Biofilm Parameters Influencing Biocide Efficacy

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The influence of biofilm areal cell density, species composition, and the presence of abiotic particles on the disinfection and removal of bacterial biofilms by monochloramine was investigated. Mono- and binary population biofilms of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were grown on stainless-steel slides in a continuous flow annular reactor. Biofilms were treated in the reactor with a pulse/step dose of 4 mg/L monochloramine for 2 h. Biofilm samples were disaggregated and assayed for colony formation on R2A agar and for total cell numbers by acridine orange direct counts. These data were used to determine apparent first order rate coefficients for the processes of disinfection and detachment. Disinfection rate coefficients exceeded detachment rate coefficients by as much as an order of magnitude and the two coefficients were poorly correlated (r = 0.272). The overall decay rate coefficient (disinfection plus detachment) depended strongly on the initial biofilm areal cell density. It displayed a parabolic dependence on cell density with a maximum near 10^8 cfu/cm^2. This result points to multiple factors influencing biofilm susceptibility to antimicrobial challenge. Decay rates of *K. pneumoniae* measured in binary population biofilms were comparable with those measured in monopopulation biofilms (p = 0.61). *P. aeruginosa* decayed more slowly in biofilms dominated by *K. pneumoniae* (p = 0.028), indicating some interaction between species. The presence of kaolin and calcium carbonate particles in the biofilm reduced disinfection efficacy. © 1995 John Wiley & Sons, Inc.

Key words: biofilm • disinfection • detachment • biofouling • ecology • biocide

INTRODUCTION

The detrimental formation of microbial biofilms afflicts diverse industrial and medical systems.[10,14] Adverse impacts of biofouling include energy losses in cooling water towers and heat exchangers;[8,25,26,31] decreased water quality and coliform contamination of drinking water distribution networks;[6,21] plugging in secondary oil recovery and other operations involving subterranean injection; corrosion or biodeterioration of equipment or pipelines[22]; pathogen contamination in food processing[7,24]; degradation of metalworking fluid properties; degradation of product quality in paper manufacturing[19]; caries and periodontal disease due to biofilm formation on teeth and gums[14]; and persistent infections associated with medical implants and catheters (Table I).[14,29]

Mechanical cleaning and antimicrobial chemicals are the most used methods of biofilm control. Mechanical cleaning is often impracticable and can be costly because it usually involves equipment downtime. Biocides and antibiotics have, therefore, been the principal weapons in combating biofouling. The magnitude of the problem can be partially gauged by the amount spent on biocides. U.S. consumption of specialty biocides for swimming pools and spas, disinfectants and sanitizers, paper manufacture, cooling water, metalworking fluids, and petroleum production totaled $315 million in 1989.[23] This figure represents only a fraction of the total economic impact of biofilm formation, because it does not cover all of the systems listed in Table I nor does it account for reduced performance, material deterioration, or increased maintenance or health care costs. Estimates of the full cost of biofouling, even for individual countries or industries, run into the billion dollar per year range.[9]

The action of antimicrobial agents against biofilm microorganisms is an excellent biochemical engineering problem. It requires integration of concepts from microbiology, engineering, and chemistry, and encompasses phenomena ranging from microbial growth and disinfection kinetics to mass transport and reactor dynamics. The problem is especially interesting in that microorganisms in biofilms are universally found to be more resistant to antimicrobial agents than their planktonic counterparts, but the underlying basis for this resistance in not well established.[5,13,14] With their long-standing involvement in the design and analysis of biofilm reactors for fermentations and wastewater treatment, engineers can contribute to the development of more effective approaches for antimicrobial application and biofilm control.

The purpose of the research reported here was to identify biofilm parameters that influence biocide efficacy in a well-characterized laboratory system. The effects of initial cell areal density, species composition, and the presence of abi-

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Table I. Detrimental effects of biofilm processes.

System	Effects
Cooling water towers and heat exchangers	Energy losses due to increased fluid frictional and heat transfer resistances
Drinking water distribution	Increased suspended solids, coliform contamination
Secondary oil recovery	Plugging of water injection wells corrosion
Process equipment	Material corrosion or biodeterioration
Food processing	Contamination
Metalworking	Degradation of metalworking fluid
Paper manufacture	Degradation of product quality
Dental plaque	Caries, peridontal disease
Medical implants, catheters	Persistent infections
Ship hulls	Increased frictional drag
Reverse osmosis membranes	Reduced permeability, material degradation
Clean surfaces (janitorial, health care, consumer)	Health risks, cosmetic degradation
Swimming pools	Health risks, cosmetic degradation

otic particles in the biofilm were investigated. Species composition and abiotic particle content are important parameters in that biofilms in industrial systems are invariably mixed microbial populations and often contain silt, corrosion products, or precipitates.[10]

MATERIALS AND METHODS

Microorganisms and Medium

Environmental isolates of *Pseudomonas aeruginosa* (ERC1) and *Klebsiella pneumoniae* were grown on a glucose minimal medium.[11] Both microorganisms are common constituents of fouling biofilms and the strains used were known biofilm formers. Glucose was the limiting substrate. The glucose feed concentration of 20 mg/L was low enough to ensure that oxygen was always in excess. This glucose concentration is representative of the organic carbon levels found in power plant cooling waters.[9] In one experiment, abiotic particles were incorporated into the biofilm by adding to the influent a suspension of kaolin and calcium carbonate. The effective influent concentration of each particulate was 50 mg/L.

Reactor Design and Operation

Biofilms were developed in a continuous flow annular reactor. The reactor and operating conditions have been described in detail elsewhere.[11,12,30] The dilution rate in the reactor of 3.5 h^{-1} was sufficiently large that planktonic growth of the organisms could be neglected. Twelve stainless-steel slides (SS304; 19 × 1.7 cm) were fitted in the reactor and could be removed to sample biofilm. Experiments were conducted at a temperature of 25 ± 1°C.

All system components were sterilized prior to each experiment by autoclaving at 121°C for 25 min. The medium supplied to the reactor was continuously formulated by mixing nutrient stock solution (1 mL/min), buffer (1 mL/min), and dilution water (30 mL/min).[11] Nutrient and buffer solutions were autoclaved. Dilution water was sterilized by passage through two 0.2-μm filters in series (Gelman Sciences). Concentrated glucose solution was also filter sterilized before addition to the nutrient stock reservoir. The reactor feed was delivered with peristaltic pumps (Masterflex 7553-30, Cole Parmer).

The reactor was inoculated with 1 mL each of a frozen stock culture (10^8 cells/mL) of the desired species and incubated in batch mode for 24 h. Influent flows were then started and the reactor was operated for 6 to 14 days. Biofilm accumulated on the wetted surfaces of the reactor, including the stainless-steel sample slides. Samples of the effluent were plated daily to check for contamination. No attempt was made to predetermine or control the proportion of each species in binary population biofilms. The biofilm species composition and areal cell density at the time of treatment varied.

Biocide Preparation and In Situ Biocide Treatment

Monochloramine was prepared by combining household chlorine bleach with excess ammonium chloride at pH 9.0 as described elsewhere.[11] The concentration of monochloramine was measured with a colorimetric test kit (Model CN-66, Hach). To initiate biocide delivery, a slug of concentrated monochloramine solution was dosed into the reactor. The amount of monochloramine added was the calculated amount required to instantaneously raise the concentration in the bulk fluid of the reactor to 4 mg Cl/L. At the same time, a continuous feed of 4 mg/L monochloramine was initiated. Biocide was applied for a period of 2 h. The monochloramine demand of the sterile reactor system was negligible.[11] Each biofilm was treated with biocide only once; that is, each data set came from independently inoculated reactors.

Ex Situ Biocide Treatment

In one experiment, biofilm covered slides were removed from the annular reactor and treated with monochloramine ex situ in magnetically stirred batch vessels. This design made it possible to treat biofilms of different areal cell densities grown in a single reactor. Two slides were removed at a time. One slide was incubated in 1 L of a 3-mg/L monochloramine solution in a pH 7.0 phosphate buffer. The second slide was incubated in the same volume of phosphate buffer without biocide as a control. The volume of the biocide solution was sufficiently large that the concentration of monochloramine did not change measurably during the treatment period. After 1 h, the slides were transferred to phosphate buffer containing 0.1% sodium

thiosulfate to neutralize the remaining monochloramine. The viable cell density on the slides was quantified as described below.

Analytical Methods

Biofilm sample slides were removed periodically from the reactor during biocide treatment.[11] Biofilm was transferred from the slide quantitatively using a cell scraper into 150 mL of phosphate buffer containing 0.1% sodium thiosulfate as a neutralizer. The solution was placed on ice and homogenized for 3 min using a tissue homogenizer (Tekmar). Viable cell numbers were assayed by plating aliquots of dilutions onto R2A agar. The two bacterial species could be distinguished on the plates by colony shading and morphology; colony counts for both species were recorded. Total cells were determined by acridine orange direct counts.[11] Biofilm cell numbers were expressed as areal cell densities (number/cm^2).

Data Analysis

Biocide action on the biofilm was interpreted by calculating first order rate coefficients from the transient reduction in biofilm cell counts. The coefficients and their standard errors were obtained by linear regression of semilog plots of areal cell density versus time. Three coefficients were determined. The detachment rate coefficient, k_{det}, was determined by the decrease in the total areal cell count during biocide treatment. This coefficient reflects only the process of detachment (physical removal), because the acridine orange method used to enumerate total cells detects nonviable as well as viable cells. The decay rate coefficient, b, was determined from the decrease in viable areal cell counts. Biofilm viable cell numbers can be reduced by disinfection as well as by detachment, so the decay rate coefficient reflects the combined effects of disinfection and detachment. The decay coefficient was calculated for both species together (in which case it is termed the overall decay rate coefficient) and for the two species individually. The disinfection rate coefficient, k_{dis}, was calculated as the difference between the overall decay rate coefficient and the detachment rate coefficient ($b - k_{det}$).

Reported p-values for regressions apply to the null hypothesis of $r^2 = 0$. That is, the p-value is the chance of observing an r^2 as large as for the data if in fact the true value of r^2 equals zero. A small p-value indicates significant correlation between the regressed variables. For tests comparing means or estimates, the p-value is the chance of observing measurements that differ as much as the data do if in fact the values are equal. A small p-value in this case indicates that the values or means are significantly different. Regression and statistical calculations were carried out using the computer software MSUSTAT.

RESULTS

Biofilms treated with monochloramine experienced a decrease in viable and total cell areal densities during the treatment period (Figs. 1 and 2, respectively). Most of the data sets for cell number versus time were approximately linear on a semilog plot. Biofilms of initial cell density less than about 10^8 cfu/cm^2 experienced a rapid decrease in viable cell numbers in the first hour that then leveled off near 10^5 cfu/cm^2. Viable cell numbers experienced a greater reduction than did total cell numbers. Disinfection rate coefficients calculated from the data generally exceeded detachment rate coefficients (Table II). Statistical analysis of the means of the two groups of rate coefficients showed that they were significantly different ($p = 0.03$). The detachment and disinfection rate coefficients were poorly correlated ($r = 0.272$). Three of the nine detachment rate coefficients reported in Table II were not statistically different from zero at the $p = 0.05$ level. Because biofilm cell counts were stable with time after 5 days of culture, detachment and disinfection rate coefficients for untreated biofilms

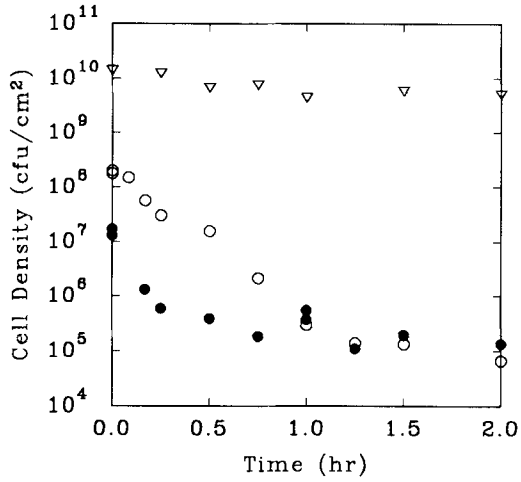


Figure 1. Viable cell areal density of binary population biofilm during treatment with monochloramine. Three different runs are shown, each with a different initial cell density.

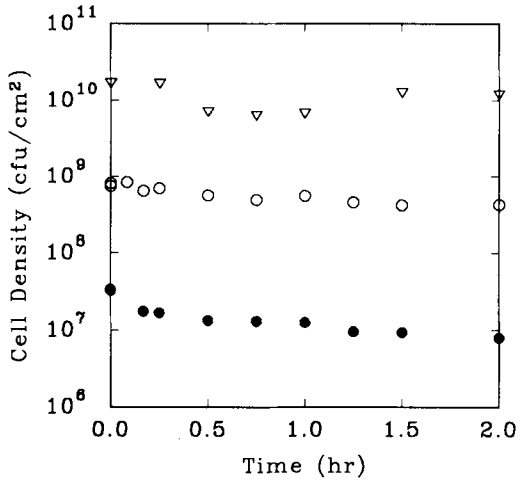


Figure 2. Total cell areal density of binary population biofilm during treatment with monochloramine. Three different runs are shown; they correspond to the data in Figure 1.

were small; from the variability in biofilm accumulation data, decay rate coefficients in untreated biofilms can be estimated to be less than approximately 0.03 h^{-1}.[30]

The overall decay rate coefficient measured in situ showed a nonlinear dependence on the initial areal cell density (Fig. 3). As the initial cell density increased from 10^8 cfu/cm^2 to 10^{10} cfu/cm^2, the overall decay rate coefficient decreased from 5.3 to 0.5 h^{-1}. As the cell density dropped to 10^7 cfu/cm^2, the overall decay rate coefficient decreased to about 1.2 h^{-1}. A fit of overall decay rate coefficient as a quadratic function of initial cell density captured the experimental data much better ($R^2 = 0.665$) than did a linear model ($r^2 = 0.003$). Because the parabolic dependence of overall decay rate coefficient on initial cell density was unexpected, we sought to confirm this result with an independent experimental design; thus, the ex situ experiment. Ex situ treatment of monopopulation biofilms of *P. aeruginosa* did indeed indicate a qualitatively similar parabolic behavior (Fig. 4). Biofilm samples removed from a single reactor at different times during biofilm accumulation showed reduced susceptibility to biocide treatment at both extremes of cell density (Fig. 4). For both in situ and ex situ biocide treatments, maximum susceptibility was observed at an initial cell areal density of approximately 10^8 cfu/cm^2.

The overall decay rate coefficient also depended on the species composition of the biofilm. To test the significance of the correlation in quantitative terms, the composition of the biofilm was expressed in terms of the fraction of the total viable cells that were identified as *P. aeruginosa*. The *p*-value for the correlation between overall decay rate coefficient and fraction of *P. aeruginosa*, f_p, was 0.025, indicating a significant dependence. Biofilms with a higher proportion of *P. aeruginosa* decayed faster than those dominated by *K. pneumoniae*. The ratio of the overall decay rate coefficients for biofilms of $f_p = 1$ to that for biofilms of $f_p = 0$, as predicted from the regressed line through the *b* versus f_p data, was 3.6. Measured decay rate coefficients of the individual species in binary population biofilms also indicated faster decay of *P. aeruginosa* than of *K. pneumoniae* (Fig. 5). The slope of the regression line through the data in Figure 5 is 2.7. These results show that *P. aeruginosa* decayed approximately three times faster than did *K. pneumoniae*. Species composition and initial cell density were not correlated ($r = 0.243$, $p = 0.53$).

The influence of biofilm species composition on the decay rate coefficients of individual species is shown in Figure 6. No dependence of the decay rate coefficient of *K. pneumoniae* on the fraction of *P. aeruginosa* in the biofilm could be discerned ($r^2 = 0.045$, $p = 0.61$). This can also be seen by comparing the decay of viable *K. pneumoniae* in monopopulation and binary population biofilms of similar initial cell densities (Fig. 7). In contrast, the decay rate coefficient of *P. aeruginosa* decreased in biofilms that were dominated by *K. pneumoniae* ($r^2 = 0.74$, $p = 0.028$; Fig. 6). A direct comparison of viable cell numbers of *P. aeruginosa* versus time in monopopulation and binary population biofilms analogous to the data for *K. pneumoniae* shown in Figure 7 was not possible, because data sets at similar initial cell densities were not attainable experimentally. Monopopulation biofilms of *P. aeruginosa* yield biofilms of significantly lower initial areal cell density than are obtained when both species are present.

Biofilm grown in the continuous presence of abiotic particles was more resistant to biocide treatment than was biofilm without the particles (Fig. 8). The overall decay rate coefficient observed without particles was 4.55 ± 0.42 h^{-1} and for biofilm with particles it was 2.51 ± 0.54 h^{-1}. A *t*-test on the equality of these two values yielded a *p*-value of 0.0005, signifying that the decay rate coefficients were statistically significantly different. Confocal microscopic imaging and cryosectioning of biofilm cultivated in the presence of particles revealed nucleic acid-free particulates, as indicated by ethidium bromide staining, dispersed throughout the biofilm.

DISCUSSION

The efficacy of chemical biocides used to control microbial growth has traditionally been measured by assays of colony formation on agar media. While this approach is sensible for studies of planktonic microorganisms, it provides an incomplete picture of biocide action against a biofilm. When biofilm fouling is the issue, the removal or detachment of biomass may be more significant than the degree of killing. Plate counting methods, however, fail to measure detachment well. Detachment and disinfection are independent processes; it is quite possible to disinfect a biofilm without removing it. In our experimental system, detachment was found to be a significantly slower process than disinfection (compare Figs. 1 and 2; Table II). The correlation between calculated detachment and disinfection rate coefficients was poor ($r = 0.272$). These results caution against sole reliance on plate counts as measures of biocide efficacy in controlling biofouling. Some analysis of total cell number, total biomass, or biofilm thickness is valuable as an indicator of the extent of actual removal.

The unexpected parabolic dependence of overall decay rate on initial viable cell areal density (Fig. 3) hints at the complexity of the biocide–biofilm interaction. These data suggest that there exist multiple mechanisms that alter the susceptibility of biofilm microorganisms to chemical challenge. One of the leading hypotheses to explain biofilm resistance to antimicrobial agents is the simple failure of the biocide to penetrate the biofilm. In the case of a reactive chemical, like chlorine or monochloramine, the failure to penetrate the biofilm is presumably due to a reaction–diffusion interaction. Evidence in support of this phenomenon in our experimental system includes a comparison of reaction and diffusion rates,[11] direct measurement of chlorine concentration profiles in biofilm using a microelectrode,[16] and demonstration of respiratory activity gradients within biofilms during monochloramine treatment.[20] Others have also measured reduced susceptibility of biofilms with increasing areal cell density[1,4,32] or investigated the reac-

Table II. Overall decay, disinfection, and detachment rate coefficients (±SE) for biofilms treated in situ with monochloramine.

log X_{vo} (cfu/cm^2)	f_p	b (h^{-1})	k_{dis} (h^{-1})	k_{det} (h^{-1})	n
10.16	0.23	0.50 ± 0.17	0.4 ± 0.3	0.10 ± 0.3	7
9.26	0.01	0.38 ± 0.20	0.03 ± 0.2	0.35 ± 0.09	8
8.57	1.00	6.0 ± 1.2	5.9 ± 1.2	0.09 ± 0.09	7
8.56	0.83	5.9 ± 0.6	5.4 ± 0.6	0.52 ± 0.04	11
8.29	0.81	4.6 ± 0.4	4.3 ± 0.4	0.34 ± 0.05	12
8.04	0	5.3 ± 1.8	3.5 ± 1.8	1.79 ± 0.26	9
7.23	0.21	2.1 ± 0.5	1.45 ± 0.51	0.65 ± 0.11	11
7.15	0	1.34 ± 0.51	1.00 ± 0.54	0.34 ± 0.17	12
6.98	0	1.28 ± 0.41	0.63 ± 0.58	0.65 ± 0.41	9

Symbols: X_{vo}, initial viable cell areal density; f_p, fraction of viable cells in biofilm that are *P. aeruginosa*; b, overall decay rate coefficient; k_{dis}, disinfection rate coefficient; k_{det}, detachment rate coefficient; n, number of data points used in the regression analysis to determine coefficients and standard errors.

tion–diffusion issue.[28,32,33] Such a transport-based explanation could account for the decreased efficacy of monochloramine against biofilms with the highest areal cell densities, but it cannot explain the poor efficacy observed against thin biofilms.

The recalcitrance of biofilms of very low areal cell density could be indicative of a resistant subpopulation. The existence of a resistant subpopulation is also supported by the biphasic decay observed for biofilms of lower initial cell density (Fig. 1). The occurrence of resistant subpopulations in planktonic cultures subjected to disinfection is common.[2] Such a subpopulation could be genotypically or phenotypically distinct, or it could be a manifestation of biofilm structural heterogeneity.[15] For example, even in an apparently thin biofilm, there could be occasional thick microcolonies that are protected by transport limitation of biocide. These colonies could constitute a resistant subpopulation. *K. pneumoniae* biofilms have been shown to be particularly patchy and heterogeneous.[27]

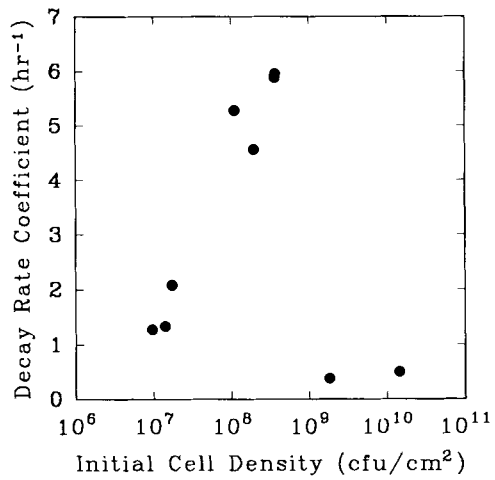


Figure 3. Dependence of overall decay rate coefficient on initial viable cell areal density (in situ treatment) for all mono- and binary population biofilms.

Another explanation for the changes in biofilm susceptibility to disinfection with initial areal cell density is the variation in physiological status of microorganisms in the biofilm at different stages of biofilm development. As the biofilm progresses from early colonization events to a thick, mature film, physiological and metabolic changes, for example, in the type and rate of polymer production and in specific growth rate, are to be expected. It is well known that such changes can alter susceptibility to antimicrobial agents.[4,5] Thus, while our correlation of experimental results with the initial areal cell density suggests an interpretation in terms of biofilm thickness or mass, this parameter could be viewed equally plausibly as an index of biofilm growth phase. Further research, including the use of techniques to characterize microbial physiology in biofilms, will be necessary to resolve these issues.

Our results indicate some evidence of species interaction.

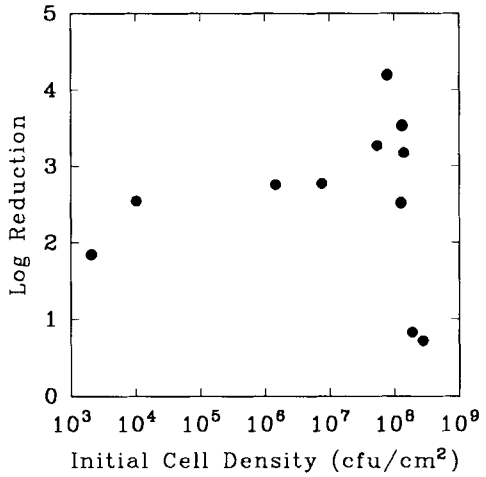


Figure 4. Dependence of biocide efficacy on initial viable cell areal density (ex situ treatment) for a monopopulation *P. aeruginosa* biofilm. Biocide efficacy is expressed in terms of the log reduction, the negative logarithm of the ratio of final and initial viable cell areal densities. Biofilms of various cell densities were obtained by sampling at different intervals during biofilm development.

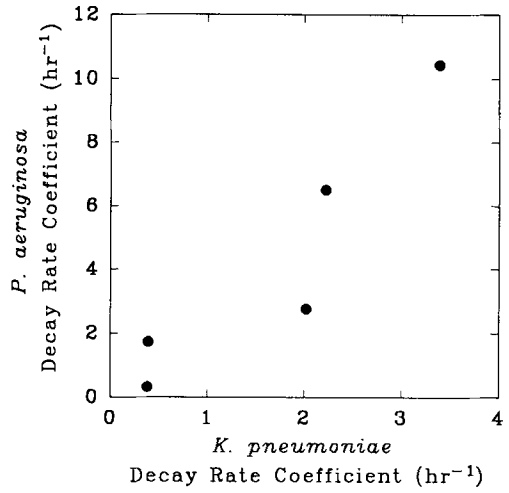


Figure 5. Comparison of decay rate coefficients for individual species in binary population biofilms. Each data point represents a separate binary population biofilm experiment.

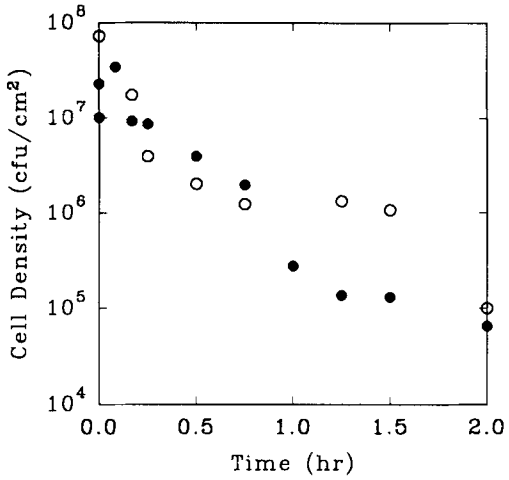


Figure 7. Viable cell areal density of *K. pneumoniae* in mono (○) and binary population (●) biofilms during treatment with monochloramine. The binary population biofilm contained 81% *P. aeruginosa*.

P. aeruginosa decayed more slowly in biofilms that contained proportionately more *K. pneumoniae* (Fig. 6). *K. pneumoniae*, on the other hand, showed no dependance on biofilm species composition. Such a commensalistic relationship could possibly arise if one species has a greater capacity to neutralize the biocide. In the present case, for example, if *K. pneumoniae* consumed monochloramine more rapidly than *P. aeruginosa*, the presence of *K. pneumoniae* could protect *P. aeruginosa* by lowering the biocide concentration. If this were the case, one would expect to see the opposite effect as well: *K. pneumoniae* should be more susceptible when it occurs in a biofilm dominated by *P. aeruginosa*. This was not observed.

An alternative explanation for the species interaction involves spatial segregation of the two species in the biofilm. For example, if the biofilm consists of microcolonies of *K. pneumoniae* floating on a sea of *P. aeruginosa*, as has been suggested by confocal microscopic examination, then the protection of *P. aeruginosa* and indifference of *K. pneumoniae* could be understood. The species structure of the biofilm could be investigated by combining fluorescently labeled oligonucleotide or antibody probes with confocal microscopy or cryosectioning.

Biofilms grown in the presence of suspended abiotic particles of kaolin and calcium carbonate were more resistant to monochloramine than those developed in clean media (Fig. 8). Biofilms capture suspended particulates, which become incorporated into the biofilm and are retained.[3,17,18] The decreased efficacy against particulate-containing biofilms could be due to destructive reactions between the particles and the biocide, hindrance of diffusive transport, alteration of local pH, or altered biofilm struc-

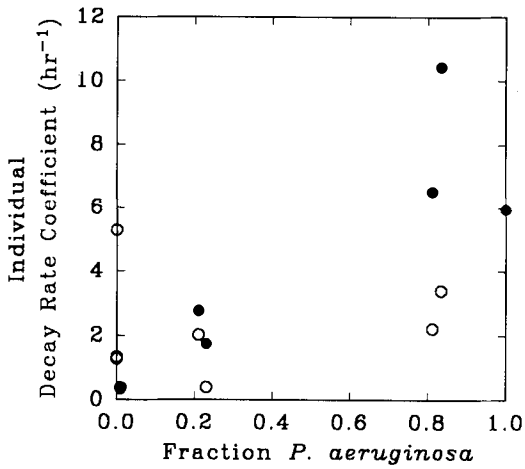


Figure 6. Dependence of individual species decay rate coefficient on biofilm species composition. Symbols indicate *P. aeruginosa* (●) and *K. pneumoniae* (○).

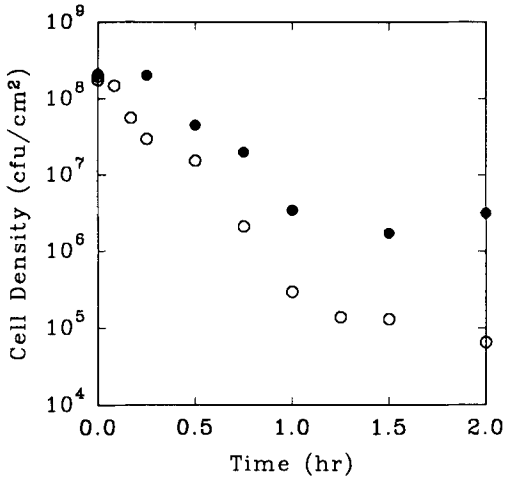


Figure 8. Comparison of viable cell areal density during monochloramine treatment of binary population biofilms with (●) and without (○) abiotic particles.

ture. The effect of inorganic particles has important practical implications because biofilms in field systems such as cooling towers or pipelines can be dominated by inorganic matter.[26,34]

The interpretation of biocide treatment efficacy in terms of simple first order rate coefficients, while adequate for the purpose of identifying major parameters and interactions, may not be sufficient for quantifying individual effects and making predictions. The first order model neglects changes in biocide concentration that occur during treatment and fails to capture the observed two-phase decay observed in some experiments. More sophisticated kinetic descriptions will be needed to be able to engineer the application of biocides and antibiotics to control biofilms.

CONCLUSIONS

In this study of monochloramine action against biofilms of *P. aeruginosa, K. pneumoniae,* and the binary population combination of the two microorganisms, the following conclusions were drawn:

1. Disinfection predominates over detachment.
2. Disinfection and detachment are poorly correlated.
3. There exists a nonlinear dependence of biocide efficacy on biofilm initial viable cell areal density.
4. *P. aeruginosa* was protected by association with *K. pneumoniae* in a biofilm while decay of *K. pneumoniae* was independent of biofilm species composition.
5. The presence of abiotic particles of kaolin and calcium carbonate in biofilms reduced biocide efficacy.

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NOMENCLATURE

b	overall decay rate coefficient for both species combined
f_p	fraction of viable cells in biofilm that are *P. aeruginosa*
k_{det}	detachment rate coefficient
k_{dis}	disinfection rate coefficient
X_{vo}	initial viable cell areal density for both species combined

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