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ORIGINAL ARTICLE

Integration and decontamination of *Bacillus cereus* in *Pseudomonas fluorescens* biofilms

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Abstract

Aims: The hypothesis that surrogate planktonic pathogens (*Bacillus cereus* and polystyrene microspheres) could be integrated in biofilms and protected from decontamination was tested.

Methods and Results: *Pseudomonas fluorescens* biofilms were grown on polyvinyl chloride coupons in annular reactors under low nutrient conditions. After biofilm growth, *B. cereus* spores and polystyrene microspheres (an abiotic control) were introduced separately. Shear stress at the biofilm surface was varied between 0·15 and 1·5 N m^{-2}. The amount of surrogate pathogens introduced ranged from approximately 10^5 CFU ml^{-1} to 10^{10} spheres ml^{-1}. The quantity of surrogate pathogens integrated in the biofilm was proportional to the amount introduced. In 14 of the 16 cases, 0·4–3·0% of the spores or spheres introduced were measured in the biofilms. The other two cases had 10% and 21% of the spores detected. Data suggested that the spores germinated in the system. The amount of surrogate pathogens detected in the biofilms was higher in the mid-shear range. Chlorine treatment reduced the quantity of both surrogate pathogens and biofilm organisms. In one experiment, the biofilms and *B. cereus* recovered when the chlorine treatment was terminated.

Conclusions: Planktonic surrogate pathogens can be integrated in biofilms and protected from chlorination decontamination.

Significance and Impact of the Study: This knowledge assists in understanding the impact of biofilms on harbouring potential pathogens in drinking-water systems and protecting the pathogens from decontamination.

Introduction

The Environmental Protection Agency stated that a 'better understanding is needed regarding which contaminants may attach to the interior of the water distribution system and how they can best be removed' as a key research need (USEPA, 2004). To gain insight into this topic, more knowledge is needed on the integration of potential pathogens in biofilms. The interiors of pipes in water distribution systems are often covered with biofilms (Ridgway and Olson 1981; Block 1992; Szewzyk *et al.* 2000). The questions that arise include: (i) if a biological pathogen is introduced into a drinking water system, will it become integrated and retained in the biofilms on the pipe-wall surfaces?, (ii) if so, what fraction of an introduced pathogen is retained in the biofilm?, (iii) once integrated into the biofilms, how long will the pathogens persist in the biofilms?, (iv) what variables impact the pathogen integration? and (v) can the biofilms protect the pathogens from disinfection or will traditional disinfection methods be able to remove the pathogens?

Pathogen integration into biofilms and subsequent survival have been studied to a certain extent, but the phenomena are not well-understood. Recent research showed that *Pseuodmonas aeruginosa* biofilms played a significant role in controlling the initial attachment, growth and survival of *Escherichia coli* in porous media (Liu and Li 2008). This may be due to the interaction between organisms; Buswell *et al.* (1998) suggested that coaggregation mediates the integration of pathogens into biofilms and

biofilms enhance the survival of pathogens. There is also a difference in persistence between species. Szabo *et al.* (2007) found that *Klebsiella pneumoniae* persisted only temporarily (9–17 days) in both chlorinated and dechlorinated drinking-water biofilms grown on corroded iron surfaces in annular reactors. In contrast, these same authors found that *Bacillus atrophaeus* subsp. *globigii*, a surrogate for *Bacillus anthracis*, persisted under the same conditions for up to 70 days with target chlorine concentrations as high as 70 mg l^{-1} (Szabo *et al.* 2007). Likewise, Långmark *et al.* (2005) found that 1-μm hydrophilic and hydrophobic microspheres, *Salmonella* bacteriophages 28B and *Legionella pneumophila* persisted in drinking-water biofilms that were monitored for over 38 days. In this case, the drinking-water biofilms were grown on a 1-km long, 50-mm diameter pilot-scale distribution system and glass coupons were used for sampling. In summary, the persistence of pathogens in biofilms is variable.

Results of experiments examining the efficacy of chlorine at disinfecting biofilms and pathogens within biofilms also differ. It is generally accepted that disinfection is more effective on planktonic organisms than organisms within a biofilm (LeChevallier *et al.* 1988a,b). However, Långmark *et al.* (2005) demonstrated that low levels of chlorine did not have a large impact on the accumulation and fate of model microbial pathogens in biofilms. They determined that chlorine impacted the culturability of *L. pneumophila* more than physical loss from the biofilm. Likewise, Vess *et al.* (1993) demonstrated the survival of a biofilm exposed to 10–15 ppm free chlorine. In contrast, both Lund and Ormerod (1995) and Thomas *et al.* (2004) found that 0·04–0·05 mg l^{-1} residual free chlorine and 2·5 mg l^{-1} free chlorine respectively were effective in eliminating biofilms. Codony *et al.* (2005) observed that discontinuous chlorination led to more robust biofilms than if there had been no chlorination at all. Butterfield *et al.* (2002) reported that mixed-population heterotrophic biofilms grown in an annular reactor with a free chlorine residual of 0·09–0·15 mg l^{-1}, yielded less biofilm mass than if they were grown without chlorine. It has been stated that the surface the biofilm grows on impacted the effectiveness of disinfection (LeChevallier *et al.* 1988a,b, 1990; Gibbs *et al.* 2004).

Clearly, universal conclusions cannot be made from any one study due to the high variability of biological and physical variables. Additional data are needed to gain a 'better understanding' stated as a key research need by the EPA. With more and more data, a better understanding of controls of contaminant attachment and removal under specific conditions will be attained. In addition, these data become useful in bounding uncertainty and quantifying variability.

This study examines the integration and retention of *Bacillus cereus* (ATCC 14579) spores and polystyrene microspheres in *Pseudomonas fluorescens* (ATCC 700830) biofilms grown on polyvinyl chloride (PVC) coupons in annular reactors in chlorine-free water. Annular reactors are commonly used to simulate drinking water systems (e.g., Gagnon and Huck 2001; Camper *et al.* 2003; Williams *et al.* 2005; Szabo *et al.* 2006, 2007). *Pseudomonas fluorescens* was chosen as the biofilm organism because it has been found in drinking water biofilms (Szewzyk *et al.* 2000) and it is commonly used as a model organism (Vieira and Melo 1995; Pereira *et al.* 2002; Busalmen and de Sanchez 2005; Delille *et al.* 2007). As *P. fluorescens* is Gram-negative, it allows for straightforward detection of the test organism, the Gram-positive *B. cereus*. *Bacillus cereus* was chosen as a surrogate for *Bacillus anthracis*, the causative agent for anthrax. The 2001 postal anthrax attacks brought attention to the ease of dissemination and potential lethality of *B. anthracis* in small doses. As an abiotic control 'pathogen', carboxylate-modified microspheres [Molecular Probes, MP 05001] were chosen because of the similarity of size (1-μm diameter) and the negative surface charge at the pH used in the experiments. PVC were chosen because water distribution systems contain PVC pipes, their ease of use and demonstration that significant biofilms could grow on the pipe material (Camper *et al.* 2003).

Three variables were examined as to their effect on surrogate pathogen integration and retention: shear stress or Reynolds's number (Re) in the system during and after surrogate-pathogen introduction, number of *B. cereus* spores and polystyrene spheres introduced to the system and initial bacterial cell density in the biofilms. These parameters were chosen because they are uncertain and/or variable in drinking water systems and have been shown to impact biofilm structure (Dunsmore *et al.* 2002; Purevdorj *et al.* 2002) and biofilm coaggregation (Rickard *et al.* 2004). After examination of the above stated parameters, preliminary experiments investigating the effects of adding sodium hypochlorite with a goal of free chlorine concentrations in the reactor between 2 and 3 mg l^{-1} were conducted. Changes in *P. fluorescens* and surrogate-pathogen surface density in the biofilms and reactor water were observed.

Materials and methods

Cultivation, preparation and enumeration of micro-organisms

Bacillus cereus (ATCC 14579) was initially grown on 2× SG sporulation agar for 5 days at 30°C (Harwood and Cutting 1990). After scraping the cells off the agar, the

remaining vegetative cells were killed through repeated washing, centrifuging and suspension of centrifuged pellets in 50% ethanol. The pellets were suspended in 50% ethanol solution with overnight storage at 4°C. Spores were washed three times in deionized (DI) water to remove the ethanol. To kill the remaining vegetative cells, the pellets were suspended in 2 ml of 20% meglumine diatrizoate (Sigma). The 2 ml of suspension was then combined with 10–20 ml of 50% meglumine diatrizoate and centrifuged for 30 min at 14 500 ***g***. The presence of spores was confirmed using phase-contrast microscopy. The spores were washed four times to remove the excess chemical and stored in DI water at 4°C until use. Prior to use, the spores were vortexed and enumerated on Trypticase™ Soy Agar (TSA) to determine the correct volume to add to the reactor. Spores were also vortexed prior to introduction to the reactor to minimize coagulation of the spores (Harwood and Cutting 1990).

For each experiment, a new stock of *P. fluorescens* (ATCC 700830) was prepared using a Cryobank™ (Copan Diagnostics Inc) bead with the *P. fluorescens* culture. The beads were stored in a −20°C freezer prior to use. The bead was placed in 9 ml of Trypticase Soy Broth (TSB) and incubated for 24 h at 30°C. Two of these samples were then vortexed and centrifuged and the pellets combined and reconstituted in 9 ml of sterile DI water.

Pseudomonas fluorescens surface density and the amount of *B. cereus* in the biofilms and reactor water were quantified using traditional pour plating methods. Pour plating was chosen because when spread or drop plating was used, the *P. fluorescens* colonies spread, which interfered with enumeration. Quadruplicate plates were prepared for each dilution. Plates were incubated at 30°C for approximately 24 h for the *B. cereus* and 48 h for the *P. fluorescens* prior to enumerating. Results are reported as the mean ± standard deviation (SD) of the counts from the four plates when the results for one sample are reported. Results for multiple samples are reported as the mean ± 1 SD of the counts from all the plates. TSA was used to culture both organisms. Triphenyl Tetrazolium Chloride (BD Biosciences) was added to the TSA at a final volume of 0·01% to facilitate *P. fluorescens* enumeration. TSA mixed with Polymyxin B (an antibiotic that inhibits Gram–negative growth) was used to plate *B. cereus*.

To enumerate cells in the biofilms, the coupons were first scraped with a sterile polypropylene cell lifter (Corning 3008) into 9 ml of sterile DI water (Camper *et al.* 2003; Heersink 2003). The coupon was then rinsed with the diluent that was transferred into a sterile test tube. One millilitre of sterile DI water was used to rinse the beaker and then water poured into the test tube. Cells were dispersed using established methods of sonication for 5–10 min and vortexing (Heersink 2003; L. Loetterle, personal communication, 2004). From this suspension, two samples of 3 ml each were used for spectrophotometric analysis and 1 ml was used for plating. The detection limit for *B. cereus* in the biofilms was 1 CFU on the first dilution, converting to 5·3 CFU cm^{-2}.

Experimental apparatus and setup

Experiments were conducted with annular reactors (BioSurface Technologies, Corporation, Model DFR 110) to simulate a water distribution system. Unused PVC coupons that had been disinfected in a 20% bleach solution were used for each experiment. Each reactor had 20 $15 \times 1{\cdot}25$ cm coupons in the inner rotating cylinder, which had a diameter of 13·9 cm. Coupons constituted approximately 25% of the surface area of the reactor. The volume of the annulus was approximately 1 L. Prior to running the experiment, the reactor was first sterilized in an autoclave at 121°C and 15 pounds per square inch (psi) for 30 min. After sterilization, the reactor was filled with 10% bleach solution and the coupons placed in the inner cylinder. Finally, the reactor was rinsed by circulating sterile DI water through the system until free chlorine concentrations in the outflow solution were $<0{\cdot}01$ mg l^{-1}.

The correlation between Re, shear stress and inner cylinder rotation speed as a function of pipe diameter was calculated based on the methods suggested by BioSurface Technologies Corporation (B. Warwood, personal communication). Shear stress at the wall surface (τ_w) was calculated assuming turbulent flow as follows:

$$\tau_w = \frac{f\rho v^2}{2}$$

where, f is the Fanning friction factor, ρ is the water density and v is the average water velocity through the pipe. The friction factor was calculated using the Blasius formula:

$$f = \frac{0.0791}{(\mathrm{Re})^{0.25}}$$

Finally,the revolutions per minute (RPM, rev min^{-1}) was calculated from the shear stress as follows:

$$\mathrm{RPM} = (1023.37\tau_w - 3.05579)^{0.77628}$$

Operation of reactor

Pseudomonas fluorescens biofilms were grown under the same conditions to form reproducible biofilms. A nutrient solution was transported through the reactors for the duration of each experiment at concentrations of 5, 2·5 and 1·25 mg l^{-1} of glucose (as Difco Dextrose, BD

Biosciences), peptone (Fisher Scientific) and yeast extract (Fisher Scientific) respectively. NaOH was also added to the reactor for the duration of the experiment from a separate container to maintain a concentration of 2·0 mg l^{-1} NaOH (Lab Chem, Inc.) to sustain a neutral pH. The reactor was first filled with the nutrient and NaOH solutions. Five millilitre of the *P. fluorescens* inoculum was then pipetted into the reactor along with 9 ml of sterile TSB. The inoculum was allowed to sit in the reactor for approximately 4 h prior to initiating flow. The flow rate was then maintained at 1·2 ml min^{-1} (residence time = 13·9 h) for 10 days. Then, to minimize the growth of planktonic bacteria in the reactor water, the flow rate was increased to 15·6 ml min^{-1} (residence time = 1 h) for another 7 days prior to adding the microspheres and spores. Previous experiments demonstrated that this was sufficient time to reach a steady-state bacterial cell density in biofilms on the order of 10^5–10^7 CFU cm^{-2} (Altman *et al.* 2005). The observed range of *P. fluorescens* colony counts was similar to those of biofilms grown with drinking water (Camper *et al.* 2003; Szabo *et al.* 2007). The rotation speed of the inner cylinder was maintained at 60 rev min^{-1} during the *P. fluorescens* biofilm growth phase.

Just prior to surrogate-pathogen introduction, two coupons were sampled from the annular reactor, the inner cylinder rotation speed was adjusted to that specified for the specific experiment and the flow rate of the reactor was decreased to 2·6 ml min^{-1} (residence time = 6 h). Micropsheres and *B. cereus* were then introduced separately and almost simultaneously into the reactor. The 6 h residence time was maintained for 24 h after surrogate-pathogen introduction to increase the initial residence time of the surrogate pathogens in the reactor. Twenty-four hours after the surrogate pathogens were introduced, the flow rate was increased again so that the residence time was 1 h.

A total of eight experiments were conducted with different initial amounts of introduced *B. cereus* spores and 1 μm-diameter TransFluoSpheres® carboxylate-modified fluorescent microspheres (Molecular Probes, # T8883) and different shear stresses, as controlled by the inner cylinder rotation speeds (Table 1). Table 2 presents the correlation between the inner cylinder rotation speed and shear stress, Re, average linear velocity and flow rate for different pipe diameters.

Samples of reactor water and biofilms were collected throughout the duration of the experiment. Ten millilitre of reactor water was collected at each sample event for analysis of *P. fluorescens*, *B. cereus* and microspheres as described below. Reactor water samples were collected periodically both before and after surrogate-pathogen introduction. After surrogate-pathogen introduction, only one coupon was collected at each time interval. Each coupon was analysed to quantify *P. fluorescens*, *B.cereus* and microspheres.

Table 1 Experiment variables

Experiment number	Average (SD) *Pseudomonas fluorescens* Surface Density (CFU cm^{-2})	Average (SD) *Bacillus cereus* Spores Introduced (CFU)	Microspheres introduced (spheres)	RPM	Start of chlorination (days after surrogate-pathogen intro)
50 RPM	$7·5 \times 10^4$ ($3·8 \times 10^3$), $n = 4$	$7·0 \times 10^7$ ($2·7 \times 10^6$), $n = 4$	$9·1 \times 10^8$	50	36·0
100 RPM #1	$2·8 \times 10^5$ ($2·5 \times 10^5$), $n = 8$	$9·9 \times 10^5$ ($9·5 \times 10^4$), $n = 4$	$1·4 \times 10^8$	100	15·0
100 RPM #2	$1·6 \times 10^5$ ($6·4 \times 10^4$), $n = 8$	$1·0 \times 10^5$ ($2·0 \times 10^4$), $n = 4$	$4·2 \times 10^9$	100	14·0
150 RPM #1	$7·9 \times 10^5$ ($4·5 \times 10^5$), $n = 8$	$4·4 \times 10^7$ ($8·1 \times 10^6$), $n = 4$	$1·2 \times 10^9$	150	14·1
150 RPM #2*	$8·3 \times 10^5$ ($9·9 \times 10^4$), $n = 8$	$2·6 \times 10^8$ ($9·3 \times 10^6$), $n = 4$	$9·5 \times 10^7$	150	3·0
150 RPM #3	$2·5 \times 10^5$ ($4·9 \times 10^4$), $n = 8$	$8·2 \times 10^8$ ($8·6 \times 10^7$), $n = 4$	$5·0 \times 10^8$	150	14·0
300 RPM #1	$3·5 \times 10^5$ ($2·5 \times 10^5$), $n = 4$	$7·6 \times 10^7$ ($3·7 \times 10^6$), $n = 4$	$7·3 \times 10^9$	300	35·0
300 RPM #2	$3·5 \times 10^5$ ($1·3 \times 10^5$), $n = 8$	$8·0 \times 10^6$ ($6·6 \times 10^5$), $n = 4$	$1·2 \times 10^9$	300	15·1

*Experiment terminated early because of clogging of outflow line.

Table 2 Correlation of inner cylinder rotation rate and Reynolds number, average linear velocity and flow rate

	Shear stress (N m^{-2}) ↓	Reynolds number (Re)			Avg. linear vel. (m s^{-1})			Flow (gpm)		
RPM ↓	Pipe → diameter (in)	2	4	8	2	4	8	2	4	8
50	0·154	10 833	12 951	52 872	0·20	0·22	0·24	6·40	28·31	125·0
100	0·372	17 939	39 621	87 457	0·33	0·36	0·40	10·60	46·84	206·8
150	0·624	24 134	53 286	–	0·44	0·49	0·54	14·26	63·0	278·2
300	1·52	40 134	–	–	0·74	–	–	23·72	–	–

Approximately 14 days after the surrogate pathogens were introduced into the reactors, chlorine treatment began for four of the experiments. The goal of the chlorine treatment was to maintain free chlorine concentrations in the reactor between 2 and 3 mg l^{-1}. This range was chosen because it is below the EPA allowable level for drinking water of 4 mg l^{-1} as Cl_2.

Chlorine was added to samples of reactor water to determine the concentration of sodium hypochlorite that would be added to the system for the chlorine treatment. Between 5 and 10 times (depending on the experiment) prior to starting the chlorine treatment, reactor water samples were collected with a sterile pipette. Sodium hypochlorite was added to the sample until the free chlorine levels were within the desired range between 2 and 3 mg l^{-1}. The amount of sodium hypochlorite that was added was recorded and used to estimate the desired sodium hypochlorite concentration in the annular reactor.

At the start of the chlorine treatment, a third container was connected to the system containing the desired concentration of a sodium hypochlorite solution. This solution was continuously pumped through the system for 7–8 days. Sampling of reactor water continued and the concentration of sodium hypochlorite was adjusted to attain the desired free chlorine range in the reactor. After chlorine treatment was terminated, additional samples of reactor water and biofilms were collected and analysed.

Unfortunately, it was difficult to maintain stable free chlorine concentrations. For this reason, our results related to the chlorine treatment should be considered preliminary. In some experiments it took approximately 1 day until the free chlorine concentrations reached the specified range. In one experiment, free chlorine concentrations exceeded 4 mg l^{-1} at one time.

One experiment was conducted to determine whether *B. cereus* alone could form a biofilm. The experiment was conducted as described in the first paragraph in this section, except that the annular reactor was inoculated with *B. cereus* spores instead of *P. fluorescens*. Samples were collected after the increase in flow rate and for 17 more days. In this case, two coupons were collected at each sampling time.

Analytical methods

Fluorescence spectrophotometry (Varian Cary Eclipse Fluorescence Spectrophotometer, system ID Eclipse–EL00073126) was used to measure the amount of microspheres in the reactor water and biofilms. Standards between 5×10^3 and 10^6 spheres ml^{-1} were used to generate linear calibration curves between log concentration (spheres ml^{-1}) and log measured intensity (arbitrary units). The detection limit for the microspheres was assumed to be the concentration of the lowest standard, which is equivalent to a surface density of $2{\cdot}7 \times 10^3$ spheres cm^{-2}. Microsphere concentrations from the biofilm samples collected prior to surrogate-pathogen introduction were consistently measured at below the detection limit.

Periodic biofilm samples were collected for visualization using a Nikon 80i Epifluorescent microscope. Samples were examined with 10×, 20× and 100× objectives. Molecular Probes LIVE BacLight Bacterial Gram Stain Kit (L-7005; Molecular Probes) was used to differentiate Gram-positive from Gram-negative organisms. SYTO®9 labels both live Gram-negative and -positive bacteria. In contrast, hexidium iodide preferentially labels Gram-positive bacteria. The hexidium iodide will displace the SYTO®9 stain, thus Gram-negative bacteria should fluoresce at a wavelength of 500 nm (green) and the Gram-positive bacteria should fluoresce at 625 nm (red). Biofilms were stained with a mixture of 25 μl of SYTO®9 and 5 μl of hexidium iodide mixed in 9970 μl of sterile DI. 0·5 ml of the stain was pipetted on the coupon and incubated for 1 h in the dark. The coupons were then rinsed three times with 500 μl of sterile DI for each rinse and viewed immediately.

Periodic pH measurements were made of the reactor water using an Orion 520A+pH meter. At the same time the pH measurements were made, the room temperature was recorded. Free chlorine measurements were made using a HACH Pocket Chlorimeter II (Hach Co., Loveland, CO).

Data analysis

The percentage of introduced surrogate pathogens that were integrated and retained in the biofilms was calculated

by multiplying the measured surface density of *B. cereus* or microspheres by the total surface area of the coupons and normalizing by the number of spores or microspheres introduced. This value represents the average percentage of the surrogate pathogen that may be captured and retained on the PVC. It does not account for the surface area of the outer cylinder of the reactor and the spaces between the coupons, which may also have biofilms on them.

A *t*-test and an *F*-test was used to compare the mean and variance respectively of the percentage of measured *B. cereus* and the microspheres in the biofilms (Microsoft Excel 2003). Results are presented as the probability that the samples from each experiment came from the same underlying population. Regression analyses were run using Kaleidagraph (ver. 4.03; Synergy Software, Reading, PA).

Results

Both plating (Table 3 and Fig. 1) and microscopy results (Fig. 2) gave evidence of surrogate pathogens (microspheres and *B. cereus*) becoming integrated and retained in biofilms in the reactor. For the most part, the amount of the surrogate pathogens in the biofilms for each individual experiment remained relatively constant (Fig. 1). For this reason, when comparisons were made between experiments, the average surface density of the surrogate pathogen in the biofilm for the samples collected after surrogate-pathogen introduction and before chlorine treatment was used (Table 3).

A clear correlation between the amount of surrogate pathogen introduced into the system and the surface density of integrated surrogate pathogens in the biofilm was observed (Fig. 3a). As the number of spores or spheres introduced into the system increased over a range of four orders of magnitude, the surface density of the spores or spheres also increased over approximately three orders of magnitude. The R^2 value of 0·73 for the regression through these data supports the positive correlation. As the regression gives a negative intercept, it is possible that a linear relationship is not the best model for fewer pathogens.

There was also an indication that, under the conditions of these experiments, a threshold amount of surrogate pathogens must be introduced to get detectable integration into the biofilm. Not included in Fig. 3 is the *B. cereus* surface density from the 100 RPM #2 experiment where $1{\cdot}0 \times 10^5$ spores were introduced into the system. *B. cereus* was only occasionally detected in the biofilms at the detection limit with 1 CFU counted in the first dilution for a surface density of 5·3 CFU cm^{-2} (Table 3). These surface densities were only measured in one of four plates for two biofilm samples collected 0·05 and 2·0 days after surrogate-pathogen introduction.

With the exception of two outliers, the percentage of surrogate pathogens measured in the biofilms was 3% or less of the amount introduced (Fig. 3b). However, a larger percentage (10% and 21%) of integration was observed for *B. cereus* colony counts from two experiments (100 and 150 RPM #1) (Fig. 3b).

The growth of *B. cereus* biofilms in annular reactors when inoculated alone gave evidence that the *B. cereus* spores can germinate to grow into biofilms. Counts

Table 3 Experiment results

Experiment number	Average (SD) *Bacillus cereus* surface density in biofilm (CFU cm^{-2})	*n*, *B. cereus*	Average (SD) microsphere surface density in biofilm (spheres cm^{-2})	*n*, microspheres	*t*-test results	*F*-test results
50 RPM	$1{\cdot}1 \times 10^3$ ($6{\cdot}3 \times 10^3$)	35	$8{\cdot}8 \times 10^3$ ($4{\cdot}1 \times 10^3$)	36	$1{\cdot}8 \times 10^{-4}$	$7{\cdot}7 \times 10^{-5}$
100 RPM #1	$5{\cdot}4 \times 10^2$ ($7{\cdot}3 \times 10^2$)	20	$1{\cdot}1 \times 10^4$ ($3{\cdot}7 \times 10^3$)	20	$1{\cdot}0 \times 10^{-2}$	$5{\cdot}0 \times 10^{-23}$
100 RPM #2	<5·3 CFU cm^{-2}, $n = 22$ 5·3 CFU cm^{-2}, $n = 2$	24	$2{\cdot}3 \times 10^4$ ($1{\cdot}2 \times 10^4$)	28	N/A	N/A
150 RPM #1	$1{\cdot}2 \times 10^4$ ($6{\cdot}0 \times 10^3$)	31	$1{\cdot}0 \times 10^5$ ($2{\cdot}9 \times 10^4$)	32	$1{\cdot}5 \times 10^{-4}$	$2{\cdot}2 \times 10^{-8}$
150 RPM #2*	$1{\cdot}7 \times 10^4$ ($1{\cdot}5 \times 10^3$)	4	Not detected	12	N/A	N/A
150 RPM #3	$2{\cdot}4 \times 10^4$ ($1{\cdot}5 \times 10^4$)	24	$2{\cdot}0 \times 10^4$ ($1{\cdot}4 \times 10^4$)	24	0·19	0·14
300 RPM #1	$2{\cdot}1 \times 10^3$ ($1{\cdot}2 \times 10^3$)	36	$2{\cdot}3 \times 10^5$ ($1{\cdot}3 \times 10^5$)	36	0·96	0·70
300 RPM #2	$1{\cdot}5 \times 10^2$ ($7{\cdot}5 \times 10^1$)	28	$1{\cdot}3 \times 10^4$ ($8{\cdot}2 \times 10^3$)	32	0·85	0·11

*Experiment terminated early because of clogging of outflow line.

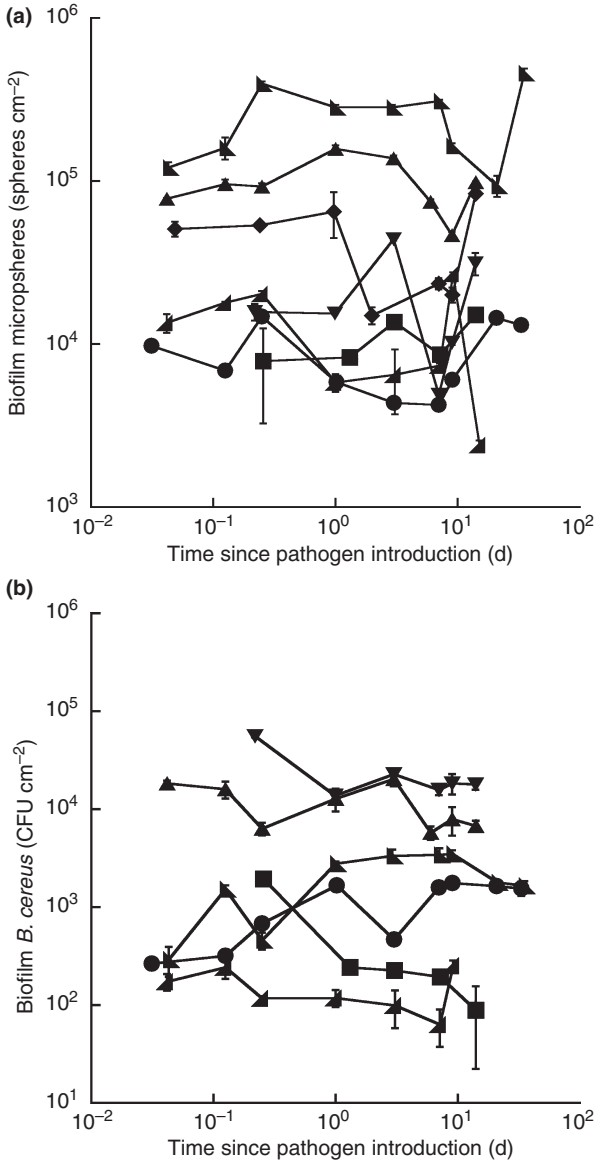

Figure 1 Surface densities of microspheres quantified by fluorescence spectrophotmetry (a) and *Bacillus cereus* quantified by pour plating (b) in biofilms over 14 days prior to chlorine treatment. Plating results are reported as mean ± SD of quadruplicate plates. Microsphere values are the mean ± SD of two measurements of two samples each. (–●–, 50 RPM; –■–, 100 RPM #1; –◆–, 100 RPM #2; –▲–, 150 RPM #1; –▼–, 150 RPM #3; –◣–, 200 RPM#1; –◢–, 300 RPM#2).

ranged from $3{\cdot}0 \times 10^5$ to $5{\cdot}7 \times 10^6$ with an average of $1{\cdot}9 \times 10^6$ and a SD of $1{\cdot}3 \times 10^6$ for the 16 coupons sampled and the 64 plates counted. The number of spores introduced into the reactor was approximately $4{\cdot}1 \times 10^5$. Strong trends in the amount of *B. cereus* measured in the biofilm over the 17 days that the biofilms were monitored were not observed.

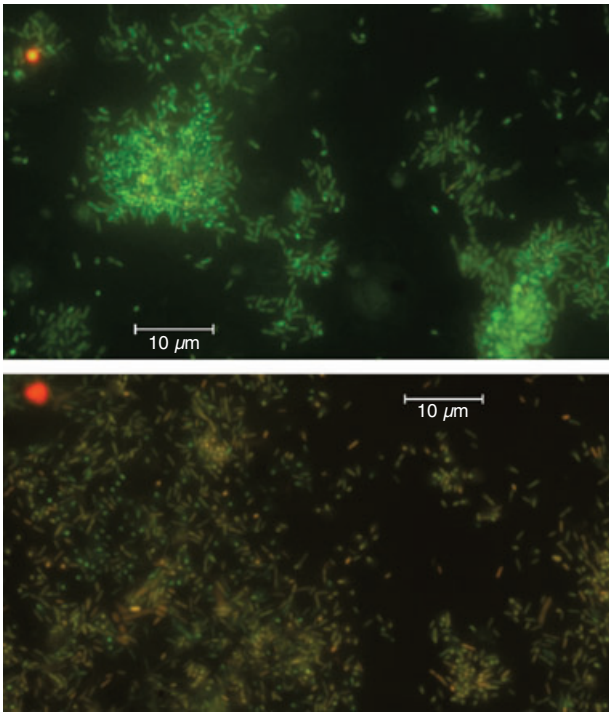

Figure 2 Epifluorescent images of *Pseudomonas fluorescens* (green) biofilms stained with LIVE BacLight Bacterial Gram Stain Kit with microspheres (red) and *Bacillus cereus* (orange) integrated within. Upper image shows clustering of biofilm organisms. Lower image shows area with concentrated *B. cereus*. Images taken with a 100× objective.

A slight correlation between the *P. fluorescens* colony counts just prior to surrogate-pathogen introduction and the percentage of surrogate pathogens measured in the biofilms was observed (Fig. 4a). Greater capture was associated with the higher *P. fluorescens* surface density. There was a positive slope for the regression through the non-outlier points and an R^2 of 0·41.

A linear relationship between shear stress at the surface of the biofilm and the amount of surrogate pathogens captured and retained in the biofilms was not observed (Fig 4b). A regression through the non-outlier points yielded a very slow R^2 value and a slope close to 0. However, a larger percentage of surrogate pathogens appeared to be captured and retained for the mid-shear stress ranges of these experiments.

Statistical analyses indicated that *B. cereus* may have behaved differently from the microspheres. In half of the six experiments that had enough data to do a statistical analysis, there was a <1% probability that the *B. cereus* and microsphere data came from the same underlying population (Table 3, *t*-test result). In other words, in these three experiments, the *B. cereus* was either captured or behaved differently in the biofilm than the microspheres. For all three of these experiments, the percentage

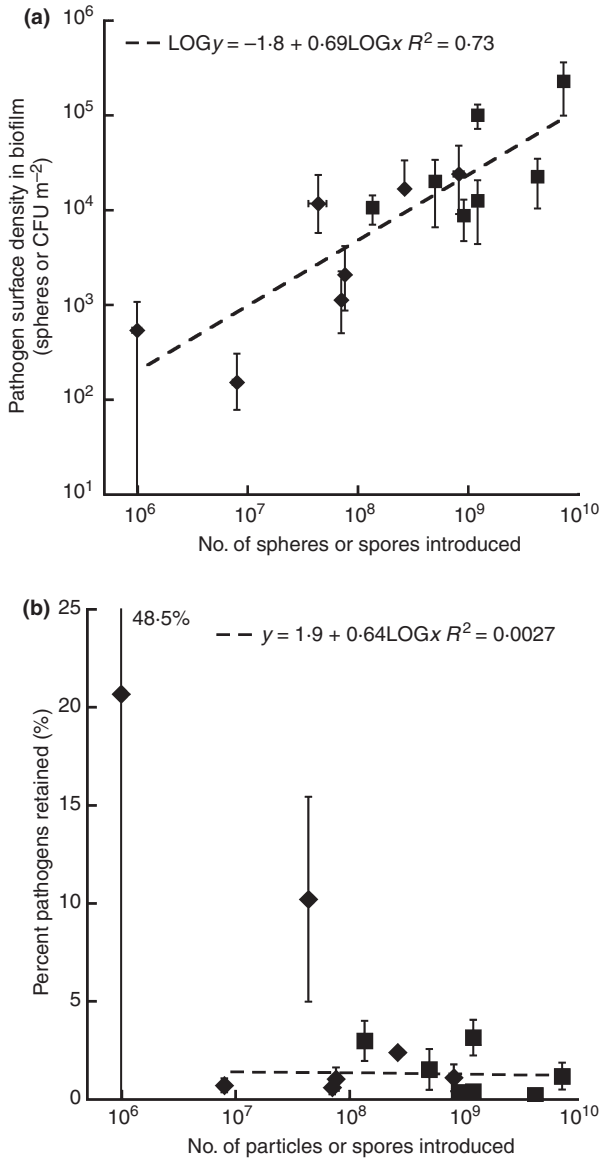

Figure 3 Relationship between the number of spheres or spores introduced into the system and the amount integrated and retained in the biofilms as surface density (a) and percentage of the quantity introduced (b). Regression line and equation in (b) are for data with percentage captured <10. Results are reported as mean ± SD of the number of measurements listed in Table 3. (■, Microspheres; ◆, *B. cereus*).

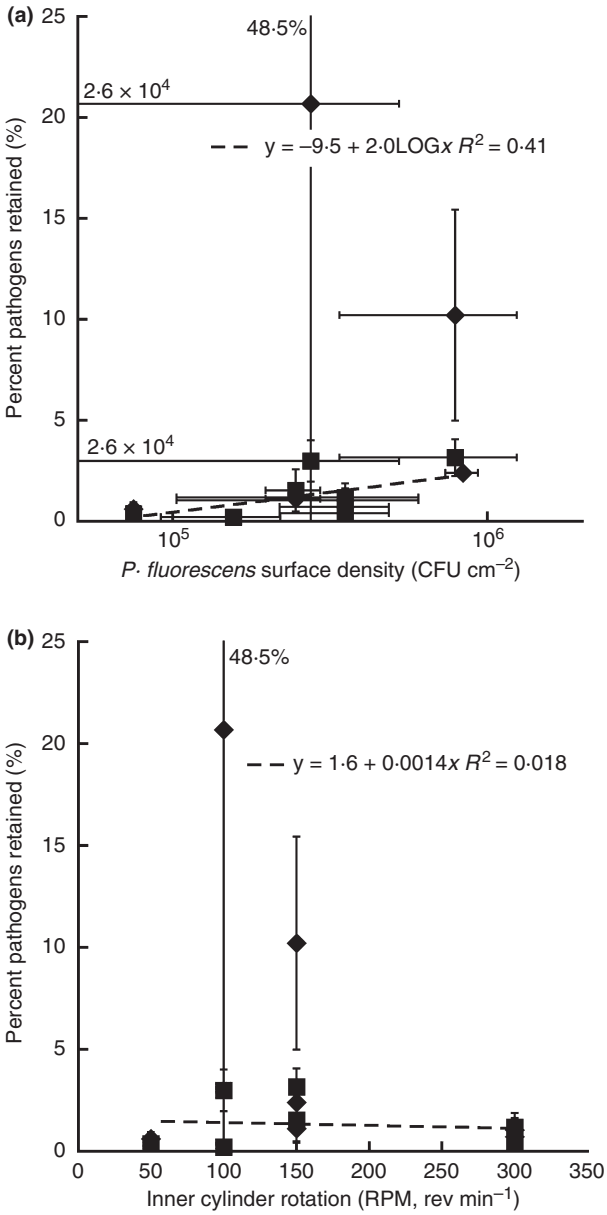

Figure 4 Percentage of surrogate pathogens introduced into the system that is integrated and retained in the biofilms as a function of the initial *Peudomonas fluorescens* surface density (a) and rotation of the inner cylinder of the reactor, an indicator of Reynolds number (Table 2) (b). Regression line and equations are for data with percentage retained <10. Results are reported as mean ± SD of the number of measurements listed in Table 3. (■, Microspheres; ◆, *B. cereus*).

of *B. cereus* measured in the biofilms was greater than the percentage of microspheres. For two of these experiments, the percentage of *B. cereus* retained includes the outliers discussed above. The third experiment, 50 RPM, had <1% of both *B. cereus* and microspheres captured. In this experiment, the amount of *B. cereus* measured in the biofilm increased by approximately one order of magnitude through the duration of the experiment. The probability that the variances of the *B. cereus* and microsphere populations came from the same underlying populations followed the same patterns as the *t*-test data (Table 3, *F*-test results).

As mentioned in the Materials and Methods Section, the attempt to maintain free chlorine levels between 2

and 3 mg l^{-1} for the duration of the chlorine treatment portion of the experiments was not entirely successful. For this reason, results from only two experiments are presented, one of which had the most constant free chlorine concentrations and the other which had the most data after the termination of the chlorine treatment. The other three experiments are discussed where applicable. Because of the small number of experiments, interpretations should be considered preliminary.

For the experiments with a sufficient concentration of free chlorine (>1 mg l^{-1}), surface density of the microspheres and *B. cereus* in the biofilms decreased during the chlorine treatment portion of the experiment (e.g. Fig. 5a,b). The *P. fluorescens* colony counts also decreased. Concurrently, the concentration of microspheres and *B. cereus* in the reactor water increased immediately after the start of the chlorine treatment, presumably due to the release of the surrogate pathogens from the biofilms (Fig. 5c). Concentrations of the surrogate pathogens then decreased in the reactor water. After termination of the chlorine treatment, surface density of the surrogate pathogens in the biofilm appeared to level off or increase slightly (Fig. 5b). The counts of *P. fluorescens* increased significantly to counts higher than that before the surrogate-pathogen introduction.

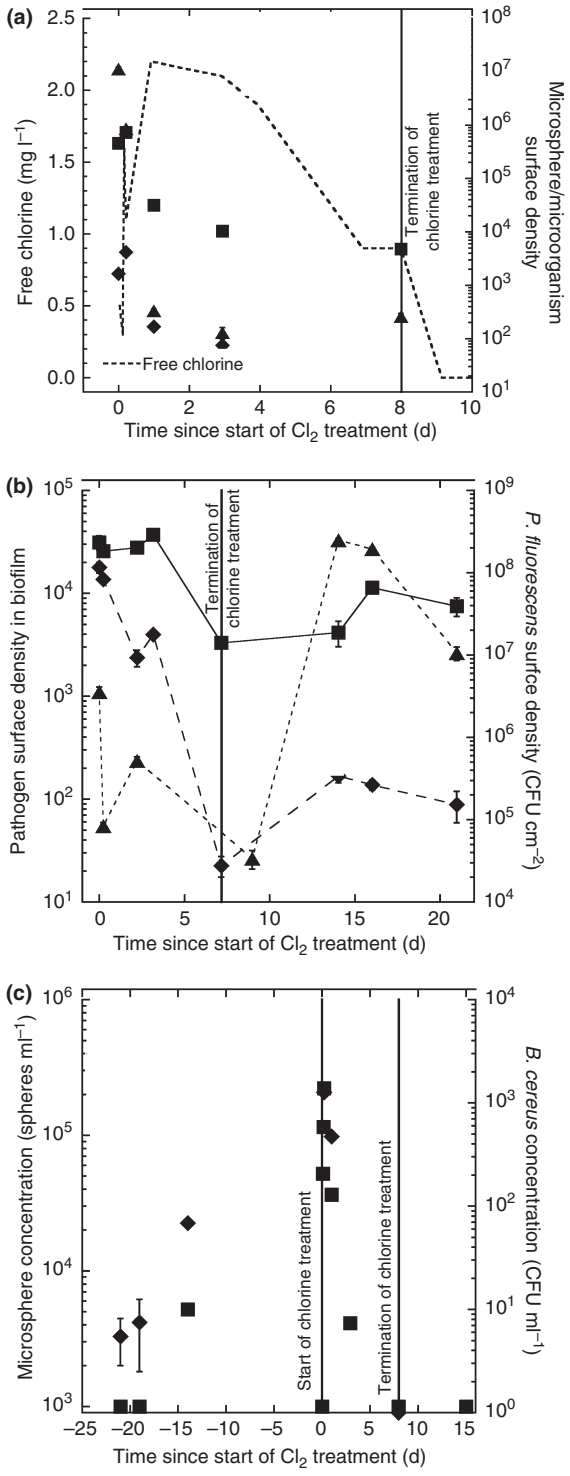

Figure 5 Impact of chlorine treatment on *Pseudomonas fluorescens* and surrogate pathogen surface density in biofilms for 300 RPM #1 (a) and 150 RPM #3 (b) experiment. Impact of chlorine treatment on surrogate pathogen concentration in reactor water for 300 RPM #1 experiment (c). Note that concentrations or surface densities below the microsphere detection limit are plotted as 1×10^3 CFU ml^{-1} or 10 spheres cm^{-2} respectively. No detection for *Bacillus cereus* is plotted as 1 CFU ml^{-1}. Plating results are reported as mean ± SD of quadruplicate plates. Microsphere values are the mean ± SD of two measurements of two samples each. [■, Microsphere (cm^{-2}); ◆, *B. Cereus* (CM^{-2}); ▲, P. fluorescense (CFU cm^{-2})].

Discussion

Results demonstrated the integration of surrogate pathogens into existing *P. fluorescens* biofilms grown in an annular reactor, with approximately 3% or less of the surrogate pathogen introduced to the system being retained. It is important to note that the absolute value of 3% should not be extrapolated to other systems. In the annular reactor, the PVC coupons accounted for approximately 25% of the available surface area on which biofilms can grow and biofilm growth on the glass of the outer cylinder or the space between the coupons was not monitored. Results also give evidence that the amount of surrogate pathogen captured may be a function of the biofilm cell density just prior to pathogen introduction. There is evidence that *B. cereus* behaves differently from the microspheres and that shear stress may impact the amount of pathogens captured and retained by the biofilms. Finally, results of one experiment demonstrated

that both biofilms and surrogate pathogens within the biofilms can recover after a chlorine treatment.

Impact of biofilm cell density

Results shown in Fig. 4a demonstrated a potential positive correlation between *P. fluorescens* surface density and the amount of surrogate pathogens detected in the biofilm. As the experiments were designed to grow repeatable biofilms, the density of bacterial cells in biofilms only spans approximately one order of magnitude and whether the measured correlation can be extrapolated to a broader range of colony counts is uncertain. These results are in contrast to the study of Långmark *et al.* (2005) that found that the accumulation of model pathogens was independent of biofilm cell density. The authors reported $1{\cdot}4 \times 10^4$ to $2{\cdot}2 \times 10^5$ cells cm^{-2} in their biofilms. Thus, with the exception of two experiments, biofilms in the current study had higher cell densities.

While the cell numbers in the reactor biofilms were in the same range of those found in drinking-water biofilms, it is recommended that future experiments are run under higher nutrient conditions to determine whether the observed increasing trend can be extrapolated. Likewise, experiments could be run under lower nutrient conditions to understand better the differences between the results of the present study and that of Långmark *et al.* (2005).

Comparison of *Bacillus cereus* to abiotic microspheres

An understanding is needed for why larger amounts of *B. cereus* were observed in the biofilms for two specific experiments (100 and 150 RPM #1). As the amount of *B. cereus* in the biofilm did not increase significantly over the duration of the experiment (Fig. 1), it is assumed for these two experiments, that there is insignificant net *B. cereus* accumulation in the biofilm. However, it is possible that growth and detachment occured at steady state with no net increase in *B. cereus*. It is possible that the *B. cereus* spores germinated in the reactor water and the planktonic cells were either captured differently from the spores or there were more cells to capture. Although heat shocking was not used to confirm germination, there are five lines of evidence that the *B. cereus* spores germinated: (i) the observation that biofilms could be grown when only *B. cereus* spores were introduced into the reactor, (ii) the fact that large clusters of *B. cereus* were stained and visualized in the biofilms (Fig. 2), which did not occur when spores were stained alone (data not shown), (iii) the observation that the stained *B. cereus* in the biofilms was more rod-shaped (Fig. 2) than the spherical-shaped spores observed with the phase-contrast microscopy, (iv) the results of the statistical analyses showing the *B. cereus* behaved differently from the microspheres and (v) the observation that, in contrast to the microspheres, *B. cereus* could not be flushed from the reactor. It is assumed that germination mostly happened in the reactor water, as significant increase in *B. cereus* colony counts in the biofilms over the time of the experiment was not observed. The exception is the 50 RPM experiment, where the amount of *B. cereus* measured in the biofilm increased by approximately one order of magnitude through the duration of the experiment. This observation suggests that either *B. cereus* multiplied in the biofilms or more *B. cereus* was captured. Why the *B. cereus* surface density in the biofilms was so much higher for the two outlier experiments remains unclear. In comparing conditions of these two experiments *vs* the others, there were no obvious trends.

Impact of shear stress

If the explanation for the *t*-statistic result of *B. cereus* behaving differently from the microspheres is that the *B. cereus* spores are germinating and multiplying, then this study suggests that the capture of planktonic cells or germination of spores in the biofilm may be more likely with lower shear stress.

Studies have been conducted showing that the shear environment can control the amount of bacteria in a biofilm (Azevedo *et al.* 2006), biofilm structure (Dunsmore *et al.* 2002; Purevdorj *et al.* 2002) and cell attachment (Duddridge *et al.* 1982) and biofilm detachment (Duddridge *et al.* 1982; Chang *et al.* 1991; Sharma *et al.* 2005). With the exception of the findings of Peyton (1996) and Soini *et al.* (2002), it is generally thought that biofilms become thinner and denser with increasing shear force (Trulear and Characklis 1982; Chang *et al.* 1991; Liu and Tay 2001a,b). In addition, the adhesive strength of a biofilm increases with increasing shear (Chen *et al.* 1998) and also increases towards the substratum to which the biofilm is attached (Ohashi and Harada 1994). In addition, shear rate has been found to impact the diversity on freshwater biofilms and autoaggregation and coaggregation correlated with shear rate (Rickard *et al.* 2004).

In this study, more surrogate pathogens were measured in the biofilm at the mid shear (100 and 150 RPM) ranges; however, not all the data were consistent. Fig. 1 and Table 3 show that the experiments with the greatest amount of *B. cereus* detected in the biofilms were both run at 150 RPM. The 150 and 100 RPM #1 experiments had the greatest percentage of captured microspheres. This correlation is consistent with the observations of Rickard *et al.* (2004) who found the largest proportion of

coaggregating bacteria at the mid-range of the shear rates of their experiments. However, their mid-range shear was closer to our low shear environment with shear stress of 0·15 N/m^2 or equivalent to 55 RPM. As the highest amounts of surrogate pathogens appear to be in the mid-range of the shear environments, it is not surprising that a regression through the non-outlier points of RPM *vs* amount of surrogate pathogens in the biofilm yielded a very low R^2 value and a slope close to 0 (Fig. 4b).

The change in shear stress immediately prior to adding the surrogate pathogens may have had some impact on the existing biofilms. Ramasamy and Zhang (2005) postulate that when the shear stress of an annular reactor increases, sloughing may occur and then the polysaccharides in the EPS increases to re-establish the biofilms. At a later time, when the biofilm has recovered, the secretion of EPS diminished to its original level and shear stress no longer has an impact on the biofilm structure. The shear environments in our experiments bound those that Ramasamy and Zhang (2005) used – 100 to 200 RPM.

It is hypothesized that for the conditions of this model system at shear stresses in the range of the 100 and 150 RPM experiments, there was some sloughing that led to an increase in EPS production that in turn led to greater surrogate pathogen capture. For the 50 RPM and the March 100 RPM experiments, the change in shear environment was not great enough to change the biofilm structure and capture was less. Finally, at the shear stress of the 300 RPM experiments, it was possible for the sloughing of the biofilm to be so large that the capture of surrogate pathogen was, for the most part, less than the 100 and 150 RPM experiments. As noted above, understanding capture of *B. cereus* is more complicated as the spores may be germinating in the reactor water or the biofilm. More studies examining the structure of the biofilms soon after the surrogate-pathogen introduction are needed to confirm this hypothesis.

Impact of chlorination

The results of preliminary tests showed that even in a simple system, the biofilms and surrogate pathogens in biofilms could persist in the presence of chlorine. The chlorine was effective in decreasing the amount of surrogate pathogens in the biofilms. However, chlorination did not always completely eradicate the surrogate pathogens from the biofilms or the biofilms themselves. In one case, *P. fluorescens* was able to recover to colony counts higher than the original counts after termination of the chlorination treatment, consistent with what Codony *et al.* (2005) observed. At the same time, the surrogate pathogen surface density in the biofilms remained relatively constant or increased slightly, as in the case of *B. cereus* after termination of the chlorine treatment (Fig. 5c). That chlorine can impact the biofilm and pathogens within the biofilm without eradicating the micro-organisms is consistent with many studies (LeChevallier *et al.* 1988a,b; Vess *et al.* 1993; Gibbs *et al.* 2004; Långmark *et al.* 2005). The studies of de Beer *et al.* (1994) and LeChevallier *et al.* (1988b) suggest that transport of the chlorine into the biofilm is the limiting factor controlling its efficacy. It is interesting to note that several studies reported that biofilms are more easily inactivated on PVC than on iron materials (LeChevallier *et al.* 1990; Gibbs *et al.* 2004).

The annular reactor system had relatively simple geometry and biology compared to drinking water distribution systems. This work also used single-species biofilms on PVC. Yet, retention of surrogate pathogens in biofilms and recovery of the surrogate pathogens and biofilms after chlorine treatment were observed. A water-distribution system will have pipes of different materials, including concrete and iron, which can corrode and generate a physically and chemically much more complicated environment. Szabo *et al.* (2007) show that spores of *B. atrophaeus* subsp. *globigii* are able to persist in corroded iron coupons in annular reactors with chlorine concentrations as high as 70 mg l^{-1}. In addition, pipe junctions and changes in flow rates can lead to stagnant regions, variable shear-stresses and differences in chlorine concentration as a function of geometry. While caution must be taken in extrapolating our results to other systems, it is the authors' opinion that the results indicate that biofilms may act as a safe harbour for bio-pathogens in drinking water systems, making it difficult to decontaminate the systems.

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