PREVALENCE, SURVIVAL AND GROWTH OF BACTERIAL PATHOGENS IN BIOFILMS IN DRINKING WATER DISTRIBUTION SYSTEMS

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Water Research Commission

PREVALENCE, SURVIVAL AND GROWTH OF BACTERIAL PATHOGENS IN BIOFILMS IN DRINKING WATER DISTRIBUTION SYSTEMS

Report to the
WATER RESEARCH COMMISSION

by

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Executive Summary

Background and motivation

Safe drinking water is one of mankind's most important basic needs. Water is a source of life but can also be a source of destruction if it contains harmful pathogens. Water may act as a conduit of a range of pathogens causing diseases of the digestive and respiratory systems, and in varying degrees of severity. With so many people of all ages suffering from immune-deficiencies, pressure is on water distributors to supply water that is of the highest quality, free from disease-causing agents at the point of delivery. This task is made increasingly difficult by large and distribution systems where bacteria grow at the pipe and reservoir surfaces, forming biofilms and thereby acting as a zone of re-growth and possible contamination post-treatment.

Treatment deficiencies may allow the passage of unharmed or sub-lethally damages organisms, especially where the source water contains elevated numbers of pathogenic bacteria. Some bacteria have a high degree of tolerance to oxidizing agents such as chlorine, surviving the treatments designed to kill bacteria such as coliforms. Microorganisms may also gain access to treated water from the air-water interface in storage tanks, or during repair and maintenance activities. With time some surviving bacteria adhere to surfaces in the systems, growing to form multicellular conglomerates called biofilms. Bacteria in biofilms are orders of magnitude more resistant to a wide range of antibacterial agents, including oxidizing agents such as chlorine. Rare pathogenic bacteria surviving treatment might join such biofilms, finding protection and an environment supporting their growth. Because biofilms are dynamic, parts are released sporadically, so that pathogens present therein would also be released. This would pose a possible gastrointestinal threat where the water is consumed and a respiratory threat where aerosols are inhaled, eg. in showers and recreational facilities.

Traditional water hygiene risk has been evaluated by the enumeration of so-called indicator groups such as the aerobic heterotrophic bacteria, total coliforms, faecal coliforms and *Escherichia coli*, with comparison to acceptable standards and guidelines. A major failing of this approach is the failing correlation with other water-associated pathogens. The water quality indicated by indicators therefore may correlates poorly with disease outcome. At the onset of this project there was very scant data on the occurrence and distribution of pathogenic bacteria in biofilms of drinking water distribution systems.

Aim

The aim of this project was to gather data on the occurrence and distribution of biofilms of drinking water distribution systems. The specific aims were to study the:

- Prevalence of pathogenic bacteria in biofilms of drinking water distribution systems and containers used for distribution and storage;
- Survival of water quality indicator bacteria and of certain bacterial pathogens in biofilms in drinking water distribution systems; and
- Fate, survival, growth and release of two selected pathogenic bacteria in biofilms in drinking water distribution systems.

Methodology

The prevalence of pathogenic bacteria in biofilms of drinking water distribution systems and containers used for distribution and storage was studied in various locations by two research teams. Biofilms (one hundred samples on two separate occasions) were sampled within pipes in residential buildings in two cities (Pretoria and Pietermaritzburg), and in various smaller towns, as well as in buckets used for storage in homes in Botshabelo. Biofilms were disturbed and a range of pathogenic bacteria quantified using selective enrichment and isolation procedures by the MPN approach. A selection of presumptive pathogens was subjected to identification by phylogenetic (16SrDNA) analysis. The associated water samples were also analyzed for aerobic heterotrophic count and faecal coliforms.

The survival of water quality indicator bacteria and of certain bacterial pathogens in biofilms in drinking water distribution systems was studied using on-line systems over a period of 18 months. For this purpose a larger, pressure-tolerant variant of the Pedersen device, the Greenwood device was designed, constructed and installed in three locations. One was in an office building after a storage tank, a two in residential buildings fed by an urban distribution network. Water flowed from an inlet through diffusers, passing over 72 slides. Water and biofilm samples were taken every 14 days and the water analyzed for residual chlorine and other parameters. The aerobic heterotrophic count was determined, and specific pathogens tested for, including *Klebsiella, Salmonella, Staphylococcus* and *Pseudomonas*.

The fate, survival, growth and release of *Salmonella* in biofilms in drinking water distribution systems were studied under simulated laboratory conditions. A pathogenic water isolate was tagged with a fluorescent marker (green fluorescent protein gene). Model drinking water biofilms were cultured in a flow cell by inoculating with seven identified water isolates and fed with sterile water containing 1000 µg per liter acetate. The colonization and subsequent growth of the *Salmonella* was studied by fluorescence microscopy, and the numbers in the biofilms as well as those released were determined by selective MPN. Attempts to study the behaviour of a second pathogenic isolate, *Aeromonas*, failed due to problems with tagging as all tagged strains were growth-impaired under low nutrient conditions as occurring in drinking water.

Results

Bacterial biofilms were detected from all surfaces of drinking water distribution systems sampled, with a mean culturable count of 8×10^5 cfu.cm^{-2}, indicating that surfaces were not completely covered in biofilm. The corresponding waters samples displayed high culturable counts (mean ~ 3×10^4.mℓ$^{-1}$). Alarmingly, 10.5% of samples tested positive for faecal coliforms, mostly coming from the distribution networks of small towns and Botshabelo. A large number of biofilm samples harboured presumptive Aeromonas, *Vibrio* and *Shigella* as well as non-tuberculoid *Mycobacterium* in high numbers, whilst some *Pseudomonas* and one *Salmonella* were detected. Non-tuberculoid *Mycobacterium* were predominant only in biofilms from the Pretoria area, but included several isolates related to the pathogenic *M. gordonae* and one *M. abscessus*. Biofilms from the two large distribution networks (Pretoria and Pitermaritzburg) revealed a very low prevalence of putative pathogens, whilst the smaller networks (small towns and Botshabelo) harboured high numbers of a variety of gram negative pathogens. The selective culture media all proved unreliable, with a high incidence of false positives. Not one putative *Vibrio*, *Shigella* or *Salmonella* could be confirmed, indicating that none of these virulent pathogens occured in drinking water-associated biofilms at detectable levels. A large

number of *Aeromonas*, one of the causative bacteria of travellers' diarrhoea, were obtained from a variety of biofilms, prompting the team to study the biofilm-physiology and fate of this group in more detail.

Biofilms developed on the surfaces in the three online systems. The results of all three units showed a variable degree of biofilm development based on the heterotrophic bacterial density. The bacterial density of the water in the storage tank at the office building was on average ten times to one hundred times higher than the heterotrophic count of the two units at the residential building. The average residual chlorine measured at the storage tank was lower at 0.2mg/L, than the target residual chlorine level for the reservoir serving the area of 0.6 – 1.2mg/L. This measurement is also much lower than the average residual chlorine measured at the residential building of 0.85 and 0.91mg/L. Although the water supplied to the residential building was within the target residual chlorine level of the local municipal authority, pathogens were still found on a number of occasions. However, a higher incidence of pathogens identified was in the biofilms at the office building than at the residential building.

Two *Salmonella* strains, isolated from fresh water sediment (viz. *S. enterica* subsp. *enterica* ser. Typhimurium and *S. enterica* subsp. *enterica* ser. Muenchen) were chromosomally tagged with the *gfp* gene encoding the green fluorescent protein. The *gfp* gene was stably maintained during continuous growth for 30 days. The *gfp*-labelled recombinant strains were not growth-rate impaired, neither in nutrient rich (LB broth) nor in nutrient poor (1/10 strength R2A broth). This was in contrast to the effect of two separate *gfp*-carrying transposon delivery system on *Aeromonas hydrophila*. *S. enterica* subsp. *enterica* ser. Typhimurium formed biofilms only poorly in drinking water supplemented with 1000 $\mu g.m\ell^{-1}$ acetate. Biofilms were patchy and detached readily under conditions of high flow. By contrast, *Salmonella* colonized established multi-species drinking water biofilms within 24h, growing to form microcolonies within the biofilm. The *Salmonella* was also released from drinking water associated-biofilm into the flow, and was seen to re-colonize elsewhere. This showed that *Salmonella* can enter into, survive and grow within, and be released from a drinking water-associated biofilm.

Conclusions

Biofilms are a real threat to water quality and can grow even in well-maintained distribution systems. To prevent or minimize biofilm development residual chlorine levels should be high enough to ensure that water is sufficiently disinfected from the start to the finish of the distribution line. Water stored in tanks before distribution should be monitored regularly and tanks cleaned and disinfected regularly to prevent the development of a biofilm on the tank walls.

Even though most heterotrophic organisms are harmless, heterotrophic organisms in distribution system water is an indication of how well or good a distribution system is managed. Bacteria escaping disinfection colonize on the surface of distribution pipes to form biofilms which in turn protect opportunistic pathogens from the effects of disinfection. The absence of faecal or pathogenic organisms in a system does not completely guarantee the absence of opportunistic pathogens. The presence of opportunistic pathogens found in the biofilms during the study provides evidence that even what is considered a well-maintained distribution system, still harbours potential harmful bacteria. It is important to understand the risk involved in supplying "contaminated" or inadequately treated water.

Recommendations for future research

Future research should investigate the effects that bacteria found in drinking water biofilms have on the quality of the water reaching the end-user. Here attention should be focussed primarily on non tuberculoid *Mycobacterium* and on *Aeromonas* as both are regularly associated with diseases and were found in high numbers in a range of drinking water biofilms. Specific questions include their occurrence and distribution in source waters, and specific treatment regimens, especially to control the often resistant *Mycobacterium* species. It may be important to investigate the infective dose of potential pathogens found in the biofilms that would cause illness in immune-deficient individuals. It is also important to focus on the formation of biofilm on metal surfaces and the health risks involved.

Capacity Building

Degrees obtained:
Sonya September (PhD Microbiology): Prevalence, survival and growth of bacterial pathogens in biofilms in drinking water distribution systems (To be completed at the end of 2005)

Jemina Ntsherwa (MTech Environmental Health): The occurrence of health-related water quality indicator bacteria associated with contaminant build-up in various types of domestic water storage containers. (Completed)

Freddie Els BSc (Hon) Rochelle Swart (Intern at CSIR)

Presentations

September, S M, Venter, S N and Brözel, V S. 2003. Construction and evaluation of a GFP labelled environmental *Aeromonas hydrophila* for use in biofilm studies. IWA Symposium on Health-related Water Microbiology, Cape Town, South Africa. September 2003.

September, S M, Els, F A, Venter, S N and Brözel, V S. 2003. Occurrence of non-TB Mycobacterium in biofilms of drinking water distribution systems in South Africa IWA Symposium on Health-related Water Microbiology, Cape Town, South Africa. September 2003.

September, S M, Els, F A, Brözel, V S and Venter, S N. 2004. Non-TB *Mycobacterium* in biofilms of drinking water distribution systems. 13th Congress of the South African Microbiology Society, Stellenbosch, South Africa, April 2004.

Franck M and Swart R. 2004. Biofilm formation in a drinking water distribution system. WISA conference, May 2004.

Some of the findings have already been published in professional journals, whilst another paper have been submitted.

September, S M, Brözel, V S and Venter, S N. 2004. Diversity of Nontuberculoid *ycobacterium* Species in Biofilms of Urban and Semiurban Drinking Water Distribution Systems. Applied and Environmental Microbiology, 70: 7571-7573.

September, S Venter, S N and Brözel, V S. Prevalence of bacterial pathogens in biofilms of drinking water distribution systems. (Submitted to Journal of Water and Health)

Technology Transfer

Findings of research team were disseminated in various professional forums, including IWA meetings.

Some of the findings have already been published in professional journals, whilst some papers are still in preparation for submission.

The Greenwood device developed during the course of the project will facilitate technology transfer as it was designed and tested to operate in actual distribution systems.

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PART A

Prevalence, survival and growth of bacterial pathogens in biofilms in drinking water distribution systems

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SECTION A: LITERATURE REVIEW

A.1 LITERATURE REVIEW

The availability of safe drinking water is of the utmost importance for the survival of life on earth. Although water is the most common compound on earth, only *ca.* 2.6% is available as potential drinking water (Szewzyk et al., 2000). Many of the water authorities throughout the world are doing their best to protect the water sources and to ensure that the water that reaches the consumer is safe for consumption and free from any substances that may be harmful to our health, including bacteria deemed pathogenic.

Water for household consumption is normally disinfected before being distributed to the different end-point users. The microbial levels of the water, when leaving the treatment plant, have to be within limits set by governments and enforced by water authorities. By the time it reaches the tap in the house of the consumer, the water quality may differ drastically from what it has been when leaving the treatment plant. This may be attributed either to recovery of sub-lethally damaged bacteria, to growth of the survivors or to the presence of biofilms within the distribution system and from which cells may be released into the flow (Camper et al., 1999, Lee and Kim, 2003).

Surfaces of drinking water distribution systems are known to harbour biofilms that may sometimes cause a drop in the overall quality of the water (Camper et al., 1999, Percival et al., 1999). Monitoring the distribution system for the presence of biofilms is not an easy task. Biofilms are also known to provide a safe haven for many bacteria and these bacteria can also become resistant to the different disinfection procedures used (Costerton et al., 1995).

A.1.1 Biofilms

Biofilms may be defined as a community of microorganisms that attach to a surface or to each other (Donlan and Costerton, 2002). These attached bacteria are embedded in exopolysaccharides (EPS) and form three-dimensional structures known as micro-colonies. Cone-shaped or mushroom-shaped micro-colonies, with water channels between them, can be observed in biofilm structures (Costerton et al., 1995).

During studies of the attachment of *Pseudomonas aeruginosa* O'Toole and Kolter (1998) observed that it would swim along the surface prior to attachment, as if looking for a perfect site. The cells would then grow to form a monolayer before forming micro-colonies and finally the mature biofilm. During maturation of the biofilm, the biofilm-associated bacteria increase their secretion of EPS and become more resistant to antibiotics and other antimicrobial factors (O'Toole et al., 2000, Donlan and Costerton, 2002). Possible mechanisms for resistance to antibiotics may be the delayed penetration through the biofilm matrix and the altered growth rate of the bacteria leading to slower uptake of the antibiotic (Donlan and Costerton, 2002, Costerton et al., 1987). In some cases the biofilm-associated bacteria may detach from the biofilm and re-enter the planktonic phase. It is thought that starvation may play a role in this detachment mechanism (O'Toole et al., 2000).

Biofilms are found on all surfaces of drinking water distribution systems, from the treatment plant through to installations in the house of the consumer (Szewzyk et al., 2000). Attachment to the pipe surfaces gives the cells a nutritional advantage in this low-nutrient environment (Camper et al., 1999). The formation of biofilms in the water distribution system is becoming problematic to water suppliers since they may harbour potential pathogens which could be released back into the flow (Szewzyk et al., 2000). Problems associated with

biofilms in the drinking water distribution system include taste and odour problems as a result of actinomycetes or fungi, corrosion of pipe material and the possible regrowth of microorganisms (Camper et al., 1999). The attached cells can also serve as a reservoir for subsequent spread through the system following detachment (Camper et al., 1999).

Initial studies of biofilms leaned heavily on the use of scanning electron microscopy, with the disadvantage that samples had to be severely dehydrated during preparation for viewing under vacuum, and leading to substantial distortion (Donlan and Costerton, 2002). With the development of the confocal laser scanning microscope (CSLM), this problem was overcome as it allows analysis of fully hydrated samples (O'Toole et al., 2000). According to Costerton et al., (1995), "CSLM may be used to provide detailed information on cell morphology, cellular metabolism, cell phylogeny, microenvironment chemistry as well as physical architecture and chemistry of the biofilm matrix and associated polymers."

Biofilms in drinking water distribution systems can be studied by setting up models of the system that have been fitted with reactors such as the Robbin's or Pedersen's devices (Manz et al., 1993; Pedersen, 1982). These devices can also be fitted in the actual drinking water distribution systems, allowing one to remove the slides that serve as a surface for biofilm growth, for analysis. The biofilm can then be analysed either by conventional culturing or by direct microscopy (Camper et al., 1999). Both of these approaches, however, have limitations. Not all organisms present in the biofilm may be culturable and organisms can generally not be identified based on their morphology alone (Camper et al., 1999).

Techniques to aid in the microscopic visualization of specific types (groups, species or strains) have been developed during the past decade. The use of fluorescently labelled ribosomal probes enabled us to identify specific organisms within a biofilm and also determine its spatial distribution within the biofilm (Donlan and Costerton, 2002). A more recent approach to study the behaviour of a specific organism in a biofilm is by tagging the cell with the gene encoding the green fluorescent protein or GFP (Eberl et al., 1997). The GFP can be detected in the organism without the addition of external substrates, and without prior treatment of samples, allowing for real-time viewing. The gene encoding GFP is reported to be very stable in a range of bacteria (Eberl et al., 1997).

A.1.2 Pathogens associated with drinking water

Drinking water should be free from any organism that might pose a health risk to the population (Article 4, European Union Council Directive 98/83/EC). Many microorganisms are known to be transmitted by water, including *Aeromonas, Campylobacter, Escherichia coli, Helicobacter, Legionella, Pseudomonas, Salmonella, Vibrio, Cryptosporidium* and Hepatitis E virus (Hunter et al., 2001; Lee and Kim, 2003, Payment et al., 2003; Szewzyk et al., 2000). Some of these organisms are able to grow in the water and were only recently recognised as relevant pathogens. They include members of the genera *Aeromonas, Legionella, Mycobacterium* and *Pseudomonas* (Szewzyk et al., 2000). Many of these organisms are also referred to as "new emerging pathogens" since they have not previously been thought to be associated with water, or could not be detected due to a lack of proper detection methods (Medema *et al.*, 2003; Szewzyk *et al.*, 2000).

The presence or increase in the numbers of immunocompromised persons might also have played a role in the emerging of these "new" pathogens since they would not necessarily have caused disease in healthy individuals (Szewzyk et al., 2000). A few of these organisms, namely *Aeromonas, Pseudomonas* and *Mycobacterium* (other than *M. tuberculosis*) will be highlighted below.

Aeromonas

Species of the genus *Aeromonas* are gram negative, non-spore forming rods with mainly polar flagella (Altweg, 1999; WHO, 2002). *Aeromonas* are found in aquatic systems including surface and treated drinking waters (Kühn et al., 1997). The health significance of *Aeromonas* in drinking water is not fully understood since no verified waterborne outbreaks of *Aeromonas* have been reported (Kühn et al., 1997; WHO, 2002). *Aeromonas* species had been linked with enteritis and wound infections in humans, with *A. hydrophila, A. caviae* and *A. sobria* being the most common (Altweg, 1999; Kühn et al., 1997).

Pseudomonas

Pseudomonas species are gram negative non-spore forming rods, motile by polar flagella (Kiska and Gilligan, 1999). They are widely spread in the environment and able to multiply by utilizing a wide range of substrates (Szewzyk et al., 2000). *P. aeruginosa* is the most important human pathogen and is readily isolated from patients with cystic fibrosis (Kiska and Gilligan, 1999; Szewzyk et al., 2000). Other infections associated with *Pseudomonas* include ear, eye, urinary tract and wound infections. *P. aeruginosa* is a known biofilm former and because of the often voluminous secretion of EPS, is difficult to eradicate. Water distribution lines, especially those to hospitals, have repeatedly been linked to nosocomial infections by *P. aeruginosa* (Kiska and Gilligan, 1999; Szewzyk et al., 2000).

Mycobacterium

The strictly pathogenic species within the genus *Mycobacterium, i.e. M. tuberculosis, M. bovis, M. africanum* and *M. leprae*, are transmitted by human and animal reservoirs only (Szewzyk et al., 2000), and should thus not be included in this study. The non-tuberculous *Mycobacterium* (NTM), also referred to as atypical *Mycobacterium*, have been isolated from various environmental sources (Falkinham III et al., 2001; Le Dantec et al., 2002a; Le Dantec et al., 2002b). *Mycobacterium* are gram positive, slightly curved or straight rods, but because of the high lipid content in the cell wall, are not readily stained by the gram method (Metchock et al., 1999). NTM can cause infections of the respiratory tract, gastrointestinal tract, skin and lymph nodes, but many of these are restricted to patients with acquired immune deficiency syndrome (AIDS) (Metchock et al., 1999; Szewzyk et al., 2000).

A.2 PREVALENCE OF POTENTIAL PATHOGENS IN DRINKING WATER DISTRIBUTION SYSTEMS

A.2.1 Introduction

Drinking water distribution systems provide an oligotrophic environment as drinking water is rigorously treated to curb the organic content thereof. Furthermore water for human consumption is generally disinfected by one or combinations of oxidizing agents (chlorine, chloramine, ozone or rarely hydrogen peroxide) in order to decimate the numbers of bacteria, especially potentially pathogenic ones. There is, however, a misconception that almost 'sterile' conditions exist in the distribution system and also that few, if any, organisms should be able to survive the disinfection procedures employed by the water authorities (Szewzyk et al., 2000). The post-treatment recovery and growth of bacteria is a concern because of the effect it can have on public health (Assanta et al., 1998; Kerr et al., 1999).

In water distribution systems, cells can attach and form biofilms on the surfaces of the piping material, irrespective of the material used (Szewzyk et al., 2000). These biofilms create niches that may provide new members with protection against residual disinfectants as well as predation (Buswell et al., 1998; Costerton et al., 1995; Thomas et al., 1999). Bacteria growing as biofilms also tend to become more resistant to treatment or disinfection, up to one thousand fold the planktonic susceptibility (Costerton et al., 1995). Therefore potentially pathogenic bacteria entering the biofilm may be afforded a protective environment, recovering and growing to greater numbers, so that the biofilm can become a reservoir for the subsequent spread of pathogenic organisms (Camper et al., 1996). In addition, the organisms in the biofilm can influence the taste and odour of the water, and if biofilms developed on ferrous metal surfaces, it may cause corrosion of the pipes and also the release of iron particles into the water (Camper et al., 1999; Cloete et al., 1992; Ridgway et al., 1981).

A number of known pathogens have emerged as 'new' pathogens associated with the water supply. These include *Campylobacter* spp., pathogenic *Escherichia coli*, *Yersinia* spp., enteric viruses and parasites like, *Cryptosporidium* and microsporidia (Szewzyk et al., 2000). In addition some of the bacteria normally present in aqueous environments, such as *Legionella*, *Pseudomonas*, *Aeromonas* and *Mycobacterium avium* have also been found to multiply in water distribution systems (Assanta et al., 1998; Brandi et al., 1999; Carson et al., 1978; Falkinham III et al., 2001; George et al., 1980). However little is currently known regarding the prevalence and distribution of these and other bacterial pathogens in biofilms of drinking water distribution systems.

A.2.2 Aims

The aim of this study was, therefore, to determine the prevalence of a range of bacterial pathogens in biofilms associated with drinking water distribution systems in various areas in South Africa.

A.2.3 Materials and methods

A.2.3.1 Sampling

Samples from the following areas, Pretoria, various small towns (Secunda, Bethal Tzaneen, Villiers, Hartbeespoort), Botshabelo (Mangaung), and Pietermaritzburg were analysed during the period ranging from September 2001 to August 2002 (Table A.1). In Botshabelo, samples were taken both from buckets and the corresponding taps reportedly used to fill the buckets.

Table A.1 List of the various sampling points analyzed for biofilm-associated pathogenic bacteria.

PRETORIA (RAND WATER)	SMALL TOWNS	BOTSHABELO	PIETERMARITZBURG
Sunnyside	TzaneenA	1273B	Khan Road 1
Hatfield cold	TzaneenB	2413B	Lotus Park
Hatfield hot	VilliersA	2514B	Mountain Rise Cemetary 1
Wapadrand	VilliersB	1119B	Darvill
Arcadia cold	SecundaA	20B	Relaot Road 1
Arcadia hot	SecundaB	20A&B	Wylie Park A
Eersterus cold	SecundaC	1119A&B	Wylie Park B
Eersterus hot	SecundaD	1273A&B	Oakleigh
Faerie GlenA	SecundaE	2413A&B	Worlds View
Faerie GlenB	HartebeespoortA	2514A&B	Waltdori
Arcadia cold	TrichardtA	S(W)A&B	QE Park
Arcadia hot	TrichardtB	S(NW)A&B	Showgrounds
Eersterus cold	SecundaF	M(O)A&B	Caravan Park
Eersterus hot	SecundaG	M(C)A&B	Joyner Road
Wapadrand	SecundaH	P(O)A&B	Bennet Road
Pretoria North	SecundaI	P(C)A&B	Radio Station
Sunnyside	Bethal BGA	C(C)A&B	Khan Road 2
Pierre van Rhyneveldt	Bethal BGB	1273T	Neptune Road
Hatfield cold	Bethal BGC	2413T	Mountain Rise Cemetary 2
Hatfield hot	Bethal BGD	2514T	Relaot Road 2
HatfieldA	Bethal BGE	1119T	
QueenswoodA	Bethal BGF	20T	
WaverleyA			
WaverleyB			
HatfieldB			
Tukkies			
ElardusparkA			
ElardusparkB			
CenturionA			
CenturionB			
QueenswoodB			

Prior to collection, water was allowed to run to waste at a uniform rate for 2 – 3 minutes. Water samples were collected in sterile bottles, containing sterile sodium thiosulphate ($Na_2S_2O_3.5H_2O$) to neutralise any free or combined residual chlorine normally occurring in the drinking water distribution system (Anon, 1982). The final sodium thiosulphate concentration in the water sample collected was 0.01% (w/v) (Le Chevalier et al., 1988).

Biofilm samples were, as far as possible, taken from the inner surface of the service pipe using a sterile cotton-tipped swab moistened in sterile ¼ strength Ringer's solution (Merck). The biofilm removed from the service pipe was then placed back into the swab container, where, prior to collection, 2 mL sterile ¼ strength Ringer's solution (Merck) was added, to prevent the sample from drying out. The area of biofilm removed in this way was assumed to be *ca.* 1 cm^2. All the samples collected were transported to the laboratory on ice and analysed within 12 to 18 hours.

A.2.3.2 Analysis of system water

Water from the systems where the biofilm samples were taken, was tested for the presence of faecal coliforms and the heterotrophic culturable counts were determined (Fig. A.1). Faecal coliforms were enumerated by filtering duplicate 100 mL volumes through 0.45 µm nitrocellulose filters and incubating on mFC agar (Merck) at 44.5°C for 22-24 hours. The heterotrophic culturable count was determined by plating serial dilutions (10^{-1} to 10^{-5}) onto R_2A agar (Oxoid) and incubating at 28°C for 5 days. Colonies were counted every day for 5 days.

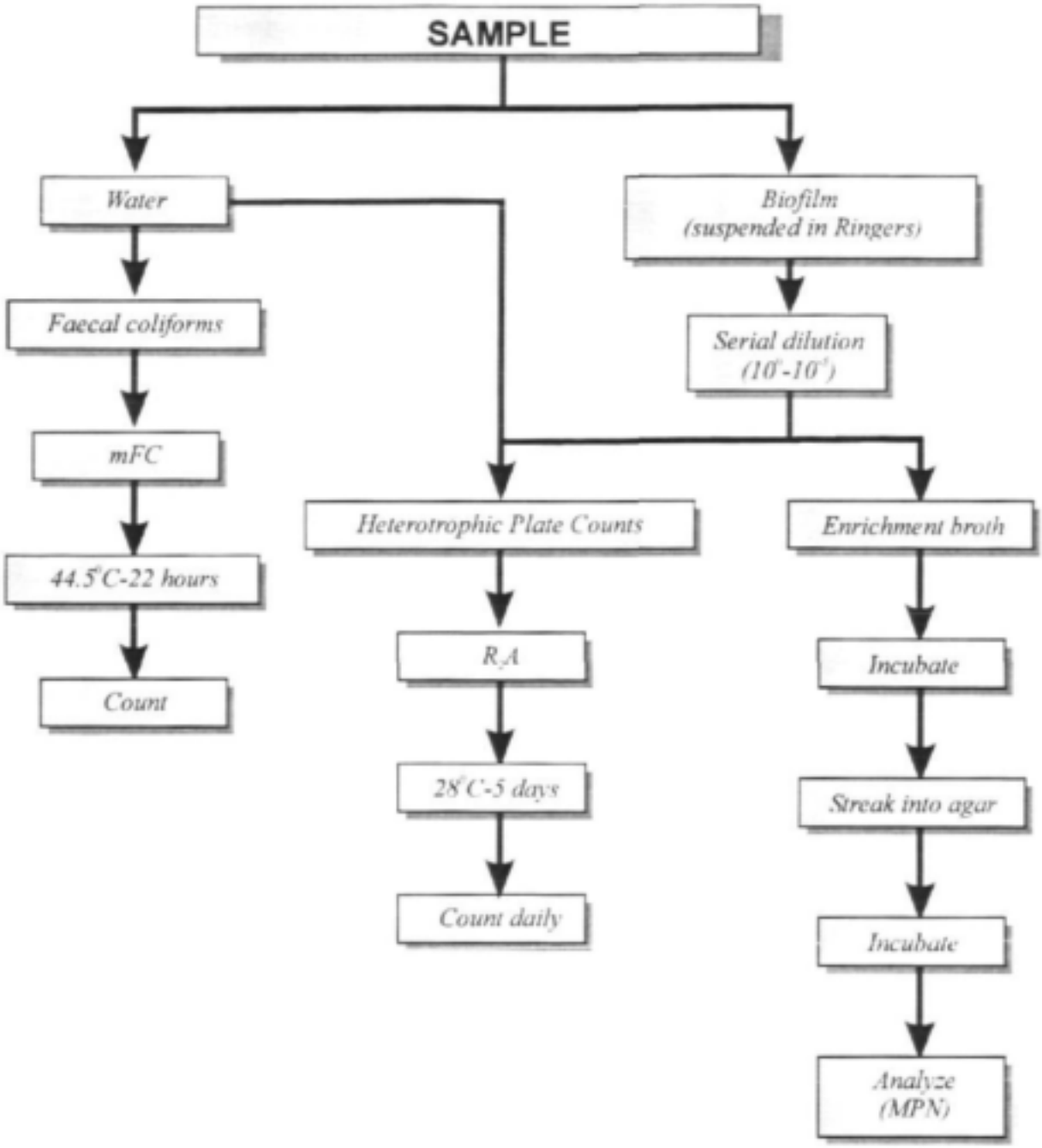


Figure A.1: Diagrammatic presentation of the procedure to be followed for screening biofilms in a water distribution system for pathogens.

A.2.3.3 Analysis of the bacterial community of biofilms

Biofilms as collected from various sites were analyzed to determine the numbers of pathogens as listed in Table A.2, and processed as outlined in Fig. A.1. These pathogens were selected based on their association with drinking water supplies in many countries (Assanta et al., 1998; Falkinham III et al., 2001; Fernández et al., 2000; Gibbotti et al., 2000; Oragui et al., 1993; Waage et al., 1999). Biofilm samples were dispersed by vigorous vortexing in sterile diluent. The heterotrophic culturable counts were determined directly from the dilution series onto R_2A agar and incubated for five days at 28°C. For the enumeration of the different potential pathogenic bacteria, the three-tube MPN technique

was employed. The 10^{-1} dilution of the biofilm samples was used to inoculate three replicate tubes of the relevant enrichment broths, as outlined in Table A.2. From this triplicate set of tubes further serial dilutions were performed to obtain a dilution series of 10^{-1} to 10^{-4}. These were then incubated for 24 hours at the respective incubation conditions (Table A.2). After 24 hours, *ca.* 10 µl from each tube was streaked onto the corresponding selective subculture medium (Table A.2). Where colony appearance corresponded to that as widely described in manuals and the literature, the respective dilution was scored as positive. For the enumeration of *Mycobacterium* spp., cetylpyridinium chloride (CPC) (Sigma) was added to the sample to a final concentration of 0.005% (w/v) and incubated at room temperature for 30 minutes before streaking on to the relevant agar and incubated for up to 21 days (Falkinham III et al., 2001).

Table A.2: Pathogens to be isolated from the biofilms in a drinking water system and the respective enrichment broths, selective agars and incubation conditions employed.

PATHOGEN	ENRICHMENT	INCUBATION CONDITIONS	SELECTIVE MEDIUM	INCUBATION CONDITIONS
Aeromonas	Tryptone Soy Broth (Oxoid)+1% Yeast Extract (Oxoid)	37°C, 24 hours	Aeromonas Agar Base (Oxoid) + Ampicillin Selective Supplement (Oxoid)	37°C, 24 hours
*Burkholderia**	Brain Heart Infusion Broth (Oxoid)	37°C, 24 hours	Tryptone Soy Agar	37°C, 24 hours
Escherichia coli	Nutrient Broth (Oxoid)	37°C, 24 hours	Chromocult Coliform-Agar (Merck)	37°C, 24 hours
*Listeria**	*Listeria* Enrichment Broth + Selective Supplement (Oxoid)	30°C, 24-48 hours	*Listeria* Selective Agar + Selective Supplement (Oxoid)	35°C, 24-48 hours
Non-TB *Mycobacterium*	Cetylpyridinium Chloride (Sigma)	Room Temperature, 30 minutes	Middlebrook 7H10 Agar (Difco) + 0.5% Glycerol + 10% Oleic Acid-Albumin	37°C, up to 21 days
*Plesiomonas**	Peptone Water	37°C, 24 hours	Macconkey	37°C, 24 hours
Pseudomonas	Buffered Peptone Water (Oxoid)	37°C, 24 hours	*Pseudomonas* Isolation Agar (Difco)	37°C, 24 hours
Salmonella	Rappaport-Vassiliadis Enrichment Broth (Oxoid)	42°C, 24 hours	XLD (Oxoid)	37°C, 24 hours
Shigella	MacConkey Broth (Oxoid)	37°C, 24 hours	Hektoen Enteric Agar (Oxoid)	37°C, 24 hours
Vibrio	Alkaline Peptone Water	37°C, 24 hours	Cholera Medium TCBS (Oxoid)	35-37°C, 24 hours
*Yersinia**	Tryptone Soy Broth + 0.6% Yeast Extract (Oxoid)	Room Temperature, Overnight	Lactose Agar (Oxoid)	37°C, 24 hours

* tested for only during the first round of sampling.

From each type of selective subculture media, single colonies were picked from the highest dilution and streaked onto Nutrient Agar (Oxoid) to obtain pure isolates. The isolates were subjected to the gram stain and KOH test (Gerhardt et al., 1994; Powers, 1995) to determine their gram status. From all the gram negative isolates obtained, glycerol stocks were prepared for the maintenance and further identification of these isolates, and *Mycobacterium* spp. were preserved on Microbank™ beads (Pro-lab Diagnostics). These were all stored at -70°C.

A.2.3.4 Molecular analysis of selected isolates

A number of the isolates from each pathogen group were selected at random for further identification. These isolates were cultured on Nutrient Agar and incubated for 24 hours at 37°C. Genomic DNA was extracted using standard procedures (Lemanceau et al., 1995). Potential *Mycobacterium* isolates were re-streaked onto Middlebrook agar and their DNA extracted using the DNeasy Tissue Kit (Qiagen).

The 16S rRNA genes were amplified from the genomic DNA extracts by PCR. Primer sets 63f (5' CAG GCC TAA CAC ATG CAA GTC 3') and 1387r (5' GGG CGG WGT GTA CAA GGC 3') (Marchesi et al., 1998) and alternatively fD1 (5' AGA GTT TGA TCC TGG CTC AG 3') and rP2 (5' ACG GCT ACC TTG TTA CGA CTT 3') (Weisburg et al., 1991). These primers have been reported to amplify respectively a 1300 bp and 1500 bp region of the 16S rRNA gene (Marchesi et al., 1998; Weisburg et al., 1991). PCR products were purified using a Geneclean™ Kit (Bio 101, Inc), but using silica instead of glassmilk (Boyle and Lew, 1995), or the QIAquick PCR Purification Kit (Qiagen). These purification steps were performed using the standard protocols as supplied by the manufacturers. The nucleotide sequences of purified PCR products were then determined. Sequencing was performed using the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) and then on an ABI PRISM 377 or ABI Prism 3100 Sequencer (Perkin Elmer).

Sequences were edited by Sequence Navigator (PE Applied Biosystems) and the identities of isolates were determined by searching known sequences in GenBank using the basic BLAST search of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences were aligned and those sections overlapping in all sequences (*Escherichia coli* numbering system) used to draw phylograms of some of the bacteria identified using Clustal X (v1.81) (Thompson et al., 1997), and viewed using TreeView (Win 32) and NJPlot (Win95) (Perrière and Gouy, 1996).

A.2.4 Results and Discussion

A.2.4.1 Analysis of water sample

The procedure for the enumeration of faecal coliforms has been used for decades as an indicator of the bacteriological safety of drinking water (Leclerc et al., 2001). Table A.3 shows the results, as the average of two determinations, obtained for the enumeration of faecal coliforms in the 95 water samples analysed.

Table A.3: Prevalence of faecal coliforms in the 95 water samples analysed (given as cfu/100mL).

SAMPLE	*cfu/ 100mL*	*SAMPLE*	*cfu/ 100mL*	*SAMPLE*	*cfu/ 100mL*	*SAMPLE*	*cfu/ 100mL*
Pretoria (Rand Water)		**Small towns**		**Botshabelo**		**Pietemaritzburg**	
Sunnyside	ND*	Tzaneen A	ND	1273B	43-54	Khan Road 1	ND
Hatfield cold	ND	Tzaneen B	ND	2413B	TNTC#	Lotus Park	ND
Hatfield hot	ND	Villiers A	ND	2514B	ND	MTN Rise Cemetary 1	ND
Wapadrand	ND	Villiers B	ND	1119B	400-600	Darvill	ND
Arcadia cold	ND	Secunda A	ND	20B	7-12	Relaot Road 1	25-30
Arcadia hot	ND	Secunda B	ND	20A&B	ND	Wylie Park 1	125-201
Eersterus cold	ND	Secunda C	ND	1119A&B	ND	Wylie Park 2	ND
Eersterus hot	ND	Secunda D	ND	1273A&B	ND	Oakleigh	ND
Faerie Glen A	ND	Secunda E	ND	2413A&B	ND	Worlds View	ND
Faerie Glen B	ND	Hartebees-poort A	ND	2514A&B	ND	Waltdon	ND
Arcadia cold	ND	Trichardt A	ND	S(W)A&B	ND	QE Park	ND

SAMPLE	*cfu/ 100mL*	*SAMPLE*	*cfu/ 100mL*	*SAMPLE*	*cfu/ 100mL*	*SAMPLE*	*cfu/ 100mL*
Arcadia hot	ND	Trichardt B	ND	S(NW)A&B	ND	Showgrounds	ND
Eersterus cold	ND	Secunda F	ND	M(O)A&B	ND	Caravan Park	ND
Eersterus hot	ND	Secunda G	ND	M(C)A&B	ND	Joyner Road	ND
Wapadrand	ND	SecundaH	ND	P(O)A&B	ND	Bennet Road	ND
Pretoria North	ND	Secundal	ND	P(C)A&B	ND	Radio Station	ND
Sunnyside	ND	Bethal BGA	2.0	C(C)A&B	ND	Khan Road 2	ND
Pierre van Rhyneveldt	ND	Bethal BGB	ND	1273T	ND	Neptune Road	ND
Hatfield cold	ND	Bethal BGC	2.0	2413T	ND	MTN Rise Cemetary 2	ND
Hatfield hot	ND	Bethal BGD	ND	2514T	ND	Relaot Road 2	ND
Hatfield A	ND	Bethal BGE	2.5	1119T	ND		
Queenswood A	ND	Bethal BGF	2.5	20T	ND		
Waverley A	ND						
Waverley B	ND						
Hatfield B	ND						
Tukkies	ND						
Elarduspark A	ND						
Elarduspark B	ND						
Centurion A	ND						
Centurion B	ND						
Queenswood B	ND						

* - Result indicates that no putative faecal coliforms were detected (or were below detection limit)
# - TNTC = too numerous to count; more than 300 cfu's on the filter

Ten (10.5%) of the 95 samples tested positive for faecal coliforms. These included four water samples from small towns (4 of the 6 points fed from boreholes in the Bethal area, *ie* 67%), 4 of 22 (18.1%) Botshabelo samples and 2 of the 20 (10%) Pietermaritzburg samples, thus a total of 10 out of 95 (01.5%) samples. No other samples tested positive for faecal coliforms. Thus, the bulk of water obtained from municipal drinking water distribution systems was implicated as safe water for human consumption. Four of five buckets tested positive for faecal coliforms in Botshabelo during the first sampling round. The high numbers obtained were confirmed by parallel analysis by the team members at Free State Technikon. As this group was also actively involved in community training and awareness programmes, the absence of detectable faecal coliforms at a later stage may serve to indicate a change in water handling and storage practices by members of the community. The faecal coliforms detected in water originating from boreholes of the Bethal district may be accounted for by faecal pollution of animal origin which infiltrated the water tables of the boreholes, mainly through rain, and due to large scale animal husbandry activities in that area.

The heterotrophic culturable counts of water samples normally serves as a measure of the effectiveness of water treatment processes such as coagulation, filtration and disinfection, and as an indication of the cleanliness of the drinking water distribution system (Reasoner et al., 1985). The heterotrophic culturable counts of the water samples are depicted in Fig. A.2. It is clear from the data shown that the heterotrophic count as determined using R_2A far exceeded acceptable levels in some of the samples from Bloemfontein / Botshabelo and Pietermaritzburg.

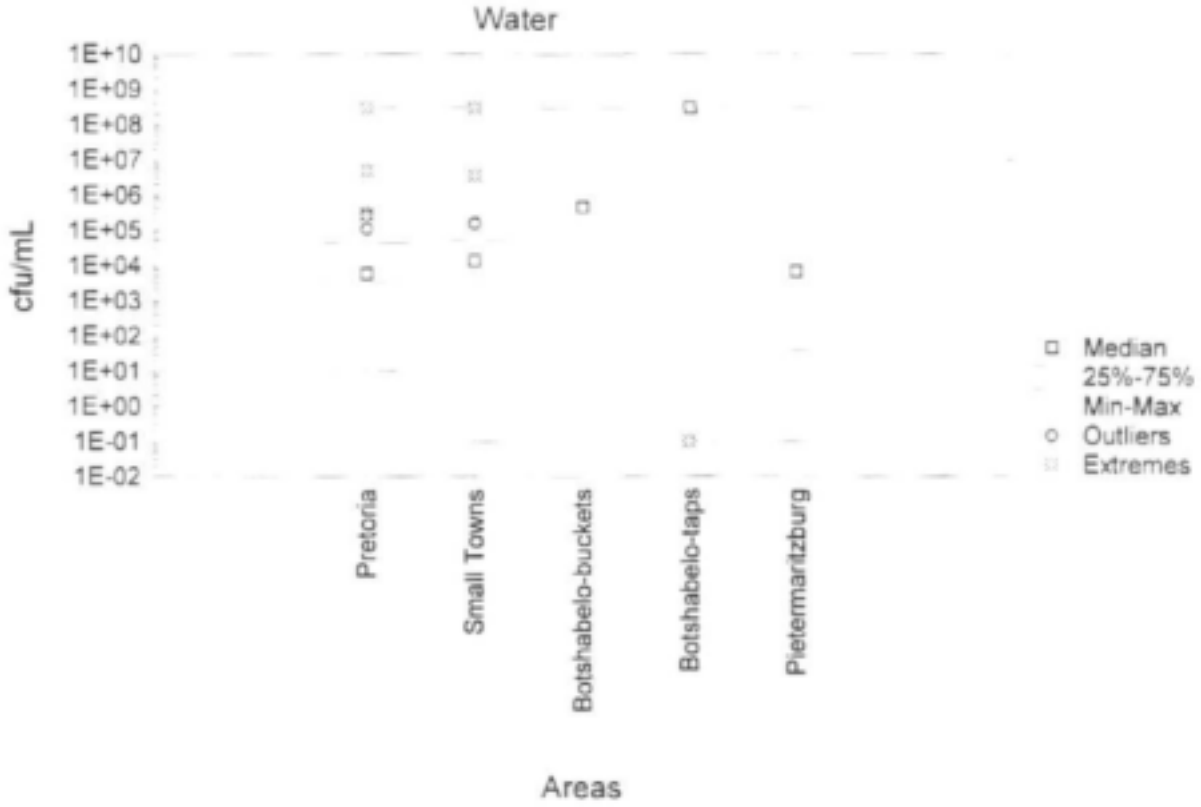
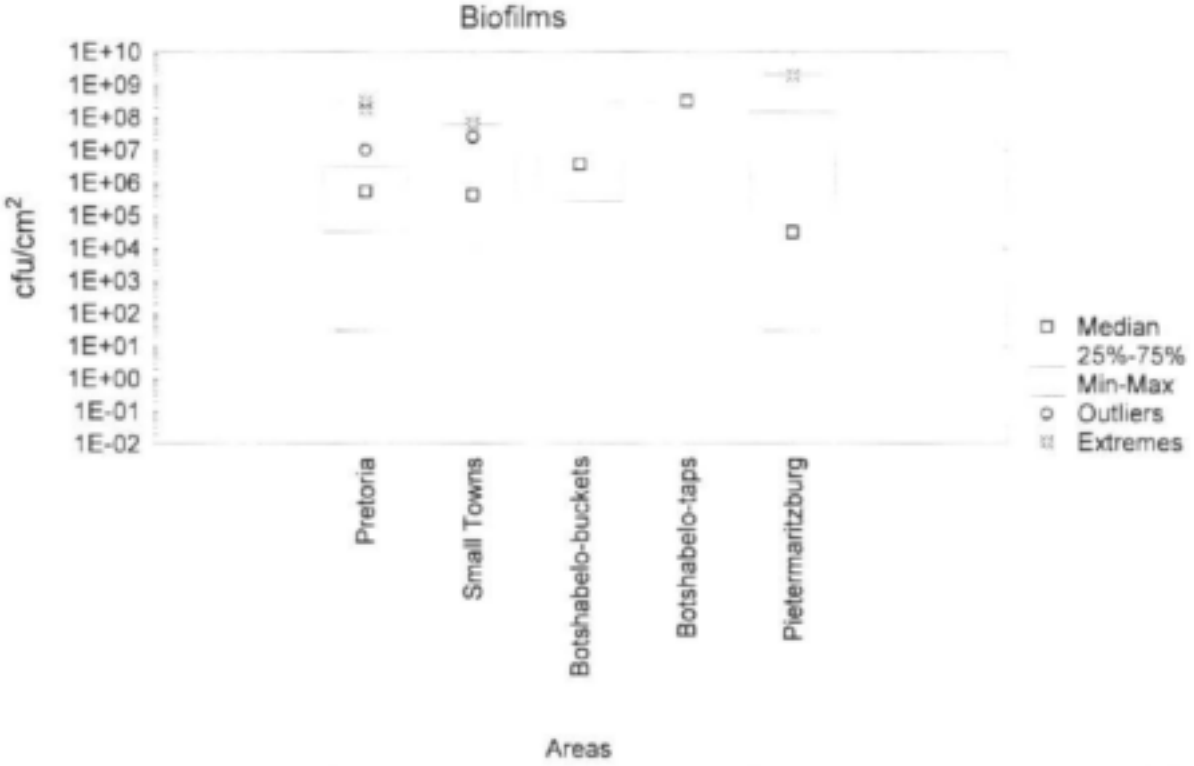


Figure A.2: Box and whisker plot showing the heterotrophic culturable counts of the water samples from the different areas sampled (Serial dilutions were plated onto R_2A and incubated for 5 days 28°C).

A.2.4.2 Analysis of bacterial community in the biofilm

The results obtained for the determination of the heterotrophic culturable counts of the biofilm samples are indicated in Fig. A.3. This analysis was included to see if any relationship could be established between the heterotrophic counts of the biofilm samples and the occurrence of potential pathogenic bacterial genera present, as well as to ascertain the degree of biofilm occurrence in drinking water distribution systems. Assuming a small average cell size (length of 2 µm and diameter of 1 µm) under low nutrient conditions, with little cell-cell spacing, complete coverage would amount to between 1 and 2.5 x 10^7 cells.cm^{-2}. The data (Fig. A.3) therefore indicates that the average surface exposed to drinking water in the various regions did harbour biofilm, but without complete coverage. Biofilms were, therefore, present, but not in excessive levels. The taps in Botshabelo constituted a noteworthy exception, and some buckets also harboured more than an average monolayer of biofilm.

Figure A.3: Graph showing the heterotrophic plate counts of the biofilms for the different areas sampled (Serial dilutions were plated onto R_2A and incubated for 5 days 28°C).



Figs A.4 to A.8 show the numbers of presumptive pathogens as found in the biofilm samples, and as based on colony appearance corresponding to that as described in the manuals and literature as obtained from the respective suppliers. During the first round of sampling it was found that the selective systems employed for the genera *Burkholderia, Listeria, Plesiomonas* and *Yersinia* yielded no isolates, so that these analyses were discontinued for subsequent samples. All-round, a large number of presumptive *Aeromonas, Vibrio* and *Shigella* as well as non-tuberculoid *Mycobacterium,* some *Pseudomonas* and one *Salmonella* were detected in biofilm samples. The numbers of presumptive members of pathogenic genera were, however, much lower than the total culturable count of the corresponding biofilm, indicating that the biofilm was composed predominantly of non-pathogenic bacteria.

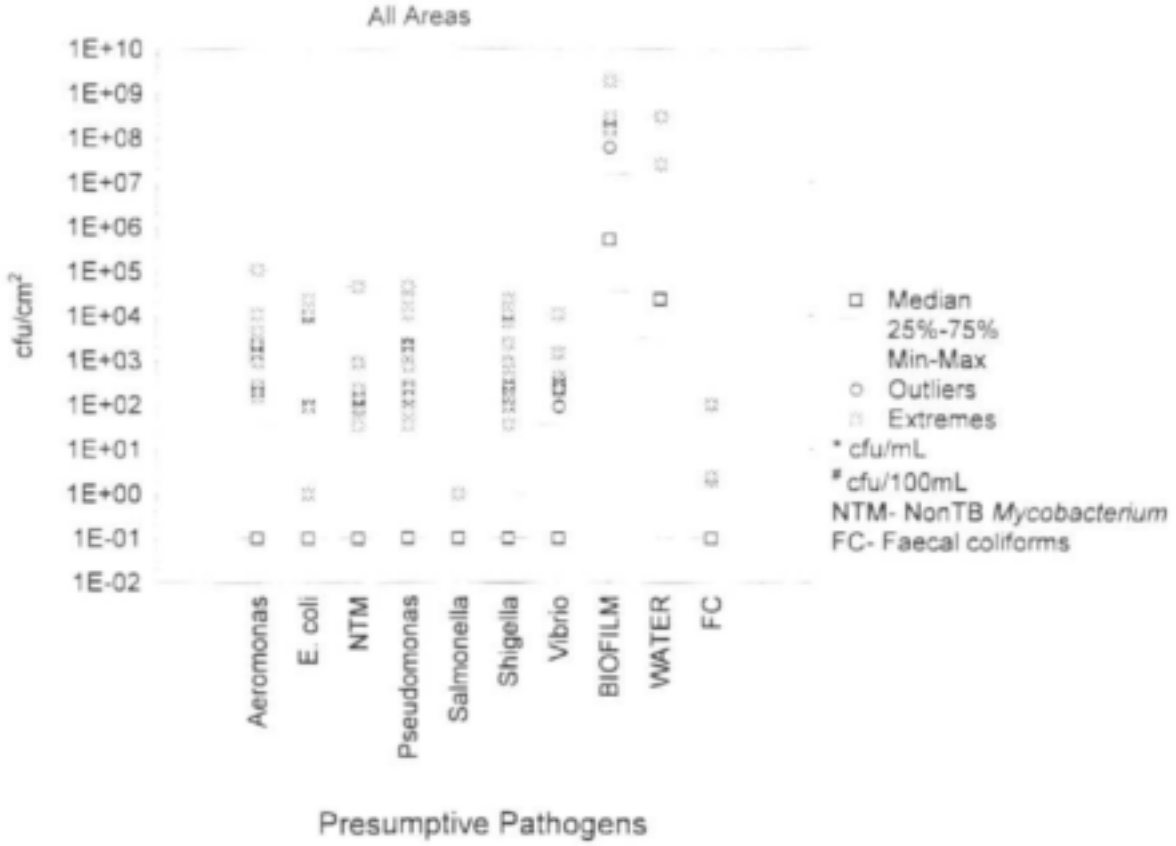


Figure A.4: The presence of the various presumptive pathogens (included are HPC and faecal coliform counts for water and biofilms) as found in all the samples.

NTM were detected primarily in the Pretoria area, with only selected occurrences in Botshabelo and small towns. This must be viewed against the near-absence of any other detectable pathogens, as well as the low overall planktonic count (Fig. A.5). By contrast, biofilms from small-town systems abounded with various gram negative pathogenic groups occurring in high numbers (Fig. A.6). The same scenario was observed in Boshabelo (Fig. A.7). Whilst the planktonic counts of Pietermaritzburg water were high, both biofilm counts and notably presumptive pathogen counts were very low.

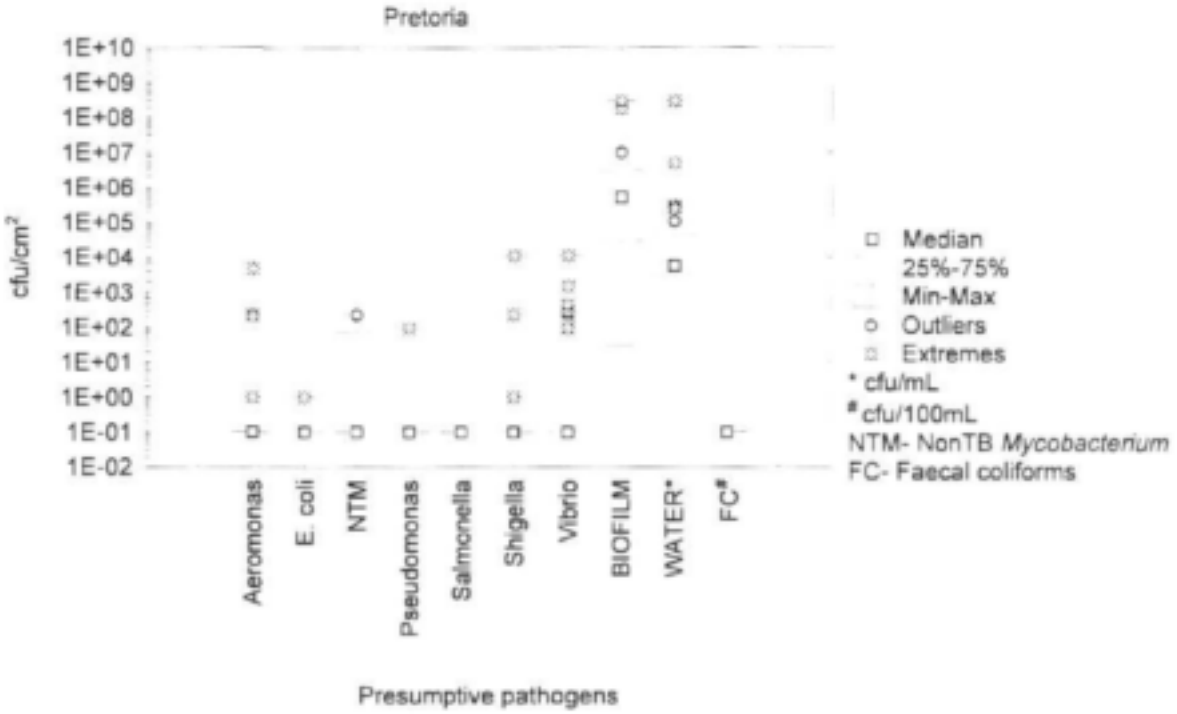


Figure A.5: The presence of the various presumptive pathogens as found in the Pretoria samples (included are HPC and faecal coliform counts for water and biofilms).

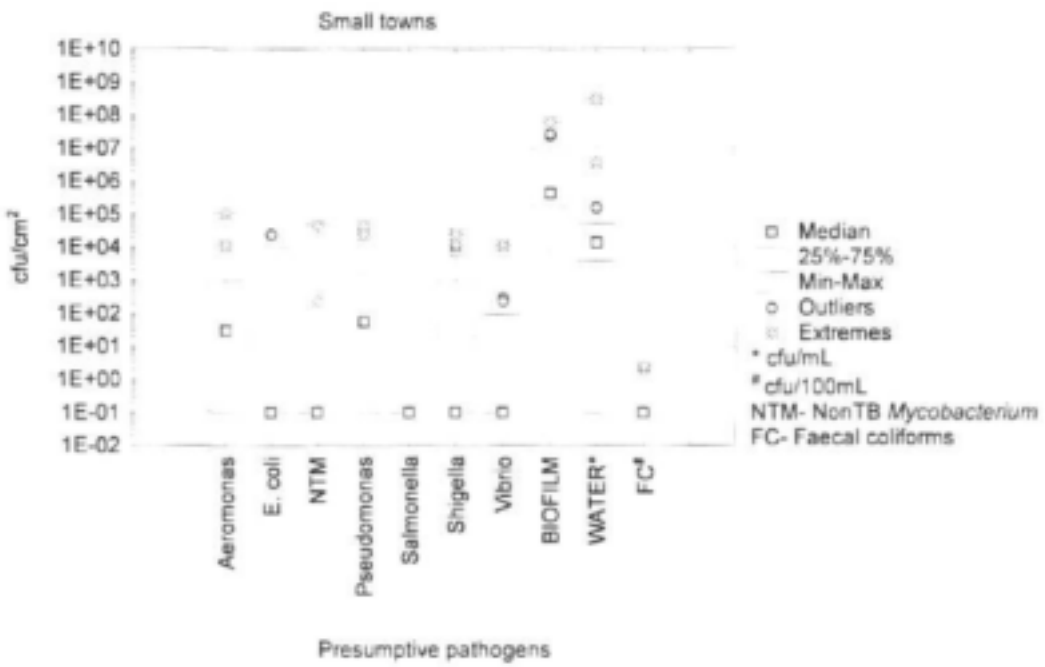


Figure A.6: The presence of the various presumptive pathogens as found in the small town samples (included are HPC and faecal coliform counts for water and biofilms).

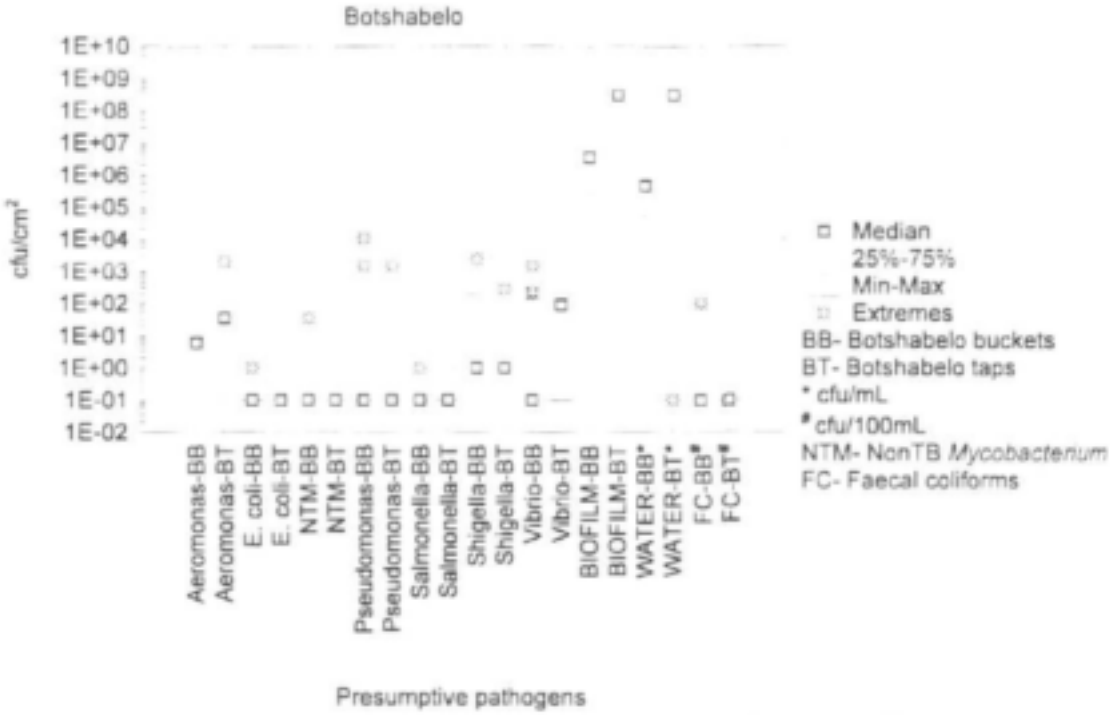


Figure A.7: The presence of the various presumptive pathogens as found in the Botshabelo samples. Samples from buckets and the taps from which these were reportedly refilled are shown separately as "BB" (bucket) and "BT" (tap) (included are HPC and faecal coliform counts for water and biofilms).

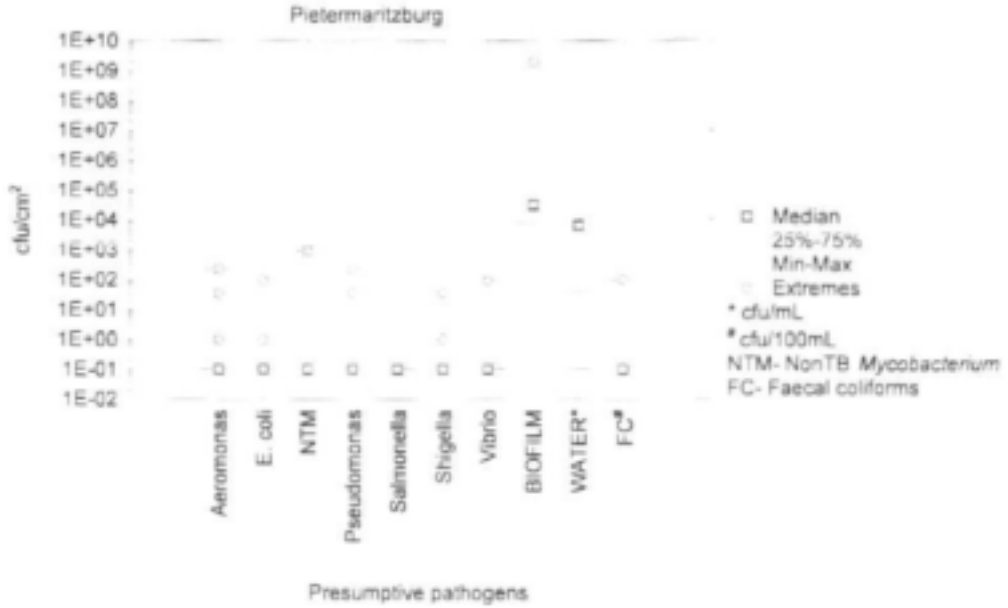


Figure A.8: The presence of the various presumptive pathogens as found in the Pietermaritzburg samples (included are HPC and faecal coliform counts for water and biofilms).

In the following figures (Figs A.9 to A.11) the distribution of the *Aeromonas*, *Mycobacterium* and *Pseudomonas* in samples from the different areas is shown.

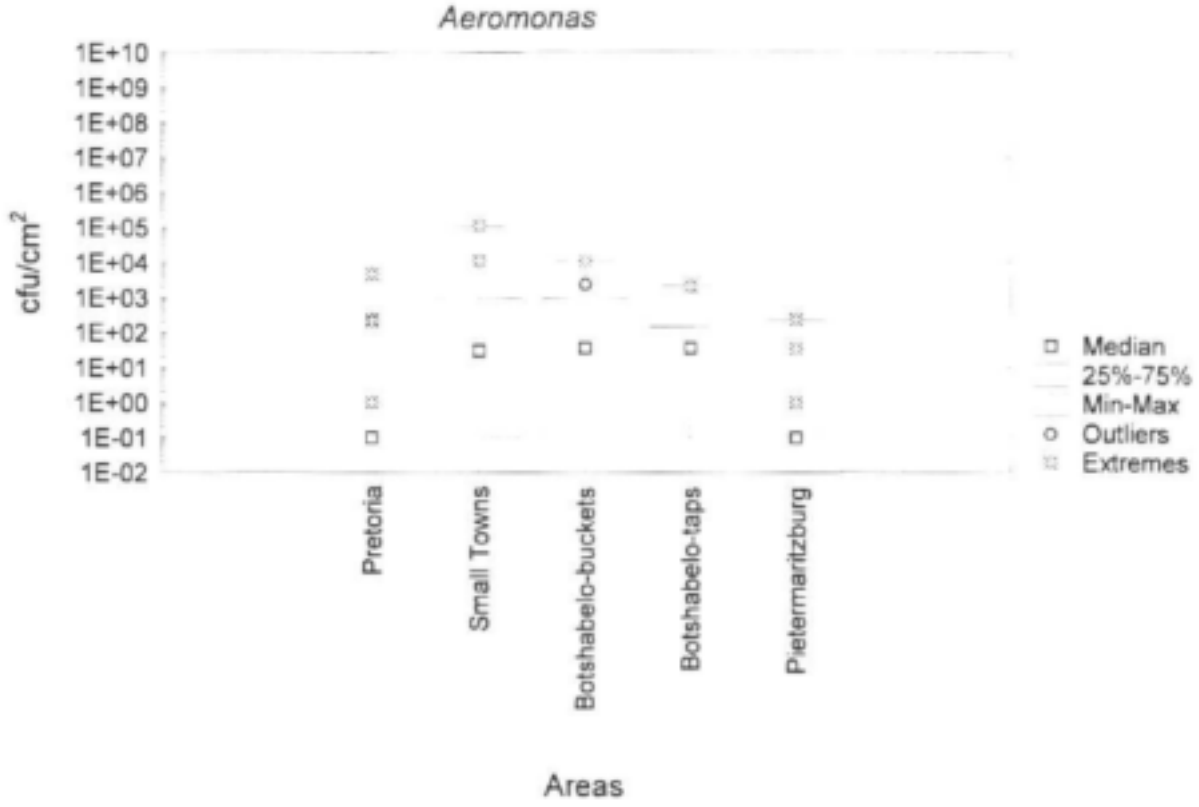


Figure A.9: The distribution of presumptive *Aeromonas* in drinking water-associated biofilms in the different areas

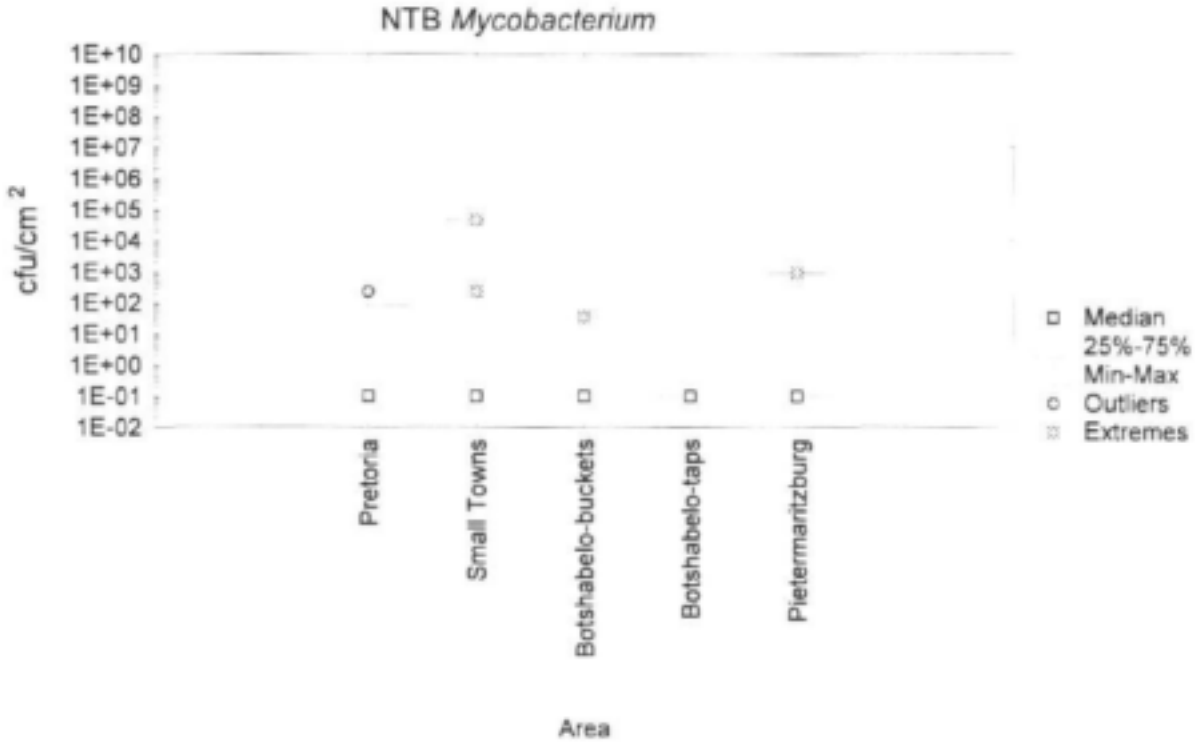


Figure A.10: The distribution of presumptive non-TB *Mycobacterium* in drinking water-associated biofilms in the different areas

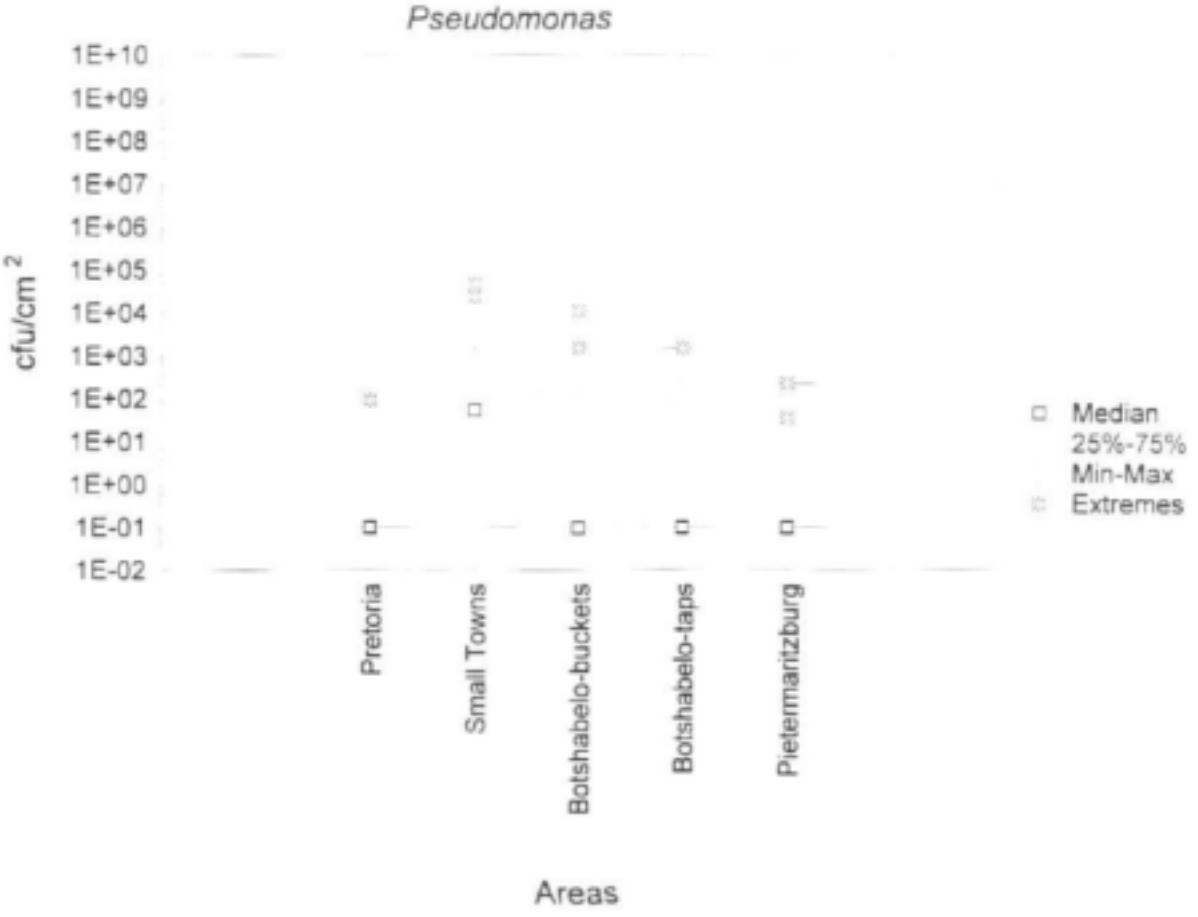


Figure A.11: The distribution of presumptive *Pseudomonas* spp. in drinking water-associated biofilms in the different areas

A.2.4.3 Sequence identity

The partial sequences of the 16S rDNA genes of 114 randomly selected isolates were determined. The results of the closest matching species as determined by BLAST search (see materials and methods) are given in Table A.4. Isolates are listed in order of their identity and then by selective medium from which obtained. It should be emphasized that isolates were picked at random, and that the identity obtained often did not match with that implied by the respective selective isolation system. All in all, 14 *Acinetobacter*, 18 *Aeromonas*,4 *Enterococcus*, 6 *Klebsiella*, 15 NTM, 6 *Pantoea*, 36 *Pseudomonas* and 3 *Staphylococcus* were identified, together with one or two representatives of various other genera. Notably, not one putative *Vibrio*, *Shigella* or *Salmonella* could be confirmed, indicating that none of these virulent pathogens did in fact occur in drinking water-associated biofilms. This does not constitute proof of absence, as not every single colony from every single dilution and sample could be identified phylogenetically.

Table A.4: Partial 16S rDNA sequence identity of randomly selected isolates

Isolate	*Isolation medium*	*16S rDNA sequence identity*
2 PTN E1	Chromocult Coliform-Agar	*Acinetobacter*
1 24B Y1	‡	*Acinetobacter*
1 12B B1	‡	*Acinetobacter*
1 25B SH1	Hektoen Enteric Agar	*Acinetobacter*
1 25B A1	Aeromonas Agar Base	*Acinetobacter*
1 12B SH1	Hektoen Enteric Agar	*Acinetobacter*
1 12T B1	‡	*Acinetobacter*
1 HAC B1	‡	*Acinetobacter*
1 HAC PL2	‡	*Acinetobacter*
1 ERC Y1	‡	*Acinetobacter*
1 ERC B1	‡	*Acinetobacter*
1 ERC PL1	‡	*Acinetobacter*
1 ERC A1	Aeromonas Agar Base	*Acinetobacter*
1 HAC PL1	‡	*Acinetobacter*
2 127 PS1	Pseudomonas Isolation Agar	*Aeromonas*
2 241 SH1	Hektoen Enteric Agar	*Aeromonas*
2 241 PS1	Pseudomonas Isolation Agar	*Aeromonas*
1 24B PL1	‡	*Aeromonas*
1 24B V1	Cholera Medium TCBS	*Aeromonas*
1 24B V2	Cholera Medium TCBS	*Aeromonas*
1 12B PL1	‡	*Aeromonas*
1 24T PS1	Pseudomonas Isolation Agar	*Aeromonas*
1 25T SA1	XLD	*Aeromonas*
1 25T PS1	Pseudomonas Isolation Agar	*Aeromonas*
1 12B A1	Aeromonas Agar Base	*Aeromonas*
1 20T A1	Aeromonas Agar Base	*Aeromonas*
1 12T Y1	‡	*Aeromonas*
2 BGF A1	Aeromonas Agar Base	*Aeromonas*
2 SEA A1	Aeromonas Agar Base	*Aeromonas*
2 SEB E1	Chromocult Coliform-Agar	*Aeromonas*
1 24B A2	Aeromonas Agar Base	*Aeromonas*
1 24B PS1	Pseudomonas Isolation Agar	*Aeromonas*
1 LOT M1	Middlebrook 7H10 Agar	*Azospirillum*
2 BGB E1	Chromocult Coliform-Agar	*E.coli*
2 BGC E1	Chromocult Coliform-Agar	*E.coli*
1 WYL A1	Aeromonas Agar Base	*Enterobacter*
1 WYL V1	Cholera Medium TCBS	*Enterobacter*
2 111 A1	Aeromonas Agar Base	*Enterobacter*
1 11T V1	Cholera Medium TCBS	*Enterobacter*
1 VIA V2	Cholera Medium TCBS	*Enterococcus*
1 24B SA1	XLD	*Klebsiella*
2 111 SH1	Hektoen Enteric Agar	*Klebsiella*
1 12B SA1	XLD	*Klebsiella*
1 11T A1	Aeromonas Agar Base	*Klebsiella*
1 20B A1	Aeromonas Agar Base	*Klebsiella*
1 20T SA1	XLD	*Klebsiella*
2 SUN M2	Middlebrook 7H10 Agar	*M. abscessus*
2 ERC M1	Middlebrook 7H10 Agar	*M. fortuitum*
2 HAC M1	Middlebrook 7H10 Agar	*M. gilvum*
2 ARC M2	Middlebrook 7H10 Agar	*M. gilvum*
2 ARC M3	Middlebrook 7H10 Agar	*M. gilvum*
2 ARC M4	Middlebrook 7H10 Agar	*M. gilvum*
1 LOT M2	Middlebrook 7H10 Agar	*M. gilvum*
2 ARW M2	Middlebrook 7H10 Agar	*M. gordonae*

Isolate	*Isolation medium*	*16S rDNA sequence identity*
2 ARW M3	Middlebrook 7H10 Agar	*M. gordonae*
2 PTN M1	Middlebrook 7H10 Agar	*M. haemophilum*
1 SUN M2	Middlebrook 7H10 Agar	*M.genomospp.3*
1 HAC M1	Middlebrook 7H10 Agar	*M.gordonae*
1 HAC M2	Middlebrook 7H10 Agar	*M.gordonae*
1 12B M1	Middlebrook 7H10 Agar	*M.parafortuitum*
1 SUN M1	Middlebrook 7H10 Agar	*M.peregrinum*
2 HAC M2	Middlebrook 7H10 Agar	*Methylobacterium*
1 LOT M3	Middlebrook 7H10 Agar	*Methylobacterium*
1 HAW M1	Middlebrook 7H10 Agar	*Microbacterium*
2 WPR M1	Middlebrook 7H10 Agar	*Micrococcus*
2 SUN M1	Middlebrook 7H10 Agar	*Micrococcus*
2 127 E1	Chromocult Coliform-Agar	*Pantoea*
2 KHA E2	Chromocult Coliform-Agar	*Pantoea*
2 KHA A1	Aeromonas Agar Base	*Pantoea*
1 24B SA2	XLD	*Pantoea*
1 24T A2	Aeromonas Agar Base	*Pantoea*
1 WYL SH1	Hektoen Enteric Agar	*Pantoea*
1 WYL PS1	Pseudomonas Isolation Agar	*Pseudomonas*
1 REL PS1	Pseudomonas Isolation Agar	*Pseudomonas*
2 PC SH1	Hektoen Enteric Agar	*Pseudomonas*
2 CC PS1	Pseudomonas Isolation Agar	*Pseudomonas*
2 127 A1	Aeromonas Agar Base	*Pseudomonas*
2 ARW V1	Cholera Medium TCBS	*Pseudomonas*
2 ERC V1	Cholera Medium TCBS	*Pseudomonas*
2 PTN V1	Cholera Medium TCBS	*Pseudomonas*
2 ARW SH1	Hektoen Enteric Agar	*Pseudomonas*
1 24T SH2	Hektoen Enteric Agar	*Pseudomonas*
1 11B SA2	XLD	*Pseudomonas*
1 11B SH1	Hektoen Enteric Agar	*Pseudomonas*
1 11B V1	Cholera Medium TCBS	*Pseudomonas*
1 20B SA2	XLD	*Pseudomonas*
1 20B SH2	Hektoen Enteric Agar	*Pseudomonas*
1 HAW V1	Cholera Medium TCBS	*Pseudomonas*
1 HAW PL1	‡	*Pseudomonas*
1 HAW A1	Aeromonas Agar Base	*Pseudomonas*
1 HAW A2	Aeromonas Agar Base	*Pseudomonas*
1 VIA A1	Aeromonas Agar Base	*Pseudomonas*
1 VIA V1	Cholera Medium TCBS	*Pseudomonas*
1 ARW B1	‡	*Pseudomonas*
1 HAW SH1	Hektoen Enteric Agar	*Pseudomonas*
1 FGA A1	Aeromonas Agar Base	*Pseudomonas*
1 FGA SH1	Hektoen Enteric Agar	*Pseudomonas*
1 FGA V1	Cholera Medium TCBS	*Pseudomonas*
1 FGB V2	Cholera Medium TCBS	*Pseudomonas*
1 HAW SH2	Hektoen Enteric Agar	*Pseudomonas*
1 VIA SH1	Hektoen Enteric Agar	*Pseudomonas*
1 VIA SH2	Hektoen Enteric Agar	*Pseudomonas*
2 BGE PS1	Pseudomonas Isolation Agar	*Pseudomonas*
2 TRA PS1	Pseudomonas Isolation Agar	*Pseudomonas*
2 SEA PS1	Pseudomonas Isolation Agar	*Pseudomonas*
2 SEG PS1	Pseudomonas Isolation Agar	*Pseudomonas*
2 SEB PS1	Pseudomonas Isolation Agar	*Pseudomonas*
2 BGA V1	Cholera Medium TCBS	*Pseudomonas*
1 LOT M5	Middlebrook 7H10 Agar	*Roseomonas*
1 VIA L1	‡	*Staphylococcus*

Isolate	*Isolation medium*	*16S rDNA sequence identity*
1 WPR PL2	‡	*Staphylococcus*
1 ARC PL1	‡	*Staphylococcus*
1 LOT M4	Middlebrook 7H10 Agar	Uncultured bacteria
2 BGE V1	Cholera Medium TCBS	*Vibrio*

‡ -All these isolates were isolated from selective systems specific for one of *Burkholderia*, *Plesiomonas*, *Listeria* and *Yersinia* used during the first round of sampling.

The genera listed all contain known pathogenic bacteria, some to a more prominent and others to a lesser degree. The *Pseudomonas* isolates were all very similar to each other, and none could be assigned to the nosocomial pathogens *P. aeruginosa* or *P. mendocina*, all belonging to the non-pathogenic *putida-fluorescens* group. Isolates belonging to the other genera were more diverse, so their partial 16SrDNA sequences were compared to each other and to those of known species in order to shed light on their possible relatedness to pathogens. Phylograms were compiled (see material and methods) for isolates allocated to the genera *Acinetobacter, Aeromonas, Klebsiella* and *Mycobacterium* as identified by their 16S rDNA, and compared to the 16S rDNA of known related species within these genera or groups. These are shown in Figs A.12 – A.15 below.

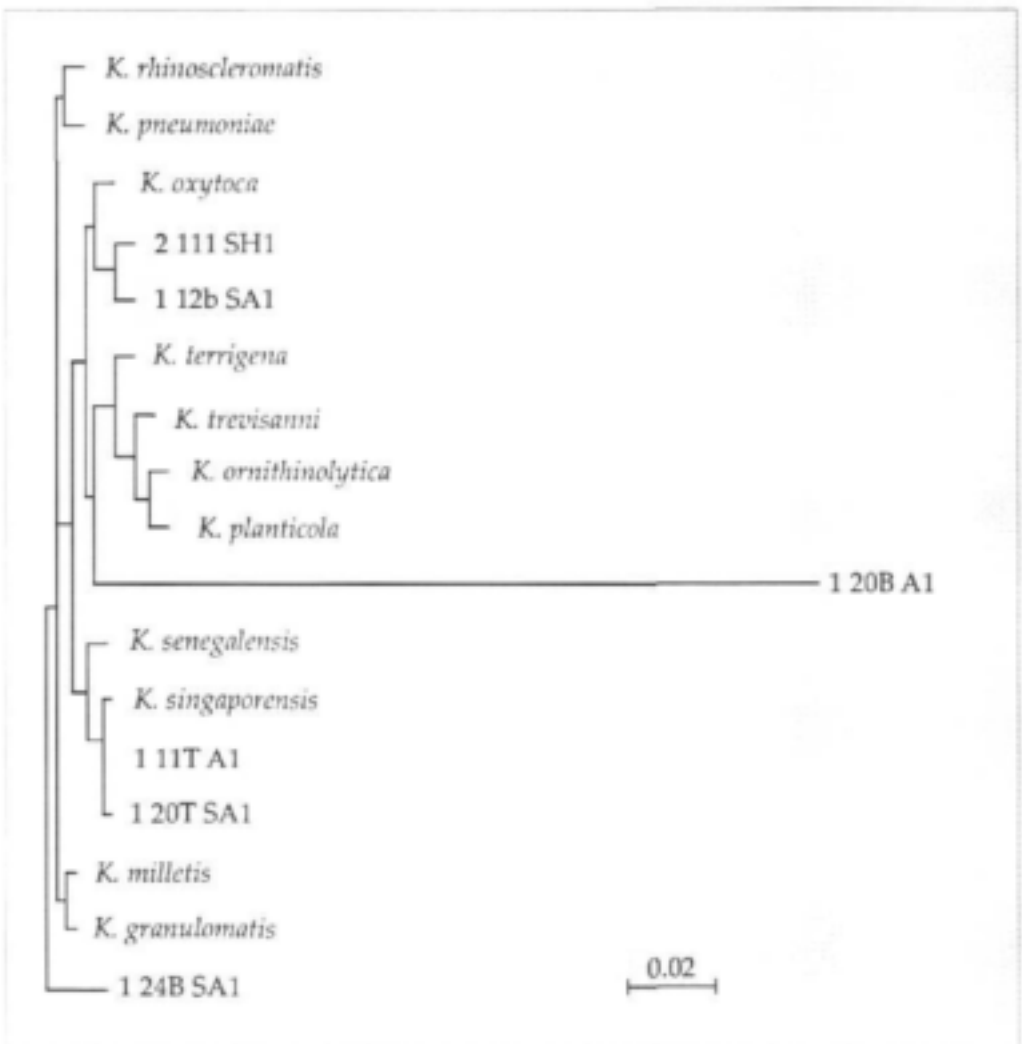


Figure A.12: Phylogenetic diagram of isolates identified as *Klebsiella*. Isolates are identified by their code, whilst the positions of known (reference) species are given by their taxonomic name. The key specifies distance as a degree of difference where 1 = 100% difference.

Four of the six *Klebsiella* isolates were obtained from XLD or Hektoen agars, recommended for isolation of *Salmonella* and *Shigella*, and the other two were presumptive *Aeromonas*. Furthermore, none of the *Klebsiella* isolates were closely related to documented pathogens such as *K. pneumoniae*.

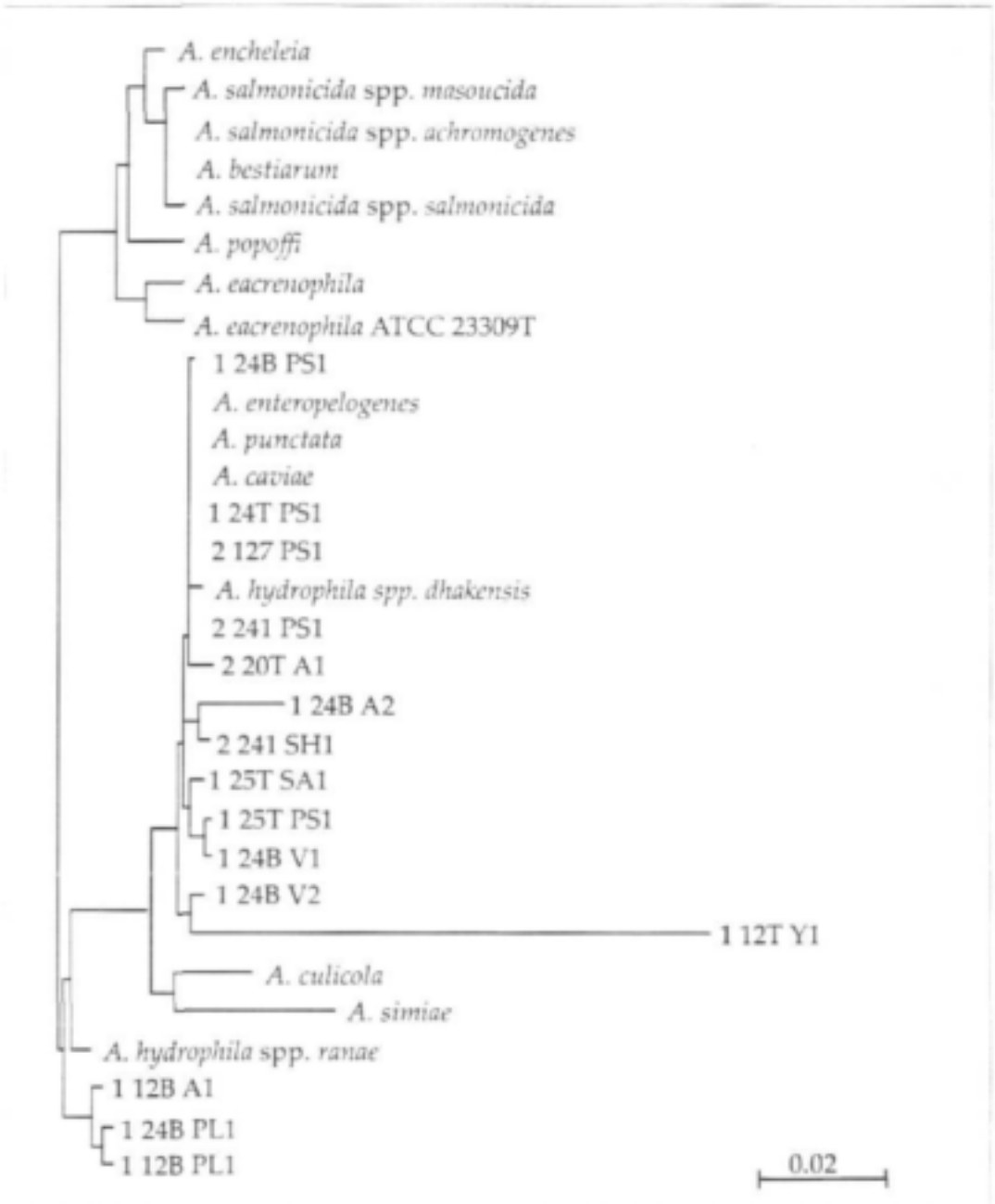


Figure A.13: Phylogenetic diagram of isolates identified as *Aeromonas*. Isolates are identified by their code, whilst the positions of known species are given by their taxonomic name.

The *Aeromonas* isolates were obtained from a range of selective media, showing the broad degree of resistance of members of this genus to the various selective agents used. Furthermore, the genus has a highly conserved rDNA gene pool, making differentiation at the species and lower level questionable. The genus is known to harbour some strains pathogenic to humans, notably *A. caviae*, *A. veronii biotype* sobria, associated among others with travellers' diarrhoea (Vila et al., 2003).

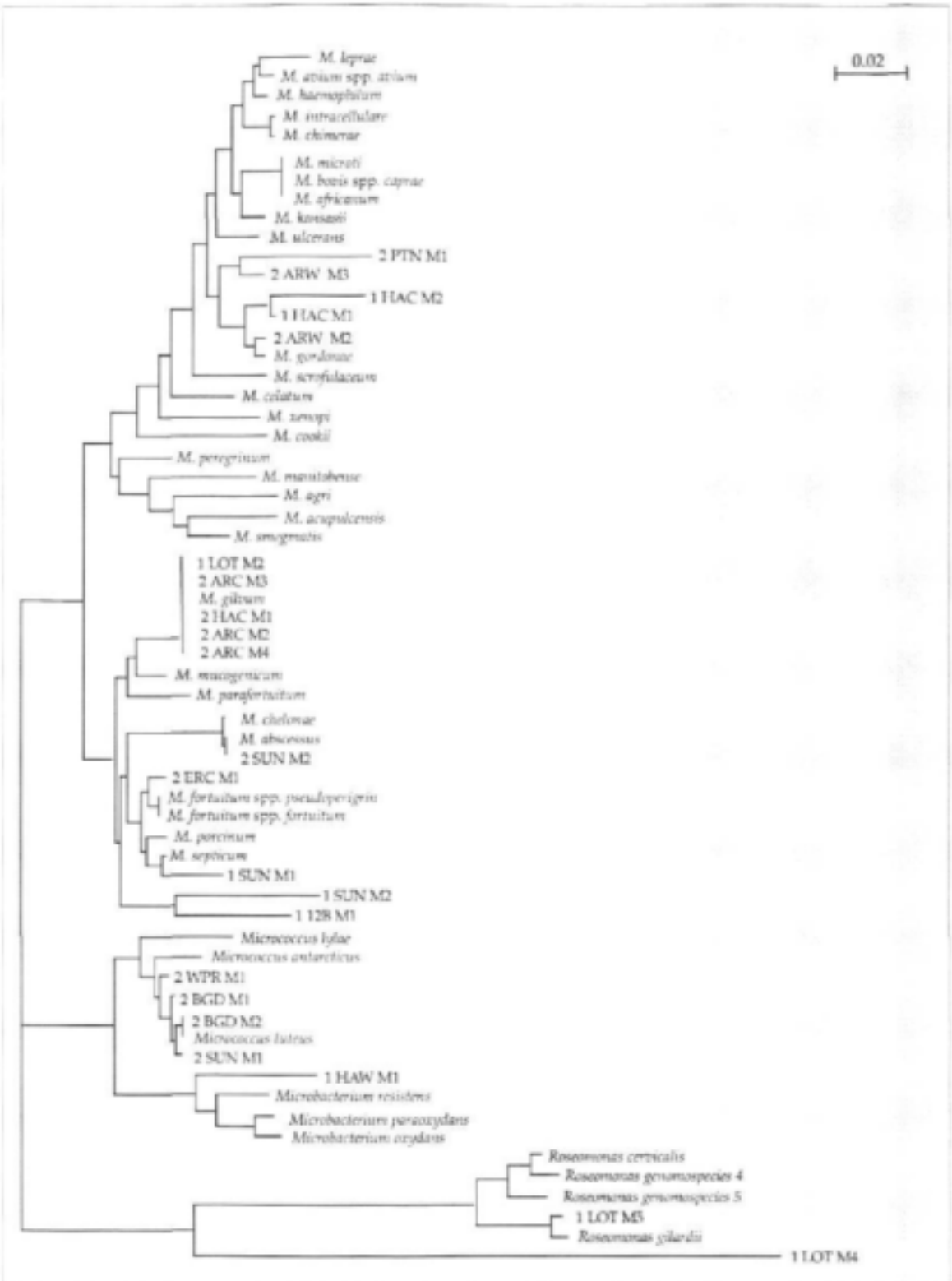


Figure A.14: Phylogenetic diagram of isolates identified as *Mycobacterium*. Isolates are identified by their code, whilst the positions of known species are given by their taxonomic name.

Middlebrook agar yielded a diverse range of isolates. Five were related to *M. gilvum*, six to *M. gordonae* and one to *M. abscessus*. *M. gordonae* is increasingly isolated from adolescents, especially the immunocompromised (Garofalo et al., 2003), and *M. abscessus* from immune-competent patients (Sungkanuparph et al., 2003). *M. gilvum* is not a reported pathogen. A number of isolates were not highly related to documented *Mycobacterium*,

indicating that there may be a number of water-associated species which are as yet not or poorly documented. A number of non *Mycobacterium* were also isolated, but all were members of the larger group within which this genus falls. None of these isolates were related to known pathogens.

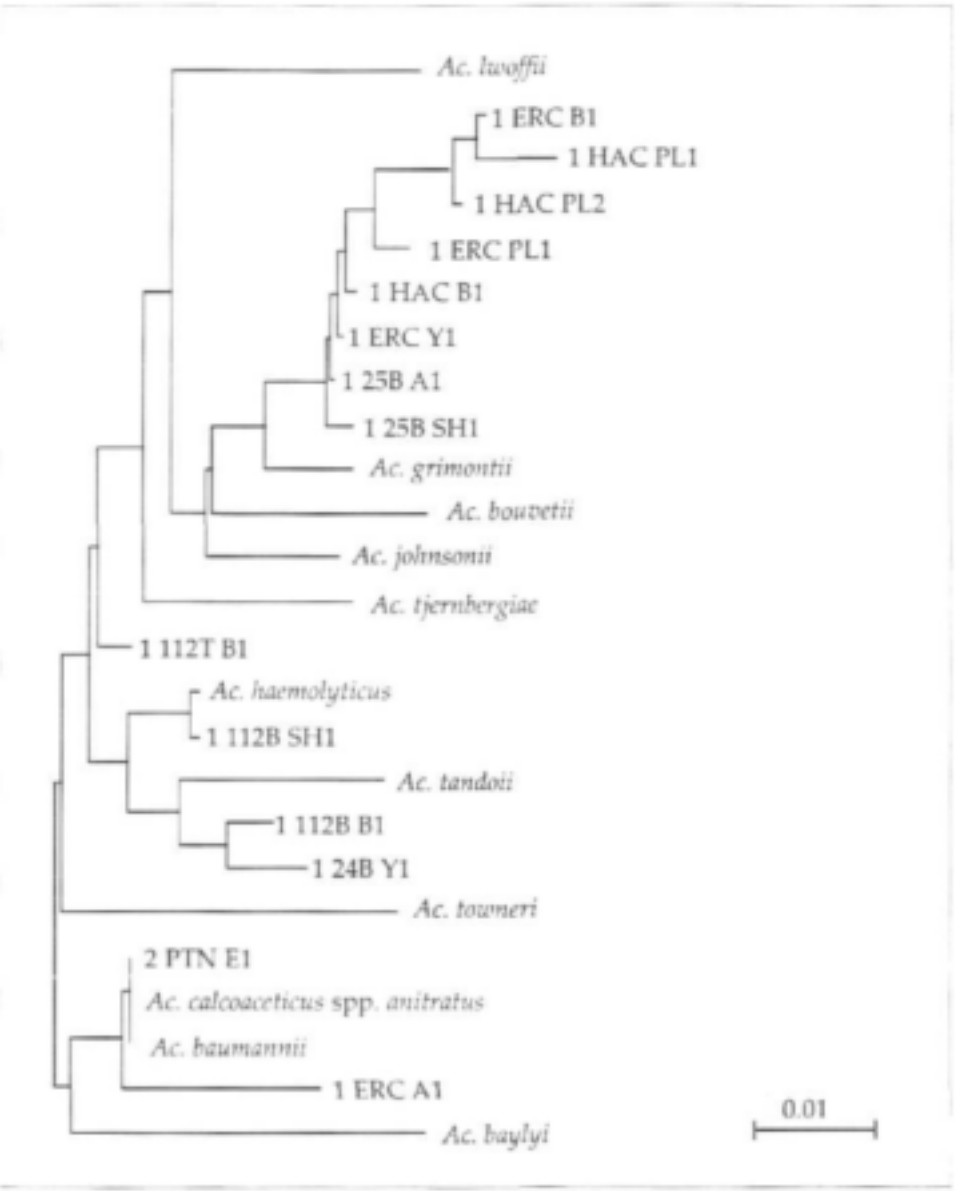


Figure A.15: Phylogenetic diagram of isolates identified as *Acinetobacter*. Isolates are identified by their code, whilst the positions of known species are given by their taxonomic name.

Only two *Acinetobacter* were related to documented pathogens, one to *A. haemolyticus* and the other to *A. baumanii*. The other isolates were all distant to documented strains, again indicating the as yet uncharted diversity of bacteria in water-associated biofilms.

A.2.5 Conclusions

- Bacterial biofilms were detected from all surfaces of drinking water distribution systems sampled, with a mean culturable count of 8 x 10^5 cfu.cm^{-2}, indicating that surfaces were not completely covered in biofilm.
- The corresponding waters samples displayed high culturable counts (mean ~ 3 x 10^4.mL^{-1}). Alarmingly, 10.5% of samples tested positive for faecal coliforms, mostly coming from the distribution networks of small towns and Botshabelo.
- A large number of biofilm samples harboured presumptive *Aeromonas*, *Vibrio* and *Shigella* as well as non-tuberculoid *Mycobacterium* in high numbers, whilst some *Pseudomonas* and one *Salmonella* were detected.
- Non-tuberculoid *Mycobacterium* were predominant only in biofilms from the Pretoria area, but included several isolates related to the pathogenic *M. gordonae* and one *M. abscessus*.
- Biofilms from the two large distribution networks (Pretoria and Pitermaritzburg) revealed a very low prevalence of putative pathogens, whilst the smaller networks (small towns and Botshabelo) harboured high numbers of a variety of gram negative pathogens.
- The selective culture media all proved unreliable, with a high incidence of false positives. Not one putative *Vibrio*, *Shigella* or *Salmonella* could be confirmed, indicating that none of these virulent pathogens occured in drinking water-associated biofilms at detectable levels.
- A large number of *Aeromonas*, one of the causative bacteria of travellers' diarrhoea, were obtained from a variety of biofilms, prompting the team to study the biofilm-physiology and fate of this group in more detail (Chapter 3).

A.3 FATE, SURVIVAL, GROWTH AND DISSEMINATION OF AEROMONAS IN A BIOFILM IN A DRINKING WATER DISTRIBUTION SYSTEM

A.3.1 Introduction

Biofilms are well known to be able to form and develop on most surfaces exposed to liquids (Costerton et al., 1995). Furthermore biofilms tend to provide protection for those organisms residing in them, either by protection from scavengers such as protozoal and other grazers, or by inducing a resistant phenotype. The pipes in a drinking water distribution system are thus no exception to the host of biofilm-colonized surfaces (Szewzyk et al., 2000). Attachment to surfaces might even give cells a nutritional advantage in this 'low-nutrient' or oligotrophic environment (Camper et al., 1999).

The distribution of *Aeromonas* species in drinking water distribution systems is well known and, and although normally killed during treatment processes, it is still being isolated from drinking water lines (Assanta et al., 1998; Kühn et al., 1997). The presence of the organism in treated water may be attributed to recovery and re-growth of sub-lethally injured cells, or alternatively it being released from a biofilm within the system (Camper et al., 1999). The adhesion of *Aeromonas* to different pipe materials has been reported by Assanta et al., (1998) who showed, with the use of scanning electron microscopy, that the organism is able to attach to copper, stainless steel and polybutylene surfaces.

The preparation of biological samples for scanning electron microscopy requires extensive dehydration, leading to distortion of the sample (Donlan and Costerton, 2002). The advent of the scanning confocal laser microscope (SCLM) opened the way for 3-dimensional study of biofilms. More importantly, advances in fluorescence microscopy have paved the way to study single cells within biological samples, provided that these bear a fluorescent marker. In order to monitor a specific organism in a mixed population biofilm, one can therefore make use of fluorescently labelled probes or antibodies specific to the organism in question (Camper et al., 1999). This allows the *in situ* detection of undisturbed cells and also gives information regarding its spatial distribution within the biofilm (Camper et al., 1999). Once labelled, however, the sample is usually no longer biologically active, rendering this approach inappropriate for real-time studies. A superior and more recently developed approach entails the prior genetic labelling of the target organism with the *gfp* gene, encoding the green fluorescent protein (GFP) (Eberl et al., 1997). The detection of GFP does not require any external substrates and is therefore a good marker system for monitoring biofilm processes over time (Eberl et al., 1997).

We report in the previous chapter that *Aeromonas* was one of the predominant bacterial pathogens associated with biofilms in drinking water distribution systems in various regions in South Africa.

A.3.2 Aims

The aims of this study were, therefore:

- to label an environmental strain of *Aeromonas* with GFP; and
- to subsequently determine its fate, survival and growth in a simulated drinking water distribution system biofilm.

A.3.3 Materials and Methods

A.3.3.1 Isolation and identification of an environmental Aeromonas *for biofilm studies*

After screening the different biofilm samples for the presence of potential bacterial pathogens, *Aeromonas* was chosen as one of two organisms to be investigated further, for its fate and behaviour in a drinking water biofilm. One strain isolated from one of the drinking water biofilms analysed during the first part of the study was chosen for further study based on its resilience under laboratory culture conditions. The isolate was identified by partially sequencing its 16S rDNA gene, as described in chapter 2. In this case, sections of both strands of the gene were sequenced using the primers fD1 and rP2 (Weisburg et al., 1991). The sequences were compared to known sequences in GenBank (NCBI).

A.3.3.2 Labelling of the environmental Aeromonas *strain with GFP*

Two approaches to labelling the *Aeromonas* isolate with the *gfp* gene were taken, one using a Tn7-based system and the other using a Tn5-based system.

A.3.3.2.1 Labelling of *Aeromonas* using a Tn7 transposon system

The Tn7 system employed was chosen due to its reported high transposition frequency, and site specific manner of integration into the chromosomes of gram negative bacteria, making it a useful delivery system for the insertion of cloned DNA (Bao et al., 1991).

The *Aeromonas* was tagged with the *gfp* gene by four parental mating. The delivery plasmid, pBK miniTn7-*gfp1* was obtained from Dr B. Koch. (Koch et al., 2001). It contains a 2kb *Not*I fragment from pJBA28 (Anderson et al., 1998), that consists of a *Lac*I-repressible promotor ($P_{A1/04/03}$), *gfp*mut3*, a synthetic ribosomal binding site, transcriptional terminators and the *cat* gene encoding chloramphenicol resistance (Fig. A.16). The plasmid was maintained in *E. coli* XL1 blue. (For a complete description on the construction of the plasmid refer to Anderson et al., 1998 and Koch et al., 2001).

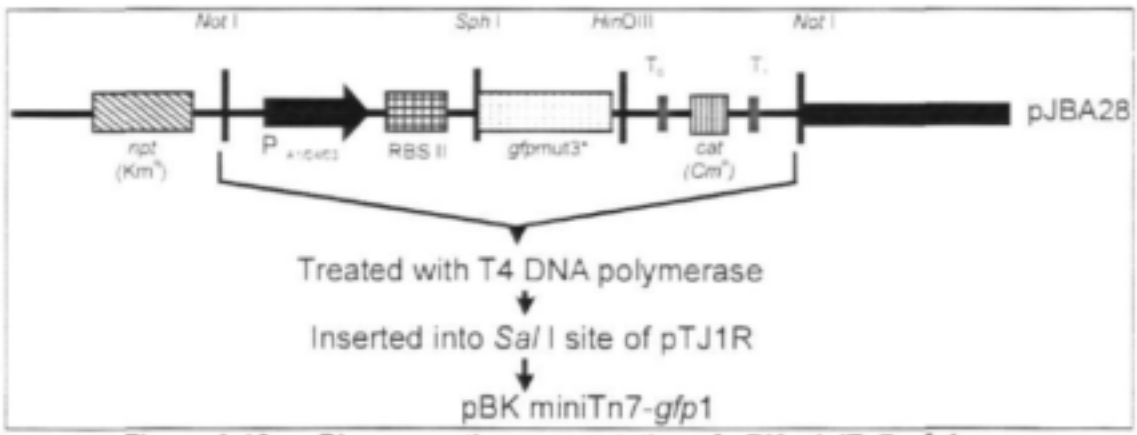


Figure A.16: Diagrammatic representation of pBK miniTn7-*gfp*1.

The helper plasmid, pUX-BF13, was maintained in *E. coli* SM10/*λpir* and pRK600 in *E. coli* was used as the mobilisor. The four parental mating was performed by mixing loopfulls of each of the four cultures (*Aeromonas*, the donor strain, helper strain and mobilizor) on LB agar and incubating at 37°C for 18 hours. A loopfull of this growth was re-streaked onto Aeromonas Agar Base (Oxoid) supplemented with chloramphenicol, (100 µg/mL) and incubated at 37°C for 18 hours. This would select for only the *Aeromonas* that has been successfully tagged with GFP. Green colonies were selected and screened for green

fluorescence using a Zeiss Axiovert 200 Inverted Microscope with excitation at 490 nm and emission at 510 nm. Three quadri-conjugant mutants were selected for further screening.

The growth dynamics of the tagged *Aeromonas* strains were compared to that of the parental *Aeromonas* under nutrient-rich (LB broth) conditions under otherwise identical conditions. This was done by growing the both the tagged and parental strains in LB broth while shaking at 37°C for 18 hours (overnights). To determine the respective growth rates, 50 mL of the respective broth in duplicate 250 mL side-arm flasks was inoculated with 0.1 mL of the corresponding overnight culture. Flasks were incubated while shaking at 37°C. The optical density at 620nm was determined at fixed time intervals throughout the experiment. The quadri-conjugant that showed the least growth impairment was selected for further evaluation.

The stability of the *gfp* in the *Aeromonas* was determined by actively growing the tagged *Aeromonas* in 1/10 strength R_2A broth for three weeks. The labeled strain was inoculated in 1/10 strength R_2A broth, incubated at 37°C and transferred to fresh broth daily for 21 days. The final culture was subjected to bright-field and fluorescence microscopy using the Zeiss Axiovert 200 Inverted Microscope (490nm excitation filter and 510 nm emission filter) to ascertain whether all cells still expressed GFP. Subsequently, the growth rate of the mutant under nutrient-poor conditions (1/10 strength R_2A broth) was compared to that of the parental as described above.

A.3.3.2.2 Labelling of *Aeromonas* using a Tn5 transposon system

The Tn5 transposon system reportedly inserts randomly on the chromosome of gram negative bacteria and may cause insertional inactivation of one of the host genes (De Lorenzo et al., 1998). By screening of the mutants, one could be selected that shows the least or no growth impairment.

Plasmid pSM1690 was used as the delivery plasmid for the *gfp*mut3b* gene (Sternberg et al., 1999). The plasmid contains, in addition to *gfp*mut3b*, the *E. coli rrnBP*1 promoter, a synthetic ribosomal binding site, stop codons in all 3 reading frames and 2 transcriptional terminators (Fig. A.17).

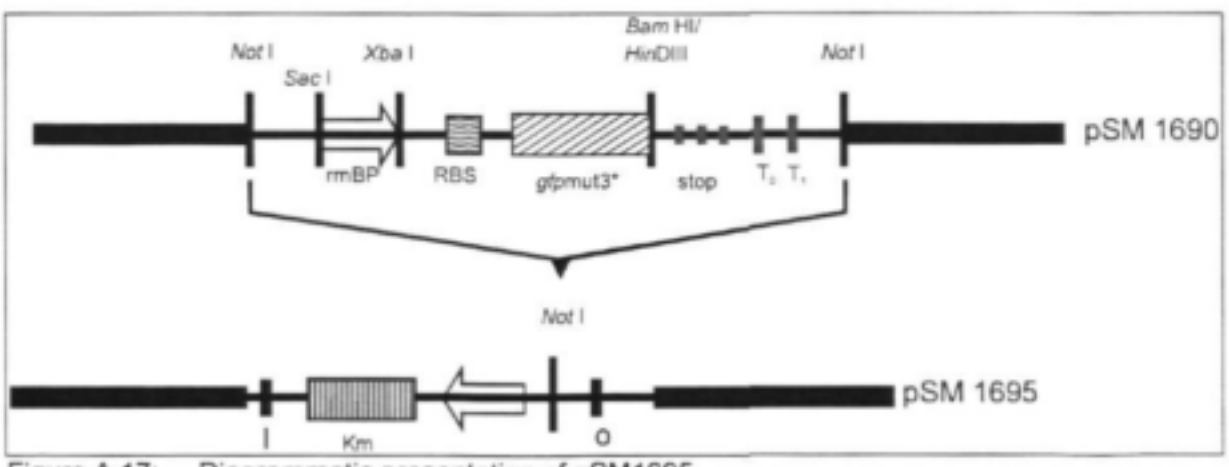


Figure A.17: Diagrammatic presentation of pSM1695.

The *gfp* gene was then transferred to *Aeromonas* by tri-parental mating. *E. coli* CC118*λpir* (pSM1695) as the donor strain and *E. coli* HB101 (pRK2013) as the helper were mixed with the environmental *Aeromonas* isolate on LB agar and incubated at 37°C for 18 hours. Growth from this plate was streaked onto Aeromonas Agar base (Oxoid) supplemented with kanamycin (100µg/mL). This was done to select for those transconjugants that had the *gfp* gene, since those that did not, would not be able to grow in the presence of kanamycin.

Plates were again incubated at 37°C for 18 hours. Single colonies were then collected and observed microscopically for fluorescence.

Three of the GFP labeled *Aeromonas* colonies were selected and their growth dynamics determined in nutrient poor media (R_2A) (as described above for the Tn7 transposon system). This was done to determine the impact of the insertion of the *gfp* gene on the growth of the organism.

The transconjugant that showed the least growth impairment was selected to determine the stability of the *gfp* gene. The stability of the *gfp* gene in the tri-transconjugant was determined by actively growing the transconjugant in non-selective 1/10 strength R_2A broth for a period of 21 days as described above.

A.3.4 Results and Discussion

The selected environmental isolate was identified as *A. hydrophila* according to its 16SrDNA sequence.

A.3.4.1 Tagging of A. hydrophila *using a Tn7 transposon system*

The *A. hydrophila* isolate appeared to be successfully and stably labelled with *gfp* using the Tn7 transposon system. The stability studies of the tagged *A. hydrophila* showed that almost all the cells were still fluorescing after the three week continuous growth period, proving that the *gfp* was stably maintained in the *Aeromonas* (Fig. A.18). The comparison of growth rate of the parental with the tagged *A. hydrophila* strains showed the tagged strain to be growth impaired under nutrient-poor but not under nutrient rich conditions (Fig. A.19). This was despite the published claim that the delivery transposon would insert at a neutral site on the chromosome (Bao et al., 1991), and that no difference could be detected in the nutrient rich medium.

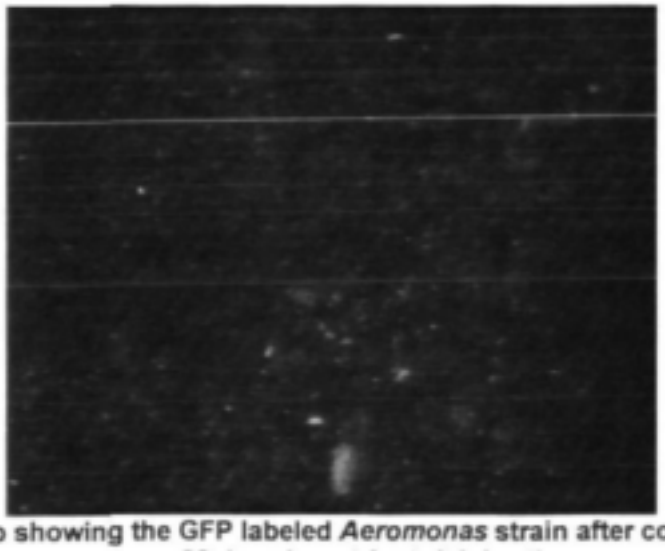


Figure A.18: Photo showing the GFP labeled *Aeromonas* strain after continuous growth for 30 days in nutrient-rich broth.

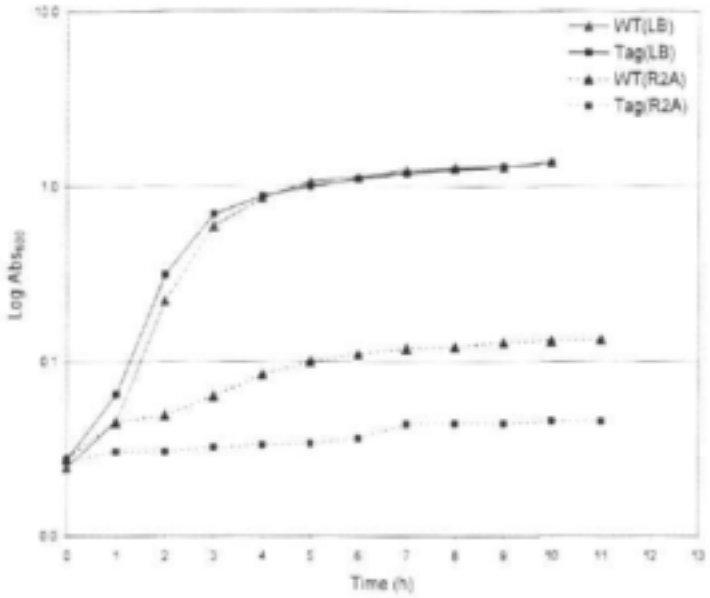


Figure A.19: Growth curve of the wild type *Aeromonas* versus the GFP labeled (Tn7 system) *Aeromonas* strain in nutrient rich (LB) and nutrient poor (1/10 strength R_2A) broths.

The results showed that whilst the *gfp* gene was stably maintained in the host, the recombinant was growth-impaired under nutrient-limiting conditions. It could therefore not realistically be used to study the fate of *A. hydrophila* in drinking water biofilms. This forced us to look at a different strategy for labelling the *Aeromonas* for the biofilm studies and it was decided to use the Tn5 transposon system to introduce the *gfp* gene onto the chromosome.

A.3.4.2 Tagging of A. hydrophila *using a Tn5 transposon system*

The labeling of the *A. hydrophila* with *gfp* using the Tn5 system produced a number of tri-transtransconjugants that were first screened for growth impairment. Screening was performed directly in the nutrient poor 1/10 strength R_2A broth, and one of the transconjugants appeared not to demonstrate any growth impairment (Fig. A.20, Tag A). This tri-transconjugant was thus selected to determine the stability of the construct.

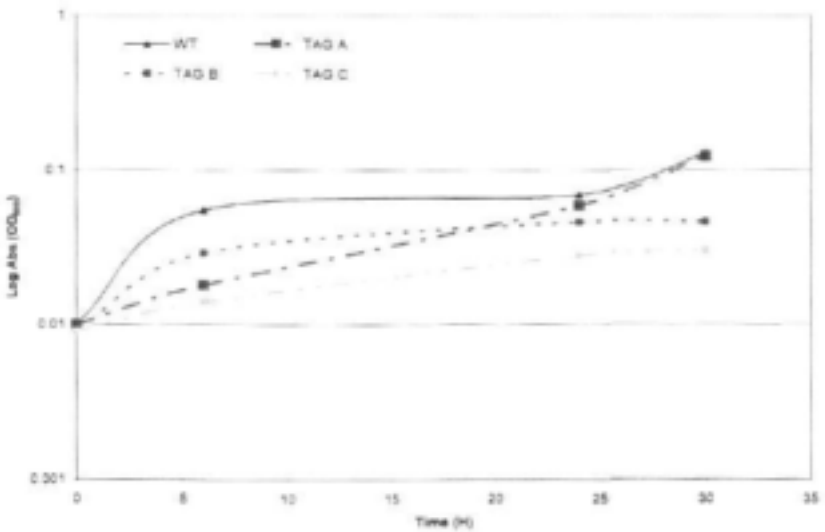


Figure A.20: Growth curve of the wild type *Aeromonas* versus three GFP labelled (Tn5 system) *Aeromonas* strain nutrient poor (10 strength R_2A) media.

The stability study in the nutrient poor medium was started but after the second day of transferring the culture, many cells appeared to loose their fluorescence and long filament-like growth was observed in the media. This experiment was repeated for a number of times to exclude the possibility of the sample becoming contaminated during transfers but the same phenomena was found with all the repeats. All isolates from the broth were confirmed as *A. hydrophila*. This indicated that the mutant selected was growth impaired in some aspect of cell division, rendering it null for long-term biofilm studies. Work to isolate new tri- and tetra-transconjugants not impaired in their long-term ability to grow under nutrient-limiting conditions is in progress before the envisioned work on the fate of this pathogen in drinking water biofilms can be further studied.

A.3.5 Conclusions

- Various chromosomally-*gfp*-tagged *Aeromonas* were obtained by two different approaches. These recombinant strains stably maintained and expressed the *gfp* gene during 30 days of continuous culture.
- Whilst some of the recombinants evaluated grew as the parental strain in standard laboratory culture media, all were severely growth-impaired in nutrient-poor media.
- Both transposon-delivery systems used may not insert as specifically into "neutral" sites, as reported in the literature. Rather they are likely to insert randomly, so that further mutagenesis and screening is in progress to obtain a mutant with a neutral insertion.
- The study of the fitness and fate of *A. hydrophila* in biofilms in water distribution systems was therefore postponed until such time as a suitable *gfp*- labelled recombinant is obtained (further research of PhD student S. September).

A.4 CONSTRUCTION AND EVALUATION OF GFP-TAGGED SALMONELLA ISOLATES FOR USE IN BIOFILM SURVIVAL AND DISSEMINATION STUDIES

A.4.1 Introduction

Salmonella is a major cause of gastroenteritis in humans, characterized by diarrhoea, cramps, vomiting, and fever. The symptoms are usually mild and self-limiting in healthy individuals. However, in the young, the elderly and in individuals with compromised immune systems, dehydration can become severe and life threatening. *Salmonella* are ubiquitous in the environment. Although food has been implicated as the major source of non-typhoidal *Salmonella* infections in the USA (Mead et al., 1999), the role of contaminated water in the transmission of the disease in developing countries is unknown.

Biofilms appear to be a common occurrence in water distribution systems (Szewzyk et al., 2000). Biofilms of potable water distribution systems have the potential to harbour enteric pathogens. Hood et al., (1997), have shown that *S. typhimurium* has the ability to grow in biofilm on inert food contact surfaces. The period of pathogen survival in water sources used for recreation, irrigation or as drinking water is of concern as it may have serious health consequences. In order to protect and manage the quality of water sources, a clear understanding of the fate, survival and transport of pathogens such as *Salmonella* is necessary.

The fate and persistence of non-typhoidal *Salmonella* in water environments has not been studied extensively due to the lack of a marked strain distinguishable *in vivo* from the rest of the bacterial community. Technologies have recently been developed which allow the tagging of bacterial strains using Green Fluorescent Protein (GFP). The *gfp* gene encoding green fluorescent protein is obtained from the jellyfish *Aequorea victoria*. GFP fluoresces green upon illumination with blue light and requires only the presence of oxygen during the initial formation of the chromophore. The chromosomal tagging of bacterial strains using GFP allows for the possibility of performing survival studies under mixed culture conditions. GFP is ideally suited to the quantification of tagged organisms in environmental samples as no substrate addition is required and the detection of fluorescence is independent of energy reserves. GFP is a very stable protein (Andersen et al., 1998) and can be detected *in situ* in single cells by fluorescence microscopy. As GFP does not reportedly interfere with the growth of the host, it is an excellent choice for non-disruptive studies of bacterial systems that require live cells to be studied at the single cell level (Andersen et al., 1998). It has been found that GFP fluorescence is maintained in viable but non-culturable cells, but is lost from dead cells (Lowder et al., 2000). Therefore, when GFP is fused with a growth-regulated promoter, a system like this will show cell growth activity, which is very useful for monitoring bacterial development in environmental samples. In this way, a more realistic indication of the growth potential of *Salmonella* is obtained due to the interaction with competing indigenous microorganisms.

A.4.2 Aim

The aims of this study were therefore:

- to isolate and identify clinically relevant *Salmonella* strains from a fresh water environment;
- to chromosomally tag two selected *Salmonella* strains with a *gfp* gene; and
- to investigate the fate and survival of *Salmonella* in an established aquatic biofilm.

A.4.3 Materials and Methods

A.4.3.1 Isolation and Identification of Salmonella *Strains*

This part of the study was performed in conjunction with a related WRC project performed together with the University of Venda. Ten grams of each of a number of sediment samples taken from rivers in the Gauteng region were pre-enriched in 50mL of buffered peptone water (BPW) and incubated for 24 hours at 37°C. Thereafter, 1mL of the pre-enrichment sample was inoculated into 9mL of Rappaport-Vassiliadis (RV) broth for selective enrichment and incubated for 24 hours at 42°C. Following incubation of the RV enrichment broth, a loopfull from each positive tube of the broth was plated on to Xylose Lysine Deoxycholate (XLD) agar and incubated for 24 hours at 37°C. A bioMérieux API 20E biochemical test strip was inoculated with a presumptive *Salmonella* suspension and incubated for 24 hours at 37°C. After the addition of the prescribed reagents to the cupules, the results were compared with the accompanying identification table. Three presumptive *Salmonella* isolates were chosen and sent for serological testing at the Onderstepoort veterinary laboratories.

A.4.3.2 Chromosomal Tagging

S. ser. Muenchen and *S.* ser. Typhimurium isolates were streaked on LB plates containing 100µg/mL of ampicillin (Amp100) and LB plates containing 100µg/mL of kanamycin (Km100) to confirm their susceptibility to these two selective agents. The pUT mini–Tn5 Km transposon (De Lorenzo et al., 1990) was used to introduce GFP onto the chromosome of the chosen *Salmonella* strains. The *Salmonella* isolates were transformed by tri-parental mating using the *E.coli* strains HB101 (pRK2013) as a helper strain and CC118λpir (pSM1695) as the donor strain (Sternberg et al., 1999) and the *Salmonella* isolates as the recipients. A loopfull of each of the donor, helper and recipient strains were mixed together on LB plates and incubated for 24 hours at 37°C. After 24 hours the growth obtained was streaked out on XLD media containing 100µg/mL of kanamycin and incubated at 37°C for 24 hours. Black colonies were selected and confirmed as tri-transconjugants by their ability to fluoresce green when exposed to blue light. Confirmed tri-transconjugant colonies were inoculated into 10mL of LB broth and incubated at 37°C. After 24 hours, serial dilutions (10^{-1} – 10^{-6}) were prepared by transferring 100µL of LB broth to 900µL of Ringer's solution. One hundred µL of the dilutions were plated out on LB Km100 plates and incubated at 37°C for 24 hours. A dilution plate was selected containing suitably spaced colonies to undergo replica plating. Sterile velvet strips were pressed lightly onto colonies on the LB Km100 plates and transferred to LB Amp100 plates by pressing the fabric on to the replica plates. The LB Amp100 plates were incubated at 37°C for 24 hours. A comparison of the colonies on the replica plates was made. Colonies that did not grow on the LB Amp100 plates were selected from the LB Km100 plates. These colonies were examined for fluorescence using a *Zeiss Axiovert* 200 Inverted Microscope. (Excitation - 490 nm and Emission - 510 nm), and one fluorescent isolate selected.

A.4.3.3 Verifying chromosomal tagging

The location of the *gfp* gene on the genome was confirmed by PCR. Chromosomal DNA was extracted from tagged strains, wild-type strains and the donor strain using the DNeasy tissue kit (Qiagen). The isolated DNA was used directly for PCR amplification. The *gfp* gene of 720 bp was amplified in a PCR reaction using the primers $P_{gfp(up)}$ (5' - ATATAGCATGCGTAAAGGAGAAGAACTTTTCA- 3') and $P_{gfp(down)}$ (5' - CTCTCAAGCTTATTTGTATAGTTCATCCATGC- 3') (Anderson et al., 1998). T he 50µL PCR reaction mixture contained 50pMol/µL of each primer, 2.5mM deoxynucleotide phosphate, 5µl of 10× buffer, 0.1µL of taq DNA polymerase (1U) and 4µL of genomic DNA. The reaction mixture was subjected to thirty amplification cycles of 92°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute. An initial start of 92°C for 5 minutes and a final elongation step at

72°C for 5 minutes was also included. The reaction product was subjected to agarose gel electrophoresis by standard methods (Sambrook et al., 1989).

A.4.3.4 Survival Studies

A.4.3.4.1 Stability of Labelling

Tagged strains and wild-type strains were inoculated separately into 10mL of LB broth (nutrient rich) and incubated with agitation at 37°C. After 24 hours, 10µl of LB broth culture was transferred to another 10mL of LB and incubated at 37°C with agitation at 180 rpm. This process was repeated for thirty days. The presence and stability of the label was verified by fluorescent microscopy initially, and after constant growth for thirty-days in both rich (LB broth) and nutrient poor medium (1/10 strength R_2A), facilitated by daily transfer into fresh broth.

A.4.3.4.2 Survival Fitness

The wild-type and tagged strains were inoculated separately into 30mL of LB broth and incubated overnight at 37°C with agitation at 180 rpm. One mL of the overnight culture was inoculated into 250 mL side-arm flasks containing 100mL LB broth. The absorbency readings of the culture were measured with a spectrophotometer set at 600nm from time 0 hours. Measurements were taken every 30 minutes. After each reading, the side-arm flasks were incubated at 37°C with agitation. This was repeated for *S.* ser Typhimurium strains (in duplicate) in side-arm flasks containing 1/10 strength R_2A medium.

A.4.3.5 Biofilm Studies

A flow cell was used to simulate a water distribution system. A glass cover-slip was fixed over the flow cell channels with silicone sealer. A pump was connected to the flow cell to allow the media to move through the flow cell channels. Initially Jik (3.5% w/v sodium hypochlorite) was pumped through the flow cell, followed by extensive rinsing with sterile culture medium. The medium chosen was sterile tap water supplemented with 1 $mg.L^{-1}$ acetate. The medium and flow cell were incubated at 37°C. A sterile needle (0.45 x 13 mm) was used to inoculate the flow cell slowly with 1mL of culture. Flow was left off for 1 hour after inoculating to allow some bacteria to attach to the inner surface of the glass slide, after which the pump was set at a flow rate of 0.4 $mm.s^{-1}$ through the flow cell. The biofilms that developed on the slides were viewed by phase contrast and epifluorescence microscopy using an inverted Zeiss fluorescence microscope.

A.4.3.5.1 Ability of *Salmonella* to form biofilms independently

In order to characterize biofilm development of *Salmonella* in mono-culture, one mL volumes of an overnight culture of tagged *Salmonella* and the wild-type strains grown in a 1/10 strength R_2A medium, and pre-diluted to 10^6 mL^{-1}, were inoculated into separate channels of the flow cell and fed with acetate-supplemented sterile water.

A.4.3.5.2 Ability of *Salmonella* to grow and survive in an established biofilm

In order to observe the entry, fate and growth in biofilm of *Salmonella* in mixed culture conditions, drinking water biofilms were cultured by two separate strategies as described below.

Approach 1: In order to obtain a mixed culture inoculum to form a biofilm, 5 L of tap water were filtered through 0.22 µm filters. These filters were sonicated in 1 mL sterile water in a

sonication bath for 5 minutes to remove adherent cells. One mL of this mixed suspension culture was inoculated into the flow cell and biofilm allowed to develop as described above.

Approach 2: Serial dilutions were made from tap water, streaked onto R_2A agar and incubated at 28°C for 48 hours. Colonies with different morphologies were selected at random and screened for auto-fluorescence by fluorescent microscopy. Isolates that did not display auto-fluorescence were inoculated separately into 10mL of R_2A media and incubated at 37°C overnight. These cultures were combined and 1mL of the combined water isolates was inoculated into the flow cell.

One mL volumes of an overnight culture of tagged *Salmonella* and the wild-type strains grown in 1/10 strength R_2A medium, and pre-diluted to 10^6 mL^{-1}, were inoculated into separate channels of the flow cell and fed with acetate-supplemented sterile water. A further channel of established biofilm remained unspiked to serve as a control.

A.4.3.5.3 Recovery of the tagged *Salmonella* from an established biofilm

In order to verify that *Salmonella* were growing in the biofilms, a section of the cover-slip was removed at the end of operation. The biofilm on the coverslip was suspended in ¼ strength Ringer's solution and vortexed. Serial dilutions were made and plated out on LB agar supplemented with Kanamycin (100 $\mu g.mL^{-1}$), and incubated at 37°C for 24 hours. The colonies were subsequently transferred to XLD. A section of the cover-slip was also suspended in BPW and vortexed. Portions of the same dilutions growing in BPW were transferred to 9mL of RV broth and incubated at 42°C for 24 hours, and subsequently plated out on XLD media. Colonies were selected and screened for fluorescence.

In order to determine whether *Salmonella* were released from the biofilm, effluent from the flow cells was collected at 72 hours of operation, and serial dilutions made in BPW and incubated at 37°C. After 24 hours, 1mL volumes of the BPW dilutions were transferred to 9 mL of RV broth and incubated at 42°C for 24 hours and subsequently plated out on XLD media. Colonies were selected and screened for fluorescence.

A.4.4 Results and Discussion

A.4.4.1 Isolation and Identification of Salmonella *Strains*

Several black colonies could be isolated from the sediment samples. The pre-enrichment in the buffered peptone water allows resuscitation of the injured or stressed bacteria (Edel et al., 1973 and Marinigo et al., 1986). Selective RV enrichment medium and XLD selective media are standard culture methods for the isolation of *Salmonella*. The high prevalence of black colonies isolated from the sediment samples suggested that *Salmonella* is widely abundant in fresh water sediments where it serves as a contamination reservoir. Colonies on the XLD that appeared black due to H_2S production were selected for further characterisation. The API 20E identification system identified all the isolates as *Salmonella* species. Metabolic and biochemical tests are the basis of presumptive identification of microorganisms. Due to the genetic variability arising from mutations and the exchange of plasmids encoding biochemical traits, the identity of *Salmonella* is widely determined by serotyping. Serotyping involves the agglutination of bacterial surface antigens with *Salmonella*-specific antibodies (Doyle et al., 1997). The isolates were typed as follows:

Isolate 1: *S. enterica* subsp. *enterica* ser. Typhimurium
Isolate 2: *S. enterica* subsp. *enterica* ser. Muenchen
Isolate 3: *S. enterica* subsp. *salamae*

Isolates 1 and 2 both belong to the enterica subspecies of *Salmonella* (group I). This is the only subspecies of *Salmonella* that is responsible for infections in warm-blooded animals and humans (Popoff et al., 1997). Isolate 3 belongs to group II of the *Salmonella* species. Serotypes belonging to this group are usually isolated from cold-blooded animals and the environment, but rarely from humans. (Farmer et al., 1984). *Salmonella* ser. Muenchen and *Salmonella* ser. Typhimurium were therefore selected for *gfp* tagging, as they are clinically relevant environmental *Salmonella* isolates. *S.* Muenchen is one of approximately 2400 *Salmonella* serotypes that cause illness in humans. It is, however, an infrequently isolated serotype, which accounts for 1.6% of human *Salmonella* reported in 1997 to the U.S. Public Health Laboratory Information System (Martin et al., 1995 and CDC, 1998). *S.* Typhimurium was the most common isolate in the United States in 1999, causing 25% of culture confirmed infections according to the CDC *Salmonella* Surveillance 1999 Annual Summary (CDC, 2003).

A.4.4.2 Chromosomal Tagging

The pUT mini-Tn5 Km transposon delivery plasmid results in integration of the marker gene onto the chromosome at a random position. Chromosomal tagging eliminates problems associated with plasmid instability. The transposase gene is adjacent to, but outside of the mobile DNA segment. Due to the loss of the transposase gene after insertion, the mini-transposon is reportedly stably inherited (Herrero et al., 1990; De Lorenzo et al., 1990). The mini Tn5 transposon's mobility is determined by two insertion sequences flanking the DNA region encoding the kanamycin resistance gene. The pUT mini–Tn5 Km transposon (De Lorenzo et al., 1990) was used to introduce GFP into the chromosome of the *Salmonella* strains. The pUT mini–Tn5 Km element has an sfil cassette containing the kanamycin resistance gene and a single NotI site outside the cassette. (De Lorenzo et al., 1998). The fragment of the pSM1690 plasmid carrying rrnBP1-RBSII-gfpmut3b*-T_0T_2 is flanked by NotI sites and is inserted into the NotI site of the pUT-mini Tn5 Km (Sternberg et al., 1999). The delivery plasmid contains gfpmut3b*, a very bright and stable variant of the green fluorescent protein. A growth-regulated 72bp promoter (rrnBP1) from *E.coli* has been inserted in front of the *gfp* gene to drive the expression of the marker gene. The delivery plasmid has a synthetic ribosome binding site, stop codons in all three reading frames and two strong transcriptional terminators (Sternberg et al., 1999).

Tri-parental mating involves donor, helper and recipient strains. The donor strain contains an R6K-based suicide delivery plasmid (Kolter *et al.*, 1978). Plasmids having the R6K origin of replication require R6K-specified replication protein π. The plasmid is only maintained in pir protein producing bacteria. The plasmid has an origin of transfer (RP4 OriT) function. Therefore, the donor plasmid is mobilized through the aid of the helper strain into target cells through RP4 conjugal transfer functions (De Lorenzo *et al.*, 1998).

Prior to tagging, *S.* ser. Muenchen and *S.* ser. Typhimurium were sensitive to both ampicillin and kanamycin. The media used to select transconjugants (XLD Km100) counter-selects the helper and donor strains, but selects recipient strains carrying the transposon marker. Once the transposon has integrated onto the chromosome, the recipient should only have resistance to kanamycin. If the suicide delivery plasmid replicates in the recipient and is not lost, the recipient will display resistance to kanamycin and ampicillin.

Following tri-parental mating, three *S.* ser. Muenchen strains were obtained and two *S.* ser. Typhimurium strains were obtained. All strains were sensitive to ampicillin, but resistant to kanamycin. All strains fluoresced green when exposed to blue light (Fig. A.21).

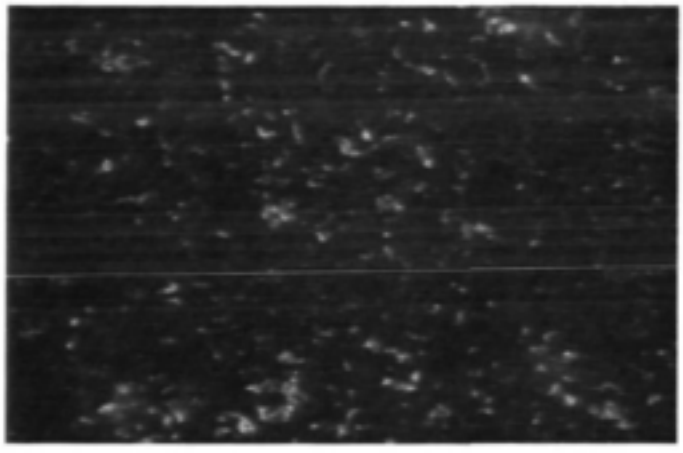


Figure A.21: Green fluorescence emitted by the recipient cells visualised by fluorescent microscopy.

A.4.4.3 Verification of chromosomal tagging

In order to verify whether transposition had taken place, a PCR detecting the *gfp* gene was performed on chromosomal DNA of the tagged strains. Genomic DNA was successfully extracted (Fig. A.22) and positive PCR reactions indicated that the *gfp* gene was present on the genome (Fig. A.23).

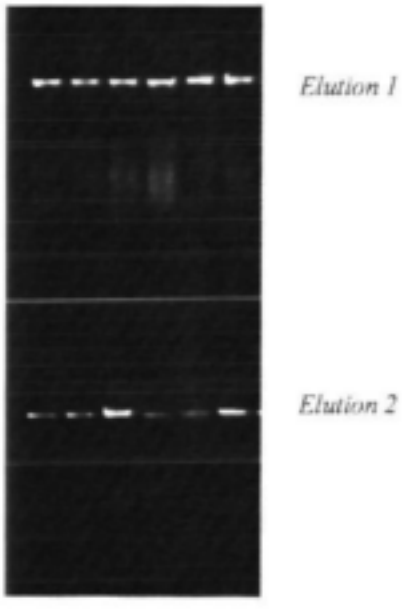


Figure A.22: Genomic extraction of *gfp*-tagged recombinant *Salmonella*.

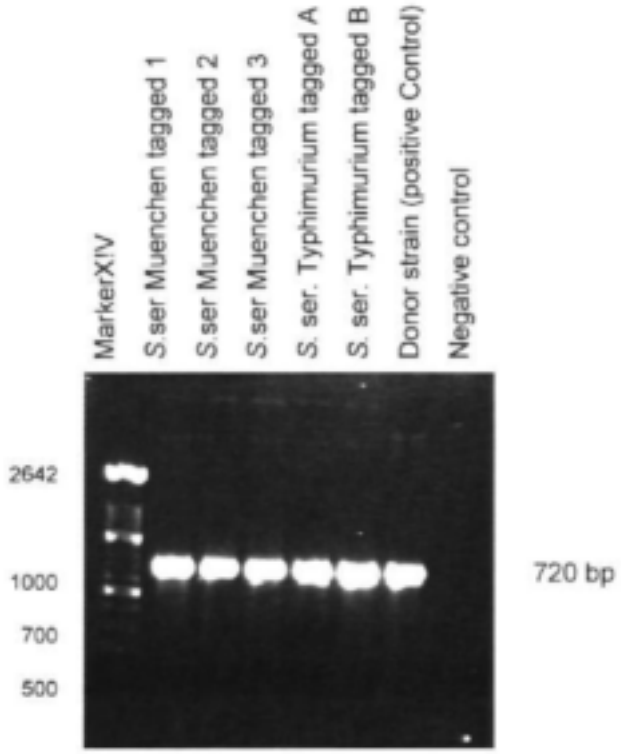


Figure A.23: Amplification of the *gfp* gene of the various recombinant *Salmonella*.

A.4.4.4 Stability of maintenance of the gfp *gene*

The stability of the *gfp* gene was tested by exposing the isolates to a thirty-day growth period under non-selective conditions. The presence of the label was verified by fluorescence microscopy. After 30 days, the GFP was visible in all tagged cells, indicating stable maintenance (Fig. A.24). The GFP fluorescence was also visible in cells growing under nutrient poor conditions (Fig. A.25), indicating that even when cells are subjected to stress, the *gfp* gene was still maintained. Nutrient poor conditions reflect the stress induced on microorganisms in aquatic environments. This indicates that the inserted fragment remains stably inherited and does not serve as a burden to the carrier strain.

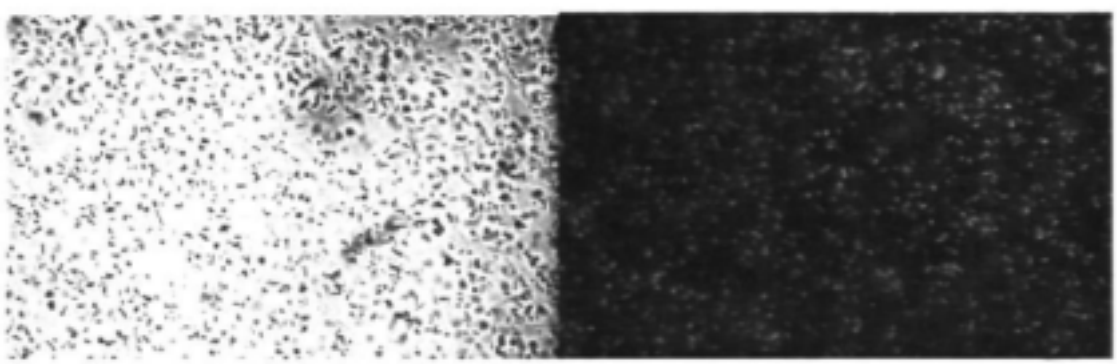


Figure A.24: Tagged isolate cultured in LB broth and viewed by phase contrast microscopy (left panel) and fluorescence microscopy (right panel).

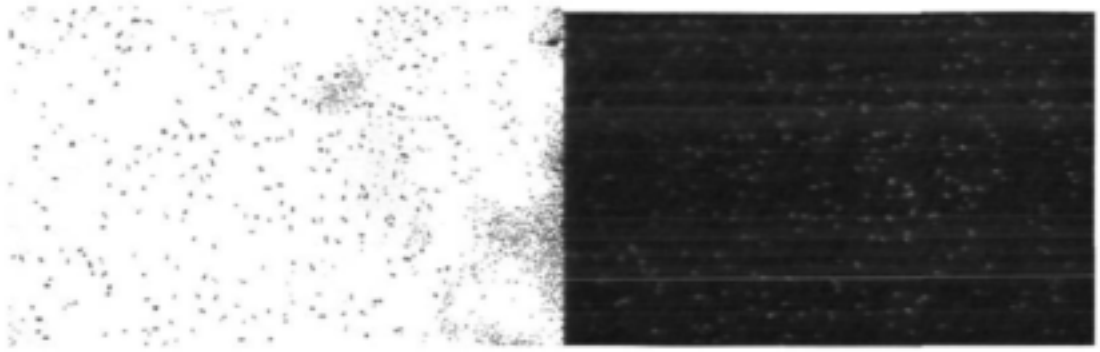


Figure A.25: Tagged isolate cultured in 1/10 strength R_2A broth and viewed by phase contrast microscopy (left panel) and fluorescence microscopy (right panel).

A.4.4.5 Fitness under nutrient-rich and nutrient-poor conditions

A general problem of the mini-Tn5 transposon is that its insertion involves the random disruption of a chromosomal sequence. Environmentally important functions can thus be affected (De Lorenzo et al., 1998). Therefore, all *gfp* strains generated from the transposon insertion were screened against growth defective lesions. The labelled strains did not display growth rate deficiencies when compared to the wildtype strains, both under nutrient rich (LB broth) and nutrient poor (1/10 strength R_2A broth) (Figs A.26 and A.27). GFP continued to fluoresce in cells cultured in nutrient poor medium (Fig. A.28). This shows that the incorporation of the *gfp* gene did not have any detrimental effects on the survival or behaviour of the isolates under the conditions used for biofilm studies.

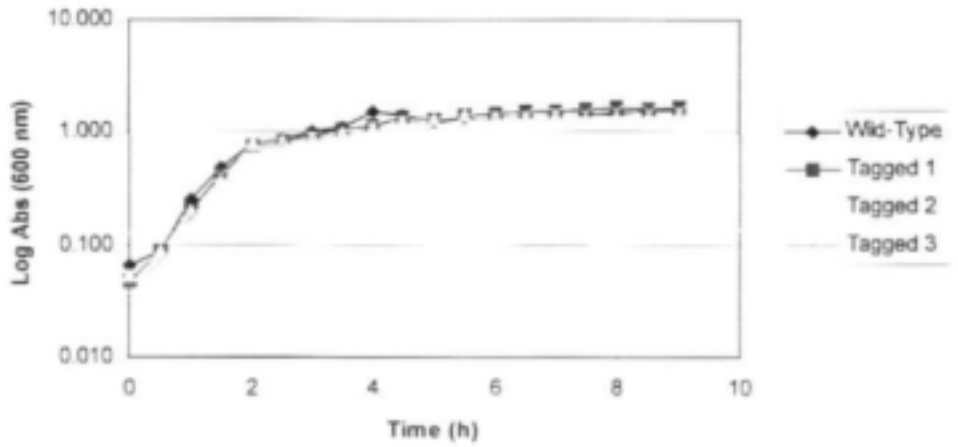


Figure A.26: Growth of *S.* ser Muenchen wild-type strains vs the tagged strains in LB broth.

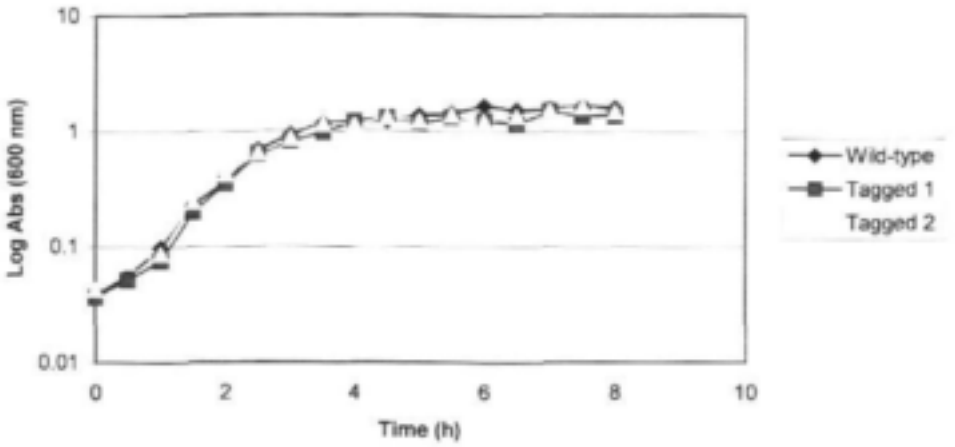


Figure A.27: Growth of *S.* ser Typhimurium wild-type strains vs the tagged strains in LB broth.

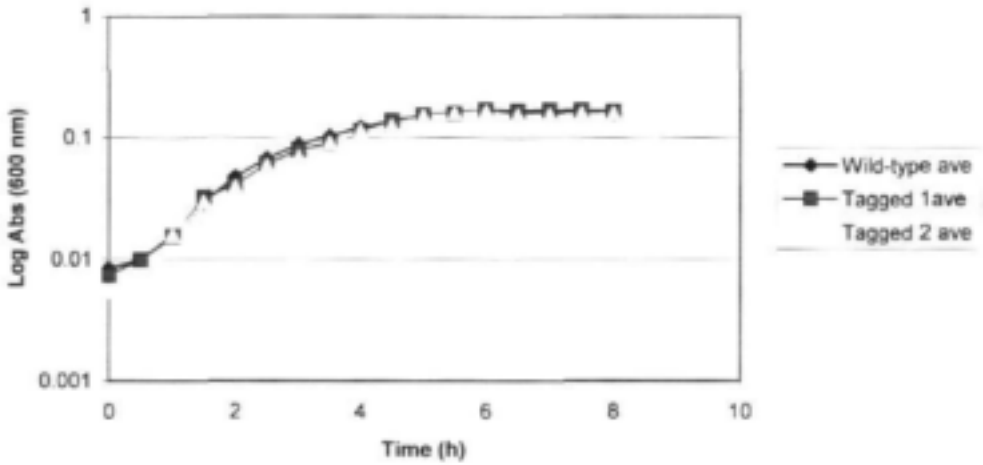


Figure A.28: Growth of *S.* ser Typhimurium wild-type strains vs the tagged strains in 1/10 strength R_2A broth.

The survival studies indicated that the incorporation of the *gfp* gene did not have any detrimental effects on the survival and fitness of the various tagged strains. These tagged strains could therefore be used *in vivo* to study the fate and survival of *Salmonella* in water environments. All further studies were performed using one of the tagged *S.* ser Typhimurium strains, as this isolate is clinically more relevant.

A.4.4.6 Biofilm studies

A.4.4.6.1 Ability of *Salmonella* to form biofilms independently

Salmonella do not appear to be suitable primary biofilm formers. Within 24 hours a thin monolayer of cells was formed (Figs A.29 and A.30). After 48 hours small micro-colonies began to develop (Figs A.31and A.32). However, the colonies did not intermix and cells did not appear to migrate from one micro-colony to another. After 72 hours, the colonies were not dispersed across the surface, but aggregated in clumps (Figs A.33 and A.34). Thick layers of intermixed micro-colonies containing water channels between them are characteristic of general biofilm architecture, but *Salmonella* failed to form the elaborate architecture of mature biofilms.

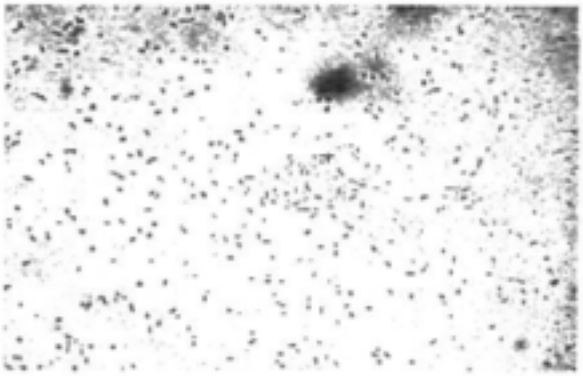


Figure A.29: Wild-type strain after 24 hours viewed by phase contrast microscopy (100x).

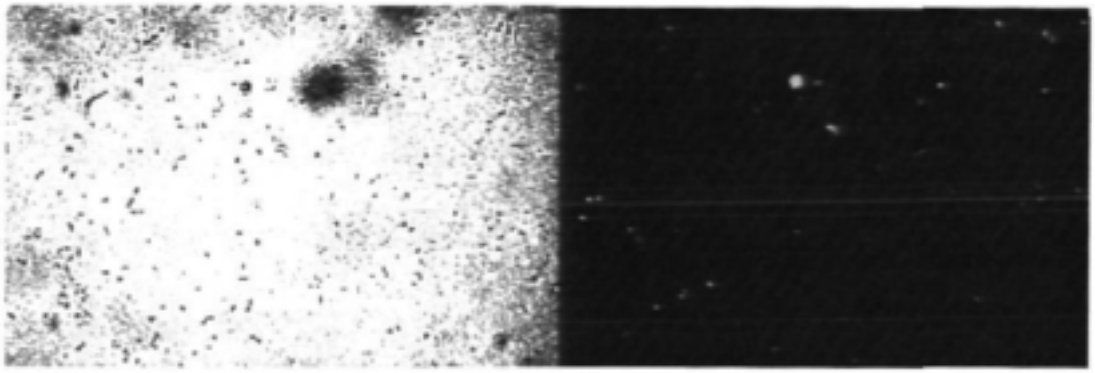


Figure A.30: Tagged strain after 24 hours viewed by phase contrast (left panel) and fluorescent microscopy (right panel).

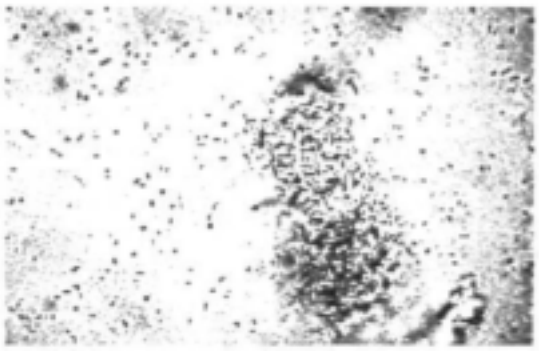


Figure A.31: Wild-type strain after 48 hours viewed by phase contrast microscopy.

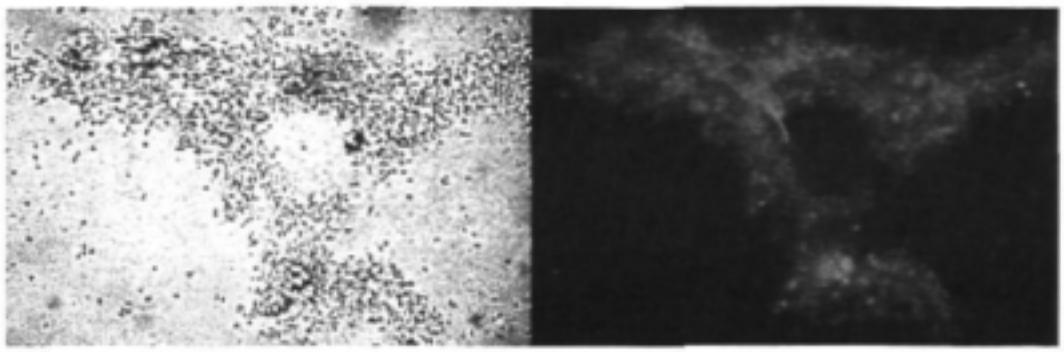


Figure A.32: Tagged strain after 48 hours viewed by phase contrast (left panel) and fluorescent microscopy (right panel).

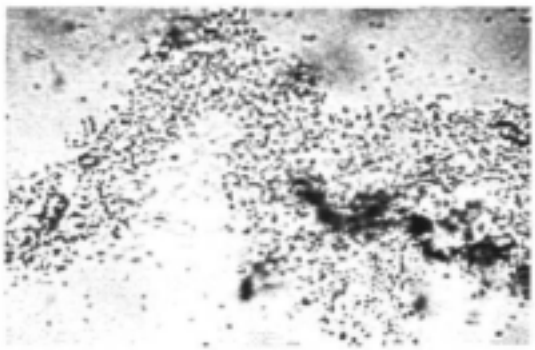


Figure A.33: Wild-type strain after 72 hours viewed by phase contrast microscopy.

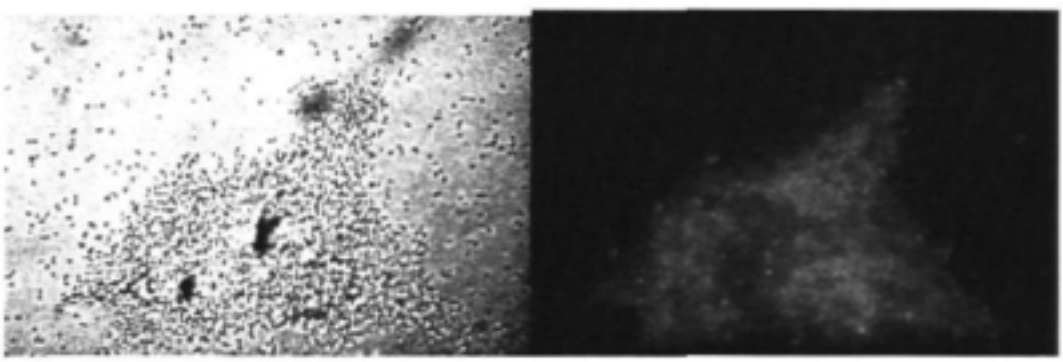


Figure A.34: Tagged strain after 72 hours viewed by phase contrast (left panel) and fluorescent microscopy (right panel).

A.4.4.6.2 Ability of *Salmonella* to grow and survive in an established biofilm

After 72 hours using a concentrated drinking water innoculum (approach 1), a thick biofilm was formed. However, when observed under the fluorescent microscope, bright green auto-fluorescence was visible (Fig. A.35). This biofilm could not be used for inoculation with the tagged strain of *Salmonella* as the auto-fluorescence of the undefined organisms was too bright to be able to distinguish GFP-labelled cells. This approach was therefore discontinued. After 72 hours, using approach 2, a thick mixed culture biofilm which did not fluoresce was formed. The tagged strain was inoculated into this biofilm and its fate and survival was investigated.

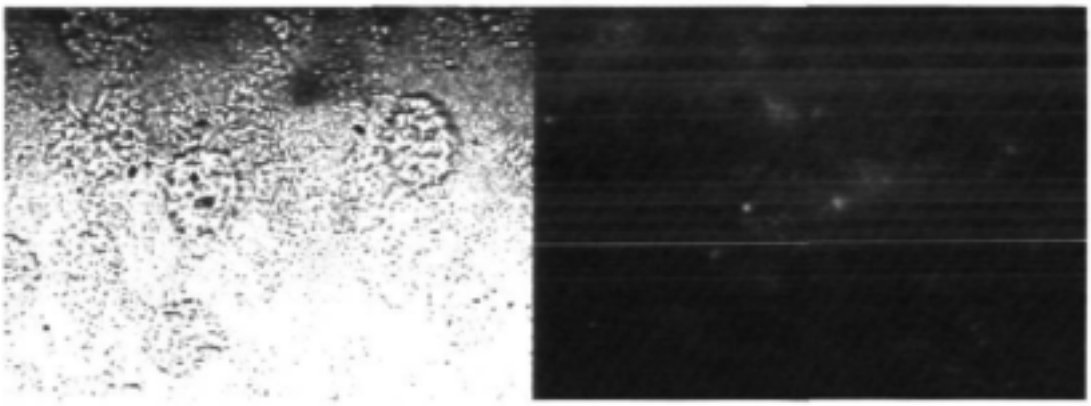


Figure A.35: Undefined mixed culture biofilm after 72 hours viewed by phase contrast (left panel) and fluorescence microscopy (right panel).

In nature most bacteria do not exist as pure cultures, and significant proportions of all microorganisms are associated with surfaces in complex multi-species communities called biofilms (Costerton et al., 1987). Although *Salmonella* do not appear to be suitable primary biofilm formers, they can attach and survive in a mixed culture biofilm. After 24 hours individual *Salmonella* cells could be visualised among the mixed culture biofilm (Fig. A.36). *Salmonella* persisted in the established biofilm, forming small micro-colonies after 48 and 72 hours (Figs.A.38 and A.39).

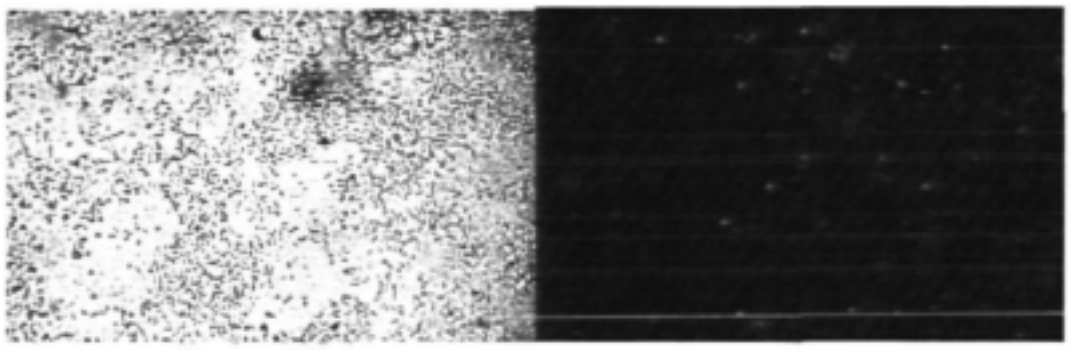


Figure A.36: Undefined mixed culture biofilm spiked with *Salmonella* after 24 hours & viewed by phase contrast (left panel) and fluorescence microscopy (right panel).

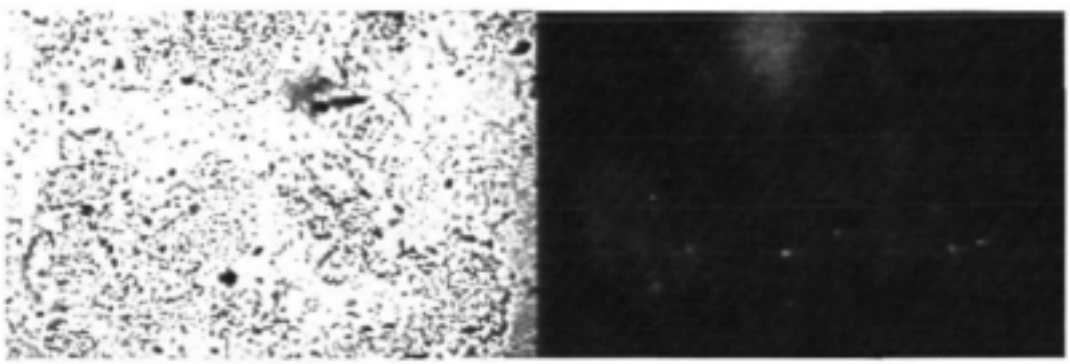


Figure A.37: Spiked mixed culture biofilm after 48 hours viewed by phase contrast (left panel) and fluorescence microscopy (right panel).

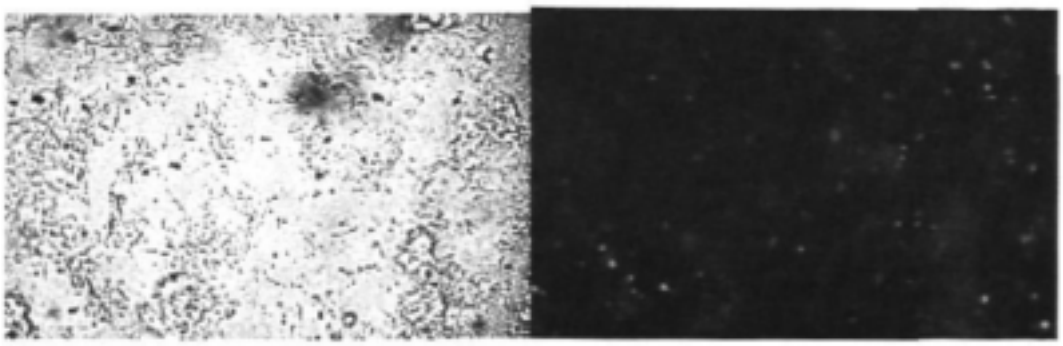


Figure A.38: Spiked mixed culture biofilm after 72 hours viewed by phase contrast (left panel) and fluorescence microscopy (right panel).

Bubbles appear from time to time in the medium feed due to permeability of the silicon tubing to atmospheric gasses. This may lead to a bubble moving through the flow cell, exerting a shear forces on the attaching cells, resulting in detachment of these cells. It was shown that once a layer of established biofilm had been removed by a bubble, *Salmonella* had the ability to recolonize in a cleared zone. This indicated that *Salmonella* growing as part of a drinking water biofilm may be disseminated and re-colonize another biofilm. The enhanced biofilm development by *Salmonella* in association with an established drinking water biofilm points to association and metabolic interactions with the indigenous organisms. The presence of fimbriae, flagella and surface associated polysaccharides or proteins may provide a competitive advantage for one organism where a mixed community is involved (Donlan, 2002). Camper et al., (1998) showed that *S. typhimurium* persisted in a model distribution system containing undefined heterotrophic bacteria from an unfiltered osmosis system for more than 50 days, which suggests that the normal biofilm flora of this water system provided niche conditions capable of supporting the growth of this organism. After 144 hours the established biofilm had formed very thick intermixed micro-colonies and the *Salmonella* could still be detected as multicellular conglomerates (Fig. A.39). This indicates that *Salmonella* can grow and survive in a mixed culture biofilm.

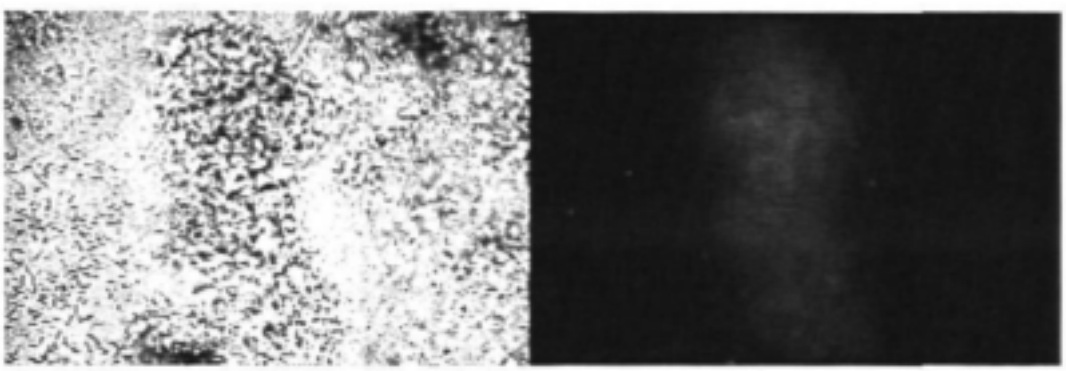


Figure A.39: Spiked mixed culture biofilm after 144 hours viewed by phase contrast (left panel) and fluorescence microscopy (right panel).

A.4.4.7 Recovery of the tagged Salmonella *from established biofilm*

Dilutions of the 144 hours old biofilm made directly in BPW all subsequently yielded black colonies on XLD, confirming the presence of *Salmonella* in the biofilm. Plating of suspensions onto Kanamycin-containing agar yielded 44 colonies on the highest dilution showing growth. All 44 colonies grew on XLD agar, producing black growth and showing that all kanamycin resistant colonies were *Salmonella*. As the *gfp* gene is directly next to the kanamycin resistance gene, the synonymity of kanamycin resistance with *Salmonella*

showed that the *gfp* gene had not been transferred to other bacterial species in the biofilm, and that all fluorescing cells were therefore *Salmonella*.

A.4.4.8 Release of Salmonella *from the drinking water biofilm*

Mature biofilms are known to shed cells, releasing them into the flow. In order to verify the notion that *Salmonella* would be released from a drinking water biofilm, isolations were performed on effluent from the flow cells. As the influent medium was sterile, presence of *Salmonella* in the effluent proves release from the biofilm. All colonies from both kanamycin plates and RV broth appeared black on XLD, showing that *Salmonella* was being released from the drinking water biofilm. Twenty-five colonies were selected and screened for fluorescence. All 25 fluoresced green, showing that the *gfp* gene had been maintained in all *Salmonella* during the 144 hours period in a biofilm environment.

A.4.5 Conclusions

- Two *Salmonella* strains, isolated from fresh water sediment (viz. *S. enterica* subsp. *enterica* ser. Typhimurium and *S. enterica* subsp. *enterica* ser. Muenchen) were chromosomally tagged with the *gfp* gene encoding the green fluorescent protein.
- The *gfp* gene was stably maintained during continuous growth for 30.
- The *gfp*-labelled recombinant strains were not growth-rate impaired, neither in nutrient rich (LB broth) nor in nutrient poor (1/10 strength R_2A broth). This was in contrast to the effect of the *gfp*-carrying transposon delivery system on *Aeromonas hydrophila*.
- *S. enterica* subsp. *enterica* ser. Typhimurium formed biofilms only poorly in drinking water supplemented with 1000 $\mu g.mL^{-1}$ acetate. Biofilms were patchy and detached readily under conditions of high flow.
- By contrast, *S. enterica* subsp. *enterica* ser. Typhimurium colonized established multi-species drinking water biofilms within 24 hours, growing to form micro-colonies within the biofilm.
- *S. enterica* subsp. *enterica* ser. Typhimurium was also released from drinking water associated-biofilm into the flow, and was seen to re-colonize elsewhere.
- This showed that *Salmonella* can enter into, survive and grow within, and be released from a drinking water-associated biofilm.
- The results indicate that once *Salmonella* has entered into a drinking water distribution system, it will be able to colonize an existing biofilm, grow in it and be released into the flow for re-colonization elsewhere, and possible subsequent infection of consumers.

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PART B

Prevalence, survival and growth of bacterial pathogens in biofilms in drinking water distribution systems

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SECTION B:

SUMMARY

Background and motivation

Safe drinking water is one of mankind's most important basic needs. Water is a source of life but can also be a source of destruction if it contains harmful pathogens. With so many people of all ages suffering from immune- deficiency diseases, pressure is on water distributors to supply water that is of the highest quality. Their task is being made increasingly difficult by water distribution systems in which organisms re-grow to form biofilms on pipes and in tanks.

Treatment deficiencies may allow the escape of either unharmed or reversibly inactivated (injured) organisms, especially when the source water contains elevated densities of coliform and pathogenic bacteria. Microorganisms can gain access to water from air-water interface in the distribution system such as in a storage tank or may remain unaffected by the chlorine treatment. Biofilms develop on the inner surface of distribution pipes and walls of reservoirs and storage tanks. Numerous different bacterial populations form part of these biofilms. Pathogens and opportunistic pathogens attach themselves to these biofilms. Biofilms can slough away into the water stream where the bacteria can either be ingested or inhaled (i.e., aerosols from showers and humidifiers). Often no disease symptoms will result. On occasion, however, health effects range from gastrointestinal illness (from ingestion) to a pneumonia-like illness (from inhalation of aerosols).

Traditionally water hygiene risk has been evaluated by the enumeration of indicator groups such as heterotrophic bacteria, total coliforms, faecal coliform and *E. coli* with comparison to acceptable standards and guidelines. A major failing of this approach is that these indicators do not directly correlate with the presence of pathogens, particularly after water/ wastewater treatment, and are therefore poorly correlated with disease outcome. A need exists for other parameters to be used to correlate with incidents of outbreaks. The heterotrophic plate count (HPC) is a useful tool for monitoring the efficiency of water treatment processes and for assessing changes in finished water quality during distribution and storage, and distribution system cleanliness.

Aim

The aim of the project was to assess

- the survival of general water quality indicator bacteria in biofilms within a water distribution system and,
- the survival of certain bacterial pathogens in biofilms within a water distribution system.

Methodology

The biofilm sampling device (Greenwood device) used for testing biofilms within a distribution system was based on the original Pedersen device described by Pedersen (1981). Water flowed from an inlet on the right hand side, through diffusers, passing over 72 slides before passing out of the device. Three Greenwood devices were set up, one device at an office building after the storage tank, and two at a residential building. The devices were sampled every fortnight for a period of one and a half years from 17 September 2001 to 26 May 2003. Temperature and residual chlorine were measured on site and turbidity was measured in the laboratory. The

distribution water and biofilms were sampled and tested for heterotrophic bacteria and specific pathogens, namely *Klebsiella* spp.; *Salmonella* spp.; *Staphylococcus* spp. and *Pseudomonas* spp.

Results

The results of all three units showed a variable degree of biofilm development based on the heterotrophic bacterial density. The bacterial density of the water in the storage tank at the office building was on average ten times to one hundred times higher than the heterotrophic count of the two units at the residential building. The average residual chlorine measured at the storage tank was lower at 0.2mg/ℓ, than the target residual chlorine level for the reservoir serving the area of 0.6 – 1.2mg/ℓ (Mackintosh, et al, 2001-2003). This measurement is also much lower than the average residual chlorine measured at the residential building of 0.85 and 0.91mg/ℓ. Although the water supplied to the residential building was within the target residual chlorine level of the local municipal authority, pathogens were still found on a number of occasions. However, a higher incidence of pathogens identified was in the biofilms at the office building than at the residential building.

Conclusions

Biofilms are a real threat to water quality and can grow even in well-maintained distribution systems. To prevent or minimize biofilm development residual chlorine levels should be high enough to ensure that water is sufficiently disinfected from the start to the finish of the distribution line. Water stored in tanks before distribution should be monitored regularly and tanks cleaned and disinfected regularly to prevent the development of a biofilm on the tank walls.

Even though most heterotrophic organisms are harmless, heterotrophic organisms in distribution system water is an indication of how well or good a distribution system is managed. Bacteria escaping disinfection colonize on the surface of distribution pipes to form biofilms which in turn protect opportunistic pathogens from the effects of disinfection. **The objectives of the project were achieved** in that the absence of bacterial indicators in a system does not completely guarantee the absence of opportunistic pathogens. The presence of opportunistic pathogens found in the biofilms during the study provides evidence that even what is considered a well-maintained distribution system, still harbours potential harmful bacteria. It is important to understand the risk involved in supplying "contaminated" or inadequately treated water.

General indicator organisms alone are not sufficient to ensure pathogen-free water and should be used in conjunction with other tests or indicators, testing larger quantities of water.

Recommendations for future research

Future research should investigate the effects that bacteria found in biofilms have on the quality of the water reaching the end-user by analyzing volumes of water larger than the specified one hundred milliliters. It may be important to investigate the infective dose of potential pathogens found in the biofilms that would cause illness in immune-deficient individuals. Interspecies communication should be investigated to give a better understanding of how biofilm formation can be prevented. It is also important to focus on the formation of biofilm on metal surfaces and the health risks involved.

Technology transfer

With the development of the Greenwood device technology transfer will be facilitated in that many other institutions can use the design to study biofilms, not just in a laboratory setting, but in an actual distribution system.

B.1 INTRODUCTION

South Africa has more HIV-positive individuals than any other country in the world (Center for Disease Control and Prevention, 2003). Together with other immune compromised individuals, the risks of infection for these people are very high. Drinking water is one route by which infections can be transmitted. Treated water may still harbour potential pathogens, even though these waters comply with stringent drinking water standards. Bacteriological indicator tests provide only limited information with regard to the hygiene quality of the water. Traditionally water hygiene risk has been evaluated by the enumeration of indicator groups such as heterotrophic bacteria, total coliforms, faecal coliform and *E. coli* with comparison to acceptable standards and guidelines. A major failing of this approach is that these indicators do not directly correlate with the presence of pathogens, particularly after water/wastewater treatment, and are therefore poorly correlated with disease outcome (Barwick et al, 2000). A need exists for other parameters to be used to correlate with incidents of outbreaks.

With the above in mind it was decided to study the occurrence, survival and growth of pathogens and opportunistic pathogens such as *Pseudomonas, Staphylococci, Salmonella* and *Klebsiella* in biofilms in drinking water distribution systems instead of focusing only on indicator organisms. Three biofilm devices, designed to study the formation of biofilms, were installed at two different locations. At the one location, an office building, water is supplied to the building via a storage tank. The other location, a residential property, receives water directly from the distribution pipeline. The biofilm development on the glass slides inside the devices was removed and the biofilms analysed every two weeks for a period of a year and a half. The water quality of the incoming water was also determined.

Motivation

With the number of immune-compromised individuals increasing daily in South Africa (Center for Disease Control and Prevention, 2003), and the water conditions here being different to many western countries, water quality is fundamentally important in South Africa. Since biofilms may harbour a number of possible pathogenic organisms research concerning biofilms is essential.

Aim

The aim of the project was to assess:

- the survival of general water quality indicator bacteria in biofilms within a water distribution system;
- the survival of certain bacterial pathogens within a water distribution system.

B.1.1 Background Information

What are biofilms?

Biofilms are a commonly occurring phenomenon found in nature. A biofilm is a layer of microorganisms in an aquatic environment held together in a polymeric matrix (slime layer) attached to a substratum. The microorganisms in the biofilm produce and excrete the matrix which consists of organic polymers referred to as extra-cellular polymeric substances (EPS) (van der Wende and Characklis, 1990). Biofilms can form on virtually any surface material and in the presence of a limited amount of nutrients. It may be a complete film, or more commonly in water systems, small patches on pipe surfaces. Microorganisms in biofilms can include bacteria including coccids, round, rod-shaped, filamentous bacteria, fungi, and higher organisms like nematodes, larvae etc. A primary reason why many water distributors become

concerned with biofilms in drinking water systems is due to growth of coliform bacteria in the pipe works (Le Chevallier, 1990).

Steps in biofilm development:

Edstrom Industries, Inc. (2003) identified the following steps in the formation of a biofilm.

1) **Surface conditioning:**

When a clean pipe comes into contact with water or any aquatic medium, organic molecules adhere to the surface. Organics neutralize the surface charge which may repel approaching bacteria. (Edstrom Industries, Inc., 2003). Bacteria grow particularly rapidly in flowing aqueous systems where a regular nutrient supply is provided to the organisms (Prescott & Harley et al, 1998).

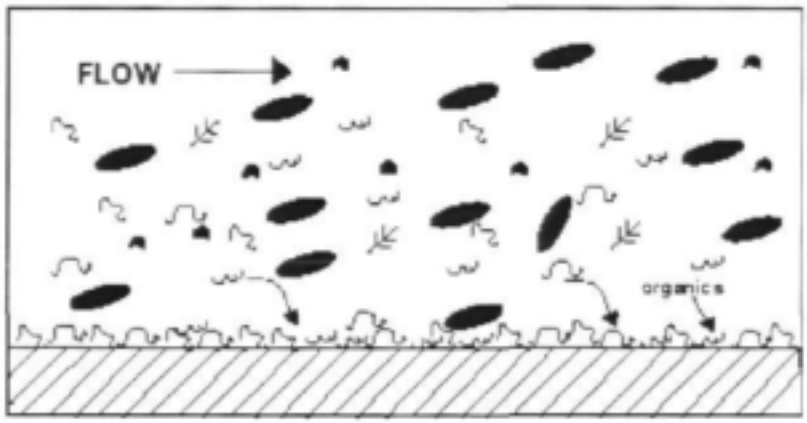

Figure B.1: **Adsorption of organic molecules on a clean surface forms a conditioning film (Characklis, 1990).**

2) **Adhesion of "pioneer" bacteria:**

Planktonic (free-floating) bacteria first attach themselves by electrostatic attraction and physical forces.

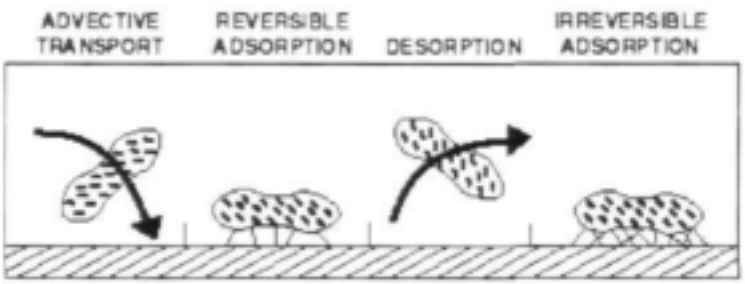

Figure B.2: **Transport of bacterial cells to the conditioned surface, adsorption, desorption, and irreversible adsorption (Characklis, 1990).**

3) **"Slime" formation:**

Nutrient materials accumulate and the cells reproduce. Extra cellular polymeric substances (EPS) consists of charged and neutral polysaccharides groups that cements the cell to the pipe wall and acts as an ion exchange system for trapping and concentrating trace nutrients from the water.

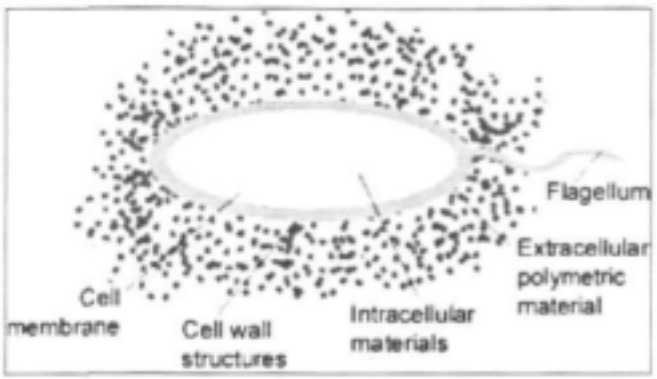

Figure B.3: Wild bacteria are "hairy" cells with extracellular polymers which stick to surfaces (Mittelman, 1985 as cited by Edstrom Industries, Inc. 2003).

As trace nutrients accumulate, the pioneer cells reproduce. The daughter cells then produce their own EPS, greatly increasing the volume of ion exchange surface. Soon a thriving colony of bacteria is established.

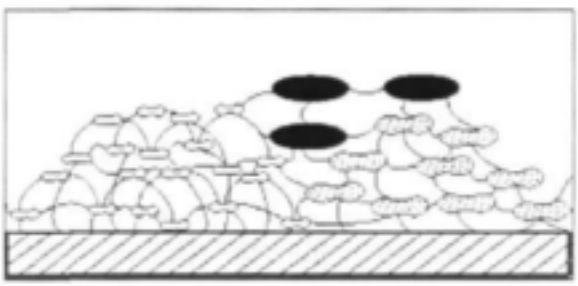

Figure B.4: Biofilm is made up of microbes and a "spider's web" of extracellular polymers (Source:Edstrom industries inc., 2003).

4) Secondary colonizers:

EPS web also snares other types of microbial cells through physical restraint and electrostatic interaction. Secondary colonizers metabolize wastes from primary colonizers and also produce their own waste which other cells can use.

5) Fully functioning biofilm:

The mature, fully functional biofilm is a complex, metabolically cooperative community made up of different species. An anaerobic layer may develop underneath the aerobic biofilm. As the film grows to a thickness that allows it to extend to a zone of more turbulent water flow, some cells are sloughed off. The released cells can then colonize downstream piping (Edstrom Industries, Inc., 2003).

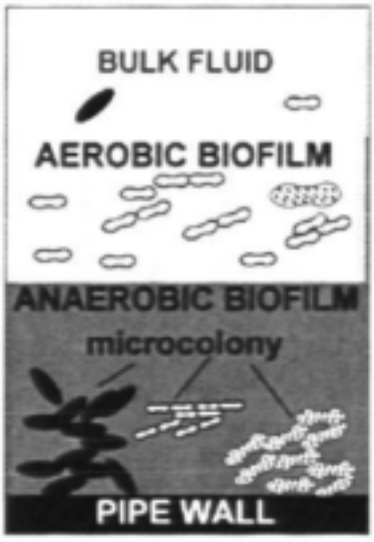

Figure B.5: Bacteria and other microorganisms develop cooperative colonies or "consortia" within the biofilm. An anaerobic biofilm may develop underneath the aerobic layer. The biofilm thickness will reach equilibrium as flowing water detaches cells extending out into turbulent flow. (Borenstein, 1994)

(Source of Figures B1-B5: Edstrom Industries, Inc., 2003)

The formation and accumulation of biofilm on pipe walls is influenced by a number of factors:

- Presence of microbial nutrients in the water.
- Characteristics of the pipe wall such as roughness and type of material.
- Type and regularity of fouling control procedures.
- Microbial and chemical quality of the finished water entering the distribution system.
- Water temperature and pH
- Chlorine disinfectant residual
- Velocity of the water
- Integrity of the distribution network. (Reiff, 1996)

According to Camper, (1995) the type of material on which a biofilm is grown is a critical factor. Camper found that regardless of the growth rate, more coliforms attached to mild steel vs polycarbonate. Mild steel consistently supported ten-fold more heterotrophs and coliforms than polycarbonate when the two systems were operated identically. When free chlorine (0.2 or 0.5 mg/ℓ) was added to polycarbonate reactors, the heterotrophic plate count (HPC) populations were decreased, while the coliforms numbers were unaffected. In mild steel reactors, a higher concentration (1mg/ℓ) did not affect the HPC populations, while the coliform numbers increased slightly. It appeared that pipe materials subject to corrosion are more likely to support coliform bacteria even if a disinfectant residual is maintained.

Interspecies interactions in Biofilms

De La Cruz, et al (2001) investigated Quorum quenching in *Bacillus cereus* to give more insight into cell to cell communication. Biofilms increase the opportunity for gene transfer between/among bacteria. This is important since bacteria resistant to antimicrobials or chemical biocides can transfer the genes for resistance to neighbouring susceptible bacteria. Gene transfer can convert a previous avirulent commensal organism into a highly virulent pathogen. Certain species of bacteria communicate with each other within the biofilm. Quorum sensing is a cell density dependent gene regulation process that allows cells to

express certain or specific genes only when they reach high cell density (De La Cruz, et al, 2001).

As their density increases, the organisms secrete low molecular weight molecules that signal when the population has reached a critical threshold. This process, called quorum sensing, is responsible for the expression of virulence factors. For example, *Pseudomonas aeruginosa* produces destructive proteinases when the numbers of these bacteria reach a high enough density in the airway biofilms of cystic fibrosis patients. Bacteria express new, and sometimes more virulent phenotypes when growing within a biofilm. Such phenotypes may not have been detected in the past because the organisms were grown on rich nutrient media under planktonic conditions. The growth conditions are quite different particularly in the depths of biofilms, where nutrients and oxygen are usually limited, and waste products from neighbours can be toxic. In short, bacteria found at the bottom of the biofilm look and act different than species located at the surface. (The Public Health Service, PHS, 1998).

Interactions among bacterial populations can have a profound influence on the structure and physiology of microbial communities. Interspecies microbial interactions begin to influence a biofilm during the initial stages of formation, during bacterial attachment and surface colonization, and continue to influence the structure and physiology of the biofilm as it develops. Although the majority of research on bacterial interactions has utilized planktonic communities, the characteristics of biofilm growth (cell positions that are relatively stable and local areas of hindered diffusion) suggest that interspecies interactions may be more significant in biofilms. (James, et al, 1995)

B.1.2 Importance of Biofilm Research

Biofilms are difficult to disinfect because the polysaccharide network protects the organisms. (Salkinoja-Salonen, 2001). Antimicrobials may be readily inactivated or fail to penetrate into the biofilm. Bacteria within biofilms have increased resistance to antimicrobial compounds (up to 1000-fold higher), even though these same bacteria are sensitive to these agents if grown under planktonic conditions. (The Public Health Service, PHS, 1998).

Monochloramines are more effective than residual chlorine at reducing biofilms. (Biofilm cells are at least 500 times more resistant to antibacterial agents.) Researchers discovered that adhesion triggers the expression of a factor that suppresses a large number of genes in the bacteria so that biofilm cells are clearly phenotypically distinct from their planktonic counterparts. Each biofilm bacterium lives in a customized microniche in a complex microbial community that has primitive homeostasis, a primitive circulatory system, and metabolic cooperativity, and each of these sessile cells reacts to its special environment so that it differs fundamentally from a planktonic cell of the same species (Salkinoja-Salonen, 2001).

Microorganisms in biofilms can include bacteria (including coccids, round, rod-shaped and filamentous bacteria), fungi and higher organisms like nematodes, larvae, and Crustacea. Recently researchers have shown that viruses and parasites like Cryptosporidium can be trapped in biofilms. Although they do not grow in a biofilm they can attach themselves to a biofilm after a contamination event (Le Chavellier et al, 1990).

What threats are biofilms to health?

The concern with biofilms is not necessarily from the organisms in the film, but from microorganisms that slough away from the biofilm and enter the main water stream. Some of these organisms can be pathogenic.

Biofilms can contain a host of different microorganisms that have potential health significance. Most notable ones are *Legionella pneumophila* and *Mycobacterium avium*

complex. These organisms can grow within a biofilm and then slough away into the water stream where they can either be ingested or inhaled (i.e., aerosols from showers and humidifiers). Often no disease symptoms will result. On occasion, however, health effects range from gastrointestinal illness (from ingestion) to pneumonia (from inhalation of aerosols). (San Francisco Public utilities commission, 2001)

According to these findings it is clear that biofilms in distribution systems may pose a health risk or cause the aesthetic appearance of water to deteriorate.

B.2 METHODOLOGY

B.2.1 Biofilm Sampling Device

The biofilm sampling device that is being used for testing biofilms within the distribution system was based on the original device described by Pedersen et al (1981) and is illustrated in Figure B.**6**. Water flows from the inlet on the right hand side of the device, through the diffusers, passing over 72 slides before passing out of the device. A modified Pedersen device was constructed (called the Greenwood device) for the purpose of this study. This device is illustrated in the following figures (Figures B.**7** & B.**8**). The new design is designed to withstand the high pressure normally encountered within certain distribution systems. The device is constructed to withstand pressure of up to12 bar.

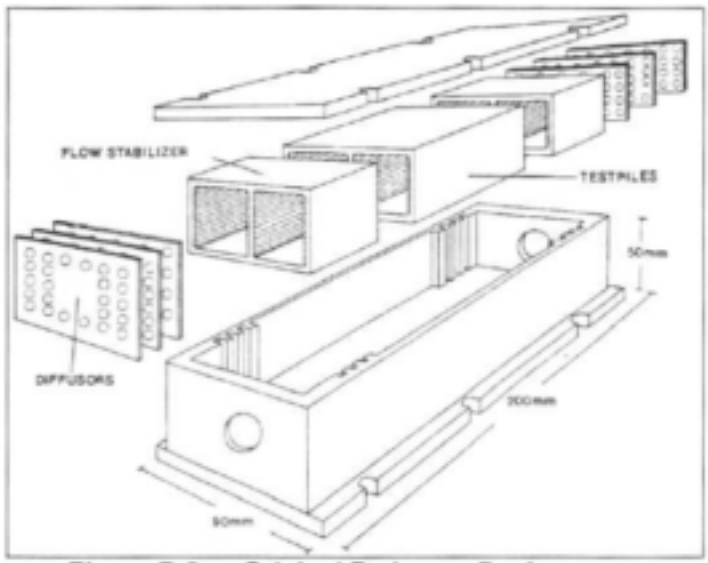

Figure B.6: Original Pedersen Device

Three of these Greenwood devices were constructed and installed. One Greenwood device was installed in an office building after the storage tank (point **A**). The device was installed where the water leaves the storage tank. From this point water is distributed through the rest of the building. Two devices were installed at a residential building situated in an upmarket residential suburb (points **B1** & **B2**). One Greenwood device was installed immediately after the water meter which is situated at the point of entry on the property (**B1**). The second device (**B2**) was installed in the water pipe servicing the bathroom and laundry of the house to allow for differences in water flow or temperature measured.

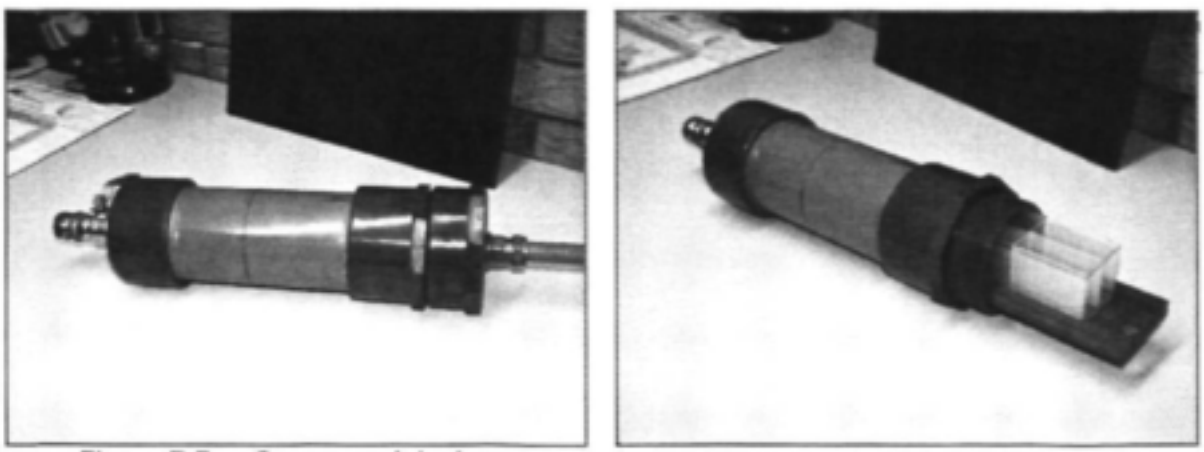

Figure B.7: Greenwood device

B.2.2 Sampling

The three Greenwood devices were sampled every two weeks for a period of one and a half years, from 17 September 2001 to 19 May 2003. At point **A**, two slides were collected. The water was turned off on both sides of the device, isolating the device from the water system. The temperature of the water flowing out of the device was measured. The cap of the device was unscrewed and the slide tray removed aseptically and placed onto a piece of sterilized foil. Two slides diagonally opposite each other were removed with sterile tweezers, and placed into two sterile Petri-dishes. The slide tray was replaced and the Greenwood device was resealed. A sample of the incoming water was collected in a sterile container before the pipes were re-connected and the faucets re-opened.

At point **B** (residential building, **B1** & **B2**), four slides were retrieved (two from each device), in the same way as described above.

B.2.3 Biofilm slide preparation

The biofilm was scraped from both sides of the slides giving a total surface area of 28.6 cm^2. The test slides were washed and the biofilm rubbed off with a sterile swab in a Petri-dish using 45mℓ of sterile saline solution. To ensure all microorganisms had been washed off, a Gram-stain was done of the slide. The 45mℓ saline solution was poured into a sterile 100mℓ bottle and made up to 60mℓ. This 60mℓ sample was used for the enumeration of the following target organisms:

Klebsiella spp.;
Salmonella spp.;
Staphylococcus spp.;
Pseudomonas spp., and
Heterotrophic organisms.

Ten millilitres of water were filtered through 0.45µ filters and placed onto differential and selective culture plates (Figure B.**9**) and incubated according to the manufacturers' instructions. The BFR-O culture plates were incubated as described by Borrego, et al (1987). Five millilitres were placed into tryptone water for qualitative enumeration of aerobic and facultative anaerobic bacteria. Another 5mℓ was enriched in malachite green broth for the enumeration of *Pseudomonas spp.* and incubated according to the manufacturer's instructions (Merck Microbiology Manual, 1996). The heterotrophic plate count was done according to Standard methods for the Examination of Water and Wastewater, (1998).

Figure B.**9** describes the methods used and the different analysis done on the biofilm-water sample.

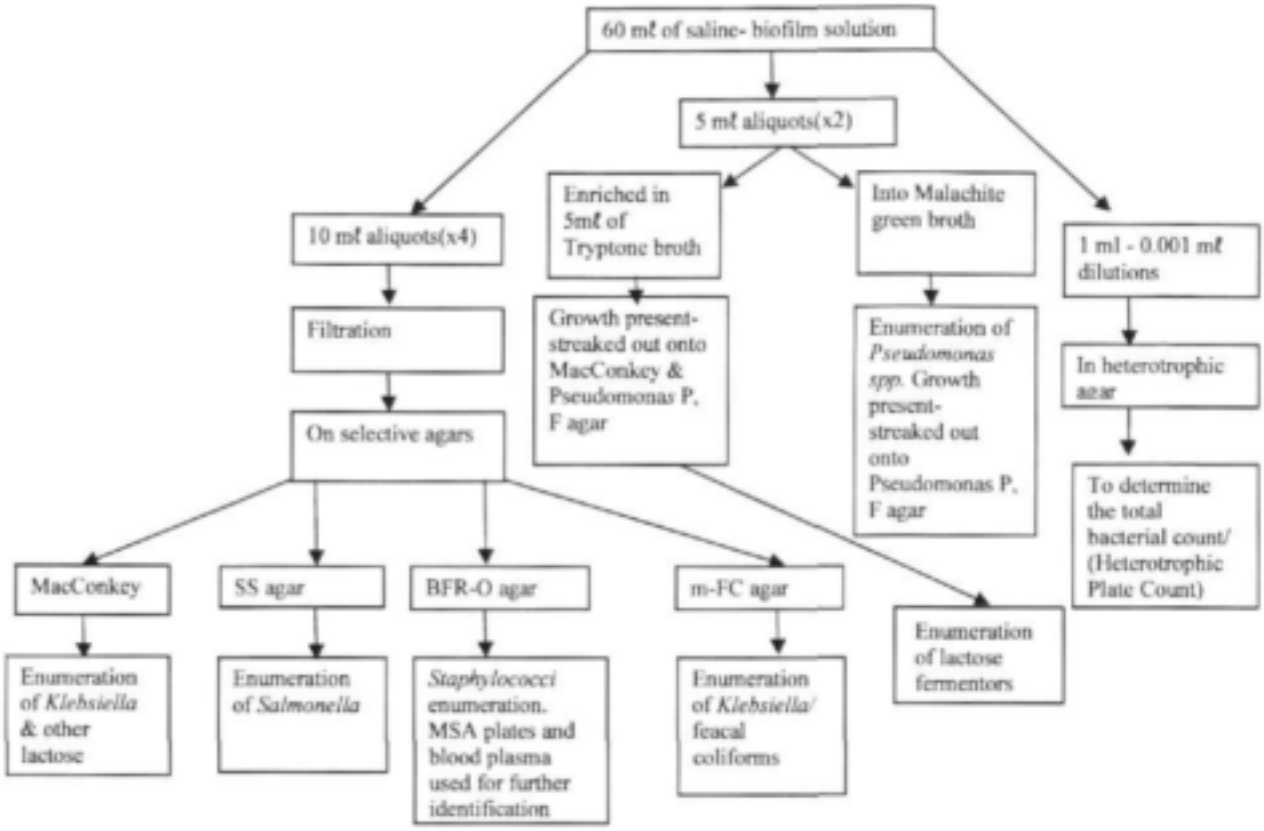

Figure B.9: Flow chart of enumeration methods for organisms in the biofilm-water.

B.2.3.1 Water Quality Indicators within the Distribution System

Incoming water was collected in a sterilized glass bottle. The quality of the water was determined by measuring the turbidity and determining the heterotrophic count. The pour plate method was used for the enumeration of heterotrophic bacteria (Standard methods for the Examination of Water and Wastewater, (1998).

B.2.4 Microorganism Identification

The main groups of bacteria were distinguished by observing their morphology macroscopically and microscopically by Gram-staining the bacterial cultures.

Lactose fermenting and oxidase-negative organisms were identified with the API 20 E system. Non-lactose fermenting organisms were identified with the API 20 NE system. Non-lactose fermenting Gram-positive bacillus and certain Gram-negative bacilli could not be identified by this laboratory. Confirmation of presumptive *Staphylococci* from the BFR-O agar was done using Mannitol Salt Agar and blood plasma. Organisms not specifically included in the research sample group, but occurred in the biofilm were inoculated onto nutrient agar slopes and stored for further identification at a later stage. These organisms can be identified through gene-typing methods providing further funding can be secured.

B.3 RESULTS

The results from the three Greenwood devices are presented below.

B.3.1 Point A: Office Building

The biofilm slides were sampled on 44 occasions. Table B.1 gives a breakdown of the organisms found in the biofilms at point **A** as well as the frequency of occurrence.

Table B.1: Isolates found at Point A.

ISOLATES	NUMBER OF OCCASIONS FOUND
Pseudomonas spp	7 occasions (15.9%)
Staphylococci spp	4 occasions (9.0%)
Micrococcus luteus	3 occasions (6.8%)
Pantoea spp	3 occasions (6.8%)
Yeast	40 occasions (90.9%)
Burkholderia cepacia	1 occasion (2.3%)

Gram-negative and Gram-positive bacilli, bacillus and cocci also found and not identified by the laboratory were inoculated onto nutrient agar slopes and stored for further identification at a later stage.

Figure B.**10** shows the heterotrophic plate count (HPC) in the **biofilm**, temperature and residual chlorine on the day of sampling.

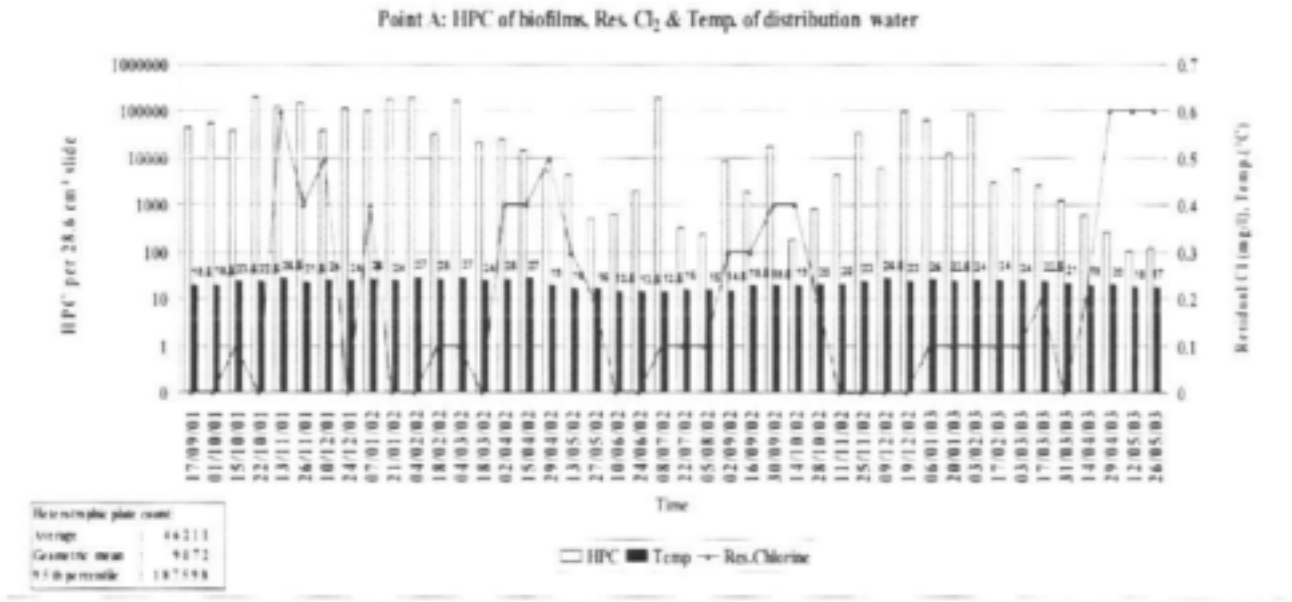

Figure B.10: Average HPC of organisms in the BIOFILM, temperature and residual chlorine of the distribution water at point A (Office building).

Note: The temperature readings are included in this figure as the trend found with the temperature is not obvious. The average temperature is indicated in Table B.2 and a summary of the raw data can be found in Appendix I.

The heterotrophic plate counts exceeded 100 000 cfu/slide (28.6 cm^2) on 8 of the 44 sampling occasions (18.2%), with an average count of 46 210 cfu/slide. The average residual chlorine levels measured was 0.19 mg/ℓ and the average temperature, 21.06 °C.

Table B.2: Summary statistics of Point A

Correlation between Average HPC & Cl_2	r = -0.21
Correlation between Average HPC & Temperature	r = 0.37
Average HPC (cfu/slide)	46210
Geometric mean HPC (cfu/slide)	9072
95th percentile HPC (cfu/slide)	187598
Average Residual Chlorine (mg/ℓ)	0.19
Average Temperature (°C)	21.06

The local municipal authority performed maintenance work on the distribution pipes on the 23rd of November 2002. The sudden increase in the heterotrophic count of the sample at the office building was attributed to this work. An increase in the number of moulds on the culture plates were also found subsequent to the maintenance work.

Figure B.**11** below shows the relationship between the residual chlorine measured by the municipality at the reservoir feeding point **A** and the residual chlorine measured at the sample point.

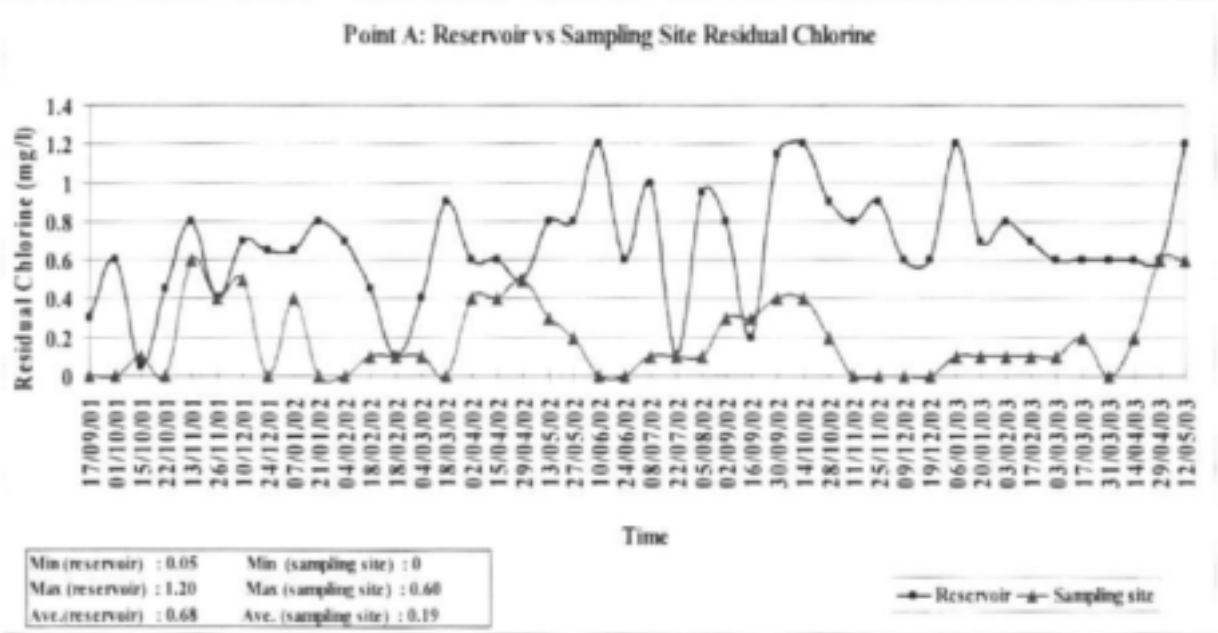

Figure B.11: Residual chlorine at reservoir and Point A

According to municipal sources the target residual chlorine level for the reservoir serving Point **A** is 0.6-1.2 mg/ℓ

B.3.1.1 Distribution Water: Point A

Figure B.**12** shows the heterotrophic plate count (HPC), residual chlorine and turbidity of the distribution water at Point **A**.

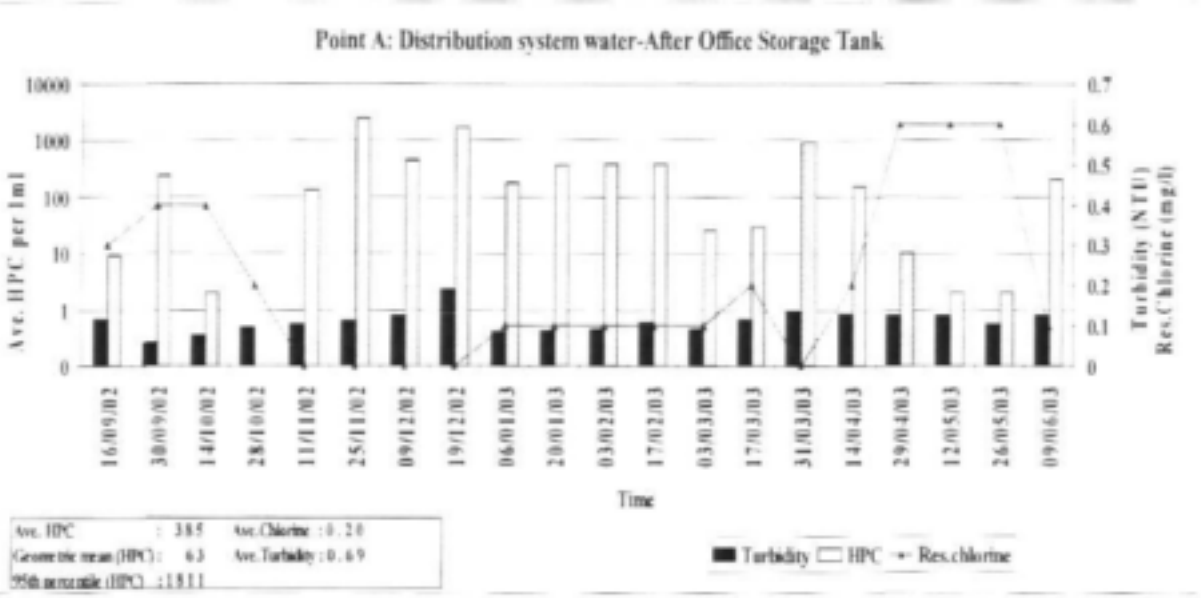

Figure B.12: Average HPC, Turbidity and Residual Chlorine of the distribution system water (tap water) on the day of sampling at Point A.

Water from the distribution system was analyzed for the above-mentioned parameters from 16/09/2002 to 09/06/2003. The heterotrophic plate counts were above 1 000 cfu/mℓ on 2 occasions, with an average count of 385 cfu/mℓ. The average residual chlorine levels measured was 0.2mg/ℓ and the turbidity below 1 NTU, except on 19/12/2002.

The summary statistics at point **A** are presented in Table B.**3.**

Table B.3: Summary statistics of the distribution system water.

Correlation between Average HPC & Cl_2	r = -0.51
Correlation between Average HPC & Turbidity	r = 0.51
Average HPC (cfu/mℓ)	385
Geometric mean HPC (cfu/mℓ)	63
95th percentile HPC (cfu/mℓ)	1811
Average Residual Chlorine (mg/ℓ)	0.2
Average Turbidity (NTU)	0.69

B.3.1.2 Local Authority Monitoring Point

Table B.**4** represents the results found by the municipality on its monthly analysis of the distribution water. The results are from a hydrant closest to and supplying water to Point **A** (Office Building).

Table B.4: Water quality indicator organisms present in distribution water and residual chlorine (municipal monitoring point).

DATE	TOTAL COLIFORMS (PER 100Mℓ)	HETEROTROPHIC PC (PER 1Mℓ)	RES.CL_2 (MG/ℓ)
11/09/01	0	1	0.90
16/10/01	4	19	0.25
13/11/01	4	2	0.25
11/12/01	0	240	0.35
22/01/02	0	5	0.10
12/02/02	0	3	0.30
12/03/02	0	9	0.20
16/04/02	0	3	0.30
15/05/02	24	7	0.85
11/06/02	0	5	0.90
23/07/02	0	4	0.95
21/08/02	0	4	0.40
18/09/02	0	2	0.45
23/10/02	0	3	0.85
27/11/02	0	1	0.30
11/12/02	1	15	0.25
22/01/03	0	1	0.35
19/02/03	0	0	0.45
19/03/03	0	15	0.25
24/04/03	0	1	0.25

Average residual Cl_2 (mg/ℓ)	0.45
Geometric mean Cl_2 (mg/ℓ)	0.34
Average HPC (cfu/mℓ)	17
Geometric mean HPC (cfu/mℓ)	5

The water failed the SABS standard of 10 total coliform bacteria for a maximum of 4% of samples on only one occasion (15/05/2002). On another occasion (11/12/2001), the heterotrophic plate count exceeded 100 colony forming units (cfu) for a minimum of 95% of samples. The water quality over the 20 months was however good.

B.3.2 Point B: Residential Building

The following data reflects the results devices **B1** and **B2**, which were installed at a residential building.

The biofilm slides at point **B1** and point **B2** were sampled on 42 and 38 occasions, respectively. Table B.**5** gives a breakdown of the organisms found in the biofilms at point **B** as well as the frequency of occurrence calculated over 42 occasions.

Table B.5: Isolates found Point B (Residential building).

ISOLATES	NUMBER OF OCCASIONS FOUND
Pseudomonas spp	3 occasions (7.1%)
Staphylococci spp	3 occasions (7.1%)
Pantoea spp	1 occasion (2.4%)
Yeast	37 occasions (88.1%)
Photobacteria damsella:	1 occasion (2.4%)
Bacilli: Gram-negative Gram-positive	 3 occasions (7.1%) 11 occasions (26.2%)
Cocci: Gram-positive Gram-negative	 7 occasions (16.7%) 2 occasions (4.8%)

Gram-negative and Gram-positive bacilli, bacillus and cocci not identified by the laboratory were inoculated onto nutrient agar slopes and stored for further identification at a later stage.

Figure B.**13** shows the heterotrophic plate count (HPC) in the **biofilm**, temperature and residual chlorine on the day of sampling at point **B1**. The average temperature is shown in Table B.**6**.

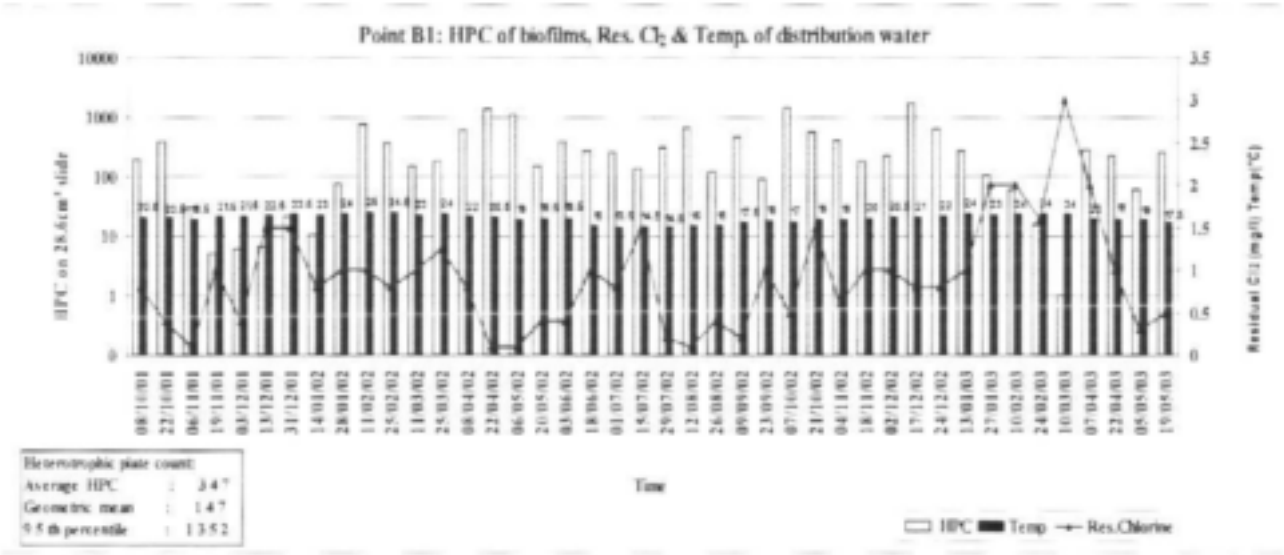

Figure B.13 Average HPC of organisms in the BIOFILM, temperature and residual chlorine of the distribution water at point B1 (residential building).

The heterotrophic plate counts exceeded 1000 cfu/slide (28.6 cm^2) on 4 of the 42 sampling occasions (9.5%), with an average count of 347 cfu/slide. The average bacterial density is 100-times less and the residual chlorine 5-times less than the results of point **A**.

Table B.6: Summary statistics at Point B1

Correlation between Average HPC & Cl_2	r = -0.36
Correlation between Average HPC & Temperature	r = -0.14
Average HPC (cfu/slide)	347
Geometric mean HPC (cfu/slide)	147
95th percentile HPC (cfu/slide)	1352
Average Residual Chlorine (mg/ℓ)	0.91
Average Temperature (°C)	20.02

Figure B.**14** shows the heterotrophic plate count (HPC) in the **biofilm**, temperature and residual chlorine on the day of sampling at point **B2**. The average temperature is shown in Table B.**7**.

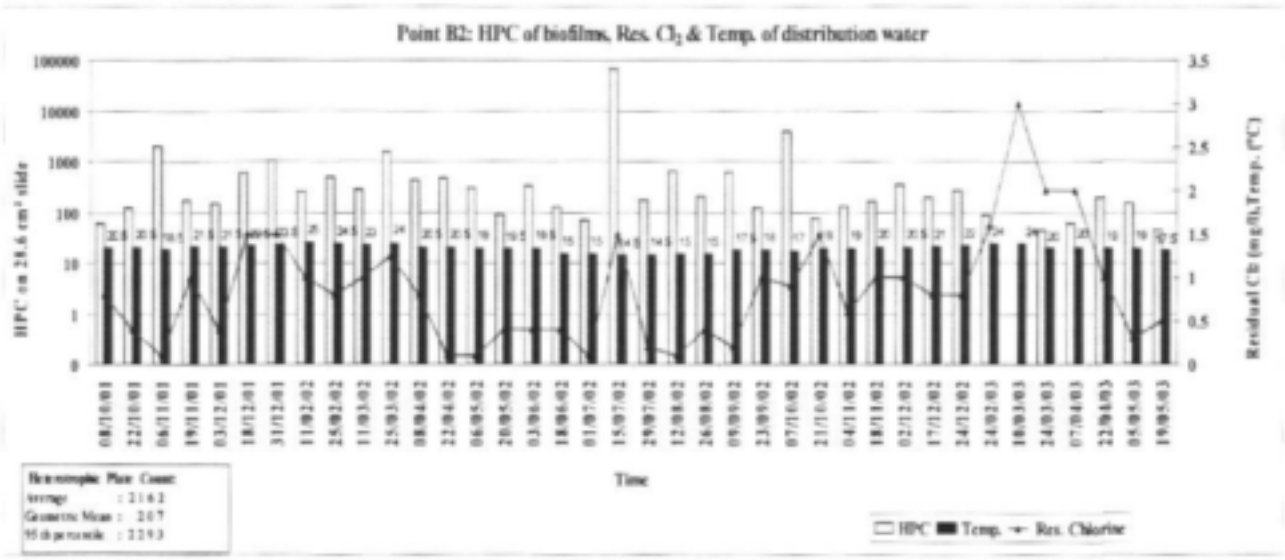

Figure B.14 Average HPC of organisms in the BIOFILM and residual chlorine of the distribution water at point B2 (residential building).

The average temperature at point B**2** was slightly lower than that at point B**1**. However, the average heterotrophic plate count was 10-times higher at this point than at point B**1** (Tables B.**6** & B.**7**).

Table B.7 Summary statistics at Point B2

Correlation between Average HPC & Cl_2	r = 0.16
Correlation between Average HPC & Temperature	r = -0.30
Average HPC (cfu/slide)	2162
Geometric mean HPC (cfu/slide)	207
95th percentile HPC (cfu/slide)	2293
Average Residual Chlorine (mg/ℓ)	0.85
Average Temperature (° C)	19.75

On 27/12/01 and 23/12/02 device **B2** broke. Both times were during December when the water pressure is higher than usual. On 10/03/2003 the heterotrophic count dropped to 1 and 0 cfu/mℓ in points **B1** & **B2,** respectively. From 13/01/2003 the residual chlorine levels steadily increased to reach a peak of 3.0 mg/ℓ on10/03/2003. After this date the chlorine levels dropped again and the bacterial count increased to the bacterial densities previously found with similar residual chlorine levels.

Both systems (**B1** & **B2**) had a large amount of what is believed to be organic and inorganic material, which settled out and formed a brown, granular deposit on the slides. The distribution system supplying water to this area is however well monitored and residual chlorine levels are usually high (statistics given in Table B.**7**). The turbidity of the incoming water can be found in Table B.**8**.

B.3.2.1 Distribution Water: Point B

Figure B.**15** shows the heterotrophic plate count (HPC), residual chlorine and turbidity of the distribution water at Point **B**.

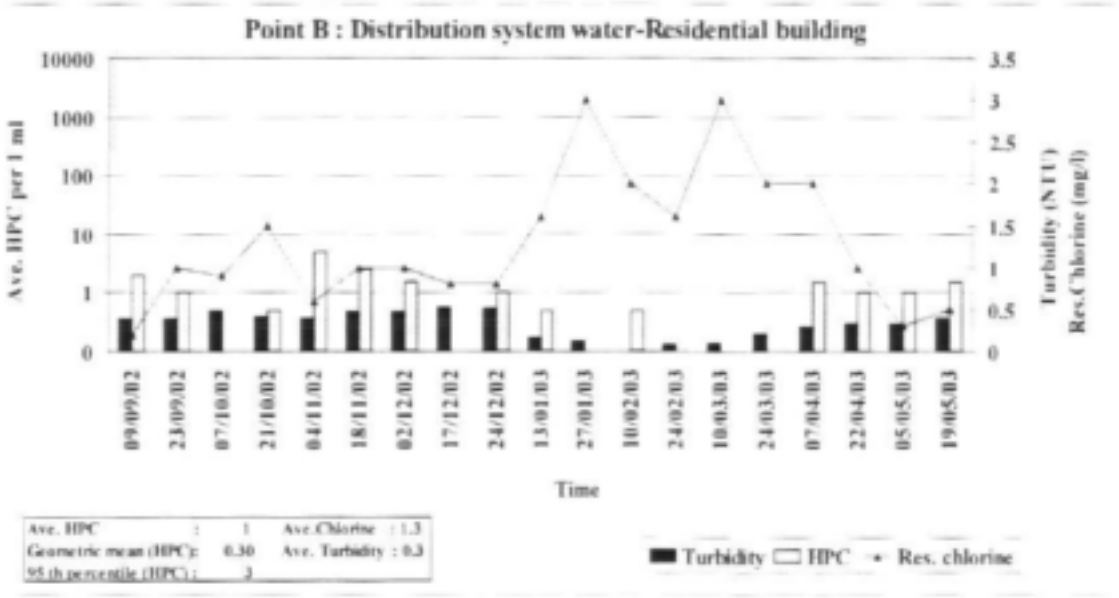

Figure B.15: Turbidity, Residual Chlorine and Average HPC of the distribution system water (tap water) on the day of sampling at Point B.

Water from the distribution system was analyzed for the above-mentioned parameters from 09/09/2002 to 19/05/2003. The heterotrophic plate counts remained at 1 cfu/mℓ or below for 13 out of the 19 sampling occasions and were always below 10 cfu/mℓ. The average turbidity of the water was also half that of the water at point **A.** The water quality in this area remained very good over the study period.

Table B.8: Summary statistics of the distribution system water.

Correlation between Average HPC & Cl_2	r = -0.48
Correlation between Average HPC & Turbidity	r = 0.28
Average HPC (cfu/mℓ)	1
Geometric mean HPC (cfu/mℓ)	0.3
95th percentile Tap water HPC (cfu/mℓ)	3
Average Residual Chlorine (mg/ℓ)	1.3
Average Turbidity (NTU)	0.3

B.3.2.2 Local Authority Monitoring Point

Table B.**9** represents the counts of the indicator organisms detected in the distribution system water taken form the point closest to sampling point **B**. This water was sampled at a residence receiving water from the same distribution source as the study site.

Table B.9: Water quality indicator organisms present in distribution water and residual chlorine (municipal monitoring point).

Date	*Total coliforms (per 100mℓ)*	*Heterotrophic PC (per 1mℓ)*	*Res. Cl_2 (mg/ℓ)*
12/09/01	0	4	0.90
17/10/01	0	3	0.50
14/11/01	0	12	0.75
12/12/01	0	86	0.90
23/01/02	0	3	2.60
13/02/02	0	6	1.00
13/03/02	1	158	0.90
17/04/02	0	61	0.35
15/05/02	28	820	2.20
12/06/02	750	93	1.60
24/07/02	0	4	0.90
21/08/02	0	6	0.70
18/09/02	0	6	0.90
23/10/02	0	0	1.20
27/11/02	0	0	0.35
11/12/02	1	2	0.05
22/01/03	0	0	0.55
19/02/03	0	1	1.00
19/03/03	0	0	2.70
24/04/03	0	0	0.25

Average residual Cl_2 (mg/ℓ)	1.00
Geometric mean Cl_2 (mg/ℓ)	0.76
Average HPC (cfu/mℓ)	60
Geometric mean HPC (cfu/mℓ)	12.93

The water failed the SABS water standards for total coliforms on 15/05/2002 and again on 12/06/2002 when total coliforms were 750 cfu/100mℓ. Both times the residual chlorine was high at 2.2 and 1.6mg/ℓ, respectively. According to municipality records, the target residual chlorine level for the reservoir serving points **B1** & **B2** is between 0.2-0.5 (Mackintosh, et al, 2001-2003).

B.4 DISCUSSION

Point A:

Sampling at point **A** (Office Building) started on 17/09/2001 which is the beginning of the summer season in South Africa. The temperature of the water on that day was 18.5°C and gradually increased over time to a high of 27°C on 02/04/2002. During the same period the heterotrophic bacterial count of the biofilm on the slides ranged between a minimum of 1.41 x 10^4 and a maximum of 1.97 x 10^5 colony forming units (cfu). The average bacterial count during this period was 9.15 x 10^4 cfu. On 15/04/2002 the water temperature decreased to 19°C and reached the lowest temperature during the study period of 13.5 °C on 10/06/2002. The temperature was again this low on 10/06/2002 and also on 08/07/2002, the middle of the winter season in South Africa. During this time the bacterial counts also decreased. The bacterial counts ranged between a minimum of 5.25 x 10^2 cfu and 1.90 x 10^4 with an average count of 5.16 x 10^3 cfu. The count of 1.9 x 10^4 was the only count during this period that was over 10 000 cfu. This count influenced the average bacterial count quite drastically and was probably due to maintenance work or a pipe break since the previous sampling date. Excluding this count, the average count for the same period is considerably lower at 2.40 x 10^3 cfu. After this time the water temperature gradually increased to 20°C on 11/11/2002 and the average bacterial count also increased to 4.29 x 10^3 cfu, somewhat higher than the average count during the middle of winter. From 25/11/2002 until 31/03/2003 the average water temperature was 23.6°C and the average bacterial count was 3.11 x 10^4 cfu. After 31/03/2003 until the last sampling date the water temperature decreased and so did the bacterial count.

The average residual chlorine during the study period was very low at 0.19 mg/ℓ. This is lower than the target residual chlorine level of 0.6 – 1.2 mg/ℓ for the reservoir serving point **A**. Although some residual chlorine was still available, it had little effect on the biofilm. The correlation between the heterotrophic count and the residual chlorine was -0.21 (Table B.**2**) and indicates that the residual chlorine had little effect on the biofilm. A time lag exists between the measured residual chlorine and the effect it has on bacteria. Taking this into account it still shows no evidence that the residual chlorine had any significant effect in reducing the bacterial counts with the biofilm at point **A**. It is difficult for antimicrobials to sufficiently penetrate through thick layers of heterotrophic organisms. Biofilm bacteria can also be up to 1000 – fold more resistant to antimicrobial compounds than when they grow under planktonic conditions (Salkinoja-Salonen, 2001).

The average heterotrohic count of the distribution water sampled at point **A** was 385 cfu/1mℓ and 1 811 cfu/mℓ at the 95th percentile (Table B.**3**). The average residual chlorine of 0.2 mg/ℓ was too low for adequate disinfection of the water. Bacteria not killed by the chlorine added to the high bacterial load in the storage tank.

The average turbidity of the incoming water at point **A** was higher than that at point **B**, 0.69 NTU and 0.32 NTU respectively. An increase in turbidity is a contributing factor to the high heterotrophic count in the storage tank (correlation of 0.51 shown in Table B.3) due to the quick depletion of chlorine.

Point B (1&2):

The average heterotrophic count of the biofilm at sample point **B2** was significantly higher (2162 cfu) than that of point B1 (347 cfu) situated at the point of entry on the property (Tables B.**6** & B.**7**). The residual chlorine at point **B1** was higher with an average of 0.91 mg/ℓ, than the average residual chlorine of 0.85 mg/ℓ at point **B2**. The correlation value between the heterotrophic plate count (HPC) and residual chlorine of -0.36 for device **B1** indicates that residual chlorine and HPC are inversely proportional to one another (Table B.**6**). The correlation value between the same parameters in device **B2** was 0.16. The average temperature at the two sampling points was almost the same at 20.2 °C and 19.8 °C for **B1** and **B2** respectively. Although the average temperature at point B**2** was slightly lower, the average heterotrophic plate count was 10-times higher at this point than at point B**1** (Tables B.**6** & B.**7**). This is also reflected by the correlation values between the average HPC and temperature. The results indicate that when the residual chlorine is relatively high, the effect of the temperature on biofilm development is less significant.

The monthly water samples taken by the municipality at a nearby residence showed total coliform counts per 100 mℓ to exceed the maximum allowable limit 100 cfu/100mℓ for 1% of the samples and 10 cfu/100mℓ for 4% of the samples (SABS 241 of 2001). It did not exceed the maximum allowable limits for the HPC. The average HPC was 60 cfu per mℓ and the average residual chlorine 1.0 mg/ℓ, indicating that residual chlorine is the significant variable in maintaining low heterotrophic organism counts in this system.

B.4.1 Importance of Heterotrophic Plate Count

Beyond treatment of water, the quality of the treated water must be maintained during storage and distribution to the consumer.

The HPC is a useful tool for:

- Monitoring the efficiency of water treatment processes, including infection.
- Obtaining supplemental information on HPC levels that may interfere with coliform detection in water samples collected for coliform compliance monitoring. (U.S.E.P.A., 1975)
- Assessing changes in finished water quality during distribution and storage, and distribution system cleanliness,
- Assessing microbial growth on materials used in construction of table water treatment and distribution systems,
- Measuring bacterial re-growth or after-growth potential in treated drinking water,
- Monitoring microbial population changes following treatment modifications such as a change in the type of disinfection used (Reasoner, 1990).

B.4.2 Significance of organisms found in the sampled biofilms

The system in the office building had a number of different bacteria developing within the biofilm, namely, *Pseudomonas spp*, *Staphylococcus spp.*, *Pantoea spp.*, *Micrococci*, Gram-negative and positive bacilli and cocci. Two of the target organisms, *Pseudomonas spp.* and *Staphylococci spp.* were routinely detected and were found growing in the biofilm of this distribution system. Some of these organisms are opportunistic pathogens that can be a health concern. Opportunistic pathogens are organisms that do not ordinarily cause disease, but multiply freely in persons whose immune systems are weakened by illness or medication. Such persons are said to be immune-compromised. Patients with AIDS have an increased risk of developing serious pseudomonas infections (Rowland, B. M., 2003).

- ***Micrococcus luteus:***
  It is associated with immune-compromised patients. There have been reports of septic shock and cavitating pneumonia in such patients. Urinary tract infections are also reported.

- ***Moulds:***
  The number of moulds introduced into the system was also worrying, as certain types of moulds can secrete mycotoxins that can be harmful. Toxicity can arise from inhalation or skin contact with toxigenic moulds.

- ***Burkholderia cepacia:***
  Burkholderia (previously known as *Pseudomonas) cepacia*, a nutritionally versatile, Gram-negative organism. *B. cepacia* is inherently resistant to multiple antibiotics, can metabolize diverse substrates, and is found in soil and in moist environments. The organism has a particular predilection for the lung in patients with cystic fibrosis (CF) and has emerged as an important opportunistic human pathogen in hospitalized and immune-compromised patients. *B. cepacia* is associated with increased illness and death among CF patients. Unlike infection with *P. aeruginosa*, *B. cepacia* infection significantly increases death rates among CF patients at all levels of lung function (Holmes, et al, 1998). The organism's unusually broad metabolic capabilities, which enable it to survive and proliferate in water-based environments, probably account for its propensity to cause nosocomial outbreaks. Because of its resistance to multiple antibiotics, once acquired, the organism can be difficult to treat. B. cepacia is rarely found in the non-CF patient; however, when it is found the organism can spread to and from CF patients (Holmes A., et al, 1998).

- ***Pseudomonas spp.***
  Pseudomonas are bacteria known for their ability to cause disease in humans, animals and plants, and for their resistance to most commonly used antibiotics. They occur naturally in soil and water, and also live on the surfaces of plants and animals, including humans. One type of the bacteria, called *Pseudomonas aeruginosa*, is often found in humans but does not always cause illness unless a person is already sick or has a weakened immune system. It can cause infections of the urinary tract, blood, lungs and airways, and any tissue that is already injured or compromised. It can also cause inflammation of the skin. Although Pseudomonas infections can be resistant to most commonly used antibiotics, there still remain effective antibiotic therapies for most strains. (AGH, 2002).

  Pseudomonas aeruginosa, a Gram-negative rod, is ubiquitous and can however cause various mild to severe symptoms. *Pseudomonas* dermatitis and otitis externa outbreaks associated with swimming pool and hot tub use are well described; at least 75 cases during six outbreaks occurred during 1997—1998 in Colorado and Maine. Dermatitis outbreaks usually occur as a result of low water disinfectant levels, a condition that also increases the risk for transmission of other chlorine-sensitive pathogens (e.g., *Escherichia coli* O157:H7 and *Shigella sonnei*) that may cause severe health consequences (Beckett G., et al, 1999-2000).

B.4.3 Target Organisms

Biofilm formation and the enumeration of potential pathogens were still possible even though the quality of the water supplied to point **B** was very good. *Pseudomonas spp.* and *Staphylococci spp.* were the most commonly observed target bacteria. There were also many Gram-positive and Gram-negative rods (as indicated in the results) of which *Bacillus subtilis and Bacillus cereus* were the most common ones (they were not identified by biochemical or serological methods).

One of the aims of the project was to assess the survival of general water quality indicator bacteria in biofilms within a distribution system. The incoming water (data from the nearby municipal monitoring point) of the system at the office building showed that during the 20 months of sampling the water failed the SABS standard of 10 total coliforms per 100mℓ for 4% of samples on only one occasion (15/05/02). Total colifoms were detected on 3 other occasions, but were within the water standards. The municipal monitoring point closest to sampling point **B** also failed the SABS water standards for total coliforms on the same day (15/05/2002) as point **A** and again on 12/06/2002 when total coliforms were 750 cfu/100mℓ. Both times the residual chlorine was high at 2.2 and 1.6mg/ℓ, respectively.

No indicator organisms were detected in the biofilms itself. Although general water quality indicators were absent in the biofilms, some opportunistic pathogens were found at both the office building and the residential home.

Considering indicator organisms give an indication that possible pathogens were present in the water, the absence of indicators and the presence of opportunistic pathogens is significant. Hence it can be deduced that relying on indicator organisms alone to determine water quality is not an adequate tool and general indicators should be used in conjunction with other indicators such as Pseudomonas and Staphylococci and analyzing larger quantities of water.

B.5 CONCLUSION

Biofilm formation in distribution systems is a real threat to water quality. It is clear that biofilms can grow even in a well-maintained distribution system. Water should thus be sufficiently dosed with chlorine to ensure that the water is effectively disinfected from the start to the finish of the distribution line.

Storing water in a tank before distribution is definitely a risk factor since bacteria can easily recover in the tank, forming a biofilm to which other organisms can attach. Pathogens and opportunistic pathogens are drawn to the biofilms and the biofilm in turn creates a safe environment for opportunistic pathogens. Storage tanks present in a distribution system should regularly receive additional disinfection if biofilm formation is to be eradicated and heterotrophic organisms kept to a minimum.

The objective of the project was achieved in that low or zero indicator organisms in a system do not completely guarantee the absence of opportunistic pathogens. Some opportunistic pathogens found in the biofilms studied, prove that they can survive in maintained distribution systems. The number of harmful organisms present in a system may not be high enough to cause illness in healthy individuals, but they certainly put immune-compromised individuals at risk. General indicator organisms alone are not sufficient to ensure pathogen-free water and should be used in conjunction with other tests or indicators, testing larger quantities of water.

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SECTION B
APPENDIX 1

POINT A – OFFICE BUILDING
SPRING/SUMMER

Date	17/09/2001	01/10/2001	15/10/2001	22/10/2001	13/11/2001	26/11/2001	10/12/2001	24/12/2001	07/01/2002	21/01/2002	04/02/2002	18/02/2002	04/03/2002	18/03/2002	02/04/2002	16/04/2002
Heterotrophic PC	44620	53610	38400	**197550**	125700	144300	37815	95625	98100	183900	188250	31935	182900	21315	25470	**14070**
HPC (average)	**91485**															
Chlorine (mg/l)	0	0	0.1	0	0.6	0.4	0.5	0	0.4	0	0	0.1	0.1	0	0.4	0.4
Chlorine (average)	**0.19**															
Temperature °C	18.5	18.5	23.5	22.5	26.5	21.5	24	24	26	24	27	25	**27**	24	25	27
Temperature (average)	**24**															

AUTUMN /WINTER

Date	29/04/2002	13/05/2002	27/05/2002	10/06/2002	24/06/2002	08/07/2002	22/07/2002	05//08/2002	02/09/2002	16/09/2002	30/09/2002	14/10/2002	28/10/2002	11/11/2002
Heterotrophic PC	5430	3450	525	645	1995	**18960**	330	240	8610	1920	17850	**180**	810	4410
HPC (average)	**5160**						**4290**							
Chlorine (mg/l)	0.5	0.3	0.2	0	0	0.1	0.5	0	0.4	0	0	0.1	0.1	0
Chlorine (average)	0.18						0.18							
Temperature °C	19	16	16	13.5	13.5	13.5	15	15	14.5	18.5	18.5	19	20	20
Temperature (average)	**15.3**						**17.6**							

AUTUMN /WINTER

Date	25/11/2002	09/12/2002	19/12/2002	06/01/2003	20/01/2003	03/02/2003	17/02/2003	03/038/2003	17/03/2003	31/03/2003	14/04/2003	29/04/2003	12/05/2003	26/05/2003
Heterotrophic PC	34260	6150	**95010**	65580	12150	86820	3090	5490	2610	1260	600	270	105	**120**
HPC (average)	**31240**										**274**			
Chlorine (mg/l)	0	0	0	0.1	0.1	0.1	0.1	0.1	0.2	0	0.2	**0.6**	**0.6**	**0.6**
Chlorine (average)	<0.1										**0.5**			
Temperature °C	23	26.5	23	26	22.5	24	24	24	22.5	21	19	20	18	17
Temperature (average)	**23.7**										18.5			

*Numbers indicated in **bold** provide significant information.*

SECTION C

Prevalence, survival and growth of bacterial pathogens in biofilms in drinking water distribution systems

Biofilm in domestic PVC water-storage containers and its effect on the health-related microbiological quality of the stored water

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SECTION C: BIOFILM IN DOMESTIC PVC WATER-STORAGE CONTAINERS

Biofilm in domestic PVC water-storage containers and its effect on the health-related microbiological quality of the stored water

SUMMARY

Studies in Southern Africa have shown that even when safe water is supplied to developing communities at communal standpipes, contamination by high numbers of pathogenic microorganisms may occur during the processes of fetching water from the supply source and storage during use at home, rendering such waters unsafe for human consumption. This study investigated the occurrence of biofilm in PVC storage containers as one possible reason for this deterioration, using heterotrophic bacteria total coliform counts as well as turbidity as indicators. A second objective was to determine whether biofilm in water-storage containers could contribute to hazardous microbiological contamination indicated by *Escherichia coli* and *Clostridium perfringens*. Results indicated that increased microbiological contamination was associated with biofilm, which harboured and released into container water, heterotrophic bacteria, total coliforms and *C. perfringens*. *E. coli* could not be associated directly with biofilm in containers but rather appeared to be introduced intermittently from the ambient domestic environment. When dislodged with the biofilm, these bacteria contributed substantially to the deterioration of the microbiological quality of supplied water stored in plastic containers.

C.1 INTRODUCTION

South African studies have shown that the microbiological quality of safe, treated water supplied to developing communities at communal stand pipes, deteriorates during the activities and processes of fetching water over distances between home and the supply source and then storing and using it at home. The extent of deterioration in especially the storage containers was such that the microbiological quality of the water generally became unsafe for human consumption and other household purposes (Daniels et al., 1990; Genthe and Seager, 1996; Jagals et al., 1997; 1999).

Several probable causes for the quality deterioration include unclean containers, unhygienic domestic water-handling practices and natural contamination from the ambient domestic environment into containers (Jagals et al., 1999; Nala, 2001; Joubert et al., 2003).

To illustrate unclean containers to community members during a water-hygiene project, Jagals et al. (1997) used a swabbing method to dislodge what appeared to be contaminant build-up adhering to inner walls of plastic (PVC) water-storage containers. The occurrence of these layers was associated with the microbiological deterioration of the container-stored water quality. Microbiological analyses of these substances subsequently revealed substantial numbers of heterotrophic bacteria, indicating that layers forming on the inside of container walls was probably biological film similar to that reported found in water distribution pipe lines (Kasti and Fisher, 1997; Camper, 2000; Geesey, 2001).

C.2 MOTIVATION

Biofilm form when bacteria adhere to surfaces in aqueous environments and begin to excrete a slimy, glue-like substance that can anchor them to all kinds of material such as metals, plastics, soil particles etc. Once anchored to a surface, biofilm-related microorganisms may cause a variety of detrimental or beneficial reactions that affect the water quality, depending on the environmental conditions (Camper, 2000; Geesey, 2001). Of the detrimental reactions would be releasing pathogens that can be harboured within biofilm in piped-water distribution systems (Schaule and Flemming, 1997).

Biofilm form on any surface exposed to bacteria and some volume amount of water. Since waters stored in open containers are far more subjected to environmental influences such as temperature and contamination by nutrients, than water in pipe distribution systems, it is reasonable to assume that biofilm could also form in storage containers, especially in those not regularly cleaned. This lead to contaminant build-up that can harbour pathogenic microorganisms that, when sloughed off into the container water, could pose a threat to consumer health.

C.3 AIM

This part of the project was conducted to gain an understanding of whether such contaminant build-up on container inner-walls could be likened to biofilm typically found in enclosed water distribution systems, and also the extent to which this could affect the health-related microbiological and aesthetic water quality in plastic containers. To achieve this, this project component consisted of two phases.

Phase one investigated whether contaminant build-up in storage containers could be biological (biofilm), thereby indicating probable sustenance and growth of bacteria on the inner walls of PVC containers. The investigation used simple indicator tests.

Phase two determined the effect of the biofilm on the health-related microbiological quality of the water in the containers by assessing the occurrence of general water quality indicator bacteria in drinking water biofilms on inner surfaces of the drinking-water storage containers.

C.4 METHODOLOGY

C.4.1 Study area

The study was conducted in Botshabelo and the Mangaung local municipalities, Free State Province, South Africa. These urban areas have a full supply-water reticulation system. However, in large parts of these cities, the supply is not tapped into dwellings, which mean that the community largely have to fetch water from public standpipes often quite far from their homes. 150 households were randomly selected in the two areas. Water samples were then taken from containers in each of these households.

C.4.2 Data analyses

Data sets were analysed in Microsoft Excel® 2000 spreadsheets for ranges, central values and compliance at the 95th percentile. The statistical computer programme SigmaStat® Version 2.0 (1997) was used to calculate and test for sample sizes and statistical significant differences between data sets using non-parametrical tests since the data were rarely normally distributed, even after log transforming the microbiological data to remove excessive variance. Approaches by Helsel and Hirsch (1995) were followed for the statistical tests used. Significant differences between more than two data sets were tested for with the

Kruskal-Wallis One-way ANOVA on Ranks test. The Mann-Whitney Rank Sum Test was used for determining statistical significant differences in water quality between two unequal sample sets e.g. of supply water and container-stored water. The Wilcoxon Signed Rank Test was applied to the paired data. SigmaPlot® for Window 2001 Version 7.00 (2001) was used to plot the data in graphs. Vertical bolded and dashed lines indicated the various guideline values for infection risk limits in the graphs.

C.4.3 Phase 1: Biofilm on inner surfaces of PVC water containers

Schaule and Flemming (1997) suggested that the extent of biofilm growth could be assessed by removing it from the surfaces in contact with the water and then analysing the content. To characterise the removed substances, literature show a variety of elaborate techniques of sampling and assaying. These were all beyond the scope of this component of the entire project since work of this nature is being carried out by other project partners.

The aim of this component of the project was to develop a simple approach that could be applied by environmental health workers to assess health-related quality of domestically-stored water supplies in communities. A method, based on a simple sampling technique, as well as an indicator-based quality suite of assays, was therefore developed to establish to what extent biofilm could contribute to the deterioration of microbiological water quality in the containers.

C.4.3.1 Phase 1 methodology

The sampling method

Jagals et al. (1997) described a dry-swab method to remove the organic layers from the inner walls of containers. This was refined in a study by Bokako (2000), into a simple technique that included the following steps:

- Water was carefully decanted into a sterile 900 ml Whirlpack® from a household container of which the contents had been left standing undisturbed for several hours. This represented the "undisturbed" state of water in the container with minimal suspended biofilm or other particles (undisturbed water sample).
- After taking the "undisturbed" water sample, the effect of biofilm being dislodged / sloughed off the sidewalls every time a household member filled or used water from an unwashed or unrinsed container was then simulated. This entailed the following:
  - The inner sidewalls of the same container were scrubbed with sterile long-handle brushes to dislodge any possible biofilm and suspend it in the container water. Care was taken to minimise introduction of any substance from the outside environment. For instance, the analysts avoided touching the water or creating excessive floating dust in the dwelling. Sampling was also not done on dusty or windy days.
  - The container was then swirled to suspend the dislodged material in the container-water content. A follow-up water sample was then taken from the mixed suspension (mixed-suspension container water).
  - The data from the sample pair (undisturbed water sample / mixed-suspension container water) were statistically compared for any changes in the indicator levels.

The water samples were collected weekly in sterile 900 mℓ Whirlpack® over a period of 12 months. Samples were also taken from the municipal water supply (communal taps) in the area every time household containers were sampled. Supply water was tested at the taps for residual chlorine levels. None were detected in any of the samples. A total 60 samples were taken of the municipal supply while 300 undisturbed water sample / mixed-suspension container water sample pairs were taken from containers, transported to the laboratory at <10°C, and analysed within 4 hours of collection.

Indicators of biofilm-related contamination

Heterotrophic bacteria, total coliforms, and turbidity were used as indicators of contaminant build-up (biofilm) in containers (Department of Water Affairs and Forestry [DWAF], 1996; Grabow, 1996, Ashbolt et al., 2001). Indicators of changes that the presence of biofilm in containers would bring about were the following:

- Heterotrophic bacteria colony counts (referred to as heterotrophic plate counts - HPC) to indicate changes in the general microbiological water quality of sampled water.
- Total coliforms (TC) indicated bacterial re-growth potential of the water in containers.
- Turbidity (NTU) measurement to indicate changes in water clarity, brought about by suspended particulate matter in container water.

Analytical methods

A spread-plate method with glucose yeast agar (Standard Methods, 1998) assessed the numbers of heterotrophic bacteria. The prepared plates were incubated aerobically at 37°C for 48hr. All visible colonies on the plates were counted as heterotrophic plate counts and expressed as colony forming units (cfu's) per 1 mℓ.

Total coliform bacteria were cultured on Chromocult® Coliform Agar, which is used for the simultaneous detection of coliforms and *E. coli* in the same water samples (Merck, 1996). The membrane filtration technique was used, and membranes were placed in triplicate on 90-mm petri-dishes before being incubated aerobically at 35°-37°C for 24h. Colonies that appeared in various shades of salmon to red were counted as total colifoms (the *E. coli* numbers included), and the numbers expressed as cfu's per 100 mℓ.

A HACH 2100 turbidity meter was used to measure turbidity levels as Nephelometric Turbidity Units (NTU's).

Maximum contamination and effect

The scrubbing technique was intended to emulate the process of potential container biofilm being dislodged during normal filling and transporting processes, and also maximised the contamination that could take place with filling as well as handling. The biofilm / container-water suspensions were therefore assumed to represent maximum contamination and could be used to assess the potential effects (infection risk as well as quality effects) based on target water quality ranges by the South African Water Quality Guidelines (DWAF, 1996), Quality of Water Supplies: Vol. 1: Assessment Guide (Water Research Commission [WRC], 1998) and Water Quality Criteria in South Africa (Aucamp and Vivier, 1990).

C.4.3.2 Phase 1 results and discussion

Figure C.1 shows the measured levels of the various indicators (NTU, HPC and TC) associated with container biofilm.

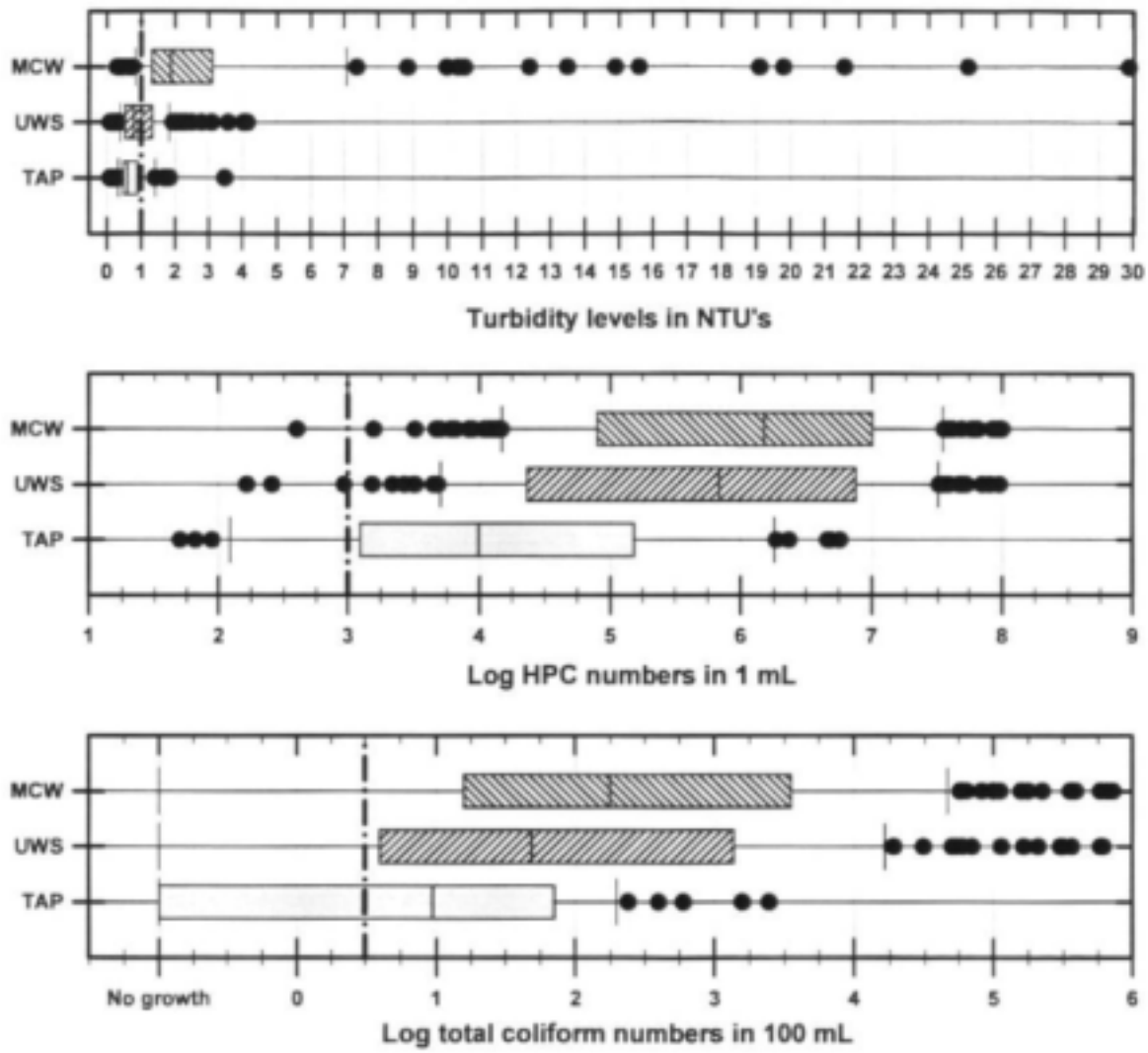

Figure C.1: Turbidity levels as well as numbers of heterotrophic bacteria and total coliforms associated with biofilm build-up in household water-storage containers.

Heterotrophic bacteria

The numbers of heterotrophic bacteria in the municipal water supply were significantly lower than those of the two container sample sets (P=<0.001). The median of log 3.9 as well as the 95th percentile (log 6.67) nevertheless indicated that the general microbiological quality of the supply water did no comply. This probably was from bacterial after-growth in the distribution systems in the areas. After-growth provides harbourage for heterotrophic bacteria (Kasti and Fisher, 1997). Another possibility is activities related to frequent pipe breaks periodically subjecting water in the distribution network to environmental pollution (Jagals et al., 1997; 1999).

These excessive levels of bacteria were released from the distribution system into the containers during filling but the significantly higher HPC levels in the containers suggested that even more bacteria were introduced during and after filling, from further growth in the containers as well as introduction from the environment during handling at home.

There were no statistically significant differences in the numbers of heterotrophic bacteria in the undisturbed water samples (median log 5.84; 95th percentile log 7.68) and mixed-suspension water samples (median log 6.18; 95th percentile log 7.81) from the container water (P=0.108) when measured by the Mann-Whitney Rank Sum Test. This is apparent in Figure C.1.

However, when comparing the data of each sample pair before and after (Wilcoxon Signed Rank Test) the dislodging process, the undisturbed water sample data were significantly lower than the mixed-suspension water data (P≤0.001). This meant that the microbiological water quality deteriorated significantly each time contaminant build-up was dislodged, adding more heterotrophic bacteria to the high levels already introduced by filling, further growth in the containers, or from the environment.

This indicated that particles dislodged from container inner-walls contained heterotrophic bacteria in greater numbers than the general undisturbed container water or the municipal supply, which suggested that after-growth, similar to that occurring in enclosed distribution systems, was also occurring in the two drinking-water container systems.

The dislodging / scrubbing process inevitably introduced additional ambient heterotrophic bacteria into the container content. It was however, uncertain to what extent and what proportion of the increase of heterotrophic plate count numbers in the mixed-suspension container water samples this would constitute. The process was therefore constantly repeated on the same day as sampling in the study areas, but from containers kept under laboratory conditions with far less potential introduction of ambient bacteria. The order of differences between the field and laboratory tests were similar (P = 0.91). It was therefore accepted that the increases in the mixed-suspension container water samples were due to increased levels of bacteria being released from the container side-walls.

Total coliforms

The median for total coliform numbers (median log 0.98) in the municipal supply was below the negligible risk limits of 5 organisms / 100 mℓ proposed by the South African Water Quality Guidelines (DWAF, 1996). Nevertheless, from Figure C.1 it is evident that more than 50% of the samples contained TC in excess of this level with log 2.76 at the 95th percentile. This confirmed organic contamination of the distribution system indicated by the heterotrophic bacteria numbers.
At the 95th percentile (undisturbed water sample log 5.06 and mixed-suspension water log 5.25), the total coliform numbers completely exceeded the target water quality guideline of ≤100 (DWAF, 1996), above which it is indicative of post-treatment contamination or definite growth in water distribution systems (Jones and Bradshaw, 1996) or as in this instance, the containers. The significantly higher total coliform numbers in the mixed-suspension container water could be assumed coming from the dislodged particles (P≤0.001), which confirmed that contaminants building up on the sidewalls of the domestic water-storage containers assessed during this study were indeed biofilm.

Turbidity

Turbidity data give a more explicit perspective on particulate water contamination since it is a more robust test not easily influenced by low input levels of ambient dust present in the dwelling at the time of scrubbing.

Comparison of the turbidity measurements showed that the median turbidity value in the supply water was 0.62 NTU, which was significantly ($P = 0.022$) lower than the median NTU of water from the undisturbed water samples (median 0.81) which in turn was significantly lower than the turbidity levels for the mixed-suspension water samples (median 1.87). This confirms that the supply water became contaminated from filling containers at the supply point, storage as well as handling in the dwelling.

The turbidity levels for the mixed-suspension water samples (median 1.87) were significantly higher than those in the undisturbed water sample ($P<0.001$). While than 30% of undisturbed water samples tested above 1 NTU, with exceedance at the 95th percentile of 2.3 NTU, it were the mixed-suspension water samples that exceeded the 95th percentile at 13.85 NTU, which meant the water showed severe aesthetic effects and also indicated associated risk of disease. The further increase in the turbidity after the dislodging operations indicated that particles had indeed built up on container inner-walls.

The laboratory-repeated process produced similar orders of differences between the field and laboratory tests ($P\leq0.001$). This confirmed that it were suspended materials dislodged from the container sidewalls that caused the significant increases in the turbidity levels.

Potential risk

From a risk perspective, the numbers of heterotrophic bacteria in all the three data sets indicated an *increased risk of infection* to consumers by completely exceeding the target water quality guideline of 1,000 organisms per 1mℓ (bold dashed line in Figure C.1) proposed by the South African Water Quality Guidelines (DWAF,1996). The numbers of heterotrophic plate count in tap water were especially disturbing since it showed that the general microbiological quality of the supply water did not comply.

Total coliform numbers also indicated *significant and increasing risks of infectious disease transmission*.

The median turbidity for the tap water samples was well within the target water quality range (indicated by the bold dashed line in each graph) of 0-1 NTU of the South African Water Quality Guidelines (DWAF, 1996), which indicated no visible turbidity or adverse aesthetic effects (appearance, taste or odour) and no significant risks of associated transmission of infectious micro-organisms. However, if, the NTU data for the tap water is compared to the water quality criteria at the 95th percentile, as suggested by the South African Water Quality Guidelines, the NTU levels did exceed the target water quality range of 0 1 NTU (1.82 NTU), which implies that while turbidity might not be visible, a slight chance of adverse aesthetic effects and infectious disease transmission existed.

At the 95th percentile, turbidity levels in the both sets of container samples exceeded the target water quality guideline of 1 NTU which meant that the water could carry severe aesthetic effects and that associated risk of disease due to infectious disease agents and chemicals adsorbed onto particulate matter (DWAF, 1996) could be expected when contaminant particles were released into the container water.

C.4.3.3 Phase 1 conclusion

Biofilms form on the inner sidewalls of domestic water-storage containers. It was demonstrated that these biofilms could release particulates into suspension with the stored container water when disturbed. This suspension contained organic matter associated with increased concentrations of microbiological contaminants indicated by higher levels of heterotrophic bacteria in the container water. These particles could therefore be assumed to harbour harmful bacteria while adhered to the container sidewalls. The significant increase in turbidity after scrubbing the inner sidewalls of water containers, and suspending the dislodged matter into the container water (mixed-suspension water), supported this. We therefore conclude that inner walls of drinking-water storage containers could harbour the classical biofilm often described to form inside water distribution pipes.

While supply water appeared to seed portions of the biofilm indicators, the increased differences between the tap water and the two container waters were too significant to conclude that the supply water had contributed meaningfully to the biofilm occurrence in the containers. Heterotrophic bacteria as well as total coliform indicator bacteria were introduced and maintained in large numbers in containers and accumulated on the sidewalls during sourcing, storage and domestic handling. Furthermore, these bacteria, as well as turbidity levels translated into hazardous microbiological contamination and associated health effects as discussed in the next part of the discussion.

C.4.4 Phase 2: Health-related water quality indicator bacteria in container biofilm

The same samples from Phase 1 as well as additional water samples were analysed to establish whether indicator bacteria were resident in the biofilm and / or incidental to the collected water volume in the container.

To further establish whether biofilm occurrence could play a role in hazardous microbiological contamination, indicators of potential health effects were used. These were:

- *Escherichia coli* (*E. coli*) to test for faecal pollution (Grabow, 1996). Target water quality range none (0) detectable per 100 mℓ proposed in the World Health Organisation Guidelines for Drinking Water Quality (WHO, 1997).
- *Clostridium perfringens* (CP) to indicate the presence of resistant microorganisms such as protozoan parasites as well as viruses (Payment and Franco, 1993). Target water quality range none (0) detectable per 100 mℓ (Aucamp and Vivier, 1990).

C.4.4.1 Phase 2 methodology

Analytical methods

E. coli (EC) bacteria were grown on Chromocult® Coliform Agar, which is used for the simultaneous detection of total coliforms in the same water samples (Merck, 1996). The membrane filtration technique was used and membranes placed in triplicate on 90-mm petri dishes before being incubated aerobically at 35°-37°C for 24 hours. Colonies that appeared in various shades of dark blue-to-violet as *E. coli*. The numbers were expressed as cfu's per 100 mℓ.

Clostridium perfringens, after sample pasteurisation, were grown in triplicate on 90-mm petri dishes, using the membrane filtration technique and supplemented Perfringens Agar (Oxoid, 1990) and the plates incubated anaerobically at 37°C for 48 hours. Oxoid® gas generating kits, producing atmospheres of 95% hydrogen and 5% carbon dioxide in anaerobic flasks,

were used to create the anaerobic conditions during incubation. Colonies that appeared as partially or fully discoloured dark brown to black were counted. These were expressed as cfu's per 100 mℓ (Aucamp and Vivier, 1990).

Colony verifications were done for total coliforms, *E. coli* and *C. perfringens*. Between 12% and 40 % of the colonies cultured on the various media were selected randomly. API® 20E (an identification system for Enterobacteriaceae and other Gram – negative rods) was used for the verification of total coliforms and *E. coli* colonies. Presumptive *C. perfringens* colonies were verified by the Rapid ID® 32A-identification system for anaerobes.

C.4.4.2 Phase 2 results and discussion

Figure C.2 shows the occurrence of *E. coli* and *C. perfringens* in container-stored water. The numbers of the indicator microorganisms were used to measure the potential risk of infection associated with increases in biofilm particles in mixed-suspension water sampled from the containers.

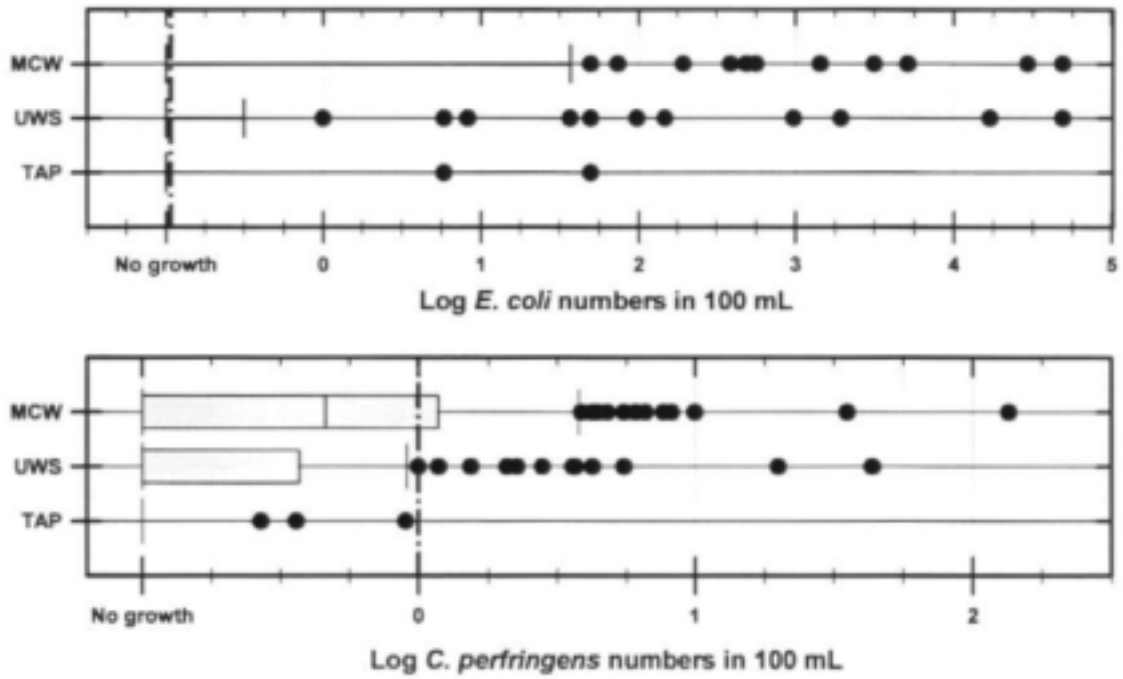

Figure C.2: Health-related microbiological indicators associated with biofilm build-up in household water storage containers.

E. coli only occurred intermittently in the tested waters as shown by the outliers on the graph, with no statistically significant differences between any of the sample sets (P= 0.22). In the containers (at the 95th percentile), *E. coli* occurred at log 1.7 and log 2.69 for undisturbed water sample and mixed-suspension water respectively which exceeded the compliance level (WHO, 1997). This indicated occasional faecal pollution in the water, which posed a slight risk of microbial infectious disease to consumers with occasional exposure. Poor personal hygiene practices during the use of water from containers, as well as unhygienic domestic environments (Theron, 2000) could be the cause for the presence of *E. coli* in the stored water. *E. coli* appeared not to be associated with the occurrence of biofilm in the water-storage containers.

All the water samples sporadically contained vegetative *C. perfringens* spores with the lowest detections in the municipal water supplies (none detected at the 95th percentile). Tap water therefore did not contribute to the significantly higher (P=<0.001) CP numbers in the container water samples. The CP numbers for municipal water were well within the target water quality range of 0 organisms per 100 mℓ, above which constituted a slight risk of microbial infectious disease to consumers suggested by the Water Quality Criteria for South Africa (Aucamp and Vivier, 1990). While most of the data for undisturbed water and mixed-suspension water samples were also within the limit, spores did occasionally occur in numbers exceeding this at the 95th percentile. CP numbers were significantly higher (P=<0.001) in the mixed-suspension container water (median 0, 95th percentile log 0.447) than in the "undisturbed" water sample (median log 0., 95th percentile log 0.781) indicating that the biofilm could readily contain and even accumulate CP spores, which implied that the biofilm could harbour pathogens as suggested by Schaule and Flemming (1997).

C.4.4.3 Phase 2 conclusion

The container-biofilm harboured and possibly propagated potentially hazardous microbiological contaminants as indicated by the numbers of *C. perfringens* spores released during the suspension process. The mixed suspension of container water and biofilm particles contained significantly higher levels of these bacteria than the undisturbed container water or tap water. It appeared however, as if *E. coli* were not effectively supported by the layers of biofilm and probably became inactivated after a resident period in the container water.

C.5 GENERAL CONCLUSION

This study had shown that contaminant build-up on the inner sidewalls of plastic domestic water-storage containers contained microorganisms and can therefore be described as container-biofilm. These biofilms also harboured health-related indicator microorganisms, which indicated the potential presence of pathogens.

The biofilms broke loose from container insides (especially during filling with no subsequent rinsing) to form particulate suspensions, which contained significant numbers of viable bacteria. The dislodging (scrubbing) technique simulated this process of biofilm being dislodged during the normal filling and transporting process. Increased turbidity after scrubbing the inner sidewalls of water containers, and suspending the loosened matter in the container water (mixed-suspension container water), supported this.

The suspended particles harboured and possibly propagated potentially hazardous microbiological contaminants as suggested by the increase in numbers of *E. coli* bacteria and viable *C. perfringens* spores. The mixed suspension of container water and biofilm particles contained significantly higher levels of these bacteria than the undisturbed container water or water from the source tap. The intermittently occurring *E. coli* as opposed to the constantly occurring higher numbers of total coliforms and *C. perfringens* indicated that *E. coli* could have been introduced from the ambient domestic environment or from handling of water with contaminated hands and utensils. *E. coli* were apparently not effectively supported by the biofilm and probably became unculturable after a while in the container water.

While the general microbiological quality of water supplied to the community did not comply with the microbiological limits in terms of water quality guidelines, the *E. coli* and *C. perfringens* levels generally did. Biofilm in the containers contributed substantially to the deterioration of the quality of supplied water due to circumstances surrounding storage and handling of such water during collection, transportation as well as at the points of use.

C.6 RECOMMENDATIONS

Domestic water-handling practices associated with container-stored water containers also need to be improved. Containers should be cleaned and disinfected thoroughly, preferably before each filling. Containers should be effectively covered or closed between water extractions. Containers should generally be stored away from the floor or windows. Children, pets, and other domestic animals should be denied access to water in containers. Water-users should wash hands before handling water (Pinfold, 1991; Joubert et al., 2003) – especially when scooped out for drinking or food handling. In previous studies (Jagals et al., 1997; 1999, Nala et al., 2003), the methods of extracting water from containers were also associated with microbiological contamination of such water. In many instances, water was scooped from the containers with mugs kept uncovered as well as with unwashed hands touching the water (Joubert et al., 2003).

In areas where safe water is supplied to communities, the ultimate way to prevent this type of contamination is to shorten the tap-to-glass sequence by supplying people with water inside their houses. In many areas, this might not be feasible in the medium to long term as public services struggle to keep up on providing treated, piped water to people. For the meantime, other ways to increase consumer water-safety should be devised in areas where water is still being fetched from communal taps and stored at home. For instance communities should be made aware on the essence of sound domestic water hygiene (including container hygiene). People should also be educated to apply sustainable precautionary anti-contamination measures during water haulage, water handling and usage during storage at home (Jagals et al., 2003; Nala et al., 2003).

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SN Venter

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Aims are to:

- Establish techniques for the detection and quantification in source and treated water of the following pathogens:
  - *Campylobacter* spp. and *Heliobacter pylori*
  - *Cyclospora cayetanensis*
  - Hepatitis E and astro-virus;
- Compare environmental isolates with clinical isolates (of *Campylobacter* spp.) to establish the importance of water in the transmission of these pathogens;
- Establish known techniques in the laboratories of participating organisations;
- Monitor various source and treated waters for the presence of the above-mentioned emerging pathogens; and
- Formulate management strategies should the pathogens be found to pose a possible health risk.

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