Microbial Pathogens in the Umgeni River, South Africa

Report to the
Water Research Commission

by

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EXECUTIVE SUMMARY

BACKGROUND

Increasing urbanization on a global scale places enormous pressure on finite freshwater supplies. South Africa is a chronically water stressed country with between 500 m^3 and 1000 m^3 of water available per person per year. Surface water is heavily committed for use, resulting in water being imported from neighbouring countries. The South African national government is concerned about the state of river water quality and the status of wastewater treatment. The Blue Drop Certification Programme was initiated in 2009 which is designed and implemented with the core objective of ensuring good quality drinking water. Decision makers, investors and researchers share the view that the declining quality of water will have a negative impact on the South African economy, in both the short and the long term. As a result, water availability is predicted to be the single greatest and most urgent development constraint facing South Africa.

As water demands increase, there are some concerns that need to be addressed to ensure protection of public health and the health of the environment. In many countries water quality of the natural resources has become an important issue. With the development of urban and industrial areas, rivers have traditionally acted as conduits of pollution through legal and illegal practices. South Africa's water resources have been under increasing threat of pollution in recent years partly due to rapid demographic changes, which have coincided with the establishment of human settlements lacking appropriate sanitary infrastructure. One of the biggest issues regarding water use is pathogen transport. Due to the complexity of contamination sources, it is still difficult to assess which pathogens can potentially be spread through the water supply. Microbial contamination of our aquatic environments poses a potential public health risk when improperly managed. Water quality issues, associated with microorganisms and physic-chemical properties, are of increasing importance because they can impact on water supplies. Accurate and comprehensive assessment of microbial water quality is of paramount importance if both existing and new water sources are to be safely employed.

Public water systems rely on bacterial indicators (i.e. coliforms) for monitoring water quality. However, it has been shown that bacterial indicators are often poorly correlated with the presence of other microorganisms. Viruses are a group of particular concern because they include highly stable pathogens that can be resistant to standard wastewater treatment

processes. The spread of viral pathogens through water is a real possibility as several studies have detected enteric viruses in treated wastewater, including Reoviruses, Astroviruses (AstVs), Saproviruses (SaVs), Rotaviruses (RV), Noroviruses (NoVs), Adenoviruses (AdVs), Hepatitis A viruses (HAVs) and Enteroviruses (EVs). The inherent resistance of enteric viruses to water disinfection processes means that they can be present in drinking water exposing consumers to the likelihood of infection. The enteric viruses, shed in large numbers in the faeces of infected individuals, enter source waterways when inadequately treated and untreated human and animal wastes are directly or indirectly discharged into rivers, streams and estuaries. Current safety standards for determining water quality typically do not specify what level of viruses should be considered acceptable. This is in spite of the fact that viruses are generally more stable than common bacterial indicators in the environment. The study of viruses is relatively poor and has historically been focusing on medical- or agricultural-related viruses. There has been little investigation of the importance of viruses in the aquatic environmental domain, where even basic information, such as their temporal dynamics and spatial distribution, is almost non-existent. Therefore, it was critical to initiate some environmental viral study in order to have an understanding of viruses in our water environment.

AIMS OF THE STUDY

The objective of this study was to evaluate the seasonal variation of viral abundance, especially human pathogenic enteroviruses in Umgeni River, South Africa.

METHODOLOGY

The Umgeni River was chosen due to it being the major water source in the Durban area. Water samples from the Umgeni River were collected during the autumn, winter, spring and summer seasons. Physico-chemical parameters including, temperature, pH, turbidity, biological oxygen demand, chemical oxygen demand, conductivity, dissolved nutrient concentrations and heavy-metals were measured. The membrane filtration (MF) technique was used for the enumeration of bacterial indicators according to standard protocol. Phage populations were determined by the plaque assays. Viral community samples (virioplankton) were concentrated using a two-step tangential flow filtration process, followed by ultra-centrifugation. SYBR Gold staining and epifluorescent microscopy was used to enumerate virus-like particles (VLPs). Transmission electron microscopy (TEM) was used to examine the structures and morphology of VLPs, by resembling known phages and human viruses. Cell-culture was utilized to determine viral infectivity on various cell lines according to the

cytopathic effect (CPE). Viral DNA and RNA were simultaneously extracted from the viral concentrates using appropriate extraction kits. The primers sequences were selected using previously published data on the different human viruses available in the GENBANK database. Statistical comparisons were performed between all datasets.

RESULTS AND DISCUSSION

The results show that the Umgeni River water quality, especially in the lower reaches, has been affected by chemical, bacterial and viral contamination.

Spatial and seasonal fluctuations of the physico-chemical environmental variables of the water samples were observed. Significant seasonal variations (p<0.05) in turbidity values were noted and ranged from 1.62 NTU (Inanda Dam; U5) in autumn to 15.64 NTU (Umgeni River mouth; U1) summer which exceeded the target water quality range of < 1 NTU for domestic water use. This might cause problems for those who drink the water directly without treatment. Large seasonal variations in BOD_5, COD and conductivity levels amongst the sampling points along the Umgeni River were also observed. The water quality of these sampling sites can only be used for most of the industrial purposes, not for the recreational and drinking purposes according to the water quality guidelines set by DWAF. The nitrite/nitrate, ammonium and phosphate levels of the water samples at the New Germany Waste Treatment Works (U3) were generally higher than other sampling points except (U1) indicating potential polluting sources in this area. The phosphate levels of the water samples from points U1, U2 (Informal settlement at Reservoir Hills) and U3 as lower reaches area are higher than South African Water Quality guideline value for the aquaculture of 0.077 mg/ℓ P. The water temperature of the Umgeni River samples showed moderate to strong correlations with turbidity (r = 0.377), pH (r = 0.572), and conductivity (r = 0.702). All above-mentioned parameters have strong correlations with the level of heterotrophic bacterial population in this river according to canonical correspondence analysis (CCA) analysis.

The downstream Umgeni River was found to be more microbially contaminated than upstream, as the river flows through the more urbanized areas of Durban. Higher nutrient concentrations and higher turbidity levels were also observed near Durban. TC and FC counts varied significantly (p<0.05) throughout all sampling points and ranged from 3.30 x 10^3 cfu/100 mℓ (U1 – winter) to 6.03 x 10^3 cfu/100 mℓ (U1 – summer) and 0.89 x 10^3 cfu/100 mℓ (U3 – winter) to 4.85 x 10^3 cfu/100 mℓ (U2 – spring), respectively. All points failed to meet the target water quality ranges of TC, FC, EC and FS levels for recreational and drinking uses according to DWAF and USEPA criteria with points U2 and U1 having the highest TC,

FC, and VC especially during the spring and summer seasons. *Salmonella* spp., *Shigella* spp. and *Vibrio cholerae* were also detected in all the water samples. Sampling site U5, which represented the upstream of the Umgeni River, had the lowest FC, VC and SHIG concentrations during all seasons. There was no significant difference in the TC, FC and VC counts ($p > 0.05$) between the seasons while EC and FS counts differed significantly ($p < 0.05$) over all seasons. Significant spearman's rho correlation coefficients were noted between the THB populations and most of other indicator organisms throughout all seasons except between FS populations with the other indicators as well as with the physico-chemical parameters as confirmed by the CCA analysis.

CCA ordination plot revealed strong correlations between the overall bacterial growths of different sites and seasons measured as well as the physico-chemical water quality variables. The temperature, BOD_5, turbidity, pH, electrical conductivity, orthophosphate and sulphate were the most prominent variables that correlate significantly with the microbial community structures at sites 1, 3 and 5 during the summer and spring seasons. The overall bacterial growths of site 2, 4, 5 during autumn and winter seasons strongly correlated with the nitrate/nitrite profiles. The results from the CCA analysis also suggest that correlations between bacterial community data and heavy metal variables exist. CCA analyses also demonstrate that the overall bacterial growths correlate with the environmental factors of different sites and vary seasonally.

In this study, the populations of somatic, F-specific coliphages and various enteric viruses were also monitored using various techniques. All sampling points throughout all seasons tested positive for the presence of somatic coliphages which ranged from 10 pfu/mℓ (U5 – autumn) to 659 pfu/mℓ (U1 – summer) especially in the lower reaches of the river and during the summer period. F-RNA coliphage counts were significantly lower compared to those of somatic coliphage counts in the literature. F-RNA coliphages from 0 pfu/mℓ (U5 – autumn) to 550 pfu/mℓ (U2 – summer) were also detected more frequently in the lower reaches of the river. Similarly, VLPs were detected using EFM at all sampling sites throughout all seasons, and increased in a way similar to coliphages, with point U1 during summer having the highest population of 2086 VLP/mℓ. Mean concentrations of somatic coliphages and F-RNA coliphages were comparable between the autumn and spring seasons and correlation analysis resulted in a strong Pearson's correlation coefficient of 0.977 ($p<0.01$). These coliphages had inverse correlations ($r = -0.536$) with the VLPs detected during autumn but correlated ($r = 0.795$) well with the VLPs found during spring. THB populations correlated ($r = 0.85$, $p< 0.05$) well with the somatic coliphages and VLP populations at all sites along the river and for both seasons. CCA ordination plots revealed strong relationships between

the overall viral populations at sites 1, 2 and 3 during the autumn, summer and spring seasons and the physico-chemical water quality variables such as temperature, BOD_5, turbidity, pH, conductivity, orthophosphate and sulphate that were the most important variables that were correlated significantly with the total bacterial community structures. The ordination plot also revealed that the Somatic phage, FRNA phage and VLPS were largely unrelated to one another. The results observed were probably due to different varieties of specific hosts involved, therefore strong relationships can only be observed between the THB communities and VLP and phage communities.

All water samples in this study contained a mixture of morphologically different tailed phage viruses, which were regarded as bacteriophages. Most of the detected phages had isometric heads and long non-contractile tails, belonging to morphotype B1 (Siphoviridae; 33%of the water samples) followed by Members of morphotypes A1 (Myoviridae; 25%), and C1 (Podoviridae; 20%). Other appendages are pertinent for the recognition of and interaction with the host cell. These accessories may be an indication that a significant proportion of the phages are suspended in the water environment as potentially infective particles.

Several presumptive virus types including Adenoviridae, Caliciviridae (Norovirus), Coronaviridae, Herpesviridae, Orthomyxoviridae (Influenza virus), Picornaviridae (Enterovirus), Poxviridae, and Reoviridae (Rotavirus) were found in the Umgeni River during all seasons based on the morphologies under TEM. The virus infectivity abilities of the above mentioned VLPs in the water samples were demonstrated using three different tissue culture assays. All the water samples from U1, U2 and U3 (except the autumn sample) were capable of inducing the cytopathic effects of all tissue cell lines. The water samples from U5 failed to produce CPE of Hep-G2 cell line except the summer sample. These results strongly indicate the potential of viruses in the water samples especially from the lower catchment areas to infect the human hosts throughout the year. These observations may have serious health care implications. Detection of these presumptive human enteric viruses from the water samples were further confirmed by PCR/RT-PCR assays using specific primers. Adenoviruses, enteroviruses, rotaviruses and Hepatitis B viruses were detected in all water samples in this study.

CONCLUSIONS

- .. This study serves as a reminder of the importance of well-functioning drinking water treatment plants. Furthermore, although river water is never managed to achieve drinking water quality, the results would also raise concerns for those who may consume water directly from the river without any form of treatment.

- .. The microbiological and physico-chemical qualities of the Umgeni River in Durban, South Africa did not meet the target water quality ranges of Total Coliforms (TC), Faecal Coliforms (FC), Enterococci (EC) and Faecal Streptococci (FS) levels for the recreational and drinking uses according to DWAF.

- .. The presumptive bacterial pathogens such as *Salmonella* spp., *Shigella* spp. and *Vibrio cholerae* were also detected in all the water samples. In addition, most of the water samples contained high populations of somatic coliphages, FRNA coliphages and many other virus-like particles (VLPs) and demonstrated their infectivity abilities of various human tissue cultures.

- .. The results of CCA analyses demonstrate that pH, electrical conductivity, temperature, and turbidity were correlated with the microbial community structures in the Umgeni River water.

- .. Many presumptive pathogenic enteric viruses such as adenoviruses, enteroviruses, rotaviruses and Hepatitis B viruses were detected in all water samples using PCR/RT-PCR. The above water samples demonstrated their infectivity abilities of various human tissue cultures.

- .. The present study highlights the importance of routine environmental surveillance of pathogenic bacteria and human enteric viruses. This can contribute to a better understanding of the actual burden of disease on those who might be using the water directly without treatment. The study also suggests a need to monitor the actual viruses present in addition to the traditional indicators.

- .. Since a “gold standard” for the detection of viruses in environmental samples is not yet defined, the incidence of viral contamination might be underestimated by the current methods.

RECOMMENDATIONS

- .. Statistical tools provide an objective interpretation of correlations between surface water quality variables, and should be incorporated into water quality monitoring systems more routinely. However, the implication of a cause and effect relationship for each correlation should not be taken for granted.

- .. Although not used in this study, it is recommended that the infectivity of viruses in river water using the Integrated Cell Culture Reverse Transcription-Polymerase Chain Reaction (ICC-RT-PCR) method be investigated. This approach apparently overcomes most of the disadvantages associated with both conventional cell culture and direct PCR assays, reducing the time needed for the detection of infectious viruses.

CAPACITY BUILDING

One PhD student participated in the current study. The study also establishes a platform in this University and the Province as a whole for more incoming students to be involved in similar studies.

KNOWLEDGE DISSEMINATION

The following publications will be prepared from the data obtained during the study:

- One doctoral thesis.
- Waterborne Human Pathogenic Viruses of Public Health Concern.
- Canononical correspondence analysis (CCA) for the microbiological, physico-chemical and heavy metal assessment of the Umgeni River -South Africa.
- Isolation and morphological characterization of virus-like particles (VLPs) from the Umgeni River and the cytopathogenic effect of these VLPs on tissue culture.
- Presence, temporal dynamics and spatial distribution of enteric viral contamination of the Umgeni River.

The results of the study were presented at the following conferences:

- .....Ganesh A. and J. Lin, Detection of human pathogenic viruses in the Umgeni river of Durban, South Africa, First Biotechnology World Congress, Dubai Men's College, Dubai, 14-15 February 2012.
- .....Ganesh A. and J. Lin, Detection of human pathogenic viruses in the Umgeni river of Durban, South Africa, 23rd congress of the South African Society of Biochemistry and Molecular Biology/ Federation of African Societies of Biochemistry and Molecular Biology (SASBMB-FASBMB 2012 congress), Champagne Sports Resort, Drakensberg, KZN, South Africa 29 January 2012-1 February 2012.
- .....Ganesh A. and J. Lin, Detection of human pathogenic viruses in the Umgeni river of Durban, South Africa, 2011 South African Society For Microbiology (SASM) Meeting, Southern Sun Cape Sun Hotel, Cape Town, South Africa, 6-9 November 2011.

Data collected during the study is available on the CD at the back of this report.

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- ....Umgeni Water Board for water samples at Krantzkloof Nature Reserve and Inanda Dam.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

A549	Human Lung Carcinoma Cell line,
ALT	Alanine Aminotransferase
APHA	American Public Health Associations
AstV(s)	Astrovirus(es)
BGMK	Buffalo Green Monkey Kidney cells
BOD_5	Biological Oxygen Demand
CCA	Canonical Correspondence Analysis
CDC	Centres for Disease Control and Prevention
COD	Chemical Oxygen Demand
CPE	Cytopathic Effect
CT	Cholerae Toxin
DWAF	Department of Water Affairs and Forestry
EC	Enterococci
EFM	Epifluorescence Microscopy
EIA	Enzyme Immunoassays
EM	Electron Microscopy
EV(s)	Enterovirus(es)
FC	Faecal Coliforms
FCM	Flow Cytometry
FIC	Frequency of Infected Cells
FS	Faecal Streptococci
FVIC	Frequency of Visibly Infected Cells
GE	Gastroenteritis
HAdV(s)	Human Adenovirus(es)
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCV	Hepatitis C virus
Hek293	Human embryonic kidney
HepG2	Human hepatocellular carcinoma
HEV	Hepatitis E virus
ICC-PCR	Integrated Cell Culture Polymerase Chain Reaction
ISO	International Organization for Standardization

MF	Membrane Filtration
MPN	Most Probable Numbers
NMMP	National Microbial Monitoring Programme
NoV(s)	Norovirus(es)
NTU(s)	Nephelometric Turbidity Unit(s)
ORF	Open Reading Frame
PAGE	Pulse-Field Gel Electrophoresis
PCR	Polymerase Chain Reaction
PEG	Poly Ethylene Glycol
pfu	Plaque-Forming Unit
PLC/PRF/5	Primary Liver Carcinoma cell
qPCR	quantitative PCR
RD	Human Rhabdomyocin sarcoma cells
RT	Reverse Transcriptase
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
RV(s)	Rotavirus(es)
SABS	South African Bureau of Standards
SAL	*Salmonella* spp.
SHIG	*Shigella* spp.
TC	Total coliforms
TCBS	Thiosulphate Citrate Bile salt Agar
TCP	Toxin-Coregulated Pilus
TEM	Transmission Electron Microscopy
TFF	Tangential-Flow Filtration
THB	Total Heterotroph Bacteria
TTV(s)	Torque teno virus(es)
USEPA	United States Environmental Protection Agency
USFDA	United States Food and Drug Administration
VC	*Vibrio cholera*
VK	Primary Vervet Monkey Kidney cells
VLP(s)	Virus-Like Particle(s)
WSPs	Water Safety Plans
WHO	World Health Organisation
WRC	Water Research Commission

1 LITERATURE REVIEW

1.1 Introduction

Microbial contamination of aquatic environments poses a potential public health risk when improperly managed (Alexander et al., 1992; Cheung et al., 1990). One of the largest issues regarding water use is pathogen transport. In spite of the advantage of technologies, it is still difficult to assess which pathogens can potentially be spread through the water supply (Salgot et al., 2001). At present, public water systems rely on bacterial indicators (i.e. coliforms) for monitoring water quality, and it has been shown that bacterial indicators are often poorly correlated with the presence of other microorganisms, such as protozoa and viruses, which can be found in various water sources including finished drinking water (Straub and Chandler, 2003). Viruses are the group of particular concern because they include highly stable pathogens that can be resistant to standard wastewater treatment processes.

In South Africa, 2.6% of all deaths are attributable to unsafe water supplies, inadequate sanitation facilities and hygiene, with significantly higher figures applying to children under five years of age and an associated treatment cost of R3.4 billion (Lewin et al., 2007; Wenhold and Faber, 2009). While the microbial safety of drinking and recreational waters has dominated the scientific and public health arena for over a century, the threat of human virus contamination to these waters has only been of interest over the past few decades. Current safety standards for determining water quality typically do not specify what level of viruses should be considered acceptable. This is in spite of the fact that viruses are generally more stable than common bacterial indicators in the environment (Okoh et al., 2010). The monitoring of water supplies and research on waterborne viruses in South Africa have been inadequate, and our understanding has been weighed down by the limited number of scientific outputs, lack of available and precise detection analyses, and imprudent suppositions with regard to virus viability, infectivity and pathogenesis (Grabow et al., 2004). Thus the key to understanding and monitoring water quality has led to the need for developing an effective and efficient method for the simultaneous collection and recovery of low levels of pathogenic human viruses that can then be rapidly identified and quantified (Craun et al., 2006).

1.2 Current Environmental Water Situation in South Africa

South Africa is opulently endowed with biodiversity, much of which lies outside of the approximately 6% of land area that falls within its protected area system (Payment et al., 1991; Turpie et al., 2008). As poverty and the demand for land for urban and agricultural use increase, habitats and therefore biodiversity are progressively more under threat. These

pressures are further exacerbated by climate change, particularly its impacts on water resources (Turpie et al., 2004). Conservation in South Africa has historically been perceived as a luxury and the concern of the wealthy, especially since almost all conservation efforts are focused on the protected areas, which tend to be geographic, economic, and socio-political enclaves (Turpie et al., 2008). Surface water is heavily committed for use with between 500 m^3 and 1000 m^3 of water available per person per year (Ashton, 2002). The current status of water quality in South Africa varies substantially, with the most contaminated water resources being the Vaal River, Crocodile West (Limpopo), Umgeni and Olifants River systems (Van der Merwe-Botha, 2009).

Like many countries in Africa, South Africa's water resources have been under increasing threat of pollution in recent years due to rapid demographic changes, which have coincided with the establishment of human settlements lacking appropriate sanitary infrastructure (Drechsel et al., 2006; Karikariay et al., 2007). This applies especially to peri-urban areas which surround the larger metropolitan towns in the country. In these areas no wastewater treatment is provided and raw sewage enters the rivers and streams directly (Mardon, 2003). Because of the lack of infrastructure in these settlements, the residents are often forced to inhabit river banks, and without adequate sanitation, this further contributes to the diminishing water quality. The lack of adequate potable water supply to many of these residents, forces them to become dependent on other water sources (Obi et al., 2002). People living in these areas, as well as downstream users, often utilize the contaminated surface water for crop irrigation, recreation as well as for domestic personal use such as washing, drinking and cooking without prior treatment, which creates a situation that poses a serious health risk to the people (Raschid-Sally et al., 2005; Verma and Srivastava, 1990). Hearings during the 2008 Parliamentary sitting reflected the lack of clarity and certainty surrounding water quality and water infrastructure in the country. With these aspects in mind and given the ever-increasing demands to prepare and plan for a water-secure future, one fact is clear – South Africans are becoming increasingly concerned about a water-secure future. Decision makers, investors and researchers share the view that the declining quality of water will have a negative impact on the South African economy, in both the short and the long term (Van der Merwe-Botha, 2009). As a result, water availability is predicted to be the single greatest and most urgent development constraint facing South Africa. The need for water is further highlighted by the fact that water scarcity in developing countries is closely linked to the prevalence of poverty, hunger, and disease (Ashton and Haasbroek, 2002, Falkenmark, 1994). While the country faces many challenges as a result of the limited and variable nature of its water resources (Figure 1.1), these challenges need not constrain sustainable growth and development, with the proviso that water management is sound.

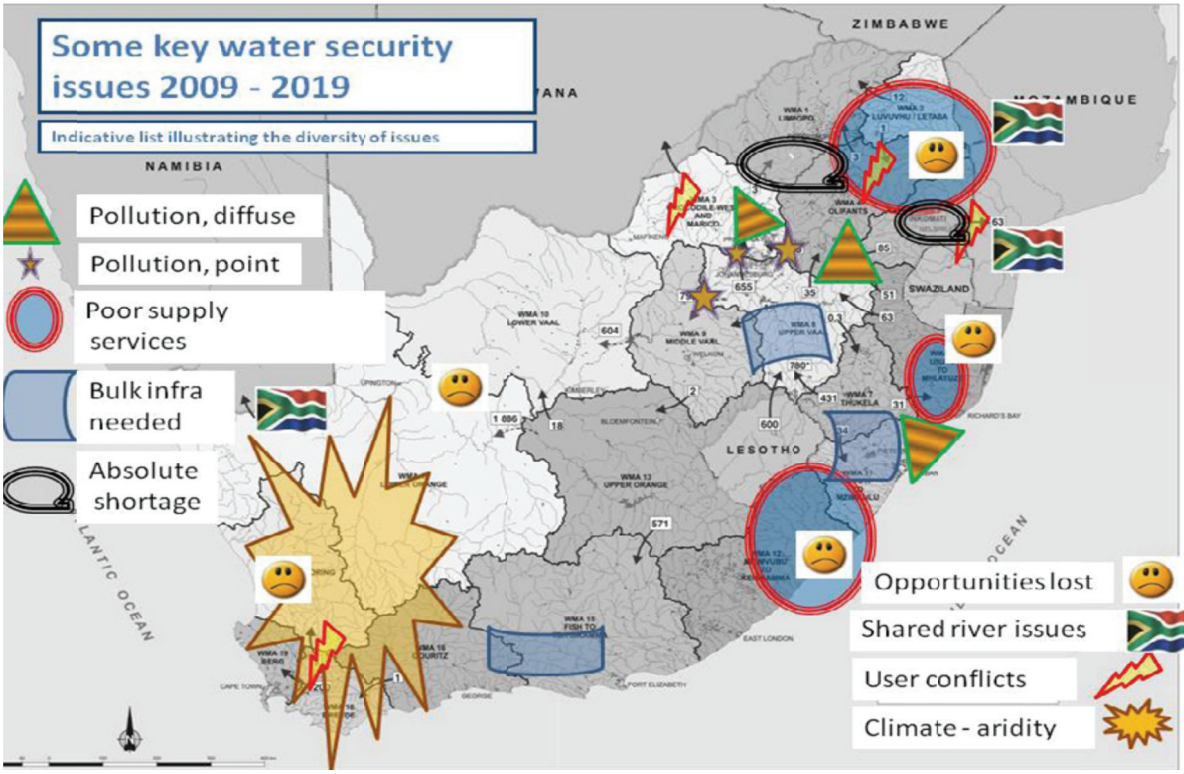

Figure 1.1 Water quality challenges and security issues facing South Africa (Muller et al. 2009).

As water demands increase, there are some concerns that need to be addressed to ensure protection of public health and the health of the environment (Levine and Asano, 2004; Rosario et al., 2009). Quality issues, predominantly associated with microorganisms, are of increasing importance as a result of environmental impacts on current water supplies and development of alternative water sources. Accurate and comprehensive assessment of microbial water quality is of paramount importance if both existing and new water sources are to be safely employed (Payment et al., 1991).

1.3 Surface Water Pollution: Sources, Indicator Organisms and Detection Methods

When referring to water quality, accessible water resources in South Africa are at times portrayed as being either “too little” (due to drought), “too much” (due to floods) or “too dirty” (due to pollution). More recently, the emphasis has shifted to water being “dirty” (Van Der Merwe-Botha, 2009). Water quality is imperative to assess the health of a watershed and to make crucial management decisions to control current and future pollution of receiving water bodies (Behbahaninia et al., 2009; Khadam and Kaluarachi, 2006). The information gained on water quality and pollution sources is important for the implementation of sustainable water-use management strategies (Nouri et al., 2008, 2009, 2011; Sarkar et al., 2007; Zhou et al., 2007).

Rivers have been utilized by mankind for hundreds of years to the extent that very few of them are now in their innate form (Chang, 2008; Masamba and Mazvimavi, 2008; Ngoye and Machiwa, 2004). With the development of urban and industrial areas, rivers have traditionally acted as conduits of pollution through legal and illegal practices (DWAF, 1996 b; Mardon, 2003). Through natural processes anything that is added to a river higher up in a catchment will eventually find its way to the coast and could lead to the pollution of the near shore region (DWAF, 2007). Water purification schemes deal with the treatment of industrial and urban wastewater (Mardon, 2003). These schemes process the wastewater and then need to dispose of the treated water – usually into rivers. The quality of discharged water is closely monitored under strict guidelines. Under normal conditions, although treated water is still polluted to a certain extent, the effect on the river is not detrimental. However problems arise when excessive rains and floods increase the volumes of untreated wastewaters beyond the capacity of the schemes. When this happens, untreated wastewater sometimes bypasses the treatment works and enters the river untreated.

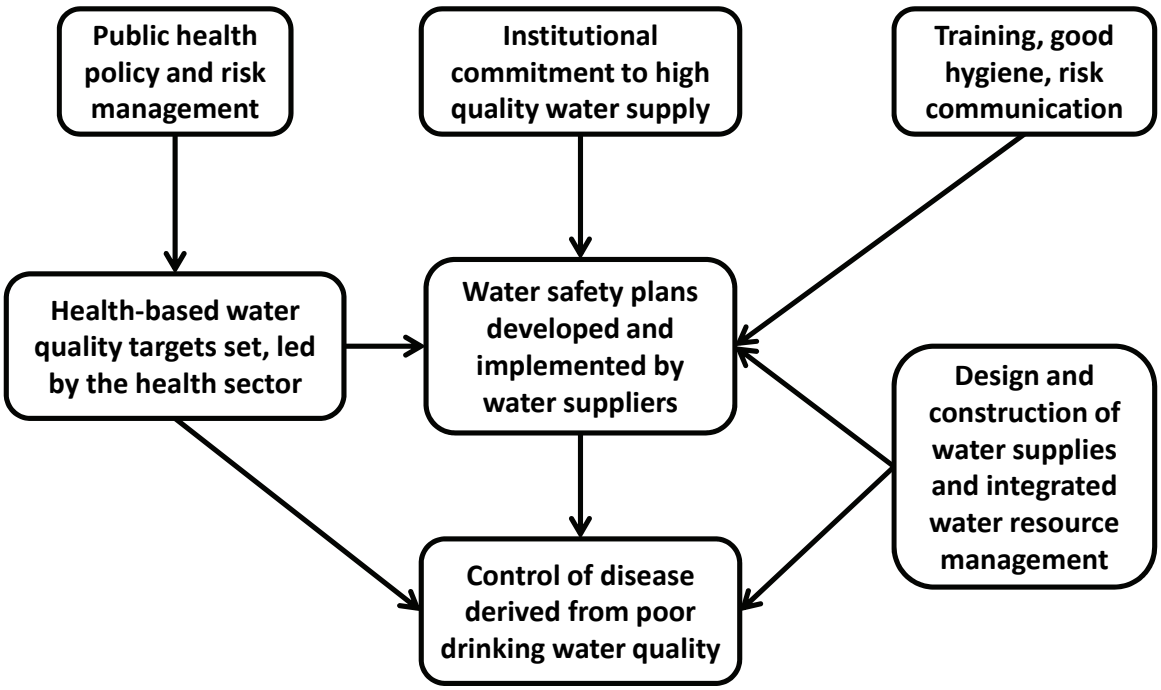

Figure 1.2 Protecting public health through ensuring drinking water quality (Davison et al., 2002)

The control of microbiological and chemical quality of drinking and recreational water requires the development of Water Safety Plans (WSPs) which, when put into practice, provide the basis for process control to make certain pathogen and chemical loads are acceptable (Deere et al., 2001). Implicit within this process is that a tolerable disease burden has been identified at national and local levels and that water quality targets have been established for each type of technology used (Davison et al., 2002). The delivery of 'safe' water, therefore, involves

actions by a variety of stakeholders who set water quality targets to improve public health, as illustrated in Figure 1.2. The Department of Water Affairs includes the sphere of risk management, operations and asset management into the Blue Drop Programme to improve the quality of drinking water (DWA, 2012).

1.3.1 Microbial Indicators and their Detection Methods

Water-borne pathogens transmit diseases to around 250 million people each year resulting in 10 to 20 million deaths around the globe (Wilkes et al., 2009; Zamxaka et al., 2004). The assessment of the microbiological quality of drinking water aspires to protect consumers from illness due to consumption of water that may contain pathogens such as bacteria, viruses and protozoa, and thus to thwart drinking-water related illnesses. An indicator of microbial water quality is generally something (not necessarily bacteria) which has entered the water system at the same time as faeces, but is easier to measure than the full range of microorganisms which pose the health risk. A useful water quality indicator should:

i. Be universally present in the faeces of humans and warm-blooded animals, in large numbers
ii. Be readily detected by simple methods
iii. Not grow in natural waters, the general environment or water distribution systems
iv. Be persistent in water and the degree to which it is removed by water treatment is comparable to those of waterborne pathogens (NHMRC-ARMCANZ, 2003; WHO, 1996).

The presence or absence of indicator organisms are key fundamentals of most drinking water quality guidelines, water supply operating licenses and agreements between bulk water suppliers and retail water companies (Colford et al., 2007). At present, the bacterial indicators used in water quality management and health risk assessments include total coliforms, faecal coliforms, *Esherichia coli*, faecal streptococci and enterococci, because they are much easier and less costly to detect and enumerate than the pathogens themselves (Meays et al., 2004). The South African water quality guidelines are centred solely on *E. coli* as being an indicator of pathogenic pollution and are subject to strict governmental regulations. Recreational waters intended for full and transitional contact are regulated by compliance to the following two limits enumerated for *E. coli*: (1) less than 20% of the samples can exceed 100 colony forming units per millilitre (cfu/ml), (2) less than 5% of the samples to exceed 2000 cfu/ml. These guidelines do not set restrictions for enterococcus concentrations nor is the sampling incidence specified (Mardon and Stretch, 2004).

None of the bacterial indicators currently used for monitoring meet all ideal criteria established for water quality (Ashbolt et al., 2001; Bitton, 2005; Stevens et al., 2003). The survival and incidence of bacterial viruses (phages) in water environments resembles that of human viruses more closely than most other indicators commonly used. The application of coliphages (bacteriophages which infect *E. coli* and certain related species) in water quality assessment is rapidly gaining ground (DWAF, 1996a; Grabow, 2001). Somatic coliphages occur in large numbers in sewage and polluted water environments and are easy to detect, but they may be replicated by host bacteria in certain water environments (Grabow, 2001). Male-specific (F-RNA) coliphages are highly specific for sewage pollution and cannot be replicated in water environments, but detection methods are more complicated (DWAF, 2004 a, b).

1.3.1.1 Total Coliforms and Faecal Coliforms

The notion of coliforms as bacterial indicators of microbial water quality is based on the hypothesis that because coliforms are present in elevated numbers in the faeces of humans and other warm-blooded animals, if faecal pollution has entered drinking water, it is probable that these bacteria will be present, even after significant dilution (Stevens et al., 2003). Total coliforms are typically described as “All facultative anaerobic, gram-negative, non-spore-forming, oxidase-negative, rod-shaped bacteria that ferment lactose to acid and gas within 48 hours at 35°C or members of Enterobacteriaceae which are β-galactosidase positive (APHA, 1998)”. The total coliform group of bacteria was originally used as a surrogate for *E. coli* which, in turn, was considered to demonstrate faecal pollution, until more specific and rapid methods became available (Kornacki and Johnson, 2001). With a few exceptions, the coliform group of bacteria themselves are not considered to be a health risk, but their presence indicates that faecal pollution may have occurred and pathogens might be present in the water environment as a result. Total coliforms signify only about 1% of the total population of bacteria in human faeces in concentrations of about 10^9 bacteria per gram (Brenner et al., 1982).

It is generally accepted that the total coliform group of bacteria is diverse and they can be considered typical inhabitants of many soil and water environments which have not been impacted by faecal pollution. Even though the presence of *E. coli* is considered a suitable and specific indicator of faecal pollution, ambiguity surrounds the use of total coliforms as a health indicator, as many authors have reported water-borne disease outbreaks in water meeting the coliform regulations (Gofti et al., 1999; MacKenzie et al., 1994; Ootsubo et al., 2003; Ottson and Stenstrom, 2003; Payment et al., 1991). Faecal coliforms have a survival pattern analogous to that of bacterial pathogens but their efficacy as indicators of protozoan or viral

contamination is limited. In addition studies have shown that *E. coli* is the only coliform almost exclusively associated with a faecal source (Tallon et al., 2005).

Detection of total and faecal coliforms in raw water can provide authorities with an indication of any changes in water quality (WHO, 1997). Classical methods for detection of total and faecal coliforms in natural waters include the Most Probable Numbers (MPN) and the Membrane Filtration (MF) techniques on selective agar (APHA, 1998). Although the tests are simple to perform, they are time-consuming, requiring 48 hours for the presumptive results and do not allow detection of all the target bacteria in natural environments.

1.3.1.2 Faecal Streptococci and Enterococci

The most commonly considered alternative indicator or adjunct to coliform bacteria is Faecal Streptococci (FS). They are regarded as suitable indicators of faecal pollution as they occur in relatively high numbers in human and other warm-blooded animals' excreta and are generally absent from natural environments having no contact with human and animal life. In addition, they persist without proliferation outside the animal host (Sinton et al., 1998). Early studies have indicated that faecal streptococci are more persistent than faecal coliforms in receiving waters and groundwater (Bitton, 2005; Evison, 1988; Evison and Tosti, 1980). The enterococcus group is a subgroup of faecal streptococci and has been used successfully as faecal pollution indicators as well as reliable as indicators of health risk in marine environments and recreational waters (Scott et al., 2002). This group of bacteria generally does not grow in the environment and have been shown to survive longer than faecal coliforms (Sinton et al., 1998). They also require greater amounts of cumulative solar radiation to be inactivated in seawater compared to faecal coliforms (Sinton et al., 1994; Sinton et al., 2002). However, it is possible that regrowth of environmental reservoirs of enterococci may occur once deposited into the environment (Desmarais et al., 2002).

Faecal streptococci/enterococci can be detected, using selective growth media in MPN or MF techniques. Media for the recovery and enumeration of faecal streptococci are usually based on their ability to grow in the presence of azide, and their fermentation of carbohydrates to produce lactic acid. Over 70 enumeration media have been proposed for faecal streptococci determination by the MF technique. Media for the recovery and enumeration of enterococci are usually based on the ability of the genus to hydrolyze the complex carbohydrate, esculin in the presence of high concentrations of bile salts. Rapid and simple methods, based on defined enzyme substrate technology, are available for the detection and enumeration of faecal streptococci and enterococci. These tests are based on the detection of the activity of two specific enzymes, pyroglutamyl aminopeptidase and β-D-glucosidase (Manafi and Sommer,

1993), using fluorogenic or chromogenic substrates incorporated into the selective media. Enterolert is a semi-automated 24-hour MPN test that is commercially available for the detection of enterococci, and is based, on the use of a methylumbelliferyl substrate (Budnick et al., 1996). The test has been used successfully to test fresh and marine waters for feacal indicator organisms (Budnick et al., 1996).

Three different approaches have been proposed for the use of faecal streptococci as indicators of the source(s) of faecal contamination, including, the faecal coliforms vs. faecal streptococci ratio (FC/FS); species identification and the FC/FS ratio shift (Cimenti et al., 2007). According to Geldreich and Kenner, (1969) a ratio of > 4.0 indicates human pollution and a ratio of ≤ 0.7 indicates animal pollution, therefore making it theoretically possible to ascribe a human or animal source to the faecal pollution based on the determined ratio. The assay is rapid, provides satisfactory results and requires minimal training to perform. However, the method is unable to accurately differentiate between human and animal sources due to differences in die-off rates between faecal coliforms and faecal streptococci as well as variability of the survival rates among faecal streptococcal species. Therefore, this ratio is no longer recommended as a viable approach to faecal source tracking unless very recent faecal pollution has occurred (Howell et al., 1995). Species identification is based on the ratio of enterococci to other streptococci in faeces among vertebrate species. Human faeces and sewage is predominantly composed of enterococci, whereas animal sources contain significant numbers of non-enterococci (Sinton et al., 1998). However, enterococci are also present in animals, and are more resistant to environmental stress than other faecal streptococci (Sinton et al., 1998) therefore; this approach to identifying sources of faecal pollution is generally regarded as unreliable (APHA, 1995). The ratio shift approach is used to determine the differential die-off coefficients of faecal coliforms and faecal streptococci in stored samples. Faecal streptococci and faecal coliform counts in receiving waters are generally correlated, although there is a shift in the ratio with time/distance from the faecal source due to unquantifiable factors (Sinton et al., 1994; Sinton and Donnison, 1994). This method requires further investigation as its application has produced variable results.

1.3.1.3 *Vibrio cholerae*

Vibrio cholerae, a motile Gram-negative curved rod shaped bacterium with a polar flagellum is the causative agent of cholera in humans (Faruque and Nair, 2008), especially in Africa. This bacterium is excreted in large numbers in the excreta of the victims suffering with cholera; is stale in an alkaline environment and can survive in environmental water bodies for several weeks at the very least (Farmer and Hickman-Brenner, 1992). The genus *Vibrio* includes more than 60 species, predominantly marine in origin, and its taxonomy is constantly restructured

due to the addition of new species. A number of *Vibrio* species, other than *V. cholerae*, may cause disease in man mainly by the ingestion of contaminated water.

Cholera has been regarded as endemic in South Africa, where between the years 2000 and 2003, 128 468 cases were reported, which resulted in 395 deaths in the country (Hemson and Dube, 2004). The major features of the pathogenesis of vibrios are well established. Infection due to vibriosis begins with the ingestion of contaminated water or food. The ability of *V. cholerae* to cause disease is reliant on several factors that allow the pathogen to inhabit the epithelium of the small intestine and produce the respective enterotoxins that interrupts ion transport. The expression of two virulence factors, the cholerae toxin (CT) which is a potent enterotoxin and a pilus-colonization factor known as the toxin-coregulated pilus (TCP) are also imperative for pathogenicity (Faruque and Mekalanos, 2003; Olaniran et al., 2011).

The detection of *Vibrio* species is based on the established phenotyping procedures as well as more recent molecular tools (Tamplin, 2001, Vandenberghe et al., 2003). Conventional culture-based methods involve a selective pre-enrichment of water samples, plating onto selective solid media by membrane filtration, followed by morphological, biochemical and serological characterization. Standard operating procedures optimized for the detection and identification of *V. cholerae* and enumeration of *V. parahaemolyticus* and *V. vulnificus* (Colwell and Huq, 1994; Tantillo et al., 2004) include an inoculation of the sample test portion into the selective enrichment medium APW (alkaline peptone water) and incubation at optimum temperatures, followed by streaking onto the selective solid medium thiosulphate citrate bile salt agar (TCBS), where yellow and green colonies are considered total presumptive *Vibrio* colonies. Molecular methods for the identification of *Vibrio* species have increased lately, especially the use of Polymerase Chain Reaction (PCR)-based techniques to amplify specific DNA sequences, as well as digestion of these fragments with restriction enzymes. Environmental factors such as pH, salinity, temperature, nutrients, and solar radiation are known to influence the survival and proliferation of *Vibrio* species directly by affecting their growth and death rates and indirectly through ecosystem interactions (Jiang and Fu, 2001).

The survival of contaminant *Vibrio* spp in water environments has been shown to decrease with elevated sunlight (Fujioka and Yoneyama, 2002; Hughes, 2003), high salinity (Sinton et al., 2002) and increased temperature. However, elevated nutrients and particle associations have been shown to promote the survival in water bodies.

1.3.1.4 Bacteriophages

Bacteriophages are viruses that infect bacteria and those that infect coliforms are known as coliphages. The survival and incidence of bacterial viruses (phages) in water environments resembles that of human viruses more closely than most other indicators commonly used. Phages are valuable prototypes for enteric viruses because they share many underlying properties and features. Among these are structure, composition, morphology, size and site of replication (Grabow, 2001). The application of coliphages (bacteriophages which infect *E. coli* and certain related species) in water quality assessment is rapidly gaining ground (Grabow, 2001; DWAF, 1996 a, b). Phage detection in environmental water samples consists of concentrating the sample, decontaminating the concentrate, and carrying out the phage (plaque) assay by the double or single-layer agar methods (Bitton, 2005). A wide range of bacterial host cells have been used as some are more efficient than others in hosting phages. Most data on the incidence of phages in water environments are on somatic coliphages. This is largely because somatic coliphages are detectable by simple, inexpensive and rapid techniques, and the phages occur in large numbers in any water environment exposed to human or animal excreta. Phages have proven to be largely valuable tools in research on viruses and have been projected as microbial indicators of water quality, as they share many fundamental properties with human enteric viruses which pose a health risk, if present in water contaminated with human faeces (Grabow, 2001).

1.3.1.5 Somatic Coliphages

Somatic coliphages belong to a variety of lytic phages that infect *E. coli* and closely related members of the bacterial family, *Enterobacteriacea.* They occur in large numbers in sewage and polluted water environments, are easy to detect, and they may be replicated by host bacteria in certain water environments (Grabow, 2001). Somatic coliphages have been found to outnumber F-RNA phages in waste water and raw water sources by a factor of about 5, and pathogenic human viruses by about 500 (Cimenti et al., 2007; Grabow et al., 2001), thus making them valuable indicators for assessing the behaviour of and the possible presence of enteric viruses in water environments like estuaries, seawater, freshwater, potable water, wastewater and bio-solids (Mocé-Llivina et al., 2003). Somatic coliphage counts in the faeces of man and animals may vary from less than 10 plaque-forming units (pfu)/g to 10^8 pfu/g, although in human faeces counts rarely exceed 10^3 pfu/g and may often be undetectable. Phages are often found in faeces of patients suffering from systemic diseases. Somatic coliphage counts in sewage range from 10^3 to 10^4 pfu/ml. In natural waters, coliphages may also be detected in high numbers, primarily due to pollution from sewage. Inactivation of coliphages is affected by similar conditions to those which determine inactivation of bacteria.

The most significant factors are temperature, suspended solids, biological activity and sunlight (Grabow, 2001).

The United States Environmental Protection Agency (USEPA) has proposed two methods (methods # 1601 and 1602) to detect somatic coliphages (host is *E. coli* ATCC 13706) in aquatic environments. Method 1601 (spot test) includes an overnight nutrient enrichment step of the water sample followed by "spotting" onto a host bacterial lawn. In method 1602 (double-overlay agar test), the water sample is supplemented with $MgCl_2$, host bacteria, and double-strength molten agar and the plaques are counted after overnight incubation (USEPA, 2001 a, b). Wild-type strains of *E. coli* are poor hosts for the detection of coliphages in wastewaters, as these strains have a complete O-antigen that conceals the mainstream phage receptor sites and their defence mechanisms which include nuclease enzymes that destroy phage nucleic acids recognized as foreign, thus preventing phage replication (Grabow, 2001). *Escherichia coli* strain C (ATCC 13706), also known as WG4, is a mutant in which the genes which code for these nuclease enzymes have been deleted. This strain of *E. coli* is susceptible to a broad range of coliphages and is the host most frequently used for detecting the presence of somatic coliphages in water environments (Grabow et al., 1998; ISO, 1998).

1.3.1.6 Male-Specific F-RNA Coliphages

F-RNA coliphages are ss-RNA phages which represent the simplest phages, include the families *Inoviridae* (F-DNA) and *Leviviridae* (F-RNA), and so represent a suitable model system for observing biological phenomena such as viral adsorption and penetration, replication and translation of the viral genome, assembly, and viral release (Grabow, 2001; Strauss and Sinsheimer, 1963). These phages infect *E. coli* (strain K12) cells, as the receptor sites for male-specific coliphages are located on the fertility fimbriae of this bacterium. These fimbriae carry the F plasmid, which codes for the F or sex pilus to which the F-RNA phage attach. The host-range of pilus-specific phages is not essentially limited to one or a few closely related species. Assembly of pili is typically encoded on the F (fertility) plasmid and the host-range of pilus-dependent phages depends mostly on the successful transfer and expression of the plasmid. Birge (1981) reported the successful transfer of the F-plasmid of *E. coli* K-12 to *Salmonella typhimurium,* as well as *Shigella* and *Proteus* species, causing these recipient cells to become susceptible to male-specific coliphages. F-RNA coliphages are classified into four serological types that are selectively excreted by humans or animals. Serogroups I and IV have to date been found solely in animal faeces and serogroup III phages in human faeces (Grabow, 2001). This phenomenon, offers an attractive tool to distinguish between faecal pollution of both human and animal origin (Cole et al., 2003).

Male-specific (F-RNA) coliphages are highly specific for sewage pollution and cannot be replicated in water environments, but detection methods are more complicated (DWAF, 2004 a, b). Grabow et al. (1998) found that F-RNA phages outnumber cytopathogenic enteric viruses by a factor of about 100 in wastewaters and raw water sources, implying that their absence from raw and treated water supplies offers a significant indication of the absence of human enteric viruses. Several studies have confirmed that the resistance of F-RNA coliphages to unfavourable environmental conditions and disinfection processes resembles or exceeds that of most human enteric viruses (Bitton, 2005; Grabow et al., 1998).

Detection of F-RNA coliphages by plaque assays is not simple as the F fimbriae are produced only by host bacteria in the logarithmic growth phase making preparation of the host cultures particularly difficult (Grabow, 2001). The USEPA has proposed the use of specific host cells such as *Salmonella typhimurium* strain WG49 or *E. coli* strain HS[pFamp]R to detect male-specific phages in aquatic environments. This highly modified strain of *S. typhimurium* is not susceptible to a large number of somatic coliphages in water environments which tend to interfere with the detection of F-RNA coliphages using *E. coli* hosts (Grabow et al., 1998; ISO, 1995). Once detected, the F-RNA phage can be additionally characterized as being a derivative of human or animal origin by immunological or genetic methods (Griffin et al., 2000; Hsu et al., 1995). In serotyping, group-specific antisera are used whereas in genotyping, hybridization with group specific oligonucleotides is used (Grabow, 2001; Sundram et al., 2006). The hybridisation assay involves plating the phage on a particular host, transferring the plaques to a nylon membrane, denaturing the phage to expose the nucleic acid, cross-linking the nucleic acid to the membrane, and then detecting group-specific nucleic acid sequences with ^{32}P- or digoxigenin-labelled oligonucleotide probes (Sundram et al., 2006). This technique is useful for identifying the four groups of F-RNA bacteriophages and therefore can be used in tracking sources of faecal pollution (Griffin et al., 2000).

1.3.2 Human Pathogenic Viruses as Potential Indicators of Water Quality

It is imperative to consider human enteric viruses in water quality studies not only because of their incidence as causal agents for diarrheal disease, but also due to their characteristics which allow them to survive in the environment for long periods of time, and tolerate changing environmental conditions (Espinosa et al., 2008; Skraber et al., 2004). Viral pathogens have been suggested as one of the most promising tools to determine the sources of faecal contaminants in aquatic environments and may be used in conjunction with bacterial indicators to assess water quality and improve public health surveillance (Fong and Lipp, 2005). However, previous studies of viral quality of coastal waters are mainly qualitative, rather than quantitative. Proper monitoring of human viruses in waters is of particular

importance, because the Centers for Disease Control and Prevention (CDC) suggest that the causative agent of nearly 50% of all acute gastrointestinal illnesses is suspected to be viral (CDC, 1988). With approximately 100 potentially water transmissible human viruses associated with human waste, it is simply impossible to detect all viruses (Berg, 1983; Jiang et al., 2001; Pina et al., 1998; Puig et al., 1994). Although it is not likely to establish a direct relationship between epidemiological and environmental data, it is imperative to consider microbial water quality in terms of water use (Espinosa et al., 2008). Furthermore, it is essential to evaluate the potential health risk to the exposed population, particularly in developing countries, considering that recycled water has been associated with the presence and re-emergence of waterborne diseases worldwide (Baggi et al., 2001).

Human Adenoviruses (HAdVs) have been proposed as a suitable index for the effective indication of viral contaminants of human origin (Bosch et al., 2008; Okoh et al., 2010). Studies conducted in Europe suggest that human adenovirus be used as an index of human viral pollution (Pina et al., 1998), since they are prevalent and very stable, they are considered human specific and are not detected in animal wastewaters or slaughterhouse sewage (Girones, 2006). HAdVs have been shown to frequently occur in raw water sources, treated drinking-water supplies urban rivers and polluted coastal waters (Castignolles et al., 1998; Chapron et al*.,* 2000; Jiang et al., 2001; Pina et al., 1998; Puig et al., 1994). HAdVs infections have been reported to occur worldwide throughout the year (Bofill-Mas et al., 2006; Flomenberg, 2005), suggesting that there are no seasonal variations in the prevalence of these viruses, thus qualifying these viruses as suitable indicators of human viral pathogens in aquatic environments. The incidence of HAdVs in such waters was surpassed only by the group of enteroviruses among viruses detectable by PCR based techniques (Chapron et al., 2000; Grabow et al., 2001). In view of their pervasiveness as enteric pathogens and detection in water, contaminated drinking and recreational-water represents a likely but unconfirmed source of HAdV infections (Grabow et al., 2001). HAdVs are also considered important because they are exceptionally resistant to some water treatment and disinfection processes, notably UV light irradiation. HAdVs have been detected in drinking-water supplies that met accepted specifications for treatment, disinfection and conventional indicator organisms (Chapron et al*.,* 2000; Grabow et al., 2001).

To this point a suitable index for the enteric viruses both in wastewater and drinking waters cannot be exclusively stated, because there are other proposed indices like Picobirnaviruses, Torque teno viruses (TTVs) (Griffin et al., 2008), and polyomavirus (Bofill-Mas et al.,2006) which show some degree of suitability as indices. However, one potential problem with the use of human viruses as indicators is that their abundance in wastewater depends on the

degree of infection and shedding in the human population at any given time. With the increasing popularity of molecular detection methods which are relatively fast and specific compared to the traditional methods, developing countries may find a solution to the problem of infectious viruses in aquatic environments if such techniques could be incorporated into part of regular monitoring programmes to assess the virus levels in wastewater effluents (Okoh et al., 2010).

1.4 Viral Studies in Freshwater Environments

Research in aquatic viral ecology has mainly focused on marine microbial ecology. It took only a few years before other environments such as lakes, rivers, sediments, as well as soils, to be studied from a virological perspective (Suttle, 2005, 2007). Viruses are essential members of aquatic ecosystems and appear to be not only the most abundant (Fuhrman, 1999) but also the most diverse biological entities (Angly et al., 2006).

In freshwater ecosystems, estimates of the abundance of viruses (or virus-like particles) have only recently begun to be documented (Wilhelm and Matteson, 2008). In part, this may be attributable to the development of more feasible approaches to enumerating total virus abundance by epifluorescence microscopy (Noble and Fuhrman, 1998; Wen et al., 2004). Some studies suggest that virus abundance in freshwaters tend to be higher relative to marine environments (DeBruyn et al., 2004; Filippini and Middelboe, 2007). In marine environments, the roles of planktonic viruses as regulators of carbon and nutrient cycling as well as microbial community structure have been a focus of numerous studies, yet the roles of freshwater virioplankton remain less studied (Suttle, 2007). Fluctuations in nutrient concentrations, temperature and community structure tend to be more significant and predictable factors (primarily due to strong seasonal cycles) in the detection of viruses in aquatic ecosystems (Farnell-Jackson and Ward, 2003; Lymer et al., 2008; Pradeep and Sime-Ngando, 2010; Sawstrom et al., 2009; Wilhelm and Matteson, 2008). Understanding the regulation and dynamics at various spatial and temporal scales is crucial to realise the viral ecology in the freshwater environment and their impact factors such as climate change.

In addition to the whole-community approach, studies in specific virus-host interactions are also important in further understanding the diversity, dynamics and regulation of viruses and host populations present in freshwater environments. There are many important gaps such as ecological consequences of viral genetic diversity and the influence of viral activity on host diversity. Additionally biogeochemical cycles still need to be explored to comprehend the importance in genetic and functional diversity in viral communities (Middelboe et al., 2008). Development of tools for analysing viruses in aquatic ecosystems is thus essential for

obtaining accurate measurements of their activity and for predicting the consequences of these activities (Miki et al., 2008).

1.5 Human Pathogenic Viruses Present in Environmental Waters

Viruses are omnipresent and extraordinarily abundant in the microbial ecosystems of water, soil, and sediment (Wommack et al., 1995). In nearly every reported case for aquatic and porous media environments (soils and sediments) viral abundance exceeds that of co-occurring host populations by 10 to 100 fold. If current estimates based on metagenomic DNA sequence data are correct, then viruses represent the largest reservoir of unknown genetic diversity on Earth (Wommack et al., 1995). Viruses are sub-microscopic inert particles of protein and nucleic acid which are unable to replicate or adapt to environmental conditions outside a living host (Van Heerden et al., 2004; Pusch et al., 2005). They vary in size from 10 to 300 nm and have a wide range of geometries including spherical, icosahedral, and rod shaped (Cann, 2003). The size, shape and other physiochemical properties of a particular virus determine its ability to survive and be transported in the subsurface of water environments (Yates et al., 1987). Possible health effects associated with the presence of such viruses in water include paralysis, meningitis, hepatitis, respiratory illness and diarrhoea (Gerba et al., 1996; Hewitt et al., 2007; Kukkula et al., 1997; Villena et al., 2003). Recreational exposure to polluted water has often been linked to viral infections (Vantarakis and Papapetropoulou, 1998). Viruses are shed in extremely high numbers in the faeces and vomit of infected individuals, and are routinely introduced into the environment (Figure 1.3) through the discharge of treated and untreated wastes, since current treatment practices are unable to provide virus-free wastewater effluents. Enteric viruses enter source waterways when treated and untreated human and animal wastes are directly or indirectly discharged into rivers, streams and estuaries (Grabow, 1991). Surface and ground waters are used for public consumption and have been implicated in waterborne outbreaks of gastroenteritis and hepatitis. In consequence viral pathogens in vomit and faeces of infected individuals contaminate the marine environment (a), fresh water (b) and ground water (c). Mankind is then exposed to these enteric viruses through various means: shellfish grown in polluted waters (d), contaminated drinking water (e) and food crops grown in land irrigated with sewage contaminated water and/or fertilised with sewage (f). Foods susceptible to be contaminated at the pre-harvest stage such as raspberries and strawberries (g) have also been implicated in outbreaks of viral diseases.

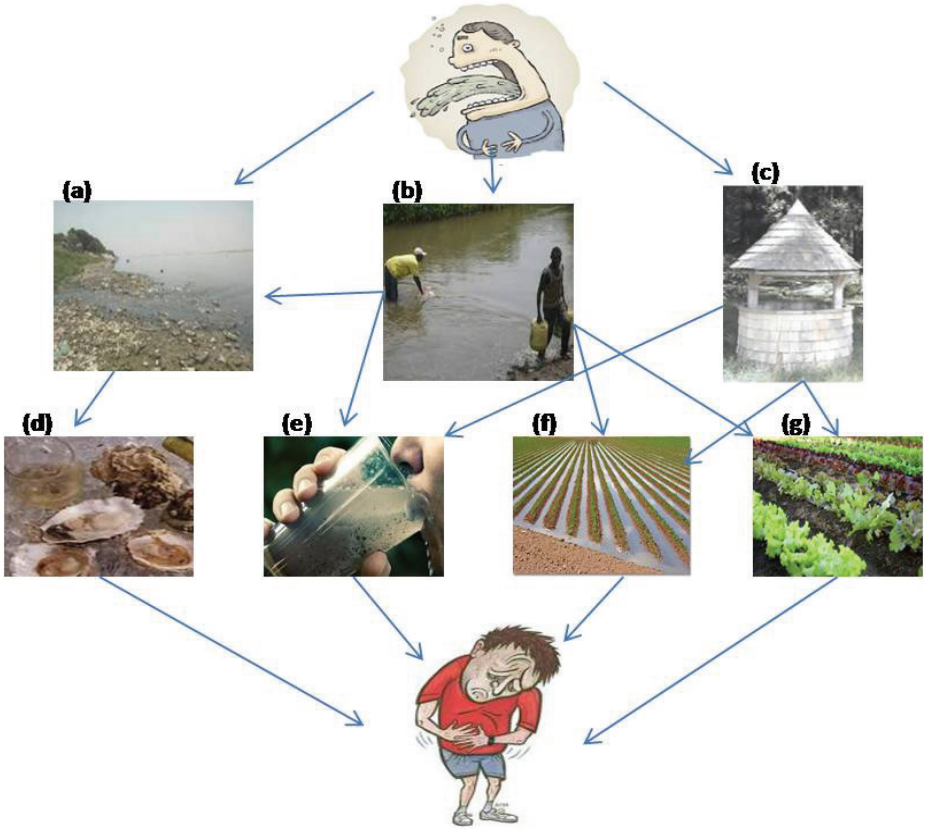

Figure 1.3 Probable routes of waterborne transmission of enteric viruses (Bosch et al., 2008)

Enteric viruses are excreted in faecal matter and may occur in recreational water as a result of storm water discharge, runoff, sewer overflows or sewage discharge. Quantitative data on the occurrence of enteric viruses in the environment are limited due to the complexity of virus recovery and detection methods. Viruses are excreted by infected individuals in numbers up to 10^{11}/g faeces (Fong and Lipp, 2005). The enteric viruses, which are shed in large numbers in the faeces of infected individuals, are stable in the environment and may survive wastewater treatment (Baggi and Peduzzi, 2000; Carter, 2005). These viruses can thus contaminate drinking water sources, recreational waters and irrigation waters thereby enabling viral transmission from person-to-person and surface-to-person to occur (Griffin et al., 2003). Since viruses cannot multiply outside a living host and are exceptionally resistant to unfavourable conditions, virus levels tend to decrease gradually after discharge into the aquatic environment and may be present in water for a long period (DWAF, 1995, 1996 a; Okoh et al., 2010). This implies that even at low levels of viral pollution, a meaningful risk of infection exists (DWAF, 2004 a). Drinking water, if ineffectively treated, can contain enteric viruses such as human adenoviruses and noroviruses derived from source water, and pose a health risk to people on consumption (Fong and Lipp, 2005; WHO, 1997).

The inherent resistance of enteric viruses to water disinfection processes means that they may likely be present in drinking water exposing consumers to the likelihood of infection (Okoh et al., 2010). Studies have revealed the presence of viruses in raw, surface and ground water and treated drinking water meeting quality standards for coliform bacteria (Cho et al., 2000; Gerba and Rose, 1990). Research carried out in Germany showed that even though microbiological parameters such as *E. coli,* enterococci and coliphages indicated acceptable microbiological water quality, the virological data suggested that surface waters might still be sources for enteric viral infections (Pusch et al., 2005). These studies also exposed that several disease outbreaks were caused by tap water contaminated with viruses in spite of compliance with water treatment procedures, an indication that viruses are being introduced into river waters and treated water (Pusch et al., 2005).

Human enteric viruses, which primarily infect and replicate in the gastrointestinal tract, have been associated with waterborne transmission (Carter, 2005; Grabow, 2007) and therefore have the potential to pollute surface (Pintó and Saiz, 2007), ground (Gerba, 2007) and drinking water (Carter, 2005). In one study, HAdVs were detected in about 22% of river water samples and about 6% of treated water samples in selected areas of South Africa excluding KwaZulu-Natal (Van Heerden et al., 2005). In another study, about 29% of river water samples and 19% of treated drinking water samples in South Africa had detectable levels of enteroviruses (Ehlers et al., 2005). Enteric viruses can cause illnesses in vulnerable individuals at low viral loads, where typically between 1 and 50 infectious viral particles is enough to cause illness (Griffin et al., 2000). Infections associated with rotavirus, in South Africa account for approximately 25% of all diarrhoeal hospital cases yearly, with 83% of infections occurring in infants less than 12 months of age (Steel et al., 2004).

Recently, more viruses, such as Aichi virus, Parechovirus, and human Bocavirus, have been considered as agents associated with diarrhoea in humans (Chow et al., 2010; Pham et al., 2007, 2010; Reuter et al., 2009). Studies also show that the virome is an important component of the environment that can interact with host genetic traits to contribute to the pathogenesis of complex diseases such as Type 1 Diabetes, inflammatory bowel disease and asthma (Foxman and Iwasaki, 2011). It can be said that the water environment (source, drinking and recreational water) thus poses a risk in the transmission of enteric viruses not only because there is doubt on acceptable virus levels but also due to the fact that enteric viruses are resistant to frequently employed disinfection methods (Dongdem et al., 2009).

1.6 Waterborne Human Pathogenic Viruses of Public Health Concern and their Associated Illnesses

Viruses, although the smallest and most numerous of all biotic agents, represent the planet's largest pool of genetic diversity and human pathogenicity (Rosario et al., 2009). All of the identified human pathogenic viruses that pose a significant public health risk in the water environment are transmitted via the faecal-oral route (Griffin et al., 2003). The significance of enteric viruses as causative agents of crucial human diseases cannot be overrated. These viruses belong primarily to the families Adenoviridae (adenovirus strains 40 and 41), Caliciviridae (Norwalk virus, astroviruses, caliciviruses, and small round structured viruses), Picornaviridae (poliovirus, coxsackieviruses, echoviruses, enteroviruses, and hepatitis A virus), and Reoviridae (reoviruses and rotaviruses). These enteric viruses are associated with a variety of diseases in humans, such as ocular and respiratory infections to gastroenteritis, hepatitis, myocarditis, and aseptic meningitis (Griffin et al., 2003).

Diarrheal diseases affect millions of people around the world and have the greatest impact on children, especially those in developing countries. Almost every child contracts a diarrhoeal disease during the first five years of their life, on an average several times per year (Bern and Glass, 1994). Diarrhoea can be caused by a number of different agents, including viruses, bacteria, parasites and toxins. However, during the past two decades, viruses have been firmly established as etiological agents of acute gastroenteritis (GE) (Bern and Glass, 1994). Diarrhoea is one of the leading causes of death in developing countries, responsible for 25-30% of deaths among children younger than five years of age (Martines et al., 1991; Snyder and Merson, 1982). In these countries the incidence of diarrhoeal cases varies between 2.5 and 3.9 episodes per child per year. In Africa, about 2.5 episodes per child per year are reported mainly among children between 6 and 11 months of age, corresponding to the introduction of weaning foods (Bern and Glass, 1994). Hepatitis, another contributor to waterborne disease, can be a seriously debilitating disease progressing from non-specific illnesses with fever, headache, nausea and malaise to vomiting, diarrhoea, abdominal pain and jaundice. Hepatitis A (HAV) represents globally approximately 50% of the total hepatitis cases and although the disease is self-limiting and rarely causing death, it may incapacitate patients for several months (Pinto and Saiz, 2007).

1.6.1 Adenovirus (AdV)

Human adenoviruses (HAdVs) are members of the genus Mastadenovirus in the Adenoviridae family, which comprises 51 serotypes classified in 6 species (A-F) (Okoh et al., 2010). They have double-stranded linear DNA and a non-enveloped icosahedral shell that has fibre-like projections from each of its 12 vertices (Stewart et al., 1993). Adenovirus serotypes, 40 and

41, are responsible for the majority of cases of adenovirus-associated gastroenteritis in children, which can be attributed to consumption of faecal contaminated water and food (Chapron et al., 2000; Dongdem, 2009; Okoh et al., 2010). Adenoviruses (subspecies B: Ad3, Ad7, and Ad21, species C: Ad1, Ad2, Ad5 and Ad6 and species E: Ad4) are responsible for 5-10% of childhood respiratory diseases and conjunctivitis (Wold and Horwitz, 2007). It is estimated that more than 90% of the human population is seropositive for one or more serotypes of adenoviruses (Fong et al., 2010).

HAdVs are excreted in large numbers in human faeces and are known to occur in sewage, raw water sources and treated drinking-water supplies worldwide (Chapron et al., 2000; Dongdem, 2009; Okoh et al., 2010). HAdVs are present at a higher frequency in sewage than are other enteric viruses (Pina et al., 1998) and are excreted in high concentrations from infected patients (up to 10^{11} viral particles per gram of faeces). Transmission routes of adenovirus infection include the faecal-oral route and inhalation of aerosols. Adenoviruses have been linked with respiratory outbreaks in various settings, including military camps (Chmielewicz et al., 2005; Kajon et al., 2007), hospitals (Hatherill et al., 2004), day care centres and schools (Fong et al., 2005)

Adenovirus identification is generally based on virus isolation in cell culture, followed by antibody or antigen detection, and visualisation by electron microscopy (Fong et al., 2005). In the past decade the progression of molecular technologies, especially the application of PCR methods, has enhanced the speed and sensitivity of adenovirus detection in water samples drastically (Ko et al., 2003; Van Heerden et al., 2003, 2005). HAdV have previously been detected in environmental samples by PCR-based techniques (Albinana-Gimenez et al., 2009 a, b; Bofill-Mas et al., 2006, 2010; Pina et al., 1998; Xagoraraki et al., 2007). Although quantitative real-time PCR (qPCR) methods for the quantification of some HAdV serotypes in diverse environmental samples worldwide have been recently described (Bofill-Mas et al. 2006; Choi and Jiang, 2005; Dong et al. 2010; Haramoto et al., 2004; He and Jiang, 2005; Jiang et al. 2005; Van Heerden et al. 2005; Xagoraraki et al., 2007). To our knowledge, quantitative data on the occurrence of HAdV in South African recreational waters is still in its infancy (Van Heerden et al., 2003, 2005). Adenoviruses, which have a high occurrence in water, have been recommended as candidates as indicator organisms for viral pathogens because they fit most criteria for an ideal indicator (Fong et al., 2005; Griffin et al., 2001; Katayama et al., 2008).

1.6.2 Enterovirus (EV)

The waterborne enteroviruses (EV) group fits into the *Picornaviridae* family, which consist of non-enveloped virus particles containing a 7,500-nucleotide single-stranded positive sense RNA genome protected by an icosahedral capsid (Nasri et al., 2007). EV sizes are very small ranging from 22-30 nm in diameter (Friedman-Huffman, 1998). Carriers of Enterovirus include raw sewage, sewage sediments, rivers receiving sewage, as well as treated sewage (Kocwa-Haluch, 2001). The sources of enteroviruses may be ground waters, river waters, coastal marine waters, aerosols emitted from sewage treatment plants and from solid waste landfills, soils and insufficiently treated drinking water (Kocwa-Haluch, 2001). Humans are the only known reservoir of enteroviruses. EVs survive in human faeces for a long time and through contact they contaminate hands, utensils, food, water, etc.

The migration of enteroviruses from the source to drinking water occurs along the following path: ill human→human faeces→sewage→wastewater treatment plant→river water receiving sewage →water intake→water treatment process →tap water (Melnick, 1976).

Enteroviral diseases occur most frequently in summer and early autumn. EVs include more than 70 distinct serotypes of human pathogens, and are known to be the main causative agent (>85%) of aseptic meningitis (Lee et al., 2004; Lee and Kim, 2002). Nonetheless, vaccinations do not exist for many serotypes, with the exception of the poliovirus, so the prevention of diseases caused by these viruses is very difficult (Lee and Kim, 2002). One of the most typical enterovirus diseases is poliomyelitis. It is almost invariably caused by one of the three poliovirus serotypes. Polioviruses may also cause aseptic meningitis or nonspecific minor illness (Hyypia et al., 1997). Coxsackieviruses, beside other illnesses, are most often connected with human heart diseases. There are no vaccines or antiviral drugs currently available for prevention or treatment of diseases caused by coxsackieviruses (Kocwa-Haluch, 2001).

The conventional diagnostic technique for enteroviruses is propagation in cell culture followed by neutralization to confirm the serotype, which is time-consuming (Kocwa-Haluch, 2001). Furthermore, several enteroviruses replicate poorly in cell cultures (Oberste et al., 2000; Wong et al., 1999). Molecular biology procedures, such as reverse transcription-polymerase chain reaction (RT-PCR), can be used for the sensitive, specific, and rapid (24 to 48 hours) detection of the enterovirus genome (Gantzer et al., 1998). Certain areas of the 5' non-coding region of the enterovirus genome are highly conserved among all serotypes. Primers binding to these areas can be used to amplify sequences common to most enteroviruses. Thus, the detection of the enterovirus genome by RT-PCR is a valuable alternative to cell culturing for

evaluating the virological status of the water environment (Gantzer et al., 1998). A multiplex real-time hybridization probe RT-PCR for detection of enterovirus 71 and Coxsackievirus A16 has been reported (Kocwa-Haluch, 2001). The results showed high specificity and sensitivity in detecting EV71 or CV-A16 from 67 clinical specimens, and no other enterovirus serotype was detected.

Enteroviruses are omnipresent in all types of water including tap water (Ehlers et al., 2005; Lee et al., 2005). They can survive drinking water treatment (Vivier et al., 2004) and have been detected in drinking water apparently free of coliform bacteria. Infections are most likely to occur during the summer and early fall coinciding with recreational activities (Fong and Lipp, 2005).76% of water samples at the river directly downstream of sewage treatment plant tested positive for enteroviruses in a German study (Pusch et al., 2005). A recent study in Morocco detected enteroviruses in only 2 of the 18 wastewater samples analysed (Hssaine et al., 2011). In South Africa, there are only few reports thus far of detecting enteroviruses (up to 16%) in treated and untreated water supplies (Ehlers et al., 2005; Vivier et al., 2004).

1.6.3 Hepatitis A Virus (HAV)

Hepatitis A virus (HAV) is endemic in South Africa with epidemiological features of both the developed and developing countries being present (Martin, 1992; Schwab et al., 1995). In high density, low socio-economic communities where sanitation is insufficient, nearly 100% of children acquire HAV immunity before the age of ten years (Martin et al., 1994; Taylor et al., 2001). Six types of hepatitis viruses have been identified (A, B, C, D, E and G), but only two types, hepatitis A (HAV) and hepatitis E (HEV), appear to be transmitted via the faecal-oral route and consequently linked with waterborne transmission (Hunter 1997; Taylor et al., 1995). Hepatitis A virus is a small (27 nm in diameter), icosahedral, non-enveloped, single-stranded, positive-sense RNA virus belonging to the family Picornaviridae (Hollinger and Emerson, 2007). The two biotypes of HAV, i.e. human HAV and simian HAV, are the only members of the genus Hepatovirus. There is only one serotype with infection conferring lifelong immunity (Hollinger and Emerson, 2007). A limited number of epidemiological studies have been applied to determine the risk posed by HAV infection after recreational exposure to contaminated surface water sources (Gammie and Wyn-Jones, 1997; Phillip et al., 1989).

Polluted drinking water has been implicated in outbreaks of hepatitis A (Hunter, 1997) and recreational exposure to faecally contaminated water has unequivocally been associated to outbreaks of HAV (Hunter, 1997; Mahoney et al., 1992), with the risk of infection increasing with increased immersion in contaminated water (Gammie and Wyn-Jones 1997, Taylor et al., 1995). HAV has also been found in surface river water and dam (impoundment) water used

for recreational, irrigational and domestic purposes in South Africa (Taylor et al., 2001). These water resources are used by the non-immune higher 'privileged' socio-economic communities for recreational activities while the predominantly immune lower 'poor' socio-economic population uses the same water for domestic, irrigation and recreational purposes (Gerba, 2000; Hunter 1997; Taylor et al., 2001). Hepatitis A virus is primarily spread by the faecal-oral route with person-to-person contact being the most significant route of infection. Maximal faecal excretion of HAV transpires two to three weeks prior to the onset of clinical symptoms (Zuckerman and Zuckerman, 1999) and remains infectious for three to four weeks after the alanine aminotransferase (ALT) levels peak (Polish et al., 1999), facilitating the spread of the virus. The infectious dose of HAV is unknown, and although Grabow (1997) suggested that one virion can cause infection, the infectious dose is presumed to be of the order of 10 to 100 virions (USFDA, 2004) which imply that even low levels of faecal pollution could pose a threat of infection.

HAV is not readily propagated in conventional cell cultures and the detection limit of routine diagnostic procedures such as electron microscopy (EM) and enzyme immunoassays (EIA) is about 10^5-10^6 viral particles ml^{-1} of test suspension (Glass et al., 1996; Nasser and Metcalfe, 1987; Taylor et al., 2001). The reverse transcriptase-polymerase chain reaction (RT-PCR) has successfully been applied for the detection of HAV in sludge and water samples (Deng et al., 1994; Graff et al., 1993; Taylor et al., 1997), and shellfish (Enriquez et al., 1992; Goswami et al., 1993), seawater (Myint et al., 1994) and environmental water samples (Marx et al., 1995, 1997, 1998). Data concerning the burden of HAV infection and disease in South Africa is limited, and consequently the contribution of treated and untreated drinking water, and recreational water to the burden of HAV infection in South Africa is unknown (Venter et al., 2007).

1.6.4 Norovirus (NoV)

Noroviruses (NoVs) are a group of non-cultivable, genetically diverse single-stranded RNA viruses which form the genus Norovirus within the Caliciviridae family (Green et al., 2001). These viruses are responsible for the majority of outbreaks of acute gastroenteritis in patients of all age groups in industrialized countries (Frankhauser et al., 2002; Glass et al., 2000; Mead et al., 1999). Genetically, NoVs are a highly diverse group of viruses and are separated into five genogroups (GI-GV) on the basis of sequence comparison of the RNA polymerase and capsid region of the genome (Pham et al., 2007) in which most of the members infecting humans are distributed in two genogroups (GI and GIV) (Green, 2001) and have about 19 genotypes in genogroup II (Vinjé et al., 2004).

Outbreaks of NoVs have been caused by contaminated food and/or drinking water, person-to-person virus transmission, and airborne droplets of infected vomitus (Ho et al., 1989; Koopmans et al., 2002; Laverick et al., 2004; McIver et al., 2001; Mead et al., 1999). Human noroviruses (HuNoVs) are shed in faeces of infected patients at a high concentration; thus the faecal-oral route via contaminated food or water is a main mode of its transmission (Green, 2001). Contaminated water poses a particularly serious health risk since results from human volunteer studies indicate that the minimum infectious dose of NoV may be as low as 10 to 100 PCR units (McIver et al., 2001). Waterborne outbreaks have been caused by contaminated surface water, ground water, drinking water, and mineral water (Abbaszadegan et al., 2003; Beuret et al., 2002; Kukkula et al., 1997; Schaub and Oshiro 2000). In waterborne outbreaks, a high proportion of the population can be affected, leading to several to hundreds of cases of gastroenteritis, followed by secondary spread and resulting in significant economic impact. NoV outbreaks are difficult to control and present a major public health challenge; thus, rapid diagnosis can be critical for the control of outbreaks.

Human noroviruses cannot be cultivated in traditional cell culture or in animal models (Duizer et al., 2004). However, progresses in molecular techniques in the last two decades have facilitated their detection in clinical and environmental samples (Atmar and Estes, 2001). Reverse transcription-PCR (RT-PCR) is currently the most extensively used assay for detection of NoVs in environmental water (Karim and LeChevallier, 2004). Furthermore, this method coupled with nucleotide sequencing techniques is able to assemble valuable information on the Norovirus genotypes occurring in the environment, thus providing epidemiological information of norovirus infections in the community. The RT-PCR primers that target the viral RdRp gene in open reading frame 1 (ORF1) or capsid gene in ORF2 have been designed to detect and genotype various norovirus strains (Kojima et al., 2002; Vinje and Koopmans, 1996; Vinje et al., 2004).

1.6.5 Rotavirus (RV)

Rotaviruses (RVs) are large (70 nm) non-enveloped icosahedral viruses that fit into the family Reoviridae (Weisberg, 2007). Rotavirus particles consist of a triple-layered protein capsid surrounding 11 segments of a double-stranded RNA genome (Dennehy, 2007). The RV genus has been divided into groups, subgroups and serotypes based on viral capsid proteins. Seven Groups [A-G] have been identified. Rotaviruses have been established as the main cause of acute gastroenteritis in young children worldwide (Kapikian and Chanock, 1996). An estimated 110,000 to 150,000 children younger than 5 years of age die annually on the African continent due to RV infection (Molbak et al., 2000; Parashar et al., 2003). Clinical symptoms of RV infection include diarrhoea, fever and vomiting. Rotaviruses have been estimated to cause

25-35% of all cases of severe diarrheal illness resulting in a significant economic impact on society in terms of direct medical costs, loss of working hours, quality of life and mortality (Glass et al., 1999, 2005). RVs have also been shown to be an imperative cause of sporadic (Wolfaardt et al., 1997) and epidemic (Sebata and Steele, 2001; Steele et al., 2004; Taylor et al., 1997) paediatric gastroenteritis in South Africa.

After replication in the gastrointestinal tract, RVs are excreted in high numbers in the faeces of infected individuals and may enter various sources of water (Santos et al., 1994), such as sewage (Baggi and Peduzzi, 2000; Dubois et al., 1997; Gajardo et al., 1995), river water (Baggi and Peduzzi, 2000; Gilgen et al., 1997), ground water (Abbaszadegan et al., 1999), and even treated drinking water (Gratacap-Cavallier et al., 2000). The stability of rotaviruses in environmental waters and their resistance to water treatment may facilitate transmission to humans via the faecal-oral route (Ansari et al., 1991; Raphael et al., 1985; Sattar et al., 1984). Group A RVs have been detected in untreated and treated drinking water samples in southern Africa (Van Zyl et al., 2004), but no data exists regarding the molecular epidemiology of RVs in contaminated water and food sources in this region. Frequently, the laboratory protocols used regularly to detect group A rotavirus, such as electron microscopy, enzyme immunoassay and PAGE, are not sensitive enough to detect the virus in concentrations represented by less than 1000 RNA molecules (Schwarz et al., 2002). Sensitive molecular assays and group- and type-specific reverse transcriptase PCR (RT-PCR) techniques have also been used to detect and genotype RVs from contaminated water sources (Hopkins et al., 1984; Van Zyl et al., 2006). In addition a number of RT-PCR protocols have been developed for the specific detection of rotaviruses of various species (Mori et al., 2001).

1.6.6 Astrovirus (AstV)

Astroviruses (AstVs) are small, non-enveloped icosahedral positive-sense, single-stranded RNA viruses, that are 28-30 nm in diameter with a smooth margin and a star-like EM appearance (Matsui and Greenberg, 1996). Seven serotypes of human astrovirus (HAstV) have been identified (Matsui and Greenberg, 1996). Outbreaks of astrovirus-associated gastroenteritis are being reported with increasing frequency (Chapron et al., 2000; Oishi et al., 1994). AstVs are transmitted via the faecal-oral route, and outbreaks have been linked with the consumption of sewage-polluted shellfish (Kurtzn et al., 1979), as well as the intake of water from streams polluted with faeces (Cubitt, 1991). The faecal-oral route is the predominant mode of transmission of AstV and has been established by numerous volunteer studies (Chapron et al., 2000, Glass et al., 1996; Taylor et al., 2001). In 1979, Kurtz and colleagues studied a filtrate from a child with mild gastroenteritis by electron microscopy and determined that it contained a large number of astrovirus particles. In a previous study 70% of

the environmental samples analysed from areas in South Africa were positive for human astroviruses (Marx et al., 1998). The prevalence of HAstV in South African patients with gastroenteritis was found to be between 5.1 and 7% (Marx et al., 1998; Steele et al., 1998). HAstV infections occur generally in young children and the elderly (Glass et al., 1996), although individuals of all age groups may be affected (Oishi et al., 1994). In one study HAstV was found to be the second most important virus associated with gastroenteritis in hospitalised patients next to rotavirus (Marx et al., 1998).

There is little data on the presence of HAstV in water sources used for domestic and recreational purposes in SA (Abad et al., 1997; Taylor et al., 1995). In temperate regions, most astrovirus infections are detected in the winter while in tropical climates, infections are noted in during the rainy season (Maldonado, 1996; Matsui and Greenberg, 1996). This temporal pattern of infection is similar to that of rotavirus infection (Maldonado, 1996). HAstV are not readily proliferated in conventional cell cultures (Matsui and Greenberg, 1996) and the detection limit of routine diagnostic procedures such as electron microscopy (EM) and enzyme immunoassays (EIA) is about 10^5-10^6 viral particles ml^{-1} of test suspension (Glass et al., 1996; Nasser and Metcalfe, 1987). Astroviruses have been detected in environmental samples by RT-PCR which has proven to be more sensitive than EM and EIA (Chapron et al., 2000; Le Cann et al., 2004; Pinto et al., 1996).

1.7 Methods for Isolating Viruses in Environmental Waters

When viruses are present in environmental waters they are often present in very low concentrations. A critical first step in the application of many virus isolation studies is obtaining a concentrate of viruses from large volumes of environmental water samples (Wommack et al., 1995). The studies undertaken to concentrate viruses used glass wool adsorption-elution filters and ultrafiltration as the tools to concentrate the viruses from different water sources before analyses (Wommack et al., 1995). Ultrafiltration is another method of concentration and makes use of size exclusion principles to concentrate water samples (Hill et al., 2007). Recent studies by Wommack et al. (2010) show that Tangential-flow filtration (TFF) procedures create high-density viral concentrates that are clear of contaminating cells and can directly feed a number of downstream analyses common to viral ecological investigations.

Suitable methods for concentrating viruses from water must fulfil a number of criteria (Wyn-Jones and Sellwood, 2001). The methods must:

i. be technically easy to complete in a short time;
ii. have a high virus recovery rate;
iii. concentrate a large range of viruses;

iv. provide a small volume of concentrate;
v. not be costly;
vi. be capable of processing large volumes of water; and
vii. be repeatable (within a laboratory) and reproducible (between laboratories) (Albinana-Gimenez et al., 2009)

1.7.1 Glass-Wool Adsorption-Elution Method

A number of approaches have been described for the recovery of viruses by techniques based on the filtration of test water through glass wool to which the phages/viruses adsorb, after which they are released from the glass wool into a small medium volume suitable for quantitative plaque assays or presence/absence testing (Lambertini et al., 2008). The principle involved in adsorption-elution methods is that viruses/phages carry a particular electrostatic charge that is primarily negative at or near neutral pH levels (Sobsey and Glass, 1980). This charge can be modified to be mainly positive by reducing the pH level to about 3.5. At this pH level viruses/phages will adsorb to negatively charged glass wool. After adsorption, a small volume of an organic solution at pH 9.5 or higher is passed through the glass wool to reverse the charge on the viruses/phages to negative. This results in the release of the viruses/phages and they can be detected by conventional and molecular methods. Filter media which carry a positive charge and hydrophobic binding sites at neutral pH levels, may be used to adsorb negatively-charged viruses/phages at neutral pH levels (Sobsey and Glass, 1980).

Regardless of the speed and efficiency of absorption-elution methods for the concentration of viruses, researchers that have focused on the ecology of autochthonous aquatic viral assemblages have not adopted these methods. The main reason is that since viruses can differ in biophysical characteristics, not all viruses are concentrated with equal efficiency through adsorption-elution (Williamson et al., 2003). All adsorption-elution techniques for viral concentration have focused on the detection of specific pathogenic viruses within freshwater, and to a lesser extent, seawater samples. Moreover, variation in the characteristics of a given water sample can influence viral recovery, and eluant buffers such as beef extract can be incompatible with downstream analyses such as molecular genetic assays and microscopy (Williamson et al., 2003).

1.7.2 Ultrafiltration and Tangential Flow Filtration

Ultrafiltration is based on the filtration of water samples through membranes of polysulphonate or related material with a nominal molecular weight cut-off limit of about 10 000 Daltons to 100 000 kiloDaltons. It is a sampling technique that is receiving increased attention as a

method for simultaneously recovering diverse microbes, including bacteria and viruses from various water samples (Grabow et al., 2001). An ultra-filter membrane is able to capture microbes and molecules above a certain molecular weight in the filter retentate while small molecules (e.g., water) pass through the fibre pores (Polaczyk et al., 2008). Filter systems include spiral wound and sheet membranes, against which the water is kept in motion by means of a recirculating pump. These yields close to 100% recovery (Grabow et al., 2001). However, the primary technical challenge in concentrating sub-micron particles from large volume aqueous samples is the prevention of filter clogging. While a number of approaches have been developed to avoid clogging of ultrafiltration membranes, the most widely adopted has been tangential-flow filtration (TFF) systems that consist of units in which filtration is enhanced by tangential flow through hollow fibres with a large total filtration surface area (Simmons et al., 2001). In water analysis procedures, filtration is performed in a tangential (i.e., cross-flow) mode where a sample is recirculated until the preferred concentration factor is attained.

TFF has been used to isolate viral particles from a variety of environments (Bench, et al., 2007; Schoenfeld et al., 2008, Thurber et al., 2009). Particles smaller than the filter pore sizes are pushed out through the filters. A backpressure is then used to force the filtrate through the holes on the filter surface. The remaining sample (retentate) is subsequently collected into a reservoir basin and repeatedly cycled through the filters. Recirculation therefore concentrates the large sample volumes. Currently a setup for the concentration of viruses from large volume water samples using a two-step tangential flow filtration process according to the method of Wommack et al. (2010) has been established in our laboratory as illustrated in Figure 1.4. Briefly 20 litres of water is filtered through a 25 µm sediment cartridge filter facilitated by a peristaltic pump to remove large particles (algae, sand and debris etc.). These pre-filtered samples are then pumped through a Millipore Pellicon Tangential Flow Filtration (TFF) cartridge filter to remove all bacteria/ contaminating cells greater than 0.22 µm in size. Virus-like particles in the 0.22 µm filtrate are then concentrated to a final volume of 500 ml using 100-kDa spiral-wound TFF filter.

Tangential-flow filtration procedures thus create high-density viral concentrates that are clear of contaminating microbial cells and particles larger than 0.22 µm. These concentrates can directly feed a number of downstream analyses common to viral ecological investigations such as isolation of new viral-host systems (Suttle, 1994) and assessing the impact of increased viral predation on host physiology (Suttle et al., 1990); detection of gene targets by PCR (Wang and Chen 2004; Zhong et al., 2002); characterization of whole virioplankton assemblages by randomly amplified polymorphic DNA-PCR (Winget and Wommack 2008) or

PFGE profiling (Wommack and Colwell, 2000). Beneficial features of this filtration approach are, the large surface area that allows large volumes of filtrate to pass through rapidly and the tangential flow that prevents clogging of the system (e.g., as with an impact filter) (Kuwabara and Harvey, 1990; Ludwig and Oshaughnessey, 1989).

The tangential flow filtration (TFF) system has significant advantages over other procedures currently used to concentrate viruses, since:

i. It does not depend on virus adsorption, and as a result it minimises virus loss resulting from antagonism for adsorption sites;
ii. It is not based on the net charge of the viral particles, thus eliminating the need for acidifying or adding polycationic salts; and
iii. It avoids the elution process (Alonso et al., 1999, 2004; Haramoto et al., 2004).

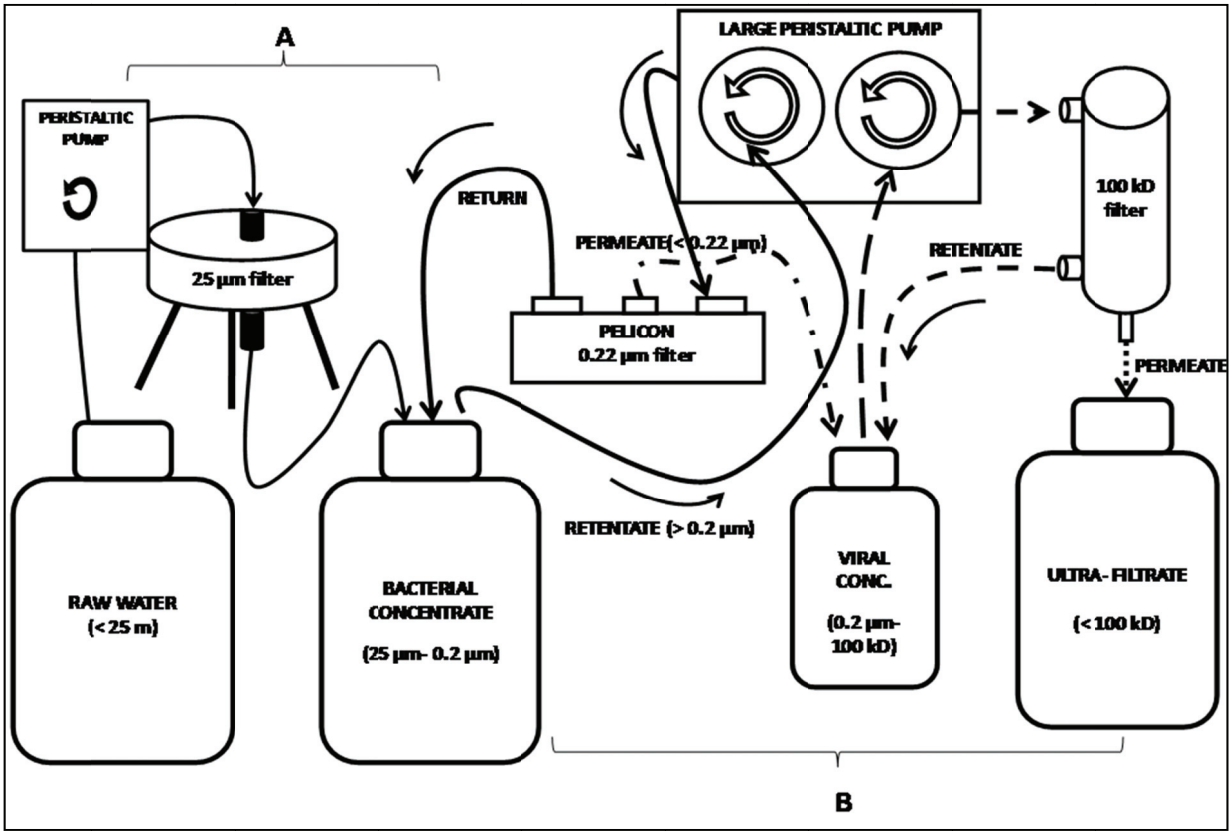

Figure 1.4 Experimental set-up for tangential flow filtration (TFF) of viruses from large volume water samples. Panel A – Pre filtration of water sample, Panel B – TFF for virus concentration.

Tangential flow filtration has been the most outstanding method used to concentrate viruses from natural waters because it reduces filter clogging and allows concentration of viruses from the large volumes (hundreds of litres) of sample that are often essential for genomic and metagenomic analyses of aquatic viral populations (Rosario et al., 2009; Wommack et al.,

2010). TFF requires expensive equipment compared to the glass-wool concentration method and several hours of processing time (Schoenfeld et al., 2008), depending on factors such as sample composition, type of TFF used, the amount of backpressure used and the operator's skill in using sample recovery techniques for back flushing of the ultrafiltration membrane.

1.7.3 Organic Flocculation

In organic flocculation, buffered beef extract is used to precipitate viruses from concentrated alkaline samples by reducing the pH to 3.5. The precipitate is then centrifuged to form a pellet before being dissolved in sodium phosphate (USEPA, 1996). In the Poly Ethylene Glycol (PEG)/NaCl precipitation procedure viral particles are precipitated from solution by addition of 0.5 M NaCl and 7% PEG 6000 to beef extract with constant stirring for 2 hours and overnight incubation at 4°C followed by centrifugation of the precipitate. The virus pellet is then resuspended in Tris-buffered saline and stored at -20°C (Enriquez and Gerba, 1995). The use of beef extract in these procedures has been reported to cause inhibitory effects in PCR assays (Arnal et al., 1999; Schwab et al., 1995).

1.8 Detecting the Presence of Viruses in Environmental Waters

The identification of a virus typically requires the application of a number of methods including physical, biological, serological and molecular methods (He and Jiang, 2005; Kreuze et al., 2009; Lee and Kim, 2002; Polaczyk et al., 2008). As a result of the low concentration of human pathogenic viruses in drinking and recreational water, it is essential to concentrate large sample volumes before detection is possible (Wommack et al., 2010). This is done using filtration techniques, flocculation or affinity chromatography and is generally associated with virus loss and at times inactivation due to the treatment (Vital et al., 2007). Thereafter the most frequent technique for viral detection after concentration is to grow viruses on susceptible cell cultures and subsequent analysis of the plaques and cytopathic effect, which are formed on the cell monolayer. Many investigations on viruses in aquatic ecosystems have been demonstrated by enumeration of these virus particles by electron microscopy. Microscopy and other molecular genetic tools have been critical in demonstrating that viruses are a dynamic component of microbial ecosystems, capable of significantly influencing the productivity and population biology of their host communities (Grabow, 2001; Okoh et al, 2010; Rosario et al., 2009).

1.8.1 Cell-Culture

Traditionally, the detection of enteric viruses in water samples had been conducted using cell culture techniques (Melnick, 1976). Cell culture can detect unsuspected viruses as well as the target viruses, whereby several different cell lines are inoculated with each environmental sample in an attempt to provide a suitable host for whichever virus might be present in that sample (Lee and Jeong, 2004; Leland and Ginocchio, 2007). Lee and Jeong (2004) found that cell culture assays demonstrated higher viral counts when compared to integrated cell culture polymerase chain reaction (ICC-PCR) despite the increased sensitivity of the ICC-PCR. After investigation, it was found that some of the cytopathic effect (CPE) in the cell culture assays was caused by reovirus not the suspected enterovirus or adenovirus. The common viral pathogens, such as the AdVs, and many of the enteroviruses, can be detected and isolated in traditional cell culture techniques.

The presence/absence and viability of viruses can be demonstrated where cytopathic effect (CPE) is observed. CPE is when the host cells are damaged or killed by the infecting virus which can be viewed under a microscope (APHA, 1998; Madigan and Martinko, 2006). Cell culture is the only technique that can assess the viability of pathogenic viruses. The number of viruses required for detection by cell culture is approximately 1-10 viral particles per gram of sample (Koopmans and Duizer, 2004) making this method suitable for environmental samples. Amongst the cell lines that can be used for human virus investigations in water are, A549 (a human lung carcinoma cell line), BGMK (buffalo green monkey kidney cells), Hek293 (human embryonic kidney), HepG2 (human hepatocellular carcinoma), PLC/PRF/5 (primary liver carcinoma cell), RD (human rhabdomyocin sarcoma cells), VK (primary vervet monkey kidney cells). The A549 human lung carcinoma cell line is currently the most commonly used cell line for adenovirus propagation and plaque titration while the Hek293 human kidney embryonic cells are mostly used as a package cell line for production of non-replicative adenovirus vectors. Unfortunately not all viruses are able to grow in culture, and with the enormous genetic diversity of virus species and the newly discovered human pathogens, many viruses cannot be isolated by the tissue culture methods.

Plaque assays are routinely available for adenoviruses, enteroviruses, rotaviruses and astroviruses (Koopmans and Duizer 2004). In this method host cells are grown in a monolayer, and the viral sample is mixed with an agarose solution and poured over this cell monolayer. Zones of clearing (plaques) are formed after incubation and these are assumed to be viral infections. The quantal method is another variation where a dilution series of the concentrated viral sample is added to the host cells which follows a most probable number

set-up. The most probable number of infectious 33-34 units (MPNIU) is calculated after the 14 day incubation period (APHA, 1998; Koopmans and Duizer 2004).

Some concerns associated with cell culture include:

i.... high running costs, up to 14 days for results,
ii.... appropriate cell lines are not available for all enteric viruses (e.g. Noroviruses),
iii.... no CPE is observed for some viruses (e.g. adenoviruses especially type 40 and 41) (Lee and Kim 2002; Van Heerden et al., 2003) and Hepatitis
iv.... the virus is slow growing (adenovirus) (APHA 1998; Greening et al., 2001; Koopmans and Duizer, 2004)
v.... some cell lines are selective of virus types (e.g. BGMK cells seem to select for enteroviruses (Lee and Kim, 2002).

Thus to overcome these issues other detection methods are often utilised in conjunction with cell culture such as PCR and more than one cell-line is often employed to ensure the study detects the maximum number of viral strains present in a sample (APHA 1998; Lee and Kim 2002).

1.8.2 Electron Microscopy

Before the dawn of electron microscopy, viruses were only identified by their ability to induce disease in susceptible hosts (Vale et al., 2010). Due to their relatively small sizes, viruses cannot be imaged with a conventional light microscope, and their particulate nature is apparent only indirectly through filtration and ultracentrifugation experiments (Kruger et al., 2000). This actuality set the stage for the rapidly mounting interest in viruses as soon as the electron microscope was invented (Ruska, 1987). Virus identification by electron microscopy has been established on the visualization and morphological identification of virus particles in samples of diseased tissues or organic fluids (Biel and Gelderblom 1999; Curry et al., 2006; Goldsmith and Miller, 2009; Vale et al., 2010). The acknowledgment that viruses are profuse in natural waters and have major effects on the mortality of heterotrophic and autotrophic microbial communities (Fuhrman, 1999; Suttle, 1994; Wommack and Colwell, 2000), has provided the impetus to develop protocols to rapidly and accurately enumerate viral particles in cultures and natural samples (Wen et al., 2004).

Direct counts provide the most basic information to assess the abundance and distribution of viruses in ecosystems. The total abundances of virus particles can be determined by transmission electron microscopy (TEM) (Maranger and Bird, 1996; Paul et al., 1993),

epifluorescence microscopy (EFM) (Drake et al., 1998; Noble and Fuhrman, 1998; Suttle et al., 1990), flow cytometry (Abad et al., 1998; Duhamel and Jacquet, 2006) and confocal laser scanning microscopy (Luef and Peduzzi, 2009). TEM has been the traditional method for viral particle counting in environmental samples in the past since it provided data on both the abundance and morphology of viruses (Wen et al., 2004). Viral counts with EFM have proven to be more reliable than TEM counts which underestimates numbers by 1 order of magnitude (Bettarel et al., 2000, Chen et al., 2001; Hennes and Suttle, 1995; Noble and Fuhrman, 1998; Weinbauer and Suttle 1997, Suttle, 2007), and have the advantage of being inexpensive and not as time-consuming as TEM. However, the drawback of using EFM alone is that it cannot describe the morphological viral diversity or the frequency of infected tissue cells as TEM does.

1.8.2.1 Epifluorescence Microscopy (EFM)

Epifluorescence Microscopy has been used to enumerate viruses in aquatic environments since the early 1990s. Virus-like particles are counted in EFM by the use of fluorescent nucleic acid stains such as DAPI, YoPro but more commonly used are SYBR Green I and SYBR Gold due to their higher sensitivity. EFM typically provides more accurate estimates (i.e. lower coefficients of variation among replicate counts) and greater counting efficiency when compared to the TEM method. At present SYBR Green I and II and SYBR Gold are most commonly used (Chen et al., 2001; Noble and Fuhrman, 1998; Middelboe et al., 2006) due to being suitable for aquatic samples within a wide range of salinity, low background staining, high stability and high emission intensity (Danovaro et al., 2001; Noble and Fuhrman, 1998). SYBR Gold is a sensitive fluorescence stain that is used to detect both double- and single-stranded DNA (ssDNA) and RNA, whereas SYBR Green I yields greatest absorbance with double-stranded DNA (dsDNA) (Fischer et al., 2005), whilst SYBR Green II gives the brightest fluorescence with RNA and ssDNA. Extracellular DNA is known to sometimes interfere with viral counts; however this effect can be circumvented by treating the samples with nucleases (Danovaro et al., 2001). In an attempt to decrease sample-processing time, the use of flow cytometers has been proposed for detecting and quantifying virus-like particles and prokaryotes (Brussaard, 2004; Marie et al., 1999).

1.8.2.2 Transmission Electron Microscopy (TEM)

Transmission electron microscopy of aquatic viruses can be performed in two ways. Firstly the sample can be pre-filtered to concentrate (ultrafiltration) the viral particles or the viral particles can be harvested directly onto TEM specimen grids by ultracentrifugation (Suttle et al., 1990). The grids with the viral sample are then negatively stained with a heavy metal salt (uranyl

acetate, phosphor-tungstic acid), to enhance the contrast on the image, and then viewed in TEM. The heavy metal salt provides a backdrop to the viral particles in the sample, thus providing an outline of the shape of the particles and also some idea of the internal structures. TEM viral morphology includes virus-like-particles, i.e. electron-dense particles with a hexagonal to round shape and a diameter of 30-200 nm. TEM can also establish the frequency of visibly infected cells (FVIC) (the number of cells containing viruses). FVIC can then be converted to the total frequency of infected cells (FIC) within the bacterial community or cell line using conversion factors (Danovaro et al., 2001). Virally induced mortality can then be calculated from the FIC values by means of additional conversion factors (Danovaro et al., 2001; Middelboe et al., 2003). Overall, TEM has proven to be a very successful tool in viral ecology morphology and will continue to play a crucial role in determining the presence of viruses in aquatic systems.

1.8.3 Flow Cytometry (FCM)

The flow cytometer is a recognised tool for clinical laboratory practice, and is fast becoming prevalent in the field of environmental microbiology. Flow cytometry allows for exceptionally swift measurements of single cells, primarily by optical means (Brussaard et al., 2000). FCM uses the principles of light scattering, and emission of fluorochrome molecules to generate specific multi-parameter data from particles and cells. Cells are characterised by light scattering based on their size, shape and density and also on the dyes that are used either independently or bound to specific antibodies or oligonucleotides that endow a fluorescent phenotype onto components of interest. As a particle flows through the beam, both light scattered by the particle and fluorescence light from the labelled particle is collected. This makes it possible to make multiple simultaneous measurements (up to six parameters) on a particle. This rapid multi-parameter evaluation of individual cells has thus made flow cytometric detection an invaluable tool for both qualitative and quantitative data analyses**.** The improvement of sensitive nucleic acid stains, in combination with flow cytometric techniques has enabled the identification and enumeration of minute particles such as viruses in aquatic systems (Brussaard et al., 2000, 2001; Chen et al., 2001; Larsen et al., 2001, Marie et al., 1999).

FCM has been shown to be capable of enumerating viruses in water by treating the samples with deep freezing or heating at 80°C in the presence of a detergent to obtain reliable results with SYBR Green I (Marie et al., 1999). FCM not only allows rapid and automated counts but also provides enough resolving power to separate different viral populations in the natural samples (Chen et al., 2001). SYBR Gold is another sensitive fluorescent stain for detecting double- or single stranded DNA or RNA nucleic acids in gels. According to the study by Chen

et al. (2001), marine viruses stained with SYBR Gold yielded a bright fluorescent signal that was much more stable than that from SYBR Green I. They established that the fluorescent signal strength of SYBR Gold stained viruses was about twice that of SYBR Green I-stained viruses, and that SYBR Gold formed at least four distinct viral subpopulations in flow cytometric signatures. In addition they illustrated that viral counts based FCM proved to be more efficient and accurate for assessing the numbers of viral particles in natural environments than direct counting by EFM (Chen et al., 2001).

1.8.4 Polymerase Chain Reaction (PCR)

Since the late 1980s, PCR has been used for the detection and quantification of viral pathogens in the environment (Saiki et al., 1985). The PCR method can be used to enzymatically amplify nucleic acid sequences that are present in low copy numbers in water samples. The analysis of viral diversity and community structure is difficult in all natural environments. Transmission Electron Microscopy that can distinguish viruses by morphotypes (Middelboe et al., 2003) offers very limited resolution. Thus the speed, specificity, low-cost and ease of this PCR procedure have led to its use in the detection of enteric viruses in water samples (Gilgen et al., 1995; Keswick et al., 1984; Reynolds et al., 1997). Application of molecular PCR assays have improved environmental virology surveys and has facilitated the examination of the diversity and ecological dynamics of specific viral populations and entire communities (Bofill-Mas et al. 2006; De Paula et al., 2007; Girones et al., 2010).

Viral genomes do not share single genes across all taxa, such as 16S or 18S rRNA in the genomes of prokaryotes and eukaryotes. A first step towards assessing viral diversity is therefore to identify conservative regions within virus-specific genes as targets for PCR primers. Once suitable primers have been identified, the genetic diversity and changes in viral community structure can be assessed (Muyzer et al., 1993). PCR of virus DNA from environmental samples requires release of the viral nucleic acid from the capsid, which is usually accomplished by extracting with guanidium thiocyanate and passing the sample through a silica column to remove the dissociated capsid proteins (Griffin et al., 2003). This method purifies both RNA and DNA, which is particularly important to the isolation of enteric viruses, most of which have RNA genomes (Fout et al., 2003; Griffin et al., 2003).

Nested PCR is a more sensitive edition of PCR in which a target sequence is amplified and the sample undergoes a second round of PCR to amplify a sequence nested within the initial amplicons (Abbaszadegan et al., 1999). This approach is taken when an extremely low concentration of template DNA (e.g. a single molecule of template in the sample) is

anticipated or when negative results are achieved using conservative PCR despite other evidence signifying presence of template DNA in the sample (Griffin *et al*., 2003).

PCR can only detect DNA sequences, so detection of enteric viruses with RNA genomes must be preceded by a process called reverse transcription, in which purified 58 retroviral reverse transcriptase (RT)-an RNA-dependent DNA polymerase-is incubated with an RNA template and free nucleotides to generate double-stranded, complementary DNA [cDNA] (Girones et al., 2010; Schwab et al., 1993).The usefulness of PCR and RT-PCR assays for routine monitoring of enteric viruses in water and sediments has also been recognised (Schwab et al., 1993). Green and Lewis (1999) detected enteroviruses, rotaviruses and hepatitis A viruses in different types of sediment samples and at various sampling times. Frontiers in PCR have allowed researchers to obtain quantitative results, higher resolution, and simultaneous detection of different pathogens. Recently, real time PCR method has been developed for quantification of human viruses and their detection in sewage and source waters (Le Cann et al., 2004, Monpoeho et al., 2002, 2004).

1.8.5 Real-Time PCR

Real-time PCR provides quantitative data for the presence of enteric viral genomes in environmental samples with the use of a fluorescent dye, such as SYBR Green (Molecular Probes, Eugene, OR), that will bind to amplified cDNA or with fluorochrome-tagged probes that fluoresce when bound to complementary sequences in the amplified region (Fong and Lipp, 2005). Real-time PCR involves the detection of a fluorescent signal emitted during the amplification reaction, where the signal intensity emitted is relative to the amount of the target DNA amplicon (Fong and Lipp, 2005; Griffin et al., 2003). By amplifying a known concentration of control DNA in parallel, the ratio of the fluorescent signals allows for quantification of the experimental target sample. Results obtained from this type of PCR inform researchers about viral concentration and in the future, may be compared to minimum infectious doses to estimate health risks (Griffin et al., 2003). Application of PCR as well as RT-PCR (reverse transcription-polymerase chain reaction) and sequencing techniques have become the standard methods for the detection and characterisation of viral pathogens (D'Agostino et al., 2011; Muscillo et al., 2008; Yan et al., 2003, 2004). Real-time quantitative PCR (qPCR) assays have also been used to detect specific viruses from a mixed population (Pal et al., 2006) and in source waters and drinking water (Albinana-Gimenez et al., 2009). The use of qPCR has proven to be rapid, sensitive, specific, and quantitative method of detecting viral genomes in low concentrations in water, but it also has a few drawbacks. Firstly, PCR (including qPCR) alone cannot differentiate between infectious and non-infectious viruses (i.e., defective virions or naked viral RNA) (Fong and Lipp, 2005). Secondly, a variety of PCR

inhibitory substances in water are concentrated together with viruses, decreasing the efficiency of PCR amplification.

A single-tube multiplex PCR for rapid detection in faeces of 10 viruses causing diarrhoea has recently been developed (Khamrin et al., 2011). Multiplex PCR enables different target DNAs to be detected in the same reaction vessel. For example, if a number of enteric virus species are assumed to exist in a water sample, they can be assayed simultaneously in the same sample vial (Formiga-Cruz et al., 2005). This approach can save time if many samples are to be processed; however, it may require a great deal of parameter optimisation in order to create conditions that are favourable for each template to denature and for each primer to anneal specifically and efficiently (Griffin et al., 2003). Many reports in the literature have reported detecting human pathogenic viruses in the freshwater systems using molecular techniques (Chen et al., 2008; Fong and Lipp, 2005) and in quantifying viruses using real time PCR (Jiang, et al., 1999; Jiang, 2006).

1.8.6 Pulse-Field Gel Electrophoresis (PFGE)

PFGE has become popular for analysing viral communities (Wommack et al., 1995). This technique allows separation of large nucleic acid fragments on agarose gels and thus the generation of fingerprinting profiles of viral communities based on differences in genome size so that major viral genotypes can be distinguished and differences in community structure resolved (Wommack and Colwell, 2000; Steward et al., 2000). PFGE analyses have also been successful in both fresh water and marine sediments (Filippini and Middelboe, 2007). However, 10^6 viruses of the same genome size are needed to obtain a visible band on a gel, and therefore the method only detects dominant strains and this technique detects only dsDNA, as RNA and ssDNA cannot be adequately represented (Wommack et al., 2000; Steward et al., 2000). Thus, PFGE reveals only a minimum estimate of the dominant genotypes present within a sample (Danovaro et al., 2007).

1.8.7 Metagenomic Sequencing

In recent year's metagenomics has proven to be a useful tool for examining viruses in a range of natural systems, revealing novel and diverse environmental viral communities. In systems with low species richness, metagenomic sequencing reveals patterns in microbial diversity and evolution (Breitbart et al., 2004). The advantage of using this method is that it surveys the complete viral community genome without selection based on host or sequence similarity to known viruses (Rosario et al., 2009). Characterisation of microbial communities using bioinformatics approaches addresses some of the limitations conventional molecular

techniques have and provides a high-resolution outlook of microbial diversity, as well as the potential functional capabilities within these assemblages (Bench et al., 2007). Metagenomics approaches circumvent the problem of the lack of general target sequences in viruses and can capture the entire diversity of viral communities. Recent developments in the application of metagenomic tools are revealing a wealth of information concerning the overall scale of the viral genetic reservoir (Rosario et al., 2009). Accordingly, metagenomic analyses have yielded 10^4-10^6 viral genotypes (Riemann and Middelboe, 2002; Steward et al., 2000; Larsen et al., 2004).

An improved understanding of the contribution of viral genomes to microbial environmental processes is just starting to be revealed through the application of these techniques. The majority of viral metagenomic studies have primarily focused on DNA isolated from material passing through filters, 0.22 µm in size, which is the fraction that contains the bulk of virus-like particles. These have revealed that viral communities are extraordinarily diverse on both local and global scales (Angly et al., 2006; Breitbart et al., 2002, 2004). Furthermore, the investigation of marine viromes across four oceanic regions proposes that viral community composition and nucleic acid type (i.e. dsDNA vs. ssDNA) is a function of geographic location and that vastly different environments support similar viral communities that differ only in the abundance of the dominant viral members (Angly et al., 2006).

1.8.8 Microfluidic Digital PCR

Even though metagenomic studies provide useful tools for revealing novel and diverse environmental viral communities in a range of natural systems, an approach to physically link single bacterial cells harvested from a natural environment with a virus is still urgently needed. Advances in microfluidic technology have enabled the isolation and analysis of single cells from nature (Marcy et al., 2007; Ottesen et al., 2006; Zare and Kim, 2010). The researchers used technique called “digital multiplex PCR” (Ottesen et al., 2006; Warren et al., 2006) to capture the hosts of an uncultured virus from the environment with a microfluidic PCR. The results demonstrated genus-wide infection patterns displaying intragenus selectivity on the bacterial community residing in the termite hindgut and limited lateral gene transfer restricted mixing of viral marker alleles between hosts despite host proximity. This approach provides a method to examine virus-bacterium interaction in many environments without culturing hosts or viruses.

2 EXPERIMENTAL PROCEDURES

2.1 Description of Study Area and Sampling Procedure

Focus in this study period was on the Umgeni River (five points along the river) due to it being the largest catchment (5000 km^2) in the KwaZulu-Natal region (WRC, 2002). The Umgeni River is approximately 230 km long, providing water to over 3.5 million people and supporting an area that is responsible for approximately 65% of the total economic production in the province (WRC, 2002). The study area stretched from upstream of the river at Inanda Dam downstream towards the river mouth that opens out to the Indian Ocean. Sampling points (Figure 2.1) were designated as follows: U1 (Umgeni River Mouth), U2 (Reservoir Hills), U3 (New Germany Wastewater Works), U4 (Krantzkloof Nature Reserve) and U5 (Inanda Dam).

Sample	GPS WAYPOINTS	
	Latitude	Longitude
U1(A)	29° 48' 43"S	31° 02' 20"E
U2(B)	29° 47' 47"S	30° 58' 03"E
U3(C)	29° 48' 13"S	30° 53' 36"E
U4(D)	29° 46' 18"S	30° 50' 11"E
U5(E)	29° 40' 29"S	30° 51'19"E

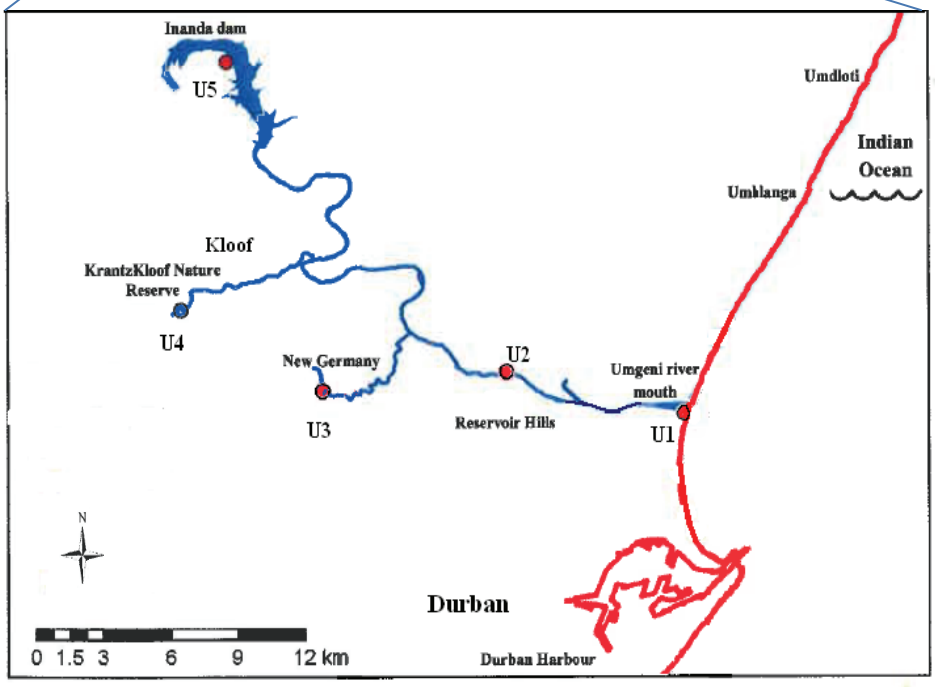

Figure 2.1 Map of the study region within Durban and surrounding areas. Shown are the locations approximate sampling points of the Umgeni River investigated in this study

Water samples from the Umgeni River were collected during the autumn (March -April 2011), winter (June-July 2011), spring (September-October 2011), and summer (December 2011-January 2012) months, to cover the four seasons of the year, so as to assess the potential effects of seasonal variations on the water quality at these sampling points. Water samples were collected in 25 ℓ plastic drums with caps. The drums were sterilised with 70% (v/v) alcohol and were rinsed with the water from the river source prior to collection. The water samples were collected by holding the container by the handle and plunging it knee deep (± 0.5 m) below the water surface facing away from the water current. If there was no water current, it was artificially simulated by pushing the container forward. All samples were protected from direct sunlight and transported to the laboratory in the Discipline of Microbiology, University of KwaZulu-Natal (Westville campus), within one hour of collection, and stored at 4°C until further analysis (Buckalew et al., 2006).

2.2 Determination of Physico-Chemical Parameters

Several physico-chemical parameters including, temperature; pH; turbidity; biological oxygen demand and chemical oxygen demand were measured. Temperature was measured *in situ*, using a temperature probe, whilst the pH and turbidity were measured at the laboratory facility, using a pH meter (Beckman Φ 50 pH meter) and portable 2100P turbidimeter (HACH), respectively. Samples were analysed for conductivity, inorganic water quality parameters, including ortho-phosphate, nitrite and nitrate, ammonia, chloride, sulphate concentrations, and heavy metals such as aluminium, lead, mercury and cadmium concentrations by the CSIR Consulting and Analytical Services (CAS) Laboratory.

2.2.1 Biological Oxygen Demand

The biological oxygen demand (BOD_5) of each water sample was measured using the OxiDirect BOD system (HACH) over a 5 day period. The selected BOD_5 range was 0-40 mg/ℓ and the corresponding sample volume according to manufacturer's instructions was used for the analysis. Essential pre-treatment steps, where necessary, were carried out prior to analysis, including modification of pH for optimum biochemical oxidation; filtering turbid samples; homogenising samples containing fibres and thorough mixing of samples. The analysis was conducted following manufacturer's instruction and the BOD measured was expressed in mg/ℓ.

2.2.2 Chemical Oxygen Demand

The chemical oxygen demand (COD) of each sample was determined photometrically using the SpectroQuant Nova 60 COD cell test (Merck) measuring in the range of 0-15000 mg/ℓ COD or O_2. Each COD test vial, containing all the required reagents, was vortexed to re-suspend the bottom sediment prior to adding 3 mℓ of each sample and mixing vigorously. The samples were digested in a thermoreactor (HACH) at 148°C for 2 hrs in the dark. Following sample digestion, the reaction cells were allowed to cool to room temperature before measuring the COD of the sample.

2.3 Isolation of Bacterial Indicator Microorganisms

The membrane filtration (MF) technique (Millipore, HANG 47 mm) was used for the isolation and enumeration of eight indicator organisms from all water samples, according to standard protocol (Standard Methods, 1992). Appropriate dilutions of the water samples were prepared with autoclaved distilled water prior to filtration. Fifty millilitre samples were filtered through 0.45 µm pore size GN-6 Metricel membrane filters (Millipore, 47 mm), held in a glass filtration unit, which were then transferred to 45 mm Petri plates containing various selective media for recovery of each indicator group (Table 2.1). After the incubation period, all the typical colonies grown on the filters were recorded as presumptive counts for the estimation of colony forming units per 100 millilitre (cfu/100 mℓ). Sample blanks were processed during MF to ensure quality of dilution water (Buckalew et al., 2006).

Table 2.1 Selective media and incubation conditions used for the isolation and enumeration of bacterial indicators.

INDICATOR MICROORGANISM	SELECTIVE MEDIA	INCUBATION CONDITIONS
Total Heterotrophs (TH)	Nutrient Agar	24hrs at 37°C
Total coliforms (TC)	M-Endo agar	24 hrs at 35°C
Faecal coliforms (FC)	M-FC agar	24 hrs at 44.5°C
Enterococci (EC)	Membrane Enterococcus Agar (MEA) Bile Aesculin Agar (BAA)	Presumptive test: 4 hrs at 37°C followed by 4 hrs at 44°C Confirmatory test: 4 hrs at 44°C
Faecal streptococci (FS)	KF-Streptococcus Agar (KFS-A)	48 hrs at 42°C
Presumptive *Vibrio cholera* (VC)	Thiosulphate Citrate Bile Salts Sucrose Agar (TCBS)	18-24 hrs at 37°C
Presumptive *Salmonella spp*. (SAL)	*Salmonella-Shigella* Agar (S-SA)	24 hrs at 35°C
Presumptive *Shigella spp.* (SHIG)	*Salmonella-Shigella* Agar (S-SA)	24 hrs at 35°C

2.4 Bacteriophage Determinations

The presence-absence spot test using the single agar layer method (ISO, 2000) was used to determine the presence of somatic and F-RNA (F-specific) coliphages. *Escherichia coli* ATCC 13786 strain (purchased from American Type Culture Collection (ATCC) was used as the host for the somatic coliphage, while *Salmonella typhimurium* WG49 (provided by Dr. Maite Muniesa, University of Barcelona, Spain) was used as the host for F-specific coliphages. The test was accomplished by spotting 10 µℓ of the TFF-concentrated water samples onto lawns of the two bacterial hosts. The presence of a clearing zone/plaque indicated the presence of phage. Bacteriophages were enumerated by the double agar layer technique following the ISO 10705-2 standard (ISO, 2000) for enumeration of somatic coliphages and ISO 10705-1 (ISO, 1995) for enumeration of F-specific RNA bacteriophages (F-RNA). One hundred microlitres of the concentrated samples was mixed with 1 mℓ of overnight bacterial host grown according to the ISO standards in 8 mℓ of soft agar and poured over agar bottom plate as described by Jiang et al. (2001). Plaques were enumerated after 12 to 24 h of incubation at 37°C and measured as plaque forming units per millilitre (pfu/mℓ).

2.5 Tangential Flow Filtration (TFF) for Viral Recovery

Viral community samples (virioplankton) were concentrated using a two-step tangential flow filtration process as given in Figure 1.4, according to the method of Wommack et al. (2010). Briefly 20 ℓ of river water was first pumped through a 25 µm string-wound polypropylene sediment cartridge filter to remove large particles at a flow rate of 230 mℓ/min. A peristaltic pump (Masterflex) was used at a flow rate of 530 mℓ/min with pressure at (P_{in} = 7psiand P_{out} = 6 psi) to pump the samples through a 0.5 m^2, 0.22 µm Pellicon TFF cartridge filter (Millipore Corp) to remove all bacterial cells. Viruses in the cell-free permeate was further concentrated to a final volume of 500 mℓ using 10 m^2 100-kDa spiral-wound TFF filter (Helicon; Millipore Corp). The retentate was allowed to re-circulate through the two step system until only 500 mℓ to 1 ℓ of sample remained in the original vessel. The virus-like particles were pelleted out from solution using an ultra-centrifuge (Beckman, LX-100) at 130 000 x g for 3hours at 4ºC. The resulting pellets were re-suspended in 15 mℓ of 1 X Phosphate Buffer (pH7) and stored at -20ºC until further use. After each experiment, the filtration unit was sterilised with a 0.4 N NaOH (pH 13) solution heated to 50°C to remove contaminants for 15 min, followed by filtration of 20 ℓ of deionised water.

Normal Water Permeability (NWP) testing had to be performed regularly on the TFF filters before and after running the system to ensure filter quality. This was calculated using the following equation:

$$NWP = \frac{R \cdot F}{A \cdot \left\{ \frac{P_{in} + P_{out}}{2} \right\} \cdot P_p}$$

Where:

R = Permeate Flow Rate in L/hour
Pin = Feed Inlet Pressure in psi
Pout = Retentate Discharge Pressure in psi
Pp = Permeate Discharge Pressure in psi
A = Total Filter area in m^2
F = Temperature correction factor

2.6 Enumeration and Visualisation of Virus-Like Particles (VLP)

2.6.1 Epifluorescent Microscopy

SYBR Gold staining coupled with epifluorescent microscopy (Chen et al., 2001; Patel et al., 2007; Shibata et al., 2006) was used to enumerate virus-like particles (VLPs) from the Umgeni River virus filtrate. After ultra-filtration (100 kDa cut-off), one millilitre of the concentrated water samples were fixed with 40 µl of a 2% paraformaldehyde solution and then filtered onto 0.02 µm Anodisc filters (Whatman, Maidstone, Kent, UK) with vacuum pressure no greater than 20 kPa. The Anodisc filters were allowed to air dry and were then stained with 2 X SYBR Gold (Invitrogen, Carlsbad, CA, USA) for 15 min in the dark. After staining the filters were wicked to remove any remaining solution and air dried. The filters were mounted onto glass slides, and counted digitally at 1000 x magnification under blue-green light excitation (Fitch filter, excitation at 480-495 nm) with an NIKON Eclipse (80*i*) epifluorescent microscope in at least eight fields of view for each sample (Chen et al., 2001). Images obtained were then analysed using the iTEM software and NIS-D Elements software (D 3.2) to digitally count the fluorescent green VLP spots and compute the results.

2.6.2 Transmission Electron Microscopy (TEM)

TEM was used to examine the structures and morphology of VLPs in the river water. These TEM images were then compared to known viral images of human origin where possible (Rosario et al., 2009). Briefly one drop of freshly prepared VLPs was spotted onto a Formvar-carbon-coated 200-mesh TEM grid (Electron Microscopy Sciences, Fort Washington, Pa). The edge of the grid was gently blotted with a piece of Whatman filter paper to drain away the

excess fluid, and the grid was then stained with a 1% Phospho-tungstic acid (PTA) solution or a 2% Uranyl Acetate solution for 30 s, washed with 1 drop of deionised water for 10 s, and dried in air before examination with a TEM (JEOL). Photomicrographs of viruses were taken at magnifications of 150 000 to 600 000 X. Morphological characteristics of VLPs were compiled from multiple photomicrographs of phage particles in order to minimize size or shape anomalies. Samples were grouped according to season.

2.7 Viral Infectivity Using Cell-Culture

Cell-culture (where cell lines were available) was used to determine infectivity of virus-like particles (VLPs).The total cultivable virus method as described in USEPA (2001) was used as the infectivity protocol. The concentrated VLPs from various water sources were fed into the various cell lines. Three known viruses: Adenovirus, Rotavirus and Coxsackievirus were used as positive controls Amongst the cell lines used for human virus investigations in water were, VERO (African green monkey kidney cells), HEK 293 (human embryonic kidney cells), and HEPG2 (human hepatocellular carcinoma cells). Cells were grown in 10% Dulbecco's modified essential medium (GIBCO) supplemented with 10% Foetal Calf serum (GIBCO) containing a penicillin/ streptomycin / fungizone mix (1:1:1) (v/v/v), to confluent monolayers in 24 well plasticplates (Corning, USA). Approximately 200 µℓ of viral concentrate was overlaid onto the monolayers of appropriate cell lines and incubated at 37°C for 5 to 7 days. The development of cytopathic effect (CPE) that is indicative of a viral infection in the cell cultures was monitored for up to 7 days. Presence or absence of CPE was confirmed as described in USEPA (2001). After three freeze-thaw cycles, CPE positive and negative samples were filtered through 0.22 µm syringe filters and were inoculated in new Vero, HepG2 and Hek 293 cells for another 7 days. Samples that showed CPE at the end of the confirmation step were reported as positive for infectivity. Cell cultures were examined under an Olympus microscope using a 400 x magnification for the cytopathic effect (CPE).

2.8 Extraction of Viral Nucleic Acids for Viral Genomic Studies

All viral concentrates were filtered through a 0.22 µm Sterivex filter (Millipore, USA) and treated with 10% chloroform to remove contaminating microbial cells. Viral concentrates were then ultra-centrifuged twice at 29 000 x *g* for 3 hours at 4°C (Rosario et al., 2009). Viral DNA and RNA were simultaneously extracted from viral concentrates using the high pure viral nucleic acid large volume kit (Roche Diagnostics) and high pure viral RNA kits respectively (Roche Diagnostics), according to manufacturer's instructions. RNA concentrations were standardised to 1 µg/ mℓ and cDNA was synthesized using the iScript cDNA Synthesis kit

(BIORAD).All PCR products were visualised using agarose gel (2%) electrophoresis and staining with ethidium bromide.

2.8.1 Detection of Human Enteric Viruses by PCR/RT-PCR Assay

The primer sequences used to amplify and to detect human enteric viral genomes were selected using previously published data (Table 2.2) (Rosario et al., 2009; Symonds et al., 2009; Thurber et al., 2009). PCR or reverse transcription-PCR for each of the targeted viral groups was performed according to Symonds et al (2009). All PCR mixtures had a total volume of 25 µℓ and contained 2 µℓ of target DNA, 1 x REDTaq PCR buffer (10.0 mM Tris-HCl [pH 8.3], 50.0 mM KCl, 1.1 mM $MgCl_2$, 0.01% gelatin; Sigma-Aldrich, St. Louis, MO), 0.25 mM each deoxynucleoside triphosphate, 1 µM of each primer, and 1 U REDTaq DNA polymerase (Sigma-Aldrich), unless otherwise stated.

Table 2.2 Primer sequences to PCR amplify three viral groups

Virus Primer	Target Gene Sequence (5'–3')	Amplicon Size (bp)	Sensitivity (no. of targets)	Reference
Adenoviruses	(Hexon gene)			(Allard et al., 1992)
AV-A1	GCC GCA GTG GTC TTA CAT GCA CAT C	300	100	
AV-A2	CAG CAC GCC GCG GAT GTC AAA GT			
AV-B1*	GCC ACC GAG ACG TAC TTC AGC CTG	143		
AV-B2*	TTG TAC GAG TAC GCG GTA TCC TCG CGG TC			
Enteroviruses				(Fong et al., 2005)
JP UP	TTA AAA CAG CCT GTG GGT TG	600	100	
ENT DOWN	ACC GGA TGG CCA ATC			
ENT UP*	CCT CCG CCC CTG AAT G	154		
JP DOWN*	ATT GTC ACC ATA AGC GAC C			
Rotaviruses (group A)	(VP7 gene)			(Gilgen et al., 1997)
RV1	GTC ACA TCA TAC AAT TCT AAT CTA AG	1059	1000	
RV2	CTT TAA AAG AGA GAA TTT CCG TCT G			
RV3*	TGT ATG GTA TTG AAT ATA CCA C	346		
RV4*	ACT GAT CCT GTT GGC CAW CC			
Hepatitis B viruses	(S gene)			(Koike et al., 1998)
HBS-1	ATC AGG ATT CCT AGG ACC C	310	10 000	
HBS-R1	AGG ACA AAC GGG CAA CAA C			
HBS-11	GCG GGG TTT TTC TTG TTG AC	1241		
HBS-R11	GAA CCA ACA AGA AGA TGA GGC			

* Primers for Nested PCR

2.8.1.1 Adenoviruses

Nested PCR was used to amplify the hexon gene of approximately 47 different adenovirus serotypes (Allard et al., 1992). Five microlitres of the product from the first round of PCR was used as a template for the second PCR reaction. Both rounds of PCR had an additional 0.4 mM $MgCl_2$ in the reaction mixture. Both adenovirus PCR conditions were 4 min at 94°C, followed by 40 cycles of 92°C for 30 s, 60°C for 30 s, and 72°C for 1 min and a final incubation step at 72°C for 5 min. In all PCR reactions a positive control (cell-cultured Adenovirus) and negative control (distilled water) were included.

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2.8.1.2 Enteroviruses

Nested PCR was used to amplify the 5'-untranslated region of approximately 25 different Enterovirus genomes (Fong et al., 2005). An additional 1.8 mM $MgCl_2$ and 1.4 mM $MgCl_2$ were added to the first- and second-round PCR reaction mixtures, respectively. The first-round of PCR conditions were 40 cycles of 95°C for 30 s, 57.7°C for 30 s, and 72°C for 45 s, followed by 5 min at 72°C. Thereafter 2 µℓ of amplified PCR product from the first round PCR was added as the template to the second round of PCR, which was amplified by 40 cycles of 95°C for 30 s, 56.5°C for 30 s, and 72°C for 30 s, followed by 5 min at 72°C. In all PCR reactions a positive control (cell-cultured Coxsackievirus) and negative control (distilled water) were included.

2.8.1.3 Rotaviruses

Nested PCR was used to amplify the VP7 gene of the group A rotaviruses (Gilgen et al*.,* 1997). The first PCR mixture had an additional 0.4 mM $MgCl_2$ and was incubated for 1 min at 94°C, followed by 25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by incubation at 72°C for 3 min. The second reaction mixture, containing an additional 2.4 mM $MgCl^2$ and was incubated for 1 min at 94°C, followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s and then incubation at 72°C for 3 min. In all PCR reactions a positive control (cell-cultured Rotavirus) and negative control (distilled water) were included.

2.8.1.4 Hepatitis B Viruses

Nested PCR was used to amplify the S gene of hepatitis B viruses (Koike et al., 1998). Both rounds of PCR had an additional 0.4 mM $MgCl_2$ added to the reaction mixture, and the PCR cycling conditions were as follows: 5 min at 95°C, followed by 30 cycles of 95°C for 30 s, 55°C for 40 s, and 72°C for 40 s. Two microlitres of product from the first reaction was used as a template in the final PCR reaction. Second round of PCR followed the same PCR conditions

as the first PCR. In all PCR reactions a positive control (serum Hepatitis B virus) and negative control (distilled water) were included.

2.9 Statistical Analysis

Pearson's and Spearman's correlation tests were used to evaluate the correlations between the bacterial indicators and environmental variables, as well as correlations between the bacteriophages, virus-like particles and environmental variables. Data comparisons were performed using analysis of variance (post hoc tests) and the Student's *t*-test. Probability (significant level) was set at 0.05. The SPSS program version 19 (SPSS, Inc., Illinois) was used for the statistical analyses (Wilkinson, 1988).Canonical correspondence analysis (CCA) was applied to elucidate the various relationships with a view to determining the important variables responsible for the observed spatial and temporal distribution of the communities. CCA ordination allowed for the assessment of the relationships between biological and environmental data, specifically the extent to which variation in biological data can be accounted for by the measured environmental variables. On CCA ordinations, environmental variables are represented by arrows whose lengths reflect their relative importance in structuring the biological sample data (Clausen and Biggs, 1997). A Monte Carlo permutation test with 499 random permutations was used to establish the environmental axis that significantly correlated with the biological variables. CCA analysis was performed using the computer programme Canoco for Windows version 4.5 (Ter Braak and Verdonschot, 1995).

3 RESULTS AND DISCUSSION

The Umgeni River catchment supports a diverse range of activities and livelihood, which contributes to approximately 20% of the gross national product of South Africa (Mardon, 2003). This diversity makes the Umgeni River catchment prone to many different types of pollution. Microbial pollutants normally enter surface waters through transport pathways such as surface runoff and discharges of raw and treated sewage (Pejman et al., 2009; Singh et al., 2004). The water quality of the lower reaches of this river catchment is being affected by many human activities and influences, which can results in increased risks to users due to microbial contamination.

3.1 Physico-chemical characteristics of water samples

Spatial and seasonal fluctuations of the physico-chemical environmental variables of the water samples and heavy metal quality are presented in Tables 3.1 and 3.2, respectively. Temperature profiles shown in Table 3.1 varied significantly (p<0.05) from all sampling sites tested along the Umgeni River and ranged from 19°C (U5 – autumn), 15.2°C (U5 – winter), 16.5°C (U4 spring) to 28.5°C (U2 – summer). Increases in water temperatures can result in higher rates of inactivation of microorganisms in water samples (Carlsson, 2003). No significant difference (p> 0.05) was observed with pH measurements across all five sampling points and seasons tested. In this study, the pH measurements of the river water samples across the four seasons ranged from pH 7.10 to pH 9.16 and fell within the South African water quality pH guideline for domestic use. It was interesting to note that point U5 during spring had the highest pH value of 9.16. Significant (p<0.05) seasonal variations in turbidity values were noted along the Umgeni River and ranged from 1.62 NTU (U5) in autumn to 15.64 NTU (U1) summer (Table 3.1). Turbidity measurements give an indication of the concentration of suspended clay, silt, organic matter, inorganic matter, plankton and other microscopic organisms in a water source (DWAF, 1996b). All water samples had turbidity values which exceeded the recommended standard value of 0.1 NTU for turbidity (SABS, 2001). High turbidity values are associated with the survival of microorganisms due to association of the microorganisms with the particulate matter (DWAF, 1996b).

BOD is a measure of the quantity of dissolved oxygen in mg/ℓ necessary for the decomposition of organic matter by microorganisms such as bacteria. COD is used to measure both the amount of biologically active substances such as bacteria and biologically inactive organic matter in water. Large seasonal variations in BOD_5, COD and conductivity levels amongst the sampling points along the Umgeni River were observed (Table 3.1). BOD, COD and conductivity values for the Umgeni River ranged from 1.4 mg/ℓ (U3 – autumn) to

9.45 mg/ℓ (U3 – spring), 10.0 mg/ℓ (U2 – autumn) to 254.0 mg/ℓ (U3 – spring) and 21.6 mS/m (U5 – spring) to 5150 mS/m (U1 – summer), respectively. Point U1 of the Umgeni River during the autumn season exhibited a very unusual and high COD measurement of 968 mg/ℓ. Some Umgeni River samples fell within the universal water quality index of 3 mg/ℓ BOD (Boyacioglu, 2007), except for points U1, U2, U3 and U4 which exceeded this value drastically during certain seasons. There is no COD guideline for aquatic systems stipulated in South African, EU, WHO and US EPA guidelines. Conductivity qualitatively reflects the status of inorganic pollution and is a measure of total dissolved solids in the water. High COD values obtained in this study suggest that organic and/or inorganic substances from domestic and non-domestic wastewaters are entering these water systems. The water quality of these sampling sites can only be used for most of the industrial purposes. The Umgeni River at point U1 had relatively high values for most of the environmental parameters tested and this could be attributed to the fact that this point of the river intercepts with the ocean.

Nutrient enrichment acutely degrades aquatic ecosystems and impairs the use of water for domestic, industrial, agricultural, and recreational purposes (Ouyang et al., 2006). The phosphate concentrations varied significantly ($p < 0.05$) for all sampling points along the Umgeni River across all four seasons tested. Phosphate concentrations of the Umgeni River varied from <0.004 mg/ℓ P (U5 – autumn) to 1.63 mg/ℓ P (U3 – summer). Point U5 during autumn, spring and summer had relatively low phosphate concentrations at <0.004 mg/ℓ P. The phosphate levels of the water samples from points U1, U2 and U3 in this study are higher than the aquaculture South African Water Quality guideline value of 0.077 mg/ℓ P (DWAF, 1996a), except for point U5 which falls below the value.

The nitrate/nitrite and ammonia concentrations for all sampling points of the river differed significantly ($p< 0.05$) and ranged from 0.087 mg/ℓ N (U5 – autumn) to 3.12 mg/ℓ N (U4 – summer) and 0.005 mg/ℓ NH_4 (U4 – summer) to 38.1 mg/ℓ NH_4 (U3 – spring). Nitrate and Nitrite are commonly present in surface water because they are the end products of aerobic decomposition of organic nitrogenous matter. The nitrite/nitrate levels of the water met the criteria for the nitrates guideline value of 6 mg/ℓ N for domestic use. In water quality studies, nitrogen and phosphorus are the nutrients most commonly identified as pollutants. The nitrite/nitrate, ammonium and phosphate levels of the water samples at point U3 were generally higher than other sampling points except point U1 indicating higher potential polluting sources in this area. Interestingly enough, U3 is located after the wastewater treatment plant and close to an informal settlement area. High concentrations of either dissolved phosphate or nitrate concentrations may lead to osmotic stress of the fish species in rivers (DWAF, 1996a).

Chloride concentrations were extremely high at point U1 of the river during all seasons tested and ranged from 9853 mg/ℓCl (autumn) to 19234 mg/ℓCl (summer). Relatively low chloride concentrations were observed between sampling points U2 and U5 during the autumn and winter seasons. Chloride is a common constituent in water, is highly soluble, and is a typical conservative substance. Typically, concentrations of chloride in fresh water range from a few to several hundred mg/ℓ Cl. The South African water quality guideline for chloride is 0-100 mg/ℓ Cl for domestic use. Point U1 exceeded this guideline by over 10 times during all seasons. High concentrations of chloride (>120 mg/ ℓ Cl) in the water are likely to cause damages to industrial equipment and structures and to interfere with several industrial processes (DWAF, 1996a). Sulphate concentrations were reasonably high at point U1 during all four seasons tested values ranged as follows: 1388 mg/ℓ SO_4 (autumn), 1405 mg/ℓ SO_4 (winter), 409 mg/ℓ SO_4 (spring) and 1512 mg/ℓ SO_4 (summer). Low sulphate concentrations were detected for the remainder of the sampling points during all seasons.

The water temperature of the Umgeni River samples showed moderate to strong correlations with turbidity (r = 0.377), pH (r = 0.572), and conductivity (r = 0.702) and weak or no correlations with COD (r = 0.282), BOD_5 (r = 0.000). Significant positive correlations was observed between pH and turbidity (r = 0.961; p = 0.039). There were inverse correlations between COD and conductivity (r = -0.228) and BOD_5 and COD (r = -0.546). Strong positive correlations between chloride and sulphate concentrations (r = 0.862) for all the river water samples were also detected. Phosphate concentrations showed strong inverse correlations (r = -0.005 to -0.913) with all chemicals measured throughout the seasons for all water samples. Nitrate concentrations in the water samples had positive correlations with chloride (r = 0.436) and sulphate (r = 0.235) for all seasons.

Most of the water samples fell within the regulatory limits for domestic use for all heavy metals tested (Table 3.2). Heavy metal concentrations for the Umgeni River ranged as follows: <0.019 mg/ℓ to 0.112 mg/ℓ for Pb; 0.00014 mg/ℓ to<0.001 mg/ℓ for Hg; <0.009 mg/ℓ to<0.006 mg/ℓ for Cd and <0.005 mg/ℓ to 0.127 mg/ℓ for Cu. Relatively low concentrations of Cu were detected throughout all sampling points and all four seasons tested and did not exceed the South African guideline value of 1 mg/ℓ Cu.

The solubility of trace metals in surface water is predominantly controlled by water pH, water temperature, and the redox environment of the river system. Heavy metal pollution in water environments is related to pH, i.e., when the pH of water decreases, the solubility of metals increases thus increasing their potential toxicity (Campbell and Stokes, 1985; Rai et al., 1993).

The metal concentrations detected during the dry seasons were higher in concentration compared to the wet seasons might be attributed to water evaporation during the dry season and dilution – due to precipitation and run-off during the rainy season. These observations have been previously reported in other studies (Singh et al., 2008).

3.2 Enumeration of Bacterial Indicators

Total Heterotrophic Bacterial counts indicate the general microbial quality of water and are used to detect a wide range of bacteria which are omnipresent in nature. According to the South African Bureau of Standards (SABS), the stipulated recommended limit for THB population for drinking water is 100 cfu/mℓ. All samples exceeded this value (SABS, 2001). Minimum THB counts were recorded during winter while the maximum values were recorded during summer and ranged as follows: 0.90 x 10^6 cfu/100 mℓ (U5 – winter) to 13.67 x 10^6 cfu/100 mℓ (U1 – summer) (Figure 3.1). The Umgeni River mouth (U1) had the highest THB counts during all seasons compared to the other sampling sites along the river. The river was found to be highly contaminated, as this river flows through the more urbanized areas of Durban and is subject to higher surface runoff. In addition high nutrient concentrations and high turbidity had significant positive correlation with the growth of bacterial indicators, thus resulting in a substantial increase of these naturally-occurring organisms. The findings from this study correspond well with studies performed elsewhere (Olaniran et al., 2009).

Table 3.1 Physico-chemical quality of the Umgeni River water samples during autumn, winter, spring and summer seasons.

Sample location	Season	Physical characteristics						Chemical characteristics				
		T (°C)	**pH**	**Turbidity (NTU)**	**BOD_5 (mg/ℓ)**	**COD (mg/ℓ)**	**Conductivity (mS/m)**	**Phosphate (mg/ℓ P)**	**Nitrate & Nitrite (soluble) (mg/ℓ N)**	**Ammonia (soluble) (mg/ℓ NH_4)**	**Chloride (soluble) (mg/ℓ Cl)**	**Sulphate (soluble) (mg/ℓ SO_4)**
*		18-24	6-9	0 .1	3	[a]na	< 700	0.077	6	1	100	200
U1	**AUTUMN**	24.5	7.84	6.51	5.64	968	2864	0.607	0.886	2.93	9853	1388
U2		22	7.78	3.60	3.23	10	39.5	0.049	0.536	0.356	52.5	19.5
U3		23	7.73	6.58	1.4	25	83.1	1.91	0.353	9.51	135	44.9
U4		21.5	7.11	3.69	4.4	21.3	27.9	0.007	1.72	0.069	53.9	13.0
U5		19	8.51	1.62	1.33	31.5	25.5	<0.004	0.087	0.011	29.4	14.3
U1	**WINTER**	19	7.1	14.9	5.55	165	3180	0.416	0.968	1.32	11052	1405
U2		18	8.33	4.42	3.21	17.4	43.3	0.099	1.90	0.228	57.4	30
U3		19	8.08	3.77	4.46	27.8	85.6	0.438	2.15	13.1	114	94.9
U4		17.1	8.02	4.38	3.82	11.1	28.9	0.205	2.23	0.092	51.6	20.1
U5		15.2	7.88	0.64	1.67	11	26.6	0.015	0.643	0.138	28.0	19
U1	**SPRING**	19.5	7.49	13.4	6.12	249	721	0.687	1.63	2.68	2544	409
U2		18	7.87	12.4	3.23	58.34	39.7	0.233	1.52	1.04	67.8	29.1
U3		21.5	8.03	15.7	9.45	172.01	82.4	0.815	0.089	38.1	124	46.7
U4		16.5	8.33	6.26	2.37	68	23.5	0.014	2.04	0.183	56.2	21.3
U5		20.5	9.16	4.08	0.95	44	21.6	<0.004	0.648	0.044	29.1	22.4
U1	**SUMMER**	27.5	8.43	15.64	6.36	254	5150	0.02	0.069	0.047	19234	1512
U2		28.5	8.55	10.91	4.12	54.34	34.3	0.087	0.733	0.289	41.7	23.2
U3		26	8.77	11.20	6.31	42.7	68.3	1.63	3.10	0.015	107	58.6
U4		22	8.63	7.63	3.32	75.1	94.5	0.005	3.12	0.005	233	47.7
U5		24	7.57	8.31	0.59	26.22	48.6	<0.004	0.677	0.092	79.5	26

* South African water quality guidelines for domestic use (DWAF, 1996a); [a] Not Applicable (no guideline for COD)

Table 3.2 Heavy metal quality of the Umgeni River water samples during autumn, winter, spring and summer seasons

Sample location	Season	Heavy metal concentrations (mg/ℓ)			
		Pb^{2+}	**Hg^{2+}**	**Cd^{2+}**	**Cu^{2+}**
*		0.004	0.0003	0.001	1.400
U1	AUTUMN	<0.019	0	<0.009	0.044
U2		<0.019	0	<0.009	0.013
U3		<0.019	0	<0.009	0.011
U4		<0.019	0	<0.009	0.009
U5		<0.019	0	<0.009	0.010
U1	WINTER	0.031	0.00018	<0.009	0.013
U2		<0.019	0	<0.009	0.009
U3		<0.019	0	<0.009	0.127
U4		<0.019	0	<0.009	0.009
U5		<0.019	0	<0.009	0.013
U1	SPRING	0.103	0.00037	<0.006	0.009
U2		<0.025	0	<0.006	<0.005
U3		<0.025	0.00090	<0.006	0.011
U4		<0.025	0.000140	<0.006	<0.005
U5		<0.025	0	<0.006	<0.005
U1	SUMMER	0.112	<0.001	<0.006	0.012
U2		<0.025	<0.001	<0.006	0.014
U3		<0.025	<0.001	<0.006	0.015
U4		<0.025	<0.001	<0.006	0.016
U5		<0.025	<0.001	<0.006	0.013

* World Average of trace elements in unpolluted rivers (Meybeck and Helmer, 1989; Schiller and Boyle, 1987)

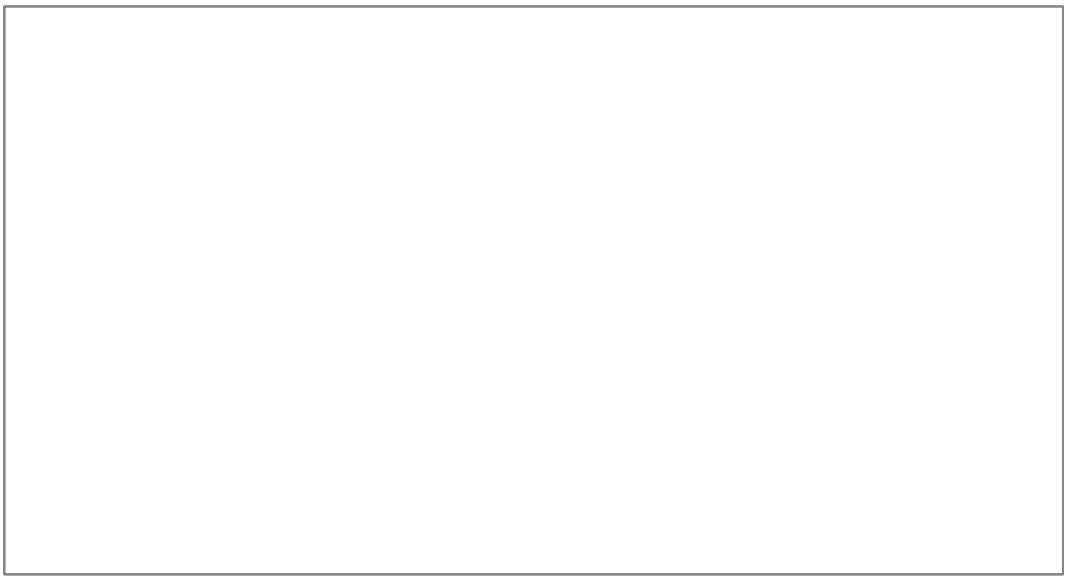

Figure 3.1 Total Heterotrophic Bacterial (THB) populations for Umgeni River at the different sampling points during autumn, winter, spring and summer seasons. Bars indicate the average of replicate samples (n = 3 or 4) while the error bars show the standard deviation.

Presumptive total coliform (TC), faecal coliform (FC), *V. cholerae* (VC), *Salmonella spp.* (SAL), *Shigella spp.* (SHIG), enterococci (EC) and faecal streptococci (FS) populations for the Umgeni River over the seasons are presented in Figure 3.2. TC and FC counts varied significantly (p<0.05) throughout all sampling points and ranged from 3.30 x 10^3 cfu/100 mℓ (U1 – winter) to 6.03 x 10^3 cfu/100 mℓ (U1 – summer) and 0.89 x 10^3 cfu/100 mℓ (U3 – winter) to 4.85 x 10^3 cfu/100 mℓ (U2 – spring), respectively (Figure 3.2). Total coliforms (TC) are frequently used to assess the general hygienic quality of water. The TC group includes bacteria of faecal origin and indicates the possible presence of bacterial pathogens such as *Salmonella spp., Shigella spp., Vibrio cholerae, Campylobacter jejuni, Yersinia enterocolitica* and pathogenic *E. coli,* especially when detected in conjunction with other FCs (Ashbolt *et al*., 2001). The South African water quality guidelines have “negligible risk” for TC and FC of 5 cfu/100 mℓ and 0 cfu/100 mℓ, respectively (DWAF, 1996). All points failed to meet the target water quality ranges for negligible risk with points U2 and U1 having the highest bacterial populations. According to NMMP (2002) drinking untreated water can pose a potential health risk if faecal coliforms are present as follows: high risk (>10 cfu/100 mℓ), medium risk (1-10 cfu/100 mℓ) and low risk (0 cfu/100 mℓ). Full or partial contact with untreated water containing faecal coliforms poses health risks of: high risk (> 2 000 cfu/100 mℓ), medium risk (600-2 000 cfu/100 mℓ) and low risk (< 600 cfu/100 mℓ) (NMMP, 2002).

VC, SAL and SHIG concentrations for the Umgeni River ranged from 1.91 x 10^3 cfu/100 mℓ (U4 – winter) to 4.97 x 10^3 cfu/100 mℓ (U2 – spring), 0.01 x 10^3 cfu/100 mℓ (U5 – winter) to 1.43 x 10^3 cfu/100 mℓ (U1 – spring) and 0.34 x 10^3 cfu/100 mℓ (U3 – winter) to 1.83 x 10^3 cfu/100 mℓ (U2 – summer), respectively (Figure 3.2).The highest population of TC, FC, and VC was observed in the water sample collected at points U1 and U2 during the spring and summer seasons, compared to the other sampling points along the Umgeni River while sampling site U5 had the lowest FC, VC and SHIG concentrations during all seasons. SAL and SHIG populations were detected at all sampling points along the river throughout the seasons. There was no significant difference in the TC, FC and VC counts ($p > 0.05$) between the seasons. EC and FS concentrations ranged from 0.22 x 10^3 cfu/100 mℓ (U3 – winter) to 1.39 x 10^3 cfu/100 mℓ (U2 – spring) and 0.02 x 10^3cfu/100 mℓ (U5 – autumn) to 2.37 x 10^3 cfu/100 mℓ (U3 – winter), respectively (Figure 3.2). EC and FS counts differed significantly ($p<0.05$) and EC had positive correlations with TC ($r = 0.343$) over all seasons. According to the USEPA criteria for EC counts (<33 cfu/100 mℓ for freshwater) (USEPA, 2004), all points along the river exceeded the guideline. DWAF specifies two guidelines for FS levels: (1) 0-30 cfu/100 mℓ for full contact recreation and (2) 0-230 cfu/100 mℓ for intermediate contact recreation. EC and FS counts differed significantly ($p < 0.05$) and EC had positive correlations with TC ($r = 0.343$) over all seasons.

Significant spearman's rho correlation coefficients were noted between the THB populations and TC and FC populations throughout all seasons ($r = 0.9$ -1.00, $p<0.05$). Positive, significant Pearson's correlations between the indicator organisms for the Umgeni River include: THB and TC ($r = 0.955$, $p = 0.045$), THB and VC ($r = 0.862$), VC and FC ($r = 0.557$), THB and SAL ($r = 0.999$, $p = 0.001$),TC and SAL ($r = 0.966$, $p = 0.34$), and EC and FC ($r = 0.986$, $p= 0.014$) with no significant correlations between FS and SHIG populations with the other indicators during the four seasons.

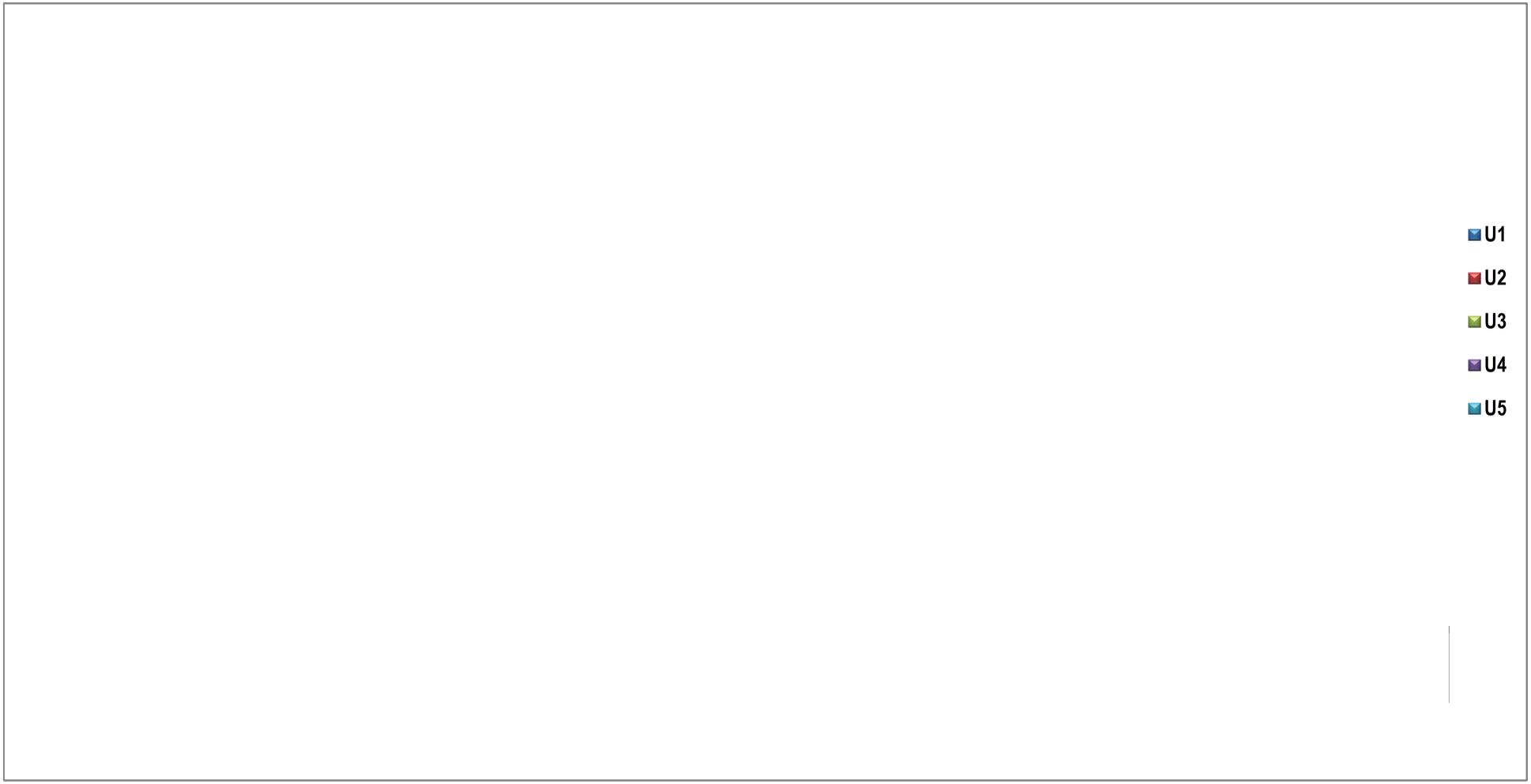

Figure 3.2 Presumptive counts of total coliforms (TC), faecal coliforms (FC), *V. cholerae* (VC), *Salmonella spp*. (SAL), *Shigella spp*. (SHIG), faecal streptococci (FS) and enterococci (EC) populations for the Umgeni River at the different sampling points during autumn, winter, spring and summer seasons. Bars indicate the average of replicate samples (n = 3 or 4) while the error bars show the standard deviation.

3.3 Canonical correspondence analysis (CCA)

Canonical correlation analysis was used to investigate the relationships between the water quality variables and the total bacterial growth, as well as the relationships between the different indicator bacteria and the physico-chemical parameters at all sites and seasons. CCA ascertained the extent to which one set of measurements was related to another and determined the particular attributes responsible for the relationships. The arrows representing the environmental variables indicate the direction of maximum change of that variable across the diagram. In essence, the length of the arrow is proportional to the rate of change, so a longer arrow indicated a larger change in environmental variable. CCA ordination plot (Figure 3.3) revealed strong relationship between the THB communities and measured physical and chemical water quality variables. The ordination plot revealed that temperature, pH and turbidity had longer arrows and were the key variables that impacted on the presence of the bacterial communities.

The exacerbated growth of the total heterotrophic bacterial populations positively correlated with the key environmental factors affecting water quality, implying that environmental relationships do exist among biological and physico-chemical data. Physical parameters, such as pH, temperature and turbidity do have a major influence on bacterial population growth (Byamukama et al., 2000; Nübel et al., 1999). The sample scores were scattered in the ordination with a number of denser clusters spread out around the origin. An interesting observation was that the presence of FS had no correlation with the other bacterial indicators or with the physico-chemical parameters (Figure 3.3). VC, SAL, FC and EC were found to be grouped similarly (green circle – Figure 3.3), with EC having a stronger correlation with COD. The nitrate concentration correlated positively with the TC populations in the environment. The relative magnitude of Eigen values for each of the CCA axis in Figure 3.4 is an indication of the relative importance of the axis.

CCA axis 1 accounted for 50.7% of total variance of the species data set and in total the species – environment relation ordination accounted for 100% (Appendix i-Table a) of the cumulative variance suggesting high correlation between bacterial community data and water quality variables. The statistical significance of the model was tested using Monte Carlo permutation test and Eigen value for axis 1 was found to be insignificant ($p > 0.05$). Pearson's correlation between species and water quality variables displayed strong positive correlation for both axes.

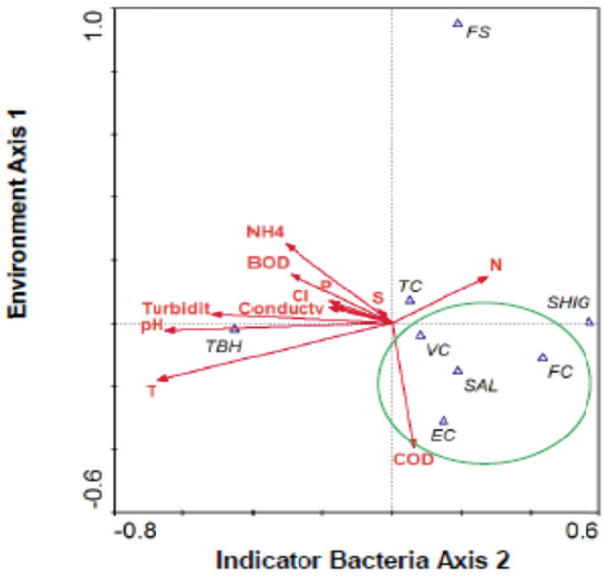

Figure 3.3 CCA ordination plot for bacterial indicators and water quality variables at the five study sites during autumn, winter, spring and summer seasons.

CCA ordination plot revealed strong relationships between the overall bacterial growths of different sites and seasons measured as well as the physico-chemical water quality variables (Figure 3.4). According to the CCA plot (blue circle), temperature, BOD_5, turbidity, pH, electrical conductivity, orthophosphate and sulphate were the most prominent variables (long arrows) that impacted the community structures significantly at Sites 1, 3 and 5 during the summer and spring seasons. The overall bacterial growths of Sites 2, 4, 5 during autumn and winter seasons strongly correlated with the nitrate/nitrite profiles (green circle). Sites 1 and 3 in winter showed no correlations with the rest of the sites and variables measured.

The relative magnitude of Eigen values for each of the CCA axis in Figure 3.4 is an indication of the relative importance of the axis. CCA axis 1 accounted for 37.9% of total variance of the species data set and in total the species – environment relation ordination accounted for 74.6% (Appendix i-Table b) of the cumulative variance suggesting that there may be a correlation between the various sites and water quality variables. Pearson's correlation between species and water quality variables displayed strong positive correlation for both axes.

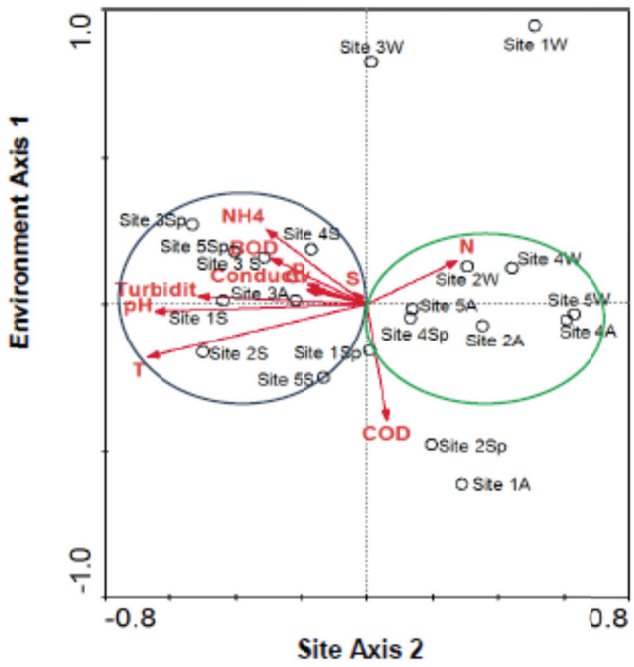

Figure 3.4 CCA ordination plot for all the water quality variables and the total bacterial growth at the five study sites and during autumn, winter, spring and summer seasons. Abbreviations: Sites 1 = Umgeni River mouth, 2 = Informal settlement at Reservoir Hills, 3 = New Germany Waste Treatment Works, 4 = Krantzkloof Nature Reserve, 5 = Inanda Dam. Seasons: A = Autumn, W = Winter, Sp = Spring, S = Summer.

CCA ordination plot (Figure 3.5) illustrated the relative abundance (long arrows) of Pb and Cu in the environment when compared to Hg (short arrow) at the five study sites during autumn, winter, spring and summer seasons. The sample scores were scattered in the ordination with Cu being spread out from Pb and Hg.VC, SHIG, FC, TC and EC were grouped similarly (yellow circle) and were clustered to the ordination center (Figure 3.5), probably giving an indication that all the heavy metals may have some correlation with them. The SAL and THB populations correlated positively with the Pb concentration in the environment. Another interesting observation was that FS was correlated with Cu and had no correlation with the other bacterial indicators or the other heavy metals tested (Figure 3.5). CCA axis 1 accounted for 18.5 % of total variance of the species data set and in total the species – environment relation ordination accounted for 73.3% (Appendix i-c) of the cumulative variance suggesting that correlation between bacterial community data and heavy metal variables does exist. Eigen value for axis 1 and axis 2 was found to be significant ($p < 0.05$). Pearson's correlation between species and heavy metal variables displayed strong positive correlation ($r = 0.775$) for axis 1.

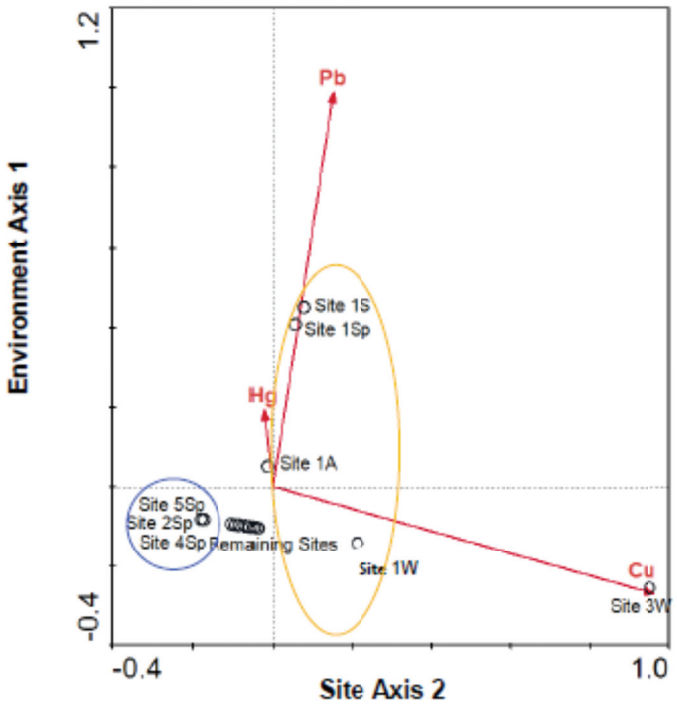

Figure 3.5 CCA ordination plot for bacterial indicators and heavy metal variables at the five study sites during autumn, winter, spring and summer seasons.

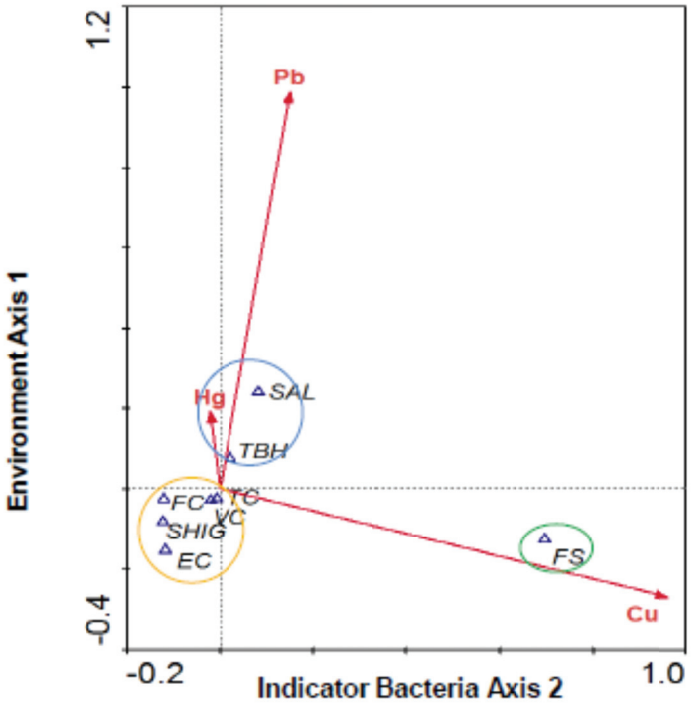

Figure 3.6 CCA ordination plot for the heavy metal quality variables and the total bacterial growth at the five study sites and during during autumn, winter, spring and summer seasons. Abbreviations: Sites 1 = Umgeni River mouth, 2 = Informal settlement at Reservoir Hills, 3 = New Germany Waste Treatment Works, 4 = Krantzkloof Nature Reserve, 5 = Inanda Dam. Seasons: A = Autumn, W = Winter, Sp = Spring, S = Summer.

CCA ordination plot revealed strong relationship between the overall bacterial growths of different sites and seasons measured as well as with the heavy metal variables (Figure 3.6). According to the CCA plot (blue circle), 80% of the sites especially during the spring season were clustered around the heavy metal ordination graph origin. Site 1 during all seasons correlated with all the heavy metal profiles (yellow circle). Site 1 during summer and spring correlated strongly with Pb, whilst during autumn and winter site 1 correlated with Hg and Cu, respectively.

Growth of bacterial indicators at Site 3 during winter showed strong correlation with the Cu. The relative magnitude of Eigen values for each of the CCA axis in Figure 3.6 is an indication of the relative importance of the axis. CCA axis 1 accounted for 63.5% of total variance of the species data set and in total the species – environment relation ordination accounted for 100% (Appendix i-Table d) of the cumulative variance suggesting that strong correlations between bacterial community data and heavy metal variables do exist at most of the sampling sites. Pearson's correlation between species and heavy metal variables displayed low positive correlation (r = 0.259) for axis 1 and no correlation for axis 2 (r = 0).

3.4 Enumeration of Bacteriophages and Virus-Like Particles (VLP)

Both somatic and F-specific coliphages were tested for in this study (Table 3.3) using *E. coli* ATCC 13876 and *S. typhimurium* WG49 as their host cultures respectively. A positive result for the presence of these phages was indicated by plaque formation on a lawn of host culture. All sampling points throughout all seasons tested positive for the presence of somatic coliphages with points U1, U2 and U3, lower reaches area, having stronger plaque formation during all seasons. As shown above, these three sites also contained higher bacterial counts and physico-chemical values. Points U1 and U3 during the autumn and spring seasons had strong F-RNA plaque formation.

The PFU results correlated well with the presence-absence spot test. Somatic coliphage and F-RNA coliphage counts varied significantly (p < 0.05) at all the sites and all seasons tested. Somatic coliphage counts ranged from 10 pfu/mℓ (U5 – autumn) to 659 pfu/mℓ (U1 – summer) and F-RNA coliphage counts from 0 pfu/mℓ (U5 – autumn) to 550pfu/mℓ (U2 – summer), respectively (Figure 3.7). VLPs were detected at all sampling sites throughout all seasons, with point U1 during summer having the highest population of 2086 VLP/mℓ and Point U4 and U5 had the lowest VLP counts of: 221.5 VLP/mℓ (U4 – winter) and190.1 VLP/mℓ (U5 – autumn), respectively. Mean concentrations of somatic coliphages and F-RNA coliphages were comparable between the autumn and spring seasons and correlation analysis resulted in a strong Pearson's correlation coefficient of 0.977 (p<0.01). These coliphages had inverse

correlations (r = -0.536) with the VLPs detected during autumn but correlated (r = 0.795) well with the VLPs found during spring. THB populations correlated (r = 0.85, p< 0.05) well with the somatic coliphages and VLP populations at all sites along the river and for both seasons.

Table 3.3 Presence – Absence spot test (based on plaque formation) for the determination of somatic bacteriophages and F-RNA coliphages in the Umgeni River water samples using host specific *E. coli* ATCC 13786 and *S. typhimurium*WG49, respectively

Sample Location		Presence – Absence Spot Test	
		Somatic Coliphage	F-RNA Coliphage
AUTUMN	U1	+++	++
	U2	++	+
	U3	+++	+++
	U4	+	+
	U5	+	-
WINTER	U1	++	+
	U2	+++	+
	U3	++	+
	U4	+	+
	U5	+	-
SPRING	U1	+++	+++
	U2	+++	++
	U3	+++	+++
	U4	++	+
	U5	+	+
SUMMER	U1	+++	+++
	U2	+++	++
	U3	++	+
	U4	+++	+
	U5	++	+

Plaque Formation (cell lysis): + : Weak Plaque; ++ : Average Plaque; +++ : Strong Plaque; -: No Plaques

All variables tested had positive correlations with each other during all seasons. Significant positive correlations were observed between the somatic phage and FRNA phage (r = 0.991, *p* = 0.001), somatic phage and VLP (r = 0.884, *p* = 0.46) and the FRNA phage and THB (r = 0.902, *p* = 0.036).

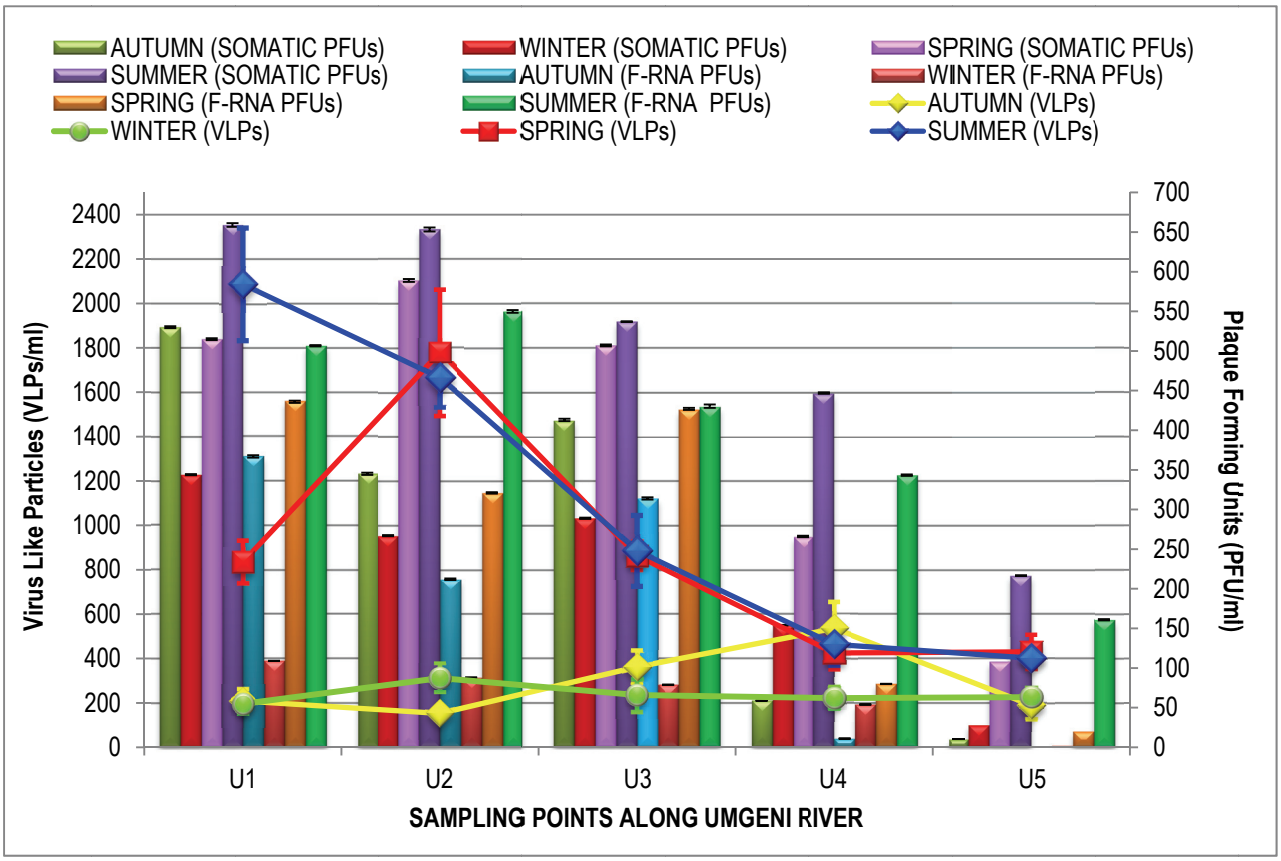

Figure 3.7 Presumptive counts of virus-like particles (VLPs) and plaque forming units counts for the Umgeni River at the different sampling sites during autumn, winter, spring and summer. Line plot indicates the average of replicate samples (n = 8 or 10) for VLPs and bars indicates the average of replicate samples for PFUs (n = 2 or 3), while the error bars show the standard deviation.

CCA ordination plot (Figure 3.8) revealed strong relationships between the overall VLP and phage populations at different sites and seasons measured as well as with the physico-chemical water quality variables. As shown in the CCA plot (blue circle), temperature, BOD_5, turbidity, pH, conductivity, orthophosphate and sulphate were the most important variables (Figure 3.8- long arrows) that impacted the community structures significantly at sites 1, 2 and 3 during the autumn, summer and spring seasons. Site 2, 4, 5 during autumn, winter and spring seasons correlated with the nitrate/nitrite profiles (green circle). Site 1 and 3 in autumn and winter showed no correlations with the rest of the sites and variables measured.

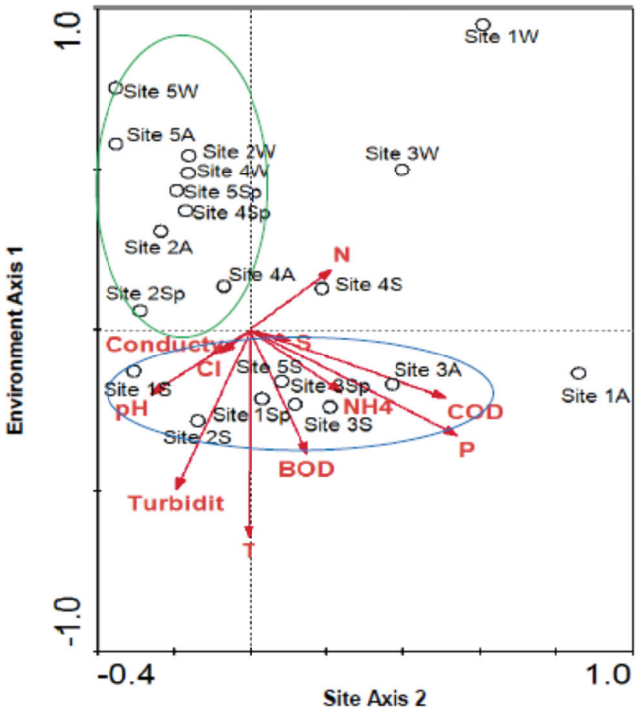

Figure 3.8 CCA ordination plot for all the water quality variables and the total viral and bacteriophage growth at the five study sites and during autumn, winter, spring and summer seasons. Abbreviations: Sites 1 = Umgeni River mouth, 2 = Informal settlement at Reservoir Hills, 3 = New Germany Waste Treatment Works, 4 = Krantzkloof Nature Reserve, 5 = Inanda Dam. Seasons: A = Autumn, W = Winter, Sp = Spring, S = Summer.

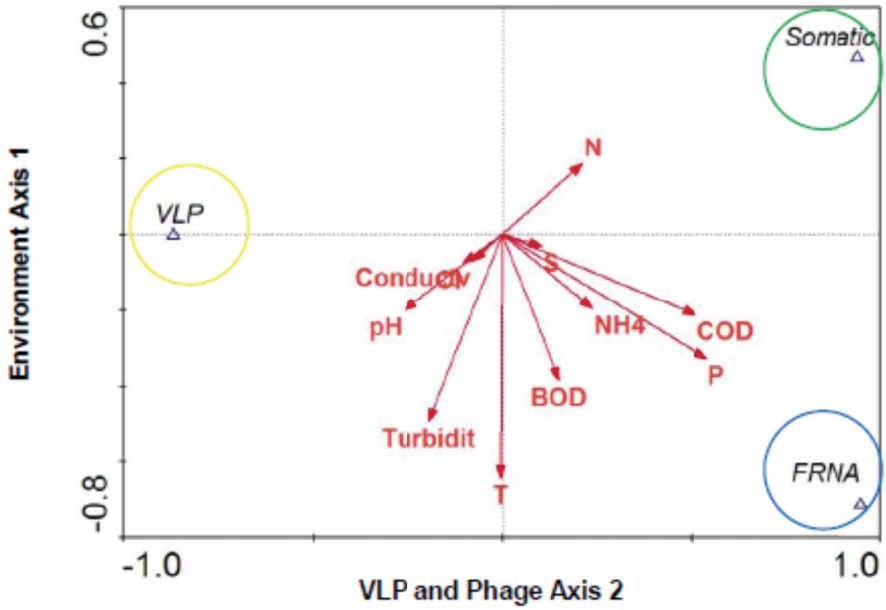

Figure 3.9 CCA ordination plot for bacteriophage and virus populations and water quality variables at the five study sites during autumn, winter, spring and summer seasons.

The ordination plot revealed that the Somatic phage, FRNA phage and VLPs were largely unrelated to one another. The sample scores were scattered in the ordination with a number of denser clusters (physico-chemical) spread out around the origin. CCA axis 1 (Figure 3.9) accounted for 60.5% of total variance of the species data set and in total, the species – environment relation ordination accounted for 83.7% of the cumulative variance. Eigen values for axis point one and two were 0.043 and 0.021 respectively. Positive significant ($p<0.05$) Pearson correlation of species and environmental scores for axis one and two were $r = 0.701$ and $r = 0.943$ respectively.

CCA shown in Figure 3.10 revealed strong relationships between the THB communities and VLP and phage communities (green circle). The VC and TC populations also had positive relationships VLP and phage communities (yellow circle). The ordination plot revealed that SHIG, EC, SAL and FS did not impact on the presence of the viral communities. The sample scores were scattered in the ordination (Figure 3.10). FC had no correlation with VLP and phage communities. Hurst et al. (1998) demonstrated that conductivity, turbidity and the number of generation of bacteria growth that a sample could support are the apparent parameters influencing viral persistence.

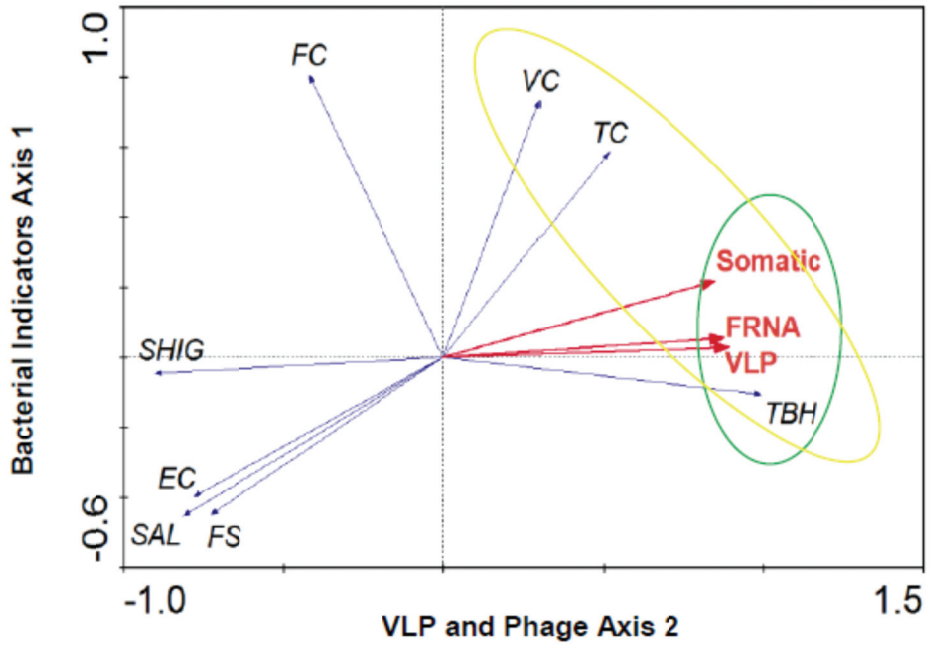

Figure 3.10 CCA ordination plot for bacteriophage and virus populations and bacterial indicators at the five study sites during autumn, winter, spring and summer seasons.

3.5 Visualisation of Virus-Like Particles (VLPs) by Transmission Electron Microscopy (TEM)

The phage/virus-like particle concentrations in every sample were high enough to view on formvar coated electron microscopic grids without enrichment. All water samples in this study contained a mixture of morphologically different tailed phage viruses, which were regarded as bacteriophages. Table 3.4 classifies the different type of bacteriophage that could be detected in the Umgeni River according to the scheme of Ackermann and Eisenstark (1974).

Table 3.4 Frequency of phage morphotypes found in the Umgeni River water, classified according to the scheme of Ackermann and Eisenstark (1974) – [International Committee on Taxonomy of Viruses (ICTV)]

	No. of phage's belonging to morphotypes:					
	A1	**B1**	**A1/B1***	**B2**	**C1**	
Sample Season						**TOTAL**
Autumn	2	1	1	0	3	7
Winter	1	1	0	0	1	3
Spring	7	4	3	2	3	19
Summer	5	14	7	0	5	31
TOTAL	15	20	11	2	12	60

*Classification uncertain, A1 – Myoviridae – short capsid contractile tail, B1– Siphoviridae- short capsid, non- contractile long tail, B2 – Siphoviridae – long capsid, non-contractile long tail, C1 – Podoviridae – short tail.

Most of the detected phages (33%) had isometric heads and long non-contractile tails, belonging to morphotype B1 (Siphoviridae) (Table 3.4). Members of morphotypes A1 (Myoviridae) were detected in 25% of the samples, and C1 (Podoviridae) was present in substantial (20%) numbers. The phages tagged as A1/B1 were not conclusive in morphological appearance and could not be classified. Most of the phages from the Umgeni River were intact, with discrete structures such as tail fibres, base plates, and other appendages which are pertinent for the recognition of and interaction with the host cell. These accessories may be an indication that a significant proportion of the phages are suspended in the water environment as potentially infective particles. Table 3.5 illustrates the size range of the bacteriophages that could be observed during all four seasons. Phage head diameters ranged from 48-59 nm (winter) to 42-79 nm (summer), with the general mean value of phage

heads being 57 nm. The tail length of the phage populations varied throughout all seasons from 69 nm in autumn to 352 nm in summer. Interesting to note was that the total length of phages varied extensively ranging from 150 nm (autumn), 181 nm (winter), 290 nm (spring) and 218 nm (summer).

Table 3.5 Size range of tailed phage's observed by electron microscopy

Sample Season	No. of Phage Observed	Head Diameter (nm)		Tail Length (nm)		Total Length (nm)	
		Range	Mean	Range	Mean	Range	Mean
Autumn	7	47-70	50	69-186	98	85-240	150
Winter	3	48-59	57	93-181	113	135-250	181
Spring	19	42-68	57	115-263	199	170-480	290
Summer	31	42-79	57	93-351	155	137-514	218

Figures 3.11, 3.12 and 3.13 resemble bacteriophages detected in the Umgeni River water samples. Phage heads examined, appeared to be hexagonal in outline, with six sided profiles which were regular, with three symmetrical axes (e.g., Figure 3.11 a, b, c, e and f; 3.12 f); others were irregular, with only one symmetrical axis (e.g., Figure 3.12 a, c and d). However, these data were not adequate to differentiate between icosahedra, octahedra, and dodecahedra classification. Figure 3.11 c and h closely resemble members of the family Myoviridae and Podoviridae, respectively. Figure 3.12 d and h resemble members of the family B2 Siphoviridae and B1 Siphoviridae, respectively. Figure 3.13 represents phage particles detected in the Umgeni River at sites U1, U2 and U3 during spring and summer seasons that resemble known bacteriophages found in literature. These include known phages of: 71A-6 of *Vibrio vulnificus* phage; Phage T4 and T4-like *Vibrio parahaemolyticus* phage. The main purposes of phage classification are generalisation and simplification, which facilitates comparisons and understanding of viruses (Ackerman, 2012). The high morphological diversity of phage communities corresponds well with the great variability and dynamics of bacterial populations obtained in this current study. Electron microscopy provides a direct insight into the morphological variability of phage present in the water environment, without being dependent on the isolation of suitable host strains. This is important, as bacteria which are not cultivable under laboratory conditions usually dominate aquatic environments and may be important phage hosts (Demuth et al., 1993).

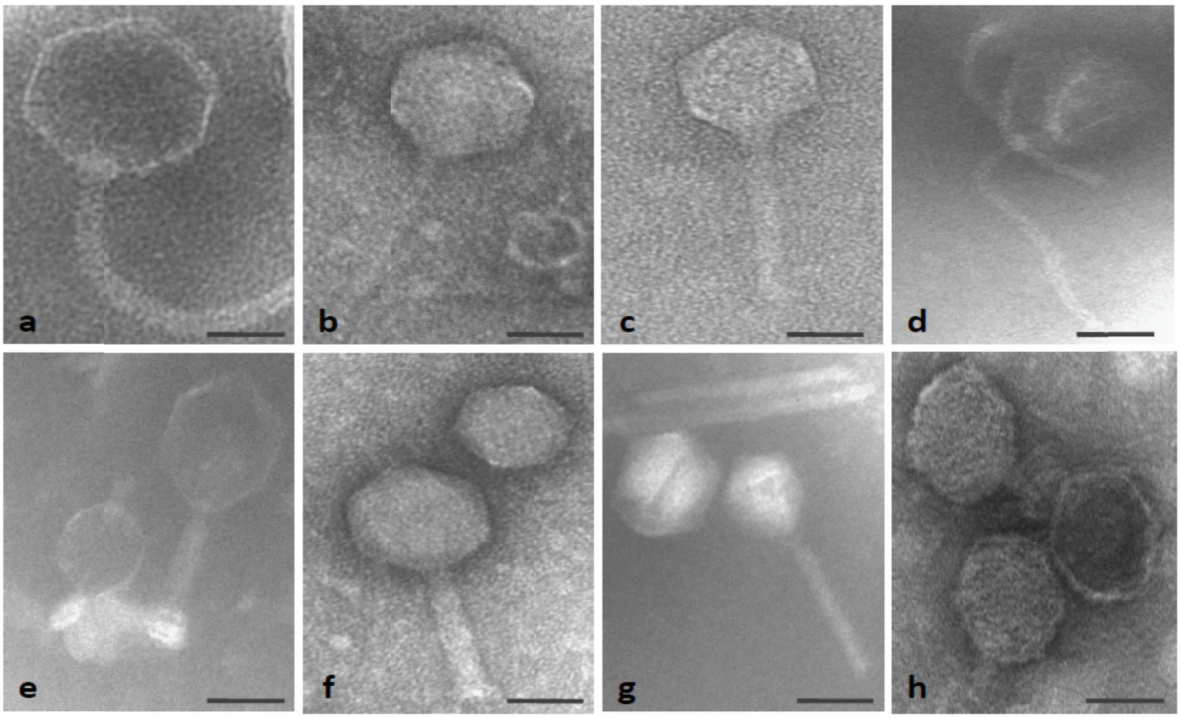

Figure 3.11 TEM images of phage particles of various morphotypes present downstream in the Umgeni River at the sampling sites U1, U2, and U3 during autumn, spring and summer seasons tested. Images captured at 300 000-400 000 x magnification. Scale bar 100 nm.

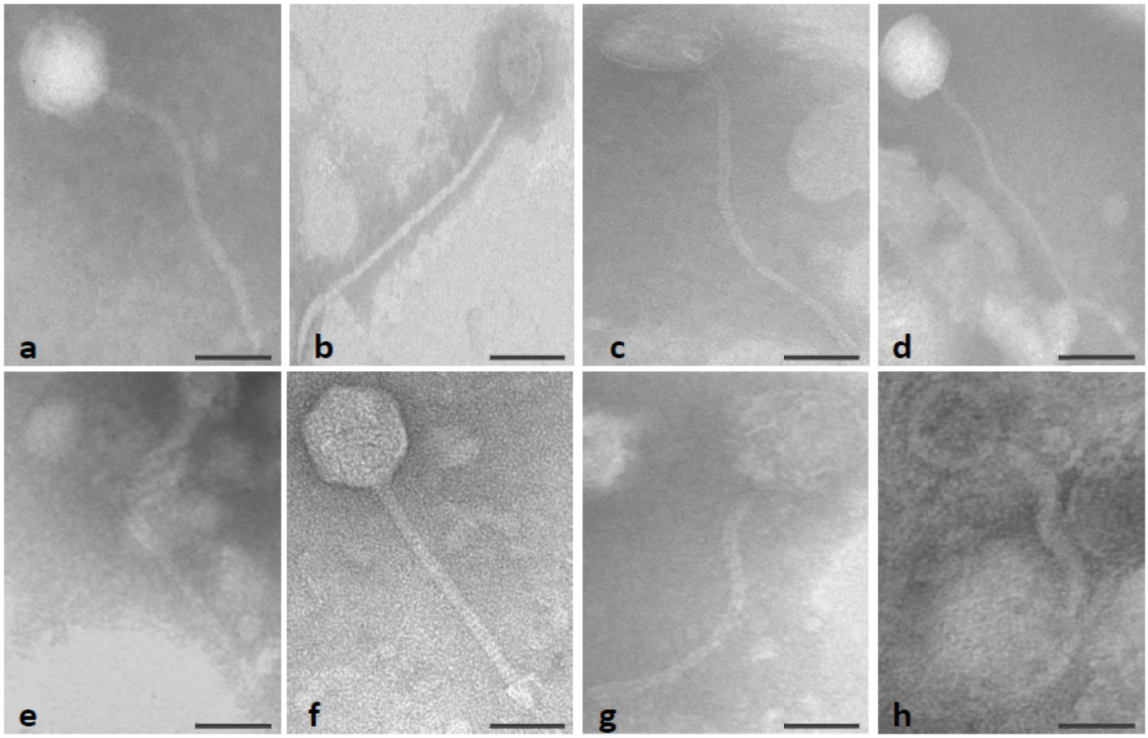

Figure 3.12 TEM images of long tailed phage particles of various morphotypes present in the Umgeni River at the sampling sites U1, U2, U3, U4 and U5 during winter, spring and summer seasons tested. Images captured at 300 000-400 000 X magnification. Scale bar 100 nm.

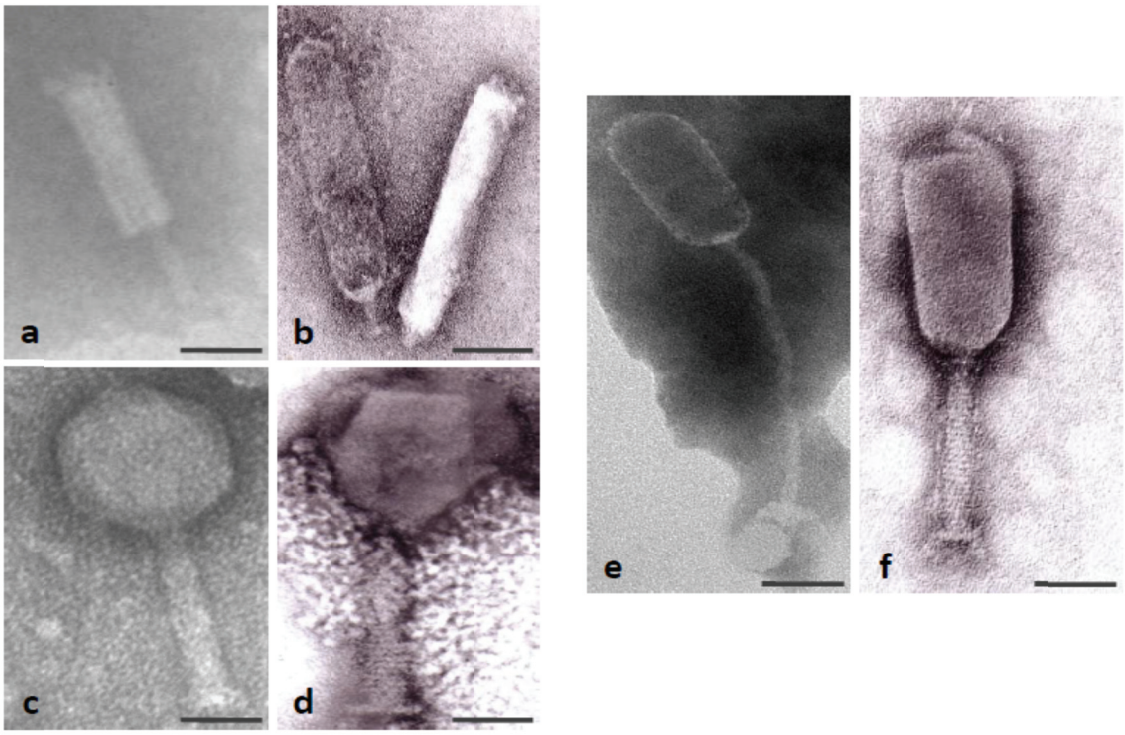

Figure 3.13 TEM images of various phage morphotypes (a, c, e) present in downstream in the Umgeni River at the sampling sites U1, U2 and U3 respectively during spring and summer seasons tested. (b) Known 71A-6 of *Vibrio vulnificus* phage (d) Known Phage T4 (f) Known T4-like *Vibrio parahaemolyticus* phage. Images captured at 300 000-400 000 X magnification. Scale bar 100 nm

Figures 3.14-3.18 illustrates several presumptive virus types that were found in the Umgeni River during all seasons. These presumptive viruses were compared to their known structures illustrated in literature. In this study, most of potential pathogenic enteric viruses as shown below have been observed under TEM especially during the summer period. Presumptive naked Adenoviridae-like particles ranging in size from 66.97-74.40 nm are shown in Figure 3.14 and these images are compared to known Adenoviruses (70-90 nm) (Figure 3.14 g). Figure 3.15 represents TEM images of presumptive naked Picornaviridae (Enterovirus)-like particles ranging in size from 26-30 nm, compared to a known Coxsackievirus (Figure 3.15 f). Poxviridae-like particles (Figure 3. 16 a, b, c) and Herpesviridae-like particles (Figure 3.16 e, f, g) were also detected in the Umgeni River water samples and these were found to be similar in literature to known Poxvirus (Figure 3.16 d) and known Herpesvirus (Figure 3.16 h). Figure 3.17 illustrates presumptive Reoviridae- like particles ranging in size from 18-20 nm (Figure 3.17 a,b,c) compared to a known Rotavirus (20-30 nm) (Figure 3.17 d). Presumptive Caliciviridae-like particles are illustrated in Figure 3.17 (e, f, g) and these had a size of 35 nm, similar to that of a known Norovirus (Figure 3.17 h) of 30-40 nm. Presumptive enveloped Coronaviridae-like particles (Figure 3.18 a, b, c) were found to be similar to a known Coronavirus (Figure 3.18 d), with presumptive Orthomyxoviridae-like particles being similar to a known Influenza virus (Figure 3.18 h).

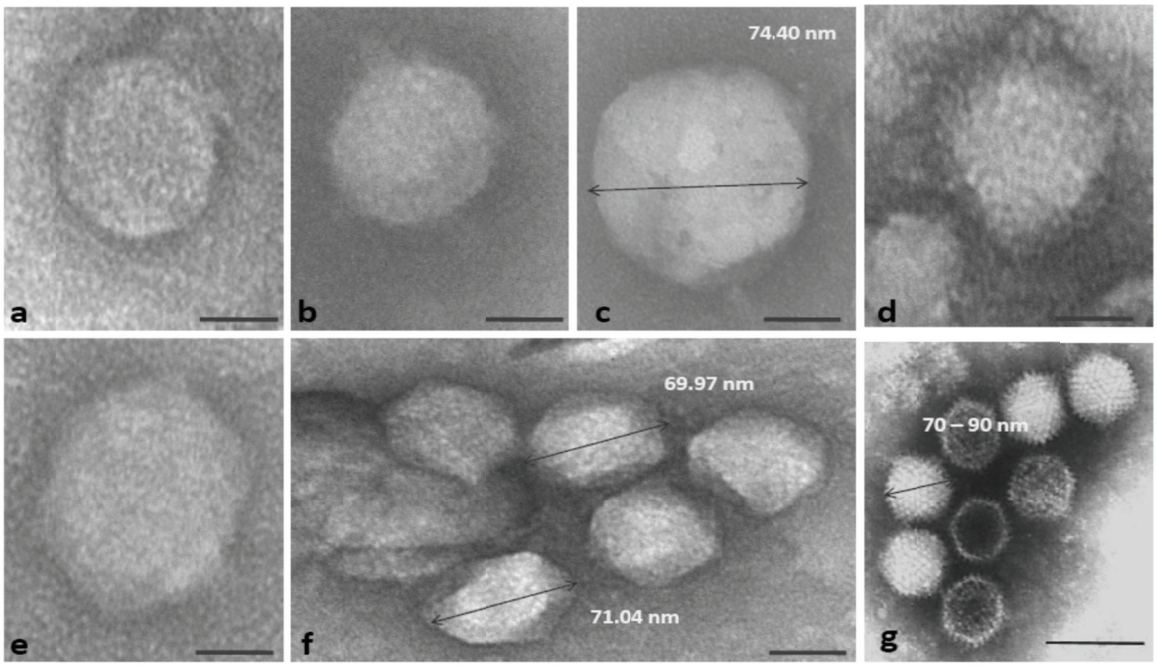

Figure 3.14 TEM images of presumptive naked Adenoviridae-like particles and (g) a known Adenovirus, present in the Umgeni River at the sampling sites U1, U2, U3, U4 and U5 during autumn, spring and summer seasons tested. Images captured at 400 000-600 000 X magnification. Scale Bar 100 nm.

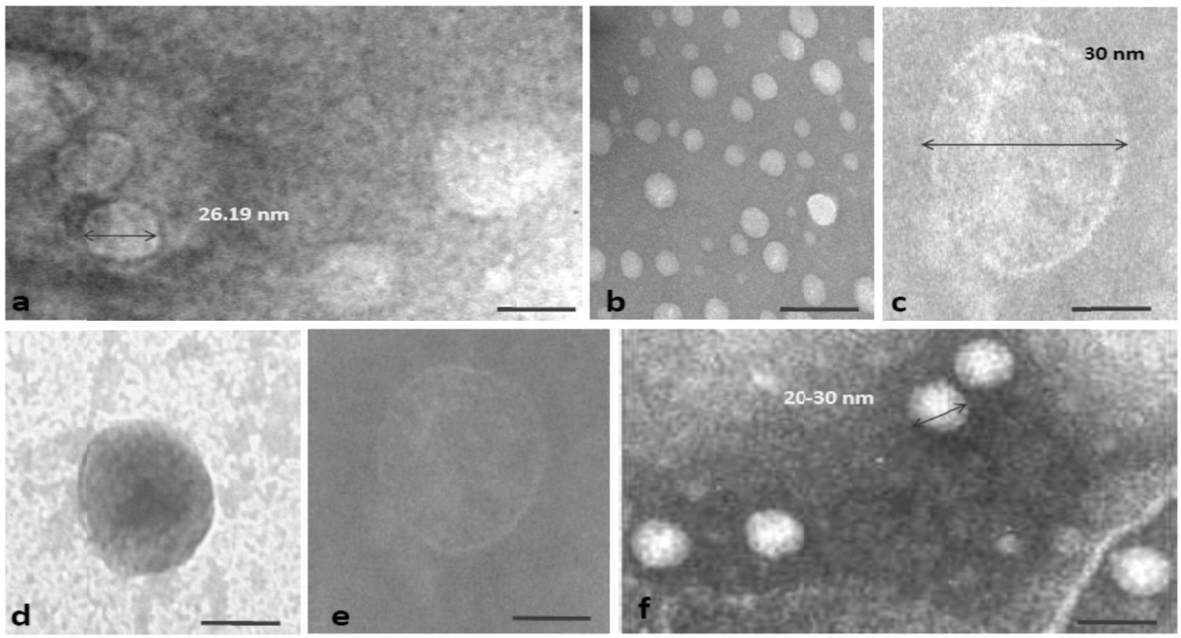

Figure 3.15 TEM images of presumptive naked Picornaviridae (Enterovirus)-like particles and f) a known Coxsackievirus, present in the Umgeni River at sampling sites U1, U2, and U3 during spring and summer seasons tested. Images captured at 500 000-600 000 X magnification. Scale Bar 50 nm.

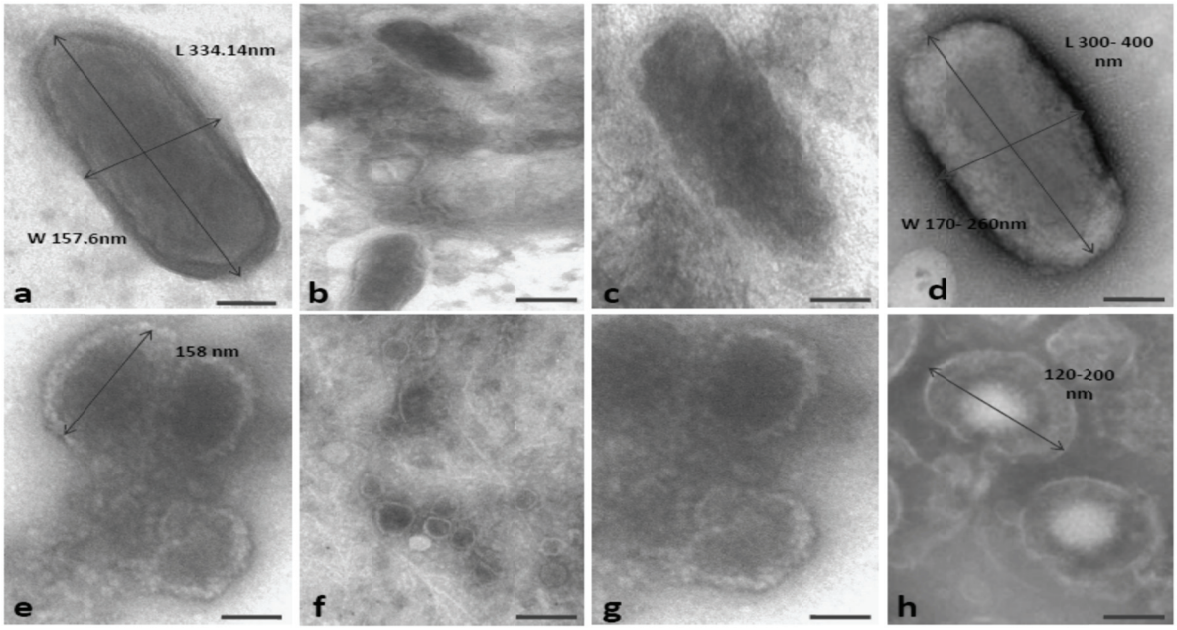

Figure 3.16 TEM images of enveloped presumptive (a, b, c) Poxviridae-like particles (d) known Pox virus and (e, f, g) Herpesviridae-like particles (h) known herpes virus, present in the Umgeni River at site U3 during the summer season. Images captured at 300 000-500 000 X magnification. Scale Bar 200 nm.

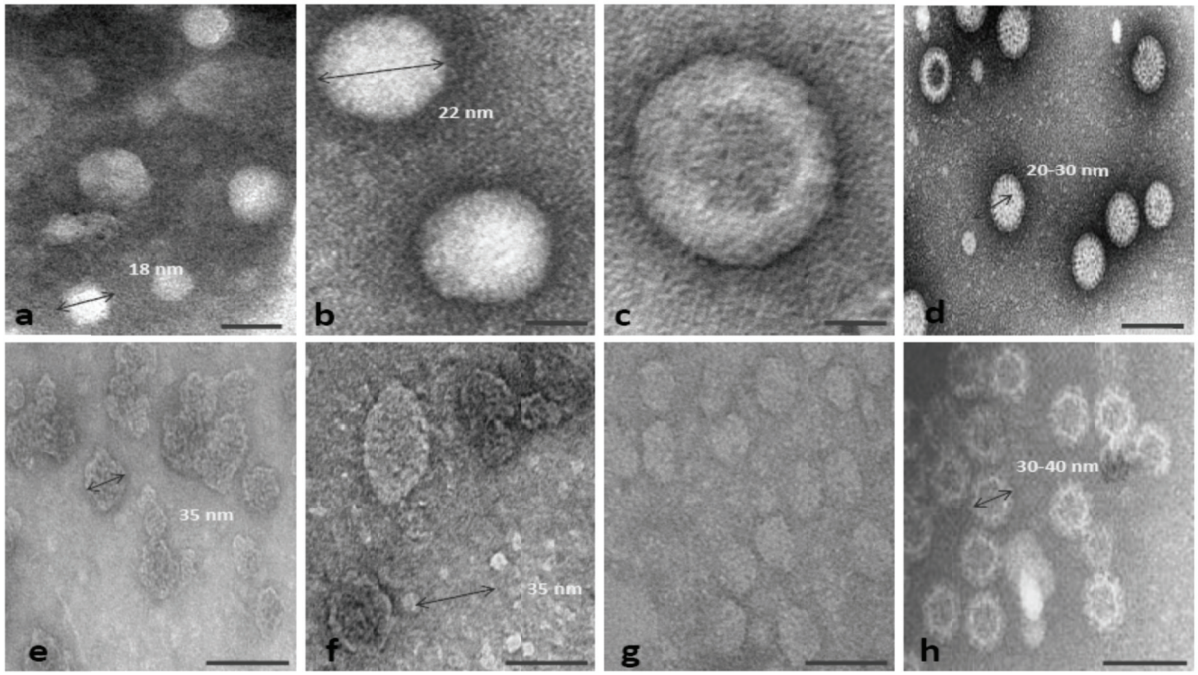

Figure 3.17 TEM images of presumptive (a, b, c) Reoviridae virus-like particles, d) known Rotavirus, (e, f, g) presumptive Caliciviridae virus-like particles, h) known Norovirus virus, present at all the sampling sites during all seasons tested. Images captured at 500 000-600 000 X magnification. Scale Bar 50 nm.

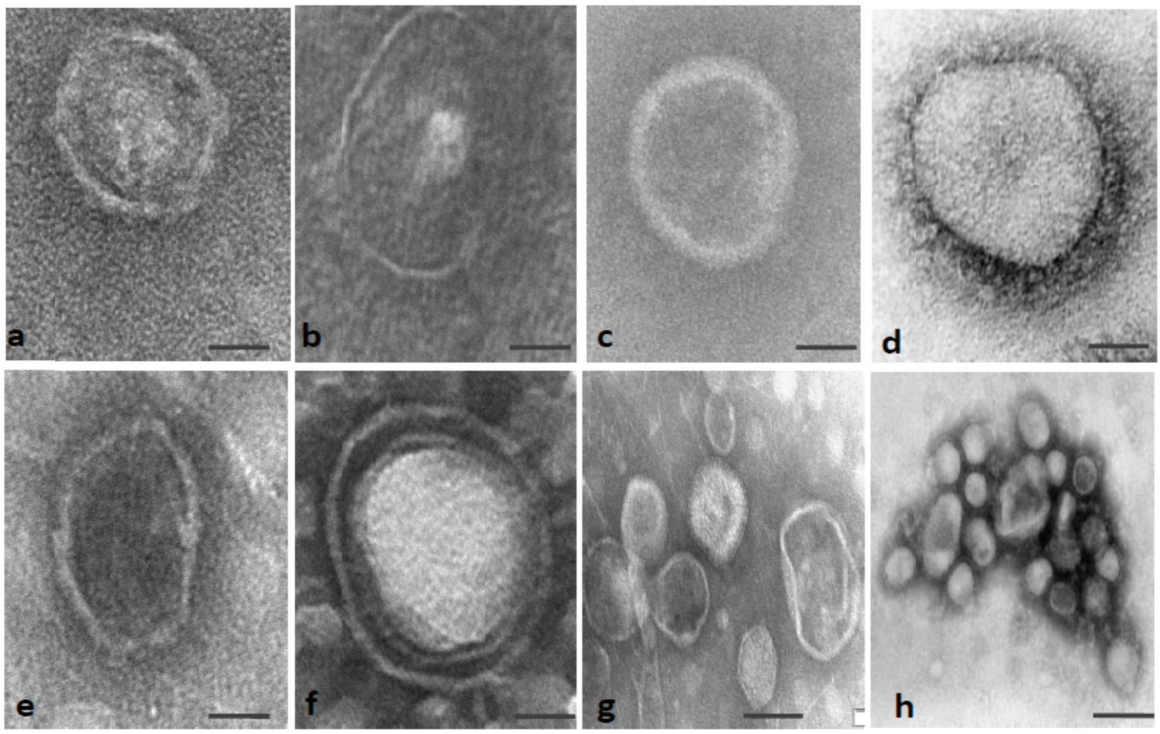

Figure 3.18 TEM images of presumptive enveloped virus-like particles (a,b,c) Coronaviridae virus-like particles, d) known Coronavirus, (e,f,g) presumptive Orthomyxoviridae virus-like particles, h) known Influenza virus, present in the Umgeni River at the different sampling sites during all seasons tested. Images captured at 400 000-600 000 X magnification. Scale Bar 100 nm.

Great morphological variability was observed in the virus assemblage's from the Umgeni River. Most of the VLPs were detected downstream of the river towards the river mouth area during the spring and summer seasons. The viral contamination of these widely used areas could be attributed to storm water discharge, surface runoff, sewage discharge and overflows, recreational exposure and other anthropogenic activities. The detection of viruses by TEM in the sampling points upstream of the Umgeni River was relatively low or non-existent, probably due to the stagnant waters and limited recreational exposure. Human viruses seen in negative stains fall into one of two major morphological categories: enveloped or naked (Zechmann and Zellnig, 2009). Enveloped viruses have a nucleocapsid (the nucleic acid held together by some structural proteins) inside, whilst naked viruses are icosahedral; their protein coat or capsid is more rigid and withstands the drying process well to maintain their spherical structure in negative stains (Ackermann and Heldal, 2010). Naked human viruses are of three size ranges: 1) 22 to 35 nm (e.g., parvoviruses, enteroviruses, and caliciviruses); 2) 40 to 55 nm (polyomaviruses and papillomaviruses) and 3) 70 to 90 nm (reoviruses, rotaviruses, and adenoviruses) (Zechmann and Zellnig, 2009). The negative staining technique for TEM allowed for the examination of particulate material including determination of structure and size of particles and has proved important in virological studies (Ackermann and Heldal,

2010). All water samples analysed were environmental in nature, thus natural degradation may have altered the morphological features of the viruses substantially.

3.6 Viral Infectivity Using Cell-Culture

The application of PCR-based molecular technology (discuss in the later section) and TEM has advanced our knowledge of the occurrence and prevalence of human viruses in water. The interpretation, in terms of health implications, of the presence of viral genome detected by PCR remains difficult due to the fact that molecular methods do not distinguish between infectious and non-infectious viral particles. The tissue culture technique has been used extensively for virus replication studies and plaque assays. Vero (BGM) cells are most often the choice for the cell culture on virus detection in aquatic environments (Lee et al., 2005) and are sensitive to infection with many different viruses (Ammerman et al., 2009; APHS, 1995; Hoyt and Margolin, 2000). Human embryonic kidney cell line 293A and human lung carcinoma cell line A549 were the most sensitive, especially to enteric adenovirus 40 and 41 (Jiang et al., 2009). HepG2 cell lines and their derivatives are often used to study Hepatitis B, C and others (Bchini et al., 1990; Zekri et al., 2011). The tissue culture assay is the only USEPA approved method for virus infectivity monitoring in the aquatic samples (Jiang, 2006).

The Cytopathic Effect (CPE) refers to observable morphological (shape) changes in tissue cells due to viral infection. Figure 3.19 and Table 3.6 illustrates the results obtained for the CPE of the virus-like particles obtained from the Umgeni River using three cell lines. Confluent monolayers of un-infected cell lines are illustrated in Figure 3.19 (a, b, c), once viral infection of the cell monolayer's occurred they started to round off and appear circular as shown in Figure 3.19 (d, e, f), an indication of cell death. All virus-like particles isolated from all water samples during all four seasons had positive cytopathic effects (cell death) on the Vero cell line (Table 3.6). All the water samples from Sites 1, 2 and 3 (except the autumn sample) were capable of inducing the cytopathic effects of all tissue cell lines. The water samples from Site 5 failed to produce CPE of Hep-G2 cell line except the summer sample. The VLPs isolated during the summer season had CPE on all three cell lines tested. These results strongly indicate the potentials of viruses in the water samples especially from the lower catchment areas to infect the human hosts throughout the year. These observations have serious health care implications.

The VLPs caused substantial CPE on the VERO cell line during all seasons and sites tested (Figure 3.20), with increased CPE of 66.37 %, 79.50%, and 79.14% at sites U1, U2 and U3 respectively during summer. It is generally known that not all viral genomes detected correspond to infectious viral particles and a high percentage of non-infectious viral particles

may be present in the environment. However, the ability of these viruses to infect susceptible host cells and to replicate their RNA presented in this study, confirms that they are viable and infectious and therefore constitute a health risk which should be investigated (Grabow, 2001; Reynolds et al., 1997).

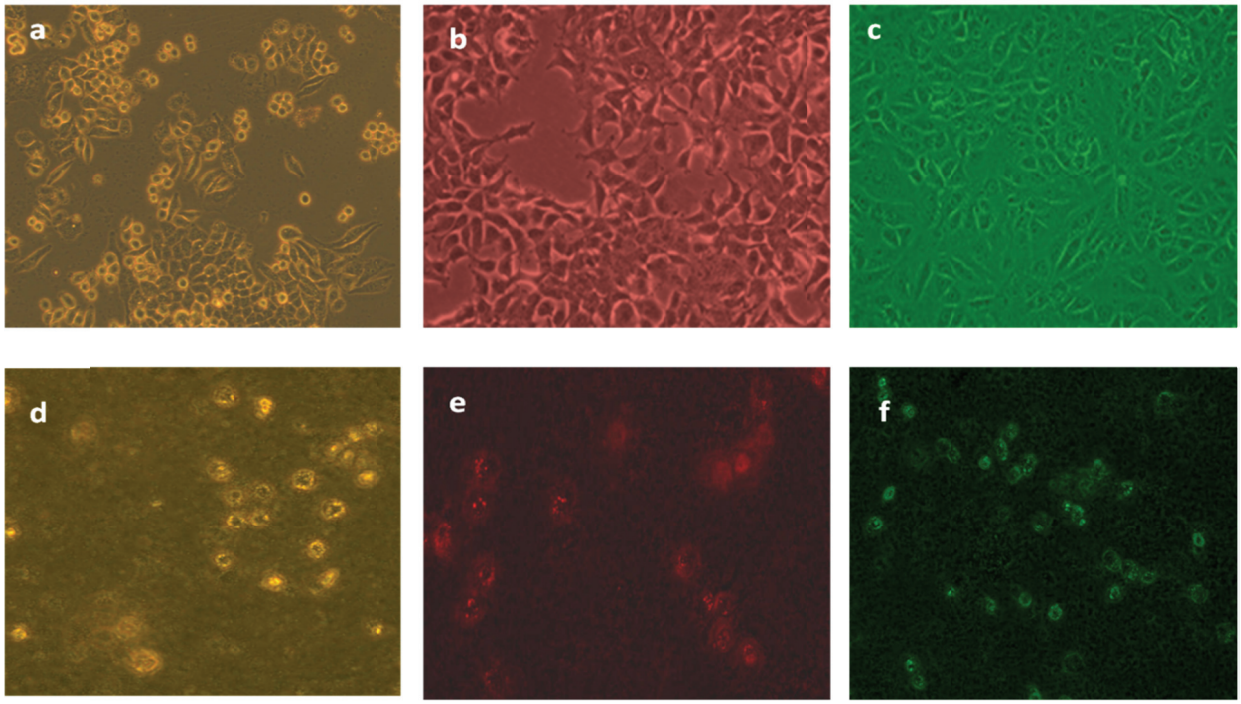

Figure 3.19 Various cell lines and their associated Cytopathic effect (CPE) from VLPs present in the Umgeni River. (a) Hep-G2 cell line, (b) HEK cell line, (c) Vero cell line. CPE of the VLPs after growth at 6 days on the (d) Hep-G2 cell line, (e) HEK cell line, (f) Vero cell line. Images captured at 400 X magnification.

Table 3.6 Cytopathic effect (CPE) of the concentrated virus-like particles on various cell lines for all sites along the Umgeni River during all seasons.

SAMPLE		CPE OF VLPS ON CELL LINES		
		VERO	HEP-G2	HEK 293
Rotavirus		+	+	-
Adenovirus		-	+	+
Coxsackievirus		+	-	+
AUTUMN	U1	+	+	+
	U2	+	+	+
	U3	+	-	+
	U4	+	-	-
	U5	+	-	-
WINTER	U1	+	+	+
	U2	+	+	+
	U3	+	+	+
	U4	+	-	+
	U5	+	-	+
SPRING	U1	+	+	+
	U2	+	+	+
	U3	+	+	+
	U4	+	+	+
	U5	+	-	-
SUMMER	U1	+	+	+
	U2	+	+	+
	U3	+	+	+
	U4	+	+	+
	U5	+	+	+

Cytopathic Effect: +: Cell Death; -: No Cell Death

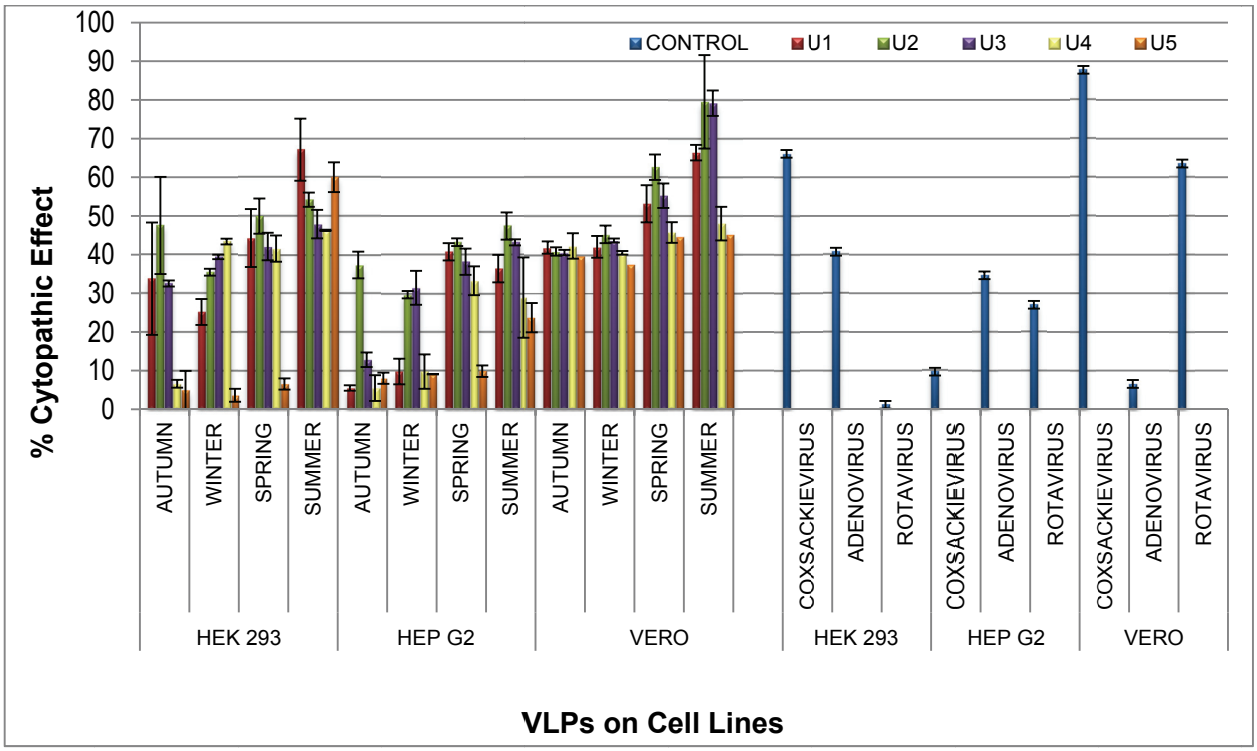

Figure 3.20 Cytopathic effect (CPE) of the concentrated virus-like particles on various cell lines for the Umgeni River at the different sampling sites during all seasons tested. Bars indicate the average of replicate samples (n = 2 or 3) while the error bars show the standard deviation.

3.7 PCR/RT-PCR

In the present study, molecular techniques were successfully used to detect human Adenoviruses, Enteroviruses, Rotaviruses and Hepatitis B viruses in the Umgeni River water samples. The sensitivities of the assays used to identify the viruses under investigation ranged from 100 targets to 10,000 targets and were similar to those reported previously (Symonds et al., 2009). The use of two phases of amplification provided higher specificity and sensitivity for the detection of different virus types. This fact was observed in the studies carried out by Puig et al. (1994) and Vantarakis and Papapetropoulou (1999), in which 11 and 9 environmental samples, respectively, were negative according to the first PCR and positive after the second amplifications. In this study, all the investigated virus types have been detected in the environmental water samples throughout the study period, thus confirming a high level of viral pollution in this water source.

Adenoviruses, enteroviruses and rotaviruses are stable in the environment with transmission occurring through ingestion of contaminated water or food and contact with contaminated surface. They are important causes of severe diarrhoea especially among infants and young children. All these enteric viruses have been detected in the Umgeni River during this study

period. Adenovirus from the river water samples were successfully amplified with both sets of primers. This resulted in DNA bands of the expected size, and semi-nested PCR allowed for their detection in less than 12 hours (Figure 3.21 a, b). PCR amplification of the 5'-untranslated region of the Enterovirus genome (Fong et al., 2005) yielded 100% PCR recovery of the expected Enterovirus genome size at all the sampling points during all seasons the Umgeni River (Figure 3.22). At the same time, the VP7 gene of group A Rotaviruses (Figure 3.23) and the S gene of Hepatitis B viruses (Figure 3.24) were also detected in all the VLPs isolated from the river during the study period. These results are strongly supported by all types of enteric viruses-like particles of the TEM results and the CPE effects of various tissue cultures demonstrated above.

Detection of Hepatitis B viruses (HBVs) in all water samples from the Umgeni River during this study period is somehow unexpected. This virus is transmitted through contact with the blood or other body fluids of infected person, not through ingestion of contaminated water or food (WHO, 2012). The hepatitis B virus is 50 to 100 times more infectious than HIV. It is estimated that 2 billion individuals worldwide are infected with this virus, which causes 620 000 deaths each year. There are currently between 3 and 4 million South African blacks who are chronically infected with HBV (Kew, 2008). Finding HBVs in water raises the concerns that HBV might be transmitted in the natural water environments via breaks in the skin.

In this study, the investigators detected the presence of adenoviruses, enteroviruses, rotaviruses as well as Heptatitis B viruses in all samples from all seasons. The results of tissue culture technique and PCR in this study strongly indicate the prevalence of enteric viruses in the natural water sources such as the Umgeni River is higher than those studies reported earlier in South Africa (Ehlers et al., 2005; Grabow et al., 2004; Van Heerden et al., 2003, 2004; Van Zyl et al., 2004, 2006; Vivier et al. 2004; Taylor et al., 2001). The high detection rate might be partially due to the efficiency of virus recovery rate using a TFF system. The results of canonical correspondence analysis in this study clearly indicate that the temperature and others have strong influences in the bacterial and viral populations in the Umgeni River. It might also be possible that increasing temperatures and other physico-chemical parameters in recent years results in changing weather patterns and stimulates the prevalence of viral populations in the natural environments. Skraber et al. (2005) point out that viruses might accumulate in biofilms from bypassing water over time. In addition, many other virus-like particles similar to the Coronaviridae, Orthomyxoviridae, pox viruses and herpes viruses were also observed in the water samples from the Umgeni River. High levels of potential pathogenic bacteria and viruses detected in this catchment area especially in the lower reach cause a serious concern in health care.

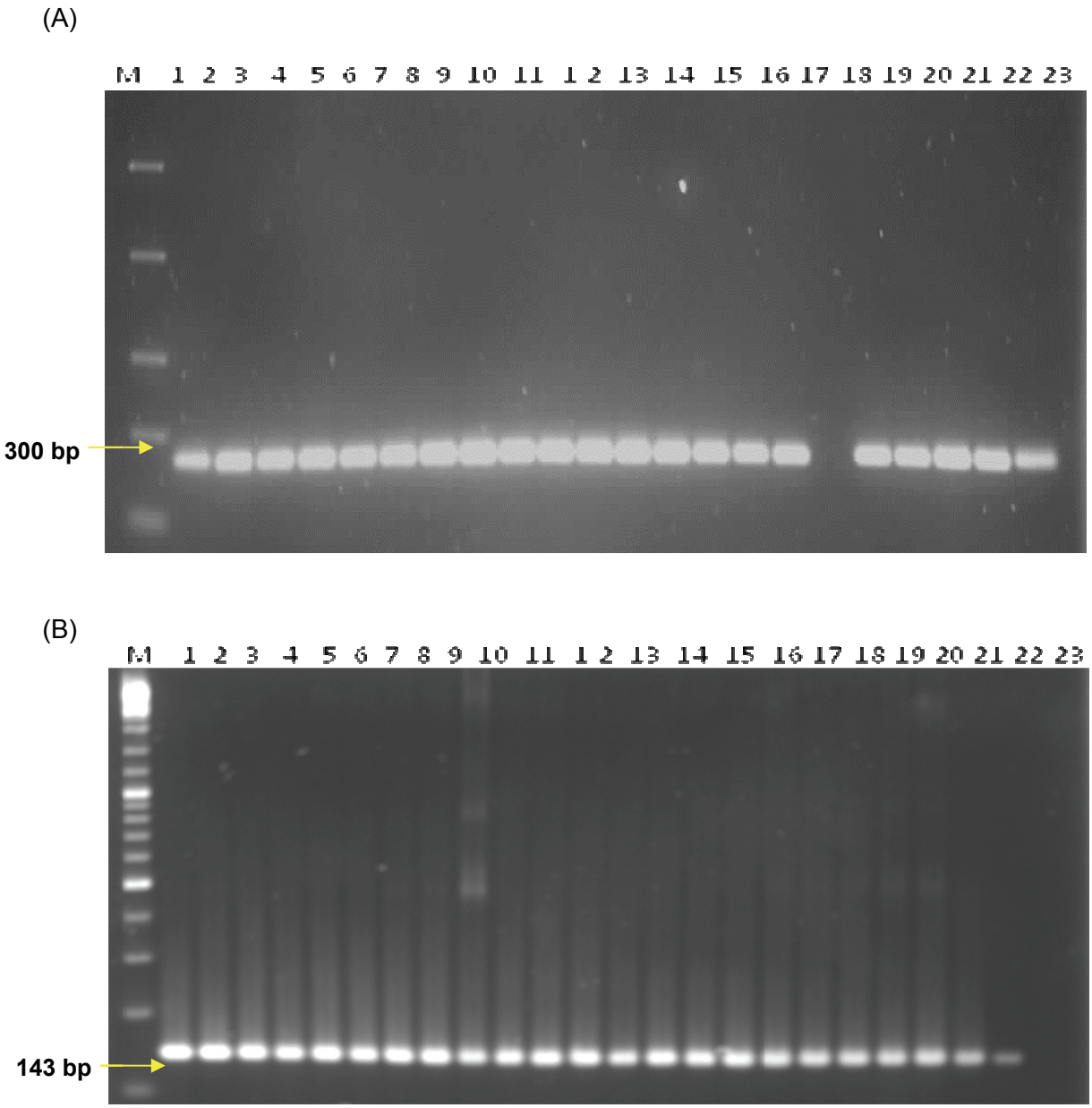

Figure 3.21 Normal PCR (A) and nested PCR (B) amplification of the hexon gene of 47 different Adenovirus serotypes. M: Molecular weight marker, L1: Adenovirus Control, L2-L6: Points for autumn season, L7-11: Points for winter season, L12 -16:-Points for spring season, L17-22: Points for summer season, L23: Negative Control.

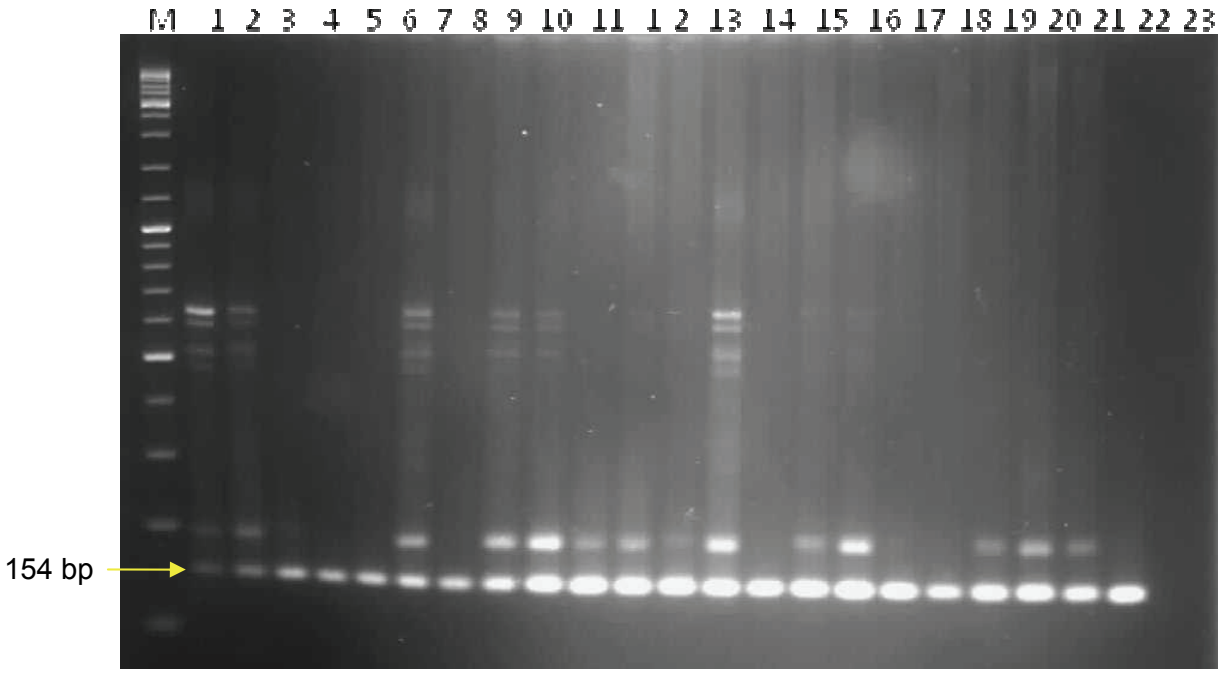

Figure 3.22 Nested PCR amplification of the 5'-untranslated region of the Enterovirus genome detecting at least 25 different Enteroviruses. M: Molecular weight marker, L1: Enterovirus Control, L2-L6: Points for autumn season, L7-11: Points for winter season, L12 -16:-Points for spring season, L17-22: Points for summer season, L23: Negative Control.

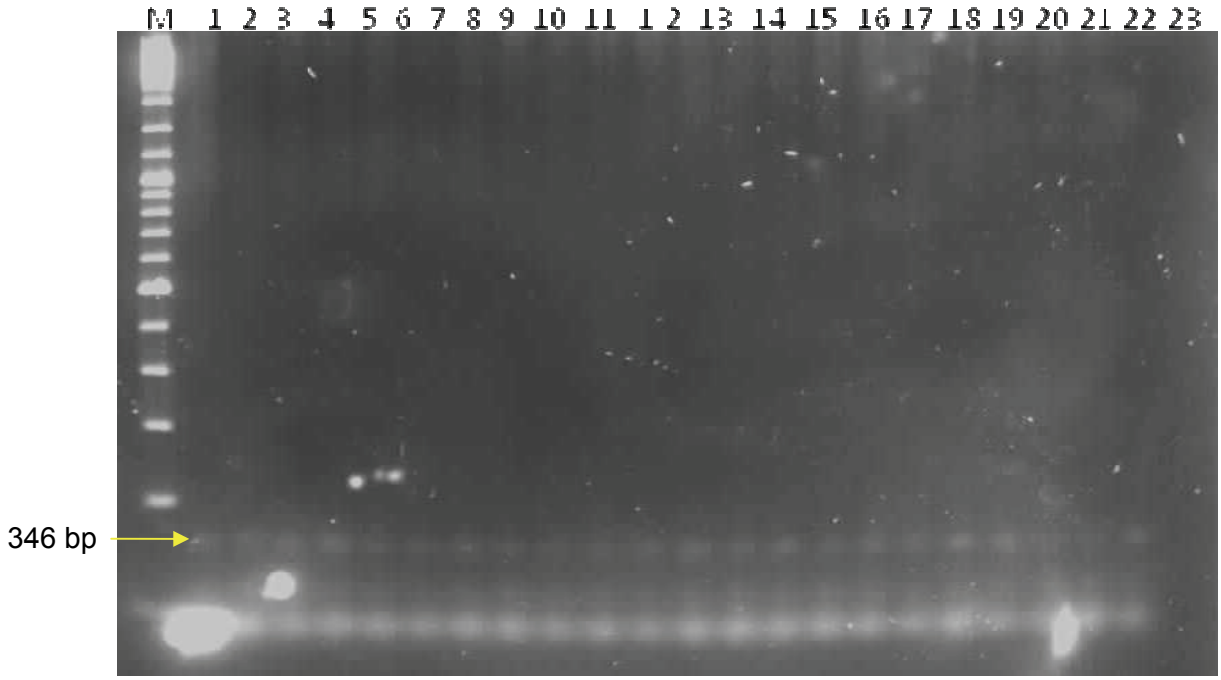

Figure 3.23 Nested PCR was used to amplify the VP7 gene of group A Rotaviruses. M: Molecular weight marker, L1: Rotavirus Control, L2-L6: Points for autumn season, L7-11: Points for winter season, L12 -16:-Points for spring season, L17-22: Points for summer season, L23: Negative Control.

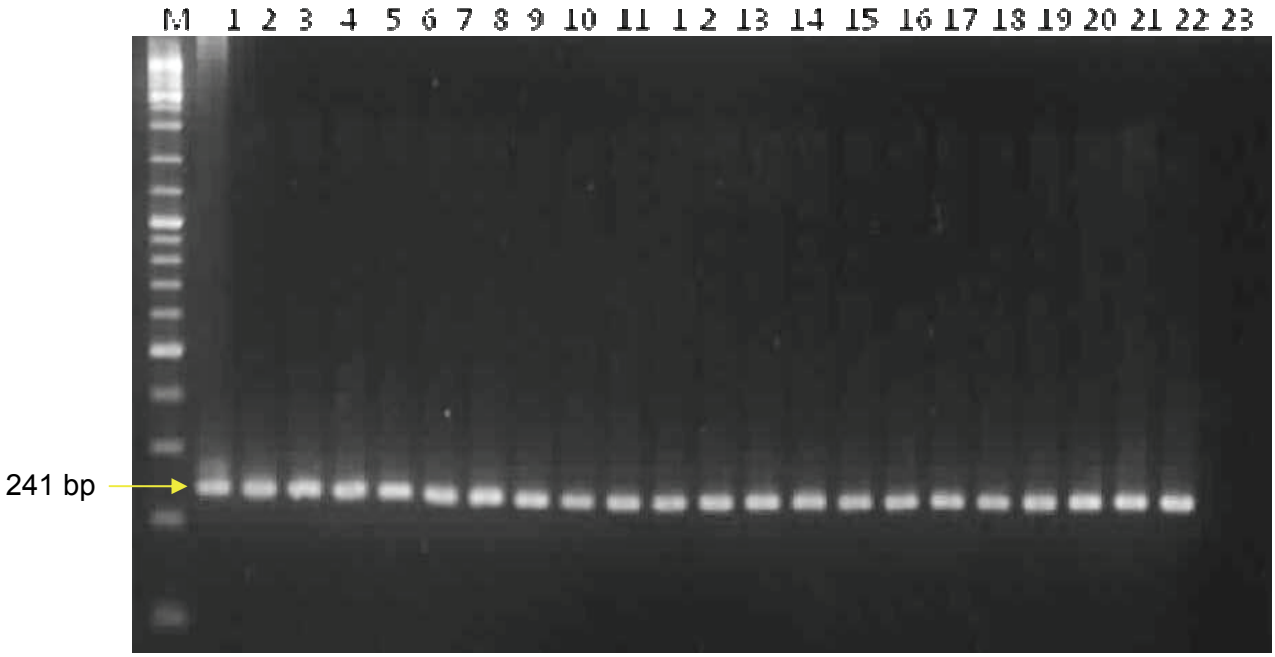

Figure 3.24 Nested PCR was used to amplify the S gene of Hepatitis B viruses. M: Molecular weight marker, L1: Hepatitis B Control, L2-L6: Points for autumn season, L7-11: Points for winter season, L12 -16: Points for spring season, L17-22: Points for summer season, L23: Negative Control.

4 CONCLUSIONS

- .. This study serves as a reminder of the importance of well-functioning drinking water treatment plants. Furthermore, although river water is never managed to achieve drinking water quality, the results would also raise concerns for those who may consume water directly from the river without any form of treatment.

- .. The microbiological and physico-chemical qualities of the Umgeni River in Durban, South Africa did not meet the target water quality ranges of Total Coliforms (TC), Faecal Coliforms (FC), Enterococci (EC) and Faecal Streptococci (FS) levels for the recreational and drinking uses according to DWAF.

- .. The presumptive bacterial pathogens such as *Salmonella* spp., *Shigella* spp. and *Vibrio cholerae* were also detected in all the water samples. In addition, most of the water samples contained high populations of somatic coliphages, FRNA coliphages and many other virus-like particles (VLPs) and demonstrated their infectivity abilities of various human tissue cultures.

- The results of CCA analyses demonstrate that pH, electrical conductivity, temperature, and turbidity were correlated with the microbial community structures in the Umgeni River water.

- Many presumptive pathogenic enteric viruses such as adenoviruses, enteroviruses, rotaviruses and Hepatitis B viruses were detected in all water samples using PCR/RT-PCR. The above water samples demonstrated their infectivity abilities of various human tissue cultures.

- The present study highlights the importance of routine environmental surveillance of pathogenic bacteria and human enteric viruses. This can contribute to a better understanding of the actual burden of disease on those who might be using the water directly without treatment. The study also suggests a need to monitor the actual viruses present in addition to the traditional indicators.

- Since a “gold standard” for the detection of viruses in environmental samples is not yet defined, the incidence of viral contamination might be underestimated by the current methods.

5 RECOMMENDATIONS

- Statistical tools provide an objective interpretation of correlations between surface water quality variables, and should be incorporated into water quality monitoring systems more routinely. However, the implication of a cause and effect relationship for each correlation should not be taken for granted.

- It is recommended that the infectivity of viruses in river water be established using Integrated Cell Culture Reverse Transcription-Polymerase Chain Reaction (ICC-RT-PCR) method. This approach overcomes most of the disadvantages associated with both conventional cell culture and direct PCR assays, reducing the time needed for the detection of infectious viruses.

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APPENDIX i:

Table a: Properties of the Canonical Correlation Analysis ordination bi-plot for bacterial indicators and water quality variables at the five study sites during autumn, winter, spring and summer seasons.

Canonical properties	Axis	
	1	**2**
Canonical Eigen value	0.059	0.011
% Cumulative variance of species data	50.7	60.5
% Cumulative variance of species – environment relation	83.7	100
Monte Carlo test p – value	0.059	0.070
Pearson correlation of species and environmental Scores	0.759	0.908

Table b: Properties of the Canonical Correlation Analysis ordination bi-plot for all the water quality variables and the total bacterial growth at the five study sites during autumn, winter, spring and summer seasons.

Canonical properties	Axis	
	1	**2**
Canonical Eigen value	0.059	0.011
% Cumulative variance of species data	50.7	60.5
% Cumulative variance of species – environment relation	83.7	100
Monte Carlo test p – value	0.059	0.070
Pearson correlation of species and environmental Scores	0.759	0.908

Table c: Properties of the Canonical Correlation Analysis ordination bi-plotfor bacterial indicators and heavy metal variables at the five study sites during autumn, winter, spring and summer seasons.

Canonical properties	Axis	
	1	**2**
Canonical Eigen value	0.023	0.006
% Cumulative variance of species data	18.5	23.7
% Cumulative variance of species – environment relation	73.3	93.7
Monte Carlo test p – value	0.023	0.07
Pearson correlation of species and environmental Scores	0.775	0.524

Table d: Properties of the Canonical Correlation Analysis ordination bi-plot for the heavy metal quality variables and the total bacterial growth at the five study sites during autumn, winter, spring and summer seasons.

Canonical properties	Axis	
	1	**2**
Canonical Eigen value	0.048	0.002
% Cumulative variance of species data	63.5	25.3
% Cumulative variance of species – environment relation	100	0
Monte Carlo test p – value	0.048	0.05
Pearson correlation of species and environmental Scores	0.259	0

Table e: Properties of the Canonical Correlation Analysis ordination bi-plot for all the water quality variables and the total viral and bacteriophage growth at the five study sites during autumn, winter, spring and summer seasons.

Canonical properties	Axis	
	1	**2**
Canonical Eigen value	0.046	0.038
% Cumulative variance of species data	77.6	89.4
% Cumulative variance of species – environment relation	83.6	94.1
Monte Carlo test p – value	0.048	0.062
Pearson correlation of species and environmental Scores	0.955	0.962

Table f: Properties of the Canonical Correlation Analysis ordination bi-plot for bacteriophage and virus populations and water quality variables at the five study sites during autumn, winter, spring and summer seasons.

Canonical properties	Axis	
	1	**2**
Canonical Eigen value	0.043	0.021
% Cumulative variance of species data	60.5	98
% Cumulative variance of species – environment relation	83.7	94.6
Monte Carlo test p – value	0.065	0.048
Pearson correlation of species and environmental Scores	0.759	0.980

Table g: Properties of the Canonical Correlation Analysis ordination bi-plot for bacteriophage and virus populations and bacterial indicators at the five study sites during autumn, winter, spring and summer seasons.

Canonical properties	Axis	
	1	**2**
Canonical Eigen value	0.039	0.024
% Cumulative variance of species data	79.7	91.1
% Cumulative variance of species – environment relation	94.2	98.2
Monte Carlo test p – value	0.051	0.042
Pearson correlation of species and environmental Scores	0.701	0.943