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Microbiological Evaluation of the Mhlathuze River in KwaZulu-Natal

by

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DECLARATION

The research herein was undertaken in the Department of Biochemistry and Microbiology, University of Zululand under the supervision of Dr Johnson Lin.

The studies represent an original undertaking by the author. Where use has been made of the work of others, it has been duly acknowledged in the text. This dissertation has not been submitted in any form for a degree to any other university.

Nompumelelo Mthembu
University of Zululand
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ABSTRACT

High levels of faecal coliforms pose a treat to the health of the rural community that uses the river water directly for domestic use without treatment. The microbiological, physical and chemical analysis of the Mhlathuze River was investigated over a twenty-one months period. Five water samples were collected along the Mhlathuze River and analysed to monitor the indicator bacteria pollution with changing seasonal patterns.

Surface water temperature and rainfall during the period of study appeared to be some of the factors affecting the increased bacterial counts. Elevated levels of indicator microorganisms (both faecal and total coliforms) and heterotrophic plate count bacteria were observed from March 1998 to November 1999. Bacteria isolated from the river included *Escherichia coli, Pseudomonas spp., Enterobacter spp., Serratia spp., Klebsiella spp.* and *Aeromonas hydrophila*. The average monthly pH values ranged between 6.5 and 8.5. The turbidity, dissolved oxygen, hardness, ortho- and total phosphates values obtained did not show any major changes that would call for caution.

A polymerase chain reaction (PCR) method was used to amplify 16S rRNA and phoP gene fragments from the isolated bacteria and directly from the water resource. Annealing temperature was adjusted to set up the optimum conditions of the PCR mixture. Performing serial dilutions of DNA carried out the sensitivity of detection for PCR products. It was deduced that amplifications with phoP and 16S rRNA primers were visualised up to 10^{-7} and 10^{-6} µg of DNA, respectively.

Multiplex PCR with the two primers generated an amplification product of approximately 755 bp for all environmental isolated used and an additional 299 bp product for *E. coli, C. freundii* and *C. diversus*. Rsa I and Hinf I restriction enzymes were also used for double digestion of *E.coli* and *P. vulgaris*.

ACROYNMS AND ABBREVATIONS

bp	base pair
CF	coliform
dNTP	Deoxynucleotide Triphosphate
DNA	Deoxyribose Neucleic Acid
GB	general bacteria
PCR	Polymerase Chain Reaction
UV	Ultra Violet
DWAF	Department of Water Affairs and Forestry
SABS	South African Bureau of Standards
WHO	World Health Organisation
WRC	Water Research Commission

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CHAPTER ONE

BACKGROUND INFORMATION

1.1. GENERAL INTRODUCTION

South Africa is one of the countries facing water scarcity and proper sanitation. Reports on microbial water quality of rives in South Africa shows that rivers have been facing increasing threat of pollution, resulting from rapid and extensive demographic changes in South Africa in recent years (DWAF, 2002 and Venter *et al.*, 1996). Many areas have improper sanitation facilities leading to high levels of microbial water pollution. This situation poses health risk to the communities that depend on such water resources for their domestic purposes. Yet the legislation, National Water Act, No. 36 of 1998 (DWAF, 1998) in place pointing out that access to sanitation facilities is a basic human right that safeguards health and human dignity.

This study was based on the water quality assessment of the Mhlathuze River. Mhlathuze River is supplying water to both rural and urban areas. Several rural communities in the Mhlathuze River catchment area are directly dependent on this river for all their basic domestic water needs. Treated drinking water is, in many cases, unavailable to these communities and their dependency on the Mhlathuze River thus includes drinking water, washing, recreation and agriculture. The communities are experiencing increasing population growth yet poor socio-economic conditions. By depending from this available and often contaminated river without any treatment, the communities are exposed to water-related and water-borne diseases such as gastroenteritis, salmonellosis, dysentery, cholera, typhoid fever and hepatitis (Pegram *et al.*, 1998).

Water-borne diseases are a serious threat to human life because they may be a carrier of death. They are mainly caused by the contamination of water by microorganisms, especially pathogens, which are associated with faecal contamination of water (Muyima and Ngcakani, 1998). There are traditional methods used for the enumeration of these faecal and pathogenic microorganisms. However, the sensitivity of these methods has been doubted due to the fact that the survival of a microbe in an environment, in which it is not indigenous, depends on its ability to tolerate a

different set of biological, physical and chemical conditions (Flint, 1987). Studies have shown that many of these microbes enter an altered physiological state called viable but non culturable (VBNC).

These VBNC bacteria are those organisms that undergo a drastic decrease in plate count but remain viable when analysed by the direct viable count (DVC) (Oliver *et al.*, 1995). Due to this state, it is clear that using routine bacteriological methods makes it very difficult to detect VBNC in the natural environment. This study adopted the molecular technique, polymerase chain reaction (PCR) to detect microorganisms that culture methods do not. It is a highly sensitive, reliable, specific and rapid method for detecting bacteria in cultures and natural waters (Bej *et al.*, 1990).

With such problems facing South Africa, there is, desperately, a need to monitor and assess level of microbial pollution in the rivers. This will help identifying areas that are at risk of contracting water-borne diseases.

1.2. THE STUDY AREA

The Mhlathuze River is a medium sized river that rises about 1280 mean sea level (msl) in the mountainous terrain around Babanango, in KwaZulu-Natal and discharges next to the Richards Bay harbour into the Mhlathuze Estuary. The river drains an area of approximately 4209km^2 (Zululand Observer, 1998).

The Mhlathuze River is the primary water resource for the whole area (Empangeni-Richards Bay). The Mhlathuze catchment and its surrounding area are experiencing a rapid industrial, agricultural and domestic development. There is not enough water available to support substantial economic growth in the region (Hughes and Smakhtin, 1999). With increasing consumption on water resources, the potential contamination of water by enteric microorganisms could be expected to increase. The persistence of human and animal pathogens and indicator organisms have increasing environmental and public health significance as water-borne outbreaks of diseases continue to occur causing a great loss in industry and in agriculture

all over the world. There is little data concerning the microbial safety of water supplies in the Mhlathuze catchment and its surrounding area.

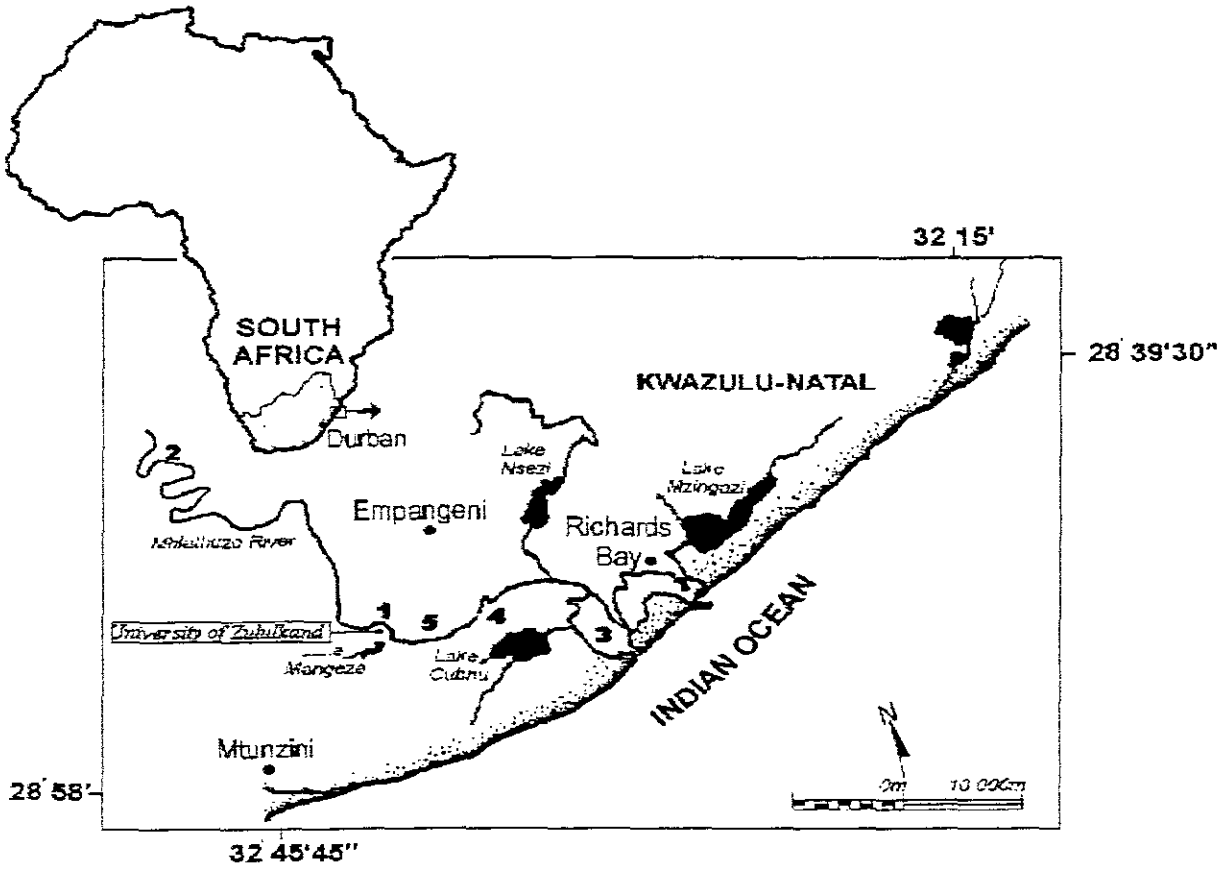


Figure 1.1: Maps of the eastern region of the Mhlathuze Catchment Area indicating sites (site 1-Kwa-Dlangezwa, site 2-Dlangubo, site 3-Mhlathuze estuary, site 4-Mhlathuze pump station and site 5-Felixton bridge)

1.3. LITERATURE REVIEW

1.3.1 Water pollution

Water pollution defines contamination of water by foreign matters such as microorganisms, chemicals, industrial or other wastes, or sewage. Pollution of water resources is on the increase in many places and such matter deteriorates the quality of the water and renders it unfit for its intended uses (Haslam, 1990). Rivers are the main sources of water in South Africa, and are also the systems most under threat form abstraction, regulation, and pollution (Dallas *et al.*, 1994.

Municipal water pollution consists of wastewater from homes and commercial establishments. For many years, the main goal of treating municipal wastewater was simply to reduce its content of suspended solids, oxygen-demanding materials, dissolved inorganic compounds (particularly compounds of phosphorus and nitrogen), and harmful bacteria (Mason, 1991). In nowadays, more effort has been based on improving the means of disposal of the solid residues from municipal treatment processes.

The pollution of rivers and streams with industrial contaminants has become one of the most critical environmental problems of the century (Haslam, 1990). The major pollutants includes:

- Exotic organic chemicals, including pesticides, various industrial products, surface-active substances in detergents, and the decomposition products of other organic compounds.
- Petroleum, especially from oil spills.
- Inorganic minerals and chemical compounds (Pelczar *et al.*, 1993 and Haslam, 1990).
- Microorganisms especially pathogenic.

Chemical pollution entering rivers and streams can be classified according to the nature of its sources: point pollution and nonpoint pollution. Point pollution involves those pollution sources from which distinct chemicals can be identified, i.e. discharge pollutants at specific

locations, for example, factories, sewage treatment plants, or oil tankers. The technology exists for point sources of pollution to be monitored and regulated, although political factors may complicate matters. Nonpoint pollution involves pollution from sources that cannot be precisely identified; such as runoff from agricultural or mining operations or seepage from septic tanks or sewage drain fields.

Agriculture, including commercial livestock and poultry farming, is the source of many organic and inorganic pollutants in surface waters and groundwater. These contaminants include both sediment from the erosion of cropland and compounds of phosphorus and nitrogen that partly originate in animal wastes and commercial fertilizers. Animal wastes are high in oxygen-demanding material, nitrogen, and phosphorus, and they often harbour pathogenic organisms. Wastes from commercial feeders are contained and disposed of on land; their main threat to natural waters, therefore, is via runoff and leaching.

Fertilizers and other nutrients used to promote plant growth on farms and in gardens may find their way into water. At first, these nutrients encourage the growth of plants and algae in water. When the plant matter and algae die and settle underwater, microorganisms decompose them. In the process of decomposition, these microorganisms consume oxygen that is dissolved in the water. Oxygen levels in the water may drop to such dangerously low levels that oxygen-dependent animals in the water, such as fish, die. This process of depleting oxygen to deadly levels is called eutrophication.

Lakes are especially vulnerable to pollution. One problem, eutrophication, occurs when lake water becomes artificially enriched with nutrients, causing abnormal plant growth. Runoff of chemical fertilizer from cultivated fields may trigger this (Mason, 1991). The process of eutrophication can produce aesthetic problems such as bad tastes and odors and unsightly green scums of algae, as well as dense growth of rooted plants, oxygen depletion in the deeper waters and bottom sediments of lakes, and other chemical changes such as precipitation of calcium carbonate in hard waters.

With almost 80 percent of the planet covered by oceans, people have long acted as if those bodies of water could serve as a limitless dumping ground for wastes. Raw sewage, garbage, and oil spills have begun to overwhelm the diluting capabilities of the oceans, and most coastal waters are now polluted. Beaches around the world are closed regularly, often because of high amounts of bacteria from sewage disposal, and marine wildlife is beginning to suffer.

Notable effects of water pollution include those involved in human health. Nitrates in drinking water can cause a disease in infants that sometimes results in death. Crops can absorb cadmium in sludge-derived fertiliser; if ingested in sufficient amounts, the metal can cause an acute diarrhoeal disorder and liver and kidney damage. The hazardous nature of inorganic substances such as mercury, arsenic, and lead has long been known or strongly suspected (Mason, 1991).

A number of chemical and physical factors common to drinking water systems are known to cause a form of sublethal and reversible injury. This injury is responsible for the failure of waterborne coliforms to grow on accepted media used in the analysis of drinking water, such as m-Endo media (Gordon, 1986). Table 1.1 shows the factors that could cause the nonculturability of microorganisms.

Table 1.1: Effects of some important natural variables on aquatic organisms

Water quality variables	Major effects
PHYSICAL FACTORS	
Temperature	• Determines metabolic rate • Determines availability of nutrients and toxins • Determines oxygen saturation level • Changes provide cues for breeding, migration, etc
Turbidity and suspended solids	• Turbidity determines degree of penetration of light, hence vision, photosynthesis. • Suspended solids reduce penetration of lights, smother and clog surfaces (e.g. gills) and adsorb nutrients, toxins, etc.
CHEMICAL FACTORS	
pH	• Ionic balance
Conductivity, salinity, individual ions	• Osmotic balance • Ionic balance • Water balance
Dissolved oxygen	• Respiration
Trace metals	• Many essential at low concentrations • Some mutagenic, teratogenic, carcinogenic

(Dallas *et al.*, 1994)

Physical and chemical components contribute to the water quality of the sample. For any sample of water, tens or hundreds of physical attributes can be measured and any number of the tens of thousands of known chemicals may conceivably be present.

All microorganisms grow well at their optimal temperature. Water with high temperature increases metabolic rate, including respiration and thus the amount of oxygen required by aquatic organisms. Turbidity in rivers often changes seasonally, the extent of the change being governed by the hydrology and geomorphology of a region (Dallas *et al.*, 1994). Turbidity is an optical property of water that causes light to be scattered and absorbed rather that transmitted in straight lines through a sample. Suspended solids such as silt, clay, algae, organic and inorganic matter, and other microorganisms cause turbidity in water.

The pH of natural waters is determined by geological and atmospheric influences. Most fresh waters are relatively well buffered and more or less neutral, with pH ranging between about 6 and 8. The concentration of dissolved oxygen is probably one of the most important abiotic determinants of the survival of most aquatic organisms. Under natural conditions the concentration of dissolved oxygen fluctuates diurnally, depending on the relative rates of photosynthesis and respiration. It is usually lowest near dawn, increasing during the day, peaking in the afternoon, and decreasing at night (Dallas *et al.*, 1994).

1.3.2 Water-borne diseases

The greatest threat to health from drinking water arises from bacterial contamination. Water that is polluted is an important vehicle for the spread of disease. It has been estimated that a total of 2,213,000 deaths in 1999 were due to other diseases related to water, sanitation and hygiene and this amounts to 4% of all deaths worldwide (Prüss, *et al.*, 2002). People that are likely to be affected by water-borne diseases are those from rural areas. Most people in these areas use sources without any treatment. It is then very important for these water sources to be examined for indicators of pollution and when results show contamination, action should be taken. This action will result in the decrease of infection diseases, and an improvement of health standards of rural communities (Nevondo & Cloete, 1999).

Water that is safe to drink is free of disease producing microorganisms and chemical substances harmful to health. In order to prevent transmission of pathogens, there must be (1) water purification methods that provide safe drinking water, (2) treatment facilities for wastewater prior to its disposal or reuse and (3) procedures whereby water can be examined to determine its microbiological quality (Pelczar *et al.*, 1993). Table 1 shows a variety of pathogens that can be introduced into waterways.

Table 1.2: Some water-related diseases and their causative bacteria (Mason, 1991)

Causative bacteria	Disease or symptoms
Salmonella typhi	Typhoid fever
S. paratyphi	Paratyphoid fever
Salmonella spp.	Gastroenteritis
Shigella spp.	Bacterial dysentery
Vibrio cholerae	Cholera
Escherichia coli	Gastroenteritis
Campylobacter spp.	Intestinal infections
Mycobacterium tuberculosis	Tuberculose

To control such diseases it is necessary to provide people with sufficient water of reasonable quality.

1.3.3 Drinking water quality

Water quality refers to those physical and chemical attributes of a sample of water that determine its value for a specific purpose. Water to be used for domestic, agricultural, industrial and recreational purposes; it has to meet a certain minimum water quality standards (Grabow, 1996). The guidelines and standards for water differ in technical details but the basic requirement specifies that drinking water should rarely if ever contain total coliforms, and never faecal coliforms or *E.coli* and should be free of pathogenic microorganisms.

Water can endanger health and life if it contains pathogenic microorganisms. The total and faecal coliform count is one of the most commonly used indicators of water pollution (Bej *et al.*, 1990 ; DWAF., 1998). Coliform bacteria have been the bacteriological tool to measure the occurrence and intensity of faecal contamination in drinking water. The presence of coliforms indicates the potential human faecal contamination of water and therefore the presence of enteric pathogens (Bej *et al.*, 1990). Coliforms are detected traditionally by culturing them on a selective medium that allows the growth of gram-negative bacteria and differentially detects lactose utilizing

bacteria, e.g. using MacConkey, m-Endo, eosin methylene blue, or brilliant-green-lactose-bile media (Pelczar *et al.*, 1993; DWAF., 1998). Coliforms that are detected include members of the genera *Escherichia, Enterobacter, Citrobacter* and *Klebsiella.*

Methods for the enumeration of faecal indicator organisms and specific pathogens from aquatic environments by traditional culture methods have been widely accepted (American Public Health Association, 1992). However, the survival of a microorganism in an environment in which it is not indigenous depends on its ability to tolerate a different set of biological, physical and chemical conditions (Flint, 1987). Studies on persistence of human enteric bacteria in aquatic environments have demonstrated that many of these organisms enter an altered physiological state called viable but non-culturable (VBNC) (Kaprelyants *et al.*, 1993; Oliver *et al.*, 1995).

Viable but non-culturable bacteria are those organisms that undergo a dramatic decrease in plate count but remain viable when analyzed by the direct viable count (DVC). These bacteria include those in genera of *Escherichia, Salmonella, Shigella, Aeromonas, Campylobacter* and *Legionella* (Byrd *et al.*, 1991). The distinction between viability and culturability may not guarantee loss of pathogenicity. Colwell *et al.*, (1985) demonstrated that VBNC *Vibrio cholerae* cells were able to induce diarrhoea in human volunteers. In a study by Singh *et al.* (1986), chlorine stressed cells of *Yersinia enterocolitica* had virulence for mice. These results cause a great concern of using viable culture methods for monitoring of the microbial safety of water supplies, including maintaining the viability of microorganisms between the time of collection and enumeration, lack of growth of VBNC organisms and time required for detection.

Accurate enumeration has been found to be a major problem in studying indicator and pathogenic bacteria within aquatic environment. Enumeration is routinely done to assess the microbiological quality of water and the presence of indicator bacteria in excessive numbers signals the possible existence of pathogens. Quantitative recovery of pathogens from source water may be important in understanding the nature of water-borne disease outbreak (Singh *et al.*, 1986).

When evaluating the problem of detecting particular microorganisms from the specific sources, proper consideration must be given to the influence of environmental factors upon detection methods. There are factors that inhibit the detection of coliform bacteria in water. These factors

include excessive numbers of heterotrophic bacteria, turbidity, and a process known as sublethal injury. These factors may lead to the underestimation of the actual number of indicator organisms (McFeters *et al.*, 1982). The concept of sublethal injury arises when it was discovered that water-borne bacteria were suppressed by membrane filtration compared to the use of multiple tube fermentation-MPN method. There are also a number of factors that lead to the injury of bacteria in the aquatic environment, e.g. time and temperature of exposure, disinfection of levels, strain of organism, concentration of nutrients, presence of heavy metal ions, and other undefined chemical and physical parameters (McFeters *et al.*, 1982).

The routine microbiological methods for the detection of indicator microorganisms should not be based on the isolation and identification of pathogenic microorganisms, for the following reasons:

1. Pathogens are likely to enter a water supply sporadically, and since they may not survive for long periods of time, they could be missed in a sample submitted in the laboratory.
2. If they are present in very small numbers, pathogens are likely to escape detection by laboratory procedures.
3. It takes 24 hours or longer to obtain results from a routine laboratory examination for pathogens. Within this time, lots of people would have been exposed to these pathogens (Pelczar *et al.*, 1993).

The above points state clearly that microbiologists do not rely on the identification of pathogenic microorganisms in their monitoring procedures, but their procedures are based on finding a bacterium whose presence indicates the possibility of the presence of pathogenic microorganisms. These microorganisms are called indicator microorganisms. Their presence in water is associated with faecal pollution excreted from humans or other warm-blooded animals. The characteristics of an indicator microorganism are:

- Present whenever pathogens are present,
- Present in the same or higher numbers than pathogens,
- Specific for faecal or sewage pollution,
- At least as resistant as pathogens to conditions in natural water environments, and water purification and disinfection processes,
- Harmless to human and other animals.

- Detectable by simple, rapid and inexpensive methods (Pelczar *et al.*, 1993; Grabow, 1996).

1.3.4 Polymerase Chain Reaction

It is very difficult to detect viable but non-culturable microorganisms in the natural environment when employing routine bacteriological methods. Any detection method that is used must be capable of detecting the low numbers of the organisms (Brauns *et al.*, 1991). Tracking such microorganisms, when and if they are released into the environment, will require methods, which are able to detect microorganisms that culture methods, do not, dead or alive (Somerville *et al.*, 1989). The development of new technology for the detection of microorganisms in the natural environment has led to a new avenue for microbiologists. One method that is usually used for detecting non-culturable cells is the Polymerase Chain Reaction (PCR) method. Application of this molecular technique offers new and potentially very powerful ways to study organisms in their natural environment (Fuhrman *et al.*, 1988).

Polymerase Chain Reaction (PCR), technique in molecular biology by which a small fragment of deoxyribonucleic acid (DNA) can be rapidly cloned, or duplicated, to produce multiple DNA copies (Somerville *et al.*, 1989). PCR can be used to identify individuals from minute amounts of tissue or blood, to diagnose genetic diseases, and to research evolution. PCR proceeds in a series of cycles, or rounds. Each successive round doubles the amount of DNA and thus more than one billion copies of a single DNA fragment can be made in just a few hours. The technique of PCR is simple enough to be used by scientists with little training in molecular biology.

Polymerase requires two additional ingredients to copy DNA. The first is a supply of the four basic building blocks of DNA, called nucleotide bases. The second is a short stretch of copied DNA, called an oligonucleotide primer, consisting of several nucleotides that initiate replication. PCR uses these same ingredients to copy DNA in a vial (Islam *et al.*, 1990). There are three phases in a polymerase chain reaction. In the first phase, called denaturation, the template, or piece of original DNA, is heated to a temperature of from 90° to 95° C for 30 seconds, which causes the individual strands to separate. In the second phase, called annealing, the temperature of the mixture is lowered to 55° C over a 20-second period, allowing the

oligonucleotide primers to bind to the separated DNA. In the third phase, called polymerization or extension, the temperature of the mixture is raised to 75° C, a temperature at which the polymerase can copy the DNA molecule rapidly.

The resulting amplified DNA is further analysed by the gel electrophoresis. The application of gel electrophoresis for the analysis of DNA range from determining base sequence to separation the DNA fragments, where the DNA is negatively charged and the fragments loaded into a well at the cathode (-) end of a gel migrate through the gel towards the anode (+). The fragment sizes are estimated from their mobilities relative to those of fragments of known sizes (Sealey and Southern, 1990).

It is hard to exaggerate the impact of the polymerase chain reaction. It is the quick, easy method for generating unlimited copies of any fragment of DNA. The relevance of PCR method in this study is based on the identification of pathogenic microorganisms from the study area as conventional methods, using growth media, may not accommodate the growth of all pathogens of interest. The success of this project will contribute to the fact that PCR is also an alternative, accurate, reliable better method for water analysis.

1.4 AIMS

- To monitor constantly the microbial safety of water supplies.
- To accumulate data on the survival and transmission of pathogens in the Mhlathuze river.

1.5 OBJECTIVES

- To evaluate the accuracy of enumeration and to detect microbial pathogens in water supplies of the Mhlathuze catchment and its surrounding area.
- To estimate the occurrence of any water-borne disease outbreaks in this area.
- To determine seasonal patterns of human pathogens and indicator microorganisms and the role of these microorganisms in this area.

- To determine if the expanding population and industries situated on the riverbanks may have an effect on the microbial population changes.
- To provide useful baseline data for further research.

1.6 APPROACH AND PURPOSE OF STUDY

1. Total and faecal coliforms are the recognized indicators throughout the world for assessing the bacteriological quality of waters. These indicators have been proven very useful for many decades, and tests for their quantification are highly sensitive and reliable.

2. Information on bacteriological water quality is lacking worldwide because of the high cost and technical sophistication required to perform the present standard tests for total and faecal coliforms on a routine basis. This situation is unlikely to change in the future unless more appropriate tests are employed.

3. Developing countries must build data records on the microbiological quality of their drinking waters. This basic information is required for the correct management of water programs (allocation of funds, determination of maintenance and upgrading schedules, evaluation of water projects, identification of problems within water treatment and distribution, and planning of corrective actions). Also, without data, the concerned authorities (from central down to municipal governments) cannot be convinced to take any type of remedial or corrective action.

CHAPTER 2

PHYSICAL, CHEMICAL AND MICROBIOLOGICAL ANALYSIS OF WATER FROM MHLATHUZE RIVER (1998 – 1999)

2.1 INTRODUCTION

Our waters are one of our most valuable resources, which we cannot survive without them. They support human, plant and animal life and the natural environment. The demand of water is increasing with increasing volumes of domestic, industrial and agricultural wastes together with ecological disturbances (DWAF, 1995). The pollution of water resulting from these activities has a high impact to poorer communities since they are utilising water directly from the running Mhlathuze River. The river might be contaminated with various types of microorganisms, namely bacteria, protozoa, fungi.

Bacteria are the most abundant of all living organisms. They are found in air, soil, water and almost all possible habitats of this planet (Pelczar *et al.*, 1993). Some bacteria have evolved to inhabit and thrive under extreme conditions such as very high and low pH, temperature, ionic conditions and pressures. These microorganisms can evolve very quickly to adapt to move to extreme conditions.

Factors like weather and usage can cause natural water systems to become turbid and contaminated by chemicals and microorganisms (Mason, 1991). Such water may become unattractive and unfit for human consumption. A large proportion of people form rural areas in third world countries depend on natural untreated waters for their daily activities. These people are exposed to polluted water and thus are vulnerable to water-borne diseases.

Human illness can result from swimming, eating seafood and drinking water contaminated by chemicals and microorganisms. One important health concern related to human use arises from the potential presence of human pathogenic organisms in water systems. Elevated levels of

bacteria such as coliform bacteria, may be an indication that other pollutants, such as bacterial or viral pathogens are be present (Farnleitner *et al.*, 2000).

Point sources of pollution, effective methods for assessing water quality and methods of disinfection need to be addressed for acceptable standards of drinking water suitable for human usage. Various methods can be used for assessing water quality, by determining the levels of pollutants. These methods all have their intrinsic advantages and disadvantages.

To protect public health, it is at times necessary to restrict use of water directly from the river while long-term solutions to these problems are being developed.

2.1.1 Sources of surface water pollutants

There are three main sources of pollution of water resources, i.e. nutrients, microorganisms and toxic substances. Nutrients are not pollutants. They are vital to plants and whole cycle of life. But when present in large quantities, they cause a negative impact on the quality of water, eutrophication (Koche *et al.*, 1990). Nutrients that are of concern are nitrogen and phosphorus. Their elevated concentrations give rise to the accelerated growth of algae and the occurrence of algal blooms. Algal blooms may cause problems associated with malodours and tastes in water and the possible occurrence of toxicity (Mason, 1991). These nutrients come from effluent pollution, released by major mines and factories, agricultural areas and sewage treatment works.

Industrial and agricultural wastes are the greatest sources of toxic pollution but in urbanized, non-industrial areas, household chemicals and pesticides can be significance source of pollution. Toxic pollutants include metals such as lead, nickel, cadmium, zinc, copper and mercury arising from many industrial processes and some agricultural uses, organic compounds, phenols, gases, anions, acids and alkalines (Mason, 1991).

Despite all the beneficial processes that microorganisms are involved, they can also be major pollutants of water. Sewage systems, human and animal wastes and soil surrounding the water system are sources of pollutants that can carry large numbers of microorganisms. The microorganisms that are likely to be present in these sources are bacteria, viruses, fungi, and protozoa. Pathogenic bacteria, fungi and protozoa can cause diseases such as intestinal infections,

dysentery, typhoid fever, cholera, hepatitis, minor respiratory and skin diseases (Mason, 1991). Viruses, excreted in human feces and urine, can cause illness at low concentrations. These diseases include paralysis, meningitis, respiratory disease, epidemic vomiting and diarrhoea, myocarditis, congenital heart anomalies, infectious hepatitis and eye infections (Pina *et al.*, 1998 and Grabow, 1996).

It is also important to determine the point sources of water pollution. Knowledge of precise pollution sources help in restoring water quality and reducing the danger of infectious disease resulting from exposure to contaminated waters (Hagedorn *et al.*, 1999).

2.1.2 Indicator organisms and pathogens

The recognized bacterial indicators for assessing water quality are bacteria of the *Enterobacteriaceae* family defined as the total coliform bacteria and the faecal coliform bacteria (American Public Health Association, 1992; Genthe and du Preeze, 1995). Both groups are identified by their ability to ferment lactose, and selective media are used to inhibit the growth of gram-positive organisms. The total coliform group includes many bacteria also of non-faecal origin. The faecal coliform group is differentiated from the total coliform bacteria by its ability to grow at elevated temperature and selective media such as m-Fc agar. The best indicator of faecal coliform activity from humans and warm-blooded animal is *Escherichia coli* (Csuros, 1994; Farnleitner *et al.*, 2000). Coliform bacteria are normally not pathogenic but only mildly infectious. For this reason they are relatively safe to work with in the laboratory. If large numbers of coliforms are detected in water it is then highly possible that pathogenic microorganisms may be present (Toranzos, 1991; Muyima and Ngcakani, 1998).

There are limitations in the use of coliform bacteria for water quality monitoring. One of them is the relatively short life span of these bacteria in the environment (Hagedorn *et al.*, 1999). The survivability of faecal coliform bacteria increases remarkably with water temperature at or above 30^{0}C. Another limitation is that there are some bacterial pathogens, which are unrelated to human wastes and therefore not detected in the routine coliform-based tests. There are also advantages in using coliform bacteria as indicator organisms. They are characteristically identified quickly and easily and present in larger numbers than pathogens. They also react to the natural environment and treatment processes in a manner and degree similar to pathogens. By

monitoring coliform bacteria, the increase or decrease of many pathogenic bacteria can be estimated (American Public Health Association, 1992).

Faecal streptococci are not part of the faecal coliform group. These organisms do inhabit the intestinal tract of warm-blooded animals and thus are another type of faecal pollution indicator. Their advantages are that they tend to persist longer in the environment than faecal coliforms and isolating and detecting faecal streptococci from sources like composed animal and poultry litter is not difficult compared to faecal coliforms.

Bacteriophages have been used as indicators of faecal contamination because they infect indicator microorganisms such as *E.coli* (Toranzos, 1991). These viruses are coliphages. Toranzos (1991) proposed a direct correlation between the concentration of bacterial indicators (faecal and total coliforms) and the concentration of coliphages. Bacteriophages that infect *Bacteriodes fragilis* are also used as indicators. Grabow (1996) discussed the risks of three hepatitis viruses, HAV, HEV and HFV, which are known to be transmitted by water.

Viruses of human origin have been identified as causative agents of water-borne diseases. The viruses of concern include enteroviruses (hepatitis A, polio, coxsackie, echo), rotaviruses, adenovirus and reoviruses (Abbaszadegan *et al.*, 1999).

Cholera and typhoid fever are some well known waterborne diseases that spread through water with contaminated matter. There are five categories of pathogens found in water: bacteria, viruses, protozoa, worms and fungi. National standards usually include only coliform determination to measure water bacterial contamination not only because coliforms are pathogenic, but because they are always present in the normal intestinal tract of humans and other warm blooded animals (Abbaszadegan *et al.*, 1999). There is a lack of epidemiological evidence on pathogenic bacteria, especially *Escherichia coli* in South Africa (Water Research Council, 1999).

Disinfection through use of chlorine or other oxidants has been shown to be effective for inactivation of most bacteria, viruses, and other microorganisms, which represent a health risk. The practice of flocculation and filtration as part of municipal water treatment of surface waters

significantly reduces the numbers of cyst-forming and oocyst-forming protozoans and enteroviruses (Pelczar *et al.*, 1993). However, there are other pathogens, most notably the protozoans, which are extremely resistant to chemical attack and may be inactivated only after high dosages or unusually long contact times (Haas *et al.*, 1993). Because of this resistance, protozoans may be present even though routine coliform monitoring indicates an otherwise safe water supply. Pathogens of note are *Giardia lamblia*, and *Cryptosporidium* (Haas *et al.*, 1993), the latter, which affected 13 000 people in Carroltown, Georgia (Mason, 1991).

There has been considerable interest in formulating risk assessment methodology for microbial pathogens in drinking water. Haas *et al.*, (1993) discussed a risk assessment approach for evaluating the water quality significance of enteroviruses in drinking water. They pointed out that the methods used for water treatment do not sterilize drinking water, nor do they necessarily reduce the concentrations of waterborne pathogens to zero in the treated water, as reported by Pelczar *et al.*, 1993. It has been also noted that the coliform standards used for drinking water are not reliable for assessing the public health risk of waterborne disease caused by viruses and pathogenic protozoans. Additional water quality assessment is needed to improve health protection from microbial pathogens that can pass through domestic water supply (Haas *et al.*, 1993).

2.1.3 Methods for detection of bacterial indicators

Bacterial contamination cannot always be detected by sight, smell or taste. Microbial and chemical methods need to be analyzed to determine the water quality. Traditional methods for water analysis rely on cultural techniques, and many selective-differential media have been developed. The commonly used traditional methods for determining water quality, using the members of the coliform group and faecal coliform group, include the membrane filtration (MF), and the most probable number (MPN) methods (American Public Health Association, 1992).

The MF method uses a fine porosity filter (0.45μm) that retains bacteria. Generally, the selective m-Endo (Muyima and Ngcakani, 1998) and m-Fc culture media (Turner *et al.*, 1997; Griffin *et al.*, 1999) are used for the isolation and enumeration of total and faecal coliforms, respectively. Advantages of this method include: usage of large sample volumes, reduced preparation time as compared to many traditional methods, allows isolation and enumeration of discrete colonies of

bacteria and provides presence or absence information within 24 hours. A disadvantage of the MF method is that it is expensive due to high cost of the membrane filters and filter manifolds.

The MPN method is relatively easy to use and positives are easily separated from negatives as a result of the accumulation of gas (Toranzos, 1991). This method uses a test tube full of media with a smaller inverted test tube (durham tube) inside which captures carbon dioxide gas released from the growth of coliform bacteria. A series of dilutions and replicates are set up, and those producing gas in 24 hrs at 35^0C are counted (Pelczar *et al.*, 1993). Statistical analysis is used to determine the most probable number of bacteria cells present in the sample.

Heterotrophic bacteria are widely enumerated by the use of nutrient agar. Alonso *et al.* (1999) demonstrated the use of media containing chromogenic and fluorogenic substrates for the enzymes β-galactosidase (LAC) and β-glucoronidase (GUD) for simultaneous detection of coliforms and *E. coli*. R2A (low nutrient medium) (Muyima and Ngcakani, 1998 and Defives *et al.*, 1999) and ¼ strength nutrient agar (Muyima and Ngcakani, 1998) were also proposed for the enumeration of heterotrophic bacteria. These bacteria showed comparable results to other methods. Assimilable organic carbon (AOC) is also used to measure the "aftergrowth potential" of bacteria present in waters (Toranzos, 1991). The disadvantage of this method is that it can take up to five days to get results.

A major disadvantage of culture methods is the time needed to produce results and also their failure to isolate viable but non-culturable (VBNC) organisms (Gunasekera *et al.*, 2000). These are some of the points that need to be taken into consideration when culture methods are formulated and when results are interpreted.

2.1.4 Physical and chemical factors influencing water quality

There are many physical and chemical factors of water that must be considered in determining water quality. The following are essential in chemical assessment of water quality (five parameters): biochemical oxygen demand (BOD), ammonia, nitrate, phosphates and dissolved oxygen (DO). BOD measures the rate of loss of oxygen in a sample incubated under standard conditions. The oxygen uptake is due to the activity of microorganisms in breaking down the organic matter present in the sample. The greater the rate of loss of dissolved oxygen the greater

the amount of organic matter present (Mason, 1991). The test also provides a measure of the level of contamination of the sampled water by biodegradable wastes.

Nitrates and phosphates are the key factors to eutrophication. Dissolved oxygen (DO) is used as an indicator of water quality associated with marinas. Several authors have demonstrated that low DO is considered to pose a significant threat to aquatic life (Scharler and Baird, 2000; Mason, 1991).

Other physico-chemical parameters that need to be considered include temperature, pH, hardness, colour, salinity, rainfall, turbidity, available nutrients and environmental pollutants that are determined almost routinely on river waters. Temperature influences the amount of dissolved gases (oxygen, carbon dioxide, nitrogen, etc.) present in the water. The cooler the water the more soluble the gas. Turbidity depends on factors such as rainfall, total dissolved solids (TDS), water flow, geological formations and vegetation cover (Ferrar, 1989).

The normal range for pH of surface water is 6.5 to 8.5, for the WHO (1996) and between 6.0 and 9.0 for South Africa (Lehloesa and Muyima, 2000). Water with pH > 8.5 could indicate that the water is hard. Hardwater does not pose a health risk, but can cause aesthetic problems (Mason, 1991). The degree of hardness becomes greater as the calcium and magnesium content increases and is related to the concentration of multivalent cations dissolved in the water (Mason, 1991). Metals such as iron, manganese, copper, zinc, lead; cadmium, chromium and nickel are analysed in cases of suspected industrial pollution.

The aim of this chapter is to determine the heterotrophic bacteria, faecal coliforms, total coliforms, physical and chemical contaminants of the Mhlathuze River for water quality assessment and to determine if it meet the South African water quality guidelines for quality of domestic use.

2.2 MATERIALS AND METHOD

2.2.1 Sample collection

Water samples were collected biweekly in sterile Schott bottles, at five different locations for the period of March 1998 to November 1999 along the Mhlathuze river (Kwa-dlangezwa (site 1), Dlangubo (site 2), Mhlathuze estuary (site 3), Mhlathuze pump station (site 4) and Felixton bridge (site 5)) (See map, Figure 1.1 in chapter 1). Once collected these samples were immediately stored on ice in a heat insulation container and transported to the laboratory. The samples were immediately stored at 4°C and analysed within 6 hours of collection.

2.2.2 Laboratory analysis

The samples were analysed in the laboratory using the methods recommended by the American Public Health Association, (1992). Analytical parameters as stipulated for microbiological, physical and chemical analysis were followed. The water quality indicators (substances) were selected based on their amenability to field-testing, their generally accepted importance to water quality, and their relevance as a measure of average water quality.

2.2.2.1 Physical and chemical analysis

The parameters examined in this study were turbidity, temperature, pH, hardness, ortho- and total phosphates and dissolved oxygen. Temperature and dissolved oxygen was measured *in situ* using a thermometer (Brannan) and dissolved oxygen meter (Brannan), respectively. The pH was determined in the laboratory by using pH-meter (Brannan). The turbidity of the five samples was measured using a Hach 2100P turbidimeter and expressed as Nephalometric Turbidity Units (NTUs). Hardness (mg/L of calcium carbonate), was measured by titrating 50ml of sample, containing 2ml buffer solution and 2 - 3 drops of Erichrome black T indicator, with EDTA (Appendix C). Ortho- and total phosphates were also analysed (Appendix C).

2.2.2.2 Microbiological analysis

a) Heterotrophic Plate counts

Nutrient agar (NA) (Merck, Germany) was used for the enumeration of heterotrophic bacteria. A series of tenfold dilution of water samples (10^{-1} to 10^{-5}) were used. The plates were incubated at 35°C for 24 hours. The analyses were performed in duplicates.

b) Total coliforms (TC)

Volumes of 1ml (undiluted sample, 10^{-1} and 10^{-2} dilutions) of each water sample were filtered through membrane filters (0.45µm pore sized membrane filter). Each volume was assayed in duplicates. The filters were placed on m-Endo agar LES (Merck, Germany) medium and incubated for 24 hours at 37°C. The colonies that produced a metallic sheen were enumerated as total coliforms according to the Standard Methods for the Examination of Water and Wastewater (American Public Health Association, 1992). The colonies were further analysed using the TSI agar (Merck, Germany) slants and test kit API 20E (bioMerieux API20E) incubated at 37°C. The TSI agar slants and API strips were analysed according to the manufacture's directions. MacConkey agar (MAC) was also used for the enumeration of the lactose fermenting bacteria. Single colonies after incubation were further analysed by using TSI agar slants and API 20E strips.

c) Faecal coliforms (FC)

Water samples were initially analysed for faecal coliforms by membrane filtration (MF) method (0.45µm pore sized membrane filters) using m-FC agar (Merck, Germany). Volumes of 1ml (undiluted sample, 10^{-1} and 10^{-2} dilutions.) of each water sample were filtered. The membrane filter was then placed on m-Fc agar and incubated for 24 hours at 44.5°C. After incubation all blue colonies were countered as faecal coliforms. Single blue colonies from the membrane were picked and streaked onto triple sugar iron agar (TSI, Merck, Germany) slants and also identified by API 20E strips (bioMerieux, France). TSI agar slants and API strips were processed and interpreted in accordance with the manufacturer's directions.

d) Isolation and enumeration of *Streptococcus, Salmonella* and *Shigella* species

The presence of *Streptococcus, Salmonella* and *Shigella* species were determined by spread plate technique using the following media: KF Streptococcus agar (KSA), and Shigella-Salmonella agar (SSA). All growth media used were manufactured by Merck, Germany. Colonies grown on these media were analysed according to their appearance and those resembling the appearance of *Shigella* and *Salmonella* species were further analysed using the test kit API 20E and Triple Sugar Iron (TSI) Agar slants. API 20E strips were processed in accordance with manufacturer's directions and incubated at 35°C.

e) MPN index

MPN technique with five tubes per water sample dilution (undiluted sample, 10^{-1}, 10^{-2} and 10^{-3}), was used for total coliforms counts using lactose broth, Merck, Germany. Test tubes (10 ml) containing the media and inverted Durham tubes were inoculated with 1 ml volumes of the respective dilutions. The tubes were incubated at 37°C. Gas and turbidity production within 48 hours was considered to be a positive response. The number of the positive tubes per dilution was recorded. The total number of positive results for all dilutions per sample was analyzed.

2.3 RESULTS

The average monthly microbiological quality and physicochemical data of the water collected at each site are summarized in Appendix A and B respectively. Sampling and analysis were performed biweekly throughout the study period (March 1998 – November 1999). Colonies on NA plates were counted for heterotrophic bacteria and those on m-Endo and m-Fc agar plates were counted and identified for total and faecal coliform bacteria respectively. Throughout the study, there was no growth in the SSA plates for the enumeration of *Salmonella* and *Shigella* species and also. *Streptococcus* agar plates for *Streptococcus* species.

Physical and chemical analysis data is tabulated in Appendix B. The changes in physico-chemical parameters (water temperature, pH, turbidity, dissolved oxygen, hardness, ortho-and total phosphates) measured are presented in Section 2.3.2.

2.3.1 Microbiological analysis

The graphs in Figure 2.1 depict the seasonal variation in heterotrophic plate count, total and faecal coliform bacteria as well as the surface water temperature and rainfall detected in the Mhlathuze River from March 1998 to November 1999. During the periods March to November (both 1998 – 1999) the average total coliform counts were generally two fold greater than the average faecal coliform counts.

However the period November 1998 – February 1999 shows an enormous (five-fold) increase in the average number of total coliforms detected, compared to the average number of faecal coliforms counted. Lowest numbers of colony forming units (CFU's) were detected during winter season (May - August) and large numbers of CFU's were detected in the summer season (November - February).

The average temperature of the water for this period (November 1998 – February 1999) was constantly between 25^{0}C and 35^{0}C. The heterotrophic plate counts detected in the Mhlathuze River during the summer period (November 1998 – February 1999) also show a peak which had four Log_{10} increases compared to winter. The changes in heterotrophic bacterial counts were more gradual than that of the indicator microorganisms. The two environmental factors (surface

water temperature and rainfall) also showed similar cyclic changes to the microbial data and may have influenced the bacteriological observations. The average monthly pH values ranged between 6.5 and 8.5. These values are well within the SA standards for potable water (DWAF, 1996) and sudden fluctuations, which are indicative of adverse conditions, were not observed.

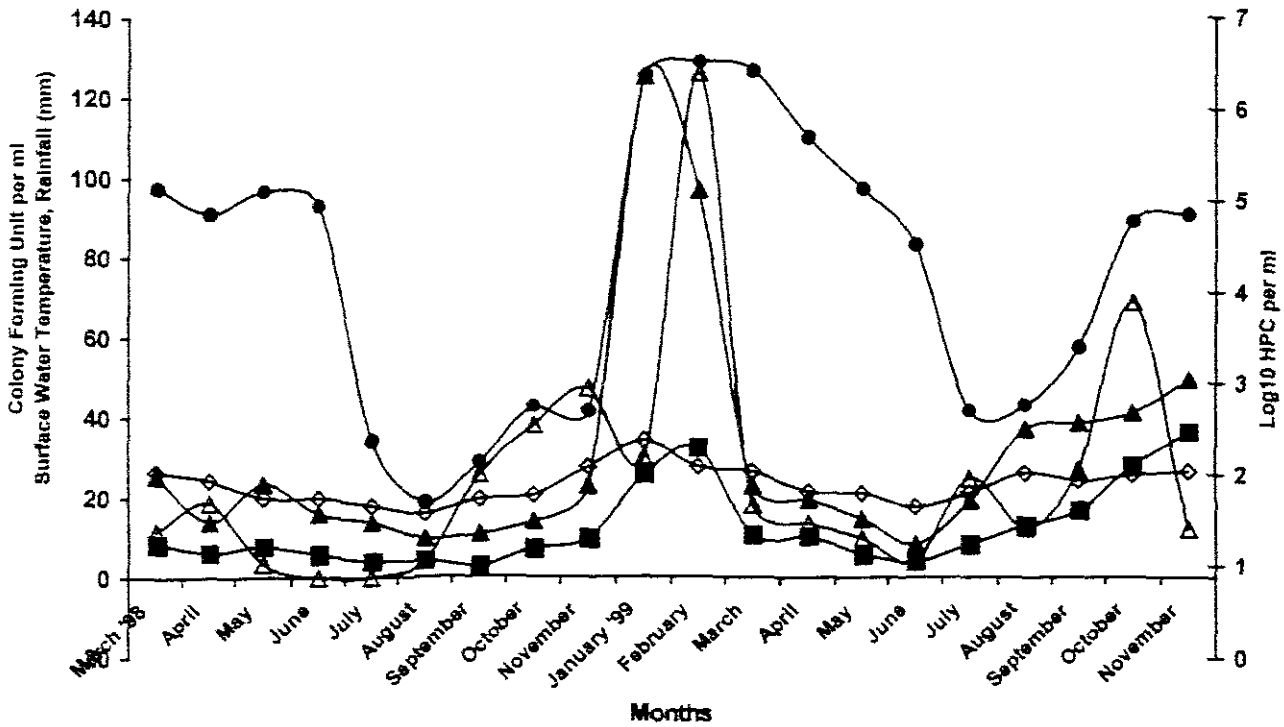
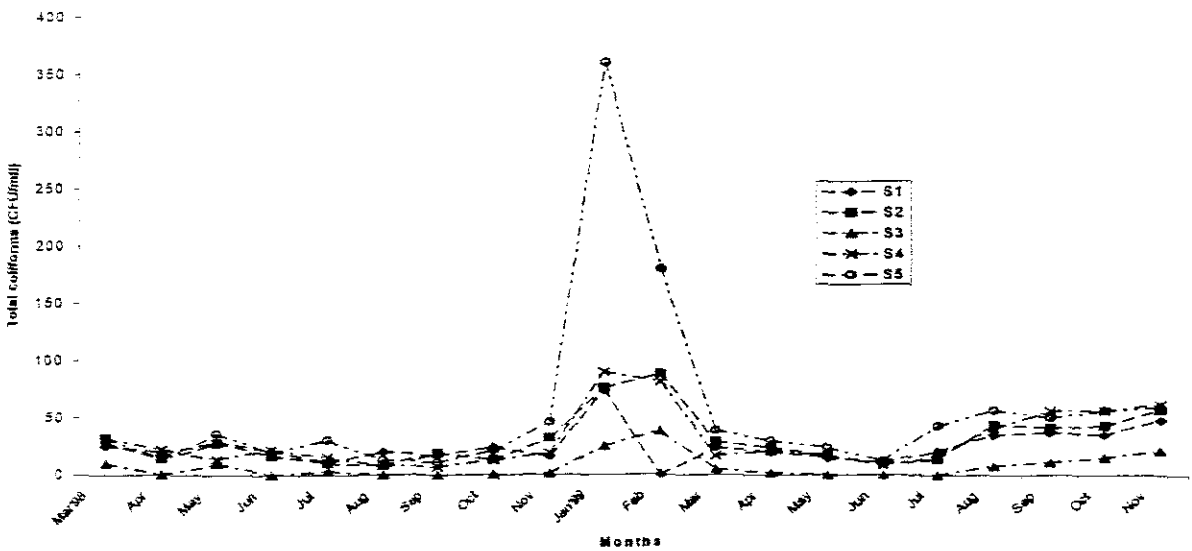


Figure. 2.1. Seasonal variation in heterotrophic plate count (•), total coliform (σ) and faecal coliform bacteria (v) in the Mhlathuze River as well as rainfall (Δ) and surface water temperature (◇) data collected from March 1998 to November 1999. The monthly values for all bacteria are the mean values of five samples per month that were sampled and analysed, in duplicate, twice per month. Temperatures were measured *in situ* and rainfall figures were supplied by the Computing Centre for Water Research of the University of Natal, Pietermartizburg.

From the graphs depicting the indicator microorganisms detected per site (Figure 2.2, total coliforms and Figure 2.3, faecal coliforms respectively) it is evident that site 5 was the major



contributor to the observed peak in CFUs especially during the summer period (November 1998 – February 1999).

Figure 2.2. Seasonal variation of the average total coliform counts taken per site from the. Mhlathuze River during the period of study (March 1998 to November 1999).

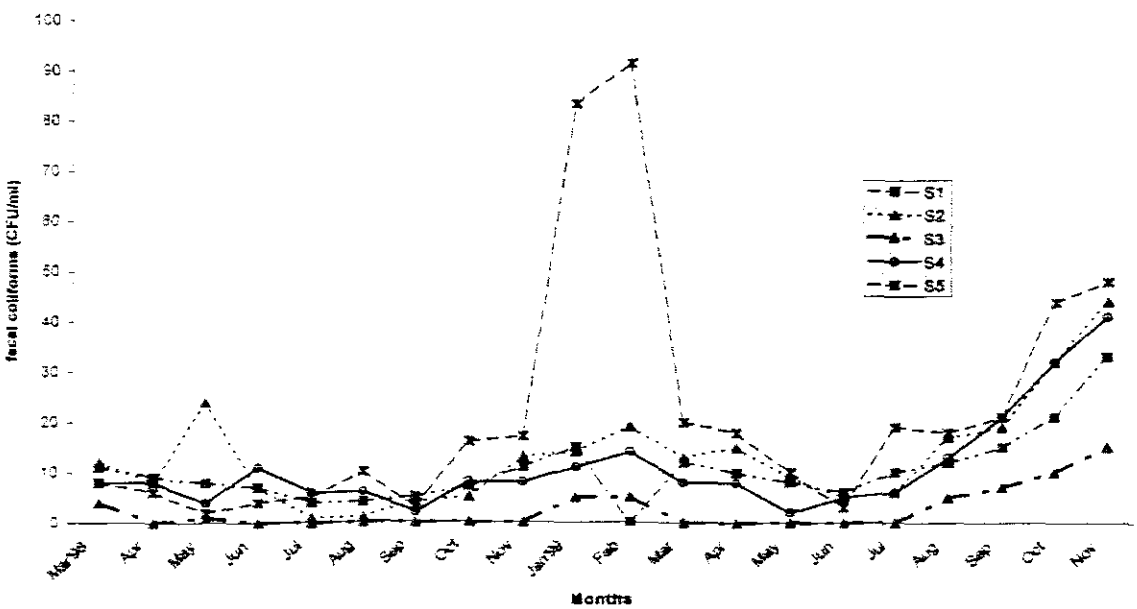


Figure 2.3. Seasonal variation of the average faecal coliform counts per site taken from the Mhlathuze River during the period of study (March 1998 to November 1999).

Monthly comparisons of the average total coliforms and faecal coliforms in 1998 to that observed in 1999 (Figure 2.4 and Figure 2.5, respectively) show that, except for May and June, greater numbers of these indicator microorganisms were detected in the Mhlathuze River in 1999 than 1998. The increase in the number of the faecal coliforms (Figure 2.5) detected in 1999 is almost four fold than in 1998.

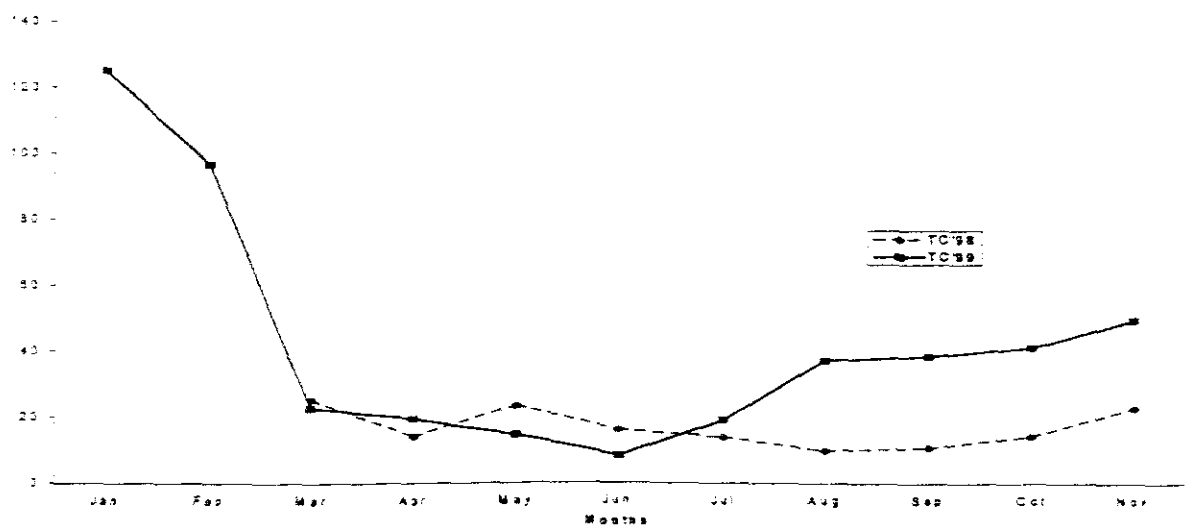


Figure 2.4. Monthly comparisons of the average total coliform counts per ml during the period of study (March 1998 to November 1999). The graph shows the growth pattern of *total coliforms*. The monthly values are the average values of the five sampling sites of the study. The dotted line represents the average monthly total coliforms detected in 1998 and the solid line represents the average monthly total coliforms detected in 1999.

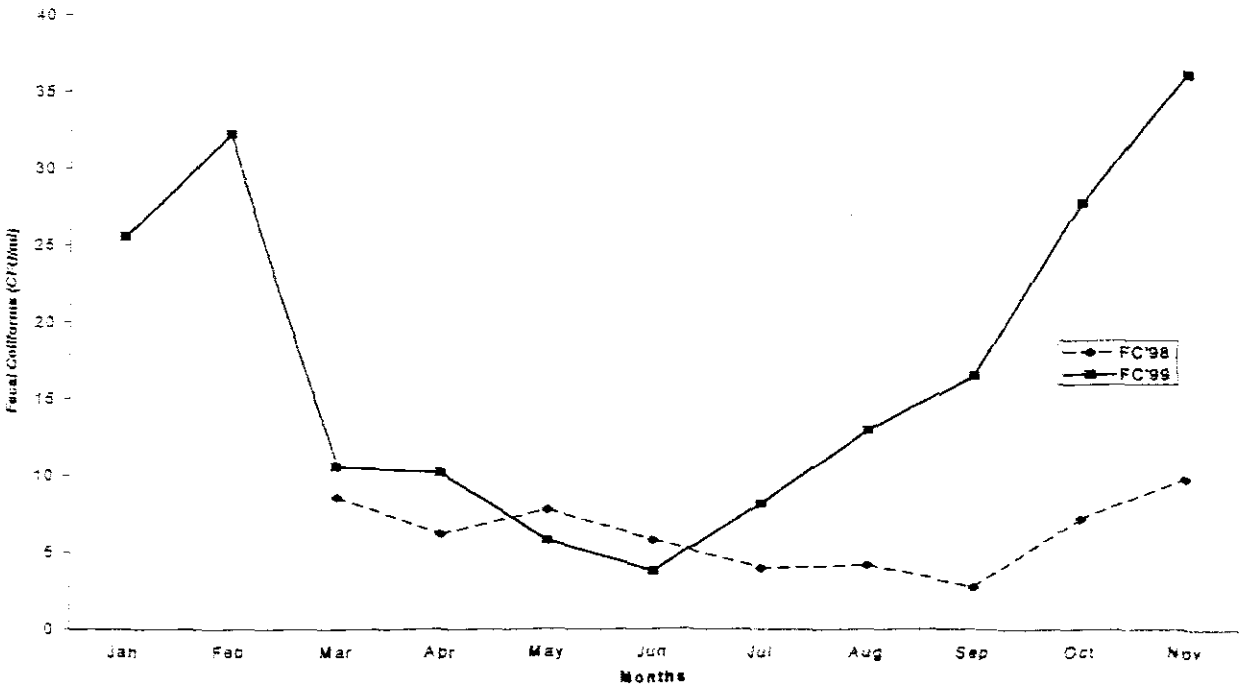


Figure 2.5. Monthly comparisons of the average faecal coliform counts taken from the Mhlathuze River during the period of study (March 1998 to November 1999). The monthly values are the average values of the five sampling sites of the study. The dotted line represents the average monthly faecal coliforms detected in 1998 and the solid line represents the average monthly faecal coliforms detected in 1999.

This phenomenon, i.e. that there is an increase in the number of microorganisms detected in 1999 when compared to 1998, is also evident in the numbers of heterotrophic plate count bacteria (Figure 2.1) detected. It must be noted that the average surface water temperatures for the corresponding period were +/- 10^{0}C greater in 1999 than in 1998 (Figure 2.6).

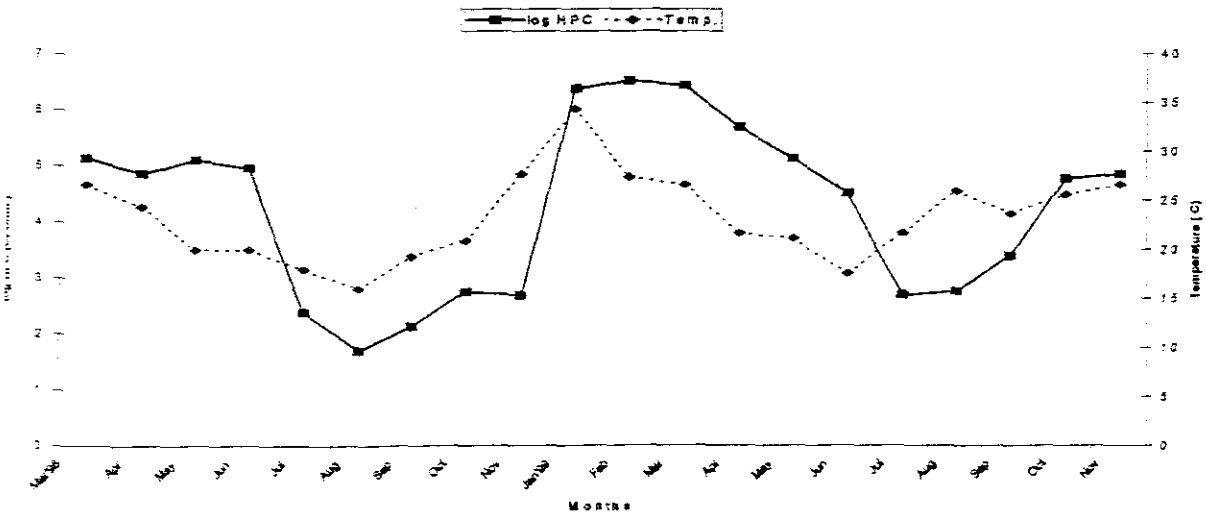


Figure 2.6. Seasonal variation of the average counts (log_{10}) of the heterotrophic bacteria and temperature taken from the. Mhlathuze River during the period of study (March 1998 to November 1999). The monthly values are the average values of the five sampling sites of the study.

In so far as hetrotrophic bacteria detected, the increases and decreases were more gradual than those of the indicator microorganisms. The sudden increase in heterotrophic bacteria detected in November 1998 and January 1999 is that the values for December 1998 were missing. It is however, clear that the heterotrophic bacterial population of the Mhlathuze River is typically and proportionally dependent on surface water temperature (Figure 2.6).

MPN index was performed concurrently with MF method for *E.coli* and coliforms enumeration. It was observed that both methods were conversely related to each other. In comparing these two methods it was also taken into account that the MPN is an estimate of the number of faecal coliforms originally present in the sample while MF technique results in an exact count (Appendix A).

Table 2.1: Bacteria isolated at five sites of the Mhlathuze River

Bacteria	Site 1	Site 2	Site 3	Site 4	Site 5
E.coli	✔	✔	✔	✔	✔
Klebsiella spp.					✔
Pseudomonas spp.	✔	✔	✔	✔	✔
Proteus spp.	✔				✔
Enterobacter spp.	✔	✔	✔	✔	✔
Citrobacter freundii		✔		✔	✔
Serratia spp.					✔
Aeromonas hydrophila			✔	✔	

Table 2.1 shows the various types of bacteria isolated and identified at the five different sites of the Mhlathuze River. These microorganisms were isolated from m-Endo Agar Les, m-Fc and SS Agars. Single colonies from each plate were randomly isolated, based on the morphology and their identity confirmed by TSI Agar and API 20E strips (BioMerieux, France) according to the manufacturer's instructions. *E.coli, Enterobacter* spp. and *Pseudomonas* spp. were constantly detected at all five sites. *Klebsiella* spp., *Proteus* spp., *Serratia* spp., *Aeromonas hydrophila* and *Citrobacter freundii* were also occasionally isolated from the various sites.

2.3.2 Physical and chemical analysis

Physical and chemical analysis was conducted as described in material and methods (Section 2.2). Surface water temperature ranged between 14^{0}C (site 1, June 1999) and 35^{0}C (sites 1, 3, 5, January 1999) (Appendix B). Average monthly temperature is shown in Figure 2.7. The figure also shows the average monthly pH values observed along the Mhlathuze River during the period of study (March 1998 – November 1999). The average monthly pH values ranged between 6.5 and 8.5 (Appendix B). These values are well within the SA standards for potable water (DWAF, 1996). pH measurements were inverse proportionate to temperature readings. This relationship is well demonstrated by the scatter graph in Figure 2.8.

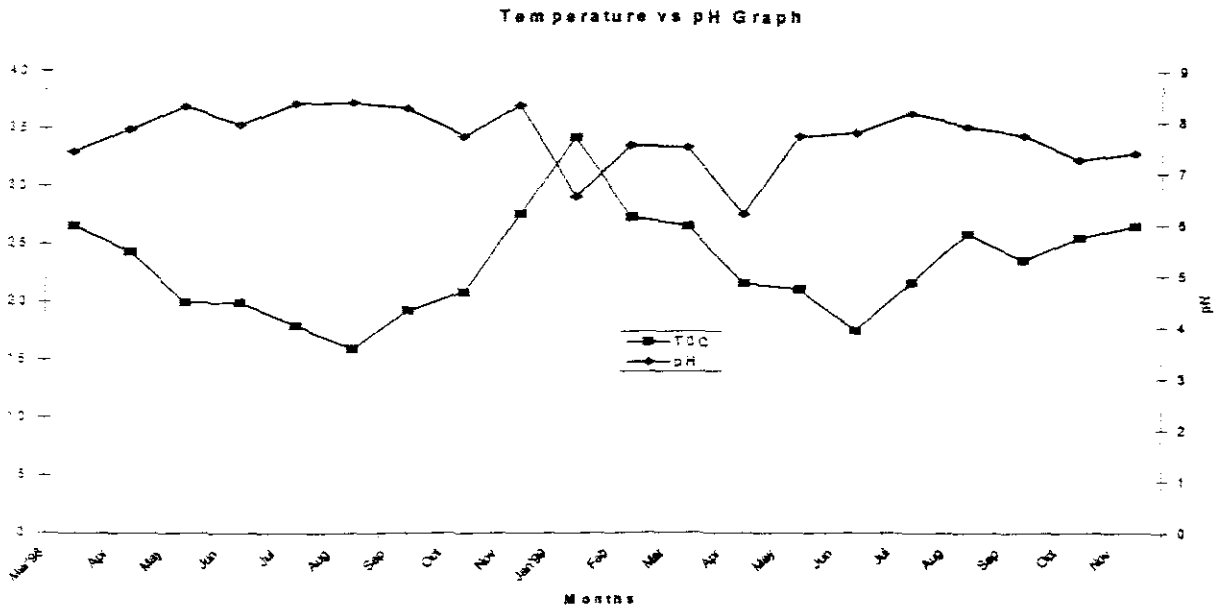


Figure 2.7. The relationship between water temperature and pH in the Mhlathuze River during the period of study (March 1998 to November 1999). The monthly values are the average values of the five sampling sites of the study.

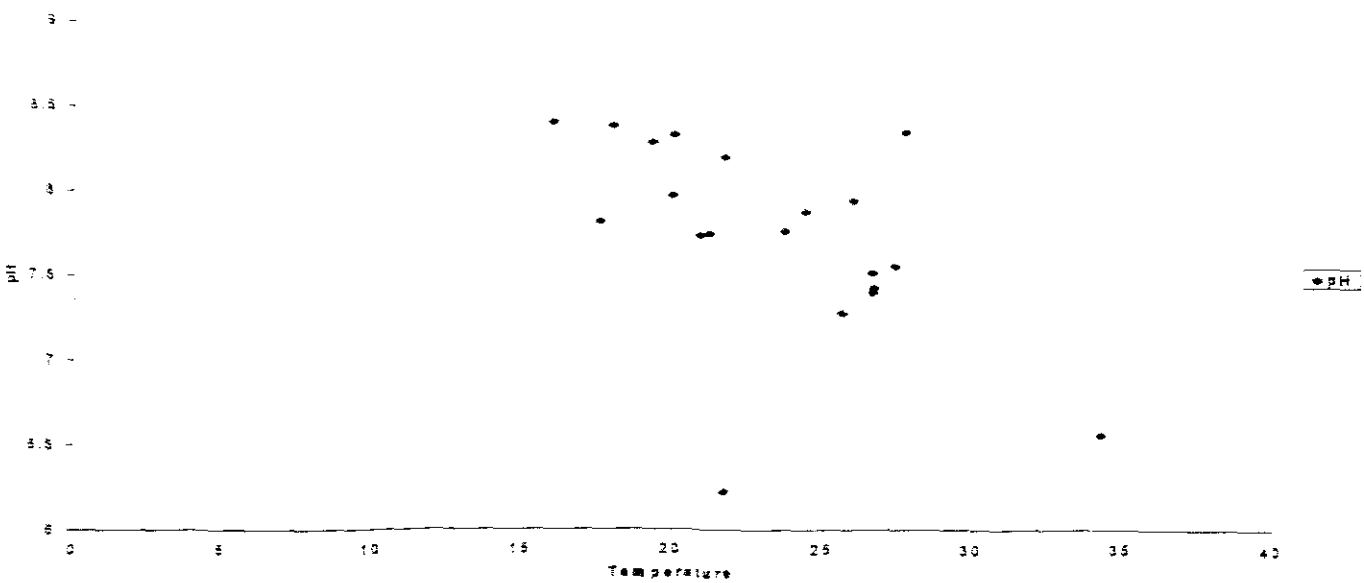


Figure 2.8. The relationship between pH and water temperature in the Mhlathuze River during the period of study (March 1998 to November 1999).

The apparatus to measure turbidity and dissolved oxygen were not readily available especially the dissolved oxygen meter. Turbidity ranged between 1 – 5 NTU (Appendix B) for period March – November 1998. In 1999 the turbidity measurements were discontinued. The appearance of the water samples was similar to those of 1998 and were thus considered to be of acceptable standards. Dissolved oxygen measurement could only be taken for March, May and July 1998. The values obtained were within the limits of SA Standards for source drinking water.

Hardness, ortho- and total phosphate values obtained over the entire period did not show any major range changes that would call for caution. The values of ortho- and total phosphates (not shown) and hardness were within the acceptable limits (SABS, 1984). Hardness of greater than 1000 mg/l calcium was constantly observed at site 3 (Appendix C). This was expected since the site is an estuary and the salinity is considerable higher than normal fresh water.

2.4 DISCUSSION

2.4.1 Microbiological analysis

For this study Nutrient Agar (NA) was the medium of choice for the recovery and enumeration of heterotrophic bacterial populations. Various media are also available for the enumeration of heterotrophic bacteria, such as Yeast Extract Agar and R2A medium. (Sartory and Watkins, 1999). R2A media have shown to allow greater numbers of bacteria when compared with Yeast Extract Agar. NA was the medium that was readily available throughout the period of study. R2A medium is widely used for this purpose. NA is cost effective and has been used and still used by many researchers because it also provide the essential nutrients for heterotrophic growth.

The membrane filtration method was used for the enumeration and identification of total and faecal coliforms. The growth media of choice were m-Endo and m-Fc agar for detection of these indicator microorganisms. These media are widely used by public health microbiology divisions in several countries due to its reliability for routine monitoring (American Public Health Association, 1992).

MPN method was also performed conjointly with MF method for comparison. The results showed that, in most instances, MPN method resulted in high levels of total coliforms than MF method. MF membrane is a more expensive technique than MPN techniques. This is because of the high cost of the membrane filters that are needed.

There are also other methods used for detection of coliform bacteria. Colilert method is one of the most known methods. Eckner (1998) discussed the comparison of membrane filtration and multiple-tube fermentation by colilert and enterolert methods for detection of waterborne coliform bacteria, *Escherichia coli*, and Enterococci. The results showed that the Colilert methods was more sensitive that Swedish standard methods for detecting coliforms. Injured coliforms are enumerated by a non-selective medium M-T7 (Muyima and Ngcakani, 1998). Some bacteria enter the viable but non-culturable state (VBNC). Defives *et al.* (1999) estimated VBNC bacteria by the difference between viable and viable cultivable (VC) counts. They used R2A medium for counting viable bacteria and viable and dead bacteria were counted using the LIVE/DEAD BacLight viable fluorescent method (Defives *et al.*, 1999).

The main focus of the study was the bacteriological assessment of the water of the Mhlathuze River. Heterotrophic bacteria, total- and faecal coliforms levels at five sites along the Mhlathuze River were determined using direct (Figure 2.1) and indirect methods. Water quality monitoring showed that water from the Mhlathuze River followed broad seasonal patterns of change. It is observed that bacterial population reached a peak in December 1998 to February 1999 and declined in June, July and August 1998 and 1999.

The microbiological analysis data reported here demonstrate that an increase in bacterial plate counts corresponded with an increase in MPN index and surface water temperature. This observation is supported by the notion that as temperature rises, chemical and enzymatic reactions in the cell proceed at more rapid rates and cell growth becomes faster (Brock and Madigan, 1988). A study by Motes *et al.* (1998) demonstrated that *Vibrio vulnificus* MPN count increased with increasing temperate (warm-weather months) and decreased with the decreasing temperature (cold-weather months).

Temperature may not be the only factor that contributed to the bacterial count changes. Lack of sanitary facilities in the informal settlements surrounding the river could be a factor contributing to this effect. Constant soil run-off and normal agricultural activities may also contribute to the increase in bacterial counts observed. There are agricultural fields along the riverbank (site 5) and recreational activities (swimming, bathing and washing) are increasing during the summer period. Domestic animals, such as cattles, also utilise the same water resource for consumption. These animals are chased into the river to drink. It is also noted (Figures 2.1, 2.4 and 2.5) that there was a tremendous increase in bacterial population during January and February. The increase is likely to be linked with the heavy rainfall, which possibly caused soil run-off at that time. The Mhlathuze river catchment is in a summer rainfall area.

The data demonstrated that influx of bacteria into the river system is more or less constant. Agricultural, human and animal activities could be considered the factors contributing most to these bacteria entering the Mhlathuze river system. Bacteriological changes occurring at sites 1,2 and 4 are more or less similar. This is evident in total coliforms per site (Figure 2.4) and faecal coliforms per site (Figure 2.5) data as well as physical and chemical data (Appendix B). Site 1,

which is next to the University of Zululand, is also one of the sites that was monitored for bacterial pollution. There is a sewage treatment plant next to this site. This sewage plant is situated upstream of site 1 and downstream of site 5. The sewage treated effluent released into the river could contain nutrients, which might have been the sole source of carbon. Some of the bacteria might have survived from the treatment and contributed to the bacterial increase in the Mhlathuze River. Site 2, which is at Dlangubo – ~45km away from site 1, is largely available for human and animal use because there are houses next to the site. Site 4 is situated ~4km away from site 5. Pump station for Mhlathuze Water Board is situated in this site. The low level of bacterial counts may be subjected to this pump station because of the flowing water. Site 3 is an estuary, boundary between seawater and river water, therefore high salt content is expected. Low level of bacterial population was detected throughout the study. This may be caused by the bacterial intolerance of salt from the seawater. Although no human activities taking place directly on banks of river estuary, low levels of total and faecal coliforms were detected.

Site 5 had the highest total and faecal bacterial population compared to other sites (Figures 2.2 and 2.3) throughout the study period (March 1998 – November 1999) and also the highest contributor to the average bacterial counts (Figure 2.1). A drastic increase in summer (November – February) is clearly demonstrated in Figures 2.2 and 2.3. The possible factors contributing these high bacterial counts at site 5 may be increased animal and human activity. This site (site 5) is also situated adjacent to Mondi (pulp and paper) factory and Hulett (sugar mill). It is possible that, although precautions are in place to prevent this, effluent rich in organic carbon may be released into the water. Even in very small quantities, organic carbon may enhance the population growth of bacteria.

The results show that the microbial quality of Mhlathuze River water is not sufficient for direct consumption by humans and animals. The faecal coliform counts should be 0 counts /100ml (Kempster *et al.*, 1997). Faecal coliforms are normally used as indicators of pathogenic organisms that may be present in the water system (Muyima and Ngcakani, 1998). If present, there is an indicative of health risk to the community using that resource. It must also be taken into consideration that utilizing these resources for domestic activities may be dangerous since the resource will be suitable for transmitting causal agents of diarrhoeal diseases.

2.4.2 Physical and chemical analysis

Water temperature is an important factor in bacterial growth pattern. Results presented in this study showed that there were essentially two seasonal episodes, i.e. one when water temperature was (14^0C) cold during winter and warm (35^0C) during summer (Figure 2.7). During winter season, the counts of heterotropic bacteria, total and faecal coliforms were low as compared to the counts during summer. This indicated that the higher the temperature (range 25^0C - 35^0C), the higher the number of bacteria being detected. Similar findings have been demonstrated by other authors, i.e. the bacterial cells generally decreases when the cells are exposed to adverse conditions, such as cold temperature and increases when conditions become favourable, e.g. high temperatures (Firth *et al.*, 1994; Whiteside and Oliver, 1997). Whitside and Oliver (1997) believed that the growth response is due to the resuscitation of viable but nonculturable (VBNC) part of population. Bacterial decrease with cold temperature has been associated with the regrowth of few surviving cells in the environment (Firth *et al.*, 1994; Bogosian *et al.*, 1998). It could not be confidently concluded that the phenomenon of changes in bacterial counts was due to temperature only, since there may be other factors that contributed too, e.g. the presence of nutrients or the absence of toxins playing a major role in the survival of bacteria in the environment.

The pH values of Mhlathuze water were within the South African recommended limits (pH6.0 – pH9.0) (Appendix B). This means there are no adverse health risks that can be related to the pH.

Total hardness in the Mhlathuze River (Appendix B) was found to be moderately hard (100 to 200 mg $CaCO_3$/l) except at site 3. At site 3 the hardness measurements were above 1000 mg $CaCO_3$/l. This site is an estuary and contains high concentrations of dissolved residues. Calcium and magnesium dissolved in water are the two most common minerals that make water hard (Mason, 1991; Lehloesa and Muyima, 2000). Soft water on the other hand has high sodium content that can affect algal growth.

There was no significant change in the turbidity of the Mhlatuze River during the period of study. Studies reveal that turbidity of water is dependent on complex interactions, between soil

characteristics and agricultural practices, within the catchment and the flow rates of rivers draining the catchment. (Ferrar, 1989; Koning and Roos, 1999). Soil run-off from agricultural activities may be factors contributing to the turbidity of various sampling sites. It was noted that site 3 had very low values of turbidity. This low level of turbidity can be accounted for by the fact that this site is the boundary of seawater and river water. River water is diluted by seawater, resulting in low levels of turbidity.

The results of dissolved oxygen (March to July 1998) did not vary (Appendix B). The limited dissolved oxygen data did not show any correlation to the seasonal trends in the number of bacteria detected. The changes in dissolved oxygen concentrations give valuable information for the measurement of environmental factors affecting aquatic life. Roos and Pieterse (1995) demonstrated that dissolved oxygen concentration in the Vaal River showed seasonal trends, with peak values coinciding with low water temperatures. It would thus have been interesting to determine if the phenomenon observed by Roos and Pieterse (1995) is also true for the Mhlathuze River.

2.4.3 Implications

High bacterial counts from the Mhlathuze River pose a health risk to the surrounding communities drinking from the river and bathers due to direct ingestion of water containing bacteria. This issue becomes more serious if comparing the bacterial counts for 1998 and 1999. It is noted that 1999 had higher microbial counts than 1998. High number of bacterial counts in 1999 can be accounted by the rise in water temperature, but also there may be some reasons, e.g. an increase in human population that may resulted in an increase in human waste or there was a large number of waste disposed. Monitoring the Mhlathuze River was also continued in our laboratory and the results showed an increase bacterial count in year 2000 in this area compared to 1998 and 1999. An increase in number of faecal coliforms in 2000 raised some questions if there was also an increase in pathogenic bacteria present in the river since high levels of faecal bacteria can indicate a possible presence of pathogenic bacteria (Toranzos, 1991). This statement is supported by cholera outbreak started at the Empangeni – Eshowe areas in August 2000. Residents of rural areas drink water directly from Mhlathuze River because they could not afford to pay for water. The health authorities suspected that the source of cholera causing bacteria is

the Mhlathuze River. About 35 000 cases of cholera have been reported and killed approximately 80 people. The outbreak has reached the whole of KwaZulu Natal, Gauteng and Mpumalanga Provinces. *Vibrio cholera* species is a pathogenic bacterium and can enter VBNC state. In this case, this species may have entered VBNC state and became resuscitated maybe due to an increase of bacterial population in the environment, which this study have proved that remarkable increase of heterotrophic, faecal and total coliform counts. According to the DWAF (1996) guidelines, the bacterial quality of the water of the Mhlathuze River posed an increased risk of infectious disease transmission to the communities that were dependent on the river for household, recreation and other purposes.

It is imperative that water should be treated before use. Sources of bacterial pollution are very important to make the process of treatment more effective. Filtration products for drinking water applications are required to help monitor the quality of municipal water systems. The possible presence of microbiological pathogens in drinking water supplies is a significant concern in the protection of public health. This risk of contaminants can be minimised through such measures as observance of required setbacks of water sources from septic systems, proper disinfection at the source where needed, and maintenance of an adequate program to prevent back-siphonage of contamination into the supply mains.

Since water remains an essential for life it serves as a potential reservoir of risk to public health. It is very important that the water industry is completely and constantly aware of those aspects of the water cycle, which carry the greatest potential health risk. It is thus important for the Water Authorities to know that microbiologists should not be confined to undertaking simple routine scientific tests for faecal indicator organisms, thus minimising the outbreaks of water borne diseases.

CHAPTER 3

IDENTIFICATION OF COLIFORMS USING POLYMERASE CHAIN REACTION

3.1 INTRODUCTION

The microbiological quality of source water is essential to determine its suitability for consumption by humans and animals. Diarrhoeal epidemics have, in many cases, been due to the poor microbiological condition of drinking water. Recent diarrhoeal outbreaks in South Africa are typical examples where poor microbial quality of water is the major contributing factor.

Pathogens such as *Salmonella, Shigella* and *Eschericia coli* are amongst the most common causes of water-borne diseases. Infections caused by these pathogens include typhoid fever, hemorrhagic colitis, and bacillary dysentery. To determine the presence of these organisms in environmental and drinking water sources, sensitive and specific detection methods are needed. Conventional methods currently used rely upon the culturing on medium that selectively permits the growth of specific bacteria. There are several problems with these viable culture methods, including lack of growth of viable but non-culturable bacteria such as those stressed by chemicals in water and time required for detection and confirmation of pathogens (Bej *et al.*, 1990). The biochemical similarity between pathogens may also be a problem using conventional detection means. *E. coli* and *Shigella* spp. are good examples of such a problem. There are no differences between *Shigella* and *E.coli* in terms of biochemical, serological, genetic and even pathogenic trait (Farnleitner *et al.*, 2000) These pathogens are causes of serious infections such as diarrhea and dysentery but can be wrongly diagnosed or remain undiagnosed because of these similarities or because of the presence of another pathogen that is capable of causing the same infection such as enteroinvasive *E. coli* 0157:H7.

The polymerase chain reaction (PCR) procedure, introduced in 1986, provides an alternative to traditional culture methods for detection of microbes from environmental samples (Atlas, 1991). The original purpose of using PCR procedure to evaluate the water quality by the researchers worldwide is to provide more sensitive approach. It is known that low total and faecal coliform

counts do not mean that it is 100% safe to consume water. Ideally the PCR procedure should be able to detect the pathogens directly from the water system.

PCR procedure is based on the recovery of the Deoxyribose Nucleic Acid (DNA) from the samples and the subsequent amplification of the target nucleotide sequences. Minute quantity of DNA associated with a pathogen or other target organism can be detected. This technique has many advantages over traditional methods, which will be discussed below. However there are also certain drawbacks associated with the technique.

3.1.1 Advantages and disadvantages of traditional and molecular techniques

Traditional methods for the detection of pathogens or indicator of faecal contamination may result in the lack of growth of bacteria due to sublethal environmental injury, inability of the target bacterial to take up nutrients and other physiological factors leading to the reduction of bacterial culturability (McFeters *et al.*, 1999). These disadvantages have led to the use of molecular techniques such as PCR for the detection of indicator and pathogenic microorganisms. PCR based methods have been demonstrated to be sensitive, reliable, specific and effective for the detection of microorganisms in environmental samples (Alvarez *et al.*, 1993; Belgrader *et al.*, 1999). Although molecular techniques have shown to be more productive than traditional techniques, there are also disadvantages to them. The polymerase reaction is very sensitive to the levels of divalent cations (especially Mg^{2+}) and nucleotides, and the conditions for each particular application must be worked out. Primer design is also important for effective amplification. Primers must be designed to amplify only specific sequences, i.e. need for sequence information. This can be expensive if not enough genetic information is available. However, more and more sequences of organisms are being published daily in Genebank. The primers must not be capable of annealing to themselves or each other, as this will result in the very efficient amplification of short nonsense DNAs. The sensitivity of PCR requires that careful laboratory techniques be employed to prevent unwanted false positives induced by carry- over mechanisms (Heinrich, 1991). Carry-over is the mechanism whereby amplification results from exogenous sources rather than the sample itself.

3.1.2 DNA Extraction

Another problem with PCR is the fact that Taq polymerase is easily inhibited by a number of substances such as fats, proteins and metal ions and also inhibited by chemicals that are required for selective enrichment of cells or DNA extraction (Schneegurt and Kulpa, 1998).

The validity of using molecular techniques depends on obtaining representative extracts of nucleic acids from entire microbial communities. There are many nucleic acid methods used each with its efficiencies in its component steps including incomplete cell lysis (Miller *et al.*, 1999). Lysis of microbial cells from environmental habitats marks a critical step in a PCR-mediated approach.

3.1.3 Molecular detection of bacteria

Molecular methods targeting nucleic acids and provide the opportunity to detect greater bacterial diversity. Basic nucleic acid methods are hybridization, restriction, amplification, and sequencing (Grimont, 1999).

Hybridization technique is mostly used for detecting specific nucleic acid sequences or genes of interest. Several variations of direct hybridization techniques have been developed by different workers. Hybridization can be used in conjunction with PCR to further examine the PCR-amplified products. A combination of PCR and southern hybridization has been used to detect genes for Staphylococcus enterotoxins A to E in strains of toxigenic *S. aureus* from various environmental sources (Sharma *et al.*, 2000). Strains that produced false positive results were further analyzed by DNA hybridization to determine whether they contained *sec* gene. According to the reference of Kwang *et al.*, (1996) (p72) they conducted a research on the use of PCR FOR *E.coli* detection. PCR amplification was performed in the presence of three primers. The PCR product with one of the primers was further verified by southern hybridization with a digoxigenin labelled probe. Sabat *et al.* (2000) reported on the detection of *E.coli* 16S rRNA genes in soil by using PCR and southern hybridization.

Restriction endonucleases recognize short specific palindromic sequences and cleave double stranded DNA at these sites. Restriction analysis is widely used in conjunction with PCR for differentiation of PCR product through restriction fragment sizes detected by gel electrophoresis.

Alternatively, a DNA fragment can be amplified and digested by a restriction endonuclease to give a simple pattern (Kilger and Grimont, 1993). Restriction methods are best applied on purified DNA extracted from pure culture (bacterial identification and typing) and are not currently used on field samples with complex bacterial flora.

Amplification is a method in which a chosen nucleic acid sequence is copied many times. Although different methods have been proposed, the polymerase chain reaction (PCR) is the most widely used. PCR uses two oligonucleotide primers hybridizing to the flanking regions of a target gene (on different strands). A DNA polymerase extends each primer so as to polymerize a strand complementary to that used as a matrix. The result is the duplication of the target sequence at each cycle. The technique is easy once the target and flanking sequences are known. The major problem is due to the extreme sensitivity of PCR, which allows to amplify contaminating polynucleotides when careful procedures are not implemented. Furthermore, killed bacteria (e.g. autoclaved) can still be detected by PCR.

Sequencing often uses a cloned or amplified gene and oligonucleotides which hybridize to part of the gene, DNA polymerase which copies the gene and nucleotide analogs which randomly stop elongation when adding a given nucleotide type. The result is a family of fragments all ending with a given nucleotide type. These are separated by electrophoresis. Sequences are now read automatically and compared with those contained in databases.

3.1.4 PCR amplification and its applications

PCR involves three phases i.e. denaturing, annealing and polymerization. In the first step of a PCR reaction, the DNA is denatured by heating at 93°C to 94°C so that the strands come apart. In the second step, the reaction is cooled to a lower temperature, typically 50°C to 72°C, such that the primers anneal selectively to the regions, which flank the target site to be amplified. Lastly, the reaction is heated to 72°C to 75°C and the DNA polymerase extends the primers such that a

copy of the target region is synthesized (Alvarez *et al.*, 1993; Schneegurt and Kulpa, 1998). Typically this three steps sequence is repeated for 25 to 35 cycles. Additional cycles, however, will often generate nonspecific products.

The annealing temperature is one of the most important parameters that need adjustment in PCR. Its flexibility allows optimization of the reaction in the presence of variable amounts of other essential ingredients, which, have to be at maximum quantity varying from reaction to reaction depending on the type and volume of the reaction mixture. The use of a thermostable enzyme Taq polymerase, derived from thermophilic bacteria *Thermus aquaticus*, allows the melting of the DNA by elevating the temperature without the denaturing of the enzyme (Schneegurt and Kulpa, 1998).

The essential components of the PCR reaction mixture are Taq polymerase, primers, deoxynucleotide triphosphates (dNTP's), template DNA and magnesium ions (Atlas, 1991). The choice of primer and nucleotide concentration has significant influence on PCR. A high primer concentration increases the probability of spurious priming and leads to the generation of nonspecific products. At the same time, it enhances the generation of primer dimers (Hosta, 1991). A substantial surplus of primer can therefore even result in a reduction of the amplification yield from the PCR target. Magnesium ion concentration has an influence on primer annealing, the melting temperature of the PCR product and product specificity. An excessively high concentration leads to a reduction in stringency, i.e. reaction specificity (Rolfs *et al.*, 1992(a)). The concentration of free Mg^{2+} ions should exceed that of the total dNTP concentration by 0.5-2.5 mM.

Gratacap-Cavallier *et al.*, (2000) demonstrated the use of reverse transcription-PCR (RT-PCR) analysis for the detection of rotaviruses in drinking water. Charpron *et al.*, (2000) worked on detecting enteroviruses, human viruses and adenoviruses types 40 and 41 in surface water samples using an integrated cell-culture (ICC)-RT-PCR procedure coupled with nested PCR. PCR is also applicable in the detection of oocysts from environmental samples. *Cryptosporidium parvum* has been recovered from environmental samples by integrated cell-culture-PCR (DiGiovanni *et al.*, 1999).

Microbial diversity and community structure can be addressed by further examination of the PCR product by various separation techniques and restriction analyses using restriction endonucleases, which are enzymes that cleave DNA at sequence-specific sites (Schneegurt and Kulpa, 1998). As stated above, PCR requires careful optimization of the parameters for maximum effectiveness.

PCR amplification can be used on other fields of study other than detection of pathogens and indicator microorganisms from the environment. It has been used for research in biology, clinical medicine and forensic science. In biological research, PCR contributes in the study of gene function, gene mapping and evolution. In medicine, it is used in prenatal testing for genetic diseases and in forensic science, it has been used to identify criminals.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains and cultures

The following strains were supplied by Dr. W. Edward, Department of Biochemistry and Microbiology to be used as reference cultures for the study, Potchefstroom University: *Eschericia coli* ATCC strain, *Klebsiella pneumoniae, Klebsiella planticolla, Pseudomonas aeuruginonsa, Pseudomonas fluorescens, Bacillus cereus, Staphylococcus aureus* and *Staphylococcus epidermidis. E.coli, Klebsiella spp., Proteus spp., Enterobacter spp., Pseudomonas spp., Citrobacter freundii.,C. diversus, Serratia spp.* and *Aeromonas hydrophila* isolated from water samples were used.

3.2.2 DNA isolation

Known cultures and isolates were grown overnight in LB broth with shaking at 37^{0}C and 1.5 ml of each was microcentrifuged for 2 minutes. Pellet was resuspended in 500µl TE buffer. Five µl of 10mg/ml of lysozyme was added and incubated at 37^{0}C for 30 minutes. Three µl of proteinase K was added and incubated at 56°C for 1 hour. Equal volume of chloroform/isoamyl (24:1) alcohol was added, mixed and microcentrifuged for 5 minutes. The supernatant was transferred to a fresh tube. The mixture was microfuged for a further 5 minutes. The top layer was transferred to fresh microfuge tube. Equal volume of icecold 100% ethanol was added and microfuged for 2 minutes. The ethanol was then poured off and tube air dried for 1 to 2 minutes.

Fifty µl of TE buffer was used to resuspend the DNA and incubated at 60°C for 5 minutes, without shaking. The mixture was cooled to 4°C and microfuged briefly. All DNA preparations were stored at –20°C until used. The concentration of the isolated DNA was determined by UV light absorbence at 260 and 280nm.

DNA was also extraction directly from water sample. Ten microlitres of water sample was filtered through the membrane filter. After filtration, the membrane was cut, dissolved to 500µl of distilled water, boiled for 5 minutes and rapidly cooled on ice. Two microlitres of the template was used for each PCR reaction.

3.2.3 Polymerase Chain Reaction

3.2.3.1 PCR components and amplification profile

PCR reaction mixture (50 µl, total volume) contained reaction buffer (50 mM KCl, 10 mM Tris-HCl [pH 9.0], 4 Mm $MgCl_2$, 0.01% gelatin), 100 pmol each of primers (Table 1): GB-1, GB-2, CF-5 and CF-6, 1 mM $MgCl_2$, 200 µM of each deoxynucleotide triphosphate (dATP, dTTP, dGTTP, and dCTTP), 2 µl (~10 ng) templated DNA, 1.0 U/µl *Taq* DNA polymerase and made up to the final volume with water (PCR grade). The mixture was overlaid with 50 µl of mineral oil to avoid evaporation. Following the preliminary trials with different annealing temperature, different concentrations of $MgCl_2$ were used for optimization.

The multiplex PCR was performed using the PCR Master as described by the manufacturer (Boehringer Mannheim) (See Table 3.1 for details). Two primer sets (GB1/2 and CF5/6) were used. See Table 1 for details. The PCR Master Mixture contained 12.5 µl master mix, 8.5 µl water, 1µl Taq polymerase, 2µl primer mix and 1µl DNA, adding up to 25µl and 25 µl of mineral oil were added:

TABLE 3.1. Primers for the PCR amplification of general bacteria and coliforms

Oligonucleotide sequence	Length (bp)	GC Content (%)	Amplification product size	Reference
CF 5, 5'-ATGCAAAGCCCGACCATGACG-3' CF 6, 5'-GTATCGACCACCACGATGGTT-3'	21 21	57.1 52.4	299	Way *et al.*, 1993
GB 1; 5'-AGACTGCTACGGGAGGCAGCAGT-3' GB 2; 5'-GTTGCGCTCGTTGCGGGACTTAA-3'	23 23	60.9 56.5	755	Villalobo and Torres, 1998

PCR amplification of the target sequence was performed in a DNA thermal cycler (Gene-Tech SPCRI MKII). The reaction mixture was subjected to PCR under the following conditions: denaturation at 94^0C for 5 minutes and then an additional 30 cycles with heat denaturation at 94^0C for 1.5 minutes, primer annealing at 62^0C for 30s, and DNA extension at 72^0C for 1.5 minutes. After the last cycle, samples were maintained at 72^0C for 5 minutes to complete synthesis of all strands.

3.2.4 Gel electrophoresis of the DNA

Fifteen µl of PCR product was mixed with 5µl gel-loading buffer (Appendix C) and loaded into the wells of the gel (Appendix C) using micropipette. A 100-bp molecular weight marker XIV (Roche) was used in all gels. The electrophoresis was carried out in a horizontal tank (ECPS 3000/IJD) at a voltage of 1-5 V/cm for 1 hour or until the desired resolution was obtained. After the electrophoresis was complete, the gel was examined by ultraviolet light (254 nm).

3.2.5 Sensitivity of PCR assay

Sensitivity of PCR amplification was determined by the use of different concentrations of DNA isolated from environmental samples. The DNA was serially diluted in double-distilled water (10^{-1} – 10^{-7}). Aliquots were assayed for amplification with CF 5/6 and GB 1/2 primer sets. The results were further analysed by gel electrophoresis.

3.2.6 Restriction endonuclease digestion

Two restriction endonuclease enzymes: *HinfI* (recognition sequence G/ANTC), and *RsaI* (recognition sequence GT/AC) were used. These enzymes were purchased from (Boehringer Manheim). Each 20 µl reaction contained 10µl PCR product, 1U of enzyme, 1 X PCR buffer and 5.5 µl dd H_2O. The reaction was then incubated at 37^0C for 4 hours. Digestion products were analysed by gel electrophoresis on 1% agarose gels at 1-5 V/cm for 1 hour.

3.3. RESULTS

3.3.1 DNA Extraction

DNA was successfully extracted from supplied strains and environmental samples. DNA concentration of each culture was measured (260 and 280nm) to check for protein contamination. All the readings were above 1.8 when dividing the 260nm reading by 280nm reading.

3.3.2 Optimizing DNA amplification for PCR and multiplex PCR

The parameters that were optimized in this study include annealing temperature and $MgCl_2$. The primers were previously used and showed reproducible results (Way *et al.*, 1993; Vollabalo and Torres, 1998). Way *et al.* (1993) used 62^0C and Vollabalo and Torres (1998) used 55^0C as the annealing temperature. Since PCR conditions between laboratories are not identical e.g. different PCR machines and reagents, it is thus essential to optimize parameters to ensure reproducibility. Determination of the optimal $MgCl_2$ concentration for PCR was carried out by titrating different concentrations of $MgCl_2$ to each PCR reaction (Figure 3.1). The DNA was amplified with GB primers at 55^0C annealing temperature. These primers produce amplicon of 755-bp as predicted by the manufacturer. The results showed that, in our environment, 1mM - 1.75mM $MgCl_2$ showed clear thick band with 15 µl PCR product loaded to each well. The results also showed that the mixture without additional $MgCl_2$ and the mixture with 0.25 mM $MgCl_2$ were not optimal for PCR amplification. The mixture with 0.5 mM $MgCl_2$ showed a very thin faint band.

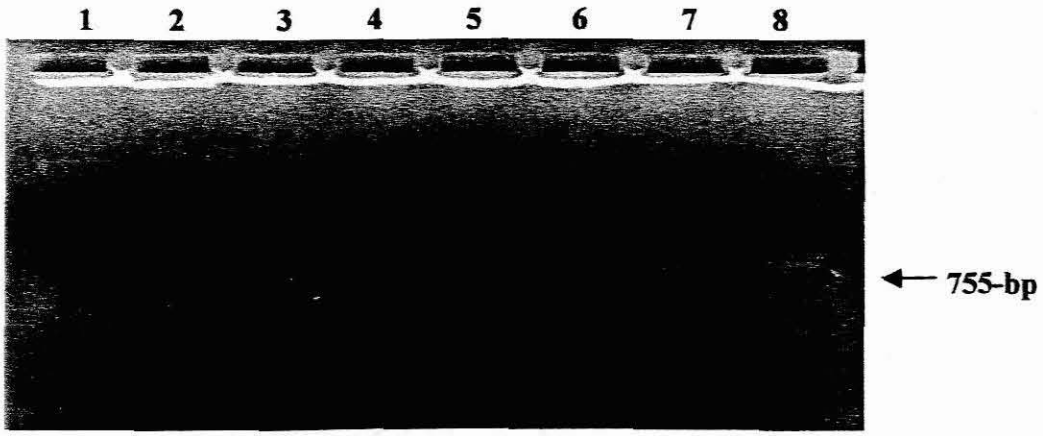


Figure 3.1. Optimization of $MgCl_2$ for PCR assay. The concentration of the $MgCl_2$-stock solution is 25mM. Lanes: 1, 100-bp DNA ladder; 2, 0.25 mM; 3, 0.5 mM; 4, 0.75 mM; 5, 1 mM; 6, 1.25 mM; 7, 1.5 mM; 8, 1.75 mM.

Primer concentration adjusted to 100 pmol yielded positive results. dNTP concentration at 200 μM also gave positive results.

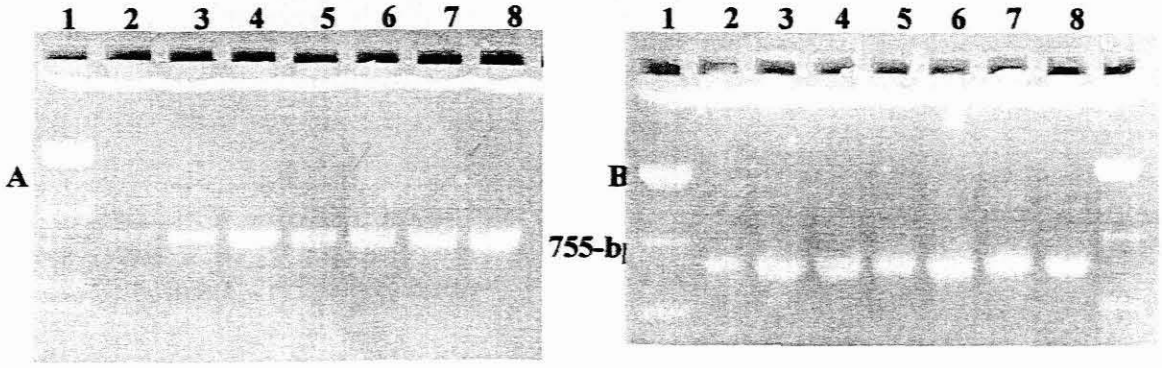


Figure 3.2. PCR amplification of DNA from supplied cultures (A) and environmental isolates (B) with GB primers. (A) Lanes: 1, 100-bp DNA ladder; 2, *E. coli ATCC strain*; 3, *K. planticolla*; 4, *K. pneumoniae*; 5, *P. fluoresens*; 6, *P. aeruginosa*; 7, *B. cereus*; 8, *S. aureus*. (B) Lanes: 1 and 9, 100-bp DNA ladder; 2, *E. coli*; 3, *Enterobacter spp.*; 4, *Klebsiella spp.*; 5, *Serratia spp.*; 6, *Citrobacter freundii*; 7, *Proteus spp.*; 8, *Pseudomonas auruginosa*

Annealing temperature is also an important parameter in achieving optimum results of PCR amplification. In this study, the annealing temperature was performed at 45^0C, 55^0C and 62^0C Annealing temperature at 45^0C gave positive results. Adjusting the annealing temperature to 55^0C was also productive with GB primers. Figure 3.2a shows the supplied cultures amplified with GB primers. The results show that all the strains were successfully amplified with GB primers, at the annnealing temperature of 55^0C, which targets 755bp DNA fragment. Figure 3.2b

shows amplified results of DNA isolated from the environmental water samples, with the annealing temperature of 55^{0}C.

DNA isolated directly from water samples was also used as a template for PCR amplification (Figure 3.3). PCR analysis of PCR products from direct DNA extractions of the five water samples from the Mhlathuze River gave consistent results at 755-bp region, with thicker bands in lanes 4, 5 and 7.

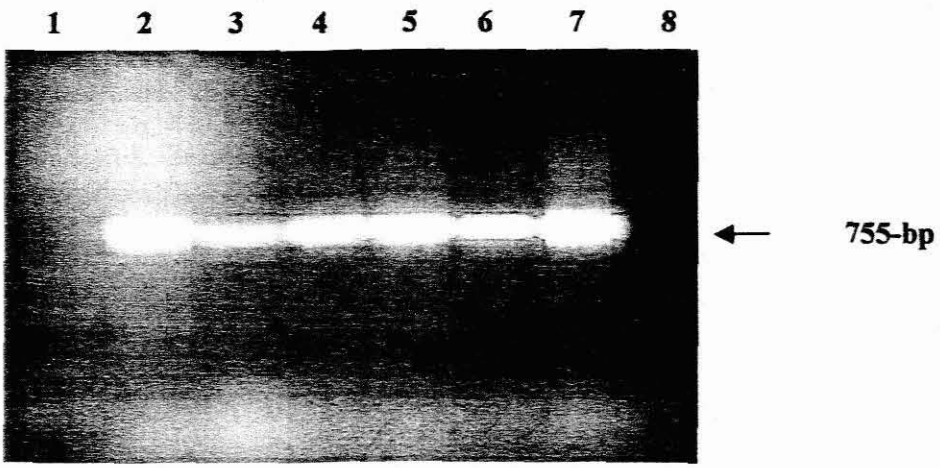


Figure 3.3. PCR amplification of DNA isolated directly from 5 water samples, collected from 5 sites of the Mhlathuze River. Lanes: 100-bp DNA ladder; 2, Site1; 3, Site 2; 4, Site 3; 5, Site 4; 6, Site 5; 7, *E.coli*; 8, blank

Annealing temperature at 55^{0}C resulted in unspecific amplification (fingerprints) when amplified with CF5/6 primers, using Boehringer Mannheim reagents. The PCR Master was able to amplify at 55^{0}C with CF5/6 primers, giving specific products at 299bp region. Figure 3.4a shows PCR analysis of DNA from the environmental samples amplified with CF5/6 primers at annealing temperature of 55^{0}C. Lanes 1 and 8 show 100bp DNA ladder. Lanes 2,4,6 and 7 show two consistent bands at ~299bp and ~250bp regions. At normal conditions the band should be amplified at ~299bp region. Lane 5 shows faint bands at ~299bp and ~320bp regions. Lane 6 has an amplification band at ~750bp region. Annealing temperature was then raised to 62^{0}C.

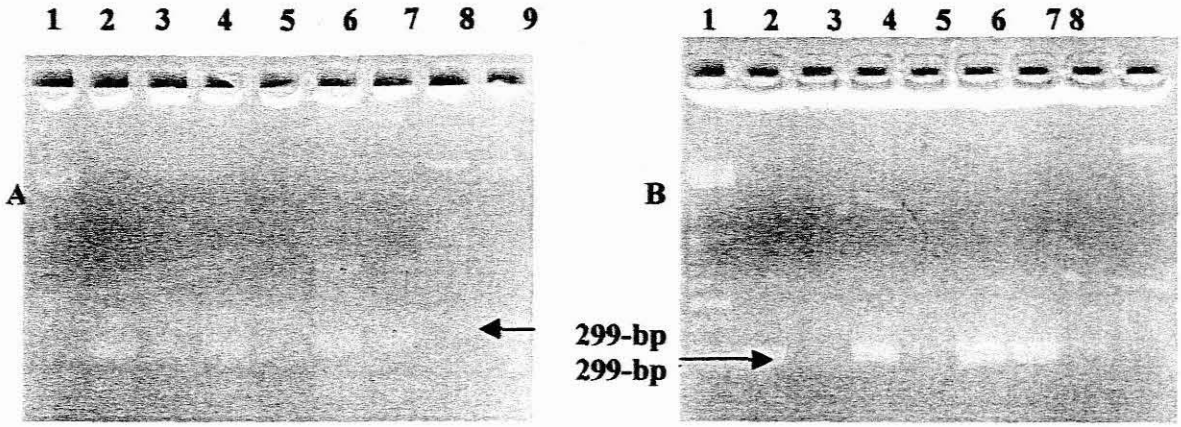


Figure 3.4. PCR amplification of DNA from environmental samples with CF primers at annealing temperatures of 55^{0}C (A) and 62^{0}C (B). A and B Lanes: 1, 100-bp DNA ladder; 2, *E. coli*; 3, *Enterobacter aerogenes*; 4, *E .coli ATCC* strain ; 5, *P. vulgaris*; 6, *C. freundii*; 7, *C. diversus* and 8, *Serratia marcescens*

Figure 3.4b shows PCR results of the PCR products, containing DNA of the environmental water samples, amplified with CF5/6 primers at 62^{0}C annealing temperature. The specific 299-bp phoP band was detected in lanes2, 4, 6 and 7. Lanes 3, 5 and 8 show no amplification. Lane 1 shows the 100bp DNA ladder. The results show that *E.coli* lane 2), *E.coli* strain (lane 4) and *Citrobacter freundii* and *C. diversus* (lanes 6 and 7) gave positive amplification products at 299-bp region. *Enterobacter aerogenes* (lane 3), *Proteus vulgaris* (lane 5), and *Serratia marcescens* (lane 8) resulted in the negative amplification products.

Multiplex PCR was carried out using the PCR Mixture and DNA extracted from environmental isolates. Figure 3.5 shows multiplex PCR results with amplicons of DNA isolated from the environmental isolates. The band 755-bp region was detected in all isolates. The band at 299-bp region was only seen with *E.coli, C. freundii* and *C. diversus*.

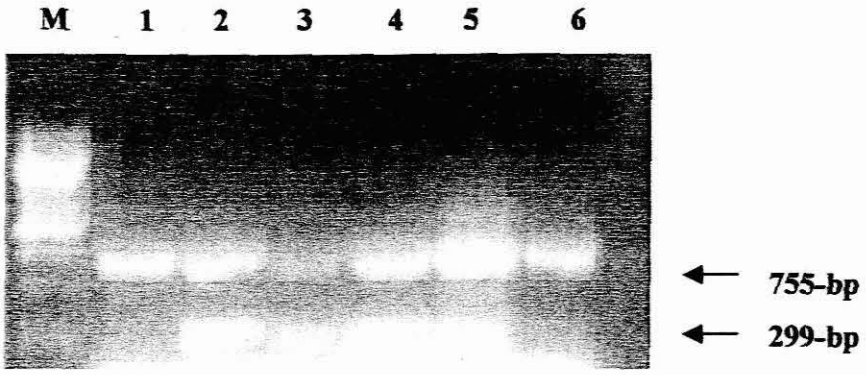


Figure 3.5. Multiplex PCR results for the specificity of *16s rRNA* and *phoP* primers on environmental isolates. Lanes: M, 100-bp DNA ladder; 1, *E. aerogenes*; 2, *E. coli*; 3, *P. vulgaris*; 4, *C. freundii*; 5, *C. diversus* 6, *K. pneumoniae*

3.3.3 Sensitivity of PCR assay

Serial dilutions of DNA from *E.coli* were used to determine the sensitivity of the PCR assay. Figure 3.6 shows the sensitivity of *E.coli* detection with phoP regions. The results show that amplification with phoP primer was visualised up to 10^{-6} concentration of DNA.

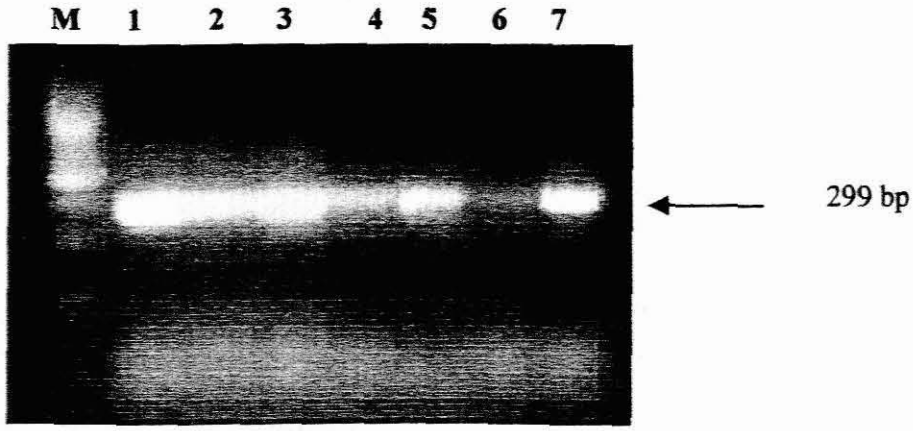


Figure 3.6. Sensitivity of the PCR in amplyfing DNA of *E.coli* with CF primers. Lanes: M, 100-bp DNA ladder; 1, undiluted DNA; 2, 10^{-1} dilution; 3, 10^{-2} dilution; 4, 10^{-3} dilution; 5, 10^{-4} dilution; 6, 10^{-5} dilution; 7, 10^{-6} dilution; 8

3.3.4 Restriction Endonuclease Digestion

Figure 3.7 shows digestion of environmental amplicons (GB primers) with Rsa I restriction enzyme. The results show restrictions on lanes 4 and 5 at ~500-bp region and ~700-bp regions. Lanes 2, 6 and 7, which contains amplicons with DNA of *B. cereus, S. aureus and S. marcescens,* show cut fragments at 500-bp region. There was no restriction fragments detected in lane 3, which has *E. aerogenes*. Figure 3.8 shows digestion of amplicons (GB primers) with Hinf I restriction enzyme the results show cut fragments at ~500-bp region at lanes 3, 4, 5 and 7. Lane 2 shows the band at 755-bp region and lane 6 at 299-bp region (thick band). Double digestion in figure 3.9 demonstrates the sizes of the fragments when cut with Hinf I, Rsa I and both enzymes. Amplicons with DNA from *E.coli* (lanes 2, 3, 4 and 5) and *P. vulgaris* (lanes 6, 7, 8 and 9) were used for this experiment. Lanes 2 and 6 contain PCR products without addition of the restriction enzyme. Thick band at 755-bp was detected in both lanes. Lanes 3 and 7 contain amplicons with Rsa I. The cut fragment is seen at ~500-bp region, retaining its original fragment at 755-bp region. Lanes 4 and 8 shows the restriction fragment at 299-bp. Double digestion is detected in lanes 5 and 9, where Rsa I and Hinf I were used. The cut fragment is seen at ~100-bp region.

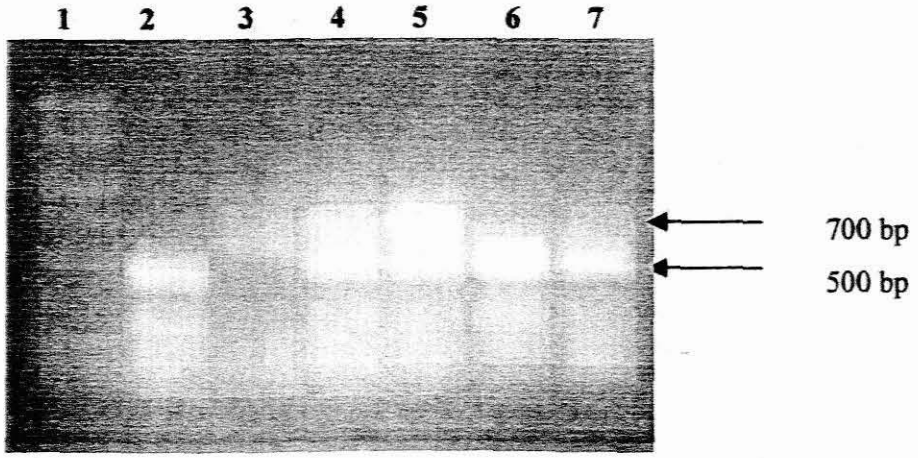


Figure 3.7. Restriction analysis of environmental isolates amplicons with Rsa I restriction enzyme. Lanel, 100-bp DNA ladder; 2, *B. cereus*; 3, *E. aerugenes*; 4, *E. coli*; 5, *P. vulgaris*; 6, *S. aureus* and 7, *S. marcescens*.

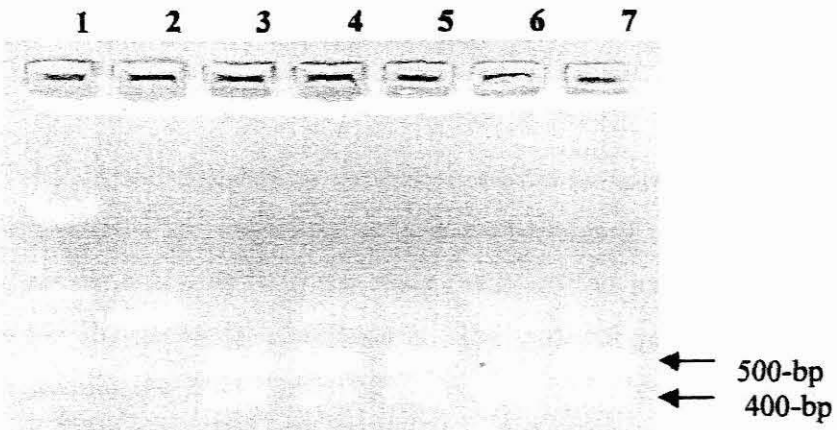


Figure 3.8. Restriction analysis of environmental isolate amplicons with Hinf I restriction enzyme. Lanel, 100-bp DNA ladder; 2, *E. aerogenes*; 3, *P. vulgaris*; 4, *E. coli*; 5, *C. freundii*; 6, *S. marcescens* and 7, *P. aeurugenosa*.

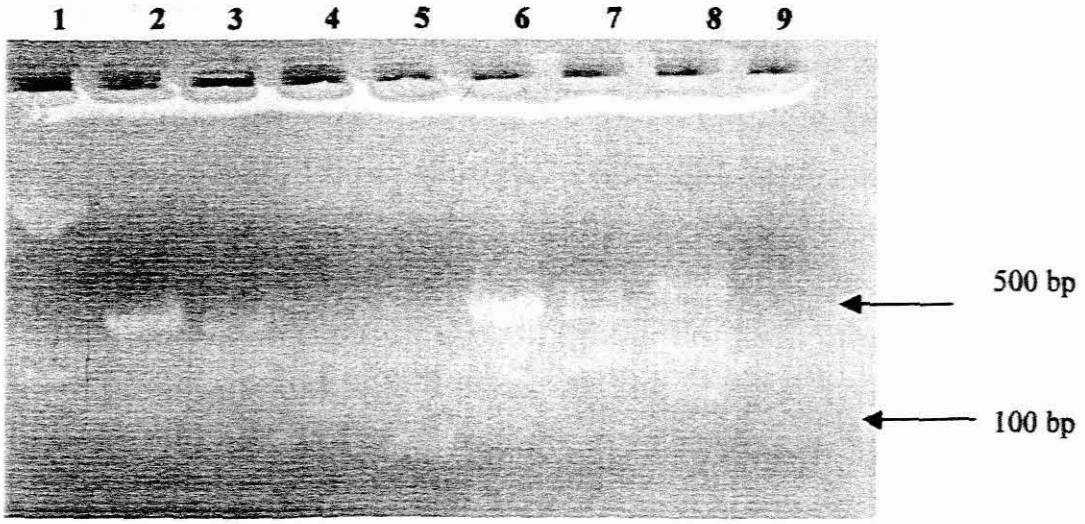


Figure 3.9. Double digestion of *E.coli* and *P. vulgaris* with Rsa I and Hinf I restriction enzymes. Lanes: 1, 100-bp DNA ladder; 2, *E.coli*; 3, *E.coli* digested with Rsa I; 4, *E.coli* digested with HinfI; 5, *E.coli* digested with RsaI and HinfI; 6, *P. vulgaris*; 7, *P. vulgaris* digested with RsaI; 8, *P. vulgaris* digested with Hinf I and 9, *P. vulgaris* digested with Rsa I and Hinf I.

3.4 DISCUSSION

3.1.4 DNA Extraction

For PCR to be applied to the environmental samples to monitor bacteria, target DNA must be recovered. There are many extraction protocols of DNA, differing mainly in lysis efficiency (Leisack *et al.*, 1991). In this study lysozyme was used to disrupt cells and the cellular proteins were digested with proteinase K. The protocol gave good DNA isolations because when measuring the DNA concentration, with UV light absorbency at 260nm and 280nmm, the ratio (A_{260}: A_{280}) was greater that 1.1. See Appendix D, Table 2.

3.4.2 Optimization

Optimization focused on levels of primers, DNA polymerase and $MgCl_2$ in the reaction mixture as well as thermal cycler programs. To test the influence of $MgCl_2$ in our experiment, a PCR with the mixture (refer to *materials and methods*), keeping the dNTP concentration at 200μM each and gradually increasing $MgCl_2$, by intervals, from 0 to 1.75mM (Figure 3.1). The overall amplification became gradually more specific and the products acquired comparable intensities at 1mM. Corless *et al.*, (2000) used 6mM $MgCl_2$, Sabat *et al*, (2000) used 2mM $MgCl_2$, and Ke *et al.*, (1999) used 2.5mM $MgCl_2$ and all of them keeping the dNTP concentration at 200μM each. This shows that the optimum concentration of $MgCl_2$ varies, depending on the individual experiment. It has been observed that $MgCl_2$ concentration has an influence on primer annealing, the melting temperature of the PCR product and product specificity (Rolfs *et al.*, 1992(a)). An excessively high concentration of $MgCl_2$ leads to a reduction in stringency, i.e. reaction specificity. Figure 3.1 also shows that in lane 8 (1.75mM) there are two faints bands detected (above 755-bp region). This shows the unspecific products with an increase in $MgCl_2$ concentrations.

The choice of primer and nucleotide concentration has a significant influence on PCR. In this study the primer concentration adjusted to 10 ng gave positive results with dNTP concentration at 200μM and $MgCl_2$ of 1mM. Figures 3.2 and 3.3 depict specific bands at 755 bp region. The figures show that DNAs were successfully isolated from the water samples and also from the

supplied cultures. The figures also show the optimal conditions that were used gave the best balance and selectivity at 10ng of primer concentration. Figures 3.2 and 3.3 also gave positive PCR products with GB primers. This was true since this primers should produce an amplification product at 755 bp if there is a presence of bacteria (Villalobo and Torres, 1998). The DNAs from cultures supplied by the laboratory were used to show the effect of amplification with GB primers and thus comparing the results with those of PCR products in the presence of DNA from the environmental isolates.

In achieving highly specific and sensitive detection of a target bacterium, the quality and the amount of the DNA is important (Saruta *et al.*, 1997). Figure 3.6 shows that as the concentration of the DNA decrease, the amplification that is depicted by the thickness of the bands also decreases. The sensitivity of the assay, which is up to 10^{-6} dilutions, shows that the assay used in this study can detect low levels of *E.coli* and this shows high degree of sensitivity.

In general, an initial denaturation process of a few minutes duration is carried out in order to insure complete denaturation of the target regions on the DNA. After the first PCR products have been synthesised, considerably shorter denaturation times can be selected in the cycles, since only a very short denaturation time is actually required (Rolfs *et al.*, 1992(b)). In this study initial denaturation was allowed for 2 minutes and heat denaturation in 30 cycles was adjusted to 1.5 minutes. There's evidence that template DNA and PCR products are degraded by extensive denaturation times (Douglas and Atchinson, 1993). Such heat damage in the DNA can, during PCR, result in a higher rate of error for nucleotide insertion (Eckert and Kunkel, 1991). Furthermore, extensive denaturation times result in a substantial loss of polymerase activity. The denaturation temperature is quite critical and there is a very little scope of deviation from 95^0C. If the temperature is high, the half-life of the *Taq polymerase* decreases drastically. In our experiments 94^0C was used as an optimum temperature and yielded positive PCR products.

Annealing temperature is one of the most important parameters that need adjustment in the PCR reaction. The flexibility of this parameter allows optimization of the reaction in the presence of variable amounts of other ingredients, especially template DNA. The PCR products depicted in figure 3.4 were amplified at annealing temperature of 55^0C and 62^0C. The figure shows that adjusting the annealing temperature to 62^0C yields good sensitivity than at 55^0C. Adjusting the

annealing temperature, for better results, has also been proven by Sabat *et al.,* (2000). They were trying to improve PCR selectivity by increasing the annealing temperature to 72^0C. The annealing temperature that was used for the experiment showed to be optimal because it did not resulted in linear amplification caused by non-specific annealing to the 16S rRNA gene.

Annealing time is also important. During annealing phase the primers are rapidly hybridized. This operation is completed within a few seconds. Very long annealing times normally do not improve yield, but rather produce an increase in spurious priming and, thus, greater amounts of nonspecific PCR products (Charlieu, 1994). In our experiments primer annealing was carried out for 30 seconds. Pabbaraju *et al.,* (2000) used annealing temperature of 56^0C for 1 minute, Meng *et al.,* (1996) used 60^0C for 1 minute and Sabat *et al.,* (2000) used 72^0C annealing temperature for 45 seconds. I personally think that annealing time depends on the individual experiment.

Extension temperature was kept at 72^0C as most authors did (Sabat *et al.,* 2000, Meng *et al.,* 1996 and Pabbaraju *et al.,* 2000). Addition of light mineral oil to the top of liquid reaction is customary. This is done to prevent fluid and heat loss due to evaporation. Evaporation and recondensation of the liquid (known as refluxing) can cause a temperature drop of a few degrees, which may cause a large decrease in efficiency of the denaturation step (Rolfs *et al.,* 1992(b)).

Multiplex PCR was also done with the annealing temperature of 55^0C using the PCR Master (Figure 3.5). The GB and CF primers were used for amplification. Greisen *et al.,* (1994) also used 16s rRNA gene sequence as one set of the primer for multiplex PCR. This sequence has also been noted to be a good target choice for multiplex PCR, since it contains conserved as well as variable domains, which can be exploited to generate family or group specific amplification (Greisen *et al.,* 1994). Multiplex PCR was performed using the PCR Master since the Boehringer Mannheim reagents gave negative PCR amplification at 55^0C annealing temperature with CF primers. Conducting multiplex PCR has some advantages. It saves considerable amounts of time and reagents, since only a single reaction must be set up and analyzed.

3.4.3 Restriction endonuclease digestion

Hinf I and Rsa I isolated from *Haemophilus influenza* R_f and *Rhodopseudomonas sphaeroides* were used for digestion to able to differentiate the isolates through restriction fragment sizes detected by gel electrophoresis. Figure 3.7 and 3.8 showed separation of Hinf I and Rsa I of supplied cultures and environmental samples on an 1% agarose gel. Fragment sizes of Rsa I at 500-bp and 700-bp regions in lanes with *E. coli* and *P. vulgaris*. For *P. aeruginosa* (lane 7)the fragment seen at 700-bp region. This shows that *E. coli* and *P. vulgaris* have same nucleotide sequences that can be cut by Rsa I enzymes, thus making it difficult to differentiate them using the Rsa I enzyme. Restriction with Hinf I (figure 3.9) showed restriction fragments at 500-bp and 400-bp regions for *P. vulgaris, E. coli, C. freundii* and *P. aeroginosa*. The restriction fragment at ~700-bp region was detected for *E. coli*. *P. aeroginosa* showed restriction fragment at ~700-bp region. *S. marsescens* showed thick fragment at 400-bp region and the other one at 200-bp region. This showed that *P. vulgaris* and *E. coli* can be differentiated by appearance of the fragment at 700-bp region.

CHAPTER 4

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

Although some of the findings of this study have already been discussed in chapters 2 and 3, the main findings are summarised in this chapter are placed in the context of the original study objectives. The original study objectives, stated in chapter 1, were: to evaluate the accuracy of enumeration and to detect microbial pathogens in water supplies of the Mhlathuze catchment and its surrounding area; to estimate the occurrence of any water-borne disease outbreaks in this area; to determine seasonal patterns of human pathogens and indicator microorganisms and to provide useful baseline data for further research.

4.1 SUMMARY OF FINDINGS

The research was based on monitoring the Mhlathuze River using physico-chemical analysis. In general, the pattern of distribution of bacteria (HPC, TC and FC) observed in the Mhlathuze River followed the seasonal trend. Fluctuations of bacteria showed a seasonal distribution with high values in summer and low indices in winter. Faecal coliforms isolated included *E. coli*, *Enterobacter spp.*, *Proteus spp.*, *Serattia spp.*,and *Klebsiella spp. Pseudomonas spp.* and *Aeromonas hydrophila* were also isolated. These organisms are normally isolated from environmental water samples. There were high levels of bacteria contamination in the Mhlathuze River, during the period of study.

The elevation of bacterial contamination from 1998 – 1999 raised some questions on the outbreak of cholera in this region, which was first isolated in this area. The questions being: Is this increase of bacterial counts has a relationship with the sudden outbreak of cholera bacteria, and if so, are health or water authorities doing the enough job to prevent the outbreak and is there enough equipment and rapid methods to detect and stop this dangerous bacteria from spreading. The outbreak is reported to have spread from KwaZulu-Natal to Mpumalanga and Gauteng provinces. *Vibrio cholera* is known to enter the viable but non-culturable state. All this years the

bactria might have entered the VBNC state. Last year, since an increase of bacteria was marked, maybe there were high levels of nutrients and also temperature supported the bacterial increase. These conditions might have been variable or suitable for the culturability of *Vibrio cholera*.

Molecular biology method, polymerase chain reaction (PCR), was used in this study to further identify coliforms in the Mhlathuze River. Molecular biology methods, especially gene-based assays, provide information about the physiological properties of cells but can be only considered reliable indicators of culturability under highly defined conditions (Barer and Harwood, 1999). Primers of coliforms and general bacteria were used and amplification was detected at specific regions. The results confirmed the presence of coliforms in the Mhlathuze River.

4.2 CONCLUSIONS

The conclusion reached in this short study is that the levels of heterotrophic bacteria, total and faecal coliform counts were very high, exceeding the standard limits that are used for evaluating the sanitary quality of drinking water. Since the communities from rural areas utilise these water resources, untreated water directly from the river, the main objective is to prevent pathogens in faecal material from reaching them. The control of faecal pollution through sewerage and full wastewater treatment remains the basic step. This will insure unpolluted drinking water sources. The government also has to make provision of supplying clean water to these areas through taps installation.

The outbreak of cholera has claimed many lives of people in this region and throughout the whole country. The community needs to be protected against this outbreak. Educational programmes to create an awareness of the dangerous bacteria and also faecal contamination of water are also necessary. Water should also be treated to inactivate the viability of microorganisms present in the system. The data presented by this study may be used to determine the levels of bacteria present in the Mhlathuze River.

Sensitive and specific techniques need to be used for the detection of either pathogens or indicator microorganisms. This calls for the necessity of combining traditional and molecular techniques, using them conjointly, in order to determine the real public health significance of laboratory findings.

4.3 RECOMMENDATIONS FOR FUTURE RESEARCH

1. The use of CF primers in this study was advantageous that coliforms were detected. Since positive results were found during the period of study, it is recommended that further research be done to detect viruses or cyst-forming protozoans as indicator microorganisms.
2. Hinf I and Rsa I restriction enzymes did not differentiate clearly the isolates because some isolates gave same fragment sizes for both enzymes. It is thus recommended that for digestion to be able to differentiate the microorganisms through restriction fragment sizes, several restriction enzymes should be used.
3. It is also recommended that the isolates be tested for antibiotic resistance to determine whether the isolates can be inhibited by the antibiotics.

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APPENDIX A

AVERAGE COUNTS OF HETEROTROPHIC BACTERIA (HPC), TOTAL COLIFORMS (TC) AND FAECAL COLIFORMS (FC) IN MHLATHUZE WATER SAMPLES, MARCH 1998 TO NOVEMBER 1999

Month	*Indicator bacteria*	S1	S2	S3	S4	S5
Mar'98	HPC	17.4×10^4	16.1×10^4	10.1×10^4	14.1×10^4	10.8×10^4
	FC	11×10^2	12×10^2	4×10^2	8×10^2	8×10^2
	TC	25×10^2	28×10^2	10×10^2	32×10^2	32×10^2
	MPN Index	8750	3625	2425	2775	2425
Apr'98	HPC	10.2×10^4	6.5×10^4	2.3×10^4	9.2×10^4	8×10^4
	FC	9×10^2	8×10^2	-	8×10^2	6×10^2
	TC	20×10^2	14×10^2	-	22×10^2	14×10^2
	MPN Index	7000	3000	3000	2800	1700
May'98	HPC	17×10^4	15.4×10^4	1.7×10^4	16.4×10^4	13×10^4
	FC	8×10^2	24×10^2	1×10^2	4×10^2	2×10^2
	TC	29×10^2	28×10^2	10×10^2	14×10^2	36×10^2
	MPN Index	8000	4225	2500	24500	1700
Jun'98	HPC	8.7×10^4	7.3×10^4	10×10^4	10.1×10^4	7.95×10^4
	FC	7×10^2	7×10^2	-	11×10^2	4×10^2
	TC	21×10^2	17×10^2	-	22×10^2	20×10^2
	MPN Index	3200	2800	350000	3400	2950
Jul'98	HPC	2.6×10^2	2.7×10^2	49×10^2	3.4×10^2	2.8×10^2
	FC	4×10^1	4×10^1	1×10^1	6×10^1	5×10^1
	TC	11×10^1	10×10^1	3×10^1	15×10^1	30×10^1
	MPN index	ND	ND	ND	ND	ND
Aug'98	HPC	76	30	21	53	69
	FC	4	1	-	6	10
	TC	20	8	-	8	13
	MPN index	ND	ND	ND	ND	ND
Sep'98	HPC	26.4×10^1	8.4×10^1	19	7.9×10^1	22.2×10^1
	FC	5	4	-	2	3
	TC	17	19	-	7	11
	MPN index	500	750	50	700	450
Oct'98	HPC	3.5×10^2	7.6×10^2	3×10^2	11.1×10^2	5.1×10^2

	FC TC MPN index	7 24 100	5 13 200	- - 50	8 13 250	16 21 600
Nov'98	HPC FC TC MPN index	9.45×10^2 11 16 800	8.1×10^2 13 32 800	1.1×10^1 - 1 250	2.8×10^2 8 19 950	3.4×10^2 17 46 1100
Jan'99	HPC FC TC MPN Index	2.26×10^6 15×10^2 74×10^2 3000	2.2×10^6 14×10^2 76×10^2 1850	3.7×10^3 5×10^2 25×10^2 355	2.19×10^6 11×10^2 90×10^2 1500	4.53×10^6 83×10^2 36×10^2 9000
Feb'99	HPC FC TC MPN Index		3.86×10^6 19×10^2 88×10^2 2200	3.5×10^3 5×10^2 38×10^2 500	3.94×10^6 14×10^2 81×10^2 1300	4.7×10^6 91×10^2 80×10^2 9000
Mar'99	HPC FC TC MPN Index	2.1×10^6 12×10^2 24×10^2 2400	3.08×10^6 13×10^2 29×10^2 1600	2.5×10^6 - 5×10^2 280	1.67×10^6 8×10^2 17×10^2 1100	3.81×10^6 20×10^2 39×10^2 9000
Apr'99	HPC FC TC MPN Index	3.1×10^5 10×10^2 20×10^2 1700	8.8×10^5 15×10^2 24×10^2 1300	1.06×10^3 - 2×10^2 220	2.8×10^5 8×10^2 21×10^2 900	8.85×10^5 18×10^2 30×10^2 3000
May'99	HPC FC TC MPN Index	14×10^4 8×10^2 15×10^2 1100	14.4×10^4 9×10^2 17×10^2 1100	2×10^3 - - 140	18.1×10^4 2×10^2 17×10^2 700	21×10^4 10×10^2 24×10^2 2400
Jun'99	HPC FC TC MPN index	8.9×10^4 6×10^2 11×10^2 ND	2.4×10^4 5×10^2 10×10^2 ND	14×10^2 - - ND	2.7×10^4 5×10^2 8×10^2 ND	2.01×10^4 3×10^2 12×10^2 ND
Jul'99	HPC FC TC MPN index	2.9×10^2 10 21 ND	5.2×10^2 6 14 ND	21×10^2 - - ND	5.6×10^2 6 17 ND	8.7×10^2 19 43 ND
Aug'99	HPC FC TC MPN	3.4×10^2 12 35 ND	7.4×10^2 17 44 ND	1.5×10^1 5 8 ND	8.1×10^2 13 41 ND	9.3×10^2 18 57 ND

	index					
Sept'99	HPC FC TC MPN index	2.6×10^3 15×10^1 34×10^1 1300	2.8×10^3 19×10^1 41×10^1 220	2.1×10^2 7×10^1 11×10^1 1100	3.1×10^3 21×10^1 56×10^1 1400	3.5×10^3 21×10^1 50×10^1 1400
Oct'99	HPC FC TC MPN index	5.5×10^4 21×10^2 35×10^2 1400	6.1×10^4 32×10^2 43×10^2 1700	6.1×10^3 10×10^2 15×10^2 220	7.8×10^4 32×10^2 56×10^2 2200	8.9×10^4 44×10^2 56×10^2 2800
Nov'99	HPC FC TC MPN index	7.6×10^4 33×10^2 48×10^2 1400	8.2×10^4 44×10^2 57×10^2 2200	7.2×10^3 15×10^2 21×10^2 350	8.8×10^4 41×10^2 62×10^2 2400	9.7×10^4 48×10^2 59×10^2 2800

APPENDIX B

AVERAGE RESULTS OF CHEMICAL AND PHYSICAL ANALYSIS 1998

Month	Sample	T°C	pH	Turbidity (NTU)	Diss.O_2 Mg/l	Hardness	Ortho-P	Total-P
Mar'98	S1	26.5	7.29	5	8.82	86.5	0.3	0.45
	S2	26	7.49	5	8.7	61.5	0.3	0.35
	S3	27.5	7.31	1	8.87	>1000	0.1	0.15
	S4	25.5	7.52	5	8.7	92.5	0.3	0.3
	S5	27.7	7.53	5	8.61	88	0.38	0.4
Apr'98	S1	25	7.38	5		124	0.35	0.41
	S2	25	7.72	5		120	0.3	0.39
	S3	23	7.71	1		>1000	0.19	0.2
	S4	25	8.27	5		168	0.30	0.36
	S5	24	8.29	5		162	0.31	0.35
May'98	S1	17	8.02	5	9.52	120	0.28	0.39
	S2	18	8.22	5	8.31	124	0.28	0.41
	S3	24	8.23	1	9.05	>1000	0.07	0.13
	S4	20	8.55	5	9.05	140	0.25	0.3
	S5	21	8.58	5	9.02	130	0.25	0.33
Jun'98	S1	17	7.80	5		122	0.25	0.365
	S2	18	8.10	5		122	0.26	0.415
	S3	24	7.95	1		>1000	0.00	0.000
	S4	20	7.93	5		152	0.27	0.39
	S5	21	8.03	5		140	0.265	0.375
Jul'98	S1	19	8.30	5	9.31	120	0.091	0.101
	S2	19	8.45	5	8.47	124	0.84	0.193
	S3	15	8.02	1	8.53	>1000	0.003	0.005
	S4	18	8.70	5	8.93	146	0.079	0.126
	S5	19	8.40	5	8.97	146	0.093	0.104
Aug'98	S1	16	8.13	5	ND	146	0.016	0.048
	S2	15	8.73	5	ND	112	0.034	0.041
	S3	17	8.41	1	ND	>1000	0.003	0.032
	S4	15.5	8.72	5	ND	150	0.002	0.036
	S5	16.5	8.01	5	ND	162	0.019	0.038
Sept'98	S1	20	8.13	5	ND	132	0.013	0.019
	S2	19.5	8.45	5	ND	110	0.007	0.014
	S3	18	8.1	1	ND	>1000	0.000	0.000
	S4	19.3	8.3	5	ND	164	0.004	0.014
	S5	19.7	8.4	5	ND	132	0.009	0.010
Oct'98	S1	21	7.47	5	ND	137	0.015	0.027
	S2	21	7.68	5	ND	114	0.023	0.023
	S3	20.5	7.70	1	ND	>1000	0.031	0.012
	S4	20.5	7.82	5	ND	150	0.010	0.028

	S5	21.5	8.00	5	ND	159	0.014	0.021
Nov'98	S1	27	8.25	5	ND	154	0.015	0.024
	S2	28	8.47	5	ND	134	0.039	0.035
	S3	29	8.09	1	ND	>1000	0.046	0.005
	S4	26	8.5	5	ND	172	0.013	0.038
	S5	28.5	8.4	5	ND	172	0.014	0.021

ND = not determined

AVERAGE VALUES OF CHEMICAL AND PHYSICAL ANALYSIS 1999

MONTH	Sample	T^0C	pH	Hardness	Ortho-P	Total-P
Jan'99	1	34.5	6.25	82	0.000	0.052
	2	34	6.16	52	0.015	0.024
	3	35	6.45	>1000	0.000	0.018
	4	33	7.00	90	0.031	0.413
	5	35	6.95	94	0.000	0.043
Feb'99	1					
	2	27	7.8	80	0.000	0.014
	3	29.5	7.29	>1000	0.000	0.005
	4	26.5	7.6	80	0.000	0.083
	5	26.5	7.53	88	0.000	0.025
Mar'99	1	24	6.83	92.5	0.026	0.028
	2	28	7.56	90	0.000	0.009
	3	26.5	7.65	>1000	0.006	0.016
	4	26	7.77	120	0.009	0.012
	5	28.5	7.78	120	0.018	0.019
Apr'99	1	19	5.76	124	0.001	0.004
	2	21	6.26	120	0.009	0.018
	3	24	6.67	>1000	0.005	0.018
	4	21.5	6.95	130	0.004	0.013
	5	23	6.50	140	0.010	0.028
May'99	1	19	7.67	132	0.180	0.220
	2	21.5	7.90	110	0.110	0.250
	3	22	7.61	>1000	0.041	0.170
	4	21	7.87	146	0.144	0.280
	5	22.5	7.67	154	0.154	0.290
Jun'99	1	14	7.78	140	0.026	0.038
	2	15	7.98	140	0.006	0.023
	3	22	7.54	>1000	0.017	0.019
	4	18	7.95	164	0.013	0.028
	5	19	7.81	154	0.014	0.032
Jul'99	1	19	8.11	146	0.000	0.048
	2	22	8.48	132	0.030	0.054
	3	23.5	8.41	>1000	0.008	0.053

	4	21	7.98	146	0.014	0.064
	5	23	7.98	146	0.041	0.062
Aug'99	1	24	8.01	162	0.000	0.028
	2	26.5	8.34	152	0.000	0.009
	3	26.5	7.98	>1000	0.000	0.010
	4	25	7.80	156	0.000	0.030
	5	28	7.56	168	0.000	0.052
Sept'99	1	23	7.95	162	0.002	0.010
	2	25	7.89	154	0.013	0.016
	3	24.5	7.65	>1000	0.009	0.010
	4	23	7.66	1172	0.008	0.014
	5	23	7.65	172	0.012	0.017
Oct'99	1	24	7.99	168	0.011	0.019
	2	26	7.95	162	0.014	0.021
	3	28	6.91	>1000	0.012	0.024
	4	25	6.71	178	0.026	0.031
	5	25	6.83	182	0.023	0.041
Nov'99	1	26	7.66	172	0.028	0.047
	2	29	7.78	162	0.025	0.053
	3	28	7.45	>1000	0.013	0.023
	4	24	7.03	182	0.031	0.052
	5	26	7.09	188	0.040	0.055

APPENDIX C

Ortho-phosphate

Reagents: 5N H_2SO_4 – (70ml/500ml)
Potassium antimony tartrate – (1.3715g/500ml)
Ammonium molybdate – (20g/500ml) – stored in plastic at 4°C
Ascorbic acid – 0.1 molar – stored at 4°C

Method: Combination reagent in sequence mix:
50ml H_2SO_4
50ml Potassium antimony tartrate
15ml Ammonium molybdate
30ml Ascorbic acid

a) 50ml sample filtered.
b) 8ml combination reagent.
c) Wait 10 minutes (<30 mins).
d) Read at 880nm spectrophotometer.

Standards: 500mg/l PO_4
0.7165g KH_2PO_4/100ml
50mg/ PO_4
10ml of 500mg/l/100ml
Working stds: 1mlof 50mg/l/50ml
1mg/l PO_4
NB: Prepare blank and standards

TOTAL PHOSPHATE

Reagents: a) Ammonium molybdate – 9.15g/500ml
b) Acid-bismuth-nitrate – 191ml HNO_3 + 1.5g bismuth nitrate made up to 500ml.
c) Combination reagent – mix a) + b) in equal volumes as needed.
d) Reducing solution – 0.375g 1-amino-2napthol-4sulphonic + 34.925g sodium meta-bisulphite + 20.975g sodium sulphite to total volume of 500ml.

Standards: 500mg/l = 1.8855g sodium hydrogen phosphate /100ml
2mg/l = 4ml of 500mg/l/1000ml

Method: a) 2ml sample or standard in test tube.
b) 2ml combination reagent.
c) Boil for 10 minutes at 100°C in water bath.
d) Remove and cool.
e) Add 10ml distilled water.
f) Add 1ml reducing solution.
g) Let stand for 10 minutes (<30 mins).
h) Read on spectrophotometer at 690nm
i) Prepare blank using distilled water.

NB: PO_4 = 30.97376 + (15.9994)4 = 94.9714g

P X 3.07 = PO_4

PO_4 / 3.07 = P

Determination of total hardness

Reagents

Buffer solution

Dissolve 16.9g ammonium chloride (NH_4Cl) in 143 ml concentrated ammonium hydroxide (NH_4OH) and dilute with distilled water to 250ml.

Indicator: Eriochrome Black T

Dissolve 0.5g of the above in 100ml 95% ethyl alcohol or methyl alcohol. Place the solution in a dropper. The solution is not stable for an unlimited period and as soon as a definite end point is no longer attained, a fresh solution has to be prepared.

Standard 0.01 molar EDTA (0.01 mol/l)

The reagent (ethylenediaminetetraacetic acid disodium salt) is known by other names as well, for example Versenate and Titriplex III. Standard solutions are available commercially which may be made up to the required strength or 3.723g EDTA may be dissolved in a little distilled water and diluted further to 1 l.

1 ml EDTA = 1.00 mg hardness as $CaCO_3$

TABLE 1: MPN index and 95% confidence limits for various combinations of positive results when various numbers of tubes are used per dilution (10 ml, 1.0 ml, 0.1 ml)

		95% CONFIDENCE LIMITS	
COMBINATION OF POSITIVES	MPN INDEX 100 ml	LOWER	UPPER
0-0-0	<2	-	-
0-0-1	2	1.0	10
0-1-0	2	1.0	10
0-2-0	4	1.0	13
1-0-0	2	1.0	11
1-0-1	4	1.0	15
1-1-0	4	1.0	15
1-1-1	6	2.0	18
1-2-0	6	2.0	18
2-0-0	4	1.0	17
2-0-1	7	2.0	20
2-1-0	7	2.0	21
2-1-1	9	3.0	24
2-2-0	9	3.0	25
2-3-0	12	5.0	29
3-0-0	8	3.0	24
3-0-1	11	4.0	29

3-1-0	11	4.0	29
3-1-1	14	6.0	35
3-2-0	14	6.0	35
3-2-1	17	7.0	40
4-0-0	13	5.0	38
4-0-1	17	7.0	45
4-1-0	17	7.0	46
4-1-1	21	9.0	55
4-1-2	26	12	63
4-2-0	22	9.0	56
4-2-1	26	12	65
4-3-0	27	12	67
4-4-0	33	15	77
	34	16	80
5-0-0	23	9.0	86
5-0-1	30	10	110
5-0-2	40	20	140
5-1-0	30	10	120
5-1-1	50	20	150
5-1-2	60	30	180
5-2-0	50	20	170
5-2-1	70	30	210
5-2-2	90	40	250
5-3-0	80	30	250
5-3-1	110	40	300
5-3-2	140	60	360
5-3-3	170	80	410
5-4-0	130	50	390
5-4-1	170	70	480
5-4-2	220	100	580
5-4-3	280	120	690
5-4-4	350	160	820
5-5-0	240	100	940
5-5-1	300	100	1300
5-5-2	500	200	2000
5-5-3	900	300	2900
5-5-4	1600	600	5300
5-5-5	>=1600	-	-

APPENDIX D

TAE Buffer

50X: 242g Tris base

57.1 ml acetic acid

100 ml 0.5M EDTA (pH 8.0)

0.5M EDTA pH 8.0

For 1 litre dissolve 186.1g of disodium ethylenediamine tetraacetate-$2H_2O$ in 800 ml H_2O. Add approximately 20g of NaOH pellets to adjust the pH. Sterilize by autoclaving.

Note: EDTA will not dissolve until the pH is close to 8.0.

Preparation of an Agarose Gel

To prepare 1% agarose, 1g agarose was dissolved in 100ml distilled water by heating in a microwave oven until the agarose dissolves. The solution was cooled to 45^0C and 2ml of 50 X buffer was added into the solution. 5µl of 0.5 µg/ml ethidium bromide was also added. The open ends of the plastic tray supplied with the electrophoresis apparatus were sealed with a tape. The gel was poured in the plastic tray and the comb 0.5 – 1.0mm was positioned above the tray so that a complete well is formed when agarose is added. The gel should be between 3 mm and 5 mm thick because thinner gels are more difficult to cast and handle. After the gel is set, the comb and the tape from the tray are removed, mounting the gel in the electrophoresis tank. The electrophoresis buffer, 1 X TAE, is filled in the electrophoresis tank to cover the gel to the depth of about 1 mm.

Table 2: Concentrations of DNA isolated from the environmental samples

Sample no.	Dilutions	260nm	280nm
2 *(E.coli)*	10^{-3}	0.0848	0.0470
3 *(Citrobacter freundii)*	10^{-4}	0.0395	0.0228
4 *(Proteus vulgaris)*	10^{-3}	0.0633	0.0352
6 *(Serratia marsescens)*	10^{-3}	0.0725	0.0425

7 *(Enterobacter aerogenes)*	10^{-3}	0.3675	0.1662
8 (*Klebsiella pneumoniae.)*	10^{-3}	0.0623	0.0342
9 (Pseudomonas aeruginosa)	10^{-4}	0.0447	0.0250
10 (*Aeromonas hydrophila.*)	10^{-3}	0.0185	0.0106