



**A COMPARATIVE MICROBIOLOGICAL ASSESSMENT OF
RIVER BASIN SITES TO ELUCIDATE FECAL IMPACT AND THE
CORRESPONDING RISKS**

**Submitted in partial fulfillment for the Degree of Master of Applied Sciences in
Biotechnology in the Department of Biotechnology and Food Technology, Durban
University of Technology, Durban, South Africa**

Ayanda Sithebe

Promoter/ supervisor:

Prof Thor Axel Stenström, SARChI

Chairholder

Co-supervisors:

Dr Gulshan Singh and Prof Faizal Bux

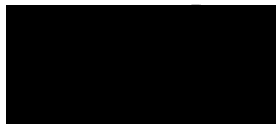
REFERENCE DECLARATION

I, Ms Ayanda Sithebe, 21005944 and Prof Thor Axel Stenström do hereby declare that in respect of the following dissertation:

Title: A comparative microbiological assessment of river basin sites to elucidate fecal impact and the corresponding risks

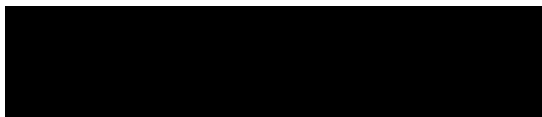
As far as we are certain:

1. No other similar dissertation exists
2. All references as detailed in the dissertation are complete in terms of all personal communication engaged in and published works consulted.



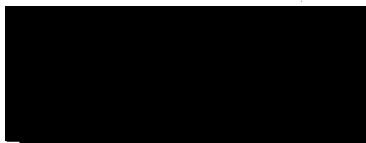
Signature of student

28/8/2017
Date



Signature of promoter/ supervisor

28/8-17
Date



Signature of co-promoter/ co-supervisor

29/8/2017
Date

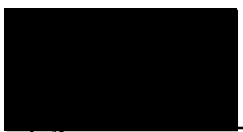


Signature of co-promoter/ co-supervisor

28/08/2017
Date

AUTHORS DECLARATION

I, **Ayanda Sithebe** declare that the study presents original work by the author. It has not been submitted before for any degree or examination to any other University. Where use was made of the work of others, it has been duly acknowledged in the text. The research described in this dissertation was carried out in the Institute for Water and Wastewater Technology, Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, South Africa, under the supervision of **Prof Thor Axel Stenström, Prof Faizal Bux and Dr Gulshan Singh.**

A solid black rectangular box used to redact the student's signature.

Student's signature

TABLE OF CONTENTS

| | |
|---|-------------|
| ACKNOWLEDGEMENTS | I |
| ABBREVIATIONS | III |
| PUBLICATIONS | VII |
| NATIONAL AND INTERNATIONAL CONFERENCES | VIII |
| TABLE OF FIGURES | IX |
| LIST OF TABLES | XI |
| CHAPTER 1 : INTRODUCTION | 1 |
| RESEARCH OBJECTIVES | 6 |
| CHAPTER 2 : LITERATURE REVIEW | 7 |
| 2.1 WATER QUALITY..... | 7 |
| 2.2 MONITORING OF WATER QUALITY IN SOUTH AFRICAN RIVERS | 7 |
| 2.3 POOR WATER QUALITY THREATENS HUMAN HEALTH..... | 10 |
| 2.4 POLLUTION SOURCES | 10 |
| 2.5 ROUTINE MONITORING AND INDICATOR ORGANISMS | 12 |
| 2.5.1 COLIFORMS | 15 |
| 2.5.2 ESCHERICHIA COLI (E. COLI) | 17 |
| 2.5.3 INTESTINAL ENTEROCOCCUS..... | 17 |
| 2.5.4 BACTERIOPHAGES | 18 |
| 2.6 FACTORS THAT AFFECT MICROBIAL CONCENTRATION..... | 19 |
| 2.6.1 TEMPERATURE..... | 19 |
| 2.6.2 RAINFALL | 20 |
| 2.6.3 SEDIMENTS | 20 |
| 2.7 CONVENTIONAL TECHNIQUES USED FOR THE DETECTION OF BACTERIAL, VIRAL | |
| INDICATORS AND PATHOGENIC BACTERIA | 22 |

| | |
|---|-----------|
| 2.7.1 MEMBRANE FILTRATION AND SPREAD PLATE METHOD..... | 22 |
| 2.7.2 DETECTION USING CHROMOGENIC SUBSTRATES | 23 |
| 2.7.3 MOST PROBABLE NUMBER METHODS | 23 |
| 2.7.4 DEFINED SUBSTRATE METHODS | 24 |
| 2.8 MOLECULAR METHODS BASED ON GENETIC SIGNATURE OF TARGET PATHOGEN | 24 |
| 2.8.1 POLYMERASE CHAIN REACTION TECHNIQUE AND QUANTITATIVE PCR..... | 25 |
| 2.8.2 DIGITAL PCR | 27 |
| 2.9 WATERBORNE OUTBREAKS IN SOUTH AFRICA | 27 |
| 2.10 QUANTITATIVE MICROBIAL RISK ASSESSMENT..... | 28 |
| CHAPTER 3 : COMPARISON OF BACTERIAL INDICATORS IN SURFACE | |
| WATER AND SEDIMENTS AT THE ISIPINGO AND PALMIET RIVERS | 31 |
| 3.1 INTRODUCTION | 31 |
| 3.2 METHODOLOGY..... | 34 |
| 3.2.1 DESCRIPTION OF STUDY AREA..... | 34 |
| 3.2.2 HISTORICAL DATA ANALYSIS: | 37 |
| 3.2.3 BACTERIAL CULTURE | 39 |
| 3.2.4 SAMPLE COLLECTION | 39 |
| 3.2.5 SEDIMENT SAMPLE PREPARATION | 40 |
| 3.2.6 PHYSICOCHEMICAL CHARACTERISTICS OF SURFACE WATER TO DETERMINE MICROBIAL QUALITY OF WATER | 40 |
| 3.2.7 ENUMERATION OF BACTERIAL INDICATORS: IDEXX COLILERT 18 AND ENTEROLERT TEST METHODS | 40 |
| 3.2.8 ANALYSIS OF THE EFFECT OF RAINFALL ON MICROBIAL CONCENTRATION TO ADDRESS VARIABILITY OVER TIME | 41 |
| 3.2.9 STATISTICAL ANALYSIS | 41 |
| 3.3 RESULTS..... | 42 |
| 3.3.1 A) HISTORICAL DATA ANALYSIS; ISIPINGO RIVER | 42 |

| | |
|---|-----------|
| 3.3.1 B) HISTORICAL DATA ANALYSIS; PALMIET RIVER | 44 |
| 3.3.2 PHYSIOCHEMICAL PROPERTIES | 46 |
| 3.3.3 E. COLI AND ENTEROCOCCI QUANTITIES IN ISIPINGO AND PALMIET RIVER’S SURFACE WATER AND SEDIMENT | 46 |
| 3.3.4 EFFECT OF RAINFALL ON MICROBIAL CONCENTRATION TO ADDRESS VARIABILITY OVER TIME | 48 |
| 3.4 DISCUSSION | 50 |
| 3.4.1 A) HISTORICAL DATA ANALYSIS; ISIPINGO RIVER | 50 |
| 3.4.1 B) HISTORICAL DATA ANALYSIS; PALMIET RIVER | 52 |
| 3.4.2 PHYSIOCHEMICAL PARAMETERS | 53 |
| 3.4.3 E. COLI AND ENTEROCOCCI QUANTITIES IN ISIPINGO AND PALMIET RIVER’S SURFACE WATER AND SEDIMENT | 53 |
| 3.4.5 EFFECT OF RAINFALL ON MICROBIAL CONCENTRATION TO ADDRESS VARIABILITY OVER TIME | 55 |
| 3.5 CONCLUSIONS & RECOMMENDATIONS | 56 |
| CHAPTER 4 : PERFORMANCE OF CONVENTIONAL AND MOLECULAR | |
| METHODS | 58 |
| 4.1 INTRODUCTION | 58 |
| 4.2 METHODOLOGY | 62 |
| 4.2A METHODOLOGY FOR COMPARISON OF CONVENTIONAL METHODS WITH COLILERT-18 AND ENTEROLERT METHOD | 63 |
| 4.2.1A DESCRIPTION OF STUDY AREA..... | 63 |
| 4.2.2A SAMPLE COLLECTION | 63 |
| 4.2.3A ENUMERATION OF BACTERIAL INDICATORS USING METHOD 1 (MEMBRANE FILTRATION (MF) / SPREAD PLATE METHOD) | 63 |
| 4.2.4A ENUMERATION OF BACTERIAL INDICATORS USING METHOD 2 (COLILERT AND ENTEROLERT IDEXX METHOD) | 64 |

| | |
|--|---------------|
| 4.2.5A STATISTICAL ANALYSIS..... | 64 |
| 4.3A RESULTS..... | 65 |
| 4.4A DISCUSSION | 66 |
| 4.5A CONCLUSION & RECOMMENDATIONS | 69 |
| 4.2B METHODOLOGY FOR PILOT STUDY CONDUCTED USING QPCR AND DDPCR FOR THE DETECTION OF SALMONELLA IN SEDIMENT SAMPLES..... | 70 |
| 4.2.1B SAMPLING SITES DESCRIPTION | 70 |
| 4.2.2B BACTERIAL STRAIN AND OLIGONUCLEOTIDES | 70 |
| 4.2.3B COMPARISON OF SENSITIVITY OF QPCR AND DDPCR IN SPIKED WATER SAMPLE | 71 |
| 4.2.3.1B QPCR: GENERATION OF STANDARD CURVE AND SENSITIVITY ANALYSIS IN SPIKED WATER SAMPLES..... | 71 |
| 4.2.3.2B DROPLET DIGITAL PCR: SENSITIVITY ANALYSIS IN SPIKED WATER SAMPLES..... | 72 |
| 4.2.3.3B COMPARISON OF QPCR AND DDPCR PERFORMANCE FOR QUANTITATIVE ENUMERATION OF SALMONELLA SPP., IN SEDIMENT SAMPLES | 73 |
| 4.3B RESULTS OF COMPARISON OF QPCR AND DDPCR | 74 |
| 4.4B DISCUSSION..... | 76 |
| 4.5B CONCLUSION | 78 |
| CHAPTER 5 : COMPARATIVE ASSESSMENT OF RISKS ASSOCIATED WITH FECAL CONTAMINATION IN SURFACE WATERS AND ON IRRIGATED CROPS | 79 |
| 5.1 INTRODUCTION | 79 |
| 5.2 MATERIALS AND METHODS..... | 84 |
| 5.2.1 DESCRIPTION OF STUDY AREA | 84 |
| 5.2.2 SURFACE WATER COLLECTION | 85 |
| 5.2.3 MICROBIAL QUALITY OF LEAFY VEGETABLES IRRIGATED WITH RIVER WATER | 85 |
| 5.2.4 QPCR METHODOLOGY | 85 |
| 5.2.5 QUANTITATIVE MICROBIAL RISK ASSESSMENT (QMRA) APPROACH..... | 87 |

| | |
|--|------------------------------|
| 5.3 RESULTS | 90 |
| 5.3.1 QUANTIFICATION OF INV A AND STX 2 GENE COPIES IN PALMIET RIVER AND ISIPINGO RIVER | 90 |
| 5.3.2 QUANTITATIVE MICROBIAL RISK ASSESSMENT (QMRA) APPROACH TO DETERMINE RISK OF INFECTION POSED BY SALMONELLA AND PATHOGENIC E. COLI EXHIBITING INV A AND STX2 GENES RESPECTIVELY AS WELL AS CAMPYLOBACTER AND SHIGELLA SPP. | 92 |
| 5.3.3 SUB-POPULATION RISK: IN RELATION TO RECREATIONAL AND OCCUPATIONAL ACTIVITIES | 96 |
| 5.3.4 QUANTITATIVE MICROBIAL RISK ASSESSMENT (QMRA) APPROACH TO DETERMINE RISK OF INFECTION POSED BY SALMONELLA EXHIBITING INV A GENE ON CROPS IRRIGATED WITH SURFACE WATER FROM ISIPINGO RIVER | 97 |
| 5.4 DISCUSSION | 98 |
| 5.4.1 STX2 AND INV A GENE DATA AND RISK RELATED TO SAMPLING SITES | 98 |
| 5.4.2 CAMPYLOBACTER SPP AND SHIGELLA SPP | 99 |
| 5.4.3 SUB-POPULATION RISK: IN RELATION TO RECREATIONAL AND OCCUPATIONAL ACTIVITIES | 100 |
| 5.4.4 CROPS IRRIGATED AT THE ISIPINGO RIVER | 101 |
| 5.5 CONCLUSION | 102 |
| 5.6 RECOMMENDATIONS | 102 |
| CHAPTER 6 : CONCLUSION AND RECOMMENDATION | 104 |
| 6.1 CONCLUSION | ERROR! BOOKMARK NOT DEFINED. |
| 6.2 RECOMMENDATIONS | ERROR! BOOKMARK NOT DEFINED. |
| REFERENCES | 107 |
| APPENDICES | 145 |
| APPENDIX 1: SUPPORTING INFORMATION FOR CHAPTER 3 | 145 |
| APPENDIX 2: SUPPORTING TABLES FOR QMRA (CHAPTER 5) | 146 |
| APPENDIX 3: FIRST PAGES OF PUBLISHED OUTPUTS | 147 |

ACKNOWLEDGEMENTS

My God almighty who has been the source of my strength through it all.

My husband Thabani Nzimande, for being my biggest supporter, motivator and best friend through the good and hard times of my research journey.

My dear grandmother Pretty Sithebe for your love and support. My greatest appreciation goes to the entire Sithebe and Nzimande families for their patience, love and encouragement throughout my research career.

I would like to appreciate my supervisors, especially Dr Gulshan Singh for all her supervision in the laboratory, hard work and always making herself available even during after working hours, and Prof Thor Axel Stenström for his constructive criticism, advice, guidance, and mentorship, as well as Prof Faizal Bux. You all have contributed immensely to my career and growth as a young researcher.

My deepest appreciation goes to the community members from the Quarry road informal settlements, which surrounds the Palmiet river, as well as the Umlazi community surrounding the Isipingo river, and the farmers especially Mr Zulu who helped with the crops. This study would not have been possible without the help from members of these two communities.

I would also like to acknowledge the Ethekewini Water and Sanitation Department (Chris Fennemore, Sibusiso Dlamini, and Noeleen) for all the help, as well as Mr Edward Mwandla and his team from the Isipingo WWTP who assisted a lot during sampling close to Isipingo.

The South African Weather services for supplying rainfall results used in this study.

Dennis Amoah, Folasade Adeyemo, Julian T. Arran and the awesome trio in-service trainees (Kholeka Mthembu, Lesantha Moodley, Busisiswe Mzindle) thank you for your constructive advice and your assistance in the lab over the years.

A special thank you to the entire IWWT department, my SARChI family, Naledi Mhlabelwana and Jabulile Zulu your friendship and advice over the years has found a special place in my heart. Thank you dearly for such priceless deposits.

Great appreciation to Durban University of Technology and South African Research Initiative (SARChI) for funding this project and providing me with a Scholarship.

ABBREVIATIONS

| | | |
|----------------|---|--|
| ANOVA | : | Analysis of Variance |
| APHA | : | American Public Health Association |
| ATCC | : | American Type Culture Collection |
| CBD | : | Central Business District |
| CDC | : | Centers for Disease Control and Prevention |
| CFU | : | Colony Forming Units |
| CSIR | : | Council for Scientific and Industrial Research |
| DAEC | : | Diffusely Adherent <i>E. coli</i> |
| DEAT | : | Department of Environmental Affairs Tourism |
| DWA | : | Department of Water Affairs |
| DWAF | : | Department of Water Affairs and Forestry |
| DNA | : | Deoxyribonucleic acid |
| DALYs | : | Disability-Adjusted Life Years |
| ddPCR | : | droplet digital PCR |
| EAEC | : | Enteraggregative <i>E. coli</i> |
| <i>E. coli</i> | : | <i>Escherichia coli</i> |
| EIEC | : | Enteroinvasive <i>E. coli</i> |

| | | |
|-------|---|--|
| EHEC | : | Enterohemorrhagic <i>E. coli</i> |
| EPEC | : | Enteropathogenic <i>E. coli</i> |
| ETEC | : | Enterotoxigenic <i>E. coli</i> |
| EWS | : | eThekweni Water and Sanitation |
| FC | : | Fecal Coliforms |
| FDIS | : | Final Draft International Standard |
| FIB | : | Fecal Indicator Bacteria |
| FISH | : | Florescence <i>in situ</i> Hybridization |
| GBD | : | Global Burden of Diseases |
| HIV | : | Human Immunodeficiency Virus |
| ICC | : | International Convention Centre |
| IFPRI | : | International Food Policy Research Institute |
| IE | : | Intestinal Enterococci |
| ISO | : | International Organization for Standardization |
| JMP | : | Joint Monitoring Program |
| KZN | : | KwaZulu-Natal |
| MF | : | Membrane Filter |
| MPN | : | Most Probable Number |

| | | |
|--------|---|--|
| MDG | : | Millennium Development Goals |
| MST | : | Microbial Source Tracking |
| MUG | : | 4- methyl-umbelliferyl- β -D-glucuronide |
| NCBI | : | National Center for Biotechnology Information |
| NMMP | : | National Microbial Monitoring Programme |
| ONPG | : | Ortho-Nitrophenyl- β -galactoside |
| PBS | : | Phosphate Buffered Saline |
| PCR | : | Polymerase Chain Reaction |
| Pinf | : | Probability of infection |
| QMRA | : | Quantitative Microbial Risk Assessment |
| qPCR | : | Quantitative Polymerase Chain Reaction |
| QRI | : | Quarry Road Informal Settlements |
| RAPD | : | Random Amplification of Polymorphic DNA |
| RFLP | : | Restriction fragment length Polymorphism |
| RHP | : | River Health Programme |
| SA | : | South Africa |
| SANS | : | South African National Standards |
| SARChi | : | South African Research Chairs Initiative |

| | | |
|--------|---|---|
| SD | : | Standard deviation |
| SEM | : | Standard error of the mean |
| ST | : | Sediment Trap |
| S/W | : | Surface Water |
| SOP | : | Standard Operating Procedure |
| STEC | : | Shiga toxin-producing <i>E. coli</i> |
| TC | : | Total Coliforms |
| TDS | : | Total Dissolved Solids |
| UNICEF | : | United Nations Children's Fund |
| USA | : | United State of America |
| USEPA | : | United States Environmental Protection Agency |
| UV | : | Ultraviolet |
| WET | : | Water Environment Technology |
| WIOMSA | : | Western Indian Ocean Marine Science Association |
| WWTP | : | Wastewater Treatment Plant |
| WHO | : | World Health Organization |
| WRC | : | Water Research Council |
| WWAP | : | World Water Assessment Programme |

YWP : Young Water Professional

PUBLICATIONS

Published book chapter:

- **Ayanda Sithebe**, Gulshan Singh and Thor Axel Stenström. 2016. A comparative microbiological assessment of the Isipingo river and Palmiet river in KZN province. In book: “Microbes in the spotlight: recent progress in the understanding of beneficial and harmful microorganism.” Brown Walker Press, Editor: A. Méndez-Vilas, ISBN-10: 1627346120, ISBN-13: 9781627346122, pages 2-6.

*The book is peer-reviewed, published and internationally distributed.

Publication refers to results presented and discussed in Chapter 3, first page is included in Appendix 3.

- Gulshan Singh, **Ayanda Sithebe**, Abimbola M Enitan, Sheena Kumari, Faizal Bux, Thor Axel Stenström. 2017. "Comparison of droplet digital PCR and quantitative PCR for the detection of Salmonella in sediments". Journal of Water and Health. Available Online 4 April 2017, wh2017259; DOI: 10.2166/wh.2017.259

Publication refers to results presented and discussed in Chapter 4, first page is included in Appendix 3.

Papers submitted/ in manuscript:

- **Ayanda Sithebe**, Gulshan Singh and Thor Axel Stenström “Prevalence and impact of rainfall on microbial variability in surface water and sediments in Isipingo and Palmiet Rivers, KwaZulu-Natal”. Submitted in Journal of Physics and Chemistry of the Earth: 17th WaterNet Symposium special issue.

Publication refers to results presented and discussed in Chapter 3, first page is included in Appendix 3.

- Gulshan Singh, **Ayanda Sithebe**, Isaac Dennis Amoah, Anthony A Adegoke and Thor Axel Stenström. “Potential Exposure Risk Associated with the High Prevalence *E. coli* O157:H7 and *Salmonella* in sediments and surface water” (To be submitted).

Popular articles/ publications:

Press release on The Mercury Newspaper titled “New light shed on pollution in two Durban rivers” on the 24th May 2016. **First page is included in Appendix 3.**

NATIONAL AND INTERNATIONAL CONFERENCES

Oral presentation

- VI International Conference on Environmental, Industrial and Applied Microbiology BioMicroWorld 2015, Barcelona (Spain), **28-30 October 2015** “A comparative microbiological assessment of the Isipingo River and Palmiet River in Kwa-Zulu Natal province to elucidate health risks” **Ayanda Sithebe**, Gulshan Singh and Thor Axel Stenström
- WISA 2016, ICC Durban, **15-19 May 2016**. “Assessment of seasonal variation in microbial quality of surface water of two rivers in KwaZulu-Natal” **Ayanda Sithebe**, Gulshan Singh and Thor Axel Stenström
- 1st Interdisciplinary Research and Postgraduate conference at Durban University of Technology, South Africa, **3-5 October 2016**. “Evaluation of the effect of rainfall on microbial river water quality” **Ayanda Sithebe**, Gulshan Singh and Thor Axel Stenström
- 17th WaterNet/WARFSA/GWPSA Symposium: Gaborone, Botswana on **26th – 28th October 2016**. “Microbiological assessment of the Isipingo and Palmiet River in Kwa-Zulu Natal province to elucidate health risks.” **Ayanda Sithebe**, Gulshan Singh and Thor Axel Stenström

Poster presentation

- 4th YWP-ZA Biennial Conference and 1st Africa-wide YWP at the CSIR International Convention Centre in Pretoria, South Africa, **16-18 November 2015**. Topic: “Fecal indicator bacteria and *Salmonella* associated with sediments and surface water” **Ayanda Sithebe**, Gulshan Singh and Thor Axel Stenström

Study visit: Chalmers University, Gothenburg Sweden (1st to 21st December 2016):

- Presented “A snapshot into South African rivers and microbial contamination” at Chalmers University of Technology to the division of Water Environment Technology (WET), on the 19th of December 2016
- Presented “A snapshot into South African rivers and microbial contamination” at Tyréns Foundation to a team of expert consultants on the 13th of December 2016.

Other outputs:

Assigned as the Durban University Young Water Professional (KZN) institutional champion for the years 2016-2018 (www.ywp-za.org/kwazulu-natal-committee.html)

TABLE OF FIGURES

| | |
|--|----|
| Figure 2.1: Examples of environmental indicator organisms of water quality. (Adopted from Shanker et al., 2013) | 16 |
| Figure 2.2: Different steps involved in Quantitative Microbial Risk Assessment tools (QMRA) analysis..... | 29 |
| Figure 3.1: An overview of Isipingo River and sampling points..... | 36 |
| Figure 3.2: An overview of the Palmiet River and the sampling sites | 37 |
| Figure 3.3: <i>E. coli</i> (Log10 MPN/ 100 mL) concentration along the Isipingo River over a period of 5 years and 6 months (2009-2014) | 42 |
| Figure 3.4: Comparison of the mean <i>E. coli</i> (Log10 MPN/100 mL) concentration along the Palmiet River over a period of 66 months (2009-2014) | 44 |
| Figure 3.5: Variability in <i>E. coli</i> and <i>Enterococci</i> concentrations in surface water over the time span of 6 hrs in case of two events AS1 (no rainfall) and AS2 (after a heavy rainfall) analysed using an auto sampler (3700C compact portable sampler, Teledyne Isco, USA). | 48 |
| Figure 3.6: Comparison of <i>E. coli</i> and <i>Enterococci</i> concentrations in surface water and sediments in AS1 (no rainfall) and AS2 (after a heavy rainfall) analysed using auto sampler and sediment trap. | 49 |
| Figure 4.1: Graphical representation of the comparison of conventional and enzyme based methods for detection of <i>E. coli</i> and <i>Enterococci</i> | 62 |
| Figure 4.2: Graphical representation of the sampling sites selected for the pilot study conducted at the Palmiet River..... | 70 |
| Figure 4.3: Schematic representation of the strategy adopted to compare qPCR and ddPCR | 74 |
| Figure 5.1: Activities that occur at the Isipingo river with potential linked exposure risks | 82 |
| Figure 5.2: Activities that occur at the Palmiet river showing a high degree of solid waste... | 83 |
| Figure 5.3: Flow diagram of methodology | 84 |

| | |
|---|----|
| Figure 5.4: Melt curve analysis of qPCR amplification product (A-B), A: Standard curve, B: Melt curve of qPCR amplified product from 10-fold serially diluted genomic DNA of <i>Salmonella Typhimurium</i> ATCC 13076 targeting <i>invA</i> gene..... | 90 |
| Figure 5.5: Melt curve analysis of qPCR amplification product (A-B), A: Amplification curve, B: Melt curve of qPCR amplified product from 10-fold serially diluted culture of <i>E. coli</i> ATCC 35150 targeting <i>stx2</i> gene. | 91 |
| Figure 5.6: Probability of infection based on <i>E. coli stx2</i> gene along the Isipingo and Palmiet Rivers at sampling sites of interest. | 93 |
| Figure 5.7: Probability of infection based on <i>Salmonella, invA</i> gene, along the Isipingo and Palmiet Rivers at sampling sites of interest. | 94 |
| Figure 5.8: Probability of infection in relation to <i>Campylobacter</i> spp. and <i>Shigella</i> spp..... | 95 |

LIST OF TABLES

| | |
|---|----|
| Table 2.1: WHO global guidelines values and DWAF South African standards, specifying the acceptable limits of indicator organisms for water intended for drinking, irrigation and recreational purposes | 14 |
| Table 3.1: Sampling point description, location, and potential pollution influences | 35 |
| Table 3.2: Summary statistics of <i>E. coli</i> Log10 MPN/100 mL from Isipingo River (66 months, 2009 -2014)..... | 43 |
| Table 3.3: Summary statistics of <i>E. coli</i> Log10 MPN/100 mL along the Palmiet River (66 months, 2009-2014) | 45 |
| Table 3.4: Mean physico-chemical parameters of the Isipingo river and Palmiet river | 46 |
| Table 3.5: Indicator organisms in surface water versus sediments at the Isipingo River | 47 |
| Table 3.6: Mean indicator organisms in surface water versus sediments along the Palmiet River | 47 |
| Table 4.1: Selective media and incubation conditions used for the detection and enumeration of indicator organisms retrieved from Isipingo and Palmiet Rivers | 64 |
| Table 4.2: Mean \pm SD comparison of conventional method vs Colilert-18/ Enterolert (IDEXX) method along Isipingo and Palmiet Rivers | 65 |
| Table 4.3: Unpaired t-test results obtained when comparing different methods. | 66 |
| Table 4.4: Quantitative analysis of <i>Salmonella</i> through <i>ttr</i> gene based qPCR in water samples spiked with serially diluted culture of <i>Salmonella enteritidis</i> ATCC 13067..... | 74 |
| Table 4.5: Quantitative analysis of <i>Salmonella</i> through <i>ttr</i> gene based ddPCR gene in water samples spiked with serially diluted culture of <i>Salmonella enteritidis</i> ATCC 13067..... | 75 |
| Table 4.6: Comparison of qPCR and ddPCR performances in sediments samples collected from upstream and downstream of Quarry road informal settlements in Palmiet River..... | 76 |
| Table 5.1: Sampling points identity as per labelling on figures and graphs | 85 |

| | |
|---|-----|
| Table 5.2: Parameters for the dose response models (beta-poisson) | 89 |
| Table 5.3: Quantitative analysis of <i>Salmonella</i> and <i>E. coli</i> through <i>invA</i> and <i>stx2</i> gene based qPCR in surface water samples over a 6 months period..... | 91 |
| Table 5.4: <i>Salmonella invA</i> gene and risk reduction rate on spinach irrigated with surface water from the Isipingo River | 97 |
| Table 6.1: Exposure assumptions relating to recreational and occupational activities..... | 146 |
| Table 6.2: Multi-barrier approach according to (WHO, 2006) | 146 |

ABSTRACT

The study aims to assess and compare the concentration of microbial contaminants, their sources and distribution in surface water and sediment, and to determine the impact of seasonal variations and corresponding risks of faecal contamination using conventional and molecular methods. Historical data analysis was conducted using *E. coli* values from the eThekweni Water and Sanitation (EWS) department for 66 months (2009-2014). *E. coli* and *Enterococci* were analysed in surface water and sediment samples using the mFC/ spread plate and Colilert-18 (IDEXX) methods. The impact of seasonal variations was assessed using *E. coli* and *Enterococci* data collected during rainfall and no rainfall events, using an auto-sampler and sediment trap in parallel. Conventional standard membrane filtration methods using mFC agar, Slanetz & Bartley/ Bile Esculin and Brilliance *E. coli* selective agar were compared to the enzymatic Colilert-18 and Enterolert (IDEXX) test methods along the Isipingo and Palmiet Rivers. In addition, comparison of the analytical performance of droplet digital PCR (ddPCR) and qPCR for the detection of *Salmonella* targeting *ttr* gene in river sediment samples collected from the four sites of the Palmiet River in Durban, South Africa was done. In order to assess the public health risk associated with exposure of men, women and children to microbial pathogens in polluted surface water during recreational activities, the QMRA tool was employed in relation to the risk exposure to pathogenic *E. coli*, *Campylobacter*, *Salmonella* and *Shigella*. Also, the risk associated with crop irrigation (on farmers) as well as the consumption of crops irrigated with surface water from the Isipingo river was determined.

Analysis of the historical data gave a baseline of the two rivers of interest, thus helps understand the current situation of the rivers enabling researchers to pick up potential gaps. In this study after the analysis of the historical data it was evident that at the Palmiet river, microbial analysis must be conducted around the QRI settlements which is a major pollution source.

Also, from this study it was found that sampling points situated close to wastewater treatment plants, pump stations or informal settlements were of major concern, thus were considered for the study. It was found that sediment exhibited higher microbial concentrations than surface water, which was observed in both rivers. Also, rainfall had a significant impact on microbial variability. Higher microbial concentrations (indicator organisms) were observed in surface water after a heavy rainfall as oppose to when there was no rainfall. This was due to contamination that is washed off into the river and sediment resuspension. Methodology comparison revealed that Colilert-18 and Brilliance *E. coli* were more selective compared to mFC agar. Brilliance *E. coli* /Coliform agar was comparable with Colilert-18 IDEXX, which was also observed with Slanetz & Bartley and Enterolert IDEXX. However, when mFC agar was compared with Colilert-18 IDEXX, significant difference was observed. In comparison of two Molecular methods, ddPCR were found to be fully amenable for the quantification of *Salmonella* and offer robust, accurate, high-throughput, affordable and more sensitive quantitation than qPCR in complex environmental samples like sediments.

Quantitative Microbial Risk Assessment (QMRA) relating to recreational and occupational exposure showed that children were at the highest risk of getting infected. Also, it was observed that the probability of infection upon exposure to surface water from the Isipingo and Palmiet rivers was significantly high, hence exceeded the WHO guidelines values. Risk assessment on crops revealed that pathogenic bacteria may pose a risk to the consumer, however, a 9-log reduction may be achieved according to the WHO multi-barrier approach which involves proper washing and proper cooking of the crop before ingestion.

Overall the sampling points that had the highest pollution level and constantly exceeded the WHO and DWAF guidelines at the Isipingo river were the points situated and named “Next to the WWTP”, and “Downstream of QRI” at the Palmiet River.

CHAPTER 1 : INTRODUCTION

Water is a fundamental natural resource that is essential to human and animal life, the environment, industrial growth, development, food production, hygiene and sanitation as well as power generation (DWA, 2013). In spite of the value of water as a precious commodity (DEAT, 2005), it suffers from pollution and overexploitation, which is of great human and environmental concern globally.

The deteriorating quality of river water is a major threat to South Africa's ability to provide adequate water of appropriate quality to meet its population's needs and to ensure a sustainable environment (Singh and Lin, 2015). The majority of drinking water in South Africa is drawn from surface water bodies such as lakes and rivers and treated for further use. It is very important that the source water for drinking water intakes as well as public water supplies in general are kept clean with minimal pollution, in order to reduce both public health risks and economic costs of treating the water. However anthropogenic activities as well as seasonal variations are determinants of the variability of the microbial water quality (Singh and Lin, 2015, Schauer et al., 2006, Cha et al., 2010). Protecting a source water body would be much more economical than treating unprotected water in order to obtain clean drinking water, this however is dependent on the local conditions (http://www.who.int/water_sanitation_health/dwq/sheet4.pdf). In spite of the general understanding and recognition that surface waters are often polluted from their surrounding environment, the protection of surface waters is often partly neglected. Catchment protection is a complex practice, that must be done both on a local and regional level with the help and involvement of the surrounding community members of a particular water body and needs to be appreciated and supported in order to obtain longstanding results (Elbag, 2006, Dufour et al., 2003).

According to the Joint Monitoring Program (JMP) report, over 90% of the world's population now has access to improved water sources (WHO/UNICEF, 2015). However, in many developing countries, like South Africa, water scarcity is a major and growing challenge (Sibanda et al., 2013). This is especially true for the rural communities where inhabitants are still using surface water of inadequate microbial quality from rivers and streams for domestic purposes such as laundry, bathing and personal hygiene and irrigational purposes (Mthembu et al., 2011, Luyt et al., 2012, Grabow, 1996). The water quality of these rivers and streams is usually significantly compromised by animal faeces, from wastewater pump stations, during pipe outbursts, industries and run-off from agricultural areas that channel their waste directly into rivers (Teklehaimanot et al., 2014, Momba et al., 2006, Okoh et al., 2007). South Africa has a population of 53 million (Stats SA, 2015), of which only an additional 31% of the total population in the year 2015 gained access to improved sanitation facilities since the year 1990 (WHO/UNICEF, 2015). Although the estimated Millennium Development Goals (MDG) target was not reached, moderate progress was achieved. This means that there is still people who practice open defecation. The inadequate sanitation and lack of access to microbiologically safe drinking water due to infrastructural problems force South Africans to supplement their drinking water consumption with water resources of poor microbial quality (WHO, 2014). Therefore, a significant proportion of residents in rural communities are exposed to pathogens that may lead to water-borne diseases. Also, the corresponding risks, due to contaminants, pathogenic organisms, as well as chemicals from industries is a major concern (Cabral, 2010). According to the World Health Organization, the global disease burden could be prevented by 10% simply by improving people's access and use of safe drinking water as well as resource management (Prüss-Üstün et al., 2008).

Utilization of water supplies of poor sanitary quality has been associated with various illnesses in the human population. The illnesses often present gastrointestinal-related symptoms such as

diarrhoea and nausea (Cabral, 2010). Organisms identified as waterborne fecal bacterial have been conventionally recognised responsible for waterborne outbreaks of gastrointestinal illnesses. Pathogenic *Escherichia coli*, *Salmonella*, *Shigella*, *Campylobacter*, *Yersinia*, *Giardia* and *Cryptosporidium* are well established fecal pathogens and parasites that have a history of being responsible for waterborne outbreaks (Pandey et al., 2014). Assessment of infectious microorganisms is based on their virulence and possibility to cause illness in humans, which also depends on the microorganisms' ability to survive in a particular environment. Depending on the type of microorganisms, the minimal infective dose (MID) varies (Leggett et al., 2012, Bitton, 2005).

According to the World Health Organization, diarrhoeal diseases is responsible for 1.5 million deaths and 3.6% of DALYs globally every year (http://www.who.int/water_sanitation_health/diseases-risks/en/). This is due to unsafe water, sanitation and hygiene resulting in 361 000 deaths yearly, of children under age five solely due to diarrhoeal diseases, especially in developing countries (<http://www.who.int/mediacentre/news/releases/2017/water-sanitation-hygiene/en/>). Children who are malnourished or have impaired immunity as well as people living with Human Immunodeficiency Virus (HIV) are at higher risk than the general population of life-threatening diarrhoea (Prüss-Üstün et al., 2008). This is partly due to the lack of access to, and use of, potable water, which has forced many inhabitants of informal settlements to rely on surface water sources for their daily needs, thus exposing them to waterborne diseases (Olaniran et al., 2009).

Sources of fecal contamination found within a river catchment can be differentiated as point source and non-point source contamination. In an urbanised area, the major point source of fecal bacteria is from wastewater treatment plants effluents and overflows, since most of the residents are connected to sewers that drive their wastewater to the municipal wastewater plant.

Fecal pollution brought to the rivers through surface runoff represents an example of a non-point source; its origin can be from informal settlements as well as from wildlife and grazing livestock faeces and also cattle manure spread on agricultural land and other cultivated areas (Wye et al., 1996, Wyer et al., 1997).

There are a number of different types of bacterial pathogens that can be present in human and animal wastes, and they can vary significantly in their distribution, depending on the sources of contamination affecting the water supply. Routine monitoring for all these organisms remains difficult and impractical, while conducting detection and identification procedures for each possible type can be very difficult and require significant resources. As a result, several indicators of fecal contamination are used to detect fecal pollution in natural waters. The abundance of these indicators may relate to the potential presence of pathogenic microorganisms from fecal origin and is thus an indication of the sanitary risk associated with the various types of water utilisations such as, domestic use, bathing and drinking (Servais et al., 2007, Bonde, 1962, Health Canada, 2013)

The United States Environmental Protection Agency (USEPA), World Health Organization (WHO) and the National Microbial Monitoring Programme (NMMP) of South Africa recommend the use of the indicator *E. coli* to assess the hygienic safety of recreational waters which a catchment represent (USEPA, 1986, Luyt et al., 2012, WHO, 2001). In society, water quality is of utmost importance, thus regular monitoring of water quality is necessary due to increased survival and persistence of microorganisms in natural water, therefore exposure to it may lead to public health concern. The hygienic quality of the natural waters is mostly assessed based on fecal indicator bacteria (*E. coli*) specifically in surface water, however the sediment is often overlooked. Thus, the potential of particle associated microorganisms to pollute the

surface water during recreational or occupational activities may pose a health risks particularly during resuspension. (Abia et al., 2016a).

The eThekweni municipality generally measure the quality of river water once a month, by collecting grab samples and analysing them according to Standard Operating Procedures (SOPs) using the Colilert-18 IDEXX test method. These cultural-based methods are widely accepted because of their relative ease of use, low cost, and demonstrated relationship to health risk.

The Isipingo and Palmiet Rivers situated in KwaZulu-Natal, Durban (South Africa) are increasingly being used for contact recreation, irrigation and other domestic purposes because of the unavailability of other fresh water. However, there is not much information that has been published in relation to the microbial pollution of these two rivers. To the best of our knowledge, no published works are available on the health risk associated with the microbial contamination of aquatic sediments and surface water of these two rivers or the impact that seasonal variation has on both. Although, a large proportion of microorganisms are particle bound, this is not reflected in normal monitoring.

Therefore, the aim of this study is to assess and compare the concentration of microbial contaminants, their sources and distribution in surface water and sediments as well as to determine the impact of seasonal variations and corresponding risks of fecal contamination.

Research objectives

In order to achieve the overall aim of this study, specific objectives (numbered) were established and detailed as follows:

1. To analyse the historical data from the eThekweni municipality of the Isipingo and Palmiet rivers at selected sites during the past five and a half years (66 months).
2. To analyse water samples for the presence of indicator organisms (thermotolerant coliforms, *Escherichia coli*, intestinal enterococci (IE) using conventional methods as well as molecular fingerprinting and make comparative methodological assessments.
3. To assess and compare the quantities of particle associated microorganisms upstream and downstream of selected sites (informal settlements and wastewater outfall affected areas) along the Isipingo and Palmiet Rivers.
4. To determine the variability of these microbial quantities due to seasonal variations, overflows, heavy rains (in order to account for pollution that is washed off into the river) and the differences in occurrence between sediments and surface water.
5. To determine the impact and corresponding risks of fecal contamination, based on the theoretical assessment of certain pathogens such as *Salmonella*, *Shigella* and *Campylobacter* on human health and as waterborne diseases.

To determine if the water from Isipingo and Palmiet rivers can be judged as suitable for crop irrigation based on current microbial guidelines for good microbial quality

CHAPTER 2 : LITERATURE REVIEW

2.1 Water Quality

Water quality is defined based on the qualitative characteristics of water in relation to guideline values which relate to its use as drinking water and whether it is considered best for consumption, basic domestic needs, recreational purposes as well as irrigation of food crops (WHO, 2008). The challenges of water quality degradation and water scarcity, have made water quality a protruding subject globally (Tundisi, 2008). Microbial (and other biological), chemical, and physical aspects are all important components and indices of water quality. The microbial aspect deals with the potential occurrence of pathogenic microorganisms that should not be present in drinking water appropriately termed as potable water (Du Plessis, 2017). These represent different potential routes of transmission of waterborne diseases to humans (Murray, 2004). Potable water is most often treated by different means, such as chemical coagulation, filtration and/ or disinfection so that it meets established drinking water guidelines or standards as set by authorities such as the World Health Organization (WHO) on a global level or national regulatory authorities such as South African National Standard (SANS) (WHO, 2008, SABS, 2011, WHO, 2004). This water is expected to be accurately free from harmful bacteria and contaminants and should be considered “safe” for drinking, cooking and other food preparation purposes. Municipal water, that has been chlorinated, filtered, distilled or purified, falls in the category of potable water. Hence, water treatment regimes including disinfection and the execution of bacteriological surveillance programs has resulted in decreased occurrence of water-related illness (DWAF, 2005).

2.2 Monitoring of Water Quality in South African Rivers

South Africa is a semi-arid country and water availability is one of our key limitations to development. The Department of Water Affairs and Forestry (DWAF) is currently responsible for water resources management at a national level and has in place a national monitoring

program with established procedures for assessing the microbiological and chemical water quality of surface waters (DWAF, 2007). In order to facilitate the management of water resources, the country has been divided into 19 catchment-based water management areas whereby the Department of Water Affairs and Forestry (DWAF) together with Government Departments is co-responsible for managing water resources (DWA, 2013). Weekly to monthly sampling is conducted at 1600 monitoring stations at various river systems across Southern Africa and the assessments form part of the River Health Program through the State of Rivers initiative (WRC, 2002, RHP, 2005). The initiative collects, stores and interprets the data in a systematic and quality-controlled manner which then allows each section of a river to be allocated different river health categories (natural, good, fair or poor) based on the water quality situation (RHP, 2005, WRC, 2002, DWAF, 2004). Monitoring points are partly located far upstream in the catchments and are generally not representative of the loads that ultimately enter the estuaries or marine environment and can be difficult in providing a national indication of the microbiological water quality (WIOMSA, 2009). In South Africa only a few rivers qualify as truly natural systems. River systems that qualify are located in protected areas such as national or provincial parks and wilderness areas. However, the downstream parts of these rivers may be impacted by upstream developments that are located outside of the protected area. A typical example is that of most rivers that flow through the Kruger National Park (DWAF, 2004).

Discharge from wastewater treatment plants has been one of the major factors that negatively affect the quality of river water in South Africa, and often contain high microbial loads (Luyt et al., 2012, Teklehaimanot et al., 2014, Okoh et al., 2007). Department of Water Affairs and Forestry (DWAF) conducts assessments on the performance of wastewater treatment plants that are run by all water authorities and service providers in South Africa. The criteria used in assessing water authorities and service providers include: assessment of operational staff,

wastewater quality monitoring, wastewater sample analysis, submission of wastewater quality results, wastewater quality compliance, management of quality failures, storm water management, review of bylaws, publication of wastewater quality performance, wastewater asset management and wastewater treatment works capacity. Water authorities and service providers that meet the criteria and achieve an overall score above 90% are awarded a “green drop” certificate for that particular treatment plant (Mupariwa, 2012). The Green Drop certification is a regulation that is incentive-based, it focuses on the entire wastewater business of the water services institutions. It is an important driver for enhancing and altering sub-standard wastewater management services, whilst recognising competency and rewarding excellence. The Green Drop certification ensures that wastewater treatment processes continue without interruption. This puts great responsibility and diligence on the wastewater services in ensuring that public health is protected and the environment is conserved for future generations (Mupariwa, 2012, DWA, 2013).

Out of 27 wastewater treatment plants that were assessed in Durban during the year 2009, only 11 were “green drop” certified and the Isipingo WWTP was one of them. In 2011, the number of WWTP that complied for a “green drop” certificate was reduced to nine when the Isipingo and Verulam treatment plants lost their green drop status (Perkins, 2013, DWAF, 2011). The Green Drop status implies excellent wastewater management and a respect for the environment and the health of the community at large, which encourages municipalities and holds them accountable for water quality monitoring (Perkins, 2013, DWA, 2013, Mupariwa, 2012).

This also ensures that effluents discharged into small tributaries like the Isipingo river are of good microbial quality, since these small tributaries eventually feed into large rivers like the Umgeni River which upstream serve as Kwa-Zulu Natal’s drinking water source.

2.3 Poor Water Quality threatens human health

The country's water resources have been severely jeopardized due to rapid demographic changes which have coincided with the establishment of informal settlements without access to basic necessities (Fatoki et al., 2001). A large part of the population do not have access to adequate domestic water supplies while millions of South Africans still lack access to appropriate sanitary infrastructure, forcing many informal settlements inhabitants to rely on poor quality surface water resources to compensate for their water requirements (Hemson and Dube, 2004, Momba et al., 2006). A significant proportion of inhabitants of these rural communities are in danger of contracting water-borne or water-related diseases (Olaniran et al., 2009). The microbial quality of river water sources located in and used by specified rural communities of South Africa, indicated that these water bodies were unsafe for human consumption with high *E. coli* levels (Abia et al., 2016a). *E. coli* in this respect both represent an indicator of fecal pollution and a potential pathogen. The upper tributaries of river ecosystems are generally of good to fair water quality whilst the lower reaches of most river systems are affected by urbanization and run-off from both point-sources and diffuse pollution sources resulting in poor water quality (Walters et al., 2011). This highly variable status of water quality within individual catchments makes it more difficult to assess the overall state of South Africa's rivers and necessitate a monitoring program which accounts for representative sampling points within the catchment.

2.4 Pollution sources

Bacterial, viral and parasitic pathogens that are present in human and animal wastes can vary significantly in their distribution, depending on the sources of contamination affecting the water supply. Surface water bodies are affected by two groups of pollution sources, namely, point-source and non-point-source pollution (Juahir et al., 2010, Mustapha et al., 2013, Simpson et al., 2002).

Point sources include sewage from wastewater treatment plants, household wastes and industrial discharges. Wastewater treatment plants often discharge oxygen deficient effluents and are considered one of the main threats to serious health. Industrial effluents often contain several different hazardous chemical and biological components, where the composition and threat to human health depends on the types of industry. According to USEPA (2005), all point source dischargers in the United States need to obtain a permit from their state, which includes both industrial and municipal dischargers, or any other facility that discharges wastewater to receiving water bodies. Such programs are put in place to greatly assist in the control of point source pollutants of anthropogenic origin as they specify the allowable flow rate of a discharge and the maximum concentration of specific pollutants (USEPA, 2005).

Non-point source pollution comes from many diffuse sources, and is typically related to runoff due to rainfall or snowmelt (in low temperature countries). As runoff flows into the water, it picks up and carries natural and human/animal related pollutants, finally depositing them in water bodies, wetlands, coastal waters, and even the underground sources of potable water where these function as recipients (WHO, 2011). The major non-point contaminants are: agricultural runoff (pesticides, excess fertilizers and pathogens), urban runoff (grease; oil), and toxic chemicals from industries and energy production. Additional non-point sources are sediment from improperly managed construction sites, eroding streambanks and acid drainage from abandoned mines; microorganisms and nutrients from livestock, and faulty septic systems as well as atmospheric deposition (USEPA, 2005, Dressing et al., 2003). Wild animals and birds have also been identified as possible non-point sources of fecal contamination of water resources (Coffey et al., 1993). Controlling non-point sources of pollution may be more difficult than pollution from point-sources (Jamieson et al., 2004). Hence, this is a major problem for surface water bodies partly due to the difficulties in identifying and rating the relative impact of these sources of pollution. Thus, land use surveys and groundwater or surface

water quality sampling are the key techniques of identifying where possible non-point sources may be located (Elbag, 2006).

The concept that involves tracing the source of fecal pollution is called Microbial Source Tracking (MST) (Harwood et al., 2014), in which endogenous markers of fecal origin are used for identification of fecal pollution in aquatic environments. Chemical MST-methods can be used to trace mainly sewage pollution, nevertheless no direct relationship was found between the used chemical targets and pathogenic bacteria (Seurinck et al., 2005). This is not the case in microbial MST-methods where source-specific bacteria or viruses are cultured or measured with molecular methods to identify fecal pollution sources (Harwood et al., 2013, Harwood et al., 2014). From time to time, microbial targets can be present in very low numbers to be detected, this can be sidestepped by using molecular assays for host-specific marker detection. Phenotypic and genotypic library-based methods are commonly used to discriminate among different fecal sources. However, according to Seurinck et al. (2005) many issues remain unresolved with regards to temporal and geographical variability. Also, the procedure is very labour-intensive due to the isolation step. In a comparative study Griffith et al. (2003), found that phenotypic methods performed better than genotypic methods amongst the library based ones.

2.5 Routine monitoring and Indicator organisms

Routine monitoring for all microorganisms still remains difficult and impractical, thus detection and identification procedures for each possible type can be very difficult and require significant resources. As a result, monitoring for broad indicators of fecal contamination is useful in verifying the microbiological quality and safety of the drinking water supply (Ashbolt et al., 2001). The World Health Organization (WHO, 2001) defines indicator and index microorganisms of public health concern as: (a) Process indicator- a group of organisms that demonstrate the efficacy of a process, such as total heterotrophic bacteria or total coliforms for chlorine disinfection, (b) Fecal indicator- a group of organisms that indicate the presence of

fecal contamination, such as thermotolerant coliforms or *E. coli*. Hence, they only assume that pathogens may be present, (c) Index and model organisms- a group/or species indicative of pathogen presence and behaviour respectively, such as *E. coli* as an index for *Salmonella* (WHO, 2001, Ashbolt et al., 2001). Therefore, for a microorganism to be declared as ‘indicator’ it must be present when pathogens are present in water, should be absent in uncontaminated water, present in higher numbers than pathogens in contaminated water and should survive better in water than pathogens with considerable ease in analysis (Horan, 2003, Lin and Ganesh, 2013, Shanker et al., 2013)

The identification and monitoring of specific pathogens at very low concentrations (especially in the presence of large numbers of background microflora) is a difficult task. Furthermore, the infective dose of different pathogenic microorganisms is in many cases low and may be masked by an abundant background microflora. (Kinnula et al., 2015). Therefore, the presence of fecal indicator organisms, generally non-pathogenic microorganisms, points towards the presence of enteric pathogens in the sample. Their quantity is then related to the density of pathogenic microorganisms of fecal origin and is thus an indication of the health risk associated with the various water uses such as, recreational, irrigation and domestic use (Servais , 2007).

Fecal indicator organisms remain at the forefront of water and wastewater microbiology. On an average, an individual will excrete roughly 10^{11} - 10^{13} organisms/g of feces (Stenström, 2004). Most of the bacteria isolated from faeces do not cause infection and in the present context they are important as far as they act as surrogate indicators of infective hazards (Horan, 2003). Different countries have permissible limits of ‘indicators’ of microbial quality of water as shown in the table below.

Table 2.1: WHO global guidelines values and DWAF South African standards, specifying the acceptable limits of indicator organisms for water intended for drinking, irrigation and recreational purposes

| Organization/ government | Water use | Bacteriological quality limit value or range | Reference |
|-----------------------------|--|--|------------------------|
| WHO | Drinking water | 0 Fecal coliforms/ <i>E. coli</i> /100 mL | (WHO, 2011, WHO, 2004) |
| WHO | Unrestricted irrigation of crops (including uncooked crops) | ≤1,000 Fecal coliforms/100 mL | (WHO, 2006) |
| DWAF | Drinking water | 0–5 Total coliforms/100 mL; 0 Fecal coliform/100 mL | (DWAF, 2005) |
| DWAF | Full-contact recreation (swimming) | 0–130 Fecal coliforms/ <i>E. coli</i> /100 mL | (DWAF, 1996c) |
| DWAF | Intermediate recreation | 0–1,000 Fecal coliforms/100 mL depending on the level of water contact | (DWAF, 1996c) |
| DWAF | Irrigation water applied to minimally processed produce | ≤1 <i>E. coli</i> /100 mL | (DWAF, 1996b) |

Indicator organisms are used for different purposes, such as (WHO, 2011):

- fecal pollution in verification and surveillance monitoring;
- the effectiveness of processes such as filtration or disinfection in validation;
- integrity and cleanliness of distribution systems in operational monitoring.

In water quality assessment, fecal indicator bacteria (FIB) are used to measure the sanitary quality of water for recreational, industrial, agricultural and water supply purposes. The FIB's are natural inhabitants of the gastrointestinal tract of humans and other warm-blooded animals. They are released into the environment with feces, and are exposed to a variety of environmental factors (Ashbolt et al., 2001). The survival time and persistence of fecal indicator bacteria in river environment is affected by environmental factors such as sunlight, temperature, competition for nutrients with autochthonous bacteria naturally inhabiting the water, predation by protozoa and other small organisms and toxic industrial wastes (Tortorello, 2003). In the indicator concept, it is assumed that the die-off of pathogens and FIB are equal. The die-off is a factor related to reduction with time (Bonde, 1962, Anderson et al., 2005,

Taylor, 2003). This is not always correct, especially where viruses and parasitic protozoa may persist extensively longer (Servais, 2007). However, the presence of relatively high numbers of FIB in the environment indicates the likelihood of the presence of pathogenic microorganisms.

2.5.1 Coliforms

Fecal indicator bacteria are represented by both total and fecal coliforms and are the most widely used in relation to water quality. Coliforms are a heterogeneous group that can originate from numerous surroundings (Ashbolt et al., 2001). Therefore, due to their long history of use, coliforms provide a reference point to which alternative indicators can be compared. These include total coliforms, fecal coliforms as well as *E. coli* which have a direct connection to fecal pollution. It is important to note that coliforms may differ in sensitivity to treatment processes such as chlorination as well as environmental stress compared to many pathogens (Bonadonna et al., 2002, Nasser et al., 2003, Ottoson, 2005) which limits their suitability to be used as water quality indicators of reuse products exclusively (Bitton, 2005). Total coliform bacteria are aerobic and facultatively anaerobic, Gram-negatives that grow, ferment lactose and produce acid or aldehyde in the presence of bile salts within 24 hours at 35–37°C. The enzyme β -galactosidase is produced as part of the lactose fermentation. The total coliform group includes both fecal and environmental species, for example the genera *Escherichia*, *Citrobacter*, *Klebsiella*, *Enterobacter*, *Serratia* and *Hafnia* (WHO 2011).

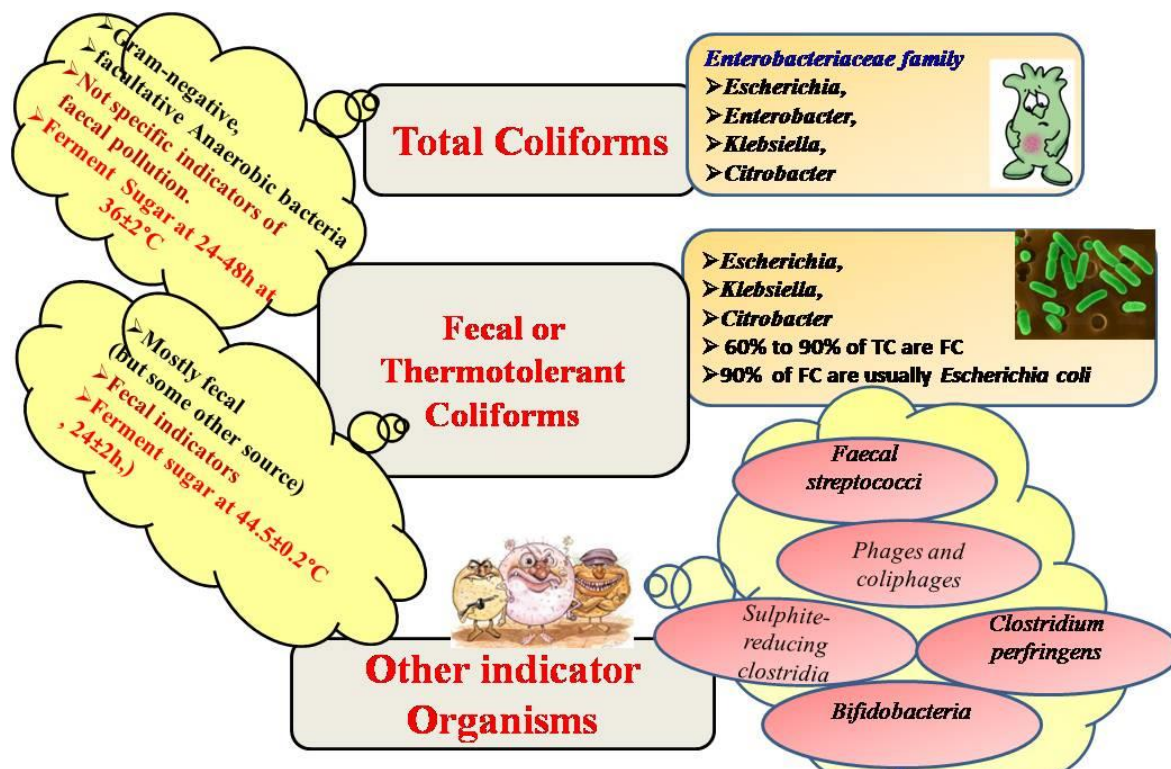


Figure 2.1: Examples of environmental indicator organisms of water quality. (Adopted from Shanker et al., 2013)

A number of environmental indicators of water quality have been described (Figure 2.1). Coliforms and related pathogens are broadly categorized as Total Coliforms (TC), Fecal Coliforms (FC) or thermotolerant coliforms and other indicator organisms Figure 2.1. Studies have shown that 60 to 90% of total coliforms are fecal coliforms and 90% of fecal coliforms are *E. coli* (Shanker et al., 2013). *E. coli* and thermotolerant coliforms ferment lactose at higher temperatures (44–45 °C). As part of lactose fermentation, total coliforms produce the enzyme β -galactosidase. *E. coli* can be differentiated from the other thermotolerant coliforms by the ability to produce indole from tryptophan or by the production of the enzyme β -glucuronidase (Wohlsen, 2011). Traditionally, coliform bacteria were regarded as fitting to the genera *Escherichia*, *Citrobacter*, *Klebsiella* and *Enterobacter* but the group is more heterogeneous and includes a wider range of genera, such as *Serratia*, *Hafnia* and others. The total coliform

group includes both fecal and environmental species as well as organisms that can survive and grow in water (WHO, 2011).

2.5.2 *Escherichia coli* (*E. coli*)

E. coli are bacteria that form part of the common microflora found in the intestines of humans and warm-blooded animals and form an important part of a healthy human intestinal tract. Most of these *E. coli* strains are not pathogenic and are therefore not harmful to the human body. It is accustomed to a pH of 7-8 and body temperature of 37 °C. *E. coli* is excreted in feces in densities between $10^5 - 10^8$ CFU g⁻¹ (Geldreich, 1978, Ottoson, 2005) although regrowth may occur. However, some *E. coli* types are pathogenic, and can lead to serious illnesses with symptoms including diarrhoea, abdominal cramping or illness outside of the intestinal tract like fever. The types of *E. coli* that cause illnesses can be transmitted through contaminated water or food, or through contact with infected animals or human feces. Independently, United States Environmental Protection Agency (USEPA) recommends *E. coli* as the best indicator of health risk in surface waters (USEPA, 2005).

According to the Centre for Disease Control and Prevention (CDC), pathogenic *E. coli* strains can be characterized into different pathotypes. Six pathotypes are associated with diarrhoea, and they are collectively referred to as diarrheagenic *E. coli*, namely: Shiga toxin-producing *E. coli* (STEC), Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC) and Diffusely Adherent *E. coli* (DAEC), (CDC, 2014). Serotypes of *E. coli* are commonly assessed using flagellar and surface antigens (H and O), and specific serotypes often cluster within specific pathotypes.

2.5.3 *Intestinal Enterococcus*

Intestinal *Enterococci* earlier called Fecal Streptococci, are Gram positive, non-spore forming members of the Streptococci bacteria generally found in the feces of warm blooded humans and animals. They are among the common microbiota of the gastrointestinal tract of humans

and animals. Thus, there are multiple strains of enterococci, many of which are not harmful to humans. *Enterococci* can be distinguished from other *streptococci* by their ability to grow in high sodium chloride concentration (6.5%), at pH values that are as high as 9.6 and at 45°C, hence they are thermotolerant (Ashbolt et al., 2001). *Enterococci* is excreted in feces in densities of $10^5 - 10^8$ CFU g⁻¹ (Geldreich, 1978, Ottoson, 2005). They are more resistant to chlorination and environmental stress and persist comparatively long in the environment. They rarely multiply in water compared to *E. coli* and other coliforms (Van Lier et al., 2008). *Enterococci* have distinctive characteristics which allow them to be isolated and used as a fecal indicator in surface water that is used for recreational, irrigation as well as drinking water intakes. *Enterococci* is recommended as an indicator of fecal contamination in management of recreational water quality as their presence correlates best with the incidence of swimming-related gastroenteritis (Ashbolt et al., 2001, Kay et al., 1994, Prüss, 1998).

As much as enterococcus plays an important role in water quality monitoring, like *E. coli*, it has significant shortfalls as a true fecal indicator, and can as well to a lesser extent regrow in the environment under favourable conditions (Desmarais et al., 2002). *Enterococci* are often less numerous than *E. coli* in human fecal material (Feachem 1983) which can make them more difficult to detect in surface waters. The ability of *enterococci* to regrow and the lower organism concentrations thus sometimes limits *enterococci*'s usefulness as a fecal indicator of recent contamination (Boehm and Sassoubre, 2014). In spite of their shortfalls, using *enterococci* as a fecal indicator has key advantages over the use of coliforms and *E. coli* (Van Lier et al., 2008).

2.5.4 Bacteriophages

Bacteriophages are viruses that infect bacteria and serve as a model for the assessment of the behaviour of enteric viruses in water treatment and disinfection processes (Ashbolt et al., 2001). According to a report by the United States Environmental Protection Agency (USEPA)

coliphages can now be considered as an indicator of viral contamination in groundwater (USEPA, 2006). There are several groups of coliphages that could be used as indicator organisms. These include somatic bacteriophages, HSP40 bacteriophages and male specific F-RNA bacteriophages (Grabow, 2001).

Somatic coliphages are viruses that infect *E. coli* through cell wall receptors (Ashbolt et al., 2001) and are very good indicators of fecal contamination because they can only infect their fecal host (USEPA, 2006). Hence, the detection of somatic coliphages indicates both bacterial and viral contamination levels in water (Harwood et al., 2013). Somatic bacteriophages are mainly used as indicators of the potential presence of enteric viruses in water due to similar survival characteristics. They occur in high counts in fecal waste, but it has been reported that they can multiply in natural waters under unusual circumstances, when their host bacteria can grow and be infected (Grabow, 2001).

Conventional methods such as the double layer plaque assay are generally used to detect somatic and FRN-specific bacteriophages (ISO, 2000a, ISO, 1995). These are conducted using the *E. coli* strain (ATCC 13706) nalidixic acid-resistant mutant WG5 and the *Salmonella typhimurium* WG 49 nalidixic acid-resistant mutant as hosts respectively.

2.6 Factors that affect microbial concentration

2.6.1 Temperature

Temperature can affect a microorganism's ability to survive and grow in water. Generally enteric microorganisms including pathogens can survive for longer time periods under low temperature conditions (-5 °C). At temperatures, above 20°C, some have the ability to regrow, but at temperatures >40 °C die off is generally rapid (Feachem et al., 1983). In a study conducted in the Lake Winnipeg, Pip and Allegro (2010) showed that higher concentrations of total coliform bacteria were associated with increased temperature. Wilkes et al. (2011) detected *Campylobacter* more often when the mean air temperatures were cooler. One study

addressed temperature (air and water) on *Bacteroides* using molecular and cultivation approaches and found that *Bacteroides* organisms were detected more often in lower temperatures than in higher temperatures. However, elevated temperatures were associated with a relatively high die-off rate (Ballesté and Blanch, 2010).

According to Blaustein et al. (2013) and Pachepsky et al. (2014) amongst others, temperature is also one of the most important physiochemical properties that affect the growth and survival of microorganisms in water. Warmer water temperatures are related to a higher microbial concentration, which was also evidence in a study conducted by (Abia et al., 2015b).

2.6.2 Rainfall

Rainfall has the potential to influence microbial water quality and relate to both point sources and non-point sources related to surface run-off. The connection between microbes in the recipient and precipitation has been demonstrated in many studies throughout the world (Cha et al., 2010, Martinez et al., 2014, Ackerman and Weisberg, 2003, Shehane et al., 2005, Holvoet et al., 2014). Whitman et al. (2008) showed that *E. coli* increased 10 and 100-fold following rainfall and snowmelt events, respectively, in an artificial stream system. Haack et al. (2003) identified a 48-72-hour lag between precipitation and elevated *E. coli* concentrations along the Grand Traverse Bay coast. Jamieson et al., (2005) found that storm events increased sediment resuspension and in turn elevated bacterial concentration in a Canadian stream. Furthermore, Whitman and Nevers (2003) identified a significant correlation between *E. coli* and barometric pressure in Lake Michigan. Collectively, these results demonstrate the impact of rainfall on natural (e.g. stream flow) and human (e.g. wastewater treatment) systems which lead to degraded surface water quality.

2.6.3 Sediments

Over the year's researchers have discovered that sediments harbor chemicals, toxins, and nutrients which probably have the potential to impact overlying water (Marvin-DiPasquale et

al., 2003, Smart and Barko, 1978, Mortimer, 1971). Although the relationship between bacteria and sediment and the corresponding impact that such bacteria have on surrounding water quality is still not as clear. It is now known that bacteria can accumulate, regrow on sediments and potentially be released to the overlying water but standards for bacteria analysis in sediments still do not exist. Sediment have been shown to harbor excessive numbers of fecal indicators and even foster regrowth of some fecal indicator bacteria (Mika et al., 2009). This is because sediments act as a reservoir for microorganisms (Abia et al., 2016a). Different types and sizes of sediments have different impact on microorganism abundance, thus smaller sized sediment particles were said to correlate positively with higher microorganism populations (Howell et al., 1996, Anderson et al., 2005). Particle associated microorganisms are also said to survive for longer periods of time in sediments compared to surface water (Whitman and Nevers, 2003). This is partly attributed to the fact that these microorganisms are not exposed to UV rays and are not easily accessible to competition (Kunkel et al., 2013, Liang et al., 2013). Resuspension due to enhanced flow, precipitation or wind can cause sediment associated bacteria to be released to the surrounding water (Whitman and Nevers, 2003). Further research is needed to elucidate the role of non-point source pollution on water quality and its association with human health risks. Such associations will require investigations at multiple spatial and temporal scales through river water bodies. Fecal indicator bacteria that had accumulated in sediments, expressively contributed to surface water quality (Abia et al., 2016b) as a result of wave action or increased flow rate (Kinzelman et al., 2004, Zikhali et al., 2015). Increased turbidity enhanced the survival of *E. coli* (Garcia-Armisen and Servais, 2009), which implicates that sediment resuspension is a source of water quality impairment.

Fecal indicator bacteria concentrations observed in beach sand and sediment are often higher than in surrounding water. Whitman and Nevers (2003) reported that sand situated nearshore were found to have higher *E. coli* concentrations (4000 CFU/100 mL⁻¹) than the surrounding

waters (43 CFU/ 100 mL⁻¹). Alm et al. (2003) made a similar observation, but also found that *E. coli* concentrations decreased with sediment depth.

Although the fate of molecular source tracking markers in sediment is not well understood, a study has attempted to investigate the persistence of source markers in water while taking into account their presence in sediments (Dick et al., 2010). However, bacteria and molecular source markers have been shown to exist in higher concentrations and survive longer in sediments than in surrounding water (Cabral, 2010). The linkage between microbial accumulation and persistence characteristics in environmental sediments to water quality change at various watershed scales are not well studied particularly given both natural and anthropogenic influences. The current study sheds some light in that respect by evaluating the surface water and sediments at the Isipingo and Palmiet Rivers using both conventional/enzymatic methods (**Chapter 3 & 4**) as well as molecular finger printing (**Chapter 5**).

2.7 Conventional techniques used for the detection of bacterial, viral indicators and pathogenic bacteria

Culture based methods have been recommended and used routinely for many years to date for the identification and detection of mainly bacterial but also viral microorganisms (plaque). When using this, bacteria are grown on specific culture medium at a specific temperature for a particular period of time. Their characterization is based on the morphology and confirmed using biochemical tests. The most common conventional culture based methods used for bacterial and viral indicators as well as pathogen detection are:

2.7.1 Membrane filtration and Spread plate Method

The membrane filter (MF) technique is used to test relatively large volumes of sample with low turbidity. This method is very useful in monitoring drinking water and a variety of natural waters (ISO, 2000b). This method involves the use of a specified pore size filters of which the water is filtered through and placed on the media of choice (depending on the target

microorganism). The incubation period is dependent on the type of media and microorganism. This method can be used for both bacterial and pathogenic microorganisms. However, the MF technique has limitations, particularly when testing waters with high turbidity as the microbial load may be too numerous to count (Buckalew et al., 2006). The spread plate technique which is a standard plate count method is often used to enumerate and isolate bacteria on highly turbid samples. The highly turbid samples are serially diluted and 0.1 or 0.2 mL of sample is spread onto the surface of the agar using a glass hockey stick. In this study, both the MF technique as well as the spread plate technique were used for the enumeration microorganisms on selective agar.

2.7.2 Detection using chromogenic substrates

Chromogenic compounds, added to the conventional or newly devised media are used in order to increase selectiveness of the media and detectability of specific organisms. These chromogenic constituents are modified either by enzymes or specific bacterial metabolites (Manafi, 2000). Once modified, the chromogenic substance changes its colour or fluorescence, thus enabling easy detection of colonies that display the metabolic activity. In this way, these substances normally limit the need for isolation of pure cultures and further confirmation tests. The time required for the determination of different indicator bacteria can be reduced from the normal 24-48 hours to a range of 14 to 18 hours (Wohlsen, 2011).

2.7.3 Most probable number methods

The most probable number (MPN) technique is an important technique in estimating microbial populations in soils and/or sediments, waters, food matrix and agricultural samples. Many samples contain heterogeneous microorganisms, therefore exact cell numbers of an individual organism are often impossible to determine. This method does not rely on quantitative assessment of individual cells; however, it relies on specific qualitative attributes

of the microorganism of interest. The MPN technique estimates microbial population sizes in a liquid substrate and results can be obtained between 24 to 48 hours (Woomer, 1994).

2.7.4 Defined substrate methods

These methods can be related to media that contain specific enzyme substrates. These substrates provide significant enhancements in recoveries and identification of target bacteria. Furthermore, the enzyme-based methods are known to pick up traditionally non-culturable microorganisms (Valente et al., 2010).

This defined substrate approach has been advocated by International Standards Organisation for miniaturised MPN based methods for coliforms/*E. coli* and enterococci (ISO/FDIS, 1999 (Warden et al., 2011)). An example of this is the IDEXX Colilert-18 and Enterolert define substrate methodology, which is used globally for the microbiological assessment of water. Colilert has been used for the enumeration of total coliforms and *Escherichia coli* simultaneously, and the Enterolert system has been used for the enumeration of *Enterococcus*, on the basis of the target organism's ability to metabolize fluorogenic or chromogenic substrate and convert it to a detectable end product (IDEXX, 2001, Kinzelman et al., 2005). Previous studies have reported false-positive results with the use of Colilert® for identification of *E. coli* (Pisciotta et al., 2002, Chao, 2006), while others reported negligible false detection rates of *E. coli* with the Colilert® reagent (Buckalew et al., 2006, Omar et al., 2010, Chao et al., 2003). In spite of these discrepancies, the Colilert® method has been proven to be comparable to other EPA approved methods for the enumeration of *E. coli* and has been highly recommended (Warden et al., 2011).

2.8 Molecular Methods based on Genetic Signature of Target Pathogen

The nucleic acid sequences are unique to all living organisms. These genetic sequences are the potential (possible) targets to differentiate one organism from another and diagnose various disease-causing agents. In the post-genomic era, large numbers of microorganisms have been

sequenced. In early, 2013, ~ 18000 prokaryotic genomes have been sequenced (NCBI Genome database, <http://www.ncbi.nlm.nih.gov/genome/browse/>). The NCBI Genome sequence database has made it possible to analyse microbial pathogens at molecular level.

The application of molecular methods should be considered in the context of quality management for potable water. The new methods influence epidemiology and outbreak investigations more than the routine testing of processed drinking water. A number of molecular approaches such as RFLP (Restriction fragment length Polymorphism), RAPD (Random Amplification of Polymorphic DNA), FISH (Florescence *in situ* Hybridization), PCR (Polymerase Chain Reaction), qPCR (Quantitative PCR) and digital PCR can be used for pathogen detection but each method has limitations.

For the fulfilment of the objectives of this study, qPCR and digital PCR were employed.

2.8.1 Polymerase Chain Reaction Technique and Quantitative PCR

The powerful molecular technique, Polymerase Chain Reaction (PCR) allows amplification of target DNA to generate multiple copies that can be detected. PCR validated by the International Organization for Standardization (ISO), is now used for testing of foodborne pathogens (Malorny et al., 2004). One of the limitations in PCR is that the assay volume is relatively small and requires the sample to be concentrated to micro-litres. The water sample has to be concentrated and purified using suitable methods as natural water samples often contain inhibitory substances such as humic acids and iron, that concentrate with the nucleic acids (Singh et al., 2017b, Sidstedt et al., 2015). Therefore, it is critical to have positive and negative controls with each environmental PCR sample in order to check for inhibition and specificity. It may also be critical to determine whether the signal obtained from the PCR is due to naked nucleic acids or living and dead microorganisms. The sensitivity of the PCR is often not sufficient and post PCR processing and analysis is required (Hayden et al., 2013).

Quantitative PCR (qPCR) also known as Real Time PCR is a fluorescence based detection format which is more sensitive than conventional PCR. Like PCR, real time PCR works on the basis of amplification of target DNA to generate multiple copies that can be detected. However, samples can be analysed in real time with higher specificity, sensitivity, reproducibility and wide quantification range. Thus, no Post PCR processing is required. The technique has been applied to locate non-point sources of pathogen contamination and environmental risk assessment (Singh et al., 2013, Singh et al., 2010).

qPCR is an often preferred method for the quantitative enumeration of pathogenic microbes in complex environmental matrices. Although qPCR is a powerful method for the quantitative enumeration of microbial communities, there are still drawbacks that often might affect the analysis of results, especially when dealing with complex sample mediums. Some of the limitations range from specificity of primers targeting a specific group, florescent probes as well as nucleic acid extraction efficiency. The quality of template nucleic acid and amplification of nonviable DNA are also important factors that affect qPCR analysis.

A recent study has shown the application of qPCR for the detection of several bacterial pathogen in a river in Southern Ontario, Canada which is the major source of drinking water (Banihashemi et al., 2015). In this study, qPCR was coupled to propidium monoazide dye for the quantitative enumeration of selected pathogens such as *Salmonella enterica*, *Campylobacter*, *Escherichia coli* O157:H7 and *Arcobacter butzleri* and suggested that qPCR coupled with propidium monoazide (PMA) dye could detect viable pathogens in surface water (Banihashemi et al., 2015). qPCR has also been used for detection of Enterotoxigenic *Escherichia coli* and *Salmonella* in environmental water and sediments (Singh et al., 2013, Singh et al., 2010). Malorny et al. (2008) also enumerated *Salmonella* bacteria in food and feed samples using qPCR in order to quantitate the microbial risk.

2.8.2 Digital PCR

Digital PCR (dPCR) is known as the third-generation PCR technology which further adds to the quantification of pathogens based upon genetic analysis. Using this technology which is developed on the workflow of qPCR, prior to PCR amplification, the sample is subjected to portioning into hundreds of millions of individual reaction chambers, so that each contains one or no copies of the sequence of interest. However, Digital PCR can be subdivided into two different types that are based on different principals, that is, the chip principal and the other is based on the droplet principal (Baker, 2012). The latter has been developed as a new platform for DNA quantification as this phenomenon has advantages over qPCR, which will be elaborated in **Chapter 4**.

The methods described above were used continuously in order to achieve various objectives. Further comparison and correlations between the results obtained using the different methods will be elaborated in **Chapter 4**.

2.9 Waterborne outbreaks in South Africa

Diarrhoeal diseases are one of the major contributors to the Global Burden of Disease (GBD) (Stenström, 2004). These include cholera, typhoid fever and shigellosis amongst others, which are transmitted through contaminated water, food, crops and direct or indirect exposure such as domestic recreational or occupational activities (Prüss, 2014) Globally, Inadequate drinking water and sanitation are estimated to cause 502 000 and 280 000 diarrhoea deaths, (http://www.who.int/water_sanitation_health/diseases-risks/gbd_poor_water/en/). They are the most severe to human health, especially those living in rural areas, informal settlements as they do not have access to proper sanitation and treated drinking water. There is sufficient evidence of developing countries relating to waterborne outbreaks demonstrating the risk related to fecal contamination of drinking water sources. Pathogenic and multi-drug resistance microorganisms are present in surface water of rivers in various parts of South Africa that are

used for household, domestic and irrigational purposes (Müller et al., 2001, Momba et al., 2006, Igbinosa and Okoh, 2009, Okoh et al., 2015, Iweriebor et al., 2015). In 2000-2001 an unexpected cholera epidemic occurred that originated in KwaZulu-Natal province whereby 114 000 cases were reported resulting in 260 deaths (Hemson, 2016). In the year 2005, another diarrhoea outbreak was reported in Mpumalanga Province (Delmas Town) which led to 528 cases resulting in four deaths and 69 people hospitalised (Oberholster and Botha, 2014).

Transmission of pathogens by exposure to contaminated water is still a major cause of infections, this is demonstrated by the number of outbreaks that have been reported globally (Medema et al., 2003). The problem is often underestimated when based solely on detected outbreaks. This is because most waterborne diseases often go undetected by the reporting systems as symptoms of gastrointestinal illnesses are usually mild and general, thus most people do not seek medical help. Also, the symptoms usually last a few days to a week (Stenström, 1994). The detection, reporting and the characterization of waterborne outbreaks is very important in the identification of the disease. However, the detection strategies should be robust and involve sensitive methods for early screening and quantitative enumeration of the targeted pathogens and should be capable of aiding in the proper assessment of the microbiological quality of water (Singh et al., 2017a).

2.10 Quantitative Microbial Risk Assessment

The risk associated with exposure to fecal contaminated water can be assessed using epidemiological studies or Quantitative Microbial Risk Assessment tools (QMRA). However, both methods have advantages and disadvantages.

In this study, emphasis was more on **QMRA** (also see further **Chapter 5**). QMRA involves identification of hazards, estimation of the risk relating to exposure to microorganisms and a

dose response relationship (Figure 2.2) (Haas et al., 1999). These steps are further used in the subsequent risk characterization.

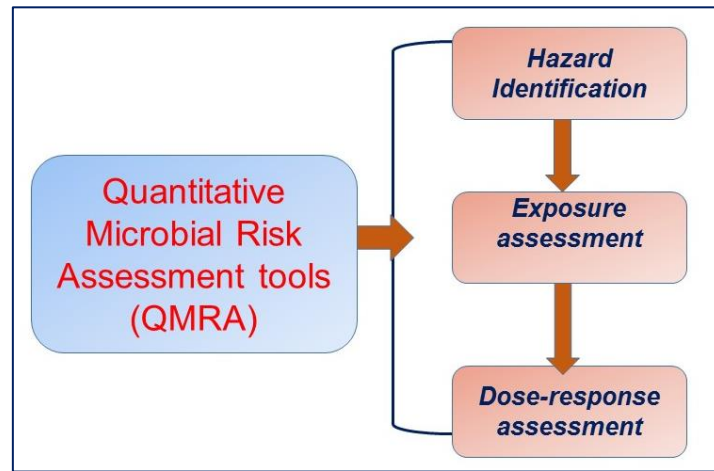


Figure 2.2: Different steps involved in Quantitative Microbial Risk Assessment tools (QMRA) analysis.

A dose response model can be used to estimate the probability of infection. QMRA is not only used to estimate the risk in water related exposures, however, it can be used to estimate the microbial risk in food (Perez-Rodriguez and Valero, 2013), water supply as well as human feces/wastewater. QMRA can be used to directly estimate the risk to human health by predicting infection rates given densities of particular pathogens. It often used in the context of drinking water in many countries. Unlike in the developed countries where QMRA is mostly associated to recreational activities involving accidental ingestion of polluted water, the risk in developing countries is usually higher due to the direct contact of the polluted waters such as domestic, irrigation, recreational and occupational activities (Gemmell and Schmidt, 2013, Chigor et al., 2014, Sibanda et al., 2013). Studies have been conducted to assess such human health risk due to microbial pollution in some South African rivers (Genthe et al., 2013, Le Roux et al., 2012, Steyn et al., 2004).

Quantitative risk assessment is further elaborated in **Chapter 5** and risk associated with recreational and occupational activities is discussed.

CHAPTER 3 : COMPARISON OF BACTERIAL INDICATORS IN SURFACE WATER AND SEDIMENTS AT THE ISIPINGO AND PALMIET RIVERS

3.1 Introduction

In settlements throughout the world, surface water is the primary available water source for human use such as for drinking and domestic purposes (Luyt et al., 2012). Surface water refers to a body of fresh water that is found flowing or standing such as streams, lakes, river basin sites, ponds and reservoirs (Cooke et al., 2016). Surface waters are often highly impacted by anthropogenic activities which is a contamination source of surface waters affecting the fast-growing informal settlements in South Africa (Cabral, 2010). The overall load of pathogenic microorganisms of human origin that enter a catchment depends on several factors, such as the total human population, the proportion of the population using sewerage systems that discharge into the river and the proportion that comes untreated due to run-off from settlements (Teklehaimanot, 2013). The level of the wastewater treatment as well as the operating efficiency of the wastewater treatment system also plays a pivotal role (Teklehaimanot et al., 2014, Sibanda et al., 2015). Therefore, the quality of the receiving water bodies varies with time (Bartram and Ballance, 1996) and is often more unreliable as compared to ground-water sources, where the water undergoes a natural purification due to physical, chemical and biological filtration through the soil (Taylor, 2003).

River basins polluted by fecal discharges from humans and animals may transport a variety of human pathogenic microorganisms (viruses, bacteria, protozoa and helminths) (Teklehaimanot et al., 2014). The human exposure relates both to direct contact to fecal material as well as oral transmission mediated both by drinking water, after recreational exposure as well as through crops that may be irrigated with polluted water (WHO, 2003). Public health is often compromised in instances where fecal matter (human/animal origin) is washed off from the

surface of the ground into the river, or when waste from pit latrines seep below the ground or are emptied directly into the river (Abia et al., 2015b). Blockage of sewage pipes also result in the discharge of waste into rivers. Direct discharges occur during periods of planned direct release of untreated wastewater or due to planned or unplanned overflow of storm water drains (Cyr et al., 2015).

Microbial contamination of potable water sources is one of the chief causes of the spread of waterborne diseases (Franz, 2005). Mortality rate due to diarrhoea in Africa is high among children under the age of 5, the immunocompromised individuals as well as the elderly (Kotloff et al., 2013, Liu et al., 2012, Walker et al., 2013). Microorganisms that enter the river either remain in the aqueous phase in surface water or become attached to suspended particles that may sediment (Gao et al., 2011). The particle bound microbial concentration increases over time whereby a vast majority of them become preferentially attached to particulate material, which promotes their downward flux to the bottom riverbed sediment where they are found in greater abundance (Droppo et al., 2009).

Increasing evidence indicate that bacteria often shows an attraction to sediment attachment (Jamieson et al., 2004, Ekwanzala et al., 2017). This is because of physical adsorption due to charge or hydrophobic interaction and that sediments further provide a beneficial environment to the microorganisms including a higher concentration of nutrients (Whitman and Nevers, 2003), food assimilation and protection from environmental stress such as predation (Liang et al., 2013, Kunkel et al., 2013).

The riverbed sediments have been reported to serve as reservoirs of pathogenic microorganisms of fecal origin (Alm et al., 2003, Luna et al., 2012, Luna et al., 2010). Sediments often undergo re-suspension due to natural events such as heavy rainfall or anthropogenic activities (Abia et al., 2017a). During these events, microorganisms may re-enter the water phase, thus increasing

the microbial load of the surface water, which implicates that sediment resuspension is a source of water quality deterioration (Griffith et al., 2010, Luna et al., 2012).

While the concentration of pathogens in surface water is a major health risk, the impact from sediment and the subsequent monitoring is still not considered in many developing countries, including South Africa (Abia et al., 2015c). Thus, the monitoring programme in place may underestimate the actual microbial load of a drinking water source and may constitute a potential human health risk, especially in an event of sediment resuspension (Donovan et al., 2008, Obasohan et al., 2010, Abia et al., 2016b). During routine monitoring, sediment analysis has often been neglected in relation to the microbiological water quality and if tested, emphases is on the chemical contaminants (Guerra et al., 2009). This may be due to lack of understanding of the importance of the relationship between particle associated microorganisms and riverbed sediments and the health risks associated with it (Droppo et al., 2009). In order to prevent microbial hazards in the surface water from causing health risk through drinking water, an understanding of the catchment and its inherent activities is needed (Ferguson et al., 2003, Abia et al., 2017a).

A highly-polluted river basin encompasses all these potential exposure scenarios, whereby the microbiological quality of Durban South river basin sites (Isipingo and Palmiet Rivers) is of potential major public health concern. The selected sites in this study are greatly influenced by anthropogenic activities such as intense agricultural practices and informal settlements discharging waste into the rivers. These activities result in fecal contamination which pose a health risk to a significant proportion of the community members, leading to water-borne diseases and related complications. Thus, the microbiological quality of the Durban South river basins has important socio-economic consequences and can function as an illustrative example of what happens in other river catchments.

In order to detect microbial hazardous events in surface waters it is necessary to carry out some type of monitoring. Monitoring involves water quality measurements and should be directed at hazardous events (Bartram and Ballance, 1996). Water quality based on fecal indicator bacteria differ from one water source to the other. The local eThekweni municipality uses *E. coli* as an indicator as well as physiochemical properties of surface water, based on grab samples.

This chapter deals with the main objectives to analyse the historical data in order to get an understanding of the catchments of interest (**Objective 1**). *E. coli* and *Enterococci* were monitored in surface water and sediment samples in order to provide an insight on the level of fecal contamination and indicate the potential presence of waterborne fecal pathogens at the sampling points of interest (**Objective 2 partly and Objective 3**). Variability associated with rainfall and sediment resuspension was evaluated in this study based on events in the presence and absence of heavy rainfall (**Objective 4**).

3.2 Methodology

3.2.1 Description of study area

The focus areas in this study was the Isipingo and Palmiet Rivers which are both situated in the KwaZulu Natal region, in Durban. The Isipingo River is located roughly 20 kilometres south of the Durban Central Business District (CBD). It is approximately 27 km long (Pillay, 2013). The river surroundings are characterized by informal settlements, formal developments as well as intensive industrial activities especially around the lower catchment, thus it is greatly influenced by anthropogenic sources.

The second catchment of interest, the Palmiet River, is a 25-km long catchment that is located in the northerly peripheral of the city of Durban, KwaZulu-Natal. It was named after the grass-plant *Prionium Serratum* known as the Palmiet plant. Palmiet River rises from Fields hill with another branch starting below Wyebank municipal dump site, and flows through the Pinetown and New Germany residential and industrial areas of which the latter is a potential pollution

source. This relatively small catchment then flows through the Quarry road informal settlements area (which is a major pollution source) just before it joins the Umgeni River under the M19 bridge. The sampling sites of interest are described below in Table 3.1 and Figure 3.1 (Isipingo River) and Figure 3.2 (Palmiet River).

Table 3.1: Sampling point description, location, and potential pollution influences

| Site ID | Site location (co-ordinates) | Site description | Potential pollution source(s) |
|---|---------------------------------------|---|---|
| Isipingo River | | | |
| Upstream WWTP | -29°59'17.8038S" 30°54'21.9630E" | Surrounded by township community members <i>Reference site not illustrated at the Fig 3.1 Map</i> | Anthropogenic activities |
| Next to WWTP Site A in Figure 3.1 | -29°59'19.4784S" 30°54'20.9340E" | This point is directly Next to WWTP, and also used for irrigation purposes by the community members | Sewage Agricultural pesticides during rain wash off. |
| Transit camps Site B in Figure 3.1 | -29°59'43.1982S" 30°55'20.8878E" | Surrounded by informal settlements/Transit camps | Anthropogenic activities |
| River mouth Site C Figure 3.1 | -29°59'39.7278S" 30°57'8.2440E" | This is where the River joins the Isipingo beach. | Pollution from upstream, industries |
| Palmiet River | | | |
| Methven road | -29°49'7.2228" S 30°54'36.8964" E | Upstream Palmiet River, surrounded by suburbs <i>Reference site not illustrated at the Fig 3.2 Map</i> | Methven pump station |
| Upstream QRI Site A Figure 3.2 | -29°48'16.4304" S 30°57'57.1968" E | This is where Palmiet River begins to flow through the informal settlement. | Anthropogenic activities (from informal settlement) |
| Downstream QRI Site B Figure 3.2 | -29°48'16.218" S 30°58'0.0552" E | This is downstream of the informal settlement | Anthropogenic activities (from informal settlement) |
| Before Palmiet River joins Umgeni River Site C Figure 3.2 | -29°48'11.3508" S 30°58'38.316" E | This is the point downstream, right before Palmiet River joins the Umgeni River. | Pollution from the QRI. |

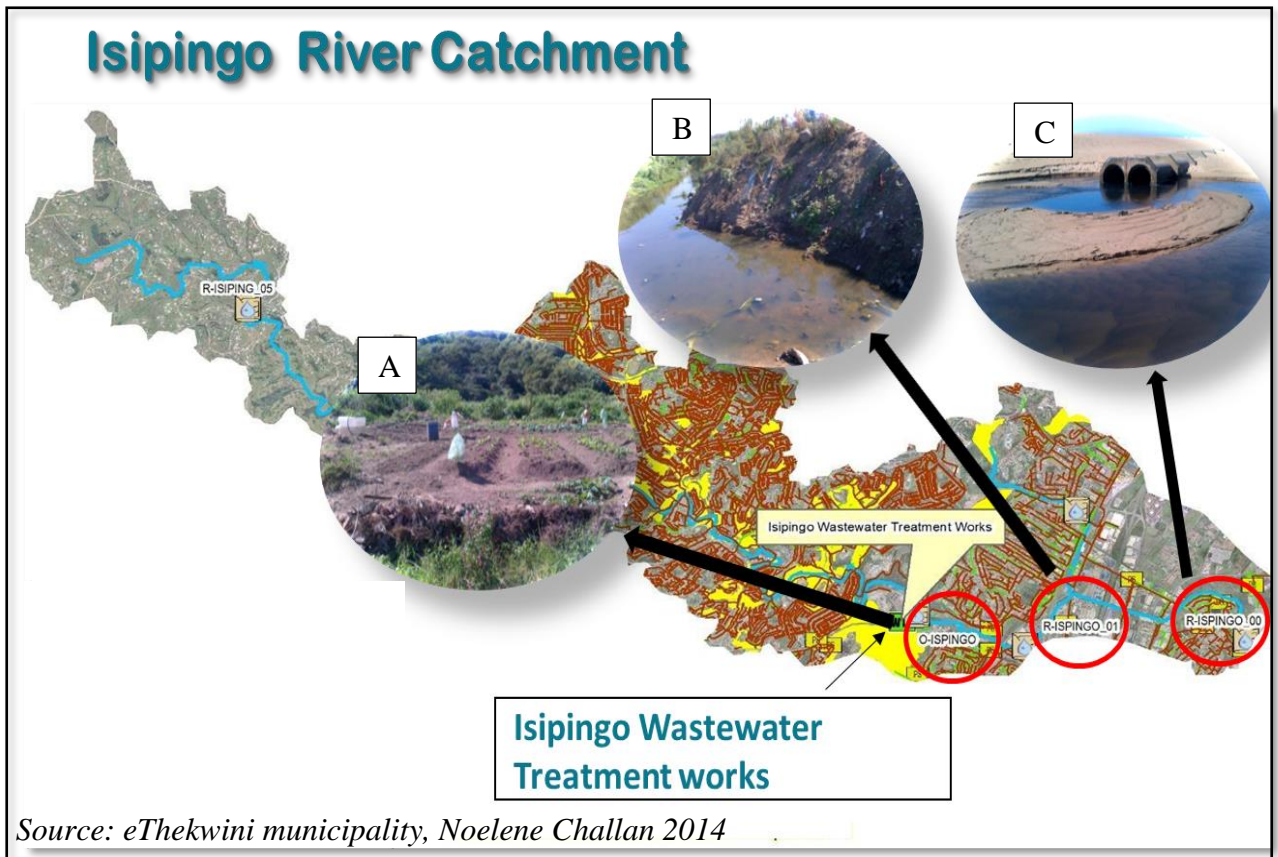


Figure 3.1: An overview of Isipingo River and sampling points

Pictures A, B, C represent the sampling points of interest for this study: A- Next to the WWTP (river water is used for irrigation), B- Transit camps (part of the river that is surrounded by informal settlements). C- River mouth (twin pipes that join the Isipingo River to the beach). The sampling point “Upstream (of WWTP)” is not part of the sampling regime of the eThekweni Water and Sanitation, therefore it is not represented in the map. However, this sampling point was considered in this study, in order to determine the impact of the WWTP (on the river).

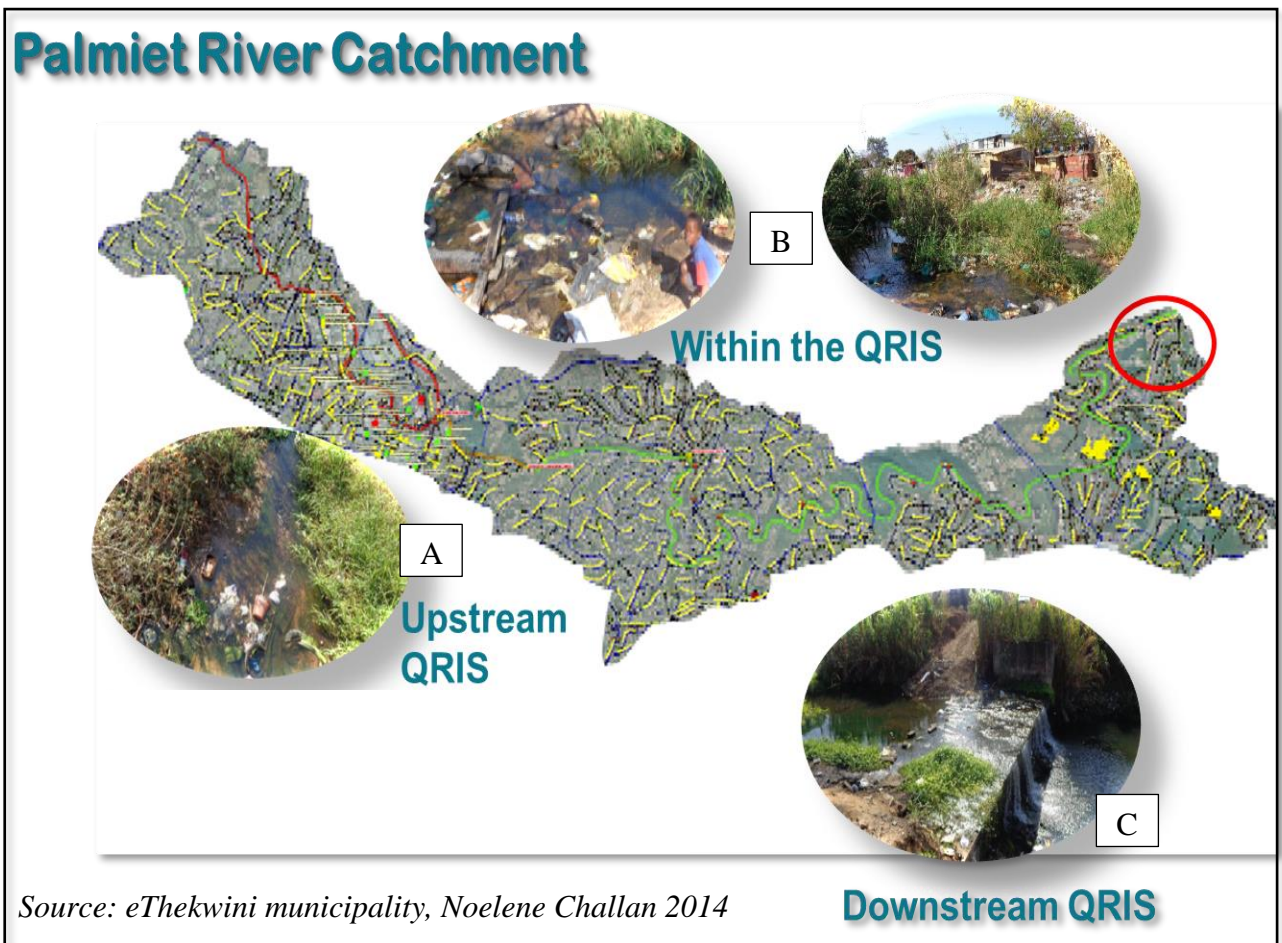


Figure 3.2: An overview of the Palmiet River and the sampling sites

Pictures A, B, C represent the sampling points of interest for this study. The main focus is on the small part of the Palmiet River around the Quarry Road Informal settlements (QRI), which is represented by the red circle: A-upstream informal settlements, B- Palmiet River flowing in the middle of the informal settlements, C-Downstream of the settlements (just before the Palmiet River joins the Umgeni River)

3.2.2 Historical data analysis:

The historical data was analysed by statistically assessing the microbiological quality of surface water, conducted on the basis of *Escherichia coli* (*E. coli*) MPN/100 mL. The values were obtained from the Municipality (eThekweni Water and Sanitation Department) who used IDEXX Colilert -18 and the Quanti-Tray Test Method for the enumeration of *E. coli* at the

Isipingo River (three sites) and the Palmiet River (six sites) collected over a 66 months period (January 2009 to June 2014). The sampling points more or less represent those that were considered for further sampling and analysis within this study. However, the main objective (**Objective 1**) of the historical data analysis was to obtain a baseline on both the rivers as well as to determine areas that needed improvement, such as the addition of sampling points along the rivers in areas that may pose a risk to human health e.g. QRI settlements. The sequence of the sampling points do not follow a numerical pattern, but rather they follow the flow of the river (upstream to downstream).

At the Isipingo River, the three points illustrated in the map were considered, due to the direct impact on human health within the river. However, other sites monitored by the municipality such as canals and those that were out of our research focus were not considered.

At the Palmiet River sampling points also followed a pattern at which the river flows from upstream to downstream (where Palmiet River joins Umgeni River). Below is a detailed description of each point considered for the historical data analysis and potential pollution sources:

- Palmiet 09 at Glenugie Road (Upstream), situated 20.80 km from the end of the Palmiet River.
- Palmiet 06 at Blair/ Otto Volek Road (18,14 km). This point is also situated next to a pump station (potential pollution source during overflows and pipe outburst).
- Palmiet 05 is situated at Birdhurst road in Westville urban area. This point is next to the Birdhurst pump station (potential pollution source during overflows and pipe outburst).
- Palmiet 08 at Crompton street/Methven (pump station near).

- Palmiet 01 is located at Varsity Drive bridge. This sampling point is situated downstream of the other sampling points, but upstream of the Quarry road informal settlements which is our main focus further for this study.
- Due to the fact that there was no sampling site on the Palmiet River that is situated within or downstream of the Quarry road informal settlements, the results obtained at Umgeni 08 sampling point were further analysed, which is situated under the M19 bridge. This is where the Palmiet River joins the Umgeni River.

3.2.3 Bacterial culture

E coli ATCC 35401 and *Enterococci faecium* ATCC 35667 were procured from Microbiologics, Inc. USA and used as positive controls in the quantitative determinations.

3.2.4 Sample collection

3.2.4.1 Surface water collection

Surface water and sediment samples were collected along the Isipingo and Palmiet rivers at the defined eight sites (Table 3.1). Samples were collected bi-monthly on each river over a period of 12 months (February 2015- February 2017). Surface water grab samples were collected, in triplicate, 1L sterilized bottles at each site. The containers were rinsed with the water from the source prior to collection and filled to a certain point, leaving about 30 mm of headspace in order to allow mixing during laboratory analysis. Surface water samples were collected by holding the sterilized container by the handle and plunging it 0.5 m below the water surface, against the water current. In the absence of a current, one had to artificially create it by pushing the container forward. All samples were protected from direct sunlight and transported on gel ice packs to the laboratory where it was stored at 4 °C until further analysis (APHA, 2005). Samples were analysed with 24 hours after collection.

3.2.4.2 Sediment sample collection

Sediment samples were collected in accordance to Singh et al., (2010) with a sterile stainless-steel scoop respectively and placed in sterile plastic bags and transported on ice to the laboratory for further analysis. Each sample represented a composite of 10-20 sub-samples. Samples were analysed with 24 hours after collection.

3.2.5 Sediment sample preparation

One gram of each sediment sample was transferred to a test tube and suspended in 4 mL of 1× phosphate-buffered saline (PBS) to obtain a 1:5 (w/v) dilution. Each sample was vortexed for 90 s to disassociate and suspend bacteria from the sediment. Aliquots of the resulting supernatant for each sample were handled aseptically for further analysis based on site specific 10-fold dilutions (Singh et al., 2010). The further analysis with the Colilert-18 are described under section 3.2.7 and for spread plate under section 4.2.3.

3.2.6 Physicochemical characteristics of surface water to determine microbial quality of water

Physico-chemical characteristics of surface water at the Isipingo and Palmiet Rivers were determined *in-situ*. Temperature (°C), Electrical conductivity (μScm^{-1}), total dissolved solids (TDS; g/L), Dissolved Oxygen (mg/L) of the river water was measured by YSI model 556 MPS equipped with multi-parameter sensor 5563 MPS (Yellow Springs, Ohio, USA). This was deployed at each sampling site in parallel with collection of water and sediment samples.

3.2.7 Enumeration of bacterial indicators: IDEXX Colilert 18 and Enterolert test methods

The presence and absence test as well as enumeration of total coliforms, fecal coliforms, *E. coli*, and enterococci were achieved using the IDEXX Colilert 18 and Enterolert test method for enterococci (according to manufacture instructions). The Colilert-18 reagent (IDEXX Laboratories) was added to samples decimal diluted with distilled water (final volume 100 mL) and mixed well to dissolve. The dilution is site specific and sediment samples are diluted further than the water samples. The mixture was poured into a Quanti-tray/2000 (IDEXX Laboratories, Westbrook, USA), sealed and incubated at 37°C for 18 hours. The Colilert-18

method simultaneously detects total coliforms by counting the number of yellow coloured wells. This is achieved when coliform bacteria metabolize the nutrient-indicator, giving a yellow coloration through the action of the enzyme β -galactosidase on ortho-nitrophenyl- β -D-galactopyranoside (ONPG). Whereas, when *E. coli* metabolizes a second nutrient-indicator through β -glucuronidase on 4- methyl-umbelliferyl- β -D-glucuronide (MUG) yellow coloured wells fluoresce at 365 nm which enables the detection of *E. coli*. The MPN was then calculated using the MPN chart provided by IDEXX (IDEXX Laboratories, Westbrook, USA). The Enterolert was prepared as described above and incubated at 44.5°C for 24 hours. Positive *enterococci* were determined by counting the yellow coloured wells were fluorescent at 365 nm, and read against the MPN chart.

3.2.8 Analysis of the effect of rainfall on microbial concentration to address variability over time

A pilot study was conducted at the Isipingo River, at the sampling point next to the WWTP, to address the variability in microbial concentrations over a period of 6 hrs, in case of no rainfall (AS1) and after a heavy rainfall (AS2). An auto-sampler (3700C compact portable sampler, Teledyne Isco, USA) and a sediment trap (self-designed) was installed (in parallel) for 6 hours when there was no rainfall (AS1) and after a heavy rainfall event (AS2). Pictures of the auto sampler and sediment trap are included in Appendix 1 (Figure 6.).

3.2.9 Statistical analysis

All microbiological data were log-transformed to fit a normal distribution. All surface water and sediment samples were statistically analysed using Microsoft Excel and GraphPad prism: Log transformation and graphs were conducted on Microsoft excel and GraphPad prism. ANOVA, Pearson correlation analysis, and unpaired t-tests were performed using GraphPad prism software with significance set at (α) 0.05.

3.3 Results

3.3.1 A) Historical data analysis; Isipingo River

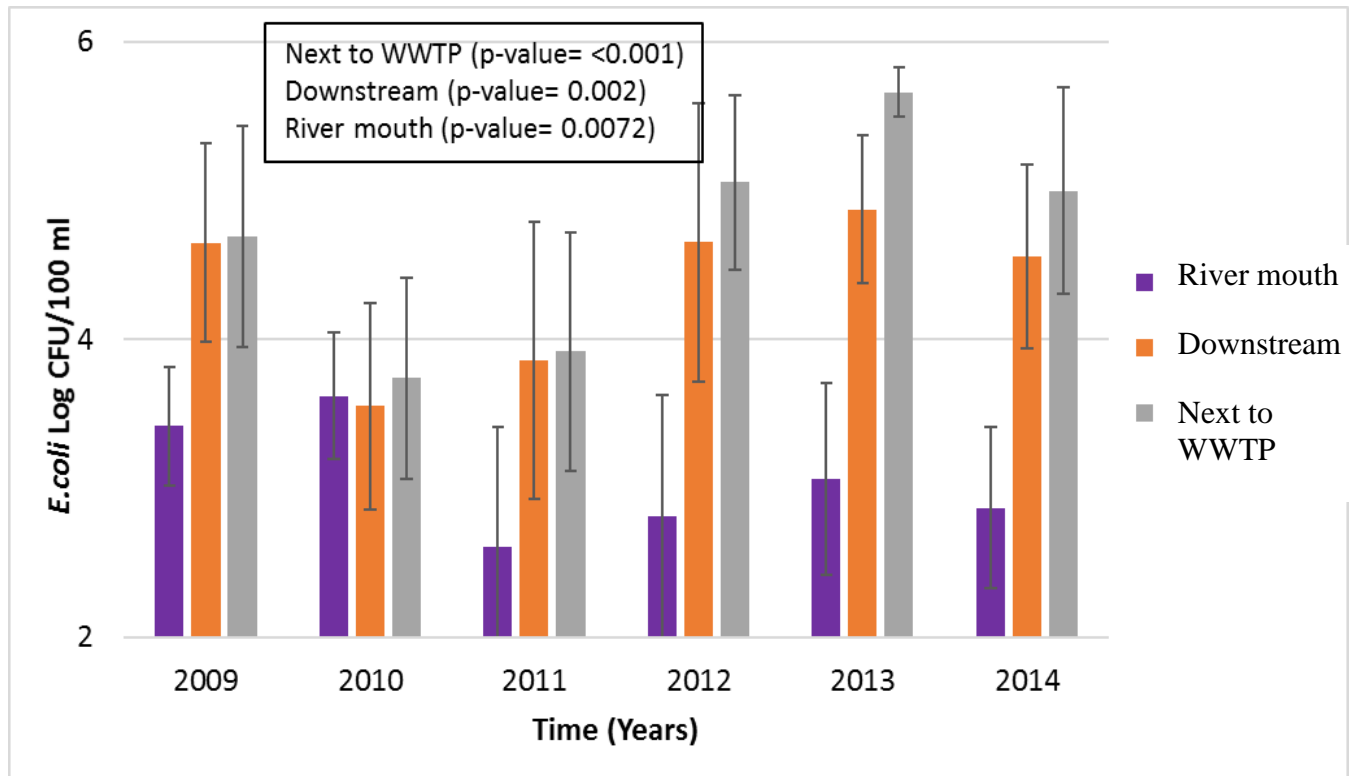


Figure 3.3: *E. coli* (Log10 MPN/ 100 mL) concentration along the Isipingo River over a period of 5 years and 6 months (2009-2014)

*P-values represent comparison of yearly means at each sampling site respectively.

*Bars represent \pm SEM.

The highest significant difference between the years 2009 to 2014 was observed at the sampling point “Next to the WWTP”. This is the sampling point that had the highest level of *E. coli* concentration, and mean range between 3.73 (2010) and 5.65 (2013). The lowest microbial load was observed at River mouth, with a mean range between 2.60 (2011) and 3.62 (2010) Log10 MPN/100 mL.

Table 3.2: Summary statistics of *E. coli* Log10 MPN/100 mL from Isipingo River (66 months, 2009 -2014)

| NEXT TO WWTP | 2009 | 2010 | 2011 | 2012 | 2013 | 2014 |
|---------------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Number of values | 11 | 9 | 10 | 4 | 10 | 4 |
| Mean | 4.69 | 3.73 | 3.91 | 5.05 | 5.65 | 4.99 |
| Std. Deviation | 0.74 | 0.67 | 0.80 | 0.58 | 0.16 | 0.69 |
| Std. Error | 0.22 | 0.22 | 0.25 | 0.29 | 0.05 | 0.34 |
| DOWNSTREAM | | | | | | |
| Number of values | 12 | 7 | 10 | 10 | 12 | 6 |
| Mean | 4.65 | 3.55 | 3.85 | 4.65 | 4.87 | 4.55 |
| Std. Deviation | 0.66 | 0.69 | 0.93 | 0.93 | 0.49 | 0.61 |
| Std. Error | 0.19 | 0.26 | 0.29 | 0.29 | 0.14 | 0.25 |
| RIVER MOUTH | | | | | | |
| Number of values | 12 | 10 | 8 | 9 | 7 | 5 |
| Mean | 3.41 | 3.62 | 2.60 | 2.81 | 3.06 | 2.86 |
| Std. Deviation | 0.39 | 0.42 | 0.80 | 0.81 | 0.64 | 0.54 |
| Std. Error | 0.11 | 0.13 | 0.28 | 0.27 | 0.24 | 0.24 |

Mean \pm SD and \pm SEM represent variation amongst yearly means. When the three sampling points were evaluated for variance, all means were significantly different with the sampling point upstream having the highest variation (p-value <0.05).

3.3.1 B) Historical data analysis; Palmiet River

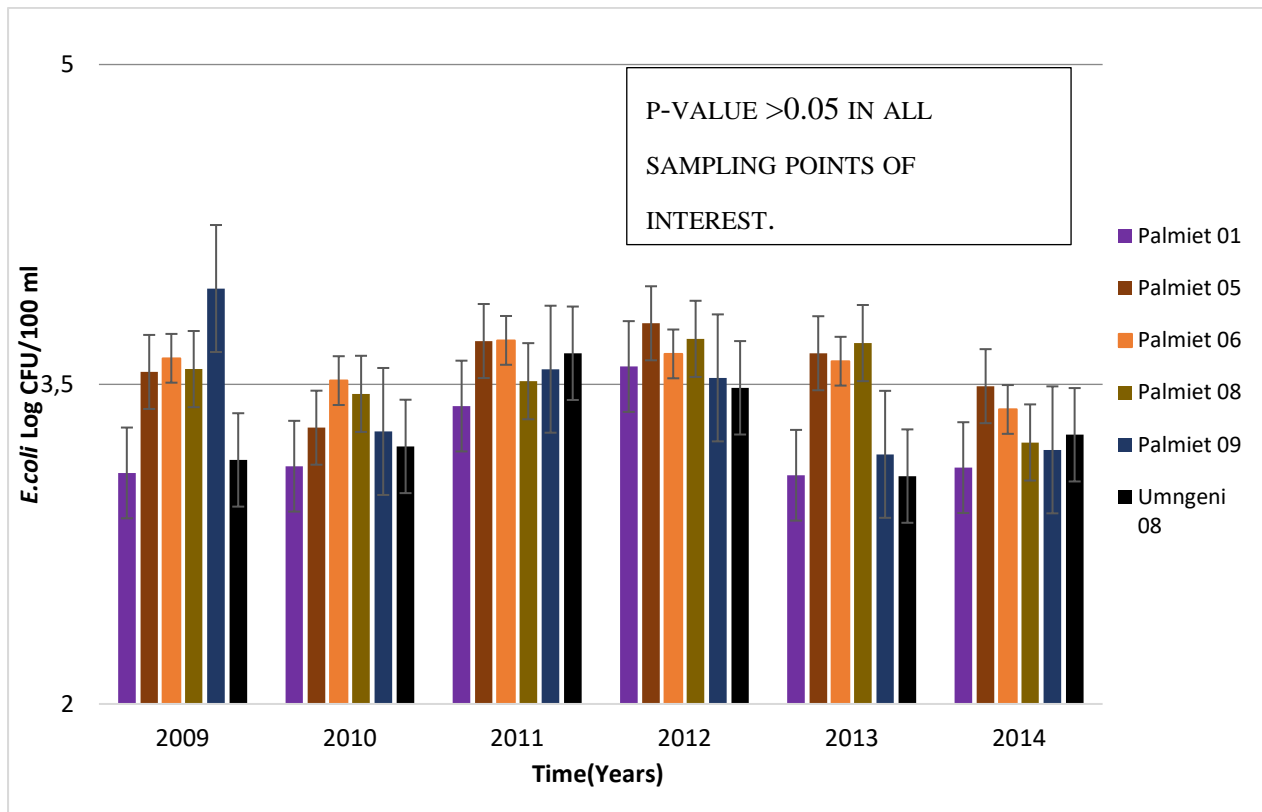


Figure 3.4: Comparison of the mean *E. coli* (Log₁₀ MPN/100 mL) concentration along the Palmiet River over a period of 66 months (2009-2014)

* P-value relates to the yearly means at each site. No significant difference was observed in the yearly means of all the sites (p-value 0.05).

*Bars represent \pm SEM

Figure 3.4 shows the six sampling sites of interest that are evaluated by the EWS on a monthly basis. When the six sampling points were evaluated for variance, there was no significant difference in the means all the sampling points. The year 2010 showed to have the lowest *E. coli* concentrations.

Table 3.3: Summary statistics of *E. coli* Log10 MPN/100 mL along the Palmiet River (66 months, 2009-2014)

| Palmiet 09 | 2009 | 2010 | 2011 | 2012 | 2013 | 2014 |
|-------------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Number of values | 6 | 9 | 12 | 7 | 10 | 7 |
| Mean | 3.95 | 3.28 | 3.57 | 3.53 | 3.17 | 3.19 |
| Std. Deviation | 1.06 | 0.43 | 0.84 | 0.31 | 0.51 | 0.64 |
| Std. Error | 0.43 | 0.14 | 0.24 | 0.12 | 0.16 | 0.24 |
| Palmiet 06 | | | | | | |
| Number of values | 7 | 8 | 9 | 10 | 11 | 5 |
| Mean | 3.62 | 3.52 | 3.71 | 3.64 | 3.61 | 3.38 |
| Std. Deviation | 0.96 | 0.56 | 0.69 | 0.43 | 0.71 | 0.14 |
| Std. Error | 0.36 | 0.20 | 0.23 | 0.14 | 0.21 | 0.06 |
| Palmiet 05 | | | | | | |
| Number of values | 10 | 7 | 12 | 11 | 11 | 7 |
| Mean | 3.56 | 3.30 | 3.70 | 3.79 | 3.65 | 3.49 |
| Std. Deviation | 0.79 | 0.37 | 0.45 | 0.91 | 0.55 | 0.64 |
| Std. Error | 0.25 | 0.14 | 0.13 | 0.27 | 0.17 | 0.24 |
| Palmiet 08 | | | | | | |
| Number of values | 11 | 6 | 10 | 11 | 11 | 7 |
| Mean | 3.57 | 3.46 | 3.51 | 3.71 | 3.69 | 3.23 |
| Std. Deviation | 0.46 | 0.49 | 0.79 | 0.49 | 0.70 | 0.40 |
| Std. Error | 0.14 | 0.20 | 0.25 | 0.15 | 0.21 | 0.15 |
| Palmiet 01 | | | | | | |
| Number of values | 9 | 9 | 8 | 11 | 12 | 6 |
| Mean | 3.08 | 3.12 | 3.40 | 3.58 | 3.07 | 3.11 |
| Std. Deviation | 0.45 | 0.38 | 0.76 | 0.76 | 0.33 | 0.43 |
| Std. Error | 0.15 | 0.13 | 0.27 | 0.23 | 0.09 | 0.18 |
| Umgeni 08 | | | | | | |
| Number of values | 10 | 8 | 10 | 12 | 11 | 5 |
| Mean | 3.15 | 3.21 | 3.65 | 3.48 | 3.07 | 3.26 |
| Std. Deviation | 0.42 | 0.48 | 1.02 | 0.59 | 0.54 | 0.38 |
| Std. Error | 0.13 | 0.17 | 0.32 | 0.17 | 0.16 | 0.17 |

Table 3.3 shows the historical data along the Palmiet river over a 66 months period (209-2014). Mean \pm SD and \pm SEM represent variation amongst yearly means along the sampling points along the Palmiet river.

3.3.2 Physiochemical properties

Table 3.4: Mean physico-chemical parameters of the Isipingo river and Palmiet river

| | Temp ^a (°C) | SpCond ^a (µS/cm) | TDS ^a (g/L) | DO ^a (mg/L) | pH ^a |
|-----------------|------------------------|-----------------------------|------------------------|------------------------|-----------------|
| Isipingo | 24.2± 3.5 | 505± 6.2 | 2.04± 2.9 | 4.54± 0.3 | 7.3± 0.2 |
| Palmiet | 22.6± 5.2 | 367± 32.5 | 0.24± 0.02 | 6.11± 1.6 | 7.4± 0.1 |

^aValues [mean ($n=24$) ±SD] temp: temperature; SpCond: electrical conductivity; TDS: total dissolved solids; DO: dissolved oxygen

The physico-chemical parameters of the Isipingo and Palmiet Rivers are given in Table 3.4. The pH value for both rivers was roughly the same at 7.3 and 7.4 respectively for Isipingo river and Palmiet river respectively. The surface water temperature was also in the same range Palmiet river 22.6 °C and Isipingo river 24.2 °C. The electrical conductivity and TDS in both rivers were generally high.

3.3.3 *E. coli* and *Enterococci* quantities in Isipingo and Palmiet River's surface water and sediment

The highest *E. coli* concentration found in surface water along the Isipingo River occurred next to the wastewater treatment plant (WWTP), with concentrations ranging between 4.2 and 6.7 log₁₀ MPN/100 mL (mean value 5.0 log₁₀ MPN/ 100 mL). High *Enterococci* concentrations were observed at the River mouth site, where concentrations ranged between 3.0 - 6.5 log₁₀ MPN/100 mL (mean value 4.6 log₁₀ MPN/100 mL). In sediment samples *E. coli* mean concentration was the least Upstream 5.3 log₁₀ MPN/100 mL, and the highest concentration in river mouth 5.8 log₁₀ MPN/100 mL. Whereas *Enterococci* had the lowest means concentration Upstream 4.7 log₁₀ MPN/100 mL and the highest Next to the WWTP 5.4 log₁₀ MPN/100 mL. A similar trend was observed in which the values Upstream were the lowest for both indicators in surface water and sediment samples, but were still above a mean of 10 000 *E coli*/100 mL as well as for *Enterococci* (Table 3.5).

Table 3.5: Indicator organisms in surface water versus sediments at the Isipingo River

| Sampling Sites | <i>E. coli</i> ^a | | <i>Enterococci</i> ^a | |
|----------------|-----------------------------|-----------|---------------------------------|-----------|
| | S/W ^b | Sediments | S/W ^b | Sediments |
| Upstream | 4.7±1.1 | 5.3±0.9 | 4.2±1.4 | 4.7±0.7 |
| Next to WWTP | 5.0±0.9 | 5.4±0.6 | 4.4±1.1 | 5.4±0.6 |
| Transit camps | 4.8±1.0 | 5.7±0.6 | 4.3±1.2 | 4.9±1.1 |
| River mouth | 4.8±1.1 | 5.8±0.9 | 4.6±1.3 | 5.3±1.4 |

^aValues [mean log₁₀ MPN/100 mL (*n* =24) ±SD]

^bS/W: Surface Water

E.coli and *Enterococci* concentrations log₁₀ MPN/100 mL were continuously higher than in sediment samples at all four sampling points. The sampling point Next to WWTP was the most polluted, and Upstream WWTP was the least contaminated.

Table 3.6: Mean indicator organisms in surface water versus sediments along the Palmiet River

| Sampling Sites | <i>E. coli</i> ^a | | <i>Enterococci</i> ^a | |
|-----------------------------------|-----------------------------|-----------|---------------------------------|-----------|
| | S/W ^b | Sediments | S/W ^b | Sediments |
| Methven | 3.4±0.9 | 4.6±1.2 | 2.8±0.6 | 3.5±0.3 |
| Upstream QRI ^c | 4.3±0.8 | 4.7±1.1 | 4.0±0.9 | 4.6±1.3 |
| Downstream QRI ^c | 4.7±0.6 | 5.9±0.9 | 4.6±0.5 | 5.1±0.7 |
| Before Palmiet joins Umgeni River | 3.7±0.7 | 5.4±1.0 | 3.5±0.7 | 4.2±0.7 |

^aValues [mean log₁₀ MPN/100 mL (*n* =24) ±SD]

^bS/W: Surface Water; ^cQRI: Quarry Road Informal settlements

In surface water samples *E. coli* mean concentration ranged between 3.4 log₁₀ MPN/ 100 mL (Methven) and 4.7 log₁₀ MPN/ 100 mL (Downstream QRI). The *Enterococci* range was 2.8 log₁₀ MPN/ 100 mL (Methven) and 4.6 log₁₀ MPN/ 100 mL (Downstream QRI). Comparative values for sediment samples, for *E. coli* mean concentration ranged between 4.6 log₁₀ MPN/

100g (Methven) and 5.9 log₁₀ MPN/ 100 g (Downstream QRI). The *Enterococci* range was between 3.5 log₁₀MPN/ 100 g (Methven) and 5.2 log₁₀ MPN/ 100 g (Downstream QRI). Overall Methven sampling site was the least contaminated, both for *E. coli* and *Enterococci* in surface water and sediment samples, whereas Downstream QRI was consistently the most polluted with high indicator concentrations in both surface water and sediment samples (Table 3.6). Higher concentrations (± 1 log₁₀ MPN/100 g greater) of *E. coli* and *Enterococci* were found in sediment samples, compared to surface water samples.

3.3.4 Effect of rainfall on microbial concentration to address variability over time

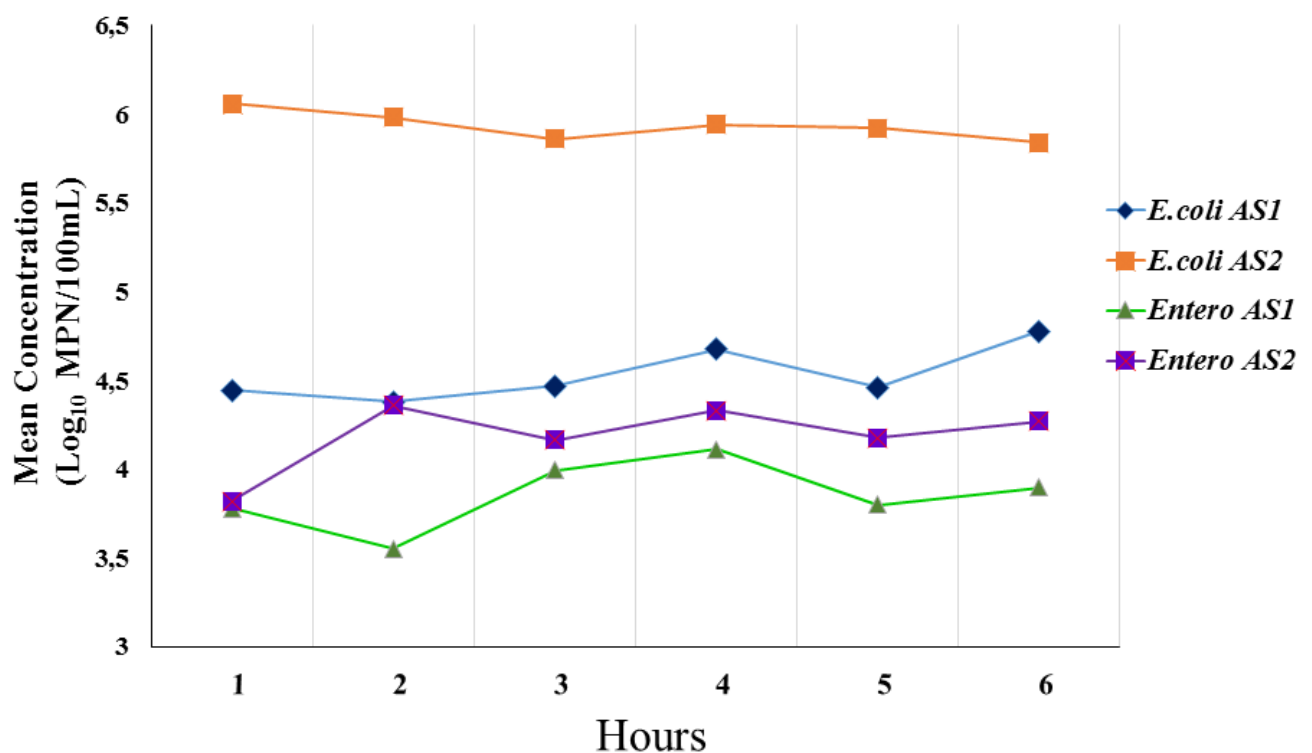


Figure 3.5: Variability in *E. coli* and *Enterococci* concentrations in surface water over the time span of 6 hrs in case of two events AS1 (no rainfall) and AS2 (after a heavy rainfall) analysed using an auto sampler (3700C compact portable sampler, Teledyne Iso, USA).

Microbial level of both indicators (*E. coli* and *Enterococci*) were higher in AS2 as compared to AS1, with a *E. coli* log difference of $\pm 1.5 \log_{10}$ MPN/100 ml between AS1 and AS2. The same trend was observed for *Enterococci*, $\pm 0.75 \log_{10}$ MPN/100 mL difference was observed between AS1 and AS2 (**Figure 3.5**). *E. coli* concentrations were more dominant than *Enterococci* in both AS1 and AS2 events (**Figure 3.5**).

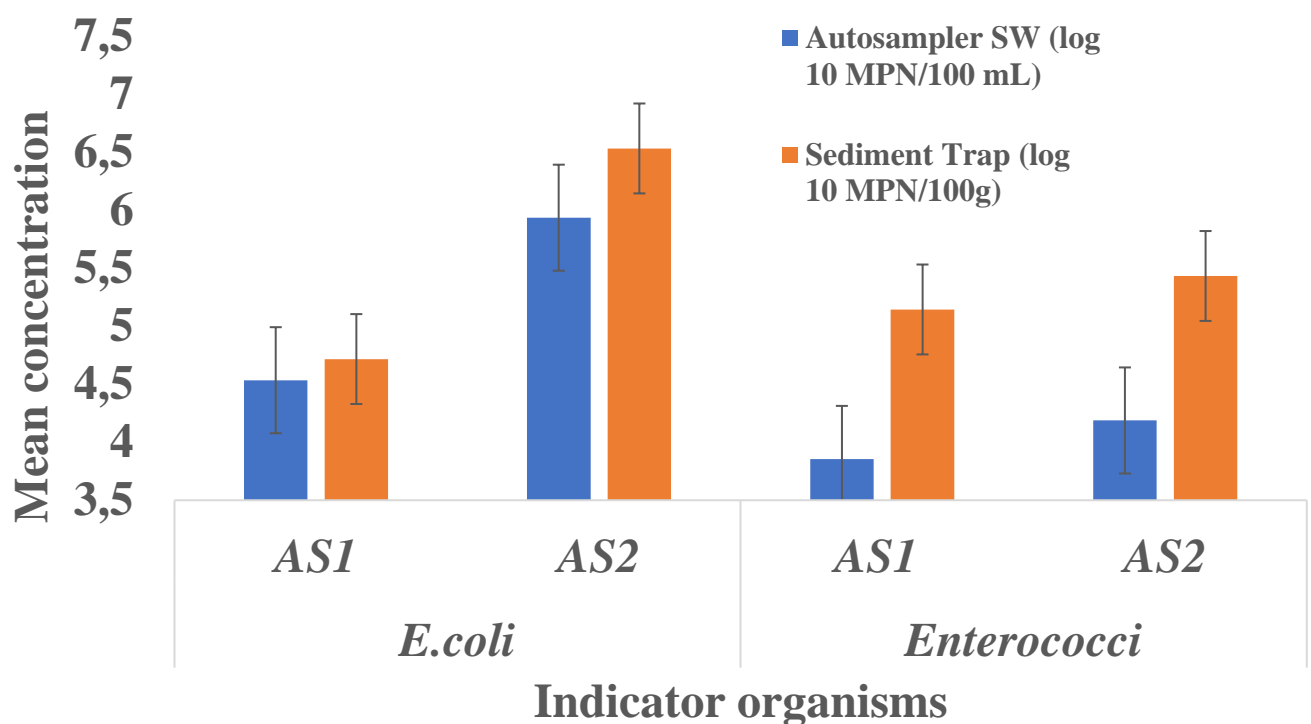


Figure 3.6: Comparison of *E. coli* and *Enterococci* concentrations in surface water and sediments in AS1 (no rainfall) and AS2 (after a heavy rainfall) analysed using auto sampler and sediment trap.

No significant difference of indicator concentration was observed in surface water over the 6-hour period as shown in Fig.3.5. Therefore, the mean values were compared with those obtained in the sediment trap, after 6 hours of installation. A similar trend was observed in AS1

and AS2 whereby the sediments samples exhibited higher concentrations of both indicators as opposed to surface water (**Figure 3.6**). Sediment samples had *Enterococci* concentrations >1 log compared to surface water in both events (**Figure 3.6**).

3.4 Discussion

3.4.1 A) Historical data analysis; Isipingo River

Historical data analysis helps researchers to establish existing conditions of a particular catchment. The historical data represents the routine monitoring which occurs once every month on the basis of surface water grab samples and results are related to the eThekweni Water and Sanitation stipulated regulatory framework. In section 3.4.5 variability was examined using an auto-sampler, which is different from the normal grab sampling. Also, the routine monitoring of sediment grab samples was analysed with surface water samples in parallel. This will be discussed in the following section 3.4.3.

When the historical data from the Isipingo River was analysed, a similar pattern was observed in all three sampling points, whereby the *E. coli* concentration decreased in 2010 and 2011, and increased again from 2012 and 2013. A slight decrease was observed again in 2014. The sampling point Next to WWTP was the most contaminated sampling point out of the 3 points, followed by the one downstream, throughout the 66 months' period, while the River mouth had the lowest level of contamination. The sampling points Next to the WWTP and Downstream consistently fell above the critical level (>4 Logs) according to EWS limits throughout the years, except for the years 2010 and 2011 even though it was in a poor state (between 3.3 – 4 logs) during those 2 years as well. Although these 2 years have the lowest concentration of *E. coli* they had the highest amount of variation, compared to the year 2013 which was extremely contaminated at 5.6 Log MPN/100 mL with very little variation (refer to Table 3.2 for standard deviation). This may be due to heavy rainfalls or unexpected events such as pipe outburst, WWTP overflows and faulty manholes.

The sampling point at the River mouth was in a poor state in 2009 and 2010, and thereafter the *E. coli* concentrations dropped, falling into the acceptable range from 2011 to 2014. The quality of the river water was consistently better (at the acceptable range according to the eThekweni water and sanitation standard limits). Analysis of Variance (ANOVA) was performed amongst the yearly means at each site. Significant difference was observed at all sites. However, the highest significant difference was observed at the sampling point Next to the WWTP, Downstream and at River mouth respectively (p-value= <0.001; 0.002; 0.0072). The high levels of *E. coli* at the Isipingo River over the years is due to the impact of its surroundings. The sampling point next to the WWTP for example is highly influenced by contamination from the Isipingo wastewater treatment works during planned/unplanned overflows into the river (which may be due to lack of timely maintenance, faulty equipment and overloaded design capacity from time to time) as well as occasional pipe outbursts. This poses a major impact on the health and wellbeing of the community (Genthe et al., 2013, Sibanda et al., 2015) as some of the people from the surrounding community use the river water for domestic use, as well as for irrigation purposes for small scale market gardens (the produce is sold in the local community). It is widely known that using fecal contaminated water can be beneficial as it provides nutrients for crops thereby saving costs (less need for fertilizers). It has further been shown that there is a potential health risks associated with the use of fecal contaminated water for irrigation (Beuchat, 2002, Gemmell and Schmidt, 2012). This creates a subtle balance where the health concerns outweigh the irrigation benefits.

This interesting topic will further be addressed in **chapter 5** whereby the risk of using fecal contaminated river water for crop irrigation is explored in detail. The sampling point situated downstream is surrounded by Transit camps/informal settlements, which also serve as a pollution source particularly due to contamination that is washed off into the river during heavy rains. The third sampling point of interest situated at the River mouth is situated next to the

Isipingo beach. The low *E. coli* concentration at this point could be due to the self-cleansing of the sea and the back flux of the sea water into the River mouth, as well as the sensitivity of *E. coli* to the high salt content. This was proven by measuring the conductivity values (extrapolated using conductivity results obtained in Feb 2015 from the municipality) of all 3 sampling points (refer to Appendix 1; Figure), and it was observed that the sampling point at River mouth had the highest conductivity value ($4.47 \mu\text{S}/\text{cm}^\circ$) which was almost double the amount of the other two sites ($\pm 2.6 \mu\text{S}/\text{cm}^\circ$).

3.4.1 B) Historical data analysis; Palmiet River

It was observed that *E. coli* concentration was consistently higher in the years 2011 and 2012. Also, when we looked at the sampling point Palmiet 01 it was consistently low throughout the years except for the year 2011 and 2012. This could be due to a certain event that could have occurred during these two years possibly a faulty WWTP/pump station. It could also be as a result of more rainfall on these two years compared to the other years. When the sites of interest along the Palmiet River were compared, there was no significant difference between the 6 sites, hence very little variation was observed (as shown in Figure 3.4). When ANOVA was performed, no significant difference was observed in the yearly means between all the sites, with p-value ranges from (0.1995 to 0.9609) over a 66 months' period. The pollution sources surrounding the Palmiet River include ablution blocks, pump stations, as well as contaminants from industries. An interesting observation was that high levels of *E. coli* were observed on sampling sites that are situated next to/ near a pump station (Palmiet 05 and Palmiet 06 and 08). This is for obvious reasons such as pipe outbursts, overflows, etc. However, none of the sites were situated along the Quarry road informal settlements which is a major health risk, thus this area will be investigated in this study.

3.4.2 Physiochemical parameters

The two rivers studied (Isipingo and Palmiet Rivers) had high total suspended solids (TDS) content, considerably higher than the WHO recommended guideline for drinking water of 20 mg/L (WHO, 2003). Although both rivers exceeded the WHO guidelines for TDS, the Isipingo River had the highest values with relatively higher microbial load than the Palmiet River. According to (Nagels et al., 2002, Johnson et al., 2010) suspended solids are directly proportional to the microbial load with high turbidity coinciding with high fecal indicator loads, in most water bodies depending on the sampling site. This is probably because turbidity may relate to a variety of factors, for example runoff from surrounding areas (Nkwonta and Ochieng, 2009). Studies conducted by Blaustein and Pachepsky (2014) showed that temperature is also one of the most important physiochemical properties that affects the growth and survival of microorganisms in water. Warmer water temperatures are related to a higher microbial concentration (Blaustein et al., 2013, Pachepsky et al., 2014). In this investigation, the water temperature was in the same range and will most probably not influence the microbial quantities found. The Isipingo is joined by a twin pipe to the sea at River mouth. At this point the highest conductivity values were observed and relate to the high salt content in the sea water that mixes with the river water due to back currents.

3.4.3 *E. coli* and *Enterococci* quantities in Isipingo and Palmiet River's surface water and sediment

As expected, and previously shown (Ishii et al., 2007, Whitman and Nevers, 2003, Abia et al., 2016a) *E. coli* and *Enterococci* were highest in sediment samples. In this study, *E. coli* and *Enterococci* concentrations were routinely ± 1 Log greater in sediments samples when compared to surface water samples at all sampling points in both rivers. Similar relationships were also shown by (Alm et al., 2003, Ishii et al., 2007, Sithebe et al., 2016, Ekwanzala et al., 2017, Whitman and Nevers, 2003), in comparable studies. Different types and size ranges of sediments will impact the microbial abundance, where finer sediment particles were found to

have a positive correlation to higher microorganism populations (Kunkel et al., 2013, Howell et al., 1996, Gao et al., 2011, Abia et al., 2015a). Particle associated microorganisms may also survive longer in sediments as compared to surface water, partly due to protection from UV rays as well as from predators (Kunkel et al., 2013, Liang et al., 2013).

Total coliforms and fecal coliforms were tested in parallel and similar trends found as portrayed by *E. coli* and *Enterococci*. These two indicators were not included since many species in these groups are normal inhabitants of sediments, plants and decaying organic material. Sampling point “Next to WWTP” was the most polluted point followed by River mouth and sampling point situated upstream was the least polluted in both the surface water and sediment samples. The difference in the microorganism concentrations at the various sampling sites shows an impacting variation at each site. For example, a higher fecal pollution at the sampling point next to the WWTP may be due to incidences such as pipe bursts or overflowing of untreated wastewater into the river, whereas lower concentrations of fecal indicators were observed in the sampling point upstream. Water at these two sampling points is used for irrigation (this was observed during the study), although the point upstream has lower fecal pollution compared to the sampling point situated next to the WWTP. Both points are not suitable for irrigational use according to the stipulated WHO guidelines: $\leq 1,000$ Fecal coliforms/100 mL and DWAF ≤ 1 *E. coli*/100 mL (Blumenthal et al., 2000, WHO, 2001). Wastewater effluent is a major factor that negatively affect the quality of river water in South Africa (Luyt et al., 2012, Teklehaimanot et al., 2014). Although the sampling point upstream was subjected to pollution from anthropogenic activities, this was to a more limited extent than sampling point surrounded by the Transit camps. The sampling point at the River mouth was found to be second most polluted after the one next to the WWTP, this is because of the additional waste discharged by industries surrounding this sampling point.

At the Palmiet River, Methven sampling point continuously showed lower concentration of the indicator organisms due to cleaner surroundings. The neighborhood where this point is located is involved in a “River watch” initiative which is conducted by a stakeholder group that exists in Westville North led by Lee DEathe. Community members monitor undesirable activities and type of pollution and report it to the municipality. Also, the community members gather occasionally to clean up the river around the Westville North area. The other three sampling points are surrounded by the Quarry road informal settlement which is a major pollution source, especially downstream as this is where most of the pollution find its way and settle. The people from the informal settlements throw waste into the river as well as faeces and domestic greywater. Sadly, the residence who are situated far from the ablution blocks sometimes opt to use this river water for domestic purposes and children play in and along this part of the river. This poses a major health risk to the community of the Quarry road informal settlements.

3.4.5 Effect of rainfall on microbial concentration to address variability over time

A study conducted by Cha et al. (2010), highlighted that variability in fecal coliform concentration tends to be positively related to precipitation, hence, an increase in total rainfall is bound to elevate the microbial contamination levels. This relationship was also evident in a study conducted by Sithebe et al. (2016) in which *E. coli* log₁₀ MPN/ 100 mL concentrations were correlated with rainfall events (on the day of sampling) from the Palmiet River. Sithebe et al. (2016) found a positive trend between *E. coli* concentrations and rainfall ($r = 0.2646$ and $p\text{-value} = 0.2724$) (Figure included in Sithebe et al., 2016) and was verified in this study when no rainfall and rainfall events were compared.

The elevated levels of *E. coli* and *Enterococci* after a heavy rainfall events is potentially due to contamination from the WWTP as described above, or run-off from surrounding grounds with their contamination sources. During heavy rains, the river’s flow is increased which results in sediment resuspension and release of particle bound microorganisms into the surface water

(**Figure 3.6**). This increases the microbial level in both the surface water as well in the sediment trapped. Thus, the overall pollution level is expected to increase, as illustrated from the findings (**Figure 3.5** and **Figure 3.6**). This is in agreement with findings that rainfall results in deteriorated microbial quality due to microbial load from non-point sources (Ackerman and Weisberg, 2003, Martinez et al., 2014, Cha et al., 2010). The use of the auto-sampler (which takes samples every hour) enabled us to determine whether there was variability over time, which is often not the case in grab samples. However, the hourly variability was low over the six hour period. We could not, unfortunately, install the auto sampler and sediment trap over longer periods due to security reasons. Inclusion of sediment sampling during routine monitoring of river quality should be considered for the future because reliance on the surface water alone may be inadequate due to potential rapid microbial water quality changes which is not reflected to the same degree in the sediment particles.

3.5 Conclusions & Recommendations

The main objective of the historical data analysis was achieved, which was to obtain a baseline on both the rivers as well as to determine areas that needed improvement, such as the addition of sampling points along the rivers in areas that may pose a risk to human health e.g. QRI settlements. The eThekweni municipality should consider sampling points in and around the Quarry road informal settlement (Palmiet River).

Overall, when the microbial water quality of the Isipingo and Palmiet Rivers were compared, it was found that the Palmiet had far better water quality than the Isipingo, however both rivers exceeded the stipulated guidelines as stated in table 2.1 (WHO, 2003, WHO, 2006, DWAF, 1996a).

The presence of high numbers of indicator organisms in sediments of both the Isipingo River and the Palmiet River indicates that the river receives high loads of fecal pollution, hence there is a high likelihood of the presence of other pathogens posing a major health risk. Therefore, there is a need to further investigate the presence of pathogens in surface water and sediments as well as to use sediment traps in order to assess particle associated microorganisms and determine the impact and corresponding risks associated with fecal contamination in surface water (**This will be addressed in chapter 5 of this study**).

Based on the findings in this study, it is recommended that sediment testing should be incorporated into the surface water microbial quality monitoring programme conducted by the municipality monthly on South African rivers.

Seasonal variations have an indirect impact in the abundance of indicator organisms in water and sediments with the wet season having a greater negative influence on the river water quality. Therefore, event monitoring programme using auto-samplers over longer time periods during rainy seasons should be employed in order to better understand this phenomenon and the linked variability in concentrations and risks.

CHAPTER 4 : PERFORMANCE OF CONVENTIONAL AND MOLECULAR METHODS

4.1 Introduction

The accessibility of water resources is essentially linked to water quality. The contamination of surface waters may prohibit its use for various activities. According to the United Nations World Water Development Report (WWAP, 2017) in the near future, the deterioration of water quality is expected to increase especially in developing countries. This will endanger human health and the environment, while constraining sustainable economic development (Veolia/IFPRI, 2015). The release of untreated wastewater from fast growing informal settlements and industries, generates biological, physical and chemical pollution, which has adverse impact on both human and environmental health (WWAP, 2017)

Therefore, monitoring and reporting of both pollutant discharge to the environment and river water quality are of utmost importance for achieving progress in relation to water quality (Murray 2004). According to the United Nations World Water Assessment Programme (WWAP) report “*If something is not measured, the problem cannot be defined and the effectiveness of policies cannot be assessed*” (WWAP, 2017). Indicator bacteria have been used for many years to determine the quality of water for human consumption, recreational activities and water for domestic purposes. According to the water quality guidelines, water deemed fit for domestic purposes should contain Total coliforms ≤ 5 CFU/100 mL and no fecal coliforms/100 mL (DWAF, 1996a). The minimum acceptable limit for South African recreational waters is ≤ 500 CFU/100 mL *E. coli* and ≤ 185 /100 mL *Enterococci* (DWAF, 1996c). The eThekweni Municipality water and sanitation department use *E. coli* as a microbial indicator on surface water of many rivers. Their guidelines stipulate that surface water with *E. coli* concentration greater than 3.2 log MPN/100 mL is in a poor state.

The most commonly used methods to detect fecal indicator bacteria include membrane filtration (Eckner, 1998, ISO 2000b), using membrane fecal coliform (mFC) agar, most probable number (MPN) techniques (Wohlsen et al., 2006) and the commercial enzymatic kits Colilert-18/ Enterolert ISO 9308: 2:2012 (ISO 2012, Wohlsen et al., 2008).

One objective of this study focuses on comparing two most widely used techniques for the enumeration of fecal indicator bacteria in surface water (**refer to Objective 2**). Culture based techniques such as the membrane filtration or spread plate technique on selective media (agar) compared to enzyme defined substrate technique (Colilert and Enterolert IDEXX). The membrane filtration is often used for less turbid samples, and the spread plate technique or other MPN methods are often used for highly concentrated samples (Murray 2004). Culture based methods entails initial incubation of samples, assessment of plates for typical colony morphology, followed by sub culturing presumptive target organisms for confirmation using a biochemical test, which leads to results in approximately 72h after initial sample analysis (Buckalew et al., 2006). Also, this type of techniques often require more labour and materials, and requires a high level of technical proficiency to read, interpret and confirm results (Ahmed et al., 2012). The enzyme defined substrate techniques however, is easy to use and produces conclusive results within 18–22h of testing. The Colilert18 and Enterolert methods make use of a multiple tube technique which is a method that gives the most probable number (MPN) of cells, and hence replication of samples is already accounted for in the technique. The Colilert-18 has a detection limit of 1 organism /100 mL of total coliforms/ *E. coli* concentrations (IDEXX, 2001) and has been shown by previous studies to have a better 95 % confidence interval than standard membrane filtration and multiple-tube lactose fermentation techniques (Valente et al., 2010, Brand and Barnes, 2014, Yakub et al., 2002, Buckalew et al., 2006). The Colilert-18 works on the basis of two nutrient-indicators, ortho-nitrophenyl galactopyranoside (ONPG) and 4-methyl-umbelliferyl-glucuronide (MUG). As coliforms grow in Colilert, they

use enzyme β -galactosidase to metabolize ONPG and change it from colourless to a yellow colour. *E. coli* uses β -glucuronidase to metabolize MUG which produces a fluorescent product under UV light at 366nm (Tortorello, 2003, IDEXX, 2001). Fricker (1997) compared Colilert to membrane filtration for surface and drinking water samples and found that Colilert detected more coliforms than membrane filtration but there was no significant difference between methods for the detection of *E. coli* (Fricker et al., 1997). The Enterolert also works on the basis of enzyme activity, like the Colilert-18 method. This method is based on the β -glucosidase enzyme activity of enterococci to produce methylumbelliferylone from 4-methylumbelliferyl- β -D-glucoside when incubated at 41°C for 24 hours. Methylumbelliferyl derivatives are known for their high sensitivity and specificity, non-carcinogenic and easily detected under UV light (Budnick et al., 1996), thus detection is based on fluorescence at 366 nm (IDEXX).

The application of molecular methods should be considered in the context of quality management for potable water. The new methods influence epidemiology and outbreak investigations more than the routine testing of processed drinking water. A number of molecular approaches such as RFLP (Restriction fragment length Polymorphism), RAPD (Random Amplification of Polymorphic DNA), FISH (Florescence *in situ* Hybridization), PCR (Polymerase Chain Reaction), qPCR (Quantitative PCR) and digital PCR can be used for pathogen detection but each method has limitations. However, for the fulfilment of the second objective of this study, qPCR and droplet digital PCR were compared (**Objective 2**).

Quantitative PCR (qPCR) also known as Real Time PCR, is a fluorescence based detection format which is more sensitive than conventional PCR. Like PCR, real time PCR works on the basis of amplification of target DNA to generate multiple copies that can be detected. However, samples can be analysed in real time with higher specificity, sensitivity, reproducibility and

wide quantification range. Thus, no Post PCR processing is required. The technique has been applied to locate non-point sources of pathogen contamination and environmental risk assessment (Singh et al., 2010, Singh et al., 2016).

Although qPCR is most preferred method for the quantitative enumeration of pathogenic microbes in complex environmental matrices, there are still drawbacks that often affect the analysis of results, especially when dealing with complex sample mediums. Some of the limitations range from specificity of primers targeting a specific group, florescent probes as well as nucleic acid extraction efficiency. The quality of template nucleic acid and amplification of DNA are also important factors that affect qPCR analysis.

The application of qPCR in sediment samples is a challenge mainly due to the presence of PCR inhibitory substances (Sidstedt et al., 2015). Even a small quantity of PCR inhibitors can delay the C_q (threshold Cycles) of complex sample in qPCR, causing erroneously low estimates of the template copy number (Sidstedt et al., 2015). However, the water emulsion technology based droplet digital PCR (ddPCR), has emerged as a direct quantitative method with the potential of overcoming the inhibitory effects, affecting qPCR (Hindson et al., 2011). An additional advantage with ddPCR over qPCR is to enable the absolute quantification of DNA concentrations without external calibrators (Hindson et al., 2011, Pinheiro et al., 2012). In digital PCR, the sample is subjected to partitioning into hundreds to millions of individual reaction chambers (depending up on the digital PCR platform) prior to the PCR cycles, so that each contains one or no copies of the sequence of interest (Baker, 2012, McDermott et al., 2013). The partitioning of the sample into multiple droplets (in the case of ddPCR) substantially reduce the susceptibility to PCR inhibitors present in the extracted DNA (Morisset et al., 2013). Recent studies have demonstrated the accuracy and precision of ddPCR in the quantitative detection of bacteria and viruses in clinical samples (Kim et al., 2014, Lui and Tan, 2014, Racki

et al., 2014, Devonshire et al., 2015, Zhao et al., 2015) but its environmental application for the detection of, for example, *Salmonella* has so far not widely done,

Therefore, **in accordance with Objective 2**, conventional standard membrane filtration methods were explored using chromogenic/fluorogenic and non-chromogenic media in comparison to the enzymatic Colilert-18 and Enterolert (IDEXX) test methods along the Isipingo and Palmiet Rivers. In addition, **for Objective 2** a comparison of the analytical performance of ddPCR and qPCR for the detection of *Salmonella* targeting *ttr* gene in river sediment samples collected from four sites of the Palmiet River in Durban, South Africa was included.

4.2 Methodology

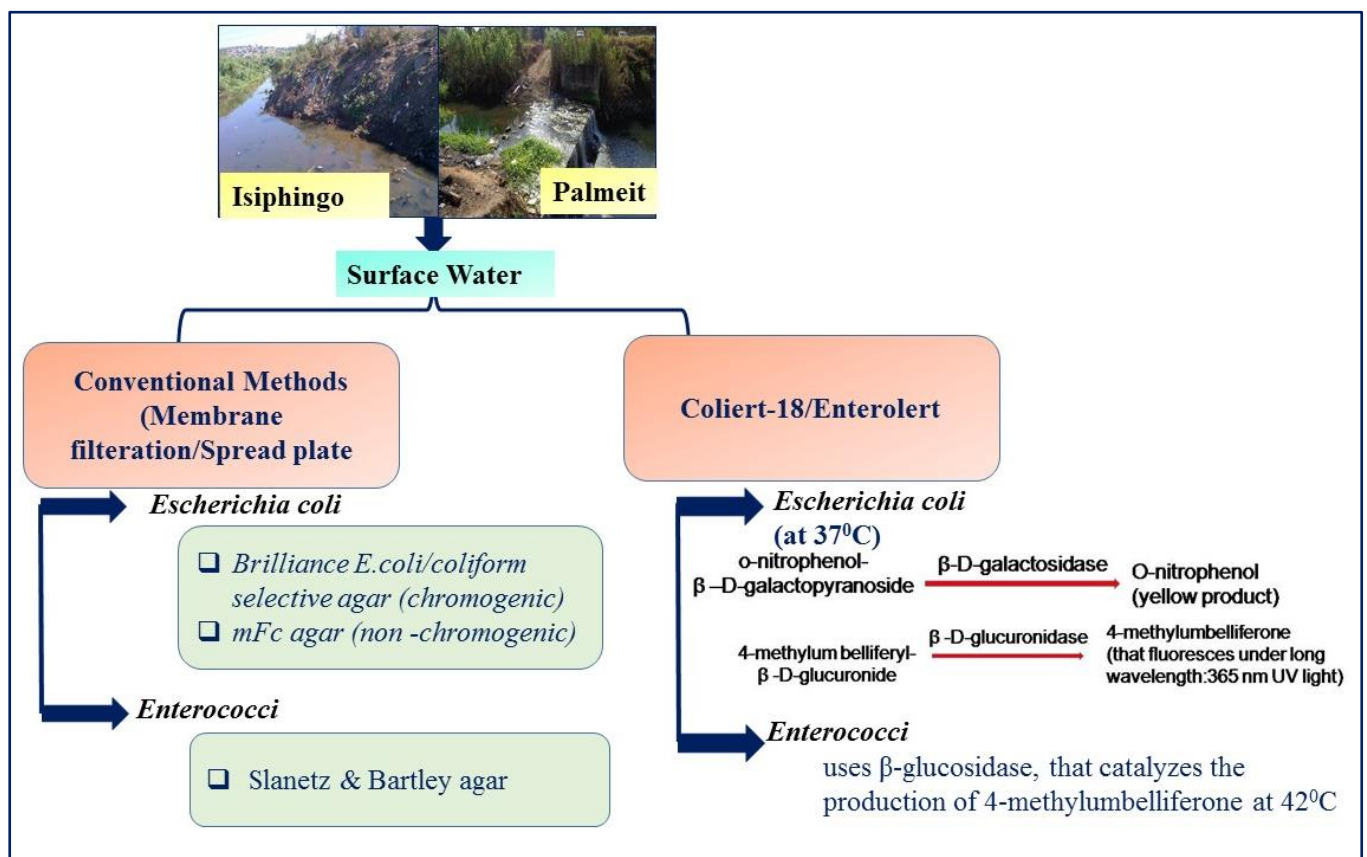


Figure 4.1: Graphical representation of the comparison of conventional and enzyme based methods for detection of *E. coli* and *Enterococci*.

4.2a Methodology for comparison of Conventional methods with Colilert-18 and Enterolert method

4.2.1a Description of study area

The area of study was as described in Chapter 3: section 3.2.1

4.2.2a Sample collection

Surface water and sediment samples were collected and stored as described in Chapter 3: section 3.2.4

Isolation and cultivation of target bacterial indicators from sediments was Preparation of sediment samples were conducted as described in Chapter 3: Section 3.2.5

4.2.3a Enumeration of bacterial indicators using method 1 (Membrane filtration (MF) / spread plate method)

Enumeration of *E. coli* and *Enterococci* were analysed using the conventional membrane filtration (MF) / spread plate method according to international standard methods (APHA, 2005, Buckalew et al., 2006). Selective media was prepared according to manufacturer's instruction in 90 mm petri plates. Appropriate serial dilutions ranging from 10^1 to 10^6 of the surface water samples were prepared with sterile distilled water prior to filtration where 100 mL samples from the series dilution were vacuum filtered through 0.45 μm pore size (47 mm) GN-6 Metrical membrane filters (Merk Millipore, USA). The filters were transferred on to 90 mm petri plates containing the specified selective media as described in Table 4.1. The Brilliance *E. coli*/Coliform Agar and Slanetz & Bartley media were procured from Thermo Scientific Oxoid™. Membrane filtration agar was procured from Merk Millipore, USA. Autoclaved distilled water was used as blank sample during the MF technique to ensure the quality of dilution. After the incubation period, all the typical colonies for indicator bacteria according to standard protocol that grew on the filters were recorded as presumptive counts for the estimation of colony forming units per 100 millilitre (CFU/100 mL), which was calculated using the following equation: (APHA 2005)

$$CFU/100mL = \frac{\text{number of colonies} \times 100}{\text{sample volume}} \times \text{dilution factor}$$

For highly concentrated/ turbid samples, the spread plate method was also used. Whereby appropriate serial dilutions ranging from 10^1 to 10^6 of the surface water samples occurred, dilutions were prepared with sterile distilled water, and 0.1 mL of the respective dilution was inoculated and spread on the appropriate selective media in triplicate (as described in Table 4.1). After the incubation period, presumptive counts were recorded and colony forming units per 100 millilitre (CFU/ 100 mL) was determined using the following equation:

$$CFU/100mL = \frac{\text{number of colonies} \times \text{dilution factor}}{\text{sample volume}} \times 100$$

Table 4.1: Selective media and incubation conditions used for the detection and enumeration of indicator organisms retrieved from Isipingo and Palmiet Rivers

| Indicator organism | Selective media | Incubation parameters | Presumptive colonies |
|---|--|---------------------------------|----------------------|
| <i>E. coli</i> | Brilliance <i>E. coli</i> /Coliform Agar | 24 hrs at 37 °C | Purple |
| Fecal coliforms (FC)/ <i>E. coli</i> | M-FC agar | 24 hrs at 44.5 °C | Blue to dark blue |
| Intestinal <i>Enterococci</i> (IE) | Slanetz& Bartley | 4 hrs at 35°C 44 hrs at 45°C | Red or maroon |
| | Bile Esculin Azide | 18-24 hrs at 35°C | Brown |

4.2.4a Enumeration of bacterial indicators using method 2 (Colilert and Enterolert IDEXX method)

E. coli and *Enterococci* was enumerated using the Colilert and Enterolert method respectively, as described in Chapter 3: section 3.2.7.

4.2.5a Statistical analysis

Data was analysed using GraphPad prism software and Microsoft excel.

4.3a Results

Table 4.2: Mean \pm SD comparison of conventional method vs Colilert-18/ Enterolert (IDEXX) method along Isipingo and Palmiet Rivers

| Indicator organisms (Log MPN/100 mL) | Isipingo River (N=20) | Palmiet River (N=16) |
|---|-----------------------|----------------------|
| ^a <i>E. coli</i> -1 | 4.46 \pm 1.01 | 3.91 \pm 1.0 |
| ^b <i>E. coli</i> -2 | 4.95 \pm 1.0 | 4.07 \pm 0.8 |
| ^a <i>Enterococci</i> -1 | 4.29 \pm 1.0 | 3.87 \pm 1.1 |
| ^b <i>Enterococci</i> -2 | 4.56 \pm 1.1 | 3.85 \pm 0.9 |

Isipingo River, n=20; Palmiet River n=16

^aConventional method (Membrane filtration/Brilliance *E. coli* agar) is represented as method 1

^bColilert-18/ Enterolert (IDEXX) method is represented as method 2

Comparative data is presented in

Table 4.2. It is evident that the MPN/Colilert-18 and Enterolert method (2) continuously yielded higher microbial means compared to the conventional culture plate method (1). Results from the Isipingo River showed that the log difference between the *E. coli* indicator using method 1 and method 2 was 0.49 Log MPN /100 mL and 0.27 Log MPN /100 mL for the *Enterococci* indicator between the two methods. A similar trend was observed in results obtained from the Palmiet River, where *E. coli* and Enterococci mean differences between the two methods was 0.16 Log MPN /100 mL and 0.02 Log MPN /100 mL respectively. No statistical significant difference was observed between the method 1 and 2 (p-value >0.05) for both *E. coli* and *Enterococci* tests.

Table 4.3: Unpaired t-test results obtained when comparing different methods.

| Method/media | Isipingo River (p-value) | Palmiet River (p-value) |
|-----------------------|--------------------------|-------------------------|
| mFC vs CM1046 | 0.0202* | 0.0481* |
| mFC vs Colilert-18 | 0.0006*** | 0.0135* |
| CM1046 vs Colilert-18 | 0.0711 | 0.3141 |

*indicates level of significance ($\alpha=0.05$); CM1046 -Brilliance *E. coli*/coliform selective agar

Unpaired t-test results showed the highest significance difference between the means of *E. coli* obtained using mFC agar and Colilert-18 (with greater sensitivity demonstrated by the Colilert-18 method) in both rivers; Isipingo (p-value= 0.0006) and Palmiet (p-value= 0.0135). Significant difference was also demonstrated between mFC and CM1046 (Table 4.3), the latter showing greater sensitivity. There was no significant difference in *E. coli* means obtained using CM1046 verses Colilert-18 method, in both the Isipingo and Palmiet rivers (p-value >0.05).

4.4a Discussion

Analyzing surface water can be a challenge since the level of background microflora can vary. Therefore, it is important that the method/ culture medium used for surface water quality is sufficiently selective. In this study CM1046 Brilliance *E. coli* /coliform selective agar, mFC agar and Colilert-18 were used for the enumeration of *E. coli* in parallel testing of surface water samples. For the enumeration of *Enterococci*, Slanetz & Bartley agar / Bile Esculin Azide agar method and Enterolert test methods were used.

Although the methods were determined as ‘different’ (

Table 4.2), there was no statistical significant difference between the two methods (membrane filtration vs enzyme activity methods). Unpaired t-test (95% CI) results (Table 4.3) obtained in

| Indicator organisms (Log MPN/100 mL) | Isipingo River (N=20) | Palmiet River (N=16) |
|---|------------------------------|-----------------------------|
| ^a <i>E. coli</i> -1 | 4.46 ±1.01 | 3.91 ±1.0 |
| ^b <i>E. coli</i> -2 | 4.95 ±1.0 | 4.07 ±0.8 |
| ^a <i>Enterococci</i> -1 | 4.29 ±1.0 | 3.87 ±1.1 |
| ^b <i>Enterococci</i> -2 | 4.56 ±1.1 | 3.85 ±0.9 |

a comparison of *E. coli* mean values from CM1046 Brilliance *E. coli*/Coliform chromogenic media and Colilert-18 (IDEXX)

showed that there was no significant statistical difference between the means (mean ±SD shown in

Table 4.2, (p-value>0.05). No statistical difference was observed between the sampling sites either. When *Enterococci* was enumerated with (CM0377 Slanetz & Bartley medium) verses Enterolert (as shown in

Table 4.2), no significant difference was observed (p-value>0.05). The same trend was observed in both the Isipingo and Palmiet Rivers. This is because the media used for both the membrane filtration and the IDEXX Colilert and Enterolert is chromogenic and therefore detects enzyme activity. CM1046 Brilliance *E. coli*/Coliform agar contains the chromogenic agent X-Glu that detects β-glucuronidase activity in *E. coli* bacteria. The Colilert-18 contains the substrate methylumbelliferyl-β-glucuronide (MUG), however, *E. coli* has the ability to cleave methylumbelliferyl-β-glucuronide (MUG), resulting in the formation of the fluorescent product 4- methylumbelliferone within 18 hours (Eckner, 1998). Other researchers also found

that Colilert and CM1046 agar were equally sensitive in detecting *E. coli*, although slightly higher *E. coli* counts were detected with Colilert-18. Wohlsen (2006) found that Colilert-18 produced the most consistent results with the lowest coefficient of variation for total coliforms and *E. coli*. Some of the studies about the comparison of the methods in relation to the enumeration of *Enterococci* were reported by Budnick et al., 1996, Adcock and Saint, 2001, Yakub et al., 2002, Valente et al., 2010, Abbott et al., 1998. Budnick (1996) concluded that the standard method of membrane filtration and the Enterolert method were equivalent for a confidence interval of 95% (Budnick et al., 1996). This was in agreement with the results obtained in this study, whereby there was no statistical significance difference between the conventional membrane filtration and Enterolert methods in both the Isipingo and Palmiet Rivers.

Thermotolerant *E. coli* enumerated with mFC agar was compared with CM1046 Brilliance *E. coli*/coliform selective agar and Colilert IDEXX using an unpaired t-test (Table 4.3). Significant difference was observed when mFC results were compared with the two chromogenic media/methods, with greater sensitivity demonstrated by the chromogenic media (CM1046 Brilliance *E. coli*/ Coliform agar and Colilert-18), in both the Isipingo and Palmiet river sites. Overall the highest significant difference was observed between mFC and Colilert-18 at the Isipingo River (p-value = 0.0006).

The sensitivity and specificity of different chromogenic media was found similar whereby several chromogenic agars, including chromogenic *E. coli*/ coliform medium (Oxoid), reported higher coliform counts than lactose fermentation methods (Wohlsen et al., 2008; Wohlsen, 2011). According to Clark (1991), this is because mFC agar underestimates the true levels of *E. coli* present. Significantly higher *E. coli* concentrations were detected in media containing MUG than with the mFC method in natural and drinking waters (Fricker et al., 1997, Eckner,

1998, Schets et al., 2011, Wohlsen, 2011, Valente et al., 2010). The Colilert-18 procedure generally yields a higher number of *E. coli* when compared with the membrane filtration method. Differences in the culture media, as well as in the substrate used by both procedures, can lead to the detection of different species within the coliform group, which can account for this different result obtained (Valente et al., 2010). A discrepancy between the numbers estimated by Colilert-18 and membrane filtration was found, with Colilert-18 having higher mean values (Chao et al., 2003, Chao, 2006, Pisciotto et al., 2002, Valente et al., 2010). This is because, as mentioned above, chromogenic media detects enzyme activity and therefore there is a probability that higher counts may be obtained with these methods (CM1046 and colilert-18) when compared with data obtained by traditional lactose fermentation methods such as membrane filtration method using mFC agar (Wohlsen et al., 2008).

Analysis of microbial indicators by an enzymatic analysis in which chromogenic/ fluorogenic substrates are used has several advantages over normal conventional methods. The most important advantage is the public health benefit of shortened analysis and response times should microbial indicators be present in the water (hence the short incubation period). Furthermore, the total time needed to accomplish the analysis is reduced to 18-24 hours, and the confirmation step for *E. coli*/ *Enterococci* (Enterolert) is not necessary. The elimination of the confirmation steps of traditional methods saves approximately 48 hours and eliminates the need to either act on presumptive, non-differentiated results or delay action in situations where remedial action is required. Also, the use of Colilert-18 e.g. allows testing with one medium that contains two substrates in order to detect both coliforms and *E. coli*.

4.5a Conclusion & Recommendations

Overall it can be concluded that the use of chromogenic/fluorogenic media yields higher results. Therefore, these methods are suitable for the detection and enumeration of microbial indicators in surface waters, with Colilert-18 as the best for *E. coli* detection and Enterolert for

enterococci detection, because of the advantages that they have over normal conventional methods.

A partial confirmation step when using the Colilert-18 method has been recommended in order to eliminate uncertainties with regards to false positive and false negative results (Pisciotta et al., 2002). Pathogen detection is also highly recommended since the indicator organisms were present in relatively high numbers. This will be addressed in the second section of this study whereby a onetime pilot study was conducted along the Quarry road informal settlement at the Palmiet River, in order to compare the qPCR and ddPCR molecular methods for the quantification of the *ttr* gene targeting *Salmonella* in sediment samples.

4.2b Methodology for pilot study conducted using qPCR and ddPCR for the detection of *Salmonella* in sediment samples.

Results have been published in: Comparison of droplet digital PCR and quantitative PCR for the detection of *Salmonella* in sediments (refer to Appendix 3)

4.2.1b Sampling sites description

Four sampling sites were considered around the Quarry road informal settlements (as described in Figure 4.2). Sediment samples were collected and process for analysis as described in chapter 3 (section 3.2.2 and 3.2.4)

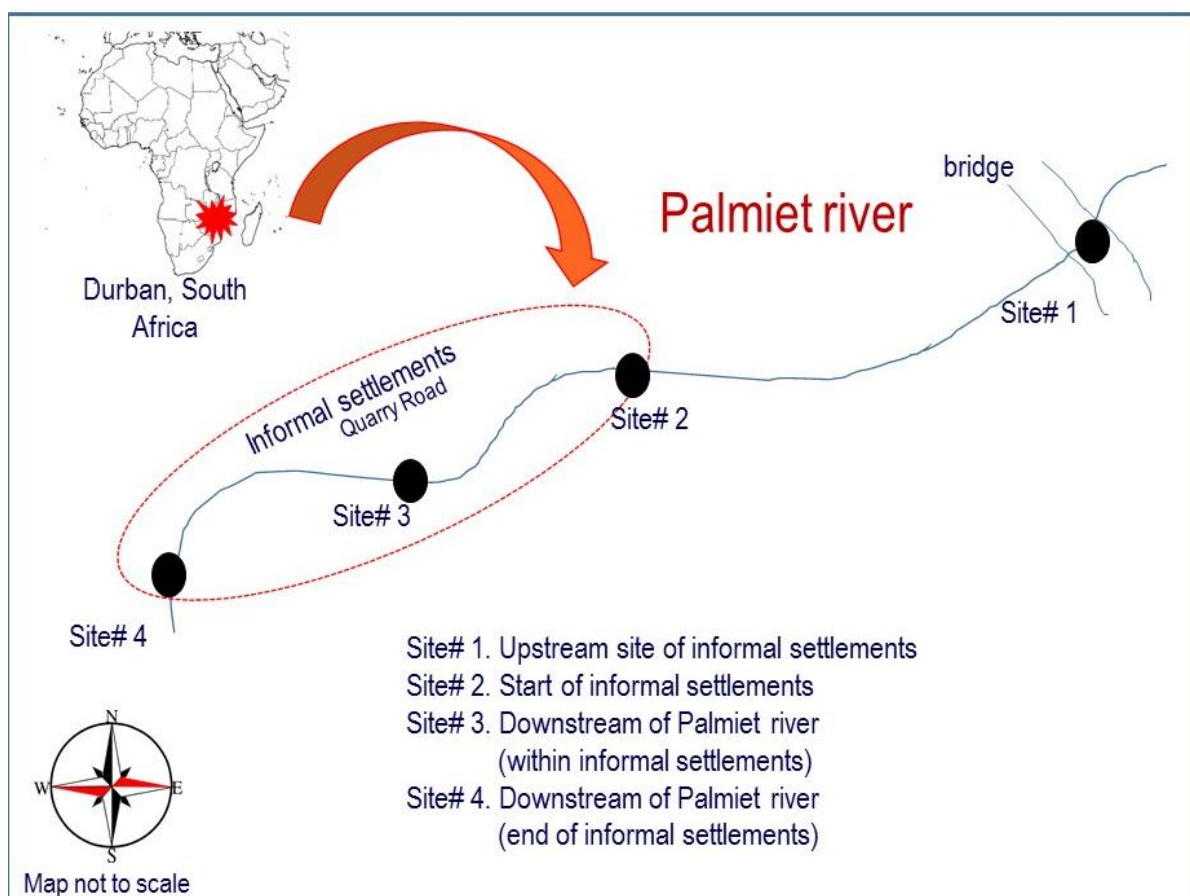


Figure 4.2: Graphical representation of the sampling sites selected for the pilot study conducted at the Palmiet River.

4.2.2b Bacterial strain and oligonucleotides

Salmonella enterica serovar *enteritidis* ATCC 13076 was procured from Microbiologics Inc, USA. The primers (forward ttr6, 5'-CTCACCAGGAGATTACAACATGG-3', position 4287-

4309 bp; reverse ttr4, 5' -AGCTCAGACCAAAAGTGACCATC-3' position 4359-4381 bp; product length 95 bp) specific for the *ttr* gene targeting *Salmonella* were adopted from Malorny et al. (2004). The primers used in this study were synthesized by Integrated DNA Technologies, USA.

4.2.3b Comparison of sensitivity of qPCR and ddPCR in spiked water sample

4.2.3.1b qPCR: Generation of standard curve and sensitivity analysis in spiked water samples

The qPCR standard curve was generated according to Jyoti et al. (2011). Briefly, *S. enteritidis* ATCC 13076 was grown at 37 ± 1 °C for 16 h in Luria-Bertani (LB) broth to an optical density of 0.8 at 600 nm (approximately 10^9 CFU /mL). The viable counts and the concentration (CFU/mL) of cells in the culture were determined according to Jyoti et al. (2011). Genomic DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN, USA) as per manufacturer's instructions. The quantity and quality of extracted DNA was measured at 260/280 nm with a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DC, USA). The total number of *Salmonella* genomic copies (GC) in purified DNA was calculated as follows: $m = n$ (1.013×10^{-21} g/bp), where m is the mass and n is the number of base pairs. The standard curve for the target gene was generated from the purified DNA extracted from the reference strain (*S. enteritidis* ATCC 13076) in the concentration range of 2- 2×10^6 GC/PCR. The qPCR assay was performed using CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD, Hercules, CA, USA). Briefly, the reaction mixture contained Maxima SYBR Green/ROX qPCR Master Mix (12.5 µL), primers (0.4 µM each, 1 µL each) and 5 µL DNA template (2 to 2×10^6 GC per PCR) in a final volume of 25 µL.

A mixture of all qPCR reagents containing 5 µL sterile Milli-Q® (Millipore, Billerica, MA, USA) water instead of DNA template in each qPCR assay served as a negative control. qPCR amplification protocol for the targeted genes consists of initial denaturation for 5 min at 95°C, followed by 45 cycles of three steps consisting of 10 seconds at 95°C, 20 seconds at 54°C and

20°C sec at 72°C. The fluorescence signals were measured at the end of each extension step. The standard curve was automatically generated by the CFX Manager™ Software v3.1. The sample concentrations were calculated from the generated standard curve. The sample was considered negative if the fluorescent signal did not increase within 45 cycles.

4.2.3.2b Droplet digital PCR: Sensitivity analysis in spiked water samples

S. enteritidis ATCC 13076 exhibiting *ttr* gene was grown in LB broth for 16 h at 37±1°C (optical density 0.8 at 600 nm). The viable counts (CFU mL⁻¹) of cell suspension were determined as stated in the above section. A serial 10- fold diluted culture (20 to 2x10⁴ CFU mL⁻¹ to get 2 to 2x10³ GC/PCR) was spiked, in triplicate, to 10 mL sterile Milli-Q® (Millipore, Billerica, MA, USA) water. DNA template was prepared from one mL spiked samples by extracting genomic DNA using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) as per manufacturer's instructions. The 5 µL of extracted DNA (range of 2 to 2x10³ GC/PCR) was used as template in droplet digital PCR for the detection and quantitative enumeration of *Salmonella*.

Droplet digital PCR was performed on the BioRad QX200 droplet digital PCR system (Bio-Rad Laboratories, CA, and USA). Briefly, *ttr* gene copies (2 to 2x10³ copies) were added with primers and Qx200™ ddPCR™ Evagreen Supermix (Bio-Rad Laboratories, CA, USA) to the reaction mixture in a final volume of 20 µL in accordance with the manufacturer's instructions (Bio-Rad Laboratories, CA, USA). The reaction mixture was then processed with 70 µL of droplet generation oil (Bio-Rad Laboratories, CA, USA) using the droplet generator (Bio-Rad Laboratories, CA, USA). The droplets generated were then transferred into a 96 well plate (BioRad, USA) and PCR amplification was performed with a thermal profile of denaturation at 95°C for 5 min, followed by 40 cycles at 94°C for 30s, 54° C for 1 min, 4°C for 5 min, 90°C for 5 min on a T100 thermal cycler (Bio-Rad Laboratories, CA, USA). Finally, the plate was

loaded onto the droplet reader (Bio-Rad Laboratories, CA, USA) and the data was generated and analysed using the Quanta Soft analysis software (Bio-Rad Laboratories, CA, USA).

*4.2.3.3b Comparison of qPCR and ddPCR performance for quantitative enumeration of *Salmonella* spp., in sediment samples*

For culture-free detection and quantitative enumeration of *Salmonella* spp., in riverine environment, sediments samples were collected in triplicate in sterile bags from four sites of Palmiet River (Figure 4.2) and transported on ice to the laboratory for further analysis.

DNA was extracted from 1g of sediments by PowerSoil® DNA Isolation Kit (Mo Bio, Laboratories) according to manufacturer's protocol. The extracted DNA (5 µL) was used as template in qPCR assays and ddPCR as described above. Quantitative enumeration of *Salmonella* in sediment samples by qPCR was carried out using standard curve prepared by 10-fold diluted genomic DNA of *S. enteritidis* ATCC 13076 (from 2 to 2×10^6 GC/PCR) while in ddPCR DNA extracted was directly subjected to droplet generation followed by PCR amplification to detect copies/µL of reaction mixture. The schematic representation of the developed strategy has been depicted below (Figure 4.3).

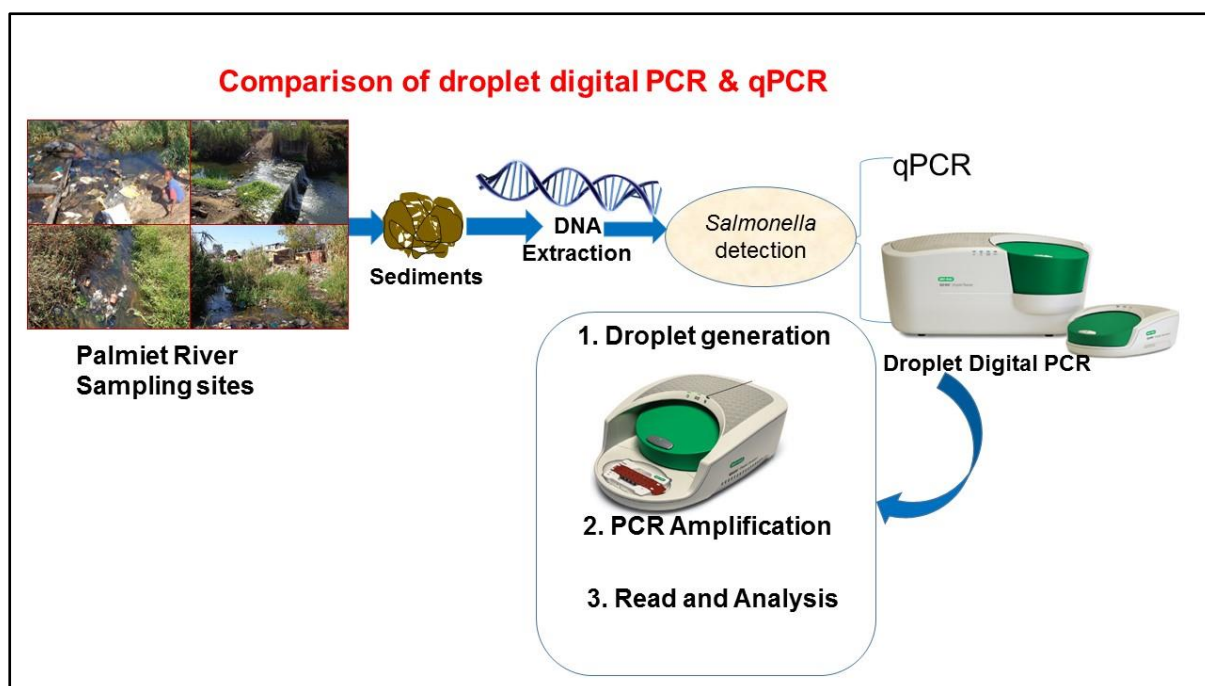


Figure 4.3: Schematic representation of the strategy adopted to compare qPCR and ddPCR

4.3b Results of comparison of qPCR and ddPCR

Table 4.4: Quantitative analysis of *Salmonella* through *ttr* gene based qPCR in water samples spiked with serially diluted culture of *Salmonella enteritidis* ATCC 13067

| <i>ttr</i> GC/PCR | Detection and quantitative enumeration of <i>Salmonella</i> spp. in spiked water samples | |
|---------------------------------|--|---|
| | Threshold | |
| | Cycles(C_T) ^a | |
| No. of <i>Salmonella</i> GC/PCR | Genomic DNA ^b | <i>Salmonella enteritidis</i> ATCC 13067 ^c |
| 2X10 ⁶ | 16.01±0.51 | 16.73±0.63 |
| 2X10 ⁵ | 19.32±0.63 | 20.40±0.71 |
| 2X10 ⁴ | 22.79±0.78 | 23.66±0.73 |
| 2X10 ³ | 26.37±1.08 | 26.99±0.71 |
| 2X10 ² | 29.69±0.89 | 30.78±0.69 |
| 20 | 33.45±1.21 | 34.88±1.28 |
| 2 | Not detected | Not detected |

^aValues are represented as mean C_T values (n= 3) ± SD

^b10 fold Standard curve generated by dilution of genomic DNA extracted from reference strain *Salmonella enteritidis* ATCC13067 (optical density 0.8 at 600nm).

^cA serial 10-fold diluted culture of *Salmonella enteritidis* ATCC 13067 (200 to 2x10⁸ CFU/mL to get 2 to 2x10⁶ GC/PCR in 25µL qPCR reaction) was spiked, in triplicate, to 10 mL sterile Milli-Q[®] water

For the generation of the standard curve, a 10-fold diluted genomic DNA (2 to 2X10⁶ GC/PCR) of *S. enteritidis* ATCC 13076 was subjected to amplification by SYBR Green based qPCR. The assay could detect and quantify 20 GC of the reference strain per PCR without any pre-enrichment Table 4.4. Quantitative PCR was found to be capable of rapidly detecting 2X10⁷ down to 20 CFU/ PCR in water samples spiked with the reference organism (*S. enteritidis* ATCC 13076) thus the qPCR assay was found to be sensitive. It can be assumed that one gene copy is present per cell of bacteria Singh et al., 2016.

Table 4.5: Quantitative analysis of *Salmonella* through *ttr* gene based ddPCR gene in water samples spiked with serially diluted culture of *Salmonella enteritidis* ATCC 13067.

| log Starting quantity ^d (log ₁₀ GC/PCR) | log positive Counts ^e (log ₁₀ GC/PCR) |
|--|--|
| 3.30 | 3.32 |
| 2.30 | 2.29 |
| 1.30 | 1.25 |
| 0.30 | 0.29 |

^d10fold serially diluted genomic DNA (from 2 to 2x10³ GC/PCR) of *Salmonella enteritidis* ATCC 13067; represented as mean value (n=3).

^eQuantities recovered (CFU/PCR reaction) in ddPCR from water samples spiked with serially diluted culture of *Salmonella enteritidis* ATCC 13067 ranging from 2 to 2x10³ GC/reaction; represented as mean value (n=3).

Droplet digital PCR was able to detect as low as log₁₀ 0.29 GC/PCR (2 GC/PCR) *Salmonella* with DNA extracted from water samples spiked with pure culture of *Salmonella enteritidis*

ATCC 13076. The results obtained were in concurrence to the limit of detection observed with 10-fold serially diluted genomic DNA (2 to 2×10^3 GC/PCR) of reference strain *Salmonella enteritidis* ATCC 13067 (as shown in Table 4.5).

Table 4.6: Comparison of qPCR and ddPCR performances in sediments samples collected from upstream and downstream of Quarry road informal settlements in Palmiet River

| Sampling Sites ^f | <i>Salmonella</i> ^g <i>ttr</i> GC/g in qPCR | <i>Salmonella</i> ^g <i>ttr</i> GC/g in ddPCR |
|-----------------------------|---|--|
| Site#1 | 59± 1.4 | 65± 0.77 |
| Site#2 | 355± 30 | 852±35 |
| Site#3 | 255± 37 | 818±30 |
| Site#4 | 75± 5.0 | 341± 31 |

^fSampling sites in Palmiet River, selected to collect sediment samples: Site#1: Upstream of Informal settlements, Site#2: Start of Informal settlements, Site#3: Downstream of Palmiet River, within informal settlements, Site #4; Downstream of Palmiet River (end of informal settlements)

^gValue represented (n=3) ±SD

Higher values of the *Salmonella ttr* GC/g were found using the ddPCR compared to the qPCR technique (in all four sites). Values obtained in qPCR ranged between 59± 1.4 and 355± 30 *Salmonella ttr* GC/g whereas in ddPCR values ranged between 65± 0.77 and 818±30 *Salmonella ttr* GC/g.

4.4b Discussion

The performance of both qPCR and ddPCR targeting *ttr* gene to detect *Salmonella*, was analysed in water samples spiked with a tenfold serially diluted culture of *S. enteritidis* ATCC 13076. Overall, the expected copy numbers measured from both the methods (qPCR and ddPCR) showed good linear regression correlation coefficients (R^2) values of 0.999 and 0.994, respectively in spiked water samples (Table 4.4 & Table 4.5).

The performance of ddPCR was further analysed to detect targeted bacteria in sediment samples collected from Palmiet River sites around Quarry road informal settlements in Durban

and subsequently compared with the qPCR (Figure 4.3). The sensitivity and linear range of ddPCR were comparable to those of qPCR in the case of spiked water samples (Table 4.4 & Table 4.5) but significantly produced higher results (GC/g) in the case of sediment samples (Table 4.6). This is probably due to the droplet partitioning in ddPCR, where, the whole PCR reaction is split into 20,000 droplets in which ideally each droplet contains 1 or less copies of targeted DNA, effectively reducing the effect of PCR inhibitors (Baker, 2012).

The first site was located upstream of the informal settlements at Quarry road and the quantities of *Salmonella* was expected to be lower as compared to the downstream sites, due to less anthropogenic activities (Figure 4.2). The sediments collected from the other three sites exhibited high numbers of *Salmonella* as compared to the upstream site 1 (Table 4.6). The *Salmonella* load was found to be significantly different in both qPCR and ddPCR for sites located at the start, within and at the end of the Quarry road informal settlements (p value= 0.0025, unpaired 't' test) (Table 4.6). Site#2, the point at which the informal settlements start, ddPCR gave 852 ± 35 *Salmonella* GC/g of sediment whereas the corresponding qPCR result was 355 ± 29.6 GC/g ($p \leq 0.0001$). The *Salmonella* load was found to be 818 ± 29.6 and 255 ± 36.6 GC/g in ddPCR and qPCR respectively (difference was statistically significant, $p \leq 0.0001$) at the site located within the informal settlement (Table 4.6).

The *Salmonella* numbers in the downstream site of the Palmiet River (at the end of informal settlements) was found to be 341 ± 30.9 and 75 ± 4.7 GC/g of sediment in ddPCR and qPCR respectively ($p \leq 0.0001$). The higher values in the sites within and downstream of the informal settlements is in agreement with the previous chapters. This is potentially due to the direct discharge of wastes into to the river streams from the community members as well as run off that is washed off into the river. The presence of significantly higher amount of *Salmonella* in the sediments of the Palmiet River gives an indication of a higher likelihood of presence of

other pathogens as well, and may pose a serious health risk to the inhabitant of the informal settlements and downstream localities.

One stated limitation of ddPCR in comparison to qPCR is the need to perform dilution of the samples as concentrations above 75,000 copies of the target molecules lead to a significant loss of linearity at high (Hayden et al., 2013). This was also evident in our results. The digital droplet PCR showed higher variability and less good precision at the higher concentrations (2×10^5 or 2×10^4) while qPCR performed well at these higher concentrations. In order to overcome this problem, the ddPCR was performed on DNA standards ranging from 2.0×10^0 *ttr* gene copies to 2.0×10^3 *ttr* gene copies (Table 4.6).

4.5b Conclusion

Droplet digital PCR may provide an opportunity to reduce the inhibitory effects of PCR inhibitors experienced with qPCR, but the methodology need to be further optimized for complex environmental samples. In conclusion, for this first comparison related to *Salmonella* and sediment samples, ddPCR is fully amenable for the quantification of *Salmonella* and offer robust, accurate, high-throughput, affordable and more sensitive quantitation than qPCR of pathogens related to this type of environmental samples.

CHAPTER 5 : COMPARATIVE ASSESSMENT OF RISKS ASSOCIATED WITH FECAL CONTAMINATION IN SURFACE WATERS AND ON IRRIGATED CROPS

5.1 Introduction

In developing countries, including South Africa, water scarcity is a major concern, which ultimately affects the quality of drinking water sources (Abia et al., 2016b). The quality of South African rivers is highly compromised due to pollution from anthropogenic activities (Nkwonta and Ochieng, 2009). Chemical pollutants such as antibiotics or heavy metals (Bunzel et al., 2013, Hamed et al., 2013); as well as pathogenic microorganisms (Tyrrel and Quinton, 2003, Economou et al., 2013, Jacob et al., 2015) find their way into the water catchments which constitute a major health risk through different types of human exposure.

In South Africa, indicator organisms (Total coliform and *E. coli*) are currently used for monitoring drinking water, surface water resources as well as the efficiency of wastewater treatment facilities. These indicators are useful for determining the quality of potable water, shellfish harvesting waters, and recreational waters (Field and Samadpour, 2007). However, the reliance on these indicators alone as the main information source of microbial quality in relation to the safety for public health is under review in many authorities (Fewtrell and Bartram, 2001). Although coliforms are used in many countries as a monitoring tool for the microbiological quality of water; water-borne disease outbreaks have been reported from water fulfilling the coliform standard limits (Medema et al., 2003). Also, low correlations between the presence of pathogens and low sensitivity of the detection methods have been reported (Savichtcheva and Okabe, 2006).

Alternatively, the major health risk associated with the exposure to pathogenic microorganisms in the water bodies can be measured through epidemiological studies (Wade et al., 2006, Mara et al., 2007, Prüss, 1998) or presumptively assessed by the quantitative microbial risk assessment (QMRA) tool (Ashbolt et al., 2010, Soller et al., 2010).

The QMRA tool is very important especially in developing countries where rural and peri-urban communities still rely on surface water bodies for their daily needs. In these countries, polluted surface waters are sometimes used as the only direct sources of water for drinking, domestic purposes and irrigation, usually without prior treatment (Gemmell and Schmidt, 2013; Teklehaimanot et al., 2014). Unlike in the developed countries where QMRA is mostly associated to recreational activities involving accidental ingestion of polluted water (Schoen and Ashbolt, 2010), the risk in developing countries is usually higher due to the direct consumption of the polluted waters (Sibanda et al., 2013). Studies have been conducted to assess human health risk due to microbial pollution in some South African rivers (Abia et al., 2016b, Chigor et al., 2014, Steyn et al., 2004, Abia et al., 2017b).

According to the WHO (Blumenthal et al., 2000), lack of resources for effective wastewater treatment in most developing countries have contributed to large volumes of wastewater remaining untreated especially in urban areas. Hence, large amounts of untreated wastewater end up being used by farmers for crop production (Qadir et al., 2010).

The use of wastewater for irrigation is either direct or indirect. The direct use of wastewater is largely in and around large cities in developing countries whereas the indirect use of wastewater involves the discharge into other water bodies such as rivers, streams and canals as irrigation water for farmers. For example, at the Isipingo river the surface water next to the WWTP is used to irrigate small scale market gardens (the produce is sold in the local community) and subsistence gardens by the community members in the surrounding area. Irrigation is conducted manually using plastic containers or buckets directly onto the produce. This potentially puts the members of the community at risk of being exposed to water-borne diseases. Both subsistence and commercial farmers potentially reduce the microbiological

quality and safety of fresh produce by using fecally contaminated water for irrigation (Qadir et al., 2010, Crush et al., 2011, Mara et al., 2007, Dickin et al., 2016).

Although it is widely known that using fecal contaminated water can be beneficial as it provides vital plant nutrients including phosphorus (Obuobie et al., 2006) and cost savings due to the reduced need for use of fertilizers the potential health risks associated with its use is a major concern (Amoah et al., 2007). According to a study conducted by Beuchat, (2002), cited by Gemmell and Schmidt, (2012), sufficient evidence exist that the presence of defecated pathogens found on the surface of vegetables irrigated with water containing fecal matter contamination. Many years ago, it was established that microorganisms (bacteria, viruses, protozoa and helminths) can survive for days, weeks or even months on crops irrigated with microbial contaminated wastewater (Rose and Clark, 1986). The presence of pathogens in wastewater and receiving water bodies is therefore a serious concern especially if used for irrigating minimally processed crops such as lettuce, spinach and parsley (Mrayyan, 2005; Agyei and Ensink, 2016).

All pathogens of viral, bacteria, parasitic and protozoan origins can be found in wastewater; and can be transmitted to farmers using the wastewater for irrigation; consumers of the wastewater irrigated vegetables and populations living in close proximity to areas where the irrigation is taking place (WHO, 2006).

There is a need to balance the risk and benefits related to irrigation with untreated or wastewater. Also, this delicate balance is essential in order to understand the health risks associated with wastewater irrigation. This will enable the advancement of effective and cost-effective interventions (Amoah, 2014).

The Isipingo and the Palmiet Rivers are highly influenced by anthropogenic activities, thus posing a major health risk to the inhabitants of these two rivers who rely on them for some of

their daily activities. The figures 5.1 and 5.2 highlight some of the activities that were observed during sampling and where linked exposure risks may occur.



Figure 5.1: Activities that occur at the Isipingo river with potential linked exposure risks

Figure 5.1 A shows the farming activity that takes place at the Isipingo River. The crops are irrigated with the surface water next to the WWTP, using the bucket system. Figure 5.1 B at the Transit camps illustrate that fishing activity occurs occasionally.



Figure 5.2: Activities that occur at the Palmiet river showing a high degree of solid waste

Figure 5.2 A: Domestic activity (A woman is using surface water from Downstream QRI to do her laundry).

Figure 5.2 B: Within the QRI informal settlement, a woman is throwing dirt into the river, as well as rinsing the bucket with surface water from the Palmiet River.

The present part of this study was conducted to assess the public health risk associated with exposure of men, women and children to microbial pathogens in polluted river water during recreational activities (**Objective 5**). The risk associated with crop irrigation (on farmers) as well as the consumption of crops irrigated with wastewater was determined (**Objective 6**). This was achieved by looking at the risk associated with pathogenic *E. coli*, *Campylobacter*, *Salmonella* and *Shigella*. Such information could be very important in fully quantifying the microbial health risk to which users of river water, especially those living in rural and peri-

urban communities, could be exposed, so as to take appropriate measures to protect their wellbeing.

5.2 Materials and Methods

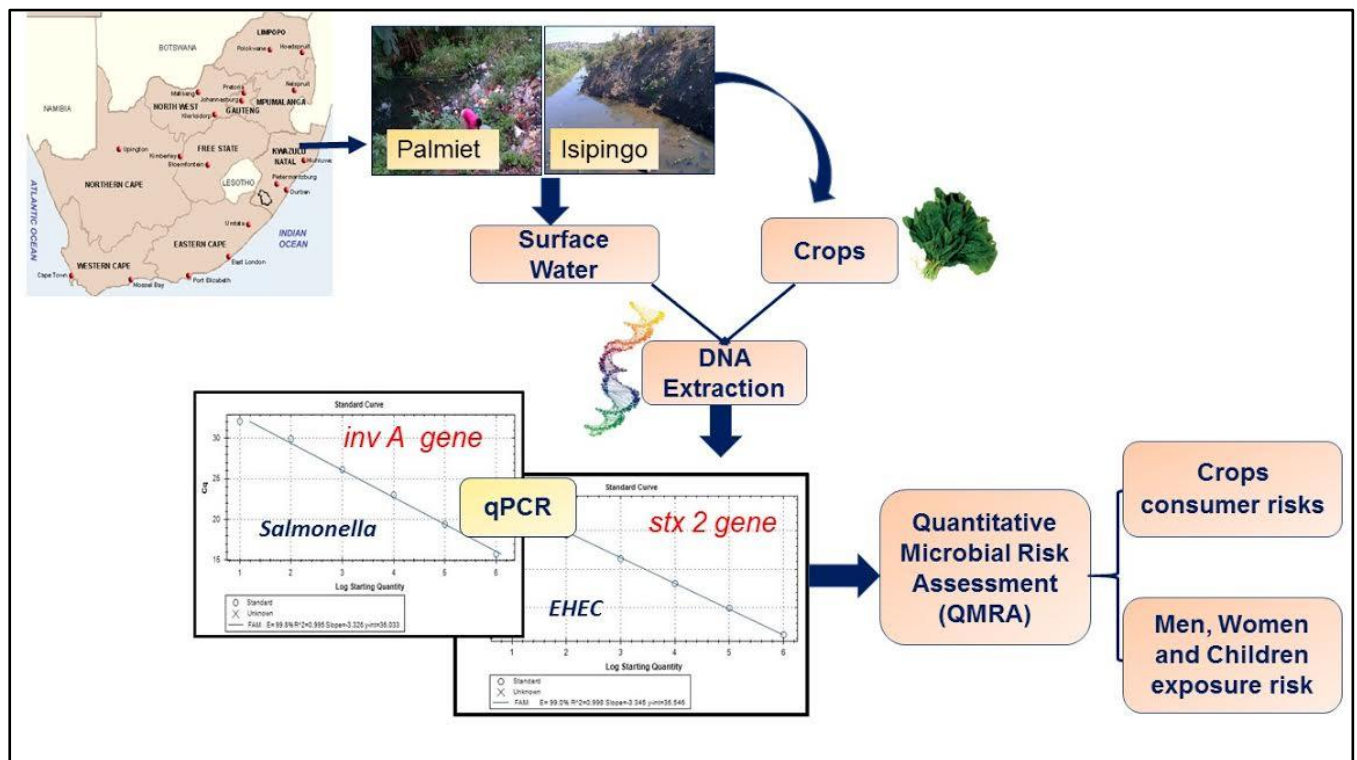


Figure 5.3: Flow diagram of methodology

5.2.1 Description of study area

The area of study was as described in Chapter 3 (section 3.2.1).

The table below illustrates the labels used to represent the sampling points of interest in the figures and tables throughout this chapter, in both rivers.

Table 5.1: Sampling points identity as per labelling on figures and graphs

| Sampling points identity | Isipingo River | Palmiet River |
|---------------------------------|-----------------------|-----------------------|
| I1/ P1 | Upstream | Downstream QRI |
| I2/ P2 | Next to WWTP | Upstream QRI |
| I3/ P3 | Transit camps | Before joining Umgeni |
| I4/P4 | River mouth | Methven Road |

5.2.2 Surface water collection

Surface water samples were collected as described in chapter 3 (Section 3.2.4) from the Isipingo and Palmiet rivers over a period of four months for each river. , samples were collected bi-monthly.

5.2.3 Microbial quality of leafy vegetables irrigated with river water

Vegetable samples (spinach leaves) irrigated with the surface water from the Isipingo River (Next to the WWTP) were collected in triplicate. Leafy samples (10g) in 100 mL of phosphate buffered saline was repeatedly washed by vigorous shaking (30 min at 200 rpm min⁻¹) on a refrigerated rotary shaker (MRC Laboratory Equipment). The supernatant of the wash solution (phosphate buffered saline containing bacteria released from vegetables) was examined using the qPCR techniques (Singh et al., 2010).

5.2.4 qPCR methodology

The primers specific for the *inv A* gene targeting *Salmonella* were adopted from Rahn et al., (1992) and primers specific for the *stx 2* gene [*stx 2*, F 5'-ACGGCAACAAATACTTTCTA CC-3''; *stx2 R* 5'-ACGGCAACAAATACTTTCTACC-3''Product size: 195 bp] targeting EHEC

were designed with in our research group (unpublished data). For the generation of standard curves for the targeted genes, reference strains *Salmonella enteritidis* ATCC 13076 and *E. coli* ATCC 35150 were grown in triplicate in one mL Luria-Bertani (LB) broth for 12 h at $37\pm1^{\circ}\text{C}$ to an optical density of approximately 0.6 at 600 nm (Genova Nano, Jenway). The number of CFU/mL of cell suspension was determined by standard plate count methods. Further, cultures of reference strains were serially diluted 10-fold to yield 10^7 down to 1 CFU/mL in phosphate-buffered saline. DNA template was prepared from each dilution as per Jyoti et al. 2010. The quantity and quality of extracted DNA was measured at 260/280 nm with a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DC, USA). The qPCR assay was performed using CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD, Hercules, CA, USA). Briefly, the reaction mixture contained Maxima SYBR Green/ROX qPCR Master Mix (12.5 μL), primers (0.4 μM each, 1 μL each) and 5 μL DNA template (10 to 10^7 GC per PCR) in a final volume of 25 μL .

A mixture of all PCR reagents containing 5 μL sterile Milli-Q® (Millipore, Billerica, MA, USA) water instead of DNA template in each qPCR assay served as a negative control. qPCR amplification protocol for the targeted genes consists of initial denaturation for 5 min at 95°C , followed by 45 cycles of three steps consisting of 10 seconds at 95°C , 20 seconds at 54°C and 20 $^{\circ}\text{C}$ seconds at 72°C . The fluorescence signals were measured at the end of each extension step. The standard curve was automatically generated by the CFX Manager™ Software v3.1. The sample concentrations were calculated from the generated standard curve. The sample was considered negative if the fluorescent signal did not increase within 45 cycles.

For the bacteriological assessment and culture free quantification of *invA* and *stx2* genes in surface water, multigenomic DNA was extracted from surface water using QIAamp® DNA Mini kit (Qiagen, Hilden, Germany) following manufacturer's instructions. The extracted

DNA (5 µL) was used as template in qPCR assays as described above. Quantitative enumeration of *Salmonella* and EHEC at each sampling location was carried out using standard curve prepared by 10 fold diluted genomic DNA of *S. enteritidis* ATCC 13076 (10 to 10⁷ GC per PCR) and *E. coli* ATCC 35150 (10 to 10⁷ GC per PCR) respectively.

5.2.5 Quantitative microbial risk assessment (QMRA) approach

The quantitative microbial risk assessment (QMRA) approach was used in the health risk assessment. QMRA involves a sequence of four interrelated steps: a) hazard identification; b) exposure assessment; c) dose-response assessment and d) risk characterization (Haas et al., 2014). This tool has been used extensively in assessing the health risk associated with several scenarios, such as health risk involved in wastewater use in agriculture (WHO, 2006), recreational use of surface water (Abia et al., 2016b). The different steps are presented as follows:

5.2.5.1 Hazard Identification

In this study, *E. coli* spp, *Salmonella* spp, *Campylobacter* spp and *Shigella* spp were chosen as the main hazard for the risk assessment, based on their prevalence and potential high concentrations in the surface water studied. The concentration of *E. coli* spp and *Salmonella* spp used for the risk assessment was based on results obtained in this study, while that of *Campylobacter* spp and *Shigella* spp were based on concentrations reported (Diergaardt et al., 2004, Wose Kinge and Mbewe, 2010). Several studies have demonstrated the relationship between pathogen contamination of surface water and adverse health outcomes for exposed populations (Doreen et al., 2015, Coffey et al., 2007, Oliver et al., 2016, Jacob et al., 2015, Yillia et al., 2009, Abbott et al., 2011, Abia et al., 2016b). Irrigation of crops (especially vegetables) with fecally contaminated surface water has also been shown to lead to higher risk

of infections for farmers as well as consumers of such crops (Amoah, 2014, Qadir et al., 2010, Fuhrmann et al., 2016, Gemmell and Schmidt, 2013).

5.2.5.2 Exposure assessment

Exposure assessment involves the determination of the “amount or number of organisms that correspond to a single exposure (termed the dose) or the concentration of *E. coli* spp, *Salmonella* spp, *Campylobacter* spp and *Shigella* spp that will constitute a set of exposures” (Haas et al., 1999).

In this study, three pathways were assessed:

- (a) Accidental ingestion of surface water by exposed community members during bathing, swimming and similar activities where direct contact with water occurs. For this exposure scenario three sub-populations were considered: men, women and children.
- (b) For women, additional exposure scenarios were considered, for example the use of the surface water for household applications such as laundry.
- (c) Consumption of vegetables irrigated with the surface water by consumers.

The different exposed populations and exposure scenarios as well as the volume of water ingested by each exposed group are presented in Appendix 2 (Table 6.1).

5.2.5.3 Dose-response assessment

The dose response assessment involves the determination of the relationship between the dose of the chosen pathogen ingested by the different exposed populations and the probability of infection. The beta-poisson dose response model represented by the equation below was chosen for this study (Haas et al., 1999).

$$p(d) = 1 - \left(1 + \left(\frac{d}{N_{50}} \right) \left(2^{\frac{1}{\alpha}} - 1 \right) \right)^{-\alpha}$$

Where ‘ $p(d)$ ’ is the risk of infection, and ‘ d ’ the concentration of pathogen ingested in a known volume of surface water or crops, ‘ N_{50} ’ is the median infection dose representing the number of organisms that will infect 50% of the exposed population and ‘ α ’ the dimensionless infectivity constant.

N_{50} is calculated using the formula:

$$N_{50} = \beta * (2^{\frac{1}{\alpha}} - 1)$$

The β values are presented in table 5.2 below.

The dose response parameters used for each of the pathogens considered are presented in table 5.2. These were adopted from dose response studies and have been used extensively in literature for quantifying risk of infections for these pathogens (Abia et al., 2016a, Eregno et al., 2016, Delignette-Muller et al., 2008, Gale, 2001, Schijven, 2011).

Table 5.2: Parameters for the dose response models (beta-poisson)

| Organism | β | A | Reference |
|----------------------|---------|-------|---------------------------|
| <i>E. coli</i> | 2473 | 0.395 | Strachan et al., 2005 |
| <i>Salmonella</i> | 49.78 | 0.21 | Meynell and Meynell, 1958 |
| <i>Campylobacter</i> | 0.011 | 0.024 | Teunis et al., 2005 |
| <i>Shigella</i> | 1480 | 0.265 | DuPont et al., 1972 |

5.2.5.4 Risk characterization

In the risk characterization, all the outcomes of the hazard identification, exposure assessment and dose response assessment were combined to characterize the probability of infection for exposed populations. The risk of infection ($P_1(A)$) associated with multiple exposures was determined using the formula:

$$P1(A)=1-(1 - P1(d))^n$$

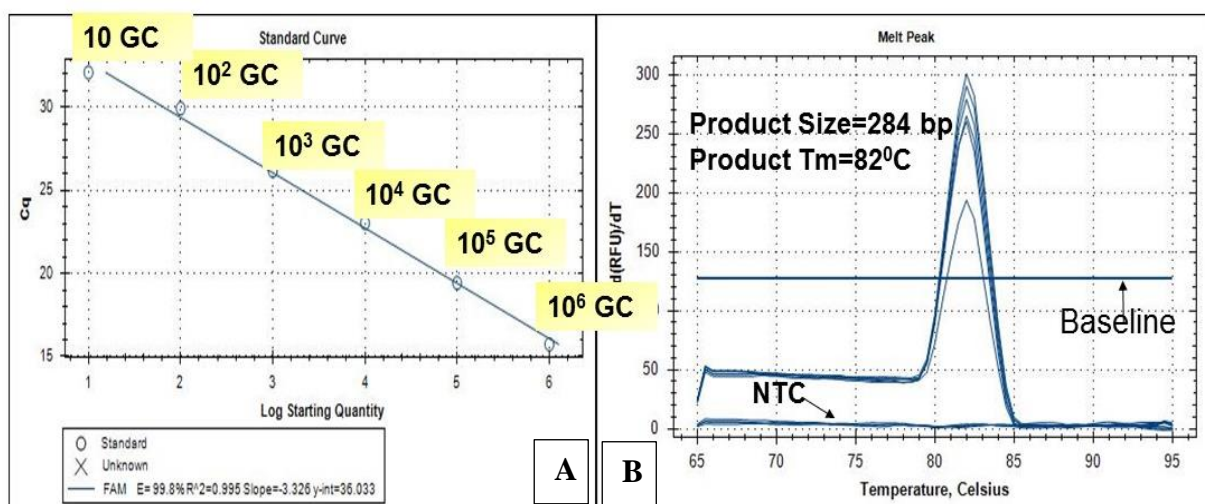
Where ‘**P₁(d)**’ is the risk of infection from a single exposure to a dose ‘**d**’ of the pathogen; and ‘**n**’ being the number of days of exposure to the single dose ‘**d**’ (Sakaji and Funamizu, 1998).

From the previous chapters 3-4, it is evident that the two rivers contain high microbial loads, thus poses a human health risk on the community members that rely on them for different activities.

5.3 Results

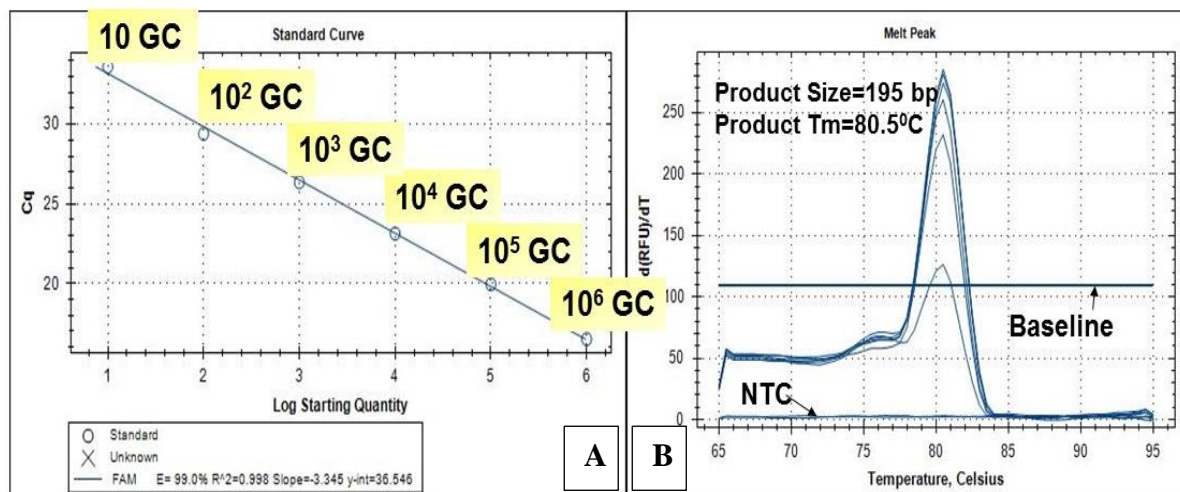
5.3.1 Quantification of *invA* and *stx 2* gene copies in Palmiet River and Isipingo River

The qPCR assays designed in this study had the detection limit of 10 genomic copies of *invA* gene (PCR efficiency 99.8%) and *stx2* (PCR efficiency 99%) genes respectively (Figure 5.4 A & B; Figure 5.5 A & B). The limit of detection of the qPCR assays has been shown in Figure 5.4 A and Figure 5.5 A whereas formation of a single PCR product is indicated in Figure 5.4B and Figure 5.5B for *inv A* gene of *Salmonella* and *stx2* gene of pathogenic *E. coli* respectively.



PCR efficiency: 99.8%, R²=0.995,
Slope=-3.326, y-int=36.033

Figure 5.4: Melt curve analysis of qPCR amplification product (A-B), A: Standard curve, B: Melt curve of qPCR amplified product from 10-fold serially diluted genomic DNA of *Salmonella Typhimurium* ATCC 13076 targeting *invA* gene



PCR efficiency: 99%, $R^2=0.998$,
Slope=-3.345, y-int=36.546

Figure 5.5: Melt curve analysis of qPCR amplification product (A-B), A: Amplification curve, B: Melt curve of qPCR amplified product from 10-fold serially diluted culture of *E. coli* ATCC 35150 targeting *stx2* gene.

It was found that the *Salmonella invA* gene was more dominant thus yielded higher concentrations than the *stx2* gene. This was observed in both the Isipingo and Palmiet rivers' surface water samples (Table 5.3).

Table 5.3: Quantitative analysis of *Salmonella* and *E. coli* through *invA* and *stx2* gene based qPCR in surface water samples over a 6 months period.

| Sampling site | <i>Salmonella invA</i> GC/ 100 mL | <i>E. coli stx2</i> GC/ 100 mL |
|------------------------|-----------------------------------|--------------------------------|
| Isipingo River | | |
| Upstream (I1) | 222.8 ± 153.1 | 37.3 ± 16.3 |
| Next to WWTP (I2) | 1530 ± 1496 | 37.1 ± 22.9 |
| Transit camps (I3) | 195.2 ± 129 | 16.8 ± 12.3 |
| River mouth (I4) | 108.2 ± 52 | 27.8 ± 12.9 |
| Palmiet River | | |
| Downstream QRI (P1) | 112 ± 70.9 | 95.5 ± 41.3 |
| Upstream QRI (P2) | 45.1 ± 14.4 | 44.8 ± 18.1 |
| B4 joining Umgeni (P3) | 51.7 ± 12 | 30.7 ± 16.5 |
| Methven Road (P4) | 78 ± 43.4 | 36.3 ± 26.4 |

At the Isipingo River, the highest *invA* gene concentration representing *Salmonella* was observed at the sampling site Next to the WWTP (1530 *invA* GC/ 100 mL) and the lowest at

River mouth (108.2 *invA* GC/ 100 mL). *Stx2* gene representing pathogenic *E. coli* was dominant at the sites Upstream and Next to WWTP (± 37 *stx2* GC/ 100 mL) and the lowest concentration was at the Transit camps (Table 5.3). At the Palmiet river, the sampling point Downstream QRI was the most contaminated with both the *Salmonella* (112 *invA* GC/ 100 mL) and *E. coli* (95.5 *stx2* GC/ 100 mL). The lowest concentrations were observed at sampling site Upstream QRI (*invA* gene) and before joining Umgeni (*stx2*), refer to (Table 5.3). *Salmonella invA* gene was more prevalent compared to pathogenic *E. coli stx2* in both rivers. At the Isipingo river *invA* concentrations ranged between (108.2 - 1530 *invA* GC/ 100 mL), whereas (45.1 – 112 *invA* GC/ 100 mL) range were observed at the Palmiet river. A rather unusual trend was observed when *stx2* concentrations ranged between (16.8 – 37.3 *stx2* GC/ 100 mL) at the Isipingo river which was a lower range than what was found at the Palmiet river (30.7 – 95.5 *stx2* GC/ 100 mL).

5.3.2 Quantitative microbial risk assessment (QMRA) approach to determine risk of infection posed by *Salmonella* and pathogenic *E. coli* exhibiting *inv A* and *stx2* genes respectively as well as *Campylobacter* and *Shigella* spp.

The probability of infection has been expressed as a function of ingested surface water from the Isipingo river and Palmiet river at various sampling points are shown in Figure 5.6. The *invA* and *stx2* gene copies were used to estimate the risk of infection upon exposure values ranging from 1-100 mL along the sampling sites of interest. Sampling point identity as per labels on the graph can be found in **Table 5.1**.

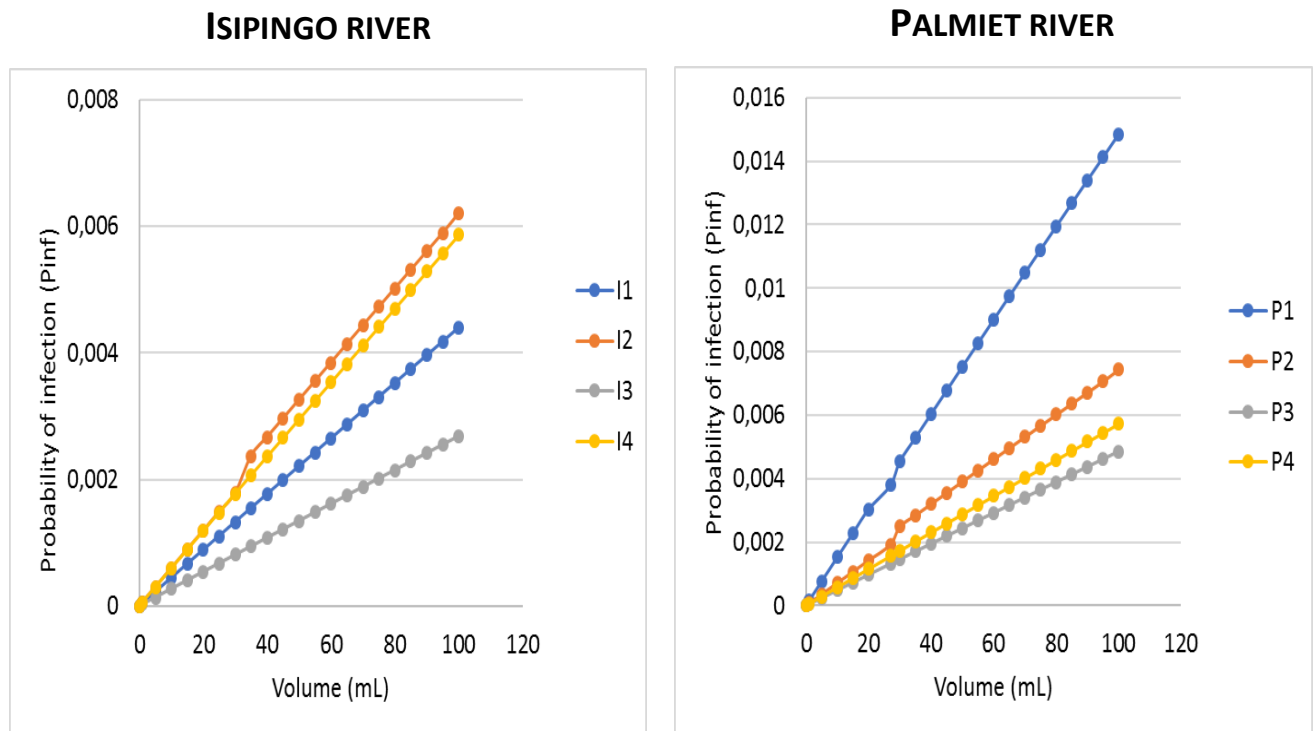


Figure 5.6: Probability of infection based on *E. coli stx2* gene along the Isipingo and Palmiet Rivers at sampling sites of interest.

From the figure above, it was found that there is a higher probability of infection at the Palmiet river, with the sampling point P1 (Downstream QRI) having the highest risk and P3 (just before the Palmiet River joins the Umgeni River) with the lowest. At the Isipingo river, the sampling point that had the highest risk of infection was I2 (Next to the WWTP). The sampling point that is surrounded by the Transit camps, I3, continuously showed the lowest probability of infection. A similar trend was observed in the sampling points I2 and I4 at surface water values between 1-30 mL there was an equal probability of infection risk at these points. As the volume increased, particularly at volumes > 40 mL, the probability of infection increased more in the sampling point I3. Assuming that a person would be exposed to 1 mL upon accidental ingestion (single exposure) of the polluted surface water from the Isipingo or Palmiet rivers, there would be no risk ($P_{inf} = 0$) at all sampling points. However, if the concentration that an individual is exposed to was 100 mL, the P_{inf} range would be P_{inf} 0.0026 – 0.0063 (I3, I2) at the Isipingo river and P_{inf} 0.005 - 0.015 (P3, P1) at the Palmiet river upon a single exposure of the untreated surface water. With pathogenic *E. coli* higher risks of infection found at the Palmiet river

Figure 5.7 below summarises the risk of infection upon accidental or intentional exposure to surface water containing *Salmonella invA* gene from the Isipingo and Palmiet rivers. The risk of infection is estimated for exposure values ranging between 1-100 mL.

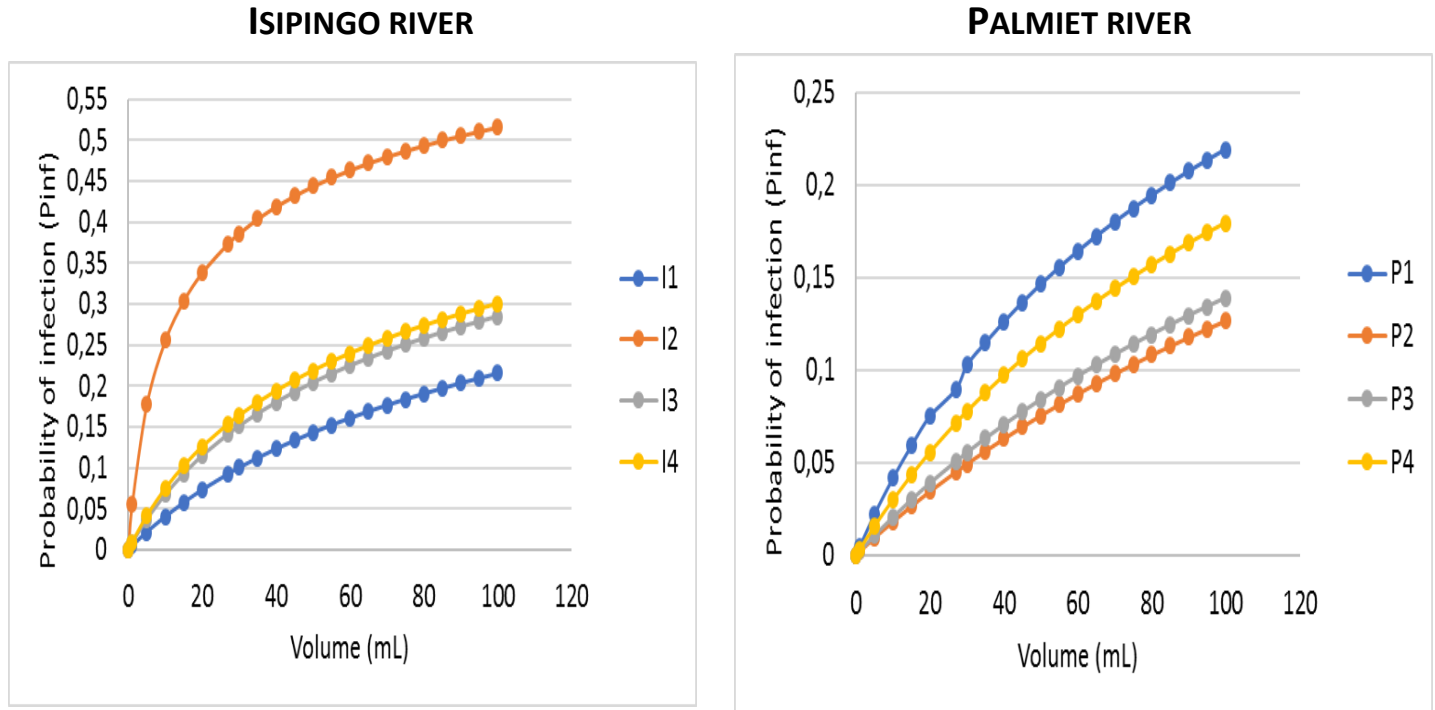


Figure 5.7: Probability of infection based on *Salmonella, invA* gene, along the Isipingo and Palmiet Rivers at sampling sites of interest.

The Isipingo river had a higher probability of infection than the Palmiet river. It ranged from Pinf 0.21 at the sampling points I1- Upstream to Pinf 0.52 at I2- Next to the WWTP. At the Palmiet river the Pinf ranged between 0.13-0.22 Pinf (P2- Upstream QRI) (P1- Downstream QRI) (Figure 5.6). The number of gene copies of *invA* gene (Figure 5.6) is much higher than *stx2* (Figure 5.7) in both the Isipingo and Palmiet rivers. This means that risk of infection is high even with relatively low exposure volumes. For example, assuming that an individual is exposed to 1 mL of water from the Isipingo river (Next to the WWTP), the probability of infection would be Pinf 0.05. Assuming that another individual is exposed to 1 mL of the polluted surface water from the Palmiet river, downstream of the QRI, the probability of infection would be Pinf 0.0046. Overall a similar trend was observed in the *invA* and *stx2* gene

based qPCR results (Table 5.3), the QMRA *stx2* results (Figure 5.6) and QMRA *invA* results (Figure 5.7) whereby the sampling point Next to the WWTP (Isipingo river) and Downstream QRI (Palmiet river) posed the highest risk (high gene copies of *invA* and *stx2* gene), as well as the highest probability of infection values.

Figure 5.8 illustrates the probability of infection relating to exposure of polluted untreated surface water contaminated with *Campylobacter* and *Shigella* spp. The concentrations used to calculate the QMRA were adopted from (Diergaardt et al., 2004, Wose Kinge and Mbewe, 2010).

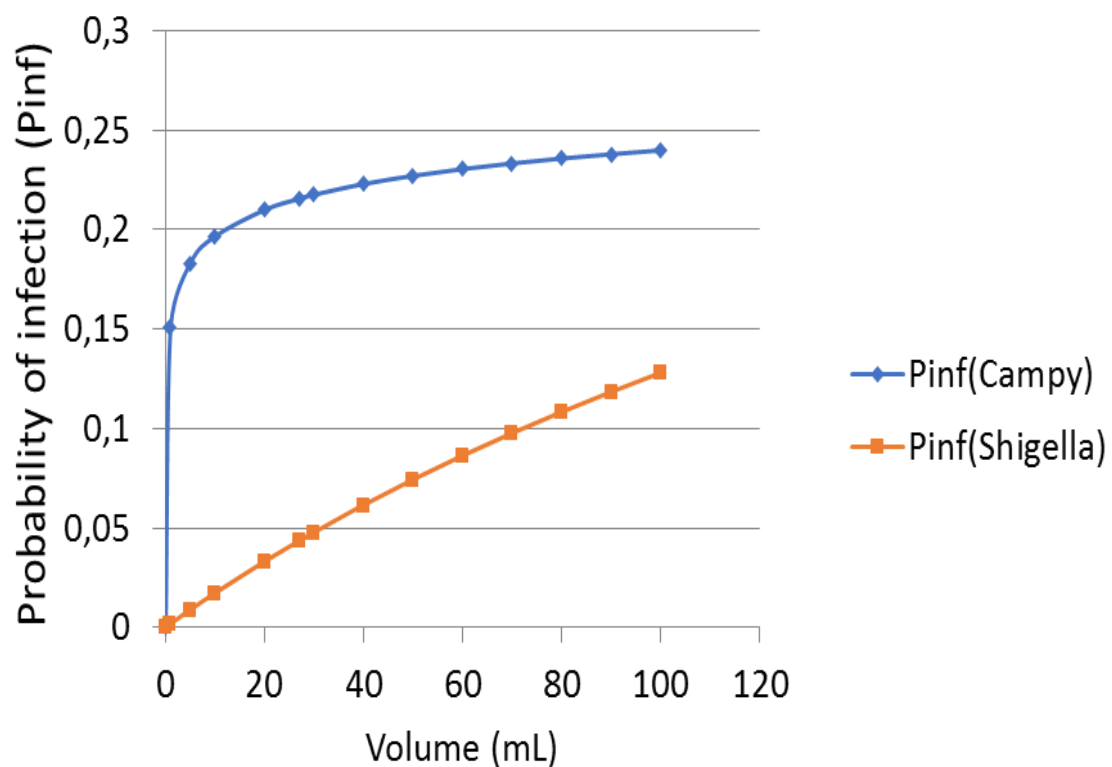


Figure 5.8: Probability of infection in relation to *Campylobacter* spp. and *Shigella* spp.

The risk of infection will differ from person to person depending on the volume that they are exposed to as well as the dose response. Therefore, the graph tells us the potential risk of

infection in relation to the volume of exposure ranging between 1-100 mL. If one person is exposed to 1mL we can deduce that the risk of infection will be 0.15 (*Campylobacter*) and 0.0017 (*Shigella*) per each exposure. Assuming that an individual is exposed to the surface water more frequently, for example the farmers during crop irrigation as illustrated (Figure 5.1) then multiple exposure occurs, (e.g. at least 3 times in a week), hence the probability of infection is increased. This means that the probability of infection of *Campylobacter spp.* or *Shigella spp.* can be obtained from the figure above, (Figure 5.8), if the volume of exposure is known (upon single exposure) as well as the frequency of exposure, in order to calculate probability of infection relating to multiple exposures.

5.3.3 Sub-population risk: in relation to recreational and occupational activities

When the three different groups (men, women and children) were analysed according to exposure volumes based on literature (Appendix 2; Table 6.1) and exposed through recreational activities, it was found that children were at the highest risk of being infected. Assuming that children were exposed to polluted surface water from the Isipingo and/ or the Palmiet river, upon a single exposure (37 mL) then the P_{inf} is as high as 0.41 *invA* and 0.002 *stx2* for children at sampling point Next to the WWTP (Isipingo river). The same trend was observed at the Palmiet River, with the highest risk at sampling point Downstream QRI (0.122 P_{inf} *invA* and 0.006 P_{inf} *stx2*) as referred (Figure 5.6 and Figure 5.7). Assuming that the water would be contaminated with *Campylobacter* and *Shigella*, the risk of infection on children per one time exposure would be 0.22 P_{inf} *Campylobacter spp.* and 0.057 P_{inf} *Shigella spp.* (refer to Figure 5.8).

The women had the lowest probability of infection upon single exposure during recreational activities but since they also use surface water for domestic/occupational purposes additional exposure are considered. An unpaired t-test was used to compare the risk of infection on women before and after the additional exposure and a significantly higher risk (p-value <0.05) resulted

both at the Isipingo and the Palmiet River sites. Although there was addition exposure amongst the women population, children were still at a higher risk of infection upon exposure due to recreational activities alone.

5.3.4 Quantitative microbial risk assessment (QMRA) approach to determine risk of infection posed by *Salmonella* exhibiting *invA* gene on crops irrigated with surface water from Isipingo River

The concentration of *Salmonella* exhibiting the *invA* gene on spinach irrigated with surface water from the Isipingo River (Next to the WWTP) as well as the Probability of infection before and after the application of the multi-barrier approach advised by the World Health Organisation (WHO, 2006) are summarized in table 5.4. Multiple barrier means that the reduction (here of organisms/genes expressed as logarithmic values) in a number of defined technical and/or behavioural steps are added together in the final calculation of risks.

Table 5.4: *Salmonella invA* gene and risk reduction rate on spinach irrigated with surface water from the Isipingo River

| Sample ID* | <i>Salmonella invA</i> GC/100 g in qPCR | Pinf in 100g Spinach. (Worst case scenario) | Pinf After risk reduction (Best case scenario) |
|-------------------|--|--|---|
| Sample 1 | 733 | 0.44 | 0.03 |
| Sample 2 | 2 547 | 0.56 | 0.01 |
| Sample 3 | 1 495 | 0.51 | 0.01 |
| Sample 4 | 826 | 0.45 | 0.03 |
| Sample 5 | 93 373 | 0.79 | 0.04 |
| Sample 6 | 130 893 | 0.81 | 0.01 |

*Sample 1-6 refers to the supernatant of the wash solution (phosphate buffered saline containing bacteria released from vegetables).

High *Salmonella* gene copy numbers show that there is a relatively high risk to human health in this case both to the farmer (who uses surface water form this sampling point which is highly

polluted, refer to Figure 5.1) as well as the consumers. The concentration of *Salmonella* exhibiting the *invA* gene on spinach irrigated with water from the Isipingo River Next to the WWTP was relatively high, ranging between 733- 130 893 *invA* GC/100g. Therefore, the probability of infection on consumers of the spinach was relatively high, where the worst-case scenario (where consumers are exposed to the highest concentration of this pathogen possible as recorded in this study) ranged between 0.44 to 0.81 Pinf. However, when the multi-barrier approach (refer to Appendix 2; Table 6.2) was applied, the risk reduced to a range of 0.1 to 0.4 Pinf posing a lower risk to the consumers of the spinach irrigated with river water as compared to the risk prior to the application of the multibarrier approach (worst case scenario), (Table 5.4).

5.4 Discussion

5.4.1 Stx2 and invA gene data and risk related to sampling sites

The high *Salmonella* and *E. coli* concentrations in the form of *invA* and *stx2* genes observed at the sampling site Next to the WWTP (Isipingo River) and Downstream QRI (Palmiet River) (Table 5.3) serve as a major human health risk to the community members that are exposed to surface water from these two rivers. This was proven by the high probability of infection values at these sampling points; in both the Isipingo and Palmiet Rivers using the *stx2* (Figure 5.6) and *invA* results (Figure 5.7). Sampling points that had the lowest risk of infection based on the two pathogens tested were River mouth and Transit camps (Isipingo River) and Upstream QRI and before joining Umgeni (Palmiet River). The high pathogenic concentrations and probability of infection is in agreement with the findings in Chapter 3 in which the sampling points next to the WWTP and Downstream QRI were constantly polluted with indicator organisms. The Isipingo River had high *Salmonella invA* concentrations thus posed a higher health risk (Figure 5.7) than the Palmiet River, which is also the general trend as was observed in the previous chapters. An interesting and unusual trend occurred when the overall probability

of infection was compared between the two rivers. The Palmiet had relatively higher infection risk of infection and *stx2* GC/100 mL than those at the Isipingo River, which is probably due to the fact that the *stx2* gene was more dominant in this river, especially at the sampling point Downstream QRI (P1). The use of the river water for domestic activities (e.g. laundry, refer to Figure 5.2A) is much more common at this point (Downstream QRI (P1)). Numerous factors such as agriculture (Walters et al., 2011), WWTWs (Naidoo and Olaniran, 2013, Abia et al., 2015c, Teklehaimanot et al., 2014, Sibanda et al., 2015) and informal settlements (Sithebe et al., 2016, Abia et al., 2016b, Abia et al., 2017b) are known as contributors to the poor microbial quality of surface water within aquatic environments. The high microbial concentrations observed were as a result of either one or a combination of these factors. As previously reported in Chapter 3 & 4, the sampling point Next to the WWTP accounted for the highest negative impact on the microbial quality of the water and sediments along the Isipingo River. Similar effects on the contribution of WWTWs to poor microbial quality of water catchments have earlier been reported in many parts of the world, including South Africa (Naidoo and Olaniran, 2013, Abia et al., 2015c, Teklehaimanot et al., 2014, Sibanda et al., 2015, La Rosa et al., 2010, Wakelin et al., 2008).

Informal settlements also played a role in enhancing the microbial concentrations and thus the risks in general as observed in the Palmiet River at the sampling point Downstream QRI. Considering the lack of sanitation facilities, the inhabitants within this informal settlement sometimes use the river water and its banks for household and fecal waste disposal which naturally also results in unfavourable effects on the microbial quality of the river (Chidamba 2015, Abia et al. 2016a, Sithebe et al. 2016, Abia et al. 2017a).

5.4.2 *Campylobacter spp* and *Shigella spp*

It is important to note that current practices to determine a possible risk of infection related to the microbiological quality of water include the assessment of the volume of water (ingested)

and the concentration of pathogenic microorganisms in water (Gerba et al., 1996). Probability of infection in relation to *Shigella* and *Campylobacter* were simulated using Figure 5.8, given that the volume of exposure is known. For the purpose of these simulations, volumes of 1mL and 100 mL were selected as representative of the best-case scenario and worst-case scenario respectively. Assuming that these volumes could be ingested accidentally or intentionally in the course of direct or indirect exposure, the daily combined risk of *Shigella spp.* (0.0017) and *Campylobacter spp.* (0.15) resulted due to ingestion of 1 mL of river water. The corresponding high-risk scenario of 100 mL ingested resulted in the corresponding figures of 0.13 (*Shigella*) and 0.24 (*Campylobacter*). The results obtained showed to be higher than the lowest acceptable risk limit of 10^{-4} estimated by the World Health Organization (WHO, 2001) for recreational water.

5.4.3 Sub-population risk: in relation to recreational and occupational activities

There are various factors that contribute to the outcome of the risk based on the different exposures as applied within QMRA. Amongst these are the general approach which assumes that the same volume of untreated water will be ingested by every individual using the water, thus the risk of infection is equal over a given population. Secondly a variability in sensitivity occurs when considering the exposures and factors like age, gender, immune status as well as previous disease history. On the other hand, researchers from different parts of the world have reported various average volumes that an individual can ingest during recreational activities, depending on the sex and age of the individual: 128mL during long distance swimming (Allen et al., 1982); 170mL amongst surfers (Stone et al., 2008); 50 mL recreational exposure (Abia et al., 2016b). In this study the volumes of women (18mL), men (27mL) and children (37mL) were considered based on Schets et al., (2011).

When sub-population risks were determined along the Isipingo and Palmiet Rivers, the highest risk due to recreational activities was observed at the sampling point next to the WWTP

(Isipingo) and Downstream QRI (Palmiet River) for men, women and children, with children having the highest risk. Risk exposure relation to *Campylobacter* and *Shigella* also showed a relatively high risk on children. The results obtained were above the WHO recreational guidelines (10^{-4}) (WHO, 2001) for all pathogens in both rivers. Thus, these rivers are not safe for children to partake in recreational activities in them.

Additional exposure (laundry) was accounted for in the women population. This is because it was observed in this study that the women especially within the informal settlements use the river water for laundry (Figure 5.2: Activities that occur at the Palmiet river). There was significantly higher difference in the risk of women prior to the additional exposure and after the additional exposure (p-value <0.05). Although the women's risk increased, the risk amongst children was continuously the highest at all the sampling points of interest, in both the Isipingo and Palmiet Rivers. This is because more water is ingested by children compared to adults, during recreational activities (Wade et al., 2008). The higher sensitivity of children is also an additional factor that puts the children at a higher risk during an event of consuming untreated river water (Abia et al., 2016b). In many parts of the world fetching water for household purposes, from the river is a chore set aside for children which is also the case in both the Isipingo and Palmiet Rivers. Ultimately this poses a relatively high risk of infection on children (Abia et al., 2016b, Musah, 2013, WHO/UNICEF, 2009).

5.4.4 Crops irrigated at the Isipingo River

The risk associated with consuming spinach that is irrigated with the river water is a major concern for all members of the community. However, the farmers are at a greater risk, because they are exposed to the river water that is highly polluted on a daily basis, given that they use the bucket system for irrigation (as seen at the Isipingo River, Figure 5.1 A). If a farmer has a one-time exposure leading to a consumption of 1mL of the untreated river water during irrigation, the resulting probability of infection due to pathogenic *E. coli stx2* (0), *Salmonella*

invA (0.0045), *Shigella spp.* (0.0017) and *Campylobacter spp.* (0.151) respectively, with the *Campylobacter spp* posing the highest risk. From the pilot study results the highest risks from the consumption of the spinach occurs based on the *Salmonella invA* gene contamination levels on the spinach. With the assumption that there is no *die off* of the pathogenic bacteria on the spinach irrigated with river water at this point (**Table 5.4**), the risk is relatively high. However, if washing and cooking is applied (refer to Appendix 2; Table 6.2), a 9-log reduction would be achieved whereby the average risk will be reduced from P_{inf} 0.64 to 0.02 thus by the time the crop is consumed there is no (“ideally”, according to the WHO approach (WHO, 2006). Thus, if the spinach is washed well and cooked well, the risk posed to the consumers is minimal.

5.5 Conclusion

The pathogens detected in the Isipingo and Palmiet Rivers pose a relatively high risk, which was proven by the probability of infection calculations and results in this study.

The sampling points Next to the WWTP (Isipingo) and Downstream QRI (Palmiet River) are highly polluted, thus pose the highest risk.

Amongst the women, men and children populations, children are at the highest risk of infection if they are exposed to surface water from the Isipingo and Palmiet Rivers, especially at sampling points Next to the WWTP and Downstream QRI.

The surface water from the Isipingo and Palmiet Rivers are above the WHO recommended target value for risk of infection

Crops irrigated with surface water from the Isipingo River (Next to the WWTP) is not safe for consumption, however handled using the WHO approach, the risk is reduced.

5.6 Recommendations

Further analysis to determine the risk in sediments as well as resuspension of sediments.

Educate farmers as well as the people surrounding the two rivers about the risk associated with the exposure to surface water from the river. As well as education on how to handle crops that are irrigated with contaminated surface water in order to reduce the risk on consumption.

CHAPTER 6 : Conclusion and Recommendation

6.1 Conclusion

The analysis of the historical data gave a baseline of the Isipingo and Palmiet rivers, which assisted in understanding the current microbial status within the two rivers. Based on the historical data analysis, it was evident that the QRI settlement area is a major pollution source within the Palmiet river, where sampling should be focussed. The analysis of the historical data for the Isipingo river further showed that upstream sampling of the WWTP is needed in order to determine the impact of the WWTP downstream. It was further clear that sampling points situated near the wastewater treatment plants, pump stations or informal settlements were of major concern and thus were considered further.

When the concentrations of microbial indicator were compared the sediment exhibited higher microbial concentrations than surface water in both rivers. This trend was observed in all the sampling points of interest. Heavy rains impacted on the variability of microbial concentrations, this was especially evident in sediment samples which exhibited higher microbial concentrations during this event than under baseline conditions. Higher microbial indicator concentrations also occurred in surface water after a heavy rainfall as compared to when there was no rainfall. Therefore, it can be concluded that rainfall had a significant impact on microbial variability both in the water and in the underlying sediment.

The municipality rely on *E. coli* as an indicator in surface water. In this study *Enterococci* were quantified in parallel. The supplementation of *Enterococci* to the *E. coli* measurements give a better reflection of the diverse and different activities that occur and the different pollution sources that affect the water quality in these two rivers. Thus, the combination of two indicators would be advantageous to assess the human health risks.

Method comparison gave a higher selectivity with the chromogenic methods than with assessments with normal conventional media. Brilliance *E. coli* /Coliform agar was comparable with Colilert-18 IDEXX. Comparable results were also obtained for *Enterococci* between Slanetz & Bartley and the Enterolert IDEXX methods. However, when mFC agar was compared with Colilert-18 IDEXX, there was a significant difference between these two methods.

Molecular methods showed to be reliable with droplet PCR giving consistently higher values in sediments, due to an assumed less impact of inhibitors, than the quantification with qPCR.

The potential risk to human health posed by pathogenic bacteria was assessed through the detection and quantification of the *E. coli stx2* gene and *Salmonella invA* gene along the two rivers. A risk assessment related the recreational and occupational exposures of children that was shown to be at the highest risk. The probability of infection upon exposure to surface water from the Isipingo and Palmiet rivers was significantly high and above the WHO target values.

A QMRA pilot study on crops irrigated with surface water collected next to the WWTP verified that pathogenic bacteria may pose a risk to the consumer. However, a 9-log reduction may be achieved when the WHO multi-barrier figures are applied, which involves proper washing and cooking of the crop before ingestion. During these circumstances, no risk prevail.

The sampling points that had the highest pollution level and constantly exceeded the WHO and DWAF guidelines (table 2.1) at the Isipingo river were situated “Next to the WWTP”, and “Downstream of QRI” at the Palmiet River.

6.2 Recommendations

- It is recommended that educational seminars are put in place with the stakeholder groups of the settlements next to Isipingo and Palmiet Rivers, in order to educate the inhabitants about the health risk associated with exposure to surface water from these two rivers.
- It is recommended that sediment sampling be incorporated in to the monthly monitoring programme conducted by the municipality on South African rivers, since these exhibit high microbial indicator concentrations.
- The use of a sediment traps and auto samplers would be recommended to further evaluate the usefulness of continuous sampling.
- The use of more than one indicator is recommended during normal monitoring. Also, pathogen testing should be incorporated in future planning

REFERENCES

- ABBOTT, B., LUGG, R., DEVINE, B., COOK, A. & WEINSTEIN, P. 2011. Microbial risk classifications for recreational waters and applications to the Swan and Canning Rivers in Western Australia. *Journal of Water and Health*, 9, 70-79.
- ABBOTT, S., CAUGHLEY, B. & SCOTT, G. 1998. Evaluation of Enterolert® for the enumeration of enterococci in the marine environment. *New Zealand Journal of Marine and Freshwater Research*, 32, 505-513.
- ABIA, A., UBOMBA-JASWA, E. & MOMBA, M. 2016a. Prevalence of pathogenic microorganisms and their correlation with the abundance of indicator organisms in riverbed sediments. *International Journal of Environmental Science and Technology*, 13, 2905-2916.
- ABIA, A. L., UBOMBA-JASWA, E., GENTHE, B. & MOMBA, M. N. 2016b. Quantitative microbial risk assessment (QMRA) shows increased public health risk associated with exposure to river water under conditions of riverbed sediment resuspension. *Science of the Total Environment*, 567, 1143-1151.
- ABIA, A. L. K., JAMES, C., UBOMBA-JASWA, E. & BENTEKE MOMBA, M. N. 2017a. Microbial Remobilisation on Riverbed Sediment Disturbance in Experimental Flumes and a Human-Impacted River: Implication for Water Resource Management and Public Health in Developing Sub-Saharan African Countries. *International Journal of Environmental Research and Public Health*, 14, 306-324.
- ABIA, A. L. K., SCHAEFER, L., UBOMBA-JASWA, E. & LE ROUX, W. 2017b. Abundance of Pathogenic Escherichia coli Virulence-Associated Genes in Well and Borehole Water Used for Domestic Purposes in a Peri-Urban Community of South Africa. *International Journal of Environmental Research and Public Health*, 14, 320-331.

- ABIA, A. L. K., UBOMBA-JASWA, E., DU PREEZ, M. & MOMBA, M. N. B. 2015a. Riverbed sediments in the Apies River, South Africa: recommending the use of both *Clostridium perfringens* and *Escherichia coli* as indicators of faecal pollution. *Journal of Soils and Sediments*, 15, 2412-2424.
- ABIA, A. L. K., UBOMBA-JASWA, E. & MOMBA, M. N. B. 2015b. Impact of seasonal variation on *Escherichia coli* concentrations in the riverbed sediments in the Apies River, South Africa. *Science of The Total Environment*, 537, 462-469.
- ABIA, L. K. A., UBOMBA-JASWA, E., SSEMAKALU, C. C. & MOMBA, M. N. 2015c. Development of a rapid approach for the enumeration of *Escherichia coli* in riverbed sediment: case study, the Apies River, South Africa. *Journal of Soils and Sediments*, 15, 2425-2432.
- ACKERMAN, D. & WEISBERG, S. B. 2003. Relationship between rainfall and beach bacterial concentrations on Santa Monica Bay beaches. *Journal of Water and Health*, 1, 85-89.
- ADCOCK, P. W. & SAINT, C. P. 2001. Development of glucosidase agar for the confirmation of water-borne *Enterococcus*. *Water Research*, 35, 4243-4246.
- AGYEI, P. A. & ENSINK, J. 2016. Wastewater use in urban agriculture: an exposure and risk assessment in Accra, Ghana. *Journal of Science and Technology (Ghana)*, 36, 7-14.
- AHMED, W., RICHARDSON, K., SIDHU, J. & TOZE, S. 2012. *Escherichia coli* and *Enterococcus* spp. in rainwater tank samples: comparison of culture-based methods and 23S rRNA gene quantitative PCR assays. *Environmental Science & Technology*, 46, 11370-11376.
- ALLEN, L. M., BRIGGLE, T. V. & PFAFFENBERGER, C. D. 1982. Absorption and excretion of cyanuric acid in long-distance swimmers. *Drug Metabolism Reviews*, 13, 499-516.

ALM, E. W., BURKE, J. & SPAIN, A. 2003. Fecal indicator bacteria are abundant in wet sand at freshwater beaches. *Water Research*, 37, 3978-3982.

AMOAHA, I. D. 2014. *Helminth infection risk associated with the use of wastewater in Urban Agriculture in Kumasi, Ghana*. Masters thesis, Department Of Theoretical And Applied Biology, College Of Science, Kwame Nkrumah University Of Science And Technology.

AMOAHA, P., DRECHSEL, P., HENSELER, M. & ABAIDOO, R. 2007. Irrigated urban vegetable production in Ghana: microbiological contamination in farms and markets and associated consumer risk groups. *Journal of Water and Health*, 5, 455-466.

ANDERSON, K. L., WHITLOCK, J. E. & HARWOOD, V. J. 2005. Persistence and differential survival of fecal indicator bacteria in subtropical waters and sediments. *Applied and Environmental Microbiology*, 71, 3041-3048.

APHA 2005. *Standard methods for the examination of water and wastewater*, Washington, DC, USA, American Public Health Association (APHA).

ASHBOLT, N. J., GRABOW, W. O. & SNOZZI, M. 2001. Indicators of microbial water quality. In: Fewtrell L, Barthram J, editors. *Water Quality: Guidelines, Standards and Health*. IWA Publishing; London, UK: 2001. Pp. 289–316.

ASHBOLT, N. J., SCHOEN, M. E., SOLLER, J. A. & ROSER, D. J. 2010. Predicting pathogen risks to aid beach management: the real value of quantitative microbial risk assessment (QMRA). *Water Research*, 44, 4692-4703.

BAKER, M. 2012. Digital PCR hits its stride. *Nature Methods*, 9, 541-544.

BALLESTÉ, E. & BLANCH, A. R. 2010. Persistence of Bacteroides species populations in a river as measured by molecular and culture techniques. *Applied and Environmental Microbiology*, 76, 7608-7616.

BANIHASHEMI, A., VAN DYKE, M. I. & HUCK, P. M. 2015. Detection of viable bacterial pathogens in a drinking water source using propidium monoazide-quantitative PCR. *Journal of Water Supply: Research and Technology-Aqua*, 64, 139-148.

BARTRAM, J. & BALLANCE, R. 1996. *Water quality monitoring: a practical guide to the design and implementation of freshwater quality studies and monitoring programmes*, London, CRC Press. Pp 400

BEUCHAT, L. R. 2002. Ecological factors influencing survival and growth of human pathogens on raw fruits and vegetables. *Microbes and infection*, 4, 413-423.

BITTON, G. 2005. *Wastewater Microbiology (Third edition)*, Hoboken, New Jersey, John Wiley & Sons, Inc.

BLAUSTEIN, R., PACHEPSKY, Y., HILL, R., SHELTON, D. & WHELAN, G. 2013. Escherichia coli survival in waters: temperature dependence. *Water Research*, 47, 569-578.

BLUMENTHAL, U. J., MARA, D. D., PEASEY, A., RUIZ-PALACIOS, G. & STOTT, R. 2000. Guidelines for the microbiological quality of treated wastewater used in agriculture: recommendations for revising WHO guidelines. *Bulletin of the World Health Organization*, 78, 1104-1116.

BOEHM, A. B. 2007. Enterococci concentrations in diverse coastal environments exhibit extreme variability. *Environmental Science & Technology (ACS Publications)*, 41, 8227–8232.

BOEHM, A. B. & SASSOUBRE, L. M. 2014. *Enterococci* as indicators of environmental fecal contamination. In: GILMORE, M.S., CLEWELL, D. B., IKE ,Y., ET AL ed.

Enterococci: From Commensals to Leading Causes of Drug Resistant Infection [Internet].

Boston: Massachusetts Eye and Ear Infirmary. Available from:

<http://www.ncbi.nlm.nih.gov/books/NBK190421/>

BONADONNA, L., BRIANCESCO, R., CATALDO, C., DIVIZIA, M., DONIA, D. & PANA, A. 2002. Fate of bacterial indicators, viruses and protozoan parasites in a wastewater multi-component treatment system. *The new microbiologica*, 25, 413-420.

BONDE G.J 1962. Bacterial indicators of water pollution. *Teknisk Forlag, Copenhagen*.

BRAND, A. S. & BARNES, J. M. 2014. Evaluation of Colilert-18® as an alternative method for monitoring total coliforms and *Escherichia coli* in some faecally polluted river waters. *Journal of Water Sanitation and Hygiene for Development*, 4, 604-611.

BUCKALEW, D., HARTMAN, L., GRIMSLEY, G., MARTIN, A. & REGISTER, K. 2006. A long-term study comparing membrane filtration with Colilert® defined substrates in detecting fecal coliforms and *Escherichia coli* in natural waters. *Journal of Environmental Management*, 80, 191-197.

BUDNICK, G. E., HOWARD, R. T. & MAYO, D. R. 1996. Evaluation of Enterolert for enumeration of enterococci in recreational waters. *Applied and Environmental Microbiology*, 62, 3881-3884.

BUNZEL, K., KATTWINKEL, M. & LIESS, M. 2013. Effects of organic pollutants from wastewater treatment plants on aquatic invertebrate communities. *Water Research*, 47, 597-606.

CABRAL, J. P. 2010. Water microbiology. Bacterial pathogens and water. *International Journal of Environmental Research and Public Health*, 7, 3657-3703.

CDC. 2014. *Chapter 3: infectious diseases related to travel* [Online]. Available: <http://www.cdc.gov/ecoli/general/index.html> [Accessed: 20-04-2017].

CHA, S. M., LEE, S. W., PARK, Y. E., CHO, K. H., LEE, S. & KIM, J. H. 2010. Spatial and temporal variability of fecal indicator bacteria in an urban stream under different meteorological regimes. *Water Science and Technology*, 61, 3102-3108.

CHAO, K. K, CHAO, C.C. & CHAO, W. L., 2003. Suitability of the traditional microbial indicators and their enumerating methods in the assessment of fecal pollution of subtropical freshwater environments. *Journal of Microbiology, Immunology, and Infection*, 36, 288-293.

CHAO, W. 2006. Evaluation of Colilert-18 for the detection of coliforms and *Escherichia coli* in tropical fresh water. *Letters in Applied Microbiology*, 42, 115-120.

CHIDAMBA, L. 2015. *Microbial Quality of Rainwater Harvested from Rooftops, for Domestic use and Homestead Food Gardens*. . PhD Thesis (Plant Pathology), University of Pretoria South Africa.

CHIGOR, V. N., SIBANDA, T. & OKOH, A. I. 2014. Assessment of the risks for human health of adenoviruses, hepatitis a virus, rotaviruses and enteroviruses in the buffalo river and three source water dams in the eastern cape. *Food and Environmental Virology*, 6, 87-98.

CLARK, D., MILNER, B., STEWART, M., WOLFE, R. & OLSON, B. 1991. Comparative study of commercial 4-methylumbelliferyl-beta-D-glucuronide preparations with the Standard Methods membrane filtration fecal coliform test for the detection of *Escherichia coli* in water samples. *Applied and Environmental Microbiology*, 57, 1528-1534.

- COFFEY, R., CUMMINS, E., CORMICAN, M., FLAHERTY, V. O. & KELLY, S. 2007. Microbial exposure assessment of waterborne pathogens. *Human and Ecological Risk Assessment*, 13, 1313-1351.
- COFFEY, S. W., SPOONER, J. & SMOLEN, M. D. 1993. *The Nonpoint Source Manager's Guide to Water Quality and Land Treatment Monitoring*. North Carolina Agricultural Extension Service, Department of Biological and Agricultural Engineering, North Carolina State University, Raleigh, NC.
- COOKE, G. D., WELCH, E. B., PETERSON, S. & NICHOLS, S. A. 2016. *Restoration and management of lakes and reservoirs*, CRC press.
- CRUSH, J., HOVORKA, A. & TEVERA, D. 2011. Food security in Southern African cities: the place of urban agriculture. *Progress in Development Studies*, 11, 285-305.
- CYR, D., HAUSLER, R. & YARGEAU, V. 2015. *Examination report by independent experts on the technical and scientific information regarding the planned discharge of untreated wastewater effluent into the St. Lawrence by the City of Montréal*, Environnement Canada.
- DEAT 2005. Environmental Reporting, Integrated Environmental Management Information Series 17. Pretoria: Department of Environmental Affairs and Tourism (DEAT).
- DELIGNETTE-MULLER, M., CORNU, M. & GROUP, A. S. S. 2008. Quantitative risk assessment for Escherichia coli O157: H7 in frozen ground beef patties consumed by young children in French households. *International Journal of Food Microbiology*, 128, 158-164.
- DESMARAIS, T. R., SOLO-GABRIELE, H. M. & PALMER, C. J. 2002. Influence of soil on fecal indicator organisms in a tidally influenced subtropical environment. *Applied and Environmental Microbiology*, 68, 1165-1172.

DEVONSHIRE, A. S., HONEYBORNE, I., GUTTERIDGE, A., WHALE, A. S., NIXON, G., WILSON, P., JONES, G., MCHUGH, T. D., FOY, C. A. & HUGGETT, J. F. 2015. Highly Reproducible Absolute Quantification of *Mycobacterium tuberculosis* Complex by Digital PCR. *Analytical Chemistry*, 87, 3706-3713.

DICK, L. K., STELZER, E. A., BERTKE, E. E., FONG, D. L. & STOECKEL, D. M. 2010. Relative decay of Bacteroidales microbial source tracking markers and cultivated *Escherichia coli* in freshwater microcosms. *Applied and Environmental Microbiology*, 76, 3255-3262.

DICKIN, S. K., SCHUSTER-WALLACE, C. J., QADIR, M. & PIZZACALLA, K. 2016. A review of health risks and pathways for exposure to wastewater use in agriculture. *Environmental Health Perspectives*, 124, 900-909.

DIERGAARDT, S., VENTER, S., SPREETH, A., THERON, J. & BRÖZEL, V. 2004. The occurrence of campylobacters in water sources in South Africa. *Water Research*, 38, 2589-2595.

DONOVAN, E., UNICE, K., ROBERTS, J. D., HARRIS, M. & FINLEY, B. 2008. Risk of gastrointestinal disease associated with exposure to pathogens in the water of the Lower Passaic River. *Applied and Environmental Microbiology*, 74, 994-1003.

DOREEN, N., OKOT-OKUMU, J. & MUYODI, F. J. 2015. Microbial safety assessment of recreation water at Lake Nabugabo, Uganda. *African Journal of Environmental Science and Technology*, 9, 773-782.

DRECHSEL, P., KERAITA, B., AMOAH, P., ABAIDOO, R., RASCHID-SALLY, L. & BAHRI, A. 2008. Reducing health risks from wastewater use in urban and peri-urban sub-Saharan Africa: applying the 2006 WHO guidelines. *Water Science and Technology*, 57, 1461-1466.

DRESSING, S. A., DAVENPOART, T., RATHKE, D., MEALS, D., DANIEL, T., HALLOCK, B., KNIGHTON, R., HATFIELD, J., GOO, R. & DEAN, R. 2003. National management measures to control non-point source pollution from agriculture. *United States Environmental Protection Agency, Washington, DC EPA*.

DROPPO, I. G., LISS, S. N., WILLIAMS, D., NELSON, T., JASKOT, C. & TRAPP, B. 2009. Dynamic existence of waterborne pathogens within river sediment compartments. Implications for water quality regulatory affairs. *Environmental Science & Technology*, 43, 1737-1743.

DU PLESSIS, A. 2017. *Freshwater Challenges of South Africa and its Upper Vaal River: Current State and Outlook*, Springer.

DUFOUR, A., SNOZZI, M., KOSTER, W., BARTRAM, J., RONCHI, E. & FEWTRELL, L. 2003. *Assessing Microbial Safety of Drinking Water: Improving Approaches and Methods*, London, IWA Publishing.WHO/OECD.

DUPONT, H., HORNICK, R., SNYDER, M., LIBONATI, J., FORMAL, S. & GANGAROSA, E. 1972. Immunity in shigellosis. I. Response of man to attenuated strains of Shigella. *The Journal of Infectious Diseases*, 125, 5-11.

DWA 2013. National Water Resource Strategy: Water for an Equitable and Sustainable Future. June 2013 Second Edition. South Africa. Pretoria: Department of Water Affairs.

DWAF 1996a. South African water quality guidelines (second edition). Volume 1: Domestic use. Pretoria CSIR Environmental Services.

DWAF 1996b. South African Water Quality Guidelines (second edition). Volume 4: Agricultural Use: Irrigation. Pretoria: Department of Water Affairs and Forestry.

DWAF 1996c. South African Water Quality Guidelines ,Second editon. Volume 2: Recreational Water Use. Pretoria: Department of Water Affairs and Forestry.

DWAF 2004. Introduction to the National Water Resource Strategy: First edition.: Department of Water Affairs and Forestry. Pretoria: Department of Water Affairs and Forestry.

DWAF 2005. Drinking Water Quality Management Guide for Water Services Authorities. Pretoria: Department of Water Affairs and Forestry.

DWAF 2007. Training Manual for Resource Directed Management of Water Quality. Pretoria: Department of Water Affairs and Forestry.

DWAF 2011. The 2010/2011 audit results for the Umlazi Wastewater Treatment Works. Durban, South Africa: Department of Water Affairs and Forestry.

ECKNER, K. F. 1998. Comparison of membrane filtration and multiple-tube fermentation by the Colilert and Enterolert methods for detection of waterborne coliform bacteria, *Escherichia coli*, and enterococci used in drinking and bathing water quality monitoring in southern Sweden. *Applied and Environmental Microbiology*, 64, 3079-3083.

ECONOMOU, V., GOUSIA, P., KANSOUZIDOU, A., SAKKAS, H., KARANIS, P. & PAPADOPOULOU, C. 2013. Prevalence, antimicrobial resistance and relation to indicator and pathogenic microorganisms of *Salmonella enterica* isolated from surface waters within an agricultural landscape. *International Journal of Hygiene and Environmental Health*, 216, 435-444.

EKWANZALA, M. D., ABIA, A. L. K., UBOMBA-JASWA, E., KESHRI, J. & MOMBA, N. B. M. 2017. Genetic relatedness of faecal coliforms and enterococci bacteria isolated from water and sediments of the Apies River, Gauteng, South Africa. *AMB Express*, 7, 1-10.

ELBAG, M. A. 2006. *Impact of surrounding land uses on surface water quality*. MSc Thesis, College of Science Environmental Engineering, Worcester. Polytechnic Institute.

EREGNO, F. E., TRYLAND, I., TJOMSLAND, T., MYRMEL, M., ROBERTSON, L. & HEISTAD, A. 2016. Quantitative microbial risk assessment combined with hydrodynamic modelling to estimate the public health risk associated with bathing after rainfall events. *Science of the Total Environment*, 548, 270-279.

FATOKI, O., MUYIMA, N. & LUJIZA, N. 2001. Situation analysis of water quality in the Umtata River catchment. *Water SA*, 27, 467-474.

FERGUSON, C., HUSMAN, A. M. D. R., ALTAVILLA, N., DEERE, D. & ASHBOLT, N. 2003. Fate and transport of surface water pathogens in watersheds. *Critical Reviews in Environmental Science and Technology*, 33, 299–361.

FEWTRELL, L. & BARTRAM, J. 2001. Water quality: Guidelines, Standards and Health: Assessment of risk and risk management for water-related infectious disease. In: FEWTRELL, L. (ed.) *Water quality: guidelines, standards and health: assessment of risk and risk management for water-related infectious diseases (World Health Organisation)*. London, UK: IWA Publishing.

FIELD, K. G. & SAMADPOUR, M. 2007. Fecal source tracking, the indicator paradigm, and managing water quality. *Water Research*, 41, 3517-3538.

FRANZ, A. 2005. *A performance study of ceramic candle filters in Kenya including tests for coliphage removal*. Massachusetts Institute of Technology.

FRICKER, E., ILLINGWORTH, K. & FRICKER, C. 1997. Use of two formulations of Colilert and QuantiTray™ for assessment of the bacteriological quality of water. *Water Research*, 31, 2495-2499.

FUHRMANN, S., WINKLER, M. S., KABATEREINE, N. B., TUKAHEBWA, E. M., HALAGE, A. A., RUTEBEMBERWA, E., MEDLICOTT, K., SCHINDLER, C., UTZINGER, J. & CISSÉ, G. 2016. Risk of intestinal parasitic infections in people with different exposures to wastewater and fecal sludge in Kampala, Uganda: a cross-sectional study. *PLoS Neglected Tropical Diseases*, 10, e0004469.

GALE, P. 2001. Developments in microbiological risk assessment for drinking water. *Journal of Applied Microbiology*, 91, 191-205.

GAO, G., FALCONER, R. A. & LIN, B. 2011. Numerical modelling of sediment–bacteria interaction processes in surface waters. *Water Research*, 45, 1951-1960.

GARCIA-ARMISEN, T. & SERVAIS, P. 2009. Partitioning and fate of particle-associated *E. coli* in river waters. *Water Environment Research*, 81, 21-28.

GELDREICH, E. E. 1978. Bacterial populations and indicator concepts in feces, sewage, stormwater and solid wastes. In: G. B. (ed.) *Indicators of viruses in waters*. Ann Arbor Michigan: Ann Arbor Science Publishers Inc.

GEMMELL, M. E. & SCHMIDT, S. 2012. Microbiological assessment of river water used for the irrigation of fresh produce in a sub-urban community in Sobantu, South Africa. *Food Research International*, 47, 300-305.

GEMMELL, M. E. & SCHMIDT, S. 2013. Is the microbiological quality of the Msunduzi River (KwaZulu-Natal, South Africa) suitable for domestic, recreational, and agricultural purposes? *Environmental Science and Pollution Research*, 20, 6551-6562.

GENTHE, B., LE ROUX, W. J., SCHACHTSCHNEIDER, K., OBERHOLSTER, P., ANECK-HAHN, N. & CHAMIER, J. 2013. Health risk implications from simultaneous exposure to multiple environmental contaminants. *Ecotoxicology and Environmental Safety*, 93, 171-179.

GERBA, C. P., ROSE, J. B. & HAAS, C. N. 1996. Sensitive populations: who is at the greatest risk? *International Journal of Food Microbiology*, 30, 113-123.

GONDO, R. 2013. *An assessment of water quality in Karoi town, Mashonaland West Province, Zimbabwe*. Bindura University of Science Education.

GRABOW, W. 1996. Waterborne diseases: Update on water quality assessment and control. *Water SA*, 22, 193-202.

GRABOW, W. 2001. Bacteriophages: update on application as models for viruses in water. *Water SA*, 27, 251-268.

GRIFFITH, J. F., SCHIFF, K. C., LYON, G. S. & FUHRMAN, J. A. 2010. Microbiological water quality at non-human influenced reference beaches in southern California during wet weather. *Marine Pollution Bulletin*, 60, 500-508.

GRIFFITH, J. F., WEISBERG, S. B. & MCGEE, C. D. 2003. Evaluation of microbial source tracking methods using mixed fecal sources in aqueous test samples. *Journal of Water and Health*, 1, 141-151.

GUERRA, R., PASTERIS, A. & PONTI, M. 2009. Impacts of maintenance channel dredging in a northern Adriatic coastal lagoon. I: effects on sediment properties, contamination and toxicity. *Estuarine, Coastal and Shelf Science*, 85, 134-142.

HAACK, S. K., FOGARTY, L. R. & WRIGHT, C. 2003. Escherichia coli and enterococci at beaches in the Grand Traverse Bay, Lake Michigan: sources, characteristics, and environmental pathways. *Environmental Science & Technology*, 37, 3275-3282.

HAAS, C. N., ROSE, J. B. & GERBA, C. P. 1999. *Quantitative microbial risk assessment*, John Wiley & Sons.

HAAS, C. N., ROSE, J. B. & GERBA, C. P. 2014. *Quantitative Microbial Risk Assessment*, John Wiley & Sons.

HAMED, Y., ABDELMONEIM, T., ELKIKI, M., HASSAN, M. & BERNDTSSON, R. 2013. Assessment of heavy metals pollution and microbial contamination in water, sediments and fish of Lake Manzala, Egypt. *Life Science Journal*, 10, 86-94.

HARWOOD, V. J., BOEHM, A. B., SASSOUBRE, L. M., VIJAYAVEL, K., STEWART, J. R., FONG, T.-T., CAPRAIS, M.-P., CONVERSE, R. R., DISTON, D. & EBDON, J. 2013. Performance of viruses and bacteriophages for fecal source determination in a multi-laboratory, comparative study. *Water Research*, 47, 6929-6943.

HARWOOD, V. J., STALEY, C., BADGLEY, B. D., BORGES, K. & KORAJKIC, A. 2014. Microbial source tracking markers for detection of fecal contamination in environmental waters: relationships between pathogens and human health outcomes. *FEMS Microbiology Reviews*, 38, 1-40.

HAYDEN, R. T., GU, Z., INGERSOLL, J., ABDUL-ALI, D., SHI, L., POUNDS, S. & CALIENDO, A. M. 2013. Comparison of droplet digital PCR to real-time PCR for quantitative detection of cytomegalovirus. *Journal of Clinical Microbiology*, 51, 540-546.

HEALTH CANADA 2013. Guidance on waterborne bacterial pathogens. In: OTTAWA, O. (ed.) *Water, Air and Climate Change Bureau*. Canada: Healthy Environments and Consumer Safety Branch.

HEMSON, D. 2016. Water, sanitation and health: South Africa's remaining and existing issues In: Padarath A, King J, Mackie E, Casciola J, ed. *South African Health Review*, Durban: Health Systems Trust. Pp 25-34.

HEMSON, D. & DUBE, B. 2004. *Water services and public health: the 2000–01 cholera outbreak in KwaZulu-Natal, South Africa*. 8th World Congress on Environmental Health, 22–27 February 2004, Durban, South.

HINDSON, B. J., NESS, K. D., MASQUELIER, D. A., ET AL., 2011. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. *Journal of Analytical Chemistry*, 83, 8604-10.

HOLVOET, K., SAMPERS, I., SEYNNAEVE, M. & UYTENDAELE, M. 2014. Relationships among hygiene indicators and enteric pathogens in irrigation water, soil and lettuce and the impact of climatic conditions on contamination in the lettuce primary production. *International Journal of Food Microbiology*, 171, 21-31.

HORAN, N. J. 2003. Faecal indicator organisms. In: DUNCAN, M., HORAN, N. (ed.) *The handbook of Water and Wastewater Microbiology*. Academic Press.

HOWELL, J., COYNE, M. S. & CORNELIUS, P. 1996. Effect of sediment particle size and temperature on fecal bacteria mortality rates and the fecal coliform/fecal streptococci ratio. *Journal of Environmental Quality*, 25, 1216-1220.

IDEXX. 2001. *Colilert-18* [Online]. Available:
<https://www.idexx.com/water/products/colilert-18.html> [Accessed 23-04-2017].

IGBINOSA, E. & OKOH, A. 2009. Impact of discharge wastewater effluents on the physico-chemical qualities of a receiving watershed in a typical rural community. *International Journal of Environmental Science & Technology*, 6, 175-182.

ISHII, S., HANSEN, D. L., HICKS, R. E. & SADOWSKY, M. J. 2007. Beach sand and sediments are temporal sinks and sources of *Escherichia coli* in Lake Superior. *Environmental Science & Technology*, 41, 2203-2209.

ISO 1995. Water Quality-Detection and Enumeration of Bacteriophages-Part 1: Enumeration of F-Specific RNA Bacteriophages. *International Organisation of Standardisation Geneva, Switzerland*.

ISO 2000a. 10705–2: Water Quality. Detection and Enumeration of Bacteriophages–Part 2: Enumeration of Somatic. Coliphages. *International Organization for Standardization, Geneva, Switzerland*.

ISO 2000b. Water Quality-Detection and Enumeration of *Escherichia coli* and Coliform Bacteria-Part 1: Membrane Filtration Method. *International Organisation of Standardisation Geneva, Switzerland*.

ISO 2012. 9308: -2:2012 (en) Water Quality- Enumeration of *Escherichia coli* and Coliform Bacteria-Part 2: Most probable number method .

IWERIEBOR, B. C., IWU, C. J., OBI, L. C., NWODO, U. U. & OKOH, A. I. 2015. Multiple antibiotic resistances among Shiga toxin producing *Escherichia coli* O157 in feces of dairy cattle farms in Eastern Cape of South Africa. *BMC Microbiology*, 15, 213-222.

JACOB, P., HENRY, A., MEHEUT, G., CHARNI-BEN-TABASSI, N., INGRAND, V. & HELMI, K. 2015. Health risk assessment related to waterborne pathogens from the river to the tap. *International Journal of Environmental Research and Public Health*, 12, 2967-2983.

JAMIESON, R., GORDON, R., JOY, D. & LEE, H. 2004. Assessing microbial pollution of rural surface waters: A review of current watershed scale modeling approaches. *Agricultural Water Management*, 70, 1-17.

JAMIESON, R. C., JOY, D. M., LEE, H., KOSTASCHUK, R. & GORDON, R. J. 2005. Resuspension of Sediment-Associated in a Natural Stream. *Journal of Environmental Quality*, 34, 581-589.

JOHNSON, C., FLOWERS, A., NORIEA, N., ZIMMERMAN, A., BOWERS, J., DEPAOLA, A. & GRIMES, D. J. 2010. Relationships between environmental factors and pathogenic vibrios in the northern Gulf of Mexico. *Applied and Environmental Microbiology*, 76, 7076-7084.

JUAHIR, H., ZAIN, S. M., ARIS, A. Z., YUSOFF, M. K. & MOKHTAR, M. B. 2010. Spatial assessment of Langat river water quality using chemometrics. *Journal of Environmental Monitoring*, 12, 287-295.

JYOTI, A., VAJPAYEE, P., SINGH, G., PATEL, C. B., GUPTA, K. C. & SHANKER, R. 2011. Identification of Environmental Reservoirs of Nontyphoidal Salmonellosis: Aptamer-

Assisted Bioconcentration and Subsequent Detection of Salmonella Typhimurium by Quantitative Polymerase Chain Reaction. *Environmental Science & Technology*, 45, 8996-9002.

MURRAY K., DU PREEZ M., KUHN A.L., VAN NIEKERK H., 2004 *A Pilot Study to Demonstrate Implementation of the National Microbial Monitoring Programme*. Water Research Commission; Pretoria, South Africa: 2004. WRC Report No. 1118/1/04.

KAY, D., JONES, F., WYER, M., FLEISHER, J., SALMON, R., GODFREE, A., ZELENKAUCH-JACQUOTTE, A. & SHORE, R. 1994. Predicting likelihood of gastroenteritis from sea bathing: results from randomised exposure. *The Lancet*, 344, 905-909.

KIM, T. G., JEONG, S.-Y. & CHO, K.-S. 2014. Comparison of droplet digital PCR and quantitative real-time PCR in mcrA-based methanogen community analysis. *Biotechnology Reports*, 4, 1-4.

KINNULA, H., MAPPEL, J., VALKONEN, J. K. & SUNDBERG, L.-R. 2015. The Influence of Infective Dose on the Virulence of a Generalist Pathogen in Rainbow Trout (*Oncorhynchus mykiss*) and Zebra Fish (*Danio rerio*). *PLoS ONE*, 10, e0139378.

KINZELMAN, J., MCLELLAN, S. L., DANIELS, A. D., CASHIN, S., SINGH, A., GRADUS, S. & BAGLEY, R. 2004. Non-point source pollution: determination of replication versus persistence of *Escherichia coli* in surface water and sediments with correlation of levels to readily measurable environmental parameters. *Journal of Water and Health*, 2, 103-114.

KINZELMAN, J. L., SINGH, A., NG, C., POND, K. R., BAGLEY, R. C. & GRADUS, S. 2005. Use of IDEXX Colilert-18® and Quanti-Tray/2000 as a rapid and simple enumeration method for the implementation of recreational water monitoring and notification programs. *Lake and Reservoir Management*, 21, 73-77.

KOTLOFF, K. L., NATARO, J. P., BLACKWELDER, W. C., NASRIN, D., FARAG, T. H., PANCHALINGAM, S., WU, Y., SOW, S. O., SUR, D. & BREIMAN, R. F. 2013. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. *The Lancet*, 382, 209-222.

KUNKEL, E. A., PRIVETTE, C. V., SAWYER, C. B. & HAYES, J. C. 2013. Attachment of *Escherichia coli* to fine sediment particles within construction sediment basins. *Advances in Bioscience and Biotechnology*, 4, 407–414.

LA ROSA, G., POURSHABAN, M., IACONELLI, M. & MUSCILLO, M. 2010. Quantitative real-time PCR of enteric viruses in influent and effluent samples from wastewater treatment plants in Italy. *Annali dell'Istituto superiore di sanita*, 46, 266-273.

LE ROUX, W., SCHAEFER, L. & GENTHE, B. 2012. Microbial water quality in the upper Olifants River catchment: implications for health. *African Journal of Microbiology Research*, 6, 6580-6588.

LEGGETT, H. C., CORNWALLIS, C. K. & WEST, S. A. 2012. Mechanisms of pathogenesis, infective dose and virulence in human parasites. *PLoS Pathogens*, 8, e1002512.

LIANG, Z., HE, Z., ZHOU, X., POWELL, C. A., YANG, Y., HE, L. M. & STOFFELLA, P. J. 2013. Impact of mixed land-use practices on the microbial water quality in a subtropical coastal watershed. *Science of the Total Environment*, 449, 426-433.

LIN, J. & GANESH, A. 2013. Water quality indicators: bacteria, coliphages, enteric viruses. *International Journal of Environmental Health Research*, 23, 484-506.

- LIU, L., JOHNSON, H. L., COUSENS, S., PERIN, J., SCOTT, S., LAWN, J. E., RUDAN, I., CAMPBELL, H., CIBULSKIS, R. & LI, M. 2012. Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000. *The Lancet*, 379, 2151-2161.
- LUI, Y. L. & TAN, E. L. 2014. Droplet digital PCR as a useful tool for the quantitative detection of Enterovirus 71. *Journal of Virological Methods*, 207, 200-203.
- LUNA, G., VIGNAROLI, C., RINALDI, C., PUSCEDDU, A., NICOLETTI, L., GABELLINI, M., DANOVARO, R. & BIAVASCO, F. 2010. Extraintestinal *Escherichia coli* carrying virulence genes in coastal marine sediments. *Applied and Environmental Microbiology*, 76, 5659-5668.
- LUNA, G. M., DELL'ANNO, A., PIETRANGELI, B. & DANOVARO, R. 2012. A new molecular approach based on qPCR for the quantification of fecal bacteria in contaminated marine sediments. *Journal of Biotechnology*, 157, 446-453.
- LUYT, C. D., TANDLICH, R., MULLER, W. J. & WILHELMI, B. S. 2012. Microbial monitoring of surface water in South Africa: an overview. *International Journal of Environmental Research and Public Health*, 9, 2669-2693.
- MALORNY, B., LOFSTROM, C., WAGNER, M., KRAMER, N. & HOORFAR, J. 2008. Enumeration of salmonella bacteria in food and feed samples by real-time PCR for quantitative microbial risk assessment. *Applied and Environmental Microbiology*, 74, 1299-304.
- MALORNY, B., PACCASSONI, E., FACH, P., BUNGE, C., MARTIN, A. & HELMUTH, R. 2004. Diagnostic real-time PCR for detection of *Salmonella* in food. *Applied and Environmental Microbiology*, 70, 7046-7052.

MANAFI, M. 2000. New developments in chromogenic and fluorogenic culture media. *International Journal of Food Microbiology*, 60, 205-218.

MARA, D., SLEIGH, P., BLUMENTHAL, U. & CARR, R. 2007. Health risks in wastewater irrigation: comparing estimates from quantitative microbial risk analyses and epidemiological studies. *Journal of Water and Health*, 5, 39-50.

MARTINEZ, G., PACHEPSKY, Y. A., WHELAN, G., YAKIREVICH, A. M., GUBER, A. & GISH, T. J. 2014. Rainfall-induced fecal indicator organisms transport from manured fields: Model sensitivity analysis. *Environment International*, 63, 121-129.

MARVIN-DIPASQUALE, M., AGEE, J., BOUSE, R. & JAFFE, B. 2003. Microbial cycling of mercury in contaminated pelagic and wetland sediments of San Pablo Bay, California. *Environmental Geology*, 43, 260-267.

MCDERMOTT, G. P., DO, D., LITTERST, C. M., MAAR, D., ET AL. 2013. Multiplexed Target Detection Using DNA-Binding Dye Chemistry in Droplet Digital PCR. *Analytical Chemistry*, 85, 11619-11627.

MEDEMA, G. J., PAYMENT, P., DUFOUR, A., ROBERTSON, W., WAITE, M., HUNTER, P., KIRBY, R. & ANDERSSON, Y. 2003. Safe drinking water: An ongoing challenge. In: Medema G.J., Payment P., Dufour A., Robertson W., Waite M., Hunter P., Kirby R., Andersson Y., ed. *Assessing Microbial Safety of Drinking Water—Improving Approaches and Methods*. WHO Press; Geneva, Switzerland.

MEYNELL, G. & MEYNELL, E. W. 1958. The growth of micro-organisms in vivo with particular reference to the relation between dose and latent period. *Journal of Hygiene*, 56, 323-346.

- MICHAEL J. ROTHROCK, KELLI L. HIETT, BRIAN H. KIEPPER, KIM INGRAM & HINTON, A. 2013. Quantification of Zoonotic Bacterial Pathogens within Commercial Poultry Processing Water Samples Using Droplet Digital PCR. *Advances in Microbiology*, 3, 403-411.
- MIKA, K. B., IMAMURA, G., CHANG, C., CONWAY, V., FERNANDEZ, G., GRIFFITH, J. F., KAMPALATH, R. A., LEE, C. M., LIN, C. C. & MORENO, R. 2009. Pilot-and bench-scale testing of faecal indicator bacteria survival in marine beach sand near point sources. *Journal of applied microbiology*, 107, 72-84.
- MOMBA, M. N. B., MALAKATE, V. K. & THERON, J. 2006. Abundance of pathogenic *Escherichia coli*, *Salmonella typhimurium* and *Vibrio cholerae* in Nkonkobe drinking water sources. *Journal of Water and Health* 04, 289-296.
- MORISSET, D., ŠTEBIH, D., MILAVEC, M., GRUDEN, K. & ŽEL, J. 2013. Quantitative Analysis of Food and Feed Samples with Droplet Digital PCR. *PLoS ONE*, 8, e62583.
- MORTIMER, C. H. 1971. Chemical Exchanges Between Sediments and Water in the Great Lakes-speculations on probable regulatory mechanisms. *Limnology and Oceanography*, 16, 387-404.
- MRAYYAN, B. 2005. Optimal utilization of reclaimed wastewater for irrigation purposes: Case of As-Samra wastewater treatment plant. *Journal of Environmental Assessment Policy and Management*, 7, 735-750.
- MTHEMBU, M., DJAROVA, T. & BASSON, A. 2011. Effect of agricultural and industrial developments on the quality of water at UMhlathuze River (Northern Coast of Kwa-Zulu Natal, RSA). *African Journal of Microbiology Research*, 5, 5780-5786.

- MÜLLER, E. E., EHLERS, M. M. & GRABOW, W. O. 2001. The occurrence of E. coli O157: H7 in South African water sources intended for direct and indirect human consumption. *Water Research*, 35, 3085-3088.
- MUPARIWA, Z. 2012. Overview of Current Green Drop Requirements: Services Master Class presentation (SALGA, IMESA, MILE). Guateng: Department of Water Affairs.
- MUSAH, B. I. 2013. Perennial Water Crisis in Ghana: Effects on Girl-Child Education. *Mediterranean Journal of Social Sciences*, 4, 193-199.
- MUSTAPHA, A., ARIS, A. Z., JUAHIR, H. & RAMLI, M. F. 2013. Surface water quality contamination source apportionment and physicochemical characterization at the upper section of the Jakara Basin, Nigeria. *Arabian Journal of Geosciences*, 6, 4903-4915.
- NAGELS, J., DAVIES-COLLEY, R., DONNISON, A. & MUIRHEAD, R. 2002. Faecal contamination over flood events in a pastoral agricultural stream in New Zealand. *Water Science and Technology*, 45, 45-52.
- NAIDOO, S. & OLANIRAN, A. O. 2013. Treated wastewater effluent as a source of microbial pollution of surface water resources. *International Journal of Environmental Research and Public Health*, 11, 249-270.
- NASSER, A., ZARUK, N., TENENBAUM, L. & NETZAN, Y. 2003. Comparative survival of Cryptosporidium, coxsackievirus A9 and Escherichia coli in stream, brackish and sea waters. *Water Science and Technology*, 47, 91-96.
- NKWONTA, O. & OCHIENG, G. 2009. Water pollution in Soshanguve environs of South Africa. *World Academy of Science, Engineering and Technology*, 56, 499-503.

- OBASOHAN, E., AGBONLAHOR, D. & OBANO, E. 2010. Water pollution: A review of microbial quality and health concerns of water, sediment and fish in the aquatic ecosystem. *African Journal of Biotechnology*, 9, 423-427.
- OBERHOLSTER, P. & BOTHA, A. 2014. Importance of water quality to the food industry in South Africa. *Understanding the Food Energy Water Nexus*. WWF-SA, South Africa.
- OBUOBIE, E., KERAITA, B., DANSO, G., AMOAH, P., COFIE, O. O., RASCHID-SALLY, L. & DRECHSEL, P. 2006. Irrigated urban vegetable production in Ghana: characteristics benefits and risks. IWMI-RUAF-CPWF, Accra, Ghana: IWMI, Pp 150.
- OKOH, A. I., ODJADJARE, E. E., IGBINOSA, E. O. & OSODE, A. N. 2007. Wastewater treatment plants as a source of microbial pathogens in receiving watersheds. *African Journal of Biotechnology*, 6, 2932-2944.
- OKOH, A. I., SIBANDA, T., NONGOGO, V., ADEFISOYE, M., OLAYEMI, O. O. & NONTONGANA, N. 2015. Prevalence and characterisation of non-cholerae *Vibrio* spp. in final effluents of wastewater treatment facilities in two districts of the Eastern Cape Province of South Africa: implications for public health. *Environmental Science and Pollution Research*, 22, 2008-2017.
- OLANIRAN, A. O., NAICKER, K. & PILLAY, B. 2009. Antibiotic resistance profiles of *Escherichia coli* isolates from river sources in Durban, South Africa. *World Journal of Microbiology and Biotechnology*, 25, 1743-1749.
- OLIVER, D. M., PORTER, K. D., PACHEPSKY, Y. A., MUIRHEAD, R. W., REANEY, S. M., COFFEY, R., KAY, D., MILLEDGE, D. G., HONG, E. & ANTHONY, S. G. 2016. Predicting microbial water quality with models: over-arching questions for managing risk in agricultural catchments. *Science of the Total Environment*, 544, 39-47.

- OMAR, K., POTGIETER, N. & BARNARD, T. 2010. Development of a rapid screening method for the detection of pathogenic *Escherichia coli* using a combination of Colilert® Quanti-Trays/2000 and PCR. *Water Science and Technology: Water Supply*, 10, 7-13.
- OTTOSON, J. 2005. *Comparative analysis of pathogen occurrence in wastewater – Management strategies for barrier function and microbial control*. PhD Thesis, Royal Institute of Technology (KTH).
- PACHEPSKY, Y., BLAUSTEIN, R., WHELAN, G. & SHELTON, D. 2014. Comparing temperature effects on *Escherichia coli*, *Salmonella*, and *Enterococcus* survival in surface waters. *Letters in Applied Microbiology*, 59, 278-283.
- PANDEY, P. K., KASS, P. H., SOUPIR, M. L., BISWAS, S. & SINGH, V. P. 2014. Contamination of water resources by pathogenic bacteria. *AMB Express*, 4, 1-16.
- PEREZ-RODRIGUEZ, F. & VALERO, A. 2013. Predictive microbiology in foods. *SpringerBriefs in Food, Health, and Nutrition (Vol 5)*. New York: Springer. Pp 1-10.
- PERKINS, P. E. 2013. *Water and Climate Change in Africa: Challenges and Community Initiatives in Durban, Maputo and Nairobi*, USA and Canada, Routledge. Pp 130.
- PILLAY, R. K. 2013. *An integrated study of the Isipingo River and Estuary: water and sediment quality, estuary-nearshore material fluxes, anthropogenic impacts and management*. Master of Science, University of KwaZulu Natal.
- PINHEIRO, L. B., COLEMAN, V. A., HINDSON, C. M., HERRMANN, J., HINDSON, B. J., BHAT, S. & EMSLIE, K. R. 2012. Evaluation of a Droplet Digital Polymerase Chain Reaction Format for DNA Copy Number Quantification. *Analytical Chemistry*, 84, 1003-1011.

- PIP, E. & ALLEGRO, E. 2010. Nearshore fluctuations in water chemistry, microcystins and coliform bacteria during the ice-free season in Lake Winnipeg, Manitoba, Canada. *Ecohydrology & Hydrobiology*, 10, 35-43.
- PISCIOTTA, J. M., RATH, D. F., STANEK, P. A., FLANERY, D. M. & HARWOOD, V. J. 2002. Marine bacteria cause false-positive results in the Colilert-18 rapid identification test for *Escherichia coli* in Florida waters. *Applied and Environmental Microbiology*, 68, 539-544.
- PRÜSS-ÜSTÜN, A., BOS, R., GORE, F. & BARTRAM, J. 2008. *Safer water, better health: costs, benefits and sustainability of interventions to protect and promote health*, World Health Organization.
- PRÜSS, A. 1998. Review of epidemiological studies on health effects from exposure to recreational water. *International Journal of Epidemiology*, 27, 1-9.
- PRÜSS, A. et al., 2014. Burden of disease from inadequate water, sanitation and hygiene in low- and middle-income settings: a retrospective analysis of data from 145 countries. *Tropical Medicine and International Health*, 19, 894-905.
- QADIR, M., WICHELS, D., RASCHID-SALLY, L., MCCORNICK, P. G., DRECHSEL, P., BAHRI, A. & MINHAS, P. 2010. The challenges of wastewater irrigation in developing countries. *Agricultural Water Management*, 97, 561-568.
- RACKI, N., MORISSET, D., GUTIERREZ-AGUIRRE, I. & RAVNIKAR, M. 2014. One-step RT-droplet digital PCR: a breakthrough in the quantification of waterborne RNA viruses. *Analytical and Bioanalytical Chemistry*, 406, 661-667.

RHP 2005. State-of-Rivers Report: Monitoring and Managing the Ecological State of Rivers in the Crocodile (West) Marico Water Management Area. Pretoria: Department of Environmental Affairs and Tourism

ROSE, J. B. & CLARK, C. S. 1986. Microbial aspects of wastewater reuse for irrigation. *Critical Reviews in Environmental Science and Technology*, 16, 231-256.

SABS 2011. South African National Standard (SANS) 241 for Drinking Water Quality. Pretoria: SABS Standard Division.

SAKAJI, R. & FUNAMIZU, N. 1998. Microbial risk assessment and its role in the development of wastewater reclamation policy. *Wastewater Reclamation and Reuse*, 10, 705-756.

SAVICHTCHEVA, O. & OKABE, S. 2006. Alternative indicators of fecal pollution: relations with pathogens and conventional indicators, current methodologies for direct pathogen monitoring and future application perspectives. *Water Research*, 40, 2463-2476.

SCHAUER, M., JIANG, J. & HAHN, M. W. 2006. Recurrent seasonal variations in abundance and composition of filamentous SOL cluster bacteria (Saprospiraceae, Bacteroidetes) in oligomesotrophic Lake Mondsee (Austria). *Applied and Environmental Microbiology*, 72, 4704-4712.

SCHETS, F. M., SCHIJVEN, J. F. & DE RODA HUSMAN, A. M. 2011. Exposure assessment for swimmers in bathing waters and swimming pools. *Water Research*, 45, 2392-2400.

SCHOEN, M. E. & ASHBOLT, N. J. 2010. Assessing pathogen risk to swimmers at non-sewage impacted recreational beaches. *Environmental Science & Technology*, 44, 2286-2291.

- SCHIJVEN, J. F., TEUNIS, P. F., RUTJES, S. A., BOUWKNEGT, M. & DE RODA HUSMAN, A. M. 2011. QMRAspot: a tool for quantitative microbial risk assessment from surface water to potable water. *Water Research*, 45, 5564-5576.
- SERVAIS, P., GARCIA-ARMISEN, T., GEORGE, I. & BILLEN, G. 2007. Fecal bacteria in the rivers of the Seine drainage network (France): sources, fate and modelling. *Science of the Total Environment*, 375, 152-167.
- SEURINCK, S., VERSTRAETE, W. & SICILIANO, S. D. 2005. Microbial source tracking for identification of fecal pollution. *Reviews in Environmental Science and Bio/Technology*, 4, 19-37.
- SHANKER, R., SINGH, G., JYOTI, A., DWIVEDI, P. & SINGH, S. 2013. Nanotechnology and detection of microbial pathogens. *Animal biotechnology: models in discovery and translation.*: American Press.
- SHEHANE, S., HARWOOD, V., WHITLOCK, J. & ROSE, J. 2005. The influence of rainfall on the incidence of microbial faecal indicators and the dominant sources of faecal pollution in a Florida river. *Journal of Applied Microbiology*, 98, 1127-1136.
- SIBANDA, T., CHIGOR, V. N. & OKOH, A. I. 2013. Seasonal and spatio-temporal distribution of faecal-indicator bacteria in Tyume River in the Eastern Cape Province, South Africa. *Environmental Monitoring and Assessment*, 185, 6579-6590.
- SIBANDA, T., SELVARAJAN, R. & TEKERE, M. 2015. Urban effluent discharges as causes of public and environmental health concerns in South Africa's aquatic milieu. *Environmental Science and Pollution Research*, 22, 18301-18317.

SIDSTEDT, M., JANSSON, L., NILSSON, E., NOPPA, L., FORSMAN, M., RÅDSTRÖM, P. & HEDMAN, J. 2015. Humic substances cause fluorescence inhibition in real-time polymerase chain reaction. *Analytical Biochemistry*, 487, 30-37.

SIMPSON, J. M., SANTO DOMINGO, J. W. & REASONER, D. J. 2002. Microbial source tracking: state of the science. *Environmental Science & Technology*, 36, 5279-5288.

SINGH, A. & LIN, J. 2015. Microbiological, coliphages and physico-chemical assessments of the Umgeni River, South Africa. *International Journal of Environmental Health Research*, 25, 33-51.

SINGH, G., MANOHAR, M., ADEGOKE, A. A., STENSTRÖM, T. A. & SHANKER, R. 2017a. Novel aptamer-linked nanoconjugate approach for detection of waterborne bacterial pathogens: an update. *Journal of Nanoparticle Research*, 19, 4-11.

SINGH, G., SITHEBE, A., ENITAN, A. M., KUMARI, S., BUX, F. & STENSTRÖM, T. A. 2017b. Comparison of droplet digital PCR and quantitative PCR for the detection of Salmonella and its application for river sediments. *Journal of Water and Health*, wh2017259.

SINGH, G., VAJPAYEE, P., BHATTI, S., RONNIE, N., SHAH, N., MCCLURE, P. & SHANKER, R. 2013. Determination of viable Salmonellae from potable and source water through PMA assisted qPCR. *Ecotoxicology and Environmental Safety*, 93, 121-127.

SINGH, G., VAJPAYEE, P., RAM, S. & SHANKER, R. 2010. Environmental reservoirs for enterotoxigenic Escherichia coli in south Asian Gangetic riverine system. *Environmental Science & Technology*, 44, 6475-6480.

SINGH, G., VAJPAYEE, P., RANI, N., AMOAH, I. D., STENSTRÖM, T. A. & SHANKER, R. 2016. Exploring the potential reservoirs of non specific TEM beta lactamase (bla TEM)

gene in the Indo-Gangetic region: A risk assessment approach to predict health hazards. *Journal of Hazardous Materials*, 314, 121-128.

SITHEBE, A., SINGH, G., AMOAH, I. & STENSTRÖM, T. 2016. A comparative microbiological assessment of the Isipingo River and Palmiet River in Kwa-Zulu Natal province to elucidate health risks. In: MÉNDEZ-VILAS, A. (ed.) *Microbes in the Spotlight: Recent Progress in the Understanding of Beneficial and Harmful Microorganisms*. Brown Walker Press, Pp 2-6.

SMART, R. M. & BARKO, J. W. 1978. Influence of sediment salinity and nutrients on the physiological ecology of selected salt marsh plants. *Estuarine and Coastal Marine Science*, 7, 487-495.

SOLLER, J. A., SCHOEN, M. E., BARTRAND, T., RAVENSCROFT, J. E. & ASHBOLT, N. J. 2010. Estimated human health risks from exposure to recreational waters impacted by human and non-human sources of faecal contamination. *Water Research*, 44, 4674-4691.

STATS SA 2015. Mid-year population estimates 2015 (Statistical release P0302). *Statistics South Africa, Pretoria, South Africa*.

STENSTRÖM, T. 1994. A review of water-borne outbreaks of gastroenteritis in Scandinavia. In: GOLDING, A. M. B., NOAH, N. AND SANWELL-SMITH, R. (ed.) *Water and Public Health*. London: Smith-Gordon & Co.

STENSTRÖM, T. A. 2004. *Guidelines on the safe use of urine and faeces in ecological sanitation systems*, Stockholm: Stockholm Environment Institute, EcoSanRes Programme.

- STEYN, M., JAGALS, P. & GENTHE, B. 2004. Assessment of microbial infection risks posed by ingestion of water during domestic water use and full-contact recreation in a mid-southern African region. *Water Science and Technology*, 50, 301-308.
- STONE, D. L., HARDING, A. K., HOPE, B. K. & SLAUGHTER-MASON, S. 2008. Exposure assessment and risk of gastrointestinal illness among surfers. *Journal of Toxicology and Environmental Health, Part A*, 71, 1603-1615.
- STRACHAN, N. J., DOYLE, M. P., KASUGA, F., ROTARIU, O. & OGDEN, I. D. 2005. Dose response modelling of *Escherichia coli* O157 incorporating data from foodborne and environmental outbreaks. *International Journal of Food Microbiology*, 103, 35-47.
- TALLON, P., MAGAJNA, B., LOFRANCO, C. & LEUNG, K. T. 2005. Microbial indicators of faecal contamination in water: a current perspective. *Water, Air, and Soil Pollution*, 166, 139-166.
- TAYLOR, H. 2003. Surface waters. In: DUNCAN, M., HORAN, N. (ed.) *The handbook of Water and Wastewater Microbiology*. Academic press, London. Pp 611-626.
- TEKLEHAIMANOT, G. Z. 2013. *Faecal water pollution loads as a function of population growth in Sedibeng and Soshanguve, South Africa*. M.Tech: Water Care, Tshwane University of Technology.
- TEKLEHAIMANOT, G. Z., COETZEE, M. A. & MOMBA, M. N. 2014. Faecal pollution loads in the wastewater effluents and receiving water bodies: a potential threat to the health of Sedibeng and Soshanguve communities, South Africa. *Environmental Science and Pollution Research*, 21, 9589-9603.

- TEUNIS, P., VAN DEN BRANDHOF, W., NAUTA, M., WAGENAAR, J., VAN DEN KERKHOF, H. & VAN PELT, W. 2005. A reconsideration of the *Campylobacter* dose–response relation. *Epidemiology and Infection*, 133, 583-592.
- TORTORELLO, M. L. 2003. Indicator organisms for safety and quality - uses and methods for detection: mini review. *Journal of AOAC International*, 86, 1208-1217.
- TUNDISI, J. G. 2008. Water Resources in the Future: Problems and solutions. *Estudos Avançados*, 22, 7-16.
- TYRREL, S. & QUINTON, J. N. 2003. Overland flow transport of pathogens from agricultural land receiving faecal wastes. *Journal of Applied Microbiology*, 94, 87-93.
- USEPA 1986. Ambient Water Quality Criteria for Bacteria: Office Of Water Regulations And Standards. US Environmental Protection Agency USEPA.
- USEPA 2005. National Management Measures to Control Nonpoint Source Pollution from Urban Areas. Washington, DC: USEPA.
- USEPA 2006. National primary drinking water regulations: Ground water rule; final rule. *Fed. Regist.*
- VALENTE, M. S., PEDRO, P., ALONSO, M. C., BORREGO, J. J. & DIONISIO, L. 2010. Are the defined substrate-based methods adequate to determine the microbiological quality of natural recreational waters? *Journal of Water and Health*, 8, 11-19.
- VAN LIER, J., MAHMOUD, N. & ZEEMAN, J. 2008. Anaerobic waste-water treatment In M. Henze, MCM van Loosdrecht, GA Ekama, and D. Brdjanovic (ed.), *Biological Wastewater Treatment Principles Modeling and Design*. IWA Publishing, London.

VEOLIA/IFPRI 2015. The Murky Future of Global Water Quality: New Global Study Projects Rapid Deterioration in Water Quality.: International Food Policy Research Institute (IFPRI).

WADE, T. J., CALDERON, R. L., BRENNER, K. P., SAMS, E., BEACH, M., HAUGLAND, R., WYMER, L. & DUFOUR, A. P. 2008. High sensitivity of children to swimming-associated gastrointestinal illness: results using a rapid assay of recreational water quality. *Epidemiology*, 19, 375-383.

WADE, T. J., CALDERON, R. L., SAMS, E., BEACH, M., BRENNER, K. P., WILLIAMS, A. H. & DUFOUR, A. P. 2006. Rapidly measured indicators of recreational water quality are predictive of swimming-associated gastrointestinal illness. *Environmental Health Perspectives*, 114, 24-28.

WAKELIN, S. A., COLLOFF, M. J. & KOOKANA, R. S. 2008. Effect of wastewater treatment plant effluent on microbial function and community structure in the sediment of a freshwater stream with variable seasonal flow. *Applied and Environmental Microbiology*, 74, 2659-2668.

WALKER, C. L. F., RUDAN, I., LIU, L., NAIR, H., THEODORATOU, E., BHUTTA, Z. A., O'BRIEN, K. L., CAMPBELL, H. & BLACK, R. E. 2013. Global burden of childhood pneumonia and diarrhoea. *The Lancet*, 381, 1405-1416.

WALTERS, S. P., THEBO, A. L. & BOEHM, A. B. 2011. Impact of urbanization and agriculture on the occurrence of bacterial pathogens and stx genes in coastal waterbodies of central California. *Water Research*, 45, 1752-1762.

WARDEN, P. S., DESARNO, M. S., VOLK, S. E. & ELDRED, B. J. 2011. Evaluation of Colilert-18® for Detection and Enumeration of Fecal Coliform Bacteria in Wastewater Using

the US Environmental Protection Agency Alternative Test Procedure Protocol. *Journal of AOAC International*, 94, 1573-1580.

WHITMAN, R. L. & NEVERS, M. B. 2003. Foreshore sand as a source of *Escherichia coli* in nearshore water of a Lake Michigan beach. *Applied and Environmental Microbiology*, 69, 5555-5562.

WHITMAN, R. L., PRZYBYLA-KELLY, K., SHIVELY, D. A., NEVERS, M. B. & BYAPPANAHALLI, M. N. 2008. Sunlight, season, snowmelt, storm, and source affect *E. coli* populations in an artificially ponded stream. *Science of The Total Environment*, 390, 448-455.

WHO 2001. Water Quality: Guidelines, Standards and Health. *In*: FEWTRELL, L., BARTRAM, J. (ed.). London, UK: International Water Association (IWA)/ World Health Organization.

WHO 2003. *Guidelines for safe recreational waters (Vol 1): Coastal and fresh waters*. Geneva: World Health Organization.

WHO 2004. *Guidelines for drinking-water quality (Vol 1). 3rd ed.* Geneva: World Health Organization.

WHO 2006. *Guidelines for the safe use of wastewater, excreta and greywater (Vol 2): Wastewater use in agriculture*. Geneva: World Health Organization.

WHO 2008. *Guidelines for drinking-water quality [electronic resource]: incorporating 1st and 2nd addenda, vol. 1, Recommendations*. Geneva: World Health Organization.

WHO 2011. *Guidelines for drinking-water quality: Fourth edition*, Geneva, World Health Organisation.

WHO 2014. UNICEF Joint Monitoring Programme for Water Supply and Sanitation: Progress on drinking water and sanitation, 2014 update. Geneva: UNICEF/WHO.

WHO 2015. *Sanitation safety planning: manual for safe use and disposal of wastewater, greywater and excreta*. Geneva: World Health Organization.

WHO/UNICEF 2009. Diarrhea: Why Children Are Still Dying and What Can Be Done? Geneva: UNICEF/WHO.

WHO/UNICEF 2015. *Progress on sanitation and drinking water–2015 update and MDG assessment*. Geneva: World Health Organization.

WILKES, G., EDGE, T., GANNON, V., JOKINEN, C., LYAUTEY, E., NEUMANN, N., RUECKER, N., SCOTT, A., SUNOHARA, M. & TOPP, E. 2011. Associations among pathogenic bacteria, parasites, and environmental and land use factors in multiple mixed-use watersheds. *Water Research*, 45, 5807-5825.

WIOMSA 2009. Regional Synthesis Report on the Status of Pollution in the Western Indian Ocean Region. United Nations Environment Programme (UNEP) ; the Council for Scientific and Industrial Research (CSIR) ; Western Indian Ocean Marine Science Association (WIOMSA).

WOHLSEN, T. 2011. Comparative evaluation of chromogenic agar CM1046 and mFC agar for detection of *E. coli* and thermotolerant coliform bacteria from water samples. *Letters in Applied Microbiology*, 53, 155-160.

WOHLSEN, T., BATES, J., VESEY, G., ROBINSON, W. & KATOULI, M. 2006. Evaluation of the methods for enumerating coliform bacteria from water samples using precise reference standards. *Letters in Applied Microbiology*, 42, 350-356.

WOHLSEN, T., BAYLISS, J., BATES, J., GRAY, B., JOHNSON, S. & SCHNEIDER, P. 2008. An evaluation of membrane faecal coliform agar and Colilert-18® for the enumeration of E. coli bacteria in surface water samples. *Journal of Water Supply: Research and Technology- AQUA*, 57, 569-576.

WOOMER, P. L. 1994. Most probable number counts. In: WEAVER, R. W. (ed.) *Methods of Soil Analysis: Part 2—Microbiological and Biochemical Properties*. SSSA Book Series 5. Madison, WI: Soil Science Society of America, Pp 59-79.

WORLD HEALTH ORGANIZATION. Water sanitation hygiene: Diseases and risks [Online]. Available: http://www.who.int/water_sanitation_health/diseases-risks/en/ [Accessed 05-05-2017].

WORLD HEALTH ORGANIZATION. Media Centre: News release [Online]. Available: <http://www.who.int/mediacentre/news/releases/2017/water-sanitation-hygiene/en/> [Accessed 22-08-2017].

WOSE KINGE, C. & MBEWE, M. 2010. Characterisation of Shigella species isolated from river catchments in the North West province of South Africa. *South African Journal of Science*, 106, 1-4.

WRC 2002. State of Rivers Report: uMngeni River and neighbouring rivers and streams. *WRC Report No. TT 200/02*, Water Research Commission, Pretoria, SA.

WWAP 2017. The United Nations World Water Development Report 2017: Wastewater: The Untapped Resource. *Wastewater: The Untapped Resource*. Paris: United Nations Educational Scientific and Cultural Organization (UNESCO).

WYE. MD, K. D., DAWSON H.M, GERRY F. JACKSON, FRANK JONES, JANETTE YEO, JOHN WHITTLE 1996. Delivery of microbial indicator organisms to coastal waters from catchment sources. *Water Science and Technology*, 37-50.

WYER MD, O. G., KAY D, CROWTHER J, JACKSON G, FEWTRELL L. 1997. Non-outfall sources of faecal indicator organisms affecting the compliance of coastal waters with directive. *Water Science and Technology* 35, 151-156.

YAKUB, G. P., CASTRIC, D. A., STADTERMAN-KNAUER, K. L., TOBIN, M. J., BLAZINA, M., HEINEMAN, T. N., YEE, G. Y. & FRAZIER, L. 2002. Evaluation of Colilert and Enterolert defined substrate methodology for wastewater applications. *Water Environment Research*, 74, 131-135.

YILLIA, P. T., KREUZINGER, N., MATHOOKO, J. M. & NDOMAHINA, E. T. 2009. Microbial risk assessment with the OAEL approach at water abstraction points in rural Kenya. *Physics and Chemistry of the Earth, Parts A/B/C*, 34, 790-798.

ZHAO, S., LIN, H., CHEN, S., YANG, M., YAN, Q., WEN, C., HAO, Z., YAN, Y., SUN, Y., HU, J., CHEN, Z. & XI, L. 2015. Sensitive detection of Porcine circovirus-2 by droplet digital polymerase chain reaction. *Journal of Veterinary Diagnostic Investigation*, 27, 784-8

ZIKHALI, V., TIROK, K. & STRETCH, D. 2015. Sediment resuspension in a shallow lake with muddy substrates: St Lucia, South Africa. *Continental Shelf Research*, 108, 112-120.

APPENDICES

Appendix 1: Supporting information for chapter 3

Appendix 1 relates to the supporting information for chapter 3, below is a graph showing the conductivity values at three sampling points of interest (Figure 6.1). Pictures of the auto-sampler and sediment trap used in this study are illustrated in (Figure 6.2).

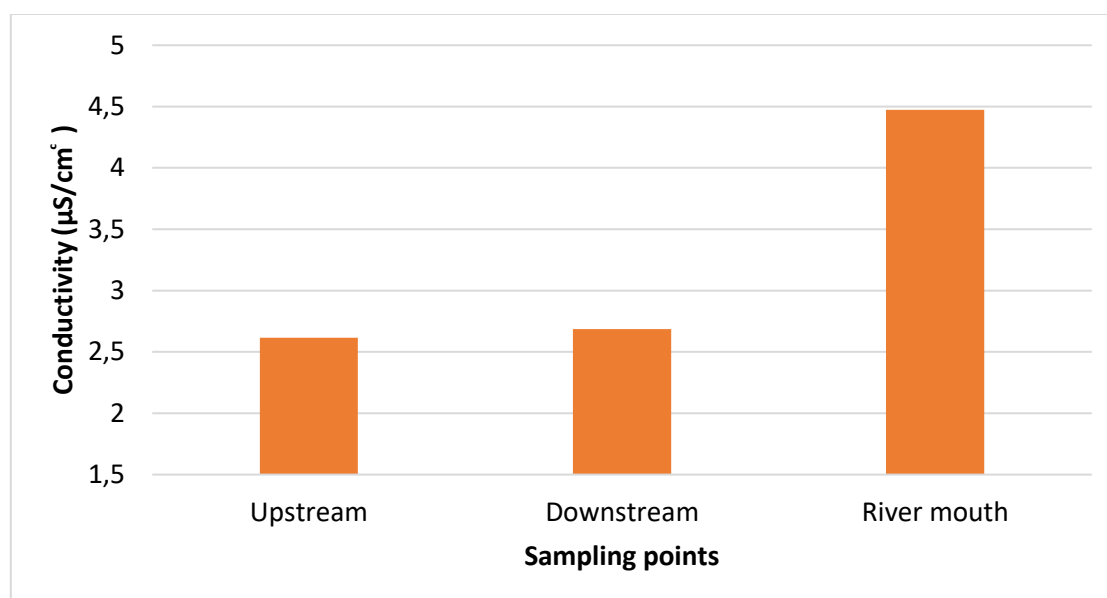


Figure 6.1: Conductivity values for sampling points along the Isipingo River (February 2015).



Figure 6.2: A- Standard auto sampler (3700C compact portable sampler, Teledyne Isco, USA); B- Sediment trap (self-designed)

Appendix 2: Supporting tables for QMRA (chapter 5)

This appendix contains additional information such as tables and figures that relates to Chapter 5 such as exposure scenarios (Table 6.1), the multi-barrier approach according to the World Health Organisation and the QMRA results relating to recreational activities.

Table 6.1: Exposure assumptions relating to recreational and occupational activities

| Group | Volume (ml) | Frequency | Reference |
|--------------------------------|-------------|-----------|---------------------|
| Recreational (Swimming) | | | |
| Men | 27 | 7 | Schets et al., 2011 |
| Women | 18 | 7 | Schets et al., 2011 |
| Children | 37 | 8 | Schets et al., 2011 |
| Occupational | | | |
| Women (Laundry) | 10 | | Steyn et al., 2004 |

Table 6.2: Multi-barrier approach according to (WHO, 2006)

| Control Measure | Reduction (log units) | Comments |
|----------------------------|-----------------------|--|
| Produce-washing with water | 1 | Washing salad crops, vegetables and fruit with clean water. |
| Produce disinfection | 2 | Washing salad crops, vegetables and fruit with a weak, often chlorine-based disinfectant solution and rinsing with clean water |
| Produce peeling* | 1–2 | Fruits, cabbage, root crops |
| Produce cooking | 6 | Immersion in boiling or close-to-boiling water until the food is cooked ensures pathogen destruction |

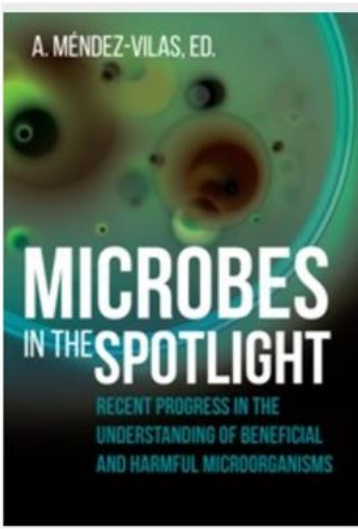
*In this instance, this control measure was not added, since we were considering Spinach and there is no peeling.

Appendix 3: First pages of published outputs

Appendix 3 contains the first page of the book chapter, publication that I have contributed to and a submitted manuscript as well as a newspaper article.

Book chapter in *Microbes in the spotlight: recent progress in the understanding of beneficial and harmful microorganisms*.

Microbes in the spotlight: recent progress in the understanding of beneficial and harmful microorganisms



A comparative microbiological assessment of the Isipingo River and Palmiet River in Kwa-Zulu Natal province to elucidate health risks

A. Sithebe^{*1}, G. Singh^{*1}, I. D. Amoah^{*1} and T. A. Stenstrom¹

¹Institute for Water and Wastewater Technology, Durban University of Technology, ML Sultan Campus
²PO Box 1334, Durban, 4000 South Africa.

^{*}Corresponding author: e-mail: sithebeayanda15@gmail.com, Phone: +27 313736704

The microbiological quality of the Palmiet and Isipingo Rivers in KwaZulu-Natal (South Africa) was investigated. The study involved an assessment of historical data collected over the past 66 months on each river based on *Escherichia coli* (*E.coli*) values obtained from the Municipality (eThekweni Water and Sanitation Dept). This was followed by microbiological quality assessments in 2015 for surface water and sediments with IDEXX Colilert 18 for coliforms and *E.coli* quantifications and Enterolert for the enumeration of *Enterococci*. Physiochemical properties included temperature, pH, conductivity, TDS, salinity and dissolved oxygen. The selected sampling points in both rivers expressed a water quality classified as *unsuitable* for crop irrigation and recreational use. The observed microbial concentrations in both rivers exceeded the World Health Organization guideline values and standard limits defined by Department of Water Affairs and Forestry (SA). It was further concluded, Isipingo River had an inferior quality than the Palmiet River.

Keywords Water quality; risk assessment; *Escherichia coli*; *Enterococci*

1. Introduction

The deteriorating quality of river water is a major threat to South Africa's ability to provide adequate water of appropriate quality to meet its population's needs and to ensure a sustainable environment. Thus, a further understanding of the quantitative variability is important for accurate and comprehensive assessment of microbial river water quality which accounts for seasonal and spatial variations. Diarrheal diseases ranked 2nd after HIV in the global burden of diseases in South Africa with an impact of 4.9% (1 138 000) life years lost per year solely due to diarrhea [1]. Children who are malnourished or have impaired immunity as well as people living with HIV are at risk of life-threatening diarrhoea. Approximately 2.2 million people die each year

Comparison of droplet digital PCR and quantitative PCR for the detection of *Salmonella* and its application for river sediments

Gulshan Singh, Ayanda Sithebe, Abimbola M. Enitan, Sheena Kumari, Faizal Bux, Thor Axel Stenström

Available Online 4 April 2017, wh2017259; DOI: 10.2166/wh.2017.259

Article

Info & Metrics

PDF

Abstract

Despite advances in microbial detection that quantitative polymerase chain reaction (qPCR) has led to, complex environmental samples, such as sediments, remain a challenge due to presence of PCR inhibitors. Aquatic sediments accumulate particle-bound microbial contaminants and thereby reflect a cumulative microbial load over time. The relatively new droplet digital PCR (ddPCR) has emerged as a direct quantitative method, highly tolerant to PCR inhibitors and relinquishing the necessity for calibration/standard curves. Information is virtually absent where ddPCR has been applied to detect pathogenic organisms in aquatic sediments. This study compared the efficacy of ddPCR with qPCR, for quantification of *Salmonella* in sediments from the Palmiet River near an informal settlement in Durban, South Africa. ddPCR significantly improved both analytical sensitivity and detection of low concentrations of *Salmonella* as compared to qPCR. The expected copy numbers measured from both qPCR and ddPCR showed good R^2 values (0.999 and 0.994, respectively). The site mostly affected by the informal settlements exhibited *Salmonella* in the range of 255 ± 37 and 818 ± 30 *Salmonella*/g ($p \leq 0.0001$) in qPCR and ddPCR, respectively. The improved detection of *Salmonella* in sediments with ddPCR makes it a promising technical method for the quantification of *Salmonella* in multifarious environmental samples.

SELECTED ISSUE



Volume 15, issue 2

[Table of Contents](#)

[Browse Archive](#)

Actions

[Email](#)

[Share](#)

[Citation Tools](#)

[View Full PDF](#)

[Tweet](#)

[Save to my folders](#)

[Alerts](#)

Manuscript Details

Manuscript number JPCE_2017_65

Title Prevalence and impact of rainfall on microbial variability in surface water and sediments in Isipingo and Palmiet Rivers, KwaZulu-Natal

Article type Full length article

Abstract

The investigated microbiological quality of the Palmiet and Isipingo rivers situated in KwaZulu-Natal (South Africa) are affected by numerous anthropogenic sources. The study involved a microbiological quality assessments of surface water and sediments collected from both rivers. The IDEXX Colilert -18 and Enterolert Quanti-Tray test methods were used for the enumeration of *E.coli* and Enterococci respectively. Further, to investigate the effect of rainfall on the microbial concentrations a pilot study was conducted in the Isipingo river water and sediment samples to address variability over time. The results indicate that sediments samples from Isipingo and Palmiet rivers had significantly higher microbial levels of *E.coli* and Enterococci than the surface water at the corresponding sites (one way Anova, $p < 0.05$). When overall microbiological parameters were compared between the rivers, Isipingo river had a mean \pm STD (log₁₀ CFU/100 mL) range in surface water of *E.coli* between 4.7 ± 1.1 - 5.0 ± 0.9 and for Enterococci between 4.2 ± 1.4 - 4.6 ± 1.3 . This was higher than for the Palmiet river surface water samples where the corresponding values were for *E.coli* : 3.4 ± 0.9 - 4.7 ± 0.6 and for Enterococci: 2.8 ± 0.6 - 4.6 ± 0.5 . The same trend was observed in sediment samples. Higher microbial concentrations occurred after heavy rainfall as compared to no rainfall event. The highest concentration of *E.coli* and Enterococci were found at the site next to the wastewater treatment plant and the lowest level of *E.coli* and Enterococci upstream of the wastewater treatment plant at the Isipingo river. Sampling sites surrounded by the Quarry road informal settlements exhibited high levels of indicator organisms. In general, the selected sampling points in both the Palmiet and Isipingo River had a water quality classified as unsuitable for crop irrigation and recreational use with an inferior quality at the Isipingo river as compared to the Palmiet river.

Keywords Fecal indicators; KwaZulu-Natal; Microbial water quality; variability

Corresponding Author Ayanda Sithebe

Corresponding Author's Institution Durban University of Technology

Order of Authors Ayanda Sithebe, Gulshan Singh, FAIZAL BUX, Thor Axel Stenström

Article on The Mercury newspaper (24 May 2016) which shed some light on the pollution state of the Palmiet and Isipingo rivers, based on the findings obtained in this study.

River water unsuitable for use» P5

SINCE 1852

THE MERCURY

R7.20 incl. VAT
R6.12 for subscribers

771016 821002

WITH BUSINESSREPORT

RACISM STOPS WITH ME

Tuesday, May 24, 2016

Dbn 15/2
Pmb 9/1
SEE PAGE 1

News

THE MERCURY
TUESDAY
MAY 24, 2016



1 In the upper reaches near Westville, close to the Palmiet Nature Reserve, the Palmiet river remains relatively pristine.



2 By the time the Palmiet river reaches the Quarry Road shack settlement, close to the junction with the Umgeni river, the watercourse is seriously polluted with litter.

New light shed on pollution in two Durban rivers

Tony Carnie

A DURBAN University of Technology (DUT) student is helping to shed new light on the sources of serious sewage and litter pollution problems in at least two Durban river systems.

Recent studies by Ayanda Sithabe, a masters student at the DUT Institute for Water and Wastewater Technology, have confirmed high levels of sewage contamination in both the Palmiet and Isipingo rivers, especially after heavy rain.

Although faecal indicator bacteria readings were much higher in the Isipingo river, E.coli levels (an indicator of human or animal sewage pollution) rendered the lower reaches of both rivers unsuitable for swimming, recreation or irrigating crops - exceed-

ing her results at a national water conference in Durban last week following a series of studies with colleague Dr Gulshan Singh, supervised by Professor Thor Axel Stenstrom.

The study analysed nearly five-and-a-half years of historical data obtained from the municipality, collected from both rivers between 2008 and 2014.

While it is understood that the pollution picture has shown some improvement since then, Sithabe told the Water Institute of South Africa conference that some of the highest readings were evident near the dense shack settlements next to both rivers, as well as next to sewage treatment plants and sewage pump stations operated by



Ayanda Sithabe prepares to take sediment samples from the Isipingo River, where a study shows high levels of E.coli sewage bacteria. Sewage contamination increases significantly after heavy rain.

of the Isipingo estuary.

In 2009, 2012, 2013 and the first six months of 2014, mean E.coli readings next to the sewage works and shack settlements were classified in the "critical" zone, with lower readings in 2010 and 2011.

E.coli readings in the lower sections of the Palmiet river were considerably lower than those in the Isipingo, falling within eThekweni's "acceptable" range of 400-2000 cfu, but still posed health risks for drinking water, recreation or watering crops.

"The observed microbial concentration in both rivers exceeded guideline values stipulated by the World Health Organisation and standard limits defined by the Department of Water Affairs," she told the conference.

A separate study by Sithabe and her colleagues has also suggested that sewage contamination in both rivers increases significantly after heavy rain. One example was a storm in September 2012, when E.coli readings shot way above 10 000 cfu.

Overall, the highest sewage bacteria counts recorded on the Palmiet river were next to pump stations "possibly due to pipe bursts and overflows".

In her conclusions section, Sithabe recommended that more surface water and sediment sampling points be set up in the vicinity of the Quarry Road informal settlement, close to the Umgeni river junction, as well as possibly to look into event rain piling.