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

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 DAPL 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.

RAPID *IN SITU* PHYSIOLOGICAL ASSESSMENT OF DISINFECTION IN BACTERIAL BIOFILMS

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APPROVAL

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Feipeng Philip Yu

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

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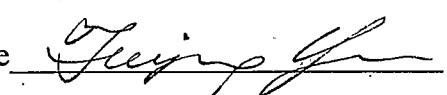
Signature 

Date 4/27/94

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/cm^2 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:

$$HOCl \rightleftharpoons H^+ + OCl^-$$

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 Cl_2 . 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:

$$NaOCl + NH_3 \longrightarrow NH_2Cl + NaOH$$

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 (NH_2Cl). The difference in potency of NH_2Cl 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, bisbenzimide (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-ditolyl 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 (Söhngen, 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