MICROBIAL COMMUNITIES AND WATER QUALITY IN THE MHLATHUZE RIVER

J Lin

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Microbial Communities and Water Quality in the Mhlathuze River

Report to the Water Research Commission
on the Project:
"A Study of the Microbial Communities and Related Water Quality of the Mhlathuze River"

by

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EXECUTIVE SUMMARY

Background

The Mhlathuze catchment supports a rapidly growing agricultural and industrial community surrounding the Empangeni/Richards Bay area in northern KwaZulu Natal. With increasing demands on water resources and contamination from industrial waste and human activities, the potential for the outbreak of water-borne diseases continues to grow. Constant monitoring of the water quality and proper resource management is crucial for the future development of the Mhlathuze catchment . In response to this need, the project leader initiated several water-related projects since 1998. Preliminary results show that untreated water from the Mhlathuze River is not suitable for human consumption. The problem is aggravated in the summer period. High water surface temperature appears to be critical to the elevated microbial population levels in general and indicator microorganisms in particular. More studies are needed to evaluate in detail, the current status of this river. Without sufficient information on all the factors contributing to, and affecting the water resource, it will be impossible to assess the isolated impact of the industrial, agricultural and human activities on the quality of the water. It is therefore urgent to monitor the microbial status of this water resource (Mhlathuze River) in order to determine the impact of current and future developments (industrial, agricultural, etc.) within the catchment area and along the banks of the river. Such information may be useful to predict the impact of future developments.

This project attempted to constantly monitor the microbial safety of the water supplies and to accumulate data on the survival and transmission of indicator microorganisms and pathogens in both the Mhlathuze River within the catchment area. The physico-chemical aspects of water from the Mhlathuze River were monitored and compared to the microbial quality of the water.

The Department of Water Affairs and Forestry (DWAF) is implementing the National Microbial Water Quality Monitoing Programme (NMMP) to address the microbial safety of the water resources in South Africa.

The Department assisted the DWAF to monitor the microbial quality of the Mhlathuze catchment, W12c-j, in the NMMP high health risk area. The particular catchment area is high on the priority list (No. 14 of 120) of the NMMP.

The conventional methodologies to study the survival and ecology of microbial communities in aquatic environments are based on culturing of viable cells. However, the potential risks derived from the viable but non-culturable (VBNC) state of enteric pathogens has been increasingly recognized as an important factor affecting public health. New approaches to the enumeration and detection of cells at the molecular level are emerging and gaining acceptance by microbiologists. One of the objectives of this project was to evaluate modern molecular biotechnology methods for the ability to analyse the microbial population structure and diversity of the Mhlathuze River. The results obtained by these methods were compared to conventional culturing methods.

Objectives

The objectives of the project were:

1. To monitor the water quality in the Mhlathuze River namely the microbial content and related physico-chemical characteristics.

2. To study the population and diversity of micro-organisms in the Mhlathuze River.

3. To accumulate data on the survival and transmission of water borne pathogens in the Mhlathuze River.

4. To improve postgraduate and undergraduate training and to develop research capacity within the Department of Biochemistry and Microbiology at the University of Zululand.

5. To help create capacity and infrastructure for the implementation of NMMP.

Methodology

Sampling Sites

Five sampling sites (namely KwaDlangezwa, KwaDlangubo, Richards Bay estuary, Mhlathuze pumping station and Felixton) along the Mhlathuze River were selected for this study.

Water sampling

All water samples were collected in sterile Schott bottles, every second week at five different locations along the Mhlathuze River and analyzed within 6 hours of collection.

Physical and Chemical analysis

The BOD, COD, total nitrogen, nitrate, nitrite, ammonium, ortho-phosphate, total phosphate concentrations and the concentrations of heavy metals of each sample were analysed. The temperature, pH, conductivity, dissolved oxygen concentration and turbidity were also monitored

Microbological analysis

The plate count and membrane filtration (Millipore, HANG 47 mm) methods as described by Chan et al. (1993) were used (where necessary) for the enumeration of bacterial counts using using nutrient, m-Endo Les, mFc and S-S agars (Merck). Experiments were performed in duplicate. The nutrient Agar, TSA, R2A plates were used to study the microbial diversity.

Isolation and identification of bacteria

Single colonies of bacteria were randomly selected from the various agar plates based on the morphology and subsequently isolated in pure form.

The isolates were then identified by Gram staining and biochemical reactions and confirmed by API strips if available according to the manufacturer's instructions (bioMerieux, France).

Antibiotic sensitivity tests

The disc diffusion method was used to determine antibiotic sensitivity (a total of the 15 antibiotics) of the isolates. The organisms were classified as sensitive, intermediate or resistant, based on the NCCLS standards.

Resuscitation of Viable But Non-Culturable (VBNC) bacteria

Resuscitation was carried out using the DVC method of Kogure (1979).

DNA Isolation

Total DNA isolation of each microorganism from the environmental sample was obtained using the heat-lysis method (Sandvang et al., 1997). The plasmid extraction was performed using the GFX™ Micro plasmid Preparation Kit (Roche). The DNA profile of the plasmid from the isolates was analyzed on a 0.7 % agarose gel.

PCR amplification of specific plasmid fragments/genes and Enterobacterial Repetitive Intergeneic Consensus (ERIC) profiles

Different sets of primers were used to determine the ERIC profiles, the presence of class 1 integron as well as the antibiotic resistant genes in each isolate. All ERIC profiles and PCR products were analyzed on agarose gel (1%) and recorded by SynGene Gel Documentation system.

Statistical Analysis

Geometric means of microbiological and of physical and chemical analysis data were used to present monthly values for these factors. All percentage data were arcsine transformed before analysis. Pearson's product moment correlations were used to correlate total and faecal coliform counts to the water temperature and the rainfall figures. Paired t-tests were used to determine the statistical significance between different species. Spatial variability in antibiotic resistance was analysed with Chi-square test.

Conjugation Experiments

Conjugation was carried out for 16 isolates representative of the different antibiotic resistance patterns (10 *E.coli*; 3 *Klebsiella spp.*; 2 *C.freundii* and 1 *S. marcesens*). Depending on the resistance pattern of the isolates, two recipient strains were used - *E.coli* HB101, containing the streptomycin resistant gene and the *E.coli* CSH56, containing the nalidixic acid resistant gene in the respective chromosomes.

Antibiotic sensitivity/resistance patterns of the transconjugants were determined and compared to those of the donors (environmental isolates) prior to conjugation. Plasmid DNA's were extracted from the successfully conjugated isolates. Molecular weights of the purified plasmids were determined using gel electrophoresis with a 0.7% agarose gel.

Summary of the Major Results

The microbial (total and faecal coliform counts) and some physico-chemical parameters of the water in the Mhlathuze River were monitored during March 2001 to November 2002 and compared to the results of the previous study conducted during 1998-1999. The results show that most of the physico-chemical values obtained were within South African standards. High concentrations of metal were detected in water samples from Felixton and the Richards Bay estuary. The water samples from the Mhlathuze pumping station and Felixton which contained higher concentrations of total nitrogen and total phosphate also had higher faecal coliform contamination. High total coliform counts in the Mhlathuze River during this study period are significantly higher than those observed in the 1998-1999 period. Larger patterns of fluctuations of total and faecal coliform counts were also observed. This phenomenon coincided with the major construction of the Mhlathuze pumping station which sought to supply additional water demands for the new mining project in the region. High water surface temperatures and rainfall figures may also have contributed to this observation. In 2002, the trend in monthly total and faecal coliform counts was smooth.

Felixton continues to be the main site of major faecal contamination similar to that observed in 1998-1999.

From the results, it can be concluded that the factors which influenced the observed elevated levels of faecal and total coliform bacteria include: (i) Activities (domestic, recreational, agricultural and industrial) along the River increase during the summer period. At Felixton, for example, industrial (a sugar-mill and paper factory is operating at full capacity) and informal agricultural activities increase. The construction at the Mhlathuze pumping station caused severe fluctuations of bacterial growth during 2001 period. (ii) The Mhlathuze River is situated in a summer rainfall area and increased run-off is thus expected in the summer period annually. (iii) Surface water temperatures of between 20°C and 31°C were regularly detected during the summer period.

The antibiotic resistance patterns of 113 identified enteric bacteria isolated from the Mhlathuze River show that 94.7 % were resistant to at least one class of antibiotic while 75.2% were multi-resistant. All isolates were sensitive to the presence of gentamycin. The levels of resistance exhibited by isolates to specific antibiotics are as follows: penicillin, 72.6%; rifampicin, 69.2%; novobiocin, 52.1%; ampicillin, 43.6% and cephalothin, 28.2 %. The antibiotic resistance gene pool especially that for □-lactamase is likely to be widely available in the environment. The antibiotic resistance profiles (ARP) for *E. coli* and non-*E. coli* groups were similar. The enteric bacteria isolated from downstream which is a mainly urban and industrial area contained a higher percentage of antibiotic resistance against several antibiotics compared to those from upstream which is rural.

Forty-three *Enterobacteriaceae* isolates, which were capable of resisting more than 4 different antibiotics, were used for the molecular characterization of antibiotic resistances. Fifty-eight percentage of these multiple antibiotic resistant isolates possess the class 1 integron. Some isolates possessed more than one copy of class 1 integrons of different sizes. Amongst these 25 isolates with positive detection of class 1 integron,

the beta-lactamase gene (*pse*) was the most commonly found and present in 44% of these integrons. The aminoglycoside resistance gene (*ant*'3) was found in in 16% of the integrons. The two genes (*sul*1, coding for sulfonamide resistance and the *QAC*Δ*E*1, coding for resistance to quaternary ammonium compounds) that are associated with class 1 integrons were detected in 24% of the integrons. In the conjugation experiment, 56% of the environmental isolates transferred their plasmids as well as their antibiotic resistance profiles to the recipient cells.

Conclusions

The results show the bacteriological quality of the water of the Mhlathuze River posed an increased risk of infectious disease transmission to the communities. The following environmental factors observed influenced the elevated levels of fecal and total coliform bacteria. (i) Activities (domestic, recreational, agricultural, industrial) along the river. (ii) Rainfall and (iii) Surface water temperatures. The resuscitation results also demonstrate strongly that the faecal coliform contamination level could not present a clear picture of the water quality in any water system.

The study suggests that the Mhlathuze River has become a major reservoir for antibiotic resistant microbes. Higher levels of antibiotic resistance amongst the clinical and heavy metal resistant isolates compared to those of the environmental isolates strongly support the idea that the sustained presence of antibiotics and/or industrial effluents enhances the ability of microbes to resist the presence of antibiotics/drugs. The study can also conclude that there exists a wide pool of antibiotic resistance genes within the Mhlathuze River. A high degree of genotypic diversity and the lack of correlation between antimicrobial resistance patterns and molecular types of the isolates suggest convergent acquisition of resistance determinants by genetically unrelated strains rather than epidemic spread of resistant isolates in the community. The ability of commensal organisms to carry resistance genes of clinical importance and their ability to transfer such genes to other bacteria is of greater concern than phenotypic measurements. The possibility of transmission of resistance genes between

bacteria (especially pathogenic) which invade human and animal populations within this River poses a health risk to the communities who are dependant on this river for consumption.

Recommendations and the Future Research

Proper management and monitoring of the human activities will be the key to improve the status of the water quality of Mhlathuze River. Provision of a proper infrastructure for the supply of drinking water and basic sanitation especially in the rural area may reduce significantly the burden of this water resource. This will ensure public health.

A continuous monitoring programme such as the NMMP is important. However, in addition to use faecal coliform bacteria as indicators, other parameters such as physical, chemical as well as the status of antibiotic resistance profiles of microbes in the natural environment should be also monitored. The impact of the VBNC state of bacteria in the environment could not be ignored. It should be equally important to develop more sensitive monitoring techniques such as the bacterial and chemical source tracing techniques or the so called biosensor to investigate the source(s) of the contamination for the proper management.

The molecular/genetic approaches provide alternative means to in-depth understanding of the water system. Our genetic studies also suggest the importance of continuing studies to determine the extent to which transmission of antibiotic resistant bacteria from humans to animals occurs and the extent to which such transfer impacts the efficacy of antibacterial use in human medicine.

The molecular/genetic approaches also can provide the species diversity in the natural environments. The early detection of trends and the identification of factors responsible for directing these trends by understanding the types, functions and diversities of organisms especially microorganisms in soil, water, land and other ecosystems are essential in the management, restoration and intervention of any ecosystem.

APPENDIX

A) Capacity Building Outcomes

Postdoctoral fellow:

Dr. C.C. Bezuidenhout – 2000-2001
Title: *The Development of Molecular Techniques to study Genetic Diversity of the Mhlathuze Catchment.*

Masters:

Mthembu N. (Miss) -2003
Title: *Use of the polymerase chain reaction for detection of pathogens from water*

Biyela P.T. (Miss) -2003
Title: *Characterization of antibiotic resistance in Enterobacteriaceae isolates from the Mhlathuze River.*

Honours:

Kunene M. (Mr.) - 2002
Title: *Isolation of Heavy Metal Resistant Microorganisms from the Mhlathuze Catchment.*

Mthethwa C.P.N. (Miss) - 2001
Title: *A study of antibiotic and chlorine resistance of Aeromonas and Pseudomonas spp. isolated from the Mhlathuze River.*

National Diplomat (Durban Institute Technology)

Mkhize N.L. (Miss) - 2002
Title: *Water Quality of the Mhlathuze River.*

B) Conference Presentations

- Biyela PT, C.C. Bezuidenhout and J. Lin, *The Role of Aquatic Ecosystem as Reservoirs of Antibiotic Resistant Bacteria and Antibiotic Resistant Genes*, A Region Conference on Water as the Key to Sustainable Development in Africa, International Water Association, Cape Town, South Africa, 14-19 September 2003.

- Kunene M. and J. Lin, *Isolation of Heavy Metal Resistant Microorganisms from the Mhlathuze Catchment*, 15th Annual Symposium of South Africa Society of Microbiology (Natal Branch), Durban, Kwa Zulu Natal, South Africa, October 2002.

- Mkhize N.L. and J. Lin, *Water Quality of the Mhlathuze River*, 15th Annual Symposium of South Africa Society of Microbiology (Natal Branch), Durban, Kwa Zulu Natal, South Africa, October 2002.

- Biyela P.T., J. Lin and C.C. Bezuidenhout *Characterization of antibiotic resistance in Enterobacteriaceae isolates from the Mhlathuze River*, 12th Biennial Congress of the South Africa Society of Microbiology, Bloemfontein Free State, South Africa, April 2002.

- Biyela P.T., J. Lin and C.C. Bezuidenhout, *Characterization of antibiotic resistance in Enterobacteriaceae isolates from the Mhlathuze River*, 14th Annual Symposium of South Africa Society of Microbiology (Natal Branch), Pietermarizburg, Kwa Zulu Natal, South Africa, October 2001.

- Mthethwa C.P.N., J. Lin and C.C. Bezuidenhout *A study of antibiotic and chlorine resistance of Aeromonas and Pseudomonas spp. isolated from the Mhlathuze River*, 14th Annual Symposium of South Africa Society of Microbiology (Natal Branch), Pietermarizburg, Kwa Zulu Natal, South Africa, October 2001.

- Bezuidenhout C.C., J. Lin, N. Mthembu, P.T. Biyela and A.K. Basson , *The Mhlathuze River Catchment: Bacterial Contamination and Antibiotic Resistance patterns of the Isolates*, Water Pollution VI: Modelling, Measuring and Prediction, Greece, 2001.

C) Articles and Papers Publications

1. Bezuidenhout CC, N Mthembu, T Puckree and J Lin. *Microbiological evaluation of the Mhlathuze River KwaZulu Natal (RSA)*, Water SA, 28(3):281-286, 2002.

2. Bezuidenhout CC, J Lin, N Mthembu, PT Biyela and AK Basson, *The Mhlathuze River Catchment: Bacterial Contamination and Antibiotic Resistance patterns of the Isolates*, Water Pollution VI: Modelling, Measuring and Prediction, pp 515-524, ed. C.A. Brebbia, WIT press, 2001.

3. Lin J, PT Biyela, T Puckree and CC Bezuidenhout, *A Study of Microbial and Related Water Qualityof the Mhlathuze River, Kwa Zulu Natal (RSA)*, Water SA, 30(1): 17-22, 2004.

4. Lin J, PT Biyela and T Puckree *Antibiotic Resistance Profiles of Environmental Isolates from Mhlathuze River, Kwa Zulu Natal (RSA)*, Water SA, 30(1): 23-28, 2004.

Archiving of data

Although this project did not form part of the National Microbial Monitoring Programme (NMMP) in this region due to the late commitment of the project, the results obtained in this study still provide valuable information for the implementation of NMMP. All the data will be deposited in the Hydrology Web site of the University of Zululand. The Resource Quality Services (former Institute for Water Quality-DWAF) also decided to include two sampling sites in our study in the future NMMP.

ACKNOWLEDGMENTS

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CHAPTER 1

INTRODUCTION

1.1 Background

The Mhlathuze catchment in northern KwaZulu Natal, South Africa supports a rapidly growing agricultural and industrial community (Steyl et al., 2000). 78.5% of the catchment population is rural. 63% of the households use dams, rivers and streams as their primary source of water and 34.8% do not have sanitation services (Census, 1996).

With increasing demands on water resources and contamination from industrial waste and human activities, the potential for outbreaks of water-borne diseases in this area continues to grow. The bacteriological quality of the water of the Mhlathuze River may pose an increased risk of infectious disease transmission to the communities that are dependent on the river (DWAF, 1996). The Institute of Water Quality Studies of Department of Water Affairs and Forestry (DWAF) states that this catchment area (W12 C-J) is high on the National Microbial Water Quality Monitoring Programme's priority list (14^{th} in a total of 120). (IWQS report, 2000)

The DWAF has adopted the Strategic Environmental Assessment (SEA) as a tool in the management and planning of all aspects (the social, economic and biophysical environment) of the Mhlathuze catchment. (Steyl et al., 2000) However, the microbiological analyses of the water specifically research related to the population of human pathogens and indicator microorganisms remain to be addressed.

1.2 Aim:

The aim of this study was to generate more extensive data on the microbial aspects of this important environmental reserve, the Mhlathuze catchment.

1.3 Objectives

The main objectives of the study on the water of the Mhlathuze River were as follows:

1. To monitor the water quality, namely the microbial content and related physico-chemical characteristics.

2. To study the population and diversity of microorganisms in the River.

3. To accumulate data on the survival and transmission of water-borne pathogens in this water resource.
4. To improve postgraduate and undergraduate training and to develop research capacity within the Department of Biochemistry and Microbiology at the University of Zululand.

5. To assist in the implementation of the National Microbial Water Quality Monitoring Programme in the Mhlathuze Catchment region.

1.4 The Structure of the Report

The report consists of three parts.

Chapter 1 provides a brief introduction of this project, the aim and its overall objectives.

Chapter 2 provides a comprehensive analysis of the water quality by monitoring the total and faecal coliform counts and the related physico-chemical characteristics.

Chapter 3 examines the heavy metal and antibiotic resistance profiles of microbial isolates (especially the *Enteriobacteriaceae* family) from the Mhlathuze River.

Chapter 4 presents the microbial population and diversity as well as the survival and transmission of antibiotic resistance genes in the Mhlathuze River.

Chapter 5 provides an overall conclusion and recommendations for the future research.

CHAPTER 2

THE MICROBIAL QUALITY AND THE RELATED PHYSICAL AND CHEMICAL VARIABLES IN THE MHLATHUZE RIVER

2.1 Background

The Mhlathuze River in northern KwaZulu Natal flows into the estuary at Richards Bay (Steyl et al., 2000). The main sources of water that make up the Mhlathuze catchment are the Goedertrouw dam, the Mhlathuze Weir and the Thukela Transfer Scheme which pumps water from the Thukela River to the Goedertrouw dam. The natural lakes in the surrounding areas also form part of the water supply system to both urban and industrial users.

The initiation of the Richards Bay-Empangeni Spatial Development Initiative stimulated industrial growth in the region. Thriving industries include the aluminum smelters, sugar mills, paper and pulp factories, fertilizer manufacturing and mineral mines as well as the largest single coal handling facility in the world. These industries provide employment opportunities for people from the area, as well as individuals from more remote areas. In the mid-catchment area, major agricultural developments, including forestry, sugarcane and citrus have flourished. The remaining parts are very rural and undeveloped with subsistence farming being an income generator and survival mechanism.

Due to the varied demands placed on this water resource and lack of infrastructure for household use and sanitation, the local rural residents who are dependent on this water face the risk of water-borne diseases. The *Vibrio cholera* epidemic (2000-2001) in the Mhlathuze catchment and other areas of KwaZulu Natal, was preceded by a diarrhoeal outbreak in the Mangeza area (May to August 2000). The Government and other organizations recognized the need to set up infrastructures to supply treated water and sanitation facilities in order to prevent any future similar outbreak in the Mhlathuze Catchment (The Mercury, RSA, 2001). Controversies about water supply from

an adjacent catchment area are also raging (Zululand Observer, RSA, 2001). In the interim, the water from the Mhlathuze River is "over allocated". It is therefore imperative that microbial and physico-chemical data of the river are collected to provide comprehensive baseline information that can be compared to other existing biological databases for the river as well as utilised for future monitoring purposes.

The quality of water is typically determined by monitoring microbial presence, especially faecal coliform bacteria, and physico-chemical properties (Gray, 1994; DWAF, 1996; USA-EPA, 1999). These parameters could be affected by external and internal factors. There is an intricate relationship between the external and internal factors in aquatic environments. Meteorological events and pollution are a few of the external factors, which affect physico-chemical parameters such as temperature, pH, salinity, hardness, dissolved oxygen and phosphates of the water. Exposure to environmental pollutants and changes in nutrient composition in agricultural run-off, sewerage and industrial effluent may lead to selection pressures favouring certain organisms or genotypes. Recent studies demonstrated positive correlations between industrial pollution and the spatial distribution of antibiotic resistance (Goñi-Urriza et al., 2000; McArthur and Tuckfield, 2000). Sudden changes of these parameters may be indicative of changing conditions in the water which affect the internal factors between and within bacterial and plankton populations in the water body (Nübel et al., 1999; Byamukama et al., 2000; Goni-Urriza et al., 2000; Lobitz et al., 2000; Nishiguchi, 2000).

Any increase in faecal pollution in source water is a problem of developing as well as developed countries (American Society for Microbiology, Colloquium Report, 1999). Water-borne bacterial pathogens such as *E.coli* 0157, *Salmonella*, *Shigella* spp. and *Vibrio cholera* can lead to diarrhoeal outbreaks that may have serious medical and economic implications (WHO report, 2000; WHO Fact Sheet, 1996). This problem is further compounded by the survival

strategies of viable but non-culturable state of bacterial flora in water systems (Colwell et al., 1985; Singh et al., 1986; Garcia-Lara et al., 1991).

2.2. Aims

The primary aim of this chapter was to determine the water quality namely total and faecal coliform counts and some physico-chemical characteristics, in the Mhlathuze River. The secondary aim was to determined seasonal changes in the bacterial population. These findings might reveal the impacts of the human and industrial activities on microbial population changes along the river.

2.3 Materials and Methods

2.3.1 Sampling Sites

Five sampling sites along the Mhlathuze River were selected for this study (Figure 2.1). Site one (KwaDlangezwa) is situated near a sewage plant at the University of Zululand. The area surrounding Site 2 (KwaDlangubo) has agricultural developments such as sugarcane. Site 3 is the Richards Bay estuary with nearby aluminium smelters and fertilizer manufacturing factories. Site 4 is the Mhlathuze pumping station, which has been expanded in 2001 to supply the water demands for new mining activities in the area. Site 5 (Felixton) contains a sugar mill and paper pulping factories as well as agricultural and informal farming activities.

2.3.2 Water sampling

All water samples were collected in sterile Schott bottles, every second week at five different locations along the Mhlathuze River. Once collected, the samples were immediately stored on ice in a dark cooler box and transported to the laboratory. The samples were stored at 4°C and analyzed within 6 hours of collection.

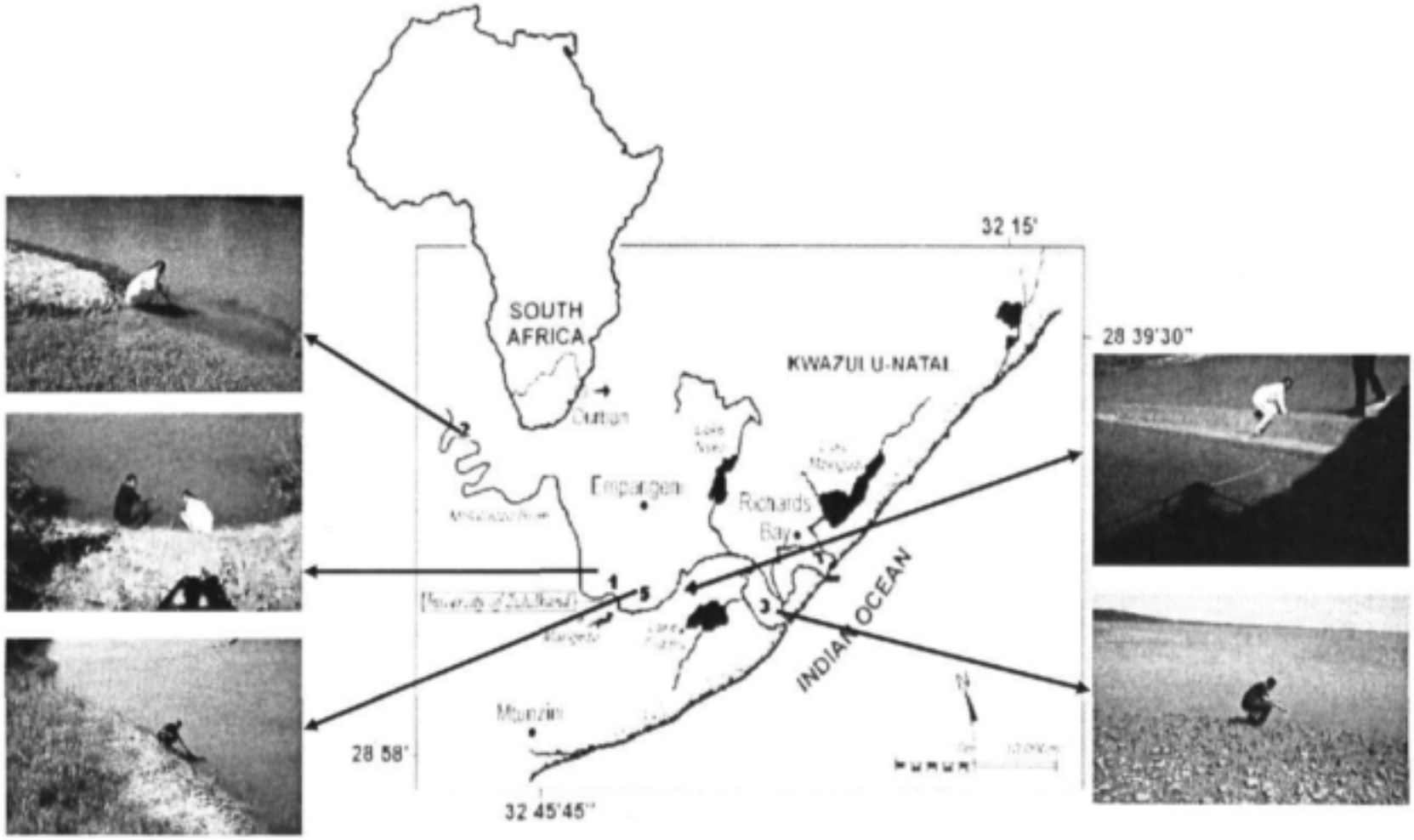

Figure 2.1 Map of the eastern region of the Mhlathuze catchment area indicating the sampling sites (site 1- KwaDlangezwa, site 2- KwaDlangubo, site 3- Richards Bay estuary, site 4- Mhlathuze pumping station and site 5-Felixton bridge).

2.3.3 Physical and Chemical analysis

The COD, total nitrogen, nitrate, nitrite, ammonium, ortho-phosphate and total phosphate concentrations of each sample were analysed using Aqualytic AL282 as described by the manufacturer. The concentrations of heavy metals (Pb^{+2}, Cd^{+2}, Cu^{+2}, Al^{+3} and Hg^{+2}) in water were measured using the Atomic Absorbance Spectrometer with the assistance of the analytical laboratory at the Mhlathuze Water Board. The temperature, pH, conductivity, dissolved oxygen concentration and turbidity were monitored on site using Corning Checkmate II with portable thermometer, pH, turbidity and Dissolved oxygen meters and Aqualytic turbidity meter respectively. BOD values were obtained based on instruction of the Aqualytic Sensomat System.

2.3.4 Microbological analysis

A series of ten-fold dilutions of the water samples were used (where necessary) for the enumeration of bacterial counts using plate count and membrane filtration (Millipore, HANG 47 mm) methods as described by Chan et al. (1993). Nutrient, m-Endo Les, mFc and S-S agars (Merck) were used to determine heterotrophic bacterial, total coliform, fecal coliform and *Salmonella* and *Shigella* spp. respectively. Plates were incubated at 35°C for 24 hours with the exception of mFc plates which were incubated at 44.5°C for 24 h. Experiments were performed in duplicate.

2.3.5 Resuscitation of Viable But Non-Culturable (VBNC) bacteria

Resuscitation was carried out using the DVC method of Kogure (1979). Nine thousand seven hundred and thirty μl of sample was resuscitated using 20 μl (0.002%) nalidixic acid and 250 μl (0.025%) yeast extract. The sample was then incubated in the dark on a shaker (165 rpm) at room temperature for 6 hours. Bacterial numeration after resuscitation

was obtained using the membrane filtration method as described above and compared with the one without resuscitation.

2.3.6 Statistical Analysis

Geometric means of microbiological and of physical and chemical analysis data were used to present monthly values for these factors. Pearson's product moment correlations were used to correlate total and faecal coliform counts to the water temperature and the rainfall figures.

2.4 Results

Table 2.1 shows the physical, chemical parameters and faecal coliform count of the water samples collected from 5 different sites along the Mhlathuze River during the study period. Most of the values obtained were within South African standards. During the rainy period, the turbidity of the Mhlathuze River, especially downstream (beyond Felixton) increased to an unacceptable level.

Water samples from Sites 4 and 5, which contained higher concentrations of total nitrogen and total phosphate, also possessed higher faecal coliform contamination. Resuscitation of water samples resulted in increases in faecal and total coliform counts up to 23 and 65 fold respectively (data not shown). Due to the high salt concentration at Site 3, the bacterial counts were much lower than those at other sites. High concentrations of metal were detected in water samples from Sites 5 (Cd^{+2}: 0.011mg/L) and 3 (Pb^{+2}: 0.056 mg/L). High concentrations of Al^{+3} (44.7 and 32.2 mg/L) were detected in Sites 5 and 3.

Figure 2.2 shows the mean values of the total coliform counts at all five sites during periods, 2001-2002 and 1998-1999. The total coliform counts of the Mhlathuze River in the present study were significantly higher than those in the previous study. During 2001 the monthly trend in total coliform populations in the Mtlathuze River showed large fluctuations. This fluctuation disappeared in 2002. The water temperature presented a typical summer and winter pattern.

Table 2.1: The geometric means of faecal coliform counts, physical and chemical characteristics measured in each site of the Mhlathuze River over the study period (February 2001-November 2002).

Site	Site 1	Site 2	Site 3	Site 4	Site 5
T(°C)	15-31.6 (23.3)	17-30.85 (25.2)	16.5-31.2 (25.9)	18-29.4 (24.2)	15-30.7 23.2)
PH	5.8-8.3 (7.6)	5.93-8.47 (7.74)	5.94-8.19 (7.51)	6.9-8.58 (7.73)	6.83-8.38 (7.70)
Turbidity (NTU)[a]	0.8 – 9.0 (5.63)	2.5-7.6 (6.1)	2.1-20.2 (7.56)	2.3-17.8 (13.4)	1.4-33.0 (7.80)
Conductivity (µS/cm)[a]	350-696 (480.9)	218-524 (391.9)	1970-51800 (25700)	263-705 (491.5)	221-717 (462.6)
DO (mg/L)[a]	36.2-51.6	33.3-51.0	32.0-43.3	32.0-41.9	31.7-43
Total N (mg/L)[b]	1.6-5.6 (2.41)	0.5-5.9 (1.43)	1.4-2.4 (1.67)	1.5-8.5 (3.33)	1.3-7.7 (2.78)
NO_2^-(mg/L)[b]	0.06-1.9 (0.20)	0.06-0.8 (0.15)	0.03-0.8 (0.17)	0.02-4.8 (0.22)	0.02-0.8 (0.15)
NO_3^-(mg/L)[b]	0.3-1.0 (0.59)	0.5-0.7 (0.59)	0.2-0.6 (0.33)	0.3-0.9 (0.62)	0.2-1.1 (0.48)
NH_4^+(mg/L)[b]	0-0.23 (0.06)	0.01-0.06 (0.03)	0.03-0.24 (0.07)	0.02-0.34 (0.08)	0-0.13 (0.04)
Total P(mg/L)[b]	1.3-2.8 (1.9)	1.8-4.8 (2.9)	3.6-32.0 (10.7)	1.0-8.3 (2.9)	1.3-10 (3.6)
Ortho-P(mg/L)[b]	0.2-0.5 (0.28)	0.19-0.87 (0.53)	0.20-0.94 (0.44)	0.46-0.80 (0.67)	0.20-0.68 (0.34)
Cd^{+2} (mg/L)	nd	0-0.001	0-0.006	nd	0-0.011
Cu^{+2} (mg/L)	nd	0.002-0.022	0.039-0.11	nd	0.004-0.047
Al+3 (mg/L)	nd	10.9-12.7	27.9-44.7	nd	13.1-32.2
Hg+2 (µg/L)	nd	0-1.19	0.52-3.29	nd	0-0.08
Pb^{+2} (mg/L)	nd	0 - 0.001	0.003-0.056	nd	0.002-0.004
BOD[b]	0-1.35 (0.21)	0-0.90 (0.23)	0-1.00 (0.41)	0-1.2 (0.48)	0-0.80 (0.24)
COD(mg/L)[b]	9-72.5 (22.3)	2.5-21.5 (11.9)	18.0-126.0 (33.6)	4.0-25.0 (12.5)	1.3-34.5 (19.7)
Faecal Coliform Count/100ml (mean)	10-900 (292)	20-2600 (642)	0-200 (54)	40-26800 (1937)	20-6120 (1118)
Ratio after resuscitation*[b]	1.5-23.3	1.5-10	1.25-4.0	1.25-7.5	1.3-2.5

* ratio of faecal coliform counts with or without resuscitation procedure.
[a] From October 2001 to present.
[b] From June 2002
[nd] not determined

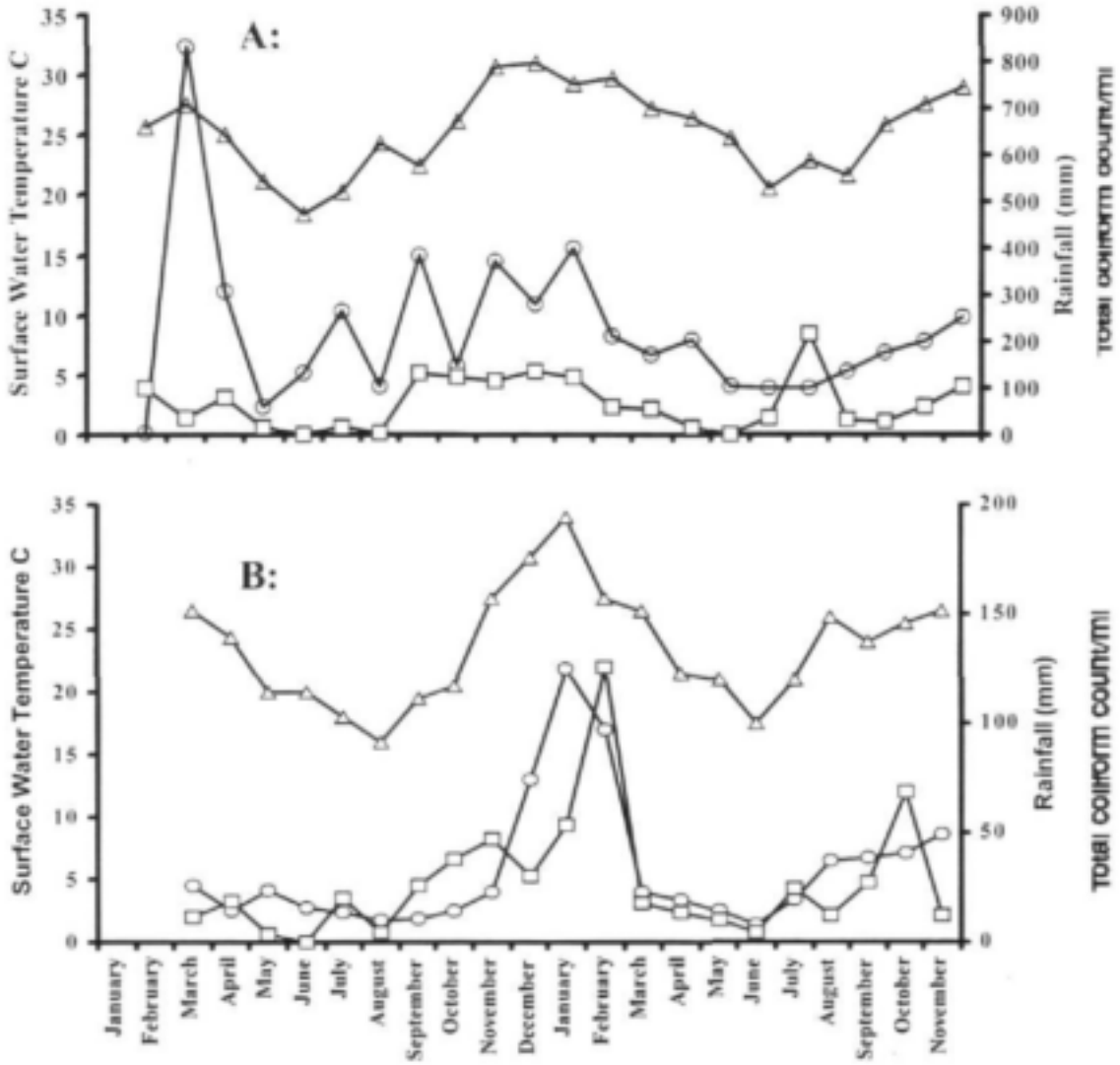

Figure 2.2: The monthly mean of total coliform counts, rainfall figures and temperature in all of the five sites along the Mhlathuze River. A) February 2001 – November 2002 period; B) March 1998-November 1999 period. (Bezuidenhout et al, 2002). ○: Total coliform count; Δ: water surface temperature; □: Rainfall figures.

However, the average water temperatures during August 2001 to May 2002 were higher than those for the same period during 1998 to 1999 with the exception of those for January. The rainfall figures were also higher in the summer period of 2001-2002 than those of 1998-1999 with the exception of that in February. However, the Pearson's correlation coefficient (r) showed weak correlations between faecal coliform counts and the water surface temperature (r=0.48) and the rainfall figures (r=0.31).

The fluctuating trend in the total coliform counts was also observed in the faecal coliform counts in the same period. Despite the increases in total coliform counts of the water system, the faecal coliform counts were similar in level to those observed in the period of 1998-1999 (Figure 2.3). However the 2001 figures fluctuated. Significant increases in faecal coliform count were seen during November and December of 2001. This sudden increase of faecal coliform count was mainly due to the significant increase at Site 4 (Figure 2.4). As observed during the 1998 and 1999 period, Site 5 continued to have higher levels of faecal contamination. Although the Mlhlathuze pumping station also emerged as a major source of faecal contamination during 2001, this situation was reversed since 2002.

A comparison of the trends of monthly faecal coliform counts at each site along the Mhlathuze River during the two study periods, shows no obvious seasonal changes as suggested in the previous study (1998-1999). This finding may be due to the pattern of fluctuation in 2001. With the exception of some sudden increases of faecal coliform counts on Sites 4 and 5 during 2001, the faecal contamination in the Mhlathuze River generally was lowered during this study period. Significant increases in faecal contamination were noted in all five sites at the end of 1999.

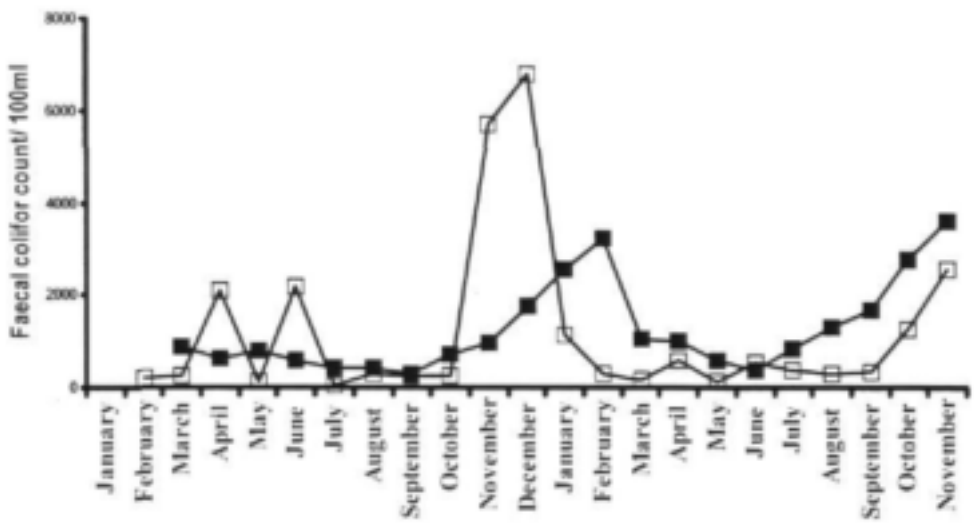

Figure 2.3: The monthly mean of faecal coliform counts at all five sites along the Mhlathuze River during two study periods. (□: Faecal coliform counts for February 2001- November 2002; ■faecal coliform count s for March 1998- November 1999).

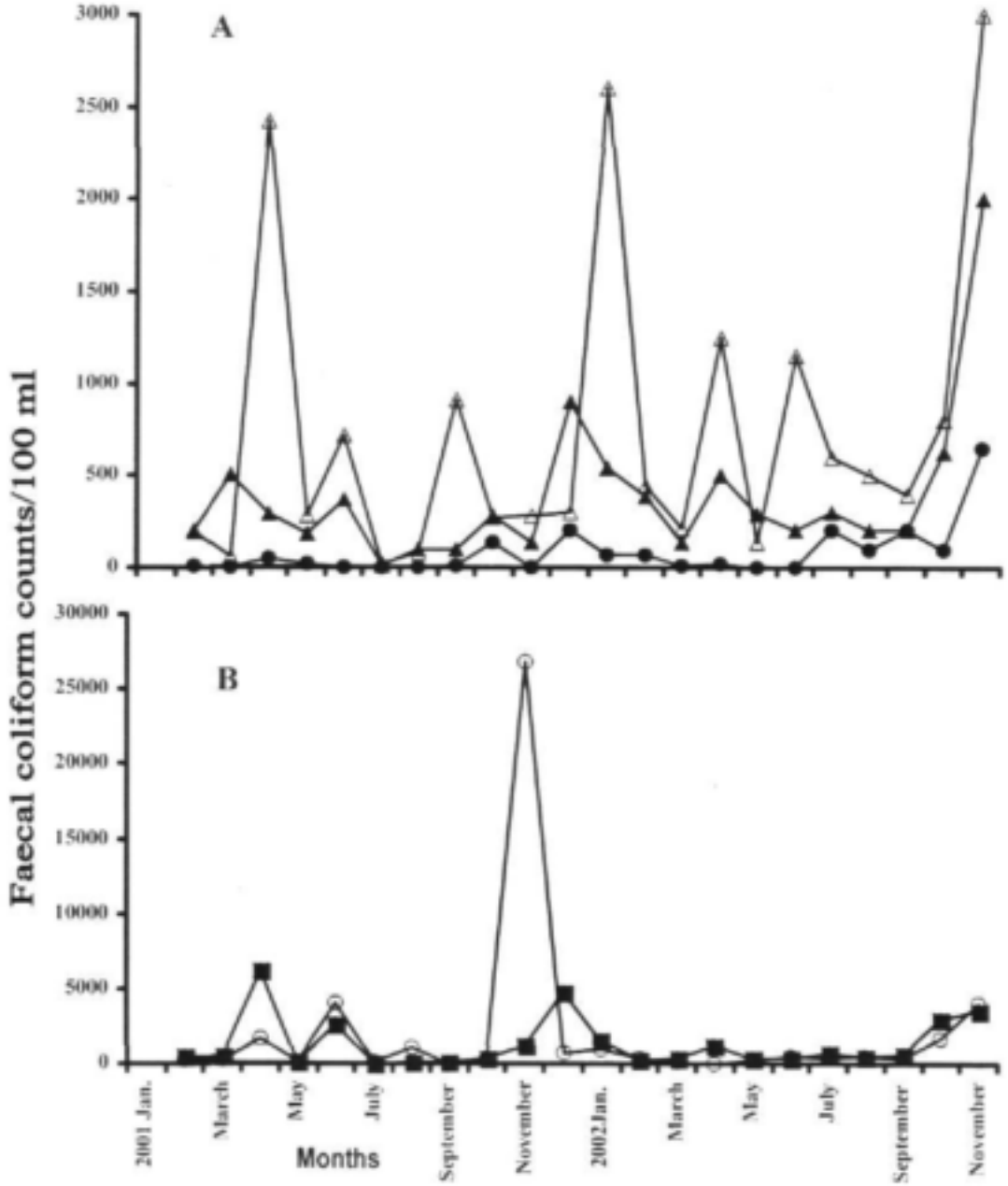

Figure 2.4: The monthly mean of faecal coliform counts at each site along the Mhlathuze River during the study period (February 2001–November 2002). (A: □ Kwa Dlangezwa Site 1; Δ KwaDlangubo Site 2; ● Richard Bay Estuary Site 3; B: ○ Mhlathuze Pumping Station Site 4; ■: Felixton Site 5)

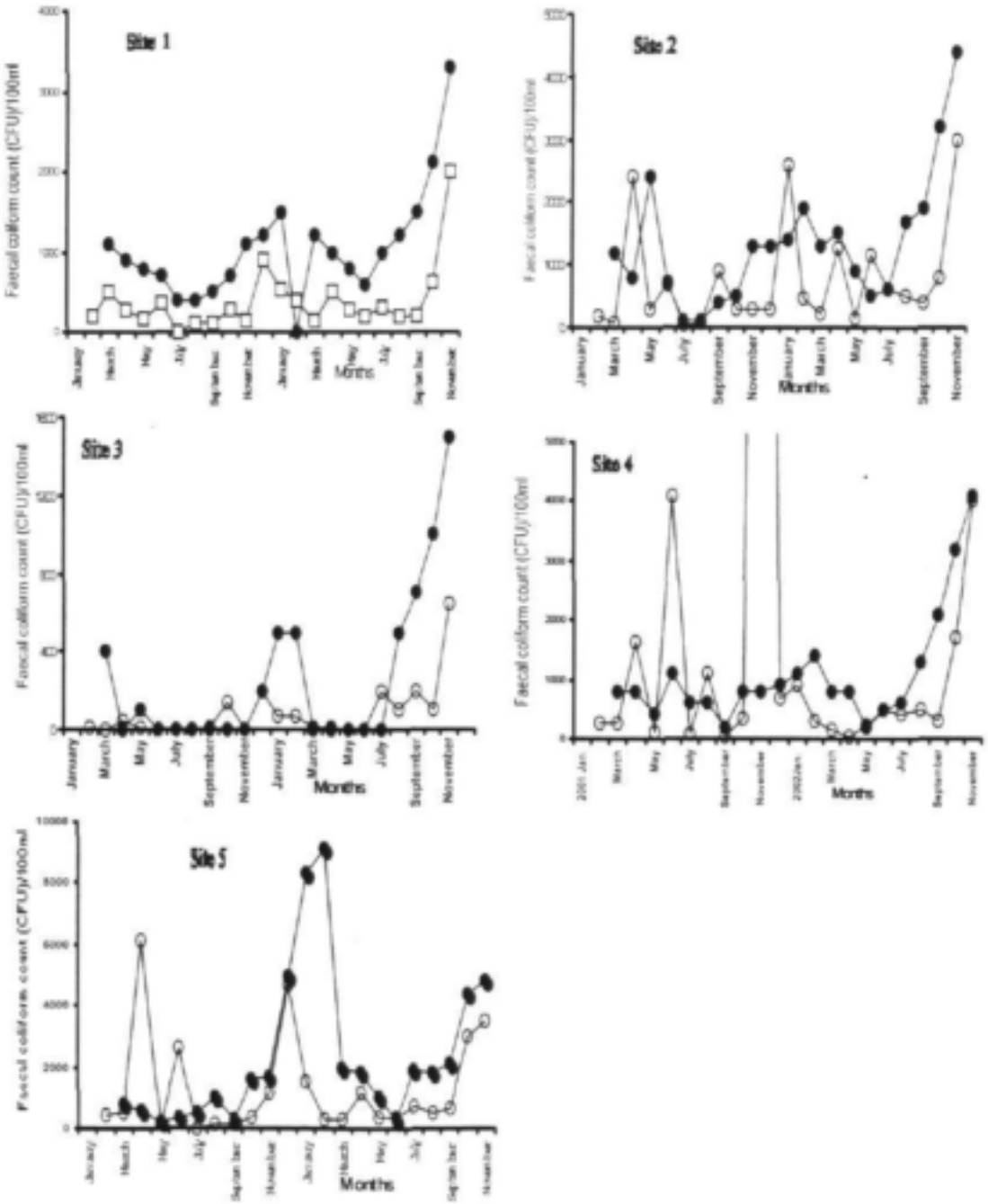

Figure 2.5: Comparison of monthly mean of faecal coliform counts at each site along the Mhlathuze River during this study period (○ February 2001 – November 2002) and the previous study period (● March 1998 – November 1999).

2.5 Discussion

2.5.1 Microbiological quality of the Mhlathuze River

This study showed seasonal patterns in faecal and total coliform counts and rainfall and surface temperatures of the Mhlathuze River (Figures 2.2-2.5). These mean values were higher during the summer season of 2001 supporting the prolonged survival of coliform bacteria. These findings are strongly supported by other studies (Nübel et al., 1999; Byamukama et al., 2000; Lobitz et al., 2000; Nishiguchi, 2000, Solo-Gabrielle, 2000). This study showed weaker correlations between bacterial counts and rainfall figures as well as water surface temperature than those during 1998-1999. This could be attributed to the fluctuation patterns of total and faecal coliform counts during 2001.

Although the present study showed significant increases in total coliform counts (Figure 2.2), the trends in total and faecal coliform counts during 2001 were fluctuating (Figures 2.2 and 2.3). This site hosts large informal gardens, swimming and the use of the adjacent bridge as a thorough-fare during the summer period. Industries such as the paper-pulping factory, the sugar mill and the sewage treatment plant outlet may also contribute towards contaminating this site. These factors could have contributed, independently or in combination, to the very high levels in bacterial counts observed at this site. Despite the substantial increase in the total coliform count, the degree of faecal contamination during 2001-2002 was similar to that in 1998.

The observed level and fluctuating trend of total coliform counts at sites 4 and 5 only (Figures 2.4 and 2.5), coincided with the major construction of the Mhlathuze pumping station. Normalization in the level of contamination coincided with the completion of the project.

Site 5 contributed significantly to the faecal contamination during both study periods (Figure 2.4). During 2001-2002 lower faecal coliform counts were observed at Sites 1 and 3 (Figure 2.5).

Mean faecal and total coliform counts throughout the study period were generally very high, but lower than those observed in other studies (Pretorius, 2000; Byamukama et al., 2000; Goni-Urriza et al., 2000). According to the DWAF (1996) guidelines, the bacteriological 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 purposes.

2.5.2 Physical and chemicals qualities of the Mhlathuze River

Studies on heterotrophic, autotrophic and other bacterial types showed that other factors such as light, salinity, predation, available nutrients and environmental pollutants are some of the key factors that influence bacterial growth and abundance in water bodies (Pernthaler et al., 1998; Lobitz et al., 2000; Solo-Gabriele et al., 2000). The results of this study (Table 2.1) show most of the physical and chemical parameters are within the acceptable levels of water quality for domestic use (WRC, DWAF and DOH, 1998). The turbidity of the Mhlathuze River was generally higher during the summer period and can increase up to 33.0 NTU, which might have serious health effects if utilized for human consumption.

Generally higher geometric means of the values of nutrients at the Mhlathuze pumping station (Site 4) coincide with the higher total coliform counts at the same site during the study period. The high concentrations of cadmium (0.011 mg/L), and lead (0.056 mg/L) detected at Felixton and the Richards Bay Estuary, respectively, might also pose health risks to sensitive groups. The higher aluminum concentrations at the Richards Bay Estuary and Felixton are most probably due to the nearby aluminum smelters. Mercury (Hg^{+2}) was also observed in the Estuary (Site 3) as well as in the KwaDlangubo (Site 2). An investigation into the prevalence of clinical symptomatlogy linked with the presence of the heavy metal in the region may lead to interesting findings.

A diarrhoeal outbreak was reported in parts of the Mhlathuze River reserve in July 2000. This episode was followed by a severe cholera epidemic. Lobitz et al., (2000) suggested sea surface temperatures, heights and phytoplankton blooms as predictors of cholera outbreaks. The influences of higher water surface temperature and rainfall observed in this study should not be underestimated. The significant increase in faecal coliform counts at all sampling sites (Figure 2.5) observed at the end of 1999 might also have been crucial in warning health authorities about the diarrhoeal outbreaks in 2000. It may be important to monitor events/phenomena (such as prolonged increased surface water temperature, rainfall) that might provide vital information as early warning signals to predict possible future cholera and/or diarrhoeal outbreaks continuously (Lobitz et al., 2000; Nishiguchi, 2000).

2.5.3 Resuscitation of viable but non-culturable bacteria

The "viable but non-culturable" (VBNC) state, an important survival strategy of several pathogenic microorganisms such as *Salmonella, Shigella* and *Vibrio* spp. has caused increasing concerns for the public health. After resuscitation, the faecal coliform counts of the water samples increased up to 23 times in this study (Table 2.1). The results indicate that the faecal contamination level in the Mhlathuze catchment is more severe than assumed. It might be important to study the real impact of this VBNC state in this water system.

2.6 Conclusion

The results from the various focus areas of this study are relevant to industrial and agricultural development as well as medical management in Northern KwaZulu-Natal. It shows that although contamination of the river is generally constant over time, there are periods when very large increases in bacterial numbers are experienced. The following factors influenced the observed elevated levels of fecal and total coliform bacteria. (i) Activities (domestic, recreational, agricultural, industrial) along the river increase during the summer period. At Felixton, for example, a sugar-mill and paper factory is operating at

full capacity and informal agricultural activities also increase. The construction at the Mhlathuze pumping station caused severe fluctuations in bacterial growth observed during 2001 period. (ii) The catchment is situated in a summer rainfall area and increased run-off is thus expected in the summer period annually. (iii) Surface water temperatures of between 20°C and 34°C were regularly detected during the summer period.

Elevated bacterial counts due to changes in environmental factors have been reported (Byamukama, 2000; Pretorius, 2000; Solo-Gabriele et al., 2000). Evidence from this and other studies (Byamukama, 2000; Goñi-Urriza 2000; Pretorius, 2000; Solo-Gabriele et al., 2000) strongly suggests that the use of faecal coliform bacteria as indicators should be expanded within a wider context and that other indicators should be also monitored for subtropical waters. In addition, more research is indicated to identify the impact of the VBNC state of pathogens in this environment.

CHAPTER 3

HEAVY METAL AND ANTIBIOTIC RESISTANCE PROFILE

3.1 Background

The Mhlathuze catchment is the principle source of water to people living in the region. It also sustains various agricultural and industrial communities (Steyl et al., 2000). Without proper infrastructure and sanitation services, the residents from the rural area that depend on the river for household purposes are prone to water-borne diseases. Pretorius (2000) illustrated how the levels of sanitation in a developing urban area affected the quality of surface water of rivers. As the demands on the water resources increases, the potential for the outbreaks of water-borne diseases also continues to grow (DWAF 1996). Evidence for this is provided by the *Vibrio cholera* epidemic (2000-2001) affecting the Mhlathuze catchment and other areas of KwaZulu-Natal, which was preceded by a diarrhoeal outbreak (May to August 2000) in the Mangeza area.

An increase of faecal pollution in source water is a problem of developing as well as developed countries (American Society for Microbiology Colloquium Report 1999). Water-borne bacterial pathogens such as *E. coli* 0157, *Salmonella*-, *Shigella* spp. and *Vibrio cholera* can lead to diarrhoeal outbreaks that may have serious medical and economic implications (WHO report, 2000; WHO Fact Sheet 1996). This problem is further compounded by the increasing incidence of pathogens with antibiotic and/or drug resistance (Ogan and Nwiika, 1993; DePaola et al. 1995). Exposure to environmental pollutants and changes in nutrient composition may lead to selection pressures favouring certain organisms or genotypes. Recent studies demonstrated positive correlations between industrial pollution and the spatial distribution of antibiotic resistance (Goñi-Urriza et al., 2000; McArthur and Tuckfield, 2000). Heavy use of antibiotics for medical and veterinary purposes (Balagué and García Véscovi, 2001; White et al., 2000) as well as the domestic and agricultural use of pesticides and related compounds (Balagué and García Véscovi, 2001) cause

significant antibiotic contamination of the natural environment and consequent development of resistance in communities of non-disease organisms. These organisms could create a widespread antibiotic resistant pool which could be transferred into human and animal disease promoting organisms. Antibiotic and drug resistance in medicine create significant health and economic impacts.

Ash et al. (2002) reported that several rivers in the U.S. have become a major reservoir for antibiotic resistant microbes. With global travel, drug and antibiotic resistance can be expected to spread to all parts of the world.

Similar to antibiotic contamination, heavy metals contamination is also a severe environmental problem as a result of the increase in human and industrial activities. Heavy metals such as lead, mercury, cadmium and nickel are metabolically poisonous at low concentration. They inhibit the activity of certain enzymes involved in the metabolic process. At high concentrations these metals form non-specific complex compounds in the cells of living organisms, which lead to toxic effects (Nies et al., 1999). Resistance to toxic heavy metals and their accumulation by bacteria is a wide spread phenomenon. The bacterial plasmid encodes the ability to resist toxic metals ions. The mechanisms of heavy metal resistance have been suggested to enhance the antibiotic resistant ability of microorganisms (Davidson, 1999; Edlund et al., 1996).

Members of the *Enterobacteriaceae* are recognised as indicators of faecal pollution of water and when present in high concentrations may indicate the presence of pathogens. There are a number of caveats in the validity of using these microbes as universal indicators of faecal pollution (Goñi-Urriza et al., 2000). Excessive use of antibiotics in medicine together with commercial pressures results in their release into the environment, thereby strengthening subsequent resistance profiles.

The data on the physical, chemical and microbiological quality of the Mhlathuze River (Chapter 2 in this report) suggests significant contamination by total and

faecal coliform microorganisms and the presence of the heavy metals in this water system. The industrial and farming activities in this region might also contribute to the survival of selected antibiotic resistant genes in bacteria that colonize human and animals. Therefore it is essential to study the level of antibiotic and metal resistances in this water system.

3.2. Aim

The aim of this chapter is to study the level of the antibiotic profiles and metal resistances of the enteric bacteria isolated from the Mhlathuze River.

3.3 Materials and Methods

3.3.1 Effects of heavy metals on microbial growth

Equal volume (0.1 ml) of water sample from each site was spread onto a nutrient agar plate or inoculated into nutrient broth containing various concentrations (0 – 0.5 mg/L) of different heavy metals. The mixture was then incubated at 37 ^{0}C overnight. The growth of microbes was measured by determining the optical density at 600 nm and confirmed with the results from the nutrient agar plate.

3.3.2 Isolation of bacteria

Single colonies of bacteria were randomly selected from the nutrient agar, m-Endo and mFC plates which were inoculated with water from each site (see Chapter 2 - Material and Methods) based on the morphology and subsequently isolated in pure form.

All colonies from the nutrient agar plates with high concentrations of heavy metal, which inhibited the microbial growth, were also isolated for identification and analysis.

3.3.3 Identification of bacteria

The isolates were then identified by Gram staining and biochemical reactions and confirmed by API strips used according to the manufacturer's instructions (bioMerieux, France). Only members of the *Enterobacteriaceae* were used for antibiotic resistance tests. All isolates from heavy-metal nutrient agar were used for antibiotic resistance tests.

3.3.4 Antibiotic sensitivity tests

The disc diffusion method was used to determine antibiotic sensitivity of the isolates. Overnight broth cultures were spread on Hilton-Mueller agar plates. The plates were dried at room temperature for 2 hours. Antibiotic discs were placed at equi-distances. The plates were incubated for 24 h at 37°C and organisms were classified as sensitive, intermediate or resistant, based on the NCCLS standards.

The antibiotic discs used correspond with those commonly used in the treatment of human infections. The 15 antibiotics used in this study can be classed into 10 different groups as shown in the table 3.1.

Table 3.1: The classes and quantity of antibiotics used in the study

Class	Antibiotic used	Quantity in µg
Fluoroquinolones	Ciproflaxazine	5
Phenicols	Chloramphenicol	30
Aminocoumarin	Novobiocin	30
Folate Inhibitors	Co-trimoxazole	1.25
Ansamycins	Rifampicin	5
Quinolones	Nalidixic acid	30
Cephalosporin	Cefuroxime; Cephalothin; Cefotaxime; Cefoxitin	30
Aminoglycosides	Streptomycin; gentamicin	10
Tetracyclines	Tetracycline	30
β-lactamase	Ampicillin; Penicillin	10

3.3.5 Statistical Analysis

All percentage data were arcsine transformed before analysis. Paired t-tests (Wilkinson, 1988) were used to determine the statistical significance between different species. Spatial variability in antibiotic resistance was analysed with Chi-square test. Pearson's correlation coefficient (r) was used to represent the relationship between the clinical isolates and the *Enterobacteriaceae* isolates. The antibiotic resistance profiles of pathogenic isolates from patients who presented with severe diarrhoea at the clinic at the KwaDlangezwa during 2000 (Biyela 2000).were compared with the antibiotic resistance profiles from environmental isolates in this study.

3.4 Results

3.4.1 Antibiotic Resistance Levels

From February 2001 to January 2002, a total of 113 enteric bacteria were isolated and identified as follows: 42 *E. coli*; 27 *Citrobacter freundii;* 23 *Klebsiella* spp.; 11 *Enterobacter* spp.; 7 *Serratia marcesens* and 3 *Proteus* spp.. In addition several *Pseudomonas* and *Aeromonas* spp. were also identified.

All isolates were sensitive to the presence of gentamicin. Ciprofloxacin inhibited all microbial isolates with the exception of one *Pseudomonas* isolate. This isolate showed the highest level (12) of resistance to tested antibiotics (15) which covered all ten different classes tested (except the aminoglycosides). Cefotaxime inhibited the growth of all enteric bacterial isolates except one *Klebsiela* and one *Enterbacter* isolates. All *S. marcesens* isolates were resistant to Rifampicin. The proportional resistance of the total enteric bacteria to the different classes of antibiotics is shown in Table 3.2. 94.7% of enteric bacterial isolates were resistant to at least one antibiotic and 75.2% to two or more classes of antibiotics. The largest proportion of the isolates was resistant to three classes of antibiotics. Four enteric bacterial isolates, namely, 1 *E. coli* and 3 *S. marcesens*, were resistant to 8

out of 15 different antibiotics tested (6 or 7 classes of antibiotics). Amongst non-*E. coli* isolates, only two *Enterobacter* isolates were sensitive to all antibiotics. Other genera (*Klebsiela* spp, *S. marcesens, Serratia* spp. *C. freundii and Proteus* spp) were resistant to at least one antibiotic.

Table 3.2: Proportional resistance (%) of total isolates of *Enterobacteriaceae* from Mhlathuze River to different classes of antibiotics

Antibiotic Class	0	1	2	3	4	5	6	7
Proportion of total isolates	5.3	19.5	22.1	32.7	18.6	6.2	2.7	1.8
E. coli	9.5	19.0	19.0	35.7	7.1	7.1	0.0	2.4
Others	2.8	19.7	23.9	31.0	25.4	5.6	4.2	1.4

Table 3.3 shows antibiotic resistance patterns (ARP) of all *Enterobacteriaceae* isolated from each and all of the five sites during the study period. ARP of *E. coli* and non-*E. coli* isolates are also shown in the Table 3.3. The highest levels of resistance were to the following antibiotics: penicillin, 72.6%; rifampicin, 69.2%; novobiocin, 52.1%; ampicillin, 43.6% and cephalothin, 28.2 %. The ARPs of *E. coli* and non-*E. coli* groups were very similar. The non-*E. coli* group showed higher resistance against nalidixic acid, novobiocin, cefuroxime and cefotaxime compared to the *E. coli* isolates.

3.4.2 Comparison of antibiotic resistance pattern (ARP) from different sites

The differences in ARPs of the enteric bacteria isolated in this study were not significant amongst all five sites. However, there were some discrepancies. The highest percentage (85.7%) of isolates from Site 1 was resistant to penicillin compared to those at other sites. Generally, a high proportion of enteric bacteria isolated from the Richards Bay estuary (Site 3) were resistant to the majority of the antibiotics with the exception of cefotaxime and nalidixic acid. A low percentage of bacteria isolated from

KwaDlangubo (Site 2), which is about 40 km upstream exhibited resistance to antibiotics. Microbes resistant to nalidixic acid, novobiocin, chloramphenicol and rifampicin were mainly isolated from downstream (below Site 5; see Figure 2.1), while those resistant to tetracycline, cefotoxime and cefuroxime were mainly isolated from upstream (Sites 1 and 2).

Table 3.3: Percentage of total and site specific enteric bacterial isolates resistant to specific antibiotics during the period of February 2001 to January 2002

Antibiotic	A	P	T	S	CXM	NA	RIP	KF	TS	C	CTX	FOX	NO
Total (%)	43.6	72.6	9.4	16.2	5.1	6.8	67.0	26.6	10.3	4.3	1.7	6.0	52.1
E. coli	46.5	69.8	11.6	18.6	2.3	2.3	67.4	27.9	11.6	4.7	0	7.0	39.5
Others	40.6	75.4	7.2	15.9	7.2	10.1	66.7	26.1	8.7	4.4	2.9	4.3	58.0
Site 1 (%)	38.1	85.7	14.3	14.3	9.5	4.7	66.7	28.6	4.8	0	4.8	4.8	52.4
E. coli	55.6	88.9	11.1	22.2	0	0	55.6	33.3	0	0	0	11.0	44.4
Others	25.0	83.3	16.7	8.3	16.7	8.3	75.0	25.0	8.3	0	8.3	0	58.3
Site 2 (%)	42.3	65.4	11.5	11.5	3.9	3.9	61.5	26.9	11.5	3.9	3.9	3.9	34.6
E. coli	54.5	63.6	18.2	9.1	0	0	63.6	0	18.2	0	0	0	27.3
Others	33.3	66.7	6.7	13.5	6.7	6.7	60.0	46.7	6.7	6.7	6.7	6.7	40.0
Site 3 (%)	62.5	81.3	12.5	31.3	6.3	0	81.3	37.5	16.7	6.3	0	6.3	76.5
E. coli	60.0	100	0	60.0	0	0	80.0	80.0	0	0	0	20.0	80.0
Others	66.7	75.0	16.7	25.0	8.3	0	83.3	33.3	25.0	8.3	0	0	75.0
Site 4 (%)	40.9	63.6	4.5	18.2	4.5	9.1	77.3	13.6	9.1	4.5	0	9.1	63.3
E. coli	42.9	57.1	14.3	28.6	14.3	0	85.7	28.6	28.6	14.3	0	0	42.9
Others	40.0	66.7	0	13.3	0	13.3	73.3	6.7	0	0	0	13.3	73.3
Site 5 (%)	42.1	73.7	0	15.8	0	10.5	78.9	36.8	10.5	10.5	0	5.3	68.4
E. coli	20.0	40.0	0	0	0	0	80.0	40.0	0	20.0	0	0	40.0
Others	50.0	85.7	0	21.4	0	14.3	78.6	35.7	14.3	7.1	0	7.1	78.6

A, ampicillin; P, penicillin; T, tetracycline; S, streptomycin; CXM, cefuroxime; NA, nalidixic acid; RIP; rifampicin; KF, cephalothin; TS, co-trimoxazole; NO, novobiocin; C, chloramphenicol; CTX, cefotaxime; FOX, cefoxitin. All isolates are sensitive to the presence of gentamicin and ciprofloxacin.

3.4.3 Resistance by *E. coli* compared to other *Enterobacteriaceae* genus

The antibiotic resistance patterns of each *Enterobacteriaceae* genus isolated from the Mhlathuze River are shown in Table 3.4. A higher percentage of *S. marcesens* isolates showed resistance to tetracycline, streptomycin, cefuroxime, cephalothin, novobiocin, chloramphenicol and 100% resistance against rifampicin. Despite the fact that the majority of isolates that were resistant to nalidixic acid and cefotaxime belonged to the genus of *Enterobacter*, very few of the isolates from the same genus exhibited resistance to other antibiotics tested.

Table 3.4: Percentage of each *Enterobacteriaceae* genus/species resistance to each tested antibiotic from February 2001 to January 2002

Antibiotic (%)	A	P	T	S	CXM	NA	RIP	KF	TS	C	CTX	FOX	NO
E. coli	46.5	69.8	11.6	18.6	2.3	2.3	67.4	27.9	11.6	4.7	0.0	7.0	39.5
Klebsiella spp	47.8	70.0	0.0	8.7	4.3	8.7	73.9	21.7	4.3	0.0	4.3	0.0	60.9
C. freundii	33.3	88.9	3.7	18.5	0.0	11.1	70.4	25.9	11.1	3.7	0.0	11.1	66.7
Enterobacter spp	41.7	58.3	16.7	0.0	16.7	16.7	25.0	0.0	8.3	8.3	8.3	0.0	25.0
S. marcesens	42.9	71.4	28.6	57.1	28.6	0.0	100	85.7	14.3	14.3	0.0	0.0	71.4

3.4.4 Comparison of antibiotic resistance patterns of *Enterobacteriaceae* isolates from the Mhlathuze River and of clinical isolates from the diarrhoeal patients in this area

A comparison of the antibiotics resistance patterns of isolates from this study with that from the diarrhoeal patients (Biyela 2000) (Table 3.5) showed a strong correlation ($r=0.97$). Despite of the similarity, the clinical isolates were highly resistant to most of the antibiotics tested.

Table 3.5: A comparison of antibiotic resistance patterns of environmental isolates, isolates with resistance to heavy metals and clinical diarrhoeal isolates:

Antibiotic (%)	A	P	T	S	CXM	NA	C	CTX	CIP	KF	GTA
Environmental isolates											
Total isolates	43.6	72.6	9.4	16.2	5.1	6.8	4.3	1.7	0	26.6	0
E. coli	46.5	69.8	11.6	18.6	2.3	2.3	4.7	0	0	27.9	0
Clinical isolates*											
	75	80	17	16.9	16.9	18.3	4.2	1.4	2.8	nd	1.4
Heavy metal resistant isolates											
E. coli	nd	71.4	57.1	28.6	nd	57.1	14.3	nd	0	71.4	0
Pseudomonas spp	nd	100	83.3	16.7	nd	83.3	100	nd	0	100	16.7
Bacillus spp	nd	83.3	50.0	0	nd	66.7	50.0	nd	0	83.3	0

Abbreviation: See Table 1; CIP: ciprofloxacin; GTA: gentamicin.
* Biyela PT (2000)

3.4.5 The effects of the presence of heavy metals on microbial growth in water samples

The presence of Hg^{+2}, Cd^{+2} and Cu^{+2} inhibited the growth of microbes in the Mhlathuze River while Pb^{+2} and Al^{+3} generated little impact (Figure 3.1). The effects of heavy metal on microbial growth were similar regardless of the sources of water samples.

Several Gram positive (6 *Bacillus* spp and 3 *Staphylococci* spp) and negative microorganisms (7 *E. coli*, 6 *Pseudomonas* spp, 3 *Enterococci* and 2 *Proteus* spp) have been isolated from the media containing high

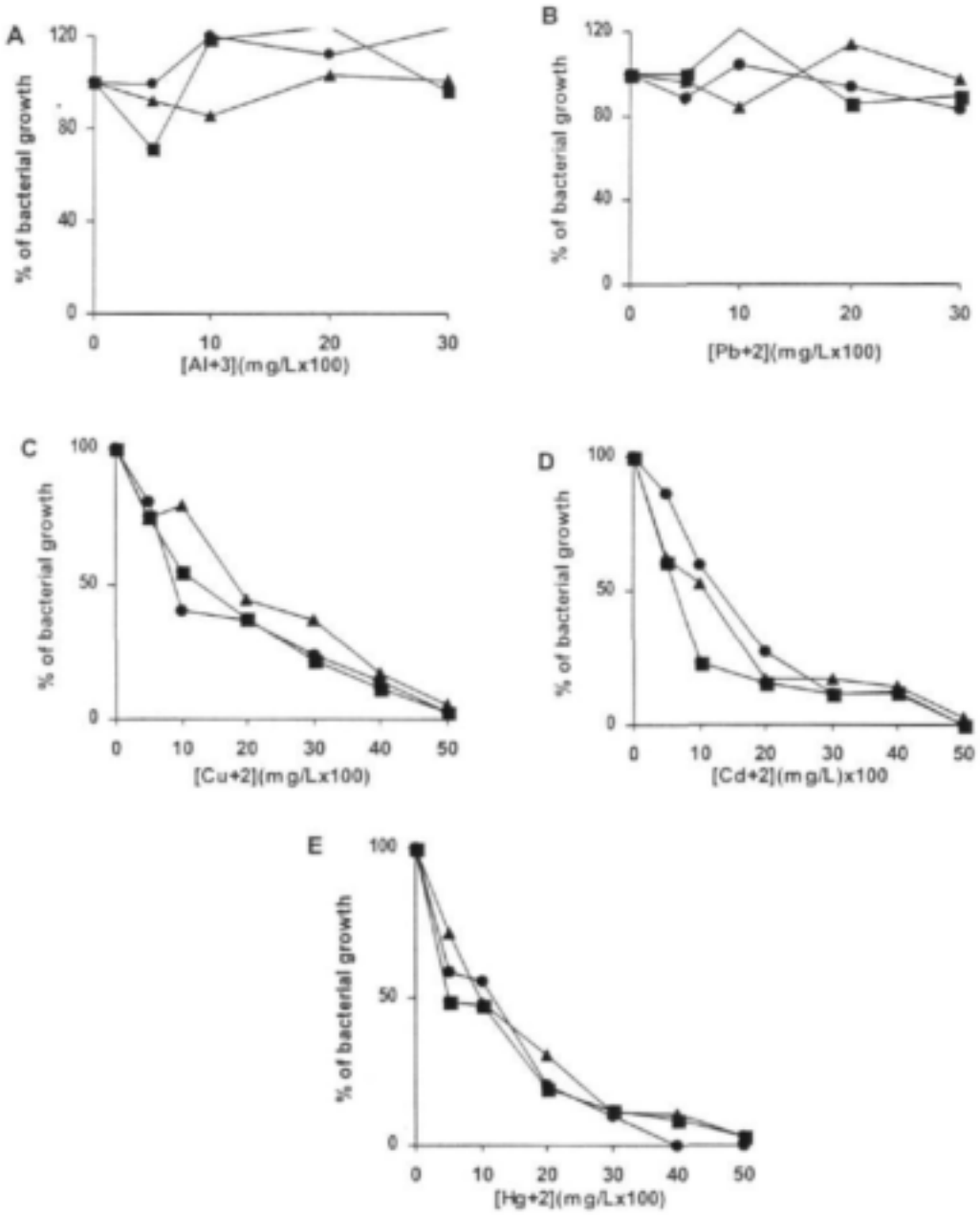

Figure 3.1: The effects of the presence of heavy metal on the microbial growth of the water samples from the Mhlathuze Rivers. ■: Site 2 KwaDlangubo; ◆: Site 4 Richard Bay Estuary; ▲: Site 5 Felixton.

concentrations of heavy metal (Hg^{+2}, Cd^{+2} and Cu^{+2}). The antibiotic resistance profile of these and other (*Enterobacteriaceae* and clinical diarrhoeal) isolates is shown in Table 3.5. The majority of the microorganisms (especially *Pseudomonas* isolates) isolated from the plates with high concentrations of heavy metal exhibited better resistance against the antibiotics tested. Gentamicin and ciprofloxacin remained the most potent antibiotics against the growth of heavy metal resistant isolates as observed in the growth of environmental isolates.

3.5 Discussion

The rural residents of the Mhlathuze catchment area who depend on the river for domestic use have been prone to water-borne diseases. The strong correlation (r=0.97) between the clinical and the environmental isolates in this region (Table 3.5) suggest a strong link of diarrhoeal incidence and the water quality in the region. Diarrhoeal outbreaks, which are strongly correlated with the water quality, continue to pose a public health risk in the community. Furthermore, this problem is compounded by the increasing incidence of pathogens with antibiotic and/or drug resistance (Ogan and Nwiika, 1993; DePaola et al., 1995). Antibiotic end-products following medical use (Balagué and García Véscovi, 2001; White et al., 2000) industrial effluents, pesticides and related compounds (Balagué and García Véscovi, 2001) and heavy metal contamination, accelerate the level of antibiotic contamination of the natural environment resulting in the development of resistance in communities of non-disease organisms. These organisms could create a widespread antibiotic resistant gene pool. (Kruse and Sorum, 1994) Antibiotic resistant genes can be transferred back to human and animal hosted pathogenic organisms, which generate significant health and economic impacts.

3.5.1 Antibiotic resistance patterns of the environmental isolates

The results from this water quality-monitoring project indicate high levels of the faecal contamination in this water system especially at Felixton (Site 5), which embraces more human, farming and industrial activities (Chapter 2). Several heavy

metals had been detected in this water system. The antibiotic resistance patterns of the enteric bacteria isolated from this study also show that 94.7 % of isolated enteric bacteria in the Mhlathuze River exhibit resistance to antibiotics. Multi-resistance is very common among these isolates (Table 3.2). Park et al. (2003) showed that 53.6% of coliform isolates from the freshwater system in Korea were resistant to one or more antibiotics tested.

The antibiotic resistance patterns (ARP) of the environmental isolates (as well as *E. coli* and non-*E. coli* groups) were very similar amongst all sites (Table 3.3) suggesting that this phenomenon is widely spread in the environment. Resistance by high proportions of all isolates against penicillin, ampicillin and cephalothin indicate that the β-lactamase genes should be widely present among the gene pool of microbes in the environment.

The results revealed some discrepancies in ARPs. Smaller percentages of the enteric bacteria isolated from KwaDlangubo (Site 2), which is about 40 km upstream, were resistant to antibiotics. Since this area has less human and industrial activities, the observations may suggest that the presence of antibiotic resistant microbes is related to the level of activity in the area. This is supported by other studies (Goñi-Urriza et al., 2000; McArthur and Tuckfield, 2000), which have shown that environmental influences such as the presence of heavy metal and industrial effluent affect the distribution of antibiotic resistant bacterial organisms. This idea is strengthened by the findings of differences in the resistance of the microbes to some antibiotics isolated from the Mhlathuze River upstream and downstream.

The sugar mill, paper pulping factories as well as agricultural and informal farming activities at site 5 may be contributory to the findings here. Greater numbers of microbes isolated from this site were resistant to chloramphenicol. Microbes which showed resistance to rifampicin were predominant downstream (from Felixton).

3.5.2 Effects of heavy metals on the survival of bacteria in the Mhlathuze River

Several heavy metals have been detected in Sites 3 and 5 (Chapter 1, Table 2.1). The Richards Bay estuary (Site 3) is brackish. The aluminum smelters and fertilizer manufacturing factories are located in the vicinity. A higher percentage of the enteric bacteria isolated from the Richards Bay estuary showed resistance against most of the antibiotics compared to those from other Sites.

The presence of the heavy metals in the environment influenced microbial growth and thereby could have posed a public health risk. Although 0.011 mg/L of Cd^{+2} was detected in the water sample (Table 2.1), this concentration did not affect the growth of microbes in this water (Figure 3.1). However, it has been reported to pose a threat to immuno-compromised individuals. (DWAF, WRC and DOH Guide, 1998)

An increase in concentration of Cd^{+2}, Hg^{+2} and Cu^{+2} to 0.5 mg/L inhibited the growth of microorganisms significantly (Figure 3.1). Several Gram-positive and negative bacteria survived even when the concentrations of heavy metals were high. The ARP results indicate that these isolates, especially *Pseudomonas* spp. which possess the ability to survive in high concentrations of heavy metals, are also better equipped to resist the presence of antibiotics.

The high resistance to rifampicin amongst the *Enterobacteriaceae* isolates in this study coincides with the finding that a higher proportion of new TB patients in this region (Ngwelezane Hospital) also showed resistance to rifampicin compared to those from the surrounding areas (Stanger and Manguzi) in the northern KwaZulu Natal (Lin et al. unpublished results). With poor sanitation facilities and the unavailability of infrastructure to supply drinking water, the high proportions of enteric bacteria which are resistant to multiple antibiotics is a major cause for concern. This will impact on medical disease management and the local economy (Lo Presti et al., 2000; White et al., 2000).

3.6 Conclusion

A large percentage of the enteric bacteria isolated from the Mhlathuze River demonstrated resistance to different classes of antibiotics. The results from this study indicate that the industrial and human activities might contribute to this phenomenon. The antibiotic resistant gene pool especially the β-lactamase genes is likely to be widely present in this environment. The microbes which showed resistance to heavy metal have also been isolated. Large numbers of these isolates also displayed resistance to antibiotics. It is thus imperative that the determination of antibiotic susceptibility/resistance patterns of isolated microbes becomes a part of the microbial monitoring process. The observations should be closely correlated with disease management practices and other possible factors that may contribute to antibiotic resistance (Harwood et al., 2000).

CHAPTER 4

MICROBIAL POPULATION AND DIVERSITY

4.1 Background

Microbial diversity is an under-valued national resource that deserves greater attention. Microbial diversity encompasses the spectrum of variability among all types of microorganisms (bacteria, fungi, viruses and many more) in the natural world and as altered by human intervention.

Microorganisms are essential for the earth to function. They play major roles to sustain the living of all species both on land and in water ((Madigan et al., 2003). Microorganisms exhibit extraordinary genetic and phenotic diversity. Despite their importance, less than 5% of the world's microorganisms have been described. Because of the revolution in microbiology, stemming from the development of new tools of molecular biology, databases are becoming more widely available as a source of molecular and macromolecular information on microorganisms.

The untapped diversity of microorganisms is a resource for new genes and organisms of value to biotechnology. The diversity patterns of microorganisms can also be used to monitor and predict environmental change, to understand biological interactions between species as well as to manage the outcome of antimicrobial therapy in future medical care and to facilitate epidemiological intervention. The early detection of trends and the identification of factors responsible for directing these trends by understanding the types, functions and diversities of organisms especially microorganisms in soil, water, land and other ecosystems are essential in the management, restoration and intervention of any ecosystem.

The results from previous parts of this study show that high levels of total and faecal coliform contamination in the Mhlathuze River occur. The majority of the

Enterobacteriaceae isolates from this water system carry antibiotic resistance gene(s) especially the β-lactamase genes. Similar observations have been reported by Ash et al. (2002) that several rivers in U.S. have become major reservoirs for antibiotic resistant microbes. Microbes containing heavy metal resistant genes also exist in the environment. Given the propensity of the *Enterobacteriaceae* family and clinically significant bacteria to acquire antimicrobial resistance, consistent surveillance of the agents commonly prescribed to treat infections arising from these organisms is imperative (Sahm et al., 2000). These organisms could create a widespread antibiotic resistant gene pool. These genes could be transferred into human and animal disease organisms (Kruse and Sorum, 1994), which subsequently could generate significant health and economic impacts.

Antibiotic resistant genes are carried on either chromosomes or mobile genetic elements e.g. plasmids; integrons etc. These mobile gene elements with antibiotic or metal resistance genes can be transferred between different species through transformation, conjugation and transduction (Madigan et al., 2003). It is therefore important to track antibiotic resistance genes in a variety of commensal; pathogenic and environmental bacteria in order to measure the environmental pool of resistance and to understand the ecology of antibiotic resistance (Aminov et al., 2001; Goni-Urriza et al., 2000). Aquatic (river) ecosystems are ideal since they may support a wide array of microorganisms e.g. indicator organisms, faecal and total coliforms; pathogens, animal and human; commensals, human and animals as well as environmental bacteria.

Until recently, studies on antibiotic resistance were only concerned with the collection of phenotypic data. However in order to study the environmental pool of resistant genes and to understand the ecology of antibiotic resistance the development of genotyping tools is indispensable. Drug resistance especially multiple-drug resistance in enteric organisms is often associated with integrons. Integrons generally contain an integrase gene (intI) and a cassette integration site

(attI), into which antibiotic resistance gene cassettes have integrated. A gene cassette contains an antibiotic resistance gene and a 59 base pair (bp), a short inverted repeat element with a recombination site. Four classes of chromosomal and plasmid–borne integrons in gram negative bacteria have been described (Bass et al., 1999). Class 1 integrons are the most common and have been found primarily in complete or truncated derivatives of the Mu- like transposon *Tn402*, which resides in a wide range of host plasmids or within *Tn21*. They generally contain the quartenary ammonium compound resistance gene qacEΔ and the sulphonamide resistance gene *sull* in the 3' conserved region. The 5' and 3' regions flank gene cassettes, which contain single or multiple antibiotic resistance gene(s). The integron acquires or exchanges antibiotic resistant genes with its specialized recombinase (Bass et al., 1999). These genetic elements are responsible for linking antibiotic resistance genes together to form large multiple loci of antimicrobial resistance within the genome.

4.2 Aims

The purpose of this chapter was firstly to study the general microbial diversity of the Mhlathuze River and secondly and most importantly, to characterize antibiotic resistance in isolates by determining (a) whether the genes responsible are chromosomal or plasmid bound (b) the stability of resistance conferring genes.

4.3 Materials and Methods

4.3.1 Microbial diversity of the Mhlathuze River

A series of ten-fold dilutions of the water samples from different sites were inoculated into different varieties of media, (Nutrient Agar, TSA, R2A). Plates were incubated at 25°C for a 2-week period. The plates were monitored everyday. The colonies with the distinct morphology from different media plates were randomly selected for further identification. The microbial identification was based on the results from the staining reactions, growing and nutritional, biochemical, physiological characteristics of each

individual microorganism. The results were confirmed using the commercial identification kits such as API 20E, API 20 NE if available.

4.3.2 Enterobacterial repetitive intergeneic consensus (ERIC) profiles of the *Enterobacteriaceae* isolates from the Mhlathuze River

The DNA sequence of ERIC primers is shown in Table 4.1. The PCR conditions for all experiments were 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 35 °C for 60 s and 72 °C for 2 min and finally for 4 min at 72 °C. All ERIC products were analyzed using 1% agarose gel and recorded by SynGene Gel Documentation system.

4.3.3 Detection of Integron 1 Gene Cassette using Polymerase Chain Reaction (PCR)

A total of 113 bacterial isolates belonging to the *Enterobacteriaceae* family and several *Pseudomonas/Aeromonas* spp were tested for their resistance/susceptibility patterns to a battery of 15 antibiotics, which are used as first line drugs in state hospitals and clinics. (Chapter 3) Forty-three isolates, which were resistant to more than 4 different antibiotics were further used for the genetic study.

4.3.4 DNA isolation

Total DNA isolation of each microorganism from the environmental sample was obtained using the heat-lysis method (Sandvang et al., 1997). In brief, one bacterial colony was suspended in 1.0 ml of phosphate buffered saline. The microorganisms were then centrifuged at 9000 g. The pellet was resuspended in 100µl of TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0). The suspension was boiled at 100°C for 10 min. The boiled suspension was stored at -20°C for future use. For each PCR reaction, 2 µl of the lysed suspension was used.

The plasmid extraction was performed using the GFX™ Micro plasmid Preparation Kit (Roche). The purified plasmid was re-suspended in 50 µl TE buffer and incubated overnight at – 4°C before use. The DNA profile of the plasmid from the isolates was analyzed on a 0.7 % agarose gel (stained with ethidium bromide) to determine their sizes. The information obtained was used to compare the plasmids isolated from the various isolates (Poirel et al., 1999).

4.3.5 PCR amplification of specific plasmid fragments/genes

Enterobacteriaceae often carry integrons that can be divided into class 1, 2 and 3. Class one integrons encode resistance to several antibacterial compounds and have been extensively studied in *Enterobacteiaceae* (Dalsgaard *et al.*, 1999).

DNAs (total and plasmid) from isolates that were resistant to 4 or more antibiotics were used for further characterization of antibiotic resistant genes, with special attention to those with class I integron-bound resistance. Different sets of primers were used to determine the presence of class 1 integron as well as the antibiotic resistant genes in each isolated plasmid. The sequences of all primers used are shown in the Table 4.1. The PCR conditions for all integrons experiments were 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 65°C for 30 s and 72°C for 2 min and finally for 5 min at 72°C.

Isolated plasmids yielding a PCR product with class 1 primers were further amplified with primers specific for certain antibiotics resistant genes which are shown in Table 4.1.

All PCR products were analyzed on agarose gel (1%). The gels were stained with ethidium bromide and visualised under UV light. A molecular weight size marker was also loaded on the gel for band size determination. Products of interest were cut and purified and sequenced for genetic diversity studies.

Table 4.1: The DNA sequences of the primers used in this study

Primer	Sequence	Reference
1.Int1 F[a] (5CS)	GGC ATC CAA GCA GCA AGC	Dalsgaard *et al.*, 1999
2.Int1B[a] (3CS)	AAG CAG ACT TGA CCT GAT	Dalsgaard *et al.*, 1999
3.QacΔE 1F	ATC GCA ATA GTT GGC GAA GT	Dalsgaard *et al.*, 1999
4.sul1 B	GCA AGG CGG AAA CCC GCG CC	Dalsgaard *et al.*, 1999
5.ant (3")	ATT GCC CAG TCG GCA GCG	Dalsgaard *et al.*, 1999
6.Ribo2-FW	GGMCAYRTGGATTTYWIGC	Aminov *et al.*, 2001
7.Ribo 2-RV	TCIGMIGGIGTRCTIRCIGGRC	Aminov *et al.*, 2001
8.VEBcasF	GGC ATC CAA GCA GCA AGC	Naas *et al.*, 1999
9.VEBcasB	CGG TTT GGG CTA TGG GCA G	Naas *et al.*, 1999
10.VEB-F	CGA CTT CCA TTT CCC GAT GC	Naas *et al.*, 1999
11.VEB-B	GGA CTC TGC AAC AAA TAC GC	Naas *et al.*, 1999
12.HS89	GGC TTG TTA TGA CTG T	Naas *et al.*, 1999
13.HS110	GGC TTA ACT CAG GCG T	Naas *et al.*, 1999
14.ORAB2 (sense)	TAT GAC GAT GTC CAG	Kern *et al.*, 2000
15.RK-3 (anti)	GAT GTA AAA AGC GCG ATT CG	Kern *et al.*, 2000

4.3.6 Conjugation Experiments

Conjugation was carried out for 16 isolates representative of the different antibiotic resistance patterns (10 *E.coli*; 3 *Klebsiella spp.*; 2 *C.freundii* and 1 *S. marcesens*). Depending on the resistance pattern of the isolates, two recipient strains were used - *E.coli* HB101, containing the streptomycin resistant gene and the *E.coli* CSH56, containing the nalidixic acid resistant gene in the respective chromosomes.

Broth cultures of the recipient and of the isolates, which were grown for 16hours at 37°C and mixed in a 1:5 (Donor:Recipient) ratio, were used for mating experiments. The cultures were precipitated by centrifugation and the pellet was resuspended in 50*u*l of nutrient broth. The suspensions were spotted onto non-selective nutrient agar plates and incubated overnight.

The mating aggregates were scraped from the agar plates and suspended in test tubes with 5ml normal saline. The tubes were vortexed for one minute to disrupt the mating pairs, and 1ml of the suspensions were precipitated again and washed 3 times in saline. The resultant cells were plated onto media with the antibiotics to select for the donors and transconjugants.

Table 4.2: The following antibiotics were used:

Antibiotic	Stock concentration	Final concentration
Ampicillin	100mg/ml	100*ug*/ml
Streptomycin	50mg/ml	50*ug*/ml
Nalidixic acid	25mg/ml	25*ug*/ml

Antibiotic sensitivity/resistance patterns of the transconjugants were determined and compared to those of the donors (environmental isolates) prior to conjugation. Plasmid DNA's were extracted from the successfully conjugated isolates. Molecular weights of the purified plasmids were determined using gel electrophoresis with a 0.7% agarose gel.

4.4 Results

All common microorganisms found in the freshwater environment were isolated using a variety of media, minimal salt agar, R2A, at room temperature and identified during the study. These microorganisms which included *E. coli, Bacillus, Chromobacterium, Corynobacterium, Alcaligene faecalis, Brucella, Neisseria, Flavobacterium, Micrococcus, Staphylcoccus and Streptococcus spp* as well as *Mycobacterium spp, Nitrobacter spp, Hafnia spp and Thiobacterillus spp* were widely distributed in the Mhlathuze River.

The microorganisms which were isolated from the nutrient agar, m-Endo and mFC plates at 37°C included mainly *Enterobacteriaceae, Pseudomonas* spp. and *Aeromonas hydrophila*. The *Proteus* spp were only isolated from the Richards Bay estuary. The populations of *E. coli* and *C. freundii* were usually small in the estuary while *Klebsiella, Pseudomonas/Aeromonas* and *Enterobacter* spp. were

well distributed at all five sites. No pathogenic *E. coli* strains or other water-borne pathogens, such as *Vibrio cholerae* and *Salmonella* spp., were detected from the samples from the Mhlathuze River during the study period.

The ERIC profiles of *E. coli* and *C. freundii* with multiple antibiotic resistance are shown in Figure 4.1. The results clearly reviewed the genetic diversity of the environmental isolates.

Of the 43 *Enterobacteriaceae* isolates that were used for the molecular characterization of antibiotic resistances, 25 yielded PCR products with the general integron 1 primer set. The size of the integrons ranged from 0.8-2.0 kb. These PCR products were purified and used to quantify the integrons with sets of diagnostic primers. The beta-lactamase gene (*pse*) was the most common, being present in 44% of the integrons. The aminoglycoside resistance gene (*ant*'3) was present in 16%. The two genes (*sul*1, coding for sulfonamide resistance and the *QACΔE*1, coding for resistance to quartenary ammonium compounds) that were associated with class 1 integrons were detected in 24% of the integrons (Table 4.1).

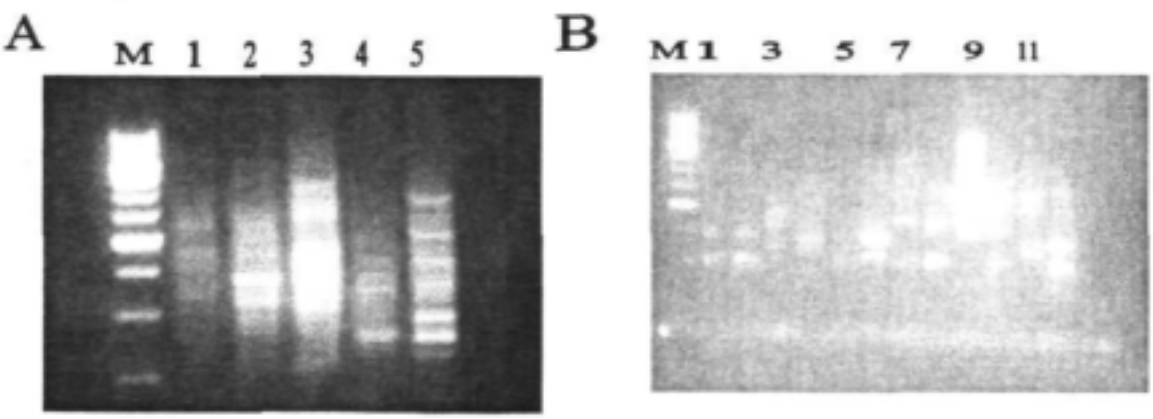

Figure 4.1: Examples of ERIC profiles of (A) *E. coli* (1: isolate 6/181601; 2: 15/141101; 3: 9/101001; 4: 9/031201 and 5: 12/031201) and (B) *C. freundii* (1: 4/031201; 2: 5/031201; 3: 3/101001; 4: 8/241001; 5: 2/180601; 6: 3/210102; 7: 8/141101; 8: 2/101001; 9: 10/260301; 10: 6/031201; 11: 3/010801; 12: 7/141001) with multiple antibiotic resistance isolated from the Mhlathuze River. M: molecular marker XVI (Roche Biochemical)

Table 4.3: The PCR detection of Class 1 Integron and the antibiotic resistance genes associated with the class 1 integron with multiple resistant environmental isolates from the Mhlathuze River

Isolate (# of R determinants)	Int1f & sul1b	Int1f & Int1B	Int1f & pseb	Int1f & ant'3	Sul1f & Qace1b
E.coli					
6/1806 (8)	+	+	+	-	-
9/0312(6)	+	+	+	-	-
10/210102(4)	+	+	-	-	-
12/0312(4)	+	+	-	-	-
9/1010(4)	+	+	-	-	-
11/0312(4)	+	+	-	+	+
15/1411(4)	+	+	-	+	+
Klebsiella spp.					
15/0312(5)	+	+	-	-	-
4/1109(4)	+	+	-	-	-
3/1806(4)	+	+	+	-	-
14/0312(4)	+	+	-	-	-
6/2304(3)	+	+	+	-	-
S.marcesens					-
9/2603(8)	+	+	+	-	-
Enterobacter spp.					
1/2603(4)	+	+	-	-	+
8/2603(3)	+	+	-	-	-
C.freundii					
4/0312 (7)	+	+	+	-	-
8/2410(6)	+	+	-	-	-
3/210102(5)	+	+	+	-	-
5/0312(5)	+	+	+	+	-
2/1806(5)	+	+	+	+	+
3/1010(5)	+	+	+	-	+
3/0108(4)	+	+	-	-	-
2/1010(4)	+	+	-	-	-
8/1411(4)	+	+	-	-	+
6/0312(4)	+	+	+	-	-

Int1f & sul1b, amplify the complete integron together with the associated genes;
int1f &int1l, amplify the variable region of the integron;
int1f &pseb amplify the gene that codes for resistance to beta-lactam antibiotics;
int1f &ant3'', amplify the gene that codes for aminoglycoside resistance;
Qacef &sul1b, amlify the two gene sequences that are associated with class 1 integrons i.e. the quartenary ammonium compound that codes for resistance to disinfectants and the sulfonamide resistance gene;
+, PCR product; -, no PCR product.

Table 4.4: The horizontal transfer of plasmid from the environmental isolates to the recipient cells through the conjugation processes

	Recipient: CSH56	Selection for donors (NA+Nal)	Selection of transconjugants (Na+Amp+Nal)
1	*E. coli* 6/180601	+	+
2	*E. coli* 5/260301	+	-
3	*E. coli* 9/031201	+	-
4	*E. coli* 4/120301	+	-
5	*E. coli* 9/210102	+	+
6	*S. marcesens* 1/241001	+	-
7	*Klebsiella spp* 15/031201	+	-
	Recipient: HB101	Selection for donors (NA+Sm)	Selection for transconjugants (NA+Amp+Sm)
1	*E. coli* 4/031202	+	-
2	*Klebsiella spp.*4/110901	+	+
3	*Klebsiella spp.*1/010801	+	+
4	*E. coli* 1/260201	+	-
5	*E. coli* 9/260201	+	+
6	*E. coli* 12/031201	+	+
7	15/141101	+	-
8	*C. freundii* 4/230401	+	+
9	*E. coli* 11/031201	+	+
10	*E. coli* 8/210102	+	+
11	*C. freundii* 5/031201	+	-

NA, nutrient agar; Nal, nalidixic acid; Amp, ampicillin; Sm, streptomycin; -, no growth observed; +, growth observed.

In the conjugation experiments, 6 of 10 *E.coli*, 2 of 3 *Klebsiella* spp and 1 of 2 *C. freundii* were successfully transferred to recipient cells. Antibiotic resistance patterns of the transconjugants were the same to those of the donors

(environmental isolates) with the exception of one *E. coli* isolate 12/031201. This *E. coli* isolate developed resistance to nalidixic acid after the conjugation process. The plasmids, which were transcongugated from the donors to the recipient cells, were about 10 Kb in size.

4.5 Discussion

Most of the microorganisms isolated from the Mhlathuze River were commonly found in the river water and soil. These microorganisms are mostly involved in the cycles of carbon, nitrogen and sulfur compounds (Madigan et al., 2003).

Enterobacteriaceae, *Pseudomonas* spp. and *Aeromonas hydrophila* were commonly isolated using nutrient agar, m-Endo and mFC agars at 37°C. (Goni-Urriza et al., 2000) *Aeromonas hydrophila*, is associated with gastroenteritis and *Pseudomonas aeruginosa*, on the other hand, reportedly affects patients that are either immuno-compromised or have metabolic or haematological disorders (Pearson et al., 2000). The presence of opportunistic pathogens including *Klebsiella* spp. may have serious implications for the consumers of the river water, especially young children, the elderly and those infected with HIV/AIDS. According to statistics of the Department of Health of South Africa, KwaZulu Natal has a HIV prevalence rate of 32.5 %, the highest in South Africa. (Health Department KZN, Annual report, 1999) In 1999 the Joshini/Empangeni region had 30 % of the state hospital bed space occupied by HIV patients (Health Department KZN, Annual report, 1999).

The results of the Enterobacterial Repetitive Intergeneic Consensus (ERIC) profiles of the *Enterobacteriaceae* isolates from the Mhlathuze River strongly suggest that the *Enterobacteriaceae* isolates possess a high degree of genetic diversity within the same species in the area. More samples are required to confirm these observations.

Other parts of this study showed high levels of enteric bacterial contamination in the Mhlathuze River. The majority of the *Enterobacteriaceae* isolates from this water system exhibited resistance to the presence of antibiotics. These organisms could

create a widespread antibiotic resistance gene pool, which subsequently can have consequences on the health system and the economics of the province. The emergence of multiple-drug resistance amongst bacterial isolates, as revealed by this study is a cause for concern amongst medical, veterinary and public health professionals. This is due in part to the ability of microorganisms to acquire new resistance genes. (Bunny et al., 1995) It is therefore important to track antibiotic resistance genes in a variety of commensal; pathogenic and environmental bacteria in order to measure the environmental gene pool of resistance and to understand the ecology of antibiotic resistance. (Aminov et al., 2001; Goni-Urriza et al., 2000).

Drug resistance especially multiple-drug resistance in enteric organisms is often associated with integrons especially Class 1 integrons. Fifty-eight percent of multiple antibiotic resistant enteric bacterial isolates in this study possessed the class 1 integron, which is much higher than that reported by Park et al. (2003) who showed that 24% of 150 coliform isolates contained class 1 integrons.

The results from this study show that some isolates possessed more than one copy of class1 integrons which varied in size. These findings are supported by Daly and Fanning (2000) who found two copies of class1 integrons with different molecular sizes present in one bacterial isolate. Integron sizes vary depending on how many cassettes are inserted adjacent to the integrase gene.

Amongst the 25 isolates with positive detection of class 1 integron, the beta-lactamase gene (*pse*) was the most common being present in 56% of these integrons. The aminoglycoside resistance gene (*ant*'3) was found in 16% of the integrons. However, there was no clear correlation between antibiotic resistance patterns and the integron/gene profiles as reported by Park et al. (2003). Park et al. (2003) suggest that in the absence of sustained antibiotic pressures, such as the natural environment, coliform bacteria may carry empty or non-functional class 1 integrons.

In the science of antibiotics resistance, the mutation rate is frequently defined as in-vitro frequency at which defective mutants arise in a bacterial population in the presence of a given antibiotic concentration. Therefore phenotypic data reports only selectively favourable bacterial mutations. The problem is complicated by the fact that the phenotype does not always reflect the same genotype in all "selected" mutants, because mutations in different genes can produce similar antibiotic resistance phenotypes. The level of expression of antibiotic resistance genes in integron associated cassettes is affected by a number of factors, e.g. cassette position. Collis and Hall (1995) reported that the expression of genes was greatest when the cassette was proximal to the promoter and reduced by the presence of preceding cassettes. This is because the efficiency of translation could be affected by the presence of upstream cassettes. Loss of upstream cassettes lead to an increase in the resistance conferred by the gene. Another factor that might influence the level of expression of antibiotic resistance genes is the copy number of the plasmid. All these factors may be significant not only in the maintenance but also in the spread of antibiotic resistance between environmental and clinical bacteria.

The results of this study show that a pool of antibiotic resistant genes exists within this water system. The difference in integron sizes and random distribution of the integrons across different genera indicates the possibility of horizontal gene transfer within this river system. A high degree of genotypic diversity and the lack of correlation between antimicrobial resistance patterns and molecular types of the isolates suggest convergent acquisition of resistance determinants by genetically unrelated strains rather than epidemic spread of resistant isolates in the community.

With the ability to transfer amongst different bacterial species, mobile genetic elements (i.e. integrons and plasmids) have the capacity to disseminate antibiotic resistance within bacteria in the aquatic environment. In this study conjugation was successful in 56% of the cases studied. The antibiotic resistance profiles of those transconjugant cells were the same with those of donors with the exception of one *E. coli* transconjugant.

Three main mechanisms of gene transfer, namely transformation, transduction and conjugation, occur naturally in aquatic environments. (Madigan et al. 2003) Aquatic environments are reservoirs for a large gene pool of bacterial DNA. Extracellular DNA can be derived from dead and viable cells. Many viable cells release DNA without cell lysis suggesting that a release of genetic materials may be a normal function of bacteria. In the aquatic environment free or bound DNA may be moved by percolation of water, flows of air, water or dust. The half-life of free DNA is longer in aquatic environments (0.017-235 hours) compared to that in terrestrial environments (9.1-28.2 hours) (Yin and Stotzky, 1997). In the natural environment, the presence of stressors e.g. antibiotics, heavy metals, decreased nutrient levels, microbial competition for the available space and nutrients etc. are capable of inducing a physiological state of competence in bacterial cells 'enabling' the bacteria to actively take up DNA from the environment.

4.6 Conclusion

From the results of this diversity study one can conclude that there exists a pool of resistance genes within the Mhlathuze River. Commensal bacteria are important reservoirs of antibiotic resistance genes. The ability of commensal organisms to carry resistance genes of clinical importance and their ability to transfer such genes to other bacteria is of greater concern than phenotypic measurements. This suggests the possibility of transmission of resistance genes from the commensal organisms to pathogenic organisms. This calls for studies to determine the extent to which transmission of antibiotic resistant bacteria occurs and the extent to which such transfer impacts on the efficacy of antibacterial use in human medicine.

CHAPTER 5

CONCLUSION and RECOMMENDATIONS

5.1 Conclusions

The present project monitored the microbial quality and the related physical and chemical variables of the Mhlathuze River, for the period, 1998 to 2002. The project also investigated the status of antibiotic resistance amongst the environmental isolates and their ability to transmit the resistance genes through the plasmid between different bacteria.

The results show that the bacteriological 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 and recreational purposes according to the DWAF (1996) guidelines while most of the physical and chemical parameters are within the acceptable levels of water quality for domestic use.

Although contamination of the river was generally constant over time, there were periods when very large increases in bacterial numbers were experienced. The following environmental factors as observed by other researchers (Byamukama, 2000; Pretorius, 2000; Solo-Gabriele et al., 2000) influenced the elevated levels of faecal and total coliform bacteria: (i) Activities (domestic, recreational, agricultural, industrial) along the river. (ii) Rainfall and (iii) Surface water temperatures. The resuscitation results also demonstrated strongly that the faecal coliform contamination level could not present a clear picture of the water quality in any water system.

Based on the results of the antibiotic resistance profiles of the environmental isolates, the Mhlathuze River has become a major reservoir for antibiotic resistant microbes. Microbes containing heavy metal resistant genes also exist in the environment. Higher levels of antibiotic resistance amongst the clinical and

heavy metal resistant isolates compared to those of the environmental isolates strongly support the idea that the sustained presence of antibiotics and/or industrial effluents enhances the ability of microbes to resist the presence of antibiotics/drugs.

Based on the results, one can also conclude that there exists a wide pool of antibiotic resistance genes within the Mhlathuze River. The commensal bacteria could be important reservoirs for these antibiotic resistance genes and their vectors. The differences in the size and random distribution of the integrons across different genera indicate the possibility of horizontal gene transfer within this river system. A high degree of genotypic diversity and the lack of correlation between antimicrobial resistance patterns and molecular types of the isolates suggest convergent acquisition of resistance determinants by genetically unrelated strains rather than epidemic spread of resistant isolates in the community. The ability of commensal organisms to carry resistance genes of clinical importance and their ability to transfer such genes to other bacteria is of greater concern than phenotypic measurements. The possibility of transmission of resistance genes between bacteria (especially pathogenic) which invade human and animal populations within this River poses a health risk to the communities who are dependant on this river for consumption.

5.2 Recommendations and the Future Research

Human (domestic, recreational, agricultural and industrial) activities along the river have been shown to be one of the dominant factors affecting the water quality. Therefore proper management and monitoring of human activities along the river will be the key to improve the status of the water quality of the Mhlathuze River. Provision of a proper infrastructure for the supply of drinking water and basic sanitation especially in the rural area may reduce significantly the burden of this water resource. This will ensure public health.

A continuous monitoring programme such as the NMMP is important. However, the use of faecal coliform bacteria as indicators should be expanded within a

wider context and other parameters such as physical, chemical as well as the status of antibiotic resistance profiles of microbes in the natural environment should be also monitored. More attention is needed to evaluate the impact of the VBNC state of pathogens in the environment. It should be equally important to develop more sensitive monitoring techniques such as the bacterial and chemical source tracing techniques or the so called biosensor to investigate the source(s) of the contamination to ensure proper management.

The molecular/genetic approaches provide alternative means to in-depth understanding of the water system. Our genetic studies also suggest the importance of continuing studies to determine the extent to which transmission of antibiotic resistant bacteria from humans to animals occurs and the extent to which such transfer impacts the efficacy of antibacterial use in human medicine.

The molecular/genetic approaches also can provide information on the diversity of species in the natural environment. The diversity patterns of microorganisms can be used to monitor and predict environmental change, to understand biological interactions between species as well as to manage the outcome of antimicrobial therapy in future medical care and to facilitate epidemiological intervention. The early detection of trends and the identification of factors responsible for directing these trends by understanding the types, functions and diversities of organisms especially microorganisms in soil, water, land and other ecosystems are essential in the management, restoration and intervention of any ecosystem.

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- The determination of the quality of water at the point of provision at selected sites;
- The examination of usage patterns, for all domestic purposes as well as farming. The quantity of water used as well as the identification of water treatment by the end-user prior to use;
- The development of surveillance techniques as well as the testing and evaluation of such techniques, incorporating sanitary inspection and water quality monitoring; and
- The prioritisation of remedial action strategies to protect the user from the risk of water-borne diseases.

The following interim conclusions have been reached:

- Intersectoral co-operation plays a key role in planning and operating a water quality surveillance programme.
- There is an urgent need for training material or guidelines for the implementation of surveillance programmes.
- Water quality problems that were noted include:
- Faecal coliform pollution of surface water is widespread in the district under surveillance. Two clinics in the district had experienced major outbreaks of water- borne disease (*Shigella*, gastro-enteritis, diarrhoea)
- Human users share water sources with domestic animals such as pigs and cattle.
- The majority of water end-users do not make use of water purification methods
- Elevated concentrations of iron and manganese and salt were identified in a significant number of dams and boreholes.
- The effects such as staining of clothing or unpleasant taste caused users to switch to unsafe surface water.
- Consumer involvement and effective transfer of information is all-important.

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