Journal of Industrial Microbiology (1995) 15, 297–304

Biofilm formation in laminar flow using *Pseudomonas fluorescens* EX101

MG Brading[1], J Boyle[2] and HM Lappin-Scott[1]

[1]*Department of Biological Sciences, Hatherly Laboratories;* [2]*Department of Engineering, University of Exeter, Devon, UK, EX4 4PS*

The relationship between biofilm formation and Reynolds number in laminar flow has been investigated using *Pseudomonas fluorescens* EX101. It was shown using a Modified Robbins Device that in laminar flow, numbers of viable cells in a developed biofilm increased with Reynolds number (Re 2, 17 and 51.5), as would be expected in a system where molecular transport to the wall is limited by diffusion. By monitoring fluorescent beads in a flowcell with a scanning confocal laser microscope at similar low Reynolds numbers, the velocity profile close to the solid surface was determined. It was shown that the presence of a thin bacterial film (up to 12 μm) displaced the flow profile away from the wall by a distance equivalent to the film thickness. Total cell counts from the Modified Robbins Device samples were not significantly different at the different flow rates but were higher than viable counts. Interruption of the flow had no significant effect on colonisation by the bacteria through the Modified Robbins Device in the first few hours. However, viable numbers were reduced when the flow was stopped at 7 h after initial colonisation.

Keywords: biofilms; laminar flow; Robbins Device; *Pseudomonas fluorescens*; bacterial adhesion

Introduction

Bacterial biofilms are found in most natural and man-made environments where bacteria are associated predominantly with surfaces rather than in a free-floating state. Biofilms are of great importance in industries such as food processing and paper production, and in the water industry, where thousands of kilometres of pipe surfaces have to be monitored and controlled for bacterial colonisation [1,14]. Bacterial biofilms reduce heat transfer efficiency, increase resistance to flow in pipes and act as a reservoir for potential pathogens [10,14].

Many factors may affect the rate of bacterial adhesion, including nutrient availability in the surrounding medium and the growth stage of the cells themselves. However, in industrial applications it is important to consider how the behaviour of the liquids flowing within the system influences the processes of bacterial attachment and detachment and the development within the biofilm.

Two contrasting extremes of flow exist, laminar flow and turbulent flow [16]. Laminar flow is the smooth flow of water through a pipe or duct with no lateral mixing [8]. The flow is considered to move in sheets or laminae [5]. Once the movement at a point in the flow becomes erratic and irregular, the flow is defined as turbulent. Fluid particles occupy different relative positions in successive cross-sections [7]. The Reynolds number (Re) is used to predict whether laminar or turbulent flow is occurring in a system [19].

Most flows in natural and engineered systems are turbulent. However, because turbulent flow is complex and difficult to predict, most experimental work by microbiologists involves laminar flow because it can be described analytically and it is of interest in studying the movement of bacteria through slow-moving or quiescent water [5]. In addition to this, it is commonly assumed that in turbulent flow a laminar sublayer exists close to the pipe wall. Hence, characteristic velocity profiles exist for laminar and turbulent flow. In a pipe the fluid actually in contact with the pipe has zero velocity and a velocity gradient exists between the fluid in the free stream and the pipe surface, the fluid moving near the centre being more rapid than fluid moving closer to the walls. In laminar flow there is no separate boundary layer as in turbulent flow, as the acceleration forces are insufficient to overcome the viscous forces present [6]. The boundary layer increases in depth as the flow develops until once fully developed, it takes up the whole of the pipe. The effect of biofilm growth on the fluid dynamics in laminar flow has not been investigated in detail in the microbiological literature.

The low velocity of fluid flow next to the surface plays an important part in the limitation of biofilm growth, due to effects on the input of cells and nutrients. These are continuously transported in the bulk motion of fluid in the pipe [6] because the fluid moves in parallel laminae and there is no bulk convection across the laminae. Most bacteria behave as colloidal particles [18]. Therefore, planktonic cells, as well as nutrients are transported between the laminae by Brownian motion or by molecular diffusion [5,16]. For some motile cells this transfer may be enhanced by cell motility. Hence, colonisation characteristics between biofilms grown at a range of flow rates would be expected to vary due to the consequent differences in diffusion rates and the resulting changes in nutrient availability and in the numbers of bacteria available for colonisation.

In laminar flow the lack of mixing and slow velocity of fluid near to the surface not only means the biofilm rapidly depletes substrate adjacent to the pipe wall but toxic metab-

Correspondence: HM Lappin-Scott, Department of Biological Sciences, Hatherly Laboratories, University of Exeter, Devon, UK, EX4 4PS
Received 23 November 1994; accepted 30 March 1995

olites and waste products also build up [3]. This leads to environmental stress on the biofilm, which not only affects the growth of developing biofilms but may also cause the shearing of already developed, thick biofilms [4].

This paper investigates interactions between flow velocity and biofilm growth at a surface. Using a scanning confocal laser microscope the effects of biofilm growth on the laminar flow pattern close to the surface has been examined in flow cells with glass coverslips. Conversely, the effects of laminar flow on biofilm growth has been investigated by examining biofilm formation in Modified Robbins Devices under different flow rates, as well as investigating the effects on colonisation due to interruption of the flow.

Materials and methods

Organisms and growth medium

A motile soil isolate, *Pseudomonas fluorescens* EX101 was used in all experiments. This strain produces mucoid colonies on Pseudomonas isolating agar. It is a good surface coloniser. Cultures were grown on sodium citrate medium containing sodium citrate 6.45 g L^{-1}, $(NH_4)_2SO_4$ 0.198 g L^{-1}, KH_2PO_4 2.72 g L^{-1}, K_2HPO_4 5.23 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 0.246 g L^{-1} and $FeCl_2 \cdot 4H_2O$ 0.0082 g L^{-1}.

Apparatus used

Flow cells were set up as described previously [2], sterilised with 60% sodium hypochlorite and irrigated with sterile deionised water before use. Dimensions of the flow cells in these experiments were 4.76 mm wide × 1 mm deep × 42 mm long.

The Modified Robbins Device (MRD) was developed from the original Robbins Device for the multisampling of biofilms [15,20]. The MRDs had internal measurements of 10 mm wide × 2 mm deep × 41.5 cm long. Silastic rubber (SAMCO) was used as the colonisation surface with backing discs of soft black rubber (Esco Rubber, Bibby Sterilin Ltd, Aldershot, UK). This allowed easy incorporation of the surfaces into the removable studs so that they were flush with the inside lumen surface. The MRDs were sterilised with ethylene oxide before use by the Derriford Hospital, Plymouth, UK.

In this paper, the Reynolds number (Re) has been used as the parameter with which to compare flow rates. This is given as:

$$\mathrm{Re} = \frac{\rho v d}{\mu}$$

where ρ is the density of the fluid (in kg m^{-3}), μ is the viscosity of the fluid (in N sm^{-2} or centipoise), v is the velocity (in m s^{-1}) and d is the diameter of the MRD (in m). In this instance because both the flow cell and MRD are rectangular rather than circular in cross-section, the hydraulic diameter (Dh) has to be substituted for d, so $Dh = 4A/P$ [19], where A is the cross-sectional area and P is the wetted perimeter.

Investigation of laminar flow at the surface of flow cells before and after biofilm formation

This was achieved using an MRC-600 Lasersharp fluorescence scanning confocal laser microscope system (SCLM), (BioRad Microscience, Mississauga, Ontario, Canada), mounted on a Nikon FXA microscope equipped with a 60×, 1.4 numerical aperture objective [13]. Computer enhanced imaging was used to track the movement of 0.5-μm fluorescent beads through the flow cells, at known distances from the flow cell surface. Perspex flow cells were set up as described previously [2] and two bulk flow values were investigated, 10 ml h^{-1} and 50 ml h^{-1} with corresponding Res of 0.74 and 5.2. The flow was maintained by a Watson and Marlow (Falmouth, UK) peristaltic pump, with medical drips being incorporated into the system to eliminate the effects of peristalsis.

The flow cell was placed on the microscope stage and sterile sodium citrate medium pumped through the apparatus. Using a sterile syringe and needle, 0.5-μm fluorescent beads (Fluoresbrite™ plain microspheres, Polysciences Inc, Northampton, UK) were injected into the system (1 drop in 10 ml sterile deionised, distilled water) and tracked via computer enhanced microscopy. As the SCLM has the ability to focus at different levels through a sample, the velocity of the beads could be measured at different sample sites and at known distances from the flow cell surface, without disturbing the sample. Three computer images were recorded at each sample time and the distance between the first and the third image was calculated. This distance was then divided by the time taken for the three frames to be completed (1.13 s per frame) to give a value for the bead velocity (in μm s^{-1}). The average velocity was determined between 0 μm and 12 μm from the surface at 2-μm intervals (N = 40 for each distance studied).

The flow cell apparatus incorporated a 0.2-μm pore size filter to prevent backgrowth. An inoculum of 1 ml of an overnight culture of *P. fluorescens* was introduced to the flow cell using a sterile syringe and the pump switched off for 5 min to allow initial bacterial colonisation. After restarting the pump, the system was left for 24 h before the fluorescent beads were injected again and the velocity gradient close to the biofilm was redetermined (N = 40 for each distance studied). The biofilm was also viewed at this time using a solution of 0.1% fluorescein (BDH, UK) in sterile distilled water as a negative stain. An estimation of the depth of the biofilm was made by focusing through the biofilm in increments of 2 μm from the flow cell surface (read as 0 μm) until cells were no longer in the field of view.

Investigation of biofilm formation at different laminar flow rates

A Modified Robbins Device was attached via a recirculating system to a 1500-ml batch culture of *P. fluorescens*. A 2% initial inoculum of an overnight culture was used and the experiments were run at ambient temperature (23° C). The flask was placed on a magnetic stirrer to maintain homogeneity and aeration of the culture. Three different flow rates were investigated, 35.5 ml h^{-1}, 270 ml h^{-1} and 810 ml h^{-1} with corresponding Res of 2, 17 and 51.5. Experiments were performed in triplicate. Samples were taken at regular time intervals and bacterial colonisation was monitored using colony forming units, scanning electron microscopy and total counts using acridine orange.

Investigation of the effect of interrupted flow on biofilm formation

The apparatus was set up as before and a flow rate equivalent to Re 51.5 maintained. The flow was stopped at 1 h, restarted at 4 h until 7 h, when it was stopped again until 18 h and then restarted for a final reading to be taken at 24 h. The experiment was undertaken in duplicate.

Biofilm enumeration

At regular time intervals studs were removed from the MRD, washed with 1/4 Ringers solution and examined. For viable cell counts, studs were scraped into 1/4 Ringers solution, together with the scalpel blade, sonicated and diluted before being plated on to nutrient agar. Total counts were determined using epifluorescence microscopy and acridine orange staining [9]. The bacteria were fixed in 0.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). Samples were filtered on to 0.2-μm pore size white polycarbonate membranes (Poretics Corporation, USA), stained with 0.01% acridine orange (in potassium phosphate buffer pH 7.5) and counted under an Olympus BH-2 epifluorescence microscope (Olympus Optical Co (UK) Ltd, London, UK).

Scanning electron microscopy

Washed discs were removed from the studs and fixed in 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h at room temperature before being dehydrated in an ethanol–distilled water series of 30, 50, 70 and 100% ethanol. The discs were then air-dried and gold sputter-coated before being viewed on a Cambridge Stereoscan (Leica, Cambridge, UK) at a 25-kV accelerating voltage. At each sample time, discs were removed in duplicate from different sites in the MRD, as representative of biofilm formation in the whole system.

Results

The SCLM and computer images successfully demonstrated biofilm formation and monitoring of flow rates close to the surfaces of the flow cells (Figure 1). However, velocity was more successfully measured at the lower flow rate (Re 0.74) than at the higher flow rate (Re 5.2). At the lower flow rate (Re 0.74) the beads travelled in straight lines and in a uniform manner, there being equidistant separation of the beads between the three computer frames used in the analysis (Figure 1a). After the *P. fluorescens* biofilm had developed for 24 h, channelling was clearly evident, the surface itself being visible in places. Variation in the depth of the biofilm was also apparent, with parts of the biofilm being up to 12 μm in thickness (Figure 1b). The velocity profile was displaced by the biofilm, with the relative velocities being maintained (Figure 2a). That is, the velocity profile seen from 0 μm to 10 μm from the surface, prior to inoculation of bacteria into the flow cell was observed from 12 μm to 20 μm from the surface after the growth of the biofilm. For example, the velocity at 2 μm from the surface before biofilm growth was the same as the velocity 14 μm from the surface after the growth of a biofilm that was 12 μm in thickness (values of 5.38 μm s^{-1} and 4.68 μm s^{-1} respectively). A calculation for the thickness

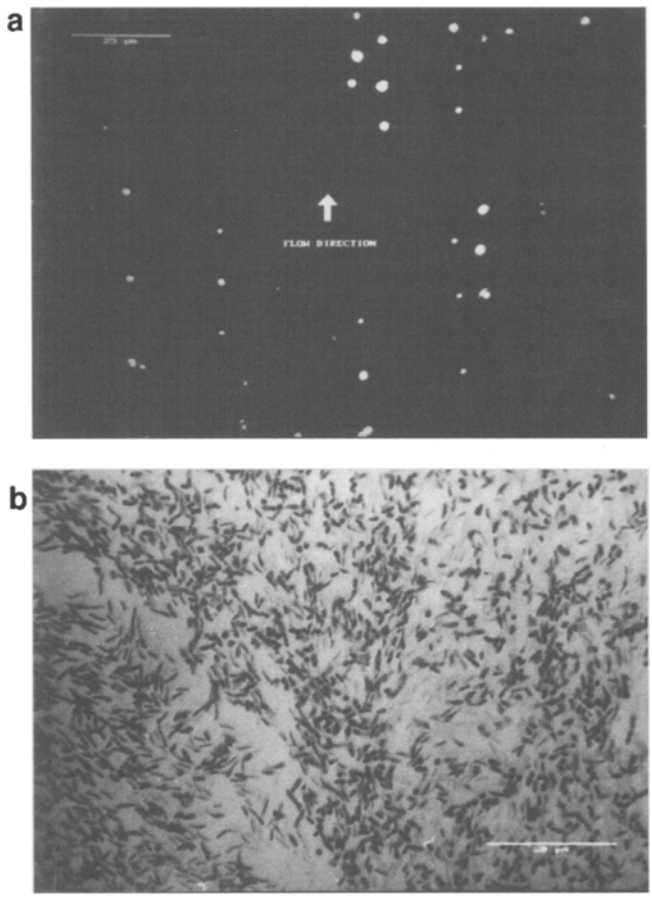

Figure 1 (a) The scanning confocal laser microscope was used in conjunction with fluorescent beads to monitor flow rates close to the surface of the flow cell. The photograph represents three superimposed computer frames to show the movement of beads 12 μm from the surface prior to biofilm formation. The scale bar represents 25 μm. (b) The scanning confocal laser microscope was used to show the structure of the *P. fluorescens* biofilm at 24 h using 0.1% fluorescein. The image was built up by superimposing images taken every 0.8 μm for a total distance of 4 μm. The scale bar represents 20 μm

of the biofilm was also made by obtaining the best-fit line for each data set and extrapolating the curves to the x-axis (Figure 2b). From this, the biofilm thickness was estimated to be 11.4 μm, compared with the visual estimation of 12 μm. However, such data could not be obtained at the higher flow rate (Re 5.2) because the beads were travelling too fast to allow accurate monitoring of their velocities. The beads were still travelling in straight lines before the bacteria were added but after the growth of the *P. fluorescens* biofilm, the beads were deflected around areas of the bacterial colonisation. This is shown in the photographs which were taken 16 μm from the surface (Figure 3). As the biofilm was on average 12 μm in thickness, the contours of the biofilm were affecting the flow laminae 4 μm from the biofilm surface itself.

There was a significant difference in biofilm formation when an MRD was used with three different laminar flow rates, specifically, a higher initial rate of colonisation at Re 51.5 and Re 17 compared with Re 2 (Figure 4). The viable cell counts for Re 2 and Re 51.5 were significantly different at the 0.01 level when data sets of the means were com-

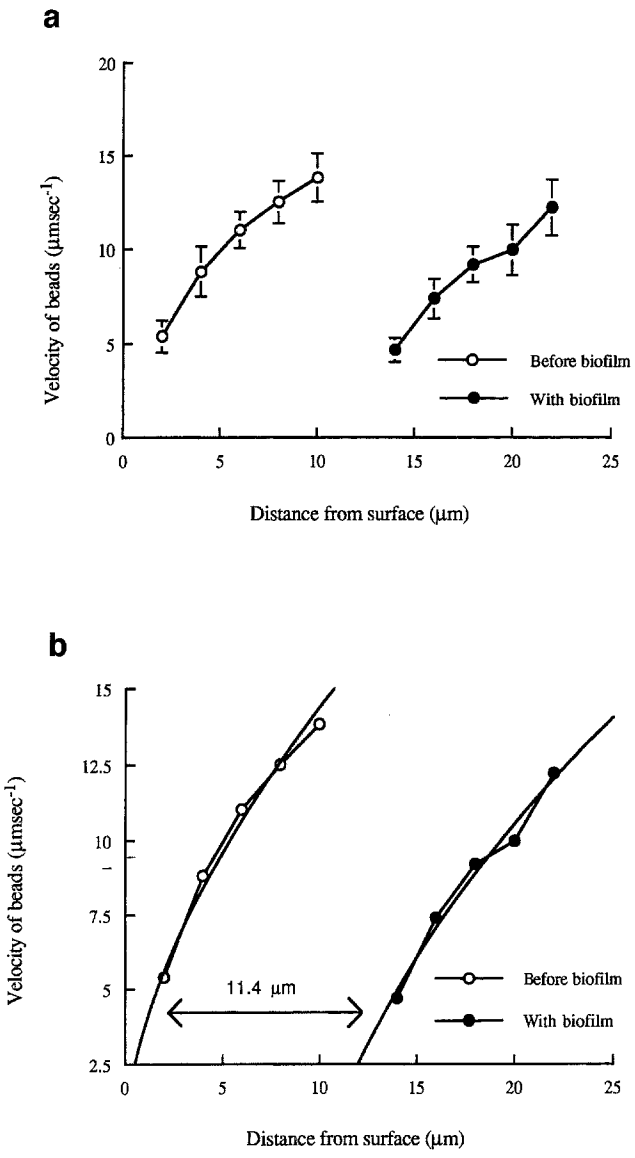

Figure 2 (a) Bead velocities are shown at known distances from the surface of the flow cell before and after growth of a biofilm estimated to be 12 μm in depth. The velocity profile was seen to be displaced by the biofilm. Error bars show standard error. (b) An extrapolation of the data in (a) was used to make a further estimation of biofilm thickness. The distance on the x-axis between the two data sets was found to be 11.4 μm

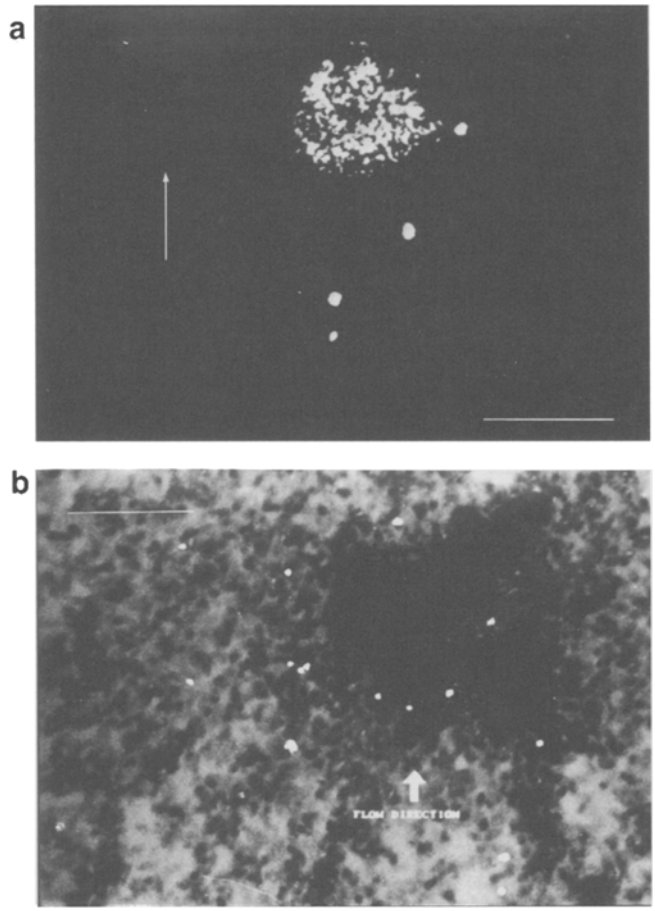

Figure 3 (a) After the growth of the *P. fluorescens* biofilm for 24 h, the flow was affected by contours in the biofilm surface. Using the scanning confocal laser microscope, three computer frames were superimposed to show the movement of a solitary fluorescent bead around a bacterial microcolony. The photograph was taken 16 μm from the flow cell surface. The arrow indicates the direction of flow. The scale bar represents 20 μm. (b) Using the same technique as in (a), the bead movement 16 μm from the flow cell surface was superimposed on to the biofilm image to show how the flow was affected by the dense colonisation in the centre of the frame. Bead movement appeared to be erratic around the bacterial colonisation. Scale bar represents 20 μm

pared individually in a one-way analysis of variance. Scanning electron microscopy (Figure 5) showed a difference in the number of cells present on the silastic rubber, a greater number of cells being seen at Re 51.5. The biofilm at the higher flow rate also appeared to have more exopolysaccharide. The total counts in both flow rates investigated (Re 2 and Re 51.5) were higher than the viable counts (Figure 6). However, there was no significant difference between the total counts themselves at the two flow rates. The data were compared at separate time intervals using a one-way analysis of variance.

Interruption of the flow (Re 51.5) in the initial hours of colonisation appeared to have a minimal effect on bacterial numbers on the surface (Figure 7a,b). When the flow was stopped at 1 h, restarted again at 4 h and stopped again at 7 h, growth of the *P. fluorescens* on the surface continued to increase and was seen to be equivalent to the organism's culture doubling time of 1 h, 34 min (Figure 7b). The numbers of bacteria in the biofilm that arose from attachment alone as opposed to those that resulted from cell division, must be represented in the difference in the colony forming units between the colonisation pattern at Re 51.5 and the numbers expected due to exponential growth (Figure 7a). After initial colonisation the flow was stopped at 7 h until 18 h when the flow was restarted. In this time the number of viable cells dropped sharply but began to level out again when the flow was resumed (Figure 7a). This contrasts with the reported pattern at Re 51.5 where bacterial numbers continued to increase. Statistically, there was only a significant difference in the numbers of bacteria present after the initial hours of colonisation. For example, after 24 h when the flow had been stopped for 17 h, the results were significantly different at the 0.01 level. Data sets were tested with a one-way analysis of variance.

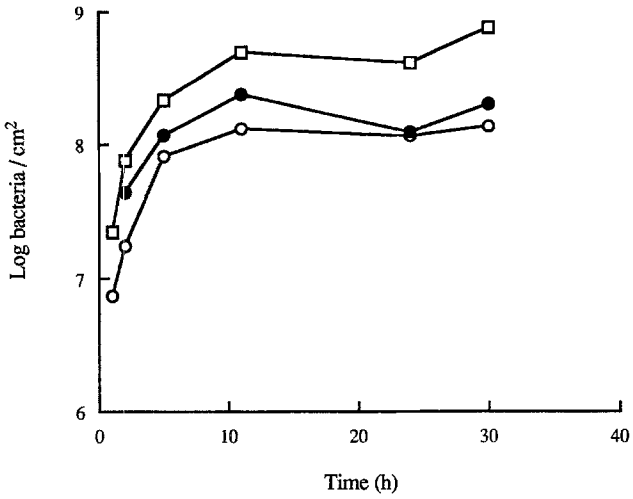

Figure 4 Colonisation by *P. fluorescens* in the MRD increased with increasing Re in laminar flow. The differences in the means were compared at each data point using a one-way analysis of variance and found to be significant at the 0.01 level. —○— Re 2; —●— Re 17; —□— Re 51.5

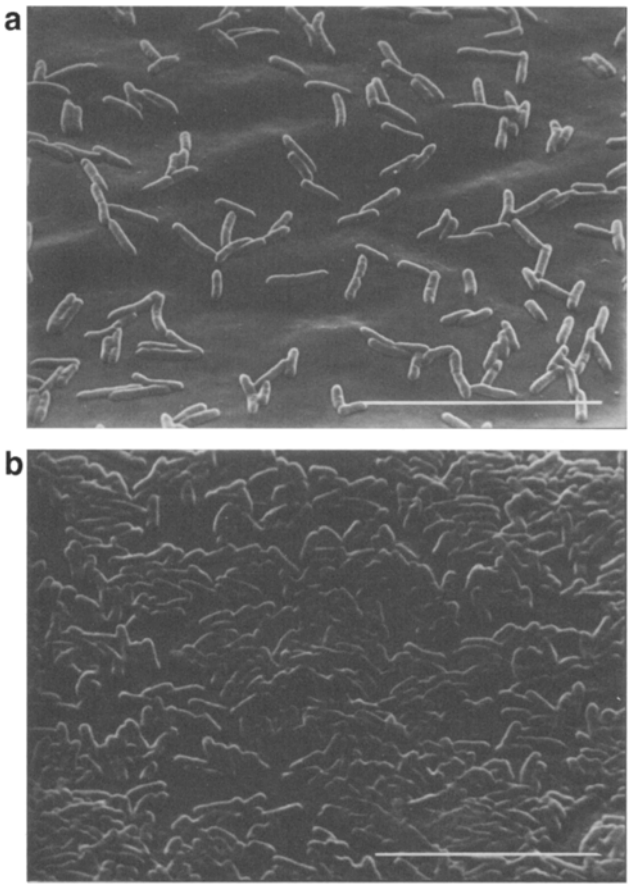

Figure 5 From the scanning electron microscope, colonisation of *P. fluorescens* at Re 2 (a) appeared to be sparse compared with the number of bacteria present at Re 51.5 (b). The bacteria at Re 2 appeared to be better defined than those at Re 51.5, indicating that there was a greater production of exopolysaccharide at the higher flow rate. Scale bars represent 10 μm

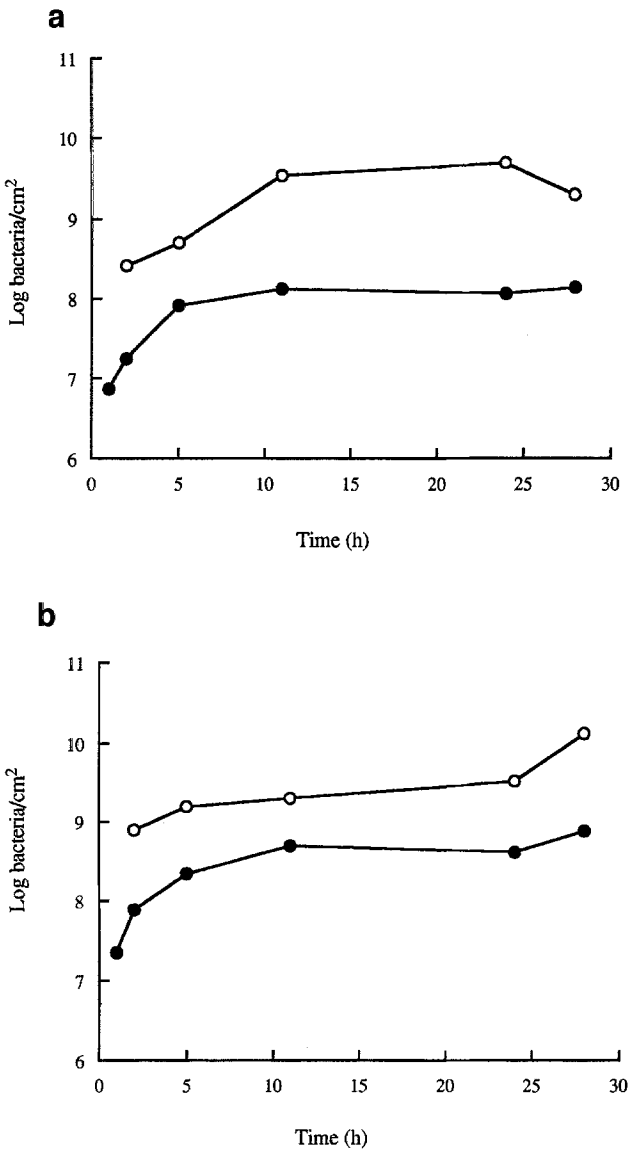

Figure 6 Total counts (○) for *P. fluorescens* colonisation in the MRD were higher than the viable cell counts (●) at both Re 2 (a) and Re 51.5 (b). However, the difference between the total and viable counts was higher at the lower Re, indicating greater cell death at this flow rate

Discussion

The use of flow cells and the Modified Robbins Device allows qualitative and quantitative data to be obtained for biofilm formation under different flow conditions.

The advantages of particle tracking of fluorescent beads by SCLM have been given previously [23] as well as the limitations to the range of velocity measurements possible due to the optics of the system and the scanning rate of the laser. Hence, in this investigation, the accuracy of this technique was limited to the laminar flow measurements of Re 0.74. At this flow rate, the beads travelled in straight lines within the flow laminae (Figure 1a), there being a gradual increase in velocity towards the centre of the lumen and away from the flow cell surface. Others [23] found that when biofilm was present the measured velocity profile had two regions, one within the biofilm and the other outside the biofilm where the profile was the same as that obtained

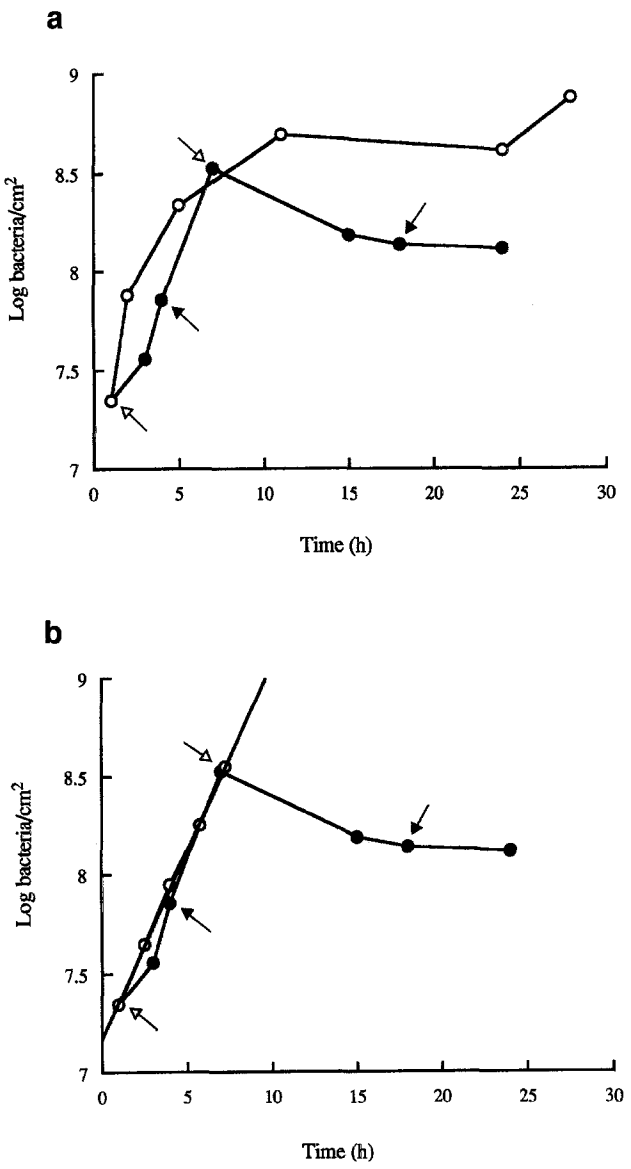

Figure 7 (a) Interruption of the flow (●) affected the rate of colonisation of *P. fluorescens* in the MRD. Stopping the flow in the initial hours of colonisation (between 1 and 4 h) did not stop viable cells increasing in number on the surface, although the numbers were reduced when compared with the expected number of viable cells present at Re 51.5 (○). However, when the flow was started again and stopped after 7 h of colonisation viable numbers decreased. Arrows indicate the times at which the flow was stopped (open arrows) and restarted (closed arrows). (b) Growth of viable cell numbers (●) in (a) was found to follow the plot of exponential growth expected for *P. fluorescens* in batch culture (○). The culture doubling time in batch culture was 1 h 34 min

in a sterile reactor. In this investigation the velocity profile had been maintained but was displaced by the growth of a maturing biofilm (Figure 2a). This displacement was measured (Figure 2b) and found to be equivalent to the average depth of the biofilm (11.4 μm to 12 μm respectively). Assuming that the effects of gravity are negligible on motile cells [12,21] and therefore that bacterial colonisation on the lower surface of the flow cell is equivalent to the upper surface then an approximate total of 24 μm in the lumen is occupied with bacterial biofilm. In a flow cell where the lumen depth is 1 mm this is equivalent to 2% of the total flow cell lumen and will have a small effect on the velocity profile in the lumen. The pump maintained the flow rate through the apparatus so there was no reduction in total volumetric flow and consequently the profile became sharpened, although in industrial systems the build up of bacterial biofilms increases drag and greatly decreases the flow through a pipe [25].

There was also an apparent reduction in the rate of change in the flow lamina for both data sets, (before and after bacterial colonisation) with increasing distance from the surface. The results obtained furthest from the surface had larger standard deviations, probably due to the speed at which the beads were travelling which may have introduced a greater degree of error. The unevenness of the biofilm may have contributed to the observed effect on the flow velocities seen after biofilm growth, due to the fact that a 3-D biofilm not only affects the vertical velocity profile but also the horizontal flow distribution.

Despite the limitations, important information can still be collected from monitoring bead movement at higher flow rates. At Re 5.2, beads travelled in straight lines before biofilm formation. After the biofilm had developed however, many of the beads present became entangled in the biofilm. While some were permanently trapped, others were released due to the fluid flow. Further from the biofilm surface at 12 μm, the beads appeared scattered and did not travel in the characteristic straight lines that were observed at the lower flow velocity. Flow laminae were restored 18 μm from the glass surface, 6 μm from the surface of the biofilm.

A number of techniques have been used to investigate the mosaic nature of biofilms [4,23,24]. Biofilm architecture is complex. Indeed, the flow pattern of beads observed around the biofilm suggested that the surface of the film was not flat. This surface disturbance can be expected to increase with increasing flow rate. As *P. fluorescens* is on average only 2 μm in length, the fact that there was movement away from the flow laminae around the biofilm microcolonies (although not complete mixing) up to 4 μm from the biofilm surface, makes this observation increasingly significant. This observation also helps to confirm the non-confluent nature of the biofilm as shown in Figure 1b. *P. fluorescens* is a motile bacterium, having a tuft of polar flagella whose action will affect the slow moving flow laminae close to the surface of the flow cell. It is generally accepted that the Re associated with the swimming of microorganisms is typically of the order of 10^{-2} or less [22]. Hence, at a very low flow rate the bacteria themselves, by swimming in close proximity to each other as well as the surface, may affect the hydrodynamic interactions occurring in the flow laminae close to the boundary of the lumen. That is, if the concentration of motile bacteria at the surface is high enough, their movement will have an effect on the flow laminae close to the surface.

Changing the flow rate in the Modified Robbins Device led to differences in the biofilm formation observed (Figures 4, 5 and 6). As all the relevant parameters in the experiments were kept constant, it can be considered that any changes in biofilm formation must be due to changes in the flow [17]. It has been demonstrated that lack of mixing and low velocity close to the surface in laminar flow

enhances the depletion of nutrients by the biofilm and the build up of toxic waste products [3]. It was postulated therefore, that there would be greater bacterial numbers at higher flow velocities due to the increased input of nutrients from the bulk flow to the film surface and a correspondingly greater rate of removal of toxic metabolites. If the flow velocity is too high, sloughing of the film may increase because of higher shear stress [17]. In this investigation viable bacterial numbers increased significantly with an increase in flow rate. Whether this was a result of increased attachment of cells or due to bacterial growth and replication was difficult to ascertain. An estimation was made from the results in Figure 7 where the flow was interrupted during the test period. At a flow rate of Re 51.5 the number of bacteria present on the silastic rubber was fewer when the flow was stopped and restarted between 1 h and 7 h, than that observed when the flow rate was maintained continuously over the same time. However, growth of the biofilm did not cease when the flow stopped, indicating that during initial stages of colonisation, the flow rate is not the important parameter for maintaining biofilm growth at a stage when space and nutrients are readily available. Interestingly, the growth rate at this time correlated closely with the culture doubling time of *P. fluorescens* (1 h, 34 min), indicating that cell growth and division take precedence over cell attachment in the initial hours of colonisation when nutrient is not limited. The difference seen between these data and the bacterial numbers observed during uninterrupted flow must be due to the proportion of cells attaching from the flow laminae and thereby contributing to the colonisation of the surfaces.

Investigation of the attachment of *Bacillus cereus* in laminar flows with Res between 0.4 and 1.6 demonstrated that deposition increased with fluid velocity [21]. In this work, although growth appeared to be the important parameter from Figure 7, colonisation which occurred in the first hour suggested that attachment was a critical factor (10^7 cells in 1 h). This exceeds the planktonic culture doubling time of *P. fluorescens*. This increased colonisation rate could be due to the motility of the cells. It has been confirmed that motility conveys a selective advantage during surface colonisation, a motile strain attaching four times more rapidly and achieving higher final cell densities on surfaces than a non-motile strain [11].

In many industrial applications the flow through pipelines is not uniform and may vary considerably, for example, in wastewater conduits or in drainage pipelines where flow through the system is dependent on rainfall. This erratic flow will have an effect on bacterial colonisation at the surface over time. In this investigation, when the flow was stopped from 7 h to 18 h it was apparent that nutrient availability was limited at this time and growth and replication of the bacteria were reduced, while viable counts decreased. This indicated that attachment is more important in a maturing biofilm to increase cell numbers rather than the growth of cells, certainly at Re 51.5, where numbers of viable cells levelled out again once flow was resumed. These results confirm those obtained previously [2], where it was found that growth was flow-dependent at a nutrient concentration of 100 mg L^{-1} and flow-independent at 1 g L^{-1} glucose, indicating the importance of flow rate to supply nutrients to a maturing biofilm in conditions where the nutrients would otherwise be limited.

Assuming the techniques used are accurate, it can be said that the flow rate at Re 2 led to starvation and subsequent death in the biofilm, due to insufficient nutrient availability compared with Re 51.5. This is shown by comparing the total counts and viable counts at each flow rate (Figure 6). Whereas the total counts for Re 2 and Re 51.5 are comparable, the viable counts show lower numbers at Re 2, indicating the presence of more non-viable bacteria in the biofilm at this flow rate. However, there appeared to be a difference in the number of bacteria present at the two flow rates (Figure 5). In addition to this, the amount of exopolysaccharide appears to be greater at Re 51.5. There is evidence that initial production of exopolysaccharide is greater at the higher Re from previous work performed in our laboratories (data not shown) indicating the role of exopolysaccharide during cell attachment and maintenance of subsequent biofilm at the higher flow rate.

Bacterial biofilm formation is a complex process and depends on prevailing environmental conditions. Low flow rates are important in a range of systems, including industrial filters and medical implants. This paper has attempted to further our understanding of the complex relationship between biofilm formation and fluid dynamics and has shown how steady state biofilm numbers can vary in laminar conditions, indicating the importance of flow considerations in many applications.

Acknowledgements

We would like to acknowledge D Caldwell at the University of Saskatoon, Saskatchewan, Canada for the use of the scanning confocal electron microscope and D Korber for his advice in using the apparatus. We also thank RA Davey for his technical assistance and for the construction of the flow cells. This work was funded by a scholarship provided by the University of Exeter.

References

1 Block JC, K Houdidier, JL Paquin, J Miazga and Y Levi. 1993. Biofilm accumulation in drinking water distribution systems. Biofouling 6: 333–343.
2 Caldwell DE and JR Lawrence. 1986. Growth kinetics of *P. fluorescens* microcolonies within the hydrodynamic boundary layers of surface microenvironments. Microb Ecol 12: 229–312.
3 Caldwell DE and JR Lawrence, 1989. Study of attached cells in continuous flow slide culture. In: A Handbook of Laboratory Model Systems for Microbial Ecosystem Research (Wimpenny JWT, ed), vol 1, pp 117–138, CRC Press, Boca Raton, FL.
4 Caldwell DE, DR Korber and JR Lawrence. 1992. Confocal laser microscopy and digital image analysis in microbial ecology. Adv Microb Ecol 12: 1–67.
5 Characklis WG, MH Turakhia and N. Zelver. 1990. Transport and interfacial transport phenomena. In: Biofilms (Characklis WG and KC Marshall, eds), pp 265–340, John Wiley and Sons, New York.
6 Cunningham AB. 1989. Hydrodynamics and solute transport at the fluid–biofilm interface. In: Structure and Function of Biofilms (Characklis WG and PA Wilderer, eds), pp 19–31, John Wiley and Sons, Chichester, UK.
7 Douglas JF, JM Gasiorek and JA Swaffield. 1984. Motion of fluid particles and streams. In: Fluid Mechanics. pp 97–117, Pitman Publishing Limited, London, UK.
8 Fletcher M and KC Marshall. 1982. Are solid surfaces of ecological

significance to aquatic bacteria? In: Advances in Microbial Ecology (Marshall KC, ed), vol 6, pp 199–236, Plenum Publishing Corp, New York.
9 Geesey GG and JW Costerton. 1981. Application of epifluorescence microscopy to the enumeration of aquatic bacteria concentrated on membrane filters. In: Membrane Filtration. Applications, Techniques and Problems (Dutka BJ, ed), pp 253–264, Marcel Dekker Inc, New York.
10 Huang CT, SW Peretti and JD Bryers. 1992. Use of flow cell reactors to quantify biofilm formation. Biotechnol Lett 6: 193–198.
11 Korber DR, JR Lawrence and DE Caldwell. 1994. Effect of motility on surface colonisation and reproductive success of *P. fluorescens* in dual-dilution continuous culture and batch culture systems. Appl Environ Microbiol 60: 1421–1429.
12 Korber DR, JR Lawrence, L Zhang and DE Caldwell. 1990. Effect of gravity on bacterial deposition and orientation in laminar flow environments. Biofouling 2: 335–350.
13 Korber DR, GA James and JW Costerton. 1994. Evaluation of fleroxacin activity against established *Pseudomonas fluorescens* biofilms. Appl Environ Microbiol 60: 1663–1669.
14 Lappin-Scott HM and JW Costerton. 1989. Bacterial biofilms and surface fouling. Biofouling 1: 323–342.
15 Lappin-Scott HM, J Jass and JW Costerton. 1993. Microbial biofilm formation and characterisation. In: Microbial Biofilms: Formation and Control (Denyer SP, SP Gorman and M Sussman, eds), pp 1–12, Blackwell Scientific Publications, UK.
16 Lappin-Scott HM, MG Brading and J Jass. 1994. How do bacteria reach surfaces? In: Bacterial Biofilms and their Control in Medicine and Industry (Wimpenny J, W Nichols, D Stickler and H Lappin-Scott, eds), pp 19–23, Bioline, UK.
17 Lau YL and D Liu. 1993. Effect of flow rate on biofilm accumulation in open channels. Water Res 27: 355–360.
18 Marshall KC. 1980. Bacterial adhesion in natural environments. In: Microbial Adhesion to Surfaces (Berkeley RCW, JM Lynch, J Melling, PR Rutter and B Vincent, eds), pp 187–196, Ellis Horwood Publishers, Chichester, UK.
19 Munson BR, DF Young and TH Okiishi. 1990. Viscous flow in pipes. In: Fundamentals of Fluid Mechanics. pp 465–559, John Wiley and Sons, New York.
20 Nickel JC, I Ruseska, JB White and JW Costerton. 1985. Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material. Antimicrob Agents Chemother 27: 619–624.
21 Powell MS and NKH Slater. 1983. The deposition of bacterial cells from laminar flows on to solid surfaces. Biotechnol Bioeng 25: 891–900.
22 Ramia M and D Tullock. 1990. Micro-organism locomotion: an application of fluid mechanics and kinematics. 5th International Conference on Rheology, Melbourne, 1990, pp 95–98.
23 Stoodley P, D DeBeer and Z Lewandowski. 1994. Liquid flow in biofilm systems. Appl Environ Microbiol 60: 2711–2716.
24 Walker JT, DE Caldwell, K Hanson and CW Keevil. 1994. Use of scanning confocal microscopy to study biofilm formation. In: Bacterial Biofilms and their Control in Medicine and Industry (Wimpenny J, W Nichols, D Stickler and H Lappin-Scott, eds), pp 69–76, Bioline, UK.
25 Watkins L and JW Costerton. 1984. Growth and biocide resistance of bacterial biofilms in industrial systems. Chemical Times and Trends 7(4): 35–40.