Investigation of biofilm resistance to antimicrobial agents
by Katherine Jean Grobe

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemical Engineering
Montana State University

Abstract:
The goal of this project was to investigate aspects of biofilm resistance to antimicrobial agents using a novel artificial biofilm system. Experiments were done with four different antimicrobial agents (chlorine, glutaraldehyde, ciprofloxacin and a quaternary ammonium compound, QAC) in an effort to explain the increased resistance to antimicrobial agents of biofilms over their planktonic counterparts. Data was collected as survival vs. time for each agent. The two mechanisms of resistance considered were 1) transport: a failure of the biocide to fully penetrate the biofilm and 2) physiology: an inherent physiological heterogeneity within the biofilm. A simple disinfection model was utilized to discern the concentration dependence of biofilm disinfection.

Bacteria in biofilms were clearly less susceptible to all biocides than were the same microorganisms when grown in a conventional suspension culture. For example, using 50 mg/L glutaraldehyde it took approximately 20 minutes to achieve a 2 log reduction in viable cell numbers in planktonic experiments but almost 600 minutes to achieve this same level of killing in the biofilm. In general, the susceptibility of bacteria in biofilms was reduced by approximately an order of magnitude compared to planktonic bacteria. Biofilm results indicated that both chlorine and glutaraldehyde penetration into the biofilms was retarded and that poor penetration likely contributed to reduced biofilm susceptibility. It was also shown that in biofilm disinfection by these two agents there is a higher dependence on concentration than in a planktonic suspension. It is more advantageous to dose a biofilm with a high concentration of these agents for a short period of time rather than a low concentration for a proportionately longer time. On the other hand results from biofilm disinfection by ciprofloxacin and QAC were biphasic and consistent with the existence of an inherently resistant subpopulation within the biofilm. The demonstration of unambiguous biofilm resistance to biocides of various chemical natures highlights the need to continue to employ biofilm testing methodologies in designing and optimizing applications of these agents. The insights obtained in this investigation suggest possible approaches for improving biofilm control.

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APPROVAL

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Katherine Jean Grobe

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

The goal of this project was to investigate aspects of biofilm resistance to antimicrobial agents using a novel artificial biofilm system. Experiments were done with four different antimicrobial agents (chlorine, glutaraldehyde, ciprofloxacin and a quaternary ammonium compound, QAC) in an effort to explain the increased resistance to antimicrobial agents of biofilms over their planktonic counterparts. Data was collected as survival vs. time for each agent. The two mechanisms of resistance considered were 1) transport: a failure of the biocide to fully penetrate the biofilm and 2) physiology: an inherent physiological heterogeneity within the biofilm. A simple disinfection model was utilized to discern the concentration dependence of biofilm disinfection.

Bacteria in biofilms were clearly less susceptible to all biocides than were the same microorganisms when grown in a conventional suspension culture. For example, using 50 mg/L glutaraldehyde it took approximately 20 minutes to achieve a 2 log reduction in viable cell numbers in planktonic experiments but almost 600 minutes to achieve this same level of killing in the biofilm. In general, the susceptibility of bacteria in biofilms was reduced by approximately an order of magnitude compared to planktonic bacteria. Biofilm results indicated that both chlorine and glutaraldehyde penetration into the biofilms was retarded and that poor penetration likely contributed to reduced biofilm susceptibility. It was also shown that in biofilm disinfection by these two agents there is a higher dependence on concentration than in a planktonic suspension. It is more advantageous to dose a biofilm with a high concentration of these agents for a short period of time rather than a low concentration for a proportionately longer time. On the other hand results from biofilm disinfection by ciprofloxacin and QAC were biphasic and consistent with the existence of an inherently resistant subpopulation within the biofilm. The demonstration of unambiguous biofilm resistance to biocides of various chemical natures highlights the need to continue to employ biofilm testing methodologies in designing and optimizing applications of these agents. The insights obtained in this investigation suggest possible approaches for improving biofilm control.

BACKGROUND

Biofilms and Biofilm Resistance

Biofilms cause myriad problems. For example, they are responsible for inefficiency in cooling towers, souring of oil fields, and clogging of drains. Many treatment applications have been developed in industry for the control of biofilms. In order to expedite transfer of effective treatments into the marketplace, a method is needed to allow biofilm resistance and resistance mechanisms to be investigated. This project investigates the utility of an artificial biofilm system by testing it against four diverse antimicrobial agents. Proposed mechanisms of resistance are examined and transport properties of the artificial biofilm system studied.

Microorganisms in biofilms are almost always found to be profoundly less susceptible to antimicrobial agents than are their freely suspended counterparts. Examples of biofilm resistance to glutaraldehyde, for example, can be found in the literature (Cheung and Beech 1998; Dewar 1986; Eagar, et al. 1998; Grab and Theis 1993; Green and Pirrie 1993; Leder 1989; McCoy, et al. 1986; Reinsel, et al. 1996; Ruseska, et al. 1982; Stewart, et al. 1998; Duguid, et al. 1992; Vess, et al. 1993; Whitham and Gilbert 1993). Because biofilms exhibit distinct physiologies and physical

properties, the conclusions drawn from antimicrobial experiments performed with planktonic (freely suspended) microorganisms may be inappropriate for biofilm applications. For example, in a comparison of two forms of chlorine, hypochlorous acid and monochloramine, it has been shown that monochloramine, which is a weaker disinfectant when tested against planktonic microorganisms, was actually the superior agent when tested against biofilms (Stewart, et al. 1999). This result underscores the importance of performing biocide efficacy tests against biofilms.

The Antimicrobial Agents Investigated

Chlorine

Chlorine has diverse applications. It is used in drinking water distribution systems, pools and spas, waste water treatment facilities, in-plant disinfection, and many common household cleaners. Its method of antimicrobial action is unknown, but some hypothesize that chlorine inhibits certain enzymes preventing particular metabolic reactions to take place (Fair et al. 1948; Dychdala 1991). The biocidal action of chlorine is dependent on a number of factors including pH and temperature. Depending on pH, two different forms of chlorine will prevail. Sodium hypochlorite will disassociate into chlorite and hypochlorite ions. It is the hypochlorite ion that is the more effective antimicrobial agent (Dychdala 1991). Chlorine treatment values typically range from 1 ppm in drinking water treatment to upwards of 20 ppm in industry.

Glutaraldehyde

In many of the application areas of glutaraldehyde, including oilfield control of souring and corrosion, industrial water treatment, and sterilization of medical instruments, biofilms are known to be present and probably represent the major target for microbial control. Glutaraldehyde was first investigated as an antimicrobial agent as an alternative to formaldehyde as a fixative. It is widely used as a chemisterilizer for medical instruments that can not be sterilized by traditional heat and pressure methods. Unhindered by organic matter, it is effective against bacteria, fungi, viruses and spores. Its action is dependent on pH, temperature and concentration. Glutaraldehyde is commonly used in the oil field for the treatment of sulfate reducing bacteria (Scott and Gorman 1991).

Ciprofloxacin

Ciprofloxacin is an antibiotic frequently used to treat infections. It is a fluoroquinolone molecule that targets DNA gyrase, a maintenance enzyme responsible for the superhelical twists of DNA (Wolfson and Hooper 1985). It is these super helical twists that regulate the binding of proteins to DNA. DNA gyrase is required for DNA replication, transcription, repair and recombination. The exact mechanism of ciprofloxacin as an antibiotic is unknown, but it is hypothesized that DNA gyrase cleaves chromosomal DNA, thereby blocking DNA replication (Wolfson and Hooper 1985). The MIC value for the bacterial strain used in this study, ERC1, was reported to be 0.25

μg/mL (Vrany, et al. 1997). There is conflicting evidence as to whether ciprofloxacin is a growth-rate dependent antibiotic. Some speculate the susceptibility of bacteria when challenged with ciprofloxacin increases with the specific growth-rate (Duguid, et al. 1992). An earlier study showed that ciprofloxacin is not transport limited in a thin *P. aeruginosa* biofilm (Vrany, et al. 1997; Anderl, et al. 1999).

Quaternary ammonium compound

Quaternary ammonium compounds (QACs) are used in slightly different applications than the aforementioned antimicrobial agents. QACs are the active ingredients in textile chemicals, oilfield chemicals, polyurethane foam catalysis and epoxy curing agents (Albemarle Corporation 1999). A surfactant long considered a biocide, QACs adsorb to the cell wall, diffuse within and bind to the cytoplasmic membrane. Once they have disrupted the membrane, it is the release of potassium ions and cell constituents that cause cell lysis. Not a very effective chemisterilizer for medical instruments, the cationic structure has been proven effective against bacteria in fabrics (which have an inherent negative charge), thus are used in detergents. QACs are also used to treat algae in swimming pools. They are also used frequently to sterilize food contact surfaces such as utensils, pasteurizing equipment and other food synthesis machinery (Merianos 1991).

Artificial Biofilms

An expedient and repeatable method of testing antimicrobial agents is needed to characterize the nature and extent of biofilm resistance to develop more practical approaches to biofilm control. Researchers at the Center for Biofilm Engineering (CBE) have devised a simple, repeatable artificial biofilm test system for characterizing the efficacy of antimicrobial agents against biofilms; alginate gel bead biofilms challenged by antimicrobial agents and sampled over time allow insight into the mechanisms of resistance displayed by biofilms.

Alginate has been used as a matrix for cell immobilization in medicinal and industrial applications. It has been used for the production of ethanol by yeast, mass production of artificial plant seeds and drug and enzyme immobilization (Smidsrod and Skjak-Braek 1990). The use of alginate and agar as a simulated matrix for artificial biofilms has been a recent development in the literature (Stewart, et al. 1998; Coquet, et al. 1998; Jouenne, et al 1994). The alginate matrix is highly hydrated and diffusion readily takes place, simulating the polymer matrix in naturally occurring biofilms (Smidsrod and Skjak-Braek 1990). Such immobilized cells are more resistant to killing than are planktonic cells (Whitham and Gilbert 1993; Keweloh, et al. 1989; Coquet, and others 1998). A particularly interesting extension of this idea is the use of implanted agar gel beads in rat lungs in an effort to model cystic fibrosis disease. The colonization of *P. aeruginosa* in the lungs of severely ill patients has recently been considered a biofilm disease (Costerton, et al. 1999). Researchers have shown that these implanted beads

effectively model the chronic pulmonary disease in rats, allowing the virulence of different strains of *P. aeruginosa* to be studied (Woods, and others 1982).

Mechanisms of Biofilm Resistance

One of the proposed mechanisms of biofilm resistance is a failure of the biocide to penetrate the biofilm. Reactive oxidants like chlorine and hydrogen peroxide are often ineffective biocides against biofilms. Transport limitation may be one explanation for their ineffectiveness. The neutralizing capacity of the components of the biofilm makes penetration of the biocide difficult. Penetration failure can be explained in two different ways. In the first model, the agent reacts stoichiometrically with biomass continually consuming the neutralizing capacity of the biofilm and penetrating deeper. In this model, the biocide must deplete all of the neutralizing capacity of the biofilm before it can penetrate fully. In concept, there are a concrete number of reactive sites within the biofilm and the agent must react with each site before it can proceed within the biofilm. The second model of penetration failure is a specific catalytic reaction within the biofilm, in which reaction-diffusion equilibrium occurs. This model suggests that the biocide only reacts with the biotic portion of the biofilm. The penetration would only be limited if the biofilm responds with an enzymatic process (Dodds, et al. 1999).

Other biofilm research has indicated that there is an inherent physiological heterogeneity within the biofilm (Huang, et al. 1998). A sensitive fraction of cells may be easily killed off by the antimicrobial agent, while a resistant fraction remains harder to

kill. Evidence for this physiological resistance mechanism explains why some antibiotics readily diffuse into biofilm, yet kill it incompletely (Vrany, et al. 1997; Anderl, et al. 1999). An observed limited zone of protein synthesis activity in biofilms also supports this mechanism hypothesis (Brown and Gilbert 1993). Cells that are not undergoing protein synthesis or growth are known to be more resistant than actively growing cells. Research at the CBE has demonstrated that there is spatial heterogeneity related to alkaline phosphatase expression, which in turn is related to starvation of the biofilm cells. It has been shown that starved cells are more difficult to kill, suggesting a less susceptible "biofilm-phenotype" (Huang, et al. 1998). Nutrient limited and slow growing, starved cells have been shown to exhibit an increased resistance to antimicrobial agents (Duguid, et al. 1992; Brown and Gilbert 1993). While these mechanisms of biofilm resistance likely do not exist independently, it should be possible to discern which mechanism may predominate in biofilm disinfection by a particular agent.

Analysis of survival versus time plots provides insight into the mechanism of resistance shown by biofilms to a particular antimicrobial agent (Dodds, et al. 1999; Figure 1). A concave down curve is characteristic of a transport limited biocide (Figure 1). The initial relatively flat portion of the kill curve suggests a period of time where the biofilm is still penetrating the biofilm. Once full penetration has been reached (indicated by the inflection point) the biocide becomes more effective. Concave up curvature suggests that physiology is the dominating mechanism of resistance (Figure 1). The initial decrease in the bi-phasic curve represents the sensitive fraction of cells within the biofilm being killed. The inflection point and subsequent flatter portion of the

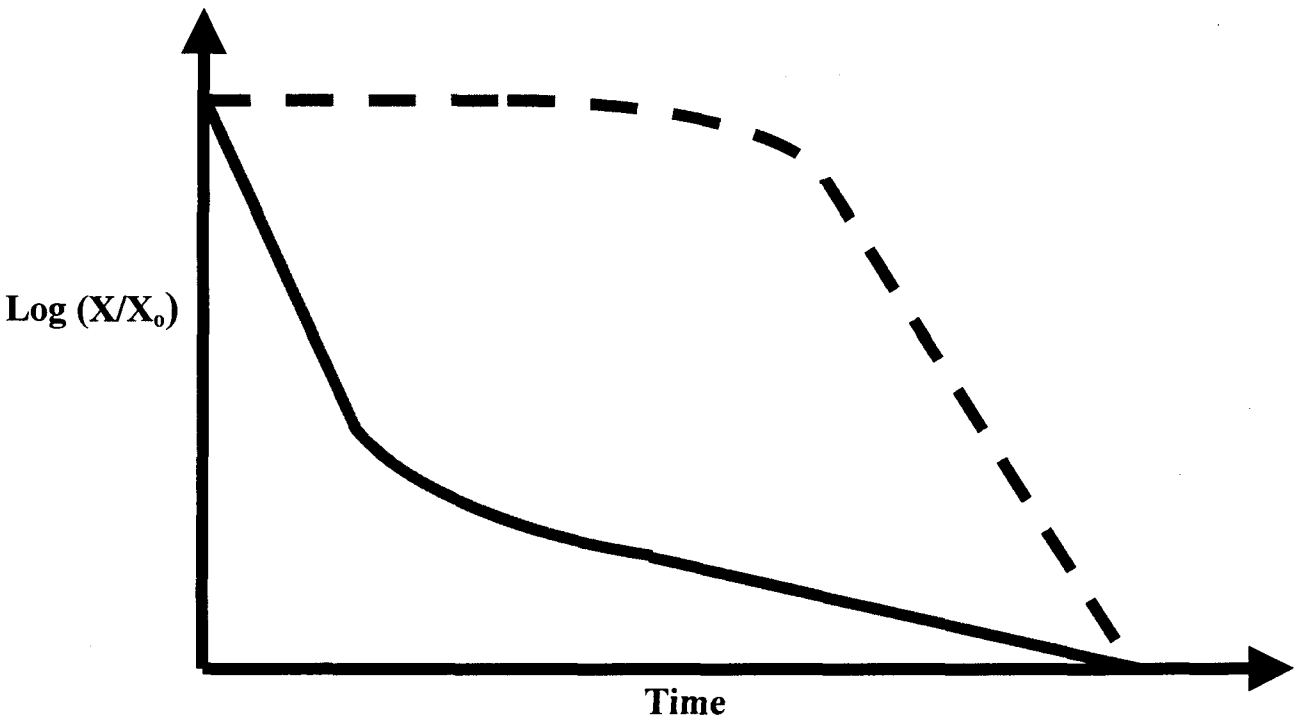

Figure 1. Predicted biofilm disinfection curve shapes (Dodds, et al. 1999). The dashed line represents a transport limitation while the solid line indicates a physiological heterogeneity within the biofilm.

physiological curve indicates a more resistant population that is more difficult to kill. A computer model created by Mike Dodds at the CBE to analyze biofilm disinfection phenomena was used in this analysis (Dodds, et al. 1999).

Traditional lab biofilms are time-consuming and tedious to grow, making collection of adequate survival versus time data difficult. This type of data is rarely found in the literature for biofilms. The artificial biofilm system is attractive because of the large number of consistent biofilm samples that can be created in a manner of minutes.

In this project an artificial biofilm disinfection system was developed to study four antimicrobial agents: chlorine, glutaraldehyde, ciprofloxacin and a quaternary ammonium compound. Objectives included analysis of concentration dependence of each particular agent, utilization of a computer model to analyze physiological model disinfection parameters, and analysis of transport properties within the artificial biofilm system.

MATERIALS AND METHODS

Artificial biofilm gel beads were created and used in disinfection experiments varying the concentration of the four biocides tested, chlorine, glutaraldehyde, ciprofloxacin, and QAC. Planktonic experiments were also performed at the same concentration tested with the artificial biofilm system. A simple disinfection model was used to capture the concentration dependence of each particular agent. Transport of chlorine and glutaraldehyde was analyzed using an observable modulus. Lastly, a computer model created at the CBE was used to gain insight into a physiological based mechanism of biofilm disinfection.

Artificial Biofilm Test System

Artificial biofilms were created by entrapping bacteria, in this case *Pseudomonas aeruginosa*, 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 the biofilm phenotype. The end result – dense microcolonies dispersed in a highly hydrated gel matrix – simulated the structure of real biofilms. Alginate gel bead artificial biofilms appear to be a flexible, reproducible experimental system for investigating antimicrobial efficacy (Stewart, et al. 1998; Whitham and Gilbert 1993; Xu, et al. 1996). Gel bead artificial biofilm preparation is described in detail below.

A plate of R_2A agar (Difco) was streaked with a lawn of *P. aeruginosa* (ERC 1) and incubated overnight (36°C). Phosphate buffer (9 mL, pH 7.2) was then added to the agar plate and the cells were gently scraped off the plate using a glass hockey stick. The buffer/bacterial suspension was mixed with an equal volume of alginic acid, (Sigma, sodium salt, from *Macrocystis pyrifera*, 4%, 9 mL) to make a final 2% alginate concentration. Next, the alginate/bacterial slurry was placed in a sterile syringe (30 mL) with an attached needle (22 gauge). A stopper attached to a compressed air tank allowed the syringe to be pressurized. When the air was turned on (20 psi), a stream of small droplets was forced out the needle and dropped into a stirred solution of calcium chloride (50 mM). The calcium cross-linked the alginate and semi-solid beads with entrapped cells were formed. The beads were allowed to stir in the calcium chloride solution (50 mM) for approximately 20 min, then rinsed in a dilute solution of calcium chloride (5 mM). The beads were incubated overnight (36°C) on a rotating shaker in 1/10 strength nutrient broth with added calcium chloride (5 mM) to maintain the bead structure. The mean gel bead diameter (2.4 mm) was measured by lining up 10 beads on a ruler and determining the mean diameter.

Biofilm Disinfection

Bacterial cells in artificial biofilm gel beads were challenged with an antimicrobial agent (chlorine, glutaraldehyde, QAC, and ciprofloxacin) at varying concentrations at room temperature (23°C). The disinfection solution was made from a

stock solution of phosphate buffer (containing 0.085g/L potassium dihydrogen phosphate and 0.4055 g/L magnesium chloride) with added calcium chloride (5 mM) to support the bead structure. The desired amount of antimicrobial agent was then added to the phosphate buffer and the concentration verified when possible. At the start of the experiment, approximately 250-300 beads were placed in the magnetically stirred disinfection solution (500-700 mL) and beads (10) were removed at various time points. The sampled beads were placed in 5 mL of a solution containing a neutralizing agent (Table 1) and sodium citrate (50 mM). The sodium citrate dissolves the bead structure.

Table 1. Neutralizing agents for the antimicrobial agents investigated.

Agent	Neutralizer
Chlorine	Sodium Thiosulfate (50 mM)
Glutaraldehyde	Glycine (1%)
Quaternary Ammonium Compound	Tryptic Soy Broth
Ciprofloxcin	None; dilution

The bead-citrate solution was refrigerated for two hours while the beads dissolved, then diluted and plated out on R_2A using the drop plate method (Hoben, et al 1948; Reed, et al. 1948). The plates were incubated overnight (36°C) and counted. A control experiment was conducted in the same manner, with the disinfection solution replaced by phosphate buffer (pH 7.4). Number of experimental replicates is tabulated in Table 2.

Planktonic Cell Disinfection

Planktonic cells were challenged with an antimicrobial agent (chlorine, glutaraldehyde and QAC) at various concentrations at room temperature (23°C). A planktonic culture of *P. aeruginosa* (ERC1) was grown on a rotating shaker (36°C) to mid-log phase in 1/10 strength nutrient broth (Difco) with added calcium chloride (5 mM, for consistency with beads experiments). Aliquots of this culture were centrifuged (7.5 min, 10,000 rpm) and the supernatant broth decanted. The bacterial pellet was resuspended with phosphate buffer (pH 7.4) by vortexing. The disinfection solution (18 mL) was made from a stock solution of the particular antimicrobial agent (chlorine: approximately 50,000 ppm, glutaraldehyde: 250 ppm, QAC: BARQUAT MB80-80% active alkyl dimethyl benzyl ammonium chloride) and phosphate buffer (pH 7.4) so that the final concentration, after the bacterial suspension was added (2 mL), was the desired concentration (the nominal concentration reported). The solution was sampled at various time points (1 mL) and the aliquot was neutralized in the appropriate neutralizer (9 mL), diluted and plated on R_2A agar using the drop plate method. The plates were incubated overnight (36°C) and counted. A control experiment was conducted in the same manner, with the disinfection solution replaced by phosphate buffer (pH 7.4).

The planktonic disinfection by ciprofloxacin was done in a slightly different manner. A planktonic culture of *P. aeruginosa* (ERC1) was grown on a rotating shaker

(36°C) to mid-log phase in 1/10 strength nutrient broth with added calcium chloride (5 mM, for consistency with beads experiments). Aliquots of this culture were centrifuged (7.5 min, 10,000 rpm) and the supernatant broth decanted. The bacterial pellet was resuspended with phosphate buffer (pH 7.4) by vortexing. The disinfection solution (45 mL) was made from a stock solution ciprofloxacin (0.0025 g/mL) and phosphate buffer (pH 7.4) so that the final concentration, after the bacterial suspension was added (5 mL), was the desired concentration (1, 5, or 25 μg/mL). Planktonic disinfection was performed at room temperature (22°C). The solution was sampled at various time points (1 mL) and vortexed (7.5 min, 10,000 rpm), the disinfection solution decanted and the challenged bacterial pellet resuspended in phosphate buffer (pH 7.4). The resuspended solution was again vortexed (7.5 min, 10,000 rpm), the wash solution decanted and the bacterial pellet again resuspended in phosphate buffer. The resuspended, washed bacterial sample was then plated on R_2A agar using the drop plate method and incubated overnight (36°C) and the culturable counts determined. Number of experimental replicates is tabulated in Table 2.

Chlorine Concentration Assay

Chlorine concentration was determined using the DPD colorimetric method (Hach). Aliquots of the chlorine disinfection solution were taken at various time points during each experiment to monitor the residual concentration.

Table 2: Number of experimental replicates for each antimicrobial agent tested. ND denotes not determined.

Antimicrobial Agent (mg/L)	# Planktonic experiment replicates	#Biofilm experiment replicates
Untreated Control:	2	2
Chlorine:		
10	4	3
20	3	3
80	4	2
Glutaraldehyde:		
25	3	2
50	4	2
100	3	3
200	ND	2
250	3	ND
Ciprofloxacin:		
1	3	3
5	7	3
25	3	3
QAC:		
50	4	4
100	3	2
250	4	5
500	ND	2
1000	ND	2

Glutaraldehyde Concentration Assay

Glutaraldehyde concentration was determined by gas chromatography. Samples (1 mL) of the glutaraldehyde disinfection solution were taken at various time points during each experiment to monitor the residual concentration and stored under refrigeration (4°C) in Target silanized vials (Fisher) until they could be injected, which was in no case longer than 24 hours. Gas chromatography analysis was performed on a Hewlett-Packard 5890 Series chromatograph. The operating temperatures were as follows: detector 250°C, injector: 190°C, oven: 185°C. The column head pressure was approximately 38 psi. Working standards were prepared from a stock solution of UCARCIDE 250 (Union Carbide Corporation) and a standard curve was constructed. The syringe (Hamilton P/N 80337) was rinsed with water between injections and several times with the sample before each injection. Working standards were injected approximately every 15 injections. The injections (2 µl) were done in duplicate for each sample.

Transmission Electron Microscopy Sample Preparation

Alginate gel beads with entrapped *P. aeruginosa* were prepared as described above and incubated for 24 hours in 1/10 strength nutrient broth (36°C). The beads were then fixed in glutaraldehyde (2.5%) in phosphate buffer (pH 7.4) with added calcium chloride (5 mM, to support bead structure). The beads were then washed (3x, 15 min) in the phosphate buffer. Next, the beads were stained with osmium tetroxide (1%) in the calcium enhanced phosphate buffer. The beads were again washed (3x, 15 min) in the

calcium enhanced phosphate buffer. The beads then underwent a series of dehydration steps: 50% ethanol, 15 minutes; 70% ethanol, 15 minutes; 1% uranyl acetate/1% phosphotungstic acid (PTA), 1 hour; 95% ethanol, 15 min; 100% ethanol, 15 minutes; 100% ethanol, 15 minutes; 100% ethanol, 15 minutes; 100% ethanol (2 parts): SPURRS (1 part, Ernest F. Fullam, Inc.), 1 hour; 100% ethanol (1 part): SPURRS (1 part), 1 hour; SPURRS epoxy resin, 8 hours or overnight. Then the beads were embedded in size 00BEEM capsules. The epoxy resin was polymerized for 14 hours (70°C) in oven. A thick section of bead was cut and stained with toluidine blue. The sections were cut and examined using a Jeol JEM-100CX electron microscope.

Computer Modeling

The computer model created by Mike Dodds at the CBE was used to determine parameters for data sets suggesting a physiological model of resistance. This model of resistance assumes that cells reside in either a resistant state or a susceptible state. Full penetration of the antimicrobial agent is assumed and it is assumed that the susceptible population is as susceptible to the antimicrobial agent as planktonic cells (Dodds, et al. 1999). The solution to this model is expressed analytically by:

$$\int_0^1 \frac{X}{X_0} \cdot d\zeta = \varepsilon_s \cdot \exp(-\psi) + (1-\varepsilon_s) \cdot \exp(-p \cdot \psi) \qquad (1)$$

where

$\psi \equiv k_{dis} \cdot C_b \cdot t_{dose}$
k_{dis} = disinfection rate constant
C_b = concentration of biocide in the bulk fluid

t_{dose} = total time biocide was applied to the biofilm
X = viable biomass density in biofilm after disinfection
X_0 = viable biomass density in biofilm before disinfection
ζ = dimensionless spatial parameter

The parameters obtained were:

ε_s = sensitive fraction of cells within biofilm
k_{dis} = disinfection rate coefficient
p = measure of the relative susceptibility of the resistant fraction

The resistant fraction, ε_r, was obtained by $\varepsilon_r = 1 - \varepsilon_s$.

Matlab computer software was used to implement the model (Dodds, et al. 1999).

Resistance Factor

A resistance factor was calculated by dividing the time needed to reach a two log reduction in the viable cell numbers in artificial biofilm by the time for a two log reduction of planktonic cell viable numbers in response to the same concentration of antimicrobial agent.

Analysis of Concentration Dependence

To analyze the antimicrobial agent concentration dependence of planktonic and biofilm killing, the Chick-Watson mathematical model of disinfection was assumed (Haas and Karra 1984)

$$\frac{dX}{dt} = -k_{dis} C^n X \qquad (2)$$

where X is the viable density, t is time , k_{dis} is a disinfection rate coefficient, and C is glutaraldehyde 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

$$\ln\left(\frac{X}{X_o}\right) = -k_{dis}C^n t \tag{3}$$

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

$$r = \frac{-\ln(0.01)}{t} \tag{4}$$

Each experimental data set was fit to a third order polynomial using an existing regression function in an Excel™ spreadsheet. The time for a two-log reduction was determined from this fit and the apparent disinfection rate calculated from Equation (4). Combining Equations (3) and (4) we obtain

$$\ln(r) = \ln(k_{dis}) + n\ln(C) \tag{5}$$

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.

There has been some speculation regarding the applicability of Chick's law to non-linear data sets (Haas and Heller 1990; Haas 1980; Haas 1988). This law was utilized in this analysis strictly as a means of comparing the concentration dependence of each agent on planktonic and biofilm cells independent of the mechanism of disinfection. The

duration of a two log reduction was chosen to minimize the non-linearity of each data set while still capturing the disinfection phenomena.

A common practice in the application of antimicrobial agents is to assume that doses in which the product of dose concentration and dose duration, C^*t, is the same will yield the same disinfection efficacy (Wichramanayake 1991; Hass et al. 1990). This method predicts plotting the surviving fraction versus the product of the bulk concentration and treatment time should collapse data from experiments using different concentrations on to a single curve. Correlations of $\log(X/X_0)$ versus the product C^*t were performed to test the applicability of the C^*t concept.

Observable Modulus

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

$$\Phi = \frac{R_{obs} L_f^{\,2}}{D_e C_o} \tag{6}$$

and R_{obs} is the overall antimicrobial disappearance rate

$$R_{obs} = \frac{\Delta C \cdot V_{sol}}{V_{bead} \cdot \Delta t} \tag{7}$$

where for chlorine:

$$\Delta C = (C_i - C_f) + k \cdot C_0 \cdot t \tag{8}$$

and for glutaraldehyde:

$$\Delta C = (C_i - C_f) \tag{9}$$

where:
C_i = initial biocide concentration (mg/L)
C_f = final biocide concentration (mg/L)
Δt = time interval (min)
V_{sol} = volume of disinfection solution (mL)
V_{bead} = volume of beads (cm^3)
L_f = effective biofilm thickness (volume to surface area radio)(radius of bead/3)
D_e = the effective diffusivity of agent in the beads (cm^2/s)
C_o = mean concentration over time interval (mg/L)
k = first order rate constant of chlorine disappearance without beads (min^{-1})

The effective diffusion coefficient, D_e, was calculated by estimating the diffusivity using correlations published in the literature (Westrin and Axelsson 1991). Different ΔC values were used for chlorine and glutaraldehyde because it was determined that chlorine's volatility (unexposed to beads) was responsible for some of the degradation of chlorine and must be accounted for. Glutaraldehyde did not significantly disperse in the absence of gel beads.

Table 3. Values of the D_{aq} (25°C) employed in observable modulus calculations (Stewart, 1999).

Antimicrobial Agent	**D_{aq} (cm^2/s)**
Chlorine	1.4×10^{-5}
Glutaraldehyde	9.3×10^{-6}

RESULTS

Survival versus time data from disinfection experiments using artificial biofilms and planktonic cells of the same organism were analyzed. Concentration dependence of biofilm and planktonic disinfection was examined using a simple mathematical model of disinfection. Resistance factor calculations were used to measure the relative resistance of the artificial biofilm cells. For chlorine and glutaraldehyde, an observable modulus was calculated as a measure of the relative rates of reaction and diffusion within the biofilm.

Artificial Biofilm Structure

Electron microscopy of gel beads revealed a structure of dense microcolonies embedded in a highly hydrated gel matrix (Figure 2). Using the electron micrograph image, 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 value of the relative effective diffusion coefficient in the gel bead, D_e/D_{aq}, accounting for the presence of polymer and cells (Westrin and Axelsson 1991), was 0.624.

The average radius of the beads was 2.42 mm $\pm$ 0.07. A distribution of initial cell

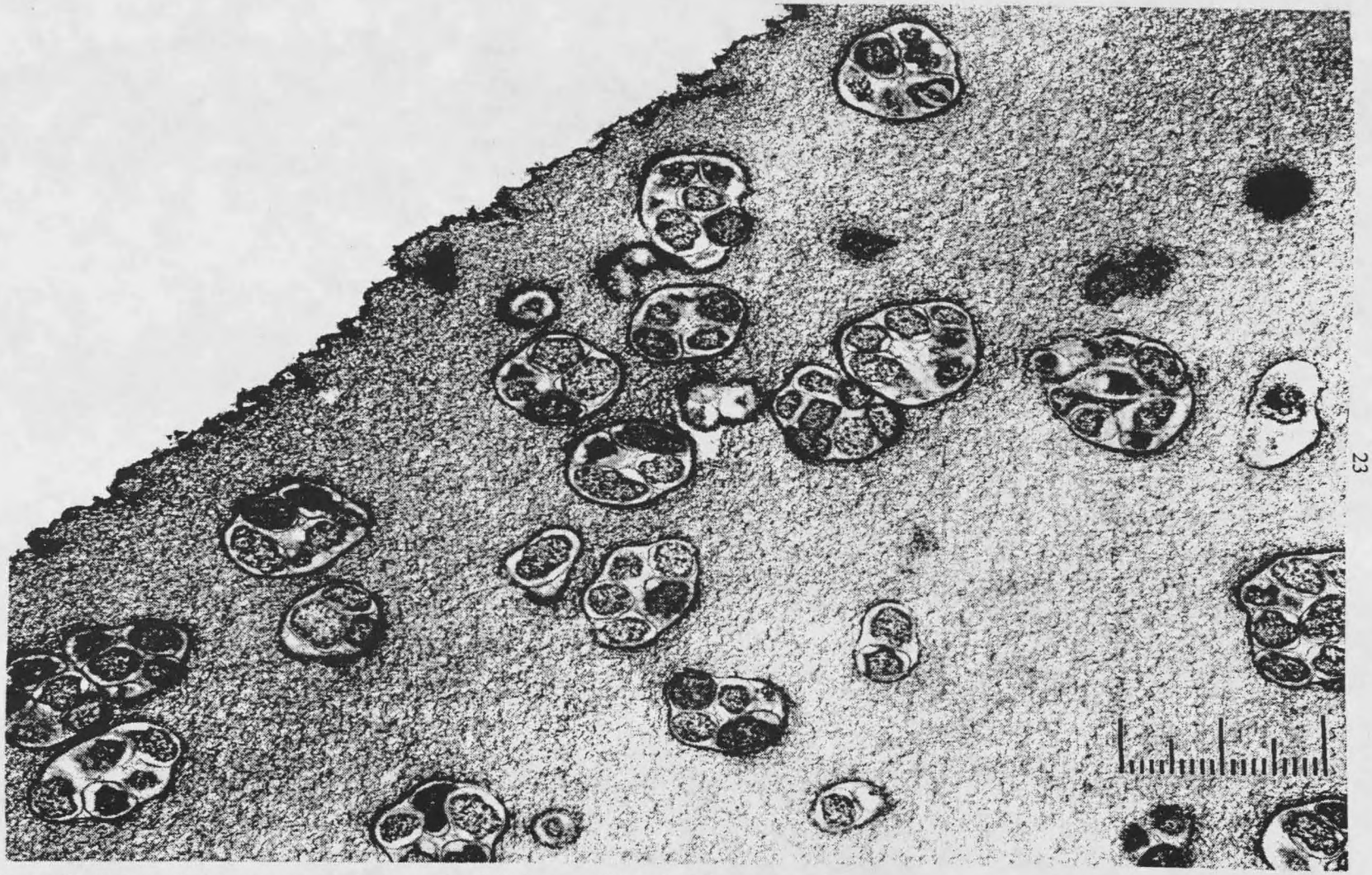

Figure 2. Transmission electron micrograph of alginate gel bead with entrapped P. Aeruginosa (scale bar = 10 μm)

density values from all of the biofilm experiments is plotted in Figure 3. The average initial cell density was 9.66 cfu/cm^3 ± 0.20.

Untreated Control

Untreated controls were performed with artificial biofilms and planktonic cells. In each case, the cell density was virtually unaffected by the control treatment solution (Figure 4 and 5).

Chlorine

Planktonic *P. aeruginosa* cells were readily killed by chlorine (pH 7.4-7.6) (Figure 6). A 2-log reduction was achieved within 2 minutes for all chlorine concentrations tested. On a plot of $\log_{10}$ of the survival fraction versus time, planktonic killing profiles were non-linear, concave up.

Biofilm cells of *P. aeruginosa* entrapped in gel beads were killed by exposure to chlorine at concentrations of 10-90 mg/L (Figure 7). At 10 mg/L a plot of $\log_{10}$ survival versus time (Figure 8) the biofilm killing profile was non-linear with a characteristic concave down shape. At 20 mg/L and 90 mg/L the killing profiles became nearly linear.

Biofilm cells were clearly less susceptible than planktonic cells to the same chlorine treatment. Killing of planktonic and biofilm bacteria by 90 mg/L chlorine is compared graphically in Figure 8. At this concentration, the highest tested, it took approximately 2 minutes to achieve a 2 log reduction in the artificial biofilm system,

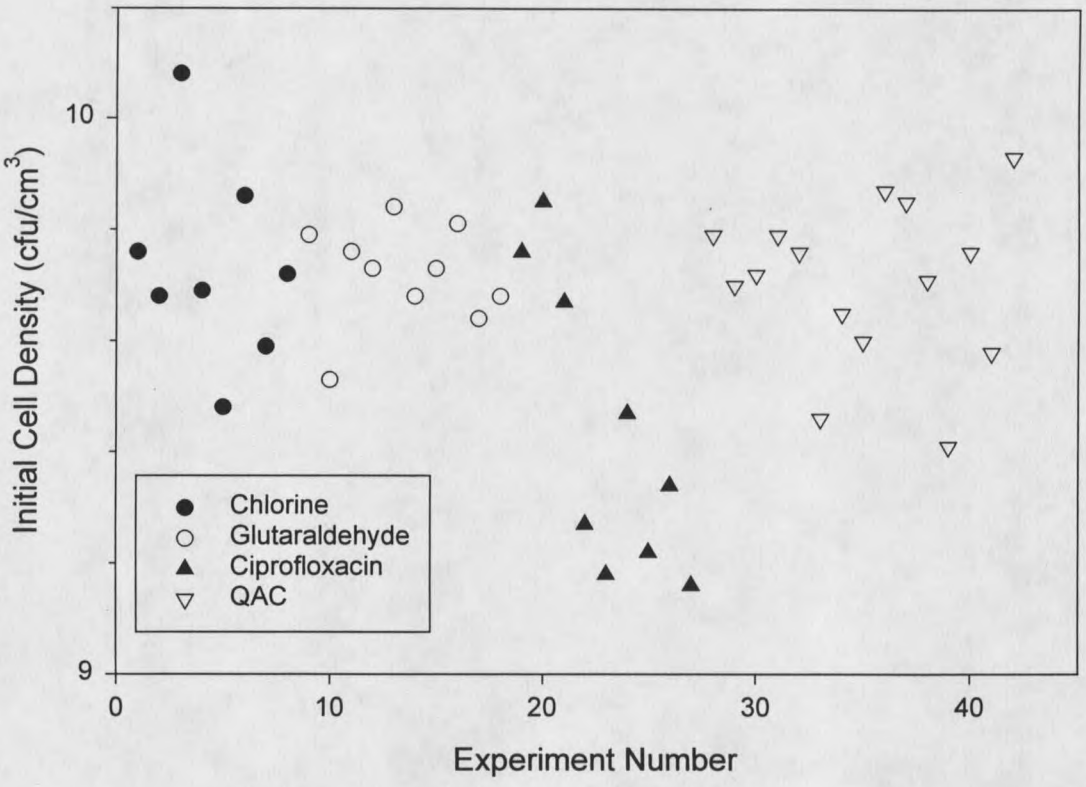

Figure 3. Distribution of initial cell density in artificial biofilm experiments.

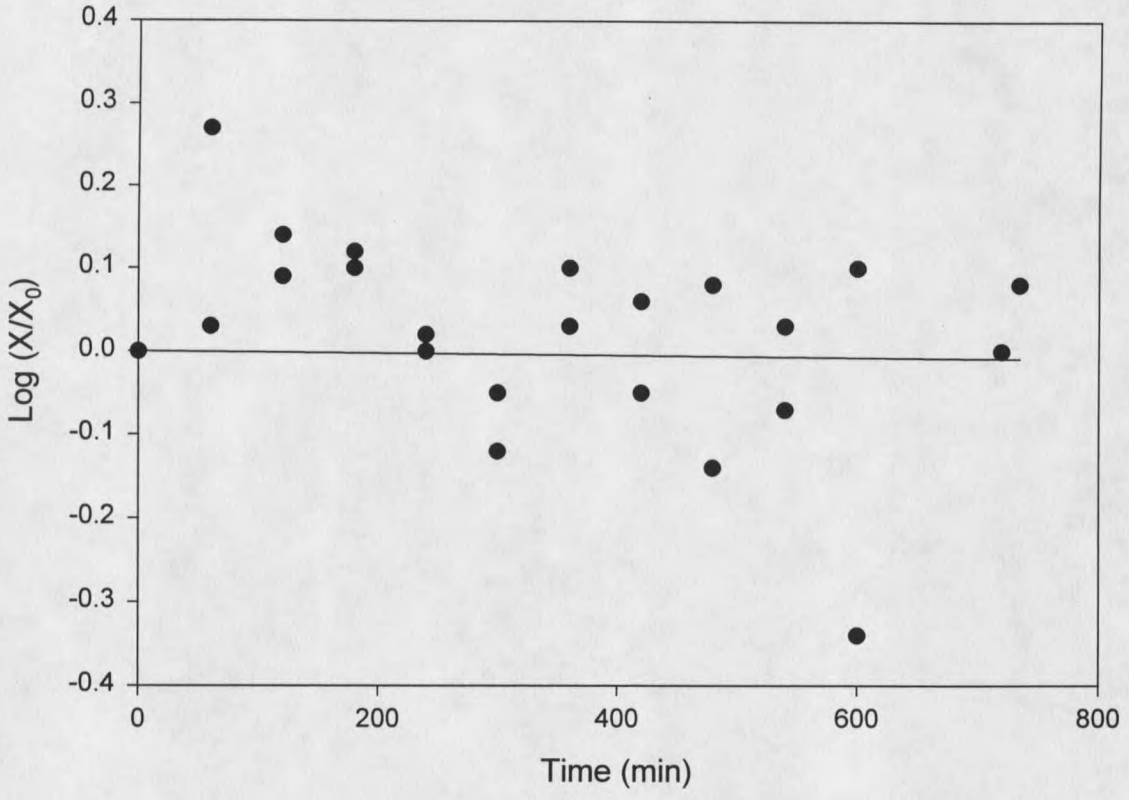

Figure 4. Artificial biofilm untreated control.

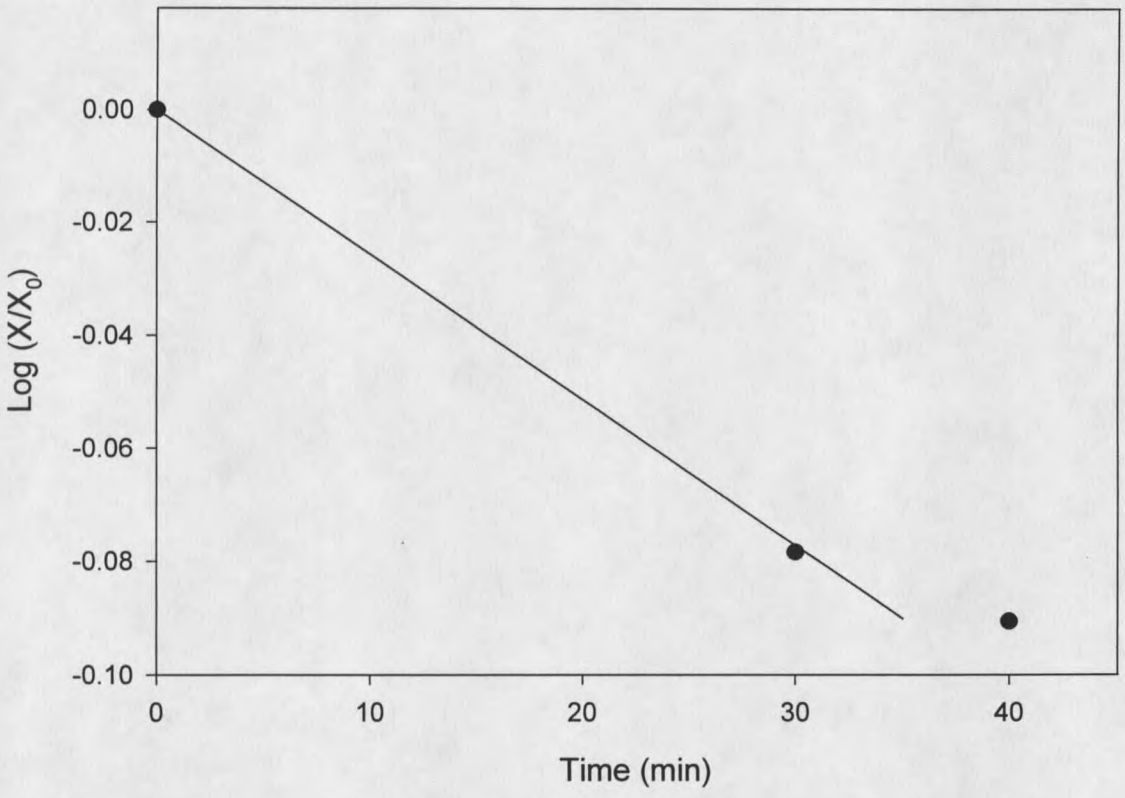

Figure 5. Planktonic untreated control.

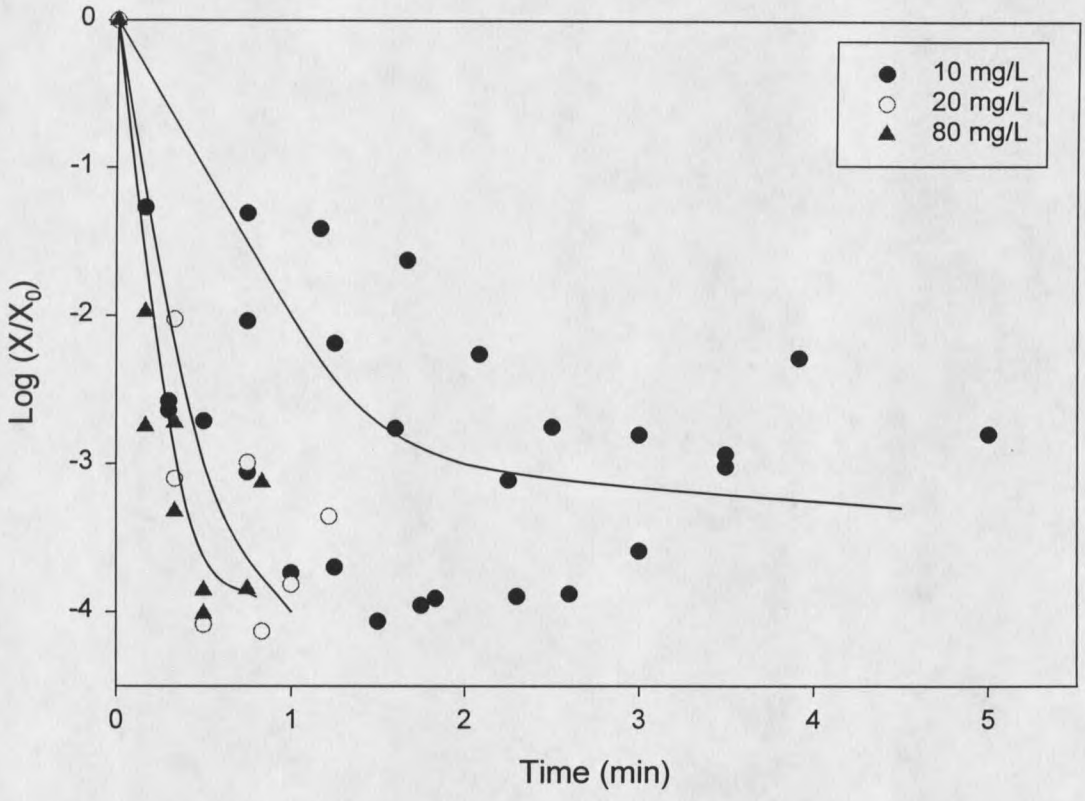

Figure 6. Killing of planktonic *P. aeruginosa* bacteria exposed to chlorine.

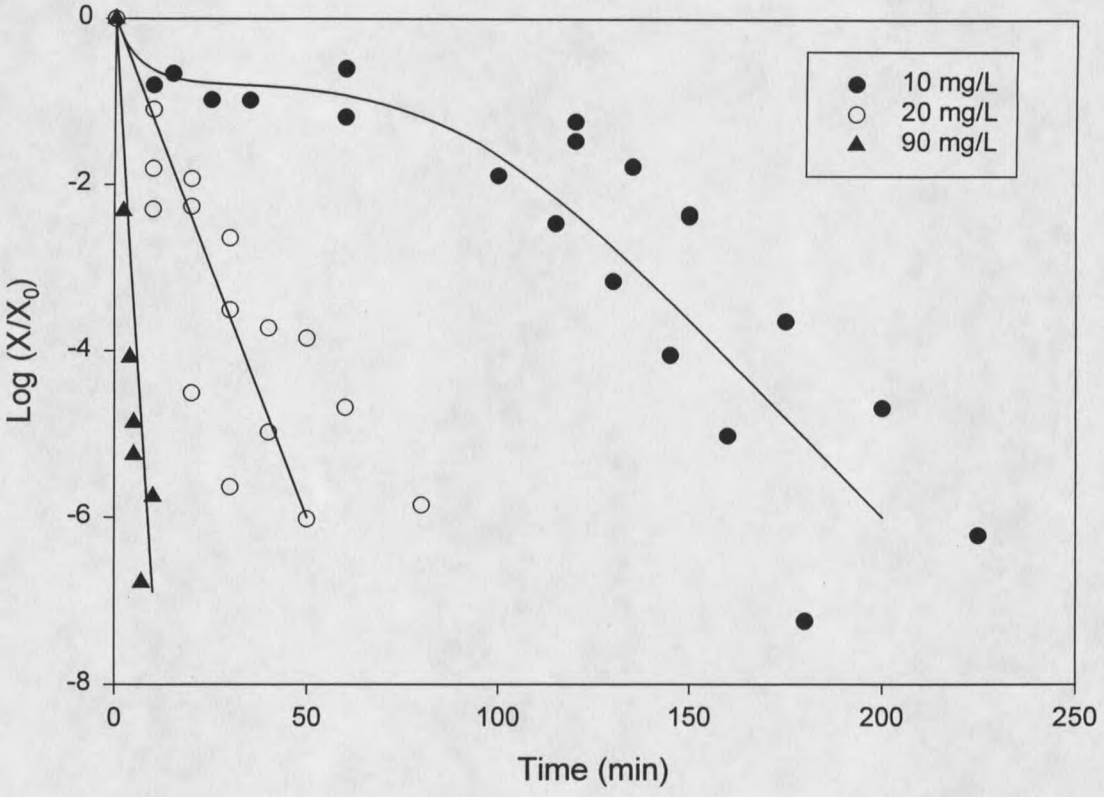

Figure 7. Killing of *P. aeruginosa* bacteria entrapped in artificial biofilms and exposed to chlorine.

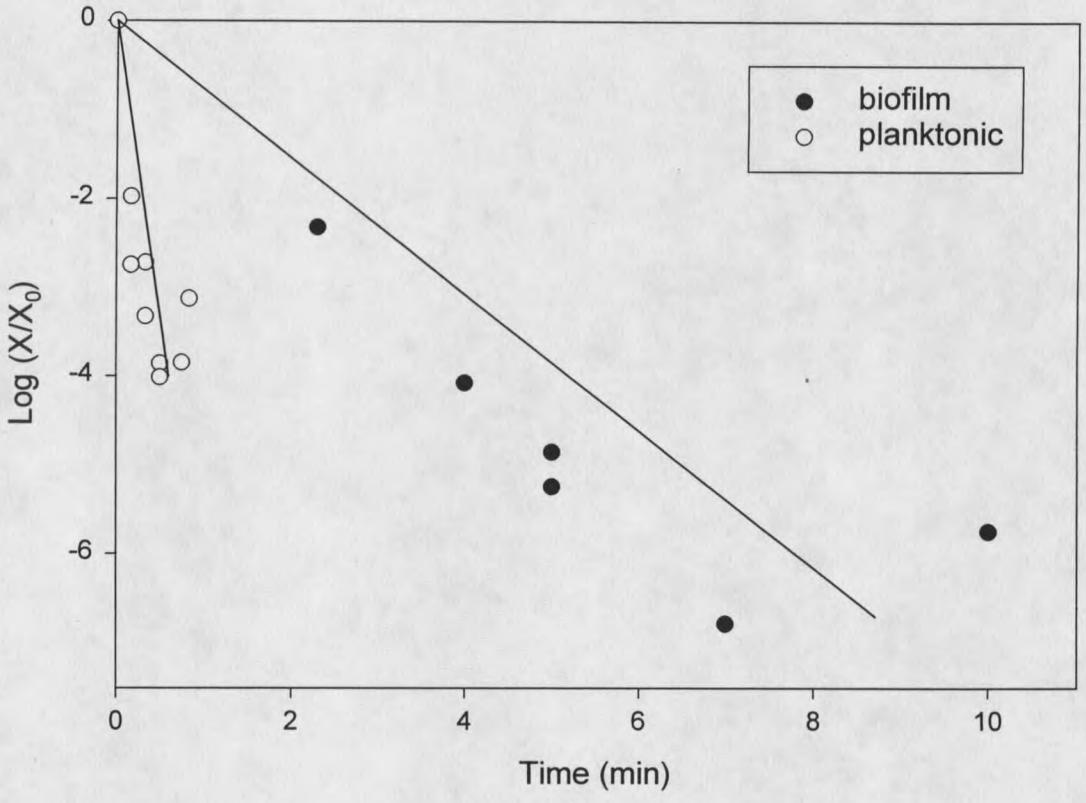

Figure 8. Comparison of planktonic and biofilm killing of *P. aeruginosa* bacteria exposed to 90 mg/L chlorine.

while planktonic disinfection took under 1 minute to achieve the same level of killing. The times needed to achieve a 2 log reduction in culturable cell numbers for planktonic and biofilm cultures are summarized in Table 4 for various treatment concentrations.

Table 4. Chlorine treatment time to attain a 2 log reduction in viable cell numbers (± values indicate standard deviation).

Concentration (mg/L)	Planktonic kill time (min)	Biofilm kill time (min)	Resistance Factor	Time for observable modulus to decrease to 1 (min)
10	0.44 ± 0.25	127 ± 8	290	133
20	0.31 ± 0.22	15 ± 6	47	79
90	0.17 ± 0.043	2 ± 0.3	11	45

Planktonic and biofilm bacteria responded to increased chlorine concentrations differently (Figure 9). The apparent kinetic order, which is given by the slope of the regressed line in Figure 9, was 0.31 ± 0.15 for planktonic cells. Increasing the chlorine concentration more rapidly accelerated biofilm killing. The apparent kinetic order of chlorine concentration dependence was 1.84 ± 0.25 for biofilm cells (Figure 9).

The measured chlorine concentration in the solution used to treat biofilm gel beads ranged from –17.5% to +7.4% of the nominal concentration in the eight experiments. On average the concentration of the experiments was 2.4% less than the

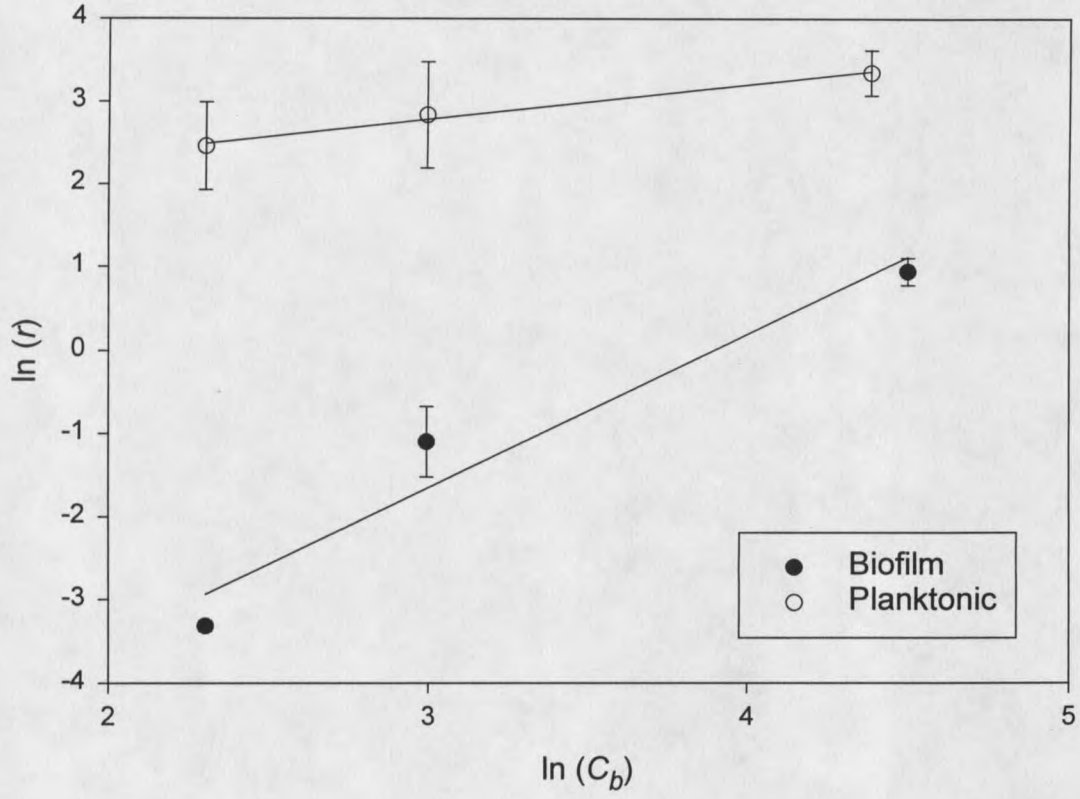

Figure 9. Analysis of the concentration dependence of the apparent rates of killing of planktonic and biofilm *P. aeruginosa* exposed to chlorine. The lines are least squares regressions with 1) planktonic, $y = 0.3086 \pm 0.15\ x + 1.9767$, $R^2 = 0.3632$ and 2) biofilm, $y = 1.0834 \pm 0.25\ x - 6.963$, $R^2 = 0.9141$. C_b has units of mg/L and r has units of min^{-1}.

nominal concentration. This suggests that there is some loss of chlorine by reaction with the artificial biofilm during treatment.

The observable modulus was calculated for each of the chlorine experiments. A plot of the modulus versus time shows that during each of the experiments, the modulus decreased over time (Figure 10). The point at which this data trend crosses a value of one indicates a shift from diffusion limitation to reaction limitation. This time is tabulated in Table 4. Increasing the concentration decreases the time to reach this reaction-diffusion point.

Plots of log (X/X_0) versus C^*t for both biofilm and planktonic cells show a non-linear and noisy relationship between the product of concentration and time and microbial survival (Figures 11 and 12). R^2 values for the linear regression of log (X/X_0) versus C^*t were 0.32 and 0.035 for biofilm and planktonic data, respectively.

Glutaraldehyde

Planktonic *P. aeruginosa* cells were readily killed by glutaraldehyde (Figure 13). A 2 log reduction in viable cell numbers was obtained within 30 minutes for all glutaraldehyde concentrations tested. On a plot of $\log_{10}$ of the surviving fraction versus time (Figure 13), planktonic killing profiles were approximately linear. Biofilm cells of *P. aeruginosa* entrapped in gel bead artificial biofilms were killed by exposure to glutaraldehyde at concentrations of 50-200 mg/L (Figure 14). 25 mg/L glutaraldehyde had little effect on biofilm cells, even after nearly 12 hours of treatment (Figure 14). On

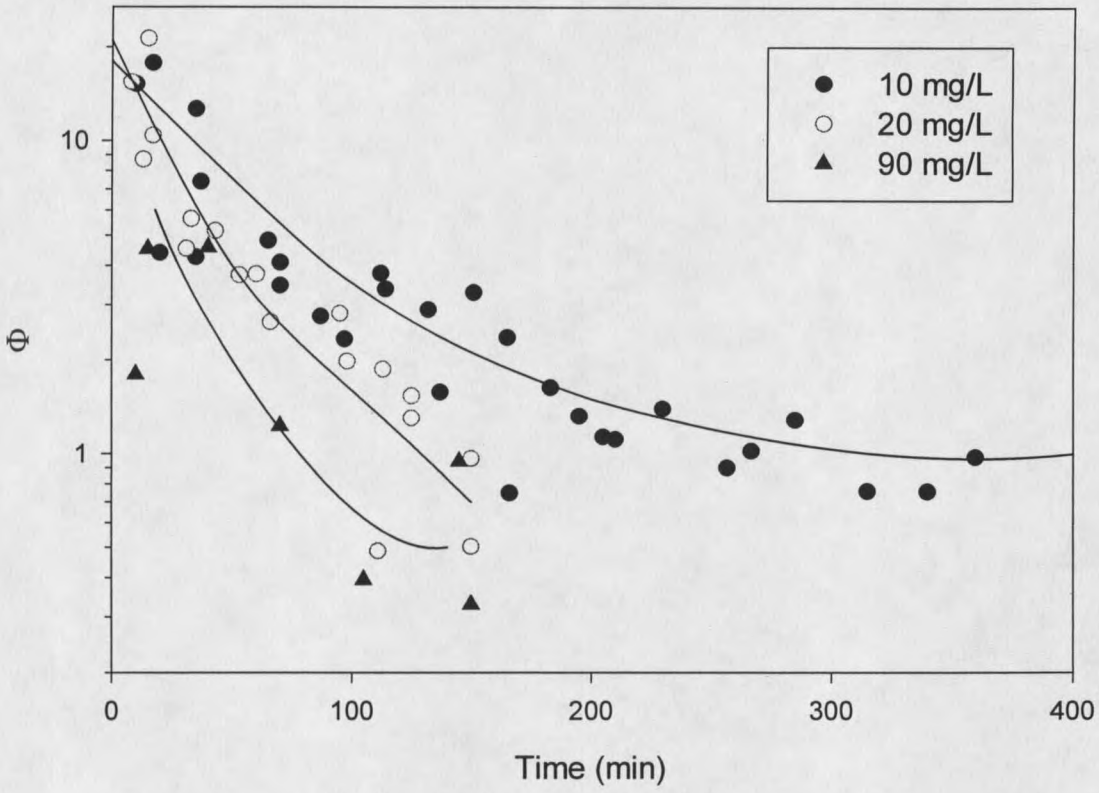

Figure 10. Analysis of the observable modulus dependence on time for biofilm exposed to various concentrations of chlorine.

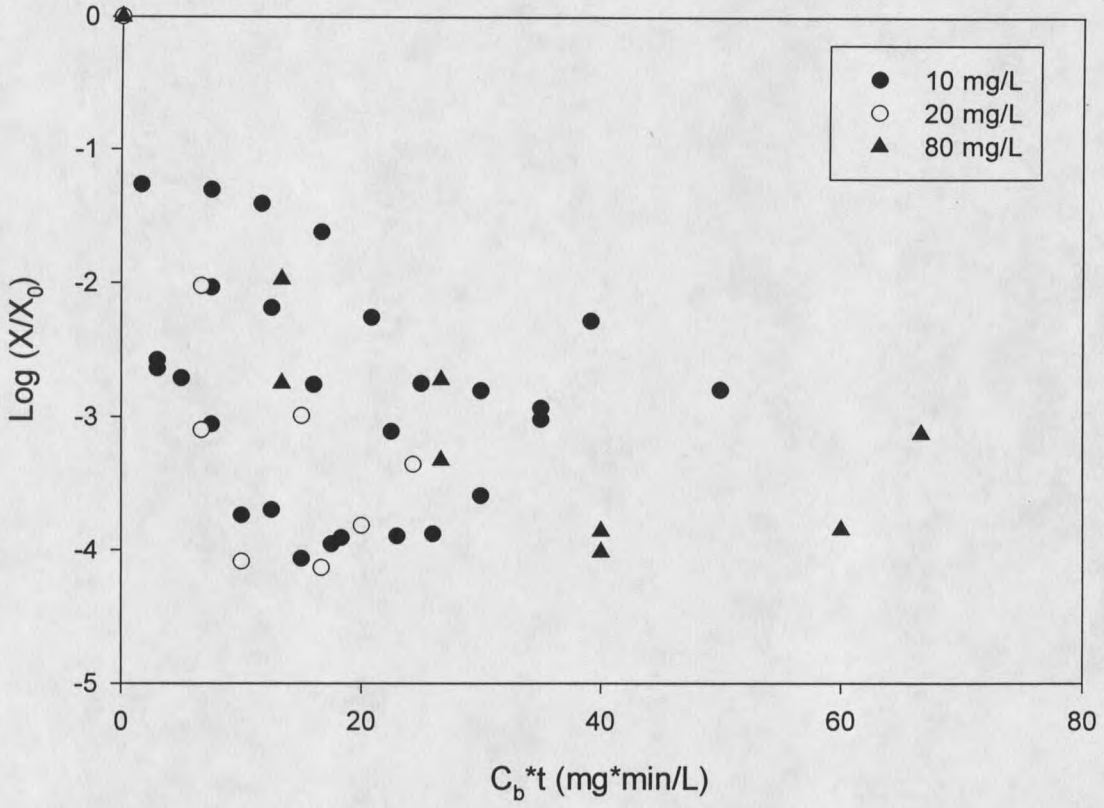

Figure 11. C*t analysis of planktonic *P. aeruginosa* disinfection by chlorine. R^2 = 0.035.

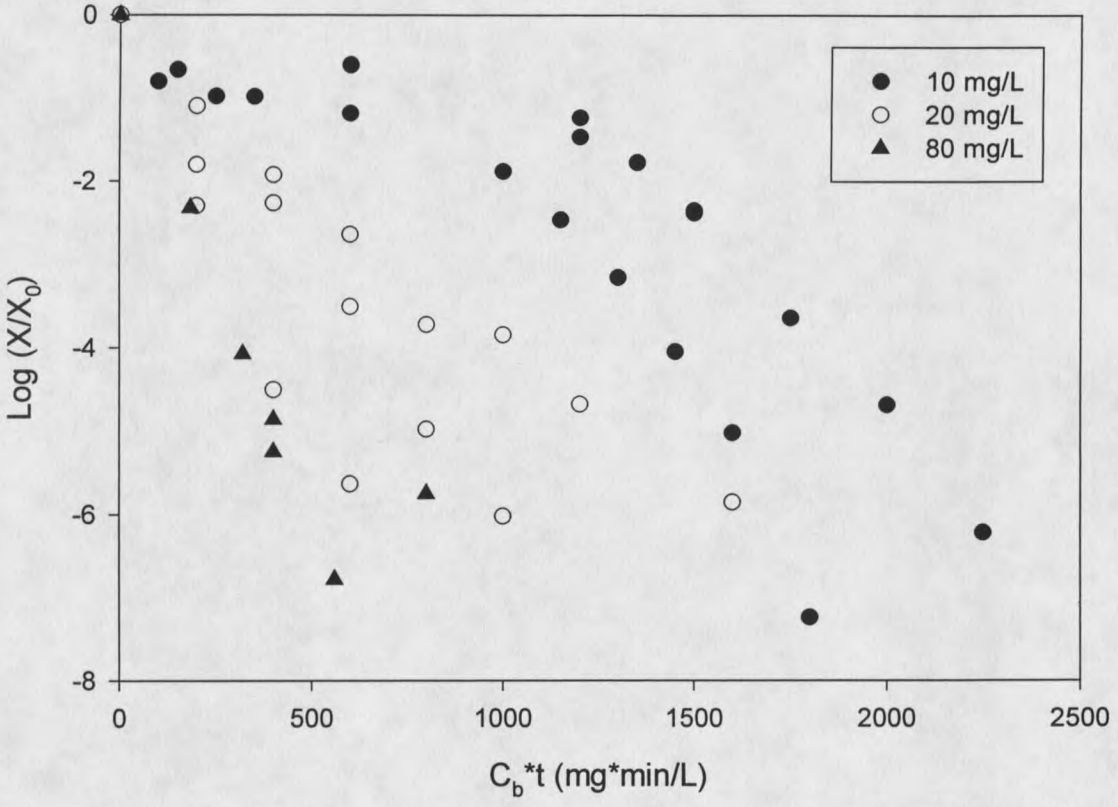

Figure 12. C*t analysis of *P. aeruginosa* artificial biofilm cells exposed to chlorine. R^2 = 0.3220.

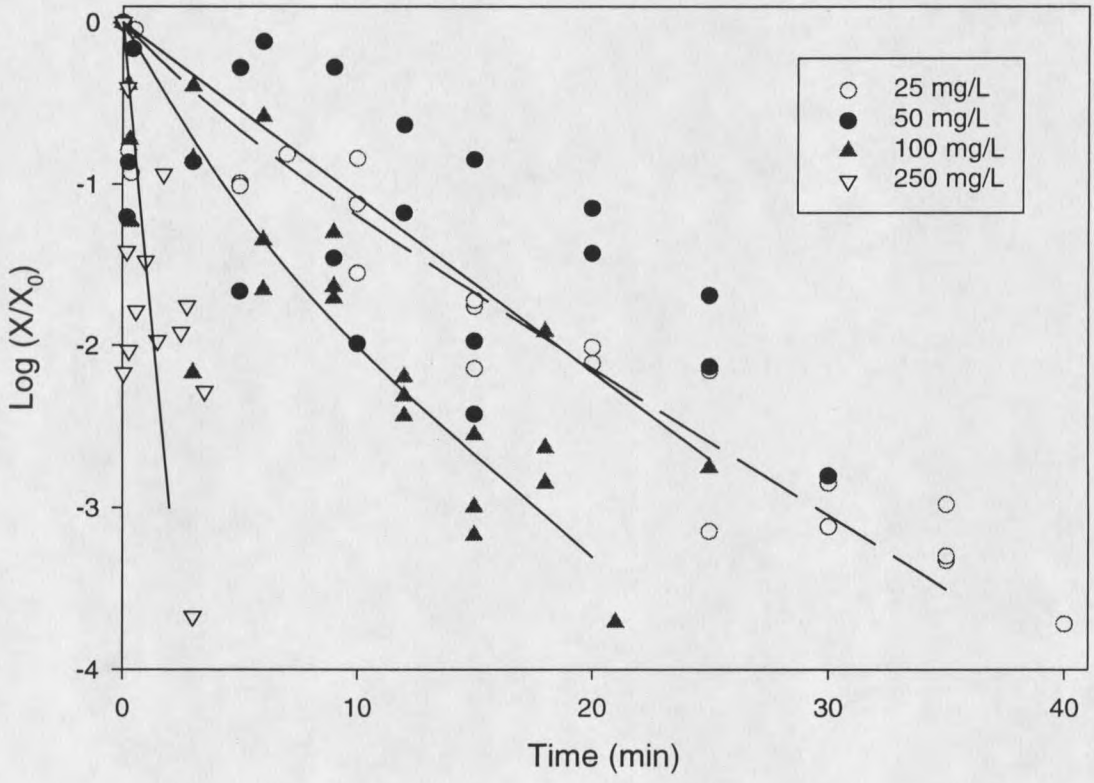

Figure 13. Killing of planktonic *P. aeruginosa* bacteria exposed to glutaraldehyde. The dashed line represents curve fit for 25 mg/L data set.

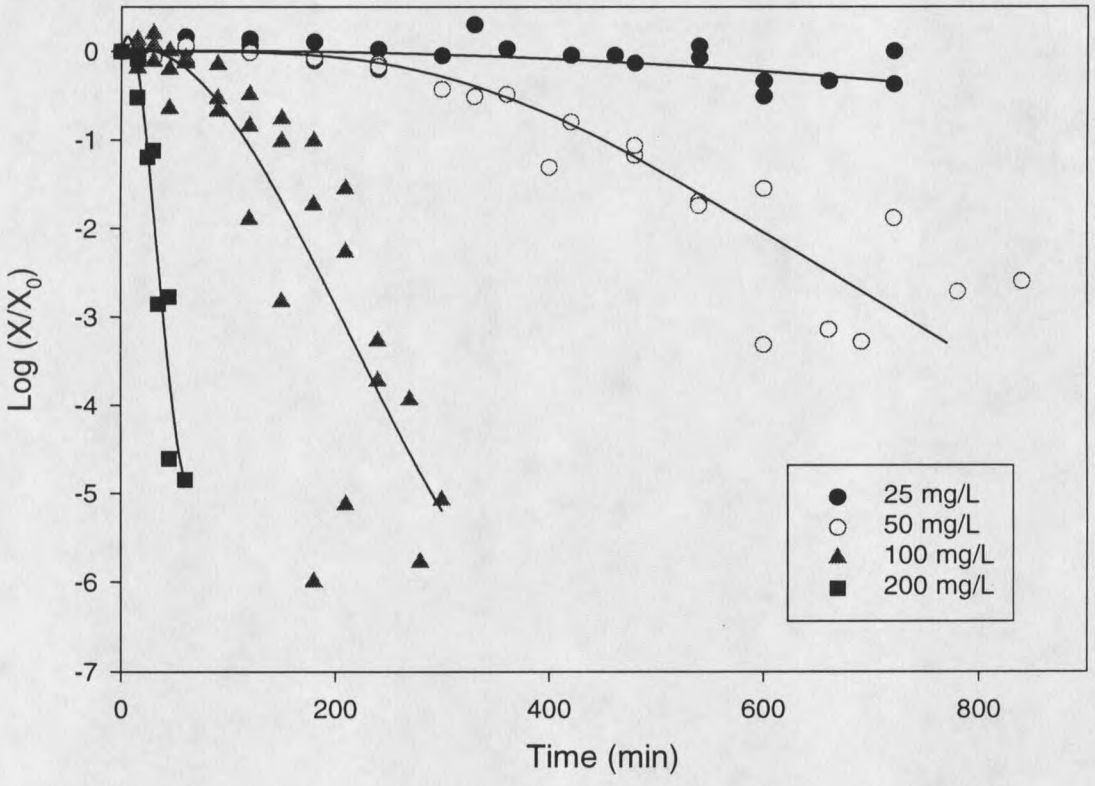

Figure 14. Killing of *P. aeruginosa* bacteria entrapped in artificial biofilms and exposed to glutaraldehdye.

a plot of the $\log_{10}$ of the surviving fraction versus time (Figure 14), biofilm-killing profiles at the concentrations exceeding 25 mg/L were non-linear with a characteristic concave down shape.

Biofilm cells were clearly less susceptible than planktonic cells to the same glutaraldehyde treatment. Killing of planktonic and biofilm bacteria by 50 mg/L glutaraldehyde is compared graphically in Figure 15. While it took only approximately 18 minutes to achieve a 2 log reduction in culturable cell numbers in the planktonic experiments using 50 mg/L glutaraldehyde, almost 650 minutes were required to achieve this same level of killing in the biofilm. This represents a 36-fold increase in the treatment time to obtain the same effect. The times needed to reach a 2 log reduction in viable cell numbers for planktonic and biofilm cultures are summarized in Table 5 for the various treatment concentrations. Increasing the concentration decreases the time required for a 2 log reduction in both cases.

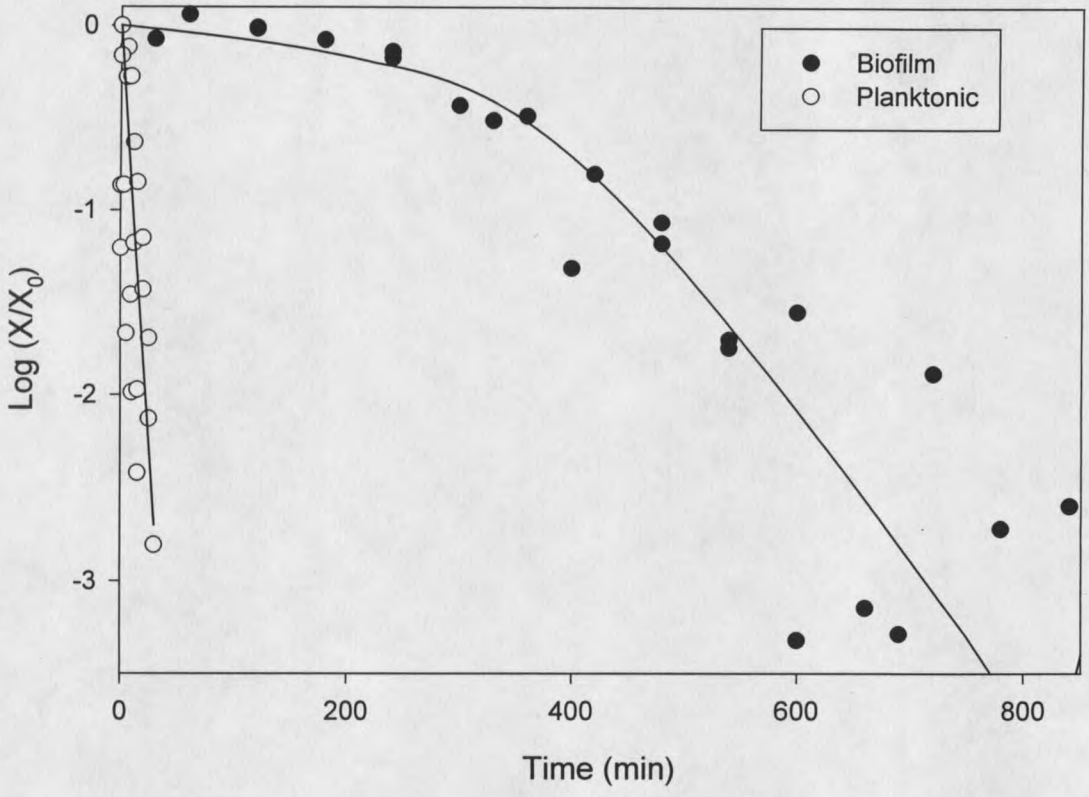

Figure 15. Comparison of planktonic and biofilm killing of *P. aeruginosa* bacteria exposed to 50 mg/L glutaraldehyde.

Table 5. Glutaraldehyde treatment time to attain a 2 log reduction in viable cell numbers (± values indicate standard deviation). ND denotes not determined.

Concentration (mg/L)	Planktonic kill time (min)	Biofilm kill time (min)	Resistance Factor	Time for observable modulus to decrease to 1 (min)
25	17.9 ± 2.5	842*	47	900
50	18.2 ± 9.2	647 ± 75	36	440
100	8.8 ± 3.9	174 ± 59	20	170
200	ND	34 ± 5.5	ND	-31
250	2.4 ± 1.1	ND	ND	ND

* indicates an extrapolated value; a 2 log reduction was not actually reached in the experiment

Planktonic and biofilm bacteria responded to increased glutaraldehyde concentrations differently (Figure 16). Planktonic killing exhibited approximately first order dependence on glutaraldehyde concentration. The apparent kinetic order, which is given by the slope of the regressed line in Figure 16, was 1.0 ± 0.19 for planktonic cells. Increasing the glutaraldehyde concentration more rapidly accelerated biofilm killing. The apparent kinetic order of glutaraldehyde concentration dependence was 2.1 ± 1.9 for biofilm cells (Figure 16).

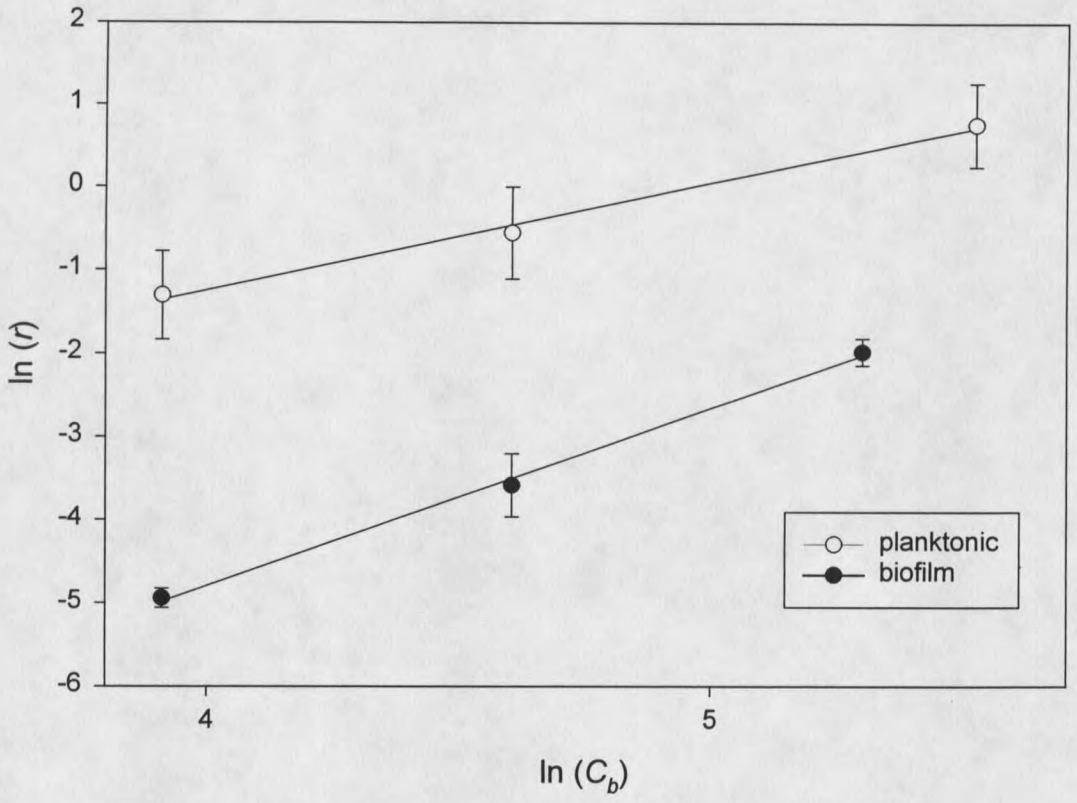

Figure 16. Analysis of the concentration dependence of the apparent rate of killing of planktonic and biofilm *P. aeruginosa* exposed to glutaraldehyde. The lines are least squares regressions with 1) planktonic, $y = 1.005 \pm 0.19\,x - 4.978$, $R^2=0.7857$ and 2) biofilm, $y = 2.134 \pm 1.9\,x - 13.3435$, $R^2 = 0.9606$. C_b has units of mg/L and r has units of min^{-1}.

The measured glutaraldehyde concentration in the solution used to treat biofilm gel beads ranged from +12% to –34% of the nominal concentration in the nine experiments. On average the concentration of the experiments was 13% less than the nominal concentrations. This reflects the fact that there was some loss of glutaraldehyde by reaction with the artificial biofilm during treatment.

The observable modulus was calculated for each of the glutaraldehyde experiments. A plot of this modulus versus time shows that during each experiment the modulus decreases over time (Figure 17). This indicates that the degree of transport limitation decreases during treatment. The time at which this trend crosses a value of one can be considered to be one measure of the time at which incomplete penetration of glutaraldehyde ceases to be limiting in the disinfection process. This time is tabulated in Table 5. Increasing the concentration of glutaraldehyde decreases the time required for complete penetration.

Plots of log (X/X_0) versus C^*t for both planktonic and biofilm cells show a non-linear and noisy relationship between the product of concentration and time and microbial survival (Figures 18 and 19). R^2 values for the linear regression of log (X/X_0) versus C^*t were 0.40 and 0.49 for biofilm and planktonic data, respectively.

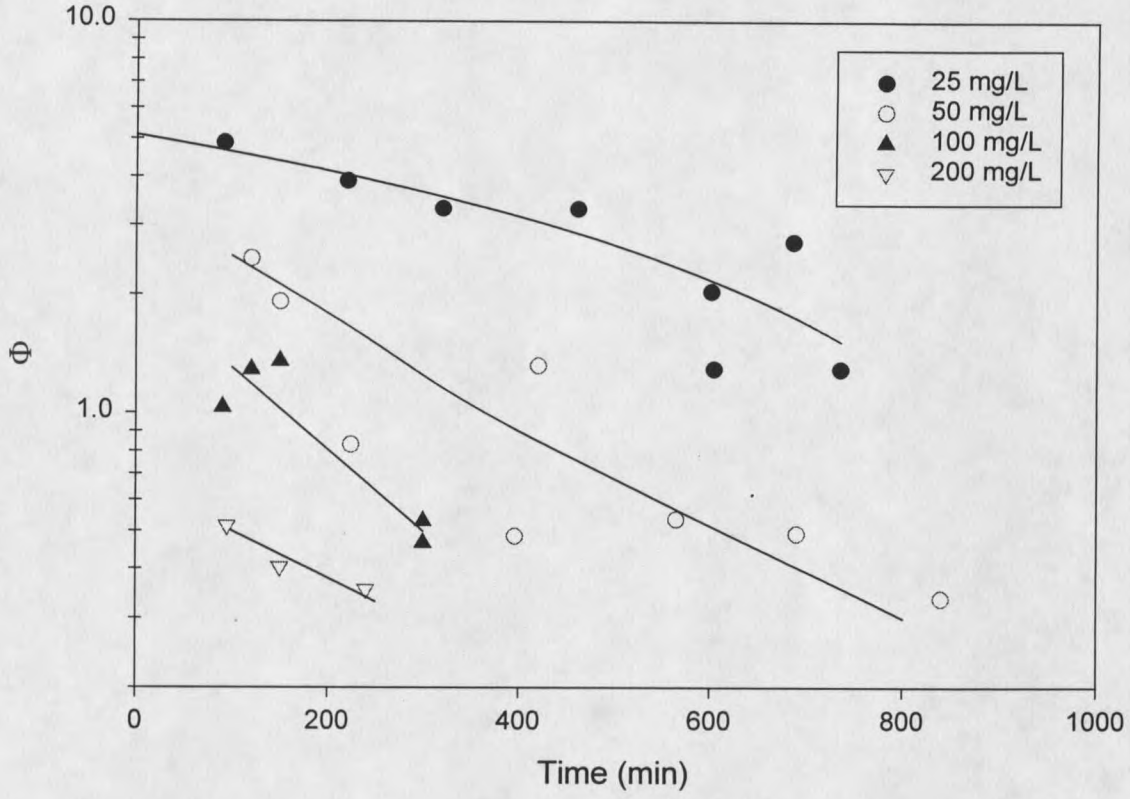

Figure 17. Analysis of the observable modulus time dependence of biofilm *P. aeruginosa* exposed to glutaraldehyde at various concentrations.

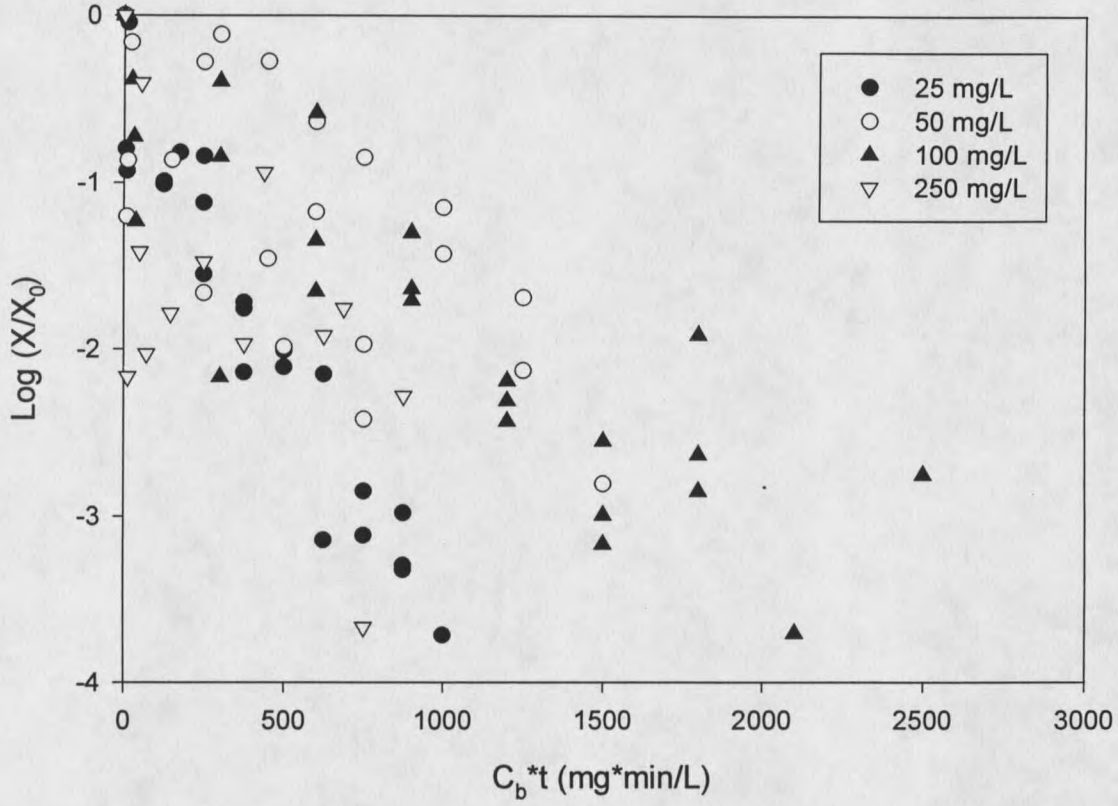

Figure 18. C*t analysis of planktonic *P. aeruginosa* exposed to glutaraldehyde. R^2 = 0.49.

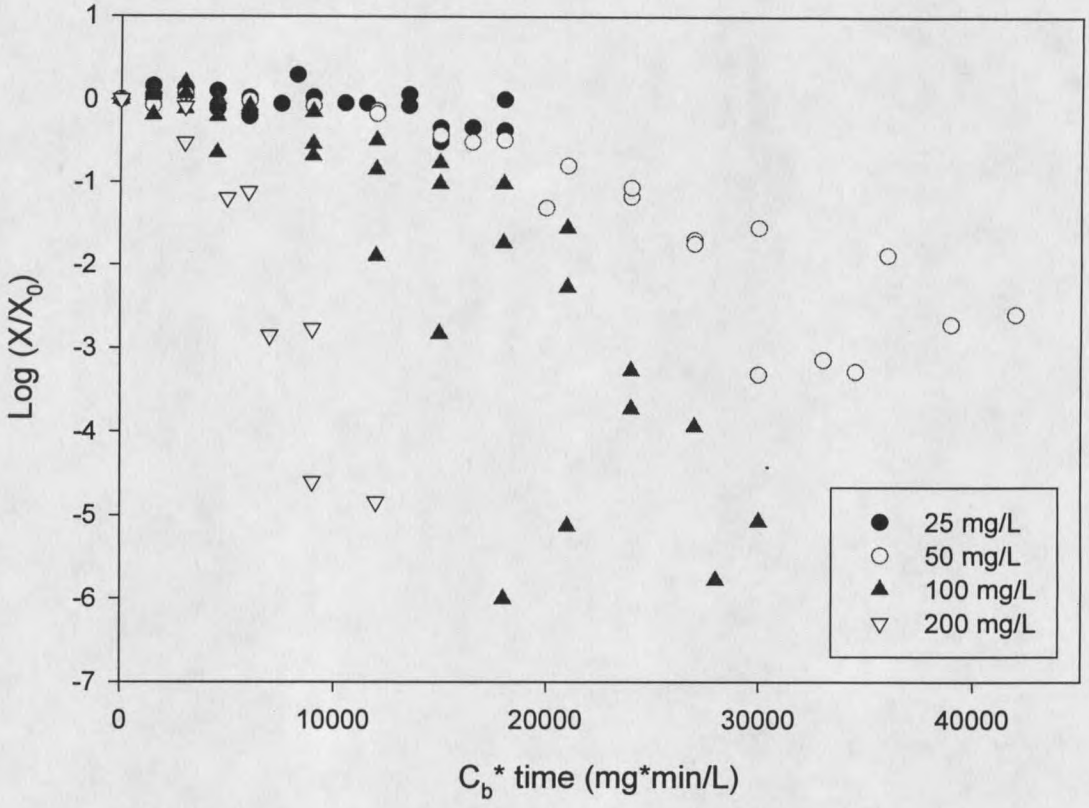

Figure 19. C*t analysis of *P. aeruginosa* artificial biofilm cells exposed to glutaraldehyde. $R^2 = 0.40$.

Ciprofloxacin

Planktonic *P. aeruginosa* cells were killed by ciprofloxacin (Figure 20). A 2 log reduction was achieved within 150 minutes for all ciprofloxacin concentrations tested. On a plot of $\log_{10}$ of the survival fraction versus time the planktonic killing profiles were non-linear.

Biofilm cells of *P. aeruginosa* entrapped in gel beads were killed by exposure to ciprofloxacin at concentrations of 1 μg/mL-25 μg/mL (Figure 21). Biofilm killing profiles were non-linear with a characteristic concave up shape on a plot of $\log_{10}$ survival versus time (Figure 21).

Biofilm cells were clearly less susceptible than planktonic cells to the same ciprofloxacin treatment. Killing of planktonic and biofilm bacteria by 1 μg/mL ciprofloxacin is compared graphically in Figure 22. It took approximately 150 minutes to achieve a two log reduction in the planktonic system, while only one of the three experiments done at this concentration in the artificial biofilm system reached a 2 log reduction (in approximately 400 minutes). The times needed to achieve a 2 log reduction in viable cell counts for planktonic and biofilm cultures are summarized in Table 6 for various treatment concentrations. Increasing the concentration of ciprofloxacin decreases the time required for a two log reduction.

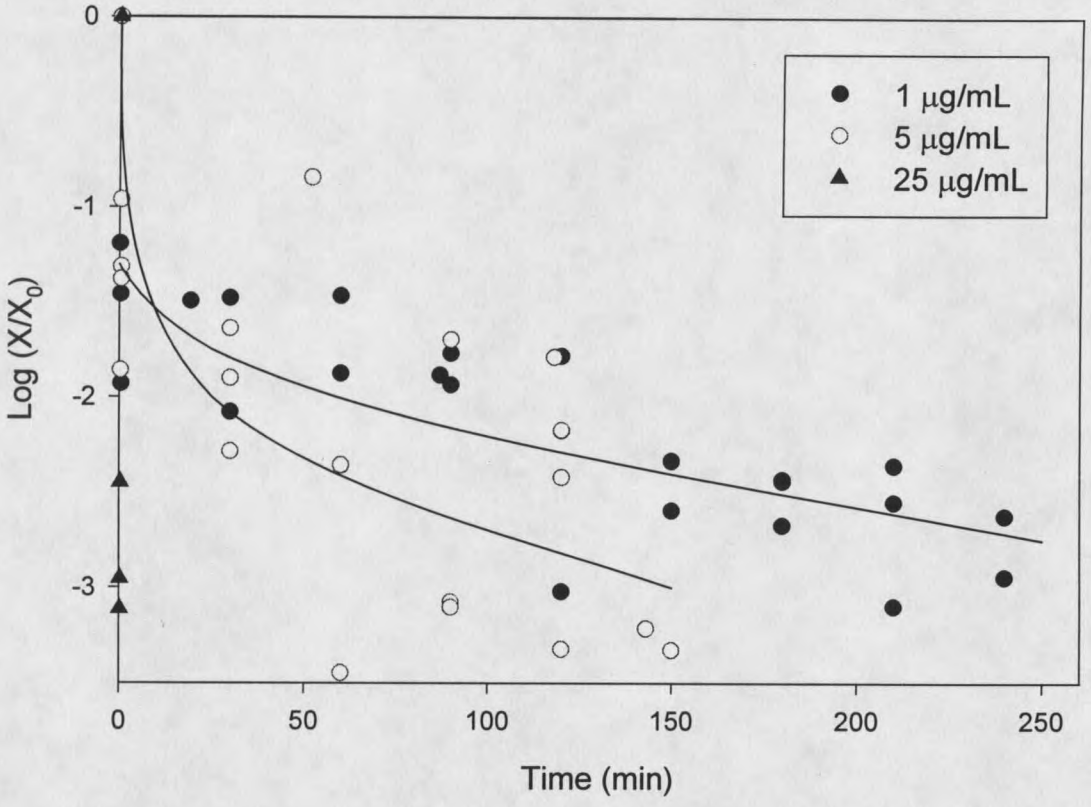

Figure 20. Killing of planktonic *P. aeruginosa* bacteria exposed to ciprofloxacin.

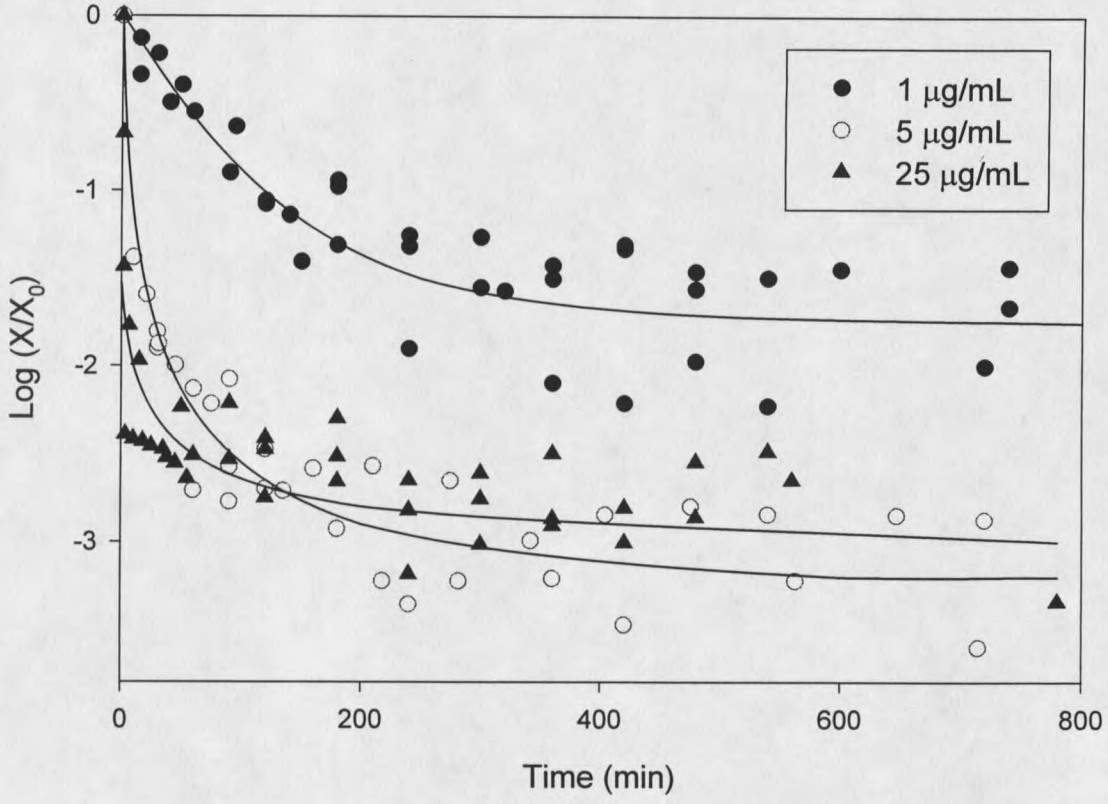

Figure 21. Killing of *P. aeruginosa* bacteria entrapped in artificial biofilms and exposed to ciprofloxacin.

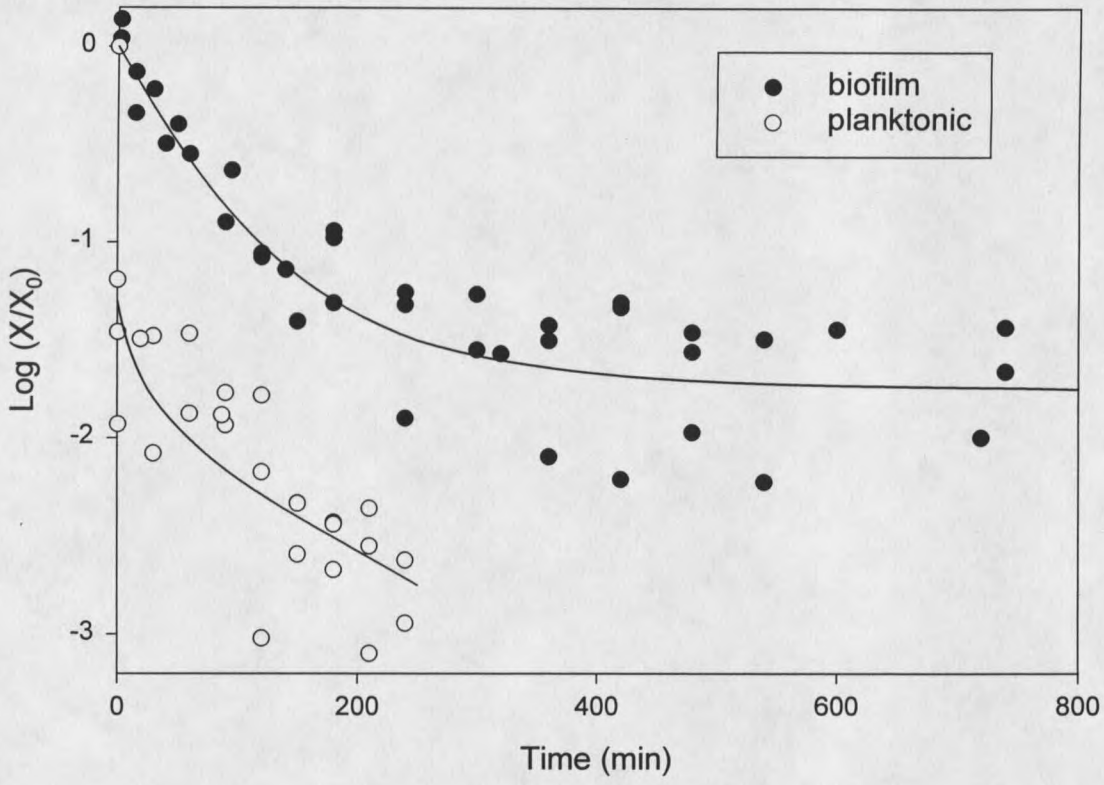

Figure 22. Comparison of planktonic and biofilm killing of *P. aeruginosa* bacteria exposed to 1 μg/mL ciprofloxacin.

Table 6. Ciprofloxacin treatment time to attain a 2 log reduction in viable cell numbers. (± values indicate standard deviation)

Concentration (μg/mL)	Planktonic kill time (min)	Biofilm kill time (min)	Resistance Factor
1	80.9 ± 30.2	1420*	17.5
5	30.1 ± 16.1	64.0 ± 18.8	1.9
25	0.18 ± 0.083	49.6 ± 30.5	271

* indicates an extrapolated value; two log reduction was not achieved in this case.

Planktonic and biofilm bacteria responded to increased ciprofloxacin concentrations differently (Figure 23). The apparent kinetic order, which is given by the slope of the regressed line in Figure 23, was 1.9 ± 0.28 for planktonic cells. Increasing concentration accelerated biofilm killing. The apparent kinetic order of ciprofloxacin concentration dependence was 0.95 ± 0.31 for biofilm cells (Figure 23). The planktonic curve only contains two data points because at the highest concentration tested, 25 mg/L, the detection limit for culturing was reached before the first sample was taken thereby skewing the data point.

All of the biofilm data obtained by disinfection by ciprofloxacin was analyzed using a model developed by Mike Dodds at the CBE (Dodds, et al. 1999). Parameters from a physiological model of biofilm resistance were obtained. A plot of the

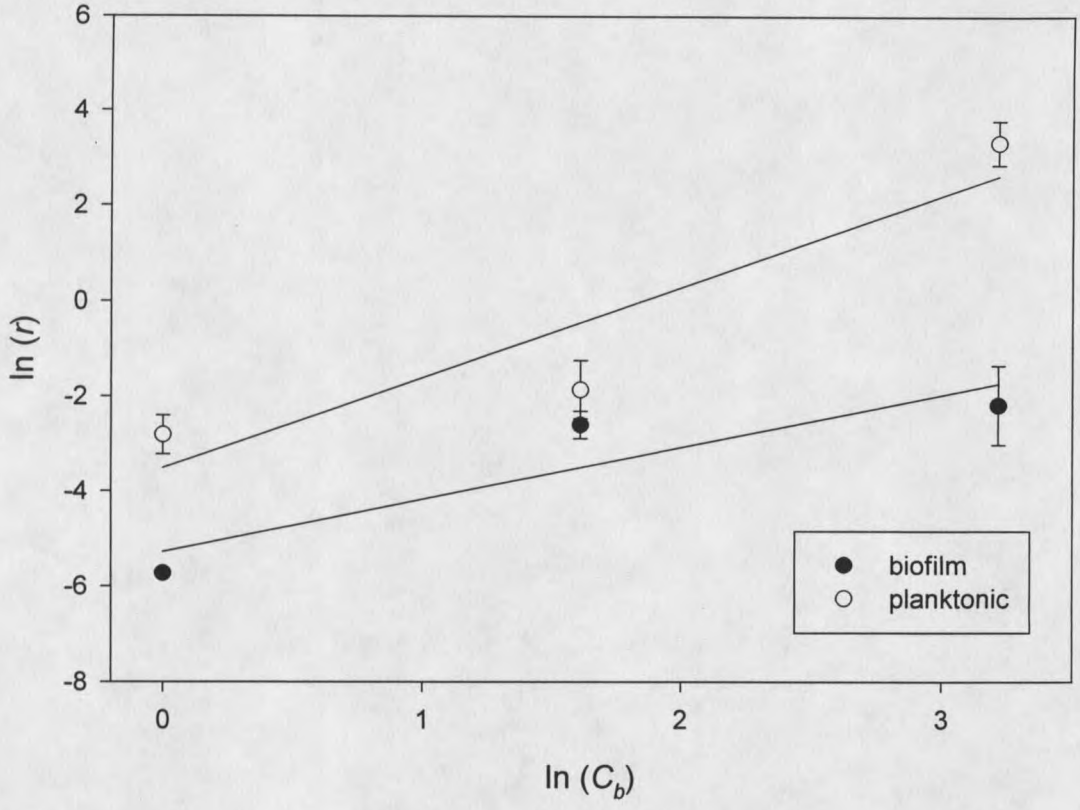

Figure 23. Analysis of the concentration dependence of the apparent rates of killing of planktonic and biofilm *P. aeruginosa* exposed to ciprofloxacin. The lines are least squares regressions with 1) planktonic $y = 1.898 \pm 0.28\ x - 3.303$, $R^2 = 0.8869$ and 2) biofilm, $y = 0.9490 \pm 0.31\ x - 4.997$, $R^2 = 0.7064$. The units of C_b are mg/L and r has units of min^{-1}.

resistant fraction of cells versus time (Figure 24) showed no statistically significant effect of the concentration of ciprofloxacin on resistant fraction remaining (P-value = 0.096; Figure 24). A plot of p-value versus time shows that while there is a decrease in the resistant fraction with concentration, the resistant fraction of cells become more difficult to kill (P-value = 0.0069; Figure 25). There is also an increase in the rate of disinfection with increasing concentration (P-value = 0.29; Figure 26).

Plots of log (X/X_0) versus C^*t for both planktonic and biofilm cells show a non-linear and noisy relationship between the product of concentration and time and microbial survival (Figues 27 and 28). R^2 values for the linear regression of log (X/X_0) versus C^*t were 0.24 and 0.32 for biofilm and planktonic data, respectively.

Quaternary Ammonium Compound

Planktonic *P. aeruginosa* cells were readily killed by QAC (Figure 29). A two log reduction was achieved within 2 minutes for all QAC concentrations tested. On a plot of $\log_{10}$ of the survival fraction versus time planktonic killing profiles were non-linear, concave up.

Biofilm cells of *P. aeruginosa* entrapped in gel beads were killed by exposure to QAC at concentrations ranging from 50 mg/L-1000 mg/L (Figure 30). At all concentrations, a plot of $\log_{10}$ survival versus time (Figure 30), biofilm killing profiles were non-linear with a characteristic concave up shape.

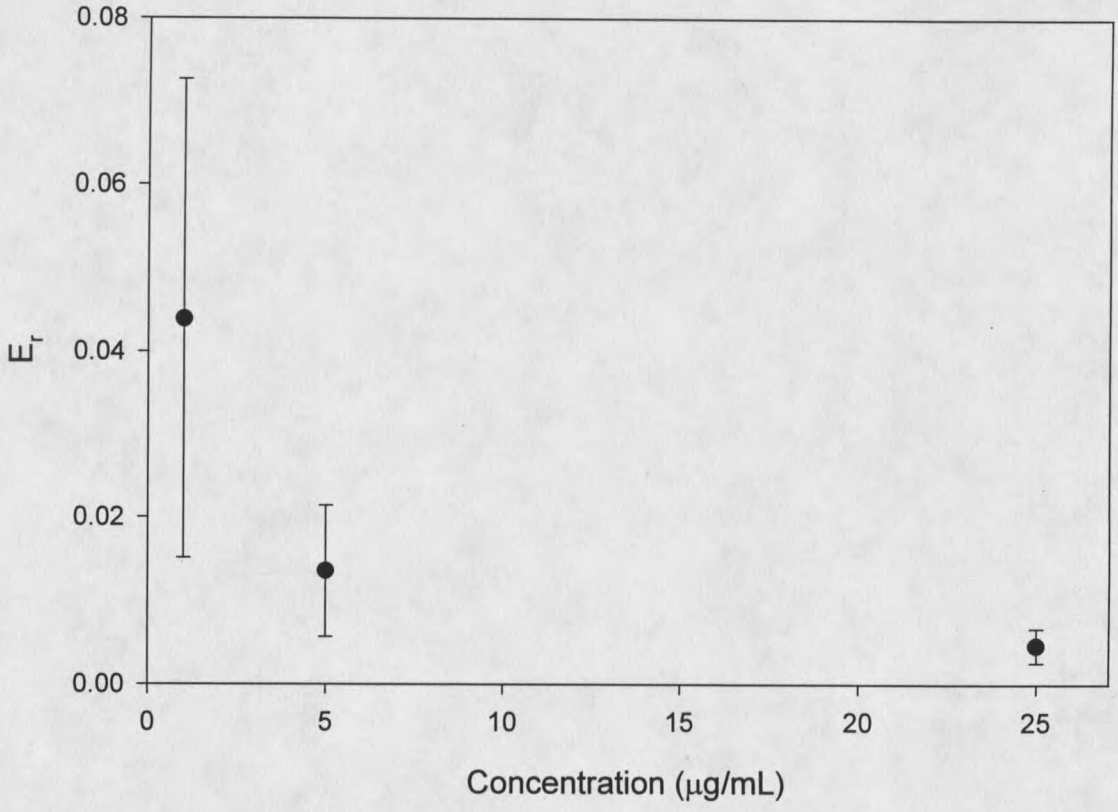

Figure 24. Analysis of the resistant fraction of viable cells in *P. aeruginosa* biofilm disinfection experiments with varying concentrations of ciprofloxacin (P-value = 0.096).

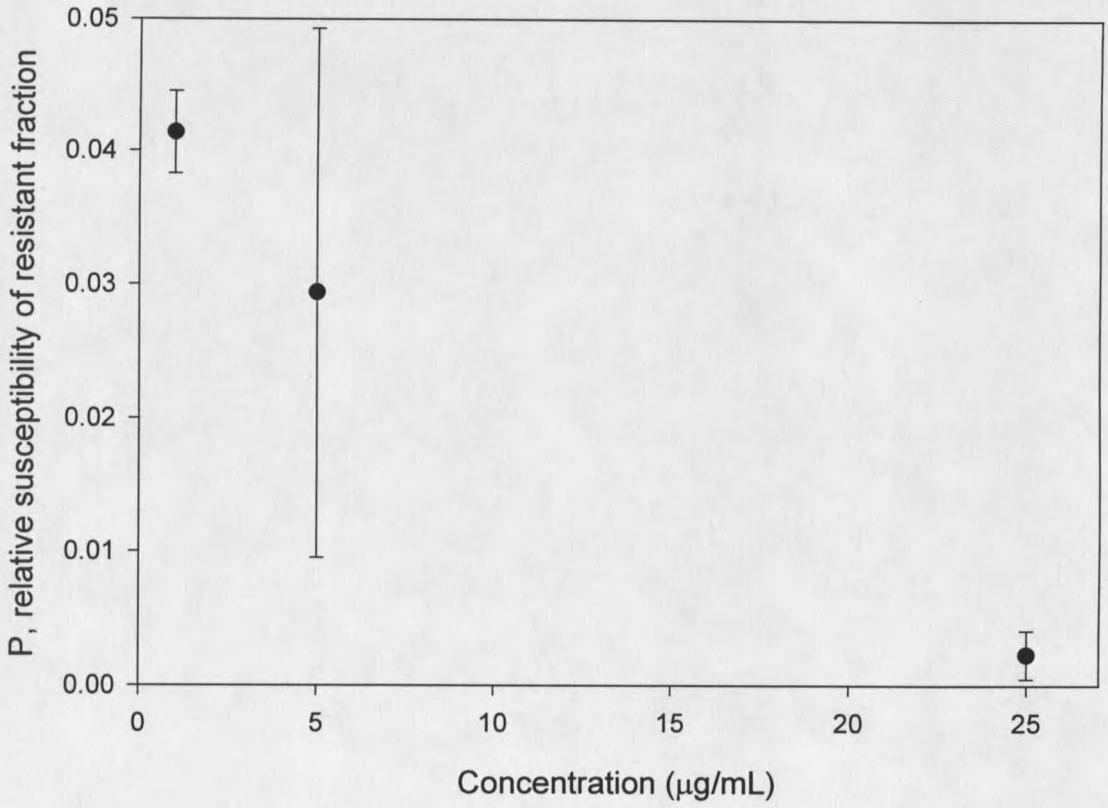

Figure 25. Analysis of P, the relative susceptibility of the resistant fraction dependence on concentration in *P. aeruginosa* biofilm disinfection by ciprofloxacin (P-value = 0.007).

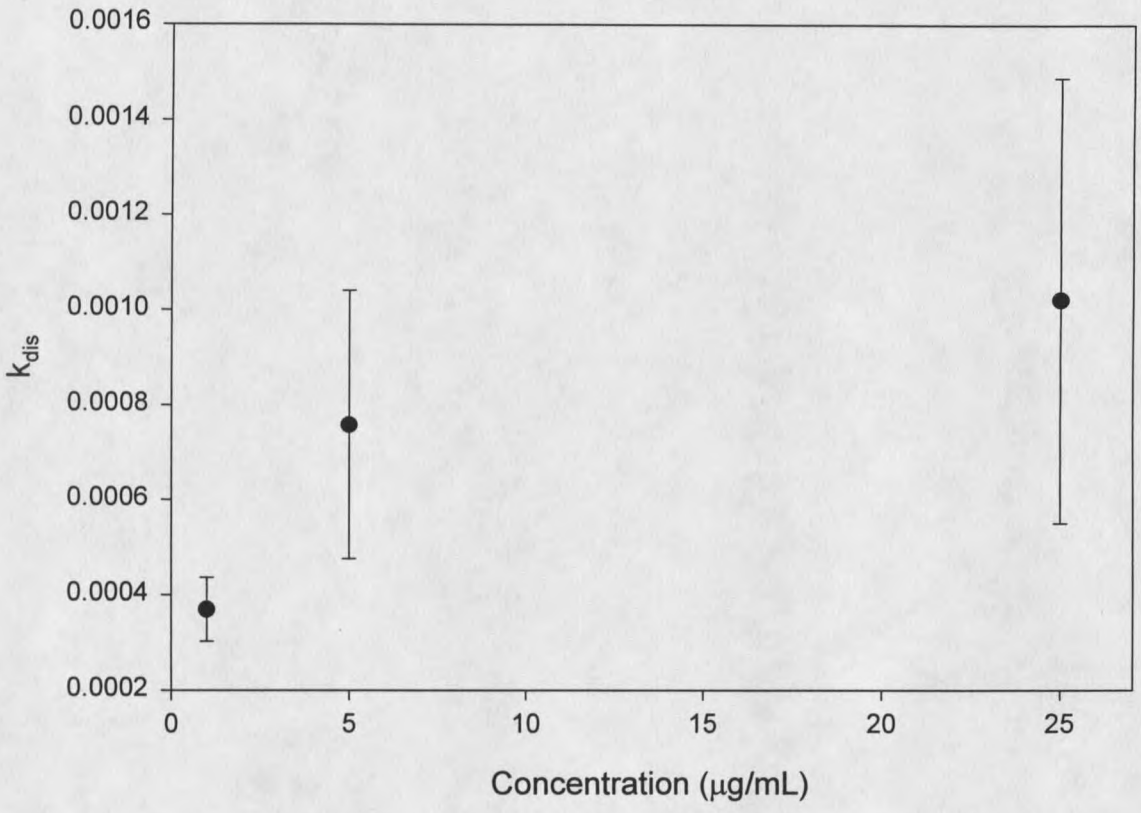

Figure 26. Analysis of the disinfection rate dependence on concentration in *P. aeruginosa* biofilm disinfection by ciprofloxacin (P value = 0.11).

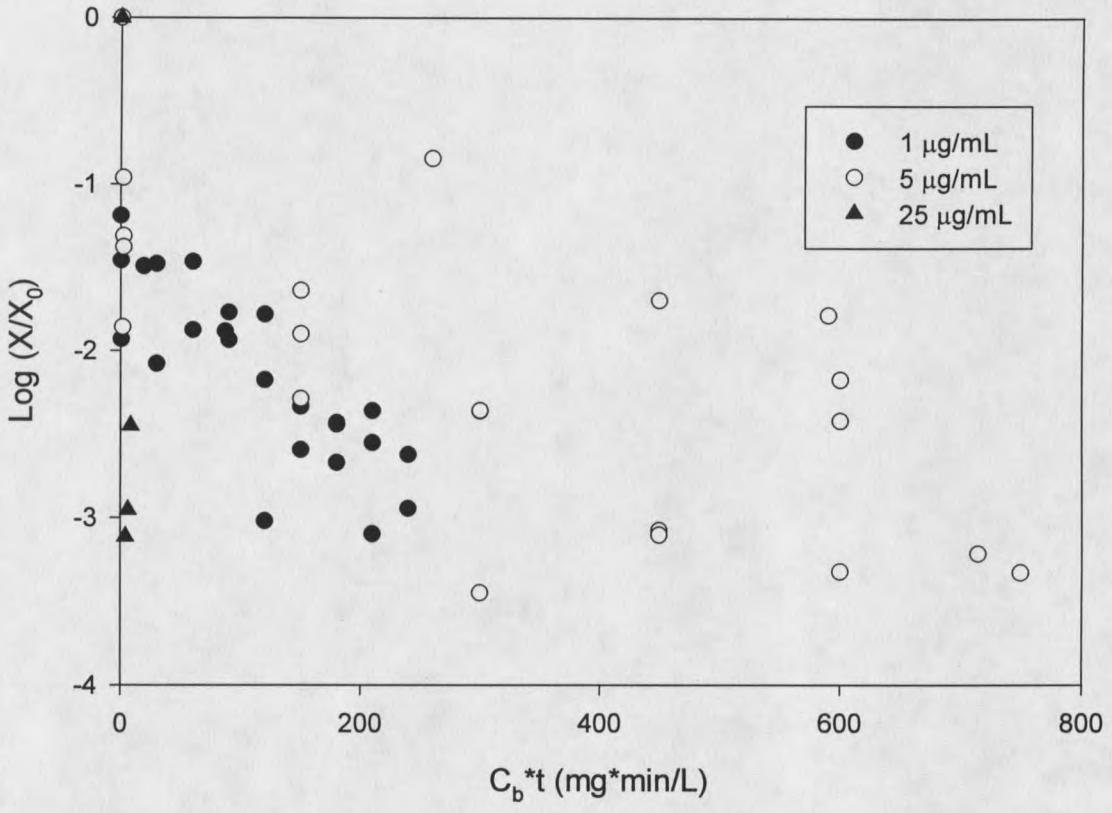

Figure 27. C*t analysis of planktonic *P. aeruginosa* exposed to ciprofloxacin. R^2 = 0.32.

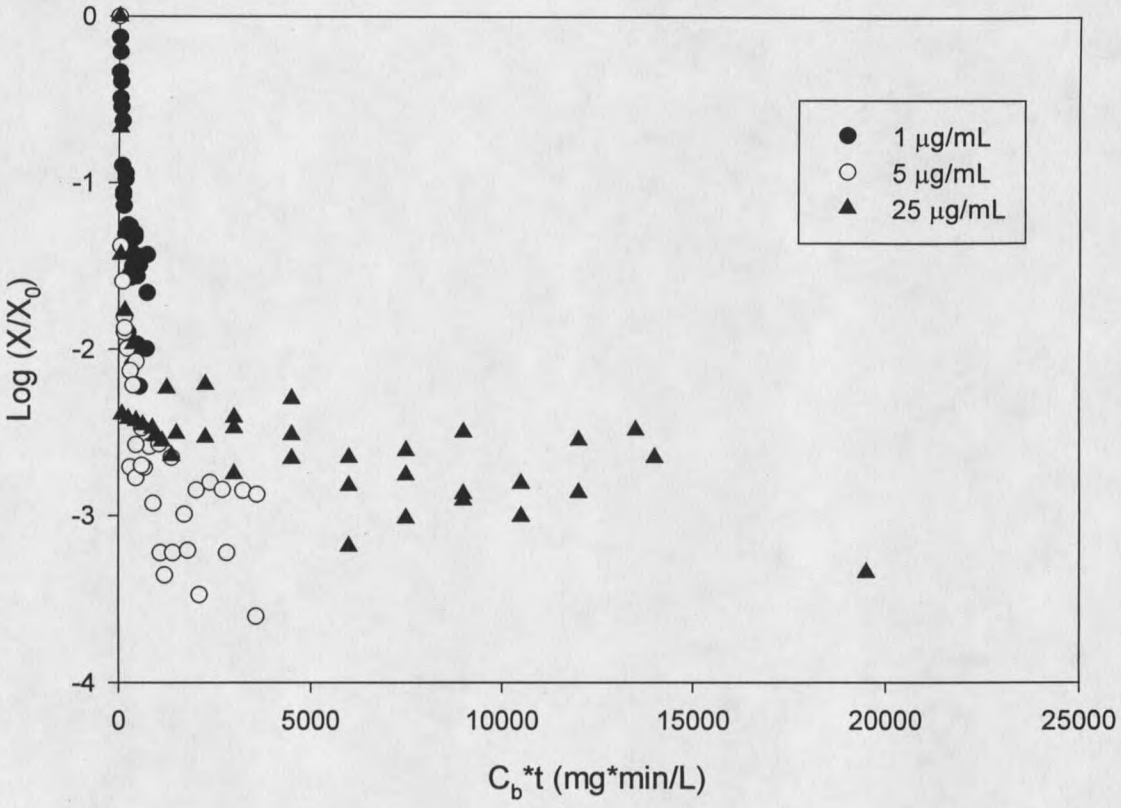

Figure 28. C*t analysis of *P. aeruginosa* artificial biofilm cells exposed to ciprofloxacin.

$R^2 = 0.24$.

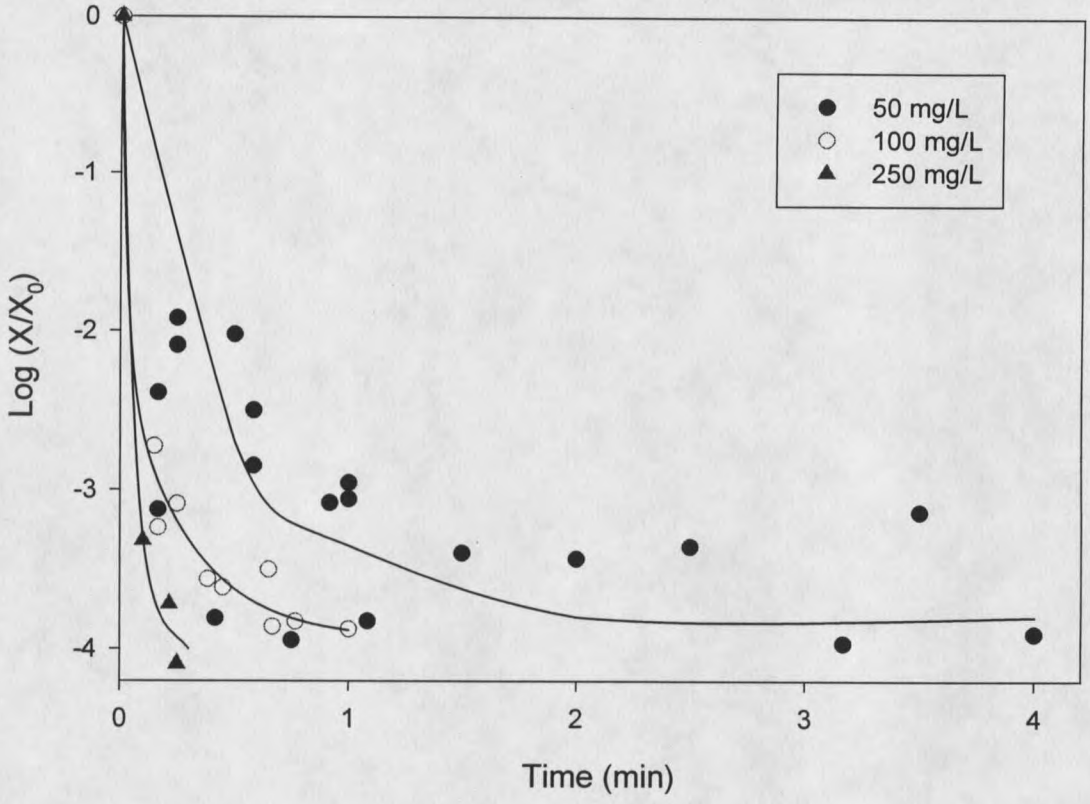

Figure 29. Killing of planktonic *P. aeruginosa* bacteria exposed to QAC.

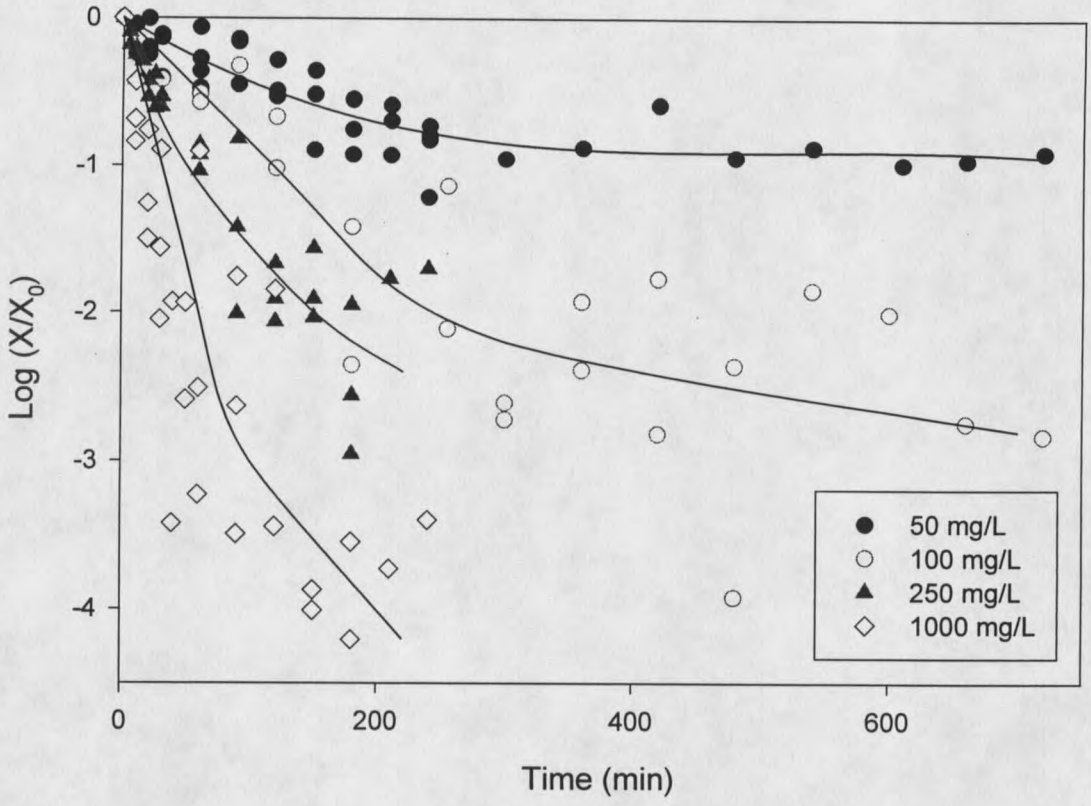

Figure 30. Killing of *P. aeruginosa* bacteria entrapped in artificial biofilms and exposed to QAC.

Biofilm cells were clearly less susceptible than planktonic cells to the same QAC treatment. Killing of planktonic and biofilm bacteria by 50 mg/L QAC is compared graphically in Figure 31. After a period of 12 hours, only a one log reduction in viable cell density was achieved in the artificial biofilm system, planktonically a 4 log reduction was achieved in less than a half hour at the same concentration. The times needed to achieve a 2 log reduction in the viable cell numbers for planktonic and biofilm cultures are summarized in Table 7 for various treatment concentrations.

Table 7. QAC treatment time to attain a 2 log reduction in culturable cell numbers (± values indicate standard deviation). ND denotes not determined.

Concentration (mg/L)	Planktonic kill time (min)	Biofilm kill time (min)	Resistance Factor
50	0.30 ± 0.16	636 ± 371	2160
100	0.11 ± 0.031	225 ± 7	2000
250	0.10 ± 030	157 ± 47	1500
500	ND	71 ± 19	ND
1000	ND	74± 28	ND

Planktonic and biofilm bacteria responded to increased QAC concentrations in approximately the same manner (Figure 32). The apparent kinetic order, which is given

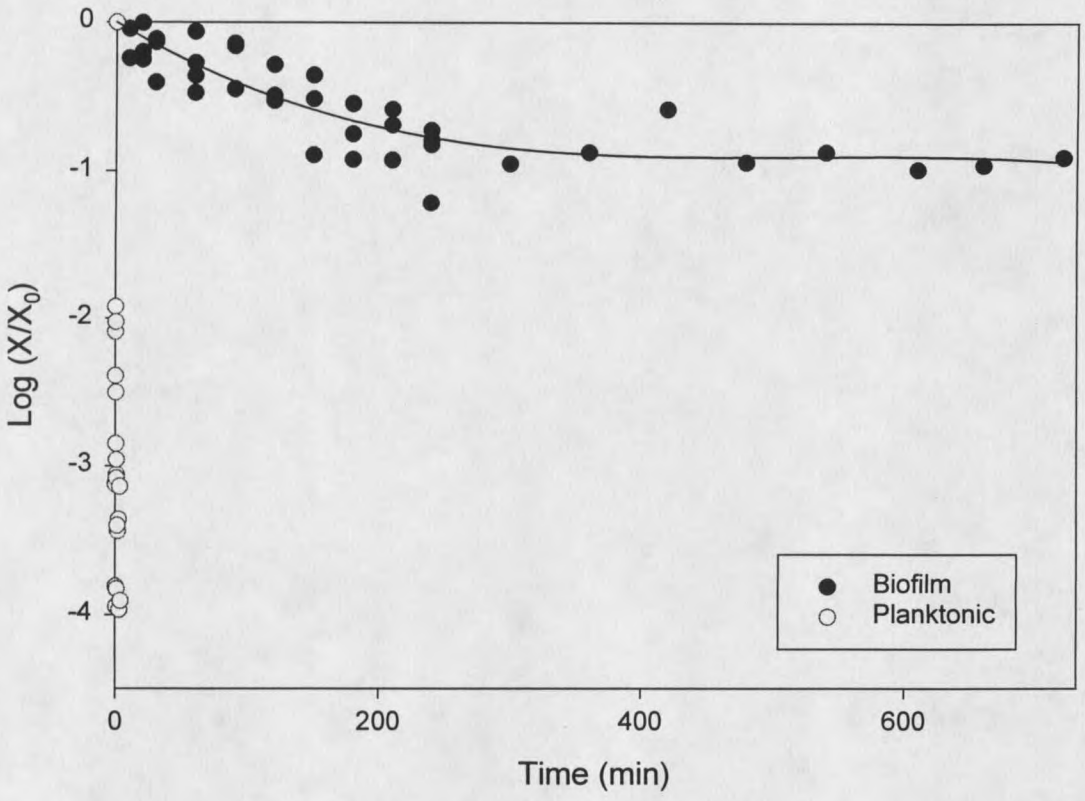

Figure 31. Comparison of planktonic and biofilm killing of *P. aeruginosa* bacteria exposed to 50 mg/L QAC.

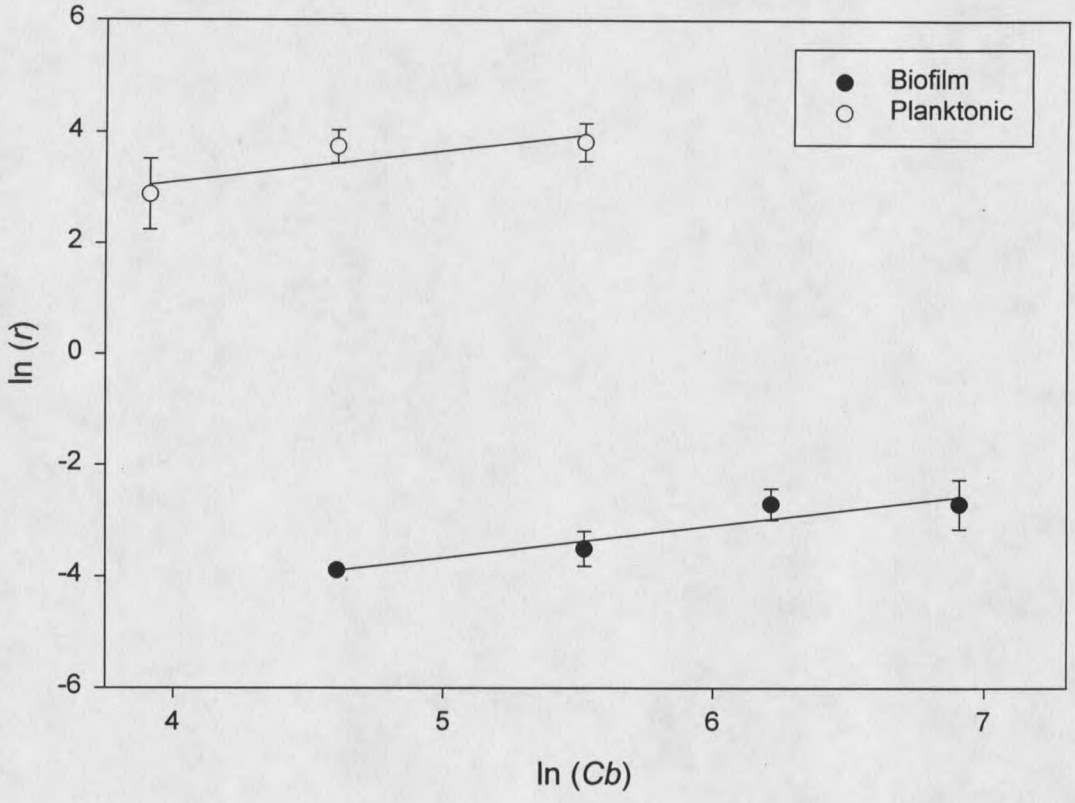

Figure 32. Analysis of the concentration dependence of the apparent rate of killing of planktonic and biofilm *P. aeruginosa* exposed to QAC. The lines are least squares regressions with 1) planktonic, $y = 0.4165 \pm 0.20\,x + 1.597$, and $R^2 = 0.350$ and 2) biofilm, $y = 0.720 \pm 0.14\,x - 7.465$, and $R^2 = 0.8018$. C_b has units of mg/L and r has unit of min^{-1}.

by the slope of the regressed line in Figure 32, was 0.42 ± 0.20 for planktonic cells. Increasing the QAC concentration does not proportionally increase the rate of planktonic killing. The apparent kinetic order of QAC concentration dependence was 0.72 ± 0.14 for biofilm cells (Figure 32).

Disinfection data from the biofilm QAC experiments were analyzed using a model of physiological resistance developed by Mike Dodds at the CBE. Resistant fraction, disinfection rate and p-value parameters were all obtained. Plotting the resistant fraction versus concentration shows that with increasing concentration, the resistant fraction remaining decreases (P-value = 0.00035; Figure 33). The QAC disinfection rate and p-value do not change with increasing concentration (P-values are 0.29 and 0.014 respectively; Figures 34 and 35).

Plots of log (X/X_0) versus C^*t for both planktonic and biofilm cells show a non-linear and noisy relationship between the product of concentration and time and microbial survival (Figures 36 and 37). R^2 values for the linear regression of log (X/X_0) versus C^*t were 0.66 and 0.46 for biofilm and planktonic data, respectively.

Resistance Factor Analysis

Calculated resistance factors of chlorine, glutaraldehyde and QAC decreased with increasing antimicrobial agent concentration (Figure 38 and 39). Ciprofloxacin resistant fraction analysis resulted in a non-linear scatter plot effectively increasing with concentration (Figure 38 and 39).

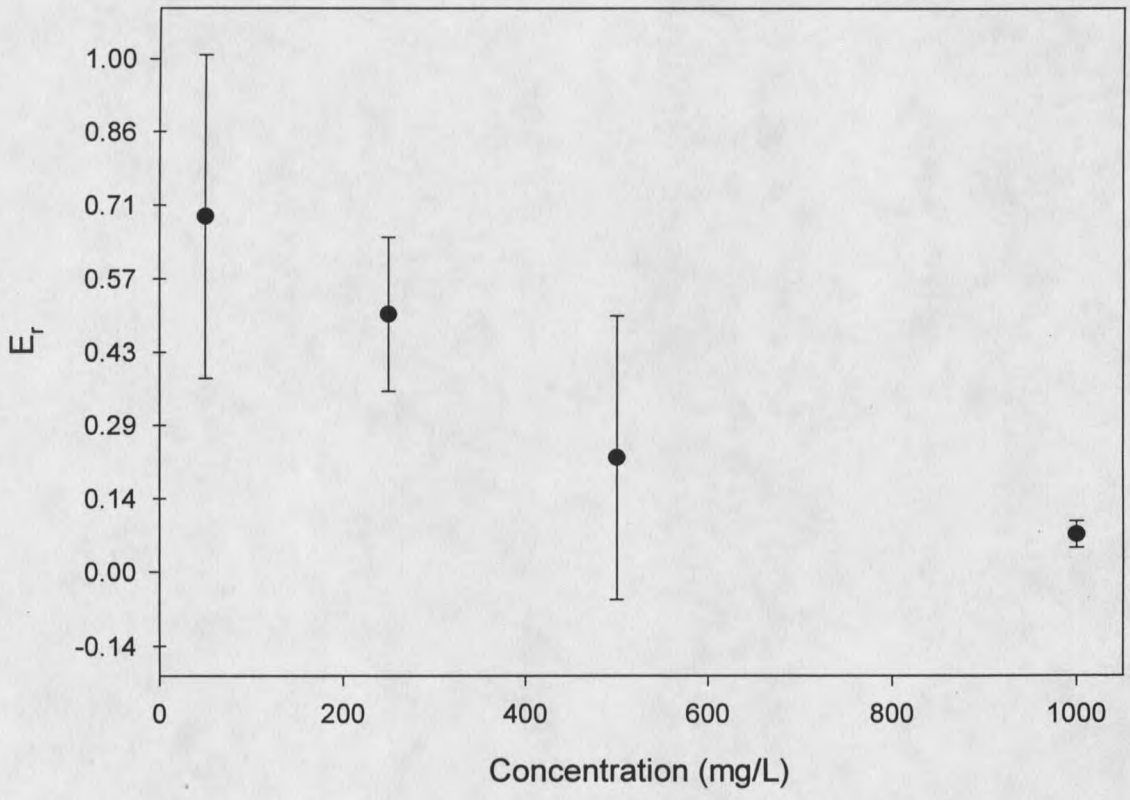

Figure 33. Analysis of the resistant fraction of *P. aeruginosa* cells in biofilm disinfection by QAC (P-value = 0.00035).

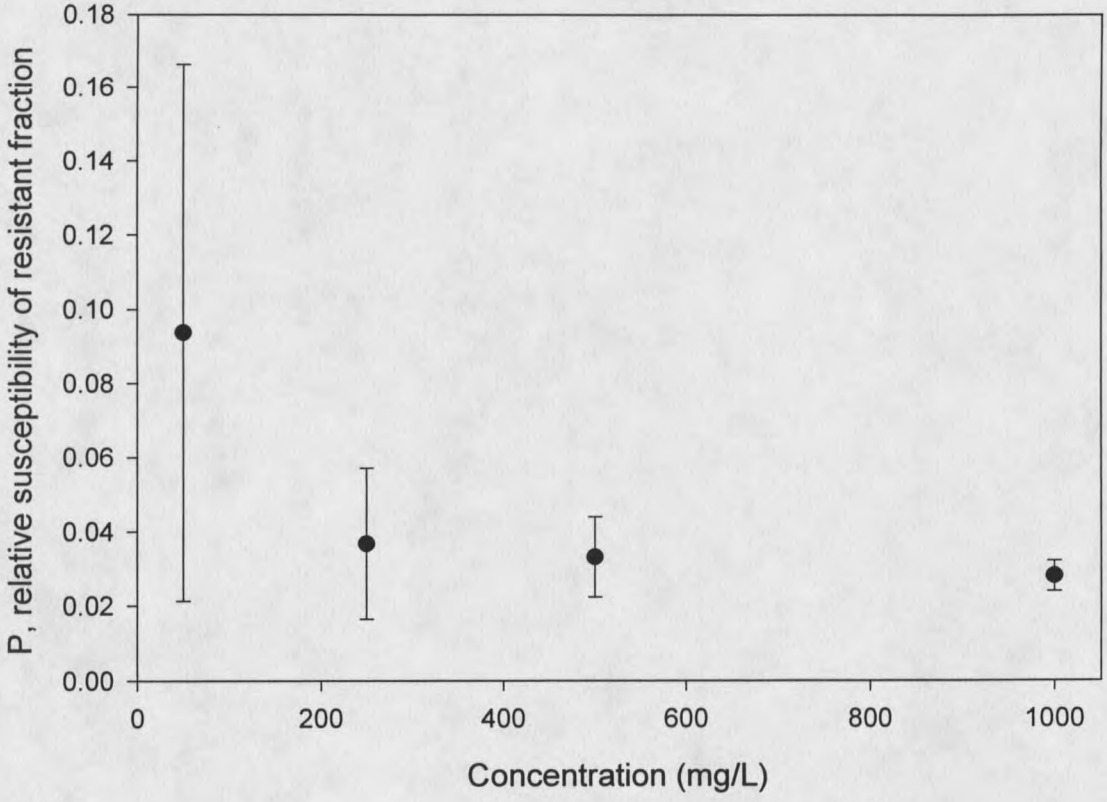

Figure 34. Analysis of P, the relative susceptibility of the resistant fraction, dependence on concentration of *P. aeruginosa* biofilm by disinfection by QAC (P-value = 0.014).

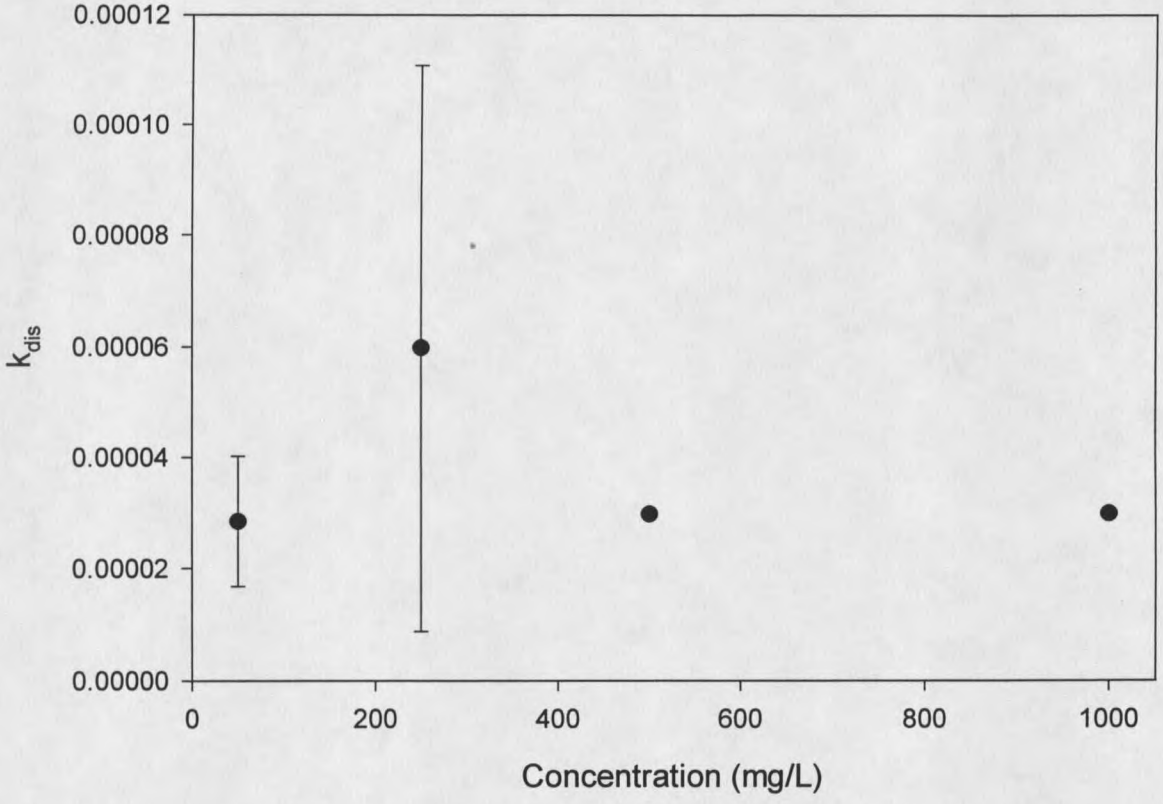

Figure 35. Analysis of the disinfection rate dependence concentration in *P. aeruginosa* biofilm disinfection by QAC (P-value = 0.29).

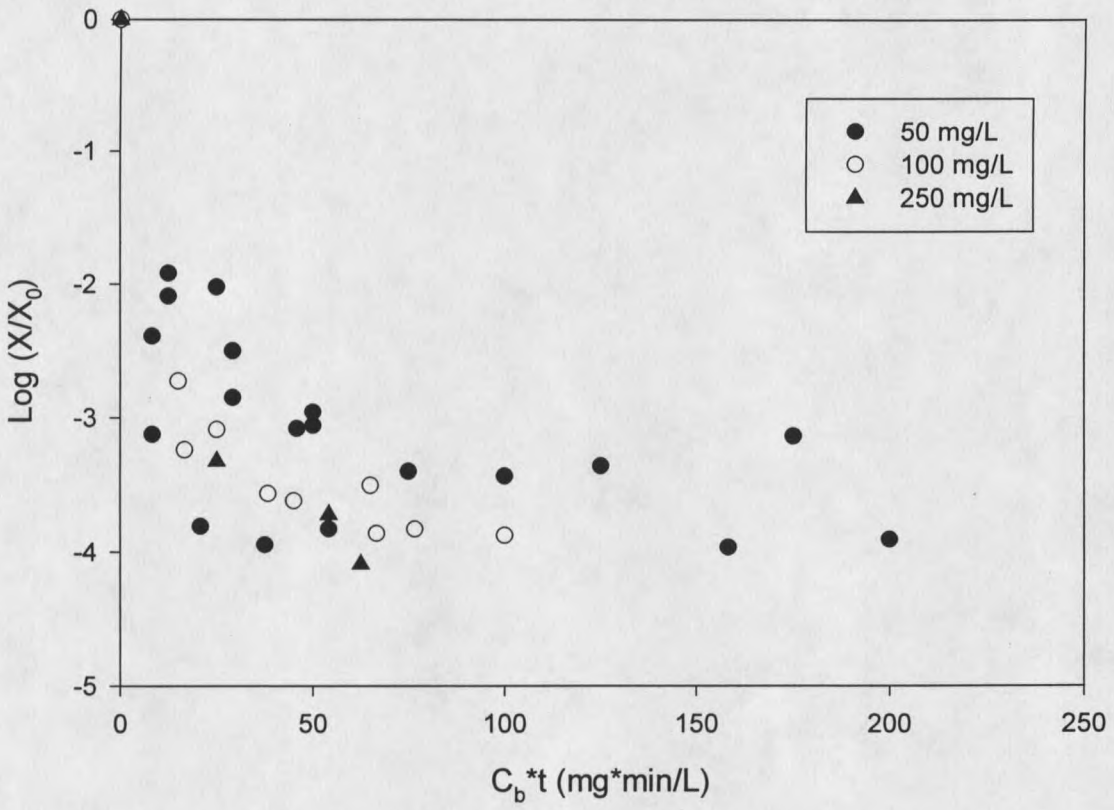

Figure 36. C*t analysis of planktonic *P. aeruginosa* exposed to QAC. $R^2 = 0.46$.

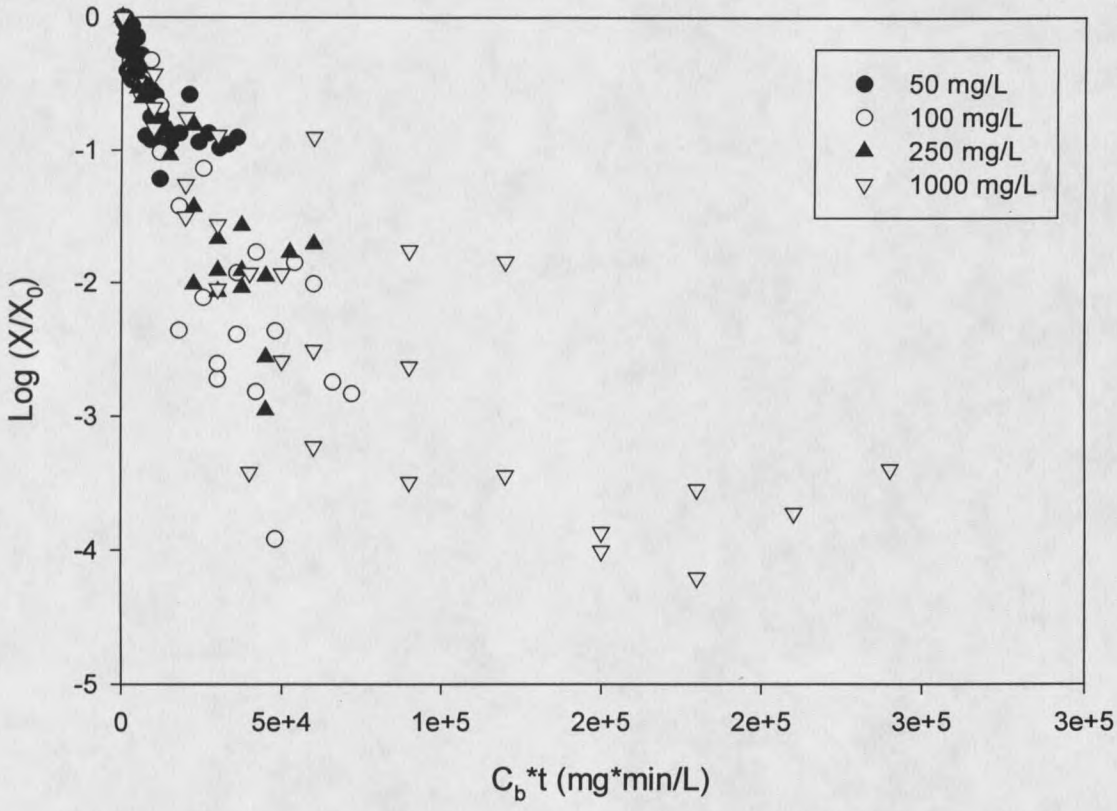

Figure 37. C*t analysis of *P. aeruginosa* artificial biofilm cells exposed to QAC. R^2 = 0.66.

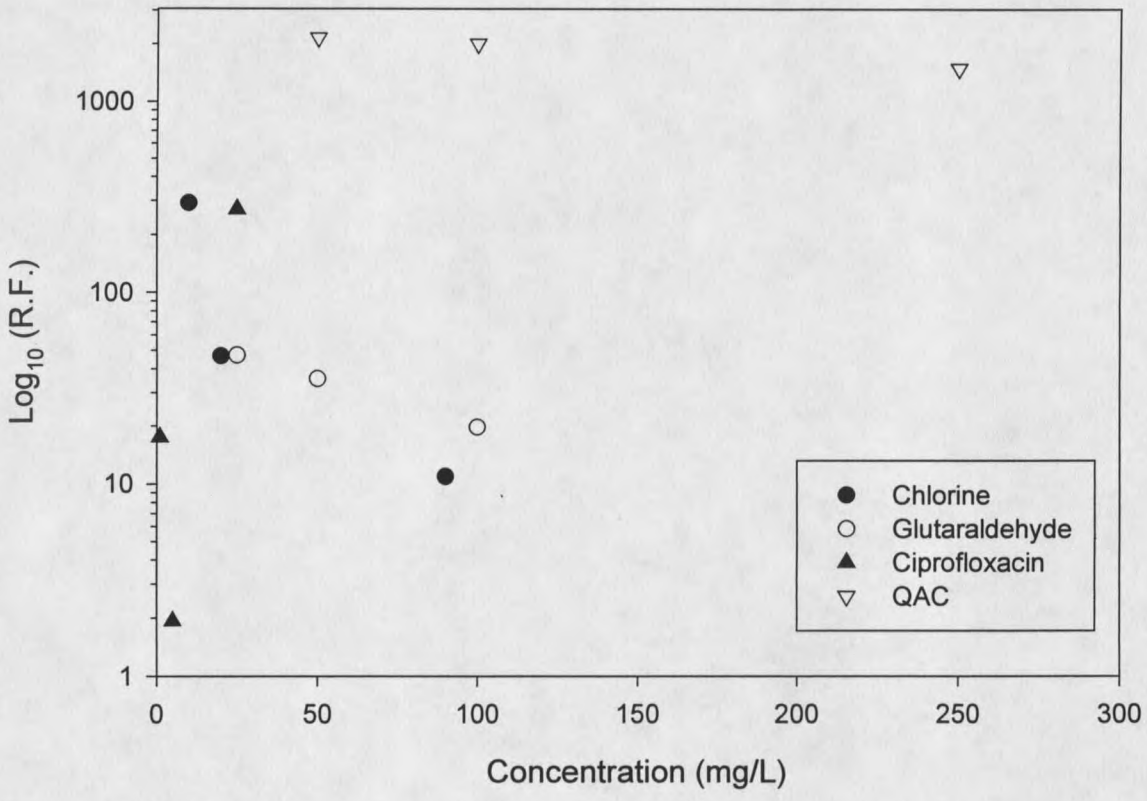

Figure 38. Analysis of a resistance factors as a function of agent concentration (mg/L).

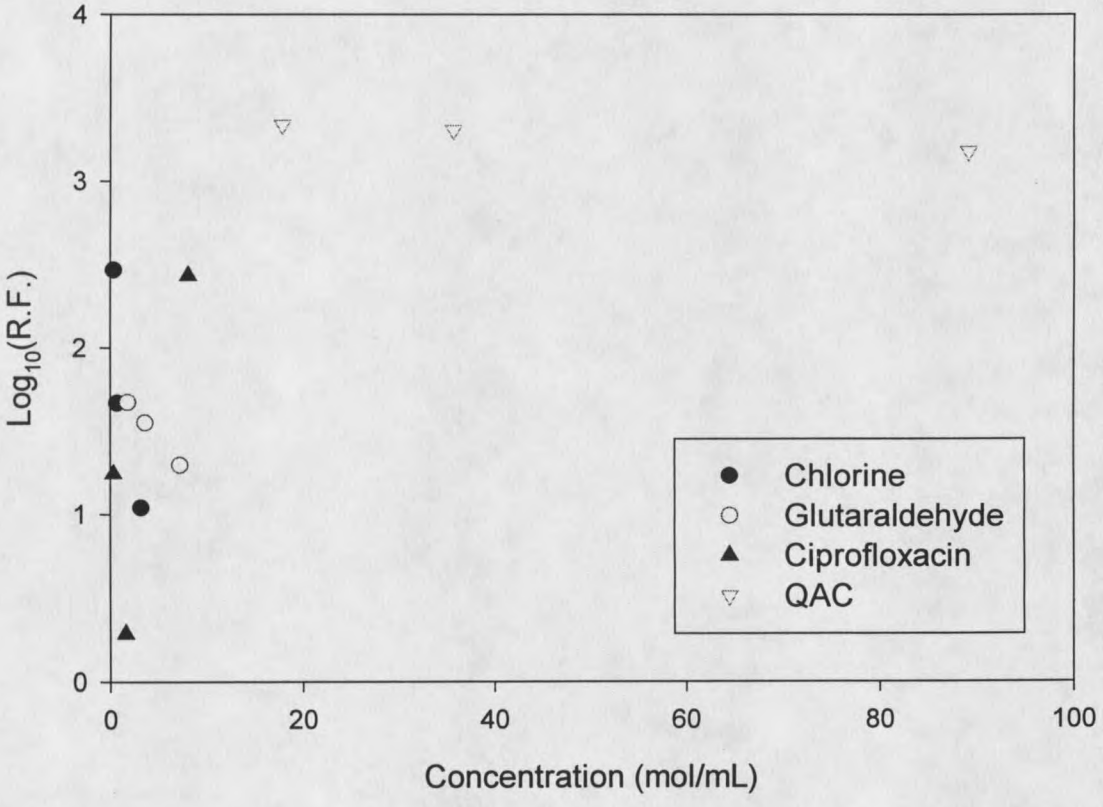

Figure 39. Analysis of a resistance factors as a function of agent concentration (mol/mL).

DISCUSSION

Artificial Biofilm System

Artificial biofilms were developed by entrapping a common environmental bacterium, *P. aeruginosa*, in hydrated alginate gel beads. The artificial biofilms mimic real biofilms in many aspects. Dense microcolonies of bacteria are interspersed randomly within a highly hydrated polymer matrix. The cell density in these artificial biofilms is similar to the densities observed in real biofilms (Sanderson 1997; Suci 1994; Vrany 1997). The equivalent slab thickness of the artificial biofilms, which is estimated as R/3, is approximately 400 μm, which is within the range of natural films (Characklis 1990). Diffusion is the predominant transport process in gel bead artificial biofilms, as it is with in the cell clusters of a real biofilm (Chen 1996; Stewart 1996; Stewart 1998;Westrin 1991; Xu 1996).

Artificial biofilm cells exhibited striking resistance to disinfection by all antimicrobial agents tested in comparison to freely suspended cultures of the same microorganism. For example, the susceptibility of bacteria in biofilms to glutaraldehyde was reduced by approximately an order of magnitude compared to planktonic bacteria (Figure 15, Table 5). Relative resistance factors ranged from 11 for chlorine to 2160 for QAC. This result shows that the artificial biofilm test system used in this investigation is

capable of capturing, at least in part, the phenomenon of biofilm resistance to antimicrobial agents. These experiments further support the use of alginate gel beads as an appropriate substitute for biofilm testing.

Resistance Mechanisms

The shape of the chlorine biofilm killing curve at 10 mg/L implies that chlorine penetration into the biofilm is retarded. The model of biofilm penetration predicts precisely this shape of killing curve (Dodds, et al. 1999). In particular, slow penetration in this case appears to be due to a stoichiometric reaction between the chlorine and constituents of the biofilm. According to this mechanism, chlorine is consumed in the surface layers of the biofilm. It does eventually penetrate the biofilm, but only once the neutralizing capacity of the biomass has been depleted. Once chlorine penetrates fully, the rate of killing accelerates markedly.

Glutaraldehyde transport into the biofilms was also retarded. While planktonic kill curves were approximately linear (Figure 13), biofilm kill curves suggest a delayed response (Figure 14). Stoichiometric transport is likely the mechanism for glutaraldehyde penetration (Dodds, et al. 1999). Again, it is the reaction between the glutaraldehyde and constituents of the biofilm that retards penetration. No evidence for a recalcitrant fraction was seen in treating with glutaraldehyde or chlorine.

The demonstration of poor penetration in the artificial biofilm system can not necessarily be extrapolated to field applications. The stoichiometric reaction mechanism

of retarded biofilm penetration, whether for glutaraldehyde or chlorine, is a strong function of the biofilm thickness. The thicker the biofilm, the more severe the penetration limitation will be. The artificial biofilms used in this investigation mimic a biofilm with a thickness of approximately a few hundred microns. In systems where the biofilms are this thick or thicker, we can anticipate that retarded penetration could contribute to biofilm resistance to glutaraldehyde or chlorine. On the other hand, in systems where the biofilm is significantly thinner, these agents could penetrate the biofilm readily. For example in unpublished research at the CBE on sulfate-reducing bacterial biofilms, glutaraldehyde was found to experience no barrier to biofilm penetration. These sulfate-reducing biofilms, however, were only about 5 microns thick. Antimicrobial penetration is also predicted to be improved at higher dose concentrations. In summary, incomplete penetration of glutaraldehyde or chlorine into microbial biofilms is likely to contribute to biofilm reduced susceptibility in thicker biofilms being treated with relatively low concentrations of antimicrobial agent. It is less likely to be important when dealing with biofilms being treated with high concentrations of these agents.

Calculation of an observable modulus comparing the relative rates of reaction and diffusion confirmed the expectation of incomplete penetration. With both glutaraldehyde and chlorine, the observable modulus decreased during treatment indicating progressively increasing extent of penetration. The point that the observable modulus decreased below one (indicating penetration) roughly corresponded to the inflection point of the

disinfection curve (Tables 4 and 5; Figures 7 and 14). No evidence of transport limitation, as would be indicated by delayed killing, was seen with either QAC or ciprofloxacin.

Sometimes the shape of biofilm killing curves indicates the presence of a subpopulation of biofilm bacteria that are resistant to the antimicrobial agent. The concave-up shape of the QAC biofilm killing curves indicates such a phenomenon. The QAC rapidly penetrates the artificial biofilm, killing off a sensitive fraction while a resistant fraction remained more difficult to kill over time. Increasing the concentration of QAC decreased the resistant fraction within the biofilm, making it more advantageous to dose a biofilm with higher concentrations.

The shape of the biofilm killing by ciprofloxacin also indicates a physiological heterogeneity within the biofilm. A sensitive fraction is killed off rapidly, while a resistant fraction remains more difficult to kill.

Concentration Dependence

Treating biofilm bacteria with high concentrations of chlorine and glutaraldehyde was shown to be more effective than using prolonged doses at lower concentrations. For example, dosing a biofilm with 10 mg/L of chlorine for 150 minutes was as effective as dosing with 20 mg/L for 40 minutes. This was also the case for planktonic cells treated with chlorine.

With glutaraldehyde, a 200 mg/L dose for 34 minutes was as effective as a 50 mg/L dose delivered for 650 minutes. 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.

The resistant fraction parameter obtained from the computer model for ciprofloxacin and QAC also supports the conclusion that a higher treatment concentration is more effective. In each case, the resistant fraction decreased with increasing concentration. In other words, as the concentration increases the biofilm can be more completely killed. The recalcitrant fraction decreases with increasing concentration.

The resistance factor analysis of chlorine, glutaraldehyde and QAC also indicates that treating a biofilm with a concentrated dose of these agents is more effective than a lower dose for proportionally longer time period. Increasing the concentration decreases the resistance factor (Figures 24 and 33). A decrease in the resistance factor means that the biofilm kill time is approaching the planktonic kill time. Therefore, increasing the concentration allows the agent to be more effective against biofilms. Ciprofloxacin's resistance factor analysis was not conclusive. The non-uniformity of the ciprofloxacin, in particular the skewed point at 25 mg/L, is likely an artifact of the sampling and analysis method. At 25 μg/mL the planktonic cells were below the detection limit at the first sampling time point (after several minutes of centrifugation) and most likely do not accurately reflect the correct time for a two log reduction at this concentration.

The product of concentration and time is widely used as an approximate method of estimating doses with equivalent efficacy. The results reported here (Table 8) indicate that the disinfection efficacy, both in planktonic suspensions and in biofilms, correlates only weakly with C*t. The use of the C*t concept is therefore difficult to justify in all cases examined.

Table 8. Comparison of R^2 values for biofilm and planktonic disinfection C*t analysis.

Antimicrobial Agent	Planktonic R^2	Biofilm R^2
Chlorine	0.035	0.32
Glutaraldehyde	0.49	0.40
Ciprofloxacin	0.32	0.24
QAC	0.46	0.66

CONCLUSION

From this experimental investigation of the susceptibility of *Pseudomonas aeruginosa* entrapped in alginate gel bead artificial biofilms to chlorine, glutaraldehyde, alkyl dimethyl benzyl ammonium chloride (QAC), and ciprofloxacin I draw the following conclusions:

- The alginate gel bead artificial biofilm system mimics many of the physical features of natural biofilms.

- The artificial biofilm system captures, at least in part, the characteristic resistance of biofilm microorganisms to disinfection. Bacteria were less susceptible to all antimicrobial agents when incorporated into artificial biofilms than they were in free aqueous suspension.

- The shape of survival versus time curves for planktonic and biofilm cells treated with the same antimicrobial agent can be very different. Planktonic killing data can not be used to predict the efficacy of treatment of a biofilm. Biofilm killing was always non-linear on a semilog plot.

- The artificial biofilm system is a relatively rapid and repeatable method for characterizing antimicrobial efficacy against biofilm microorganisms.

- Increasing the antimicrobial agent concentration improved biofilm killing for all four antimicrobial agents. The relative resistance of biofilm cells, as compared to planktonic cells, decreased as the treatment concentration was increased. Concentrated brief doses of antimicrobial agents are more effective against biofilm than are diluted and proportionally longer treatments.

- The penetration of chlorine and glutaraldehyde into artificial biofilm is retarded and this slow penetration contributes to biofilm resistance to these agents. No evidence of incomplete biofilm penetration was found for either ciprofloxacin or QAC.

- Killing curves of biofilm bacteria treated with ciprofloxacin or QAC suggest that a subpopulation of cells in the biofilm is highly resistant.

The alginate gel bead disinfection experimental method developed in this project is very flexible allowing many different variations of the experiments performed in this project. Varying the size of the beads will allow further insight into transport limitations observed. Multi-species alginate gel beads would allow further practical extensions of real biofilm applications. Future recommendations include the development of traditional laboratory biofilm survival versus time experiments for a basis of comparison.

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APPENDICES

APPENDIX A: Untreated Control Data

Biofilm Untreated Control:

A1: Biofilm Control-3/11/99

Time (min)	Average log10 Cell Density (cfu/cm^3)	log(X/X0)
0	9.62	0.00
110	9.62	0.01
155	9.65	0.03
255	9.59	-0.03
370	9.58	-0.04
427	8.62	-1.00
503	9.66	0.04
604	9.73	0.11
736	9.57	-0.05

A2: Biofilm Control-3/15/99

Time (min)	Average log10 Cell Density (cfu/cm^3)	log(X/X0)
0	9.63	0.00
60	9.90	0.27
120	9.72	0.09
180	9.75	0.12
240	9.63	0.00
300	9.51	-0.12
360	9.73	0.10
420	9.69	0.06
480	9.71	0.08
540	9.66	0.03
600	9.73	0.10
735	9.71	0.08

Planktonic Untreated Control Data:

A3: Planktonic Control-3/9/99

Time (min)	Average log10 Cell Density (cfu/mL)	log(X/X0)
0	5.98	0
40	5.89	-0.09

A4: Planktonic Control-3/20/99

Time (min)	Average log10 Cell Density (cfu/mL)	log(X/X0)
0	6.89	0
30	6.81	-0.08

APPENDIX B: Chlorine Data

Chlorine Biofilm Data:

B1: 9.33 mg/L- 6/18/98

Time (min)	Average log10 Cell Density (cfu/cm^3)	log(X/X0)	Time (min)	Absorbance at 515 nm	Concentration of Chlorine (mg/L)
0	9.76	0.00	0	0.248	9.35
60	9.16	-0.60	10	0.17	6.41
120	8.29	-1.47	15	0.248	9.35
135	7.98	-1.78	35	0.201	7.58
150	7.36	-2.39	37	0.268	10.11
175	6.11	-3.65	70	0.202	7.62
200	5.07	-4.69	72	0.262	9.88
225	3.54	-6.22	97	0.227	8.56
			100	0.265	10.00
			112	0.237	8.94
			114	0.269	10.15
			137	0.246	9.28
			138	0.259	9.77
			166	0.246	9.28
			167	0.27	10.18
			210	0.24	9.05
			215	0.27	10.18
			257	0.246	9.28

B2: 9.36 mg/L- 6/24/98

Time (min)	Average log10 Cell Density (cfu/cm^3)	log(X/X0)	Time (min)	Absorbance at 515 nm	Concentration of Chlorine (mg/L)
0	9.68	0.00	0	0.246	9.28
60	9.08	-0.60	20	0.198	7.47
120	8.44	-1.24	25	0.227	8.56
135	7.89	-1.79	35	0.166	6.26
150	7.31	-2.36	37	0.221	8.34
200	3.74	-5.94	45	0.26	9.81
			65	0.205	7.73
			67	0.268	10.11
			87	0.234	8.83
			90	0.277	10.45
			132	0.205	7.73
			133	0.295	11.13
			165	0.245	9.24
			166	0.289	10.90
			195	0.263	9.92
			200	0.286	10.79
			230	0.258	9.73

B3: 9.65 mg/L- 6/26/98

Time (min)	Average log10 Cell Density (cfu/cm^3)	log(X/X0)	Time (min)	Absorbance at 515 nm	Concentration of Chlorine (mg/L)
0	10.08	0.00	0	0.257	9.69
10	9.29	-0.80	17	0.118	4.45
15	9.43	-0.66	20	0.258	9.73
25	9.11	-0.97	37	0.189	7.13
35	9.10	-0.98	40	0.301	11.35
60	8.90	-1.18	70	0.222	8.37
100	8.19	-1.89	73	0.31	11.69
115	7.61	-2.47	114	0.22	8.30
130	6.92	-3.16	116	0.291	10.98
145	6.03	-4.05	151	0.219	8.26
160	5.06	-5.02	155	0.301	11.35
180	2.84	-7.24	183	0.269	10.15
			205	0.253	9.54

B6: 21.47 mg/L -7/10/98

Time (min)	Average log10 Cell Density (cfu/cm^3)	log(X/X0)	Time (min)	Absorbance at 515 nm	Concentration of Chlorine (mg/L)
0	9.69	0.00	0	0.282	26.59
10	8.60	-1.09	15	0.123	11.60
20	7.76	-1.93	16	0.247	23.29
30	7.04	-2.65	33	0.195	18.39
40	5.97	-3.73	35	0.256	24.14
50	5.84	-3.85	60	0.203	19.14
60	5.01	-4.68	63	0.246	23.20
80	3.84	-5.85	95	0.197	18.58

B7: 21.36mg/L -7/16/98

Time (min)	Average log10 Cell Density (cfu/cm^3)	log(X/X0)	Time (min)	Absorbance at 515 nm	Concentration of Chlorine (mg/L)
0	9.48	0.00	0	0.247	23.29
10	7.17	-2.30	8	0.182	17.16
20	4.97	-4.51	10	0.244	23.01
30	3.84	-5.64	17	0.204	19.24
			22	0.251	23.67
			43	0.192	18.11

B8: 20.73 mg/L 7/16/98

Time (min)	Average log10 Cell Density (cfu/cm^3)	log(X/X0)	Time (min)	Absorbance at 515 nm	Concentration of Chlorine (mg/L)
0	9.86	0.00	0	0.242	22.82
10	8.06	-1.80	13	0.183	17.26
20	7.59	-2.27	15	0.238	22.44
30	6.35	-3.51	31	0.199	18.77
40	4.88	-4.98	33	0.244	23.01
50	3.84	-6.02	53	0.203	19.14
			55	0.224	21.12

B4: 97.37 mg/L -7/2/98

Time (min)	Average log10 Cell Density (cfu/cm^3)	log(X/X0)	Time (min)	Absorbance at 515 nm	Concentration of Chlorine (mg/L)
0	9.59	0.00	0	0.279	105.24
5	4.73	-4.86	15	0.236	89.02
10	3.84	-5.75	18	0.313	118.07
			40	0.244	92.04

B5: 82.47mg/L 7/9/98

Time (min)	Average log10 Cell Density (cfu/cm^3)	log(X/X0)	Time (min)	Absorbance at 515 nm	Concentration of Chlorine (mg/L)
0	9.72	0.00	0		93.90
2.3	7.40	-2.32	2	0.208	78.46
4	5.65	-4.08	10	0.199	75.07
5	4.48	-5.25			
7	2.94	-6.78			
19	2.84	-6.88			

Chlorine Planktonic Data:

B9: 10 mg/L-10/28/98

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	6.95	0.00
0.30	4.37	-2.58
0.75	3.89	-3.06
1.25	3.25	-3.70
1.75	2.99	-3.96

B10: 10 mg/L-10/28/98

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	6.95	0.00
0.30	4.30	-2.64
0.75	4.91	-2.04
1.25	4.76	-2.19
1.60	4.18	-2.76
2.25	3.83	-3.11
2.60	3.07	-3.88
3.00	3.35	-3.59
3.50	4.01	-2.94

B11: 10 mg/L-10/30/98

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	6.95	0.00
0.50	4.23	-2.71
1.00	3.21	-3.74
1.50	2.88	-4.07
1.83	3.04	-3.91
2.30	3.05	-3.90

B12: 10 mg/L-11/4/98

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	6.93	0.00
0.17	5.66	-1.27
0.75	5.62	-1.31
1.17	5.52	-1.41
1.67	5.30	-1.62
2.08	4.67	-2.26
2.50	4.17	-2.75
3.00	4.12	-2.81
3.50	3.91	-3.02
3.92	4.64	-2.28
5.00	4.13	-2.80

B13: 20 mg/L-11/5/98

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	6.93	0.00
0.33	4.90	-2.02
0.75	3.93	-2.99
1.22	3.57	-3.36

B14: 20 mg/L-11/8/98

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	6.93	0.00
0.50	2.84	-4.09
1.00	3.11	-3.82

B15: 20 mg/L-11/11/98

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	7.07	0.00
0.33	3.97	-3.10
0.83	2.94	-4.13

B16: 77 mg/L -11/13/98

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	6.87	0.00
0.33	4.15	-2.72
0.83	3.75	-3.13

B17: 82 mg/L -11/17/98

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	6.79	0.00
0.33	3.46	-3.32
0.75	2.94	-3.85

B18: 78 mg/L-11/18/98

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	6.84	0.00
0.17	4.87	-1.97
0.50	2.99	-3.86

B19: 78 mg/L -11/19/98

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	7.01	0.00
0.17	4.26	-2.75
0.50	3.00	-4.01

B20: Chlorine 2 log reduction rate analysis:

Time for 2 log kill	kdis*C^n	ln(kdis*C^n)	ln(Cb)	Average ln(kdis*C^n)	Standard Deviation
10 mg/L Biofilm					
136.17	0.03	-3.39	2.30	-3.32	0.06
124.52	0.04	-3.30	2.30		
121.12	0.04	-3.27	2.30		
20 mg/L Biofilm					
20.48	0.22	-1.49	3.00	-1.10	0.42
14.62	0.32	-1.16	3.00		
8.85	0.52	-0.65	3.00		
90 mg/L Biofilm					
1.61	2.86	1.05	4.50	0.93	0.16
2.03	2.27	0.82	4.50		
10 mg/L Planktonic					
0.72	6.43	1.86	2.30	2.46	0.53
0.26	18.06	2.89	2.30		
0.34	13.67	2.61	2.30		
20 mg/L Planktonic					
0.57	8.08	2.09	3.00	2.83	0.65
0.19	24.24	3.19	3.00		
0.18	25.16	3.23	3.00		
80 mg/L Planktonic					
0.17	27.25	3.31	4.38	3.35	0.28
0.22	21.12	3.05	4.38		
0.17	27.41	3.31	4.38		
0.11	41.12	3.72	4.38		

Chlorine observable modulus calculations:

B21: 10 mg/L-6/18/98

Time	Concentration (mg/L)	C*k*t	Robs	Φ
0	9.35	0		
10	6.41	-0.02	0.04	15.19
15	9.35	-0.03	-0.08	
35	7.58	-0.07	0.01	4.26
37	10.11	-0.07	-0.18	
70	7.62	-0.14	0.01	3.46
72	9.88	-0.14	-0.16	
97	8.56	-0.19	0.01	2.33
100	10.00	-0.20	-0.07	
112	8.94	-0.22	0.01	3.78
114	10.15	-0.23	-0.09	
137	9.28	-0.27	0.01	1.58
138	9.77	-0.28	-0.07	
166	9.28	-0.33	0.00	0.75
167	10.18	-0.33	-0.13	
210	9.05	-0.42	0.00	1.11
215	10.18	-0.43	-0.03	
257	9.28	-0.51	0.00	0.90
260	10.45	-0.52	-0.06	
315	9.43	-0.63	0.00	0.76
320	10.34	-0.64	-0.03	
340	9.96	-0.68	0.00	0.76

B22: 10 mg/L-6/24/98

Time	Concentration (mg/L)	C*k*t	Robs	Φ
0	9.28	0		
20	7.47	-0.04	0.01	4.40
25	8.56	-0.05	-0.03	
35	6.26	-0.07	0.03	12.63
37	8.34	-0.07	-0.15	
45	9.81	-0.09	-0.03	
65	7.73	-0.13	0.01	4.81
67	10.11	-0.13	-0.17	
87	8.83	-0.17	0.01	2.76
90	10.45	-0.18	-0.08	
132	7.73	-0.26	0.01	2.90
133	11.13	-0.27	-0.48	
165	9.24	-0.33	0.01	2.36
166	10.90	-0.33	-0.24	
195	9.92	-0.39	0.00	1.32
200	10.79	-0.40	-0.02	
230	9.73	-0.46	0.01	1.40
235	10.83	-0.47	-0.03	
285	9.24	-0.57	0.00	1.29
295	10.98	-0.59	-0.02	
360	9.39	-0.72	0.00	0.97

B23: 10 mg/L- 6/26/98

Time	Concentration (mg/L)	C*k*t	Robs	Φ
0	9.69	0		
17	4.45	-0.03	0.04	17.75
20	9.73	-0.04	-0.25	
37	7.13	-0.07	0.02	7.39
40	11.35	-0.08	-0.20	
70	8.37	-0.14	0.01	4.10
73	11.69	-0.15	-0.16	
114	8.30	-0.23	0.01	3.37
116	10.98	-0.23	-0.19	
151	8.26	-0.30	0.01	3.28
155	11.35	-0.31	-0.11	
183	10.15	-0.37	0.01	1.63
205	9.54	-0.41	0.00	1.13
207	11.32	-0.41	-0.13	
267	9.73	-0.53	0.00	1.02
270	11.17	-0.54	-0.07	
320	10.90	-0.64	0.00	0.19

B24: 20 mg/L- 7/10/98

Time	Concentration (mg/L)	C*k*t	Robs	Φ
0	26.59	0		
15	11.60	-0.18	0.14	21.05
16	23.29	-0.19	-1.70	
33	18.39	-0.40	0.04	5.18
35	24.14	-0.42	-0.44	
60	19.14	-0.72	0.02	3.22
63	23.20	-0.76	-0.23	
95	18.58	-1.14	0.02	2.12
97	24.42	-1.16	-0.50	
125	21.97	-1.50	0.00	0.60
126	23.86	-1.51	-0.49	
150	22.54	-1.80	0.00	-0.35

B25: 20 mg/L- 7/16/98

Time	Concentration (mg/L)	C*k*t	Robs	Φ
0	23.29			
8	17.16	-0.10	0.11	15.18
10	23.01	-0.12	-0.43	
17	19.24	-0.20	0.07	9.82
22	23.67	-0.26	-0.13	
43	18.11	-0.52	0.03	4.68
45	22.92	-0.54	-0.38	
66	19.99	-0.79	0.01	1.93
68	23.58	-0.82	-0.31	
98	20.37	-1.18	0.01	1.25
99	23.58	-1.19	-0.63	
125	21.69	-1.50	0.00	0.27
150	21.03	-1.80	-0.01	-0.87

B26: 20 mg/L-7/16/98

Time	Concentration (mg/L)	C*k*t	Robs	Φ
0	22.82	0		
13	17.26	-0.16	0.06	8.45
15	22.44	-0.18	-0.38	
31	18.77	-0.37	0.03	4.08
33	23.01	-0.40	-0.33	
53	19.14	-0.64	0.02	3.12
55	21.12	-0.66	-0.19	
78	21.22	-0.94	-0.01	-0.86
81	21.41	-0.97	-0.06	
111	20.65	-1.33	0.00	-0.37
113	20.46	-1.36	-0.08	
150	20.46	-1.80	-0.01	-0.97

B27: 90 mg/L-7/2/98

Time	Concentration (mg/L)	C*k*t	Robs	Φ
0	105.24	0		
15	89.02	-0.27	0.15	4.46
18	118.07	-0.32	-1.40	
40	92.04	-0.72	0.16	4.46
42	106.75	-0.76	-1.10	
70	98.08	-1.26	0.04	1.05
85	98.45	-1.53	-0.02	
105	96.57	-1.89	0.00	0.00
110	101.47	-1.98	-0.20	
145	93.55	-2.61	0.02	0.63
150	93.17	-2.70	-0.07	
180	66.01	-3.24	0.11	
185	96.94	-3.33	-0.98	
233	107.88	-4.19	-0.05	-1.25

B28: 90 mg/L-7/9/98

Time	Concentration (mg/L)	C*k*t	Robs	Φ
0	93.90	0		
2	78.46	-0.04	1.10	36.37
10	75.07	-0.18	0.046	1.70

APPENDIX C: Glutaraldehyde Data

Glutaraldehyde Biofilm Data:

C1: 25 ppm-3/17/99

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)	Time (min)	Area of GC Peak	Concentration (mg/L)
0	9.79	0.00	0	53807	21.66
60	9.82	0.03	0	49114	19.77
120	9.93	0.14	126	52005	20.94
180	9.89	0.10	126	51024	20.54
240	9.81	0.02	246	46815	18.85
300	9.74	-0.05	246	47681	19.2
360	9.82	0.03	352*	55132	22.2
420	9.74	-0.05	352	54968	22.13
480	9.65	-0.14	484*	77196	31.09
540	9.71	-0.07	484	74468	29.98
600	9.45	-0.34	604	63254	25.47
720	9.79	0.00	604	61218	24.65
			735	50497	20.33
			735	49875	20.08
				Average:	22.635

C2: 25 ppm-3/29/99

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)	Time (min)	Area of GC Peak	Concentration mg/L)
0	9.53	0.00	0	45097	18.16
60	9.69	0.16	0	43637	17.57
120	9.59	0.06	91	25063	10.09
180	9.42	-0.11	91	24665	9.93
240	9.32	-0.21	106*	49076	19.76
330	9.83	0.30	106	45731	18.41
461	9.48	-0.05	220	26399	10.63
540	9.59	0.06	220	26696	10.75
600	9.02	-0.51	230*	45883	18.47
660	9.20	-0.34	230	44559	17.94
720	9.16	-0.37	320	29675	11.95
			320	31973	12.87
			325*	46530	18.73
			325	44059	17.74
			461	25807	10.39
			461	24613	9.91
			462*	44031	17.73
			462	44242	17.81
			601	31730	12.77
			601	29661	11.94
			609*	68953	27.76
			609	71463	28.77
			686	55172	22.21
			686	52313	21.06
				Average:	16.39

C3: 50 mg/L-2/15/99

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)	Time (min)	Area of GC Peak	Concentration (mg/L)
0	9.76	0.00	0	135867	54.70
30	9.69	-0.07	0	132573	53.37
60	9.82	0.06	120	92675	37.31
120	9.75	-0.01	120	90890	36.59
180	9.69	-0.08	240*	100112	40.30
240	9.63	-0.14	240	104177	41.94
300	9.33	-0.43	420	74210	29.88
360	9.28	-0.49	420	76619	30.85
400	8.45	-1.31	480*	109364	44.03
480	8.58	-1.18	480	110890	44.64
540	8.06	-1.70	690	97211	39.14
600	6.44	-3.32	690	95942	38.63
660	6.62	-3.15		Average:	40.95
690	6.48	-3.29			

C4: 50 mg/L-2/24/99

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)	Time (min)	Area of GC Peak	Concentration (mg/L)
0	9.73	0.00	0	120097	48.35
120	9.72	-0.02	0	116233	46.79
240	9.56	-0.17	224	94431	38.02
330	9.22	-0.51	224	92003	37.04
420	8.93	-0.80	396	82543	33.23
480	8.67	-1.07	396	85138	34.28
540	7.99	-1.75	397*	118782	47.82
600	8.18	-1.55	397	121867	49.06
720	7.85	-1.89	565	105729	42.57
780	7.01	-2.72	565	108911	43.85
840	7.14	-2.60	566*	92487	37.23
			566	96307	38.77
			840	83574	33.65
			840	84392	33.98
				Average:	40.33

C5: 100 mg/L-1/29/99

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)	Time (min)	Area of GC Peak	Concentration mg/L)
0	9.84	0	0	202037	81.34
15	9.66	-0.19	0	203948	82.11
30	9.73	-0.11	150	156556	63.03
45	9.64	-0.20	150	156771	63.11
60	9.73	-0.11	300	141654	57.03
90	9.31	-0.53	300	144990	58.37
120	9.00	-0.85		Average:	67.50
150	8.83	-1.02			
180	8.11	-1.74			
210	7.57	-2.27			
240	6.57	-3.27			
270	5.90	-3.94			
300	4.77	-5.07			

C6: 100 mg/L-2/3/99

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)	Time (min)	Area of GC Peak	Concentration mg/L)
0	9.68	0.00	0	248074	99.87
15	9.81	0.13	0	234930	94.58
30	9.75	0.07	90	217163	87.43
45	9.04	-0.64	90	212071	85.38
60	9.55	-0.13	150*	240643	96.88
90	9.00	-0.68	150	253023	101.87
120	7.78	-1.90	300*	285227	114.83
150	6.85	-2.83	300	234688	94.48
180	3.68	-6.00		Average:	96.92
210	4.55	-5.13			
240					
270					
300					

C7: 100 mg/L-2/10/99

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)	Time (min)	Area of GC Peak	Concentration (mg/L)
0	9.73	0.00	0	219528	88.38
15	9.78	0.05	0	220760	88.88
30	9.93	0.20	120	181264	72.98
45	9.74	0.00	120	180409	72.63
60	9.65	-0.08	150*	249822	100.58
90	9.59	-0.15	150	243939	98.21
120	9.25	-0.49	300	219941	88.55
150	8.97	-0.76	300	226364	91.13
180	8.72	-1.02		Average:	87.67
210	8.18	-1.55			
240	6.01	-3.73			
280	3.96	-5.78			
300					

C8: 200 mg/L-2/19/99

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)	Time (min)	Area of GC Peak	Concentration mg/L)
0	9.81	0.00	0	553119	222.68
15	9.72	-0.09	0	554126	223.09
30	8.69	-1.12	150	503446	202.68
45	7.04	-2.78	150	521919	210.12
60	4.97	-4.84	151*	577779	232.61
			151	616312	248.12
			240	585973	235.91
			240	561384	226.01
				Average:	225.15

C9: 200 mg/L-2/22/99

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)	Time (min)	Area of GC Peak	Concentration (mg/L)
0	9.64	0.00	0	518970	208.93
15	9.12	-0.52	0	503122	202.55
25	8.44	-1.20	95	480164	193.31
35	6.78	-2.85	95	480423	193.41
45	5.03	-4.61		Average:	199.55

Glutaraldehyde Planktonic Data:

C10: 25 mg/L-3/9/99

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	6.89	0.00
0.50	6.85	-0.04
7.00	6.08	-0.82
10.00	5.77	-1.12
15.00	5.13	-1.76
25.00	3.75	-3.14
35.00	3.57	-3.32

C11: 25 mg/L-3/16/99

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	6.15	0.00
0.37	5.22	-0.93
5.00	5.15	-0.99
10.00	5.31	-0.84
15.00	4.01	-2.14
20.00	4.04	-2.11
25.00	3.99	-2.15
30.00	3.03	-3.12
35.00	2.85	-3.30

C12: 25 mg/L-3/18/99

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	6.96	0.00
0.25	6.17	-0.79
5.00	5.95	-1.01
10.00	5.41	-1.55
15.00	5.24	-1.72
20.00	4.95	-2.01
30.00	4.11	-2.85
35.00	3.98	-2.98
40.00	3.25	-3.72

C13: 50 mg/L

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	5.98	0.00
0.25	5.12	-0.87
5.00	4.32	-1.67
10.00	4.00	-1.99
15.00	3.56	-2.42

C14: 50 mg/L-3/30/99

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	5.76	0.00
0.20	4.55	-1.21
3.00	4.90	-0.86
6.00	5.64	-0.12
9.00	4.30	-1.46
12.00	4.58	-1.18
15.00	3.78	-1.97
20.00	4.33	-1.43
25.00	4.06	-1.69

C15: 50 mg/L-3/30/99

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	5.76	0.00
0.20	4.55	-1.21
3.00	4.90	-0.86
6.00	5.64	-0.12
9.00	4.30	-1.46
12.00	4.58	-1.18
15.00	3.78	-1.97
20.00	4.33	-1.43
25.00	4.06	-1.69

C16: 50 mg/L-4/2/99

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	6.94	0.00
0.43	6.77	-0.16
5.00	6.66	-0.28
9.00	6.66	-0.28
12.00	6.30	-0.63
15.00	6.09	-0.85
20.00	5.78	-1.15
25.00	4.81	-2.13
30.00	4.13	-2.81

C17: 100 mg/L-4/5/99

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	6.02	0.00
0.38	4.78	-1.23
3.00	3.85	-2.17
6.00	4.36	-1.66
9.00	4.31	-1.71
12.00	3.71	-2.31
15.00	2.85	-3.17
18.00	4.10	-1.91

C18: 100 mg/L-4/6/99

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	6.52	0.00
0.32	5.80	-0.72
3.00	5.68	-0.84
6.00	5.17	-1.35
9.00	5.22	-1.30
12.00	4.09	-2.43
15.00	3.53	-2.99
18.00	3.89	-2.63

C19: 100 mg/L-4/7/99

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	6.53	0.00
0.25	6.14	-0.38
3.00	6.13	-0.39
6.00	5.95	-0.58
9.00	4.89	-1.64
12.00	4.33	-2.19
15.00	3.98	-2.55
18.00	3.68	-2.85
21.00	2.82	-3.71
25.00	3.77	-2.75

C20: 250 mg/L-4/8/99

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	6.67	0.00
0.22	6.27	-0.40
3.00	3.00	-3.67

C21: 250 mg/L-4/12/99

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	5.30	0.00
0.20	3.88	-1.42
1.00	3.82	-1.48
1.75	4.36	-0.94
2.75	3.54	-1.76
3.50	3.02	-2.28

C22: 250 mg/L-4/13/99

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	5.99	0.00
0.05	3.82	-2.17
0.28	3.96	-2.03
0.58	4.20	-1.79
1.50	4.02	-1.97
2.50	4.07	-1.92

C23: Glutaraldehyde 2 log reduction rate analysis:

Time for 2 log kill	kdis*C^n	ln(kdis*C^n)	ln (Cb)	average ln(kdis*C^n)	standard deviation
25 mg/L Biofilm					
841.96	0.01	-5.21	3.22	-5.21	
50 mg/L Biofilm					
699.77	0.01	-5.02	3.91	-4.94	0.12
593.54	0.01	-4.86			
100 mg/L Biofilm					
219.60	0.02	-3.86	4.61	-3.58	0.38
194.06	0.02	-3.74			
107.27	0.04	-3.15			
200 mg/L Biofilm					
37.51	0.12	-2.10	5.30	-2.10	
25 mg/L Planktonic					
15.00	0.31	-1.18	3.22	-1.35	0.15
19.40	0.24	-1.44			
19.25	0.24	-1.43			
50 mg/L Planktonic					
24.72	0.19	-1.68	3.91	-1.31	0.53
11.72	0.39	-0.93			
100 mg/L Planktonic					
11.49	0.40	-0.91	4.61	-0.56	0.55
10.65	0.43	-0.84			
4.28	1.08	0.07			
250 mg/L Planktonic					
3.29	1.40	0.34	5.52	0.75	0.51
1.23	3.76	1.32			
2.54	1.81	0.60			

Glutaraldehyde observable modulus calculations:

C24: 25 mg/L-3/17/99

Time	Concentration (mg/L)	Robs	Φ
0	20.72		
126	20.74		
246	19.03	0.002	0.57
352	22.17		
484	30.54		
604	25.06	0.008	1.29
735	20.21	0.006	1.29

C25: 25 mg/L-3/29/99

Time	Concentration (mg/L)	Robs	Φ
0	17.87		
91	10.01	0.015	4.88
106	19.09		
220	10.69	0.013	3.90
230	18.21		
320	12.41	0.011	3.31
325	18.24		
461	10.15	0.010	3.30
462	17.77		
601	12.36	0.007	2.04
609	28.27		
686	21.64	0.015	2.72

C27: 50 mg/L-2/15/99

Time	Concentration (mg/L)	Robs	Φ
0	54.04		
120	36.95	0.024	2.47
240	41.12		
420	30.36	0.010	1.32
480	44.34		
690	38.88	0.004	0.49

C28: 50 mg/L-2/24/99

Time	Concentration (mg/L)	Robs	Φ
0	47.57		
224	37.53	0.008	0.83
396	33.75	0.004	0.49
397	48.44		
565	43.21	0.005	0.54
566	38.00		
840	33.81	0.003	0.34

C29: 100 mg/L-1/29/99

Time	Concentration (mg/L)	Robs	Φ
0	81.72		
150	63.07	0.021	1.35
300	57.70	0.006	0.47

C30: 100 mg/L-2/3/99

Time	Concentration (mg/L)	Robs	Φ
0	97.23		
90	86.40	0.021	1.03
150	99.37		
300	104.66		

C31: 100 mg/L-2/10/99

Time	Concentration (mg/L)	Robs	Φ
0	88.63		
120	72.80	0.023	1.29
150	99.39		
300	89.84	0.011	0.53

C32: 200 mg/L-2/19/99

Time	Concentration (mg/L)	Robs	Φ
0	222.88		
150	206.40	0.019	0.40
151	240.37		
240	230.96	0.018	0.35

C33: 200 mg/L-2/22/99

Time	Concentration (mg/L)	Robs	Φ
0	205.74		
95	193.36	0.022	0.51

APPENDIX D: Ciprofloxacin Data

Ciprofloxacin Biofilm Data:

D1: 5 ug/mL-6/9/99

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)
0	9.76	0.00
30	7.86	-1.90
60	7.62	-2.13
90	7.68	-2.08
120	7.28	-2.47
160	7.17	-2.58
210	7.19	-2.57
275	7.11	-2.65
342	6.76	-2.99
404	6.91	-2.84
476	6.96	-2.80
540	6.91	-2.84
647	6.91	-2.85
720	6.88	-2.87

D2: 5 ug/mL-6/14/99

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)
0	9.85	0.00
30	8.05	-1.80
60	7.14	-2.71
90	7.08	-2.77
135	7.14	-2.71
218	6.63	-3.22
282	6.63	-3.22
360	6.64	-3.21
420	6.38	-3.47
563	6.63	-3.22
715	6.25	-3.60

D3: 5 ug/mL-6/16/99

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)
0	9.67	0.00
2	9.70	0.03
10	8.29	-1.38
21	8.07	-1.59
31	7.79	-1.87
45	7.67	-2.00
60	7.54	-2.13
75	7.45	-2.22
90	7.09	-2.57
120	6.97	-2.70
180	6.74	-2.93
240	6.31	-3.36

D4: 25 ug/mL-6/25/99

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)
0	9.27	0.00
3.5	6.89	-2.39
10	6.86	-2.42
18	6.85	-2.43
25	6.82	-2.46
35	6.80	-2.47
45	6.72	-2.55
55	6.64	-2.64
90	6.74	-2.53
120	6.53	-2.75
180	6.62	-2.66
240	6.09	-3.18
300	6.26	-3.01
360	6.41	-2.87

D5: 25 ug/mL-7/7/99

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)
0	9.18	0.00
1.5	8.51	-0.67
6.5	7.41	-1.77
15	7.21	-1.97
50	6.95	-2.23
90	6.97	-2.21
120	6.71	-2.47
180	6.88	-2.30
240	6.53	-2.65
300	6.43	-2.75
360	6.28	-2.91
420	6.18	-3.00
480	6.64	-2.54
540	6.70	-2.48
780	5.84	-3.34

D6: 25 ug/mL-7/9/99

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)
0	9.47	0.00
2	8.04	-1.43
38	6.95	-2.52
60	6.96	-2.51
120	7.06	-2.41
180	6.96	-2.51
240	6.65	-2.82
300	6.86	-2.61
360	6.97	-2.50
420	6.66	-2.81
480	6.61	-2.86
560	6.82	-2.65

D7: 1 ug/mL-7/12/99

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)
0	9.22	0.00
2	9.26	0.04
40	8.72	-0.50
60	8.67	-0.55
120	8.16	-1.06
180	8.24	-0.98
240	7.90	-1.32
300	7.95	-1.26
360	7.79	-1.42
420	7.91	-1.31
480	7.76	-1.46
740	7.79	-1.43

D8: 1 ug/mL-7/14/99

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)
0	9.34	0.00
2.33	9.48	0.14
15	9.21	-0.13
50	8.94	-0.40
95	8.70	-0.63
140	8.20	-1.14
180	8.39	-0.94
240	8.08	-1.26
320	7.77	-1.57
360	7.84	-1.50
420	8.01	-1.33
480	7.78	-1.56
540	7.84	-1.49
600	7.89	-1.44
740	7.68	-1.66

D9: 1 ug/mL-7/16/99

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)
0	9.16	0.00
15	8.82	-0.34
30	8.94	-0.22
90	8.26	-0.90
120	8.08	-1.08
150	7.75	-1.40
180	7.85	-1.31
240	7.26	-1.90
300	7.61	-1.55
360	7.06	-2.10
420	6.95	-2.21
480	7.18	-1.97
540	6.93	-2.22
720	7.16	-2.00

Ciprofloxacin Planktonic Data:

D10: 1 ug/mL-7/15/99

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0	6.07	0.00
0.17	4.88	-1.19
30	4.59	-1.48
60	4.60	-1.47
90	4.30	-1.77
120	3.05	-3.02
150	3.74	-2.33
180	3.40	-2.67
210	2.97	-3.10

D11: 1 ug/mL-7/21/99

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0	5.94	0.00
0.37	4.49	-1.46
30	3.87	-2.08
60	4.07	-1.88
90	4.01	-1.94
120	3.77	-2.17
150	3.61	-2.33
180	3.51	-2.43
210	3.59	-2.36
240	3.00	-2.94

D12: 1 ug/mL-7/22/99

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0	5.64	0.00
0.5	3.71	-1.93
19.33	4.15	-1.49
87	3.76	-1.88
120	3.86	-1.78
150	3.05	-2.59
180	3.20	-2.44
210	3.09	-2.55
240	3.02	-2.62

D13: 5ug/mL-5/25/99

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0	6.02	0.00
0.32	4.21	-1.82
10.5	4.20	-1.82
19.95	4.53	-1.50
29.1	4.16	-1.86
39.5	3.85	-2.17
50.75	3.80	-2.22
57.83	3.99	-2.03

D14:5 ug/mL- 5/26/99

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0	6.60	0.00
0.5	6.14	-0.46
11	5.81	-0.80
21.5	5.51	-1.09
32.75	5.47	-1.13
41.85	5.20	-1.40
51.5	5.16	-1.44
61	5.02	-1.58
72	5.34	-1.26
80.5	4.87	-1.73
90.5	5.21	-1.39

D15: 5 ug/mL-6/2/99

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0	5.27	0.00
0.5	3.96	-1.31
30	3.63	-1.64
60	2.91	-2.36
120	2.85	-2.42

D16: 5 ug/mL-6/3/99

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0	4.69	0.00
0.5	3.31	-1.38
52	3.84	-0.85
90	2.99	-1.70
118	2.90	-1.79

D17: 5 ug/mL-6/7/99

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0	6.14	0.00
0.42	5.18	-0.96
30	4.24	-1.90
60	3.78	-2.36
90	3.07	-3.08
120	2.82	-3.32
143	2.93	-3.21

D18: 5 ug/mL-6/11/99

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0	5.82	0.00
0.28	2.91	-2.91
30	2.85	-2.97

D19: 5 ug/mL-7/7/99

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0	6.27	0.00
0.33	4.38	-1.86
30	3.95	-2.29
60	2.79	-3.45
90	3.13	-3.10
120	4.06	-2.17
150	2.91	-3.33

D20: 25 ug/mL-7/2/99

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0	5.81	0.00
0.17	2.70	-3.11

D21: 25 ug/mL-7/6/99

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0	5.65	0.00
0.25	2.70	-2.95

D22: 25 ug/mL-7/6/99

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0	5.15	0.00
0.33	2.70	-2.45

D23: Ciprofloxacin 2 log reduction rate analysis:

Time for 2 log kill	kdis*C^n	Cb	ln(Cb)	Average ln(kdis*C^n)	Standard Deviation
5 ug/mL Biofilm					
47.72	0.097	5.00	1.61	-2.60	0.29
84.50	0.054				
59.72	0.077				
25 ug/mL Biofilm					
75.23	0.061	25.00	3.22	-2.19	0.83
57.54	0.080				
15.91	0.289				
1 ug/mL Biofilm					
1418.00	0.003	1.00	0.00	-5.73	
5 ug/mL Planktonic					
39.96	0.12	5.00	1.61	-1.86	0.61
14.72	0.31				
44.57	0.10				
1 ug/mL Planktonic					
85.83	0.115	1.00	0.00	-2.81	0.41
48.58	0.313				
108.41	0.103				
25 ug/mL Planktonic					
0.27	0.054	25.00	3.22	3.30	0.47
0.17	0.095				
0.11	0.042				

D24: Ciprofloxacin Physiological Model Parameters:

Ciprofloxacin Disinfection (1 mg/L) of P.Aeruginosa

Method	AIC	Kdis	Es	p	Er
Phys	-33.19	4.41E-04	0.92922	4.05E-02	0.07078
Phys	-36.37	3.09E-04	0.95275	4.48E-02	0.04725
Phys	-22.38	3.59E-04	0.98641	3.87E-02	0.01359
Average:		3.70E-04		3.70E-04	0.043873
Standard Deviation:		6.67E-05		0.003127	0.028744

Ciprofloxacin Disinfection (5 mg/L) of P.Aeruginosa

Method	AIC	Kdis	Es	p	Er
Phys	-22.31	5.33E-04	0.9949	1.49E-02	0.0051
Phys	37.15	6.66E-04	0.98516	2.12E-02	0.01484
Phys	-20.79	1.08E-03	0.97932	5.21E-02	0.02068
Average:		7.58E-04		2.94E-02	0.01354
Standard Deviation:		0.000282		0.019879	0.007871

Ciprofloxacin Disinfection (25 mg/L) of P.Aeruginosa

Method	AIC	Kdis	Es	p	Er
Phys	-30.8	1.42E-03	0.99644	1.89E-03	0.00356
Phys	-17.79	5.04E-04	0.99299	4.25E-03	0.00701
Phys	-27.34	1.13E-03	0.9966	7.41E-04	0.0034
Average:		1.02E-03		2.29E-03	0.004657
Standard Deviation:		0.000469		0.001787	0.00204

APPENDIX E: QAC Data

QAC Biofilm Data:

E1: 50 mg/L-9/3/98

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)
0	9.79	0.00
10	9.87	0.09
20	9.78	0.00
30	9.67	-0.11
60	9.72	-0.06
90	9.63	-0.16
120	9.50	-0.28
150	9.43	-0.36
180	9.24	-0.54
210	9.20	-0.58
240	8.96	-0.82

E2: 50 mg/L-9/10/98

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)
0	9.70	0.00
10	9.45	-0.24
20	9.45	-0.25
30	9.56	-0.13
60	9.42	-0.27
90	9.25	-0.45
120	9.21	-0.49
150	8.81	-0.89
180	8.78	-0.92
210	8.77	-0.93
240	8.48	-1.22

E3: 50 mg/L-9/10/98

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)
0	9.72	0.00
10	9.68	-0.04
20	9.52	-0.20
30	9.59	-0.13
60	9.36	-0.36
90	9.58	-0.14
120	9.20	-0.53
150	9.21	-0.51
180	8.97	-0.75
210	9.03	-0.69
240	8.94	-0.79

E4: 50 mg/L-10/5/98

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)
0	9.79	0.00
30	9.39	-0.40
60	9.32	-0.47
120	9.30	-0.49
180	9.24	-0.55
240	9.06	-0.73
300	8.84	-0.95
360	8.92	-0.87
420	9.21	-0.58
480	8.85	-0.94
540	8.92	-0.87
610	8.80	-0.99
660	8.83	-0.96
720	8.89	-0.90

E5: 100 mg/L-4/28/99

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)
0	9.76	0.00
30	9.35	-0.42
60	9.25	-0.51
90	9.44	-0.32
120	9.10	-0.66
180	7.41	-2.36
255	8.62	-1.14
300	7.04	-2.72
360	7.37	-2.39
420	7.99	-1.77
480	7.40	-2.36
540	7.91	-1.85
600	7.75	-2.01
660	7.02	-2.75
720	6.93	-2.83

E6: 100 mg/L-4/29/98

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)
0	9.46	0.00
60	8.89	-0.57
120	8.44	-1.02
180	8.04	-1.42
255	7.35	-2.11
300	6.85	-2.61
360	7.53	-1.93
420	6.64	-2.82
480	5.54	-3.92

E7: 250mg/L-8/19/98

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)
0	9.65	0.00
5	9.05	-0.60
10	8.83	-0.82
15	8.74	-0.91
30	6.82	-2.83
60	5.22	-4.43
90	7.19	-2.46
120	6.32	-3.33
150	6.26	-3.39
180	5.14	-4.51

E8: 250 mg/L-8/26/98

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)
0	9.60	0.00
5	9.29	-0.31
10	9.02	-0.58
15	8.97	-0.63
20	8.18	-1.42
25	7.52	-2.08
30	6.96	-2.64
180	5.94	-3.66

E9: 250mg/L-8/27/98

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)
0	9.87	0.00
5	9.78	-0.09
10	9.67	-0.20
15	9.58	-0.29
20	9.34	-0.53
25	9.49	-0.38
30	9.27	-0.60
60	8.95	-0.92
90	8.45	-1.42
120	8.20	-1.67
150	8.31	-1.56
180	6.92	-2.95

E10: 250mg/L-8/29/98

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)
0	9.85	0.00
5	9.67	-0.18
10	9.60	-0.25
15	9.43	-0.42
20	9.43	-0.42
25	9.25	-0.60
30	9.33	-0.52
60	8.82	-1.03
90	7.84	-2.01
120	7.94	-1.91
150	7.94	-1.91
180	7.90	-1.95

E11: 250 mg/L-9/2/98

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)
0	9.71	0.00
10	9.30	-0.41
20	9.18	-0.53
30	9.18	-0.53
60	8.86	-0.85
90	8.90	-0.81
120	7.65	-2.06
150	7.68	-2.03
180	7.15	-2.56
210	7.94	-1.77
240	8.01	-1.70

E12: 500 mg/L-5/12/99

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)
0	9.41	0.00
22	7.03	-2.38
40	8.21	-1.20
60	8.08	-1.33
90	7.64	-1.77
126	4.95	-4.46
150	5.88	-3.53
207	4.95	-4.46
255	6.57	-2.84

E13: 500mg/L-9/18/98

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)
0	9.76	0.00
10	9.33	-0.43
20	9.01	-0.75
30	8.87	-0.89
60	8.86	-0.90
90	8.00	-1.76
120	7.92	-1.84
150	5.75	-4.01
180	6.22	-3.55
210	6.04	-3.73
240	6.36	-3.40

E14: 1000 mg/L-9/15/98

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)
0	9.59	0.00
10	8.91	-0.68
20	8.09	-1.50
30	7.54	-2.05
40	6.17	-3.42
50	7.00	-2.59
60	6.36	-3.23
90	6.96	-2.63

D15: 1000mg/L-9/16/98

Time (min)	Average log10 Cell Density (cfu/cm^3)	Log (X/X0)
0	9.93	0.00
10	9.10	-0.83
20	8.67	-1.26
30	8.37	-1.56
40	8.00	-1.93
50	8.00	-1.94
60	7.42	-2.51
90	6.44	-3.49
120	6.49	-3.44
150	6.06	-3.87
180	5.72	-4.21

QAC Planktonic Data:

E16: 50mg/L-11/26/98

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	6.80	0.00
0.17	3.67	-3.13
0.42	2.99	-3.81
0.75	2.85	-3.95
1.08	2.97	-3.83

E17: 50 mg/L-11/27/98

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	6.99	0.00
0.25	4.90	-2.09
0.58	4.49	-2.50
1.00	3.94	-3.06
2.50	3.64	-3.36

E18: 50 mg/L-11/30/98

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	6.92	0.00
0.25	5.00	-1.92
0.50	4.90	-2.02
1.00	3.97	-2.96
2.00	3.49	-3.44
3.17	2.96	-3.97
4.00	3.02	-3.91

E19: 50 mg/L-12/9/98

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	6.70	0.00
0.17	4.31	-2.39
0.58	3.85	-2.85
0.92	3.61	-3.08
1.50	3.30	-3.40
3.50	3.56	-3.14

E20: 100 mg/L-11/25/98

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	6.96	0.00
0.25	3.87	-3.09
0.67	3.10	-3.86

E21: 100 mg/L-4/26/99

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	6.32	0.00
0.17	3.08	-3.24
0.38	2.76	-3.56
0.65	2.82	-3.50

E22: 100 mg/L- 4/27/99

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	6.80	0.00
0.15	4.08	-2.73
0.45	3.19	-3.62
0.77	2.98	-3.83
1.00	2.93	-3.88

E23: 250mg/L-11/23/98

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	6.85	0.00
0.25	2.70	-4.16

E24: 250mg/L-4/16/99

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	6.96	0.00
0.22	3.24	-3.72

E25: 250 mg/L-4/22/99

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	6.85	0.00
0.25	2.76	-4.09

E26: 250mg/L-4/23/99

Time (min)	Average log10 Cell Density (cfu/mL)	Log (X/X0)
0.00	6.02	0.00
0.10	2.70	-3.32

E27: QAC 2 log Reduction Rate Analysis:

Time for 2 log reduction	Kdis*C^n	ln(kcis*C^n)	ln(Cb)	Average ln(kdis*C^n)	Standard Deviation
50 mg/L Biofilm					
898.79	0.01	-5.27	3.91	-5.27	
373.45					
100 mg/L Biofilm					
219.55	0.02	-3.86	4.61	-3.89	0.03
229.63	0.02	-3.91			
250 mg/L Biofilm					
190.36	0.02	-3.72	5.52	-3.50	0.31
123.36	0.04	-3.29			
500 mg/L Biofilm					
84.40	0.05	-2.91	6.21	-2.71	0.28
56.90	0.08	-2.51			
1000 mg/L Biofilm					
41.53	0.11	-2.20	6.91	-2.71	0.45
84.40	0.05	-2.91			
94.68	0.05	-3.02			
50 ppm Planktonic					
0.49	9.38	2.24	3.91	2.88	0.64
0.31	15.05	2.71			
0.28	16.69	2.81			
0.11	43.04	3.76			
100 ppm Planktonic					
0.14	32.43	3.48	4.61	3.74	0.30
0.08	58.29	4.07			
0.12	40.04	3.69			
250 ppm Planktonic					
0.06	76.75	4.34	5.52	3.82	0.35
0.12	39.36	3.67			
0.12	37.75	3.63			
0.12	38.38	3.65			

E28: QAC Physiology Model Parameters:

QAC Disinfection (50 mg/L) of P. aeruginosa Biofilm

Model	AIC	Kdis	Es	p	Er
Phys	-31.86	1.16E-05	0.001	1.89E-01	0.999
Phys	-25.35	3.44E-05	0.13435	1.06E-01	0.86565
Phys	-29.18	3.78E-05	0.11655	6.23E-02	0.88345
Phys	-32.77	3.03E-05	0.67087	1.88E-02	0.32913
Average:		2.85E-05	0.23069	9.38E-02	0.69274
Standard Deviation:		1.2E-05	0.29935	0.07249	0.29935

QAC Disinfection (250 mg/L) of P. aeruginosa Biofilm

Model	AIC	Kdis	Es	p	Er
Phys	-18.68	3.08E-05	0.36029	5.51E-02	0.63971
Phys	-6.72	1.19E-04	0.47609	1.49E-02	0.52391
Phys	4.22	2.99E-05	0.65751	4.00E-02	0.34249
Average:		5.98E-05	0.49796	3.67E-02	0.50204
Standard Deviation:		5.1E-05	0.14981	0.02029	0.14981

QAC Disinfection (500 mg/L) of P. aeruginosa Biofilm

Model	AIC	Kdis	Es	p	Er
Phys	-8.81	2.96E-05	0.58176	4.08E-02	0.41824
Phys	19.45	2.99E-05	0.97287	2.55E-02	0.02713
Average:		2.98E-05	0.77732	3.31E-02	0.22269
Standard Deviation:		1.6E-07	0.27656	0.01079	0.27656

QAC Disinfection (1000 mg/L) of P. aeruginosa Biofilm

Model	AIC	Kdis	Es	p	Er
Phys	11.72	3.00E-05	0.94557	3.10E-02	0.05443
Phys	-2.93	2.99E-05	0.90837	2.51E-02	0.09163
Average:		2.99E-05	0.92697	2.80E-02	0.07303
Standard Deviation:		8.4E-08	0.0263	0.00412	0.0263