Journal of Industrial Microbiology & Biotechnology (2002) 29, 10–15

www.nature.com/jim

Role of dose concentration in biocide efficacy against *Pseudomonas aeruginosa* biofilms

KJ Grobe, J Zahller and PS Stewart

Center for Biofilm Engineering and Department of Chemical Engineering, Montana State University, Bozeman, MT 59717-3980, USA

Pseudomonas aeruginosa **entrapped in alginate gel beads to form artificial biofilms resisted killing by chlorine, glutaraldehyde, 2,2-dibromo-3-nitrilopropionamide (DBNPA), and an alkyl dimethyl benzyl ammonium compound (ADBAC). The degree of resistance was quantified by a resistance factor that compared killing times for biofilm and planktonic cells in response to the same concentration of antimicrobial agent. Resistance factors averaged 120 for chlorine, 34 for glutaraldehyde, 29 for DBNPA, and 1900 for ADBAC. In every case, resistance factors decreased with increasing concentration of the antimicrobial agent. An independent analysis of the concentration dependence of the apparent rates of killing of planktonic and biofilm bacteria showed that elevating the treatment concentration increased bacterial killing more in the biofilm than it did in a suspension culture. Calculation of a transport modulus comparing the rates of biocide reaction and diffusion suggested that at least part of the biofilm resistance to chlorine, glutaraldehdye, and DBNPA could be attributed to incomplete or slow penetration of these agents into the biofilm. Time-kill curves were nonlinear for biofilm bacteria in some cases. The shapes of these curves implicated retarded antimicrobial penetration for chlorine and glutaraldehyde and the presence of a tolerant subpopulation for DBNPA and ADBAC. The results indicate that treating biofilms with a concentrated dose of biocide is more effective than using prolonged doses of a lower concentration.**
Journal of Industrial Microbiology & Biotechnology (2002) **29,** 10–15 doi:10.1038/sj.jim.7000256

Keywords: biofilm; *Pseudomonas aeruginosa*; biocide; penetration; resistance

Introduction

Bacteria in biofilms escape killing by biocides and antibiotics even though these agents can be shown to control the same microorganisms effectively when grown in a conventional suspension culture [15]. The mechanisms of biofilm resistance to antimicrobial agents are just beginning to emerge, and it remains difficult to select and dose antimicrobial agents for use against biofilms. It is clear that data on antimicrobial efficacy obtained with planktonic bacteria are not reliable indicators of performance when a biofilm is present. Data from biofilm systems are therefore needed to yield insights into underlying resistance mechanisms and guide the practical application of antimicrobial agents.

Natural biofilm samples are labor intensive to generate and are inherently heterogeneous. Researchers have consequently devised artificial biofilm model systems that capture the resistant phenotype of biofilm bacteria while providing numerous consistent biofilm samples. In particular, a variety of artificial biofilms formed by entrapping bacteria in hydrogels have been used to study the effects of antimicrobial agents on biofilm cells [3,5,8,14,17-19,21-23]. These systems have employed different hydrogels and diverse microorganisms, but have consistently found that gel-entrapped microorganisms exhibit reduced susceptibility to antimicrobial agents. The magnitude of the resistance measured in artificial biofilms resembles that reported for natural biofilms. Gel beads containing bacteria have also been used in rat and murine models of lung infection [2,9].

Correspondence: Dr PS Stewart, Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717, USA
Received 29 October 2001; accepted 18 March 2002

We describe in this article the application of an artificial biofilm system to quantify the resistance mounted in the biofilm state, to characterize the dependence of biofilm killing on the applied concentration of antimicrobial agent, and to diagnose the extent of biocide penetration into the biofilm. Each of these properties is an important aspect of the rational design of biofilm control programs.

Materials and methods

Artificial biofilm model

Artificial biofilms were created by entrapping bacteria, in this case *Pseudomonas aeruginosa* strain ERC1, in alginate gel beads. The gel beads were then suspended in a nutrient medium overnight to allow for growth of microorganisms in the beads and adoption of a biofilm phenotype. A plate of R_2A agar (Difco, Detroit, MI) was streaked with a lawn of *P. aeruginosa* and incubated overnight at 36°C. Nine milliliters of pH 7.2 phosphate buffer was then added to the agar plate and the cells were gently scraped off the plate using a glass hockey stick. This bacterial suspension was mixed with an equal volume of 4% sodium alginate (Sigma, St. Louis, MO) to make a final alginate concentration of 2%. The alginate and bacteria slurry was placed in a sterile 30-ml syringe with a 22-gauge needle attached. A stopper attached to a compressed air tank allowed the syringe to be pressurized. When 20 psig pressure was applied, a stream of small droplets was forced out of the needle and dropped into a stirred solution of 50 mM calcium chloride. The calcium cross-linked the alginate, and semisolid beads with entrapped cells were formed. The beads were allowed to stir in the calcium chloride solution for approximately 20 min, then rinsed in a dilute solution of calcium chloride (5 mM). These beads were incubated overnight at 36°C on a

rotating shaker in 1/10 strength nutrient broth (Difco) with 5 mM added calcium chloride to maintain the bead structure. The mean gel bead diameter was measured by lining up 10 beads on a ruler.

Antimicrobial treatment of biofilm cells

Bacterial cells in artificial biofilm gel beads were challenged with each of four antimicrobial agents. These antimicrobials were prepared in a pH 7.4 phosphate buffer containing 0.085 g l^{-1} potassium dihydrogen phosphate, 0.4055 g l^{-1} magnesium chloride, and 5 mM calcium chloride. Chlorine stock solution was prepared by diluting sodium hypochlorite (Sigma). Glutaraldehyde was a gift of Union Carbide (Danbury, CT). 2,2-Dibromo-3-nitrilopropionamide (DBNPA) was a gift of The Dow Chemical and was provided as a liquid with approximately 20% by weight of the active ingredient. The quaternary ammonium compound (ADBAC) was Barquat MB80-80% (Lonza, Fairlawn, NJ), which contains predominantly $C_{12}-C_{14}$ alkyl groups.

At the start of the experiment, approximately 250–300 beads were decanted from the nutrient broth in which they were cultured and placed into a magnetically stirred vessel containing 500 to 700 ml of the desired disinfection solution. The antimicrobial challenge was performed at room temperature of 23°C. The antimicrobial solution was replenished every 15 to 20 min by turning off the stirring, allowing the beads to settle, decanting the old solution, and replacing it with fresh antimicrobial solution. Ten beads were removed at various time points and placed in 5 ml of a solution containing an appropriate neutralizing agent and 50 mM sodium citrate (50 mM). The neutralizing agents were 50 mM sodium thiosulfate for chlorine and DBNPA, 1% glycine for glutaraldehyde, and tryptic soy broth for ADBAC. The sodium citrate functions to dissolve the alginate gel and release bacteria into the fluid. The bead–citrate solution was refrigerated for 2 h while the beads dissolved, then diluted and plated out on R_2A using the drop plate method [7,11]. This method of recovering alginate-entrapped bacteria has been widely used [12] and has little effect on bacterial viability. The plates were incubated overnight at 36°C and counted. Control experiments were conducted in the same manner with phosphate buffer lacking antimicrobial agent. Each experimental condition was repeated between two and five times.

Antimicrobial treatment of planktonic cells

Planktonic cells were challenged with the same antimicrobial agent solutions. A planktonic culture of *P. aeruginosa* was grown on a rotating shaker at 36°C in 1/10 strength nutrient broth (Difco) with 5 mM added calcium chloride. When the culture was still in exponential phase, 2-ml aliquots of this culture were centrifuged for 7.5 min at 10,000 rpm in a Denver Instrument (Arrada, CO) Micro 14 microcentrifuge. The supernatant was removed and the bacterial pellet was resuspended by adding 2 ml of pH 7.4 phosphate buffer, formulated as described above, and vortexed. Two milliliters of bacterial suspension was mixed with 18 ml of a stock antimicrobial solution, which was designed to yield the desired final concentration of antimicrobial agent. The antimicrobial challenge was performed at room temperature of 23°C. The suspension was sampled at various time points by taking 1 ml into 9 ml of the appropriate neutralizer. Serial dilution, plating, enumeration, and controls were conducted as described above.

Antimicrobial agent concentration determination

Aliquots of the treatment solution were sampled periodically to monitor the residual antimicrobial concentrations. Chlorine and DBNPA concentrations were determined using the *N,N*-diethyl-*p*-phenylenediamine (Hach, Loveland, CO) colorimetric method (APHA). Glutaraldehyde was determined by gas chromatography. One-milliliter samples were stored under refrigeration at 4°C in Target silanized vials (Fisher, Pittsburgh, PA) until they could be injected. Gas chromatography was performed on a Hewlett-Packard (Roseville, CA) 5890 Series chromatograph. The operating temperatures were as follows: injector: 190°C, oven: 185°C, detector 250°C. The column head pressure was approximately 38 psi. ADBAC concentrations were not determined.

Analysis of concentration dependence of bacterial killing

To analyze the antimicrobial agent concentration dependence of planktonic and biofilm killing, the Chick–Watson mathematical model of disinfection was assumed [6]

$$\frac{dX}{dt} = k_{dis} C^n X \tag{1}$$

where X is the viable density, t is time, k_{dis} is a disinfection rate coefficient, and C is antimicrobial agent concentration. The exponent n on the concentration captures the concentration dependence of killing. Assuming a constant biocide concentration, the solution to this model is found by integration to be

$$\ln\left(\frac{X}{X_0}\right) = -k_{dis} C^n t \tag{2}$$

The apparent rate of disinfection over the interval from zero to two-log reduction was defined as

$$r = \frac{-\ln(0.01)}{t} \tag{3}$$

Each experimental data set was fit to a third-order polynomial using an existing regression function in a spreadsheet. The time for a two-log reduction was determined from this fit and the apparent disinfection rate was calculated from Eq. (3). Combining Eqs. (2) and (3) we obtain

$$\ln(r) = \ln(k_{dis}) + n \ln(C) \tag{4}$$

A plot of $\ln(r)$ versus $\ln(C)$ should yield a straight line with slope n. A least squares linear regression of this type was performed to calculate n.

Analysis of antimicrobial transport limitation

The relative rates of reaction and diffusion within the gel beads were evaluated by calculating an observable modulus, Φ, where

$$\Phi = \frac{R_{obs} L_f^2}{D_e C_o} \tag{5}$$

This dimensionless parameter indicates incomplete penetration when Φ is greater than one and effective penetration when Φ is less than one. The constituent parameters include R_{obs}, the observed overall rate of antimicrobial reaction (mg l^{-1} s^{-1}); L_f, the effective biofilm thickness (cm), which was taken as the volume to surface area ratio or the bead radius divided by three; D_e, which is the effective diffusion coefficient of the antimicrobial agent in the

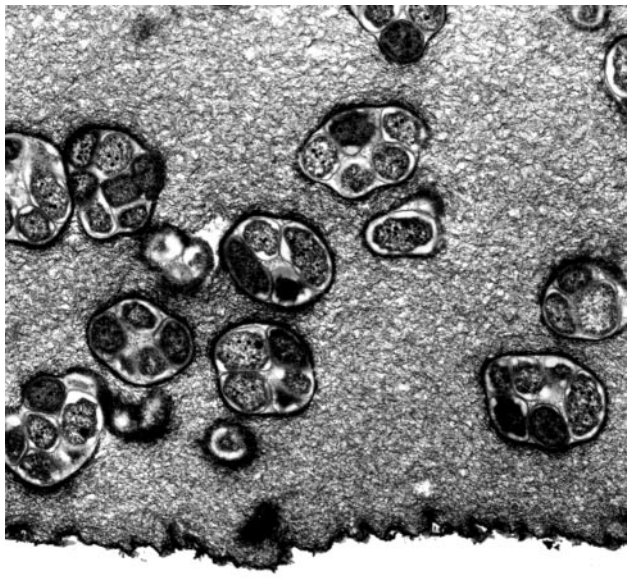

Figure 1 Transmission electron micrograph of alginate gel bead artificial biofilm containing entrapped *P. aeruginosa*. A location near the bead surface is shown. The scale bar is 1μm.

beads ($cm^2\ s^{-1}$); and C_o, the mean antimicrobial agent concentration over a particular time interval (mg/l). The reaction rate of antimicrobial agent was calculated by

$$R_{obs} = \frac{\Delta C \cdot V_{sol}}{V_{bead} \cdot \Delta t} \quad (6)$$

with

$$\Delta C = (C_i - C_f) \quad (7)$$

where C_i is the initial antimicrobial concentration ($mg\ l^{-1}$), C_f is the final antimicrobial concentration ($mg\ l^{-1}$), t is the time interval (min) between the initial and final antimicrobial concentration determinations, V_{sol} is the volume of antimicrobial solution (ml), and V_{bead} is the combined volume of gel beads dispersed in the antimicrobial solution (ml). For chlorine, the volatility of the agent independent of reaction was corrected for by a first-order rate constant for chlorine loss in the absence of beads. The effective diffusion coefficient, D_e, was calculated by estimating the diffusivity using published correlations [10,20].

Transmission electron microscopy

Alginate gel beads with entrapped *P. aeruginosa* were prepared as described above and incubated for 24 h in 1/10 strength nutrient broth at 36°C. The beads were then fixed in 2.5% glutaraldehyde in phosphate buffer amended with 5 mM calcium chloride. The beads were washed three times for 15 min each in the phosphate buffer. Next, the beads were stained with osmium tetroxide (1%) in the calcium-enhanced phosphate buffer. The beads were again washed (3×, 15 min) in the same buffer. Specimens then underwent a series of dehydration steps: 50% ethanol, 15 min; 70% ethanol, 1.5 min; 1% uranyl acetate/1% phosphotungstic acid (PTA), 1 h; 95% ethanol, 15 min; 100% ethanol, 15 min; 100% ethanol, 15 min; 100% ethanol, 15 min; 100% ethanol (2 parts): SPURRS (1 part, Ernest F. Fullam, Latham, NY), 1 h; 100% ethanol (1 part): SPURRS (1 part), 1 h; SPURRS epoxy resin, 8 h or overnight. The epoxy resin was polymerized for 14 h at 70°C. Thin sections were cut and examined using a Jeol (Peabody, MA) JEM-100CX electron microscope.

Results

Electron microscopy of artificial biofilm gel beads revealed a structure of dense microcolonies of bacteria dispersed in a faintly stained matrix (Figure 1). The cell density was higher near the bead surface than it was in the bead interior. Using the electron micrograph images, the cell volume fraction was estimated to be 0.23 in the region near the bead surface. The polymer volume fraction was taken as 0.02. The estimated value of the relative effective diffusion coefficient in the gel bead, D_e/D_{aq}, which accounts for the presence of polymer and cells, was 0.624. The average diameter of the gel beads was 2.42±0.07 mm. The average viable cell density after overnight culture but before antimicrobial treatment was 9.7±0.2 log cfu cm^{-3}. This density was based on the gel bead volume and therefore represents the average cell density throughout the bead.

Planktonic *P. aeruginosa* were readily killed by chlorine, glutaraldehyde, DBNPA, or ADBAC (Figure 2). The times needed to attain a two-log reduction in viable cell numbers for planktonic cells treated with each agent are tabulated in Table 1. These times were less than 20 min for every agent tested even at the lowest concentrations. The initial cell density in experiments with planktonic cells was 6.5±0.1 log cfu ml^{-1}. There was less than a 0.1 log change in the number of viable cells in untreated control experiments.

Biofilm bacteria were less susceptible to killing compared to planktonic cells for all four antimicrobial agents (Figure 2, Table 1). For the lowest concentrations of glutaraldehyde (25 mg l^{-1}) and DBNPA (1 mg l^{-1}) tested, no killing of biofilm bacteria could

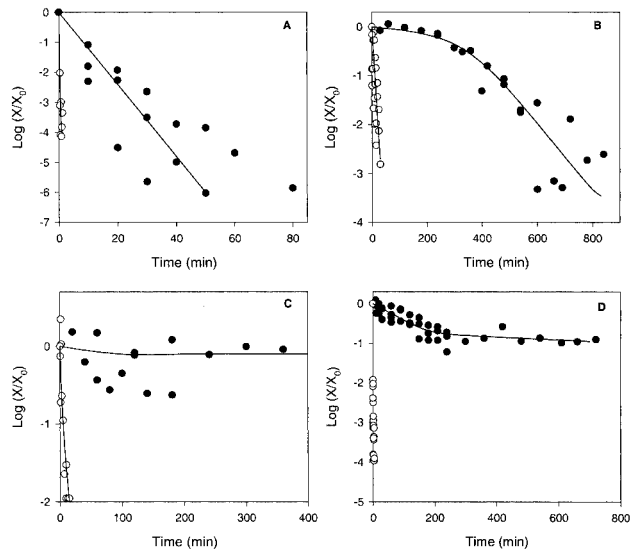

Figure 2 Comparison of bacterial killing in the planktonic (open symbols) and biofilm (filled symbols) states in response to treatment by 20 mg l^{-1} chlorine (A), 50 mg l^{-1} glutaraldehyde (B), 1.0 mg l^{-1} DBNPA (C), and 50 mg l^{-1} ADBAC (D). The ratio X/X_0 is the viable cell density at a particular time divided by the viable cell density at time zero.

Table 1 Biofilm resistance factors of *P. aeruginosa* artificial biofilms

Antimicrobial concentration (mg l^{-1})	Planktonic kill time (min)	Biofilm kill time (min)	Resistance factor
Chlorine			
10	0.44±0.25	127±8	290
20	0.31±0.22	15±6	47
90	0.17±0.04	12±0.3	11
Glutaraldehyde			
25	17.9±2.5	842[a]	47
50	18.2±9.2	647±75	36
100	8.8±3.9	174±59	20
200	ND	34±5.5	
250	2.4±1.1	ND	
DBNPA			
1.0	12.0±5.1	540[a]	45
1.8	ND	303±95	
2.4	6.0	72±39	12
4.0	ND	8.7±7.7	
12.6	ND	5.4	
ADBAC			
50	0.30±0.16	636±371	2160
100	0.11±0.03	225±7	2000
250	0.10±030	157±47	1500
500	ND	71±19	
1000	ND	74±28	

Kill time indicates the time required to achieve a two-log reduction in viable cell numbers. The resistance factor is the biofilm kill time divided by the planktonic kill time.
[a]Extrapolated. ND denotes not determined.

be discerned even after prolonged exposure to the agent for nearly 10 h (Figure 3). The same concentrations of these biocides were sufficient to kill free-floating bacteria quickly (Table 1). The degree of biofilm resistance was quantified by a resistance factor that

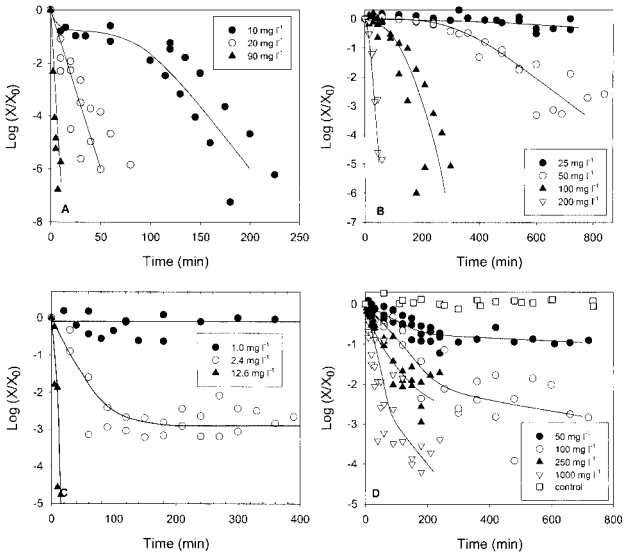

Figure 3 Bacterial killing in gel bead artificial biofilms in response to treatment with chlorine (A), glutaraldehyde (B), DBNPA (C), and ADBAC (D). The ratio X/X_0 is the viable cell density in the gel bead at a particular time divided by the viable cell density at time zero. The untreated control is shown as open squares in panel D.

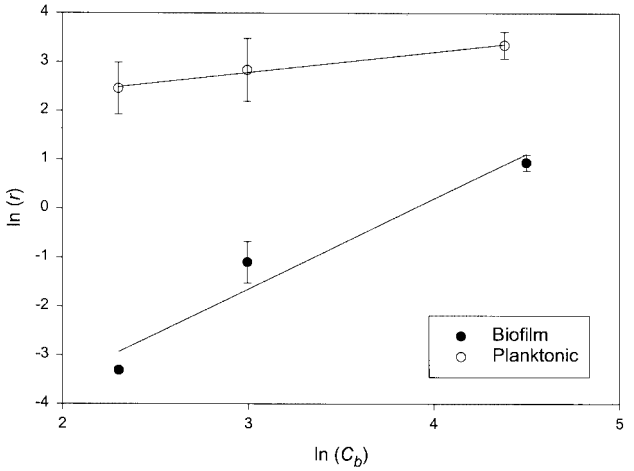

Figure 4 Analysis of the apparent rate of bacterial killing by chlorine (r) as a function of applied chlorine concentration, (C_b). The lines are least squares regressions and the slope of each line is the parameter n reported in Table 2. C_b has units of mg l^{-1} and r has units of min^{-1}.

compared killing times for biofilm and planktonic cells in response to the same concentration of antimicrobial agent (Table 1). For example, while it took approximately 6 min to achieve a two-log reduction in viable cell numbers of planktonic cells in experiments using 2.4 mg l^{-1} DBNPA, more than an hour was required to achieve this same level of killing in the biofilm. Resistance factors averaged 120 for chlorine (range 11 to 290), 34 for glutaraldehyde (20 to 47), 29 for DBNPA (12 to 45), and 1900 for ADBAC (1500 to 2160). In an untreated control, the number of viable bacteria in artificial biofilms remained unchanged over 10 h ($p=0.58$).

Biofilm bacteria became progressively more susceptible to killing as the concentration of the antimicrobial agent increased (Figure 3). This phenomenon was evident in the values of biofilm resistance factors, which decreased with increasing concentration of the antimicrobial agents (Table 1). An independent analysis of the concentration dependence of the apparent rates of killing of planktonic and biofilm bacteria showed that elevating the treatment concentration increased bacterial killing more in the biofilm than it did in a suspension culture. An example of this analysis is shown in Figure 4 and the n values thus derived are summarized in Table 2. The parameter n describes the dependence of the killing rate on antimicrobial concentration. For example, if n equals 1, then the rate of killing increases in direct linear proportion to the biocide concentration. On the other hand, if n equals 2, the rate of killing is proportional to the square of the biocide concentration. Doubling the antimicrobial concentration in this case would quadruple the rate of kill. Whereas the n values for planktonic bacteria were all less than or equal to 1, n values for all of the antimicrobials except ADBAC exceeded 1 for biofilm bacteria (Table 2). Values of n for biofilm bacteria were greater

Table 2 Values of the antimicrobial agent concentration dependence parameter, n

Antimicrobial agent	n, planktonic	n, biofilm
Chlorine	0.31±0.15	1.08±0.25
Glutaraldehyde	1.01±0.19	2.13±1.9
DBPNA	0.72	1.92±0.31
ADBAC	0.42±0.20	0.72±0.14

The uncertainty given is the standard error of the mean.

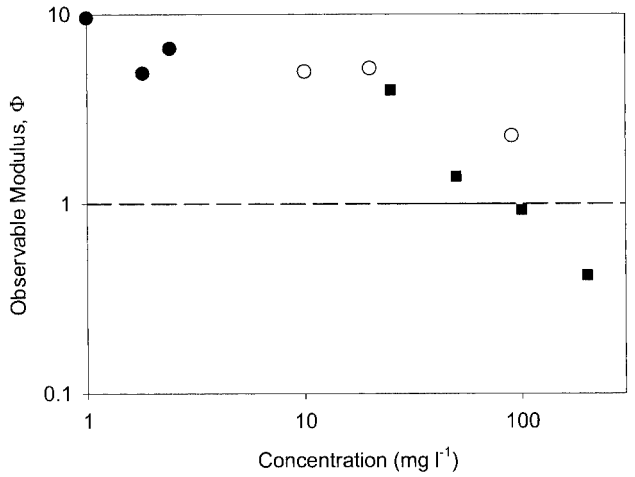

Figure 5 Mean values of the observed modulus, Φ, for chlorine (open circle); glutaraldehyde (filled square), and DBNPA (filled circle).

than the corresponding values for planktonic bacteria for each antimicrobial agent.

Antimicrobial concentrations of chlorine, glutaraldehyde, and DBNPA decreased continuously when antimicrobial solutions were incubated with artificial biofilms. In control experiments in the absence of gel beads, glutaraldehyde and DBNPA concentrations were stable over several hours. It was concluded from these results that glutaraldehyde and DBPNA react with constituents of the artificial biofilm and are neutralized by these reactions. Measurements of antimicrobial agent concentrations were used to calculate observed reaction rates for these agents. In the control experiment with chlorine, a slight loss of antimicrobial was measured in the absence of gel beads. This loss was approximated by a first-order decay term and this was subtracted from the overall loss of chlorine when incubated with artificial biofilm to estimate the reaction rate between chlorine and gel bead biofilm. Reaction rates for ADBAC were not determined as no reliable assay for the active concentration of this chemical was readily available.

The decrease in measured bulk fluid antimicrobial agent concentration never exceeded more than about 20% of the nominal applied concentration because the solution was periodically replenished. The bulk fluid biocide concentration can therefore be considered to be approximately constant in time for the purposes of analysis of rates of bacterial killing.

Experimentally determined reaction rates were used to calculate an observable modulus that compares the relative rates of reaction and diffusion. Calculation of this modulus is one way to diagnose the extent of penetration of the antimicrobial agent into the biofilm. As Φ exceeds 1, incomplete penetration of the agent is indicated. Mean values of Φ for chlorine, glutaraldehyde, and DBNPA generally exceeded 1 (Figure 5). The value of Φ decreased with increasing concentration for chlorine and glutaraldehyde but not for DBNPA. The value of Φ decreased as experiments progressed for chlorine and glutaraldehyde but remained relatively constant in experiments with DBNPA (data not shown).

Discussion

Bacteria entrapped in alginate gel beads captured the phenotypic tolerance to antimicrobial agents that is nearly universally observed in natural biofilms. The mean diameter of these artificial biofilms was 2.4 mm. From the standpoint of reaction-diffusion interactions, this spherical particle can be considered to be equivalent to a flat slab biofilm of thickness one-third the radius of the sphere [1]. The equivalent thickness in this case would therefore be 400 μm. Bacteria were densely aggregated in the gel beads, especially near the surface, just as they are in natural biofilms. The gel bead artificial biofilms described here capture the reduced susceptibility to killing by antimicrobial agents that is a hallmark of the biofilm mode of growth. The magnitude of bacterial resistance measured in alginate gel beads is comparable to the degree of resistance measured in other biofilm models [15] and is consistent with previous investigations using gel-entrapped biofilms [5,8,13,21-23]. Gel bead artificial biofilms provide a flexible, efficient, and repeatable, experimental system for investigating some aspects of antimicrobial efficacy against biofilms. These aspects include, as discussed below, the extent of transport limitation of biocide penetration into the biofilm and the concentration dependence of biocide action.

Chlorine, glutaraldehyde, and DBNPA react with constitutents of alginate gel bead biofilms and are neutralized by these reactions. While the chemistry of these reactions remains uncharacterized, their effect is to retard the penetration of the antimicrobial into the biofilm. This phenomenon appears to be consistent with the reaction-diffusion interactions of antimicrobial agents in biofilms that have been discussed elsewhere [13,14,16]. At least part of the observed biofilm resistance to chlorine, glutaraldehdye, and DBNPA can be attributed to incomplete or slow penetration of these agents into the biofilm. This interpretation is supported by the shapes of time-kill curves, which were markedly nonlinear in some biofilm experiments (Figure 3). Mathematical modeling of two different biofilm resistance mechanisms predicts distinct killing trajectories [4]. The shapes of the time-kill curves measured in this work implicated retarded antimicrobial penetration for chlorine and glutaraldehyde and the possible presence of a resistant subpopulation for DBNPA and ADBAC. The presence of a resistant subpopulation is indicated for low concentrations of DBNPA, but is not evident at the higher concentrations of this agent that were tested.

The relative efficacy of the four biocides studied against biofilm bacteria was concentration dependent. Treating biofilm bacteria with elevated concentrations of an antimicrobial agent was more effective than using prolonged doses of lower concentration. For example, a 200 mg l^{-1} dose of glutaraldehyde for 34 min was as effective as a 50 mg l^{-1} dose delivered for 650 min. The dose that was four times more concentrated was able to achieve the same effect as the low concentration dose in less than one-tenth the time. This phenomenon was consistently observed for biofilms treated with glutaraldehyde but was not evident for planktonic cells treated with this antimicrobial. Improved bacterial killing in biofilms by the higher concentrations of biocides was observed qualitatively for all four biocides. This leads us to suggest that biofilms are best controlled by using relatively high concentrations of antimicrobial agents rather than prolonged doses of lower concentration.

We caution against reliance on the *Ct* concept in dealing with biofilms. This familiar rule posits that the product of biocide dose concentration and dose duration can be used as a predictor of equally effective treatments. Our data do not support this concept in the context of biofilm control.

Acknowledgements

This work was supported through cooperative agreement EEC-8907039 between the National Science Foundation and Montana State University, by The Dow Chemical and Union Carbide.

References

1 Bird RB, WE Stewart and EN Lightfoot. 1960. Transport Phenomena. Wiley, New York.
2 Cash HA, DE Woods, B McCullogh, WG Johanson and JA Bass. 1979. A rat model of chronic respiratory infection with *Pseudomonas aeruginosa*. *Am Rev Respir Dis* 119: 453–459.
3 Chen X and PS Stewart. 1996. Chlorine penetration into artificial biofilm is limited by a reaction-diffusion interaction. *Environ Sci Technol* 30: 2078–2083.
4 Dodds MG, KJ Grobe and PS Stewart. 2000. Modeling biofilm antimicrobial resistance. *Biotechnol Bioeng* 68: 456–465.
5 Gilbert P, MV Jones, DG Allison, S Heys, T Maira and P Wood. 1998. The use of poloxamer hydrogels for the assessment of biofilm susceptibility towards biocide treatments. *J Appl Microbiol* 85: 985–990.
6 Haas CN and SB Karra. 1984. Kinetics of microbial inactivation by chlorine — I. *Water Res* 18: 1443–1449.
7 Hoben HJ and P Somasegaran. 1982. Comparison of the pour, spread, and drop plate methods for enumeration of *Rhizobium* spp. in inoculants made from presterilized peat. *Appl Environ Microbiol* 44: 1246–1247.
8 Jouenne T, O Tresse and G-A Junter. 1994. Agar-entrapped bacteria as an *in vitro* model of biofilms and their susceptibility to antibiotics. *FEMS Microbiol Lett* 119: 237–242.
9 O'Reilly T. 1995. Relevance of animal models for chronic bacterial airway infection in humans. *Am J Respir Crit Care Med* 151: 2101–2107.
10 Perry RH and CH Chilton. 1973. Chemical Engineer's Handbook, 5th ed. McGraw-Hill, New York.
11 Reed RW and GB Reed. 1948. "Drop plate" method of counting viable bacteria. *Can J Res* 26: 317–326.
12 Smidsrod O and G Skjak-Braek. 1990. Alginate as immobilization matrix for cells. *Trends Biotechnol* 8: 71–78.
13 Stewart PS. 1996. Theoretical aspects of antibiotic diffusion into microbial biofilms. *Antimicrob Agents Chemother* 40: 2517–2522.
14 Stewart PS, L Grab and JA Diemer. 1998. Analysis of biocide transport limitation in an artificial biofilm system. *J Appl Microbiol* 85: 495–500.
15 Stewart PS, C-T Huang and GA McFeters. 2000. Biofilm control by antimicrobial agents. In: Bryers JD (Ed), Biofilms, 2nd ed., Wiley, New York, pp. 373–405.
16 Stewart PS and JB Raquepas. 1995. Implications of reaction-diffusion theory for the disinfection of microbial biofilms by reactive antimicrobial agents. *Chem Eng Sci* 50: 3099–3104.
17 Tresse O, T Jouenne and GA Junter. 1994. Susceptibility of agar-entrapped *Escherichia coli* cultures to cefotaxime as assessed by the potentiometric measurement of lipoic acid reduction. *J Chemother* 6: 388–391.
18 Tresse O, T Jouenne and GA Junter. 1995. The role of oxygen limitation in the resistance of agar-entrapped sessile-like *Escherichia coli* to aminoglycoside and betalactam antibiotics. *J Antimicrob Chemother* 36: 521–526.
19 Tresse O, T Jouenne and GA Junter. 1997. Antibacterial efficacy of tobramycin against anaerobic *Escherichia coli* cultures in the presence of electron acceptors. *J Antimicrob Chemother* 40: 419–421.
20 Westrin BA and A Axelsson. 1991. Diffusion in gels containing immobilized cells: a critical review. *Biotechnol Bioeng* 38: 439–446.
21 Whitham TS and PD Gilbert. 1993. Evaluation of a model biofilm for the ranking of biocide performance against sulphate-reducing bacteria. *J Appl Bacteriol* 75: 529–535.
22 Wirtanen G, S Salo, DG Allison, T Mattila-Sandholm and P Gilbert. 1998. Performance evaluation of disinfectant formulations using poloxamer-hydrogen biofilm-constructs. *J Appl Microbiol* 85: 965–971.
23 Xu X, PS Stewart and X Chen. 1996. Transport limitation of chlorine disinfection of *Pseudomonas aeruginosa* entrapped in alginate beads. *Biotechnol Bioeng* 49: 93–100.