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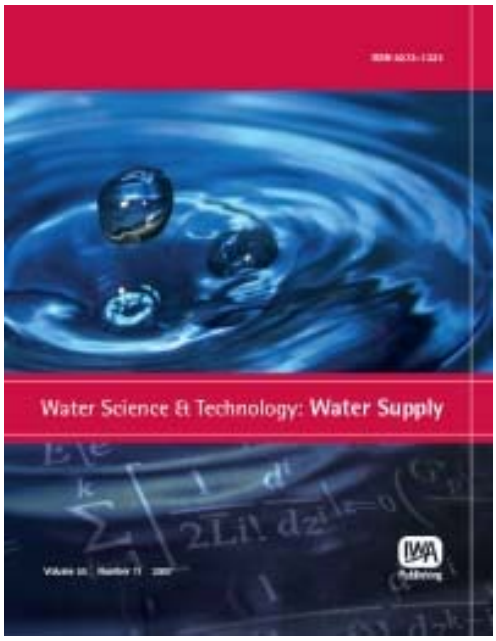

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A comparative study of drinking water biofilm monitoring with flow cell and Propella™ bioreactors

Lúcia Chaves Simões, Manuel Simões and Maria João Vieira

ABSTRACT

Monitoring of drinking water (DW) biofilm formation under different process conditions was performed using two distinct bioreactors: a Propella™ and a flow cell system. Biofilms were grown on polyvinyl chloride (PVC) and stainless steel (SS) coupons under laminar (Reynolds number: 2000) and turbulent (Reynolds number: 11000) flow. The parameters analysed were the numbers of total and cultivable bacteria. The impact of different process conditions was assessed after the biofilms reached steady-state. The number of total bacteria was mostly higher than those cultivable. Biofilm steady-state was achieved in 3 days in both bioreactors with adhesion surfaces under turbulent flow. Under laminar flow it was only achieved in 6 days. The numbers of total and cultivable bacteria in turbulent flow-generated biofilms were similar in both bioreactors, regardless of the adhesion surface tested. Under laminar flow, the Propella™ bioreactor allowed the formation of steady-state biofilms with a higher number of total and cultivable bacteria than the flow cell system. Comparing the effects of the flow regime on biofilm accumulation, only turbulent flow-generated biofilms formed on the flow cell system had a higher amount of total and cultivable bacteria than those formed under laminar flow. In terms of adhesion surface effects, a higher number of total and cultivable cells were found on PVC surfaces compared to SS when biofilms were formed in the flow cell system. Biofilm formation on PVC and SS was similar in the Propella™ system for both flow regimes.

Key words | adhesion surfaces, bioreactors, drinking water biofilms, hydrodynamics

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INTRODUCTION

The provision of microbiologically safe supplies of drinking water (DW), following treatment, is one of the main goals that both DW companies and governments worldwide try to achieve, and represents one of the cornerstones for the maintenance of high standards of public health (Szewzyk *et al*. 2000; Deines *et al*. 2010). However, the occurrence of waterborne diseases by ingestion of contaminated DW is still a major economic and, in some cases, social burden all around the globe. According to the World Health Organization, diseases associated with unsafe water, sanitation and hygiene cause approximately 1.7 million deaths each year (Prentice 2002). Disinfectant residuals, typically chlorine based, are normally used to reduce the numbers of microorganisms in drinking water distribution systems (DWDS). Nevertheless, increases in microbial numbers during distribution of DW have long been recognized (Baylis *et al*. 1930), with microbial mediated processes contributing to the deterioration of water quality (Camper 2004; Emtiazi *et al*. 2004).

Biofilms are suspected to be the main source of microorganisms, including pathogens, in DWDS that are fed with treated water (LeChevallier *et al*. 1987; Percival & Walker 1999; Szewzyk *et al*. 2000; Batté *et al*. 2004; Codony *et al*. 2005). The microorganisms in biofilms have a number of advantages over their counterparts, namely the production of an extracellular polymeric matrix that enables resistance to a number of control strategies (antimicrobial agents and shear stress conditions) (Simões *et al*. 2005a, b, 2007a). Although DWDS disinfection significantly reduces the numbers of planktonic bacteria, it has

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little to no effects on the numbers of biofilm bacteria (Gagnon *et al.* 2005).

The dynamics of microbial growth in DW networks is very complex, as a large number of interacting processes are involved. Even though numerous environmental factors will influence biofilm formation in DWDS, including water temperature and pH, disinfectant type and residuals (Lund & Ormerod 1995; Gagnon *et al.* 2005), organic matter (Norton & LeChevallier 2000), nutrient concentrations (Volk & LeChevallier 1999; Chu *et al.* 2005), surface material (Camper *et al.* 1996) and hydraulics (Lehtola *et al.* 2006), a complete understanding of how these factors act in concert to influence and control compositional changes during biofilm formation and detachment within DWDS remains a key challenge. The amount of biofilm in a given system after a certain period of time depends on a dynamic biofilm formation process, which has been defined as the balance between bacterial attachment from the planktonic phase, bacterial growth within the biofilm and biofilm detachment from the surface (Stoodley *et al.* 1999). When that balance is null, the biofilm is said to have reached a steady-state. The final amount of biofilm in that state, which can be assessed by cell counts or biofilm mass, is directly related to the biofilm formation potential of that system (van der Kooij 1999).

Research on DW biofilms has been performed in a wide variety of systems or biofilm monitoring bioreactors that should mimic the *in situ* situations with reproducible results; thus, important information is assessed about biofilm behaviour within the real DWDS. Several bench-top laboratory biofilm reactor systems, such as the rotating disc reactor (Murga *et al.* 2001; Möhle *et al.* 2007), the CDC biofilm reactor (Goeres *et al.* 2005), the biofilm annular reactor (Batté *et al.* 2003a, b), the Propella™ reactor (Parent *et al.* 1996; Appenzeller *et al.* 2001), the Robbins device (Manz *et al.* 1993; Kalmbach *et al.* 1997), the modified Robbins device (McCoy *et al.* 1981; Kharazmi *et al.* 1999; Millar *et al.* 2001), flow cell systems (Simões *et al.* 2006), the Prévost coupon (LeChevallier *et al.* 1998; Prévost *et al.* 1998), the Bioprobe monitor (LeChevallier *et al.* 1998), the Pipe sliding coupon holder (Chang *et al.* 2003), the biofilm sampler (Juhna *et al.* 2007) and PWG coupon (Deines *et al.* 2010), have been used for studying DW biofilms. The complexity of the microenvironment under study and even the use of different methodologies and biofilm reactor systems has led in some cases to ambiguous or not easily comparable results. However, most studies assess only one variable at a time (Pedersen 1990; Rogers *et al.* 1994; Kerr *et al.* 1999; Niquette *et al.* 2000; Zacheus *et al.* 2000; Dunsmore *et al.* 2002; Soini *et al.* 2002), and apart from notable exceptions (Block *et al.* 1993; Stoodley *et al.* 1999; Simões *et al.* 2006), few attempts have been made so far to study their inter-relationships and compare the relative importance of these different factors.

The purpose of the present study was to evaluate biofilm formation by DW autochthonous bacteria on stainless steel (SS) and polyvinyl chloride (PVC), two support materials commonly used on DW networks, under different water flow rates, using a Propella™ bioreactor and the flow cell system. These bioreactors provide effective equipment to permit biofilm growth in a potable water system under environmental conditions mimicking real scenarios. The use of granular activated carbon (GAC) upstream of the biofilm bioreactors allows their inoculation with uniform cell densities under the low nutrient conditions encountered in DW (Morin & Camper 1997). This strategy avoids heterogeneity in results from independent experiments.

MATERIAL AND METHODS

Bioreactors and biofilm monitoring

Monitoring of DW biofilm formation under different conditions was performed using two distinct bioreactors: flow cell system and Propella™. The configurations of these bioreactors are presented in Figure 1.

Biofilms were grown on PVC and SS ASI 316 2R coupons. The water flow rate through the bioreactors was controlled by recirculating the water by means of centrifugal pumps (flow cells) or by means of a motor and a propeller for water agitation (Propella™). The biofilms were developed under laminar (Reynolds number: 2000) and turbulent (Reynolds number: 11000) flow. Temperature in both bioreactors was maintained at $20 \pm 1\ ^{\circ}C$ by an external refrigeration mechanism (Thermomix® BU, B. Braun – Biotech SA) in order to simulate the conditions found in real DWDS.

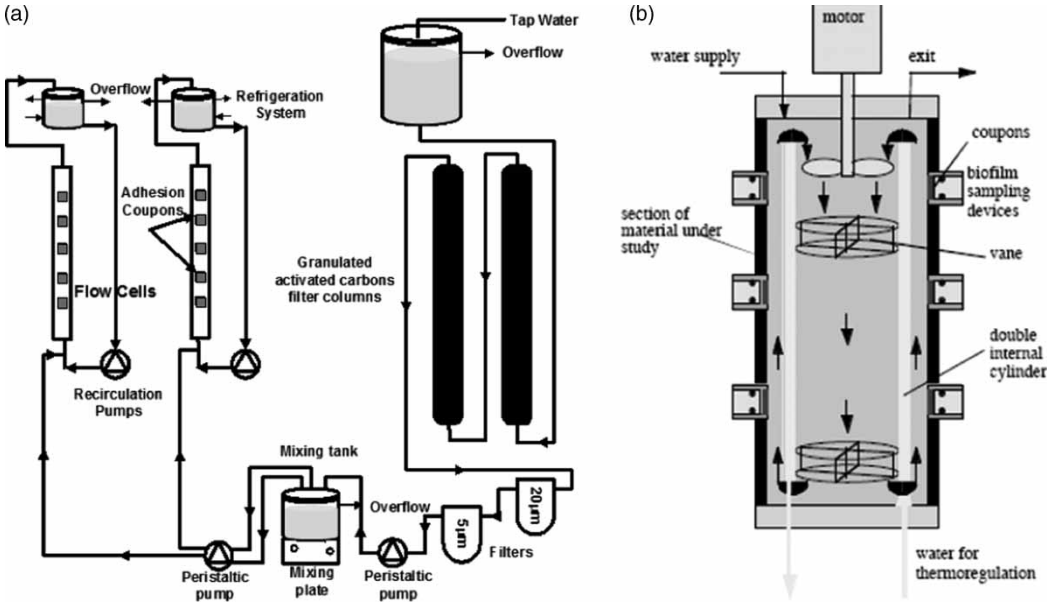

Figure 1 | Experimental set-ups, showing the GAC filter columns and the flow cell system (a); and the Propella™ bioreactor (b).

The Reynolds number was calculated as a function of the duct design, using the hydraulic equivalent diameter (D_h), defined as (Tosun *et al.* 1988):

$$D_h = 4 \times \text{Flow area/wetted perimeter} \quad (1)$$

For the flow cell system:

$$D_h = 4 \times (\pi_{/8} \times d^2)/(\pi_{/2} \times d + d) \quad (2)$$

where d is the semicircular duct diameter (1 cm).

For the Propella™ bioreactor:

$$D_h = (d_1 - d_2)$$

where d_1 is the internal diameter of the external cylinder (9.34 cm) and d_2 is external diameter of the internal cylinder (7.25 cm).

The Reynolds number, based on the hydraulic diameter, is:

$$\text{Re} = (D_h \times u \times \rho)/\mu \quad (3)$$

where u is the flow velocity (m/s), ρ is the fluid density (Kg/m^3) and μ the fluid viscosity (Kg/m.s). For this study, the fluid characteristics were considered for water at the operational temperature.

The biofilm experiment was carried on for at least 2 days after the biofilm reached a steady-state (considered to occur when constant over time values were obtained both for coloning forming units (CFU) and total bacterial cell counts (TB)), after which the experiment was terminated and the bioreactors disinfected.

Drinking water source

The DW source was from the public network in Braga (northern Portugal). Briefly, tap water (9 ± 4 CFU ml^{-1} and $1 \times 10^5 \pm 3 \times 10^4$ TB ml^{-1}) was collected in a reservoir, which was connected to one of two consecutive GAC filter columns. It has been shown elsewhere that the first GAC filter eliminates free chlorine and biodegradable matter contained in the tap water, while the second is a biological activated filter providing a continuous bacterial inoculum to the bioreactor (Morin & Camper 1997). To avoid the presence of large carbon particles released from the columns, two filters (pore sizes 20 and 5 µm) were placed between the second GAC filter and the mixing tank. This tank supplied a constant inoculum ($6 \times 10^4 \pm 2 \times 10^4$ CFU ml^{-1} and $1 \times 10^6 \pm 2 \times 10^5$ TB ml^{-1}) at a flow rate of approximately 0.02 l h^{-1} into each of the flow cells

or 1.12 l h^{-1} into the Propella™, in order to obtain an adequate dilution rate, similar in both bioreactor systems. Absence of free chlorine in the mixing tank was certified by regular sampling, using a free chlorine ion specific meter HI-93701 (Hanna Instruments, USA).

Flow cell system

The flow cell bioreactor is a pipe, with half-circle section, and adhesion coupons are placed on its inner flat surface. The flow cell may be directly connected to the tap and operates as a plug flow reactor or connected to a vessel that recirculates the water approaching a perfectly mixed reactor. This reactor system was designed to uncouple system residence time and fluid velocity by allowing water recirculation between the flow cell unit and a vessel. In the flow cell bioreactor, several coupons, with adhesion materials, are attached to the inner surface and may be replaced gradually without affecting the remaining system.

In this study, two flow cells were used in parallel, according to the procedure described by Pereira *et al.* (2002). Each one consists of a semicircular perspex duct 43 cm in length and with 1 cm of equivalent diameter (internal diameter of the half cylinder is 1.6 cm), where the biofilm coupons can be inserted. These rectangular coupons (2.4 cm length × 1.4 cm width), consisting of either SS or PVC, were glued to pieces of perspex that could be properly fitted in the apertures. Biofilms were formed on those coupons whose upper faces were in contact with the tap water circulating in the flow cell reactor system. It was possible to remove each of the rectangular coupons separately without disturbing the biofilms formed on the others and without stopping the flow. This was managed because outlet ports were disposed on the round face of the flow cell between each two adjacent removable pieces of perspex which allowed the deviation of the circulating flow from the point where the reactor was opened.

Propella™ bioreactor

The Propella™ bioreactor is a perfectly mixed continuous reactor in which a propeller pushes the liquid down through the internal tube (external diameter of 7.25 cm) and up through the annular section between the two tubes (internal diameter of 9.34 cm). The flow rate inside the pipe was controlled by the rotation speed of the propeller and the residence time is proportional to the fresh inlet flow rate. In this reactor, the internal velocity and the hydraulic residence time may be chosen independently.

In this study, the Propella™ was made essentially of PVC and allowed 20 screwed biofilm sampling points to be placed in the inner reactor surface. On each sampling port, a circular coupon of SS and PVC surface material was glued. Biofilms were formed on those coupons whose upper faces were in contact with the tap water circulating in the bioreactor.

Biofilm sampling

Biofilm sampling was made from the top to the bottom of the bioreactors under aseptic conditions and the coupons removed were substituted with new ones that were previously cleaned, immersed in ethanol (70% v v^{-1}) for 30 min, and rinsed in sterile distilled water. The removed coupons were gently washed with sterile sodium phosphate buffer (pH = 7.0) to remove loosely attached microorganisms and scraped with a scalpel into 15 ml glass tubes containing 10 ml of sterile phosphate buffer. Before serial dilutions, biofilm suspensions were vortexed for 2 min and used to assess both CFUs and TB.

Cultivable and total cell counts

CFUs were evaluated by standard culture on R2A (Oxoid, UK) prepared according to the manufacturers instructions. Triplicate plates were used for each dilution and for each sampling time. CFUs were counted after 15 days of incubation at 20 ± 3 °C, and the results were expressed as CFU cm^{-2}. TB were obtained by filtering the adequate volume (up to 10 ml as a function of the bacterial concentration) through a 25 mm black Nucleopore® polycarbonate membrane with a pore size of 0.2 μm (Whatman, UK). Before the filtration step, 2% (v v^{-1}) formaldehyde (Merck, Germany) was added to the solution for sample fixation and preservation. After filtration, cells in the membrane were stained with 100 μg ml^{-1} of 4,6-diamino-2-phenylindole (DAPI) (Sigma, Portugal) for 5 min and the preparations were stored at 4 °C for up to 7 days in the

dark, before visualization. No significant decay of fluorescence was noticed during this time span. Cells were visualized under an epifluorescence microscope (Carl Zeiss, Germany) equipped with a filter sensitive to DAPI fluorescence (359 nm excitation filter in combination with a 461 nm emission filter). A total of 20 fields were counted and the average of three membranes was used to calculate total cells per cm^2.

Statistical analysis

Paired t-test analyses were performed to estimate whether or not there was a significant difference between the results obtained. Statistical calculations were based on a confidence level equal or higher than 95% (a P value <0.05 was considered statistically significant).

RESULTS AND DISCUSSION

Biofilm formation is a significant problem in a wide variety of fluid handling systems. In DWDS the presence of biofilms can compromise the final product quality and public health safety (Simões *et al.* 2007b). Due to the complexity of these systems, the *in situ* study of biofilms in DWDS is almost impossible. Therefore, the use of bioreactors is a key strategy to understand the dynamics of biofilm formation under particular environmental conditions. In this study, a flow cell system and a Propella™ bioreactor were used to monitor biofilm formation from DW bacteria to PVC and SS surfaces, under two distinct hydrodynamic conditions. The flow cell system operates in vertical position in a continuous recycling mode, and provides controlled environmental conditions for the study of DW bacterial adhesion and biofilm formation. The Propella™ bioreactor also provides an effective way to permit biofilm growth in a potable water system. The water within the reactor is perfectly mixed and flows along the vessel in the same way as water does in a real pipe. This is due to the internal propeller, the speed of which controls the flow rate close to the reactor walls. In both reactors, the design of the sampling ports allows coupons to be added and removed without emptying or even stopping the system. This means that the reactor can operate for long term studies with coupons being constantly added and removed with little or no disruption of the system.

In this study, biofilm accumulation in all experiments, expressed both in CFU and TB, increased markedly in the first few days, following a sigmoidal curve (Figure 2). Biofilm steady-state was achieved 3 days after the starting of operating conditions for turbulent flow conditions and for both bioreactors and adhesion surfaces. Under laminar flow conditions, it was only achieved 6 days after. Steady-state conditions were considered when the numbers of CFU or TB were statistically similar over time ($P > 0.05$). For those cases, the number of total bacteria was invariably higher than the cultivable cells (differences always higher than 2 log). The heterotrophic plate count is the reference procedure for estimating the number of viable heterotrophic bacteria in water and measuring quality changes during water treatment (APHA, AWWA & WPCF 1992). This method only includes the assessment of cultivable bacteria which are able to initiate cell division at a sufficient rate to form colonies, being very sensitive to culture conditions (temperature, media, duration of incubation, etc.) and responses may require from 24 h to more than 1 week. It has long been recognized that the use of culture-based enumeration techniques may significantly underestimate the actual numbers of the viable population. Several reasons may account for this difference: the presence of starved or injured cells or potentially viable but non-cultivable cells that are not able to initiate cell division at a sufficient rate to form colonies; inadequate culture conditions; aggregation of bacteria that can lead to the formation of one colony from more than one cell, thereby underestimating the total number of cells (Banning *et al.* 2002). For comparative purposes, the biofilm population was characterized in terms of TB (both viable and non-viable bacteria) using the DAPI stain and epifluorescence microscopy. This method is interesting to assess the overall cell population, but does not provide information on the bacteria that survived the DW disinfection process and that are able to multiply.

Total and cultivable bacteria in turbulent flow-generated biofilms were similar in both bioreactors, regardless of the adhesion surface ($P > 0.05$). This result suggests that increased hydrodynamic stress favours biofilm bacteria cultivability. In fact, studies analysing the electron transport system have shown that high shear stress can stimulate

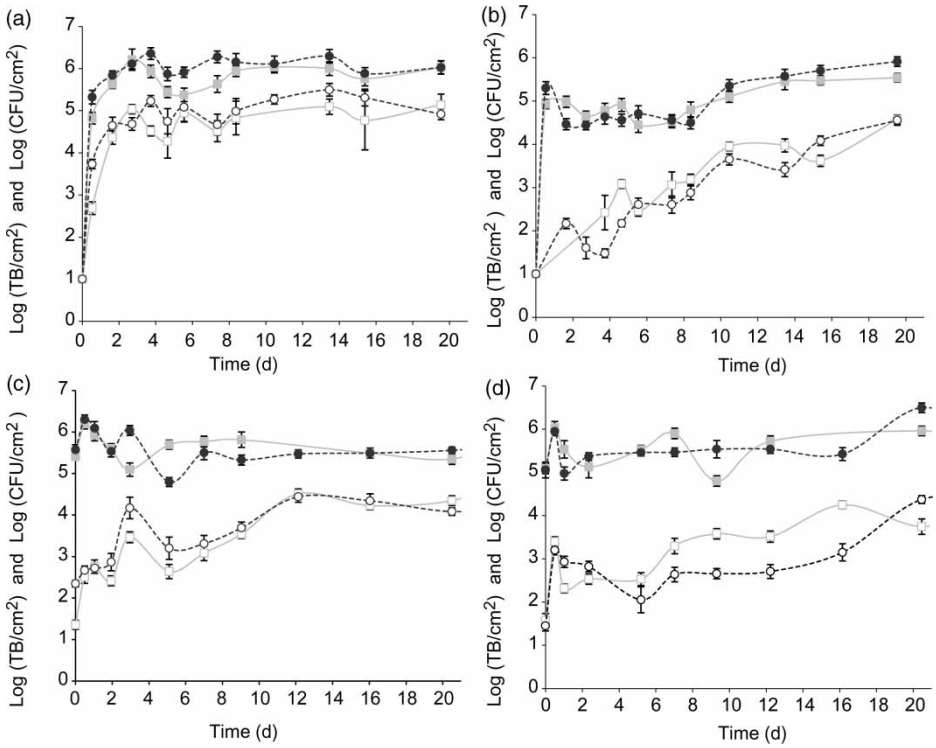

Figure 2 | Biofilm accumulation over time (TB and CFU) on SS and PVC surfaces. (a) turbulent flow and (b) laminar flow in the flow cell system. (c) turbulent flow and (d) laminar flow in the Propella™ bioreactor. SS/TB; SS/CFU; PVC/TB; PVC/CFU. The means ± standard deviations for at least three replicates are illustrated.

biofilm catabolic activity (Liu & Tay 2001). Hydrodynamic conditions may determine the rate of transport of cells, oxygen and nutrients to the surface, as well as the magnitude of the shear forces acting on a developing biofilm, with turbulent hydrodynamic conditions allowing the formation of biofilms with higher cell density (Simões *et al*. 2007c). Vieira *et al*. (1993) found that mass transfer limitations existed to a higher extent in biofilms formed under laminar flow than for turbulent conditions. Other authors (Stoodley *et al*. 1997; Wäsche *et al*. 2002) have also demonstrated the correlation between nutrient mass transfer in biofilms and flow velocities. Consequently, the higher transport of substrate and oxygen, even if at very low levels in DWDS, from the fluid to the biofilm (mass transfer effects) should favour microbial metabolism and cell replication. Comparing the effects of the flow regime on biofilm accumulation, only in the flow cell system was it found that turbulent flow-generated biofilms had a higher amount of total and cultivable bacteria than those formed under laminar flow ($P < 0.05$). This result is in agreement with previous studies (Stoodley *et al*. 1999; Simões *et al*. 2007c), with single and mixed species biofilms formed on flow cell systems, showing that biofilms formed under turbulent flow had a significant higher cell density than laminar counterparts. Turbulent and laminar flow-generated biofilms formed on the Propella™ bioreactor had comparable cell densities ($P > 0.05$). Moreover, the Propella™ system allowed the formation of steady-state laminar flow-generated biofilms with a higher number of total and cultivable bacteria than those formed on the flow cell system ($P < 0.05$). In fact, there are significant differences in the design of the bioreactor systems used that can account for the differences obtained. For example, hydrodynamic stress is obtained by distinct mechanisms when using a Propella™ bioreactor (agitation by means of a rotating device system) and the flow cell system (fluid flow). Under the hydrodynamic conditions studied, a fully developed/uniform flow, mimicking the DWDS, is more likely to be achieved in the flow cell system. Teodósio *et al*. (2011) demonstrated full development of the flow by analysis of velocity profiles and by monitoring

the maximum and average wall shear stresses in a flow cell system. One of the key reactor design issues concerns the inlet conditions which dictate the length required for flow development (Bakker *et al*. 2003). However, further work is required to characterize the fluid dynamics inside the reactors. In terms of adhesion surface effects, in the flow cell system bacteria formed biofilms with higher cell densities on PVC surfaces compared to SS ($P < 0.05$). Biofilm formation on PVC and SS was similar ($P > 0.05$) in the Propella™ bioreactor, regardless of the flow regime. In a previous study (Simões *et al*. 2007b), it was demonstrated that the tested materials had similar physico-chemical characteristics, such as hydrophobicity, and both are prone to colonization by DW isolated bacteria. Consequently, taking into account the physico-chemical characteristics, low biofilm data variability was expected as a consequence of the adhesion surface differences.

CONCLUSIONS

The development and validation of reliable biofilm monitoring techniques is required in order to mimic real environmental situations using laboratorial systems. This work demonstrates that distinct bioreactor configurations provide different biofilm data. In fact, the use of PVC or SS as adhesion surfaces and distinct hydrodynamic conditions lead to biofilm accumulation variability in terms of CFU and TB when using the Propella™ or flow cell bioreactors. Moreover, this study highlights the need for a deeper understanding of how the large spectrum of conditions interact and affect biofilm formation potential and accumulation with the final purpose of predicting the total and cultivable bacteria attached to real DW distribution pipes, based on the system characteristics. Although the practical use of these conclusions by DW network companies is still limited, the information provided here demonstrates the potential of the flow cell system and Propella™ bioreactors for DW biofilm monitoring and might be a prospective framework for future studies on bioreactors and DW biofilms. It should be emphasized that taking into account previous studies, a fully developed flow was only characterized for a flow cell system, proposing that this bioreactor can simulate the fluid dynamics found in DWDS more accurately than the Propella™ bioreactor.

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