Reproducibility of biofilm processes and the meaning of steady state in biofilm reactors

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Abstract The need for reproducing biofilm processes is undisputable – the quality of biofilm research depends on this reproducibility. However, as many biofilm researchers know, long-term biofilm processes are notoriously difficult to reproduce. To avoid problems related to biofilm reproducibility two strategies are used: (1) to study very young biofilms that have accumulated for a few hours to a few days only, and (2) to run biofilm experiments only once. The first approach trades reproducibility for relevance because natural biofilms are usually older, often much older than a few days. This approach can be applied to answer questions relevant to initial events of biofilm formation but not questions relevant to long-term biofilm accumulation. The second approach conceals the problem of biofilm reproducibility.

To assure reproducibility of biofilm processes, we methodically followed a procedure for growing biofilms in terms of microbial makeup, media composition, temperature, surface preparation, etc. Despite all this effort the reproducibility of our results for long term growth is unimpressive. Consequently, the question had to be asked: Are biofilm processes reproducible? The experiments described in this paper address this question. Biofilms grown in two identical and identically operated biofilm reactors had comparable structure only until the first sloughing event. After that, biofilms had different patterns of accumulation.

Keywords Biofilm processes; reproducibility; steady state

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Introduction

Attempting to develop a model biofilm relevant to environmental studies we have isolated from environmental biofilms three microorganisms: *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Klebsiella pneumoniae*, and registered them in the American Type Culture Collection, ATCC, as *Pseudomonas aeruginosa* (ATCC 700829), *Pseudomonas fluorescens* (ATCC 700830), and *Klebsiella pneumoniae* (ATCC 700831). Consequently, each member of the model biofilm consortium can be obtained from an independent source and our results can be reproduced in other laboratories. To prepare the model biofilms, we always use the selected microorganisms at the same stage of growth. To assure reproducibility of biofilm processes, we closely follow a protocol for inoculating and growing the model biofilms in terms of media composition, surface preparation, etc.

To test if the model biofilms can be grown reproducibly, we have operated two identical flat plate biofilm reactors under identical and well defined flow regimes. The reactors were operated in parallel, were inoculated with microorganisms from the same vessel, and the biofilms were grown using the growth media from the same container. As the criterion of biofilm reproducibility we tested reproducibility of biofilm areal porosity measured at the level of the bottom. We assumed that structurally identical biofilms grew as a result of identical biofilm processes. Therefore, if the biofilm processes are identical in both reactors, biofilm areal porosity in both reactors should be identical.

Materials and methods

Figure 1 shows the experimental setup that was used in the study. Two identical flat plate

reactors were attached to the inverted microscope. The reactors were polycarbonate channels with glass bottoms, 2.5 cm wide, 4.0 cm deep and 34.5 cm long. Each reactor had a working volume of 150-mL, including the volume in the tubing. The tubing for the growth media was Masterflex® 6402-14 (Cole Parmer, Chicago, IL.), while the recycle, air and waste lines were all Masterflex® 6402-16 (Cole Parmer, Chicago, IL.). Peristaltic pumps (Cole-Parmer, Chicago, IL.) were used to maintain nutrient flow and recycle rates. The airline had an in-line bacterial air vent filter (Pall – Gelman Laboratory, Ann Arbor, MI, USA) with a pore size of 1 μm. The lid of the reactor was sealed with silicon rubber to prevent contamination, and the entire system is sterilized with bleach before each experiment. The tubing, connectors, and air filters were all autoclaved at 121°C to ensure sterilization.

The biofilms were composed of *P. aeruginosa* (ATCC#700829), *P. fluorescens* (ATCC#700830), and *K. pneumoniae* (ATCC#700831). A frozen stock sample of each species was inoculated into separate flasks and the cultures were grown for 24 hours in 100 mL of growth medium with a modified amount of yeast extract (2 g/L) on a shaker set to 150 rpm at room temperature (approximately 25°C). The reactor was inoculated with 30 mL of each culture, aseptically, via needle and syringe, through the line in which the growth medium enters the reactor. It was assumed that the number of cells introduced into the reactors was the same since both reactors were inoculated from the same source. Just before inoculation, the sterile water in the reactor was replaced with freshly prepared sterile growth medium. During the inoculation the flow of growth media, the recycle loop, and the airflow were stopped, and the waste line was clamped. Approximately 15 minutes after the inoculation, the recycling was resumed. Twelve hours later the feed pump was turned on and the airflow was restored to the reactor, the effluent clamp was removed, and the reactor was operated in continuous mode. 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 for the duration of the experiments. This high recycle ratio also provided uniform substrate concentration along the reactor (Smith, 1970). Each day before imaging, the reactor was flushed with sterile, de-ionized water to remove suspended microorganisms. The reactor was operated at room temperature (~25°C). The reactor was continuously aerated from the surface using filtered air at 3 L/h.

The growth medium consisted of (reagents from Fisher®): Na_2HPO_4 (1.825 g/L), KH_2PO_4 (0.35 g/L), $MgSO_4 \cdot 7H_2O$ (0.01 g/L), $(NH_4)_2SO_4$ (1.0 g/L), glucose (5 g/L) and yeast extract (0.1 g/L). The solution was autoclaved at 121°C for 3 hours per 20 L of solution. The glucose and yeast were autoclaved separately and then added after sterilization. One mL of micronutrient solution per litre of growth medium was added aseptically through a 0.2 μm syringe filter (Corning Inc, New York). The micronutrient solution con-

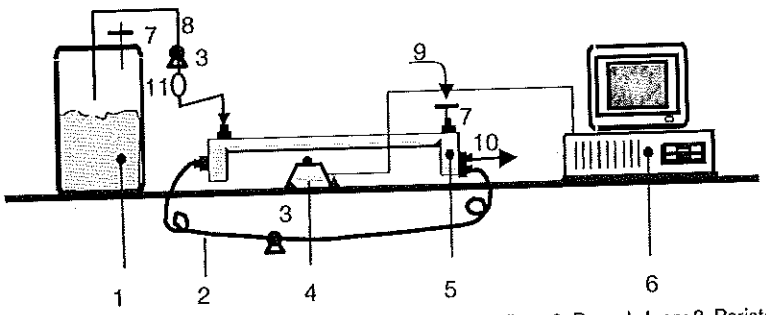

Figure 1 Schematic view of the experimental set-up. 1. Growth medium; 2. Recycle loop; 3. Peristaltic pump; 4. Inverted microscope integrated with a digital camera; 5. Flat plate reactor; 6. Computer; 7. Air filter; 8. Feed line; 9. Air In; 10. Outflow; 11. Flow breaker

sisted of $MnCl_2 \cdot 4H_2O$ (527 mg/L), $CuCl_2 \cdot 2H_2O$ (228 mg/L), $CoCl_2 \cdot 6H_2O$ (317 mg/L), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (231 mg/L), $Na_2B_4O_7 \cdot 10H_2O$ (127 mg/L), $ZnCl_2$ (363 mg/L), $CaCl_2$ (3.7 g/L), and $FeCl_3$ (2.16 g/L), dissolved in 1 L of 0.1 M HCl.

The biofilms were grown in reactors with glass bottoms and placed on the Olympus CK2 inverted microscope. The best quality images we could obtain were just after the reactor was flushed with sterile water. The images were taken through the bottom of the reactor using 40× magnification and were captured by a COHU® camera using Flashpoint, a frame grabber integrated with a computer. The images were in 8-bit gray-scale TIFF format, consisting of 640 × 480 pixels. Each day, 30 images were taken at random locations within the biofilm. Areal porosity of the biofilms was evaluated from the images using the ISA3 software and compared in Figure 2. The areal porosity (AP) was defined as the ratio of void area to total area (Yang *et al.*, 2000):

$$Areal\ porosity = \frac{Number\ of\ void\ pixels}{Total\ number\ of\ pixels}$$

To assign statistical significance to this comparison of biofilm areal porosities in both reactors, and thereby evaluate reproducibility of the biofilms, we used the two-tailed t-test using 95% confidence level, $p = 0.05$ (Figure 3).

Results and discussion

The results are in Figures 2 and 3. The biofilms in both reactors had similar structure for the first 20 days. On day 20 of operation a sloughing event occurred in reactor 2, and for the remaining time of operation the biofilms in both reactors were different.

The results indicate that biofilm structure in both reactors remained identical for 20 days, until the first sloughing event happened in reactor 2. After that each reactor showed a different pattern of biofilm accumulation, and each was indicating existence of sloughing and regrowth cycles. This result was reproduced a few times in our laboratory, and we are confident that biofilms in two identical and identically operated reactors are similar only until the first sloughing event, and then the biofilms in each reactor show different patterns of accumulation. However, this pattern of accumulation can be characterized by cycles of growth-sloughing-regrowth cycles rather than steady state. We hypothesize that these cycles may be more reproducible than the steady state. However, the data collected at this time do not permit testing this hypothesis.

Biofilm structure in both reactors was continually changing during the entire time of biofilm accumulation. This observation questions the assumption that biofilm structure reaches steady state, and has further consequences. If biofilm structure does not reach steady state, than steady state of biofilm activity in the reactor is questionable. The existence of steady state in biofilm reactors has always been a convenient assumption for modeling biofilms. However, it is not an experimentally demonstrable fact. On the contrary, experimental evidence is accumulating indicating that biofilms never reach steady state. For example, Bakke *et al.* (1989) demonstrated that older biofilms were continuously increasing their density, even though their thickness remained constant. Our data corroborate these observations although using different types of measurements. The concept of steady state applies to the continuous culture of suspended microorganisms, but it is not clear why it should apply to the continuous culture of attached microorganisms. Mathematical models of biofilm accumulation continue to operate on the assumption that biofilms do reach steady state (Noguera *et al.*, 1999; Wood and Whitaker, 1998; Picioreanu *et al.*, 1998 a,b; Horn and Hempel, 1997; Wimpenny and Colastini, 1997; Wanner and Reichert, 1996; Wanner, 1995; Reichert, 1994; Elmaleh, 1990; Livingston and Chase, 1989, and many others) because this assumption simplifies

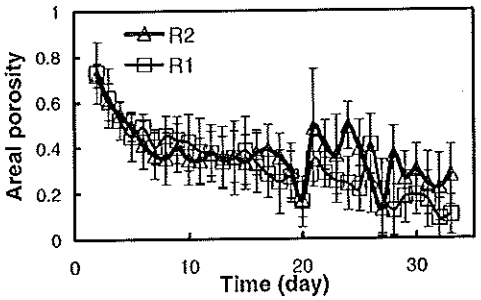

Figure 2 The average areal porosities, and standard deviations from the averages, were evaluated daily from thirty images of each biofilm taken at randomly selected locations. The data show that the biofilms in two parallel reactors are similar only until the first sloughing event. After that each biofilm has a different growth pattern. R1 and R2 refer to the reactors number one and number two

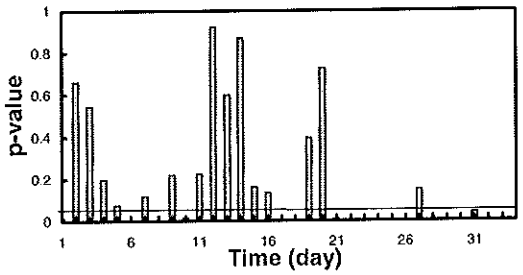

Figure 3 Statistical analysis, using the two tailed *t*-test, of biofilm areal porosities shows that after about 20 days the identically inoculated and grown biofilms acquired different growth patterns. The *p*-values exceeding 0.05 indicate that the biofilms were the same and the *p*-values less than 0.05 indicate that the biofilms were different. The continuous horizontal line marks the *p*-value of 0.05, which was accepted as the criterion for testing the null hypothesis that the biofilms were identical

computations, not because the existence of steady state has been experimentally demonstrated.

The assumption that biofilm processes reach steady state leads to the expectation that if the environmental conditions remain the same, biofilm processes should reach the same steady state every time. This is equivalent to expecting that such variables as biofilm thickness, density, cell count, amount of extracellular polymer deposited by the microorganisms, etc., are reproducible as long as environmental conditions are the same. Since this expectation resists experimental verification, biofilm processes are renowned for being difficult to reproduce. Perhaps, however, as we hypothesize, these are not the steady states that are reproducible under a given set of conditions, but those are the fluctuations in biofilm structure.

The concept of steady state is invaluable in flow reactors where microbial growth occurs in suspension. In such reactors, the interplay between the growth rate, biomass concentration, and hydraulic retention time leads to a steady state where process variables do not change in time. The meaning of steady state in biofilm reactors is less clear because of the intricate interplay between the microscale steady state (processes in the biofilm) and the macroscale steady state (processes in the reactor). When chemical profiles are measured across biofilms using microelectrodes, e.g. oxygen microelectrodes, it is assumed that the biofilm is at pseudo steady state because the shapes of the profiles do not change in time. This assumption (see Horn and Hempel, 1997) is useful because it simplifies mathematical treatment of the results. However, this assumption implies only that the profiles do not change during the time of the measurement, which lasts a few minutes. Concentration

profiles indeed remain constant during such measurements, which can be verified by taking another profile a few minutes later. Extrapolating this microscale pseudo steady state which refers to a particular location in the biofilm to the true macroscale steady state in the entire biofilm reactor is not warranted because demonstrating that substrate concentration profiles remain constant for a short time does not mean that they remain constant for an unspecified length of time. In summary, it appears that the concept of steady state in biofilm reactors should be revisited. These conclusions coincide with other recently published results. Specifically, Picioreanu *et al.* (2001) mathematically modeled biofilm growth and attachment to evaluate effects of detachment on biofilm structures and concluded that biofilm structure was continuously changing, and that detachments and erosion affect biofilm structure. These effects bring the questions: if biofilm structure is continuously changing, then what is the meaning, and how do we define steady state in biofilm reactors? These questions are closely related to the problem of reproducibility of biofilm processes. Without a clear notion what steady state in biofilm reactors really means, it is difficult to properly formulate the problem of reproducibility of biofilm processes. Extrapolating the concept of steady state from the continuous flow reactors with suspended microorganisms leads to a conclusion that biofilm structure, and possibly biofilm activity, are not reproducible, which may question validity of many research projects.

Conclusions

1. Biofilm structure, evaluated as areal porosity, is reproducible only for a limited time of reactor operation, until the first sloughing event. After that biofilm structure changes unpredictably.
2. Biofilm structure changes continuously as a result of sloughing and re-growth cycles.
3. As a result of perpetual changes in biofilm structure, and possibly in the accumulated biomass, and the overall microbial activity, the meaning of steady state in biofilm reactors is unclear. Consequently, it is also unclear how to define reproducibility of biofilm processes.

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