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RAPID COMMUNICATION

NOTES ON BIOFILM POROSITY

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Abstract—Difficulties have been encountered attempting to use porosity as a parameter for quantifying biofilm heterogeneity. Some of those difficulties are technical in nature—measurement of biofilm porosity and interpretation of the results—while other are more fundamental and result from using the well-known concept of rigid porous bed porosity to describe the porosity of a gelatinous biofilm matrix. Possible remedies are suggested and discussed. © 2000 Elsevier Science Ltd. All rights reserved

Key words—biofilms, biofilm heterogeneity, confocal microscopy

INTRODUCTION

Despite the growing popularity of the model of heterogeneous biofilms, the overall effect of biofilm heterogeneity on biofilm activity remains unclear. This lack of clarity will persist until means are developed to correlate the magnitude of biofilm heterogeneity with the magnitude of other parameters that may be influenced by biofilm heterogeneity, e.g. local mass transport rates and local microbial activity. It is expected that correlating the magnitude of biofilm heterogeneity with other quantifiable factors characterizing biofilm processes will lead to understanding the physiological significance of biofilm heterogeneity and will expose the relations between heterogeneity and the overall performance of biofilm reactors. Since images of a biofilm's inner space resemble images of porous media, porosity is a parameter of choice for quantifying the extent of biofilm heterogeneity. The main reasons supporting the choice of porosity are: (1) porosity is a well established concept; (2) the influence of porosity on biofilm performance is intuitively obvious; and (3) biofilm porosity is a part of some recently introduced 3-D models of biofilm activity (in fact biofilm porosity has always been implicitly used in biofilm models, as effective diffusivity). However, conceptual difficulties arise in translating the well-known concept of porosity from porous media reactors to biofilms. The porosity of a porous bed is defined as the ratio of pore volumes to the total volume of the bed. An image associated with this definition is that of a packed bed reactor where the porous material constitutes the solid phase and the fluid is confined to the pores. However, biofilm matrix is not solid—it is a highly hydrated gel—and this difference is the source of our difficulties. Simply put, if the biofilm matrix itself is more than 90% water, then how can we distinguish the water within the matrix as different from the water in the pores?

Despite the conceptual difficulties associated with biofilm porosity, there is a strong tendency among biofilm researchers to use it anyway because it is believed that the advantages of using a single, easy-to-understand parameter outweigh the disadvantages. This tendency is strongly amplified by the hope that the concept of biofilm porosity may establish an additional link between mathematical modeling of biofilm processes and experimental verification of those models. Older biofilm models, like AQUASIM, which accept heterogeneous structure of biofilms require porosity as an input parameter (Horn and Hempel, 1997; Wanner and Reichert, 1996). The new approach to biofilm modeling, cellular automaton (Picioreanu *et al.*, 1998a,b; Wimpenny and Colasanti, 1997) allows predicting biofilm porosity from first principles, although the link between models and experiments has not yet been established. The tendency to use biofilm porosity has its merit; however, the consequences of adopting porosity as *the* parameter characterizing biofilm heterogeneity should be considered.

RESULTS AND DISCUSSION

I shall identify the main difficulties with using the porosity concept in biofilm engineering and discuss

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possible remedies. Two approaches to quantifying biofilm porosity will be implicated: (1) use of biofilm slices and (2) use of microscope images of biofilms. The first approach is well represented by the work of Zhang and Bishop (1994) and the second approach is exemplified by one of our papers (Yang *et al*., 2000). I shall refer in detail only to quantifying biofilm porosity from microscope images and refer to quantifying porosity from biofilm slices as to a possible way of verifying the results obtained from optical measurements. For routine measurements, microscopy combined with image analysis has advantages over the more tedious biofilm slicing because it is a noninvasive technique that permits in situ analysis of living, fully hydrated biofilms.

Confocal images of heterogeneous biofilms demonstrate that thin optical slices of biofilms have non-uniform distribution of the biomass, meaning areas of condensed biomass are separated by areas with no biomass at all. In the colloquial sense such slices are porous and have a well developed structure. We conjecture that there exist a finite number of parameters that uniquely describe the structure of a heterogeneous 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, we rely 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 our microscopic field of view. If a parameter appears to approach steady state, it is behaving in the expected manner, and we accept it as a parameter describing biofilm heterogeneity. Initial experiments indicate that areal porosity, fractal dimension, diffusional distance, and textural entropy are such parameters (Lewandowski *et al*., 1999).

Areal porosity, one of the parameters we calculate from confocal images, is the ratio of the combined areas of the voids to the total area of the image. It should be noted that the term ''areal porosity'' is a misnomer because porosity characterizes three-dimensional space, while the calculated parameter is evaluated from two-dimensional images. Therefore, the first question that needs attention is how the areal porosity is related to the true, 3-D porosity of the biofilm.

Figure 1 shows a sequence of confocal images of a biofilm evaluated at different distances from the bottom. Using several such images, the biofilm is reconstructed in 3-D (Fig. 2) and stored by the computer memory for further analysis. At this point, the images of real biofilms are replaced by their digital analogs, which may be retrieved at any time and compared with results of other analyses. Areal porosity is then calculated from these reconstructed images. Another advantage of using reconstructed biofilms is that, with appropriate software, they can be sliced in any direction and, therefore, allow better insight into the inner space of the biofilm. Figure 3 shows a series of such slices, taken from the reconstructed biofilm in Fig. 2. Areal porosity values calculated from these slices are arranged into a depth profile shown in Fig. 4.

The depth profiles of areal porosity are directly related to biofilm porosity. To expose this relation, we integrate the areal porosity of all slices along the distance, from the bottom of the biofilm to the biofilm surface, and multiply that value by the area of the image (the field of view). The result is equal to the volume of voids for this field of view. If we

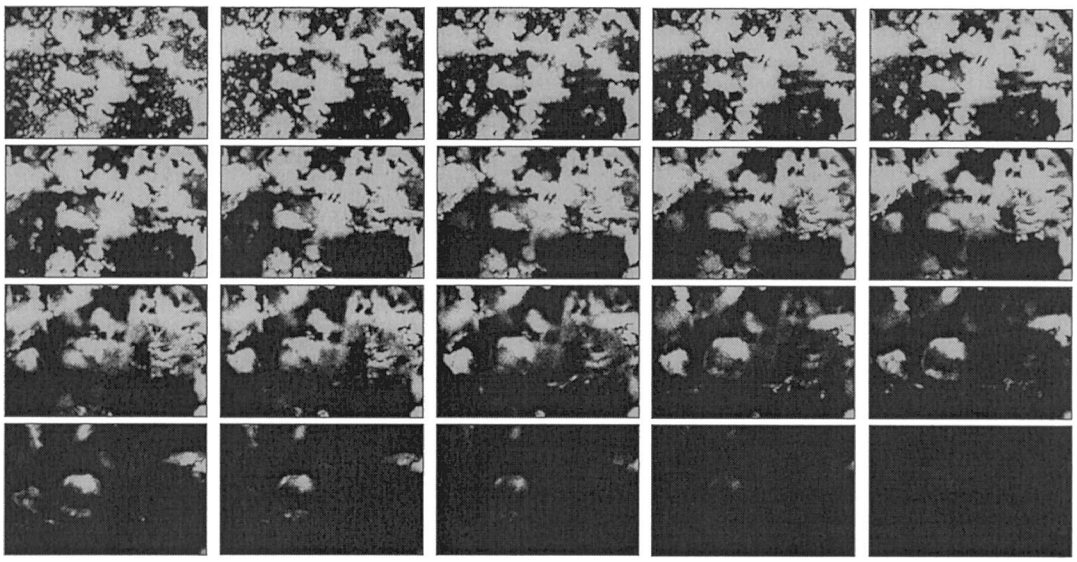

Fig. 1. A series of confocal images of a biofilm taken at different depth from the bottom of the biofilm, upper left corner, to the top, lower right corner.

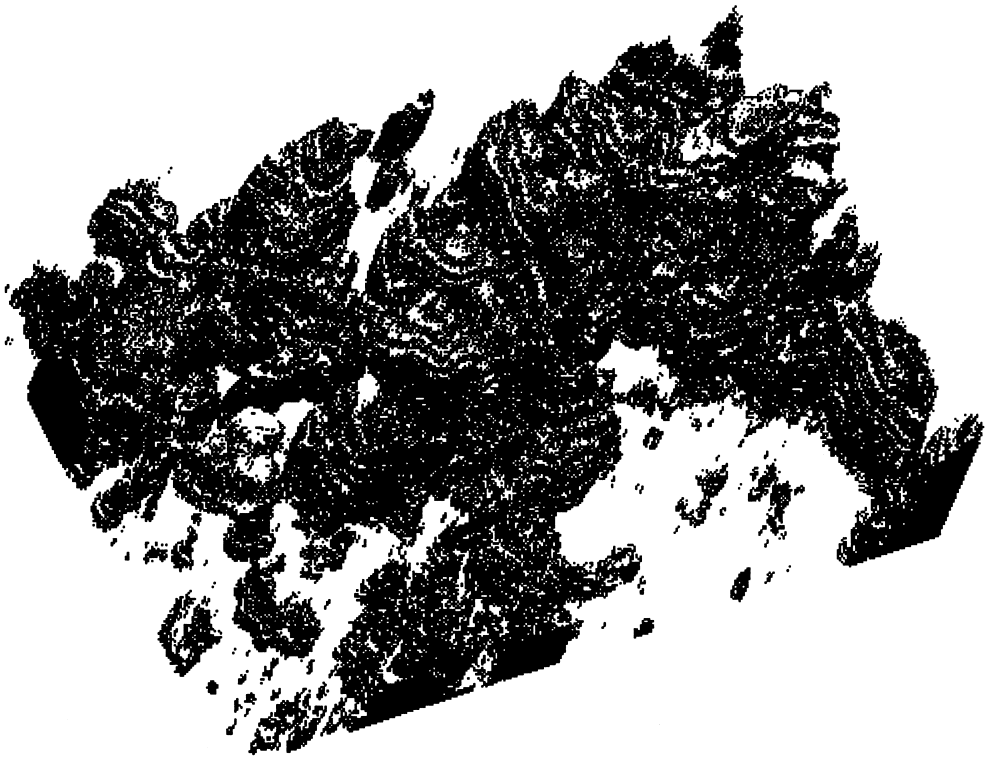

Fig. 2. Three-dimensional image of the biofilm, reconstructed from the series of confocal images in Fig. 1.

divide this combined void volume by the volume of the sample (the field of view multiplied by the biofilm thickness), the result is biofilm porosity:

$$\text{Biofilm Porosity} = \frac{(\text{Field of View}) \times \sum_{\text{for all images}} (\text{Areal Porosity} \times \text{Slice Thickness})}{(\text{Volume of the Biofilm Sample})} \tag{1}$$

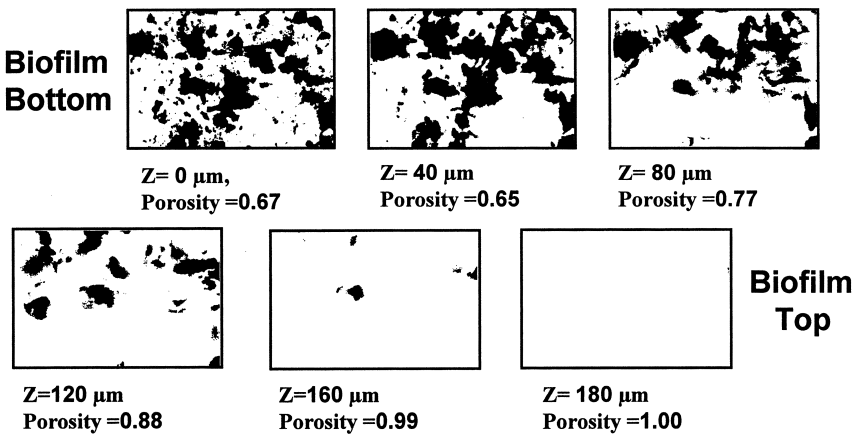

Fig. 3. Biofilm slices cut at planes parallel to the surface they adhered to, extracted from the reconstructed biofilm image shown in Fig. 2.

We then assign the following symbols to each variable: AT = total area of the image (field of view); $(AP)_x$ = areal porosity at the distance x from the bottom; Th = biofilm thickness.

$$\text{Biofilm Porosity} = \frac{(AT)\int_0^{Th}(AP)_x\,dx}{(AT)(Th)} = \frac{\int_0^{Th}(AP)_x\,dx}{(Th)} \quad (2)$$

The area of the image (value AT) cancels out, indicating that biofilm porosity does not depend on the surface area of the image. More important, the final result is the average value of the function in Fig. 4, thus biofilm porosity is equal to the average areal porosity measured across the biofilm.

This finding lends credibility to using areal porosity measured from confocal images. However, the doubts about using biofilm porosity as a parameter characterizing biofilm heterogeneity remain. The procedure presented here involves steps that are, at present, arbitrary and greatly influence the final result. The first of those steps is related to the image manipulation procedure called thresholding. In essence, the images taken by a camera are gray scale images, while the images processed to extract areal porosity are binary, having two colors only. Thresholding relies on an arbitrary selection of a gray scale value to segment a gray scale image into a binary image. To extract statistically meaningful heterogeneity parameters from a series of images, the thresholding has to be reproducible. The problem is that there are no rules for setting the threshold value and the operator manipulates the image until it looks good. The numerical value prescribed to areal porosity depends on this step.

Variance in image magnification is the second difficulty we find, and it is related to the assertion that biofilm porosity is an independent parameter. If we imagine a very small area of a biofilm, an area of the size of a single pixel, then regardless whether this is an area of a cell cluster or a void the areal porosity equals zero. If we use somewhat lesser magnification, we will see detail structure of the biofilm with well-defined pores and the areal porosity will be higher than zero. If we go even further and use very low magnification, most fine features within the biofilm become indiscernible and the areal porosity will decrease. The question is: What magnification should be used in these measurements?

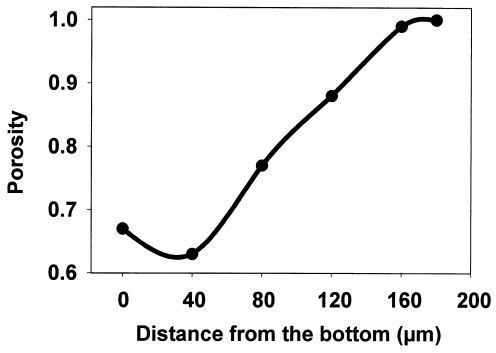

Fig. 4. Depth profile of areal porosity calculated from the results in Fig. 3.

We find a third difficulty related to the measuring of biofilm thickness. Equation (2) uses biofilm thickness (Th) in the denominator to calculate the volume of the sample. But Fig. 2 shows that the estimate of biofilm thickness is arbitrary, so depending on the value accepted as biofilm thickness the results of porosity calculation will vary.

CONCLUSIONS

The three problems I have identified must be prioritized and solved before implementing the procedure of estimating biofilm porosity from confocal images. The utmost priority is finding a suitable technique for reliable and reproducible thresholding of biofilm images. From our experience it appears that an algorithm for automatic thresholding may help. Once a reliable thresholding technique is achieved, we need to examine the effect the field of view (magnification) has on the resulting biofilm porosity. Finally, estimating biofilm thickness in calculating the volume of the sample can be solved by asserting that a certain percentage of the biomass volume has to be confined within the sample volume. Such a procedure can be easily designed and implemented because it is possible to calculate biomass volume from the porosity depth profiles.

The first two problems we discussed (thresholding and image magnification) are specific to image analysis, and it is interesting to notice that techniques that rely on biofilm slicing do not have these problems. It is conceivable that, even though evaluating porosity from biofilm slices is based on assumptions that need to be verified (see Zhang and Bishop, 1994 for details), porosity evaluated from biofilm slicing can be used to calibrate the more time-efficient procedures relying on confocal microscopy and image analysis.

Finally, if the effort to quantify porosity from biofilm images succeeds, it is expected that biofilm porosity will be used in biofilm modeling. For that purpose we tacitly assume that there is a single number characterizing biofilm porosity, as is the case with a porous media reactor. Equation (2) gives such a single number, but Fig. 3 shows that an infinite number of possible areal porosity depth profiles of areal porosity can give the same porosity (the same average value of the function). Porosity appears to be a continuous function of biofilm depth and one can expect nutrients to penetrate biofilms at different rates, depending on the slope of the areal porosity profiles, even if the biofilms have the same porosity calculated from equation

(2). The effect that the slope of the areal porosity profile may have on the intra-biofilm nutrient transport rates needs to be quantified to decide whether biofilm models should include porosity as a single number or as a function of biofilm depth.

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