Presence of potentially pathogenic heterotrophic plate count (HPC) bacteria occurring in a drinking water distribution system in the North-West Province, South-Africa

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Submitted in partial fulfilment of the requirements for the degree

MAGISTER OF SCIENCE

Microbiology

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May 2010

ABSTRACT

There is currently growing concern about the presence of heterotrophic plate count (HPC) bacteria in drinking water. These HPC may have potential pathogenic features, enabling them to cause disease. It is especially alarming amongst individuals with a weakened immune system. South Africa, the country with the highest incidents of HIV positive individuals in the world, mainly uses these counts to assess the quality of drinking water in terms of the number of micro-organisms present in the water. These micro-organisms may be present in the bulk water or as biofilms adhered to the surfaces of a drinking water distribution system. The current study investigated the pathogenic potential of HPC bacteria occurring as biofilms within a drinking water distribution system and determined the possible presence of these micro-organims within the bulk water. Biofilm samples were taken from five sites within a drinking water distribution system. Fifty six bacterial colonies were selected based on morphotypes and isolated for the screening of potential pathogenic features. Haemolysin production was tested for using sheep-blood agar plates. Of the 56, 31 isolates were β-haemolytic. Among the 31 β-haemolytic positive isolates 87.1% were positive for lecithinase, 41.9% for proteinase, 19.4% for chondroitinase, 9.7% for DNase and 6.5% for hyaluronidase. All of the β-haemolytic isolates were resistant to oxytetracycline 30 µg, trimethoprim 2.5 µg and penicillin G10 units, 96.8% were resistant to vancomycin 30 µg and ampicillin 10 µg, 93.5% to kanamycin 30 µg, 74.2% to chloramphenicol 30 µg, 54.8% to ciprofloxacin 5 µg, 22.6% to streptomycin 300 µg and 16.1% to erythromycin 15 µg. Nineteen isolates producing two or more enzymes were subjected to Gram staining. The nineteen isolates were all Gram-positive. These isolates were then identified using the BD BBL CRYSTAL™ Gram-positive (GP) identification (ID) system. Isolates were identified as *Bacillus cereus, Bacillus licheniformis, Bacillus subtilis, Bacillus megaterium, Bacillus pumilus* and *Kocuria rosea.* 16S rRNA gene sequencing was performed to confirm these results and to obtain identifications for the bacteria not identified with the BD BBL CRYSTAL™ GP ID system. Additionally identified bacteria included *Bacillus thuringiensis, Arthrobacter oxydans* and *Exiguobacterium acetylicum.* Morphological properties of the different species were studied with transmission electron microscopy (TEM) to confirm sequencing results. All the isolates displayed rod shaped cells with the exception of *Arthrobacter oxydans* being spherical in the stationary phase of their

life cycle. Bulk water samples were taken at two sites in close proximity with the biofilm sampling sites. The DNA was extracted directly from the water samples and the 16S rRNA gene region was amplified. Denaturing gradient gel electrophoresis (DGGE) was performed to confirm the presence of the isolates from the biofilm samples in the bulk water samples. The presence of *Bacillus pumilus* and *Arthrobacter oxydans* could be confirmed with DGGE. This study demonstrated the presence of potentially pathogenic HPC bacteria within biofilms in a drinking water distribution system. It also confirmed the probable presence of two of these biofilm based bacteria in the bulk water.

Keywords: Biofilms, HPC, drinking water distribution system, DGGE

Ek dra graag hierdie werk op aan my wonderlike ouers, broer en suster, grootouers, my vriendinne Jeanné, Lanie en Karen en my Hemelse Vader. Sonder jul liefde, ondersteuning en motivering sou hierdie studie nie moontlik gewees het nie.

Mag hierdie die begin wees van ‘n wonderlike nuwe hoofstuk van my lewe.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to the following persons and institutions for their contributions and support towards the completion of this study:

Prof. C.C. Bezuidenhout, for allowing me to be part of the North West University team, his support, input and time;

Karen Jordaan, for all her assistance and time with the molecular section of the study;

Me. W. Pretorius, for her assistance with the TEM;

Midvaal Water Company, for allowing me to use their drinking water distribution system as sampling site;

Stefan Ferreira, for his support, motivation and assistance with this thesis;

My parents, for their patients, love and financial support;

My family for years of support, love and motivation.

All my friends at the North West University; Abraham, Charné, Danie, Herman, Ina, Jerry, Karen, Lanie, Simoné and Wesley. Thank you for your support!

DECLARATION

I declare that the dissertation for the degree of Master of Science in Microbiology at the North-West University: Potchefstroom Campus hereby submitted, has not been submitted by me for the degree at this or another University, that it is my own work in design and execution, and that all material contained herein has been duly acknowledged.

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Leandra Venter **Date**

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CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION AND PROBLEM STATEMENT

The quality of drinking water has been known to deteriorate during transport through distribution systems. This is a major problem for drinking water suppliers (Momba *et al.,* 2000). Reasons for this include, biofilm formation in pipe lines (Hu *et al.,* 2005), discoloration of water (Vreeburg and Boxall, 2007) and corrosion of pipe lines (Zhang *et al.,* 2008).

A biofilm is defined as a group of organisms occurring in water, bound together by a polymeric matrix, and attached to a solid surface such as pipe lines of drinking water distribution systems (Escher and Characklis, 1990). Biofilms can either be in a planktonic or a sessile phase. It is estimated that for each cell of planktonic bacteria there are 1000 cells of sessile bacteria present in the water (Van der Kooij and Zoetemann, 1978; LeChevallier *et al*., 1987; Momba *et al*., 2000). These sessile bacteria, present in the water, are the most likely cause of infection upon exposure or ingestion by consumers. Sessile bacteria generally include: *Acinetobacter* spp., *Aeromonas* spp., *Flavobacterium* spp., *Klebsiella* spp., *Legionella* spp., *Moraxella* spp., *Mycobacterium* spp., *Pseudomonas* spp., *Serratia* spp. and *Xanthomonas* spp. (Rusin *et al*., 1997; Kudinha *et al*., 2000; Pavlov *et al*., 2004).

The biofilm formation and the diversity of micro-organisms within the biofilm will be influenced by different parameters. These include; fluctuating temperatures due to seasonal changes (Moll *et al.,* 1999), the type of pipe materials used for the distribution system (Van der Wende and Charaklis, 1990), the type and concentration of disinfectants used (Gilbert, 1988) and the availability of biodegradable compounds as energy source for microbial growth (Van der Kooij, 1999).

There is a definite need to identify the micro-organisms present in the biofilms as waterborne pathogens are able to colonize these biofilms or form a new biofilm, adding to the persistence of pathogens (LeChevallier and McFeters, 1985). Different techniques are employed for the identification of bacteria such as plating and isolation (Martiny *et al.,* 2005), microscopy (Gamby *et al.,* 2008), 16S rRNA gene sequencing (Martiny *et al.,* 2003) and polymerase chain reaction – denaturing gradient gel electrophoresis (PCR-DGGE) (Wu *et al.,* 2006). Plating techniques have mostly been replaced by molecular techniques due to the high numbers of non-culturable micro-organisms present in the biofilms and bulk water samples (Farnleitner *et al.*, 2004).

South Africa currently uses the heterotrophic plate count (HPC) bacteria standard to evaluate drinking water quality. The standard stipulates that drinking water may not contain more than 100 cfu/ml of HPC bacteria (SABS, 2001). Previous studies, done by Rusin *et al*. (1997) and Pavlov *et al*. (2004) suggested that these HPC bacteria may be harmful to infants, the elderly or immuno-compromised individuals. It may cause secondary infections in those patients whose immune system has been compromised by a primary infection (Grabow, 1996; Rusin *et al*., 1997; Barbeau *et al*., 1998; Pavlov *et al*., 2004).

In 2008 it was estimated that 5.6 million South Africans are HIV positive (Nicolay, 2008). This number exceeds any number in any country in the world. The North-West province has the fourth largest number of HIV positive individuals in South Africa (HSRC, 2005; Department of Health, 2006). It is estimated that 496 000 people in North-West are HIV positive, thus 13% of the population. A total of 43 000 people get infected per annum with 34 000 people dying of AIDS per year in this province (Nicolay, 2008). An updated report for 2009 indicated that 501 066 people in the North-West province is HIV positive (Nicolay and Kotze, 2009). It is to these individuals that safe drinking water is of crucial importance.

1.2 RESEARCH AIM AND OBJECTIVES

The aim of the study was to determine whether samples taken from pipe lines in a drinking water distribution system contain HPC bacteria with a potential health risk to consumers.

Objectives were to:

i. isolate and purify HPC bacteria taken from a drinking water pipe line;

ii. screen isolates for the production of haemolysin and selected enzymes potentially involved with pathogenesis;

iii. conduct antibiotic susceptibility tests on haemolytic positive isolates;

iv. group organisms as Gram-positive or Gram-negative and identify the organisms using a biochemical test method as well as 16S rRNA gene sequencing;

v. determine morphological characteristics of the different species isolated with TEM;

vi. detect and compare bacteria present in bulk water samples with isolates from biofilm samples using PCR-DGGE.

CHAPTER 2

LITERATURE OVERVIEW

2.1 General overview

South Africa has an ever increasing number of immuno-compromised individuals (Shisana *et al.*, 2009), adding to the pressure on drinking water supply companies to provide safe drinking water. According to the ASSA2003 (Provincial) AIDS and demographic projections for 2009, 501 066 people in the North-West province are living with HIV (Nicolay and Kotze, 2009). Studies done by September *et al.* (2004) and Pavlov *et al*. (2004) suggest that heterotrophic plate count (HPC) bacteria might not be as harmless as initially thought. These micro-organisms may cause secondary infections especially in hosts with a weakened immune system, classifying them as potential pathogens (Rusin *et al.,* 1997; September *et al.,* 2004; Pavlov *et al.,* 2004).

Biofilms are continuously being studied within drinking water distribution systems in terms of (i) microbial community diversity (Kalmbach *et al.,* 1997; Martiny *et al.,* 2003; Lethola *et al.,* 2004), (ii) parameters influencing biofilm formation (Block, 1992; Niquette *et al.,* 2000 and Chenier *et al.,* 2006) and (iii) problems caused by biofilms (Hu *et al.,* 2005; Vreeburg and Boxall, 2007; Zhang *et al.,* 2008). Methods such as the haemolysin assay (Payment *et al.,* 1994) and enzyme profiling (Janda and Bottone, 1981; Pavlov *et al.,* 2004) are used to determine the pathogenic potential of bacteria present in biofilms within drinking water distribution systems. Identification of bacteria conducted by biochemical methods such as the BBL CRYSTAL^{TM} identification system (Balows *et al*., 1991; Baron *et al*., 1994; Mandell *et al*., 1990; Murray *et al*., 1995) or molecular methods such as 16S rRNA gene sequencing (Burtscher *et al.,* 2009). Culture independent methods such as PCR-DGGE are also used to provide insight into microbial community structure in drinking water distribution systems (Wu *et al*., 2006; Wu and Zhao, 2009).

2.2 Drinking water quality framework

According to the World Health Organization (WHO) drinking water should at all times be suitable for consumption by humans in terms of personal hygiene and domestic purposes (WHO, 2002). Drinking water should, thus be safe for food preparation, showering or washing, and for consumption. Consumption of drinking water should be safe across all life stages for an unlimited period of time (Hodgson and Manus, 2006). This is however, not necessarily the case for immuno-compromised individuals (Bartram *et al*., 2003).

With the ever increasing number of immuno-compromised individuals, the WHO *Guidelines for Drinking-water Quality* has embarked on a "water safety plan" (WSP) for the management of piped water supplies (WHO, 2002). The aim is to reduce the risk factor of opportunistic pathogens for individuals with a weakened immune system. The plan is divided into five categories; Setting targets, evaluating the water supply system, critical point evaluation, communicating and documenting the evaluation and independent inspection of the targets (WHO, 2002; Bartram *et al*., 2003).

South Africa has also set up a framework for the quality of drinking water (Hodgson and Manus, 2006). It is based on a preventative approach by implementing risk management. Managing a drinking water distribution system includes knowledge of the functioning of the entire distribution chain, incidents that can cause failure in the system to provide good quality water and putting operational control strategies in place for ensuring the quality of drinking water and public health (Hodgson and Manus, 2006; WHO, 2010).

2.3 Drinking water distribution system challenges

A major problem faced by drinking water suppliers is the decline in water quality during its transport through a drinking water distribution system (Momba *et al*., 2000; Lethola *et al.,* 2004). It is the responsibility of each drinking water supply company to ensure that their customers receive clean water, free from any factors that might be harmful after consumption (Hodgson and Manus, 2006).

2.3.1 Problems occurring in drinking water distribution systems

Three major problems occur in drinking water distribution systems that may affect the quality of the water. These problems include: discoloration of the water (Vreeburg and Boxall, 2007), formation of biofilms in the pipe lines (Hu *et al.,* 2005) and corrosion of the pipe lines (Zhang *et al*., 2008).

Discoloration is caused by the presence of particulate matter. There is a relation between the presence of particles and biological activity (Gauthier *et al*., 1999), which immediately compromises the safety of the drinking water (Vreeburg and Boxall, 2007). Husband and Boxall (2010) designed a model to study and verify parameters influencing the discoloration of water. Conclusions drawn were that material layers have a tendency to develop on all water distribution pipes with special reference to iron and plastic pipes. Discoloration is mainly caused by materials being present on pipe walls which are then mobilized by shear stress caused by the force of the water flowing through the water distribution system (Husband and Boxall, 2010).

One of the most significant and frequent biological processes occurring within a water distribution system is the formation of biofilms (Van der Kooij, 2002). Although complex and poorly understood, biofilm formation may have great implications for the quality of drinking water (Vreeburg and Boxall, 2007).

Two main factors may cause an increase in bacterial numbers during the distribution of water. These are, mechanical failure, which includes mains break (this will occur during the aging of a system), disturbances of the distribution system during installation of new pipe lines, a decrease in water flow pressure causing back siphonage of the water and open reservoirs (Rossie, 1975; Momba *et al*., 2000). Bacterial numbers can also increase within the distribution system due to regrowth or aftergrowth of bacteria resulting in the formation of biofilms. Biofilm formation results in a decrease in water quality due to a subsequent change

in odor or color as well as an increase in the rate of pipe line corrosion (Nagy and Olson, 1985; Zhang *et al*., 2008).

Two types of metal corrosion have been reported, microbiologically influenced corrosion (MIC) and electrochemical corrosion (Lin *et al*., 2001; Teng *et al*., 2008). A number of risks are associated with corrosion such as the provision of a favorable environment for pathogens and opportunistic pathogens (Tuovinen and Hsu, 1982; Teng *et al*., 2008). Numerous studies indicate that certain biofilm forming bacteria are able to increase the rate of corrosion (Little *et al.,* 1997; Gonzalez *et al.,* 1998; Gu *et al.,* 1998; Starosvetsky *et al.*, 2001), as is the case for some sulfate-reducing bacteria (SRB) (Hamilton, 1985; Seth and Edyvean, 2006).

2.3.2 Techniques for cleaning drinking water distribution systems

Sub-standard drinking water quality in a distribution system may have a negative effect on consumer confidence. They may also lose trust in the company to supply good quality services (Vreeburg and Boxall, 2007). There are, however, ways in which these obstacles can be overcome or even prevented. Companies should aim at understanding the process and mechanisms in which these problems occur. The framework for solving these problems should shift from a reactive manner to a preventative one (Bartram *et al.,* 2003; Vreeburg and Boxall, 2007).

Establishing the problems in a water distribution system is as important as the management thereof. Cleaning techniques will lower the risk elements occurring within a drinking water distribution system. Most drinking water supply companies use swabbing/pigging or water flushing as a method for controlling biofilms in the system (Satterfield, 2007).

Pigging is a process where bullet-shaped poly pigs are inserted into pipe lines. The pig consists of a soft foam type material that is specifically formulated for a certain size and type of pipe line. The pipes are cleaned by forcing the pig forward using hydraulic pressure

(Satterfield, 2007). Water flushing is a cost effective and simple way of cleaning a distribution system. It will, however, not remove all the materials from the pipes, as would a method like pigging (Satterfield, 2007). Two criteria are considered when flushing a system. These are the velocity of the flush, and the shear stresses on the inner surfaces to remove the biofilms from the pipe lines (Vreeburg and Boxall, 2007).

2.4 Biofilms in a drinking water distribution system

"Biofilm" is a descriptive term referring to a group of micro-organisms forming a layer on a surface within an aquatic environment, bound together by an insoluble extracellular polymeric matrix (Costerton *et al*., 1995). As much as 95% of the biomass present in a distribution system will be present on the pipe surfaces. The planktonic state of bacteria is outnumbered to such a degree, due to the large surface to volume ratio in a pipe line (Flemming, 1998; Hu *et al.,* 2005). Micro-organisms present in biofilms are protected from antimicrobial agents (chemical and biological), and from unfavorable environmental conditions by the polymeric matrix (Lappin-Scott and Costerton, 1989; De Saravia *et al.,* 2003).

2.4.1 Biofilm formation

Water distribution systems are colonized by bacteria in a very specific manner (Wolfaardt and Archibald, 1990). The persistence, community biostability and discharge of microbial cells, into the supply system, are determined by this colonization type. Waterborne pathogens will interact with the biofilm, increasing the persistence of the pathogens (LeChevallier and McFeters, 1985) when they colonize an existing biofilm or form a new one (Bartram *et al*., 2003).

Apart from mechanical failure there are three terms describing the entry of bacteria into a water distribution system: "breakthrough", "regrowth" and "aftergowth" (Nagy and Olson, 1985). Breakthrough occurs when disinfection process is surpassed by viable bacteria, these then multiply within the water distribution system. Regrowth occurs when bacteria have the ability to recover from injury caused by the treatment process, and then multiply within the

distribution system. Aftergrowth refers to the growth of bacteria occurring naturally in the water distribution system (Van der Wende and Characklis, 1990; Momba *et al*., 2000).

2.4.2 Parameters influencing biofilm formation

There are a number of factors that will either promote, or retard, the development of biofilms. Only a few bacterial cells will be able to survive the treatment process, or a small number of bacteria will be present in the distribution system. These bacteria then require optimal growth conditions to multiply. The parameters include: pipe material, temperature, disinfectants used and the presence of biodegradable compounds (Block, 1992; Niquette *et al.,* 2000; Chenier *et al.,* 2006).

2.4.2.1 Pipe materials

There are three generic types of pipe materials available; metallic, cementitious and plastic. These materials all have both advantages as well as limitations (Lion *et al*., 1998; Momba and Makala, 2004). Micro-organisms have the ability to colonize the internal surface of different types of materials within a water distribution system, as it is continuously in contact with water (Momba and Makala, 2004). A characteristic of materials that may influence the rate of biofilm formation is the irregularity of the material (Pederson, 1990; Percival *et al.,* 1998).

Pederson (1990) found that more micro-organisms were present after 167 days on a rough stainless steel compared to electro-polished steel. Van der Wende and Charaklis (1990) reported that smooth surfaces may retard initial development rate of biofilm formation, but after a period of time the amount of biofilm will be the same for smooth or rough surfaces. Momba and Makala (2004) found a definite correlation between the generic type of the pipe material and the bacterial density within the water system. It was found that plastic-based materials supported more fixed bacteria than cement-based materials. They recommended that cement or asbestos-cement pipes are used especially where water is treated with chlorine- and monochloramine.

Lethola and co-workers (2004) studied a pilot drinking water distribution system to determine the chemistry, microbiology and biofilm development for two types of pipe lines, copper and polyethylene (PE). They documented that initial biofilm formation is faster for PE pipes than for copper pipes, but after 200 days, microbial numbers on both materials were similar. Virus-like particle numbers were higher for PE pipes than for copper pipes in both the biofilm and outlet water samples. Lethola *et al.* (2004) also concluded that the microbial community structure was influenced by the pipe material used for both the water and biofilm samples.

The above mentioned studies indicated that the initial development of biofilms within the pipe lines of drinking water distribution systems are influenced by the roughness of the material used. However, over a period of time the developed biofilm will be the same independent of the material used for the pipe lines. The generic type of the pipe material does play a role in the attachment of bacteria to the pipe surfaces but the microbial numbers will stabilize over a period of time for most types of pipe material used.

2.4.2.2 Temperature

Some bacteria grow within a narrow temperature range whereas others are able to grow at a wide range of temperatures. Seasonal changes might have an influence on the composition of the biofilm as demonstrated by LeChevallier *et al*. (1980). The effects of temperature have however not been studied intensively. It is mostly based on seasonal changes in temperatures (Moll *et al*., 1999).

Moll and co-workers (1999) investigated the effect of temperature on (i) performance of biofilters within a drinking water purification process and on (ii) community structure. They performed tests at 5, 20 and 35 °C and found that low operation temperatures during winter caused a decrease in biofilter performance as well as a decrease in biomass. A decrease in temperature also changed the microbial community structure. Substrate metabolism also significantly decreased. They concluded that changes observed were solely due to

temperature and that it was not the result of seasonal variation in influent microbial communities and biodegradable organic matter (Moll *et al.,* 1999).

Microbial community structure is affected by temperature fluctuations that are due to seasonal changes. Biofilm compositions might change over time. Different bacteria might thus dominate the biofilm at different stages during the year depending on the temperature range at which the bacteria are able to grow. Such temperature fluctuations will affect drinking water pipe lines that are surface based.

2.4.2.3 Disinfectants

There are two disinfectant factors that affect the formation of biofilms within drinking water distribution systems. These include the effectiveness of the disinfectant, and the resistance of bacteria to the disinfectant (Momba *et al.,* 2000).

It is very important to use the correct disinfectant at the optimum concentration, as micro-organisms are able to use biodegradable organic substances as an energy source, and in so doing enhance the formation of biofilms within the distribution system (Gilbert 1988; Van der Kooij, 1999; Momba *et al*., 2000). Van der Wende and Characklis (1990) demonstrated that less reactive compounds such as chloramines has better persistence than for instance, free chlorine. These less reactive compounds will keep the disinfectant residual level higher throughout the distribution system. Chloramine also penetrates the biofilm and biofilm organisms may be better controlled.

The resistance of certain bacteria to disinfectants, even at relatively high concentrations, is another obstacle to overcome. A number of research groups have investigated the possibility of bacteria becoming resistant to certain compounds used as drinking water disinfectants (Ridgeway and Olson, 1982; Olivieri *et al*., 1985; LeChevallier *et al*., 1988). Reports have indicated that bacteria, present in biofilms, are more resistant to disinfectants than the same

cells in a planktonic state (LeChevallier *et al.,* 1988; Srinivasan *et al.,* 1995; Cochran *et al.,* 2000; Boe-Hansen *et al.,* 2002; Morato' *et al.,* 2003).

In recent years, chlorine has been replaced with ozone as a disinfectant or added during the treatment process (Zacheus *et al.,* 2000). Ozone serves as an oxidant for elimination of odor and taste. Total organic carbon (TOC) present in the water will determine the concentration of ozone required (Albidress *et al.,* 1995). Ozone can either be added at the beginning of the treatment process or just before the filtration step (Zacheus *et al.,* 2000). Clark and co-workers (1994) conducted experiments where ozone was used during the purification process of water. Results showed that ozone has the ability to degrade recalcitrant organic compounds to more easily degradable nutrients, enhancing biofilm growth within a distribution system.

Some bacteria have the ability to withstand disinfectants enabling them to survive the treatment process. This could be due to genetic elements or features. These bacteria are then able to colonize the distribution system and form biofilms. Caution should be exercised when disinfectants are selected for the water purification process. It might eliminate unwanted odors or tastes but at the same time enhance the growth of biofilm based bacteria as is the case for ozone when the dissolved organic levels are high.

2.4.2.4 Presence of biodegradable compounds

Biodegradable compounds are either present in the chemicals used during the treatment procedure, or in the water being contaminated by other materials. These compounds are then utilized by the bacteria for regrowth in a distribution system (Van der Kooij, 1999). Micro-organisms differ in their types of cellular energy source (organic or inorganic hydrogen), hydrogen acceptor (nitrate, carbon dioxide, oxygen, organic C or sulfate) and carbon source (organic or inorganic carbon) they use (Van der Kooij, 1982).

Van der Kooij *et al.* (1989) and Albidress *et al*. (1995) reported that the addition of ozone increased degradable organic carbon (DOC) concentrations significantly. DOC includes BDOC (biodegradable organic carbon) and AOC (assimilable organic carbon). The BDOC fraction of the DOC depicts the fraction that is assimilated and mineralized by the heterotrophic flora present in the drinking water (Escobar and Randall, 2001). AOC mainly consists of small molecular weight compounds which is readily degradable (Van der Kooij, 1990). Certain combinations of bacteria of specific strains are able to utilize AOC, causing an increase in biomass within the drinking water distribution system (Escobar and Randall, 2001).

Zacheus and co-workers (2000) investigated the formation of biofilms when ozone was added during the treatment process. They measured biofilm accumulation on three pipe materials, stainless steel, polyethylene (PE) and polyvinyl chloride (PVC). Results showed an increase in AOC concentrations when ozone was added. There was also an increase in number of viable heterotrophic bacteria and cell volume for ozonated water vs. non-ozonated water. Although PVC displayed the highest initial bacterial numbers when exposed to ozonated water, all three pipe materials displayed similar biofilm accumulation.

Even though ozone removes odors and unwanted tastes from the water, it does increase the DOC concentrations significantly. Due to the increased availability of DOC, bacterial numbers might increase. High bacterial numbers will in turn enhance the development of biofilms within the drinking water distribution system.

2.4.3 Identification of bacteria present in biofilms

The identification of bacteria present in a distribution system has received very little attention and research prior to 2002 (Wagner and Loy, 2002). Several groups have, however, been investigating the diversity and complexity of bacteria involved in the formation of biofilms (Kalmbach *et al*., 1997; Martiny *et al*., 2003; Lethola *et al*., 2004; Berry *et al.,* 2006). This

knowledge is of great importance as it provides information for improvement of drinking water quality (LeChevallier *et* al., 1996).

Plating and isolation have mostly been used for the identification and monitoring of bacteria occurring in biofilms within a distribution system (Martiny *et al*., 2005). The difficulty with these techniques is that many of the micro-organisms present may be non-culturable, leading to an underestimation of the quantity of cells present. A collection of cells, due to the scrapping sample procedure, may be presented by a single colony (Colwell, 1984; September *et al.,* 2004). Each planktonic cell detected in a distribution system may be present due to the presence of up to a 1000 sessile cells, thus biofilms are regarded by some as the main source of contamination of water within a system (Van der Kooij and Zoeteman, 1978; LeChevallier *et al*., 1987; Pavlov *et al.,* 2004).

More recently Gamby and co-workers (2008) used methods such as microscopy (atomic force microscopy (AFM) and scanning electron microscopy (SEM)), electrochemistry (rotating disk electrode (RDE)) and spectroscopy (polarization modulation infrared reflection absorption spectroscopy (PM-IRRAS)) for the detection and characterization of biofilms present in drinking water. They concluded that a combination of these techniques will provide usefull results to study the steps in biofilm formation, as well as an indication of the dominant bacterial morphology present in the biofilm.

Culture-independent methods can also be used to identify bacteria present in biofilms. Martiny *et al.* (2003) used terminal restriction fragment length polymorphisms and 16S rRNA gene sequencing to study the structure and diversity of biofilms in a model drinking water distribution system. The bacteria present were identified as *Acidobacterium, Nitrospira, Planctomyces* and *Pseudomonas.* Lee *et al.* (2004) investigated the bacterial species diversity within biofilms in a drinking water distribution system. PCR-DGGE and DNA sequencing were the methodologies applied. *Sphingomonas* sp. and *Rhodobacter* sp. were amongst the identified bacteria.

Culture-dependant and culture-independent methods are currently used for studying the dynamics and structure composition of biofilms. Although culture-dependent methods are fast and easy to perform, there is a definite need to identify bacteria that play a role in biofilm development that cannot be cultured. Culture independent methods provide more representative results in terms of the actual composition of bacteria present in biofilms.

2.5 Heterotrophic plate count (HPC) and heterotrophic plate count bacteria

It is important to distinguish between heterotrophic bacteria and heterotrophic plate count bacteria as they are not synonymous. Heterotrophic bacteria are present in air, soil, vegetation, food and water and use organic nutrients as their energy source (Edberg and Allen, 2004). HPC bacteria include all the microbes isolated by using a specific method under a predetermined set of conditions. These conditions can differ in terms of incubation time and temperature, the composition of the media used and the way in which the medium is inoculated (Reasoner, 1990). Thus, HPC bacteria represent a subpopulation of heterotrophic bacteria within a certain sampling area (Allen *et al.,* 2004).

Drinking water quality is assessed using heterotrophic bacteria as indicator organisms (Grabow, 1996). These organisms are considered to be part of the natural microbiota of water and are described as non-hazardous (Bartram *et al*., 2003).

Many groups characterized the HPC bacteria present in drinking water and obtained almost the same spectrum of predominant species. These species include: *Acinetobacter* spp., *Aeromonas* spp., *Alcaligenes* spp., *Bacillus* spp., *Comamonas* spp., *Enterobacter* spp., *Flavobacterium* spp., *Klebsiella* spp., *Moraxella* spp., atypical *Mycobacterium* spp., *Nocardia* spp., *Pseudomonas* spp., *Sphingomonas* spp., and *Stenotrophomonas* spp. (Burligame *et al*., 1986; LeChevallier *et al*., 1987; Payment *et al.,* 1988; Payment 1989; Reasoner *et al*., 1989; Manaia *et al.,* 1990; Edberg *et al.,* 1997; Rusin *et al*., 1997; Norton and LeChevallier, 2000; Pavlov *et al.,* 2004).

2.5.1 HPC test methods and applications for water management

Water microbiologists have been using HPC testing for a long period of time as an indication of water quality and in ascertaining the correct functioning of certain phases in the purification system (Payment *et al.,* 2003). It has, however, been replaced mostly by specific fecal indicator bacteria (WHO, 2002). A reason for this is that it is very expensive and intensive treatment is required to adhere to HPC specifications. These specifications as set by the South African Bureau of Standards (SABS), specify that drinking water may not contain more than 100 cfu HPC per millilitre of drinking water (SANS 241: 2006). There is also a difference in opinion on whether these HPC results only include harmless organisms which pose no risks to human health (Bartram *et al.,* 2003; September *et al.,* 2004; Pavlov *et al*., 2004).

HPC test methods entail a range of culture-based tests that are simple to perform with a recovery of a wide variety of micro-organisms. Test conditions are varied to obtain a diversity of qualitative and quantitative results (WHO, 2002; Bartram *et al.,* 2003)). Nutrient rich, non-selective media are used to detect the widest range of micro-organisms. Incubation temperatures vary between 20°C and 37°C for 24 to 48h. Visible bacterial colonies are counted which then represents the HPC used to examine the quality of drinking water (Grabow, 1996; Pavlov *et al*., 2004). The methods have been standardized but a universal "HPC measurement" does not exist (WHO, 2002; Bartram *et al.,* 2003).

A downside to these test methods are that only a fraction of the micro-organisms present will be detected at a certain set of incubation conditions (Amann *et al.,* 1995; Hammes *et al.,* 2008). Different methods will have different results in terms of the population recovered. Furthermore, HPC testing used for organism recovery will provide varied results between seasons, between repeated sampling at the exact same location and between different locations. The test results are thus not always reproducible (Norton and LeChevallier, 2000; WHO, 2002).

Despite these objections, many countries still use HPC measurements as guidelines in water management. The uses include: evaluation of the proper functioning of the treatment process (WHO, 2002; Bartram *et al.,* 2003), measurement of the number of micro-organisms present due to regrowth in the system (WHO, 2002; Bartram *et al.,* 2003) and evaluation of changes in water quality during the distribution and storage (Sartory, 2004).

It is important to consider that the infection rate due to the presence of micro-organisms, including specific heterotrophic micro-organisms in water, has increased dramatically (Huang *et al*., 2002). High-risk areas, including hospitals treating immuno-compromised individuals, have to pay special attention to ensure that the quality of drinking water is satisfactory. Authorities use HPC in these areas to determine the risk of opportunistic pathogens present in the drinking water distribution system (Hargreaves *et al*., 2001).

Although authorities have shifted towards fecal indicator bacteria as a measure for water quality, HPC test methods still remain a good indication of the functioning of the water purification process. HPC test methods may also provide informative results in terms of the opportunistic pathogens present within a drinking water distribution system.

2.5.2 HPC bacteria involved in health aspects

Water samples have been tested for the presence of health-related micro-organisms for more than 100 years (Ashbolt *et al*., 2001). Several studies have demonstrated that pathogens are able to grow as biofilms within pipe lines (Jones and Bradshaw, 1996; Barbeau *et al*., 1998; Buswell *et al.,* 1998; Falkinham *et al.,* 2001).

Serious public health concerns have been associated with the presence of biofilms in the pipe lines of a distribution system (Buswell *et al*., 1998; Percival *et al*., 1999; Bressel *et al*., 2003). Studies have presented evidence that bacteria obtained through heterotrophic plate counts may not all be harmless through all life stages (Keynan, 2007; Kalpoe *et al.,* 2008). This is the

case for individuals with a weakened immune system where secondary infections may be caused by these potentially pathogenic micro-organisms (Rusin *et al*., 1997; Pavlov *et al*., 2004). These individuals include the children younger than 5 years, the elderly, organ transplant and cancer patients receiving medical treatment and patients with AIDS (Grabow, 1996; Rusin *et al*., 1997; Barbeau *et al*., 1998; Pavlov *et al*., 2004).

The opportunistic pathogens that may be found among the naturally occurring HPC microbiota include: *Acinetobacter* spp., *Aeromonas* spp*., Bacillus* spp., *Flavobacterium* spp., *Klebsiella* spp*., Legionella* spp., *Moraxella* spp*., Mycobacteria* spp*., Pseudomonas* spp., *Serratia* spp. and *Xanthomonas* spp.(Rusin *et al*., 1997; Kudinha *et al*., 2000; Bartram *et al*., 2003). Low exposure to *Aeromonas* spp. present in drinking water poses an infection risk of 7.3 per billion, whereas high level exposure to *Pseudomonas* spp. poses an infection risk of 98 per 100 for individuals on antibiotic treatment (Rusin *et al*., 1997).

September and co-workers (2004) investigated the presence of nontuberculous mycobacteria (NTM) in a distribution system. NTM were considered as non-pathogenic but reports have shown that many can be characterized as opportunistic pathogens (Collins *et al*., 1984; Emmerson, 2001; Mangione *et al*., 2001). Immuno-compromised individuals are not the only people at risk, but also the otherwise healthy individuals (Graham, 2002). NTM can cause, amongst others, lymphadenitis, cutaneous and pulmonary diseases (Le Dantec *et al*., 2002). September *et al.* (2004) confirmed the presence of NTM in drinking water distribution systems in South Africa and recommended sporadic testing as part of the quality control measures, especially in areas with high numbers of immuno-compromised individuals. A study done by Emtiazi *et al.* (2004) also revealed the presence of NTM within the biofilms of a drinking water distribution system in Germany.

The presence of *Bacillus* spp. in particular *Bacillus cereus, Bacillus subtilis, Bacillus licheniformis, Bacillus thuringiensis* and *Bacillus pumulis* is of concern as it is associated with food poisoning (Ostensvik *et al.,* 2004). Due to the production of endospores these micro-organisms are widely spread (Fritze, 2002) and may be able to withstand the disinfection

process during drinking water treatment. The surviving bacteria may contaminate food and cause food poisoning in the individual consuming the food (Ostenvik *et al,* 2004).

There is reason for concern when it comes to the presence of HPC bacteria in drinking water as it has been associated with health related issues. However, the magnitude of concern is related to the abundance of the specific organism in the water as well as the health status of the individual consuming the water.

2.6 Antibiotic resistance of bacteria

The misuse of antibiotics has contributed greatly to the presence of antibiotic-resistant (AR) bacteria in the environment. Selection pressure on bacteria is created by the prescription of antibiotics to patients with a non-bacterial infection, the use of anti-bacterial disinfectants in and around households and also the use of antibiotics in animal farming and agricultural uses (Klare *et al.,* 1995; Aarestrup *et al.,* 1996; Schwartz, 2003). A number of possible explanations have been proposed as to why biofilm based bacteria have an elevated resistance to antimicrobial agents. These include: i) genetic adaption, ii) outer membrane structures, iii) efflux pump, iv) biofilm matrix limiting antimicrobial agent diffusion, v) resistance due to enzymes being produced, vi) metabolic activity of the biofilm based bacteria and vii) biofilm matrix interacting with the antimicrobial agent (Stewart and Costerton, 2001; Cloete, 2003).

2.6.1 Health implication of AR bacteria

Antibiotic resistance genes (ARGs) of AR bacteria are widely spread in water systems (Cooke, 1975; Gonzal *et al.,* 1979; Kummerer, 2004; Baquero *et al.,* 2008; Martinez, 2008; Zhang *et al.,* 2009a; Zhang *et al.,* 2009b). This has been recognized for a long time and is of growing concern in terms of public health challenges (Klare *et al.,* 1995; Xi *et al.,* 2009).

Xi *et al.* (2009) made use of culture-dependant (R2A) and quantitative molecular techniques (Real-time PCR) to test for the presence and abundance of ARGs and AR bacteria in water. These tests were conducted on source water, finished water and tap water. AR bacteria numbers were higher for tap water than for finished water which indicated regrowth within the drinking water distribution system. An increased resistance to certain antibiotics was observed after treating the water and for tap water than what was observed for source water. This suggested that the treatment process might be selecting for antibiotic resistance of bacteria, particularly those injured during the treatment process. Thus, drinking water distribution systems aid in the spread of these AR bacteria and ARGs. They suggested further research on what the impact of the spread of AR bacteria and ARGs would be on the health of particularly the immuno-compromised individuals. From this example and other studies listed it is evident that there is need to determine the health implications that these two elements impose on the individuals consuming the drinking water.

2.6.2 Antibiotic resistance as a method for bacterial source tracking

Water environments are constantly contaminated with bacteria from animal and human sources. Some of these bacteria contain ARGs which at some stage might be inserted into mobile genetic elements such as plasmids. These mobile genetic elements are able to spread amongst the naturally occurring bacteria enabling them to be resistant to certain antibiotics (Alonso *et al.,* 2001). Antibiotic resistance evolves from four main genetic reactors, i) animal and human microbiota, ii) hospital and farms (agricultural), iii) waste water from sewage treatment plants and iv) soil and surface or ground water contaminated with bacteria from the previous genetic reactors (Baquero *et al.,* 2008).

Due to advanced molecular techniques antibiotic-resistance can be used for bacterial source tracking (Baquero *et al.,* 2008). Casarez *et al.* (2007) conducted a study to compare four bacterial source tracking methods. These methods included pulsed-field gel electrophoresis (PFGE), enterobacterial repetitive intergenic consensus sequence polymerase chain reaction (ERIC-PCR), Kirby-Bauer antibiotic resistance analysis (KB-ARA) and automated ribotyping using *HindIII*. They used the same collection of water for all four methods. KB-ARA data could

however not be used to differentiate between highly similar isolates. They suggested a combination of these methods for accurate source tracking. Thus, although the KB-ARA method will not be able to differentiate between highly similar isolates it still provides an indication of the reactor, whether it is from human or animal related sources.

2.7 Methods employed to establish the pathogenic potential of HPC bacteria

Disease-causing micro-organisms are referred to as pathogens. The ability of a micro-organism to cause disease in a plant, insect or animal is termed pathogenicity. Pathogenicity is expressed by the virulence of the microbe (Edberg *et al.,* 1996). The virulence of the micro-organism is thus the degree of pathogenicity. Virulence is determined by any structural, genetic or biochemical features enabling the pathogen to cause disease within the host (Todar, 2009).

Pathogenic bacteria can cause disease by either producing toxins or they could invade tissues. Toxigenesis is facilitated by the production of endo- or exotoxins. Invasiveness is accomplished by producing extracellular substances for invasion, colonization or bypassing defence mechanisms of the host (Payment *et al.,* 1994; Edberg and Allen, 2004). Pathogenic potential of the bacteria are established by methods such as the haemolysin assay (Hoult and Tuxford, 1991) and enzyme production analysis (Janda and Bottone, 1981).

2.7.1 Haemolysin assay

Haemolysin production by micro-organisms is responsible for the lysis of red blood cells (Hoult and Tuxford, 1991) and causes haemoglobin to be released. Haemolysin can either be lecithinases, phospholipases or channel-forming proteins (Todar, 2009). These proteins will either disrupt the membrane structure or make a hole in the membrane. The production of haemolysin is tested for by growing the bacteria on blood agar. Blood agar is high in nutrient content and support the growth of a wide range of bacteria. It normally contains sheep or rabbit red blood cells (Atlas, 1997; BookRags Staff, 2005).

Haemolysis is divided into three types (alpha, beta or gamma; Payment *et al.,* 1994). Alpha haemolysis is described as a green discoloration forming on the agar around the inoculation spot. This is due to haemoglobin being partially decomposed. Beta haemolysis involves the complete decomposition of haemoglobin causing a zone of clearing around the spot of inoculation. Gamma haemolysis represents no haemolysis. It appears brownish which is the normal reaction displayed by blood at 37°C in the presence of carbon dioxide (BookRags Staff, 2005).

Payment and co-workers (1994) used blood agar for the detection of virulence factors possessed by heterotrophic bacteria present in drinking water. They used tryptic soy agar containing sheep blood as test media to detect cytolytic bacteria. Twenty five percent of the isolated bacteria were cytolytic and possessed other virulence factors. Thus, many heterotrophic bacteria found in drinking water have an increased disease-causing potential due to the presence of virulence factors.

2.7.2 Enzyme production analysis

Extracellular enzymes are produced by micro-organisms to facilitate the spread and growth of pathogens and/or damage host cells and promote the invasion potential of bacteria (Todar, 2009). Amongst these are hyaluronidase, lecithinase, protease, lipase, DNase and chondroitinase.

Hyaluronidase is described as a spreading factor, as it will promote pathogen spread by affecting the physical properties of intercellular spaces and tissue matrices. It depolymerizes hyaluronic acid found in the interstitial barrier of connective tissue. By doing so it initiates infection at the skin surface enabling pathogen spread (Rogers, 1948). Disaccharides are the end products of depolymerised hyaluronic acid. These disaccharides provide nutrients to the pathogens needed for spread and replication (Hynes and Walton, 2000). Lecithinase on the other hand, affects the cell membrane by destroying the lecithin (Hoult and Tuxford, 1991), creating pores in the membrane for micro-organisms to gain access to the cell.

Proteases and lipases have not clearly been linked to pathogenesis and invasion. However, it has been suggested that these enzymes may play a role in bacterial metabolism or nutrition and may have a direct or indirect role in invasion of host cells (Todar, 2009). Chondroitin is a major constituent of the connective tissue protecting the joints (Busci and Poor, 1998), thus chondroitinase will catalyze the hydrolysis of chondroitin. DNase production by bacteria is responsible for the degradation of DNA (deoxyribonucleic acid). It is however, not clear as to what the exact role of this enzyme is. Proposed roles are the use of the degraded DNA as an energy source by the pathogen (MacFaddin, 1985) or to shut down the phagocyte genetic machinery when a DNase producing bacteria is engulfed (Janda and Bottone, 1981).

Janda and Bottone (1981) performed enzyme profiling on *Pseudomonas aeruginosa* to determine their invasive potential and to apply it as an epidemiological tool. They tested for the production of the following enzymes: fibrinolysin, haemolysin, lipase, DNase, proteinase, hyaluronidase, gelatinase, elastase, chondroitinase and lecithinase. It was observed that clinical isolates produced 9 of the 11 enzymes tested for, whereas environmental samples were enzymatically, relatively inert. They also found that these enzyme profiles could be used to distinguish clinical strains from different sources, thus a sort of fingerprint could be formulated for epidemiological studies. Their observations were based on the differences in enzyme profiles of *Pseudomonas aeruginosa* isolated from different clinical samples. For instance the elastase enzyme was more readily produced by systemic isolates than by sputum or genitourinary isolates.

In another study, Pavlov *et al.* (2004) made use of enzymes involved in pathogenecity to determine the pathogenic potential of HPC bacteria present in drinking water. DNase, gelatinase, lecithinase, proteinase, hyaluronidase, lipase, coagulase, fibrinolysin, chondroitinase and elastase were produced by many of the haemolytic positive isolates but none of the isolates produced pyocyanin and fluorescein. Potentially pathogenic genera that were isolated included; *Acinetobacter, Aeromonas, Aureobacterium, Bacillus, Chryseobacterium, Corynebacterium, Klebsiella, Moraxella, Pseudomonas, Staphylococcus, Tsukamurella* and *Vibrio* (Pavlov *et al.,* 2004).

Haemolysin, together with extracellular enzyme production analysis, provides informative results as to whether bacteria are potentially pathogenic. Bacteria that are haemolytic positive and produces two or more extracellular enzymes are generally regarded as potentially pathogenic and it is for these isolates that further analysis and identifications are needed.

2.8 Biochemical method employed for identification of HPC bacteria

The first reported use of biochemical identification micromethods dates back to 1918 (Bronfenbrenner and Schlesinger, 1918). This biochemical method was used to detect pathogenic bacteria present in stools of patients with intestinal infection symptoms. It was based on the inability of pathogens to ferment lactose, whereas non-pathogens produce acid on lactose containing media. Each of the suspended colonies was plated in an agar drop containing lactose. Acid production could be observed after six to eight hours, thus eliminating non-pathogenic strains within hours (Bronfenbrenner and Schlesinger, 1918).

Micro-tube and reagent-impregnated paper discs have been used by many study groups for the differentiation between species of enteric bacteria (Sanders *et al*., 1957; Soto, 1949). Due to the growing interest in these identification systems many commercial systems were available by the end of the 1960's (Cowan *et al*., 1974; Hartman, 1968). One of the earliest test panels used for the identification of closely related bacteria within a certain group was the Analytical Profile Index (API) (Shoeb, 2006).

The API system for bacterial identification is a simplified biochemical test kit. Anaerobes, enterobacteria and lactobacilli are amongst the groups of bacteria that can be identified with the different API kits. The kits consist of dehydrated chemicals each in an individual cupule. A bacterial suspension is then used to inoculate each cupule (Janin, 1976). Each reaction is awarded a plus or minus which is then converted into a numerical code. This numerical code provides a profile for the organism. The profile number is entered into a computer identification model which then provides the identification of the organism (Holmes *et al.,* 1978). The API 20E system does, however, not work very well for Gram-positive isolates

(Juang and Morgan, 2001). The need for an alternative system such as the BBL CRYSTAL™ Gram Positive Identification System arose from this predicament.

2.8.1 BBL CRYSTAL™ Identification System

The BBL CRYSTAL™ Gram Positive (GP) Identification (ID) system makes use of modified chromogenic, conventional and fluorogenic substrates. It is a miniaturized identification method used to identify Gram-positive bacteria (Balows *et al*., 1991; Baron *et al*., 1994; Mandell *et al*., 1990; Murray *et al*., 1995). The advantages in using miniaturized identification systems include its simplicity in use, ease of storage, standardized quality control and long shelf life.

The BBL CRYSTAL™ GP ID systems consist of 29 dehydrated enzymatic and biochemical substrates. The inoculum fluid containing the bacterial cells in suspension is added to the substrates and then rehydrated. Microbial degradation and utilization of substrates are the basis for the tests. The fluorogenic substrates contain either coumarin derivatives 7-amino-4-methylcoumarin (7-AMC) or 4-methylumbelliferone (4-MU) which is enzymatically hydrolyzed by the bacteria causing fluorescence to increase and be observed under UV light. Color-changes are observed for the wells containing chromogenic substrates (Maddocks and Greenan, 1975; Manafi *et al*., 1991; Mangels *et al*., 1993; Moncia *et al*., 1991).

2.9 Molecular Methods used for the identification of HPC bacteria

Microbial communities can now be analyzed in terms of structure and composition by using molecular biological techniques (Muyzer *et al*., 1993; Muyzer and Smalla, 1998). These techniques are also employed for the identification of non-culturable micro-organisms (Ward *et al.,* 1990; Farnleitner *et al.,* 2004). A polymerase chain reaction (PCR) is performed to amplify ribosomal DNA, after which it is subjected to sequence determination (Giovannoni, 1990; Burtscher *et al.,* 2009) and a BLAST search in GENBANK.

Sequencing is a valuable tool when it comes to studying communities and individuals within the communities. It provides information on genetic differences between different strains of the same organisms, differences that can not be detected with phenotypic studies (Mignard and Flandrois, 2006). It is especially valuable when studying biofilms and individuals within the biofilms as genetic differences can be detected and relatedness of the organisms can be determined (Eisen, 2007).

The 16S rRNA gene is used for identification purposes as this gene is present in all prokaryotes (Priest and Austin, 1993). Thus, this gene contains regions that differ at species level. Bacteria can be identified at genus and/or species level by using databases for comparison with organisms in the public domain (Vandamme *et al.,* 1996). This is very useful when identifying bacteria from environmental samples and community diversity needs to be established (Mignard and Flandrois, 2006). Sacchi *et al.* (2002) used 16S rRNA gene sequencing to differentiate between *Bacillus anthracis, Bacillus cereus* and *Bacillus thuringiensis.* These three *Bacillus* spp. share a 99% sequence similarity adding to the difficulty of differentiating between them (Ash *et al.,* 1991a; 1991b; Helgason *et al.,* 2000b). They were however, able to demonstrate a distinct 16S sequence associated exclusively to each of the different *Bacillus* spp. (Sacchi *et al.,* 2002).

Martiny and co-workers (2003) made use of 16S rRNA gene sequencing to compare bulk water samples with biofilm samples taken from the pipe lines of a model drinking water distribution system. They observed that micro-organisms found in the bulk water attached to the pipe lines contributing to the species richness of biofilm samples. The dominating species include; *Acidobacterium* spp., *Nitrospira* spp. *Planctomyces* spp.*,* and *Pseudomonas* spp*.* Another study done by Tokajian *et al.* (2005) 16S rRNA gene sequencing was used to do a phylogenetic assessment of HPC bacteria present in a drinking water distribution system. Dominating bacteria present was identified as *Bacillus* spp., *Mycobacterium* spp., *Pseudomonas* spp. and *Sphingomonas* spp.

Molecular methods provide the means of studying organisms without the need to culture them. This provides more accurate information on community structure and diversity as identities can also be obtained for the non-culturable organisms. However, molecular methods are also critical to identify and characterize culturable bacteria.

2.10 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE is considered a molecular fingerprinting technique that was initially designed to study micro-organisms that are difficult to culture. It is also used to study these organisms as they occur in their natural habitat, such as soil and water, without having to culture them prior to any experimental procedures (Muyzer and Smalla, 1998). DGGE provides an indication of the diversity and succession of the community of microbes and usually exploits the comparison of rDNA of different microbes (Hori et al., 2006).

The principle of the method relies on the PCR-amplified 16S rRNA gene fragments being electrophoresed in an increasing solution gradient of polyacrylamide gel (Fischer and Lerman, 1979). The gradient is provided by denaturants such as urea and formide (Ercolini, 2004). One of the PCR primers usually contains a GC-rich sequence which is referred to as the GC-clamp. The function of the GC-clamp is to maintain the integrity of the one end of the heteroduplex, thus maintaining a partially double stranded molecule (Myers *et al.,* 1985; Sheffield *et al.,* 1989). Certain regions show discrete melting preferences and are thought to be melting domains. These are stretches of base pairs possessing the same melting temperature. When the region with the lowest melting temperature achieves its melting temperature at a certain position in the DGGE gel, the migration of the DNA molecule will be retarded due to the partially melted conformation. When the base sequences differ, so will the melting temperature and the migration distance of the different fragments of DNA. DNA fragments of similar size but different sequences can be separated by this technique (Muyzer *et al*., 1993).

The visualization of DNA bands under UV light is made possible by ethidium bromide staining of the gel (Muyzer and Smalla, 1998). SYBR Green I (Muyzer *et al.,* 1997) and silver staining (Felske *et al.,* 1996) can also be used. SYBR Green I allows for the visualization of low concentration DNA fragments because of less background staining (Muyzer *et al*., 1997). Increased sensitivity is achieved by silver staining but single stranded DNA is also stained (Heuer and Smalla, 1997).

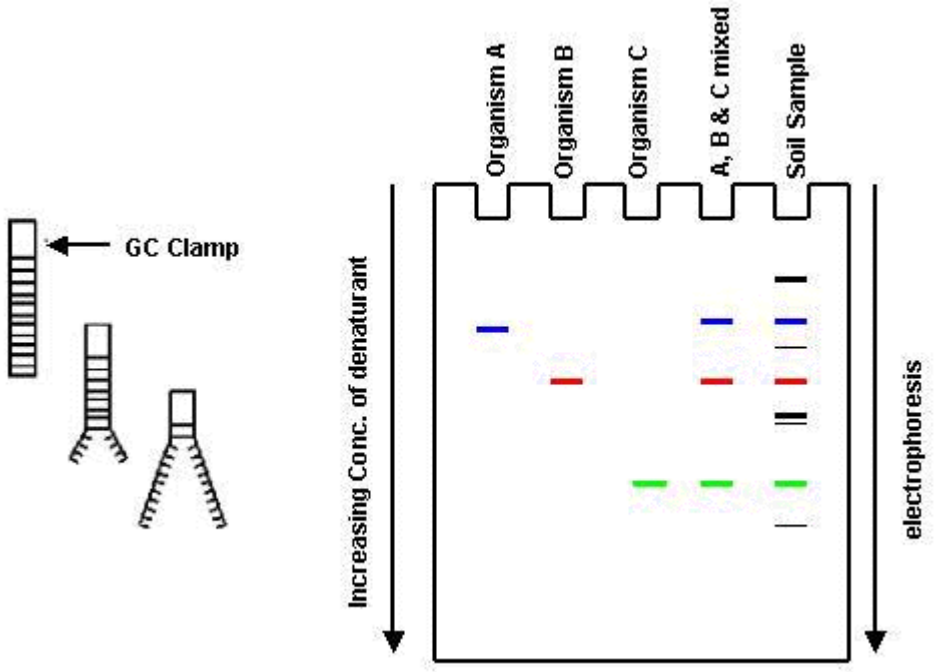

Figure 2.1: A simplified diagram on the principles of DGGE. Image was obtained from www.environmental-expert.com. [Date of access: 4 February 2010].

Wu *et al.* (2006) used PCR-DGGE as a tool to determine the bacterial diversity in drinking water. DNA was isolated directly from the water samples after which the 16S rDNA was amplified. DGGE was performed, certain prominent bands excised and the extracted DNA was sequenced. Results have shown the presence of amongst others, *Bacillus* sp., *Brevibacillus* spp., *Escherichia coli* and *Shigella flexneri.* They concluded that the extraction of total DNA, directly from the water sources, can be done more rapidly and accurately than traditional plating and isolating techniques. It will also provide information on the diversity of

bacteria present in the drinking water. In another study Wu and Zhao (2009) made use of DGGE to compare the diversity of bacteria present in biofilms and drinking water. Bulk water and biofilm samples were taken at the same time. Fewer bands were observed for the bulk water samples than for the biofilm samples and all the bands presented by the bulk water samples were present in the biofilm samples. They concluded that at identical sampling times, bacterial diversity is less for bulk water samples than for biofilm samples as indicated by PCR-DGGE.

Burtscher *et al.* (2009) compared the community structure of heterotrophic plate count agar with the community structure obtained with PCR-DGGE analysis. They obtained a completely different community structure for each of the methods. The HPC community consisted of cultivable copiotrophic micro-organisms such as *Bacillus* spp. Community structure obtained with DGGE analysis consisted of mostly uncultured bacteria found in oligotrophic water environments. Thus, different methods displayed different results which need to be taken into account when distribution systems are monitored.

PCR-DGGE is a method which provides information on community structure and diversity without the need to culture the micro-organisms. Thus, more accurate analysis can be done in terms of the presence and diversity of bacteria within a biofilm in drinking water distribution systems.

2.11 Summary of literature

In the preceding literature review it was demonstrated that HPC bacteria are either present in bulk water or as biofilms on the inner surface of the pipe lines of drinking water distribution systems. When present in biofilms, they are protected from adverse environmental conditions and antimicrobial agents (Lappin-Scott and Costerton, 1989). These biofilms can cause problems such as corrosion of pipe lines (Zhang *et al.,* 2008) or discoloration of water (Vreeburg and Boxall, 2007). Parameters that might influence the formation of biofilms include; pipe materials (Momba and Makala, 2004), temperature (LeChevallier *et al.,* 1980),

disinfectants (Momba *et al.,* 2000) and the presence of biodegradable compounds (Van der Kooij, 1999).

Heterotrophic plate counts are generally used as a tool to determine the quality of drinking water in many countries. Recent studies have provided evidence that these micro-organisms, usually present within drinking water distribution systems, might not be as harmless as previously accepted. It is especially alarming for countries with high incidences of immuno-compromised individuals including the elderly, the very young, AIDS patients and cancer patients (Pavlov *et al*., 2004). The pathogenic potential of these micro-organisms need to be established in order to revise the guidelines for drinking water quality and to implement management strategies for the prevention of biofilm build up within the drinking water distribution systems. The literature overview also dealt with several methods that could be used to study the diversity and characteristics of HPC occurring in drinking water distribution systems.

CHAPTER 3

MATERIALS AND METHODS

3.1 Study site

The drinking water distribution system investigated was that of Midvaal Water Company (Figure 3.1). It is located in the North-West province of South-Africa. The company has been supplying drinking water since 1954. Midvaal Water Company serves industrial and mining sectors as well as residential areas. Their supply area covers about 900 km^2.

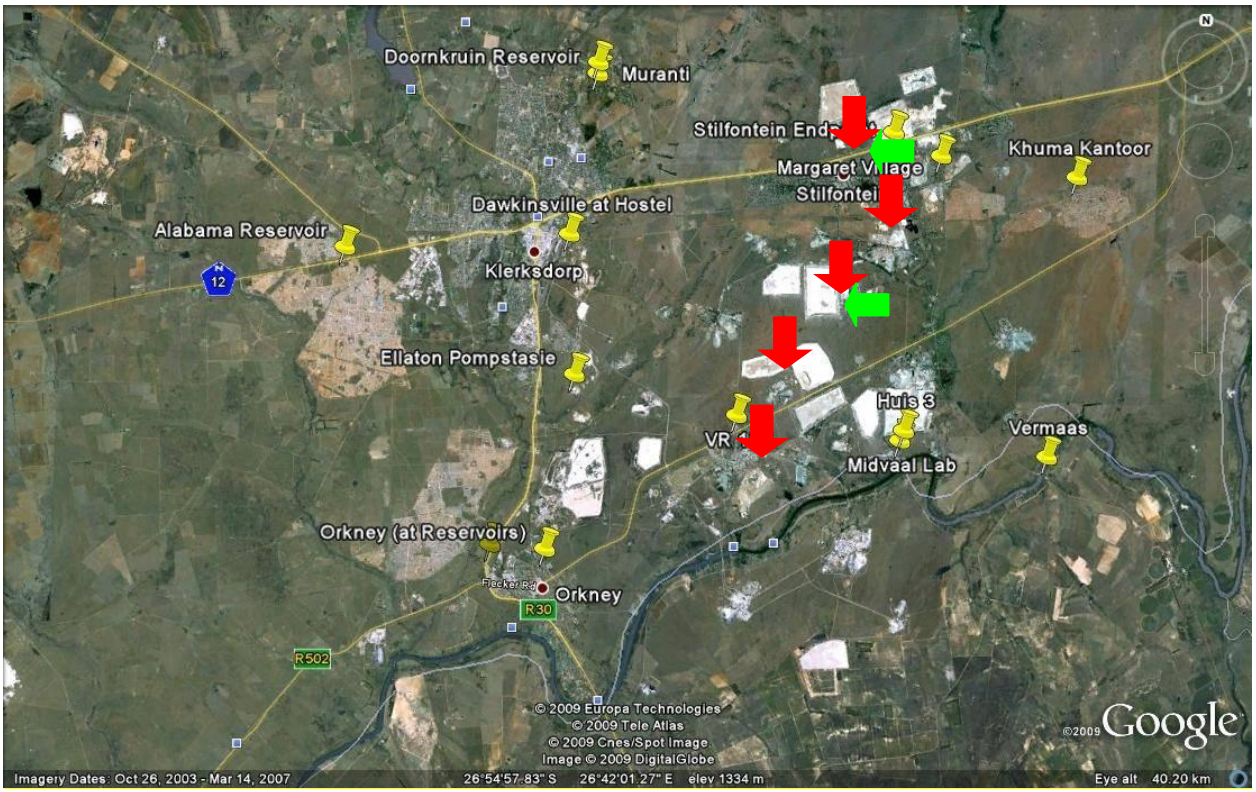

Figure 3.1: A Google Earth map of the Midvaal water distribution system used for sampling (www.google.co.za). [Date of access: 10 February 2010]. Red arrows indicate the biofilm sampling points with site 1 closest to the Midvaal lab, which is where the treatment plant is situated, and site 5 at the Stilfontein endpoint. Green arrows indicate the bulk water sampling sites.

Water is pumped from the Vaalriver. The quality of the raw water is largely dependent on seasonal changes, as rainfall affects the mineral and nutrient levels as well as the turbidity of the water. Water from the Vaalriver is considered “hard” water due to the presence of a number of minerals such as magnesium and calcium. The water is then treated by the addition of chemicals in the form of flocculants. Dissolved air flotation follows for the removal of particles such as algae and organic material. Water is then ozonated to oxidize organic matter and dissolved matter. Flocs are then subjected to sedimentation to remove any remaining particles. The next step involves filtration of the water to remove small particles not removed by sedimentation. Disinfectant in the form of chlorine is then added. Water is stored and distributed (www.midvaalwater.co.za/home.html#Overview).

Pigging used to be the preferred method for cleaning the distribution system. This was done once a year. The pig consisted of a sponge, scraping the pipes from unwanted build up and flushing the water to the next reservoir. Pigging was however stopped as new pipes contained valves not allowing the pig to be pushed through (Mr Jan Pietersen, Chief Technical Manager, Midvaal Water Company). Five sites were designated downstream from the treatment plant of the Midvaal water distributions system for biofilm sampling. The sites are indicated by red arrows in Figure 3.1. Site 1 is the closest to the treatment plant and site 5 is the furthest distance away. Bulk water sampling areas are indicated by green arrows (Figure 3.1).

3.2 Sample collection and culture conditions

Swab samples were taken using sterile cotton swabs (Charis Laboratory Supplies, SA). The swabs were submerged in nutrient broth (Merck, US) and spread-plated on R2A agar (Sigma Aldrich, US). Plates were incubated at 37°C for 2 days. Colonies were selected based on differences in colony morphology and purified by streak plating on new R2A agar. Plates were incubated at 37°C for 2 days. These steps were repeated 3 times.

3.3 Production of haemolysin on blood agar

Isolates were aseptically streaked onto 5% sheep blood agar plates (National Health Laboratories, SA) as the first measure in establishing the pathogenic potential of the HPC isolates. Blood agar plates (National Health Laboratories, SA) were incubated at 37°C for 24h. β-haemolysin production was indicated by clear zones around the bacterial colonies, α-haemolysin production was indicated by a green zone around the bacterial colonies and γ-haemolysis referred to no haemolysis (Atlas, 1997; Pavlov *et al*., 2004).

3.4 Enzyme production

Infectious micro-organisms possess virulence factors assisting in the pathogenicity of the micro-organism (Edberg *et al*., 1996; Pavlov *et al*., 2004). HPC isolates that produced α- and β-haemolysin were selected to test for the production of various extracellular enzymes.

3.4.1 Proteinases

A growth medium was prepared by first preparing 50ml of a 3% (w/v) skim milk (Oxoid, UK) solution, 50ml Brain Heart Infusion Broth (Merck, US) and 100ml agar (Merck, US). These were then mixed and poured into plates. The organisms were spot inoculated onto the plates and incubated at 37°C for 48h. A positive result was seen as a clear zone around the inoculation spot.

3.4.2 DNase

DNase agar (Merck, US) was prepared according to manufacturers specifications and supplemented with (0.01%) toluidine blue O (Sigma Aldrich, US). Plates were incubated for 48 h at 37°C after which it was flooded with a 0.1% 1M HCl (Merck, US) solution. A positive result was indicated by a zone of clearing or a pink halo around the bacterial colonies.

3.4.3 Lipase

Tryptone soy agar (Merck, US) was prepared and supplemented with 1% Tween 80 (Sigma Aldrich, US). A turbid halo around the bacterial colony indicated a positive result.

3.4.4 Hyaluronidase

Brain Heart Infusion Broth (Merck, US) was prepared to which 1.0g of Noble agar (Difco Laboratories, US) was added for each 100ml. Minisart filter units (0.20-µm; Separations, US) were used to filter sterilize an aqueous solution of 5% bovine albumin fraction V (Sigma Aldrich, US) and 2mg/ml of hyaluronic acid (Sigma Aldrich, US). A positive result was indicated by a zone of clearing around the bacterial colonies.

3.4.5 Chondroitinase

The growth medium was prepared by adding 1g of Noble agar (Difco Laboratories, US) to 100 ml of Brain Heart Infusion Broth (Merck, US). This was supplemented with 5% bovine albumin fraction V (Sigma Aldrich, US) and 4mg/ml chondroitin sulphate A obtained from bovine trachea (Sigma Aldrich, US). Both these aqueous solutions were sterilized using 0.20-µm Minisart filter units (Separations, US). A zone of clearing around the bacterial colonies was indicative of a positive result.

3.4.6 Lecithinase

Bacto McClung Toabe Agar Base (Difco Laboratories, US) was supplemented with a 50% egg yolk (Merck, US) solution at a 9:1 ratio to serve as the growth media in determining the production of lecithinase. A positive result was indicated by a white precipitate around or beneath the spot of inoculation.

3.5 Antibiotic susceptibility tests

The Kirby-Bauer quality-controlled disk diffusion method (Raphael *et al*. 1983; Atlas, 1997) was used to test for the antibiotic susceptibility of the haemolytic positive HPC isolates. The following antibiotics were tested; Oxytetracycline 30µg, Streptomycin 300µg, Vancomycin 30µg, Chloramphenicol 30µg, Trimethoprim 2.5µg, Ampicillin 10µg, PenicillinG 10 units, Kanamycin 30µg, Ciprofloxacin 5µg and Erythromycin 15µg (Mast Diagnostics, UK). The HPC isolates displaying resistance to the antibiotics were recorded.

3.6 Identification of the HPC isolates

Gram staining was performed to group the organisms into Gram-positive or Gram-negative. Nineteen HPC isolates were selected for identification according to enzyme production. Isolates producing two or more extracellular enzymes were identified. The BD BBL CRYSTAL^{TM} Gram-Positive (GP) Identification (ID) system was used for the identification of the organisms.

3.7 16S rRNA gene sequencing

The identification of the selected organisms obtained with the BBL CRYSTAL^{TM} GP ID system was confirmed using 16S rRNA gene sequences.

3.7.1 DNA isolation

Overnight cultures were obtained by inoculating nutrient broth (Merck, US) with each isolate and incubating it at 37°C for 18h. A genomic DNA isolation kit (Nexttec, Germany) was used to extract the DNA using the protocol of the manufacturer. The protocol consisted of two main steps, lysis of the cells and purification of the DNA. Lysis was achieved by the addition of two lysis buffers, lysozyme and RNase A. DNA was purified by centrifugation of the lysate through a column. DNA concentrations and quality (A_{260nm}:A_{280nm} ratios) were determined by a NanoDropTM 1000 Spectrophotometer (Thermo Fischer Scientific, US).

3.7.2 Agarose gel electrophoresis of extracted DNA

The integrity of the DNA was determined by electrophoresis of 5µl of the extracted DNA mixed with 5µl 6 x Orange Loading dye (Fermentas Life Science, US). A 1% (w/v) agarose gel in 1 x TAE buffer (20mM Acetic acid (Merck, US), 40mM Tris (Sigma Aldrich, US) and 1mM EDTA (Merck, US), pH 8.0) was used. Electrophoresis conditions were set at 80V for 40-60 min. Bands were visualized under a UV light with the addition of 10mg/ml EtBr (Bio-Rad, UK) to the agarose gel.

3.7.3 DNA amplification

An ICycler thermal cycler (Bio-Rad, UK) was used to amplify the DNA. A final reaction volume of 25µl consisted of 12.5µl double strength PCR master mix (0.05U/µl *Taq* DNA Polymerase in reaction buffer, 0.4mM of each dNTP, 4mM $MgCl_2$) (Fermentas Life Science, US), 11µl PCR-grade water (Fermentas Life Science, US), 100ng sample DNA and 0.5µl 27F and 1492R primer mix (Table 3.1; Inqaba Biotech, SA). Cycling conditions were set at 95°C for 300 sec denaturing; 30 sec at 95°C for melting, 30 sec at 52°C for annealing, 60 sec at 72°C for extension, these conditions were repeated for 35 cycles; final extension was done at 72°C for 600 sec.

Table 3.1: Primer sets employed for this study

Gene	Primer Sequence	Size (bp)	Reference
16S rRNA GC	**341F-GC**: 5'-CGCCCGCCGCGCGCGGCGGGCGGGGC GGGGGCACGGGCCTACGGGAGGCAGCAG-3'	566	Muyzer *et al.*, 1993
	907R: 5'-CCGTCAATTCCTTTGAGTTT-3'		
16S rRNA	**27F:** 5'-AGAGTTTGATCMTGGCTCAG-3'	1465	Lane, 1991
	1492R: 5'-TACGGYTACCTTGTTACGACTT-3'		

3.7.4 Agarose gel electrophoresis of PCR products

Confirmation of DNA amplifications was done by electrophoresis of PCR products on a 1.5% (w/v) agarose gel (PeQlab, Germany) in 1 x TAE buffer [20mM Acetic acid (Merck, US), 40mM Tris (Sigma Aldrich, US) and 1mM EDTA (Merck, US) at pH 8.0]. The gel contained 10mg/ml of ethidium bromide (Bio-Rad, UK). Bands were visualized under a UV light. A mixture of 5µl PCR product and 5µl 6 x Orange Loading dye (Fermentas Life Science, US) was loaded into each well of the gel. A 100bp molecular marker (O'GeneRuler, Fermentas Life Science, US) was used to confirm the fragment sizes by loading it into each gel. Electrophoresis conditions were set at 80V for 40-60 min. A Gene Bio Imaging System (Syngene, Synoptics, UK) and GeneSnap (version 6.00.22) software was used to capture the gel images (Bezuidenhout *et al*., 2006).

3.7.5 PCR clean-up for sequencing

A PCR clean-up kit (NucleoSpin Extract II, Separations, US) was used to remove any unwanted products resulting from the PCR process. The quality of the PCR product after cleansing was determined using a NanoDropTM 1000 Spectrophotometer (Thermo Fischer Scientific, US). A 260:280 ratio, of between 1.7 and 1.9 was deemed acceptable values.

3.7.6 Second round amplification for sequencing

The second round of amplification was performed by using a Cycle Sequencing BigDye Terminator Kit (Zymo Research, US). The reaction mixture had a final volume of 20µl consisting of 4µl Ready Reaction Premix (2.5X), 2µl BigDye Sequencing Buffer (5X), 3.2pmol 27F primer (Inqaba Biotech, SA), 1µl Template (10-40ng) and 9.8µl PCR-grade water (Fermentas Life Science, US). Cycle conditions were set at 96°C for 60 sec; 25 cycles of 10 sec of denaturation at 96°C, 5 sec of annealing at 50°C and 240 sec of extension at 60°C after which it is kept at 4°C for 600 sec.

3.7.7 Sequencing

Amplicons were sequenced by Karen Jordaan (North-West University, Potchefstroom Campus, South Africa) using an ABI 3130 Genetic Analyzer (Applied Biosystems, UK). Chromatograms were viewed with Geospiza Finch TV (version 1.4) software. Amplified sequences were identified using BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST).

3.8 Transmission electron microscopy

Overnight cultures on nutrient agar (Merck, US) were used for transmission electron microscopy. A suspension of bacteria was transferred to a grid and stained with 1 % uranyl acetate. Bacteria were examined with a Philips CM10 Transmission Electron Microscope. Micrographs were taken at different magnifications ranging from 15 500 to 27 000.

3.9 Analysis of bulk water

3.9.1 Collection of water samples

Water samples were collected in sterile 1 litre glass bottles from outside service connections at 2 sites close to the sites where swab samples were taken (Figure 3.1). On site measurements were taken for dissolved oxygen, pH, temperature and electric conductivity using a multi probe (Multi 350i/Set, Merck, Germany). Water samples were transported to the lab in a thermo-insulated container at 4°C. The membrane filter procedure was used to filter 700ml water through 0.45-µm-pore-size membrane filters (Separations, US).

3.9.2 DNA extraction from membrane filters

Membrane filters were placed in sterile 100ml glass bottles. Glass beads and 3ml lysozyme (10mg/ml) were added and incubated at 37°C for 10 min in a shaking incubator. Three millilitre (ml) proteinaseK (10 mg/ml) was added and incubated at 37°C, for 30 min, in a shaking incubator. The same DNA extraction kit and procedure was followed as described in Section 3.7.1.

3.10 Amplification of biofilm and pure culture DNA

The same procedure was followed as described in Section 3.7.3 with the exception of the addition of 0.5µl $MgCl_2$ (Fermentas Life Science, US) and 0.5µl butane (Sigma Aldrich, US) to the bulk water samples reducing the PCR-grade water to 10µl. A 566bp 16S primer mix (Inqaba Biotech, SA) containing a GC-clamp was used (Table 3.1).

3.11 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was performed to establish whether HPC isolates that were present in the biofilm were also present in the bulk water of a drinking water distribution system. Four of the pure cultures from the biofilm were used to construct a ladder against which the bulk water samples could be tested.

DGGE analysis was done using a DCode Universal Mutation Detection System (Bio-Rad, UK). An 8% (w/v) acrylamide gel was used for electrophoresis at 80V for 16h. A 40-60% denaturing gradient was used. Staining was done using 1 x TAE containing ethidium bromide (10µg/ml) (Bio-Rad, UK) for visualization of bands. Gene Genius Bio Imaging System (Syngene, Synoptics, UK) and GeneSnap software (version 6.00.22) were used for visualization.

CHAPTER 4

RESULTS

4.1 Colony characteristics and growth

The samples from five sites selected for biofilm sampling were plated out on five R2A agar plates for each site. The sites displayed different degrees of growth and varied colony characteristics. Results were noted according to the distance from the treatment plant, with site 1 closest to the treatment plant, and site 5 the furthest distance away from the treatment plant.

The R2A plates from site 1 displayed very little growth. Either a few colonies or a single colony were observed on the plates. Only 2 morphotypes were observed. The second most growth was observed on plates of site 2. However, there were at least 4 morphotypes, not all the plates had equal growth. The third most growth was observed on plates from site 3. Two morphologies were observed on these plates. The majority were yellow. No growth was observed on any of the plates from site 4. The R2A plates from site 5 displayed the most growth. At least 5 different morphotypes were observed on the 5 plates. Colonies varied from small and large white colonies, cream colonies to small yellow and orange colonies. A total of 56 colonies were purified. Details for each of the sites are listed in Table 4.1.

4.2 Haemolysin assay

Fifty six pure colonies from all the biofilm sampling sites were tested for the production of haemolysin. Thirty one of the HPC isolates displayed β-haemolysis indicated by a zone of clearing around the bacterial colony. Twenty five HPC isolates displayed γ-haemolysis which indicated no haemolysis. None of the isolates displayed α-haemolysis. Results are displayed in Table 4.1.

Table 4.1: Results obtained for haemolysin production for each individual site**.**

Sample	Number streaked out	Number β-Haemolysis	Number γ-Haemolysis
Site 1	10	4	6
Site 2	12	8	4
Site 3	12	7	5
Site 5	22	12	10

The number of isolates streaked out for each site was to represent the different morphotypes obtained on the R2A agar. For sites 2, 3 and 5 more than 50% of the isolates displayed β-haemolysis. Forty percent of the isolates from site 1 displayed β-haemolysis.

4.3 Extracellular enzyme production

The ability of the 31 β-haemolytic HPC isolates to produce enzymes was tested against a group of 6 enzymes. These enzymes include DNase, lipase, proteinase, hyaluronidase, chondroitinase and lecithinase and methods are described in Section 3.4. The results are depicted in Figure 4.1.

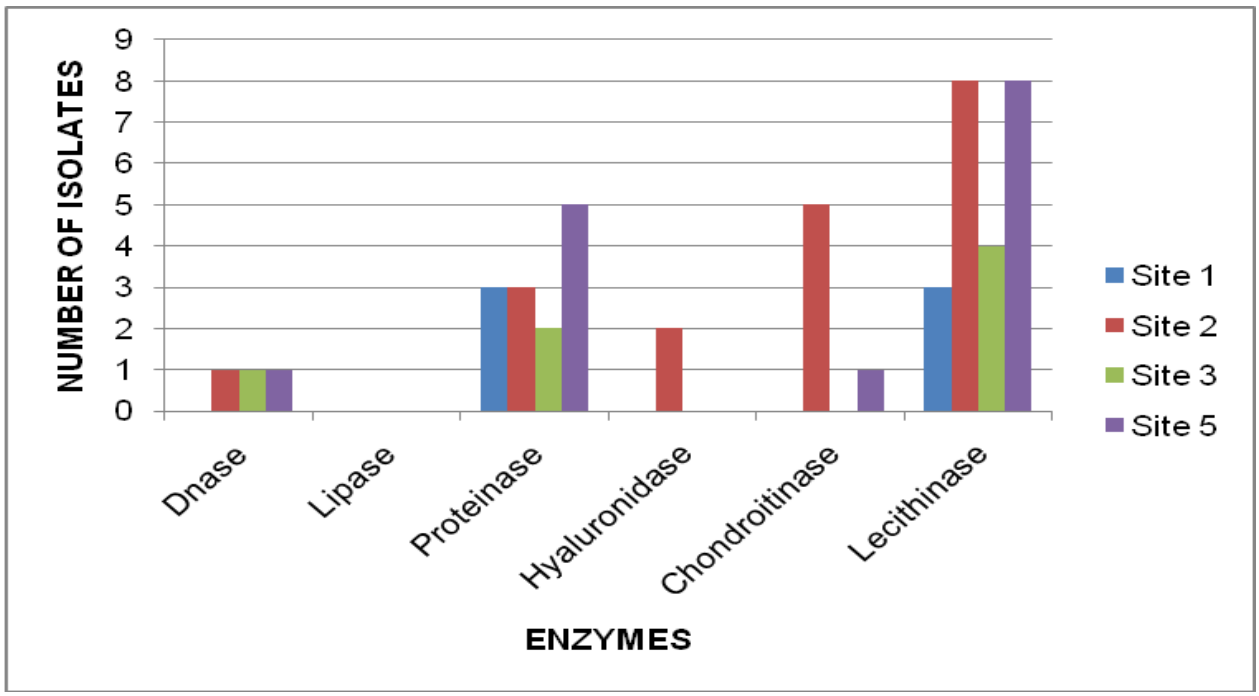

Figure 4.1: The number of isolates testing positive for each enzyme at the individual sites.

Lecithinase was produced by isolates from all four sites, three isolates from site 1, eight isolates from site 2, four isolates from site 3 and eight isolates from site 5 (Figure 4.1). Lipase was not produced by any of the isolates at any site. DNase was produced by a single islolate from sites 2, 3 and 5. Proteinase was produced by three isolates from sites 1 and 2, two isolates from site 3 and five isolates from site 5 (Figure 4.1). Hyaluronidase was only produced by two isolates from site 2. Chondroitinase was produced by five isolates from site 2 and one isolate from site 5 (Figure 4.1).

The overall percentages of isolates that produced the various enzymes varied. Of the 31 haemolytic positive isolates, DNase was produced by 9.7%, proteinase by 41.9%, hyaluronidase by 6.5%, chondroitinase by 19.4% and lecithinase by 87.1%. The individual production of enzymes by the nineteen identified isolates is depicted in Table 4.2. All the isolates except for one produced 2 enzymes. Isolate LV 19 from site 2 produced four enzymes. Site 2 was the most diverse with 5 different enzyme expression profiles. Site 1 had only one expression profile. Site 3 had two expression profiles and site 5 had three expression profiles. The most dominant enzyme profile was P, Le (Table 4.2).

4.4 Antibiotic susceptibility of the HPC isolates

The antibiotic test results were recorded for haemolytic HPC isolates only. All 31 isolates were resistant to oxytetracycline 30µg, trimethoprim 2.5µg and penicillinG 10 units. Large numbers were resistant to vancomycin 30µg (96.8%), chloramphenicol 30µg (74.2%), ampicillin 10µg (96.8%) and kanamycin 30µg (93.5%). More than half the isolates were resistant to ciprofloxacin 5µg (54.8%). The lowest numbers of resistance were observed for streptomycin 300µg (22.6%) and erythromycin 15µg (16.1%).

The antibiotic phenotypes for the individual isolates that were identified are presented in Table 4.2. The most observed antibiotic phenotype for isolates from site 1 was AMP, KAN, PEN, OT, VAN, TM. Isolates from site 2 displayed the same phenotype with the exception of two isolates being also resistant to ERY. One *Bacillus thuringiensis* isolate with a phenotype of AMP, KAN, PEN, ERY, OT, STR, VAN, CHL, TM, was also the only one that produced four enzymes. Isolates from site 3 displayed resistance to CHL and one isolate to CIP. The dominant antibiotic phenotype for isolates from site 5 was AMP, KAN, PEN, OT, VAN, CHL, TM with the exception of two isolates being resistant to ERY and *Arthrobacter oxydans* displaying resistance to STR.

4.5 Identification of HPC isolates with a biochemical test method

Gram staining was done prior to identification of the isolates to determine the type of identification system to be used. All the isolates were Gram-positive. The nineteen isolates that produced two or more extracellular enzymes were subjected to identification. A “No identification” was obtained for four of the isolates when BBL Crystal^{TM} GP ID system was used. 16S rRNA gene sequencing were used for conformation and comparison to the results obtained with the BBL Crystal^{TM} GP ID system. Organisms were identified as *Bacillus licheniformis, Bacillus subtilis, Bacillus cereus, Bacillus megaterium, Bacillus pumilus* and *Kocuria rosea.* Unidentifiable isolates had to be identified with 16S rRNA gene sequencing. Results are indicated in Table 4.2.

Table 4.2: A summary for the results obtained for the isolates from each site in terms of the identification with the biochemical test method and 16S rDNA sequencing, enzyme production and antibiotic phenotypes.

Site	Isolate	*BBL Crystal ID*	*Sequencing ID*	D	L	H	C	P	Le	Antibiotic phenotype
1	LV 2	*Bacillus licheniformis*	*Bacillus thuringiensis*					X	X	AMP,KAN,PEN,OT,VAN,TM
	LV 5	*Bacillus subtilis*	*Bacillus thuringiensis*					X	X	AMP,KAN,PEN,OT,VAN,TM
	LV 17	*No identification*	*Exiguobacterium acetylicum*					X	X	AMP,KAN,PEN,OT,VAN,TM
2	LV 4	*Bacillus cereus*	*Bacillus cereus*				X		X	AMP,KAN,PEN,OT,VAN,TM
	LV 10	*Bacillus cereus*	*Bacillus cereus*			X			X	AMP,KAN,PEN,ERY,OT,VAN,TM
	LV 11	*No identification*	*Bacillus thuringiensis*			X			X	AMP,KAN,PEN,OT,VAN,TM
	LV 15	*Bacillus cereus*	*Bacillus cereus*					X	X	AMP,KAN,PEN,OT,VAN,TM
	LV 16	*Bacillus cereus*	*Bacillus cereus*				X		X	AMP,KAN,PEN,OT,VAN,TM
	LV 18	*Bacillus cereus*	*Bacillus cereus*				X		X	AMP,KAN,PEN,ERY,OT,VAN,TM
	LV 19	*No identification*	*Bacillus thuringiensis*	X			X	X	X	AMP,KAN,PEN,ERY,OT,STR,VAN,CHL,TM
3	LV 7	*Bacillus megaterium*	*Bacillus megaterium*					X	X	AMP,PEN,OT,VAN,CHL,TM
	LV8	*Bacillus cereus*	*Bacillus cereus*	X					X	AMP,CIP,K,PEN,OT,VAN,CHL,TM
5	LV 1	*Bacillus cereus*	*Bacillus cereus*					X	X	AMP,KAN,PEN,OT,VAN,CH,TM
	LV 3	*Bacillus cereus*	*Bacillus cereus*					X	X	AMP,KAN,PEN,ERY,OT,VAN,CHL,TM
	LV 6	*Bacillus subtilis*	*Bacillus thuringiensis*					X	X	AMP,KAN,PEN,OT,VAN,CHL,TM
	LV 9	*Bacillus pumilus*	*Bacillus pumilus*	X			X			AMP,KAN,PEN,OT,VAN,TM
	LV 12	*Bacillus cereus*	*Bacillus cereus*					X	X	AMP,KAN,PEN,OT,VAN,CHL,TM
	LV 13	No identification	*Bacillus thuringiensis*				X		X	AMP,KAN,PEN,ERY,OT,VAN,TM
	LV 14	*Kocuria rosea*	*Arthrobacter oxydans*					X	X	AMP,KAN,PEN,OT,STR,VAN,CHL,TM

***D-DNase, L-Lipase, H-Hyaluronidase, C-chondroitinase, P-Proteinase, Le-Lecithinase, AMP-Ampicillin, KAN-Kanamycin, PEN-Penicillin, OT-Oxytertracycline, ERY-Erythromycin, VAN-Vancomycin, STR-Streptomycin, TM-Trimethoprim, CHL-Chloramphenicol.**

4.6 DNA extractions

4.6.1 DNA extraction of pure HPC cultures

Genomic DNA was extracted from nineteen pure overnight cultures, isolated from biofilm samples, incubated in nutrient broth using the genomic DNA isolation kit from Nexttec (Section 3.8.1). Figure 4.2 is an ethidium bromide stained 1% (w/v) gel. It illustrates the quantity and quality of the DNA extracted. The quantity of the extracted DNA was rather low as observed on the gel, especially for samples in lanes 4, 5, 9, 10 and 19. There was, however, no fragmentation or RNA observed.

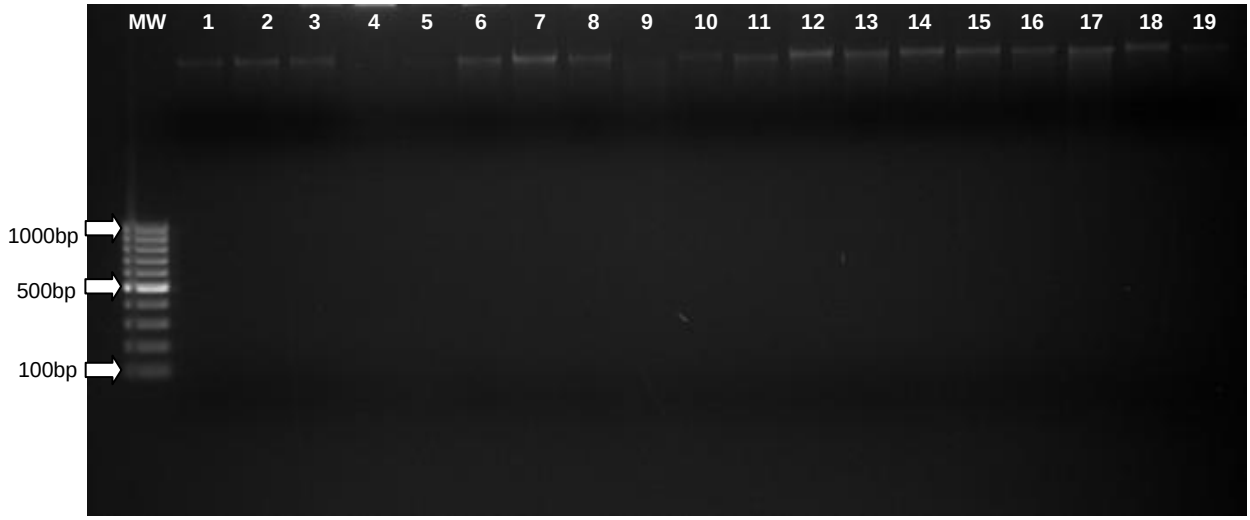

Figure 4.2: An ethidium bromide stained agarose gel (1% w/v) indicating DNA isolated from pure cultures.

These results were confirmed by determination of A_{260nm}:A_{280nm} ratios and DNA concentrations making use of a NanoDropTM 1000 Spectrophotometer. The 260:280 ratios varied between 1.30 and 1.50. Low 260:280 ratios normally indicate protein contaminations. DNA concentrations ranged between 150 and 400ng/µl.

4.6.2 DNA extraction from bulk water samples

DNA was extracted as described in Section 3.9.2. Due to the very low concentrations of DNA, bands could not be visualized on agarose gel. Results are thus not shown. Very low 260:280 ratios were obtained in the range of 0.70 to 0.90. DNA concentrations varied from 45 to 70ng/µl.

4.7 DNA amplification

4.7.1 DNA amplification of HPC isolates for sequencing

Isolated bacterial genomic DNA was amplified using PCR under conditions described in Section 3.7.3. The genomic DNA of the isolates amplified without any problems when 16S primer sets (Table 3.1) were used. Amplification products were of the expected (1465bp) size (Figure 4.3).

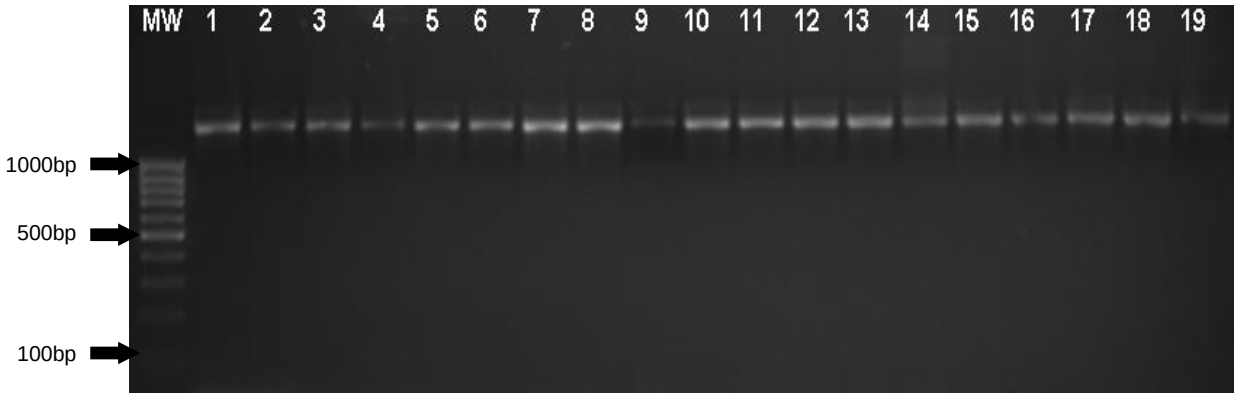

Figure 4.3: A 1.5% (w/v) agarose gel stained with ethidium bromide illustrating the amplified products of the nineteen pure cultures. MW represents the 100bp molecular size marker (O'GeneRuler^{TM} 100bp DNA ladder, Fermentas Life Science, US). Lanes 2, 5 and 17 contain samples from sampling site 1. Lanes 4, 10, 11, 15, 16, 18 and 19 contain samples from sampling site 2. Lanes 7 and 8 contain samples from sampling site 3. Lanes 1, 3, 6, 9, 12, 13 and 14 contain samples from sampling site 5.

Product yield was very similar for all the isolates except for isolates in lanes 4, 9 and 19. Although product yield was lower for these isolates, it was still sufficient for sequencing

analysis. Very little traces of smears were observed, thus no optimization of the amplification process was needed. No primer dimers or non-specific products were observed under the amplification conditions described in Section 3.7.3.

4.7.2 DNA amplification of bulk water samples and pure cultures for DGGE

Isolated bacterial DNA, from both the pure cultures and bulk water samples, were amplified under conditions described in Section 3.10. Fragment lengths were of the expected size (566bp) when 16S primer set (Table 3.1) was used.

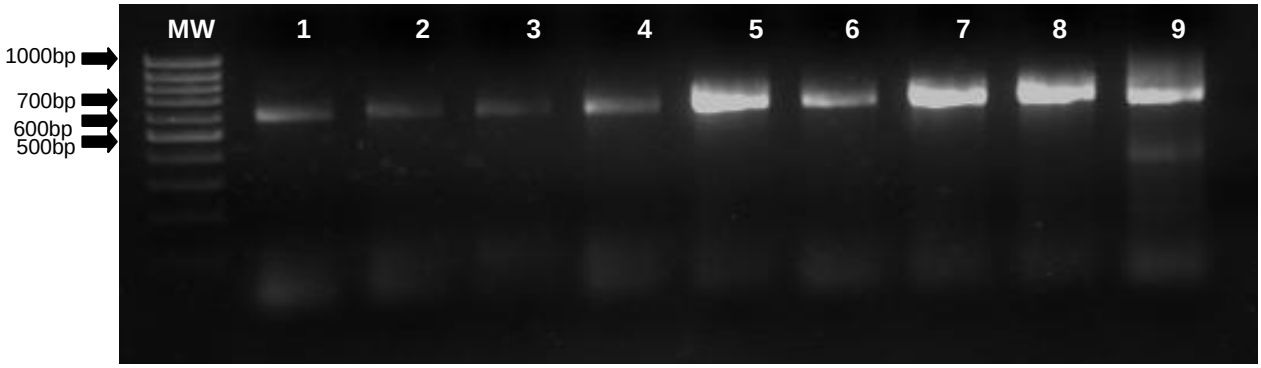

Figure 4.4: A 1.5% (w/v) agarose gel stained with ethidium bromide illustrating the amplified products of the bulk water samples and pure cultures of the biofilm samples. MW represents the 100bp molecular size marker (O'GeneRuler^{TM} 100bp DNA ladder, Fermentas Life Sciences, US). Lanes 1 and 2 contain bulk water samples from site 1. Lanes 3 and 4 contain bulk water samples from site 2. Lane 5 contains *Bacillus thuringiensis* (pure culture 2). Lane 6 contains *Bacillus cereus* (pure culture 4). Lane 7 contains *Bacillus pumilus* (pure culture 9). Lane 8 contains *Arthrobacter oxydans* (pure culture 14). Lane 9 contains *Exiguobacterium acetylicum* (pure culture 17).

Product yield was satisfactory for bulk water samples (lanes 1 to 4) using the protocol described in Section 3.10. High product yield was obtained for pure cultures in lanes 5 to 9. Primer dimers were present for all the samples despite an increased annealing temperature and decreased quantity of primer used. The presence of non-specific products can be observed in lane 9, even after multiple optimization and clean-up attempts.

4.8 Sequencing

The sequencing reaction was done as described in Sections 3.7.6 and 3.7.7. DNA as illustrated in Figure 4.3 (Lane 1 to 19) was used for the sequencing reaction. GenBank was used to identify the amplified sequences by BlastN searches (http://www.ncbi.nlm.nih.gov/BLAST). Approximately 450 nucleotides (Table 4.3) were selected to obtain identifications. Identifications based on the 16S rRNA gene are illustrated in Table 4.3 for each of the different sites sampled. BlastN searches revealed the domination of *Bacillus* spp. within the biofilm. Background noise was very low for all the sequences. Exact matches were obtained for 15 of the isolates and high sequence similarities were obtained for the remaining 4 isolates (Table 4.3). These identifications can be considered very close to definite species obtained. All the E values were below 0, thus indicating high similarity between the input sequences and the matched sequences (http://www.ncbi.nlm.nih.gov/). The identities obtained were not all in accordance with identities obtained with the BBL Crystal^{TM} GP ID system (Table 4.3). Identification systems can be observed in Table 4.3. Potential reasons for these conflicts will be discussed in Chapter 5 (Section 5.5.2)

Table 4.3: GenBank identification of the amplified pure samples from the different sites

Samples	Nucleotides used	Background noise		Genbank ID	% Similarity	Sequence ID	E value
		Yes	No				
Site 1							
LV 2	120 – 590		X	*Bacillus thuringiensis* strain CCGE 2286 EU867374.1	100	508/508	0.0
LV 5	100-575		X	*Bacillus thuringiensis* strain ZJOU-010 GU384894.1	100	475/475	0.0
LV 17	100-570		X	*Exiguobacterium acetylicum* strain TSWCSN13 GQ284374.1	100	470/470	0.0
Site 2							
LV 4	90-590		X	*Bacillus cereus* strain YH-10-2 GU384894.1	100	371/371	0.0
LV 10	110-560		X	*Bacillus cereus* strain MUJ GU325714.1	100	451/451	0.0

LV 11	110-590	X	*Bacillus thuringiensis* strain ML10 GU562000.1	100	482/482	0.0
LV 15	100-590	X	*Bacillus cereus* strain YUPP-4 FJ696709.1	100	491/491	0.0
LV 16	90-560	X	*Bacillus cereus* strain w10 GU332642.1	100	472/472	0.0
LV 18	100-570	X	*Bacillus cereus* strain EB16 GU321330.1	100	471/471	0.0
LV 19	95-580	X	*Bacillus thuringiensis* strain Pd1T GU391524.1	99	488/490	0.0
Site 3						
LV 7	100-577	X	*Bacillus megaterium* strain EH44 GU339272.1	99	477/480	0.0
LV 8	110-570	X	*Bacillus cereus* strain ZQP3 GU384226.1	100	461/461	0.0

Site 5						
LV 1	120-590	X	*Bacillus cereus* strain SU-6 GU395988.1	100	486/486	0.0
LV 3	120-510	X	*Bacillus cereus* strain YM-4-6 GU369812.1	99	386/388	0.0
LV 6	90-580	X	*Bacillus thuringiensis* strain CCGE2049 EU867367.1	100	491/491	0.0
LV 9	90-580	X	*Bacillus pumilus* strain EH62 GU339301.1	100	471/471	0.0
LV 12	90-530	X	*Bacillus cereus* strain JS-2-2 GU369809.1	100	441/441	0.0
LV 13	120-580	X	*Bacillus thuringiensis* strain 1P03AC EU977819.1	100	461/461	0.0
LV 14	110-580	X	*Arthrobacter oxydans* strain ZF3 EU709759.1	95	450/470	0.0

4.9 Transmission electron microscopy (TEM)

TEM was performed to differentiate between the morphologies on species level for confirmation of BBL Crystal^{TM} GP ID system and sequencing results. The method is explained in Section 3.8.

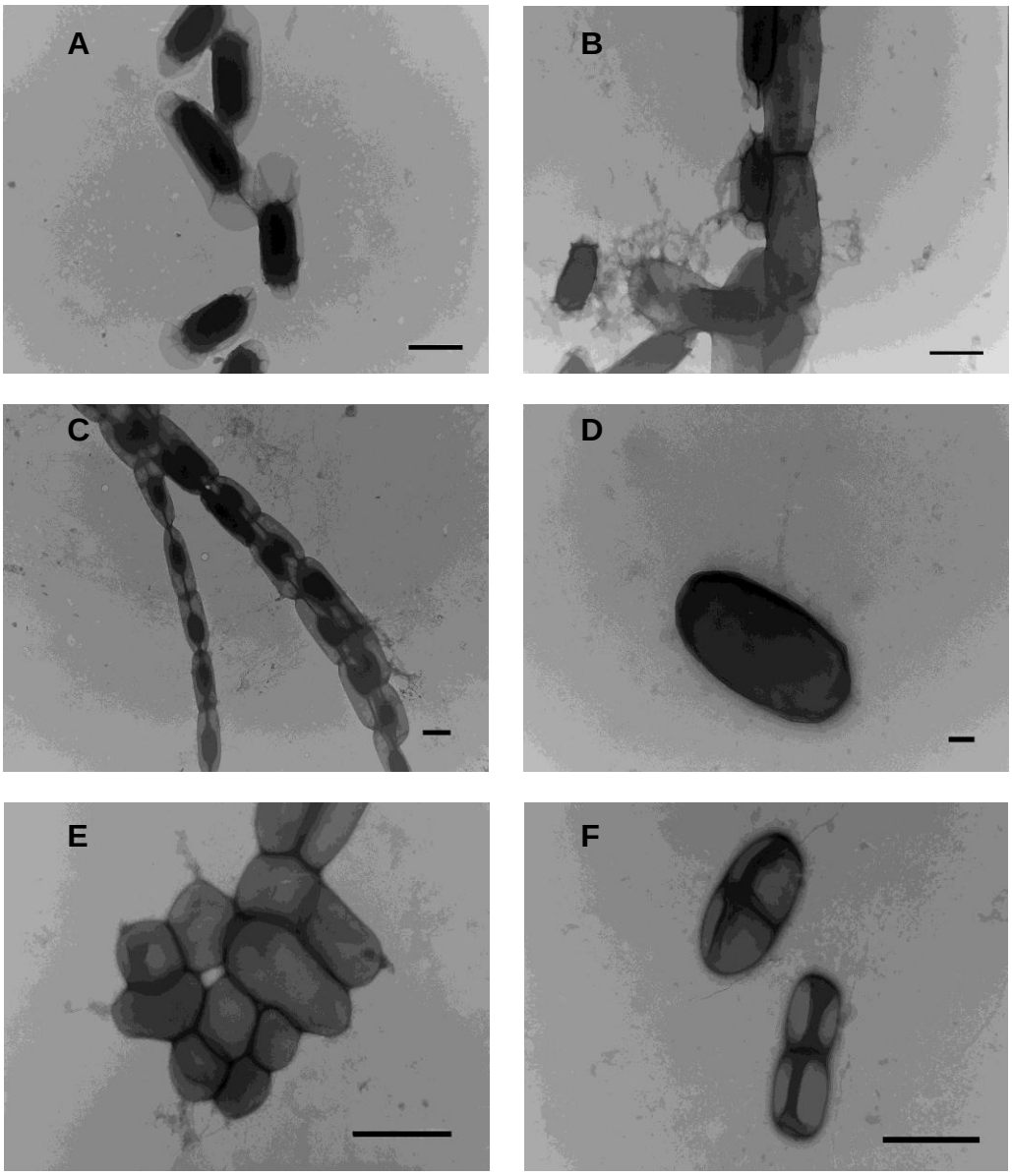

Figure 4.5: Transmission electron microscope photos illustrating morphological differences between various species. A – *Bacillus thuringiensis,* B – *Bacillus cereus,* C – *Bacillus megaterium,* D – *Bacillus pumilus,* E – *Arthrobacter oxydans* and F – *Exiguobacterium acetylicum.* The bar represents 0.3 - 1µm.

From the micrographs it is evident that all cells were rod-shaped. *Bacillus thuringiensis* (Figure 4.6A) occurred in clusters while *Bacillus cereus* (Figure 4.6B) isolates had a more chain-like structure. Endospores could be observed on the micrographs in Figure 4.6C. *Bacillus megaterium* was arranged in very neat chains. *Bacillus pumilus* were observed as remote single cells (Figure 4.6D). *Arthrobacter oxydans* displayed a more spherical shape than the normal rod shape that would be expected (Figure 4.6E). *Exiguobacterium acetylicum* was also observed as rod shaped cells (Figure 4.6F).

4.10 On site quality measurements of bulk water samples

The physical properties of the bulk water samples were determined on site using a multi probe analyser. The two sites selected were in close proximity with the biofilm sampling areas. Bulk water at sampling site 1 had a pH of 8.17, the water temperature was measured at 21.1 °C, oxygen content of 8.18 mg/l and an electric conductivity of 88 mS/m. Bulk water sampling site 2 had a pH of 8.07, a temperature of 21.6 °C, an oxygen content of 8.65 mg/l and electric conductivity of 82.9 mS/m. These values were compared to SANS 241: 2006 as discussed in Chapter 5 (Section 5.7.1).

4.11 Denaturing gradient gel electrophoresis of bulk water samples and pure cultures

DGGE was employed to determine whether the HPC isolates present in the biofilm samples also occur in the bulk water. A 40-60% denaturing gradient polyacrylamide gel was employed for the analysis. The details of the procedure are described in Section 3.11. Results are depicted in Figure 4.7.

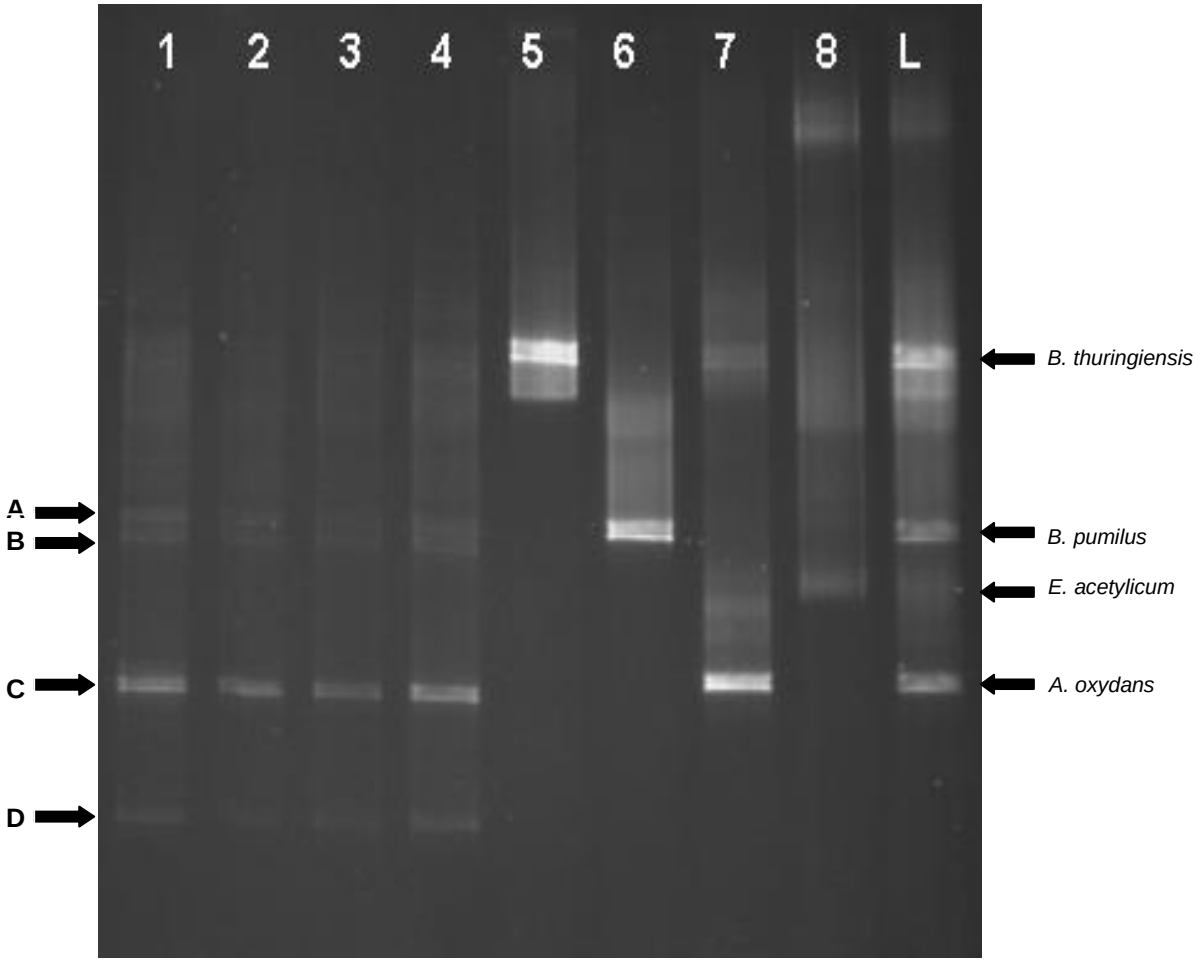

Figure 4.6: DGGE analysis of 500bp 16S rDNA fragments of bulk water samples and pure cultures isolated from biofilm samples. A 40-60% denaturing gradient was used on an 8% polyacrylamide gel. Electrophoresis was carried out at 100V for 16 hours. Lanes 1 and 2 – bulk water samples from site 1; lanes 3 and 4 – bulk water samples from site 2; lane 5 – *Bacillus thuringiensis;* lane 6 – *Bacillus pumilus;* lane 7 – *Arthrobacter oxydans;* lane 8 – *Exiguobacterium acetylicum;* and lane L contains a ladder constructed from all the pure cultures present in lanes 5 to 8.

Figure 4.7 depicts an ethidium bromide stained 8% (w/v) polyacrylamide DGGE gel. Four isolates from the biofilm samples were selected to construct the ladder based on their migration potential. These four isolates migrated to distinct positions on the gel. The isolates included *Bacillus thuringiensis, Bacillus pumilus, Arthrobacter oxydans* and *Exiguobacterium acetylicum.* The selection was made after initial trial runs on DGGE gels

(results not shown). *Bacillus cereus* and *Bacillus thuringiensis* migrated to the exact same position on the gel. Thus, *Bacillus cereus* was excluded for DGGE analysis.

Even though pure cultures were used in lanes 7 and 8, multiple bands could be observed. This could be due to non-specific products being present in the form of heteroduplexes and chimers. PCR conditions were optimized several times with no success of eliminating the non-specific products.

It is evident from the gel (Figure 4.7) that the HPC isolates found in lanes 6 (*Bacillus pumilus)* and 7 (*Arthrobacter oxydans*) were also present in bulk water samples (Lanes 1 to 4). The band representing *Exiguobacterium acetylicum* (Figure 4.7, Lane 8) was not present in the bulk water samples. The ladder constructed, confirmed the positions of the isolates on the gel. A band was observed at position D in Figure 4.7. This represents possibly a bacterial species that was present amongst the original isolates but that were not haemolytic. On the other hand, it could represent a completely different species that might be viable-but-not-culturable.

4.12 Summary

In this study, biofilm samples were collected at 5 different sites from the pipe lines of a drinking water distribution system. HPC bacteria were isolated on R2A agar. The pathogenic potential of these isolates were determined with the haemolysin assay and a series of enzymatic test. Results indicated that 31 of the 56 HPC isolates were able to lyse red blood cells. Five of the six enzymes, including DNase, proteinase, chondroitinase, hyaluronidase and lecithinase were produced by the HPC isolates from the four sites being evaluated. The highest number of isolates produced lecithinase and proteinase. No lipase was produced. The Kirby-Bauer quality-controlled disk diffusion method was employed to determine the resistance of the 31 haemolytic isolates against 10 antibiotics. The percentage resistance ranged from 100% to oxytetracycline 30 µg, trimethoprim 2.5 µg and

penicillin G 10 units to 16.1% for erythromycin 15 µg. The most frequently observed antibiotic phenotype was AMP, KAN, PEN, OT, VAN, TM.

Nineteen isolates were selected for identification based on their ability to produce two or more of the screened enzymes. Gram stains were performed to determine which biochemical test method should be used. All of the isolates were Gram-positive. The BBL Crystal^{TM} GP ID system was employed for the identification and results indicated the presence of *Bacillus cereus, Bacillus subtilis, Bacillus licheniformis, Bacillus pumilus* and *Bacillus megaterium* and *Kocuria rosea.* Four of the isolates could not be identified. 16S rRNA gene sequencing were performed to confirm the results obtained with the biochemical test method and to obtain identities for the unidentified isolates. *Bacillus subtilis* and *Bacillus licheniformis* were identified as *Bacillus thuringiensis* with 16S rRNA sequencing. *Kocuria rosea* was identified as *Arthrobacter oxydans.* One of the isolates that the BBL Crystal^{TM} GP ID system could not identify was identified by 16S rRNA gene sequences as *Exiguobacterium acetylicum.*

Bulk water samples were collected at two sites, in close proximity of the sites where biofilm samples were collected. DNA was extracted and amplified. DGGE was employed to determine whether HPC isolates present in the biofilm samples were also present in the bulk water of the water distribution system. Four HPC isolates from the biofilm samples were selected for ladder construction due to their electrophoresis migration differences. DGGE profiles revealed the presence of *Bacillus pumulis* and *Arthrobacter oxydans* and the possible presence of *Bacillus thuringiensis*. *Exiguobacterium acetylicum* was not present in the bulk water samples.

CHAPTER 5

DISCUSSION

5.1 Introduction

This study was concerned with the isolation of HPC bacteria from the biofilm of a section of a drinking water distribution system. The bacteria were isolated on R2A agar and selected based on colony morphology. They were purified by streak plating and tested for haemolytic capacity. Haemolytic positive isolates were then tested for pathogenic potential by determining their ability to produce certain virulence-associated enzymes. Only those that produced two or more of these enzymes were tested for antibiotic susceptibility and were identified using a biochemical method (BBL Crystal^{TM} GP ID system). Identifications were confirmed using 16S rRNA gene sequences as well as transmission electron microscopy. Furthermore, PCR-DGGE was used to demonstrate that some of the biofilm-associated bacteria species were also present in the bulk water.

5.2 Levels and diversity of HPC

The five sampling sites were at increasing distances from the water purification plant. Levels and diversity of HPC bacteria isolated from these sites varied. No HPC bacteria were isolated from site 4. It would be expected that the number of HPC bacteria adhering to the inner surfaces of the pipe lines would be constant throughout the distribution system as exposure to water would not vary topographically over a certain period of time. Studies have demonstrated that after only one year of constant exposure to drinking water one could expect a uniform biofilm along a distribution system (Van der Wende and Charaklis, 1990; Lethola *et al.,* 2004). Lethola *et al.* (2004) demonstrated that this was the case for copper and PE pipe lines, after 200 days microbial numbers were similar for both the materials. Van der Wende and Charaklis (1990) demonstrated that even if the surface roughness was different, it could be expected that over a period of time a biofilm would contain HPC bacteria of uniform level and diversity throughout a drinking water distribution system. According to Pietersen (Mr. Jan Pietersen, Chief Technical Manager, Midvaal Water Company) the

distribution line was older than 50 years. Considering this and the literature examples mentioned it was thus expected that a more uniform diversity at the various sites exists. The fact that no HPC bacteria were isolated from site 4, 6 km from the purification plant, was inconsistent with expected results. This could have been due to a sampling or a laboratory error.

A problem encountered by drinking water supply companies is the dissipation of disinfectants as it flows through the distribution system, allowing micro-organisms to multiply (LeChevallier *et al.,* 1980). This might have been a reason for HPC abundance being higher at site 5 than observed at site 1. Hoefel *et al.* (2005b) applied culture-independent techniques, such as PCR, DGGE, cloning and sequencing, to detect bacteria present in distribution systems due to the loss of chloramine residual during the transport of water. They discovered that no active bacteria were present as the water left the treatment plant, but 12 km downstream the chloramine residual levels had dropped and active bacteria could be detected. These culture-independent techniques can thus be applied by water treatment facilities to evaluate the efficacy of the disinfectants during the transport of water. In the current study, site 1 and site 5 were 10 km apart.

Different morphotypes were observed at each individual site but remained relatively similar in diversity through all the sites, indicating the presence of a possible regularity in the species diversity throughout the system. The most dominating morphologies were yellow, cream and white colonies of different sizes. R2A plates were only incubated at 37 °C as this study focused on the presence of potential human pathogens. The variety of HPC isolates were thus limited, compared to studies that incubated R2A plates at different temperatures (20 °C to 40 °C) to obtain all the HPC bacteria present within a system for quality control purposes.

5.3 Haemolysin assay and other extracellular enzyme production

The haemolysin assay was the first step in establishing the pathogenic potential of the isolated HPC bacteria. Thirty one of the 56 isolates displayed β-haemolysis which was

indicated by a zone of clearing around the bacterial colony. These isolates produce haemolysin. Lyses of red blood cells cause the release of haemoglobin (Todar, 2009), and is a cytotoxicity phenomenon (Edberg and Allen, 2004). The 31 isolates represented sites all the sites with the exception of site 4.

Analysis of HPC isolates possessing virulence factors, from drinking water, were done by Lye and Dufour (1991). They concluded that HPC isolates generally lack virulence factors. In 1994 Payment and co-workers also investigated the presence of virulence factors associated with HPC bacteria isolated on R2A media and blood agar. They found that HPC bacteria isolated on blood agar was more likely to possess virulence factors than HPC bacteria isolated on R2A media. Based on the conclusion of Payment *et al.* (1994), HPC bacteria isolated in the present study were tested for potential cytotoxicity by inoculating on blood agar. In this way non-haemolytic HPC bacteria could be eliminated.

Edberg and co-workers (1996) stated that a micro-organism can only be regarded as virulent if it possesses two or more extracellular virulence enzymes. They also mentioned that these extracellular enzymes might not be solely accountable for bacterial virulence but do indeed play a role in the pathogenic process. Several studies have investigated the production of extracellular enzymes because of the role they play in pathogenesis (Rogers, 1948; Kushner, 1956; Esselmann and Liu, 1961; Shah and Wilson, 1963; Smith and Willett, 1968; Janda and Buttone, 1981; Kouker and Jaeger, 1987; Lior and Patel, 1987; Calander *et al.,* 2003; Pavlov *et al.,* 2004). Most of these enzymes are involved in damaging host macromolecules including immunoglobulins, mucus, hyaluronic acid and lipoprotein membranes all of which are part of the host defence system. Invasion of tissues and body cells are thus mediated by these enzymes, enabling the pathogen to disrupt the functioning of cells (Edberg *et al.,* 1996).

Nineteen of the 31 haemolytic isolates produced two or more extracellular enzymes in this study. These included *Bacillus cereus, Bacillus megaterium, Bacillus thuringiensis, Bacillus pumilus, Arthrobacter oxydans* and *Exiguobacterium acetylicum.* Pavlov and co-workers

(2004) also isolated *Bacillus* species capable of producing two or more extracellular enzymes and rated them amongst the most virulent HPC bacteria isolated from their sampling sites. In a study by Molva *et al.* (2009) large numbers of isolates from *Bacillus cereus* and *Bacillus thuringiensis* produced DNase, lecithinase and protease. These enzymes were also produced by *Bacillus cereus* and *Bacillus thuringiensis* isolated from the biofilm samples in the current study. Boominadhan *et al.* (2009) investigated the protease production potential of *Bacillus* spp. They found that *Bacillus megaterium* was amongst the isolates that produced proteases. The production of protease enzyme by *Bacillus megaterium* was also observed in the current study.

Bacillus spp. are well known to produce the enzyme lecithinase (McGaughey and Chu, 1948; Colmer, 1948). This enzyme has been used to group some of the members of the genus *Bacillus* (Colmer, 1947). In the current study, lecithinase was produced by almost all isolates. Janda and Bottone (1981) observed that environmental isolates were more likely to produce lecithinase than clinical isolates. Thus, lecithinase production is not unusual for bacteria isolated from water samples. In the current study the *Bacillus pumilus* isolate did not produce lecithinase. This observation was also made by Hoult and Tuxford (1991) when two strains of *Bacillus pumilus* were tested for the production of lecithinase. One strain produced a very small zone of clearing around the inoculation spot after 24 hours of incubation. The other strain did not produce any zone even after a prolonged incubation period (Hoult and Tuxford, 1991).

More than 50% of isolates in the present study produced haemolysin and 34% of the isolates produced 2 or more other extracellular virulence-associated enzymes. The various species that produced these extracellular enzymes were isolated in studies on HPC from drinking water distribution systems in South Africa (Pavlov *et al.,* 2004) and elsewhere in the world (Edberg *et al.,* 1996). Furthermore, *Bacillus* spp. extracellular enzyme production profiles were similar to previous studies (Janda and Buttone, 1981; Hoult and Tuxford, 1991; Pavlov *et al.,* 2004; Molva *et al.,* 2009). Thus, results obtained in the present study were not uncommon and were previously reported. However, all these studies were concerned with

isolates from bulk water. The present study, on the other hand, was only concerned with HPC isolated from biofilms that occurred in a drinking water distribution system.

5.4 Antibiotic susceptibility of HPC isolates

The haemolytic positive HPC isolates were all resistant to oxytetracycline (30 µg), trimethoprim (2.5 µg) and penicillin G (10 units). Tetracycline prevents the tRNA from binding to the ribosome, specifically the A site, thus protein translation is inhibited. Inhibition is overcome by ribosomal mutations or resistance genes carried on plasmids or transposons (Snyder and Champness, 2003). Penicillin G is a β-lactam antibiotic. β-lactam antibiotics inhibit the synthesis of the cell wall by binding to the transpeptidase enzyme. The transpeptidase reaction cannot be catalyzed resulting in a weakened cell wall. Penicillin-resistant bacteria are able to produce β-lactamase enzyme which cleaves the β-lactam ring of penicillin. Ampicillin is semi synthetic penicillin. Thus, resistance to one may also result in resistance to the other. Vancomycin also affects cell wall synthesis (Madigan and Martinko, 2006). High numbers of isolates were resistant to these three antibiotics. Trimethoprim blocks the synthesis of precursors needed for DNA replication by inhibiting the enzyme dihydrofolate reductase. The cell is then depleted from tetrahydrofolate, a compound needed for a spectrum of biosynthetic reactions. Bacteria overcome this inhibition by having transposons or plasmids encoding for dihydrofolate reductase enzyme that is less sensitive to trimethoprim. Biosynthetic reactions can thus continue normally (Snyder and Chapness, 2003).

Only 16.1% of the haemolytic positive isolates were resistant to erythromycin (15 µg). Erythromycin is part of the macrolide antibiotics which binds to the 23S rRNA and by doing so inhibits protein translation. Transposons or plasmids carried by some bacteria confer resistance to the bacteria by methylating the 23S rRNA. The antibiotic will thus not be able to bind (Snyder and Chapness, 2003).

Pavlov *et al.* (2004) observed that lower percentages of HPC isolated by them were susceptible to ciprofloxacin (5 µg) and gentamicin (100 µg). In the current study 54.8% of the isolates were resistant to ciprofloxacin (5 µg). The difference in results could be ascribed to the micro-organisms occurring in biofilms for this particular study. Pavlov *et al.* (2004) however, isolated HPC bacteria from the bulk water. Brown and Gilbert (1993) concluded that the resistance of bacteria occurring in a biofilm may be extremely different from the same bacteria existing as a planktonic cell. These biofilm based bacteria may be more resistant to antibiotics (Korber *et al.,* 1997). The increased resistance may be achieved by the transfer of mobile genetic elements in the form of plasmids (Alonso *et al.,* 2001).

In the current study the antibiotic phenotypes remained relatively consistent throughout the distribution system. The most frequently observed antibiotic phenotype was AMP, KAN, PEN, OT, VAN, TM. All the *Bacillus* spp. from site 1 and 2 were resistant against these antibiotics. Isolates from site 3 and 5 were resistant to a broader spectrum of antibiotics. A reason for this might be the genetic adaption of the bacteria based in the biofilm against certain antibiotics in the form of a plasmid. The biofilms present in the system at site 3 and 5 might have been more established than the biofilms at site 1 and 2.

5.5 Identification of HPC isolates

5.5.1 BBL Crystal™ GP ID system

All the isolates from the biofilm of the drinking water distribution system were Gram-positive. Isolating large quantities of Gram-positive bacteria from drinking water is not uncommon. Stelma *et al.* (2004) also isolated a high percentage (73%) of Gram-positive HPC bacteria from drinking water. *Bacillus cereus* was amongst the identification obtained (Stelma *et al.,* 2004). In the present study, identities obtained with the BBL Crystal™ GP ID system (specific for Gram-positive bacteria) for the nineteen isolates included *Bacillus cereus, Bacillus subtilis, Bacillus pumilus, Bacillus licheniformis, Bacillus megaterium* and *Kocuria rosea.* Four of the isolates could not be identified. Reasons for this may include one of the following, (i) the species tested for is not included in the taxa provided by the system, (ii) atypical reactions

might have been produced by the isolate or (iii) the level of identity confidence for the specific isolate was too low.

Stelma *et al.* (2004) demonstrated that the BBL Crystal™ GP ID system is a reliable identification system since the 36 identifications out of 45 isolates (80%) could be made successfully. In the present study 15 out of 19 (80%) identifications were also obtained. The BBL Crystal™ GP ID system identifications were confirmed using 16S rRNA gene sequencing. Furthermore, Gram-positive isolates that could not be identified by the BBL Crystal™ GP ID system were also identified using 16S rRNA gene fragments.

5.5.2 16S rRNA gene sequencing

DNA was successfully isolated with the Nexttec™ genomic DNA isolation system using the instructions of the manufacturer. The DNA concentrations varied from 150 to 400 ng/µl and 260:280 ratios from 1.30 to 1.50. Isolated DNA was not fragmented and there was no RNA (See Figure 4.3). In previous studies (Tokajian *et al.,* 2005; Burtscher *et al.,* 2009) it was demonstrated that the quality of isolated DNA was important for successful 16S sequencing identifications. Thus DNA isolated in the present study was of sufficient quantity and of high enough quality for successful amplification of the 16S rRNA gene. In the current study the annealing temperature was 52 °C. This is higher than the 46°C and 50°C used by Tokajian *et al.* (2005) and Hoefel *et al.* (2005a) respectively, for the same primer set. The higher annealing temperature in the present study enhanced the specificity of the PCR reaction.

The sequencing results for 11 of the isolates were in accordance with the results obtained with the BBL Crystal™ GP ID system. The four HPC isolates that could not be identified by the biochemical system were *Bacillus thuringiensis* and *Exiguobacterium acetylicum*. These two organisms are not in the list of identifiable taxa of the BBL Crystal™ GP ID system. All the isolates that were identified as *Bacillus subtilis* and *Bacillus licheniformis* with the Crystal™ GP ID system were identified as *Bacillus thuringiensis* when 16S rRNA gene sequencing was used. The reason for misidentification could possibly be ascribed to *Bacillus*

thuringiensis not being listed in the BBL Crystal^{TM} GP ID system. More closely related species such as *Bacillus subtilis* and *Bacillus licheniformis* were thus suggested by the system for *Bacillus thuringiensis.*

Identifications with 16S rRNA gene sequencing revealed a 100% sequence similarity for 15 of the isolates, 99% sequence similarity for 3 of the isolates and 95% for one of the isolates. These are satisfactory percentages for accurate identification. Sequencing results were taken as the correct identifications as the margin for human errors is much lower than the biochemical test methods (Clarridge, 2004). 16S rRNA gene sequencing has been used by many research groups for the identification of HPC bacteria (Farnleitner *et al.,* 2004; Hoefel *et al.,* 2005b; Tokajian *et al.,* 2005; Burtscher *et al.,* 2009).

5.5.3 Transmission electron microscopy

TEM was performed to differentiate between the morphologies of the various isolates and to determine if this is in agreement with previous studies and typed cultures. Juang and Morgan (2001) also applied TEM to confirm identifications of dominant species obtained with a biochemical test method (API). *Arthrobacter* spp. was amongst the species. *Arthrobacter oxydans* cells displayed a spherical shape rather than a rod shape. This is however, not an unusual observation as *Arthrobacter* spp. changes cell shape during different growth stages. Spherical shapes are observed in the stationary phase (Keddie *et al.,* 1986). Crocker *et al.* (2000) observed the same phenomenon for their *Arthrobacter* isolates.

All the *Bacillus* cells were rod shaped but differ in the arrangement for each of the species. *Bacillus thuringiensis* cells were arranged in clusters. *Bacillus cereus* cells were arranged in irregular strings. *Bacillus megaterium* cells occurred in neat strings. *Bacillus pumilus* were observed as single cells. These observations were in accordance with Fritze (2002). *Exiguobacterium acetylicum* was observed as rod shaped cells. Collins *et al.* (1984) also described *Exiguobacterium acetylicum* as a rod shaped organism.

5.6 Significance and implications of HPC bacteria isolated from the drinking water distribution system

Bacillus spp. is widely spread in nature due to endospores being distributed. These spores enable the organism to prevail in sub-optimal conditions (Fritze, 2002). *Bacillus* spp. in the form of vegetative cells or spores is expected to be present in water (Østensvik *et al.,* 2004). Ichimatsu *et al.* (2002) isolated *Bacillus thuringiensis* from 49.5% of their fresh water samples from natural environments. In the current study, *Bacillus* spp. was isolated from the biofilm samples at four sites within the drinking water distribution system. This indicated the dominance of *Bacillus* spp. in this particular system. *Bacillus* spp. is commonly isolated from drinking water as demonstrated by various researchers (Edberg *et al.,* 1996; Edberg *et al.,* 1997; Pavlov *et al.,* 2004; Stelma *et al.,* 2004; Tokajian *et al.,* 2005; Burtscher *et al.,* 2009).

One of the major concerns associated with *Bacillus* spp. within a drinking water distribution system is the persistence of spores (Morrow *et al.,* 2008).Thus enabling the organisms to withstand the disinfection process and colonize the distribution system when conditions are condusive. It was documented by Rice *et al.* (2005) and Rose *et al.* (2005; 2007) that *Bacillus* spores are generally more resistant than vegetative cells against disinfectants such as free chlorine and monochloramine. It was mentioned in Chapter 3 that the source water for the drinking water distribution system examined in the current study, is the Vaal River. It is thus possible that *Bacillus* spp. may enter the distribution system from the source water in the form of spores. Spores tend to be more resistant to the disinfection process as mentioned above. Thus allowing *Bacillus* spp. to colonize and form biofilms within the distribution system.

Exiguobacterium acetylicum was also amongst the species isolated in the current study. The genus *Exiguobacterium* was first described by Collins *et al.* (1984). According to Keynan *et al.* (2007) *Exiguobacterium acetylicum* displayed golden-yellow to orange colonies and could not be identified with biochemical test methods (API Coryne). In the present study the colonies were also golden yellow (results not shown) and the BBL Crystal^{TM} GP ID system

could also not identify the organism. The correct identification was only obtained when 16S rRNA gene sequencing were used.

Arthrobacter oxydans is frequently isolated from soil samples (Smit *et al.,* 2001), indicating that soil might have been the source of contamination of the drinking water distribution system in the current study. *Arthrobacter* spp. only gained interest from the mid 1990's when it was isolated from human clinical specimens (Funke *et al.,* 1996). Mages *et al.* (2008) reported the isolation of 50 *Arthrobacter* and *Arthrobacter*-like strains from human clinical samples. *Arthrobacter oxydans* were amongst the isolates, isolated from wound swabs, vaginal swabs, eyes, lungs and blood (Mages *et al.,* 2008). These results emphasize the need for investigations into HPC bacteria as they are more frequently isolated from human clinical samples.

HPC bacteria are mostly harmless to normal healthy individuals with intact immune systems. However, cases have been reported where these bacteria caused infections amongst individuals with weakened immune systems (Keynan, 2007; Torregrossa *et al.,* 2000). A study was done in the Palermo Burns Centre in Italy to determine the presence of opportunistic pathogens in the air and water supplies (Torregrossa *et al.,* 2000). *Bacillus cereus* was amongst the species isolated from the water and it was isolated from blood and skin lesions of two of the patients. In another case study, *Exiguobacterium acetylicum* was reported to cause catheter-related bacteraemia in a 92-year-old woman (Keynan, 2007). *Bacillus thuringiensis* has been associated with wound infections (Hernandez *et al.,* 1998), periodontitis (Helgason *et al.,* 2000a), diarrhea caused by food-poisoning (Jackson *et al.,* 1995), burn infections (Damgaard *et al.,* 1997) and corneal ulcer (Samples and Beuttner, 1983). Cases such as these confirm the concern of HPC bacteria present in drinking water especially for immuno-compromised individuals.

The WHO proposed the re-evaluation of HPC for determining the hygienic quality of drinking water (Bartram *et al*., 2003). These HPC bacteria might be potentially pathogenic. Bacteria of concern are divided into 4 catagories: (i) emerging bacteria, (ii) commensal bacteria, (iii)

bioterrorist threat agents and (iv) recognized pathogens (Lightfoot, 2003). None of the bacteria isolated in the current study were mentioned by the WHO as bacteria of concern, but they displayed virulence factors enabling them to potentially cause disease. The WHO suggested the continued use of HPC for evaluation of the changes in flora within the drinking water (Bartram *et al.,* 2003). Such changes must then be further analysed with molecular techniques to detect any pathogens present (Lightfoot, 2003).

5.7 Analysis of bulk water

Bulk water was analysed using PCR-DGGE to confirm the presence of some of the biofilm-associated bacteria. This analysis indicated either that bacteria survive the drinking water treatment process and colonize the pipe lines of the drinking water distribution system or the biofilm-associated bacteria disperse from the pipe lines and end up in the drinking water.

5.7.1 Physical-chemical analysis

Two sites, close to biofilm sampling site 3 and 5, were used for bulk water sampling (Figure 3.1). The pH levels of the water were 8.17 and 8.07 respectively for the 2 sites. These values were within the standard of 6.0 – 9.0 as set out by SANS 241 (2006) for class I water. The water temperatures for the 2 sites were 21.1°C and 21.6°C respectively. *Bacillus* spp. can grow at temperatures as low as 4 °C and as high as 55 °C (Granum, 1997) and thus the isolation thereof can be guaranteed all year round. Oxygen levels were measured as 8.18 mg/l and 8.65 mg/l for the two respective sites. The majority of the bacteria isolated are facultative anaerobes, which prefer to grow in the presence of oxygen but can also grow anaerobically. Oxygen levels will thus not have had a great effect on their growth. Electric conductivity was 88 mS/m and 82.9 mS/m. The electric conductivity measurements were above the ideal levels (Class I) as set by SANS 241 (2006). It did however adhere to the class I criteria which indicate an acceptable water quality.

5.7.2 Culture independent microbiological analysis: DGGE

Due to growing lack of confidence in HPCs to give an accurate assessment of the quality of drinking water, many researchers have shifted to culture-independent techniques to study microbial diversity (Farnleitner *et al*., 2004; Hoefel *et al.,* 2005a; Hoefel *et al*., 2005b; Tokajian *et al.,* 2005; Burtscher *et al.,* 2009). In this study, PCR-DGGE was employed to determine if species occurring as biofilms in the drinking water distribution system were also be present in the bulk water. DNA fragments from the pure cultures isolated from the biofilm samples were used as a ladder against which the bulk water samples could be tested.

All the isolates could not be used in the construction of the ladder as many of them migrated to the same position. Co-migration could have been due to species being closely related, 16S rRNA sequence were not sufficiently did not provide enough sequence different to allow separation of amplified DNA of all species on a DGGE gel (Ercolini, 2004). Some of the pure isolates used for ladder construction produced multiple bands as observed in Figure 4.7. This could have been due to the presence of multiple copies of the 16S rRNA, hence multiple bands were observed for the same species (Ercolini, 2004).

For analysis the following was done. DNA was isolated directly from bulk water using the method described in Section 3.9.2. and concentrations were between 45 and 70 ng/µl. However, the 260:280 ratios were between 0.70 and 0.90. Even so, the PCR-DGGE analysis was not affected by the lower quality DNA. Hoefel *et al.* (2005a) extracted DNA from bulk water (total DNA) to determine the survival of bacteria through the treatment and distribution of water. They used a freeze-thaw DNA isolation method. In the current study, DNA was isolated using an enzymatic and chemical spin-column based method. A 500bp 16S fragment was amplified. The primer contained a GC-clamp ensuring the DNA molecule remains partially intact when it reached a point in the DGGE gel where most of the fragment is single stranded (Sheffield *et al.,* 1989). The annealing temperature was set at 56°C. This was much lower than the annealing temperature used by Hoefel *et al.* (2005a) which was 65°C. However, they used a nested PCR approach because PCR was performed directly on the crude DNA. Burtcher *et al.* (2009) also used a direct DNA isolation method to obtain DNA.

The DNA was also analysed by PCR-DGGE. The advantage of this culture-independent method is that the diversity of culture-dependent and non-culturable bacteria can be determined and studied.

It is evident from Figure 4.7 that *Bacillus pumilus* and *Arthrobacter oxydans* might be present in the bulk water due to the migration patterns of these species correlating with the constructed ladder. One of the drawbacks of this particular application of DGGE is that there is no absolute guarantee of unambiguous identification of species (Ercolini, 2004). This is due to the co-migration of different species (Muyzer *et al.,* 1993; Ercolini *et al.,* 2001; Sekiguchi *et al.,* 2001; Meroth *et al.,* 2003). This is however technically, a relatively uncomplicated and cost effective technique in establishing the presence of certain bacteria. Farnleitner *et al.* (2004) demonstrated that prominent bands can be excised and sequenced to obtain identities of the various isolates.

From these results it is clear that there is indeed a correlation between the organism present in the bulk water samples and bacteria present in the biofilms. Either these organisms enter the drinking water distribution system by bypassing the treatment process or they enter from external sources. They then adhere to and colonize the distribution system internal surfaces to form biofilms which then in turn disassociate from the pipe lines to form new biofilms further downstream. Thus the presence of the bacteria in the bulk water could be due to bacteria entering the system or biofilms disassociate from the pipe lines. Such results were also observed by Srinivasan *et al.* (2008).

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The aim of this study was to establish the pathogenic potential of HPC bacteria isolated from the pipe lines of a drinking water distribution system and to determine the possible presence of these isolates in the bulk water.

Seven well-structured objectives were formulated to achieve the aim of this study. These objectives could be divided into 2 main goals: to isolate and characterize HPC bacteria adhering to the pipe lines of a drinking water distribution system, and to determine the possible presence of these in the bulk water.

(i) Isolation and purification of HPC bacteria from pipe lines

R2A agar was successfully employed for the isolation of HPC bacteria from biofilms obtained from 4 of the 5 sites. Fifty six isolates could be purified for further analysis. Thus, the drinking water distribution system had biofilm build-up throughout the system with a diversity of HPC bacteria. Such a build-up of biofilm could cause corrosion to the pipe lines. Even though a thin layer of biofilm might be present, it could increase the energy required to transport the water through the distribution system.

(ii) Screening for the production of haemolysin and extracellular enzymes

Thirty one of the 56 HPC isolates displayed β-haemolysis. Therefore, HPC bacteria isolated from biofilms present in this drinking water distribution system were potentially cytotoxic, assisting in the pathogenic potential of the isolates. Two or more extracellular enzymes were produced by *Bacillus* species, *Arthrobacter oxydans* and *Exiguobacterium*

acetylicum. The most commonly produced enzymes included, proteinase and lecithinase. Thus, these isolates may be pathogenic, especially when they are able to multiply within the host. A *Bacillus thuringiensis* isolate was the most pathogenic as it produced four of the extracellular enzymes. The risk factor of the HPC bacteria have not yet been established as quantitative analysis must still be done. The question remains as to what exactly the dose should be for the HPC bacteria to confer health implications.

(iii) Antibiotic susceptibility of haemolytic isolates

A large number of the isolates were resistant to multiple antibiotics. The most frequently observed antibiotic phenotype was AMP, KAN, PEN, OT, VAN, TM. Individual isolates were also resistant to erythromycin, chloramphenicol and streptomycin. It might be possible that antibiotic resistance was obtained by the isolates from the biofilms through the transfer of mobile genetic elements. This data set can be useful to the medical field as to which antibiotic would be more effective against infections by these organisms.

(iv) Identification of isolates with a biochemical test method, 16S rRNA gene sequencing and TEM

Fifteen of the 19 HPC isolates were identified using a biochemical test method. These identifications included *Bacillus cereus, Bacillus licheniformis, Bacillus subtilis, Bacillus megaterium, Bacillus pumilus* and *Kocuria rosea*. Four of the isolates could not be identified with the biochemical test method. 16S rRNA gene sequencing provided some identification results that were conflicting with the BBL Crystal^{TM} GP ID system results. *Bacillus subtilis* and *Bacillus licheninformis* were identified as *Bacillus thuringiensis* with 16S rRNA gene sequencing. The unidentified isolates were identified as *Bacillus thuringiensis* and *Exiguobacterium acetylicum.* It was demonstrated that these results were in accordance with previous studies. TEM confirmed the morphologies of the identified isolates. All 19 identified isolates displayed rod shaped cell with the exception of *Arthrobacter oxydans* being spherical in the stationary phase of their life cycle. Although identities could be obtained by a single identification method, it still remains necessary to confirm identities with other identification systems.

(v) Analysis of bulk water samples

The physico-chemical measurements were within the SANS 241 standards for drinking water. PCR-DGGE revealed the presence of *Bacillus pumilus* and *Arthrobacter oxydans* in the bulk water samples. There was a clear correlation between bacteria present in the bulk water and the bacteria present in the biofilms. The water provided by the Midvaal Water Company is of good quality as the physico-chemical measurements met the standards for drinking water and no indicator organisms were isolated. There is however, the problem of biofilm build-up which needs further analysis in terms of the entry of these organisms into the system.

6.2 Recommendations

1) A broader spectrum of extracellular enzymes should be tested for to prevent elimination of isolates that might have produced two or more enzymes if more enzymes were tested for.

2) Samples should be taken at point of abstraction of source water and after the treatment process. It should be tested for spores and vegetative cells. This will allow one to determine whether *Bacillus* spp. enters the water distribution system by spores or vegetative cells. It may also provide some indication of the effectiveness of the treatment process.

3) Bands obtained with DGGE analysis should be excised from the gel and sequenced. This might provide answers of DNA co-migrating to identical positions on the gels.

4) RNA analysis should be done to determine the levels and diversity of viable organisms after the water treatment process and compared to the levels and diversity of viable organisms prior to treatment.

5) The ability of the isolated species to form biofilms should be determined. Bacterial species that easily form biofilms are able to colonize medical devices, for instance artificial implants and catheters.

6) Midvaal Water Company uses ozone as part of the water treatment process. Ozone degrades complex organic substances into easily assimilated organic material. This could have a positive on aftergrowth (regrowth) in the distribution system. Thus, effects of ozone on growth potential of bacteria should be investigated.

7) Plasmids are mobile genetic elements that can easily spread in aquatic environments. This is particular the case in biofilms. Plasmids are renowned for the spread of antibiotic resistance and virulence genes. It is thus possible that if plasmids carrying bacteria enter a drinking water distribution system that these could easily be spread and maintained in the system. Determining the diversity and dynamics of plasmids in a water distribution system will thus be a useful study.

8) The effect of polluted source water on the efficiency of the treatment process should be investigated. During such a study the impacts that deteriorating source water quality has on biofilm formation should also be investigated.

This study has demonstrated that potentially pathogenic HPC bacteria is adhered to the internal surfaces of a drinking water distribution system and might be present in the bulk water. Thus, the aim was achieved by the successful execution of the seven objectives.

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