Journal of Industrial Microbiology & Biotechnology (2002) 29, 331–338

www.nature.com/jim

Binary and mixed population biofilms: Time-lapse image analysis and disinfection with biocides

KT Elvers[a], K Leeming[b] and HM Lappin-Scott

Department of Biological Sciences, University of Exeter, Hatherly Laboratories, Prince of Wales Road, Exeter EX4 4PS, UK

Simultaneous binary population biofilm formation by a bacterium and filamentous fungus was demonstrated by time-lapse image analysis in a flow cell system. The accumulation of attached bacterial cells followed an S-shaped graph similar to batch culture bacterial growth, with continual attachment, detachment, rotation, and movement of bacteria over the surface. An extensive hyphal network formed on the surface of the flow cell, protruding into the bulk flow, which subsequently detached. Multiple species mixed fungal–bacterial model biofilms were tested for isothiazolone biocide susceptibility. Biofilms were less susceptible to biocide treatment than planktonic cells of the same organisms. Mixed species biofilms, particularly for the bacterial species, offered greater protection against the action of the biocide compared to single species biofilms. Microbial loss as a result of biocide activity was shown by reduced cell surface coverage in electron micrographs.
Journal of Industrial Microbiology & Biotechnology (2002) **29,** 331–338 doi:10.1038/sj.jim.7000318

Keywords: multiple species biofilms; isothiazolone biocide; Robbins Device; flow cells; fungal species

Introduction

In many industrial processes such as water distribution systems [20], cooling water towers [2], oil recovery [30], and heat exchanger surfaces [24], microorganisms attach to surfaces, forming biofilms with detrimental consequences that incur significant financial losses. Adverse impacts of biofouling include impedance of heat transfer processes, decreased water quality, mechanical blockages, corrosion, and product spoilage. Biocides, in combination with mechanical cleaning, are often employed to overcome biofilm formation in industries. It has become widely accepted that biofilm-grown cells are more resistant to killing by biocides when compared with the same cells grown in planktonic phase [3,8]. Excessive use of biocides at high concentrations is likely to lead to environmental, ecological, and toxicological problems if water contaminated with biocide is discharged directly into natural water or effluent treatment plants.

Biofilm resistance to biocides may result from the exopolysaccharide that embeds cells in the biofilm community, imposing a physical barrier that excludes the access of biocides to the underlying cells. Physiological heterogeneity develops within biofilms from nutrient and oxygen gradients and accumulation of waste products. Thus, cells within the biofilm encounter different microenvironments, responding with alterations in growth rate or by changes in gene expression that infers a biofilm-specific phenotype and increased resistance to biocides [3,4,6,22,32].

Chlorine is routinely used to control biofouling in many industrial water systems, often with little effect [17]. There is strong evidence that reduced efficacy results from limited penetration of chlorine into the biofilm matrix and reduced activity at high concentrations of organic matter [11,33,37]. Other biocides used to control biofilms include monochlorine, ozone, chlorine dioxide, and isothiazolones. The latter are effective against a broad range of bacteria and fungi, and are widely used in cooling water towers, paper systems, cosmetics, and textiles industries [7,15].

In most industrial environments, biofilms are complex communities consisting of many different microbial species functioning in a consortium. Complex interspecies and intraspecies interactions occur in these diverse microbial communities, such as coaggregation shown among aquatic biofilm bacteria [5] and interactions that influence initial stages of biofilm formation and subsequent development. Multiple species biofilms may be thicker and more stable than single species biofilms. That further influences their susceptibility to biocides.

This study reports on surface colonization behaviour *in situ*, using microscopic monitoring and image analysis, of a novel binary biofilm system consisting of a bacterium and filamentous fungus isolated from fouling of industrial photoprocessors. Secondly, these and additional bacteria and fungi from the same industrial system are used to investigate the efficacy of isothiazolone biocide treatment by destructive sampling of biofilms formed in a modified Robbins Device.

Materials and methods

Microorganisms and culture conditions

The bacteria and fungi used represented the biofilm community present in photographic processing tanks as previously reported [13]. The bacterial species were *Alcaligenes denitrificans*, *Pseudomonas alcaligenes*, *Stenotrophomonas maltophilia*, *Flavobacterium indologenes* and fungal species *Fusarium solani*, *Fu. oxysporum*, and *Rhodotorula glutinis*. *S. maltophilia* and *Fu. oxysporum* were selected as representative species from the model to study the development of dual species biofilms. All species were

Correspondence: Dr KT Elvers, School of Biomedical and Life Sciences, University of Surrey, Guildford, Surrey GU2 7XH, UK
[a]Present address: School of Biomedical and Life Sciences, University of Surrey, Guildford, Surrey GU2 7XH, UK.
[b]Present address: Kodak European Research and Development, Kodak, Harrow, Middlesex HA1 4TY, UK.
Received 11 March 2002; accepted 8 August 2002

routinely cultured in R2A medium [28] at 30°C. Nutrient agar (NA; Oxoid, Basingstoke, UK) and 2% malt extract agar (MEA; Oxoid) were used for viable counts of bacteria and fungi, respectively, at 30°C. Mixed cultures were plated on NA, NA with streptomycin (2.5 μg ml^{-1}), MEA, and MEA with tetracycline (50 μg ml^{-1}) to allow enumeration of each individual species [13].

In situ analysis of simultaneous binary species biofilms in a flow cell system

The flow cell system was adapted from that designed by Stoodley *et al* [34]. It consisted of square glass tubing (0.3×0.3×9 cm; Camlab, Cambridge, UK) incorporated into a recirculating loop to a reservoir containing 2 l of half-strength R2A medium. The reservoir was constantly agitated using a magnetic stir bar. The flow cell was mounted onto a perspex holder and clamped to the microscope stage. Colonization of the flow cells was initiated by inoculating the reservoir with 10 ml of *S. maltophilia* and 10 ml of *Fu. oxysporum* from 18-h cultures. The peristaltic pump was calibrated to a laminar flow rate of 50 ml h^{-1}. After 1 h, a single area was selected by focusing the microscope to the top surface of the flow cell, and biofilm formation and behaviour were monitored *in situ* at regular intervals for 24 h at 22°C. In order to observe attached fungi and bacteria in the same field of view, the square glass tubing of the flow cell was inverted by rotation through 180° on the microscope stage after 4 h of attachment.

Microscopy and image analysis

Colonization of the binary culture was observed using transmitted light on an Olympus CH-2 (Olympus Optical, Southall, UK) microscope, with a 40× objective and neutral density filter (LBD-2). Images were captured using a Cohu CCD camera (Cohu, San Diego, CA, USA) and a Scion framestore board. Images were processed on a Macintosh 7200/90 computer (Apple Computer, Cupertino, CA, USA) using NIH-Image 1.59 and printed directly to a laser printer (Hewlett Packard Laserjet 6MP, Bracknell, UK). A video (Panasonic NV-FS88 HQ, London, UK) and tape were used to make real-time recordings in the same field of view for several periods during the course of the experiment. Cell numbers and hyphal measurements were made from the printed images.

Biocide

The isothiazolone compound Kathon™ LX (Rohm and Haas, Philadelphia, PA, USA) was used to study its efficacy against single and mixed species biofilms. Kathon™ has two active molecules comprising 14% of the solution: 5-chloro-2-methyl-4-isothiazolin-3-one (Cl-MIT) and 2-methyl-4-isothiazolin-3-one (MIT). Prepared stock solutions were added to the test media to give final concentrations of the active molecules.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MICs of Kathon™ for the bacterial and fungal species were determined by the standard macrodilution method [16] in R2A medium. The final concentrations of biocide tested for Kathon™ ranged from 20 to 0.016 μg ml^{-1}. Cultured bacterial and fungal suspensions in R2A medium were adjusted to 0.5 McFarland standard and enumerated by viable counts on appropriate agar (10^5 cfu ml^{-1} for bacteria and 10^3 cfu ml^{-1} for fungi). Media containing different concentrations of biocide were inoculated with 0.1 ml of the cell suspensions and incubated at 30°C for 24 h. The MIC was recorded as the lowest concentration of biocide that completely inhibited visible growth. The MBCs were determined by plating 0.1 ml from the tubes with no visible growth onto appropriate agar.

Effect of biocide on single and multiple mixed species biofilms

Mono species biofilms for each of the seven microorganisms were formed on PVC (Simona extruded rigid smooth dark grey) surfaces using a flow-through MRD batch culture system allowing 24 h colonization of two devices in parallel at 22°C. Reservoirs containing 2 l of half-strength R2A medium were inoculated with 30 ml of bacterial or fungal culture, which was continuously pumped over the PVC surfaces at a rate of 50 ml h^{-1} into a waste flask. Biofilms formed after 24 h were either continuously treated with Kathon™ (5 μg ml^{-1}) or left untreated. This was achieved by changing the reservoirs to media containing Kathon™ and media only, respectively. Surfaces were sampled at regular intervals over 24 h for viable counts and scanning electron microscopy (SEM). Surfaces for SEM were prepared following the procedure described by Elvers *et al* [13].

The effect of Kathon™ on mixed culture biofilms of all seven species was carried out as described above, except the reservoirs were inoculated with 5 ml of culture from each of the four bacteria and three fungi. Twenty-four hour biofilms were formed at room temperature and continuously treated with Kathon™. Biofilms were sampled over 24 h for viable counts and SEM.

Statistical analysis

The data were analysed by analysis of variance (ANOVA). The ANOVAs were conducted in MINITAB® using a restricted model with Treatment and Time (covariate) as fixed effects and Replicate as a random effect nested within Treatment. A P value of <0.05 was considered to be statistically significant. The data are shown as means with standard errors of the mean from duplicate or triplicate experiments.

Results

Binary culture biofilm formation in situ

Colonization of the glass flow cell under laminar conditions by a binary culture of *S. maltophilia* and *Fu. oxysporum* is shown by a representative series of captured images (Figure 1). Initial attachment was observed only for the bacterium *S. maltophilia* within the chosen field of view. Focusing the microscope objective through the lumen to the bottom flow cell surface determined that the fungus *Fu. oxysporum* attached in higher numbers to the bottom rather than the top surface (data not shown). Thus, the flow cell was inverted after 4 h to obtain clearer images of attachment of both species assuming that the bacteria attached equally to both surfaces. For the first 4 h, there was a slow attachment of bacterial cells followed by a rapid rise in numbers to 593 attached cells in 12 h per sample area of 16,000 μm^2 (Figure 2a). The rate of attaching cells appeared to slow hereafter, with a resulting S-shaped graph similar to that of a typical batch culture growth curve (Figure 2a). The data show an overall net increase in attached cells over 24 h.

Hyphal growth from the attached conidia as indicated (Figure 1) was measured and represented graphically until the hyphae

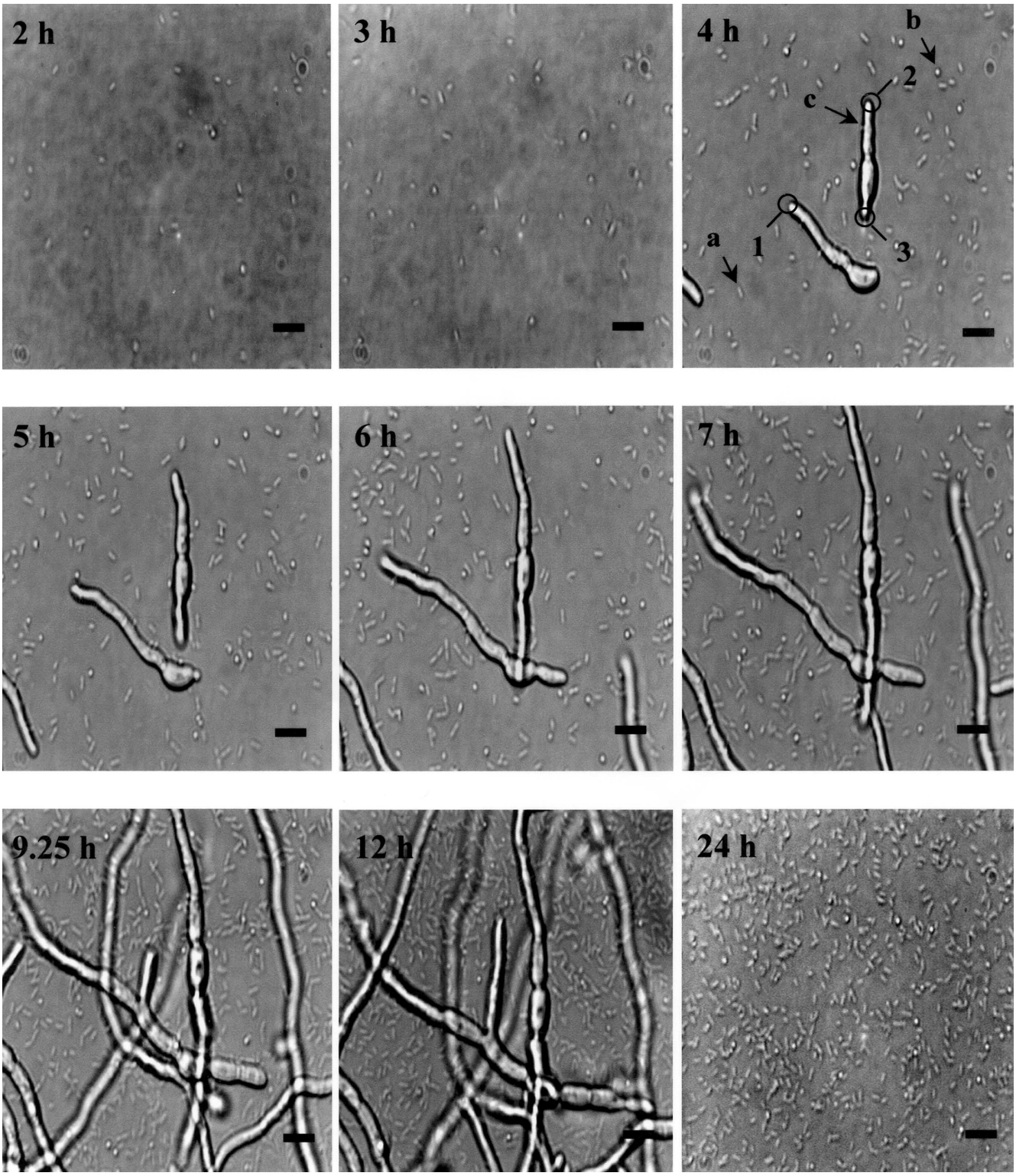

Figure 1 Simultaneous attachment of *S. maltophilia* and *Fu. oxysporum* to the glass surface of a flow cell at 50 ml h^{-1} over 24 h. These images show increased attachment of bacterial cells and germinating fungal conidia (indicated by arrows with letters and numbers). (a) Bacterial cells attached side on "rods"; (b) bacterial cells end on "circles"; and (c) attached fungal conidia, where 1, 2, and 3 indicate hyphal branches measured for growth. Direction of flow was right to left. Observed area (grey box) = 16,000 μm^2. All bars = 10 μm.

extended beyond the field of view (Figure 2b). The hyphal growth rates (between 13.9, 12.5, and 17. 8 $\mu m\ h^{-1}$) differed slightly from the ends of germinating conidia and from conidia to conidia.

The real-time video showed different types of surface colonization behaviour by the motile bacterium, *S. maltophilia*. Cells were seen attaching, detaching, and moving across the surface

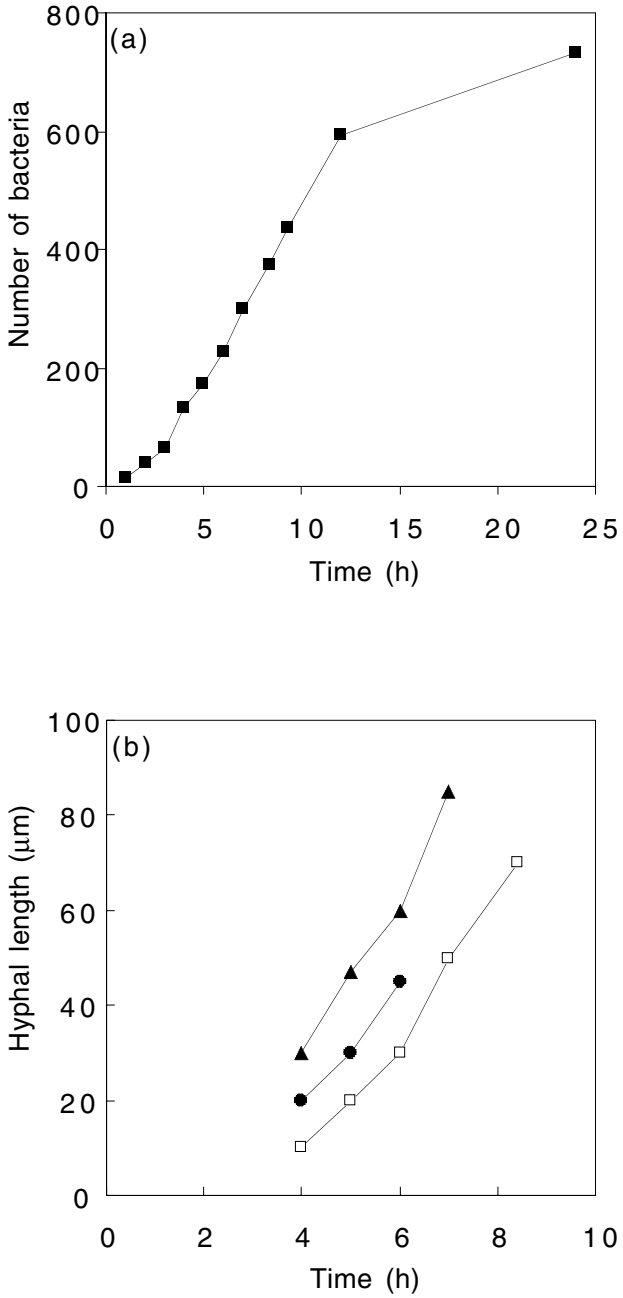

Figure 2 (a) Accumulation of attached cells within the flow cell over 24 h and (b) hyphal growth from three branches shown in Figure 1, where 1, 2, and 3 are represented by (▲), (●), and (□), respectively.

between other attached cells both upstream and downstream of the laminar flow conditions. Some attached cells rotated about their point of attachment in either a clockwise and anticlockwise direction. Some cells attaching on their ends were observed as circular, whilst others attaching on their sides were observed as rods (Figure 1). Detaching cells moved beyond the observed field or reattached nearby. It was not until the later stages of colonization that the cells remained in a more fixed position and some became trapped between the hyphal branches (Figure 1). As colonization progressed, *Fu. oxysporum* grew extensively over the surface with some branches becoming out of focus as they projected into the bulk flow. After 24 h, the fungus had detached from the top surface of the lumen. This detachment was concluded from the absence of the fungus in the chosen field of view. By moving and inverting the flow cell at the end of the experiment, the fungus was observed attached to other areas of both top and bottom surfaces (data not shown).

MIC and MBC

The MIC and MBC of Kathon™ for the model community species after 24 h are shown in Table 1. The MIC of Kathon™ for the species ranged from <0.016 to 1.25 μg ml^{-1}, with the MBC at higher concentrations than the MIC. These data showed that all the species were susceptible to Kathon™. The data were used to select a concentration suitable for studies on the efficacy of Kathon™ against biofilm-grown microorganisms accounting for the increased resistance of biofilm organisms reported in the literature [4]. Kathon™ was used at 5 μg ml^{-1}, double the highest observed MBC.

Effect of biocide on single species biofilms

Figure 3 shows the effect of Kathon™ (5 μg ml^{-1}) on established biofilms grown as single species after 0, 12, and 24 h of biocide dosing. All biofilms were grown for 24 h before exposure to a flow of fresh medium with or without biocide as efficacy of biocide against established biofilms is relevant to industrial applications (Figure 3a). At this point, cell densities of the bacterial species were between 10^6 and 10^8 cfu cm^{-2} and the fungal species between 10^4 and 10^6 cfu cm^{-2}. After 12 h of exposure to Kathon™ (Figure 3b), there were no surviving cells detected for *Fu. oxysporum* single culture biofilms and this species remained undetectable after 24 h of exposure (Figure 3c). Biofilms of *Fl. indologenes* were not affected by Kathon™ even after 24 h of exposure (Figure 3c). *S. maltophilia*, *A. denitrificans*, and *P. alcaligenes* showed a 0.5- to 2-log reduction in cell densities over 24 h biocide treatment. *R. glutinis* and *Fu. solani* were reduced by 1 and 2 logs, respectively, over 24 h (Figure 3c). Only single culture biofilms of *Fu. oxysporum* were eradicated by biocide dosing. Statistical analysis using a restricted model ANOVA showed that all species except *Fl. indologenes* (P=0.564) had significantly (P<0.05) lower cell densities per square centimeter as a result of biocide treatment.

Effect of biocide on multiple mixed species biofilms

Kathon™ at 5 μg ml^{-1} also decreased the number of viable cells per square centimeter of each species in 24 h mixed culture biofilms (Figure 4). Mixed culture biofilms including all seven species were grown for 24 h before exposure to a flow of fresh medium with or without biocide (Figure 4a). Cell densities of the

Table 1 The MIC and MBC of the model community species after 24 h in R2A medium at 30°C in the presence of Kathon™

Species	MIC (μg ml^{-1})	MBC (μg ml^{-1})
P. alcaligenes	0.313	1.25
A. denitrificans	1.25	2.5
S. maltophilia	1.25	2.5
Fl. indologenes	<0.016	0.313
R. glutinis	0.313	0.313
Fu. oxysporum	0.313	0.625
Fu. solani	0.313	0.313

Number of replicates (n)=2.

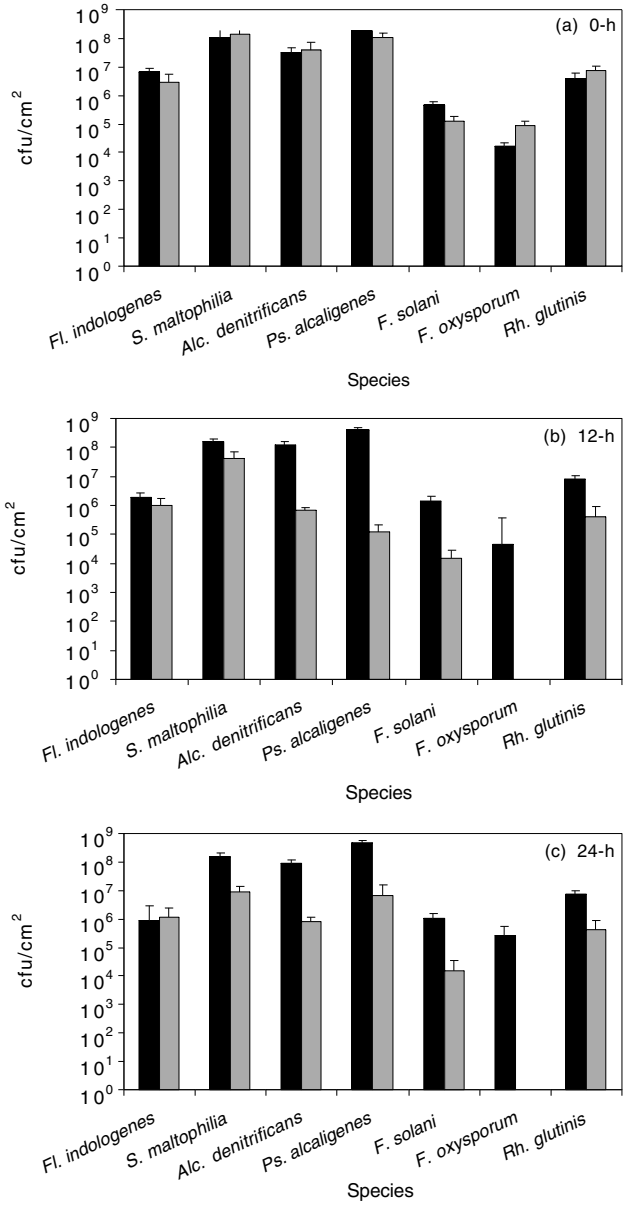

Figure 3 Effect of biocide on 24 h single species biofilms of *Fl. indologenes*, *S. maltophilia*, *P. alcaligenes*, *A. denitrificans*, *R. glutinis*, *Fu. oxysporum*, and *Fu. solani* in half-strength R2A medium at 22°C. (a) At 0 h, (b) 12 h, and (c) 24 h of exposure to Kathon™ (▨ 5 μg ml^{-1}) compared with untreated (■) biofilms. The absence of bars indicates that no viable counts were detected. Bars = 1 SE; $n = 2$.

bacterial species in mixed culture biofilms were 10^7 cfu cm^{-2}. Viable counts for the yeast within the biofilm were approximately 10^5 cfu cm^{-2}, intermediate to the counts for bacteria and filamentous fungi (10^3 cfu cm^{-2}). Cell densities of all species except *Fl. indologenes* were significantly ($P<0.05$) lower in mixed biofilms than as single biofilms. *Fu. solani*, *Fu. oxysporum*, and *R. glutinis* had no viable counts after 24 h of exposure to biocide in mixed culture. Cell densities for *Fu. oxysporum* after 12 h of biocide treatment in mixed biofilms were 10^1 cfu cm^{-2}, whereas there were no surviving cells in single species biofilms. The bacterial species were shown to have a 1- to 1.5-log difference in cell densities between treated and untreated in mixed culture biofilms at 24 h. All species in mixed species biofilms had significantly ($P<0.05$) lower cell densities per square centimeter as a result of biocide treatment.

SEM

SEM of single species biofilms showed a reduction in attached cells between untreated and biocide-treated films. There was some evidence for loss of exopolysaccharide in treated biofilms but no differences in cell morphology. Untreated biofilms formed by *Fl. indologenes* consisted of small microcolonies of cells scattered over the surface coated in exopolysaccharide. *S. maltophilia* formed monolayer biofilms and both *P. alcaligenes* and *A. denitrificans* formed biofilms with single cell surface coverage

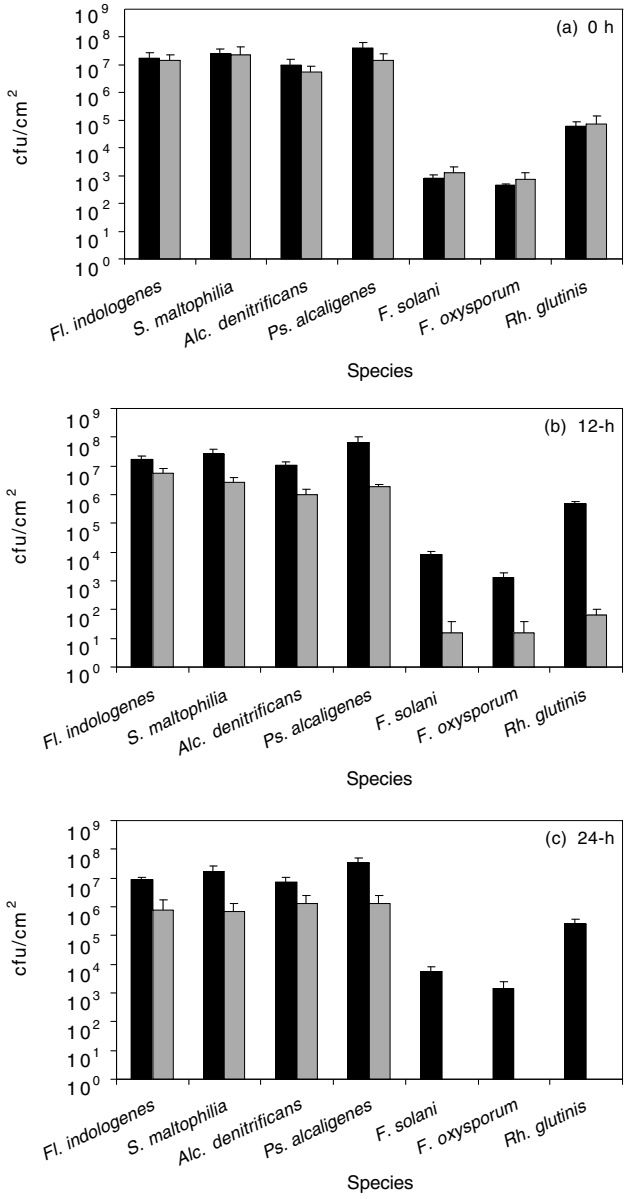

Figure 4 Effect of biocide on 24 h mixed species biofilms of *Fl. indologenes*, *S. maltophilia*, *P. alcaligenes*, *A. denitrificans*, *R. glutinis*, *Fu. oxysporum*, and *Fu. solani* in half-strength R2A medium at 22°C. (a) At 0 h, (b) 12 h, and (c) 24 h of exposure to Kathon™ (▨; 5 μg ml^{-1}) compared with untreated (■) biofilms. The absence of bars indicates that no viable counts were detected. Bars = 1 SE; $n = 2$.

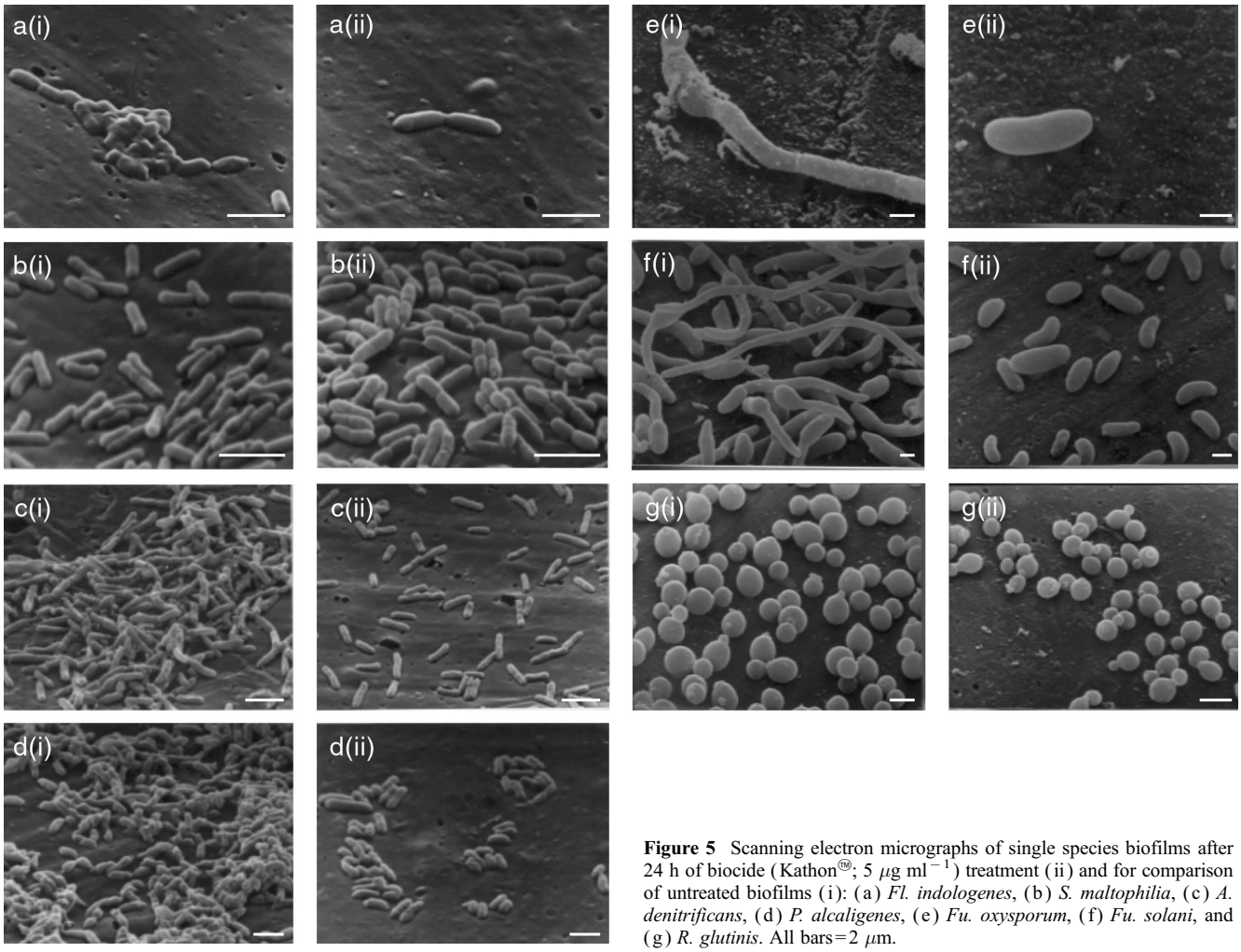

Figure 5 Scanning electron micrographs of single species biofilms after 24 h of biocide (Kathon™; 5 μg ml^{-1}) treatment (ii) and for comparison of untreated biofilms (i): (a) *Fl. indologenes*, (b) *S. maltophilia*, (c) *A. denitrificans*, (d) *P. alcaligenes*, (e) *Fu. oxysporum*, (f) *Fu. solani*, and (g) *R. glutinis*. All bars=2 μm.

with microcolonies of several cells thick. SEM showed untreated biofilms of *Fu. solani* to consist of numerous germinating conidia attached to the surface, forming an extensive hyphal network. This was also shown for untreated biofilms of *Fu. oxysporum*; but overall, fewer conidia were attached. However, biocide-treated biofilms of both fungal species showed less attached conidia with no germination. *R. glutinis* untreated biofilms formed a monolayer of budding yeast cells over the surface with an apparent loss of cell numbers in biocide-treated biofilms (Figure 5).

SEM of untreated mixed species biofilms was composed mainly of yeast cells surrounded by bacterial cells and of surface areas covered with the bacterial species. No clear visual differences were seen between untreated and biocide-treated multiple mixed species biofilms.

Discussion

There has been no published literature on simultaneous binary culture biofilm formation between a bacterium and filamentous fungus within a flow cell system as described here. In fact, the presence of fungi within biofilms has received limited attention, with the exception of a few reports of their characterization in water distribution systems [12,27]. However, sequential attachment studies between *S. maltophilia* and *Fu. oxysporum* showed that glass or PVC surfaces had no significant effect on total cell numbers in the biofilm and that the attachment of the second colonizer was also not significantly influenced by the previous attachment of the first colonizer [14]. Similar studies between pairs of bacterial species have shown that the attachment of each species was increased, decreased, or not affected by simultaneous or previous attachment of another species [21,29,31].

The use of flow cells and microscopic image capture has become increasingly important for observing a field of view for initial attachment events, for monitoring species interactions, and for determination of biofilm structure [9,35]. Parallel plate flow chambers have been used extensively to examine adhesive interactions between yeasts and oral bacteria [23,25,26]. This noninvasive technique allows much better understanding of these events over more conventional techniques such as viable cell counts, as they do not result in destruction of the biofilm at the time of sampling. The work reported here using nondestructive image analysis of flow cells shows that *S. maltophilia* and *Fu. oxysporum* coexisted within binary biofilms.

Coadhesion between conidial attachment by *Fu. oxysporum* and cell attachment by *S. maltophilia* in a flow cell cannot be visualised to the same extent as the literature describes for bacterial coadhesion [5]. This makes conclusions about interactions between

these more diverse species difficult and may be a result of size and mass differences between a single conidium and a bacterial cell. The larger size and mass of fungal conidia may explain the greater attachment of fungi to the bottom of the flow cell through sedimentation under the laminar flow conditions examined. Conidial attachment and subsequent germination forming extensive hyphal networks may contribute to the susceptibility of the fungus to detachment. The extensive hyphal growth projects out of the hydrodynamic boundary layer further into bulk flow, where it would be subjected to fluid forces resulting in detachment. Similarly, Stoodley *et al* [36] showed biomass ranging from single cells to 500-μm aggregates detaching from mature mixed bacterial species biofilms grown under turbulent flow. The detachment reported here may be reflected by the lower abundance of fungi in the natural biofilms within photoprocessors and is important when considering fouling problems within this industrial system. Different hyphal growth rates may be a response to the direction of flow of the bulk fluid or chemotactic responses to nutrient or oxygen gradients on the surface.

Bacterial attachment by *S. maltophilia* appeared exponential after a lag (3 h) during initial attachment. This was followed by slowing of attachment rate, presumably as uncolonized areas of the surface were reduced because of increased attached biomass that is evenly distributed over the surface. It was clearly seen from the real-time video images that there was a constant movement of attaching and detaching cells and that the general belief that cells attach and divide to form microcolonies is overly simplistic. This and other works (e.g., Ref. [19]) clearly show that bacterial cells following attachment are by no means fixed and are frequently seen swimming between the more quiescent cells that rotate about their axis.

Industrial biofilm communities are colonized by a mixed consortium of microbial species. While it is easier to study interactions in binary species biofilms, these systems may be oversimplified when monitoring disinfection efficacy of biocides. The effects of Kathon™ on the bacterial and fungal cells were investigated in suspension by the quantitative determination of MICs and MBCs. This determined a level of activity for the isothiazolone biocide that was bactericidal to the species studied under defined planktonic conditions. However, growth media, inoculum size, and environmental conditions such as temperature and pH affect MIC determinations [1]. The disinfection efficacy of Kathon™ was compared between the species grown as single culture biofilms to seven-species mixed culture biofilms. Pure culture biofilms of three bacterial and three fungal species were affected by treatment with Kathon™, but the efficacy of treatment varied with species. Cell densities of *Fl. indologenes* were not significantly reduced by biocide treatment, whereas no viable cells were detected for *Fu. oxysporum* after 12 h. In mixed culture biofilms, the bacteria were more resistant to biocide treatment. On the other hand, the fungal species appeared less resistant. These results indicate that the bacteria and, to a lesser extent, the fungi growing in biofilms are more resistant to biocide than cells grown in planktonic phase. The concentration of Kathon™ used in the biofilm studies was bactericidal to all the species in planktonic phase; thus, the biofilm must offer protection against the biocide particularly for the bacterial species and to a greater extent in mixed biofilms than as single biofilms. This reduced efficacy of biocides against biofilms compared to its action against planktonic cells may be a result of limited penetration of the biocide into the biofilm matrix, which leads to gradients of biocide concentration, or that the biofilm biomass effectively neutralizes the action of the biocide. For example, alginate gel beads-entrapped *Enterobacter aerogenes* artificial biofilms were less susceptible to chlorine, glutaraldehyde, an isothiazalone, and a quaternary ammonium compound than were planktonic bacteria [33]. The degree of kill measured in this artificial biofilm system decreased as gel bead radius or cell density increased. In a similar system, thin *P. aeruginosa* biofilms also exhibited a reduced susceptibility to monochloramine and hydrogen peroxide [6]. This was hypothesized not to be due simply to reduced penetration of the biocide but also to expression of new genes when bacteria attach to the surface whose gene products somehow reduce the susceptibility of the cells to biocides.

The scanning electron micrographs presented in this work for single species biofilms before and after biocide treatment clearly showed a loss in attached cell numbers. Biocide-induced removal of biofilm was also demonstrated by Koenig *et al* [18] who challenged *Burkholderia cepacia* biofilms grown in flow cells with oxidants and recorded up to 50% loss of biofilm in 1 h of continuous biocide treatment. Furthermore, it was reported that the loss of sloughed bacteria from actively growing biofilms can reach levels of 10^6–10^7 cfu ml^{-1} eluted medium and that while in this instance free chlorine treatment greatly reduces sloughing, there is still significant bacterial loss into the planktonic phase [10]. Subsequently, biofilm recovery was rapid once the biocide was removed, which was demonstrated by resumed sloughing ability from the biofilm. Thus, even though biocide dosing may reduce recovery, biofilm numbers after biocide removal may result in further damage to industrial systems. It is not known whether the mixed biofilms studied here would resume growth after removal of biocide dosing, but it is hypothesised to be a likely scenario.

In conclusion, bacteria and filamentous fungi can coexist as binary culture biofilms with neither species adversely affected by the presence of the other species, although future work might identify specific interactions that may be occurring between these species. Mixed species biofilms treated with biocide appear to offer greater protection to the bacterial species than the fungi. Although biocide dosing is effective at reducing microbial numbers through biocidal activity and possibly biocide-induced detachment, biocide removal may result in rapid recovery of the biofilm, which poses additional problems towards eradication of industrial fouling.

References

1 Bloomfield SF, M Arthur, B van Klingeren, W Pullen, JT Holah and R Elton. 1994. An evaluation of the repeatability and reproducibility of a surface test for the activity of disinfectants. *J Appl Bacteriol* 76: 86–94.
2 Bott TR. 1994. The control of biofilms in industrial cooling water systems. In: Wimpenny JWT, W Nichols, D Stickler and HM Lappin-Scott (Eds), Bacterial Biofilms and Their Control in Nature and Medicine. Bioline, Cardiff, pp. 173–180.
3 Brown MRW and P Gilbert. 1993. Sensitivity of biofilms to antimicrobial agents. *J Appl Bacteriol Symp Suppl* 74: 87S–97S.
4 Brown MRW, JW Costerton and P Gilbert. 1991. Extrapolating to bacterial life outside the test tube. *J Antimicrob Chemother* 27: 565–567.
5 Buswell CM, YM Herlihy, PD Marsh, CW Keevil and SA Leach. 1997. The role of coaggregation among aquatic biofilm bacteria. In: Wimpenny J, P Handley, P Gilbert, HM Lappin-Scott and M Jones (Eds), Biofilms: Community Interactions and Control. Bioline, Cardiff, pp. 95–102.

6 Cochran WL, GA McFeters and PS Stewart. 2000. Reduced susceptibility of *Pseudomonas aeruginosa* biofilms to hydrogen peroxide and monochloramine. *J Appl Microbiol* 88: 22–30.
7 Collier PJ, AJ Ramsey, P Austin and P Gilbert. 1990. Growth inhibitory and biocidal activity of some isothiazolone biocides. *J Appl Bacteriol* 69: 569–577.
8 Costerton JW, K-J Cheng, GG Geesey, TI Ladd, JC Nickel, M Dasgupta and TJ Marrie. 1987. Bacterial biofilms in nature and disease. *Annu Rev Microbiol* 41: 435–464.
9 Dalton HM, PE March and KC Marshall. 1997. Community interactions in marine bacterial biofilms. In: Wimpenny J, P Handley, P Gilbert, HM Lappin-Scott and M Jones (Eds), Biofilms: Community Interactions and Control. Bioline, Cardiff, pp. 129–132.
10 Daly B, WB Betts, AP Brown and JG O'Neill. 1998. Bacterial loss from biofilms exposed to free chlorine. *Microbios* 96: 7–21.
11 DeBeer D, R Srinivasan and PS Stewart. 1994. Direct measurement of chlorine penetration into biofilms during disinfection. *Appl Environ Microbiol* 60: 4339–4344.
12 Doggett MS. 2000. Characterization of fungal biofilms within a municipal water distribution system. *Appl Environ Microbiol* 66: 1249–1251.
13 Elvers KT, K Leeming, CP Moore and HM Lappin-Scott. 1998. Bacterial–fungal biofilms in flowing water photo-processing tanks. *J Appl Microbiol* 84: 607–618.
14 Elvers KT, K Leeming and HM Lappin-Scott. 2001. Binary culture biofilm formation by *Stenotrophomonas maltophilia* and *Fusarium oxysporum*. *J Ind Microbiol Biotechnol* 26: 178–183.
15 Fuller SJ, SP Denyer, WB Hugo, D Pemberton, PM Woodcock and AJ Buckley. 1985. The mode of action of 1,2-benzisothiazolin-3-one on *Staphylococcus aureus*. *Lett Appl Microbiol* 1: 13–15.
16 Jones RN, AL Barry, TL Gavan and JA Washington. 1985. Susceptibility tests: microdilution and macrodilution broth procedures. In: Lennette EH, A Balows, WJ Hausler and HJ Shadomy (Eds), Manual of Clinical Microbiology. ASM Press, Washington, pp. 972–977.
17 Keevil CW and CW Mackerness. 1990. Biocide treatment of biofilms. *Int Biodeterior* 26: 169–179.
18 Koenig DW, SK Mishra and DL Pierson. 1995. Removal of *Burkholderia cepacia* biofilms with oxidants. *Biofouling* 9: 51–62.
19 Lawremce JR, PJ Delaquis, DR Korber and DE Caldwell. 1987. Behaviour of *Pseudomonas fluorescens* within the hydrodynamic boundary layers of surface microenvironments. *Microb Ecol* 14: 1–14.
20 LeChevallier MW, TM Babcock and RG Lee. 1987. Examination and characterization of distribution system biofilms. *Appl Environ Microbiol* 53: 2714–2724.
21 Leriche V and B Carpentier. 1995. Viable but nonculturable *Salmonella typhimurium* in single and binary species biofilms in response to chlorine treatment. *J Food Prot* 58: 1186–1191.
22 Mah T-FC and GA O'Toole. 2001. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* 9: 34–39.
23 McKee AS, AS McDermid, DC Ellwood and PD Marsh. 1985. The establishment of reproducible complex communities of oral bacteria in the chemostat using defined inocula. *J Appl Bacteriol* 59: 263–275.
24 Melo LF and MM Pinheiro. 1992. Biofouling in heat exchangers. In: Melo LF, TR Bott, M Fletcher and B Capdeville (Eds), Biofilms: Science and Technology. Kluwer Academic Publishing, The Netherlands, pp. 499–509.
25 Millsap KW, HC Van Der Mei, R Bos and HJ Busscher. 1998. Adhesive interactions between medically important yeasts and bacteria. *FEMS Microbiol Rev* 21: 321–336.
26 Millsap KW, R Bos, HC Van Der Mei and HJ Busscher. 2000. Dot assay for determining adhesive interactions between yeasts and bacteria under controlled hydrodynamic conditions. *J Microbiol Methods* 40: 225–232.
27 Nagy LA and BH Olson. 1982. The occurrence of filamentous fungi in drinking water distribution systems. *Can J Microbiol* 28: 667–671.
28 Reasoner DJ and EE Geldreich. 1985. A new medium for the enumeration and subculture of bacteria from potable water. *Appl Environ Microbiol* 49: 1–7.
29 Sasahara KC and EA Zottola. 1993. Biofilm formation by *Listeria monocytogenes* utilizes a primary colonizing microorganism in flowing systems. *J Food Prot* 56: 1022–1028.
30 Shaw JC, B Bramhill, NC Wardlaw and JW Costerton. 1985. Bacterial fouling in a model core system. *Appl Environ Microbiol* 49: 693–701.
31 Skillman LC, IW Sutherland and MV Jones. 1999. The role of exopolysaccharide in dual species biofilm development. *J Appl Microbiol* 85: 13S–18S.
32 Slack MPE and WW Nichols. 1982. Antibiotic penetration through bacterial capsules and exopolysaccharides. *J Antimicrob Chemother* 10: 368–372.
33 Stewart PS, L Grab and JA Diemer. 1998. Analysis of biocide transport limitation in an artificial biofilm system. *J Appl Microbiol* 85: 495–500.
34 Stoodley P, I Dodds, JD Boyle and HM Lappin-Scott. 1999. Influence of hydrodynamics and nutrients on biofilm structure. *J Appl Microbiol* 85: 19S–28S.
35 Stoodley P, Z Lewandowski, JD Boyle and HM Lappin-Scott. 1999. The formation of migratory ripples in a mixed species bacterial biofilm growing in turbulent flow. *Environ Microbiol* 1: 447–455.
36 Stoodley P, S Wilson, L Hall-Stoodley, JD Boyle, HM Lappin-Scott and JW Costerton. 2001. Growth and detachment of cell clusters from mature mixed-species biofilms. *Appl Environ Microbiol* 67: 5608–5613.
37 Walker JT, J Rogers and CW Keevil. 1994. An investigation of the efficacy of a bromine containing biocide on an aquatic consortium of planktonic and biofilm microorganisms including *Legionella pneumophila*. *Biofouling* 8: 47–54.