



Relative activities of cells in suspension and in biofilms

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Relative Activities of Cells in Suspension and in Biofilms

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Abstract. This paper stresses that the activities of cells in biofilms are likely to differ qualitatively from those in homogeneous suspension due to the heterogeneous microenvironments and structured microbial communities which are fundamental characteristics of mixed culture biofilms. Even with simpler systems comprised of only adsorbed organisms or a thin monospecies biofilm, the extrinsic features of attachment and detachment, polymer accumulation, and diffusional resistance may affect directly the apparent magnitude of intrinsic cellular properties. In assessing, therefore, the relative activities of cells in suspension and in biofilms, it is necessary to establish which cellular processes are being compared, and to take full account of the extracellular physicochemical and biological variables inherent within the structural and temporal organization of the biofilm.

INTRODUCTION

Although it is generally assumed that cells are at an advantage when attached to a surface, the experimental evidence for this and for an associated higher metabolic activity is inconclusive (Hamilton 1987). Over a wide range of organisms and experimental systems, processes (e.g., growth, sporulation) and activity assays (e.g., substrate assimilation, nutrient catabolism, respiration) have variously been found to be increased, decreased, or unaffected by adsorption to a solid surface. Therefore, to reach any conclusion that might possibly be of validity to adsorbed organisms and biofilm systems in general, it is clearly necessary to define with considerable care exactly what

we mean by "activity" and "biofilm," and to have at our disposal the means to measure the requisite parameters (see Walch et al.; Stal et al., both this volume).

CELLULAR PROCESSES AND METABOLIC ACTIVITY

There is a vast array of different types of metabolic activities associated with microorganisms. Whereas many of these, such as energy-generating redox reactions or protein synthesis, are common to all life forms, others are found more selectively only within particular species: methanogenesis or sulfate reduction during anaerobic growth, oxidation of inorganic sources of metabolic energy, assimilation of CO₂ as sole carbon source. Each of these activities, however, is only of limited significance when considered in isolation. It is the integration of all metabolic activities and their orchestration within certain fundamental cellular processes which is the true measure of biological activity. These processes are:

1. growth—with an increase in biomass and/or cell numbers;
2. maintenance—where cell numbers and viability remain constant;
3. death—in which viable cell numbers decrease, with or without parallel decrease in total cell numbers.

Other cellular processes which are less universal, but may in certain circumstances and with particular organisms be of major importance, are the synthesis of extracellular polymeric substances (EPS) and sporulation. Ideally, therefore, any comparative study of cells in suspension or as component organisms within a biofilm should (a) maintain all other potential variables constant and (b) examine one or more of the fundamental cellular processes.

A further important feature of these processes is that they are measures of cellular activity with direct reference to the experimental environment in which that activity is taking place; growth in a biofilm cannot be determined by removal of cells from the biofilm and determining the rate or extent of their growth in some other experimental setup. This can, however, be a major practical obstacle which must be overcome in both the construction of model systems and the experimental design. Either it becomes necessary to remove samples of biofilm for analysis of cell numbers, viability, etc. without perturbing the continuing cellular processes within the biofilm itself, or data must be obtained from substrate and product fluxes through the system with extrapolation to deduce the kinetics and stoichiometry of the biofilm processes.

As indicated above, each of the fundamental cellular processes is itself a composite of many individual reactions and more complex metabolic

sequences such as, for example, substrate catabolism and energy generation by substrate-level, oxidative, or photosynthetic phosphorylation. In many situations it may be both simpler and more direct to measure nutrient uptake or respiration than to attempt to quantify an increase or decrease in growth rate or degree of sporulation. Can any experimental advantage gained from such an approach be justified theoretically (see Prosser, this volume)?

For the very reason that cellular processes are composites of many separate individual reactions, it is not possible to analyze any recorded effects in terms of molecular mechanisms without first having data relating directly to specific reaction sequences rather than simply to the overall process. Being able to describe the effects of a biofilm habitat in terms of one or a number of separate measures of biological activity can therefore add immensely to our understanding of the mechanisms involved and consequently take the analysis beyond the purely descriptive.

On the other hand, metabolic activities such as nutrient uptake or respiration are not uniquely associated with any one particular cellular process so that, taken on its own, a stimulatory effect on respiration, for example, cannot be assessed with reference to the fundamental processes of cellular growth and development. Once again we are reduced to simple statements of observed effects.

What is required, therefore, for the meaningful comparison of the activities of cells in suspension and in biofilms are quantitative *in situ* measurements of fundamental cellular processes, ideally supported by assays of relevant metabolic activities so that an interpretation in terms of the molecular mechanisms involved can be added to the kinetic and stoichiometry analysis of the cellular processes.

Such an analysis of cellular activity has as its basic assumption the tenet that microorganisms, and their activities, are not fundamentally altered by their adsorption to a surface or inclusion within a biofilm. Where this may hold true, at least as a first approximation, for microcolonies of adsorbed bacteria or thin monospecies biofilms, it is likely to be less applicable to more complex biofilms which tend to be characterized by mixed microbial flora, copious quantities of EPS, entrapped inorganic inclusions, and microenvironments with, in particular, regions of anaerobiosis (Costerton et al. 1987). The qualitative nature of the environment, the cells, and their activities may be further modified where the substratum on which the biofilm accumulates also serves as a substrate, supplying the organisms of the biofilm with at least a portion of their nutrient requirements. In examining the relative activities of cells in suspension and in biofilms, therefore, we must be acutely aware that we are not necessarily comparing like with like.

At this point, therefore, we must examine the nature of biofilms and in particular note the manner in which their various characteristics might be expected to influence the activities of their constituent microorganisms.

BIOFILMS

The structural and functional nature of biofilms has been the subject of a number of reviews in recent years (Characklis and Cooksey 1983; Hamilton 1987; Costerton et al. 1987) and is, of course, the central theme of this present volume. Here we wish to consider different levels of structural complexity within biofilms with particular reference to their direct effects on the biological activities demonstrable by those biofilms and by their constituent organisms.

Adsorbed Organisms

It is arguable whether or not isolated microorganisms or their growth as microcolonies on a surface constitute a true biofilm, but such a level of structural organization is useful in allowing us to introduce certain concepts central to the development of our main thesis.

In the case of stalked oligotrophic bacteria, where adsorption to an inert surface is an integral part of the species' natural life cycle, there is evidence for a phenotypic increase in enzymic activity due to differentiation in response to sessile growth (Dow et al. 1983). Growth of organisms at a surface can also give an apparent increase in activity due simply to the increase in cell numbers, with the activity of each individual cell being unaltered. (This would be a case in point where a true understanding would depend on measuring the fundamental process of growth in addition to carrying out convenient assays of specific metabolic activities such as substrate catabolism or respiration.) Although the concept of the concentration of nutrient substances at a surface has insubstantial theoretical and practical support (Marshall 1984), it is true to say that organisms fixed at a surface in a moving stream of a dilute nutrient will "see" more of that nutrient and so be capable of greater metabolic activity in terms of its turnover.

Where adsorbed organisms can demonstrate not so much increased but rather different or "new" activities is where the substratum is also a substrate for the organisms in question. This arises, for example, with the biodegradation of insoluble polymeric materials such as cellulose or hydrocarbons (Rosenberg, this volume).

Thin Monospecies Biofilms

A biofilm is generally considered to exist where there is complete or at least broad coverage of the solid substratum by microorganisms and their associated EPS. Whereas in nature such biofilms are most usually multispecies in character, monospecies biofilms are readily demonstrable and have been the subject of experimental study (Characklis 1984).

The rates of cellular processes and individual metabolic reaction sequences within biofilms are liable to be controlled by substrate limitation arising from mass transfer resistance between the bulk liquid phase and the biofilm and/or diffusional resistance within the biofilm itself. These considerations refer not only to organic sources of carbon and energy but also to inorganic ions, where they may be growth-limiting, and to terminal electron acceptors, including oxygen with aerobic species.

In a well-mixed system or with turbulent flow of the bulk liquid, it is unlikely that mass transfer resistance will be a significant factor in determining the overall rate of biofilm processes, but this cannot be assumed at low fluid velocities or in the presence of low nutrient concentrations. Under these conditions the absence of mass transfer resistance must be established experimentally in order that true values for biofilm rate processes may be obtained.

Diffusional resistance is influenced by substrate concentration and by biofilm thickness. Characklis and his colleagues (Characklis 1984) have noted that the substrate removal rate increases in proportion to biofilm thickness up to a critical or "active" thickness and that this critical thickness increases with increasing substrate concentration. Clearly where the biofilm thickness is greater than the critical or active thickness and substrate removal rate therefore becomes constant and independent of biofilm thickness, that substrate removal rate cannot be used to determine rate constants for the biofilm fundamental cellular processes.

Diffusion rates through biofilms have variously been estimated at between 60–100% of the value for that substance in pure water (Nielsen 1987). A further complication must be that at the same time as the nutrient or inorganic component is diffusing through the active thickness of the biofilm it is being utilized and reduced in concentration as a direct result of these activities.

In a biofilm community these effects reach their most dramatic point with the development of anaerobiosis within aerobic biofilms, which may in turn lead to such effects as sloughing or the growth of anaerobic species such as the sulfate-reducing bacteria.

With monospecies *Pseudomonas aeruginosa* biofilms in a well-mixed reactor, biofilm thickness does not exceed 50 μm and diffusional resistance has been shown to be not significant (Bakke et al. 1984). This cannot be assumed, however, for other experimental or natural biofilms and, as with mass transfer resistance, it is critical that the characteristics of diffusional resistance be determined experimentally for each system individually.

Multispecies Biofilms

Biofilms are ubiquitous in nature and equally found widespread in man-made and industrial environments. Most usually, such biofilms display

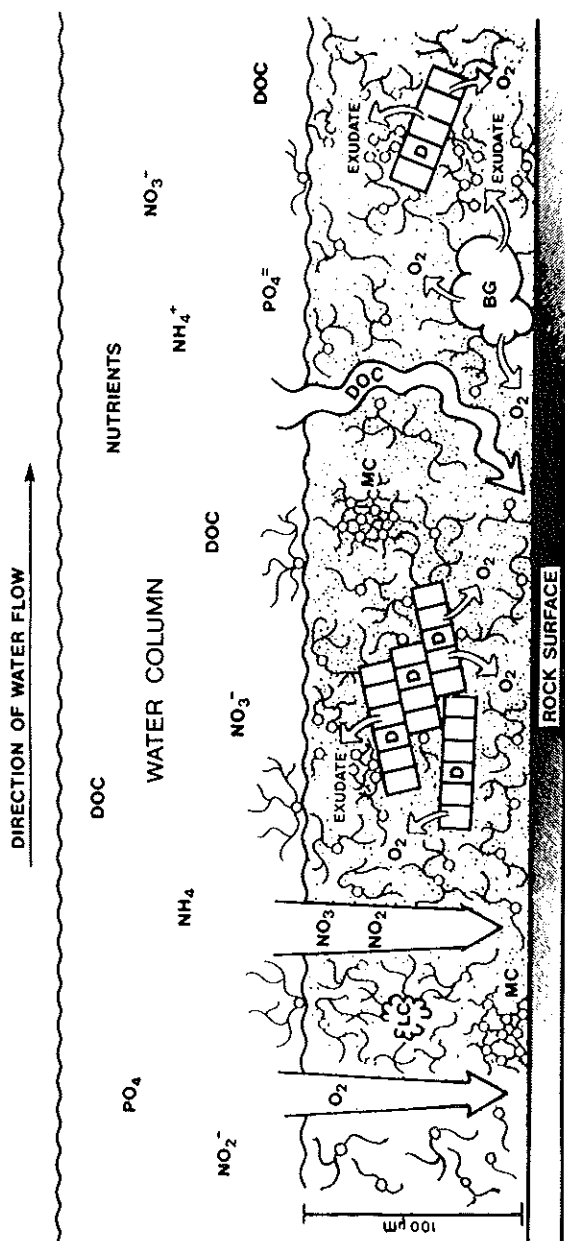


Fig. 1—Diagrammatic representation of a natural biofilm. BG: blue-green bacteria (cyanobacteria); D: diatoms; DOC: dissolved organic carbon; LC: lysed cells; MC: microcolony. Reproduced with the permission of Plenum Press (Costerton et al. 1985).

considerable heterogeneity, both with respect to the organisms present and the physicochemical microenvironments generated by the activities of these organisms within the physical matrix created by the EPS and inorganic inclusions, which constitute the main bulk of the biofilm biomass (Costerton et al. 1985; Costerton et al. 1987; Hamilton 1987). A representation of such a biofilm from a flowing stream is given in Fig. 1.

The dominating factors determining the quantitative and qualitative features of metabolic activities and other processes of the biofilm are the biological and physical heterogeneities present within the biofilm. The metabolic potential and requirements of the organisms present, their interactions and interdependencies, the creation of microenvironments, and the influence of these on subsequent biological activities all determine that biofilms demonstrate a level and complexity of activity which allow only trivial comparisons to be made with that of the individual organisms present in homogeneous suspension.

As biofilm thickness and density increase, diffusional resistances become major factors influencing the rate and indeed the nature of biofilm processes.

The characteristic features of biofilm heterogeneity and organism interaction can be illustrated with reference to Fig. 1. There are concentration gradients of oxygen, essential inorganic nutrients, and dissolved organic carbon throughout the depth of the biofilm. There may be a heterogeneity of organisms present with cross-feeding from both photosynthate exudate and cell lysis. This may be fortuitous and serve only to increase the metabolic versatility of the biofilm or, in nutrient-depleted conditions, it may be an obligatory requirement for the activity and growth of heterotrophic bacteria (Haack and McFeters 1982).

In enclosed and other systems from which light is excluded, similar tight interdependencies can occur between nonphototrophic species. As the nature of interactions within communities of mixed microbial species has become more fully appreciated (see, for example, review articles by Zeikus 1983; Hamilton 1985, 1987), the term consortium has come into use, carrying with it the direct implication that the community of organisms has an identifiable and characteristic spectrum of metabolic activities that is unique and more than just the sum of the activities of the constituent organisms operating in isolation. This is a most powerful concept which extends, of course, to other ecosystems such as algal and cyanobacterial mats (Ward et al. 1987) and which is likely to be one of the main directive forces for the next generation of researchers in microbial physiology and ecology.

One of the consortium's most striking manifestations arises when diffusional resistance, absence of oxygenic phototrophy, and high levels of oxygen-requiring heterotrophy ensure that an anaerobic microenvironment is generated within the depths of an aerobic biofilm. Under these conditions, nutrient successions can become established with aerobic and anaerobic

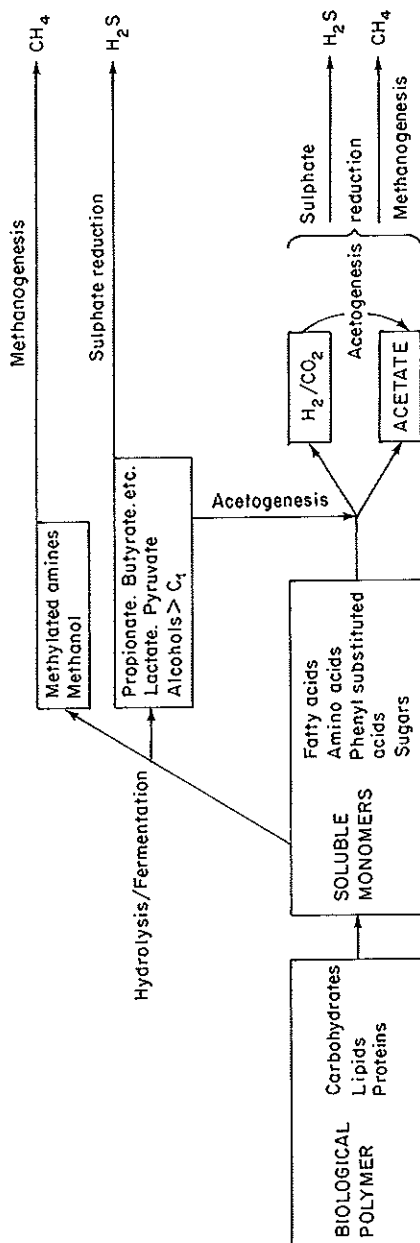


Fig. 2—A scheme of carbon flow through an anaerobic ecosystem showing some of the functional metabolic groups and their interrelationships. Reproduced with the permission of Society of General Microbiology (Parkes 1987).

species initiating the degradation of complex polymeric materials, including, for example, hydrocarbons or structural polysaccharides followed by the action of fermentative and acetogenic organisms carrying the catabolic activity through to the level of bacterial fermentation products, of which the key components are hydrogen and acetate. Thereafter, the terminal stages of biodegradation depend upon the presence or absence of sulfate. In its presence, the sulfate-reducing bacteria oxidize hydrogen, acetate, and other fermentation products with the concomitant production of sulfide. In the absence of, or under limiting sulfate concentrations, the terminal oxidative steps are the responsibility of the methanogenic bacteria. These metabolic patterns are depicted in Fig. 2, while Fig. 3 represents the application of such a scheme to serve as a model for the involvement of the sulfate-reducing bacteria in anaerobic microbial corrosion.

In these cases, therefore, although diffusional resistance and other factors may have "limited" the activity of aerobic species within the biofilm, they have made possible a completely new range of activities where sulfate reduction not only can increase the overall carbon flux through the system, but is itself driven by the oxidation of primary substrates toward which this group of anaerobic bacteria are themselves quite inactive.

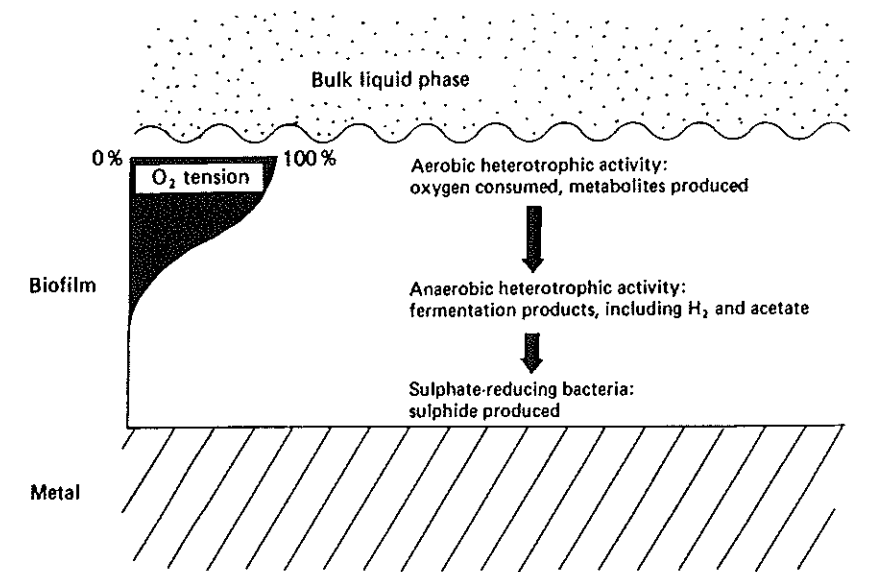


Fig. 3—Model for anaerobic microbial corrosion. Reproduced with permission from *Annual Review of Microbiology*, vol. 39 © 1985 by Annual Reviews Inc. (Hamilton 1985).

A similar complex of interacting organisms, physicochemical conditions, and metabolic activities (although without either sulfate reducers or methanogens) is characteristic of dental plaque and caries. Where the substratum is in fact also the biofilm's substrate, as with cellulolytic digestion in the rumen and hydrocarbon degradation, an intriguing situation arises where there are equivalent patterns of organism and nutrient succession but with gradients directed outwards through the biofilm (Costerton et al. 1987; Rosenberg, this volume).

Each of these heterogeneities represents a level of spatial organization in the vertical dimension, relative to the biofilm supporting surface. In certain instances, an equivalent heterogeneity is evident in the horizontal plane of the biofilm. This latter is referred to as patchiness, and it has been suggested that such an uneven distribution of biomass and biofilm activity may have a stimulatory effect on microbial corrosion. Heterogeneity can also result due to the geometry of the system as, for example, in plug flow reactors (Bouwer, this volume).

In addition to its major role in mass transfer and diffusional resistance in biofilms, the EPS can have other more direct effects on biofilm processes and observed metabolic activities. EPS is both negatively charged and highly hydrated so that it can act as an effective cation exchanger, serving to increase local concentrations of ionic species such as heavy metals, ammonium, potassium, or lysine while having the opposite effect on anionic groups, and none at all on uncharged potential nutrients such as the sugars. These effects may be masked, however, as the binding will in fact reduce the chemical activity of the species in question. At least in part linked with this phenomenon is the well-documented resistance of biofilm organisms to antibiotics and chemical biocides. On the other hand, recent studies have suggested that in certain cases adsorption to or decreased diffusion through EPS cannot fully explain the observed antibiotic resistance of biofilm bacteria (Nichols, this volume).

Dynamic Nature of Biofilms

The above arguments relate to the mature biofilm, although inherent in the discussion of organism and nutrient succession is an acknowledgement of the development of a biofilm over time, with consequent alterations in the activities of that biofilm during the phases of its development. The phases of biofilm development have been characterized as:

1. transport of organic molecules and cells to surface;
2. adsorption of organic molecules to give "conditioned" surface;
3. adsorption of cells to conditioned surface;
4. growth of adsorbed cells with associated synthesis of EPS;
5. detachment of portions of the biofilm.

Each of these phases can and should be subject to a process analysis (Characklis 1984) designed to establish the kinetics and stoichiometry of the fundamental processes involved at each stage.

In the present analysis, we are primarily concerned with the fourth or growth phase, although attachment and detachment cannot be ignored. In particular, it is necessary to establish whether the biofilm is in a steady state and to determine the exact nature of that steady state. Does it represent a stable nongrowing population with only maintenance energy being expended and/or cryptic growth, i.e., limited growth dependent on and balancing death and cell lysis within the stable population? Or is the steady state the result of a dynamic equilibrium with growth of cells and synthesis of polymer both balanced by detachment? While any increase in biomass is relatively easy to measure, it is only with the establishment of the actual nature of the apparent steady state that it becomes possible to identify the fundamental cellular processes occurring and to deduce their kinetic and stoichiometric constants.

Conclusions

The following points establish the concepts and methodology necessary to achieve a meaningful analysis of biological activity associated with a biofilm.

1. Fundamental cellular processes should be identified and their kinetic and stoichiometric characteristics determined quantitatively. Additional information regarding molecular mechanisms may be obtained from assay of specific metabolic activities.
2. All biofilm measurements should preferably be made *in situ* and cause minimal disturbance to the system.
3. It should be appreciated that the biofilm habitat may induce qualitative alterations in the patterns of metabolic activity.
4. Mass transfer to the biofilm and diffusional resistances within the biofilm are major determinant factors controlling biofilm activity.
5. Physical and biological heterogeneities introduce unique characteristics to biofilm processes and individual metabolic activities. Of particular importance is the aerobic/anaerobic interface when this occurs within the biofilm.
6. The existence of microbial consortia negates earlier concepts of complex biofilm processes based on the activities of individual species operating in isolation.
7. The biofilm steady state must be established and its nature identified.

MODEL SYSTEMS

In attempting to develop theoretical and experimental models of biofilms, one is faced with the classic dilemma found with all biological systems and

their analyses. Where the subject of study (be it complex natural ecosystem, biofilm, or bacterial cell population) is maintained whole and undisturbed in order that any findings may be of maximal relevance to the naturally occurring system as a whole, there is an inevitable loss of detail in experimental data and loss of rigor in their interpretation. On the other hand, where an experimental system is devised that can be subjected to unequivocal experimental analysis, it is likely to be far removed from the complexity of the physical and biochemical interactions that characterize the natural system being modeled. The techniques employed to minimize these inherent limitations center on both simplifying the structural and functional complexities of natural systems and on building complexity in a defined and controlled manner into simplistic model systems. Both approaches are evident in biofilm studies and it will be the judicious marrying of their respective concepts and experimental findings that will determine our increasing knowledge and understanding of biofilm processes.

Pseudomonas aeruginosa Monospecies Biofilm

Characklis and co-workers have made extensive studies of monospecies biofilms in an annular reactor operated as a continuous flow stirred tank biofilm reactor known as a RotoTorque (Fig. 4), using *P. aeruginosa* as their test organism (Robinson et al. 1984; Bakke et al. 1984, 1989). Their purpose

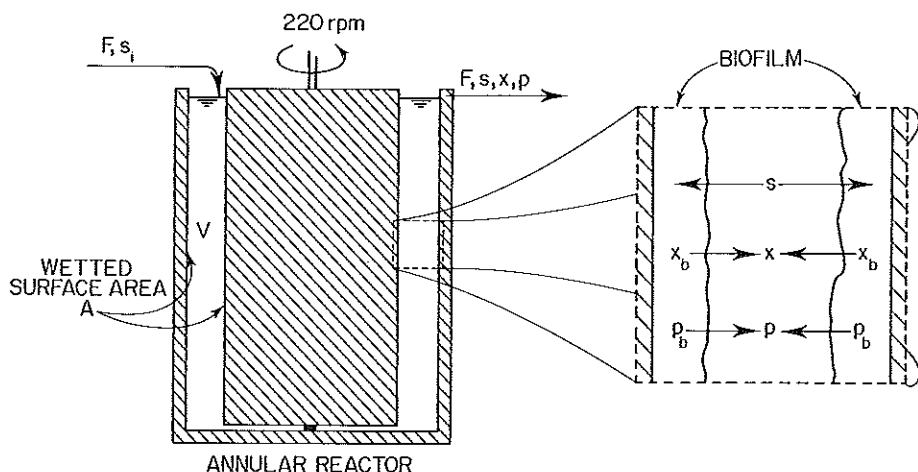


Fig. 4—The RotoTorque annular reactor operated as a continuous flow stirred tank reactor. Reproduced with the permission of *Biotechnology and Bioengineering*. (Bakke et al. 1984).

was to present a predictive model of the kinetics and stoichiometry of biofilm processes. The major impetus for modeling an environmentally atypical monospecies biofilm is the facility it offers to elucidate the fundamental processes leading to accumulation and persistence, without the confounding factors of population dynamics and species interactions.

The model is based on the constitutive equations of materials balance, along with consideration of mass transfer into and diffusion through the biofilm itself. The validity of the model has been tested by comparing theoretical predictions with experimental data. In particular, intrinsic properties determined in chemostat experiments have been shown to apply also to biofilm processes, provided that there are minimal mass transfer and diffusional resistances. Mass transfer resistance has been shown to be negligible in the RotoTorque at rotational speeds in excess of 150 rpm, and measurement of the so-called effectiveness factor (ratio of observed specific substrate consumption rate in biofilm to calculated rate presuming no diffusional resistance) gave values never less than 0.9 for biofilms up to 50 μm thick. Under these conditions, the intrinsic properties $\mu_{\text{max}} = 0.37 \text{ h}^{-1}$ and $K_s = 2 \text{ mg glucose carbon l}^{-1}$ determined in the chemostat applied equally to the biofilm. Values for the intrinsic yield coefficients were also obtained; they were estimated to be 0.34 g g^{-1} and 0.27 g g^{-1} for cells and EPS, respectively. It was further noted that in both the chemostat and the RotoTorque biofilm studies there existed the same linear relationship between growth rate, μ , and the specific cellular substrate removal rate, q_s (Fig. 5). This does not apply, however, where the parameters are rather total biomass production rate and substrate removal rate per unit of biofilm biomass. That is, the relationship holds for cells but not for cells plus EPS. This is a direct consequence of the accumulation of EPS due to the prolonged retention time of individual cells within the biofilm, as compared to the chemostat.

In parallel studies, in a rectangular duct reactor with laminar flow, it was found that although the biofilm reached a constant thickness within 24–40 h, cell density continued to increase for up to 15 days. In the RotoTorque, the model allows prediction of the increase with time of biofilm cell mass against both influent substrate concentration and dilution rate. Predictions of substrate, cells, and polymer in the bulk phase, and of cells and polymer in the biofilm, have been compared with experimental data. With the possible exception of polymer in the biofilm, there is good agreement between theory and experiment.

The detachment or cellular erosion rate is directly proportional to biofilm cellular density rather than thickness (and is influenced by hydrodynamic shear force) and is independent of the substrate loading rate, growth rate, and dilution rate. The steady state in these RotoTorque biofilms occurs where the growth rate is balanced by the rate of materials loss (cells plus EPS) by erosion.

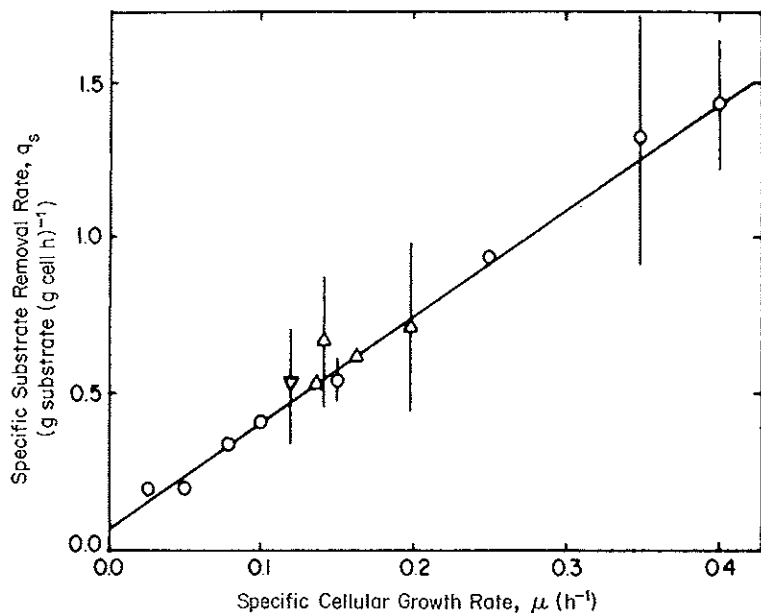


Fig. 5—Substrate removal rate, q_s , versus growth rate, μ , in a steady-state biofilm. Δ : $D = 6 \text{ h}^{-1}$; ∇ : $D = 3 \text{ h}^{-1}$; O: data from chemostat applied to biofilm model. Reproduced with the permission of *Biotechnology and Bioengineering* (Bakke et al. 1984).

In the model, homogeneity of distribution of cells and EPS throughout the biofilm has been assumed, along with constant biofilm density. We know, however, that there can be surface irregularities in mature biofilms and that biofilm density does in fact change with time. With other species, notably *Klebsiella pneumoniae*, biofilm thickness can be extremely variable (Siebel 1987). Such factors may have major influences on mass transfer and diffusional resistances, and on biofilm processes themselves. Clearly, therefore, each experimental model must be fully characterized and direct extrapolation from one system to another cannot be taken for granted. Equally, each theoretical model must have its assumptions explicitly stated and be subject to continuous refinement and development in the light of data obtained from experimental analyses.

Sulfate-reducing Bacterial Biofilms

The RotoTorque annular reactor has also been used in a study of biofilm kinetics with an anaerobic sulfate-reducing biofilm (Nielsen 1987). Although, not strictly a monospecies biofilm, the inoculum (enrichment from sewage),

nutrient (excess lactate, with sulfate), and physicochemical conditions (anaerobic) made it likely that the biofilm would be predominantly made up of one or a number of closely related species of sulfate-reducing bacteria. During the development phase over 10 to 12 days there were exponential increases in biofilm thickness up to 300 μm and in sulfide production rate, giving an estimated doubling time for the sulfate reducers of less than 30 h. Estimates of K_m and V_{max} for sulfate reduction were made with homogenized samples of biofilm removed from the reactor. Not surprisingly, therefore, the data obtained were comparable with other estimates of these intrinsic properties in suspended cells; $K_m = 1.4 \text{ } \mu\text{mol l}^{-1}$, $V_{\text{max}} = 1.4 \text{ mmol SO}_4^{2-} \text{ g}^{-1} \text{ h}^{-1}$. These values and an estimate for the molecular diffusion coefficient of sulfate through the biofilm were applied to a mathematical predictive model of biofilm kinetics and the data obtained compared with experimental findings for sulfide production rate against sulfate concentration at two biofilm thicknesses (Fig. 6).

Two interesting practical observations were that (a) diffusional resistance became significant with biofilms of less than 300 μm when low sulfate concentrations (0.1 to 0.5 mmol l^{-1}) were employed and (b) the activity of biofilms appeared to be two or three orders of magnitude greater than that experienced in marine sediments. This was taken to be due to the high cell concentration within biofilms rather than to any higher sulfate reduction activity in individual cells.

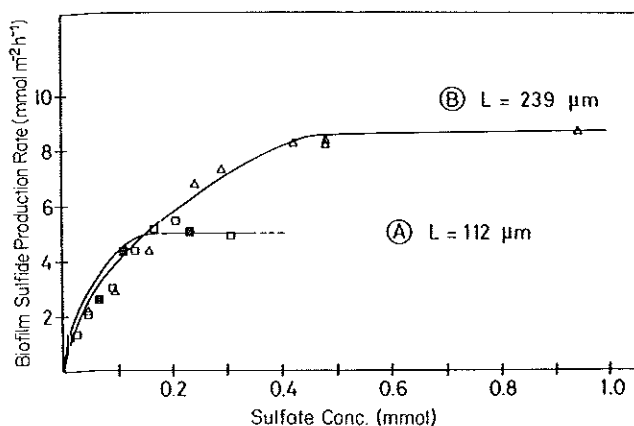


Fig. 6—Biofilm sulfide production rate at various concentrations of sulfate. A: experimental data for sulfide production, $\square$, and sulfate reduction, $\blacksquare$, in 112 μm thick biofilm; B: experimental data for sulfide production, $\triangle$, in 239 μm thick biofilm. The two curves are predictions from the model for sulfate uptake in the biofilm, based on data taken from cells in suspension. Reproduced with permission from *Applied and Environmental Microbiology* (Nielsen 1987).

Pelobacter acidigallici Monospecies Biofilm

The surface colonization and attendant life cycle of the bacterium *P. acidigallici* was studied in a continuous flow microchamber with gallic acid as sole carbon source (Szewzyk and Schink 1988). It was noted that the development of a biofilm was markedly influenced by available substrate concentration. Whereas at high gallic acid concentrations ($> 5 \text{ mmol l}^{-1}$) there was a high number of suspended cells leading to a fast deposition rate with growth resulting in a homogeneous biofilm within 3–6 days, at low gallic acid concentrations ($0.1\text{--}1.0 \text{ mmol l}^{-1}$) there were fewer suspended cells and a lower deposition rate leading to microcolonies remaining isolated for a longer period. Even after 25 days the biofilm retained a heterogeneous structure with well-developed colonies and smaller microcolonies or even single cells in the intervening spaces. The actual growth rates at the surface, over a wide range of substrate concentrations ($0.1\text{--}10 \text{ mmol l}^{-1}$), are comparable with rates calculated for suspended cells.

Szewzyk and Schink (1988) point out that the formation of heterogeneous biofilms at low substrate concentration may have far-reaching consequences since such conditions are characteristic of most natural environments. The majority of biofilm models on the other hand make an explicit assumption of structural homogeneity.

Aerobic Multispecies Biofilms

A constant depth thin film fermentor has been developed by Wimpenny and his colleagues (Wimpenny, this volume) and used to study the kinetics of biofilm growth resulting from an inoculum of a mixed aerobic enrichment from river water. The design of the fermentor with the scraper bar ensuring constant biofilm thickness gives rise to an experimentally determined steady state. At least for periods of up to 10 days after the biofilm has attained maximal protein content, this steady state is maintained with constant cell and total biomass.

Low nutrient concentration (5 mg l^{-1} glucose) was employed and growth was determined by increase in cells (protein) and in total biomass (dry weight). Using media flow rates of 2 ml min^{-1} and 7 ml min^{-1} , steady-state values of biofilm protein were achieved in approximately 14 days, allowing estimates of a doubling time of around 60 h. In later experiments, slower growth rates were determined but the composition of the enrichment inoculum may have altered, and in any case no logically sensible comparison can be made with the growth rate of the individual species in pure suspended culture. Assuming constant geometry (i.e., film pans always filled to maximum depth with biofilm), this increase in protein with time represents increasing cellular density of the biofilm.

The fermentor operates aerobically but it is interesting to know at what depth within the biofilm anaerobic conditions will be created by the balance

of diffusion of oxygen into the biofilm against its utilization and exhaustion by virtue of the metabolic activity of the aerobic species constituting the biofilm. The authors have attempted to answer this point using an oxygen microelectrode attached to a micromanipulator. Unfortunately, the film pan must be removed from the fermentor and, to date, it has not proved possible to measure oxygen concentrations under shaken or stirred conditions that would remove possible limitations of mass transfer resistance affecting the passage of oxygen from the bulk aqueous phase into the biofilm. Nonetheless, it is clear from preliminary studies that anoxic conditions prevail at or very close to the open surface of the biofilm.

Using a much less-defined system, biofilm development with dental plaque bacteria has been examined with acrylic tiles suspended in a chemostat (Keevil et al. 1987). From earlier work these authors demonstrated that conditions of nutrient limitation could influence the cellular composition of mixed culture biofilms and concluded that surface-associated organisms grow at approximately twice the rate of the same organism in the surrounding medium. In the present study, they noted variation in cellular composition of the biofilm with time and concluded that the biofilm community and its consequent activities were qualitatively different from the mixed culture in which the acrylic surface had been suspended.

Sulfate-reducing Bacteria in Biofilm Communities

Hamilton and his co-workers have studied the presence and activity of sulfate-reducing bacteria in biofilm communities formed from marine inocula using two different experimental procedures. When metal test coupons (1.5×3.0 cm) made of structural steel were exposed in a mildly polluted estuarine environment there was a rapid colonization by aerobic heterotrophic bacteria: 10^4 – 10^5 being culturable from each coupon after 1 day with a plateau value of 10^7 – 10^8 being reached after 20 days. Sulfate-reducing bacteria could also be cultured from these aerobic biofilms but only after 3 days, and they took longer to reach a maximum value of 10^2 – 10^3 . The results were even more striking when *in situ* assays of sulfate reduction within the undisturbed biofilm were obtained by $^{35}\text{SO}_4^{2-}$ radiorespirometry (Maxwell and Hamilton 1986). Measurable activity could only be demonstrated after the coupons had been exposed for 20 days. That is to say, after a short lag of 3 days it was possible to culture strictly anaerobic bacteria from these biofilms formed in an aerobic environment. A longer period of exposure was necessary, however, before sufficiently reduced conditions had developed within the biofilm to allow the sulfate reducers to become metabolically active *in situ*. Care must be exercised, however, in interpreting such data as the relative sensitivities of the assays for cell numbers and activities could be at least partially responsible for the difference between 3 and 20 days for the first positive results.

Information was also gained regarding the nature of that activity. When mature biofilms (64 days) were incubated for 24 h in the sulfate reduction assay they became substrate-limited; this limitation could be overcome by the addition of either lactate or acetate to the incubation (Hamilton and Maxwell 1984). Lactate and acetate are not found in significant amounts in seawater and they must therefore be produced in the biofilm by the hydrolytic and fermentative action of the general heterotrophs and so ultimately support the growth of lactate- and acetate-utilizing sulfate-reducing bacteria. The organisms making up the biofilm therefore demonstrate positive interactions resulting in biochemical activities which are absent from the bulk liquid.

Similar studies have been carried out in a laboratory flow system inoculated with seawater enrichments for general aerobic (facultative) heterotrophs, general anaerobic heterotrophs, and sulfate-reducing bacteria (S.M. Parkinson and W.A. Hamilton, unpublished). Here the biofilm was formed under anaerobic conditions and with relatively high levels of nutrient in the circulating medium: 1.34 mmol l^{-1} glucose and 1.7 mmol l^{-1} lactate. Mature biofilms were formed within 10 to 12 days and the numbers of all three types of bacteria were in the region of 10^7 – 10^8 per removable stud. Again it was possible to demonstrate substrate limitation in the sulfate reduction assay and a much wider range of potential substrates was tested for stimulation of the activity. Positive results were obtained with lactate and acetate but also with benzoate, ethanol, formate, fumarate, hexanoate, propionate, pyruvate, and possibly with succinate, laurate and malate. It remains to be shown whether all of these substrates are directly supporting the growth of what would therefore have to be a very extensive range of sulfate-reducing species (although they were originally enriched from lactate-based media) or whether they must themselves be further metabolized by the general heterotrophs to give a more limited range of substrates such as lactate and acetate to support sulfate reducer activity and growth.

Sulfate-reducing Bacteria in a Constructed Multispecies Biofilm

The RotoTorque has also been used in an effort to establish more controlled biofilms containing sulfate-reducing bacteria (G.F. Gammack and W.A. Hamilton, unpublished). The rationale has been to introduce into the RotoTorque two organisms whose individual physiological characteristics in suspended culture are well known and to observe then the nature of their interactions within a biofilm ecosystem. The organisms chosen were a marine *Vibrio* sp. as a facultative heterotroph and the sulfate reducer *Desulfovibrio desulfuricans*; conditions were anaerobic. In batch culture with glucose as sole carbon source it was shown that *D. desulfuricans* grew on the small

molecular weight mixed acid fermentation products from the growth of the *Vibrio* on the primary substrate.

It has so far proved difficult to establish a stable bispecific steady-state biofilm. Initial lack of success was due to poor colonization rates by the sulfate reducer ($\leq 7 \times 10^5$ cells cm^{-2}), combined with generalized sloughing and nonreplacement of the biofilm on the surface. Recently, however, colonization rates by *D. desulfuricans* have been improved dramatically ($\leq 4 \times 10^8$ cells cm^{-2}) by serial subculture of the two organisms in a batch culture prior to RotoTorque inoculation. This procedure presumably allows the sulfate reducer to adapt its metabolism for growth on the *Vibrio* sp. fermentation by-products. Using this technique, combined with a reduction in growth rate from $D = 0.25$ to 0.15 h^{-1} , resulted in a stable biofilm, with respect to protein content, over a period of seven weeks. The *D. desulfuricans* numbers reached a maximum, however, at around 3.5 weeks, followed by their disappearance by 6 weeks. This "washout" from the biofilm may be indicative of the dynamic nature of multispecies biofilms and highlights the difficulty of achieving in this type of system steady-state conditions, as defined in chemostat terms.

Even when conditions have been modified to allow the development of a steady-state biofilm, considerable problems still remain in terms of analytical procedures capable of identifying the biochemical activities and quantifying their integration in the various biofilm processes. Ideally, for example, the RotoTorque should be operated aerobically in order to allow characterization of the role of the aerobic/anaerobic interface in the biofilm, but autooxidation of produced sulfide would further restrict respirometric analysis. A further major limitation of this and other biofilm systems remains the inability to measure *in situ* activities, not only of the whole biofilm but of specific areas within it, spatially differentiated in both the horizontal and vertical dimensions. In this regard, cytochemical and microprobe techniques may eventually allow at least partial answers, but only where the basic experimental design allows direct incorporation and observation of such analytical tools.

Summary

The various model systems described demonstrate a spectrum of conceptual approaches and experimental methodology. None of them can claim to meet all of the seven requirements specified earlier but all of them meet some of these requirements. Putting it another way, no model has all the answers but taken as a group such models can lead us to a greater understanding and so ultimately to more direct control of biofilm processes. In working toward this ultimate goal we must not only define what we mean by "activity" and "biofilm," we must be very clear exactly which questions our

particular model can answer, and which it cannot. Furthermore, we must both make the assumptions of our model explicit and be certain of the validity of these assumptions. These conditions rely on a comprehensive understanding of our present knowledge of biofilms and will in turn lead to an increase in that knowledge and understanding.

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