Pathogens in model distribution system biofilms
by Malcolm Robert Warnecke

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

Abstract:
Little is known about the behaviour of specific pathogenic microorganisms within a drinking water distribution system. This is particularly the case with surface-associated organisms, which have previously been demonstrated to have survival advantages over organisms in the bulk phase, and can serve as a reservoir of contamination. The drinking water environment can bring about physiological changes in organisms, leading to difficulties in their detection and changes in behaviour to that seen in other, more laboratory-oriented environments.

This study involved annular reactors operated to simulate a drinking water pipe environment. The reactors were initially seeded with a monoculture of one of two enteric pathogens (Salmonella typhimurium and Escherichia coli O157:H7) or two other bacterial strains of interest (Aeromonas hydrophila and Klebsiella pneumoniae). Following a period of establishment, a heterotrophic population was continuously added to the reactor. After the biofilm population was allowed to reach a pseudo-steady state, some reactors were treated with chlorine at levels typically observed in end-reaches of a distribution system. Samples of the bulk phase and the biofilm of the reactors were taken over the course of the experiment. These were examined using nonselective and selective media, and also by confocal microscopy using fluorescently labelled antibodies, DNA staining, and viability staining. Some colony identification was performed using the polymerase chain reaction.

The different bacteria studied showed significantly different behaviour under the tested conditions. Salmonella typhimurium rapidly became nondetectable with culture methods, but was still detectable using microscopy. It was found to persist in the biofilm, with at least some of these cells still viable. Escherichia coli O157:H7 did not persist well in the test reactors. Aeromonas hydrophila demonstrated variable behaviour between experiments, generally showing declining persistence and a loss of culturability in both bulk phase and biofilm, but an increase in culturable counts from growth or resuscitation in one replicate. Klebsiella pneumoniae showed a slow decline in culturability and overall numbers, rather than the growth expected with this organism. Chlorination reduced the numbers of test organisms in the reactors; cells in the biofilm were less affected by disinfection. Numbers of target cells determined by selective media were usually reduced to below detectable levels by chlorination, while immunofluorescence counts were less affected. Reactor conditions were shown to be similar between experiments by the levels of heterotrophic bacteria present.

Because the observed behaviour of similar bacterial pathogens was shown to be quite different in the test environment, it is unlikely that a single organism or group of organisms could be monitored to predict pathogen behaviour. Changes in bacterial physiology resulted in poor detection of pathogens using selective media. Surfaces can give pathogens various survival advantages, and are a likely reservoir for some organisms within a drinking water distribution system.

PATHOGENS IN MODEL DISTRIBUTION SYSTEM BIOFILMS

by

Malcolm Robert Warnecke

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Microbiology

MONTANA STATE UNIVERSITY-BOZEMAN
Bozeman, Montana
August 1996

APPROVAL

of a thesis submitted by

Malcolm Robert Warnecke

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.

Anne Camper _____ 9/3/96
(Signature) Date

Approved for the Department of Microbiology

Al Jesaitis _____ 9/3/96
(Signature) Date

Approved for the College of Graduate Studies

Robert Brown _____ 9/16/96
(Signature) Date

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a master's degree at Montana State University-Bozeman, I agree that the Library shall make it available to borrowers under rules of the Library.

If I have indicated my intention to copyright this thesis by including a copyright notice page, copying is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for permission for extended quotation from or reproduction of this thesis in whole or in parts may be granted only by the copyright holder.

Signature M Darnell

Date 4th Sep, 1996.

ACKNOWLEDGEMENTS

I would like to acknowledge the support of the American Water Works Association Research Foundation and the National Science Foundation for providing major funding for this project, and Australian Water Technologies (Ensight) for partial transport funding.

A big "thank you" for the many people who have assisted during this project.

Anne Camper, as project initiator and organizer, academic advisor, committee chair, and overall prime mover.

Gordon McFeters and Cliff Bond as committee members.

These guys made a great committee. I really appreciated the advice and proof-reading.

The rest of the AWWARF group, particularly Brian Ellis and Pierre Morin, for methods advice, general assistance, and attitude.

Guy Cooke and Paul Stoodley, imaging and confocal gurus.

Marty Hamilton for advice on statistics and data analysis.

Nick Ashbolt and Peter Cox, as the Australian connection.

Last but definitely not least, Norma and Heinz, Susan, Peter, and of course Tina, for understanding, and for being there.

TABLE OF CONTENTS

TABLE OF CONTENTS - Continued

TABLE OF CONTENTS - Continued

LIST OF FIGURES

LIST OF FIGURES - Continued

LIST OF FIGURES - Continued

LIST OF FIGURES - Continued

LIST OF FIGURES - Continued

LIST OF FIGURES - Continued

ABSTRACT

Little is known about the behaviour of specific pathogenic microorganisms within a drinking water distribution system. This is particularly the case with surface-associated organisms, which have previously been demonstrated to have survival advantages over organisms in the bulk phase, and can serve as a reservoir of contamination. The drinking water environment can bring about physiological changes in organisms, leading to difficulties in their detection and changes in behaviour to that seen in other, more laboratory-oriented environments.

This study involved annular reactors operated to simulate a drinking water pipe environment. The reactors were initially seeded with a monoculture of one of two enteric pathogens (*Salmonella typhimurium* and *Escherichia coli* O157:H7) or two other bacterial strains of interest (*Aeromonas hydrophila* and *Klebsiella pneumoniae*). Following a period of establishment, a heterotrophic population was continuously added to the reactor. After the biofilm population was allowed to reach a pseudo-steady state, some reactors were treated with chlorine at levels typically observed in end-reaches of a distribution system. Samples of the bulk phase and the biofilm of the reactors were taken over the course of the experiment. These were examined using nonselective and selective media, and also by confocal microscopy using fluorescently labelled antibodies, DNA staining, and viability staining. Some colony identification was performed using the polymerase chain reaction.

The different bacteria studied showed significantly different behaviour under the tested conditions. *Salmonella typhimurium* rapidly became nondetectable with culture methods, but was still detectable using microscopy. It was found to persist in the biofilm, with at least some of these cells still viable. *Escherichia coli* O157:H7 did not persist well in the test reactors. *Aeromonas hydrophila* demonstrated variable behaviour between experiments, generally showing declining persistance and a loss of culturability in both bulk phase and biofilm, but an increase in culturable counts from growth or resuscitation in one replicate. *Klebsiella pneumoniae* showed a slow decline in culturability and overall numbers, rather than the growth expected with this organism. Chlorination reduced the numbers of test organisms in the reactors; cells in the biofilm were less affected by disinfection. Numbers of target cells determined by selective media were usually reduced to below detectable levels by chlorination, while immunofluorescence counts were less affected. Reactor conditions were shown to be similar between experiments by the levels of heterotrophic bacteria present.

Because the observed behaviour of similar bacterial pathogens was shown to be quite different in the test environment, it is unlikely that a single organism or group of organisms could be monitored to predict pathogen behaviour. Changes in bacterial physiology resulted in poor detection of pathogens using selective media. Surfaces can give pathogens various survival advantages, and are a likely reservoir for some organisms within a drinking water distribution system.

INTRODUCTION

Purpose of this study

A drinking water distribution system is a complex heterogeneous environment, potentially serving as both a conduit and a growth site for pathogenic bacteria. These organisms have frequently been well characterized in the clincal microbiological setting, but their behaviour can change in the environment of drinking water systems. This can lead to various difficulties in recognition of an organism as a public health threat, as well as its detection and control in the distribution system environment. The overall aim of this project is to determine what behaviour can be expected of selected bacteria of concern within this system, with regard to their persistance, survival, physiological changes, and interactions with other organisms. Of particular interest is the ability of cells to persist in or form biofilms, and to then subsequently detach into the bulk phase of distributed water.

There are many considerations influencing the behaviour of microorganisms within a distribution system. This introduction aims to briefly summarize the current understanding of various factors influencing pathogens in the drinking water environment, such as surfaces, nutrient levels and disinfection. Particular emphasis is given to the various bacterial pathogens studied in this project, and their relevance in waterborne disease and behaviour in distribution systems. Various detection methodologies under recent development are also briefly discussed, and compared with the more standardized methods currently in common usage.

Coliform testing

Since early in the 20th century, coliform enumeration has been the primary method of monitoring water quality (Pipes, 1990). Coliforms are defined as aerobic and facultative gram-negative nonspore-forming rod-shaped bacteria which ferment lactose with gas and acid production at 35°C within 48 hours (APHA, 1992). In practical terms this includes several members of the bacterial family *Enterobacteriaceae*. As these organisms are normal flora in the enteric tract of warm-blooded animals, water containing them may have been contaminated with fecal material, including pathogenic organisms.

The intended significance of coliforms in water is therefore as indicators of fecal pollution. There are many pathogenic organisms with demonstrated fecal-oral spread and potential for waterborne transmission. To verify water supplies as free of them all would be impractical, and in some cases the task would be impossible. This is due to the sheer number of pathogens that would have to be tested for, usually with expensive and time-consuming methods that are frequently unreliable and require very large sample volumes. For example, the standard method for *Cryptosporidia* detection in water requires experienced technicians, over 100 liters of water sample volume, and frequently results in recovery of less than 5% (Vesey et al, 1993a). Using the coliform test, and the fecal or thermotolerant coliform test (fermentation of lactose with gas production at 44.5°C within 24 hours - APHA, 1992) to indicate fecally compromised water has been of much benefit to public health. The major waterborne disease threats at the turn of the century, such as

typhoid fever and cholera, have been largely controlled in developed countries by use of coliform monitoring and chlorine disinfection.

Coliforms are thus well accepted as potential indicators for recent fecal pollution in drinking water supplies, and of the efficiency of water treatment processes in removing this pollution. However, care must be taken when interpreting the indicative value of such data. To predict the behaviour of different pathogens under a wide variety of circumstances, indicator organisms would have to mimic the pathogen's physiology almost perfectly. These circumstances would include, for example, potential for regrowth in the system, tolerance to chlorine-induced injury, and ability to survive for extended periods in the drinking water environment. It seems likely the predictive role of indicators could be limited in these applications.

Physiological differences between coliforms and waterborne pathogens

Parasitic pathogens

The most obvious differences between some types of fecally-derived waterborne pathogens and coliforms is on a kingdom level, specifically with viruses and protozoa. A recent example of the importance of this was the failure of coliform testing to indicate a waterborne outbreak of approximately 403,000 cases in Milwaukee, Wisconson, with the etiologic agent being the protozoan *Cryptosporidia* (MacKenzie et al, 1994). This organism has the capacity to exist as an oocyst, giving it long term persistance and chlorine resistance. The protozoan *Giardia* has demonstrated similar chlorine resistance in

its cyst form; this is emerging as the leading identified cause of waterborne disease in the USA today (Craun, 1986; Craun et al 1991; Herwaldt et al 1992; Moore et al 1994). These organisms have long-term environmental persistance, show some association with turbidity, and usually are not well correlated with coliform levels. They can demonstrably pass disinfection barriers in the distribution system, and their behaviour once in the system is largely unknown. Some initial studies have indicated oocysts adhere very well to preexisting biofilm (Rogers et al, 1996). Hence, the subsequent detachment of biofilm containing large numbers of oocysts could explain infectivity and difficulties in the detection of these organisms in water. *Entamoeba histolytica* is an infrequent but still endemic waterborne pathogen in the USA today. It infects the gastrointestinal tract, frequently asymptomatically (Craun, 1986).

Viral pathogens

Over 120 different viruses can be excreted in feces and urine, and become water pollutants leading to a wide spectrum of diseases (Rao and Melnick, 1986; Kapikian et al, 1996). This includes such groups as the picornaviruses including enteroviruses and hepatitis A virus, caliciviruses including the Norwalk agent, and others such as rotaviruses. Agents such as the hepatitis A virus have demonstrated extended survival in the aquatic environment (Gerba and Rose, 1990).The prevalence and ability of viruses to persist in water distribution systems is generally unknown, along with the effects their survival has upon the community. However, viruses were identified as the cause in 12% of waterborne outbreaks in the USA between 1946 and 1980 (Gerba and Rose, 1990), and that given the

difficulties in isolation this figure is undoubtably under-reported.

Bacterial pathogens

Bacterial enteric pathogens identified from waterborne outbreaks include *Salmonella* spp., *Shigella* spp., *Campylobacter jejuni,* enterotoxigenic and enterohemorrhagic *Escherichia coli* (ETEC and EHEC), *Yersinia enterocolitica,* along with *Vibrio* spp. and *Salmonella typhi* in developing countries (Craun, 1986). Survival of these organisms varies greatly, ranging from the poor survival of *Campylobacter* to an estimated survival of *Y. enterocolitica* in well water of 540 days (Highsmith et al, 1977).

Environmental stress responses within the bacterial cell

Injury

The importance of and processes related to injury from various sources in coliforms have been summarized by McFeters (1990). Because indicator organisms and pathogenic bacteria are not well adapted to conditions in water, they can become physiologically damaged with aquatic exposure. This results in an inability to reproduce under selective or restrictive conditions tolerable by uninjured cells. Highly selective media, such as those usually employed to enumerate coliforms and fecal coliforms, will therefore incorrectly estimate numbers of target cells, as it will restrict growth of cells injured past a certain level. However, this cellular injury is reversible, and debilitated cells can be resuscitated under appropriate conditions. In drinking water, disinfection is

probably the most significant cause of injury, particularly at sub-optimal (non-lethal) levels. Metal ions, UV radiation, acid conditions, and biological interactions are also noted as potentially injurious stresses, causing damage primarily to the cell membrane. Studies have shown the levels of injury to be high in distribution systems, and highly selective methods may be underestimating coliforms by orders of magnitude. The overall significance of this is the potential generation of false-negative coliform results from water testing, obscuring the coliform's usefulness to signal health hazards.

The effects of injury upon pathogenic bacteria, particularly when related to coliforms, are also of significance, and have been summarized by Singh and McFeters (1990). The primary question is whether injured pathogens retain their ability to cause disease. There may be a temporary loss or reduction of virulence, caused by damage to surface components responsible for attachment or invasiveness. Damage can be repaired under suitable conditions, such as enrichment media or the small intestine of a host; and thus the health hazard of the organism may be undiminished. Other consequences are also possible, including altered physiology towards dormancy. The behaviour of different bacterial pathogens under stressful conditions varies widely, and is influenced by other factors such as cell concentration, contact time, and temperature. Pathogens such as *Y. enterocolitica, S. typhimurium*, and *Shigella* sp. were found to be more chlorine resistant than coliform bacteria, whereas *Campylobacter jejuni* and enterotoxigenic *E. coli* (ETEC) were found to be inactivated and injured at lower chlorine doses.

Starvation

A similar phenomenon is that of reduced culturabilty and therefore detectability of organisms taken from a low-nutrient or "oligotrophic" environment. This appears to be an active reaction to physiological stress by many bacteria, taking the form of the "starvation" response, or an apparently related response leading to "viable but nonculturable" (VBNC) cells.

Many waterborne pathogenic bacteria are adapted to growth at high nutrient levels. When confronted with nutrient limitation they have been shown to undergo certain physiological and morphological changes, collectively known as the starvation response (Matin et al, 1989). This response is under global genetic control in the cell. Some observed responses have included macromolecular degradation of cellular RNA and protein, a decrease in size and increases in adhesive properties to take advantage of surface-congregating nutrients (Marshall, 1988; Matin et al, 1989). These changes appear dependant upon an intense period of metabolic activity at the start of the response (Morita, 1988), and are reversible following nutrient addition to the cells (Morita, 1988; Marshall, 1988), although with a lag time proportional to the period of starvation.

Of major significance in drinking water distribution systems is the observation that cells undergoing the starvation response are markedly more disinfectant resistant than rapidly growing cells (Matin and Harakeh, 1990). It is likely that this resistance is conferred by the production of stress proteins in starved cells which help protect them against a variety of environmental stresses. The amount of resistance is generally determined by the extent of nutrient limitation, and other factors such as temperature. The

longer the cell has been starved, the greater the disinfection resistance conferred. This has been observed in several organisms of concern to the drinking water industry, such as *Y. enterocolitica, E. coli,* and *L. pneumophila.* The exact mechanism of protection by stress proteins is not known, although it is thought to involve factors such as the regulation of metabolism in the cell, and nutrient uptake affinity mechanisms.

Viable but nonculturable cells

A related phenomonen is the formation of viable but nonculturable, or VBNC cells, recently extensively reviewed by Oliver (1993). This differs from injury in that cell behaviour is related to starvation, and results in reduced culturability on all media rather than just selective media. Affected cells remain metabolically active, and can "resuscitate" to a culturable state given appropriate conditions such as human passage. VBNC studies have examined predominantly marine *Vibrio* spp., although this condition has been observed in many of the gram-negative pathogen and indicator bacteria. It appears to be triggered by stresses such as temperature shifts rather than xenobiotic compounds, although the inducing environmental conditions vary widely depending on the bacterium. It differs from the starvation response in that resuscitation of VBNC cells can take hours or days rather than a rapid reversal of minutes to hours. However, there appears to be some relationship between these conditions, with starved cells demonstrating a slower entry into the VBNC state than log-phase cells, following appropriate triggering conditions for inducing VBNC cells.

The significance of the VBNC condition is much the same as that of injured cells,

with the potential for indicators and pathogens to be undetected by commonly used culture media.

Heterotrophs and opportunistic pathogens in distribution systems

The heterotrophic population

Distribution system waters are known to have the potential for containing a wide variety of heterotrophic organisms in large numbers. These organisms are routinely enumerated by water utilities using broad spectrum, low nutrient growth media such as R2A agar (Reasoner and Geldreich, 1985), or by total count methods. Little is known of the gastrointestinal pathogenic potential of this heterotrophic population.

Infections caused by general heterotrophs may be quite specific and generally unnoticed. For example, they could cause sub-clinical symptoms, or only affect vulnerable parts of the consumer population such as the immunocompromised. Bodily areas other than the gastrointestinal tract may also be the site of infection such as the ear, nose or throat (Reasoner, 1991), or the respiratory tract in the case of Legionnaires Disease (see below). In the bulk of recent waterborne outbreaks of gastroenteritis in the USA, the etiologic agent has remained unidentified (Craun et al, 1991; Herwaldt et al 1992; Moore et al 1994), often along with the absence of coliform indicator bacteria using conventional media. These infections could be caused by unrecognized pathogens amongst the heterotrophic population, as well as viral or protozoan pathogens.

It has been shown (Payment, 1991) that consumers of distribution system water in a metropolitan system had a greater risk of low-level gastrointestinal illness than a control group consuming the same water after reverse osmosis filtration. This was manifested as an increase in number of episodes of gastrointestinal illness amongst susceptible individuals, rather than increasing the number of people with illness. The data from this study were further analysed (Payment, 1993), and while there was no correlation of illness with coliform levels, there was association with heterotrophic plate counts incubated at 35°C, and with increasing distance from the water treatment plant. This correlation with distance was attributed to regrowth of bacteria, as sewage cross-connections would have resulted in an epidemic distribution of cases, and viruses or parasites would have been further inactivated with increased exposure time in the distribution system. From this epidemiological study, it could be concluded that the enumeration of heterotrophs should be included with coliform monitoring of water supplies. It has been further suggested (Payment et al, 1994) that assaying the virulence of heterotrophic bacteria may be of more practical use than merely assessing their numbers.

This potential for low-level endemic infection is becoming increasingly important particularly with a growing immunocompromised proportion of the community, as a result of aging and spread of the HIV virus. There remains the possibility that organisms thought to be benign in a distribution system might be the etiologic agent in waterborne outbreaks of disease. For example, both *Klebsiella pneumoniae* and *Aeromonas hydrophila* may be isolated from well-maintained systems, and be considered harmless. However, both of these organisms are capable of expressing virulence factors, and are the causitive agents

for other, non-waterborne human infections (see the later section on these bacteria).

In addition to potentially containing opportunistic pathogens, the heterotrophic population can also be a considerable nuisance. Heterotrophs can cause false positives to the coliform test, mask coliforms by overcrowding on filter membranes (APHA, 1992), or generate taste and odor problems (O'Connor et al, 1975; Servais et al, 1993).

Legionella

An atypical opportunistic pathogen which has acquired some notoriety in recent years is *Legionella pneumophila*. This organism produces an opportunistic respiratory disease (Legionnaire's Disease) as opposed to a gastrointestinal one, and is spread by aerosolized infective particles, originating from sources such as air conditioning cooling towers and shower heads (Edelstein and Meyer, 1984). The organisms itself is quite fastidious with regards to growth requirements, but is widespread in its natural aqueous environment. It is not readily isolated from drinking water, although in some cases drinking water is able to support growth of added *Legionella* following chlorine neutralization (States et al, 1987). *Legionella* incidence is complicated by its niche as an intracellular pathogen of free-living protozoa, which themselves graze upon bacteria usually congregated in the biofilm of a distribution system. The infection of protozoa can lead to production of vesicles laden with legionellae which serve as infective particles, or protozoan cysts containing legionellae which are highly resistant to disinfection (Rowbotham, 1986). *Legionella* is a temperature dependant genus that can cross-feed with other microbial populations to meet its growth requirements (Tison et al, 1980;

Rogers et al, 1994a and 1994b); this type of community interaction is likely only in the biofilm of a water distribution system. Events which detach biofilm, such as maintenance of household hot water systems, have potential to release large numbers of *Legionella* into the water phase. Detachment as a source of infection appears to be frequently unrecognized. Epidemiologic efforts to isolate the mode of exposure frequently end inconclusively (Fraser, 1985), presumably due to lack of biofilm sampling.

Bacteria studied in this project

Aeromonas hydrophila

Aeromonads in drinking water systems have been the subject of much scrutiny and conjecture in recent years. They are considered water-based rather than water-borne organisms, since they are indigenous to aquatic environments and physiologically adapted for growth within the drinking water system (Rippey et al, 1979). Aeromonads appear to be a small and variable component of the overall heterotrophic population (Havelaar et al, 1990), with conflicting evidence sometimes supporting the correlation of aeromonads with the overall heterotrophic plate count population (Havelaar et al, 1990; LeChevallier, 1982). They can use a wide range of biopolymers; this may be important for aeromonads to maintain viability or grow in a distribution system, particularly if the aeromonads are growing in the biofilm using products from more predominant bacteria (Van der Kooij, 1988). It has been shown that the presence of other microflora such as pseudomonads in bottled water enhanced the survival of *Aeromonas* (Warburton, 1993).

The factors governing aeromonad regrowth appear highly complex and are not well understood. In some studies, positive correlations have been made with temperature and residence time (Havelaar et al, 1990), although temperature has been shown to have no correlation in other investigations (Burke et al, 1984b). However, these studies collectively agree that aeromonad numbers have no correlation to coliform levels.

Aeromonad taxonomy is also highly confusing. Based on phenotype and DNA-DNA reassociation kinetics (Janda, 1991), three species are generally accepted as most clinically important (*Aeromonas hydrophila, A. caviae,* and *A. sobria*). The different species have been typically associated with a wide range of opportunistic skin and soft tissue infections. The source is usually water or soil (Khardori and Fainstein, 1988; Altwegg and Geiss, 1989). All three species have been found in potable water distribution systems, and isolated from stools of patients with diarhhea. *A. caviae* the most commonly isolated aeromonad from such patients (Van der Kooij, 1988).

The potential for waterborne aeromonads to act as etiological agents in a disease outbreak is also poorly understood. It has been shown (Burke et al, 1984a and 1984b) that aeromonad numbers in the distibution system correlated with the numbers of clinical gastroenteritis cases associated with aeromonads. In a survey of studies investigating the presence and absence of aeromonads in feces, particularly of diarrhea patients (Van der Kooij, 1988), it was found that isolation rates varied widely (<1% to 20%), with highest isolation frequencies in tropical regions and lowest in Europe and the USA. The most common isolate was *A. caviae.* He also notes that the isolation of aeromonads in the absence of other pathogens is not adequate evidence of it being the etiologic agent.

Virulence factors in aeromonads have been demonstrated by many studies (LeChevallier et al, 1982; Burke et al, 1984a; Altwegg and Geiss, 1989; Janda, 1991), and include adherence factors, various hemolysins, cytotoxins, enterotoxins and proteases. Together with data from other varied aeromonad infections, these organisms can clearly be pathogenic. When isolated from drinking water systems, they often possess these virulence factors (above studies, also Gray et al 1990). However, it has been observed (Van der Kooij, 1988) that despite the widespread occurance of aeromonads in drinking water, epidemic outbreaks of *Aeromonas*-caused diarrhea have not been reported. There has also been a noted lack of correlation between the known virulence factors in aeromonads and human pathogenicity (Morgan et al, 1985).

Concerns associated with aeromonads have led to some attempts at regulation of these organisms. For example, health authorities in the Netherlands have defined 20 CFU/100mL in drinking water at the production plant and 200 CFU/100mL during distribution as maximum allowable values (Van der Kooij, 1988). In Canada, a limit of 0 CFU/100mL in bottled water has been proposed (Warburton et al, 1994).

Overall, it can be concluded that while *Aeromonas* demonstrably has a niche in distribution system microbiota and posesses the potential for causing waterborne disease in humans, it is unclear as to exactly what role it plays in both of these fields of interest.

Salmonella typhimurium

Salmonella is well recognized as an organism of fecal origin. It therefore should not be present in a well-run potable water distribution system. If found in water, it is regarded as more a case of survival rather than proliferation for the organism in such conditions. This survival possibility has been demonstrated in the outbreaks at Riverside, California, and at Gideon, Missouri, as described below.

The importance of attachment to environmental surfaces in this survival phenomenon has been previously investigated. Camper et al (1985) found *Salmonella* readily colonized and persisted on granular activated carbon in water, although attachment was at a lower rate and the organism decreased in numbers more rapidly in the presence of other heterotrophic bacteria. Suspended pathogen cells died away faster than attached cells. Longer term survival of attached *Salmonella* in water conditions appears to be a common observation from this study.

With the exception of some very large outbreaks such as Riverside, there seems to be a level of about 1-100 cases per year of waterborne salmonellosis (Craun, 1986). There is considerable potential for non-detection, given phenomena such as viable non culturable (VBNC) cells with *Salmonella*. In an investigation with *Salmonella enteritidis* in river water microcosms (Roszak et al, 1983), salmonellae rapidly became nonculturable. This was initially reversible following nutrient addition, but after 3 weeks resuscitation failed to give culturable cells, although the cells remained viable. Therefore, even if water during a disease outbreak tests negative for salmonellae, there remains the possibility of transmission by VBNC cells.

A significant *S. typhimurium* outbreak in Gideon, Missouri was reported by Clark et al, 1996. This waterborne outbreak was attributed to a large municipal storage tank in poor repair, with bird's feathers and droppings present in the tank. A temperature inversion led to the mixing of tank sediments with the water column, resulting in consumer taste and odor complaints. This led to a city-wide flushing of the supply system with the contaminated stagnant tank water.

Tank sediments were found to contain *Salmonella* of the same serovar as outbreak patients. Water samples taken through the outbreak were generally negative for coliform and fecal coliform detection, although some were positive at low levels. The *Salmonella* strain was shown to survive well in the town water, demonstrating only a 30% drop in numbers after 4 days at 15°C.

There seems a good case here for a long-term buildup of sediment-associated salmonellae, spread by a sudden release of the resuspended sediment. The uncertain correlation of indicator bacteria with the salmonellae in this case could be due to differing survival potentials of these organisms in this environment. If numbers of contaminating indicators had dropped sufficiently over time, they could not indicate the presence of surviving salmonellae.

A major *S. typhimurium* outbreak in Riverside, California is described by Boring et al, 1971, and a collaborative report, 1971.This outbreak was traced to a single well in the town supply system, although no contamination event was established. Prior to and during the outbreak this well supplied the town system intermittently, with several periods of 12-

48 hours when the pumps were not working.

Coliform test results before and during the outbreak revealed no significant contamination. *Salmonella* of the same type as the outbreak cases was isolated from the water supply in the vicinity of the suspect well. It was estimated that the *Salmonella* source was present for about 12 days and then disappeared when chlorination was begun, although the two events were not necessarily linked.

The limited *Salmonella* quantification performed revealed only low numbers (10^1 - 10^3 cells per liter), whereas food-borne *Salmonella* usually requires a large infective dose (~10^6 cells). This apparent discrepancy was postulated as being possible, as water has a shorter residence time in the stomach than foodborne *Salmonella* infections, thus requiring a lower infective dose.

It could be speculated that this outbreak was caused by sediment- or biofilm-associated salmonellae being released into or within the distribution system. Resuspension of the bacteria could have been caused by the recorded changes in hydraulics, or some other environmental change. The apparently low infective dose could also be explained if large numbers of organisms were associated with particles or a released "bolus" of biofilm, resulting in a highly uneven distribution through the water phase, producing large numbers of cells in single infectious particles which were protected from stomach acids.

Escherichia coli

The standardized use of the coliform and thermotolerant coliform enumeration indicator tests to assess microbial water quality has stimulated many studies on the

behaviour of *E. coli* in water systems, as it can make up a significant proportion of coliforms, and almost all of the thermotolerant coliforms. Its use as an indicator assumes that *E. coli* originates exclusively from fecal contamination, and survives in a fashion approximating that of fecal pathogens. The former assumption is becoming increasingly challenged from results in warm climatic areas of the world, where environmental isolates of *E. coli*, perhaps associated with the phyllosphere, are commonly isolated from water. This has occurred in dune systems near Rio de Janeiro, Brazil (Hagler et al, 1993), source waters in Puerto Rico (Rivera et al, 1988), and water storages in Sydney, Australia (personal observations, unpublished). The use of *E. coli* as an indicator of fecal pollution is thus of less use in these systems.

Some strains of *E. coli* such as the enterohemorrhagic O157:H7 strain (ECO157) have a demonstated pathogenicity to humans. This strain has several physiological differences from typical isolates, including a lack of the enzyme ß-glucuronidase (often used for *E. coli* detection) and poor or no growth at 45°C (Rice et al, 1992). Therefore, this strain is non-detectable by standard methods used for the routine detection of *E. coli*. It has been shown to persist at a similar rate as typical *E. coli* strains under drinking water conditions, confirming the premise that typical *E. coli* strains would be effective in indicating the ECO157 presence (Rice et al, 1992). However, this study did not account for differential survival on particulates or in biofilms. Survival on surfaces has been shown to be the major long-term persistance mechanism of *E. coli* in lake waters (Brettar and Höfle, 1992). Pathogenic and non-pathogenic *E. coli* strains have been shown to have similar growth rates to an environmental isolate of *E. coli* under growth conditions

relevant to drinking water distribution systems (Camper et al, 1991).

Colonization by an environmental *E. coli* isolate of a preexisting biofilm has been demonstrated under water distribution system conditions (Robinson et al, 1995). A fecal origin, non-benzoate degrading *E. coli* has been shown to be able to colonize a reactor containing a biofilm of benzoate degrading bacteria, and subsequently re-enter the water phase . In this study, 5mM benzoate was the sole carbon source, demonstrating consortial feeding by this organism (Szewzyk et al, 1994).

A significant *Escherichia coli* O157:H7 outbreak in Cabool, Missouri was reported by Geldreich et al, 1992; and Swerdlow et al, 1993. Prior to this outbreak, sections of a water main in Cabool were replaced followed by flushing but without hyperchlorination. This area was also in the vicinity of various sewage overflows. A single event of system contamination by backflow during repairs was concluded as the likely source of the outbreak. ECO157 was not isolated from the water supply, although this is not unusual for a waterborne outbreak.

Cases of ECO157 infections decreased after a boil order was issued and chlorination of the distribution system began. Towards the end of the outbreak the incidence of bloody as opposed to non-bloody diarhhea amongst case patients decreased, which was attributed to ingestion of lower doses of ECO157. It was estimated that ECO157 survived up to 2 weeks in the distribution system.

From this outbreak report it may be concluded that the possibility exists that there was a single large inoculum of ECO157 into the distribution system. This contamination

was then disseminated through and flushed out of the system by routine water usage. It may have not persisted in the system, as seen by the change of patient symptoms resulting from diminishing doses of infection.

Klebsiella pneumoniae

This organism has caused some concern in the drinking water industry, due to its demonstrated capacity to colonize and regrow in distribution systems and also by causing a positive coliform test (Geldreich and Rice, 1987; unpublished data). This suggests the possibilty that a bloom of *Klebsiella* could generate a false indication of fecal contamination within the system that could potentially mask a true coliform positive result. Most *Klebsiella* species have been detected from water systems by the coliform test, although some *K. pneumoniae* elicit a positive result with the fecal coliform test as well. The latter organisms can be regarded as of either fecal or environmental origin, as this species is associated with the gastrointestinal tract of many warm-blooded animals and also with plant products (Geldreich and Rice, 1987; Geldreich, 1991a and 1991b).

The public health significance of environmental *Klebsiella* is not clear. Using mouse infectivity models, *Klebsiella* from diverse environmental origins, regardless of fecal coliform response or biotype, have been demonstrated to be potentially as pathogenic as isolates of clinical origin (Bagley and Seidler, 1978). This contrasted with tested *E. coli* and *Salmonella* isolates, which demonstrated diminished virulence upon entry into the environment. The presence of multiple antibiotic-resistant *Klebsiella* in water would be of potential concern for susceptible consumers (Geldreich and Rice, 1987); although it would

appear that these strains are found in nosocomial infections rather than acquired in the community (Smith et al, 1982). It has been observed that klebsiellae infections are seen mainly in the hospital and are rare in the community; this is attributed to the colonization of susceptible patients rather than the hospital environment itself (Montgomerie, 1979). It is possible that a susceptible population within the community could be vulnerable to waterborne exposure to this opportunistic organism.

There is a lack of evidence of increased illness in a community during a biofilm regrowth event of klebsiellae (Geldreich, 1991b), potentially due to difficulties in gathering reports of water related illness amongst susceptible individuals in the community. The epidemiological difficulties of linking generalized opportunistic infections (gastrointestinal, respiratory, urinary, wound) with such an event would be immense, and probably render such an exercise impractical.

The water distribution system as a microbial growth environment

An environment containing low levels of nutrients and often biocides at levels designed to kill microorganisms would not seem like a good growth environment. However, the universal presence of heterotrophic bacteria in distribution systems would indicate that growth is very possible in these systems, or that organisms from source waters are persisting through the system.

Surfaces

Interaction with surfaces in these systems is one of the more important growth determinants. In pilot plant studies it has been shown that growth in the bulk liquid phase is negligable and that planktonic increases are due primarily to detachment of biofilm cells and "breakthrough" of cells through the water treatment plant barriers (Van der Wende et al, 1989; Bucklin et al, 1991; Block, 1992). A biofilm has been defined as a surface accumulation of cells immobilized at a substratum, frequently embedded in an organic polymer matrix of microbial origin, which may contain a significant fraction of inorganic or abiotic substances (Characklis and Marshall, 1990). Bacterial attachment to suspended particles or to the pipe wall can give substantial advantages to these cells. As summarized by Fletcher and Marshall (1982), (1) substrata-attached cells do not have to waste energy searching for food, as water containing fresh nutrients flows over them and removes waste products at the same time; (2) they are prevented from washing through the system; (3) the substrata attached to may also be a growth substrate; and (4) nutrients tend to adsorb to surfaces and are thus more available to surface attached cells.

The protective effect of attachment from disinfection is also very significant. Attachment led to the single greatest increase in disinfection resistance in one study of several potential factors (LeChevallier et al, 1988). Disinfectant-resistant cells can potentially be transported through water treatment barriers and throughout the distribution system by suspended particles (LeChevallier et al, 1984). It was demonstrated in this study that cells colonized cracks and crevices of granular activated carbon grains, produced extracellular polymeric substances, and were effectively resistant to 2.0 mg/L of chlorine.

These findings agree with Herson, et al (1987), where it was found that cells readily colonized suspended particles, and that attachment gave enhanced chlorine resistance. With the increasing usage of carbon to filter organics from source waters, the opportunity for pathogen breakthrough into the water system on carbon particles is also increased. Attachment to pipe surfaces can give protection in the same fashion as attachment to particles. An examination of distribution system biofilms found no correlation between biofilm heterotrophic plate counts and the bulk phase free chlorine residual (Nagy and Olson, 1985). A pilot system study (Van der Wende et al, 1989), found that chlorine influenced biofilm location within a system, with it tending to form later in the system once the chlorine residual was lower.

Temperature

Water temperature is usually correlated with heterotrophic growth rates. It is attributed as a major factor in seasonal regrowth events, although there is the possibility of other seasonal effects such as increased nutrient levels affecting water quality. Donlan and Pipes (1988) found that the attached microbial population density was directly related to water temperature. LeChevallier et al (1991) found coliform regrowth increased at temperatures over 15°C. Camper and Jones (1996) found growth rates of heterotrophs higher at 20°C than 10°C under distribution system conditions. This could be expected due to increased cellular enzyme reaction rates at the higher temperatures.

Corrosion products

The role of corrosion products in the population dynamics of bacteria in potable water distribution systems is not entirely clear. In cast-iron mains, it is contended that microbial induced corrosion is largely responsible for rusting (Victoreen, 1984a). The resultant corrosion formations or "tubercles" have been found to harbour large numbers of microorganisms including coliforms. Pulverized tubercle material, largely consisting of rust, has been found to enhance growth of heterotrophs and coliforms in some studies (Allen and Geldreich, 1977; Victoreen, 1984b) but not in others (Camper et al, 1991). This could be explained by the rust acting as a biological catalyst (Victoreen, 1984b) or as nutrient source, dependant upon the chemistry of the water and substratum, and the microflora present. The protective effect of tubercles from predation and disinfection could also explain their preferential colonization.

Substrata

The original pipe surface properties greatly influence the subsequent growth upon it, and hence the water quality within the pipe. Comparative testing of pipe surfaces under drinking water conditions to assess biofilm development has been performed in several studies (Block, 1992; Rogers et al, 1994a and 1994b; Camper and Jones, 1996). The results of these studies indicate that the smoother and less corrodable surfaces are less attractive for bacterial colonization. Leaching from the substrata of potentially inhibitory ions or of organic nutrients also affects colonization (Rogers, 1994a and 1994b).

Nutrient levels

Nutrient levels in the water itself are an obvious determinant of microbial growth. For example, there has been an association of the level of assimilable organic carbon (AOC) with microbial growth in some studies. LeChevallier et al (1991) found that an AOC level of greater than 50μg L^{-1} of acetate-carbon equivalents correlated with the occurance of coliforms in one distribution system, and concluded AOC levels should be kept below this concentration to limit coliform regrowth. AOC was the only nutrient parameter observed to decline as water moved through the distribution system, and AOC levels also correlated with heterotroph numbers. Van der Kooij (1992) found a significant correlation between concentration of AOC leaving the water treatment plant and heterotroph counts within the water phase, suggesting that AOC uptake by biofilm cells is potentially reflected by the number of suspended cells. He concluded that AOC concentrations of less than 10μg L^{-1} acetate-carbon equivalents could be used to limit bacterial growth potential. Camper and Jones (1996), in a pilot plant study, found the number of organisms to be elevated at higher substrate levels, but found that the overall heterotrophic growth rate was not affected by carbon substrate concentration.

"Typical" AOC levels in water systems are difficult to determine. A recent single-sampling survey of large participating US utilities found 18-322 μg L^{-1} of C equivalents in water supplies (Kaplan et al, 1994). A study of a particular system over a period of time by Huck, et al (1991) found much seasonal variability, with raw water AOC values peaking as high as 610 μg L^{-1} during early spring and usually lower during the rest of the year. It was suggested that waters having different temperature regimes, concentrations

of, or sources of organic matter would influence AOC levels and regrowth. An example would be preciptation causing runoff into source water resulting in the introduction of nutrients (LeChevallier et al, 1991).

There is a need for using AOC as a growth determinant parameter, as opposed to simpler and more easily determinable parameters such as dissolved organic carbon (DOC). DOC levels have no correlation with heterotroph numbers in a distribution system (Van der Kooij, 1992). However, DOC can be measured before and after an incubation with indigeous bacteria, in order to measure biodegradable DOC (BDOC) as an alternate method of nutrient measurement. Reduction of BDOC has been shown to decrease bacterial numbers in a distribution system after disinfection depletion (Servais et al, 1993). AOC measurements may underestimate growth potential, since they are based on a two species inoculum, and are consistently lower than BDOC measurements (APHA, 1992). BDOC may serve as a more accurate assessment of growth potential.

Nutrient limitation in a water distribution system may not necessarily take the form of carbon limitation. One study has reported a system containing high levels of organic carbon which appeared to be phosphorous limited (Miettinen et al, 1996).

Indicator and pathogen detection and enumeration methods

The use of selective media

Traditionally, bacterial isolation and identification has been performed using using selective media, relying upon the target cell's ability to form colonies upon it. This practice

originated with clinical microbiology for diagnosis of pathogenic bacteria, and was subsequently extended to environmental health microbiology, including drinking water microbiology. Plate counts are one of the standard methods (APHA, 1992) of enumerating the indicator groups of coliforms, using m-Endo medium for "total coliforms" and m-FC medium for fecal coliforms following concentration of the bacteria from a water sample by membrane filtration. This method takes about 24 to 48 hours, is reasonably specific, can be very sensitive, inexpensive, is familiar to water industry workers and is able to provide historical background data.

This is not to imply this methodology is without faults. Due to the processing time required, the test is a retrospective indication of potential contamination. Potential relevance of the results can be of limited value, for reasons given elsewhere in this review. The test is also underpinned by measuring the cells' culturability rather than viability or infectivity.

Reduction of culturability seen in some waterborne bacteria can be compensated for by use of appropriate culture media. An example of this is the use of mT7 medium for coliform isolation from chlorinated waters (LeChevallier et al, 1983). This is a less restrictive medium than m-Endo or m-FC and allows recovery and growth of injured coliforms, giving a more accurate representation of their numbers.

The recent development of chromogenic presence/absence tests for coliforms (APHA, 1992) has provided greater simplification and standardization of microbiological water testing. These media rely upon the selective growth of bacteria and the production of indicative enzymes such as ß-galactosidase and ß-glucuronidase that catalyze a color

change or fluorescence within 24 hours. This methodology has potential drawbacks in generating a non-quantitative result unless performed in a multiple tube fashion. However, it is easier to perform than membrane filtration, has forced a more standardized sample volume on the U.S. water industry, and is more applicable to automation and on-line sampling.

Rapid on-line monitoring of water supplies is very desirable in the control of a contamination event. With almost continuous automated sampling and rapid results, corrective action might be taken before contaminated water reaches consumers. One potential approach to this is using electrical impedance monitoring (Colquhoun et al, 1995; Silley and Forsythe, 1996). The method involves adding sample water to a specific substrate medium, then monitoring electrical impedance during incubation. In this system, the growth of the target organisms gives a typical change of the impedance profile over time, within 14 hours for coliform detection (Colquhoun et al, 1995). The application of this technology to drinking water is still in the formative stages, but represents an interesting potential development for the industry. Impedance may be more rapid than other growth-based techniques, but still may be unable to deliver results in the time frame needed to prevent water reaching consumers.

Using selective plating for detection of bacterial pathogens has many of the same advantages and drawbacks as described for coliforms. In addition, larger sample sizes must be taken to allow for the usual low densities of these organisms even in contaminated waters. Detection methods often involve enrichment steps that can extend the time of analysis and lower the quantitative aspect of gathered data. Given the wide range of

potential bacterial pathogens, testing for them all by their various culture plating methods on a routine basis is impractical for even the best equipped water utility laboratory.

There exists no culture media test for detection of viruses or protozoa. The analogous traditional method of virus detection employs concentration methods usually based upon the surface adsorptive properties of the viral particles to harvest the particles from 400 litres or more of water. Detection is by performing a cytopathogenicity assay of the concentrate upon a host cell culture (Rao and Melnick, 1986; Hurst et al, 1989). These methods are limited by (1) the failure of some viruses to produce cytopathogenic effects, (2) lack of growth in cell culture, (3) long incubation time, and (4) a requirement for skilled laboratory staff. Detection of pathogenic protozoa is dependant on non-culture methods such as immunofluorescence.

Alternative detection approaches to selective media

Perhaps the most readily applicable alternative detection methodology to selective culturing is that of immunofluorescent staining followed by direct microscopy. Field testing this method for enteropathogenic *E. coli* detection in water samples has been reported as far back as 1960 (Bohlool and Schmidt, 1980). Today, antibodies are available to a wide range of epitopes from pathogenic organisms, and have wide applications in ecological studies and routine confirmation of food safety. As outlined by Bohlool and Schmidt (1980) there remain some limitations with using this method. These are (1) antibody specificity and nonspecific staining; (2) autofluorescence or nonspecific adsorption to background; (3) antigen stability under different growth conditions and

environments; (4) distinguishing between live and dead cells; and (5) efficiency of cell recovery of samples for quantification. These limitations apply to any application of immunofluorescence methodology.

Antibody detection of *E. coli* O157:H7 in water has been recently investigated by Pyle, et al (1995). Immunofluorescence was combined with cyanoditolyl tetrazolium chloride (CTC) staining to detect viable cells; this procedure was able to be completed within 3-4 hours.

Another way to detect immunologic staining is by use of an enzyme-linked immunosorbent assay (ELISA). An example is the use of an enterobacterial common antigen in an ELISA assay to detect *Enterobacteriaceae* in drinking water samples (Hubner et al, 1992).

An alternate method for visualization of specific target cells is the use of fluorescently labelled oligonucleotide probes to bind with specific regions of cellular ribosomes. Given the advances in sequence databases over recent years, the target regions can be chosen to tailor specificity from kingdom level to individual strains. This has been applied previously to the analysis of microbial communities (DeLong et al, 1989; Ward 1989). The method has some of the same limitations as antibody staining, although targeting ribosomes with a relatively small molecule has advantages in the stability of the target molecule, and ribosome numbers can reflect the physiological status of individual cells. Model drinking water biofilms have been examined using this method to determine general bacterial groups (Manz et al, 1993) and to detect pathogenic *E. coli* (Szewzyk et al, 1994).

A limitation of using fluorescent labelling to detect cells is the direct microscopy time required to examine samples for target organisms. This can be alleviated with the use of the flow cytometer, an automated microscope which examines a continuous stream of sample with respect to particle properties and previous labelling with fluorochromes. Modern flow cytometers are also capable of sorting suspect particles such as bacterial cells for further examination. This type of automation can radically cut subjectivity and labour involved with microscopic examination. This seems particularly relevant when examining large sample volumes for very low numbers of target organisms (Vesey et al 1993a and 1993b).

The rapid evolution of the polymerase chain reaction (PCR) as an analytical technique over the past decade offers much promise as a microbial detection method. Correct choice of primers can give specificity of amplification as desired, including that of nonculturable as well as nonviable organisms. Studies using spiked samples of drinking water have indicated excellent sensitivity (Bej et al, 1991a and 1991b; Kapperud et al 1993; LeChevallier et al 1994), often to the detection of a single cell. Processing can be "rapid", giving accurate results with a low cost within a few hours. Despite this potential, problems still remain in application of this technology as a primary detection method for environmental samples. Concentration of the necessary large sample volumes while eliminating environmental substances known to be inhibitory to the polymerase enzyme, all in a timely and cost-effective method, still remains a major problem. Optimization of reactions including cycling temperature protocols and magnesium ion concentration is crucial, along with strict quality control. Another consideration is the significance of

detected DNA, and whether it comes from viable or nonviable organisms.

An immediate application of PCR is as a bacterial identification methodology. Given currently available primer sets and thermal cycler technology, a suspect colony from selective medium can be prepared, processed and examined by gel electrophoresis in under 2 hours to give a definitive identification (unpublished data). This approach has been taken by other investigations when source water has required an enrichment step prior to PCR of the sample (Way et al 1993; Kapperud et al 1993).

Another methodology showing potential for future application is the profiling of extracted fatty acid methyl esters (FAMEs) of bacterial cells using gas chromatography and mass spectroscopy (GC/MS). This has been applied successfully for the identification of colonies grown under specific conditions, using the Microbial ID Inc. (MIDI) system.The method involves comparison of the isolate's whole-cell GC/MS FAME profile to a database of known profiles. Some work has been performed in applying this methodology to FAME profiles of microbial communities rather than individual isolates (Haack et al, 1994). In this investigation, specific signature FAMEs or ratios of key groups of FAMEs were used to indicate the presence of target organisms. This method currently appears to be of most use in assessing the relative similarities and differences of communities with respect to their constituent organisms. Detection of specific organisms depends on growth conditions for quantities of specific cellular FAMEs. In the case of pathogens such organisms can be numerically minor members of a community, and the signature FAMEs thus only present in minute quantities.

Determination of the physiological status of a cell by means other than selective