



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

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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 ($\mu\text{gC/L}$); 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 $\mu\text{gC/L}$ 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 ($\mu\text{gC/L}$), 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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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

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 ($\mu\text{gC/L}$); 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 $\mu\text{gC/L}$ 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 ($\mu\text{gC/L}$), 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]) \pm SE	0.10 \pm 0.007	0.18 \pm 0.013	0.19 \pm 0.008	0.29 \pm 0.030	0.29 \pm 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 $\mu\text{g/L}$ (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 ($\mu\text{g/L}$) acetate equivalents
Kemmy et. al.	Filter Sterilization	Four Species (P17, Curtobacterium sp., Corynebacterium sp., & unidentified coryneform	6	20	cfu/mL	AOC ($\mu\text{g/L}$)
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	$\cong 20$	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 ($\mu\text{g/L}$)
Billen-Servais	Filter Sterilization	Treatment plant	10-30	$\cong 20$	Bacterial number and size	BDOC (mg/L)
Billen-Servais	Filter Sterilization	Water from same environment as sample	28	20 ± 0.5	DOC	$\Delta\text{DOC} = \text{BDOC}$
Joret-Levi	none	Biologically active sand from a water treatment plant that does not use prechlorination	Until no change in DOC	$\cong 20$	DOC	$\Delta\text{DOC} = \text{BDOC}$
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	$\Delta\text{DOC} = \text{BDOC}$

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 $<10 \mu\text{g acetate-C eq/L}$ 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 Hamsch, 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 $\mu\text{gC/L}$ for sodium acetate and 50, 100, and 500 $\mu\text{gC/L}$ 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 $\mu\text{g/L}$ 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); $Mg_2SO_4 \cdot 7H_2O$ 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:

$$\text{AOC} = (\text{P17 AOC value} + \text{NOX AOC value}) - \text{the control AOC value.}$$

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 μ l of sample was placed into the Turner TD 20e Luminometer (Model 20, Turner Designs, Sunnyvale, CA).

8) 100 μ l 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 μ g/L AOC and for NOX as 3.4 μ g/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:

$$\text{AOC} = (\text{P17 AOC value} + \text{NOX AOC value}) - \text{the control AOC value.}$$
 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 $\mu\text{gC/L}$ (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

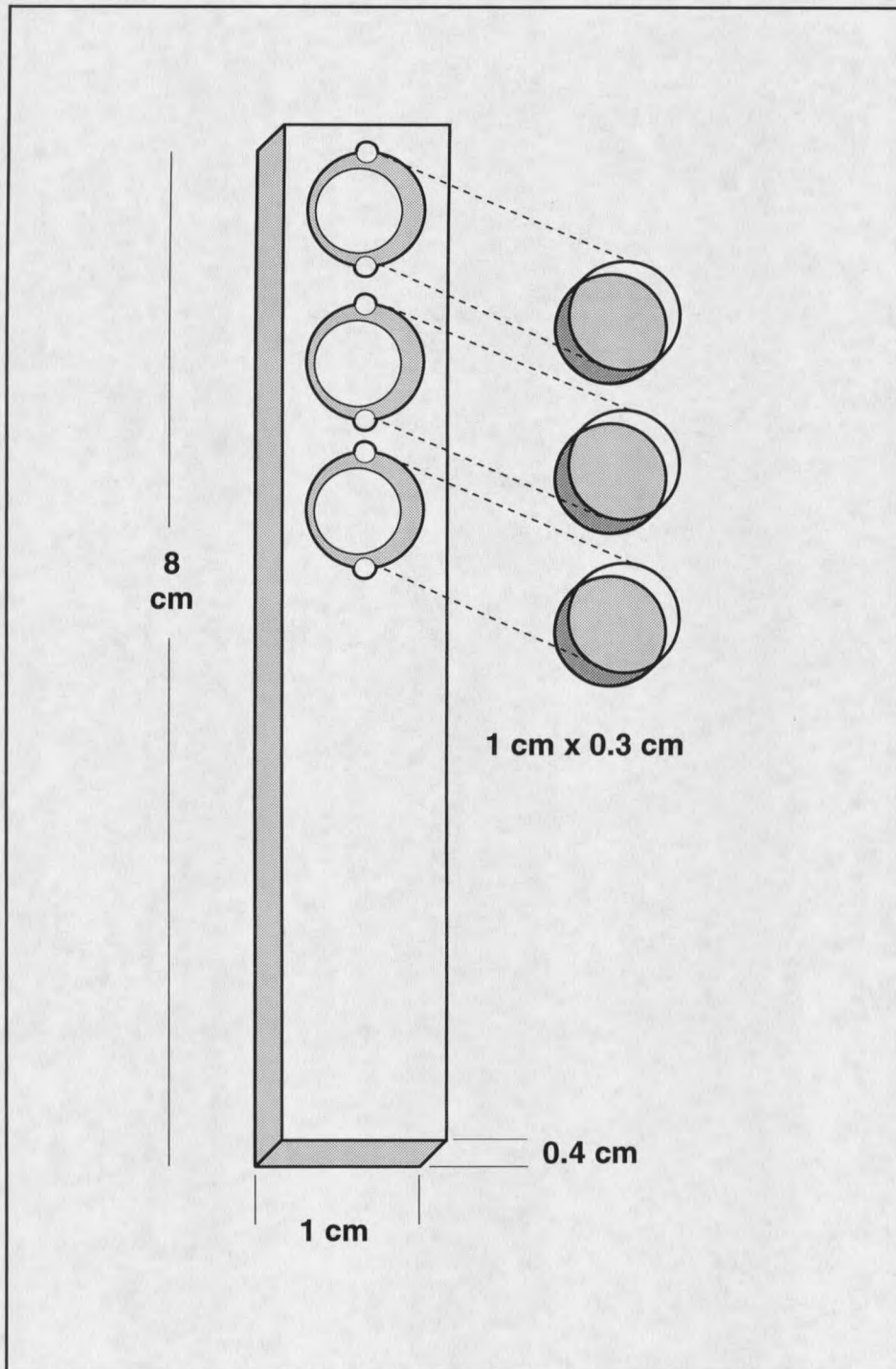


Figure 1. Schematic of biofilm coupon holder and inserts.

are 1 cm in diameter. The inserts are seeded with a monolayer of bacteria (5 μ l 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_2PO_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).

The bacterial suspension was then placed on either gel pad surface in 0.005 ml drops (one drop per insert). The silica gel pads were dried at room temperature for thirty minutes and the two percent noble agar gel pads were dried for fifteen to twenty minutes at 35° C. A piece of the gel pad with the bacterial suspension was then cut from the plate using a sterile 0.3 cm diameter plastic tube. The pad was inverted and then gently pushed into the coupon insert so that the bacteria were in close contact with the bottom surface of the insert. The insert was then filled with melted (50° C) three percent noble agar (See Figure 2). The bacteria in ten grids per coupon insert were counted directly under an Olympus bright-field microscope (Model BH-2, Olympus Optical Company, Ltd., Japan) at an overall magnification of 600x.

Viability Tests

CTC & DAPI Staining on Organisms in the Inserts

5-Cyano-2, 3-di 4-poly-tetrazolium chloride (CTC) (Polysciences, Inc., Warrington PA) staining was attempted on freshly prepared biofilm coupons using both *K. pneumoniae* and *P. fluorescens* (P17). A CTC solution was prepared by sonicating 0.025 g CTC in 50 ml of filtered nanopure water on the day of use. A stock solution of 4,6 diamidino-2-phenylindole (DAPI) (Sigma Chemical Co., St. Louis, MO) was prepared by dissolving 10 mg of DAPI into

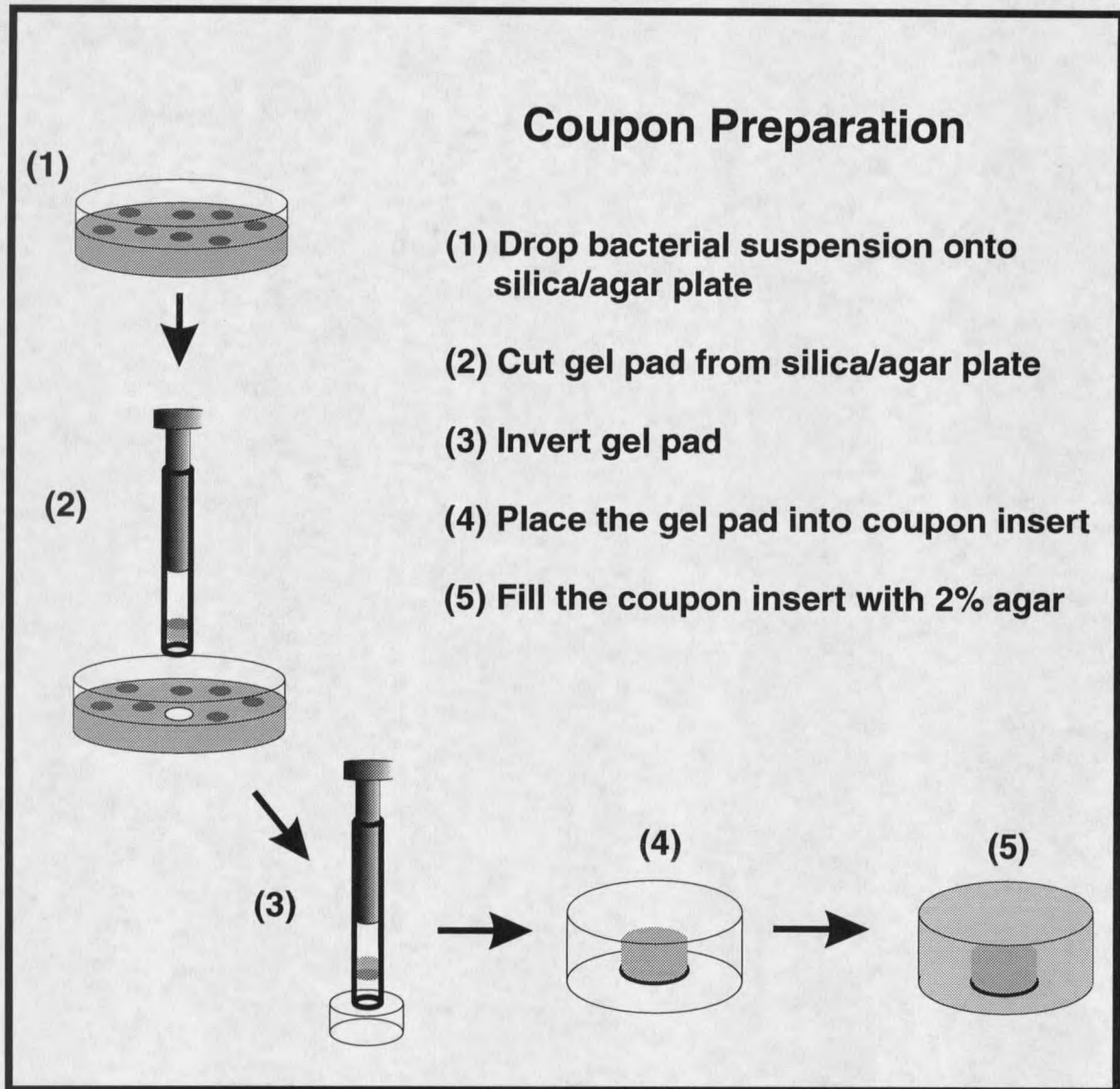


Figure 2. Schematic diagram of the procedure for biofilm coupon preparation developed by Xiaoming Xu (Xu, 1993)

100 ml of filtered nanopure water. The inserts were incubated in 20 ml of CTC solution for 4-6 hours. The CTC was siphoned off and the coupons were placed in 20 ml of DAPI with 0.4 ml of formalin and allowed to incubate for 4 hours. The inserts were then removed from the DAPI/formalin solution and observed under the epifluorescent microscope. The CTC either did not reach the cells or the cells did not reduce the CTC to CTC-formazan in the biofilm coupon. In addition, the DAPI reacted with the gel matrix so cells could not be distinguished from background. Modifications were then made.

CTC & DAPI Staining of Planktonic Organisms

K. pneumoniae (Kp) and P17 were prepared for use in the biofilm coupon as described above. After the four hour incubation period, one ml from each bacterial solution was placed in a 150 ml oven sterilized carbon free glass beaker with 5 ml of the CTC solution. The sample was allowed to stand in the CTC solution at room temperature (20-25° C) for two hours. Cells were then fixed by the addition of 0.3 ml of 37% formalin. The 6.3 ml solution was retained in a membrane filtration tower (Millipore Corporation, Cambridge, MA) for two to three minutes prior to filtration through a 0.22 µm black polycarbonate filter (Fisher Laboratory Supplies, Santa Clara, CA). The membrane was then covered with 0.1-0.2 ml of the DAPI solution. This was allowed to stand for five to ten minutes and the DAPI was removed by filtration. Once the DAPI was removed, the membrane was allowed to dry and applied to a slide. Bacteria in

ten grids per slide were counted using an Olympus model BH-2 microscope with an Olympus model BH2-RFL-T2 mercury lamp, an Olympus model UG1 filter for DAPI, an Olympus model BP490 filter for CTC, and an Olympus D Plan APO UV series 160/1.30 oil immersion objective (all equipment from Olympus Optical Company, Ltd., Japan). Both CTC counts and DAPI counts were done in the same grid area. Cells that reduced CTC had crystals that fluoresced orange-red, while the DAPI cells fluoresced blue. The number of cells with CTC crystals was divided by the number of total cells to calculate the percent of cells respiring. This work was done in triplicate.

CTC & DAPI staining were also conducted on log phase planktonic cells of both test organisms to compare the percent respiration of actively growing cells with the percent respiration of cells depleted of endogenous reserves. Both *K. pneumoniae* and *P. fluorescens* were grown in batch using a similar protocol as described for the growth curve work. Once the cells were in log growth, the CTC protocol described above was followed for both organisms. This work was done in triplicate.

INT Staining

A p-iodonitrotetrazolium violet (INT) (Sigma Chemical Co., St Louis, MO) solution was prepared by sonicating 0.126 grams of INT in 50 ml of filtered nanopure water. The biofilm coupon inserts were prepared as described above. Three inserts were placed into 10 ml of INT and allowed to incubate for 24 hours

in the dark at room temperature (20-25° C). After 24 hours, 0.4 ml of 37% formalin was added to stop the reaction. Bacteria in ten grids per coupon insert were counted using an Olympus bright-field microscope (Model BH-2, Olympus Optical Co., Ltd., Japan). Cells with red INT crystals and total cells (cells in the grid with or without a red INT-formazan crystal) were counted. The number of cells with INT crystals was divided by the number of total cells per grid to calculate the percent of cells respiring. The INT staining was done on three inserts per organism per flowing reactor experiment to make sure there were actively respiring cells in each experiment.

Assessment of Utilizable Carbon Leaching from Insert Materials

The biofilm coupon inserts were washed, heated for three days at 100° C, then UV sterilized for forty-five minutes. The coupons were prepared as described above using the silica gel pad and agar gel overlay, except there were no added organisms. One insert was placed into 40 ml of double-glass-distilled water (12 different coupons were put into 12 separate 40 ml vials per experiment) and incubated for four days at room temperature (20-25° C). The AOC of the double-glass-distilled water prior to addition of the biofilm coupons was determined (by both van der Kooij's plate count method and LeChevallier's

ATP method) and compared to the AOC of the double-glass-distilled water after incubation of the coupons. These experiments were done in triplicate.

The above procedure was repeated with the methanol/hexane sonication washed inserts for comparison. In addition to changes in the cleaning process, the gel pad was changed from a silica gel pad to a two percent noble agar gel pad. These experiments were done in duplicate.

Biological Stability Studies

The biofilm coupon inserts were prepared as described above. Bacteria in six inserts per organism were counted using bright-field microscopy. Three counted inserts of *K. pneumoniae* (Kp) and three counted inserts of P17 were placed into each of two continuous flow reactors per experiment (both a control and a supplemented reactor) with a residence time of ten minutes and a flow rate of 1.06 ml min^{-1} (See Figure 3). The control was double-glass-distilled water with added mineral salts. The supplemented reactor had double-glass-distilled water with added mineral salts (diluted in double glass distilled water 1:1000 for 15-50 $\mu\text{gC/L}$ and 1:100 for 100-500 $\mu\text{gC/L}$) and various concentrations (15-145 $\mu\text{g/L}$) of sodium acetate or (50-500 $\mu\text{gC/L}$) of a defined carbon cocktail (composition consisted of the following: ethyl alcohol (100%), 6.42 ml/L; sodium acetate, 0.342 g/L; sodium benzoate, 0.172 g/L; p-hydroxy benzoic acid, 0.164 g/L; and propionaldehyde, 0.2 ml/L; the stock solution was

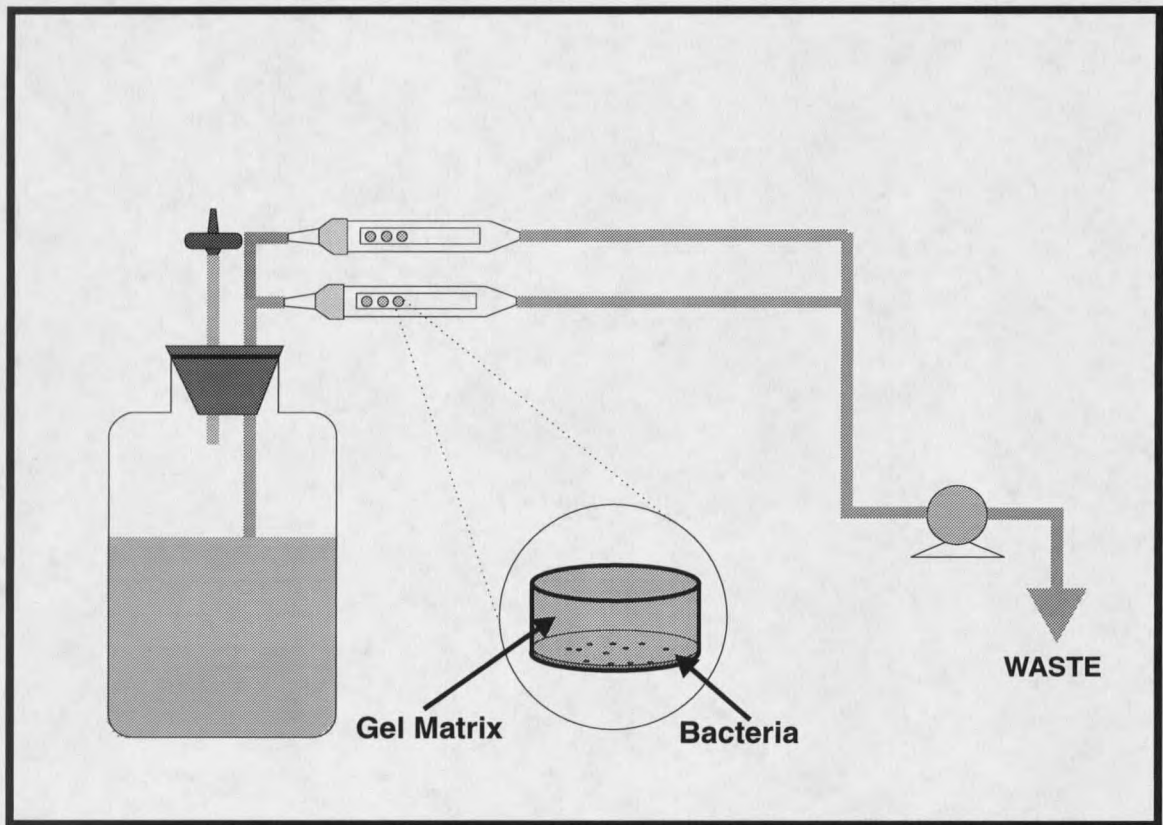


Figure 3. Schematic diagram of the continuous flow reactor used in the biostability experiments.

diluted to 50-500 $\mu\text{gC/L}$). The water for both the control and supplemented reactor was pumped over the coupons and collected for an initial AOC value. Flow continued through both reactors for seventy-two hours. The coupons were counted after 48 hours by bright-field microscopy, and then replaced in the system for 24 hours if the average number of cells was less than 100 per grid. The growth rate was calculated using the following equation:

$$\mu = \frac{\ln(N_t) - \ln(N_0)}{\text{Time}}$$

where (N_t) = the average highest cell count and (N_0) = the average initial cell count. The AOC of the control and supplemented water after passage through the coupon holder was determined at the end of the experiment. The pH for both the control and supplemented water was measured using an Accumet pH/ion Meter (Model 950, Fisher Scientific, Santa, Clara, CA) for each experiment. The pH ranged from 5.9-6.7. Replicate experiments were conducted for each different concentration of sodium acetate and the carbon cocktail.

Carbon Leaching in the Flowing System

In three of the flowing reactor experiments, the AOC of the control water was measured (using LeChevallier's ATP method) at the beginning of the experiment, after twenty-four hours, after 48 hours and at the end of the

experiment. The AOC of the supplemented water in the three corresponding experiments was determined at the beginning of the experiment and at the end of the experiment. These measurements were done in triplicate.

Rates of Acetate Uptake

Radiolabelled Sodium Acetate

Acetic acid-2-¹⁴C, sodium salt (Sigma Chemical Co., St Louis, MO) with a specific activity of 41.8 mCi/mmol was used as the sole added carbon source for these experiments. The concentration of sodium acetate was either 5 µg/L or 50 µg/L. The amount of isotope needed for a concentration of 5 µg/L produced approximately 5.6056×10^4 dpm when run as a standard in the liquid scintillation analyzer (Model A1900, Packard Instrument Company, Meriden, CT). For the 50 µg/L experiments the amount of the radiolabel added was multiplied by ten.

Organisms

K. pneumoniae (Kp) and P17 were streaked for purity onto separate R2A plates 24 and 48 hours respectively prior to the sampling day. A portion of one colony of P17 or Kp was suspended in 10 ml of mineral salts with no added carbon (composition of standard mineral salt solutions for this work consisted of the following (g/L): KH₂PO₄, 7; K₂HPO₄, 3; (NH₄)₂SO₄, 1.0; Mg₂SO₄ • 7H₂O 0.1;

the stock solution was diluted 1:1000 for 5 µg/L and 1:100 for 50 µg/L with double-glass-distilled water). The cell suspension was homogenized for one minute with the Tissumizer (Model SDT 1810, Tekmar Company, Cincinnati, OH); Tissumizer probe (Model S25N-8G, Tekmar Company, Cincinnati, OH). The cell suspension was plated in triplicate to determine the initial number of CFU/ml (approximately 10^7 to 10^8 CFU/ml).

Sample Design

Twenty-five mm glass fiber filters (Fisher Laboratory Supplies, Santa Clara, CA) were cut into eight sections, dipped in methyl-benzethonium hydroxide (Sigma Chemical, Co., St. Louis, MO) and allowed to dry for sixty minutes. The filters were used to trap the $^{14}\text{CO}_2$ that was respired.

The biotic samples included mineral salts, 1 ml of organisms and 2.5 µl of acetic acid-2- ^{14}C from the stock solution. The abiotic samples contained mineral salts and 2.5 µl of acetic acid-2- ^{14}C . The Control A sample included mineral salts and 1 ml of the organisms, but no radiolabel (See Table 3). All sample vials were oven sterilized and carbon free.

At the beginning of the experiment all sample vials were prepared as listed in above. Wheaton straight plug stoppers (with methyl-benzethonium hydroxide $^{14}\text{CO}_2$ traps inserted) (Fisher Laboratory Supplies, Santa Clara, CA)

Table 3. Design of sampling procedure for each experiment.

Types of Samples	Number of Samples	Mineral Salts	acetic acid- 2- ¹⁴ C	Organisms added
Biotic	3	9 ml	2.5 μ l	1 ml
Abiotic	2	10 ml	2.5 μ l	0
Control A	1	9 ml	0	1 ml

with 20 mm aluminum rings (Fisher Laboratory Supplies, Santa Clara, CA) were used to seal the vials. At the end of each incubation period (Time 0, 2, 4, 6, 8, 12 hours) 0.1 ml of 4N H₂SO₄ was added to all six samples (biotic, abiotic, and control). The sample vials were left sealed for 30 minutes after the addition of H₂SO₄ so that all of the ¹⁴CO₂ would be released from the solution. After thirty minutes, the six vials were destructively sampled. Each filter with the trapped ¹⁴CO₂ from the biotic, abiotic and Control A vials was put into 10 ml of Ultima Gold scintillation cocktail (Packard Instrument Company, Meriden, CT). One tenth of a ml of the biomass and unconverted substrate sample from the three biotic samples and the Control A sample was added to 9.9 ml of scintillation cocktail. The rest of the combined sample was centrifuged in a microcentrifuge (Model 5A9, Fisher Scientific, Santa Clara, CA) for fifteen minutes at the maximum speed (10,000 x g) to separate the biomass sample (pellet) from the unconverted substrate sample (supernatant). Once the supernatant and pellet

were separated, 0.1 ml of supernatant was then added to 9.9 ml of scintillation cocktail. One ml of scintillation cocktail was also added to each pellet, vortexed, and all the pellets for one sample were combined in a scintillation vial with 3 ml of scintillation cocktail. One tenth of a ml from each abiotic sample was placed in a scintillation vial with 9.9 ml of scintillation cocktail. This procedure was repeated for all sampling times.

At the end of the experiment all scintillation vials were analyzed in the liquid scintillation analyzer. The counts of ^{14}C were measured for five to ten minutes per sample and the DPM's were recorded. A quench curve and a luminescence correction were included in the protocol.

CHAPTER 4

RESULTS**Growth of *K. pneumoniae* in 100 mg/L Sodium Acetate****Planktonic Growth**

The growth characteristics of *K. pneumoniae* were observed to determine whether it would grow planktonically with sodium acetate as the sole carbon source. 10^4 CFU/mL were inoculated into 100 ml of mineral salts medium with 100 mg/L added sodium acetate. Cell numbers were measured at various intervals. The results of each experiment are presented as separate growth curves. There were two replicate flasks in two experiments. It was observed that log phase growth began within the first twenty-four hours and by 72 hours there was a three to four log increase in the number of colony forming units in all four flasks (Figure 4). Thus, *K. pneumoniae* is able to grow planktonically with sodium acetate as the sole carbon source.

Attached Growth

The growth characteristics of *K. pneumoniae* immobilized in the biofilm coupon grown with sodium acetate as the sole added carbon source was also observed. *K. pneumoniae* was depleted of endogenous reserves and inoculated

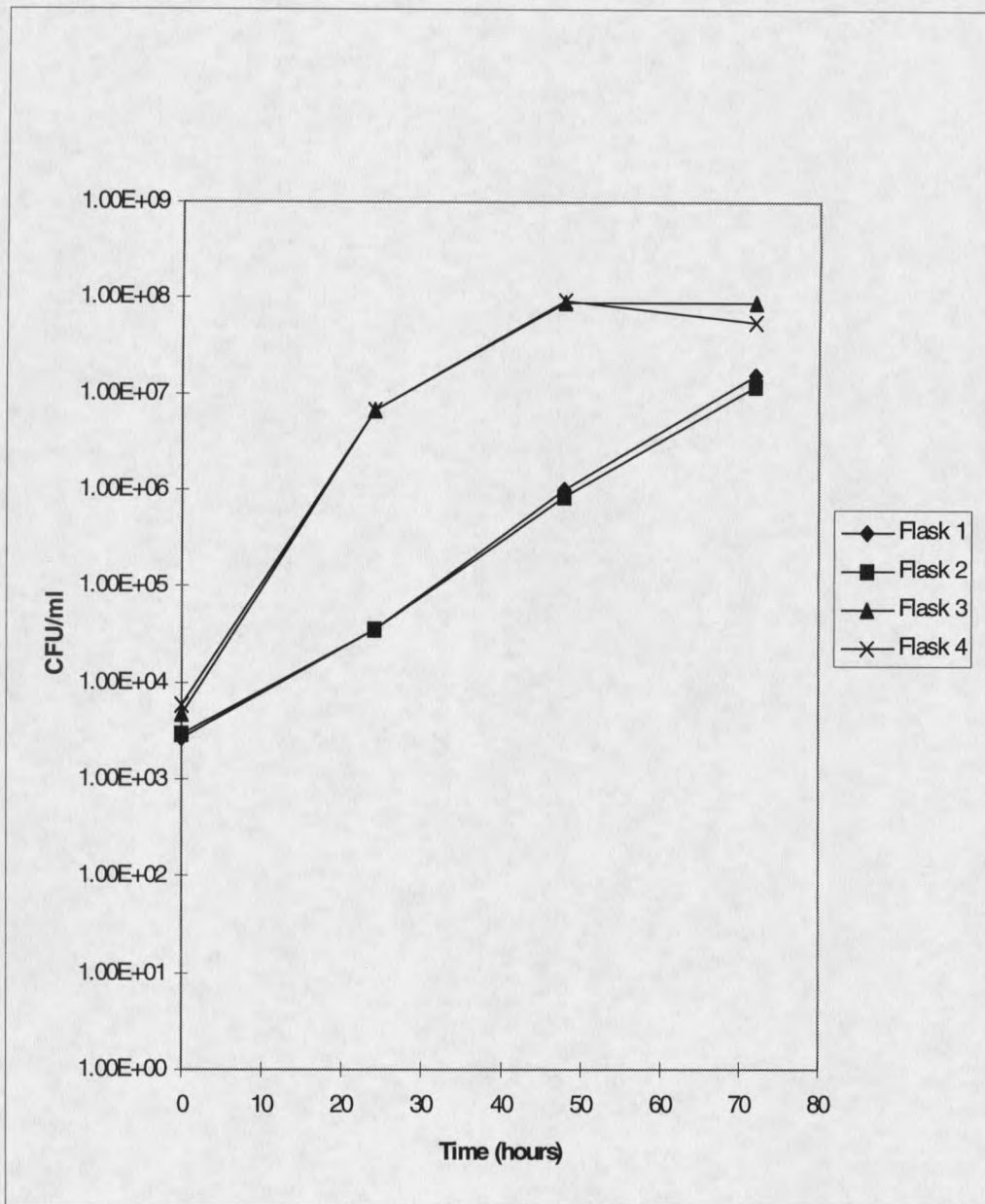


Figure 4. A growth curve of *K. pneumoniae* grown in a mineral salts solution with 100 mg/L sodium acetate. Flasks 1 & 2 represent one experiment and Flasks 3 & 4 are a replicate experiment. Each point represents the average of three plate counts.

into the biofilm coupon. The biofilm coupon inserts were incubated in batch in 100 ml mineral salts medium with 100 mg/L added sodium acetate. The inserts were observed microscopically at different time periods for up to 66 hours and the number of cells were counted at each time period. The results of these experiments are presented graphically as the ratio of the number of cells at each time to the initial number. Each line represents one insert of the biofilm coupon. The data points from each experiment represent the relative increase in the average number of cells in ten counted grids with time. It was observed that in the first 0-6 hours the organisms were in lag phase; from 6-48 hours the organisms were in log phase; and after approximately 48 hours the organisms entered stationary phase (Figure 5). There was at least a seven fold increase in cells in forty-eight hours. Thus, *K. pneumoniae* is also able to grow immobilized in the biofilm coupon with sodium acetate as the sole carbon source.

Measurements of Double-Glass-Distilled Water

Standard AOC Methodologies

Initially, it was necessary to determine the AOC concentration of double-glass-distilled water using the standard planktonic methods (LeChevallier's ATP method and van der Kooij's plate count method). Comparisons were made

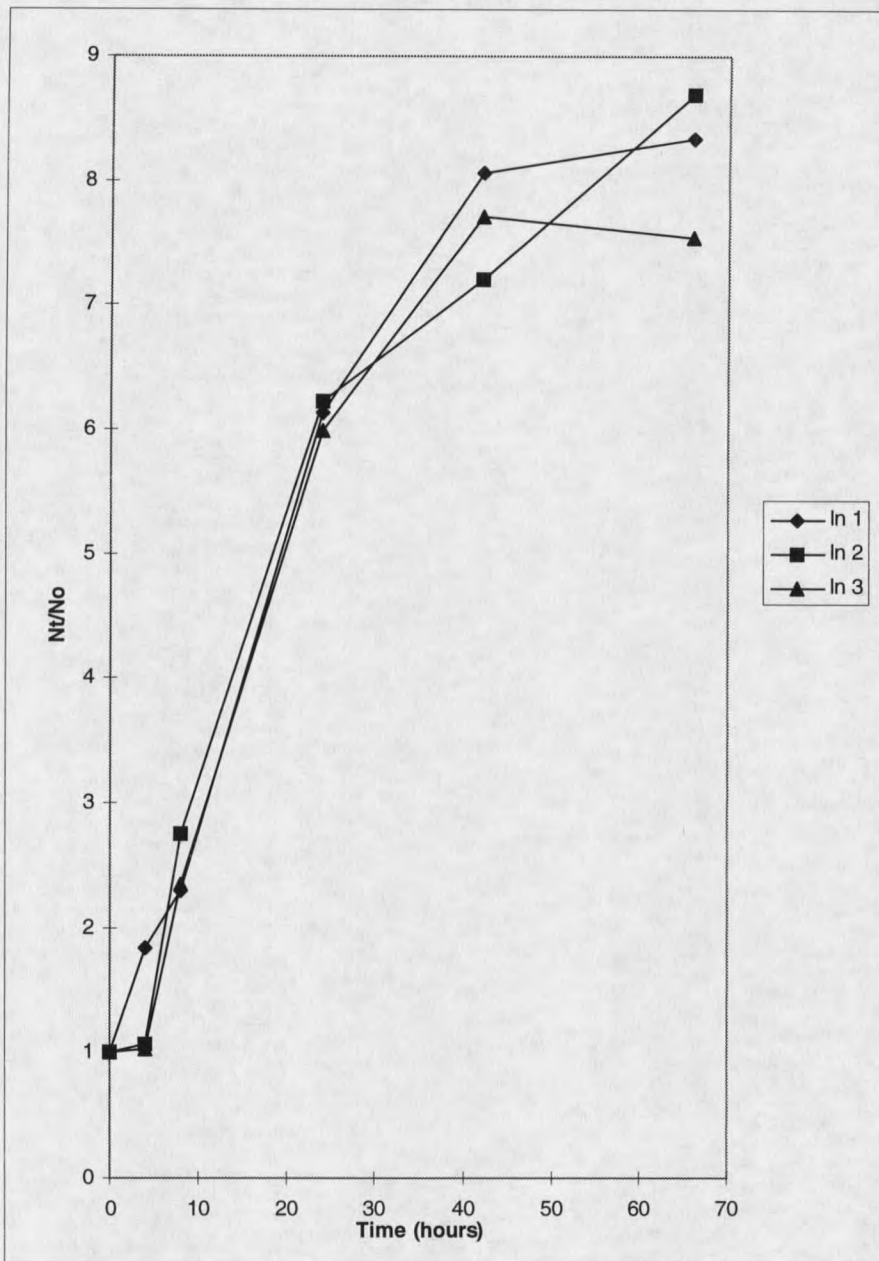


Figure 5. Growth of *K. pneumoniae* immobilized in the biofilm coupon in mineral salts solution with 100 mg/L sodium acetate. Inserts (In 1, In 2, In 3) are the separate wells in one coupon holder. Each point represents the average number of cells in 10 counted grids divided by the initial count (time 0).

between the assimilable organic carbon (AOC) measurement determined by LeChevallier's ATP method and van der Kooij's plate count method in order to determine which procedure would be used for this research. The results of this work are presented in bar graphs. Each bar represents one measurement by either LeChevallier's ATP method or van der Kooij's plate count method. There were three different experiments conducted with three different samples of double-glass-distilled water. Replicate experiments were not conducted. It was observed that each method yields different AOC measurements (Figure 6). The largest difference occurred in experiment one where LeChevallier's ATP measurement indicated an AOC of 11 $\mu\text{gC/L}$, while van der Kooij's plate count measurement indicated an AOC of 18 $\mu\text{gC/L}$. Though the differences were small, the measurements were conducted on double-glass-distilled water which typically has a low AOC value. Thus, the 1-7 $\mu\text{gC/L}$ difference may be important.

Comparisons of the two methodologies continued on water from the annular reactors at the pilot plant because this water typically has higher AOC values than double-glass-distilled water (20-350 $\mu\text{gC/L}$; Camper, *Submitted*). All water was sampled at the same time on the same day. The water was sampled from annular reactors 1-1, 1-2, 2-1, and 2-2 which did not receive chlorine or added AOC. The results of this work are presented in bar graphs (Figure 7).

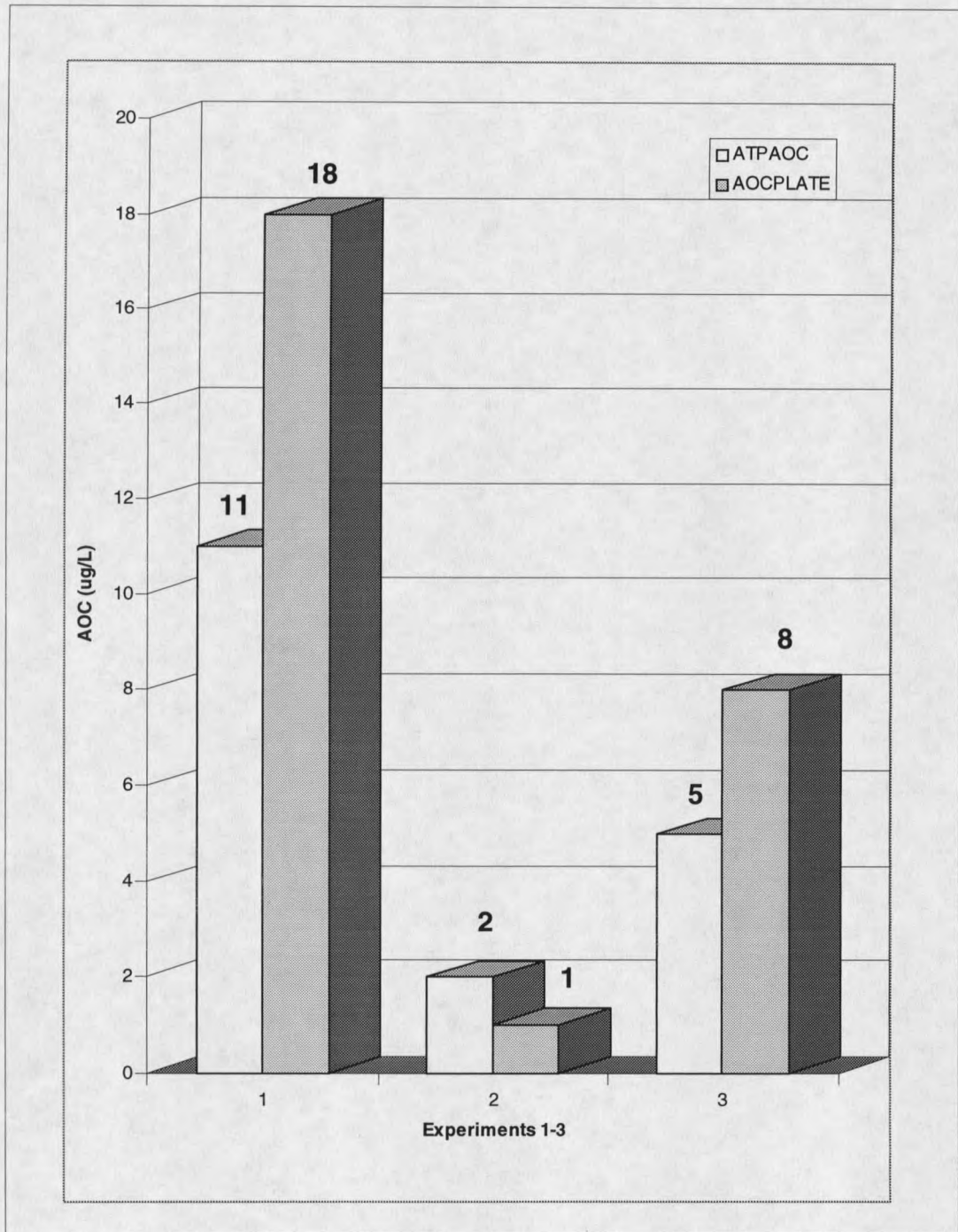


Figure 6. Comparisons of the AOC concentration of double-glass distilled water using LeChevallier's ATP method (ATPAOC) and van der Kooij's plate count method (AOCPLATE). The experiments were not replicated.

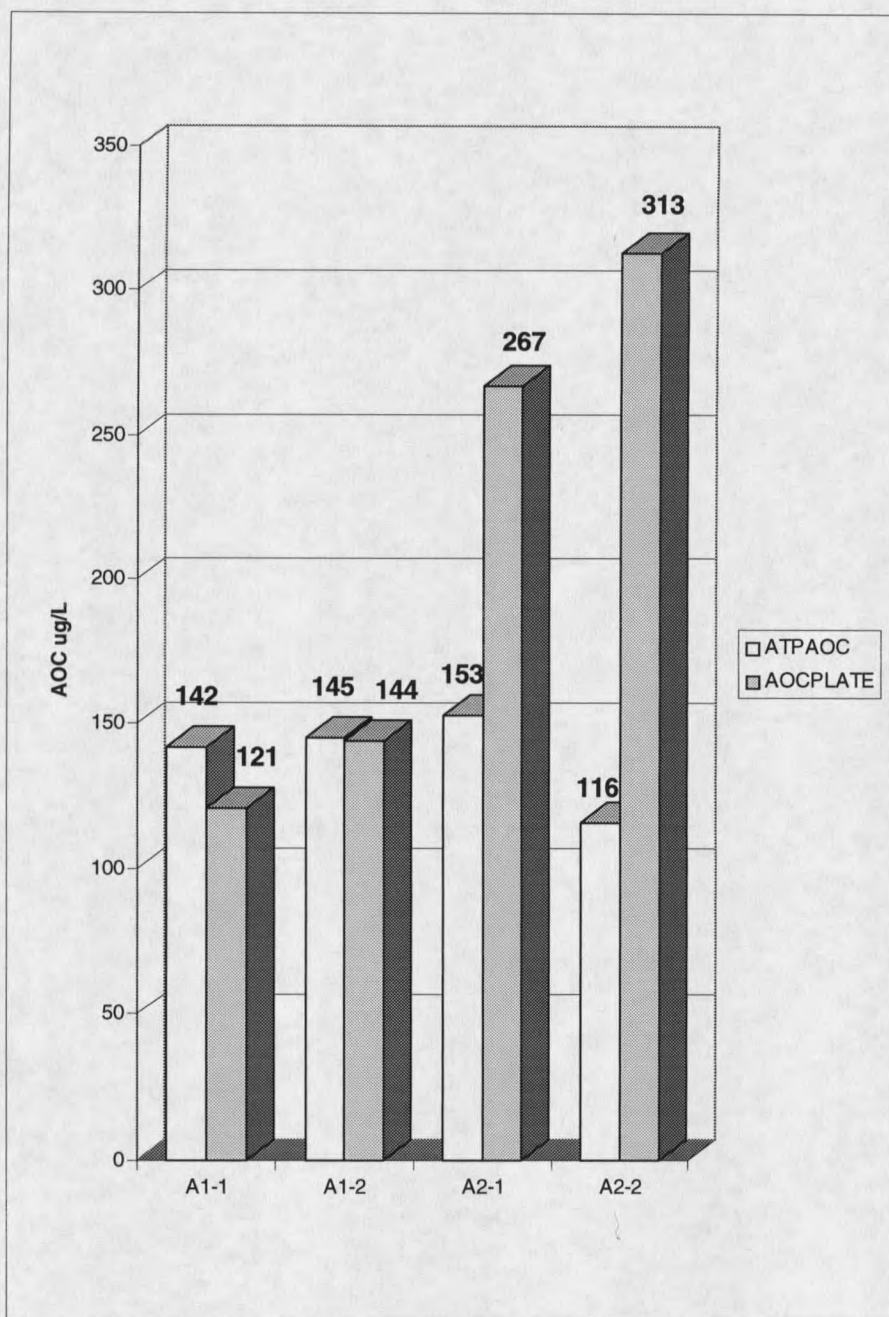


Figure 7. Comparisons of the AOC concentration in annular reactors A1-1, A1-2, A2-1, and A2-2 in the pilot plant at Bozeman's drinking water treatment plant using LeChevallier's ATP method (ATPAOC) and van der Kooij's plate count method (AOCPLATE). Each bar represents one measurement by either method. This work was not replicated.

Each bar represents one measurement of the AOC concentration using either LeChevallier's ATP method or van der Kooij's plate count method. Once again differences occurred between the AOC concentrations indicated by the two methodologies. In reactors 1-1 and 1-2 the discrepancy between the AOC values ranged from 1-21 $\mu\text{gC/L}$, and in reactors 2-1 and 2-2 from 114-197 $\mu\text{gC/L}$. The differences were once again considered important.

An experiment was devised to measure the AOC of the same sample of double-glass-distilled water in triplicate using LeChevallier's ATP method. Two experiments were conducted. The results of these experiments are listed in Table 4. The data are expressed as an average and standard error of three different AOC measurements of the same water. It was observed that the AOC of the first water supplemented was 58.7 $\mu\text{gC/L}$ with a standard error of ± 6.7 $\mu\text{gC/L}$. The AOC of the second water sample was 26.7 $\mu\text{gC/L}$ with a standard error of ± 7.1 $\mu\text{gC/L}$. Based on the simplicity, time to conduct the AOC analysis, and the medium to low variability between the measurements, LeChevallier's ATP method was chosen to measure the AOC of all water in this research.

Table 4. Three replicate AOC measurements on two samples of double-glass-distilled water.

	Exp. 1 AOC values ($\mu\text{gC/L}$)	Exp. 2 AOC values ($\mu\text{gC/L}$)
Replicate 1	72	13
Replicate 2	51	37
Replicate 3	53	30
Average ($\mu\text{g/L}$)	58.7	26.6
Standard Error ($\mu\text{g/L}$)	± 6.7	± 7.1

Growth Potential of Double-Glass-Distilled Water

The growth of *K. pneumoniae* and *P. fluorescens* (P17) immobilized in the biofilm coupon in double-glass-distilled water was measured. Biofilm coupon inserts with each organism were placed in 100 ml of double-glass-distilled water with an AOC of 4 $\mu\text{gC/L}$ as measured using LeChevallier's ATP method. The inserts were observed microscopically at different time periods for up to 124 hours and the number of cells were counted at each time period. The results of these experiments are presented graphically as the ratio in the number of

cells with time (Nt/No) (Figure 8). Each line represents one insert from the coupon holder. The data points represent the ratio in the average number of cells of ten counted grids with time. It was observed that both *K. pneumoniae*(Kp) and *P. fluorescens* (P17) were able to grow in double-glass-distilled water. There were at least 3-5 times more cells after 124 hours. In addition, P17 grows at a faster rate.

Viability of Immobilized Test Organisms in Coupons

CTC and DAPI Staining

CTC staining was attempted to determine which cells were actively respiring in freshly prepared biofilm coupon inserts using both *K. pneumoniae* (Kp) and *P. fluorescens* (P17). In two separate experiments neither CTC nor DAPI fluorescing cells were observed, though cells were present as indicated by bright field microscopy.

An alternate staining method was devised using planktonic *K. pneumoniae* (Kp) and *P. fluorescens* (P17). Prior to placement in the biofilm coupon, both Kp and P17 were depleted of endogenous nutrients by placing approximately 10^7 organisms into 10 ml of mineral salts medium with no added carbon for at least four hours. Since the viability of the planktonic test organisms directly affects the viability of the test organisms in the biofilm coupon, the percent of respiring planktonic test cells depleted of endogenous nutrients was

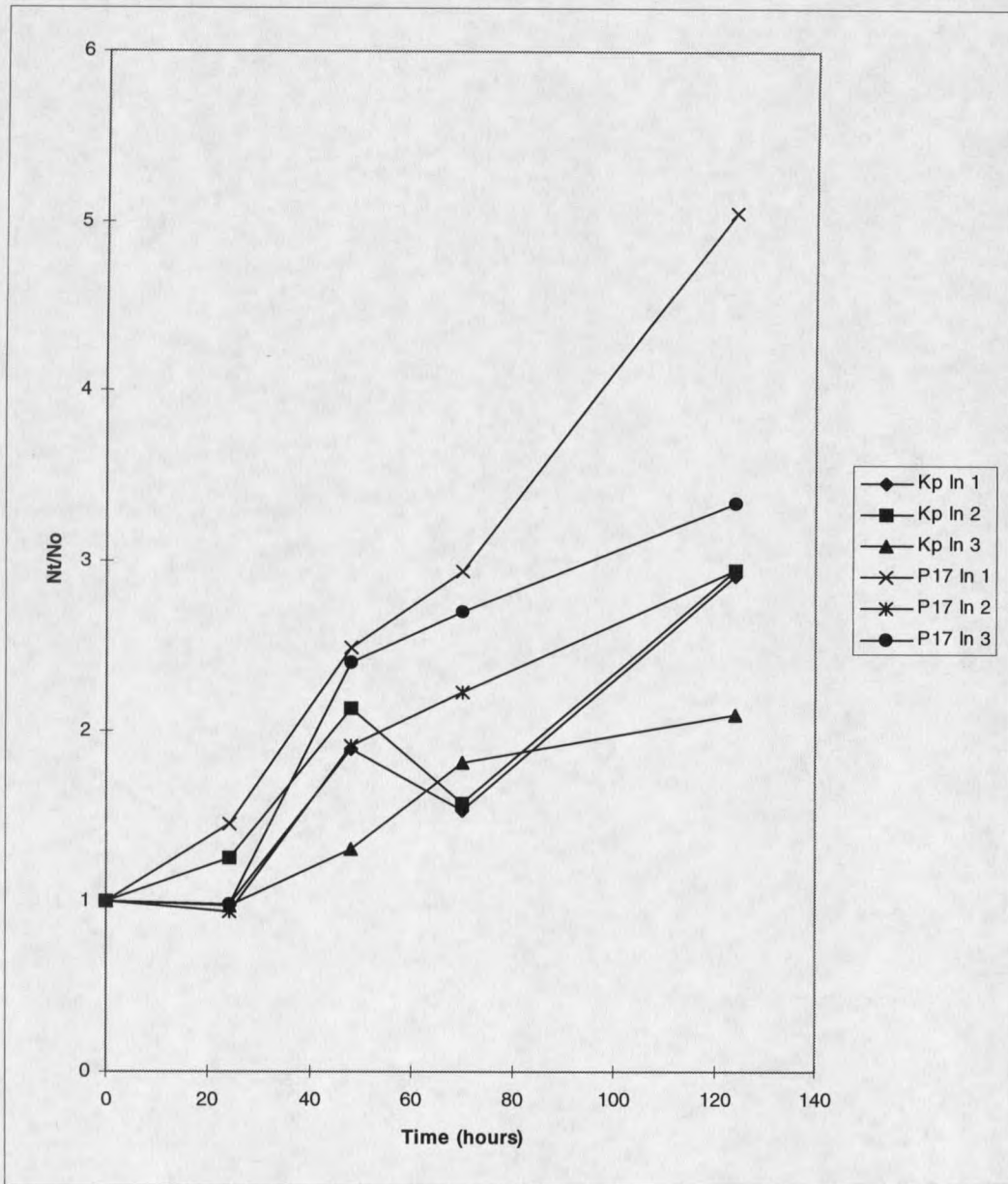


Figure 8. Growth of *K. pneumoniae* (Kp) and *P. fluorescens* (P17) immobilized in the biofilm coupon in double-glass-distilled water. Inserts (In 1, In 2, In 3) are the separate wells in one coupon holder.

determined using CTC and DAPI staining. For comparison, the percent of respiring planktonic test cells in log phase growth was also determined using CTC and DAPI. The results of these experiments are presented in a bar graph (Figure 9). Each bar represents the percent of CTC (+) cells determined using the average number of cells respiring per ten counted grids. Three different experiments were conducted for both organisms depleted of endogenous nutrients and both organisms in log phase growth. It was observed that approximately fifty percent of the test organisms depleted of endogenous nutrients were respiring. By comparison, ninety percent of the test organisms in log phase growth were respiring.

INT Staining

Since the one of the goals of this work was to determine the viability of the immobilized test organisms in the biofilm coupon, an alternate stain, INT, was utilized. INT, like CTC, is a tetrazolium salt that is reduced by the electron transport system of respiring organisms to INT-formazan. The red INT-formazan crystals are deposited intracellularly in bacterial cells and can be seen by light microscopy. This allows for easy observation of actively respiring cells in the biofilm coupon. INT staining was conducted for three coupons of each organism per flow reactor experiment. The average percent of actively respiring *K. pneumoniae* cells immobilized in the biofilm coupon was calculated as 47% ±

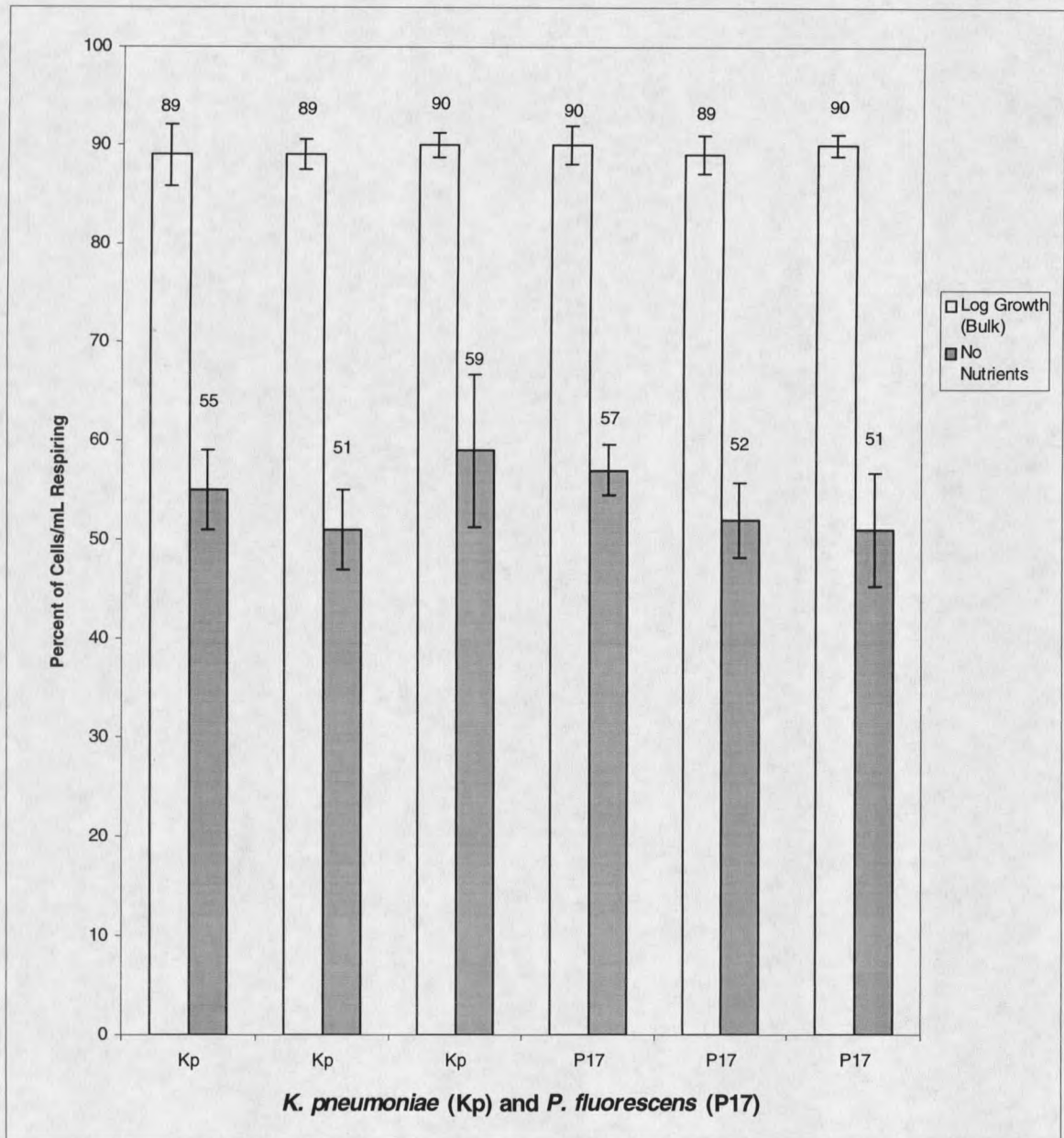


Figure 9. Percent of cells in culture exhibiting respiration as indicated by CTC fluorescence in logarithmic growth (Log growth (Bulk)) and following stationary phase incubation to exhaust organisms of endogenous reserves (No nutrients). Results are shown for triplicate tests with both *K. pneumoniae* (Kp) and *P. fluorescens* (P17).

1.8%. The average percent of actively respiring *P. fluorescens* cells was $43\% \pm 2.2\%$. The averages are for all INT measurements including certain instances where the cells did not reduce the INT to INT-formazan which resulted in very low to zero percentages (See Appendix B).

Utilizable Carbon Leaching From Insert Materials

Batch Studies

Comparisons continued between the AOC of double-glass distilled water prior to incubation with the biofilm coupon inserts and the AOC of water after a four day incubation with the biofilm coupon. Twelve separate inserts were placed in 12 separate 40 ml vials of double-glass-distilled water per experiment and incubated for four days at room temperature. The results of these experiments are presented in a bar graph (Figure 10). Each bar represents the average difference in the AOC prior to incubation and the AOC after incubation with the biofilm coupon inserts. Four different experiments were conducted using two cleaning techniques and two gel pad gels. The error bars represent the standard error between replicate experiments. It was observed that the largest increase in the AOC concentration ($237 \pm 30 \mu\text{gC/L AOC}$) occurred from the water exposed to the inserts cleaned by the detergent washing procedure and made with silica gel pads. The next highest difference occurred in the water

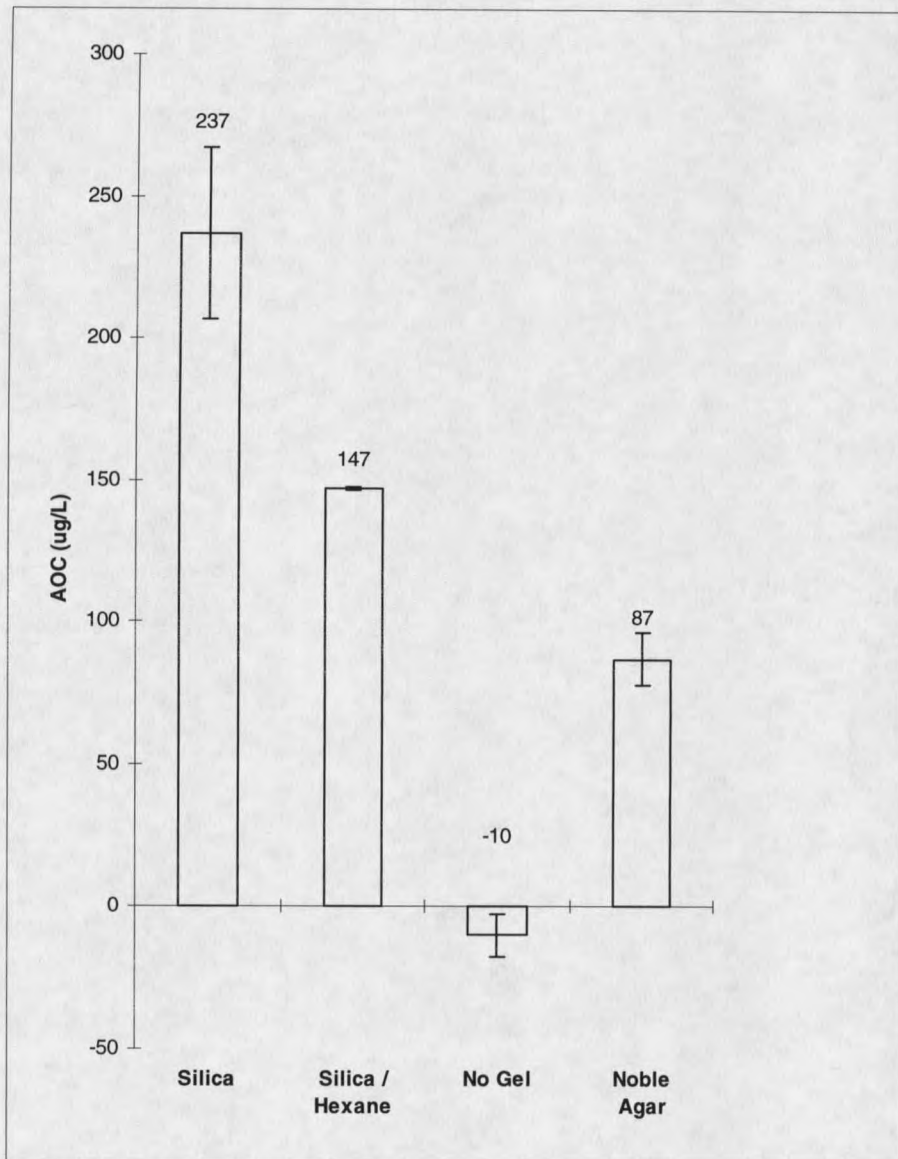


Figure 10. The differences between the AOC ($\mu\text{gC/L}$) leaching from the biofilm coupon inserts cleaned with and without methanol/hexane and made with either silica or agar gel pads. The error bars represent the standard error between replicate experiments.

containing inserts cleaned using the methanol/hexane sonication cleaning procedure and made with silica gel pads ($147 \pm 0.5 \mu\text{gC/L AOC}$). Since the methanol/hexane sonication cleaning technique showed a smaller amount of AOC leaching from the inserts, this cleaning technique was used for the remainder of the experiments. The lowest amount of AOC leaching from the inserts occurred with the methanol/hexane cleaned inserts with the noble agar gel pad ($87 \pm 9 \mu\text{gC/L AOC}$). Since this value was statistically lower than the value obtained with the silica gel pad inserts, the inserts were made with agar gel pads for the remainder of the experiments. A fourth experiment was conducted to measure the difference between the AOC that might be leaching from the coupon inserts without a gel matrix. It was observed that the difference in the AOC value is negative ($-10 \pm 7.5 \mu\text{gC/L AOC}$), but statistically, the AOC before and AOC after are not different. Thus, the coupon inserts without the gel leach little if any utilizable carbon as measured by AOC. The carbon leaching from the coupon inserts either comes from the gel matrix or from contamination during preparation and/or sampling.

Flow System

The purpose of these experiments was to determine if the carbon leaching from the coupons remained constant or decreased with time in the flow system. Comparisons of the difference in the AOC values of the control water

(double-glass-distilled water with added mineral salts) in the continuous flow reactor experiments at time zero, 24 hours, 48 hours, and 72 hours were made. The prepared biofilm coupons were placed in the continuous flow reactors and the control water was pumped over the coupons and collected in 40 ml EPA sample vials for an initial AOC measurement. The coupons were removed, counted, and replaced in the system. After 24 hours water was again collected in 40 ml EPA sample vials for a second AOC measurement. This procedure was repeated at 48 hours. After 48 hours the coupons were removed, counted, and replaced in the system for the final 24 hours. Water was collected for the final AOC measurement at 72 hours and the system was shut down. The AOC of the water in the continuous flow reactor receiving added acetate carbon ($15 \mu\text{gC/L}$) was measured at both the beginning of the experiment and after 72 hours. The results are presented in a combined bar (control AOC) and line graph (supplemented AOC) (Figure 11). Three replicate experiments were conducted for the AOC values from both the control and supplemented reactors. The general trend for the control system seems to be an initial increase of AOC followed by a decrease in AOC after 72 hours. The AOC of the supplemented system shows a decrease in AOC after 72 hours. Thus, the carbon leaching from the coupons decreases with time.

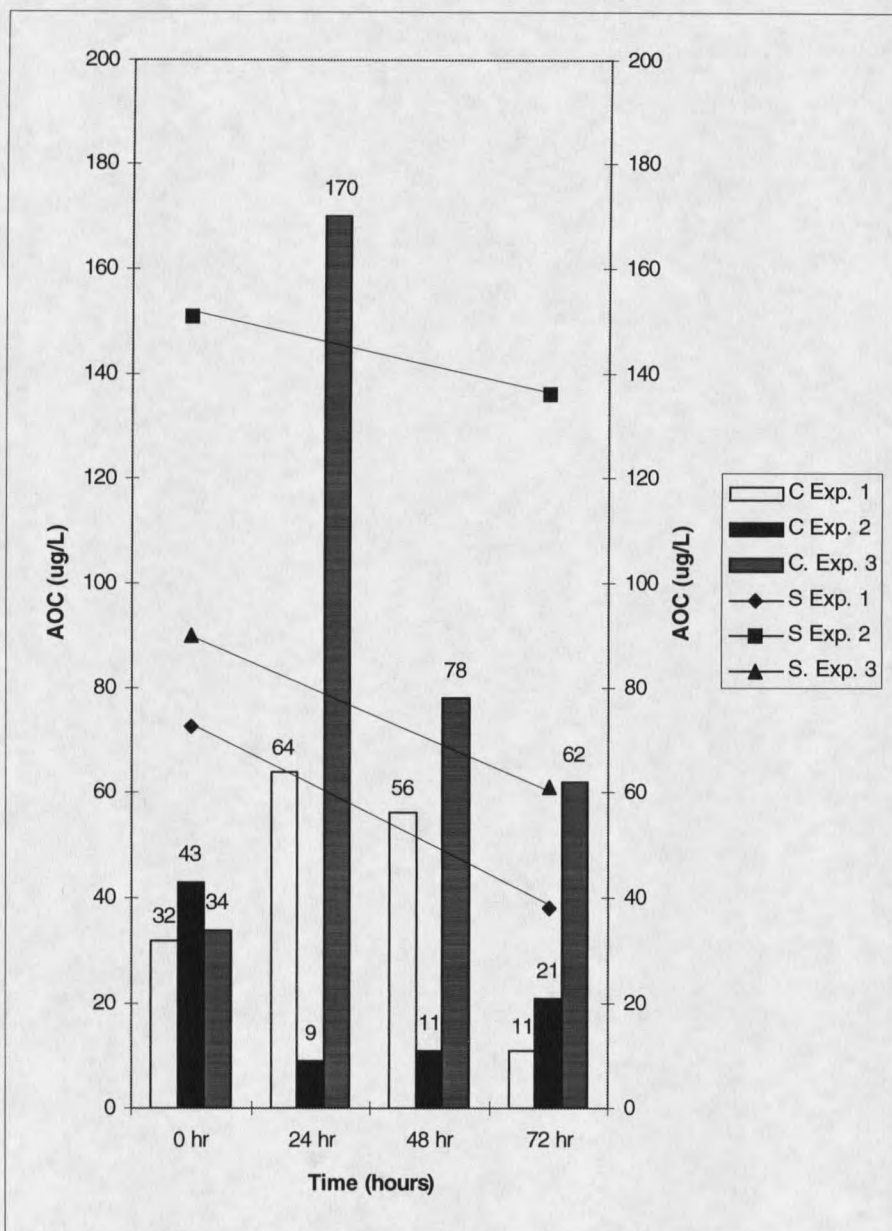


Figure 11. Comparisons of the AOC values of the control (C) and supplemented (S) from 0-72 hours in three continuous flow reactor experiments.

Biological Stability Studies

Sodium Acetate as the Sole Added Carbon Source

All figures in this section represent data from the continuous flow reactor experiments with the biofilm coupon using sodium acetate as the sole added carbon source for the supplemented reactor. Both reactors received mineral salts so that nitrogen and phosphorous were not limiting. The AOC of the control and supplemented continuous flow reactors was measured at the beginning and at the end of each experiment. The average AOC was then calculated from these two values for each experiment. Comparisons were made between the measured AOC value and the added sodium acetate value in order to determine if these values were similar. The results are presented in a scatter graph with a linear regression line indicating the correlation between the two values (Figure 12). Each point represents the average AOC value from the initial and final AOC measurements plotted against the added sodium acetate value for eight different experiments. The added sodium acetate concentration was at 0, 15, 29, and 145 $\mu\text{gC/L}$. It was observed that the two values are correlated ($R^2 = 0.7262$). Statistical analysis revealed the measured AOC is significantly different from the added sodium acetate concentration at a 95% confidence interval. For the hypothesis that the slope = 1, the p-value is 0.00046. The 95% confidence interval for this slope is (1.001, 3.462).

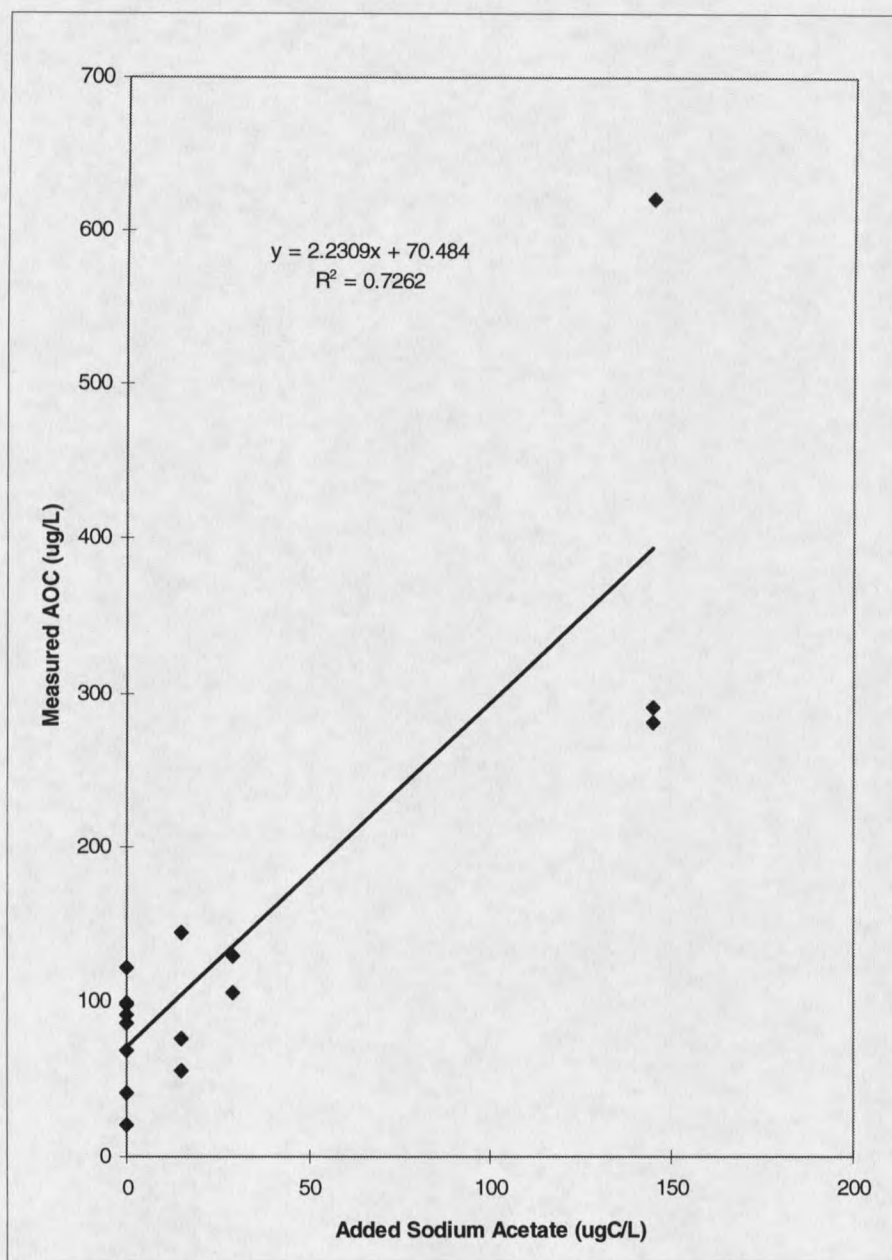


Figure 12. The measured AOC ($\mu\text{gC/L}$) versus the added sodium acetate value ($\mu\text{gC/L}$) from the continuous flow reactor experiments.

The biofilm coupon was utilized to indicate the response of immobilized *K. pneumoniae* and *P. fluorescens* to the measured AOC ($\mu\text{gC/L}$) and varying concentrations of added sodium acetate. The growth rates of *K. pneumoniae* and *P. fluorescens* in the supplemented continuous flow reactors were compared to the measured AOC values ($\mu\text{gC/L}$). The results of these experiments are presented in a scatter graph with linear regression lines through *K. pneumoniae* growth rates and *P. fluorescens* growth rates at the measured AOC concentrations (Figure 13). Each point represents the average growth rate of the three coupon inserts for one experiment at the measured AOC value in the supplemented continuous flow reactor system. It was observed that as the measured AOC ($\mu\text{gC/L}$) value increased the growth rate also increased for both organisms. However, there was not a tight correlation between growth rate and measured AOC concentration ($\mu\text{gC/L}$) for either organism ($R^2 = 0.4479$ for *K. pneumoniae* and $R^2 = 0.2312$ for *P. fluorescens*).

The growth rates of immobilized *K. pneumoniae* and *P. fluorescens* in both the control (no added sodium acetate) and the supplemented continuous flow reactor experiments at the measured AOC values were then compared. The results are presented in a scatter graph with linear regression lines through *K. pneumoniae* and *P. fluorescens* growth rates at the measured AOC values (Figure 14). Each point from the control and supplemented reactors represents the average growth rate of the three coupon inserts for one experiment at the

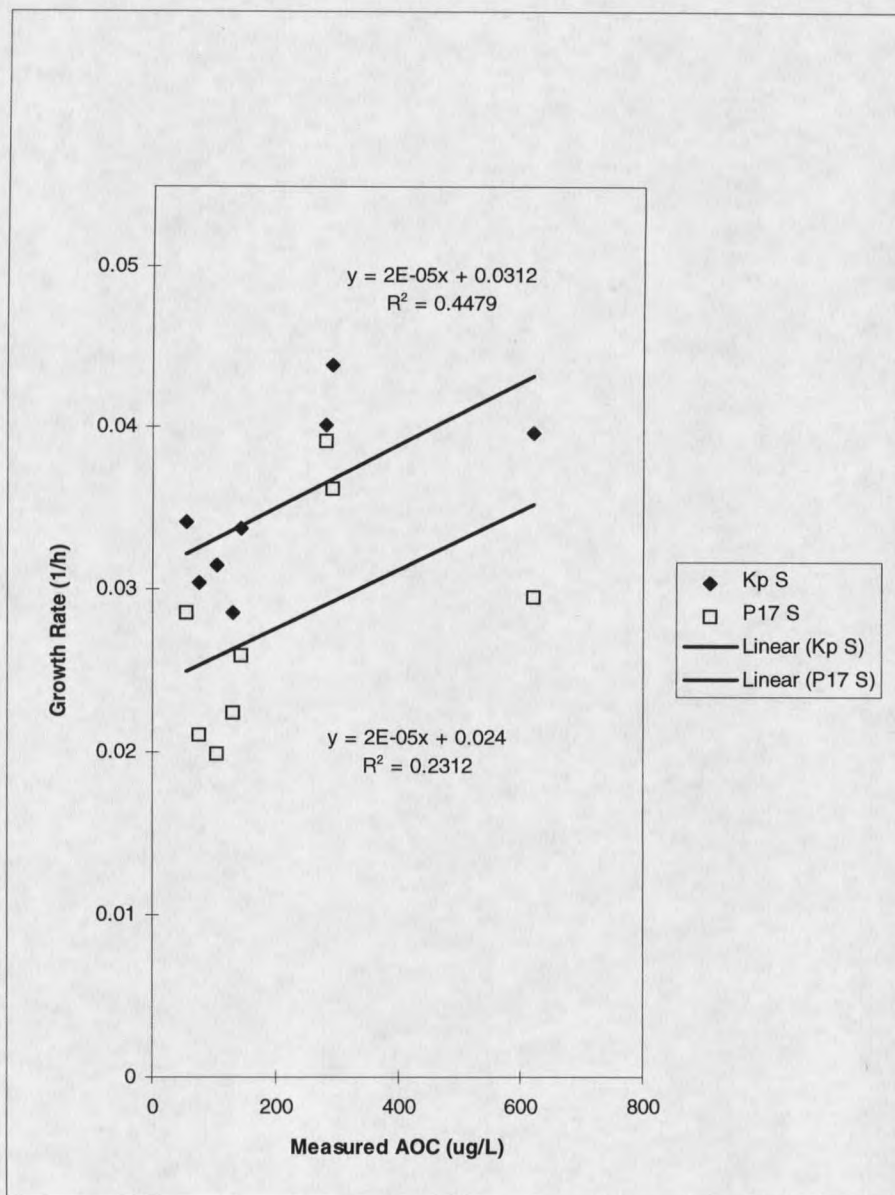


Figure 13. The growth rates (h^{-1}) of *K. pneumoniae* (Kp S) and *P. fluorescens* (P17 S) from the supplemented continuous flow reactors at the measured AOC values ($\mu\text{gC/L}$).

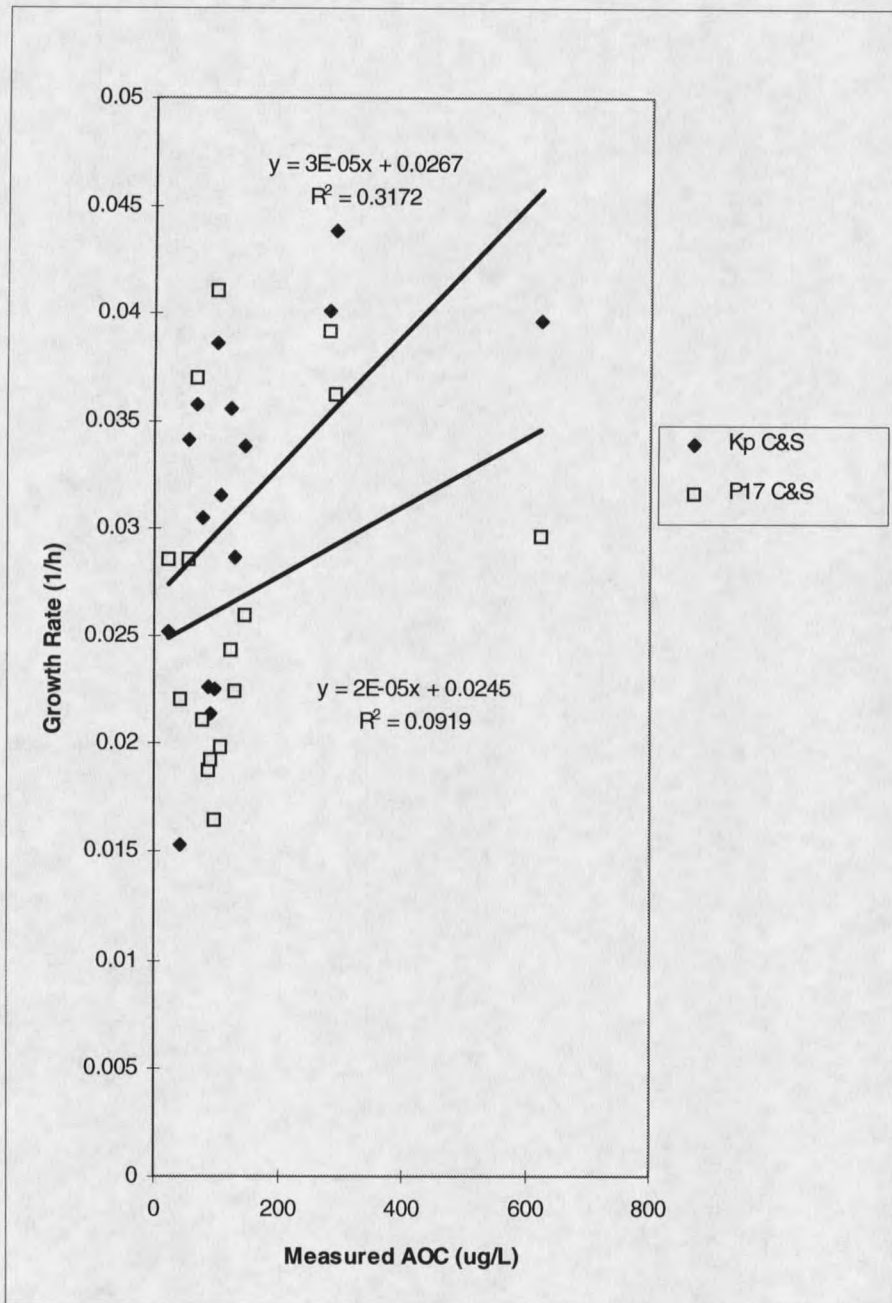


Figure 14. The growth rates (h^{-1}) of *K. pneumoniae* (Kp C&S) and *P. fluorescens* (P17 C&S) in both the control and supplemented continuous flow reactor experiments at the measured AOC values ($\mu\text{g/L}$).

measured AOC value. It was observed that the growth rate seems to increase with increasing concentration of measured AOC; however, with the inclusion of the control measured AOC values, the growth rates of *K. pneumoniae* and *P. fluorescens* become less correlated with the measured AOC values ($R^2 = 0.3172$ versus 0.4479 for *K. pneumoniae* and $R^2 = 0.0919$ versus 0.2312 for *P. fluorescens*).

The growth rates of *K. pneumoniae* and *P. fluorescens* in the supplemented continuous flow reactor experiments were also compared to the various added sodium acetate concentrations. These results are presented in a scatter graph with linear regression lines through *K. pneumoniae* growth rates and *P. fluorescens* growth rates at the various added sodium acetate concentrations (Figure 15). Each point represents the average growth rate in three coupon inserts for one experiment at the added sodium acetate concentrations of 15, 29, 145 $\mu\text{gC/L}$. The data indicate that the growth rates of both *K. pneumoniae* and *P. fluorescens* increase with increasing concentrations of added sodium acetate. The growth rates for both organisms are more correlated with the added sodium acetate values ($R^2 = 0.8004$ for *K. pneumoniae* and $R^2 = 0.6689$ for *P. fluorescens*) than the measured AOC values

The growth rates of *K. pneumoniae* and *P. fluorescens* in both the control (no added sodium acetate) and supplemented continuous flow reactor experiments at the added sodium acetate values were then compared. These results are presented in a scatter graph with linear regression lines through *K.*

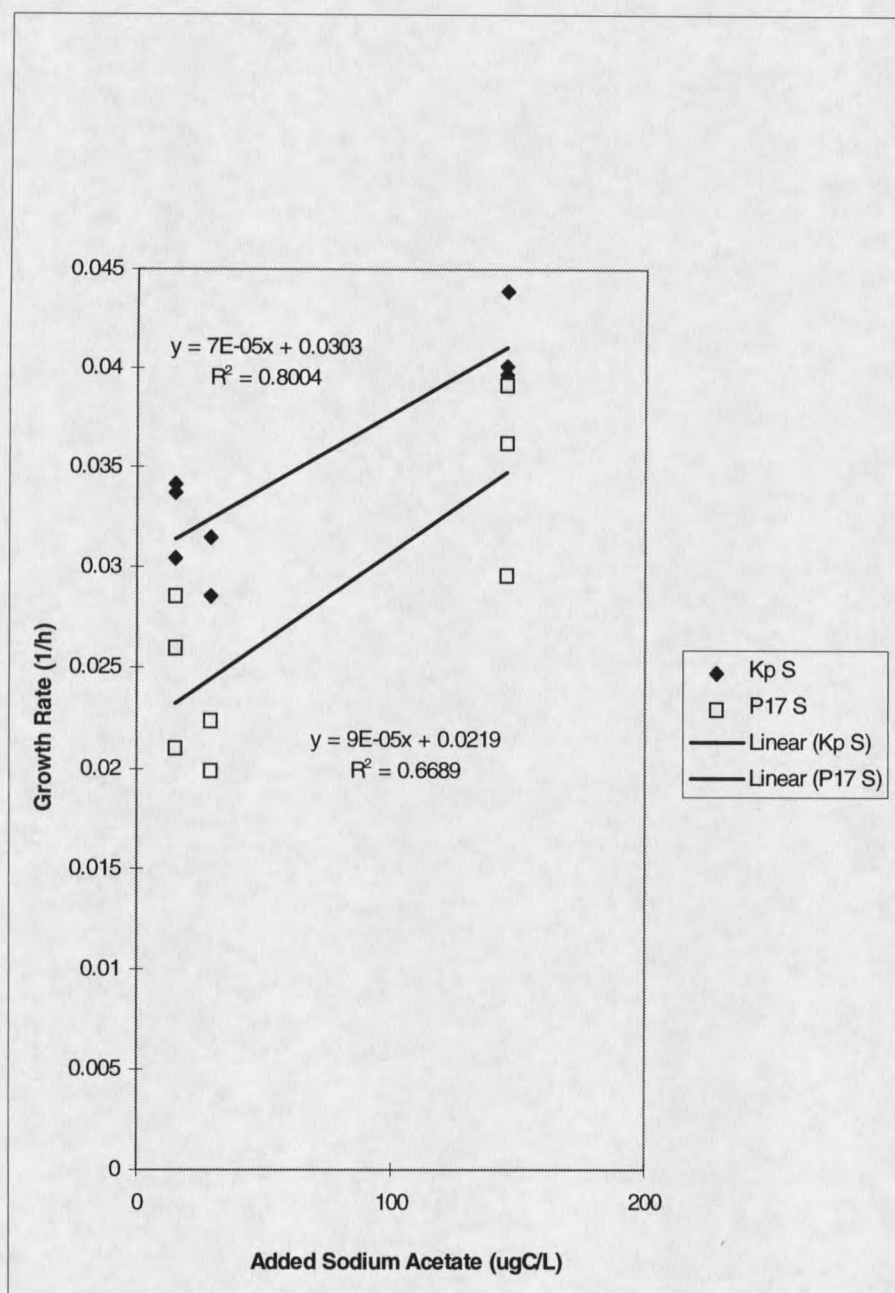


Figure 15. The growth rates (h^{-1}) of *K. pneumoniae* (Kp S) and *P. fluorescens* (P17 S) from the supplemented continuous flow reactor experiments at the added sodium acetate ($\mu\text{gC/L}$) values.

pneumoniae growth rates and *P. fluorescens* growth rates at the added sodium acetate values (Figure 16). Each point from the control and supplemented experiments (0, 15, 29, 145 $\mu\text{gC/L}$) represents the average growth rate of the three coupon inserts for one experiment at the added sodium acetate concentration and the corresponding control. It was observed that the growth rate seems to increase with increasing concentrations of sodium acetate; however, with the inclusion of the 0 $\mu\text{gC/L}$ acetate points, the growth rates of *K. pneumoniae* and *P. fluorescens* are not highly correlated with the added sodium acetate concentration ($R^2 = 0.4188$ for *K. pneumoniae* and $R^2 = 0.2220$ for *P. fluorescens*). Therefore, addition of points at 0 $\mu\text{gC/L}$ sodium acetate decreases the correlation coefficient when compared with results when they are omitted ($R^2 = 0.4188$ versus 0.8004 and 0.2220 versus 0.6689).

The growth rates of *K. pneumoniae* and *P. fluorescens* in the supplemented flow reactor systems minus the growth rates of both organisms in the corresponding control was calculated to determine if increasing concentrations of sodium acetate yielded faster growth for either *K. pneumoniae* or *P. fluorescens*. The results of these experiments are presented in a scatter graph with a linear regression line through *K. pneumoniae* growth rates (supplemented (-) control) and *P. fluorescens* growth rates (supplemented (-) control) at the added sodium acetate value (Figure 17). Each point represents the mean growth rate of three inserts from the supplemented reactor minus the

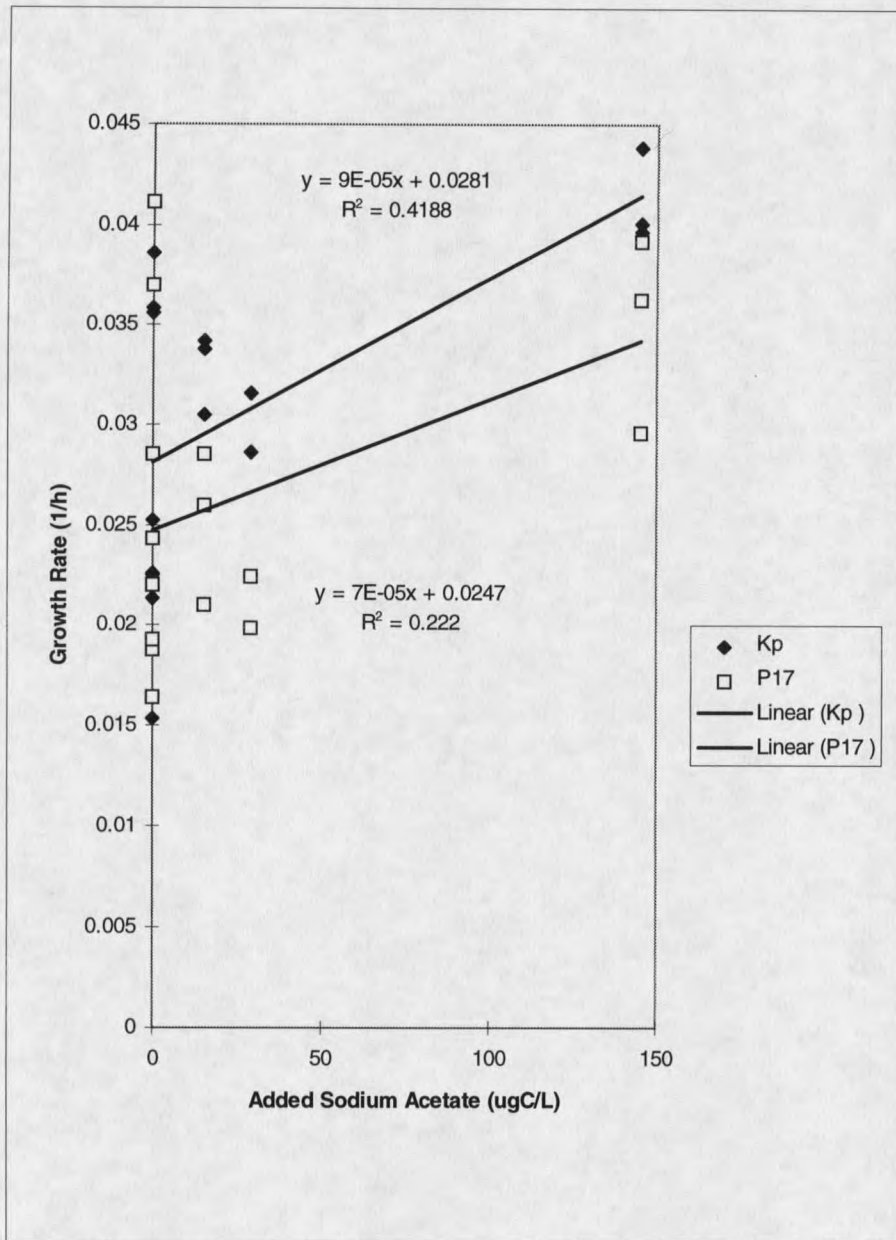


Figure 16. The growth rates (h^{-1}) of *K. pneumoniae* (Kp) and *P. fluorescens* (P17) in both the control and supplemented continuous flow reactor experiments at the added sodium acetate values ($\mu\text{gC/L}$).

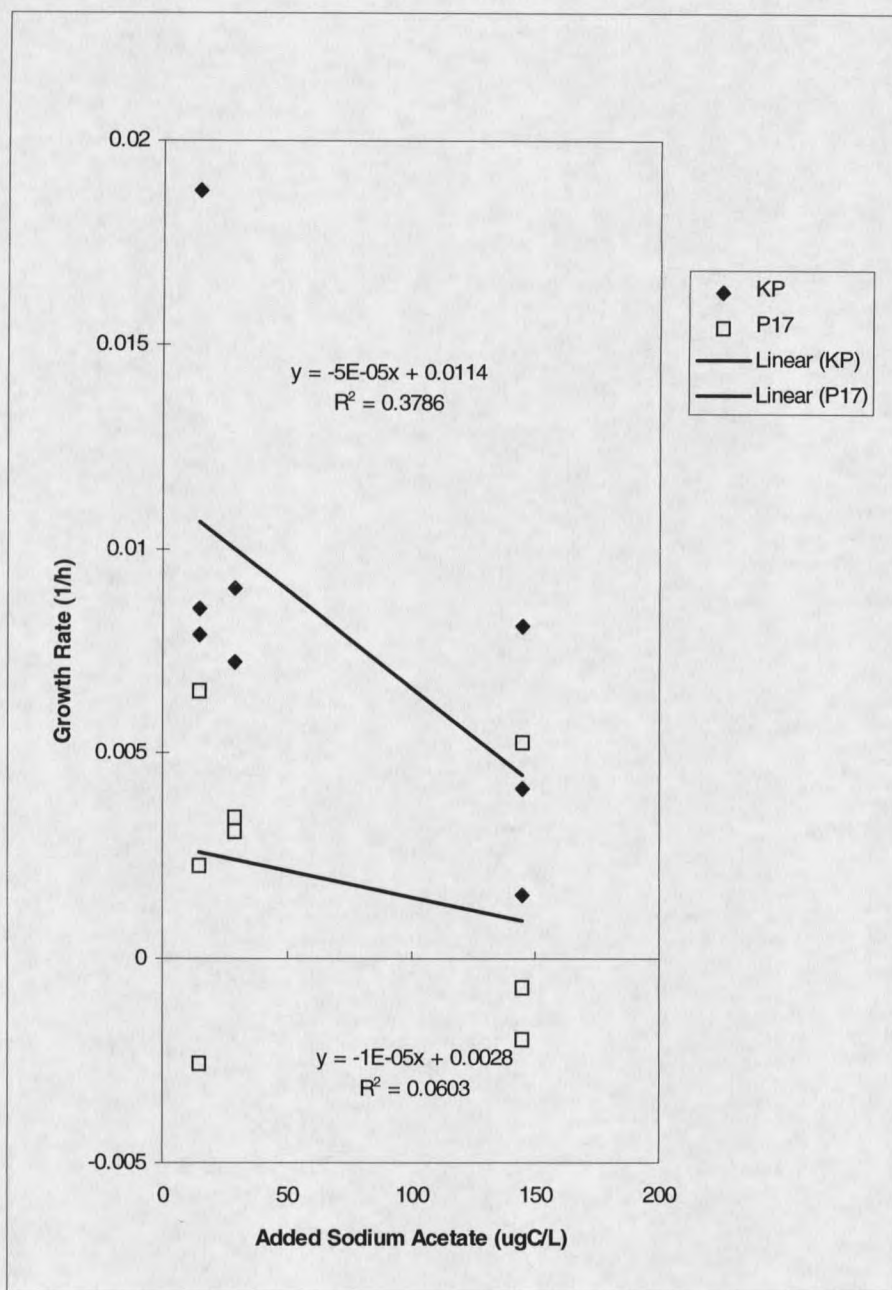


Figure 17. The difference between the growth rates (h^{-1}) of the supplemented and control for both *K. pneumoniae* (Kp) and *P. fluorescens* (P17) at the added sodium acetate concentration ($\mu\text{gC/L}$) in the continuous flow reactor experiments.

mean growth rate of three inserts from the corresponding control reactor. The difference between the supplemented and control growth rates for *K. pneumoniae* were positive at all added acetate concentrations, indicating that acetate addition increased the growth rate of this organism. A similar statement cannot be made for *P. fluorescens* as three of the eight difference were negative. It appears that as more sodium acetate is added, the growth rates for both *K. pneumoniae* and *P. fluorescens* minus the background growth decrease. However, the growth rate difference for both *K. pneumoniae* and *P. fluorescens* is not statistically correlated with the added sodium acetate values ($R^2 = 0.3786$ for *K. pneumoniae* and $R^2 = 0.0603$ for *P. fluorescens*).

A comparison was made between the growth rates of *K. pneumoniae* and *P. fluorescens*. These results are presented in a scatter graph with an orthogonal least squares line fit through the data (Figure 18). Each point represents the mean growth rate from three inserts of *K. pneumoniae* and *P. fluorescens* from both control and supplemented reactors. The data indicate that the growth rate of *K. pneumoniae* is loosely correlated with the growth rate of *P. fluorescens* ($R^2 = 0.567$). From the regression parameters (slope = 0.978 and intercept = -0.0036 h^{-1}), it is tempting to conclude that there was no statistically significant difference between the growth rates of the two immobilized organisms. This relationship was actually explored using a different analytical approach with the data.

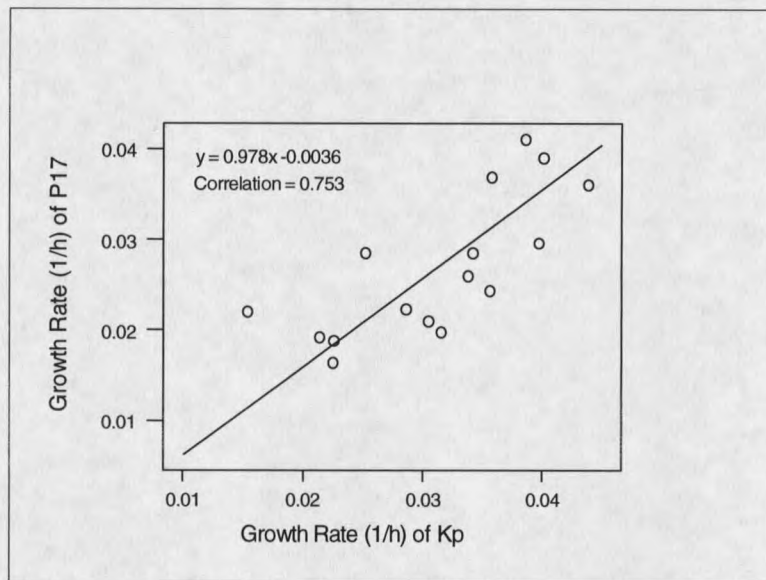


Figure 18. The growth rate (h^{-1}) of *P. fluorescens* (P17) versus the growth rate (h^{-1}) of *K. pneumoniae* (Kp) in the continuous flow reactor experiments.

The difference between the growth rate of *K. pneumoniae* and *P. fluorescens* in both reactors was calculated. The results are presented in a scatter graph (Figure 19). Each point represents the mean difference of the growth rates of *K. pneumoniae* and *P. fluorescens* from three inserts at each added sodium acetate value (the control data are given at 0 $\mu\text{gC/l}$). The data are highly variable for each sodium acetate concentration. The growth rate values indicated that *K. pneumoniae* seemed to grow at a faster rate than *P. fluorescens* in most control and supplemented reactor experiments. One interesting point is that in four of the control points the growth rate of *P. fluorescens* is higher than that of *K. pneumoniae*.

The mean growth rate for all inserts from each experimental set (9 inserts for three experiments with 15 and 145 $\mu\text{gC/L}$ added sodium acetate concentrations; 6 inserts for two experiments with 29 $\mu\text{gC/L}$ added sodium acetate; and there were 24 inserts for the control value) was calculated. The results are presented in a scatter graph with error bars representing the standard error between the replicate experiments (Figure 20). Each point is the mean for the entire experimental set (0, 15, 29 and 145 $\mu\text{gC/L}$). The difference in the growth rates of the control experiments is significantly lower than the difference in the growth rates from the supplemented experiments and is not significantly different from zero. The growth rate differences for the supplemented systems are all significant at the 90% level. The trend in the data

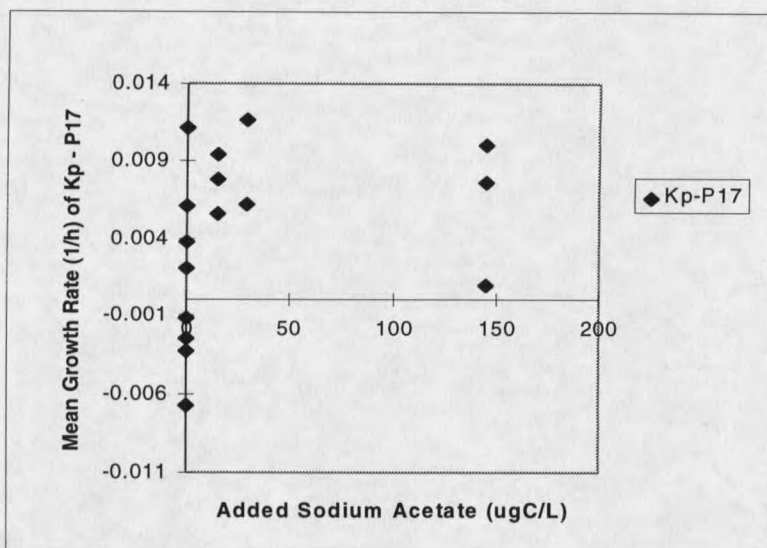


Figure 19. The mean growth rates (h^{-1}) of *K. pneumoniae* (Kp) minus the mean growth rates (h^{-1}) of *P. fluorescens* (P17) in the control and supplemented continuous flow reactor experiments.

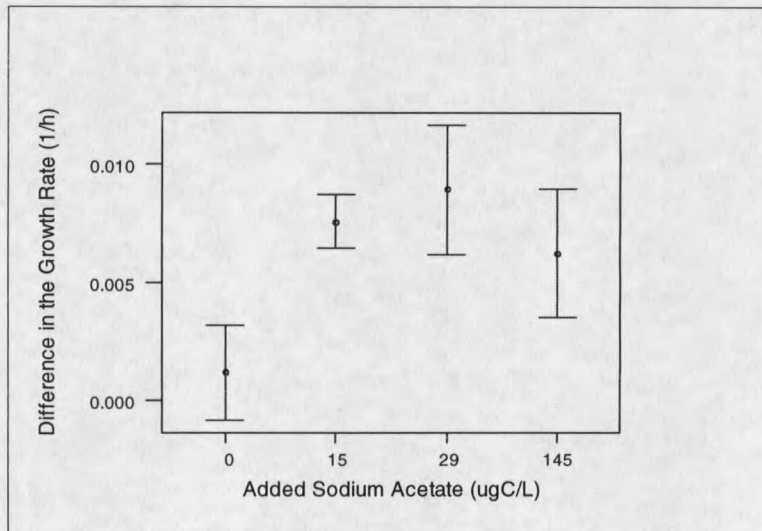


Figure 20. The mean (of 9 inserts per experiment for the 15 and 145 $\mu\text{gC/L}$ experiments, 6 inserts of the 29 $\mu\text{gC/L}$ experiments and 24 inserts of the control experiments (0 $\mu\text{gC/L}$)) of the growth rates (h^{-1}) of *K. pneumoniae* (Kp) minus the means of the mean growth rates (h^{-1}) *P. fluorescens* (P17) for the continuous flow reactor experiments. Error bars represent the standard error.

seems to indicate that the difference in the growth rate increases when 29 $\mu\text{gC/L}$ of sodium acetate is added and then decreases when the sodium acetate concentration is increased to 145 $\mu\text{gC/L}$. However, the differences in the growth rates at the three added sodium acetate concentrations are not significantly different. Thus, the data suggests that *K. pneumoniae* grew at a faster rate than *P. fluorescens* when supplemented with low concentrations of sodium acetate.

Carbon Cocktail as the Sole Added Carbon Source

All figures in this section represent data from continuous flow reactor experiments with the biofilm coupon using the carbon cocktail (composed of ethyl alcohol, sodium acetate, sodium benzoate, p-hydroxy benzoic acid, and propionaldehyde) as the sole added carbon source. The AOC of the control and supplemented continuous flow reactor systems was measured at the beginning and at the end of each experiment. The average AOC was then calculated from the two values for each experiment. Comparisons were made between the measured AOC value and the added carbon cocktail value to determine if the values are similar. The results are presented in a scatter graph with a linear regression line to indicate the correlation between the two variables (Figure 21). Each point represents the average AOC value from the initial and final AOC measurements plotted against the added carbon cocktail values for six different experiments. The added carbon cocktail concentration was 50, 100, and 500

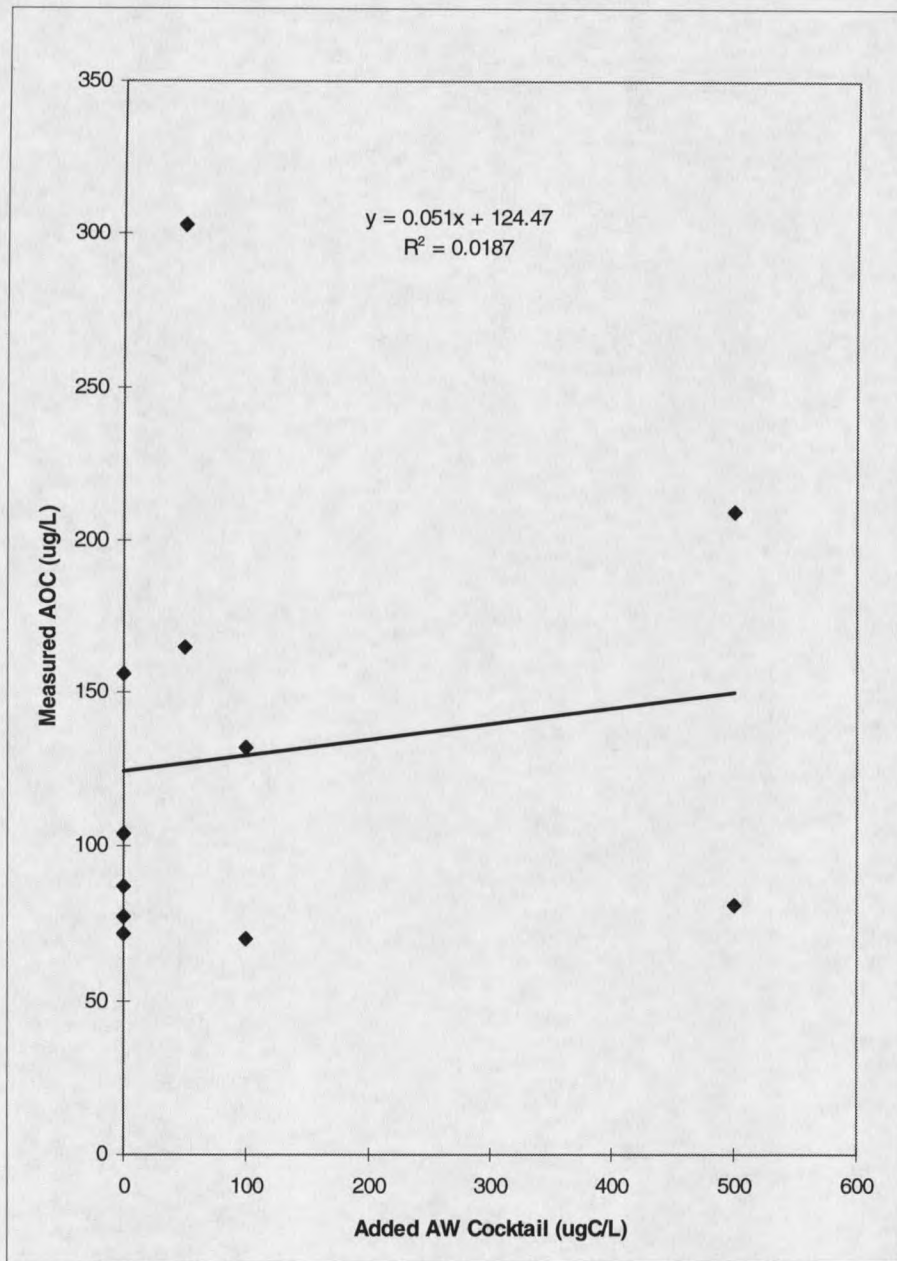


Figure 21. The measured AOC ($\mu\text{gC/L}$) versus the added carbon cocktail ($\mu\text{gC/L}$) from the continuous flow reactor experiments.

$\mu\text{gC/L}$. The data indicate that the two variables are not correlated ($R^2 = 0.0187$). Statistical analysis revealed that the measured AOC is significantly different from the added carbon cocktail concentration at a 95% confidence interval. For the hypothesis that the slope = 1, the p-value is 0.000. The 95% confidence interval for the slope is -0.898 to 1.0.

The biofilm coupon was utilized to indicate the response of immobilized *K. pneumoniae* and *P. fluorescens* to the measured AOC ($\mu\text{gC/L}$) and to varying concentrations of added carbon cocktail ($\mu\text{gC/L}$). The growth rates of *K. pneumoniae* and *P. fluorescens* in the supplemented continuous flow reactor experiments were compared to the measured AOC values ($\mu\text{gC/L}$). The results are presented in a scatter graph with linear regression lines through *K. pneumoniae* growth rates and *P. fluorescens* growth rates at the measured AOC concentrations (Figure 22). Each point represents the average growth rate from three coupon inserts per experiment at the measured AOC value in the supplemented continuous flow reactor system. It was observed that as the measured AOC value increased, the growth rate increased for both organisms, but there was no statistical correlation between the growth rate and the measured AOC concentration ($\mu\text{gC/L}$) ($R^2 = 0.2735$ for *K. pneumoniae* and $R^2 = 0.1399$ for *P. fluorescens*).

The growth rates of *K. pneumoniae* and *P. fluorescens* in both the control (no added carbon cocktail) and supplemented continuous flow reactors at the

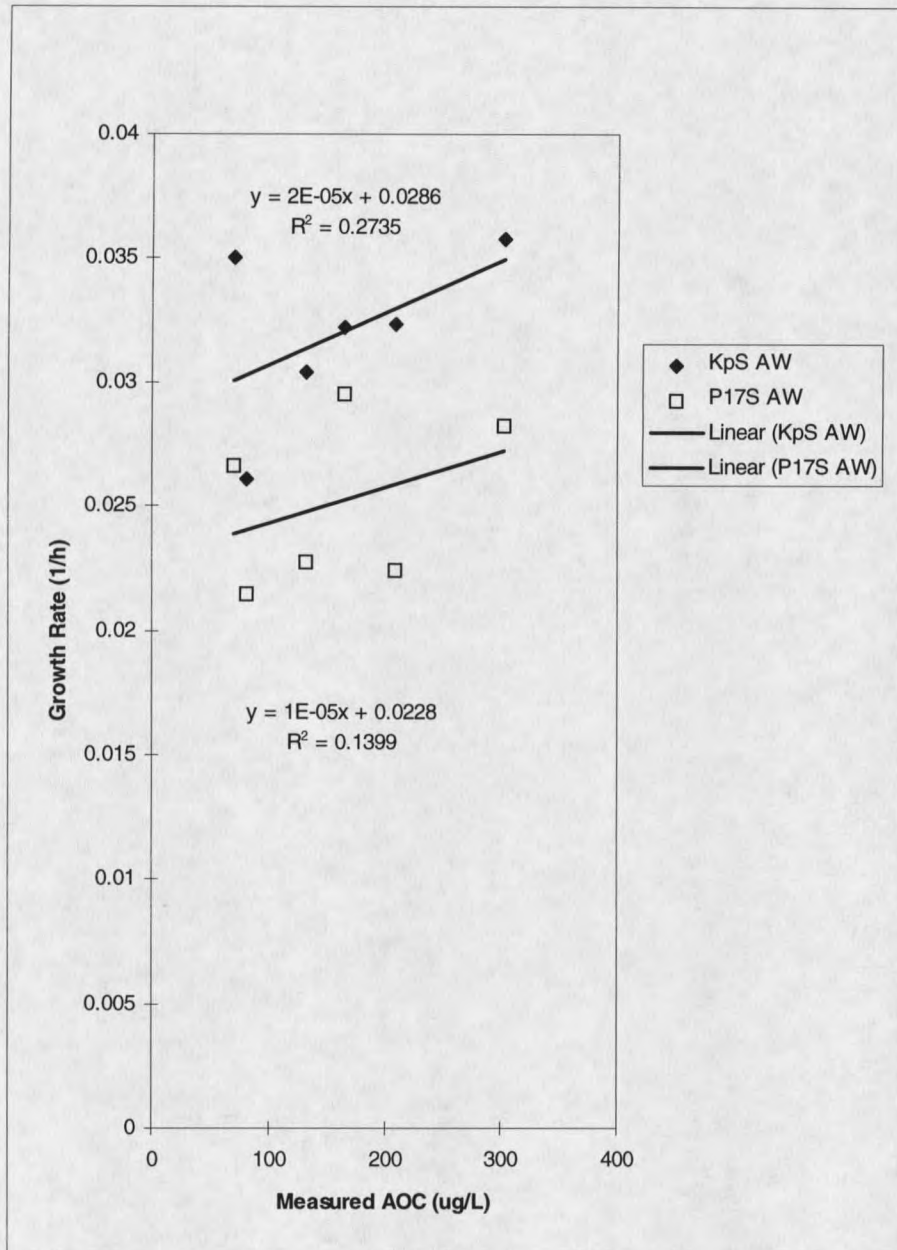


Figure 22. The growth rates (h^{-1}) of *K. pneumoniae* (Kp S AW) and *P. fluorescens* (P17 S AW) from the carbon cocktail supplemented continuous flow reactors at the measured AOC values ($\mu gC/L$).

measured AOC values were then compared. These results are presented in a scatter graph with linear regression lines through *K. pneumoniae* and *P. fluorescens* growth rates at the measured AOC values (Figure 23). Each point from the control and supplemented experiments represents the average growth rate of the three coupon inserts for one experiment at the measured AOC values. It was observed that the growth rate seems to increase with increasing concentrations of measured AOC. The inclusion of the control measured AOC values decreases the correlation of *K. pneumoniae* with the measured AOC values and the correlation of *P. fluorescens* growth rates with the measured AOC values increased somewhat ($R^2 = 0.2341$ versus 0.2735 for *K. pneumoniae* and $R^2 = 0.2408$ versus 0.1399 for *P. fluorescens*).

The growth rates of *K. pneumoniae* and *P. fluorescens* in the supplemented continuous flow reactor experiments were compared to the various added carbon cocktail concentrations. The results are presented in a scatter graph with linear regression lines through *K. pneumoniae* and *P. fluorescens* average growth rates of three coupon inserts per experiment at the various added carbon cocktail concentrations (50, 100, or 500 $\mu\text{gC/L}$) (Figure 24). The data indicates that the growth rate for both organisms decreases with increasing amounts of added carbon cocktail. The growth rates of *K. pneumoniae* are poorly correlated with the added carbon cocktail concentration

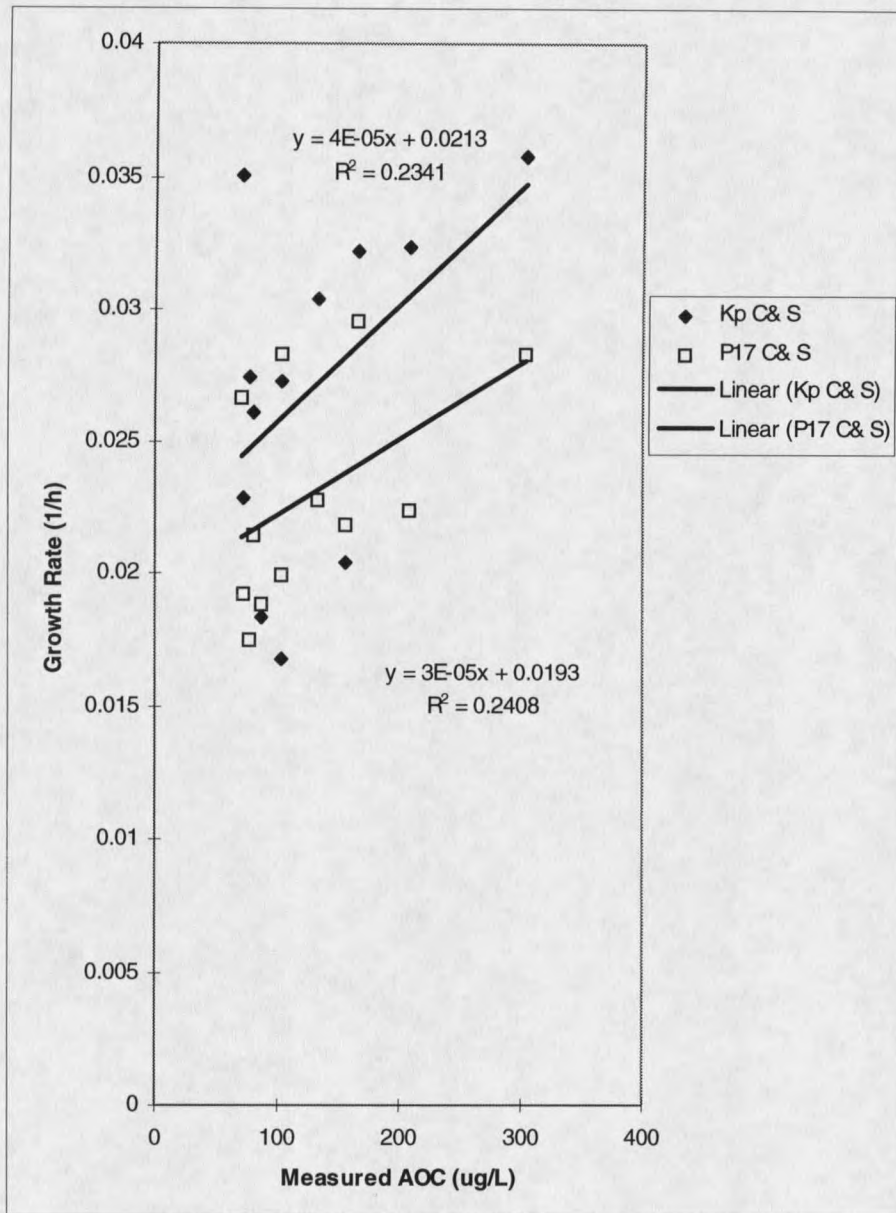


Figure 23. The growth rates (h^{-1}) of *K. pneumoniae* (Kp C&S) and *P. fluorescens* (P17 C&S) in both the control and supplemented continuous flow reactor experiments at the measured AOC values ($\mu\text{g/L}$).

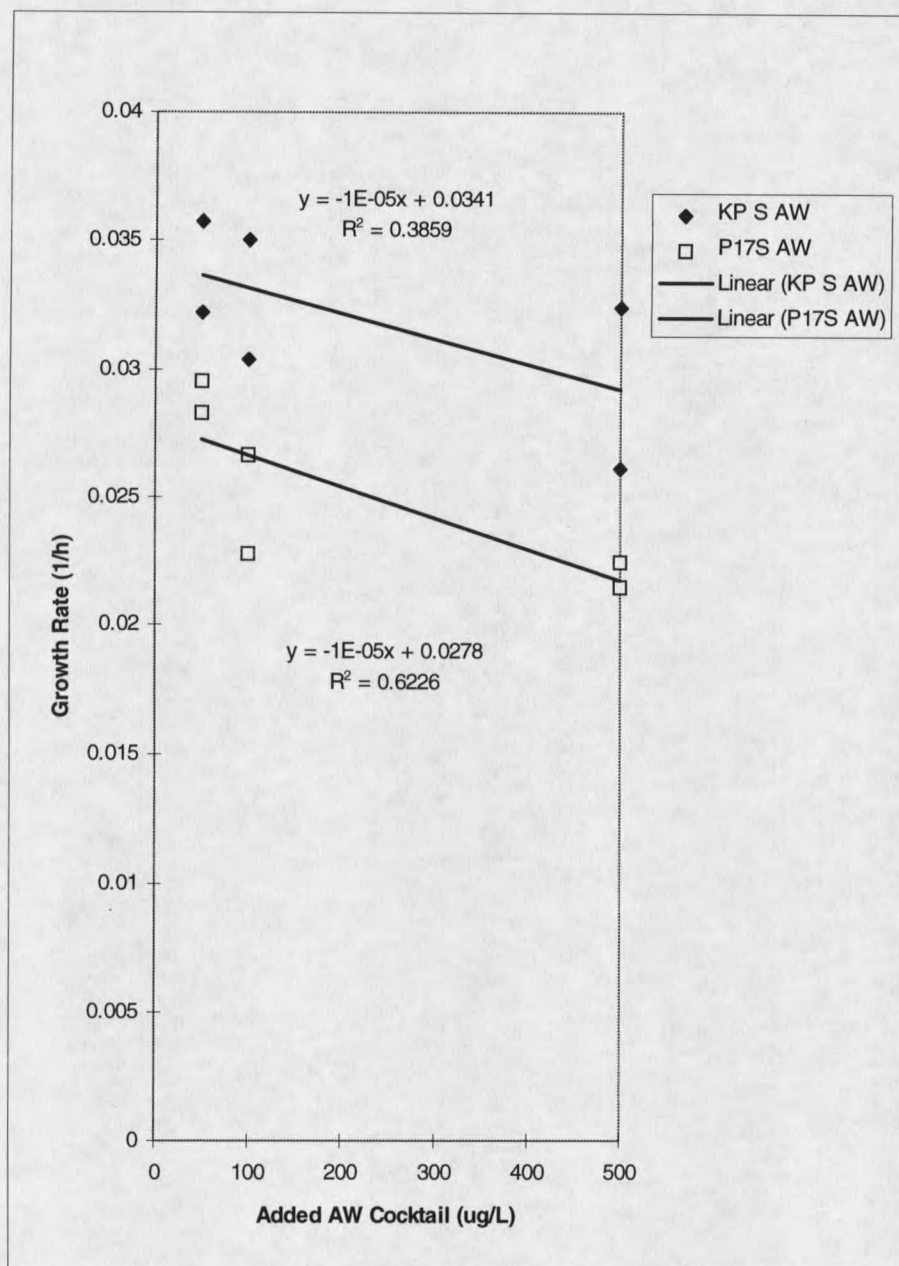


Figure 24. The growth rates (h^{-1}) of *K. pneumoniae* (Kp S AW) and *P. fluorescens* (P17 S AW) from the supplemented continuous flow reactor experiments at the added carbon cocktail values ($\mu\text{gC/L}$).

($\mu\text{gC/L}$) ($R^2 = 0.3859$); while growth rates of *P. fluorescens* are somewhat correlated ($R^2 = 0.6226$).

The growth rates of *K. pneumoniae* and *P. fluorescens* in both the control and supplemented continuous flow reactor experiments were then compared. These results are presented in a scatter graph with linear regression lines through *K. pneumoniae* and *P. fluorescens* growth rates (Figure 25). Each point from the control and supplemented data (0, 50, 100, and 500 $\mu\text{gC/L}$) represents the average growth rate of the three coupon inserts for one experiment. It was observed that the growth rates for *K. pneumoniae* seemed to increase with increasing carbon cocktail concentrations, while the growth rates of *P. fluorescens* are unchanged. The growth rates of *K. pneumoniae* and *P. fluorescens* are not correlated with the added carbon cocktail values when the control growth rate values are included ($R^2 = 0.0912$ for *K. pneumoniae* and $R^2 = 0.0006$ for *P. fluorescens*).

The growth rates of *K. pneumoniae* and *P. fluorescens* in the supplemented flow reactor systems minus the growth rates of both organisms in the corresponding control was calculated in order to determine if increasing concentrations of carbon cocktail yielded faster growth. The results of these experiments are presented in a scatter graph with linear regression lines through *K. pneumoniae* (supplemented (-) control) and *P. fluorescens* growth rates (supplemented (-) control) at the added carbon cocktail values (Figure 26). Each

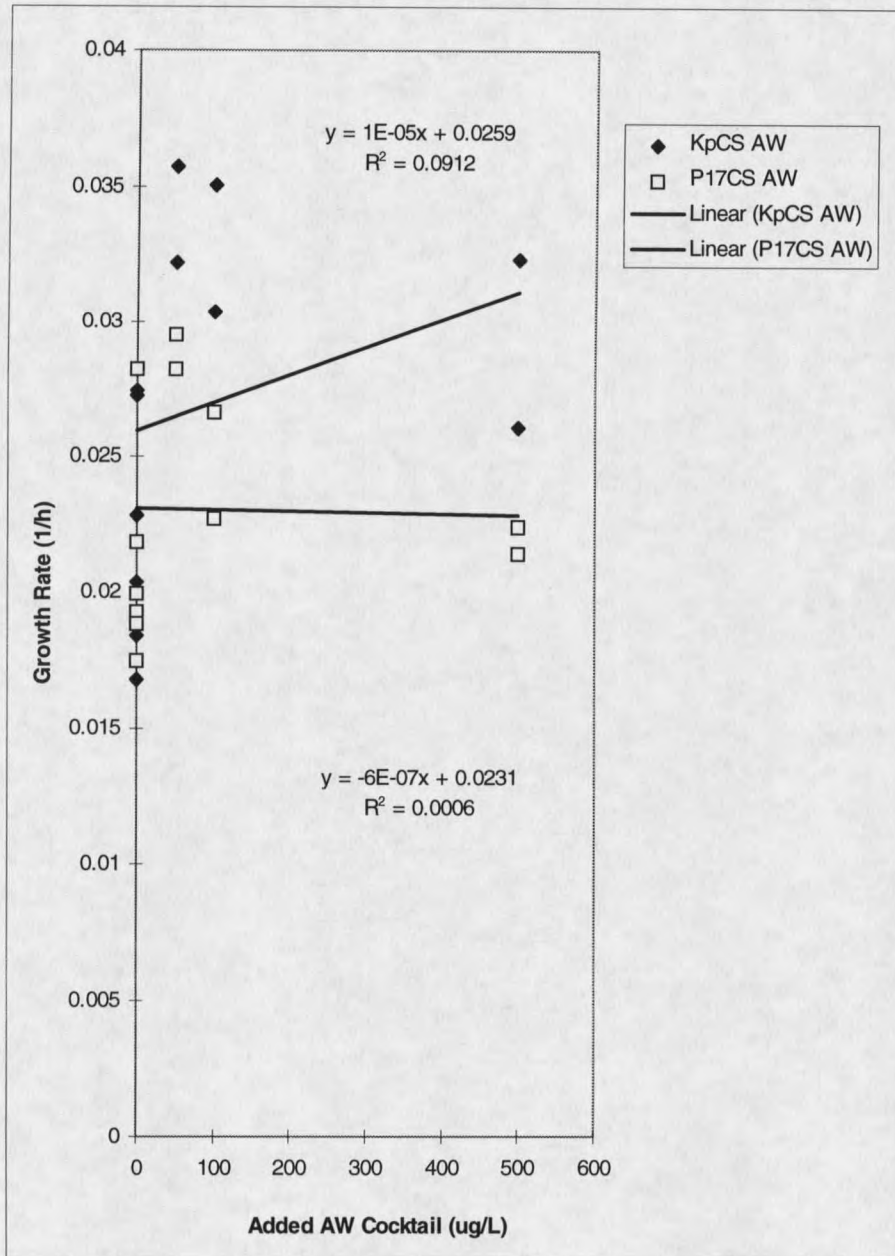


Figure 25. The growth rates (h^{-1}) of *K. pneumoniae* (KpCS AW) and *P. fluorescens* (P17CS AW) in both the control and supplemented continuous flow reactor experiments at the added carbon cocktail (AW cocktail) values ($\mu\text{gC/L}$).

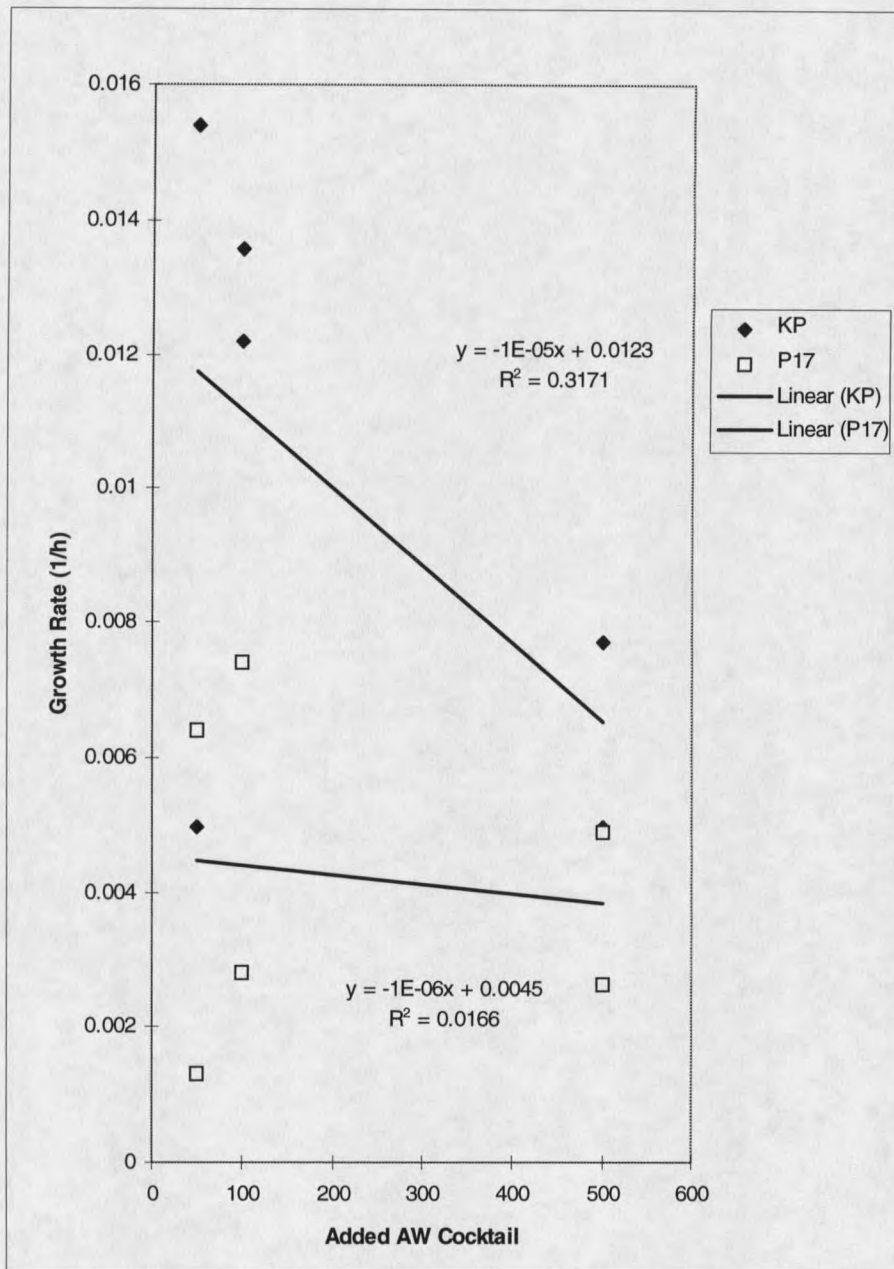


Figure 26. The difference between the growth rates (h^{-1}) of the supplemented and control for both *K. pneumoniae* (Kp) and *P. fluorescens* (P17) at the added carbon cocktail concentration ($\mu\text{gC/L}$) in the continuous flow reactor experiments.

point represents the mean growth rate of three inserts from the supplemented reactor minus the mean growth rate of the three inserts from the corresponding control reactor. It was observed that as the concentration of added carbon cocktail increases, the growth rates for both *K. pneumoniae* and *P. fluorescens* minus the background decrease. However, the difference between the supplemented and control growth rates for both *K. pneumoniae* and *P. fluorescens* is not correlated with the added carbon cocktail values ($R^2 = 0.3171$ for *K. pneumoniae* and $R^2 = 0.0166$ for *P. fluorescens*). It should be noted, however, that all growth rate differences are positive indicating that the addition of carbon cocktail produced an increase in the growth rate of both organisms at all concentrations tested when compared to an otherwise identical control.

A comparison was made between the growth rates of *K. pneumoniae* and *P. fluorescens*. The results are presented in a scatter graph with an orthogonal least squares line fit through the data (Figure 27). Each point represents the mean growth rate from three inserts of *K. pneumoniae* and *P. fluorescens* from both the control and supplemented reactors. The data indicate that the growth rate of *K. pneumoniae* is not tightly correlated with the growth rate of *P. fluorescens* ($R^2 = 0.4597$) when the carbon cocktail was the sole added carbon source.

The growth rate values from most of the supplemented experiments indicated that *K. pneumoniae* grew faster than *P. fluorescens*. The difference between the growth rates of *K. pneumoniae* and *P. fluorescens* in both the

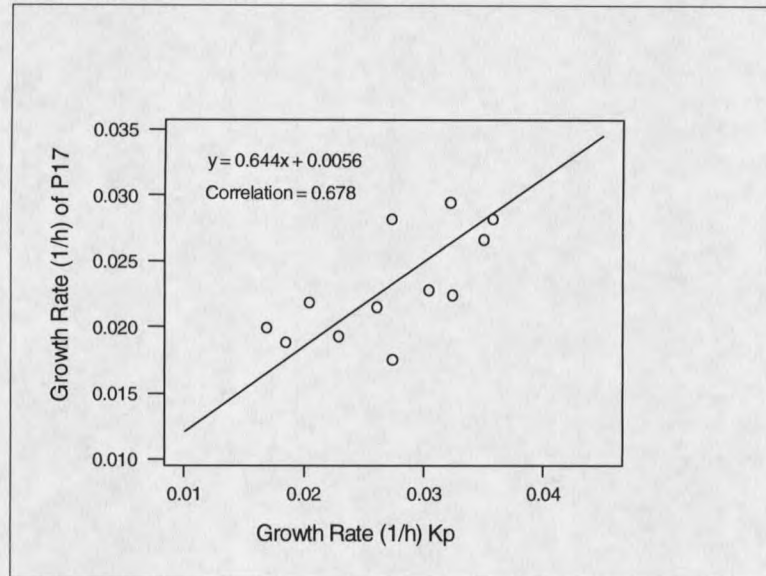


Figure 27. The growth rate (h^{-1}) of *P. fluorescens* (P17) versus the growth rate (h^{-1}) of *K. pneumoniae* (Kp) in the continuous flow reactor experiments.

control and supplemented reactors was calculated. The results are presented in a scatter graph (Figure 28). Each point represents the mean growth rate from three inserts at each added carbon cocktail value (the control data are presented at 0 $\mu\text{gC/L}$). The data are highly variable. It was interesting to note that the growth rate of *K. pneumoniae* was lower than *P. fluorescens* for four of the six control experiments. This indicated that *P. fluorescens* is able to grow in double-glass-distilled water with no added carbon at a slightly higher rate than *K. pneumoniae*. The difference between the means of all the inserts (6 inserts for each experiment conducted at 50, 100, and 500 $\mu\text{gC/L}$ added carbon cocktail and 18 inserts for the control value) of *K. pneumoniae* minus *P. fluorescens* was calculated. The results are presented in a scatter graph with error bars representing the standard error between the replicate experiments (Figure 29). The data indicate that the difference in the growth rates from the control experiments is significantly lower than the difference in the growth rates from the 100 and 500 $\mu\text{gC/L}$ experiments. The general trend is an increasing difference between the growth rate of *K. pneumoniae* and *P. fluorescens* with increasing added carbon cocktail values until the concentration reached 500 $\mu\text{gC/L}$. At 500 $\mu\text{gC/L}$ there was a slight decrease. Thus, *K. pneumoniae* grows at a slightly faster rate than *P. fluorescens* with increasing carbon cocktail concentrations up to 500 $\mu\text{gC/L}$ added carbon cocktail.

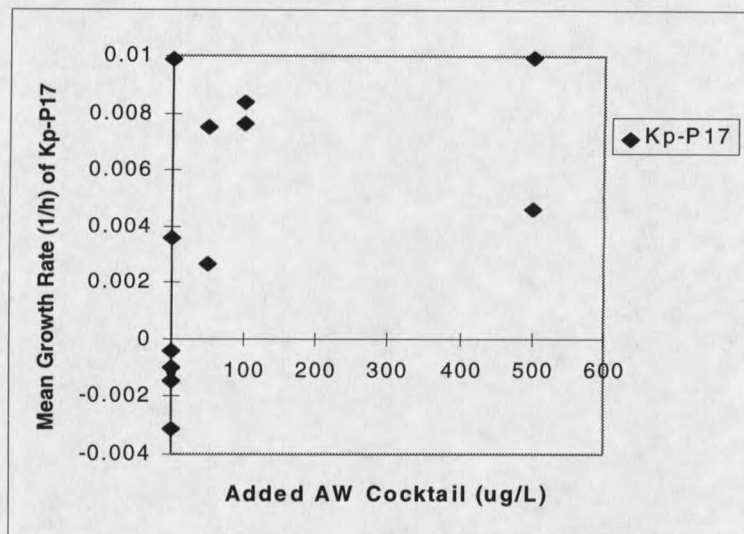


Figure 28. The mean growth rates (h^{-1}) of *K. pneumoniae* (Kp) minus the mean growth rates (h^{-1}) of *P. fluorescens* (P17) in the control and supplemented continuous flow reactor experiments.

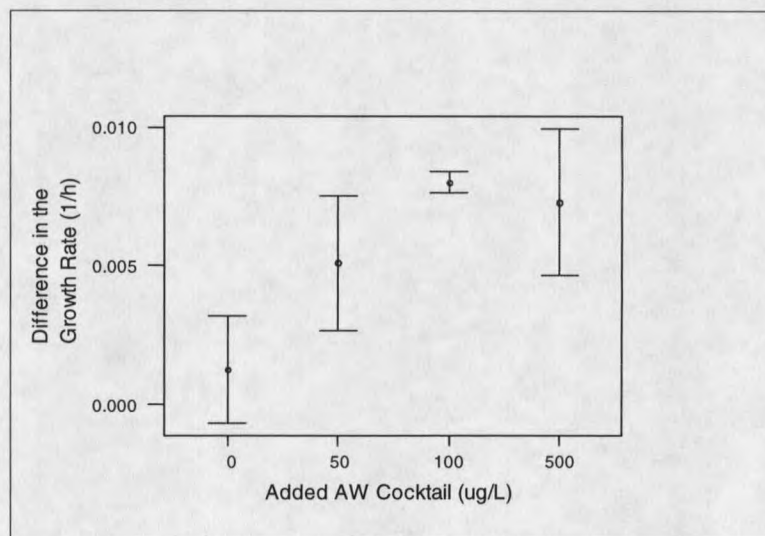


Figure 29. The mean (of 6 inserts per experiment for the 50, 100, and 500 $\mu\text{gC/L}$ added carbon cocktail and of 18 inserts for the control (0 $\mu\text{gC/L}$)) of the growth rates (h^{-1}) of *K. pneumoniae* (Kp) minus the mean of the growth rates (h^{-1}) of *P. fluorescens* (P17) for the control and supplemented continuous flow reactor experiments. Error bars represent the standard error.

Rate of Acetate Uptake

The uptake rates of both *K. pneumoniae* and P17 were determined based on the unconverted substrate supplemented at 5 and 50 $\mu\text{g/L}$. Radiolabeled acetic acid-2- ^{14}C was inoculated into each biological sample at time zero. The vials were destructively sampled at time 0, 2, 4, 6, 8, and 12 hours and the DPM's of the unconverted substrate, respired $^{14}\text{CO}_2$, and the biomass were recorded. The results of these experiments are presented graphically as the $\ln(\text{DPM})$ of the unconverted substrate sample versus time with a linear regression line (Figures 30, 31, 32, 33). Each point represents the average of three samples taken at each time point and includes standard error bars. The decreasing DPM's are highly correlated for *K. pneumoniae* at 5 and 50 $\mu\text{g/L}$ ($R^2 = 0.9898$ and 0.9489) and *P. fluorescens* at 5 and 50 $\mu\text{g/L}$ ($R^2 = 0.9202$ and 0.8351) with time.

The slope of the line was used to calculate the rate constant (μg acetate carbon/cell \cdot hour). The rate constants of both *K. pneumoniae* and *P. fluorescens* for both substrate concentrations are presented in a bar graph (Figure 34). Each bar represents the rate constant for the average of three samples per organism at 5 or 50 $\mu\text{g/L}$ acetic-acid-2- ^{14}C . The rate constant for *K. pneumoniae* decreases at 50 $\mu\text{g/L}$ acetic-acid-2- ^{14}C as compared to the rate constant at 5 $\mu\text{g/L}$ acetic-acid-2- ^{14}C . The rate constant of *P. fluorescens* is

similar at both 5 and 50 $\mu\text{g/L}$ acetic-acid-2- ^{14}C . Thus, *K. pneumoniae* is more sensitive to the added carbon concentration than *P. fluorescens*.

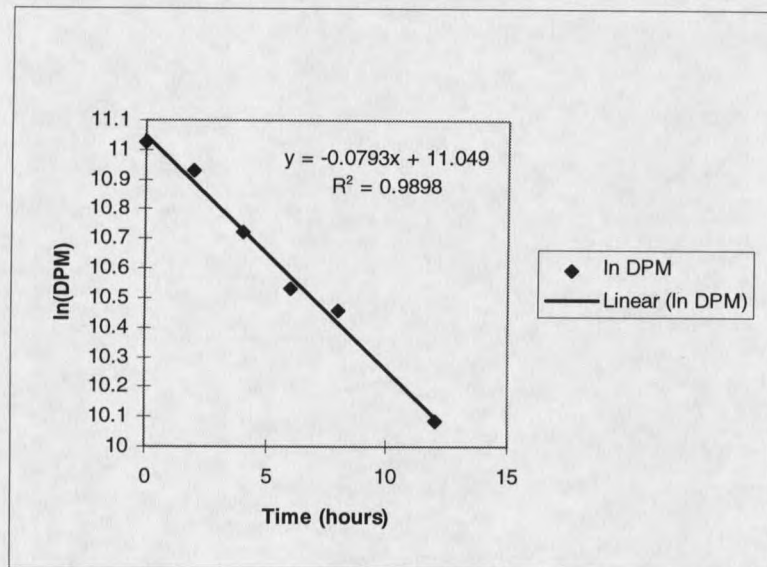


Figure 30. The change in the concentration of unconverted substrate with time for *K. pneumoniae* grown on 5 $\mu\text{gC/L}$ acetic acid-2- ^{14}C sodium salt. Each point is an average of three biological samples per sampling time.

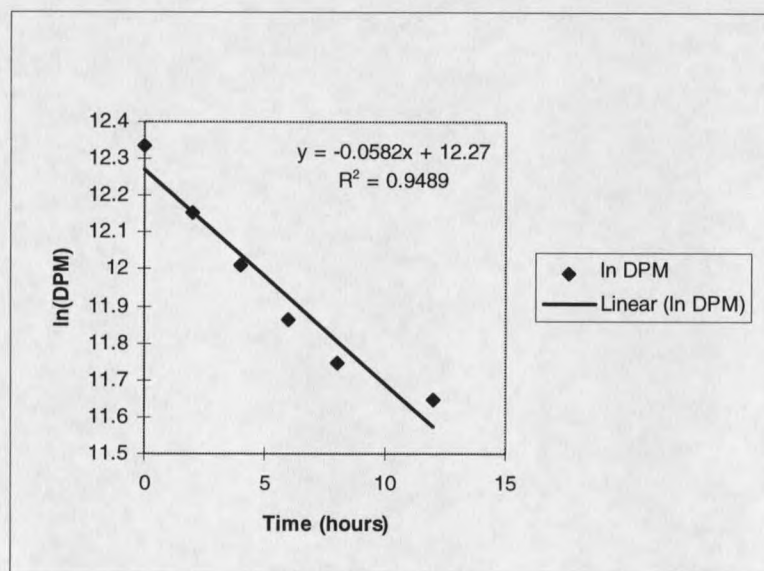


Figure 31. The change in the concentration of unconverted substrate with time for *K. pneumoniae* grown on 50 $\mu\text{gC/L}$ acetic acid-2- ^{14}C sodium salt. Each point is an average of three biological samples per sampling time.

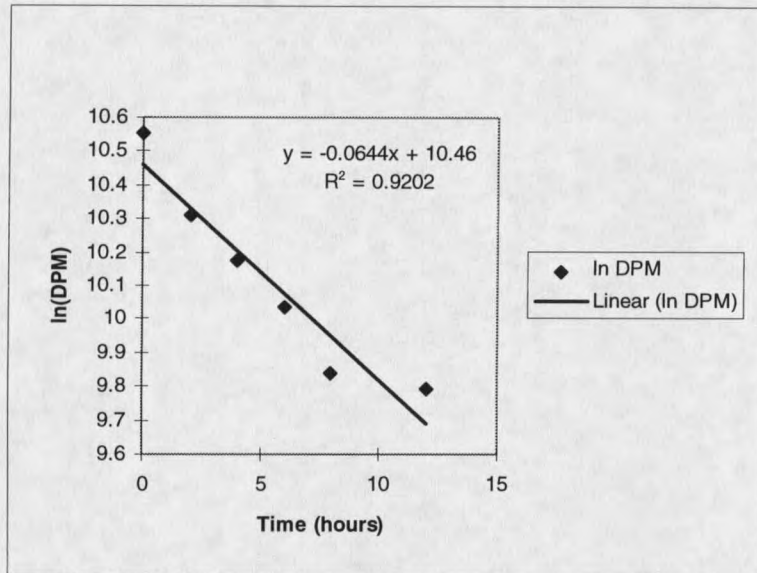


Figure 32. The change in the concentration of unconverted substrate with time for *P. fluorescens* grown on 5 $\mu\text{gC/L}$ acetic acid-2- ^{14}C sodium salt. Each point is an average of three biological samples per sampling time.

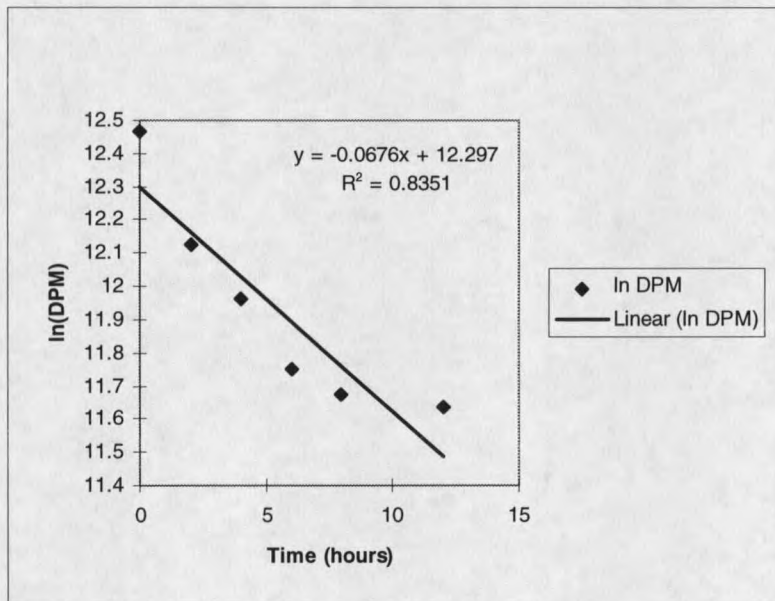


Figure 33. The change in the concentration of unconverted substrate with time for *P. fluorescens* grown on 50 $\mu\text{gC/L}$ acetic acid-2- ^{14}C sodium salt. Each point is an average of three biological samples per sampling time.

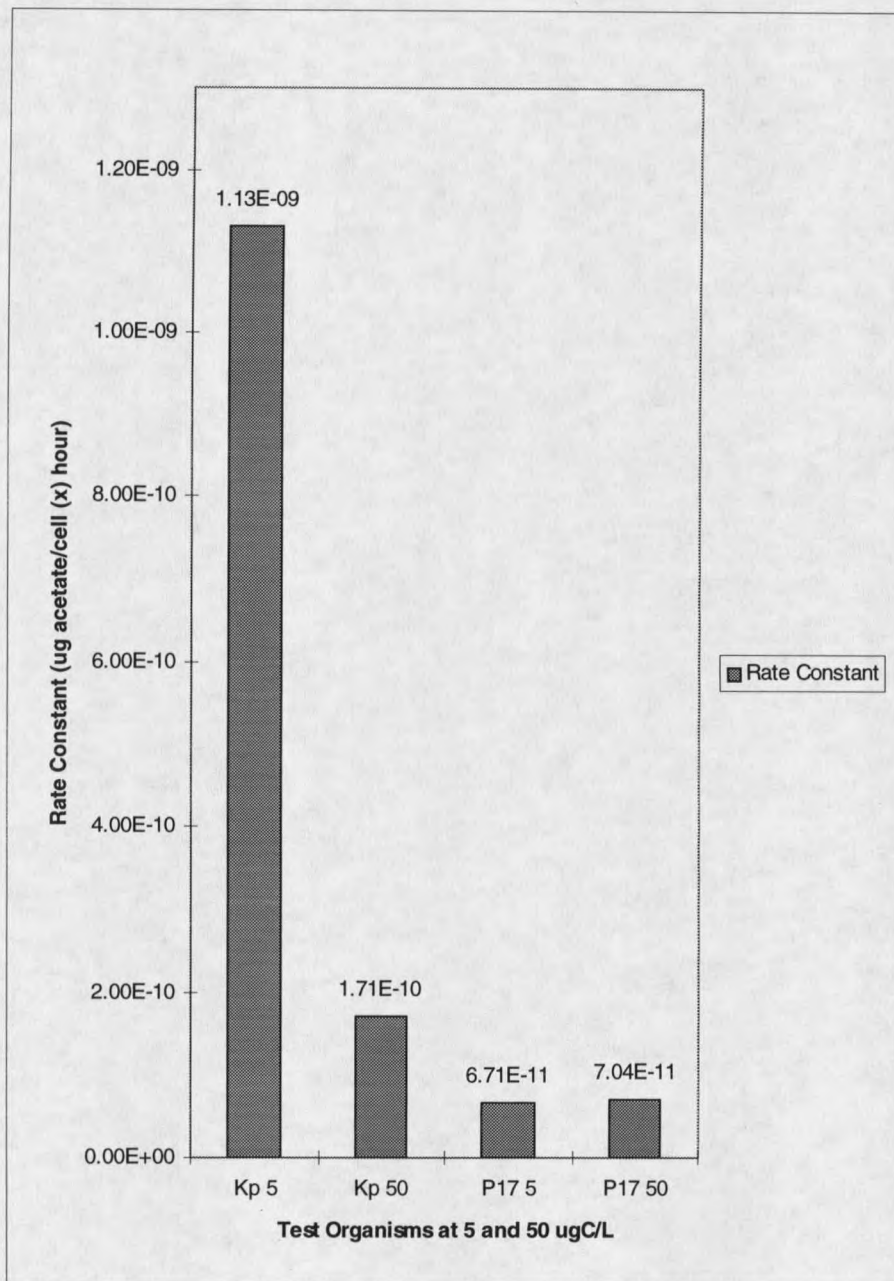


Figure 34. Comparison of the rate constants for *K. pneumoniae* and *P. fluorescens* at 5 and 50 $\mu\text{gC/L}$ sodium acetate.

CHAPTER 5

DISCUSSION

The growth and regrowth of bacteria in drinking water distribution systems is determined in part by the concentration of assimilable organic carbon (AOC) (Frias, *et al*, 1994; Huck, 1990; van der Kooij *et al.*, 1982). 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 immobilized on a surface. There is no method currently available to relate the growth and persistence of biofilm bacteria to AOC uptake. 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. This research evaluated certain technical problems concerning the use of the biofilm coupon as a monitoring device as well as the application of the biofilm coupon as a bioassay for measuring the growth potential of drinking water. The results from the previous chapter will be analyzed and discussed in this section.

Comparisons of Two AOC Methodologies

Assimilable organic carbon (AOC) is a measurement of the biodegradable substances that support the growth of heterotrophic and coliform bacteria in water. Comparisons were made between the AOC concentration of double-

glass-distilled water using both van der Kooij's plate count method and LeChevallier's ATP method. The AOC was measured using P17 alone. Results indicate that the two methodologies produce different AOC values (Figures 6 & 7). During development of the ATP methodology, LeChevallier indicated that the two methods may produce different AOC values; "there were no significant differences between AOC values determined using strain P17 for the ATP and plate count procedures; for strain NOX the plate count procedure underestimated bacterial levels in some samples, (LeChevallier, 1991(a)). LeChevallier (1991(a)) attributed the difference to an underestimation of bacterial levels by plate counts. However, the results presented in Figures 6 & 7 indicate that typically, the plate count AOC was higher than the ATP AOC, which directly contradicts LeChevallier's study. In addition, since only P17 was utilized in these tests, the two methods should have produced similar AOC measurements. The difference in the AOC values determined in double-glass-distilled water may not be significant; however, the difference in the AOC values from annular reactors 2-1 and 2-2 were greater than 100 µgC/L (Figure 7).

It has been found that AOC values can be highly variable on the same water samples (Prévost, *et al.*, 1989; Clark and Olson, 1991). The reproducibility of the AOC value using LeChevallier's ATP method was determined. The data in Table 4 indicate that LeChevallier's ATP method was fairly reproducible when measuring the AOC of the same water sample (Table 4). This method was then

utilized to measure the AOC concentration of all water samples in the rest of the work.

Viability of Test Organisms in the Biofilm Coupon

Tetrazolium salts such as 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) and 5-cyano-2,3-ditoly tetrazolium chloride (CTC) are often used to indicate the respiratory activity of microorganisms. The two stains directly compete with molecular oxygen as an artificial electron acceptor (Bitton and Koopman, 1986; Dutton, *et al.*, 1983; Iturriaga, 1979; Zimmerman, *et al.*, 1978). The reducing power generated by the electron transfer system converts INT and CTC into insoluble INT-(CTC) formazan crystals (through dehydrogenase-catalyzed reactions) which accumulate in metabolically active bacteria (Rodriguez, *et al.*, 1992). The INT-formazan crystals can be observed with bright field microscopy as red intracellular deposits. The red fluorescent CTC-formazan crystals can be observed under longwave UV light (> 350 nm).

One concern raised during Xu's work (1993) was that only a certain portion of the immobilized bacteria in the biofilm coupon were viable. Spotty distribution and non-uniform growth indicated that there may be differences in the physiological state of the organisms immobilized in the biofilm coupon. This study evaluated the viability of the test organisms immobilized in the biofilm

coupon using CTC and DAPI staining. However, the gel matrix in the biofilm coupon inserts interfered with the CTC reduction by the test organisms. The use of CTC in reducing environments where the *in situ* redox potentials are lower than the $E_{1/2}$ values of the tetrazolium salts will lead to non-biological tetrazolium reduction (Rodriguez, *et al.*, 1992). It has been found that CTC can be reduced in the presence of agar gel without viable cells (Smith, 1995).

The respiration of planktonic *K. pneumoniae* and *P. fluorescens* depleted of endogenous reserves (just prior to placement in the coupon inserts) was then determined using CTC and DAPI (Figure 9). The viability of the planktonic test organisms directly affects the viability of the test organisms in the biofilm coupons. Approximately fifty percent of the test organisms were actively respiring prior to placement in the biofilm coupon (Figure 9). The percent of respiring bacteria in log growth was also measured in order to compare the respiration rates of organisms depleted of endogenous reserves and organisms in log growth. Approximately ninety percent of the test organisms in log growth were actively respiring. Two conclusions can be drawn from this information : 1) fewer organisms are actively respiring after depletion of endogenous reserves and/or 2) fewer organisms in endogenous growth are able to reduce CTC (Smith, 1995). Unfortunately this approach necessitates measuring or assuming something about the viability or respiring percentage of the increased cell numbers after incubation. A third method was attempted using INT staining on test organisms immobilized in the biofilm coupon.

One important feature of INT is that endogenous respiration optimally promotes INT reduction. The test organisms are placed in the biofilm coupon inserts after four hours of endogenous respiration. In addition, the coupon inserts are incubated in the INT solution without added carbon for twenty-four hours. INT was able to diffuse through the gel matrix to the test organisms. Approximately 47 ± 1.8 percent of the *K. pneumoniae* and 43 ± 2.2 percent of the *P. fluorescens* cells were actively respiring. This number is somewhat lower than the percent of respiring planktonic bacteria prior to placement in the coupon (Figure 9). The difference may reflect disturbance of the organisms during preparation of the coupon or may reflect the inability of INT to be reduced in certain circumstances. Blenkinsopp and Lock (1990) found that under low growth, incubation times may need to be increased for INT-formazan formation to occur. In addition, INT deposits in cells smaller than $0.4 \mu\text{m}$ may have been too small to be observable (Harvey and Young, 1980). The cells of *K. pneumoniae* and *P. fluorescens* depleted of endogenous reserves may be quite small. Finally, the test organisms lacking formazan spots may actually be in a state of activity below the detectable level (Zimmerman, *et al.*, 1978).

Utilizable Carbon Leaching from Insert Materials

Batch Systems

The cleaning procedure of the inserts, all of the materials used in the preparation of the biofilm coupon (water, silica gel, agar gel, and polycarbonate), and the counting procedure may introduce organic carbon. The levels of introduced organic carbon may have a significant impact on the growth rate of both *K. pneumoniae* and *P. fluorescens* in the biofilm coupon. This could result in high background noise in the experimental results when low concentrations of carbon are added (as seen in Figures 17 and 26).

The initial cleaning procedure involved washing, oven drying at 100° C overnight, and UV sterilization of the inserts for forty-five minutes prior to use. Surface organics were not removed, nor were the inserts completely sterile. The second cleaning procedure used methanol/hexane and sonication to clean the surfaces of the inserts. The methanol solubilizes the polar organics and the hexane solubilizes the non-polar organics (Johnston, 1995). This method is widely utilized to clean poly(tetrafluoroethylene-co-hexafluoropropylene) (FEP, Teflon) (Vargo, *et al.*, 1992) and was recommend to surface clean the polycarbonate inserts (Baty, 1995). This cleaning procedure resulted in a decrease in the AOC leaching from the biofilm coupon inserts as indicated by LeChevallier's ATP method (Figure 10). The methanol/hexane sonication surface cleaning procedure was used for the rest of the tests. The AOC leaching

from the silica gel pads and the noble agar gel pads was also compared. The coupon inserts made with noble agar gel pads leached less AOC than the silica gel pad inserts. The noble agar is purified so that most trace organics are removed. The silica gel used for this work was not purified, which may explain why the silica gel leaches more AOC than the noble agar (Davies, 1995). The polycarbonate insert without any gel matrix did not leach any AOC which was expected as polycarbonate should not leach organics into the system. It is recommended that the biofilm coupon inserts be surface cleaned using the methanol/hexane sonication procedure and prepared using noble agar or purified silica gel pads.

Flow Systems

The carbon leaching from the biofilm coupon inserts should decrease with time in a flowing system. The carbon leaching from the biofilm coupon inserts decreased over 72 hours in both the control and supplemented reactors experiments 1 and 2 (Figure 11). The AOC leaching from the supplemented reactor in experiment 3 also decreased by 72 hours (experiments 2 & 3 are replicates of experiment 1) (Figure 11). The increase in the AOC values of the control water at intermediate times may be an artifact of the handling and counting procedure. The coupons are removed from the control reactor, placed in an oven-sterilized glass plate, and then counted under a bright-field microscope. The coupon holders and inserts are exposed to the air for at least

an hour per counting time. During this time, volatile organic substances in the air which are capable of supporting growth of microorganisms may contaminate the holders and inserts (Geller, 1983).

Biological Stability Studies

AOC Measurements With Sodium Acetate

The rate and the extent of growth of microorganisms in drinking water are related to the concentration of compounds serving as energy sources, such as total organic carbon (van der Kooij and Hijnen, 1988). Only a small portion of the total organic carbon is assimilable for bacteria (Frias, *et al.*, 1994). This carbon cannot be measured by simple chemical methods due to the complexity of the carbon compounds. AOC is measured by the maximum bacterial growth (N_{max}) using the yield value of the organisms on acetate. The AOC bioassay assumes that 1) 10 $\mu\text{gC/L}$ organic carbon is limiting to the growth of the bioassay organism, 2) the yield of the bioassay organism on naturally occurring AOC is constant over a wide range of concentrations, and 3) the bioassay organism is as capable as the native microflora in a water distribution system in utilizing AOC (Kaplan and Bott, 1990). Van der Kooij, *et al.*, (1982) found a good linear relationship from values of acetate between 0 and 50 $\mu\text{gC/L}$, while Frias *et al.*, (1994) found a very good relationship for values of acetate between 0 and 100

$\mu\text{gC/L}$. The results from this study indicated that there is a linear relationship between the measured AOC using both P17 and NOX and the concentration of added acetate values from 15 to 145 $\mu\text{gC/L}$ (Figure 12). Statistical analysis revealed that the slope of a regression line between AOC and acetate was near 2.0, with a ninety-five percent confidence interval of 1.001 to 3.462. Frias, *et al.*, (1994) found that higher concentrations of acetate resulted in a non-linear relationship with very low values of R^2 .

AOC Measurements with the Carbon Cocktail

The carbon cocktail used for this work is composed of five carbon sources that represent five major classes of compounds found in drinking water distribution systems (ethyl alcohol, sodium acetate, sodium benzoate, p-hydroxy benzoic acid, and propionaldehyde (Camper, 1995)). An important assumption in the AOC bioassay is that the yield of P17 on acetate-C is equivalent to the yield of P17 on naturally occurring AOC (Kaplan and Bott, 1990). If phosphorous is not limiting in the system, Kaplan and Bott (1990) found that the yield on naturally occurring AOC approximates the yield on acetate. The measured AOC value was not correlated with the added carbon cocktail value in this research (Figure 21). This result has two implications. First, the yield of P17 on the carbon compounds in the cocktail is not constant with varying concentrations. Second, the yield is not equivalent to the yield on acetate. The lack of correlation may be due to the types of substrates available. Van der Kooij, *et al.*,

(1982) did not show that an equivalent amount of organic carbon in the form of complex natural biodegradable substrates yields an identical maximum number of bacteria grown with a single carbon source. NOX utilizes acetate, oxalate, formate, glyoxylate, glycolate, propionate, pyruvate, lactate, malonate, malate, fumarate, and succinate as its carbon sources (Huck, *et al.*, 1991; van der Kooij and Hijnen, 1984). NOX does not preferentially grow with any of the compounds in the carbon cocktail. It can grow on sodium acetate, but not very well (van der Kooij and Hijnen, 1984). *P. fluorescens* (P17) is a nutritionally versatile organism that grows well on "easily biodegradable compounds: amino acids, carboxylic acids, hydrocarboxylic acids, alcohols, and carbohydrates (polysaccharides excluded)" (Huck, *et al.*, 1991). Growth of P17 is probably slower on complex carbon compounds (Servais, *et al.*, 1987). There are a variety of other reasons why P17 does not grow well on the carbon cocktail: 1) perhaps P17 is not able to utilize these carbon compounds very readily, 2) the concentrations of the compounds are too high for the growth of this oligotrophic organism, or 3) the compounds themselves or their metabolic products become inhibitory at the high concentrations of carbon cocktail. Studies to improve the AOC determination should consider the special characteristics of the oligotrophic bacteria used in this assay.

Growth in Double-Glass-Distilled Water

Certain organic compounds such as amino acids, carboxylic acids, carbohydrates, and long-chain fatty acids typically found in drinking water can be utilized by bacteria at concentrations of a few micrograms of carbon per liter (van der Kooij and Hijnen, 1988). Species of the genera *Pseudomonas* occurring in drinking water have substrate affinities for these compounds as low as a few micrograms per liter (van der Kooij, 1990). Consequently, such organisms attain a maximum growth rate at concentrations of 10 to 25 $\mu\text{gC/L}$ of these compounds (van der Kooij and Hijnen, 1988). Coliforms can also utilize a variety of low molecular weight compounds at low levels (as low as 1 $\mu\text{gC/L}$); but these organisms typically multiply more slowly at low concentrations than heterotrophic bacteria (van der Kooij and Hijnen, 1988). Double-glass-distilled water supported the growth of both *K. pneumoniae* and *P. fluorescens* immobilized in the biofilm coupon (Figure 8). The *K. pneumoniae* strain used in this study was an environmental isolate and previous work indicated it is able to grow well in double-glass-distilled water (Camper, *et al.*, 1991). The nutritional versatility of *P. fluorescens* allows it to grow in water that contains trace amounts of organic carbon.

The growth rates of both organisms immobilized in the coupon inserts were also determined in double-glass-distilled water with added mineral salts in the continuous flow reactor experiments. The results indicate that the growth

rates of *K. pneumoniae* and *P. fluorescens* are highly variable in the control reactors (double-glass-distilled water with added mineral salts and no added carbon) (Figures 16 and 25). The measured AOC values of the control water that corresponded with the sodium acetate experiments ranged from 21 to 122 $\mu\text{gC/L}$, while the measured AOC values for the control reactors that corresponded with the carbon cocktail experiments ranged from 72 to 156 $\mu\text{gC/L}$. Both ranges of AOC values are capable of supporting significant growth of both organisms (van der Kooij, *et al.*, 1982; van der Kooij and Hijnen, 1988; van der Kooij, 1992; LeChevallier, 1991(b); Servais, *et al.*, 1987; and Camper, *et al.*, 1991).

Sodium Acetate as the Sole Added Carbon Source

The energetic cost of aerobic growth on acetate is high. There is one central metabolic pathway that includes the Embden-Meyerhof-Parnas (EMP) pathway, the tricarboxylic acid (TCA) cycle, and the pentose phosphate cycle. All other pathways link to, reverse, or bypass the central pathway through anaplerotic reactions which are more costly to the organisms. If acetate is the sole carbon source, the organism must reverse the entire central pathway in order to synthesize the 12 precursor metabolites, ATP, and reducing power necessary for synthesis of cell materials. A lot of energy is produced, but a large amount of energy is expended, making growth on acetate inefficient.

Aerobic growth is dependent on the amount of oxygen available per amount of carbon added to the system. The theoretical oxygen demand for 15, 50, 100, and 500 $\mu\text{gC/L}$ was calculated as 0.016, 0.0531, 0.107, and 0.531 mg/L oxygen respectively. These values are much below the oxygen saturation concentration in the double-glass-distilled water.

K. pneumoniae is facultatively anaerobic, having both a respiratory and fermentative type of metabolism. This organism has no special growth factor requirements and most strains use citrate and glucose as the sole carbon source (Orskov, 1984). The strain used in this study was a drinking water isolate that was capable of growth in very low-nutrient environments. Though acetate is not a preferred carbon source of *K. pneumoniae*, acetate is found in typical distribution systems. For example, acetate is produced by the reaction of ozone with humic acids (Ahmed and Kinney, 1950). My research indicates that planktonic and immobilized *K. pneumoniae* grow aerobically with acetate as the sole added carbon source (Figures 4, 5, 15, 16 & 17). This is consistent with previous research (Xu, 1993).

Organic carbon in drinking water that can be converted to cell mass is the assimilable organic carbon (AOC) fraction. Since most of the methodologies are biomass based, the AOC measurement should indicate the amount of bacterial growth the water can sustain (Huck, 1990). The trend in the data (Figures 13 & 14) suggests that as the measured AOC concentration increases, the growth rates of both *K. pneumoniae* and *P. fluorescens* also increase. However, the

growth rates of both organisms are more tightly correlated with the added sodium acetate concentration from 15 to 145 $\mu\text{gC/L}$ than the measured AOC ($\mu\text{gC/L}$) (Figure 15 & 16). This indicates that the AOC concentration as determined using P17 and NOX may not indicate the growth of *K. pneumoniae* or P17 immobilized in the biofilm coupon. This contradicts the assumption made by van der Kooij and Veenendaal (1992) that the AOC value as measured using planktonic organisms should be indicative of the growth of the attached organisms. It has been observed that a measured AOC value may not actually reflect growth of microorganisms in distribution systems. (Clark and Olson, 1991; Palmer, C.J., *et al.*, 1991; LeChevallier, 1990(a); and Opheim and Smith, 1990).

The Carbon Cocktail as the Sole Added Carbon Source

The growth rates of *K. pneumoniae* and P17 immobilized in the biofilm coupon with the carbon cocktail as the sole added carbon source seem to increase with increasing concentrations of measured AOC (Figure 22 & 23). However, the growth rates of both organisms are not correlated with the measured AOC ($R^2 = 0.2375$ and 0.2341 (control values included) for *K. pneumoniae* and $R^2 = 0.1399$ and 0.2408 (control values included) for *P. fluorescens*). In addition, the value of the measured AOC is not equal to the added carbon cocktail value.

The growth rates of *K. pneumoniae* and P17 decreased when the added carbon cocktail concentration increased (Figure 24). The growth rate of *K. pneumoniae* seems to increase when both the control and supplemented values are included (Figure 25); however, there is no correlation between the two variables. Though *K. pneumoniae* has no particular growth requirements, the carbon compounds found in the cocktail are not preferred carbon sources for this organism. Like acetate, the energetic cost of growth with these compounds as the sole carbon source is quite high. When the growth rates of immobilized P17 for the control values are included, the growth rate increases from 0-50 $\mu\text{gC/L}$ and then remains the same from 50 to 500 $\mu\text{gC/L}$ (Figure 25). Thus, planktonic and immobilized P17 show similar tendencies in response to the carbon cocktail.

Added Substrate Considering Background Growth

The response to the added sodium acetate or carbon cocktail concentration minus the background (control) growth rate on double-glass-distilled water was also determined. These results indicate that the growth rates for *K. pneumoniae* and *P. fluorescens* generally are higher when acetate is added than when it is not (Figure 17). Concentrations greater than 29 $\mu\text{gC/L}$ of sodium acetate yield lower growth rates for both organisms when the background growth was subtracted. This may be due to the significant growth rates of *K. pneumoniae* and *P. fluorescens* in double-glass-distilled water. The

lower growth rate of *K. pneumoniae* can also be explained by the different rate constants observed at 5 and 50 $\mu\text{g/L}$ acetic acid-2- ^{14}C . The rate constant decreases at the higher value (Figure 34).

The growth rate of both organisms (minus the background growth) was always positive, but that of *K. pneumoniae* decreased with increasing concentrations of carbon cocktail (Figure 26). This indicates that the growth rates of both organisms are stimulated by low concentrations of carbon cocktail, but decrease with increasing amounts of cocktail. This may be due to the significant growth rates of *K. pneumoniae* in the control reactors or may be due to the nature of the carbon compounds in the cocktail. The growth rate for P17 remains fairly constant regardless of the concentration of carbon cocktail (Figure 26). The constant growth of *P. fluorescens* may be due to the similar rate constants of this organism when grown at 5 and 50 $\mu\text{g/L}$ acetic acid-2- ^{14}C (Figure 34). Though AOC levels in drinking water are fairly low, this work indicates that even at values of 50-500 $\mu\text{gC/L}$, immobilized P17 and *K. pneumoniae* may be inhibited.

P17 as an Indicator of the Growth of Coliforms

The use of P17 and a coliform bacterium, *K. pneumoniae*, provided insight into the assumption that the growth response of P17 in the biofilm coupon is indicative of the growth response of *K. pneumoniae* in the biofilm coupon. The

use of heterotrophs as indicators of the regrowth potential of coliforms has been questioned due to the differences in the nutritional requirements of the organisms (Clark and Olson, 1991; Rice, *et al.*, 1991). Clark and Olson (1991) found that the AOC value did not correlate with the coliform growth response. However, Rice *et al.*, (1991) found that the coliform growth response was correlated with the AOC value. Results from this study indicate that the growth rate of *K. pneumoniae* is highly correlated with the growth rate of *P. fluorescens* (Figure 18), though *K. pneumoniae* grows at a faster rate than *P. fluorescens* (Figures 19 and 20) when grown with sodium acetate as the sole added carbon source. The results also indicate that the growth rates of *K. pneumoniae* are somewhat correlated with the growth rates of *P. fluorescens* with the carbon cocktail as the sole added carbon source (Figure 27). However, the growth rates are not as tightly correlated with the carbon cocktail as they were with sodium acetate as the sole added carbon source ($R = 0.753$ for sodium acetate and 0.678 for the carbon cocktail). *K. pneumoniae* also grows at a faster rate than P17 using the carbon cocktail as the substrate (Figures 28 & 29). Thus, the growth of *P. fluorescens* may indicate the growth response of *K. pneumoniae* though at a lower rate. The orthogonal least squares lines fit through the data was used because there is a high degree of uncertainty for both the x and y variables (Barker, 1988).

CHAPTER 6

CONCLUSIONS

- 1) Comparisons made between the AOC values measured with van der Kooij's plate count method and LeChevallier's ATP method on the same water samples indicate that the two methods may not produce the same AOC value for sample water with greater than 100 $\mu\text{gC/L}$.
- 2) LeChevallier's ATP method is fairly reproducible on the same water sample.
- 3) INT can be used in the biofilm coupon to measure the percent of respiring bacteria. The average percent of actively respiring cells of *K. pneumoniae* and *P. fluorescens* immobilized in the biofilm coupon were 47 ± 1.8 percent and 43 ± 2.2 percent respectively.
- 4) The methanol/hexane cleaned biofilm coupon inserts prepared with noble agar gel pads leached the lowest amount of AOC as measured with LeChevallier's ATP method.
- 5) The measured AOC value was correlated with the added sodium acetate concentration; however, the slope of the line is closer to two than one. The measured AOC was not correlated with the added carbon cocktail concentrations and was significantly different from the added carbon cocktail concentration at a ninety-five percent confidence interval. These results have two implications: 1)

the yield of P17 on the carbon compounds in the cocktail is not constant with varying concentration and 2) the yield is not equivalent to the yield on acetate.

6) *K. pneumoniae* is able to grow with sodium acetate as the sole added carbon source both planktonically and immobilized in the biofilm coupon. The growth rate response of immobilized *K. pneumoniae* is more tightly correlated with the added sodium acetate value (not including the background growth rates from the control reactors) than the measured AOC. Thus, the measured AOC values indicated for sodium acetate may not indicate the growth response of immobilized *K. pneumoniae*.

7) The growth rate of P17 immobilized in the biofilm coupon is more correlated with the added sodium acetate concentration than the measured AOC value. The AOC value (as determined using P17 and NOX grown planktonically) is not indicative of the growth of P17 immobilized in the biofilm coupon. This finding suggests that the AOC value measured with planktonic organisms is not indicative of the growth of biofilm bacteria.

8) While the growth rates of both *K. pneumoniae* and *P. fluorescens* are stimulated by the addition of small amounts of added carbon cocktail, continued addition has no significant effect; growth rates were not well correlated with AOC added as carbon cocktail.

9) The growth rates of *K. pneumoniae* and *P. fluorescens* in the control reactors (double-glass-distilled water with added mineral salts) were highly variable. This may be due to the low to medium concentration of measured AOC values which

ranged from (21 to 156 $\mu\text{gC/L}$). These AOC values are capable of supporting significant growth of both of these organisms.

10) The growth rates of *K. pneumoniae* and P17 are correlated for both carbon sources; though *K. pneumoniae* grows at a faster rate.

11) Acetate uptake rates (as indicated by apparent first order rate constants) were significantly lower for *P. fluorescens* than for *K. pneumoniae* and were nearly constant for *P. fluorescens*, while dropping significantly for *K. pneumoniae* with higher acetate concentrations. Thus, *K. pneumoniae* is more sensitive to the added carbon concentration.

12) The growth response of the test organisms immobilized in the biofilm coupon is highly correlated with the added sodium acetate concentration ($\mu\text{gC/L}$). In addition, the growth response of the test organisms in the biofilm coupon is less variable than AOC measurements and reflects the growth response of immobilized bacteria. Thus, 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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APPENDICES

Appendix A

Standard Curves for LeChevallier's AOC Method

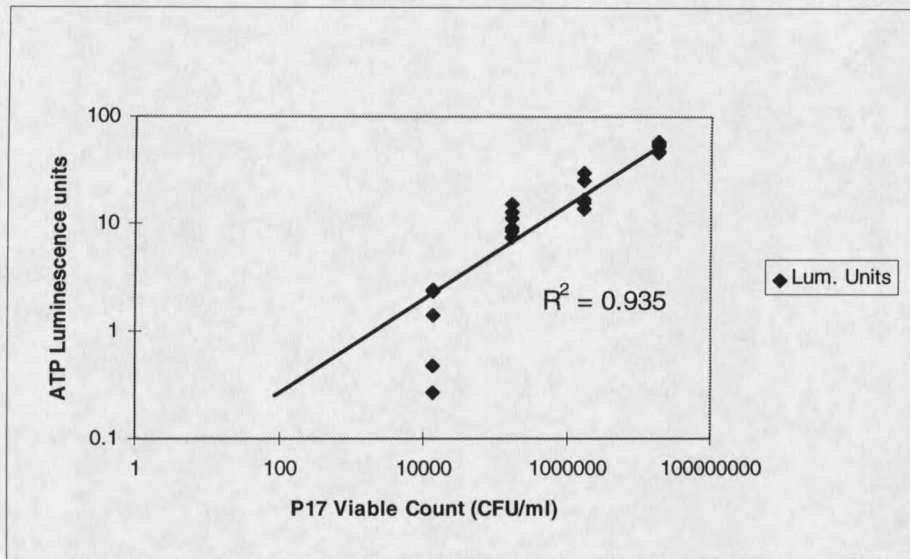


Figure 35. Relationship between luminescence units and strain P17 viable counts.

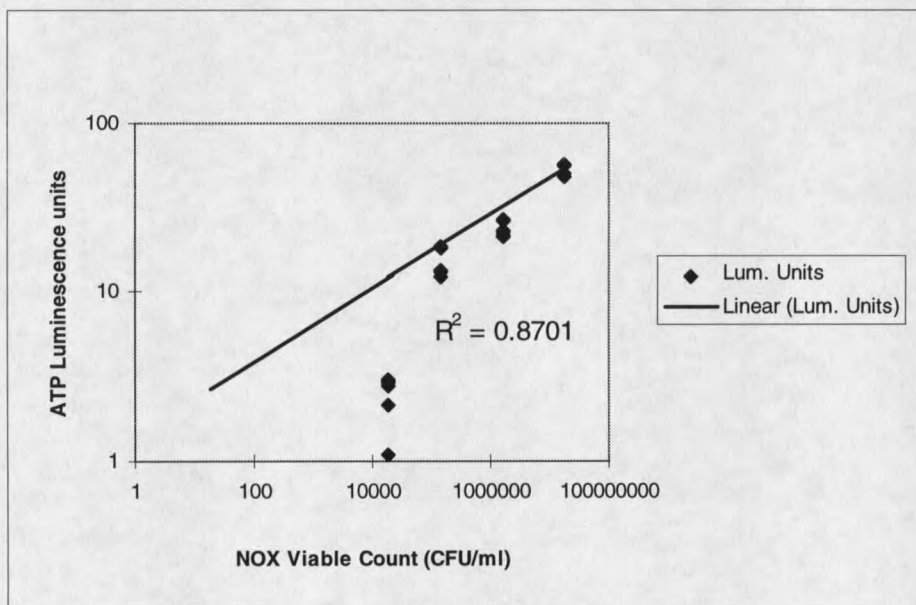


Figure 36. Relationship between luminescence units and strain NOX viable counts.

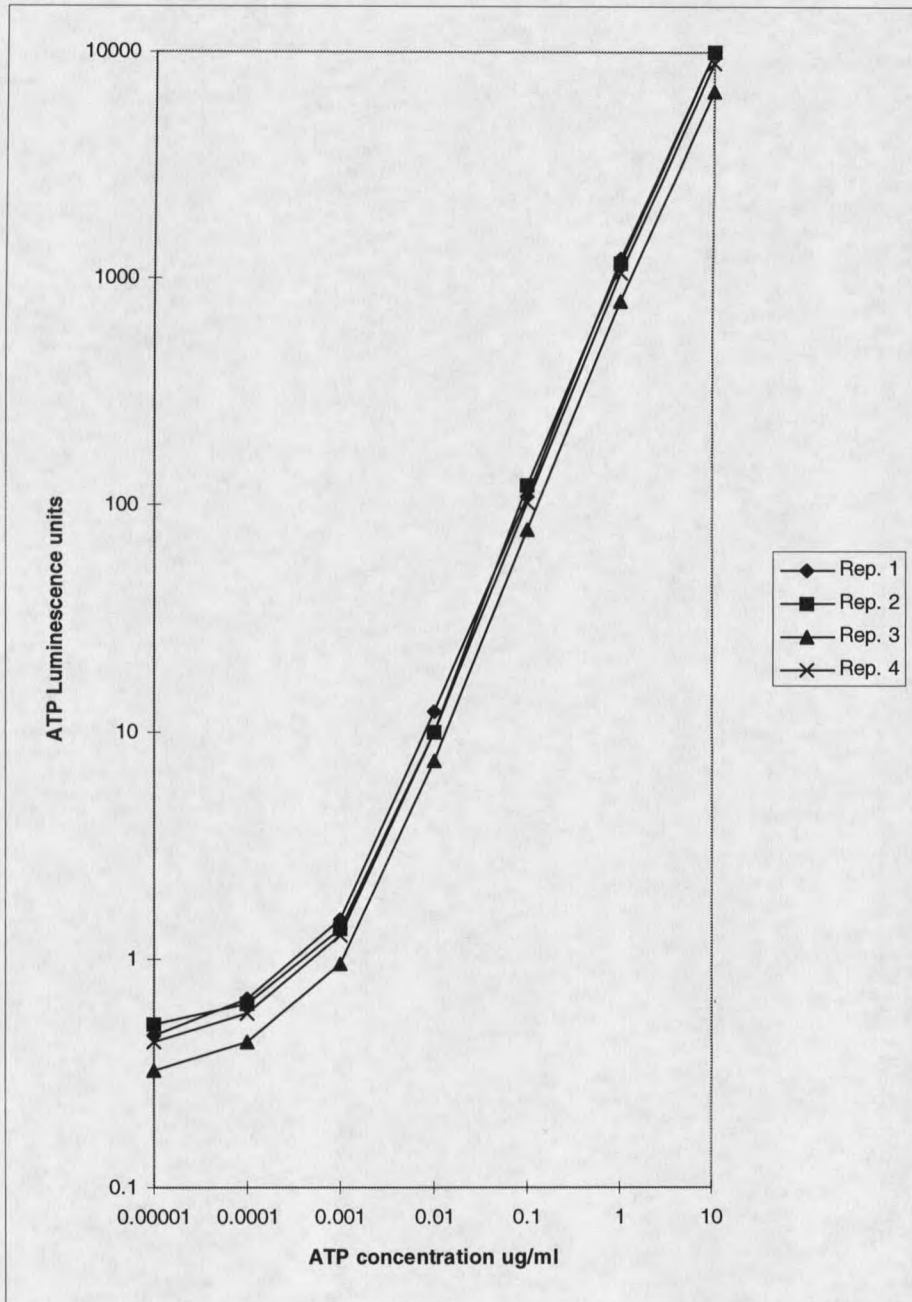


Figure 37. Standard curve of luciferin-luciferase activity versus the concentration of ATP.

Appendix B

INT Data from all Continuously Flowing Reactor Experiments

Table 5. The percent of respiring *K. pneumoniae* and *P. fluorescens* cells (three inserts per experiment) immobilized in the biofilm coupon as determined with INT.

Experiments	<i>K. pneumoniae</i> % Respiring (Three Inserts)	<i>P. fluorescens</i> % Respiring (Three Inserts)
1	56, 54, 51	56, 52, 56
2	51, 55, 54	53, 49, 49
3	53, 47, 51	27, 50, 52
4	57, 4, 51	45, 6, 0
5	0, 52, 54	52, 41, 0
6	48, 59, 44	41, 44, 48
7	48, 52, 49	41, 49, 46
8	45, 45, 50	46, 51, 49
9	0, 42, 67	52, 51, 51
10	54, 51, 53	50, 52, 50
11	51, 51, 50	51, 53, 54
12	50, 52, 49	0, 0, 0
13	49, 49, 41	49, 50, 45
14	47, 47, 47	48, 46, 51
15	48, 16, 49	47, 48, 49
16	48, 46, 48	49, 49, 48
17	49, 46, 47	48, 49, 46
18	52, 52, 51	49, 51, 50

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