

Biofilm monitoring: a perfect solution in search of a problem

Z. Lewandowski and H. Beyenal

Center for Biofilm Engineering, P.O. Box 173980, Room 366 EPS, Montana State University, Bozeman, MT 59717-3980, USA

Abstract The main problem with monitoring biofilms is data interpretation. Biofilm heterogeneity causes monitored parameters to vary from location to location in the same biofilm, and it is difficult to assess to what extent these variations are caused by biofilm heterogeneity and to what extent they reflect other properties of the biofilm. We have used the concept of discretized biofilms, which is an integrated system of biofilm monitoring and data interpretation, to assess the effect of biofilm heterogeneity on biofilm activity. Using this approach we have estimated that a heterogeneous biofilm can be ten times more active, in terms of glucose consumption rate, than a homogeneous biofilm of the same thickness but with uniformly distributed density.

Keywords Biofilms; biofilm activity; biofilm monitoring; biofilm structure; discretized biofilms

Introduction

Biofilm monitoring is a useful, colloquial term that helps to communicate ideas among biofilm researchers and practitioners. However, it has to be explicitly stated that “monitoring biofilms” is a misnomer and one monitors biofilms only in the sense that one monitors the weather. Strictly speaking, we can monitor temperature, humidity, pressure, etc. and based on these parameters, we can generate a subjective opinion to determine if the weather is good or bad. The same holds true for biofilms; we can only monitor physical quantities in biofilms, such as thickness, activity, density, etc., and use the measurements to generate opinions about specific functions of these biofilms. For example, how thick, active, dense, etc., should a biofilm be to accelerate corrosion or significantly decrease heat transfer resistance? Progressing from monitoring physical quantities in a biofilm to quantifying biofilm functions is critical to biofilm process analysis, however, there are no clear notions of how to do so. Meanwhile, a host of tools has been developed to quantify various parameters in biofilms. They are useful tools, but they do not solve the problem of what parameters should be monitored. Even a superficial analysis indicates that the lack of suitable mathematical models of biofilm accumulation and activity, which could accept the monitored quantities as variables, aggravates the problem.

If we examine the approach to monitoring industrial fermenters, we quickly detect a pattern: the operators monitor the parameters that can be used in mathematical models to predict the system performance. For example, the operators monitor the respiration rate, biomass concentration, substrate concentrations, pH, and dilution rate in the fermenter, and use the data in the models to predict the rates of substrate consumption or product generation. In contrast, most so-called biofilm monitors generate data that is somehow related to the presence of the biofilm, or to an effect caused by the presence of the biofilm. However, the existing mathematical models cannot directly use this data, and relate them to the biofilm activity or to the effects caused by the presence of the biofilm.

Because the lack of suitable mathematical models prevents extrapolating the monitored data, the next best strategy is to monitor the parameters that are evidently related to biofilm accumulation or an effect of biofilm accumulation, and to select the intensity of the

measured signal that triggers a warning system; e.g. if the readout exceeds a certain number, add a biocide. Many biofilm monitoring systems follow such a preventive strategy, and they act as action triggers.

The most popular parameters to monitor in biofilms are: light density, heat transport resistance, electrical conductivity, torque, pressure drop, and frequency of oscillation of piezoelectric crystals. Although it is clear that all of these parameters are affected by biofilm accumulation, these parameters are also affected by other factors that are not accounted for. For example, light density, dispersed or reflected, changes with biofilm thickness, but it also depends on the concentration of particulate matter in the system, color (biofilms have different colors), or chemical composition of the water. Monitoring lump parameters such as light density or pressure drop may be quite useful, particularly when they are monitored for an extended time period at the same location; given that the operator understands, from experience, the meaning of the readouts. The expectation, that the monitored parameters can be unambiguously related to a defined biofilm function, is unrealistic.

Some difficulties encountered when monitoring biofilms are general in nature, and common in monitoring physical quantities. For example, contrary to popular belief, dissolved oxygen electrodes do not measure dissolved oxygen, and pH electrodes do not measure pH. The users must calibrate the responses of these sensors, and convert the quantity that they really measure to the quantity that they desire to know. For oxygen and pH, it is well known what affects the selectivity of these measurements, and how to calibrate the sensors. We, unfortunately, do not have such knowledge when monitoring biofilms, particularly when monitoring lump parameters like dispersed light, or heat transfer resistance. Furthermore, biofilm monitors are not selective, and it is often not even possible to assess their selectivity because they monitor lump parameters by design.

A common problem in biofilm monitoring, is the spatial distribution of the measured parameters. Biofilms are often described not as physical entities, but as descriptions of the mode of microbial growth – the attached as opposed to the planktonic (suspended) mode of microbial growth. While techniques of monitoring planktonic growth are well established, monitoring biofilm growth is at the beginning stages. Attempts to analyze biofilms by using monitors that are developed for planktonic growth causes numerous misunderstandings. Many parameters that are unambiguously determined in planktonic cultures cannot be determined as such in biofilms. For example, oxygen concentration in planktonic cultures can reach any value between zero and saturation, while oxygen concentration in biofilms often reaches *all* values between zero and saturation simultaneously, depending on the location of the measurement. This fact, often referred to as spatial distribution of the measured parameters, clearly affects the selection of parameters for monitoring.

Two terms, measuring and monitoring, are used in this paper, interchangeably, when referring to the evaluation of various parameters in biofilms. Without attempting to define the term precisely, for conversational purposes only, we define biofilm monitoring as measuring a parameter repeatedly over a period of time. Meanwhile, measuring is a one-time activity. To prevent an over-interpretation of the data acquired by a biofilm monitoring system, it is useful to contemplate three simple questions:

What quantity do we desire to know about the monitored system?

What quantity does the monitoring system *really* measure?

Is it possible to convert the monitored quantity and express it in terms of the quantity we need to know?

The number of parameters that can be measured and monitored to quantify various processes in biofilms at the micro- and macro-scale is impressive. These parameters roughly fall into four categories: (1) microbial, physiology, ecology, and genetics; (2) biofilm morphology; (3) mass transport and flow velocity; and (4) biofilm chemistry. Even though

each of the measured parameters is informative, few of them can be correlated, and it is not clear which parameters are more useful than others.

This contribution comes from the Biofilm Structure and Function Group at the Center for Biofilm Engineering, Montana State University. In our research, we routinely attempt to relate microscale biofilm structure and activity to the macroscale performance of biofilm reactors. Part of these attempts necessarily involves monitoring selected features of a biofilm at the microscale, and predicting the system response at the macroscale. Although we measure parameters belonging to all four specified categories, the purpose of this text is to document that these measurements can be used to produce a consistent system that can be used to interpret the monitored data. As stated, the primary obstacle is the lack of appropriate mathematical biofilm models that can directly use the measured data as control parameters. We bypass this obstacle by using discretized biofilms. The basis for this approach is dividing a biofilm into a finite number of layers, parallel to the substratum, and measuring the interesting parameter in each of these layers; a procedure that is well known in analyzing sediments (Berg and Petersen, 1998). To test the effect of biofilm heterogeneity, we generate two sets of data. For the first data set, we assume that the biofilm is heterogeneous, for the second set, we assume that the biofilm is homogeneous. The two sets of data give two biofilm activities, and these activities are then compared. If they are reasonably close, then the biofilm can be considered uniform. The procedure we use is illustrated later in the text, by evaluating the effect of diffusivity gradient in a biofilm on glucose consumption.

Testing whether the monitored parameters can be averaged has pragmatic significance. It is expected that the simplest biofilm monitors should be able to monitor a single parameter in a biofilm, and that it should ignore spatial distribution of this parameter within the space occupied by the biofilm. The question is: how to examine the parameters that can be monitored, and select those that will provide valuable information about the system, despite ignoring their spatial distribution. Discretizing biofilms can serve as a tool to quantify the consequences of ignoring the spatial distribution of the measured parameters, and help in the selection of parameters that are suitable for monitoring.

Discretized biofilms – the concept

Mass transport and microbial activity in heterogeneous biofilms are two, intricately interrelated processes. Using individual microcolonies as units of biofilm structure is not very productive in biofilm modeling because the number and the sizes of microcolonies vary from one biofilm to another, and even from one location to another within the same biofilm. At the same time, many biofilm researchers emphasize that structural heterogeneity of biofilms should not be ignored because it affects the spatial distribution of almost all measurable parameters. To quantify the effect of biofilm heterogeneity, we use discretized biofilms. In this approach, biofilms are subdivided into a finite number of discrete, uniform, and continuous layers. The non-uniformity of the heterogeneous biofilm is prescribed to the different average properties of each layer. Since the average properties of each layer are determined within certain limits, and are quantified by the standard deviations from the average, the model solutions can, in principle, be expressed within confidence limits, and the researcher can control these limits by selecting the number and the size of the layers. The effect under study, e.g. substrate consumption rate, is then quantified in each layer, and the results are integrated over the entire thickness of the biofilm. These principles are well exemplified using the measurement of diffusivity in biofilms.

To quantify the variations in mass transport rates between locations in heterogeneous biofilms, we use the concepts of a local mass transport coefficient, which is the mass transport coefficient at a single point within the biofilm (Yang and Lewandowski, 1995), and local diffusivities (Beyenal *et al.*, 1998; Beyenal and Lewandowski, 2000). As expected,

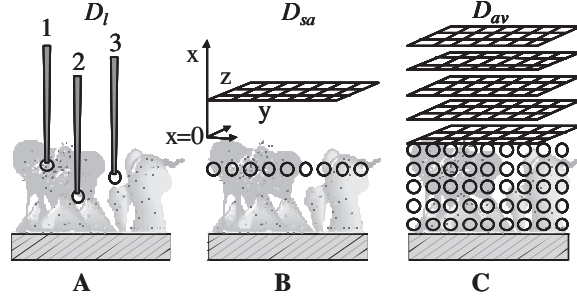


Figure 1 Discretized biofilms: concept and implementation. The system of diffusivity measurements in heterogeneous biofilms. A) Local relative effective diffusivity (D_l) measured by microelectrodes at arbitrarily selected locations at different distances from the bottom. B) The D_l are measured at grid points equally distant from the bottom. The measured D_l are then averaged, which gives the surface-averaged relative effective diffusivity, $D_{sa} = \sum_{n=1}^k D_{ln}/k$. C) The average relative effective diffusivity, $D_{av} = \sum_{n=1}^p D_{lkn}/p$, is an average of all local measurements for the entire biofilm (Beyenal and Lewandowski, 2000)

local diffusivities vary between locations, and the average diffusivity decreases toward the bottom of the biofilm. As a specific example, horizontal distributions of local relative effective diffusivities (D_l) for biofilms grown at a flow velocity of 3.2 cm/s and a glucose concentration of 50 mg/L at distances of 60 μm , 120 μm , and 180 μm from the bottom were measured. The surface-averaged relative effective diffusivities were 0.409, 0.426, and 0.449, and the standard deviations were 0.0179, 0.0195, and 0.0284, respectively (Beyenal and Lewandowski, 2000).

The effect of discretizing biofilms can be generalized. On the macroscale, microbial activity in biofilms is controlled by the rates of nutrient transfer to the biofilm and consumption within the homogeneous biofilm. Eq. (1) equates the biofilm activity with the internal mass transport, assuming constant effective diffusivity and constant biofilm density (uniform biofilm or biofilm layer).

$$D_f \frac{d^2C}{dx^2} = \frac{\mu_{\max} CX_f}{Y_{X/S}(K_S + C)} \quad (1)$$

where

- D_f = averaged effective diffusivity of growth-limiting nutrient (m^2/s)
- X_f = averaged biofilm density (kg/m^3)
- $Y_{X/S}$ = yield coefficient ($\text{kg biomass}/\text{kg nutrient}$)
- μ_{\max} = maximum specific growth rate (s^{-1})
- K_S = Monod half-rate constant (kg/m^3)
- C = growth limiting substrate concentration (kg/m^3)

Following the approach used in modeling uniform biofilms, we assume that the mass transport is one-dimensional, and that the nutrients are transferred only toward the bottom of the biofilm and are consumed by the microorganisms located in the biofilm. Using this assumption, the mass balances for nutrients around a differential element shown in Figure 2 are described below.

As described in previous sections, we attempted to calculate the average effective diffusivity over a representative surface area of the biofilm, Δx thick. By accepting the average properties of the biofilms over the differential volume ($\Delta y \times \Delta x \times \Delta z$), we ignore the variations in the z - and y -directions (they are lumped as an average into our discrete layers), but

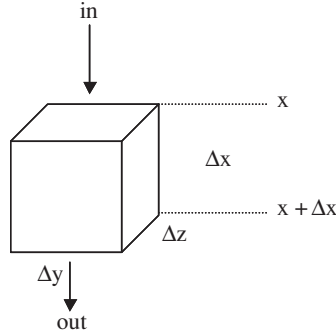


Figure 2 A differential element of a biofilm

not in the x-direction. Using this assumption, we will model the activity of discrete biofilms and test if it is meaningfully different from the activity of uniform biofilms.

If N is the flux of the nutrient, the rate of the nutrient supply into the differential element along x , is

$$\text{Nutrient(in)} = N\Delta y\Delta z \quad (2)$$

Similarly, the rate of nutrient mass flow rate from the differential element at $x+\Delta x$ is:

$$\text{Nutrient(out)} = N\Delta y\Delta z + \frac{dN}{dx}\Delta y\Delta z\Delta x \quad (3)$$

The nutrient consumption rate in the differential element is:

$$\frac{dN}{dx}\Delta y\Delta z\Delta x = \frac{\mu_{\max}CX_{fl}}{Y_{X/S}(K_S + C)}\Delta y\Delta z\Delta x \quad (4)$$

where X_{fl} is the average cell density in the differential element.

In summary, the difference between the rates of nutrient in and out from the differential element equals the nutrient consumption rate within the volume of the differential element, and the mass balance around the differential element is:

$$N\Delta y\Delta z - (N\Delta y\Delta z + \frac{dN}{dx}\Delta y\Delta z\Delta x) = \frac{\mu_{\max}CX_{fl}}{Y_{X/S}(K_S + C)}\Delta y\Delta z\Delta x \quad (5)$$

Since we have assumed one-dimensional mass transport, the diffusive flux is given by Fick's first law (Bird *et al.*, 1960):

$$N = -D_f \frac{dC}{dx} \quad (6)$$

where D_f is the effective diffusivity. However, in discrete biofilms, diffusivity changes in the x-direction, and should be represented by the variable effective diffusivity which is averaged over $\Delta y \times \Delta z$:

$$D_f = D_f(x) = D_{fx} \quad (7)$$

D_{fx} represents the average effective diffusivity in the differential volume element ($\Delta y \Delta x \Delta z$). In similar fashion, biofilm density also changes in the x-direction: biofilms are denser near the bottom than near the surface. Biofilm density can be approximated from the following equation (Fan *et al.*, 1990).

$$D_{fx} = 1 - \frac{0.43X_{fl}^{0.92}}{11.19 + 0.27X_{fl}^{0.99}} \quad (8)$$

When we insert Eqs (6) and (7) into Eq. (5), we have the following:

$$\frac{dN}{dx} = \frac{d}{dx} \left(D_{fx} \frac{dC}{dx} \right) = D_{fx} \frac{d^2C}{dx^2} + \frac{dD_{fx}}{dx} \frac{dC}{dx} = \frac{\mu_{max}CX_{fl}}{Y_{X/S}(K_S + C)} \quad (9)$$

Since, experimentally, we found that the variation of effective diffusivity by distance is constant (see Figure 3), we define the diffusivity gradient (ξ) by the following equation:

$$\frac{dD_{fx}}{dx} = \xi \quad (10)$$

which results in a mass transport continuity equation, which may be used to compute nutrient concentration profiles in discrete biofilms:

$$D_{fx} \frac{d^2C}{dx^2} + \xi \frac{dC}{dx} = \frac{\mu_{max}CX_{fl}}{Y_{X/S}(K_S + C)} \quad (11)$$

Figure 3 shows changes in diffusivity across a heterogeneous biofilm. These results are average diffusivities, and their standard deviations are determined from the results presented in Beyenal and Lewandowski (2000).

The following illustrates how important it is to discretize biofilms by comparing fluxes of nutrients to: (1) a nondiscretized uniform biofilm, and (2) a biofilm that has been discretized. Eq. (11) is a second order, nonlinear differential equation, and can be solved using numerical techniques using two boundary conditions:

$$\text{at } x=0 \quad dC/dx=0 \quad (12)$$

$$\text{at } x=L_f \quad C=C_s \quad (13)$$

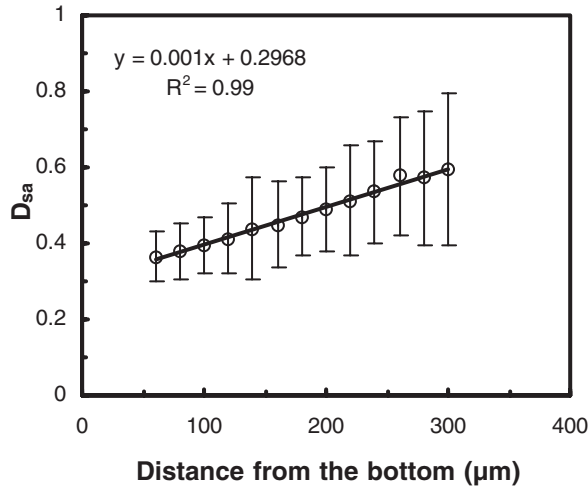


Figure 3 D_{sa} versus the distance from the bottom for biofilms grown at 7.5 cm/s flow velocity; $D_{sa} = (D_{fx}/D_w)$ is relative effective diffusivity, where D_w is molecular diffusivity of the nutrient and ξ is equal to the slope $\times D_w$. The standard deviations increase toward the biofilm surface, which reflect the fact that biofilms are more heterogeneous near the surface than near the bottom. It is also worth noticing that the effective diffusivity gradient (ξ) has the same dimensions as the mass transport coefficient ($\text{length} \cdot \text{time}^{-1}$); we termed ξ , a secondary mass transport coefficient (Beyenal and Lewandowski, 2002)

The first boundary condition (12) states that there is no nutrient flux across the boundary, at the bottom. The other boundary condition (13) states that the biofilm defines the position of the second boundary, the biofilm surface, and gives the concentration of nutrient at this location. These two cases, a non-discretized and a discretized biofilm, are represented by the following adjustments in Eq. (11):

1. For non-discretized biofilms (diffusivity is constant throughout the biofilm):

$$\xi = 0 \text{ and } D_{sa} = D_{av} \quad (14)$$

2. For discretized biofilms (diffusivity forms a profile):

$$D_{sa} = (a + bx) \quad (15)$$

where Eq. (15) represents the linear correlation between the surface-averaged relative effective diffusivity and the distance, and the numerical values are taken from the data in Figure 3. The effective diffusivity for each layer was calculated as:

$$D_{fx} = D_{sa} \times D_w \quad (16)$$

To quantify the differences between discrete and uniform biofilms, we calculated fluxes of glucose using the biokinetic parameters given by Bailey and Ollis, (1986) as $K_s = 22 \text{ mg/L}$ and $\mu_{\max}/Y_{x/s} = 6.37e-5 \text{ s}^{-1}$. Assuming that the biofilm is $1,000 \text{ }\mu\text{m}$ thick and the concentration of glucose at the biofilm–liquid interface is 500 mg/L , the predicted glucose fluxes for uniform and for discrete biofilms are compared in Table 1.

To solve Eq. (11) we used Matlab’s boundary value solver function (bvp4c). The bvp4c is a finite difference code that implements the 3-stage Lobatto IIIa formula, and we used Matlab’s defaults to control the precision of the solution.

The predicted flux of glucose to the discretized biofilm is ten times higher (!) than the flux of glucose to a uniform biofilm, which reflects the magnitude to which biofilm heterogeneity affects biofilm activity. This, unexpectedly strong effect is caused, to a large extent, by the fact that decreasing effective diffusivity (Eq. (11)) is associated with increasing biomass concentration in deeper layers of the biofilms (Eq. (8)), and increasing reaction rate.

Measurements in discretized biofilms

Several existing measurements can be used in such a way that their results can be interpreted in the framework of discretized biofilms. Some of them are presented here:

An important step toward understanding relationships between biofilm structure and the underlying biofilm processes, e.g. mass-transport dynamics, nutrient concentration, and microbial species distribution, is by quantifying biofilm heterogeneity. We have developed image analysis techniques and integrated them to software that measures structural elements from microscope images of biofilms. Structural elements possess characteristics such as size, shape, intensity, and texture that may be expressed quantitatively. Biofilm structure is monitored by successively acquiring images of a growing biofilm. From these

Table 1 The predicted glucose fluxes for a uniform and a discrete biofilm

	Predicted glucose flux at the biofilm surface ($\text{mg}\cdot\text{s}^{-1}\cdot\text{cm}^{-2}$)
Uniform biofilm	3.78×10^{-5}
Discrete biofilm	3.98×10^{-4}

images we compute several parameters using a custom made software available at <http://www.erc.montana.edu> (Yang *et al.*, 2000, 2001). Thus far, the most promising parameters are areal porosity and biofilm porosity (volumetric). It is useful to notice that relative biomass coverage equals $(1 - \text{porosity})$. Therefore, monitoring biofilm porosity gives the volume of accumulated biomass, which is a useful parameter. Typical results are shown in Example 1.

Example 1. Computing structural parameters from biofilm images: see Figure 4 and Table 2.

From the areal porosity (ratio of the area of the voids to the total area of the image) monitored at a selected location in a biofilm, we evaluate rates of biofilm accumulation (Example 2).

Example 2. Areal porosity monitored near the bottom of three biofilms grown at various glucose concentrations: see Figure 5.

From areal porosity measured at several distances from the bottom, we compute biofilm porosity (volume of voids/volume of biofilm) as shown in Example 3.

Example 3. Calculating biofilm porosity requires an acquisition of confocal images at different distances from the bottom and a computation of areal porosity for each image: see Figure 6. Biofilm porosity is calculated as:

$$\text{Biofilm porosity} = \frac{(AT) \int_0^{\text{Th}} (\text{AP}) dx}{(AT)(\text{Th})} = \frac{\int_0^{\text{Th}} (\text{AP}) dx}{(\text{Th})} \quad (17)$$

where (AT) is total area of the image (field of view); (AP) is areal porosity measured from an image taken at the distance x from the bottom (Figure 6D); (Th) is the biofilm thickness.

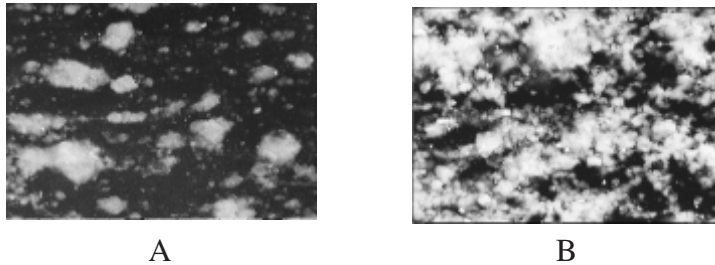


Figure 4 (A) third-day biofilm (B) seventh-day biofilm

Table 2 Structure parameters calculated from the images of 3- and 7-day old biofilms

Parameter	3-day old biofilm	7-day old biofilm
Threshold value	159	147
Areal porosity	0.66	0.17
Textural entropy	3.0014	3.3963
Angular second moment	0.0868	0.0475
Inverse difference moment	0.7079	0.6680
Fractal dimension	1.3720	1.3650
Average horizontal run length	10.59	37.94
Average vertical run length	9.56	35.01
Average diffusion distance	6.93	12.91
Maximum diffusion distance	44.05	58.24

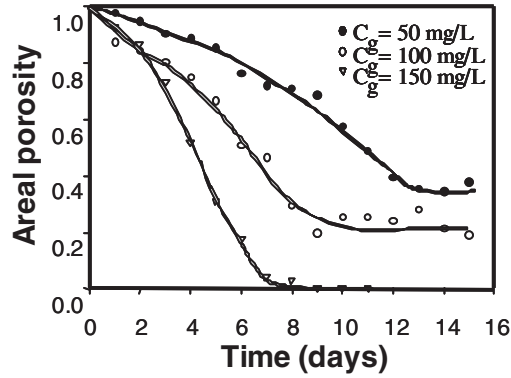


Figure 5 When glucose concentration increases, the rate of biofilm accumulation near the bottom increases as well (i.e. areal porosity decreases)

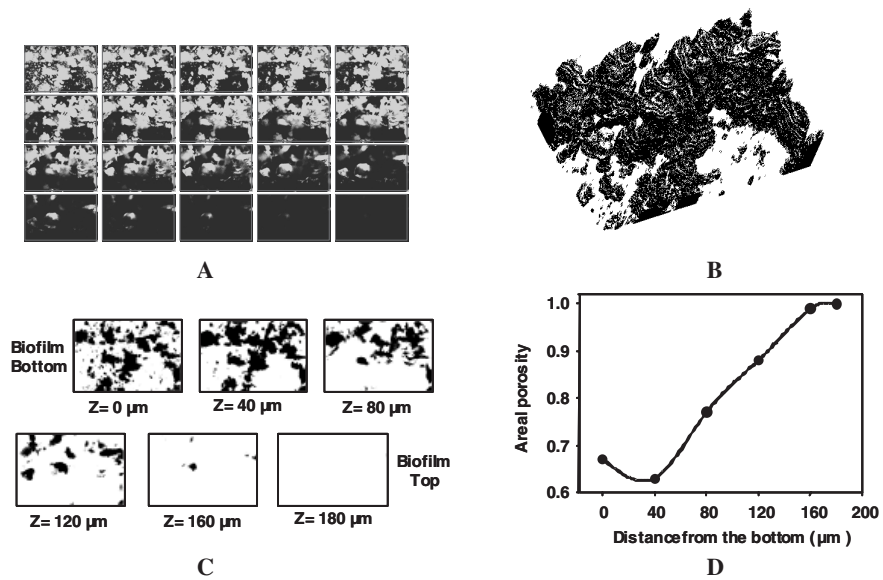


Figure 6 Evaluating biofilm porosity. A) A series of confocal images of a biofilm are taken at different heights from the bottom of the biofilm, upper left corner, to the lower right corner. B) A three-dimensional image of the biofilm is reconstructed from the series of confocal images. C) Biofilm slices cut at planes parallel to the surface from the reconstructed biofilm image. D) Depth profile of areal porosity

Conclusions

Monitoring biofilms exposes several weak areas of biofilm research. It appears that even if the monitoring process is technically successful, little can be done with the acquired results. At the bottom of this problem, is the lack of mathematical models that can directly accept the monitored parameters. In addition, many biofilm monitors measure lump parameters that do not seem to be related to any defined biofilm function, and conceal the fact that parameters characterizing biofilms are spatially distributed. Monitoring biofilms, when properly executed, can provide insight into fundamental biofilm processes. However, one needs to be fully aware of what is actually measured, and how the measured quantities correlate with biofilm functions, such as rates of biofilm accumulation or substrate conversion. It remains unclear which parameters should be monitored to make such correlations. For those reasons biofilm monitoring is well portrayed by the adage about computers – a solution in search of a problem.

Biofilm monitoring, in its simplest form, ignores the effects of biofilm heterogeneity. We advocate here discretizing biofilms to evaluate if this is acceptable. Although discretizing biofilms by subdividing the volume occupied by the biofilm into a finite number of layers conceals the effects of lateral mass transport in biofilms, it gives a rough estimate of the effects of biofilm heterogeneity on the measured parameter.

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