Evidence of Bacterial Adaptation to Monochloramine in *Pseudomonas aeruginosa* Biofilms and Evaluation of Biocide Action Model

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Abstract: A mathematical model of biocide action against microbial biofilm was tested experimentally by measuring the response of *Pseudomonas aeruginosa* biofilm to various doses of monochloramine. Pure culture biofilm was developed in continuous flow annular reactors for 7 days, then treated with a 2-, 4-, or 8-h dose of 2 or 4 mg L^{-1} monochloramine. Some experiments investigated repeated treatment. Disinfection and regrowth of the biofilm were observed by sampling the biofilm for viable and total cell areal densities for up to 100 h following the biocide treatment. A phenomenological mathematical model was fitted to experimental data sets and captured overall trends, but it could not simulate certain experimentally observed features. The model did simulate rapid disinfection followed by steady regrowth. It correctly predicted a much greater decrease in viable than in total cell densities and also correctly captured the shapes of these trajectories. Discrepancies between the model and data included the following: the model predicted faster regrowth than was experimentally observed, the model predicted that a second dose would be more effective than the first dose but the opposite was observed in the experiments, and parameters estimated by fitting one dose concentration could not be used to predict the results of a different dose concentration or a second dose. Discrepancies between model and the experiment were hypothesized to be due to an adaptive stress response by the bacteria, a process not included in the model. A practical implication of this work is that it is more effective to deliver monochloramine in a short concentrated dose as opposed to a longer dose of lower concentration. *Biotechnol Bioeng* **56:** 201–209, 1997.

Keywords: adaptation; biofilm; biocide; disinfection; model; monochloramine; *Pseudomonas;* stress response

INTRODUCTION

Antimicrobial agents are one of the principal means of controlling fouling and corrosion problems associated with microbial biofilm formation (Cloete et al., 1992; Costerton et al., 1987). Literature reviews illuminate the diversity of systems in which biofilm fouling problems occur as well as the wide range of antimicrobial agents in use (Brown and Gilbert, 1993; Cloete et al., 1992; Costerton et al., 1987; Nichols, 1989). Although the use of antimicrobial agents is widespread, quantitative methods for biocide selection and for the design of efficient biocide delivery protocols do not exist. This gap represents an opportunity for biochemical engineers to become involved in developing engineering approaches for analyzing and predicting biofilm control phenomena. Engineering contributions should be helpful particularly in view of the fact that transport processes play a role in limiting biocide efficacy against biofilm in some cases (Chen and Stewart, 1996; de Beer et al., 1994; Xu et al., 1996).

One such engineering approach is mathematical modeling of the action of a biocide or antibiotic against a biofilm. Stewart et al. recently described a model that integrates key constituent phenomena of this interaction: microbial growth, detachment, disinfection, solute transport, and reaction (Stewart, 1994; Stewart et al., 1996). The purpose of the work reported in the present article was to evaluate this mathematical model experimentally. We have done this by performing a series of experiments in which a pure culture *Pseudomonas aeruginosa* biofilm was treated with monochloramine doses of various concentrations and durations.

MATERIALS AND METHODS

Microorganism and Culture Conditions

Pseudomonas aeruginosa (ERC1) was grown in pure culture on a minimal salts medium with 20 mg L^{-1} glucose as the sole carbon source (Chen et al., 1993). Experiments were conducted at room temperature, 25 ± 1°C.

Reactor System and Operation

Biofilms were grown on a dozen 316L stainless steel slides fitted in recesses in the outer cylinder of a continuous flow

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rotating annular reactor. A detailed description of the reactor and operating conditions can be found elsewhere (Chen et al., 1993). Tracer studies have shown that the mixing characteristics in this system closely approximate those of an ideal continuous stirred tank reactor (Gjaltema et al., 1994). Biofilm was sampled without interruption of nutrient flow or inner cylinder rotation by removing the steel slides through stoppered holes in the top of the reactor. The inner cylinder rotated at 150 rpm giving rise to turbulent flow and good mixing. The dilution rate of 3.2 h^{-1} exceeded the maximum specific growth rate of the microorganism (0.35 h^{-1}) sufficiently to ensure that biofilm activity predominated over that of planktonic cells. Concentrated glucose and mineral salt solution, phosphate buffer, and dilution water were continuously fed to the reactor at rates of 60, 60, and 1800 mL h^{-1}, respectively. The reactor was inoculated with 1.0 mL of thawed stock culture (10^8 cells mL^{-1}) and grown in batch mode for 24 h. Influent flows were restarted and the system was run for 7 days before sampling or dosing biocide.

Monochloramine Preparation and Treatment

A fresh monochloramine stock solution was prepared for each experiment by combining ammonium chloride with household chlorine bleach as described previously (Chen et al., 1993). Monochloramine concentration was spectrophotometrically determined in reactor effluent samples using the *N*,*N*-diethyl-*p*-phenylenediamine (DPD, Hach Co.) method. Double distilled water was used to dilute the stock solution to the required concentration. Monochloramine was added by replacing the flow of buffer solution with the monochloramine stock solution for a predetermined dose period. The monochloramine demand of the sterile system was negligible (Griebe et al., 1994).

Cell Enumeration

Biofilm covered slides were removed using aseptic sampling techniques at various times during the experiments. They were scraped into 100 mL of phosphate buffer containing 0.1% sodium thiosulfate to neutralize residual monochloramine. The solution was homogenized for 2 min on full power using a tissue homogenizer. Viable cells were enumerated by performing serial dilutions and plating on Difcobacto R2A agar using the drop plate method. Total cells were determined by acridine orange direct counts (Chen et al., 1993). Cell numbers were reported as areal cell densities. Effluent samples were obtained through a 3-way stopcock in the effluent tubing.

Data Analysis

Experimental data were analyzed to determine the regrowth rate of the biofilm following biocide treatment, the overall reaction rate of monochloramine in the reactor, and the degree of transport limitation of monochloramine penetration into the biofilm. Regrowth rates were calculated as the slope of the least-squares regressed line through natural log transformed viable cell count versus time data for the post biocide dose period.

The overall reaction rate of monochloramine was calculated from the following material balance:

$$V\frac{dC_b^*}{dt} = Q(C_b^i - C_b^*) - r_B, \tag{1}$$

where C_b* is the experimentally measured bulk monochloramine concentration, C_b^i is the influent monochloramine concentration, and r_B is the desired overall reaction rate of monochloramine. The time derivative was estimated from a forward difference formula.

The importance of diffusive transport limitation was assessed by calculating an observable modulus (Bailey and Ollis, 1986):

$$\Phi = \frac{R_o L_f^2}{C_b^* D_b}, \tag{2}$$

where

$$R_o = \frac{r_B}{AL_f}. \tag{3}$$

The observable modulus was calculated at three points during the biocide dose corresponding to one-quarter, one-half, and three-quarters of the dose duration. The interpretation of Φ is that diffusive transport becomes a factor as this dimensionless group exceeds 1; when Φ is less than 1, diffusion does not significantly limit the overall reaction rate.

Modeling

A previously described phenomenological computer model of biocide action against biofilm was modified slightly for this investigation (Stewart et al., 1996). The model incorporated processes of bulk flow in and out of a well-mixed reactor, transport of dissolved species into the biofilm, substrate consumption by bacterial metabolism, bacterial growth, advection of cell mass within the biofilm, cell detachment from the biofilm, biocide neutralization by reaction with the biofilm, and biocide concentration-dependent disinfection. In the original model, the rate of biocide neutralization was assumed to depend only on the local biocide concentration. The neutralizing capacity of the biomass was not diminished by its reaction with the biocide. In the present model, the rate of biocide neutralization was modeled as first order in both biocide concentration and the concentration of a component of biomass termed the ''neutralizer.'' In the new version of the model, the disappearance of biocide and neutralizer is stoichiometrically coupled. This formulation is supported by experimental results for the interaction of free chlorine with *P. aeruginosa* biomass

(Chen and Stewart, 1996). The revised biocide balance within the biofilm is

$$\frac{\partial C_b}{\partial t} = D_b \tau \frac{\partial^2 C_b}{\partial z^2} - k_r C_b \varepsilon_n \rho_x, \quad (4)$$

which replaces equation (12) in Stewart et al. (1996). The balance on the neutralizer is

$$\frac{\partial \varepsilon_n}{\partial t} = 0.0417 \frac{\mu_{max} C_s}{K_s + C_s} \varepsilon_n - \frac{\partial}{\partial z}(v \varepsilon_n) - \frac{k_r C_b \varepsilon_n}{Y_{bn}}, \quad (5)$$

where the terms on the right-hand side reflect production by growth, particulate displacement or advection, and consumption by reaction with biocide, respectively. The initial condition on the neutralizer arbitrarily assumed that it constituted 4% of biofilm biomass and was uniformly distributed within the biofilm. The factor of 0.0417 in Eq. (4) simply ensures that the neutralizer content of biomass is maintained at 4% in the absence of biocide.

The model was implemented with the program AQUASIM (Reichert, 1994; Wanner and Reichert, 1996). Parameter values and their sources are listed in Table I.

Parameter Estimation by Data Fitting

One of the attractive features of AQUASIM is its ability to perform parameter estimation by fitting imported experimental data. This is done with a weighted least-squares method. The parameter estimation component of the program was used to generate simultaneous estimates of three parameters: k_r, k_b, and Y_{bn}. Parameter values were estimated independently for each experiment by fitting viable and total cell versus time data for the disinfection and regrowth periods. Effluent total chlorine versus time data for the dose and washout period were simultaneously fit.

RESULTS

Disinfection and Regrowth

P. aeruginosa biofilm was treated with monochloramine doses of different concentrations and durations in nine experiments summarized in Table II. Three of the experiments implemented double dose protocols. In all cases, monochloramine treatment caused transient reductions in viable and total cell areal densities.

Figure 1 illustrates the behavior observed in duplicate experiments using a 4 mg L^{-1}, 2-h dose. Good reproducibility was attained. With this particular dosing protocolan initial 3–4.5 log decrease in viable cell numbers was observed within the first 4 h after biocide addition. The reduction in total cells was much less, only about 1 log, and the minimum in the total cell areal density occurred nearly 40 h after the minimum in viable cells. Without additional treatment, the biofilm regrew to its initial areal density after approximately 3 days.

Different biocide dosing protocols resulted in different reductions in areal cell densities, even when the total amount of biocide added was the same. For example, a 2 mg L^{-1}, 8-h dose gave rise to a 0.9 log reduction in viable cell areal density, whereas a 4 mg L^{-1}, 4-h dose resulted in a 5.3 log decrease. This difference in efficacy between doses con-

Table I. Parameter input values for biofilm modeling.

Parameter	Symbol	Value	Source
maximum specific growth rate	μ_{max}	0.3 hr^{-1}	Characklis, 1990
yield coefficient	Y_{xs}	0.34 g g^{-1}	Robinson et al, 1984
Monod coefficient	K_s	2.0 g m^{-3}	Robinson et al, 1984
cell volume fraction	ε_c	0.096	Drury et al, 1993
cell intrinsic density	ρ_x	44,500 g m^{-3}	Peyton and Characklis, 1993
initial biofilm thickness	L_f	30 μm	Murga et al, 1995 Stewart et al, 1993
conc. boundary layer thickness	L_L	10 μm	Christensen and Characklis, 1990
substrate influent concentration	C_s	20 g m^{-3}	Experimental parameter[a]
biocide influent concentration	C_b	2, 4 g m^{-3}	Table II
biocide dose duration	t_b	2, 4, 8 hr	Table II
substrate diffusion coefficient	D_s	2.5×10^{-6} m^2 hr^{-1}	Perry and Chilton, 1973
biocide diffusion coefficient	D_b	6.0×10^{-6} m^2 hr^{-1}	Chen et al, 1993
biofilm/bulk diffusivity ratio	τ	0.9	Westrin and Axelsson, 1991
reactor liquid volume	V	5.96×10^{-4} m^3	Peyton and Characklis, 1993
biofilm surface area	A	0.184 m^2	Gjaltema et al, 1994 Peyton and Characklis, 1993
volumetric flow rate	Q	1.92×10^{-3} m^3 hr^{-1}	Gjaltema et al, 1994 Peyton and Characklis, 1993
biocide disinfection rate coeff.	k_b	m^3 g^{-1} hr^{-1}	b
biocide reaction rate coefficient	k_r	m^3 g^{-1} hr^{-1}	b
neutralizer/biocide yield coeff.	Y_{bn}	g g^{-1}	b

[a]Experimentally set operating parameter.
[b]Parameter value estimated by fitting; see text.

Table II. Biocide dosing protocols.

Experiment no.	1st dose		2nd dose		Time between doses (h)
	C_b^i (mg L^{-1})	t_b (h)	C_b^i (mg L^{-1})	t_b (h)	
3	4	2	—	—	—
4	4	2	—	—	—
6	0	2	—	—	—
10	2	4	—	—	—
12	2	8	—	—	—
14	4	4	—	—	—
17	4	2	4	2	40
18	2	4	4	2	0
19	4	2	4	2	60

C_b^i is the influent monochloramine concentration and t_b is the dose duration.

taining the same total amount of monochloramine is illustrated graphically in Figure 2, which plots the maximum observed log reduction in viable cells for each biocide dose versus the product of concentration and time ($C_b{}^i t_b$). Very little reduction in either viable or total cells occurred with the low concentration dose (2 mg L^{-1}), regardless of the dose duration.

Table III presents the maximum reductions in viable and total cells and the times that these minima occurred for all experiments. The reduction in total cells was always much less (average 0.8 log) than the reduction in viable cells (average 2.5 log). The minimum value of total cell areal density always occurred later (average 26 h after dose) than the minimum value of viable cell areal density (average 9 h after dose).

In double dose experiments, the second dose was always less effective than if the same dose was applied to previously untreated biofilm (Table III, Fig. 2). For example, when two 4 mg L^{-1}, 2-h doses were administered to a biofilm 40 h apart, the second dose resulted in only about half as much disinfection as was achieved by the first dose (Fig. 3). A similar reduction in efficacy of a 4 mg L^{-1}, 2-h dose was observed when it was immediately preceded by a 2 mg L^{-1}, 4-h dose (see Table III, Exp. 18).

Decreases in viable cell areal density after biocide treatment were followed by relatively steady regrowth. Biofilms returned to their initial cell densities approximately 3 days after biocide treatment. Regrowth rates ranged from 0.046 to 0.21 h^{-1} and averaged 0.10 h^{-1} (Table III). Regrowth rates in single dose experiments or following a first dose (average 0.12 h^{-1}) were somewhat higher than the regrowth rates observed after a second dose (average 0.075 h^{-1}).

Biocide Reaction

Added monochloramine was partially neutralized by reaction with the biofilm. Representative effluent total chlorine versus time data are plotted in Figures 4 and 5 along with the reaction rate of monochloramine that was calculated from this data. Very different trends in the monochloramine

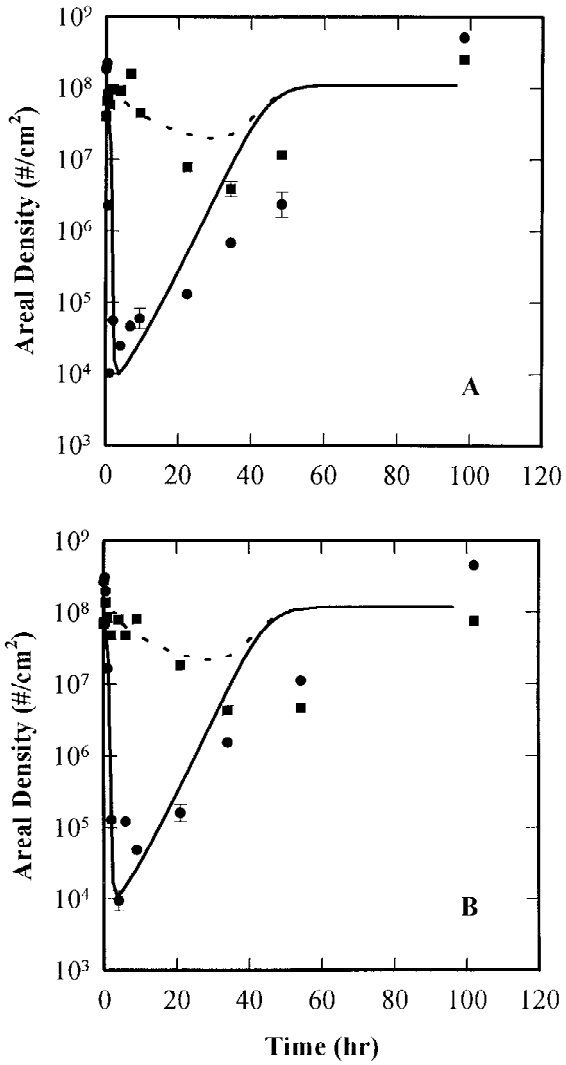

Figure 1. Experimental and fitted model results for a 7-day-old *Pseudomonas aeruginosa* biofilm treated with 4 mg L^{-1} monochloramine for 2 h: (A) experiment 3; (B) experiment 4. Experimental cell areal densities are denoted by symbols and model fits are denoted by lines: (●, —) viable cell areal density; (■,---) total cell areal density. Error bars are shown on three points to indicate the statistical reliability of the plotted means. (A) The first error bar on a viable cell point represents the SEM for three dilutions. The second error bar on a viable cell point represents the SEM for two dilutions. The total cell error bar represents the SEM for 10 fields counted. (B) The first error bar on a viable cell point represents the SEM for two dilutions. The second error bar on a viable cell point represents the SEM for three dilutions. The total cell error bar represents the SEM for 10 fields counted.

reaction rate were observed depending on the dose concentration. With a 4 mg L^{-1} dose (Fig. 4), a characteristic breakthrough curve that peaked close to the influent concentration was seen. The monochloramine reaction rate decreased over time. The observation that monochloramine concentration was increasing at the same time that the reaction rate was decreasing suggests that the reaction rate was controlled by the depletion of neutralizing capacity in the biofilm. In contrast, when a 2 mg L^{-1} dose (Fig. 5) was applied, the monochloramine concentration in the effluent

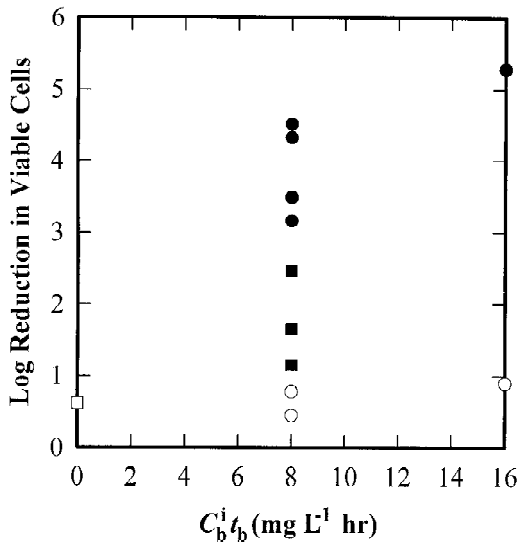

Figure 2. Maximum log reduction in viable cell areal density in response to a monochloramine dose as a function of the product of the dose concentration and duration (CT). The symbols indicate a (●) 4 mg L^{-1} dose, (○) 2 mg L^{-1} dose, (□) untreated control, and (■) second 4 mg L^{-1} dose.

never rose above 25% of the influent concentration. In fact, after the first hour of treatment, the monochloramine concentration decreased even though monochloramine was still being added. The reaction rate rose during the first hour of treatment then remained approximately constant through the remainder of the dose. The behavior observed with the 2 mg L^{-1} dose suggests that the neutralizing capacity of the biofilm at least remained constant, and possibly even increased, during the treatment.

Overall monochloramine reaction rates (r_B, mg h^{-1}) during second doses were lower than during first doses. Specific monochloramine reaction rates (mg h^{-1} divided by total cell areal density) were actually higher, by factors ranging from 1.7 to 3.9, during second doses than during a first dose (Fig. 6).

Modeling

Model fits to experimental data sets captured overall trends but could not simulate certain experimentally observed features. The model did simulate rapid disinfection followed by steady regrowth (Fig. 1). It correctly predicted a much greater decrease in viable than in total cell densities and also the delayed minimum in total cell density. Discrepancies between the model and data included the following: the model predicted faster regrowth than was experimentally observed (Fig. 1); the model predicted that a second dose would be more effective than the first dose, but the opposite was observed in experiments (Fig. 3); parameters estimated by fitting one dose concentration could not be used to predict the results of a different dose concentration or a second dose; and the model could not simulate the shape, even qualitatively, of the effluent total chlorine versus time data for the 2 mg L^{-1} monochloramine dose experiments (Fig. 5).

The regrowth rate predicted by the model was essentially the maximum specific growth rate of the microorganism, 0.30 h^{-1}. The magnitude of this growth rate has been independently confirmed by several experimenters (Characklis, 1990). Experimentally observed regrowth rates averaged 0.10 h^{-1}, just one-third of the maximum specific growth rate.

When the parameters extracted from the fit to a first dose are used to make an a priori prediction of the response of the biofilm to an identical second dose, the model overestimates the efficacy of the second dose (Fig. 3). The model predicts that the second dose should be more effective than the first dose because the second dose is timed to occur when the biofilm has not yet fully recovered. Because the biofilm is thinner when the second dose is applied, there is less capacity to neutralize biocide and the system is expected to experience higher biocide concentrations. The experimentally measured biocide concentration during the second dose was indeed the same or higher than that found for the first dose, but the degree of disinfection effected by the second dose was always less than that effected by the first dose.

Data fitting generated estimates of three parameters for each experiment: the disinfection rate coefficient (k_b), the biocide/neutralizer reaction rate coefficient (k_r), and the biocide/neutralizer yield coefficient (Y_{bn}). Estimates of these parameters, especially k_r and Y_{bn}, varied widely from experiment to experiment (Table IV). The estimated value of the disinfection rate coefficient (k_b) was relatively stable, ranging from 0.9 to 2.0 m^3 g^{-1} h^{-1} and averaging 1.7 ± 0.3 m^3 g^{-1} h^{-1}. The estimated value of the reaction rate coefficient (k_r) ranged by a factor of nearly 8 from 27 to 200 m^3 g^{-1} h^{-1}. It was highest for the 4 mg L^{-1} dose concentration experiments and lower for the 2 mg L^{-1} experiments. The estimated yield coefficient Y_{bn} (grams monochloramine consumed per gram of neutralizing biomass consumed) ranged from 2 to 35 g g^{-1}, a factor of 17 difference. Y_{bn} was low for the 4 mg L^{-1} dose concentration experiments and higher for the 2 mg L^{-1} experiments. This last result indicates that the biofilm was able to neutralize more monochloramine when exposed to a lower concentration. As mentioned above, when parameters estimated by fitting one dose concentration were used to make a priori predictions of the results of a different dose concentration or a second dose, very poor agreement was obtained (not shown).

The model was able to capture well the shape of the effluent total chlorine versus time data for the 4 mg L^{-1} monochloramine dose concentration (Fig. 4). The model was not able to reproduce, with any parameter combination, the decrease in monochloramine concentration that was experimentally observed after the first hour of biocide addition for the 2 mg L^{-1} monochloramine dose concentration (Fig. 5).

Transport Limitation

Average values of a dimensionless modulus comparing the relative rates of reaction and diffusion ranged from 0.01 to

Table III. Summary of experimentally observed total and viable cell minima in biofilm, regrowth rates, average total chlorine concentrations in reactor, and observable modulus values.

Experiment no.	Maximum log reduction		Time after dose of minimum values (h)		Regrowth rate (h^{-1})	Avg. chlorine concn. (mg L^{-1})	Observable modulus, Φ
	Total cells (cells cm^{-2})	Viable cells (cfu cm^{-2})	Total cells	Viable cells			
3	1.62	4.33	34.3	1.0	0.11	2.99	0.16
4	1.51	4.52	34.0	4.0	0.10	2.19	0.34
6	0.44	0.62	6.3	6.3	—	0.00	—
10	0.26	0.45	4.0	2.0	0.21	0.11	1.43
12	0.85	0.90	6.0	12.2	0.12	0.19	1.16
14	0.87	5.28	10.3	22.0	0.10	3.02	0.01
17, 1st dose	0.16	3.16	39.5	15.0	0.09	1.87	0.18
17, 2nd dose	0.76	1.65	63.5	12.0	0.07	2.62	0.02
18, 1st dose	0.18	0.78	3.8	3.8	—	0.31	0.54
18, 2nd dose	0.65	1.93	20.0	2.0	0.05	1.68	0.13
19, 1st dose	1.04	3.49	59.5	14.0	0.08	1.65	0.20
19, 2nd dose	0.48	2.47	58.5	12.0	0.11	3.10	0.01
Average	0.77 ± 0.45	2.5 ± 1.7	26 ± 23	8.9 ± 6.6	0.10 ± 0.02	—	—

1.43 (Table III). In general, the observable modulus was less than 1, indicating that penetration of monochloramine into the biofilm was not limiting in these experiments. In two of the 2 mg L^{-1} monochloramine dose experiments the average modulus was slightly higher than 1. The maximum value of the modulus during any experiment was 2.4 during one of the 2 mg L^{-1} dose experiments. Model simulation of the monochloramine concentration profile within the biofilm for a 2 mg L^{-1} dose experiment indicated no more than an 11% drop in monochloramine concentration from the bulk fluid to the biofilm substratum (not shown).

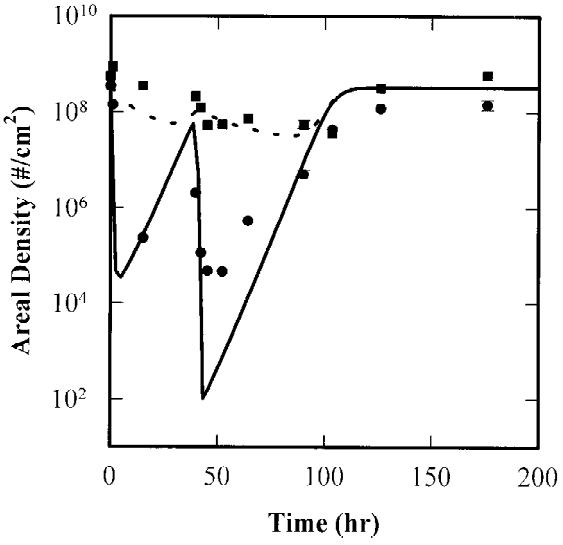

Figure 3. Experimental and model results for a 7-day-old *Pseudomonas aeruginosa* biofilm treated with two monochloramine doses 40 h apart, experiment 17. Experimental cell areal densities are denoted by symbols and model fits are denoted by lines: (●, —) viable cell areal density; (■,---) total cell areal density. The model results are fits to the data up to the beginning of the second dose (at 40 h) and a priori predictions using the same parameter values for the remainder of the data. Error bars (barely discernable) are shown on three points to indicate the statistical reliability of the plotted means. The first error bar on a viable cell point represents the SEM for two dilutions. The second error bar on a viable cell point represents the SEM for three dilutions. The total cell error bar represents the SEM for 10 fields counted.

DISCUSSION

A phenomenological computer model of biocide action against microbial biofilm captured qualitative trends of experimental evaluation data but was not predictive. The model did simulate the rapid decrease of viable cells immediately following a biocide dose and the subsequent regrowth of the biofilm. The model also captured the general

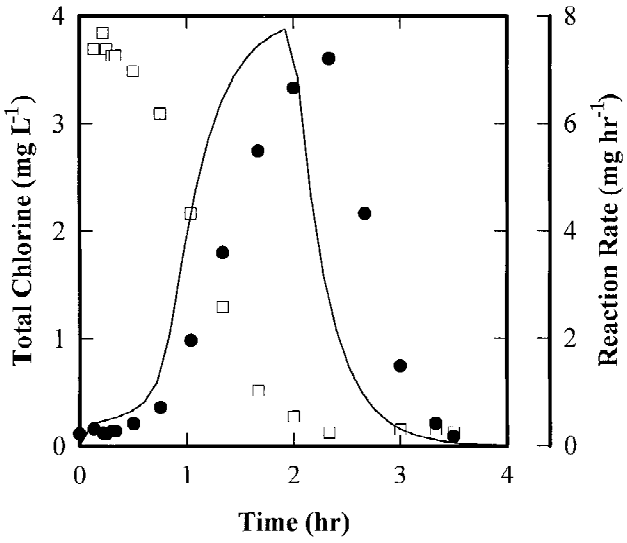

Figure 4. (●) Effluent total chlorine detected during a 4 mg L^{-1}, 2-h monochloramine dose and (□) the overall reaction rate of monochloramine with the biofilm during treatment, experiment 4. (—) The model fit to the effluent total chlorine data.

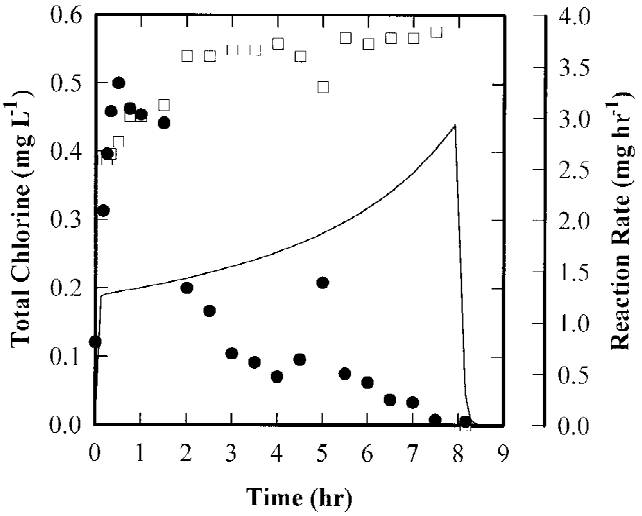

Figure 5. (●) Effluent total chlorine detected during a 2 mg L^{-1}, 8-h monochloramine dose and (□) the overall reaction rate of monochloramine with the biofilm during treatment, experiment 12. (—) The model fit to the effluent total chlorine data.

shape of the total cell trajectory. Specific failings of the model included overestimation of the biofilm regrowth rate, overestimation of the efficacy of second doses, inability to extract a set of universally applicable values for key parameters, and inability to capture even the qualitative shape of the effluent total chlorine versus time data for a 2 mg L^{-1} dose. The level of agreement seen between model and experiment suggests that certain parts of the model are probably adequate but other parts are inadequate or missing altogether.

We hypothesize that all of the deficiencies of the model can be attributed to an adaptive stress response by the bacteria, a process not included in the model. This putative adaptation has two aspects: increased biocide neutralizing

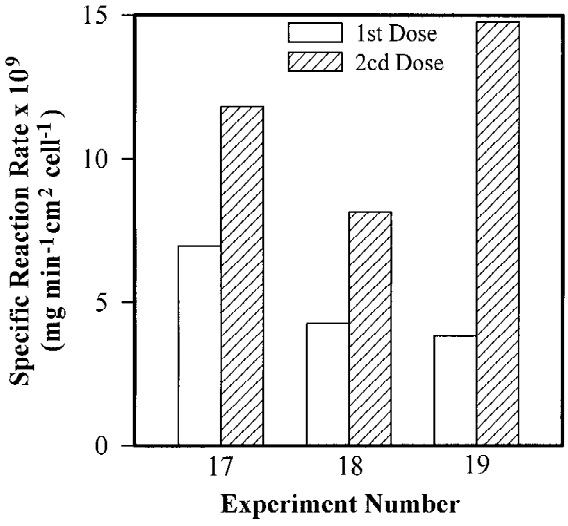

Figure 6. Comparion of specific monochloramine reaction rates during first and second doses.

Table IV. Parameter values estimated by data fitting.

Experiment no.	k_r (L mg^{-1} h^{-1})	k_b (L mg^{-1} h^{-1})	Y_{bn} (g g^{-1})
3	100	1.95	4.50
4	100	1.95	4.50
10	29	2.00	35.0
12	27	1.70	35.0
14	200	0.90	2.00
17, 1st dose	100	1.95	4.50
17, 2nd dose	50	1.70	8.00
18, 1st dose	29	2.00	35.0
18, 2nd dose	40	1.40	7.00
19, 1st dose	100	1.95	4.50
19, 2nd dose	50	1.50	6.00

capacity and reduced disinfection susceptibility. The first of these features we propose has the following characteristics. When the monochloramine concentration is low, less than approximately 0.5 mg L^{-1}, the bacteria are able to induce increased production of monochloramine-neutralizing biomass constituents. When the monochloramine concentration is above approximately 0.5 mg L^{-1}, the bacteria are overwhelmed and the induction of increased neutralizing capacity is prevented or is ineffectual. This scenario would explain why monochloramine concentration decreases during the dose period for 2 mg L^{-1} doses (Fig. 5). This feature of the 2 mg L^{-1} dose experiments is impossible to simulate with any model that assumes constant or decreasing neutralizing capacity in the biofilm. Induction of increased neutralizing capacity in the 2 mg L^{-1} dose experiments is consistent with the increased values of the biocide/neutralizer yield coefficient, Y_{bn}, obtained in these experiments. Estimates of Y_{bn} averaged 35 g g^{-1} in 2 mg L^{-1} dose experiments and 5 g g^{-1} in 4 mg L^{-1} dose experiments (Table III). Finally, induction of neutralizing capacity explains why the 2 mg L^{-1} doses are less effective at disinfecting the biofilm compared to the 4 mg L^{-1} doses (Fig. 2). The time scale for this induction to occur is approximately 1–2 h, based on the time after dose initiation when the monochloramine concentration begins to decrease (Fig. 5).

The observation that specific monochloramine reaction rates are higher during second doses than during first doses (Fig. 6) further supports the hypothesis of an induced increase in neutralizing capacity. These data suggest that the induced state is maintained for at least 40–60 h after the first exposure to monochloramine.

A second feature of the hypothesized adaptive response is that cells that have been exposed to monochloramine acquire reduced susceptibility to disinfection. Evidence for this is most clearly seen in the double dose experiments (Fig. 2). The estimated value of the disinfection rate coefficient, k_b, averaged 2.0 L mg^{-1} h^{-1} for 4 mg L^{-1}, 2-h first doses and 1.5 L mg^{-1} h^{-1} for 4 mg L^{-1}, 2-h second doses. Prolonged doses at both dose concentrations (2 mg L^{-1}, 8 h, and 4 mg L^{-1}, 4 h) also gave rise to reduced estimates of k_b compared to shorter doses (Table IV). For example, the disinfection rate coefficient estimated for the 4 mg L^{-1}, 4-h

dose was 0.9 L mg^{-1} h^{-1}, which is just half the value estimated for 4 mg L^{-1}, 2-h doses. One interpretation of this result is that the disinfection rate coefficient decreased dramatically in the second half of the 4 mg L^{-1}, 4-h dose. Based on the prolonged dose experiments, the time scale of the phenotypic response is as fast as 2–4 h. This suggests that the phenomenon is indeed an adaptive response rather than selection of a mutant strain.

We speculate that the relatively slow experimentally measured regrowth rates, which were only one-third of the maximum specific growth rate, were also due to a bacterial stress response. Microorganisms that express new genes to be able to counteract a chemical challenge must divert resources away from the process of reproduction; it would not be surprising if they were not able to grow at their maximum rate.

The phenomenon of bacterial adaptation to industrial biocides has been extensively investigated by Brözel and coworkers (Brözel et al., 1993). Although they have not studied monochloramine or free halogens in particular, they do report significant adaptation to most agents. Thus, there is a good precedent for adaptation to disinfectants. Bacterial oxidative stress response systems are also well characterized in *Escherichia coli* and *Salmonella typhimurium* (Farr and Kogoma, 1991). It would be interesting to know if an analogous response is invoked in *P. aeruginosa* upon exposure to monochloramine. The postulated adaptive response could also be related to the phenomenon of injury (McFeters, 1990) or glutathione production (Chesney et al., 1996).

Diffusive transport of monochloramine into the biofilm was not a significant factor in limiting biocidal efficacy in the 4 mg L^{-1} dose concentration experiments. The observable modulus (Table III) averaged 0.13 for these experiments, which is small enough to rule out any significant transport limitation. In the 2 mg L^{-1} dose experiments, transport limitation was possibly a significant or transiently significant factor in determining biocidal efficacy. The observable modulus averaged 1.04 for these experiments and its maximum value was 2.4. While these numbers do not exceed 1 by much, the decrease in disinfection efficacy with the onset of transport limitation can be dramatic (Stewart and Raquepas, 1995). These conclusions regarding transport limitation of monochloramine concur with those reached in earlier work in this particular system (Chen et al., 1993). The absence or borderline presence of transport limitation of biocide efficacy in this system can be largely attributed to the fact that the biofilm was relatively thin, 30 μm.

CONCLUSIONS

Experimental investigation of monochloramine disinfection of *P. aeruginosa* biofilms showed that these bacteria adapt to prolonged or repeated treatments. The data indicate that the biofilm microorganisms increase their capacity to neutralize monochloramine and that they become less susceptible to killing by this agent after continued exposure. Quantitative kinetic descriptions of this adaptive response and extensions to mixed-population biofilms need to be formulated to be able to construct an improved mathematical model of biofilm disinfection. An immediate and practical implication of this work is that it is more effective to deliver monochloramine in a short concentrated dose as opposed to a longer dose of lower concentration. This would be expected to be the case for any biocide that microorganisms can adapt to.

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NOMENCLATURE

A	biofilm surface area (m^2)
C_b	concentration of biocide (g m^{-3})
C_s	concentration of substrate (g m^{-3})
D_b	diffusion coefficient of biocide in water (m^2 h^{-1})
D_s	diffusion coefficient of substrate in water (m^2 h^{-1})
k_b	biocide disinfection rate coefficient (m^3 g^{-1} h^{-1})
k_r	biocide reaction rate coefficient (m^3 g^{-1} h^{-1})
K_s	substrate Monod half-saturation coefficient (g m^{-3})
L_f	biofilm thickness (m or μm)
L_L	concentration boundary layer thickness (m or μm)
Q	volumetric flow rate (m^3 h^{-1})
r_B	overall reaction rate of biocide (g h^{-1})
R_o	overall reaction rate of biocide per biofilm volume (g m^{-3} h^{-1})
t	time (h)
t_b	duration of biocide dose (h)
v	particulate advective velocity (m h^{-1})
V	volume of bulk liquid compartment (m^3)
Y_{bn}	yield coefficient of biocide on neutralizer (g g^{-1})
Y_{xs}	yield coefficient of biomass on substrate (g g^{-1})
z	distance coordinate normal to the substratum (m)

Greeks

ε_c	cell fraction of total biofilm volume
ε_n	neutralizer volume fraction
μ_{max}	maximum specific growth rate (h^{-1})
ρ_x	cell intrinsic density (g m^{-3})
τ	biofilm/bulk fluid effective diffusivity ratio
Φ	observable modulus comparing reaction and diffusion rates

Superscripts

i	influent value
*	bulk fluid value

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