NON-INVASIVE METHODS FOR MONITORING BIOFILM GROWTH IN INDUSTRIAL WATER SYSTEMS

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Abstract. **Microbiological control in industrial environments is frequently restricted to monitor the number of planktonic (suspended) cells. However, this number is not often related with the microorganisms attached to the surfaces (sessile bacteria). The aim of this research work was to develop a method for monitoring biofilm growth and the biocidal efficacy through simple non-invasive ways. With this purpose, biofilms were formed on stainless steel coupons and after preset periods were removed and immersed in sterile solutions with and without biocide. The number of sessile cells attached on the metal surface and of planktonic cells that were shed from the biofilmed coupons (pcb) and grow in the initially sterile solutions were determined. Sessile cells were scrapped from the metal surface to be enumerated. The relation between the degree of growth of pcb and the reactivation capacity of the biofilm was evaluated. It could be observed that pcb growing in a nutrient non aggressive medium was related to the number of sessile cells that remain alive after the biocidal treatment. The early stages of the biofilm growth, the thickness of the biofilms and their microstructural characteristics before and after the biocidal treatment could be followed through optical microscopy using a non-invasive technique recently developed in the laboratory. Microscopic observations showed that the biofilm thickness varied to obliterate the unevenness of rough surfaces.**

Keywords. **Biofilm, Biocide, Biocidal Treatment, Optical Microscopy, *Pseudomonas fluorescens***

I. INTRODUCTION

Biofilm mode of microbial growth is predominant in aquatic environments and protects the microorganisms from adverse environmental conditions and from biological and chemical antimicrobial agents. It prevails in virtually all nutrient–sufficient aquatic systems independent of its type and geometry (Lappin-Scott and Costerton, 1989). Biofilms are dynamic structures, which consist of cells, their secreted matrix of insoluble extracellular polymers matrix (EPM) and inorganic materials entrapped in it. Planktonic cells are usually shed from established biofilms and lose their protection so that new fresh habitats can be colonized by new biofilms (Costerton *et al*., 1995).

During several decades biofilms were thought as uniform layers of cells embedded in an EPM without heterogeneities inside them. Traditional microscopic methods of sampling and culture did not allow examining the biofilm microstructure. Planktonic cells often represents only 0.1 % of bacteria in an ecosystem (Geesey *et al*, 1978). Phenotypic changes that bacteria undergo when they adhere to surfaces have been reported (Davies *et al*., 1993). The number of cells in the biofilm is frequently underestimated because the aggregates obtained by scrapping the sample produce only a single colony on the plates. Moreover, the biofilm population in many aquatic ecosystems may be viable but nonculturable (Colwell, 1984). Notwithstanding this, many researchers in microbiology still study microbial ecosystems by extrapolation from planktonic samples. The quantitative numerical analysis available must be tested against the direct microscopic examination. Thus, if we are to understand biofilm processes, living biofilms microscopy must be made.

Confocal Scanning Laser Microscope (CSLM) (Costerton *et al*., 1995) revealed the complex biofilm architecture of some ecosystems in which microcolonies are enclosed in EPM separated by water-filled channels. The discovery of convective flow within the water channels has revolutionized the concept of bacterial growth within biofilms. Through these channels the nutrients are carried throughout the biofilm like a primitive circulatory system (McFeters *et al*., 2000).

Unfortunately, some of the high-resolution techniques are not a dairy tool in many laboratories. On the other side, some microscopic techniques frequently used by researchers require extensive sample preparation ex situ including dehydratation, use of chemicals to fix the biofilm to the surface, use of fluorescent stains, coated by a conductive layer, etc. (Beech *et al*, 1996). Morever, some of them cannot be used for observations at real time.

Biofilms are the cause of several operational problems associated with industrial and drinking water systems (Geesey and Bryers, 2000). They originate not only economical looses but health diseases as well. The control of biofilms is a real challenge within engineered systems. Engineers are familiar with the effects of biofilms in heat exchangers, trickling filters and anaerobic digestors caused by biofouling formation

(increase in heat exchange resistance and pressure drop) or microbial induced corrosion. The main biofilm control relies on chemical biocides.

Glutaraldehyde (GA) is an antimicrobial agent frequently used in industrial and medical environments (Grobe and Stewart, 2000). The diffusion of the biocide through the polymeric matrix may be restricted. However, even thin biofilms can show higher resistance than unattached bacteria. This would suggest that the physiological status of sessile cells is altered and renders them less susceptible to biocidal action (Beyenal *et al*., 1998, Costerton *et al*., 1999, Costerton and Stewart, 2000, Dodds *et al*., 2000). Thus, the extrapolation made from studies about biocidal efficacy against planktonic samples towards sessile cells may be often invalid.

With the purpose of providing a simple non–invasive way to visualize the early stages of thin biofilm growth at real time, a special assemblage developed in the laboratory was used (Cortizo and Mele, 2000). This technique combines conventional optical microscopy with a glass flow cell. In this way, information about some microstructural characteristics of the biofilm can be obtained without pretreatments like freezing and staining the samples (Huang *et al*., 1995). The aim of this research work is to monitor the biofilm growth and the biocidal efficacy of GA through this method and to compare these results with sessile and planktonic cells counts.

II. EXPERIMENTAL

Pure cultures of *Pseudomonas fluorescens* (*P. fluorescens*) were used in the experiments. They were isolated from an industrial environment. *P. fluorescens* was maintained in Cetrimide agar at 28°C and its purity was periodically checked by means of Gram stain, the oxide test, and other biochemical tests and plating. *P. fluorescens* inoculum was prepared by suspending a Cetrimide agar slant (24 h old) in 2 mL of sterile nutrient medium. The inoculum was later poured into an Erlenmeyer flask containing 200 mL of the nutrient broth medium and kept on a rotary shaker for 3 h.

Thin stainless steel coupons (area: 3 cm^2) were immersed for 3 h in an Erlenmeyer flask inoculated with *P. fluorescens* under static conditions and biofilms formed on their surfaces. The number of planktonic bacteria at the beginning of the experiment was in the order of c.a. 10^7 cells/mL.

The biofilmed samples were treated in different ways:

Treatment *Ia*: Two biofilmed samples were removed from the Erlenmeyer flask and afterwards they were immersed in 30 mL flasks containing a buffer solution (NaCl 8 g/L, KH_2PO_4 0.34 g/L, K_2HPO_4 1.21 g/L) with different GA concentrations (25, 50, 100, 200 and 500 ppm) and a control without GA. Planktonic cells that were shed from the biofilm and then reproduced (pcb) during GA treatment were enumerated after different periods (contact times: 1, 2, 4 and 6 h) using standard plating method (spm) and double side paddles with TTC agar (pTTC). Sessile cells were enumerated by spm after scrapping the metal samples and suspending the cells in physiological solution.

Treatment *Ib*: After performing treatment *Ia* another two biofilmed samples were removed from the buffer + GA solution and were immersed in a 1/10 nutrient broth medium. The planktonic and sessile cells (suspended in physiological solution after the scrapping) were enumerated by spm and pTTC. The relation between the degree of growth of pcb and the reactivation capacity of the biofilm was evaluated.

The early stages of the biofilm growth were observed through a simple non-invasive optical microscopy method previously developed (Cortizo and Mele, 2000) using a glass flow cell and a thin substratum. The substratum was visualised as an opaque surface and the biofilm and the flowing bathing solution as a bright area using a X40 objective.

III. RESULTS AND DISCUSSION

The different processes that result in biofilm formation were followed through continuous optical microscopy of biofilms on thin substrata (OMBT) at real time. The transport of cells from the bulk towards the substratum/solution interface followed by the attachment of the pioneer cells could be observed. Although random displacement was expected for motile cells that were approaching to the interface a preferential approximation to the attached pioneer cells was actually observed. Afterwards, a particular motion of planktonic bacteria around the border of the colonies was noticed. A higher planktonic bacterial density in this region, compared to that around the uncolonized substratum or within the bulk solution was detected. Probably there was an inter-cells communication mechanism (Singleton *et al*. 1997; Davies *et at*., 1998). It was noticed that *P. fluorescens* motility played an important role during the colony formation. The behaviour of the motile cells was very different from that of colloids during an aggregation process. Thus, the use of colloids to simulate the behaviour of bacteria may be invalid.

Two stages of the growth of a *P. fluorescens* colony can be seen in Figure 1. It can be observed that the colony shape is like a hill at shorter periods and seems to be a finger later. Streamers like this could be seen by OMBT stretching, relaxing and oscillating in agreement with observations reported by other authors (Lewandowski, 1998; Stoodley *et al*., 1999). Oscillations of the biofilm in the turbulent flow could be a contributor to the energy losses observed in conduits because of their viscous action. Sometimes the detachment of a portion of the biofilm resulted in a wide streamer.

Through a careful examination of the field it could be noticed that there was a change in the viscosity of the bulk fluid within the biofilm surroundings. The displacement of the cells in this viscous region was

more sluggish than in the rest of the fluid in agreement with previous results (Cortizo and Mele, 2000). The higher viscosity is probably produced by the EPM exuded by the bacteria towards the bulk fluid. A great number of cells that are moving slowly within this viscous interface could be seen.

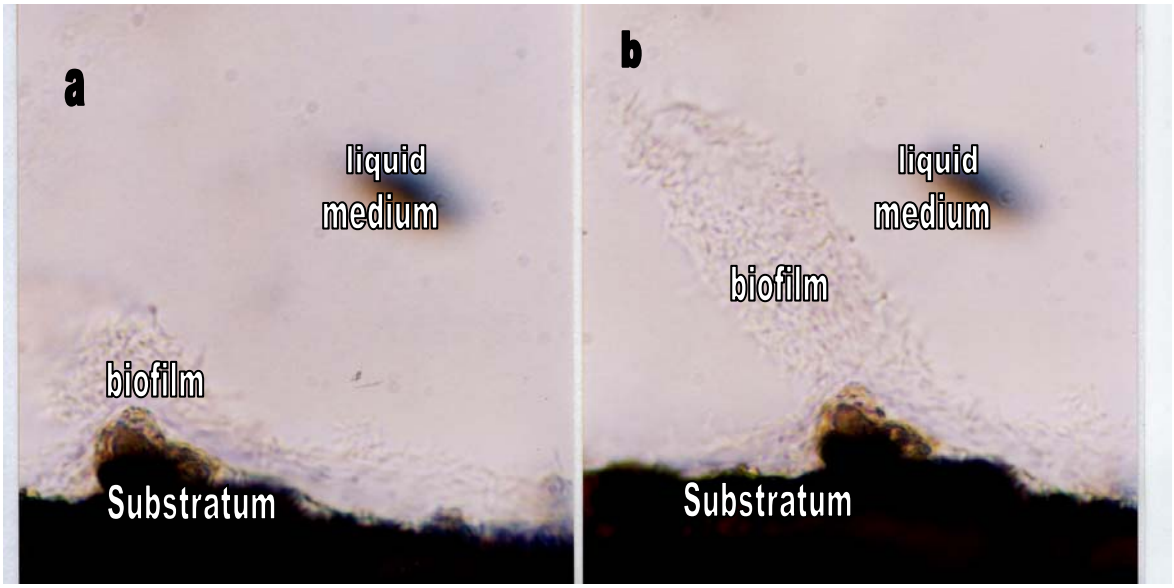

Fig. 1. Microphotograph of a *P. fluorescens* microbial biofilm after 1 h (a) and 4 h (b) of immersion in a buffer solution with 100 ppm GA. (Bar = 80 μm).

It has been measured (Zelver *et al*., 1985) that there is an increase in the shear stress when a 3 days old biofilm is growing on a surface. However it was noticed that there was not an increase of the friction factor during the first 30 hours of biofilm growth on a rough surface. The increase in the friction factor should not occur until the biofilm accumulated beyond the roughness elements. Figure 2a confirms this suggestion showing that the roughness of the substrate is smoothed by the *P. fluorescens* biofilms in agreement with previous results (Cortizo and de Mele, 2003). The biofilm thickness varied to obliterate the unevenness of the rough surface (Figures 2a). The flow lines around the substrate surface are flatter because heterogeneities are filled with bacteria and EPM and the liquid medium is in contact with the apparently smoother surface. The motility of *P. fluorescens* cells seems to facilitate the attachment of the cells inside the grooves (Scheuerman *et al*., 1998).

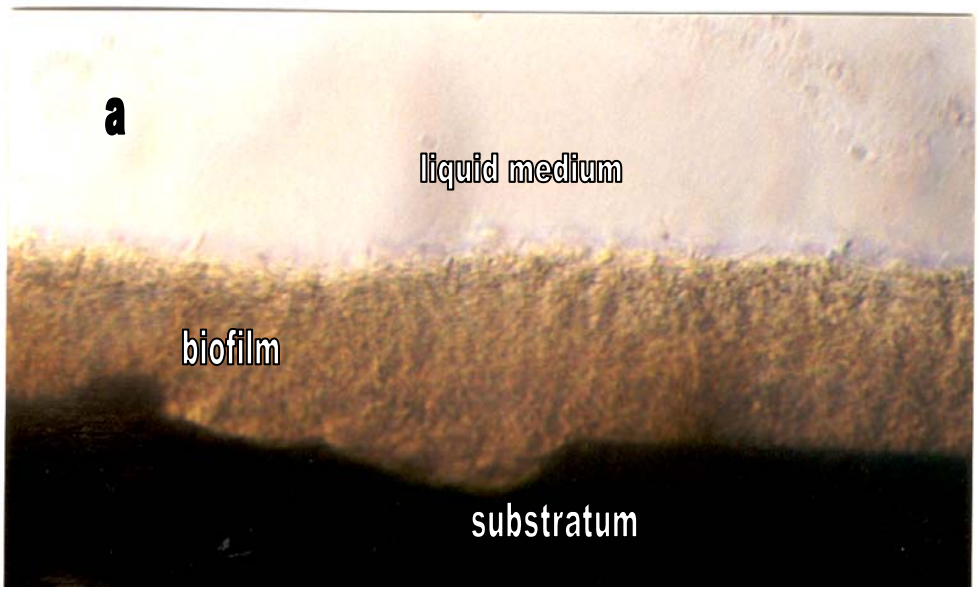

Fig. 2a. Microphotograph of a microbial biofilm after 22 h of immersion in a buffer solution with 100 ppm GA. Biofilm is very compact and its thickness varies smoothing the rough surface (Bar = 80 μm).

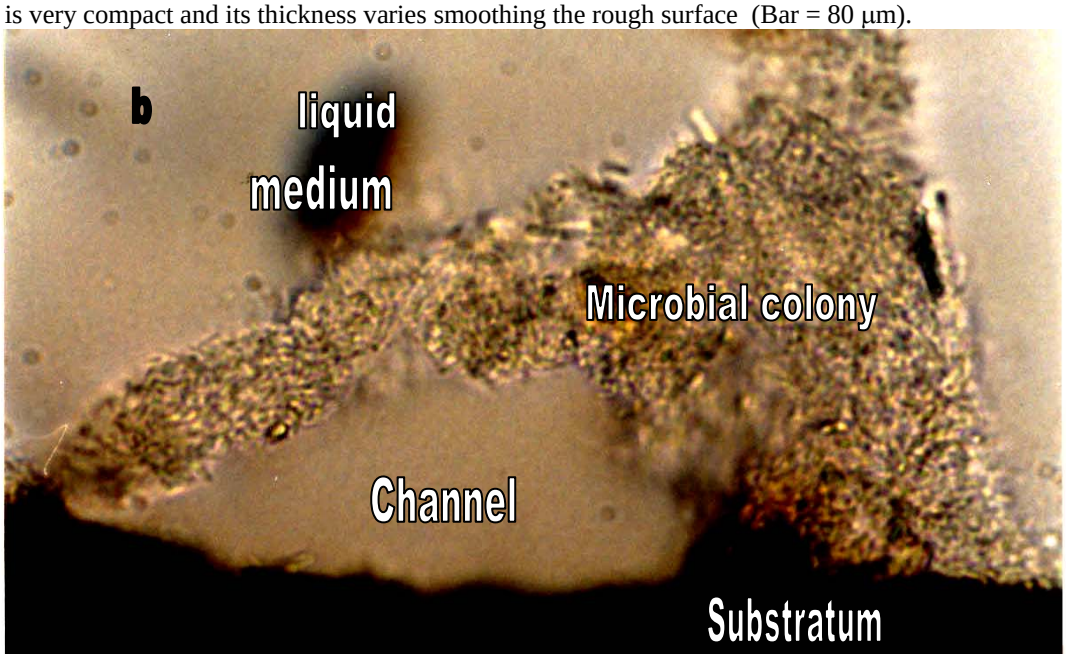

Fig. 2b. Microphotograph of a microbial biofilm after 3 h of immersion in a *P. fluorescens* culture, showing a channel within the colony (Bar = 80 μm).

The actual thickness of the thin biofilms can be evaluated at real time by OMBT. Figure 3 shows that the thickness of the biofilm can be easily measured. A significant reduction of the thickness of the biofilm is observed in SEM microphotographs because of the dehydration of EPM occurring during pretreatments (Cortizo and de Mele, 2003).

Microstructural characteristics like pores, interstitial channels (Figure 2b) and bridges can be distinguished using OMBT. Conversely, SEM pretreatments impede the visualization of these characteristics because the biofilm constricts and channels and pores are blocked during dehydratation (Cortizo and de Mele, 2003). Water channels are frequently formed when neighboring colonies coalesced at the upper side, close to the solution while the inner side, close to the substratum, remained separated (Yang *et al*., 2000). The microstructural details of the biofilms obtained using OMBT are similar to those previously reported employing more sophisticated microscopic techniques (de Beer *et al*, 1994; Huang *et al*, 1995,; Rasmussen and Lewandowski, 1998; Beyenal *et al*, 1998; Wattanakaroon and Stewart, 2000). Channel depth evaluation through OMBT is limited to the depth of the field of the microscope objective and it is useful to take photographs at different focal planes.

A colour and transparency gradient which may be attributed to a change in the bacterial and/or EPM densities is observed in Figure 2a. A darker colour and less transparency in the inner older layer than in the outer younger layers were detected.

In agreement with previous results (Grobe and Stewart, 2000) planktonic cells were readily eraducated by GA in the concentration range assayed (25, 50, 100, 200 and 500 ppm) in buffered solutions.

The killing action of GA against biofilms was analysed by enumerating the sessile cells after 4 h of immersion in the GA containing medium. The number of sessile cells was obtained after scrapping the metal surface, suspending the cells in a physiological solution and enumerating these cells (treatment *Ia*). It could be noticed that there was a decrease less than one order in the number of cells that remained alive (from 7×10^6 to 1.1×10^6 cellscm^{-2}) when 100 ppm GA was used. The efficacy of GA did not significantly increased when 500 ppm GA was employed. Commercially available paddles with TTC agar were also used to estimate the number of suspended cells with similar results. Figure 2a shows that the biofilm is more compact than that of Figure 1. The higher density is probably related with the long period (22 h) in contact with GA.

The number of pcb that were able to grow in the sterile medium with or without GA was evaluated to investigate if it was related with the number of sessile cells that remained alive. After 4 h of exposure the $\log_{10}$ pcb number decreased at least 3 with respect to the control (100 and 200 ppm GA, Figure 3). A complete killing of pcb could be achieved after 6 h of exposure.

A set of biofilmed samples treated with *Ia* during 4 h were immersed in a 1/10 nutrient broth culture medium (treatment *Ib*) and the pcb growing from the biofilms previously treated and untreated with GA were enumerated by spm and pTTC. The number of pcb was in this case at least two orders higher than in the case of treatment *Ia* for all the GA concentrations assayed. Thus, a notorious regrowth of microorganisms occurred

when the availability of nutrients was higher. This was in agreement with the high level of sessile alive cells enumerated after scrapping the biofilms (For example: 1.1 x 10^6 cells cm^{-2} for treatment *Ia* and 2 x 10^9 for treatment *Ib*, both treated with 100 ppm GA).

It could be observed that when the biofilmed samples were treated with GA in a buffer solution and then transferred to a medium with higher nutrient level (*Ib*) a wide layer of cells that seem to be floating in a viscous liquid was formed around the colonies (Fig.4). The density of the cells was lower than that of the biofilm.

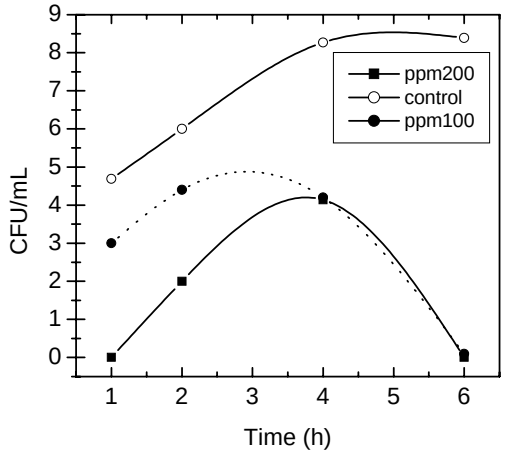

Fig. 3. Log_{10} planktonic cell number vs. exposure time. Planktonic cells were shed from a biofilmed sampled that was immersed in a buffer solution without glutaraldehyde (o) and treated with 100 (•) and 200 ppm glutaraldehyde (■).

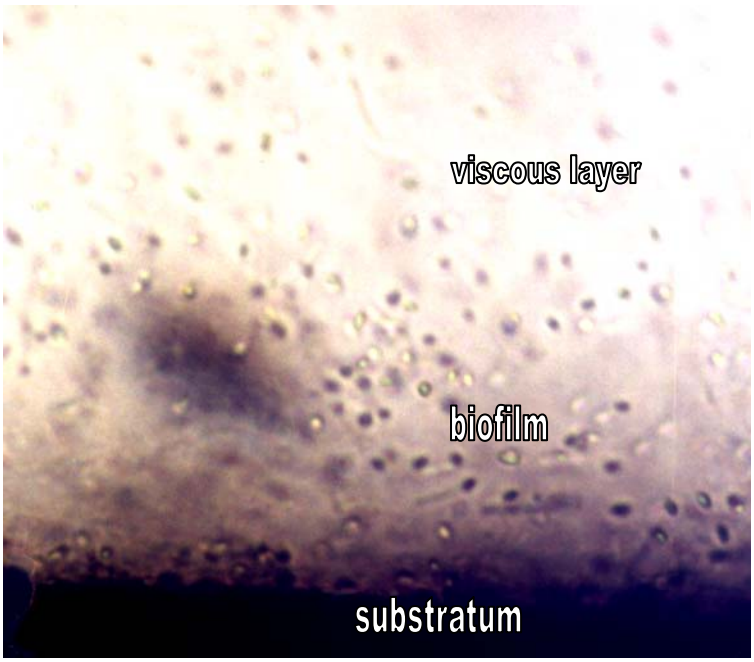

Fig. 4. Biofilmed sample treated with glutaraldehyde (200 ppm) in a buffered solution and then transferred to a medium with a higher nutrient level. A layer of cells that seem to be floating in a viscous liquid formed around the biofilm can be seen (Bar = 80 μm).

Biofilms can resist the antimicrobial challenges by three main mechanisms (Stewart *et al*, 2000): a) decrease of the antimicrobial concentration in the surroundings of the biofilm, b) reduction of the antimicrobial penetration within the biofilm, c) adoption a resistant physiological state or phenotype by a group of cells. It seems that there is often a combination of these three types of resistant behaviours (Stewart *et al.*, 2000). Our results show that the biofilms became more compact after the GA treatment (Fig 2a) and probably

reduces the antimicrobial penetration. As it was expected, after 6 h of exposure to a saline phosphate buffer solution the growth of pcb was lower than in the case of the diluted nutrient broth. When GA was added no pcb was detected. However, there was a high number of sessile cells both under nutrient limitation (in a saline phosphate buffer solution) and under aggressive conditions (with GA addition). Sessile cells remained alive in a slow-growing state. In this state they seem to be less susceptible to antimicrobial action. When the nutrient limitations and aggressive conditions disappeared an important regrowth of sessile cells and pcb could be observed.

IV. CONCLUSIONS

GA is able to eradicate *P. fluorescens* planktonic cells at concentrations as low as 25 ppm. However, at initial concentrations up to 500 ppm GA is unable to exterminate *P. fluorescens* sessile cells. Those that remain alive were the cause of the high reactivation of the biofilm growth when the nutrient level was increased. The new layers of cells were wider and had lower bacterial density than the older biofilm.

Results show that the absence of planktonic cells is not an indicator of a high biocidal efficacy when a biofilm is present because sessile cells remain alive in a slow-growing state. The number of pcb treated with a biocide and then immersed in a sterile media has proved to be an indicator of the reactivation capacity of the biofilms at different nutrient and biocidal concentrations. Commercially available double side paddles with TTC-agar can also be used to enumerate pcb.

The use of conventional optical microscopy together with a flow cell and an opaque thin substratum allows the visualization of details of biofilms microstructure during the early stages of biofilm formation. *P. fluorescens*. Biofilms show a complex microstructure with pores, channels and streamers. The displacement of planktonic bacteria was not random showing a preferential motion towards the attached cells. The roughness of the substratum and of the biofilm as well as the thickness of the biofilm can be easily evaluated by OMBT. It could be observed that under laminar flow the thickness of the biofilm varied to obliterated the unevenness of the rough surface, in coincidence with the decrease of the friction factor reported previously.

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