Potential pathogenicity of heterotrophic plate count bacteria isolated from untreated drinking water

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Dissertation submitted in fulfilment of the requirements for the degree *Magister Scientiae* in Environmental Sciences at the Potchefstroom Campus of the North-West University

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May 2014

ACKNOWLEDGEMENTS

The following institutions contributed financially towards this study:

- The National Research Foundation (NRF) (grant-holder bursary).
- The Water Research Commission (WRC) of South Africa (K5/1966).
- The North-West University (post-graduate bursary)

I would also like to express my sincere gratitude to the following people for their contribution and support towards the completion of this study:

- My supervisor and co-supervisor, Dr. Rialet Pieters and Prof. Carlos Bezuidenhout.
- I appreciate your time, patience, guidance, encouragement and contributions.
- Fellow graduates for their overall support and motivation.
- A special thanks to my family and friends, especially Heinrich Horn, and my parents for their love, support and encouragement.
- My Creator for blessing me with faith, knowledge, strength and the gift to do research.

"*Water — gathered and stored since the beginning of time in layers of granite and rock, in the embrace of dams, the ribbons of rivers — will one day, unheralded, modestly, easily, simply flow out to every South African who turns a tap. That is my dream.*"

(President Thabo Mbeki, quoting poet Antjie Krog at the launch of the 2006 UNDP Development Report, Cape Town, November 2006).

DECLARATION

I declare that this dissertation submitted for the degree of *Master of Science in Environmental Sciences* at the North-West University, Potchefstroom Campus, has not been submitted by me for a 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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ABSTRACT

Water is considered the most vital resource on earth and its quality is deteriorating. Not all residents living in South Africa's rural areas have access to treated drinking water, and use water from rivers, dams, and wells. The quality of these resources is unknown, as well as the effects of the bacteria in the water on human health. The heterotrophic plate count (HPC) method is a globally used test to evaluate microbial water quality. According to South African water quality guidelines, water of good quality may not contain more than a 1 000 coliforming units (CFU)/mℓ. There is mounting evidence that HPC bacteria may be hazardous to humans with compromised, underdeveloped, and weakened immune systems.

In this study the pathogenic potential of HPC bacteria was investigated. Samples were collected from boreholes in the North West Province and HPCs were enumerated with a culture-based method. Standard physico-chemical parameters were measured for the water. Different HPC bacteria were isolated and purified and tested for *α*- or *β*-haemolysis, as well as the production of extracellular enzymes such as DNase, proteinase, lecithinase, chondroitinase, hyaluronidase and lipase, as these are pathogenic characteristics. The isolates were identified with 16S rRNA gene sequencing. The model for the human intestine, Hutu-80 cells, were exposed to the potentially pathogenic HPC isolates to determine their effects on the viability of the human cells. The isolates were also exposed to different dilutions of simulated gastric fluid (SGF) to evaluate its effect on the viability of bacteria. Antibiotic resistant potential of each isolate was determined by the Kirby-Bauer disk diffusion method. Three borehole samples did not comply with the physico-chemical guidelines. Half of the samples exceeded the microbial water quality guideline and the greatest CFU was 292 350 CFU/mℓ. 27% of the isolate HPC bacteria were *α*- or *β*-haemolytic. Subsequent analysis revealed the production of: DNase in 72%, proteinase in 40%, lipase and lecithinase in 29%, hyaluronidase in 25% and least produced was chondroitinase in 25%. The HPC isolates identified included: *Alcaligenes faecalis, Aeromonas hydrophila* and *A. taiwanesis*, *Bacillus* sp., *Bacillus thuringiensis, Bacillus subtilis, Bacillus pumilus, Brevibacillus* sp.*, Bacillus cereus* and *Pseudomonas* sp. All the isolates, except *Alcaligenes faecalis,* were toxic to the human intestinal cells to varying degrees. Seven isolates survived exposure to the most diluted SGF and of these, four isolates also survived the intermediate dilution but, only one survived the highest SGF concentration. Some isolates were resistant to selected antibiotics, but none to neomycin and vancomycin. Amoxillin and oxytetracycline were the least effective of the antibiotics tested. A pathogen score was calculated for each isolate based on the results of this study.

Bacillus cereus had the highest pathogen index with declining pathogenicity as follows: *Alcaligenes faecalis* > *B. thuringiensis* > *Bacillus pumilus* > *Pseudomonas* sp. > *Brevibacillus* > *Aeromonas taiwanesis* > *Aeromonas hydrophila* > *Bacillus subtilis* > *Bacillus* sp. The results of this study prove that standard water quality tests such as the physico-chemical and the HPC methods are insufficient to provide protection against the effects of certain pathogenic HPC bacteria.

Keywords: HPC bacteria; Extracellular enzymes, Cytotoxicity; Simulated gastric fluid, MTT assay, Antibiotic resistance.

CONTENTS

LIST OF ABBREVIATIONS AND ACRONYMS

A	
AGI	acute gastrointestinal illness
AIDS	acquired immunodeficiency syndrome
B	
BLAST	Basic Logic Alignment Search Tool
BHIB	brain heart infusion broth
C	
C	chondroitinase
CFU	coliforming units
CI	cell Index
Cl	chloride
COD	chemical oxygen demand
D	
D	DNase
DHF	dihydrofolic acid
DMEM	Dulbecco’s modified Eagle’s medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DOC	dissolved organic carbon
DO	dissolved oxygen
E	
EC	electrical conductivity
F	
FFA	free fatty acids
FV	fold viability
G	
GIS	Geographical Information System
H	
h	hours
H	hyaluronidase
HIV	human immunodeficiency virus infection/
HPC	heterotrophic plate count

L	
Le	lecithinase
Li	lipase
M	
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
N	
Na	sodium
NW	North West Province
O	
OD	optical density
P	
P	proteinase
PCR	polymerase chain reaction
R	
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RTCA	Real Time Cell Analysis
S	
SANS	South African National Standards
SGF	simulated gastric fluids
T	
t	time
TDS	total dissolved solids
THF	tetrahydrofolic acid
TWQR	Target Water Quality Ranges
V	
VBNC	viable but not culturable
W	
(w/v)	weight/volume

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

1.1 General overview and problem statement

A common problem in rural areas of developing countries, such as South Africa, is the poor quality of drinking water (Momba *et al.*, 2006). Due to limited availability of drinking water, rural communities often use water directly from untreated sources such as dams, streams, rivers, wells and ponds. Sixty five percent of the North West Province's population resides in rural areas (NWPG, 2002). Eighty percent of the North West Province's groundwater resources are used by these rural communities (NWDACE-SoER, 2008). Access to safe drinking water is a basic human right and should not pose a health hazard when ingested. There are major health risks associated with consumption of untreated water, it may cause diseases such as shigellosis, cholera, salmonellosis, diarrhoea and a variety of other bacterial, fungal, parasitic and viral infections (Zamxaka *et al.*, 2004). People who are particularly susceptible to these diseases include those with underdeveloped, compromised or weakened immune systems, such as very young children, individuals living with HIV/AIDS, and the elderly respectively (Pavlov *et al*., 2004).

The quality of water is expressed in terms of chemical, physical and microbiological characteristics (Oparaocha *et al.*, 2010; WRC, 1998). In South Africa the heterotrophic plate count (HPC) bacteria standard is one of the measures to evaluate the microbiological quality of drinking water. According to SANS 241 (2011) the amount of HPC bacteria in good quality drinking water should not exceed 1 000 CFU/mℓ. Heterotrophic bacteria are those bacteria that utilize organic nutrients for survival. This group of bacteria include those that can be counted when cultured on specific culture media and under specific culture conditions (Allen *et al.*, 2004). HPC bacteria are considered harmless with no meaningful risk to human health. However, studies by Rusin *et al*. (1997) and Pavlov *et al*. (2004) suggest that HPC bacteria may be opportunistic pathogens and may cause adverse health effects to individuals with compromised health, even when present at low and acceptable levels (Stelma *et al.*, 2004; De Wet *et al.*, 2002). According to Bartram *et al.* (2003) there are a few opportunistic pathogens present among natural occurring HPC bacteria and those include: *Aeromonas* spp., *Acinetobacter* spp., *Bacillus* spp., *Klebsiella* spp., *Moraxella* spp., *Flavobacterium* spp., *Mycobacteria* spp., *Pseudomonas* spp., *Serratia* spp. and *Xanthomonas* spp.

Limited research has been conducted to determine the effect of HPC bacteria on human health and the South African National Standards (SANS) 241 and Target Water Quality Ranges (TWQR) standards do not consider all the effects that these bacteria may have on the health of different individuals. The practice of depending on the abiotic parameters such as pH, electrical conductivity, and dissolved oxygen only to indicate water quality, overlooks the biological impact.

In this study the HuTu-80 cell line (human duodenum adenocarcinoma) acted as a model for the human intestine and cell viability was determined to predict whether microbes (specifically HPC bacteria) in the water affect the viability of cells in culture. The pathogenic potential of HPC bacteria were determined by standard methods such as the haemolysin assay (Hoult and Tuxford, 1991) and enzyme production analysis (Janda and Bottone, 1981). In one study the HPC isolates were also subjected to simulated gastric fluid (SGF), which represent the acidic conditions of the human stomach and form an integral part of assessing the risk that bacterial proteins capable of surviving the stomach pose for the intestines (Schnell & Herman, 2009). Mimicking gastric fluids enables a more direct comparison to the human body, because gastric fluids act as an important first line of defence against consumed pathogens, especially when an individual lacks a fully functioning immune system.

It is important that the pathogenic potential of HPC bacteria is investigated and that the extent to which they may influence human health, is determined. The hypothesis of this study is that HPC bacteria in untreated drinking water are potentially pathogenic and that these HPC bacteria survive exposure to gastric fluids. The overall goal is to determine whether this hypothesis holds.

1.2 Research aims and objectives

The first aim of the study was to investigate the type of enzymes produced by HPC bacteria isolated from untreated drinking water sources in the North West Province.

The objectives were to:

- measure the physico-chemical quality of groundwater sources
- isolate and purify HPC bacteria using R2A agar
- test for *alpha*- or *beta*-haemolysis and enzyme production that are associated with pathogenicity
- identify HPC isolates that are potentially pathogenic with molecular methods
- investigate susceptibility of HPC isolates to antibiotics

The second aim was to measure cytotoxicity caused by HPC bacteria in untreated drinking water on the viability of a duodenum adenocarcinoma cell culture, which acted as a model for the human small intestine.

The objective was to:

- determine the cytotoxic effects of the HPC isolates

The third aim of the study was to investigate whether potentially pathogenic HPC isolates will survive exposure to simulated gastric fluid.

The objectives were to:

- expose isolates to different SGF dilutions
- use the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay to evaluate the survival of exposed HPC bacteria

2. LITERATURE REVIEW

2.1 Water quality and drinking water

Water is vital for the maintenance of all forms of life. Although water occurs in a dynamic cycle of rain, evaporation and runoff influenced by temporal and spatial variation, only 3% of water resources on earth are of good and usable quality (Phiri *et al.*, 2005; Rijsberman, 2006). These resources include surface water such as rivers, lakes, streams, and groundwater. Not only do we depend on water for life, but it is an essential resource for manufacturing, industry, transportation and many other human activities (Phiri *et al.*, 2005). Despite its importance, water is the most poorly managed resource in the world (Sobsey, 2006).

Aquatic resources are highly susceptible to various forms of pollution that may affect the quality of water and limit water uses. Major sources of contamination include fertilizers in agricultural areas, careless disposal of industrial effluents and other waste from urban areas (Phiri *et al.*, 2005).

Drinking water that is adequate in quantity and of acceptable quality is a fundamental human need and a basic human right (Momba *et al.*, 2006; NWA, 1998). The quality of drinking water directly influences human and animal health and safe water is vital to the well-being of the global population (Thomas *et al.*, 2006). A considerable amount of research worldwide is focused on ensuring safe drinking water (Wu *et al.*, 2011a).

2.2 The water situation in South Africa and North West Province

Water is a scarce resource, especially in South Africa with its high temperatures and seasonal rainfall. The availability and quality of water are limited and should therefore be managed carefully and used wisely (Dallas & Day, 2004). Water fit for human consumption is defined as water without any significant health risks over a lifetime of consumption and that is free from harmful organisms and organic substances (DWAF, 2005). In South Africa good quality drinking water should comply with the South African National Standards (SANS) 241 drinking water specifications in order to be fit for consumption. Water to be used for domestic, irrigation or recreational purposes should comply with the TWQR (DWAF, 1996b).

The most alarming issue regarding the water situation is the fact that people do not always have access to water suitable for human consumption (Rijsberman, 2006). Approximately 15 % of the

world's population lives in areas with water stress, around 1.1 billion people worldwide do not have access to good quality drinking water and 2.4 billion people do not have access to basic sanitation (WHO, 2003). In South Africa more than 7 million people do not have access to potable water, 54% of the population lack basic sanitation and 3.7 million people have no access to water supply infrastructure (Kahinda *et al.*, 2007; DWAF, 1996a).

Residents from rural areas lack access to treated drinking water which contributes to the increase in use of untreated water. Nearly 80% of people in South Africa rely on surface water as their main water source, indicating that there are many people that rely on untreated water for domestic uses (Zamxaka *et al.*, 2004). Due to the increase in contamination of surface water, there is an increase in demand for using groundwater as a drinking water resource. Because of the high demand for groundwater it is important to determine the quality of untreated water that are present in wells, so frequently used because of lack of good quality surface water (Strauss *et al.*, 2001).

Many residents in rural areas have a low income and also have no access to water for their livelihoods. The lack of access to safe drinking water and sanitation influences the well-being of humans and in combination with poor personal hygiene may increase health hazards (Rijsberman, 2006). The water that occurs in nature normally contains a variety of substances that cannot be seen by the naked eye. In this study the focus is on microbiological quality of water. Drinking water and food reservoirs are considered as important sources of human infections due to bacteria present that produce extracellular enzymes and toxic compounds (Tantillo, *et al.*, 2004).

The quality and risks associated with consuming untreated surface and groundwater is unknown. Consumption of polluted surface water is linked to burden of illness and there is substantial literature to support this. However, this is not the case for consumption of untreated groundwater. The perception with groundwater is that since it percolated through several layers of sand, soil and rocks it is free of any contaminant and is thus safe to drink. This may be true in an unpolluted world, but not in a scenario where industrial, agricultural, municipal sewage, and mining pollution affects the quality of water sources. The water resources in developing countries suffer either from chronic shortages of fresh water or accessible water resources that are already polluted (Zamxaka *et al.*, 2004).

2.3 Human health

2.3.1 Immune-compromised individuals

The widespread occurrence of Human Immunodeficiency Virus Infection and the consequent Acquired Immunodeficiency Syndrome (HIV/AIDS) has reached a crisis point in developing countries (Obi & Bessong, 2002). In 2009 it was estimated that 13% of the population residing in the North West Province are HIV positive (Nicolay, 2008). Many of these infected persons have an inadequate supply of potable water, good sanitation and lack good hygienic practices. The available water sources (mainly groundwater in rural communities) are known to be highly polluted, not treated, and thus serve as a medium for spreading waterborne diseases because there is a great number of waterborne pathogens present in groundwater (Ferreira, 2011; Momba *et al.*, 2006; Obi & Bessong, 2002).

The relationship between infections and various forms of illnesses is not well understood. Only about 50% of infections result in illness (Macler & Merkle, 2000). Individuals with immunosuppressive conditions are at the greatest risk of infection when consuming water containing waterborne pathogens (Sheffer *et al.*, 2005). Apart from people infected with HIV/AIDS, these individuals include patients with HIV/AIDS, leukeamia, diabetes, cancer patients receiving medical treatment, people with advanced age and children younger than 5 years (Pavlov *et al*., 2004; Barbeau *et al*., 1998; Rusin *et al*., 1997; Grabow, 1996).

2.3.2 Waterborne diseases

As a consequence of poor water quality in rural areas of South Africa, a considerable number of residents living there are exposed to waterborne pathogens that cause diseases. Microbial contamination is responsible for the most health related water quality problems (Smith *et al.*, 2006). More than 100 viral and several bacterial pathogens have been found to contaminate groundwater. Waterborne diseases resulting from infection depend on the causal agent. This will also affect the severity of the infection. Examples of diseases that can be contracted through consumption of contaminated water include shigellosis, cholera, salmonellosis, yersiniosis, diarrhoea and a variety of fungal, parasitic (eg. bilharzia) and other bacterial and viral infections (Oparaocha *et al.*, 2010; Phiri *et al.*, 2005; Zamxaka *et al.*, 2004). The predominant illness caused by waterborne pathogens is referred to as generalized acute gastrointestinal illness (AGI), resulting in fever, nausea, diarrhea, and/or vomiting (Macler & Merkle, 2000). Most of the AGI are acute, self-resolving and do not have major consequences to healthy individuals. This is, however, not the case for immuno-compromised individuals. They may suffer from chronic, severe or fatal AGI (Macler & Merkle, 2000).

There are currently a large number of known waterborne and water-based pathogens. Viruses (Enteroviruses, Hepatitis A and E, Norwalk viruses, Rotaviruses, Adenoviruses, Astroviruses) (Adetunde & Glover, 2010; Thomas *et al.*, 2006), Bacteria (*Salmonella, Shigella, Escherichia coli* O157:H7, *Legionella pneumophila*), Protozoa (*Naegleria, Cyclospora, Septata* spp.), Cyanobacteria (*Microcystis, Anabaena*) and Helminths (*Ascaris lumbricoides, Taenia saginata*) are only a few pathogens, not including potential pathogens or emerging pathogens (Straub & Chandler, 2003). All the human pathogenic bacteria are heterotrophic, hence an increased concern regarding the pathogenic potential of the other representatives, not yet classified as definite pathogens, of this group of microorganisms to human health (Stine *et al.*, 2005; Rusin *et al.*, 1997).

Unsafe drinking water or inadequate sanitation is responsible for the deaths of more than 5 million people per annum worldwide. There are around 4 billion cases of diarrhoea reported worldwide each year and 2–2.5 million people die per year due to diarrhoeal diseases caused by poor quality of water sanitation and hygiene (Poté *et al.*, 2009; Fenwick, 2006). Of these deaths, 90% are children from developing countries. Some researchers are of the opinion that the number of individuals that report their illnesses are an underestimation of the actual levels of microbial diseases associated with drinking water (Macler & Merkle, 2000).

There are a few factors that influence the magnitude and the spreading rate of waterborne disease outbreaks. These include the type of pathogen, the load of pathogens, their survival and infectivity in raw water, the speed at which it enters the water, the nature of water treatment if applicable, the rate of consumption by humans, as well as their susceptibility to the pathogen. There are many routes for faecal contamination to reach groundwater. Some concentrated point sources are of particular concern and include leaking sewer lines, cesspools and failed septic systems. Other sources are dairy farms, animal feedlots and animal-husbandry operations (Macler & Merkle, 2000). The weather is often an external factor that is responsible for creating favourable conditions for pathogen survival, its growth, and reproduction in the water (Thomas *et al.*, 2006).

Several factors are contributors to the emergence and spread of disease agents. These include ecologic changes (including those caused by human activity), international travel and trades, technology, human actions and demographics, microbial evolution, and the breakdown of public health systems (Hunter *et al.*, 2001). Some humans living in rural areas do not have access to other water resources and are forced to drink the “safe” untreated water from wells. These people are often exposed to small amounts of bacteria and low levels of chemicals in the water, which only show effects after long terms chronic exposure.

2.3.3 Antibiotic resistance

Bacteria present in water bodies are constantly exposed to antibiotics and chemicals also present in the water. This leads to the increased prevalence of bacteria that are now resistant to the antibiotics that were previously used to kill them. Individuals with compromised immune systems greatly rely on antibiotics to treat infections, due to their lack of natural immunity (Zhao & Drlica, 2002).

There are several types of antibacterial drugs available and they are extensively used to protect the health of humans and animals by treating infections. These drugs are divided into groups based on their mechanisms of action (Willey *et al.*, 2008; Gutmann *et al.*, 1988). Some inhibit the synthesis of the bacteria's cell wall, proteins and nucleic acids. Several antibiotics disrupt cell membranes and act as antimetabolites (Kohanski *et al.*, 2010; Willey *et al.*, 2008).

Although antibiotics are produced in laboratories for commercial purposes, many of them were first discovered as products of naturally occurring micro-organisms. However, the same micro-organisms that produce the antibiotic also has to be resistant to their own antibiotic, which implies the existence of antibiotic resistance genes in the natural environment (Martinez, 2008). Many micro-organisms harbour antibiotic-resistant genes that are able to spread among water and soil bacterial communities (Baquero *et al.*, 2008) through horizontal gene transfer (Martinez, 2009), making a bacterial species previously susceptible to antibiotics, resistant.

Another method through which bacteria may develop resistance to antibiotics is by evolving under strong selective pressure during the antibiotic treatment of infections in humans (Martinez, 2009). A situation that may contribute to the development of resistance within a bacterial species is the fact that due to the increased usage of antibiotics, large quantities are released into the wastewater treatment plants (Martinez, 2008). Since most of the antibiotics excreted by humans enter the environment unchanged (Zhang *et al.*, 2009), it is possible that antibiotic resistance might evolve in the aquatic systems due to these increased levels of still bio-active antibiotics. The increased prevalence of antibiotic resistance pathogenic bacteria is a growing concern worldwide (Van den Bogaard & Stobberingh, 2000; Martinez, 2009).

Bacteria resistant to antibiotics have modified target sites (sites targeted by the antibiotic) and/or enzymes capable of destroying or deactivating antibiotic compounds, preventing them from entering the bacterium (Bax *et al.*, 2000; Crabbe & Mann, 1996). An example of an antibiotic that destroys the bacterium wall, is the *β*-lactam antibiotics (Rawat & Mair, 2010). However, Gram-negative bacteria have a natural resistance against first generation *β*-lactam

antibiotics. A large percentage of the acquired resistance occur by secreting *β*-lactamase that hydrolyze the *β*-lactam ring and deactivates the molecules' antibacterial properties. Gram-negative bacteria also have a natural up-regulated impermeability and efflux that assist them in inhibiting the antibiotic activity of *β*-lactams. Examples of diseases caused by waterborne bacteria to which antibiotic resistance have been described, include *Shigella* spp, *Salmonella*, *Vibrio cholerae*, *E. coli*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa* (Alanis, 2006; Jansen *et al.*, 2006).

The most often used test to determine microbial resistance is the Kirby-Bauer disk diffusion method (Joseph *et al.*, 2011). This method is performed by placing disks containing different concentrations of antibiotics on agar plates that have been inoculated with the bacterium of interest. Depending on the bacteria's growth it is classified as susceptible, intermediate or resistant (Willey *et al.*, 2008). Susceptible bacteria are effectively treated with the prescribed antimicrobial agent, whereas intermediate bacteria have a buffer zone. This buffer zone implies that the antibiotic will be most effective if the drugs are physiologically concentrated or when a high dosage of a drug can be used without serious adverse effects to the patients. Resistant bacteria are not inhibited by prescribed antimicrobial dosages and antibiotics will be ineffective in patient treatment (Willey *et al.* 2008).

2.4 Methods to determine water quality

There are various tests available that can be applied to examine the quality of drinking water. The two main classes include physico-chemical and microbiological parameters.

2.4.1 Physico-chemical parameters

The physical and chemical qualities of drinking water contributes to its acceptability to the general consumer (WHO, 2006). There are a number of physico-chemical parameters that are measured to determine water quality or to detect problems. The measurement of physico-chemical parameters forms part of standard water quality tests. SANS 241 (2011) and the TWQR (DWAF, 1996b) are guidelines used in South Africa to assess water quality.

Determining the pH, electrical conductivity (EC), total suspended solids (TDS), salinity, nitrates, chemical oxygen demand (COD) and biological oxygen demand (BOD) all give an indication of the quality of the water sample (Laluraj & Gopinath, 2006). Often conventional water quality analysis includes measuring the temperature, EC, dissolved oxygen (DO), dissolved organic carbon (DOC), total nitrogen and turbidity apart from the already mentioned characteristics (Zhou *et al.*, 2010). The chemical quality directly influences the microbial quality with regard to

supplying certain microbes with nutrients or sources. These parameters will briefly be described to show their potential effect on human health.

2.4.1.1 Temperature

The water temperature plays an important role with regard to physico-chemical equilibriums and biological reactions (Delpla *et al.*, 2009). It may directly or indirectly affect physical parameters such as pH, redox potential, microbial activity and dissolved oxygen (Park *et al.*, 2010). High temperatures lead to increased microbial growth and low temperatures can slow down microbial growth (Zamxaka *et al.,* 2004). Water temperature can be measured manually with a thermometer or electronic multiprobes.

2.4.1.2 pH

The pH value of water is a logarithmic expression of the hydrogen ion concentration in water, measured with the appropriate probe. It reflects the extent to which the water is acidic (pH < 7) or alkaline (pH > 7). Measuring the pH of water resources is useful as the pH controls the solubility and biological availability of nutrients (phosphorus, nitrogen, carbon) and heavy metals (copper, lead, cadmium) in natural water. These chemical constituents are pH-dependent (Banks *et al.*, 2004) and only when they are in solution they become bioavailable and might have biological effects like becoming toxic. Extreme pH levels can have adverse health effects (DWAF, 1998). Consumption of water with low pH values may cause gastrointestinal disorders such as ulcers, stomach pain, hyper-acidity and create a burning sensation. The pH of drinking water should be between 5 and 9.7 and for domestic use vary between 6 and 9 (TWQR) (DWAF, 1996b).

2.4.1.3 TDS

Total dissolved solids (TDS) include compounds such as the ions of sodium, calcium, bicarbonates, chlorides, magnesium, potassium, sulphates and also a small percentage of organic matter (WHO, 2011; Heydari & Bidgoli, 2012). TDS are transferred into water resources from urban runoff, industrial wastewater, as well as sewage. Chronic exposure to TDS through consumption of water may have severe health effects, but overall TDS affects the aesthetic quality of the water (Hohls *et al.*, 2002; DWAF, 1996a). TDS levels in water are determined by using automated meters (multimeter probes) or the gravitational method, in which the sample is evaporated and the remaining solids aremeasured (Atekwana *et al*., 2004). Drinking water

guidelines state that TDS levels should be less than 1 200 mg/ℓ (SANS 241: 2011) and for domestic use (TQWR) it should be less than 450 mg/ℓ (DWAF, 1996b).

2.4.1.4 EC

The ability of water to conduct electricity is reflected by the electrical conductivity (EC) (DWAF, 1996b). Water with high levels of salt conducts electricity more effectively. The EC, TDS and salinity levels in water are closely related. The electrical conductivity in water is directly proportional to the concentration of TDS by a factor of 6.5 at 25°C (Atekwana *et al*., 2004). Elevated EC levels can affect human health by disrupting the salt and water balance in heart patients, people with high blood pressure and infants (Memon *et al.*, 2008). The TWQR standard for EC is ≤ 70 mg/ℓ and ≤ 170 mg/ℓ for SANS 241 (2011) in drinking water. EC levels are measured with a multiprobe.

2.4.2 Microbiological parameters

Some pathogenic microbes do originate from human and other warm-blooded animal faeces that are released into the aquatic environment, mostly through surface runoff, soil leaching and wastewater effluents. The risk these pathogens pose to human health is associated with the uses of the water and the pathogen concentration in the water (Quattara *et al.*, 2009). The primary goal for quality management of drinking water from a health perspective is to ensure that consumers are not exposed to pathogens that are likely to cause disease (Zamxaka *et al.*, 2004). In developing countries it is especially difficult to evaluate and regulate the quality and impact of such waste on drinking water supply due to the lack of demographic information and statistics, particularly of rural areas. It is therefore very important to know the incidences when disease occurs in rural areas due to pollution.

It is difficult, costly and time consuming to detect all the pathogenic microbes present in untreated drinking water due to the large diversity of pathogens and the low abundance of each species. There are, however, various standard methods to detect the degree of contamination in water resources. The basic microbiological technique to monitor water quality requires the detection and enumeration of indicator organisms. Microbiological indicators are used as an indication of faecal pollution and potential risk of infectious diseases in the water (Szewzyk *et al.*, 2000). Indicator bacteria or viruses are not necessarily pathogenic, but originate from the same faecal sources as pathogenic microbes.

Indicator bacteria or viruses should comply with the following criteria: 1) they should be easy to detect using basic methods; 2) be present in the faeces of warm blooded animals; 3) be present

in water together with pathogenic organisms; 4) should not multiply in water sources or any other environmental setting; 5) have the same or longer life span than pathogenic organisms and should not cause adverse health effects in humans (WRC, 1998; DWAF, 1996a). There are four categories of indicator organisms generally used that comply with this criteria, namely *E.*coli, faecal coliforms, total coliforms, faecal streptococci and heterotrophic plate count bacteria (HPC) (Zamxaka *et al.*, 2004).

An increased number of indicator bacteria means a high probability for the presence of pathogens excreted via faeces (Szewzyk *et al.*, 2000). There are guideline levels (SANS:241 &TWQR) available for these indicator organisms in water, although this does not necessarily mean that if water complies with quality standards, it is free of potential pathogens. Laboratory conducted tests detect potentially toxic substances, contaminants and microbes. However, these tests cannot determine the direct effects on human health and predict what effect the pathogens may have should humans consume this water (Zamxaka *et al.*, 2004).

2.4.2.1 Heterotrophic plate count (HPC) method

Culture-based methods are used to determine the amount of bacteria present in a sample of food, air, soil, sputum, wastewater and drinking water. The most basic bacterial enumeration method is the plate count method. A standard method used to determine microbiological water quality is heterotrophic plate counts (HPC's) expressed as colony forming units (CFU's). Heterotrophic bacteria are present in soil, air, water and food and utilize organic nutrients as their energy source (Edberg & Allen, 2004). HPC bacteria represent the levels of bacteria present in water and can be isolated by using different culture-based methods under a predetermined set of conditions. These conditions include incubation time and temperature, the medium and also the way in which the medium is inoculated (WHO, 2002). HPC bacteria is therefore a subset of the heterotrophic bacteria within a sample (Allen *et al.*, 2004). The heterotrophic plate count (HPC) method has been used for more than 100 years and was developed by Robert Koch in 1881 as one of the first techniques to analyse drinking water.

This test is recommended and included the drinking water quality guidelines worldwide (Chowdhury, 2012; Siebel *et al.*, 2008). HPC bacterial numbers are also used to monitor the efficiency of treatment and disinfection processes (WHO, 2006). Higher levels of HPC bacteria in treated water is an indication of a decline in microbiological water quality, bacterial regrowth, possible stagnation and formation of biofilms (Bartram *et al.*, 2003; Szewzyk *et al.*, 2000). Over the years the plate count method has been optimised to culture all of the culturable HPCs,

considering the versatility and variety of bacteria present in an aquatic environment (Jeena *et al.*, 2006).

According to SANS:241 (2011) good quality drinking water should not exceed HPC bacteria of 1 000 CFU/mℓ (SANS, 2011). The TWQR however, states that HPC values of <100 CFU/mℓ has a negligible risk of infection, counts of 100–1 000 CFU/mℓ has a slight risk of infection and >1 000 CFU/mℓ has an increased risk of infectious disease transmission (DWAF, 1996b).

On the other hand the HPC method has some shortcomings. Incubation of the plate takes a long time and colonies will have different cell numbers and may crowd neighbouring colonies. Another factor to consider is the fact that the tens of thousands of different bacterial species each have different metabolic states and they all have different requirements for growth and detection. It is impossible to have a set of conditions that allows growth of all bacteria present. For this reason plate counts may vary by several log units and often underestimate the cell number (Zhou *et al.*, 2010). Because HPC results are influenced by various factors such as cultivation medium, incubation time and temperature and the selective culturability of bacteria (Van Der Wielen & Van Der Kooij, 2010; Siebel *et al.*, 2008) there are strict precautions to follow when the method is used and when results are interpreted (Hammes *et al.*, 2008). Despite the large variation of HPC bacterial numbers in drinking water this method is still commonly used and is still considered as very useful worldwide to obtain information about: 1) the effectiveness of drinking water treatment processes, 2) microbial water quality during distribution and storage, 3) microbial regrowth and after growth events (Van Der Wielen & Van Der Kooij, 2010; WHO, 2002).

2.5 Potentially pathogenic HPC bacteria

There are a variety heterotrophic bacteria present in water that are not yet well characterized. These bacteria have specific growth requirements and require certain media for culturing. It is generally believed that HPC bacteria are not harmful to healthy individuals, but there is a growing recognition that some of the bacteria may be pathogens and have the capability to cause adverse health effects on individuals with compromised health (Kalpoe *et al.*, 2008; Keynan *et al.*, 2007; Pavlov *et al.*, 2004; Lye and Dufour, 1991).

The list of opportunistic pathogens is increasing. Lye and Dufour (1991) used a membrane filter method to determine pathogenicity of microbes in drinking water. Heterotrophic bacteria were isolated and tested positive for characteristics associated with virulence. Similar results were obtained from a study done at Yale University by Edberg *et al.*, (1997). HPC bacterial isolates expressed virulence factors, suggesting that a significant number of bacteria in potable water

have pathogenic potential. However, Stelma *et al.* (2004) found that HPC isolates were not pathogenic to immuno-compromised mice, but suggested that more *in vitro* screening test should be done because bacterial virulence is multi-factorial and not well understood.
As can be expected there is a lot of controversy regarding the pathogenicity of HPC bacteria and will this study contribute to resolving the issue.

2.6 Methods to determine pathogenicity of micro-organisms

Consumed microorganisms that cause gastrointestinal diseases share a number of virulence characteristics such as secretion of extracellular enzymes, cytotoxicity to cells and adherence to cells, and survive passing through the gastric fluids of the stomach (Yuk & Marshall, 2004; Janda & Bottone, 1981). Pathogenicity is the organism's potential to cause disease and virulence refers to the degree of intensity of pathogenicity (Willey *et al.*, 2008). Degree of infection is calculated as the product between the number of organisms and virulence/host resistance (Willey *et al.*, 2008).

Micro-organisms secrete extracellular enzymes that act as toxins and are responsible for pathogenicity and cause diseases in a host. Pathogens have many ways of entering a host and consuming contaminated food and water is but one (Kashid & Ghosh, 2010).

A membrane filter technique was established by Lye and Dufour (1991) to determine the effects of bacteria and viruses on cell viability. Cultured cells are an *in vitro* investigative method to determine the cytotoxic responses when exposed to different extracellular toxins produced by bacteria. It is expected that bacteria will cause similar effects when *in vivo,* but whole animals do not have to be sacrificed (Lye & Dufour, 1991). It is particularly in this regard that the study will contribute to elucidating the possible effects of pathogenic bacteria on human intestinal cells.

2.6.1 Enzyme production

Micro-organisms can produce two types of toxins: endo- and exo-toxins. Endotoxins are lipopolysaccharides that are cell associated (Kashid & Ghosh, 2010). Exotoxins are soluble heat-labile proteins, some of which are enzymes, and are released into the surroundings as the bacterial pathogen grows.

2.6.1.1 Haemolysin

Haemolysin is responsible for lysis of erythrocytes and make iron available for microbial growth (Willey *et al.*, 2008). Haemolysin is one of the first toxins tested for when screening for pathogens or pathogenic potential. Bacteria are grown on blood agar containing sheep, horse or rabbit blood cells to determine whether they produce haemolysins. There are three types of haemolysins: alpha, beta or gamma (Payment *et al.,* 1994). Alpha haemolysins partially break down blood cells and beta haemolysins are responsible for full lysis of blood cells. Brownish growth on blood agar represents no haemolysis (gamma haemolysis). Other enzymes include hyaluronidase, chondroitin sulfatase, protease, lipase and lecithinase, which are hydrolytic enzymes and play a role in the infectious processes to some extent (Steffen & Hentges, 1981).

2.6.1.2 DNase

DNase induces the degradation of nucleic acids and is DNA-specific (Pavlov *et al.*, 2004). MacFaddin (1985) suggests that pathogens then use the degraded DNA as an energy source.

2.6.1.3 Proteinase

Proteinase, also known as protease, is identified as a virulence factor in a variety of diseases caused by microbes. This group of enzymes are responsible for the initiation of protein catabolism and thereby breaks down peptide bonds of long protein chains that link amino acids. However, its most important effect is the degradation of proteins that function in a host defence *in vivo*, which enables bacteria to enter the host . The digestion of proteins can be measured by growing the target bacterium on agar containing a protein substrate. Broken down proteins can be seen by a clear zone around the inoculum.

2.6.1.4 Lipase

Lipase are responsible for the reduction of triacylglycerols into monoacylglycerols, diacylglycerols, free fatty acids (FFA) and glycerol. The enzyme also catalyses the trans-esterification reaction, inter-esterification and esterification between a fatty acid and alcohol, which is the reverse reaction of hydrolysis (Kumar *et al.*, 2012; Sharma *et al.*, 2012). These enzymes play a major role in pathogenesis via host cell damage/modulation, inflammation and cell signalling (Bender & Flieger, 2010).

2.6.1.5 Hyaluronidase and chondroitinase

Hyaluronic acid is a constituent of the extracellular matrix that cements cells together and chondroitin forms part of the connective tissue (Willey *et al.*, 2008). Hyaluronidase and chondriotinase are classified as virulence factors because they make it possible for infecting microbes to penetrate tissue. These factors cause depolymerisation of the basic constituents of tissue: hyaluronic acid and chondroitin sulphate that are incorporated into agar plates to detect hyaluronidase and chondriotinase. The latter creates and therefore promotes invasiveness of some microbes (De Assis *et al.*, 2003).

2.6.1.6 Lecithinase

Lecithin is a group of fatty substances present in animal and plant tissue. Included in this group are: choline, fatty acids, glycerol, phosphoric acid, triglycerides and phospholipids. Lecithinase destroys lecithin in plasma membranes allowing pathogens to spread by forming pores in the membranes for bacteria to gain access to the cell (Willey *et al.*, 2008; Hoult and Tuxford, 1991). Lecithinase activity is recognized by the formation of phosphorus and choline, with precipitations of fat after bacteria were grown on agar containing fatty substrates (Esselman & Liu, 1961).

2.7 Molecular methods to identify bacteria

It is important to identify pathogens accurately because this aids in the selection of the appropriate and specific antibiotic, as well as the identification of the possible source of contamination (Saglani *et al.*, 2005). The conventional culturing methods may not be helpful in the identification of fastidious bacteria that need very specific culturing conditions. Traditional culturing methods also fail when antibiotic treatment has already begun because of low numbers of viable bacteria. The same is true for samples transported in poor conditions: too few viable bacteria survive the trip and as a result they do not grow in the culturing conditions (Rosey *et al.*, 2007).

The molecular technique of polymerase chain reaction (PCR), during which small pieces of genetic material is amplified (Willey *et al.*, 2008), and the subsequent identification of the nucleic acid based sequences of the amplified DNA make it possible to identify bacterial strains. This would not have been possible with the traditional culturing methods. For the identification of bacteria, 16S ribosomal RNA is targeted for amplification because it is present in prokaryotes (Janda & Abbott, 2007; Priest & Austin, 1993). The PCR technique is simple, accurate, time and

cost effective and widely used. There are three key stages to this method 1) denaturation of double stranded DNA by increased temperature; 2) annealing of primers to single strand DNA at lower temperatures and 3) extension of the primers into new complimentary DNA (Saglani *et al.*, 2005). This third step is carried out by Taq-polymerase enzymes, which synthesize complimentary copies of the initial single strand. These steps are repeated until enough of the product is formed, usually after 20 cycles. The amplified DNA is subjected to sequence determination, and a basic logic alignment search tool (BLAST) in the GENBANK (Burtscher *et al*., 2009).

Today, it is possible to extract rDNA from a water sample and amplify these DNA fragments in a PCR in a single day with the aid of commercial kits. Sequencing can also be done within 48 hours.

2.8 Cell based assay/model

The array of microbiological tests used to measure water quality does not necessarily indicate pathogenicity of micro-organisms or supply information about the human health effects. In essence, a model such as cultured cells from human origin, could be used to predict human health related effects and enable a more direct comparison as to whether certain HPC bacteria are in fact harmful. Cells in culture do not have an immune system like a human being, making this model more sensitive than a whole animal would have been. This is beneficial when the effect of microbes on immune-compromised individuals is investigated.

Cells are considered the most basic unit of living organisms. Specific techniques are used to isolate pure populations of particular cell types from human or animal tissue. These cells grow in a laboratory under desirable and controlled conditions with essential nutrients. They are maintained at a temperature of 37°C, and in the case of mammalian cells in a very sterile environment. Although long-term exposure to animals remains a fundamental tool for toxicology studies, cultured cells are often used as a screening tool to evaluate toxicity (Derfus *et al.*, 2004). The practice of using isolated human cell lines to evaluate cytotoxicity *in vitro* has been increasing (Sambruy *et al.*, 2001). The human cell line used in this study is the intestinal epithelial cell line, HuTu-80, derived from a duodenum adenocarcinoma obtained from a 53-year-old Caucasian male (Reidling *et al.*, 2006). These cells acted as a model for the human intestine in this study to determine the effects of bacteria in untreated drinking water on the viability of the cells. Cells from the alimentary canal were chosen because that is one of the first avenues of exposure.

2.9 Gastric fluid

Gastric fluid plays an important role in the first line of defence against ingested pathogens as a bactericidal barrier and was therefore included this study to determine the degree of survival of potential pathogens. The human gastrointestinal (GI) tract is a tube in the body running from the mouth to the anus and is approximately 9 meters long. The interior of the GI tract is lined with epithelium cells that act as a partial barrier to invasion. If pathogens breach this layer the immune system acts as the next defence system. Ingested food and water are exposed to enzymes from the salivary glands, thereafter it travels through the GI tract where it is digested and exposed to the hydrochloric environment of the stomach, bile from the liver and cells in the stomach, and the pancreas (Schnell & Herman, 2009). To mimic gastric fluids a simulated gastric fluid version can be prepared and consists of several components such as proteose-peptone, D-glucose, bile salts, lysozyme from chicken egg white, pepsin, NaCl, KH_2PO_4, $CaCl_2$, and KCl dissolved in distilled water and contributing to pH-hydrochloric acid-dependent environment of the stomach. The gastric environment typically has a pH of 1–3 (Just & Daeschel, 2006). The acidity of the human stomach may vary due to physiological variables such as food intake and drinking water.

The conditions in the stomach can be simulated in the laboratory by preparing an artificial mixture of enzymes, proteins and hydrochloric acid. Simulated gastric fluid (SGF) is a mixture prepared with different compounds known to be present in the human stomach. These *in vitro* SGF methods do not reproduce exact *in vivo* gastric conditions, but represents a good standardized model system for investigating interactions in the stomach (Schnell & Herman, 2009; Herman *et al.*, 2005). For exposures it is important to note that a fasting stomach has only 25 mℓ of gastric fluids that may increase to 200–250 mℓ during eating (Vertzoni *et al.*, 2005). A realistic volume to simulate the total fluids of the stomach falls in the range of 250–300 mℓ (Vertzoni *et al.*, 2005).

In spite of its low pH, a healthy human stomach contains about 2 500 microbes per millilitre (Just & Daeschel, 2006).There are many factors that restrict bacterial growth in the small intestine. The factors include activities associated with normal gastrointestinal tract physiology, gastric acidity, digestive enzymes, bile salts, mucus and exfoliation of enterocytes during epithelial renewal (Ouellette, 2004). However, there are pathogens that have evolved to cope with acidic environments and can survive for hours *in vivo* (Crittenden *et al.*, 2006).

2.10 Chapter summary

There may be a large number of pathogens present in untreated water sources. Standard tests only measure physico-chemical parameters and make use of indicators organisms, such as the HPC test and coliform tests to gauge microbial water quality. According to SANS:241 and TWQR guideline levels, water containing less than a 1 000 CFU/mℓ of HPC bacteria, are considered safe for human consumption but according to the literature HPC bacteria may be potentially pathogenic. It is therefore careless to assume that low levels of HPC bacteria pose no health risk. Potential pathogens pose a definite health risk especially to individuals with compromised health. The increasing prevalence of antibiotic resistant pathogens causes an even greater health risk. Enzyme production and cytotoxic tests have been used to qualify the pathogenicity of HPC's. The following sections present the approach of this study, determining pathogenic potential using established and new tests.

3. MATERIALS AND METHODS

To enable a logical flow for this section the methods are summarised in figure 3.1.

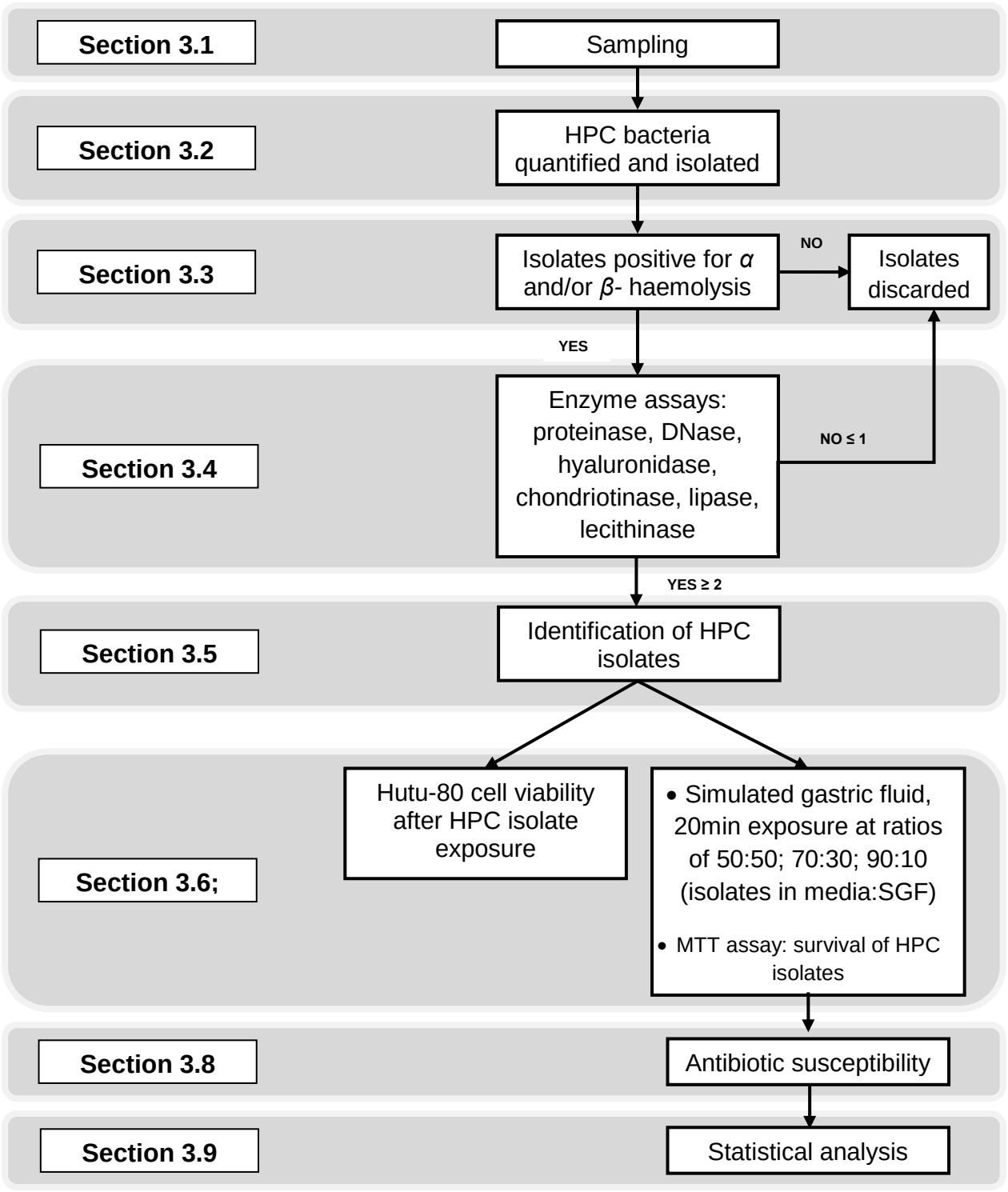

Figure 3.1: Sequence of the procedures and methods performed.

3.1 Sampling

3.1.1 Sampling sites

Groundwater samples were collected from various boreholes in the North West Province, South Africa (Table 3.1 & Fig. 3.2). The depth at which water was sampled ranged from 5 m to 75 m and varied for every borehole. Sampling took place in November 2012 (summer/high rainfall) and May 2013 (winter/low rainfall). Boreholes were selected on the grounds that they were regularly used by residents living in rural areas of the NW province and the residents consumed the untreated drinking water. The boreholes were sampled once to obtain different heterotrophic plate count bacteria from various locations that would enable a broad spectrum of isolates for investigation. Coordinates were taken at each sampling point to assemble a GIS-map indicating the locations of the boreholes sampled (Fig. 3.2).

3.1.2 Sampling method

Taps or pipes were purged for approximately 5 minutes prior to sampling to prevent collection of stagnant water in the pipe lines. Approximately 1 ℓ of water from each of the sources was sampled in sterilised glass bottles and thereafter capped immediately. Room was left in the bottle to allow mixing of the samples before laboratory experimentation. Water quality parameters such as pH, temperature, electrical conductivity (EC), salinity and total dissolved solids (TDS) were measured on site (Laluraj & Gopinath, 2006). The probe of the Multi-Parameter Testr 35 Series (Eutech Instruments, Singapore) was rinsed with distilled water before placing it in a sterile glass beaker containing the water sample. All the readings were recorded in a field notebook. The samples were kept on ice and protected from ultraviolet radiation during transportation to the laboratory, where it was stored at 4°C until analysis commenced, but not longer than 24 hours.

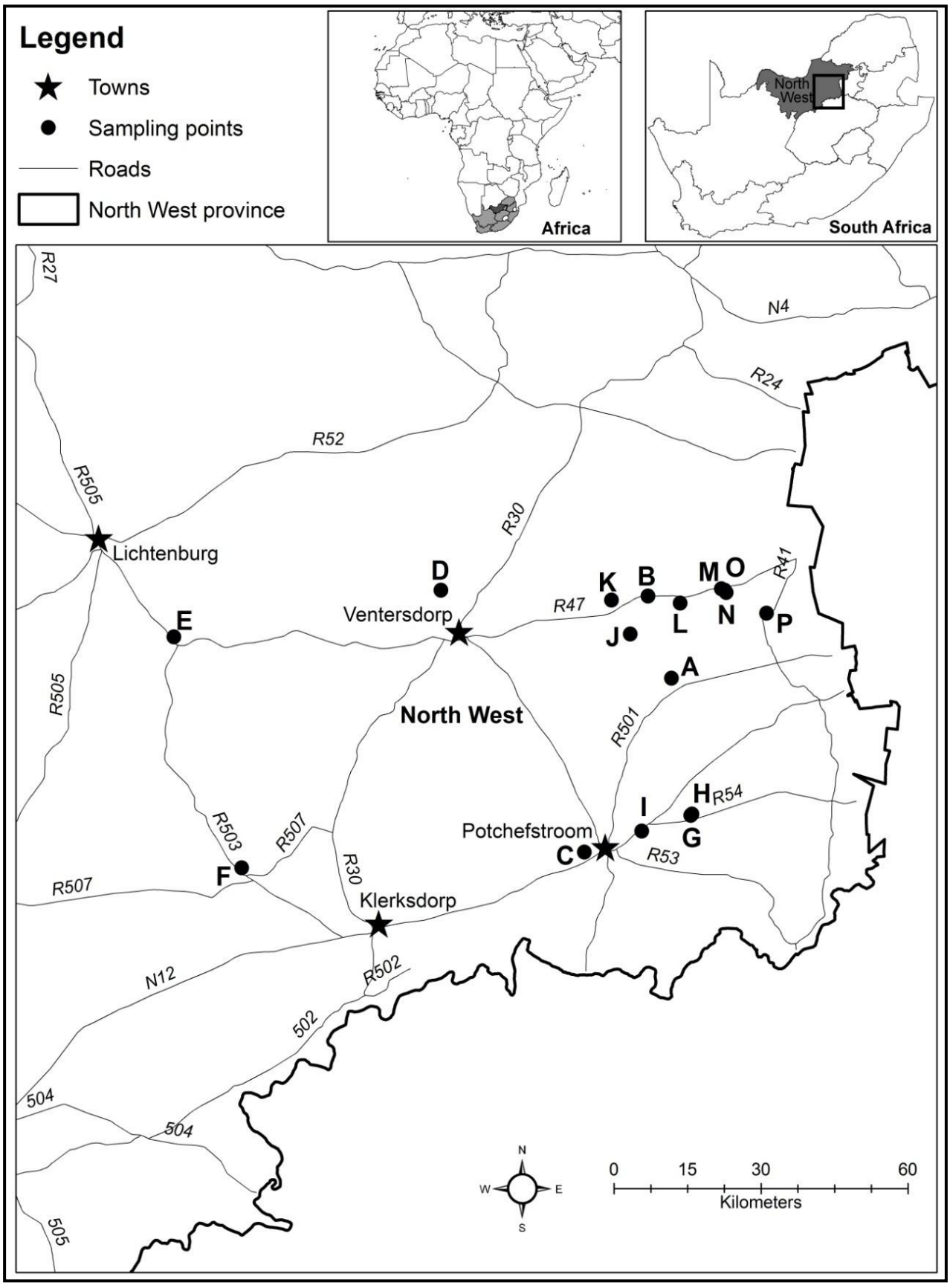

Figure 3.2: Map illustrating the distribution and location of all the sampling points in the North West Province

Table 3.1: Information on the location of the sites, as well as the time of sampling

Sites	GPS-coordinates	Site description	Sampling date
A	S26° 24' 4.99" E27° 12' 43.27"	40 km north-east from Potchefstroom, left hand side of R501, in a veld on a cattle farm.	November 2012
B	S26° 15' 10.28" E27° 10' 7.80"	Near Klerkskraal dam, surrounded by cattle grazing.	November 2012
C	S26° 42' 54.73" E27° 3' 7.79"	In Ikageng near an old gypsum dam (dolomitic area).	November 2012
D	S26° 14' 32.45" E26° 47' 19.70"	10 km N from Ventersdorp at a school situated in an informal settlement.	April 2013
E	S26° 19' 36.30" E26° 17' 55.60"	North-west direction from Coligny, at a school situated in an informal settlement.	April 2013
F	S26° 44' 36.50" E26° 25' 23.99"	At a school of the informal settlement in Hartbeesfontein.	April 2013
G	S26° 38' 47.25" E27° 14' 58.77"	Located north-east from Potchefstroom in the Vyfhoek area (agricultural activities).	May 2013
H	S26° 38' 56.20" E27° 14' 51.24"	Located north-east from Potchefstroom in a veld, cattle grazing in the area.	May 2013
I	S26° 40' 37.81" E27° 9' 26.39"	North-east from Potchefstroom at Vyfhoek Primary school.	May 2013
J	S26° 19' 17.94" E27° 8' 11.33"	Located on a farm, surrounded by agricultural activities.	June 2013
K	S26° 15' 36.60" E27° 6' 6.42"	Borehole located on farm with agricultural activities in the area.	June 2013
L	S26° 15' 56.00" E27° 13' 40.32"	On a farm, surrounded by agricultural activities and cattle grazing	June 2013
M	S26° 14' 23.58" E27° 18' 12.40"	East from Ventersdorp, area surrounded with agricultural activities	June 2013
N	S26° 14' 32.58" E27° 18' 35.02"	On a farm, near commercial slaughtering activities.	June 2013
O	S26° 14' 47.00" E27° 18' 45.53"	North-east from Potchefstroom, agricultural activities as well as a piggery in the area	June 2013
P	S26° 17' 2.35" E27° 23' 10.98"	10 km east from Carletonville, area surrounded by venues and houses.	June 2013

All the experiments were conducted under strict sterile conditions to prevent any contamination. Precautionary measures were taken to ensure that I am not exposed to the isolates and that they were not released into the laboratory environment.

3.2 HPC

3.2.1 Isolation of heterotrophic plate count bacteria

A dilution series (10^{-1} to 10^{-5} mℓ) of the sampled water was prepared and 100 µℓ of each diluent was spread plate inoculated onto the surface of solidified R2A agar (Merck, Germany) (Zhou *et al.*, 2010). Duplicates were prepared for each diluent. The plates were incubated at 37°C for 48 h. This temperature was selected because it is human body temperature and this study aims to determine effects of HPC isolates on human health. Colonies were counted and expressed as HPC colony forming units (CFU) per mℓ water (Venieri *et al.* 2010). More than 300 colonies on a plate were considered as too-many-to-count, and for conservative statistical analysis, these plates were assigned a value of 300 colonies (White *et al.*, 2010). A few colonies of each water sample were selected as representatives of that site and described based on morphological differences (Fig. 3.3) (Pepper *et al.*, 2004). The large size refers to a colony with a 15 mm diameter and the other sized colonies were scored in relation to the large colonies (Fig. 3.3). These isolates were purified by streak plating them on fresh agar plates and preserving them for further analysis.

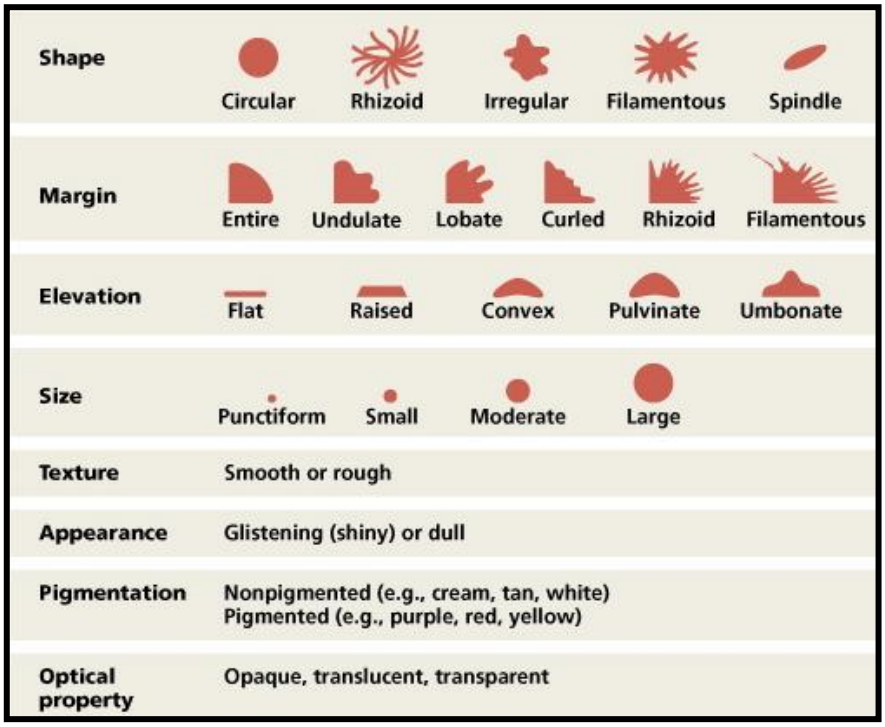

Figure 3.3: Illustration of the terminology used to describe microbial colony morphology (Pepper *et al.*, 2004).

3.2.2 Gram staining

The purity of isolates selected was confirmed with Gram staining. Smears were prepared from cultures grown overnight at 37°C. The slides were stained with 1% Gram's crystal violet for 1 min, rinsed, stained with Gram's iodine for 1 min, decoloured with acetone alcohol and counter stained with Gram's safranin for 1 min (Pandolfi & Pons, 2004). The slides were inspected at 1 000 times magnification under the oil immersion objective of a light microscope (Nikon E200, China). Purple and red stained organisms were regarded as Gram-positive and Gram-negative organisms respectively.

3.3 Selecting bacterial isolates capable of haemolysis

Haemolysin is a cytolytic protein responsible for lysis of red blood cells, and the ability to secrete haemolysin is a characteristic of virulent bacteria. Blood agar, which is rich in nutrients, and red blood cells are used to select the isolates with haemolysis capability (Payment *et al.*, 1994). There are three types of haemolysis: alpha (*α*), beta (*β*) or gamma (*γ*) (Valenzuela *et al.*, 2008). The presence of a distinct greenish-black ring around the inoculums indicates *α*-haemolytic activity (Şeker, 2010). A clear and colourless zone surrounding the colonies indicates *β*-haemolysin production (Xiao *et al.*, 2009; Hoeffler, 1977). The absence of haemolysis (only bacterial growth) was considered as *γ*-haemolysis (Tetlock *et al.*, 2012; Şeker, 2010).

HPC isolates were streaked on 5% sheep blood agar (BioMérieux, SA) and incubated at 37°C for 24 h (Xiao *et al.*, 2009). The isolates that produced *α*- and *β*-haemolysin were selected for further testing of extracellular enzyme production (section 3.4), as these characteristics are associated with infections and their increased severity in animal models (Upadhyaya *et al.*, 2010).

3.4 Selecting bacterial isolates with extracellular enzyme production

Media that contain substrates specific for particular enzymes were used for the assessment of different extracellular enzymes (Israil *et al.*, 2011). The bacterial production of the following enzymes was determined: proteinase, lipase, DNase, hyaluronidase, chondroitinase and lecithinase. These results, together with the results of haemolysin production, provided information about the potential pathogenicity of HPC bacteria present in untreated drinking water (Pavlov *et al.,* 2004).

3.4.1 Proteinase

Isolates were screened for proteolytic enzyme production on skimmed milk agar plates (Sen *et al.*, 2010; Janda & Bottone, 1981). The plates contained equal volumes of 3% (w/v) skimmed milk (Oxiod, England) and Brain Heart Infusion Broth (BHIB) (Merck, Germany) with the addition of 3 g agar (Merck, Germany) per 100 mℓ (Pavlov *et al.*, 2004). The ingredients were prepared separately, autoclaved and 20 mℓ of the culture medium were poured into plates and left to solidify (Saran *et al.*, 2007). Isolates were inoculated and the plate incubated at 37°C for 48 h. The development of transparent zones around the colonies indicated proteolytic activity (Boominadhan *et al.*, 2009).

3.4.2 Lipase

A medium containing tryptone soy agar (prepared according to manufacturer's instructions) (Merck, Germany) was supplemented with 1% Tween-80 (Sigma, Germany) and served as the substrate for lipase production (Kumar *et al.*, 2012; Israil *et al.*, 2011). A positive reaction for lipase is the appearance of a turbid halo around the inoculation spot after a 72 h incubation period (Pavlov *et al.*, 2004; Janda & Bottone, 1981).

3.4.3 DNase

DNase agar (Merck, RSA) was used and prepared according to manufacturer's instructions. Toluidine blue (Sigma, Germany) was added as a supplement and acted as an indicator by binding to hydrolyzed DNA and forming bright zones on a bright blue background (Sen *et al.*, 2010). The plates were incubated for 24–48 h at 37°C. After incubation the plates were flooded with 1 mol HCl (Merck, US) and scanned for the presence of a clearing zone around the growth, which indicated DNase activity (Gündoğan *et al.*, 2006).

3.4.4 Hyaluronidase

The secretion of hyaluronidase was tested by preparing medium containing 1 g Noble agar (Difco, France) for every 100 mℓ of BHIB (Merck, Germany). The medium was autoclaved and allowed to cool down. A second aqueous substrate containing 2 mg/mℓ hyaluronic acid (Merck, Germany) and 5% bovine albumin fraction V (final concentration) (Roche, Germany) was prepared. The solution was sterile filtered with a 0.22 µm filter (Corning, USA) before the two substrates were mixed together and poured into plates (De Assis *et al.*, 2003). Isolates were inoculated and the plates incubated at 37°C for 48 h. A positive result was indicated by a clear

zone around that inoculums which produced enzymes to attack the hyaluronidate (Pavlov *et al.*, 2004).

3.4.5 Chondroitinase

Chondroitin sulphatase activity was evaluated by incorporation of a chondroitin sulphatase aqueous solution into 1 g Noble agar for every 100 mℓ of BHIB (Pavlov *et al.*, 2004). The plates contained 4 mg/mℓ chondroitin sulphate A from bovine trachea (ROTH, Germany) and 5% bovine albumin fraction V (final concentration) (Roche, Germany) that were poured through a 0.22 µm filter to sterilize (De Assis *et al.*, 2003; Steffen & Hentges, 1981). After inoculation, incubation followed for 48 h at 37°C. A clear zone around the inoculation spot against a cloudy background is the evidence for chondroitinase activity (Xiao *et al.*, 2009).

3.4.6 Lecithinase

The secretion of lecithinase by a bacterium was determined by McClung-Toabe egg yolk agar (Steffen & Hentges, 1981). McClung-Toabe agar (Difco, France) was prepared according to manufacturer's instructions and sterilized. After cooling 1 part of the 50% egg yolk mix (Merck, RSA) was added to 9 parts of agar and plates were prepared. Plates were examined for evidence of egg yolk degradation after 72 h of incubation at 37°C (Esselman & Liu, 1961). A distinct zone of opacity around or beneath the inoculums spot on the egg yolk agar indicated the production of lecithinase (Jula *et al.*, 2011; Rossignol *et al.*, 2009).

3.5 Identification of HPC isolates using molecular methods

3.5.1 DNA extraction

Purified HPC colonies grown on agar plates were scraped from the plates using a sterile pipette tip and suspended into 20 µℓ PCR-grade nuclease-free water (Fermentas Life Science, US). The mixture was centrifuged at 13 400 rpm for 90 seconds. The tubes were subjected to microwave irradiation at high power (500 W) for 60 seconds and again centrifuged at 13 400 rpm for 90 s (Carturan *et al.*, 2008). One microlitre of supernatant was added to 24 µℓ of PCR reaction mixture to be amplified (section 3.5.2).

3.5.2 Polymerase chain reaction (PCR)

PCR reaction mixture contained 12.5 µℓ double strength PCR master mix [(0.05 U/µℓ Taq DNA Polymerase in reaction buffer, 0.4 mM of each dNTP (deoxyribonucleotide triphosphate); 4 mM

$MgCl_2$ (Fermentas Life Sciences, US)]; 10 pmol of each primer (Applied Biosystems, UK); 50 ng DNA template; 0.5 µℓ $MgCl_2$ (Fermentas Life Science, US) and PCR-grade nuclease-free water (Fermentas Life Science, US) to fill up the volume to 25 µℓ.

The primers used targeted certain regions of the 16S rRNA gene and were designed to amplify DNA of most bacterial species (Dempsey *et al.*, 2007). The primers used were 5'-AGAGTTTGATCMTGGCTCAG-3' (27F) and 5'-TACGGYTACCTTGTTACGACTT-3' (1492R), which would give an expected amplification product of about 1 465 base pairs (Weisburg *et al.*, 1991). The amplification reaction took place in a T100 thermal cycler (BioRad, UK) under the following conditions: 300 s at 95°C; followed by 35 cycles of 30 s at 95°C, 30 s at 52°C, 60 s at 72°C; and final extension of 300 s at 72°C (Fenollar *et al.*, 2006). The resultant PCR product was then analysed by agarose gel electrophoresis (section 3.6.1).

3.6 Characterisation of amplified DNA

3.6.1 Electrophoresis

To assess the quality of the PCR product and to detect amplicons, electrophoresis was performed. A mixture of 5 µℓ PCR product and 3 µℓ Gel Red loading dye (Biotium, US) was loaded into wells in a 1.5% (w/v) agarose gel prepared in TAE (Tris-acetate-EDTA) buffer [40 mM Tris (Sigma Aldrich, US); 20 mM acetic acid (Merck, US); 1 mM ethylenediaminetetraacetic acid (Merck, US); (Carturan *et al.,* 2008)]. Before loading the samples the agarose gel was placed into a tank containing TAE buffer. Electrophoresis then proceeded at 80 V for 50 minutes (Orsini and Romano-Spica, 2001). A 1 kb ladder as a DNA marker (Fermentas Life Sciences, US) was also run on the gel along with the PCR amplicons to enable identification of the sizes of these amplicons. Afterwards the amplicons were made visible using the BioRad Gel Doc imaging system (BioRad, UK). The expected amplicon was 1 465 base pairs long and a positive reaction was therefore defined with the presence of a band of this size (Rosey *et al.*, 2007)

3.6.2 Sequencing

DNA sequencing was performed on the samples that yielded a positive result with the electrophoresis step. A NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Germany) was used to purify the DNA amplicons and remove traces of primer-dimers and PCR reagents. The concentration and 260:280 ratio of the purified DNA amplicons were determined using the NanoDrop™ 1000 Spectrophotometer (Thermo Fischer Scientific, US). For pure DNA the

260:280 ratio should be between 1.6 and 1.9. The pure DNA amplicons served as the template for sequencing PCR. The BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, UK) was used for the sequencing PCR. The reaction mixture consisted of 2.5 X Ready Reaction Premix (Applied Biosystems, UK), 5 X BigDye Sequencing Buffer (Applied Biosystems, UK), 3.2 pmol of either the forward or reverse primer (Applied Biosystems, UK) — the same as was used in the original PCR reaction. The amount of DNA template used was determined according to manufacturer's instructions and differed depending on DNA concentration. The volume was filled up to 20 µℓ with PCR-grade nuclease-free water (Fermentas Life Science, US). The reaction took place in an iCycler thermal cycler (BioRad, UK) under the following conditions: 60 s at 96°C; 10 s at 96°C for 25 cycles; 5 s at 50°C; and extension for 420 s at 60°C followed by holding at 4°C.

The products of this reaction were purified again using the Zymo Research DNA Sequencing Clean-up Kit™ (Zymo Research, US) as per the manufacturer's instructions (Dashti *et al.*, 2009). The eluent was transferred to a 96-well plate and placed in the 3130 Applied Biosystems Genetic Analyser, where sequencing electrophoresis took place using a 36 cm capillary array and POP-7™polymer (all Applied Biosystems, UK). Analysis of the data was done by the 3130 Genetic Analyser Data Collection software (Applied Biosystems, UK). The resulting chromatograms were analysed in BioEdit v7.1.3 software. For identification of bacteria the sequences were compared to existing sequences in the GenBank database using the BLAST program (Basic Local Alignment Search Tool, http://www.ncbi.nlm.nih.gov) (Fenollar *et al.*, 2006).

3.7 Cell culture

3.7.1 Maintenance of the cells

HuTu-80 cells (HTB-40™) were obtained from the American Type Culture Collection (ATCC) (Manassas, USA). They were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, Germany) with a pH of 7.1 and supplemented with 10% foetal bovine serum (FBS) (Thermo Scientific Hyclone, UK) and 0.08 mol $NaHCO_3$ (Sigma, Germany). The cells were grown in tissue culture dishes (90 mm x 20 mm) (Tooltech Pty, SA) in a humidified atmosphere at 37°C and 5% CO_2 with media changes every 2–3 days (Reidling *et al.*, 2006). HuTu-80 cells are adherent cells and were isolated from the human intestine, which is one of the sections of the body that would be directly exposed to consumed water and where infections could be initiated.

3.7.2 xCELLigence RTCA system

The xCELLigence Real time cell analyser (RTCA) system (Roche, 2008) was used to determine cell viability during exposure to individual HPC isolates (section 3.2). This system consists of four components: the RTCA analyser, the RTCA single plate station, disposable 96-well E-plates and a computer with integrated software. The RTCA station is placed inside an incubator under standard culturing conditions (depending on cell-line specifications) and connected to the analyzer and laptop on the outside (Urcan *et al.*, 2010). This system measures electrical impedance across microelectrodes integrated with the bottom of the E-plates where the cells attach and can provide information on the cell numbers, morphology and viability in real-time (Atienza *et al.*, 2006). The cell-sensor impedance is expressed as the cell index (CI). The more cells attached to the surface of the E-plate, the higher the CI reading (Wu *et al.*, 2011(b)). Thus, a change in cell index suggests a change in cellular viability (Kloetzel *et al.*, 2013). To quantify the response of the cells, the data was exported to Excel and a cell index was calculated from a single time point resistance, from which the background resistance (only containing cell medium) were subtracted and percentage viability was calculated by using the following equation (Wu *et al.*, 2011(b); Zhu *et al.* 2006) %viability = (CI exposed cells/CI unexposed cells) x 100. There were two set of controls, one set of cells were not exposed to anything except for their growth medium, DMEM for the whole exposure period. The second set was exposed to sterile broth, to control for a possible effect that the broth might have on the cells.

3.7.3 Determining cell viability due to isolate exposure

At this point, isolates that tested positive for haemolysin production and that produced more than 2 extracellular enzymes were regarded as potentially pathogenic as gathered from previous studies (Pavlov *et al.*, 2004). The extent of their pathogenicity was further investigated by determining the ability of the microbes to be cytotoxic. The influence the individual isolates exerted on the viability of the HuTu-80 intestinal cells was subsequently determined. It was important to control for the bacterial concentration in the viability tests. The absorbance of the turbidity caused by microbial growth in nutrient broth was measured using a spectrophotometer and indirectly reflects the amount of microbial growth present.

Isolates under investigation were inoculated into nutrient broth by using sterile swabs. The broth was briefly mixed and 150 µℓ of the suspended inoculum was transferred to a 96-well plate. Absorbance of the nutrient broth containing the individual isolates was measured with a Powerwave X Select Microplate Spectrophotometer (Bio-Tek, US) at 520 nm every 30 min with periodic shaking for 48 h at 37°C (Parkkinen *et al.*, 2000). A growth curve of each isolate was

generated and reflected the specific time point at which each isolate reached their log growth phase at an optical density (OD) value of 0.4 (Sondi & Salopel-Sondi, 2004).

The effect that each bacterial isolate has on cell viability was determined using the xCELLigence RTCA system. Background impedance was measured after adding 100 µℓ of DMEM cell medium into the E-plate (Ryder *et al.*, 2010; Galante *et al.*, 2012). The HuTu-80 cells were seeded at 80 000 cells/mℓ, in E-plates, which is the ideal seeding density and ensured that the observed effects were due to the exposure of microbes and not to stress associated with over confluence (Freshney, 2005). The cells were allowed to adhere for 13.5 h before exposure while being monitored for effective attachment and proliferation (Yedjou & Tchounwou, 2007; Handfield *et al.*, 1996). After reaching approximately 90% confluency (CI value measured xCELLigence RTCA system) the HuTu-80 cells were exposed to 10 µℓ of nutrient broth containing the different isolates. The OD values were confirmed to be 0.4 which means all the isolates were all in the same concentration range when given to the cells. The cells were exposed in triplicate and incubated for 24 h. From seeding to the end of exposure, cell proliferation, spreading and attachment were registered as CI at 15 min intervals. The control cells received sterile nutrient broth. The real-time cell analysis was performed under cell culture conditions (37°C, 5% CO_2, 95% humidity) (Bondzio *et al.*, 2013).

3.8 The effect of simulated gastric fluid on bacterial viability

It is well-known that human gastric fluids act as an important first defence against ingested pathogens and are supposed to kill or inactivate these pathogens (Yuk & Schneider, 2006). The effect of simulated gastric fluid on HPC bacterial survival was determined by exposing these isolates to a mixture containing 8.3 g/ℓ proteose-peptone (Conda Pronadisa, Spain), 3.5 g/ℓ D-glucose (Merck, RSA), 0.05 g/ℓ bile salts (Difco, France), 0.1 g/ℓ lysozyme from chicken egg white (Merck, Germany), 13.3 mg/ℓ pepsin (Merck, Germany), 2.05 g/ℓ NaCl (Merck, RSA), 0.6 g/ℓ KH_2PO_4 (Merck, RSA), 0.11 g/ℓ $CaCl_2$ (Merck, RSA) and 0.37 g/ℓ KCl (Merck, RSA) in distilled water. The final pH of the SGF was adjusted to 2.5 with sterile 5.0 mol HCl (Yuk *et al.*, 2006). The solution was sterilized by filtering through 0.22 µm bottle top filters (Corning, USA). The HPC isolate suspensions were exposed to different dilution of SGF for 20 min at 37°C. The average time it takes water to move from the stomach to the duodenum is 20 min (Chavanpatil *et al.*, 2006). The total volume was 10 mℓ and dilutions were 50:50; 70:30; 90:10 (HPC bacterial suspension:SGF) to represent actual amounts when water is consumed. The concentration of the HPC bacterial cells in suspension were monitored to ensure that the cells are in the same growth phase at OD values of 0.4. A fasting stomach has 25 mℓ of gastric fluid, when a glass of water (250 mℓ) is consumed the gastric fluids are diluted, hence the 90:10 dilution. When

humans are about to eat or drink the stomach produces 250 mℓ gastric that is diluted 50:50 when a glass of water is consumed. The 70:30 dilution was chosen as an intermediate to investigate possible effects that could have been overlooked between the highest (50:50) and lowest (90:10) concentration of SGF.

The survival of the HPC bacterial isolates was determined by using the MTT viability assay, which is a colorimetric method based on the metabolic ability of the cells to reduce the yellow tetrazolium salt (MTT) to a blue crystalline formazan product (Mosmann, 1983). At the end of the SGF exposure period 100 µℓ of the mixture (SGF and HPC bacteria) was transferred to a 96-well plate to perform the MTT assay. Sterile media acted as the control and the assay was done in triplicate. Each well received 100 µℓ of 0.5 mg/mℓ MTT (Sigma, Germany) and were incubated at 37 °C. After 30 minutes 200 µℓ of dimethyl sulphoxide (Merck, RSA) was added and the plates were incubated at room temperature for 30 minutes to dissolve the newly formed formazan crystals**.** Absorbance was measured at 540 nm (Térouanne *et al*., 2000) in a micro plate reader (Berthold TriStar LB 941, Germany). The optical density of the solubilized formazan is directly proportional to the number of viable cells per well (bacterial cells in this instance) (Madsen *et al*., 2008). Optical density (OD) values obtained of each exposed isolate were expressed in terms of the OD values of the sterile broth for the same dilution to give fold viability (FV) values i.e. the number of times the OD of the bacteria containing mixture was greater than the OD of the control. A FV > 1 would imply surviving and viable bacteria. A FV ≤ 1 would mean no surviving bacteria capable of metabolising MTT into its blue product. Statistically significant differences were calculated by comparing the OD value of the exposed HPC cells to the OD values of the control that contained sterile broth and no bacteria.

3.9 Antibiotic susceptibility of HPC isolates

Antibiotic treatment is the last line of defence against infections if a person lacks an immune system and if the potential pathogen has the ability to survive passing through ones gastric fluids. Knowing which of the pathogenic isolates are antibiotic resistant strengthens the argument for improved microbial tests for drinking water quality: if HPC bacteria are both pathogenic and antibiotic resistant, then people with compromised immune systems are definitely at risk.

The antibiotic resistancy/susceptibility of the HPC bacterial isolates was determined using the Kirby-Bauer disk diffusion method (Pavlov *et al.*, 2004). HPC isolates grown in nutrient were spread plated on Mueller-Hinton (Merck, RSA) agar plates (Oparaocha *et al.*, 2010). Various antibiotics from different classes based on their modes of action were selected to be tested

(Kohanski *et al.*, 2010). Cell wall synthesis inhibitors included: Ampicillin 10 µg (AP10), Amoxillin 10 µg (A10), Vancomycin 30 µg (VA30) and Cephalothin 30 µg (KF30). Protein synthesis inhibitors included: Neomycin 30 µg (NE30), Tetracycline 30 µg (T30), Oxytetracycline 30 µg (OT30) and Streptomycin 25 µg (S25) for the 30 S ribosomal subunit and Chloramphenicol 30 µg (C30) as a 50 S ribosomal subunit inhibitor. Trimethoprim 2.5 µg (TM2.5) were chosen as a folic acid synthesis inhibitor. Antibiotic disks (diameter 6 mm) (Mast Diagnostics, UK) were placed onto the Mueller-Hinton spread plates and incubated for 24 h at 37°C. After incubation the growth inhibition zones were measured and compared with an interpretative chart to classify the isolates as resistant, intermediate or sensitive to the antibiotics (Jeena *et al.*, 2006).

3.10 Statistical analysis

Basic statistics were performed by using SPSS version 20, property of International Business Machines Corporation (IBM®). Statistics were used to test whether EC and TDS values correlate and if differences in viability were statistically significant for the cytotoxic tests and SGF exposures. Sample size dictated that non-parametric tests should be performed. The non-parametric tests include: Mann-Whitney and Spearman's test. *p*-values less than 0.05 were considered statistically significant.

4. RESULTS AND DISCUSSION

This chapter gives the results obtained after sampling groundwater from 16 different boreholes in the North West Province and offers an interpretation of these results. An battery of tests was performed to investigate the features of potential pathogenic HPC bacterial isolates. The chapter is structured along the lines of the chronology of the process, meaning that a results section is both presented and discussed before progressing to the next section. The reason for this choice is that to a large extent, the results of an analysis determined how to progress further.

4.1 Physico-chemical analysis

The physico-chemical parameters were measured as a first indication of the samples' water quality. These parameters included the pH, temperature, electrical conductivity (EC), total dissolved solids (TDS) and salinity (Table 4.1). The values were compared to guideline levels for drinking water recommended by SANS 241 (2011), as well as the Target Water Quality Ranges (TWQR) (DWAF, 1996b). The latter are specifically aimed at giving guideline values for water quality for different purposes, such as domestic and agricultural use.

4.1.1 Temperature

Water samples were collected during 2 different seasons, representing the wet and warm season, and the dry and cold season of 2012–2013 (section 3.1). Sites A–F were sampled in the wet and warm season and sites G–P were sampled in the dry and cold season. Although the overarching aim of this study was to determine the pathogenicity of HPC bacteria— and not how HPC activity changes over time in the same well — it is important to note the season in which water was sampled from a well as this most likely would influence the presence of bacteria. The water temperatures, however, only ranged from a minimum of 13.4°C in the winter (site I) to a maximum of 23.2°C in the summer (site C) (Table 4.1). Neither SANS 241 (2011), nor the TWQR (DWAF, 1996b), have guideline levels for temperature.

4.1.2 pH

The pH values of all the sites complied with the drinking water standard (SANS 241, 2011), as well as the TWQR for domestic use (Table 4.1). Water sampled from site A had the highest pH with a value of 7.94 and site L had the lowest pH with a value of 6.22. The pH values were in general considered to be neutral and will not likely have a negative effect on human or animal health when consumed.

Table 4.1: The physico-chemical characteristics of the water samples

Sites	pH	Temp (°C)	TDS (mg/ℓ)	Salinity (mg/ℓ)	EC (mS/m)
Drinking water quality (SANS 241, 2011)	**5–9.7**	**N/A**	**≤ 1 200**	**N/A**	**≤ 170**
TWQR: Domestic water quality (DWAF, 1996b)	**6–9**	**N/A**	**≤ 450**	**N/A**	**≤ 70**
A	7.94	20.6	260	181	36
B	6.85	22.8	237	166	33
C	6.95	23.2	900*	652*	127*
D	6.80	19.2	667*	474*	92*
E	7.44	20.4	128	84	18
F	7.84	18.7	424	298	60
G	7.47	14.9	478*	337	68
H	7.52	14.8	370	254	55
I	7.48	13.4	440	306	62
J	6.82	17.5	388	271	55
K	7.84	15.3	148	101	22
L	6.22	19.7	83	60	12
M	6.70	18.7	105	73	15
N	6.73	16.7	51	38	7
O	6.73	14.6	100	68	14
P	7.13	18.8	366	259	53

Temp = Temperature; * = Does not comply to TWQR

4.1.3 TDS

TDS levels in the water ranged from 51 mg/ℓ (site N) to 900 mg/ℓ (site C) (Table 4.1). Water from three sites (C, D and G) exceeded the TWQR for domestic use of the water and site I came close. However, none of the sites exceeded the SANS:241 TDS guidelines for drinking water (SANS 241, 2011) (Table 4.1).

4.1.4 Salinity

There is no drinking water TWQR (DWAF, 1996b) or SANS 241 (2011) guideline for salinity, but salinity is sometimes reported as total salt concentration or TDS (Grattan, 2002). The values obtained for TDS and salinity correlated ($p < 0.05$), and therefore salinity values were compared to the TDS drinking TWQR (DWAF, 1996a) and SANS 241 (2011). Salinity values are generally less than the TDS values. This is usually ascribed to the fact that there are solubilised compounds other than salts present. Again, sites C and D had salinity values greater than the TDS guideline from the TWQR (≤ 450 mg/ℓ) with values of 652 mg/ℓ and 474 mg/ℓ respectively (Table 4.1). The rest of the sites had salinity levels lower than the TDS guideline of TWQR. Not one of the sites' salinity was greater than the SANS 241 (2011) TDS guideline.

4.1.5 EC

Many salts are water soluble, increasing water's ability to conduct electricity and therefore the salt content is directly proportional to the EC (Grattan, 2002). The EC values from the samples correlated well (Spearman's test) ($p < 0.05$; $r = 0.99$) (Figure 4.1) with the TDS results and follow the same tendencies: the lowest value were from site N with 7 mS/m; the highest value were from site C with 127 mS/m. The EC for both sites C and D (Table 4.1) exceeded the TWQR for domestic use (≤ 70 mS/m). The EC for site G almost reached the maximum TWQR guideline level as well. None of the TDS, salinity, EC values exceeded the SANS guideline levels for drinking water (SANS 241, 2011).

According to the results of the physico-chemical analysis, the overall water quality of the samples was good and within acceptable levels except for EC, TDS and salinity of sites C and D. Finding only three of the sixteen sites with high levels for EC, TDS and salinity was unexpected, seeing that all of the sites are situated in an area with many agricultural activities such as maize crop farming and the consequent application of fertilizers. Water from sites C, D and G were, however, not fit for domestic use at the time of sampling as none of these samples complied with the TWQR with regard to TDS, salinity and EC. It would be worthwhile to monitor

all the physico-chemical qualities of the water in site G, as it was higher than the TWQR guideline and its EC is almost 50.

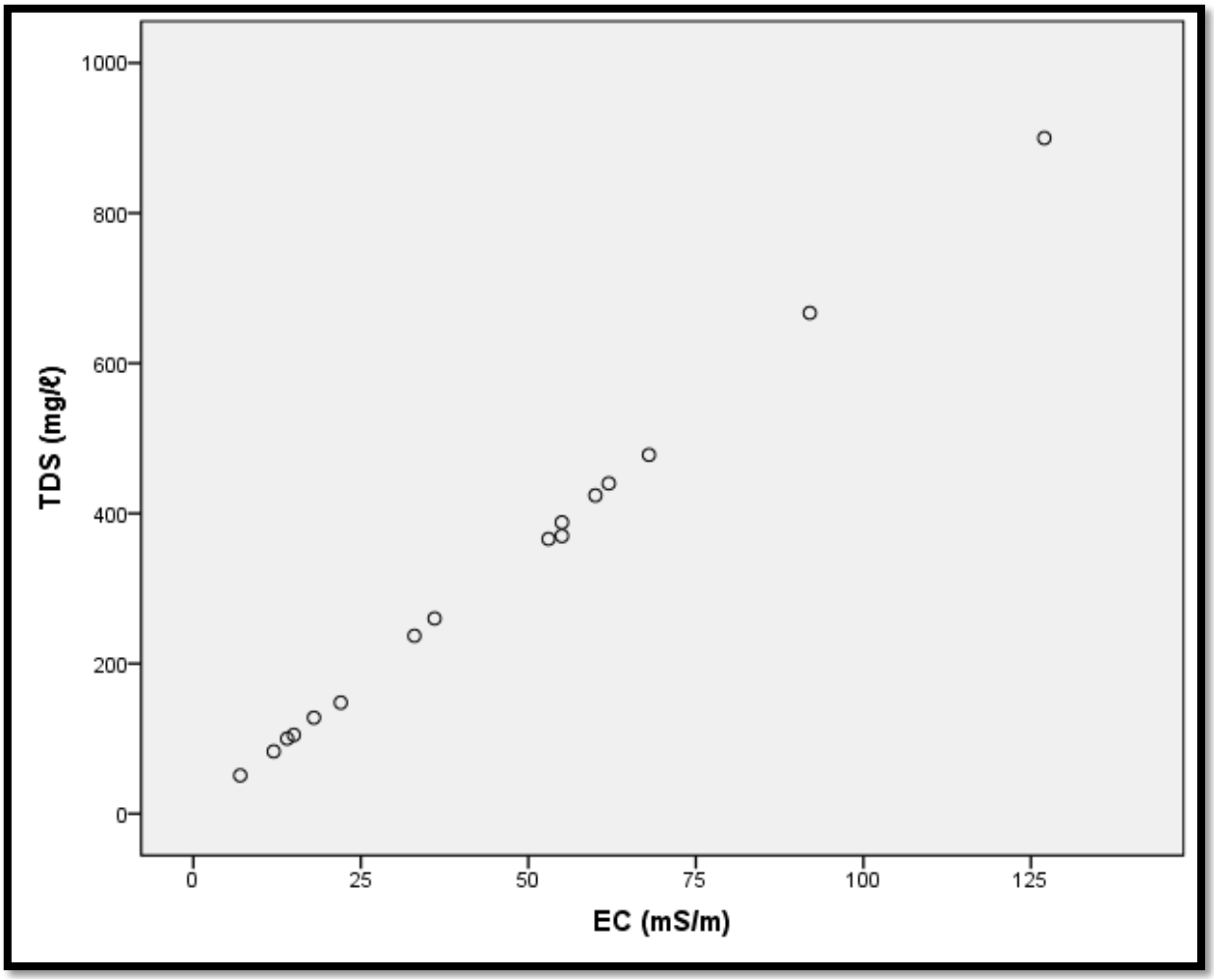

Figure 4.1: Correlation between TDS and EC for all the samples

4.2 Heterotrophic plate count

Heterotrophic plate counts (HPC) were performed by incubating the bacteria from untreated water samples at 37°C for 48 h on R2A medium. Microbiological water quality was evaluated by interpreting HPC results in terms of the SANS 241 guideline for drinking water and TWQR for domestic use, which state that good quality drinking water may not exceed 1 000 CFU/mℓ (SANS 241, 2011; DWAF, 1996a). Water that does not comply with this guideline may cause severe infections in humans when consumed by susceptible individuals. The TWQR have 2 other categories which state that HPC counts between 100 and 1 000 CFU/mℓ have a slight risk of infection and HPC counts < 100 CFU/mℓ pose a negligible risk of infection (DWAF, 1996b).

HPC bacteria were present in all the water samples (Table 4.2). Water sampled from sites A, B, C, E, F, G, I and N did not comply with the drinking water standard (SANS 241, 2011) or the TWQR for domestic use as the CFUs exceeded the standard of a 1 000 CFU/mℓ. Samples from site I contained 292 350 ± 476 501 CFU/mℓ which was the highest of all the boreholes sampled, even though it was sampled in the dry and cold season. Less HPC bacteria was detected from site K with only 5 ± 1 CFU/mℓ and was also sampled in the dry and cold season. Sites L, M, O and P had moderate levels of HPC bacteria ranging from 335–207 CFU/mℓ which were below SANS 241 levels, but within the TWQR guideline, 100–1 000 CFU/mℓ, indicating the slight risk to cause infection (TWQR) (DWAF, 1996b).

Table 4.2: Heterotrophic plate count results expressed as CFU/mℓ

Site	CFU/mℓ
Drinking water quality (SANS 241, 2011)	**≤ 1 000**
TWQR: Domestic water quality (DWAF, 1996)	**≤ 1 000**
A	^41 290 ± 34 179
B	^9 284 ± 21 210
C	^3 092 ± 3 290
D	30 ± 100
E	^2 078 ± 9 959
F	^88 287 ± 97 485
G	^9 598 ± 19 983
H	60 ± 123
I	^292 350 ± 476 501
J	85 ± 12
K	5 ± 1
L	225 ± 81
M	207 ± 128
N	^2 606 ± 9 932
O	335 ± 205
P	320 ± 52

^ = Does not comply to SANS 241 nor to TWQR

The HPC levels for sites D, H, J and K satisfied the SANS 241 (2011) guideline for drinking water quality and also the three TWQR guidelines for domestic use with HPC values below a 1 000 CFU/mℓ. Consequently, one can assume that if water complies with the guidelines, it is safe for human consumption. However, these guidelines do not take into consideration the degree of virulence of HPC bacteria. High levels of HPC bacteria are a concern due to the fact that some primary and secondary pathogens known to cause diseases in humans are represented by heterotrophic bacteria (Chowdhury, 2012).

HPC bacteria have been used as indicators of water quality for decades. There are however, indications that these bacteria may not be as harmless as previously accepted (Pavlov *et al.*, 2004; Payment *et al.*, 1994). Previous studies conducted in South Africa investigated the potentially pathogenic features of HPC isolates (Pavlov *et al.*, 2004). Characteristics that were analysed included haemolysis, capability to invade human cells, potential adherence to human cells as well as the production of extracellular enzymes responsible for bacterial virulence. Pavlov *et al.* (2004) concluded that HPC isolates in the drinking water invaded human cells and are thus potentially pathogenic to humans, especially to immuno-compromised individuals. There is much controversy with regard to the usefulness of HPC bacteria as indicators of microbial water quality (Donskey, 2006; Stelma *et al.*, 2004; Ford, 1999; Quiroz, 1999). HPC bacteria are used as indicators, and are therefore supposed to be non-pathogenic according to the criteria of indicator bacteria. Previous studies have shown that the latter is not true for all HPC bacteria (Pavlov *et al.*, 2004; Payment *et al.*, 1994). Some HPC bacterial representatives have previously proven to possess virulent characteristics, making them potentially pathogenic when exposed to a susceptible individual. The current study aims to enlighten this subject by determining whether HPC bacterial isolates from this study have virulence features.

From the 16 borehole water samples a total of 128 HPC bacterial isolates were selected and purified on the grounds of their morphologies and appearance. Different colony morphologies were observed among these HPC isolates. Some isolates were pigmented (yellow, orange, pink and red) and others were non-pigmented (white) colonies. Tokajian *et al.* (2005) commented that some pigmented colonies found in drinking water may be opportunistic pathogens.

4.3 Summary of physico-chemical and microbiological results

The results obtained from the physico-chemical (section 4.1) and microbiological (section 4.2) analysis revealed the general water quality status of the samples collected. These tests are standard methods used to evaluate and screen the water quality (DWAF, 1996a; DWAF, 1996b). Sites C and D failed to comply with TWQR in terms of TDS, EC, salinity this was also the case for site G's TDS levels. Sites A, B, C, E, F, G, I, L, M, N, O and P did not comply with TWQR and SANS 241 with regard to the amount of HPC bacteria present.

The water sampled at sites C and G failed both the physico-chemical and microbiological guidelines. The water from site C and G were therefore compromised and after only performing standard tests one should already know that using or consuming this water may pose a health risk to humans.

Sites H, J and K complied with the guideline levels for both the physico-chemical and microbiological analysis and are at this stage of the investigation regarded as safe for human consumption. There were a few HPC bacteria present in these samples, although not at high levels.

There are, however, other factors contributing to water quality and its effects with regard to health related risks that are not detected by the standard water quality tests. One of these factors is that HPC bacteria may be pathogenic. The rest of this chapter is dedicated to proving that judging water quality on standard water quality criteria is perhaps not enough.

4.4 Haemolysis

Bacteria that produce haemolysin are regarded as virulent. Purified HPC bacterial isolates were grown on blood agar plates to determine whether they are haemolytic. Of the 128 HPC bacteria isolated, 35 (27%) were haemolytic (Table 4.3). Thirteen (37%) and 22 (63%) isolates were responsible for *α*- and *β*-haemolysis respectively.

The number of isolates per water sample purified from a specific site is reflected in table 4.3. This number gives no indication of the number of bacteria, merely those that were isolated due to their morphological appearance. Isolates that proved positive for *α*- and *β*-haemolysis ranged from 1 to 6 isolates per water sample. There were no haemolytic bacteria present among the isolates from site J, K, L and M. According to these results and the results of the physico-chemical and microbiological tests, water from sites J and K are of good quality.

Up to this point, the water quality for site H was considered to be good according to the physico-chemical (Table 4.1) and microbiological (HPC) (Table 4.2) analysis, but there were haemolytic bacteria present in the samples (Table 4.3). This raises doubt about the quality of the water from site H. It was evident from the physico-chemical (section 4.1) and microbiological (section 4.2) results that the water quality of the samples from sites A, B, C, D, E, F, G, I and N were poor. Now adding to the list factors indicating poor water quality are the presence of haemolytic bacteria (Table 4.3).

Table 4.3: Number of isolates that were *α*- and *β* haemolytic

Site	Number of isolates tested	Isolates positive for *α*- or *β*-haemolysis
A	13	6
B	14	6
C	15	6
D	5	1
E	13	1
F	11	3
G	13	4
H	7	2
I	10	2
J	3	0
K	1	0
L	3	0
M	3	0
N	6	1
O	9	1
P	2	1
Total	128	35

Although the percentage of haemolytic bacteria may seem small, the aim of the study was to identify different types of HPC bacteria, to have a higher diversity and to determine their pathogenic potential, rather than a large number of the same isolates. There may be more haemolytic bacteria present in the sample that was not selected for further investigation. A more detailed study could be conducted to test all the isolates.

Inomata *et al.* (2009) found 32.5% and Lye and Dufour (1993) 36% haemolytic isolates among HPC bacteria isolated from treated drinking water. These values are in the same range as the 27% haemolytic bacteria found in the current study. However, in this study untreated drinking water was tested. Pavlov *et al.* (2004) found 55.5% haemolytic HPC bacteria from treated and untreated water, which is double the percentage of what this study revealed. The ratio of *α*- and *β* haemolytic bacteria were similar to that of the study of Pavlov *et al.* (2004). In both cases, a third of the HPC bacteria were *α*-haemolytic and the rest *β*-haemolytic. This is contrary to the findings of Inomata *et al.* (2009), who reported 39 and 3 isolates responsible for *α*- and *β* haemolysis respectively. Almost a third of the HPC isolates tested in the present study have demonstrated pathogenic potential based on results from the haemolysin assay.

In conjunction with the haemolysin assay, extracellular enzyme production was determined. Bacteria have the ability to produce more than one extracellular enzyme. If they are haemolytic and produce more than one such enzyme, they were regarded as potentially pathogenic.

4.5 Enzyme production and identification of bacteria

The ability of the 35 haemolytic HPC isolates to produce other extracellular enzymes was further investigated against a panel of 6 enzymes. These enzymes included: proteinase, DNase, hyaluronidase, chondroitinase, lecithinase and lipase. The results are depicted in figure 4.2 and illustrate the frequency at which certain enzymes were produced by the different HPC isolates. Those isolates that were able to produce more than one enzyme were classified as potentially pathogenic (Edberg *et al.*, 1996). Of the 35 haemolytic bacteria, 22 (63%) produced more than one extracellular enzyme.

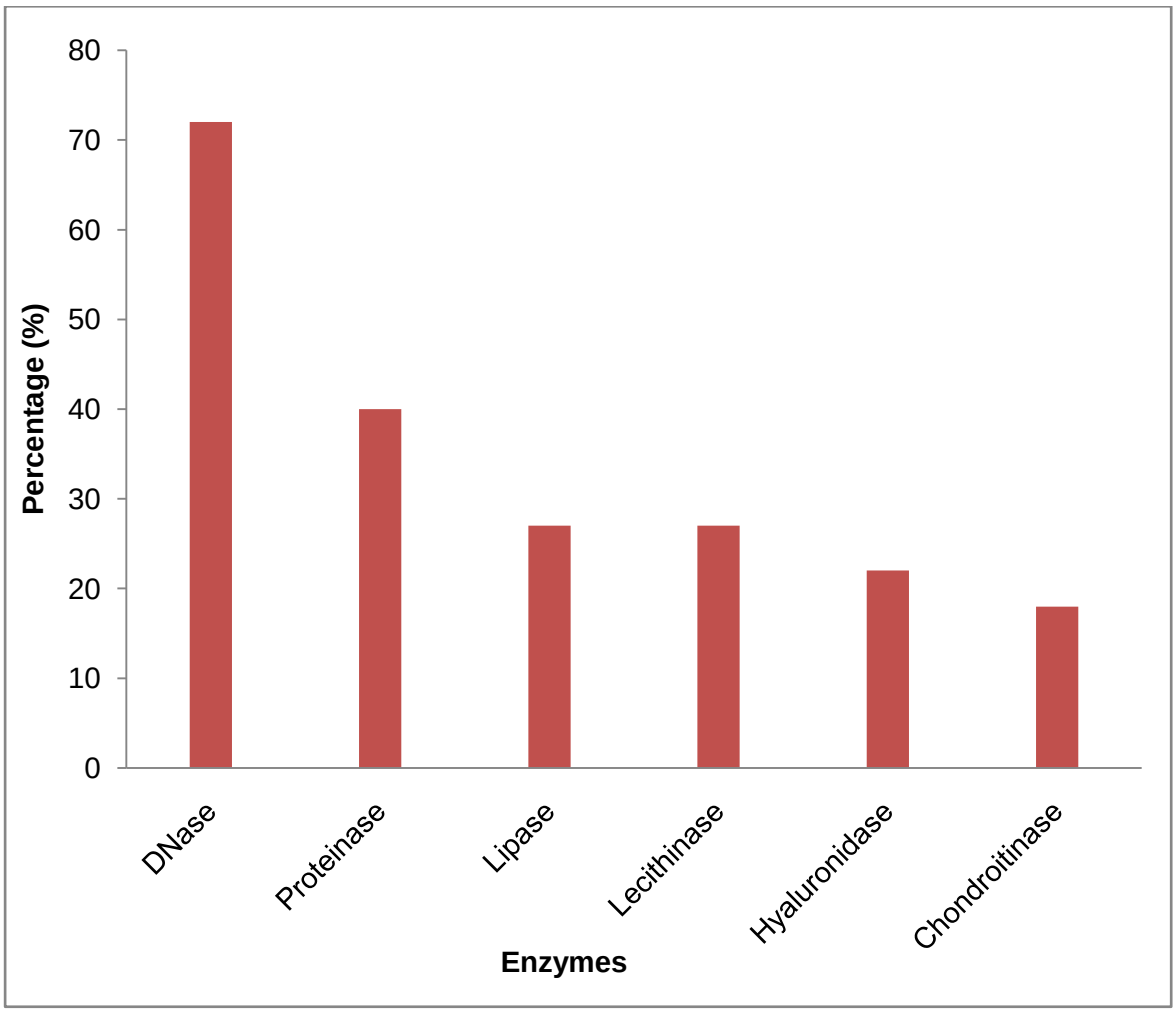

Figure 4.2: Total percentages of enzymes produced by haemolytic HPC isolates

Pavlov *et al.* (2004) isolated HPC bacteria from treated and untreated water sources to measure pathogenicity potential and obtained the following enzyme production results: proteinase 64.4%, DNase 60.6%, lipase 54.8%, lecithinase 47.9%, hyaluronidase 21.3% and chondroitinase 5.3%. In contrast with these results the current study revealed that DNase was produced by most of the isolates (72%), followed by proteinase (40%). For the remaining enzymes the trend of most to least produced was: lipase > lecithinase > hyaluronidase > condroitinase. This agreed with results from Pavlov *et al.* (2004).

DNase induces the degradation of cell components such as DNA (Pavlov *et al.*, 2004). MacFaddin (1985) suggests that pathogens then use the degraded DNA as an energy source. DNA is present in humans and almost all other organisms (except RNA viruses), therefore concern arises when DNase is the most dominant enzyme produced by HPC bacteria isolated from drinking water.

The results however, reflect the enzyme production of culturable bacteria, specifically the HPCs. It is well-known that environmental samples have high bacterial numbers, but only a small percentage of them are culturable and therefore detectable with this method (Leclerc and Moreau, 2002). This leads to an underestimation of the real threat and are pathogens present in water sources in much higher numbers than anticipated with culturable methods.

The HPC isolates that were *α*- or *β*-haemolytic and produced 2 or more extracellular enzymes up to this point were already regarded as potentially pathogenic. These isolates were identified by molecular methods (section 3.8). Out of the 22 isolates there were 10 different species identified (Table 4.4). HPC colonies were selected based on morphological differences and replicates of the same organism were not isolated. So, these isolates only represent a portion of the HPC bacteria present in the water samples. The identified bacteria and the enzymes they excrete are summarised in table 4.4. From this section onwards the emphasis is on these HPC bacterial isolates and their characteristics. Two extracellular enzymes were produced by *Pseudomonas* sp., *Bacillus pumilus, Aeromonas taiwanesis* and *Brevibacillus. Alcaligenes faecalis*, *Bacillus thuringiensis, Aeromonas hydrophila, Bacillus cereus, Bacillus sp.* and *Bacillus subtilis* each produced 3 extracellular enzymes.

Table 4.4: The identity and enzyme production of the different HPC isolates

Isolate	Identity	Haemolysis	Extracellular enzymes produced					
			P	D	H	Le	Li	C
A1	*Alcaligenes faecalis*	*α*	x	x	x			
A2	*Bacillus thuringiensis*	*α*		x	x	x		
B1	*Aeromonas hydrophila*	*β*		x	x			x
C1	*Bacillus cereus*	*β*		x		x	x	
D1	*Bacillus sp.*	*β*	x	x			x	
F2	*Pseudomonas* sp.	*β*	x			x		
F3	*Bacillus subtilis*	*β*	x	x			x	
G2	*Bacillus pumilus*	*β*		x		x		
H2	*Aeromonas taiwanesis*	*α*				x	x	
O1	*Brevibacillus* sp.	*α*	x			x		

P=Proteinase, D=DNase, H=Hyaluronidase, Le=Lecithinase, Li=Lipase, C=Chondroitinase

4.6 Enzyme production by the different HPC isolates

Every HPC bacterium produces specific extracellular enzymes which contribute to their pathogenic potential. In the following section each isolate and its enzyme producing profile is discussed. The role that temperature might have on type of enzyme produced, was not investigated in this study. All isolates were incubated at 37°C to imitate human body temperature.

4.6.1 *Aeromonas* spp.

Aeromonas taiwanesis was responsible for the production of lecithinase and lipase. DNase, chondroitinase and hyaluronidase were produced by *Aeromonas hydrophila* (Table 4.4). Although these two isolates originated from the same morphological group, they produced two different sets of extracellular enzymes. According to Handfield *et al.* (1996) *A. hydrophila* strains are able to produce proteinases. However, no proteinase were produced by the strain isolated in the current study. Furthermore, Cumberbatch *et al.* (1979) found *Aeromonas hydrophila* positive for cytotoxin and haemolysin production. Both Cumberbatch *et al.* (1979) and Mateos *et al.* (2008) commented on the high prevalence of the virulent environmental strains of *A. hydrophila* because they produce proteinase, enterotoxins and cytotoxins.

Aeromonas hydrophila is commonly found in aquatic habitats, although it is not their only habitat. This bacterium is widely distributed and therefore has a high capacity to adapt to different environments. *A. hydrophila* is known to be an opportunistic pathogen and can grow in temperatures ranging from 4°C to 42°C. Mateos *et al.* (2008) found that haemolytic and cytotoxic effects are more severe at 37°C rather than environmental temperatures that range from -5°C−25°C, which highlights the relevance of the role of these extracellular enzymes in the pathogenic process.

The presence of these strains in drinking water is a major health concern. Some cases of human infections associated with high numbers of *A. hydrophila* in water have been reported (Mateos *et al.*, 2008). This organism has previously been isolated from 3% of healthy people (Cumberbatch *et al.*, 1979). This shows that the organism could be non-virulent in such healthy subjects.

4.6.2 *Alcaligenes faecalis* sp.

The isolate identified as *A. faecalis* produced proteinase, DNase and hyaluronidase (Table 4.4). In a study by Thangam and Rajkumar (2000) *A. faecalis* produced proteinase, as in the current study.

A. faecalis are present in a wide variety of ecological niches such as water, soil and various clinical samples, such as faeces, blood and other body fluids (Kahveci *et al.*, 2011). *A. faecalis* may be present in the alimentary canal of humans, but is not likely to cause infections in healthy humans, although this organism has been known to cause infections in humans with compromised and uncompromised immune systems. The majority of the infections are due to contamination of medical devices (Kaliaperumal *et al.*, 2006). A study done by Simmons *et al.* (1980) found that *A. faecalis* isolated from broiler chicks were likely responsible for respiratory disease in the chickens. This organism has been isolated from human and non-human hosts and has pathogenic potential. Many studies have been conducted on avian and mammalian species and Kahveci *et al.* (2011) proposed that these pathogenic mechanisms may be carried out in human hosts.

4.6.3 *Bacillus* spp.

The *Bacillus* sp. found in this study produced proteinase, DNase, lecithinase and lipase (Table 4.4). Proteinase, DNase and lipase were produced by *Bacillus* sp. According to previous studies most *Bacillus* spp. are known for their production of lecithinase (Kushner & Heimpel, 1957). The results from this study concurred with this as four from the six *Bacillus* isolates produced lecithinase (Table 4.4).

Bacillus thuringiensis was responsible for the production of DNase, lecithinase and hyaluronidase. This is slightly different from the results reported by Molva *et al.* (2009) where *B. thuringiensis* also produced DNase and lecithinase, but had proteinase as the third enzyme. This organism forms crystal proteins (Cry toxins) that are widely used in agriculture for lepidopteran pest control. The production of the insecticidal crystal protein distinguishes *B. thuringiensis* from *B. cereus* strains.

B. thuringiensis is known to cause diarrhoea and emesis, as well as some non-gastrointestinal infections in humans (Kotiranta *et al.*, 2000). The diarrhoeal disease is caused by enterotoxins, produced by *B. cereus* in the small intestine. Due to the increased use of biopesticides, *B. thuringiensis* is released into food and water sources, to which humans are then exposed. Only

a few cases of *B. thuringiensis* causing gastrointestinal diseases have been reported for humans (Hansen & Hendriksen, 2001).

Bacillus cereus produced DNase, lecithinase and lipase (Table 4.4). In a previous study *B. cereus* also produced DNase and lecithinase, which agree with the enzymes produced in the current study (Molva *et al.*, 2009). Chaves *et al.* (2011) and Cadot *et al.* (2010) investigated *B. cereus* from various kinds of foods, and found that *B. cereus* produced lecithinase and the majority were *β*-haemolytic, as reflected in this study. The pathogenicity of *B. cereus* is closely related to secretion of toxins as haemolysins, emesis-inducing toxins, phospholipases and enterotoxins such as non-hemolytic enterotoxins and cytotoxin K (Bottone, 2010). This organism is a spore-forming, aerobic-to-facultative, Gram-positive rod and widely distributed. Its natural environment includes fresh and marine waters, decaying organic matter, soil, vegetables and the intestinal tract of invertebrates. This organism has close genetic (16S rRNA) and phenotypic relationships to other *Bacillus* species. Spores germinate within an insect or animal host, enhancing chances of survival for this potential pathogen (Bottone, 2010). It was previously determined that lecithinase plays a role in the pathogenicity of *B. cereus* towards the larch sawfly (Kushner & Heimpel, 1957).

Proteinase, DNase and lipase were produced by *Bacillus subtilis.* This organism's enzyme production profile is the same as *Bacillus sp*.

Bacillus pumilus produced DNase and lecithinase. The production of lecithinase is in contrast with previous studies conducted by Hoult and Tuxford (1991). These authors did not report lecithinase production by *Bacillus pumilus*.

4.6.4 *Brevibacillus* sp.

Proteinase and lecithinase were produced by *Brevibacillus* sp. (Table 4.4). This species produced the same enzymes as *Pseudomonas* sp. *Brevibacillus* sp. was formerly known as *Bacillus brevis*. The habitat of *Brevibacillus* is closely related to *Bacillus* sp. These organisms are abundant in soil habitats (Sanders *et al*., 2006), commonly found in air, water and decaying organic matter. *Brevibacillus* sp. is a Gram-positive spore-forming organism and grows optimally at 35°C–55°C. Huang *et al.* (2005) found that *Brevibacillus laterosporus* G4 produce proteinase.

According to Park *et al*. (2009) *Brevibacillus* spp. isolated from medical waste are opportunistic pathogens. In accordance with the last two statements, the *Brevibacillus* sp. isolated in this

study has shown signs of pathogenesis through haemolytic activity and the production of two extracellular enzymes.

4.6.5 *Pseudomonas* sp.

Pseudomonas sp. produced 2 enzymes: proteinase and lecithinase (Table 4.4). Kida *et al.* (2011) found that the production of proteinase and haemolysin by *P. aeruginosa* play an important role in this organism's pathogenic potential. *P. aeruginosa* is an opportunistic pathogen and known to cause infections in humans (Kida *et al.*, 2011). The secretion of proteinase is a major virulence factor in this organism and the isolated proteinase produced by *P. aeruginosa* were proven to be involved in pathogenesis. Proteinases are responsible for hydrolysis of proteins and peptides, and cause tissue damage in hosts, which contributes to the survival of this pathogen in a host (Kida *et al.*, 2011). In previous studies (Brözel *et al.*, 2007) *Pseudomonas* were non-haemolyitc, which was not the case for the *Pseudomonas* isolated in the current study.

Pseudomonas strains were tested for the production of various enzymes, cell adherence and also antibiotic susceptibility by Sasikala and Sundararaj (2012). They found that all the strains produced *β*-haemolysin, which is also what the current study revealed. Sasikala and Sundararaj (2012) reported that from the *Pseudomonas* they isolated, 81% produced proteinase, 77% lipase and 13% lecithinase. The *Pseudomonas* HPC isolate in this study also produced proteinase and lecithinase, however, no lipase production was observed.

4.7 Cell viability after exposure to individual isolates

In this section the effect these bacterial isolates had on the survival of the human intestinal model, the HuTu-80 cells, is presented. According to Lye and Dufour (1993) the eukaryotic cell culture cytotoxicity test is the most reliable indicator of bacteria expressing virulence factors. HuTu-80 cells (human intestinal cells) were exposed to HPC isolates to determine whether they cause cytotoxicity. Cell viability was monitored every 15 min with the xCELLigence system over a period of 36 h. The degree of cytotoxicity was based on the time frame in which cell viability was significantly decreased (Mann-Whitney, $p \leq 0.05$). Cytotoxicity was recognised when cell viability of the exposed cells differed significantly from the unexposed cells. The degree of pathogenicity between isolates was based on the time it took for cell viability to decrease significantly. Statistically significant differences were calculated by determining cell viability (section 3.6.2) and then comparing the cell viability of the exposed cells to that of unexposed

cells. The quicker the isolate caused a significant decrease in cell viability, the more pathogenic it was considered to be.

The percentage viability of the cells for the total experimental period (before and after exposure) is given in figure 4.3. The cell index values (CI) obtained from the exposed cells were expressed in terms of the unexposed cells (control; i.e. cells receiving DMEM only) and converted to percentage viability. It was assumed that these cells were 100% viable (Prinsloo *et al.*, 2013). Another control was added at the same time as the HPC isolates to compensate for the effect of sterile broth (in which the HPC isolates were grown).

The HuTu-80 cells were seeded into gold-plated 96 well E-plates at t = 0 h. Initially the percentage viability values ranged from 130% to– 115%. This is attributed to the fact that the cells began attaching to the surface of the plate, hence the wide range of %viability (Fig. 4.3). The cells' growth were stabilised after approximately 1 h.

From t = 0 h–13.5 h the cells were all unexposed, they received the same 'treatment' and were 100% viable. Between 2 h and 13.5 h cell viability was rather stable with slight increases or decreases in percentage viability (Fig. 4.3). The viabilities ranged from 75%–130% (Fig. 4.3).

Because of the range in percentage viability at the time of exposure (t = 13.5 h) between the various isolates, each graph was adjusted to represent 100% viability at t = 13.5 h. This was done by adding or subtracting the amount needed to move each line to the 100% mark. Doing this made the comparison easier. Figure 4.4 displays the 100% viability-normalised graphs at the time of exposure to the HPC isolates at t = 13.5 h–36 h.

At the approximate end of the exposure period (t = 34–36 h) all the cell cultures showed a gradual decrease in viability (Fig 4.3). Since this was observed for every isolate, it could be ascribed to the long exposure period during which nutrients became depleted. At t = 13.5 h the HPC isolates grown in the nutrient broth were added to the cells.

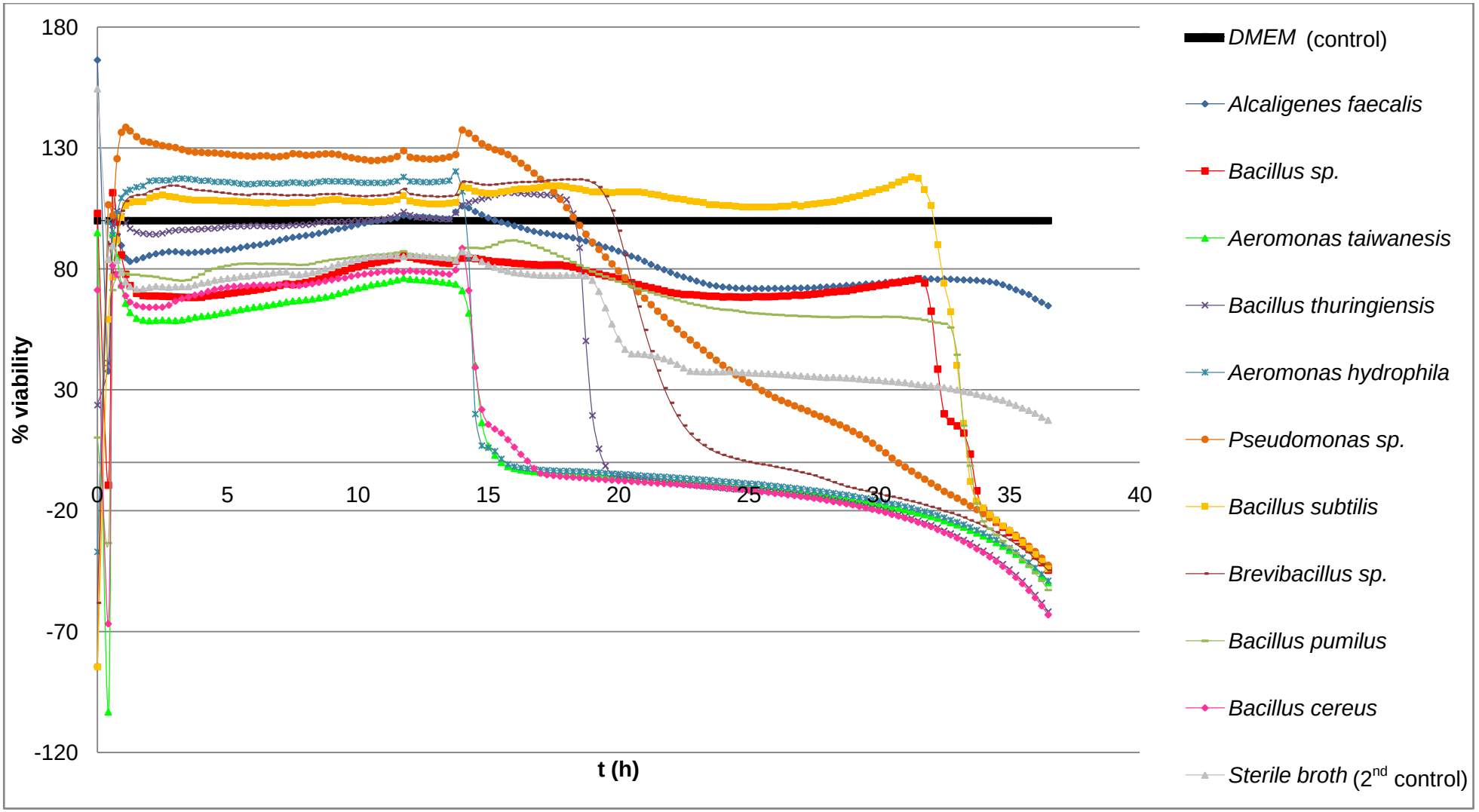

Figure 4.3: Percentage cell viability for the whole cytotoxicity experiment (before and after exposure to HPC isolates)

The initial slight decrease (t = 13.5–19.5 h) could be explained by the change in the impedance due to the added broth, but at t = 19.5 h the viability dramatically decreased to below 60% within a 90 minute period (Fig 4.3), followed by a further gradual decrease until the cells were only 30% viable (t = 33 h). This sudden decrease in cell viability caused by the sterile broth was not observed for the cells exposed to those bacteria that had a delayed decrease in cell viability, such as *Alcaligenes faecalis, Bacillus* sp., *Bacillus pumilus* and *Bacillus subtilis* (Fig 4.3). It could not be observed for those bacteria that had caused an almost immediate decline in cell viability, i.e. *Aeromonas hydrophila, A. taiwanesis*, as well as *Bacillus cereus*, because these isolates' impacts were so severe that it masked the less dramatic impact of the broth. The results obtained for the sterile broth could therefore not be used to control for an effect of the broth, as the supposed sterile broth must have been contaminated.

Statistically significant differences were therefore calculated by comparing the CI values of the exposed cells to the CI values of the cells exposed to DMEM (and not the sterile broth).

Aeromonas *hydrophila* and *A. taiwanesis*, as well as *Bacillus cereus* caused an immediate decline in cell viability after adding them to the cells (Fig. 4.4). The decrease in viability caused by *Aeromonas hydrophila* was quick and caused cell death within 60 minutes (Fig 4.4). *Aeromonas taiwanesis* and *Bacillus cereus* instantly decreased the cell viability to below 40% (t = 15) within an hour, followed by a more gradual decrease to 20% (t = 19), after which a steady decline in viability could be observed until the end of the experiment (t = 36 h) (Fig 4.4). *Aeromonas hydrophila* were responsible for the quickest significant decrease in cell viability (t = 13.75 h). *Aeromonas taiwanesis* and *Bacillus cereus* both caused a statistically significant viability decrease at t = 14.5 h.

The effects caused by the HPC bacteria on the viability of the HuTu cells were divided into different categories:

No effect		DMEM, *Alcaligenes faecalis*
Immediate effect	Steep	A. *hydrophila* and *A. taiwanesis*, *Bacillus cereus*
	Gradual	*Pseudomonas*
Delayed effect	Steep	*Brevibacillus* sp., *Bacillus thuringiensis*
	Gradual	Sterile broth
	Late, steep	*Bacillus* sp., *Bacillus pumilus, Bacillus subtilis*

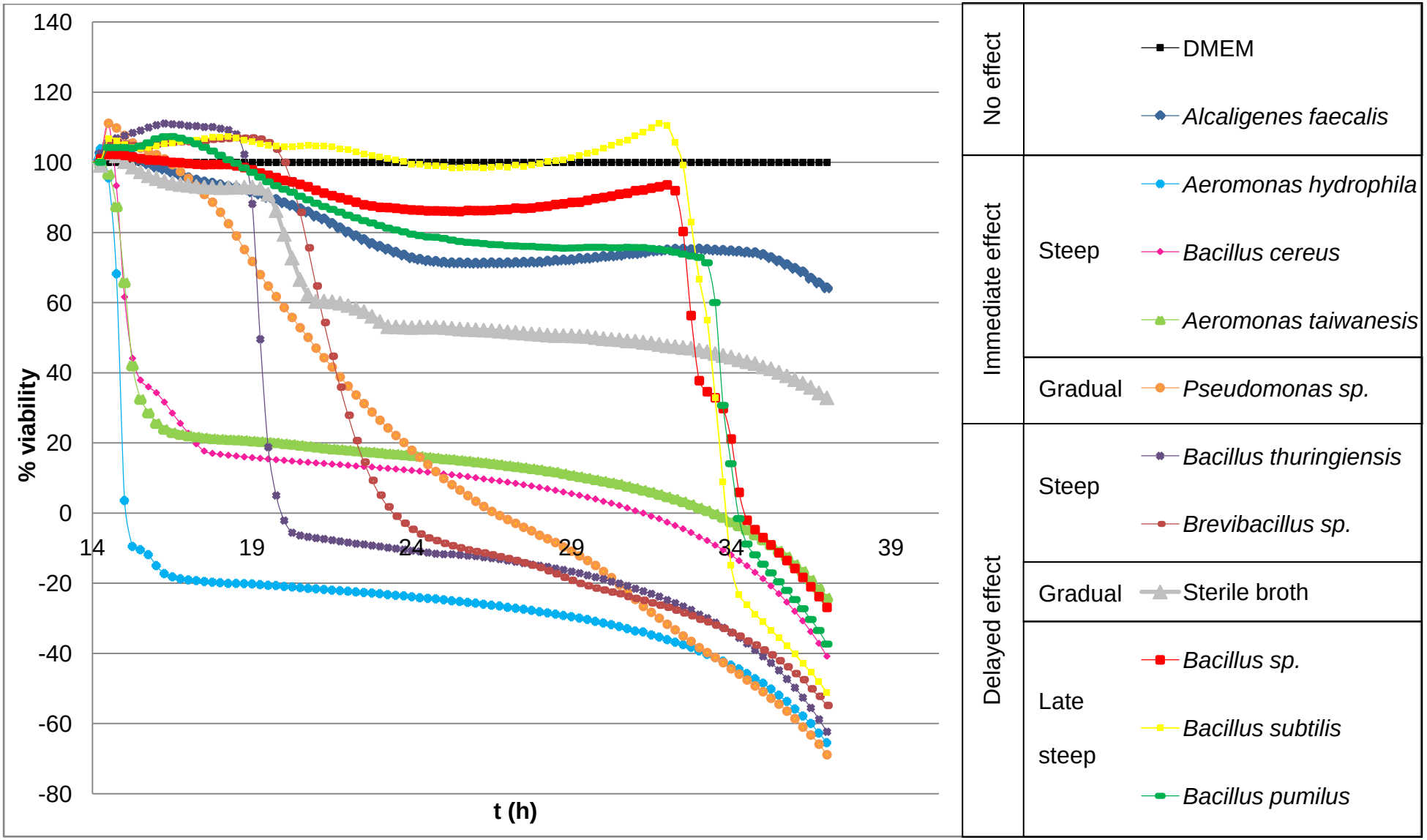

Figure 4.4: Percentage viability of cells after exposure to HPC isolates

Pseudomonas sp. influenced cell viability directly after being added to the cells. Cell viability decreased gradually from the beginning to the end of the exposure (Fig. 4.4). However, cell viability was only statistically significantly decreased at t = 19.5 h.

The cells exposed to *Bacillus thuringiensis* responded with a slight increase in viability initially and at t = 19 h cell viability began to decrease. After 1 h of decreasing cell viability the cells were all dead. The decrease in cell viability compared to the DMEM control were statistically significantly different at t = 18.75 h.

Brevibacillus sp. had almost the same effects on cell viability as *Bacillus thuringiensis.* However, this organism began to decrease cell viability a few minutes later than the latter and statistically significant differences were only caused at t = 20.25 h.

Alcaligenes faecalis, Bacillus sp., *Bacillus pumilus* and *Bacillus subtilis* did not influence cell viability until t = 32 h. The cell viabilities were constant and varied from 70% to 110%. At t = 32 h *Bacillus* sp. and *Bacillus subtilis* began to cause a decrease in viability. Within 2 h these organisms had caused the cells to completely die off. However, statistically significant differences caused by *Bacillus* sp. and *Bacillus subtilis* when compared to the DMEM control were only observed at t = 32.24 h and 33.24 respectively. The viability of the cells exposed to *Bacillus pumilus* decreased at t = 32.5 h and these cells also died at t = 34 h, with significant differences at t = 32.99 h.

Alcaligenes faecalis did not cause any decrease in HuTu cell viability during the exposure period. It might be possible that this isolate is slow growing and only decreases cell viability after a longer exposure period. One should also keep in mind that if an isolate does not immediately cause cytotoxicity, the effects can be overlooked, and when one least expects it, the isolate may cause cytotoxic effects.

A study conducted by Pang *et al.* (2010) showed that *B. thuringiensis* isolates were cytotoxic to Chinese hamster ovary cells, as is the case in the current study. These strains also harboured enterotoxins, also known as extracellular enzymes that are released by microorganisms and that target the human intestine and induce diarrhoea, vomiting and abdominal pain. It has therefore been demonstrated (Pang *et al.,* 2010) that some *B. thuringiensis* strains may cause disease symptoms. Handfield *et al.* (1996) conducted a study in which cytotoxicity caused by *Aeromonas* were investigated in seven different cell lines: human lung carcinoma cells, Chinese hamster ovary cells, human epitheloid carcinoma cells, human foreskin fibroblasts, human colon adenocarcinoma cells, African green monkey kidney cells and mouse adrenal tumour cells.

Cytotoxic activity was caused by 73% of the *Aeromonas* isolated from water. Both the *Aeromonas* spp. isolated in this study caused a significant decrease in cell viability, which is consistent with results of previous studies. According to Cumberbatch *et al.* (1979) the production of the cytotoxins correlate with diarrhoeal disease ($p = 0.004$). The survey conducted by Handfield *et al.* (1996) confirmed that *A. hydrophila* strains isolated from food and water source produce different virulence factors.

This test could also discriminate between the different HPC isolates and the effects they had on the cells' viability. Previously Prinsloo *et al.* (2013) concluded that human intestinal cells acted as a good model to determine the effects of bacteria present in water on the viability of cells. However, a direct link to human health still needs to be established. It is important to keep in mind that these cells grown in tissue culture dishes, without having the benefit of an immune system. Individuals with compromised health might be at risk.

4.8 Bacterial survival after exposure to SGF

Gastric fluids act as a natural defence mechanism against ingested pathogens (Yuk, *et al.* 2006). The survival ability of the potentially pathogenic HPC bacteria isolated and identified was investigated by exposing the HPC isolates to simulated gastric fluid. The effect of SGF on survival of the bacteria was determined with the MTT assay. The MTT viability assay was developed and used for many years to determine viability of tissue culture cells. The dehydrogenase activity of living cells reduces the yellow MTT into a blue-purple formazan product (Foongladda *et al.*, 2002). Live bacteria do the same (Prinsloo *et al.*, 2013).

HPC isolates were exposed to different dilutions of SGF (pH 2.5) for 20 min (section 3.6). The dilutions were 50:50; 70:30; 90:10 (HPC bacterial isolate:SGF). After exposure the MTT assay was performed. Sterile broth exposed to SGF acted as a control containing no viable bacteria (section 3.7). Fold viability (FV) was calculated by expressing the OD of the HPC bacterial isolates in terms of the sterile broth containing no viable bacteria (FV=OD HPC/OD broth). A FV > 1 would imply surviving and viable bacteria. A FV ≤ 1 would mean no surviving bacteria capable of metabolising MTT into its blue product. The statistical differences were determined with the Mann-Whitney test ($p \leq 0.05$) by comparing OD values of the isolates exposed to SGF to the sterile broth exposed to the SGF.

All of the isolates, except *B. subtilis,* had a FV value > 1 indicating that all of them survived the 90:10 (HPC bacterial isolate:SGF) exposure. The OD values for most of the isolates were statistically significantly higher than the control (Figure 4.5). The ODs of only *B. subtilis, Bacillus* sp. and *Brevibacillus* sp. were not statistically significantly different (Fig. 4.5).

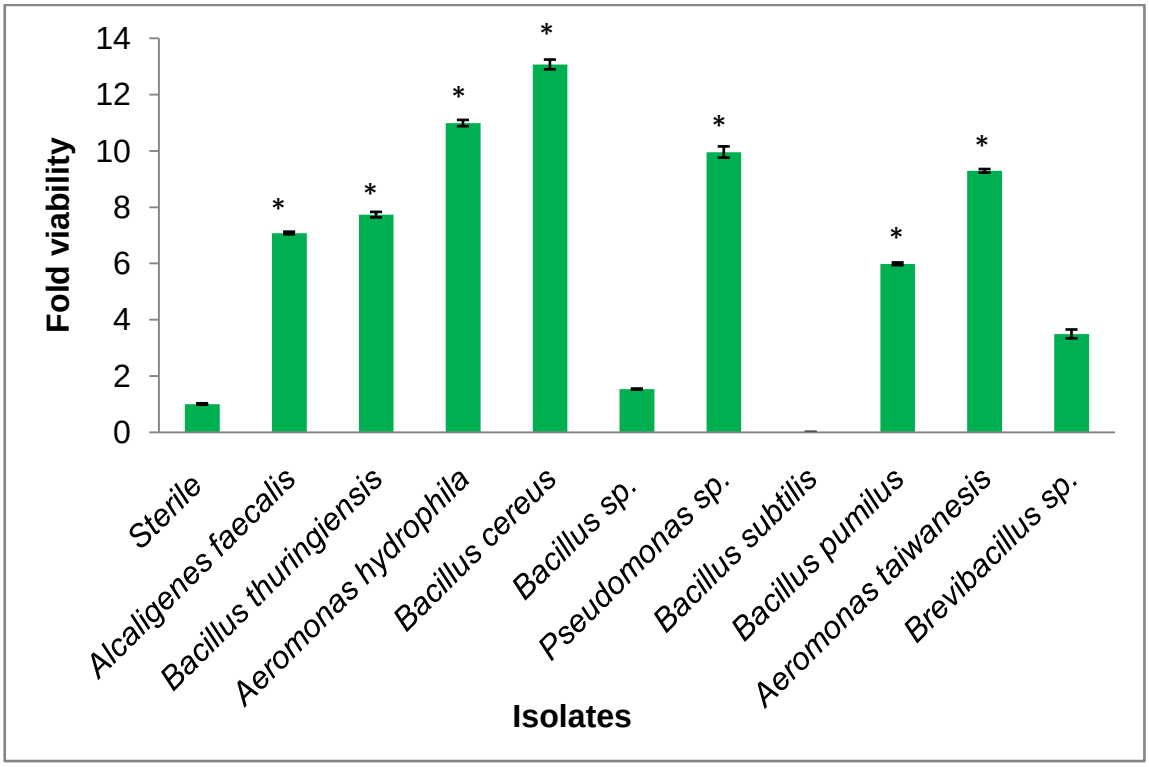

Figure 4.5: Fold viability of isolates after 90:10 (isolate:SGF) exposure

* indicates when the OD differed statistically from the FV of the control ($p \leq 0.05$)

Thus, for the lowest concentration SGF i.e. 90:10 (HPC bacterial isolate:SGF) 9 isolates had a FV > 1. However, only 7 isolates: *Alcaligenes faecalis*, *Bacillus thuringiensis*, *Aeromonas hydrophila*, *Bacillus cereus*, *Pseudomonas* sp., *Bacillus pumilus* and *Aeromonas taiwanesis* differed statistically significantly (Fig. 4.5).

Most of the isolates had a FV > 1 for the 70:30 (HPC bacterial isolate:SGF) exposure, except for *Alcaligenes faecalis, Bacillus thuringiensis, Bacillus subtilis and Brevibacillus* sp. Statistically significant differences were once again obtained from *Aeromonas hydrophila*, *Bacillus cereus, Pseudomonas* sp. and *Aeromonas taiwanesis* (Fig 4.6). The latter also survived the 90:10 (HPC bacterial isolate:SGF) exposure.

For the 50:50 dilution fifty percent of the isolates had FV > 1, indicating the survival of these species. However, only *Bacillus cereus* was statistically significantly different when compared to the sterile mixture (Fig 4.7). *Aeromonas hydrophilia*, *Pseudomonas* sp. and *A. taiwanesis* survived both the 90:10 and 70:30 (HPC bacterial isolate:SGF) exposures, but not the highest concentration (50:50). *Bacillus cereus* survived all three exposures.

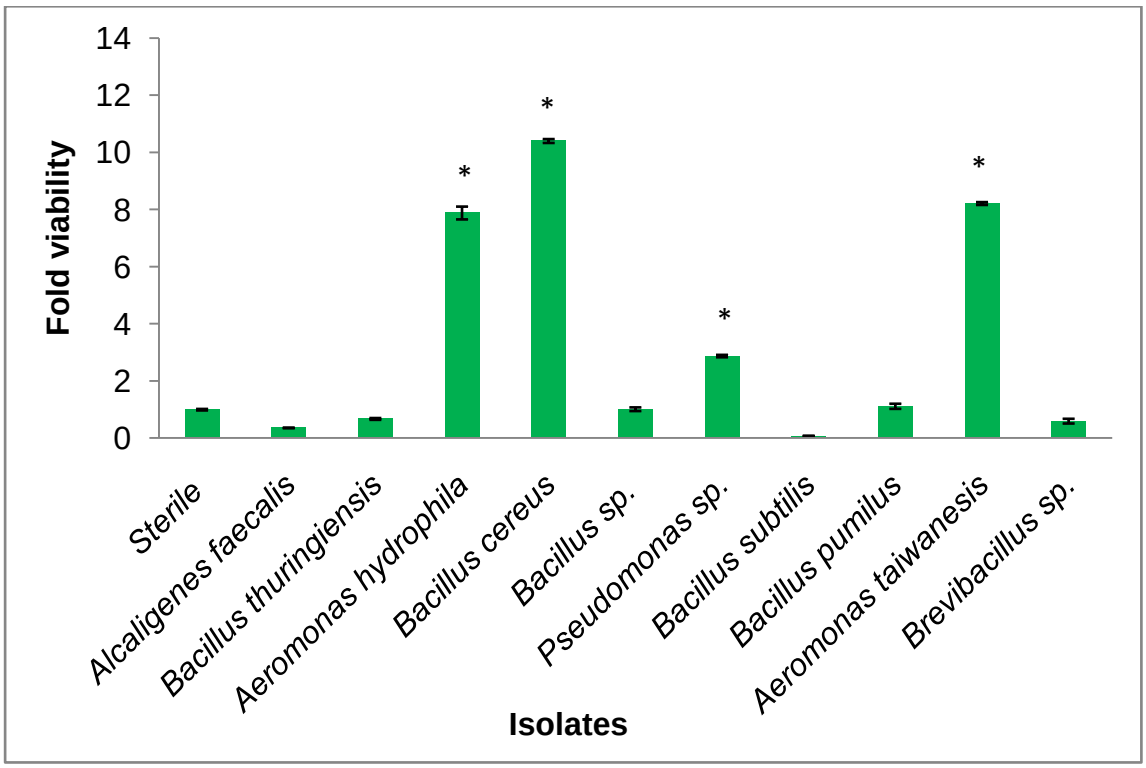

Figure 4.6: Fold viability of isolates after exposure to 70:30 (isolate:SGF)

* indicates when the OD differed statistically from the FV of the control ($p \leq 0.05$)

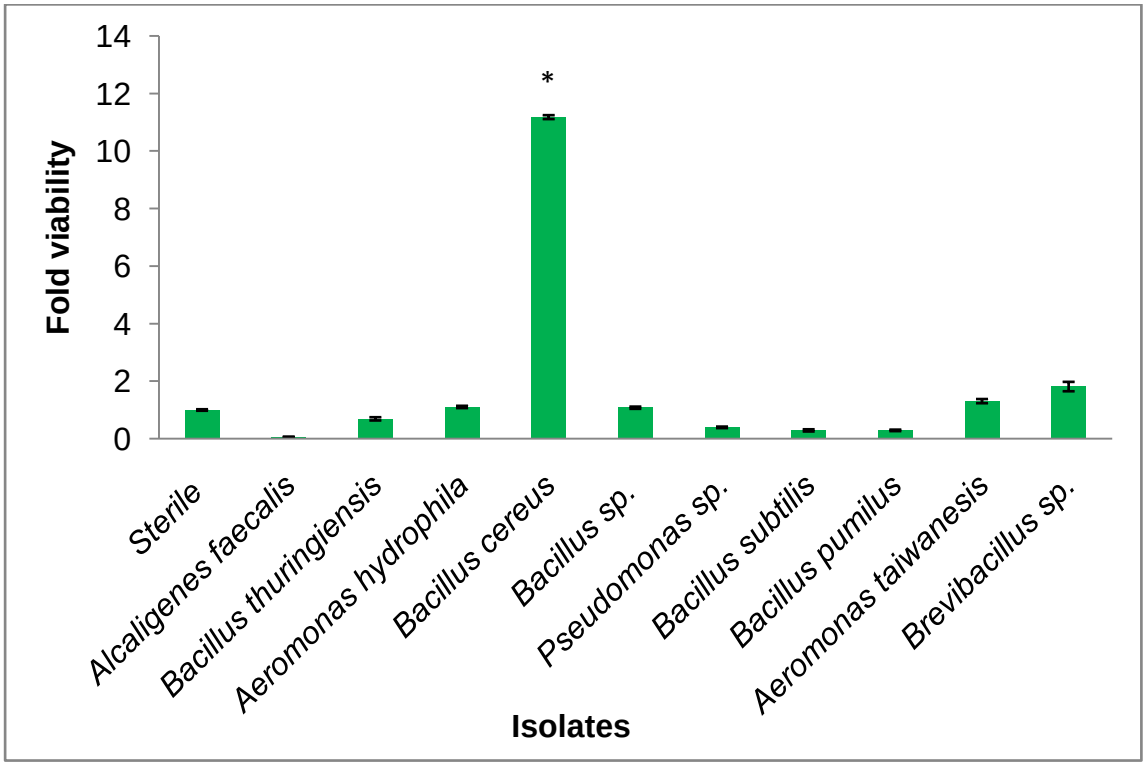

Figure 4.7: Fold viability of isolates after exposure to 50:50 (isolate:SGF)

* indicates when the OD differed statistically from the FV of the control ($p \leq 0.05$)

The HPC bacteria isolated in this study were only representatives of the large variety of the HPC bacteria in the water sampled. In section 4.2 it was shown that these HPC bacteria were present in water at levels up to 293 000 CFU/mℓ. Results obtained for the HPC method only reflect the number of culturable HPC bacteria and it does not take into consideration the amount of bacteria present in water sources that are viable, but not culturable (VBNC). Some of the VBNC bacteria may be able to cause diseases.

After studying the survival rate of the HPC isolates in various concentrations of SGF, it was clear that some of these isolates showed tolerance to acidic conditions similar to what is found in the human gastric environment. Bacteria are known to evolve and adapt readilly to unfavourable conditions and environments (Valentine, 2007; Meyers & Bull, 2002). *Bacillus* sp. for example has the ability to form spores that survive unfavourable conditions. In this study 50% of the HPC bacteria isolated were *Bacillus* sp. Four out of five *Bacillus* spp. isolates tested survived exposure to the lowest concentration SGF and *Bacillus cereus* survived exposure to all three concentrations of SGF tested. A fasting human stomach contains 25 mℓ of gastric fluid (Vertzoni *et al.*, 2005). Every time humans are about to eat or drink the stomach produces 250 mℓ of gastric fluids in response to this stimulus. The amount of gastric fluid produced do not compensate for the amount of water or food to be consumed. It is therefore possible when large amounts of water is consumed, that the gastric fluids are diluted and this may lead to HPC bacteria surviving exposure, as was the case in the 90:10 exposure of this study. If a glass of water is consumed the gastric fluids will be diluted 50:50.

4.9 Antibiotic resistance profile of HPC isolates

The previous section demonstrated that HPC bacteria have the ability to survive exposure to a low pH, which in turn increases their ability to cause infections. In the next section the antibiotic resistant profiles of the HPC isolates are presented. The identified HPC isolates were subjected to the Kirby-Bauer method to determine whether they have antibiotic resistant or susceptible properties (Forster *et al.*, 2013).

The HPC isolates tested in this study were obtained from environmental water samples where they could have been exposed to unknown substances, possibly contributing to their antibiotic profiles. Therefore, a wide range of antibiotics were tested based on their different modes of action. The four major groups of antibiotics tested were the cell wall synthesis inhibitors, 30S and 50S protein synthesis inhibitors and a folic acid inhibitor.

Table 4.5: Percentage resistance, intermediate resistance and susceptibility of the HPC isolates to antibiotics

	Antibiotic	*Alcaligenes faecalis*	*Bacillus thuringiensis*	*Aeromonas hydrophila*	*Bacillus cereus*	*Bacillus* sp.	*Pseudomonas* sp.	*Bacillus subtilis*	*Bacillus pumilus*	*Aeromonas taiwanesis*	*Brevibacillus* sp.
	Gram reaction	-	+	-	+	+	-	+	+	-	+
Cell wall synthesis inhibitors	Ampicillin 10 µg (AP10)	R	R	R	R	S	S	S	S	R	S
	Amoxillin 10 µg (A10)	R	R	R	R	IR	IR	S	R	R	S
	Cephalothin 30 µg (KF30)	IR	R	R	R	S	R	S	S	R	S
	Vancomycin 30 µg (VA30)	R	S	R	S	S	R	S	S	R	S
Protein synthesis inhibitors 30 S ribosomal subunit	Tetracycline 30 µg (T30)	R	R	IR	R	S	S	S	IR	IR	R
	Oxytetracycline 30 µg (OT30)	R	R	IR	R	IR	IR	IR	R	IR	R
	Streptomycin 25 µg (S25)	R	S	S	S	IR	IR	IR	S	S	IR
	Neomycin 30 µg (NE30)	S	S	S	S	S	S	S	S	S	S
Protein synthesis inhibitors 50 s ribosomal unit	Chloramphenicol 30 µg (C30)	R	S	S	S	S	S	S	R	S	S
Folic acid inhibitor	Trimethoprim 2.5 µg (TM2.5)	R	R	IR	R	S	R	S	S	IR	R

R = resistant, IR = Intermediate resistance, S = susceptible

In two instances (cell wall and 30S ribosomal subunit protein synthesis inhibitors) there were 4 types of antibiotics per major group. The antibiotic resistant profiles for each individual HPC isolate are provided in table 4.5. Some bacterial strains might have multiple resistances to two or more types of antibiotics from different antibiotic groups. Gram-negative bacteria have natural resistance to *β*-lactam antibiotics such as vancomycin, therefore the results obtained regarding resistance of Gram-negative bacteria towards vancomycin was not considered as evidence of newly developed resistance in a strain previously susceptible and therefore not considered a virulent characteristic *per se*.

Six out of ten of the isolates were resistant to amoxillin (Table 4.5). Half of the isolates were resistant to trimethoprim, cephalothin, ampicillin and oxytetracycline (Table 4.5). Two and one of the isolates were resistant to chloramphenicol and streptomycin respectively. None of the isolates were resistant to neomycin (Table 4.5) and none of those that could develop resistance against vancomycin (Gram-positive strains) (Table 4.5) showed any resistance.

Therefore, vancomycin and neomycin were the most effective antibiotics tested. All the tested isolates were susceptible to them. Amoxillin was the least successful with 6/10 isolates being resistant, 2/10 being intermediate resistant and 2/10 susceptible. Oxytetracycline was also not that effective, since 50% of the isolates tested were resistant and the other 50% were all intermediate resistant.

Although the various antibiotics belonging to the same group have the same mechanism of action towards an isolate, the isolates responded differently towards them: ampicillin, amoxillin, cephalothin, vancomycin are all cell wall synthesis inhibitors, and *Bacillus thuringiensis* were resistant against ampicillin, amoxillin and cephalothin, but susceptible to vancomycin. It is evident that certain types of antibiotics in the same group have different effects on the isolates, as seen in table 4.5.

Bacillus thuringiensis and *Bacillus cereus* revealed the same antibiotic resistant profile which included resistance against tetracycline, amoxillin, trimethoprim, oxytetracycline, cephalothin and ampicillin. These two *Bacillus* strains have very similar genetic profiles, which can be the reason for the identical antibiotic resistant profile. *Alcaligenes faecalis* were resistant to tetracycline, streptomycin, trimethoprim, oxytetracycline and chloramphenicol. *A. faecalis* was the only organism resistant to streptomycin. Growth of *Brevibacillus* was not inhibited by tetracycline, trimethoprim, oxytetracyclin and *Bacillus pumilus* was resistant to amoxillin, oxytetracycline and chlormaphenicol. *Pseudomonas* sp. was only resistant to one antibiotic namely trimethoprim. *Bacillus* sp. and *Bacillus subtilis* were not resistant to any of the antibiotics

tested. *Aeromonas hydrophila* and *Aeromonas taiwanesis* are Gram-negative bacteria which explains their inherent resistance against vancomycin.

Both Kiyomizu *et al*. (2008) and Savini *et al.* (2009) reported that the *B. cereus* isolated in their respective studies were resistant to tetracycline. In the present study a similar observation was made. Bottone (2010) concluded that *B. cereus* is always resistant to different generations of *β*-lactam antibiotics such as penicillin, cephalosporin, ampicillin and trimethoprim and frequently susceptible to erythromycin, clindamycin, vancomycin, chloramphenicol, the aminoglycosides, and tetracycline. The trend observed by Bottone (2010) was similar to observations in the present study. In contrast to a study conducted by Sasikala and Sundararaj (2012), *Pseudomonas* sp. isolated in the present study was not resistant to multiple antibiotics.

4.10 Pathogen score based on virulence characteristics of each isolate

The HPC bacteria isolated from the untreated water sources in the North West Province had various virulence characteristics that may contribute to their pathogenic potential. This provided the opportunity to combine the outcomes of all the tests performed to determine and indicate pathogenicity and convert this into a pathogen index. This index was used to compare and evaluate the degree to which these organisms have the potential to cause disease in humans. Each isolate was responsible for a unique set of virulent properties (Table 4.6).

Numbers were assigned to the different virulent factors (Table 4.6) depending on the weight of its contribution to a pathogenic profile. The following was proposed: Isolates responsible for *α*- and *β*-haemolysis allocated 0.1 and 0.2 respectively. Beta-haemolyisis cause complete lysis of red blood cells (Pakshir *et al.*, 2013) and are therefore regarded to have more severe effects for the host. Alpha-haemolysis is responsible for incomplete lysis of red blood cells (Miyake *et al.*, 2010). For every enzyme produced 0.1 was added to the pathogen score. Pathogen scores for cytotoxicity ranged from 1.2 (0 h) to 0 (24 h). If cytotoxicity was evident within the first 2 h of exposure an isolate was awarded 1.2. The score decreased with 0.1 increments every 2 h. Evidence of cytotoxicity was deemed valid when it was statistically significant.

Isolates that survived the most concentrated SGF (50:50) received a value of 0.5, the second highest (70:30) a value of 0.3 and survival of the lowest concentration (90:10) a value of 0.1. A value of 0.1 was also added to the score for resistance against all the different types of antibiotics (Table 4.5). If an isolate showed resistance against antibiotics from different groups based on their modes of action, a value of 1, out of possibly 4, was additionally added. The highest pathogen score indicated the most pathogenic HPC bacteria isolated in this study (Figure 4.8).

Table 4.6: Summary of the results obtained showing the virulence factors of HPC isolates

HPC isolate	Haemolysis	Enzyme production						Cytotoxicity (h)	SGF survival			Antibiotic resistance
		P	D	H	Le	Li	C		50:50	70:30	90:10	
*Pathogen weights	*α=0.1, β=0.2*			0.1				0-1.2	0.5	0.3	0.1	1-4
Alcaligenes faecalis	*α*	x	x	x				None			✓	A10,AP10, T30, OT30, C30, S25, C30, TM2.5
Bacillus thuringiensis	*α*		x	x	x			18.75			✓	T30, A10, OT30, KF30, AP10, TM2.5
Aeromonas hydrophila	*β*		x	x			x	14.5		✓	✓	A10, AP10, KF30
Bacillus cereus	*β*		x		x	x		14.5	✓	✓	✓	T30,A10,OT30,KF30,AP10, TM2.5
Bacillus sp.	*β*	x	x			x		32.24				--
Pseudomonas sp.	*β*	x			x			19.5		✓	✓	KF30, TM2.5
Bacillus subtilis	*β*	x	x			x		33.24				--
Bacillus pumilus	*β*		x		x			32.99			✓	A10, OT30, C30
Aeromonas taiwanesis	*α*				x	x		13.75		✓	✓	A10, AP10, KF30
Brevibacillus sp.	*α*	x			x			20.25				T30, OT30, TM2.5

*See text for explanation of weights

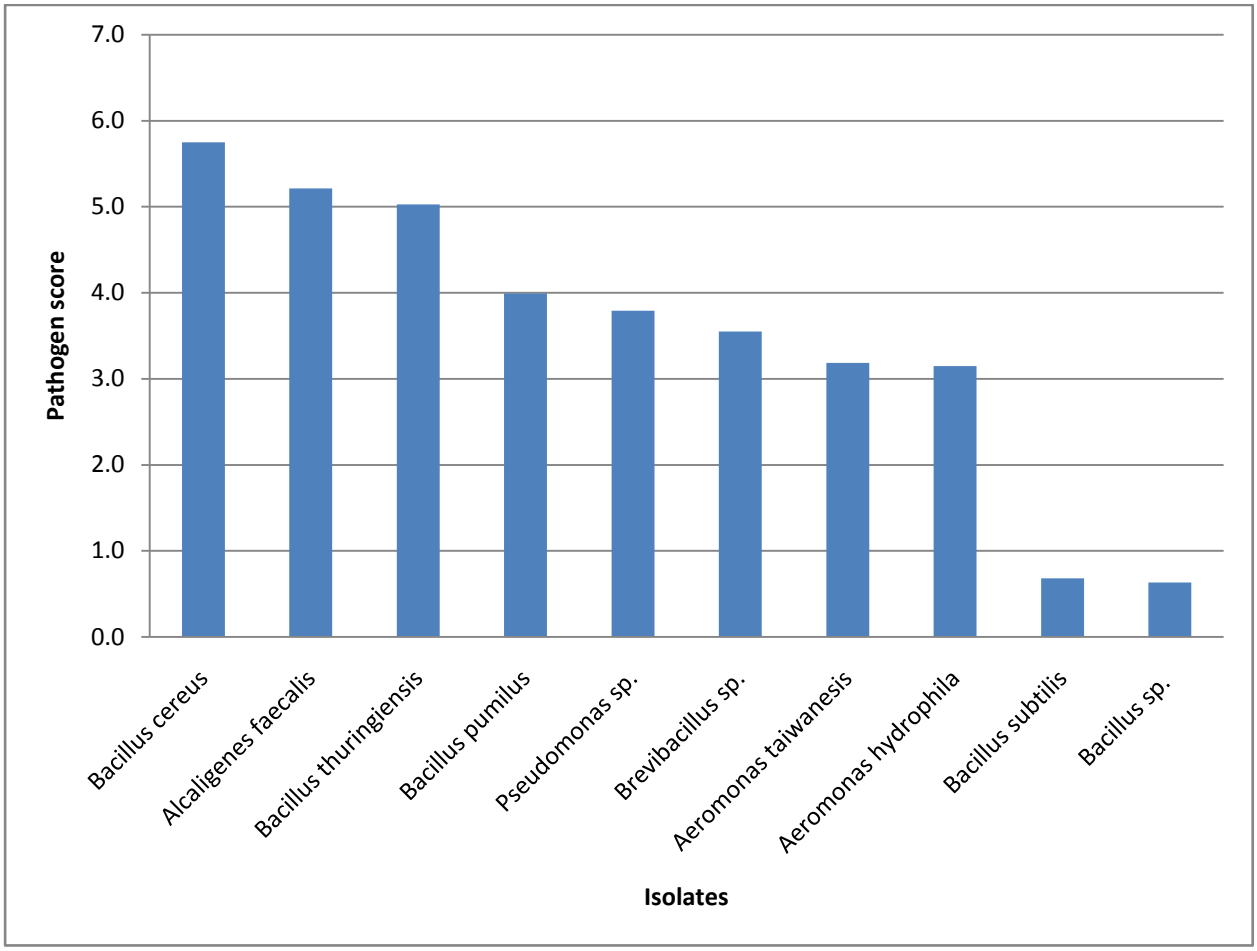

Figure 4.8: Illustration of the pathogen score for each HPC isolate

Bacillus cereus was given the highest pathogenic score of all the bacterial strains isolated in this study. *B. cereus* is a spore-forming organism (Kotiranta *et al.*, 2000) and can therefore survive harsh environments, adapt easily to changing nutritional requirements and grow at a broad range of temperatures (Finlay *et al.*, 2000). *Alcaligenes faecalis* obtained the second highest pathogen score with 5.2, where-after it was *B. thuringiensis* with a score of 5. *Bacillus pumilus* and *Pseudomonas* sp. were assigned scores of 4 and 3.8 respectively. In sixth place was *Brevibacillus* sp. given a score of 3.6. *Aeromonas taiwanesis* and *Aeromonas hydrophila* were scored 3.2 and 3.1 respectively. These two organisms may share some virulence characteristics that are exclusive to the *Aeromonas* spp. The isolates with the lowest scores were regarded as having the least pathogenic potential and were *Bacillus subtilis* and *Bacillus* sp. 0.7 and 0.6. These scores were very low in relation to the other isolates. Although the latter was not considered to have a high pathogenic potential, these organisms still possessed virulence characteristics which indeed can lead to health consequences when individuals are exposed.

5. CONCLUSION

Drinking water guidelines act as a reference to compare water quality with and are aimed at protecting public health. Current measurements to determine water quality include analysis of physico-chemical characteristics and counting indicator bacteria in the HPC method. Guidelines state that good quality drinking water may not contain more than a 1 000 HPC CFU/mℓ. These levels do not necessarily consider the pathogenic potential of the HPC bacteria. Heterotrophic plate count bacteria occur naturally in the aquatic environment and are continuously exposed to a variety of factors such as temperature, chemicals and antimicrobial compounds which might influence them to adapt and change accordingly (Hu *et al.*, 2005).

Water from untreated sources is not subjected to tests to measure the water quality. The quality and risks associated with consuming this water is therefore unknown. A broad range of primary and secondary pathogens are also HPC bacteria. However a definite opinion regarding their pathogenicity is still to be formed and broadly supported by the scientific community (Chowdhury, 2012). A number of studies reported HPC bacteria to have potential virulent characteristics associated with pathogenicity such as secretion of extracellular enzymes (Pavlov *et al.*, 2004), which causes them to be cytotoxic to cells (Lye & Dufour, 1991), to adhere to cells, and to survive passing through the gastric fluids of the stomach (Yuk & Marshall, 2004; Janda & Bottone, 1981).

Currently there is not just one test available to predict the effect of HPC bacteria in untreated drinking water on human health. However, in this study a battery of tests were performed to obtain answers regarding whether these ‘safe’ HPC bacteria used in the determination of water quality, are potentially pathogenic.

The physico-chemical quality of the water samples measured was average. Three of the sixteen boreholes did not comply with SANS:241 (2011) and the TWQR (DWAF, 1996b) for EC, TDS and salinity. HPC bacteria were isolated with the appropriate method. Half of the boreholes did not comply with the two guideline levels and had more than a 1 000 HPC CFU/mℓ.

27% of the HPC bacteria isolated were haemolytic and 10 different HPC isolates produced 2 or more extracelullar enzymes. These isolates were considered to be potentially pathogenic at this stage.

The cytotoxic effects of these isolates on human intestinal cells were measured with the real time cell analyser (xCELLigence-instrument). This system has the advantage of determining the amount of time it takes the HPC isolates to completely kill the intestinal cells. Pavlov *et al.* (2004) concluded that HPC isolates that can invade cell lines were potentially pathogenic especially to immuno-compromised individuals. The results of the current study moved beyond showing invasiveness and have shown clear evidence of the cytotoxicity of HPCs, whereas evidence of invasiveness could also include bacteria living in symbiosis with the mammalian cells.

According to Salyers *et al.* (2004) human intestinal bacteria have the ability to acquire or donate antibiotic resistant genes to, bacteria passing through the human intestine. It would therefore be possible for antibiotic susceptible bacteria to become resistant on their way from the mouth to the intestine which in the case of already pathogenic bacteria would be even more dangerous to the health of immuno-compromised individuals.

Ten representatives of HPC bacteria were identified as: *Aeromonas hydrophila and taiwanesis, Bacillus* sp., *B. thuringiensis, B. cereus, B. subtilis, B. pumilus, Pseudomonas* sp., *Alcaligenes faecalis* and *Brevibacillus* sp. Of these, *Aeromonas, Bacillus, Pseudomonas* and *Alcaligenes* have previously been found in untreated water sources (Pavlov *et al.*, 2004). Pathogenic features of *Aeromonas, Bacillus* and *Pseudomonas* have also been established with existing tests (bloodagar, enzyme tests, cell adherence and cell invasiveness (Pavlov *et al.*, 2004; Payment *et al.*, 1994; Lye and Dufour, 1991).

Another component of this study is the exposure of bacterial isolates to SGF. These conditions mimic human gastric fluids which act as a natural defence mechanism. Some HPC isolates were able to survive exposure to gastric fluids. These results predict the possible increase of waterborne diseases in immuno-compromised individuals that rely on gastric fluids for protection against bacterial pathogens.

The cytotoxic effects of these isolates were investigated on human intestinal cells. Nine out of the ten isolates proved to have statistically significant cytotoxic effects. Some isolates survived exposure to SGF at different dilutions. The MTT-assay was proven successful in determining the survival of bacteria after exposure to SGF. Different antibiotic resistant profiles were determined for every isolate.

In conclusion, the HPC bacteria found in this study were potentially pathogenic. It was a novel approach to summarise the virulence degree of each isolate according to its virulent characteristics by assigning a score to each HPC isolate i.e. the pathogen score. The pathogen

score to which they exert virulent characteristics differed between species. These tests made use of new techniques to obtain answers regarding the pathogenicity of HPC bacteria.

Recommendations:

1. To further validate the results of and methods used in this study, future studies should include a bacterial strain with well-known and characterised virulent characteristics. This can act as a positive control for the methods used to test for the various virulence characteristics, but can also be used as a strain with which the other strains are compared with to help gauge their virulence.
2. The HPC method has the ability to only assess the number of culturable HPC bacteria that form visible colonies on a solid medium, under specific predetermined conditions (R2A agar for 48 h at 37°C). Therefore, the effect of unculturable bacteria was not determined with the techniques used in this study. In the event of consumption of untreated water sources, it may be beneficial to test the effects of SGF on the survival of the unculturable bacteria.
3. In light of the survival of bacteria after exposure to SGF, it would be worthwhile to investigate whether the bacteria were in a survival mode, not metabolising, and able to revive when exposed to favourable conditions again. To test for this it is proposed that the researcher grows the isolates on fresh agar plates after exposure to SGF. The bacteria can also be exposed to a wider range of SGF dilutions, and then enumerated on agar plates to compare survival rates between various isolates.
4. Another recommendation would be to distinguish between bacteriostatic (kill the bacterial cells) and bacteriocidal (temporarily inhibit growth of bacteria) characteristics of the antibiotics used to determine resistance in bacteria.
5. To learn the effect of temperature on bacterial populations in sampling sites, the same sites should be sampled in consecutive seasons.
6. Since human settlements might contribute to bacterial load in underground water, is would be wise to determine whether distance between a human settlement and a sampled well has an effect on the bacterial numbers, Both suggestions at number 5 and 6 should be done in conjunction with geological studies of the area and groundwater behaviour.

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