Application of the biofilm coupon as a direct measure of the in situ growth potential of water
by Shannon Gaylord Bakich

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

Abstract:
Coliform bacteria that grow and persist in drinking water distribution systems affect the delivered water quality for the consumer. The coliforms that are most responsible for regrowth events are biofilm organisms growing at the expense of nutrients in the bulk water. The research project "Factors Limiting Microbial Growth in the Distribution System" funded by the American Water Works Research Foundation and NSF Center funding worked with select utilities across the United States to establish strategies for limiting microbial growth, particularly biofilms, in drinking water distribution systems. One of the objectives of the study was to determine conditions that allow coliforms to establish and persist in mixed population biofilms. Nutrient loading, particularly assimilable organic carbon (AOC) is one of the key variables. Several methodologies have been developed to measure AOC. However, these methodologies are labor-intensive, time-consuming, and they describe the activity of planktonic organisms, not organisms attached to the surface. The biofilm coupon, a patented device of the Center for Biofilm Engineering has been improved and used as a bioassay for measuring the growth potential of drinking water as it relates to the AOC concentration using immobilized bacteria.

Comparisons of AOC values as indicated by two AOC methodologies (van der Kooij's plate count and LeChevallier's ATP method) on the same water sample did not produce the same AOC values. However, the AOC value measured with LeChevallier's ATP method is fairly reproducible on the same water sample. The measured AOC value using LeChevallier's ATP was not significantly different from the added sodium acetate concentration (μgC/L); however, it was significantly different from the added carbon cocktail concentration at a ninety-five percent confidence interval. The carbon cocktail at concentrations ranging from 100-500 μgC/L may be inhibiting to immobilized K. pneumoniae and planktonic and immobilized P17. The growth rates of K. pneumoniae and P17 are highly correlated; however, K. pneumoniae grows at a faster rate than P17. The present study demonstrates that the AOC value may not be indicative of the growth of immobilized bacteria grown on single substrates. However, the growth response of K. pneumoniae and P17 immobilized in the biofilm coupon was correlated with the sodium acetate concentration (μgC/L), not the measured AOC value. Based on these observations, the biofilm coupon could be useful as a monitoring device for the growth potential of immobilized bacteria in drinking water distribution systems.

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ABSTRACT

Coliform bacteria that grow and persist in drinking water distribution systems affect the delivered water quality for the consumer. The coliforms that are most responsible for regrowth events are biofilm organisms growing at the expense of nutrients in the bulk water. The research project "Factors Limiting Microbial Growth in the Distribution System" funded by the American Water Works Research Foundation and NSF Center funding worked with select utilities across the United States to establish strategies for limiting microbial growth, particularly biofilms, in drinking water distribution systems. One of the objectives of the study was to determine conditions that allow coliforms to establish and persist in mixed population biofilms. Nutrient loading, particularly assimilable organic carbon (AOC) is one of the key variables. Several methodologies have been developed to measure AOC. However, these methodologies are labor-intensive, time-consuming, and they describe the activity of planktonic organisms, not organisms attached to the surface. The biofilm coupon, a patented device of the Center for Biofilm Engineering has been improved and used as a bioassay for measuring the growth potential of drinking water as it relates to the AOC concentration using immobilized bacteria.

Comparisons of AOC values as indicated by two AOC methodologies (van der Kooij's plate count and LeChevallier's ATP method) on the same water sample did not produce the same AOC values. However, the AOC value measured with LeChevallier's ATP method is fairly reproducible on the same water sample. The measured AOC value using LeChevallier's ATP was not significantly different from the added sodium acetate concentration (μgC/L); however, it was significantly different from the added carbon cocktail concentration at a ninety-five percent confidence interval. The carbon cocktail at concentrations ranging from 100-500 μgC/L may be inhibiting to immobilized *K. pneumoniae* and planktonic and immobilized P17. The growth rates of *K. pneumoniae* and P17 are highly correlated; however, *K. pneumoniae* grows at a faster rate than P17. The present study demonstrates that the AOC value may not be indicative of the growth of immobilized bacteria grown on single substrates. However, the growth response of *K. pneumoniae* and P17 immobilized in the biofilm coupon was correlated with the sodium acetate concentration (μgC/L), not the measured AOC value. Based on these observations, the biofilm coupon could be useful as a monitoring device for the growth potential of immobilized bacteria in drinking water distribution systems.

CHAPTER 1

INTRODUCTION

Coliform bacteria that grow and persist in drinking water distribution systems affect the quality of water delivered to the consumer. The numbers of coliform bacteria present in drinking water are regulated. When coliforms are detected in the system, water utilities may be required to notify the public and issue boil orders. This has the potential to cause a decline in public confidence in the water utility. While coliforms may be the results of events of public health concern, they may arise from the detachment of biofilm organisms growing at the expense of nutrients in the bulk water.

The prevention and control of bacterial biofilms in drinking water distribution systems have become extremely important water quality objectives. The research project "Factors Limiting Microbial Growth in the Distribution System" funded by American Water Works Association Research Foundation (AWWARF) and the National Science Foundation (NSF) worked with select utilities across the United States and utilized laboratory and pilot distribution systems in order to establish strategies for limiting microbial growth, particularly biofilms, in drinking water distribution systems. One objective of the study was to experimentally determine conditions that allow coliforms to establish and persist in mixed population biofilms (Camper, 1991). Two of the key variables of the research were biocide efficacy and substrate loading, specifically assimilable

organic carbon (AOC). Several methodologies have been developed to measure AOC. However, these methodologies are labor intensive, time-consuming, and they describe the activity of planktonic organisms, not organisms attached to the surface. The majority of microbial growth occurs in the biofilm formations associated with pipe surfaces, not in the bulk water (Rice, *et al.,* 1991). Van der Kooij (1992) discussed the importance of the development of a measurement to indicate the growth of immobilized bacteria as related to AOC uptake.

The biofilm coupon, a monitor for measuring microbial growth, was developed and patented (Patent 5,051,359) by researchers at the Center for Biofilm Engineering in 1991. The biofilm coupon is used to measure the "in situ" activity of surface associated microorganisms within industrial and municipal water systems. Bacteria are seeded and immobilized into the coupon. The bacteria grow and respond to factors in the system such as nutrients, biocides, and other growth stimulating or inhibiting substances. Thus, the biofilm coupon has the potential to be an effective monitor for the detection of the growth potential of water for immobilized bacteria in drinking water distribution systems.

Since the use of the biofilm coupon as a monitoring device has been quite limited, this research will evaluate certain technical problems concerning the use of the biofilm coupon as a drinking water monitoring device. Two of these problems include viability of the test organisms in the biofilm coupon and the suitability of the gel pad components. The goal of this research is to utilize the

biofilm coupon as a bioassay for measuring the growth potential of drinking water. The objectives of this research are:

1) To determine the effects of varying concentration of AOC on the immobilized test bacteria.

2) Determine the limiting amount of AOC required for growth of the test organism.

3) Compare the response of the immobilized test bacteria with AOC measurements utilizing planktonic organisms.

CHAPTER 2

LITERATURE REVIEW

Indicator Organisms in Drinking Water Distribution Systems

Drinking water utilities have been conducting microbiological monitoring of distributed drinking water since the early 1900's. One of the main tasks in microbiological monitoring is the development of laboratory methods which can be used to detect microbiological contaminants in drinking water. A key issue for monitoring is the selection of an indicator organism. The indicator organism should (a) "be present whenever pathogens are present and present in the same or higher numbers than pathogens; (b) be at least as resistant as pathogens to water treatment and disinfection; (c) grow readily on a selective medium and have easily identifiable characteristics; (d) be specific for fecal contamination; and (e) preferably be nonpathogenic" (Poole and Hobson, 1979). Indicator organisms are associated with the intestinal tract, and their presence may indicate fecal contamination of the water supply. The first indicator organism that was chosen was *Bacterium coli* which was defined in the 1914 and 1925 standards as an organism that ferments lactose and produces acid and gas (Pipes, 1990). Later, several species from several different genera were also included which produce gas from the fermentation of lactose. The group

became known as the Coliform group. As defined by Brock and Madigan (1988) for water bacteriology this group includes all the aerobic and facultatively anaerobic, Gram-negative, nonspore-forming, rod shaped bacteria that ferment lactose with gas formation within 48 hours at 35° C. Members of the coliform group are suitable as indicators because 1) they are common inhabitants of the intestinal tract, 2) when coliforms are excreted into the environment the coliform organism usually dies, but not at a faster rate than the pathogenic bacteria, and 3) it is likely that if coliforms are found in a water sample, the water may have received fecal contaminants and could be unsafe for drinking purposes (Brock and Madigan, 1988). However, the coliform group consists of both fecal and nonfecal source organisms. Recent studies have shown that nonfecal coliforms may grow attached to the interior surfaces of water pipes, so the presence of coliforms in the drinking water does not always indicate fecal contamination in the system (Herson, *et. al.,* 1987; Brock and Madigan, 1988; van der Wende, *et. al.,* 1989; LeChevallier, 1990(a); and Block, 1992). These attached organisms have not been found to cause health problems, but when they are sloughed into the drinking water the standards are violated. Thus, Pipes (1990) believes that "water utilities are reluctant to spend a great deal of time and money eliminating what they consider to be a "non-problem" and thus do not support the use of the coliform standard anymore".

Included in the coliform group is the opportunistic pathogen *Klebsiella pneumoniae*. According to Seidler (1981), environmentally derived *Klebsiella*

should be considered as valid a fecal coliform as *Escherichia coli* due to the prevalence of the fecal biotype. Thus, *K. pneumoniae* has been widely monitored in drinking water distribution systems and been used extensively in drinking water studies (LeChevallier, *et. al.*, 1987). As described in Bergey's Manual, (Orskov, 1984) *K. pneumoniae* is a straight rod 0.3-1.0 μm in diameter and 0.6-6.0 μm in length, capsulated, gram-negative, non-motile, facultatively anaerobic with both a respiratory and a fermentative type of metabolism, and has few particular growth requirements. The strain of *K. pneumoniae* that is widely used at the Center for Biofilm Engineering was originally isolated from the New Haven Connecticut system during a coliform regrowth episode (Camper, 1991; Xu, 1993). This strain is capable of significant growth under low nutrient concentrations, including double-glass-distilled water (Table 1) (Camper, *et. al.*, 1991).

Table 1. Kinetic parameters for *K. pneumoniae* (Water Isolate) grown on various concentrations of yeast extract at 25°C (Camper, *et. al.*, 1991).

Yeast Extract Concentration (mg liter $^{-1}$)	0	0.1	0.25	0.5	1.0
Specific Growth Rate (μ [h^{-1}]) ± SE	0.10 ± 0.007	0.18 ± 0.013	0.19 ± 0.008	0.29 ± 0.030	0.29 ± 0.011

Since most drinking water has low nutrient concentrations, and this strain of *K. pneumoniae* shows significant growth under low nutrient conditions, it is a relevant organism for studies concerning drinking water. *K. pneumoniae* (New

Haven Isolate) was chosen for this research based on these criteria: 1) it is an indicator organism, 2) there are no particular growth requirements, 3) it shows significant growth under low nutrient conditions, 4) it is non-motile, and 5) it has been used in the biofilm coupon in previous research (Xu, 1993).

Pseudomonas fluorescens

Pseudomonas fluorescens is another organism that is found in drinking water distribution systems. Van der Kooij (1977) showed that fluorescent Pseudomonads constitute 1 to 10% of the heterotrophic bacterial population in both surface water and tap water. According to Bergey's Manual, (Palleroni, 1984) *Pseudomonas fluorescens* is characterized as a rod-shaped organism, 0.5-1.0 μm in diameter and 1.5 - 4.0 μm in length, Gram-negative, motile by polar flagella, strict aerobe, and grows with acetate as the sole carbon source. This organism is potentially pathogenic, but virulence is low (von Graevenitz and Weinstein, 1971). The bacterium grows at temperatures near 18°C, and is able to grow in very low nutrient environments (Frias, *et. al.*, 1994). *Pseudomonas fluorescens* has great nutritional versatility; it multiplies on simple sources of nitrogen, it does not require special growth factors, and it grows rapidly on non-selective media (Frias, *et. al.*, 1994). Due to the nutritional versatility of strain P17 of this organism, it has become the standard strain for conducting

assimilable organic carbon (AOC) tests in drinking water (van der Kooij, *et al.,* 1982). Since the work done for this thesis involved comparing results from the biofilm coupon with results using P17 as an indicator of AOC, it was only reasonable to choose this organism as the second organism of study.

Growth in Oligotrophic Environments

Growth is defined as an increase in the number of cells or an increase in cellular mass. Growth rate is the change in the number of cells or mass per unit time. Bacterial growth occurs in a cycle of 1) initial stationary phase, 2) lag or growth acceleration, 3) logarithmic growth, 4) negative growth acceleration, 5) stationary phase, 6) accelerated death, and 7) logarithmic death (Roszak and Colwell, 1987). The latter stage is termed cryptic growth which occurs when a portion of a starved microbial population dies, releasing products of lysis and leakage that support the growth of survivors (Postgate, 1976). The survivors tend to be smaller cells. These small cells are usually the first to attach to surfaces (Roszak and Colwell, 1987). Once attached, active growth may resume.

Growth is measured by following changes in number of cells or weight of cell mass either by direct microscopic counting, viable counts, or cell mass. In a continuous culture, two elements control bacterial growth; the flow rate and the concentration of the limiting nutrient. Nutrient concentration can affect both

growth rate and total growth. At very low concentrations of nutrient, the rate of growth and the total growth is reduced (Brock and Madigan, 1988). In drinking water distribution systems, utilizable organic carbon is typically the limiting nutrient (van der Kooij, *et. al.,* 1982; Lucena, *et. al.,* 1990; and Frias, *et. al.,* 1994).

The low concentrations of biodegradable organics in drinking water favor the growth of oligotrophic microorganisms. Oligotrophic organisms are defined as those bacteria that grow in a medium containing < 1 mg/L of organic carbon (Geesey, 1976). Oligotrophs have a high surface-to-volume ratio and exhibit high affinity uptake systems to scavenge the utilizable energy sources (Geesey, 1976). Uptake systems appear to be maintained by the bacteria even when the compounds they transport are too dilute to be taken up into the cell. The microbial cell can adapt readily to changes in environmental parameters by means of genotypic and phenotypic accommodations such as modifications of enzyme synthesis to take up growth-limiting nutrient, modulation of uptake rates for nutrients in excess, rerouting metabolic pathways to avoid blockages due to nutrient limitation, and coordination of synthetic rates to maintain balanced growth (Roszak and Colwell, 1987). Some studies have indicated that bacteria can change their uptake components depending on the concentration of nutrients (Geesey and Morita, 1979; Dawes, 1976; Kurath and Morita, 1983; and Novitsky and Morita, 1978;). Oligotrophic organisms have relatively small maximum growth rates (Bouwer and Crowe, 1988).

When growth-promoting resources are not available in sufficient concentrations to support cell reproduction, microorganisms employ a variety of strategies to optimize their survival until conditions become more favorable (Geesey, 1976). Since most aquatic habitats are energy limiting, the strategies employed for survival are likely to be present more often than not and need to be considered as important adaptations for survival. One of the key adaptations is attachment of bacteria to surfaces resulting in biofilm formation.

Biofilms in Drinking Water

A biofilm consists of cells immobilized at a substratum and held together by an extracellular polymeric substance (EPS) of microbial origin (Characklis and Marshall, 1990). Most pipe surfaces in drinking water distribution systems are colonized by microorganisms (LeChevallier, *et. al.*, 1987), while suspended growth is minimal. Biofilm accumulation in drinking water distribution systems leads to a decrease in water quality and the potential for increased health risks if pathogenic bacteria become embedded in the matrix.

Biofilm formation in drinking water distribution systems is primarily governed by two processes: 1) growth and 2) detachment. Growth refers to cell elongation and multiplication which occurs at the expense of nutrients in the water. Detachment is a process by which the biofilm organisms are transferred to the bulk liquid, thus allowing interaction between planktonic cells and sloughed

biofilm cells (Characklis and Marshall, 1990). Detachment typically occurs as the result of increasing biofilm thickness combined with fluid shear stress at the surface-liquid interface or through the addition of an artificial stimulus. A regrowth event may occur when the bacteria detach from the biofilm.

The microenvironment inside a biofilm is much different from the environment the planktonic organisms live in. Attached bacteria in flowing oligotrophic environments have certain advantages over planktonic organisms : 1) organic films tend to accumulate on clean surfaces, 2) the high flow rates transport large quantities of nutrients to fixed microorganisms, 3) the EPS produced by the bacteria allows for firm adhesion of the bacteria to the surface and helps to trap other nutrients, and 4) the bacteria embedded in the EPS matrix are protected from the action of disinfectants by a combination of physical and transport phenomena (Geesey, 1976; Fletcher, 1982; Herson, *et. al.*, 1987 and Ridgway and Olson, 1981). Thus, attachment of bacteria to the inner surfaces of pipes could significantly enhance their survival and regrowth potential in distribution systems (Ridgway and Olson, 1981).

Regrowth Events

Regrowth is associated with the proliferation of bacteria in drinking water distribution systems either in the bulk liquid or on the pipe wall (Camper, 1994). Several genera of bacteria have been described as representatives of regrowth,

including *Flavobacterium, Klebsiella, Xanthomonas, Arthrobacter, Corynebacterium, Bacillus, Aeromonas,* and *Pseudomonas.* These genera have also been frequently isolated from drinking water (LeChevallier *et. al.*, 1980; Reasoner *et. al.*, 1989; van der Kooij *et. al.*, 1982; van der Kooij and Hijnen, 1984, 1988; and Frias, *et. al.*, 1994). The major problems associated with regrowth are 1) the multiplication of potentially pathogenic bacteria, 2) taste, odor, and the color problems, and 3) corrosion of pipe materials (van der Kooij and Veenendaal, 1992). Bacterial regrowth is affected by a variety of factors including temperature, hydraulic regime, presence of dormant or injured bacteria, disinfection, and nutrients in the distribution system. However, Baylis, *et al.,* (1930) concluded that that microbial regrowth can be avoided by maintaining a free chlorine residual, while van der Kooij, *et al.,* (1982) suggested that regrowth may be prevented by controlling the nature and concentration of biodegradable compounds.

Temperature

The temperature of the water plays a major role in bacterial growth because the water temperature influences the growth rate and the observed cell yield (Fransolet, *et. al.*, 1985). Coliform bacteria have optimum growth temperatures that range between 25-35°C, which are well above those found in drinking water distribution systems. The temperature of drinking water varies

throughout the year as the external temperature varies. Several studies have indicated that an increased temperature in the distribution system leads to a faster growth rate and/or higher numbers of bacteria (Rice, *et. al.*, 1991, LeChevallier, *et. al.*, 1991(b), and Donlan and Pipes, 1988; Camper *et. al.*, 1991).

Hydraulic Regime

The design of the distribution system and the velocity of water flowing through the pipes influences microbial colonization and growth within the pipes of the distribution system. Sections of pipe where there are high velocities of flowing water tend to limit microbial growth, while dead-end sections and slow flowing areas have been "statistically correlated with water quality deterioration" (LeChevallier, *et. al.*, 1987).

Injured Bacteria

Coliform organisms not resulting from regrowth that are detected in drinking water distribution systems typically come from two different sources: 1) a contamination event and/or 2) organisms that have survived treatment and disinfection. In the second case, survivors enter the finished water in an injured state. A key characteristic of injured organisms is their inability to grow on selective media which separates them from non-injured organisms (McFeters, 1990). This may lead to inaccurate results in the monitoring of coliform bacteria.

In addition, the injured bacteria may recover later in the planktonic state or attached to the pipe surface (Camper, 1994; Bucklin, *et al.,* 1991). The recovered bacteria may contribute in part to regrowth.

Disinfection

Disinfection efficacy depends on the system parameters including the type of disinfectant, transport factors, composition of the pipe materials, and accumulation of corrosion deposits (Camper, 1994; Xu, 1993). Mechanisms by which bacteria are resistant to disinfection include attachment to particles, formation of aggregates, and capsule production (Stotsky, 1967; Ridgway and Olson, 1981; Kjellberg, *et. al.,* 1983; LeChevallier, *et. al.,* 1984; 1987; and 1988; Herson, *et. al.,* 1987; Berman, *et. al.,* 1988; and van der Wende, *et. al.,* 1989). Disinfection of biofilm bacteria is more difficult than disinfection of planktonic organisms.

The most commonly used disinfectants in the drinking water industry are chlorine and monochloramine. Since 1910, drinking water distribution systems have adopted chlorine as the disinfectant of choice due to its effectiveness in inactivating planktonic cells. Chlorine causes the death of most microorganisms within thirty minutes as well as reacting with other organic materials to eliminate many of the taste and odor problems associated with them. However, there are some problems associated with the use of chlorine as a disinfectant: 1) chlorine reacts with organic matter in water to form trihalomethanes (THM's) and other

halogenated byproducts, which are suspected carcinogens or mutagens and are toxic at high concentrations (Bouwer and Crowe, 1988), 2) chlorination usually causes increased AOC concentrations (van der Kooij and Hijnen, 1984), and 3) in the more recent past, it has been shown that the standard chlorine concentrations (residual does not usually exceed 0.2-0.6 mg/ml) have a decreased effectiveness on biofilm bacteria. "Chlorine doses up to 12 mg l^{-1} were inadequate in attempts to control coliform regrowth" (Earnhardt, 1980; and Lowther and Moser, 1984). Several studies have indicated that chlorine and other disinfectants are not effective for controlling regrowth for the following reasons: 1) the chlorine demand, 2) their limited effect on attached organisms and 3) the survival of chlorine resistant species (van der Kooij, 1992). It has been shown that monochloramine, a slower acting biocide, is more effective for disinfection of biofilm bacteria. In addition, monochloramine treatment did not yield significant biofilm detachment whereas chlorine does (LeChevallier, 1990(b); Camper, 1994; Griebe, *et. al.*, 1994). Finally, monochloramine is not as reactive toward system components as free chlorine. Nevertheless, at this point in time there is no evidence that supports the use of disinfection alone to control regrowth events.

Growth-Promoting Factors

The extent of regrowth is also regulated by the concentration of organic and inorganic compounds serving as nutrients for bacteria. Microorganisms require carbon, nitrogen, and phosphorous in a ratio of approximately 100:10:1 for balanced growth (C:N:P). The organic carbon is necessary as an energy source and for the production of new cellular material, while the nitrogen and phosphorous are required for biosynthesis (LeChevallier, *et. al.,* 1991(b); Camper, 1994). Generally, the energy source (carbon-containing compounds) is the limiting nutrient in drinking water distribution systems. Most carbon-containing compounds in the water supply include humic acids, fulvic acids, polymeric carbohydrates, proteins, and carboxylic acids. In a National Organic Reconnaissance Survey, conducted by the US EPA, the nonpurgeable total organic carbon concentration in drinking water in 80 separate locations ranged from 0.05 to 12.2 mg/L (Symons, *et. al.,* 1975). The utilizable organic carbon, sometimes called the biodegradable dissolved organic carbon (BDOC) or the assimilable organic carbon (AOC) is the portion of total organic carbon (TOC) that can be used by microorganisms for growth. The utilizable organic carbon (BDOC or AOC) typically ranges between 1 and 2000 µg/L (LeChevallier *et. al.*, 1987, LeChevallier *et. al.*, 1991(b), and Bouwer and Crowe, 1988). Recently, measurements of utilizable organic carbon (BDOC or AOC) have been widely used to indicate water quality. "The potential regrowth of bacteria in drinking water distribution systems is determined by the concentration of assimilable

organic carbon (AOC) or (BDOC)" (Huck, 1990; van der Kooij *et. al.*, 1982; and Frias, *et. al.*, 1994). The relationship between the AOC or BDOC levels and bacterial growth indicates that coliform growth occurs in water containing greater than 50 µg/L utilizable carbon (LeChevallier, *et. al.*, 1991(b)).

The amount of utilizable organic carbon that may be available in drinking water cannot be assessed by simple chemical methods because of the low concentrations and unknown composition of many of the compounds. Thus, in the past few years several techniques to measure the utilizable organic carbon have been developed. These methods are based on the following: 1) measurement of the amount of bacterial biomass produced after incubation of the water sample by direct counts, DNA measurements, ATP measurements, or by colony counts; 2) measurement of the growth rate of bacteria (biomass must also be determined by this methodology); and 3) measurement of the decrease in the concentration of organic compounds such as dissolved organic compounds (DOC) (van der Kooij, 1990) (Table 2) (Huck, 1990).

Table 2. Comparison of procedures: Biomass based methods and DOC based methods (modified from Huck, 1990)

Method	Sample Preparation	Source of Inoculum	Incubation Time (days)	Temp. ° C	Parameter Measured	Expression of Results
van der Kooij	Pasteurization	Pure Cultures (P17 and Nox)	Up to 20	15	cfu/mL	AOC (µg/L) acetate equivalents
Kemmy et. al.	Filter Sterilization	Four Species (P17, Curtobacterium sp., Corynebacterium sp., & unidentified coryneform	6	20	cfu/mL	AOC (µg/L)
USEPA (coliform growth response)	Filter Sterilization	Three coliforms (E. Coli, Enterobacter cloacae, and Klebsiella oxytoca)	5	20	cfu/mL	CGR = log (N_5/N_0)
Werner	Filter Sterilization	Sample	2.5 or 5	≅ 20	Slope and Height of Curve	µ (growth rate); log (y/y_0) (amount of substrate)
Jago-Stanfield	Filter Sterilization	Raw water of treatment plant or distribution system	Until max. ATP concentration reached	20	ATP	AOC (µg/L)
Billen-Servais	Filter Sterilization	Treatment plant	10-30	≅ 20	Bacterial number and size	BDOC (mg/L)
Billen-Servais	Filter Sterilization	Water from same environment as sample	28	20 ± 0.5	DOC	ΔDOC = BDOC
Joret-Levi	none	Biologically active sand from a water treatment plant that does not use prechlorination	Until no change in DOC	≅ 20	DOC	ΔDOC = BDOC
Kaplan	none	Indigenous species from each particular water utility	Approximately one-year to set-up; then readings can be taken every two hours	Water temp. in system	DOC	ΔDOC = BDOC

Methods to Study Microbial Regrowth Potential

Biodegradable Organic Carbon

Biodegradable organic carbon (BDOC) is the portion of organic carbon in water that can be mineralized by microorganisms (Huck, 1990). BDOC is typically based on measuring a difference in dissolved organic carbon (DOC) before and after sample incubation. The BDOC is the difference between the initial DOC and the minimum DOC concentration. Several investigators have developed methods to determine the BDOC in drinking water samples. The first includes a biomass-based method by Billen-Servais (Servais, *et. al.*, 1987). In this methodology the biomass is determined as a function of time. The experiment is continued until the total bacterial mortality (total mortality equals total biomass production) during the incubation period can be reliably determined (Huck, 1990). The total mortality is then divided by the growth yield to give an estimate of BDOC. This method is quite laborious and the time to get results is quite long, so the method was modified by Billen-Servais. In the modified method, BDOC is defined as a difference between initial DOC and the DOC after a four week incubation in the dark. Joret and Levi developed a more rapid assay for BDOC (Joret, *et. al.*, 1988). This method uses biologically active sand as the inoculum that is washed until there is no detectable DOC released. The sample is then incubated with the sand in an erlenmeyer flask under aerated conditions. The DOC is measured daily until there is no further change. The BDOC is taken

as the change in DOC during the test. The use of a support medium in the Joret-Levi test decreases the incubation time (Huck, 1990). A recent BDOC method using a flow through bioreactor, a bioassay technique, has been developed by Kaplan *et al.,* 1993). This method uses indigenous organisms as the inoculum. The bioreactors require a period of colonization that can take about one year. Once the bioreactors are colonized, BDOC readings can be measured every two hours by subtracting the inflow DOC from the outflow DOC. This method allows for rapid and reproducible BDOC measurements.

A key issue with the DOC based methods is their applicability to water with low levels of BDOC. A difference between 0.1 or 0.2 mg/L could not be reliably detected (Huck, 1990). Since most utilizable organic carbon in drinking water distribution systems is in much lower concentrations than mg/L, these methodologies can not be used for routine monitoring. In addition, DOC measurements have been shown to be unreliable for predicting microbial growth (van der Kooij, *et. al.*, 1982 and van der Kooij, 1992).

Assimilable Organic Carbon

Assimilable organic carbon (AOC) is that portion of biodegradable organic carbon that can be converted to cell mass and expressed as a carbon concentration by means of a conversion factor (Huck, 1990). The AOC concentration has been defined as, "an index of the biological stability of drinking water, with values <10 µg acetate-C eq/L indicating a limiting growth potential"

(van der Kooij, 1992). AOC is measured by the maximum bacterial production (N_{max}) of each sample, which can be measured by plate counts, ATP, or turbidity. The first method for determination of the AOC of drinking water was developed by van der Kooij, *et. al.*, in 1982. The AOC in the water sample is determined by measuring the growth of *Pseudomonas fluorescens* (strain P17), and a *Spirillum* species (strain NOX). P17 was originally isolated from drinking water. P17 can use a variety of compounds at both low and high concentrations, does not need specific growth factors, and can use nitrate and ammonia as nitrogen sources (Huck, 1990). However, P17 cannot use oxalic acid, one of the compounds frequently produced during ozonation. Thus, the NOX species, which is capable of using oxalate was incorporated into the test. Growth of P17 and NOX is determined by daily plate counts. Van der Kooij's AOC method assumes a linear relationship between the maximum level of growth of P17 and NOX and the concentration of acetate-C added to a tap water sample. Based on known yield coefficients for P17 and NOX, the equivalent amount of carbon is calculated and expressed as µg acetate C eq/L. This method takes between 5 and 25 days to complete. The method is standardized (Frias, *et. al.*, 1994).

Most of the other methods developed to determine AOC involve modifications of the van der Kooij method such as using a mixed consortia of known bacteria (Kemmy, *et. al.*, 1989) or coliforms (Rice, *et. al.*, 1991) to inoculate the sample; filter sterilization instead of pasteurization, (Kemmy, *et. al.*, 1989; Werner and Hambsch, 1988; Rice, *et. al.*, 1991; and Stanfield and Jago,

1987); turbidity measurements (Werner and Hambsch, 1988); and ATP analysis (Stanfield and Jago, 1987; and LeChevallier, *et. al.*, 1993) (Refer to Table 2). These various modifications have specific ramifications, so the choice of methodology is up to the investigator.

LeChevallier modified van der Kooij's method in order to simplify the test and to decrease the incubation time. LeChevallier uses a higher initial inoculum to decrease the incubation time and his method utilizes the measurement of ATP as an indirect measure of bacterial cells rather than performing plate counts (LeChevallier, *et. al.*, 1993). The stationary phase (N_{max}), as determined by luminescence units, is proportional to the amount of limiting nutrients in the water. LeChevallier, *et al.*, (1993) found that there was no significant difference between AOC values determined using the ATP or plate count procedures. Using this modification of the van der Kooij method, the AOC of the sample can be determined within 2-4 days. Due to the rapid availability of results and the ease of this modified test, LeChevallier's method was chosen to measure the AOC concentration in this research.

Limitations of Current Methodologies

In evaluating and comparing the methods to determine AOC or BDOC it is important to decide the purpose of the measurement. If the concern is with bacterial regrowth or growth of coliforms, the parameter to measure is the

bacterial biomass or an AOC measurement (Huck, 1990). However, if the concern is reducing chlorine demand or disinfection by-product formation potential through a biological treatment process, then a more closely related parameter is DOC. In general, the biomass based assays are more labor intensive than the DOC-based measurements. However, the DOC measurements require a good low-level TOC analyzer. All methods require a good technique for cleaning glassware, both types of methodologies are time-consuming, and the levels of biodegradable matter below 0.2 mg/L typically require a biomass-based approach. The most important limitation of most of the methods developed is that they describe the activity of planktonic organisms. This presents a dilemma, in that many of the water quality problems that occur are due to the presence of organisms attached to the pipe wall. The AOC method developed by van der Kooij includes the assumption that "the AOC uptake by biofilm bacteria is reflected in the number of suspended bacteria" (van der Kooij, 1992). However, previous research has failed to establish a correlation between van der Kooij's AOC value and the growth rate or quantity of attached coliforms or HPC's (Camper, *Submitted*). To further explain the regrowth phenomenon, studies relating the presence of attached bacteria to AOC uptake are needed. In addition, none of the current methods are ready for routine use in water treatment facilities. "Development of such a monitor is a high priority" (Huck, 1990).

The Biofilm Coupon as a Monitor

The biofilm coupon is an innovative device that was developed at the Center for Biofilm Engineering by David Davies and patented (Patent 5,051,359) in 1991 by William Characklis (Characklis, *et. al.*, 1991). This device can be used to monitor the *in situ* activity of surface associated microorganisms within industrial and municipal water systems. The biofilm coupon is constructed of polycarbonate and has the same dimensions as a typical corrosion coupon, 8 cm long by 1 cm wide. Each holder contains three cylindrical inserts which are 1 cm in diameter. The inserts are seeded with a monolayer of indicator organisms, which stick to the inside surface of the inserts. Select organisms can be installed in the inserts to quantify their response to particular environments. The bacteria are then covered with a gel matrix which can be composed of silica gel and/or noble agar gel. The gel allows passage of nutrients, biocides, and other soluble additives that may be present in the water system. The waste products produced by the bacteria can also pass through the gel so inhibition or enhancement of growth from these products does not occur. The seeded bacteria however, cannot pass through the gel matrix nor can indigenous species enter the coupon. The cells within the coupon are counted using a bright field microscope. The optically clear discs of the biofilm coupon inserts allow for observation without staining or destruction of the sample using bright-field microscopy. Cell numbers prior to incubation in the system are compared to

cell numbers after exposure to the system. The seeded bacteria respond to factors such as nutrients, biocides, or other growth/inhibition promoting factors in the system.

The use of the biofilm coupon has several potential advantages as a methodology for determining the sustained utilizable organic carbon in the system: 1) the biofilm coupon is easy to sample, observe, and is fairly inexpensive for routine monitoring; 2) any organism can potentially be seeded into the biofilm coupon, thus indigenous organisms can be used to evaluate the water quality in each system; 3) results from the coupon show the *in situ* potential for bacterial growth because bacterial growth in the biofilm coupon is in response to nutrients, biocides, and other non-transient growth/inhibition promoting factors in the system water; and 4) the biofilm coupon more closely simulates an actual biofilm (Xu, 1993) so the response of the test organisms in the coupon may be indicative of attached, not planktonic growth. Thus, the biofilm coupon has the potential to be an effective monitor for the detection of the growth potential of immobilized bacteria in drinking water distribution systems.

In light of the previous literature, several experiments were designed to address the use of the biofilm coupon as a bioassay for measuring the growth potential of drinking water using *K. pneumoniae* and *P. fluorescens* (P17) as the test organisms.

1) Initially it was important to determine if *K. pneumoniae* would grow with sodium acetate as the sole carbon source.

2) Since double-glass-distilled water was used in all media, solutions, and feed jugs, the AOC of this water was measured using van der Kooij's plate count method and LeChevallier's ATP method.

3) The growth potential of the double-glass-distilled water with and without added nutrients was measured using both test organisms immobilized in the biofilm coupon.

4) Based on results from the experiments using double-glass-distilled water, the utilizable carbon leaching from the cleaning method or the materials used to prepare the biofilm coupon was determined.

5) The viability of the test organisms immobilized in the biofilm coupon was determined using CTC/DAPI staining and INT staining.

6) For the biological stability studies, two continuous flow reactors were designed to compare the response of both test organisms immobilized in the biofilm coupon to three concentrations of added sodium acetate or carbon cocktail. The control was double-glass-distilled water with added mineral salts solution but no added carbon, while the supplemented reactor had added carbon at concentrations of 15, 29, or 145 μgC/L for sodium acetate and 50, 100, and 500 μgC/L for the carbon cocktail. The AOC of both the control and supplemented reactors was determined.

7) The rate constants for uptake of 5 and 50 μg/L radiolabelled sodium acetate for both *K. pneumoniae* and *P. fluorescens* was determined.

CHAPTER 3

MATERIALS AND METHODS

Microorganisms

The *Klebsiella pneumoniae* strain used for this study was originally isolated from the drinking water distribution system of New Haven, CT, and was obtained from Anne Camper, Center for Biofilm Engineering, Montana State University, Bozeman, MT. The *Pseudomonas fluorescens* strain used for this study was received from Mark LeChevallier at American Water Works Service Company.

The cultures of *K. pneumoniae* and *P. fluorescens* were both stored on R2A agar (Difco Laboratories, Detroit, MI) at 4° C in the refrigerator after resuscitation from frozen stocks maintained in 2% peptone 20% glycerol at -70 ° C. One day prior to preparation of the biofilm coupon, *K. pneumoniae* was refreshed on a new R2A agar plate. *P. fluorescens* was refreshed on a new R2A agar plate two days prior to use. Bacterial suspensions of both organisms were prepared by selecting one colony from the new R2A plates with a sterile inoculating loop and suspending the colony in a mineral salt solution (composition of standard mineral salt solutions for this work consisted of the following (g/L): KH_2PO_4, 7 (Fisher Laboratory Supplies, Santa Clara, CA);

K_2HPO_4, 3 (Fisher Laboratory Supplies, Santa Clara, CA); $(NH_4)_2SO_4$,1.0 (J.T. Baker Chemical Co., Phillipsberg, NJ); $Mg_2SO_4 \circ 7H_2O$ 1.0 (Aldrich Chemical Co., Inc., Milwaukee, WI); the stock solution was diluted 1:1000 with double-glass-distilled water). The cultures were plated in triplicate to determine the CFU/ml prior to preparation of the biofilm coupon (approximately 10^7 CFU/ml). The bacterial suspensions were then homogenized and held at room temperature (20-25°C) for four hours to deplete the bacteria of endogenous nutrients prior to coupon preparation.

P. fluorescens (P17) and NOX, a *Spirillum* organism were used in the AOC tests. Preparation and storage of these organisms were done in the same manner as described by LeChevallier, (1991(a)). The organisms were streaked for purity on R2A plates and then incubated at room temperature (20-25°C) for 3-5 days. One colony of each organism was then inoculated into 100 ml of sterile chlorine-neutralized tap water (using sodium thiosulfate) and incubated for 7 days. The cultures were then plated in triplicate on R2A agar to determine the colony forming units (CFU) after conditioning. The bacteria from the tap water were then suspended in 100 ml of double-glass-distilled water plus mineral salts and sodium acetate (J.T. Baker Chemical Co., Phillipsberg, NJ) (11.34mg/L final concentration) and incubated for seven days. The cultures were plated in triplicate to determine the CFU (approximately 10^6 CFU/ml). The cultures were stored in the dark and used for one month. Then 1 ml was subcultured into a

fresh mineral salts solution plus 11.34 mg/L sodium acetate. The new cultures were then incubated for seven days and plated in triplicate to determine the CFU/ml prior to each use.

AOC Methodology

Initially, both van der Kooij's modified plate count protocol (van der Kooij, *et. al.,* 1982) and LeChevallier's ATP protocol (LeChevallier, 1991(a)) and LeChevallier, *et. al.,* 1993) were used to determine the AOC concentration of all test water. Van der Kooij's modified plate count protocol is summarized below:

1) Sample water was divided into 12 precleaned carbon-free (washed, then oven-sterilized at 500° C for 4-24 hours in an Isotemp Muffle Furnace, (Model 182A, Fisher Scientific, Santa Clara, CA)) 40 ml EPA sample vials with teflon septa (Fisher Laboratory Supplies, Santa Clara, CA). The septa were washed for one hour in a ten percent solution of potassium persulfate (Fisher Laboratory Supplies, Santa Clara, CA) and then dried before use.

2) The samples were pasteurized for 30 minutes at 70° C and then cooled to room temperature.

3) Separate vials were inoculated with approximately 10^4 CFU/ml of strain P17 or NOX (four vials each). The initial inoculum was plated at the beginning of every AOC test to verify that there were approximately 10^4 CFU/ml of each strain. The

sample vials containing P17 and NOX were stored at room temperature (20-25°C).

4) The uninoculated control vials were stored at 4° C.

5) On days 0, 2, 3, & 4, 0.1 ml of control, P17 and NOX suspensions were plated in triplicate onto R2A agar with the following dilutions -2, -3, & -4. Cells were allowed to grow on the plates for 3-4 days and then counted.

6) The plate counts were averaged and the values were then converted to acetate carbon equivalents (the AOC value): P17 conversion factor (2×10^{-4} µgC/CFU) and NOX conversion factor (5×10^{-5} µgC/CFU).

7) The P17 (NOX, or control) AOC value is equal to the stationary phase (N_{max}) P17 (NOX, or control) value (times the appropriate conversion factor) minus the initial P17 (NOX or control) value (times the appropriate conversion factor).

8) The total AOC is calculated as follows:

AOC = (P17 AOC value + NOX AOC value) - the control AOC value.

Thus, the stationary phase (N_{max}), as determined by plate counts, is proportional to the amount of limiting nutrient in the water.

LeChevallier's protocol is very similar to van der Kooij's:

Steps 1-4 are the same as van der Kooij's (see above).

5) On days 0, 2, 3, & 4, 40 ml of the control, P17, and NOX were filtered through a 0.22 µm cellulose acetate membrane (Fisher Laboratory Supplies, Santa Clara, CA).

6) Each filter was placed in 1.0 ml of releasing buffer (0.5 ml releasing agent + 0.5 ml HEPES buffer (both from Turner Designs, Sunnyvale, CA)) and incubated for twenty minutes.

7) 150 µl of sample was placed into the Turner TD 20e Luminometer (Model 20, Turner Designs, Sunnyvale, CA).

8) 100 µl of luciferin-luciferase (Turner Designs, Sunnyvale, CA) (standard curve of luciferin-luciferase activity in Appendix A) was injected into the sample.

9) The full integral of light was measured and recorded.

10) The luminometer reading was then converted to acetate-carbon equivalents (AOC value) using a P17 or NOX standard curve (See Appendix A). The correction factor was determined for P17 as 1.3 µg/L AOC and for NOX as 3.4 µg/L AOC.

11) The P17 (NOX, or control) AOC value is equal to the stationary phase (N_{max}) P17 (NOX, or control) value minus the initial P17 (NOX or control) value.

12) The total AOC is calculated as follows:

AOC = (P17 AOC value + NOX AOC value) - the control AOC value. As above, the stationary phase (N_{max}), as determined by luminescence units, is proportional to the amount of limiting nutrient in the water. After approximately 6-7 experiments only LeChevallier's method was used to determine the AOC of all waters.

Comparison of Two AOC Methodologies

The AOC concentration of three different samples of double-glass-distilled water was measured using LeChevallier's ATP method and van der Kooij's plate count method as described above. Both methods were used on each water sample to see if the two methods yielded similar results. Comparisons of the AOC values indicated by the two methodologies were also conducted on water from annular reactors 1-1, 1-2, 2-1, and 2-2 at the pilot plant. These annular reactors did not receive chlorine or added AOC. The water at the pilot plant has AOC values that range from 20-350 μgC/L (Camper, *Submitted*) which are higher values than can typically be found in double-glass-distilled water. All water was sampled on the same day at the same time. The water was then brought to the lab, filtered into oven sterilized EPA sample vials, and pasteurized for 30 minutes at 70 ° C. The AOC of each sample was measured using LeChevallier's ATP method and van der Kooij's plate count method as described above.

The reproducibility of LeChevallier's ATP method was also determined. The AOC of one water sample of double-glass-distilled water was measured three times using LeChevallier's ATP method. These experiments were replicated with a second water sample of double-glass-distilled water.

Growth Curve Methodology

K. pneumoniae (Kp) was streaked for purity onto an R2A plate one day prior to inoculation. The following day, one colony of Kp was added to 9 ml of phosphate buffer and vortexed for 30 seconds. The initial suspension was then diluted by a factor of 100. One ml of the final dilution of Kp was inoculated into 100 ml of mineral salts solution. Sodium acetate solution (100 mg/L) was then added to a 250 ml carbon-free erlenmeyer flask that was oven sterilized at 500° C for 4-24 hours. There were two flasks prepared per experiment. At time zero, the cultures were plated in triplicate to determine the CFU/ml in each flask (approximately 10^3 CFU/ml). The flasks were then incubated at 25° C and sampled at 6-24 hour intervals for a total of 140 hours. At the chosen intervals, 0.1 ml from each flask was plated in triplicate to determine the CFU/ml. Several replicate experiments were conducted.

Biofilm Coupon Preparation

Description of Biofilm Coupon

The biofilm coupon holder and inserts are constructed of polycarbonate. The holder has the same dimensions as a typical corrosion coupon (8 cm long by 1 cm wide) (Figure 1). Each holder contains three cylindrical inserts which

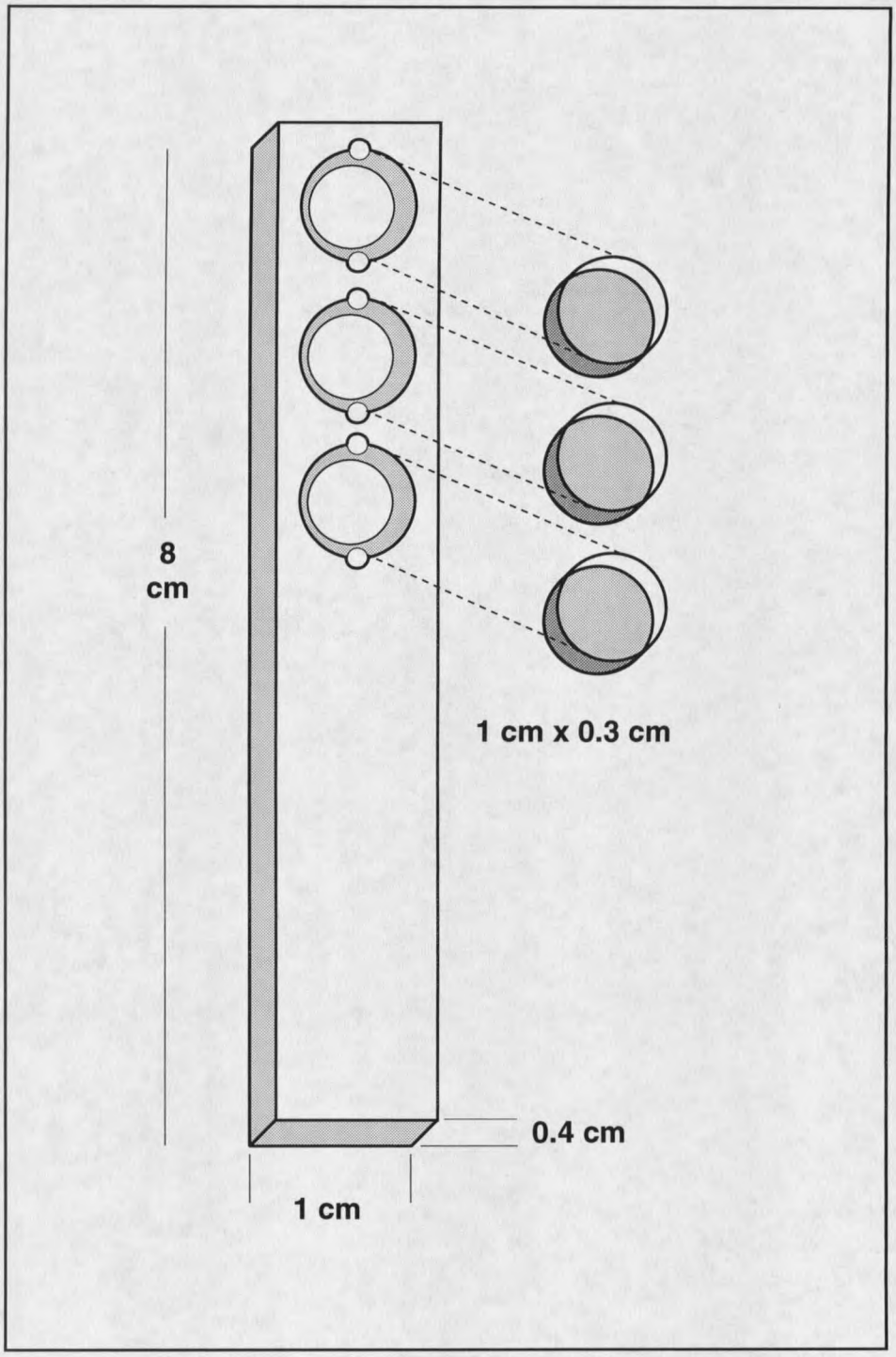

Figure 1. Schematic of biofilm coupon holder and inserts.

are 1 cm in diameter. The inserts are seeded with a monolayer of bacteria (5 µl of the bacterial suspension is initially placed on a 1-2 mm gel pad made of either silica or noble agar), and then covered with a gel matrix. The gel pad and gel matrix allow passage of nutrients, biocides, and soluble additives in the water.

Silica Gel Pad

The silica gel pad was prepared using three different components: potassium silicate solution, twenty percent phosphoric acid, and phosphate buffer. The potassium silicate solution was prepared by dissolving 10 g of powdered silica acid (reagent grade) (Sigma Chemical Co., St. Louis, MO) into 100 ml of 7 percent (w/v) KOH by heating. The twenty percent aqueous phosphoric acid was prepared by adding 11.5 ml of phosphoric acid to 38.5 ml of double-glass-distilled water. The phosphate buffer was prepared by dissolving 4.3 g K_2HPO_4 and 3.4 g $KH_2 PO_4$ into one liter of double-glass-distilled water by heating. To make the gel pads, 5 ml of phosphate buffer was mixed with 5 ml of potassium silicate solution in a 100 x 20 mm glass plate (oven sterilized at 500° C for 4-24 hours). One ml of phosphate buffer was added to the 10 ml solution in the plate. The plate was then stirred gently so the gel would be evenly spread. The gel pads were allowed to harden at room temperature for approximately thirty minutes prior to the addition of ten ml of phosphoric buffer. The prepared gel pads were stored at 4° C.

Agar Gel Pad

In later experiments, agar gel pads were used instead of the silica gel pad. These pads were prepared by dissolving two grams of noble agar into 100 ml of double-glass-distilled water by heating. Eleven ml of the two percent agar solution was added to a 100 x 20 mm glass plate (oven sterilized at 500° C for 4-24 hours) and allowed to solidify at room temperature for approximately fifteen minutes. The gel pads were then stored at 4° C.

Agar Gel Overlay

A three percent agar gel overlay was used for all coupon inserts. Three grams of noble agar were dissolved into 100 ml of double-glass-distilled water by heating. The agar was allowed to harden and then stored at room temperature in a sealed glass jar (oven sterilized at 500° C for 4-24 hours) until needed.

Cleaning Procedure of the Coupons (Inserts)

Initially, the biofilm coupon inserts were washed with detergent (Versa-Clean, Fisher Laboratory Supplies, Santa Clara, CA), heated at 100° C for three days, and just prior to use, UV sterilized in a Steril Gard Hood Class II (Model A/BE, The Baker Company, Inc., Sanford, ME) for forty five minutes. Later, changes in the cleaning procedure were made. The inserts were washed, placed into an acid washed vial, sonicated for five minutes in methanol, rinsed

with methanol, sonicated in hexane for five minutes, rinsed with methanol, and then stored in methanol until one day prior to use. The day before the coupons were prepared, the inserts were placed in an oven sterilized (at 500° C for 4-24 hours) 100 x 20 mm glass plate and placed in a 35° C Stabil Therm Incubator (Model B2730-Q, Blue-M, Blue Island, IL) overnight to remove the methanol. Just prior to use, the inserts were UV sterilized as above.

Test Organisms

The test organisms were streaked for purity onto an R2A plate and incubated until the organisms were actively growing: *K. pneumoniae* (Kp) for 24 hours and *P. fluorescens* (P17) for 48 hours. One colony was then suspended in 10 ml of mineral salts solution without added carbon and incubated for four hours at room temperature (20-25° C). The organisms were then ready to be applied to the silica or agar gel pads.

Procedure for Coupon Preparation

All the coupons for this research were prepared using the gel pad technique developed by Xiaoming Xu (Xu, 1993). The phosphate buffer on the silica gel pads was removed and the pads were allowed to dry for approximately thirty minutes. When the two percent noble agar was used, these pads were removed from the refrigerator and allowed to reach room temperature (20-25°C).