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Biofilm Structure and Influence on Biofouling under Laminar and Turbulent Flows

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ABSTRACT

A flow system was designed so that biofilms could be grown simultaneously in parallel flow cells under laminar and turbulent flows using shared nutrients and inocula. The flow cells were made from rectangular glass tubing chosen to simulate flow in industrial pipes. The hydrodynamics in the flow cells were characterized using dye tracers and the relationship between the Fanning friction factor (*f*) and Reynolds number (Re). Flow was laminar at Re 100 and turbulent at Re 3000. Transition between flows occurred at approximately Re 1000. Biofilms from environmental inocula were grown on tap water or a minimal salts medium with 40 ppm glucose. Biofilms grown under laminar flow were patchy and consisted of cell clusters separated by interstitial voids. Biofilms grown under turbulent flow were filamentous. The filaments had a complex structure and were formed by the colonization of filamentous sheathed bacteria with microcolonies of non-filamentous bacteria. The filamentous bacteria were often tangled together and subsequent colonization by microcolonies resulted in the formation of cohesive structures that we termed "biofilm streamers". The frictional loss coefficient (k_f) across the flow cell colonized with biofilm grown under turbulent flow was almost twice the k_f across the flow cell colonized with biofilm grown under laminar flow. There was little difference in k_f across the flow cell colonized with biofilm grown under laminar flow and the k_f across a clean flow cell.

1 INTRODUCTION

Biofilms growing in tubular flow cells demonstrate two distinct types of morphology. Reported experimental conditions suggest that hydrodynamics may be a determining factor for biofilm morphology. Under low shear, laminar conditions biofilms are often patchy with an apparently non-uniform surface distribution[1-6] but under high shear, turbulent conditions biofilms are often filamentous.[7-10] The influence of filamentous biofilms grown under turbulent conditions on biofouling has been well described.[7-10]

However, detailed *in situ* microscopic examination of these biofilms is limited. Recently we reported the formation of filamentous biofilm "streamers" under turbulent flow.[8] The streamers were flexible and oscillated from side to side in the flowing water. For biofilms grown under laminar conditions structure is well described but the influence of these biofilms on biofouling is limited. The recent application of confocal microscopy to biofilms grown under laminar conditions showed that these biofilms were generally heterogenous and consisted of cell clusters separated by interstitial voids.[2-6] The cell clusters were formed from discrete aggregates of microbial cells in a slime matrix, and the voids were open channels connected to the bulk liquid. Convective flow was discovered in the biofilm channels.[11]

It was the goal of this research to compare the structure of biofilms accumulated under laminar or turbulent flows and assess the influence of the biofilms on biofouling. We had four objectives. First, to design a flow system with parallel flow cells so that biofilms could be grown simultaneously under laminar and turbulent flows using shared nutrients and inocula. The hydrodynamic conditions in the flow cells had to be well characterized and the flow cell had to be able to fit on a microscope stage. Second, to examine the developing biofilms microscopically *in situ* under flow conditions. Third, to determine the structure of biofilm filaments and streamers, and finally, to determine the influence of the biofilms on biofouling. We used the relationship between the Fanning friction factor (f) and the Reynolds number (Re) to characterize the hydrodynamics in the flow cells and a frictional loss coefficient (k_f) as a measure of biofouling. The k_f is used by engineers to compare frictional losses across different fittings.[17] To our knowledge k_f has not been used to compare losses across fittings colonized with fouling biofilms.

2 MATERIALS AND METHODS

2.1 Reactor system and microscopy

The reactor system consisted of two flow cells (1 and 2), which were incorporated into a recycle loop with a mixing chamber for aeration and nutrient addition (Figure 1). The flow cells were made from sections of square glass tubing (S-103 Camlab Ltd., Cambridge, UK.) that were 3 mm wide and 3 mm deep. Nutrients were delivered by peristaltic pump (Masterflex, Cole Parmer, Niles, Il. USA) and the recycle flow rate was controlled with a vane head pump (Masterflex, Cole Parmer, Niles, Il. USA). The volume (V) of the mixing chamber and recycle loop, including the flow cells, was approximately 175 ml. The nutrient influent flow rate (Q_n) was 40 ml/min giving a resulting residence time ($\theta = V/Q_n$) of 40 minutes. The flow rate through each of the flow cells (Q_f) was measured using flow meters (McMillan Flo-sensor model 101T # 3724 and 3835 supplied by Cole-Parmer, Niles, IL). The pressure drop (ΔP) across each flow cell was measured using differential pressure transducers (RS Components, Corby, Northants, UK. model 286-686). The pressure transducers were calibrated using a pressurized water manometer. Q_f through each flow cell was controlled independently by tightening or loosening clamps on the inlet tubing The flow cells were positioned on a polycarbonate holder which was mounted on the stage of an Olympus BH2 upright microscope with epifluorescence capabilities. The biofilm could be imaged *in situ*

without interrupting flow. Under operating conditions the water temperature in the reactor system was 25°C and all experiments were performed at this temperature.

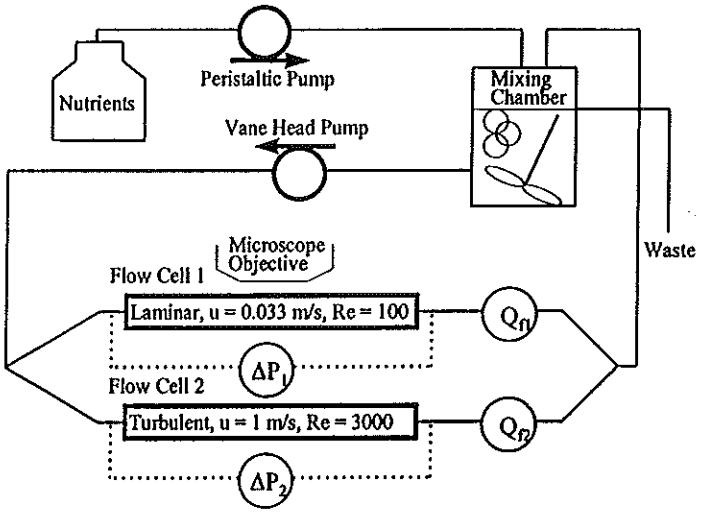

Figure 1 *Biofilm reactor system consisting of parallel flow cells in a recycle loop attached to a mixing chamber. The flow rates in the flow cells were measured by flow meters (Q_f) and the pressure drop across each of the flow cells was measured by pressure transducers (ΔP). The mixing chamber was aerated and the level maintained by overflow to waste.*

2.1.1 Image analysis A COHU 4612-5000 CCD camera (Cohu, Inc., San Diego, CA. USA) was used to collect images and a Scion VG-5 PCI framestore board (Scion Inc. Frederick, MD. USA) was used to capture the images. Image processing was done on a Macintosh 7200/90 computer using the public domain NIH-Image 1.59 program (developed at the National institutes of Health and available from the Internet by anonymous FTP from zippy.nimh.nih.gov or a floppy disk from the National Technical information service, Springfield, Virginia, USA, part number PB95-500195GEI). Biofilm length measurements (in a plane parallel to the substratum) were made using the "line tool" function and calibrated against a 1mm graticule with 10 μm divisions (Ref. # CS990, Graticules Ltd., Tonbridge, Kent, UK).

2.2 Hydrodynamic conditions in the flow cells

The flow system was designed so that there would be laminar flow in one flow cell and turbulent flow in the other flow cell. We determined the hydrodynamic characteristics of the flow cells using dye tracer studies and the relationship between the Fanning friction factor (f) and Reynolds number (Re). The f and Re were found from ΔP, average flow velocity ($u_{(ave)}$), and flow cell geometry. Two lengths of flow cell were studied; 10 cm and 20 cm. The distances between the pressure ports (l_f) were 15 and 24 cm respectively.

2.2.1 Pressure drop - velocity relationship ΔP was measured at different $u_{(ave)}$ across clean flow cells. The $u_{(ave)}$ was found by dividing the flow rate by the cross sectional area of the flow cell. The Reynolds number (Re) was calculated from [12]:

$$Re = \frac{u_{(ave)} D_h}{\nu} \qquad [1]$$

where D_h, was the hydraulic diameter and ν was the kinematic viscosity of water (0.897×10^{-6} m^2/s at 25 °C). D_h was (3×10^{-3} m) based on the cross sectional area and the wetted perimeter[13]. The f was found from[14]:

$$f = \frac{\Delta P \times D_h}{2 l_f \rho_w u_{(ave)}^2} \qquad [2]$$

where ρ_w was the density of water (997 kg/m^3 at 25°C). The relationship between f and Re from the experimental data was compared to theoretically and empirically derived relationships for flow through smooth pipes.

2.2.2 Dye trace study A syringe needle (size $21^1/_2$G) was inserted through a side port into a 10 cm long flow cell. India ink was injected into the flow cell using a peristaltic pump at the same $u_{(ave)}$ as the bulk liquid. Dye dispersion was observed microscopically.

2.3 Experimental runs

Two biofouling experimental runs were performed. The flow cells used for these experiments were 10 cm long. The friction factor data and the dye trace data were used to determine the flow rates through the flow cells. Flow was laminar at $u_{(ave)}$ 3.35×10^{-2} m/s (Re = 100) and turbulent at $u_{(ave)}$ 1 m/s (Re = 3000). Details are given in the results section.

2.3.1 Biofouling experiment 1 In this preliminary experiment the reactor was inoculated with non-sterile tap water which was recirculated through the flow system for 24 hours to allow attachment of bacteria in the flow cells. Tap water was then fed continuously to the reactor system for five days and biofilm accumulation monitored microscopically. Pressure drop data were not collected for this experiment. Flow cell 1 was maintained at Re 100 and flow cell 2 at Re 3000. After 5 days the biofilm was fixed with 1% paraformaldehyde (30 min.) and stained with the nucleic acid stain propidium iodide (0.4 %, at 25°C for 30 min.). The biofilms were imaged using a confocal scanning laser microscope (CSLM, Bio-Rad MRC600) attached to an inverted Olympus IMT-2 microscope using the Bio-Rad COMOS operating software.

2.3.2 Biofouling experiment 2 The reactor system was cleaned by exposure to a 1% household bleach solution for 24 hours. The bleach was removed by rinsing with 60 volumes of sterile tap water, 60 volumes of sterile sodium thiosulfate (100 ppm), and finally 60 volumes of sterile tap water. The nutrient feed and associated tubing were sterilized by autoclaving at 121°C for 15 minutes. The reactor was inoculated with stock cultures (1ml) of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Klebsiella pneumoniae* and initially run as a batch culture for 24 hours before switching to

continuous culture. Because of the complexity of the flow system aseptic conditions were not maintained and the resulting biofilms were undefined mixed cultures. A minimal salts medium (glucose 40 ppm, potassium phosphate monobasic (KH_2PO_4) 70 ppm, potassium phosphate dibasic (K_2HPO_4) 30 ppm, ammonium sulfate ($(NH_4)_2SO_4$) 10 ppm, and magnesium sulfate ($MgSO_4.7H_2O$) 1 ppm) was used to grow the biofilm. Biofilm accumulation was monitored daily by transmitted light microscopy. Flow cell 1 was maintained at Re 100 and flow cell 2 at Re 3000 for 17 days. ΔP was measured at different $u_{(ave)}$ in each of the flow cells on day 17. The friction loss coefficient[12] (k_f) was found by linear regression of the $\log_{10}$ - $\log_{10}$ plot of the ΔP - $u_{(ave)}$ data from:

$$k_f = \frac{2\Delta P}{u_{(ave)}{}^m \rho_w} \qquad [3]$$

where m is a dimensionless exponent dependent on pipe roughness. Flows were then switched so that flow cell 1 was at Re 3000 and flow cell 2 was at Re 100 and the reactor system was operated for a further 7 days.

2.3.3 Biofilm structure and metabolic activity The structure of biofilm streamers grown in experiment 2 was examined using the metabolic stain 5-cyano-2,3-ditolyl tetrazolium chloride (CTC). The application of CTC is described elsewhere.[15] After 24 days the flow cells were removed from the reactor system and stained with CTC (0.04 % w/v) for 30 minutes at 25°C in a shaker incubator. The flow cells were imaged using transmitted light microscopy and epifluorescent UV microscopy.

3 RESULTS AND DISCUSSION

3.1 **Hydrodynamic characteristics of the flow cells**

3.1.1 *Pressure drop - velocity relationship* The friction factor associated with the flow cells decreased with increasing Re (Figure 2). The data indicated two regions associated with laminar and turbulent flows. The transition between laminar and turbulent flow occurred between Re 1000 and Re 2000 which is within the established range for flows through smooth pipes.[17] The f values for the 10 cm flow cell in the laminar region were widely scattered indicating flow instability in this region. The predicted friction factor for laminar flow through a smooth pipe[16] (from the Hagen-Poiseullie equation) is:

$$f = \frac{16}{\mathrm{Re}} \qquad [4]$$

In the turbulent region the observed f values were approximately twice those predicted from the Blasius formula for turbulent flow (also for a smooth pipe)[16]:

$$f = 0.0791/\mathrm{Re}^{0.25} \qquad [5]$$

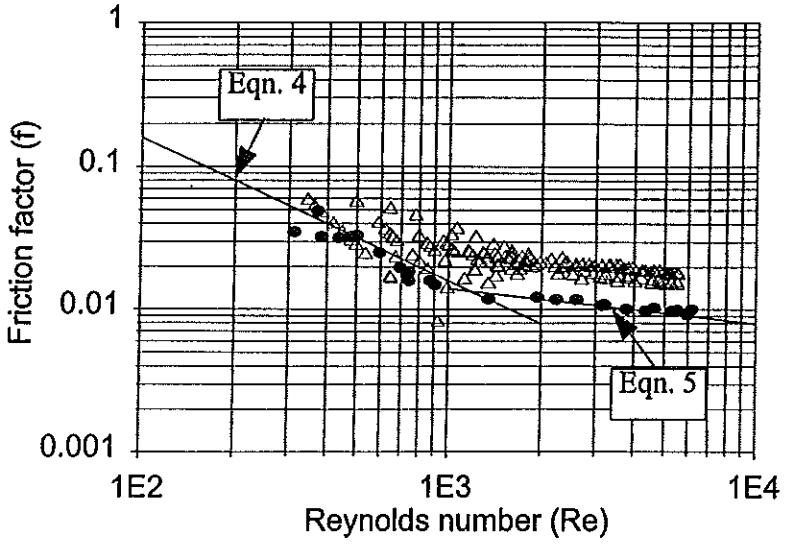

Figure 2 *Friction factor chart showing the data from the flow cells. 10 cm sections are indicated by open triangles and 20 cm sections are indicated by closed circles. The friction factor curves for the laminar region (Eqn. 4) and the turbulent region (Eqn. 5) are shown as solid lines for reference*[16].

Both of these effects could be caused by an inadequate flow cell entry length. Pressure gradients are greater across entry length regions than sections of pipe where flow is fully developed[17]. Pressure drop data could only be collected at Re > 300 because of the sensitivity limits of the pressure transducers (0.04 kPa). The *f* values of the 20 cm flow cell were very close to the predicted values from Eqns. 4 and 5. These data indicate that the length of a section of the square tubing should be at least 20 cm to simulate hydrodynamic conditions in larger scale pipe.

3.1.2 Dye tracer study When dye was injected into the 10 cm long flow cell at Re 100 the dye described a parabolic profile which was maintained down the entire length of the flow cell (data not shown). At Re 3000 the dye became dispersed after approximately 6 mm (data not shown). These data corroborate the *f*-Re relationship and demonstrated that flow was laminar at Re 100 and turbulent at Re 3000.

3.2 **Biofouling experiments**

3.2.1 *Biofouling experiment 1* After five days the biofilm in flow cell 1 (Re 100) was composed of single cells and small cell clusters (Figure 3a). The cell clusters were approximately circular in plan view. The diameter of the largest cell clusters were approximately 40 μm. The biofilm in flow cell 2 (Re 3000) was significantly different in appearance (Fig. 3b). The biofilm again contained single cells and cell clusters but there were also many filaments present. The cell clusters were larger than in flow cell 1 and were up to 100 μm in diameter. The filaments were composed of rod shaped bacteria that were approximately 3 μm long evenly spaced down the filament. The bacteria were separated from one another by gaps of approximately 3 μm. The filaments

were greater than 300 μm in length. The bacteria were brightly stained and the gaps between them were slightly stained. Transmitted light microscopy showed that the bacteria were sheathed (data not shown). Some of the filaments were tangled together and microcolonies were attached to the filaments.

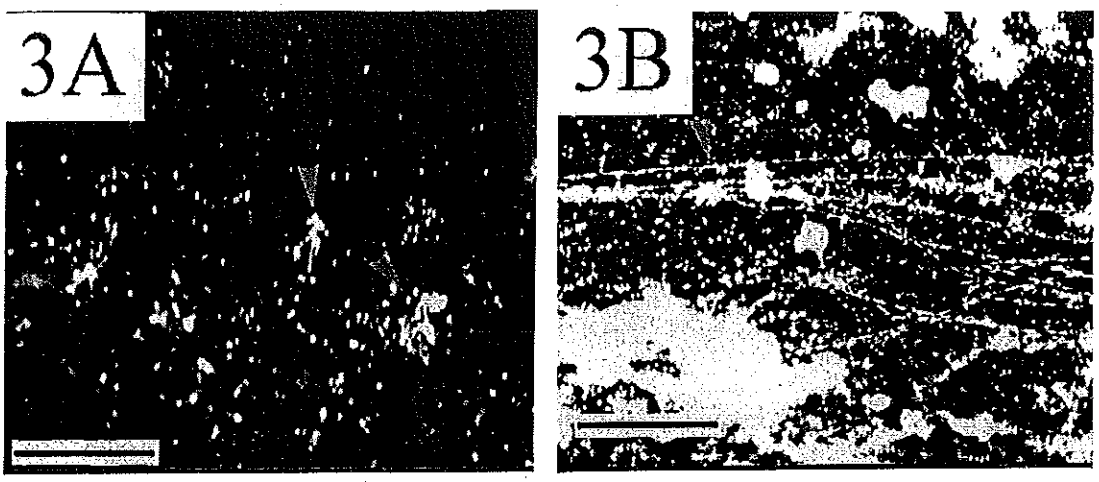

Figure 3 *Epifluorescent images showing biofilm accumulation after five days in biofouling experiment 1. Biofilm cells were stained with propidium iodide and appeared white. (A) The biofilm in the laminar flow cell was composed of single cells and cell clusters (indicated by arrow). (B) The biofilm in the turbulent flow cell had filamentous growth. The filaments were colonized with cell clusters. Single bacterial rods were spaced along the filaments (indicated by arrow). Scale bar = 50 μm.*

3.2.2 *Biofouling experiment 2* After 17 days the biofilm in flow cell 1 (Re 100) was composed of patches of cell clusters that were up to approximately 50 μm in diameter (Figure 4a). The cell clusters sparsely covered the flow cell. The biofilm in flow cell 2 (Re 3000) was composed of cell clusters with filaments trailing from the downstream edge of the cell clusters (Figure 4b).

The filaments were up to 2 mm in length and at low magnification appeared to have microcolonies growing around them. The filaments were free to oscillate from side to side in the flow.

The flow conditions in the two flow cells were then switched and after a further 7 days the biofilm in flow cell 1 (now Re 3000) had begun to develop streamers which were up to 2 mm in length (Figure 4c). The biofilm in flow cell 2 (now Re 100) was similar in appearance to that at day 17 but the streamers had lengthened so that some were greater than 3mm in length (Figure 4d). The biofilm streamers were composed of filaments trailing from the downstream edge of cell clusters which had become tangled and colonized with microcolonies forming a cohesive structure (Figure 5).

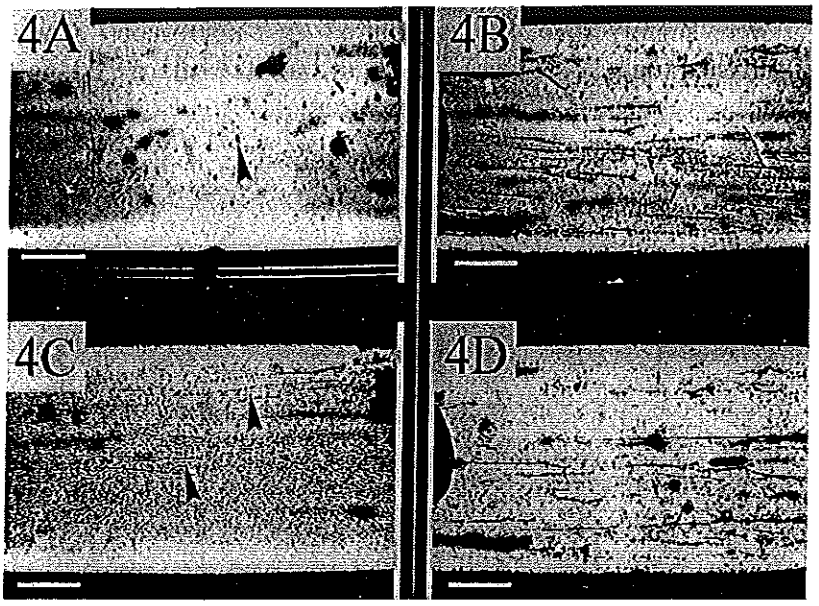

Figure 4 *Low power transmitted light images showing biofilm accumulation in biofouling experiment 2. Flow is from right to left. After 17 days growth in flow cell 1 (initially Re 100) the biofilm was composed of single cells and cell clusters (indicated by arrow) (A). The biofilm in flow cell 2 (initially Re 3000) was composed of single cells, cell clusters and filamentous streamers (B). Seven days after flow conditions were switched the biofilm in flow cell 1 began to develop filaments (indicated by arrows) (C) but there was little change to the biofilm in flow cell 2 (D). Scale bar = 500 μm.*

In both experiments filamentous biofilms only formed in the turbulent flow cells even though nutrient conditions were markedly different. This indicates that filament formation was more dependent on shear forces than nutrient loading. The results from the biofouling experiments allowed us to formulate a hypothesis for the development of the streamers. First, cell clusters form on the substratum. Second, high shear conditions stimulate the downstream growth of filamentous bacteria which are anchored to the cluster. Third, the filaments become tangled and provide a support matrix for the growth of microcolonies. Finally, the microcolonies merge to form a single structure.

3.2.3 *Influence of laminar and turbulent biofilms on frictional losses* After 17 days of biofilm accumulation the ΔP across the flow cells was found for different $u_{(ave)}$ (Figure 6). The ΔP increase across flow cell 1 (laminar) was similar to that for a clean flow cell for $u_{(ave)}$ less than 1.25 m/s. Above $u_{(ave)}$ of 1.25 m/s the increase in ΔP was more rapid in the clean flow cell than flow cell 1. The ΔP increase across flow cell 2 (turbulent) with increasing $u_{(ave)}$ was greater than that for the clean flow cell and flow cell 1. This difference may be caused by the different structures of the two biofilms. Linear regression analysis of the $\log_{10} \Delta P$ - $\log_{10} u_{(ave)}$ curves was used to determine the loss coefficients (k_f) and the exponent m in Eqn. 3 for flow cell 1 and 2 and a clean flow cell (Table 1).

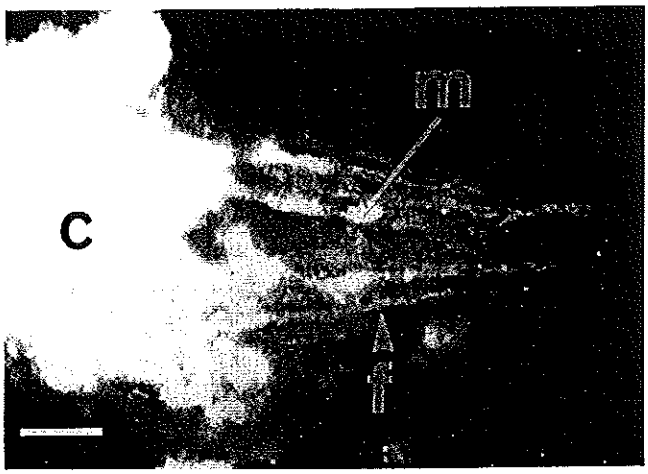

Figure 5 *Epifluorescent image of the biofilm from flow cell 2 in expt. 2 shows structural detail of a biofilm streamer. The biofilm was stained with CTC and appeared light against the dark background. A streamer is attached to the downstream side of cell cluster "C". The streamer was composed of filaments ("F") which had been subsequently colonized with bacteria microcolonies ("M"). Scale bar = 10 microns.*

Table 1 *Values for the loss coefficient (k_f) and velocity exponent (m) in Eqn. 3 for a clean flow cell and flow cells with 17 day old biofilms grown under laminar and turbulent flows. The associated regression correlation coefficient (r^2) and number of data points (n) are also shown.*

	k_f	m	r^2	n
Clean Tube	1.38	2.23	.997	14
Biofilm grown under laminar flow	1.44	1.69	.994	13
Biofilm grown under turbulent flow	2.68	1.67	.995	14

Only data in the turbulent region (above Re 2000) was used for the regression analysis. The k_f of flow cell 1 was 4.3 % higher than the clean flow cell and k_f of flow cell 2 was 94 % higher than the clean flow cell. The frictional losses associated with the turbulent biofilm were nearly twice those of the laminar biofilm. These results are consistent with the research of McCoy and Costerton who found that their biofilms only caused an increase in friction factor when filaments were formed.[18] The value of m for the clean flow cell was 2.23. For fully rough surfaces m has been shown to have a value of two.[16] The higher value that we found can be explained by an inadequate flow cell entry length as discussed previously in section 3.1 of this paper. The value of m for the biofilm colonized flow cells was approximately 1.7. This indicates that the fouled flow cells were behaving as if they were less rough than the clean flow cell. A study of velocity profiles in biofilm colonized flow cells by Lewandowski *et al.*[13] showed that their

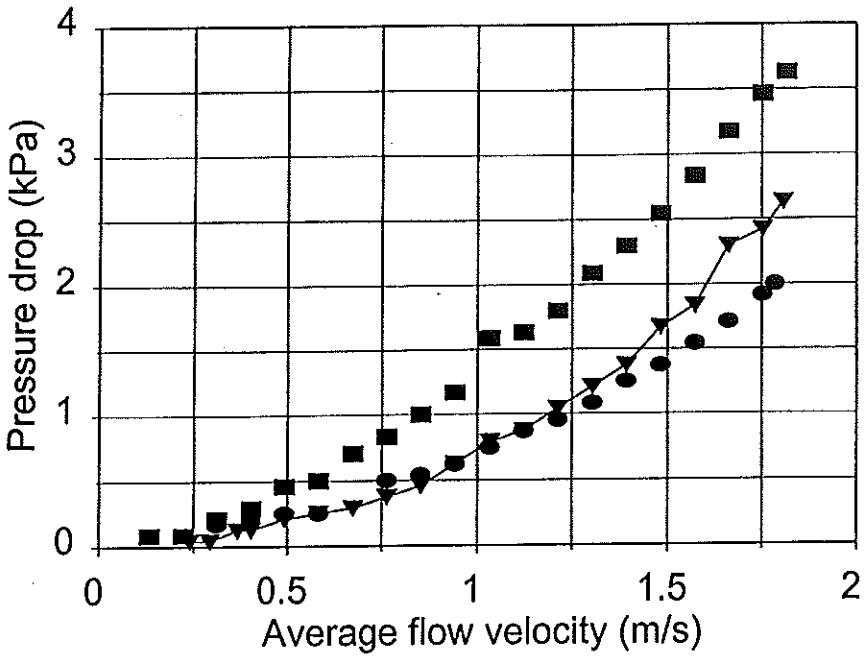

Figure 6 *Influence of $u_{(ave)}$ on ΔP across a clean flow cell (triangles), a flow cell colonized with a biofilm grown under laminar flow (circles) and a biofilm grown under turbulent flow (squares).*

4 CONCLUSIONS

1 Flow velocity had a strong influence on biofilm structure.
2 Filamentous biofilms formed under turbulent flow.
3 Biofilm streamers were formed by microcolony colonization of filamentous sheathed bacteria.
4 The filamentous biofilms which developed under turbulent flow caused greater frictional losses than the non-filamentous biofilms which developed under laminar flow.

5 ACKNOWLEDGMENTS

This research was supported in part by the co-operative agreement EEC-8907039 between the National Science Foundation and Montana State University.

NOMENCLATURE

Dimensions in terms of mass (M), length (L), and time (T).

D_h	hydraulic diameter (L)
f	friction factor (dimensionless)
k_f	friction loss coefficient (dimensionless)
l_f	distance across pressure ports (L)
m	average flow velocity exponent in Eqn. 3 (dimensionless)
Q_f	flow rate in the flow cells (L^3T^{-1})
Q_n	nutrient flow rate (L^3T^{-1})
r^2	regression correlation coefficient (dimensionless)
Re	Reynolds number (dimensionless)
$u_{(ave)}$	average flow velocity (LS^{-1})
ΔP	pressure drop ($ML^{-1}T^{-2}$)
V	volume (L^3)
θ	hydraulic residence time (T)
ρ_w	density of water (ML^{-3})
ν	kinematic viscosity (L^2T^{-1})

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Page 17
Equation [5] should read: $f = 0.0791/\mathrm{Re}^{0.25}$

Page 21
The final paragraph has been cut off. The last two sentences on the page should read:

"A study of velocity profiles in biofilm colonized flow cells by Lewandowski *et al.*[13] showed that their biofilms effectively reduced Re and stabilized flow up to Re 2000. It is possible that the biofilm had a similar effect in our experiments".