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ORIGINAL ARTICLE

Real-time quantification of *Pseudomonas fluorescens* cell removal from glass surfaces due to bacteriophage φS1 application

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Abstract

Aims: To study the efficacy of the lytic phage φS1 in eliminating *Pseudomonas fluorescens* in the early stage of biofilm formation, using an *in situ* and real time methodology for cell quantification.

Methods and Results: Cell adhesion and phage infection studies were carried out in a parallel plate flow chamber under laminar conditions. Cells were allowed to adhere until reaching $1{\cdot}7–1{\cdot}8 \times 10^6$ cells cm^{-2} and phage infection was performed with two different phage concentrations (2×10^9 PFU ml^{-1} and 1×10^{10} PFU ml^{-1}). Phage concentration clearly affects the speed of infection. The less concentrated phage solution promoted a three times slower rate of cell removal but did not affect the overall percentage of cell removal. In fact, after a longer infection period the less concentrated phage solution reached the same 93% cell removal value.

Conclusions: Phages are efficient in the eradication of bacterial cells at the early stage of biofilm formation and their presence at the surface did not allow bacterial recolonization of the surface.

Significance and Impact of the Study: To date, no published studies have been made concerning *in situ* and real time quantification of cell removal from surfaces due to phage action.

Introduction

Microbial adhesion to surfaces is the first step in the process of biofilm development. This initial adhesion process occurs mainly due to physico-chemical interactions (Busscher *et al.* 1987; Van Loosdrecht *et al.* 1990; Cunliffe *et al.* 1999) and the micro-organisms attach first, for a short time period, reversibly and afterwards irreversibly. The adhesion step is frequently underestimated in the biofilm formation process as it is always followed by a multitude of complex steps which at the end result in the mature biofilm structure (Busscher *et al.* 1995). The irreversibly adhered micro-organisms are crucial for the maintenance of the biofilms in the substratum surface (Busscher and van der Mei 2006) and therefore studies regarding the adhesion should be considered essential when studying biofilms. Microbial adhesion experiments can be well studied using parallel plate flow chambers which offer good control of the hydrodynamic conditions (Sjollema *et al.* 1989).

There are different approaches in order to remove and kill surface attached biofilms; nevertheless these methods usually rely on the use of chemical agents. With the increase of resistance of bacterial strains to the common biocides and also the known fact that biofilm bacteria are more resistant to these agents than the same strain in the planktonic form (Brown and Gilbert 1993), there is a need to study and develop other strategies. The use of bacteriophages (phages) as biofilm control agents has already been suggested as an alternative to conventional chemical agents (Doolittle *et al.* 1995, 1996; Hughes *et al.* 1998; Tait *et al.* 2002; Sillankorva *et al.* 2004; Curtin and Donlan 2006). In a previous work, the podoviridae phage φS1 was used to infect *Pseudomonas fluorescens* mature biofilms (Sillankorva *et al.* 2004) and under ideal infection conditions this phage resulted in higher biomass

removal efficiencies than obtained with chemical agents (Simões *et al.* 2003).

In this paper, the same phage, φS1, was studied for its ability to infect *P. fluorescens* cells at the early stage of biofilm formation. To our knowledge, this is the first work that attempts bacterial elimination before an actively growing biofilm could be formed and also the first using a parallel plate flow chamber which enabled the study of phage infection *in situ* and on real time. Two phage concentrations were used in order to study the influence of phage titre in the lytic activity, when infection occurs under a flow regime.

Materials and methods

Bacteria, phage and media

Pseudomonas fluorescens ATCC 27663 and the respective phage φS1 from the American Type Culture Collection were used.

Nutrient broth medium (NBM) contained 10 g l^{-1} Nutrient broth, 1 g l^{-1} glucose, 1·45 g l^{-1} $K_2HPO_4{\cdot}3H_2O$ and 0·49 g l^{-1} KH_2PO_4. Nutrient broth agar (NBA) contained NBM with the addition of 1·5% (w/v) agar and nutrient broth soft agar (NBSA) contained 10 g l^{-1} Nutrient broth, 2% (w/v) glucose, 1·45 g l^{-1} $K_2HPO_4{\cdot}3H_2O$, 0·49 g l^{-1} KH_2PO_4 and 0·6% (w/v) agar. The phage buffer (PB) used for phage dilution and preservation contained 0·73 g l^{-1} Trizma base, 0·5 g l^{-1} of gelatine, and 2·5 g l^{-1} of $MgSO_4{\cdot}7H_2O$.

Bacteriophage propagation and purification

Phage propagation was performed as described before (Sillankorva *et al.* 2004) and the purified phage solution was stored at 4°C until required.

Bacteriophage enumeration

Phage enumeration was done according to the soft agar overlay technique described by Adams (1959) and all the analyses were performed in triplicate.

Cell adhesion and infection assays

Parallel plate flow chamber

In this study, a parallel plate flow cell, with laminar flow, developed by Sjollema *et al.* (1989) was used to study cell adhesion and phage infection. The flow cell was mounted in an inverted microscope (Nikon, Diaphot 300, Cambridge, MA, USA) with a digital CCD camera (Sony, AVC-D5CE) connected to allow image capture throughout the assays. The camera was coupled to an image analyser (Image Proplus 3·0; Media Cybernetics, Bethesda, MD, USA) and a ×40 magnification contrast phase objective was used. The number of cells present on glass surfaces was enumerated using Sigma Scan Pro 5 and the respective programs Image Math and Measure Objects.

Flow chamber and glass surface preparation

The parallel plate flow chamber as well as all the silicone tubes were autoclaved (121°C, 20 min) prior to cell adhesion and phage infection experiments.

Glass surfaces were immersed in 50% methanol for 30 min in order to remove all existent grease and cellular debris. Afterwards, the surfaces were carefully washed with commercial detergent solution and thoroughly rinsed with distilled water and let dry at 65°C. Dry surfaces were then sterilized at 121°C for 20 min. Cleaned and dried glass surfaces were used for determining the hydrophobicity parameters of the surface and to test if the washing procedure influenced these parameters. The hydrophobicity parameters were obtained as described by Busscher *et al.* (1984). Briefly, the contact angle was obtained using the sessile-drop contact angle technique and water as the reference liquid. All experiments were done in triplicate, with four repeats and the contact angle value obtained for glass after the methanol and detergent washing steps was 23·9 ± 0·8°.

Cell adhesion experiments

Pseudomonas fluorescens cultures were grown for 24 h at 26°C. The cells were harvested by centrifugation at 7000 ***g*** for 10 min at 4°C, and suspended in fresh NBM. The optical density used was approximately 1·0, which corresponds to $6{\cdot}9 \times 10^9$ cells ml^{-1}. Before each adhesion assay the system was flowed, without recirculation, during one hour with sterile deionised water and afterwards 30 min with NBM. The flow was then switched to the *P. fluorescens* cell suspensions, prepared as described previously, and the system was operated under recirculation at a flow rate of 14·5 ml min^{-1}, to allow bacterial adhesion. Images were captured at the centre of the flow chamber during the period of adhesion. Cell suspension feeding was ended after approximately 50 min that corresponded to the time when the number of adhered cells reached about $1{\cdot}7–1{\cdot}8 \times 10^6$ cells cm^{-2}.

Phage infection

After cell adhesion the system was washed for 30 min with NBM, without recirculation, in order to remove any nonadhered cells from the surface of the flow cell and silicone tubes. After the removal of nonadhered cells, the feed was shifted from NBM to a solution containing phage. This phage solution consists of 30 ml of phage stock solution and 30 ml of NBM in order to have

2×10^9 and 1×10^{10} PFU ml^{-1}or of 30 ml of NBM and 30 ml of PB in the control experiments. The solution was allowed to recirculate through the system and images were captured during the infection process. When the number of adhered cells was about $0{\cdot}3 \times 10^6$ cells cm^{-2} the flow of phage solution was ended.

Recolonizaion assays

Following phage infection of adhered cells the system was flowed with PB during 60 min, in order to remove the existing phages present in the liquid flow. After, recolonization of the glass surfaces was ascertained with fresh *P. fluorescens*. A bacterial cell suspension, prepared as described previously, was again allowed to flow throughout the system at a flow rate of 14·5 ml min^{-1} and images were captured during approximately 90 min.

Resistance assays

After phage infection and recolonization experiments, sterile swabs were used to collect some of the cells that remained on the surfaces. The swabs were used to streak directly the bacteria on NBA dishes which were incubated for 18 h at 26°C. Colonies were picked and grown for 7 h on 50 ml of NBM. Three millilitres of NBSA was added to 100 μl of cell suspension and poured on a NBA plate. Then, 10 μl of each of the stock phage solutions, with 2×10^9 and 1×10^{10} PFU ml^{-1}, were put on the bacterial lawn and the drop was allowed to dry. The NBA dishes were incubated for 18 h at 26°C and checked for presence of phage plaque.

Samples for scanning electron microscopy observations

For scanning electron microscopy (SEM) observations, a special lower glass plate for the parallel plate flow chamber was prepared. This new plate had two round recesses made for the insertion of two 1 cm round glass cover slides. Attachment and infection experiments were performed on these surfaces according to the methodology previously described. The round cover slides were removed for SEM observations that were performed using a Leica S360 scanning electron microscope at 10 kV EHT.

Statistical analysis

To compare the results of the rate of cell lysis using different phage concentrations, one-way analysis of variance (one-way ANOVA) was used. Then *post hoc* testing using Tukey's test was performed to assess statistically significant differences. In all the analyses performed the confidence interval used was 95%. These tests were performed using SPSS 11·5 for Windows.

Results

Cell adhesion assays

Pseudomonas fluorescens, a well known biofilm forming micro-organism, was used to study the first step involved in the biofilm formation process that is the adhesion to a surface. Throughout the adhesion of cells to glass surfaces it was necessary to monitor and enumerate the number of cells present in the surface in order to study the kinetics of cell deposition (Fig. 2). Some exemplifying micrographs are presented showing the coverage of the glass surface in different time periods (Fig. 1). In the four different assays, cells began to adhere to the glass surface as soon as the bacterial suspension started flowing through the system. It is noticeable that in some time points there were more cells adhered than in the following captured image and this is due to reversible adhesion of cells which momentarily attach to the surface of the parallel plate flow chamber (Fig. 2). In the four adhesion assays performed the maximum cell number was reached after approximately 50 min of the adhesion experiment.

Phage infection assays

Glass surfaces with adhered *P. fluorescens* were infected with phages to study the cell lysis and elimination from the surface. Triplicate studies with two phage solutions with different concentrations were used to test the performance of the phage and micrographs were taken at different infection steps (Fig. 3) and also the kinetics of cell removal from the surface were studied (Fig. 4). The two different phage concentration solutions resulted in clearly distinct performances. Nevertheless, for both phage concentrations used in the infection experiments, the cell lysis started after approximately 22 min (Fig. 4). The higher concentrated phage solution led to approximately 90% reduction in the number of cells adhered 25 min after the beginning of cell lysis, while the less concentrated phage solution took 60 min to achieve the same percentage of cell reduction. In the control experiments, in which the parallel plate flow cell with adhered cells was flowed with a solution of NBM and PB (1 : 1, v/v), there was not observed a decrease in the number of cells adhered (Fig. 4). The rate of cell removal (Table 1) was significantly higher and statistically different ($P < 0{\cdot}05$) when a more concentrated phage solution was used. Phage performance was clearly influenced by the concentration of the phage solution used nevertheless similar removal percentages were obtained with the two different

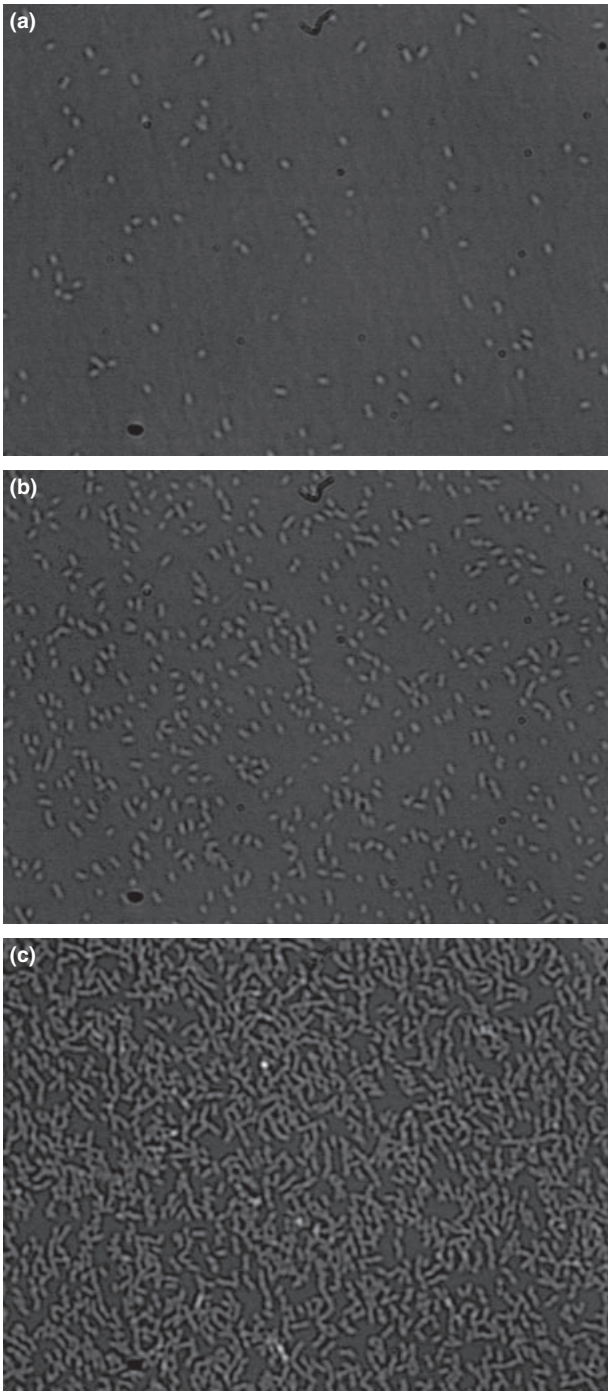

Figure 1 Micrographs of *Pseudomonas fluorescens* cells adhered to glass surfaces at different time points: (a) 1 min; (b) 15 min and (c) 50 min.

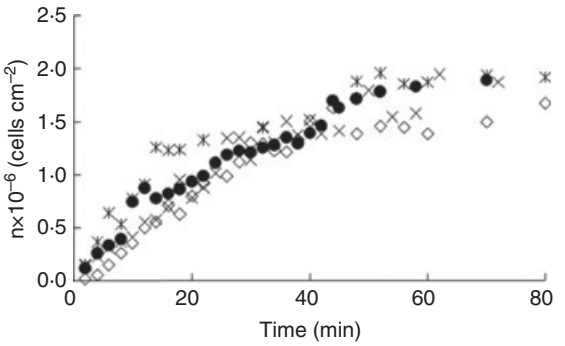

Figure 2 Deposition kinetics of *Pseudomonas fluorescens* onto glass as a function of time in a parallel plate flow chamber from four different adhesion experiments.

Table 1 Effect of phage titre in the rate of cell removal per cm^2 (R) and percentage of cell removal (P) obtained in experiments with adhered cells

Phage titre (PFU ml^{-1})	2×10^9	1×10^{10}
R (cells cm^{-2} min^{-1}) (± SD)	0·024 (0·002)	0·073 (0·027)
P (%) (± SD)	93·4 (0·010)	93·5 (1·510)

SD, standard deviations for data collected from three infection experiments.

phage concentrations (Table 1). In all phage infection assays performed, at the end of the assay, no matter how long the phage solution was allowed to circulate in the system, there were always some cells that were not eliminated and therefore remained intact on the glass surface. Also, comparing the micrographs taken before (Fig. 1) with the ones taken after phage infection (Fig. 3), it is possible to visualize in Fig. 3, the place where cells used to be attached before phage infection was performed. The glass surfaces were observed also by SEM observations (Fig. 5) which also confirmed the presence of foot-prints as well as it allowed the observation of phages adhered to the surface which correspond to the small white spots (Fig. 5b).

Recolonization assays

After phage infection assays and the complete rinsing of the system with buffer to remove the greatest amount of phages, the recolonization of the glass surfaces with fresh *P. fluorescens* cells was evaluated. Even though the rinsing eliminated the phages from the flow, there were still approximately 10^3 PFU ml^{-1} phages that remained in the system, most likely attached to the glass surfaces, as observed in the SEM micrographs (Fig. 5b), as well as to the silicone tubes used in the system. The bacterial recolonization studies were performed with *P. fluorescens* suspensions with the same concentration as the ones used in the adhesion assays. The recolonization was monitored during 100 min and it was observed that cell adhesion after a phage

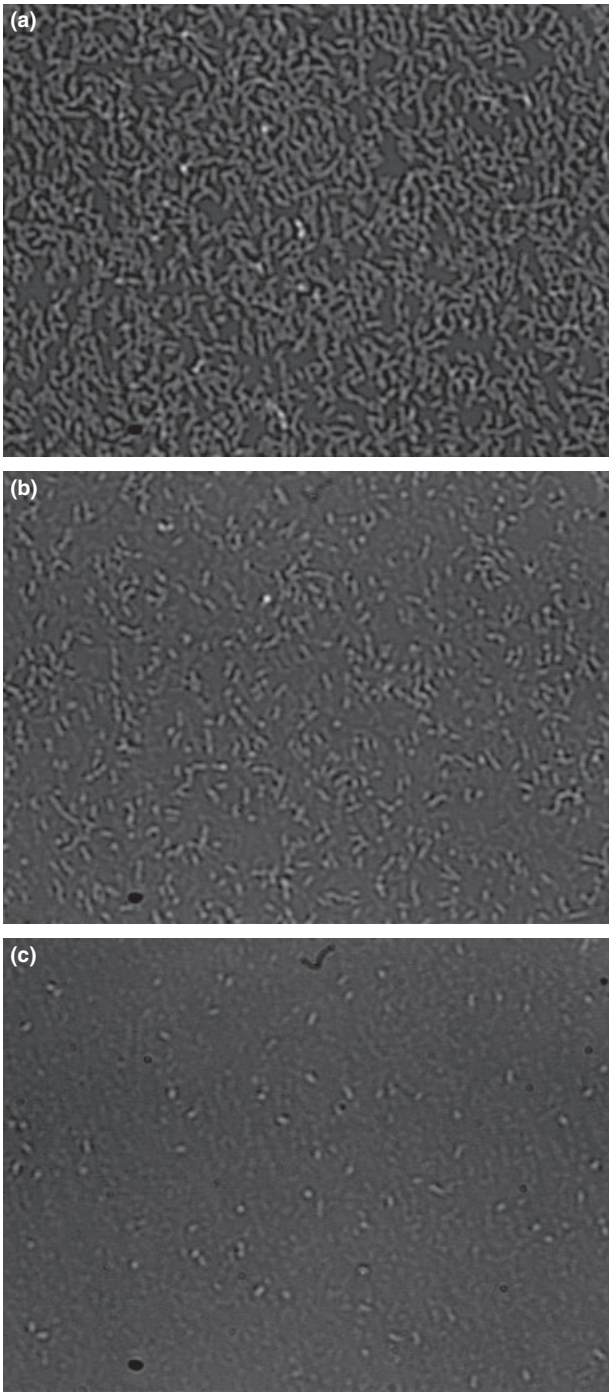

Figure 3 Micrographs of *Pseudomonas fluorescens* cells adhered to glass surfaces after phage infection: (a) beginning; (b) middle and (c) end of infection.

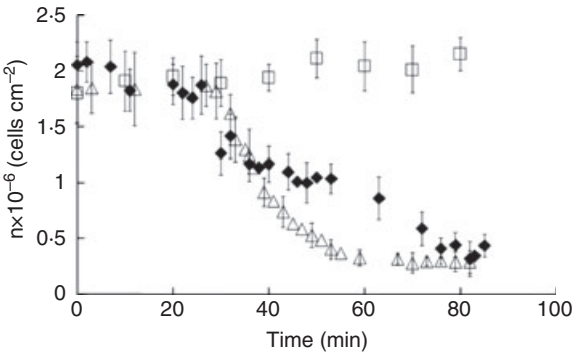

Figure 4 Cell removal kinetics after phage infection with a solution of 2×10^9 PFU ml^{-1} (♦), 1×10^{10} PFU ml^{-1} (△). Control samples (□) consist of adhered cells flowed with a solution of NBM and PB (1 : 1, v/v). Error bars represent ± SD from triplicate infection experiments.

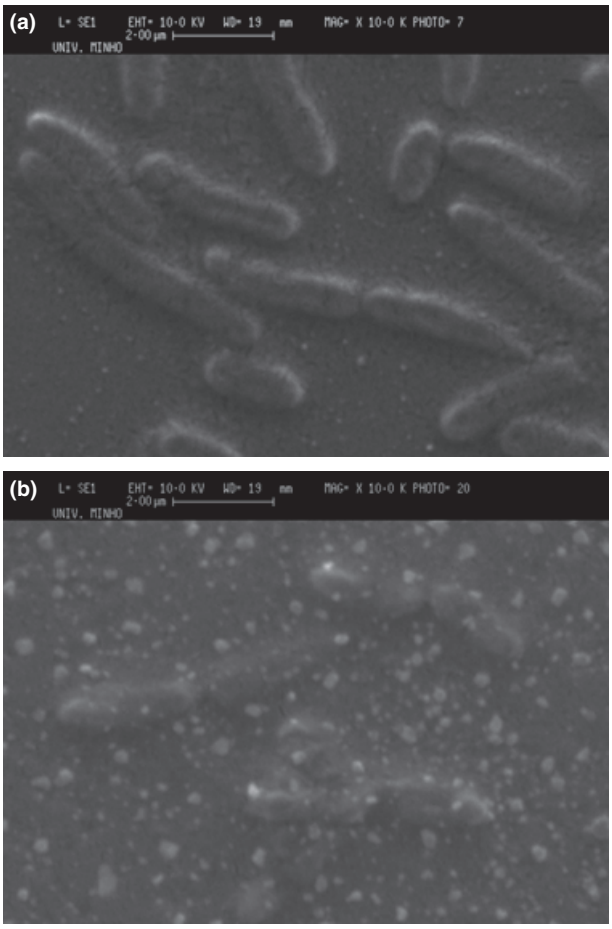

Figure 5 SEM observations at magnification of 10 000 ***g*** of glass surfaces: (a) biofilm before phage infection and (b) biofilm after phage infection.

infection process was no longer achieved (Fig. 6). The variation obtained regarding the number of cells attached to the glass surface was possibly due to cells that adhered reversibly for a short time but could not attach irreversibly. After the recolonization experiments, phage sensitivity tests were performed for the bacteria that remained on the surfaces (Fig. 3). The remaining

bacteria present in the glass surface, were collected and streaked on NBA plates and afterwards, some colonies (5–10) were picked and regrown in different Erlenmayers with NBM. After all phage infection experiments performed the bacteria picked for phage sensitivity test never showed resistance to the phage stocks as in all tested bacterial lawns the phage was able to produce clear plaques (data not shown).

Discussion

Bacterial adhesion to surfaces is found in several environments and has been subject of investigation for many years. Some of the negative impacts of bacterial adhesion are, for instance: economic losses, contamination of medical settings and reduction of product quality. The removal of the adhered bacteria from surfaces tend to be very difficult to evaluate, nevertheless, the use of flow devices, such as a parallel-plate flow chamber, provides reliable data on the kinetics of microbial adhesion to various substrata (Bos *et al.* 1996). In these flow cells, the hydrodynamic conditions are well controlled and it is possible to monitor, *in situ* and in real time, the number of cells adhered throughout an assay (Azeredo *et al.* 2003).

Previous work has shown that the application of phage φS1, a *Podoviridae* phage, to *P. fluorescens* biofilms resulted in a great decline on the cell number and also that phage infection of *P. fluorescens* biofilms was able to disrupt the biofilm matrix (Sillankorva *et al.* 2004). Phage studies to control bacterial populations usually focus on planktonic cultures or mature biofilm cells; therefore this work presents a novel approach in the phage infection field as it concerns phage infection in an early stage of biofilm formation or simply adhered cells.

Pseudomonas fluorescens cells were allowed to adhere to glass surfaces and phage infection was later on studied using two different phage concentrations. Cell lysis started approximately after 22 min of phage φS1 infection which is consistent with typical *Podoviridae* infection parameters as this phage family has characteristically short latent periods. The rate of cell lysis due to phage performance was influenced by the phage concentration applied (Fig. 4 and Table 1). A higher phage concentration was responsible for a higher rate of cell lysis and therefore for a faster cell reduction. As phages rely on random encounters with the host cells, it is most likely that when a more concentrated phage solution was used, the probability of a phage to find a cell was higher which consequently promoted a faster cell lysis. Using a lower phage concentration but allowing phage infection to occur for a longer period resulted in similar cell removal percentages (Table 1). However, phages where not able to promote the complete eradication of the bacteria (Figs 3c and 4), even when a more concentrated phage solution was applied. This might be due to the lower number of cells that remained on the surface, after phage infection, which results in a more difficult contact between the adhered hosts cells and the phages present in the flow.

Comparing this phage infection processes with some studies performed with *P. fluorescens* and cationic surfactants and biocides, it is possible to withdraw many positive conclusions on phage application. For instance, conversely to phage infection, some biocides and surfactant treatments do not promote cell removal and even enhance further bacterial recolonization due to the cell debris that remain on the surface (Azeredo *et al.* 2003; Simões *et al.* 2003). Biocides have very low removal rates and usually it is necessary to use highly concentrated solutions. In order to investigate whether phage was also responsible for enhancing bacterial redeposition on the surfaces, it was necessary to perform a study on cell adhesion after phage treatment. This recolonization study (Fig. 6) proved that, even after 100 min of bacteria feeding to the system no further cell adhesion was observed. Several studies have demonstrated that surfaces with adhered cells when submitted to sonication or the passage of an air/liquid interface are difficultly recolonized (Neu 1996; Goméz-Suárez 2001, Goméz-Suárez *et al.* 2002). According to Neu (1996) when bacterial cells are detached from surfaces, they leave on the surface polysaccharide nature substances designated by foot-prints that could inhibit or induce bacterial adhesion. Also, a study regarding the pretreatment of catheters with phage showed that phages were able to prevent *Staphylococcus epidermidis*

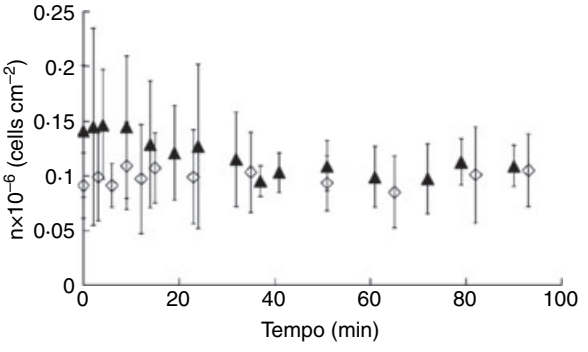

Figure 6 Number of *Pseudomonas fluorescens* cells adhered during the process of recolonization of a glass surface after cell removal using two phage concentrations: 2×10^9 PFU ml–1 (◇) and 1×10^{10} PFU ml^{-1} (▲). Error bars represent ± SD from triplicate *P. fluorescens* recolonization experiments.

adhesion to the catheter surfaces (Curtin and Donlan 2006). So, the failure of *P. fluorescens* in recolonizing the glass surface may be due to the presence of footprints and also phages that inhibit the bacterial re-attachment process (Fig. 5b).

This work demonstrates the ability of phage φS1 to lyse *P. fluorescens* cells adhered to glass surfaces using a well known *in situ* and real time methodology commonly used to assess bacterial adhesion and detachment kinetics. This is a novel strategy for phage infection studies and should be considered to study phage infection of biofilms in the initial stages of formation.

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