APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1993, p. 327–329
0099-2240/93/010327-03$02.00/0

Vol. 59, No. 1

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Quantitative Observations of Heterogeneities in *Pseudomonas aeruginosa* Biofilms

PHILIP S. STEWART,* BRENT M. PEYTON,† WILLIAM J. DRURY, AND RICARDO MURGA

Center for Interfacial Microbial Process Engineering, College of Engineering, 409 Cobleigh Hall, Montana State University, Bozeman, Montana 59717

Received 28 July 1992/Accepted 14 October 1992

Heterogeneity in a *Pseudomonas aeruginosa* biofilm was quantified by measuring distributions of thickness in biofilm samples and a distribution of particle sizes in effluent samples. The mean steady-state thickness was approximately 33 μm, but individual measurements ranged from 13.3 to 60.0 μm. Particles exceeding 100 μm^3 were observed in the reactor effluent. The results reveal a rough biofilm surface and indicate that most biomass detaches in the form of multicellular particles.

With rare exceptions (14), mathematical models of biofilm activity treat the biofilm as a planar aggregate with a uniform thickness. The assumption of uniform thickness conflicts with numerous microscopic observations of biofilms that, in the words of various authors, suggest heterogeneity in the forms of pores, ridges, and crevices, etc. (4, 8, 11, 13). Another indication of heterogeneity in biofilms is manifest in the widely acknowledged, if poorly understood, phenomenon of sloughing (3, 7, 10). Sloughing refers to the detachment of multicellular, even macroscopic, particles from a biofilm. As with the microscopic observations, much of the evidence of sloughing is either anecdotal or qualitative in nature. We report in this article quantitative measurements of heterogeneity in biofilm thickness and effluent particle size from a well-characterized experimental biofilm system.

Pure culture biofilms of an environmental isolate of *Pseudomonas aeruginosa* were grown on a glucose minimal medium in a continuous-flow, annular biofilm reactor (Rototorque). The reactor system and general operating conditions have been described elsewhere previously (2). The data reported in this article were collected by using several Rototorques operating under similar, but not identical, conditions. The reactors included removable polycarbonate slides, which are used to characterize biofilm. Biofilm thickness was measured at eight points on each sample slide by an optical method (1). This technique involves focusing first on the substratum and then on the biofilm surface and recording the distance between the two focal planes as read from a scale on the fine-focus knob of the microscope. Microscopic observations were made with an Olympus BH-2 microscope.

A biofilm thickness distribution is presented in Fig. 1 in the form of a cumulative percentage plot. This type of plot gives for any value on the abscissa the percentage of all measurements that are less than or equal to that value. For example, Fig. 1 shows that 90% of the thickness measurements were less than or equal to 47 μm. The thickness distribution shown in Fig. 1 compiles 48 measurements from three replicate experiments. All of the data represent measurements taken after the attainment of steady-state biofilm thickness (after 140 or more hours of reactor operation). Biofilm thickness is broadly distributed. The mean and standard deviation of the measurements are 31.9 ± 10.6 μm. In earlier works (2, 12), such biofilms have been characterized by their average thicknesses, a description that fails to communicate the truly rough structure of the film.

A second type of biofilm thickness measurement was obtained from the distribution of tracer particles added to a biofilm reactor in a short-pulse addition. The particles attached to the biofilm and essentially mapped its surface; 86% of the particles lay within 2 μm of the biofilm surface. Fluorescent latex microbeads with a nominal diameter of 1 μm (no. 15702, Polysciences) were added for an 8-min period to the influent of the reactor at a concentration of 7.2×10^{11} beads per liter. A reactor slide was removed 3 min after the end of the bead addition period. A polycarbonate membrane had been taped to the Rototorque slide prior to sterilization and inoculation in the reactor. After the slides were removed from the reactor, the biofilm-membrane specimen was separated from the slide. Specimens were fixed in 2.5% glutaraldehyde in phosphate-buffered saline, dehydrated in ethanol, and embedded in JB-4 plastic resin (no. 18570, Polysciences). Thin sections (2.5 μm) were cut with a microtome by using a glass knife. Sections were stained with Giemsa stain and examined microscopically by using simultaneously transmitted white and epifluorescent light. The spatial distribution of tracer microbeads in the biofilm sample was constructed from distance measurements made by using an image analysis system (Videometric 150, American Innovision).

The distribution of positions measured for 1,113 beads is shown in Fig. 1. The distribution has a mean and standard deviation of 33.8 ± 9.8 μm, and it is similar to that obtained from optical thickness measurements. Although the agreement between the two distributions is good, it should be noted that the tracer beads do not necessarily attach to the biofilm surface randomly. It is conceivable, for example, that the beads do not always fully penetrate pores in the biofilm. There is some evidence of this in the slight discrep-

* Corresponding author.
† Present address: Pacific Northwest Laboratories, Battelle Boulevard, Richland, WA 99352.

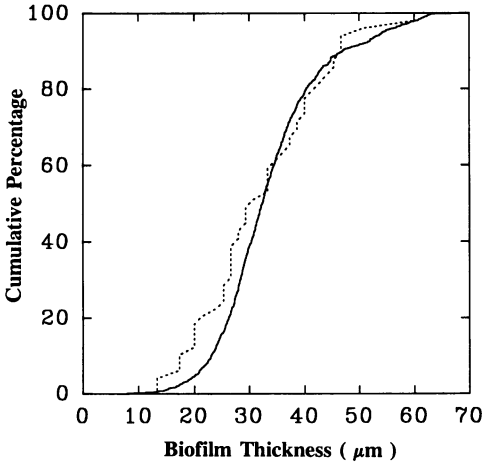

FIG. 1. Biofilm thickness (- - -) and tracer particle (——) spatial distributions.

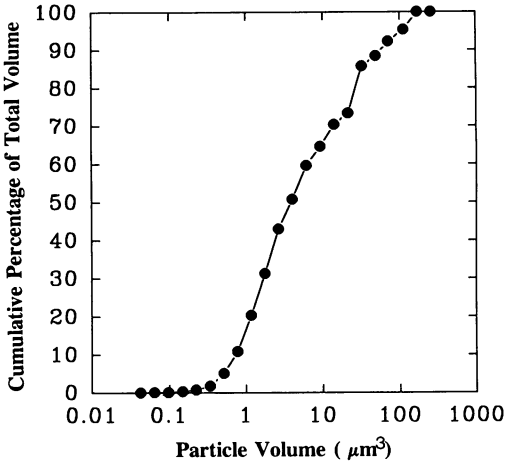

FIG. 3. Biomass particle size distribution.

ancy between the two distributions at smaller thicknesses. Once again, the principal message of these thickness distributions is that the biofilm surface is rough.

The rough surface of the biofilm can also be visualized by plotting measurements of biofilm thickness along a linear path across the substratum. Figure 2 presents such a biofilm thickness profile determined from thin cross sections by the image analysis system. These measurements do not rely on bead position; the biofilm surface was located by the presence of stained biomass. Thickness was measured at approximately 2-μm intervals along a 1,000-μm path. Gaps in the profile occur where there were wrinkles in the thin section. Significant changes in biofilm thickness occur over lateral distances as small as 5 to 10 μm.

To demonstrate the discrete nature of biomass removal from the biofilm, we measured biomass particle sizes by microscopic examination of particles retained on a microporous filter. A 10-ml sample of reactor effluent was diluted 1:1 with phosphate-buffered formalin and refrigerated at 4°C until it was analyzed. Biomass was stained by adding to the preserved sample 1 ml of a 56-mg/liter solution of Hoechst stain 33342. The sample was briefly vortex mixed, covered, and allowed to stand for 45 min. The sample was diluted with 1 to 4 ml of sterile water, and then a 1- to 5-ml aliquot was filtered through a 25-mm-diameter, 0.2-μm-pore-size Irgalan black-stained filter. The filter was then examined microscopically by using the image analysis system to determine particle areas. Thirty frames on each of three filters were analyzed. Aggregation of the cells during refrigerated storage was not investigated, and it cannot be ruled out.

The effluent particle size distribution is reproduced in Fig. 3. Particle sizes have been converted from areas to an equivalent cubic volume to better illustrate the amount of biomass in particulate form. Particles ranged in size from less than 0.1 to greater than 100 μm^3, with a mean particle volume of 2.2 μm^3. The mean volume of a single cell of this organism was measured separately to be 0.6 μm^3. Approximately 80% of the total biomass volume that detached from the biofilm was in the form of particles with volumes greater than 1 μm^3. Most of the detached biomass was, therefore, in the form of multicellular aggregates. Because of possible shearing of larger particles in the turbulent flow environment of the reactor, these measurements probably underestimate the actual extent to which biomass detaches as multicellular particles.

In summary, *P. aeruginosa* biofilms grown in a laboratory system exhibit substantial heterogeneity in thickness and in the size of their detached biomass particles in the reactor effluent. Biofilm heterogeneity may be even more pronounced in other systems. For example, in a previous experiment with an undefined mixed population, the coefficient of variation (9) of biofilm thickness was 83%, whereas with the *Pseudomonas* biofilms it was approximately 30%.

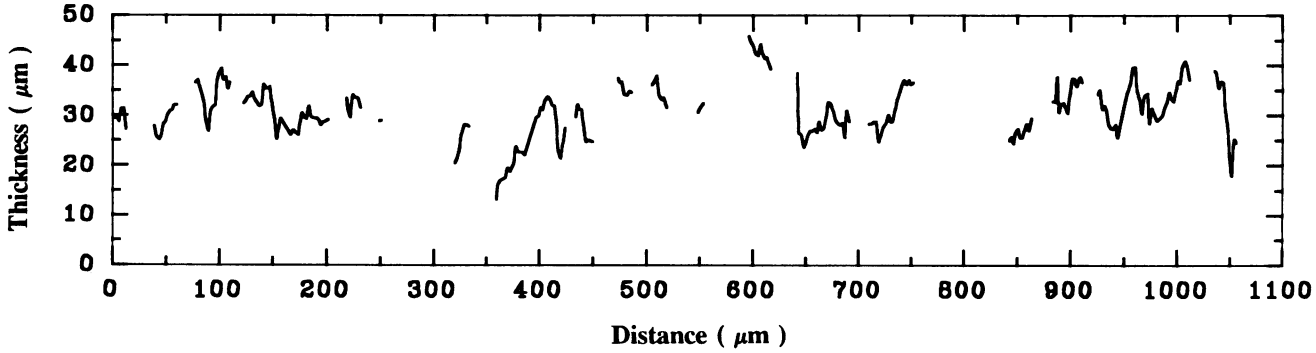

FIG. 2. Biofilm thickness profile.

Others have also previously reported relatively large coefficients of variation for the biofilm thicknesses of mixed-population films (4, 8, 12).

One possible explanation for the occurrence of heterogeneity in biofilms is that detachment occurs as discrete multicellular aggregates of varying sizes (12a). This mechanism could explain variations in thickness as well as in detached-particle size. An important step toward refining biofilm models based on such concepts will be the development of experimental and theoretical approaches to characterizing these heterogeneities quantitatively (5, 6, 14). While the use of an average biofilm thickness may be adequate for some purposes, such as predicting overall substrate consumption rates, the assumption of uniform biofilm thickness is probably inadequate for modeling such phenomena as population dynamics and localized, microbially influenced corrosion.

This work was supported by the National Science Foundation and by the Industrial Associates of the Center for Interfacial Microbial Process Engineering. Cooperative agreement ECD-8907039 between the National Science Foundation and Montana State University is acknowledged.

We thank Martin Hamilton for statistical guidance.

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