Limits To Growth And What Keeps A Biofilm Finite

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ABSTRACT

Two of the factors, shear erosion and diffusive mass transfer, which limit the growth of heterogeneous biofilms are considered. For permeable beds of particulates, with a regulated throughflow, equating shear induced erosion and biofilm growth, leads to estimates of biofilm thickness and activity which conform with experimental measurements. In the more open environments of pipes and channels, increased thickness of biofilm is not directly balanced by increased cell erosion from the biofilm surface. However increasing thickness leads to growth limitations as diffusion limits the rate of mass transfer to cells deep in the film. For heterogeneous biofilms , consisting of complex clusters intersected by channels, mass transfer into the biofilm is by a combination of advective flow in the channels and diffusive transfer in clusters. In this paper we have considered mass transfer into simplified cluster forms, that is cylinders and hemispheres. Using the concept of critical dimension we have explored some of the implications of these simplified structures. We discuss the limitation to this approach as fluid shear alters the form of these simplified clusters. The viscoelastic properties of the biofilm clusters are being investigated and should allow better prediction of the effect of lateral shear on simple forms. The advection in biofilm channels and the related mass transfer processes needs further investigation.

KEYWORDS
Biofilms, heterogeneity, clusters, channels, diffusion, advection, limits.

1. LIMITS TO GROWTH

Micro-organisms appear to be present wherever we look, but the physical dimensions of these colonies of microorganisms are always limited. When we find them in biofilms or in floc, we are accustomed to consider that a thick biofilm may extend to one centimetre or so and be fluffy or filamentous. A thin biofilm is anything from a monolayer of a micron or so up to 30 or 40 microns; many authors describe films of 50-500 microns thick. When respirometric activity of river biofilms is determined,

Oxygen Uptake Rate(OUR) seems to reach a limit of between 10-16 g m^{-2} day^{-1} based on plane surfaces with higher rates associated with the extended surfaces present in permeable sand and gravel beds (Boyle 1984). Flowcell studies show biofilm reaching a limiting thickness. Observations of trickling filters and other extended surface systems indicate that growth in biofilm systems is normally limited. For homogeneous films, we can calculate the thickness of the aerobic layer if we know the OUR and the oxygen diffusion coefficient. For heterogeneous films, where channels around clusters contribute to film porosity, the clusters are microporous and the morphology of the film seems random, it is not so clear how the mean film thickness will be controlled.

Grazing, predation and scour contribute to controlling natural biofilm communities. Cleaning processes, using combinations of detergents, acids, alkalis, biocides and scour are used to control biofilm growth in food and drink processing equipment. Much medical effort is employed to prevent or remove biofilm infections on implants often requiring the use of much stronger antibiotic doses than would be necessary if the infection were not associated with attached micro-organisms.

In this paper, we will consider how fluid shear or availability of nutrient or oxygen would limit growth of a model heterogeneous aerobic biofilm in two idealised laboratory conditions, a permeable bed and a pipe. We can make some progress with our analysis of the effect of fluid shear and nutrient and oxygen availability on complex biofilm development if we can represent the form of a biofilm in a simplified but realistic way. A better understanding of the factors limiting growth may allow us to develop improved methods for controlling biofilms.

2. PROPERTY VARIATIONS IN HETEROGENEOUS BIOFILMS

Although it has been recognised that biofilms seldom, if ever, grow naturally as flat homogeneous films of uniform thickness, there are only a limited number of published studies quantifying the internal variation of physical or biological properties in heterogeneous films. At a previous Gregynog meeting, BBC1, Keevil (1993) presented a video showing stacks and water channels in a film grown under the conditions simulating those in water. The Bozeman group have described and measured flow, oxygen gradients, and effective diffusion coeffecients in the channels which form within biofilms (deBeer and Stoodley 1996). Zhang and Bishop (1994a,b), using a micro-slicer, provided data on the spatial properties of mixed species biofilms fed on a synthetic wastewater (COD 350mg/l) which ranged in thickness from 180-2530 microns. Their data suggest that for biofilms up to a thickness of 500 microns, that the mean porosity increases linearly from about 20% at the support surface to zero at the outer edge of the film. Their data also suggest that in the microbial masses, the surface area for absorption is uniform and directly related to the number of viable cells.

3. BIOFILM STRUCTURES AND INTERNAL MASS TRANSFER PROCESSES

3.1 Water related diffusion and advection.

If biofilms were sold in supermarkets, and their ingredients had to be written on the side of a packet, at the top of the list would be the pricincipal component, water. This could be subdivided into the free water in the channels and the water of hydration which forms the gel round the extracellular polymeric material and the water enclosed

within the cell membrane. While it is clear that the water of hydration is probably not irreversibly attached to the polymer, and in most electron micrographs of biofilm it has been removed, the "free water" in the pores is mobile but the channels through which it moves may be obstructed by hydrated polymer strands. Measurements of diffusion coefficients provide evidence that diffusion in these channels is not significantly different from that in open water. Similar measurements in the microbial colonies/clusters, Bryers and Drummond(1998), show that diffusion coefficients of a range of molecules in *Pseudomonas putida* biofilm ranged from 15 to 90% of the value in pure water. They also include a figure showing spatial differences in the diffusion coefficient of dextrin in a cross section of their 200 micron thick biofilm. This clearly indicates the presence of channels which are about 50 microns wide close to the support surface and more than 100 microns wide at the upper interface around a microbial gel mass which appears to be 500 microns long and about 200 microns deep with a tunnel through it about 2/3 of the way to the top. The voids (porosity) of a heterogeneous biofilm include the voids in the channels as well as the voids in the micropores running through the clusters.

Definitions of the water content of a biofilm may include the volume of water contained in the channels and micropores as well as the water of hydration and the intercellular water. Typical overall figures are between 80 and 95% of biofilm space occupied by water. Stewart (1998) reviewed measurement of diffusion coefficients and diffusive permeabilities in biofilms varying from dental plaque, monoculture pseudomonads to mixed microbial films from biofilters. The data he collected showed that the ratio between diffusion coefficient measured in biofilm is most likely to lie between 10 and 90% of that in pure water for a wide range of solutes. Stewart sketches a conceptual model of a biofilm in which scattered "cells are surrounded by reduced permeability envelopes and embedded in an EPS matrix."

3.2 The Extra Cellular Polymeric Substances (EPS) Matrix

The second main component of total biofilm space is the EPS. Christensen and Characklis (1990) discuss the characteristics of EPS and suggest that the physical properties of biofilms are related to those of the main components. They report that the EPS matrix can account for as much as 50-90% of the biofilm organic carbon. They do not discuss how the EPS matrix relates to the enclosed cells. Brading (1996) measured the mass of carbohydrate per 10^6 bacterial cells in a P. *fluorescens* biofilm developing in laminar flow in a modified Robbins Device (Table 1). Her data show that the mass of carbohydrate per unit cell declines as a film develops and is dependent on the flow conditions. Interpreting these data by assuming that all cells have a diameter of 1 micron and that the densities of the polymer, cell and water are the same, leads to the following estimates of EPS volume per cell and the corresponding equivalent diameter of a glycocalyx before hydration.

Re	Time hrs	μg/10^6 cells	EPS vol/cell vol	Glycocalyx diameter μm
51	2	12	24	2.9
2	2	6	12	2.35
51	5	4	8	2.1
2.5	12+	2	4	1.7

Table 1.

Transmitted electron micrographs (TEM) of stained biofilm (Geesey et al. 1977, Costerton and Cheng. 1981) show cells separated by two or three cell diameters. For some of the cells, the capsule or glycocalyx extends out two or three cell diameters and the images show fibres, apparently attached to the cell wall, radiating thickly out to the edge of the glycocalyx. Bradings data are consistent with these images and the question arises about how the individual cells in their glycocalyx envelope are attached to other envelopes. Do the individual polymer strands from one envelope penetrate others or are the envelopes attached in some other way?

3.3 Mechanical and Hydraulic Properties of Biofilm

The mechanical properties of biofilm are largely dependent on the structure and properties of the EPS matrix. Stoodley et al. (1999a) has shown that biofilm in-situ behaves viscoelastically, it may stretch elastically when subject to shear stress, but if the shear stress exceeds a particular value, comparable to the elastic limit, the biofilm flows and when the stress is relieved, the unstressed material is found to be permanently stretched.

We have considered various structures for the EPS fibres including a layered structure as suggested by various authors and an extended 3D matrix with cells distributed within the lattice. There seems little evidence for the layered structure, and biofilms seem to exist without a surrounding skin (membrane). Extended matrices imply very long fibres, with frequent cross links; conceptually, it is difficult to reconcile this with production of the EPS fibres by individual cells and the ability of daughter cells to emerge and move through the matrix. Our preferred model based on the TEM images and the measurements of Zhang and Bishop (1994b) is an assemblage of individual cells surrounded by their hydrated glycocalyx sticking together where their glycocalyces touch. The analogy is of a pile of balls of a springy, sticky fibre, the "sticky balls" model. This system would be microporous, having interstitial spaces with dimensions of about one tenth of the ball diameter ie about 0.3 microns; daughter cells could develop by pushing out adjacent cells.

The mechanical behaviour of a "sticky balls" cluster would differ from that of recrystallised EPS. It might be different for different cell communities if those communities produced EPS with different properties, but the overall mechanical properties would depend on the bonding between the glycocalyx envelopes. Shear induced erosion of individual cells is easily understood, but such cells would leave the cluster with an intact glycocalyx and would be expected to adhere to other clusters more easily than a naked cell. Fracture and loss of chunks of biofilm can be predicted, however,; we cannot yet explain our recent observations of ripple formation in

biofilms (Stoodley et al. 1999b) as there is limited data on the dynamic behaviour of groups of sticky particles. However, there is a great deal of published work on the mechanical behaviour of assemblages of non-sticky particles in flow conditions and this non-sticky particle behaviour is one of the asymptotes for the behaviour of sticky balls.

4. LIMITS TO GROWTH IN PERMEABLE BEDS.

Microbial growth on the particulate surfaces in sand and gravel beds is significant in the self purification of some rivers where it can enhance BOD decomposition rates by an order of magnitude. It can play a part in the filtration of water in water treatment plants by reducing dissolved organic carbon(DOC) and in tertiary treatment of wastewater by reducing BOD or nitrate concentration. Bacterial growth in sand beds has been modelled both experimentally and mathematically by authors including Taylor and Jaffe (1990). However they did not consider how flowrate affected biofilm activity and they used measured values of biofilm thickness. Dodds(1999), extended their model by equating the rate of erosion of cells from the biofilm surface to the growth rate at a point in the bed. Using an expression for erosion from Rittmann (1982), he obtained estimates for the variation of thickness of the film in a particulate bed, (Fig. 1), variation of oxygen uptake for a given fixed flowrate and nutrient concentration, (Fig. 2), variation of uptake rate as particle size is varied and the minimum nutrient concentration at which a stable biofilm could establish in a given bed as a function of flowrate (Fig. 3). All this information can be predicted for the case where with constant volumetric flowrate through a bed, an increase in biofilm thickness leads to increased local velocities within the bed and consequential increased rate of shear induced erosion. For other situations, where there is a constant pressure drop across a permeable bed, increased biofilm growth may lead to reduced interstitial velocities and eventual complete plugging of the bed. In this case velocity reductions, reduce cell erosion below the rate at which cells are generated.

5. LIMITS TO GROWTH IN PIPES AND CHANNELS.

In pipes and channels where the spaces are much larger than the thickness of a biofilm, an increase in biofilm thickness does not normally lead to increased surface shear. There is therefore no consequential increase in the erosion of individual cells from the biofilm outer surface to compensate for the cell growth from the thicker film, unlike the situation in permeable beds with constant volumetric flow. With cell numbers increasing as the film thickens without a compensating increase in erosion, the film would thicken at an exponentially increasing rate until the growth rate of the cells deeper in the film declines However we know that as a film thickens, the transport of nutrients and for aerobic films also oxygen becomes limiting at least for aerobic cells. Atkinson et al. (1970) developed an expression for concentration profiles in homogeneous uniformly thick biofilms and Harremoes(1977) further developed the kinetics of substrate removal in a flat, homogeneous biofilm. They both appreciated that in flat biofilms the calculated depth of an aerobic layer is dependent on the concentration of oxygen at the biofilm outer surface, the diffusion coefficient of oxygen through the film and the rate of removal of oxygen per unit volume of film which is dependent on the nature of the kinetic equation.

At previous BBC meetings, Wimpenny in Cardiff and Picioreanu at Gregynog in 1997 discussed the modelling of biofilms in still fluid using differential-discrete cellular automaton approaches. Van Loosdrecht et al (1995) discussed the factors which control biofilm accumulation and structure, arguing that high loading and low shear there would be thin, smooth and dense biofilms and high loading and low shear, biofilms would develop low density, extended surfaces. The complexity of heterogeneous films has meant that modelling of activity, prediction of density, biomass per unit surface area and morphology have presented problems akin to determining the size shape and orientation of every leaf on a beech tree. We have explored a simplifying approach from which we believe that we can obtain a better understanding of the factors which limit heterogeneous biofilms.

6. SIMPLIFYING BIOFILM MORPHOLOGIES.

In order to make progress with understanding the limits to the growth in heterogeneous biofilms in flowing systems we propose a simplified model for a heterogeneous biofilm, which accounts for some of the observed phenomena. The base for the model are that heterogeneous biofilms comprise clusters separated by channels, which provide a path for the superfluent water through the biofilm. We are making three simpliofying assumptions. The first is that the advective flow is sufficiently fast to ensure that nutrient and oxygen concentrations in the water in the channels are everywhere at the same value as the bulk flow. For biofilm systems with porosity greater than about 40-50% in turbulent flow and channel widths of similar thickness to the thickness of the clusters, this assumption can be easily justified. As biofilms thicken or channels narrow and deepen, then the rate of removal of nutrient or oxygen at the biofilm surfaces becomes significant compared with the advective flux of the same component entering the channels and the concentrations decline through each channel.

The second assumption is that the clusters are all uniform in size and shape and are uniformly distributed across the support surface. The third assumption is that oxygen uptake by each cell in the clusters is independent of the position of the cell in the cluster and is not affected by changes in oxygen or nutrient concentration. Most authors assume that cell activity in biofilms is determined by Monod type kinetics, and than they make assumptions about mean properties in heterogeneous films.

For our model system , we wish to explore the effect of cluster shape and in common with Harremoes (1978) and Rauch et al (1999), we use zeroth order kinetics initially in order to simplify the mathematics, accepting that it will be necessary to investigate whether conclusions drawn using our simplified model also apply if a more realistic kinetic model is used.

We have considered three forms in which biofilm structures might grow, as

1. homogeneous flat films,
2. hemispheres
3. cylinders.

For each of these we have used diffusion with biochemical reaction equations to predict the aerobic thickness or radius for films with a constant oxygen diffusion coefficient D, 0.000021cm^2 s^{-1}, in which all bacteria have a constant demand for oxygen per unit volume of biomass at a rate G, 0.000617 mg O_2 cm^{-3} s^{-}.

Initially we assume that the concentration of oxygen at the outer biofilm surface is C_0. For flat films the aerobic thickness is $\sqrt{(2DC_0/G)}$. For spherical clusters, the critical radius R_a at which oxygen is just exhausted in a sphere of radius R_0 is given by the solution of the equation :-

$$DC_0/G + [R_a^2/6 - R_0^2/2 + R_0^3/3R_a + (R_0^3 - R_a^3)(1/R_0 - 1/R_a)/3] = 0$$

and for cylinders

$$DC_0/G - [(R_0^2 - R_a^2)/4 - R_a^2 \ln(R_a/R_o)/2 = 0$$

The Oxygen Uptake Rates per unit area of biofilm surface can be calculated from these equations.

Shapes	Aerobic limits	Surface uptake	Biofilm surface	Biofilm oxygen uptake
	μm	/ unit area of biofilm	/unit area of support	/ unit area support
Flat plate	257 (thickness)	0.158	1	0.158
Cylinders	368 (radius only)	0.114	limited by stability	limited by stability
Spheres	452 (radius)	0.093	3.63	0.337

Table 2. Limiting conditions for aerobic films. (The radii given for cylinders and spheres are the radii for which the oxygen concentration is just zero at the centre.)

The values included in table 2 were calculated assuming that the diffusion coefficient of oxygen in biofilm mass is the same as that in water. Stewart's (1998) data suggest that it is usually lower and Bryers and Drummond (1998) found that in the biofilm masses, the diffusion coefficient was about 0.26 of that in water. Fig.4 shows the critical radius for cylinders and spheres as D is varied. Because the expression DC_0/G reappears in each expression, a reduction in external dissolved oxygen concentration from 10 mg/l has the same effect as a reduction in D from 0.000021 to 0.0000105, the critical radii being reduced as Fig. .5 illustrates. A reduction in oxygen uptake rate per unit volume of biofilm, would tend to increase the critical radii. Less active bacteria can grow thicker films.

Most authors, Atkinson(1970), Harremoes(1977) assume that bacterial respiration can be described using a Monod equation. In this work, we have simplified the model and used zero order kinetics for oxygen uptake. This greatly simplifies the mathematics but we recognise that the critical radii that we calculate are under-estimates of those we should find using Monod kinetics. However, we are interested in relative dimensions and will assume that the general principles relating critical dimensions to shape, external concentration, diffusion coefficient and bacterial uptake rates can be inferred from simplified kinetic expression and applied to the more complex Monod kinetic systems.

7.1 Surface Coverage

The actual surface area of biofilm hemispheres growing on a flat surface depends on how the hemispheres cover the surface. If the hemispheres are closely packed, then their biofilm surface area per unit support surface is $2\pi/\sqrt{3}$, which is 3.63. The support surface coverage of these hemispheres which each touch six others, would be 90.7%; the limiting porosity, or voidage close to the support is 9.3%.
Cylinders standing on end may extend vertically to infinity if there are no limiting mechanical factors. From table 2, we see that a biofilm consisting of hemispheres would have roughly twice the number of active aerobic cells compared to a flat film. This assumes that the concentration of oxygen in the fluid surrounding the hemispheres is uniform and equal to that in the bulk fluid. This will probably be the case for the film in turbulent flow. Thus we might wonder whether because a film formed of equal diameter hemispheres, a sort of biofilm bubblepack, can sustain twice the number of active cells as the flat film.

7.2 Transitions between forms

If we further consider that vertical cylinders, whose diameters are less than the hemispheres, could sustain much higher biofilm numbers per surface area of support than hemispheres providing they were stiff and that the concentration of oxygen over all their surface was not influenced by their height. A hemispherical cluster might grow by stretching upwards. If it did, the centre of the developing structure would become anaerobic unless the cylinder contracted. The upper section could have a spherical cap, with a larger diameter than the lower section, a sort of mushroom shape. If the base contracted then the porosity close to the support surface would increase. If cylinders of diameter 368 microns replaced hemispheres of 452 microns, the surface coverage would be reduced to 60 %. If the cylinders are considered to be of height h with a hemispherical cap the same diameter as the cylinder, then the cylinders would need to be to have a cylindrical height of 103 microns, which with the cap would give a total height of 471 microns. Because the surface flux for the cylindrical section is 22% greater than for spheres, this body could sustain a higher bacterial population than the hemisphere from which it grew.

Increasing height of cylinders will lead to a reduction of oxygen concentration towards the roots of the cylinders. The consequence of this would be reduction in the critical diameter towards the base of the cylinder. We have now identified two factors which would lead to the growth of mushroom shaped clusters. However, stalked clusters with bulbous heads made of jelly like material are not well adapted to withstand transverse fluid shear. Even without thickening at the top, any flow over a group of vertical cylinders which have little structural strength will bend them in the direction of flow. At the 1997 BBC3 Gregynog meeting, The authors (Boyle et al 1997) explained that transverse shear across a flexible heterogeneous biofilm, reduced the porosity of the biofilm. While a reduction in porosity implies a reduction in the cross sectional area of channels through which advective transfers can occur, an increase in fluid shear implies an increase in pressure gradient along the surface of the biofilm. Increased pressure gradient would increase internal advection, decreased porosity would decrease it.

Another possible transition in biofilm development can be considered. If a film grows uniformly thick and homogeneous on a flat surface, the critical thickness using

our parameter values is 247 microns. If this film could be transformed into cylindrical stacks, then the number of sustainable cells would increase. If we consider the steps necessary for the development of one cylinder, we start to see that the metamorphosis requires a thinning of the flat film in a circle round the point at which the cylinder starts to develop. At the point where the cylinder leaves the base film it must be thinner than the critical radius; the actual radius would depend on the thickness of the base film. The radius of this aerobic vertical cylinder would increase the further it grew away from the base until it reached the critical value we can calculate for an infinite cylinder. If the base film contracted so that the cylinder rose directly from the suppport surface, then the cylinder should grow out to the critical radius at its base.

This is another of the limitations of the simplification process . While we are beginning to build up information on the mechanical properties of some biofilms which relate how biofilm materials respond to shear stress, this has still to be applied to predict how a simple cylindrical biofilm structure responds to transverse shear either on its own or when it is closely surrounded by other similar bodies. There has been some work published on mass transfer and gas concentrations in standing crops, Jenkins(1982) investigated mass transfer and hydraulic resistance in bryophytes from several Devon rivers and she showed that mass transfer and pressure drop depended on the stiffness of a model of the bryophytes constructed from vertical cylinders. Our ideal gelatinous cylinders will respond to transverse shear by bending. adjacent cylinders may touch and stick together, subsequently the forms must change as parts of the modified film are now further from the fluid surface and will become anoxic. If the shear is removed, the cylinders may separate and return to the vertical, or depending on the viscoelastic properties, they may have been stretched and now conform with the new configuration. Using the information which we have for the visco-elastic properties of living biofilms, we should be able to make estimates of the effect of flow on individual and groups of biofilm clusters of simple form.

In this paper, we have analysed the limitations on biofilm growth which are inherent in diffusion or shear limited systems. For the packed bed system , we have shown how balancing erosion by shear against growth leads to a model capable of describing how the oxygen uptake rate varies with flowrate, concentration and particle diameter. For homogeneous biofilms on surfaces in open channels or large diameter pipes, the theoretical maximum active population of cells per unit surface area of surface is dependent on the biofilm morphology. We can predict this for some simple morphologies . However we are at present unable to extend this analysis to cover real systems subject to transverse shear firstly because of the difficulty involved in prediction of the flow over and through dense assemblages of vertical cylinders, altough it is a conceptually simple system and secondly, how the shape of gelatinous vertical cylinders would respond to the transverse shear. Our improved awareness of the viscoelastic properties of biofilm material should allow us to predict the behaviour of single vertical cylinders; we would hope that this might lead us to a better understanding of how heterogeneous biofilms develop and respond to changing conditions.

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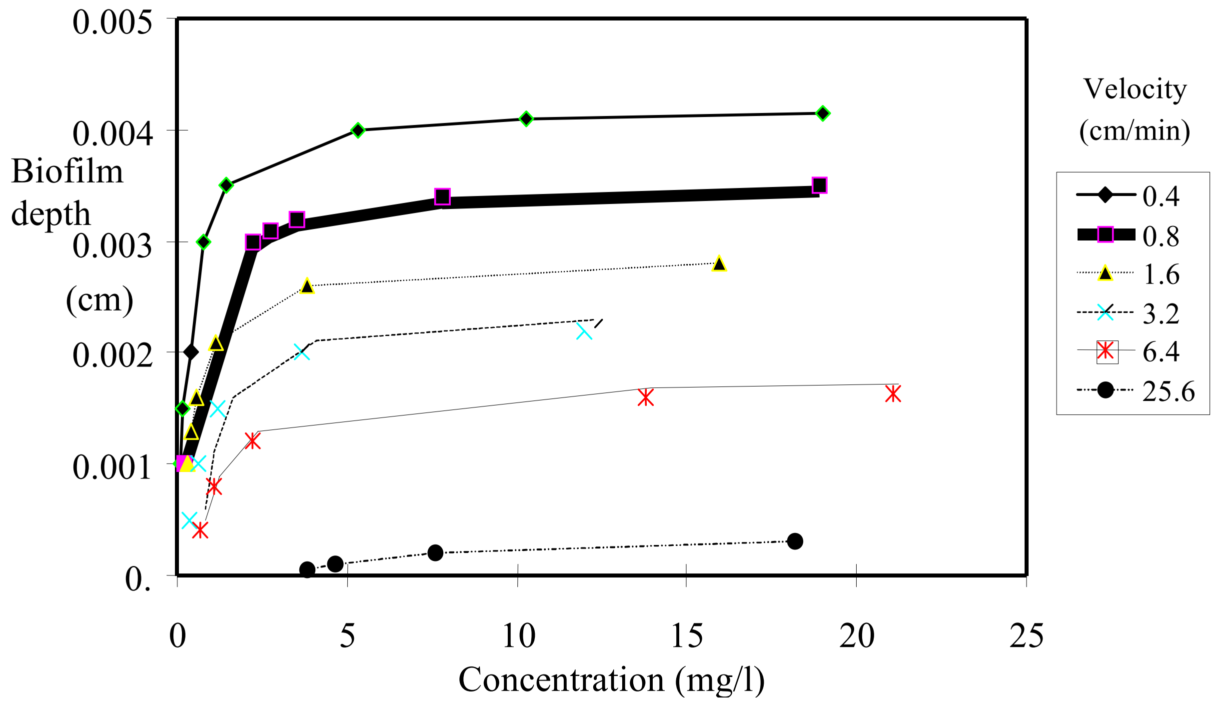


Fig. 1. Relationship between steady state biofilm thickness and flow velocity for a range of nutrient concentrations.

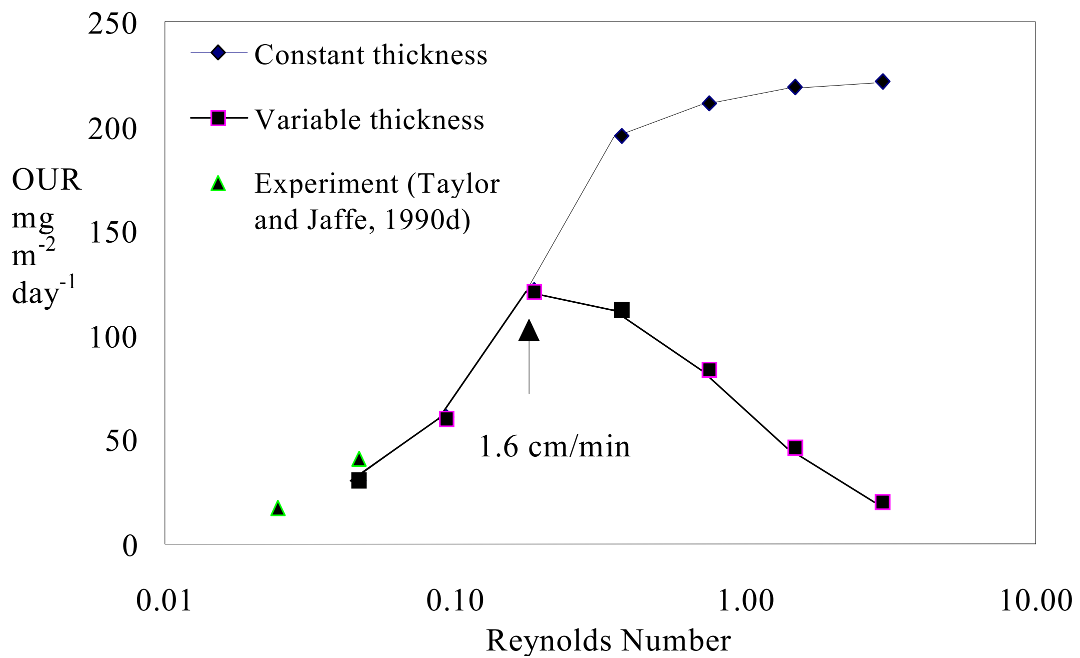
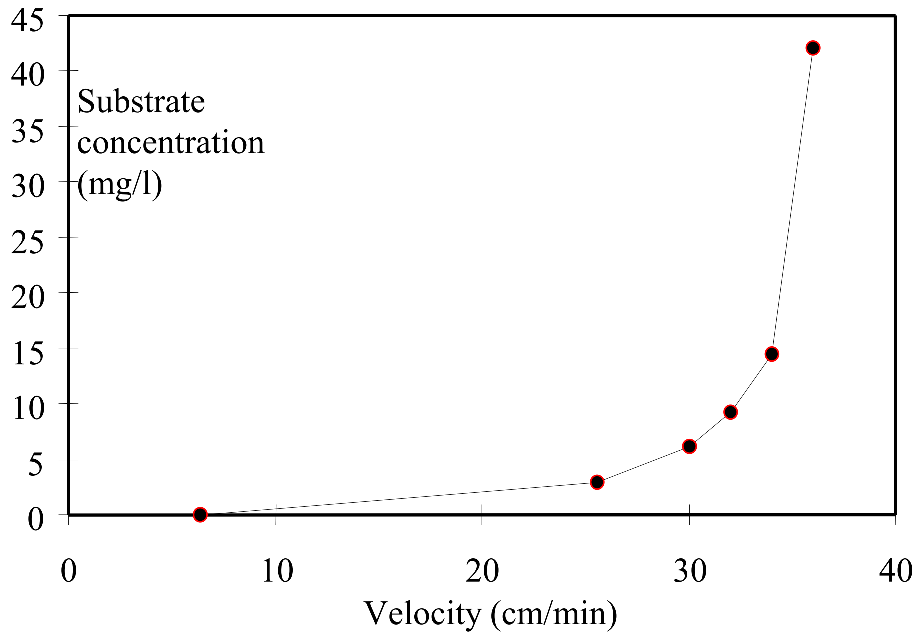


Fig. 2. Variation of Oxygen Uptake Rate(OUR) and Reynolds number in packed bed.



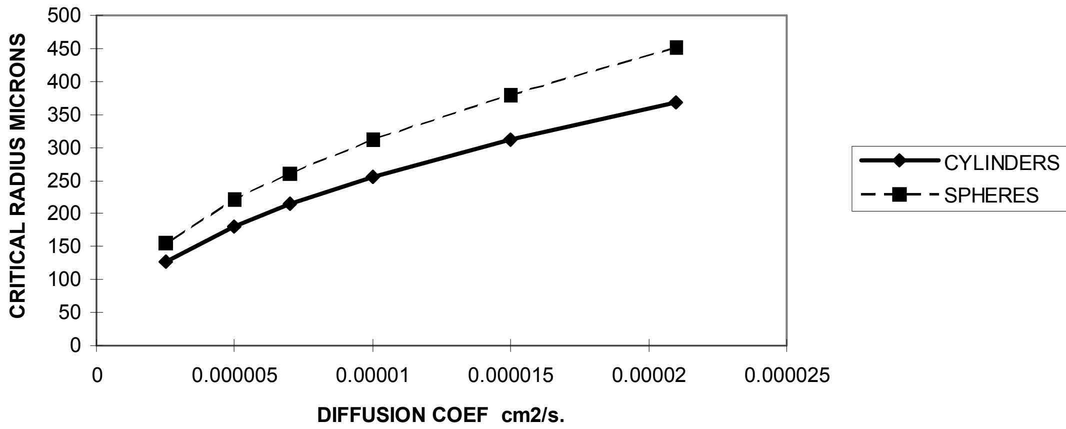


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Fig. 4. Prediction of Critical Radius at which all the cluster is aerobic for cylindrical and spherical clusters as diffusion Coefficient varies.

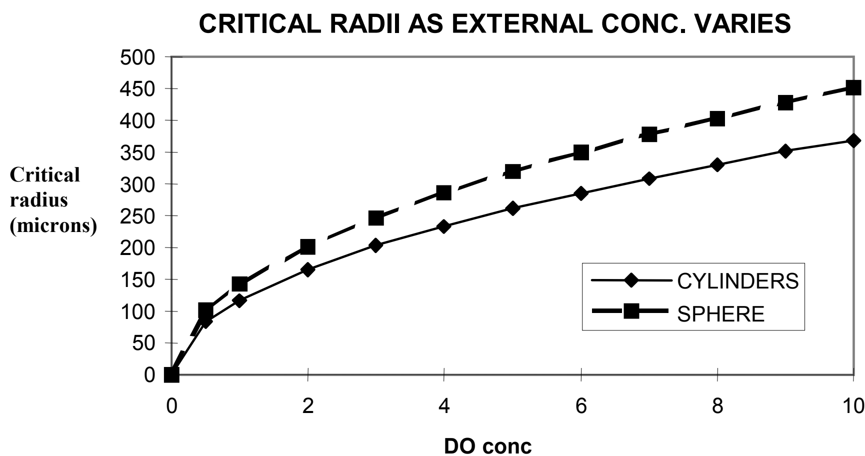

Fig. 5. Prediction of Critical Radius for Cylindrical and Spherical Clusters as surface oxygen concentration varies.