomass-membrane combination placed in fluor cocktail in a 20 mℓ glass scintillation vial. Disintegrations per minute were determined using a Packard Tri-Carb 1900 CA liquid scintillation counter.

3.4.2. Biofilm Species Spatial Profiles. Biofilm species distributions were determined with depth, in either experimental series, by removing a section of biofilm-covered glass slide then radiolabelling the cells within the intact biofilm. In series I, biofilm samples were submerged in a phosphate buffer containing both $NaH^{14}CO_3$ and ^{3}H-acetate (50 Ci/mmol) for two hours. In series II, biofilm samples were submerged in phosphate buffer containing both ^{14}C-methanol and ^{3}H-glucose (50 Ci/mmol) for one hour. After these incubations, biofilm covered slides are rinsed

in phosphate buffer then fixed with a cacodylate buffered (0.1 M, pH=7.2) glutaraldehyde solution (2.5% wt), air dried for only 15 minutes then embedded in Spurr resin. The entire resin embedded biofilm slide combination is placed into a modified base assembly of a microtome which provided biofilm sections appx. 20 μm thick. Resultant biofilm sections were placed in fluor cocktail in 20 mℓ glass liquid scintillation vials and both labels simultaneously counted.

4. RESULTS

4.1 Model Predictions

Model simulations for the two ecosystems studied were carried out using the kinetic and stoichiometric parameters given in Table 1; biofilm physical parameters and simulated liquid phase initial conditions are provided with the appropriate results below.

TABLE 1

Parameters employed in model simultations of studied ecosystems.

Parameters	Exp. Series I		Exp. Series II	
	Heterot: Nitrifiers		P.putida:	Hyphomicr.
$\mu^*(h^{-1})$	0.2	0.039	0.54	0.24
K_s(mgS/ℓ)	5 (acetate)	1(NH_4^+-N)	10.0	0.01
K_{O2}(mgO_2/ℓ)	0.1	0.1	1.0	0.10
$Yx/_s$(mgX/mgS)	0.45	0.1	0.42	0.30
$k_e(h^{-1})$	0.008	0.004	0.005	0.002
$D_{eff}(cm^2/sec)$	acetate: $9.6x10^{-6}$		methanol:	$15.0x10^{-6}$
	ammonia: $17.2x10^{-6}$		glucose:	$5.0x10^{-6}$
	oxygen: $20.2x10^{-6}$		oxygen:	$20.2x10^{-6}$

Figure 4 exemplifies one type of predictions available from the model; the relative bacterial species composition with depth and time expressed as a fraction of the biofilm density. Similar profiles are available for the other three cases considered (HET:NIT - different acetate concn.; P. putida: Hyphom.-different initial surface conditions). Due to page constraints and for comparison to experimental results, it is more expedient to convert relative compositions to actual bacterial concentrations (mgC/cm^2) within the biofilm depth. Experimental results were converted from cell numbers to equivalent mass-carbon concentrations per area.

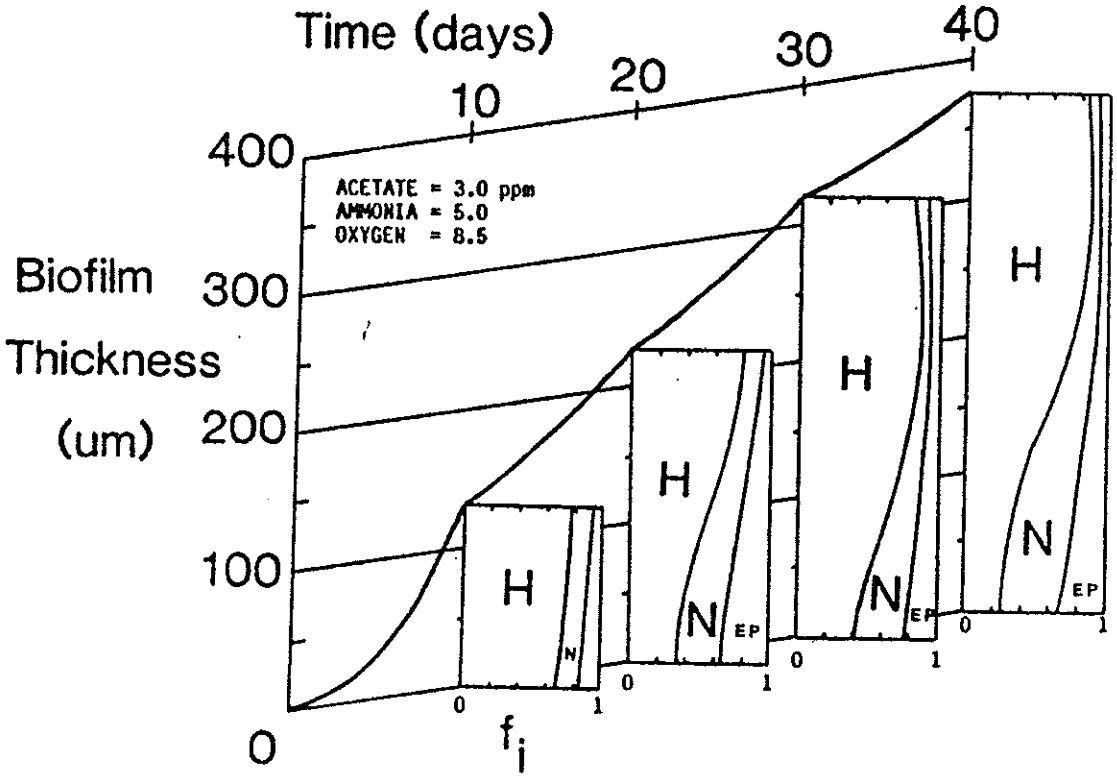

Fig. 4. Model predictions of a heterotrophic: nitrifier biofilm species profile. f_i = fraction of biofilm density that is particular species concn. Feed conditions: Acetate = 3.0, Ammonia = 5.0, Oxygen = 8.5 ppm. EP = extracellular polymer fraction.

4.2 Experimental Series I.

Figs. 5A and 5B illustrates both model predictions and experimental data for heterotrophic: autotrophic biofilms initiated at identical surface conditions but cultivated at different inlet liquid phase acetate concentrations. At the lower acetate concentration (Fig. 5A), biofilm formation and

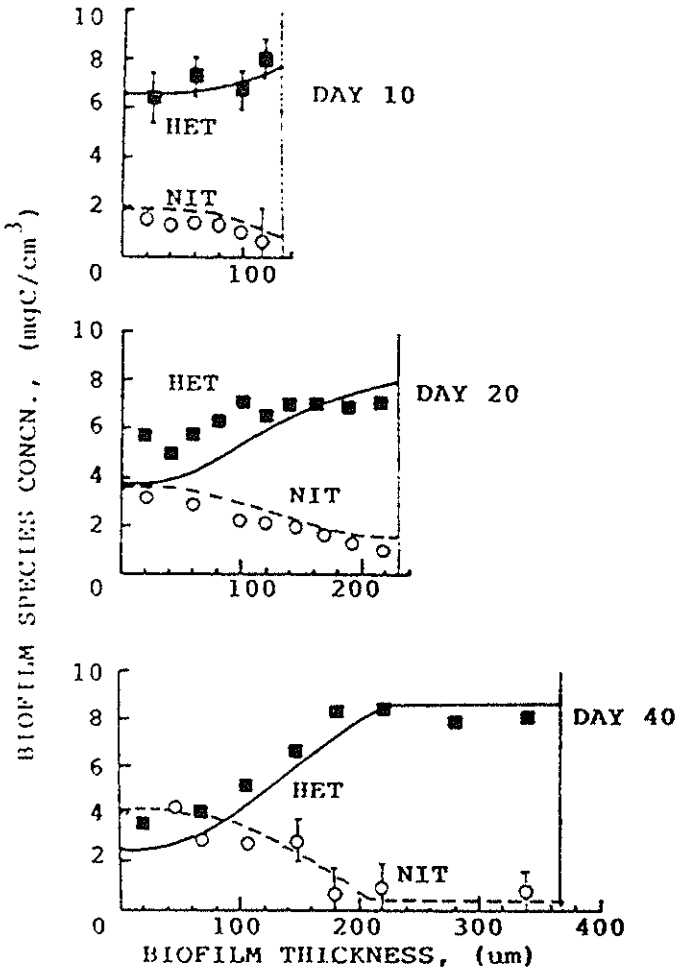

Fig. 5A. Predicted vs experimental heterotroph:nitrifier biofilm species distributions at an inlet NH_4-N = 5.0 ppm, O_2 = 8.5 ppm, and acetate = 3.0 ppm.

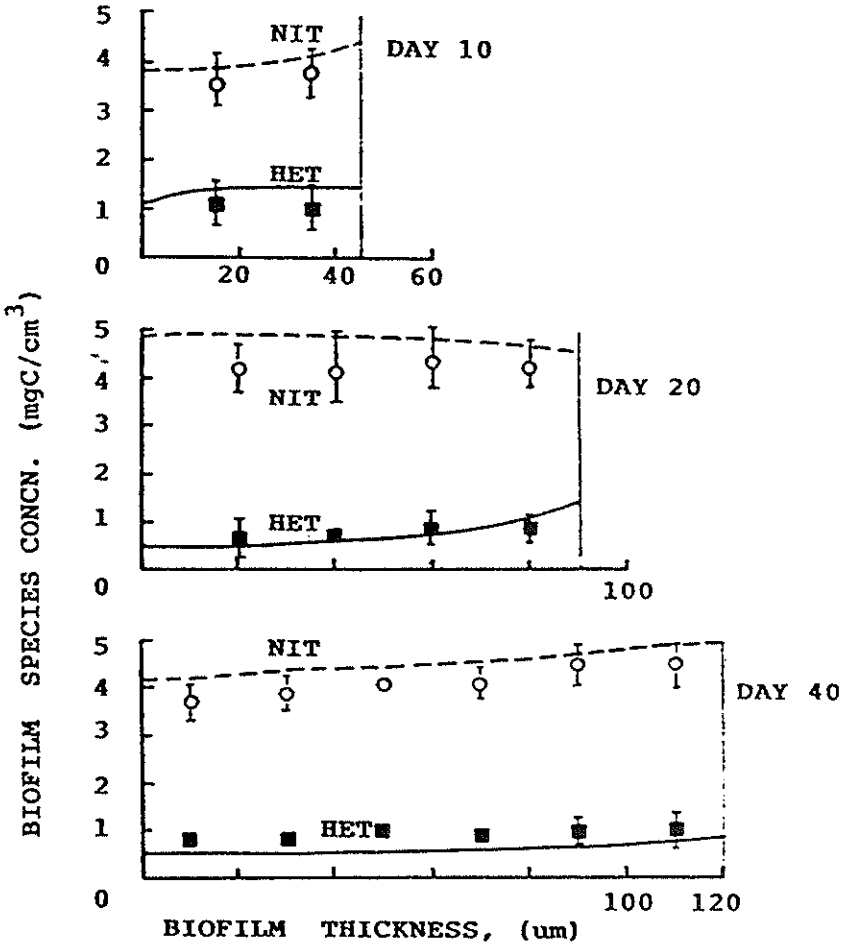

Fig. 5B. Predicted vs experimental heterotroph: nitrifier biofilm species distributions at an inlet NH_4-N = 5.0 ppm, O_2 = 8.5 ppm, and acetate = 1.0 ppm.

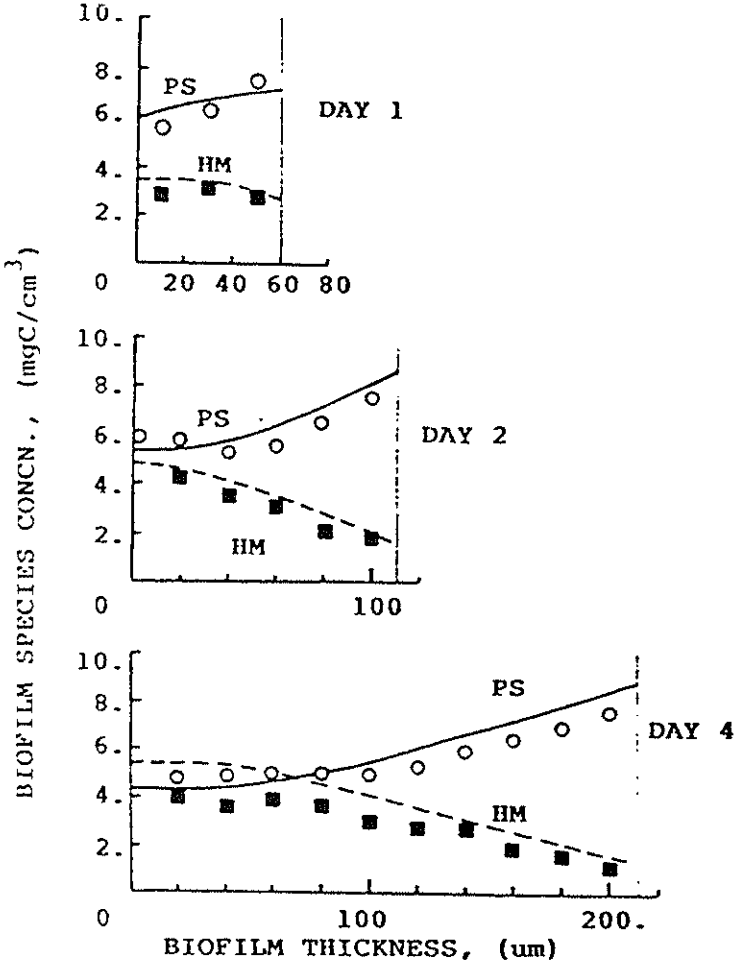

Fig. 6A. Predicted vs experimental biofilm distributions of P. putida & Hyphomicrobium. Intentional bi-layer biofilm construction.

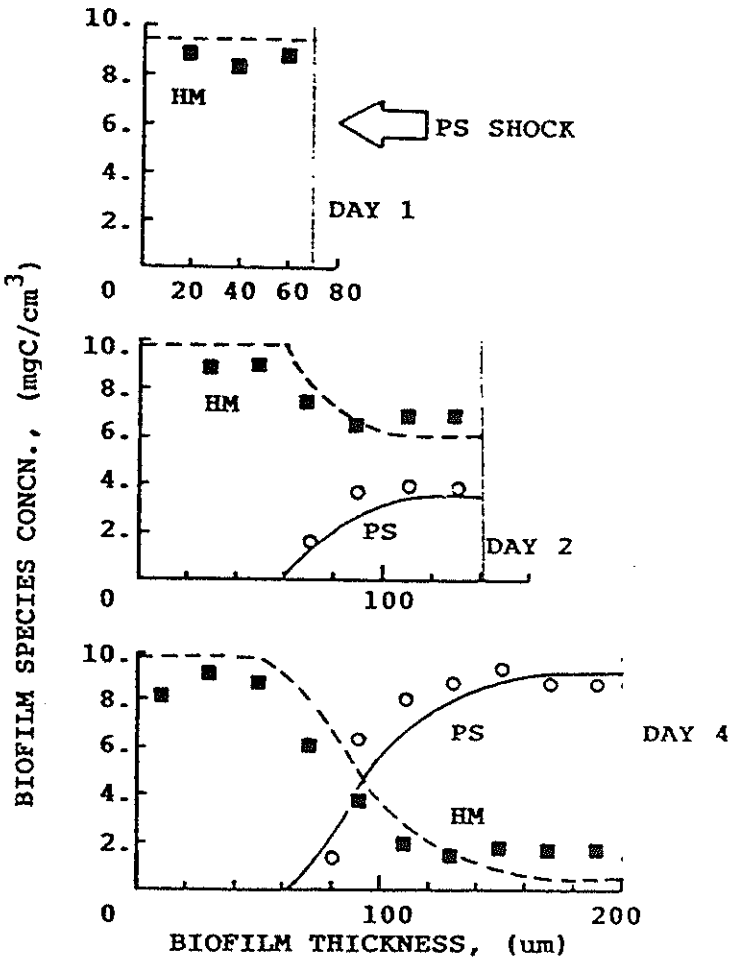

Fig. 6B. Predicted vs experimental biofilm distributions of P. putida & Hyphomicrobium. Binary culture initial surface inoculation.

spatial composition is dictated by the autotrophs, producing at day 40 a biofilm thickness of 110 μm and density of 6.0 mgDW/cm^3. Conversely, at an acetate concentration of 3.0 mg/ℓ (Fig. 5B), heterotrophs dominate the biofilm and dictate the rate of biofilm thickness increase which at day 40 yields a thickness of 370 μm and a density = 9.5 mgDW/cm^3.

4.3 Experimental Series II.

Figs. 6A and 6B illustrates both model predictions and experimental data for P. putida: Hyphomicrobium biofilms cultivated under identical liquid phase conditions but initiated with a different surface composition. Fig. 6A illustrates the artificial construction of a bi-layer biofilm, starting with a pure culture Hyphomicrobium biofilm then exposing it for 6 hours to a suspension of P. putida. Due to its growth kinetics, P. putida completely "overgrows" the Hyphomicrobium sublayer. The analytical technique used is able to discern the evolution of these species profiles over a biofilm of just over 200 μm. Figure 6B illustrates a binary biofilm cultivated from an initial surface inoculum affected by exposure to a cell suspension of 50:50 P. putida: Hyphomicrobium. By day 3, the biofilm reaches 210 μm and a biofilm density of 10.5 mgDW/cm^3; similar to values observed for the experiment in Fig. 6A. However, dual-labelling results indicate that species profiles in each case were dramatically different. Analytical "sectioning" coupled with specific radiolabelling of cell mass provides estimates of species distributions that agree well to predictions of existing mathematical models.

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