



Rapid in situ physiological assessment of disinfection in bacterial biofilms
by Feipeng Philip Yu

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in
Microbiology

Montana State University

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Abstract:

This report describes the development of a rapid in situ enumeration approach with the adaptation of a direct viable count (DVC) method, utilization of fluorescent probes and a radioisotope labelling technique to assess the physiological responses of bacteria in biofilm disinfection studies without disturbing the integrity of the interfacial community. The experiments were performed in two stages using two model biofilm systems. In the first stage, an environmental isolate (*Klebsiella pneumoniae* Kp1) was used to form monolayer biofilms on stainless steel coupons in a stirred batch reactor. Fluorescent dyes, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and rhodamine 123 (Rh 123), were chosen to perform this study. CTC is an indicator of bacterial respiratory activity and Rh 123 is incorporated into bacteria in response to transmembrane potential. The intracellular accumulation of these fluorescent dyes can be determined using epifluorescence microscopy. The incorporation of uridine represents the global RNA turnover rate. Conventional enumeration methods, the plate count (PC) and DVC methods were also applied for comparison. Chlorine (0.25 mg/L, pH 7.2) and monochloramine (1 mg/L, pH 9.0) were used to determine the susceptibilities of attached and planktonic bacteria to disinfection. The attached cells did not reveal any more resistance to disinfection than planktonic cells in this system. The results indicated that viable cell densities within biofilms determined by the three in situ methods were comparable and always showed approximately 2-fold higher values than those obtained with the PC method. As an additional advantage, the results were observed within 4 h instead of the 24 h incubation time required for colony formation. The results acquired following disinfection exposure showed a range of responses with these methods and suggested different physiological responses in biofilms exposed to chlorine and monochloramine. The DVC response and respiratory activity were affected more by disinfection than the transmembrane potential and RNA turnover rate, on the basis of comparable efficiency evaluated by PC enumeration. Although being a weaker disinfectant, monochloramine was more effective in removing attached bacteria from the substratum than free chlorine. In the second stage, binary population biofilms of Kp1 and *Pseudomonas aeruginosa* grown on stainless steel surfaces in continuous flow annular reactors were applied. Biofilms were treated for 2 h with chlorine (1 mg/L) or monochloramine (4 mg/L) then stained with 0.05% CTC and counterstained with DAPI. Biofilms were cryoembedded and removed from the substratum followed by cryosectioning to 5 μ m frozen sections. Cross sections (xz) of the biofilm matrix were examined by an epifluorescence microscope equipped with a color CCD camera and quantitated by an image analysis system. Penetration of biocides into the biofilm matrix with time was indicated by a color change from red-orange CTC-formazan to green DAPI, which represented the loss of respiratory activity due to disinfection. The results showed higher removal and respiratory impairment were achieved by monochloramine treatment. Overall, our approach not only allowed the rapid efficacy assessment of disinfection but also provided more descriptive information on mechanisms of biocide action within biofilms than the conventional approach.

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A thesis submitted in partial fulfillment
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of

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MONTANA STATE UNIVERSITY
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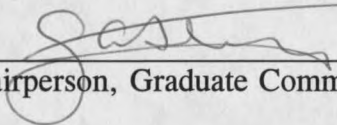
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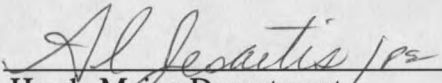
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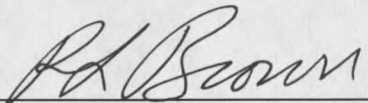
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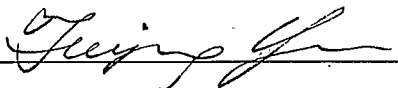
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This work is dedicated to my beloved late grandfather.

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ABSTRACT

This report describes the development of a rapid *in situ* enumeration approach with the adaptation of a direct viable count (DVC) method, utilization of fluorescent probes and a radioisotope labelling technique to assess the physiological responses of bacteria in biofilm disinfection studies without disturbing the integrity of the interfacial community. The experiments were performed in two stages using two model biofilm systems. In the first stage, an environmental isolate (*Klebsiella pneumoniae* Kp1) was used to form monolayer biofilms on stainless steel coupons in a stirred batch reactor. Fluorescent dyes, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and rhodamine 123 (Rh 123), were chosen to perform this study. CTC is an indicator of bacterial respiratory activity and Rh 123 is incorporated into bacteria in response to transmembrane potential. The intracellular accumulation of these fluorescent dyes can be determined using epifluorescence microscopy. The incorporation of uridine represents the global RNA turnover rate. Conventional enumeration methods, the plate count (PC) and DVC methods were also applied for comparison. Chlorine (0.25 mg/L, pH 7.2) and monochloramine (1 mg/L, pH 9.0) were used to determine the susceptibilities of attached and planktonic bacteria to disinfection. The attached cells did not reveal any more resistance to disinfection than planktonic cells in this system. The results indicated that viable cell densities within biofilms determined by the three *in situ* methods were comparable and always showed approximately 2-fold higher values than those obtained with the PC method. As an additional advantage, the results were observed within 4 h instead of the 24 h incubation time required for colony formation. The results acquired following disinfection exposure showed a range of responses with these methods and suggested different physiological responses in biofilms exposed to chlorine and monochloramine. The DVC response and respiratory activity were affected more by disinfection than the transmembrane potential and RNA turnover rate, on the basis of comparable efficiency evaluated by PC enumeration. Although being a weaker disinfectant, monochloramine was more effective in removing attached bacteria from the substratum than free chlorine. In the second stage, binary population biofilms of Kp1 and *Pseudomonas aeruginosa* grown on stainless steel surfaces in continuous flow annular reactors were applied. Biofilms were treated for 2 h with chlorine (1 mg/L) or monochloramine (4 mg/L) then stained with 0.05% CTC and counterstained with DAPI. Biofilms were cryoembedded and removed from the substratum followed by cryosectioning to 5 μm frozen sections. Cross sections (xz) of the biofilm matrix were examined by an epifluorescence microscope equipped with a color CCD camera and quantitated by an image analysis system. Penetration of biocides into the biofilm matrix with time was indicated by a color change from red-orange CTC-formazan to green DAPI, which represented the loss of respiratory activity due to disinfection. The results showed higher removal and respiratory impairment were achieved by monochloramine treatment. Overall, our approach not only allowed the rapid efficacy assessment of disinfection but also provided more descriptive information on mechanisms of biocide action within biofilms than the conventional approach.

CHAPTER 1

GENERAL INTRODUCTION

Biofilms are composed of microorganisms attached to an inert surface, termed the substratum. These form three dimensional communities within a matrix of extracellular polymers secreted by the bacteria. This structure traps dissolved organic macromolecules and particles providing sufficient nutrients for the microorganisms. During the process of biofilm formation, microorganisms colonize surfaces and proliferate, thus optimizing their survival in the system and permitting continual release of microbes into the bulk liquid (Characklis and Marshall, 1990; Blenkinsopp and Costerton, 1991; Bryers, 1993). The existence of biofilms was first recognized in soil (Söhngen, 1913), and then later studied in aquatic environments (Cholodny, 1930; Henrici, 1933; Zobell, 1943). Since then much progress has been made in understanding the ecology and physiology of adherent microorganisms (Characklis and Marshall, 1990; Lappin-Scott *et al.*, 1992). The adhesion event exerts a profound effect on bacteria, in that it alters their physiological processes (Costerton *et al.*, 1987; Davies and McFeters, 1988; Kölbel-Boelke and Hirsch, 1989), their surface structures (Costerton *et al.*, 1981; Costerton and Lappin-Scott, 1989; Rosenberg *et al.*, 1967; Wolfaardt and Cloete, 1992; Marshall, 1992) and their relationships to the bulk fluid (Wolfaardt and Cloete, 1992; Lau and Liu, 1993).

Both biofouling and biocorrosion are phenomena which are linked to the

existence, properties and activities of biofilms. Biofilm processes are manifested in many forms and are studied by researchers from a wide variety of disciplines. There are numerous industrial environments where corrosion and biofouling processes are potentially troublesome including cooling water systems, storage tanks, water and wastewater treatment facilities, filters, piping, and drinking water distribution systems (Flemming and Geesey, 1990). Control of biofilm problems has usually been attempted by the application of biocides in water systems. It was initially assumed in general that the disinfection kinetics of attached bacteria would be similar to their planktonic counterparts. Many industrial systems have experienced the inevitable problems caused by biofilms, even in the presence of an effective disinfectant residual. It is now recognized that strategies to control attached bacteria must be based on data generated from biofilm studies.

Numerous reports have shown that attachment of microorganisms to various surfaces is strongly related to increased disinfection resistance (LeChevallier *et al.*, 1981; Ridgway and Olson, 1982; LeChevallier *et al.*, 1984a; Camper *et al.*, 1986; Herson *et al.*, 1987; Costerton *et al.*, 1987; LeChevallier *et al.*, 1988; Brown and Gilbert, 1993). The microorganisms entrapped in particles or adsorbed to surfaces seem to be shielded from disinfection and become more resistant to antimicrobial agents than planktonic cells (Ridgway and Olson, 1982; LeChevallier *et al.*, 1984a; Camper *et al.*, 1986). This troublesome characteristic has caused difficulties for disinfection of biofilms in drinking water systems. It has been reported that the bacterial density in an aqueduct biofilm was as high as 10^4 CFU/cm² in the presence

of 1 to 2 mg of chlorine residual per liter (Nagy *et al.*, 1982). The dosages required to kill the microorganisms in drinking water distribution systems can be varied from 1 to 10 mg/L of free chlorine (Ridgway and Olson, 1982). *Klebsiella pneumoniae* has been recovered in a potable water supply one week after scrubbing redwood tank biofilms with a solution of 200 mg of chlorine per liter (Seidler *et al.*, 1977). A study with reverse osmosis membranes (Ridgway *et al.*, 1984) shows that 15 to 20 mg of chlorine per liter is required to control biofilm fouling of reverse osmosis membranes.

It has been recognized that environmental factors can change an organism's susceptibility to disinfection. Organisms grown in aquatic environments, whether source water or distribution water, are physiologically different than those cultured under laboratory conditions using rich nutrient media (Morita, 1985; Roszak and Colwell, 1987; Martin *et al.*, 1989; McFeters, 1990; Kjelleberg *et al.*, 1993). This phenomenon has led to the agreement that reduced disinfection susceptibility is caused by a variety of factors, such as penetration (LeChevallier *et al.*, 1990), physiology (LeChevallier *et al.*, 1984a; LeChevallier *et al.*, 1988; Pyle and McFeters, 1989; Pyle and McFeters, 1990a; Pyle and McFeters, 1990b; Pyle *et al.*, 1992; Olson and Stewart, 1990; Berman *et al.*, 1992; Cargill *et al.*, 1992; Jones *et al.*, 1992; Pyle *et al.*, 1994) and electrical properties (Matsunaga *et al.*, 1992; Blenkinsopp *et al.*, 1992).

The use of most established culture media often leads to serious underestimations of bacterial population density and the complexity of natural and

engineered systems. For example, this is explained by instances when known culture methods fail to detect autochthonous bacteria in certain environments (Ward *et al.*, 1992) and the reduced culturability (Roszak and Colwell, 1987), injury (McFeters, 1990) and dormancy (Kaprelyants and Kell, 1993b) of allochthonous bacteria exposed to sublethal stresses. Hence bacteria that sometimes are regarded as nonculturable in the literature might be more realistically considered as uncultivated (Ward *et al.*, 1992). Although these considerations present very real and practical constraints on the reliability of established media, the possibility of developing novel cultural approaches with appropriate nutrients for the detection of bacteria that have been affected by environmental exposure (Desmonts *et al.*, 1990; Amann *et al.*, 1992; Binnerup *et al.*, 1993) or sublethal injury (LeChevallier *et al.*, 1983; LeChevallier *et al.*, 1984b; Camper and McFeters, 1979; McFeters *et al.*, 1982; Singh and McFeters, 1986; Singh *et al.*, 1990) should be acknowledged.

Disinfection Kinetics

The most common model for inactivation of microorganisms by disinfectants has been derived from the work of Chick (Chick, 1908) and Watson (Watson, 1908). The "Chick-Watson law" is $\ln(N/N_0) = -kC^n t$, where N/N_0 is the ratio of the number of surviving organisms (N) at time t to initial cell numbers (N_0). C is the disinfectant concentration, and k and n are empirical constants (n is also called the coefficient of dilution). The Chick-Watson law is the basis for all other models which can be

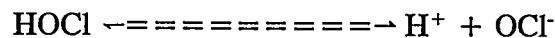
considered as derivations of this formula. The model implies that disinfectant concentration and contact time are the two key variables determining disinfection efficacy. The equation is based on the observation that inactivation of microorganisms generally follows first order kinetics (Hass and Karra, 1984a; Hass and Karra, 1984b). These concepts are so influential that current disinfection regulations of drinking water are based on a CxT concept.

One criticism of the kinetic model has been that much of the data used for development was based on laboratory studies of monodispersed microorganisms that do not reflect realistic conditions from operating systems. In a distribution system, the presence or absence of chlorine in the water seems to bear little relationship to the number of organisms that are present in a given sample (Ridgway and Olson, 1982). The observations of biphasic or nonlinear inactivation curves previously described by Moats (Moats, 1971) are based on findings reported in the disinfection literature. Theories to explain nonlinear inactivation curves are numerous but generally include resistant subspecies, aggregated organisms, multiplicity of cellular inactivation sites, and changes in the disinfectant. Even in laboratory studies, observations of these nonlinear curves are common.

Chlorination

Chlorine has been used commonly as a disinfectant in water treatment. When chlorine gas is dissolved in water, it hydrolyzes rapidly and forms hypochlorous acid

(HOCl). By a specially controlled process, using a caustic solution, this procedure produces a hypochlorite bleach. The bleach solution is used by diluting with water to form hypochlorous acid as a disinfectant. The most important reaction in the chlorination of aqueous solution is the formation of hypochlorous acid. This species of chlorine is the most bactericidal of all chlorine compounds with the possible exception of chlorine dioxide (White, 1992). HOCl is a weak acid, it tends to undergo a partial dissociation as follows:

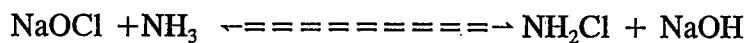


to produce a hypochlorite ion (OCl⁻) and a hydrogen ion. The dissociation of hypochlorous acid depends on pH, and the equilibrium between HOCl and OCl⁻ is maintained, even though HOCl is constantly consumed through its bactericidal function (Baker, 1959) and oxidizing ability. The amount of hypochlorite ion becomes appreciable above pH 6, while molecular chlorine is nonexistent. The percentage distribution of undissociated hypochlorous acid over various temperatures and pH values has been described (White, 1992). This study clearly shows that the ratio of OCl⁻ to HOCl increases as pH rises during the dissociation reaction. The oxidizing capacity of the HOCl is equal to two equivalents of chlorine or one mole of Cl₂. HOCl and OCl⁻ have very different bactericidal efficiencies. The OCl⁻ ion is a poor disinfectant because of its inability to diffuse through the cell envelope of microorganisms. It appears that the disinfecting efficiency of chlorine decreases with

an increase in pH, which is parallel to the concentration of undissociated hypochlorous acid. This indicates that HOCl must be far stronger in bactericidal action than OCl⁻, thus the pH is a determining factor during chlorine disinfection.

Chloramination

The monochloramine is produced in accordance with the following equation:



The observation that chloramines are slower to kill microorganisms than free available chlorine has been common knowledge for some time. They have been categorized as a poor disinfectant compared to free chlorine, as US Environmental Protection Agency (EPA) labelled chloramines a secondary disinfectant in 1978. For the same conditions of contact time, temperature and a pH range of 6-8, it will take at least 25 times more combined available chlorine than free chlorine to produce the same bactericidal efficiency. Furthermore, it can be assumed that if the chlorine to ammonia nitrogen ratio is less than 5 : 1 and if the pH is 7.5 and higher, the combined residual will probably be 100 percent monochloramine (NH₂Cl). The difference in potency of NH₂Cl and HOCl might be explained by the difference in their oxidation potentials, assuming that the action of chloramine is of an electrochemical nature rather than one of diffusion, as seems to be the case with

hypochlorous acid (White, 1992).

Recent studies have examined the comparative disinfection efficiency of free chlorine and monochloramine for controlling biofilm organisms in a model pipe system (LeChevallier *et al.*, 1990). The results show that a biofilm grown on iron pipes treated with free chlorine doses of 4 mg/L for two weeks did not show significant changes in viability. However, when the treatment time extended to two weeks, these biofilms exhibited more than a 3-log die-off (i.e. > 99.9%). It is believed that monochloramine, despite being a weaker disinfectant, is able to penetrate and inactivate bacteria within biofilms growing on a variety of surfaces (LeChevallier, 1990).

Methods for Studying Biofilm Disinfection

The conventional approach used to study biofilm disinfection has relied on scraping the biofilm from the substratum surface after disinfection followed by plate counting (PC) of the viable bacteria. Differences in physiological activity between attached and free-living bacteria (Fletcher, 1984) may explain the diverse susceptibilities and growth requirements after cells are removed from the substratum. In addition, enumeration of viable bacteria by plate count methods may not detect all viable cells, particularly those injured by environmental stress (Camper and McFeters, 1979; McFeters *et al.*, 1982; Roszak and Colwell, 1987), and detached bacteria that are aggregated may be problematic in the plate counting technique. As a consequence,

these approaches not only require 24 h incubation but often underestimate viable cell density in certain microbial communities (Morita, 1985; Brock, 1987; Wayne *et al.*, 1987).

Fluorescence Microscopy and Nucleic Acid Stains

Fluorescence microscopy is a technique whereby fluorescent substances are examined. Because fluorogenic compounds are widely available, fluorescence microscopy can be applied to a variety of studies (Rost, 1991). The use of incident or epi-illumination fluorescence microscopy is of particular utility in microbiology since objects may be viewed on opaque surfaces. This alternative design, referred as epifluorescence microscopy, has been widely used (Ploem, 1993).

Currently, direct microscopic enumeration after staining with acridine orange (AO) is one of the most commonly used methods in microbial ecology and environmental microbiology (Daley, 1982; van Es and Meyer-Reil, 1982). This fluorogenic dye, AO, has been used for over one hundred years and is applied as part of the acridine orange direct count (AODC) or the direct total microbial count method (American Public Health Association, 1992). It has been suggested that the reaction of bacteria with AO will allow the discrimination between physiologically active and dead cells. This hypothesis is based on the different AO complexes formed between single and double stranded nucleic acids resulting in red-orange and green fluorescence, respectively. However, a tempered confirmation study (McFeters *et al.*, 1991) indicates that investigators applying the AO staining reaction as an

indicator of physiological activity should understand the relevant variables and validate their conclusions using independent indices.

Other fluorescent nucleic acid stains have been applied by microbiologists to determine the "direct total count" in a range of circumstances. The DNA stain 4',6-diamidino-2-phenylindole (DAPI), for example, has been used in this approach (Porter and Feig, 1980; Hoff, 1988; Wolfaardt *et al.*, 1991; Robertson and Button, 1989; Swannell and Williamson, 1988). When excited at wavelength of 365 nm, the DNA-DAPI complex fluoresces a bright blue, while unbound DAPI and DAPI bound to non-DNA material may fluoresce a weak yellow. Bacteria can, therefore, be distinguished from the other particulate material. Additional fluorochromes including acriflavine, bisbenzimidazole (Bergstorm *et al.*, 1986), Hoechst 33258 (Paul, 1982; Paul and Myers, 1982; Ellenbroek and Cappenberg, 1991), Hoechst 33342 (Paul, 1982; Monger and Landry, 1993) and ethidium bromide (Roser, 1980; Swannell and Williamson, 1988) have been utilized in microbiological applications.

The Direct Viable Count (DVC) Method

This technique was first developed by Kogure (Kogure *et al.*, 1979; Kogure *et al.*, 1984), and it has given higher viable cells counts than the traditional PC (viable count) method in some studies (Liebert and Barkay, 1988; Singh *et al.*, 1989; Hoff, 1989). Accordingly, samples are incubated in a nutrient medium containing small quantities of nalidixic acid (NA) and yeast extract. NA, an inhibitor of DNA synthesis, prevents cell division in gram-negative bacteria while other synthetic

pathways like RNA and protein formation continue. In the presence of appropriate nutrients, this leads to the formation of elongated cells that are considered viable. The use of image analysis technology further improves the DVC method to determine viability and injury and provide accurate and quantitative data about cell morphology and cell number (Hoff, 1989; Singh *et al.*, 1990). However, minor modifications, such as the concentrations of the nalidixic acid used, have to be incorporated to optimize this method to different microorganisms (Singh *et al.*, 1990).

Other Fluorochromes for Different Physiological Properties

Cell biologists have extensively used indicators of membrane potential for nearly 20 years (Wu and Cohen, 1993; Loew, 1993). The use of rhodamine 123 (Rh 123), which has been employed extensively by cell biologists, has been reported in studies of bacteria (Matsuyama, 1984; Bercovier *et al.*, 1987) and more recently by microbiologists using flow cytometry (Kaprelyants and Kell, 1992; Diaper *et al.*, 1992; Morgan *et al.*, 1993) and spectrofluorocytometry (Resnick *et al.*, 1985). However, the gram-negative envelope is only slightly permeable to Rh 123 and a permeation pretreatment is used to obviate this problem (Diaper *et al.*, 1992). Other fluorogenic compounds, such as *n*-phenyl-1-naphthylamine (Cramer *et al.*, 1976), merocyanine (Kashket and Wilson, 1974) and dansyl galactoside (Schuldiner *et al.*, 1975), have also been proposed as general indicators of membrane energy level or energy linked parameters in bacteria and vesicles.

Fluorescein diacetate (FDA) has been suggested as a vital stain because it is

sensitive to both periplasmic and membrane-bound enzymes. FDA, which is uncharged and non-fluorescent is passively transported into cells and then deacetylated by nonspecific endogenous esterases to fluorescein which is charged and fluorescent. In viable cells, the membrane is permeable to the charged molecules which therefore accumulate intracellularly and can be detected by fluorescence microscopy (Soderstrom, 1979; Brunius, 1980; Jarnagin *et al.*, 1980; Chrzanowski *et al.*, 1984; Manafi *et al.*, 1991). However, organisms with leaky membranes do not retain fluorescein. This index of activity has been closely correlated with cellular ATP concentration and glucose-stimulated respiration rate in one report (Stubberfield and Shaw, 1990) while that relationship is not apparent in a study of bacteria in wastewater and activated sludge (Jørgensen *et al.*, 1992). It is possible that the low level of bacterial enzyme activity, when compared with those cells exhibiting electron transport activity in some systems, may be attributed to the low permeability of the FDA through the gram-negative envelope.

The study of bacterial respiratory activity through the reduction of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to iodonitrotetrazolium formazan (INT-formazan) via succinate dehydrogenase in the electron transport system has been reported in a wide range of ecological and environmental studies (Zimmermann *et al.*, 1978; Iturriaga, 1979; Tabor and Neihof, 1982; Herson and Baker, 1982; Bitton and Koopman, 1982; Dutton *et al.*, 1983; Bright and Fletcher, 1983a; Jeffrey and Paul, 1986a; Jeffrey and Paul, 1986b; Swannell and Williamson, 1988; King and Parker, 1988; Pérez-Rosas and Hazen,

1989; Muñiz *et al.*, 1989; Stubberfield and Shaw, 1990; Blenkinsopp and Lock, 1990; Dodd and Waite, 1992; Thom *et al.*, 1993). One important obstacle in using this method is that the INT-formazan cannot be visualized microscopically on opaque surfaces in the study bacterial biofilms, without removal of the cells. Although the combination of fluorescent-antibody (FA) and INT reduction has been applied successfully to study cellular activity (Baker and Mills, 1982), the tedious procedures of preparing and testing FA has limited this approach as a general application. Recently, another ditetrazolium salt, 5-cyano-2,3-ditoly tetrazolium chloride (CTC), has been applied successfully to study physiological activity within eukaryotic (Stellmach, 1984) and prokaryotic (Rodriguez *et al.*, 1992; Kaprelyants and Kell, 1993a; Schaule *et al.*, 1993) cells. It should be noted that, unlike INT, the reduction product of CTC is a fluorescent formazan, which enhances its sensitivity for detection.

Radioisotope Labelling Techniques

The measurement of radioisotope incorporation in bacteria has been used extensively to determine the bacterial activities in various ecosystems (Karl, 1979; Fuhrman and Azam, 1982; Bell and Albright, 1982; Bell *et al.*, 1983; Winn and Karl, 1984; Jeffrey and Paul, 1986b; Robarts *et al.*, 1986; Davis, 1989). The effect of chlorine on metabolite uptake in *E. coli* has been reported using ^{14}C -labeled glucose and algal protein hydrolysate (Camper and McFeters, 1979). RNA turnover rate is also a useful indicator of physiological activity since it has been used to

determine *in situ* growth rate and the response of bacteria to changes in environmental conditions (Karl *et al.*, 1981).

The determination of cellular activity in interfacial environments is important to our understanding of the ecology in aquatic ecosystems. Physiological deviation between attached and free-living bacteria has been studied using several different approaches, including amino acid assimilation, glucose utilization, and respiration (Bright and Fletcher, 1983b; Fletcher, 1986; Bell and Albright, 1982; Simon, 1985; Ludwicka *et al.*, 1985; Camper and McFeters, 1979). In many of these cases, attachment to surfaces appears to increase cellular activity, although it may have no effect or decrease activity. Much ambiguity exists regarding the physiology of bacteria within biofilms. It is necessary to determine the physiological heterogeneity within biofilms while studying disinfection of attached microorganisms.

Scanning Confocal Laser Microscopy (SCLM)

The structural analysis of biofilms has only recently been practical with the development of SCLM techniques. Extensive reviews of its application to biological materials have recently been published (Agard *et al.*, 1989; Brakenhoff *et al.*, 1988; Carlsson and Liljeborg, 1989; Carlsson *et al.*, 1989; Shotton, 1989; Shotton and White, 1989; Wilson, 1989). Its application in microbiology offers a non-destructive imaging of vertical (*xz*) sectioning of microbial biofilms (Lawrence *et al.*, 1991; Caldwell *et al.*, 1992a; Caldwell *et al.*, 1992b). However, the resolution of confocal images in the *xz* plane decreases when thicker biofilms are examined, and most

SCLM images have been obtained with translucent glass slides as the substrata. Therefore, some issues are unresolved and might be problematic when applying SCLM to studies of biofilms on opaque surfaces. In addition, the cost of installing the whole SCLM system is rather expensive and might thus limit its general application in scientific research.

Research Goal and Objectives

The main goal of this study is to characterize the physiological responses with time and in space of surface-associated bacteria after disinfectant treatment. A meaningful comparative analysis of attached and planktonic bacteria was performed using stainless steel as the substratum. The specific aims are as follows:

1. Determine the susceptibility of biofilm bacteria to antimicrobial agents with methods utilizing direct microscopic examination.
2. Develop and validate techniques utilizing different fluorogenic compounds which can reveal various cellular activities to assess the susceptibility of biofilm bacteria to antimicrobial agents.
3. Correlate bacterial susceptibility to disinfectants with their physiological activities and properties in a model system.
4. Develop techniques for visualization of spatial heterogeneity within bacterial biofilms grown in a continuous flow system.
5. Analyze quantitatively the physiological responses of biofilm bacteria to

biocides with image analysis technology.

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CHAPTER 2

A DIRECT VIABLE COUNT METHOD FOR THE ENUMERATION OF ATTACHED BACTERIA AND ASSESSMENT OF BIOFILM DISINFECTION

Introduction

The activities of surface-associated microorganisms, frequently called biofilms, were first studied in soil (Söhnngen, 1913), and then later by microbiologists who submerged slides in aquatic environments (Cholodny, 1930; Henrici, 1933; Zobell, 1943). Since then, progress has been made in understanding the ecology and physiology of adherent microorganisms (Characklis and Marshall, 1990). In addition, attachment of bacteria to surfaces has important practical implications since biofilms may result in a significant increase in disinfection (Tracy *et al.*, 1966; LeChevallier *et al.*, 1984; Olivieri *et al.*, 1985; LeChevallier *et al.*, 1988b; Costerton and Lappin-Scott, 1989) and heavy metal (Costerton *et al.*, 1981) resistance. Attachment to surfaces also changes the physiology of microorganisms in a variety of ways as reviewed by van Loosdrecht *et al.* (van Loosdrecht *et al.*, 1990), including substrate uptake (Bright and Fletcher, 1983; Davies and McFeters, 1988; Costerton and Lappin-Scott, 1989), and one report (McFeters *et al.*, 1990) indicated that sand-associated bacteria can adapt more quickly to and have a greater degradative activity for nitrilotriacetate (NTA).

Difficulties associated with studying surface-associated cells have hindered

work characterizing the activities of adherent bacteria, compared to the progress made with free-living bacteria. For instance, it is necessary to remove the cells from the substratum prior to the enumeration of viable bacteria attached to surfaces.

Differences in physiological activity between attached and free-living bacteria (Fletcher, 1984) may explain the diverse susceptibilities and growth requirements after cells being removed from the substratum. In addition, enumeration of viable bacteria by plate count (PC) methods may not detect all viable cells, particularly those injured by environmental stress (Camper and McFeters, 1979; McFeters *et al.*, 1982; Roszak and Colwell, 1987). Also, detached bacteria that are aggregated may be problematic in the plate counting technique.

Chlorine is the most widely used disinfectant for the chemical treatment of water. Monochloramine (NH_2Cl) penetrates biofilms and inactivates bacteria more effectively than hypochlorous acid (HOCl), hypochlorite (OCl^-) and chlorine dioxide (ClO_2) (LeChevallier *et al.*, 1988a). Therefore, we chose to use free chlorine (hypochlorous acid) and monochloramine as disinfectants in this study. Hypochlorous acid tends to undergo partial dissociation to produce a hypochlorite and a hydrogen ion, and the equilibrium is maintained mainly by the hydrogen ion concentration. Thus, pH is believed to have a strong influence on the antimicrobial activity of chlorine in solution (White, 1986). In water between pH 6.5 and 8.5, the reaction is incomplete and both species (HOCl and OCl^-) are present in varying ratios. Because the bactericidal activity of HOCl is believed to be far stronger than OCl^- (Baker, 1959), an increase in pH (lower $[\text{H}^+]$) substantially decreases the biocidal activity of

chlorine with the formation of higher OCl^- concentrations, while lower pH values increase the biocidal activity. Thus, we selected two pH conditions (6.0 and 7.2) in this study to determine if higher activity for biofilm disinfection would be found at a lower pH.

This study was initiated to seek an *in situ* method to quickly enumerate viable attached bacteria and assess the effect of bactericides on surface-associated bacterial cells without disrupting the integrity of biofilms. This was based on our previous studies (Singh *et al.*, 1989), which applied image analysis and the conventional direct viable count (c-DVC) method to obtain a rapid enumeration of viable planktonic bacteria, as well as a report by Lytle *et al.* (Lytle *et al.*, 1989) who used a DVC method to examine bacteria in a gravel filter. We adapted the c-DVC method (Kogure *et al.*, 1979) to examine the susceptibilities of attached bacteria after disinfection. This novel approach offers a non-destructive means to study the biofilm community *in situ*. An environmental isolate (*Klebsiella pneumoniae* Kp1) was used to form biofilms on stainless steel coupons, which were limited to monolayers in order to be compatible with enumeration using epifluorescence microscopy. The disinfectants were used as tools to evaluate the efficiencies of the three enumeration methods. The *in situ* DVC method showed higher viable cell counts than the PC and c-DVC methods without the bias caused by aggregated bacteria. As an additional advantage, it took 4 h to complete the enumeration process in contrast to the 24 h period required for plate counting.

Materials and Methods

Biofilm Apparatus

The vessel used to grow bacterial biofilms was a wide-mouth pint Mason jar (Kerr, 450 ml), which was redesigned for this application. This device is described elsewhere (Cargill *et al.*, 1992). The jar containing stainless steel coupons, inoculated culture and a magnetic stir bar was incubated on a thermally insulated magnetic stirrer with a constant speed (285 rpm).

Bacterial Strains and Growth Conditions

Klebsiella pneumonia Kp1, isolated from drinking water, was obtained from Dr. D. Smith, South Central Connecticut Water Authority, New Haven, CT. The culture was stored in a solution that consisted of 2% peptone (Difco) and 20% glycerol (Sigma Chemical Company) in reagent grade water at -70°C.

Frozen cultures were inoculated into 1/10 trypticase soy broth (TSB) (Difco) and incubated at 35°C for 24 h. Cultures were then inoculated (1:100) into sterile medium and incubated for another 24h, followed by dilution in the biofilm apparatus with sterile 1/10 TSB at a final cell density of ca. 10^2 CFU/ml. The diluted cultures were stirred at room temperature (25°C) for various time intervals to allow accumulation of cells on coupon surfaces. Biofilms were formed on coupons (12x76 mm²) of 316 stainless steel.

Procedures for Coupon Cleaning

A cleaning method was used that yielded coupons with the same surface characteristics after being reused. Coupons were dipped into acetone to remove grease and allowed to air dry, then transferred to fresh RBS 35 (Pierce) working solution and heated to 50°C for 5 min and sonicated (Sonogen Automatic Cleaner, Branson Instruments Inc.) for 5 min. The coupons were then rinsed with sterile reagent grade water (Milli-Q, Millipore Corp.) until foaming ceased (approximately 5 times), sonicated again for 5 min then rinsed three more times. Coupons were transferred to a vial containing 30 ml of reagent grade water, sealed and autoclaved for 20 min then stored at room temperature in the same sealed vial until used.

Preparation of Disinfectants

The disinfectants used were chlorine (sodium hypochlorite) and monochloramine. The chlorine solution was prepared as described earlier (Singh *et al.*, 1986) using sodium hypochlorite, and the monochloramine solution was made according to LeChevallier *et al.* (LeChevallier *et al.*, 1988a). The bacterial cells were exposed to disinfectants in chlorine-demand-free sterile phosphate buffered water (PBW) (American Public Health Association, 1992) without $MgCl_2 \cdot 6H_2O$ (Pyle and McFeters, 1989) at different pH values for 10 min at 25°C.

Disinfection

The planktonic cells within the reactor were diluted and transferred to filtered

PBW (final concentration 10^5 - 10^6 CFU/ml) in an acid-washed bottle, then treated with disinfectants (0.25 mg/L hypochlorous acid and 1 mg/L monochloramine) at 25°C. The dilution process reduced the chlorine demand caused by medium carryover. Samples were removed after 5 and 10 min and the disinfectant was neutralized with sodium thiosulfate (final concentration 0.01%). Surviving bacteria were enumerated by the PC and DVC methods (Singh *et al.*, 1990).

Bacteria attached on the metal coupons were rinsed by transferring coupons to another biofilm apparatus filled with sterile distilled water to remove loosely attached cells. Biofilms were disinfected in chlorine-demand-free sterile PBW at 25°C in an acid-washed bottle mixed with a magnetic stir bar. The procedures for disinfection were similar to those described above. Biofilm bacteria on coupons were either removed from the coupons by scraping with a sterile rubber policeman or processed directly with *in situ* DVC incubation (described below) for enumeration.

The disinfection exposure was performed at different pH values in PBW (6.0 and 7.2 for chlorine and 9.0 for monochloramine). Tests were also done to determine the effect of pH on cell viability in PBW before disinfectants were added. The Hach DPD free chlorine and total chlorine test kit (Hach Co. Loveland, CO.) was used to measure the concentrations of residual chlorine before and after disinfection.

Optimization of the Direct Viable Count Method

The conventional direct viable count (c-DVC) (Kogure *et al.*, 1979) method was optimized as described by Singh *et al.* (Singh *et al.*, 1989). The optimal

concentrations of nalidixic acid (Sigma) used for *Klebsiella pneumoniae* strain Kp1 were 15 $\mu\text{g/ml}$ for untreated cells and 12 $\mu\text{g/ml}$ for disinfectant-treated cells. The planktonic cells in suspension and the attached cells on the coupons did not show any difference in the concentrations of nalidixic acid required for optimal elongation without cell division.

The removal of attached cells from coupons with a sterile rubber policeman had a 99.9% efficiency based on examination of the scraped and unscraped coupons stained with acridine orange under the epifluorescence microscope.

Determination of Viable Bacteria

The PC count method was performed by a modified drop plate method (Miles and Misra, 1938) using five 10 μl drops per dilution with tryptone lactose yeast extract agar medium (TLY) as described by McFeters *et al.* (McFeters *et al.*, 1982). Colonies were counted after 24 h incubation at 35°C.

After disinfection treatment, coupons were placed flat in a Petri dish containing the medium used for c-DVC incubation, which prevented further sloughing of attached cells during the 4 h incubation. The c-DVC method was performed using phosphate buffered saline (PBS) containing 0.3% casamino acids (Difco), 0.03% yeast extract (Difco) and different concentrations of nalidixic acid to incubate attached bacteria scraped from the coupons and planktonic cells at 35°C for 4 h. Samples were then withdrawn, fixed with formalin (5%, final concentration) and stained with acridine orange according to the method described by Hobbie *et al.* (Hobbie *et al.*,

1977). Before staining, the bacteria in suspension were filtered onto 25-mm diameter (0.2- μm pore size), black polycarbonate membranes (Nuclepore).

The *in situ* DVC method was similar to the c-DVC method. Coupons with attached cells were transferred into medium without scraping. After incubation, coupons were withdrawn and fixed with formalin followed by immersing in 0.02% acridine orange solution for 2 min. The coupons were then allowed to air-dry, placed on glass slides and examined using epifluorescence microscopy (Leitz Ortholux II). Both total and elongated cells in 10 microscopic fields were counted. If the total cell number was less than 400, more than 10 fields were examined.

Statistical Analysis

Statistical analyses were performed on all data from three replicates using mathematical functions within SigmaPlot™ (Version 4.1 by Jandel Scientific) and one-way analysis of variances (ANOVA) with InStat™ (Version 1.1 by GraphPAD) computer software.

Results

Enumeration of Surface-Associated Bacteria with Disinfection

To enumerate viable biofilm bacteria, the *in situ* DVC assessment was used and compared with approaches where surface-associated cells were removed from the substratum followed by PC and c-DVC enumeration. Different concentrations of

disinfectants (0.25 mg/L for free chlorine and 1 mg/L for monochloramine) were used to treat Kp1 biofilms grown on stainless steel coupons. Decreases in viable cell numbers were measured after 5 and 10 minutes exposure to disinfectants in order to describe the patterns of disinfection. Both PC and c-DVC methods were used to enumerate surviving cells after scraping attached bacteria from coupon surfaces. Results obtained with these two approaches were used to evaluate the efficiency of the non-destructive *in situ* DVC method. The viable cell densities are expressed as \log_{10} cfu per mm^2 (on coupons) \pm S.E. for the PC method, and \log_{10} cells per $\text{mm}^2 \pm$ S.E. for the c-DVC and *in situ* DVC methods. Comparisons of disinfection efficiency were determined by $\log N/N_0$ values (the log ratio of surviving bacterial numbers to initial bacterial numbers) after biocide exposure for 5 and 10 minutes.

Figure 1 shows that the viable cell numbers determined by three different methods at time 0 were similar. The counts were 4.22 ± 0.08 (*in situ* DVC), 4.03 ± 0.02 (c-DVC) and 3.98 ± 0.02 (PC) logs respectively. After 10 minutes of chlorine disinfection, the viability of attached cells on coupons decreased by 2.4 logs when determined by the *in situ* DVC assessment. A slightly larger reduction (2.6 logs) was observed when the attached cells were scraped off then enumerated with the c-DVC method. However, the conventional procedure of scraping followed by PC enumeration showed a much larger (3.3 logs) decline in bacterial viability within the disinfected biofilms. These data reveal a large portion of the biofilm bacteria that were not enumerated by the PC method since there was a 1.2 log discrepancy (0.65 ± 0.56 vs. 1.88 ± 0.11) in bacterial enumeration between the PC and *in situ* DVC

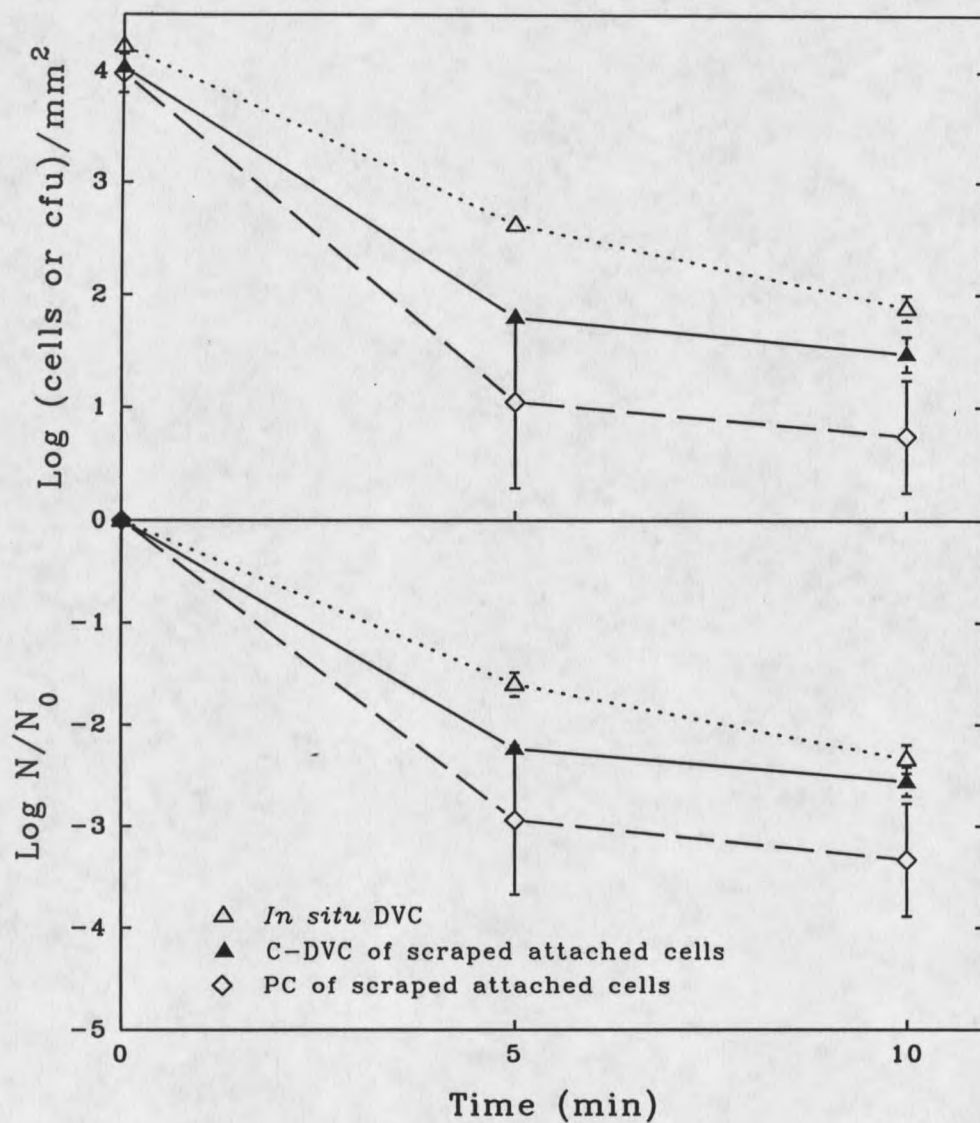


Figure 1. Effect of chlorine treatment (0.25 mg/L) on *Klebsiella pneumoniae* Kp1 biofilms at pH 7.2. Surviving bacteria were enumerated by three different methods. ($n = 3$, bars = S.E.)

methods after 10 minutes of chlorine disinfection, whereas the difference at time 0 (3.98 vs. 4.22) was not significant ($p > 0.05$). Furthermore, comparing the viable counts determined with the PC and c-DVC methods after removing attached bacteria by scraping, there was still a 0.82 log difference (0.65 ± 0.56 vs. 1.47 ± 0.16) which implied scraping was not the sole factor influencing the enumeration efficiencies. However, the differences between these three enumeration methods are statistically significant ($p < 0.05$).

The results of a similar experiment where bacterial biofilms were treated with monochloramine are shown in Figure 2. The initial viable cell densities on the coupons were 4.16 ± 0.10 (*in situ* DVC), 3.80 ± 0.01 (c-DVC) and 3.51 ± 0.15 (PC) logs respectively. Following 10 minutes of disinfection, a 1.8-log decrease was observed by the *in situ* DVC assessment. With c-DVC and PC methods, lower counts that were similar (1.14 ± 0.08 and 1.13 ± 0.48) were observed following enumeration of attached cells after scraping. Nevertheless, statistical analyses showed that these three methods used for enumeration following monochloramine treatment gave significantly different results after 10 minutes of disinfectant exposure ($p < 0.05$).

Utilizing the *in situ* DVC method, we were able to enumerate the remaining surface-associated cells following disinfection. The data (Figure 3) indicate that monochloramine removed approximately three times more attached bacteria from the substratum than free chlorine.

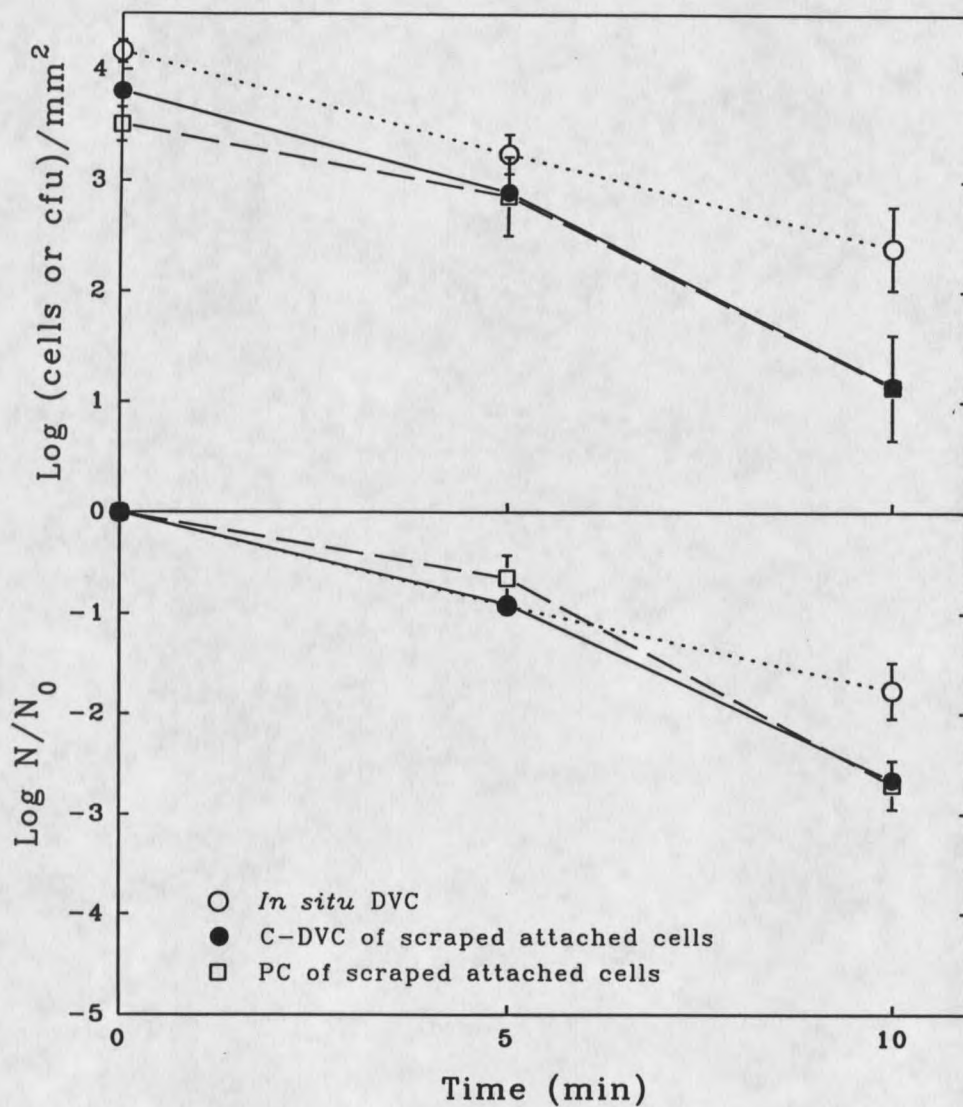


Figure 2. Effect of monochloramine treatment (1 mg/L) on *Klebsiella pneumoniae* Kp1 biofilms at pH 9.0. Surviving bacteria were enumerated by three different methods. ($n = 3$, bars = S.E.)

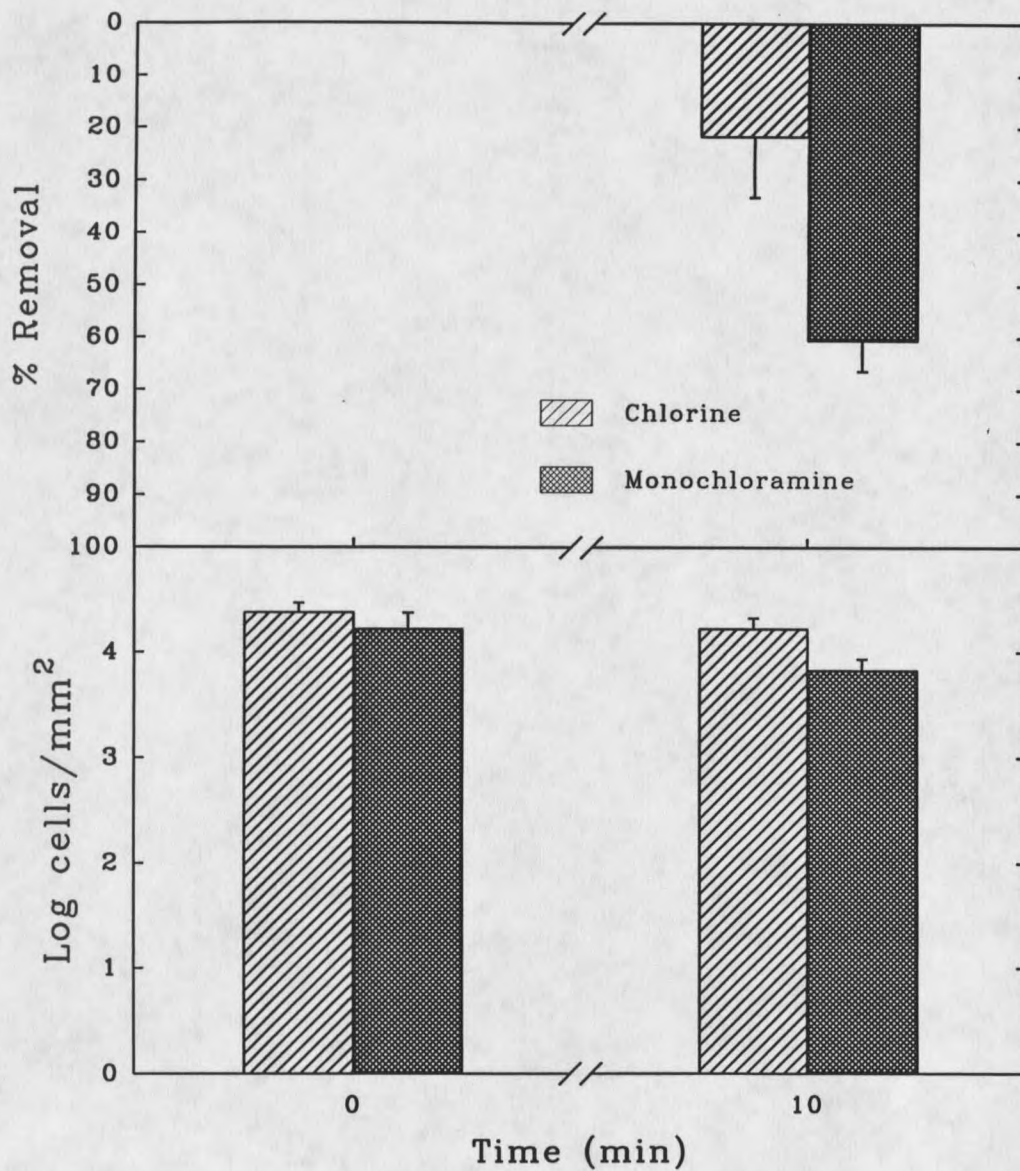


Figure 3. Removal of attached *Klebsiella pneumoniae* Kp1 cells from substratum after 10 minutes treatment with disinfectants. The total cell numbers are displayed on the lower panel. ($n = 3$, bars = S.E.)

Enumeration of Planktonic Cells with Disinfection

The PC and c-DVC methods were also used to assess the viability of planktonic bacteria following disinfection. Prior to incubation, the \log_{10} counts/ml of planktonic cells enumerated with c-DVC and PC methods were 5.54 ± 0.06 and 5.08 ± 0.12 respectively, and they were inactivated 2.29 ± 0.17 and 3.09 ± 0.24 logs after 10 minutes exposure to free chlorine (Figure 4). During the period of biofilm formation in the reactor, the culture medium contained planktonic plus aggregated cells detached from the coupons, which were observed microscopically. Following 43 h incubation in the biofilm apparatus, the \log_{10} counts/ml of planktonic cells were 6.11 ± 0.11 and 6.08 ± 0.04 , with inactivation of 2.11 ± 0.24 and 3.63 ± 0.05 logs when enumerated with c-DVC and PC methods under the same conditions. As expected, the viable cell numbers determined by the c-DVC method were significantly higher than those by the PC method ($p < 0.05$), but not by the three-orders of magnitude as previously reported (Kogure *et al.*, 1979). This population did not show any statistical difference in susceptibility to chlorine treatment ($p > 0.05$) when enumerated either by the c-DVC or PC methods.

For the monochloramine treatments, the \log_{10} counts/ml of planktonic cells were 5.47 ± 0.04 (c-DVC) and 5.12 ± 0.05 (PC) before attachment (Figure 5). Following 10 minutes exposure to 1 mg/L monochloramine at pH 9.0, planktonic bacteria were inactivated 1.74 ± 0.16 (c-DVC) and 2.84 ± 0.41 logs (PC). After biofilm attachment, the total numbers (\log_{10} counts/ml) were 5.97 ± 0.01 (c-DVC) and 6.05 logs (PC) respectively. The disinfectant reduced the viable cell numbers

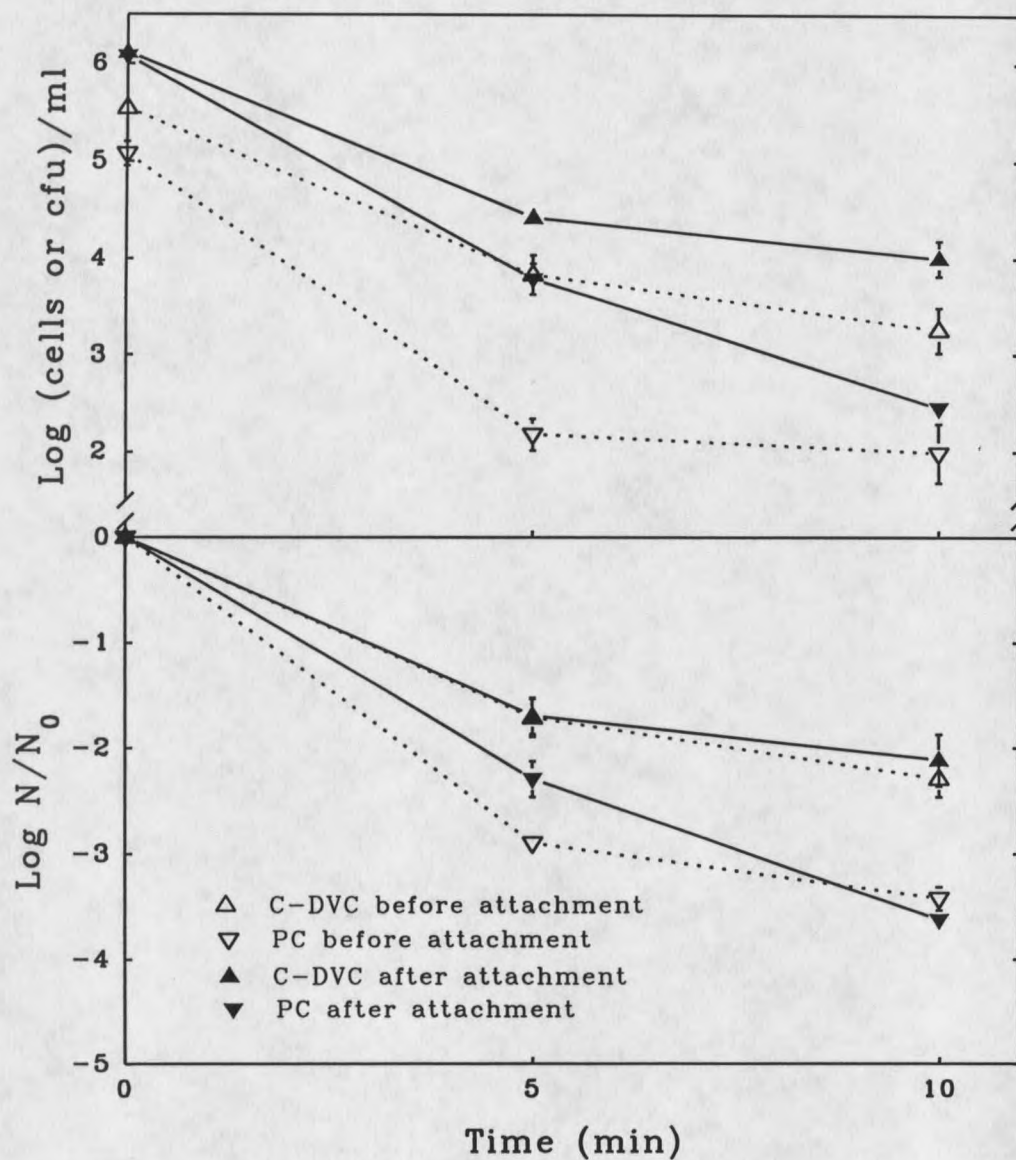


Figure 4. Enumeration and survival of planktonic *Klebsiella pneumoniae* Kp1 before and after attachment when treated with chlorine (0.25 mg/liter) at pH 7.2. ($n = 3$, bars = S.E.)

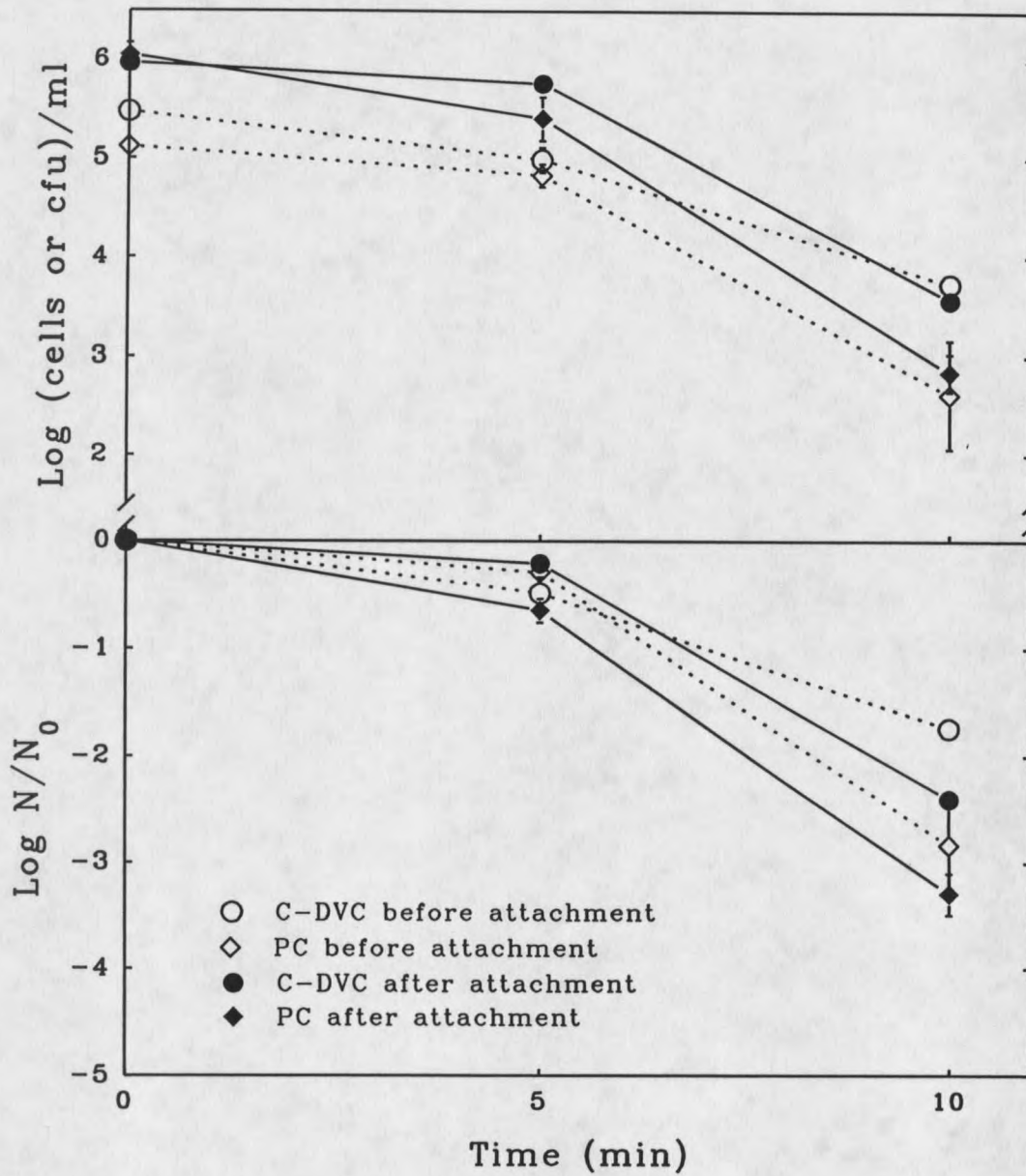


Figure 5. Enumeration and survival of planktonic *Klebsiella pneumoniae* Kp1 before and after attachment when treated with monochloramine (1 mg/liter) at pH 9.0. ($n = 3$, bars = S.E.)

by 2.40 ± 0.11 (c-DVC) and 3.21 ± 0.22 logs (PC). Statistical analyses indicated that planktonic cells became slightly more susceptible to monochloramine disinfection during the process of biofilm attachment when enumerated by the c-DVC method ($p < 0.05$). However, that difference was not apparent when the cells were enumerated by the PC method ($p > 0.05$).

Effect of pH on Chlorine Disinfection

The pH of PBW (see Materials and Methods) was varied to study activity of chlorine disinfection under different conditions. The attached and planktonic cells were examined using different procedures (Table 1). Most of the results showed that

Table 1. One-way analysis of variance (ANOVA) of the effect of pH on chlorine disinfection (0.25 mg/L for 10 min) of *Klebsiella pneumoniae* Kp1. ($n=3$, \pm S.E.)

Procedure ^a	Log N/N_0		ANOVA
	pH 6.0	pH 7.2	
#1	-2.10 ± 0.33	-2.56 ± 0.14	P = 0.2687
#2	-2.04 ± 0.36	-3.80 ± 0.52	P = 0.0497
#3	-2.30 ± 0.44	-2.29 ± 0.17	P > 0.8 ^b
#4	-3.79 ± 0.51	-3.09 ± 0.24	P = 0.2821

^a Procedure #1: DVC method with scraped attached cells.
 Procedure #2: PC method with scraped attached cells.
 Procedure #3: DVC method with planktonic cells before attachment.
 Procedure #4: PC method with planktonic cells before attachment.

^b The exact p-value was not given by the InStat program.

at 25°C, the bactericidal activity of chlorine under pH 7.2 and pH 6.0 was comparable ($p > 0.05$). The exception was from procedure #2 which indicated higher bactericidal activity at pH 7.2 when attached cells were enumerated with the PC method.

Tests were also done to determine the effect of pH on detachment and bacterial viability in the absence of disinfectant (data not shown). No significant changes were observed ($p > 0.05$) in total cell numbers and viable cell numbers which determined by the *in situ* DVC method.

Discussion

Without environmental stresses such as disinfectants, the enumeration results of bacterial biofilms obtained using the three methods that were studied (*in situ* DVC, c-DVC and PC) were comparable. However, differences between the methods became apparent after the introduction of disinfectant, as illustrated in Figures 1 & 2. The *in situ* DVC enumeration up to the 10 min exposure showed higher bacterial densities than those determined with either the c-DVC or the PC methods after removal of attached bacteria from coupon surfaces. Furthermore, the large error bars (Figure 1) obtained with the PC method after chlorine treatment implied aggregation of scraped bacteria may have caused greater fluctuations in colony forming units. That conclusion was supported by microscopic observations. The significant difference in viable cell numbers determined by the c-DVC and the PC methods after

the removal of attached bacteria from the coupons was only observed following chlorine and not monochloramine treatment. This may be explained by the suggestion that monochloramine is more effective in dispersing aggregated cells (Dychdala, 1991).

Our findings with the DVC analytical approach might be used to assess the physiological status of the bacteria under conditions of biofilm formation. An examination of Table 2 as well as a comparison of the lower panels in Figures 1 and

Table 2. Comparison of $\log N/N_0$ values between planktonic and attached cells after 10 minutes disinfection obtained with two DVC methods. ($n=3$, \pm S.E.)

		Log N/N_0	
		Chlorine (0.25 mg/L)	Monochloramine (1 mg/L)
Planktonic cells	c-DVC ^a (before attachment)	-2.29 \pm 0.17	-1.74 \pm 0.08
	(after attachment)	-2.11 \pm 0.24	-2.41 \pm 0.10
Attached cells	<i>in situ</i> DVC	-2.40 \pm 0.12	-1.66 \pm 0.13
	c-DVC	-2.56 \pm 0.14	-2.34 \pm 0.15

^a Conventional direct viable count method.

4 indicate that the attachment of *K. pneumoniae* to stainless steel coupons does not markedly alter their susceptibility to free chlorine when either of the DVC enumeration methods were used. This suggests that bacterial attachment in early biofilm formation did not result in a significant change in cellular physiology relating to chlorine susceptibility although a slight increase in susceptibility to monochloramine was seen (Table 2, lower panels of Figures 2 and 5). These results are in

contrast to the findings of others showing that attached bacteria are more resistant to disinfection (Tracy *et al.*, 1966; LeChevallier *et al.*, 1984; Olivieri *et al.*, 1985; LeChevallier *et al.*, 1988b; Pyle and McFeters, 1990), but those workers either did not compare phenotypic responses before and after attachment as we have done, or used different experimental circumstances (high level of nutrients, longer incubation time, unencapsulated bacterium and glass microscopic slides as substratum) as reported by LeChevallier *et al.* (LeChevallier *et al.*, 1988b). It should be noted however that biofilm formation was limited to bacterial monolayers in this study and it is likely that more mature biofilm communities respond differently. Such altered properties of surface-associated bacteria have been attributed to both changes in cellular physiology with biofilm formation (Fletcher, 1984; Costerton *et al.*, 1987) and protection by extracellular polymers (LeChevallier *et al.*, 1988b).

Results of similar experiments using planktonic bacteria provided a perspective for describing the kinetics of disinfection in biofilms. During the biofilm colonization process in the reactor, surface-associated bacteria detached from the substratum and became a part of the planktonic cell population which were observed microscopically as cell aggregates. Therefore, these formerly surface-associated bacteria, which are more resistant to disinfection (Tracy *et al.*, 1966; LeChevallier *et al.*, 1988b; Olivieri *et al.*, 1985), could have influenced the susceptibility of the planktonic populations in the reactor to disinfection.

There were no obvious changes in the susceptibility of planktonic bacteria to free chlorine following biofilm formation (Table 2 and lower panel of Figure 4).

However, there was a slight increase in the susceptibility of the planktonic cells to monochloramine with biofilm formation (Table 2 and lower panel of Figure 5).

These observations might also be indicative of a lack of a significant physiological change with biofilm initiation by *K. pneumoniae*, although it is important to note that the experimental design used here with cellular monolayers may have underestimated any physiological responses in the bacteria as they attached to the substratum.

From the shapes of the inactivation curves of chlorine disinfection over time, we repeatedly found that disinfection was more rapid for the first 5 min (Figures 1 and 4). With monochloramine, it appeared that there was less rapid disinfection for the first 5 min followed by a period of more rapid disinfection (Figures 2 and 5). These different reaction kinetics might result from distinct reaction mechanisms with the two disinfectants used, as suggested by other investigators (Kreft *et al.*, 1985; Haas and Karra, 1984; Jacangelo and Olivieri, 1985).

The disinfecting efficiency of chlorine is considered to be directly related to the concentration of undissociated hypochlorous acid, which is greater at pH 6.0 (97.18%) than at pH 7.2 (68.52%) at 25°C (White, 1986). Aqueous solutions of chlorine exhibits rapid bactericidal action by the formation of hypochlorous acid (White, 1986). Hypochlorous acid tends to undergo a partial dissociation to produce hydrogen and hypochlorite (OCl^-) ions in water at pH values between 6.5 and 8.5. HOCl is far stronger in bactericidal action than OCl^- , thus, the disinfecting efficiency of chlorine theoretically decreases with increasing pH values (Dychdala, 1991). However, results in Table 1 indicated that pH was of negligible effect on the

bactericidal activity of chlorine disinfection with the possible exception of one procedure (#2). In addition to pH, numerous other environmental factors, alone or in combination, determine the antimicrobial action of chlorine compounds (Dychdala, 1991).

The relationship between disinfectant concentration (C) and contact time (t) might appear simple in bacterial disinfection. Nevertheless, the empirical $C^n \times t$ equation (Chick, 1908) does not adequately predict the exponential rate of disinfection. Higher concentrations are not always more effective. Differences in antimicrobial resistance between species, levels of aggregation, physiological status, and prior growth conditions are all factors that affect the outcome. In addition, the properties of biofilms vary with environmental factors (e.g. population distribution in the film, nutrient loading rate, shear stress, etc.) and physiological properties of the bacteria. Therefore, the conclusions of this study apply only to the model system used.

The DVC method (Kogure *et al.*, 1979) distinguished viable cells by their ability to elongate while cell replication is inhibited by nalidixic acid. Although the *in situ* DVC method has limits in assessing the viability of bacterial biofilms due to certain constraints imposed by optical microscopy, this approach provided rapid and accurate enumeration of viable cells without disturbing the biofilm integrity. Accordingly, this technique is valuable in studying the effect of disinfectants on attached bacteria *in situ*. In addition, this technique can reveal not only viable cell numbers within thin biofilms but also the total number of bacteria remaining on the

substratum throughout the disinfection period. The resulting data may be applied to studies comparing the physiological status and disinfection kinetics of attached and planktonic cells. However, bacteria in thicker mature biofilms may have dramatically different responses when compared with free-living cells.

Our previous studies (Singh *et al.*, 1989; Singh *et al.*, 1990) indicated that image analysis can be used for rapid enumeration and is able to determine cell viability as well as injury in planktonic suspensions of bacteria. With the aid of image analysis, the *in situ* DVC method would provide further quantitative information that may be used in determining the viability of bacteria within intact biofilms. Recently, progress has been reported on the use of scanning laser confocal microscopy, especially with the software developed for 2- and 3-dimension image processing (Lawrence *et al.*, 1991). It, therefore, might be possible to utilize the *in situ* DVC method in mature biofilms with the assistance of scanning laser confocal microscopy.

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CHAPTER 3

RAPID *IN SITU* ASSESSMENT OF PHYSIOLOGICAL ACTIVITIES IN
BACTERIAL BIOFILMS USING FLUORESCENT PROBESIntroduction

The ability of microorganisms to grow and form colonies on culture media has been used as the traditional approach to study bacterial viability. Conventional microbiological methods for assessing the viability of bacteria within biofilms are currently based on the mechanical removal of cells from substrata followed by enumeration by colony formation. However, these methods not only require at least 24 h incubation but often underestimate bacterial viability (Morita, 1985; Brock, 1987; Wayne *et al.*, 1987).

The direct microscopic enumeration method using acridine orange (AODC) usually has a reasonable correlation with viable counts when applied in exponentially growing cultures. However, AODC data can exceed the plate counts (PC) by several orders of magnitude (Roszak and Colwell, 1987) when used to examine bacterial populations in natural environments. The direct viable count (DVC) method developed by Kogure *et al.* (Kogure *et al.*, 1979; Kogure *et al.*, 1984), has been employed successfully in enumerating bacteria within environmental samples (Maki and Remsen, 1981; Xu *et al.*, 1982; Liebert and Barkay, 1988; Rollins and Colwell, 1986; Singh *et al.*, 1990). The DVC method was later applied as a direct *in situ*

enumeration method for thin biofilms (Yu *et al.*, 1993), where the results indicated that this adaptation of the DVC method can provide rapid (4 h) as well as more accurate information regarding bacterial number and viability within biofilms.

This study was initiated to seek other *in situ* methods to quickly determine the bacterial physiological activity of surface-associated bacteria without disrupting biofilm integrity. The acridine orange (AO) stain used in the DVC method has been suggested as an index of physiological activity or viability (McFeters *et al.*, 1991; Kolter, 1992; Zambrano *et al.*, 1993). However, the validity of this approach is variable and needs to be defined for each organism and set of culture conditions (McFeters *et al.*, 1991). Hence, AO has limited application as a vital stain. Other fluorescent stains allow the selective microscopic examination of particular activities or physiological properties of bacteria. After entering the cell envelope, the fluorochromes can be observed directly using appropriate excitation & emission filters with epifluorescence microscopy. The fluorescent probes we chose to employ in this study were 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and rhodamine 123 (Rh 123).

The reduction of tetrazolium salts to formazan has been used for many years in histo-, cyto-, and biochemical determinations of oxidase and dehydrogenase activities. Zimmermann *et al.* (Zimmermann *et al.*, 1978) used the redox dye, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) to study respiratory activity in aquatic bacteria. However, the reduced form of INT (INT-formazan) can only be observed within bacteria by light microscopy, which can not be applied to study

biofilm bacteria on opaque substrata without removal of the cells. Although the combination of fluorescent-antibody (FA) and INT reduction has been applied successfully to study cellular activity (Baker and Mills, 1982), the tedious procedures of preparing and testing FA has limited this approach as a general application. CTC has been applied successfully to study physiological activity within eukaryotic (Stellmach, 1984) and prokaryotic (Rodriguez *et al.*, 1992; Kaprelyants and Kell, 1993) cells. Rh 123 is a cationic fluorescent dye which is concentrated in mitochondria by the relatively high negative potential across the energized mitochondrial membrane (Johnson *et al.*, 1981). In bacterial cells Rh 123 is accumulated in an uncoupler-sensitive fashion via transmembrane potential (Haugland, 1992). This fluorochrome has been utilized to assess the physiological states of *Micrococcus*, *Escherichia coli* (Kaprelyants and Kell, 1992) and *Aeromonas* (Morgan *et al.*, 1993) by flow cytometry. In this study, we compared the *in situ* performance of these two physiologically dependent fluorochromes with the *in situ* DVC and PC in the determination of bacterial viability within pure culture biofilms.

Materials and Methods

Biofilm Apparatus

Bacterial biofilms were grown in wide-mouth pint Mason jars (Kerr, 450 ml) described elsewhere (Cargill *et al.*, 1992). Jars containing 6 stainless steel coupons, inoculated medium and a magnetic stir bar were incubated on a thermally insulated

magnetic stirrer with a constant speed (285 rpm).

Bacterial Strains and Growth Conditions

Klebsiella pneumonia Kp1, isolated from drinking water, was obtained from Dr. D. Smith, South Central Connecticut Water Authority, New Haven, CT. The bacterial culture was grown in 1/10 Trypticase soy broth (TSB, Difco) for 24 h at 35°C, then transferred (1%) to sterile medium for another 24 h before being used as inoculum. Biofilms were formed on 316 stainless steel (12x76 mm²) coupons incubated at 25°C for 36 h (Yu *et al.*, 1993).

Fluorochromes and Staining Procedures

The fluorochromes utilized in this study were 5-cyano-2,3-ditolyl tetrazolium chloride (CTC, Polysciences, Inc.) and rhodamine 123 (Rh 123, Eastman Kodak Co.). Bacteria attached on the coupons were transferred to a staining box containing 0.04% (approx. 4 mM) CTC in reagent-grade water or Rh 123 (5 µg/mL, final concentration) in phosphate buffered saline (PBS, pH 7.2) to perform assays for physiological activity. The preparation of CTC was different from that reported by Rodriguez *et al.* (Rodriguez *et al.*, 1992). After incubation at 35°C for up to 2 h, the CTC solution was removed and replaced with 5% formalin to fix the biofilms for 5 min, then counterstained with 4',6-diamidino-2-phenylindole (DAPI) (1 µg/mL, final concentration) for 3 min. A pretreatment of immersing biofilms in Trizma hydrochloride (50 mM) and disodium EDTA (5 mM) at pH 8.0 (Kaprelyants and

Kell, 1992) for 5 min was required for staining with Rh 123. No further treatment or counterstain was required for Rh 123 staining (at 35 °C) after the solution was withdrawn. However, in order to determine the total cell numbers on coupons, a modified acridine orange direct count (AODC) technique using 0.02% AO solution (Yu *et al.*, 1993) was applied to half of the biofilm area after coupons were stained with Rh 123. Coupons were then air-dried prior to examination by epifluorescence microscopy.

Epifluorescence Microscopy

A Leitz Ortholux II microscope fitted with an epifluorescence illumination system and a 100-watt mercury lamp was used. Different Leitz Pleomopak filter blocks (B for DAPI; H for AO, DAPI, CTC and Rh-123; and N2.1 for CTC) were used as appropriate for individual fluorochromes. The filter combination used for visualizing biofilms under the epifluorescence microscope was a Leitz "H" filter block with excitation filter (BP420-490), dichromatic mirror (RKP 455) and a suppression filter (LP 515).

Enumeration

The results obtained using the two different fluorescence stains were compared with an *in situ* DVC enumeration method (Yu *et al.*, 1993) and a traditional approach where surface-associated cells were removed from the substratum with a sterile rubber policeman followed by plate count (PC) enumeration. The viability of attached

bacteria in the DVC method was determined by cell elongation after 4 h incubation with nalidixic acid, yeast extract and casamino acid in PBS (Yu *et al.*, 1993). Plate counts were determined by a modified drop plate method (Miles and Misra, 1938) using five 10 μ l drops per dilution with tryptone lactose yeast extract agar medium (TLY) (McFeters *et al.*, 1982). Colonies were counted after 24 h incubation at 35°C. The *in situ* DVC method was performed as described by Yu *et al.* (Yu *et al.*, 1993).

Statistical Analysis

The microscopic enumeration results of each experiment were obtained from counts of 10 microscopic fields. If the total cell number was less than 400, more than 10 fields were examined. Statistical analyses were performed on all data from three replicates using mathematical functions within SigmaPlot™ (Version 4.1 by Jandel Scientific) and one-way analysis of variance (ANOVA) with InStat™ (Version 1.1 by GraphPAD) computer software. The level of significance was set at $p < 0.05$ for all comparisons.

Results

Activity Determined by CTC Reduction

Respiring cells within Kp1 biofilms reduced CTC to crystalline CTC-formazan, which could be visualized as red crystals inside the bacteria by epifluorescence microscopy (Figure 6). Optimal time for CTC reduction was 1 to 2 h

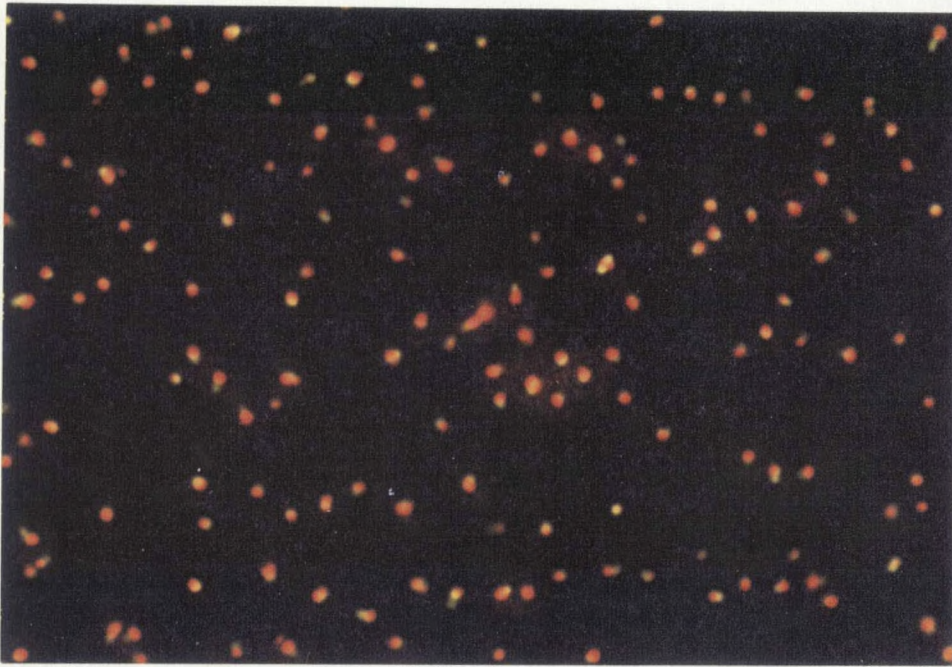


Figure 6. Epifluorescence micrograph of *Klebsiella pneumoniae* (Kp1) biofilm bacteria on stainless steel stained with CTC-formazan (red crystals) inside, counter stained with DAPI (green) using Leitz filter block "H" (1,000 X).

incubation at 35 °C (Figure 7). The Leitz "H" filter block with a suppression (barrier) filter (LP 515) gave good color contrast for both nonrespiring cells, which stained green with DAPI, and respiring cells, which were green but contained red CTC-formazan crystals (Figure 6). Respiratory activity of the Kp1 cells in biofilms was determined by comparing the cells containing intracellular CTC-formazan with total cell numbers stained by DAPI. The fraction of the total population in a growing biofilm that demonstrated respiratory activity, determined by CTC reduction, was $94.9 \pm 2.6 \%$ (Table 3).

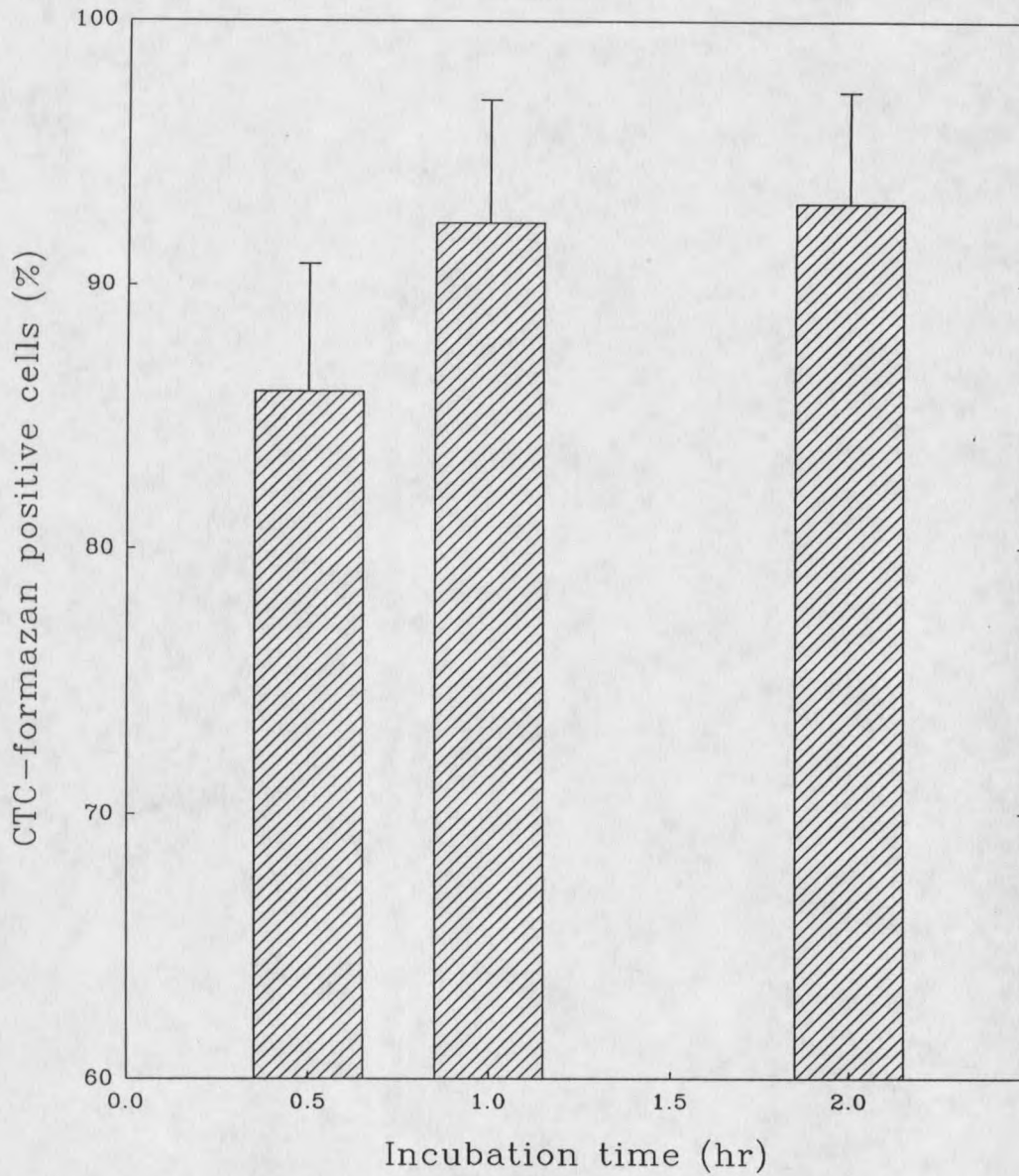


Figure 7. Reduction of CTC by *Klebsiella pneumoniae* (Kp1) biofilms at 35°C with time. ($n = 3$, bars = S.D.)

TABLE 3. Activity of *K. pneumoniae* population in biofilm determined by various enumeration methods. ($\bar{X} \pm \text{S.D.}$, $n=3$)

Assay	Total cells	Active or viable cells (cells/mm ²)	% Activity or viability (cells or cfu/mm ²)
CTC reduction	2.23×10^4	2.11×10^4	94.6 ± 4.4
Rh 123 staining	2.01×10^4	1.74×10^4	86.6 ± 6.1
<i>in situ</i> DVC	2.33×10^4	2.15×10^4	92.3 ± 4.4
PC method	$2.33 \times 10^{4*}$	1.01×10^4	43.3 ± 7.9

* Value was based on AODC data.

Activity Determined by Rhodamine 123 Staining

Rh 123 is not readily absorbed by Gram negative bacteria because of a permeability limitation in their outer membrane (Nikaido and Vaara, 1985). However, treatment with Tris and EDTA at alkaline pH (Kaprelyants and Kell, 1992) eliminates this barrier. The accumulation of Rh 123 into Kp1 biofilm bacteria was observed within 30 minutes, and incubation for 2 h yielded optimal staining results (Figure 8). After staining with Rh 123 for 2 h, active biofilm cells appeared green by epifluorescence microscopy when using a Leitz "H" filter block (Figure 9). The level of activity within Kp1 biofilms determined by Rh 123 staining was 86.6 ± 6.1 % (Table 3). The fraction of the biofilm bacterial population that stained by Rh 123 was compared with total cell numbers obtained by AODC on different areas of the same coupon.

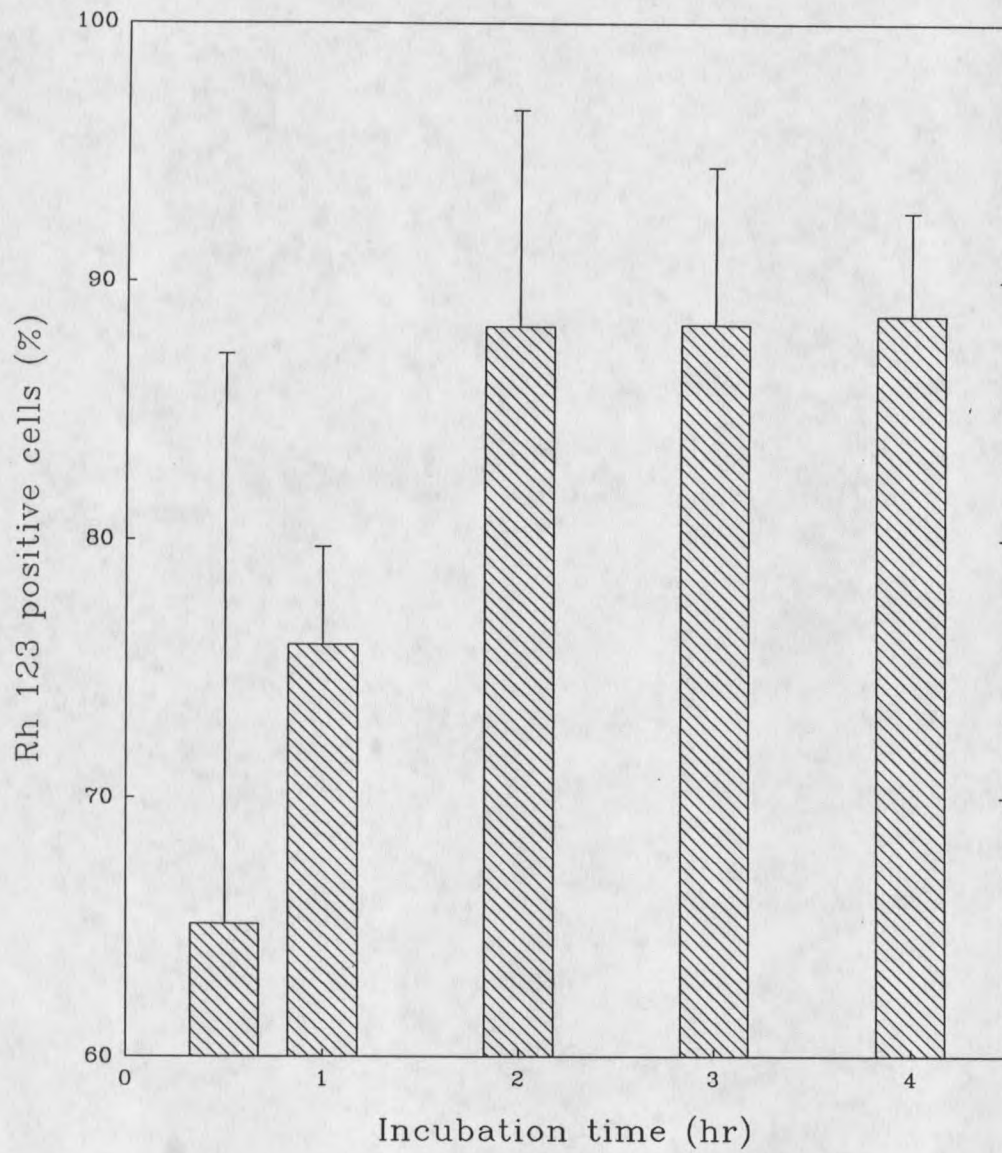


Figure 8. Staining of *Klebsiella pneumoniae* (Kp1) biofilms with Rh 123 at 35°C with time. ($n=3$, bars = S.D.)

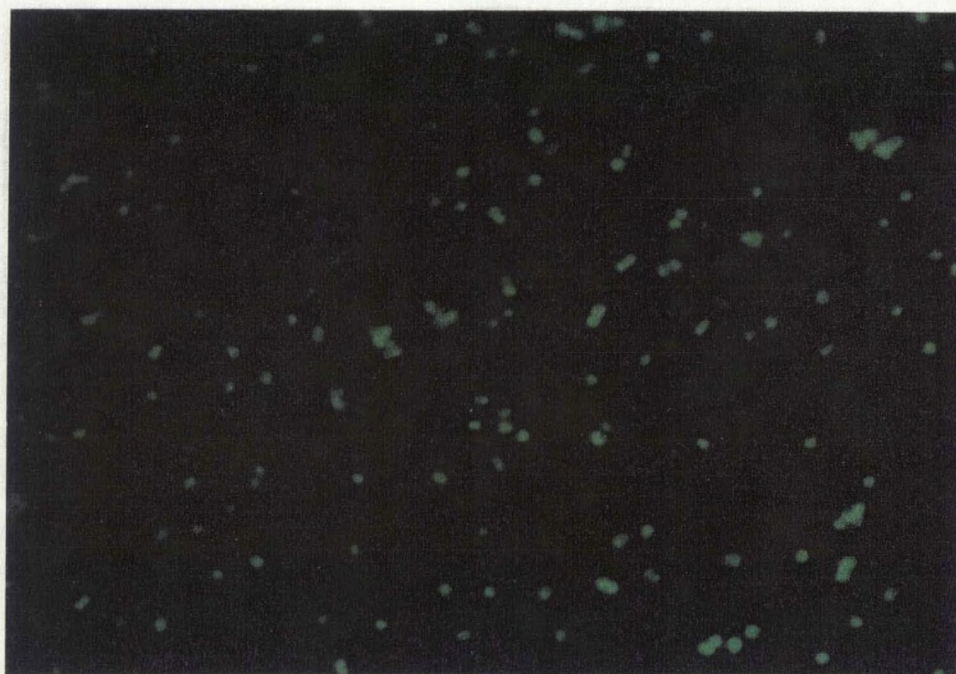


Figure 9. Epifluorescence micrograph of *Klebsiella pneumoniae* (Kp1) biofilm bacteria on stainless steel stained with Rh 123 under Leitz filter block "H" (1,000 X).

Viability Determined by *in situ* DVC and PC Methods

After 4 h incubation with nalidixic acid and nutrients, the elongated cells on coupons that stained with AO were counted as viable cells. The total cell numbers were the sum of the elongated and non-elongated cells observed. The viability of biofilm bacteria determined by the *in situ* DVC method was 92.3 ± 4.4 % (Table 3), and the total cell numbers on coupons obtained by AODC and DAPI staining were statistically comparable ($p < 0.05$).

The PC method only revealed bacteria capable of forming colonies on TLY plates, expressed as viable cells in Table 1, however, this technique cannot reveal

total cell populations. Thus, the percent viability calculated for this approach, $43.3 \pm 7.9 \%$, was based on total counts obtained by the AODC method (Table 3).

Discussion

The enumeration of viable bacteria by the PC method may not include all viable cells, particularly those exposed to environmental stress (Camper and McFeters, 1979; McFeters *et al.*, 1982; Roszak and Colwell, 1987; McFeters and Singh, 1991; McFeters, 1990). Also, removal of sessile bacteria and the quantitative measurement of aggregated populations present problems when using plate counting techniques. The direct microscopic examination of biofilm bacteria with specific fluorochromes as vital stains overcomes these persistent disadvantages.

CTC has potential as a vital stain in biofilm study since it allows the detection of respiratory activity by epifluorescence microscopy. Although the results of incubation for 1 h and 2 h showed similar fractions of CTC reduction ($92.4 \pm 3.8\%$ vs. $93.1 \pm 3.4\%$), we observed that the size of CTC-formazan crystals formed inside the bacteria after 1 h incubation were smaller, and tended to fade more rapidly when examined with epifluorescence microscopy than those incubated for 2 h. Thus, we chose 2 h as the optimal incubation time for the CTC reduction assay. Only cells with electron transport system (ETS) activity possess the membrane-bound respiratory chain dehydrogenase to reduce tetrazolium salts (Kaprelyants and Kell, 1993; Packard and Taylor, 1968). Extracellular reduction of CTC was not observed within Kp1

biofilms.

Rh-123 is a proton motive force (PMF)-driven dye, which is only taken up by viable cells. The optimal staining of Kp1 biofilms by Rh 123 was observed after 2 h of incubation (Figure 8). Unexplained fluctuations were observed in the fraction of bacteria that took up Rh 123 during the first half hour of incubation as reflected by a large standard deviation ($65.2 \pm 22.1\%$, \pm S.D.). The uptake of Rh 123 increased to $75.9 \pm 3.8\%$ after 1 h, and reached a plateau at 2 h ($88.3 \pm 8.3\%$ at 2 h; $88.3 \pm 6.1\%$ at 3 h; $88.7 \pm 4.0\%$ at 4 h) with diminished fluctuation as incubation proceeded. However, differences in Rh 123 uptake between 2,3,4 h were not significant ($p > 0.05$). The membrane permeability and cellular retention of the fluorescent probes depend on many physical, chemical, physiological and biochemical factors, including nonspecific binding, hydrophobicity, size and net charge (Haugland, 1992). Many of the cell-permeant probes enter the cell and associate with cellular structures by intrinsic physiological process (Haugland, 1992). Bacteria are extremely heterogeneous with respect to their ability to accumulate the lipophilic dye Rh 123, although this process is correlated with viability and membrane potential (Kaprelyants and Kell, 1992; Morgan *et al.*, 1993). However, a pretreatment of biofilms with Trizma hydrochloride and disodium EDTA (Nikaido and Vaara, 1985) before staining overcame this problem.

The viability data reported here on bacteria in the absence of environmental stress, such as disinfectants, indicated that enumeration results obtained using PC and the three *in situ* direct microscopic methods were comparable. Levels of

activities/viabilities determined by the three methods under investigation (CTC, Rh 123, and *in situ* DVC) varied somewhat (Table 3), but the one-way ANOVA test showed the differences between methods were not significant ($p > 0.05$). However, the results of a previous study indicate that the differences between PC and *in situ* DVC enumeration become apparent when disinfectants (i.e. chlorine and monochloramine) are introduced into the system (Yu *et al.*, 1993).

The choice of appropriate filter combinations to obtain distinctive excitation and emission spectra for different fluorochromes is of crucial importance in epifluorescence microscopy. Leitz filter block "D" (BP 355-425, RKP 455, LP 460) is suggested by the manufacturer to visualize cells stained with DAPI. This combination of filters is similar to that reported by Rodriguez *et al.* (Rodriguez *et al.*, 1992). However, DAPI stained cells appeared blue with the red CTC-formazan crystals hardly visible when that filter combination was used. Leitz filter block "H" allowed the visualization of DAPI stained cells (green) and CTC-formazan (red-orange) simultaneously (Figure 6). The size of the CTC-formazan crystals inside the bacteria varied and smaller crystals sometimes were hard to identify. This introduced some personal bias in enumerations using filter block "H". Another filter block ("N 2.1") with a different combination (BP 515-560, BKP 580, LP 580) gave the optimal visualization of CTC-formazan with epifluorescence microscopy. This is similar to the combination suggested by Rodriguez *et al.* (Rodriguez *et al.*, 1992). The disadvantage of using this filter block is that the emission of DAPI was cut off by the barrier filter and no counterstain could be observed. As a result, bacteria with

multiple CTC-formazan crystals inside might be counted more than once, giving incorrect enumeration results. Consequently we suggest using filter block "H" or a similar combination for different microscope systems, which allows observation of DAPI and CTC-formazan at the same time, as shown in Figure 6.

This paper demonstrates that CTC and Rh 123 provided novel *in situ* methods for making physiological observations of bacteria in pure culture biofilms without disrupting community integrity. Our previous study (Yu *et al.*, 1993) reveals the utility of an *in situ* DVC method to enumerate viable bacteria within biofilms in 4 h. The use of CTC and Rh 123 further expedites data collection from 4 h to 2 h, and is significantly faster than the 24 h incubation time required for colony formation. However, it should be noted that biofilm formation was limited to bacterial monolayers in this study, and it is likely that thicker biofilms respond differently. CTC and Rh 123 staining methods accomplish the rapid and accurate *in situ* enumeration of active bacteria as well as the assessment of different physiological properties of bacteria within biofilms to an extent that is not possible by conventional techniques.

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CHAPTER 4

PHYSIOLOGICAL RESPONSES OF BACTERIA IN BIOFILMS
TO DISINFECTIONIntroduction

Methods to determine disinfection efficacy have relied on viability assays using conventional plate counting techniques for decades. One problem associated with this practice is that certain microorganisms are unable to form colonies on established media (Winogradsky, 1949; Brock, 1987; Atlas, 1984; Ward *et al.*, 1992). This is especially problematic when examining microbial communities with a significant species diversity representing a wide range of physiological activities. Also, exposure to disinfectants under suboptimal conditions results in bacteria that become unable to form colonies on selective media due to reversible injury (McFeters, 1990). This phenomenon has been documented in operating drinking water systems (McFeters *et al.*, 1986). Environmental exposure in the absence of disinfectants can also decrease culturability (Roszak and Colwell, 1987). These inadequacies are more acute when dealing with biofilms or microbial communities where spatial heterogeneities are established and may be complex. Therefore, the traditional approach of describing microbial populations based on colony-forming ability is inadequate in many systems since established culture media often underestimate the actual bacterial population density and provide no information on spatial heterogeneities within communities such

as biofilms.

Numerous alternative approaches have been applied to obtain quantitative information on bacteria or their physiological activities within environmental systems. Direct examination employing traditional microscopy (Rodrigues and Kroll, 1989; Meyer-Reil, 1978; Marshall, 1986) and, more recently, scanning confocal laser microscopy (SCLM) (Caldwell *et al.*, 1992) have made progress in visualizing attached microbial communities. A number of different approaches, including 16S rRNA analysis (Ward *et al.*, 1992; McSweeney *et al.*, 1993; Ramsing *et al.*, 1993), flow cytometry (Allman *et al.*, 1992; Monger and Landry, 1993; Morgan *et al.*, 1993; Kaprelyants and Kell, 1993a), and probes for the expression of specific genes (Stewart and Williams, 1992; Walker *et al.*, 1992) have also been applied in such studies. However, most of these techniques either demand complex preparation processes or sophisticated and expensive equipment. As a result, the assessment of biofilm disinfection still mainly relies on the conventional approach of scraping and enumeration by plate counting techniques with their inherent problems.

In a previous paper, two rapid *in situ* methods for assessing physiological activities of biofilm bacteria were presented (Yu and McFeters, 1994) using the fluorogenic probes, 5-cyano-2,3-ditoly-tetrazolium chloride (CTC) and rhodamine (Rh 123). These compounds reflect cellular respiratory activity and membrane potential. RNA turnover rate is also a useful indicator of physiological activity since it has been used to determine *in situ* growth rate and the response of bacteria to changes in environmental conditions (Karl *et al.*, 1981). In this paper, we followed

changes in physiological activities of attached bacteria with exposure to disinfectants without disrupting biofilm integrity using CTC, Rh 123 and the incorporation of tritiated uridine. The results obtained provided more precise information describing the physiological consequences of biocides within bacterial biofilms than is accomplished by the conventional colony enumeration approach.

Materials and Methods

Biofilm Apparatus

Bacterial biofilms were grown in a simple reactor made from wide-mouth pint Mason jars (Kerr, 450 ml) as described elsewhere (Cargill *et al.*, 1992). Jars containing 6 stainless steel coupons, inoculated medium and a magnetic stir bar were incubated on a thermally insulated magnetic stirrer with a constant speed (285 rpm). The preparation of the coupons is described in chapter 2.

Bacterial Strains and Growth Conditions

Klebsiella pneumonia Kp1, isolated from drinking water, was obtained from Dr. D. Smith, South Central Connecticut Water Authority, New Haven, CT. The bacterial culture was grown in 1/10 Trypticase soy broth (TSB, Difco) for 24 h at 35°C, then transferred (1%) to sterile medium for another 24 h before being used as inoculum for the biofilm reactors. Biofilms were formed on 316 stainless steel (12x76 mm²) coupons incubated at 25°C for 36 h (chapter 2).

Preparation of Disinfectants

The disinfectants used were chlorine and monochloramine. The chlorine solution was prepared from sodium hypochlorite (Singh *et al.*, 1986), and the monochloramine solution was made with sodium hypochlorite and ammonium chloride (LeChevallier *et al.*, 1988a). The biofilm coupons were exposed to disinfectants in chlorine demand-free sterile phosphate buffered water (PBW) without $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ as described in chapter 2. The pH value of PBW was adjusted to 7.2 for chlorine and 9.0 for monochloramine. The concentrations of chlorine and monochloramine were measured with the Hach DPD chlorine test kit and Hach digital amperometric titrator (Hach Co., Loveland, CO).

Disinfection of Biofilms

The biofilm coupons were rinsed in sterile distilled water (chapter 2) then transferred to an acid-washed quart wide-mouth Mason jar (Kerr, 900 ml) containing chlorine-demand free PBW and a magnetic stir bar. The biofilm coupons were then treated with disinfectants (0.25 mg/L chlorine at pH 7.2 and 1 mg/L monochloramine at pH 9) at 25 °C. The concentrations of the disinfectants were chosen to allow comparisons of the data with previously published results obtained from plate counts (PC) on TLY medium (McFeters *et al.*, 1982) and DVC methods (Yu *et al.*, 1993). Coupons were removed after 5 and 10 min and the disinfectant neutralized by immersion in sodium thiosulfate (0.01%, final concentration). Surviving biofilm bacteria were enumerated using the Rh 123 and CTC staining (Yu and McFeters,

1994) methods.

Fluorochromes and Staining Procedures

The fluorochromes utilized in this study were 5-cyano-2,3-ditolyl tetrazolium chloride (CTC, Polysciences, Inc.) and rhodamine 123 (Rh 123, Eastman Kodak Co.). Bacteria attached to the coupons were rinsed (see chapter 2) and transferred to a staining vessel containing 0.04% (approx. 4 mM) CTC in reagent-grade water or Rh 123 (5 $\mu\text{g}/\text{mL}$, final concentration) in phosphate buffered saline (PBS, pH 7.2). The details of the CTC reduction assay and the Rh 123 staining procedures are described elsewhere (Yu and McFeters, 1994). Coupons were then air-dried prior to examination by epifluorescence microscopy.

Tritiated Uridine Incorporation Assay

Uridine uptake was employed to determine the RNA turnover rate in biofilm bacteria. [5,6- ^3H]uridine (40.1 Ci/mmol; 1483.7 Tbq/mmol; Dupont-NEN) was used in the tritiated uridine incorporation assays. The biofilm coupons withdrawn at timed intervals during disinfection were placed in a vessel containing 4 ml uridine solution (0.2 $\mu\text{Ci}/\text{ml}$) to allow incorporation. After 4 h incubation at 25 °C, the coupons were retained in the vessel and the attached bacteria removed from the coupons by scraping with a sterile rubber policeman followed by collection in a vial and sonication (Sonogen Automatic Cleaner, Branson Instruments Inc.) for 5 min to disperse aggregates. An aliquot of the suspension (100 μl) was added to an eppendorf tube

with 0.1 ml lysis buffer [Tris HCl (50 mM), EDTA (10 mM), SDS (4%), pH 12.4] and vortexed for 5 min, then transferred onto a Whatman glass fiber filter (GF/C). The filter was washed 3-5 times with 5% cold trichloroacetic acid (TCA) to precipitate the bacterial RNA. The filter was washed again with cold 95% ethanol (4°C) to remove residual TCA then dried in an oven (100°C) overnight before being transferred to a small vial with 3 ml scintillation fluid (0.5% PPO in xylene). The quantitation of radioactivity was done with a Packard Tri-Carb 460 CD liquid scintillation counter. Quenching analysis was done using [5,6-³H]uridine to determine the effects of lysis buffer and glass fiber filters. No significant quenching effect was detected from lysis buffer or glass fiber filters.

Enumeration with Epifluorescence Microscopy

A Leitz Ortholux II microscope fitted with an epifluorescence illumination system and a 100-watt mercury lamp was used. Different Leitz Pleomopak filter blocks were used as appropriate for individual fluorochromes. The H filter block was used for visualizing attached bacteria stained with Rh 123, AO, and intracellular CTC formazan, whereas filter block B was applied in the enumeration of total cell numbers stained with DAPI by epifluorescence microscopy. After 2 h incubation time at 35 °C, respiring cells within Kp1 biofilms reduced soluble CTC to CTC-formazan deposits, which could be visualized as intracellular red crystals in bacteria by epifluorescence microscopy. The barrier filter (LP 515) in the Leitz "H" filter block gave distinctive color contrast on the biofilm coupons. The nonrespiring bacteria

appeared green when stained with DAPI, whereas the respiring cells were also green but contained red CTC-formazan crystals. Filter block N2.1 was used to visualize the red CTC-formazan crystals without DAPI fluorescence. The accumulation of Rh 123 into Kp1 cells within biofilms appeared green using filter block "H". The results obtained using the two different fluorescence stains were compared with the enumeration data from plate count (PC) and *in situ* DVC methods (Yu *et al.*, 1993).

Statistical Analysis

The microscopic enumeration results were obtained in each experiment from counts of 10 microscopic fields. If the total cell number was less than 400, more than 10 fields were examined. Statistical analyses were performed on all data from three replicates using mathematical functions within SigmaPlot™ (Version 5.0 by Jandel Scientific) and one-way analysis of variance (ANOVA) with InStat™ (Version 1.1 by GraphPAD) computer software. The level of significance was set at $p < 0.05$ for all comparisons.

Results

Biofilm Chlorination

The initial unexposed mean total bacterial density on the coupons was $1.80 \pm 0.27 \times 10^4$ cells/mm² (\pm S.E.) as determined microscopically by staining with DAPI while the mean number of viable cells, which was measured by colony formation on

TYL plates after removing the attached bacteria from the coupons, was $1.44 \pm 0.74 \times 10^4$ cfu/mm². The cell population demonstrating respiratory activity, determined by CTC reduction, was $1.71 \pm 0.20 \times 10^4$ cells/mm². The initial total cell density obtained by AODC enumeration on a different area of the same coupon was $1.90 \pm 0.48 \times 10^4$ cells/mm² and the number of cells retaining transmembrane potential, which stained by Rh 123, was $1.64 \pm 0.26 \times 10^4$ cells/mm².

Free chlorine (0.25 mg/L) was used to treat attached cells grown on stainless steel coupons. Biofilms of *Klebsiella pneumoniae* Kp1 were exposed to chlorine or monochloramine for timed intervals and assayed to determine the effects of these disinfectants on cellular respiration and membrane potential. Comparison of disinfection effects was determined using log N/N_0 values (the log ratio of surviving bacterial numbers to initial bacterial numbers, which gave a positive indication with each assay) after disinfectant exposure for 5 and 10 min. During chlorine disinfection, the respiratory activity of attached cells on coupons decreased by 1.93 logs after 10 min (Figure 10). However, the fraction of the biofilm bacteria that retained a membrane potential decreased less after chlorination showing only a decline of 0.93 log at 10 min (Figure 10). A larger decrease was observed in the viability of the biofilm bacteria determined by the DVC method (2.34 logs after 10 min). The largest decrease was seen in the PC results, where bacterial viability declined 3.30 ± 0.50 logs and the confidence interval became larger after 10 min chlorination (Figure 10). The differences between the four enumeration methods were statistically significant ($p < 0.05$).

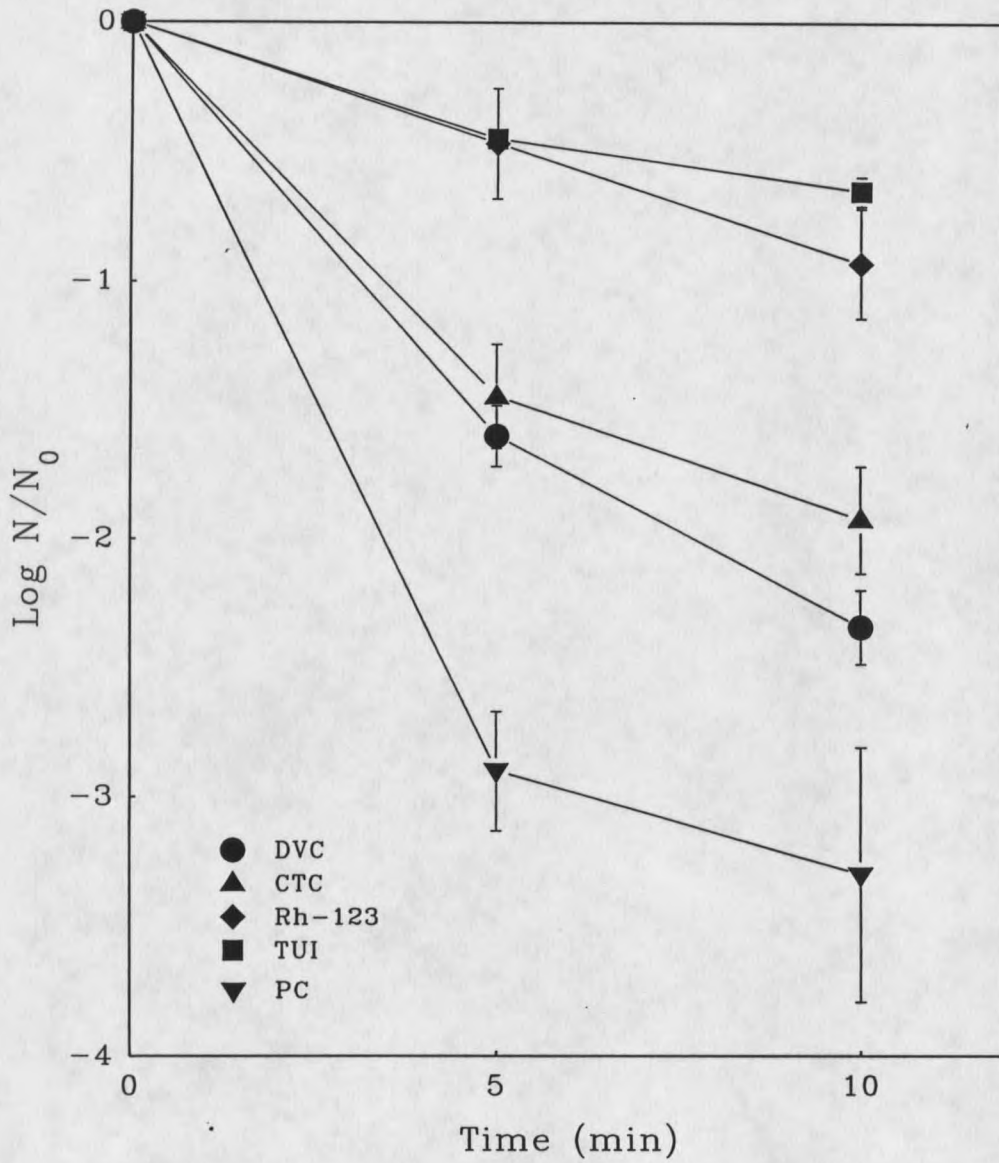


Figure 10. Effect of chlorine treatment (0.25 mg/L, pH 7.2) on *Klebsiella pneumoniae* KP1 biofilms. The activity of surviving bacteria was assessed by four different methods ($n=3$, bars = S.E.).

Biofilm Chloramination

The results of a similar experiment where monochloramine (1 ppm) was used to treat Kp1 biofilms are shown in Figure 11. The initial mean density of respiring cells on the coupons was $1.67 \pm 0.27 \times 10^4$ cells/mm², and the cell population stained with Rh 123 was $1.57 \pm 0.29 \times 10^4$ cells/mm². Following 10 min of chloramination, a 1.59-log decrease was observed in the fraction of the biofilm bacteria that reacted in the CTC reduction assay (Figure 11). With the Rh 123 staining method, higher counts were observed (0.53 log reduction) after monochloramine treatment for 10 min. The DVC method showed a 1.76-log reduction, which is comparable with the data acquired from the CTC reduction assay. Statistical analyses showed there was no significant difference between results obtained with the CTC and DVC methods ($p > 0.05$), but the results from Rh 123 method were significantly different than the other three methods ($p < 0.05$). The PC results again showed the largest decline (2.71 ± 0.50 logs) in bacterial culturability and increase in confidence interval after 10 min exposure to monochloramine. However, it should be noted that the log N/N_0 values obtained with the PC method did not show any significant difference ($p > 0.05$) between chlorine and monochloramine after 10 min exposure (Figures 10 & 11).

Tritiated Uridine Incorporation in Biofilms after Disinfection

Tritiated uridine incorporation was used to estimate the RNA turnover rate in the attached cells. The incorporation of [5,6-³H]uridine by Kp1 biofilms after

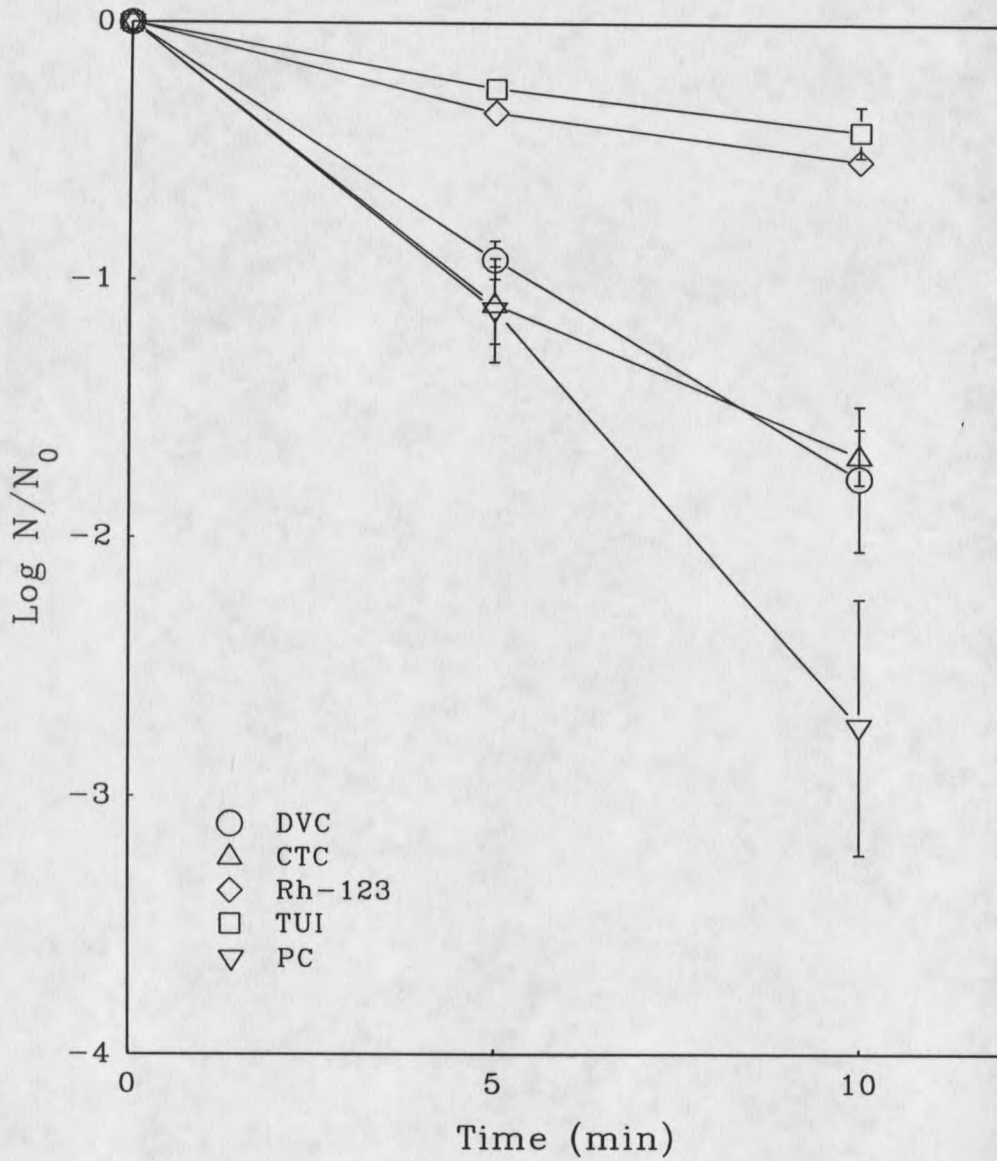


Figure 11. Effect of monochloramine treatment (1 mg/L, pH 9.0) on *Klebsiella pneumoniae* KP1 biofilms. The activity of surviving bacteria was assessed by four different methods ($n=3$, bars = S.E.)

Table 4. Incorporation of [5,6-³H]uridine by *Klebsiella pneumoniae* biofilms after chlorine (0.25 mg/L, pH 7.2) and monochloramine (1 mg/L, pH 9.0) treatment. ($n=3$, \pm S.E.)

Treatment time	[5,6- ³ H]uridine incorporation			
	Chlorine	% Control	Monochloramine	%
Control				
Control	103784 \pm 4194 cpm	100	78573 \pm 4050 cpm	100
5 min	37030 \pm 2118 cpm	35.7	44566 \pm 6220 cpm	56.7
10 min	23749 \pm 3507 cpm	22.9	32167 \pm 7630 cpm	40.9

treatment with biocides is shown in Table 4. In order to compare the RNA turnover rate with the other physiological activities measured in this study, the uptake of TUI by biofilm bacteria was also expressed as $\log N/N_0$ (the log ratio of cpm by surviving bacteria to cpm of initial bacteria) at 5 and 10 min (Figures 10 & 11). The incorporation of [5,6-³H]uridine by attached bacteria was significantly higher after 5 and 10 min ($p < 0.05$) disinfection with monochloramine than with chlorine exposure.

Discussion

The results indicated a large variation in the responses of different physiological activities examined within Kp1 biofilms after disinfection. Control data obtained without exposure to biocides demonstrated that the DVC, CTC and Rh 123 methods yielded results that were comparable as described previously (Yu *et al.*,

1993; Yu and McFeters, 1994). It should also be noted that these results were all in relatively close agreement with microscopic (AODC) enumerations to detect the total bacterial population. However, differences between the various physiological processes that were examined became apparent after exposure to the disinfectants, as illustrated in Figures 10 and 11. Among all the methods that were studied (DVC, CTC, Rh 123, and TUI), chlorine always caused a greater reduction in the physiological activities assayed, although the PC method yielded comparable levels of bacterial culturability. In addition, the fraction of the active cell population detected by colony formation (PC) was always at least 1 log lower than data obtained with the other methods. These results agree with earlier reports (McFeters, 1990; Yu *et al.*, 1993) suggesting that reliance on PC methods overestimates the efficacy of disinfection treatment.

The results reported here provide a comparison of the relative effects of biocides on different physiological processes within *Klebsiella pneumoniae* biofilms while PC results suggested comparable efficiency was achieved by chlorine and monochloramine. With chlorine, the effect on culturability and viability (PC and DVC) was greater than its consequences on respiratory activity (CTC) which was greater than transmembrane potential (Rh 123) and RNA turnover rate (TUI). The action of monochloramine showed comparable results between DVC and CTC and between Rh 123 and TUI. As with chlorine, the greatest consequence of monochloramine exposure was to decrease viability, respiratory activity and substrate responsiveness (DVC) to a greater degree than its effects on transmembrane potential

and RNA turnover rate. Although monochloramine appeared to cause slightly less damage to the cellular processes studied, the overall pattern of physiological consequences is similar for both disinfectants and some processes displayed significant differences in susceptibility. By way of a possible explanation, it is reasonable that tests such as the DVC and PC which depend on the comprehensive integrity of cellular physiology would be more susceptible to biocides while discrete physiological processes would be more likely to display variable and reduced susceptibility. However, both disinfectants are considered effective in the control of biofilms although monochloramine penetrates biofilms more effectively (LeChevallier *et al.*, 1988a) and removes biofilm bacteria better (Yu *et al.*, 1993).

The experimental system described here used a pure bacterial culture of an environmental isolate grown as thin biofilms (approx. 1~2 μm) under controlled laboratory conditions. It should be noted that thicker and more complex biofilm communities might respond differently to disinfection due to variations in cellular physiology (Fletcher, 1984; Costerton *et al.*, 1987) and the greater production of extracellular polymers (LeChevallier *et al.*, 1988b). However, the physiological approach reported here to assess the efficacy of biocides might be used in the study of mixed culture systems and thicker biofilms.

One noteworthy drawback associated with the DVC method is the difficulty in differentiating the elongation of individual cells within biofilms or aggregates although, image analysis technology can be useful (Singh *et al.*, 1990). By contrast, the utilization of CTC and Rh 123 allowed the clear resolution of individual cells

using epifluorescence microscopy. This can be facilitated with combinations of different excitation and barrier filters to allow the colors of stains and counterstains to be differentiated without causing confusion when enumerating the bacteria. As an additional advantage, staining with either CTC or Rh 123 can be completed in 2 h, although additional time might be needed for data analysis. This approach provides more timely and useful information for the formulation of effective responses directed towards the control of biofilms.

CTC and Rh 123 have also been used recently with flow cytometry to determine respiratory activity (Kaprelyants and Kell, 1993a) and dormancy (Kaprelyants and Kell, 1993b) in bacteria as well as respiring autochthonous bacteria in drinking water and biofilms (Schaule *et al.*, 1993). There is a large number of other fluorogenic reagents utilized in biological and physiological applications with eukaryotic cells (Haugland, 1992; Mason, 1993). However, there are only a few microbiological uses of this analytical approach (Caldwell *et al.*, 1992; Stewart *et al.*, 1994; McFeters *et al.*, 1994), and there is a great potential for the expanded application of fluorogenic compounds to determine bacterial physiological and metabolic activities at the cellular level. This development is further facilitated by recent advances in SCLM, digital image analysis systems and high resolution cameras (Caldwell *et al.*, 1992).

The use of fluorogenic indicators of metabolic activities by microscopy provides information describing the physiological status of individual cells and the assessment of specific cellular processes *in situ* within attached microbial

communities. This approach is more accurate and goes beyond what the conventional strategy, which is dependent on culturability, can offer. This study indicated that the use of fluorescent probes can reveal different physiological insights within bacterial biofilms that may be applied in the evaluation of disinfection efficacy.

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CHAPTER 5

CRYOSECTIONING OF BIOFILMS FOR MICROSCOPIC EXAMINATION

Introduction

Most structural studies of microbial biofilms have relied on light and electron microscopy (Kinner *et al.*, 1983; Robinson *et al.*, 1984; Costerton *et al.*, 1987; Eighmy *et al.*, 1983; Stewart *et al.*, 1993; Lappin-Scott *et al.*, 1992; Ganczarczyk *et al.*, 1992). Problems associated with these techniques include disruption of biofilm structure during removal from the substratum, laborious preparations, and extensive sample processing that may introduce artifacts. Light microscopy used in combination with computer-enhanced microscopy is an effective tool, but it is best applied during the early phases of biofilm development (Lawrence *et al.*, 1989). Visualization of bacterial monolayer biofilms can also be easily accomplished by either light or fluorescence microscopy (Yu *et al.*, 1993; Yu and McFeters, 1994). Due to the resolution limits of optical microscopy, studies on thicker biofilms require mechanical removal of biofilms from the substratum prior to further analysis.

The development of scanning confocal laser microscopy (SCLM) enables researchers to visualize reconstructed 2-D and 3-D images without physically disrupting the biofilm (Lawrence *et al.*, 1991; Caldwell *et al.*, 1992a; Caldwell *et al.*, 1992b). SCLM eliminates out-of-focus haze and allows horizontal and vertical optical

sectioning (Lawrence *et al.*, 1991). The reconstruction of images is based on optical sections that can be applied nondestructively to living tissue in a matter of minutes. This technology involves relatively expensive instrumentation and has limited resolution when applied to thicker biofilms. These aspects restrict it from general application.

Cryoembedding and cryotomy (cryosectioning) of human and animal tissues for light and fluorescence microscopy are well-established histotechniques (Bancroft, 1982; Troyer, 1980; Carson, 1990; Elias, 1990). Cryosections, commonly known as frozen sections, have medical applications in the rapid diagnosis of pathological tissue lesions found during surgical applications (Sawady *et al.*, 1988). This technique has also been utilized with immunoperoxidase and immunofluorescent stainings of tissues for diagnostic and research purposes (Troyer, 1980; Elias, 1990). This report describes the application of cryoembedding and cryosectioning techniques to visualize the structure of bacterial biofilms.

Materials and Methods

Binary population biofilms of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were grown on 316L stainless steel slides in an annular reactor (Siebel and Characklis, 1991). The annular reactor was a continuous flow stirred tank reactor in which 12 removable slides were positioned around the inside wall of the outer drum. Minimal salts medium (van der Wende, 1991) with 20 mg/L glucose and phosphate

buffered water were used to grow the biofilms. The reactors were operated at a dilution rate of 3.2 h^{-1} to ensure that the growth of the planktonic cells was negligible. The effluent was sampled daily and plated on R2A (Difco) agar to monitor the growth of biofilm bacteria and to check for contamination.

The preparation of biofilm samples is illustrated in Figure 12. Biofilms were collected by withdrawing slides from the reactor and placing them in a staining container with the biofilm side up. Formalin (5%, final concentration) was added to fix the specimens which were then immediately stained with $1 \mu\text{g/mL}$ 4',6-diamidino-2-phenylindole (DAPI) (Sigma Chemical Co.). The liquid was removed from the staining container after 5 min. Cryoembedding was performed with Tissue-Tek[®] OCT compound (Miles Inc.) by placing the slide on top of a dry ice slab (Callis *et al.*, 1991), and immediately dispensing a thick layer of OCT on top of the biofilm. Alternatively, the specimen was allowed to infiltrate under the OCT layer for 10 min before transferring the slide onto dry ice. The embedded biofilm was allowed to rapidly freeze until the specimen turned opaque white. The specimen was then separated from the substratum by gently bending the slide to remove the frozen sample. The embedded biofilm was then turned over to embed the side that was previously attached to the substratum. The whole process was performed on dry ice to prevent the samples from thawing. The second step of cryoembedding created a layered frozen matrix in which biofilm was completely surrounded by OCT. The specimen was then wrapped in aluminum foil and stored at -70°C before cryotomy.

In order to determine the efficiency of biofilm removal by the cryoembedding

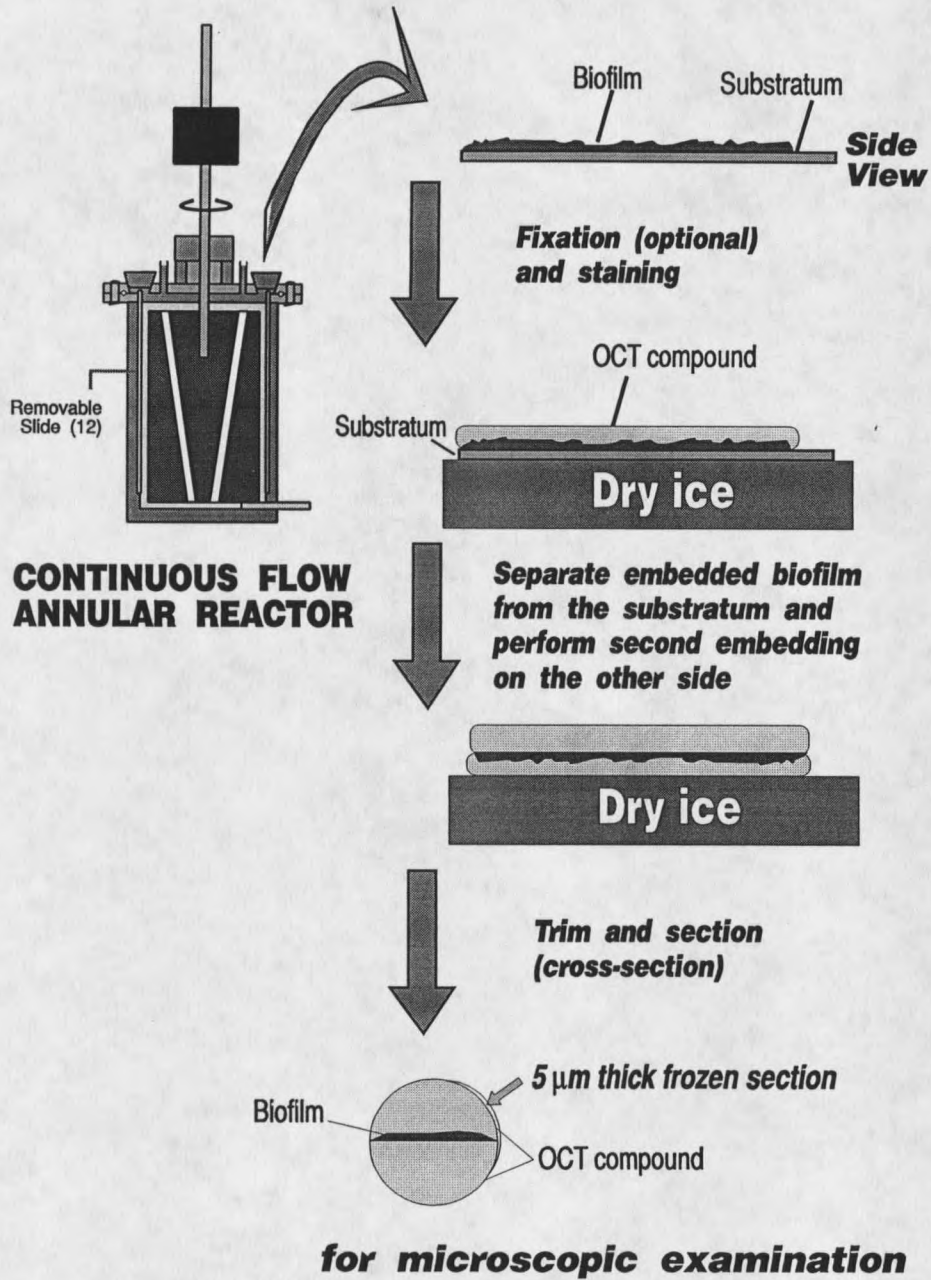


Figure 12. Diagram of cryoembedding and cryosectioning procedures.

technique, biofilm slides before and after cryoembedding were scraped, disaggregated and suspended in reagent-grade water. The suspension was then filtered onto a 25-mm diameter (0.2 μm pore size), black Nuclepore polycarbonate membrane (Costar). The total direct microscopic count procedure described by Hobbie *et al.* (Hobbie *et al.*, 1977) was used with minor modification. Because the biofilms were already stained with DAPI, staining with acridine orange was omitted.

Frozen sections were cut with a cryostat (Reichert-Jung Cryocut 1800, Leica) operated at -19°C . The frozen specimen was placed in the cryostat for 15 min prior to sectioning to allow thermal equilibration. A small portion (2 cm long) of the matrix was bisected for mounting on a precooled (-19°C) specimen chuck. This was done by pouring a small amount of OCT on the chuck and pressing the specimen into the embedding medium. When the OCT solidified, the sample was fixed on the chuck and ready for sectioning. The specimen was oriented perpendicular to a disposable microtome blade (#815, Reichert-Jung, Leica). Trimming was necessary to create a smooth surface prior to cryosectioning. Each 5 μm thick frozen section was collected on a glass slide for microscopic examination.

The sections were examined using an Olympus BH-2 microscope with epifluorescence illumination (100-watt mercury lamp). An Olympus U excitation filter cubic unit with excitation filter (UG-1), a dichroic mirror (DM 400) and a barrier filter (L420) were used for visualizing the sectioned biofilms.

Results

Cryosectioning allowed visualization of cross-section images of biofilms with resolution to the cellular level. An example micrograph (Figure 13) reveals the internal structure of a biofilm grown on a stainless steel substratum. The heterogeneous structure of the biofilm, which consists of microcolonies and water channels can be clearly seen in this specimen. Individual cells are easily resolved. There is some blurring of the image, presumably due to overlapping bacterial cells in the 5- μm thick section. The interface between the substratum and base of the biofilm is also well defined. The embedding technique yielded intact sections with equally good feature preservation for biofilm samples at variable thickness up to 500 μm . It should be noted that the thickness of the biofilm and the frozen section are different in orientation. The thickness of the biofilm is measured in the direction perpendicular to the substratum, whereas the 5 μm thick frozen slices were cross-sections of the embedded biofilm that were also perpendicular to the substratum. The thickness of the frozen sections was, therefore, in a direction parallel to the substratum (Figure 12).

The cryoembedding technique cleanly removed the biofilm from the substratum. The average cell density on a slide before embedding was $3.72 \pm 1.70 \times 10^{11}$ cells/m² ($n=3$), when analyzed by scraping, disaggregation and total direct microscopic counting. After cryoembedding and removal the cell density was $1.66 \pm 1.41 \times 10^3$ cells/m². Direct microscopic examination of slides before and after

biofilm embedding and removal visually confirmed good removal efficiency (Figure 14). When examined at high magnification, occasional cells or aggregates of cells

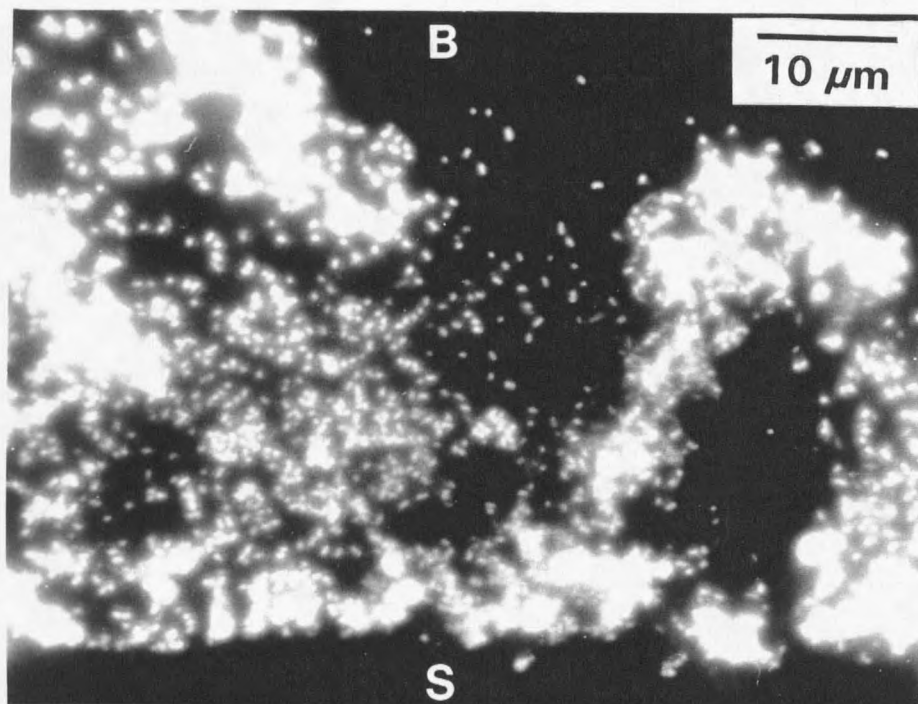


Figure 13. Fluorescence micrograph of frozen section of a mixed *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* biofilm grown on stainless steel. S indicates the location of the substratum and B the location of the bulk fluid.

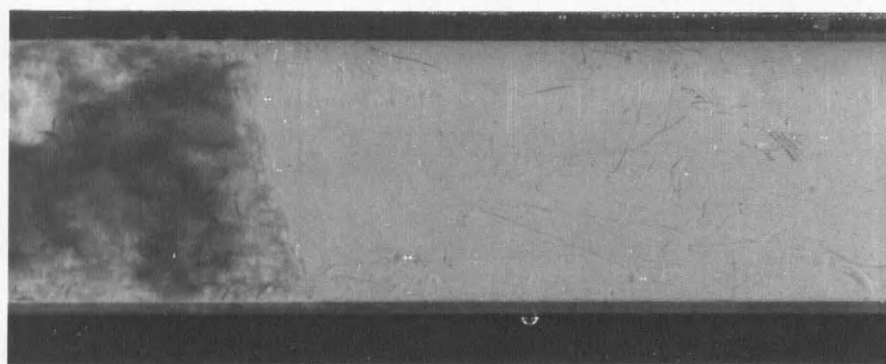


Figure 14. Plan view of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* biofilm distribution on stainless steel slide before (left) and after (right) removal by cryoembedding. The width of the slide is 17 mm.

were found on the substratum after embedding and removal. This indicates that while removal was good, it was not perfect.

Discussion

The non-destructive structural analysis of biofilms has only recently been practical with the development of SCLM techniques. Preparation of samples for electron microscopy tends to cause morphological changes due to shrinkage during fixation and processing (Woldringh *et al.*, 1977). In addition, there have not been good procedures for cleanly separating biofilm from the supporting substratum. The cryoembedding and cryosectioning results described in this study demonstrated the application of a simple histological technique for the imaging of biofilm cross-sections with minimal disruption. The frozen sections of biofilm provided images of biofilm bacteria with reasonably high resolution and demonstrated that the biofilm was maintained in a fully hydrated state during the cryoembedding procedure. The cell boundaries were clearly resolved without the shrinkage problems encountered in electron microscopy (Woldringh *et al.*, 1977). A major advantage of the cryosectioning technique is its ability to examine the biofilm regardless of the type of substratum employed. The residual water left in slide mounted frozen sections tended to quench DAPI fluorescence and resulted in excessive bleaching. Therefore, overnight air drying is recommended before examining biofilm frozen sections by fluorescence microscopy.

The OCT compound used in this study to provide support for the biofilm during quick freezing and sectioning is a mixture of water soluble polymers including polyvinyl alcohol and polyethylene glycol. It was originally developed to surround and support tissues during cryoembedding and cryosectioning. OCT maintained the intact structure of biofilm with minimal sample fixation and preparation. Quick freezing using dry ice minimized ice crystal formation in the specimen and allowed the embedded biofilm to be easily separated from the substratum. Liquid nitrogen cooled isopentane was not recommended for this quick freezing process because the lower temperature (-150°C) creates cracks in the OCT (Carson, 1990; Elias, 1990). Cracked OCT causes difficulties during cryosectioning resulting in split sections which are hard to collect on microscope slides.

The timing of OCT addition was critical to ensure appropriate cryoembedding and eventually allow a clean separation between the frozen biofilm and the substratum. The high viscosity of OCT tended to dislodge some of the biofilms when placed directly on unfrozen biofilms. In order to retain the biofilm integrity, the slides were first placed on dry ice and OCT immediately added to the biofilm. This permitted an initial freezing of biofilm components (i.e. water molecules, exopolymers, and bacteria), which stabilized and strengthened the biofilm structure from artifacts caused by forces associated with the addition of OCT. However, if biofilms stayed in contact with dry ice too long before covering with OCT, the interface between OCT and biofilm became unstable and poor removal of biofilms resulted. Simultaneous embedding and freezing preserved biofilm structure while

permitting effective removal from the substratum (Figure 14).

Sometimes, a visible gap was seen between the two layers of OCT. This indicates a poor binding of OCT to the sample, which causes sections to split (Callis *et al.*, 1991). This could also be due to excessive water in the samples resulting in ice layers that shatter during sectioning (Troyer, 1980). The existence of inorganic corrosion products in certain biofilms might destroy knife edges, and could also result in difficulties in sectioning samples.

Different embedding techniques have been applied to biofilms for measurement and evaluation of morphological parameters. Embedding media including paraffin (Li and Ganczarczyk, 1990), plastic resin (glycol methacrylate) (Stewart *et al.*, 1993; Ganczarczyk *et al.*, 1992) and agar (Ganczarczyk *et al.*, 1992) have been utilized. The cryoembedding technique described here involves less sample processing and is more rapid than any of these procedures. The whole process could be completed in less than 24 hours. The cryoembedding procedure should be compatible with specialized staining or labeling techniques, such as double staining, immunofluorescence staining, oligonucleotide probing and radioisotope labeling. Fixatives and stains may also be applied to frozen sections after air drying (Bancroft, 1982; Elias, 1990).

The cryosectioning technique described here offers an alternative minimally disruptive approach for biofilm microscopy. Individual cells, microcolonies, void areas and biofilms thickness were observed through the application of this technique. It is a simple, relatively rapid technique which required minimal sample preparation,

and can be applied in different areas of biofilm research at reasonable expense.

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CHAPTER 6

ASSESSMENT OF BACTERIAL RESPIRATORY ACTIVITY DURING BIOFILM
DISINFECTION BY IMAGE-ANALYZED
EPIFLUORESCENCE MICROSCOPYIntroduction

Biofilms cause a wide range of problems in various industrial water systems; such as increased frictional resistance, decreased heat transfer and induced corrosion. The accumulation of attached microorganisms can also lead to degradation of water quality resulting in product-related illness or violation of regulatory standards.

The control of biofilms has usually been implemented by the application of biocides. Many industrial systems have experienced persistent biofilm problems, even in the presence of significant residuals of disinfectants. Published reports have shown that the attachment of microorganisms to various surfaces confers increased resistance to disinfection (LeChevallier *et al.*, 1981; Ridgway and Olson, 1982; LeChevallier *et al.*, 1984; Camper *et al.*, 1986; Herson *et al.*, 1987; Costerton *et al.*, 1987; LeChevallier *et al.*, 1988a; Pyle and McFeters, 1990a; Brown and Gilbert, 1993). It has been recognized that environmental factors can also change the susceptibility of microorganisms. Bacteria grown in aquatic environments, whether source water or distribution water, are physiologically different from those cultured under laboratory conditions using rich nutrient media (Morita, 1985; Roszak and Colwell, 1987; Pyle and McFeters, 1989; Martin *et al.*, 1989; McFeters, 1990; Pyle and McFeters,

1990a; Pyle and McFeters, 1990b; Kjelleberg *et al.*, 1993, Pyle *et al.*, 1994).

Chlorine has been used most commonly as a disinfectant in water systems. However, the maintenance of a chlorine residual does not always correlate with reduced bacterial counts in the potable water systems (Reilly and Kippen, 1983; Olivieri *et al.*, 1985; LeChevallier *et al.*, 1987) and environmental exposure in the absence of disinfectants can also decrease culturability (Roszak and Colwell, 1987). These concerns are more acute when dealing with biofilms or microbial communities where spatial heterogeneities are established and may be complex. Therefore, a novel approach other than the conventional plate counting technique is needed to describe the dynamics of disinfection within bacterial biofilms.

Visualization of bacterial monolayer biofilms can be easily accomplished by either light or fluorescence microscopy (chapters 4 and 5). Due to the resolution limits of optical microscopy, studies on thicker biofilms require mechanical removal of biofilms from the substratum prior to analysis. The structural analysis of biofilms has recently been improved with the development of scanning confocal laser microscopy (SCLM). Its application offers visualization of reconstructed 2-D and 3-D images without physically disrupting the biofilm (Lawrence *et al.*, 1991; Caldwell *et al.*, 1992a; Caldwell *et al.*, 1992b). However, this technology involves relatively expensive instrumentation and has limited resolution when applied to thicker biofilms on opaque surfaces. These characteristics have restricted it from general application.

In a previous chapter, a new approach to visualize the structure of bacterial biofilms with the application of cryoembedding and cryosectioning techniques was

reported (chapter 5). Chapter 4 described an *in situ* method for assessing physiological activities of biofilm bacteria using the fluorogenic probes, 5-cyano-2,3-ditoly-tetrazolium chloride (CTC) and Rhodamine 123. This chapter describes the application of the CTC staining and cryoembedding-cryosectioning techniques to study changes in cellular respiratory activities of biofilm bacteria with exposure to disinfectants. The results were analyzed by epifluorescence microscopy and image analysis to provide qualitative and quantitative information describing the physiological consequences and penetration of biocides within bacterial biofilms.

Materials and Methods

Biofilm Apparatus

Binary population biofilms of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were grown on 316L stainless steel slides in an annular reactor. The operation conditions of this biofilm reactor system have been described in detail previously (Siebel and Characklis, 1991; Chen *et al.*, 1993a; Chen *et al.*, 1993b). The annular reactor was a continuous flow stirred tank reactor in which 12 removable slides were positioned around the inside wall of the outer drum. The reactors were operated at a dilution rate of 3.2 h^{-1} to ensure that the growth of planktonic cells was negligible.

Bacterial Strains and Growth Conditions

Klebsiella pneumoniae Kp1, isolated from drinking water, was obtained from Dr. D. Smith, South Central Connecticut Water Authority, New Haven, CT. An environmental isolate of *Pseudomonas aeruginosa*, ERC1, was taken from the culture collection at the Center for Biofilm Engineering. A phosphate buffered (pH 7.2) minimal salts medium (van der Wende, 1991; Chen *et al.*, 1993a) with 20 mg/L glucose as the sole carbon source was used to grow the biofilms. The inner cylinder of the annular reactor was rotated at 150 rpm to produce a uniform shear stress over the area on which biofilms were grown. The effluent was collected and followed by homogenization to break cell aggregates then plated on R2A (Difco) agar to monitor the growth of biofilm bacteria and to check for contamination on a daily basis. Both *K. pneumoniae* (Kp1) and *P. aeruginosa* (ERC1) were recognized by the shading and morphology of the colonies. The colonies of Kp1 on R2A plates appeared white, round with smooth convex surface, while ERC1 arose as cream white, irregular and rough flat colonies. Steady state was reached after 7 to 10 days of operation, as reflected by a steady concentration of cells in the reactor effluent. The average area on the stainless slide covered with biomass was 3145 mm².

Disinfection of Biofilms

The disinfectants used were chlorine (1 mg/L) and monochloramine (4 mg/L). The chlorine solution was prepared from sodium hypochlorite (Singh *et al.*, 1986), and the monochloramine solution was made with sodium hypochlorite and ammonium

chloride (LeChevallier *et al.*, 1988b). A pulse of concentrated disinfectant was added to bulk fluid (600 ml in volume) in the reactor which was immediately followed by steady addition of biocide to maintain the appropriate concentration. The biofilm slides were exposed to disinfectants in the reactor at pH 7.2 for chlorine and pH 9.0 for monochloramine at 25°C. The concentrations of the biocides were measured with a Hach DPD chlorine test kit (Hach Co., Loveland, CO).

Respiratory Activity Assessment

Biofilms were collected by withdrawing slides after 15, 30, 60, 90 and 120 min of disinfection. Slides were then placed in a staining container with the biofilm side up, and the disinfectant was neutralized by immersion in sodium thiosulfate (0.01%, final concentration). Respiratory activity within biofilms was determined using 5-cyano-2,3-ditolyl tetrazolium chloride (CTC, Polysciences, Inc.) by a procedure reported by Rodriguez *et al.* (Rodriguez *et al.*, 1992) with minor modification (chapter 3). Biofilm slides were immersed in 0.05% CTC solution for 2 h at 25°C. The specimens were fixed with formalin (5%, final concentration) then immediately stained with 1 µg/ml (final concentration) 4',6-diamidino-2-phenylindole (DAPI) (Sigma Chemical Co.) for 5 min. The biofilms were removed from the substrata by a cryoembedding technique (Yu *et al.*, 1994) using Tissue-Tek® OCT compound (Miles Inc.). The specimen was then wrapped in aluminum foil and stored at -70°C before cryotomy. Frozen sections were cut with a cryostat (Reichert-Jung Cryocut 1800, Leica) operated at -19°C. Frozen sections (5 µm thick) were collected

with glass slides for examination by epifluorescence microscopy.

The sections were examined using an Olympus BH-2 microscope with epifluorescence illumination (100-watt mercury lamp). An Olympus B filter cubic unit with excitation filter (BP490), a dichroic mirror (DM500) and a barrier filter (AFC+O515) were used to simultaneously visualize the CTC-formazan and DAPI fluorescence within the sectioned biofilms by the distinctive colors of each stain. The nonrespiring bacteria appeared green when stained with DAPI, whereas the respiring cells were green but contained red intracellular CTC-formazan crystals. Filter block G fitted with a O590 barrier filter could be used to visualize the red CTC-formazan crystals by excluding DAPI fluorescence, whereas the U excitation filter cubic unit with excitation filter (UG-1), a dichroic mirror (DM 400) and a barrier filter (L420) was used for visualizing the DAPI fluorescence.

Image Analysis

An American Innovision (AI) image processing system was used that consisted of a Pulnix color CCD camera (1.5 lux) with 512 x 400 pixels resolution for image capture. The software was run under an IBM-clone 80486 PC unit, which converted the epifluorescence micrograph into a digitalized image (a matrix of pixels assigned RGB values ranging from 0 to 255). Conversion of pixel unit to length (in μm) was accomplished by calibrating with an on-stage micrometer. The whole image area of the frame grabbed by the buffer was 45740 μm^2 .

The stored images from the AI system were converted to a TIFF file format

then analyzed with "MARK" software on a Hewlett Packard 9000/730 workstation. The "MARK" program was developed by Dr. Gary Harkin of the Center for Biofilm Engineering at Montana State University to be used on a UNIX operating system for image processing and analysis. Selected areas of the DAPI and CTC-formazan images were bracketed and superimposed to compare each pixel and determine the ratio of intensities of the two fluorochromes using the built-in "cross-section" function of the "MARK" program. The "% blackness" function of the image analysis software was employed to calculate the intensities of fluorescence in biofilm images captured by the video camera. The data processed by the UNIX system were stored as ASCII files which could be analyzed on PC-based systems.

Enumeration

Surviving biofilm bacteria were also assayed by scraping the biofilms from the slides followed by disaggregation of the cell suspensions in an ice bath for 3 min with a homogenizer (Tekmar) and enumeration using R2A agar and the plate counting method. The areal density of biofilm bacteria on the substratum is expressed as cfu/mm². Total cell numbers were obtained by enumerating DAPI stained samples collected on black polycarbonate membranes (0.2 μ m, Nuclepore).

Statistical Analysis

The microscopic enumeration results were obtained in each experiment from counts of 10 microscopic fields. Statistical analyses were performed on all data from

two to ten replicates using mathematical functions within SigmaPlot™ (Version 5.0 by Jandel Scientific) and one-way analysis of variance (ANOVA) with InStat™ (Version 1.1 by GraphPAD) computer software. The level of significance was set at $p < 0.05$ for all comparisons.

Results

Biofilm Chloramination

Chloramination of the binary population biofilms grown on stainless steel slides was performed using 4 mg/L monochloramine (pH 9.0) for a 2 h period. The total direct count (TDC) determined microscopically by the DAPI staining technique and the plate count (PC) results of attached cells removed from the slides are shown in Figure 15. The initial cell density (TDC) on the slide before monochloramine treatment was $1.50 \pm 0.50 \times 10^7$ cells/mm² (\pm SE), while the mean concentrations of viable cells determined by colony formation on R2A plates were $9.26 \pm 1.95 \times 10^6$ cfu/mm² for *Klebsiella pneumoniae* Kp1 and $2.40 \pm 0.94 \times 10^6$ for *Pseudomonas aeruginosa* ERC1, respectively (Figure 15). The differences in the enumeration of unexposed cell populations at time zero obtained with TDC and PC methods were not statistically significant ($p > 0.05$). After 2 h chloramination, a 2.31-log decrease in viable count (PC) was observed for KP1, whereas a 2.06-log reduction was detected in the ERC1 subpopulation. The TDC only showed a 0.47-log reduction.

The cryosectioning technique was employed to determine the effect of

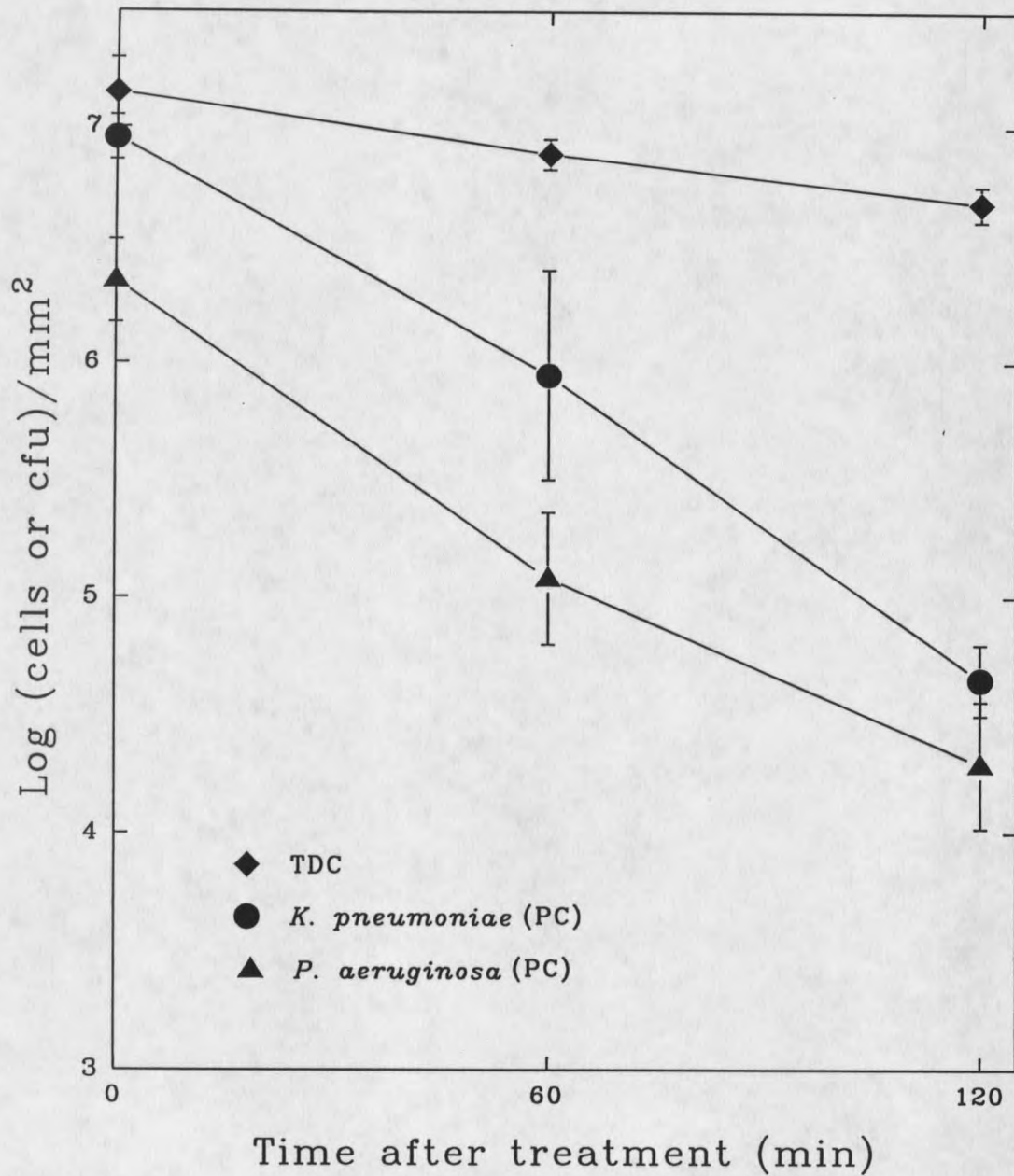
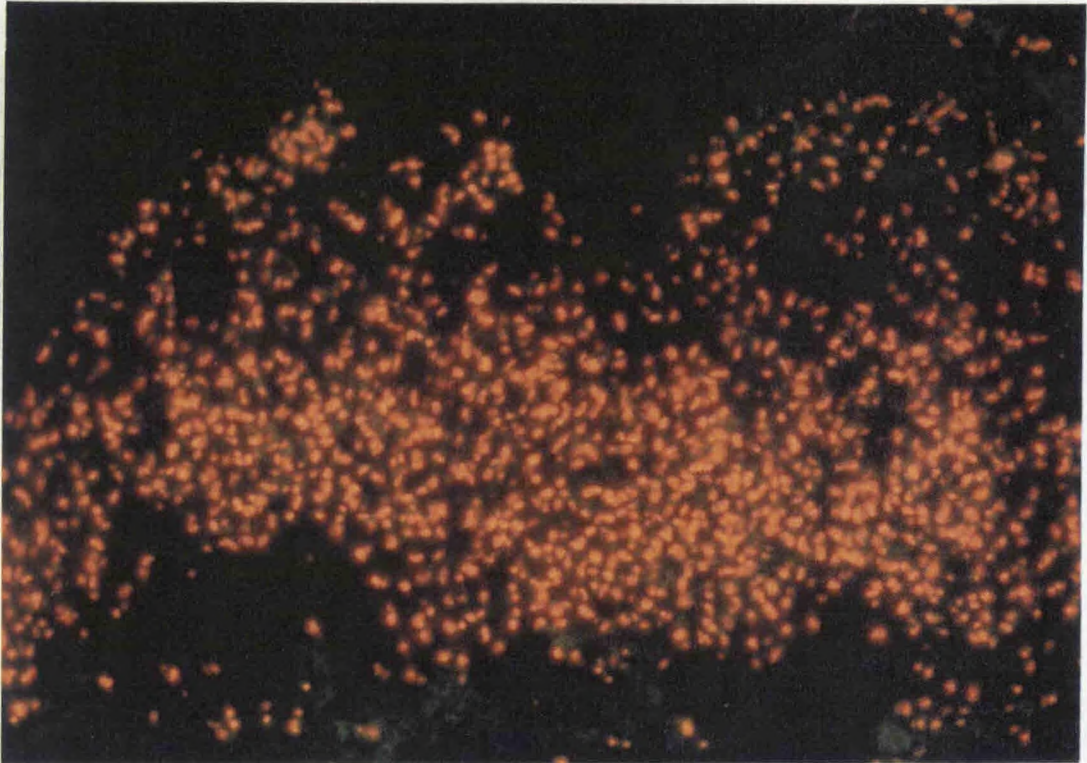


Figure 15. Total direct count (TDC) and plate count (PC) results of mixed *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* biofilms treated with 4 mg/L monochloramine (pH 9.0). ($n=2$, \pm S.E.)

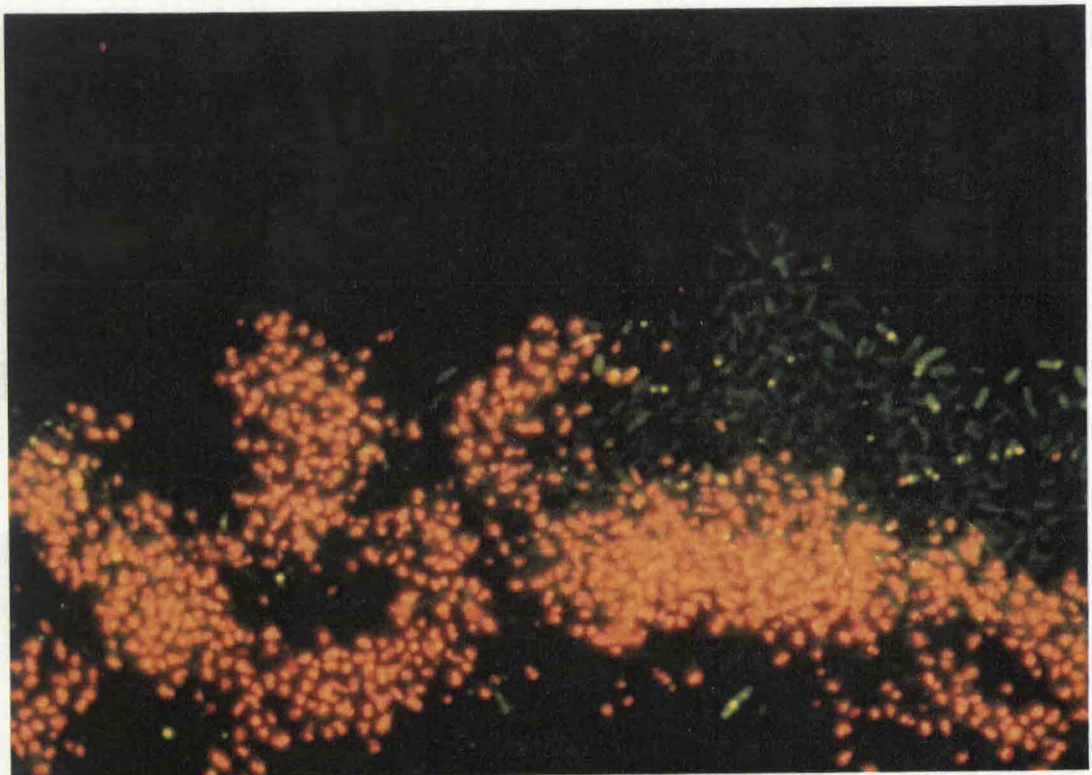
disinfection on bacterial respiratory activity within biofilms. The fraction of the bacterial population in a growing biofilm that demonstrated respiratory activity was determined by intracellular CTC reduction. The changes of CTC-formazan and DAPI intensities within biofilm bacteria observed at timed intervals (30, 60, 90 and 120 min) exhibited decreasing respiratory activity with time of chloramination (Figure 16). The unexposed biofilms shown in Figure 16(a) demonstrate active respiration within the xz cross-section of the biofilms by red-orange intracellular formazan crystals. During the disinfection treatment, a decline in respiratory activity was observed. Nonrespiring cells stained green with DAPI and respiring cells were also green but contained red-orange CTC-formazan as shown in Figure 16(b)-(e).

The ratio of fluorescence intensities of CTC-formazan/DAPI was employed as a parameter to describe the changes of respiratory activity within the biofilm cross-sections examined. Figure 17 shows the distribution of the two fluorochromes within a biofilm cross-section before and after 2 h chloramination. The thickness of untreated biofilms was approximately 72 μm . The peak intensity of fluorescent CTC-formazan was observed in the central area of the sample. The CTC-formazan/DAPI ratios detected were near 1.0 throughout most of the biofilm depth. This distribution of the two fluorochromes was similar to the image revealed in the epifluorescence micrograph as shown in Figure 16(a). An apparent decrease in respiratory activity, indicated by the loss of CTC-formazan fluorescence was observed after 120 min of monochloramine treatment (Figure 16(e)) while the CTC-formazan/DAPI ratio dropped to 0.1 and the thickness of biofilm was reduced to 30 μm (Figure 17).

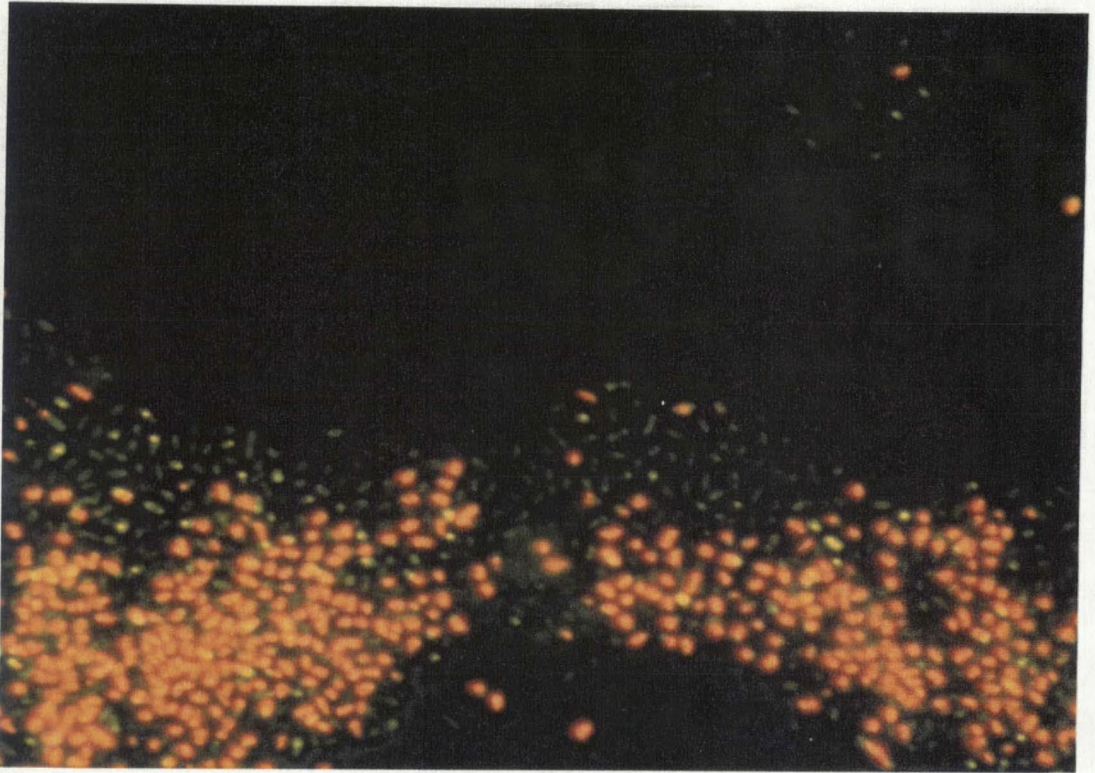


(a)

————— 10 μm

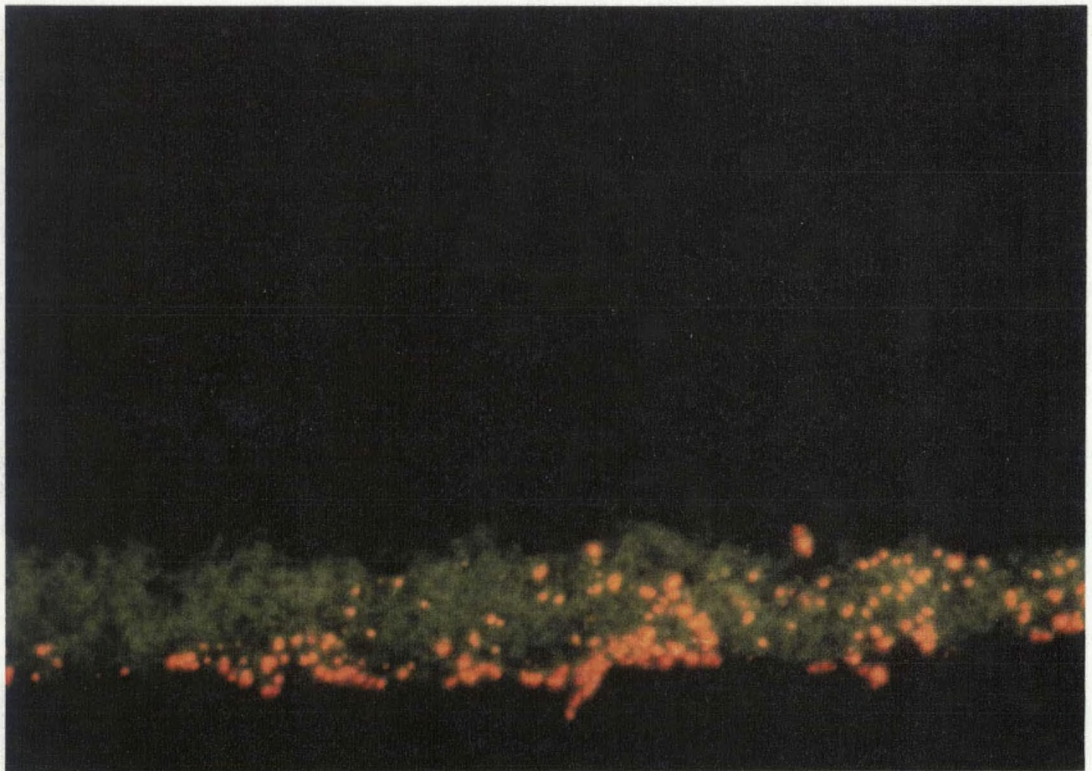


(b)



(c)

— 10 μm



(d)



Figure 16. Epifluorescence micrographs of frozen sections of a mixed *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* biofilm grown on stainless steel treated with monochloramine (4 mg/L). Disinfection intervals are as follows: (a) time 0, (b) 30 min, (c) 60 min, (d) 90 min and (e) 120 min. The biofilm bacteria were positioned so that the substratum was at the bottom, and bulk liquid interphase on top of the micrographs.

The comparison of relative intensities at timed intervals during chloramination, each representing the average of 10 different frames, is shown in Figure 18. The fluorescence intensities of CTC-formazan and DAPI were comparable within untreated biofilms as shown by the overlapping data points at time zero (Figure 18). However, differences in two fluorescent compounds became apparent as chloramination proceeded. The results also showed a reduction in total biomass (DAPI) and a greater loss of respiratory activity (CTC) within the treated biofilms.

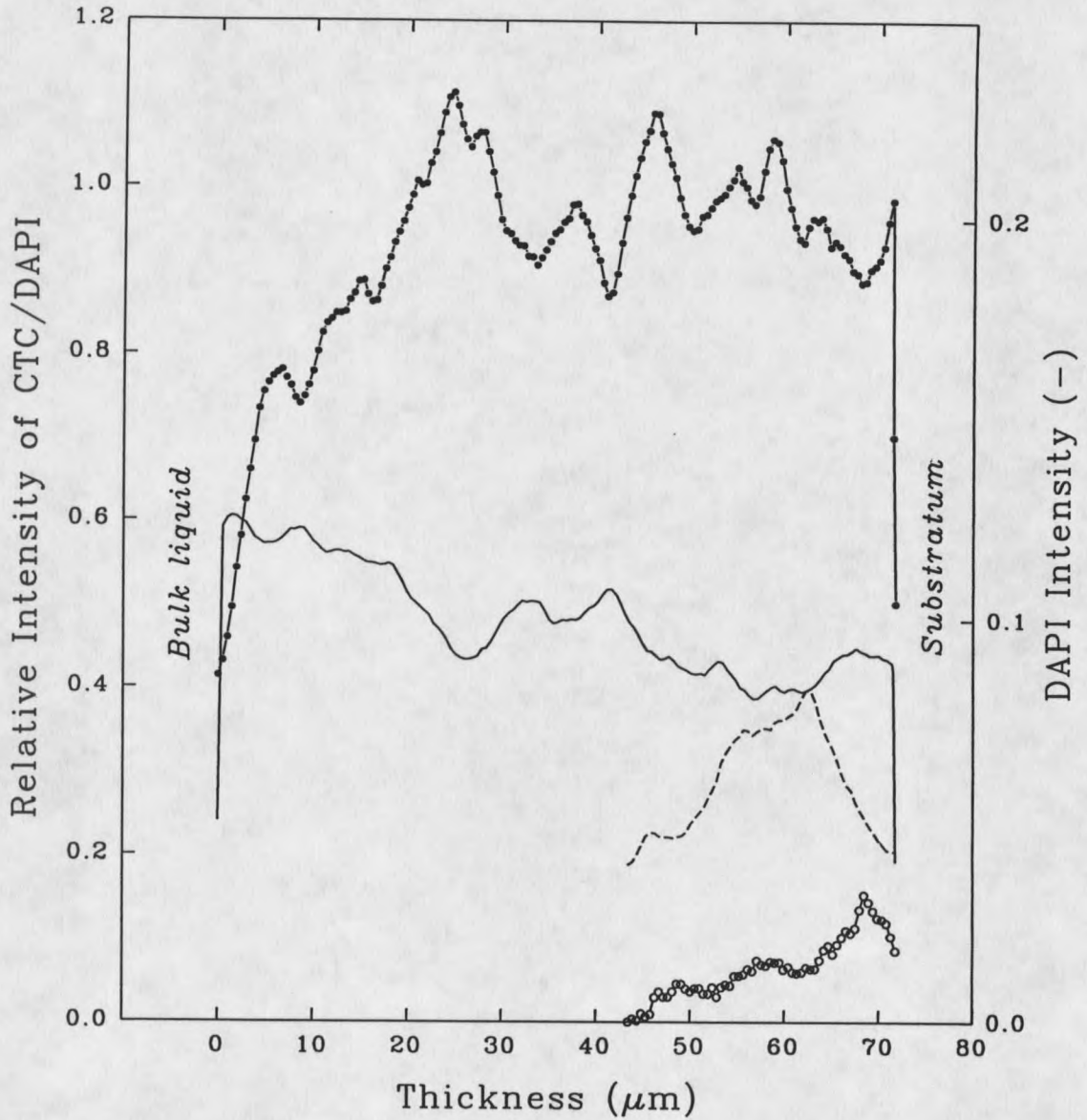


Figure 17. Comparison of CTC-formazan:DAPI fluorescence intensities obtained from 5 μm frozen sections between disinfected and untreated control of mixed *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* biofilms. The ratios of CTC/DAPI were expressed as follows: (-●-, time zero control; -○-, 2 h chloramination). The solid line was used to show the intensities of DAPI for untreated biofilms, while short dash line was for disinfected biofilms.

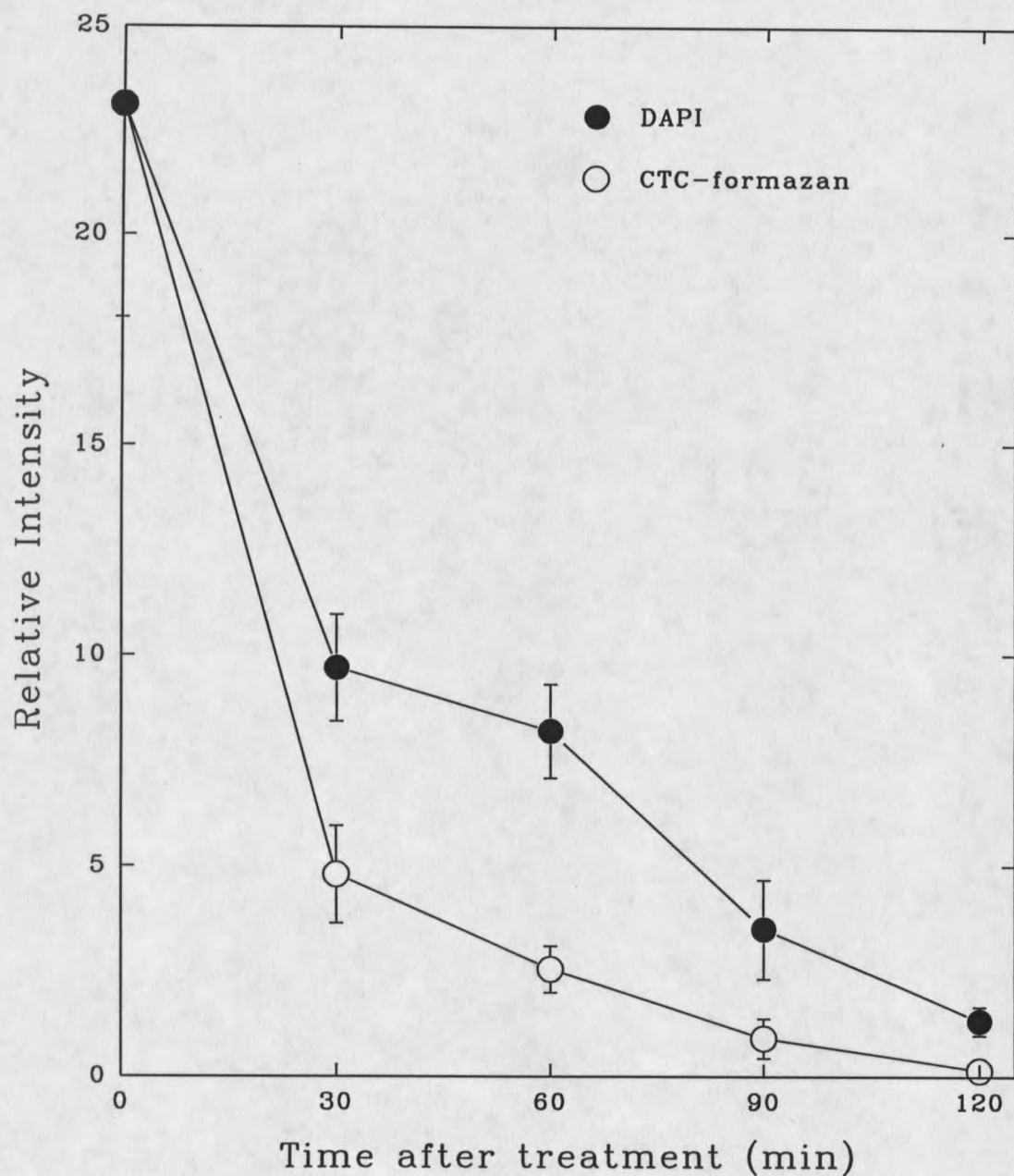


Figure 18. Changes in relative intensities between DAPI and CTC-formazan within the mixed *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* biofilms during 2 h chloramination (4 mg/L, pH 9.0) treatment. ($n=10$, \pm S.E.)

Biofilm Chlorination

The PC and TDC results of similar experiments using chlorine (1 mg/L, pH 7.2) to treat mixed-culture (Kp1 and ERC1) biofilms are shown in Figure 19. A reduction in the density of viable cells of both strains after 120 min chlorination was observed. There were 2.17-log and 2.54-log decreases detected for KP1 and ERC1, respectively. Statistical analysis showed no significant difference ($p > 0.05$) in the susceptibility of the two bacterial strains to chlorine. The total areal density of biofilms on the substratum showed a 0.47-log reduction after 120 min chlorination. This is comparable to what was observed following chloramination (Figure 15).

The changes in bacterial respiratory activity within biofilm sections for timed intervals (0, 30, 60, 90 and 120 min) of chlorination showed a similar pattern as seen in Figure 16 (micrographs not shown). The comparison of relative intensity ratio of CTC-formazan/DAPI for chlorination show a behavior similar to that seen for chloramination as presented in Figure 17 (data not shown). However, the mean areal density of biofilm cross-sections showed that significantly higher fluorescence intensities ($p < 0.05$) were detected both for DAPI and CTC-formazan after chlorination (Figures 18 & 20). This indicated greater biofilm removal and respiratory impairment was achieved by chloramination than by chlorination using these measurements.

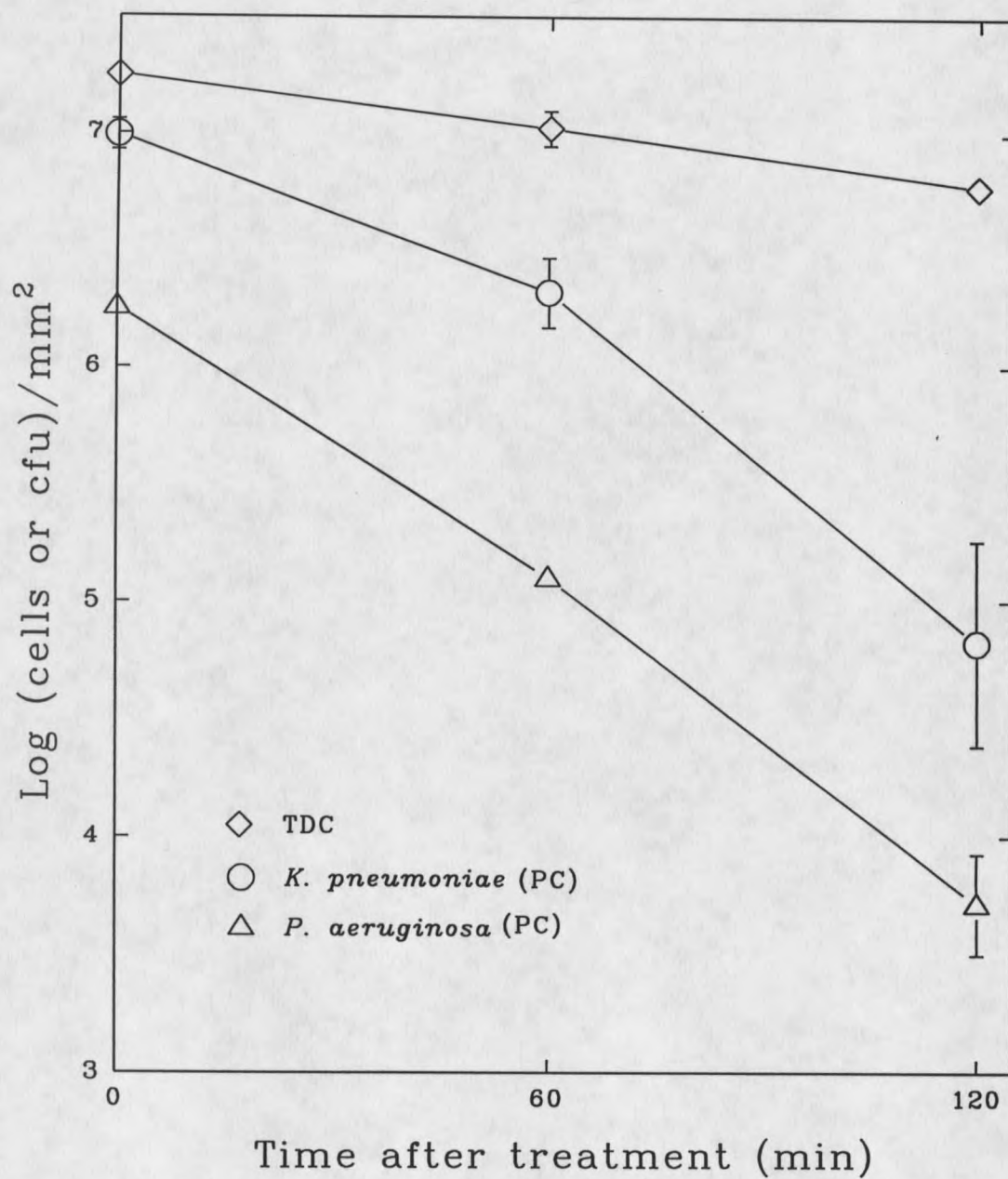


Figure 19. Total direct count (TDC) and plate count results of mixed *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* biofilms treated with 1 mg/L chlorine (pH 7.2). ($n=2$, \pm S.E.)

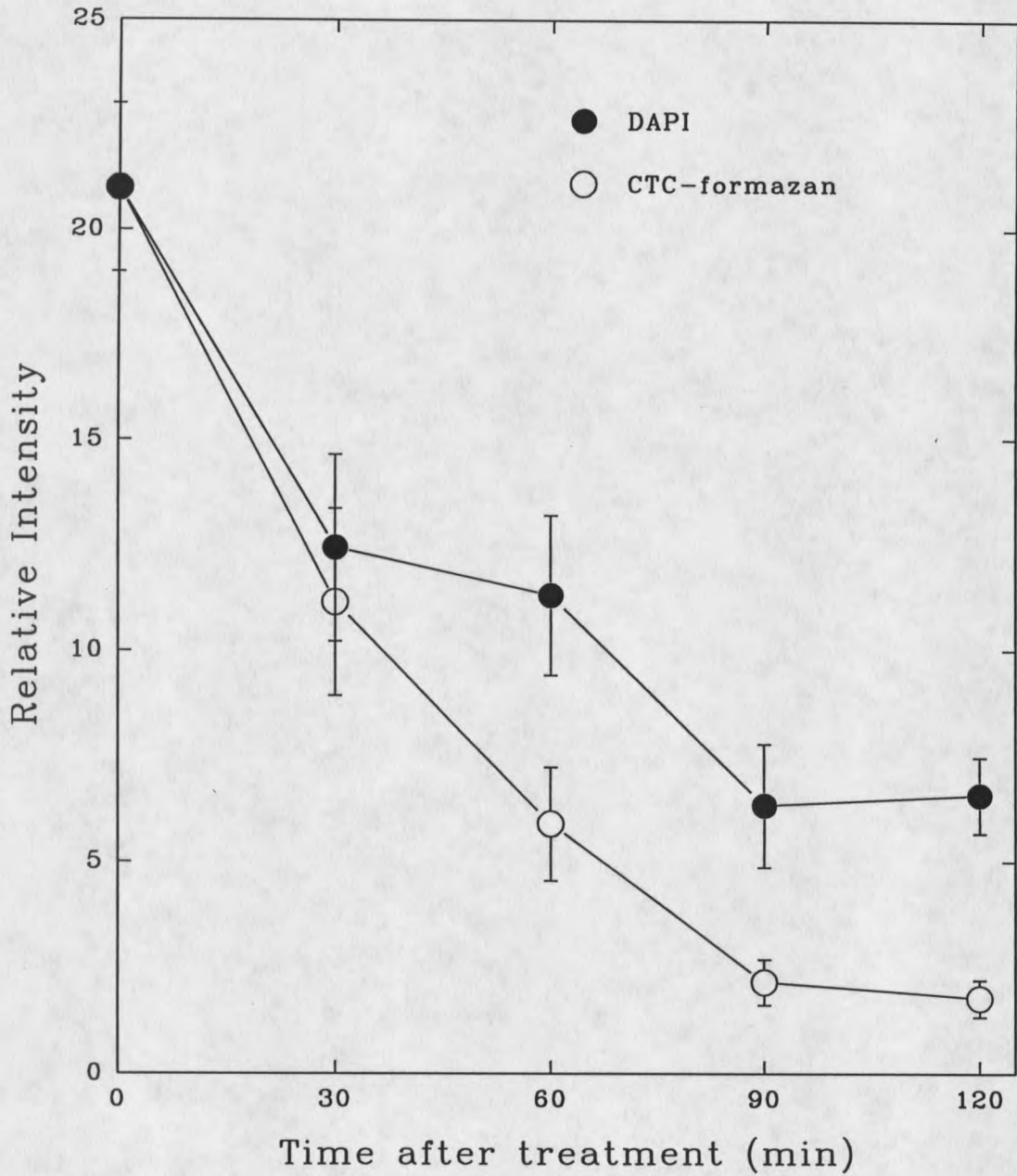


Figure 20. Changes in relative intensities between DAPI and CTC-formazan within the mixed *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* biofilms during 2 h chlorination (1 mg/L, pH 7.2) treatment. ($n=10$, \pm S.E.)

Discussion

The application of image analysis technology provides quantitative information from complicated micrographs and is now routinely used as a rapid means for the enumeration of microorganisms (Korber *et al.*, 1989b; Sjollem *et al.*, 1989; Korber *et al.*, 1990; Lawrence *et al.*, 1992). Fluorescence microscopy further extends the range of information that can be obtained by image analyses from size, shape and behavioral data to the identification cellular macromolecules (Sieracki *et al.*, 1985; Singh *et al.*, 1989; Sieracki *et al.*, 1989; Singh *et al.*, 1990; Wynn-Williams, 1992; Caldwell *et al.*, 1992b).

Conventional methods using plate counting and direct enumeration techniques gave comparable results regarding disinfection efficacy when two different biocides were applied (Figures 15 and 19). There were no significant differences ($p > 0.05$) either in total cell densities (TDC) or viable cell populations (PC) between 2 h. treatment with monochloramine (4 mg/L) and chlorination (1 mg/L). This approach did not reveal the mechanism of disinfectants reacting with the biofilm bacteria. However, with the utilization of the biofilm cryosectioning technique and a fluorescent probe (CTC) for bacterial respiratory activity, we were able to observe how monochloramine affected respiratory activity within a heterogeneous biofilm community (Figure 16). It should be noted that untreated biofilm bacteria did not express 100 % respiratory activity, even in the presence of unlimited nutrients in a controlled laboratory system, as shown in Figure 16(a). The penetration of biocide

into biofilms was indicated by the loss of respiratory activity, as evidenced by CTC-formazan fluorescence (red-orange) while the non-physiological DAPI fluorescence (green) was retained, Figure 16(b)-(e). The epifluorescence micrographs obtained with chlorine treatment did not show a significantly different pattern of changes in intensities of CTC-formazan and DAPI. Therefore, those results were not included in this report. Partial removal of biofilm bacteria by the disinfectants was also observed in Figure 16 during the 2-h treatment time, although the TDC data (Figures 15 and 19) did not show significant reduction in total cell numbers ($p > 0.05$). This apparent contradiction might be explained by the structural heterogeneity of biofilms (Stewart *et al.*, 1993). That factor might affect the action of biocides and the bacteria within the microcolonies could be more difficult to remove. As pointed out previously in chapter 4, the thickness of the biofilms that were examined ranged from 60 to 500 μm . Also, it should be noted that the epifluorescence micrographs (Figure 16) shown were only a very small portion of the entire biofilm community that was studied. The images were arbitrarily chosen to represent the distinctive physiological responses in biofilms during different treatment times. In addition, mechanisms cannot be inferred for biofilms exposed to monochloramine and chlorine without the collection of a larger quantity of images and additional statistical analysis.

A comparison of DAPI and CTC-formazan gradients within biofilms during disinfection (Figures 17, 18 and 20) showed a dramatic decrease in fluorescence intensities of both fluorochromes. This indicated reductions in both total biomass (stained by DAPI) and respiratory activity (CTC reduction) with disinfection. The

spatial distribution of CTC-formazan was evenly distributed in all areas of unexposed biofilms (Figure 17). However, after 2 h chloramination the intensity of CTC-formazan decreased but displayed a gradient indicating a stronger fluorescence and greater respiratory activity remained in bacteria near the substratum. This evidence from image analysis suggests that diffusion limits biocide penetration near the substratum interface and agrees with what was observed from the timed-series epifluorescence micrographs (Figure 16). The observation of CTC-formazan/DAPI ratios that sometimes exceeded 1.0 was due to the stronger fluorescence observed for CTC-formazan and by the different images captured by the color CCD camera. This problem might be corrected by reducing the incubation time for CTC staining from 2 h to 1 h, which would allow smaller amounts of formazan crystals to form.

Image analysis involves a number of functions linked together to form a path of computer operations including image acquisition, image processing, image segmentation, object recognition, object measurement and data output (Caldwell *et al.*, 1992a). During the conversion of digitalized color images stored on the AI system into a TIFF file format, the RGB palette values were merged into gray values ranging from black to white. However, the gray level distribution is not normally partitioned over the usable level range (Korber *et al.*, 1989a), and some resolution was lost during the image conversion process. In addition, the low signal intensity and tendency of fluorescent molecular probes to fade, requires the use of a light-sensitive video camera, and extensive image processing to accurately define cell boundaries (Sieracki *et al.*, 1989). The "MARK" software was originally designed

for general application in image processing and analysis. However, parameters for additional analysis not supplied with the software package may be programmed for specific purposes.

By comparing 10 image frames collected for each timed interval of biocide exposure, we were able to differentiate between the antimicrobial mechanisms seen with chloramination and chlorination (Figures 18 and 20). Statistical analysis of the results indicates that monochloramine removed more total biomass and caused higher decline in respiratory activity ($P < 0.05$) within the biofilm community. However, both treatments showed comparable results when determined by the TDC and PC methods. Using 10 images of biofilm frozen sections would not be a good representation of the whole biofilm community grown on the slide. Therefore, in the absence of enough micrographs for further statistical analysis, our findings somewhat are inconclusive.

Overall, the image analysis system offers a very powerful capacity for quantitatively analyzing micrographs and providing numerical data in addition to the qualitative information from the images. Statistical analysis can also be performed to determine the level of significance. Using this capability, there are many novel approaches that can be attempted to study the physiological activities and responses of bacteria within biofilm to disinfection. This report offers alternative *in situ* approaches for the measurement of biocide efficacy. It is more rapid than the plate counting technique, and provides additional qualitative and quantitative information describing spatial variations within biofilm communities after biocide treatment.

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CHAPTER 7

SUMMARY AND DISCUSSION

General Conclusions

It has been eighty-one years since research on biofilms was initiated (Söhnngen, 1913). Biofilm science/technology is still a relatively new technical discipline and continues to emerge in response to the tremendous opportunities and significant costs resulting from pervasive microbial activities at interfaces (Characklis and Marshall, 1990). The influences of biofouling have been recognized in a wide range of areas including most industries, medicine and public health. Much progress has been made in understanding the physiology and ecology of attached microorganisms. Lately, the establishment of the Center for Biofilm Engineering (CBE) at Montana State University and a colloquium held in 1993 American Society for Microbiology General Meeting have further demonstrated the importance of biofilm study in scientific research.

The main goal of this study was to develop novel methodologies for monitoring the chemical control of microbial biofouling in municipal and industrial water systems. It has been recognized that environmental factors can change bacterial susceptibility to disinfection. Microorganisms grown in aquatic environments, whether source water or distribution water, are physiologically different from those

cultured under laboratory conditions using rich nutrient media (Morita, 1985; Roszak and Colwell, 1987; Martin *et al.*, 1989; McFeters, 1990; Kjelleberg *et al.*, 1993). The choice of appropriate nutrients for the detection of bacteria has been affected by environmental exposure (Desmots *et al.*, 1990; Amann *et al.*, 1992; Binnerup *et al.*, 1993) and sublethal injury (Camper and McFeters, 1979; McFeters *et al.*, 1982; LeChevallier *et al.*, 1983; LeChevallier *et al.*, 1984; Singh and McFeters, 1986; Pyle and McFeters, 1989; Singh *et al.*, 1990; Pyle and McFeters, 1990a; Pyle and McFeters, 1990b). As a result, the use of established culture media often leads to serious underestimations of the bacterial population density and complexity of natural and engineered systems. These considerations present very real and practical constraints on the reliability of established media and suggest the need for developing novel cultural approaches.

An environmental isolate, *Klebsiella pneumoniae* (Kp1) was initially employed to grow biofilm on 316L stainless steel (SS) surfaces in a stirred batch reactor as the model system (Chapters 2, 3 and 4). The growth of biofilm bacteria relied on a limited supply of undefined nutrients (1/10 trypticase soy broth) for a relatively short period (within 48 h). This experimental design was not intended to mimic the natural environments but to grow biofilms under controlled laboratory conditions in a short time. Our preliminary study determined that 0.25 mg/L chlorine (pH 7.2) and 1 mg/L monochloramine (pH 9.0) gave comparable disinfection results when judged by the plate count (PC) method using TLY agar. It should be noted that biofilm formation was limited to bacterial monolayers for assessing biocide action with direct

microscopic examination in this stage of the research.

Different approaches have been attempted in this study to investigate additional alternative methods to effectively evaluate the efficacy of biofilm disinfection.

Nalidixic acid, an antibiotic and DNA gyrase inhibitor, was used to estimate substrate utilization and viability (Hadrys *et al.*, 1992; Kogure *et al.*, 1980; Kogure *et al.*, 1984) of bacteria (chapter 2) with the addition of appropriate nutrients. Fluorogenic compounds, 5-cyano-2,3-ditolyl-tetrazolium chloride (CTC) and rhodamine 123 (Rh 123) were employed to assess the physiological responses of bacteria within biofilms. CTC is a soluble redox indicator which can be reduced by respiring bacteria to fluorescent intracellular CTC-formazan crystals (Stellmach, 1984; Rodriguez *et al.*, 1992). Rh 123 is incorporated into bacteria in response to transmembrane potential (Matsuyama, 1984; Resnick *et al.*, 1985). Nucleic acid dyes, acridine orange (AO) and DAPI were used to stain intact bacterial cells for microscopic examination. The intracellular accumulation of these fluorochromes can be observed using epifluorescence microscopy. A Radioisotope labeling technique using tritiated uridine incorporation (TUI) was also utilized to determine the global RNA turnover rate in this study.

Binary populations of Kp1 and *Pseudomonas aeruginosa* (ERC1) were then used to grow thicker and more mature biofilms in a continuous flow annular reactor with defined nutrients during the second stage of research (chapters 6 and 7). This later experimental design was intended to simulate the growth of biofilms in pipe systems. It is likely that thicker biofilms respond differently to disinfection.

Therefore, the dosage of disinfectants was raised to 1 mg/L for chlorine and 4 mg/L for monochloramine, respectively. The *in situ* enumeration approach using fluorescent probes developed with the batch reactor system was successfully applied to biofilm studies in a continuous flow system.

Relative to this time-course study of biofilm disinfection, the results from the preceding chapters are summarized as follows:

1. The direct viable count (DVC) method was adapted and applied directly to assess the viability of bacteria in thin biofilms. The results could be observed after 4 h instead of the 24 h incubation time required for colony formation (chapter 2).
2. *In situ* enumeration methods using fluorogenic probes (CTC and Rh 123) and radioisotope (tritiated uridine) labelling techniques showed that the DVC response and respiratory activity were affected more by disinfection than the transmembrane potential and RNA turnover rate. These approaches provided different physiological insights that may be used in evaluating the efficacy of biofilm disinfection (chapters 3 and 4).
3. The biofilm removal by the biocides was determined by enumerating total cell numbers that remained on the substratum (chapters 2 and 4).
4. A method for rapid and minimally disruptive embedding and sectioning of biofilms was developed. The resulting sections could be examined by light or epifluorescence microscopy. Heterogeneous structures within the biofilm matrix including irregular interfaces with the bulk liquid, water channels, local protrusions up to 500 μm thick, and a well-defined substratum interface were visualized (chapter

5).

6. The utilization of image analysis provided a quantitative evaluation of epifluorescence micrographs of biofilm cross-sections and allowed a more descriptive assessment of disinfection efficacy than the conventional plate count technique (chapter 6).

7. Greater removal of bacteria from biofilms was observed in monochloramine treatment than in chlorination (chapters 2, 4 and 6).

By characterizing the physiological responses of adherent bacteria during disinfection, we were able to obtain information regarding cell numbers and physiological activities of bacteria, as well as mechanisms of biocides within biofilms. These advances enabled a more thorough evaluation of disinfection efficiency than with conventional methods.

Discussion

The common way to determine whether a bacterium is alive or dead is to examine its capacity to multiply on a suitable medium. It is generally agreed that a dead bacterium is one that has irreversibly lost its ability to reproduce. Since bacteria might temporarily lose their reproducibility and become dormant or injured, many arguments have arisen concerning the definition of viability. The classical definition of death in procaryotes largely ignores the physiological state of the microorganism. A bacterium may lose its capacity to reproduce but still respire, take up nutrients and

metabolize them, as well as maintain membrane integrity. In addition, of the vast number of microorganisms in natural habitats including soil, water and other microenvironments, only a very small portion has been cultivated and characterized. Therefore, the concepts including viability, death, dormancy and survival based on our present understanding in procaryotes are somewhat arbitrary.

The results from different physiological activity assessments indicated various responses during the disinfection of biofilm bacteria. Specifically, respiratory activity, determined by CTC reduction, was affected to a larger extent than the transmembrane potential (Rh 123 staining) and RNA turnover rate (tritiated uridine incorporation). However, these findings did not imply that any one of these methods is better than another in evaluating efficacy of biofilm disinfection.

CTC reduction is only an indicator of respiratory enzyme activity in the electron transport system. The exact enzyme reduction mechanism of CTC is not clear yet, and is still under investigation. However, transmembrane potential can be contributed from different processes (e.g. by the operation of the electron transport chain; by the hydrolysis of ATP by a proton ATPase; by the hydrolysis of pyrophosphate by a proton pyrophosphatase; by end-product efflux; or by extracytoplasmic oxidation). When the enzyme(s) responsible for CTC reduction is inactivated during disinfection, bacteria might be able to utilize an alternative pathway to maintain the transmembrane potential. As for the RNA turnover rate, there are a lot more processes involved in its regulation, which is less likely to be significantly affected by biocides. Therefore, it is reasonable that CTC reduction is more

susceptible to the action of biocides that are known to disrupt bacterial respiration (Camper and McFeters, 1979) than membrane potential and RNA turnover rate. In addition, it is probably unwise to rely solely on one physiological process to judge the activity or viability of microbial populations. All the approaches described in this report can be used to thoroughly evaluate the efficacy of disinfection.

The development of advanced computer technology has become useful in the automation of microbiological laboratories. Image processing and analysis are routinely used as a rapid means for the study of microbial accumulation on substrata (Korber *et al.*, 1989; Sjollem *et al.*, 1989; Korber *et al.*, 1990; Lawrence *et al.*, 1992). Our previous reports have shown that rapid detection of viable bacteria and chlorine-induced injury can be accomplished by the utilization of image analysis with the DVC method (Singh *et al.*, 1989; Singh *et al.*, 1990). Other applications include determination of bacterioplankton (Bjørnsen, 1986), analysis of bacterial growth as well as behavior on surfaces (Lawrence *et al.*, 1989), and the assessment of antimicrobial activity of antibiotics (Hammonds and Calliford, 1991). The introduction of scanning confocal laser microscopy (SCLM) and 3-D image analysis has established a new analytical capacity by eliminating interference from out-of-focus objects and creating computer reconstructed 2-D or 3-D images of biofilms and bioaggregates (Caldwell *et al.*, 1992).

Unresolved Issues

The regrowth and recovery of biofilm bacteria after disinfection were not included in this study. Such experiments would provide valuable insights into the eventual consequences of biocide application on the reestablishment of a growing biofilm. In addition, the Rh 123 staining technique might provide different information describing the disinfection of biofilms grown in a continuous flow annular reactor. This approach was also not sustained in this study. Numerous fluorogenic compounds for assessing different physiological activities (Haugland, 1992; Mason, 1993) are available. There is a very good potential for further exploration in this field of microbiological study with some of these other fluorogenic compounds (McFeters *et al.*, 1994).

Using such fluorescent probes, SCLM, computer image analysis, and other approaches, it should be possible to visualize not only the organisms and their positioning but also their behavior, growth, metabolism, gene expression, genetic sequencing as well as the surrounding ambient physicochemical and biochemical environments. The drawback of this advanced technology is the cost of the instruments that may not be affordable for most microbiological laboratories. Our approach was thus focused on the utilization of fluorescence microscopy to achieve nondestructive, *in situ* analyses of the physiological (i.e. especially respiration using CTC) heterogeneities within biofilms. That goal has been realized. Our approach is simple, relatively rapid, requires minimal sample preparation, and can be applied in

different areas of biofilm research at reasonable cost.

Industrial Relevance

The traditional technique of assessing biocide efficacy has relied on enumerating surviving bacteria by colony formation following removal from the substratum. This approach usually overestimates the efficiency of antimicrobial chemicals since it underestimates the number of surviving bacteria. Our study used a different approach to reveal changes in physiological responses inside biofilms with biocide exposure. This method represents an alternative *in situ* approach for the analysis of biocide efficacy. It is more rapid than the plate counting technique, and provides additional information about spatial variations of biocide action within biofilms.

A major goal of scientific research in the academic environment is to provide knowledge and practical tools that might be applied in other disciplines and areas of potential use. The methodology development in this study is applicable in a range of industries, such as drinking water treatment. In fact, three Industrial Associates affiliated with CBE have already applied our cryoembedding-cryosectioning and fluorescent probe techniques in their systems. In addition, a workshop session in the application of image analysis has recently been offered by CBE to introduce the latest methodologies and knowledge that has been developed in the center.

To quote from Albert Einstein, "It stands to the everlasting credit of science

that by acting on the human mind it has overcome man's insecurity before himself and before nature." It is hoped that the research which has been accomplished in this study will make a significant contribution to the understanding of biofilms and the control of biofouling.

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APPENDIX

Application in Fourier Transform Infrared Spectroscopy

Recently, the chemical changes that occur within microbial biofilms during colonization have been monitored using attenuated reflection Fourier transform infrared spectroscopy (ATR/FT-IR) (Bremer and Geesey, 1991; Mittleman *et al.*, 1990; Naumann *et al.*, 1991a; Naumann *et al.*, 1991b; Nivens *et al.*, 1993). Chemical information describing biofilm components such as proteins and extracellular polysaccharides is obtained from the interphase between a germanium internal reflection element (IRE) and the growth medium (Bremer and Geesey, 1991). This technique was utilized to monitor penetration of a fluoroquinolone antibiotic, ciprofloxacin, to the base of *P. aeruginosa* ERC1 biofilms (Suci *et al.*, 1994). The biofilms were grown on a germanium IRE within a Micro-Circle cellTM of a Perkin Elmer Model 1800 FT-IR spectrophotometer (Figure 21). The resulting biofilms were treated with ciprofloxacin (100 $\mu\text{g/ml}$) for 21 min. The penetration of the antibiotic was examined by means of the CTC staining and cryoembedding-cryosectioning techniques reported in chapters 5 and 6. Epifluorescence micrographs of the control and treated biofilm cross-sections are shown in Figure 22. The colonization of the cylindrical surface by ERC1 cells (Figure 21) was fairly confluent. Some detachment of interfacial bacteria was indicated by the ATR/FT-IR results (Suci *et al.*, 1994). The spatial structure of the unexposed biofilm cross-section is shown in

Figures 22(a), with a mean thickness of $27.1 \pm 8.2 \mu\text{m}$. The treated biofilms were measured as $15.1 \pm 6.2 \mu\text{m}$ (Figure 22(b)), which indicated the antibiotic caused some detachment of attached bacteria. The penetration of ciprofloxacin into ERC1 biofilms was indicated by the loss of respiratory activity with depth as shown in Figure 22(b), which was also observed by signals detected by ATR/FT-IR (Suci *et al.*, 1994). Other possible applications utilizing the techniques developed in this study are still under investigation.

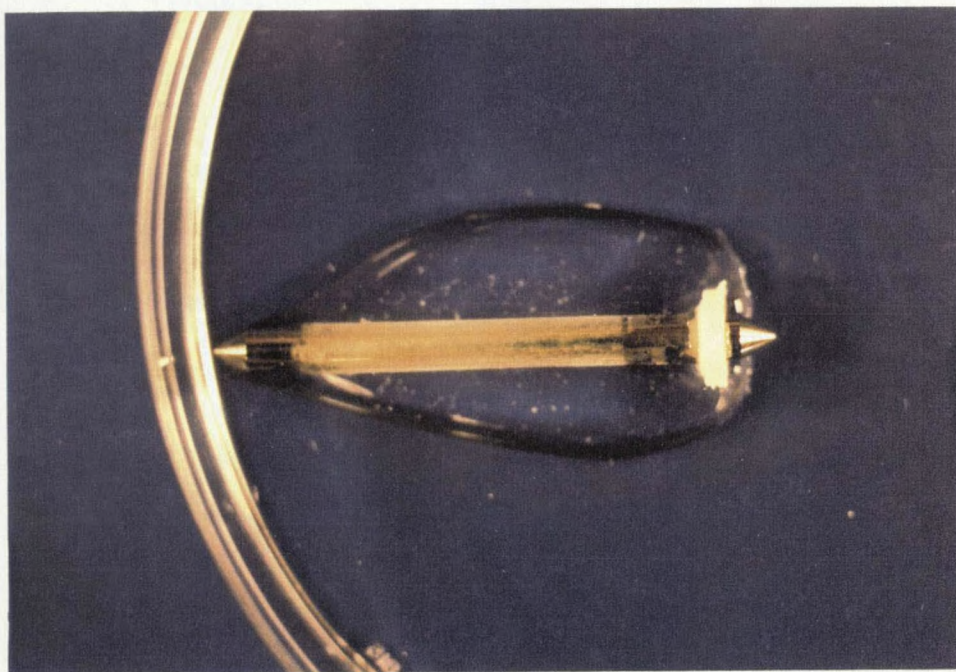


Figure 21. Plan view of *Pseudomonas aeruginosa* ERC1 cells colonized on the surface of a cylindrical germanium internal reflection element (IRE).

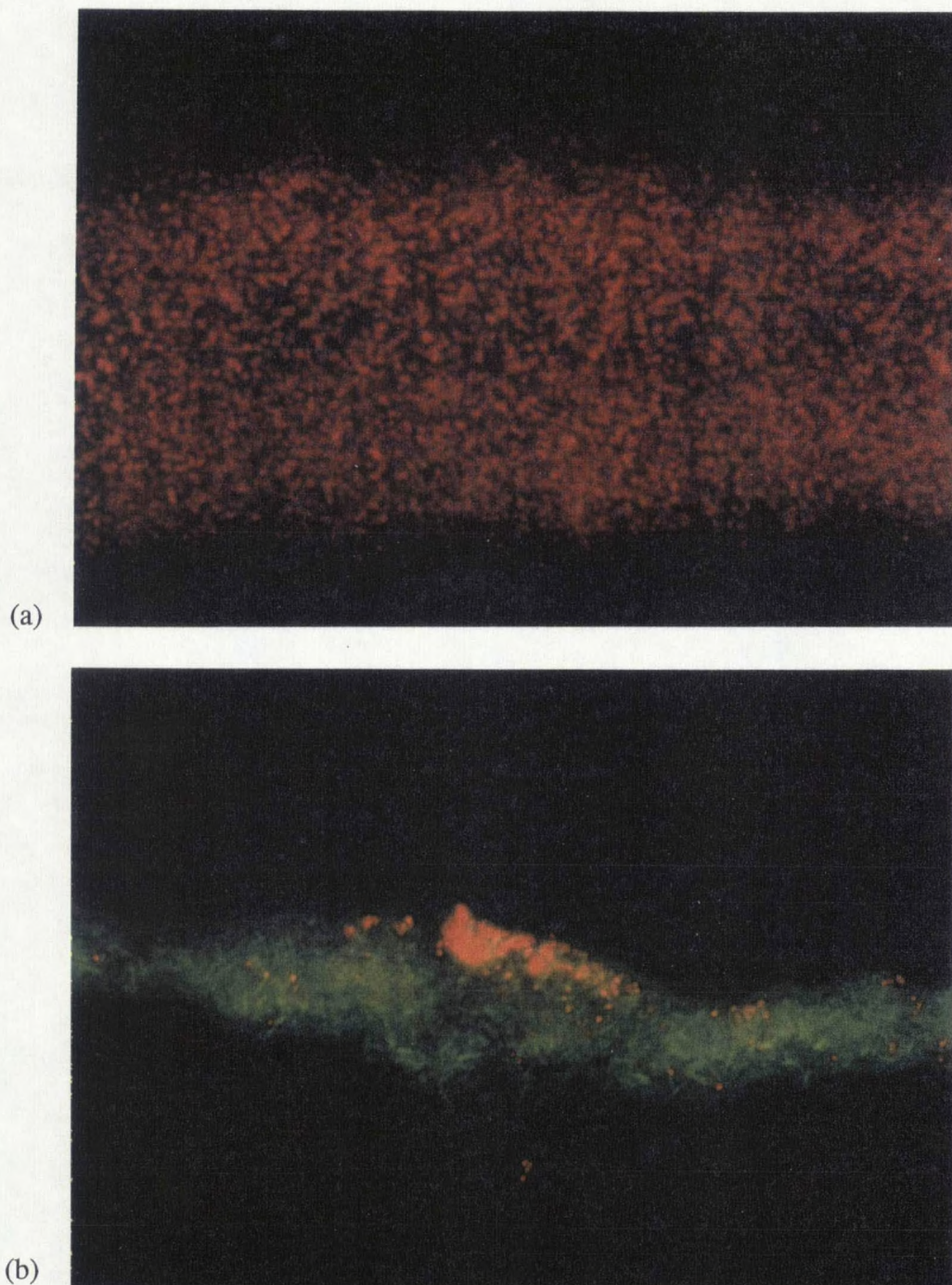


Figure 22. Epifluorescence micrographs of frozen sections of *Pseudomonas aeruginosa* biofilm grown on a cylindrical germanium internal reflection element (IRE) treated with ciprofloxacin (100 $\mu\text{g}/\text{ml}$) and stained with CTC. (a) unexposed control and (b) treated biofilms.

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