A 3D model of antimicrobial action on biofilms

S.M. Hunt, M.A. Hamilton and P.S. Stewart

Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717-3980, USA
(E-mail: *phil_s@erc.montana.edu*)

Abstract A three-dimensional cellular automata model of biofilm dynamics was adapted to simulate the protection from killing by antimicrobial agents afforded to microorganisms in the biofilm state. The model incorporated diffusion and simultaneous utilization of a single substrate, growth and displacement of cells, detachment, and killing by an antimicrobial agent. The rate of killing was assumed to be directly proportional to the local concentration of substrate available to the microorganisms. Some of the features predicted by this model included development of dynamic, heterogeneous biofilm structures, gradients in substrate concentration leading to regions of substrate depletion in the interior of large cell clusters, variable killing by an antimicrobial agent from one simulation to the next, greater killing of cells at the periphery of cell clusters compared to those cells which were more deeply embedded, and reduced overall antimicrobial susceptibility of cells in the biofilm. These simulations show that substrate limitation can contribute to the protection from antimicrobial agents in biofilms but cannot explain the long-term persistence of biofilm viability that is often observed in practice.

Keywords Antimicrobial; biofilm; growth rate; model; tolerance

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Introduction

Microorganisms within biofilms have a remarkable tolerance to killing by antimicrobial agents. The reduced susceptibility of bacteria and yeast in biofilms is recognized as an important factor in the persistence of some chronic infections and the troublesome recurrence of fouling in industrial systems. The possibility of substrate limitation leading to inactive, and less susceptible, cells remains an attractive explanation for the recalcitrance of biofilm cells to antimicrobial agents. It is now clear that gradients in substrate concentrations exist within biofilms. These concentration gradients give rise to corresponding gradients in microbial growth rate and activity as observed by researchers using fluorescent probes and reporter genes. Since antimicrobials are thought to be more effective in killing actively growing cells, it seems reasonable that in substrate limited regions of a biofilm, microorganisms could better tolerate the presence of an antimicrobial agent by virtue of their inactivity. However, one would expect that as growing cells within the biofilm are killed, substrate would penetrate into regions that were previously substrate depleted. Thus, microorganisms that were previously dormant would lose their tolerance for the antimicrobial agent as substrate becomes available. Using a three-dimensional computer model of biofilm dynamics, this study investigates the level of protection that could be explained by substrate limitation when a biofilm is exposed to a substrate-dependent antimicrobial agent.

Several multidimensional computer models of biofilm dynamics have been described (Hermanowicz, 1998; Kreft *et al.*, 1998; Kreft *et al.*, 2001; Picioreanu *et al.*, 1998; Chang *et al.*, 2003; Picioreanu *et al.*, 2004; Lapsidou and Rittmann, 2004). This article presents the first example of incorporation of the action of an antimicrobial agent into such models. Earlier studies have employed 1D biofilm models to investigate biocide or antibiotic effects on biofilms (Dibdin *et al.*, 1996; Roberts and Stewart, 2004).

Methods

The computer model, BacLAB, used in this study has been described in detail elsewhere (Hunt *et al.*, 2003; Hunt *et al.*, 2004). This model uses a hybrid modeling approach in which all soluble components are modeled using discretized differential equations and the individual microorganisms that compose the biofilm are modeled discretely using a cellular automata algorithm. The advantage to the hybrid approach is the ability to separate different biofilm processes according to their natural time scales and also that the aggregate behavior of the biofilm emerges from the local interactions between individual microorganisms. Some of the processes simulated by the computer model include diffusion of soluble components into the biofilm, substrate consumption, microbial growth, and biofilm detachment resulting from substrate starvation. The model implemented in this study treats the biofilm as a single species system. Parameter values are given in Table 1.

To probe the degree of protection that could be expected from a substrate-dependent antimicrobial agent, antimicrobial efficacy was simulated to be proportional to the amount of substrate available to the microorganism. That is:

$$P = \frac{P_{\mathrm{MAX}}}{C_{S0}} \cdot C_S \tag{1}$$

where P_{MAX} is the maximum probability of killing and equal to the probability of killing used for cells in the bulk fluid, C_{S0} is the substrate concentration in the bulk fluid, and C_S is the local substrate concentration at a particular cell. Thus cells in substrate-rich regions of the biofilm have the highest antimicrobial susceptibility, whereas cells in substrate-depleted regions will be less susceptible to antimicrobial killing.

Results and discussion

The computer model used in this investigation simulated biofilm development, and the response to antimicrobial treatment, over a period of 500 h. Some of the features captured in

Table 1 Kinetic and diffusion parameters used in the simulations

Parameter	Symbol	Value	Unit(s)
Bulk substrate concentration	$C_{S,\mathrm{bulk}}$	8.0	gm^{-3}
Diffusivity of the antimicrobial in the aqueous phase (including the liquid, channels and voids)	$D_{A,\mathrm{aq}}$	1.51×10^{-6}	$\mathrm{m}^2\mathrm{h}^{-1}$
Relative effective diffusivity of the antimicrobial in biofilm	$D_{A,e}/D_{A,\mathrm{aq}}$	0.2	
Diffusivity of substrate in the aqueous phase (including the liquid, channels and voids)	$D_{S,\mathrm{aq}}$	$7.20{\cdot}10^{-6}$	$\mathrm{m}^2\mathrm{h}^{-1}$
Relative effective diffusivity of substrate in biofilm	$D_{S,e}/D_{S,\mathrm{aq}}$	0.55	
Local nutrient concentration threshold	$C_{S,\mathrm{min}}$	1.0	gm^{-3}
Monod half-saturation coefficient	K_S	0.1	gm^{-3}
Average cell mass	m_{avg}	1.75×10^{-13}	g
Number of initial colonies	N_c	28	
Number of nodes in *x*-direction	N_x	300	
Number of nodes in *y*-direction	N_y	300	
Radius of initial colonies	R_c	8.55	μm
Limiting substrate	S	Oxygen	
Duration of time below $C_{S,\mathrm{min}}$ before detachment	t_{detach}	24	h
Maximum antimicrobial killing probability	P_{MAX}	0.6838	
Yield coefficient	Y_{XS}	0.24	$\mathrm{g_x\ g_s^{-1}}$

these simulations included dynamic development of three-dimensional, heterogeneous biofilm structures, gradients in substrate concentration leading to regions of substrate depletion in the interior of large cell clusters, variable killing by antimicrobial agent from one simulation to the next, greater killing of cells at the periphery of cell clusters compared to those cells which were more deeply embedded, and reduced overall antimicrobial susceptibility of cells in the biofilm. A representative video of one simulation can be viewed at http://www.erc.montana.edu/Res-Lib99-SW/Movies/Database/MD_DisplayScript.asp.

We selected a value for the maximum antimicrobial killing probability, P_{MAX}, that corresponded to a 6 log reduction in viable cell numbers after 12 h exposure of planktonic cells to the antimicrobial agent in the absence of cell growth. Base case simulations with the biofilm model demonstrated that when the rate of antimicrobial killing was assumed to be independent of the substrate concentration, inclusion within a biofilm provided no additional protection.

When antimicrobial efficacy was assumed to be proportional to the local substrate concentration, the predicted time course of biofilm killing and recovery was as shown in Figure 1. When a 12 h antimicrobial dose was administered at both 100 h (Figure 1A) and 200 h (Figure 1B) of biofilm development, dead cell numbers rapidly increased within the biofilm. At the end of the antimicrobial dose period, the dead cell numbers remained constant for a period of approximately 37 to 117 h. During this time, surviving cells grew and little detachment occurred. The extent of killing at the end of the 12 h dose, expressed as a log reduction, was approximately 0.5 when antimicrobial treatment was initiated at 100 h (Figure 1C) and ranged from 0.45 to 1.6 when treatment began at 200 h

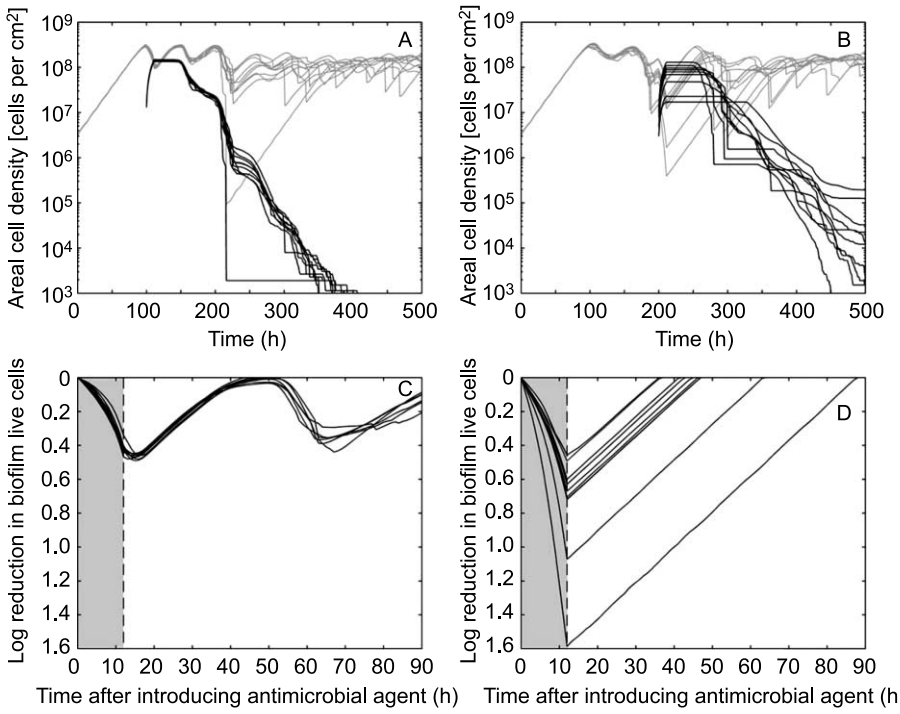

Figure 1 Simulated killing and recovery of cells in a biofilm exposed to an antimicrobial agent expressed in terms of areal cell density (A and B) and log reduction (C and D). In panels A and B, gray lines denote total cell numbers and black lines denote dead cell numbers. The antimicrobial treatment was applied for 12 h (shading) after either 100 h (A and C) or 200 h (B and D) of biofilm development. Ten replicate simulations are plotted

(Figure 1D). These log reductions are considerably less than those calculated for free-floating cells exposed to the bulk fluid concentration of substrate (log reduction = 6).

Figure 1C reveals killing during the antimicrobial dose (time 0 to 12 h) followed by a regrowth period (approximately 15 to 50 h), followed by a reproducible period of sloughing (50 to 60 h). Figure 1D reveals surprising variability in the log reduction achieved in replicate simulations.

These results are consistent with the idea that microorganisms within a biofilm are afforded some degree of protection from antimicrobial agents whose action is dependent on the availability of a metabolic substrate. The model predicted that lower substrate concentrations prevail closer to the substratum and in the vicinity of cell clusters (data not shown).

Consistent spatial patterns of killing within the denser biofilms, those that were 100 h old at the onset of treatment, were noted (Figures 2A–C). There was an apparent higher rate of killing near the surface when compared to the cell cluster interiors. This is explained by the higher substrate concentration at the cluster–bulk fluid interface and the depletion of substrate in the cell cluster interiors. In the 200 h old biofilms, no spatial organization of killing patterns could be discerned when examining cross-sections (Figures 2D–F). These biofilms were characterized by significant voids and loosely tethered clusters in which the substrate concentration was more uniformly distributed.

In simulations where antimicrobial treatment continued until biofilm extinction, complete killing of the biofilm took 43.4 ± 2.2 h and 43.9 ± 5.9 h (mean ± standard deviation, $n = 10$) when treatment began at 100 h and 200 h respectively. In other words, the model predicts that the biofilm does eventually succumb to the antimicrobial treatment (Figure 3). Since many real biofilms survive even prolonged antimicrobial exposure, substrate-limited killing may not be a sufficient explanation for the antimicrobial tolerance of biofilms in engineered systems and medical settings. Simulations in this and an earlier 1D model investigation (Roberts and Stewart, 2004) suggest that substrate limitation has the potential to retard antimicrobial killing but cannot explain sustained tolerance. One of the features predicted by the model is that killing should accelerate at

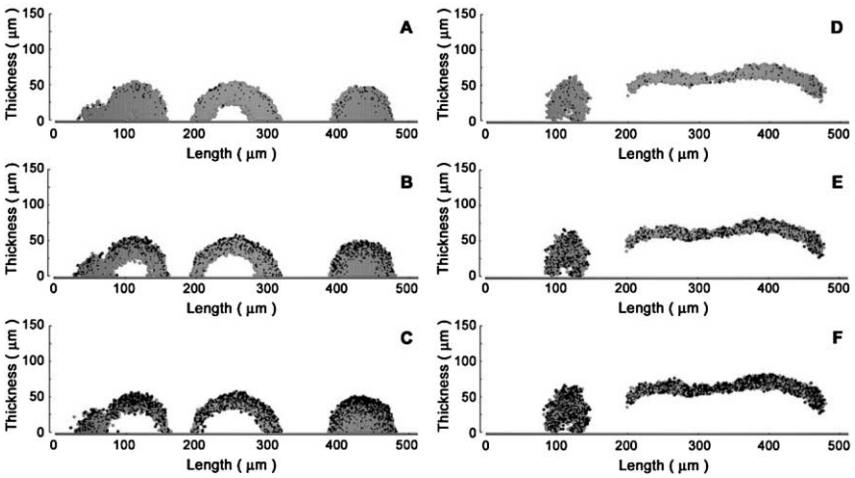

Figure 2 Live (gray) and dead (black) cell distributions in biofilm cross sections at 1, 6, and 11 h after introduction of the antimicrobial agent. Figures A–C and D–F are representative of simulations when treatment begins at 100 h and 200 h, respectively. The cell cluster in panels D–F that appears to be floating above the substratum is actually tethered to the surface at locations outside of this 2D section

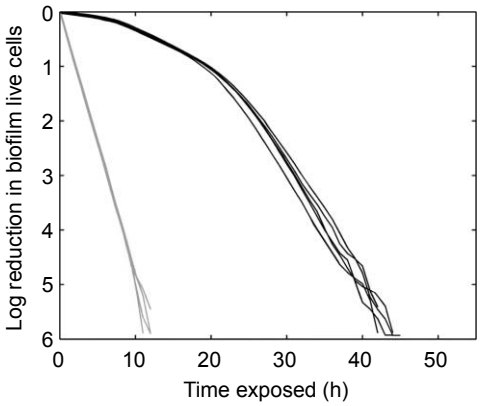

Figure 3 Biofilm killing by continuous antimicrobial exposure. Gray lines denote simulations in which killing was independent of substrate concentration and black lines denote simulations in which killing was substrate concentration dependent. Five replicate simulations of each condition are plotted

some point during the treatment. This behavior is rarely observed experimentally. We therefore suggest that substrate limitation in biofilms is a plausible contributing factor to the reduced antimicrobial susceptibility in biofilms, but is probably not adequate by itself to account for the full degree of protection afforded to microorganisms in biofilms.

Conclusions

This model of antimicrobial agent action on a biofilm predicts:

- Variable killing by an antimicrobial agent in replicate simulations using identical parameter values. This variability can be attributed to the stochastic elements of the model.
- Greater killing of cells at the periphery of cell clusters compared to more deeply embedded cells.
- An increasing rate of killing during antimicrobial treatment when killing is dependent on the local concentration of the metabolic substrate.

Acknowledgements

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