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LABORATORY GROWTH SYSTEMS IN BIOFILM RESEARCH

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Abstract

The huge variety of ecosystems that we collectively refer to as "biofilm" is reflected by the numerous different systems available to grow them in the laboratory. The relationship between *in situ* systems, microcosms and laboratory models is defined and discussed. The first two represent holistic approaches designed to assess the structure and function of **particular** biofilms: the last is analytical and reductionist, aiming to isolate specific functions of biofilms in order to understand properties that can apply to biofilm in general. Properties of a model can be completely understood whilst this is unlikely with natural ecosystems because of the possibility of unculturable species which could play an unrecognised but important part in its structure and function. A range of systems is reviewed. These include simple surfaces exposed to nutrient in different ways, flow systems such as the Robbins device and constant shear devices such as the Rototorque and the Fowler cell adhesion measurement module. The constant depth film fermenter (CDFF) is described as are membrane based models including the membrane biofilm and the perfused biofilm reactors. Some examples of microcosms are described. The concept and value of "steady state" biofilm is introduced in terms of the CDFF and of fluidised bed reactors. A number of commercially available film fermenters are listed in the appendix.

Key Words: Biofilm, model, microcosm.

Introduction

Definition of Biofilm

Biofilm is so ubiquitous and yet so varied that to produce a good general definition almost defies reason. The simplest view is that a biofilm is a collection of microorganisms bound together within a polysaccharide matrix and forming at an interface between different phases. Such a definition can apply to a monolayer of organisms attached to a solid surface, to a dense thick jelly like mass appearing in the reservoirs of water cooling systems and to a tangled biomass forming around the impellors of a large scale fermentor. The phases at which biofilm can form may be liquid/solid, perhaps the most common, for example epilithon appearing on rocks in a stream; liquid/liquid, for example the biomass forming at a gas-oil water interface in a storage tank; gas/solid -here the surface can be a nutrient source (microbial film on meat or on the surface of agar) or it can be gas/liquid such as neuston which appears as a surface film on different water bodies. One characteristic of biofilm is that, in general, it is a structure in which diffusion gradients of substrates and products appear and it is the latter which can cause community differentiation to occur within it. Thus, a dental plaque community may be aerobic at its surface but anaerobic in lower levels leading to the development of strictly anaerobic types, for example *Veillonella* or *Porphyromonas* species.

Biofilm can be regular or as is being increasingly reported now, highly irregular in structure. Thus a natural epilithic community can consist of pillars of biomass through which water channels and grazing protozoa move (deBeer *et al.*, 1994; Lewandowski *et al.*, 1994). Other "biofilms" are largely filamentous and very rough in texture. Many such structures are hybrids in that they have rough textured fringes on the outside of what is a regular relatively homogeneous biofilm at the base (Characklis and Wilderer, 1988). The relationship between substrate concentration and structure has recently been assessed by Wimpenny and Colasanti (1997).

A most important question in microbial ecology, indeed in science in general, is whether to study a natural phenomenon *in situ* where it is found or whether to investigate simplified systems in the laboratory.

Models, microcosms and reality

There is no easy answer to the question posed in the last section. The approach taken strongly depends on the psychological make-up of the investigator. On the one hand researchers of a holistic inclination believe that there is no substitute for investigating the complete system. The main problems with this approach is the difficulty with which one can make generalisations regarding a family of ecosystems, since the variability of natural systems is so great. The next step away from reality but only slightly so is to generate a "microcosm." A microcosm can be defined as a part of a natural ecosystem which is separated off from its origins either on a large scale like the huge plastic Lund tubes inserted into freshwater lakes (Jones, 1977) or on a small scale, for example growing plaque on an extracted tooth under controlled conditions in the laboratory (Russell and Coulter, 1975, 1977). A microcosm, although a homologue of the natural system, is separated from it and investigated under controlled conditions so that it is possible that it evolves away from the original state (see Wimpenny, 1988). At all events it retains much of the complexity of the natural system.

The next stage (away from reality!) is the generation of model systems. These are analogues of reality rather than reality itself. To grow *Streptococcus sanguis* from a pure culture on a sterile surface using a defined growth medium under controlled conditions in the laboratory is to generate one kind of a model of dental plaque. It could be made more sophisticated by adding other bacteria found in natural plaque. The medium could be altered to make it a more faithful copy of natural saliva. The substratum on which the film is growing could be changed to more closely reflect the natural enamel surface of teeth. The system will always be a model since all its constituents **are known** whereas there is always some doubt about a natural system. For example there are almost certainly novel bacterial species that have never been isolated from natural habitats: consequently there is no possibility of understanding any subtle effects that such uncultured and possibly unculturable species can contribute as these are absent from the model system.

Models have a most important part to play. In the biofilm field we may want to isolate and investigate the part played by molecular diffusion in regulating metabolism or in controlling community development. The production of a relatively homogeneous simplified model film community can facilitate this by allowing the deployment of microelectrodes, by allowing cryo-sectioning and by making it easy to perform light, laser confocal and electron microscopy.

In the final analysis of course we need to approach the investigation of natural systems from every conceivable route. The holistic approach is complemented by the more analytical route taken by the modelers. The author was asked to review systems for growing biofilm in the laboratory. There are innumerable different approaches, almost as many as there are investigators. Some are clearly microcosm systems others are models. Many systems designed primarily as models become microcosms when they are inoculated with a natural population. Conversely what may be seen to be microcosms when a natural population is employed become models using pure culture inocula. What must be accepted is that they are all systems to facilitate the development of such a collection of bacteria as we would include in our very loose definition of "biofilm."

What is a "growth system"?

It may be simpler sometimes to move away from definitions of microcosm and model and regard all such phenomena as systems designed for growing microbes under appropriate cultural conditions. What is a microbial growth system? It can be defined as an apparatus which provides a suitable environment for the proliferation of microbes. Growth systems can be closed or open, homogeneous or heterogeneous, pure or mixed culture(s), environmentally controlled or uncontrolled, aseptic or "septic."

A closed growth system allows the formation of a batch culture of bacteria. Thus a fresh medium is inoculated with an appropriate organism or organisms. After a lag period these grow, exponentially at first. As conditions become unfavourable and nutrients exhausted, growth rate slows, stops and finally if left long enough the population dies.

In contrast to this, the open system has an input of fresh medium and an outlet for spent medium and cells. This allows a steady state population to develop and continuous culture systems like the chemostat are powerful research tools.

Growth systems can be homogeneous, in other words, well mixed systems like the chemostat or a continuously stirred tank reactor in biochemical engineering terms, or it can be spatially heterogeneous. Biofilm systems are nearly all the latter because physicochemical conditions vary from point to point throughout them. An interesting question is whether it is possible to have a growth system that is both heterogeneous and capable of entering a steady state. This will be discussed in more detail later.

Growth systems can be environmentally controlled,

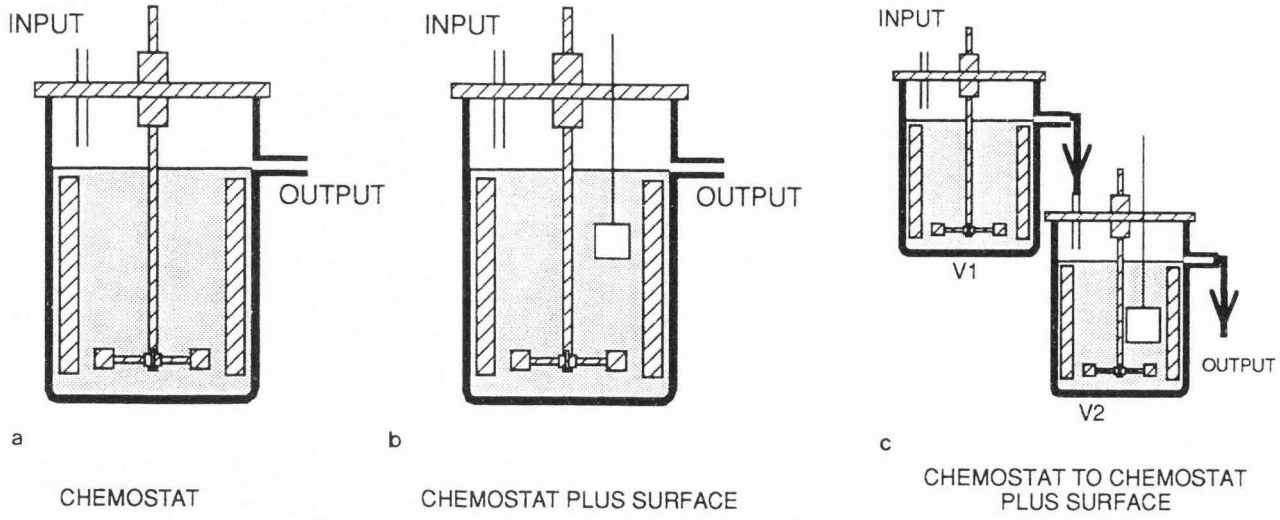

Figure 1. Chemostat based biofilm models. (a) Growth of a "homogeneous" model biofilm in which a population derived from a biofilm is grown as a planktonic culture. (b) Immersed test surfaces. Here a steady state culture is developed in the chemostat and some or all components adhere to test pieces immersed in the system. (c) A two-stage chemostat system. Organisms are grown as a planktonic culture in the first stage and are then transferred to a second stage where they are able to adhere to test samples.

for example temperature, pH, oxygen partial pressure, redox potential, etc., can be monitored and regulated, or they may be allowed to grow in an unregulated fashion, generating appropriate physicochemical conditions in the growth vessel.

Finally, the majority of model growth systems are established using known pure cultures of bacteria in equipment that is initially sterilised and which is secure from contamination from outside the container. Other systems, generally microcosms, may not be operated under sterile conditions. The (often unstated) assumption is that a "natural" community is robust and contaminating bacteria will not become important members of it at least during the life cycle of the experiment.

Growth Systems for Biofilm Research

Simple systems: Glass or mineral plates or beads

One of the simplest methods of growing a biofilm is to expose a surface to a culture of bacteria.

Some of the earliest experiments on cell adhesion were carried out by ZoBell (1943, 1946) who noted that much higher concentrations of bacteria from sea water were associated with the container surface than free swimming in the water itself. Later, other workers investigated adhesion to glass in more detail (Wood, 1950; Larsen and Dimmick, 1964).

A simple but powerful biofilm growth model is to use a glass plate flow cell which can be located under a microscope so that growth can be monitored visually or as a refinement using image capture and processing techniques. An important contribution to this field as far as microbial, attachment and early colony growth is concerned is due to Caldwell and his colleagues (Caldwell and Lawrence, 1988). Continuous flow slide culture was used by Wolfaardt *et al.* (1994) to grow biofilm capable of degrading the herbicide diclofop. The biofilm formed had identifiable and reproducible cell structures which were different from structures seen when the film was grown on more labile substrates. Busscher and his colleagues have developed this concept to investigate biofilm growth and behaviour in a parallel plate flow chamber which also allows monitoring biofilm behaviour using image analysis (Bos *et al.*, 1995). Bos *et al.* (1994) used such a system to monitor quantitatively the coaggregation of oral bacteria.

A standard technique for culturing biofilm is to establish a steady state in a chemostat and either to immerse test surfaces directly into a single stage chemostat or to use a multistage system and to suspend tiles in later stages (Fig. 1). Good examples of this approach have been described in dental plaque studies (Keevil *et al.*, 1987; Keevil, 1989; Marsh, 1995). Such systems were also used to investigate corrosion in water transmission systems (Keevil *et al.*, 1993; Walker *et al.*, 1995). In all these systems, the tiles are always bathed in a culture, either (in a single stage system) in one that is growing, or (in multistage devices in a second or third stage) where cells are no longer growing or are exposed to different regimes than in the first growth vessel.

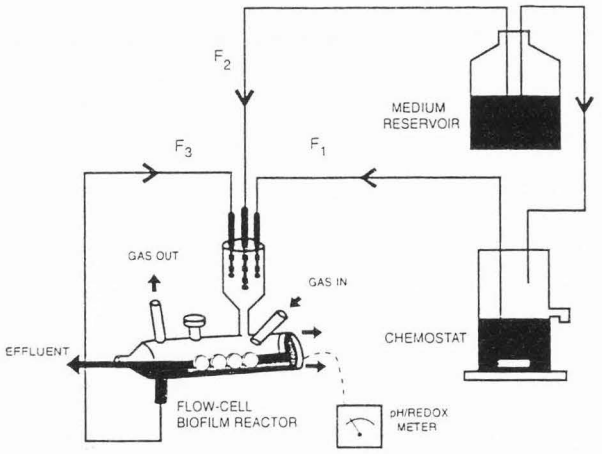

Figure 2. A sophisticated version of the irrigated disc system (from Watson *et al.*, 1995).

Such systems allow one to investigate the importance of different surfaces. For example Keevil *et al.* (1993) used a range of surfaces from copper and glass through a number of different plastics to a latex elastomer to report on colonisation by a natural flora and by *Legionella pneumophila*. In these experiments Thames river water was used as the basic nutrient solution.

Herles *et al.* (1994) used a chemostat to feed six flow cells each containing two types of surface one hy droxyapatite, the second germanium. The latter was used to allow the analysis of growth using attenuated total reflectance fourier transform infra red (ATR/FT-IR) spectroscopy. Li and Bowden (1994) used glass surfaces pre-conditioned with mucin to monitor the adhesion of oral gram positive bacteria. Cell accumulation on these surfaces followed four distinct phases from attachment without growth through to the formation of a mature biofilm.

Another approach is to take a disc of appropriate material and to irrigate this with nutrient solution by simply dropping it onto the surface. This technique has been used to study oral biofilms by Sutton *et al.* (1994) who investigated the structure of *Streptococcus crista* biofilms using different electron microscopic techniques. They emphasise the different results that can be obtained and the ever present dangers of artifacts. The group at Unilever Port Sunlight laboratories, have developed the system to a more sophisticated level (Fig. 2) where the discs are held in a chamber which can be irrigated either with a chemostat culture, or with fresh medium or with effluent from the growth chamber (Watson *et al.*, 1995).

In all these systems, film generation and life history is "natural" even though it is generally established using pure culture inocula. It is not clear whether such films approximate to a steady state or whether they go through

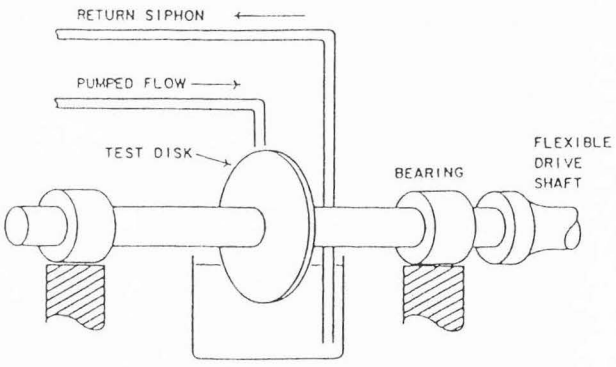

Figure 3. The rotating disk fermenter (from Characklis, 1988).

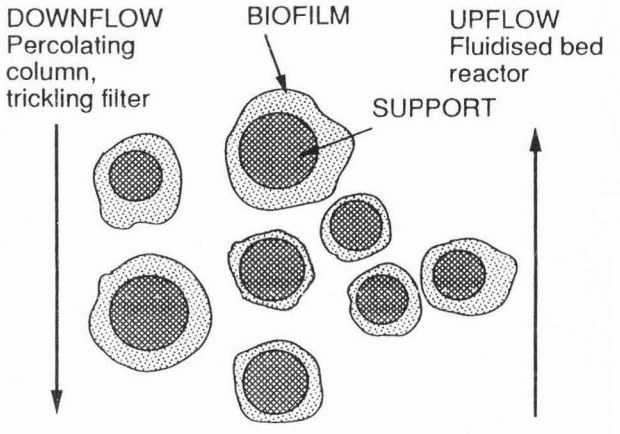

Figure 4. Trickling filter/fluidised bed systems showing biofilm growth surrounding a solid support.

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a standard "batch" culture life history of attachment growth, maturation and detachment.

Rotating disk fermenters, used mainly in the effluent treatment industry, consist of a stack of discs which are partially immersed in the material being treated. Biofilm grows on the disk surface which is periodically exposed to oxygen in the atmosphere before being submerged again in the largely anaerobic effluent (Fig. 3).

Solid supports with (roughly) spherical symmetry have been used in numerous applications for growing biofilm (Fig. 4). These range from the very crude coke or stone supports used in trickling filters in the effluent treatment industry to glass beads often used in percolating columns and sand particles used in fluidized bed fermentation systems. In each system biofilm forms over the surface of the support. Packed bed reactors can be used as straight through systems or as employed by Speitel and Leonard (1992) as a sequencing system, in this case the organism a methanotroph *Methylosinus trichosporium* was grown on methane and oxygen, a

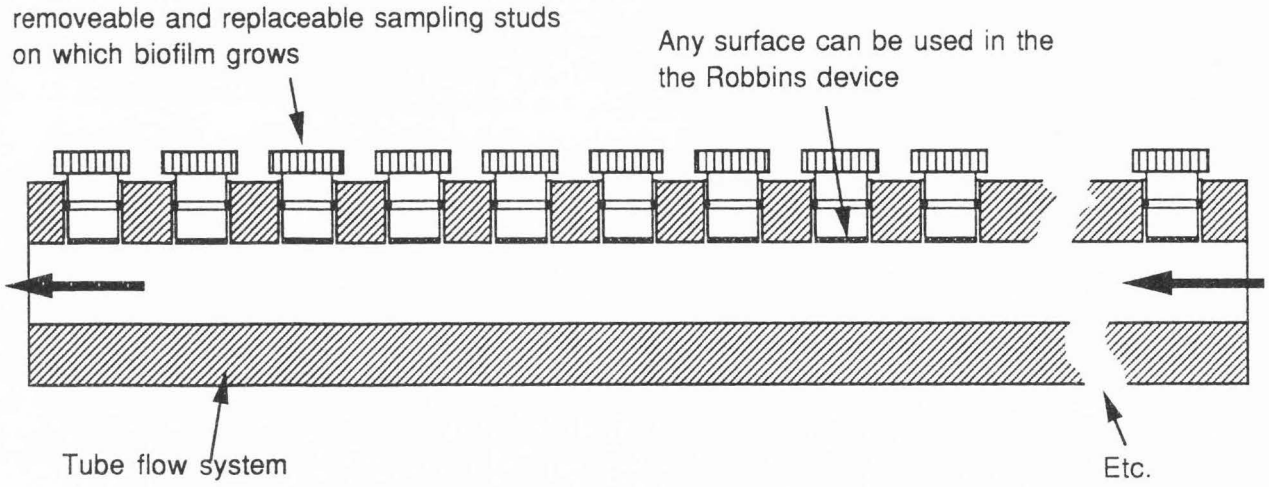


Figure 5. The Robbins device. This is a tubular system into which are let a number of removable plugs on which biofilm grows. Note growth takes place on the inner surfaces of the tube as well as on the plug test surfaces. Arrows indicate flow through the system. Flow can be straight through or recycled via a reservoir. Almost any configuration and any number of sample studs are possible.

mixture that was then alternated with an aqueous chloroform mixture in the absence of methane. The system degraded chloroform for several days before the activity declined and the system was changed back to oxygen-methane again.

The importance of fluidized bed systems is at least two-fold. Excellent mixing means that the film is exposed to aerated medium and nutrients and maximises mineralisation of the organic compounds present. The other main advantage is that the film soon reaches a steady state since any excess growth is removed by attrition due to the rapid motion of the particulate support. Coelhoso *et al.* (1992) investigated waste water denitrification in a fluidised bed reactor in which the substratum was 1.69 mm diameter activated carbon beads. The system was grown using molasses as nutrient and a thick biofilm (800 μm) developed after one week. Other configurations include biofilm growth on suspended particles in the related airlift fermenters (Tijhuis *et al.*, 1994).

Flow systems

Many natural biofilms are found in flow systems including streams and rivers, pipes and channels and in water cooling towers. Whilst such systems have been used directly a popular model system is the Robbins device (McCoy *et al.*, 1981; Ruseska *et al.*, 1982) which has become a standard weapon in the armoury of the biofilm researcher. This is a flow system in which are inserted a number of plugs whose composition may be determined by the researcher (Fig. 5). These plugs are located flush with the inside wall of the tube. The tube system is generally a closed loop so that culture is recycled at different velocities around the system. It may be connected to a reservoir or to a chemostat and can be replenished from a sterile medium reservoir.

The Robbins device is a very flexible system. It can be operated at high recycle rates where the latter greatly exceeds the dilution rate for the system. Under these conditions the system is completely mixed and concentration gradients over the length of the channel are virtually absent. If the tube is long enough and pumping is single pass and reasonably slow the system can behave as a plug flow reactor, and here concentration gradients become established. This can be useful if one wants to investigate biofilm growth as a function of nutrient con centration, especially if the latter can be accurately monitored at different positions along the tube. If one wishes to reduce the concentration of cells in the bulk liquid the fluid residence time can be decreased, however, this is likely to increase the shear stress which might alter the film characteristics. Of course the Robbins device is an excellent tool for investigating the effects of shear stress *per se*.

A multiplate laminar flow device was developed by Cote *et al.* (1992). This used a synthetic textile as support for the growing biofilm. The latter developed within a few days and was able to remove high levels of pollutants quickly. Because of the thickness of the film aerobic mineralisation took place at the surface whilst anaerobic digestion processes occurred deeper into it.

Constant shear systems

If shear stress is of major importance in any biofilm investigation the Rototorque (Trulear and Characklis, 1982; Bakke *et al.*, 1984) is probably the best model to use. This device consists of two concentric cylinders;

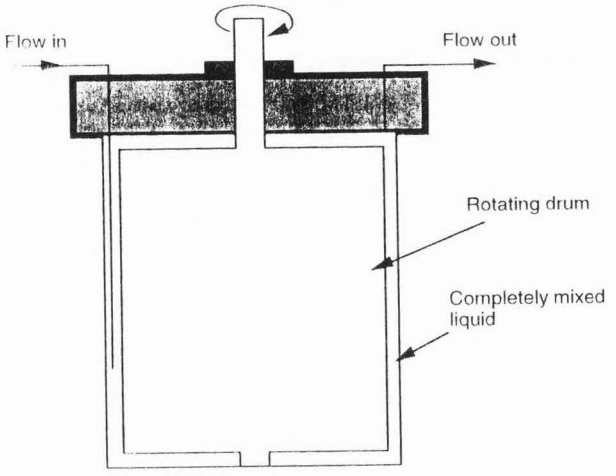


Figure 6. The Rototorque. Growth takes place in a constant shear field. Removable slides are used to sample the biofilm (from Characklis, 1988).

the outer of these is stationary whilst the inner rotates (Fig. 6). A torque converter mounted between the drive unit and the inner cylinder monitors drag forces which, together with rotational speed, enables fluid frictional forces to be determined. The walls of the outer cylinder are fitted with from 4 to 12 removable slides on which biofilm grows. Nutrient medium is fed to the device which is equipped with draft tubes that help to provide good mixing. In this system liquid residence time is independent of shear stress.

The Rototorque was used by Gjaltema *et al.* (1994) to characterise biofilms of pure and mixed cultures. Careful examination revealed that the structure was always very heterogeneous. They concluded that small imperfections and changes in the flow pattern rather than cell motility were the most important causes of the observed heterogeneity.

The Fowler Cell Adhesion Measurement Module (CAMM) was devised to measure the strength of cellular attachment to a surface (Fowler and Mackay, 1980; Fowler, 1988). The system (Fig. 7) consisted of two precisely parallel discs the upper of which is made from the material under test. Medium enters at its centre and spreads outwards. Since the flow area increases across the disc the fluid velocity decreases with a consequent reduction in shear stress at the fluid surface interface. Cells are generally allowed to attach under zero shear conditions for example by immersing the test disc in a culture in a Petri dish. The disc is then placed in the CAMM and a shear gradient established. At shear rates higher than the attachment strength of the cells a clear zone is seen near the centre of the device where shear rate is highest. Surface shear stress may be calculated as:

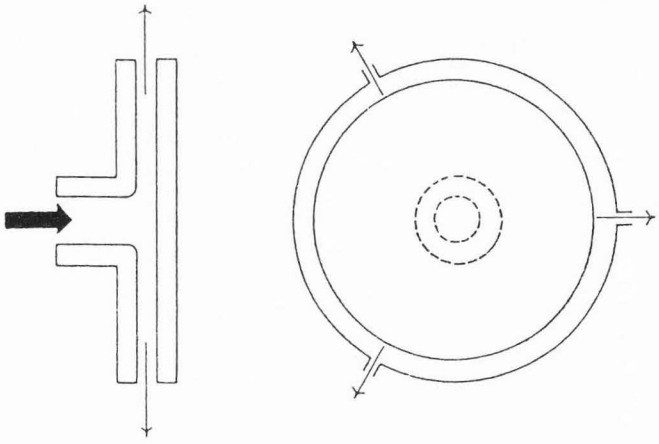


Figure 7. The Fowler radial flow reactor (from Characklis, 1988).

$$t = 3Q\mu/\pi rh^2 \qquad (1)$$

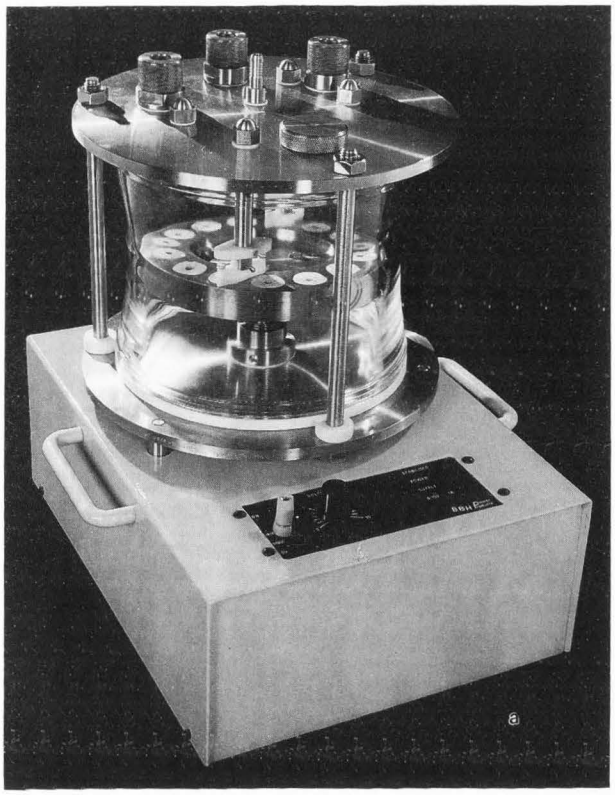
where Q is the volumetric flow rate through the radial flow chamber, μ is the viscosity of the fluid, h is the disk separation distance, and t is the surface shear stress at radius r.

Constant depth biofilm reactors

The first attempt at developing a constant depth biofilm was by Atkinson and Fowler (1974); two models were devised. The first was a roughened glass surface whilst the second used a thin metal template attached to a surface. The template had recessed areas within which biofilm grew. Both systems used a scraper blade which removed excess biofilm from above the surface. Unfortunately these systems were not developed further. A similar concept was used to produce the constant depth film fermenter (CDFF) (Coombe *et al.*, 1981, 1984; Peters and Wimpenny, 1988a,b).

The CDFF consists of a circular stainless steel disc around whose rim are located 15 polytetrafluoroethylene (PTFE) film pans containing either five 5 mm or six 4.7 mm diameter film plugs recessed to a set depth which can be about 50-500 μm in depth (Fig. 8). The plugs on which film grows can be of any material, hydroxyapatite and PTFE have commonly been used. The mother disc rotates at about 2 rpm beneath a spring loaded PTFE scraper blade. The surface of the mother disc is irrigated with sterile medium. Growth takes place within the recess formed between the plug surface and the scraper blade. Excess growth removed by the latter is carried away in the effluent stream. The system is enclosed in a borosilicate glass tubing section with stainless steel top and bottom plates and is easy to sterilise and operate completely aseptically.

The CDFF has been used in a number of different experiments both in our and in other laboratories.



A *Pseudomonas aeruginosa* biofilm was grown in the CDFF on an amine:carboxylayte medium (representing common constituents in metal working fluids); see more details of these experiments in Wimpenny *et al.* (1993). The biofilm reached a steady state in terms of protein and viable count. Oxygen and pH microelectrodes were used to measure gradients of these in the biofilm. A technique for cryo-sectioning the film was used to map the distribution of viable cells across the film profile. Since the organism is an obligate aerobe viability in the lower zone (shown to be anoxic using the pO_2 microelectrode) was only 1% of the maximum value which was found to be towards the upper surface of the film. Cryo-sectioning was also used to determine the profile of adenylates and adenylate energy charge across the biofilm (Kinniment and Wimpenny, 1992). Energy charge values, though low compared with values reported in planktonic cultures, rose towards the top of the film.

A community of nine different oral bacteria (Bradshaw *et al.*, 1989) was established in a chemostat gassed with 5% CO_2 in nitrogen and this, once it had reached a steady state, was used to inoculate a CDFF. After a period of 400-500 hours the biofilm community also reached a steady state as judged by protein concentration and viable counts both for the whole community and for each constituent member. The film was also grown under aerobic conditions. The two steady states were quite different. In the anaerobic chemostat the dominant

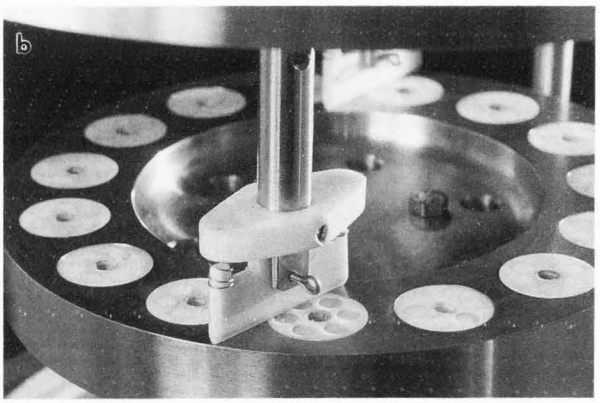


Figure 8. The Constant Depth Film Fermenter. **(a)** A view of the complete fermenter. **(b)** Detail to show scraper bar and film pans.

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organism was *Fusobacterium nucleatum* whilst least numerous was the *Streptococcus mutans*. Growth of the anaerobic chemostat culture under aerobic conditions in the CDFF led to significant changes. The anaerobe numbers fell dramatically at first when exposed to air, however all three recovered well and *Porphyromonas gingivalis* became the dominant organism in the steady state biofilm. The aerobic *Neisseria subflava* grew quickly at first and in the end became the second most common organism in this community.

Transmission electron microscopy across the biofilm showed high numbers of kidney shaped cocci, assumed to be the neisseria, in the upper half of the biofilm. Whilst cocci and rods could easily be differentiated the only other species recognisable by its morphology was the anaerobic fusobacterium. This appeared only to be present towards the base of the film.

Membrane systems

Several workers have used permeable membranes as substratum for biofilm growth. One virtue of such a system is that reactants can be deployed from opposite sides of the membrane. A common configuration is to have the carbon source/reductant on one side of the membrane, whilst oxidant is provided on the other (Fig. 9). This model is related to hollow fibre fermentation systems where cells are located on one side of a fibre wall whilst reactants are located on the other. A good example of the membrane reactor is described by Rothemund *et al.* (1994). The Membrane Biofilm Reactor (MBR) is used to treat waste water: the film forms on the waste water side of a permeable membrane whilst the other side is exposed to air. A biofilm membrane reactor has been used to grow white rot fungi *Phanerochaete chrysosporium*, that can degrade lignin through the extracellular enzyme lignin peroxidase (Venkatadri *et al.*, 1992).

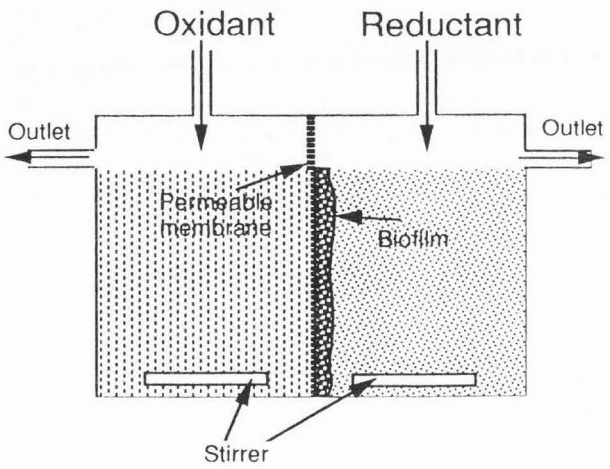

Figure 9. The principle of operation of a membrane biofilm fermenter. Note: many such models are based around permeable tubular systems.

Quite a different membrane reactor is based on the Helmstetter "baby factory" originally developed to secure a synchronised cell population for cell cycle studies. The Perfused Biofilm Fermenter (PBF) (Gilbert *et al.*, 1989) consists of a membrane to which a population of bacteria attach through filtration. The membrane is then inverted and perfused with culture medium (Fig. 10). Three populations of bacteria can be identified: (i) truly planktonic cells grown in a chemostat; (ii) young daughter cells eluted from the membrane; (iii) biofilm cells attached to the membrane and then removed for analysis. The biofilm that forms is very thin, at any rate less than 10 μm in thickness, so that it is unlikely that significant physicochemical gradients form. The key factor that these cells show is that they are an **attached** population. One intriguing aspect of the PBF is that changing the substrate concentration and flow rate can alter the growth rate of the attached population. It is therefore possible to perform experiments with attached cells growing at different μ values. The PBF has revealed interesting and complex changes in adherent cell populations ranging from changes in surface properties to variations in the sensitivity of cells to antibiotics and other antimicrobial compounds. It was shown that young daughter cells were highly electronegative and unable to attach to surfaces easily until they had "matured." Changes in surface chemistry, charge, exopolysaccharide production, etc., were most often associated with growth rate rather than attachment *per se*. Gilbert and Brown (1995) consider that at least one factor involved in the increased resistance of biofilm bacteria to antimicrobials is their reduced growth rate.

Microcosms

It is not possible to do justice to the numerous systems which come under the heading of "microcosm" as defined earlier in this paper. They are united only in that each system retains as much of the complexity of the natural system as possible yet it is removed from the latter and placed under more controlled conditions suitable for laboratory examination.

It has been estimated (Stickler and Winters, 1993) that 53 million indwelling urethral catheters are used annually worldwide. These constitute a major source of infection and are often colonised with thick biofilms of pathogenic bacteria. Stickler *et al.* (1994) have described a catheterized bladder model which feeds artificial or natural urine under controlled flow rates and temperature through catheter sections. To investigate antimicrobial activities two such models were run in parallel, one without the antimicrobial the other with it. Electron microscopy plus viable counts of sonicated section of colonised catheter confirmed that antibiotics like ciprofloxacin at normal concentrations had little or no effect on biofilm populations of *Pseudomonas aeruginosa*, *Escherichia coli*, *Providencia stuartii* or *Proteus mirabilis* after a period of 48 hours incubation.

Perhaps the simplest model system that closely represents "reality" is to deploy specific surfaces at the site where biofilm is growing. Hamilton and his colleagues (McKenzie and Hamilton, 1992; Hamilton, 1995) have done this using steel coupons in sea bed sediments associated with oil production platforms where corrosion processes were studied.

Fry and his colleagues (Bale *et al.*, 1987; 1988; Fry and Day, 1993) have also tried to make experimental systems that replicate as faithfully as possible conditions in the natural environment. In this case they were investigating the transfer of natural plasmids in riverine biofilms. In one system, donor and acceptor strains were mixed together on membrane filters which were incubated on stones in the river or in laboratory stream microcosms. A second system used donors and acceptors on separate membranes which were incubated on river stones with or without preexisting biofilm for 24 hours. After this time the two stones were placed so that inoculated zones were touching, and after further incubation the numbers of transconjugants were assessed by the usual methods.

There have been numerous *in vitro* systems used to investigate the growth of oral communities. These come really under the heading of microcosms. That is, they transfer dental plaque for example from the mouth to an *in vitro* surface often extracted teeth, sometimes hydroxyapatite coupons or even platinum wires. Where teeth are used the common name for these systems are "artificial mouths" (Russell and Coulter, 1975, 1977; Dibdin *et al.*, 1976). Such systems have been comprehensively reviewed by Tatevossian (1988).

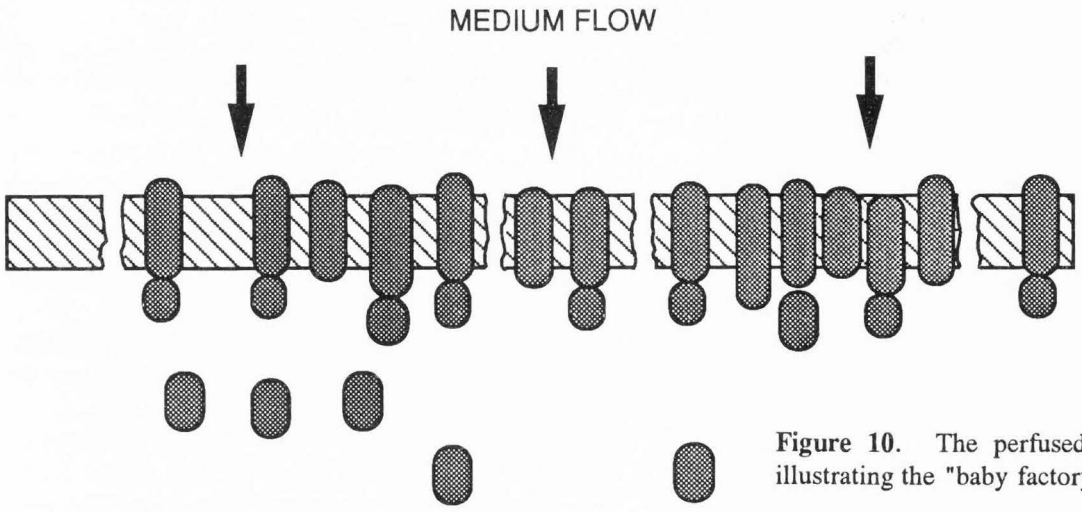

Figure 10. The perfused biofilm fermenter illustrating the "baby factory" principle.

Miscellaneous systems

Agarose gel and gel entrapped bacteria have sometimes been used as model systems for biofilms. Blakehaskins *et al.* (1992) used the gel system without added organisms to investigate calcium uptake and carieous lesion formation on enamel blocks after undergoing mineralisation-demineralisation cycles. Jouenne *et al.* (1994) trapped *Escherichia coli* within an agar layer and incubated this for 2 days exposed to a glucose mineral salts medium. They were then incubated for a further three weeks under metal ion depletion. Examination of cell distribution in this artificial biofilm revealed extensive heterogeneity in cell distribution.

Deep gel-stabilised model systems have been used in a number of applications including modelling growth of *Bacillus cereus* and some other bacterial species in oxygen-glucose counter gradients, growth of *Beggiatoa* species in oxygen sulphide counter gradients, modelling estuarine sediments and so on. These and other examples were reviewed by Wimpenny (1988). The possibility of using a gel-stabilised system as a **stretched** model of a biofilm needs to be explored. Rhian Mustow (personal communication) has used such a model to discern positional differences between streptococci and veillonella in a dental plaque system.

Rotating drums have been used as biofilm reactors. For example, Zhang *et al.* (1994) and Zhang and Bishop (1994) used microslicing as well as microelectrodes (to investigate oxygen, ammonia, nitrate and pH gradients) to investigate spatial distribution and competition in a mixed culture biofilm grown in such a laboratory model system. Results suggested that stratification plus a basically heterogeneous distribution of organisms meant that traditional biofilm modelling assuming a simple one dimensional structure, was doomed to failure.

Conclusions

The question of steady state operation was raised earlier in this article. Clearly, steady state systems are desirable from an experimental point of view. The notion that a system is in some kind of dynamic equilibrium provides a good baseline for experiments which perturb the steady state. This has been a most powerful tool in homogeneous culture systems like the chemostat. Is it possible to generate a steady state heterogeneous growth system? The multistage chemostat where different vessels are connected in sequence in a unidirectional fashion goes some way to achieving this. Linking a number of vessels **bidirectionally** as in the gradostat (Lovitt and Wimpenny, 1981), provides a good model of a steady state, spatially heterogeneous system, even though each vessel is itself homogeneous. The CDFF takes a step further into the realms of the steady state. Here, the system is a typical biofilm except that it is maintained at a constant depth by the removal of any growth that extends above the film pan surface. The system is complex because it is clear that as nutrient diffuses from the upper surface into the film so there will be a gradient of growth rates until a point is reached where net growth is zero. Cells at the base of this film may be dying. There are two possibilities: (i) if there is exchange of material all the way to the base of the film then a steady state is possible; (ii) If a "compartment" exists (say of dead bacterial remains) where there is no significant exchange of materials then this section of the film will be excluded from the steady state. Although growth rates vary from positive to negative across the system, it eventually reachs a stable steady state. The time to steady state will depend on the depth of the film and the gradient of growth rates and may be quite long.

Determining whether a system has attained a steady state is a matter of definition. At its simplest it can be based on protein per unit of film volume. Other components such as dry weight and total cell or viable cell counts or any other component whose concentration is proportional to biomass, can also be used. In our ex-

perience the time to steady state can be quite different depending on which parameter is determined. Once all have reached a stable value we can assume that the system is really in some sort of dynamic equilibrium. In the end the more parameters determined the better.

An important caveat to assessment of steady states must be the stability of the genotype or types involved. It is clear from work with the chemostat that mutation and selection occur continually throughout the "steady state." This will be equally true in biofilm models and more so if mixed cultures are involved. Finally, the claim to steady state must always be accompanied by a description of the parameters by which the steady state was assessed.

Questions have been raised concerning whether *in vitro* model systems are valuable in solving **practical** problems. I believe that the answer is a definite "yes," largely because the reproducibility of model systems makes them well suited as test systems for practical treatments. For example the CDFF is used by organisations concerned with oral health care, in order to test the effects of antimicrobials under well controlled conditions. Systems like the Rototorque and the Fowler cell adhesion system give precise data on the effects of shear forces on biofilm attachment, results which can be directly translated to flow in pipes. Perhaps the most important function of model systems, however, is to obtain generalisations through simplification which can then be applied to a wide range of different biofilm systems.

This paper has been largely methodological and it is apparent that numerous different systems exist for investigating biofilm growth. This should not be surprising since the range of biofilms found naturally is huge. The recent reports that biofilm can no longer be considered a simple layered structure but rather many natural examples are highly irregular structures, penetrated by pores through which the bathing water phase and predatory protozoa can move freely only add to the difficulty in selecting a system. The type of device selected will depend very much on the type of questions asked. If the problems are "local" and apply particularly to a single natural system from which practical solutions are urgently needed, then the microcosm may be the best solution since all that is required is that the system should behave as closely as possible to the natural biofilm. If more general answers are needed to problems that may be common to a wide range of biofilms then a model system where the conditions are simplified and results are reproducible may be the answer.

In the end every approach has its own validity and it seems sensible that any given scientific problem should be approached in as many different ways as possible to finally get at the truth.

Appendix 1

Some of the commercially available biofilm systems known to the author.

(1). PS Biofilm Technology, Moselstrasse 56, 63452 Hanau, Germany. This firm specialises in equipment for examining growth and corrosion on water flow systems using a tubular device containing removable coupons, which is functionally similar to the Robbins device (see **Flow systems**).

(2). Bridger Scientific, P.O. Box 1923, Sandwich, Massachusetts 02563 USA. Bridger Scientific markets the DATS II Fouling Monitor System.

(3). Biosurfaces Technologies, Corp., 920 Technology Boulevard, Suite C, Bozeman, MT 59715 USA. This organisation provide many different services concerning biofouling. They market the Annular Reactor (described in this paper as the Rototorque).

(4). University of Cardiff Consultants Ltd., Univ. Wales Cardiff, Box 915, Cardiff, CF1 3TL, Wales, UK. The Constant Depth Film Fermenter is available in two versions: plain or water jacketed. Also in two sizes containing 6 or 15 separate film pans each fitted with 5 X 5 mm diameter or 6 X 4.7 mm diameter film plugs.

(5). Dr. Hilary Lappin-Scott, Hatherley Labs., Univ. Exeter, UK. Dr. Lappin Scott can provide all Perspex (Plexiglass) Robbins devices to order.

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Editor's Note: All of the reviewer's concerns were appropriately addressed by text changes, hence there is no **Discussion with Reviewers**.