



Factors influencing biofilm growth in drinking water distribution systems  
by Anne Kosteczko Camper

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in  
Civil Engineering  
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Abstract:

Conditions contributing to the growth of biofilm bacteria, particularly coliforms, in simulated drinking water distribution systems were investigated. Key experimental variables were substrate loading, temperature, hydraulic residence time, presence of chlorine, pipe material, and the initial growth rate of the introduced coliforms. Pilot distribution systems comprised (a) annular reactors with carbon steel pipe as the outer cylinder and (b) a five segment pipe loop of 4" carbon steel. The pilot systems and laboratory annular reactors were operated to simulate water quality and operational conditions typical of drinking water distribution systems.

A mathematical model was developed to calculate growth rates of the suspended and attached heterotroph and coliform populations using experimental data. Inclusion of a potential for planktonic growth to that of the biofilm increased the precision of the model.

The initial growth rate of the coliforms was critical to their ability to persist longterm in mixed population biofilms. The longest chemostat residence time tested (20 hr) produced the most competitive organisms.

The influence of substratum material was tested in laboratory systems. Biofilms grown on mild steel contained more heterotrophs and coliforms than those sampled from polycarbonate. However, growth rates on the polycarbonate were higher.

In lab and pilot reactors, higher substrate loading resulted in increased bacterial numbers, but not in increased growth rate. No correlation was found between substrate loading, organism numbers or growth rate.

Temperature increase from 10° to 20° C did not result in elevated coliform numbers or growth rates. Heterotroph growth rate was enhanced.

Chlorination was not effective in controlling coliforms, regardless of the substratum composition. The numbers of culturable heterotrophs on polycarbonate were reduced when chlorine was present; this effect was not seen on mild steel.

Coliforms were more abundant at the shortest (2 hr) hydraulic residence time while heterotrophs were unaffected at increased residence times of 4, 8, and 16 hr.

Comparisons between data from parallel annular reactors and pipe loops showed that the qualitative response of the organisms to operational parameters was similar. The annular reactors are recommended as the preferable tool for experimental and industrial use.

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IN DRINKING WATER DISTRIBUTION SYSTEMS

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APPROVAL

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Anne Kosteczko Camper

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

Conditions contributing to the growth of biofilm bacteria, particularly coliforms, in simulated drinking water distribution systems were investigated. Key experimental variables were substrate loading, temperature, hydraulic residence time, presence of chlorine, pipe material, and the initial growth rate of the introduced coliforms. Pilot distribution systems comprised (a) annular reactors with carbon steel pipe as the outer cylinder and (b) a five segment pipe loop of 4" carbon steel. The pilot systems and laboratory annular reactors were operated to simulate water quality and operational conditions typical of drinking water distribution systems.

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Comparisons between data from parallel annular reactors and pipe loops showed that the qualitative response of the organisms to operational parameters was similar. The annular reactors are recommended as the preferable tool for experimental and industrial use.

## CHAPTER 1

## INTRODUCTION

When coliform bacteria are present in treated drinking water in the absence of cross-connections, disinfection barrier breakthrough, or other sources of immediate public health concern, the water utility is said to be experiencing a regrowth event. Regrowth is presumed to be associated with the proliferation of bacteria in the distribution system, either in the bulk fluid or on pipe surfaces. Evidence suggests that the coliform bacteria must be growing on pipe surfaces in biofilms and subsequently detaching into the bulk water. When these detached bacteria are detected in routine monitoring, the microbiological quality of the water is considered unnecessarily compromised. Presence of these indicator bacteria in water regardless of their source is regulated by federal law through the Coliform Rule of the Safe Drinking Water Act; thus the utility must initiate a response action, including public notification, and "boil water" orders. These actions may seriously erode public confidence in the water supply. Utilities are forced to select a "best guess" control measure which in fact may have little chance of controlling the regrowth event. Coliforms arising from a regrowth event may also conceal the presence of coliforms that arise from sources of genuine health concern.

In response to the urgent needs of water utilities, the American Water Works Association Research Foundation issued a request for proposals for research on "Factors

Limiting Microbial Growth in the Distribution System." This thesis reports results of a research project that was subsequently awarded by AWWARF. A comprehensive research plan was developed to investigate those variables that govern the growth of bacteria, particularly coliforms, in distribution system biofilms. The approach included (a) laboratory studies to determine the importance of bacterial physiology and substratum composition (pipe materials) on the surface colonization potential of coliforms; (b) controlled experiments in pilot scale distribution systems to determine the influence of temperature, substrate loading and measured assimilable organic carbon (AOC) concentration, disinfectant concentration, and hydraulic residence time on bacterial colonization; and (c) a field study of 31 drinking water distribution systems to identify field conditions that correlate with regrowth events. Laboratory and pilot data were collected by the project's principal investigator and are presented in this thesis. The field data were gathered and processed by Dr. Mark LeChevallier of the American Water Works Service Company and Darrell Smith of the South Central Connecticut Regional Water Authority.

The goal for the project was to establish criteria to limit microbial growth in drinking water distribution systems. Project objectives were the following:

- (1) Determine the extent of coliform growth in mixed population biofilms.
- (2) Experimentally determine conditions that permit coliforms to establish and persist in mixed population biofilms in model distribution systems.

Important variables included a) growth rate of the introduced coliforms, b) nutrient loading, particularly assimilable organic carbon, c) temperature,

- d) hydraulic regime, particularly residence time, e) the presence of disinfectant, and f) pipe surface composition.
- (3) Establish correlations between operational and environmental variables and the establishment and persistence of coliforms on pipe surfaces by experimentation. Verify laboratory and pilot observations with field results.
  - (4) Design, construct, and evaluate model distribution system(s) for investigating biofilm accumulation on pipe walls.
  - (5) Establish the validity of the pilot distribution system(s) for simulating actual distribution systems.

### Research Approach

Developing relevant information on distribution system microbial growth that can be effectively applied by the drinking water industry requires an integrated research effort. Microbial and chemical processes occur in the entire treatment plant/distribution system, in individual pipes, and on the surface of the pipes in biofilms. Research for the AWWARF project and this thesis was defined in terms of scales of observation (Fig. 1.1). As illustrated, microscale research focuses on measurements and interactions within the biofilm. At the mesoscale, fluid dynamics and system geometries become important, since biofilm processes are dependent on mass transfer phenomena. The macroscale, or treatment plant/distribution system network, is influenced by system operating parameters and environmental variables including influent water quality, water demand,

treatment methods, and regulations regarding water quality. Experiments at the micro- and mesoscale must be performed under relevant conditions so that the results can be extrapolated to the macroscale.

Because it is difficult to identify cause-and-effect relationships in full scale distribution systems, the research effort was coordinated to include laboratory and pilot scale experiments to test perceived relationships between factors and regrowth events. A parallel effort, which is not reported here, was conducted in the form of a field survey of thirty one utilities. A systematic examination of data from the utilities was performed to determine if coliform regrowth events could be correlated with measured parameters. An overview of the research plan for the entire project is illustrated in Figure 1.2. This figure also provides a platform for describing the manner in which the individual papers written for this thesis are integrated. First, the literature review "Coliform Regrowth and Biofilm Accumulation in Drinking Water Systems: A Review," which has been published in the book *Biofouling and Biocorrosion in Industrial Water Systems*, provided the input for determining the important variables for investigation. The microscale research results are described in the paper "Effect of Growth Conditions and Substratum Composition on the Persistence of Coliforms in Mixed Population Biofilms" submitted to *Applied and Environmental Microbiology*. Results from the microscale experiments were used to design colonization strategies for the pilot plant and to interpret responses seen in mesoscale experiments. These results satisfy objectives 1 and part of objective 2 of the research plan. Mesoscale work is described in two papers "Physical Distribution System Models for Assessing Regrowth: Pipe Loops and Annular Reactors" and "Influence of

Water Quality Parameters on Biofilm Growth in Model Drinking Water Distribution Systems,” both submitted to the Journal of the American Water Works Association. The first of these provides a physical description of the pilot plants and gives a statistical comparison of the reactor types on the basis of the response of biofilm organism to operational parameters. This paper specifically addresses objective 4. The second paper describes the response of the biofilm organisms to the tested parameters in fulfillment of objectives 2 and 3. The final paper, “Estimated Biofilm Organism Growth Rates as Influenced by Distribution System Parameters” has been submitted to Water Research and describes modeling efforts conducted to explain the behavior of the organisms in the simulated distribution systems. Integration of all results from the entire project has been provided as a final chapter of this thesis.

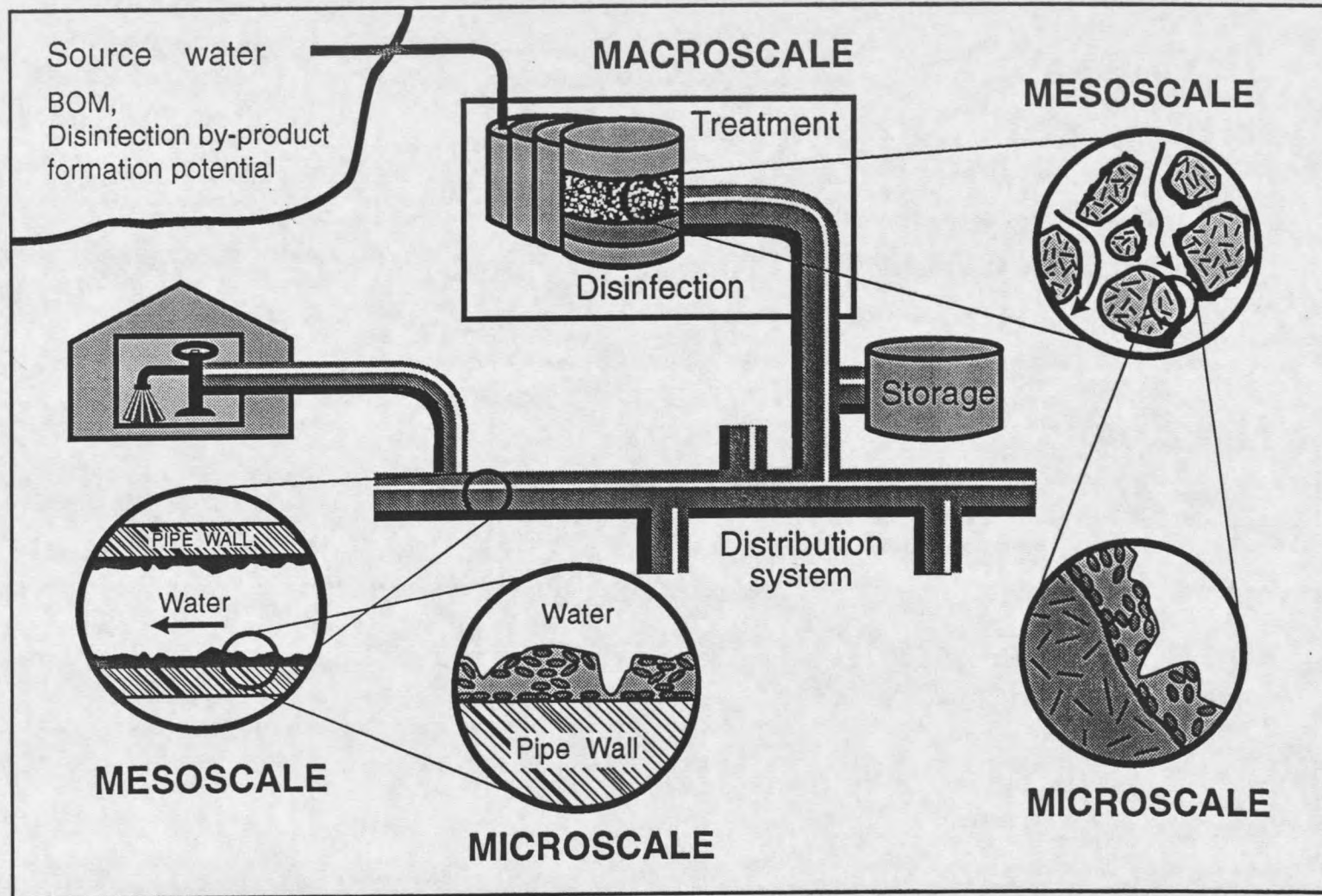
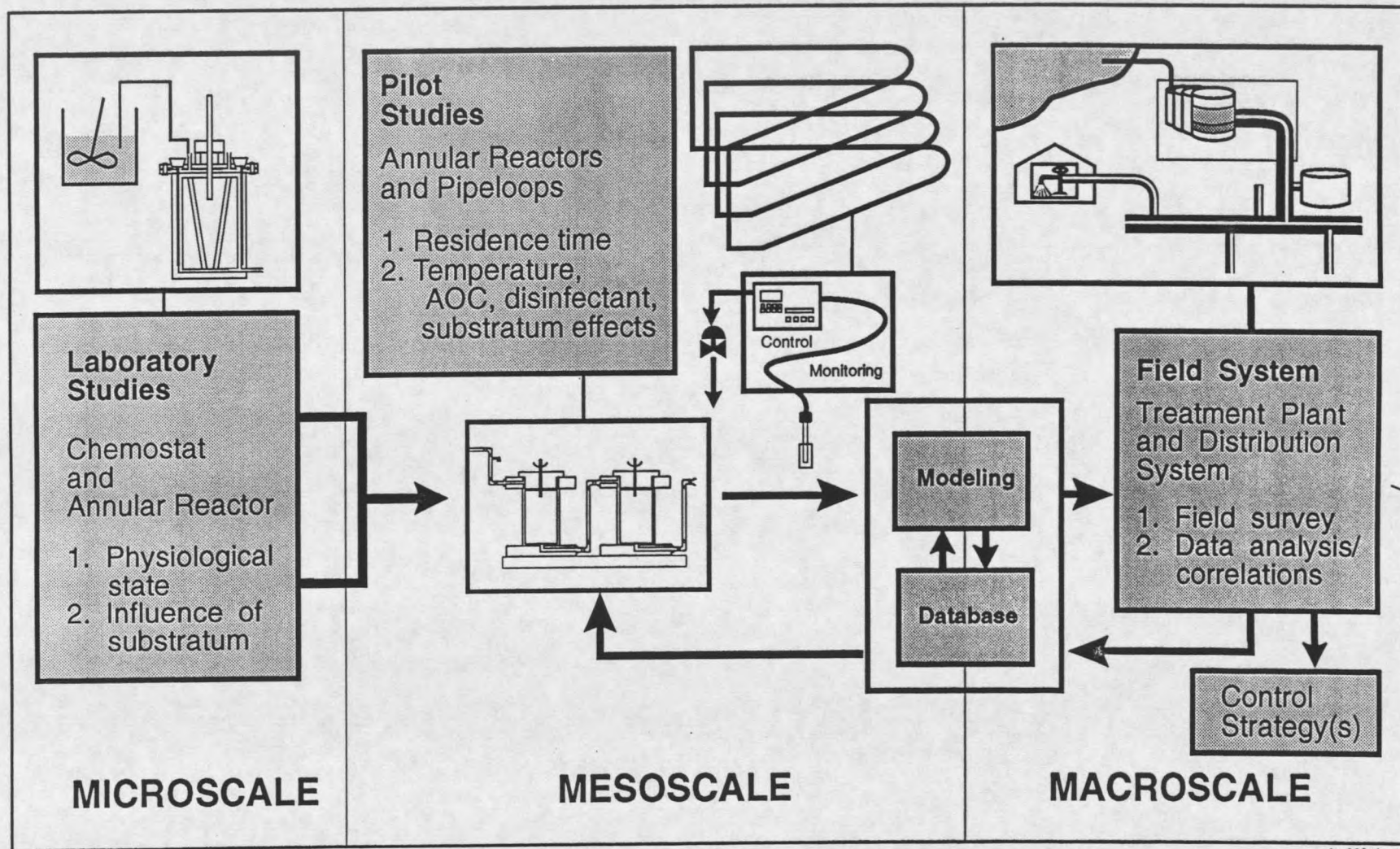


Figure 1.1. Scale-up based on observations at the micro-, meso- and macroscales. At the microscale, biofilm activity is similar regardless of system configuration. System geometry and related hydrodynamics become important at the mesoscale. Investigations at the macroscale provide insight on the importance of system operations and the environment on regrowth.



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Figure 1.2. Illustration of the integrated research approach.

## CHAPTER 2

COLIFORM REGROWTH AND BIOFILM ACCUMULATION  
IN DRINKING WATER SYSTEMS: A REVIEWIntroduction

Coliforms in finished potable water are of concern to the U.S. drinking water industry for a variety of reasons. These bacteria are used as indicator organisms; their presence in water is interpreted as evidence of contamination by fecal material. When coliforms are present in the absence of cross-connections or disinfection barrier breakthrough, the microbiological quality of the water is unnecessarily questioned. Since the presence of coliforms is regulated, the utility must initiate a response action, including public notification and boil water orders, which can seriously erode public confidence. The utility must also select a control measure based on unproven technology which may in fact have little chance of controlling the regrowth event. Coliforms which have proliferated in the system may also conceal the presence of those which arise from sources of genuine public health concern.

A recently completed survey of 164 water utilities (Smith, et al., 1990) indicates that a relatively high number of water distribution systems in the United States experience recurring coliform episodes. Many occurrences have been shown to be independent of obvious disinfection barrier breakthrough or cross-contamination. It has

generally been assumed that these coliforms grow in biofilms on pipe surfaces at the expense of nutrients in the bulk water. Biofilm-associated coliform growth in oligotrophic environments such as drinking water distribution systems is plausible, as there are sufficient nutrients present to support the growth of surface-associated bacteria but not suspended organisms (Characklis, 1988; van der Wende, et al., 1989; van der Wende and Characklis, 1990; Haudidier, et al., 1988).

The European drinking water industry is required to limit the presence of heterotrophic plate count bacteria (HPC) in water. The HPC are a much broader class of organisms than the coliforms, and are therefore more numerous and may arise from diverse sources. However, the same phenomenon of increases in HPC counts in finished water after adequate treatment have been seen and the implication of growth in biofilms has been made. In addition to regulatory concerns, certain bacteria, such as members of the genus *Actinomyces*, have been found on distribution surfaces (Olson, 1982), and may contribute to taste and odor problems. It may also be hypothesized that nitrification in monochloraminated systems, which may contribute to taste and odor problems as well as increased HPC growth, is the result of bacterial activity on pipe walls.

A biofilm is an accumulation of cells immobilized at a substratum and frequently embedded in an organic polymer matrix of microbial origin (Characklis and Marshall, 1990). Biofilms are ubiquitous on surfaces in aqueous environments and growth of coliforms and other nuisance organisms in biofilms occurs under as of yet undefined environmental conditions; regrowth events are detected when these organisms leave the biofilm and enter the bulk water. Because of the low nutrient conditions, drinking water

distribution system biofilms may be very thin and occur nonuniformly on the pipe walls (Allen, et al., 1980; Ridgway and Olson, 1981). Heterogeneities also occur in the distribution of species within the biofilm itself (Amann, et al., 1992; Rogers and Keevil, 1992; Siebel and Characklis, 1991; Zambon, et al., 1984). For example, coliforms are not uniformly distributed on pipe surfaces but have been detected in tubercle materials from water distribution systems (LeChevallier, et al., 1987; Opheim, et al., 1988).

A wealth of circumstantial information exists, generally anecdotal, which relates operational and environmental variables with the appearance of coliforms in finished drinking water. This information is most often system-specific and does not establish a relationship between variables and coliform presence. Factors which may contribute to distribution system microbial growth are 1) seasonal variation in water temperature; 2) availability of growth-promoting nutrients; 3) passage of dormant and/or injured organisms through the treatment process; 4) occurrence of distribution system corrosion products; 5) distribution system disinfection practices; and 6) distribution system hydrodynamics (Smith, et al., 1990). These factors have been addressed in previous research (Characklis, 1988; LeChevallier, et al., 1987; van der Wende, et al., 1988; Martin, et al., 1982; Reilly and Kippin, 1983; Clark, 1984; Wierenga, 1985; Donlan and Pipes, 1988; Fransolet, et al., 1985; Oliveri, et al., 1985; LeChevallier, et al., 1990b; LeChevallier, et al., 1991) and two review articles (LeChevallier, 1990; Block, 1992).

The broad range of experimental and field conditions reported in the literature, together with the inherent heterogeneities of biofilm systems, make it difficult to derive cause-and-effect relationships between variables and coliform/HPC occurrences in

finished drinking water. An integrated systems approach with standardized, relevant parameters and analyses will be required to generate information which can be used to successfully control regrowth events. Hence, multiple factors which may be important in regrowth as investigated in field studies, pilot scale studies, and laboratory investigations are addressed in this review.

### Terminology

The current literature uses numerous terms to describe the presence of bacteria in finished drinking water. "Occurrences," "episodes," and "events" are used synonymously to describe the phenomenon wherein coliforms in excess of standards are detected in finished water. Chronic or periodic appearance of bacteria in water has been termed "regrowth." Regrowth is presumed to be associated with the proliferation of bacteria within distribution systems, either in the bulk water or at the pipe walls. This contrasts with "breakthrough," which describes bacteria passing through the treatment and disinfection barrier. Regrowth and breakthrough are related, since bacteria must inoculate the system (breakthrough) in order to proliferate or grow at the expense of nutrients in the system (regrowth).

Other conceptual terms are "operational" and "environmental." Operational variables are those which can be controlled by the operator or system designer, e.g., disinfection and assimilable organic carbon (AOC). Environmental variables or parameters are outside the realm of control by the operator, and include seasonal effects

(temperature, consumer demand, raw water quality), hydraulic regime and pipe composition.

Two terms have been developed to describe the organic carbon fraction in drinking water which is utilized by bacteria as a carbon source. These are operationally based and depend on the type of analysis performed to obtain the information. Assimilable organic carbon (AOC) represents the small readily degradable fraction of dissolved organic carbon and is traditionally defined as a calculated quantity based on the number of organisms which grow in the test water. Biodegradable organic carbon, biologically degradable organic carbon and biodegradable dissolved organic carbon (BDOC) refer to the change in total organic carbon in the water after it has supported the growth of microorganisms. The BDOC content of a given water may be greater than the AOC value due to the more diverse microbial population used in the assay. The various procedures used to obtain AOC and BDOC values are summarized in a review article by Huck (1990).

### Field Studies

#### Biofilms on Distribution System Materials

Large numbers of bacteria were shown to be associated with distribution system materials as early as 1979 when Allen, et al. (1980) reported that organisms were found in and on water distribution main tubercles and encrustations. These findings were supported by Tuovinen, et al. (1980, 1982) and more recently by other investigators (Characklis, 1988; LeChevallier, et al., 1987; Opheim, et al., 1988). More direct

evidence has come through scanning electron microscopic observations of drinking water distribution system surfaces (Olson, 1982; Ridgway and Olson, 1981; LeChevallier, et al., 1987; Ridgway, et al., 1981).

There is little conclusive evidence for the proliferation of coliforms on pipe surfaces, even though significant numbers of these bacteria may be found in the water. This is most likely due to the difficulty in sampling distribution systems and the patchy or heterogeneous manner in which the coliforms may proliferate on these surfaces. However, Olson (1990) has recovered *Escherichia coli* from pipe surfaces. LeChevallier, et al. (1987) has enumerated coliforms from iron tubercles in one distribution system, and has concluded that the indicator organisms found in the bulk fluid originated from surface-associated growth.

Organisms that colonize the pipe materials in distribution systems originate from a variety of sources, but the majority are those which can be found in the system's source water. This may also include coliform bacteria, especially total coliforms. These bacteria may pass the treatment and disinfectant barriers (breakthrough), enter the finished water in an injured state, and later recover either in the planktonic state or in biofilms. The injured bacteria may be undetected if bacteriological surveillance media such as m-Endo are used to evaluate the quality of drinking water. For example, Bucklin, et al. (1991) reported that filter effluent produced immediately after filter backwash could be characterized by high levels of turbidity and injured coliforms. Likewise, McFeters, et al. (1986) demonstrated that a significant number of injured coliforms (5.7 to 67.5 cfu per 100 ml) were present in various drinking water samples while <1 per 100 ml were

detected using m-Endo LES medium. Additional information on the significance of injured coliforms may be found a book chapter by McFeters (1990).

### Cause-and-Effect Investigations of Regrowth in Distribution Systems

When evaluating field data, it is important to recognize that separation of variables is often impossible. Distribution systems must provide drinking water to their consumers, and as such, cannot be operated as experimental systems. It is difficult to determine direct cause-and-effect relationships in these systems when several variables change at once. For example, elevated summer temperatures are often accompanied by increased nutrient levels and turbidities; all have been implicated in influencing regrowth events. Comparisons between systems is also complicated by differences in hydraulic regime, distribution system piping materials, raw and finished water quality, and disinfectant type and concentration. Reported general categories of variables which may control regrowth are chemical constituents and physical parameters.

Chemical Constituents. The growth of heterotrophic and coliform bacteria requires the presence of various nutrients, including carbon, nitrogen, and phosphorous. A general rule of thumb is that these substances must be present in a ratio of approximately 100:10:1, respectively, for balanced growth. Carbon-containing compounds are the source of energy (electron donor) and carbon (for anabolic processes) for these bacteria, while nitrogen and phosphorous are required for biosynthesis. Other growth factors such as trace metals and organic cofactors may also be necessary for bacterial growth, although these are generally presumed to be nonlimiting in a mixed

microbial population. Heterotrophs also require a terminal electron acceptor, which in these systems is typically oxygen. It is possible, however, for the fermentative organisms such as coliforms to use an internal organic compound as their terminal electron acceptor in the absence of oxygen.

Because carbon must be present in significantly greater quantities than the other nutrients, a major focal point in recent investigations on regrowth has been the concentration of the utilizable organic carbon fraction in drinking water, which ranges from 3 to > 1000  $\mu\text{g l}^{-1}$  (LeChevallier, et al., 1991; Bouwer and Crowe, 1988; Servais, et al., 1987). This is an operational parameter which could be controlled at the treatment plant, particularly through the use of biological treatment. van der Kooij (1992) has found a significant correlation between AOC concentrations and the regrowth of HPC in Dutch distribution systems. Growth at levels < 10  $\mu\text{g C l}^{-1}$  was limited. In an investigation at a New Jersey facility, LeChevallier, et al. (1991) determined that the occurrence of coliform bacteria in the distribution system could be related to a number of factors, including AOC concentrations in excess of 50  $\mu\text{g C l}^{-1}$ . In an earlier study (LeChevallier, et al., 1987), it was shown that AOC concentrations decreased with travel time of the water from the treatment plant. It was hypothesized that this decrease was due to utilization of the carbon by biofilm organisms. The loss of utilizable organic material with travel time from the plant has also been demonstrated with the BDOC procedure (Levi and Joret, 1990). In contrast, Opheim and Smith. (1990) demonstrated no correlation between AOC concentrations and the presence of coliforms in their distribution system.

Nitrogen is present in water in the form of organic nitrogen, ammonia, nitrate and nitrite. All forms are utilizable by different microorganisms. Studies of nitrogen concentrations in distribution systems as related to microbial regrowth events are limited. Donlan and Pipes (1988) indicated that there was no correlation between organic nitrogen, ammonia, nitrate or nitrite with attached microbial population density. In another investigation, concentrations of nitrogen in water which were adequate for balanced microbial growth did not change with travel through a distribution system, whereas the AOC concentration decreased (LeChevallier, et al., 1987; LeChevallier, et al., 1991). It is generally assumed that nitrogen is not limiting in distribution systems due to turnover of existing cellular nitrogen and the low concentrations which are required for growth and maintenance.

Similar to nitrogen, phosphorous is required in very low concentrations for microbial growth. It is also not presumed to be limiting in drinking water systems (Donlan and Pipes, 1988), although one study has shown that low phosphorous concentrations may have restricted microbial growth in a section of distribution main (Herson, et al., 1984).

It is generally recognized that simply raising the chlorine concentration in drinking water often does not control biofilms or the presence of indicator organisms (LeChevallier, et al., 1987; Martin, et al., 1982; Reilly and Kippen, 1983; Oliveri, et al., 1985; Ludwig, 1985; Centers for Disease Control, 1985; Hudson, et al., 1983). In extreme cases chlorine doses up to 12 mg l<sup>-1</sup>, which are far in excess of usual concentrations, were inadequate in attempts to control coliform regrowth (Earnhardt,

1980; Lowther and Moser, 1984). When distribution system biofilms were examined, no correlation was found between free chlorine residuals and the number of HPC organisms per unit surface area (Ludwing, 1985).

Chloramines have become increasingly popular as a drinking water disinfectant. In a recent distribution system comparison, chloramines were more effective at reducing the number of biofilm total coliforms and HPC than chlorine (Neden, et al., 1992). In another study where statistical evaluation of the influence of chloramine concentration on attached microbial populations in a distribution system was made, an inverse relationship was established (Donlan and Pipes, 1988).

The reasons for the differences in disinfectant performance cannot be assessed in drinking water distribution systems due to the multitude of variables which influence disinfectant efficacy. However, these variables can be controlled in pilot and/or laboratory experiments, and their importance will be addressed in the following sections on pilot plant studies and laboratory investigations.

Physical Attributes. As an interdependent variable, temperature has been implicated as contributing to increased microbial populations in distribution systems. Most, but certainly not all regrowth events occur in summer months when water temperatures are highest. LeChevallier, et al. (1991) demonstrated an association between coliform regrowth and water temperatures above 15° C. In London, it was reported that coliform occurrences increased when the water temperature exceeded 20° C and declined when the temperature was less than 14° C (Colbourne, et al., 1991).

Likewise, Donlan and Pipes (1988) reported a direct relationship between water temperature and the attached microbial population density.

Increases in nutrients associated with runoff from rainfall have been proposed by some investigators as a contributing factor to regrowth events (LeChevallier, et al., 1991; Lowther and Moser, 1984). The nutrients are collected in surface water sources, pass through the treatment plant, and are then available for microbial growth in the distribution system.

Various pipe materials are used in distribution systems, including cement lined, asphaltic coated, mild steel, ductile iron, and PVC. When examining steel and iron surfaces, the interaction between the ferrous metal and disinfectants must be considered, as well as the production of corrosion products and tubercles. As stated before, coliforms have been found in association with tubercles in some distribution systems (LeChevallier, et al., 1987; Opheim, et al., 1988), while not in others. When pipe materials have been compared, the number of bacteria on an unlined cast-iron pipe were the highest, with PVC pipe biofilm densities the lowest (Neden, et al., 1992). The relative importance of corrodible pipe materials in regrowth events has been shown indirectly by the reduction or control of coliform bacteria by application of corrosion inhibitors (Martin, et al., 1982; Lowther and Moser, 1984), although this is not universally true (LeChevallier, et al., 1987).

Hydraulic regime in distribution systems is controlled by original design and layout, but is also heavily influenced by customer water demand. Large daily fluctuations can occur between peak use periods (mornings, evenings) and night. Low

flow conditions can lead to decreased disinfectant concentrations and nutrient transport. Higher flow rates result in increased nutrient transport to attached organisms, but also increases disinfectant flux and shear stresses at the surface. Associations between flow rates in distribution systems and regrowth events are therefore difficult to establish, although one study has shown an inverse relationship between flow rates and biofilm density (Donlan and Pipes, 1988).

Residence time of the water in a system may also be an important factor in determining where organisms are most likely to occur on pipe surfaces since AOC and BDOC concentrations have been shown to decrease as water moves through a distribution system (LeChevallier, et al., 1987; LeChevallier, et al., 1991; van der Kooij, 1992; Levi and Joret, 1990).

#### Pilot Plant Studies

A pilot system provides flexibility in conducting controlled experiments and accessing data under conditions which are far more controlled than those found in an operating system of water mains. Pilot systems must be carefully designed to simulate critical parameters in operating drinking water distribution systems and maximize separation of variables. These systems provide an important "proving ground" for relevancy of laboratory findings or the clarification of presumed phenomena in the field. Unfortunately, the presence of well-constructed and operated pilot facilities for research on regrowth are limited due to expense of construction and operation.

Two basic types of pilot pipe systems are found in the literature: 1) once-through and 2) recirculating. Examples in a review on pipe loop systems for investigating corrosion by Levin and Shock (1991) and others (LeChevallier, et al., 1990a; Gardels and Sorg, 1989; Heumann, 1989; Birden, et al., 1985; Treweek, et al., 1985; AWWA-DVGW, 1985) indicate a majority of pilot systems have once-through flow. Characklis (1988) has developed a staged pilot water distribution system using RotoTorques with controllable residence time to represent plug-flow conditions and residence times up to several days, as found in the field. Once-through pilot systems lack this important capability.

Haudidier, et al. (1988) describe the use of six pilot pipeline recirculation loops, configured in series, in a pilot system developed in Nancy, France to conduct studies of attached microbial films in water distribution systems. The Nancy system is designed so that each loop can be operated as a perfectly mixed reactor and, when operated in series, can simulate the plug flow of a pipeline with a high axial dispersion coefficient. An additional benefit of the latter system is the ability to separate residence time effects from those of shear stress.

#### Cause-and-Effect Investigations

Few published papers are available on the use of pilot systems to investigate cause-and-effect relationships between drinking water quality parameters and biofilm accumulation. Using RotoTorque systems, it was demonstrated that the amount of biofilm which accumulated in sequential reactors depended upon chlorine concentration (Characklis, 1988; van der Wende, et al., 1988). When no chlorine was present, most

biofilm accumulation occurred at the entrance to the system. It was hypothesized that this occurred due to rapid consumption of available nutrients. At low chlorine concentrations, biofilm accumulation was reduced at the entrance to the system where chlorine concentrations were higher; as chlorine was consumed at greater residence times in the reactor, biofilm accumulation increased at the expense of the nutrients. In similar experiments with the same system, it was shown that a chlorinated backwash of the upstream pilot mixed media (anthracite/sand) filter decreased the amount of biomass on the filter media with a subsequent increase in the amount of total organic carbon in the filter effluent. There was a concomitant increase in the amount of biofilm within the RotoTorques, indicating that the amount of available carbon influenced biofilm accumulation (Characklis, 1988). Using a pipe loop system, Levi and Joret (1990) report that virtually all BDOC was consumed within 24 hours. Both groups of investigators, although using different systems, concluded that the numbers of planktonic organisms in the water can originate only from detached biofilm organisms and not growth in the bulk phase. In a study using the French recirculating pipe loop system, biofilm accumulation and AOC depletion were greatest in the first loop (40 hour residence time) (Haudidier, et al., 1988). LeChevallier, et al. (1990a) utilized a model pipe system to determine the influence of chlorine and monochloramine disinfectant efficacy on biofilms which had accumulated on several pipe materials. Biofilms on galvanized, copper, or PVC surfaces were readily disinfected by free chlorine or monochloramine ( $1 \text{ mg l}^{-1}$ ). Iron pipe surface-associated bacteria were more susceptible to monochloramine than free chlorine,

but higher monochloramine concentrations ( $4 \text{ mg l}^{-1}$ ) were required than on the other surfaces.

### Laboratory Investigations

Although an abundance of basic laboratory research has been performed on biofilms (Characklis and Marshall, 1990), the focus of this section will be restricted to those directly related to drinking water research. In well designed laboratory investigations, proper control of variables is possible. However, the scope of these studies is often so narrow that applicability of the results to actual distribution systems is difficult. Proper design of experiments which takes into account features relevant to pipe systems is critical.

Determining the response of bacteria to potential nutrients has been of key importance, and has been described in the section on AOC, BDOC and regrowth potential methods. This has not been the only area of interest. The ability of coliforms isolated from drinking water to grow on extremely low carbon concentrations and temperatures has been established (Camper, et al., 1991). The source of growth-stimulating compounds has also been investigated. Fedorak and Huck (1988) demonstrated that cyanobacterial products, which may be a source of nutrients in drinking water, were readily mineralized by HPC bacteria. The importance of crushed tubercle material in enhancing coliform growth has been substantiated by several researchers (Allen, et al., 1980; Martin, et al., 1982; Victoreen, 1980; Victoreen, 1984) and refuted by one (Camper, et al., 1991).

Much of the fundamental research on efficacy of disinfectants against biofilms has been conducted in laboratory studies. Initially, it was shown that attachment of organisms to surfaces resulted in decreased disinfection by chlorine (LeChevallier, et al., 1984; Ridgway and Olson, 1982; Herson, et al., 1987; Berman, et al., 1988). In more elaborate studies using biofilms on relevant surfaces, Berman, et al. (1988) reported that monochloramine was no more effective than chlorine. In another study, LeChevallier, et al. (1988a) showed that there was decreased sensitivity to chlorine conveyed to organisms simply by being attached to a surface, and that this effect was enhanced in older biofilms. When these biofilms were treated with monochloramine, more disinfection was observed than with free chlorine. In a related study, the interaction of oxidizing disinfectants, biofilms, and various pipe surfaces was investigated (LeChevallier, et al., 1988b). Monochloramine was less effective than free chlorine against suspended organisms, but the reverse effect was seen with biofilm bacteria. In analyses were the CT values (concentration-time of biocide exposure for 99% inactivation) of biofilms and suspended (dispersed biofilm) cells of *Pseudomonas aeruginosa* exposed to chlorine and monochloramine were calculated, the same effect was seen (Griebe, et al., 1994). A laboratory system for assaying the efficacy of monochloramine against biofilms containing coliforms has also been developed (Colbourne, et al., 1991). This device supports a stable heterotrophic bacteriological population representative of that found in their distribution system. When challenged with a coliform, it became incorporated into the biofilm and remained as a component. Monochloramine concentrations of 0.6 mg l<sup>-1</sup> caused a loss of the coliform; at lower disinfectant concentrations it persisted.

### Integrating Scales of Observation

Developing relevant information on distribution system microbial growth which can be effectively applied by the water industry requires an integrated research effort. Microbial and chemical processes occur in the water distribution system as a whole, within individual pipes, and in biofilms at the surface of the pipe. To investigate the problem, research must be defined in terms of scales of observation. Microscale research, e.g. millimeters in size ( $<10^{-3}$  m) focuses on measurements and interactions at the cellular level within the biofilm, and is represented in the above material by laboratory studies. At the mesoscale (1 mm to 10 m), fluid dynamics and system geometries become important, since the activity of the biofilm is dependent on the transport and interfacial transfer phenomena created by these conditions (within a pipe). Well-designed pilot scale research falls within this category. The macroscale ( $>10$  m), or field system, is influenced by system operating parameters and environmental variables such as influent water quality and water demand as well as regulations regarding water quality. Experiments at the micro- and mesoscale must be carefully designed so the results can be "scaled up" to the macroscale. For example, microscale observations in a biofilm must be done under conditions relevant to the fluid dynamics in a pipe system (mesoscale) in order to model an operating distribution system (macroscale).

While the overview presented in this paper touches on the relevance of phenomena, parameters, and processes at each scale of observation, it is clear that

integration of scales has yet to be accomplished. Nevertheless, some complimentary conclusions appear by comparing results at the various scales:

- Organic carbon is probably the most important nutrient in a distribution system, as it has been shown to support coliform growth in the laboratory as well as being correlated with regrowth in pilot and full scale systems.
- While little work on the effect of temperature has been done at the micro- or mesoscale, and macroscale phenomena are often linked with other variable variations (e.g., nutrients, turbidity), it is reasonable to expect higher coliform proliferation with increased temperature due to increased enzyme kinetics. These organisms often have optimum growth temperatures well above those found in distribution systems, and are quite sensitive to changes in the lower temperature range.
- Disinfection efficacy appears to vary widely with system parameters and variables as well as disinfectant type. It is tempting to conclude that slower acting biocides (e.g., monochloramine) are superior to highly reactive compounds (e.g., free chlorine) in treating biofilms. However, there is insufficient supporting evidence at this time. Nevertheless, it is also clear that disinfectant alone may not be capable of controlling regrowth events.

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## CHAPTER 3

EFFECT OF GROWTH CONDITIONS AND SUBSTRATUM COMPOSITION  
ON THE PERSISTENCE OF COLIFORMS IN MIXED POPULATION BIOFILMSIntroduction

Over the past decade, drinking water utilities have reported unexplained occurrences of coliform bacteria in finished drinking water, termed regrowth. After thorough investigation of the potential source of these bacteria, the opinion has been that these organisms must be growing on the pipe walls of the distribution system at the expense of the low concentrations of carbon present in the water, detaching from the surface, and being detected as suspended bacteria. It is likely that biofilm growth is responsible for the presence of the coliforms, as there is insufficient useable organic carbon present to support the proliferation of suspended cells (Characklis, 1988; van der Wende, et al., 1989; Haudidier, et al., 1988). Although coliforms are generally perceived to be copiotrophic and mesophilic, isolates from drinking water have been shown to be capable of growth in batch cultures under oligotrophic conditions and temperatures relevant to distribution systems (Camper, et al., 1991). In a limited number of cases, investigators have reported coliforms in association with deposits from full scale distribution system materials (Emde, Smith and Facey, 1992; LeChevallier, et al., 1987).

Locating these organisms has been difficult even in systems plagued with coliform regrowth problems presumably because they are present non-uniformly on the surfaces.

The drinking water industry is interested in knowing what water quality parameters and environmental conditions lead to coliform growth in biofilms. Investigators have had limited success in establishing these organisms in mixed population biofilms for extended periods of time (Block, 1992). Therefore, the systematic study of conditions in laboratory and pilot systems that result in the presence of these bacteria in the field has been impossible. One purpose of this research was to provide guidelines to design experiments on coliform growth using pilot scale distribution systems (Camper, et al., 1995).

The research described here was carried out to determine conditions that allowed indicator bacteria to successfully colonize and persist in laboratory reactors operated to simulate conditions found in municipal distribution systems. Variables selected for study were the initial growth rate of the introduced coliforms, the composition of the substratum, and the presence of disinfectant. It seemed plausible that organisms with low growth rates would be more likely to persist in these reactors, since oligotrophic environments favor slow growing organisms (Bouwer and Crowe, 1988). Laboratory reactors were ideal for the comparison of surfaces for supporting coliforms. A comparison was made between a non-reactive (polycarbonate) and reactive (mild steel) material. Past research has shown that coliforms were found in association with iron tubercles in pipes (Emde, Smith and Facey, 1992; LeChevallier, et al., 1987). Presence/absence of free chlorine was selected since practical operations have shown that

the presence of coliforms in finished water is frequently not controlled by increasing the concentration of disinfectant (Martin, et al., 1982; Reilly and Kippen, 1983; Centers for Disease Control, 1985; Hudson, et al., 1983). In pilot distribution systems with varied materials, it has been demonstrated that organisms growing on ferrous metal surfaces were less susceptible to free chlorine than when present on other materials (LeChevallier, et al., 1990 and 1993). Because conditions in the laboratory reactors could be easily controlled, the structure of coliform-containing biofilms established under low-nutrient conditions was also investigated.

## Materials and Methods

### Reactor Design and Operation

Parallel polycarbonate annular reactors were used to simulate conditions found in a drinking water distribution system. The reactors consist of a stationary outer cylinder with twelve removable flush mounted slides for biofilm sampling (Fig. 3.1). Slides used in these experiments were either polycarbonate or mild steel. The rotation of the inner cylinder was set to simulate the shear stress found in a four inch pipe with a fluid velocity of 0.3 m/s (1 ft/s). Draft tubes in the inner cylinder facilitate mixing so that the reactors can be modeled as continuously stirred tank reactors (CSTRs). The residence time was set at two hours. This time was found to be optimal for coliform growth under similar water conditions (Camper, et al., 1995) and also to be less than the growth rate of the organisms (Camper, et al., 1991). Because planktonic growth was minimal, any

increased number of bacteria in the effluent of the reactor as compared to the influent is expected to be the result of detached biofilm bacteria.

The influent to the reactor consisted of separate feeds of nitrate/phosphate (100  $\mu\text{g/L}$  each), a carbon source (250  $\mu\text{g C/L}$ ), dilution water, and in some experiments, a free chlorine stock solution. The carbon source consisted of equimolar concentrations on the basis of carbon of sodium acetate, sodium benzoate, propionaldehyde, parahydroxybenzoic acid, and ethanol. These compounds were chosen because they represent major classes of compounds found in drinking water, and also because they are relatively non-reactive with chlorine. All chemicals were supplied by Sigma Chemical Co. Dilution water was Bozeman tap water that was passed in an upflow mode through two columns. The first column contained granular activated carbon (12 x 40 mesh Nuchar, Westvaco) to remove chlorine, and the second was filled with biologically activated carbon from a full-scale water treatment plant (City of Laval, Quebec, Canada) to decrease the concentrations of naturally occurring organics that may support microbial growth. Empty bed contact time for each filter was 15 minutes. Effluent from these columns consistently contained  $< 25 \mu\text{g/L}$  assimilable organic carbon as determined by the method of van der Kooij (1982) and modified by LeChevallier, et al. (1993b). The dilution water also contained a substantial number of heterotrophic bacteria (ca.  $10^4$  cfu/ml) that could colonize the annular reactor surfaces, and essentially no coliforms ( $< 1$  cfu/ml). The nitrogen/phosphorous and substrate feed supplies were made with the carbon column effluent in large glass carboys and autoclaved prior to use. Chlorine solutions were prepared by adding an appropriate concentration of bleach to nanopure

water. All influent materials were supplied to the reactors via peristaltic pumps (Cole-Parmer).

Temperature was maintained at 20° C by immersing the reactors in a waterbath attached to a Fisher Isotemp recirculating cooling device.

Prior to each experiment, the reactors were disassembled, completely cleaned, reassembled, and autoclaved with the exception of the mild steel slides. Because autoclaving resulted in excessive corrosion, the mild steel slides were sterilized by UV light for 45 minutes before installation in the reactor. All tubing was replaced and the pumping rates recalibrated.

The chlorine demand of sterile polycarbonate reactors was determined by exposing the systems to an influent concentration of 1.0 mg/L and measuring the concentration in the effluent over several days. Demand from the tubing, medium, and reactor surfaces was found to be approximately 0.1 mg/L.

### Coliforms

The coliforms were originally isolated from drinking water distribution systems and were identified by physiological profiles to be *Klebsiella pneumoniae*, *K. oxytoca*, *Enterobacter aerogenes*, *E. cloacae*, and *Escherichia coli*. Each organism exhibited the fecal biotype at 44.5° C in EC broth (Difco). To minimize the chance of genetic or phenotypic drift, the organisms were streaked for isolation and then frozen in glycerol/peptone (20% and 2% respectively) at -70° C. The frozen stocks were used to start each experiment.

The organisms were pre-conditioned in batch cultures before each experimental run in 100 ml of sterile column effluent containing 5000  $\mu\text{g C/L}$  substrate and 1000  $\mu\text{g/L}$  each of nitrate and phosphate. Incubation was at 20° C in a shaking incubator for 48 hr.

### Chemostats

One ml of each pre-conditioned coliform culture was added to duplicate sterilized 500 ml chemostats fed the same concentrations of substrate and nutrients as the starter cultures. Dilution rate varied depending upon the experiment. The total flow was split between two sources (substrate and nitrate/phosphate) and provided by a doubleheaded peristaltic pump. The chemostats were aerated with filtered air supplied by an aquarium pump. A steady-state population was reached within three residence times; the organism numbers were determined on mT7 and/or R2A agars (Difco) and identifications conducted using API-20E strips (Analytab Corp.). All five organisms were routinely found in the chemostat effluent. Typical cell numbers were  $10^5$  cfu/ml.

### Reactor Inoculation

The entire contents of the chemostats were rapidly pumped to the parallel empty reactors while the inner drums were rotating. The chemostat feed supply was transferred to the annular reactors that were then operated at the same dilution rate as the chemostat for one residence time. The chemostat feed was then terminated and the reactors operated continuously at a residence time of 2 hr using dilution water from the carbon columns, 250  $\mu\text{g C/L}$  influent substrate and 100  $\mu\text{g/L}$  nitrate and phosphate. The dilution water

acted as the source for heterotrophic organisms. If chlorine was used, it was started one week after the dilution water addition commenced.

### Fluid Sampling

Influent and effluent samples were collected in sterile tubes from both reactors at each sampling time (weekly for the first four weeks, every other week for two additional sampling periods). These were analyzed for coliform numbers and heterotrophic plate counts. No coliforms were ever detected in the dilution water from the granular activated carbon columns. In some instances, samples were also collected for total and respiring cell counts. If the reactor was chlorinated, samples for bacterial determinations were neutralized with sterile sodium thiosulfate.

The concentrations of free and combined chlorine in the reactor influent and effluent were measured colorimetrically (DPD method) using a Hach kit.

Culturable bacterial numbers were obtained using the spread plate technique. Samples were homogenized using the method of Camper, et al. (1985), diluted appropriately, and placed on triplicate mT7 (for coliforms) or R2A plates (for heterotrophs). Incubation was at 35° C for twenty four hours or room temperature for seven days, respectively.

### Biofilm Sampling

Whenever the bulk fluid was sampled, slides were removed from the reactors for biofilm analyses and then replaced. Two slides per sampling time were taken to determine the reproducibility of results; this was reduced to one per time when it was

found that there no significant difference in bacterial numbers. Deposits on the slides were scraped using a flame-sterilized utility knife into ten ml sterile water in a 100 ml beaker. The contents of the beaker were homogenized and bacterial numbers determined as above. Results were reported as the number of colony forming units per square centimeter of slide surface.

Total and respiring cell counts were done in the disinfection studies to provide a basis for comparison with culturable counts. Slides were scraped and the cells dispersed by homogenization. 5-cyano-2,3-ditolyl tetrazolium chloride (CTC, Polysciences, Inc.) was added to obtain a final concentration of 0.05% to test for respiratory activity. Following incubation at 25° C for 2 hours, 5% formalin (final concentration) was added to fix the bacteria. The bacterial suspension was filtered onto a 25 mm black 0.2 µm pore size polycarbonate membrane (Poretics). A 1 µg/L solution of 4',6-diamidino-2-phenylindole (DAPI; Sigma Chemical Co.) was added to the filter funnel and allowed to incubate for 5 min. The DAPI was removed and the filter washed with sterile water. The membrane was air dried prior to examination by epifluorescence microscopy. An Olympus BH-2 microscope fitted with an epifluorescence illumination system, a 100 watt mercury lamp, and filter cube units U and G was used. The number of blue (DAPI) and red (CTC) cells in 10 microscopic fields were counted at a total magnification of 1000X. If the total cell number was less than 400, more than 10 fields were examined. Results were reported for total cells (those fluorescing at the DAPI wavelength) and respiring cells (those visible at the emission wavelength of CTC).

If intact biofilms were examined, the slides were removed from the reactor, placed in a flat shallow chamber, and immersed in the CTC solution as above. The biofilm was rinsed, fixed in paraformaldehyde, and counterstained with DAPI.

In some instances, slides were removed, the biofilm fixed in 1% paraformaldehyde, and the cells stained with 5 mg/L propidium iodide for 15 minutes. The biofilm was then counterstained with a monoclonal antibody developed in-house for *Klebsiella pneumoniae*. The staining method consisted of exposing the slide to skim milk as a blocking agent to reduce non-specific staining, rinsing with phosphate buffered saline, incubation in the presence of a monoclonal antibody produced against the organism, rinsing, and secondary staining with a fluorescein conjugated antibody directed against the monoclonal. The slides were observed using an Olympus BH-2 microscope attached to a BioRad MRC 600 confocal laser system. The images produced indicated total cells by a red fluorescence and antibody-labeled cells with a green emission.

At the end of the eight week experiments, the biofilm on the inner drum of the annular reactor was removed with a teflon cell scraper directly into 100 ml sterile water. The cells were dispersed by homogenization, and the coliforms and heterotrophs enumerated by plate counts as for the fluid samples. In some instances, replacement slides from the first few weeks were removed and the biofilms analyzed.

### Data Analysis

Plate count data were entered into spreadsheets in QuattroPro© and reported as the average of the three plates from the appropriate dilution on a per ml (fluid) or per cm<sup>2</sup>

(biofilm) basis. Microscopic counts (total and respiring cells) from the dispersed samples were developed in a similar fashion.

## Results

The research was organized to provide information on the importance of (1) the initial growth rate of the coliforms on their ability to persist in mixed-population biofilms, (2) substratum composition on coliform and heterotrophic biofilm populations, and (3) disinfectant interactions with the substratum and bacterial biofilms. It was also possible to obtain information on the distribution of heterotrophs and coliforms in the reactors with destructive and in situ methods.

### The Importance of Initial Growth Rate

A total of four chemostat residence times (5, 7.5, 10 and 20 hr) were selected to determine the impact of initial coliform growth rate on the ability of coliforms to colonize and persist long term in mixed population biofilms. Initial coliform concentrations in the inocula to the annular reactors were similar at  $10^6$  cfu/ml with the exception of the experiment conducted with chemostat effluent from the 5 hr reactor. In this case, cell numbers were 1.5 logs lower.

Polycarbonate slides were used in the annular reactors. Sampling continued for eight weeks or until no coliforms were detected. Duplicate slides were removed and analyzed for the number of culturable heterotrophs and coliforms. The standard error between

slides in both bacterial counts was less than 10%, indicating good replication at different locations within the reactor.

In replicate experiments with parallel reactors inoculated with coliforms grown in a five hour residence time chemostat, the indicator bacteria were eliminated within the first week after exposure to heterotrophs in the water (Fig. 3.2). When the residence time was increased to 7.5 hours, coliforms were not detected after the first week. If the coliforms were initially cultured at a residence time of 10 hr, they persisted for the entire eight week time period, albeit at very low levels. The longest initial growth rate of  $0.05 \text{ hr}^{-1}$  enabled the coliforms to successfully compete and remain in the biofilms for the entire experimental run time at a level approximately one log higher than observed with the 10 hr grown cells. In both cases, the indicator organisms declined to a pseudo-steady state between three and four weeks after inoculation. In all experiments, the heterotrophs in the biofilms were constant at ca.  $10^6 \text{ cfu/cm}^2$  regardless of the sampling time.

The suspended coliforms followed the same trend with initial growth rate as the biofilm bacteria. Planktonic cells were approximately an order of magnitude lower ( $\text{cfu/ml}$ ) than the biofilm counts ( $\text{cfu/cm}^2$ ) for the 10 and 20 hr. experiments, and not detected in the 5 and 7.5 hr runs. Heterotrophs in the effluent were always present at concentrations of approximately  $10^5 \text{ cfu/ml}$ .

Comparisons of coliform colonization potential in reactors with mild steel were also conducted with 10 and 20 hr residence time chemostat effluents. Biofilm numbers were at least twice as high when the reactors were inoculated with bacteria grown at the slower growth rate.

### Coliform Distribution in Biofilms

Destructive sampling at the end of two experiments was conducted to determine if the coliform numbers on the inner drums of the annular reactors were similar to those found on the slides. In one experiment, parallel 10 and 20 hr chemostat inoculated reactors were operated. Organism numbers from the inner drums and the slides taken from the last sampling time (week eight) were similar, suggesting that there was no preferential association with the inner or outer cylinders.

Concurrently, the coliforms isolated from the inner drums were identified and their metabolic profiles compared to those found in the chemostat effluent used to inoculate the reactors. All five organisms were detected in the chemostat contents that were originally added to the annular reactors. Of eighteen colonies isolated from the 20 hr reactor, fifteen were *K. pneumoniae*, two were *K. oxytoca* and one was *E. cloacae*. All coliforms from the 10 hr reactor were *K. pneumoniae*. A similar analysis was conducted on isolates from the inner drum at the end of a replicate run using a 20 hr chemostat inoculum. Of the 35 isolates tested, 33 were identified as *E. cloacae*, with the remainder being *K. pneumoniae* and *K. oxytoca*. The metabolic profiles of the isolated biofilm organisms were identical to those found in the chemostats.

It was of interest to know how clean surfaces introduced into the reactors after a week or more of operation would be colonized with the coliforms. Slides that had been replaced after the first sampling time (one week after coliform addition) were sampled again at the end of the experiment. The numbers of coliforms on these surfaces were

similar to those from slides that had been undisturbed for the entire eight week run time, suggesting that coliforms emanating from the biofilms could recolonize clean substrata.

To examine changes in biofilm "morphology" over time, intact biofilms from the parallel 10 and 20 hr experiments were visualized after nonspecific (DNA) staining with propidium iodide. To provide information on the distribution of a coliform, fluorescent antibody staining for *K. pneumoniae* was used. The results were intended to be qualitative rather than quantitative. In both reactors, samples from the first three weeks revealed a heterogeneous distribution of individual *K. pneumoniae* and non-specifically stained cells on the surface. At week four, the organism was present primarily as discrete microcolonies with a few individual cells. The remainder of the population was present as clumps up to 20  $\mu\text{m}$  in height, strings of cells, and as individuals. Bacteria were found in association with small pieces of what was believed to be granular activated carbon from the biological filter. The majority of the bacteria were present as distributed cells in direct contact with the substratum. Approximately 60% of the total surface was covered with microorganisms. The overall appearance of the biofilm did not change substantially for the remainder of the experiment. *K. pneumoniae* was detected for the entire eight weeks, and was the most abundant coliform identified with scraped and plated biofilm samples.

#### Substratum Effects

The relative importance of a reactive (mild steel) or inert substratum (polycarbonate) on biofilm accumulation and coliform persistence was determined using

inocula from 10 or 20 hr residence time chemostats. Substantial amounts of corrosion products accumulated on the steel slides with time. Regardless of the initial growth rate, the mild steel reactors supported approximately tenfold more heterotrophs and coliforms per square cm than similar reactors containing only polycarbonate (Fig. 3.3). This increase was also seen in the reactor effluents, indicating that the increased numbers were due to an enhancement of growth rather than merely from greater accumulation on the mild steel. Mild steel slides taken from the reactor for sampling were replaced with polycarbonate. When the runs were terminated, the polycarbonate slides that had been in place for seven weeks were also analyzed for heterotrophic and coliform cell numbers. When coliform numbers on the replacement polycarbonate slides were evaluated, the values were similar to those from the final mild steel slides (98 vs 110/cm<sup>2</sup>, respectively). Heterotroph densities on the replacement slides approximately a half log lower than on the mild steel, but were still greater than numbers on polycarbonate slides from reactors that had never been operated with mild steel. The biofilms scraped from the polycarbonate replacement slides contained large accumulations of corrosion products.

#### Chlorination Effects

Three separate experiments were conducted with mild steel or polycarbonate slides to evaluate the potential interaction of chlorine and the substratum on biofilm and suspended cell numbers. Parallel reactors were operated for four weeks after coliform addition before disinfection was begun in one of the two reactors. The second acted as

the unchlorinated control. Influent free chlorine concentrations were either 0.2 or 0.5 mg/L in the polycarbonate systems and of 1 mg/L when mild steel was used.

Influent chlorine concentration in the first experiment was initially set to 0.2 mg/L and subsequently increased to 0.5 mg/L on day 7 because there was no response from the biofilm coliforms (Fig. 3.4). The increased dose did not result in a decline in biofilm coliforms. Similarly, effluent coliforms were unaffected by the chlorine (ca. 50 cfu/ml). At the lower chlorine concentration, there was approximately a two log reduction in the number of culturable biofilm heterotrophs with no further reduction when the concentration was increased. Effluent heterotrophs declined from near  $10^5$  cfu/ml to  $1.5 \times 10^3$  at the lower chlorine dose and further decreased to  $1.0 \times 10^3$  after the dose was increased.

Data were also collected on the numbers of respiring and total cells in scraped biofilm samples. Values from these measurements and the culturable cells in the chlorinated reactors were compared to those in the parallel system by taking the log of their ratio (Fig. 3.5). Culturable coliforms were the least affected, with nearly the same numbers in the control and chlorinated reactors (ratio approximately 1). The heterotrophic enumeration was the most impacted measurement. Total cells decreased slightly in the chlorinated reactor. The ratio of respiring cells declined through day 7 and then increased. The percent of respiring cells (compared to total counts) in the chlorinated biofilm increased from less than 10% to 70% at the end of the experiment.

A replicate chlorination experiment was conducted with polycarbonate as the substratum. The influent chlorine concentration was held constant at 0.5 mg/L for the

duration of the run. Similar results to the first run were obtained, with the exception that a 0.5 log reduction in culturable coliforms was seen. In addition to the dispersed biofilm analyses, intact biofilms were stained with CTC and DAPI in an attempt to locate the position of respiring cells. Qualitative evaluation of the observations suggested that the largest numbers of respiring cells were in association with particulate material in the biofilm.

When the experiment was terminated, slides replaced at one week and on day 15 were resampled. Not only were the coliform numbers the same on both slides, there was no difference between the chlorinated and control values. The results suggest that even under constant chlorination, the coliforms arising from the reactor biofilms could successfully colonize clean materials.

The third disinfection study used an influent concentration of 1 mg/L and mild steel as the substratum. Because of the interference of the corrosion products with direct microscopic observations, only plate counts were performed. Even though there was an average residual of 0.3 mg/L in the chlorinated reactor effluent, there was no difference in heterotrophic plate counts between the two reactors. Numbers varied throughout the eight week experiment from  $1.4 \times 10^6$  to  $1.0 \times 10^7$  cfu/cm<sup>2</sup>. Coliform counts were slightly higher in the chlorinated system and remained constant at approximately 10 cfu/cm<sup>2</sup>, while the control reactor numbers ranged from a high of 10 cfu/cm<sup>2</sup> from the first sampling to a low of 0.2 cfu/cm<sup>2</sup> on week 6.

### Discussion

The initial growth rate of coliforms appears to have a long-term impact on their ability to effectively compete in a mixed population biofilm. Regardless of the initial growth rate, the coliforms attached to the surfaces and were detected for the first week; only with the slower growth rates were the bacteria present for the entire run time. Also of considerable interest was that the initial growth rate influenced the long-term relative abundance of these bacteria. At growth rates of 0.10 and 0.05/hr, a steady-state level was attained at approximately 3-4 weeks after inoculation. However, the organisms grown more slowly maintained a higher population on both an inert and reactive substratum for the entire eight week run time. This effect was also apparent on surfaces introduced to the reactors after the initial colonization period. Organisms on the replacement slides resulted from the reattachment of bacteria detached from the biofilms elsewhere in the reactor and attained the same steady-state numbers as their source populations. The slow-growing bacteria may have been pre-conditioned in the chemostats to the low nutrient conditions found in the reactors. It has been shown that bacteria can change their uptake mechanisms depending on the available substrate concentrations as a survival mechanism in low-nutrient conditions (Dawes, 1976; Novitsky and Morita, 1978; Geesey and Morita, 1979; Kurath and Morita, 1983). Other possibilities, as described by Roszak and Colwell (1987) are the rerouting of metabolic pathways to avoid blocks from nutrient limitation, better coordination of biosynthesis to stabilize balanced growth, and the adjustment of uptake of nutrients in excess. Regardless of the mechanism, the initial growth rate has a

profound influence on the long-term behavior of these bacteria. Results may explain why it has been difficult to establish certain bacteria in biofilms when they have been grown under rich nutrient conditions in batch culture. In the one reported instance where coliform maintenance in a mixed-population biofilm has been reported (Colbourne, et al., 1991), a drinking water isolate of *E. coli* was introduced to a continuous flow reactor containing tiles of distribution system materials for colonization operated at a dilution rate of 0.05/hr. The practical implication of these results is that slow-growing organisms from environmental sources are more likely to colonize pipe surfaces. Conversely, rapidly growing organisms from a source such as fecal contamination may be less likely to become established in distribution system biofilms.

Even though coliform numbers were similar in replicate experiments, the predominant coliform changed from *K. pneumoniae* to *E. cloacae*. These are the two organisms most frequently communicated by utilities to be present in regrowth events. It is unclear what environmental pressures predisposed the prevalence of one organism over the other under identical conditions.

The spatial distribution of classes and subclasses of organisms in drinking water biofilms has been performed using rRNA probes (Manz, et al., 1993). In these studies, there was intermingling of various organisms on the surface and within microcolonies. With the exception of one report on the location of *E. coli* by rRNA probes in a dual population biofilm fed with benzoate (Szewzyk, et al., 1994), there is no information on the distribution of coliforms. A monoclonal antibody was used in this research to characterize the distribution of *K. pneumoniae* in mixed population biofilms. These

bacteria were present most frequently as microcolonies, which is in agreement with information obtained in other laboratory experiments using defined mixed populations (Siebel and Characklis, 1991; Murga, et al., 1995). The general microbial population was dispersed on the surface; the substratum was not uniformly covered with bacteria. The low colonization density reflects the oligotrophic conditions present in the reactor.

There is some indication that ferrous metal surfaces support more organisms than other pipe materials. Neden, et al. (1992) found that bacterial populations on unlined cast were the highest while PVC were colonized with the lowest number. Block (1992) also determined that there was a decrease in bacterial numbers when the surfaces were cast iron, tinned iron, cement lined cast, or stainless steel. Indirect evidence for the involvement of ferrous metals in coliform regrowth events is proposed when corrosion control measures reduced or eliminated a distribution system's regrowth problem (Martin, et al., 1982; Lowther and Moser, 1984; Hudson, et al., 1983). The importance of pipe material on organism numbers, including coliforms, was substantiated in our research. The mild steel surfaces were consistently colonized by nearly ten-fold more heterotrophs and two to ten fold more coliforms than polycarbonate when the reactors were operated under the same conditions. The impact extended to the effluent bacterial concentrations as well; elevated counts were found in reactors with mild steel even though only 10% of the reactor surface area is encompassed by the slides. Further, the presence of mild steel affected population densities on polycarbonate surfaces in the same reactor. These surfaces supported the same numbers of bacteria as seen on the steel itself. These results may be extrapolated to aging distribution systems, where old ferrous metal pipes tend to

be highly corroded. Exposed metal surfaces may impact biofilms on new plastic or cement lined surfaces.

The interaction of chlorine with the substratum has been demonstrated to be important in biofilm disinfection. In one study, biofilms on galvanized, copper or PVC surfaces were readily disinfected with 1 mg/L free chlorine, while bacteria associated with iron were much less susceptible (LeChevallier, Lowry, and Lee, 1990). Free chlorine residual concentrations of 3.3 mg/L did not disinfect biofilms on black iron pipes (LeChevallier, et al., 1993a). There is no information, however, on the impact of chlorination on coliforms in mixed population biofilms grown on an inert (polycarbonate) or reactive mild steel surface. On an inert substratum, chlorine residuals of 0.05 to 0.15 mg/L did not affect coliform numbers in the bulk fluid or biofilm, while heterotrophs were impacted. With a chlorine residual of 0.3 mg/L and a reactive substratum, there was no obvious effect of disinfectant on either biofilm population or on the suspended coliform numbers. In parallel experiments conducted on mild steel surfaces in two pilot distribution systems, it was found that there was an enhancement of coliforms in biofilms and bulk fluids when free chlorine was applied (Camper, et al., 1995). These results may explain why utilities are typically unsuccessful in reducing coliform numbers with elevated chlorination during a regrowth event.

Further insight on the impact of chlorination was gained from staining of dispersed biofilm populations from the polycarbonate reactors. Changes in total numbers on polycarbonate when chlorinated reactors were compared to the control revealed that there was little removal. Respiratory activity as measured by CTC incorporation was

initially impacted, but the remaining population regained CTC reducing capabilities with time. In short term biofilm disinfection studies, it has been shown that there is a significant retention of respiratory activity with a concurrent substantial loss of culturability (Stewart, et al., 1994). The recovery of respiratory activity with time in the constant exposure to disinfectant was unexpected. Possible explanations include selection and gradual replacement of impacted organisms with those less susceptible to chlorine or activation of a stress response. Srinivasan, et al. (1995) hypothesize that thin biofilms may be less susceptible to disinfection than thicker films due to the presence of resistant subpopulations resulting from phenotypic variation or structural heterogeneity. Structural heterogeneity exhibited by *K. pneumoniae* in the form of microcolonies may protect the interior organisms by transport limitation of the disinfectant.

The impact of particles in the biofilms on organism distribution was observed in both control and disinfected reactors. There was a concentration of organisms around particulate matter in the biofilm. Since these reactors received water processed by the carbon filters, it is likely that the solids were released carbon fines. Pilot and full-scale treatment plant observations have shown that particles released from filters are frequently colonized with bacteria (Camper, et al., 1987; Stringfellow, Mallon and DiGiano, 1993). It is unknown whether the bacteria were present on the particles when they became imbedded in the biofilms of the laboratory reactors or if growth in the vicinity of the carbon was enhanced. Regardless of the source of the bacteria, respiratory activity in the vicinity of the particles was enhanced when the biofilms were chlorinated. The presence

of particles in other biofilms has been shown to reduce disinfectant efficacy (Srinivasan, et al., 1995).

Other information obtained from these experiments indicated that coliforms released from biofilms in the reactors were capable of reestablishing in biofilms on newly introduced surfaces. This occurred whether the substratum was mild steel or polycarbonate and in the presence of a free chlorine residual. The practical implication is that distribution systems may be constantly recolonized with coliforms, even if treatment practices are optimized. Since chlorination appears to be ineffective and may actually enhance coliform numbers, alternative control strategies for regrowth are indicated.

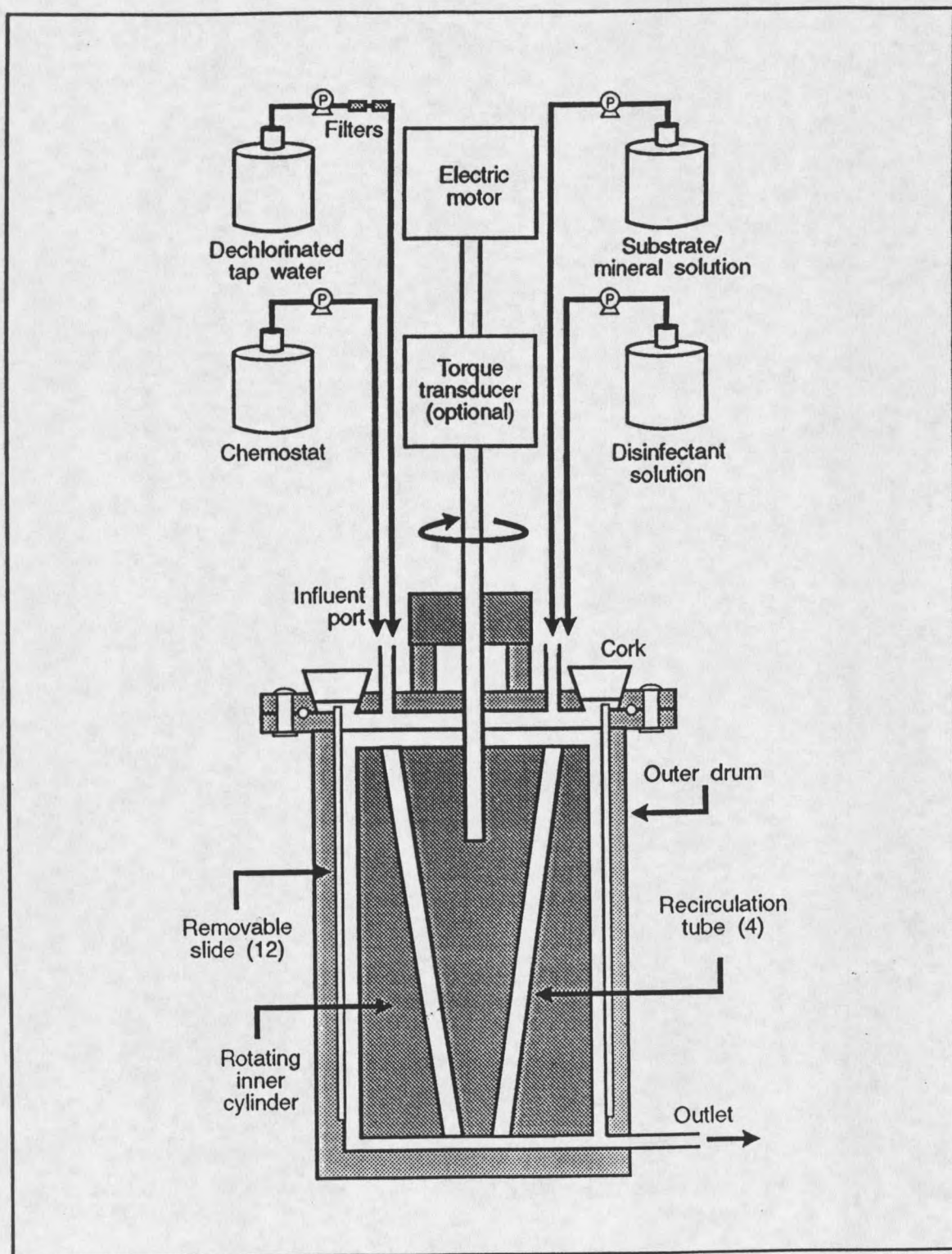


Figure 3.1. Schematic of the laboratory annular reactor.

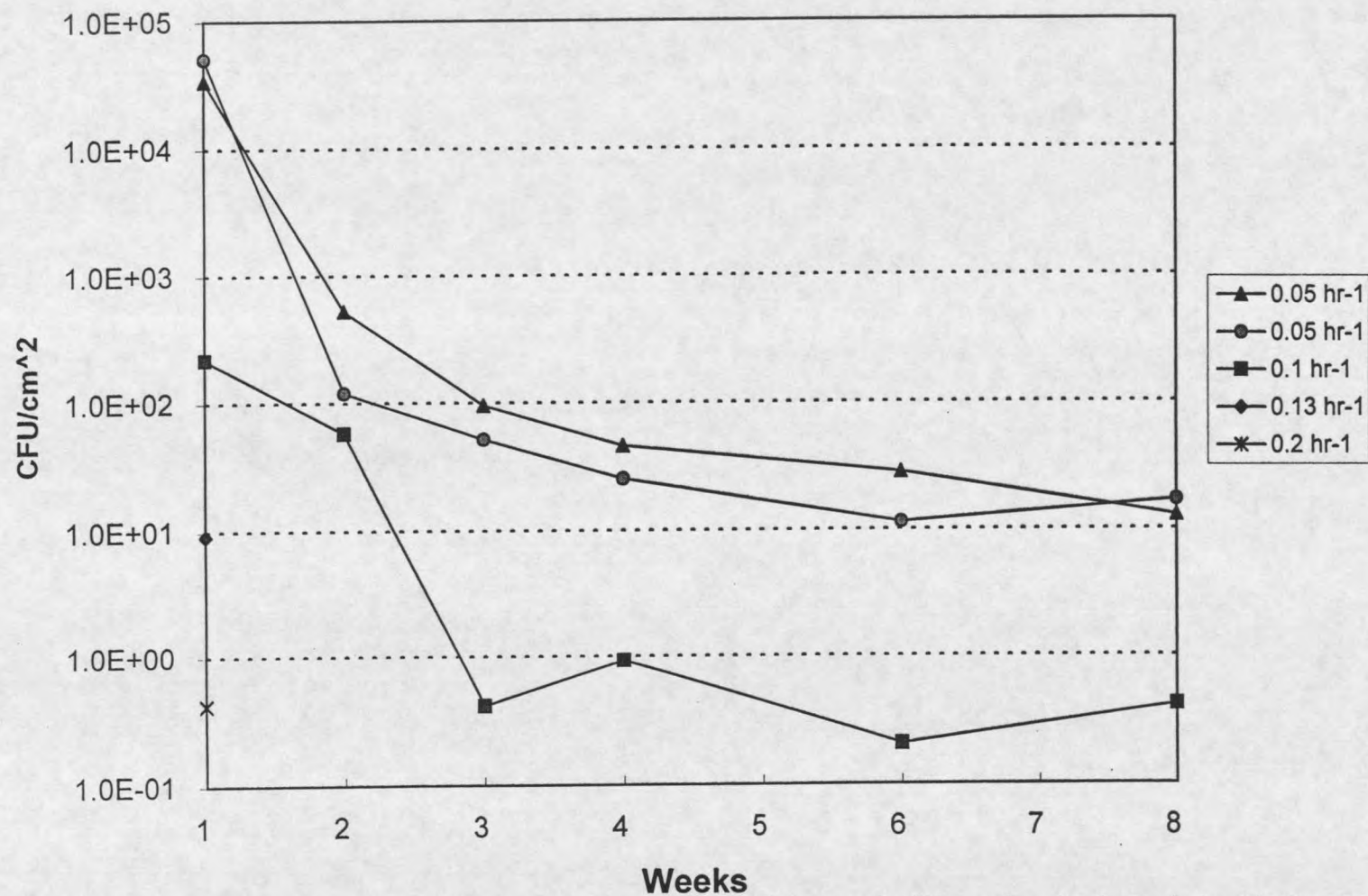


Figure 3.2. Impact of initial growth rate on the long term persistence of coliforms in mixed population biofilms on a polycarbonate substratum. Coliforms grown in chemostats with a residence time of 20, 10, 7.5 or 5 hr were used to inoculate the reactors.

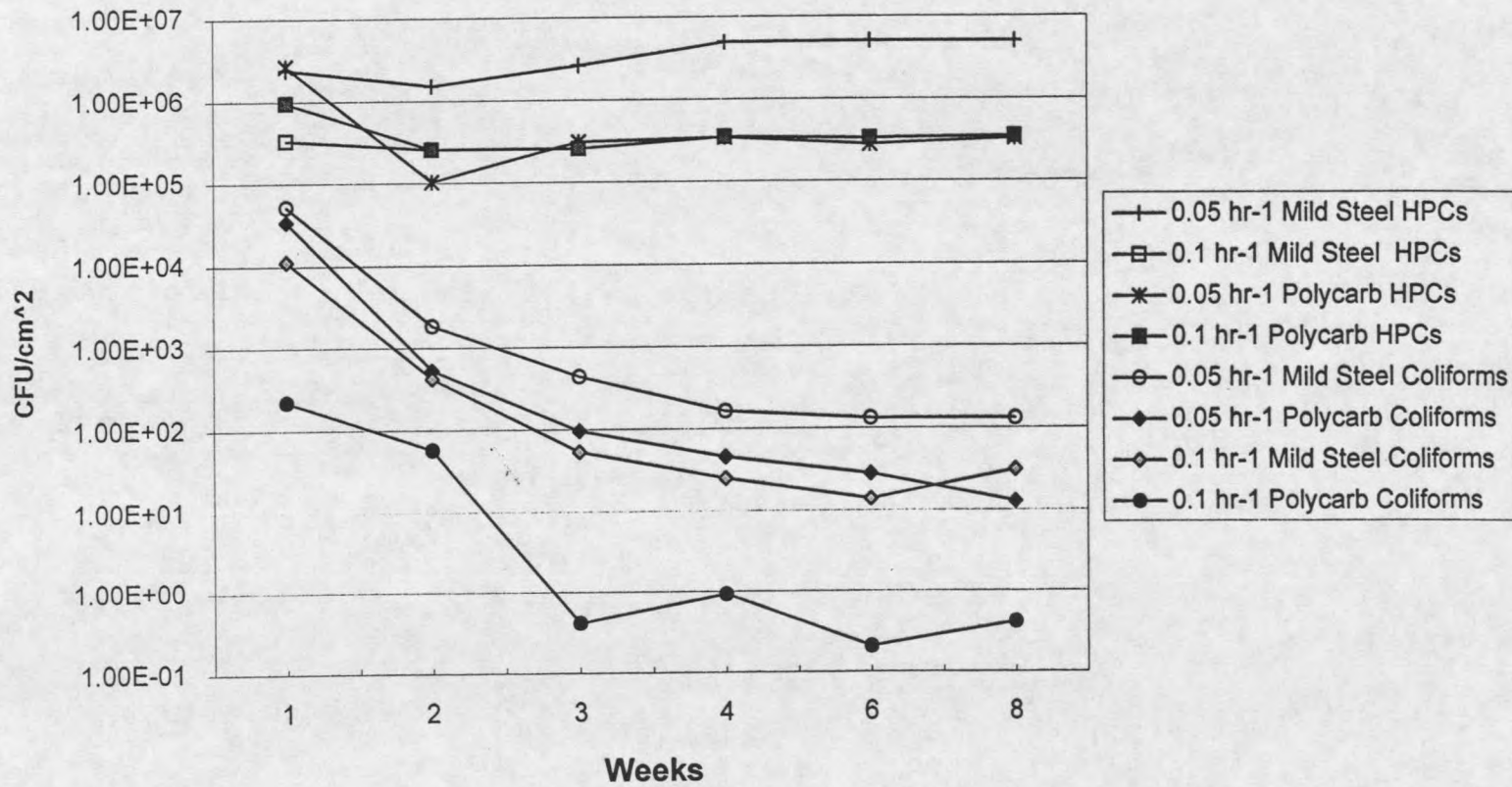


Figure 3.3. Effect of substratum composition and initial coliform growth rate on heterotrophs and coliforms in mixed population biofilms.

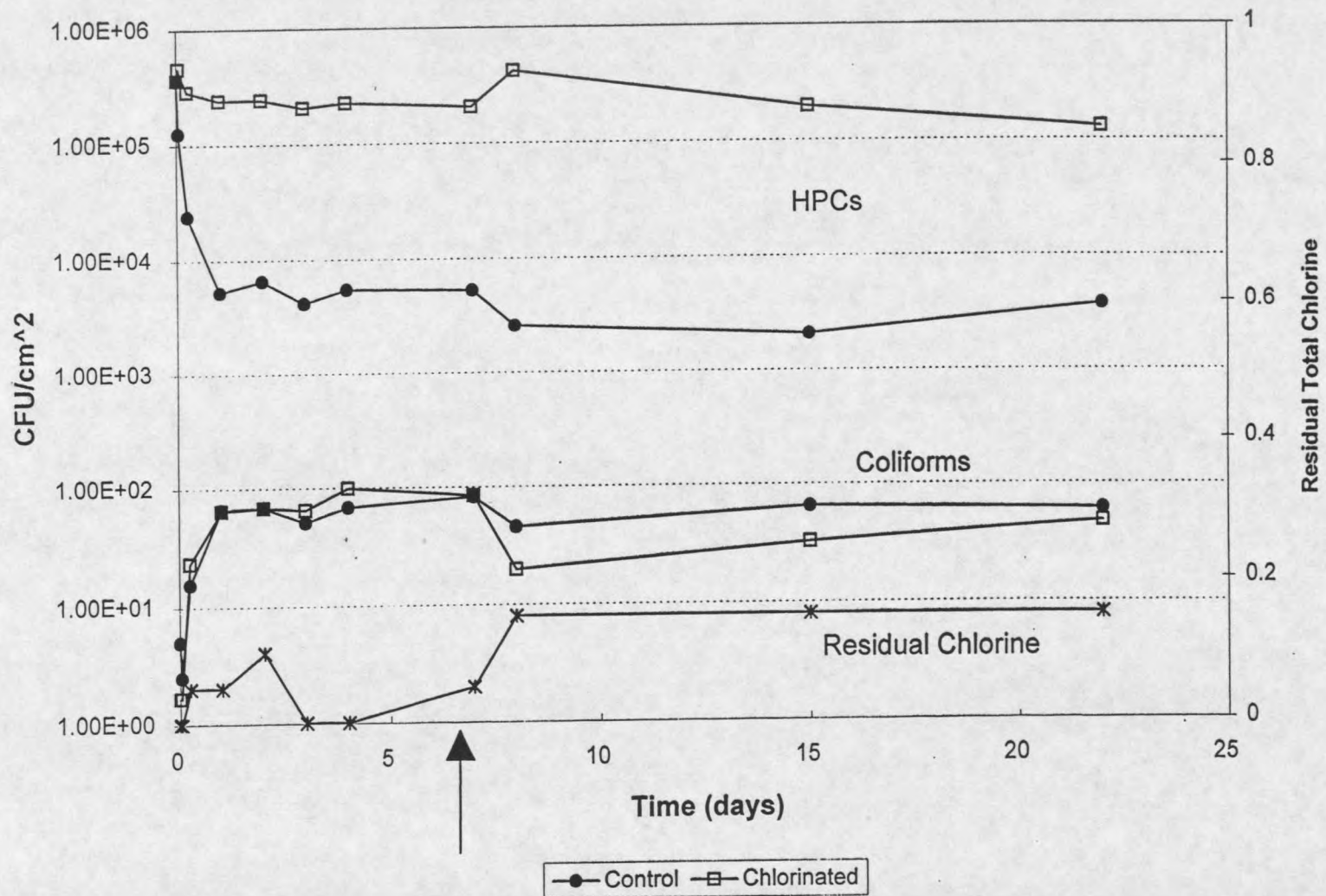


Figure 3.4. Numbers of biofilm heterotrophs and coliforms in parallel control and chlorinated polycarbonate reactors. Initial influent chlorine concentration was 0.2 mg/L; this was increased to 0.5 mg/L on day 7 (arrow).

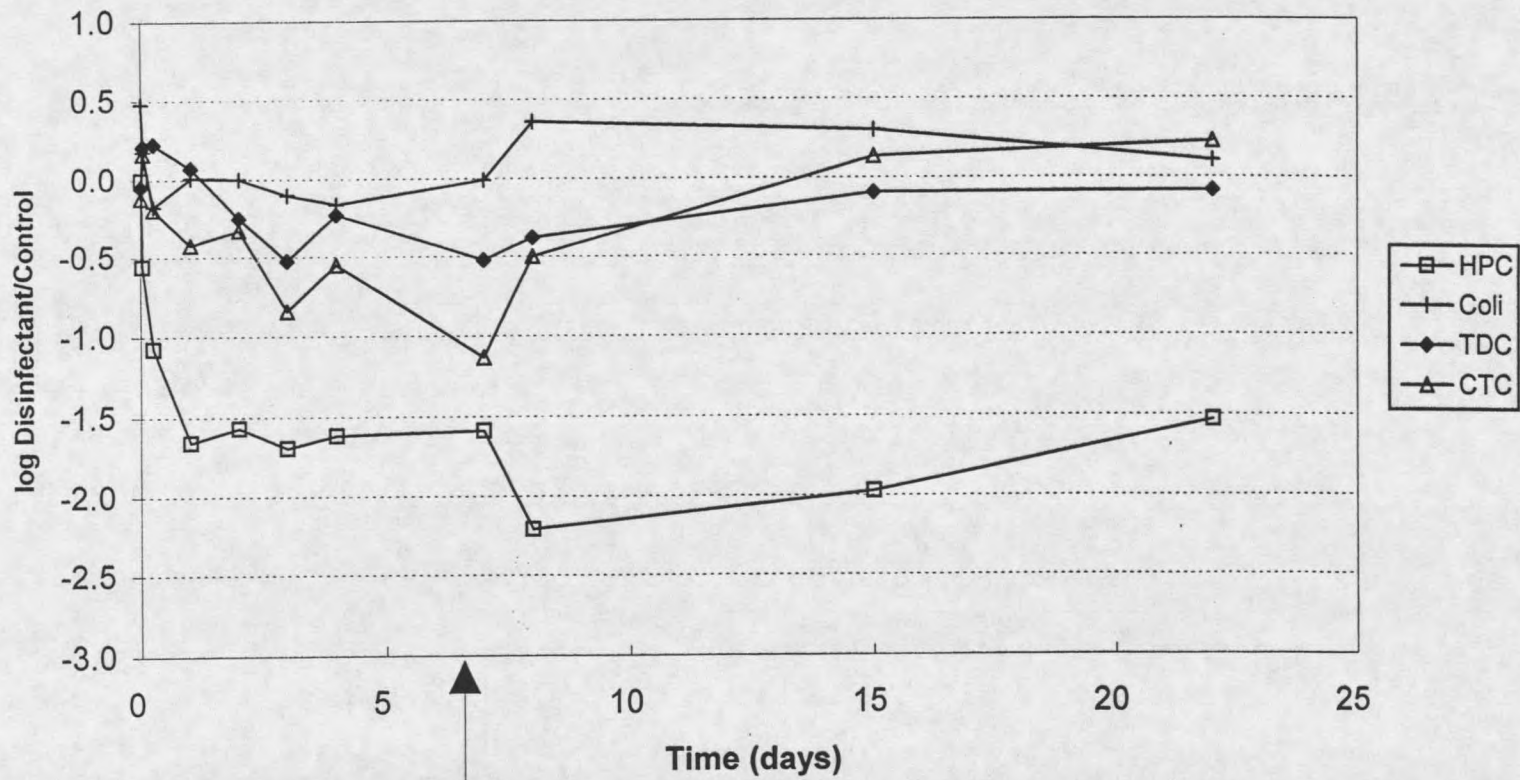


Figure 3.5. Ratio of biofilm organism numbers in parallel chlorinated and control polycarbonate reactors. Initial chlorine concentration was 0.2 mg/L was increased to 0.5 mg/L on day 7 (arrow). HPC is heterotrophs by plate count, coli is coliforms by plate count on mT7, TDC is total direct count using DAPI, and CTC represents respiring cells measured by CTC staining.

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## CHAPTER 4

PHYSICAL DISTRIBUTION SYSTEM MODELS FOR ASSESSING REGROWTH:  
PIPE LOOPS AND ANNULAR REACTORSBackground

Pilot-scale distribution facilities are typically constructed to provide a platform for determining the influence of water quality parameters on full-scale distribution system performance. This is required when access to the full scale system is limited, when the parameters to be tested may significantly alter the quality of water delivered to the consumer, or if more tightly controlled conditions are required (flow, water quality, pipe composition, etc.). Since pilot system results are extrapolated to the distribution system, it is critical that the reactors be designed to simulate relevant operational conditions, including shear stress, pipe materials, temperature, disinfectant types and concentrations, and other water quality parameters such as organic carbon concentrations. Coupons for sampling surfaces should be flush mounted to reduce perturbations in the local hydrodynamics. Ideally, the reactors should be relatively compact, easily controlled, have minimal water demand, and be inexpensive to construct, maintain, and operate. An additional consideration is the manner in which the pilot system can be mathematically modeled, i.e., either plug flow or completely mixed reactor behavior.

Historically, pilot systems have been designed to provide information on corrosion (Levin and Shock, 1991; Gardels and Sorg, 1989; Heumann, 1989; Birden, Calbrese and Stoddard, 1985; Treweek, et al., 1985; AWWARF/DVGW, 1985). In some instances, they have been constructed for the evaluation of various operational parameters on biofilm formation under conditions similar to those found in distribution systems (LeChevallier, Lowry and Lee, 1990; Haudidier, et al., 1988; Characklis, 1988). The majority of these reactors were operated in a plug-flow mode. Disadvantages of this configuration can be significant water usage and an inability to simulate different residence times at the same flow rate. Only the last two pilot systems were used to simulate residence times found in distribution systems under relevant flow conditions.

The system described by Haudidier, et al. (1988) was constructed in Nancy, France and consisted of six individual pipe loops composed of cement lined pipe. Each loop can be operated as a perfectly mixed reactor, or when operated in series, can simulate discrete residence times along a pipeline. Test coupons of a desired material are inserted flush to the inner pipe wall and removed to assess biomass accumulation or other desired parameters. Additionally, the bulk fluid from that loop can be sampled at the same residence time. Experiments can be designed to examine biofilm accumulation and detachment of bacteria; disinfectant decay and efficacy against biofilm and suspended organisms; and disinfection by-product formation. Advantages of this configuration are that (1) relevant distribution pipes (composition, diameter) were used, (2) residence time can be simulated independently of shear stress, (3) temperature can be controlled, (4) the individual loops are well-mixed and can be mathematically modeled as completely stirred

tank reactors (CSTRs), and (4) water quality can be altered to simulate conditions in actual distribution systems. Disadvantages are (1) size, (2) capital and operating cost, (3) limited number of coupons for analysis, and (4) the inability to change pipe materials with the exception of coupons.

As an alternative to pipe loops, Characklis (1988) and van der Wende and Characklis (1990) utilized annular reactors in series to simulate the hydraulic conditions found in a distribution system. Annular reactors consist of a stationary outer cylinder and a rotating inner cylinder with an annular space occupied by the water. Deposit/biofilm samples can be taken by removing coupons threaded into the outside wall of the annular reactors and bulk fluid samples from the effluent. If the residence time for each reactor is sufficiently short, the proliferation of bacteria in the bulk fluid can be ignored and the contribution of detached bacteria to the suspended population determined. Rotation of the inner drum controls shear stress, which can be scaled to that found in a circular pipe. Annular reactors are also well-mixed, and when staged, the entire series can simulate long residence times typical of municipal systems. Advantages of the annular reactors are: (1) the outer cylinder can be easily changed to test various pipe materials, (2) they can be completely cleaned between experiments, (3) they are more compact than pipe loops, (4) water use and chemical addition is minimal, (5) they are well mixed (Gjaltema, et al., 1995) and can be modeled individually as CSTRs, (6) residence time (dilution rate) in each reactor can be set independently from shear stress (rotation of inner drum) and (7) water quality can be amended as necessary to assess the importance of changes in water quality. Disadvantages include a much larger surface area to volume ratio than found in

pipes, and a lack of information on the correlation of results from these reactors to those from a pipe loop or distribution system. It is also possible to have heterogeneous distribution of biofilm on the surfaces, depending upon operational conditions (Gjaltema, et al., 1995).

Researchers at the Center for Biofilm Engineering at Montana State University designed and built both a parallel pipe loop system and a series of annular reactors to assess the relative importance of various water quality parameters and hydraulic residence time on biofilm accumulation and coliform regrowth potential. These reactor systems were designed based on the above criteria, with modifications made to simplify construction, operation, and interpretation of the results. An additional emphasis of the research was to directly compare the two systems to determine if the more compact annular reactors were an appropriate alternative model for piping in a distribution system. The following is a description of the two systems and an overview of results, especially those comparing the two types of reactors.

### System Description

Both reactor systems were constructed to permit experimentation under relatively well-defined conditions to assess the influence of hydraulic residence time, disinfectant type and dose, biodegradable organic carbon (measured as assimilable organic carbon), and temperature on the ability of coliforms to colonize and persist in biofilms. Such pilot scale systems were required since it is nearly impossible to adequately sample biofilms in full-scale distribution systems. We were fortunate to be allowed access to space and

water at the City of Bozeman Water Treatment Plant for our research platform (Fig. 4.1). The Bozeman plant treats high-quality surface water from two sources. The plant operates by direct filtration through mixed media (anthracite/sand) following ferric chloride coagulation. pH ranges from approximately 8.0 in the winter to 7.5 in summer. Alkalinity (as mg/L CaCO<sub>3</sub>) has a similar seasonal trend from 95 down to 60. Disinfection is by free chlorination. Water from the full-scale plant was removed from the clearwell and treated prior to use in the pilot distribution system.

An important variable in the experimental design was the amount of carbon substrate carbon added to the influent water of the pilot facilities. It was therefore necessary to remove the background concentrations of carbon from city water. This was accomplished by passing water from the clearwell through a granular activated carbon column to remove the chlorine. The column was a 1.83 m (6 ft) section of 15.25 cm (6 in) schedule 80 PVC pipe capped top and bottom with side ports at the top for influent and bottom for effluent. Influent pressure was regulated. Following dechlorination, the water was passed through two parallel swimming pool filters filled with biologically active carbon from the full-scale plant in Laval, Quebec, Canada. Two filters were constructed to permit sequential backwashing in case of significant head loss, but this has not been necessary in three years of continuous operation. Empty bed contact time was approximately fifteen minutes. Effluent from these filters consistently contained approximately 20 µg/L AOC as determined by the plate count method of van der Kooij (1982) as modified by LeChevallier, et al. (1993). This low AOC water flowed into an insulated drum where the water was amended with nitrogen and phosphorous. After

passing through a tankless flash heater, the water was used in the parallel pipe and annular reactor systems.

An important consideration in the construction of the pilot distribution system was the choice of pipe wall material. To simulate "worst case" conditions in distribution systems, highly corrodible mild steel was used. This choice is supported by information in the literature. For example, in a comparison between pipe materials, unlined cast iron supported the highest number of attached bacteria, while PVC was colonized the least (Neden, et al., 1992). Coliforms have been found associated with tubercles from actual distribution systems (LeChevallier, Babcock and Lee, 1987; Opheim, Grochowski and Smith, 1988). The importance of ferrous metals in supporting bacterial growth in distribution systems has been documented indirectly by the reduction or control of coliforms by implementation of a corrosion control scheme in some cases (Martin, et al., 1982; Lowther and Moser, 1984), but not in others (LeChevallier, Babcock and Lee, 1987). The pipe loops, annular reactors, and sampling coupons were constructed of mild steel.

### Pipe Loop

Five pipe loops were build to be operated independently or in series (representing sequential hydraulic residence times), depending on the experimental design. A total of five loops were constructed to permit a nested experimental design when the loops are operated independently. A simplified schematic of one loop is presented in Fig. 4.2.

Pipe material (welded seam thin wall mild steel) and diameter (10.16 cm; 4") were selected to represent relevant materials and surface area to volume ratios found in distribution systems, recognizing that there was a restriction on excessively large pipes. Pipe size was partially constrained by the intended support of the pipe loop system, which was on the struts of the existing filters at the treatment plant. Each loop was constructed of twenty sections, two feet in length. This configuration provided flexibility in case large surface areas were required for biofilm/deposit sampling, for ease of repair in case of leaks, and to permit cleaning between experiments. The individual pipe pieces were welded to flanges at each end, with care taken to ensure that there was a minimal gap between pipe pieces when the sections were secured together. Sections were bolted together with gasket material between the flanges to isolate the welds from the water as well as to seal the joint.

A significant component of the pipe loop system was the flush-mounted coupons of identical material. Four circular sections  $8.84 \text{ cm}^2$  ( $1.37 \text{ in.}^2$ ) were cut equidistant from each other in a straight line along the length of each two foot section, creating a total of eighty coupons per loop. The large number of coupons allows for extensive sampling over long experimental run times. Metal cut-outs were backed with rubber gaskets and secured to rectangular pieces of pipe of a slightly larger diameter with small bolts (flush with coupon on inside) and nuts (on exterior of backing piece). The original intent was to use adhesives, but no satisfactory compound was found. This configuration allowed the coupons to be flush-mounted with the pipe wall when inserted into the holes and held in place with metal hose clamps. The hose clamps also provided electrical contact with the

pipe sections. When the sections were connected, care was taken to ensure that all coupons were located in a straight line along the entire length with the midpoint of the coupon approximately  $10^\circ$  from vertical.

At the midpoint of each loop, one coupon was replaced with a spigot for fluid sampling and system draining. It was fastened to the hole via a gasket, rectangular coupon backing, and two hose clamps.

Return lines for each loop were constructed of 3.81 cm (1.5 inch) thin walled mild steel pipe to reduce both volume and weight. Valves were placed at the junction between the two sized pipes to isolate segments in case of repair or to permit partial emptying for coupon removal. When the system is in operation, both valves are completely open to reduce the chance for disturbances in the hydrodynamics. Immediately upstream of each four inch pipe loop section, an in-line flow meter was installed to measure flow velocity in the system. After the valve on the effluent end of the four inch line, a simulated dead-end section was installed into the 1.5 inch return line at a  $90^\circ$  angle. This two foot long section was tapped with ports for twelve standard 0.5" mild steel pipe plugs. The dead-end coupons were removed only at the end of an experimental period due to the shallow threads in the pipe material which made reinsertion difficult.

Each loop has a recycle and water storage tank consisting of a 55 gallon steel oil drum insulated on the exterior with spray-foam. The drums had the top removed and were pressure washed to remove residual oil before use. Water from the return line enters at the top of the barrel approximately 5 cm above the steady-state water level. The temperature in each drum is further controlled with a household water heater element or

cooling coil, depending on the experimental conditions. When operated independently, each drum also receives added carbon source or disinfectant. Dilution water is provided from a sixth water storage tank with pre-temperature control from a flash heater operated on a recycle. Peristaltic pumps supply the dilution water to each of the pipe loops. When cumulative residence time was investigated, the first loop received the added substrate and dilution water, and a portion of the effluent from the previous loop served as the influent dilution water for the next loop. The volume of the barrel (and of the entire pipe loop) is maintained constant at approximately 240 L (70 gal) by a gravity overflow opening 18 cm down from the top of the barrel. Approximately 5 cm from the bottom of each pipe loop storage tank, a fitting was installed for a pipe to remove water through a recirculating 3/4 hp bronze body swimming pool pump. A bypass loop with a valve was installed between the pump intake and discharge to control flow rate in the pipe sections. Overflow water from all the loops is collected in a separate plastic barrel, chlorinated using a floating swimming pool device with solid calcium hypochlorite, pumped to a second identical chlorination vessel, and returned to the head of the treatment plant.

### Annular Reactor

Annular reactors are so named because they consist of inner (rotating) and outer (stationary) cylinders forcing an annulus of fluid between. When equipped with a torque monitor to obtain measurements of hydraulic shear stress, they are called RotoTorques and have been described by Characklis (1990). The reactors designed for this research consisted of a stationary outer cylinder of 15.25 cm (6 in) mild steel and an inner drum of

high density polycarbonate and a total volume of one liter (Fig. 4.3). The inner cylinder contains four diagonal draft tubes to reduce the potential for the formation of Taylor vortices in the annular space by facilitating vertical mixing. It is rotated by means of a top mounted motor set at a speed to develop the desired shear stress. The outer cylinder contains twenty tapped coupons with an individual surface area of  $2.27 \text{ cm}^2$  (1.25 cm pipe plugs); the threads are covered with teflon tape to prevent leakage and facilitate removal as corrosion occurs (Fig. 4.4). Since completion of this research, the reactors have been redesigned and are available commercially. Improvements include a bottom drive, which significantly reduces the space requirements. The new coupons are encased in a nylon sleeve and held in place by a steel clip to facilitate removal.

Two "trains" each with four reactors were constructed. The trains are housed on separate wheeled tables in temperature controlled plastic tents.

Dilution water for the annular reactors is from the same source as that used for the pipe loops. When the reactors are operated independently, dilution water, substrate and/or disinfectant are fed separately to each parallel reactor via a peristaltic pump. Residence time is controlled by the influent volumetric flow rate and a gravity overflow. For hydraulic residence time experiments, four reactors were arranged in series (Fig. 4.5) with the first reactor receiving substrate and fresh dilution water, and subsequent reactors provided with effluent from the previous reactor. In this case, the two trains acted as replicates.

### System Operation

Since the pipe loop is operated with a high recycle, it can be modeled as a CSTR. The same modeling approach can be taken with the annular reactors. If the residence time in the reactors is less than the growth rate of the organisms, a bacterial cell balance across an annular reactor reflects biofilm processes of growth and detachment (Trulear and Characklis, 1982; Bakke, et al., 1984). The same can be said for the pipe loops. At longer system residence times, the cumulative processes of bacterial detachment and planktonic growth are simulated.

A significant advantage of the annular reactors and the high recycle pipe loop design is that hydraulic residence time can be set independently from shear stress. Hydraulic residence time can be easily determined for the two reactor types by knowing the volume of the system and the total influent volumetric flow rate. The scaling parameter used to set hydrodynamic conditions between the two types of reactors was shear stress. The flow in the larger pipe was set at 0.305 m/s (1 ft/s). The procedure for setting the rotational speed of the inner drum of the annular reactor is described briefly as follows.

The relationship between head loss in a pipe, its dimensions, the flow velocity, and the resulting mean shear stress on the wall were developed using the Darcy-Weisbach frictional head loss equation, the Moody diagram for friction factor, and a simple force balance on the fluid. The Reynolds number in a 4" diameter pipe with a mean velocity of 1 ft/sec at 20°C is 31,000. For smooth pipes and surface roughnesses up to about 0.0002,

the corresponding friction factor is about 0.024. Higher surface roughnesses (e.g., for mildly corroded surfaces) yield friction factors as high as 0.04. The Darcy-Weisbach frictional head loss equation,

$$\frac{h_f}{L} = \frac{fv^2}{2Dg} \quad (1)$$

is substituted into the force balance to produce a wall shear stress given by:

$$\tau_0 = \gamma \frac{h_f D}{4L} = \frac{\gamma v^2 f}{8g} \quad (2)$$

where  $D$  is the pipe diameter,  $L$  is the pipe length,  $f$  is the friction factor,  $v$  is the fluid velocity, and  $h_f$  is the head loss. Using the friction factors between 0.024 and 0.04, the resulting shear stress at the wall is between 0.27 Pa and 0.45 Pa. In the annular reactor, the shear stress at the outer wall is related to the torque applied to the outer cylinder by the moving fluid according to:

$$\tau = \frac{M}{RA} \quad (3)$$

where  $M$  is the moment,  $R$  is the radius of the outer cylinder, and  $A$  is the wetted surface area of the outer cylinder. For the dimensions of the annular reactors used in this study, a torque of between 0.00076 Nm and 0.00126 Nm corresponds to the shear stresses give above. Schlichting (1968) showed that the fluid dynamics of the rotating annular reactor

vary from laminar to turbulent according to the Taylor number analogous to the Reynolds number criteria. The Taylor Number is given by:

$$Ta = \frac{\omega R_i d}{\nu} \left( \frac{d}{R_i} \right)^{1/2} \quad (4)$$

where  $R_i$  is the radius of the inner cylinder,  $d$  is the dimension of the annular space,  $\omega$  is the angular velocity, and  $\nu$  is the kinematic viscosity. Further, a specific relationship between Taylor Number and a dimensionless torque coefficient was presented. Based on the analysis presented by Schlichting, moments in the range shown above produce Taylor numbers between 420 and 1260, which correspond with rotation speeds between 30 and 90 rpm. For this study, a rotational speed of 60 rpm was chosen.

A total of four experiments, each thirteen weeks in length, were carried out to evaluate the importance of various operational parameters on coliform regrowth. Between experiments, the reactors were thoroughly cleaned to remove corrosion products and provide as nearly identical starting conditions as possible for the next experiments. All coupons were removed from the pipe loops and beadblasted. The drums and pipes were scraped. The entire inner surface of the annular reactors were beadblasted as well. Although detailed methods and results are presented elsewhere (Camper, et al., 1995), a general overview of the experimental design is presented here since the data were used to assess the comparability of the two pilot systems.

Experiment 1 was designed to evaluate the importance of hydraulic residence time on regrowth. The pipe loops and annular reactors were operated in series to represent

cumulative residence times of 2, 4, 8, and 16 hours. Two loops were operated at the same residence time (2 hr), and received an influent concentration of 500  $\mu\text{g/L}$  (as carbon) substrate, and 100  $\mu\text{g/L}$  each potassium phosphate and sodium nitrate. No additions were made to subsequent loops. The reactors were initially inoculated with a known concentration of a mixture of five chemostat-grown coliforms, scaled to the total volume. Following inoculation, the attached coliforms were challenged with the autochthonous heterotrophic bacteria present in the dilution water. Coupons and bulk fluid samples were removed from the reactors weekly for the first five weeks and then every other week for a total of thirteen weeks. A portion of the water in the loop was withdrawn via the mid-loop valve and retained in a bucket before the coupons were removed. The water was replaced just after the coupons were replaced and flow resumed. For the first experiment, each loop was divided into five sections and a coupon selected at random along its length. On alternating weeks, duplicate coupons were sampled from the annular reactors. Annular reactor inner drums were removed during sampling to drop the water level sufficiently for coupon removal. The pipe loop and annular reactor coupons were scraped into sterile water, labeled to prevent inadvertent resampling, and replaced at the same location. The biofilm/deposit samples and the bulk fluid were then homogenized and the numbers of heterotrophs and coliforms determined in triplicate by the spread plate method on R2A and mT7 agar, respectively.

The second experiment was designed to determine the influence of carbon substrate loading and temperature on regrowth. All reactors were operated independently with a hydraulic residence time of two hours. Two loops received 500  $\mu\text{g/L}$  and water

temperature was maintained at 20°C. A third was operated with the same influent carbon but at 10°C, the fourth with no added carbon at 20°C, and the fifth with no carbon at 10°C. Duplicate annular reactors on each train received the same influent concentration of carbon. One train was housed in a 20°C tent, and the second at 10°C. Inoculation and sampling was identical to the first experiment with the exception that the loops were divided into three sections and one coupon per section sampled at each time point.

The third and fourth experiments used a residence time of two hours, a set temperature of 20°C, influent carbon concentration of 500 µg/L or no added carbon, and an influent chlorine concentration of 1 mg/L or no chlorine. Reactor configuration and sampling was the same as above, with the exception that one train of annular reactors received carbon and the other did not. Two reactors in each train were fed chlorine.

### Statistical Evaluations

Three replicate measurements were taken for the bacterial counts of the biofilm and effluent (bulk fluid) for each sample. The counts were converted into the number of organisms per square centimeter (biofilms) or per milliliter of water (fluids) and averaged. A second average was made for results from replicate samples from one reactor. To stabilize the variance of residuals, transformations of these numbers were required. Because the coliform counts have a variability that increased with the mean, Poisson distributions were assumed. For Poisson distributions, square root transformations are used. HPC data were the result of serial dilutions, and a  $\log_{10}$  transformation was used.

Statistical procedures were performed using the PROC GLM or PROC MIXED protocols in SAS© software (1992). Evaluations of the reproducibility of replicate reactors within an experiment and the comparison between annular reactors and pipe loops were made. Probabilities reported as  $p_{\text{bio}}$  for biofilms and  $p_{\text{bf}}$  for bulk fluids (effluents) were obtained from the analysis of variance tables produced by SAS©.

### Comparisons between Pipe Loop and Annular Reactors

#### Operational Comparisons

When costs of construction and operation are considered, the annular reactors are significantly less expensive. Labor and material amounts for the pipe loop system were approximately \$60,000. An unexpected expenditure was nearly \$5,000 per year for electricity if the loops were operated continually, primarily for the pumps. Individual annular reactors were \$4500 with an additional \$500 for each peristaltic pump.

Ease of sampling was comparable. The pipe loop required partial draining; in contrast, the inner drum was removed from the annular reactor. A potential advantage of the pipe loop coupon was its larger surface area.

Space requirements for the annular reactors were considerably less than that for the pipe loop. Use of a wheeled table also made them somewhat portable.

Maintenance and cleaning was generally more difficult for the pipe loops than the annular reactors. Exceptions were the necessity for calibrating very small volumetric flow rates for the annular reactors and the potential for their effluent lines to plug with corrosion products.

## Data Comparisons

Reproducibility within One Reactor. Literature information exists on the applicability of the CSTR model to the annular reactors (Trulear, 1983). Previous research for this project conducted in the laboratory also indicated that there was no difference in biofilm numbers with position in the reactor. To substantiate that there were no gradients in biofilm along the pipe loop length, five replicate samples per loop per sampling time were taken during the first experiment. Fig. 4.6 presents HPC biofilm results from one sampling time during the experiment. As can be seen, there was very little variability between HPC numbers on coupons with position along the pipe length. This trend was consistent throughout the experiment.

Reproducibility between Duplicate Reactors. An important component of the research was to demonstrate that there was statistical or practical reproducibility between replicate pipe loops or annular reactors. For all experiments, two loops were operated identically and either the two trains or duplicate annular reactors were run as pairs.

In the residence time experiment, reproducibility was tested by comparing data from pipe loops 1 and 2 and between individual reactors representing different residence times in the two parallel trains of annular reactors. For coliform counts, there was no significant difference between the two loops for both the biofilm and detached (effluent) bacteria ( $p > 0.50$ ) for any comparisons tested (Table 4.1). HPC counts were comparable between loops for the biofilm and bulk fluid data ( $p_{\text{bio}} = .13$  and  $p_{\text{bf}} = .87$ ). Comparison between annular reactors with the same residence time in the two parallel trains showed

no significant difference in coliform and HPC biofilm numbers ( $p > 0.10$ ). Similarity in bulk fluid counts was less significant with  $p$  values between 0.05 and 0.06. This was because one train typically had higher HPC counts and lower coliforms than the other train.

Table 4.1. Residence time (Experiment 1)  $p$ -values for reproducibility in pipe loops and annular reactors for entire 13 week run time

	<i>(coliform)<sup>1/2</sup></i>		<i>log(HPC)</i>	
	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid
Loops	.8240	.6727	.1286	.8690
Annular Reactors	.2225	.0519	.1344	.0517

The remainder of the experiments had a residence time set to two hours, and all reactors were operated in parallel rather than in series. Again, two loops were operated identically but individual annular reactors were run in pairs. A description of the statistical analyses (Table 4.2) for the coliforms and HPC are presented in separate paragraphs.

In the substrate/temperature experiment, one replicate loop consistently contained higher biofilm coliform numbers, but the counts between loops were highly correlated. When the loops were run for the substrate/chlorine tests, there were no significant differences in coliform biofilm data for the replicate loops in both experiments. The bulk fluid coliform counts in all three trials were extremely low, and no statistical evaluation

could be made. However, it can be said that there is no practical evidence for a lack of reproducibility. In replicate annular reactor biofilm and bulk fluid coliform counts in the substrate/temperature investigation, there were differences ( $p_{\text{bio}} = 0.03$  and  $p_{\text{bf}} = 0.04$ ). Practically, however, the differences between the four biofilm and four effluent pairs are  $< 1.3$  fold (Camper, et al., 1995). In the two experiments using substrate/chlorine levels as the variables, all coliform p-values were  $> 0.19$ , indicating no difference between annular reactor pairs either for biofilm or suspended bacteria.

Table 4.2. p-values for the pilot system from three 13 week experiments all operated with a two hour residence time

Effect	Pipe Loop Data				Annular Reactor Data			
	$(\text{coliform})^{1/2}$		$\log(\text{HPC})$		$(\text{coliform})^{1/2}$		$\log(\text{HPC})$	
	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid
C/T <sup>a</sup>	.0114	.7046	.0200	.2622	.0283	.0402	.8835	.0763
C/Cl <sup>b</sup>	.3212	.1907	.2619	.0124	.1903	.6553	.3497	.3028
C/Cl <sup>c</sup>	.7566	.7370	.2584	.2798	.9549	.8232	.1148	.5717

<sup>a</sup>Carbon Substrate/Temperature Experiment (Exp. #2)

<sup>b</sup>Carbon Substrate/Chlorine Experiment (Exp. #3)

<sup>c</sup>Replicate Carbon Substrate/Chlorine Experiment (Exp. #4)

With two exceptions, there were no significant differences between replicate loops or replicate annular reactors in terms of biofilm and bulk fluid HPC for the three 2 hour residence time experiments (all p values  $> 0.075$ ). One exception was in the

substrate/temperature experiment when p value for biofilm HPC in the replicate loops was 0.02. The number in one loop was higher than the other, but in contrast to that observed with the coliforms, the trend was not consistent. The second exception was in the pipe loop bulk fluid HPC data from the first substrate/chlorine experiment ( $p_{bf} = 0.01$ ).

Comparisons between Annular Reactors and Pipe Loops. The reactor effluent and biofilm HPC and coliform data from all experiments were compared to determine if there was a statistical difference in the same measured parameters between the two reactor types. Although the qualitative results were the same (Camper, et al., 1995), there were consistently larger numbers of both organisms in biofilms and effluents of the annular reactors. To provide quantitative comparisons, coliform and HPC biofilm and bulk fluid data from the three constant residence time experiments were segregated into chlorinated and non-chlorinated sets. Regressions were performed to calculate the ratio of all annular reactor numbers in a set to all pipe loop data in the same set. The results are shown in Table 4.3. For chlorinated systems, a ratio of approximately 4:1 for annular reactor vs. pipe loop coliforms was observed for both the biofilms and bulk fluid. The  $r^2$  value was 0.84, indicating that this trend was also consistent between and for the duration of the experiments. The ratio for the non-chlorinated bulk fluid coliforms was less than one (0.58) with a low  $r^2$  (0.51) indicating considerable scatter in the data. In contrast, the biofilm coliform ratio for the two reactor systems was nearly the same for the chlorinated and non-chlorinated sets (3.55 with  $r^2 = 0.81$ ). The comparison between reactors based

on HPC counts were relatively constant for all data sets. These varied between 11.8 to 15.3 regardless of whether the organisms were from the biofilm or bulk fluid or if chlorine was present. These ratios are considerably higher than those for the coliforms; the HPC comparison is approximately 10:1 while that for coliforms is 4:1.

Table 4.3. Proportionality constants (annular reactor:pipe loop ratio) for bulk fluid and biofilm samples of both populations. p-values were all less than 0.01. Correlation coefficients ( $r^2$ ) are given for coliforms, while standard errors (on the logarithmic scale) are given for the HPCs.

System (population)	proportionality			$r^2$ (coli) or S.E. (HPC)		
	Cl <sub>2</sub> +	Cl <sub>2</sub> -	both	Cl <sub>2</sub> +	Cl <sub>2</sub> -	both
Bulk Fluid (coli)	4.88	0.58	2.78	.85	.16	.55
Biofilm (coli)	4.20	3.55	3.88	.84	.78	.81
Combined (coli)	4.46	2.75	3.60	.84	.63	.74
Bulk Fluid (HPC)	15.3	12.1	13.1	.026	.013	.013
Biofilm (HPC)	11.8	12.5	12.2	.010	.011	.008
Combined (HPC)	13.4	12.3	12.7	.016	.008	.008

No immediate answer is available to determine the cause of the higher counts in the annular reactor in these experiments. Research is planned to address this observation. It is interesting to note that in another investigation where the dry mass accumulation in an annular reactor was related to that of a 1.27 cm (0.5 in) stainless pipe, a correlation existed on a one to one basis with an  $r^2$  value of 0.94 (Roe, et al., 1994). Regardless of

the quantitative difference, the two reactor types did exhibit similar qualitative responses to changes in temperature, substrate, and chlorine.

### Conclusions

A goal of our research plan was to develop and compare reactors that could be used to assess the impact of operational and environmental variables on bacterial regrowth in distribution systems. Two distribution system physical models, a five loop pipe loop system and two identical trains of four annular reactors, were constructed for this purpose.

The pilot distribution systems have been in service for three years, and data from several experiments have been compared. Both reactor types are well mixed, and it is possible to set shear stress set independently from residence time. Each has flush-mounted coupons for deposit analysis. Cumulative hydraulic residence time can be simulated by operating individual loops or reactors in series. The annular reactors have the lowest capital and maintenance costs, and in general are easier to operate. Additionally, they are more compact and have lower water requirements than the pipe loops. They are more flexible, in that different types of pipe surfaces can be compared by changing the outer cylinder. A potential disadvantage is the large surface to volume ratio, which is approximately 17 times greater than in the pipe loop. Consequently, results from disinfection reaction experiments conducted in the annular reactors should be interpreted with caution. Advantages of the pipe loops are a more realistic surface area to volume ratio when compared to an actual distribution system, larger coupon surface area

for sampling, more coupons, and easier temperature control. However, their large size, capital cost, and operational costs will be prohibitive for most utilities and research groups.

When data from four long-term experiments were compared, it was found that there was good reproducibility in biofilm and reactor effluent bacterial counts in replicate pipe loops or annular reactors. Therefore, the choice of reactor is not limited by the ability to replicate results. Even though the actual counts in biofilms and effluents from the annular reactors were approximately ten fold higher for heterotrophs and four times greater for coliforms in biofilms and effluents from the annular reactors, the qualitative response (trends) of the bacteria to operational conditions tested simultaneously in both systems were comparable.

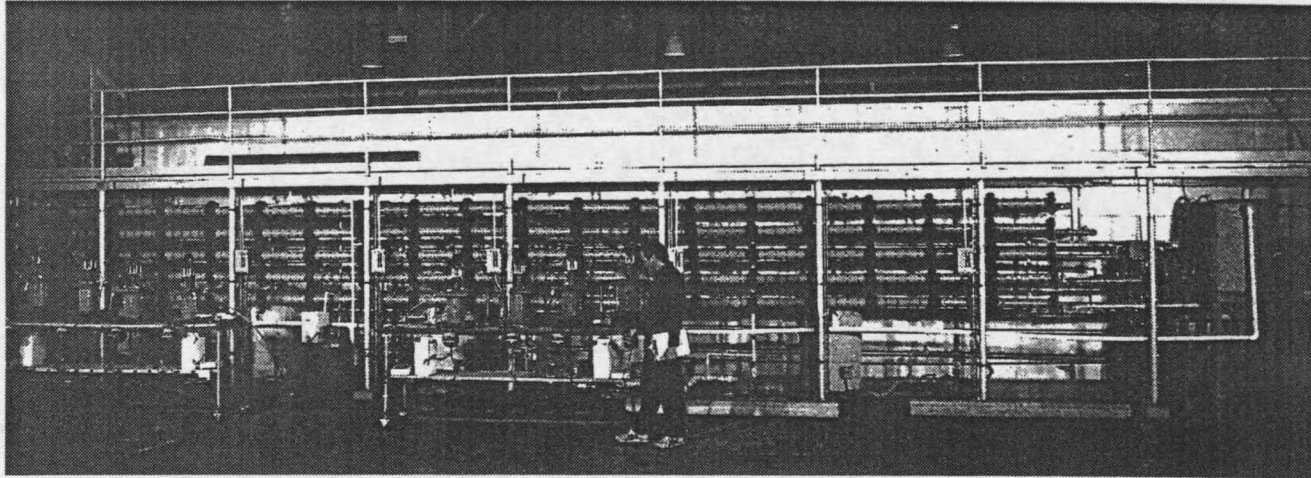


Figure 4.1 Photograph of the pipeloops and two trains of annular reactors at the City of Bozeman Water Treatment Plant.

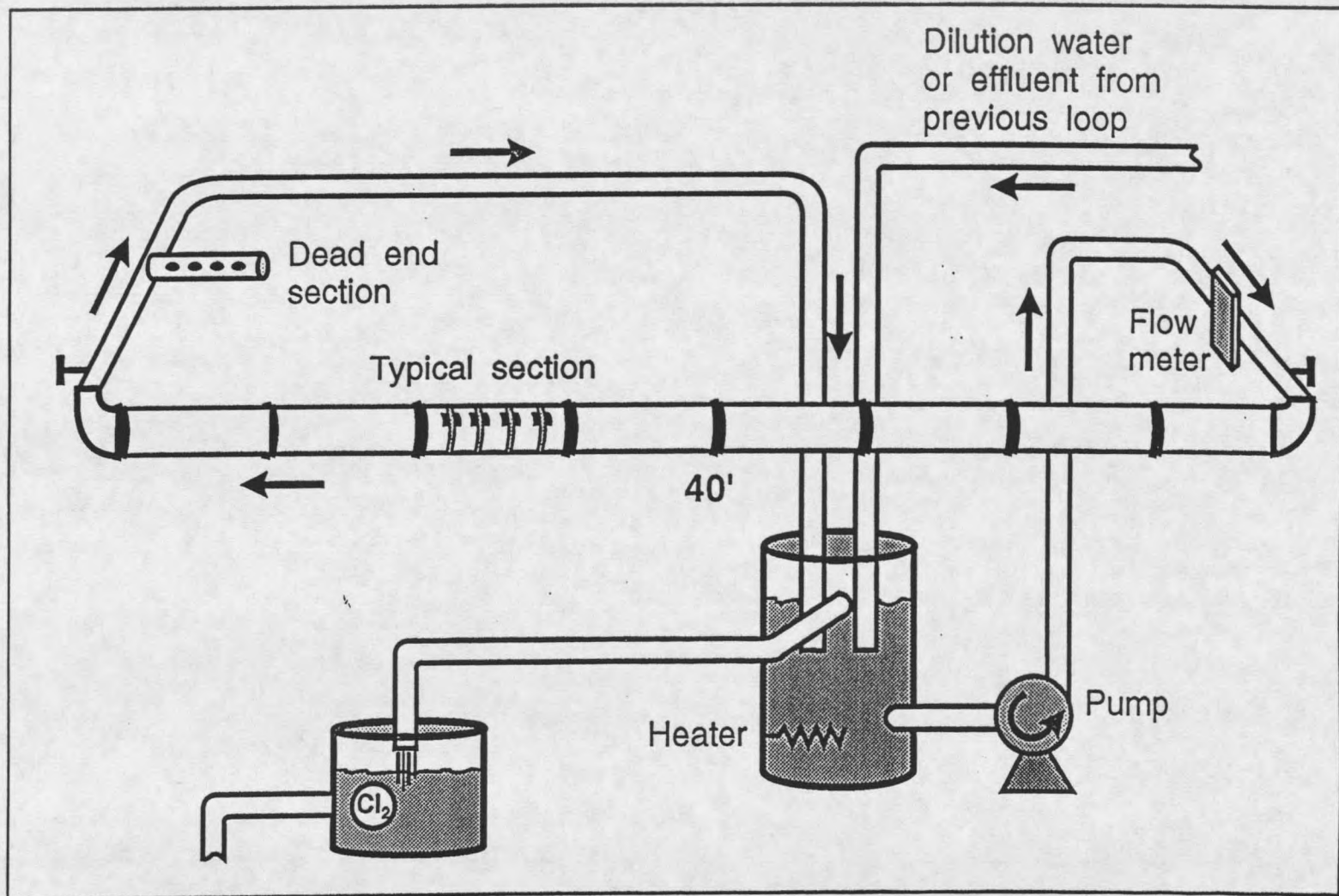


Figure 4.2. Schematic of one pipe loop.

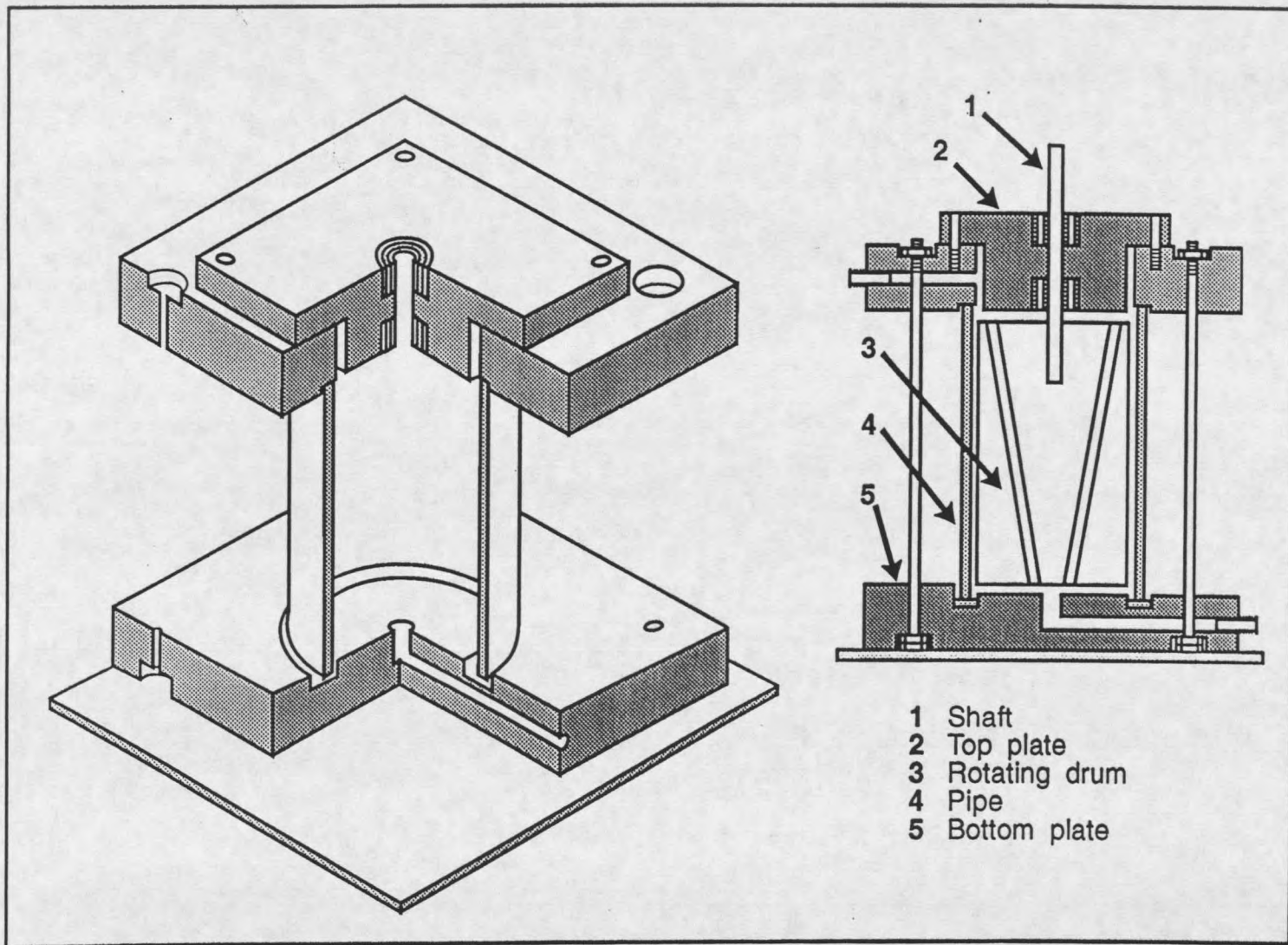


Figure 4.3. Schematic of the annular reactor.

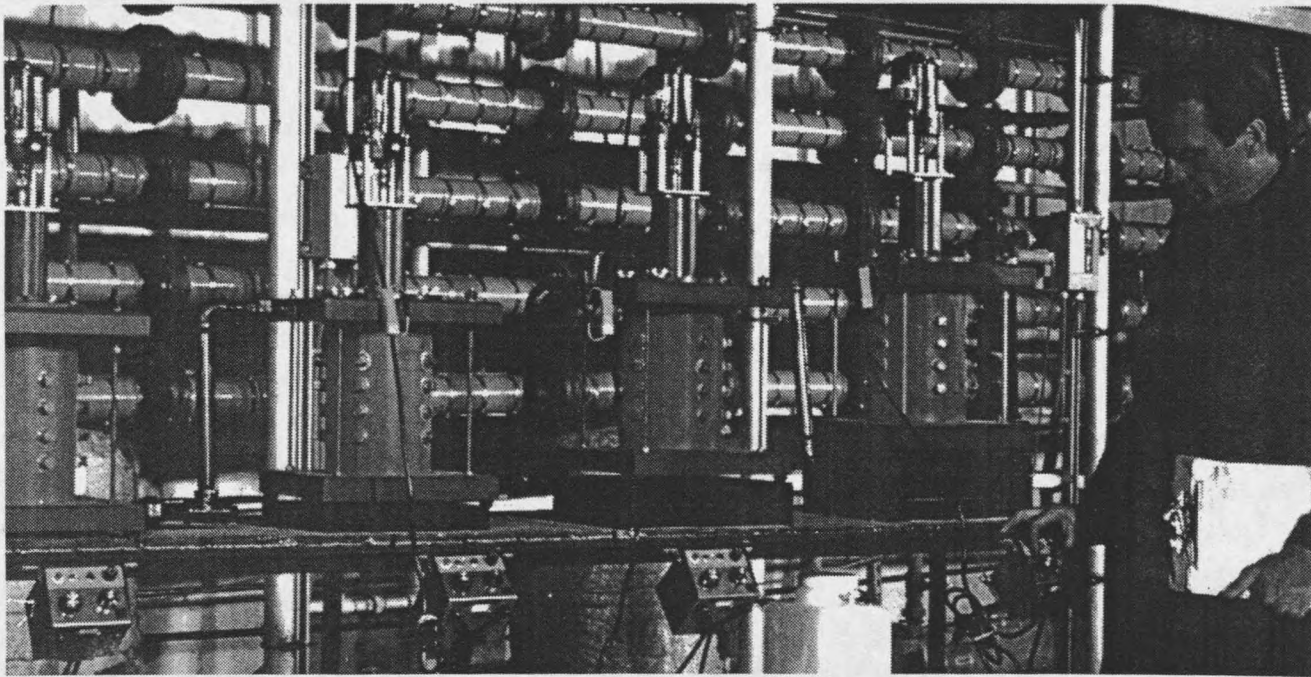


Figure 4.4 Photograph of the annular reactors at the City of Bozeman Water Treatment Plant.

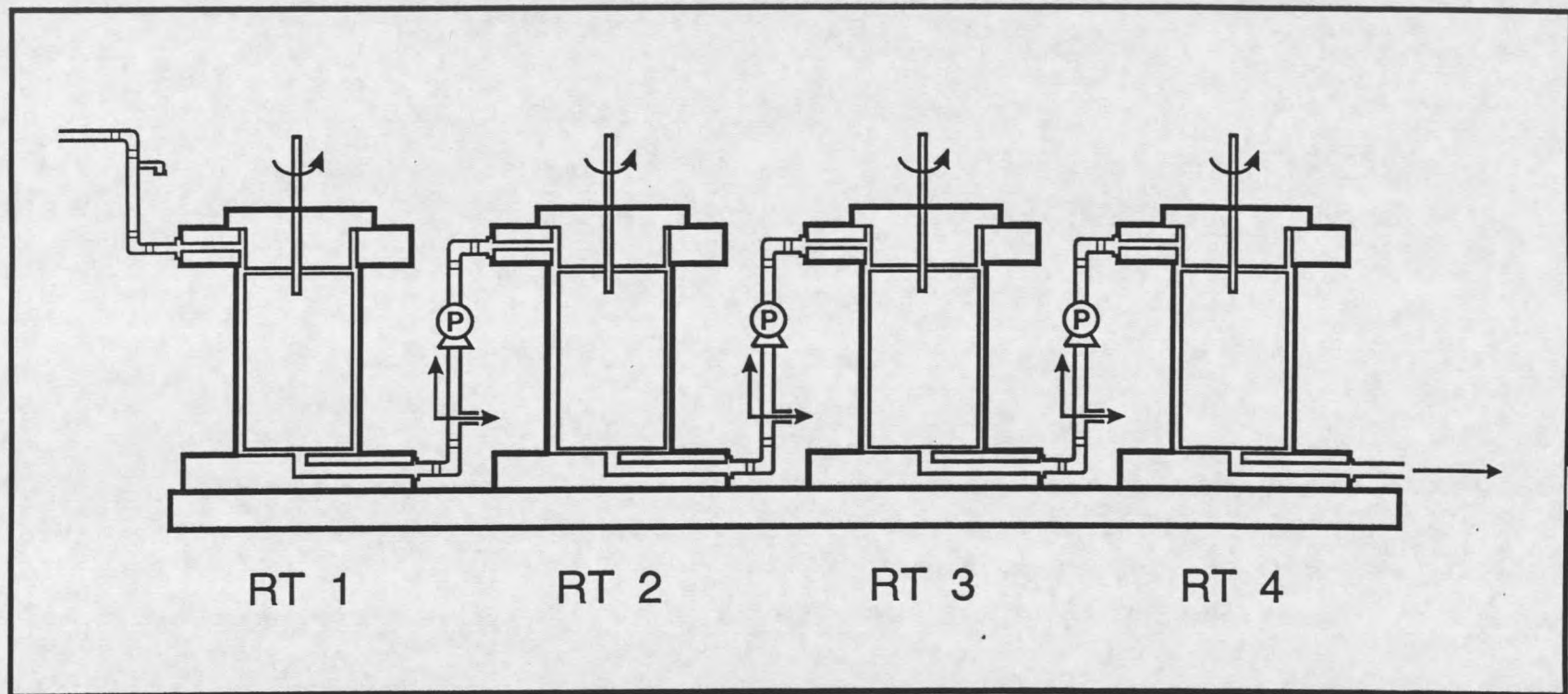


Figure 4.5. Schematic of annular reactors in series.

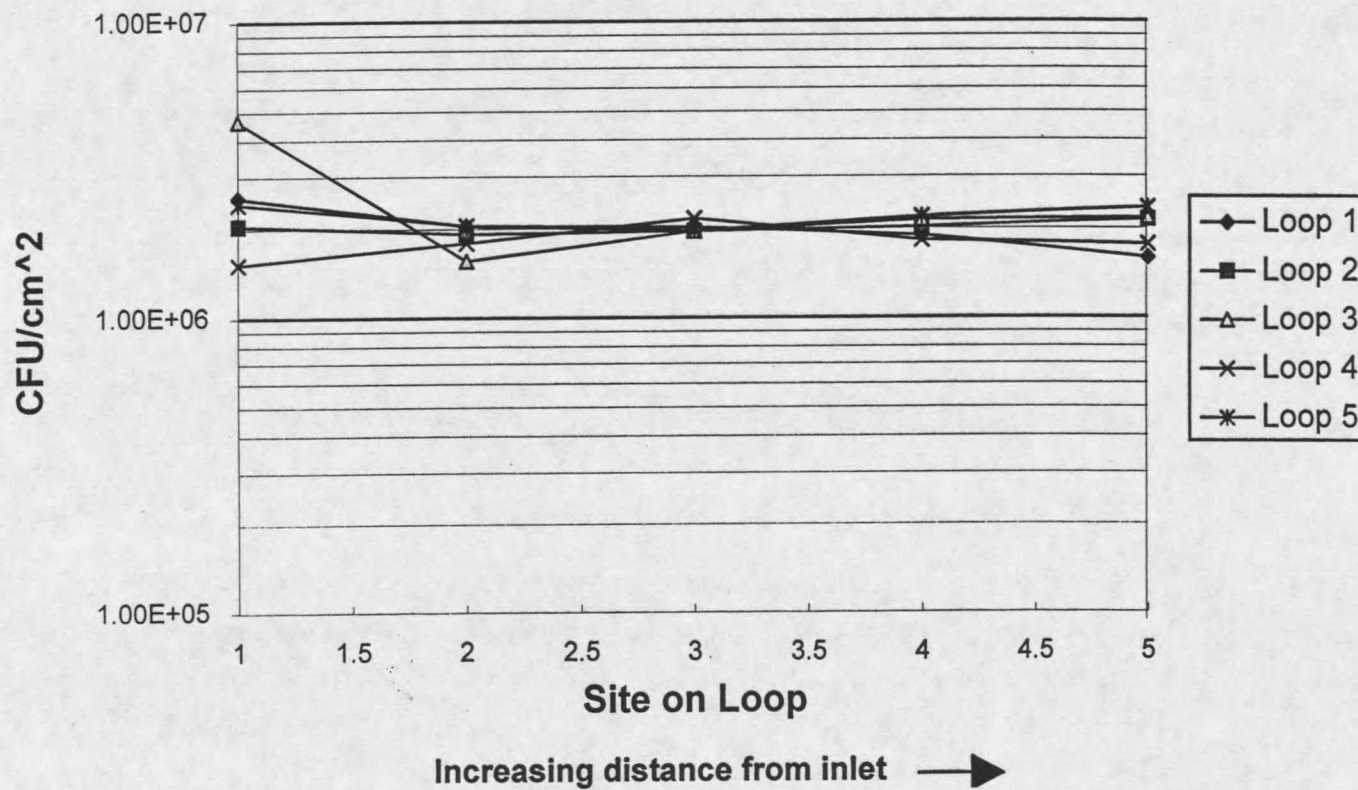


Figure 4.6. Variation in biofilm heterotrophic plate counts within loops after seven weeks run time. The loops were divided into five eight-foot sections with one coupon removed randomly per section. Site 1 is the influent end, while site 5 is at the effluent. Cumulative hydraulic residence times in loops were 2 hr (Loops 1 and 2), 4 hr (Loop 3), 8 hr (Loop 4) and 16 hr (Loop 5).

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## CHAPTER 5

INFLUENCE OF WATER QUALITY PARAMETERS ON BIOFILM GROWTH  
IN MODEL DRINKING WATER DISTRIBUTION SYSTEMSIntroduction

The appearance of coliform bacteria in finished drinking water when there has been no breach in system integrity or breakthrough from treatment has been termed regrowth. It has been presumed that regrowth is due to the replication and subsequent detachment of bacteria from biofilms present on pipe walls and sediments in the distribution system, since there is insufficient substrate present in water to support planktonic growth (Characklis, 1988; van der Wende, Characklis and Smith, 1989; van der Wende and Characklis, 1990; Haudidier, et al., 1988). Proving that coliforms arise from biofilms has been difficult. In most cases when distribution system pipe materials have been analyzed for the presence of coliforms, the results have been negative (Characklis, 1988). Emde, et al. (1992) found the coliforms *Escherichia coli*, *Enterobacter aerogenes*, and *Klebsiella* spp. in iron tubercles from a distribution system pipe surface. When one distribution system was intensively studied, LeChevallier, Babcock and Lee (1987) found coliforms in iron tubercles and ascribed a regrowth event to them.

Regrowth has been attributed to a host of variables including: (1) temperature effects, especially warm water conditions; (2) the amount of utilizable carbon for substrate; (3) inefficiencies in the removal/disinfection of organisms in treatment; (4) the presence of corrosion products in distribution systems; (5) disinfectant dose/type; and (6) distribution system hydrodynamics (Smith, Hess and Hubbs, 1990). These topics have been discussed in review articles by Camper (1994), LeChevallier (1990) and Block (1990).

Hydraulic residence time may be an important factor in determining which portions of the distribution system may be prone to regrowth since the concentration of growth-limiting substrates should be highest leaving the treatment plant. Previous research has shown that the concentrations of assimilable organic carbon (LeChevallier, Babcock and Lee, 1987; LeChevallier, Schulz and Lee, 1987; van der Kooij, 1992) decrease as water is transported through a distribution system. In a pipe loop system, it was shown that the biodegradable organic carbon concentration was depleted in 24 hr (Levi and Joret, 1991). Using sequential pipe loops, Haudidier, et al. (1988) demonstrated that the highest biomass accumulation and greatest carbon substrate depletion occurred in the first loop (shortest residence time of 40 hr). In distribution systems the growth due to the presence of substrate is balanced with disinfectant concentrations, which also tend to be highest leaving the plant. This was demonstrated in a staged annular reactor pilot system, where the amount of biofilm that accumulated in sequential reactors (representing increasing discrete residence times) depended on initial

chlorine concentration (Characklis, 1988; van der Wende, Characklis and Grochowski, 1988). When no chlorine was present, the biofilm was most abundant in the first reactor.

A typical response to a regrowth event is to raise the chlorine concentration. In several reported cases, this has been inadequate to control either biofilms or coliforms (LeChevallier, Babcock and Lee, 1987; Martin, et al., 1982; Reilly and Kippin 1983; Oliveri, et al., 1985; Ludwing, 1985; Centers for Disease Control, 1985; Hudson, Hankins and Battaglia, 1983), even at extremely high concentrations of 12 mg/L (Earnhardt, 1981; Lowther and Moser, 1985). Ludwing (1985) found no correlation between free chlorine concentrations and biofilm heterotrophs in a distribution system.

Temperature is also believed to be an important variable in regrowth events. Utilities perceive that they are at higher risk during the summer when water temperatures are highest. It has been reported that coliform regrowth is associated with water temperatures above 15° C (LeChevallier, Schulz and Lee, 1991) or between 14° and 20° C (Colbourne, et al., 1991). A direct relationship between biofilm heterotroph numbers and water temperature has also been shown (Donlan and Pipes, 1988).

The water quality parameter most commonly believed to be associated with regrowth events is biodegradable organic carbon (BOM) measured either as BDOC or AOC. The concentration of BOM in finished water is dependent upon source water, and varies from 3 to >1000 µg/L (Bouwer and Crowe, 1988; LeChevallier, Schulz and Lee, 1991; Servais, Billen and Hascoet, 1987). In Dutch systems, van der Kooij (1992) found a concentration of 10 µg/L AOC to be limiting to heterotrophs. At a New Jersey site,

LeChevallier, Schulz and Lee (1991) reported that AOC levels greater than 50  $\mu\text{g/L}$  were associated with coliform regrowth.

Determining the cause-and-effect relationship between variables and coliform regrowth in full-scale distribution systems is difficult for a variety of reasons. First, regrowth events are sporadic and difficult to predict. Obtaining pipe samples is expensive and the number of samples is limited. It is often impossible to separate variables; for instance, when water temperatures are high, the amount of utilizable carbon is often elevated as well. Perhaps most importantly, distribution systems cannot typically be operated to test a hypothesis.

To provide a platform for regrowth experimentation under controlled conditions, two pilot scale distribution systems were constructed at the City of Bozeman Water Treatment Plant. These reactors were designed to be operated under relevant drinking water distribution system conditions and to provide information on suspended organisms, substrates, and disinfectant concentrations as well as biofilms.

Key variables selected for study included hydraulic residence time, temperature, utilizable carbon concentration, and chlorination. Two variables, temperature and residence time, cannot be controlled by a utility. Utilizable carbon and chlorine concentrations, however, can be manipulated through changes in treatment practice or dosing. These variables were selected on the basis of discussions with water treatment personnel as well as literature information.

## Materials and Methods

### Reactors

Two mild steel pilot systems were constructed to simulate conditions found in drinking water distribution systems. These systems have been described in detail elsewhere (Camper, et al., 1995b); a brief discussion is provided here.

The first reactor is a pipe loop consisting of five 12.18 m (40 ft) sections of 10.16 cm (4 in) pipe. Each pipe contains eighty flush mounted coupons for biofilm sampling, a recycle tank, and temperature control. The second reactor system consisted of four annular reactors, constructed with flush-mounted coupons on an outer cylinder of 15.25 cm (6 in) mild steel. Four reactors were housed together in a temperature-controlled plastic tent.

Individual pipe loops or annular reactors can be modeled as continuous flow stirred tank reactors, while pipe loops or annular reactors in series simulate plug flow. If dilution rate in the reactors is greater than the growth rate of the organisms, the biofilm processes of growth and detachment will govern organism numbers in the bulk water. Shear stress in the annular reactors is controlled by the rotational speed of the inner cylinder, while the recycle flow rate determines shear in the pipe. In both systems, the addition of make-up water regulates residence time.

Dilution water is provided to both systems from two biological contactors designed to remove the background BOM from dechlorinated Bozeman domestic water. A tank upstream from both types of reactors received dilution water that was

supplemented with 100 µg/L sodium nitrate and 100 µg/L potassium phosphate to ensure that the microbial population would not be nitrogen or phosphorous limited. Ammonia was not used due to its reactivity with chlorine. This water was pumped via vane pumps to each barrel of the pipe loop and a holding tank for the annular reactors. The annular reactors were supplied with dilution water with peristaltic pumps. When used, carbon substrate and/or disinfectant were added to the drums of the pipe loops or individual annular reactors with peristaltic pumps.

With the exception of one experiment, the reactors were operated individually with a hydraulic residence time of two hours. The exception was a residence time experiment, where two sets of four annular reactors and four of the pipe loops were run in series. In this instance, a portion of the effluent from one annular reactor or pipe loop was used as the influent for the subsequent reactor. Cumulative residence times were 2, 4, 8, and 16 hrs.

Between experimental runs, the reactors were completely cleaned. The coupons were removed from the pipe loop and bead blasted. The annular reactors were disassembled and the outer cylinder bead blasted. All tubing was replaced and the flow rates recalibrated.

### Organisms

The coliforms (*Klebsiella pneumoniae*, *K. oxytoca*, *Enterobacter cloacae*, *E. aerogenes*, *Escherichia coli*) used in these experiments were originally isolated from distribution systems during regrowth events and were stored at -70° C in 2% peptone 20%

glycerol. Each organism was checked for purity on R2A agar prior to inoculation of starter cultures.

The organisms were inoculated individually into 100 ml mineral salts solution containing 1000  $\mu\text{g/L}$  each of sodium nitrate and potassium phosphate and 5000  $\mu\text{gC/L}$  of an assimilable organic carbon solution composed of equimolar concentrations on the basis of carbon of ethyl alcohol, propionaldehyde, parahydroxybenzoic acid, benzoic acid, and acetate. After 48 hr incubation at 20° C, the cultures were added to a sterile 20 L chemostat with an air sparger. The chemostat was filled with mineral salts containing 5000  $\mu\text{g/L}$  carbon and operated with this as the influent for at least three 20 hr residence times prior to inoculating the pilot reactors. Final cell numbers in the chemostat were approximately  $10^6$  cfu/ml.

#### Pilot Plant Inoculation

After the reactors had been cleaned, they were operated for one week with unsupplemented filtered water to inoculate the surfaces with heterotrophs. The dilution water consistently contained ca.  $10^3$  heterotrophs/ml as determined by the spread plate technique on R2A agar incubated at 20° C for one week. Just prior to addition of the coliform suspension, the pumps supplying dilution water were turned off. Fifty ml of annular reactor volume were removed and replaced with the coliform suspension and 3.9 L were added to each pipe loop recycle barrel. The systems were run batch-wise for 24 hr. At the end of the incubation period, the dilution water supply was resumed. When the reactors were supplemented with AOC, addition began at this time.

### Sampling Techniques and Measurements

During operation of the pilot plant, temperature, flow rates, and disinfectant concentration were routinely monitored. Chlorine levels were analyzed using a Hach kit by the DPD colorimetric method.

Deposit/biofilm and bulk fluid samples were collected on weeks 1, 2, 3, 4, 5, 7, 9, 11, and 13. During the first experiment, a coupon was removed at random from five segments in each loop. This was reduced to three segments and coupons in later experiments. Either single or duplicate coupons were removed from the annular reactors. Coupons were scraped into 10 ml of sterile water at the pilot plant and transported to the laboratory. The coupons were labeled to prevent inadvertent resampling and reinstalled in the loops or annular reactors. Influent and bulk fluid samples were taken from the loops and annular reactors. In the systems fed chlorine, the effluent and coupon samples received sodium thiosulfate.

During the final sampling on week 13, dead end coupons from the loops were extracted and coupons that had been sampled the first week were resampled. The latter analysis provided an opportunity to determine the extent to which the surfaces could be recolonized by coliforms that had been released from the existing biofilm, rather than from the initial inoculum.

Presence of substantial corrosion products in the coupon scrapings precluded the use of direct microscopy. Therefore, all bacterial enumerations were performed using plate counts. Following homogenization to disperse the bacteria, appropriate dilutions were applied to triplicate R2A (heterotrophs) and mT7 (coliform) agar plates. The R2A

plates were incubated for one week at room temperature and the mT7 plates for 24 hr at 35° C. Colonies were counted, averaged, and reported as cfu/ml (effluent and influent samples) or cfu/cm<sup>2</sup> (biofilm samples).

### AOC Analyses

Concentration of assimilable organic carbon in the pilot plant influent (following the biological filters) and in each of the reactors was determined whenever coupons were removed. The plate count method of van der Kooij (1982) as modified by LeChevallier, et al. (1993a) was used.

### Statistical Evaluations

Data from the triplicate plate counts for each observation were averaged, and in the case of coupon analyses where duplicate, triplicate, or quintuplicate samples were taken in one reactor, these were also averaged. These averages were then transformed to stabilize the variance. Since ten-fold serial dilutions were used for the HPC counts, a  $\log_{10}$  transformation was used. The coliform counts exhibited variability that increased with the mean, suggesting a Poisson distribution. Therefore, a square root transformation was applied.

The statistical procedures PROC GLM or PROC MIXED in the SAS© statistical software were used to analyze the data. For more information, see SAS©/STAT User's Guide Volume 2 (1992). p values were determined from the analysis of variance tables produced by SAS©. Biofilm and bulk fluid probabilities were assigned the nomenclature  $p_{\text{bio}}$  and  $p_{\text{bf}}$  respectively.

### Experimental Design

A total of four separate experiments were completed to evaluate the effects of variables on suspended and biofilm organism numbers. The first investigated the importance of hydraulic residence time and the associated decrease in substrate, the second the influence of temperature and substrate loading, and the third and fourth the interaction of substrate loading and chlorine.

For the residence time experiment, the first loop was operated with a two hour hydraulic residence time and received 500  $\mu\text{g/L}$  carbon substrate. Effluent water from the first loop was fed to the second loop for a cumulative residence time of four hours. The third and fourth reactors represented accumulative system hydraulic residence times of 8 and 16 hr, respectively. Two loops at the shortest residence time were operated as replicates. Two replicate trains containing four annular reactors in series with identical residence times were assembled. Temperature was set at 20° C.

For the substrate/temperature experiment, reactors and loops were operated independently with a residence time of two hours. Again, two loops acted as internal replicates and received 500  $\mu\text{g/L}$  substrate and were maintained at 20° C. The third loop was at 20° C with no added substrate, the fourth at 10° C with no added substrate, and the fifth was fed 500  $\mu\text{g/L}$  substrate and held at 10° C. One set of four annular reactors was housed in a tent controlled at 10° C and the second set at 20° C. Two reactors in each tent were fed 500  $\mu\text{g/L}$  substrate and the remaining two were unsupplemented.

When the influence of substrate loading and chlorine was evaluated, the reactors were run independently with a residence time of 2 hr. Temperature was constant at 20° C.

Two pipe loops were fed 500  $\mu\text{g C/L}$  and 1 mg/L free chlorine, one received 1 mg/L chlorine and no substrate, the third had no chlorine and 500  $\mu\text{g C/L}$ , and the fourth was unsupplemented. As in the substrate/temperature experiment, two sets of annular reactors were run; one set received carbon and the other did not. In each set, two reactors were chlorinated (1 mg/L) and two were not. Chlorination was begun immediately after the first week's sampling. Twenty-four hours later, an additional set of samples were taken to determine the short-term effect of chlorination on the suspended and biofilm populations.

### Results

As reported elsewhere (Camper et al., 1995b), the response of the bacteria in both types of reactors were qualitatively similar, but differed quantitatively. The annular reactors supported approximately ten-fold more heterotrophs in the bulk fluid and biofilms than the pipe loops. Coliforms were enhanced four-fold. Actual numbers of heterotrophs in the biofilms were ca.  $10^6$  (pipe loops) to  $10^7$  cfu/cm<sup>2</sup> (annular reactors). In general, the bulk fluid heterotrophic plate counts were an order of magnitude lower than those on the surfaces. The following results are from both types of reactors.

Extensive statistical evaluations of the data were performed for each of the four experiments. These included the influence of individual variables, interaction of the variables, interaction of variables with the type of reactor, and the interaction of the variables with real time (weeks). Many of these comparisons varied widely in their

statistical significance. Therefore, only results that have a phenomenological basis or provide additional information in explaining important findings are presented.

### Residence Time

The residence time experiments were developed to determine if coliform proliferation was favored at shorter hydraulic residence times when substrate concentrations should be highest. Under conditions where no chlorine is used, it is reasonable to assume that the readily available carbon substrates should be utilized rapidly, resulting in decreased concentrations of the limiting substrate with travel time in the pipe. Also of interest was whether the concentration of heterotrophs and coliforms in the bulk fluid increased with residence time which would occur if bacteria were continually detaching from the biofilm without significant reattachment or lysis.

The first hypothesis tested was that there was no influence of residence time on biofilm and suspended heterotroph and coliform counts. Statistical analysis of pipe loop and annular reactor data for the heterotrophs indicated that biofilm HPC numbers increased with the system hydraulic residence time, but the trend did not occur in the bulk fluid ( $p_{\text{bio}} = .0002$ ,  $p_{\text{bf}} = .2706$ , Table 5.1). This is reflected by the slight increase in the actual mean across all sampling times for the biofilm heterotrophs from a  $\log_{10}$  value of 6.01 at HRT = 2 hr to 6.07 at HRT = 16 hr (Table 5.2). From a practical standpoint, however, this increase is not important. In the annular reactors, there was a statistically significant upward trend in suspended heterotrophic counts with system residence time ( $p_{\text{bf}} = .0001$ , Table 5.1) that represented nearly a full log increase (Table 5.2).

Conversely, biofilm heterotrophs were essentially the same for all residence times ( $p_{\text{bio}} = .0780$ ). Biofilm coliforms in both reactors decreased significantly with increased residence time ( $p_{\text{bio}} < .01$  for the four loops,  $p_{\text{bio}} = .0001$  for the four annular reactors). A graphical representation of the biofilm coliforms in the pipe loops is shown in Fig. 5.1. Variability in the data is present, but the trend towards decreasing coliforms with increasing residence time is obvious. There was also a decreased number of suspended coliforms with residence time in the pipe loops from a value of 0.63 at a HRT = 2 hr to 0.04 at HRT = 16 hr ( $p_{\text{bf}} < .01$ ), but not in the annular reactors ( $p_{\text{bf}} > .5$ ). The annular reactor data were strongly influenced by the particularly large value in the fourth annular reactor in train 1, which exhibited an unexplained high number of organisms during the final sampling on week 13. The overall conclusion is that the biofilm coliforms are more prevalent at shorter residence times, while the trend with suspended coliforms is less clear.

Table 5.1. *p*-values from the residence time experiments based on the hypothesis that the stated parameter had no effect on the corresponding organism counts.(A) Pipe loop *p*-values

Parameter	<i>(coliform)</i> <sup>1/2</sup>		<i>log (HPC)</i>	
	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid
Loops ( <i>L</i> )	.0001	.0068	.0002	.2706
Weeks ( <i>W</i> )	.0001	.0051	.0955	.5375
<i>L</i> * <i>W</i>	.0119	.0001	.0221	.0001

(B) Annular reactor *p*-values

Parameter	<i>(coliform)</i> <sup>1/2</sup>		<i>log (HPC)</i>	
	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid
Trains ( <i>T</i> )	.2225	.0519	.1344	.0517
AR (Train)	.0001	.5910	.0780	.0001
Weeks ( <i>W</i> )	.0664	.1101	.0016	.6220
<i>T</i> * <i>W</i>	.0001	.1437	.9767	.1208

Table 5.2. Mean transformed organism counts as functions of residence time in each system.

Res. Time (hrs)	Pipe Loop Means				Annular Reactor Means					
	Pipe Loop	<i>(coliform)</i> <sup>1/2</sup>		<i>log(HPC)</i>		AR (Train)	<i>(coliform)</i> <sup>1/2</sup>		<i>log(HPC)</i>	
		Biofilm	Bulk Fluid	Biofilm	Bulk Fluid		Biofilm	Bulk Fluid	Biofilm	Bulk Fluid
2	1	2.12	0.60	5.90	4.56	1(1)	10.75	4.68	6.01	5.16
	2	2.09	0.63	6.01	4.58	1(2)	8.42	3.07	6.08	5.25
4	3	0.97	0.21	5.97	4.67	2(1)	10.21	4.45	6.17	5.31
						2(2)	6.85	3.71	6.12	5.74
8	4	0.82	0.12	6.00	4.71	3(1)	7.75	3.62	6.05	5.81
						3(2)	5.36	2.31	6.03	5.93
16	5	0.80	0.04	6.07	4.80	4(1)	4.46	5.49	6.05	5.98
						4(2)	3.34	2.58	5.93	5.99

### Interaction of Substrate Loading and Temperature

Anecdotal information from water utilities implicate warm water conditions with coliform regrowth events. Increased temperatures may favor the growth of coliforms over those of the heterotrophic population. During the summer months when water temperatures are higher, surface water sources may also be impacted by events that contribute to higher BOM concentrations including algal blooms and run-off. Therefore, the separation of temperature and substrate effects on regrowth in distribution systems is difficult. These experiments were designed to determine the importance of these variables independently and in combination.

Of the potential interactions, three were determined to be of the most interest. These are (1) effect of substrate alone, (2) effect of temperature alone, and (3) interaction of temperature and substrate. Transformed statistical means of data taken across the 13 weeks of sampling are summarized in Table 5.3.

When substrate was fed to the pipe loops, there was a statistically significant increase in biofilm coliforms ( $p_{\text{bio}} = .0041$ , Table 5.4b) over those observed in unsupplemented loops. When the results in each loop were averaged with time, two of the three supplemented loops supported more coliforms than the loops that did not receive carbon (Table 5.3) with composite transformed coliform biofilm values of 3.49 vs 2.24, respectively (Table 5.4a). The substrate loading effect on biofilm coliforms was also observed in the annular reactors ( $p_{\text{bio}} = .0227$ ). Here the actual numbers were considerably higher in the amended vs unamended reactors (transformed values of 7.35 and 3.10, respectively; Table 5.4a). In both reactor types, the suspended coliforms did

not change significantly with added substrate ( $p_{bf} = .4525$  for loops and  $.0790$  for annular reactors).

Table 5.3. Transformed mean organism counts at specified carbon substrate and temperature levels.

C Level	Temp Level	Pipe Loop	Pipe Loop Means				AR (Train) <sup>c</sup>	Annular Reactor Means			
			<i>(coliform)</i> <sup>1/2</sup>		<i>log(HPC)</i>			<i>(coliform)</i> <sup>1/2</sup>		<i>log(HPC)</i>	
			Biofilm	Bulk Fluid	Biofilm	Bulk Fluid		Biofilm	Bulk Fluid	Biofilm	Bulk Fluid
No <sup>a</sup>	20	1	2.27	0.18	5.88	4.54	1(2)	3.21	0.72	6.81	5.16
							2(2)	3.93	0.71	6.78	5.29
No	10	2	2.20	.029	6.24	4.56	1(1)	2.29	0.86	6.65	5.03
							2(1)	2.98	0.80	6.59	4.89
Hi <sup>b</sup>	10	3	4.09	0.37	6.39	4.86	3(1)	6.67	1.14	7.02	5.10
							4(1)	6.24	1.13	6.97	5.03
Hi	20	4	2.26	0.37	5.98	4.63	3(2)	7.97	2.29	7.32	5.76
							4(2)	8.53	1.08	7.32	5.55

<sup>a</sup> No = no added substrate

<sup>b</sup> Hi = 500 µg C/L added substrate

<sup>c</sup> represents Train 1 (10° C) or Train 2 (20° C)

When the influence of temperature was evaluated, there was no apparent difference in biofilm coliform concentrations between 10 and 20° C loops that did not receive substrate (Table 5.3). When means were taken of temperature data regardless of substrate supplementation, the 10° C values were higher (Table 5.4a), and the p-value suggested marginal significance to this trend ( $p_{bio} = .0596$ ). Similar results were seen in the annular reactors, as there was a slight decrease in biofilm coliforms with increasing

temperature (Table 5.4a), but the effect was marginal ( $p_{\text{bio}} = .0828$ ). For both reactors, there was no significant association of suspended coliforms with temperature.

Table 5.4. Transformed means and corresponding  $p$ -values across substrate loading and temperature levels.  $P$ -values correspond to the hypothesis that the indicated parameter had no effect on organism count transformed means.

(A) transformed mean counts.

C Level	Pipe Loop Means				Annual Reactor Means			
	$(\text{coliform})^{1/2}$		$\log(\text{HPC})$		$(\text{coliform})^{1/2}$		$\log(\text{HPC})$	
	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid
NO	2.24	0.23	6.06	4.55	3.10	0.77	6.71	5.09
HI	3.49	0.38	6.21	4.76	7.35	1.41	7.16	5.36

Temp Level	Pipe Loop Data		Pipe Loop Data		Annual Reactor Data		Annual Reactor Data	
	$(\text{coliform})^{1/2}$	$\log(\text{HPC})$	$(\text{coliform})^{1/2}$	$\log(\text{HPC})$	$(\text{coliform})^{1/2}$	$\log(\text{HPC})$	$(\text{coliform})^{1/2}$	$\log(\text{HPC})$
	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid
10	3.14	0.33	6.31	4.71	5.91	0.98	6.81	5.01
20	2.88	0.32	6.04	4.65	4.54	1.20	7.06	5.44

(B)  $p$ -values.

Parameter	Pipe Loop Data				Annular Reactor Data			
	$(\text{coliform})^{1/2}$		$\log(\text{HPC})$		$(\text{coliform})^{1/2}$		$\log(\text{HPC})$	
	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid
Carbon (C)	.0041	.4525	.0143	.0412	.0227	.0790	.0001	.0157
Temp (T)	.0596	.3687	.0099	.0341	.0828	.4243	.0013	.0037
C*T	.0759	.4190	.5018	.2338	.3205	.3617	.2663	.2667

The interaction between substrate loading and temperature was also assessed. The  $p$ -values for the interaction indicate that there is no significant interaction between these two variables on either biofilm or suspended coliforms ( $.0759 < p > .4190$ ).

The effect of AOC and temperature can also be seen in Figure 5.2, where there are obviously higher coliform numbers in the supplemented reactors. Note that the data were not "smooth," with an increased number of coliforms observed in each loop on week 5. Earlier in that week, the treatment facility reconfigured their inlet/outlet on the clearwell, resulting in a flushing of accumulated particulates. Even with pretreatment of the dilution water, this operational "upset" had an impact on the pilot plant results.

Statistically, heterotrophic populations in the pipe loops were significantly enhanced by the addition of substrate, both in the biofilm and bulk fluid ( $p_{\text{bio}} = .0143$ ,  $p_{\text{bf}} = .0412$ ). This trend also appeared in the annular reactors ( $p_{\text{bio}} = .0001$ ,  $p_{\text{bf}} = .0157$ ). The actual values were less than 0.5 log higher for biofilm organisms in the annular reactors and less than that in the pipe loops (Table 5.3). Temperature effects were also statistically significant, but somewhat in the opposite direction from the coliforms. Increased heterotrophs in the biofilm and bulk were associated with higher temperatures in the annular reactors with p values of .0013 and .0037. An opposite trend seen in the pipe loop biofilm and suspended heterotrophs (Table 5.4a) was statistically significant. It is important to note, however, that the practical differences in heterotroph numbers due to temperature effects are quite small.

There was no evidence for a significant interaction between substrate loading and temperature on either bulk fluid or biofilm heterotrophs ( $p > .20$  for all data, Table 5.4b).

### Interaction of Substrate Loading and Chlorine

Information from full-scale distribution systems has indicated that there is an unpredictable response of regrowth bacteria to disinfection. The potential interaction of free chlorine and assimilable organic carbon was investigated to provide insight as to why variable degrees of coliform control have been obtained by elevated disinfectant doses.

Two replicate pilot plant runs were conducted to evaluate the impact of chlorination in the presence (500  $\mu\text{g/L}$ ) and absence of added substrate. An influent chlorine concentration of 1 mg/L was selected as a relevant concentration leaving a treatment plant and entering a distribution system. Residual free chlorine measurements were taken routinely and the concentrations varied between 0.05 and 0.5 mg/L.

There was a consistent pattern of elevated coliforms in reactors that received both chlorine and carbon (Tables 5.5 and 5.7) in the replicate experiments. The data were sufficiently noisy to mask statistical significance (Tables 5.6b, 5.8b) with the exception of the coliforms in the pipe loop biofilms in the replicate experiment ( $p_{\text{bio}} = .0197$ ) and the marginal value for suspended coliforms in the first run ( $p_{\text{bf}} = .0431$ ). The means of coliform data for the reactors that received substrate either with or without chlorine tend to be higher than those that were not fed carbon (Tables 5.6 and 5.8). Of interest was the observation that the presence of chlorine in the absence of carbon did not result in substantially lower numbers of indicator bacteria. The influence of substrate loading alone was not obvious.

Table 5.5. Transformed mean organism counts at specified substrate and chlorination levels, replicate 1.

C level	Cl <sub>2</sub>	Pipe Loop Means				Annular Reactor Means					
		Pipe Loop	Biofilm ( <i>coliform</i> ) <sup>1/2</sup>	Bulk Fluid <i>log</i> (HPC)	Biofilm <i>log</i> (HPC)	Bulk Fluid	AR (Train) <sup>c</sup>	Biofilm ( <i>coliform</i> ) <sup>1/2</sup>	Bulk Fluid <i>log</i> (HPC)	Biofilm <i>log</i> (HPC)	Bulk Fluid
Hi <sup>a</sup>	No	1	7.54	3.53	6.31	4.74	1(1)	23.89	8.01	6.56	5.56
							2(1)	21.63	7.90	6.54	5.51
Hi	Yes <sup>b</sup>	2	10.60	5.88	6.20	4.75	3(1)	25.29	17.26	6.66	5.57
							3	11.30	7.26	6.37	4.13
No	No	4	9.00	4.11	5.96	4.17	1(2)	19.52	7.42	6.57	4.82
							2(2)	21.10	8.11	6.49	5.11
No	Yes	5	8.89	4.19	6.42	4.66	3(2)	23.19	11.03	6.46	5.08
							4(2)	23.06	13.29	6.41	5.09

<sup>a</sup> Hi C = 500 µg C/L added

<sup>b</sup> Yes chlorine = 1 mg/L added

<sup>c</sup> represents Train 1 (with substrate) or Train 2 (no substrate)

Table 5.6. Transformed means and corresponding  $p$ -values across substrate loading and chlorine levels, replicate 1.  $P$ -values correspond to the hypothesis that the indicated parameter had no effect on organism count transformed means.

(A) transformed mean counts.

C Level	Pipe Loop Means				Annular Reactor Means			
	$(coliform)^{1/2}$		$\log(HPC)$		$(coliform)^{1/2}$		$\log(HPC)$	
	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid
No	8.95	4.15	6.19	4.41	21.72	10.03	6.48	5.02
Hi	9.81	5.56	6.29	4.54	23.84	12.50	6.64	5.55

Chlorine Level	Pipe Loop Means				Annular Reactor Means			
	$(coliform)^{1/2}$		$\log(HPC)$		$(coliform)^{1/2}$		$\log(HPC)$	
	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid
No	8.27	3.82	6.13	4.45	21.53	7.87	6.54	5.25
Yes	10.26	5.78	6.33	4.51	24.02	14.60	6.59	5.32

(B)  $p$ -values

Parameter	Pipe Loop Data				Annular Reactor Data			
	$(coliform)^{1/2}$		$\log(HPC)$		$(coliform)^{1/2}$		$\log(HPC)$	
	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid
Carbon (C)	.7061	.1175	.1881	.1261	.4133	.1254	.0021	.0001
Chlorine (Cl)	.1035	.0411	.2503	.1085	.0861	.1995	.5650	.4094
C*Cl	.1166	.0431	.0251	.0281	.7395	.1343	.0153	.7123

Table 5.7. Transformed mean organism counts at specified substrate and chlorination levels, replicate 2.

C Level	Cl <sub>2</sub>	Pipe Loop Means					Annular Reactor Means				
		Pipe Loop	<i>(coliform)</i> <sup>1/2</sup>		<i>log(HPC)</i>		AR (Train) <sup>c</sup>	<i>(coliform)</i> <sup>1/2</sup>		<i>log(HPC)</i>	
			Biofilm	Bulk Fluid	Biofilm	Bulk Fluid		Biofilm	Bulk Fluid	Biofilm	Bulk Fluid
Hi <sup>a</sup>	No	1	2.28	0.14	6.42	5.24	1(1)	1.76	0.73	6.98	4.96
							2(1)	1.68	0.73	6.80	4.99
Hi	Yes <sup>b</sup>	2	3.67	0.31	6.71	4.48	3(1)	2.39	0.79	7.31	5.23
			3	3.54	0.26	6.81	4.44	4(1)	2.28	1.00	7.26
No	No	4	2.47	0.30	6.46	4.41	1(2)	1.37	0.46	6.52	4.92
							2(2)	1.51	0.52	6.62	4.81
No	Yes	5	1.46	0.45	6.13	5.16	3(2)	2.21	0.80	6.83	4.72
							4(2)	2.25	0.52	6.63	4.74

<sup>a</sup> Hi C = 500 µg C/L added

<sup>b</sup> Yes chlorine = 1 mg/L added

<sup>c</sup> represents Train 1 (with substrate) or Train 2 (no substrate)

Table 5.8. Transformed means and corresponding *p*-values across substrate loading and chlorine levels, replicate 2. *P*-values correspond to the hypothesis that the indicated parameter had no effect on organism count transformed means.

(A) transformed mean counts.

C Level	Pipe Loop Means				Annular Reactor Means			
	$(coliform)^{1/2}$		$\log(HPC)$		$(coliform)^{1/2}$		$\log(HPC)$	
	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid
No	1.97	0.38	6.30	4.72	1.84	0.57	6.65	4.80
Hi	3.16	0.24	6.65	4.79	2.03	0.81	7.09	5.10

Chlorine Level	Pipe Loop Data		Annular Reactor Data	
	$(coliform)^{1/2}$	$\log(HPC)$	$(coliform)^{1/2}$	$\log(HPC)$
	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid
No	2.38	0.22	6.44	4.83
Yes	2.89	0.34	6.55	4.69

(B) *P*-values.

Parameter	Pipe Loop Data				Annular Reactor Data			
	$(coliform)^{1/2}$		$\log(HPC)$		$(coliform)^{1/2}$		$\log(HPC)$	
	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid
Carbon (C)	.1230	.3672	.0120	.7585	.2362	.3533	.0022	.0117
Chlorine (Cl)	.2825	.3980	.8200	.3187	.0134	.4542	.0019	.5500
C*Cl	.0197	.9844	.0103	.0001	.6559	.9871	.0898	.0698

In the heterotrophic populations, bulk fluid counts increased when substrate was present alone, and decreased in the presence of substrate when chlorine was added (Tables 5.5, 5.7). Combining the effects masked the trends (Tables 5.6a, 5.8a). Statistical significance was mixed depending on the reactor type and whether the organisms were attached or suspended (Tables 5.6b and 5.8b).

The numbers of coliforms present in the summer experiment were higher than those in the winter, but the general behavior was the same.

#### Steady-state Behavior

Since the coliforms were inoculated once at the beginning of the experiment, it was of interest to determine the amount of time required to reach a steady-state population. In no instances did the influent water contain coliforms, so it is probable that all coliforms in the reactor systems originated from the initial inoculum. As can be seen with the experimental averages for the unchlorinated experiments (Table 5.9), there was a substantial decline in coliforms as averaged across all loops or annular reactors in the first few weeks of operation. The numbers tended to stabilize at week 5-7, but variability did occur. Statistics were performed only for the residence time experiment. In the pipe loops, the difference in coliforms averaged over all the loops for a point in real time stabilized at week 7 ( $p_{\text{bio}} = .0001$  and  $p_{\text{bf}} = .0051$ , Table 5.1a). A similar situation occurred in the annular reactors, but a large increase in coliforms on the last week of sampling caused the real time effect to be insignificant ( $p_{\text{bio}} = .0664$ ,  $p_{\text{bf}} = .1101$ , Table 5.1b).

Table 5.9. Influence of real time on organism numbers in experiments with no disinfectant.

## (A) Residence time experiment

Pipe Loop Means					Annular Reactor Means				
Week	$(coliform)^{1/2}$		$log(HPC)$		Week	$(coliform)^{1/2}$		$log(HPC)$	
	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid		Biofilm	Bulk Fluid	Biofilm	Bulk Fluid
1	2.47	0.83	5.82	4.55	1	10.85	4.82	5.98	5.63
2	1.50	0.09	5.97	4.84	2	8.44	7.29	6.01	5.96
3	1.73	0.57	6.06	4.86	3	6.30	4.79	6.07	5.69
4	0.95	0.67	6.04	4.58	4	5.50	4.14	6.12	5.63
5	0.90	0.09	6.14	4.72	5	4.63	2.69	6.17	5.46
7	0.92	0.00	6.25	4.70	7	5.80	2.17	6.15	5.68
9	0.65	0.00	5.96	4.62	9	3.07	2.65	6.12	5.85
11	0.93	0.00	5.89	4.62	11	2.45	1.79	5.93	5.58
13	0.47	0.00	6.00	4.74	13	17.25	3.07	5.92	5.63

## (B) Substrate/Temperature experiment

Pipe Loop Means					Annular Reactor Means				
Week	$(coliform)^{1/2}$		$log(HPC)$		Week	$(coliform)^{1/2}$		$log(HPC)$	
	Biofilm	Bulk Fluid	Biofilm	Bulk Fluid		Biofilm	Bulk Fluid	Biofilm	Bulk Fluid
1	10.71	2.41	6.11	5.02	1	17.21	6.26	6.68	5.29
2	2.51	0.12	6.23	4.58	2	3.56	1.07	7.16	5.44
3	1.29	0.00	6.22	4.59	3	2.69	0.68	7.07	5.36
4	1.13	0.25	6.12	4.59	4	1.61	0.73	6.96	4.95
5	7.74	0.12	6.38	4.69	5	9.28	0.26	7.04	5.12
7	1.01	0.00	6.17	4.71	7	7.67	0.37	7.00	5.26
9	0.84	0.00	6.04	4.54	9	1.91	0.45	6.98	5.17
11	0.75	0.00	5.89	4.62	11	1.39	0.00	6.89	5.07
13	0.91	0.00	6.19	4.71	13	1.72	0.00	6.62	5.39

The influence of real time on heterotrophs was also investigated. In all experiments, there was little variability with time, implying that a pseudo-steady state condition was attained in the first week. For example, in the residence time experiments,

the four pipe loops in series contained biofilm populations that varied by less than 0.33 log (Table 5.9). The p-values for the biofilm and bulk fluid heterotrophs were all greater than 0.9, indicating that there was no influence of real time on the heterotrophic populations. In the annular reactors, there was no effect of real time on the suspended heterotrophs ( $p_{bf} = .6220$ ). A statistically significant value was obtained for the biofilms ( $p_{bio} = .0016$ ), but there was no consistent trend either up or down between sampling times.

At the end of the runs, coupons that had been removed, scraped, and reinserted into the reactors during the first week were again sampled to determine if they were colonized with the same numbers of heterotrophs and coliforms coupons that had been undisturbed for the entire time. Coupons that had been scraped and replaced after system inoculation were colonized to the same level with heterotrophs and coliforms as coupons that had been in place for the entire run time of thirteen weeks (data not shown).

Although there were limited number of samples, coupons from the dead end sections of the pipes also contained comparable numbers of organisms to those found in the four inch line.

#### Biofilm vs Bulk Fluid Measurements

With the exception of the first experiment where long residence times may allow organisms to reproduce in the bulk fluid, the organisms present in the water should be primarily the result of detached biofilm bacteria. Comparisons were made between the suspended and biofilm counts in the three experiments when the residence time was set at

two hours. The data were segregated into two sets; chlorinated and unchlorinated.

Regressions of the form  $s_{\text{bio}} = k \cdot s_{\text{bf}}$  were used for the coliform data, and intercept terms were excluded. If the constant  $k$  is squared, it represents the ratio of biofilm to bulk fluid counts. For the heterotroph counts, regressions of the form  $l_{\text{bio}} = K + l_{\text{bf}}$  were performed, and a dummy variable used to set the slope to zero. The antilogarithm of the constant  $K$  (i.e.,  $10^K$ ) is the ratio of the biofilm to bulk fluid heterotroph counts. Separate values of the coefficient ( $k$  and  $K$ ) and corresponding  $r^2$  values were determined for the combined and independent data sets (chlorinated and unchlorinated). The results are shown in Table 5.10.

Table 5.10. Ratio of biofilm to bulk fluid organism numbers for both systems and both populations.  $p$ -values were all less than 0.01. Correlation coefficients ( $r^2$ ) are given for coliforms, while standard errors (on the logarithmic scale) are given for the HPCs.

System (population)	biofilm:bulk fluid ratio			$r^2$ (coliforms) or S.E. (HPC)		
	Cl <sub>2</sub> +	Cl <sub>2</sub> -	both	Cl <sub>2</sub> +	Cl <sub>2</sub> -	both
Annular Reactor (coliforms)	2.00	2.02	2.01	.89	.37	.64
Pipe Loop (coliforms)	2.06	2.81	2.26	.77	.76	.76
Combined (coliforms)	2.01	2.02	2.04	.87	.41	.66
Annular Reactor (HPC)	44.1	42.4	43.0	.070	.037	.034
Pipe Loop (HPC)	104.	25.1	54.7	.079	.074	.063
Combined (HPC)	63.9	37.4	46.4	.059	.034	.031

The ratio of coliforms in the biofilm to the bulk fluid (2:1) from both types of reactors were essentially unchanged whether chlorine was present or not. Correlation

coefficients for the chlorinated systems were higher than in the nonchlorinated reactors. For the heterotrophs, the ratios were considerably higher (ca. 40:1) than for the coliforms. In the annular reactors, the ratios were similar regardless of the presence or absence of disinfectant. In contrast, the ratio in the pipe loop was influenced by the presence of chlorine, with a difference of approximately four-fold.

### AOC Concentrations

AOC measurements were taken each time biofilm and bulk fluid samples were removed. The data were highly variable, and no correlation could be found between AOC concentration in the CSTRs with biofilm or suspended organism numbers.

### Discussion

Evaluations of the influence of various factors on regrowth in distribution systems is hampered by (1) complex interactions between operational and environmental variables, (2) inability to control variables, and (3) inability to collect biofilm samples. Experiments were designed using pilot reactors to examine the relative importance of four factors. To simulate worst-case conditions, the pilot reactors were made of highly corrodible mild steel. Variables under potential control of the treatment plant included chlorine level and assimilable organic carbon, while temperature and hydraulic regime are essentially uncontrolled.

An important component of the work was to establish relatively reproducible coliform-containing biofilms in the reactors. Laboratory experiments determined the

conditions necessary to permit the coliforms to successfully colonize and persist in biofilms (Camper, et al., 1995a), and the results were applied to pilot studies. The behavior of the indicators was similar at both scales; numbers decreased to a pseudo steady-state within a few weeks. It was also shown that the organisms that were released from the biofilms within the reactors could recolonize clean surfaces, as indicated when coupons were resampled. Heterotrophs reached a pseudo steady-state within the first two weeks of operation at levels of  $10^6$  to  $10^7$  cfu/cm<sup>2</sup>. These numbers are similar to those observed in another ferrous metal system (LeChevallier, et al., 1993b).

When individual pipe loops or annular reactors were run in series, conditions representing increasing hydraulic residence time or water age were simulated. No chlorine was present, and the carbon source consisted of small molecules that should be reasonably degradable. Under these conditions, it was noted that the coliforms were most abundant at the shortest residence time (two hours). In a mixed population biofilm the coliforms may be more successful competitors where the substrate concentration is highest. These results are in agreement with the findings of LeChevallier, Babcock and Lee (1987), where the majority of coliform growth in a distribution system occurred close to the treatment plant. It was anticipated that there should also be a decrease in biofilm heterotroph numbers with increased residence time, but this trend was not observed. These results are in contrast to those of Characklis (1988) and Haudidier, et al. (1988) where the largest numbers of organisms were present at the shortest residence times. A potential explanation is that both of the cited references reported results from concrete lined pipes, while our research utilized steel. There was also no correlation between the

highly variable circulating AOC levels and bacterial numbers. A similar result has been reported by Kerneis, et al. (1994) where there was no correlation between distribution system BDOC measurements, suspended bacteria, and fixed biomass. In contrast, decreases in BDOC (Levi and Joret, 1990) and AOC (van der Kooij, 1992) with water age have been noted; AOC levels were associated with heterotrophic regrowth.

Experiments conducted at a fixed residence time (2 hr) and variable organic carbon concentrations and temperatures were conducted to separate variables associated with summer regrowth events. It is known that pure cultures of coliforms isolated from drinking water can proliferate at the expense of extremely low concentrations of carbon at low temperatures (Camper, et al., 1991), although growth was enhanced at higher temperatures. In distribution system studies where suspended coliforms were measured, investigators have suggested that coliform regrowth events were more prevalent at higher temperatures (LeChevallier, Shultz and Lee, 1991; Colbourne, et al., 1991). Donlan and Pipes (1988) showed a direct relationship between temperature and biofilm population density. However, in each of those distribution studies, the amount of carbon substrate was not controlled. Target AOC levels for the control of coliforms and heterotrophs have been suggested as 50  $\mu\text{g/L}$  (LeChevallier, et al., 1991) and 10  $\mu\text{g/L}$  (van der Kooij, 1992), respectively. Servais, et al. (1991) have associated biological stability of water with a BDOC level of 0.2 mg/L. More recently, Joret, et al. (1994) have stated that BDOC values for biological stability of water are temperature dependent (0.15 mg/L at 20° C and 0.30 mg/L at 15° C). Data from the pilot plant experiments showed that biofilm coliform and heterotroph counts increased when substrate (500  $\mu\text{g C/L}$ ) was added. The

temperature effect was less dramatic; in fact, the coliform levels appeared to decrease with increased temperature (10 and 20° C). The heterotrophs increased slightly with increasing temperature in the annular reactors, but declined marginally in the pipe loops. There was no interaction between both increased substrate concentration and temperature on organism numbers. Therefore, the regrowth may be more the result of elevated substrate than temperature. These results are somewhat encouraging, as treatment can be designed to control carbon levels while temperature is impossible to influence.

Historically, utilities have relied on increased disinfectant doses to control regrowth events, with mixed success. It is known that biofilm organisms are less susceptible to disinfectants than suspended cells, especially if they are present on reactive iron surfaces (LeChevallier, Lowry and Lee, 1990; LeChevallier, et al., 1993b; Chen, et al., 1993). However, higher levels of disinfectants have also been unable to reduce the number of suspended organisms believed to have originated from biofilms. The results from the pilot studies showed that there was a consistent trend towards increased coliform numbers when both chlorine and substrate were added to the reactors. It was also observed that the accumulation of corrosion products was the most pronounced on coupons from systems receiving both substrate and chlorine. LeChevallier, et al. (1993b) described results where increased corrosion rates decreased the efficacy of free chlorine on biofilm bacteria. In our systems, when chlorine was present in the absence of added carbon, there was no decrease in coliform numbers. Some measure of control of both suspended and biofilm heterotrophs was obtained with the addition of chlorine. These results were confirmed when the ratios between biofilm and bulk fluid organism numbers

were calculated. Chlorinated reactors contained the same ratio of coliforms as the controls, while the heterotrophs in the bulk fluids were considerably reduced when chlorine was present. It therefore appears that the biofilm and suspended coliforms are less susceptible to chlorination than the general heterotrophic population. Evaluation of the correlation coefficients for biofilm/bulk fluid ratios suggests that when chlorine is present, its effects dominate the coliform response. When absent, the correlation coefficients were lower, implying that an interaction of the remaining variables may be more important than chlorine as a single parameter.

Three hypotheses are proposed to explain the enhanced numbers of coliforms in the presence of chlorine. First, the disinfectant may decrease the metabolic activity of the heterotrophs and therefore give the coliforms a selective advantage. Second, the disinfectant may oxidize some of the more recalcitrant fractions of the organic matter in the water, resulting in higher levels of AOC. This has been shown to occur in chlorinated wastewater (El-Reahili, 1995) and drinking water (van der Kooij and Hijnen, 1984). Results from the substrate/temperature experiment showed that coliforms were enhanced in the presence of elevated substrate, but that heterotrophs were essentially unaffected. Third, the coliforms have been shown to exist in microcolonies on the surface (Camper, et al., 1995a) and may therefore be protected from the action of the chlorine. In studies where defined mixed population biofilms containing *K. pneumoniae* were grown, detachment of large aggregates of the organism occurred (Murga, Stewart, and Daly, 1995), which may also result in decreased disinfection efficacy.

Based on the results of this study and the findings of others, the following recommendations are made:

- (1) When available substrate (biodegradable organic matter) is maintained at low levels, organism numbers in distribution system biofilms are decreased and disinfection efficacy is increased.
- (2) If regrowth is occurring in systems that contain thick deposits due to corrosion products or excessive organism numbers resulting from high concentrations of biodegradable organic matter, disinfection is less likely to be an effective remedy.
- (3) Disinfection may, in some cases, worsen the severity of a coliform regrowth event if the biodegradable organic matter levels are uncontrolled.

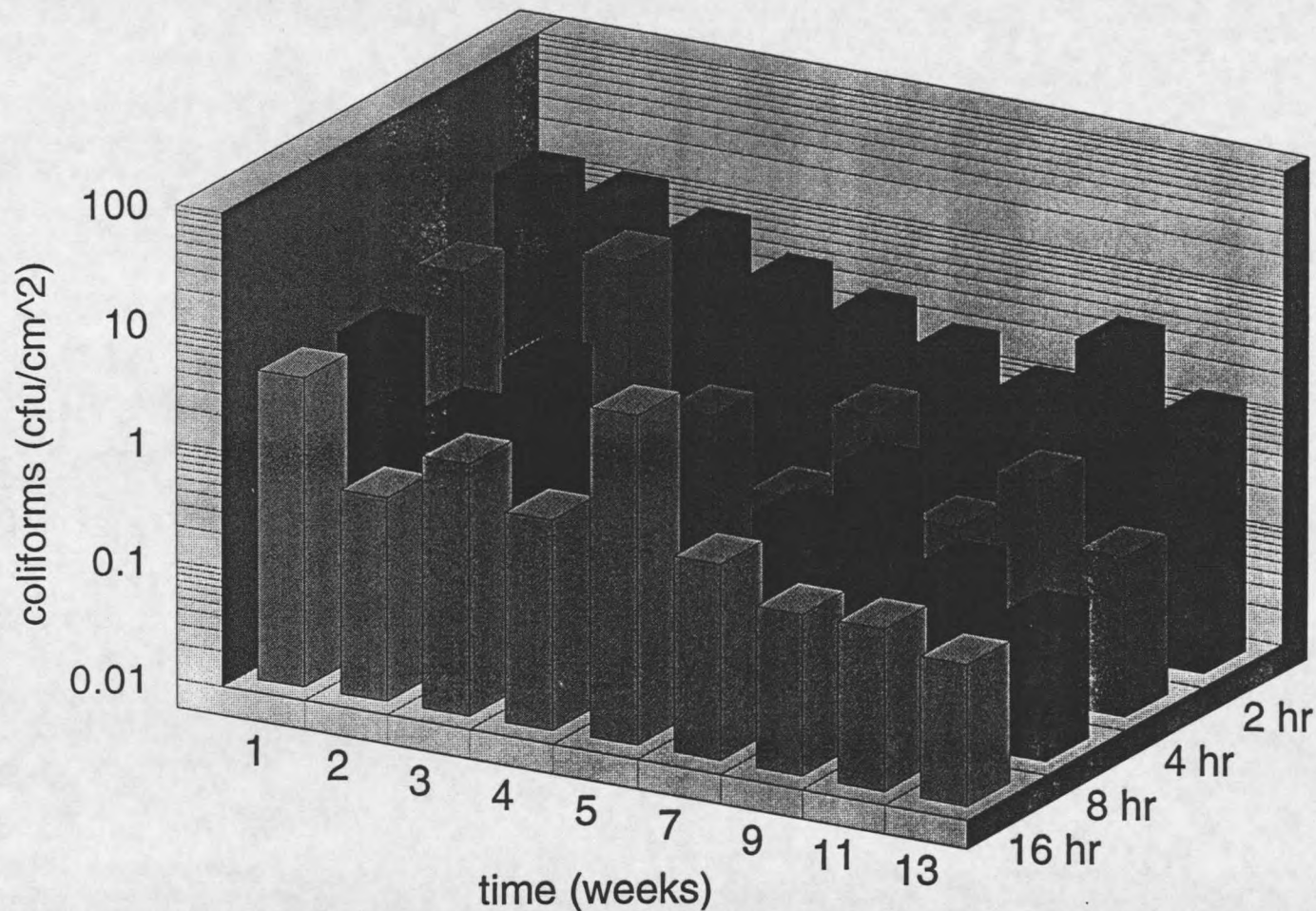


Figure 5.1. Influence of hydraulic residence time on biofilm coliforms from the pipe loop. Coliforms were added at week 0; sampling began on week 1. The loops were operated in series, with the first loop (2 hr residence time) receiving 500  $\mu\text{g C/L}$ . No subsequent additions were made to the remaining loops.

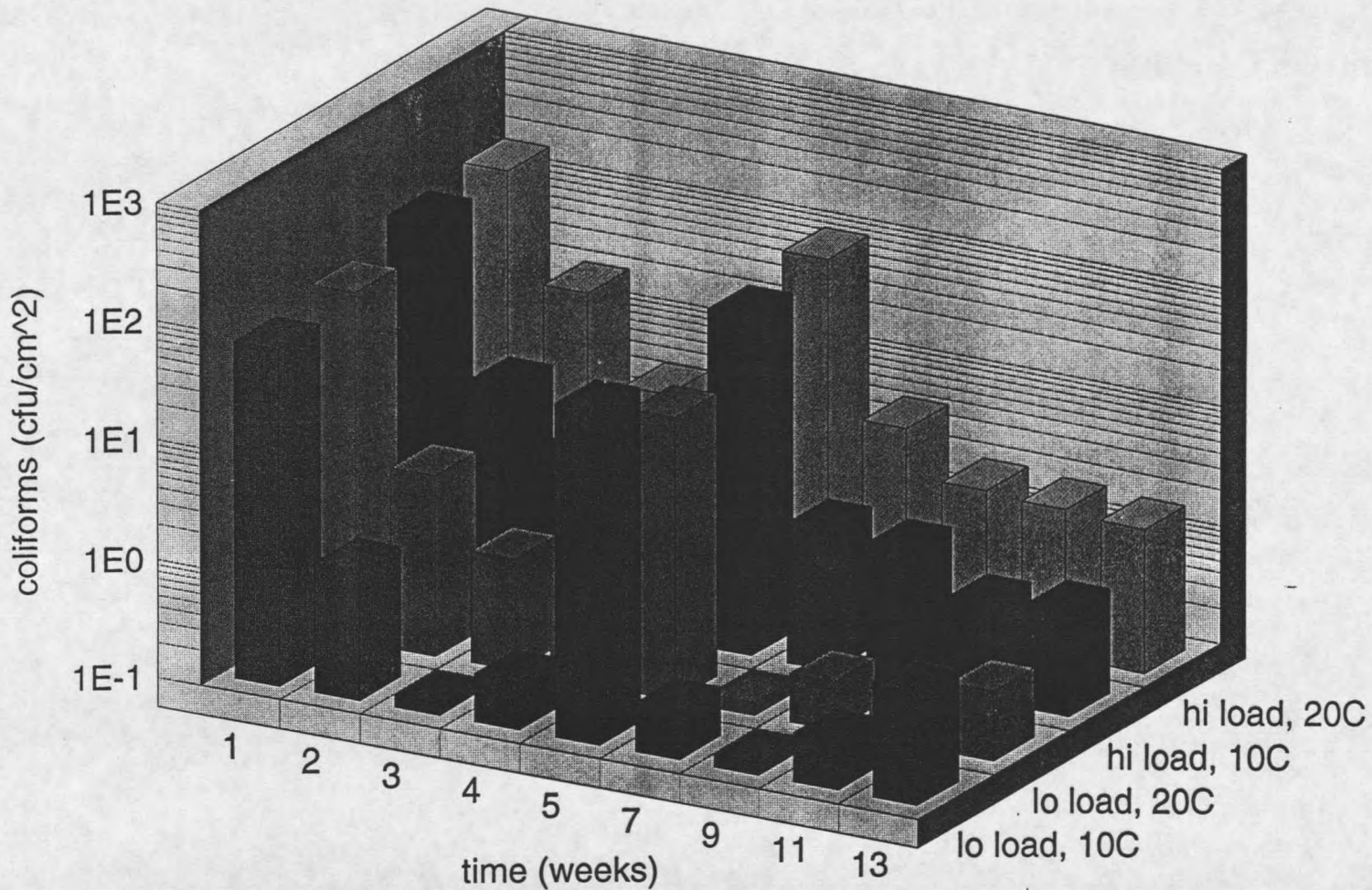


Figure 5.2. Influence of temperature and substrate (AOC) on biofilm coliforms in the pipe loops. Loops were operated in parallel with a residence time of 2 hrs. Temperatures were set at 20 or 10° C and the influent substrate (AOC) concentration at 500 µg/L or no added carbon.

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## CHAPTER 6

ESTIMATED BIOFILM ORGANISM GROWTH RATES  
AS INFLUENCED BY DISTRIBUTION SYSTEM PARAMETERSIntroduction

In monitoring of complex biofilm systems, samples of suspended and biofilm cell counts can be taken at intervals to provide information on the state of the system at that particular time. These data can then be subjected to a variety of statistical analyses, many of which can lead to conclusions on cause-and-effect relationships (Camper, et al., 1995a). A limitation to this approach is that frequently only one set of data at a time can be analyzed (e.g., the suspended cell population) or at best, arithmetic relationships between observations obtained. These approaches, while useful, do not provide any phenomenological insight into the relationships between the bulk and biofilm populations. To this end, the use of growth and detachment rates as analytical tools is proposed in this manuscript.

Calculation of growth rates in biofilm systems is difficult because of the complex interaction of the various processes which govern biofilm formation. Cellular growth is only one of the many phenomena contributing to biofilm accumulation; adsorption or attachment, desorption or detachment, and cellular decay are also involved. Typically, the growth rate in a steady-state biofilm is believed to be equivalent to the detachment rate. If the residence time in the reactor is sufficiently short and there are no influent

cells, the suspended cells should be the result of detached biofilm organisms. However, no clear relationship between biofilm growth rate and that of planktonic cells has arisen, and it may be inappropriate to assume that the growth rate/substrate concentration relationship is the same for both populations (van Loosdrecht, et al., 1990).

A second approach is to compare substrate utilization with growth rate estimated from cell production. A significant limitation to this method in dense biofilms is the potential for considerable mass transfer effects; cells deep within the biofilm do not "see" the same substrate concentration as those near the biofilm-liquid interface. In oligotrophic environments such as drinking water distribution systems (and in those model systems discussed in this paper), biofilms tend to be very patchy and are typically less than a monolayer, so that diffusional resistances can effectively be neglected. These biofilms are very heterogeneous, both physically and ecologically (Camper, et al., 1995b; Robinson, et al., 1995; Manz, et al., 1993; Rogers and Keevil, 1992), and the substrates on which organisms thrive are often poorly characterized (Malcolm, 1991; Kaplan and Bott, 1990; Meyer, et al., 1987; Tranvik and Hofle, 1987). Hence, *a priori* prediction of biofilm growth rate from substrate data is also next to impossible.

This paper describes population balances on biofilm and suspended cells in well-characterized reactors used to develop estimates of growth and detachment rates from biofilm and suspended organism cell count data. The analysis is based on the concept that the difference between cells entering and leaving the overall reactor (after accounting for accumulation between sampling periods and, in some cases, growth of planktonic cells) must be equal to the number of cells detaching from the reactor surface. In the

biofilm, the processes of growth and detachment lead to a measurable accumulation.

These two balances are combined to produce estimates of the specific detachment and growth rates of organisms in the biofilm.

Two groups of organisms were chosen for study, the heterotrophs and coliforms. The heterotrophic population represents a diverse group that will proliferate at the expense of organic carbon in the water. The coliforms are a subset of the heterotrophs, and are of specific interest to the drinking water industry because their presence is used as an indicator of the microbiological safety of finished water. Utilities are interested in understanding the conditions that allow coliforms to grow in distribution system biofilms and cause non-compliance with the Total Coliform Rule of the Safe Drinking Water Act.

Three model distribution systems were operated in the absence of disinfectant for periods ranging from 8 to 13 weeks, data on biofilm and suspended coliform and heterotrophic organisms were collected, and the results were subjected to analysis using a cell balance approach. This method is similar to that provided by van der Wende, et al. (1989) and by Jones, et al. (1995), but significant changes in the assumptions leading to the model have been made, as described below.

It is important to note that although the measurements and organisms used to develop the model are specific to drinking water systems, the model itself is applicable to calculating the growth rates of biofilm organisms in any industrial system where mass transfer is not limiting.

Model Development

A population balance on a type of organism (e.g., heterotrophic plate count bacteria - HPC or mT7-positive bacteria - coliforms) states that the accumulation of that organism results from the net sum of the processes of transport and production. In a well-mixed system such as a rotating annular reactor or recirculating pipe loop system (Camper et al., 1995c), concentrations and fluxes of cells may effectively be averaged over the entire volume and/or surface of the system. Under these conditions, a population balance written on the bulk fluid of the reactor system yields:

$$\frac{dX_b}{dt} = D(X_{b0} - X_b) + q_d a + \mu_b X_b \quad (1)$$

where

$a$  = specific surface area ( $L^{-1}$ )

$D$  = dilution rate ( $t^{-1}$ )

$q_d$  = net detachment flux (cells  $L^{-2} t^{-1}$ )

$X_b$  = bulk fluid cells (cells  $L^{-3}$ ) in effluent

$X_{b0}$  = bulk fluid cells (cells  $L^{-3}$ ) in influent

$\mu_b$  = net specific growth rate of cells in the bulk fluid ( $t^{-1}$ )

A similar population balance written on the biofilm compartment yields equation (2):

$$\frac{dX_f}{dt} = r_{gf} - q_d \quad (2)$$

where:

$r_{gf}$  = net growth rate of attached cells (cells cm<sup>-2</sup> hr<sup>-1</sup>) :

$X_f$  = attached cells (cells cm<sup>-2</sup>)

The bulk fluid cell balance (equation 1) may be further modified by assuming that the dilution rate far exceeds the growth rate of suspended cells in the reactor ( $D \gg |\mu_b|$ ), as was done by van der Wende, et al. (1989). Provided that the specific growth rate of cells in the system is less than one tenth the dilution rate, this assumption is reasonable. Also, this assumption precludes the use of disinfectant in the system, since the cell death caused by the disinfectant can result in significant (and negative) net growth rates. The growth and attachment rates can be expressed as:

$$q_d = k_d X_f \quad \text{and} \quad r_{gf} = \mu_f X_f \quad (3)$$

where

$k_d$  = specific detachment rate (hr<sup>-1</sup>)

$\mu_f$  = net specific growth rate of biofilm organisms (hr<sup>-1</sup>)

Equations (1), (2) and (3) may be combined to yield equations (4) and (5) for the specific growth and detachment rates of biofilm organisms.

$$\mu_f = \frac{\frac{1}{a} \left( \frac{dX_b}{dt} - D(X_{bo} - X_b) \right) + \frac{dX_f}{dt}}{X_f} \quad (4)$$

$$k_d = \mu_f - \frac{1}{X_f} \frac{dX_f}{dt} \quad (5)$$

Similar expressions have been described by van der Wende, et al. (1989), Block, et al. (1993) and Bauda, et al. (1993) and applied successfully to simulated distribution systems and packed bed bioreactors. An alternative to equations (4) and (5) may be made by assuming that the biofilm growth rate ( $\mu_f$ ) and the bulk fluid growth rate ( $\mu_b$ ) are equal, rather than neglecting growth in the bulk fluid balance. This approach yields:

$$\mu_f = \frac{\frac{1}{a} \left( \frac{dX_b}{dt} - D(X_{b0} - X_b) \right) + \frac{dX_f}{dt}}{X_f + X_b/a} \quad (6)$$

and the same expression (equation 5) for the specific detachment rate coefficient,  $k_d$ . The only pragmatic difference between equations (4) and (6) is in the appearance of the term  $X_b/a$  in the denominator of equation (6). It should also be pointed out that the assumption of equal growth rates in the bulk liquid and biofilm requires more stringent assumptions than merely assuming that growth is less than dilution. These include: 1) no disinfection (shown to be much more effective at killing bulk fluid than biofilm cells), 2) similar population structure in the biofilm and bulk fluid, and 3) mass transfer resistance between the bulk fluid and biofilm is negligible.

The above equations were fit to the experimental data by expressing the time derivatives as finite differences, e.g.,

$$\frac{dX_f}{dt} \approx \frac{X_{fi} - X_{fi-1}}{t_i - t_{i-1}} \quad (7)$$

and by averaging the cell count measurements ( $X_p$ ,  $X_b$ ) over each the time step between time  $t_i$  and  $t_{i-1}$ .

### Materials and Methods

Data collected for the modeling efforts were obtained from laboratory and pilot scale reactors operated to simulate conditions found in drinking water distribution systems. Detailed methods and results from other analyses are presented elsewhere (Camper, et al., 1995a, b, c). An overview of the reactors and pertinent methods relevant to data used specifically for the model are provided below.

#### Reactor Design and Operation

Laboratory System. Microscale work was conducted in polycarbonate rotating annular reactors. These systems consist of a stationary outer cylinder containing twelve removable slides for biofilm analysis. The inner drum rotates, generating the shear stress between the liquid and the reactor surfaces. A shear stress roughly equivalent to that produced in a 4" pipe with a fluid velocity of 1 ft/s was used ( $0.27 \text{ N/m}^2$ ). To facilitate mixing, the inner drum has four inclined draft tubes which recirculate water when the drum rotates. Because the reactors are well mixed, they can be modeled as continuously

stirred tank reactors (CSTRs). Total fluid volume of the filled, assembled reactors is approximately 670 ml, and the total surface area available for colonization (including draft tubes and bottom surfaces) is about 1750 cm<sup>2</sup>, producing a specific surface area of 2.61 cm<sup>-1</sup>. In all laboratory experiments, the dilution rate was set at 0.5 hr<sup>-1</sup> (residence time 2.0 hr). This dilution rate was selected based on (1) pilot results indicating that biofilm coliform numbers were highest at short residence time and (2) the dilution rate is sufficiently high to preclude significant replication of planktonic cells. To control temperature, the laboratory reactors were immersed in a large waterbath. Water was circulated through the bath and a bench-top chilling unit set to maintain a temperature of 20 ± 0.5° C.

After inoculation with coliforms as described previously (Camper et al., 1995b), the reactors were fed continuously with dilution water emanating from laboratory scale biologically active carbon filters. These small filters were designed to remove chlorine and assimilable organic carbon from tap water used as dilution water. These columns also provided a steady supply of suspended heterotrophic plate count bacteria that colonized the test reactor surfaces. Dilution water was supplemented with nitrogen and phosphorus sufficient to ensure that these nutrients were not stoichiometrically limiting to growth. In all laboratory studies, the dilution water was also amended with a mixture of five compounds representative of those found in drinking water at a final concentration of 250 µg C/L.

Pilot Scale Systems. Two types of pilot scale systems were employed - a rotating annular reactor system and a recirculating pipe loop system. These have been described in detail elsewhere (Camper, et al., 1995c). Briefly, the rotating annular reactor system utilizes a section of 6" diameter pipe as the outer drum, and coupons have been inserted in the outer wall to obtain biofilm samples. The total wetted surface area within a pilot-type annular reactor is 2700 cm<sup>2</sup>, and the volume is 1.10 L, producing a specific surface area of 2.46 cm<sup>-1</sup>. In all other respects, these are similar to the systems described above. The recirculating pipe loop system consists of five individual loops, each fitted with 20 two-foot sections of 4" mild steel tubing containing four flush-mounted coupons, a dead end section and return line of 2" pipe, and a 55-gallon drum. The total volume of the pipe loop is 270 L, and the wetted surface area is 6.65 m<sup>2</sup>, of which 65% is contained in the 4" tubing test section. The total specific surface area is 0.244 cm<sup>-1</sup>. Velocity in the 4" section is maintained at 0.3 m/sec (1 ft/sec), yielding a residence time of under one minute. However, the presence of the mixing vessel allows dilution water addition at any desired rate to fix the residence time of the system. The loop systems may be operated in series to simulate sequential sections (and residence times) in the distribution system or in parallel to simultaneously test different operating and water quality characteristics on biofilm growth and detachment in the loop.

### Experimental Design

Although some pilot and laboratory experiments did test the effects of chlorination, the results discussed below will be limited to those experiments where a

disinfectant was not added . As indicated in the model development section, the cell death caused by the addition of disinfectant can be significant, and is likely to occur at different rates in the bulk liquid and biofilm. Variables assessed include coliform inoculum growth rate and substratum composition (from the lab studies), and of the effects of temperature, substrate addition, and residence time (from the pilot scale studies) as well as a comparison of the performance of the three types of reactors (substrate added, 20° C, mild steel substratum) (Table 6.1).

#### Bacterial Enumerations

The majority of the measurements were taken from systems using mild steel as the substratum for biofilm growth. Because of the large amount of corrosion products and the interference introduced by their presence in most enumeration methods, plate counts were used. Biofilm cell numbers were determined by scraping the coupon from the reactor into ten ml sterile water, homogenizing the deposit to disperse the bacteria, serial dilution as necessary, and spread plating in triplicate on R2A (heterotrophs) or mT7 medium (coliforms). R2A was incubated at room temperature for 7 days and mT7 at 35° C for 24 hrs. The numbers of colonies on the three plates were counted, averaged, and reported as colony forming units per unit surface area (cfu/cm<sup>2</sup>). Bulk fluid samples were also homogenized prior to plating in triplicate on the same media, the counts averaged, and the data recorded as cfu/ml.

Table 6.1. Experimental conditions and design for the pilot and laboratory studies from which growth rates were calculated.

System	Experiment	Reactor No.	Conditions			Comments	
			Substrate <sup>a</sup>	Temp (C)	Residence		
					Time (hr)		
Pipe Loop	1	1,2	added	20	2		
		3	added	20	2	fed from loop 1	
		4	added	20	4	fed from loop 3	
		5	added	20	8	fed from loop 4	
	2	1	no	20	2		
		2	no	10	2		
		3	added	10	2		
		4,5	added	20	2		
		5	added	20	2		
Annular Reactor (Pilot scale)	1	1:1,2:1 <sup>b</sup>	added	20	2		
		1:2,2:2	added	20	2	fed from reactor 1	
		1:3,2:3	added	20	4	fed from reactor 2	
	2	1:4,2:4	added	20	8	fed from reactor 3	
		1:1,1:2	no	10	2		
		1:3,1:4	added	10	2		
		2:1,2:2	no	20	2		
		2:3,2:4	added	20	2		
Annular Reactor (Lab scale)	1,2	mild steel <sup>c</sup>	added	20	2	Coliform inoculum grown at 0.05 1/hr	
	3,4	polycarb	added	20	2		
	5	mild steel	added	20	2	Coliform inoculum grown at 0.1 1/hr	
	6	polycarb	added	20	2		

<sup>a</sup>In pilot experiments, 500 µg/L was used (added) or not as a supplemental nutrient.

<sup>b</sup>Numbers refer to Train No:Reactor No.

<sup>c</sup>material of construction of removable slides.

### Assimilable Organic Carbon (AOC) Analysis

The amount of carbon substrate remaining in the reactors was measured by the AOC method developed by van der Kooij, et al. (1982) and modified by LeChevallier, et al. (1993b). Briefly, the method consists of collecting water samples in critically cleaned 40 ml glass vials sealed with a Teflon septum, pasteurizing the sample at 70° C for 30 min to kill the indigenous organisms, and cooling to room temperature. Vials are then inoculated with a known concentration of a *Pseudomonas sp.* P17 and a *Spirillum* designated as NOX. Samples are removed from the vials on a daily basis, diluted as necessary, and the numbers of organisms determined in triplicate by the spread plate method on R2A agar. The maximum number of organisms produced is converted to carbon equivalents using a factor obtained from a growth curve of the test organism on acetate.

### Results

Growth rate calculations using both models described above were made using the pipe loop and annular reactor data from experiments 1 and 2 of the pilot scale system (residence time and substrate load/temperature variations, respectively) and from laboratory studies on the effect of substratum type and coliform inoculum growth rate. The numbers of organisms in the various experiments in the biofilm and bulk phases have been reported previously, and subjected to a rigorous statistical analysis (Camper, 1995a and c). In general, the HPC numbers in the bulk fluid ranged from  $10^6$  to  $10^7$  cfu/ml in the pilot scale experiments and from  $10^5$  to  $10^6$  in the laboratory systems. Numbers of

coliforms in the bulk fluid ranged from none detected to over 100 cfu/ml. Biofilm cell counts for the two organisms in both lab and pilot systems were about an order of magnitude higher, but with units of cfu cm<sup>-2</sup>. The two models outlined above were applied to the time series data collected from these systems. The modeling results were used to evaluate the ability of a single parameter (the population growth rate) to describe phenomena observed by separately evaluating the individual populations at the substratum and in the bulk fluid. It was also of interest to determine if the growth rate parameter might provide further insight into the behavior of the two biofilm populations in these various systems in response to changes in the environmental and operating conditions.

A number of preliminary comparisons were carried out to determine the general performance of the model. First, the growth rate calculated using the assumption of negligible bulk growth (the "no bulk" assumption) was compared to the growth rate calculated using the assumption that the biofilm and bulk fluid growth rates were equal (the "bulk=film" assumption). This comparison was made for all of the systems and for both types of organisms. Results of such a comparison for the combined HPC and coliform data from all the lab system calculations are shown in Figure 6.1. At high growth rates, the calculated values with the "no bulk" assumption are significantly higher than those using the "bulk=film" assumption. At low values, the two methods produce approximately equal results. Similar results were found in all of the pilot scale study calculations. The assumption of no bulk fluid growth was made based on the comparison of bulk fluid growth rate to dilution rate. Growth rates on the order of 0.1 to 0.3 hr<sup>-1</sup> are

obviously not negligible in comparison to a dilution rate of  $0.5 \text{ hr}^{-1}$ . Since both methods produced approximately the same results at low growth rates, and the high growth rates invalidated the assumption of no bulk growth in comparison to the dilution rate, the superior "bulk=film" assumption was used for all further calculations.

Replicate sampling of reactor fluids and coupons removed from the systems further demonstrated that pipe loop results were reproducible to within 20%, and that annular reactor results were often within 10%. Based on this degree of variability, a sensitivity analysis of the model output was conducted. Mean values of the time derivatives (both bulk fluid and biofilm) and of the time-averaged cell counts (bulk fluid effluent and influent; and biofilm) were all calculated from the measurements of loops 4 and 5 of experiment 2, representing  $20^\circ \text{ C}$  operation at high substrate load and a residence time of 2 hours. These mean values were then used to calculate the growth rate of each set of organisms. This result is presented in Table 6.2 along with the actual means and standard deviations of the calculated growth rates. Each of the input parameters (time derivatives and cell counts) were then individually incremented or decremented by 20% to observe the effect on the calculated growth rate. The results of this sensitivity analysis are presented in Figure 6.2 as the ratio of the new calculated growth rate to that of the datum case. From this figure, it may be seen that the calculated growth rates are very insensitive (<2%) to variations in the values of the time derivative and influent cell counts (Note: no coliforms were ever detected in the system influent). Conversely, calculated growth rates were approximately directly affected by changes in the bulk fluid cell counts, and inversely affected by changes in the biofilm cell counts.

Table 6.2. Mean growth rate, standard deviation, and calculated growth rate ( $\text{hr}^{-1}$ ) from mean parameter values for loops 4 and 5 from experiment 2.

	Heterotrophs	Coliforms
Mean population growth rate	0.063	0.025
Standard deviation of the mean	0.027	0.046
Growth rate from parameter means	0.062	0.043

As can be seen from equation (5), the difference between the specific growth and detachment rates is also related to the time derivative. When linear regressions were made of  $\mu$  versus  $k_d$ , the slopes were never significantly different from 1.0. Standard errors were  $0.003 \text{ hr}^{-1}$  for the heterotrophs and  $0.006 \text{ hr}^{-1}$  for the coliforms. Thus, the influence of the time derivative becomes important only at low growth rates well below the means measured in this experiment (Table 6.2).

After the performance of the model had been investigated, it was used with experimental data to determine if the tested variables influenced the growth rates of the coliforms and/or heterotrophs in laboratory annular reactors, pilot annular reactors, and pilot pipe loops. The intent was to provide practical information on growth rates that could be used to explain regrowth events.

Comparisons of calculated growth rates were made with laboratory reactor data from experiments where the systems were fed  $250 \mu\text{g C/L}$  and the substratum material was either mild steel or polycarbonate. Both coliforms and heterotrophs tended towards

higher growth rates on the inert substratum. Regardless of the substratum composition, the calculated heterotroph growth rate exceeded that of the coliforms (Fig. 6.3).

Because the materials of construction and the raw water feed were identical in the two pilot systems (pipe loops and annular reactors), calculated growth rates from these two systems were compared. Because scatter plots did not produce any useful information, the ranges of calculated growth rates were compared (Table 6.3). In the first experiment, where individual reactors had residence times of 2 to 8 hours (up to 16 hours cumulative residence time), the range of HPC growth rates in the pipe loop system was approximately twice as wide as in the annular reactors, while the range of coliform growth rates was about 10 times higher in the pipe loops than in the annular reactors. In experiment 2, where residence time was fixed at 2 hours for all experiments, and temperature and substrate loading were varied, the range of growth rates of HPCs was about 10 times higher in the loops than in the annular reactors, and the range of coliform growth rates was only about 8 times higher. It is also interesting to note that the range of coliform growth rates was significantly higher than the heterotrophs in the first experiment, but that the ranges for the two types of organisms were similar in the second experiment.

Growth rates were also calculated for heterotrophs and coliforms within the temperature/substrate experiment to provide insight on the relative importance of the two variables on organism growth. There was no apparent influence of substrate loading on the growth rates of the heterotrophs (Fig. 6.4), although the quantity of biofilm and suspended numbers were enhanced (Fig. 6.5). Coliform growth rate was insensitive to

substrate load, but the number of organisms was higher (Fig. 6.5). The higher temperature appeared to cause an increase in the growth rate of the heterotrophs in both types of reactors (Fig. 6.6). There was no observable trend in the coliform results (Fig. 6.7).

Table 6.3. Ranges of calculated growth rate values ( $\text{hr}^{-1}$ ) for the pilot scale systems (mean +/- one standard deviation).

Experiment/ System	HPCs		coliforms	
	mean - std. dev.	mean + std. dev.	mean - std. dev.	mean + std. dev.
Exp. 1 Loops	-.03	.04	-.5	.5
Exp. 1 A. R.	-.01	.02	-.04	.04
Exp. 2 Loops	.02	.08	-.01	.05
Exp. 2 A. R.	-.003	.006	-.003	.008

Since growth rate was unaffected by substrate loading, an analysis was performed to assess the importance of measured assimilable organic carbon. In laboratory experiments, there was no association of higher measured AOC concentrations with increased growth rates for either organism (Fig. 6.8). One could infer an inverse relationship between substrate and heterotrophic growth rate in the pilot residence time experiment (Fig. 6.9).

Discussion

At least three other reports of growth rate calculations from steady-state biofilm systems appear in the literature (van der Wende, et al., 1989; Bauda, et al., 1993; Block, et al., 1993). Bauda, et al. (1993) considered time-variant behavior in some of their ancillary studies, but the shocks to which the system was subjected (increases and decreases in salinity and substrate loading) have been shown to generate sloughing and/or detachment events which effectively invalidate application of their results to typical steady flow conditions. In none of those studies was the unsteady-state nature of biofilm development considered. This consideration was necessary in this study because coliform numbers in the biofilm continually declined after inoculation for the first 3 to 5 weeks of reactor operation (Camper, et al., 1995a and b).

Results of the sensitivity study (Fig. 6.2) show that a change of 20% in a parameter results in less than a 25% variation in the growth rate. The least sensitive parameters are the derivatives, leading to the conclusion that the unsteady-state terms are of little consequence. This is probably because the time increments in the derivatives are one or two weeks. Greater sensitivity may be obtained if the sampling interval is decreased. The biofilm and suspended cell counts are marginally more important. Considering that the variability between replicate samples is 10-20%, one would expect that the growth rates would fall into a fairly narrow range ( $\pm 20\%$ ), provided that the organism numbers were not particularly low. This was not the case in any of the systems

(see Table 6.3), where standard deviations typically exceeded the mean. An explanation of the wide range of growth rates was sought.

Inherent variability between samples has been ruled out as indicated above. Time variations on the order of +/-20% do not have any significant effect; time derivatives would need to be several orders of magnitude higher in order to produce the variations in growth rate observed in this study. We believe that the explanation lies in large measure in the method used to enumerate the organisms. Plate counts are inherently selective for organisms that can grow and reproduce on that medium. Further, it has been shown that plate counts typically only detect 1-10% of a population. Since these systems were dominated by an uncharacterized mixed population, significant variations in populations could have occurred between sampling periods, resulting in highly variable plating efficiencies and the resulting high variations in calculated growth rates. It is also possible that an underlying parameter not isolated in the experimental design dominates the growth rate of the organisms.

The pilot and laboratory experiments were designed to provide information on organism response to a suite of distribution system variables. The growth rate analysis was applied to the data to provide phenomenological insight on the response of the organisms to the experimental parameters.

A few general comments can be made about the growth rates in the experimental systems. First, heterotroph growth rates typically exceeded those of the coliforms. This would be expected, since these organisms originated from drinking water and were acclimated to that environment. However, the difference in growth rates between the

types of organisms was surprisingly small, which may explain why the coliforms were capable of successfully competing with the heterotrophs and being retained in the biofilms. Second, in all of the experimental situations, coliform growth rates were relatively insensitive to changes in any of the system parameters. It is believed that this is the result of the extremely low number and high variability in the counts.

Two types of pilot reactor systems, the annular reactors and pipe loops, were built and operated in parallel. The annular reactor systems are more compact and considerably less expensive to operate, and would be the physical model of choice provided that biofilm response is similar to that in a pipe. Previous results have shown that the populations in the two reactors have a similar quantitative response to changes in water quality or operating conditions, but that the annular reactors consistently support approximately ten fold more heterotrophs and four fold more coliforms than the pipe loops (Camper, et al., 1995a). The growth rate calculations from two experiments revealed that heterotrophs in the pipe loops typically grew more rapidly than in the annular reactors. Since these biofilms are near steady state, the specific detachment rate is approximately the same as the specific growth rate. Thus, the biofilms in the pipe loops experience a higher specific detachment rate, resulting in less dense biofilm.

The influence of temperature on the growth rates was investigated as a potential explanation for utility observations that regrowth problems tend to occur in summer when water temperatures are elevated (LeChevallier, et al., 1991; Colbourne, et al., 1991). It has also been shown that biofilm heterotroph numbers increase with water temperature (Donlan and Pipes, 1988). The growth rate analysis supported this observation, with

heterotroph replication accelerated at 20° C over 10° C. The statistical evaluation of the organism numbers was not as conclusive; there were higher numbers of heterotrophs in the annular reactors with increased temperature but a slight decrease in the pipe loops. The difference in actual numbers, however, was relatively small (Camper, et al., 1995a).

Attempts have been made to correlate the amount of carbon substrate entering a distribution system with the potential for biofilm growth. Using the AOC method, van der Kooij (1992) determined that a concentration of 10 µg C/L is the threshold level for heterotrophs, while LeChevallier, et al. (1991) recommended a concentration of < 50 µg C/L to control coliforms. Data analyzed from pilot plant experiments where the added substrate concentration was 0 or 500 µg C/L showed that there were statistically higher numbers of biofilm heterotroph and coliform counts at the higher influent concentration (Camper, et al., 1995a). The growth rate analysis on the same data indicated that although the numbers of biofilm organisms were elevated under higher substrate loading, the growth rate was unaffected. When attempts were made to correlate either numbers of bacteria or the calculated growth rate with the amount of AOC actually measured in the plot systems, there was no correlation. In these experiments, then, the measurement of AOC within the system itself did not appear to carry any significance. This is somewhat in contrast to the findings of van der Kooij and LeChevallier, et al, but it must be remembered that their analysis examined the effect of AOC *entering* a section of distribution system, not what remained after the biofilm had acted upon it. No clear indicator of nutrient availability as it relates to intrinsic growth rate has yet been found.

It is possible that growth rate is affected by the substratum to which the bacteria are attached. This is particularly true of thin biofilms, where the majority of the bacteria will be in direct contact with the substratum. In laboratory experiments where direct comparison between substrata at a similar substrate concentration was possible, both the heterotrophs and coliforms had a higher specific growth rate on polycarbonate than mild steel. When the actual counts are compared, the mild steel reactors supported ten-fold more biofilm bacteria (Camper, et al., 1995b). Block, et al. (1993) compared growth rates and culturable biofilm cells on PVC and cement lined pipe; the growth rates were higher and cell counts lower on PVC. Similarly, Neden, et al. (1992) found that the lowest numbers of biofilm bacteria occurred on PVC pipes. Apparently the plastic surfaces have higher growth rate/detachment characteristics, which result in thinner biofilms.

The growth rates calculated in this study ( $0.01$  to  $0.1 \text{ hr}^{-1}$ ) are considerably higher than those reported by others. At a similar hydraulic residence time in bitumastic-coated pipe annular reactors using plate counts as the detection method, van der Wende (1989) found a growth rate of  $0.006 \text{ hr}^{-1}$  (1989). Block's results (1993) from cement lined pipe were even lower ( $0.0017 \text{ hr}^{-1}$  to  $0.0001 \text{ hr}^{-1}$ ). His values were based on epifluorescent counts, which tend to be much more stable than plate counts. However, total direct count methods do not distinguish between viable and non-viable cells, and may have severely underestimated the growth rate in their systems. Both of these investigators used unsupplemented, unchlorinated finished drinking water as their source.

It was originally thought that the disparities in growth rate may be attributed to the addition of substrate in our experiments. However, growth rates in our reactor systems were unaffected when the water was amended with additional carbon source. The major difference between the literature systems and those reported here is the substratum material (relatively unreactive cement and bitumastic lined pipes vs. highly reactive mild steel). It is possible that the mild steel supported a microbial population with a capable of substantially higher growth. Another hypothesis is that the populations are similar, but growth rates of organisms on mild steel are enhanced. There is some utility evidence to support this hypothesis. In three instances, implementation of corrosion control resulted in the reduction or elimination of coliform regrowth in full-scale distribution systems (Martin, et al., 1982; Lowther and Moser, 1984; Hudson, et al., 1983). Pilot-scale investigations have also implicated corrosion as a major factor contributing to biofilm development (LeChevallier, et al., 1993a).

### Conclusions

- Inclusion of a term allowing for growth of planktonic cells improves the precision of the growth rate model.
- The model was insensitive to changes in time derivatives and moderately sensitive to changes in bulk fluid and biofilm cell numbers.
- When the model was applied to biofilm and bulk fluid data from two types of pilot distribution systems (pipe loops vs annular reactors), it was found that biofilm heterotrophs in the pipe loop grew faster.

- Increased temperature resulted in higher heterotroph growth rates.
- Neither added or measured carbon substrate concentrations were correlated with specific growth rates of the organisms.
- An inert substratum, polycarbonate, supported biofilm organisms with a higher growth rate than those colonizing mild steel. The mild steel surfaces were colonized with more organisms than the polycarbonate.
- Coliform growth rates were generally slightly lower than those for the heterotrophs and insensitive to any of the tested variables.
- Growth rates from this study were considerably higher than those from other drinking water investigations. The major difference in experimental conditions that could contribute to the difference is the pipe material.

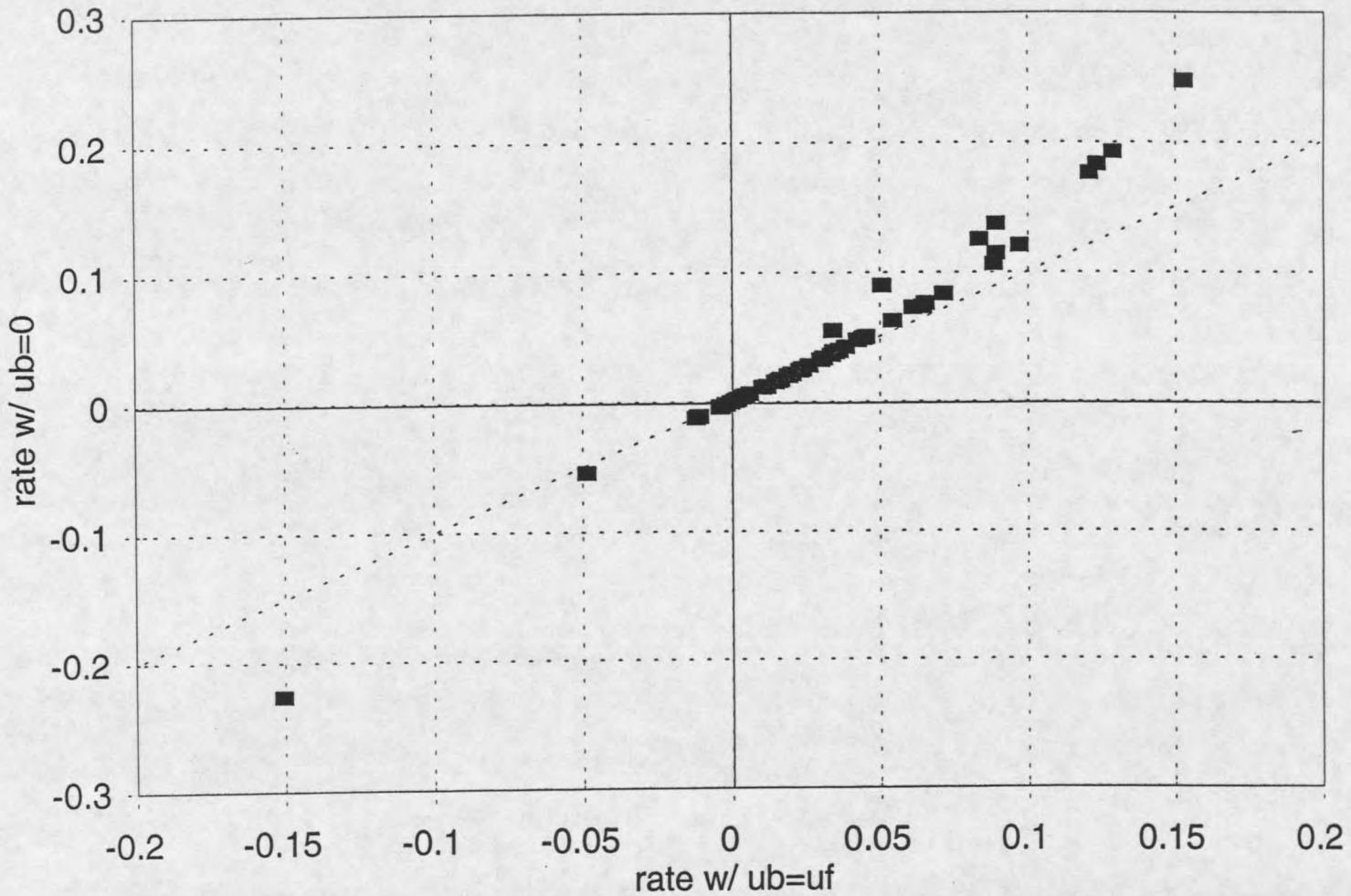


Figure 6.1. Comparison of growth rate values computed from laboratory annular reactor data using the assumption that the bulk growth rate was negligible ( $u_b = 0$ ) or that bulk and biofilm growth rate were the same ( $u_b = u_f$ ). Line of 1:1 slope is shown for comparison.

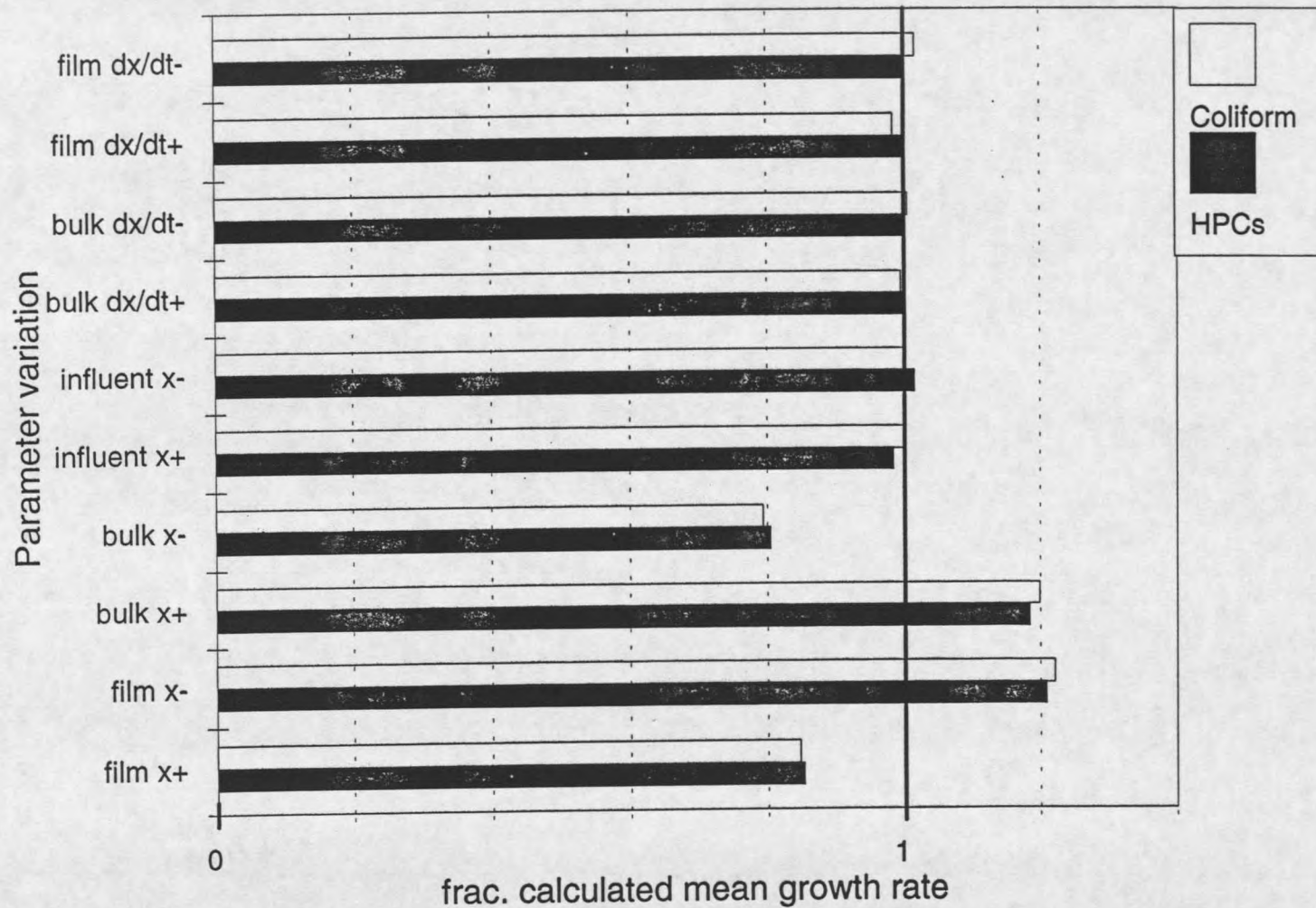


Figure 6.2. Fractional change of calculated growth rate in response to 20% increments (+) or decrements (-) in the designated parameter.

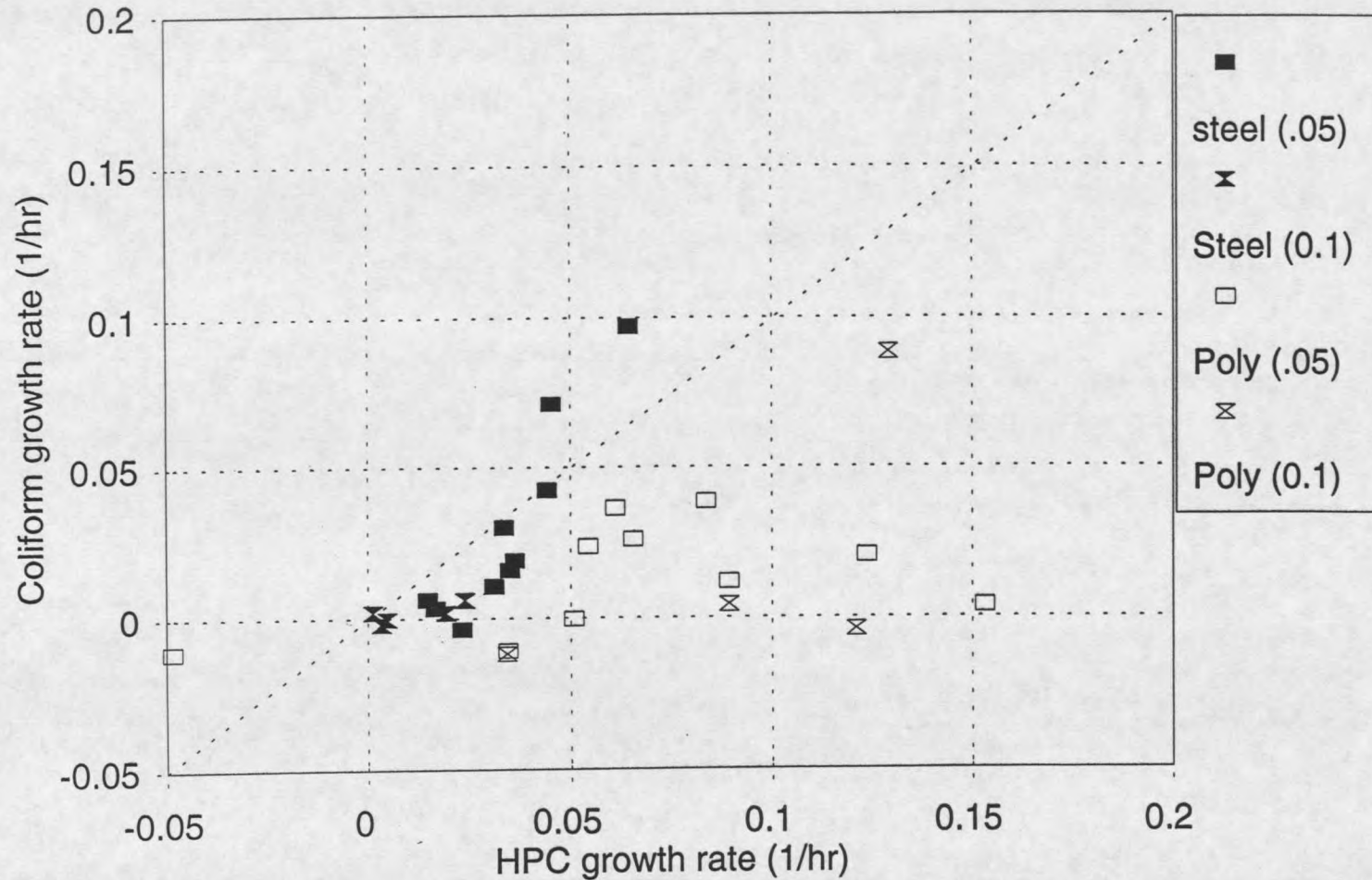


Figure 6.3. Comparison of the growth rates of heterotrophs and coliforms calculated from laboratory annular reactor data from experiments where the initial growth rate of the coliforms (0.1 or 0.05/hr) and the substratum composition (mild steel or polycarbonate) were varied. Line of 1:1 slope is shown for comparison.

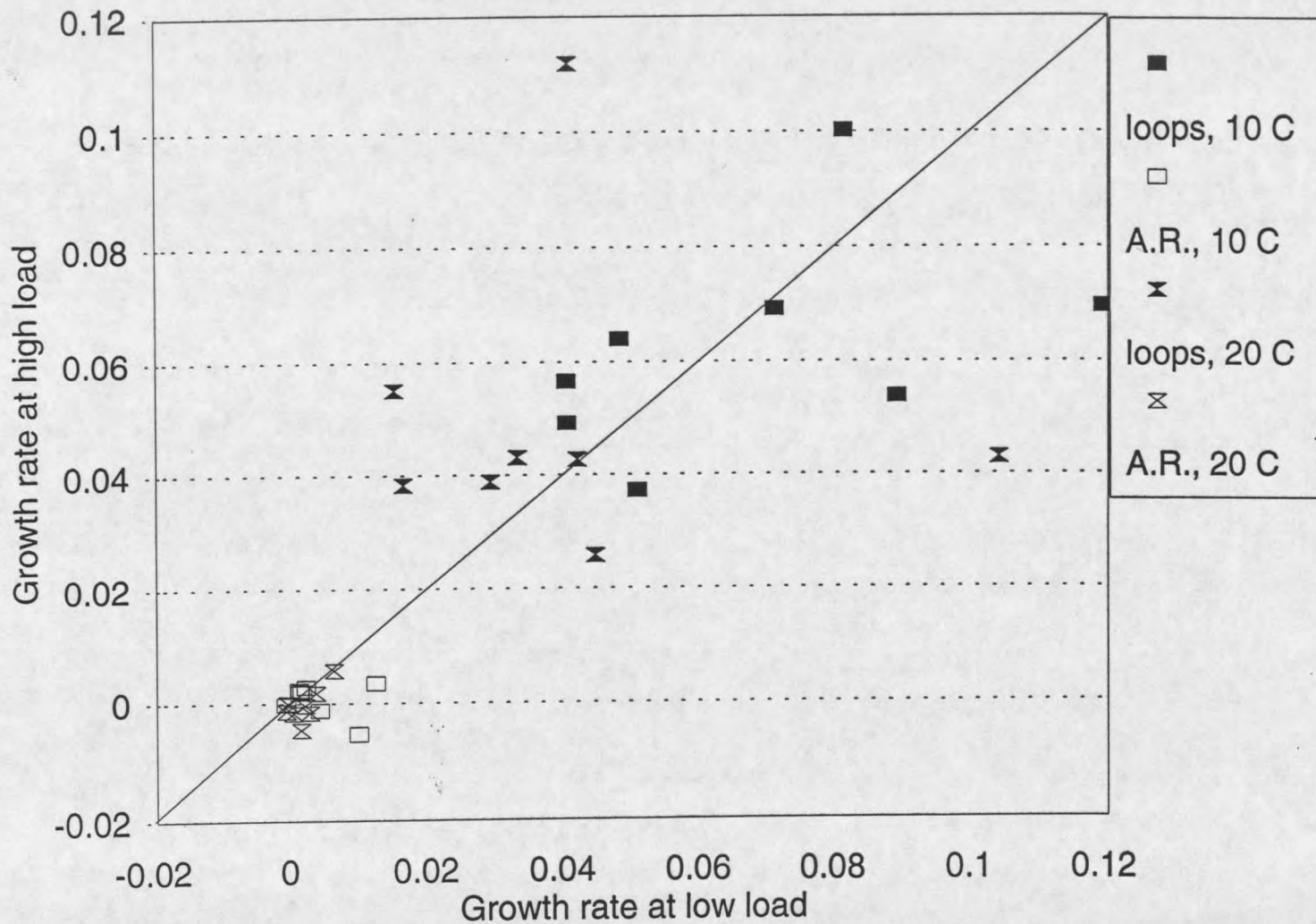


Figure 6.4. Comparison of heterotroph growth rates in pilot annular reactors (A.R.) or pipe loops (loops) operated at 10 or 20° C supplemented with 500 µg C/L (high load) or no added carbon substrate (low load). Line of 1:1 slope is shown for comparison.

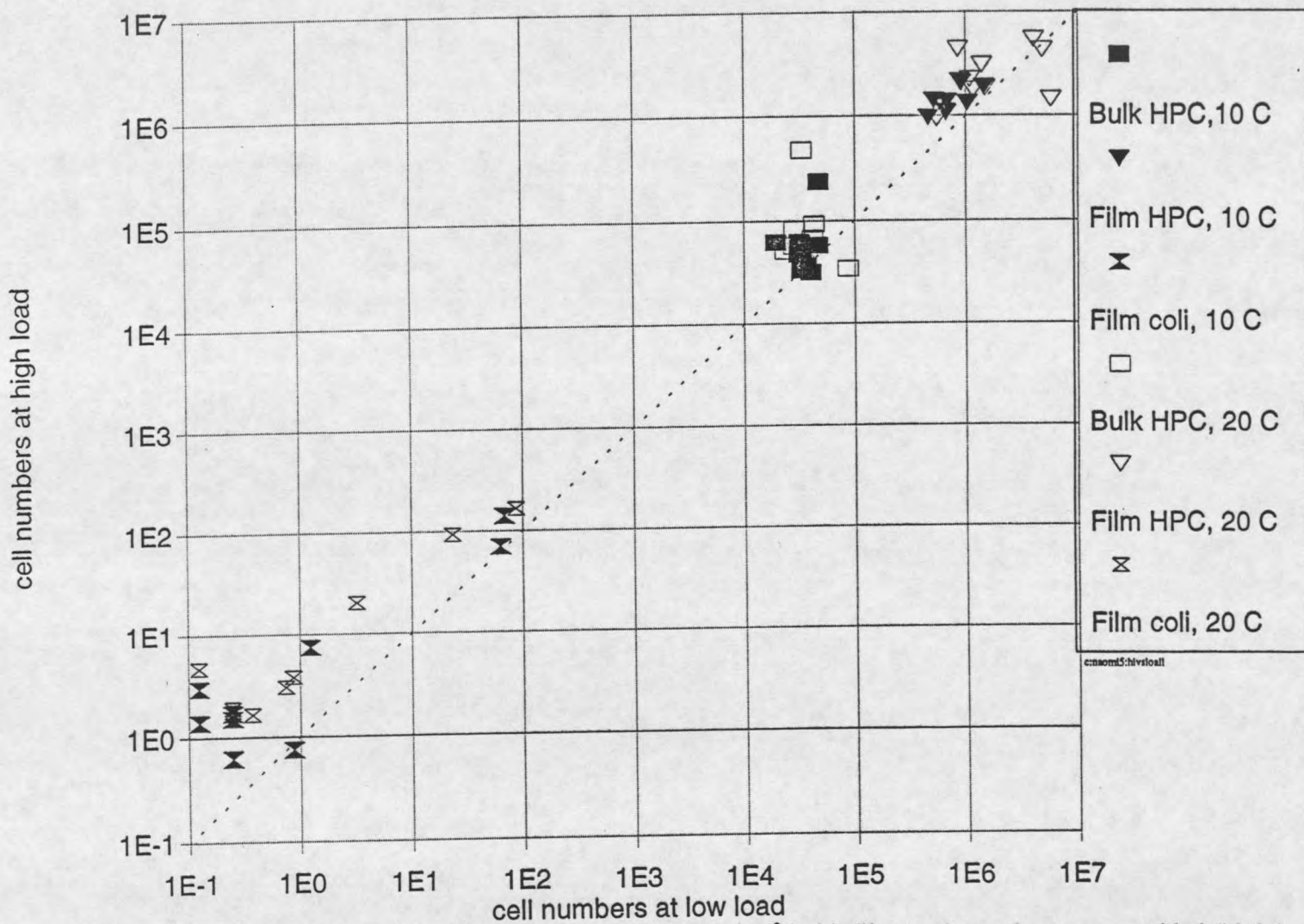


Figure 6.5. Comparison of organism numbers (cfu/ml in bulk fluid, cfu/cm<sup>2</sup> in biofilms) when substrate was added (high load) versus when it was not (low load). Line of 1:1 slope is shown for comparison.

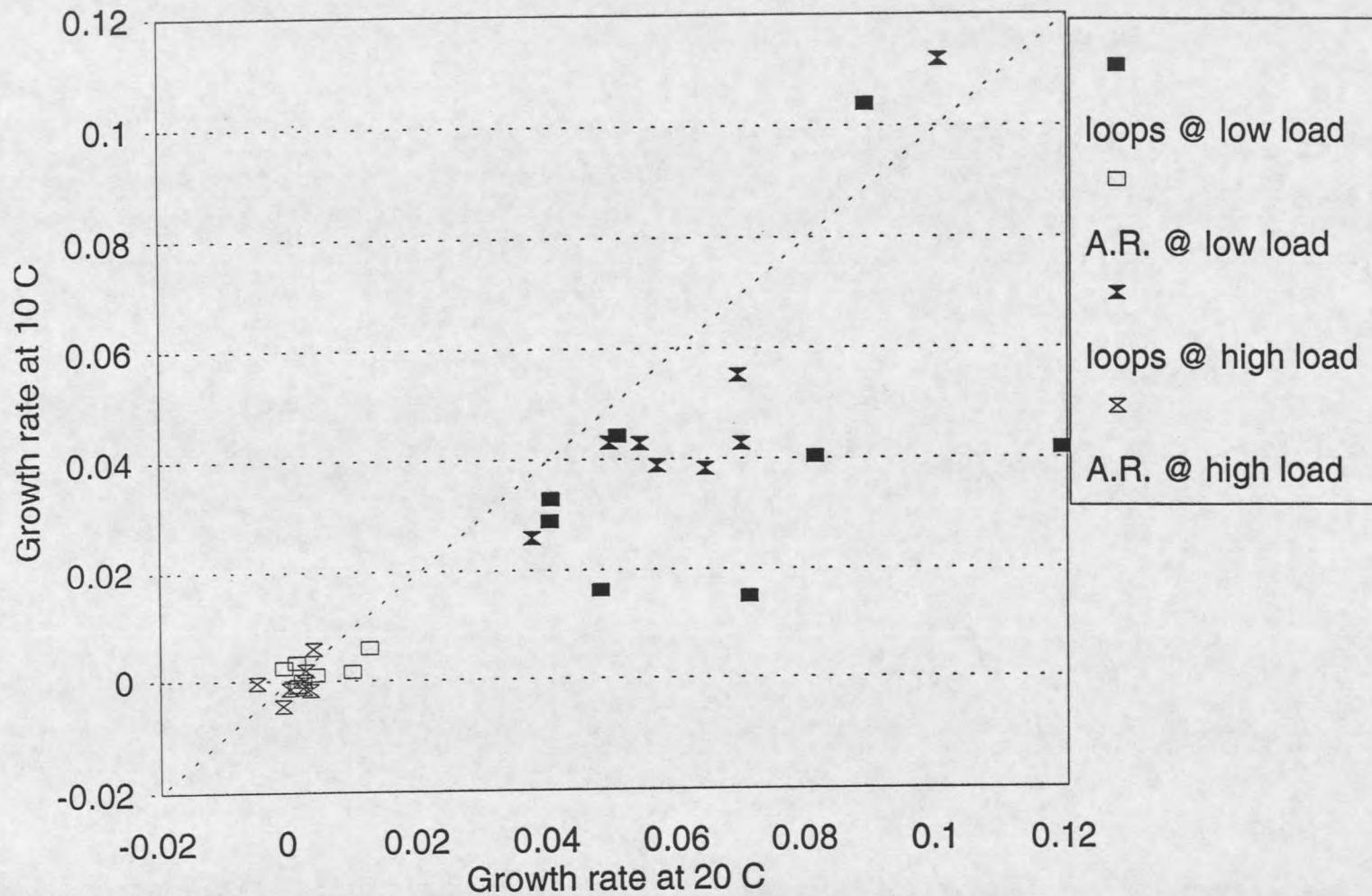


Figure 6.6. Effect of temperature on the growth rate of heterotrophs in pipe loop (loops) or annular reactor (A.R.) pilot systems receiving either 500  $\mu\text{g C/L}$  (high load) or no added organic carbon (low load).



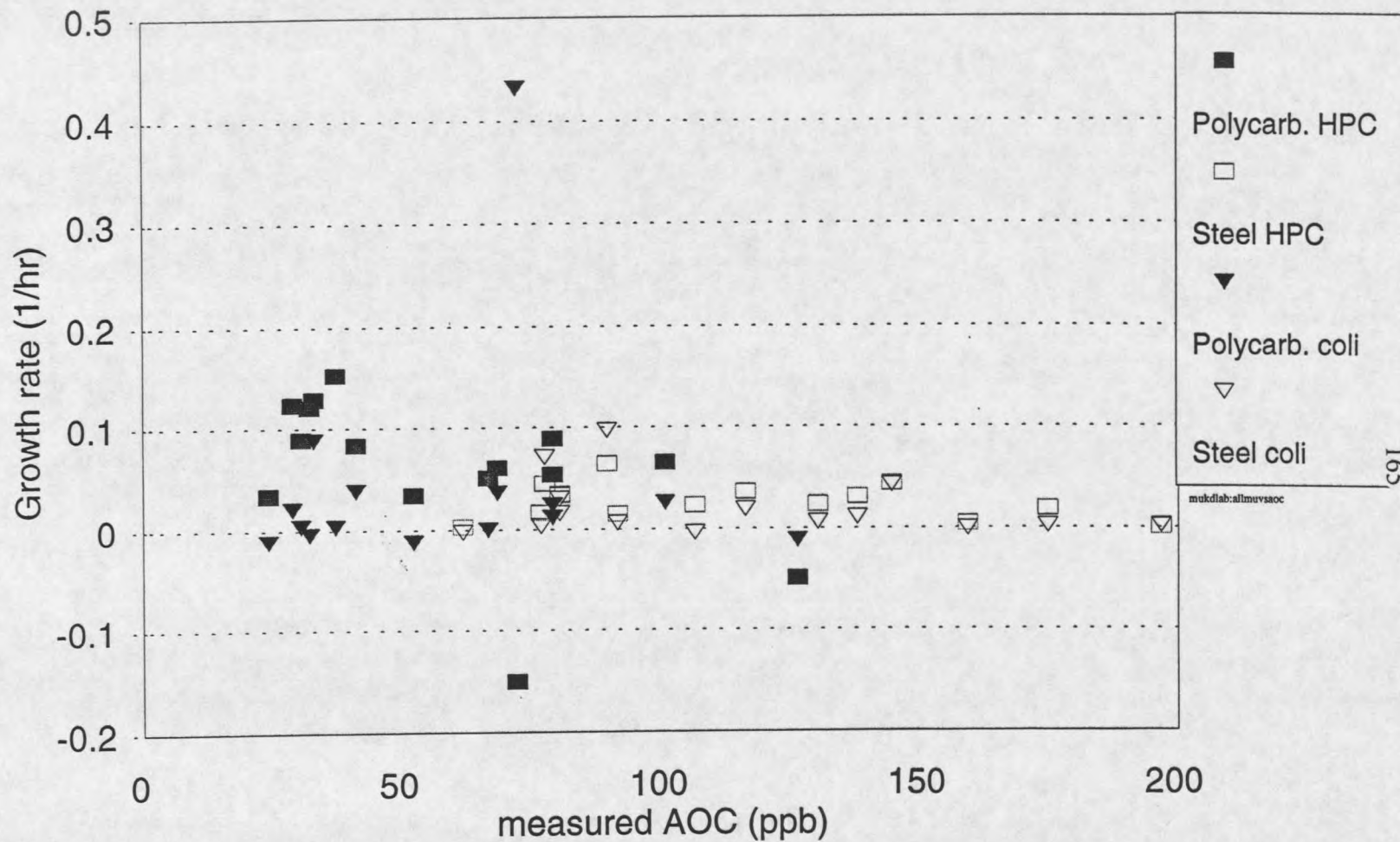


Figure 6.8. Influence of measured substrate (assimilable organic carbon, AOC) on the growth rates of heterotrophs and coliforms in laboratory reactors containing either mild steel (steel) or polycarbonate (polycarb) slides.

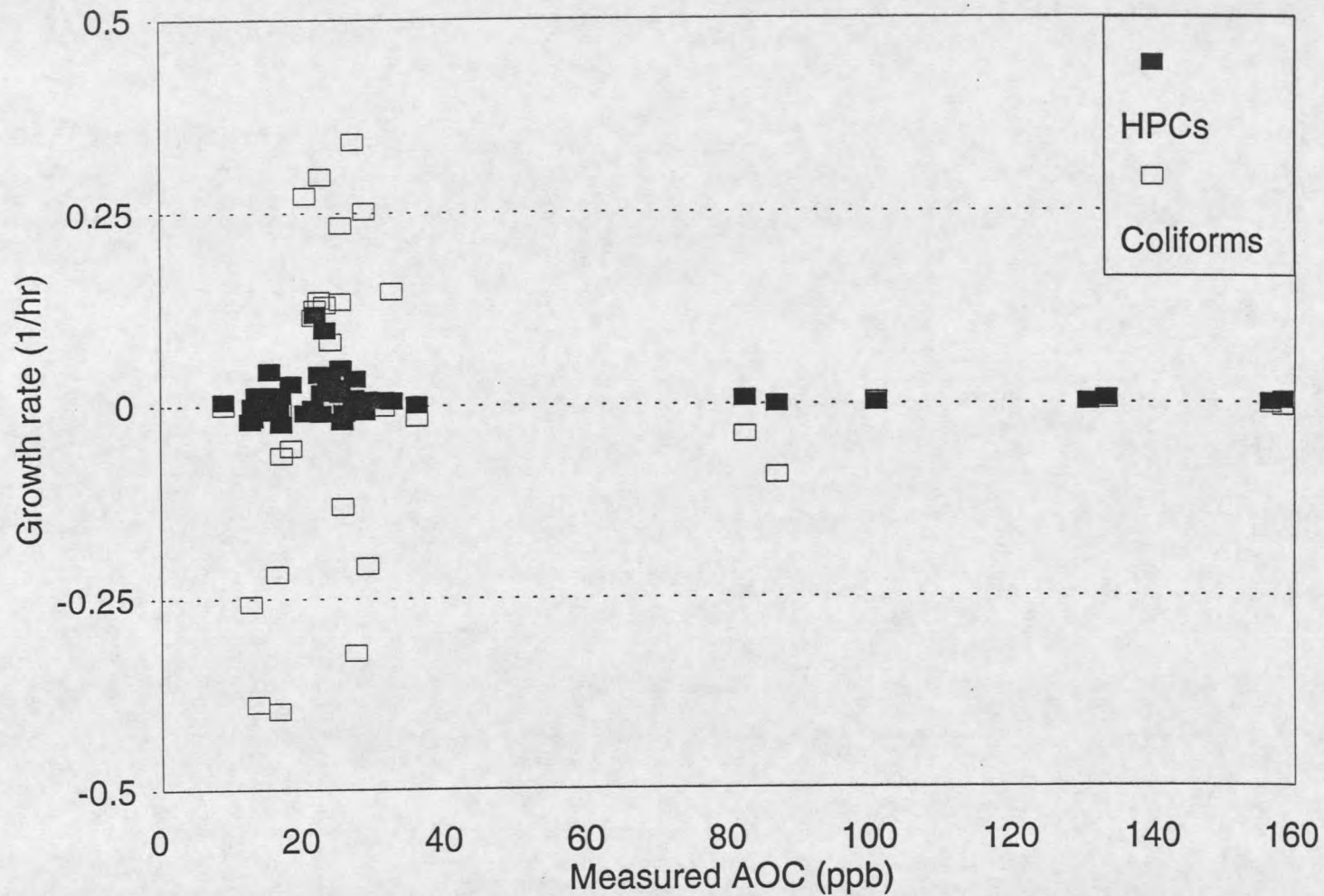


Figure 6.9. Influence of measured substrate (assimilable organic carbon, AOC) on growth rates of heterotrophs and coliforms in pilot pipe loop reactors operated at several hydraulic residence times.

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## CHAPTER 7

## SUMMARY

A comprehensive research plan was developed to investigate factors which influence the growth of microorganisms, particularly coliforms, in drinking water distribution system biofilms. The approach included laboratory studies to determine the importance of initial growth rate and substratum composition (pipe material) on the colonization potential of coliforms in mixed population biofilms; pilot-scale experimentation in two model distribution systems to assess the impact of temperature, carbon substrate levels, and presence of chlorine on biofilm accumulation/behavior; and modeling efforts to quantify the growth rates and response of the bacteria to these various parameters. This research was part of a project that also contained a major field study of drinking water distribution systems to identify field conditions that correlate with regrowth events. Integration of methods results across the three scales of observation (laboratory microscale, pilot mesoscale, field macroscale) provided information that can be used by the drinking water industry in their efforts to attain compliance with complex federal regulations.

At the laboratory and pilot scales, research was integrated so that direct comparisons could be made between the systems. For example, shear stress, temperature, substrate composition, coliform inoculum preparation, and most measurement techniques

were identical, regardless of the system. Some experiments and methods were more easily performed in one system with the results applied to the other. The influence of initial growth rate on coliform persistence was carried out in laboratory systems and the optimum conditions used to prepare the inoculum for the pilot reactors. Because substratum materials could be varied in the laboratory units, they were used to evaluate the impact of pipe materials on colonization. Conversely, pilot systems were used to establish the hydraulic residence times used at the laboratory scale. Although the conclusions are divided into laboratory and pilot sections for clarity, they should be viewed as parts of a consolidated effort.

#### Laboratory Studies

Microscale or laboratory polycarbonate annular reactors were used for experiments not practical in the pilot system, to obtain preliminary information for pilot plant experiments, and to provide fundamental information of biofilm behavior under relevant drinking water conditions. A mixture of five carbon sources representative of classes of compounds found in drinking water were used as the carbon source at a final influent concentration of 250  $\mu\text{g C/L}$ . Reactor temperature was maintained at 20° C, hydraulic residence time was set at 2 hr, and a shear stress of 0.27  $\text{N/m}^2$  was developed at the reactor wall. Research was performed to determine conditions required by a mixture of five coliforms originally isolated from drinking water to successfully colonize and persist in mixed-population biofilms on two substrata (mild steel and polycarbonate).

Important findings were:

- The initial growth rate of introduced coliforms affects their ability to persist in mixed-population biofilms. A net growth rate of 0.05/hr, the lowest examined in these experiments, was optimal. Long term colonization was also obtained when the growth rate was 0.1/hr, but not with values of 0.13 and 0.2/hr.
- The influence of initial growth rate persisted for the entire eight week experimental run time, and was apparent even when clean substrata were introduced into previously colonized reactors.
- Biofilm surface coverage was never exceeded approximately 60%. The species *Klebsiella pneumoniae*, as observed directly with a fluorescently tagged monoclonal antibody, existed as small microcolonies dispersed over the surface.
- Biofilm coliforms reached a steady-state concentration after approximately three or four weeks. Heterotrophs were at steady-state at two weeks.
- Although the numbers of coliforms per unit surface area at the end of two replicate experiments were the same, the predominant organism was different (*K. pneumoniae* vs. *Enterobacter cloacae*).
- Substratum composition influences the level at which both heterotrophs and coliforms persist in biofilms. Mild steel supported approximately ten fold more of each type of bacteria than polycarbonate.
- Influent concentrations of 0.2 and 0.5 mg/L free chlorine in reactors with polycarbonate slides decreased the number of viable biofilm and effluent heterotrophs by approximately two logs, but had little effect on coliforms.

Respiring biofilm cell numbers initially decreased and then attained levels comparable to those in a parallel non-chlorinated control reactor.

- When biofilms were grown on mild steel, an influent concentration of 1.0 mg/L  $\text{Cl}_2$  had no influence on biofilm heterotrophs and slightly stimulated the coliforms.

### Pilot Studies

Mesoscale or pilot studies were carried out to evaluate the influence of hydraulic residence time, temperature, substrate loading, and the presence of chlorine on regrowth events. Whenever possible, operational conditions were identical to those used in the lab to provide continuity between the two scales. To more closely simulate conditions found in full-scale distribution systems, two pilot-scale facilities were built and utilized for the research. These consisted of two trains of four annular reactors each and a five section pipe loop system, both composed of mild steel. An evaluation of the comparability of results between the two reactor types was completed. Significant findings were:

- Each pipe loop was well mixed and could be modeled as an ideally mixed reactor. This was substantiated by an absence in spatial gradient of biofilm density. Annular reactors were similarly uniform.
- In general, reproducibility of biofilm and suspended coliforms and heterotrophs between paired annular reactors or pipe loops was good.
- Ratios of annular reactor to pipe loop organism numbers were surprisingly constant within each population (10:1 for heterotrophs, 4:1 for coliforms). The

trends of bacterial numbers in response to changes in temperature, substrate, and chlorine were similar. Coupled with ease of operation and lower expense, the annular reactors are recommended as a pilot distribution system.

- Coliforms were more abundant at the shortest (2 hr) hydraulic residence time while heterotroph levels were unaffected.
- Increased substrate loading resulted in elevated coliform and heterotroph numbers in biofilms and the bulk fluid. An independent decrease in temperature resulted in a slightly enhanced number of biofilm coliforms. There was no cooperativity between the two variables.
- Chlorination somewhat reduced the numbers of biofilm and suspended heterotrophs, but was ineffective at controlling coliforms. There were consistently higher numbers of coliforms in reactors that had elevated levels of substrate and chlorine as opposed to those with substrate alone.

#### Modeling of Growth Rates

A model was developed to calculate the growth rates of heterotrophs and coliforms using data from the laboratory and pilot experiments. The model was based on cell mass balances and incorporated the opportunity for growth of the planktonic cells at the same net cell growth rate as the biofilm organisms. A sensitivity analysis of the model determined that it was insensitive to changes in the time derivative and moderately sensitive to differences in the biofilm and bulk fluid bacterial counts. Calculation of the

growth rates of heterotrophs and coliforms in the reactors lead to the following observations:

- When the model was applied to biofilm and bulk fluid data from two types of pilot distribution systems (pipe loops vs annular reactors), it was found that heterotrophs in the pipe loop had a higher net growth rate. Conversely, the annular reactors were more heavily colonized.
- Increased temperature resulted in higher heterotrophic growth rates in both reactors.
- Neither added nor measured carbon substrate concentrations correlated with specific growth rates of the organisms.
- An inert substratum, polycarbonate, was associated with heterotrophic populations with a higher growth rate than seen on mild steel, but the latter was more heavily colonized.
- Coliform growth rates were generally slightly lower than those for the heterotrophs and insensitive to any of the tested variables.
- Growth rates from this study were considerably higher than those calculated in other drinking water investigations. The major difference in experimental conditions that could contribute to the difference is the pipe material.

### Industrial Relevance

There are several conclusions from the research that are of particular relevance to the drinking water industry as it seeks understanding of coliform regrowth events. It was demonstrated that coliforms can become established as members of mixed population biofilms grown in reactors operated under relevant drinking water distribution system conditions. Growth rate of the organisms when they are introduced appears to be a critical factor in their ability to effectively compete and remain on the surface long-term. Slower growing coliforms are more likely to persist, and have growth rates in the system comparable to those of the heterotrophs. Although further research is needed for confirmation, it is probable that slow-growing coliforms are likely to arise from environmental sources and are potentially of minimal public health concern. Conversely, organisms arising from fecal contamination are growing much faster, and are less likely to persist in distribution system biofilms.

It was also determined that coliforms resulting from detached biofilm cells are capable of recolonizing the reactor surfaces. This may explain why utilities have recurrent problems with coliforms even when no known "reinoculation" of the system has occurred.

Biofilm organisms were not distributed uniformly on the substratum. *Klebsiella pneumoniae* persisted in microcolonies, producing a pattern that was more heterogeneous across the surface than the other attached bacteria. This configuration may partially explain why coliforms are often not controlled by chlorination.

Pipe composition appears to be a critical factor. Although the growth rates of heterotrophs were higher on polycarbonate, there were more heterotrophs and coliforms on mild steel surfaces. When biofilms on mild steel were exposed to chlorine, there was no effect on the organisms. Growth rates from the mild steel reactors in these investigations were orders of magnitude higher than those from other investigators' studies where the surfaces were coated with bitumen or cement.

Increased substrate loading results in higher numbers of both types of organisms in the biofilm and bulk fluid. However, measured concentrations of assimilable organic carbon in the reactors were not correlated with added substrate, bacterial numbers or growth rates. This may explain why there has been an inability in many instances of associating regrowth with concentrations of utilizable carbon within distribution systems. Since treatment plants can be designed and/or operated to reduce the amount of carbon entering the distribution system, this may be an appropriate way to control regrowth events.

Increased temperature did not appear to result in higher numbers of coliforms whereas it was associated with higher heterotroph growth rates. Since utilities cannot control the temperature of their water, these results are encouraging.

Chlorination was not effective in controlling coliforms in the bulk fluid or biofilm. On polycarbonate surfaces in the presence of chlorine, culturable heterotrophs declined and coliforms were unaffected. Mild steel afforded a protective niche for both groups of biofilm organisms from chlorination. When chlorine was added to systems with elevated levels of carbon substrate, there were more coliforms than in reactors

receiving carbon without disinfectant or those operated with no added carbon or disinfectant. This may explain why utilities are generally unsuccessful in controlling regrowth events by simply raising the amount of chlorine in the distribution system. Comparisons between data from parallel annular reactors and pipe loops used as simulated distribution systems showed that the qualitative response of the organisms to operational parameters was similar. Bacterial numbers in the annular reactors tended to be higher, while growth rates in the pipe loops were greater. When considering the cost, ease of operation, flexibility in experimental design, size, and qualitative similarity in data between the systems, the annular reactors are a preferable tool.

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