00-029

1 Structure and Function of Biofilms

Zbigniew Lewandowski

Biofilms consist of microcolonies separated by interstitial voids and are heterogeneous in many respects *e.g.* structurally, chemically, and physiologically. A new model of biofilm structure, declaring microcolonies as building blocks of biofilms, is used to interpret experimental results and to verify hypotheses about the relations between biofilm structure and function. Microscale chemical profiles, intrabiofilm hydrodynamics, and intrabiofilm mass transport mechanisms are all affected by biofilm heterogeneity.

KEY WORDS: biofilm structure, chemical gradients, intrabiofilm mass transport, intrabiofilm hydrodynamics

INTRODUCTION

During the last decade it became obvious that the researchers who studied biofilms at the microscale accumulated a collection of experimental results that were impossible to interpret using the traditional conceptual model of biofilms where microorganisms are uniformly distributed in a continuous matrix of extracellular polymers. Some of these results are discussed later in this chapter. As a solution, a new conceptual model of heterogeneous biofilms has been suggested. There are several versions of that model now, all conveying the same message, *viz.* that biofilms consist of microcolonies separated by interstitial voids. It soon became evident that the basic declaration of the new model, that the building blocks of biofilms are microcolonies, may have implications going much further than initially expected. Reported evidences of spontaneous microbial coaggregation and cell-cell recognition (Kolenbrander and London, 1992; 1993), and cell-cell communication in biofilms (Davies *et al.*, 1998) were quickly associated with the new biofilm model and hypotheses were suggested regarding the possible role of microcolony structure and internal cell organization in biofilm activity and survival. The journal *New Scientist* expanded on these hypotheses in an article published in August 1996, and emphatically compared biofilms to cities built by microorganisms. General expectation among biofilm researchers is that these elaborate microorganism-formed structures have meaning. However, notions endowing biofilms with abilities to intentionally control these structures, and their environment, should be approached with caution, as there is little experimental evidence

to support them. Nevertheless, the unusual propensity of microorganisms to form complex structures on surfaces has been noted by many researchers (Keevil and Walker, 1992; Costerton *et al.*, 1994, Massol-Deya *et al.*, 1994; Wolfaardt *et al.*, 1994; Bishop and Rittmann, 1995), although the reasons for this remain unclear. In natural and engineered systems a spectrum of structurally heterogeneous biofilms is observed ranging from dense, amorphous biofilms, which are less structurally heterogeneous, to biofilms demonstrating robust, well developed structures.

This chapter discusses the relations between the structure and function in biofilms. The popular term biofilm structure means, more often implicitly than explicitly, spatial distribution of biomass density in biofilms, or, sometimes, the complementary distribution of biofilm porosity. Biofilms with well-developed microcolonies, separated by wide interstitial voids are considered "structurally heterogeneous", and most of their biomass is concentrated within microcolonies. The terms structurally heterogeneous and morphologically heterogeneous are used interchangeably and should be considered synonyms. Usually a heterogeneous biofilm means a structurally heterogeneous biofilm while other heterogeneities, when referred to, carry appropriate adjectives, *e.g.* physiological, chemical, ecological. Defining biofilm function is more challenging because it means different things to different people, depending on their interest in biofilms. To construct such a definition biofilms may be looked at as agents of certain activities and the term function associated with the main human activity the biofilm impacts; biofilm function is then realized through the agency of the biofilm. For example, the main function of biofilms forming dental plaque is tooth decay, while the main function of biofilms contributing to microbially influenced corrosion is accelerated anodic dissolution of metals, and the main function of biofilms responsible for bioremediation of toxic compounds is converting toxic compounds to a more benign form. Biofilm function may also refer to their ability to exhibit specific physiological reactions, *e.g.* nitrification, denitrification, or sulfate reduction (function: removal of ammonia, nitrate, sulfate). Clearly, biofilm function is an obscure term covering a broad spectrum of meanings and should be defined each time it is used. For the purpose of this chapter biofilm function is identified with substrate conversion. From such a definition biofilm function can be evaluated as the substrate conversion rate, overall or local, and quantitatively related to biofilm structure.

Many concepts and all the experimental results presented in this chapter originated in the Biofilm Structure and Function group of the Center for Biofilm Engineering, Bozeman, Montana. Experimental results have been selected to illustrate the opinions of that research group which, consequently, may be biased toward promoting their definition of biofilm function and toward exposing relations between biofilm structure and biofilm activity, the main area of study. Important aspects of biofilm research, related to other definitions of biofilm function, as well as relationships between biofilm structure, biofilm ecology, and genetics have been omitted.

BIOFILM STRUCTURE AND THE CONCEPT OF HETEROGENEOUS BIOFILMS

The model of heterogeneous biofilms was needed to interpret experimental results difficult to explain using the model of homogeneous biofilms. Examples of such results follow.

Example 1

Drury (1992) introduced small (1 μm diameter), fluorescent latex particles during biofilm growth and studied their fate. The expectation was that these particles, after settling on the biofilm surface, would be pushed off by the growing and exfoliating bacteria. According to one of the assumptions accepted for biofilm modeling, the displacement would be perpendicular to the substratum (Wanner and Gujer, 1986). In a sense the beads should have imitated bacteria having a growth rate equal to zero and should have been pushed out by faster growing microrganisms. After the experiment was terminated, the biofilm was sectioned and the beads were recovered. Many of them were found at the bottom of the biofilm, near the substratum. This contradicted one of the assumptions of the model. If the biofilms were a continuous gelatinous layer, as the model stipulated, how did the beads get to the bottom of the biofilm? Clearly, the model of homogeneous biofilms could not give a satisfactory explanation. On the other hand, in heterogeneous biofilms such an effect is expected; the beads penetrate and are trapped in interstitial channels that frequently reach the bottom of the biofilm. This technique is now used to study flow rate within biofilm (see Figure 1a); fluorescent beads are injected into individual voids/channels in a biofilm and their movement followed with confocal scanning laser microscopy (CSLM) (DeBeer *et al.*, 1994a; Stoodley *et al.*, 1994).

Example 2

An argument often used when discussing microbially influenced corrosion (MIC) was that if biofilms are forming continuous layers on metal surfaces, they should decrease the corrosion rate, not increase it, because they deplete oxygen, the principal cathodic reactant, near the surface. The experimental observations indicating the opposite are difficult to interpret. In heterogeneous biofilms, on the other hand, oxygen freely penetrates to the substratum through the voids and, therefore, causes formation of differential aeration cells and accelerates corrosion. Differential aeration cells cannot explain the whole complexity of MIC but they are a known phenomenon, and do not raise immediate objections. Besides, their role has been demonstrated experimentally (Roe *et al.*, 1996). Oxygen consumption within microcolonies also promotes growth of SRB whose sulfidogenic activity influences the chemistry and electrochemistry near metal surfaces and initiates a second, longer phase of active corrosion (Lee, W. *et al.*, 1995; Hamilton, 2000).

Example 3

The use of microelectrodes to measure chemical profiles in biofilms was expected to help verify models of biofilm activity. Microelectrodes with a tip diameter of the order of a few microns are driven across biofilms, measuring concentration profiles with high spatial resolution. These measurements provide valuable insight into the chemistry of the inner space of a biofilm. However, their use to verify mathematical models of biofilm activity has been impeded by the fact that profiles measured at different locations were different to an extent that could not be justified by experimental error only. As long as single profiles were analyzed, which was the case in most early

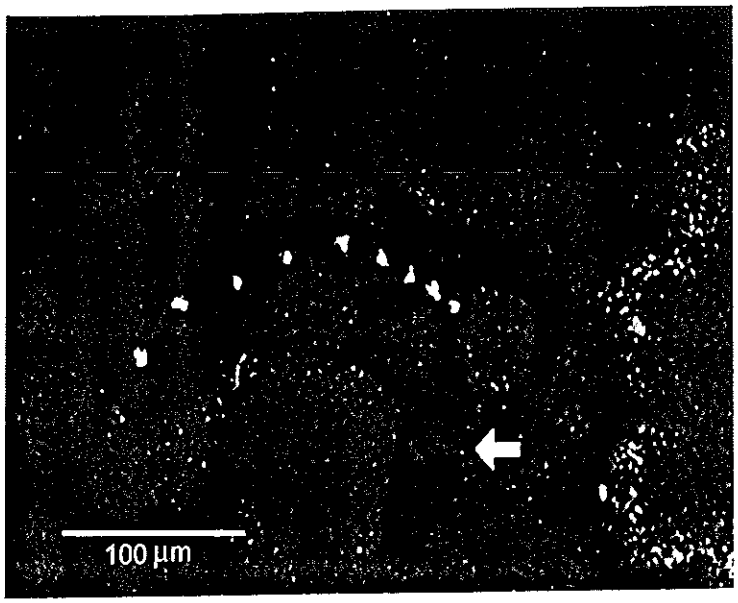


Figure 1a Confocal image of biofilm structure. A small fluorescent latex bead was injected into the biofilm and has moved along the network of channels. The arrow indicates the direction of water flow. (Reproduced from Stoodley *et al.*, 1994, with permission.)

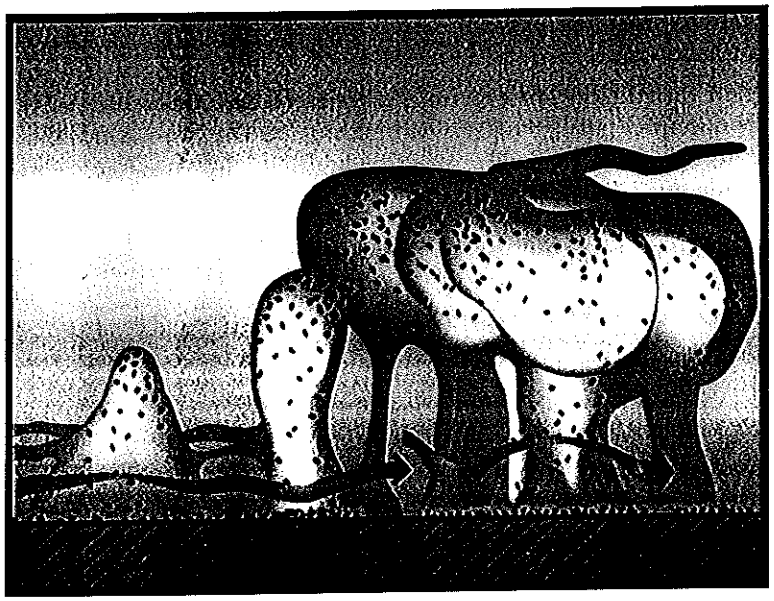


Figure 1b Diagrammatic representation of the structure of a hypothetical bacterial biofilm drawn from CSLM examination of a large number of mixed-species biofilms. The discrete microcolonies of microorganisms are surrounded by a network of interstitial voids filled with water. The arrows indicate convective flow within the water channels.

publications, everything worked as expected. Mathematical models of biofilm activity, however, describe biofilm activity over a certain surface area, not only at a single point. Attempts to find an average substrate consumption rate over a certain area often generated unexpected variability in that parameter. Since there was no independent technique to verify the microelectrode measurement, the suspicion was that the microelectrodes were not accurate. Therefore, the first confocal images of the inner space of biofilms were most welcomed by those who had invested time and effort in developing microelectrode technology; the biofilms on those images appeared to be quite heterogeneous (Lawrence *et al.*, 1991, see also Figure 1a). This could, possibly, explain why chemical profiles were so different at different locations in biofilms. Indeed, biofilm heterogeneity strongly influenced the shape of chemical profiles measured by microelectrodes, as was demonstrated by combining microelectrode measurements with confocal microscopy (DeBeer *et al.*, 1994b).

Example 4

Flow velocity distribution in biofilms was first studied using nuclear magnetic resonance imaging (NMRI) (Lewandowski *et al.*, 1992, 1993a; 1993b). It was expected that the biofilms covering walls of conduits would behave as if they were merely decreasing the dimensions of the conduits. Flow velocity was expected to decrease on approaching the biofilm surface and to finally reach zero at the biofilm surface. However, careful measurements of flow velocity distribution in biofilms demonstrated that the flow velocity was reaching zero at the conduit surface, instead of the biofilm surface as expected (Lewandowski *et al.*, 1993b). Again, such an effect is expected in heterogeneous biofilms where water can move in the interstitial voids. Flow velocity is now routinely measured in individual pores of heterogeneous biofilms (DeBeer *et al.*, 1994a; Stoodley *et al.*, 1994; Xia *et al.*, 1998).

The foregoing examples are samples of experimental results which lead to the change in the conceptual model of biofilm structure. According to the new model, biofilms are made of microcolonies separated by interstitial voids. Microcolonies are compact aggregates of extracellular polymers with densely packed microorganisms. Voids between these aggregates are filled with water and, perhaps, with strands of extracellular polymers connecting individual microcolonies. The shape of the microcolonies appears to be different in different biofilms, a fact that became an issue among different research groups. However, despite some differences in opinion about the shape of microcolonies, the extent of heterogeneity, and the somewhat different terminology used by different research groups the general conclusion is that biofilms are structurally heterogeneous. Figure 1a shows an image of a heterogeneous biofilm with microcolonies separated by interstitial voids, while Figure 1b shows an idealized model of biofilm structure derived from images such as the one in Figure 1a, *i.e.* mushroom shaped microcolonies separated by interstitial voids.

According to Figure 1b, heterogeneous biofilms are composed of 1) densely compact sublayers, 2) roundly-shaped microcolonies, 3) streamers, which are long strands of extracellular polymers extending the microcolonies, and 4) interstitial voids. The sublayer is not continuous and, at places, exposes the substratum. Above the sublayer are dense, roundly-shaped microcolonies, filled with extracellular polymers,

densely packed with microorganisms, and finished with elongated streamers extending downstream. The microcolonies are separated by interstitial voids forming a network of interconnected channels, giving biofilms their characteristic, porous structure. Water can freely move within the network of these channels.

It is now known that as biofilms get older the semicontinuous sublayer tends to get denser, thicker, and accumulates various particles from the system while the upper layer consisting of characteristic roundly-shaped microcolonies and streamers remains the same. New advances in quantifying biofilm structure (see later) show that, as time progresses, the porosity of the bottom layers is much lower than the porosity of the upper layers. It is not clear whether that decrease in porosity is the result of biofilm growth and expansion near the bottom or the effect of accumulated debris and pieces of biofilm sloughed upstream. Free flowing particles can be trapped in biofilm pores, in the same way as fluorescent beads were trapped in the experiment of Drury (1992).

Structural heterogeneity presents different challenges for microbial ecologists and for those who mathematically model biofilm activity. For microbial ecologists it is important to define how, and why, the microorganisms are organized into such structures. For those who model biofilm activity it is important to determine the relations between structural heterogeneity, mass transport rates and mechanisms, and microbial activity. At present, mathematical description of heterogeneous biofilms is inadequate, which impedes progress in understanding of the significance of biofilm heterogeneity. For modeling purposes, it does not seem useful, nor even possible, to describe the spatial distribution of activity in each microcolony and in each interstitial void. Some simplifying assumptions will have to be made. The important issue is that these simplifying assumption are made as a result of careful experimentation, not merely for computational convenience. Ultimately, this is mathematical modeling that will quantify the relations between biofilm processes at the microscale and biofilm system performance at the macroscale.

SUBSTRATE CONCENTRATION PROFILES IN HETEROGENEOUS BIOFILMS

Dissolved substrates are transported from the bulk solution to the biofilm along concentration gradients, and are used in relevant biochemical reactions. The waste products of these reactions are transported back to the bulk solution *via* the same path. Consequently, substrate utilization in biofilms may be limited by the biofilm activity, (rate limiting reactions) or by the intensity of substrate delivery to the cells (mass transport limited to the surface of the biofilm or through the biofilm matrix). Most biofilms are mass transport limited because the dissolved substrates are delivered at a slower rate than they can be consumed. The intricate interplay between factors influencing substrate concentration profiles is best interpreted using one-dimensional profiles in homogeneous biofilm, for example oxygen concentration profiles. Mechanisms determining substrate concentration profiles in heterogeneous biofilm are the same; they act in three dimensions because of the microcolony structure. Assuming that the biofilm matrix is continuous, homogeneous, flat, and that there is no substrate consumption above the biofilm surface, three major factors influence oxygen concentration profiles, *viz.* hydrodynamics, mass transport, and biofilm activity. Transport

of oxygen within the system is caused by convection and by molecular diffusion; the ratio of those two processes varies between locations and depends on the local flow rate. Spatial distribution of oxygen across the system, from the bulk liquid to the bottom, is presented as oxygen concentration profiles. At steady state, the concentration of oxygen at each point within the system is constant and determined by the rate of oxygen delivery to that point from the bulk solution equal to the rate of oxygen removal from that point, along concentration gradient. There are two boundary layers above the biofilm surface, *viz.* the hydrodynamic boundary layer and the mass transfer (diffusion) boundary layer; their presence is a direct consequence of water viscosity and substrate diffusivity. The kinematic viscosity of water solutions exceeds oxygen diffusivity (they both have the same dimension and can be numerically compared) by a few orders of magnitude and, therefore, the hydrodynamic boundary layer is thicker than the mass transfer boundary layer. Consequently, the latter is imbedded in the hydrodynamic boundary layer and remains proximate to the biofilm surface. Oxygen must travel across these two boundary layers to reach the biofilm. In the main stream, away from the hydrodynamic boundary layer, flow velocity is high, convective mass transport rate is high, and oxygen is uniformly distributed throughout the liquid. Near the biofilm surface, within the hydrodynamic boundary layer, and even more so within the mass transfer boundary layer, flow velocity slows down and the rate of convection decreases. As a result, the oxygen transport rate decreases in that zone, which is reflected by the curvature of the oxygen profile just above the biofilm surface. Below the biofilm surface a new factor, consumption of oxygen, changes the curvature of oxygen profiles from concave up, above the biofilm surface, to concave down below the biofilm surface. Mathematically, these two parts of the oxygen profile, above and below the biofilm surface, are described by different equations bound together by the requirement that oxygen flux across the common boundary, the biofilm surface, must be the same. Below the biofilm surface mass transport is said to be entirely due to molecular diffusion. Using these requirements, the shape of oxygen profiles can be analyzed numerically and relevant procedures have been developed to calculate the kinetic parameters of biofilm reactions from oxygen profiles (Lewandowski, 1991; 1994).

One-dimensional conceptual models of biofilms, such as the one described, assume that the dissolved substrates are transported perpendicularly to the substratum and that mass transport rates along the substratum are negligible. This is not true in heterogeneous biofilms. In these each microcolony acts as an entity, and the shape of boundary layers and local concentration profiles are influenced by the activity and spatial distribution of individual microcolonies, and by local hydrodynamics. That can make the substrate concentration profiles convoluted, as shown in Figure 2, using oxygen concentration profiles as an example.

HYDRODYNAMICS IN HETEROGENEOUS BIOFILMS

Hydrodynamics influences biofilms at all stages of their development. Biofilm accumulation is a net effect of cell attachment, detachment, and growth, and hydrodynamics influence all these processes. Bouwer (1987) points out that increased surface irregularity due to biofilm formation can influence particle transport rate and biofilm

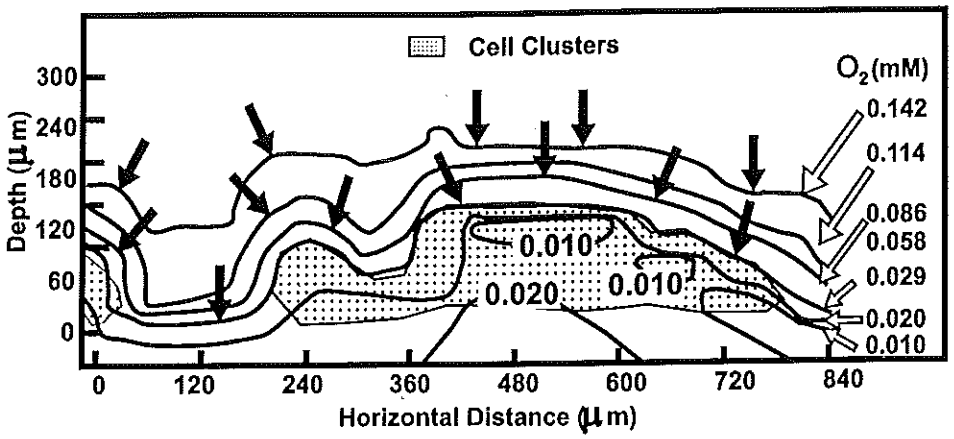


Figure 2 Oxygen concentration around a microcolony. Continuous lines = isobars; arrows = the direction of oxygen fluxes, always perpendicular to the active surface. Note that the microcolony is anoxic in the middle while oxygen is still detectable at the bottom, which demonstrates that oxygen near the bottom was transported there *via* channels and voids, not merely by diffusion through the microcolony. (Reproduced from DeBeer *et al.*, 1994b, with permission.)

attachment rate by 1) increasing convective mass transport near the surface, 2) providing shelter from shear forces, and 3) increasing surface area for attachment. In mature biofilms, the rate of substrate metabolism is controlled by the mass transport rate, which is influenced by hydrodynamics. Hydrodynamics is known to influence biofilm erosion and, possibly, sloughing.

Mathematical models of biofilm activity make assumptions about adjacent flow velocity distribution. It is important to verify these assumptions experimentally, using NMRI and flat plate biofilm reactors – rectangular conduits. The flow velocities employed were on the order of a few centimeters s^{-1}, because NMRI could not be used for higher velocities. Figure 3 shows the distribution of flow velocity in two identical reactors, one with a biofilm.

It was reasonable to expect that if biofilm accumulation increased surface roughness it should also increase the entry length to the reactor. (Entry length is the distance water must flow from the reactor entrance to the place where flow is fully developed, which, in the case of laminar flow, means parabolic distribution of flow velocity.) Entry length in the reactor with biofilm was expected to be longer than that in the sterile reactor for the same flow velocity. The flow velocity profiles were measured in both reactors at the same distance. Figure 3 shows that the flow velocity profile in the reactor with the biofilm is already parabolic while in the reactor without biofilm there still is a jet in the middle of the conduit. This result shows that the flow is already stable in the biofilm reactor while it remains unstable in the absence of the biofilm; this is opposite to expectations. This effect is believed to be caused by the viscoelastic nature of biofilms. By definition, biofilms are microorganisms imbedded in viscoelastic extracellular polymers. The expectation that biofilm increases surface roughness was based on the concept of rigid roughness elements. However, this concept does not

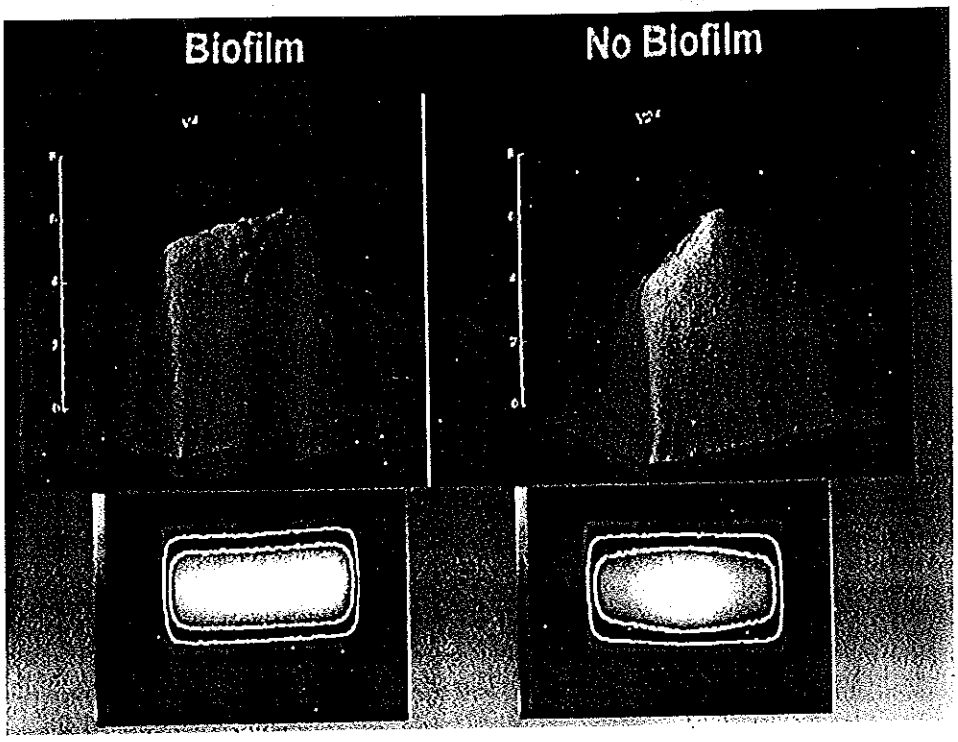


Figure 3 NMRI image of flow velocity distribution in rectangular conduits with and without biofilm. (Reproduced from Lewandowski and Altobelli, 1994, with permission.)

seem to apply to biofilms and the viscoelastic polymers do not behave as rigid roughness elements. They do not dissipate kinetic energy because the roughness elements protrude through the boundary layer. Instead, being elastic, they have the ability to move in the stream of water. Such surfaces, actively interacting with boundary layers, are called compliant surfaces and their deformations have the ability to stabilize flow in conduits (Lee T. *et al.*, 1995). Therefore, biofilms may delay the onset of turbulence in the main conduit.

When the image in Figure 3 was digitized, another unexpected result was noticed. The thickness of the biofilm in the reactor was on the order of 200 to 250 μm and it was expected that the flow velocity would reach zero at that distance from the wall. Instead, it reached zero at the reactor surface, some 200 μm below the surface of the biofilm (Lewandowski *et al.*, 1993b). Consequently, it was concluded that water was moving within the biofilm. This conclusion was later corroborated by injecting fluorescent beads to a biofilm and tracking them with CSLM.

Most biofilms, particularly those in industrial installations, are exposed to turbulent flow, while most biofilms in laboratories are grown in laminar flow. To fully appreciate the effects of hydrodynamics on biofilm growth, several flat plate reactors were operated at high flow velocities of a few m s^{-1} (Lewandowski and Stoodley, 1995). As biofilm accumulated the pressure drop across the rector was monitored for several days.

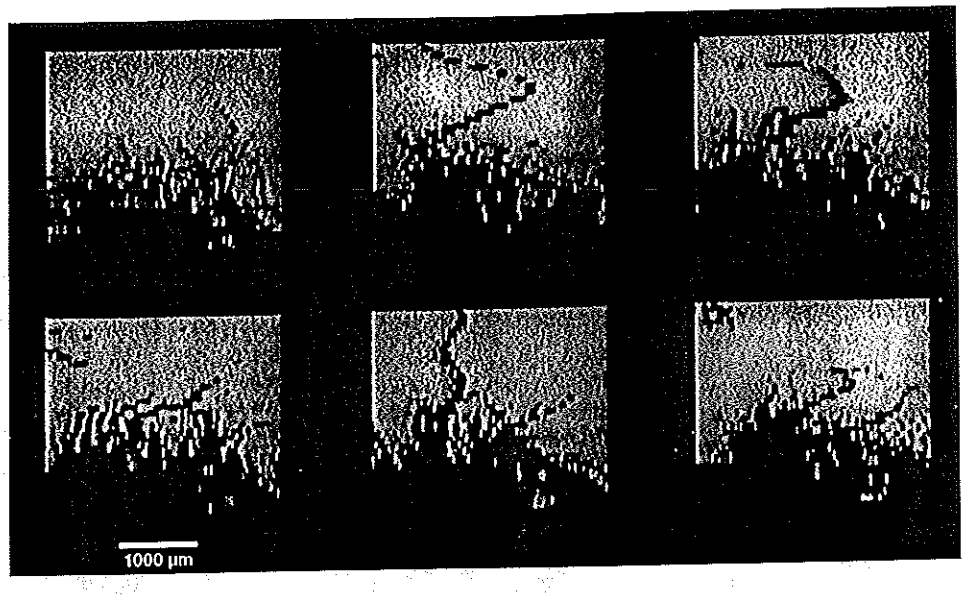


Figure 4 Surface of a biofilm with well developed streamers. The time sequence of images gives a planar view looking at the biofilm attached to the side wall of a reactor. (Reproduced from Lewandowski and Stoodley, 1995, with permission.)

As expected, the pressure drop increased with time. However, some reactors operated at lower flow velocities showed pressure drops higher than reactors operated at higher flow velocities (Lewandowski and Stoodley, 1995). This effect was, again, a consequence of the elastic and viscoelastic properties of biofilms. Microcolonies are made of bacterial cells imbedded in gelatinous extracellular polymers that can change shape under stress. At high flow velocities the hydrodynamic boundary layer separates from the microcolonies causing pressure drag which pulls the microcolonies downstream. The microcolonies, being made of viscoelastic materials, slowly flow under the strain, forming elongated shapes called streamers (Figure 4). Such streamers are often seen when biofilms grow at high flow velocities. The streamers move rapidly and dissipate the kinetic energy of the flowing water, which is reflected by the pressure drop. The movement of the streamers is transmitted to the underlying microcolonies, which oscillate rhythmically (Stoodley *et al.*, 1998). It would be expected that as the flow velocity increases, the streamers, eventually, lose mechanical stability, and separate from the parent microcolonies. Because of that, it would also be expected that streamers formed at high flow velocities should be shorter than streamers formed at lower velocities. Further, if the pressure drop is proportional to the streamers' length, that, hypothetically, explains why biofilm reactors operated at low flow velocities can sometimes show higher pressure drop than reactors operated at high flow velocity.

In conclusion, it is important, when discussing hydrodynamics in biofilms, to remember that 1) biofilms are made of viscoelastic polymers and 2) that hydrodynamics can actively change the biofilm structure. Based on present understanding, at low flow velocities biofilms would be expected to behave as compliant surfaces, to hydraulically smooth surfaces, and to stabilize the flow. However, when these

biofilms are exposed to high flow velocities they oscillate faster and, eventually, the frequency of their oscillation can not follow the frequency of the eddies. At that point biofilm oscillation is "out of phase" and the biofilm not only fails to damp the flow instabilities but actively contributes to them by randomly oscillating at a different frequency. At that point the pressure drop rapidly increases, exceeding that expected for rigid roughness elements be a few times. Such an effect was monitored before it was properly explained (Picologlou *et al.*, 1980).

MASS TRANSPORT IN HETEROGENEOUS BIOFILMS

Most mathematical models of biofilm activity assume that the transport of dissolved substrates in the bulk liquid is controlled by convection and within the biofilm by molecular diffusion (Rittmann and McCarty, 1980). This assumption, reflecting the fact that flow velocity decreases near surfaces, causing convection to become negligible just above the biofilm, was also used to interpret chemical gradients measured in biofilms by microelectrodes (Lewandowski *et al.*, 1991). However, this assumption is not consistent with experimental evidence or with the model of heterogeneous biofilms. Considering relations between intra-biofilm mass transport and microbial activity, two important factors distinguish heterogeneous biofilms from homogeneous biofilms, *viz.* 1) heterogeneous biofilms have a much larger active surface area than the surface they cover, and 2) water can move within heterogeneous biofilms delivering nutrients to the deeper layers. Quantification of the dynamics of mass transport in such biofilms has been attempted.

To study the mechanisms of mass transport in biofilms microelectrodes have been developed based on the limiting current technique, to measure local mass transport coefficients (Yang and Lewandowski, 1995), and local effective diffusivity (Beyenal *et al.*, 1998). The term local refers to the mass transport rate in the vicinity of the microelectrode tip. To evaluate local effective diffusivity, cathodically polarized microelectrodes with a tip diameter of 10 μ are used to measure local consumption of ferricyanide introduced to the biofilm reactor. The solution of ferricyanide, $Fe(CN)^{-3}_6$, dissolved in a suitable electrolyte, replaces the nutrient solution in the reactor, and is allowed to equilibrate with the biomass until there is no detectable reduction of the ferricyanide by the biomass. Then, cathodically polarized microelectrodes are driven across the biofilm and the ferricyanide is reduced at their tips to ferrocyanide, $Fe(CN)^{-4}_6$, which generates current. The current is proportional to the reaction rate (ferricyanide reduction), which is proportional to the mass transport rate in the vicinity of the microelectrode tip. From such measurements local mass transport coefficient is calculated. Local limiting current density can be also directly related to the local effective diffusivity by calibrating the microelectrodes in layers of agar of known densities and effective diffusivities for the ferricyanide (Beyenal *et al.*, 1998). Using microeletrodes is simpler and less time consuming than using microinjections of fluorescent dyes, a technique used previously (DeBeer *et al.*, 1997) to evaluate local mass transport rates in biofilms. The result of such measurements is a map representing spatial distribution of effective diffusivity of ferricyanide to the tip of the microelectrode. It would be expected that molecules of similar size to ferricyanide would have similar diffusivities in biofilms. Having independent measurements of mass transport coefficients in biofilms helps understanding chemical gradients in

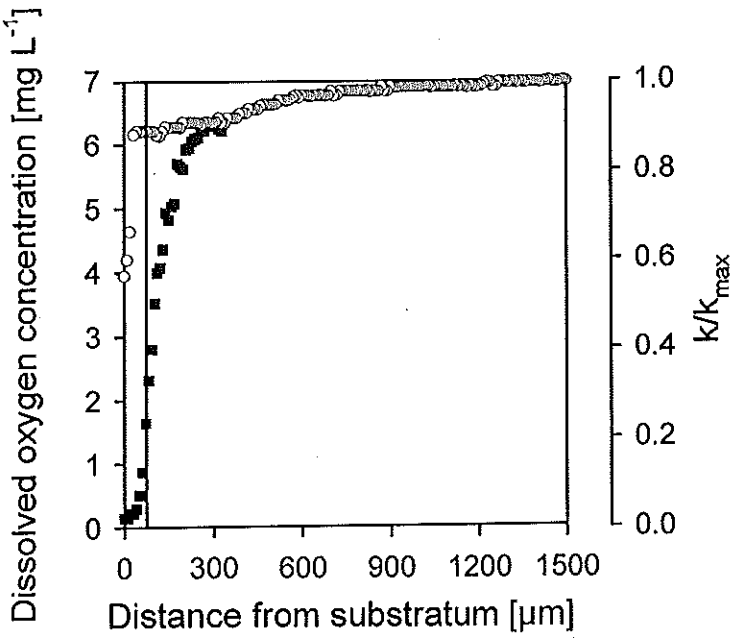


Figure 5 Profiles of dissolved oxygen (■) and local mass transfer coefficient (○) through a thin biofilm cluster. The vertical line marks the observed thickness of the biofilm. At distances less than 30 µm the wall effect caused the local mass transport coefficient to decrease. The biofilm thickness was 70 µm in this location. k/k_{max} was only slightly affected by the presence of the biofilm until a distance less than 30 µm from the substratum. (Reproduced from Rasmussen and Lewandowski, 1998, with permission.)

biofilms. Also, these measurement have helped in understanding the fact that the unusual resistance of biofilms to antimicrobial agents is not necessarily due to the high diffusion resistance of biofilm; signs of unusually low diffusivity that could be responsible for such an effect have not been seen. Parameters determining intra-biofilm mass transport can be also measured at a microscale level using fluorescence recovery after photobleaching (Bryers and Drummond, 1996), and confocal microscopy (Lawrence *et al.*, 1994).

A comparison of profiles of local mass transport coefficient and profiles of oxygen in a biofilm (Figure 5) shows that, for thin biofilms, the decrease in oxygen concentration near the biofilm surface is not correlated with the decrease in mass transport coefficient. The oxygen concentration decreases rapidly near the biofilm surface, due to oxygen consumption by the biofilm, while the mass transport coefficient remains relatively constant, due to convective mass transport within the mass transport boundary layer and water movement within the biofilm. For thicker biofilms these profiles are more complicated but, generally, mass transport coefficient profiles are poorly correlated with oxygen profiles near the biofilm surface (Rasmussen and Lewandowski, 1998).

Microelectrodes, similar to those used to measure local mass transport rates, have been calibrated to measure local flow velocity in interstitial voids of heterogeneous

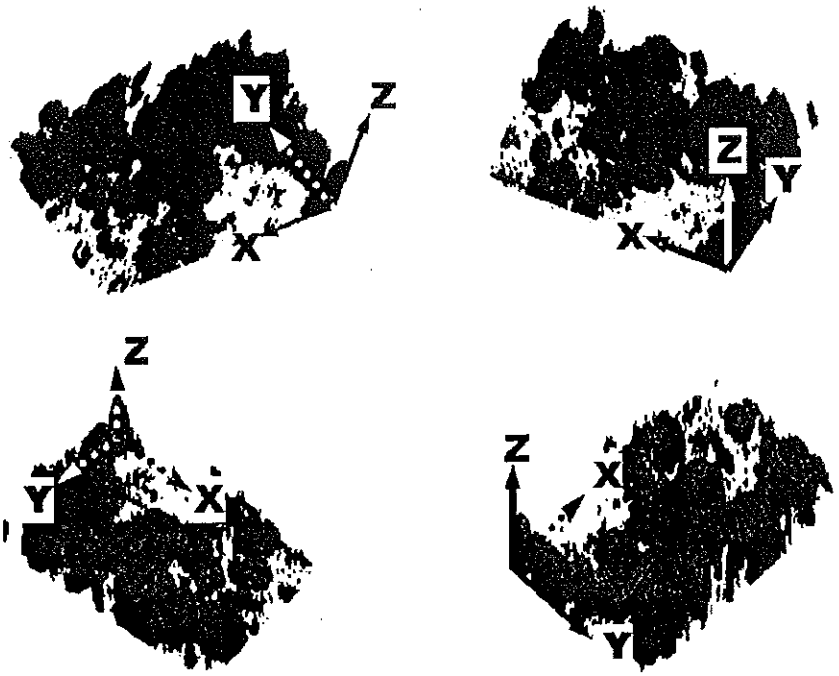


Figure 6 A digitally reconstructed 3-D image of a biofilm. To examine biofilm structure the image can be rotated around horizontal axes X and Y, and the vertical axis Z.

biofilms. This is a particularly useful and promising development since the procedures previously used to study flow velocity distribution in biofilms were either expensive (NMRI) or tedious (fluorescent particle velocimetry with confocal microscopy). Using such microelectrodes sensitive to water movement it is possible to see differences in flow velocity in biofilm channels differently oriented with respect to the flow direction in the main conduit (Xia *et al.*, 1998).

RECONSTRUCTING BIOFILMS AND QUANTIFYING HETEROGENEITY

Transport of substrates, hydrodynamics, and microbial activity are all interrelated with the structure of biofilms (Van Loosdrecht *et al.*, 1995). To study these relations, biofilms are reconstructed in three dimensions from their confocal images and chemical gradients and local diffusivity profiles superimposed on these constructs. A large number of confocal images is collected at different distances from the substratum and collated with an aid of a computer (Figure 6).

Such collated images can be rotated and sliced digitally to provide insight into the inner space of the biofilm and to understand chemical profiles measured by microelectrodes. Figure 7 shows how a convoluted effective diffusivity profile can be explained when it is superimposed on the biofilm structure. Without information about the biofilm structure such a profile could have been rejected as an artifact.

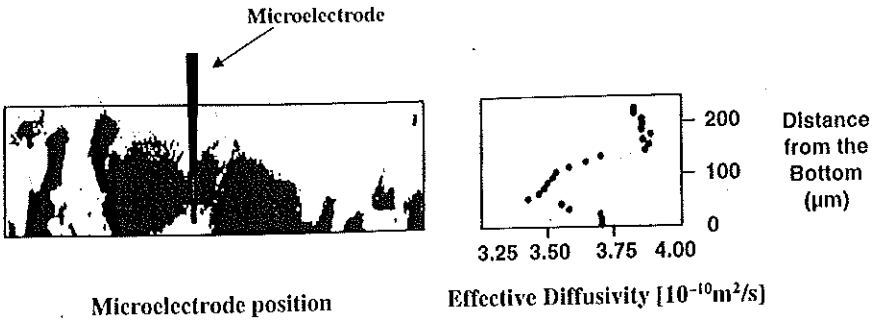


Figure 7 An effective diffusivity profile (right) is analyzed by comparing it side by side with an image of the internal structure of the biofilm (left). The internal structure can be visualized at any location by digitally slicing images such as the one in Figure 6. The results demonstrate that when density increases (within the microcolony), effective diffusivity decreases.

To study the relationships between local biofilm activity and biofilm heterogeneity it is necessary to quantify biofilm heterogeneity, and to use heterogeneity as a variable that can be correlated with other system variables such as mass transport rates and flow velocity. Attempts to quantify biofilm heterogeneity have already been made with respect to biofilm density, porosity, pore structures, tortuosity (Zang and Bishop, 1994a; 1994b) and fractal structure (Hermanowicz *et al.*, 1995). Also, attempts have been made to introduce some of these parameters to mathematical modeling of biofilm activity (Wimpenny and Colasanti, 1997; Picioreanu *et al.*, 1998).

Biofilm heterogeneity is best visualized by confocal microscopy combined with image analysis and 3-D reconstruction (Figure 6). To extract quantitative parameters from confocal images a computer program has been designed for the Unix computer operating system (Yang X *et al.*, 2000). Using these parameters, biofilm heterogeneity can be placed on a numerical scale and correlated, for example, with mass transport rates. Several parameters characterizing the shape of microcolonies and interstitial voids are calculated; four are particularly promising, *viz.* areal porosity, diffusional distance, fractal dimension, and textural entropy. Areal porosity is the ratio of the void area to the total area of an image and ranges in value from 0 to 1. The lower the value, the higher the surface coverage. Diffusion distance is the minimum distance from each cluster pixel to its nearest void pixel. The average diffusion distance and the maximum diffusion distance are calculated for a given image. Fractal dimension reflects the degree of raggedness of biofilm cell clusters and for a two dimensional image it can range from 1 to 2, where the higher the number, the more ragged the edge of the cluster. Textural entropy measures biofilm homogeneity; it has a minimum value of 0 and no theoretical maximum value. High values represent more heterogeneous structures. It is conjectured that there exists a finite number of parameters that uniquely describe the structure of a biofilm and contain enough information either to reflect variations in the growth dynamics or to predict the functional characteristics of the biofilm. To find these parameters reliance is placed on the fact that biofilms achieve steady state conditions, where the physical structure is dynamic at the molecular level, but static at the scale corresponding to the microscopic field

of view. If a parameter appears to approach steady state, it is behaving in the expected manner, and it is accepted as describing biofilm heterogeneity. Initial experiments indicate that porosity, fractal dimension, difusional distance, and fractal dimension are such parameters and appear to reach steady state for some biofilms (Lewandowski *et al.*, 1998).

CONCLUDING REMARKS

Microscale biofilm research has demonstrated that biofilms are structurally heterogeneous. However, except for the simple case of differential aeration cells in microbially influenced corrosion, it is still not clear how these structures influence the overall biofilm performance at the macroscale. The most exciting hypotheses presently tested include the active role of biofilms in controlling their own structural heterogeneity; greater structural heterogeneity encourages delivery of substrates to deeper layers of the biofilm. Therefore, if substrate concentration decreases in the bulk water, biofilms may actively increase their heterogeneity, conceivably, using cell-cell communication. Whether this is true or not is another matter. The state of the art in microscale biofilm research is such that hypotheses of this kind can be put forward and there are technical means to verify them experimentally.

ACKNOWLEDGEMENTS

This work was partially funded by Cooperative Agreement #EEC-8907039 between the National Science Foundation Engineering Research Centers Program and Montana State University, and by the Industrial Associates of the Center for Biofilm Engineering.

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