Characterization of heterotrophic plate count (HPC) bacteria from biofilm and bulk water samples from the Potchefstroom drinking water distribution system

By

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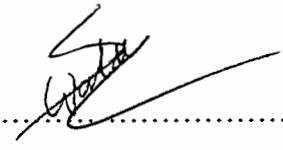
This study is dedicated to my parents, Fritz and Alta Walter.

"Water – gathered and stored since the beginning of time in layers of granite and rock, in the embrace of dams, the ribbons of rivers – will one day, unheralded, modestly, easily, simply flow out to every South African who turns a tap. That is my dream." (President Thabo Mbeki, quoting poet Antjie Krog at the launch of the 2006 UNDP Development Report, Cape Town, November 2006).

DECLARATION

I, Sunette Walter, declare hereby that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institution of higher education.



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ABSTRACT

The presence of heterotrophic plate count (HPC) bacteria in drinking water distribution systems is usually not considered harmful to the general consumer. However, precautions must be taken regarding the immunocompromised. All water supply authorities in South Africa are lawfully required to provide consumers with high-quality drinking water that complies with South African- and international standards. This study mainly focused on the isolation, identification and characterization of HPC and other bacteria from biofilm- and bulk water samples from two sampling points located within the Potchefstroom drinking water distribution system. Based on five main objectives set out in this study, results indicated that the bulk water at the J.S. van der Merwe building was of ideal quality fit for lifetime consumption. Application of enrichment- and selective media allowed for the isolation of 12 different bacterial morphotypes. These were identified by way of biochemical- and molecular methods as *Bacillus cereus*, *Bacillus subtilis*, *Brevundimonas* spp., *Clostridiaceae*, *Corynebacterium renale*, *Flavobacteriaceae*, *Kytococcus sedentarius*, *Leuconostoc lactic*, *Lysinibacillus sphaericus*, *Pseudomonas* spp., *Staphylococcus aureus* and *Staphylococcus capitis*. The greatest diversity of bacteria was detected early autumn 2008, while the lowest diversity occurred during mid-winter 2007. *Bacillus cereus*, *Kytococcus sedentarius* and *Staphylococcus capitis* displayed potential pathogenic properties on blood agar. *Kytococcus sedentarius* could be classified as potentially the most pathogenic among the isolates. All isolates displayed multiple-resistant patterns towards tested antibiotics. *Corynebacterium renale* and *Staphylococcus aureus* were least resistant bacterial species and *Lysinibacillus sphaericus* the most resistant. All isolates were susceptible to ciprofloxacin (CIP) and streptomycin (S), but most were resistant to erythromycin (E). Transmission electron microscopy (TEM) allowed for detailed examination of *Brevundimonas* spp., *Pseudomonas* spp. and *Staphylococcus* spp. The capability of *Brevundimonas* spp. to produce slime and store nutrients within inclusion bodies, suggests the ability of this bacterium to form biofilm

and persist in the drinking water for prolonged periods. Despite the inhibitory or toxic effect of copper against bacterial growth, scanning electron microscopy (SEM) revealed the presence of biofilms as well as diatoms on red-copper coupons. Biofilm activity was also observed on reverse-osmosis (RO) filters. Since corrosion was evident on red-copper coupons, it is recommended that prospective studies also look into the significance of microbial induced corrosion (MIC) within the Potchefstroom drinking water distribution system. Other prospects include determining minimum inhibitory concentrations of isolates against antibiotics and the application of culture independent methods such as SSCP and DGGE to investigate biofilm development. The use of diatoms as an index of the drinking water quality is also suggested.

Keywords: Heterotrophic plate count bacteria, drinking water quality, biofilms, red-copper coupons, drinking water distribution systems

OPSOMMING

Die teenwoordigheid van heterotrofiese plaattellings (HPC) bakterieë in drinkwater stelsels is meestal onskadelik vir die gewone verbruiker. Voorsorg moet nietemin getref word rakende verbruikers met verswakte immuunstelsels. Alle drinkwater verskaffers in Suid-Afrika word regtelik verplig om hoë-kwaliteit drinkwater aan verbruikers te voorsien. Hierdie studie het hoofsaaklik gefokus op die isolasie, identifikasie en karakterisering van HPC en ander bakterieë vanuit biofilm- en drinkwater monsters vanuit twee monsternemings punte in die Potchefstroom drinkwater sisteem. Gebasseer op die vyf hoof uitkomste van die studie, het resultate getoon dat die drinkwater van die J.S. van der Merwe gebou van ideale gehalte was en geskik is vir langdurige gebruik. Toepassing van verrykings- en selektiewe media het dit moontlik gemaak om 12 verskillende bakteriese morfotipes te isoleer. Hierdie isolate is geïdentifiseer deur middel van biochemiese- en molekulêre metodes as *Bacillus cereus*, *Bacillus subtilis*, *Brevundimonas* spp., *Clostridiaceae*, *Corynebacterium renale*, *Flavobacteriaceae*, *Kytococcus sedentarius*, *Leuconostoc lactic*, *Lysinibacillus sphaericus*, *Pseudomonas* spp., *Staphylococcus aureus* en *Staphylococcus capitis*. Die grootste diversiteit van bakterieë is vroeg herfs 2008 aangetref, terwyl die laagste diversiteit middel winter 2007 voorgekom het. *Bacillis cereus*, *Kytococcus sedentarius* en *Staphylococcus capitis* het potensiële patogeniese eienskappe op bloed agar getoon. *Kytococcus sedentarius* is geklassifiseer as die potensieel mees patogeniese isolaat. Alle isolate het meervoudige weerstandbiedenheidspatrone teen getoetsde antibiotikums getoon. *Corynebacterium renale* en *Staphylococcus aureus* was die minste weerstandbiedende spesies en *Lysinibacillus sphaericus* die mees weerstandbiedende spesies. Alle isolate was vatbaar vir siprofloksasien (CIP) en streptomisien (S), maar meestal weerstandbiedend teen eritromisien (E). Transmissie elektron mikroskopie (TEM) het dit moontlik gemaak om *Brevundimonas* spp., *Pseudomonas* spp. en *Staphylococcus* spp. noukeurig te bestudeer. Die vermoë van *Brevundimonas* spp. om slym te produseer en nutriënte te stoor, suggereer dat hierdie

bakterium in staat is om biofilm te vorm en vir langdurige periodes in die drinkwater te oorleef. Ten spyte van die inhiberende of toksiese effek van koper op bakteriese groei, het skanderings elektron mikroskopie (SEM) die teenwoordigheid van biofilm en diatome op rooi-koper koeponne getoon. Biofilm aktiwiteit is ook op tru-osmose (RO) filters aangetref. Aangesien korrosie duidelik was op rooi-koper koeponne, word aanbeveel dat toekomstige studies die belangrikheid van mikrobies geïnduseerde korrosie (MIC) in die Potchefstroomse drinkwater stelsel sal ondersoek. Ander vooruitsigte sluit in die bepaling van minimum inhiberings konsentrasies van isolate teen antibiotikums en die gebruik van kultuur onafhanklike metodes soos SSCP en DGGE om biofilm ontwikkeling te ondersoek. Die gebruik van diatome as 'n indeks van drinkwater kwaliteit word ook voorgestel.

Kernwoorde: Heterotrofiese plaattellings bakterieë, drinkwater kwaliteit, biofilms, rooi-koper koeponne, drinkwater verspreidingssisteme

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

In 1883, Robert Koch's article "About detection methods for microorganisms in water" was published. This paper introduced the use of microbial indicators for testing water hygiene, and, for the first time, methods for measurement of heterotrophic plate count (HPC) bacteria in water were described (WHO, 2003). It is important to distinguish between heterotrophs and HPC bacteria, since these two terms are not the same. The WHO (2002) defines heterotrophs as bacteria, yeasts and molds that need organic carbon for growth. The term "heterotrophic plate count" refers to the different culture-based tests used to recover various microorganisms from water (WHO, 2002). Despite the strict precautions taken during production and distribution of drinking water, it will always contain microorganisms. As a result, there is deterioration in the quality of potable water in its transport from water works to consumer (Boe-Hansen, 2002).

South African guidelines recommend a HPC concentration of 0-100 CFU/mL for domestic water (DWAF, 1996). Low, consistent levels of HPC in finished drinking-water shows that the treatment processes are working well (WHO, 2003). De Wet *et al.* (2002) however maintain that, due to evident potentially pathogenic properties of HPC bacteria in drinking water (even at low, acceptable levels), these bacteria might pose a health risk to consumers, especially the immunocompromised.

According to the WHO (2006), HPC bacteria include opportunistic pathogens such as *Acinetobacter* spp., *Aeromonas* spp., *Flavobacterium* spp., *Klebsiella* spp., *Moraxella* spp., *Pseudomonas* spp., *Serratia* spp. and *Xanthomonas* spp. With so many people of all ages suffering from immuno-deficiencies, there is great pressure on water distributors to supply

water that is of high quality and free from disease-causing agents at the point of delivery (Brözel *et al.*, 2007). It is the duty of water services providers to provide water services as set out in the Constitution (1996) and the Water Services Act (108/1997). All water services authorities in South Africa are legally obliged to monitor drinking water quality on a monthly basis. The Water Services Act (108/1997) contains a compulsory national standard for potable water quality. The South African National Standards (SANS 241: 2006) classifies water into three categories in terms of physical, chemical and microbiological parameters. These categories are Class 0 (an ideal standard comparable to international standards), Class I (water fit for whole lifetime consumption) and Class II (water allowable for short-term consumption) (Mackintosh and Uys, 2006).

Johnson (2001) recommended the use of reverse osmosis (RO) systems as a treatment option for consumers who suffer the adverse effects of unacceptable levels of inorganic contaminants in their drinking water. A study undertaken in Montreal, Canada (Moe, 2007) involved the provision of RO filters to households as additional treatment to treated municipal water. Over a 15-month period, a 35% higher rate of gastrointestinal infections occurred in households without RO treatments systems compared to households supplied with RO filters. This study demonstrated the usefulness of such filter systems to prevent adverse gastrointestinal conditions (Moe, 2007).

Heterotrophic plate count bacteria rely on reduced organic carbon as their energy source (Harley *et al.*, 2002). Heterotrophs are the most important bacteria found in biofilms, which suggests that they are able to get sufficient energy from water that flows past surfaces (Characklis *et al.*, 1990). According to Dreeszen (2003) 99% of bacteria in a water-distribution system are likely to be contained within biofilms. Feng *et al.* (2005) regard biofilm growth within water distribution systems as problematic, since it could lead to

operational problems (e.g., pipe corrosion and clogging water filters), water quality deterioration and other adverse impacts.

Heterogeneity of biofilm renders it ideal for development of different microenvironments and thereby different ecological niches containing specialized microorganisms, including pathogens (Boe-Hansen, 2002). The dynamic nature of biofilms causes parts of it to be released sporadically, thus also releasing pathogens or opportunistic pathogens present therein. This would increase chances for gastrointestinal outbreaks where water is consumed and respiratory illness where aerosols are released (e.g., in showers and recreational facilities) (Brözel *et al.*, 2007). Various investigators (Allen *et al.*, 1979; Gunning *et al.*, 1996; Botha, 2005) have employed scanning electron microscopy (SEM) and transmission electron microscopy (TEM) to study the structure of biofilms and associated bacteria.

Drinking water is usually highly oligotrophic and bacteria occurring in this environment experience oxidative stress (Baudart *et al.*, 2002). Such conditions could result in bacteria present in distribution systems to enter a viable but non-cultivable (VBNC) state (Harley *et al.*, 2002). Pathogens in this VBNC state could remain virulent or produce enterotoxins. These bacteria cannot be detected by conventional culture-based methods (Baudart *et al.*, 2002).

It may be important to include enrichment steps when bacteria from drinking water systems are studied. Methods used to determine these characteristics include growth characteristics on blood agar to test for pathogenicity potential and the Kirby-Bauer method for antimicrobial sensitivity testing (Andermark *et al.*, 1991; Harley and Prescott, 2002). Experimental results from De Wet *et al.* (2002) and Pavlov *et al.* (2004) indicated hemolytic activity and other

positive pathogenic tests of HPC bacteria isolated from selected South African drinking water supplies. These were also resistant to multiple antibiotics (Pavlov *et al.*, 2004).

1.2 BACKGROUND TO THE PRESENT STUDY

A previous study (Vos, 2007) involved the characterization of HPC bacterial isolates yielded from red-copper coupons in an in-stream biofilm development device connected to the main domestic distribution system of the J.S. van der Merwe academic building located at North-West University, Potchefstroom. Bacteria isolated from activated carbon- and RO home water filter systems located at two residences in Potchefstroom were also characterized (Vos, 2007). A major problem that was identified from this prior study was the difficulty that was experienced in cultivating HPC bacteria. It was found that bacterial counts and diversity were relatively low and that bacterial cultivation was rather tedious (Vos, 2007). The inability to detect certain bacteria in drinking water samples does not necessarily mean that they are not present elsewhere in the distribution system. They might be in a viable-but-non-cultivable (VBNC) state. To ensure that one gets a more representative sample of HPC, additional nutrients (usually not present in palatable water) is provided. This is an attempt to elevate their numbers, diversity and rate of growth. Enrichment strategies included selective enrichment broths and incubation at optimal conditions prior to inoculation of these onto various selective agar plates (Brözel *et al.*, 2007).

1.3 RESEARCH AIM AND OBJECTIVES

The main aim of this study was to isolate, identify and characterize HPC and other bacteria from biofilm- and bulk water samples within the Potchefstroom drinking water distribution system.

Objectives were:

- To classify the tap water according to physico-chemical measurements and guideline values.
- To isolate and identify HPC bacteria from bulk water and biofilms of a reverse-osmosis (RO) filter system as well as an in-stream biofilm development device.
- To determine the diversity of isolates.
- To characterize bacteria in terms of (a) pathogenicity potential, (b) antibiotic resistance patterns and (c) their appearance in transmission electron micrographs.
- To make use of scanning electron microscopy (SEM) to detect and study the structure of biofilms in RO filters and red-copper coupons.

CHAPTER 2

LITERATURE REVIEW

2.1 PHYSICAL AND CHEMICAL WATER QUALITY PARAMETERS

The physical and chemical quality of potable water may determine its acceptability to the general consumer (WHO, 2006). A selected number of physical and chemical parameters may be measured to detect possible water quality problems within a distribution network. Results of measurements should be evaluated according to guideline values (WHO, 2006).

2.1.1 Hardness

Hardness can be defined as all the polyvalent cations present in water and is expressed as an equal quantity of calcium carbonate ($CaCO_3$). Water can be regarded as "soft" when it has less than 75mg/L $CaCO_3$, and "hard" when it has more than 150mg/L $CaCO_3$ (Arnold and Tate, 1990). In some instances, hardness can benefit the health of consumers since it contains the essential elements calcium and magnesium. It is however recommended that sensitive groups (persons with a history of kidney or gall-bladder stones and babies under the age of one year) should avoid excessive hardness. Increased hardness weakens the lathering of soap and also makes water less tasteful (Manyaka and Pietersen, 1998). Depending on pH and alkalinity, hardness exceeding 200mg/L can cause scale deposition in pipes and hot water systems (Manyaka and Pietersen, 1998; WHO, 2006). Hardness of less than 100mg/L may be corrosive to water pipes (WHO, 2006). Classifications of total hardness are contained within Annexure A. Home treatment kits that function on the principle of ion-exchange processes are commercially available, but these kits are expensive and only small volumes of water can be treated (Manyaka and Pietersen, 1998).

2.1.2 Chlorine

Chlorine kills bacteria in the water treatment plant and continues to disinfect all the way to the taps of consumers (WHO, 2006). Measurement of free available chlorine will indicate if the disinfection process was efficient and is therefore also a good indicator of microbiological safety of drinking water (Manyaka and Pietersen, 1998). Municipal water supplies are usually disinfected with 0.5mg/L-3.0mg/L chlorine to control bacterial growth within distribution systems (Arnold and Tate, 1990). Absence of free available chlorine shows that the water was not treated with chlorine, or that not enough chlorine was used for successful disinfection. A high residual chlorine concentration (>1.5mg/L) can cause health problems such as irritation of mucous membranes, nausea and vomiting (Manyaka and Pietersen, 1998). Aesthetic effects of elevated chlorine levels include bad taste and odour of the water. Consumers who object to the chlorine taste of their drinking water may use activated carbon filters as a treatment option (Manyaka and Pietersen, 1998). This removes the chlorine but also other organic and inorganic contaminants.

2.1.3 Total dissolved solids (TDS)

One of the main domestic water quality problems in South Africa is related to widespread increased TDS levels. Elevated TDS will give the water a salty taste and does not quenche thirst (Hohls *et al.*, 2002). Generally, water with TDS less than 1200mg/L is acceptable to consumers, but levels less than 650mg/L are preferred (Arnold and Tate, 1990). No acute negative health effects are expected to arise with consumption of water with high TDS, but it is possible that sensitive persons might suffer salt overload in the long term (Hohls *et al.*, 2002). Ideally the TDS should be less than 450mg/L (SANS 241: 2006).

2.1.4 pH

The pH of water has to be known, since more alkaline water needs a longer chlorine contact time or a higher free residual chlorine level for sufficient disinfection. Effective disinfection requires a dosage of 0.4-0.5mg/L chlorine at pH 6-8, up to 0.6mg/L chlorine at pH 8-9. Above pH 9, chlorination may be ineffective (WHO, 2006). The pH of drinking water should be between 6 and 9 (SANS 241: 2006). According to Manyaka and Pietersen (1998) direct health effects of a too-high pH arise from irritation or burning of mucous membranes. Indirect effects are related to the health effects of corrosion products that form during cooking or from distribution pipes. Very high pH levels give water a soapy taste while low pH levels makes water sour (Manyaka and Pietersen, 1998).

2.2 HETEROTROPHIC BACTERIA: A GENERAL OVERVIEW

It is important to differentiate between heterotrophic bacteria and HPC bacteria. The WHO (2002) describes heterotrophs as bacteria, yeasts and molds that need organic carbon for growth. Heterotrophic plate count bacteria represent the level of general bacteria in water. These bacteria might not be harmful themselves, but can conceal the presence of pathogens/potential pathogens (Liee and Lubout, 2008). The term "heterotrophic plate count" refers to the different culture-based tests used to recover various microorganisms from water (WHO, 2002). It is a subset of heterotrophic bacteria and is used as a microbiological water quality parameter (Allen *et al.*, 2004).

Within water distribution systems, HPC bacterial numbers are used to monitor the efficiency of treatment- and disinfectant processes, as well as to determine the cleanliness and integrity of the system (WHO, 2006). Elevated HPC levels are indicative of deteriorated microbiological quality, possible stagnation, bacterial regrowth and potential formation of biofilms (WHO, 2006). It is also used to indicate whether biofilms are potentially present in

the distribution system (Bartram *et al.*, 2003). Drinking water quality guidelines globally recommend HPC limits from 100 to 500 CFU/mL (Pavlov *et al.*, 2004). The DWAF (1996) has criteria of 0 to 100 CFU/mL, while the SANS 241 standard for HPCs is <100 CFU/mL (SANS 241: 2006).

Various heterotrophic bacterial species have been isolated from biofilm samples collected from chlorinated and non-disinfected water distribution systems (Colbourne *et al.*, 1988). Some of these isolates include *Acinetobacter* spp., *Actinolegionella* spp., *Aeromonas* spp., *Alcaligenes* spp., *Arthrobacter* spp., *Bacillus* spp., *Caulobacter* spp., *Citrobacter* spp., *E. coli*, *Enterobacter* spp., *Klebsiella* spp., *Prosthescomicrobacterium* spp., and *Pseudomonas* spp., (Ridgway and Olson, 1981; Olivieri *et al.*, 1985; Herson *et al.*, 1987; Schindler and Metz, 1991). Engelhart *et al.* (2003) and the WHO (2006) maintain that most heterotrophic bacteria found in drinking water are not pathogenic to humans.

2.3 SIGNIFICANCE OF PATHOGENS IN DRINKING-WATER DISTRIBUTION SYSTEMS

According to the USEPA (2002), drinking water is not sterile. Various microorganisms can pass through the treatment process, of which some are biofilm producers (USEPA, 2002; Bessong *et al.*, 2006). These organisms may include primary pathogens that cause disease in healthy persons, or opportunistic pathogens that cause disease in immunosuppressed persons (USEPA, 2002). Distribution pipe biofilms can concentrate various microbial pathogens, thus acting as a potential source of persistent microbial exposure (Aśhbolt *et al.*, 2005). Changes in the flow rate of the water within the distribution system can cause sloughing of biofilm pathogens into the bulk water (USEPA, 2002). Most waterborne pathogens can persist in water, but mostly do not grow or proliferate (WHO, 2006). Moe (2007), Pretorius (2003) and Bessong *et al.* (2006) listed *Aeromonas* spp., *Legionella* spp., *Mycobacterium avium* and

Pseudomonas aeruginosa as infectious agents that can both survive and proliferate in drinking water. After leaving the body of the host, pathogens usually lose their viability and become unable to cause infection (Pretorius, 2003; Bessong *et al.*, 2006; Moe, 2007). Pathogens having low persistence in water are required to quickly find new hosts and will rather be spread by person-to-person contact or improper personal hygiene than by drinking water (WHO, 2006).

2.4 OPPORTUNISTIC PATHOGENS AND THE IMMUNOCOMPROMISED

Von Graevenitz (1977) defines an opportunistic pathogen as one that makes use of the opportunity arising from weakened immune defense mechanisms to cause damage to the host. Organisms such as *Aeromonas* spp., *Legionella* spp., and *Pseudomonas aeruginosa*, for instance, rarely inflict disease in normal immunocompetent individuals. These organisms can be regarded as opportunistic, causing disease only in persons who are, for some reason, immunosuppressed (e.g., infants and the elderly, organ transplant recipients, people who receive antibiotic treatment, malnourished children and persons suffering from HIV/AIDS) (Bessong *et al.*, 2006; Moe, 2007).

According to Engelhart *et al.* (2003), opportunistic pathogens have lower infectious doses in the immunocompromised. However, drinking water containing these bacteria will increase the risk of infection. Based on the South African National HIV Survey of 2005, the estimated HIV prevalence for South Africans over 2 years old was 10.8% (AVERT, 2008). According to this survey, the North-West Province (including Potchefstroom) had the fourth highest HIV prevalence (10.9%) out of the 9 South African provinces (AVERT, 2008). Igumbor *et al.*, (2007) undertook a study that entailed the scope and frequency of enteric bacterial pathogens isolated from HIV/AIDS patients and their household drinking water in the Limpopo Province. Bacteria isolated were significantly linked to HIV/AIDS patients suffering from

diarrhoea as a result of their domestic drinking water. The bacteria isolated included *Aeromonas* spp., *Campylobacter* spp., *Escherichia coli*, *Salmonella* spp., and *Shigella* spp..

Moe (2007) explains that, since data derived from most studies usually come from healthy, adult volunteers, care must be taken when findings are applied to those with weakened immune systems. Immunocompromised individuals (especially those suffering from HIV/AIDS) have a greater need for potable water than healthy persons. Water service providers in South Africa must consider the HIV/AIDS epidemic in their design and operations (Bessong *et al.*, 2006). The Centers for Disease Control (CDC) recommend that people with severely weakened immune systems should avoid drinking tap water (Olson, 2003). They are advised to boil their drinking water and to make use of water filters in order to destroy possible infectious opportunistic agents (DWAF, 1995).

2.5 THE CONCEPT OF "VIABLE-BUT-NON-CULTIVABLE (VBNC)"

Many bacteria possess the ability to enter a viable-but-non-cultivable (VBNC) state (Sardessai, 2005). The VBNC state is defined as a metabolically active state of the cells but they are unable to divide on normal media, producing colonies. This means that the bacteria enter a dormant state, in which they are still viable and able to metabolize and respire, but cannot be detected as colony-forming units on agar plates (Oliver, 1993; Colwell and Walch, 1994; Sardessai, 2005).

According to Oliver (1993) and Sardessai (2005), only 0.01% of waterborne microorganisms are cultivable heterotrophic bacteria, and 1% of viable bacteria are not cultivable. Baudart *et al.* (2002) assume that the highly oligotrophic nature of drinking water as well as oxidative stress, imply that drinking-water distribution systems may contain bacterial cells in a VBNC state. Starvation and low-nutrient conditions are two of the main factors that can trigger the

VBNC response (Fricker, 2003). Other factors involved include lethal or sub-lethal injury of cells, low temperature, adaptation and differentiation, nutrient substances accelerating death and lysogenic bacteriophages (Colwell and Walch, 1994). According to Oliver (2000), studies regarding virulence of VBNC pathogens strongly indicate that they are hazardous to public health, since they can remain virulent or produce enterotoxins.

For cells to remain of public health significance, they must retain virulence while in the VBNC state and be able to resuscitate to the metabolically active state (Oliver, 2000). In a case study reported by Sardessai (2005), sudden re-emergence of tuberculosis years after a patient was declared cured has been caused by resuscitation of VBNC *Mycobacterium* cells. Some of the organisms capable of entering a VBNC state from which they regain the ability to become infectious after passing through animal hosts have been identified in previous studies as *Escherichia coli*, *Campylobacter jejuni*, *Helicobacter pylori*, *Mycobacterium tuberculosis*, *Vibrio cholerae*, and *Vibrio vulnificus* (Sardessai, 2005) as well as *Aeromonas* spp. and *Legionella* spp. (Fricker, 2003).

2.6 BACTERIAL ANTIBIOTIC RESISTANCE

Bacteria are regarded resistant to antimicrobial substances when they are insensitive to concentrations of anti-bacterial agents used in medical therapy (Cloete, 2006). According to Crabbe and Mann (1996), bacteria can be resistant to antibiotics by mechanisms that involve modification of target sites or enzymes, prevention of access for the antibiotics into cells and production of enzymes that destroy or inactivate the antibiotic. Once a bacterium has developed resistance, it may remain resistant. Newly gained plasmids might become the site for additional resistant genes. Resistance could lead to increased illness and death, and use of more toxic and expensive treatment (Calandra *et al.*, 1995).

Lewis and Spoering (2001) suggest that the tolerance of biofilm bacteria towards certain antibiotics, as well as the relapsing nature of biofilm infections, is attributable to the presence of persister cells. According to Costerton *et al.* (1990) and Heilmann *et al.* (1999) biofilm sheds planktonic cells that are responsible for the onset of a disease. Certain antibiotics (e.g., ofloxacin) will eliminate most planktonic and biofilm cells without having any effect on persister cells. The human immune system in turn will eliminate any remaining planktonic persisters, while the biofilm persisters will be protected from the immune system by means of the EPS matrix. These biofilm persisters will therefore persevere (Costerton *et al.*, 1990; Heilmann *et al.*, 1999). As soon as the antibiotic level drops, persister cells will cause regrowth of the biofilm (Lewis, 2000; 2001).

2.7 RELEVANCE OF BIOFILMS IN DRINKING WATER DISTRIBUTION SYSTEMS

2.7.1 The importance of studying drinking water biofilms

Drinking water systems may contain biofilms, despite the oligotrophic conditions and application of disinfectants such as chlorine (Boe-Hansen, 2002). It is important to investigate the nature of these biofilms, as well as their modes of formation and proliferation. Extensive knowledge of drinking water biofilms will make it easier to control them within a distribution system (Costerton *et al.*, 2002). The following sections briefly describe the basic structure of biofilms, the general mechanism of biofilm formation, as well as the pros and cons of biofilm formation within water distribution systems.

2.7.2 General structure and development of biofilms

In aqueous environments with low nutrient levels, microorganisms prefer to colonize surfaces rather than occur in the planktonic phase (Westwood, 2002). Surfaces contain higher nutrient levels and also protect the bacteria from adverse environmental conditions. Biofilm layers in

water distribution systems are generally relatively thin, not more than a few hundred micrometers (Westwood, 2002).

Characklis and Marshall (1990) describe a biofilm system as cells attached to a substratum and embedded in an organic polymer matrix that originates from microbes, along with an overlaying gas and/or liquid layer. Franklin and Stewart (2008) describe biofilms as containing many bacterial cells in different physiological states. They go on to indicate that a biofilm population consists of cells with distinguished genotypes and phenotypes that express different metabolic pathways, stress responses and other specific processes. According to Boe-Hansen (2002), drinking water biofilms generally form micro-colonies held together by EPS. The heterogenic nature of biofilms allows for development of different microenvironments and niches harbouring specialized microorganisms (Boe-Hansen, 2002).

The process of biofilm formation is instantaneous, beginning immediately when water enters a clean pipe (Dreeszen, 2003). Biofilm development occurs gradually by means of the following physical, chemical and biological processes (Characklis, 1990; Dreeszen, 2003) (Also see Figure 2.1):

1. *Substratum conditioning*: Organic molecules accumulate and adsorb to the surface; these molecules will later serve as a source of nutrients for bacteria.
2. *Attachment of pioneer bacteria, adsorption and desorption*: A fraction of planktonic bacteria from the bulk water approach the surface and reversibly adhere to it, some of the reversibly adsorbed cells desorb and another fraction becomes irreversibly adsorbed. The irreversibly adsorbed biofilm cells grow and proliferate in the bulk water (at the expense of substrate and nutrients) and form other metabolic products.

3. *Formation of slime*: Sessile bacteria excrete EPS. These are sticky substances that hold the biofilm together, act as a nutrient trap and protect the bacteria from biocides.
4. *Secondary colonization*: Other types of bacteria (secondary colonizers) become entrapped in the slime by means of physical restraint and electrostatic interaction. These secondary colonizers metabolize waste products excreted by primary colonizers and also excrete their own waste products that are in turn utilized by other cells.
5. *Mature biofilm*: The biofilm is now a fully functioning system within the distribution system, inhabiting a complex consortia of different species each living in their own microniche.
6. *Detachment of cells*: The biofilm reaches a point of growth where some of the cells are sloughed off and re-enters the bulk water, once again becoming part of the planktonic phase.

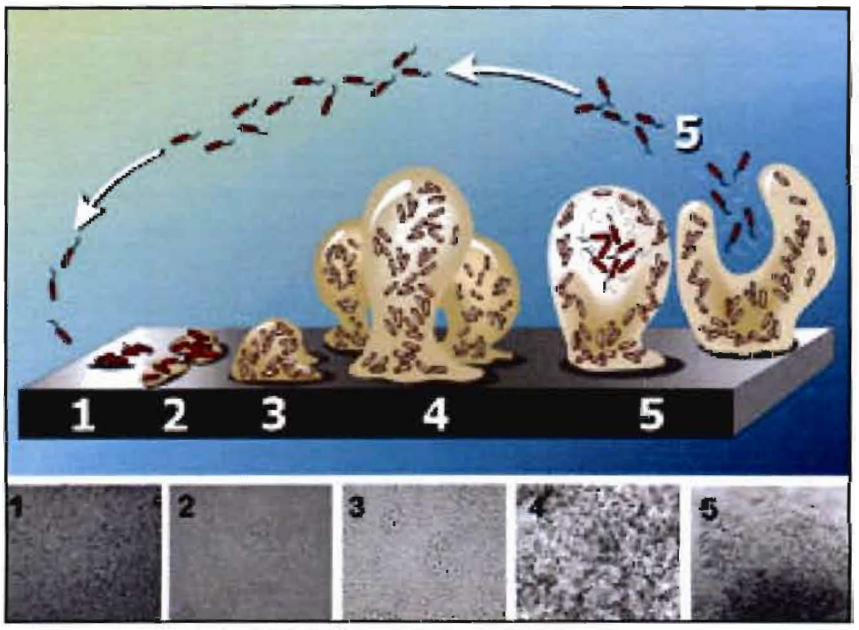

Figure 2.1 A graphical representation of the stages of biofilm development in *Pseudomonas aeruginosa*, as adapted from Costerton *et al.* (2002). Numbers in the graphic represent corresponding photomicrographs of a *Pseudomonas aeruginosa* biofilm, shown below. Stage

1: Reversible attachment of bacterial cells to the matrix. Stage 2: Irreversible attachment of cells with the help of EPS and accompanied by loss of flagella-driven motility. Stage 3: Initial development of biofilm architecture marks the first maturation phase. Stage 4: The second maturation phase is reached in which the biofilm architecture increases in complexity. Stage 5: Detachment stage in which single motile cells (red cells in the figure) detach from microcolonies.

2.7.3 Biofilm accumulation and water quality

Biofilms can lead to many problems within water distribution systems (Westwood, 2002). Some of these problems can be of a technical nature, such as those described by Cloete *et al.* (2000), Westwood (2002) and Feng *et al.* (2005). These authors all demonstrated that biofilm accumulation within distribution networks could cause operational problems such as biocorrosion and biofouling of pipes and other technical equipment. Biocorrosion and biofouling are induced by a deterioration of the microbiological quality of the water (Fleming and Patching, 2003). Another operational-related problem caused by biofilms is the increased demand for chlorine as a disinfectant (Momba *et al.*, 2008). According to Westwood (2002), formation of biofilms can have unpleasant aesthetic effects such as discoloration of water. Deteriorated water quality could also cause an increased health risk (Characklis and Marshall, 1990). Ketley *et al.* (1998), Dreeszen (2003) and Westwood (2002) all support this statement by demonstrating that biofilms within a water distribution system may harbour waterborne pathogens. Furthermore, biofilm increase the persistence of these pathogens, allow for regrowth of some strains of coliforms and protect bacteria from biocides and disinfectants (Westwood, 2002).

All of the previously mentioned aspects will have a negative impact on both water suppliers and consumers. Suppliers will suffer financial losses due to increased costs resulting from

energy losses, replacement of damaged pipes and other operational equipment. They also face the need for additional use of biocides, chlorine and other disinfectants. Consumers will be negatively affected when water tariffs are raised.

2.8 EFFECT OF PIPING MATERIAL ON BIOFILM DEVELOPMENT WITHIN DISTRIBUTION SYSTEMS

Some materials will support or promote bacterial growth more than others (Westwood, 2002). By using construction materials that do not promote growth, water quality can be maintained during water distribution. Makala and Momba (2004) compared the effect of different pipe materials on biofilm accumulation. Commonly used piping materials in South African drinking-water distribution systems are plastic-based (polyvinyl chloride (PVC), unplasticised polyvinyl chloride (UPVC) and medium density polyethylene (MDPE)) and cement-based (cement and cement asbestos). Results showed that microorganisms colonized all these pipe materials under the chlorination process within the initial 20 minutes and over the rest of the study period (Makala and Momba, 2004). Heterotrophs and coliforms were removed from test pipe surfaces by adding monochloramine to the chlorinated water system 24 hours after chlorination. It was also found that less than 1 colony forming unit/cm^2 remained viable (with the exception of PVC) on test pipe surfaces between 48- and 168 hours. Regrowth took place on all piping surfaces between 168- and 672 hours (Makala and Momba, 2004). It was evident that cement-based materials greatly supported less attached bacteria than plastic-based materials. Based on results obtained, the authors recommended that cement and asbestos be used for distribution of chlorine-monochloramine treated water. They further suggested that the existence of an effective monochloramine residual in chlorinated water networks is one of the best ways to control the effect that piping materials have on biofilm development. The study of Makala and Momba (2004) is supported by a recent review (Chaudhuri, 2008).

Rogers *et al.* (1994) compared chlorinated polyvinyl chloride, polybutylene and copper at various temperatures with regard to growth of *Legionella* spp. and biofilm formation. Measurements over 21 days indicated that there were more biofilm accumulation and growth of *Legionella* spp. in the plastics than in copper. It was concluded that copper surfaces inhibit biofouling and growth of *Legionella* spp.

In a study by Parsek and Teitzel (2003), it was demonstrated that copper does not limit biofilm growth, but that it does however, limit growth of planktonic bacteria. The authors suggested that one of the reasons for this might be that EPS found in biofilms protect cells from heavy metal stress by binding the heavy metals and delaying their diffusion through the biofilm.

Van der Kooij and Veenendaal (1999) studied the biofilm development potential of different pipe materials, including polyethylene (PEX) and copper. Measurements from a continuous flow system were recorded at different times up to a period of 140 days. Although biofilm concentrations for copper were a little higher than that for PEX, the amount of biofilm formation in these two types of piping materials were not significantly different.

In another study Van der Kooij *et al.* (2005) investigated biofilm accumulation and growth of *Legionella* spp. on tubing surfaces of stainless steel, PEX and copper. After two years, *Legionella* spp. concentrations were similar for all three materials. Furthermore, in a study by Lehtola *et al.* (2004), changes in water quality and biofilm development were investigated for polyethylene (PEX) and copper. Even though biofilm formation in copper pipes was more tedious than in polyethylene pipes, there was no difference in bacterial numbers between the two materials after 200 days.

Lehtola *et al.* (2005) investigated how piping material can change the effectiveness of chlorine- and UV disinfection on bacteria present in biofilms and potable water. Copper and polyethylene were used as test materials in a pilot scale water distribution system. They demonstrated that UV-disinfection decreases planktonic numbers, but had little effect on sessile bacterial numbers. Chlorine was effective in decreasing bacterial numbers in bulk water and biofilm on polyethylene surfaces. The effect of chlorination was weaker in the outlet water from copper pipes. It only took a few days for bacterial numbers to increase back to the level it was prior to chlorination (Lehtola *et al.*, 2005). In the biofilms found on copper pipes, chlorine decreased bacterial numbers only in transport components preceding the pipeline. The authors (Lehtola *et al.*, 2005) hypothesized that one of the possible explanations for weaker effectiveness of chlorine in copper pipes might be that its concentration decreased quicker in copper pipes than in polyethylene pipes, since chlorine reacts with copper. This finding suggests that a higher chlorine dosage is needed in the case where water flows through copper pipes than water that flows through plastic pipes.

Vos (2007) investigated the effect of red-copper, yellow-copper and galvanized steel on growth of biofilm within an in-stream biofilm development device connected to a domestic water distribution system. Two discs of each type of material were exposed to domestic water flowing through the device over a period of four weeks. Scanning electron micrographs revealed that biofilm accumulation was more dense on red-copper than on yellow-copper or galvanized steel (Vos, 2007). Since red-copper is commonly used as a construction material within drinking-water distribution networks, and sufficient bacterial growth took place on the red-copper coupons, it was decided by the supervisor of that study (Vos, 2007) that further investigations of biofilm development should be done using red-copper coupons.

2.9 AN OVERVIEW OF THE METHODS (PRINCIPLES AND APPLICATIONS) AVAILABLE

2.9.1 Physico-chemical analysis of bulk water

Water quality parameters [pH, chlorine (Cl_2 - free residual chlorine), total hardness (TH), carbonate hardness (CH) and total dissolved solids (TDS)] could be measured using Spectroquant® multi-test strips (Merck, Germany), a chlorine cell test kit (Merck, Germany) and TDS using a hand held TDS meter such as the Water Pro TDS meter (Sprite Industries, California), respectively. According to the manufacturer (Merck, Germany), the pH zone on the test strip is impregnated with phenol red and changes color depending on the pH. The calcium and magnesium ions responsible for TH react with a blue indicator and form a red-violet complex. In the CH test, hydrogen carbonate and carbonate ions react with acid and the consequent change in pH influences the color of a mixed indicator. Measurement values are determined semi-quantitatively by visually comparing the reaction zone on the test strip to a color scale. The chlorine cell test is based on the principle that, in weakly acidic solution, free chlorine reacts with dipropyl-p-phenylenediamine (DPD) to form a red-violet dye that is determined photometrically (Macherey-Nagel, 2009). The amount of TDS in a solution is proportional to dissolved ionized solids such as salts and minerals. These substances contribute to the electrical conductivity of the solution. The TDS meter measures the conductivity of the solution which is then converted to a TDS reading (DWAF, 1996). The relevance of these parameters are discussed in Section 2.1.

2.9.2 Isolation and identification of bacteria

Cultivation methods, combined with microscopy and molecular analysis, is a powerful method to investigate microbial populations (Ogunseitan, 2005). When samples containing mixed bacteria are analysed, it is useful to make use of enrichment- and selective media that

will inhibit growth of competitors, while facilitating growth of a target bacterial species (Bajeva, 2006).

Various biochemical as well as molecular methods can be used to aid in the identification of bacteria. One such biochemical system is the BBL Crystal™ Rapid Gram-Positive (RGP) Identification (ID) System for aerobic Gram-positive bacteria. According to Becton Dickinson (2004), this kit includes ID panels containing 29 dried enzymatic and biochemical substrates. The substrates are rehydrated by means of a bacterial suspension in inoculum fluid. Tests used are based on microbial use and degradation of specific substrates detected by a variety of indicator systems. Enzymatic hydrolysis of fluorogenic substrates that contain coumarin derivatives of 4-methylumbelliferone (4MU) or 7-amino-4-methylcoumarin (7-AMC), causes increased fluorescence that is detected visually with a UV light. Chromogenic substrates upon hydrolysis cause visible color changes. The resulting pattern of the 29 reactions is converted into a ten-digit profile number that is used as the basis for identification. An appropriate code book is then used to aid in the identification.

Analysis of 16S rDNA fragments is useful for identification of prokaryotes (Harley *et al.*, 2002). These are amplified using specific universal primers (Hayashimoto *et al.*, 2005). The sequences of these amplicons are determined and then BLASTN searched against GenBank (http://www.ncbi.nlm.nih.gov/BLAST/). A query submitted to BLASTN search results in a list of sequences in the database which are judged as related to the specific target sequence. "Bit scores" and "E-values" are statistical values used to evaluate the relevance of the sequence matches. BLASTN presents related sequences in descending order according to bit scores. The higher the bit score, the more closely the sequence is related to the target sequence. The E value acts as an estimate of the chance occurrence of identified matches in the database. The smaller the E value, the higher the level of confidence that similarities

between two sequences are more likely caused by common descent than by chance (Ogunseitan, 2005).

2.9.3 Scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

Scanning electron microscopy is used to provide detailed images of outer surfaces of microorganisms (Andermark *et al.*, 1991). The specimen is put on a stub and sputter-coated with platinum or gold under a vacuum. Thereafter, the stub is put in an electron microscope containing a probe that scans the specimen.

There are various examples in literature of investigators that used SEM in their studies. Cortizo *et al.* (2007) employed SEM to investigate the adhesion of motile *P. fluorescens* on different materials, including copper. Feng *et al.* (2005) used SEM to observe the structure of biofilms present in a local drinking water distribution system located in Singapore. In a study by Jacques *et al.* (2006), SEM was used to visualize collection system biofilms that formed on inner surfaces of tubing samples during the production of maple syrup. These investigators (Jacques *et al.*, 2006) observed predominantly rod shaped biofilm bacteria that were embedded in an EPS layer. Babic *et al.* (1996) investigated the use of low temperature SEM (LTSEM) to observe bacteria on spinach leaves. They concluded that frozen, hydrated leaf tissue containing bacteria could be visualized using LTSEM, processed for studying structural details of cells by TEM or recovered to culture pathogenic bacteria for additional investigations.

According to Dykstra (1993), negative staining with phosphotungstic acid (PTA) is a common method for various particulate samples. A formar-coated grid containing particulate materials is coated with electrons, with only a slight stain surrounding the particulates. This allows for

easy visualization of external structures (such as bacterial flagella) of particulate samples with the aid of transmission electron microscopy (TEM).

Lei *et al.* (2008) conducted a study that involved the inactivation of bacteria in oil-field reinjection water by pulsed electric field (PEF) process. Observation of TEM micrographs confirmed that PEF technology could cause severe external damage and shredding of bacterial cells. Fagerbakkel *et al.* (1996) studied the abundant populations of iron (Fe) and manganese (Mn) sequestering bacteria in coastal water. They (Fagerbakkel *et al.*, 1996) employed TEM to obtain bacterial cell counts and also to observe different morphotypes of Fe-Mn bacteria. Iron-manganese bacteria could easily be observed at magnifications of as low as 3000X due to the presence of high electron density, metal-containing structures on these bacteria (Fagerbakkel *et al.*, 1996). Another example of a study that involved the use of TEM to study bacterial structure was that of Delphin *et al.* (2008), who used this method to analyze different bacterial morphotypes from subsurface environments. These authors (Delphin *et al.*, 2008) observed intracellular grains abundantly enriched with lead and phosphorus.

2.9.4 Determination of pathogenic potential and antibiotic resistance patterns of bacterial isolates

Some bacterial colonies cultivated on blood agar plates are able to lyse erythrocytes in the culture medium, a process known as hemolysis. There are three types of hemolysis: (1) alpha hemolysis (α-hemolysis) that is apparent when the area underneath colonies becomes green, (2) beta hemolysis (β-hemolysis) where the area around and underneath colonies are lightened and transparent, and (3) gamma-hemolysis (γ-hemolysis) where no lysis occurs and the area around and underneath colonies remains intact (Ryan and Ray, 2004). The type of hemolysis displayed by an organism is an indication of whether it is potentially pathogenic or not (Harley and Prescott, 2002).

After cultivation of a pathogen, its sensitivity to certain antibiotics acts as a guide for choosing appropriate antimicrobial treatment (Andermark *et al.*, 1991). Some pathogens have predictable sensitivity to specific antibiotics. Others, such as Gram-negative rods, enterococci and staphylococci have unpredictable sensitivity patterns to a variety of antibiotics, and need susceptibility testing to decide on what antimicrobial therapy to use (Champe *et al.*, 2007). A popular qualitative method for determining susceptibility to antibiotics is the Kirby-Bauer disk-diffusion method (Harley and Prescott, 2002). This type of testing entails placing disks containing exact amounts of various antibiotics on culture media that are seeded with the bacteria to be tested. Growth of the bacterium (resistance to the antibiotic) or lack of growth (sensitivity to the antibiotic) is recorded (Champe *et al.*, 2007). According to Andermark *et al.* (1991), it is important to test a number of colonies from a culture to avoid the selection of colonies that have for some reason lost resistance.

An important question that arises is how pathogenic potential and antibiotic resistance patterns are linked. Levy (2000) and McDermott *et al.* (2003) mention the use of antibiotic resistance spectra of bacteria as a way to assess their potential pathogenicity. According to Biedenbach *et al.* (2003) infections caused by resistant bacteria lead to more frequent cases of hospitalization, illness and death. Antibiotic resistance genes are mostly located on horizontally mobile elements such as conjugative plasmids, integrons, transposons (Heinemann, 1999; Dionisio *et al.*, 2002). These genes are easily transferred from one bacterium to another by the mobile elements (Heinemann, 1999; Dionisio *et al.*, 2002). It is thus possible that a nonpathogenic/potentially pathogenic bacterium may transfer resistance factors to more infectious bacteria, thereby making the illness caused by the specific pathogen more severe (Bedekovic and Dzidic, 2003).

2.10 INCREASING DEMAND BY CONSUMERS FOR THE USE OF FILTER SYSTEMS TO TREAT DOMESTIC WATER

According to the DWAF (1995), people living in regions where their drinking water is of such quality that it meets national water standards, do not have to use water filters. In those regions where drinking water is unfit for domestic use, consumers have the option to make use of commercially available home-filtration units for water purification. Olson (2003) explains that household filtering devices make sense for pregnant women, the immunocompromised and those living in regions with unsafe drinking water. The DWAF (1996) says that RO is effective in removing nitrates, other ions and organic compounds from domestic water. The DWAF (1995) and Olson (2003) emphasize the need for regular replacement of filters as directed by the manufacturer. Severely clogged filters pose a risk for "breakthrough" of potential pathogens and other contaminants back into the bulk water and causing illness in individuals who drink the tap water (Olson, 2003). Water consumers of Potchefstroom are in the fortunate position in that safe drinking water is supplied. The Tlokwe municipality that is responsible for the water supply was recently awarded a blue-drop. The latter is a new reward system recently introduced by the South African Department of the Environment and Water Affairs (DEWA). The reward is for drinking water quality and accompanying management systems (DWAF 2009). However, aesthetically the water is not always pleasing and consumers constantly complain about this aspect. This particular scenario is also used by water filter vendors to promote their products. Several of these companies are successfully doing business in the Potchefstroom area.

2.11 SUMMARY

Literature presented in the preceding sections demonstrated that physico-chemical and microbiological parameters are important for classifying the quality of drinking water. Water authorities must analyse for both sets of parameters using SANS 241 (2006) criteria.

Heterotrophic plate count bacteria may occur in drinking water. It is an important parameter to determine efficiency of water treatment. Consistent high levels of HPC in drinking water may indicate poor treatment efficiency and the presence of biofilms that potentially could harbour opportunistic pathogens. It is thus important to study biofilms in distribution water systems and understand their structure and the organisms occurring in them. The literature review also discussed various physico-chemical as well as microbiological methods available to study water quality. It also compared some methods that could be used to characterize HPC bacteria isolated from distribution water systems as well as biofilms developing in such systems.

CHAPTER 3

MATERIALS AND METHODS

3.1 DETERMINATION OF PHYSICO-CHEMICAL PARAMETERS OF BULK WATER

3.1.1 Physical water quality parameters: pH and TDS (Total Dissolved Solids)

The pH and TDS of the bulk water (tap water) from the J.S. van der Merwe building were measured twice weekly over a period of eight weeks during Winter 2007 (June/August 2007). Test strips (Merck, Germany) were used to measure pH. Total dissolved solids were measured using a Water Pro (Sprite Industries, California) TDS meter. The pH, TDS, Cl_2, TH and CH of tap water were measured once weekly for 12 weeks during Summer/Autumn (February – April 2008). TDS was measured with the Water Pro TDS meter (Sprite Industries, California) and Cl_2 was measured photometrically by means of the Spectroquant chlorine cell test kit (Merck, Germany). All the other parameters were measured by using multi-test strips (Merck, Germany). The water was classified according to a colour-coded classification system (Manyaka and Pietersen, 1998). This classification system is presented in Annexure A.

3.2 SAMPLING

Two sampling sites in Potchefstroom were selected. The one sampling site was at a residence (RO home water filtering system) while the other site was at the J.S. van der Merwe academic building at NWU-Potchefstroom Campus (in-stream biofilm development device).

3.2.1 In-stream biofilm development device

There were three sampling periods (March 2007, July 2007 and March 2008) and red-copper coupons were exposed continuously to drinking water for four months at a time to allow for biofilm formation. Upon sampling, the water supply was turned off on both sides of the

device, isolating unit from the main water system. Red-copper coupons (20 x 15 mm) containing the biofilm were removed with sterilized tweezers and placed into sterile 50mL centrifuge tubes containing 20mL sterile 0.8% (w/v) NaCl solution. A 100mL planktonic sample was collected, using a sterile syringe, and placed into a sterile 50mL centrifuge tube prior to replacement of copper coupons into the biofilm development device, re-sealing and turning on of the water supply.

Figure 3.1 shows the set-up of the in-stream biofilm development device that was designed, constructed and inserted into the main water supply of the J.S. van der Merwe building at the North-West University, Potchefstroom in 2006, initially as part of a previous study (Vos, 2007). Up to six copper coupons (15 x 20 mm) were placed horizontally in the device (Figure 3.1 B). Water flow direction was controlled by three main valves (1, 2 and 3) (Figure 3.1 A). During times of operation, valve 1 was closed while 2 and 3 remained open, exposing the copper discs to water flow.

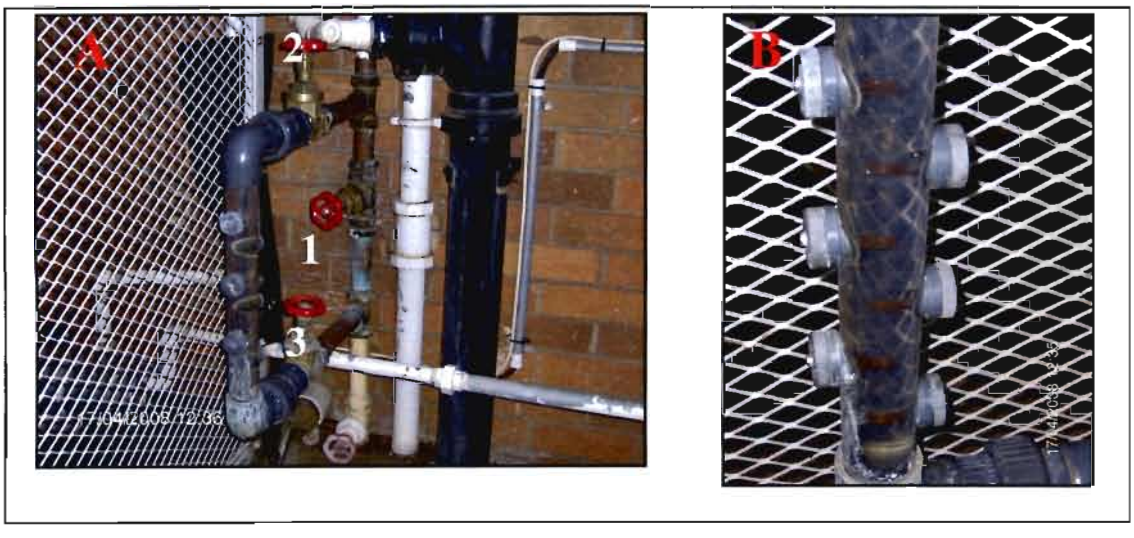

Figure 3.1 In-stream biofilm development device connected to the main water supply of the J.S. van der Merwe building (A), and a close-up photo showing the horizontal positioning of copper coupons (B)

3.2.2 RO home water filtering system

There was only one sampling period (May 2007). Samples were collected after an operational period of 12 months. Pieces of each of the three filters were cut with a sterile scalpel and put into sterile 50mL centrifuge tubes containing 20mL sterile 0.8% (w/v) NaCl solution.

3.3 MICROBIAL ANALYSIS OF BULK WATER AND BIOFILM SAMPLES

The biofilm was scraped off from the red-copper coupons and filter pieces with sterile cotton swabs (within the same 50mL centrifuge tube containing the 20mL sterile 0.8% (w/v) NaCl solution). The biofilm-saline (0.8% w/v NaCl) solution suspension was vortexed for about a minute to ensure sufficient cells coming off, and used for the enumeration of target bacteria as indicated in Table 3.1. Some of the original coupons were set aside for SEM.

One millilitre aliquots of planktonic and biofilm samples were enriched and spread plates were prepared from a dilution series of 10^0 - 10^{-5} on various selective media (see Table 3.1). After initial incubation of the plates at the temperatures specified in Table 3.1, streak plates were prepared to obtain pure cultures.

Bulk water (tap water) was tested two weeks prior to the fourth sampling period to see whether organisms found in biofilm and planktonic samples also appeared in the bulk water. A sterile 50mL microfuge tube was filled with tap water, and 2mL volumes were transferred into the various enrichment broths. After 36 hours of incubation at 37°C, 0.1mL was spread plated onto selective agar plates and incubated at the appropriate conditions, after which further analyses were performed. Morphological features of colonies and isolates were used for preliminary grouping of the bacteria. Gram stains were performed on all isolates according to the protocol described in Bajeva (2006).

Table 3.1 Enrichment broths, selective agars and incubation conditions used (adapted from Brözel *et al.*, 2007) for specific bacterial genera potentially present in bulk water and biofilms. All enrichment broths were incubated at 37°C for 36 hours.

Target bacteria	Enrichment	Selective medium	Incubation conditions
Aeromonas spp.	Tryptone Soy Broth + 1% Yeast Extract	Aeromonas Isolation Agar + Ampicillin Selective Supplement	37°C, 24 hours
Burkholderia spp.	Brain Heart Infusion Broth	Tryptone Soy Agar	37°C, 24 hours
Enterobacter spp.	Tryptone Soy Broth + 1% Yeast Extract	Eosine Methylene Blue Agar	37°C, 24 hours
Enterococcus spp.	Nutrient Broth	Enterococcus Selective Agar	37°C, 24 hours
Escherichia coli	Nutrient Broth	mFC & MacConkey Agar	45°C (mFC); 37°C, 24 hours
Heterotrophs	Nutrient Broth	R2A Agar	Room temperature, up to 5 days
Pseudomonas spp.	Buffered Peptone Water	King's B Agar	37°C, 24 hours
Staphylococcus spp.	Nutrient Broth	Mannitol Salts Agar	37°C, 24 hours

3.4 IDENTIFICATION OF BACTERIA

3.4.1 Biochemical identification: The BBL Crystal™ Rapid Gram-positive identification system for aerobic Gram-positive bacteria

The BBL Crystal RGP ID kit consists of (i) BBL Crystal RGP ID panel lids, (ii) BBL Crystal bases and (iii) BBL Crystal ANR, GP, RGP, N/H ID Inoculum Fluid (IF) tubes. The lid contains 29 dehydrated substrates and a fluorescence control on tips of plastic prongs. Test inoculum was prepared with the inoculum fluid (following the manufacturer's – Becton Dickinson, USA – protocol) and was used to fill the 29 wells contained within the base. The lid was aligned with the base and snapped in place, for the test inoculum to rehydrate the dried reagents. After an incubation period of 4 hours at 37°C, the wells were examined for

color changes or fluorescence that resulted from metabolic activities of the bacteria. The pattern obtained from the 29 reactions was converted into a ten-digit profile number and bacteria identified using a manual codebook (Becton Dickinson, 2004).

3.4.2 Molecular identification of isolates

A peqGOLD Bacterial DNA Mini Kit was used to extract DNA from isolates, according to the method described in the instruction manual (peqGOLD, Germany). Briefly: Overnight cultures were harvested by centrifugation. The bacterial cell wall was removed by lysozyme digestion, and protoplasts lysed by protease K digestion. Following lysis, binding conditions were adjusted and the sample applied to a HiBind® DNA spin-column. Two rapid wash steps removed trace salt and protein contaminants and finally DNA was eluted in a TE buffer. To determine the quality and estimate the quantity, purified genomic DNA samples were electrophoresed on a 1% (w/v) ethidium bromide containing agarose gel (submerged in 1X TAE buffer consisting of 40mM Tris-HCl, 20mMNaOAc and 1mM EDTA) at 100 volts for 45 minutes. Gels were viewed using a Gene Genius Bio Imaging System and GeneSnap gel imaging software (version 6.00.22) from Syngene (Synoptics, UK).

DNA of samples that were clearly visible on agarose gel was selected for amplification by PCR. PCR reagents consisted of: (i) 12.5µl 2x PCR Master Mix (0.05 U/µl *Taq* polymerase, 4 mm $MgCl_2$, and 0.4 mm dNTPs; Fermentas, USA), (ii) 0.5µl mixed 16S rDNA primers (GM5F: 5'-CCT ACG GGA GGC AGC AG-3' and 907R: 5'-CCG TCA ATT CCT TTG AGT TT-3') synthesized by Inqaba Biotech (Pretoria), (iii) 11.0µl PCR water (Fermentas, USA) and (iv) 1.0µl genomic template DNA (~50ng) to make up a total volume of 25µl. Mixtures were briefly (3 seconds) centrifuged to ensure sufficient mixing of reagents before using the Bio-RAD C1000™ Thermal Cycler (BioRAD, UK) for amplification. The reaction mixtures were subjected to the following operational conditions: Cycle 1 (1x): 95°C for 300

seconds, 60°C for 30 seconds; cycle 2 (35x): 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 60 seconds; cycle 3 (1x): 72°C for 300 seconds. Amplified PCR products were electrophoresed on a 1.5% (w/v) agarose gel at 100 volts for 45 minutes, and viewed using a Gene Genius Bio Imaging System and GeneSnap gel imaging software (version 6.00.22) from Syngene (Synoptics, UK). Representatives of the various isolates were selected and the 16S rDNA fragments sent to Inqaba Biotech (Pretoria) to be sequenced. Sequenced data were used to determine the identities of the isolates. The sequences were viewed in ChromasPro (version 1.34) software and the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) was used to determine the identities.

3.5 CHARACTERIZATION OF ISOLATES

3.5.1 Hemolysis on blood agar (pathogenicity testing)

To test isolates for a potentially pathogenic feature, pure cultures were streaked aseptically onto blood agar plates [5% (w/v) sheep blood], incubated for 24 hours at 37°C and checked for hemolytic activity. The different types of hemolytic activities were classified as follows: α-hemolysis showed a greenish darkening of agar under colonies, β-hemolysis caused the area around and under colonies to be lightened and transparent; γ-hemolysis left the agar under and around colonies unchanged (Ryan and Ray, 2004). Isolates were classified as being potentially pathogenic or non-pathogenic according to their ability/lack thereof to produce hemolysins that lyse red blood cells.

3.5.2 Antibiotic resistance/susceptibility test (Kirby-Bauer technique)

Antibiotic resistance/susceptibility patterns of isolates towards a selection of nine antibiotics (from seven different classes) were determined by means of the Kirby-Bauer disc diffusion method (Andermark *et al.*, 1991; Harley and Prescott, 2002). Five representative colonies for each isolate were tested so that percentage resistance could be calculated. Only Gram-

positive isolates were tested for resistance against vancomycin. Isolates could be classified as either being susceptible, resistant or intermediate towards each antibiotic, according to the diameter (in millimeters) of their zones of inhibition (see Table 3.2). Guidelines of NCCLS (1999) were used and isolates ranked according to their antibiotic resistance patterns. Antibiotics were in turn ranked according to the level of susceptibility of bacteria.

Table 3.2 Antibiotics used in this study and interpretation of inhibition zones of test cultures (Harley and Prescott, 2002; NCCLS, 1999).

Chemical class	Antibiotic	Disk symbol	Disk content	Resistant	Intermediate	Susceptible
ß-Lactams	Ampicillin	AP	10µg	≤16	-	≥17
Chloramphenicols	Chloramphenicol	C	30µg	≤12	13-17	≥18
Quinolones	Ciprofloxacin	CIP	5µg	≤15	16-20	≥21
Macrolides	Erythromycin	E	15µg	≤13	14-22	≥23
Aminoglycosides	Kanamycin	K	30µg	≤13	14-17	≥18
	Neomycin	NE	30µg	≤12	13-16	≥17
	Streptomycin	S	300µg	≤6	7-9	≥10
Tetracyclines	Oxytetracycline	OT	30µg	≤14	15-18	≥19
Glycopeptides	Vancomycin	VA	30µg	≤14	15-16	≥17

3.5.3 Bacterial structure

Transmission electron microscopy (TEM) was employed to investigate the structure of selected isolates more closely. This was done especially where it was difficult to distinguish between rods and cocci by conventional light microscopy. Electron microscopy also provided the opportunity to study surface appendages such as flagella. For this particular study, the negative staining method with phosphotungstic acid (Dykstra, 1993) was used for preparation of TEM samples. A Philips CM10 microscope (Philips, Germany) was used to view samples at enlargements of 11 500X and 15 500X.

3.6 BIOFILM STRUCTURE

Scanning electron microscopy (SEM) was used to study the structure of biofilms on the RO filters and red-copper coupons. For SEM, biofilm samples were fixed in sequentially increasing ethanol concentrations (70% ethanol for 6 hours; 90% ethanol for 30 minutes; 100% ethanol for 1 hour), dehydrated and critically dried in liquid carbon dioxide. Samples were then mounted on SEM stubs with double-sided carbon tape. Finally, samples were sputter-coated with gold/palladium and viewed under enlargements ranging from 63X up to 20 000X (Tiedt, 2008), using a Philips XL30 SEM (Philips, Germany).

3.7 STATISTICAL ANALYSIS

Inhibition zone data as well as numbers of isolates that were resistant to various antibiotics were analysed using Microsoft Excel® 2000 software. Where appropriate, averages, percentages and standard deviations were calculated and presented in tables or figures.

CHAPTER 4

INTERPRETATION OF RESULTS

4.1 PHYSICO-CHEMICAL ANALYSIS OF BULK WATER

The pH and total dissolved solids (TDS) of the bulk water (tap water) were measured twice weekly over a period of eight weeks during the Winter 2007. Average results are given in Table 4.1. From Table 4.1 it is evident that the pH remained relatively constant over the eight-week period, with a slight drop of 0.3 units during weeks 1 and 5. The pH was considered neutral and one would thus not expect excessive corrosion of system piping or other unpleasant technical, health-related or aesthetic effects. Total dissolved solids (TDS) ranged from the lowest concentration (245mg/L) recorded during week 6, to the highest concentration (268mg/L) recorded during week 1 (Table 4.1). These values are what is expected from adequately treated water. According to the overall results and the colour-coded classification system (see Annexure A) of physical water quality parameters for domestic water (Manyaka and Pietersen, 1998; SANS 241: 2006), the bulk water could be classified as "blue; class 0; ideal water quality" fit for lifetime consumption.

Table 4.1 pH and TDS values of bulk water at the J.S. van der Merwe building (Winter 2007).

	Average pH (units)	Average TDS (mg/L)
Week 1 (28 June)	7.5	261
Week 2 (5 July)	7.8	256
Week 3 (12 July)	7.8	257
Week 4 (19 July)	7.8	251
Week 5 (26 July)	7.5	251
Week 6 (2 August)	7.8	245
Week 7 (9 August)	7.8	258
Week 8 (16 August)	7.8	268
Mean	7.725	255.875
Standard Deviation	0.138873	7.019107

The pH, TDS, Cl_2, TH and CH were measured once a week for 12 weeks during Summer/Autumn (February - April 2008). These results are given in Table 4.2. The pH was lowest (7.2 units) during week 11, and highest (8.0 units) during weeks 2 and 9. As in 2007, the tap water was within recommended pH limits of 6 to 9 for ideal water quality (Manyaka and Pietersen, 1998; SANS 241: 2006). The TDS concentration was lowest (231 mg/L) during week 9, and highest (321mg/L) during week 6. These values were within the recommended limits for ideal water quality (< 450 mg/L) as specified by Manyaka and Pietersen (1998) and SANS: 241 (2006).

Free residual chlorine remained very low over the 12-week period with the lowest concentration (0.012mg/L) being recorded in week 4. Free chlorine was detected in the highest concentration (0.047mg/L) in week 5. These very low residual concentrations were alarming since it could be an indication of possible regrowth within the distribution system. Total hardness (Table 4.2) ranged from 12mg/L (week 7) to 20mg/L (weeks 1, 8, 9, 10 and 12). The CH concentration was the lowest (9mg/L) during weeks 2 and 7. The highest CH concentration (16mg/L) was found during week 6. These values classify the bulk water as "soft" (Arnold and Tate, 1990). Once again – according to guidelines (Manyaka and Pietersen, 1998; SANS 241:2006) – the bulk water was classified as "blue; class 0; ideal water quality."

Table 4.2 Physico-chemical measurements for bulk water at the J.S. van der Merwe building (Summer/Autumn 2008).

	pH (units)	TDS (mg/L)	Cl_2 (mg/L)	TH (mg/L)	CH (mg/L)
Week 1 (13 February)	7.8	252	0.036	20	14
Week 2 (20 February)	8.0	260	0.014	16	9
Week 3 (27 February)	7.5	274	0.024	15	14
Week 4 (5 March)	7.8	317	0.012	15	14
Week 5 (12 March)	7.5	276	0.047	18	14
Week 6 (19 March)	7.8	321	0.025	15	16
Week 7 (26 March)	7.5	268	0.027	12	9
Week 8 (2 April)	7.8	264	0.034	20	14
Week 9 (9 April)	8.0	231	0.019	20	14
Week 10 (16 April)	7.5	296	0.039	20	14
Week 11 (23 April)	7.2	244	0.041	18	12
Week 12 (30 April)	7.8	252	0.027	20	14
Mean	7.683333	271.25	0.02875	17.41667	13.16667
Standard Deviation	0.24058	27.80492	0.010889	2.74552	2.124889

4.2 MICROBIAL ANALYSIS

4.2.1 Isolation and identification of bacteria

The various enrichment broths and selective media presented in Table 3.1 allowed for the isolation of 12 different bacterial morphotypes. These were isolated from the biofilm development device, RO filters and bulk water from the two sampling points in the Potchefstroom drinking water distribution system over four sampling periods. The various morphological features, including colony morphology, pigmentation, Gram reactions and shapes of bacterial colonies are summarized in Table 4.3. The majority of morphotypes appeared shiny, translucent and smooth on agar plates. Three morphotypes were shiny, opaque and smooth whereas two appeared dull, opaque and smooth. One of the morphotypes grew as dull, translucent, smooth colonies, while another type was dull, opaque and rough.

Among the shiny, translucent, smooth colonies were three that were different shades of purple, while two were tan-colored (Table 4.3).

Table 4.3 Characterization of bacteria in the Potchefstroom drinking water distribution system.

Identity of isolate	Method of ID	Habitat/Phase	Appearance of colonies on agar	Pigment	Gram reaction and sbape	(a) Type of hemolysis	(b) Antibiotic resistance pattern	(c) Sampling 1;March 2007; 4 months' growth	(d) Sampling 2; May 2007; 12 months' growth	Sampling 3;July 2007; 4 months' growth	Sampling 4;March 2008; 4 months' growth
Bacillus cereus	16S rDNA sequencing	Biofilm development device/biofilm	Irregular, flat, undulate, dull, opaque, rough	White	+ bacilli	α	**(D)** AP, E, NE, OT	√			
Bacillus subtilis	16S rDNA sequencing	Biofilm development device/planktonic	Spindle, flat, entire, dull, translucent, smooth	Tan	+ bacilli	γ	**(C)** AP, E, OT	√			√
Brevundimonas spp.	16S rDNA sequencing	Biofilm development device, bulk water/biofilm, planktonic	Punctiform, flat, entire, shiny, translucent, smooth	Tan	- bacilli	γ	**(F)** AP, C, E, K, OT				√
Clostridiaceae	16S rDNA sequencing	Filters/biofilm	Irregular, flat, undulate, shiny, translucent, smooth	Purple	- bacilli	γ	**(H)** C, E, K, NE, OT, VA		√		
Corynebacterium renale	BBL System	Biofilm development device/biofilm, planktonic	Punctiform and circular, convex, entire, shiny, translucent, smooth	Light purple	+ bacilli	γ	**(B)** K, OT			√	
Flavobacteriaceae	16S rDNA sequencing	Filters/biofilm	Circular, convex, erose, shiny, opaque, smooth	Fluorescent yellow	- bacilli	γ	**(G)** AP, C, E, K, NE, OT		√		

(a) α = alpha hemolysis; - = no hemolysis; (b) Letter in brackets is an arbitary one representing the dominant antibiotic resistance pattern of the species.

(c) √ indicates that these species were detected ; (d) This represents the sampling from the RO filters.

Identity of isolate	Method of ID	Habitat/Phase	Appearance of colonies on agar	Pigment	Gram reaction and shape	(a) Type of hemolysis	(b) Antibiotic resistance pattern	(c) Sampling 1;March 2007; 4 months' growth	(d) Sampling 2; May 2007; 12 months' growth	Sampling 3;July 2007; 4 months' growth	Sampling 4;March 2008; 4 months' growth
Kytococcus sedentarius	BBL System	Biofilm development device, bulk water/biofilm, planktonic	Punctiform, raised, undulate, shiny, translucent, smooth	Dark purple	+ cocci	α	(G) AP, C, E, K, NE, OT				√
Leuconostoc lactic	BBL System	Biofilm development device, bulk water/biofilm, planktonic	Punctiform, flat, entire, dull, opaque, smooth	Dark green	+ cocci	γ	(E) E, K, OT, VA				√
Lysinibacillus sphaericus	16S rDNA sequencing	Filters, biofilm development device, bulk water/biofilm, planktonic	Punctiform, flat, entire, shiny, opaque, smooth	Cream	+ bacilli	γ	(I) AP, C, E, K, NE, OT, VA		√		√
Pseudomonas spp.	16S rDNA sequencing	Biofilm development device, bulk water/biofilm, planktonic	Punctiform, raised, entire, shiny, translucent, smooth	Tan	- bacilli	γ	(G) AP, C, E, K, NE, OT				√
Staphylococcus aureus	16S rDNA sequencing	Filters/biofilm	Irregular, raised, entire, shiny, opaque, smooth	Cream	+ cocci	γ	(A) AP, E		√		
Staphylococcus capitis	BBL System	Biofilm development device, bulk water/biofilm, planktonic	Punctiform, flat, entire, dull, opaque, smooth	Dark green	+ cocci	α	(E) E, K, OT, VA				√

(a) α = alpha hemolysis; - = no hemolysis; (b) Letter in brackets is an arbritary one representing the dominant antibiotic resistance pattern of the species.

(c) √ indicates that these species were detected; (d) This represents the sampling from the RO filters.

Shiny, opaque, smooth colonies included two types with a creamy pigmentation, and one that appeared fluorescent yellow. The dull, opaque, smooth group consisted of dark green colonies. Dull, translucent, smooth colonies were tan-colored, while those with a dull, opaque, rough appearance were white. Purple colonies comprised Gram-positive cocci, Gram-positive- and Gram-negative bacilli. Tan-colored colonies included Gram-positive as well as Gram-negative bacilli (Table 4.3). Creamy-colored isolates were observed as Gram-positive cocci- and bacilli. The isolate with the fluorescent yellow pigmentation consisted of Gram-negative bacilli. Dark-green colonies were all Gram-positive bacilli while the white colonies consisted of Gram-positive bacilli (Table 4.3).

Isolates were identified by 16S rDNA sequencing (both Gram-positive and Gram-negative isolates) and four Gram-positive isolates were identified with the BBL Crystal Rapid Gram-Positive ID System. The BBL Crystal results sheets are presented in Annexure B. Chromatograms of the 16S rDNA sequences for selected isolates are given in Annexure C. Such sequence details were used to identify the isolates by means of ChromasPro software and BLASTN searches in GenBank (http://www.ncbi.nlm.nih.gov/BLAST/). Identities of isolates, the means by which they were identified as well as their origin (biofilm development device, filters or bulk water) and phases of existence (biofilm and/or planktonic/bulk) are given in Table 4.3. Figure 4.1 is an example of a PCR gel image demonstrating that the PCR fragments were of the expected sizes (between 500bp and 600bp). It is also evident from Figure 4.1 that there were no additional or non-specific bands present. The chromatograms in Annexure C of the amplicons also demonstrate that there was no sequencing background that could have interfered with the results. The BLASTN search results could thus be regarded as credible.

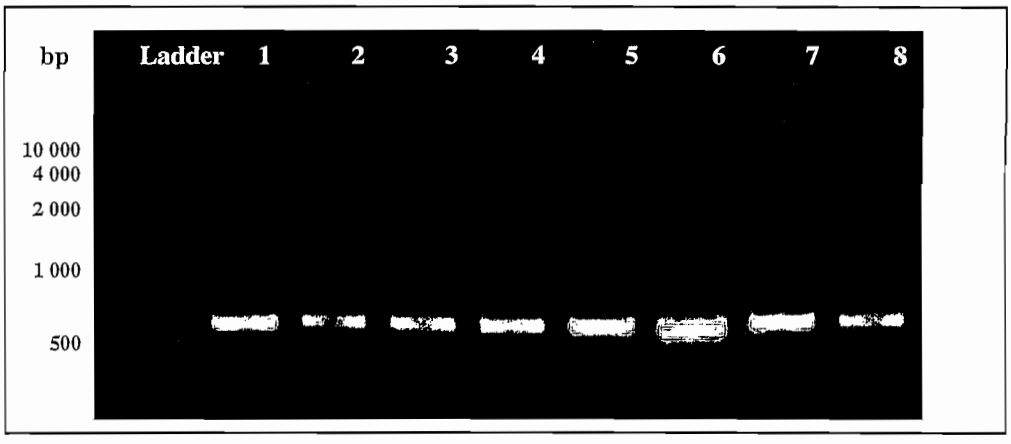

Figure 4.1 An ethidium bromide stained, agarose gel image (40ms exposure time) of PCR amplified 16S rDNA gene fragments for selected isolates. The agarose gel concentration was 1.5% (w/v), electrophoresed at 100V for 45min, using a High Range FastRuler 10 000bp DNA ladder (Fermentas Life Sciences, US). The electrophoresis buffer was 1 x TAE.

4.2.2.1 Biofilm isolates on red-copper coupons

During the first sampling period (biofilm device, March 2007) 2 species were isolated and identified. These were *Bacillus cereus* and *Bacillus subtilis*. *Bacillus subtilis* was isolated from the planktonic phase only whereas *Bacillus cereus* was only isolated from the biofilm. *Corynebacterium renale* was isolated in mid-Winter 2007 (July 2007). It was the only isolate that could be cultivated during this sampling period and occurred in both biofilm and planktonic phases of the biofilm device. More than half of the isolates occurred in early Autumn 2008 (Table 4.3). This period then shows the greatest diversity. The various species isolated were *Bacillus subtilis*, *Brevundimonas* spp., *Kytococcus sedentarius*, *Leuconostoc lactic*, *Lysinibacillus sphaericus*, *Staphylococcus aureus* and *Pseudomonas* spp. One of these isolates, *Lysinibacillus sphaericus*, was previously detected on the RO filter biofilm as well. With the exception of *Bacillus subtilis*, these species were isolated from all three compartments tested. *Bacillus subtilis* was only isolated from the planktonic phase. The same scenario was observed during the first sampling period.

4.2.2.2 Biofilm isolates on RO filters

A quarter of the morphotypes isolated in this study were from the RO filters that were analysed during May 2007. *Lysinibacillus sphaericus* was the only species isolated from the biofilm device (biofilm and planktonic) as well as bulk water. The other species isolated from the RO filter included *Clostridiaceae*, *Flavobacteriaceae* and *Staphylococcus aureus*.

4.3 CHARACTERIZATION OF ISOLATES

4.3.1 Characterization of isolates based on their pathogenic features (hemolytic activity) and antibiotic resistance patterns

Hemolysis types (α-hemolysis) and antibiotic resistance patterns (designated A-I) of isolates are presented in Table 4.3. Average zones of inhibition for isolates are given in Table D1, Annexure D. *Kytococcus sedentarius*, *Bacillus cereus* and *Staphylococcus capitis* were the only isolates that produced hemolysins on blood agar (thus potential pathogens).

Figure 4.2 is a graph that ranks the species from the least to the most multiple antibiotic resistant, based on the numbers/percentages (five representative colonies per isolate were tested for each antibiotic) that were resistant towards the respective antibiotics. Eight different antibiotic resistance patterns (A-I) were observed (Table 4.3). All isolates were resistant towards at least two antibiotics from different chemical classes and were thus considered multiple resistant. *Lysinibacillus sphaericus* demonstrated the greatest multiple resistance, while *Staphylococcus aureus* and *Corynebacterium renale* were least multiple resistant. *Flavobacteriaceae*, *Kytococcus sedentarius* and *Pseudomonas* spp. had the same antibiotic resistance pattern **G** (AP, C, E, K, NE, OT). *Staphylococcus capitis* and *Leuconostoc lactic* also shared the same pattern **E** (E, K, OT, VA). Results suggests that, although *Lysinibacillus sphaericus* was the isolate that demonstrated resistance towards the

greatest number of antibiotics it did not show any hemolytic activity on blood agar and can thus cannot be considered being the most pathogenic. Taking both the hemolytic activity (α-hemolysis) and antibiotic resistance pattern (resistance against six antiobiotics) of *Kytococcus sedentarius* into account, it seems that this bacterium was the one with the highest potential of causing disease. This was followed by *Staphylococcus capitis* and *Bacillus cereus*. These characteristics of the isolates are a cause for concern particularly if infection of immune compromised individuals of the community of Potchefstroom is considered.

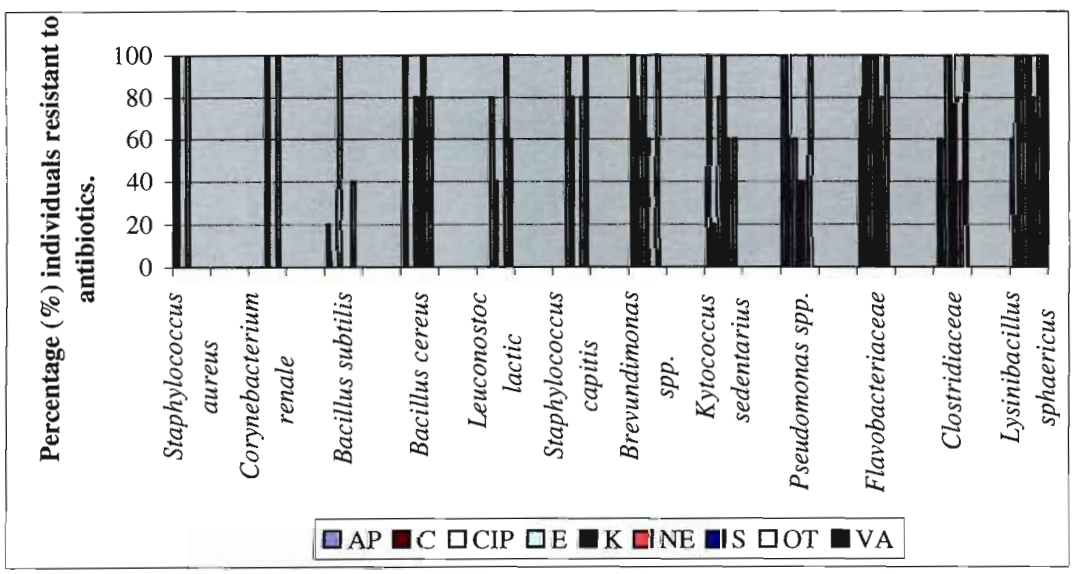

Figure 4.2 Percentage of isolates resistant to between two and nine antibiotics. In this study isolates resistant to more than one antibiotic were considered multi-resistant.

Table 4.4 ranks the tested antibiotics from most effective to least effective according to the numbers/percentages of colonies out of 60 (five representative colonies per isolate) that were resistant. This ordination is based on values from Figure 4.2. From the results, it is evident that ciprofloxacin (CIP) and streptomycin (S) were the most effective, since all the isolates were susceptible to both. In contrast, erythromycin (E) was the least effective. A total of 50 representatives (21.8%) were resistant to this agent. Results indicated CIP to be the most

effective since all isolates were susceptible to this antibiotic. Resistance to tetracyclines (OT) was also quite high (19.7%). Furthermore, 50% of Gram-positive isolates were resistant to vancomycin (VA). This is a further cause for concern. Based on the literature review and the results of this study, it can be concluded that the wider the resistance spectra of a specific nonpathogenic/potentially pathogenic/pathogenic bacterium (i.e., the more multiple-resistant the bacterium), the more difficult and expensive it will be to treat the specific illness caused by the bacterium. The adverse effects of antibiotic treatment on patients are another concern.

Table 4.4 Ranking of antibiotics from most effective to least effective according to the gross numbers/percentages of resistant representative colonies of isolates.

Chemical class	Antibiotic	Number of resistant colonies	Percentage of resistant colonies (%)
Quinolones	CIP	0	0
Aminoglycosides	S	0	0
Glycopeptides	VA	20	*50.0
Aminoglycosides	NE	22	9.6
Chloramphenicols	C	23	10.0
ß-Lactams	AP	33	14.4
Aminoglycosides	K	36	15.7
Tetracyclines	OT	45	19.7
Macrolides	E	50	21.8

* The percentage resistance for VA were calculated for Gram-positive isolates only; 20/40 Gram-positive isolates tested were resistant against VA.

4.3.2 TEM analysis of bacterial structure

4.3.2.1 *Pseudomonas* spp.

Figure 4.3 depicts a TEM micrograph (11 500X enlargement) of the negatively-stained potentially non-pathogenic *Pseudomonas* spp. that was isolated from the red-copper coupons, as well as planktonic and bulk water samples during early Autumn 2008. This image shows closely associated rod-shaped cells that have polar flagella. Flagella are important for attachment of planktonic *Pseudomonas aeruginosa* cells to abiotic surfaces during the initial stages of biofilm formation (Costerton *et al.*, 2002; also see Figure 2.1).

Figure 4.3 Transmission electron micrograph (magnification 11 500X) of *Pseudomonas* spp.

4.3.2.2 *Brevundimonas* spp.

A TEM micrograph (15 500X enlargement) of *Brevundimonas* spp. is shown in Figure 4.4. Inclusion bodies, possibly acting as a reserve of nutrients for bacteria, and slime threads are clearly visible. Slime formation is a well-known characteristic of biofilm bacteria. Slime helps bacteria to adhere to smooth surfaces, protects the bacteria from environmental stressors such as antibiotics and prevents loss of moisture. The presence of slime within water distribution networks can cause problems such as clogging of pipes and filters (Lazcano, 2008).

Figure 4.4 Transmission electron micrograph (magnification 15 500X) of *Brevundimonas* spp.

4.3.2.3 *Staphylococcus* spp.

The characteristic single, diploid, triad and tetrad arrangements of *Staphylococcus* spp. are illustrated in Figure 4.5. Pavlov *et al.* (2004) explained how these groupings of cocci allows for high numbers of bacteria to adhere to human cells. This contributes to increased health risks, especially in the immunosuppressed.

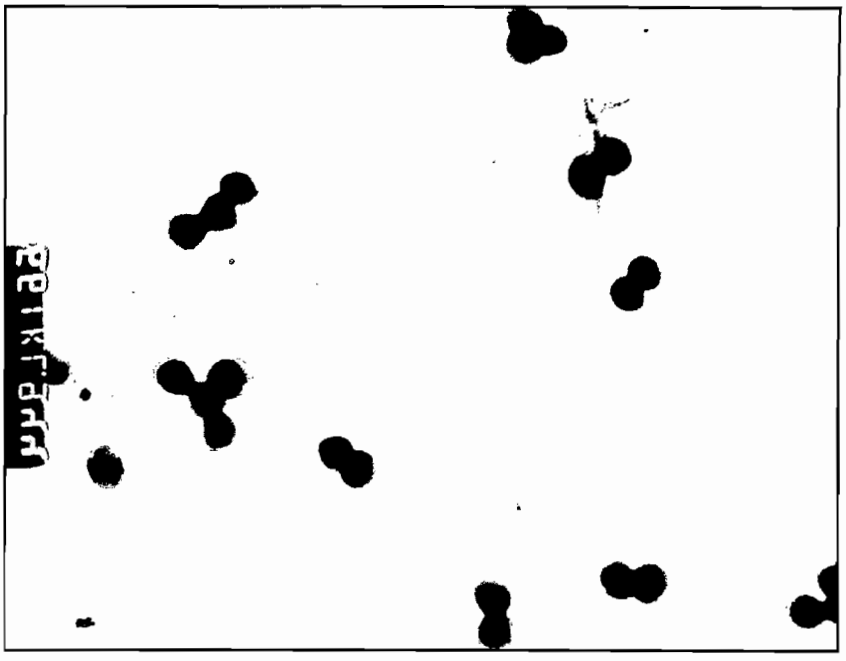

Figure 4.5 Negatively-stained TEM image (magnification 11 500X) showing different arrangements of *Staphylococcus* spp.

4.4 SEM ANALYSIS OF BIOFILM STRUCTURE

Figures 4.6 and 4.7 depicts two sections on the surfaces of two red-copper coupons after being exposed to bulk water at the J.S. van der Merwe building for 4 months. When these two coupons were removed from the biofilm development device, they had a dirty-green rust-like appearance. These corrosion products are visible in Figures 4.6 and 4.7 as crystal-like substances. Figure 4.6 also shows the presence of various diatoms (white arrows). The species observed during this particular study are associated with polluted waters (Taylor *et al.*, 2007).

Single-cell attachment of vibrios (red arrows) and rods (blue arrows) in association with corrosion products (green arrows), polymer-like substances (brown arrows) and EPS (yellow arrows) are visible in Figure 4.7. Figures 4.6, 4.7 and 4.8 show stages of biofilm formation. The surface seen in Figure 4.7, for instance, might act as a "conditioning" layer for bacteria,

whereas the single bacterial cells in Figure 4.8 are possibly primary and/or secondary colonizers.

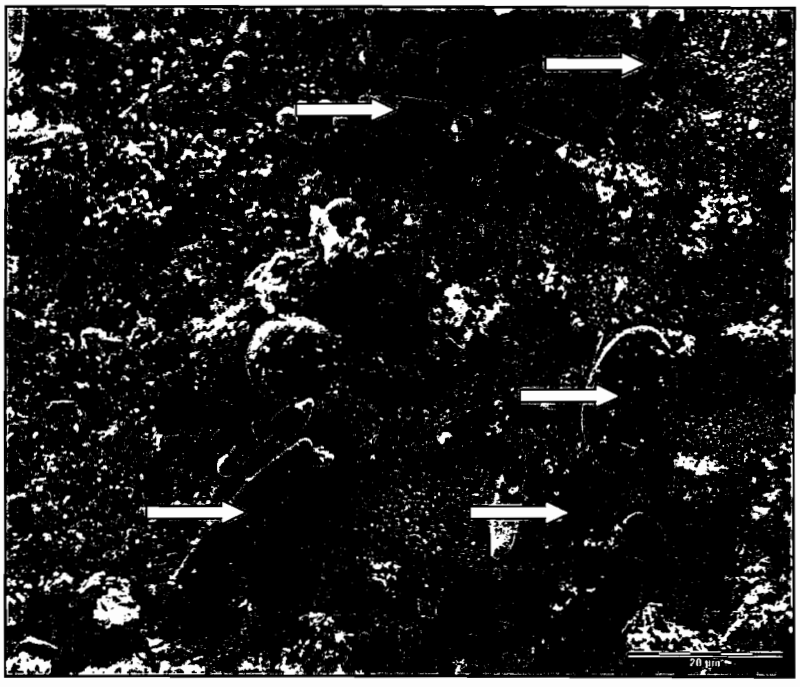

Figure 4.6 Scanning electron micrograph of the external surface (magnification 2 500X) of a red-copper coupon (coupon 1) from the biofilm development after 4 months of operation (sampling period 1).

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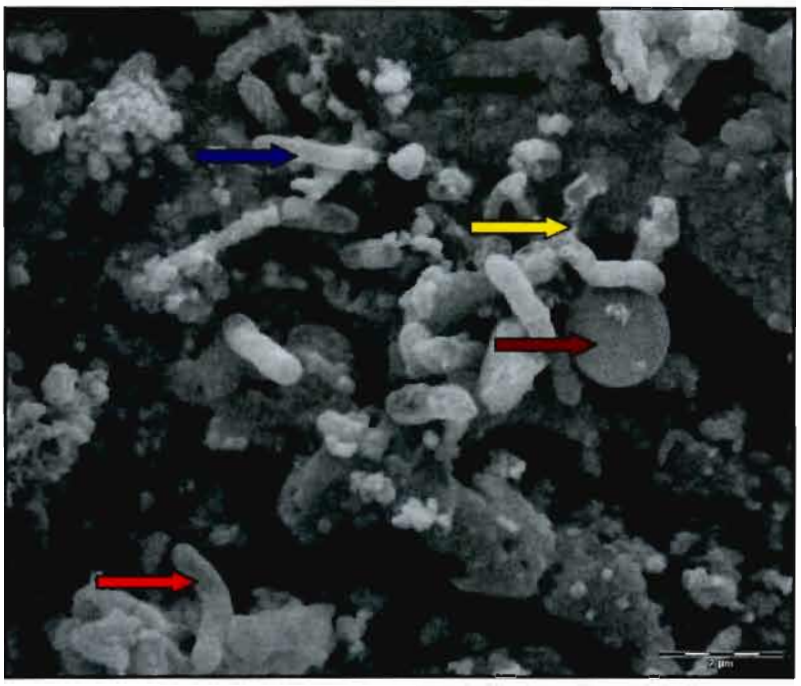

Figure 4.7 Scanning electron micrograph (magnification 20 000X) of the outer surface of coupon 2 after 4 months of exposure to bulk water (sampling period 1).

Figures 4.8 and 4.9 shows some evidence of biofilm formation (still at one of the earlier stages) on the surface of two copper coupons after 4 months of exposure (sampling period 4). An extensive "sponge-like" EPS (yellow arrows) layer can be seen in Figure 4.8. This is in association with a few bacterial cells (orange arrows). It is not impossible that a bacterial biofilm might be present underneath this EPS layer. The aggregation of rod-shaped bacteria (blue arrows) attached to the crystal-like surface (green arrows) (Figure 4.9) might be the beginning of the development of a true biofilm. Some of the bacilli present in Figures 4.7 - 4.9 might include *Bacillus cereus*, *Bacillus subtilis*, *Brevundimonas* spp., *Lysinibacillus sphaericus* and *Pseudomonas aeruginosa* listed in Table 4.3.

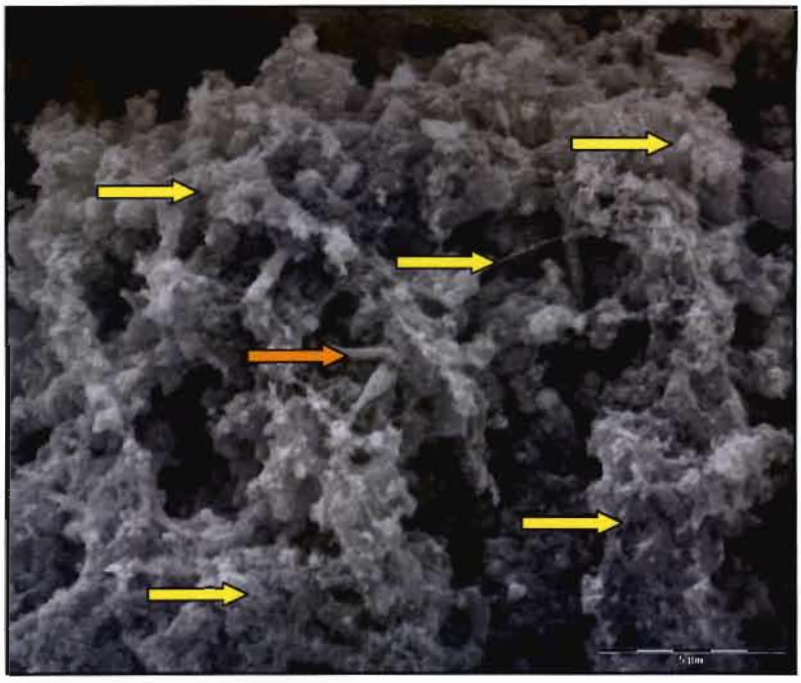

Figure 4.8 Scanning electron micrograph (magnification 12 000X) showing the outer surface of a copper coupon after 4 months of exposure to bulk water (sampling period 4).

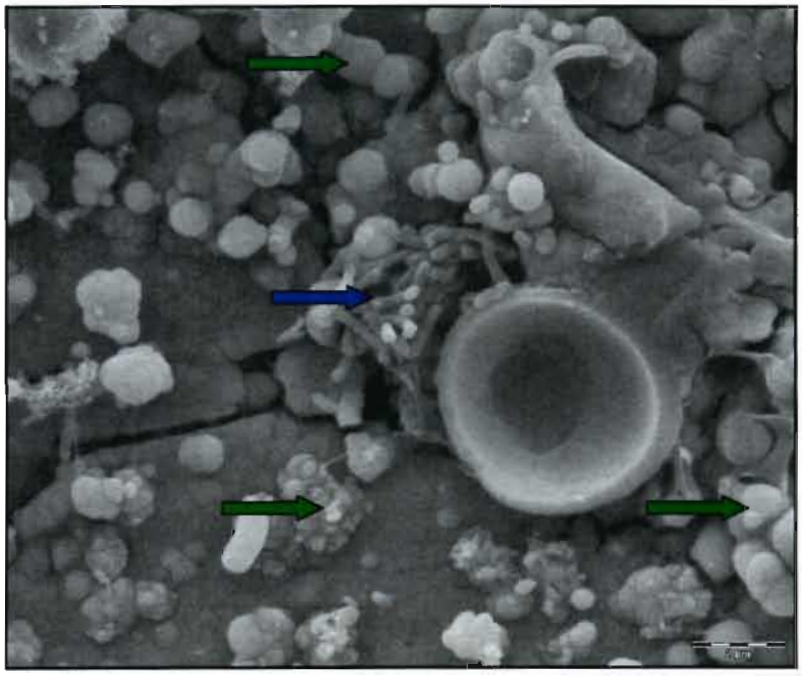

Figure 4.9 Scanning electron micrograph of the external surface of a coupon (magnification 15 000X) after 4 months of growth (sampling period 4).

Biofilm accumulation on one of the RO filters is illustrated in Figure 4.10. This is an example of a more "mature" biofilm. This biofilm was more than 12 months old. Various bacteria (orange arrows) entangled in thread-like EPS (yellow arrows) are clearly visible. The biofilm community on this filter might, amongst others, include *Lysinibacillus sphaericus*, *Flavobacteriaceae*, *Clostridiaceae* and *Staphylococcus aureus* listed in Table 4.3.

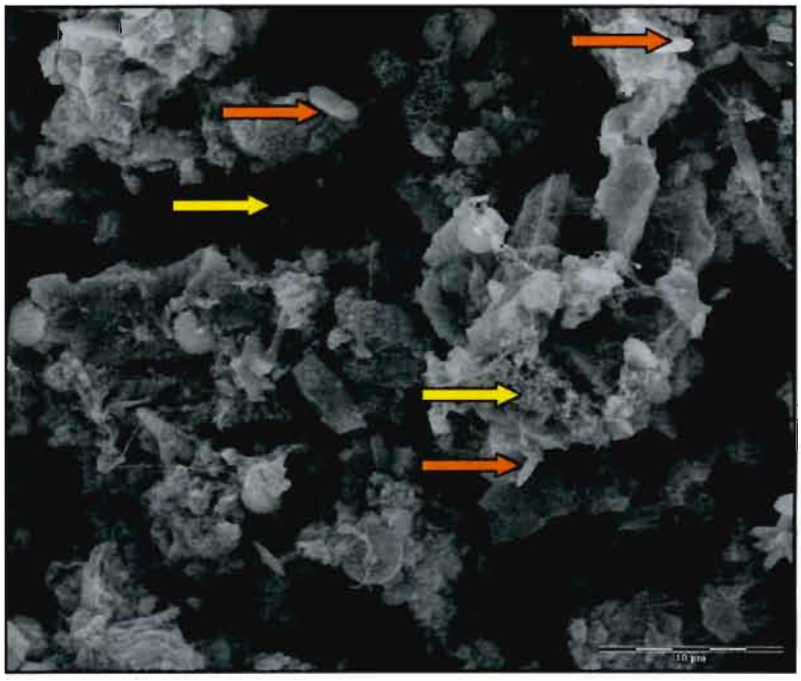

Figure 4.10 Scanning electron micrograph (magnification 6 000X) depicting the surface of one of the RO filters after 12 months of operation (sampling period 2).

The biofilm matrix of one of the red-copper coupons that were exposed to the bulk water over a 4-month time period (sampling period 3) is presented in Figures 4.11 and 4.12. Part of the matrix shown in Figure 4.11 comprised a variety of bacterial morphotypes (orange arrows), EPS (yellow arrows), filaments (purple arrows) and corrosion products (green arrows). Figure 4.12 illustrates a part of the matrix that consisted of an extensive EPS layer (yellow arrows) (similar to that of Figure 4.8), along with bacteria (orange arrows) and polymer-like substances (brown arrows) entrapped within this layer. The presence of crystals and other

corrosion products on this coupon made it difficult to detect coccoid bacteria. Cocci are generally very small and since crystals and other substances have a similar round or ovoid appearance as these bacteria, the cocci that might be present are "camouflaged". Bacteria visible (and hidden) on the surface of this coupon might include *Corynebacterium renale* (Table 4.3).

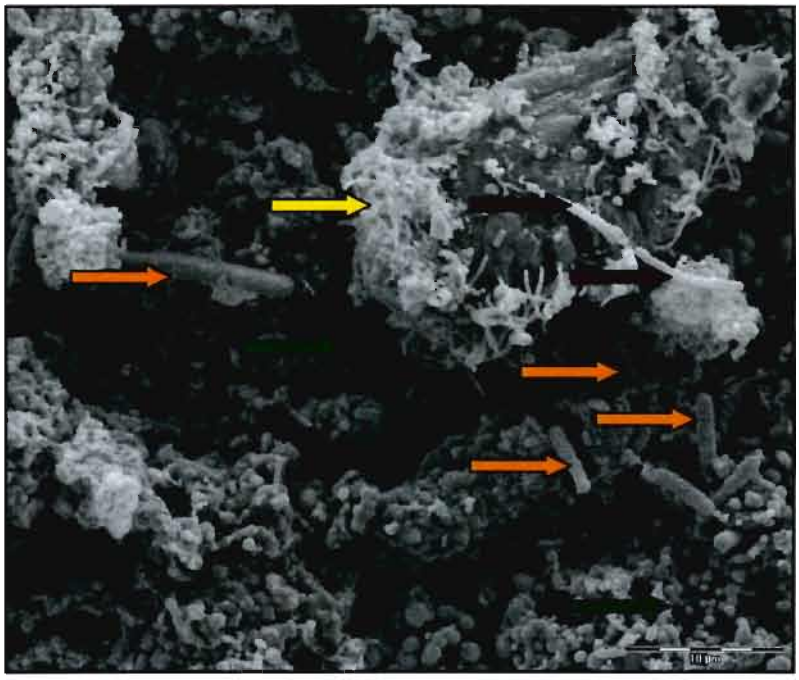

Figure 4.11 Scanning electron micrograph (magnification 5 000X) depicting a 4-month old biofilm on red-copper (sampling period 3).

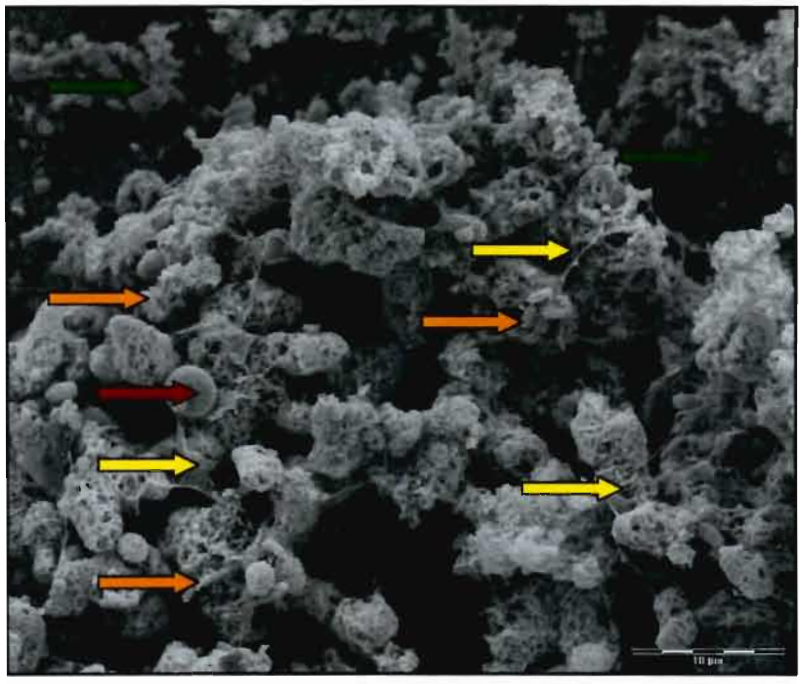

Figure 4.12 Scanning electron micrograph (magnification 5 000X) depicting a 4-month old biofilm on red-copper (sampling period 3). Extensive EPS (yellow arrows) is shown.

4.5 SUMMARY OF RESULTS

The bulk water at the J.S. van der Merwe building, based on physico-chemical characteristics and microbiological parameters, could be classified as "blue; class 0; ideal water quality" fit for lifetime consumption. However, 12 different HPC bacterial morphotypes were isolated from the two sampling points in the Potchefstroom drinking water distribution system. All isolates were pigmented. These were isolated using selective media. Eight isolates were Gram-positive and four were Gram-negative. Among the 12 morphotypes, eight were rod-shaped and four were coccoid. Isolates were identified by means of 16S rDNA sequencing and by the BBL Crystal identification system. The greatest diversity of isolates came from the biofilm development device and bulk water in late Summer/early Autumn 2008, while the lowest diversity occurred mid-Winter 2007. *Kytococcus sedentarius*, *Bacillus cereus* and *Staphylococcus capitis* were characterized as potential pathogens. *Kytococcus sedentarius* could be considered as potentially the most pathogenic. Eight different antibiotic resistance

patterns were observed. All isolates were resistant towards two or more antibiotics. *Staphylococcus aureus* and *Corynebacterium renale* were least multiple antibiotic resistant and *Lysinibacillus sphaericus* the most multiple antibiotic resistant. All isolates were susceptible to ciprofloxacin (CIP) and streptomycin (S). Most isolates were resistant to erythromycin (E). Transmission electron microscopy allowed for more detailed examination of *Pseudomonas* spp., *Brevundimonas* spp., *Staphylococcus aureus* and bacterial endospores. Scanning electron microscopy revealed the presence of both immature and mature biofilms on red-copper and RO filters. At low magnification, diatoms were also observed.

CHAPTER 5

DISCUSSION

5.1 PHYSICO-CHEMICAL ANALYSIS OF BULK WATER

The pH (ranging from 7.5-7.8) and TDS (ranging from 245mg/L-268mg/L) of bulk water at the J.S. van der Merwe building during June-August 2007 complied with South African standards as set out in the guidelines for domestic water (Manyaka and Pietersen, 1998; SANS 241: 2006). The tap water could be classified as "blue; class 0; ideal water quality" fit for lifetime consumption. These guidelines specify a pH value of 6-9 and a TDS value of <450mg/L for water to be classified as "blue-ideal" for drinking, food preparation, bathing and doing laundry. Vos (2007) previously monitored pH and TDS at the J.S. van der Merwe building and found that the pH ranged from 8.06-8.31, while TDS ranged from 641mg/L - 764mg/L (a concentration of almost twice as much as that of the present study). Based upon pH values monitored by Vos (2007), the tap water could previously also be classified as "Blue-Ideal" for all household purposes. TDS values obtained by Vos (2007) showed the tap water to be "Green-Good" for drinking and food preparation, and "Blue-Ideal" for bathing and laundry.

The pH (ranging from 7.2-8.0), TDS (ranging from 231mg/L-321mg/L), Cl_2 (ranging from 0.012mg/L-0.047mg/L), TH (ranging from 12mg/L-20mg/L) and CH (ranging from 9 mg/L - 16mg/L) that were monitored at the J.S. van der Merwe building over 12 weeks during February-April 2008 were within the recommended limits as set out in the guidelines by Manyaka and Pietersen (2008) and SANS 241 (2006). The tap water was classified as "Blue-Ideal" for all domestic purposes. Free Cl_2 concentrations were very low (0.012mg/L - 0.047mg/L). Experiments by McFeters and Singh (1991) revealed that disinfection with chlorine at concentrations of 0.25-0.5mg/L for 10-30min were required to cause 90% injury

to coliforms. Levels of 0.9-1.5mg/L were needed to injure the same percentage of pathogens. Barnick *et al.* (1989) state that municipal water supplies are easily disinfected with Cl_2 of 0.3 -3.0mg/L to control bacterial growth within distribution systems. According to the *WHO* (2006) chlorine, in most disinfected drinking water distribution systems, should be at concentrations of 0.2mg/L-1.0mg/L. Manyka and Pietersen (1998) recommend that Cl_2 residual must be 0.2mg/L-0.6mg/L for adequate disinfection. Any concentration below this could mean that disinfection is compromised. This increases the chance of bacterial regrowth and biofilm formation within the distribution system. The very low Cl_2 residual that was observed in the present study may indicate that the bulk water or piping had a high oxidant demand due to bacterial growth (WHO, 2006). A possible explanation for the very low residual Cl_2 concentrations at point-of-use might be that Cl_2 was scavanged by the biofilm as well as the pipe material of the distribution system. The latter phenomenon was observed by Lehtola *et al.* (2005). They (Lehtola *et al.*, 2005) found that Cl_2 concentrations decreased much faster in copper pipes than plastic pipes, because Cl_2 reacts with copper.

In a South African study conducted by Brouckaert *et al.* (2006), two major factors that lead to high bacterial numbers in drinking-water distribution systems were inefficient chemical (coagulant and chlorine) dosing and high turbidity. This in turn caused low residual chlorine in the systems. These investigators observed chlorine residuals of 0.07mg/L-3.16mg/L and 0.01mg/L-1.15mg/L at point of treatment and distribution systems, respectively, in 55 plants across the Eastern Cape Province.

5.2 MICROBIAL ANALYSIS

5.2.1 Isolation and identification of bacteria

The various enrichment broths and selective media (see Table 3.1) allowed for the isolation of 12 types of pigmented HPC bacteria from red-copper biofilms, RO filter biofilms, planktonic (bulk water) samples from the Potchefstroom drinking water distribution system. Cream, white, tan, fluorescent-yellow, green and purple colonies were cultivated. Barnick *et al.* (1989) investigated the presence of pigmented bacteria in a municipal drinking water system and isolated high numbers heterotrophic yellow, orange and pink colonies. They (Barnick *et al.*, 1989) were of the opinion that regular monitoring of certain groups of pigmented bacteria throughout the year could allow for additional tracking of water quality changes and characterization of the distribution system. These investigators furthermore argued that the occurrence of high levels of pigmented bacteria within distribution systems could pose increased health risks to immunocompromised individuals.

Having knowledge of which organisms are capable of surviving in distribution systems and understanding their growth needs will help operators and managers to control biofilm organisms. Very few bacteria inhabiting distribution system biofilms are harmful to the average consumer. Only opportunistic pathogens are of concern with special reference to the immunocompromised within a population (Pavlov *et al.*, 2004; WHO, 2006).

Twelve different bacteria were successfully isolated from the biofilm development device, filters and bulk water from the two sampling points in the Potchefstroom drinking water distribution system. Four of the Gram-positive isolates were identified by means of the BBL Crystal Rapid Gram-Positive Identification System as *Corynebacterium renale*, *Kytococcus sedentarius*, *Leuconostoc lactic* and *Staphylococcus capitis*. The remaining eight isolates

were identified by 16S rDNA sequencing as *Bacillus cereus*, *Bacillus subtilis*, *Brevundimonas* spp., *Clostridiaceae*, *Flavobacteriaceae*, *Lysinibacillus sphaericus*, *Staphylococcus aureus* and *Pseudomonas* spp. The relevance of these isolates with respect to health risks and operational conditions of drinking water distribution systems are discussed in section 5.2.2.

5.2.2 Characteristics and diversity of isolates

5.2.2.1 *Bacillus cereus*

This bacterium was isolated from the biofilm development device as part of the biofilm phase during the first sampling period (early Autumn, March 2007). Colonies were white, irregular, flat, undulate, dull, opaque and rough on R2A agar. Gram stains indicated the Gram-positive, rod-shaped cells of this microbe (Ryan and Ray, 2004). Furthermore, this microorganism could be characterized as potentially pathogenic, according to its α-hemolytic behavior on blood agar. This finding coincides with that of Ryan and Ray (2004) who demonstrated that *Bacillus cereus* causes foodborne illness. These authors however characterize the bacterium as β-hemolytic, which is a contradictory observation to the present study. *Bacillus cereus* isolated from Potchefstroom was resistant to four antibiotics [resistance pattern **D** (AP, E, NE, OT)]. Beuchat and Ryu (2005) investigated biofilm formation by *Bacillus cereus* on stainless steel coupons, sporulation in the biofilm and resistance of vegetative cells and spores in the biofilm. They found that the total counts in biofilm formed on coupons that were immersed in tryptic soy broth at 12°C and 22°C consisted of 99.94% vegetative cells and 0.06% spores. The capability of *Bacillus cereus* to produce endospores (Ryan and Ray, 2004) might be one of the factors contributing to its survival in the distribution system.

Beuchat and Ryu (2005) found that treatment of biofilm on coupons that were immersed in tryptic soy broth at 22°C with 50µg/mL chlorine for 5 minutes decreased total cells counts (vegetative cells and spores) by 4.7 log CFU per coupon. This could mean that *Bacillus cereus* could be controlled within the distribution system if sufficiently high chlorine levels are maintained. However, the Cl_2 levels in the Potchefstroom distribution system were extremely low. This may explain why *B. cereus* was isolated from this system.

5.2.2.2 *Bacillus subtilis*

Bacillus subtilis was isolated from the biofilm development device as part of the planktonic phase in early Autumn, 2007 (sampling 1), early Autumn, 2008 (sampling 4) as well as the bulk water. In the present study, colonies appeared tanned, spindle, flat, entire, dull, translucent and smooth on R2A media. Colonies were Gram-positive. Furthermore, the representative from this study was non-pathogenic as determined by its non-hemolytic nature and was resistant towards only three antibiotics – AP, E and OT (pattern **C**). Babcock *et al.* (1987) isolated *Bacillus* spp. as part of the HPC biofilm bacteria from distribution pipe surfaces, while Kim and Lee (2003) found *Bacillus* spp. to persist in biofilm communities in the Seoul (Korea) water distribution system.

5.2.2.3 *Brevundimonas* spp.

Brevundimonas spp. formed part of the biofilm and planktonic communities in the biofilm development device, as well as the bulk water during the fourth sampling period (March 2008). Colonies were tan-colored, punctiform, flat, entire, shiny, translucent, and smooth on TSA. Representative individual cells were Gram-negative rod-shaped cells (Harley and Prescott, 2002). Isolates seemed to be non-pathogenic, with an antibiotic resistance pattern of (**F**) AP, C, E, K, OT. Albrechtsen *et al.* (2005) managed to isolate *Brevundimonas* spp. from

the bulk water of a non-chlorinated model drinking water distribution system. Dutkiewicz *et al.* (2008) isolated *Brevundimonas vesicularis* and *Brevundimonas diminuta* from tap water samples of six hospitals in Lublin, Poland by using tryptic soy agar. On the other hand, Domingo *et al.* (2004) revealed the presence of *Brevundimonas* spp. in a chlorinated water works distribution system of greater Cincinatti. The present study was conducted in the chlorinated distribution system of Potchefstroom, and the isolation of *Brevundimonas* spp. is consistent with the studies of Dutkiewicz *et al.* (2008) and Domingo *et al.* (2004).

The transmission electron micrograph (Figure 4.4) of *Brevundimonas* spp. depicted a group of rods surrounded by slime threads. Various inclusion bodies (Oliver, 2000) could be observed within the cytoplasm of these cells. According to Salle (2007) slime-formation benefits bacteria by helping them adhere to surfaces and to neighboring cells. Slime (Oliver, 2000) also protects the bacterium from chemical substances such as antibiotics and prevents loss of moisture or dessication. Although slime is beneficial to bacteria, its accumulation within distribution systems can have many adverse effects such as clogging of pipes and obstructing water flow. Inclusion bodies are thought to act as nutrient reservoirs for bacteria during times when nutrients are scarce (Salle, 2007).

5.2.2.4 *Clostridiaceae*

Clostridiaceae was present on the RO filter biofilm during the second sampling (late Autumn, May 2007). It was not possible to classify *Clostridiaceae* up to a species level in this study. Franco and Payment (1993) and Ashbolt *et al.* (2001) explained that the occurrence of *Clostridium perfringens* in drinking water may be indicative of intermittent faecal contamination. Chalmers *et al.* (2007) detected *Clostridium perfringens* in treated

water of Galway City water supply, with a significant high level of 54 per 100mL being recorded. This high level was associated with disease outbreaks.

Colonies isolated in the present study were purple, punctiform, convex, entire, shiny, translucent and smooth on EMB agar. Isolates were non-hemolytic (i.e., non-pathogenic) on blood agar and thus dissimilar to the disease causing *Clostridium perfringens* strain(s) detected by Chalmers *et al.* (2007) in the Galway City water supply. This species was resistant towards C, E, K, NE, OT and VA (resistance pattern **H**). Since the genus *Clostridium* is known to produce endospores (Barrow and Feltham, 1993), these highly resistant structures might be one of the main reasons for this isolate being able to survive in a chlorinated distribution system.

5.2.2.5 *Corynebacterium renale*

BBL Crystal results revealed a 93% chance of *Corynebacterium renale* occurring as the sole isolate in mid-Winter 2007. This isolate came from the biofilm development device as part of both the biofilm and planktonic phase.

Critchley *et al.* (2003) isolated various heterotrophic bacteria, including *Corynebacterium* spp., from copper plumbing biofilms and tested their potential for cuprosolvency. Statistical evidence indicated that biofilms of *Corynebacterium* spp. significantly increased copper release by coupons in Glover's Hill, summit filtered water and summit chlorinated water. The study also demonstrated the impact of water chemistry to influence cuprosolvency by biofilms and its contribution to corrosion problems within water distribution systems.

Colonies were light purple on EMB agar, punctiform and circular, convex, entire, shiny, translucent, and smooth. Gram staining indicated Gram-positive bacilli. Barlough *et al.*, (1988) describe *Corynebacterium renale* as a relatively large Gram-positive short, stumpy bacillus, thicker at one end. According to Allen *et al.* (2006) Gram-stained cultures tend to aggregate, forming characteristic "Chinese letter" arrangements. The bacteria can exist as single cells or form biofilm colonies (Olivieri *et al.*, 1985; Lehman *et al.*, 2007). Transmission electron micrographs in this study, at magnifications of 21 000X and 28 500X, did reveal bacterial cells possessing these distinctive characteristics and could possibly be *Corynebacterium*. These micrographs are however not shown in this document, since the true identities of the bacteria could not be confirmed. This bacterium could furthermore be characterized as non-pathogenic, and with a resistance pattern of **B** (K, OT), was one of two isolates that were the least resistant against the various antibiotics tested. According to Gerba *et al.* (1997), Corynebacteria are normally non-pathogenic with the exception of *C. diphtheriae*.

5.2.2.6 *Flavobacteriaceae*

Fluorescent yellow colonies from RO filter biofilm samples (Autumn 2007) were identified as *Flavobacteriaceae*. In another South African study carried out in the Eastern Cape Province, Muyima *et al.* (2004) isolated *Flavobacterium oryzihabitans* from drinking-water in the Nkonkobe rural area in Autumn 2002. According to Olson *et al.* (1985) and Franzblau *et al.* (1985), *Flavobacteriaceae* are chromogenic bacteria often detected in water supplies and isolated on agar plates as bright yellow colonies. Critchley *et al.* (2003) isolated *Flavobacterium* spp. as part of the HPC spectrum of bacteria from copper plumbing biofilms. Colonies of this isolate from the present study had a fluorescent yellow, circular, convex, erose, shiny, opaque and smooth appearance on King's B agar. The cells were Gram-

negative bacilli. Boone and Castenholz (2001) classify *Flavobacteriaceae* as Gram-negative and rod-shaped. Isolates from the present study were non-pathogenic (Coffin *et al.*, 1994) also isolated non-pathogenic *Flavobacterium* spp. from chlorinated tap water in Montreal) and shared an antibiotic resistance pattern with *Kytococcus sedentarius* and *Pseudomonas aeruginosa* – **G** (AP, C, E, K, NE, OT). Gerba *et al.* (1997) determined health risks associated with pigmented bacteria present in drinking water. According to them (Gerba *et al.*, 1997) *Flavobacterium* is an opportunistic pathogen. Furthermore, *Flavobacterium meningosepticum* is the clinically important member of *Flavobacteriaceae* and causes meningitis.

5.2.2.7 *Kytococcus sedentarius*

BBL Crystal identification indicated the presence of *Kytococcus sedentarius* (99%) in the tap water, as well as in biofilm and planktonic samples during the fourth sampling period. Colonies were dark purple, punctiform, raised, undulate, shiny, translucent and smooth on EMB agar. Bacterial cells were Gram-positive cocci. Gurcharan *et al.* (2005) describe *Kytococcus sedentarius* as a Gram-positive bacterium that can be cultivated on tryptase-soy agar. The isolate in the present study was resistant against the same antibiotics as *Flavobacteriaceae* and *Pseudomonas aeruginosa*. Since this bacterium was α-hemolytic and resistant to six out of nine antibiotics, it seems to be of the biggest health concern in comparison to the other isolates. Gurcharan *et al.* (2005) demonstrated that their isolates were β-hemolytic on horse blood agar. According to Cappiello *et al.* (1987), *Kytococcus sedentarius* is one of the pathogenic organisms that cause pitted keratolysis (a skin infection). Michel and Ramsay (1996) prescribe daily application of erythromycin solution or gel as a treatment option for pitted keratolysis. However, the *Kytococcus sedentarius* strain isolated in this study was α-haemolytic and resistant to several antibiotics including erythromycin.

This implies that if individuals from Potchefstroom are diagnosed with pitted keratolysis, treatment with erythromycin will be futile. Such information must be brought to the attention of the medical and veterinary fraternity.

5.2.2.8 *Leuconostoc lactic*

Leuconostoc lactic (96%) were present in the bulk water, biofilm and planktonic samples during the last sampling period (March 2008, after 4 months' growth). This was surprising, since none of the literature cited could provide any examples of *Leuconostoc* spp. being isolated from drinking water. The colonies from this isolate appeared dark green, punctiform, flat, entire, dull, opaque and smooth on *Aeromonas* isolation agar. These cells were Gram-positive cocci. Garvie (1984) demonstrated that *Leuconostoc lactic* spp. is Gram-positive and belongs to the family *Streptococcaceae*. Although the representative isolated in the present study did not show any haemolytic features it was resistant against the same four antibiotics (E, K, OT and VA) as *Staphylococcus capitis*. In contrast to the present study, Chatsuwan *et al.* (2007) observed α- and β hemolysis of *Leuconostoc* spp. from clinical isolates. Studies by Acar *et al.* (1985) and Orberg and Sandine (1984) showed that vancomycin resistance is a common trait in *Leuconostoc* spp. Results by these investigators further indicated that *Leuconostoc* spp. were susceptible to AP, E, K, S, and T, a finding that differs from results obtained in the present study. Observations by Acar *et al.* (1985) strongly suggest that *Leuconostoc* spp. may be opportunistic pathogens, causing severe infections in compromised individuals. In case studies by Ling (1992), *Leuconostoc* spp. were isolated from blood samples of 19 patients in Singapore General Hospital. These isolates were all susceptible to ampicillin and penicillin, but resistant to vancomycin. *Leuconostoc* spp. were isolated as pure cultures from the blood specimens of 11 patients. Blood samples from the remaining eight patients contained *Leuconostoc* spp. along with other bacteria. Five patients recovered

without any antibiotic treatment, 13 were successfully treated and one patient died within 2 hours of submission. This patient was immunosuppressed due to glioma and bronchopneumonia.

5.2.2.9 *Lysinibacillus sphaericus*

Lysinibacillus sphaericus was isolated from the RO filters, biofilm development device and bulk water during sampling 2 and sampling 4. Brinkman *et al.* (2003) and Ahmed *et al.* (2007) mentioned the isolation of *Lysinibacillus sphaericus* from a mosquito breeding site in China in 1987. Some strains of *Lysinibacillus sphaericus* are pathogenic/toxic for mosquito larvae and are extensively used as a biological control agent (Brinkman *et al.*, 2003; Ahmed *et al.*, 2007). No examples from literature could be found where *Lysinibacillus sphaericus* was isolated from drinking water. Colonies from the bacterium in the present study were cream-colored, punctiform, flat, entire, shiny, opaque and smooth on King's B agar and cells were Gram-positive bacilli. The genus *Lysinibacillus* consists of Gram-positive, spore-forming rods (Euzéby, 2007). The isolate from the present study was non-haemolytic, but was resistant to seven of the nine antibiotics (AP, C, E, K, NE, OT, VA). Somewhat contradictory to the results of the present study, the five strains of *Lysinibacillus* spp. studied by Balloy *et al.* (2002) were susceptible to most antibiotics that they were tested against including AP and T. Coffin *et al.* (1994) investigated virulence factors (cytotoxicity, hemolysis, cell adherence, cell invasiveness) from heterotrophic bacteria, including cytolytic *Lysinibacillus sphaericus*, from chlorinated tap water samples in Montreal. Their results indicated that this bacterium was γ-hemolytic, non-adherent and non-invasive to human Caco-2 cells. One would thus not expect any health risks to consumers who happen to drink water that contains *Lysinibacillus sphaericus*.

5.2.2.10 *Pseudomonas* spp.

Pseudomonas spp. was detected in the tap water, biofilm on copper coupons and planktonic samples from the biofilm device, during the last sampling period (March 2008). In another South African study, Muyima *et al.* (2004) isolated *Pseudomonas aeruginosa* from rural drinking water supplies in the Eastern Cape during all four seasons. Brouckaert *et al.* (2006) isolated *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* and *Pseudomonas putida* from water samples from municipal water treatment plants located in the Eastern Cape Province, South Africa. Albrechtsen *et al.* (2005) isolated *Pseudomonas* spp. from both biofilm and bulk water samples from a nonchlorinated model drinking water distribution system located in Denmark. These authors consider *Pseudomonas* as the most abundant bacterium in water supply networks, regardless of water source. Critchley *et al.* (2003) detected *Pseudomonas* spp. in copper plumbing biofilms. In the present study, colonies that grew on King's B agar were tan-colored, punctiform, raised, entire, shiny, translucent and smooth. Gram stained cultures were Gram-negative rods. Bajeva (2006) characterized the genus *Pseudomonas* as Gram-negative, non-sporulating rods that are motile by polar flagella. *Pseudomonas* spp. isolated during this study did not demonstrate hemolytic activity. However, it was resistant to six antibiotics (AP, C, E, K, NE and OT). This antibiotic resistance pattern was similar to that of *Flavobacteriaceae* and *Kytococcus sedentarius* – **G** (AP, C, E, K, NE, OT). Brözel *et al.* (2007) identified non-pathogenic *Pseudomonas* spp. from biofilm samples from a South African drinking-water distribution system. According to Gerba *et al.* (1997), *Pseudomonas aeruginosa* is an opportunistic pathogen responsible for nosocomial infections. Ingestion of 10^6 - 10^8 cfu leads to colonization of the gastrointestinal tract but do not cause death in healthy persons. Amadi *et al.* (2009) demonstrated that *Pseudomonas* spp. may be resistant to multiple antibiotics and can cause infections in persons with open wounds or sores. One

common health risk is eye infections caused by contact lens users when lenses become contaminated with *Pseudomonas aeruginosa* (Cannon *et al.*, 2000).

A negatively stained pure culture of *Pseudomonas* spp. (Figure 4.3) viewed under TEM illustrated the presence of rods with polar monotrichous flagellation. This observation fits the description given for flagellation types in *Pseudomonas* spp. (Leifson, 1960). According to this author (Leifson, 1960) motile strains of *Pseudomonas* possess either polar monotrichous or polar multitrichous flagella. *Pseudomonas aeruginosa* is a well-known example of a bacterium with a single polar flagellum (Madison and Martinko, 2006). Costerton *et al.* (2002) explained that the importance of flagella-mediated motility in planktonic cells of *Pseudomonas aeruginosa* is for adhering to surfaces during biofilm development. Stanley (1983) demonstrated a 90% decrease in the attachment of *Pseudomonas aeruginosa* cells after removal of the flagella. However, the *Pseudomonas* spp. isolated in this study did not show any of the features of *Pseudomonas aeruginosa*.

5.2.2.11 *Staphylococcus aureus*

Staphylococcus aureus was isolated from the RO filters. Faria *et al.* (2009) isolated coagulase negative *Staphylococcus aureus* from a drinking water distribution network and a wastewater treatment plant located in Portugal. Colonies from the present study that grew on R2A appeared cream-colored. Todar (2002) differs by saying that colonies *Staphylococcus aureus* are yellow-pigmented, irregular, raised, entire, shiny, opaque and smooth. Gram staining indicated coccoid cells that were Gram-positive. *Staphylococcus* spp. comprises Gram-positive spherical bacteria occurring in grape-like clusters and should always be considered a potential pathogen (Todar, 2002). According to Irlinger (2008) some coagulase negative staphylococci may be opportunistic pathogens. Bradshaw (1992) describes the β-

hemolysin of *Staphylococcus areus* as potently toxic, contributing to the pathogenicity of the species. By demonstrating resistance against only two antibiotics – AP and E (pattern **A**) – this bacterium was among the isolates that were resistant to the least number of antibiotics. According to Todar (2002), hospital strains of *S. aureus* are usually resistant to various antibiotics. A few strains are resistant against all clinically useful antibiotics, including vancomycin. Although methicillin was not one of the antibiotics used in this study, it is worth mentioning the occurrence of Methicillin Resistant *Staphylococcus aureus* (MRSA). Methicillin Resistant *Staphylococcus aureus* (MRSA) is a major concern in the medical field, and much research still has to be undertaken to find ways to control this so-called "superbug". Dusé *et al.* (2007) undertook a study to explain the molecular epidemiology of MRSA in South Africa. Methicillin resistant *Staphylococcus aureus* isolates were collected from clinical laboratories from each of the nine provinces in the country and standard microbiological techniques were employed to confirm the presence of *S. aureus* strains and their resistance against methicillin. Of the 342 *S. aureus* isolates, 314 (91,8%) were resistant towards methicillin. Faria *et al.* (2009) concluded that the presence of antibiotic resistant coagulase negative staphylococci in drinking water distribution systems could be hazardous under conditions favouring their overgrowth, for instance biofilm formation.

5.2.2.12 *Staphylococcus capitis*

Staphylococcus capitis was isolated from tap water, biofilm as well as planktonic samples in early Autumn 2008. Faria *et al.* (2009) isolated coagulase negative *Staphylococcus capitis* from a drinking water distribution network in Portugal. Hassenpflug (2008) isolated *Staphylococcus capitis* from bodies of water from two Lynchburg (Virginia) parks. This author (Hassenpflug, 2008) showed organisms that grew on TSA as smooth, colorless colonies, while those colonies on EMB varied in pigmentation from maroon to fuchsia.

Colonies from the present study isolated on *Aeromonas* isolation agar were dark green, punctiform, flat, entire, dull, opaque and smooth. Cells were Gram-positive cocci. This species could be characterized as potentially pathogenic due to its α-hemolytic properties on blood agar. The bacterium was resistant to the same antibiotics as *Leuconostoc lactic*, namely E, K, OT and VA (resistance pattern **E**). According to Faria *et al.* (2009), the presence of coagulase negative *Staphylococcus* spp. (including *Staphylococcus capitis*) in drinking water under conditions causing their overgrowth, e.g. biofilm formation, could be a health concern to consumers.

The various cellular arrangements of *Staphylococcus* spp. are displayed in Figure 4.5. As described by Csuros and Csuros (1999) and Bajeva (2006), the characteristic single, diploid, triad and tetrad configurations of this genus could be observed. This species forms clusters, which enables it to attach to human cells in high numbers, possibly contributing to disease.

5.3 IMPLICATIONS AND HEALTH RISKS OF IDENTIFIED BACTERIA WITH RELEVANCE TO SOUTH AFRICA

Of the 12 genera that were isolated in the present study, five (*Bacillus*, *Corynebacterium*, *Flavobacteriaceae*, *Pseudomonas* and *Staphylococcus*) were previously detected within South African chlorinated drinking water (Muyima *et al.*, 2004; Pavlov *et al.*, 2004; Brouckaert *et al.*, 2006; Brözel *et al.*, 2007). Potentially pathogenic features of these HPC isolates that were analysed by Pavlov *et al.* (2004) included haemolysis, adherence to human cells, invasiveness of human cells, as well as production of enzymes responsible for bacterial virulence.

According to Pavlov *et al.* (2004), Gram-negative isolates that displayed haemolysis on human- and horse-blood agar included *Pseudomonas* spp., while Gram-positive isolates included *Staphylococcus* spp. and *Corynebacterium* spp. *Pseudomonas* spp. isolated by Brözel *et al.* (2007) were non-pathogenic. In the present study, *Bacillus cereus* and *Staphylococcus capitis* were hemolytic on sheep-blood agar, while *Pseudomonas* spp. and *Corynebacterium renale* were non-hemolytic.

Pavlov *et al.* (2004) analysed adherence properties of bacterial species to HEP-2 and Caco-2 HTB-37 (human colorectal adenocarcinoma) cells. These investigators found that Gram-negative isolates such as *Pseudomonas* attached to cells in larger numbers than Gram positives (e.g. *Bacillus*). An exception was *Staphylococcus* spp., where their grouping in pairs or tetrads of cocci allowed for attachment of 30-40 bacteria per HEP-2 cell. In contrast, Gram-positive rods adhered in smaller numbers to HEP-2 cells. This is probably due to their shape and grouping in chains of single, thick rods compared to the relatively small size of Gram-negative bacilli (Pavlov *et al.*, 2004).

According to Pavlov *et al.* (2004), *Corynebacterium*, *Pseudomonas* and *Staphylococcus* spp. were potentially invasive to HEP-2 cells, while *Bacillus*, *Corynebacterium*, *Pseudomonas* and *Staphylococcus* were invasive HPC bacteria to Caco-2 cells. The investigators concluded that HPC isolates from drinking water samples were invasive to both cell lines and are thus potentially pathogenic to humans, especially in the immunocompromised.

Bacteria can be classified as virulent when they contain more than one extracellular enzyme that is linked to pathogenesis (Edberg *et al.*, 1996). Pavlov *et al.* (2004) found that *Bacillus* spp., *Corynebacterium*, *Flavobacterium*, *Pseudomonas* and *Staphylococcus* produced

disease-causing extracellular enzymes that included DNase, fibrinolysin, gelatinase, lipase and proteinase. These isolates can thus be regarded as virulent. Although the various extracellular enzyme activities of the isolates from this study were not determined, the presence of the various species identified is cause for concern.

5.4 BIOFILM STRUCTURE

Both immature and mature biofilm were observed on red-copper coupons that were exposed to bulk water within the Potchefstroom drinking water distribution system. Coupons were removed after 4 months of exposure. The two copper coupons used for SEM analyses during the first sampling period, had a dirty-green rust-like appearance. These corrosion products appeared on the SEM images (Figures 4.6 and 4.7) as crystal-like substances. Similar results were observed by De La Iglesia *et al.* (2008) when they investigated microbiologically induced corrosion of copper pipes in low-pH water. They (De La Iglesia *et al.*, 2008) used scanning electron microscopy to examine copper piping in rural houses supplied with non-chlorinated water. The exterior of copper pipes in two of the residences was covered with bluish-green corrosion products that could be identified as malachite by X-ray diffraction (XRD) analysis. According to Allen *et al.* (1979), corrosion products increase the surface area and protect bacteria from the shear force of flowing water.

Structures of immature 4 month-old biofilms were also observed on the copper coupons (sampling period 4, March 2008). An extensive "spongy" EPS layer also covered the substratum (Figure 4.8). According to Dreeszen (2003) the main purpose of EPS is to help bacteria to attach to surfaces. The anionic nature of most EPS provides biofilms with cation exchange properties, allowing bacteria to concentrate and use cationic nutrients such as amines, especially in oligotrophic environments (Characklis and Christensen, 1990). Small

microcolonies of bacteria observed in Figure 4.9 might indicate the beginning of the development of advanced biofilm formation. This micrograph (Figure 4.9) fits the description given by Allen *et al.* (1979) who observed a complex of crystals and microcolonies of similar shaped bacteria on pipe coupons. Similar to the findings by Allen *et al.* (1979), the microcolonies of cells in the present study formed on a rough surface comprising cracks and crevices on a corroding surface.

Various diatoms were observed on one of the red-copper coupons during the early Autumn 2007 sampling period (see Figure 4.7). According to the Kentucky Department Division of Water (2008), the presence of certain diatoms in drinking water can cause taste and odor problems. This division also indicated that diatoms such as *Tabellaria*, *Synedra* and *Melosina* will reduce the lifetime of filters, and the rigid walls of these diatoms may cause clogging of filters.

The biofilm that formed on the RO filter was a 12-month old biofilm. In the image of this biofilm EPS is clearly observed. This observation is supported by studies such as that of Characklis and Christensen (1990) who suggested that the EPS frequently seen in scanning electron micrographs as an extracellular matrix, is responsible for the integrity of the biofilm. In such instances (as seen in Figure 4.10) the polymers exist as condensed fibers extending from bacterial cells. The SEM biofilm structure observations correspond with that of Jacques *et al.* (2006), who observed predominantly rod shaped biofilm bacteria that were embedded in an EPS layer.

CHAPTER 6

CONCLUSIONS AND PROSPECTS

The main aim of this study was to isolate, identify and characterize HPC and other bacteria from biofilm- and bulk water samples within the Potchefstroom drinking water distribution system. Five objectives were set for this study. The results of these were presented and discussed in chapters 4 and 5, respectively. Based on these objectives, general conclusions were made, which are briefly discussed in the following sections.

6.1 CLASSIFICATION OF TAP WATER ACCORDING TO PHYSICO-CHEMICAL MEASUREMENTS AND GUIDELINE VALUES

The first objective as set out in Section 1.3 was achieved and physico-chemical data were obtained as outlined in Section 3.1.1. According to guideline values as set out by Manyaka and Pietersen (1998) and SANS 241 (2006), the bulk water, based on physico-chemical data, at the J.S. van der Merwe building were classified as "blue; class 0; ideal water quality fit for lifetime consumption". Free residual chlorine levels were unusually low, implicating possibility of bacterial regrowth within the distribution system. It is however recommended that additional tests be performed to monitor these chlorine levels, to rule out experimental error.

6.2 ISOLATION AND IDENTIFICATION OF HPC BACTERIA FROM BULK WATER AND BIOFILMS OF A RO FILTER SYSTEM AND AN IN-STREAM BIOFILM DEVELOPMENT DEVICE

Twelve types of pigmented HPC bacteria from the two selected sampling points in the Potchefstroom drinking water distribution system were isolated by means of enrichment techniques and selective media. The occurrence of high levels of pigmented bacteria within

distribution systems is often associated with increased health risks in the immuno-compromised individuals within a community. Biochemical- and molecular identification methods identified these 12 morphotypes as *Bacillus cereus*, *Bacillus subtilis*, *Brevundimonas* spp., *Clostridiaceae*, *Corynebacterium renale*, *Flavobacteriaceae*, *Kytococcus sedentarius*, *Leuconostoc lactic*, *Lysinibacillus sphaericus*, *Staphylococcus aureus*, *Staphylococcus capitis* and *Pseudomonas* spp.

6.3 DIVERSITY OF ISOLATES

Overall, the highest level of diversity was observed early Autumn 2008, with 7/12 isolates that were detected during this period. The lowest diversity occurred during mid-Winter 2007, when only *Corynebacterium renale* was isolated.

6.4 FEATURES AND CHARACTERISTICS OF ISOLATED SPECIES

According to their hemolytic behavior on blood agar, *Bacillus cereus*, *Kytococcus sedentarius* and *Staphylococcus capitis* were characterized as potential pathogens. Based on both its α-hemolytic nature on blood agar and since it was resistant to six of the nine antibiotics tested, *Kytococcus sedentarius* was classified as potentially the most pathogenic among the isolates.

All isolates were resistant to two or more antibiotics. *Staphylococcus aureus* and *Corynebacterium renale* were resistant to only two antibiotics, namely **A** (AP, E) and **B** (K, OT), respectively. Displaying a resistance pattern of **I** (AP, C, E, K, NE, OT, VA), *Lysinibacillus sphaericus* was resistant to the most antibiotics. *Leuconostoc lactic* and *Staphylococcus capitis* shared a resistance pattern of **E** (E, K, OT, VA). *Flavobacteriaceae*, *Kytococcus sedentarius* and *Pseudomonas* spp. were all sensitive to AP, C, E, K, NE and OT

(pattern **G**). All isolates were susceptible to CIP and S. In contrast, most isolates resisted E. CIP and S should thus be considered as treatment options in the case of water-related disease outbreaks.

The structure of *Brevundimonas* spp., *Pseudomonas* spp. and *Staphylococcus aureus* were determined by means of TEM. The evident slime-forming capability and ability to store nutrients in this particular isolate of *Brevundimonas* spp., suggests that this organism might be a persistent biofilm-former within the distribution system of Potchefstroom. The characteristic polar flagellation pattern of *Pseudomonas* spp. was also illustrated. The single, diploid, triad- and tetrad arrangement of *Staphylococcus* cells could also be observed.

6.5 THE STRUCTURE OF RO FILTER- AND RED-COPPER BIOFILMS BASED ON SEM

Although copper is generally considered to be inhibitory or toxic to bacteria, SEM revealed the presence of both immature and mature biofilms on red-copper coupons. Biofilms were also detected on RO filters. The occurrence of corrosion products on the copper coupons suggests the phenomenon of microbial induced corrosion (MIC) within the distribution system. Various diatoms were observed on the coupons.

6.6 PROSPECTS OF THIS STUDY

The present study indicated that a period of 4 months is too short for development of a fully mature biofilm. It is therefore suggested that coupons be exposed to the bulk water for at least 12 months. Regarding antibiotic sensitivity testing, it is recommended that minimum inhibitory concentrations of isolates be determined. Molecular biofouling of biofilm should be investigated using culture independent methods such as SSCP and DGGE. Prospective

studies should include testing for the presence of iron-reducing bacteria (IRB's) and sulphate reducing bacteria (SRB's) usually involved in MIC. Future investigators should consider identifying the diatoms present in the distribution system and their implications for water quality. Finally, it can be concluded that the aim and various objectives of this study were achieved as set out in Section 1.3.

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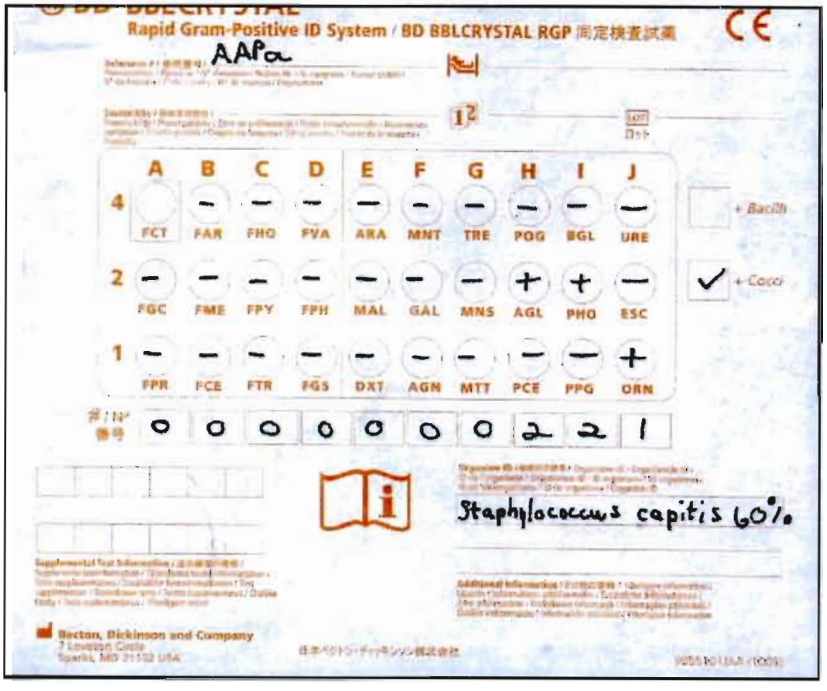
ANNEXURE A

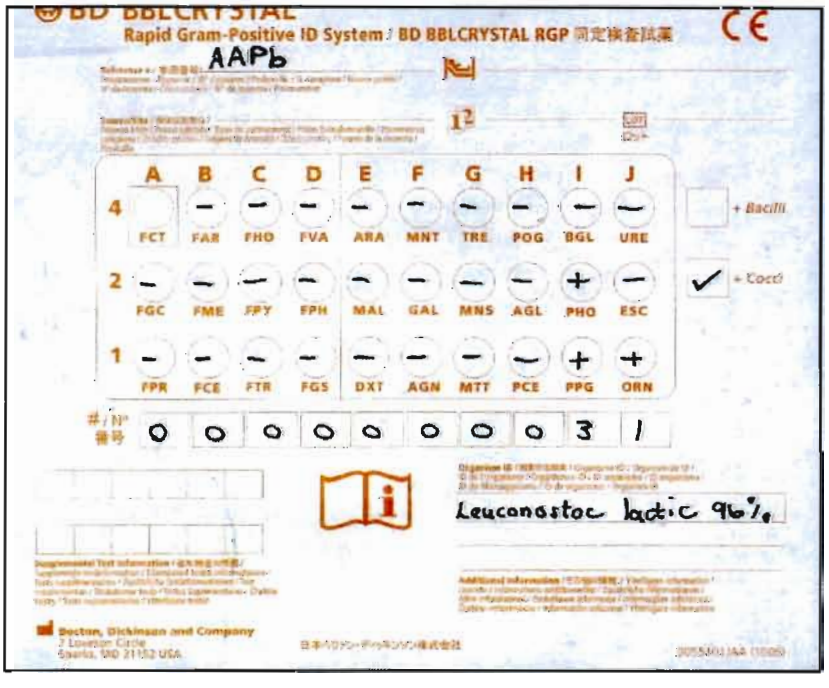
Table A1 Colour - coded classification system for domestic water (adapted from Manyaka and Pietersen, 1998 and SANS, 2006).

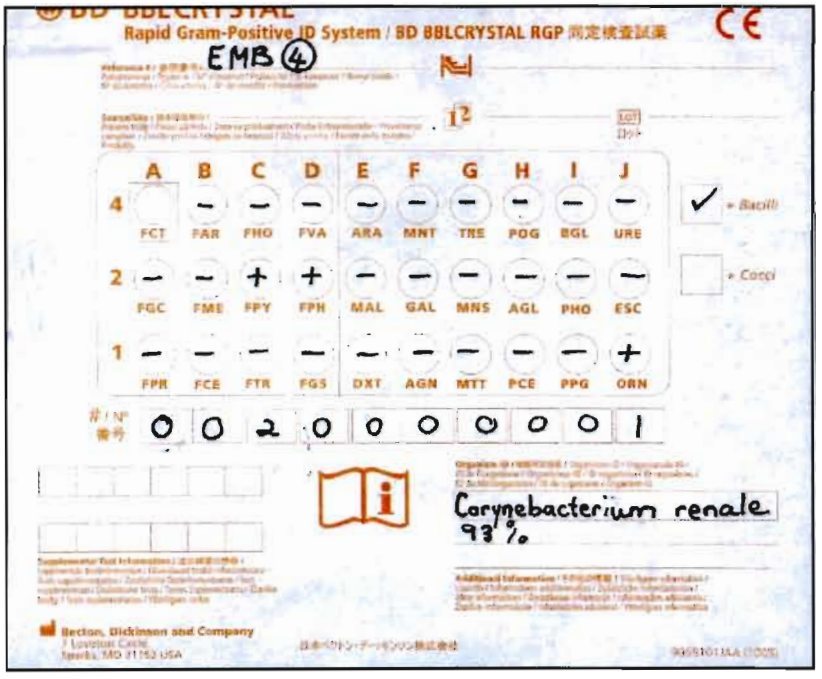
pH: 6-9.5 **TDS:** <450mg/L **TH:** 0-100mg/L **Residual Cl_2:** 0.3-0.6mg/L	**Blue**	Class 0	Ideal water quality comparable to international standards – suitable for lifetime consumption
pH: 4-6;9.5-10 **TDS:** 450-1000mg/L **TH:** 100-200mg/L **Residual Cl_2:** 0.2-0.3; 0.6-0.8mg/L	**Green**	Class I	Good water quality – suitable for consumption, rare cases of adverse effects
pH: 4-4.5; 10-10.5 **TDS:** 1000-2400mg/L **TH:** 200-300mg/L **Residual Cl_2:** 0.8-1.0mg/L	**Yellow**	Class II	Marginal water quality – conditionally acceptable for short-term consumption, possible adverse effects in sensitive groups
pH: 3-4; 10.5-11 **TDS:** 2400-3400mg/L **TH:** 300-600; >600mg/L **Residual Cl_2:** 0.05-0.1;1-1.5mg/L	**Red**	Class III	Unsuitable for consumption without treatment, chronic effects may occur
pH: <3; >11 **TDS:** >3400mg/L **TH:** N/A **Residual Cl_2:** >1.5mg/L	**Purple**	Class IV	Totally unsuitable for consumption, acute effects may occur

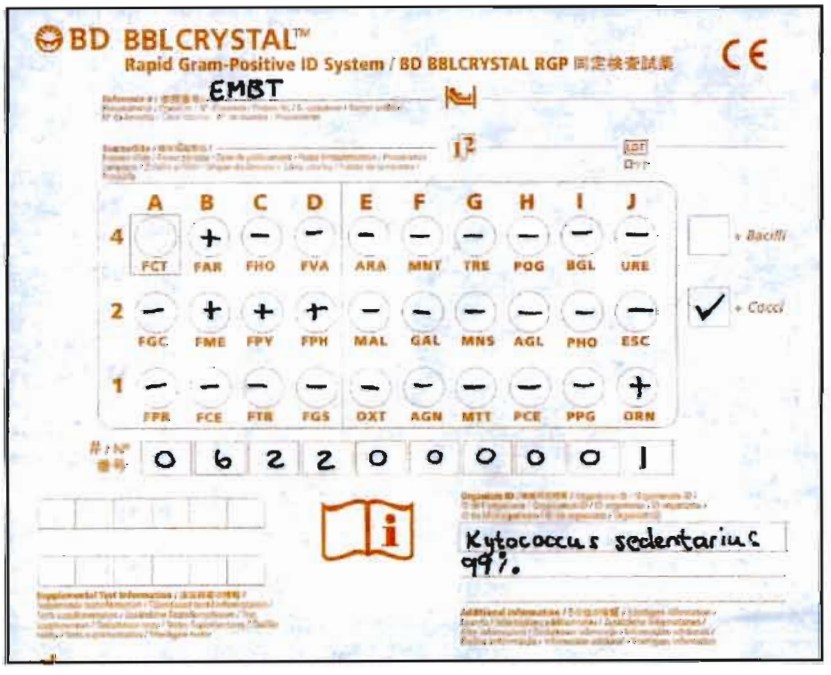
ANNEXURE B

BBL CRYSTAL RESULTS SHEETS






Rapid Gram-Positive ID System / BD BBLCRYSTAL RGP
EMB ④
A B C D E F G H I J
4 – – – – – – – – –
FCT FAR FHO FVA ARA MNT TRE POG BGL URE
2 – – + + – – – – – –
FGC FME FPY FPH MAL GAL MNS AGL PHO ESC
1 – – – – – – – – – +
FPR FCE FTR FGS DXT AGN MTT PCE PPG ORN
+ Bacilli
+ Cocci
# / N°
0 0 2 0 0 0 0 0 0 1
Corynebacterium renale
93%
Becton, Dickinson and Company

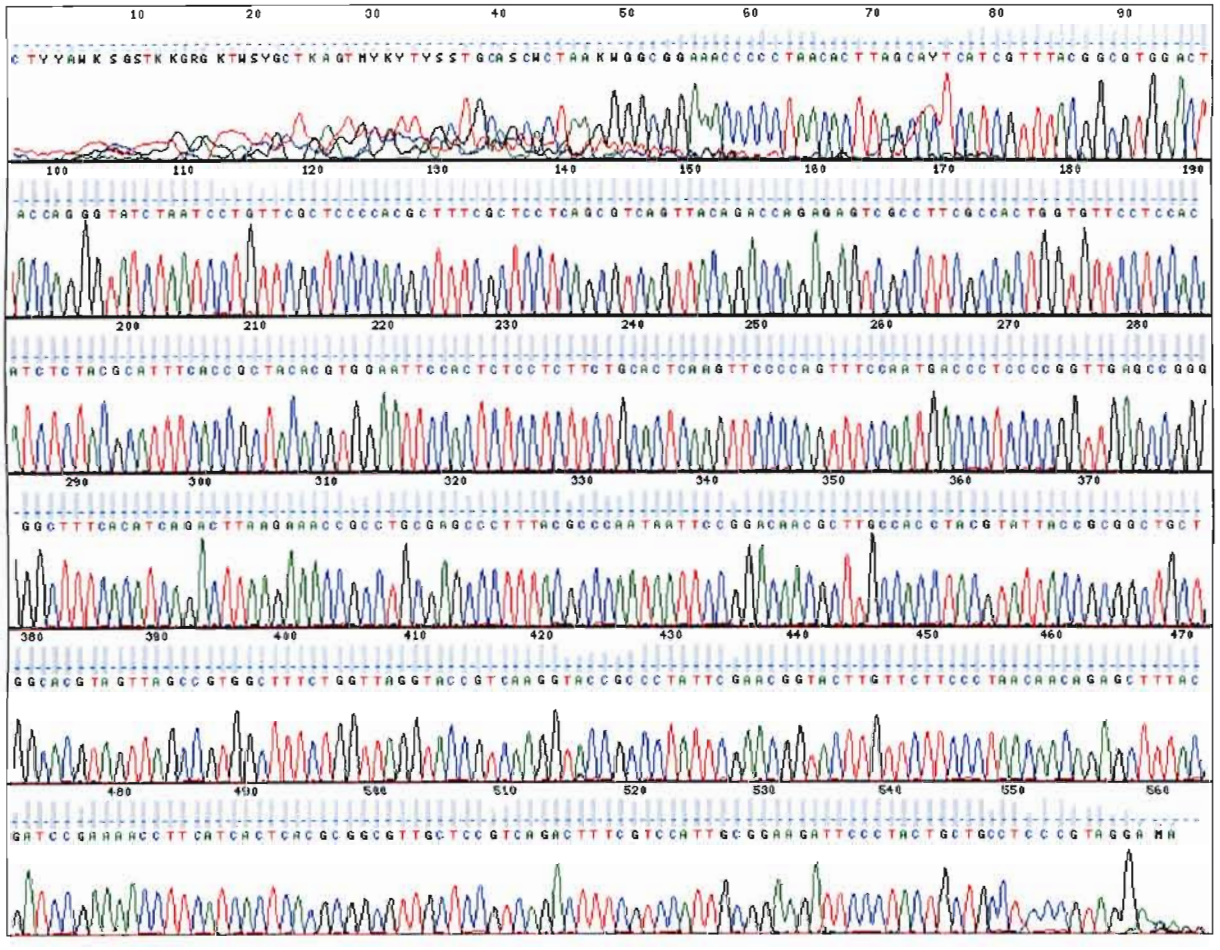

BD BBLCRYSTAL™
Rapid Gram-Positive ID System / BD BBLCRYSTAL RGP
EMBT
A B C D E F G H I J
4 + – – – – – – – –
FCT FAR FHO FVA ARA MNT TRE POG BGL URE
2 – + + + – – – – – –
FGC FME FPY FPH MAL GAL MNS AGL PHO ESC
1 – – – – – – – – – +
FPR FCE FTR FGS DXT AGN MTT PCE PPG ORN
+ Bacilli
+ Cocci
# / N°
0 6 2 2 0 0 0 0 0 1
Kytococcus sedentarius
99%

ANNEXURE C

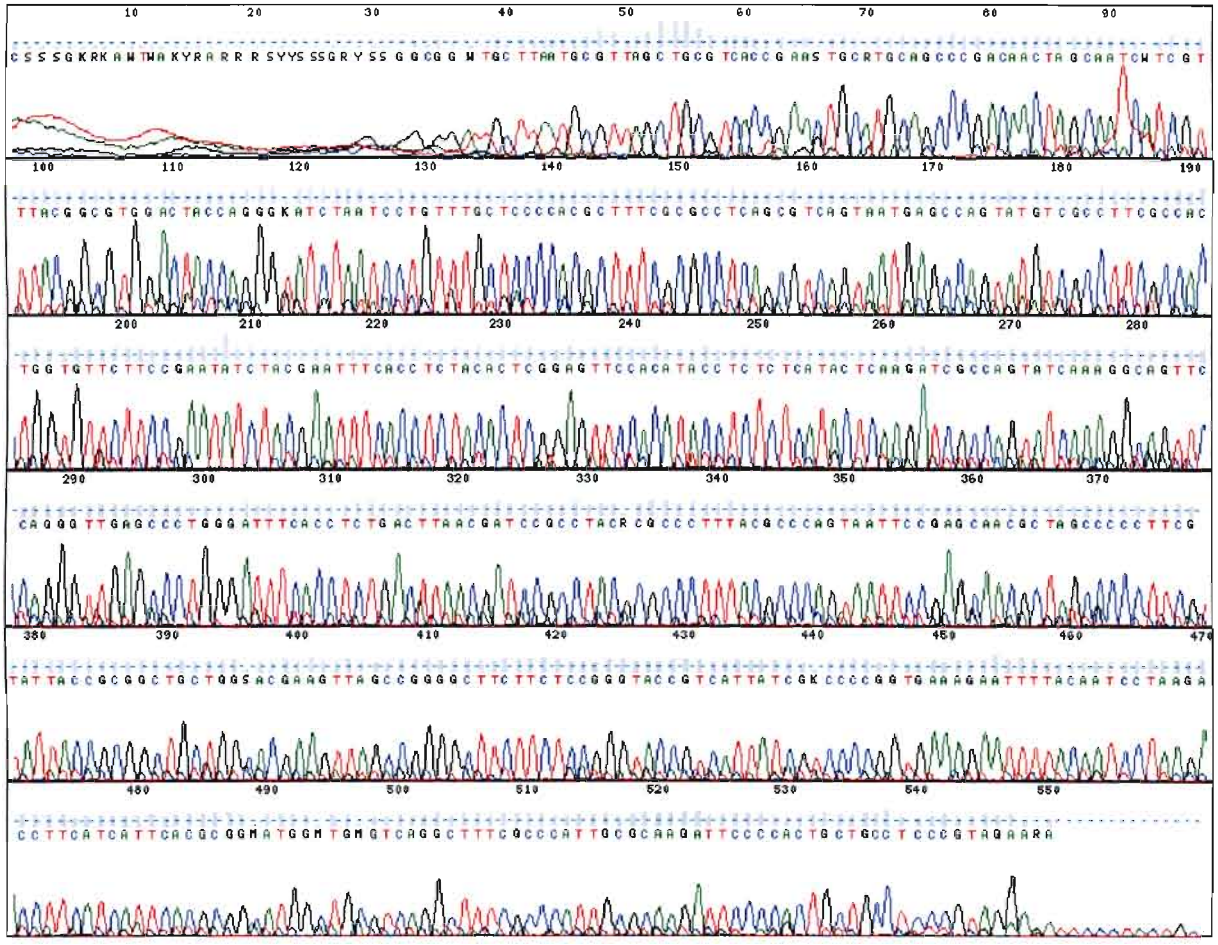
WRAPPED VIEWS OF CHROMATOGRAM TRACES

FOR SELECTED ISOLATES

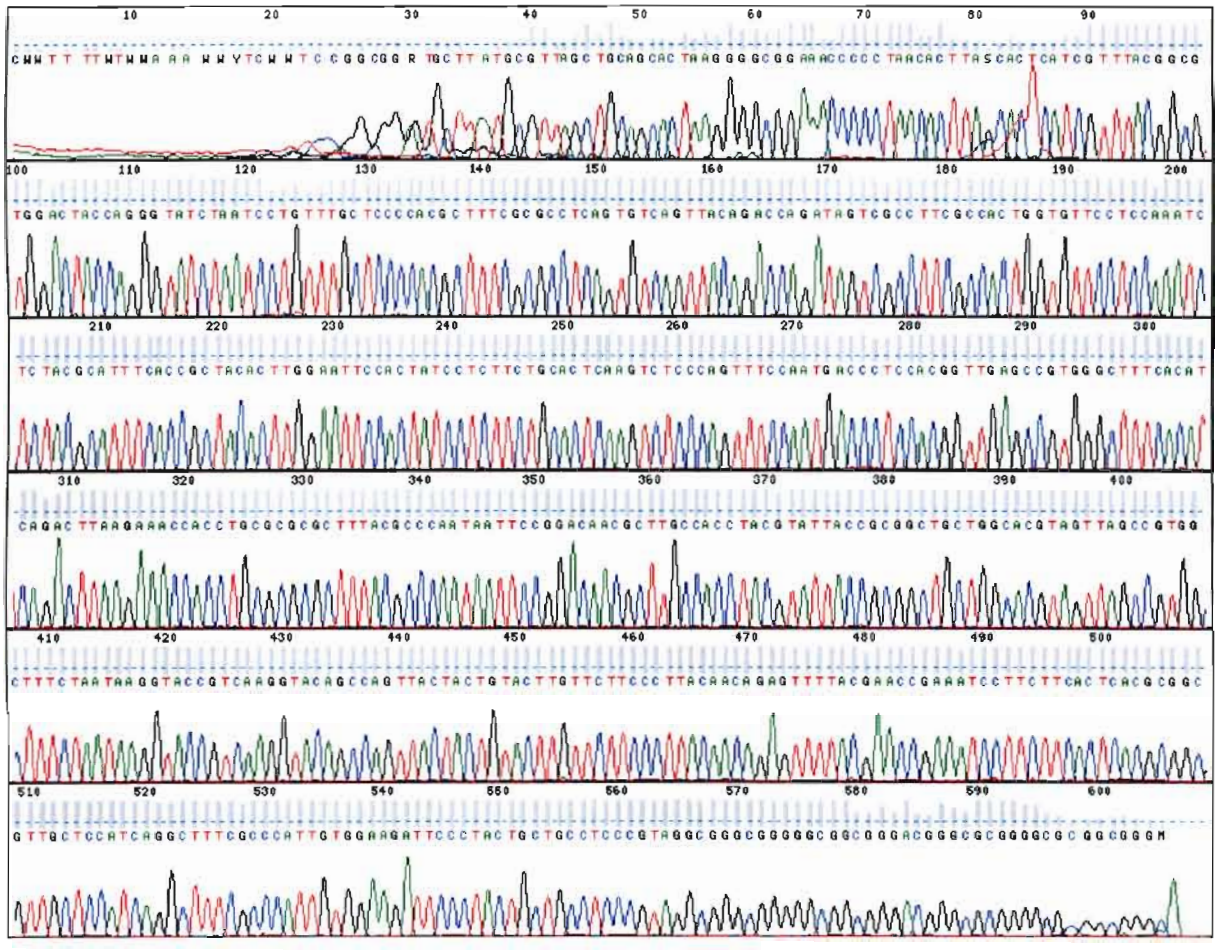
Bacillus subtilis



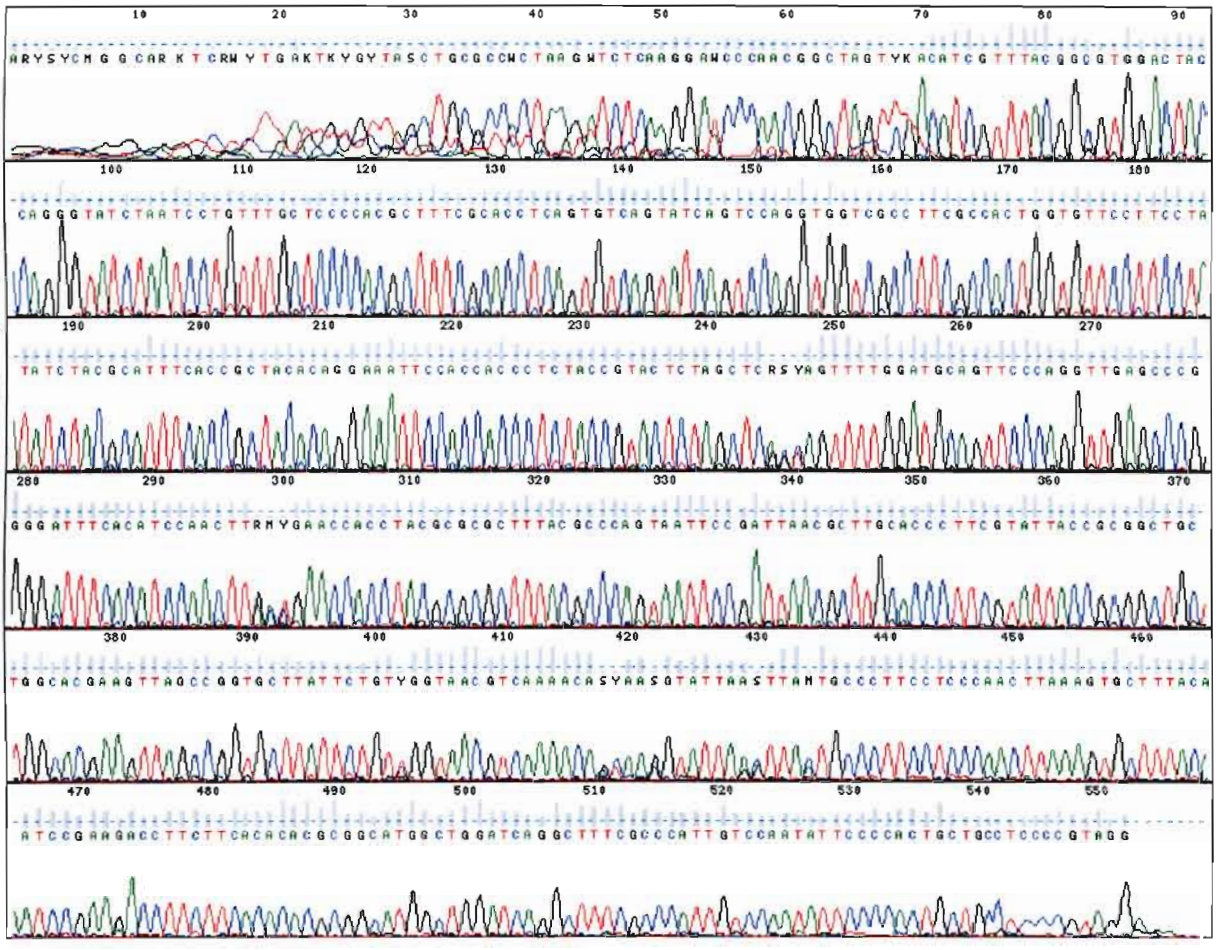
Brevundimonas spp.



Lysinibacillus sphaericus



Pseudomonas spp.



ANNEXURE D

ANTIBIOTIC RESISTANCE DATA

Table D1 Resistance (red), intermediacy (blue) and susceptibility (green) of isolates towards selected antibiotics according to their average (five colonies tested per isolate) zones of inhibition (in millimeters).

Identity of isolate	AP	C	CIP	E	K	NE	OT	S	VA
Lysinibacillus sphaericus	0	0	28	0	0	12	18	22	0
Flavobacteriaceae	0	0	26	7	7	11	7	26	-
Kytococcus sedentarius	0	0	24	0	0	13	6	24	14
Clostridiaceae	20	8	26	0	0	13	7	22	17
Bacillus cereus	0	15	30	0	19	10	0	22	17
Staphylococcus capitis	16	16	31	8	10	15	11	20	0
Leuconostoc lactic	22	24	36	0	0	15	8	24	0
Bacillus subtilis	0	16	33	0	16	21	0	24	17
Staphylococcus aureus	0	26	28	12	18	16	30	27	15
Corynebacterium renale	17	20	24	30	11	24	14	30	16
Brevundimonas spp.	10	6	21	13	5	18	0	10	18
Pseudomonas aeruginosa	12	10	27	10	11	0	0	15	17