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A new technique for the performance evaluation of clean-in-place disinfection of biofilms

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A concentric cylinder reactor (CCR) is described that enables the steady-state kinetics of microbial biofilms to be evaluated under conditions of constant nutrient flow and variable shear-stress. The reactor has been used to evaluate the influence of fluid dynamic shear on the extent and mode of detachment of bacteria from biofilms. Using a food factory isolate of *Pseudomonas aeruginosa*, a general increase in the overall growth rate and detachment of the biofilms (cfu cm^{-2} min^{-1}) with time was shown for each biofilm, regardless of the prevailing shear. As the shear rate was increased beyond 0.123 ms^{-1}, populations tended toward a pseudo steady-state. Sudden changes in shear force, however, caused dramatic changes in the productivity of steady-state populations. The CCR provides an effective means of testing disinfectant activity, particularly for clean-in-place situations, and allows for an examination of the residual effects of a cleansing programme on a treated surface for three different chemical classes of disinfectant. Utilisation of the CCR would, therefore, provide enhanced ability to determine the efficacy and efficiency of chemical products for use in sanitation protocols. *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 235–241.

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Introduction

The ability of bacteria that are attached to surfaces as biofilms to transfer to fresh surfaces and to their surrounding medium is not only imperative for long-term survival within a manufacturing plant, but is also central to the problems of product contamination. This is of particular importance to the food industry where processing environments have an abundance of exposed surfaces on which microorganisms may attach, grow and develop into biofilms [11,17,20]. Within product pipelines these biofilms are sometimes able to resist complete removal not only during the process flow but also by cleansing chemicals and disinfectants [16]. They are capable of rapid regeneration following such treatments and thereby provide a source of contamination for the next product-line [8]. Failure to eradicate biofilms is part of their notorious recalcitrance toward a panoply of antimicrobial agents and is associated with their ability to survive relatively harsh hygienic cleaning protocols [3,10,11,17]. Changes in food-hygiene legislation and increased public awareness of product quality makes cleanability testing an important facet of food manufacturing.

The hygienic condition of equipment and processing surfaces is controlled by the application of cleansing and disinfection systems that assist in the removal or control of biofilms [18]. Such sanitation processes are designed to reduce surface contamination initially through cleansing with detergent formulations and secondarily to reduce the viability of the residual attached population [12]. The overall aim of sanitation programmes is to prevent microbial growth from occurring during the interproduction period [23].

It is well established that biofilm populations are more resistant to biocides than are organisms growing in suspension [9]. Although previous studies have noted that both the adhesiveness of planktonic bacteria and the strength of attachment of biofilm populations to surfaces can be affected by treatment with disinfectants [8,15], such studies have not examined the effects of fluid shear on the adhesion of attached bacteria. Moreover, although a variety of annular biofilm reactors have been used to observe the formation and properties of biofilms [6], none allow the simultaneous generation of different shear rates on the same inoculating population. Indeed, few studies have examined the ease with which colonising bacteria may be removed or the extent and manner of their detachment from surfaces [21]. It is unlikely that cell attachment will be unaffected by fluid flow across a surface. Therefore, increased understanding of the impact of fluid flow dynamics associated with sanitation processes is required. In this paper a new experimental rig designed to model the effects of fluid flow in pipework is described. The device provides a utility model for testing and improving upon the effectiveness of product cleaning-in-place (CIP) procedures. The approach utilises a rig comprising concentric steel cylinders constructed from food-grade stainless-steel piping, which may be rotated at variable speeds, within a multichambered, continuously fed, culture vessel. This enables the steady-state kinetics of a microbial biofilm formed on the surfaces of each cylinder to be evaluated under conditions of constant nutrient flow and variable shear-stress. The potential use of the device to assess the effectiveness of different biocide formulations over a range of fluid dynamic shear rates is demonstrated.

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Materials and methods

Organisms and culture maintenance

Pseudomonas aeruginosa PaENV, isolated from the internal face of a stainless-steel pipe used to transfer raw milk within a dairy, was used throughout the study. This organism was collected post cleansing of the system. Cultures were identified using Vitek and maintained on nutrient agar (NA, Oxoid) slants, in the dark at 4°C, after overnight incubation at 30°C. Overnight cultures were prepared from the slants by inoculating volumes (50 ml) of dextrose peptone broth (10 g l^{-1} dextrose; 20 g l^{-1} peptone; 5 g l^{-1} NaCl, pH 7.4), contained in Erlenmeyer flasks (250 ml), and incubating the flasks at 30°C for 16 h in an orbital incubator (200 rpm).

Media and chemicals

Dehydrated culture media were obtained from Oxoid (Basingstoke, UK). The biocide formulations were (i) a buffered (pH 10) tertiary alkyl amine formulation in an amphoteric surfactant (TAAS, 1.4% v/v), (ii) a hydrogen peroxide, peracetic acid, acetic acid (pH 1) formulation (HPPA, 1.0% v/v) and (iii) sodium hypochlorite/sodium hydroxide (pH 13, HYPO, 0.84% v/v) obtained from DiverseyLever (Turku, Finland). All other reagents were of the purest available grade and were obtained from BDH Ltd (London, UK) or the Sigma Chemical Co., (Poole, UK).

Experimental rig

Biofilms were produced in the experimental rig shown in Figure 1. The rig consists of four cylindrical sections constructed from food grade stainless steel that may be rotated, at variable speeds, within four concentric chambers. At any given angular velocity the fluid dynamic forces experienced by the stationary and rotating surfaces are proportional to their radius. The 101-mm cylinder is, therefore, subject to a greater shear force than are the smaller (76-, 50-, and 26-mm) cylinders. This allows shear forces in the range 0.024–0.53 ms^{-1} to be generated. The assembled rig, termed the concentric cylinder reactor (CCR), can be sterilised by autoclaving before being mounted into a rigid support and aseptically connected to a medium reservoir. Each chamber of the CCR is independently supplied with fresh medium from its base at controlled flow rates *via* a peristaltic pump and maintained at a constant volume *via* pumped reservoir overflows. Separate sampling ports are also provided for each chamber.

Attachment of microorganisms and development of biofilms

To establish a biofilm population within the CCR, the four chambers were filled with fresh dextrose–peptone broth, equilibrated to 25°C, and each chamber was inoculated by syringe and needle with a stationary phase culture of *P. aeruginosa* PaENV to give $\sim 10^5$ cfu ml^{-1} in each chamber. Stationary-phase cultures

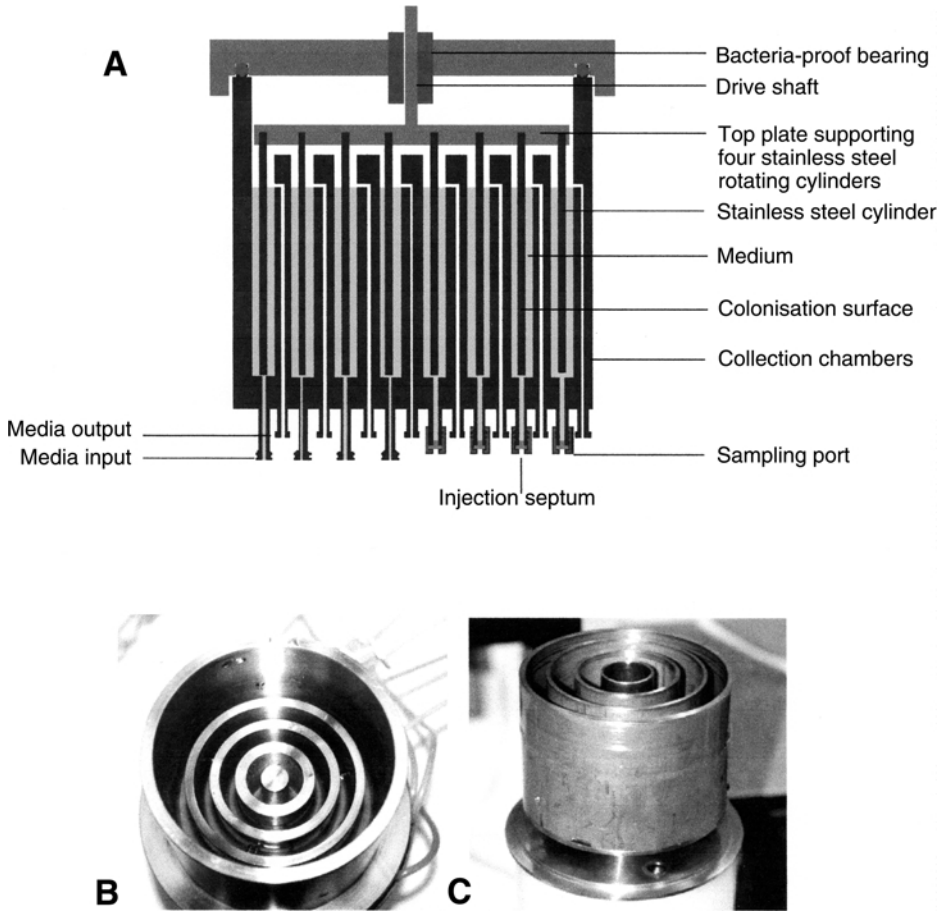

Figure 1 The concentric cylinder reactor (CCR). Section A illustrates a cross-sectional view of the four cylinders interlocked within the collecting chambers. Sections B and C show the collection chambers and rotating cylinders, respectively.

were used because they possess a greater strength of attachment than logarithmic-phase cells [8]. The inoculated rig was then left at 25°C, without medium flow but with the rotators revolving for 16 h. After this time the medium inlet and outlet pumps were switched on and adjusted such that washout of the planktonic phase cells occurred in the chamber at flow rates greatly in excess of the maximum dilution rate (D_{max}) of the organism. This was intended to dramatically reduce the planktonic count. For *P. aeruginosa* PaENV, doubling times of approximately 90 min were obtained in dextrose–peptone broth. Media flow rates corresponding to 4.2 ml min^{-1} for the 101-mm (56 ml) and 76-mm (34 ml) chambers and 1.5 ml min^{-1} into the 50-mm (24 ml) and 26-mm (9 ml) cylinder chambers, respectively, were sufficient to provide a minimum of two volume changes per hour. After 24 h, media input rate was altered to a flow rate slightly in excess of D_{max} (2.1 ml min^{-1} into 101- and 76-mm cylinders and 0.75 ml min^{-1} into the 50- and 26-mm cylinders) to maintain the washout of planktonic organisms during the experimental run. Because any remaining planktonic cells would, theoretically, contribute less than one colony-forming unit (cfu) per plate, under these conditions the colony-forming count corresponds to the detached population. Bioburden levels formed on the chamber surfaces were quantified at the end of each run by dismantling the CCR and removing the biofilm from the rotator and stator surfaces using a sterile, damp alginate swab. Organisms were released from the swab by vigorously shaking it in 10 ml sterile saline (0.9% NaCl, w/v) for 10 min and plated out onto predried nutrient agar plates and incubated for 48 h at 30°C. Detached cells were sampled by inserting a sterile syringe through the inoculation port and withdrawing 1-ml volumes, which were then serially diluted in saline and plated out and incubated as above. Release rates (productivity) of attached cells to the liquid phase were calculated as colony forming units per square centimeter per minute (cfu cm^{-2} min^{-1}).

CIP study

An overnight culture of *P. aeruginosa* PaENV (1% chamber working volume) was inoculated into each compartment of the device and left for 2 h without medium flow to allow bacterial attachment to occur. Biofilms of this age (2 h) were used to mimic those typically generated during a food-manufacturing run [16,17]. Following this attachment period, planktonic organisms were washed out for 24 h at a rate greater than D_{max} before initiating the disinfection process (zero time on graphs). After determining an initial viable count, biocide solutions (or water for the control) were used to replace the growth medium. Peristaltic pump speed was increased to give the fastest possible substitution of biocide for medium. These flow rates corresponded to dilution rates of 7.12, 11.73, 5.16 and 21.61 h^{-1} for the 101-, 76-, 50- and 26-mm cylinder chambers, respectively. Biocide flow was maintained for 90 min and samples were taken at 45 and 90 min. Biocide solutions were replaced with nutrient medium once again the fast flow rate was maintained for a further 90 min before reverting to the original value (2.1 ml min^{-1} into 101- and 76-mm cylinders and 0.75 ml min^{-1} into the 50- and 26-mm cylinders). Samples were taken to a neutraliser solution (10°C) at regular intervals immediately after cessation of treatment up to 24 h. The neutraliser solution, which contained lecithin (0.6% w/v), Tween 80 (6% w/v), sodium thiosulphate (0.8% w/v), L-histidine hydrochloride (0.5% w/v), bovine serum albumen (0.72% w/v), in Sorenson's phosphate buffer (1.25 mM) was sterilised by filtration through a 0.45-μm pore size filter. Preliminary studies demonstrated the effectiveness of the neutraliser in all instances [22]. After 5 min incubation serial dilutions were made and viable counts estimated as before.

Enumeration and statistical analysis of viable microorganisms

Viable counts of cell suspensions were measured by preparing suitable dilution series in sterile saline (0.9% NaCl). Aliquots (0.1 ml) of appropriate dilutions were spread, in triplicate, onto the surfaces of nutrient agar plates and incubated for 48 h at 30°C. Plates containing between 30 and 300 cfus were counted. Results were subjected to analysis of variance (ANOVA) test.

Results and discussion

The aim of these experiments was to evaluate the effects of fluid dynamic forces at solid:liquid interfaces upon the detachment and dispersal of bacterial cells from biofilms formed at surfaces within the manufacturing plant. Whether such detachment is as single cells moderated through erosion of the biofilm or is driven by the cell-division cycle, or whether it corresponds to sporadic sloughing, has profound implications both upon the quality and shelf-life of the product [21] and upon the QA procedures needed to monitor it. Although there have been many studies that have examined the ability of organisms to adhere to different surfaces, few have examined the ease with which the colonising bacteria may be removed or have investigated the extent and manner of their dispersal from the surface. The influence of fluid-shear on detachment and dispersal processes is one aspect that is pertinent to food manufacturing plants and CIP regimens.

In a manufacturing plant shear rates will vary according to pipe geometry (bends, branches, diameter) and fluid flow rates. Normally fluid flow is maintained at less than 1 ms^{-1} during manufacturing cycles but during CIP higher flow rates of around 1.5 ms^{-1} are often achieved. In this respect, rotational speeds of 0–100 rpm and cylinder diameters of 26–101 mm enable the rig to duplicate the likely range of conditions found in manufacturing pipework during both use and CIP.

Influence of shear rate on biofilm productivity

In the present study, conditions were established such that biofilms were formed on both surfaces of both the stator (static cylinder) and rotator in each chamber. Employment of medium flow rates in excess of D_{max} would ensure that any planktonic cells detected in the chambers were derived from the attached population.

Initial experiments investigated the influence of fluid shear on the steady-state biofilm population and upon detachment and dispersal of cells from surfaces (Figure 2). Because biofilms were constant (Table 1), detachment can be expressed as productivity. These data show a general trend of increase in productivity, over time, for each biofilm regardless of shear rate. From start to finish there were no consistent trends in the relationship between productivity and the speed of rotation for shear rates less than 0.123 ms^{-1}. Biofilms grown at higher speeds of rotation ($\geq$0.123 ms^{-1}), however, generally showed a lower level of productivity than did those grown at the slower speeds. As shear rate was increased beyond 0.123 ms^{-1}, a transition toward steady state occurred. At a single time point (40 h) the range of productivity between the reactor chambers

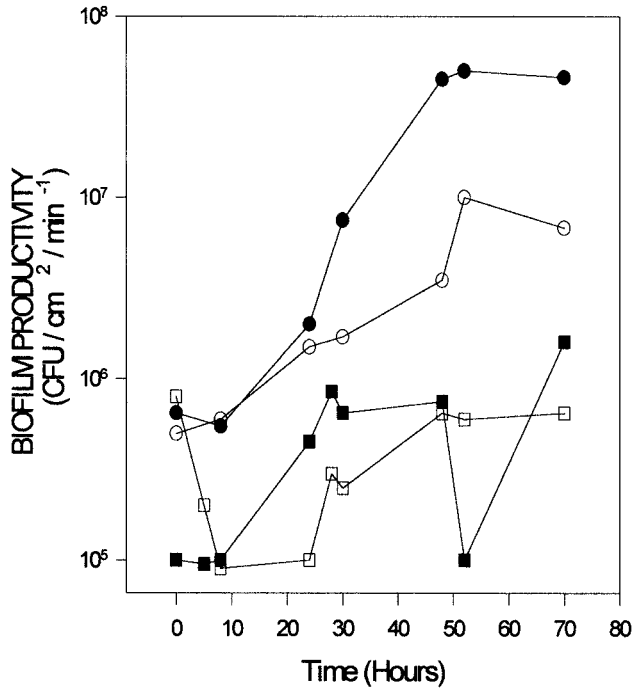

Figure 2 Effect of constant shear force on *P. aeruginosa* PaENV biofilm productivity formed on the stainless-steel cylinders of the CCR at various speeds of rotation: 0.024 ms^{-1} (○), 0.097 ms^{-1} (●), 0.123 ms^{-1} (■) and 0.194 ms^{-1} (□).

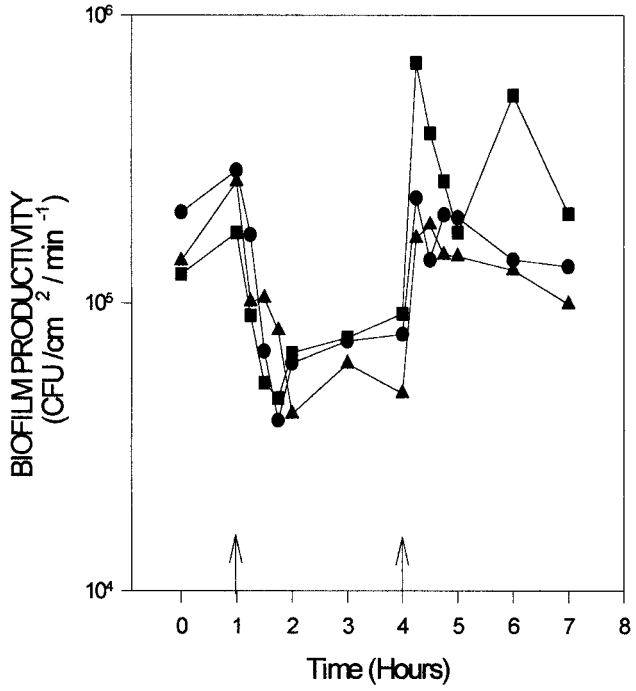

Figure 3 Effect of rapid decrease in shear rate upon the productivity of a steady-state *P. aeruginosa* PaENV biofilm established at different angular velocities. Shear was altered, as indicated, at 1 and 4 h, from a high–low–high setting corresponding to velocities of 0.529–0.097–0.529 ms^{-1} (●), 0.399–0.074–0.399 ms^{-1} (■) and 0.254–0.047–0.254 ms^{-1} (▲).

was almost 1000-fold. Indeed, when the time profile of each individual biofilm chamber was studied, productivity over time was often chaotic, i.e., large increases and decreases were observed in productivity that were not related to external stimuli. If, however, steady-state biofilms formed at a constant shear were subjected to a sudden change in shear rate, a dramatic change in productivity was observed (Figure 3). Here, steady-state biofilms ($\sim 3\times10^5$ cfu cm^{-2} min^{-1}) established after 1 h at high shear rates produced a marked drop in productivity as the shear rate was altered to the lowest setting. At this value a new steady-state productivity level was established ($\sim 5\times10^5$ cfu cm^{-2} min^{-1}, reaching the minimum level after 45 min, after which time productivity steadily increased. After 4 h, shear force was increased back to the original high level, which was followed by a rapid release of cells. Maximum detachment was reached almost immediately, followed by a decrease back toward the original steady-state level. A similar, but mirrored, pattern was observed when steady-state biofilm populations were sequentially altered from low-to-high-to-low shear rates (data not shown). These results could suggest that although population dynamics might be similar at different shear forces, the nature of the biofilm population is not. Alternatively, this may simply reflect changes in sloughing rates. Recently, it has been shown that changes in detachment from a steady-state biofilm due to a rapid change in shear force are independent of the initial shear rate and can cause temporary, elastic deformation of biofilm structures [19]. At steady-state, the rate of growth equals the rate of detachment. An increase or decrease in shear rate modifies the steady-state population by increasing or decreasing the rate of detachment compared to rate of growth of the biofilm. Biofilm stability is

Table 1 Bioburden present on the stator and rotator surfaces of the CCR

Shear rate (ms^{-1})	Bioburden (cells cm^{-2})
0.024	2.78×10^9
0.097	5.25×10^9
0.123	2.85×10^9
0.194	6.93×10^9

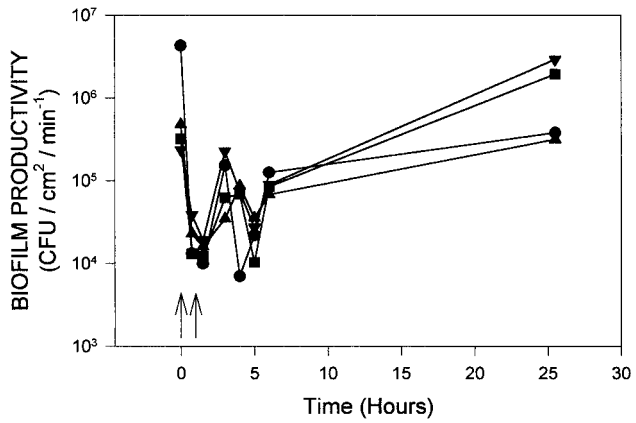

Figure 4 Effect of water on productivity of *P. aeruginosa* PaENV biofilms established for 2 h on the stainless-steel surfaces of the CCR at shear rates of 0.063 ms^{-1} (▼), 0.123 ms^{-1} (▲), 0.194 ms^{-1} (■) and 0.257 ms^{-1} (●). Start and end of disinfectant treatment are indicated by the two arrows.

possibly a function of exopolymer dissolution rate [20] and the prevailing fluid dynamics. Within manufacturing pipework systems, sudden changes in shear forces are inevitable, for example, due to the mechanism of action of filling heads, the incorporation of bends within the pipework system and production down-time requiring lines to be stopped. The evidence provided from this study indicates that any sudden changes to shear force during manufacturing should be avoided in order to limit biofilm disruption. On a more applied front, however, such sudden changes in shear rate might prove to be advantageous in CIP procedures whereby pulsing of fluid flow for an initial rinsing fluid may disrupt and displace the resident biofilm population to a greater extent than continuous flow. Indeed, although large clumps of biofilm-derived cells were observed passing down the outlet tubing, it was not possible to record these without dismantling and sacrificing the CCR. Nevertheless, given the overwhelming view that biofilm bacteria more readily resist antimicrobial treatments than do cell suspensions [4,10], disruption of biofilm architecture in this manner may, as a consequence, lead to greater penetration and hence action of the applied disinfectant solution [14].

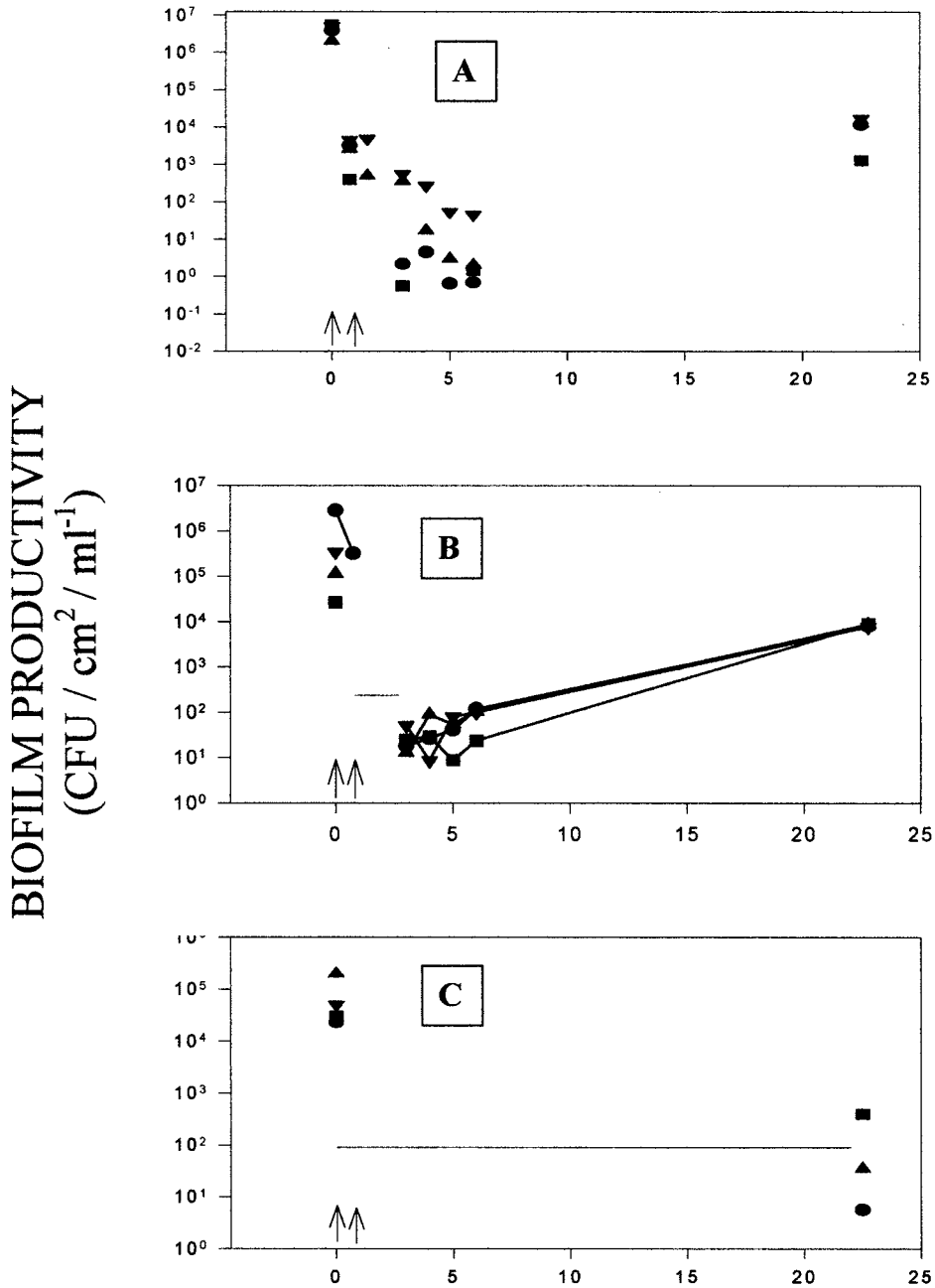

Figure 5 Effect of (A) HYPO (0.84%), (B) TAAS (1.4%) and (C) HPPA (1.0%) on productivity of *P. aeruginosa* PaENV biofilms established for 2 h on the stainless-steel surfaces of the CCR at shear rates of 0.063 ms^{-1} (▼), 0.123 ms^{-1} (▲), 0.194 ms^{-1} (■) and 0.257 ms^{-1} (●). Start and end of disinfectant treatment are indicated by the two arrows. The horizontal lines in graphs B and C represent a period (135 min and 24 h, respectively) when no viable cells were detected.

CIP study

Changes in biocide susceptibility occur almost immediately upon attachment of cells to surfaces that could affect disinfection efficacy [5]. In food-processing environments insufficient time elapses between the end of a manufacturing run and the start of a CIP programme to allow substantial biofilms to develop. Thus, the short period of CIP used in this study is representative of the time period between the end of a manufacturing run and post production cleansing period in the food industries [16,17]. Results from the challenge of steady-state biofilms with commercial disinfectant formulations are presented in Figures 4–5 as biofilm productivity. Arrows on the graph denote the onset and end of challenge in each system. The control (Figure 4) shows productivity to be slightly reduced when water replaces nutrient medium and when the flow rate is increased. Productivity within the control system rapidly increases after nutrient is replaced (at 90 min), and within 3 h it has almost returned to the initial rate. This clearly indicates that organisms detaching to the liquid phase are doing so as a result of growth and division rather than physical instability due to shear. In this respect it is noteworthy that a number of studies have linked detachment of cells to a phase of the division cycle [1,2,7].

Challenge with HYPO (0.84%) show productivity profiles similar to that of the control (Figure 5A). Productivity decreased to a lower level than the control ($\sim 10^2$ to 10^0 cells cm^{-2} min^{-1}) depending on shear rate, indicating either a killing of the biofilm or sublethal injury resulting in a lag in growth following removal of the hypochlorite. This effect was not immediate, however, as the minimum level was reached 3 h after termination of biocide treatment. Within 24 h the residual biofilm had, however, regenerated to release approximately 10^4 cells cm^{-2} min^{-1}.

Significantly greater biocidal action was observed when biofilms were challenged with TAAS (1.4%). It can be seen from Figure 5B that productivity immediately reduced to zero (below detectable levels) on challenge with the biocide. Within 90 min of post biocide treatment, however, the biofilm had revived and productivity increased such that after 24 h a level of $\sim 10^4$ cells cm^{-2} min^{-1} was reached. This strongly suggests that the action of TAAS biocide on the biofilm was only temporary.

The most effective of the formulations was HPPA (1%). In this instance productivity reduced rapidly to zero (below detectable levels) following biocide challenge (Figure 5C). Moreover, although the exact length of time for biofilm regeneration is not known, recovery after 24 h was much less ($\sim 10^1$ to 10^3 cells cm^{-2} min^{-1}) than for the other two biocides.

In CIP procedures it is recovery of surviving cells post treatment that is the important measure. Hence, in this study we examined the ability of three different disinfectant formulations to delay or prevent the re-emergence of microorganisms following CIP treatment. Because a high flow rate was employed, recovery can only relate either to an imposed dormancy on surviving cells or immediate re-growth of a small surviving fraction. Moreover, following removal of the disinfectant there is no longer a planktonic effect. Equally, strength of attachment would only have been affected during the disinfection phase and not post treatment.

The strength with which organisms attach to surfaces directly affects the ease with which they can be removed by disinfection and other hygienic treatments. Moreover, some chemical agents can cause the attachment strength to surfaces to increase, thereby making disinfection and cleansing less effective [8]. Conventional disinfection testing based not only on killing action but also on the ability to remove cells from a surface often fails to discriminate between different formulations [13]. The CCR device described in this study can be used to predict the efficiency of formulations based not only on their killing action but also on their ability to remove cells from a surface as a function of shear rate. In this manner, the device can be used to evaluate not only killing effectiveness, but also the residual effects of a formulation on a cleansing programme on a treated surface. Generally, an agent which leaves small residual populations of viable cells attached to a surface, but which exerts a high level of killing, will be less effective than one which exerts a lesser killing effect but for which few cells, surviving or not, remain attached. In addition, the device can be used to determine the time period post cleaning for which the surface remains clean. This is critical in the timing of cleaning procedures after the end of one production run and the start of the next. Because sanitation is critical in any industry that manufactures products for human or animal consumption or use, advanced modelling of the effect of chemical agents on attached micro-organisms is required to enable selection of the optimum disinfectant regime.

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