Measuring and modeling chlorine penetration into artificial biofilms
by Xiao Chen

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemical Engineering
Montana State University
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Abstract:
The penetration of chlorine into artificial biofilms of Pseudomonas aeruginosa entrapped in agarose gel was investigated. A chlorine microelectrode was used to measure transient chlorine concentration profiles in artificial biofilms in a flow cell. While chlorine penetrated relatively quickly (<30min) into a film of pure agarose, when cells are added to the biofilm, chlorine penetration was greatly retarded. A 773μm thick film containing 1.0 mg/1 of cells was not fully penetrated by 14 mg/1 chlorine within the two hour treatment period. The slow penetration was shown to be consistent with reaction-diffusion theory. Biomass-chlorine reactions were studied using well mixed suspensions. Kinetic and stoichiometric coefficients for the reactions of agarose and cell mass with chlorine were obtained by fitting a simple first order (in both reactants) kinetic model to chlorine versus time data. The reaction rate constant for chlorine-cell reaction (6.7x10^-4mg/l*min) exceeded that for the chlorine-agarose reaction (2.2x10^-4mg/l*min) by 2 orders of magnitude. The yield coefficient relating the amount of biomass consumed to the amount of chlorine consumed varied from 0.6 to 4.3mg/mg, depending on the duration of the experimental measurement. A mathematical model of the transient reaction-diffusion interaction correctly captured the qualitative behavior of the experimentally measured chlorine concentration profiles. Using independent estimates of parameter values and adjusting the value of the biomass-chlorine yield coefficient to a value (~1.8mg/mg) midway between the experimentally determined values a good match to experimental data was obtained. This study shows that a reaction-diffusion interaction could explain the poor efficacy of reactive antimicrobial agents such as chlorine when used against biofilm microorganisms.

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APPROVAL

of a thesis submitted by

Xiao Chen

This thesis has been read by each member of the 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.

April 17, 1995
Date

Philip A. Stewart
Chairperson, Graduate Committee

Approved for the Major Department

April 17, 1995
Date

John T. Sears
Head, Major Department

Approved for the College of Graduate Studies

4/21/95
Date

R. L. Brown
Graduate Dean

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TABLE OF CONTENTS

Page

LIST OF TABLES....................vii

LIST OF FIGURES....................viii

ABSTRACT....................x

INTRODUCTION....................1
  Problem Overview....................1
  Goal and Objectives....................1

LITERATURE REVIEW....................2

MATERIALS AND METHODS....................5
  Microorganism and Culture Method....................5
  Artificial Biofilm....................5
  Biofilm Thickness Measurement....................7
  Chlorine Assay....................7
  Chlorine Microelectrode....................8
  Current Measurement....................9
  Suspended Biomass-Chlorine Reaction Kinetics....................10
  Chlorine Profile Measurement in Artificial Biofilm....................10
  Reaction Kinetics Data Analysis....................13
  Biofilm-Chlorine Interaction Model....................14

RESULTS....................16
  Microbial Culture....................16
  Characteristics of the Chlorine Microelectrode....................16
  Suspended Biomass-Chlorine Reaction Kinetics....................26
  Chlorine Concentration Profiles in Artificial Biofilms....................34
  Modeling Results....................43

DISCUSSION....................51
  Artificial Biofilm....................51
  Chlorine Microelectrode....................51
  Biomass-Chlorine Reaction Rate and Yield Coefficients....................53
  Chlorine Penetration....................54

CONCLUSIONS....................56

RECOMMENDATIONS FOR FUTURE WORK 57

REFERENCES 58

APPENDIX 61

LIST OF TABLES

Table Page

1. Composition of culture medium....6
2. Composition of phosphate buffer....6
3. Kinetic data of chlorine-cell reaction, short duration experiment No. 1....27
4. Kinetic data of chlorine-cell reaction, short duration experiment No. 2....28
5. Kinetic data of chlorine-cell reaction, short duration experiment No. 3....29
6. Kinetic data of chlorine-cell reaction, short duration experiment No. 4....30
7. Kinetic data of chlorine-agarose reaction experiment....31
8. Kinetic data of chlorine-cell reaction, long duration experiment....33
9. Chlorine-cell reaction rate constants and yield coefficients from short duration experimental data regressions....35
10. Chlorine-agarose reaction rate constants and yield coefficients from experimental data regressions....36
11. Chlorine-cell reaction rate constants and yield coefficients from long duration experimental data regressions....36
12. Parameter values used in biofilm-chlorine interaction model....49
13. Results from Case A, chlorine concentration profiles measurement....61
14. Results from Case B, chlorine concentration profiles measurement....64
15. Results from Case C, chlorine concentration profiles measurement....65

LIST OF FIGURES

Figure Page

1. Apparatus for measurement of suspended biomass-chlorine reaction kinetics 11

2. Apparatus for chlorine concentration profile measurement inside artificial biofilms.... 12

3. *Pseudomonas aeruginosa* ERC-1 growth curve 17

4. Calibration curves of chlorine microelectrodes with different tip diameters 18

5. pH dependence of microelectrode (25μm) signal in chlorine solution (18.6mg/l)........ 19

6. pH dependence of hypochlorous acid and hypochlorite ion distribution 20

7. Effect of phosphate buffer capacity on the sensitivity of a chlorine microelectrode........ 22

8. The stirring effect on the signals of chlorine microelectrodes with different tip diameters 23

9. Sensitivity and selectivity of chlorine microelectrode at different applied potentials...... 24

10. Effect of dissolved oxygen on the chlorine microelectrode signal 25

11. Standard curve of DPD colorimetric method for chlorine measurement 32

12. Kinetic data from chlorine-cell reaction experiment No. 1 and regression results at different initial cell concentrations 37

13. Kinetic data from chlorine-cell reaction experiment No. 2 and regression results at different initial cell concentrations 38

14. Kinetic data from chlorine-cell reaction experiment No. 3 and regression results at different initial cell concentrations 39

15. Kinetic data from chlorine-cell reaction experiment No. 4 and regression results at different initial cell concentrations 40

16. Kinetic data from chlorine-agarose reaction experiment and regression results at different initial chlorine concentrations 41

17. Chlorine concentration profile control experiments....42

18. Chlorine concentration profiles, Case A....44

19. Chlorine concentration profiles, Case B....45

20. Chlorine concentration profiles, Case C....46

21. Chlorine concentration profiles from the model predictions, Case D....50

ABSTRACT

The penetration of chlorine into artificial biofilms of *Pseudomonas aeruginosa* entrapped in agarose gel was investigated. A chlorine microelectrode was used to measure transient chlorine concentration profiles in artificial biofilms in a flow cell. While chlorine penetrated relatively quickly (<30min) into a film of pure agarose, when cells are added to the biofilm, chlorine penetration was greatly retarded. A 773μm thick film containing 1.0 mg/l of cells was not fully penetrated by 14 mg/l chlorine within the two hour treatment period. The slow penetration was shown to be consistent with reaction-diffusion theory. Biomass-chlorine reactions were studied using well mixed suspensions. Kinetic and stoichiometric coefficients for the reactions of agarose and cell mass with chlorine were obtained by fitting a simple first order (in both reactants) kinetic model to chlorine versus time data. The reaction rate constant for chlorine-cell reaction (6.7×10^{-2}mg/l*min) exceeded that for the chlorine-agarose reaction (2.2×10^{-4}mg/l*min) by 2 orders of magnitude. The yield coefficient relating the amount of biomass consumed to the amount of chlorine consumed varied from 0.6 to 4.3mg/mg, depending on the duration of the experimental measurement. A mathematical model of the transient reaction-diffusion interaction correctly captured the qualitative behavior of the experimentally measured chlorine concentration profiles. Using independent estimates of parameter values and adjusting the value of the biomass-chlorine yield coefficient to a value (~1.8mg/mg) midway between the experimentally determined values a good match to experimental data was obtained. This study shows that a reaction-diffusion interaction could explain the poor efficacy of reactive antimicrobial agents such as chlorine when used against biofilm microorganisms.

INTRODUCTION

Problem Overview

The undesirable accumulation of microorganisms in the form of biofilm has been a longstanding problem in industry and medicine. Biocides and antibiotics, while successful in controlling planktonic microbial populations have been commonly found to be less effective against biofilms or cell aggregates (Costerton 1987, LeChevallier 1988). One hypothesis to explain biofilm recalcitrance is that antimicrobial agents fail to fully penetrate through the biofilm during the time of treatment. Depletion of an antimicrobial agents by reaction with microorganisms or other biofilm constituents near the surface of the biofilm could result in microorganisms in the biofilm interior not being exposed to effective concentrations of the antibiotic or biocide (Nichols, 1989a).

Goal and Objectives

The long term goal of the research program of which this thesis is a piece is to elucidate mechanisms of biofilm resistance to disinfectants. The particular objectives of this project were to: a) demonstrate the failure of chlorine penetration as result of chlorine-biomass interaction, b) compare experimental chlorine concentration profiles in artificial biofilms with the predictions of reaction-diffusion theory. Four specific items needed to be addressed. They were: 1) develop an artificial biofilm system for chlorine penetration studies, 2) measure and model the kinetics of cell or agarose reactions with chlorine, 3) measure chlorine concentration profiles at different time in artificial biofilms, 4) compare these profiles with simulations based on transient reaction-diffusion theory.

LITERATURE REVIEW

Chlorine has been used for over a century as a strong disinfectant. Chlorine mainly refers to aqueous hypochlorous acid and hypochlorite ion. Because of its strong tendency to acquire extra electrons, chlorine is able to oxidize many inorganic and organic materials in water. Therefore, the chlorine demand, the difference between the amount of chlorine applied and the amount that remains in solution, has been a concern for a long time. Attention was mainly focused on the impurity of water that consumed chlorine, rather than the chlorine demand of biomass. Nevertheless, there is some research that can be related to biomass-chlorine interactions. It has been widely recognized that protein, a major part of microbial cell, reacts readily with chlorine, and consequently may be responsible for the fast microbial inactivation by chlorine disinfection (Green 1946, Knox 1948). Amino acids, a building block of protein, have been investigated for their reactivity with chlorine in terms of chlorine demand (Pereira 1973, Hureiki 1994). Nucleic acids are also able to react with chlorine and rapidly inactivate the transforming activity of DNA and the infectivity of RNA by chlorine (Olivieri 1980).

Recent research of chlorine disinfection have shifted from planktonic to biofilm systems. A report from Characklis *et. al.* (1976) shows that slime or extracellular microbial polysaccharide exhibits more rapid and greater ultimate uptake of hypochlorite. They also recorded the transient change of chlorine concentration, but no kinetic analysis was given. By assuming reacted biofilm is soluble, Characklis (1980) was able to measure the stoichiometric coefficient and the reaction rate constant of biofilm reaction with chlorine,

though the rate constant is a lumped parameter including the intrinsic rate coefficient and the biomass concentration.

Because of the cell aggregate that occurs in biofilms, cells are not equivalently exposed to chlorine at same time. Diffusion due to the concentration gradient coupled with reaction occurs in biofilm systems. Therefore, a reaction-diffusion theory has been used to guide the investigations of biofilm disinfection. Chen (1993) interpreted the differing disinfection efficacy of monochloramine against *Pseudomonas aeruginosa* biofilm at different biocide concentrations by using the observable modules Φ to assess the magnitude of mass transfer effects on overall reaction kinetics. He argued that the low efficacy of 2ppm monochloramine treatment was the result of high mass transfer resistance in the biofilm ($\Phi>2.97$). Nichols (1989b) used a sorption theory coupled with diffusion to predict a penetration time for tobramycin and cefsulodin in *P. aeruginosa* biofilm. Recently Stewart (1994, 1995) gave theoretical explanations of low efficacy of antimicrobial agents against biofilms by adapting a computer model of biofilm dynamics. According to the model, the failure penetration of these reactive agents and the slow microbial growth rate deep in the biofilm are both plausible mechanisms of biofilm resistance. The lack of tools to directly reveal the penetration of these chemicals in biofilms has impeded the progress of biofilm disinfection research. Tashiro (1990) tried to measure the penetration depth of several biocides by relating it to the remaining reducing activities of TTC (1,3,5-trichlorophenyl tetrazolim chloride), a indicator of the electron transport activity.

Different kinds of chlorine electrodes have been successfully developed (Tsausis 1985, Ge 1990, Morrison 1990) to record the chlorine concentration in real time. In our labs

a modified miniature chlorine microelectrode was recently developed based on the same principle. This microelectrode enables us to directly measure chlorine penetration with time inside the biofilms. De Beer (1994) first demonstrated a profound chlorine penetration resistance by recording the chlorine concentration profiles using this microelectrode in a *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* binary culture biofilm.

MATERIALS AND METHODS

Microorganism and Culture Method

Pseudomonas aeruginosa ERC1 was used throughout in pure culture. The strain is an environmental isolate and was stored in glycerol peptone solution as a frozen culture at -70^{0}C. To generate a culture, a shake flask containing 100ml of medium was inoculated with 0.1 ml of frozen culture. The medium composition is given in Table 1. Medium components were autoclaved except for glucose which was sterilized by filtration.

The culture was grown for 20 hours in a shaker at 27.5±0.5^{0}C. Because of the appearance of some floc in the culture, a homogenizer (Tekmar) was used to disperse them. 20ml of the solution then was transferred to each of four centrifuge tubes. The culture was centrifuged at 5^{0}C and 12,500 rpm for 15 minutes. The supernatant was decanted and the cell pellet washed with 4.59mM phosphate buffer (Table 2). Two tubes of pelletized cell mass marked with C and D were resuspended in 5ml phosphate buffer by using the homogenizer for 1.5 minutes at full power, and the other two tubes marked with A and B were used to measure the dry weight by membrane (0.4µm, 47mm in diameter) filtration after they were resuspended with 5ml buffer by vortexing at low power. The dry weight is the weight difference of the predried membrane and the membrane after filtration and drying for 2 hours at 105^{0}C.

Artificial Biofilms

Artificial biofilms were prepared by depositing an agarose-cell mixture in a thin

Table 1. Composition of culture medium.

Nutrients	Concentrations
Nutrient Broth	4 (g/l)
Yeast Extract	1 (g/l)
Glucose	5 (g/l)

Table 2. Composition of phosphate buffer (pH 7.4).

Chemicals	Concentrations
KH_2PO_4	0.236 (g/l)
Na_2HPO_4	0.405 (g/l)

layer on a stainless steel slide and allowing the mixture to gel. Agarose solution was prepared by melting 2% agarose in a 65^{0}C water bath for 30 minutes. The solution was then cooled down to about 40^{0}C. 5ml of agarose solution was added to tube C, one of the resuspended *Pseudomonas aeruginosa* cell suspensions, and was mixed by vortexing. 0.1ml of agarose-cell mixture was dropped on an autoclaved prewarmed stainless steel slide (17cm x 2.1cm) and a sterilized razor blade was used to spread the drop to an even thickness before the gel set.

Biofilm Thickness Measurement

The artificial biofilm thickness was measured by light microscopy according to Bakke and Olsson *et. al.* The actual thickness of biofilm is 1.33 time the optical distance between the biofilm surface and the slide substratum determined by microscopy. Since there is some expansion of the ell-agarose gel during experimentation, each thickness was the average of two measurements before and two after the chlorine profiles measurement experiments.

Chlorine Assay

Chlorine concentration measured either by chlorine microelectrode or DPD colorimetric method was calibrated against a chlorine standard solution whose concentration was determined by the DPD ferrous titrimetric method (4500-Cl F, Standard methods, APHA 1992). Each concentration of a chlorine standard solution was the average of three titrations whose errors between any two were less than 1%.

Chlorine Microelectrode

A glass covered platinum wire was used as a chlorine sensitive probe. A 100μm diameter platinum wire was dipped into 2M KCN solution 40 to 50 times with applied potential of +0.25V with respect to a graphite bar. After this treatment the wire tapered off with a tip diameter less than 10μm. This wire was then inserted into a 1mm diameter glass case which had been pulled by hand in a projected propane fire to get a tapered shape. After the tip position of the wire inside the glass case was marked, a puller (Micro Electrode Puller, Stoelting Co.) was used to seal the wire by melting the glass case, which was done by adjusting the tip mark 1.5cm above the heat loop, then fastening the glass case, attaching a weight to the suspended end of the glass case and setting the heating power at 75% of full power. About 15 seconds later, the glass case elongated and finally fell down, sealing the electrode. The next step was to grind away the tip of the electrode to expose the platinum wire. The exposure area then was recessed about 2μm by quickly touching a fresh 2M KCN solution with an applied potential half of the previous one. After it was carefully washed with acetone and distilled water three times and dried by a heat gun, the electrode was dipped into a acetone solution with 1g/ml cellulose acetate for 20 seconds and pulled out with the tip coming out of the liquid first. Three hours later the polymer coating dried and a chlorine microelectrode was ready. Most electrodes needed to stabilize in a phosphate buffer at neutral pH with applied +0.2V potential with reference to a saturated calomel electrode (SCE) for two hours before they could fully function. Without this stabilization, some electrodes gave a negative signal in the absence of chlorine when they should have a

response of zero current.

Current Measurement

An amperometric method was used to measure the chlorine concentration in the liquid. The electrochemical cell in this case consisted of a stable voltage source (adjustable modified 1.5V battery), an ammeter (picoammeter, Keithley 480, Keithley Instruments) two electrodes which were the working electrode (chlorine microelectrode) and the reference electrode (saturated calomel electrode, type D, Cole-Parmer), electroactive species (hypochlorous acid and hypochlorite), and some nonactive species (phosphate buffer) at the applied potential (+0.2 V vs. SCE) in the solution.

The amperometric method measures the current flow in the electrochemical cell at a certain applied potential and then relates it to the concentration of electroactive specie(s). The current measured by an ammeter at a certain potential may include some non-faradaic current, which cannot be related to the concentration of concerned species, such as charging current, migration current, adsorption current, or catalytic current. Charging current comes from the formation of the double-layer at the electrode-solution interface from a polarized working electrode. Once present, it remains a constant for a stationary electrode and is small enough to be negligible. Migration current is caused by the electrostatic attraction between electrode and the oppositely charged ions. It can be eliminated by adding a large excess of a supporting electrolyte like phosphate buffer. Adsorption and catalytic current both come from the impurity of the sensing surface. The appearance of catalytic current can be avoided by coating the surface with an additional semipermeable layer to restrict the access of interfering species.

Suspended Biomass-Chlorine Reaction Kinetics

The kinetics of the reaction between chlorine and planktonic biomass were measured in batch experiments. The experimental setup is shown schematically in Fig. 1. For these experiments a chlorine microelectrode with a large tip diameter (about 40-50μm) was used and first stabilized with +0.2V applied potential in 100ml phosphate buffer for 20 minutes. Then a desired bulk chlorine concentration was obtained by adding a fresh known concentration (determined by DPD ferrous titrimetric method) stock solution. The chlorine concentration was recorded with time after a known amount of cell suspension (from tube D) or agarose (200mg/l) was added into the continuous stirred batch reactor.

Chlorine Profile Measurement in Artificial Biofilm

Chlorine concentration profiles were measured in artificial biofilms placed in a flow cell (Figure 2). Two carboys were used to provide the influent flow by a steady gravity feed. One had a phosphate buffer solution and the other a chlorine solution which was made by diluting sodium hypochlorite reagent (Clorox bleach, Clorox) with phosphate buffer. A flow cell was used as a reactor and soaked with chlorine solution prior to the experiment. The effluent was removed by suction. Two carboys were connected to the reactor via a 3 way valve that only allowed one designated solution to flow through reactor at one time, i.e. either buffer or chlorine solution. The volume of the flow (385ml/min to 410ml/min) was controlled by a valve and measured using a volumetric cylinder and timer.

Approximately 1μm accuracy of the microelectrode movement in the z-direction can be achieved by using a step motor (Stepper Mike 18503, Oriel) mounted on a micro-

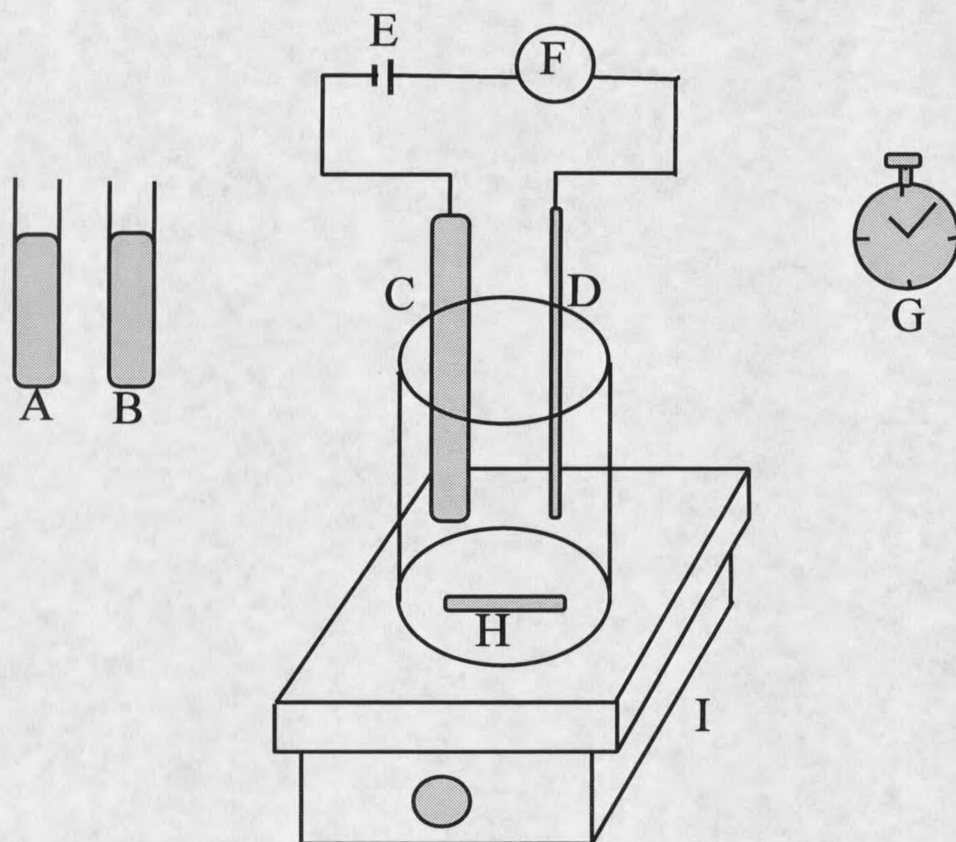

Figure 1. Apparatus for measurement of suspended biomass-chlorine reaction kinetics. A, chlorine stock solution; B, *Pseudomonas aeruginosa* cell stock solution; C, SCE reference electrode; D, chlorine microelectrode; E, modified battery; F, ammerter; G, stop watch; H, stirbar; I, stirrer.

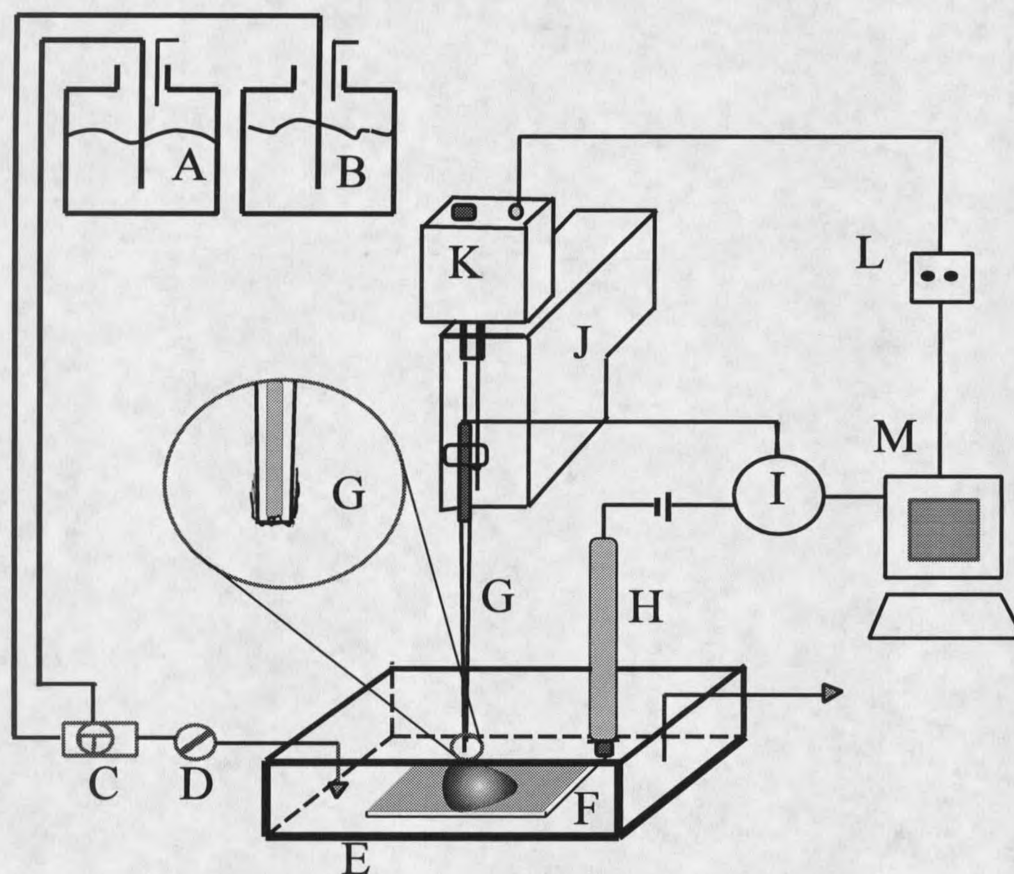

Figure 2. Apparatus for chlorine concentration profile measurement inside artificial biofilms. A, chlorine solution carboy; B, phosphate buffer solution carboy; C, three way valve; D, flow rate control valve; E, flow cell; F, stainless steel slide with artificial biofilm; G, chlorine microelectrode; H, SCE reference electrode; I, ammeter; J, micro-manipulator; K, step motor; L, step motor controller; M, computer for data acquisition and step motor automation.

manipulator (World Precision Instruments Ins.). The movement was automated by connecting the step motor controller (Oriel 20010, Oriel) to a computer with a software written by Dong Chen.

When a slide, either with or without agarose cell gel, was placed in the flow cell, a horizontal microscope with a magnification of 7 to 15 times and a pin-point illuminator were used to determine when the microelectrode reached the surface of the artificial biofilm. Each of the chlorine microelectrodes used in these experiments had a tip diameter less than 20μm.

Reaction Kinetics Data Analysis

Because the actual interaction between chlorine and biomass is expected to be complicated and involve multiple reactions, a lumped one step reaction approach was used to capture the overall behavior. A simple two constituent first order reaction kinetics was used to simulate the chlorine-biomass interactions. The reaction rate was assumed to be first order with respect to each of chlorine and biomass (cell or agarose) concentrations. Differential mass balance on chlorine and biomass are:

$$\frac{dC}{dt} = -KXC$$

$$\frac{dX}{dt} = -YKXC$$

where the variables are: C, concentration of chlorine; X, concentration of cell mass or agarose; t, time, and the parameters are K, reaction rate constant and Y, yield coefficient, defined as the ratio of the amount cell mass consumed to the amount of chlorine reacted.

The solution of this system of equations is:

$$C=\frac{X_o-YC_o}{-Y+\exp[\ln\frac{X_o}{C_o}+(X_o-YC_o)Kt]}\text{..........................(3)}$$

$$X=X_o+Y(C-C_o)\text{................................(4)}$$

where C_o and X_o are the initial values of C and X, respectively.

A nonlinear least squares method was used to get the regressions for each individual run. All chlorine concentration data from experiment were fit to follow equation (3). The Marquardt-Levenberg algorithm within SigmaPlot software (Jandel) was used to find these two values of the two parameters Y and K for each set data that give the best fit between the equation and the data.

Biofilm-Chlorine Interaction Model

The penetration of chlorine into a biofilm is amenable to description by reaction-diffusion theory. Inside the agarose-cell artificial biofilm, diffusion is the only mass transport process. Since reactions between chlorine and biomass occur, the interaction between reaction and mass transport can lead to the formation of a concentration gradient inside the biofilm. Because the dimension of the artificial biofilm in the z-direction was far smaller than in the x-direction, along which chlorine solution flowed, a one dimensional model with the substratum as the origin was used to simulate the chlorine concentration profile. Starting with a differential mass balance on chlorine and biomass, and assumptions of uniform biofilm density, constant bulk concentration of chlorine, no cell growth and no film

detachment, the model then can be written as:

$$\frac{\partial C}{\partial t}=D_e\frac{\partial^2 C}{\partial z^2}-K_1XC-K_2AC$$

$$\frac{\partial X}{\partial t}=-Y_1K_1XC$$

$$\frac{\partial A}{\partial t}=-Y_2K_2AC$$

with initial conditions:

$$X\,(z,0)=X_o,\quad A\,(z,0)=A_o$$

and boundary conditions:

$$\frac{\partial C}{\partial z}\Big|_{(0,t)}=0$$

$$D_e\frac{\partial C}{\partial z}\Big|_{(Lf,t)}=\frac{D_{aq}}{\delta}(C_o-C^*)$$

where the new variables are A, concentration of agarose, and z, the distance, and new parameters are: A_o, initial value of A; D_{aq}, chlorine diffusion coefficient in water; D_e, chlorine diffusion coefficient in gel; K_1, cell-chlorine reaction rate constant; K_2, agarose-chlorine reaction rate constant; Y_1, yield coefficient of cell-chlorine reaction; Y_2, yield coefficient of agarose-chlorine reaction; δ, thickness of the fictitious stagnant liquid layer from film theory.

RESULTS

This section presents experimental results including cell culturing, characterization of the chlorine microelectrode, suspended biomass-chlorine reaction kinetics, chlorine concentration profiles in artificial biofilms, and modeling results.

Microbial Culture

A rich medium was used to attain high cell concentration of approximately $5x10^9$ (cfu/ml) in the culture broth (Fig. 3). Cells were consistently harvested after 20hr when the culture was in the late exponential phase. After centrifuging and loading bacteria into the artificial biofilm, the cell density in the gel was approximately $1x10^{10}$ (cfu/ml).

Characteristics of the Chlorine Microelectrode

Several preliminary studies were conducted to characterize the chlorine microelectrode. The first of these was a chlorine sensitivity measurement, which is shown in Fig. 4. The signal current was linear with chlorine concentration.

This probe is quite sensitive to the solution pH, as shown in Fig. 5. The relative proportions of chlorine species, normally hypochlorous acid and hypochlorite ion, are pH sensitive. The percentage of these two species at different pH is calculated and plotted in Figure 6. Cl_2 was believed not existed in pH greater than 5.5. The chlorine probe may be sensitive to both species. Hypochlorous acid has a higher standard reduction potential (+1.49V) than hypochlorite ion (+0.90V). This probably explains why a higher signal was measured at low pH where the hypochlorous acid dominates. Another experiment was

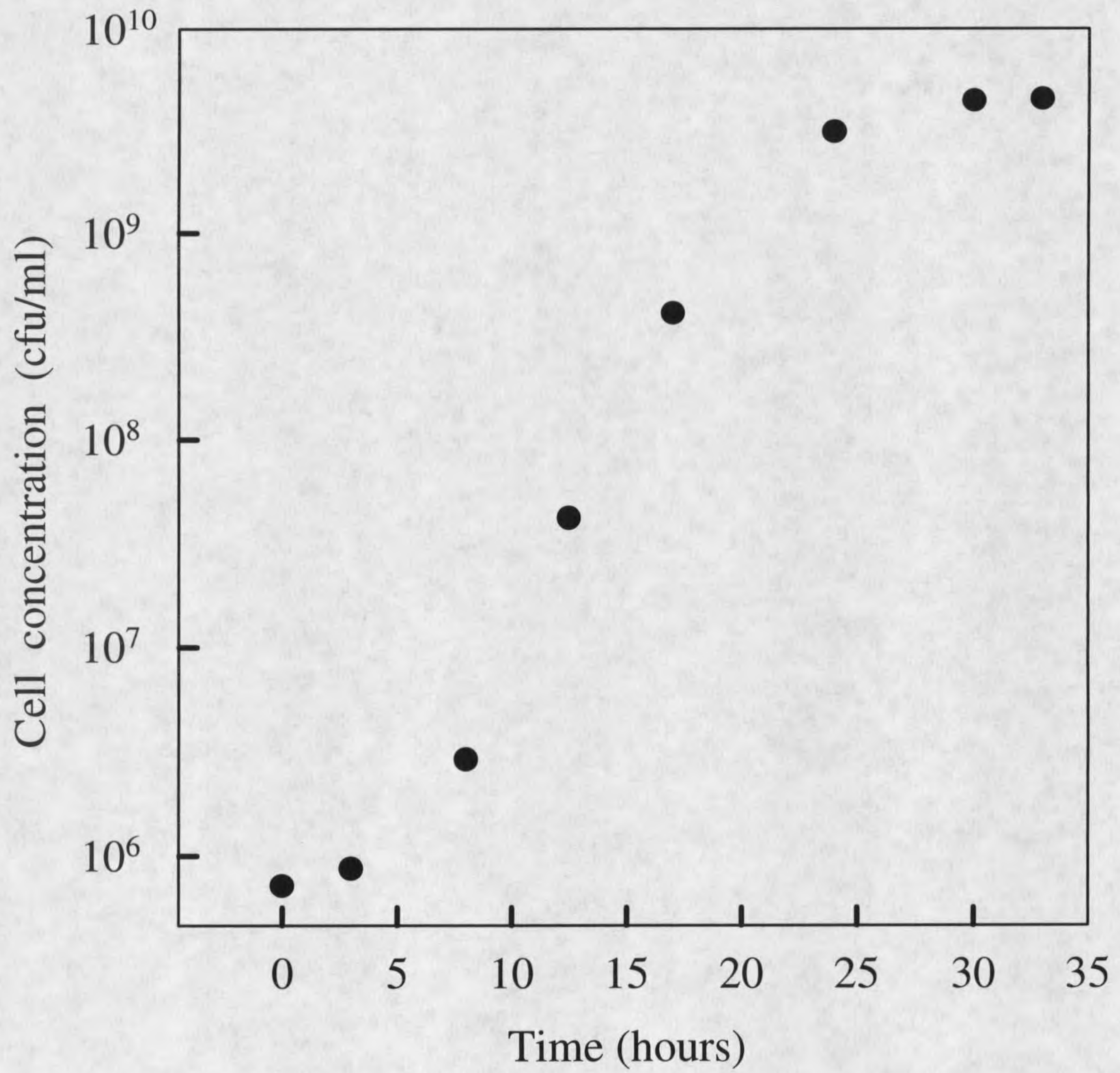

Figure 3. *Pseudomonas aeruginosa* ERC-1 growth curve.

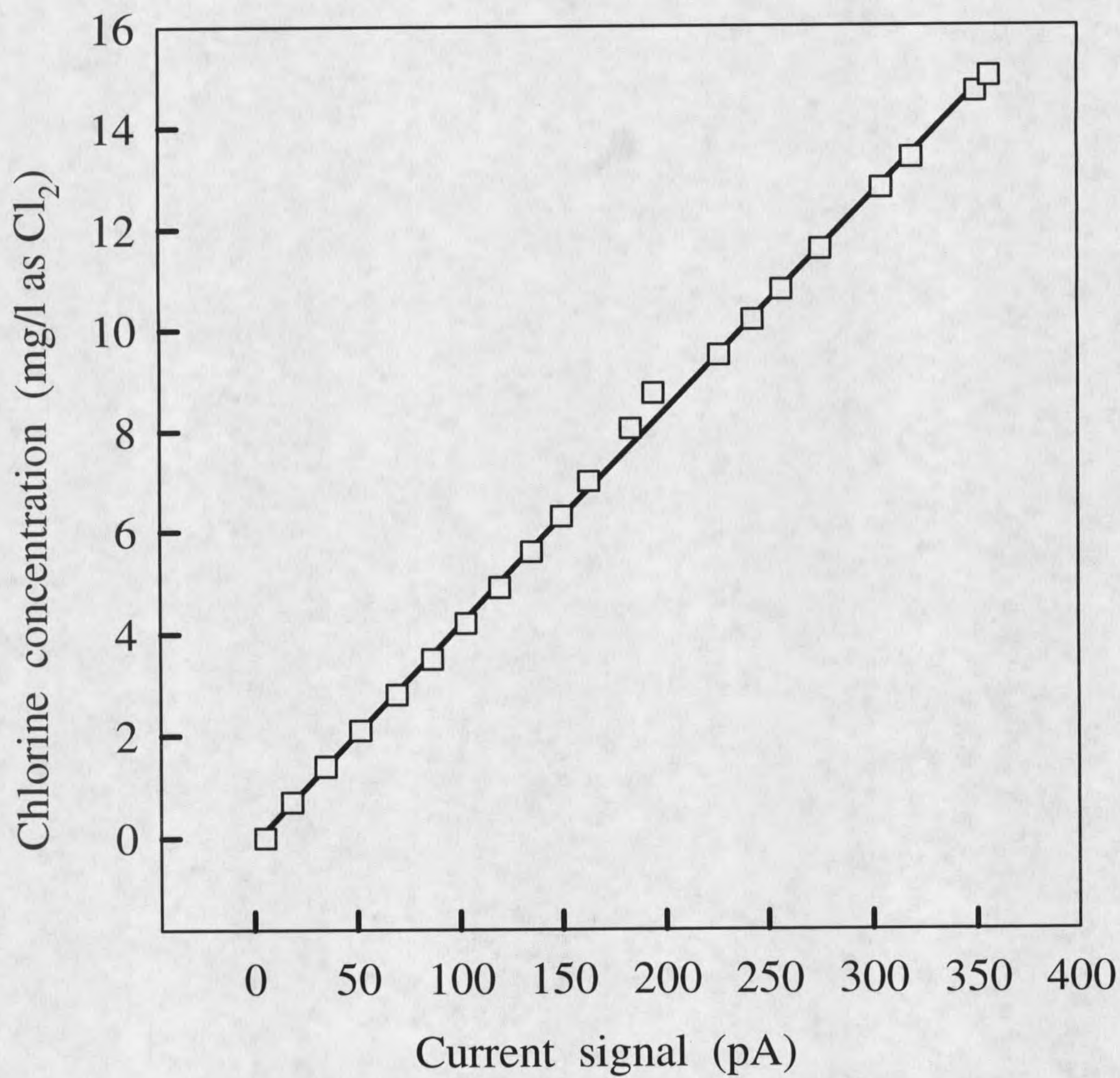

Figure 4. Calibration curve of chlorine microelectrode with tip diameter of 20μm.