

The effect of detachment on biofilm structure and activity: the oscillating pattern of biofilm accumulation

Z. Lewandowski^{***}, H. Beyenal^{*}, J. Myers^{*} and D. Stookey^{*}

^{*}Center for Biofilm Engineering, Montana State University, Bozeman, 59717, USA
(E-mail: zl@erc.montana.edu; beyenal@erc.montana.edu)

^{**}Department of Civil Engineering, Montana State University, Bozeman, 59717, USA

Abstract In our previous papers we have demonstrated that biofilm structure never reaches a steady state in biofilm reactors; in this paper we link this fact to biofilm detachment and to the oscillating pattern of biofilm accumulation. In one respect reactors supporting suspended microbial growth and reactors supporting attached microbial growth (biofilms) are similar: in both the biomass accumulates in the reactor and is disposed of with the effluent. However, while in reactors with suspended microbial growth biomass accumulation and disposal occur simultaneously, in biofilm reactors these two processes are separated in time. Biomass accumulation in biofilm reactors shows a distinct pattern composed of three phases: (1) growth, (2) detachment, (3) regrowth. Despite this distinct pattern of biofilm accumulation observed at the microscale, biofilm reactors do reach a steady state of substrate removal.

Keywords Biofilm; biofilm accumulation; biofilm activity; biofilm reproducibility

Introduction

For each biofilm reactor we can estimate the biofilm activity at two scales of observation, the macroscale and the microscale. It is impossible to ascribe a specific linear dimension separating these scales of observation because any selected dimension would have to be chosen arbitrarily. Instead, it is better to base this distinction on the type of information generated and the tools routinely applied at each scale. Macroscale observations generate information about the average, overall properties and activity of the entire biofilm system, evaluated from chemical analysis of the bulk solution with the aid of mass balances. Microscale observations generate information about the spatial distribution of factors affecting local microbial activity, local microbial community structure, local mass transport dynamics, local biofilm structure, etc.

Biofilm activity at the macroscale is reported as a single number, while biofilm activity at the microscale is reported as a set of numbers, because at the microscale biofilm activity depends on the location of the measurement. The existing mathematical models of biofilm activity have difficulty reconciling the results of measurement of biofilm activity at the macroscale with those at the microscale. It is expected that the two types of measurements should give the same overall biofilm activity. However, this expectation can be fulfilled only if the biofilm is uniformly distributed in the reactor and the microbial activity is uniformly distributed in the biofilm. Neither of these two conditions is satisfied in real biofilms, and the biofilm activity estimated from microscale measurements, in general, is not equal to that estimated from macroscale measurements. It is not clear how to compare these results or how to integrate the results of biofilm activity measurements at the microscale so that we can achieve an overall biofilm activity equal to that measured at the macroscale. From our observations, we have concluded that

one reason for these difficulties is the cyclic pattern of biofilm accumulation observed at the microscale: it is difficult to translate this effect to the macroscale.

The cyclic pattern of biofilm development was postulated as a result of our (Jackson *et al.*, 2001) and other researchers' failure to grow biofilms of reproducible structure and activity (Bester *et al.*, 2005; Xavier *et al.*, 2005). One of the conclusions of our study was that biofilms do not reach a steady state, but instead exhibit a cyclic pattern of growth characterised by growth, detachment and regrowth (Lewandowski *et al.*, 2004). Figure 1 shows an example of this pattern (Lewandowski *et al.*, 2004). After a few days of accumulation, the first sloughing event initiates the cycle. After that the biofilm structure oscillates between regrowth and detachment.

The cyclic pattern of accumulation is clearly visible when biofilm structure and activity are evaluated at the microscale, but each location may show a different pattern. To characterise the pattern of biofilm accumulation in the entire reactor we have characterised the biofilm structure, at the microscale, at several locations and averaged the results. In our previous reports (Jackson *et al.*, 2001; Beyenal *et al.*, 2004) we demonstrated that averaging approximately 15 measurements at randomly selected locations is sufficient to characterise biofilm structure at the macroscale. Averaging more than 15 measurements does not change the average significantly. To be on the safe side, we usually double this number and average data from 30 locations, as we do in this paper.

We believe that the cyclic pattern of biofilm accumulation may be one of the reasons why we have difficulty predicting the rates of macroscale biofilm processes from microscale observations: the predictions depend on the phase of the cycle in which the microscale observations were made. If this is true, then to predict macroscale biofilm system

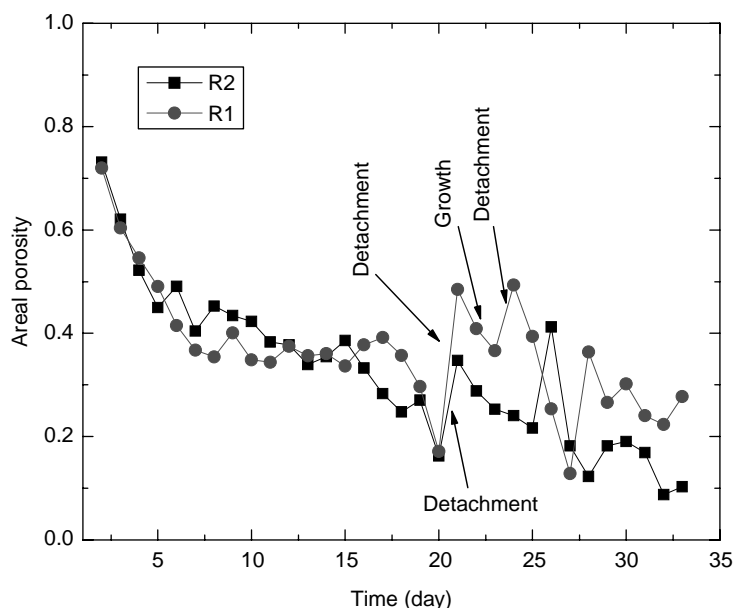


Figure 1 Cyclic pattern of biofilm growth. The average areal porosities and standard deviations from the averages were evaluated daily from 30 images of a biofilm taken at randomly selected locations. The biofilm was the three-species model biofilm, composed of *Pseudomonas aeruginosa* (ATCC #700829), *Pseudomonas fluorescens* (ATCC #700830) and *Klebsiella pneumoniae* (ATCC #700831), and the growth medium was based on glucose and inorganic nutrients (Lewandowski *et al.*, 2004). R1 and R2 refer to reactors 1 and 2. It takes several days for a biofilm to reach the mature stage of cyclic development. For the purpose of this paper, the data demonstrate that: (1) the biofilm structure had not reached a steady state and (2) the biofilm had entered the phase of cyclic growth characterised by growth, detachment and regrowth

performance from microscale observations, we need to determine the “relevant” scale of observation, and perhaps the “relevant length of time” for which the data need to be collected and averaged, both sufficiently large to be useful for making predictions about the behaviour of the biofilm system at the macroscale.

It has been hypothesised that the oscillations in biofilm structure and activity at the microscale do not affect the substrate conversion rate at the macroscale. To test this hypothesis, two experiments were performed by two students: four identical biofilm reactors were operated and each student operated two of these reactors. Temporal variations in the structure of the biofilm were evaluated by taking 30 biofilm images through the bottom of each reactor daily. Biofilm porosity was evaluated from each of these images. Each set of 30 results was averaged daily, and the results were plotted *versus* time. The number of images acquired daily for each reactor (30 at randomly selected locations) was selected so that the averaged result of the microscale measurements would reflect the biofilm structure in the entire reactor, at the macroscale. The activity of the biofilm was quantified at the macroscale, by measuring the inlet and outlet concentrations of the carbon source, glucose.

Materials and methods

Biofilm reactors and growing biofilms

Single-species biofilms composed of *Pseudomonas aeruginosa* PAO1 were grown in four identical flat-plate flow reactors under well-controlled conditions. The reactors were polycarbonate channels with glass bottoms 2.5 cm wide, 4.0 cm deep and 34.5 cm long (Jackson *et al.*, 2001) and they were placed on the Olympus CK2 inverted microscope during the experiments. To prevent contamination, the components of the experimental setup, except for the reactors, were autoclaved before the operation. The reactors were sealed with silicone rubber (ACE[®] Hardware Corp., Oak Brook, IL, USA), connected to the autoclaved components (i.e. tubings, recycle lines and air filters), filled with 20% Clorox[®] and left for two hours. Then, the bleach was flushed from the reactor by passing at least 10 L of autoclaved (3 hours at 121 °C and 1 atm) de-ionised water. The sterile water was allowed to flush the reactor overnight. Then the water was replaced with the growth medium. Figure 2 shows the setup, and the components of the setup are described in the following sections.

Growth medium

The growth medium was composed of Na₂HPO₄ (0.9125 g/L), KH₂PO₄ (0.175 g/L), MgSO₄·7H₂O (0.005 g/L), (NH₄)₂SO₄ (0.5 g/L), glucose (1 g/L) and yeast extract

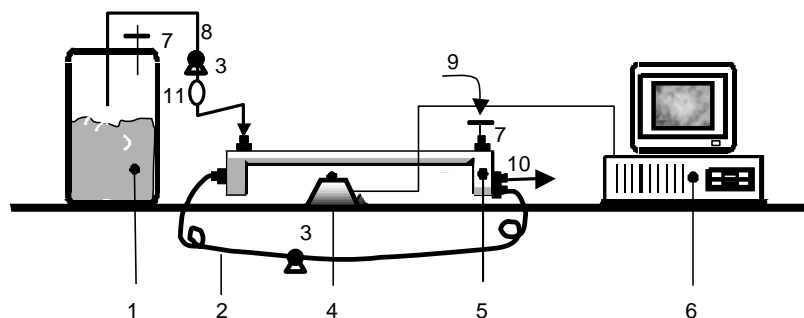


Figure 2 The experimental setup: 1, growth medium; 2, recycle loop; 3, peristaltic pump; 4, inverted microscope integrated with a digital camera; 5, flat-plate reactor; 6, computer; 7, air filter; 8, reed line; 9, air inlet; 10, outflow; 11; flow breaker

(0.05 g/L). The pH was adjusted to 7.2. Glucose and yeast extract were autoclaved separately and then added to the autoclaved growth medium.

Reactor inoculation

One millilitre of a frozen stock sample of *Pseudomonas aeruginosa* PAO1 was inoculated into a flask, and the culture was grown for 24–30 hours in 100 mL of growth medium on a shaker set to 150 rpm at room temperature ($\sim 25^\circ\text{C}$). Each reactor was inoculated with 20 mL of culture, aseptically, *via* needle and syringe, through the line through which the growth medium entered the reactor. Just before inoculation, the sterile water in the reactor was replaced with freshly prepared sterile growth medium. During the inoculation the flow of growth medium, the recycle loop and the airflow were stopped, and the waste line was clamped. Approximately 15 minutes after the inoculation the recycling was resumed and continued for 8 hours, until the feed pump was turned on. Also, at this point, the airflow was restored to the reactor, the effluent clamp was removed and the reactor continued to operate as described below.

Operating the reactors

The fresh feed flow rate was 0.4 ± 0.1 mL/min. To maintain a reasonable flow velocity and shear stress, the recycle ratio was set to 300. The high recycle ratio also provided a uniform substrate concentration along the reactor. Before taking the biofilm images, the reactors were flushed with sterile, de-ionised water to remove suspended microorganisms. The reactors were operated at room temperature ($\sim 25^\circ\text{C}$). The reactor was continuously aerated from the surface using filtered air (3 L/h).

All reactors were operated under identical conditions, i.e. feed substrate concentrations and feed and recycle flow rates. There were two operators and four reactors, and each operator operated two reactors. This experimental design was chosen so that the reproducibility of the results could be estimated between samples (the results from the two reactors operated by each operator were compared) and between operators (the results from the reactors operated by different operators were compared). The reactors were operated for more than a month.

Biofilm imaging

To capture images of biofilm structure, the reactor was placed on the Olympus CK2 inverted microscope. UV light (Cambridge Instruments, Buffalo, NY, USA) was used to illuminate the reactor. The best quality images were obtained just after the reactor was flushed with sterile water. The images were taken through the bottom of the reactor using $40\times$ magnification and were captured by a COHU[®] camera using Flashpoint, a frame grabber integrated with a computer. The images are in 8-bit grey-scale TIFF format, consisting of 640×480 pixels, and are viewed with a customised software package, ImagePro Plus[®] (Media Cybernetics, Version 3). Each day of operation, 30 biofilm images were taken at random locations though the bottom of each reactor. From these images we computed biofilm areal porosity using image structure analyser (ISA).

Quantifying biofilm structure

We selected areal porosity as the parameter quantifying biofilm structure. Areal porosity is the ratio of the area covered by voids (empty space) to the total area of the image. When areal porosity reaches one, there is no biofilm on the surface, and when areal porosity reaches zero, the entire surface is covered with the biomass.

Substrate removal rate

The glucose concentration in the reactor was measured using procedure 510 by Sigma[®] Diagnostics. The glucose removal rate was quantified as:

$$\text{Glucose removal rate} = Q(C_{\text{ginfluent}} - C_{\text{geffluent}}) \quad (1)$$

where C_g and Q refer to the glucose concentration and the volumetric feed flow rate, respectively. If the substrate removal rates do not vary by more than 2% during several days, we consider that the substrate removal has reached a steady state.

Results

The temporal variation of biofilm areal porosity is shown in Figure 3; each data point resulted from averaging 30 measurements from randomly selected locations.

From the results in Figure 3, we plotted a histogram, Figure 4, showing the frequencies with which the specified areal porosities occurred in each reactor during the entire time the reactors were operated.

As shown in the frequency diagram, Figure 4, the biofilm areal porosity was most of the time between 0.5 and 0.7. The diagram is denser on the right-hand side than on the left-hand side, which demonstrates that during the operation the biofilms had porosities reaching one, but rarely had porosities lower than 0.4. Once the areal porosity of the biofilm reached values lower than 0.4, a sloughing event occurred, and biofilm regrowth promptly followed.

Although this was not the main objective of our study, it is worth noting that the histogram in Figure 4 indicates an interesting possibility, that there exists a predominant areal porosity for a biofilm grown under a certain set of conditions. This observation does not contribute to the answer to the main question, but it does contribute to the answer to another question: what is reproducible in biofilm reactors operated at a certain set of operational conditions? Perhaps the predominant areal porosity is reproducible.

Unfortunately, it is not possible to operate the biofilm reactors in the same way as the chemostats, controlling the biomass concentration by changing the dilution rate. In biofilm reactors, substrate concentration decreases to quite low values, as it does in chemostats operated at low dilution rates. The data in Figure 5 do not indicate that the substrate concentration in the effluent followed the spikes of biofilm accumulation seen in Figure 3. Instead, they indicate that the substrate removal reached a steady state; the glucose removal rates quantified from equation 1 do not vary more than 2%.

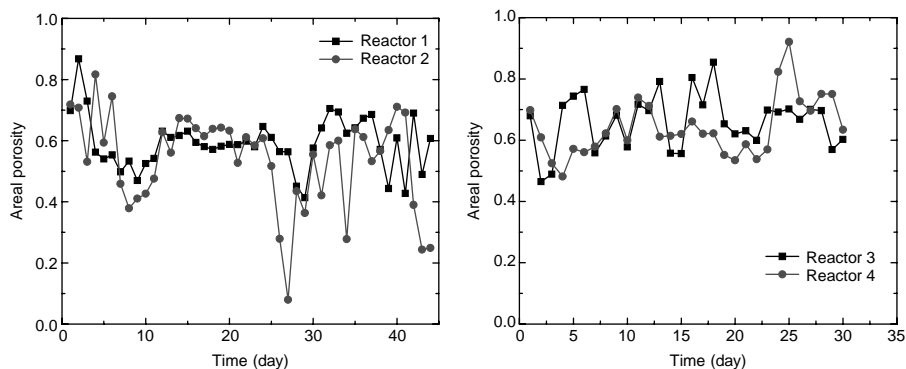


Figure 3 Temporal variation in biofilm porosity in four reactors operated in parallel. Reactors 1 and 2 were operated by one operator and reactors 3 and 4 were operated by another operator. Qualitatively, the results resemble those in Figure 1: after a few days of initial colonisation, the biofilms exhibited a cyclic pattern of accumulation

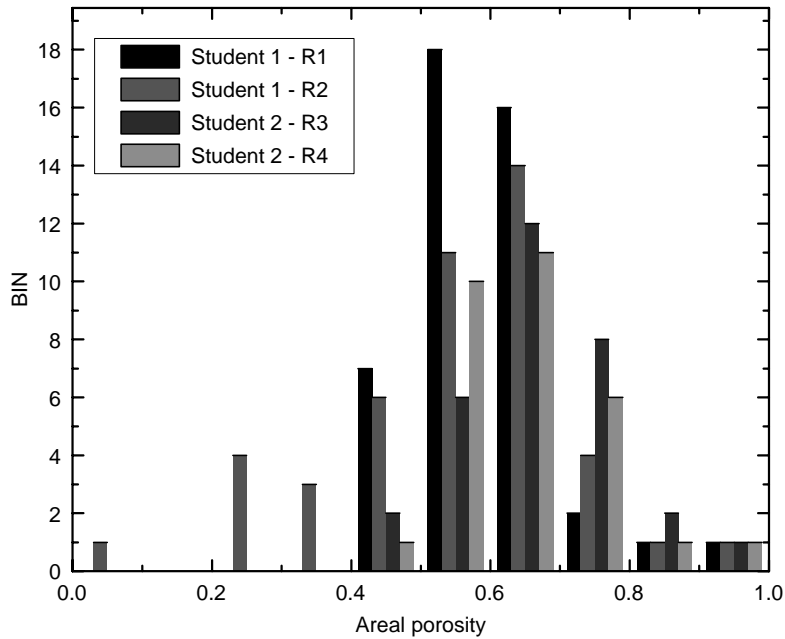


Figure 4 Frequencies with which specified areal porosities occurred in each biofilm reactor. R1, R2, R3 and R4 refer to reactors 1, 2, 3 and 4, respectively

To address the original question, whether the substrate removal reaches a steady state, we quantified the influent and effluent glucose concentrations. [Figure 5](#) shows the results for the two reactors shown on the left-hand side in [Figure 3](#).

It is interesting, and unexpected, to see that despite the observed swings in biofilm areal porosity in reactors 1 and 2, shown in [Figure 3](#), the rate of substrate removal, shown in [Figure 5](#), was practically unaffected. Apparently, sloughing did not affect the

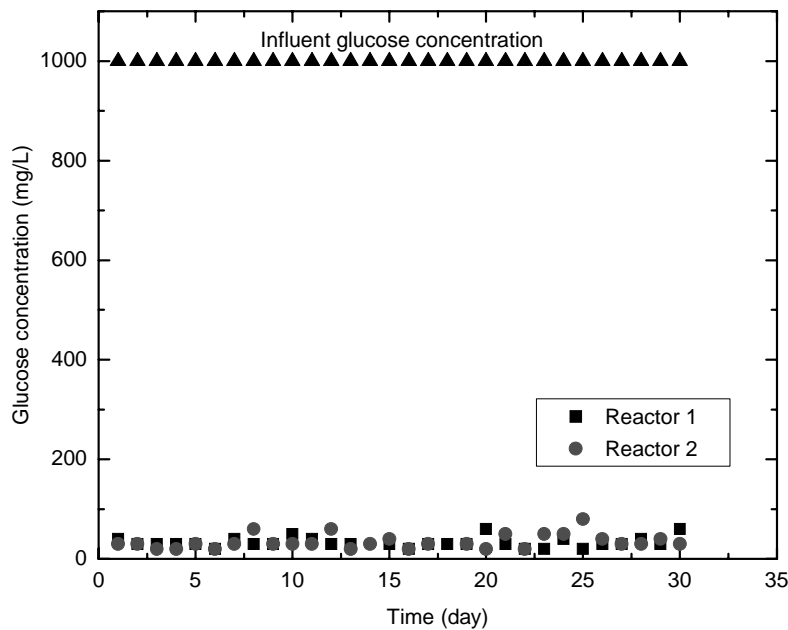


Figure 5 Glucose concentration in the influent and effluent of the two reactors. The influent glucose concentration was 1,000 mg/L

overall substrate removal rate. This can be explained if we accept that the amount of biofilm accumulated on the surface exceeds the amount of biomass needed for complete substrate removal, so that the sloughing removes the excess of the biofilm, leaving the overall substrate removal rate unaffected. The biomass retention time in the reactor is much higher than the liquid retention time, which allows the accumulation of large quantities of biomass in the reactor. It is well known that bacteria in deeper layers of the biofilm can be dormant and do not consume significant amounts of substrate. When sloughing exposes these microbes to fresh nutrient solution, they rapidly increase their metabolic activity (Keren *et al.*, 2004), which may also account for the unchanged overall substrate removal despite the variations in the accumulated biomass.

Discussion

The fact that biofilm structure and activity at the microscale do not reach steady states should not come as a surprise: why should they? The only reason we might expect biomass accumulation in biofilms to reach a steady state is that we think in terms of the models describing the performance of reactors with suspended biomass and use these models to describe the performance of biofilm reactors. These expectations are ill founded. Biomass removal in biofilm reactors is mostly caused by the sudden detachment of large chunks of biofilm, rather than by erosion. Therefore, biomass removal in biofilm reactors should be described by functions characterising catastrophic events rather than by the well-behaving functions describing the shearing of biofilm surface by flowing water.

Based on the results presented here, we conclude that it is possible for biofilm reactors to reach a steady state of substrate removal and, at the same time, exhibit continuously changing biofilm structure. We have demonstrated that even though the biofilm reactor does not reach a steady state, because of the oscillating pattern of biofilm accumulation at the microscale, the nutrient removal from it can reach a steady state: the oscillating patterns of local biofilm accumulation are averaged on the scale of the reactor. This conclusion has a corollary: it appears that there is more biomass accumulated in the biofilm reactor than is needed for substrate removal and the oscillating pattern of accumulation removes the excess biomass only, which does not affect the overall substrate conversion.

Conclusions

- Biofilm structure, as quantified by areal porosity, did not reach a steady state, as expected. Instead, it varied continually and the biofilm developed in cycles: growth, detachment and regrowth.
- When the areal porosity reached a critical value, which was 0.4 in our reactors, the biofilm detached and the regrowth phase promptly followed. Most of the time, the areal porosity of the biofilm was between 0.5 and 0.7.
- Biofilm detachment did not affect the overall substrate conversion rate as evaluated from the difference in substrate concentration between the influent and the effluent of the reactor.
- The biofilm structure in the reactor did not reach a steady state, but the substrate conversion rate did reach a steady state.

Acknowledgements

Jarred Myers was supported by the Undergraduate Scholars Program (USP), Montana State University. Deanne Stookey was supported by an award from the W. M. Keck Foundation.

References

- Bester, E., Wolfaardt, G., Joubert, L., Garny, K. and Saftic, S. (2005). Planktonic-cell yield of a pseudomonad biofilm. *Appl. Environ. Microbiol.*, **71**, 7792–7798.
- Beyenal, H., Lewandowski, Z. and Harkin, G. (2004). Quantifying biofilm structure: Facts and fiction. *Biofouling*, **20**, 1–23.
- Jackson, G., Beyenal, H., Rees, W.M. and Lewandowski, Z. (2001). Growing reproducible biofilms with respect to structure and viable cell counts. *J. Microbiol. Meth.*, **47**, 1–10.
- Keren, I., Shah, D., Spoering, A., Kaldalu, N. and Lewis, K. (2004). Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli*. *J. Bacteriol.*, **186**, 8172–8180.
- Lewandowski, Z., Beyenal, H. and Stookey, D. (2004). Reproducibility of biofilm processes and the meaning of steady state in biofilm reactors. *Wat. Sci. Tech.*, **49**(11–12), 359–364.
- Xavier, J.D., Picioreanu, C. and van Loosdrecht, M.C.M. (2005). A general description of detachment for multidimensional modelling of biofilms. *Biotechnol. Bioeng.*, **91**, 651–669.