# Surveillance of Microbial Pathogens in the Umgeni River, Durban South Africa

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**Submitted in fulfilment of the academic requirements for the degree of Doctor of Philosophy in the Discipline of Microbiology; School of Life Sciences, College of Agriculture, Engineering and Science at the University of KwaZulu-Natal, Westville Campus.**

**As the candidate's supervisor, I have approved this thesis for submission.**

**Signed:_____ Name: Professor Johnson Lin Date:_____**

**PREFACE**

The experimental work described in this dissertation was carried out in the Discipline of Microbiology, School of Life Sciences, College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Durban, South Africa from March 2010 to November 2012, under the supervision of Professor Johnson Lin.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

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I, **Mrs**. **Atheesha (Ganesh) Singh**, declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

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**DECLARATION 2 - PUBLICATIONS**

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication)

**Publication List**

**Publication 1**

**Title:** Waterborne Human Pathogenic Viruses of Public Health Concern

**Authors:** Atheesha Ganesh and Johnson Lin

**Journal:** International Journal of Environmental Health Research

**Published:** online 2013- http://dx.doi.org/10.1080/09603123.2013.769205

**Contributions:** Miss Atheesha Ganesh performed the writing of the review article. Professor Johnson Lin performed the editing of the article and submission to the journal. The article forms part of Chapter One of the thesis.

**Publication 2**

**Title:** Water Quality Indicators: Bacteria, Coliphages, Enteric Viruses

**Authors:** Johnson Lin and Atheesha Ganesh

**Journal:** International Journal of Environmental Health Research

**Published:** online 2013- http://dx.doi.org/10.1080/09603123.2013.769201

**Contributions:** Both authors contributed equally to the writing of the review article. Professor Johnson Lin submitted the article to the journal. The article forms part of Chapter One of the thesis.

**Publication 3**

**Title:** Canonical Correspondence Analysis (CCA) for the microbiological, physico-chemical and heavy metal assessment of the Umgeni River-South Africa

**Authors:** Atheesha Ganesh and Johnson Lin

**Journal:** Water SA

**Published:** Submitted Revision

**Contributions:** Miss Atheesha Ganesh performed all experimental work and the writing of the article. Professor Johnson Lin performed the editing of the article and submission to the journal. The article forms Chapter Two of thesis.

**Publication 4**

**Title:** Microbial Pathogens in the Umgeni River- WRC Report No. K8/990

**Authors:** Johnson Lin, Atheesha Ganesh and Moganavelli Singh

**Journal:** Water Research Commission- Technical Report

**Published:** In Press

**Contributions:** Miss Atheesha Ganesh performed all experimental work and the writing of the report. Professor Johnson Lin performed the editing of the report and submission to the journal. Dr Singh assisted in tissue culture experimentation. The technical forms part of chapter One, Two, Three and Four of the thesis.

**Publication 5**

**Title:** Detecting the Presence of Virus-like Particles (VLPs) from the Umgeni River-South Africa

**Authors:** Atheesha Ganesh and Johnson Lin

**Journal:** Clean-Soil, Air, Water

**Published:** Submitted Revision

**Contributions:** Miss Atheesha Ganesh performed all experimental work and the writing of the article. Professor Johnson Lin performed the editing of the article and submission to the journal. The article forms chapter three of the thesis.

**Publication 6**

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**Authors:** Atheesha Ganesh and Johnson Lin

**Journal:** Environment International

**Published:** Submission

**Contributions:** Miss Atheesha Ganesh performed all experimental work and the writing of the article. Professor Johnson Lin performed the editing of the article and submission to the journal. The article forms part of Chapter Three and Four of the thesis.

**Conference Presentations**

**Conference 1**

**Title:** Detection of human pathogenic viruses in the Umgeni River of Durban, South Africa

**Authors:** Atheesha Ganesh and Johnson Lin

**Conference:** 2011 South African Society For Microbiology (SASM) Meeting, Southern Sun Cape Sun Hotel, Cape Town, South Africa, 6-9 November 2011

**Contributions:** Oral Presentation

**Conference 2**

**Title:** Detection of human pathogenic viruses in the Umgeni River of Durban, South Africa

**Authors:** Atheesha Ganesh and Johnson Lin

**Conference:** 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 congress), Champagne Sports Resort, Drakensberg, KZN, South Africa 29 January-1 February 2012.

**Contributions:** Poster Presentation

**Conference 3**

**Title:** Detection of human pathogenic viruses in the Umgeni River of Durban, South Africa

**Authors:** Atheesha Ganesh and Johnson Lin

**Conference:** First Biotechnology World Congress, Dubai Men's College, Dubai, 14-15 February 2012

**Contributions:** Poster Presentation

**Conference 4**

**Title:** Detecting the Presence of Virus-like Particles (VLPs) from the Umgeni River-South Africa

**Authors:** Atheesha Ganesh and Johnson Lin

**Conference:** School of Life Sciences and Engineering First Research Day, College of Agriculture, Engineering and Science, PMB Campus- UKZN, 29 October 2012.

**Contributions:** Poster Presentation

**Conference 4**

**Title:** Evaluation of the virus Population in the Umgeni River-South Africa

**Authors:** Atheesha Ganesh and Johnson Lin

**Conference:** 2nd International Water Research Conference, Singapore, 20-23rd January 2013

Contributions: Poster Presentation

Signed:

_____

Atheesha (Ganesh) Singh

*Declaration Publications FHDR 22/05/08 Approved*

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**DEDICATION**

***This work is dedicated to my beloved parents, father Diplall Ganesh and mother Reetha Devi Ganesh, for their love, endless support and prayers. Thank you for always believing in me and giving me the opportunity to dream big…***

**ABSTRACT**

This study assessed the quality of the Umgeni River in Durban South Africa seasonally from March 2011 to January 2012, according to standard protocol. Water samples were collected from Inanda dam-U5, KrantzKloof Nature Reserve-U4, New Germany-U3, Reservoir Hills –U2 and River mouth – U1 areas of the Umgeni River. A two-step tangential flow filtration (TFF) process was setup for the concentration of viruses from water samples. Virus like particles (VLPs) was detected using electron microscopy. Canonical correspondence analysis (CCA) was used to statistically evaluate the data sets. All water samples had turbidity values which exceeded the South African water quality guideline value of 0.1 NTU for turbidity. Large seasonal variations in $BOD_5$, COD and conductivity levels were observed. Chloride concentrations were extremely high at point U1 (19234 mg/ℓ) Cl during summer. Total heterotrophic bacterial (THB) population was highest at 13.67 x $10^6$ cfu/100ml (U1 – summer). Enterococci (EC) concentrations were detected at points U1, U2, U3, and U4 during the autumn and spring period. pH, electrical conductivity, temperature, and turbidity positively correlated with the microbial communities, and were the key parameters responsible for water pollution according to CCA. Most water samples contained high populations of somatic (659 pfu/mℓ, U1 – summer) and F-RNA coliphages (550 pfu/mℓ, U2 – summer). VLPs were detected throughout all seasons, with point U1 (summer) having the highest population of 2086 VLP/mℓ. Several presumptive viruses including Adenoviridae, Picornaviridae, Poxviridae, and Reoviridae were detected based on their morphologies. Six cell culture lines were used to determine cytopathic effect (CPE) of the VLPs. VLP samples produced CPEs on the Vero, Hek 293, Hela and A549 cell lines. Integrated cell culture (ICC) - PCR confirmed the presence of infectious VLPs in the river water samples. Adenoviruses, Enteroviruses, rotaviruses and Hepatitis B viruses were detected and quantified in all water samples by nested PCR/RT-PCR and Real-Time PCR respectively, against positive control viruses. These results 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 have public health care implications and establish a need to monitor the viral population in addition to traditional water quality indicators.

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# CHAPTER ONE

## 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 *et al.,* 2007; 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.

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). 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. 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). 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.,* 1991a,b; 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.,* 2003). 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; Karikari *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.

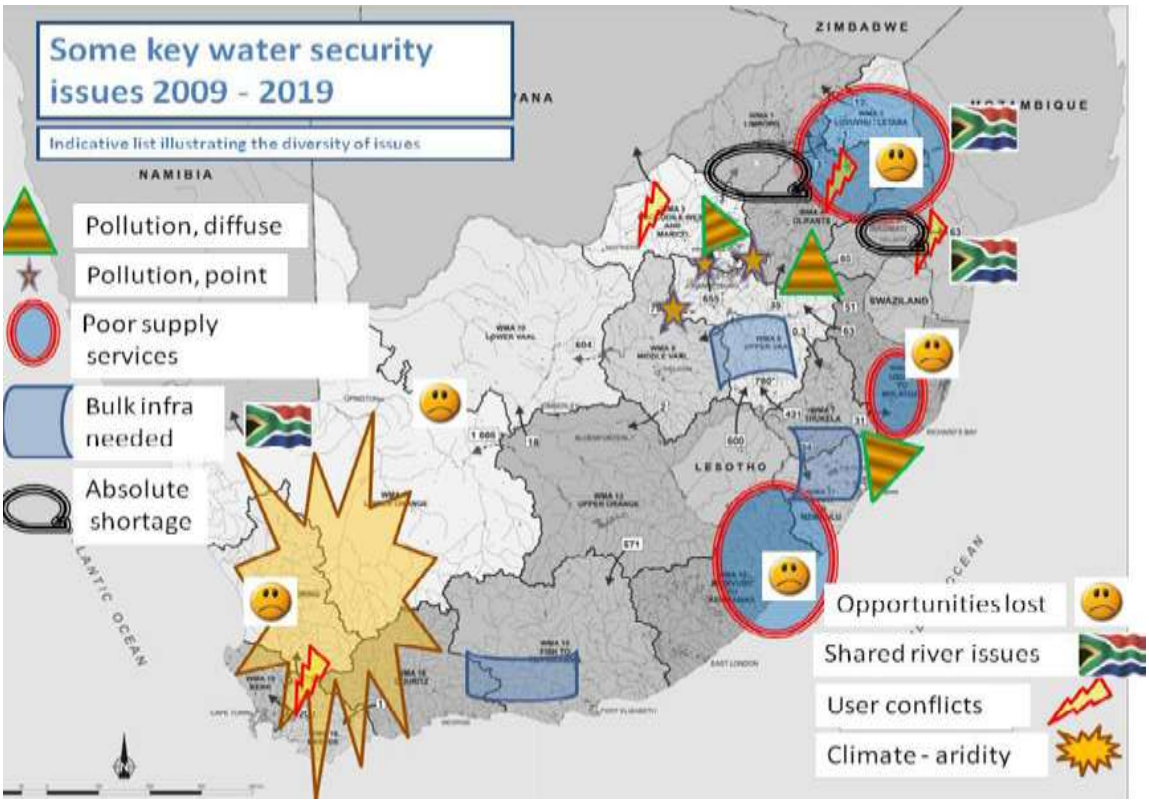


**Figure 1.1.** Water quality challenges and security issues facing South Africa (Muller *et al.*, 2009).

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.*, 1991b).

## 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 Kaluarachchi, 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, 1996b; 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.

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 that set water quality targets to improve public health, as illustrated in Figure 1.2.

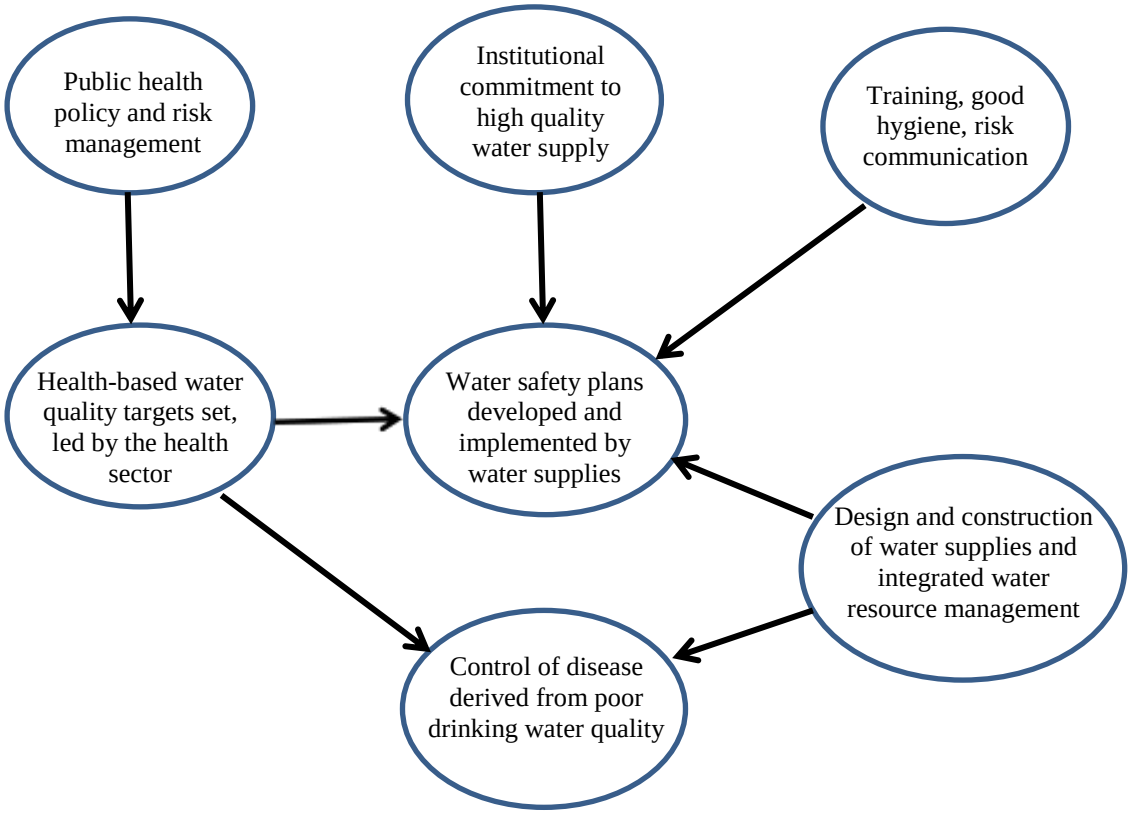


**Figure 1.2.** Protecting public health through ensuring drinking water quality (Davison *et al.*, 2002).

### 1.3.1 Microbial Indicators of Water Quality 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:

a. Be universally present in the faeces of humans and warm-blooded animals, in large numbers
b. Be readily detected by simple methods
c. Not grow in natural waters, the general environment or water distribution systems
d. 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, *E. 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 *Esherichia 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 describe as "All facultative anaerobic, gram-negative, non spore-forming, oxidase-negative, rod-shaped bacteria that ferment lactose to acid and gas within 48 h 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.,* 1991a,b). 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 hrs 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

To increase the assurance of water quality results, especially when monitoring for faecal pollution, analysis for enterococci has been used (Stevens *et al.,* 2003). The enterococci are the group of bacteria most often recommended as alternatives to coliforms, and importance in their use as a water quality indicators dates back to 1900 when they were found to be common commensal bacteria in the gut of warm-blooded animals (Gleeson and Gray, 1997). The enterococci were integrated in the functional group of bacteria known as "faecal streptococci" and now largely belong in the genus *Enterococcus* which was formed by the splitting of *Streptococcus faecalis* and *Streptococcus faecium*,

along with less important streptococci, from the genus *Streptococcus* (Schleifer and Klipper-Balz, 1984). There are now 19 species that are included as enterococci (Topley, 1997). In addition, other *Enterococcus* species and some species of *Streptococcus* (namely *S. bovis*, and *S. equinus*) may occasionally be detected in waters.

The term "faecal streptococci" refers to that streptococci commonly present in the faeces of humans and animals. The genus *Enterococcus* has recently been defined to include all streptococci sharing certain biochemical properties and having wide tolerance of adverse growth conditions (Stevens *et al.,* 2003). For water examination purposes enterococci/ faecal streptococci can be regarded as indicators of faecal pollution, since they have a number of advantages as indicators over total coliforms and even *E. coli* (Topley, 1997). These advantages include that they generally do not grow in the environment (WHO, 1993) and they have been shown to survive longer (McFeters *et al.,* 1974). Faecal streptococci rarely multiply in polluted waters and despite being approximately an order of magnitude less numerous than faecal coliforms and *E. coli* in human faeces (Feacham *et al.,* 1983), they are still numerous enough to be detected after significant dilution. Their main value in assessing water quality is therefore as an additional indicator of treatment efficiency. Furthermore, streptococci are highly resistant to drying and may be valuable for purpose of routine controls after new mains have been laid or distribution systems repaired, or for detecting pollution by surface runoff to ground water or surface waters. Rapid and simple methods, based on defined substrate technology, are available for the detection and enumeration of faecal streptococci/enterococci in MPN or MF techniques, based on their ability to grow in the presence of azide, and their fermentation of carbohydrates to produce lactic acid (WHO, 1993).

#### 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 (Igbinosa, 2010)

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 know 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 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). Somatic coliphages have been found to outnumber F-RNA phages in waste water and raw water sources by a factor of about 5, and cytopathogenic 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; Olivieri *et al.,* 1999).

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.,* 2006). 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 (CDCP) suggest that the causative agent of nearly 50% of all acute gastrointestinal illnesses is suspected to be viral (CDCP, 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, 2008; Okoh, 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; Tani *et al.,* 1995). 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 virus (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. The predominant factors affecting virus survival in the water environment are temperature, virus association with solids, exposure to UV and the presence of microbial flora (Bosch *et al.,* 2008). The effect of temperature on viral perseverance in water may be due to several mechanisms including virion protein denaturation, RNA damage, and influence on microbial or enzymatic activity (Bosch *et al.,* 2008).

Several studies have pointed out the role of groundwater as a source of viral outbreaks in countries of different economic level (Fong *et al.,* 2005). Enteric viruses are the most probable human pathogens to contaminate groundwater, due to their extremely small size, which allows them to penetrate soils from contamination sources such as broken sewage pipes and septic tanks, eventually reaching aquifers (Okoh *et al.,* 2010). Enteroviruses, noroviruses, rotaviruses and hepatitis A viruses

have been detected in many groundwater supplies, including private household wells, municipal wells and unconfined aquifers (Borchardt *et al.,* 2003). In these studies, the authors identified nearby surface waters or septic tanks as the most probable sources of contamination. Groundwater has been associated as a common transmission route for waterborne transferable disease in the United States with about 80% of viral waterborne outbreaks being attributed to drinking contaminated well water (Borchardt *et al.,* 2003). The enteric viruses regularly associated with these outbreaks were noroviruses and hepatitis A virus.

Potential adsorbents of viruses in natural waters include sand, pure clays (e.g., montmorillonite, illite, kaolinite, and bentonite), bacterial cells, naturally occurring suspended colloids, and estuarine silts and sediments (Okoh *et al.,* 2010). Removal rates depend to a great extent on the pH, substrate saturation, and redox potential and dissolved oxygen of the system.

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 analyzing 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, 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 metagenome DNA sequence data are correct, then viruses represent the largest reservoir of unknown genetic diversity on Earth (Wommack, 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).

Surface water can be contaminated with enteric viruses by a variety of sources. Enteric 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.

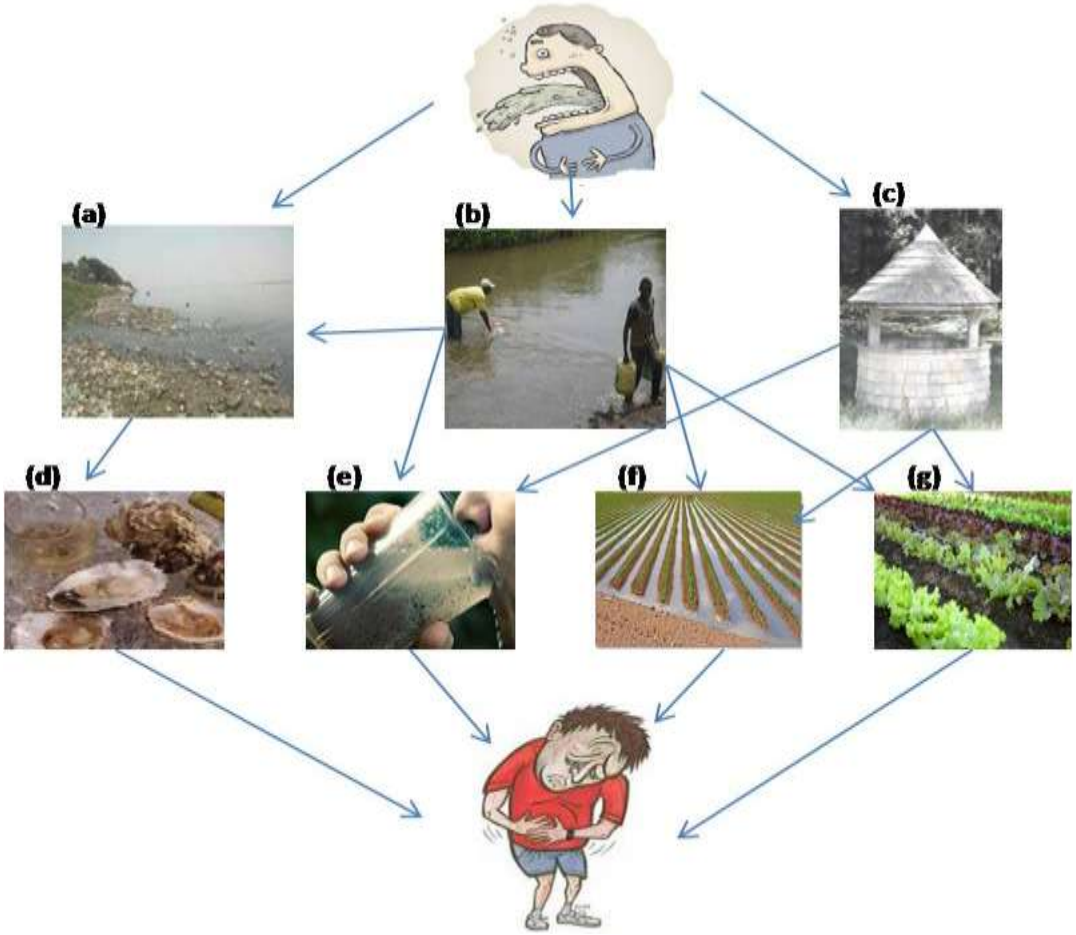


**Figure 1.3.** Probable routes of waterborne transmission of enteric viruses (Bosch *et al.,* 2008). In consequence viral pathogens in vomit and faeces of infected individuals contaminate the marine environment (a), fresh water (b) and ground water (c). Mankind is then exposed to these enteric viruses 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

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 (Hollinger and Emerson, 2007), virus levels tend to decrease gradually after discharge into the aquatic environment and may be present in water for a long period (Okoh *et al.*, 2010). This implies that even at low levels of viral pollution, a meaningful risk of infection exists (DWAF, 2004 a). Current treatment practices are unable to provide virus-free wastewater effluents (Dongdem *et al.*, 2009). Wastewater treatment processes pertaining to the activated sludge process, oxidation ponds, activated carbon treatment, filtration, and lime coagulation and chlorination merely eradicate between 50% and 90% of viruses present in wastewater (Okoh *et al.*, 2010), thus allowing for a significant viral load to be released in effluent discharge. Due to their steadiness and perseverance, enteric viruses consequently become pollutants in environmental waters resulting in human exposure through pollution of drinking water sources and recreational waters, as well as foods. 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, 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; however 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). Viruses are accountable for 14% of gastroenteritis occurrences and 38% of illnesses associated with drinking water in the United States (U. S.) from 1999 to 2002 (Li *et al.*, 2010), and are on the 'contaminant candidate list' of the U.S. Environmental Protection Agency (EPA) for regulatory consideration for drinking water program (Federal Register, 2005). The outbreak of waterborne viral diseases has been reported extensively worldwide (Ye *et al.*, 2012). Approximately 655,965 human cases of infectious diarrhoea (excluding cholera, dysentery, typhoid, and paratyphoid) were reported in China during 2009, with 33,087 cases being diagnosed definitely in etiology, of which 92.79 % were caused by virus infection (Ye *et al.*, 2012). Rotavirus was the main infectious agent accounting for 92.58 % of the diarrheal cases, tailed by adenovirus accounting for 3.29 % (Ma *et al.*, 2010). Simultaneously, there were 488,955 cases of hand, foot, and mouth disease in China during 2008 and 1,155,525 cases in 2009 which were primarily caused by enterovirus 71 and coxackievirus A16 (Chang *et al.*, 2011). During this period, 0.2–1.2 % of the diseased cases expressed severe symptoms, with 2.6–10.8 % being fatal (Chang *et al.*, 2011).

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). Enteric viruses can cause a meaningful risk of infection in vulnerable individuals even at low levels of viral pollution (Fong and Lipp, 2005; Teunis *et al.,* 2008). The high prevalence of enteric viruses in surface water highlights the importance of assessing the water sources used for domestic purposes for viral contamination (Kiulial *et al.,* 2010). In one study, human AdVs 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.,* 2005b). 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 (African Rotavirus Symposium, 2002). In South Africa, the government loses about R3.4 billion annually, due to approximately three million diarrhoeal cases and 50 000 mortalities (Momba *et al.,* 2010). Diarrhoea is also considered as a signature hallmark of HIV/AIDS because over 80% of patients in developing countries suffer chronic diarrhoea (Momba *et al.,* 2010). A recent survey across seven of the nine provinces of South Africa revealed the failure of the majority of water treatment plants to produce drinking water at the points of treatment and in distribution systems (Momba *et al.,* 2009). The surveillance of surface water samples for enteric viruses becomes an important indicator of the level of human faecal pollution regardless of the socio-economic status of a country. This information can be used to further assess the public health risks associated with exposure to these water sources (van Heerden *et al.,* 2005; Venter *et al.,* 2007; Espinosa *et al.,* 2008).

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, and caliciviruses), *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), as shown in Table 1.1. The primary site of viral infection and replication is the intestinal tract.

**Table 1.1.** Common human enteric viral pathogens shed in faeces and found in aquatic environments (adapted from Pulford, 2005).

| Family | Genus | Popular Name | Disease caused |
|---|---|---|---|
| ***Picornaviridae*** | Enterovirus | Poliovirus | Fever, Meningitis, Paralysis |
| | | Coxsackievirus A, B | Fever, Heart Anomalies, Myocarditis, Respiratory Disease, Meningitis, Hand- Foot-and-Mouth Disease |
| | | Echovirus | Meningitis, Fever, Rush, Gastroenteritis, Respiratory Disease |
| | Hepatovirus | Hepatitis A | Hepatitis |
| ***Reoviridae*** | Orthoreovirus | Human reovirus | Unknown |
| | *Rotaviridae* | Human rotavirus | Gastroenteritis |
| ***Adenoviridae*** | Mastadenovirus | Human adenovirus | Conjunctivitis, Gastroenteritis, Respiratory Disease |
| ***Caliciviridae*** | Calicivirus | Human calicivirus | Gastroenteritis |
| | | Norwalk virus | Gastroenteritis, Fever |
| | | SRSV | Gastroenteritis |
| ***Astroviridae*** | Mamastrovirus | Human astrovirus | Gastroenteritis |
| | Parvovirus | Human parvovirus | Gastroenteritis |
| ***Coronoviridae*** | Coronavirus | Human coronavirus | Gastroenteritis, Respiratory Disease |
| | Torovirus | Human torovirus | Gastroenteritis |

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). Adenoviruses species 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). Adenovirus species F (Ad 40/41) has been recognized as an agent of 5–20% of acute gastroenteritis cases among infants and young children (Haramoto *et al.,* 2010), which can be attributed to consumption of faecal contaminated water and food (Chapron *et al.,* 2000; Dongdem *et al.,* 2009; Okoh *et al.,* 2010). It is estimated that more than 90% of the human population is seropositive for one or more serotypes of adenoviruses (Fong *et al.,* 2010). Pathogenicity differs according to species and serotype, and organ specificity and disease patterns appear to be serotype dependent (Wold and Horwitz, 2007; Larranaga *et al.,* 2007, Madisch *et al.,* 2006).

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 (Dongdem *et al.,* 2009; Okoh *et al*., 2010). HAdVs are present at a higher frequency in sewage than 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 faecal–oral, oral–oral and hand–eye contact transmission, as well as indirect transfer through contact with contaminated surfaces or shared utensils and inhalation of aerosols (Boone and Gerba, 2007; WHO, 2004). Adenoviruses have been linked to 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 and Lipp, 2005). Adenovirus resistance to purification and disinfection processes (ie. Chlorine and ultra violet (UV) inactivation) and the virus's long perseverance in the environment have increased the importance of monitoring adenoviruses from water (Thompson *et al.* 2003; Jiang, 2006). The increased UV resistance showed by AdVs may be related with the double stranded nature of their DNA genome, which if damaged, may be repaired by the host cell DNA-repair mechanisms (Jiang, 2006).

Adenovirus identification is generally based on virus isolation in cell culture, followed by antibody or antigen detection, and visualisation by electron microscopy (Fong and Lipp, 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 (van Heerden *et al.,* 2003; 2004; 2005a). HAdV have previously been detected in environmental samples by PCR-based

techniques (Albinana-Gimenez *et al.,* 2009; Bofill- Mas *et al.,* 2006; 2010). Although quantitative real-time PCR (qPCR) methods for the quantification of some HAdV serotypes in diverse environmental samples worldwide have been described (Bofill-Mas *et al.* 2010; Dong *et al.* 2010; Haramoto *et al.*, 2010), 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; 2005b). 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 and Lipp, 2005; Griffin *et al.,* 2003; Katayama *et al.,* 2008). Adenoviruses have been detected in surface water in Germany (Pusch *et al.*, 2005), southern California (Jiang and Chu, 2004) and in sewage and polluted river and dam water, as well as treated drinking water in Pretoria (van Heerden *et al.*, 2005b).

### 1.6.2 Enterovirus (EV)

The waterborne enteroviruses (EVs) 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). EVs are small RNA viruses with sizes ranging from 22-30 nm in diameter (Friedman-Huffman, 1998). Human enteroviruses are sub classified into polioviruses (PV, serotypes 1–3), coxsackieviruses group A (CAV, serotypes 1–22 and 24), coxsackieviruses group B (CBV, serotypes 1–6, echoviruses (ECV, serotypes 1–7, 9, 11–27, and 29–33) and enteroviruses 68–71 (EVs, 4 serotypes) (Ehlers *et al.* 2005b). Molecular techniques of enterovirus typing are becoming progressively accessible, thus allowing new enteroviruses to be continually identified, with enteroviruses 79-101 having been recently described (Oberste *et al.*, 2000). Carriers of EVs include raw sewage, sewage sediments, rivers receiving sewage, as well as treated sewage (Kocwa-Haluch, 2001). Enteroviruses are regularly found in several water types including natural water, 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; Vivier *et al.,* 2004). Surface and ground waters are used as a supply of domestic drinking water throughout most of the world, as well as for leisure and recreational activities; this often leads to the unintended ingestion of microbiologically contaminated water (Melnick,1976). Jean (1999) stated that the severity of the enterovirus outbreak in Taiwan in 1998 might in part have been related to the contamination of soil and groundwater. 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 and water. Enteroviruses are highly resistant to disinfection, tolerant to residual chlorine from sewage treatment (Wang *et al.,* 2005) and a wide range of salinities (Skraber *et al.,* 2004), enabling their survival in environmental waters. Most enteroviruses are readily inactivated at 42°C, although some sulfhydral reducing agents and magnesium cations can stabilize these viruses so that they are relatively stable at 50°C (Wang *et al.,* 2005).

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; Pang *et al.,* 2012). 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-based assays such as PCR, RT-PCR and real-time RT-PCR have been shown to be more advantageous than cell culture-based assays for the detection of enteric viruses in faecally contaminated water (Denis-Mize *et al.,* 2004; Greening and Hewitt, 2008; Miagostovich *et al.,* 2008). 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 were detected in river water samples tested in Brazil (Miagostovich *et al.,* 2008), France (Hot *et al.,* 2003), Germany (Pusch *et al.* 2005), Italy (Grassi *et al.,* 2010), Keyan (Kiulial *et al.,* 2010) and Netherlands (Rutjes *et al.,* 2009). The presence of enteroviruses (predominantly coxsackie B viruses) was also detected in various types of water samples (Ehlers *et al.,* 2005a; Vivier *et al.,* 2001) including treated drinking water samples (Ehlers *et al.,* 2005b; Vivier *et al.,* 2004). Human enteroviruses have been included in the European Union (EU) guidelines governing water quality as a parameter for assessing the degree of viral pollution of a water body (Kocwa- Haluch, 2001).

### 1.6.3 Hepatitis A Virus (HAV)

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 to 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). Hepatitis A virus (HAV) is prevalent to South Africa with epidemiological features of both the developed and developing countries being present (Schwab *et al.,* 1995). In high density, low socio-economic and predominantly black African communities where sanitation is insufficient, the infection is acquired sub-clinically in early

childhood where nearly 100% seropositivity by school going children acquire HAV immunity before the age of ten years, while in the higher socio-economic and predominantly white community more clinical infections are noted and immunity rises to about 50–70% in adults (Taylor *et al.*, 2001).

The two biotypes of HAV, i.e. human HAV and simian HAV, are the only members of the genus Hepatovirus (Hunter, 1997). HAV strains isolated from around the world constitute a single serotype and are divided into six genotypes based on phylogenetic analysis of nucleotide sequences in the VP1/2A region (Lu *et al.*, 2004). Many of the human HAV strains studied belong to genotypes I or III (Lu *et al.*, 2004). The majority of HAVs (80%) belong to genotype I (Vaidya *et al.*, 2002). There is only one serotype with infection conferring lifelong immunity (Hollinger and Emerson, 2007).

Patients infected with hepatitis A virus may excrete up to $10^5$ to $10^{11}$ virus particles per gram of stool (Koopmans *et al.*, 2002). Studies have indicated that faecal excretion of HAV may be prolonged in HIV-infected individuals thus serving as an added reservoir of infection (Koopmans *et al.*, 2002). Food or waters can be contaminated with HAV directly by faecal matter and vomitus or indirectly by exposure to contaminated surfaces (Kovać *et al.*, 2009; Lamhoujeb *et al.*, 2008). 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), 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, ground or subsurface 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 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 which imply that even low levels of faecal pollution could pose a threat of infection (Venter *et al.*, 2007).

Hepatitis A virus's stability against chemical and physical disinfection plays a key role in its persistence and spread in the environment (Venter *et al.*, 2007). HAV is resistant to concentrations of free residual chlorine of 0.5-1.5 mg/l for 1 h, and exposure to 2-2.5mg /l for at least 15 min is recommended to inactivate any infectious HAV (Feinstone and Gust, 2002). The virus can withstand temperatures of 60-80ºC for a minimum of 1 h (Koopmans *et al.*, 2002), low relative humidity (~25% for 7 days) (Mbithi *et al.*, 1991) and pH values as low as pH 1 for 2 h at room temperature and retains its infectivity for up to 5 h, which explains how HAV can pass through the human or primate stomach (Feinstone and Gust, 2002). Hepatitis A virus has been shown to survive for months in experimentally

contaminated fresh water, surface waters, seawater, marine sediments, wastewater, soils, and oysters (Hollinger and Emerson, 2007) and depending on conditions, can be stable in the environment for months (CDC, 1988). However it has been shown that HAV can be inactivated by ultraviolet radiation, autoclaving, formalin, iodine, or chlorine, with specific thresholds for duration and intensity (Hollinger and Ticehurst, 1996).

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 (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 (Taylor, 1997), and shellfish (Goswami *et al.,* 1993), seawater (Myint *et al.,* 1994) and environmental water samples (Marx *et al.,* 1998). HAV was detected in the river and the dam water samples in South Africa where microbiological indicators of faecal pollution were absent (Taylor *et al.,* 2001). 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)

Norwalk-like viruses (NLVs), now called 'noroviruses's (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 similar in size and morphology and exhibit nucleotide sequence homology but are antigenically distinct (Atmar and Estes, 2001). Norwalk-like viruses are responsible for the majority of outbreaks of acute gastroenteritis in patients of all age groups in industrialized countries (Frankhauser *et al.,* 2002; Mead *et al.,* 1999). NoVs demonstrate high genetic diversity and are currently divided into five genetically discrete genogroups I (GI) to V (GV) on the basis of sequence comparison of the RNA polymerase and capsid region of the genome (Atmar and Estes, 2001, Phan *et al.,* 2007). Three groups GI, GII and GIV are known to infect humans (Logan *et al.,* 2007). Within the genogroups, norovirus strains can be further separated into genetic clusters, or genotypes, with genogroup II/genotype4 (GII/4, Bristol/ Lordsdale group) virus strains being the most leading worldwide and are endemic in hospitals and long-term care facilities (Noel *et al.,* 1999). Investigations across Europe have recognized norovirus GII/4 as the predominant viral strains (Lindell *et al.,* 2005), with reports of epidemic spread of GII/4 norovirus variants (Siebenga *et al.,* 2007).

Outbreaks of NoV have been caused by contaminated food and/or drinking water, person-to-person virus transmission, and airborne droplets of infected vomitus (Koopmans *et al.,* 2002; Laverick *et al.,* 2004; McIver *et al.,* 2001; Mead *et al.,* 1999). In 2002, 84% of outbreaks of infectious intestinal disease in Ireland were either confirmed or suspected to be norovirus (NDSC, 2003). Of these outbreaks, 70% occurred in hospitals and other healthcare settings placing an enormous burden on the healthcare system (Logan *et al.,* 2007). Human noroviruses (HNoVs) 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 *et al.,* 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). Noroviruses are environmentally stable, able to endure both freezing and heating (although not thorough cooking), are resistant to several chemical disinfectants, and can persist on surfaces for up to 2 weeks (Hall *et al.,* 2011). Two studies have confirmed the presence of RNA specific for NoV in different brands of European mineral water, thus indicating that bottled water could also be an important source of the viral infection (Beuret *et al.,* 2002; Kovać *et al.,* 2009). Noroviruses have now become the leading cause of endemic diarrheal disease across all age groups (Hall *et al.,* 2011), the leading cause of foodborne disease (Scallan *et al.,* 2011), and the cause of half of all gastroenteritis outbreaks worldwide (Patel *et al.,* 2009). In the United States alone, Noroviruses are responsible for an estimated 21 million cases of acute gastroenteritis annually, including >70 000 hospitalizations and nearly 800 deaths (Hall, *et al.,* 2011; 2012; Lopman *et al.,* 2011; 2012). Whilst in developing countries, where the greatest burden of diarrheal disease occurs, NoVs have been estimated to cause up to 200 000 deaths each year in children <5 years of age (Patel *et al.,* 2008). 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. GI NoV strains have been detected repeatedly in sewage, effluent, and surface waters (Katayama *et al.,* 2008; Myrmel *et al.,* 2006), which adds to the view that many norovirus infections are symptomless, with GI viruses being under-represented among those found in clinical cases.

Human Noroviruses cannot be cultivated in traditional cell culture or in animal models (Duizer *et al.,* 2004). Traditional methods of electron microscopy and enzyme immunosorbent assay, used in clinical diagnosis, lack adequate sensitivity for analysis of environmental samples (Greening and Hewitt, 2008). 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 (Greening and Hewitt, 2008; Karim and LeChevallier, 2004), in the Netherlands (Lodder and de Roda Husman, 2005) and in Germany (Pusch *et al.* 2005). 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 *et al.,* 2004). The RT-PCR format offers the ability to detect lower levels of the virus,

as well as a broad-spectrum of viruses from both genogroup I and genogroup II, thus for these reasons, RT-PCR testing for noroviruses can be valuable in the evaluation of outbreaks (Kojima *et al.,* 2002).

### 1.6.5 Rotavirus (RV)

Rotaviruses (RV) 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. Rotaviruses are classified into at least five groups (A to E), and there are possibly two more groups (F and G) based on the re-activities of the VP6 middle layer protein (Estes, 2001). Rotaviruses contain 11 segments of double-stranded RNA within a core shell. Each segment encodes a single viral polypeptide, for a total of five non-structural and six structural proteins (Estes, 1996). The two outer capsid proteins VP7 and VP4, which independently elicit neutralizing antibodies, are the basis of a binary classification system for rotaviruses: G types (derived from the VP7 glycoprotein) and P types (derived from the protease sensitive VP4 protein). Thus far, 15 different G genotypes and 21 different genotypes have been reported (Rao *et al.,* 2000). Group A rotaviruses are generally associated with human infections (Kapikian, 2001). Rotaviruses have been established as the main cause of acute gastroenteritis in young children worldwide (Kapikian and Chanock, 1996). Rotaviruses are accountable for an estimated 500,000 deaths each year in developing countries (Parashar *et al.,* 2003). 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 diarrhoeal 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 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, such as sewage (Baggi and Peduzzi, 2000; Dubois *et al.,* 1997), river water (Baggi and Peduzzi, 2000), ground water (Abbaszadegan *et al.,* 2003), 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). RVs were detected in surface water in Germany (Pusch *et al.,* 2005), Italy (Grassi *et al.* 2010) and the Netherlands (Rutjes *et al.* 2009). Group A RVs have been detected in untreated and treated drinking water samples in southern Africa (van Zyl *et al.,* 2004; 2006). van Zyl *et al.* (2006) also demonstrated the similarity between the environmental types to those clinical RV strains in patients. 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). In South Africa, infections related to rotavirus account for approximately 25 % of all diarrheal hospital cases yearly, with 83 % of infections occurring in infants > 12 months of age (African Rotavirus Symposium, 2002). Results have shown that 95% of the RV-positive environmental water samples is: the Group A RV G type in Kenya (Kiulial *et al.,* 2010) and in South Africa (van Zyl *et al.,* 2006), as compared to the typing rate of 70 and 43.6% reported for Egypt and Spain, respectively (Villena *et al.,* 2003), and 20% reported for Brazil (Miagostovich *et al.,* 2008).

### 1.6.6 Astrovirus (AstV)

Astroviruses (AstVs), classified as genus *Mammoastrovirus,* 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). Astroviruses are divided into eight serotypes (HAst1-8) that comprise two genogroups (A and B) capable of infecting humans (Carter, 2005).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 intake of water from streams or raw sewages (Cubitt, 1991; Liu *et al.,* 2006; Meleg *et al.,* 2008). 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; Martin, 1992; Taylor *et al.,* 2001). In 1979, 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). 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). AstVs were detected in sewage sources from the Tshwane (Pretoria) Metropolitan Area in South Africa and these viruses are closely related to clinical AstV isolates based on phylogenetic analyses (Nadan *et al.,* 2003). In temperate regions, most astrovirus infections are detected in the winter while in tropical climates, infections are noted during the rainy season (Matsui and Greenberg, 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). 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).

### 1.7 Hepatitis B Virus

Hepatitis B virus (HBV) belongs to the genus *Orthohepadnavirus*, family *Hepadnaviridae*, it has a circular genome of partially double-stranded DNA, ≈3.2 kb in length, that contains 4 genes with partially overlapping open reading frames (ORFs) (Zuckerman, 1996). These ORFs encode the polymerase protein (Pol gene); core antigen and e antigen (C gene); large, medium, and small surface-antigen proteins (S gene); and the X protein (X gene) (Coleman, 2006). HBVs replicate through an RNA intermediate form by reverse transcription (Locarnini, 2004). The replication of HBVs takes place in the liver, the virus spreads to the blood where viral proteins and antibodies against them are found in infected people (Custer *et al.,* 2004) This viral infection puts people at high risk of death from cirrhosis of the liver and liver cancer (WHO, 2012). The hepatitis B virus is 50 to 100 times more infectious than HIV, thus making it an important occupational hazard for health workers (WHO, 2012). There are currently between 3 and 4 million of the South African black population who are chronically infected with HBV (WHO, 2008). Routes of infection include vertical transmission (such as through childbirth), early life horizontal transmission (bites, lesions, and sanitary habits), and adult horizontal transmission (sexual contact, intravenous drug use) (O'Connor, 2007). According to the literature, hepatitis B viruses cannot be spread routinely through food or water and by holding hands, sharing eating utensils or drinking glasses or breastfeeding (WHO, 2012). However, Hepatitis B viruses (HBVs) was detected in water samples from the Umgeni River in this laboratory (this study). Finding HBVs in water raises the concern that HBV might be transmitted in the natural water environments via breaks in the skin, loss of blood through cleansing of the body, washing of soiled clothing and further investigation into this incidence is imperative. The diagnosis and clinical monitoring of HBV infection are based on the detection of viral antigens, antibodies to viral proteins, and circulating viral genomes (HBV DNA) (Gitlin, 1997).

### 1.8 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-Gimeneza *et al.,* 2009)

### 1.8.1 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. 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.,* 2008). 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.,* 2008).

### 1.8.2 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 ultrafiltration 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 ultrafiltration systems yield 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 re-circulated 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.

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 *et al.,* 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; Haramoto *et al.,* 2004).

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 (Colombet *et al.,* 2007; 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.

### 1.8.3 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 h 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).

## 1.9 Methods for 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, 2010; Rosario *et al.,* 2009).

### 1.9.1 Cell-Culture Techniques

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; Lewis and Sequeiros 2005)
(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).

### 1.9.2 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; Wilhelm and Suttle, 1999; 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 (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; Suttle, 2007; Weinbauer and Suttle 1997), 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.

#### 1.9.2.1 Epifluorescence Microscopy (EFM)

EFM 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; Middelboe *et al.,* 2006; Noble and Fuhrman, 1998) 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).

#### 1.9.2.2 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.,* 2006). 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.

### 1.9.3 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).

### 1.9.4 Polymerase Chain Reaction (PCR)

Since the late 1980s, PCR has been used for the detection and quantitation 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 amplicon

(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).

### 1.9.5 Real-Time (RT) 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; 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).

### 1.9.6 Pulsed-Field Gel Electrophoresis (PFGE)

PFGE has become popular for analysing viral communities (Wommack *et al.,* 1999). 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 *et al.,* 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).

### 1.9.7 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).

### 1.9.8 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). Tamor *et al.* (2011) recently presented an alternative approach to the classical phage enrichment technique. 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.

## 1.10 Scope of Present Study

South Africa is one of most species-diverse countries in the world. The study of viruses is relatively poor and has historically been focusing on medical- or agricultural-related viruses. There has been little investigation of their importance in the aquatic environmental domain, where even basic information, such as their temporal dynamics and spatial distribution, is almost non-existent. Therefore, it is critical to initiate some environmental viral study in order to have a comprehensive understanding of viruses in our environment (Rosario *et al.*, 2009; Turpie *et al.*, 2004).

South Africa has suffered several viral disease outbreaks in recent years. The possibility of viral contamination of aquatic environments can never be underestimated. Information regarding viruses in the various water sources will help regulatory agencies to make informed decisions about water use to minimise negative impacts upon human and environmental health. The ultimate goal in South Africa's water quality management is to keep the water resources suitable for their designated users (Mardon, 2003). 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, 2010). The monitoring of water supplies and research on waterborne viruses (mainly in Gauteng) in South Africa have been inadequate (Grabow *et al.*, 2004). This viral study thus focused on the Umgeni river catchment in (KwaZulu-Natal) South Africa, as this water resource is widely used for recreational, agricultural and domestic activities (DWAF, 1996 a; b). The river provides water to over 3.5 million people and supports an area that is responsible for approximately 65% of the total economic production in the province (WRC, 2002). The main objective of this study was to set up a virus concentration system and to evaluate the abundance of viruses found in the Umgeni river water samples from Durban, South Africa.

### 1.11 Hypothesis

It was hypothesized that pathogenic viruses are present in the Umgeni River in Durban, South Africa. It was further hypothesized that monitoring these viruses using advanced molecular techniques will provide relevant information for improved water resource management and may be a promising tool to assess water quality and direct surveillance for viral pathogens may be warranted to better protect public health.

### 1.12 Research Objectives :

a) To monitor the seasonal changes of the microbiological quality and physico-chemical aspects from the Umgeni River water in Durban, South Africa.

b) To set up a virus concentration system to optimize viral recovery from the Umgeni River.

c) To study four virus types along the Umgeni River throughout the seasons.

d) To monitor the presence, temporal dynamics and spatial distribution of enteric viral contamination of the Umgeni River using real time PCR.

### 1.13 Experimental Design

The content of this thesis has been organized into five chapters that are presented as follows:

- **Chapter *one*** presents the introduction and literature review. The review begins with a general overview of the current water situation in South Africa, with specific reference to anthropogenic action, lack of infrastructure for water, and lack of potable water supplies and sanitation, that affect the environment and public health. In addition surface water pollution is discussed in terms of its sources, and indicator organisms involved. Human pathogenic viruses present in water environments are also described with emphasis on viruses contaminating South Africa's waters. Isolation and detection methods of the viruses are also presented. The final section of the literature review summarizes the relevant molecular techniques used for detecting the presence of viruses in water, with emphasis on PCR technologies.

- **Chapter *two*** presents the first part of the study; here the quality of the Umgeni River water is examined in terms of its microbial load, and physico-chemical aspects. In addition, the effects of seasonal variability on the microbial load and physico-chemical qualities of the River water were established using canonical correspondence analysis (CCA).

- **Chapter *three*** describes the recovery of virus like particles (VLP) using TFF. In the first instance the somatic and F-RNA phage load is described. Thereafter several techniques such as TEM, EM and Tissue culture were employed to detect and enumerate the VLPs. Statistical comparison of viral counts to phage counts are presented using CCA.

- **Chapter *four*** presents the final component of the study based on molecular identification of viral concentrates obtained from the water samples. In this section conventional PCR, nested PCR, ICC-PCR and Real time PCR are discussed.

- **Chapter *five*** concludes with a general discussion and a summary of this study's research findings. Future recommendations to further develop this study are also presented.

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# CHAPTER TWO

## Microbial and Physico-Chemical Quality of the Umgeni River in Durban, South Africa

## 2.1 Introduction

Water is one of the most imperative resources for human life (Miernik, 2004). Water quality in rivers is vital to humans, animals and to the maintenance of environmental integrity (Caraco *et al.*, 2003).The expansion of industry, new technologies absorbing and producing huge amount of chemicals, organic and inorganic compounds, and increasing urban developments have resulted in increasingly sewage polluted natural waters (Drechsel *et al.,* 2006). Currently, almost one billion people, worldwide, are deprived of safe water supplies while 2.5 billion people live without proper sanitary facilities, majority (at least 80%) of whom live in under developed areas (JMP, 2008). In South African Water Law, a river system and its riverine biodiversity (i.e. the complete aquatic ecosystem) is regarded as the 'water resource' that provides ecosystem goods and services to society (van Wyk *et al.,* 2006). Like many countries in Africa, South Africa's water resources have been under the spotlight due to the increasing threat of pollution in recent years brought about by rapid demographic changes, which have coincided with the establishment of human settlements lacking appropriate sanitary infrastructure (Drechsel *et al.,* 2006; Karikari *et al.,* 2007). Given that freshwater resources are the single most limiting resource for expansion in South Africa (Davies and Wishart, 2000), it is inevitable that the country's rivers will continue to be exploited to meet human needs and demands for water (DWAF, 1996b). While increased water removal and effluent discharges create immediate threats to riverine biodiversity, potential augments in temperature and reduced precipitation caused by climate change are likely to aggravate the situation in the long-term (Davies *et al.,* 1993; Driver *et al.,* 2005; Tyson, 1987). Water physico-chemistry variation of rivers are dependent on and influenced by the regions in which it occurs, as a result of different climate, geology, soils and biotic composition (Dallas and Day, 2004). Dissimilarities in freshwater microbial communities transpire temporally and spatially between and within habitats in response to different environmental factors such as varying water chemistry, temperature, pH, solar radiation, quality and quantity of dissolved organic matter (Dominik and Hofle, 2002; Logue and Lindström, 2008; Yannerell and Triplett, 2005; Zwisler *et al*., 2003). Evidence in the literature suggests that seasonal changes as well as anthropogenic activities affect both the water quality and physico-chemistry in aquatic ecosystems thus influencing bacterial abundance, community structure and activities (Crump *et al*., 2003; Kent *et al*., 2004; Parnthaler *et al*., 1998; Schauer *et al*., 2006; Shade *et al*., 2007; Wu and Hahn, 2006).

South Africa is one of the driest countries on Earth with an average rainfall of about 18 inches per annum of which only 9% of its rainfall reaches the river streams, which is lower than the average rainfall of 31% from around the rest of the world (DWAF, 2002; Otieno and Ochieng, 2004). This water scarcity is further compounded by the strong seasonality of rainfall, as well as high within-season variability over almost the entire country which results in highly variable surface runoff (Otieno and Ochieng, 2004).The rising deficit of good quality water in South Africa has prompted the need to consume not only subterranean waters but also fresh waters at maximal risk of microbiological and chemical pollution (Ashton, 2002; 2007). Majority of the rural communities are poverty-stricken, lack access to potable water supplies and rely mainly on river, stream, well and pond water sources for their

daily water needs (Nevondo and Cloete, 1999). As a result, a significant proportion of residents in these rural communities are exposed to water-borne disease and their associated complications (Schalekamp, 1990). These water-borne diseases are due to the considerable numbers of human microbial pathogens that are present in municipal sewage and may be considered environmental contaminants (Girones *et al.,* 2010). Although most microbial pathogens can be removed by sewage treatment, many are discharged into the effluent and enter receiving waters (Stewart *et al.,* 2008).

In urban areas, faecal microorganisms are mainly transported to the aquatic environments through the discharge of domestic wastewater and some industrial wastewater. In rural areas, faecal pollution in rivers is caused through non-point sources (surface runoff and soil leaching); its origin can be the wild life animals and grazing livestock faeces and also cattle manure spread on cultivated fields (Stewart *et al.,* 2008). These polluting sources have had a significant effect on the quality of surface water bodies in Durban, South Africa, especially the Umgeni and Umdloti Rivers, where the lower reaches of these river catchments are being affected by urbanization which poses a high risk to users due to microbial contamination (Naicker, 2010; Olaniran *et al.,* 2009). People living in these areas, as well as downstream users, often utilise the contaminated surface water for drinking, recreation and irrigation, which creates situation that poses a serious health risk to the people (Olaniran *et al.,* 2009).

Faecal pollution problems are common to all nations regardless of economic position although pollution level and type of contamination vary among countries (Stewart *et al.,* 2007). Global estimates suggest that drinking, swimming and bathing in faecally contaminated water, together with the consumption of shellfish harvested from polluted waters results in an excess of 175 million cases of infectious disease each year, whilst in developing countries 1.8 million people, mostly children, die every year as result of water-related disease (WHO, 1996).

In aquatic ecosystems, the detection and enumeration of all pathogenic/ disease causing microorganisms potentially present, is difficult due to the large diversity of pathogens, the low abundance of each species and the absence of standardized methods for their detection (DWAF, 2002; Rajeshwari and Saraswathi, 2009; Stewart *et al.,* 2008). Since it is not practical to test water supply for all pathogens related to water-borne diseases due to the complexity of the testing method as well as the time and cost involved (Lehloesa and Muyima, 2000), indicator microorganisms are used (Grabow, 2001). However, there exists no one simple indicator that complies with all the water quality guidelines available; hence more than one indicator organism is employed (Genthe and Seager, 1996). The presence of enteric pathogens in drinking waters is of great concern, and thus, legislation either in Europe, USA and other countries requires analysis of *E.coli*, total coliforms, faecal coliforms and faecal streptococci indicators to determine the microbiological quality of these waters (Figueras and Borrego, 2010).

The South African national government is concerned about the state of river water quality and the status of wastewater treatment in South Africa (van der Merwe-Botha, 2009). The deteriorating river water quality is one of the major threats to South Africa's capability to provide sufficient water of appropriate quality to meet its population's needs and to ensure environmental sustainability. Thus

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). Thus in this Chapter, microbial indicators of pollution as well as several physico-chemical parameters were used to determine the microbial quality of water from the Umgeni River in the Durban areadue to it being the largest catchment 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

## 2.2 Materials and Methods

### 2.2.1 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 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-estuarine/ brackish water), U2 (Reservoir Hills-informal settlement/ domestic waste), U3 (New Germany Wastewater Works-treated water after chlorination that enters the adjoining Umgeni River water ), U4 (Krantzkloof Nature Reserve-vegetation and conservation area) and U5 (Inanda Dam-restricted water containment).

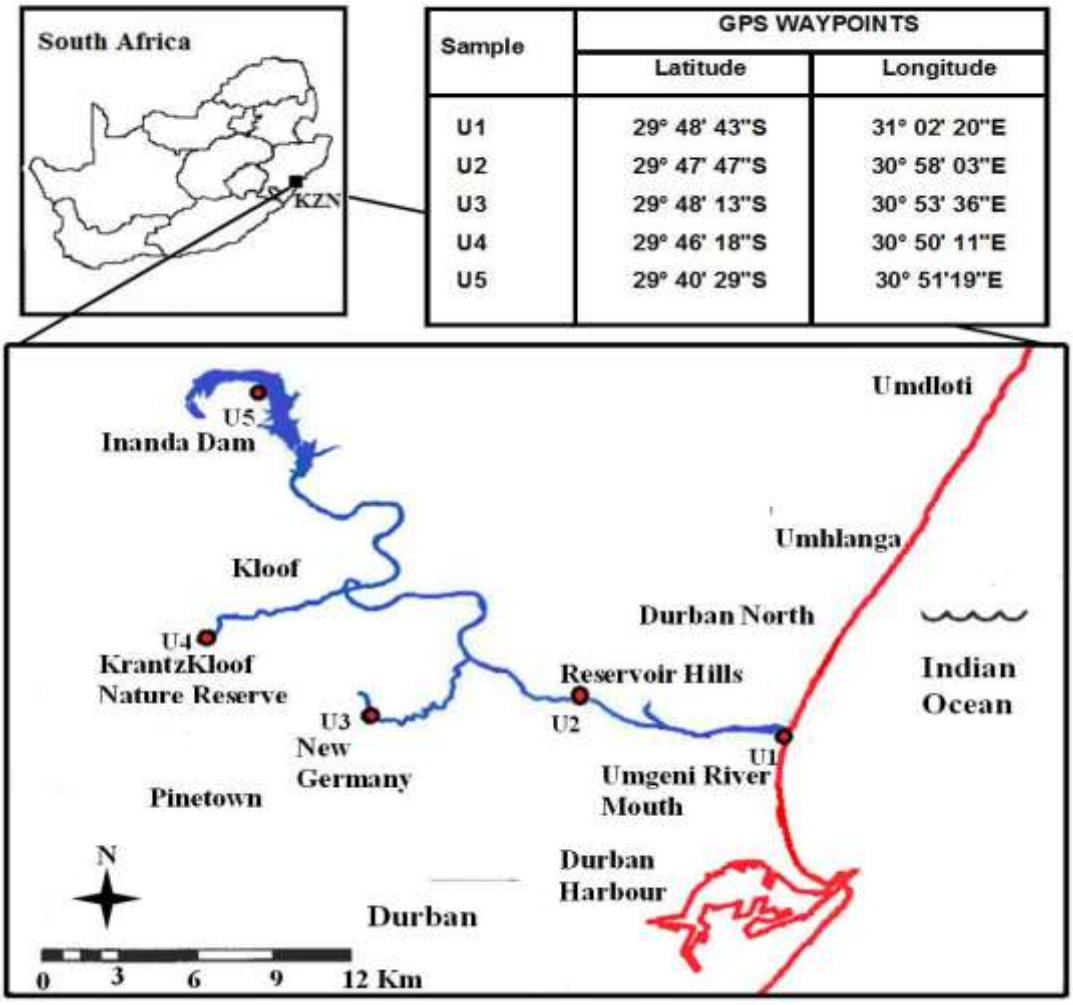



**Figure 2.1**Map of the study region within Durban (South Africa) and surrounding areas. Shown are the GPS locations of sampling points of the Umgeni River investigated in this study.

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 25ℓ 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.The container was filled leaving about 50 mm of headspace to allow mixing during laboratory analysis. 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).

### 2.2.2 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. pH measurements were determined in 100 mℓ volumes of water samples using a pH meter (Beckman Φ 50 pH meter) and the turbidity of each water sample was measured using the portable 2100P turbidimeter (HACH), at the microbiology laboratory facility. The pH and turbidity meters were calibrated according to the manufacturers' instruction. 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. Table 2.1 provides an overview of the methods employed by the CSIR laboratory for analyses.

**Table 2.1** Chemical analysis methods utilised by the CSIR laboratory (Certificate of Analysis: 11237).

| Parameter | Method | Method No. |
|---|---|---|
| **Dissolved Nitrate, Nitrite, Ammonia and Ortho Phosphate** | Colorimetric analysis, using a Bran and Luebbe Auto Analyser III | MM001 to MM004 |
| **Sulphate** | Turbidimetric method | MM-016 |
| **Chloride** | Titration with silver nitrate. | MM-013 |
| **Electrical Conductivity** | Potentiometry | MM-FW006 |
| **Dissolved metals** | ICP-OES analysis | MM-017 |
| **Mercury** | ICP-OES –VGA analysis | MM-Hg |

#### 2.2.2.1 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 (3 mℓ) according to manufacturer's instructions was used for the analysis. 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.

#### 2.2.2.2 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 water 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.

### 2.2.3 Enumeration of Bacterial Indicator Microorganisms

The membrane filtration (MF) technique (Millipore, HANG 47 mm) was used for the enumeration of eight indicator organisms from all water samples, according to standard protocol (Standard Methods, 1992). Selective media was prepared according to manufacturers instruction in 45mm Petri plates [Appendix i]. Appropriate serial dilutions of the water samples were prepared with autoclaved distilled water prior to filtration. Fifty millilitre samples from the dilution series were vacuum filtered through 0.45 µm pore size GN-6 Metricel membrane filters (Millipore, 47 mm), held in a glass filtration unit (GLASCO). These filters were then transferred with the right side up onto 45 mm Petri plates containing various selective media for recovery of each indicator group (Table 2.2). The incubation conditions for each indicator organism are listed in Table 2.2. After the incubation period, all the typical colonies for indicator bacteria according to standard protocol that had grown on the filters were recorded as presumptive counts for the estimation of colony forming units per 100 millilitre (cfu/100 mℓ). Sample blanks (autoclaved distilled water) were processed during MF to ensure quality of dilution water (Buckalew *et al.,* 2006). All water samples were processed in duplicate to ensure accuracy of the method.

**Table 2.2** Selective media and incubation conditions used for the isolation and enumeration of the bacterial indicator organisms.

| INDICATOR MICROORGANISM | SELECTIVE MEDIA | INCUBATION CONDITIONS |
|---|---|---|
| **Total Heterotrophs (TH)** | Nutrient Agar | 24 hrs 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 cholerae* (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 |

## 2.3 Statistical Analysis

Pearson's and Spearman's correlation tests were used to evaluate the correlations between the bacterial indicators 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 relationship between the bacterial indicator communities and the measured physical and chemical water quality variables at the various sites and seasons, with a view to determining the important variables responsible for the observed spatial and temporal distribution of the bacterial community. CCA ordination allows 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 (terBraak and Verdonschot, 1995).

## 2.4 Results

### 2.4.1 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 2.3 and 2.4 respectively. Temperature profiles shown in Table 2.3 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). No significant difference ($p$>0.05) was observed with pH measurements across all five sampling points and seasons tested. All river water samples were more or less neutral to alkaline in nature with values ranging from pH 7.11 (U4 – autumn), pH 7.1 (U1 – winter), pH 9.16 (U5 – spring) to pH 8.77 (U3 – summer). Significant ($p$<0.05) seasonal variations in turbidity values were noted along the Umgeni River and ranged from 1.62 NTU (U5) to 6.58 NTU (U3) in autumn, 0.64 NTU (U5) to 14.9 NTU (U1) in winter, 4.08 NTU (U5) to 15.7 NTU (U3) in spring and 7.63 NTU (U4) to 15.64 NTU (U1) summer (Table 2.3). An interesting observation was that Umgeni River point U1 (downstream of river) experienced high turbidities throughout all four seasons as compared to point U5 (upstream of river) that had relatively low turbidities. Large seasonal variations in $BOD_5$, COD and conductivity levels amongst the sampling points along the River were observed (Table 2.3). 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.

The phosphate concentrations varied significantly ($p$ < 0.05) for all sampling points along the River across all four seasons tested. Phosphate concentrations of the 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 nitrate/nitrite and ammonia concentrations for all sampling points of the river differed significantly ($p$<0.05) and ranged from 0.087 mg/ℓN (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). Chloride concentrations were extremely high at point U1 of the river during all seasons tested and ranged from 9853 mg/ℓ Cl (autumn) to 19234 mg/ℓ Cl (summer). Relatively low chloride concentrations were observed at sampling points U2 and U5 during the autumn and winter seasons. Sulphate concentrations were reasonably high at point U1 during all four seasons tested, with values 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 Pearson's correlation matrices of the physico-chemical properties of the water samples were performed. The correlation coefficients were interpreted with caution as they are affected simultaneously by spatial and temporal variations. The water temperature of the 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) (Appendix ii).

**Table 2. 3** 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 $SO_4$) |
| **Guideline** | | 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)

Significant positive correlation was observed between pH and turbidity ($r$ = 0.961; $p$ = 0.039). Interesting observation was inverse correlation between COD and conductivity(r = - 0.228) and $BOD_5$ and COD ($r$ = -0.546) [Appendix ii]. 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 all heavy metals tested (Table 2.4). 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 <0.001mg/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. At point U1 Pb concentration increased from 0.031 mg/ℓ Pb (winter) to 0.112 mg/ℓ Pb (summer). Relatively low concentrations of Cu were detected throughout all sampling points and all four seasons. Hg concentrations reached: 0.00018 mg/ℓ Hg (U1- winter), 0.00037mg/ℓ Hg (U2 – spring), 0.0009 mg/ℓ Hg (U3 – spring) and <0.001mg/ℓ Hg (U1 – summer). Pb had positive correlations with conductivity ($r$= 0.669, $p$= 0.001), THB ($r$= 0.584, $p$=0.007) and turbidity ($r$= 0.557, $p$=0.011). Hg had significant positive correlations with BOD ($r$= 0.650, $p$= 0.002), and turbidity($r$= 0.530, $p$= 0.016).

**Table 2.4** Heavy metal quality of the Umgeni River water samples during autumn, winter, spring and summer seasons

| Sample location | Season | Heavy metal concentrations (mg/ℓ ) | | | |
|---|---|---|---|---|---|
| | | $Pb^{2+}$ | $Hg^{2+}$ | $Cd^{2+}$ | $Cu^{2+}$ |
| **Guideline** | | 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) and Schiller and Boyle (1987))

### 2.4.2 Enumeration of Bacterial Indicators

The Total Heterotrophic Bacterial (THB) population of the Umgeni River water samples over the four seasons is presented in Figure 2.2. The general trend observed was a significant increase ($p$<0.05) in the levels of THB at all sampling points along the River during spring and summer followed by a gradual decline through autumn and winter ($p$<0.05). 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/100ml (U5 – winter), to 13.67 x $10^6$ cfu/100ml (U1 – summer) (Figure 2.2). The Umgeni River mouth (sampling point U1) consistently had the highest THB counts during autumn, spring and summer as compared to the other sampling sites along the river during these seasons. Interesting to note is that point U3 (New Germany treatment works) had the highest THB count of 3.92 x $10^6$ cfu/100ml during winter.

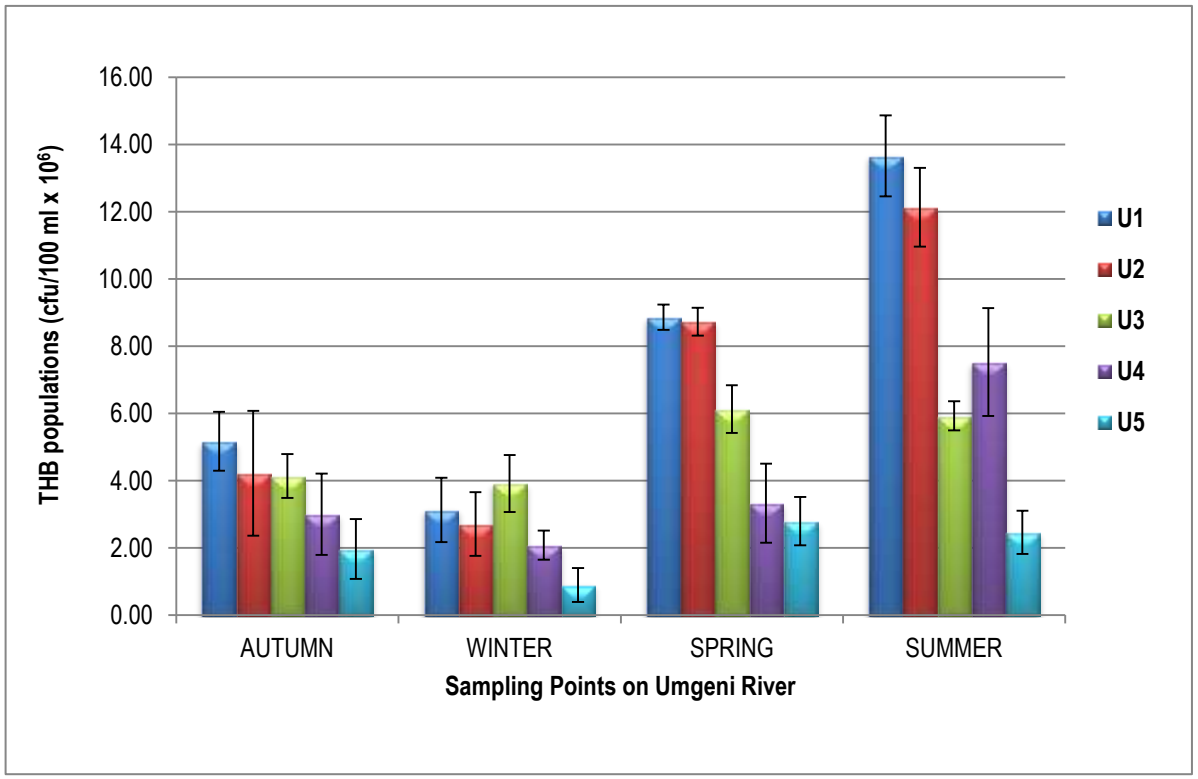



**Figure 2.2** Total Heterotrophic Bacterial (THB) populations for Umgeni River at the different sampling points during autumn, winter, spring and summer seasons. Bars indicate the average of replicate samples (n = 3 or 4) while the error bars show the standard deviation.

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 2.3. TC and FC counts varied significantly ($p$<0.05) throughout all sampling points and ranged from 3.30 x $10^3$ cfu/100mℓ (U1 – winter) to 6.03 x $10^3$ cfu/100mℓ (U1 – summer) and 0.89 x $10^3$ cfu/100mℓ (U3 – winter) to 4.85 x $10^3$ cfu/100mℓ (U2 – spring), respectively (Figure 2.3).VC, SAL and SHIG concentrations for the Umgeni River ranged from 1.91 x $10^3$ cfu/100mℓ (U4 – winter) to 4.97 x $10^3$ cfu/100mℓ (U2 – spring), 0.01 x $10^3$ cfu/100mℓ (U5 –

winter) to 1.43 x $10^3$ cfu/100mℓ (U1– spring) and 0.34 x $10^3$ cfu/100mℓ (U3 – winter) to 1.83 x $10^3$ cfu/100mℓ (U2 – summer), respectively (Figure 2.3).There was no significant difference ($p$>0.05) in the TC, FC and VC counts between the seasons. 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 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. EC and FS concentrations ranged from 0.22 x $10^3$ cfu/100mℓ (U3 – winter) to 1.39 x $10^3$ cfu/100mℓ (U2 – spring) and 0.02 x $10^3$cfu/100mℓ (U5– autumn) to 2.37 x $10^3$ cfu/100mℓ (U3 – winter), respectively (Figure 2.3). 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 were noted between the THB populations and TC and FC populations throughout all seasons ($r$ = 0.9 -1.00, $p$<0.05) [Appendix ii]. 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.

### 2.4.3 Canonical correspondence analysis (CCA)

CCA was used to illustrate the variation in the data; and was chosen because of its ability to reveal ecological trends in community data and to relate environmental factors to them. 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 indicates a larger change in environmental variable. CCA ordination plot (Figure 2.4) 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 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 whatsoever with the other bacterial indicators as well as with the physico-chemical parameters (Figure 2.4). VC, SAL, FC and EC were found to be grouped similarly (green circle- Figure 2.4), 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 2.5 is an indication of the relative importance of the axis.

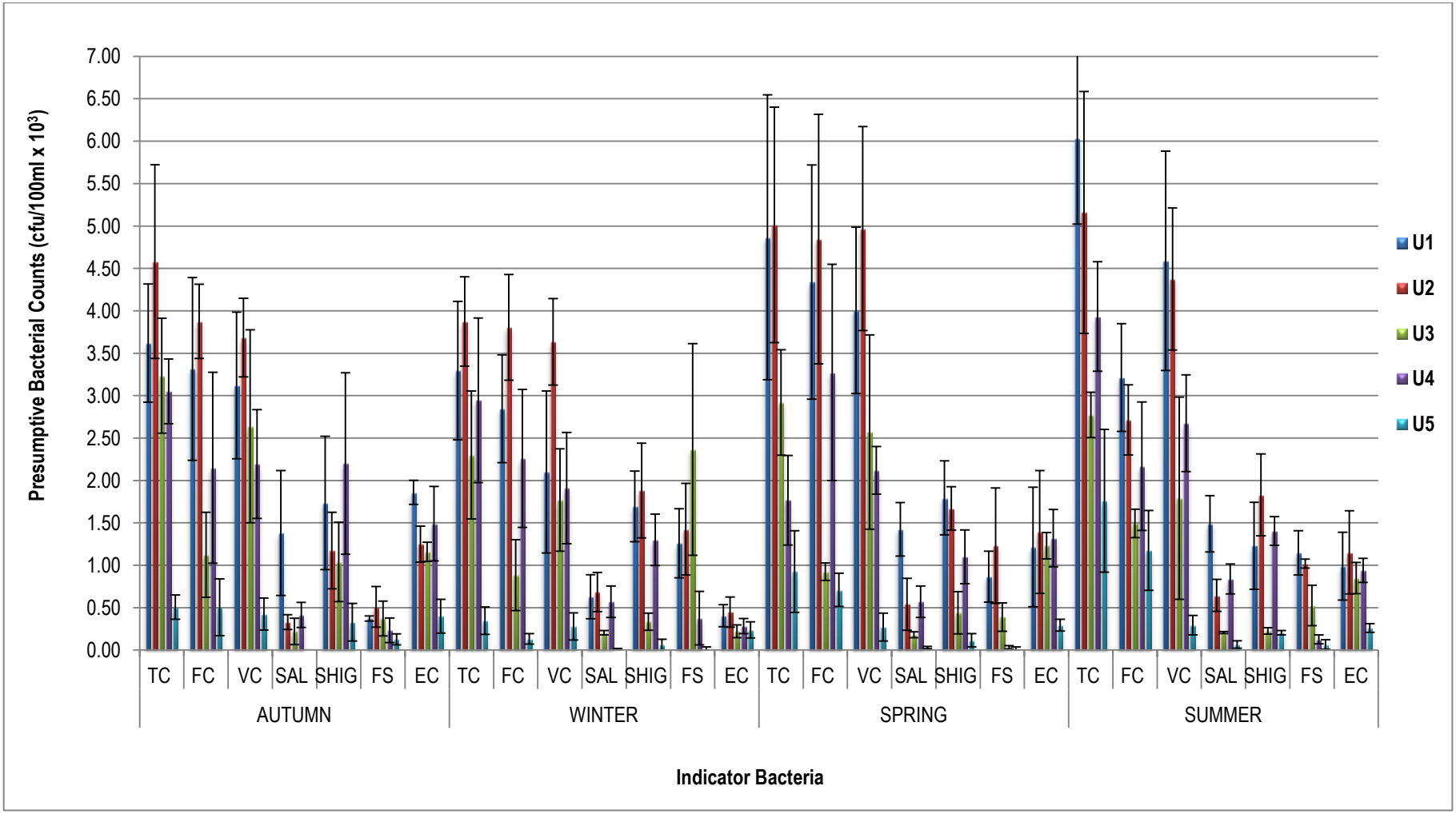



**Figure 2.3** Presumptive counts of total coliforms (TC), faecal coliforms (FC), *V. cholerae* (VC), *Salmonella spp.* (SAL), *Shigella spp.* (SHIG), faecal streptococci (FS) andenterococci (EC) populations for the Umgeni River at the different sampling points during autumn, winter, spring and summer seasons. Bars indicate the average of replicate samples (n = 3 or 4) while the error bars show the standard deviation.

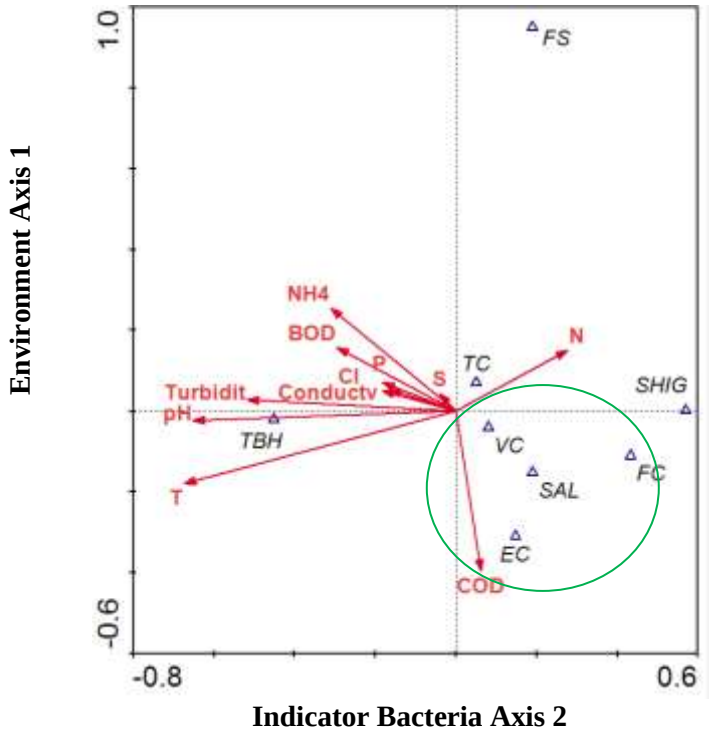



**Figure 2.4** CCA ordination plot for bacterial indicators and water quality variables at the five study sites during autumn, winter, spring and summer seasons.

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% (Table 2.5) of the cumulative variance suggesting high correlation between bacterial community data and water quality variables.

**Table 2.5** 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 |

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) (Table 2.5). Pearson'scorrelation between species and water quality variables displayed strong positive correlation for both axes (Table 2.5).

CCA ordination plot revealed strong relationship between the overall bacterial growth of different sites and seasons measured as well as the physico-chemical water quality variables (Figure 2.5). 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. Site 2, 4, 5 during autumn and winter seasons strongly correlated with the nitrate/nitrite profiles (green circle). Site 1 and 3 in winter showed no correlations with the rest of the sites and variables measured.

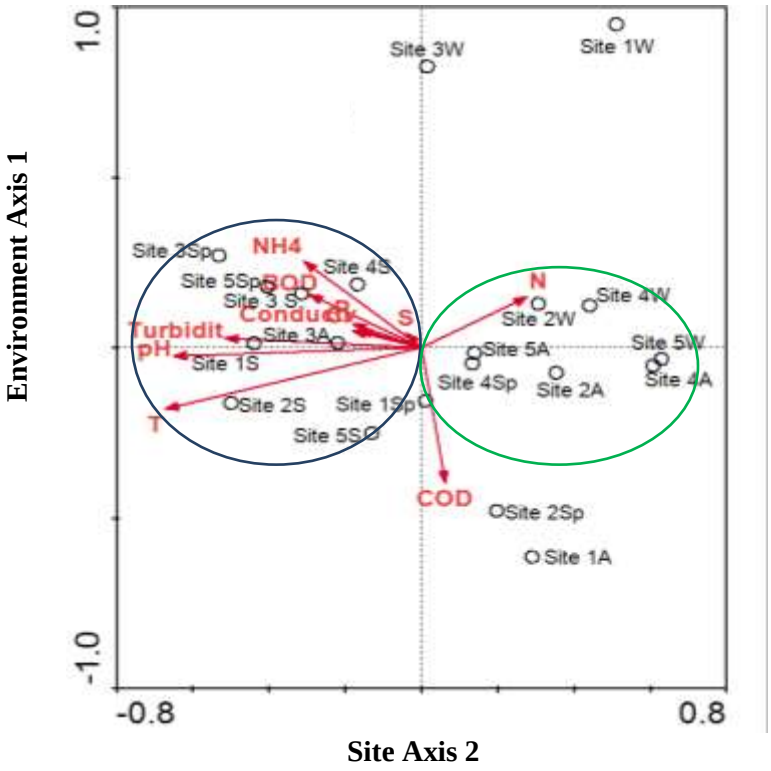



**Figure 2.5** CCA ordination plot for all the water quality variables and the total bacterial growth at the five study sites and during autumn, winter, spring and summer seasons. Abbreviations: Sites 1= Umgeni River mouth, 2= Informal settlement at Reservoir Hills, 3= New Germany Waste Treatment Works, 4= Krantzkloof Nature Reserve, 5= Inanda Dam. Seasons: A=Autumn, W= Winter, Sp= Spring, S= Summer.

The relative magnitude of Eigen values for each of the CCA axis in Figure 2.5 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% (Table 2.6) of the cumulative variance suggesting that there may be a correlation between the various sites 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 significant (p<0.002) (Table 2.6). Pearson's correlation between species and water quality variables displayed strong positive correlation for both axes (Table 2.6).

**Table 2.6** 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 | 37.9 | 50.5 |
| % Cumulative variance of species – environment relation | 74.6 | 80 |
| Monte Carlo test p – value | 0.001 | 0.070 |
| Pearson correlation of species and environmental Scores | 0.759 | 0.908 |

CCA ordination plot (Figure 2.6) 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. These ordination curves impacted on the presence of the bacterial communities. 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 2.6), probably giving an indication that all the heavy metals do 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 had positive relations with Cu and had no correlation whatsoever with the other bacterial indicators as well as with the other heavy metals tested (Figure 2.6).

The relative magnitude of Eigen values for each of the CCA axis in Figure 2.6 is an indication of the relative importance of the axis. 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% (Table 2.7) of the cumulative variance suggesting that correlation between bacterial community data and heavy metal variables do exist. Monte Carlo permutation test was used toassess the statistical significance of the model and Eigen value for axis 1 and axis 2 was found to be significant ($p< 0.05$) (Table 2.7). Pearson's correlation between species and heavy metal variables displayed strong positive correlation ($r= 0.775$) for axis 1 (Table 2.7).

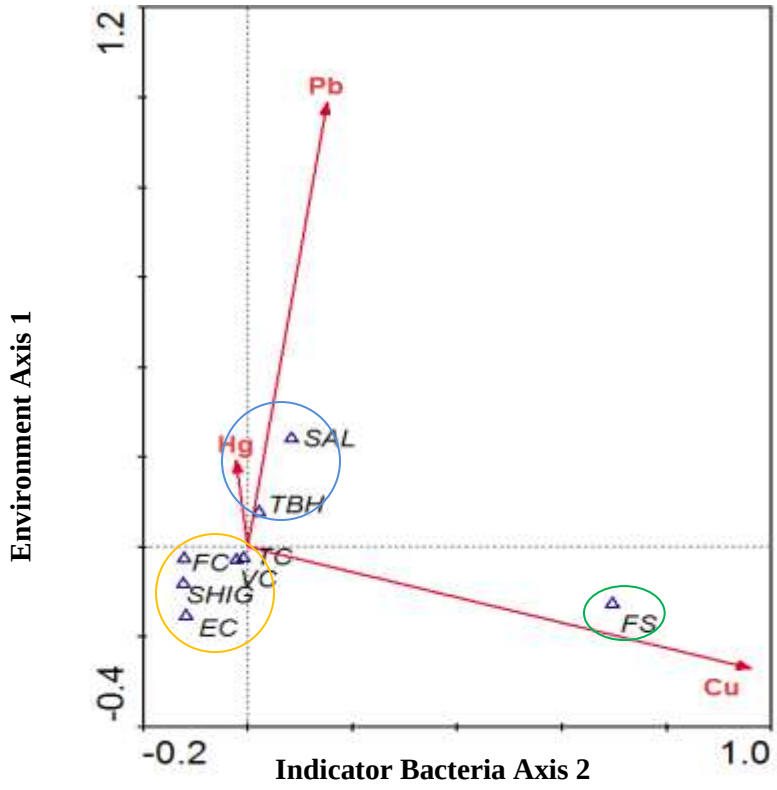



**Figure 2.6** CCA ordination plot for bacterial indicators and heavy metal variables at the five study sites during autumn, winter, spring and summer seasons.

**Table 2.7** Properties of the Canonical Correlation Analysis ordination bi-plot for bacterial indicators and heavy metal variables at the five study sites during autumn, winter, spring and summer seasons.

| | | |
|---|---|---|
| | **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 |

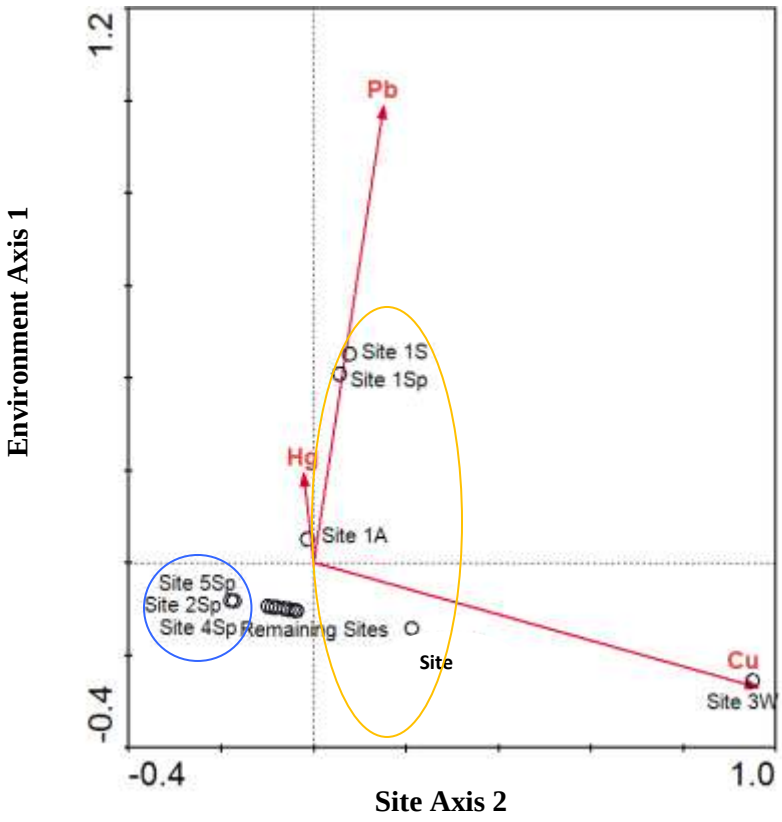



**Figure 2.7** CCA ordination plot for the heavy metal quality variables and the total bacterial growth at the five study sites and during during autumn, winter, spring and summer seasons. Abbreviations: Sites 1= Umgeni River mouth, 2= Informal settlement at Reservoir Hills, 3= New Germany Waste Treatment 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 growth of different sites and seasons measured as well as with the heavy metal variables (Figure 2.7). 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). Interesting to note however is that site 1 during summer and spring correlated strongly with Pb, whilst during autumn and winter site 1 correlated with Hg and Cu respectively. Site 3 in winter showed strong correlation with Cu.

The relative magnitude of Eigen values for each of the CCA axis in Figure 2.7 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% (Table 2.8) of the cumulative variance suggesting that strong correlations between bacterial community data and heavy metal variables do exist at most of the sampling sites. Monte Carlo permutation test was used to assess the statistical significance of the model and Eigen value for axis 1 and axis 2 was found to be significant ($p< 0.05$) (Table 2.8). 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 ($r= 0$) (Table 2.8).

**Table 2.8** 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.

| | | |
|---|---|---|
| | **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 |

## 2.5 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 and Stretch, 2004), thus making the river prone to almost every possible type of pollution. 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 are being affected by urbanisation (human activities and influences) which poses a high risk to users due to microbial contamination.

In this study the pH of the river water samples across the four seasons ranged from 7.10 to 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. 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).Shittu and colleagues (2008) found that high turbidity observed in the surface waters they studied could be associated with higher levels of disease causing microorganisms such as bacteria and other parasites. A significant relationship between turbidity and microbiological quality of the Umgeni river water samples implied that turbidity can be viewed as a supporting factor for microbiological contamination in drinking and recreational waters. CCA analysis reinforced this relationship and showed that temperature, pH and turbidity were the key variables that impacted on the presence of the bacterial communities (Figure 2.4). This finding is consistent with reports whereby 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 a water quality measure used to measure both the amount of biologically active substances such as bacteria and biologically inactive organic matter in water. It is the oxygen equivalent of the total organic matter in a water sample that is susceptible to oxidation by a strong chemical oxidant such as dichromate. The COD and BOD values are generally meant for effluent samples not for drinking water. It means there should not be any trace of COD or BOD values for drinking water. 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 stipulated COD guideline for aquatic systems in South Africa, EU, WHO and US EPA. In South Africa, A COD target water quality range value of < 10 mg $O_2$/ℓ is considered safe for industrial use in the cooling water, stream generation and the process water from pharmaceutics (DWAF, 1996a). Wang *et al.* (2007) demonstrated that mortality due to liver cancer for men and women was positively correlated with the COD in drinking water in a nationwide study in 2007.

High COD values obtained in this study suggests that both organic and inorganic substances from domestic and non-domestic wastewaters are entering these water systems, as well as organic substance from municipal sewage plants (Olaniran *et al.,* 2012). The water quality of these sampling sites can only be used for most of the industrial purposes. Relatively high COD values and conductance levels were obtained for point U1 during all seasons with an exceptionally high COD value being detected during autumn (969 mg/ℓ) and high conductance level detected during summer (5150mS/m). Conductivity qualitatively reflects the status of inorganic pollution and is a measure of total dissolved solids and ionised species in the water (Iqbal *et al.,* 2004). The Umgeni River at point U1 had relatively high values for the environmental parameters tested and this could be attributed to the fact that this point of the river intercepts with the ocean hence the water is naturally more brackish in nature.

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 levels of the water samples from points U1, U2 and U3 in this study are higher for aquaculture with a South African Water Quality guideline value of 0.077 mg/ℓ P (DWAF, 1996a), except for point U5 which falls below the value. 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 samples varied from 0.087 to 1.70 mg/ℓ N thereby meet the criteria for the nitrates guideline value of 6 mg/ℓ N. In water quality studies, nitrogen and phosphorus are the nutrients most commonly identified as pollutants. Nitrogen in the form of ammonia ($NH_3$) and nitrates ($NO_3^-$) and phosphorus are essential nutrients to plant life, but when found in excessive quantities; they can stimulate excessive and undesirable plant growth such as algal blooms (Igbinosa and Okoh, 2009). Chloride is a common constituent in water, is highly soluble, and is a typical conservative substance (Igbinosa and Okoh, 2009). 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-fold during all seasons making it a cause for concern. Sulphate concentrations at sampling point U1 was the highest for all points in the river and reached 1400 mg/ℓ S in the autumn and 1512 mg/ℓ S in summer. The guideline value for sulphate according to the South African Water Quality guideline for domestic use is 200 mg/ℓ.

Heavy metals are some of the most common environmental pollutants, and their occurrence in surface waters and biota indicate the incidence of natural or anthropogenic sources. Natural sources of metals in surface waters include the chemical weathering of minerals and soil leaching, whilst anthropogenic sources are associated with industrial and domestic effluents, urban storm runoff, landfill leachate, and mining of coal and ore (Maanan *et al.,* 2004; Zarazua *et al.,* 2006). Heavy metals in surface waters can exist in colloidal, particulate, and dissolved phases (Miller *et al.,* 2003). Rivers are a dominant pathway for metal transport with many reports on temporal changes, especially seasonal variations, in heavy metal concentrations in river waters (Iwashita and Shimamura, 2003; Miller *et al.,* 2003). The metals most commonly associated with most river water systems are

lead (Pb), copper (Cu), iron (Fe), cadmium (Cd), aluminium (Al), mercury (Hg), arsenic (As), zinc (Zn) and manganese (Mn) (Wright and Welbourne, 2002).

The solubility of trace metals in surface water is predominantly controlled by water pH, water temperature, the river flow and the redox environment of the river system. Metal pollution in water environments is related to pH, i.e., when the pH of water decreases, the solubility and speciation of metals are improved thus increasing their toxicity (Campbell and Stokes, 1985; Rai *et al.*, 1993). This effect was evident at sampling points U1, U3 and U4 where a decrease in water pH from winter to spring resulted in increased concentrations of mercury. However, Hg had little or no impact on the microbial growth due to the low concentrations of Hg detected in the water systems (Table 2.4 and Figure 2.6). Mercury is known to occur at high concentrations in water bodies subject to industrial pollution. Mercury serves no known beneficial physiological function in humans, and exposure to mercury takes the form of neurological (organic mercury) and renal (inorganic mercury) disturbances (Guzzi and La Porta, 2008; Paasivirta, 2007).

The higher metal concentrations detected during the dry seasons were higher in concentration compared to the wet seasons could also 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). The presence of cadmium in the aquatic environment is a cause for concern because it bioaccumulates, and at elevated concentrations cadmium is acutely toxic to humans and can cause severe renal damage with renal failure (Paasivirta, 2007). In this study cadmium was not found to be present above the detection limit and CCA did not depict cadmium as an environmental variable that is of concern.

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 FC (Ashbolt *et al*., 2001). The South African water quality guidelines have (negligible risk) for TC and FC of 5 cfu/100mℓ and 0 cfu/100mℓ, respectively (DWAF, 1996a). Bacterial pathogens such as *E. coli, V. cholera* and *Salmonella typhimurium* are among the major health risks associated with water in general and wastewater in particular (Dungeni *et al.*, 2010). According to NMMP (2002) drinking untreated water can pose a potential health risk if faecal coliforms are present as follows: high risk (>10cfu/100mℓ), medium risk (1-10 cfu/100mℓ) and low risk (0 cfu/100mℓ). Full or partial contact with untreated water containing faecal coliforms poses health risks of: high risk (> 2 000 cfu/100mℓ), medium risk (600-2 000 cfu/100mℓ) and low risk (< 600 cfu/100mℓ) (NMMP, 2002).All points failed to meet the target water quality ranges for negligible risk with points U2 and U1 having the highest TC and FC populations. Since the sampling points were in the close proximity of human activities, the quality of the river sources depend on local circumstances. The high TC and FC load of the Umgeni River is an indication of poor sanitation and hygiene conditions as well as lack of environmental awareness among the people of the river bank communities. This observation was also highlighted in a similar study by Olaniran *et al*. (2009) where they found the highest total and faecal coliform counts to be at sampling points of Palmiet River and Umgeni River, where informal

settlements lacking proper sanitation facilities lined the banks of the rivers. The likely sources of contamination of the river waters found in the study area were the presence of pit latrines close to the river water sources and poor catchment management (Olaniran *et al.,* 2009). Sibanda *et al*. (2012) found counts of TC and FC increasing from upstream to downstream in the Tyume River, with microbial source tracking showing that faecal pollution was predominantly of human origin.

Investigators have now reported that the presence of faecal coliforms in surface water sources may not be definitive for a faecal origin of the bacteria (Obi *et al.,* 2002). For this reason, we employed an additional faecal indicator, enterococci (EC). *Enterococci* may be better indicators of human faecal pollution in water (Levin *et al.,* 1975; Rice *et al.,* 1993) as they are reportedly better indicators of risk to recreational users of contracting gastrointestinal illness, due mainly by enteric viruses in sewage contaminated waters (Paul *et al.,* 1995). According to the USEPA (2004) criteria for EC counts (<33 cfu/100mℓ for freshwater), all points along the Umgeni River exceeded this guideline value during all seasons tested, representing a cause of concern.. CCA ordination (Figure 2.5), found that FS did not correlate and form any relationships with any of the other bacterial indicators and physico-chemical factors in the environment. It can therefore be assumed that FS populations can exist independently in water environments. DWAF specifies two guidelines for FS levels: (1) 0 – 30 cfu/100mℓ for full contact recreation and (2) 0 – 230 cfu/100mℓ for intermediate contact recreation (DWAF, 1996a).

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). The Umgeni River mouth (sampling point U1) had the highest THB counts during all seasons compared to the other sampling sites along the river during these seasons. The Umgeni 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 previous studies (Olaniran *et al.,* 2009; Shittu *et al.,* 2008). Previous reports on the microbial quality of river water in some rural communities of South Africa showed that the water sources were unsafe for human consumption and *Escherichia coli* was one of the predominant potential pathogens isolated (Obi *et al.,* 2002). Probable causes of contamination of the river water sources include human and animal faeces or introduction of microorganisms by livestock, birds and insects (Nevondo and Cloete, 1999; Paul *et al.,* 1995; Sibanda *et al.,* 2012). Most river sources are reportedly prone to higher bacterial levels due to heightened ecological activities, and are thus inappropriate for domestic use and human consumption (Lazorchak *et al.,* 1998). These sources of contamination are further compounded by limited environmental awareness in rural communities surviving on surface waters (Obi *et al,*. 2002).

Canonical correlation analysis was used to find out the relationship between all the water quality variables and the total bacterial growth, as well as the relationship 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 current study showed that temperature, pH, electrical conductivity and turbidity were the key environmental factors affecting all sites especially during the spring and summer seasons. This positive correlation was further highlighted by the increased growth of the total heterotrophic bacterial populations at the same sites during spring and summer seasons. 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 (Nübel *et al.,* 1999; Byamukama *et al.,* 2000; Nishiguchi, 2000).The findings of this study are in agreement with the research performed by Noori *et al.* (2010), where they used CCA to determine relationship between physical and chemical water quality parameters. In their study, the researchers found that EC, TDS, and Temperature of physical variables and $BOD_5$, and $NO^{3-}$ of chemical parameters have high correlation (Noori *et al.,* 2010). In another study CCA ordination plot revealed a strong relationship between chironomid (macro invertebrate) communities and measured physical and chemical water quality variables (Odume, 2011).

Since catchment conditions and water quality change seasonally, a proper investigation of the chemical and microbial surface water quality was performed to assess samples taken both during the rainy and the dry seasons, to take annual variation into account. The study highlighted the poor microbiological and physical quality of the Umgeni River in Durban, South Africa which raises concerns regarding public health. In addition, in this study, CCA was used to evaluate the Umgeni River water data sets.

Water quality and water resource protection are of paramount importance in South Africa as rapid population growth and economic progression are both placing colossal demands on the countries diminishing water resources. Issues concerning water quality have been a problem as long as urbanization proceeds at a fairly rapid rate. An integrated water management system is therefore required to handle such demands while communities and authorities should become more involved with their environment by ensuring that their environment is clean and not a health hazard risk to any individual.

The study highlighted a need for a continuous pollution monitoring programme of the surface waters in rural, sub-urban and urban communities in the KwaZulu-Natal Province of South Africa. In addition the provincial government and all agencies concerned with environmental matter in South Africa should evolve measures to ascertain and ensure that wastewater discharge effluents comply with laid down rules and regulations. In areas lacking in adequate tap water as in rural communities, educative programmes must be organized by health care providers and government officials to enlighten the villagers on the proper use of surface water. As such, water managers and government officials should embark on identifying periods when water quality is poor and issue advisory notices warning the public of increased risk of use.

Actions to protect public health may include permanently discouraging recreational activity in contaminated water, for example by fencing or signposting. In addition campaigns to improve health literacy and

create an awareness of the dangers when using raw-water sources may also need to be conducted in communities who directly rely on surface waters for domestic uses. These campaigns should be aimed at reducing the risks of contracting waterborne illnesses.

# Chapter Three

## Detection of virus-like particles (VLPs) from the Umgeni River- South Africa

## 3.1 Introduction

Despite the advancement in water and wastewater treatment technology, faecal pollution of surface waters is still a cause of concern for the general public and can lead to waterborne diseases which have public health and socioeconomic implications in both the developed and developing world (Hamza *et al.,* 2011; Wong *et al.,* 2012). Waterborne diseases are frequently caused by enteric pathogens, which belong to the group of organisms transmitted by the faecal-oral route (Redwan *et al.,* 2008). According to the World Health Organization (WHO), every year there are ~2.2 million deaths associated with unsafe water conditions, sanitation and hygiene, and millions more suffer multiple episodes of non-fatal diarrhoea (WHO, 2008). Water quality concerns have increased in recent years, in part due to recurrent contamination of surface water resources by waterborne bacterial, viral and protozoan pathogens (Savichtcheva and Okabe, 2006).

Microbiological quality standards for water include only bacterial faecal indicators, which are not correlated with the presence of waterborne viruses (Melnick and Metcalf 1985). Routine viral monitoring is not required for drinking or recreational waters and neither is it required for wastewater that is discharged into the environment. This lack of a monitoring effort is due largely to the lack of methods that can rapidly and sensitively detect infectious viruses in environmental samples (Li *et al.,* 2010). Phages have been recommended as alternate microbial indicators as they behave more like the human enteric viruses which pose a health risk to water consumers if water has been contaminated with human faeces (Grabow, 2001; Havelaar, 1993). Phages share many properties with human viruses, particularly composition, morphology, and structure (Contreras-Coll *et al.,* 2002).

Somatic coliphages have been described as a heterogeneous group of viruses, which could originate from faecal sources (Havelaar, 1993; Calci *et al.,* 1998). The presence of these viruses in faecally contaminated water means that they can serve as indicators of faecal pollution and may indicate the coexisting presence of pathogenic viruses. Due to their resistance against environmental factors, somatic coliphages are more applicable than faecal bacteria for indicating faecal contamination of water (Contreras-Coll *et al.,* 2002; DWAF, 1996a). The occurrence of F-RNA phages in surface waters generally indicates pollution by human or animal faeces (IAWPRC, 1991; DWAF, 1996a; Leclerc, 2000; Schaper and Jofre, 2000). It was shown that for monitoring purposes, F-specific RNA bacteriophages can indicate the possible presence of human pathogenic enteric viruses as they behave like waterborne viruses and their morphology and survival characteristics closely resemble that of some of the important human enteric viruses (Havelaar *et.al.,* 1993; Turner and Lewis, 1995). The phage plaque assay has the advantages of technical simplicity and low cost but the choice of a suitable bacterial host is of paramount importance (Yee *et al.,* 2006).

Human enteric viruses are imperative causative agents of waterborne illness; however, viral diseases are difficult to identify by current diagnostic techniques (Horman *et al.,* 2004; Winfield and Groisman, 2003). The Centers for Disease Control and Prevention (CDCP, 1993) projected that viral

infection may be the causative agent of nearly 50% of all acute gastrointestinal illnesses, and therefore, viral contamination of recreational coastal water is of paramount importance and is a rising public health concern (Jiang *et al.,* 2007). Enteric viruses have a relatively low infectious dose and can be responsible for a large range of human illnesses such as paralysis, meningitis, respiratory diseases, myocarditis, congenital abnormalities, epidemic vomiting, diarrhoea, and hepatitis (Fleisher *et al.,* 1998; Kopecka *et al.,* 1993). Nevertheless, it is now accepted that viral contamination should be definitely taken into account in the microbiological analysis of water and food (Metcalf, 1995). The requirement for the virological analysis of water is an efficient and simple virus concentration method, as viruses may be present in very low numbers, thus making it essential to start with a large water sample volume and concentrate it to several orders of magnitude (Ehlers *et al.,* 2005a; Griffin *et al.,* 1999; Lipp *et al.,* 2001; Lodder *et al.,* 2005; van Heerden *et al.,* 2003). It is well recognized that monitoring the presence of enteric viruses could be challenging due to the relatively low level of viral particles and small viral particle size existing in environmental waters. However, this limitation can be overcome by the use of improved methods for aquatic sample concentration, viral infectivity, and more sensitive viral detection techniques (Fong and Lipp, 2005). Tangential-flow and hollow-fiber ultrafiltration (UF) has been used to investigate microbial contamination of drinking water (Hewitt *et al.,* 2007a; O'Reilly *et al.,* 2007). The virus concentration method should be applicable to a broad spectrum of enteric viruses to facilitate concurrent concentration (van Heerden *et al.,* 2003).

Transmission electron microscopy (TEM) and Epiflourescence microscopy (EFM) is used for the detection, identification and enumeration of viral particles (Zechmann and Zellnig, 2009).With these methods, virus types and diseases can be diagnosed reliably since size and ultrastructural features are specific for each group of viruses . Negative staining of viruses followed by visualization by TEM can provide rapid and accurate results, and, in most cases they are sufficient for the identification of viraldiseases including determination of structure and size of virus particles (Baumeister and Steven, 2000; Harris, 2007; Marshall, 2005; 2012; Wild, 2008).

Cell culture is the only technique that can assess the viability of pathogenic viruses, whereby the cytopathic effect (CPE- cell death) is observed. Many viruses produce cytopathic effects in their host cells during a productive infection. While some viral infections can be assayed by the assembly of plaques, many viruses, while producing cytotoxicity, do not easily form plaques, or do not form plaques at all (Heldt *et al.,* 2006). A tetrazolium salt assay (MTT assay) has now been applied to measure CPEs produced by viral infection for different virus families. The MTT assay is a high-throughput, process that can reproduce consistent titers for a variety of viruses (Heldt *et al.,* 2006).

It is important to consider viruses in water quality because of their incidence as causal agents for diarrheal disease, and due to their characteristics, which allow them to survive in changing environmental conditions indefinitely. 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, 2010). The monitoring of water supplies and research on waterborne viruses (mainly in Gauteng) in South Africa have been inadequate (Grabow *et al.*, 2004).Therefore this study assessed the viral quality of the Umgeni River water seasonally. The present study was conducted to optimize procedures to extract and enumerate indigenous virus likeparticles and to determine the community structures and cytotoxicity of these viruses from the river water. A current technique for filtering viruses from water was setup. Here ultra-filtration of viruses from large (20 l) volume water samples using a two-step tangential flow filtration (TFF) process was established. The presence of virus like particles (VLPs) was determined using epifluorescence microscopy (EM), transmission electron microscopy (TEM) and their infectivity was determined on cell culture.

## 3.2 Materials and Methods

### 3.2.1 Sample Collection

Water samples were collected as described in Section 2.2.1 of Chapter two.

### 3.2.2 Bacteriophage Determinations

#### 3.2.2.1 Preparation of Bacterial Hosts for Bacteriophage Detection

*Escherichia coli* ATCC 13786 was used as the host for the somatic coliphage. The bacterial host was grown overnight at 37 °C on a shaking incubator at 100 rpm, in nutrient broth (Appendix i). *Salmonella typhimurium* WG49 (provided by Dr. Maite Muniesa, University of Barcelona, Spain) was used as the host for F-specific coliphage. The *S. typhi* host was grown in Tryptone Yeast Extract broth (Appendix i) and incubated at 37 °C with shaking at 100 rpm until the F-pili developed (ISO 1995). This growth suspension was monitored at 30 min intervals from time 0 against a blank reference at 560 nm until an absorbance of 0.75 nm was obtained indicating that the sex pili were produced (Grabow, 2001).

#### 3.2.2.2 Presence –Absence Spot Test

The presence- absence spot test using the single agar layer method was to determine the presence of somatic and F-RNA (F-specific) coliphages. The test was accomplished by spotting 10 µℓ of 0.45µm filtered water samples onto lawns of the two bacterial hosts. The plates were incubated at 37 °C for 24 hr and zones of cell lysis (plaques) were considered as positive and indicated the presence of phage.

#### 3.2.2.3 Double Agar Overlay Plate Assay

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 (Appendix i) and poured over agar bottom plates (Appendix i) 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).

### 3.2.3 Tangential Flow Filtration (TFF) for Viral Recovery

Viral community samples (virioplankton) were concentrated using a two-step tangential flow filtration process as shown in Figure 3.1, according to the method of Wommack *et al.* (2010), with some modifications. Concentration of virus particles from large volume water samples occurred in four steps: (1) Twenty litres of river water was first pumped through a 25 µm string-wound polypropylene sediment cartridge filter to remove large particles and cells at a flow rate of 230 mℓ/min; (2) A peristaltic pump (Masterflex) was used at a flow rate of 530 mℓ/min with pressure at ( $P_{in}$ = 7 psi and $P_{out}$ = 6 psi) to pump the pre-treated water samples through a 0.5 $m^2$, 0.22 µm Pellicon TFF cartridge filter (microporous filtration membrane) [Millipore Corp] to remove all bacterial cells; (3) Viruses in the cell-free permeate was then further concentrated to a final volume of 500 mℓ using 10 $m^2$ 100-kDa spiral-wound TFF filter (Helicon; Millipore Corp); (4)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. Retentates were then reconcentrated by ultracentrifugation. For ultracentrifugation, 3 runs of 3 hr 30 min each at 130 000 × *g* (i.e. 29 000 rpm), 4°C with a SW-32 Ti rotor (Optima L-100 XP, Beckman Coulter Ultracentrifuge) were necessary to ultracentrifuge 500 mℓ (6 tubes of 30 m ℓ filled with 28 mℓ of samples per run) of the ultrafiltered retentate (Colombet *et al.*, 2007). Viral pellets were then resuspended in 5 mℓ of 1 x Phosphate Buffered Saline (pH 7).

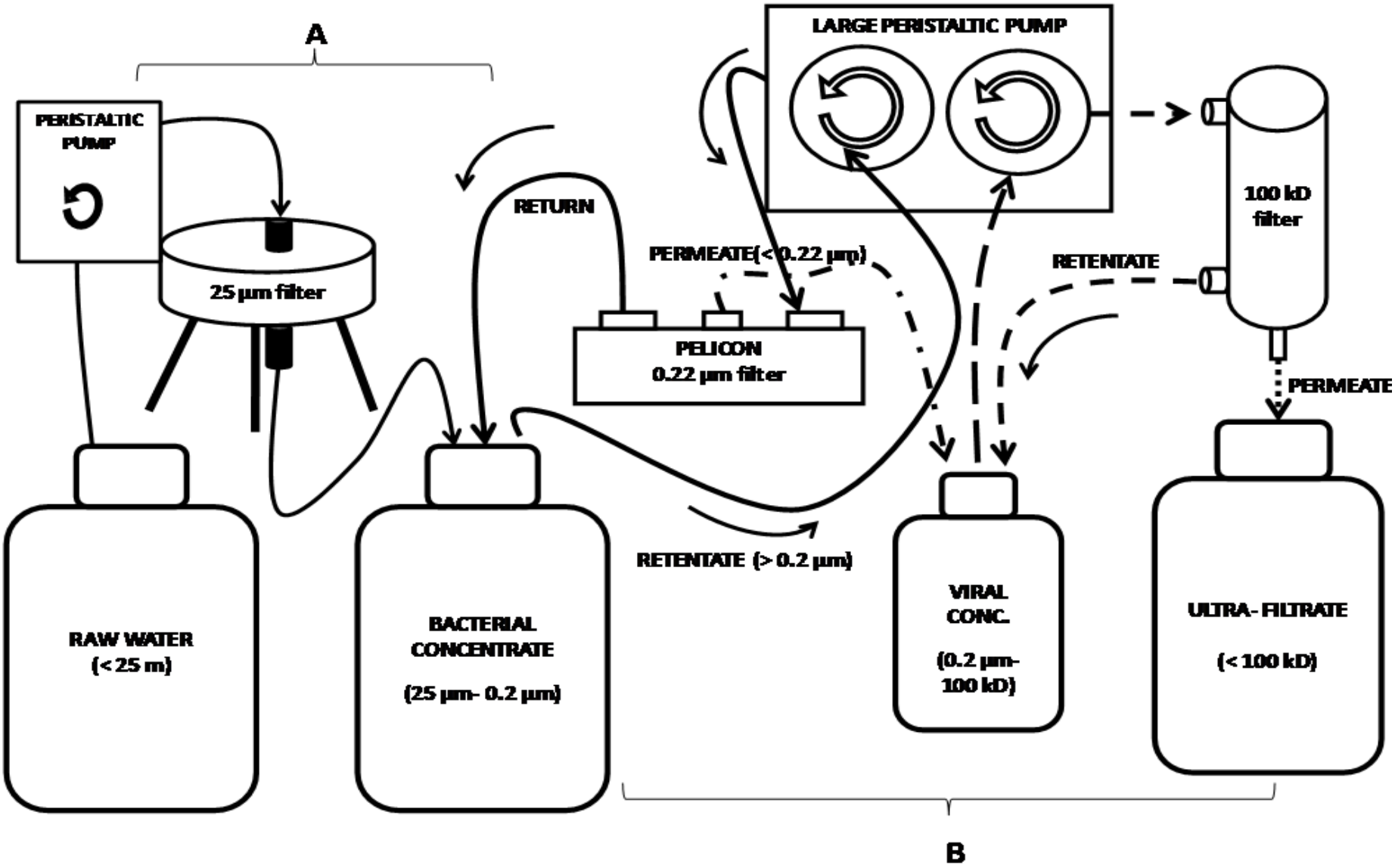



**Figure 3.1** Experimental set-up for tangential flow filtration concentration of viruses from large volume water samples. Pre-filtration of initial water sample illustrated in panel A and panel B illustrates the two-step TFF procedures for virus concentration

### 3.2.4 Normal Water Permeability (NWP)

Normal Water Permeability (NWP) testing was performed regularly on the TFF filter membranes before and after running the water samples through the system to ensure filter quality. Proper maintenance and preservation of filter quality was carried out according to manufactures instruction with some modifications [Appendix i].

NWP 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 | $P_{in}$ | = Feed Inlet Pressure in psi |
| $P_{out}$ | = Retentate Discharge Pressure in psi | $P_p$ | = Permeate Discharge Pressure in psi |
| A | = Total Filter area in $m^2$ | | |
| F | = Temperature correction factor (based on Water Fluidity Relative to 25°C) | | |
| T | = Water Temperature in °C (used to determine F, Millipore Pellicon Filters) | | |

### 3.2.5 Enumeration and Visualisation of Virus-Like Particles (VLP)

#### 3.2.5.1 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 ultrafiltration 1 mℓ (100 kDa cut-off) 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 480-495nm) with a 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.

#### 3.2.5.2 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 air dried 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.

### 3.2.6 Viral Infectivity Assay 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 (2001b) 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, the A549 (adenocarcinomic human alveolar basal epithelial cell), HEK 293 (human embryonic kidney), Hela (Henrietta Lacks – cervical cancer), HepG2 (human hepatocellular carcinoma), PLC/PRF/5 (human primary liver carcinoma cells) and Vero (African green monkey kidney 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 plastic plates (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 (2001b). After three freeze-thaw cycles, CPE positive and negative samples were filtered through 0.22 µm syringe filters and were inoculated in new A549, HEK 293, Hela, HepG2 and PLC/PRF/5 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). Wells were considered to have CPE when there was loss of cell to cell contact and detachment. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, MTT) assay was performed using the cell lines mentioned above (Heldt *et al.,* 2006). When performing the MTT assay for all VLP samples, the cells were plated onto 24-well plates and infected approximately 24 h later. Each VLP sample was added to the plates in triplicate. After 6 - 7 days the MTT solution was added to the plates, and the solubilization agent added 4 h after adding the MTT

solution. The reaction was stopped by the addition of dimethyl sulfoxide (DMSO) and the plates were then read using a spectrophotometer (Vacutec Micro Plate Reader, South Africa). The optical density (cell viability %) was defined as: Optical density = (Well absorbance/Blank absorbance) × 100% (Heldt *et al.*, 2006). The percentage of cell death was calculated as: 100 - Optical density. The replicate samples were averaged and a plot of the CPE (%) was generated.

## 3.3 Statistical Analysis

Pearson's correlation test was used to evaluate the 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) as explained in Section 2.3 of Chapter 2 was applied to elucidate the relationship between the bacteriophages, virus-like particles and the measured physical and chemical water quality variables at the various sites and seasons, with a view to determining the important variables responsible for the observed spatial and temporal distribution of the communities.

## 3.4 Results

### 3.4.1 Enumeration of Bacteriophages and Virus-Like Particles (VLPs)

Both somatic and F-specific coliphages were tested for in this study (Table 3.1). A positive result for the presence of somatic and F-specific coliphages was indicated by plaque formation on a lawn of host culture. All samples 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 (Table 3.1). These three sites also contained higher bacterial counts and physico-chemical values (Chapter 2). Points U1 and U3 during the autumn and spring seasons and Point U1 and U2 during summer had strong F-RNA plaque formation.

**Table 3.1** Presence – Absence spot test (based on plaque formation on a lawn of host culture) for the determination of somatic bacteriophages and F-RNA coliphages in the Umgeni River water.

| 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 | ++ | + |

Intensity of Plaque Formation (cell lysis) based on visualisation: + : Weak Plaque; ++ : Average Plaque; +++ : Strong Plaque; -: No Plaques

The plaque forming units (PFU) results obtained 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 using epifluorescence microscopy (EFM) 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) and 190.1 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 well with the somatic coliphages and VLP populations ($r = 0.85$, $p< 0.05$) at all sites along the river and for both seasons.

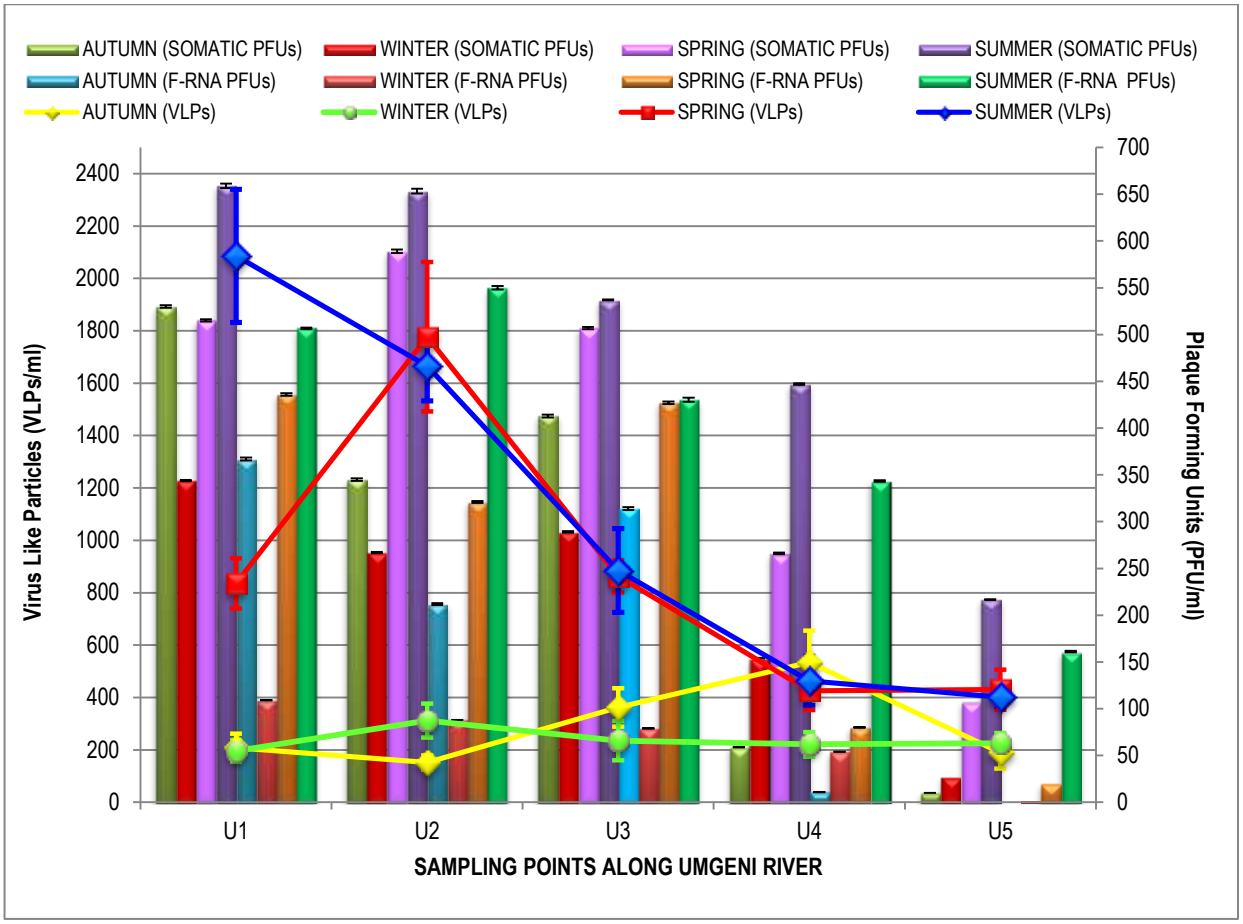



**Figure 3. 2** Presumptive counts of virus like particles (VLPs) and plaque forming units for the Umgeni River at the different sampling sites during autumn, winter, spring and summer. Line plot indicates the average of replicate samples (n = 8 or 10) for VLPs and bars indicates the average of replicate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

### 3.4.2 Canonical correspondence analysis (CCA)

CCA is a multivariate statistical analysis used to elucidate the relationships between biological community and their environment (ter Braak and Verdonschot, 1995). In this study CCA was used to investigate the relationships between the water quality variables and the virus like particles, as well as the relationships between the bacteriophages and the physico-chemical parameters at all sites and seasons. 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 relationships between the overall VLP and phage populations at different sites and seasons measured as well as with the physico-chemical water quality variables.

CCA plot (blue circle), temperature, $BOD_5$, turbidity, pH, conductivity, orthophosphate and sulphate were the most important variables (Figure 3.3- 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.

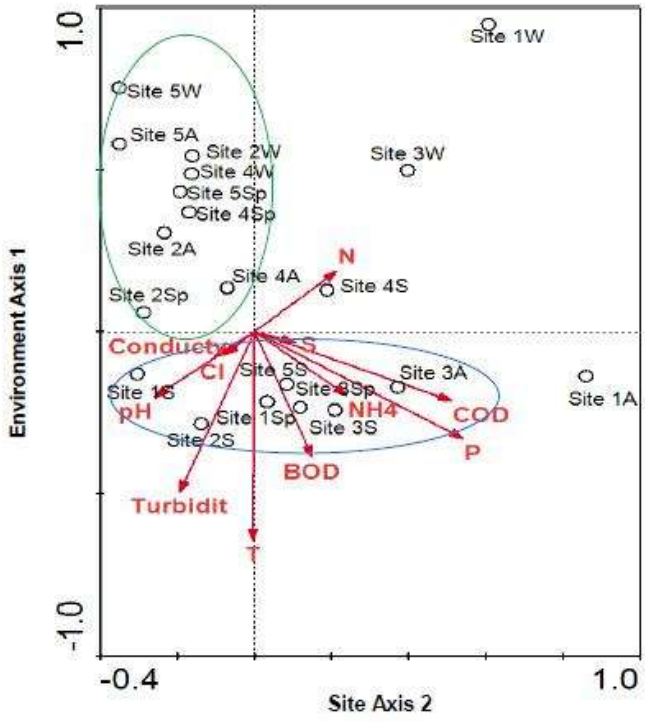



**Figure 3. 3** CCA ordination plot for all the water quality variables and the total viral and bacteriophage growth at the five study sites and during autumn, winter, spring and summer seasons. Abbreviations: Sites 1= Umgeni River mouth, 2= Informal settlement at Reservoir Hills, 3= New Germany Waste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

CCA axis 1 (Figure 3.3) accounted for 77.6 % of total variance of the species data set and in total the species – environment relation accounted for 94.1% of the cumulative variance suggesting that there may be a correlation between the VLP and phage populations at the various sites and water quality variables during all seasons. Positive significant ($p<0.05$) Pearson correlation of species and environmental scores for axis one and two were $r = 0.955$ and $r = 0.962$ respectively (Table 3.2).

**Table 3. 2** 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 |

The ordination plot revealed that the Somatic phage, FRNA phage and VLPs had no relationships witheach other and existed independently (Figure 3.4). 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 3.4) 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.

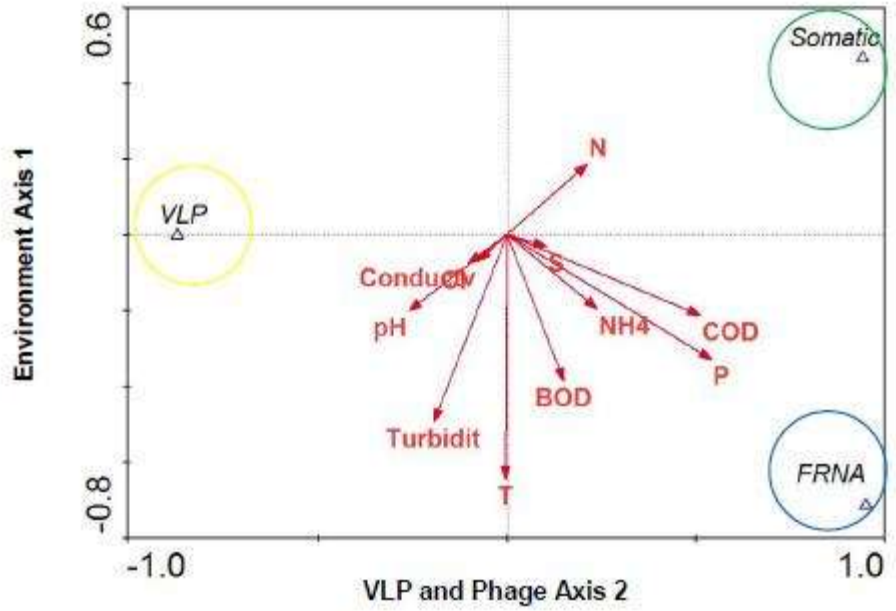



**Figure 3. 4** CCA ordination plot for bacteriophage and virus-like particle populations and water quality variables at the five study sites during autumn, winter, spring and summer seasons.

Eigen values for axis point one and two were 0.043 and 0.021 respectively (Table 3.3). Positive significant ($p<0.05$) Pearson correlation of species and environmental scores for axis one and two were $r = 0.759$ and $r = 0.980$ respectively.

**Table 3. 3** Properties of the Canonical Correlation Analysis ordination bi-plot for bacteriophage and virus like particle 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 |

CCA shown in Figure 3.5 revealed strong relationship between the THB communities and VLP and phage communities (green circle). The VC and TC populations also had positive relationships with+ 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 3.4). FC had no correlation with VLP and phage communities.

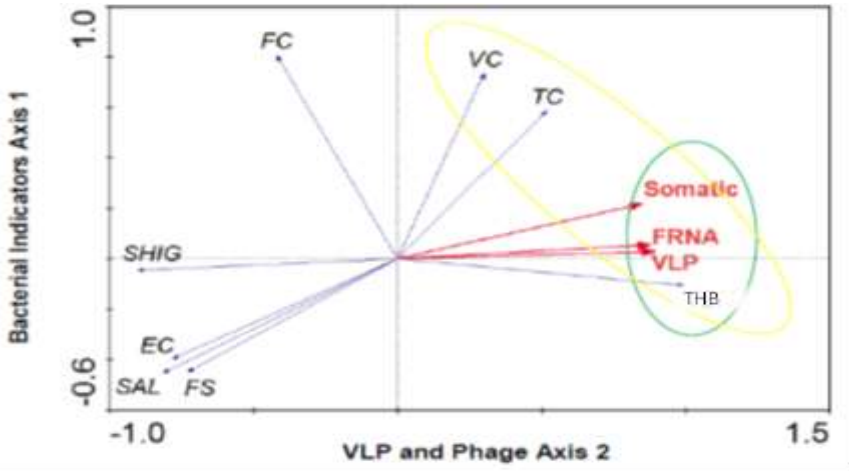



**Figure 3.5** CCA ordination plot for bacteriophage and virus populations and bacterial indicators at the five study sites during autumn, winter, spring and summer seasons.

CCA axis 1 (Figure 3.5) accounted for 79.7% of total variance of the species data set and in total the species – environment relation ordination accounted for 98.2% of the cumulative variance suggesting high correlation between bacterial community data and VLP and phage communities. Eigen values for axis point one and two were 0.039 and 0.024 respectively (Table 3.4). 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.

**Table 3.4** 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 |

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

Table 3.5 classifies the different type of bacteriophage that could be detected in the Umgeni River by TEM according to the scheme of Ackermann and Eisenstark (1974). Most of the detected phages (33%) had isometric heads and long non-contractile tails, belonging to morphotype B1 (*Siphoviridae*) (Table 3.5). 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. Table 3.6 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), to 218 nm (summer).

**Table 3. 5** 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)].

| Sample Season | No. of phage's belonging to morphotypes: | | | | | |
|---|---|---|---|---|---|---|
| | **A1** | **B1** | **A1/B1 *** | **B2** | **C1** | **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.

**Table 3.6** Size range of tailed phages 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 3.6, 3.7 and 3.8 represent 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 3.6 a, b, c, e and f; 3.7 f); others were irregular, with only one symmetrical axis (e.g., Figure 3.7 a, c and d). However, these data were not adequate to differentiate

between icosahedra, octahedra, and dodecahedra classification. Figure 3.6 c and h closely resemble members of the family *Myoviridae* and *Podoviridae*, respectively. Figure 3.7 d and h resemble members of the family B2 *Siphoviridae* and B1 *Siphoviridae* respectively. Figure 3.8 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 (Ackermann and Heldal, 2010).

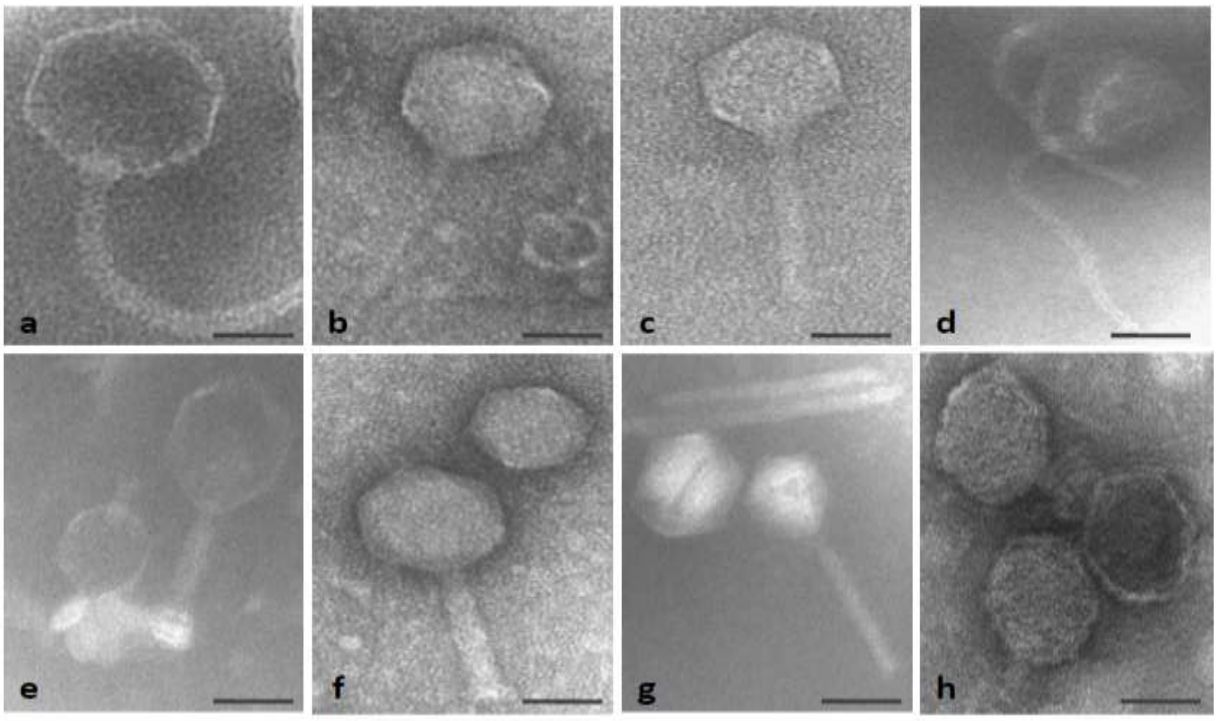



**Figure 3.6** TEM images of phage particles of various morphotypes present downstream of the Umgeni River at the sampling sites U1, U2, and U3 during autumn, spring and summer seasons tested. Images captured at 300 000 – 400 000 x magnification. Scale bar 100 nm.

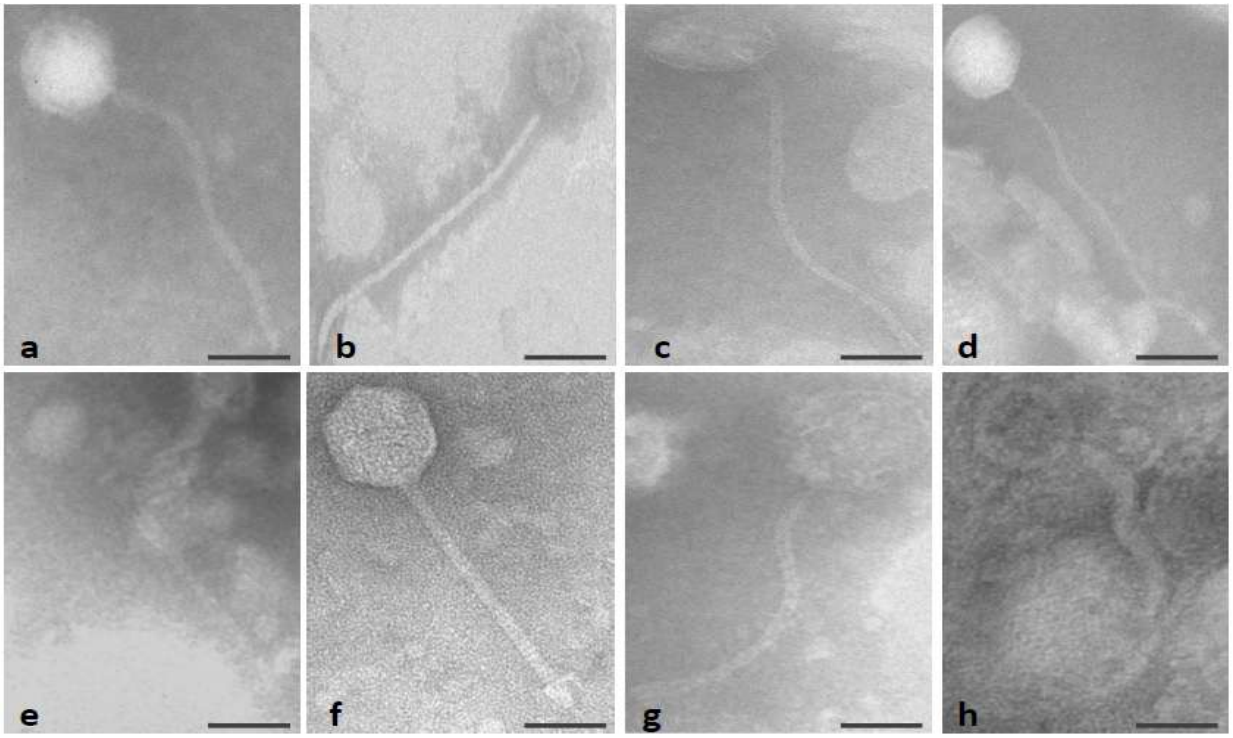



**Figure 3.7** TEM images of long tailed phage particles of various morphotypes present in the Umgeni River at the sampling sites U1, U2, U3, U4 and U5 during winter, spring and summer seasons tested. Images captured at 300 000 – 400 000 X magnification. Scale bar 100 nm.

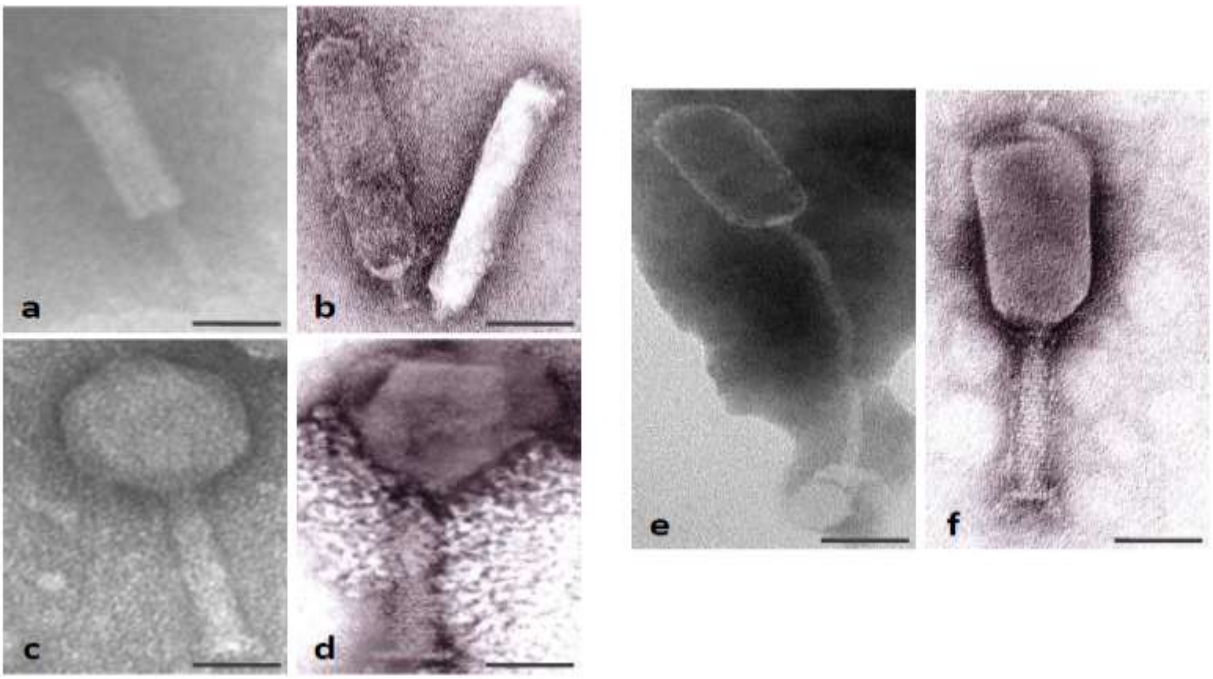



**Figure 3.8** TEM images of various phage morphotypes (a, c, e) present in downstream of the Umgeni River at the sampling sites U1, U2 and U3 respectively during spring and summer seasons tested. (b) Known 71A-6 of *Vibrio vulnificus* phage (d) Known Phage T4 (f) Known T4-like *Vibrio parahaemolyticus* phage, (Ackermann and Heldal, 2010). Images captured at 300 000 – 400 000 X magnification. Scale bar 100 nm

Figures 3.9 – 3.13 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. Presumptive naked *Adenoviridae*-like particles ranging in size from 66.97- 74.40 nm are shown in Figure 3.9 and these images are compared to known Adenoviruses (70-90 nm) (Figure 3.9 g).

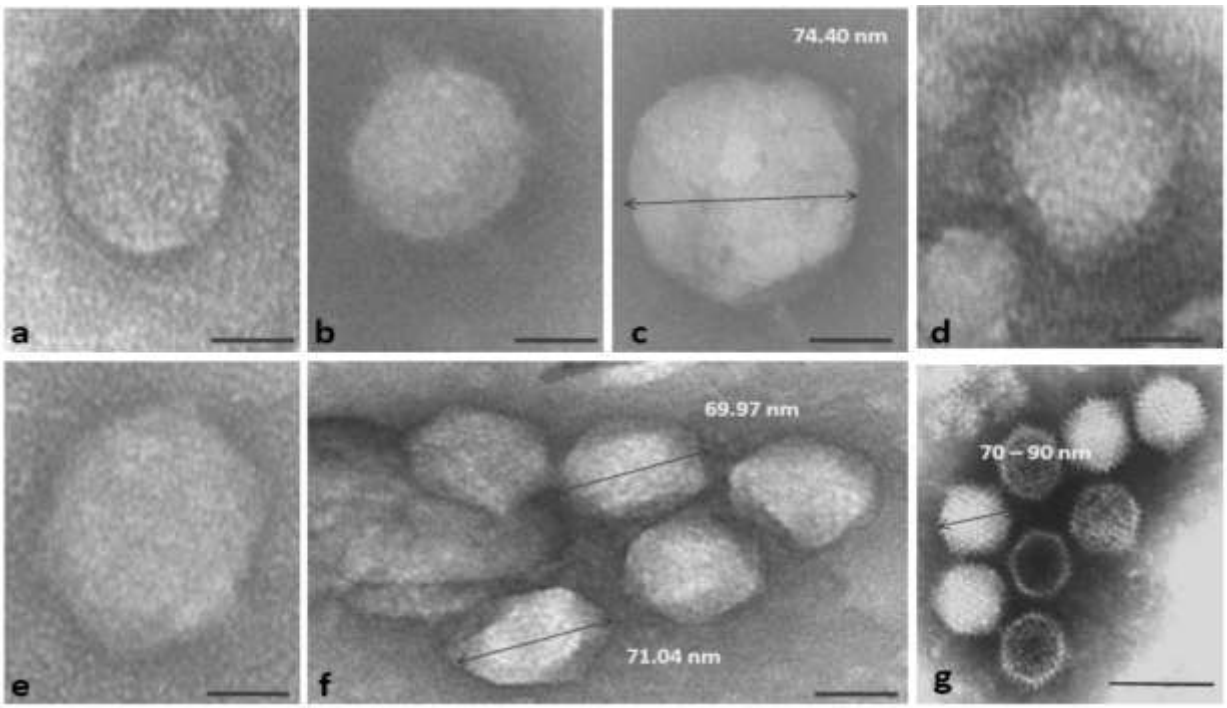



**Figure 3. 9** TEM images of presumptive naked *Adenoviridae*-like particles and (g) Adenovirus (Steffens, 1998), present in the Umgeni River at the sampling sites U1, U2, U3, U4 and U5 during autumn, spring and summer seasons tested. Images captured at 400 000 – 600 000 X magnification. Scale Bar 100nm.

Figure 3.10 represents TEM images of presumptive naked *Picornaviridae* (Enterovirus)-like particles ranging in size from 26 – 30nm, compared to a known Coxsackievirus (Figure 3.10 f). Poxviridae-like particles (Figure 3. 11 a, b, c) and *Herpesviridae*-like particles (Figure 3.11 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 3.11 d) and known Herpesvirus (Figure 3.11 h). Figure 3.11 illustrates presumptive *Reoviridae*-like particles ranging in size from 18 – 20 nm (Figure 3.12 a, b, c) compared to a known Rotavirus (20 – 30 nm) (Figure 3.12 d). Presumptive *Caliciviridae* - like particles are illustrated in Figure 3.12(e, f, g) and these had a size of 35 nm, similar to that of a known Norovirus (Figure 3.12 h) of 30 – 40 nm. Presumptive enveloped *Coronaviridae* -like particles (Figure 3.13 a, b, c) was found to be similar to a known Coronavirus (Figure 3.13 d), with presumptive *Orthomyxoviridae*-like particles being similar to a known Influenza virus (Figure 3.13 h).

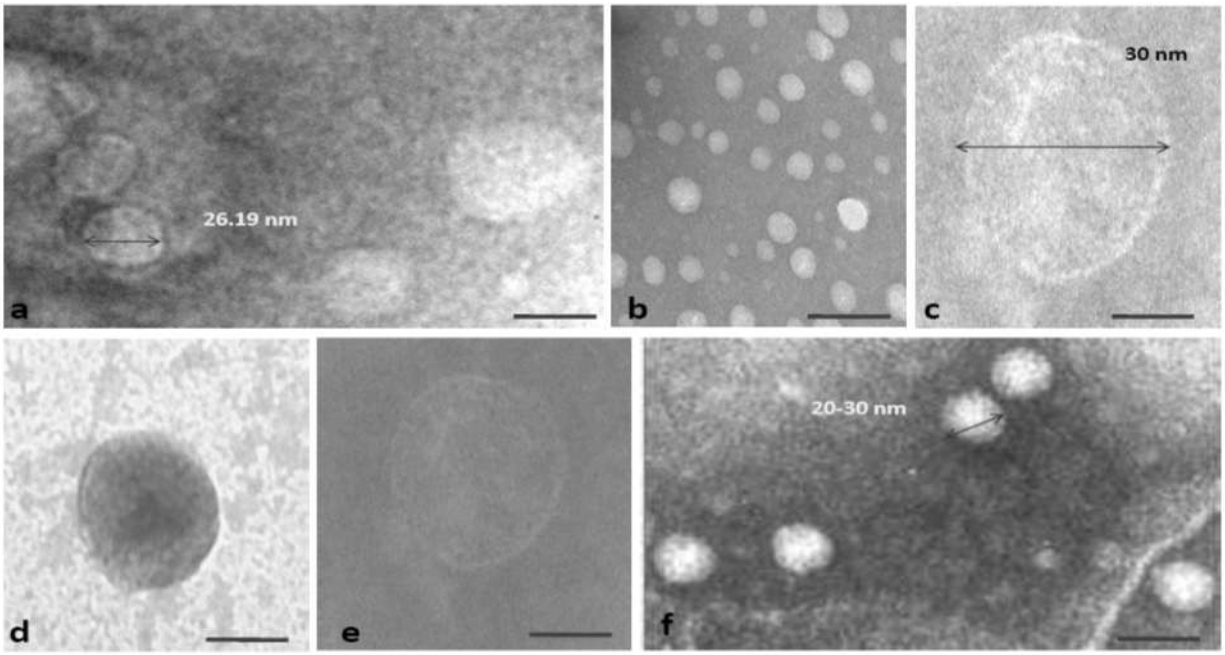



**Figure 3.10** TEM images of presumptive naked *Picornaviridae* (Enterovirus) -like particles (a-e) and f) Coxsackievirus (Schramlová *et al.*, 2010), present in the Umgeni River at sampling sites U1, U2, and U3 during spring and summer seasons tested. Images captured at 500 000 – 600 000 X magnification. Scale Bar 50nm.

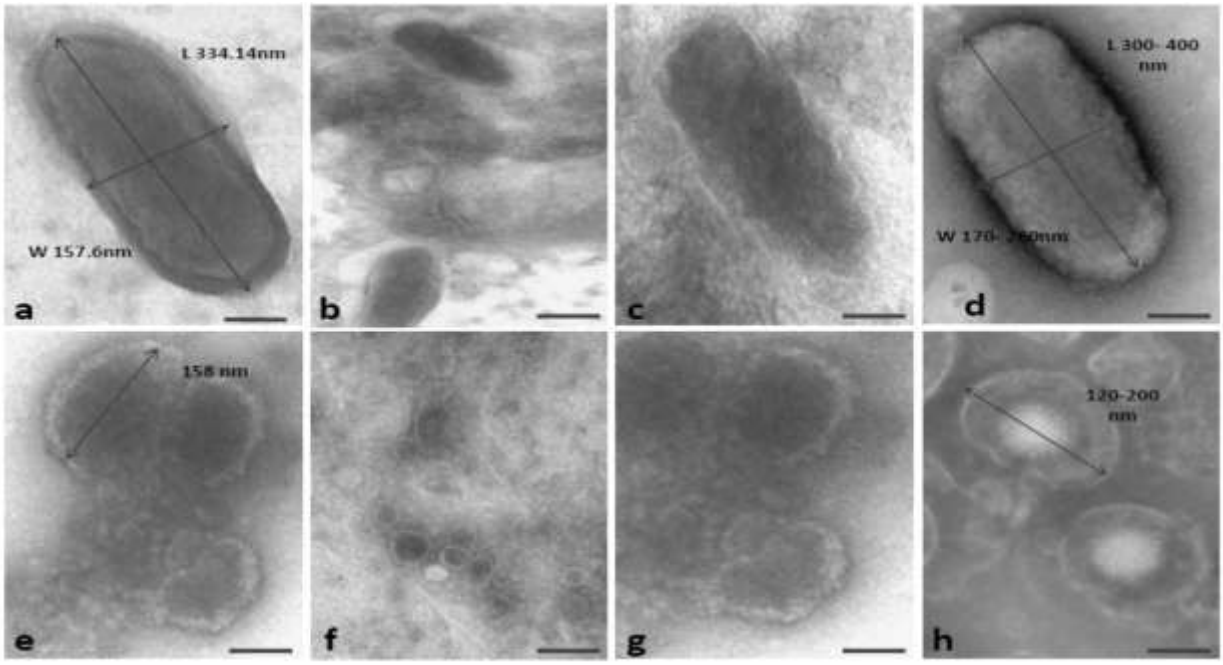



**Figure 3.11** TEM images of enveloped presumptive (a, b, c) *Poxviridae*-like particles (d) known Pox virus (Goldsmith and Miller, 2009) and (e, f, g) *Herpesviridae*-like particles (h) Herpes virus (Goldsmith and Miller, 2009), present in the Umgeni River at site U3 during the summer season. Images captured at 300 000 – 500 000 X magnification. Scale Bar 200nm.

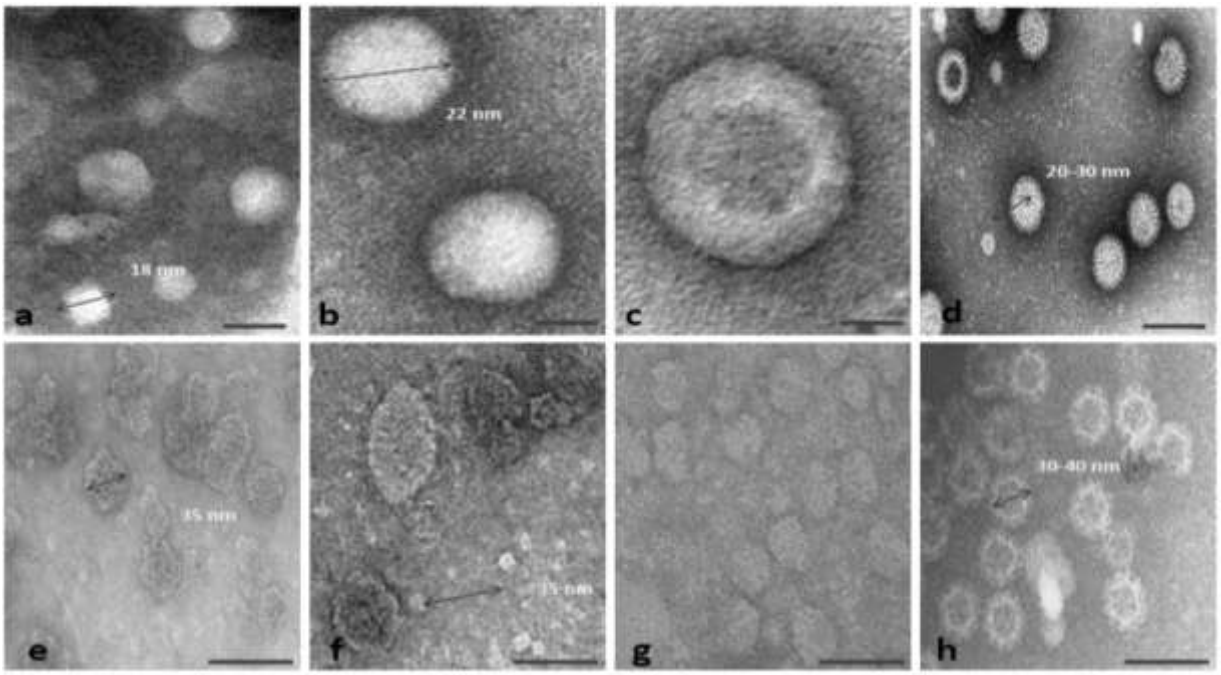



**Figure 3.12** TEM images of presumptive (a, b, c) *Reoviridae* virus like particles, d) known Rotavirus (Marshall, 2005), (e, f, g) presumptive *Caliciviridae* virus like particles, h) Norovirus (Humphrey, 2008), present in the Umgeni River at the different sampling sites during all seasons tested. Images captured at 500 000–600 000 X magnification. Scale Bar 50nm.

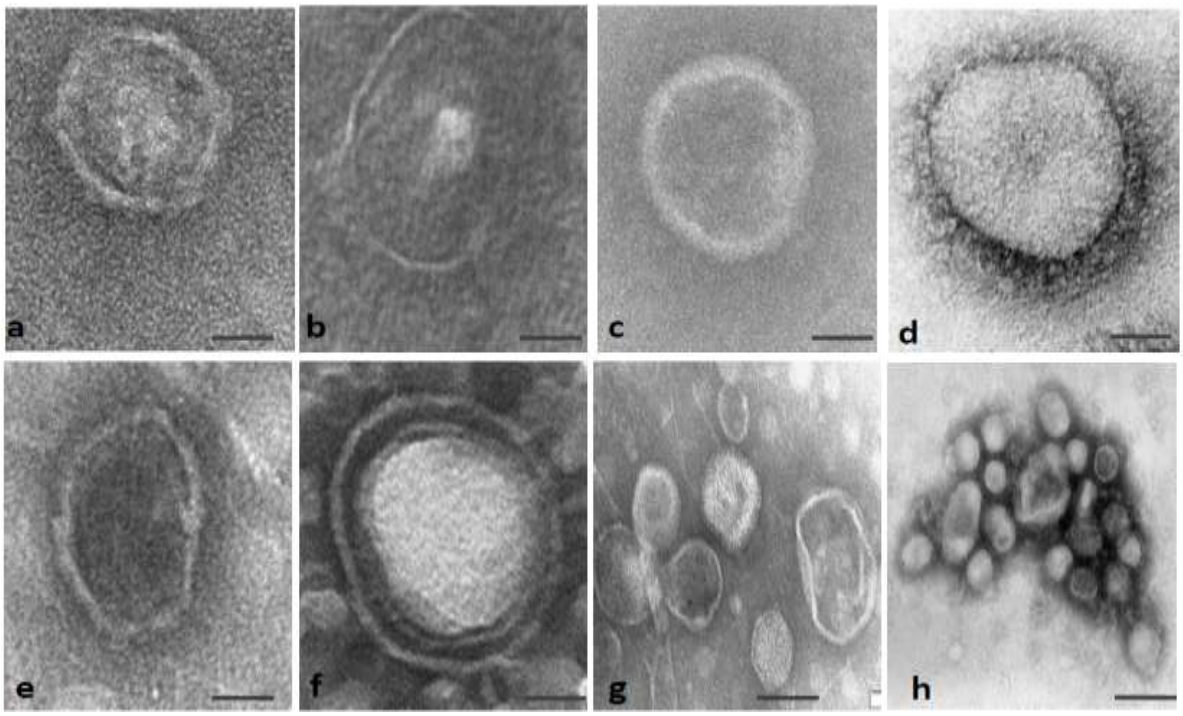



Figure 3.13 TEM images of presumptive enveloped virus like particles (a, b,c) *Coronaviridae* virus like particles, d) Coronavirus (Schramlová *et al.*, 2010), (e, f, g) presumptive *Orthomyxoviridae* virus like particles, h) known Influenza virus (Schramlová *et al.*, 2010), present in the Umgeni River at the different sampling sites during all seasons tested. Images captured at 400 000 – 600 000 X magnification. Scale Bar 100nm.

### 3.6 Viral Infectivity Using Cell-Culture

The Cytopathic Effect (CPE) is an observable morphological (shape) change in tissue cells due to viral infection. Table 3.7 and Figure 3.14 illustrate the results obtained for the CPE of the virus-like particles isolated from the Umgeni River using six tissue cell lines. These cell line were chosen due to their ability to propagate a wide range of viral particles. Confluent monolayers of un-infected cell lines are illustrated in Figure 3.14 (a, b, c, d, e, f), and once viral infection of the cells monolayer's ensued and the cells began to round off (appear circular) and lose consistent morphology as shown in Figure 3.14 (g, h, i, j, k, l), an indication of cell death. The identification of CPE in the cell cultures included the occurrence of elongated, granulated cells, loss of cell to cell contact, and the appearance of vacuoles.

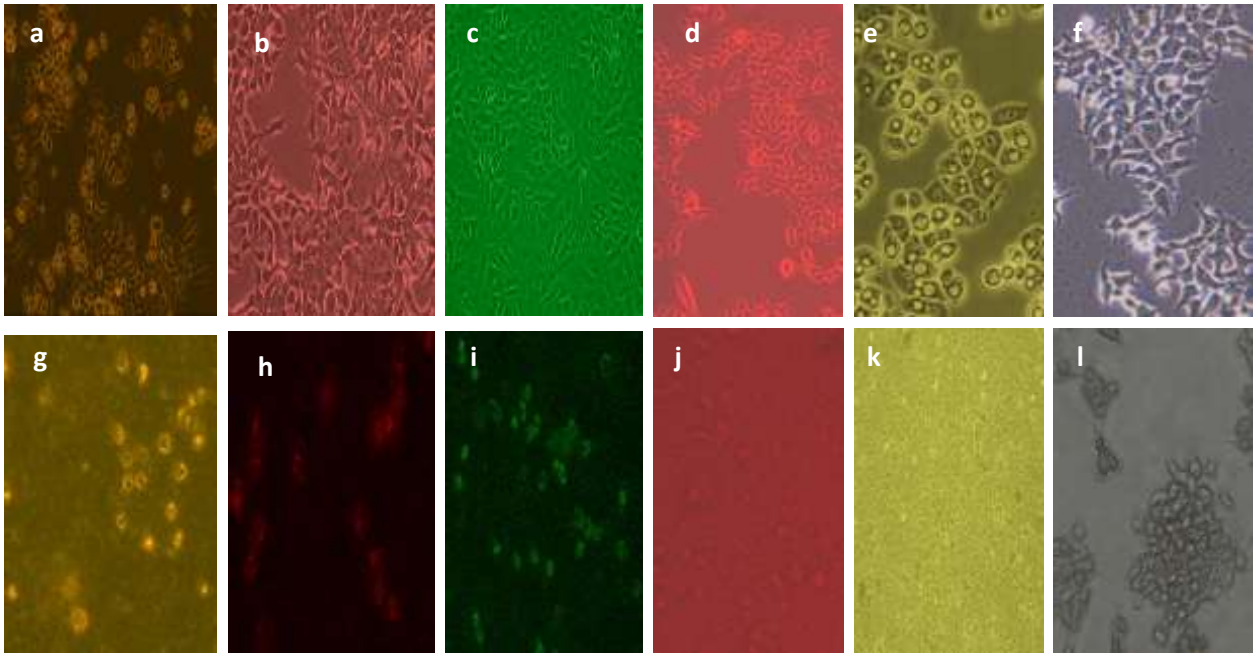



**Figure 3.14** Six different cell lines and their associated CPE from virus like particles present in the Umgeni River. (a) Hep-G2, (b) HEK, (c) Vero, (d) PLC, (e) HELA, (f) A549. CPE of the VLPs on the (g) Hep-G2, (h) HEK, (i) Vero, (j) PLC, (k) HELA, (l) A549, cell line after 2$^{nd}$ passage at 6 days. Images viewed at 400 X magnification.

All virus-like particles isolated from all water samples during all four seasons were capable of inducing some CPE (cell death) on all tissue cell lines tested (Table 3.7). Most of the VLP samples were capable of inducing the cytopathic effects on all tissue cell lines. The VLP samples from site U3, U4 and U5 produced no CPE on the Hep-G2 cell line during the autumn season (Table 3.7).VLPs from site U5 produced CPE on the Hep-G2 during the summer season only and failed to cause CPE on the PLC cell line during autumn season. The VLPs isolated during the summer season had CPE on all six cell lines tested. All VLP samples from all sites and seasons produced CPEs on the Vero, HEK 293, Hela and A549 cell lines.

**Table 3.7** 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 | HELA | PLC | A549 |
| 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

The VLPs caused substantial CPEs on the Vero; Hela and A549 cell lines during all seasons and sites tested (Figure 3.14). CPE values for Vero cells ranged from 66.37%, 79.50%, and 79.14% at sites U1, U2 and U3 respectively during summer. Site U4 (autumn), U1 (winter) and U3 (summer) seasons had 93.28%, 89. 28% and 71.19% CPE respectively on the Hela cell line (Figure 3.14). Sites U1, U2, U3, U4 and U5 had relatively high CPEs for the A549 cell line during the winter season as compared to the other cell lines during that season. Sites U1, U2 and U3 had CPEs of 83.97%, 90.29% and 99.38% respectively on the A549 cell line during the summer season. HEK 293 and HepG2 cell lines showed minimal cytopathic activity from the VLPs. Propagation of Coxsackievirus, Rotavirus A and Adenovirus controls were favoured on the Vero; Hela, PLC and A549 cell lines (Figure 3.14).

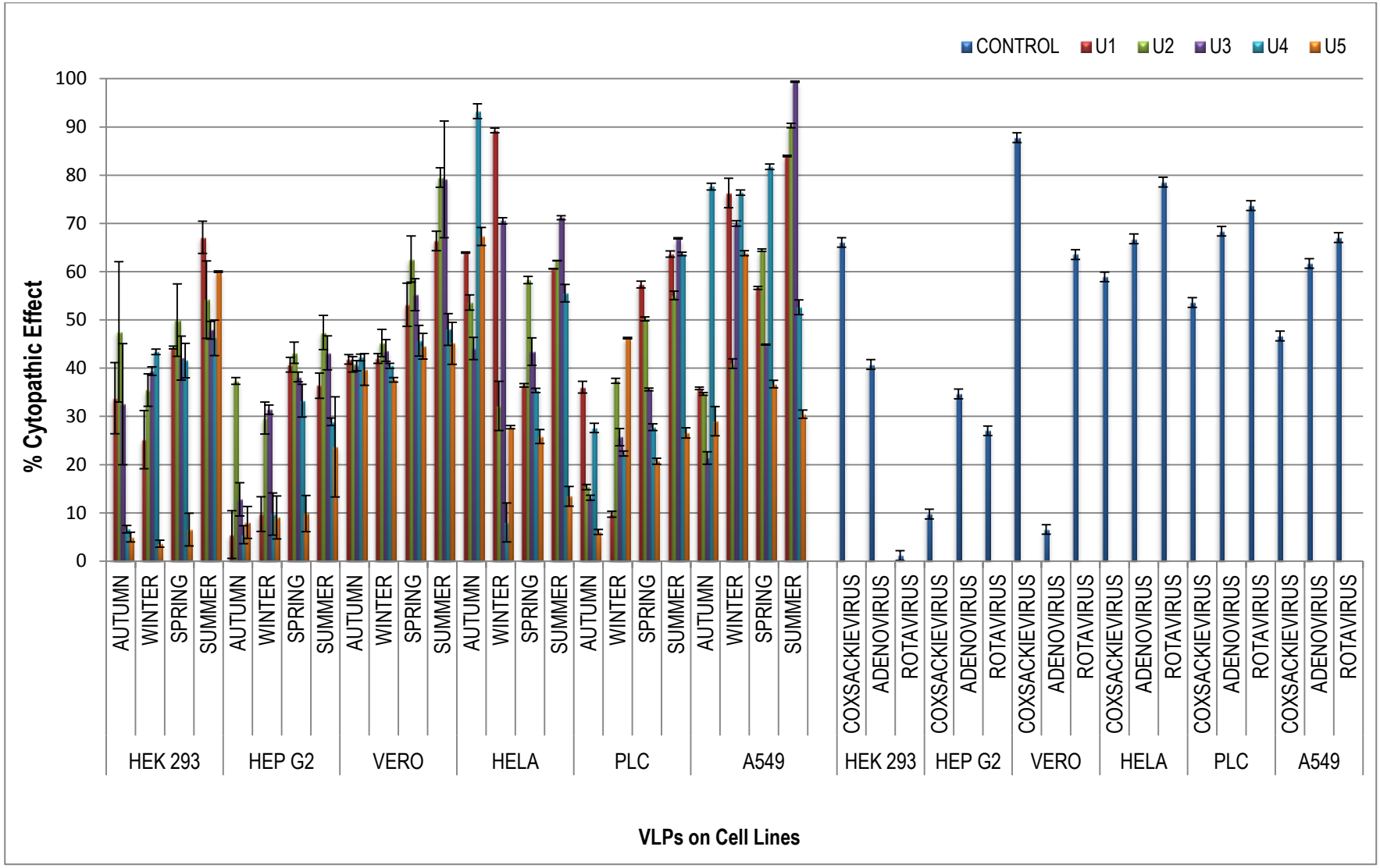



**Figure 3. 15** Cytopathic effect (CPE) of the concentrated virus like particles on six cell lines for the Umgeni River at the different sampling sites during all seasons tested. Bars indicate the average of replicate samples (n = 2 or 3) while the error bars show the standard deviation.

## 3.7 Discussion

Microbiological contamination of water has long been a concern to the public and contamination of water resources, intended for use by general population, with enteric viruses is a great public health problem. Although the occurrence of human enteric viruses has been demonstrated in surface water bodies as well as in drinking water supplies throughout the world and several outbreaks of enteric viral diseases (attributed to either contamination of drinking water or vegetables and seafood grown in such waters) have been recorded (Croci *et al.,* 2000), routine examination of water samples for the presence of enteric viruses is not largely performed in both developed and developing countries (Croci *et al.,* 2000). In this study, the populations of somatic and F-RNA coliphages as well as various enteric viruses were monitored using traditional techniques such as electron microscopy and tissue culture.

Samples at all 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 based on this study. 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. 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 ($p<0.01$). Bacteriophages are not a stable part of water micro flora and their presence is usually associated with wastewater inflow rich in animal and human excrement (Miernik, 2004). Their survivability depends upon physico-chemical environmental conditions (El-Abagy *et al.,* 1988). Somatic coliphages have been found to outnumber F-RNA phages in waste water and raw water sources by a factor of about 5, and cytopathogenic 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. 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, 2004b). Phages have been proposed 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).

SYBR gold staining combined with direct counting by epifluorescent microscopy provided a rapid and inexpensive method for studying the total number of virus like particles in the river water samples. SYBR Gold has been established to be a stable stain for counting viruses and bacteria in aquatic environments, as it is capable of brightly staining both DNA and RNA (Chen *et al.* 2001). VLPs were detected using epifluorescence microscopy at all sampling sites throughout all seasons, with point U1 during summer having the highest population of 2086 VLP/mℓ. Umgeni river points U1, U2 and U3 were more contaminated with phage and virus like populations than points U4 and U5. This was probably

because the river flows through the more urbanised areas of Durban and is subject to higher surface runoff (Olaniran *et al.*, 2009). It can be speculated that increased human activity as well as informal settlements and trading activities in the storm water drain catchment areas contributed to the high levels of phage populations downstream the river at points U1, U2 and U3. It was interesting to note that both the Krantzkloof Nature Reserve (U4) and Inanda dam (U5) had the lowest phage and VLP populations for all seasons compared to the other sampling stations along the rivers. This was probably due to the surrounding thicket and bushland that enclosed the river and the dam, which served as vegetated pervious buffers that acted as passive treatment systems allowing agricultural and storm water runoff to reach the river and dam in a less impaired state (Naicker, 2010). An increase in the phage and VLP populations were observed in all seasons from upstream to the river mouth. Mallin *et al.* (2000) found that human development along the land–seawater interface is considered to have significant environmental consequences resulting in different abundance and distribution of the enteric pathogen indicator microbes and can often pose an increased human health risk.

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 ($r = 0.902$, $p = 0.036$). A correlation between phage quantity and degree of faecal pollution has been found elsewhere (Primrose *et al.*, 1982). Similar results were also obtained by Armon and Knott (1995) who found positive correlations of phage number and bacterial pollution indicator factors.

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 most significantly correlated 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.

The isolation of waterborne virus particles requires concentration from large volumes of water samples as these particles are usually present in low numbers (Watanabe *et al.*, 1988). In this study, tangential flow filtration (TFF) with the Pellicon Cassette System (Millipore Corp.) for concentrating virus like particles from twenty litre river water samples was evaluated. Tangential-flow filtration procedures create high-density viral concentrates that are clear of contaminating cells and particles larger than 0.22 μm (Wommack *et al.*, 2010). The two-step filtration procedure (Figure 3.1) evaluated in our laboratory involved fluid flowing parallel to the 0.22 μm TFF filter surface. Particles with molecular weights (Mw) smaller than the exclusion size (0.22 μm) of the filter passed through and collected (filtrate) in a reservoir which was further concentration through a 100 kDa spiral wound TFF unit. Particles with larger molecular

weights were retained and recycled to the original reservoir (retentate). Thus as the filtration continued, the retentate volume decreased until the desired volume was attained. The TEM study demonstrated that the two-step TFF procedure coupled with ultracentrifugation produced a viral concentrate devoid of bacteria, with most viruses being intact. 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. It was possible to observe viral particles with long tails (B1-*Siphoviridae*), short tails (A1-*Myoviridae* and C1- *Podoviridae*), and without tails. The phages particles observed without tails may belong to a wide range of hosts, including eukaryotes (Alonso *et al.,* 1999). 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. 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).

Great morphological variability was observed in the virus assemblages from the Umgeni River, which suggests that these viruses may also be diverse in terms of the hosts that they infect. Most of the viruses 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 (Olaniran *et al.,* 2009). 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. 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 (Figures 3.9 – 3.13). 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). As a general pattern, the virus population was dominated by small forms (<40 nm), and tails were rarely seen, which is in agreement with the results reported by several authors (Ackermann, 1992; Alonso *et al.*, 1999, Hara *et al.*, 1991; Wommack *et al.*, 1992). 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.

The application of PCR-based molecular technology and TEM has advanced our knowledge of the occurrence and prevalence of human viruses in water. However, it has provided no information on viral viability and infectivity in a specific environment. The tissue culture assay is the only USEPA approved method for virus infectivity monitoring in aquatic samples (Jiang, 2006). Virology laboratories have traditionally used tissue culture cell lines to amplify the amount of virus present in a water sample, express the viral antigens and in many cases monitor the cell death as a consequence of the viral infection producing characteristic cytopathic effects in the cell monolayer. The tissue culture technique has been used extensively for virus replication and infectivity studies and plaque assays (Lee *et al.*, 1965). It has been established that for the recovery of a maximum number of infectious viruses, it is often necessary to inoculate each sample type into several different cell culture lines because no single cell culture is available with susceptibility to all enteric virus types (Lee *et al.*, 1965). In the present study six cell lines (the A549, HEK 293, Hela, HepG2, PLC/PRF/5 and Vero) were used to determine CPE of the VLPs from the Umgeni River during four seasons. All VLP samples from all sites and seasons produced substantial CPEs on the Vero, HEK 293, Hela and A549 cell lines (Figure 3.14). This effect was tested against positive controls of three known viruses (Coxsackie virus, Rotaviruses and Adenovirus) in the same cell lines. Vero cells are sensitive to infection with many different viruses (WHO, 2008). 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). Grabow *et al.* (1992) reported the propagation of both enteric adenoviruses 40 and 41 in the primary liver carcinoma (PLC/PRF/5) cell line. Hep-G2 cell lines are often used to study Hepatitis B, C and other viruses (WHO, 2008). Cytopathic effect (CPE) of the VLPs was monitored by microscopic examination daily for the first week of incubation to maximize the detection of viral growth. Some viruses take 1 day to 3 weeks to produce a CPE depending on the initial concentration and virus type. CPE can be swelling, shrinking, rounding of cells to clustering or complete destruction of the monolayer (Leland and Ginocchio, 2007). All the VLPs isolated from water samples were capable of inducing the cytopathic effects on six tissue cell lines. The ability of these viruses to infect susceptible host cells and to replicate their DNA/RNA, confirms that they are viable and infectious (Grabow, 2001; Reynolds *et al.*, 1997).and therefore constitute a health risk which is due to be investigated in future studies.

In this study, different approaches to determine viral pollution of the Umgeni River during four seasons and at five sampling points were comparatively evaluated. A setup for the concentration of viruses from large (20 L) volume water samples using a two-step tangential flow filtration (TFF) process was successfully established. Electron microscopy illustrated the presence of virus like particles in concentrated water samples at all sites sampled, and their infectivity was established based on their CPE on various cell lines. The results produced suggest the infectious potentials of the VLPs in the Umgeni River water samples, especially river water from the lower catchment areas, to infect the human hosts throughout the year. These observations have serious health care implications if raw untreated water is used for human consumption. However, it should also be considered that not all viral particles detected in this study correspond to infectious viral particles and a high percentage of non-infectious viral particles may be present in the environment (Grabow, 2001; Reynolds *et al.,* 1997). The present study thus highlights the importance of routine environmental surveillance of human enteric viruses for a better understanding of the actual burden of these viral infections on those who might be using the water directly without treatment.

# CHAPTER FOUR

## Temporal Dynamics and Spatial Distribution of Human Viral Contamination of the Umgeni River over Four Seasons

## 4.1 Introduction

Increasing attention is being directed to the contamination of waters and wastewaters by viruses on a global scale. Surface waters and rivers that serve as sources for both domestic and recreational uses carry varying amounts of wastewaters, which may sometimes reach a proportion of 50% and more during periods of low flow (WHO, 2004). This coupled with rapid urbanization in developing countries such as South Africa, has raised critical issues of water supply and waste disposal. The increasing demands on available water in both developed and developing worlds and the concurrent expansion of industrial activity make the recycling of domestic wastewater inevitable (WHO, 1996).Water quality, and consequently human health, has become extensively affected by the occurrence of pathogenic enteric microorganisms (Howard *et al.,* 2006; Poma *et al.,* 2012). Over150 enteric viruses, the most frequently reported enteric viruses in faecal-polluted water are adenoviruses (AdV), Enteroviruses (EV), noroviruses (NoV), rotaviruses (RV), hepatitis viruses (HepV) and polyomavirus (PyV) (Howard *et al.,* 2006).

Enteric viruses are present in the faeces and vomit of infected individuals in high concentrations, and are transmitted primarily through the faecal–oral route, either directly through person to person contact or by consumption of contaminated food or water (Wong *et al.,* 2012). Protection of public welfare from severe gastro-intestinal infections by enteric viral pathogens depends on the ability to identify a given virus as pathogenic, limit viral exposure and ultimately destroy it (Poma *et al.,* 2012). The identification of viruses in environmental samples allowsfor quantitative risk assessment and monitors subsequent disinfection efforts (Wobus and Nguyen, 2012).

Currently, there are four key approaches for detection of waterborne viruses. The first approach is the application of a suitable virus isolation technique in obtaining a concentrate of viruses from large volumes of environmental water samples (Wommack *et al.,* 2010). In this study (as explained in chapter three), a tangential flow filtration TFF with the Pellicon Cassette System (Millipore Corp.) was used for the concentration of virus like particles from large volume water samples. Thereafter a critical second step for viral detection after concentration is to propagate viruses on susceptible cell cultures and subsequent analysis of their cytopathic effects (CPE). While this approach yields information about the infectivity of a virus, it is largely expensive, labour-intensive, and time-consuming (Fout *et al.,* 1996). More rapid and sensitive techniques such as standard PCR, nested PCR (Reynolds 2004), integrated cell culture PCR (ICC-PCR) (Lee *et al.* 2005; Reynolds 2004) and quantitative PCR (qPCR) (Choi and Jiang, 2005) have been developed and offer a range of potential interpretations. Thus the third approach for virus detection is by PCR, which can be performed with and without reverse transcription for RNA and DNA viruses, respectively (Fout *et al.,* 1996). PCR is an *in vitro* method for primer directed enzymatic amplification of specific target DNA sequences which provides specific and high sensitivity in the detection of viruses in water samples (Kittigul *et al*., 2006). Nested PCR amplification is second round of PCR which is often

applied to ensure the specificity of detection, eliminate any false-positive results, and increase the amplification signal, providing the method with the highest sensitivity which allows for the detection of a small number of viral contaminants in an environmental sample. Pina *et al.* (1998) suggested that PCR has led to higher rates of detection of adenoviruses in environmental samples. Borchardt *et al.* (2003) detected enteric viruses (enteroviruses, rotavirus, Norwalk-like virus [norovirus], and hepatitis A virus) from 4 (8%) of 50 household wells by PCR, while no virus was detected by cell culture. PCR, however, detects viral nucleic acids of both infectious and non-infectious virus particles, which limits decisions regarding the significance for public health surveillance (Reynolds *et al.,* 1996). The detected genomic material may be present in otherwise defective virus particles that are not able to attach to or replicate in the host cells. Due to this the infection or disease threat due to consumption of drinking water may be overestimated (de Roda Husman, 2009). In order to assess the infection or disease risk for these non-cultivable pathogenic viruses, assumptions need to be made based on so-called PCR-detectable units.

A fourth approach for virus detection is integrated cell culture PCR (ICC-PCR). This technique combines the advantages of both conventional tissue culture and direct PCR assays, while overcoming some of the limitations of each of these methods (Chapron *et al.,* 2000; Reynolds, 2004). Detection relies on viral replication in cell culture for short incubation periods followed by PCR amplification, which substantially reduces the time necessary for viral detection (Reynolds, 2004). Viruses that replicate but do not produce cytopathic effects can potentially be detected; however, ICC-PCR does not currently detect viruses that cannot grow in a monolayer such as the Norovirus group (Parshionikar *et al.,* 2010). The ICC/RT-PCR is an established method for the rapid detection of infective Enteroviruses in environmental waters and it allows low viral concentrations to be propagated in cell culture to increase target nucleic acid (Parshionikar *et al.,* 2010). The advantage of ICC-PCR, is that it detects virus infectivity faster than cell culture alone which is vital for public health concern and risk management of viral infections, however, this method is still labour-intensive, and it requires at least 2 or more days before results can be obtained. PCR is a rapid, sensitive, and specific technique that can be made quantitative by use of real-time quantitative PCR (qPCR) techniques. Quantification of HAds by RT-PCR has been described for HAds extracted from clinical specimens such as blood, serum, faeces, sputum, urine, eye swabs and nasopharyngeal swabs (Heim *et al.,* 2003). The real-time qPCR/ qRT-PCR is now mostly used for the detection and quantification of virus densities in environmental waters. The technique is highly sensitive and rapid, thus making it useful in assessing human health risk associated with recreational and domestic use of environmental waters. In addition, molecular identification of viral sequences by phylogenetic inference complements the RT-PCR diagnosis and supplies information about the viral types circulating in the community (Muscillo *et al.,* 2001), as well as the suitability of the cell lines used for these viruses (Kok *et al.,* 1998)

There is a strong focus on safe water supplies and water reclamation in South Africa. However, the studies that have assessed for viral agents in South Africa's waters have occurred only in a limited number

of locations and provinces (Chigor and Okoh, 2012), and no records exist of similar investigations in KwaZulu-Natal. However, there are only limited surveillance data and knowledge of the actual burden of waterborne viruses in the KwaZulu-Natal region. It was hypothesized that monitoring waterborne viruses of enteric origin in the Umgeni River in Durban, South Africa, using advanced molecular techniques will provide relevant information for improved water resource management. Therefore, this study evaluated the ease and efficacy of PCR, nested PCR, RT-PCR, nested RT-PCR, ICC-PCR and qPCR for the detection and quantification of pathogenic viruses in the surface waters of the Umgeni River samples collected throughout four seasons. At the same time, phylogenetic analysis of virus-positive samples was conducted to identify some of the dominant strains circulating in the environment.

## 4.2 Materials and Methods

### 4.2.1 Sample Collection

Umgeni River water samples were collected as described in Section 2.2.1 of Chapter two.

### 4.2.2 Tangential Flow Filtration (TFF) for Viral Recovery

Viral community samples (virioplankton) were concentrated using a two-step tangential flow filtration process with ultra-centrifugation as described in Section 3.2.3 of Chapter three.

### 4.2.3 Extraction of Viral Nucleic Acids from Water Samples and cDNA Synthesis

All viral concentrates were filtered through a 0.22 µm Sterivex filter (Millipore, USA) and treated with 10% chloroform to remove contaminating microbial cells before use. Viral DNA and RNA were extracted separately from 1 mℓ viral concentrates each using the High Pure Viral Nucleic Acid Large Volume Kit (for the isolation of viral DNA for PCR and RT-PCR, Roche Diagnostics, Germany) and High Pure Viral RNA Kit (for the isolation of viral RNA for RT-PCR, Roche Diagnostics, Germany) respectively, according to manufacturer's instructions with no modifications. DNA and RNA quality and quantity was measured using the NanoDrop 2200 Spectrophotometer (Thermo Scientific) and the extracts were stored at -70°C.

RNA concentrations were standardised to 1 µg/ mℓ with RT-PCR grade water before cDNA was synthesized using the iScript$^{TM}$ cDNA Synthesis kit (BIORAD, South Africa). Each 20 µℓ reaction contained 4x iScript Reaction Mix, iScript Reverse Transcriptase (RNAse $H^+$), Nuclease-free water, and approximately 8-10 µℓ template RNA (100 fg to 1µg Total RNA). The complete reaction mix was incubated for 5 minutes at 25 ºC, 30-40 minutes at 42 ºC followed by 5 minutes at 85ºC. The resulting cDNA was stored at –70 °C.

### 4.2.4 Detection of Human Viruses from the Umgeni River Water 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 4.1) (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). Primer stocks (100 µM) (Inqaba Biotech) were prepared by adding an appropriate amount of RT-PCR grade water (Ambion) to the lyophilized oligo pellets, while working primer solutions were prepared to a final concentration of 10 µM using RT-PCR grade water and stored at –20°C.

**Table 4.1** Primer sequences used for PCR amplification of four viral groups.

| 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 | 1241 | 10 000 | |
| HBS-R1 | AGG ACA AAC GGG CAA CAA C | | | |
| HBS-11* | GCG GGG TTT TTC TTG TTG AC | 310 | | |
| HBS-R11* | GAA CCA ACA AGA AGA TGA GGC | | | |

* Primers for Nested PCR

All PCR mixtures had a total volume of 25 µℓ and contained 5 µℓ of target DNA/ cDNA, 12.5 µℓ of 1 x JumpStart™ REDTaq® ReadyMix™ Reaction Mix for High Throughput PCR (20 mM Tris-HCl, pH 8.3, 100 mM KCl, 4 mM $MgCl_2$, 0.002 % gelatin, 0.4 mM each dNTP (dATP, dCTP, dGTP, dTTP), inert dye, stabilizers, 0.03 unit/mℓ Taq DNA polymerase, and JumpStart Taq antibody; Sigma-Aldrich, St. Louis, MO), RT-PCR grade water and 10 µM of each primer, unless otherwise stated.

#### 4.2.4.1 Adenoviruses

Nested PCR was used to amplify the hexon gene conserved in 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.

#### 4.2.4.2 Enteroviruses

Nested PCR was used to amplify the 5'-untranslated region conserved in 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 5 µℓ of amplified PCR product from the first round PCR was added as the template for 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.

#### 4.2.4.3 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, contained an additional 2.4 mM $MgCl_2$, 2 µg/mℓ Bovine serum albumin and 10 µℓ of amplified PCR product from the first round PCR was added as the template to the second round of PCR, 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.

#### 4.2.4.4 Hepatitis B Viruses

Nested PCR was used to amplify the S gene of hepatitis B viruses (HBV) (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 microliters 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.

### 4.2.5 Detection and Sequencing of PCR/RT-PCR Positives

Seven microliters of the PCR amplicons were visualised directly by electrophoresis on 2% (w/v) agarose (Seakem®LE Agarose, BioWhittaker Molecular Applications, Rockland, ME, USA) gels in 1 x Tris-Acetate-EDTA (TAE) running buffer (Appendix i) with an applied voltage of 90 V for 90 min. After electrophoresis, the gels were stained using ethidium bromide (1 µg/mℓ) and visualized by UV

transillumination with the Chemi Genius$^2$ BIO Imaging System and Gene Snap software (Syngene, UK). Molecular weight marker, O'GeneRuler 100bp DNA Ladder mix (Fermentas) was used to determine amplicon size. If more than one band was present, the band of the PCR product of the correct size was gel extracted, purified using the UltraClean Gelspin kit (Mo Bio Laboratories Inc.) and re-amplified as before.

To verify the identity of positive nested PCR products , the amplicons were sequenced with their respective primer sets by Inqaba Biotech (South Africa). The obtained nucleotide sequences were analysed by BLAST program at the NCBI (National Centre for Technology Control, NIH, USA) website: http://www.ncbi.nlm.nih.gov/BLAST, under the nucleotide sequences database, to obtain identities of the positive PCR products.

Amino acid alignment of the sequences was performed in ClustalX (Larkin *et al.,* 2007). A phylogenetic tree was constructed using the neighbour-joining method for each virus type. The branching confidence was estimated by bootstrapping with 1000 re-samplings in MEGA version 5 (Tamura *et al.,* 2011). The accession numbers of the virus prototypes used for alignment and neighbour joining tree phylogenetic analysis were retrieved from the BLAST search GenBank database. Nucleotide sequences in the present study are designated according to the sampling location and season for different genotypes or variants.

### 4.2.6 Integrated Cell Culture PCR (ICC PCR)

#### 4.2.6.1 Extraction of Viral Nucleic Acids

The virus like particles from the Umgeni River water sources were propagated in the A549, HEK 293, Hela, HepG2, PLC/PRF/5 and Vero cell lines and incubated as previously described in Chapter three section 3.2.6. The viral RNA and DNA were extracted from 500 µℓ cell culture supernatants using the TRISure$^{TM}$ Reagent (Bioline, Germany) according to manufacturer's instruction with no modification. The RNA and DNA pellets were dissolved in 60 µℓ TE Buffer (Applied Biosystems), and stored at –70°C. Unfortunately due to unforeseen circumstances the tissue culture samples of Hela, Vero and A549 cell lines for viral DNA isolation was absent.

#### 4.2.6.2 cDNA Synthesis

Viral RNA concentrations were standardised to 1 µg/ mℓ with RT-PCR grade water before cDNA was synthesized using the DyNamo$^{TM}$ cDNA Synthesis kit for qRT-PCR (Finnzymes, Thermo Fisher Scientific, Finland). Each 20 µℓ reaction contained 2x RT Buffer, 300 ng/µℓ Random Hexamer Primer set, M-MuLV RNaseH$^+$ Reverse Transcriptase, Nuclease-free water, and approximately 8-10 µℓ template RNA (100 fg to 1µg Total RNA). The reaction mix was incubated for 10 minutes at 25 ºC, 40-60 minutes at 37 ºC followed by 5 minutes at 85 ºC. The resulting cDNA was stored was stored at –70 °C.

#### 4.2.6.3 Detection of Human Enteric Viruses from Cell Culture by PCR/ RT PCR Assay

PCR/RT-PCR for the viral community samples grown on cell culture were performed as described above in section 4.4 using the primer sequences outlined in Table 4.1. Ten to fifteen microliters of the ICC-PCR amplicons were analysed directly by electrophoresis on 2-3% (w/v) agarose (Seakem®LE Agarose, BioWhittaker Molecular Applications, Rockland, ME, USA) gels in 1 x Tris-Acetate-EDTA (TAE) running buffer (Appendix i) with an applied voltage of 90-100 V for 90 min. After electrophoresis, the gels were stained using ethidium bromide (10 µg/mℓ) and visualized by UV transillumination with the Chemi Genius2 BIO Imaging System and Gene Snap software (Syngene, UK). Molecular weight marker, O'GeneRuler 100bp DNA Ladder mix (Fermentas) was used to estimate amplicon size. No sequencing was performed on the ICC-PCR positive samples, as this technique was only executed to verify that the cytopathic effect visualised on the cell culture samples were in fact as a result of viral infection.

### 4.2.7 Real-time PCR amplification of Human Viruses from the Umgeni River Water

Custom TaqMan® Gene Expression Assays (containing a primer and probe mix) were developed by Applied Biosystems (South Africa) using previously published primer and probe sequences used to quantify the four human viral genomes (Table 4.2). TaqMan MGB probes, characterised by a minor groove binder (MGB) at the 3' end, were selected since the MGB increases the melting temperature (*T*m), allows the use of probes shorter than 20 nucleotides and differentiates two sequences with only one mismatch (Ogorzaly and Gantzer, 2006). The reporter FAM was attached to the 5' end. Each Real time reaction sample was analysed in triplicate wells for all four viral targets. Multiple negative and positive controls were included for each PCR assay. The TaqMan® Fast Virus 1-Step master mix [AmpliTaq® Fast DNA polymerase, MMLV enzyme, dNTPs, RNaseOUT™ Recombinant Ribonuclease inhibitor, ROX™ dye, and buffer components, Applied Biosystems, USA] was utilised to perform one-step RT-PCR/ qPCR of viral RNA and DNA directly. A typical 20µℓ fast real time assay reaction contained, 5µℓ of 4X TaqMan® Fast Virus 1-Step master mix, 1 µℓ of Custom TaqMan® Gene Expression Assay (having a combination of approximately 18µM primer and 5µM probe concentration), RT-PCR grade water (Ambion) and RNA or DNA samples up to the maximum allowed by each reaction volume. Real-time PCR was performed on an ABI 7500 Fast (Applied Biosystems) using default fast universal thermal cycling conditions of 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The entire reaction was completed in less than 45 minutes.

**Table 4.2** Primer and probe sequences for TaqMan custom gene expression assays designed for real-time RT-PCR detection of four viral groups

| Virus | Primer /Probe | Primer/Probe Sequence (5'→ 3') | Amplicon size (bp) | Reference |
|---|---|---|---|---|
| **Hepatitis B Virus** | HBV F | 5'-GGACCCCTGCTCGTGTTACA-3' | 89 | Pas *et al.*, 2000 |
| | HBV R | 5'-AGAGAAGTCCACCTCGAGTCTAGA-3' | | |
| | HBV probe | 5'-FAM-TGTTGACAAGAATCCTCACCAT ACCRCAGA-MGB-3' | | |
| **Enterovirus** | ENTV F | 5′-CCCTGAATGCGGCTAAT-3′ | 143 | Gregory *et al.*, 2006 |
| | ENTV R | 5′-TGTCACCATAAGCAGCCA-3′ | | |
| | ENTV probe | 5′-FAM–ACGGACACCCAAAGTAGTCGGT TC-MGB-3′ | | |
| **Adenovirus** | ADV F | 5'-GCCACGGTGGGGTTTCTAAACTT-3' | 69 | Van Heerden *et al.*, 2005 |
| | ADV R | 5'-GCCCCAGTGGTCTTACATGCACATC-3' | | |
| | ADV Probe | 5'-FAMTGCACCAGACCCGGGCTCAGGTAC TCCGA-MGB-3' | | |
| **Rotavirus** | ROT F | 5'-ACCATCTACACATGACCCTC-3' | 87 | Pang *et al.*, 2004 |
| | ROT R | 5'-GGTCACATAACGCCCC-3' | | |
| | ROT probe | 5'-FAM-ATGAGCACAATAGTTAAAAGCT AACACTGTCAA-MGB-3' | | |

#### 4.2.7.1 Analysing Real-Time PCR Data

The 7500 Fast software for the 7500 Fast Real Time Machine (Applied Biosystems, USA) was used to analyse data. Real time quantities were taken and a threshold cycle (Ct) value for each sample was calculated by determining the point at which the fluorescence exceeded a certain threshold limit. Rhodamine X (ROX dye) was used as a passive reference to which the signal of the reporter dye was normalized during data analysis, which reduced non-PCR-related fluorescence fluctuation from well to well. Triplicate reactions of a known positive virus sample was used to generate standard curves with dilutions ranging from $10^0$ to $10^5$ dilutions were used for each run. Several negative controls were run as no template controls. All viral DNA and RNA samples were run in triplicate. Sample copy numbers were calculated (automatically by the 7500 Fast software) by interpolation of the experimentally determined standard curve. The slope (*s*) of the standard curve was used for determining the PCR efficiency (E) in conformity with $E=10^{(-1/s)} - 1$ (Kubista *et al.*, 2006). Thus, a standard curve with a slope of −3.33 corresponded to a reaction with an efficiency value of 100% (Ogorzaly and Gantzer, 2006**).** After determination of RNA and DNA concentration by spectrophotometry, the copy number of standard curve RNA and DNA molecules was calculated using the following formulae(QIAGEN, 2004 b):

RNA copies were calculated as : **(X g/(µl) RNA / [transcript length in nucleotides × 340]) × 6.022 × $10^{23}$**= Y molecules/µl

Where: (X) g/(µl) RNA: Concentration of RNA determined spectrophotometrically,

Transcript length in nucleotides: length of target gene sequence in base pairs.

DNA copies were calculated as: **(X g/(µl) DNA / [DNA or plasmid DNA length in base pairs × 660]) × 6.022 × $10^{23}$ = Y molecules/µl**

Where: (X) g/(µl) DNA: Concentration of DNA determined spectrophotometrically,

Plasmid length in nucleotides: length of Plasmid in base pairs.

### 4.2.8 Quality Control

Standard precautions were applied in all the manipulations to reduce the possibility of cross-contamination between samples, and sample reaction contamination by DNA amplicons. A separate biohazard laboratory, equipped with its own pipettes, filter-tips and reagent tubes, etc. was used for sample processing, reagent preparation, reaction preparation and manipulation of amplified viral fragments. Each reaction test was accompanied by two overall controls. A positive control consisting of a spiked sample containing a known viral concentration and a no template control (NTC) consisting of sterile water and master-mix in order to detect possible contaminations and to monitor for false-positive and false-negative reactions. The risk of contamination during the preparation of master-mixes for PCR was minimized by using a laminar flow hood. In addition a separate RNA laboratory was used to handle RNA viral fragments.

## 4.3 Results

### 4.3.1 Detection of Pathogenic Human Viruses using PCR / RT-PCR

This study used PCR and RT-PCR to determine the presence of Adenovirus, Enterovirus, Rotavirus and Hepatitis B virus in Umgeni river water samples. The nested PCR profiles of the viral communities of the five sampling stations along the Umgeni River (U1 – U5), during a seasonal cycle, are illustrated against the molecular weight marker O'GeneRuler 100bp DNA Ladder in Figures 4.1 to 4.4,. The temporal distribution of each virus type showed that all viruses under investigation were present in the 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 (Table 4.1). The hexon gene of 47 different Adenovirus serotypes was amplified by nested PCR (Figure 4.1) and yielded 143 bp product for the positive control and all the water samples tested. The sensitivity of the number of targets of the primer pairs used for Adenovirus PCR was 100. The 5'-untranslated region of the Enterovirus genome detecting at least 25 different Enteroviruses was amplified by nested RT- PCR (Figure 4.2) and yielded 154 bp products for the positive control and all the river water samples tested. The sensitivity of the number of targets for the primer pairs used for Enterovirus PCR was 100. It was interesting to note several PCR banding patterns occurring throughout the winter season samples probably indicating the spatial seasonal distribution of other Enteroviral genomes.

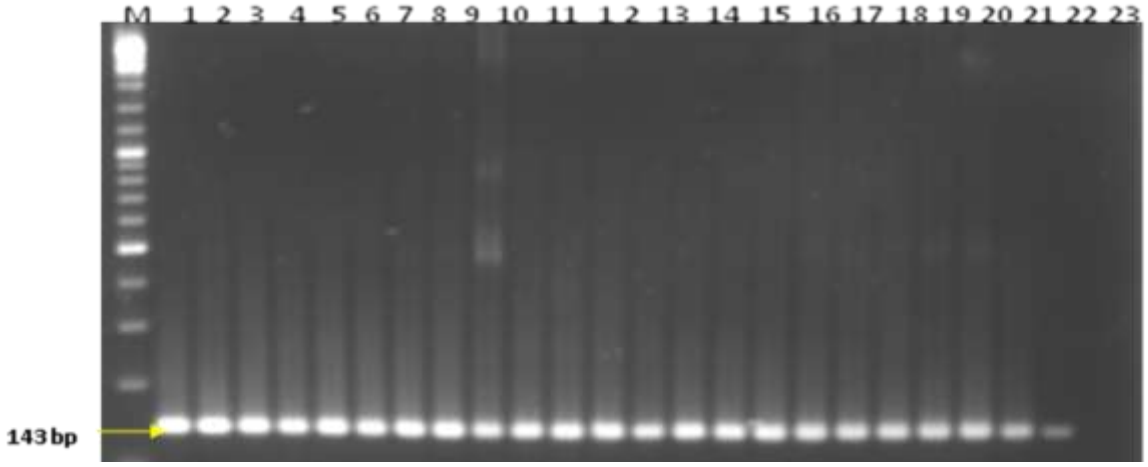



**Figure 4.1** Nested PCR amplification of the hexon gene of 47 different Adenovirus serotypes. M: Molecular weight marker, L1: Adenovirus Control, L2-L6: Points for autumn season, L7-11: Points for winter season, L12 -16:- Points for spring season, L17-22: Points for summer season, L23: Negative Control.

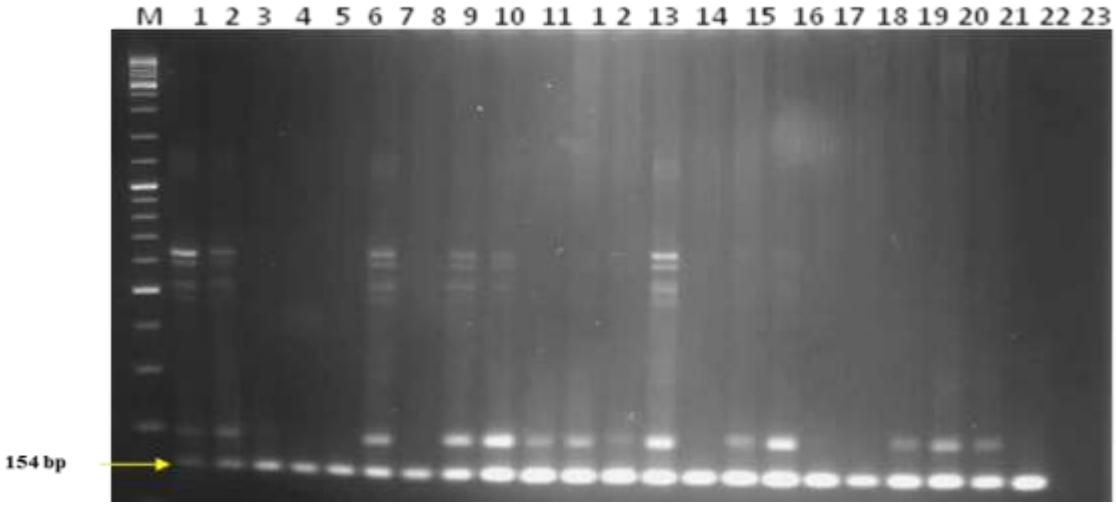



**Figure 4.2** Nested PCR amplification of the 5'-untranslated region of the Enterovirus genome detecting at least 25 different Enteroviruses. M: Molecular weight marker, L1: Enterovirus Control, L2- L6: Points for autumn season, L7-11: Points for winter season, L12 -16:-Points for spring season, L17-22: Points for summer season, L23: Negative Control.

Rotavirus nested RT-PCR had a sensitivity of 1000 RV targets for the primer pairs used. The VP7 gene of the group A Rotavirus genome was successfully amplified (Figure 4.3) and yielded 346 bp products for the positive control and some of the river water samples tested.

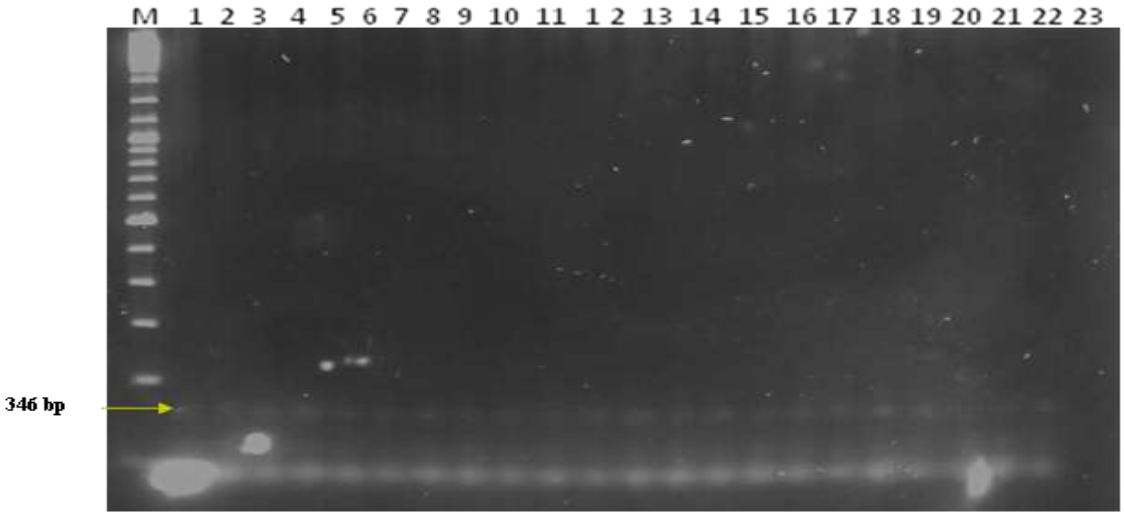



**Figure 4.3** **N**ested PCR was used to amplify the VP7 gene of group A Rotaviruses. M: Molecular weight marker, L1: Rotavirus Control, L2-L6 : Points for autumn season, L7-11: Points for winter season, L12 -16:-Points for spring season, L17-22: Points for summer season, L23: Negative Control.

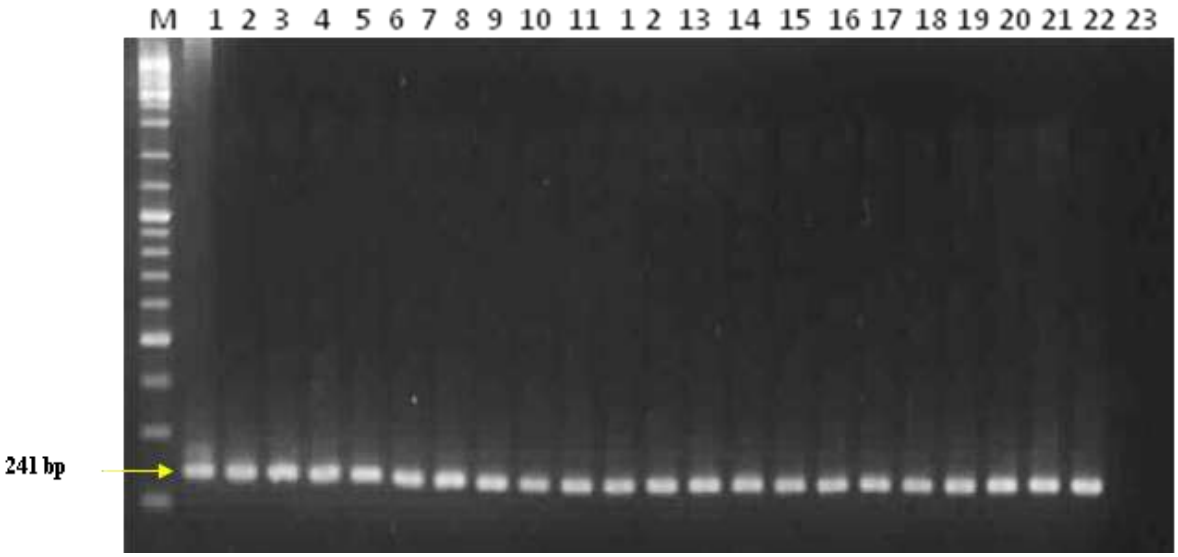



**Figure 4.4** Nested PCR was used to amplify the S gene of Hepatitis B viruses. M: Molecular weight marker, L1: Hepatitis B Control, L2-L6: Points for autumn season, L7-11: Points for winter season, L12 -16:-Points for spring season, L17-22: Points for summer season, L23: Negative Control.

An alarming occurrence was the presence of Hepatitis B virus in the river water samples. Nested PCR for this viral group amplified the S gene of the HBV genome (Figure 4.4). The primer pairs designed

for a target sensitivity of 10 000, amplified a 241 bp nested PCR product for all water samples tested. Nested PCR for this viral group amplified the S gene of the HBV genome (Figure 4.4). The primer pairs designed for a target sensitivity of 10 000, amplified a 241 bp nested PCR product for all water samples tested.

### 4.3.2 Detection of Human Enteric Viruses by Integrated Cell Culture PCR (ICC-PCR)

The Umgeni river water samples were analysed by integrated cell culture PCR to provide sensitive detection and confirmation of infectious Adenovirus, Rotavirus, Enterovirus and Hepatitis B virus particles in various cell culture lines. Figure 4.5 illustrates the percentage of positively identified infectious surface water occurring human pathogenic viruses in the Umgeni river water. Nested ICC-PCR (gel images Appendix iii- page 228-237) confirmed the presence of infectious Adenovirus particles in approximately 80%, 85% and 100% of the HEK 293, PLC/PRF/5 and Hep G2 cell lines respectively (Figure 4.5). No infectious Adenovirus particles occurred at point U4 during the winter and summer seasons on the HEK 293 cell line and at point U5 on HEK 293 during spring and on the PLC/PRF/5 cell during autumn and spring seasons (Appendix iii). Infectious Rotavirus particles was positively detected on 100% of the A549, Vero and HeLa cell lines, contrary to no infection being detected on the HEK 293 and PLC/PRF/5 cell lines. Nested ICC-RT PCR confirmed the presence of infectious Enterovirus particles on all six cell lines tested (Figure 4.5), with most of the infections occurring on the HEK 293, Vero and HeLa cell lines.

Infectious Hepatitis B virus particles were detected on 20% of the Hep G2 cell line, 40% of the HEK 293 cell lines, and 55% on the PLC/PRF/5 cell line. Interesting to note was that infectious HBV particles on the PLC cell line occurred mainly at sites U3, U4 and U5 during autumn, winter and spring seasons.

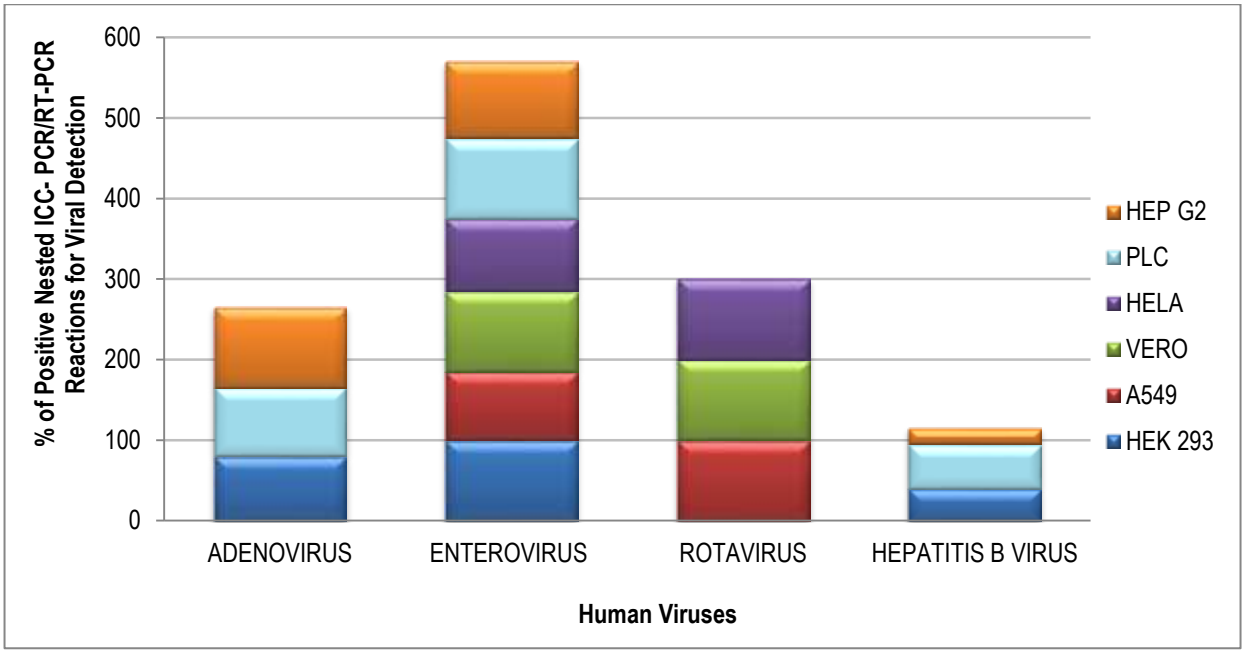



**Figure 4.5** Detection of naturally occurring Adenovirus, Rotavirus, Enterovirus and Hepatitis B virus genomes in the various infected cell lines. Bars indicate the positive detection of each viral group by nested ICC-PCR/ RT-PCR.

### 4.3.3 Phylogenetic Analysis

The genetic relationships between the viral groups of Adenovirus, Enterovirus and Hepatitis B virus are represented in Figure 4.6, 4.7 and 4.8 respectively. Profiles of the virus phylogenetic treeswere obtained by comparing the sequences obtained from environmental samples with reference sequences stored in GenBank. Not all positive PCR samples could be sequenced as the amount of target RNA in some cases was too low. The GenBank BLAST analysis results including E-values and % Maximum Identity are shown in Appendix iii (page 238-250). It was found that all three viral groups gene sequences (ie. Adenovirus, Rotavirus and Hepatitis B virus) had relatively low E-values with sequence identities ranging from 91% to 100% to their known counterparts on the GenBank database. All water samples VLPs tested, had 100% PCR positivity against their known viral controls. The PCR products for the Rotavirus positive samples had relatively low template concentrations and could not be sequenced after several attempts of amplification.

Neighbour-joining trees grouped isolates into clades with viruses of the same viral type verifying the identity obtained by BLAST searching. From the neighbour joining phylogenetic tree in Figure 4.6, it can be seen that 96% of the Adenovirus genogroups were clustered together into one clade with the known Adenovirus control. Human Adenovirus strain 2 at point U5 during summer was separated from this clade. Most of the identified samples belonged to Human Adenovirus C strain and Human Adenovirus 2 strain. Human Adenovirus 41 was detected at point U1 during the winter season.

Figure 4.7 represents the phylogenetic tree of the Enterovirus genogroups, showing varying distributions of these viral groups into 14 clades. Interesting to note clade 1 grouped U2 (autumn, spring and summer) with points U1 (summer) and U4 (spring). Clade 3 and 4 had groupings during winter and clade 5 was grouped with autumn and winter. Half(50%) of the samples were identified as Coxsackievirus B5 Genomic RNA, with 25% of the samples being Coxsackievirus B5 strain Faulkener and Human Enterovirus 82 strain USA (10 %). Figure 4.8 represents the phylogenetic tree of Hepatitis B virus genogroups. The genogroups were separated into 9 clades. It was found that 70% of the samples were identified as being Hepatitis B virus isolate SHH043A (S), with 15% of the samples belonging to Hepatitis B virus.

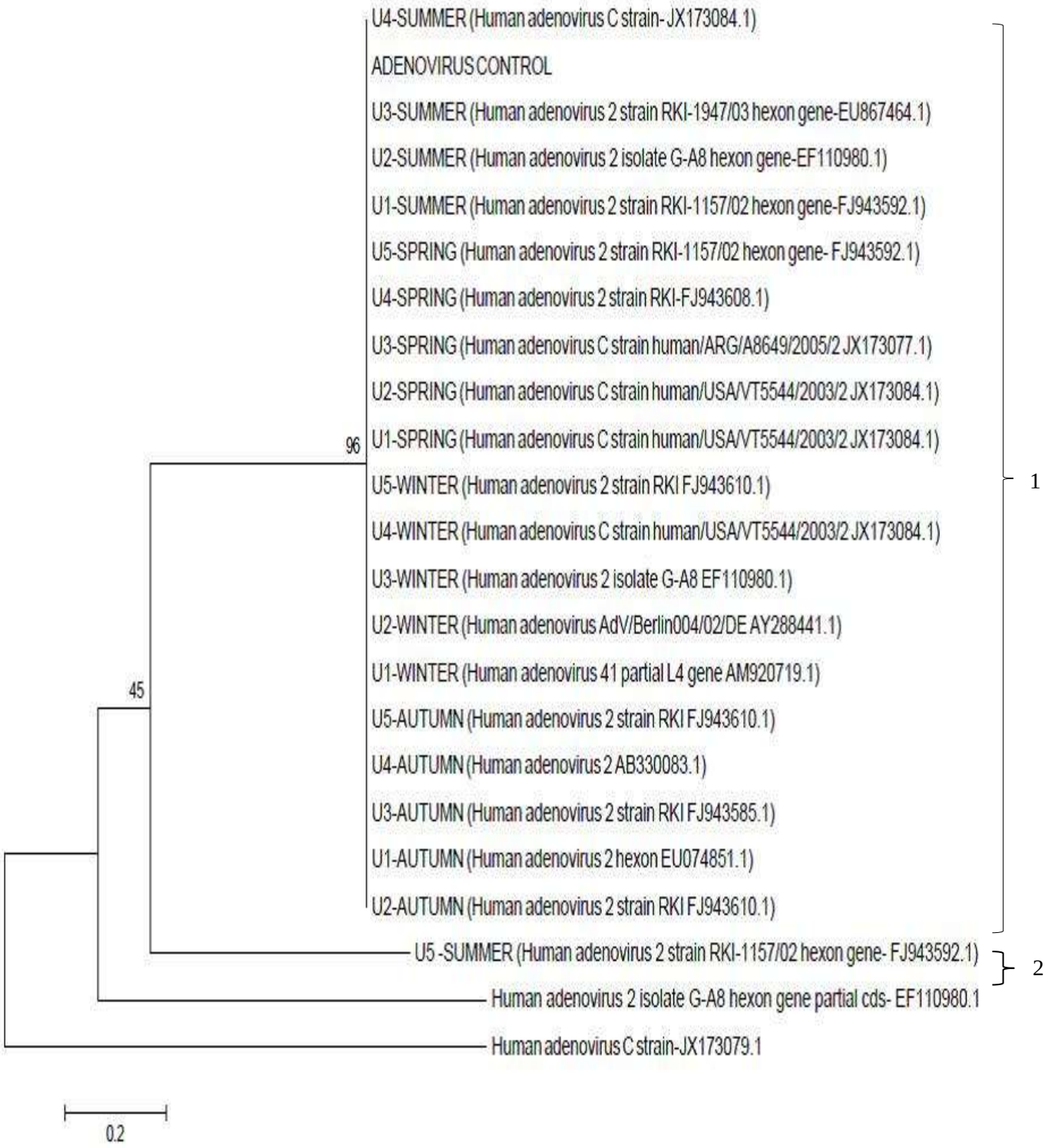



**Figure 4.6** Neighbour joining tree representing the phylogenetic relationship between nucleotide sequences of amplicons (143 bp) of the hexon gene of Adenovirus genome from different river water samples (U1-U5, Autumn, Winter, Spring and Summer). Each branch represents a sequence or a group of sequences, with identities and sequences being selected from GenBank database using BLAST search of the obtained sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

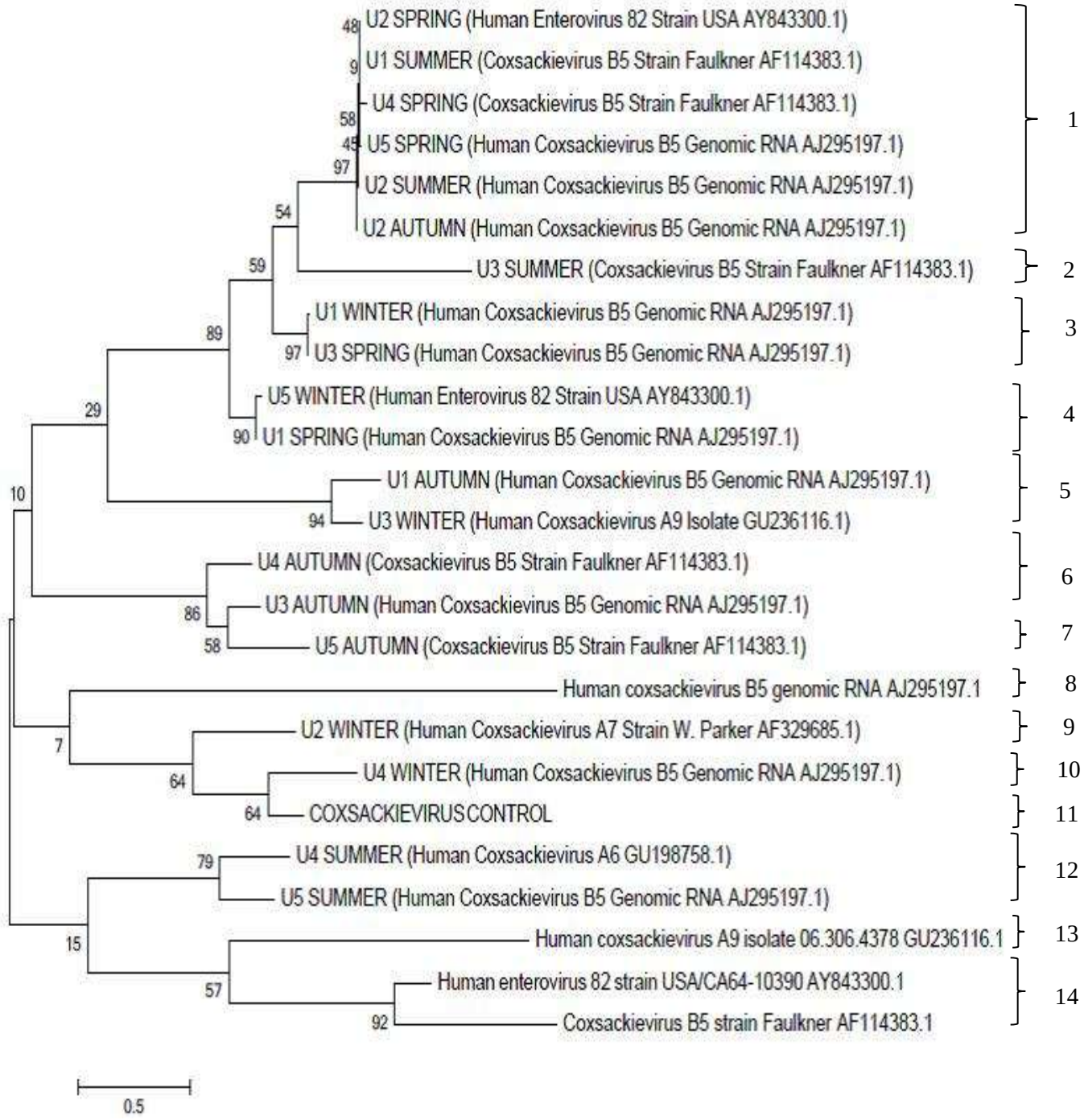



**Figure 4.7** Neighbour joining tree representing the phylogenetic relationship between nucleotide sequences of amplicons (154 bp) of the 5'-untranslated region of the Enterovirus genome from different river water samples (U1-U5, Autumn, Winter, Spring and Summer). Each branch represents a sequence or a group of sequences, with identities and sequences being selected from GenBank database using BLAST search of the obtained sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

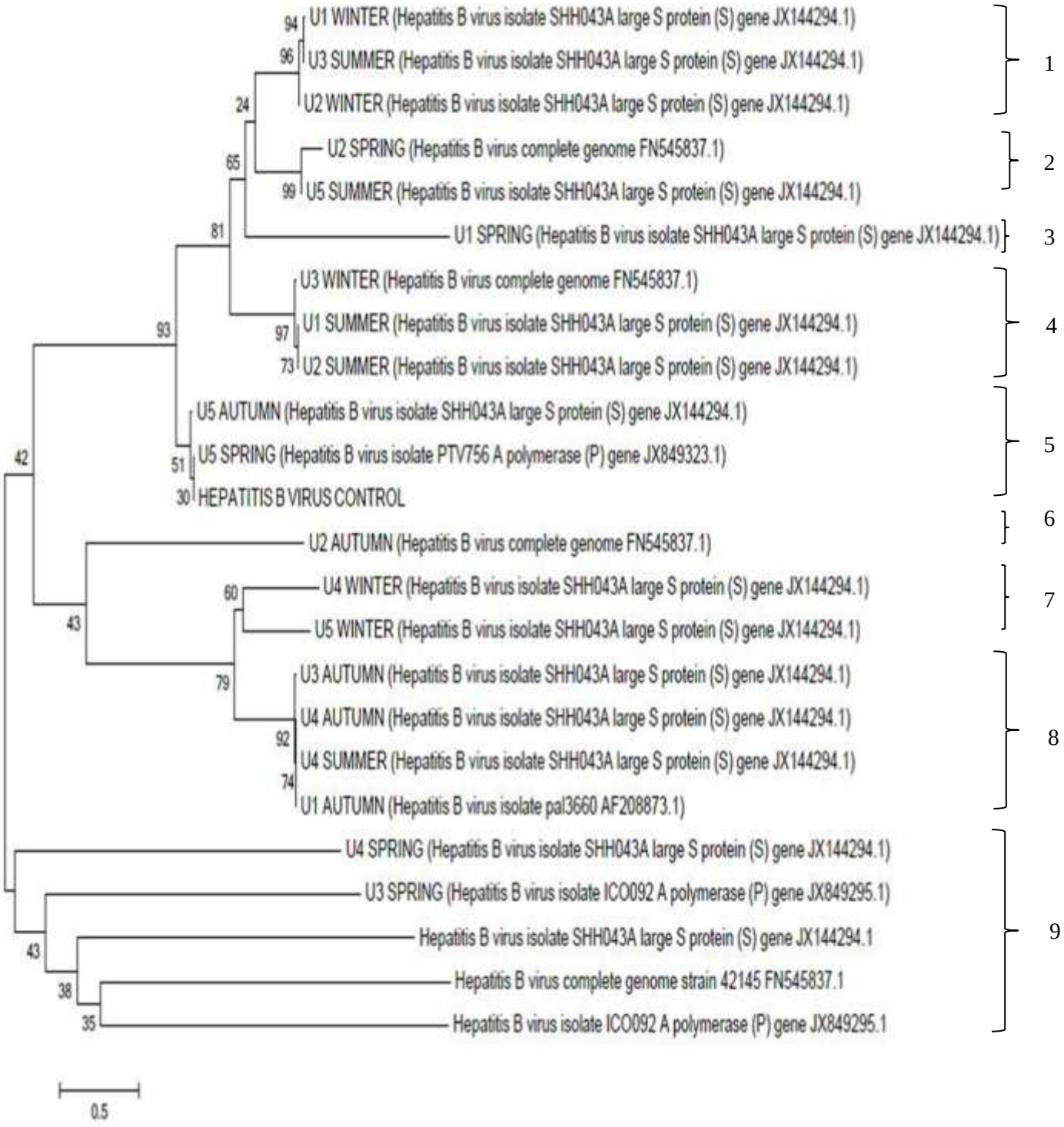



**Figure 4.8** Neighbour joining tree representing the phylogenetic relationship between nucleotide sequences of amplicons (241 bp) of the S gene from different river water samples (U1-U5, Autumn, Winter, Spring and Summer). Each branch represents a sequence or a group of sequences, with identities and sequences being selected from GenBank database using BLAST search of the obtained sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

### 4.3.4 Real-Time PCR Absolute Quantification of Detected Viral Nucleic Acids

To validate the real-time PCR assays prior to application to environmental samples, the detection limit and amplification efficiency of each reaction were determined. The nucleic acids were standardised to approximately 1 µg/mℓ where necessary. Standard curves with 10-fold serial dilutions of plasmid DNA and RNA controls were prepared and assayed in triplicate with copy numbers ranging from 11.3 x $10^{11}$ copies/µℓ , 2.1 x $10^{11}$ copies/µℓ , 64 x $10^{11}$ copies/µℓ and 3.8 x $10^{9}$ copies/µℓ for Enterovirus, Adenovirus, Rotavirus and Hepatitis B virus respectively. The resulting standard curves are illustrated in Figures 4.9 a-d. PCR amplification efficiency (E) for each assay was calculated from the slope of the standard curves (as indicated previously) and are as : 96.693% (Adenovirus), 101.608% (Enterovirus), 102.174% (Rotavirus) and 128.297% (Hepatitis B virus).

TaqMan®Fast Virus 1-Step Master Mix protocol was used because it has the ability to assay both types of nucleic acid, from either RNA or DNA virus. The primers and TaqMan probes applied in this study were chosen from previously published data, with oligonucleotide sequences targeting the specific genes of interest for each virus type as indicated in Table 4.2. These primer and probe sequences were created into optimised gene expression assays (copyright Applied Biosystems) for use with a Fast Real Time PCR system. The amplification was performed under universal conditions. Table 4.3 shows the copy numbers obtained from the real-time PCR amplification of Adenovirus, Enterovirus, Rotavirus and Hepatitis B virus, using known copies of genomic equivalent as a standard. Ct values for each viral Real-Time assay are shown in Appendix iv. Due to the high specificity and simplicity of the Real-Time assays all viral samples were quantified. Adenovirus copy numbers ranged from: 1.99 x $10^{4}$ copies/µℓ (U5) to 8.41 x $10^{5}$ copies/µℓ (U1) during autumn, 1.91 x $10^{4}$ copies/µℓ (U4) to 2.92 x $10^{5}$ copies/µℓ (U2) during winter, 2.04 x $10^{4}$ copies/µℓ (U1) to 1.79 x $10^{7}$ copies/µℓ (U4) during spring and 3.02 x $10^{4}$ copies/µℓ (U3) to 1.24 x $10^{5}$ copies/µℓ (U1) during summer (Table 4.3). High adenovirus copy numbers were observed during autumn and spring seasons indicating high spatial distribution. Enterovirus copy numbers ranged from: 3.22 x $10^{8}$ copies/µℓ (U3) to 7.59 x $10^{10}$ copies/µℓ (U5) during autumn, 2.24 x $10^{6}$ copies/µℓ (U3) to 3.84 x $10^{10}$ copies/µℓ (U2) during winter, 5.19 x $10^{5}$ copies/µℓ (U5) to 3.76 x $10^{10}$ copies/µℓ (U1) during spring and 1.25 x $10^{8}$ copies/µℓ (U2) to 1.83 x $10^{9}$ copies/µℓ (U1) during summer (Table 4.3). Relatively high spatial distribution of Enterovirus occurred throughout all samples and seasons tested. Rotavirus copy numbers were low in the water environment and ranged from: 3.81 x $10^{1}$ copies/µℓ (U3) to 8.51 x $10^{1}$ copies/µℓ (U1) during autumn, 5.47 x $10^{1}$ copies/µℓ (U1) to 7.08 x $10^{2}$ copies/µℓ (U4) during winter, 4.51 x $10^{1}$ copies/µℓ (U3) to 1.78 x $10^{2}$ copies/µℓ (U5) during spring and 4.03 x $10^{1}$ copies/µℓ (U5) to 4.04 x $10^{7}$ copies/µℓ (U3) during summer (Table 4.3). The HBV copy numbers of the Umgeni water samples which were calculated using known copies of genomic equivalent as a standard and rectified with the concentration factor of 4000, ranged from 33.7 copies/µℓ (U5) to 1.12 x $10^{10}$ copies/µℓ (U1) during autumn, 0.39 copies/µℓ (U3) to 2.13 x $10^{8}$ copies/µℓ (U2) during winter, 8.22 x $10^{6}$ copies/µℓ (U4) to 1.49 x $10^{9}$ copies/µℓ (U3) during spring

and 7.82 x $10^7$ copies/µℓ (U5) to 1.13 x $10^9$ copies/µℓ (U4) during summer (Table 4.3). HBs gene copy numbers of hepatitis B virus in the Umgeni water samples were found to be alarmingly high. In all seasons, the virus copy numbers were usually higher in Sites U1 and U3 than those of other sites (Table 4.3).

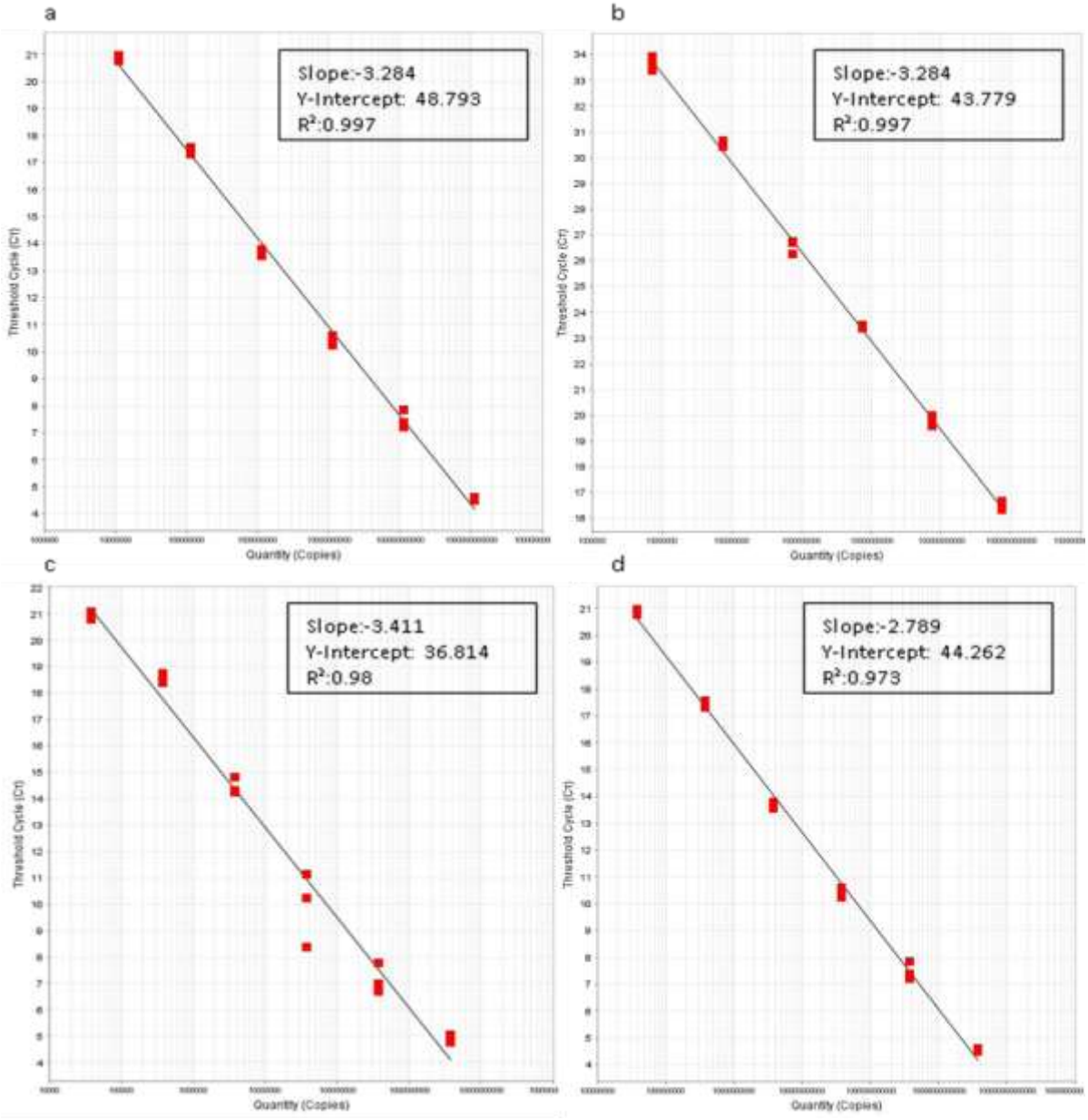



**Figure 4.9** Standard curves of a) Adenovirus; b) Enterovirus; and c) Rotavirus; d) Hepatitis B virus genogroups using TaqMan real-time PCR. Means and standard deviation of Ct are shown (triplicate).

**Table 4.3** Number of copies of human viruses detected by TaqMan primer/probe sets in the Umgeni River water samples containing mixtures of viral DNA and RNA.

| SAMPLING POINTS | | ADENOVIRUS | | ENTEROVIRUS | | ROTAVIRUS | | HEPATITIS B VIRUS | |
|---|---|---|---|---|---|---|---|---|---|
| | | Quantity Mean* | SD | Quantity Mean* | SD | Quantity Mean* | SD | Quantity Mean* | SD |
| | | copies/µℓ | | | | | | | |
| AUTUMN | U1 | 8.41E+05 | 4.10E+05 | 4.48E+08 | 4.42E+07 | 8.51E+01 | 4.11E+01 | 1.12E+ 10 | 1.59E+ 10 |
| | U2 | 5.66E+05 | 1.71E+05 | 5.55E+09 | 2.34E+09 | 7.85E+01 | 5.82E+01 | 1.91E+ 7 | 1.98E+ 7 |
| | U3 | 3.06E+05 | 4.64E+04 | 3.22E+08 | 1.86E+08 | 3.81E+01 | 1.02E+04 | 3.32E+ 8 | 4.35E+ 8 |
| | U4 | 1.19E+05 | 1.31E+05 | 1.89E+09 | 1.35E+09 | 7.45E+01 | 1.23E+01 | 4.47E+ 8 | 3.88E+ 8 |
| | U5 | 1.99E+04 | 1.04E+05 | 7.59E+10 | 9.43E+09 | NA | NA | 33.5 | 13.2 |
| WINTER | U1 | 2.91E+04 | 1.66E+04 | 1.96E+08 | 2.60E+08 | 5.47E+01 | 2.91E+01 | 2.13E+8 | 3.9E+8 |
| | U2 | 2.92E+05 | 6.69E+04 | 3.84E+10 | 6.62E+10 | 8.35E+01 | 3.69E+01 | 0.39 | 0.33 |
| | U3 | 9.25E+04 | 4.17E+04 | 2.24E+06 | 3.34E+06 | 1.24E+02 | 8.42E+01 | 5.02E+8 | 8.7E+8 |
| | U4 | 1.91E+04 | 2.44E+04 | 5.76E+08 | 1.40E+08 | 7.08E+02 | 3.44E+02 | 1.15E+8 | 1.97E+8 |
| | U5 | 6.41E+04 | 4.25E+04 | 3.91E+08 | 3.37E+08 | NA | NA | 5.68E+7 | 6.02E+7 |
| SPRING | U1 | 2.04E+04 | 3.44E+03 | 3.76E+10 | 6.51E+10 | 1.63E+02 | 1.68E+02 | 2.55E+8 | 4.40E+8 |
| | U2 | 9.56E+04 | 6.68E+04 | 3.62E+09 | 5.63E+09 | 8.16E+01 | 4.35E+01 | 2.52E+8 | 4.40E+8 |
| | U3 | 1.11E+06 | 4.62E+05 | 8.98E+09 | 3.56E+09 | 4.51E+01 | 1.25E+01 | 1.49E+9 | 2.05E+9 |
| | U4 | 1.79E+07 | 3.99E+06 | 1.72E+10 | 2.95E+10 | 9.90E+01 | 2.50E+01 | 8.22E+6 | 1.42E+7 |
| | U5 | 9.84E+06 | 2.72E+06 | 5.19E+05 | 3.91E+05 | 1.78E+02 | 8.90E+01 | 5.05E+8 | 5.55E+8 |
| SUMMER | U1 | 1.24E+05 | 8.76E+04 | 1.83E+09 | 2.20E+09 | 2.00E+02 | 2.38E+02 | 9.1E+8 | 7.9E+8 |
| | U2 | 3.80E+04 | 3.74E+04 | 1.25E+08 | 1.71E+08 | 1.05E+02 | 3.31E+01 | 7.82E+7 | 1.20E+8 |
| | U3 | 3.02E+04 | 2.19E+03 | 2.80E+08 | 1.81E+08 | 4.04E+07 | 7.01E+07 | 9.07E+8 | 1.28E+9 |
| | U4 | 6.46E+04 | 3.75E+04 | 3.57E+08 | 2.25E+08 | 1.72E+02 | 3.32E+01 | 1.13E+9 | 1.23E+9 |
| | U5 | 7.56E+04 | 7.17E+04 | 1.73E+08 | 6.00E+07 | 4.03E+01 | 3.37E+01 | 2.29E+8 | 1.65E+7 |

* n= 2 or 3; NA= No Amplification

## 4.4 Discussion

The present study highlights the importance of routine environmental surveillance of human enteric viruses for a better understanding of the actual burden of these viral infections to receiving water bodies and the population that uses this resource. The study of viral diversity in surface waters has greatly advanced with the introduction of molecular techniques. To the best of our knowledge, this is the first study in KwaZulu-Natal (South Africa) to examine the viral diversity of the Umgeni River water, over a seasonal cycle using several molecular techniques including PCR, ICC-PCR and Real-Time PCR. A seasonal cycle of sampling provided a template for the temporal dynamics and spatial distribution of the viral community, since samples from the same season are expected to have a more similar fingerprint than samples that are distant in time as described by Sánchez *et al.* (2007). Several studies have assessed the recovery of viruses from different water samples and established that the recovery rate depends not only on concentration and detection methods, but also the source of the environmental water that is tested (Albinana-Gimenez *et al.*, 2009, Haramoto *et al.*, 2009, Victoria *et al.*, 2010). It is imperative to highlight that the detection of high or low levels of virus in a specific geographic area depends not only on virus emission patterns in the population, but also the procedure that is used for virus detection (Girones *et al.*, 2010).

In the present study, molecular techniques were effectively used to detect the presence and infectivity of human Adenoviruses, Enteroviruses, Rotaviruses and Hepatitis B viruses in the Umgeni River water samples. The first round conventional PCR/ RT-PCR failed to give visible bands on an agarose gel. However, the conventional nested PCR/RT-PCR step detected viral particles in 100% of the concentrated river water samples (Figures 4.1 to 4.4). The use of two phases of amplification was necessary as it 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. Our results, consistent with other studies, suggest that some viruses, known to be pathogenic for humans (adenoviruses, enteroviruses), can be commonly found in environmental waters by molecular biology techniques (Schvoerer *et al.*, 2000).

PCRs have been considered as valuable tools in environmental virology studies, especially due their specificity and sensitivity to detect a few viral genomic copies in several environmental matrices (Girones *et al.*, 2010). Virus like particles from the river water samples were successfully amplified with all viral primer sets, resulting in PCR/RT-PCR amplification products of the expected size, and nested PCR/RT-PCR allowed for their detection in less than 12 hours. The hexon gene region of Adenovirus was successfully amplified from 100 % of the VLPs isolated from the water samples (Figure 4.1). Nested RT-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 4.2). At the same time, nested RT- PCR of the VP7 gene of group A Rotaviruses

(Figure 4.3) and nested PCR of the S gene of Hepatitis B viruses (Figure 4.4) were detected in 100 % of the VLPs isolated from the Umgeni River during the study period. Nested PCR is an *in vitro* method for direct enzymatic amplification of specific targeted DNA and cDNA sequences that ensures very sensitive, specific and rapid detection of viruses with relative ease. This technique enables the analysis of a large number of samples and also has potential to eliminate false-positive results encountered with microscopy (Dongdem *et al.,* 2009). The high prevalence of enteric viruses in the river water suggests the circulation of these viral pathogens at a high frequency among the low income population in Durban.

Adenoviruses are associated with numerous disease outbreaks and are responsible for many recreational water outbreaks (Enriquez and Gerba, 1995). Children and immune-compromised patients are at greater risk for contracting adenovirus infections. Subsequently, adenovirus has been included in EPA's Drinking Water Contaminant Candidate Lists. Adenoviruses have been detected in various waters worldwide (Pina *et al.,* 1998) and typically outnumber the Enteroviruses, when both are detected in surface waters. Previous detection of adenoviruses from water sources in South Africa has been limited (van Heerden *et al.,* 2003; 2004), 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). . Human AdVs have been 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). Our results showed that human Adenoviruses were detected in 100% the river water samples tested which is significantly higher than the previous report. Nested PCR assays for adenoviruses as reported by Allard et al. (1992) and Van Heerden *et al.* (2003) were shown to have increased sensitivities compared to conventional PCR, with detection limits of one adenovirus particle and $10^{-2}$ PFU, respectively.

Enteroviruses (EVs) are ubiquitous in all types of water including recreational water, tap water and sea water (Ehlers *et al.,* 2005a; Lee *et al.,* 2005). They can survive drinking water treatment and have been detected in drinking water apparently free of coliform bacteria (Vivier *et al.,* 2004). Infections are most likely to occur throughout the year coinciding with recreational activities (Fong and Lipp, 2005). The high prevalence of enteric viruses in surface water highlights the importance of assessing the water sources used for domestic purposes for viral contamination (Kiulial *et al.,* 2010). About 29% of river water samples and 19% of treated drinking water samples in South Africa had detectable levels of EVs (Ehlers *et al.,* 2005a,b). In this study, Enteroviruses were detected at all sampling points during all seasons, with 90 % of the EVs being found to be infectious by ICC-PCR. This occurrence should raise the alarm to authorities as further epidemiological investigation is required to determine the risk of disease to the population exposed to contaminated water.

The World Health Organization (WHO) estimates that globally 527,000 deaths occur each year among children as a result of rotavirus infection especially in sub-Saharan Africa (WHO, 2007). In South

Africa approximately six children die each day from severe rotavirus gastroenteritis (Parashar *et al.,* 2003). Their survival characteristics can be demonstrated by the presence of large amounts of infectious particles in wastewater (Dubois *et al.,* 1997), in environmental waters (van Zyl *et al.,* 2006) and in drinking water (van Zyl *et al.,* 2006). Ansari *et al.* (2001) reported that although waterborne outbreaks of rotavirus gastroenteritis are often recorded, other vehicles such as air, body contact and food may be a vehicle for this infection. Although rotavirus is a common cause of diarrhoea clinically in South Africa, studies by van Zyl *et al.* (2004; 2006) were the only reports on the presence of rotaviruses in raw and treated water in South Africa prior to this study. This study detected the presence of Rotavirus in water samples throughout all seasons of the year. Rotavirus infection has dire consequences with prevention incidence being necessary to limit the spread of disease.

Detection of Hepatitis B viruses (HBVs) in all water samples from the Umgeni River during this study period is unexpected as this virus is not waterborne. HBV is transmitted through direct contact with blood or other body fluids of an infected person, and not through ingestion of contaminated water or food (WHO, 2007). However, it should be known that HBV can survive outside the body for at least 7 days and still be capable of causing infection, thus any blood spills - including dried blood can still be infectious (WHO, 2007).The hepatitis B virus is 50 to 100 times more infectious than Human Immunodeficiency-virus, and it is estimated that 2 billion individuals worldwide are infected with this virus, which causes 620 000 deaths each year (WHO, 2007). There are currently between 3 and 4 million of the South African black population who are chronically infected with HBV (WHO, 2008). Finding HBVs in water raises the concerns that HBV might be transmitted in the natural water environments via breaks in the skin, and further investigation into this incidence is imperative.

The disadvantage of the PCR-based assays is that they do not allow a conclusion on the infectivity of the water samples analysed, since free viral particle nucleic acids would also be detected by the PCR-based assay. Currently, the amalgamation of PCR and cell culture offers the unsurpassed approach to assess viral infectivity, including the detection of slow-growing viruses. Methods that combine features of cell culture and molecular methods for a rapid, sensitive detection of infective virus particles detected in water samples have been developed (Cantera et al., 2010) and is found promising to be used in the future. The cell culture procedure applied for the PCR study was performed as described in Chapter three section 3.2.6. The cell lines used in this study for human virus investigations in water were, the A549 (adenocarcinomic human alveolar basal epithelial cell), HEK 293 (human embryonic kidney), Hela (Henrietta Lacks – cervical cancer), HepG2 (human hepatocellular carcinoma), PLC/PRF/5 (human primary liver carcinoma cells) and Vero (African green monkey kidney cells). In brief the cell lines amplified the amount of virus present in the water samples, expressed the viral antigens and in many cases die as a result of the viral infection producing characteristic cytopathic effects in the cell monolayer. The augmented viruses were further identified by molecular techniques.

The use of Integrated Cell Culture (ICC)-PCR has been described for the detection of infectious EVs (Reynolds *et al.,* 1996), hepatitis A virus (Jiang *et al.,* 2004; Reynolds *et al.,* 2001), enteric adenovirus (Lee and Kim, 2002), and astrovirus (Grimm *et al.,* 2004). The integrated use of cell culture with PCR has demonstrated a wide distribution of infectious viruses in water sources, since it allows for the detection of non-CPE-producing enteric viruses (Lee *et al.,* 2005). *Lee et al.* (2005) demonstrated the simultaneous detection of both Enteroviruses and adenoviruses in the same cell line with this approach. The Umgeni river water samples were analysed by integrated cell culture PCR to provide confirmation of infectious Adenovirus, Rotavirus, Enterovirus and Hepatitis B virus particles in various cell culture lines. Nested ICC-PCR (Figure 4.5) confirmed the presence of infectious Adenovirus particles in 80%, 85% and 100% of the HEK 293, PLC/PRF/5 and Hep G2 cell lines respectively. Infectious Rotavirus were detected on 100% of the A549, Vero and HeLa cell lines, with no infection being detected on the HEK 293 and PLC/PRF/5 cell lines. Nested ICC-RT PCR confirmed the presence on infectious Enterovirus particles on all six cell lines. Infectious Hepatitis B virus particles were detected on 20% of the Hep G2 cell line, 40% of the HEK 293 cell lines, and 55% on the PLC/PRF/5 cell line. Sensitive detection of infectious viruses is needed for effective examination of air, water, and food samples in which concentrations of viruses in these sample types are typically very low. Combining cell culture and PCR as reported here will allow researchers to detect more rapidly infectious viruses in clinical and environmental samples. Lee *et al*. (2004) evaluated a cell culture-PCR assay based on a combination of A549 and BGMK cell lines as a tool to monitor infectious adenoviruses and Enteroviruses in river water. They found that the number of samples positive for adenoviruses was higher with A549 cells (13 samples) than with BGMK cells (1 sample); and that the numbers of samples positive for enteroviruses were similar with both types of cells. The cell culture-PCR assay established in their study with a combination of A549 and BGMK cells and molecular identification was shown to be a useful tool for monitoring infectious adenoviruses and enteroviruses in aquatic environments (Lee *et al.,* 2004).

Nucleotide sequences of Adenovirus, Enterovirus and Hepatitis B virus particles were BLAST searched and identified based on their similarity to GenBank database entries. Multiple alignments and phylogenetic analyses were performed by using ClustalX and MEGA version 5 respectively. The neighbour joining phylogenetic trees illustrated vast spatial and temporal distribution of each virus type (Figure 4.6 to 4.8). Majority of the identified samples belonged to Human Adenovirus C strain, Coxsackievirus B5 Genomic RNA and Hepatitis B virus isolate SHH043A.

In this study, a real-time Fast TaqMan® RT-PCR assay was described to improve further molecular methods for sensitive and rapid detection of the human viruses. A rapid, sensitive, and specific detection method was used for the examination of Adenovirus, Rotavirus, Enterovirus and Hepatitis B virus particles in the Umgeni River water samples. A standard curve of viral concentration versus threshold cycle was

generated for each virus type based on copy number (Figure 4.9). The slope of a standard curve provides a signal of the efficiency of the real-time PCR. A slope of –3.322 means that the RT- PCR has an efficiency of 1, or 100%, and the amount of PCR product doubles during each cycle (Qiagen, 2004a). A slope of less than –3.322 (e.g., –3.8) is suggestive of a reaction efficiency of <1. A slope of greater than –3.322 (e.g., –3.0) indicates a PCR efficiency, which seems to be greater than 100%. This can transpire when values are measured in the non-linear phase of the reaction or it can indicate the presence of inhibitors in the reaction. (Qiagen, 2004a). The hexon gene was targeted to develop a broadly reactive TaqMan assay for detection of all adenovirus species. In this study PCR efficiencies ranged from 96.693 % (Adenovirus) to 128.297%(Hepatitis B virus). Adenovirus copy number was highest at 1.79 x $10^7$ copies/µℓ (U4) during spring (Table 4.3). Sibanda and Okoh (2012) found that adenovirus detection rate increased with distance downstream the Tyume river, and the likelihood of this was due to the anthropogenic activities contributing to contamination of natural water sources with enteric pathogens being discharged from partially/untreated wastewater effluents from domestic and municipal sewage into riverwaters. High copy numbers and spatial distribution of Enterovirus occurred throughout all samples and seasons tested. In South Africa, Ehlers *et al*. (2005b), used a combination of cell culture and nested-PCR, and reported the presence of enteroviruses in 42.5% of sewage, 18.7% of treated drinking water, 28.5% of river water, 26.7% of dam/spring water and in 25.3% of borehole water samples. Rotavirus copy numbers were low in the river water environment. In a similar study, Chigor and Okoh (2012) detected RVs in 14% (10/72) of the samples and EVs in 9.7% of the samples in the Buffalo River of South Africa. In a study from Kenya, Kiulia *et al*. (2010) detected Group A rotavirus in 10 (100%) of samples collected from a river located in urban area and in three (25%) of rural river water samples. Hepatitis B virus copy numbers were found to be disturbingly high which is a cause of concern. The higher occurrence of viruses in the downstream stretch of the river could be explained in terms of increased human pressure on the environment (Sibanda and Okoh, 2012). It is difficult to compare the values of virus concentrations determined in in this study to different studies employed elsewhere due to the differences in Real Time PCR assays employed (Hamza *et al.*, 2009). The adenovirus DNA copy numbers, for example, as determined by one quantitative PCR assay differed by more than a factor of 1-log from those determined by another PCR assay (Bofill-Mas *et al.*, 2006). The broadly reactive TaqMan PCR assay used in this study does provide a more sensitive and specific approach for viral detection and can be a useful tool for performing environmental surveys to determine whether any of the detected virus serotypes are present in water to which humans are exposed.

The VLPs from the water samples analysed in this study represent only a single time point in the Umgeni River; therefore, it is possible that the types of viruses found could differ if samples were collected at different periods of the year. In addition, the results of this study may have been biased due to the differential recovery efficiency of the concentration and nucleic acid isolation methods for different viral groups. This could have transpired due to viruses adhering to particles may have been lost in the ultra-

filtration steps. It is also possible that inefficient reverse transcription or the degradation of DNA and RNA could have skewed the results. Thus further studies need to be completed to verify the absence of undetected or under detected viruses.

In conclusion, 100% AdVs, EVs, RVs, and HBVs were found in all the Umgeni River water samples collected from five points along the river seasonally. Viral infectivity was confirmed by ICC-PCR with 90 % of VLPs being detected. Adenoviruses, Enteroviruses and Hepatitis B viruses were the only viruses identified by sequencing and BLAST search. The molecular characterization of these viruses confirmed that majority of environmental isolates were of human origin. Real-Time TaqMan PCR quantified each viral group, with relatively high copy numbers being observed for the EV and HBV populations. The results of this study suggest that adenoviruses and enteroviruses have potential as viral indicators of faecal contamination in water environments because they were detected in all water samples tested. However further validation studies in this regard are required tomeet the criteria required for an indicator of faecal pollution.In addition the presence of HBV in the river water is a serious consequence and requires further investigation, as this virus is not known to be waterborne. These results emphasise the necessity of assessing the sources of environmental contamination in interconnected ecosystems to effectively enhance the safety of recreational waters and to demonstrate the need to include virological parameters when determining water quality to reduce the potential exposure of users. The present study identifies potential viral hazards of river water for domestic water supply and recreational activities. Further research will be needed to determine if these candidate viruses detected in this study have the necessary characteristics of a microbial water quality indicator. This will enable scientific decisions to be made regarding the selection and prioritization of human pathogenic viruses to be included in the future risk assessment and to determine how the incidence of these enteric viruses in river waters correlates with their presence in contaminated wastewaters.

# CHAPTER FIVE

## Research in Perspective and Future Recommendations

## 5.1 Research in Perspective

Microbial contamination of surface waters and coastal waters, in South Africa, is one of the primary challenges fronting the water industry and regulatory authorities (DEAT, 2006). Deteriorating water quality due to anthropogenic and natural effects has restricted the country's capability to provide sufficient water to meet its requirements and to ensure environmental sustainability (DWAF, 2007). In developing countries such as South Africa, rivers play a pivotal role in the life of the people for social, cultural and religious reasons, and for these reasons, such dependent populace are directly exposed to prevailing poor quality of river water thus necessitating the need for the regulation of biological quality of both wastewater effluents and the receiving water bodies (Igbinosa, 2010). During routine environmental monitoring, coliform bacteria are normally used as microbiological parameters of water quality, which does not evidence its contamination by viruses. Assessment of virological safety of water is an imperative concern for the consumers as well as public health authorities worldwide, with developing countries and third world countries representing the main sufferers from enteric diseases (Reynolds *et al*., 2008). Although bacterial contamination of surface water and soils and the related health risks have been carefully studied, attention is now progressively being focused on the threats associated with virus contamination of water (Verma *et al.,* 2009; WHO, 2004). Bacteria used as conventional indicators to monitor the safety of potable water provisions have been shown to be significantly less resistant than viruses to environmental factors and to water and wastewater treatment processes (Okoh *et al.,* 2010). As a result, human viruses may be present in water that manifests little or no sign of bacterial pollution (Reynolds *et al*., 2008).

In South Africa and other industrialised areas of the world, wastewater from municipalities is generally treated at municipal treatment plants (Igbinosa, 2010). All collected wastewaters including – storm water from roof gutters, streets and other hard surfaces, blackwater from toilets, greywater from kitchen sinks and washing machines, bathrooms and washing facilities and industrial wastewater; are transported to the treatment plant. Hypothetically more than 100 different types of pathogenic viruses, bacteria and parasites that may cause diseases and clinical symptoms in humans such as diarrhoea, meningitis and hepatitis, can be present in the faecally-polluted water (Pegram *et al.,* 1998; Obi *et al.,* 2002).

The quality of surface waters (both fresh and marine) in Durban has not been adequately investigated, despite the fact that the microbiological qualities of other water sources in South African provinces have been widely reported. The study presented here was thus undertaken to examine the prevalence of microbial and human viral pathogens in the Umgeni-River. Since catchment conditions and water quality change seasonally, a proper investigation of the chemical and microbial surface water

quality was performed to assess samples taken both during the rainy and the dry seasons, to take annual variation into account. The sampling seasons and sites included five vast areas of the river starting from a Dam water source, passing through: a nature reserve, waste treatment works, informal human settlement and finally ending of at the river mouth (were the river and ocean meet). The samples were collected seasonally during a period of 12 months.

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. Large seasonal variations in $BOD_5$, COD and conductivity levels amongst the sampling points along the Umgeni River were also observed. Water from 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 the 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 were also demonstrated to have strong correlations with the level of heterotrophic bacterial population in this river according to Canonical correspondence analysis (CCA) analysis.

The microbiological 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 the DWAF (1996). TC and FC counts varied significantly ($p$<0.05) throughout all sampling points and ranged from 3.30 x $10^3$ cfu/100mℓ (U1 – winter) to 6.03 x $10^3$ cfu/100mℓ (U1 – summer) and 0.89 x $10^3$ cfu/100mℓ (U3 – winter) to 4.85 x $10^3$ cfu/100mℓ (U2 – spring), respectively. All points failed to meet the target water quality ranges of TC, FC, EC and FS levels for the recreational and drinking uses according to the DWAF and USEPA criteria with points U2 and U1 having the highest TC, FC, and VC especially during the spring and summer seasons. The potential bacterial pathogens such as presumptive *Salmonella spp.* and *Shigella spp.* 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 significantly correlate with the microbial community structures at sites 1, 3 and 5 during the summer and spring seasons. The overall bacterial growths at site 2, 4, 5 during autumn and winter seasons correlated with the nitrate/nitrite profiles. The results from the CCA analysis also suggest that correlations exist between bacterial community data and heavy metal variables exist. CCA analyses also demonstrated that the overall bacterial growths depend on the environmental factors of different sites and vary due to seasonal changes.

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. Similarly, VLPs were detected using EFM at all sampling sites throughout all seasons, and increased in similar trends with 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 affected the total bacterial community structures significantly. 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 six different tissue culture assays. All concentrated water samples produced substantial cytopathic effects on the Vero, HEK 293, HeLa and A549 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. Approximately 90% of Adenoviruses, Enteroviruses, Rotaviruses and Hepatitis B viruses were found in all the Umgeni River water samples collected from five points along the river seasonally. Viral infectivity was confirmed by ICC-PCR with 90 % of infectious VLPs being detected. Adenoviruses, Enteroviruses and Hepatitis B viruses were the only viruses identified by sequencing and nucleotide BLAST search. The molecular characterization of these viruses confirmed that majority of environmental isolates were of human origin. Real-Time TaqMan PCR quantified each viral group, with relatively high copy numbers being observed for the EV and HBV populations at $7.59 \times 10^{10}$ copies/µℓ (U5) during autumn and $5.89 \times 10^{12}$ copies/µℓ (U3) during spring respectively.

The working hypothesis of this research was that pathogenic viruses are present in the Umgeni River of Durban South Africa. To this end, our findings revealed that the Umgeni River is polluted both microbially and virally. The quality of the river water did not meet recommended South African guidelines for recreational and domestic use, therefore suggesting that the water body posed a significant health and environmental risk on the biota including the informal communities relying on this receiving watershed for domestic purposes. This raises concerns over the management of these water resources and the subsequent deleterious effects these environments have on the end users. This study, detected the presence of adenoviruses, enteroviruses, rotaviruses as well as Heptatitis B viruses in all water samples from all seasons. The high detection rate might be partially due to the efficiency of virus recovery rate

using a TFF system. However, the results of tissue culture technique and PCR 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; Taylor *et al.,* 2001; van Heerden *et al.,* 2003, 2004; van Zyl *et al.,* 2004, 2006; Vivier *et al.,* 2004).

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 present study also 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.

## 5.2 Future Recommendations

Given the paucity of viral guidelines in surface waters, it is now time to contemplate the establishment of virus standards for potable and non-potable waters. Such guidelines are unquestionably necessary for enhanced wastewater effluent treatment and for the planned use of reclaimed wastewater for domestic and industrial usage. Where virological facilities can be provided, it is necessary to monitor wastewater effluents, raw-water sources and drinking-water for the presence of viruses. This will provide baseline data to evaluate the health risk that could be faced by the receiving population. 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.

Further research is vital into the health risks associated with viruses in water and soil. These studies should include the development and assessment of methods of detecting viruses and alternative indicators of virus pollution (e.g., phages) and the enhancement of treatment methods for the inactivation and removal of viruses from water and wastewater. The dissemination and survival of viruses in the natural environment should also be investigated to understand the actual prevalence of viruses in the river catchment area, because most urban rivers receive effluents from multiple wastewater treatment plants that contain viruses shed from all patients in the catchment area.

A standard method should be established for the concentration and detection of viruses in large volumes of drinking-water (e.g., 20-100ℓ) based on a full evaluation in different laboratories of present

techniques (WHO, 2004). Such an attempt would facilitate the development of virus-monitoring programmes and would ensure a maximum degree of comparability of results. More reliable approaches to ensure acceptable safety of drinking water supplies may be based on control by multiple-barrier principles from catchment to tap using hazard assessment and critical control point (HACCP) principles. A laboratory quality-control system should be developed to enable participating laboratories to standardize their procedures.The significance of enteric viruses as causative agents of crucial human diseases cannot be overestimated, and hence continuous monitoring of water environments cannot be overrated. Since this project appears to be the first of its kind in the KwaZulu-Natal Province and coupled with the interesting revelations from the study, there is a need to extend this investigation to determine the nature and extent of pollution in other rivers and recreational water bodies in the Province. Future studies are needed to provide effective and reproducible methods for the detection of waterborne viral pathogens in order to govern the extent of contamination of water environments, the types of pathogens involved and the association between viral contamination and environmental factors. Quantitative microbial risk assessment (QMRA) analysis should be performed, using water quality data, generating: pathogen-specific characteristics, prevalence data, and exposure data. This information will lead to a better understanding of the health risks related to water systems, and to improved methods of control. The victims of poor water quality are ordinary people, thus a national hotline is also proposed to which people can report complaints to give them the means to highlight problems before they turn into a national crisis.

# APPENDIX i

## Media and Buffers

**Composition of commercial media and buffers used in this study**

**1. Media**

| | | |
|---|---|---|
| • *Bile aesculin agar* (Merck) | 54.65 | g/L |
| • *KF- Faecal streptococcus agar* (Fluka BioChemika) | 76 | g/L |
| • *Membrane filter enterococcus selective agar* ( Merck) | 41.5 | g/L |
| • *m-Endo Les agar* (Biolab) | 51 | g/L |
| • *m-FC agar* (Biolab) | 50 | g/L |
| • *Nutrient agar* (Biolab) | 31 | g/L |
| • *Salmonella- Shigella agar* (Merck) | 75 | g/L |
| • *Thiosulphate citrate bile salts sucrose agar* (Fluka BioChemika) | 88 | g/L |
| • *Nalidixic acid solution* | | |
| Nalidixic acid (Sigma Aldrich) | 2.5 | g |
| 2M Sodium Hydroxide (Merck) | 2 | ml |
| De-ionised water | 98 | ml |
| • *Calcium glucose solution* | | |
| Calcium chloride ($CaCl_2$. $2H_2O$) (Merck) | 3 | g |
| Glucose | 10 | g |
| De-ionised water | 100 | ml |
| • *Nutrient broth with Sodium Chloride* | | |
| Nutrient broth (Biolab) | 16 | g |
| Sodium chloride (NaCl) (Saarchem) | 1 | ml |
| Water | 1000 | ml |
| • *Trypticase yeast extract broth* | | |
| Trypticase peptone (Biolab) | 10 | g |
| Yeast extract powder (Biolab) | 1 | g |
| Sodium chloride (NaCl) | 8 | g |
| Water | 1000 | ml |
| • *Bottom agar for somatic phage* | | |
| Bacto agar (Biolab) | 14 | g |
| Tryptone powder (Biolab) | 13 | g |
| Glucose (Merck) | 1.5 | g |
| Sodium chloride (NaCl) | 8 | g |
| Water | 1000 | ml |
| Nalidixic acid solution (Sigma Aldrich) | 1 | ml |
| • *Bottom agar for F-RNA phage* | | |
| Bacto agar | 12 | g |

| | | |
|---|---|---|
| Trypticase peptone | 10 | g |
| Sodium chloride (NaCl) | 8 | g |
| Yeast extract | 1 | g |
| Water | 1000 | ml |
| Adjust pH 7.2 | | |
| Calcium glucose solution | 8 | ml |

- *Top agar for somatic phage*

| | | |
|---|---|---|
| Bacto agar | 8 | g |
| Tryptone powder | 10 | g |
| Glucose | 3 | g |
| Sodium chloride (NaCl) | 8 | g |
| 1M Sodium carbonate (Saarchem) | 5 | ml |
| 1M Magnesium chloride (Merck) | 1 | ml |
| Water | 1000 | ml |
| Nalidixic acid | 1 | ml |

- *Top agar for F-RNA phage*

| | | |
|---|---|---|
| Bacto agar | 12 | g |
| Trypticase peptone | 10 | g |
| Sodium chloride (NaCl) | 8 | g |
| Yeast extract | 1 | g |
| Water | 1000 | ml |
| Adjust pH 7.2 | | |
| Calcium glucose solution | 10 | ml |
| Nalidixic acid | 4 | ml |

## 2. Buffers

- *0.5 M Disodium ethylenediaminetetraacetate (EDTA)*

| | | |
|---|---|---|
| EDTA (Saarchem) | 186.12 | g |
| De-ionised water (bring up) | 1000 | ml |
| pH adjustment (sodium hydroxide pellets ~20 g) | pH 8 | |

- *1 M Tris-hydroxymethyl-aminomethane (Tris)*

| | | |
|---|---|---|
| Tris base (Roche) | 121.14 | g |
| De-ionised water (bring up) | 1000 | ml |
| pH adjustment (hydrochloric acid ~42 ml) | pH 8 | |

- *Tris-EDTA buffer (TE)*

| | | |
|---|---|---|
| 0.5 M EDTA stock solution | 2 | ml |
| 1 M Tris stock solution | 10 | ml |
| De-ionised water (bring up) | 1000 | ml |

- *50 X Tris-acetate EDTA buffer (TAE)*

| | | |
|---|---|---|
| Tris base | 242 | g |
| Glacial acetic acid (Merck) | 57.1 | ml |
| 0.5 M EDTA (pH 8) | 100 | ml |
| De-ionised water (bring up) | 1000 | ml |
| pH adjustment (sodium hydroxide pellets/glacial acetic acid) | pH 8 | |

- *Phosphate buffered saline (PBS)*

| | | |
|---|---|---|
| Sodium chloride | 8 | g |
| Potassium chloride (KCl) (Saarchem) | 0.2 | g |
| Disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$) (Saarchem) | 3.58 | g |
| Potassium dihydrogen phosphate ($KH_2PO_4$) (Saarchem) | 0.24 | g |
| De-ionised water (bring up) | 1000 | ml |
| pH adjustment (hydrochloric acid) | pH 7.4 | |

- *Ethidium bromide stain (EtBr)*

| | | |
|---|---|---|
| Ethidium bromide (Sigma) | 50 | µl |
| De-ionised water | 500 | ml |

# APPENDIX ii

## Numerical Data & Statistical Results

**Table 1: Presumptive bacterial indicator counts for water samples collected along the Umgeni River during March-April 2011 (Autumn)**

| **Total Heterotrophic Bacterial Count** | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **U1** | | | **U2** | | | **U3** | | | **U4** | | | **U5** | | |
| | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-6}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-6}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-6}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-6}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-6}$** |
| **$10^{-4}$** | 234 | 468.0 | 4.7 | 178 | 356.0 | 3.6 | 163 | 326.0 | 3.3 | 113 | 226.0 | 2.3 | 69 | 138.0 | 1.4 |
| | 221 | 442.0 | 4.4 | 167 | 334.0 | 3.3 | 186 | 372.0 | 3.7 | 118 | 236.0 | 2.4 | 77 | 154.0 | 1.5 |
| **$10^{-5}$** | 32 | 64.0 | 6.4 | 23 | 46.0 | 4.6 | 29 | 58.0 | 5.8 | 22 | 44.0 | 4.4 | 15 | 30.0 | 3.0 |
| | 26 | 52.0 | 5.2 | 27 | 54.0 | 5.4 | 19 | 38.0 | 3.8 | 36 | 72.0 | 7.2 | 23 | 46.0 | 4.6 |
| **$10^{-6}$** | 21 | 42.0 | 42.0 | 8 | 16.0 | 16.0 | 1 | 2.0 | 2.0 | 15 | 30.0 | 30.0 | 2 | 4.0 | 4.0 |
| | 18 | 36.0 | 36.0 | 4 | 8.0 | 8.0 | 2 | 4.0 | 4.0 | 11 | 22.0 | 22.0 | 5 | 10.0 | 10.0 |
| **Average (x $10^{-6}$)** | | | 5.2 | | | 4.2 | | | 4.1 | | | 3.0 | | | 2.0 |
| **SD** | | | 0.8787 | | | 0.9569 | | | 0.3736 | | | 1.2077 | | | 0.8927 |
| **Total Coliforms Count** | | | | | | | | | | | | | | | |
| | **U1** | | | **U2** | | | **U3** | | | **U4** | | | **U5** | | |
| | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** |
| **$10^{-1}$** | 158 | 316.0 | 3.2 | 158 | 316.0 | 3.2 | 56 | 112.0 | 1.1 | 134 | 268.0 | 2.7 | 28 | 56.0 | 0.6 |
| | 148 | 296.0 | 3.0 | 145 | 290.0 | 2.9 | 67 | 134.0 | 1.3 | 98 | 196.0 | 2.0 | 34 | 68.0 | 0.7 |
| **$10^{-2}$** | 99 | 198.0 | 19.8 | 89 | 178.0 | 17.8 | 13 | 26.8 | 2.7 | 14 | 27.4 | 2.7 | 2 | 4.8 | 0.5 |
| | 78 | 156.0 | 15.6 | 77 | 154.0 | 15.4 | 14 | 28.6 | 2.9 | 14 | 28.6 | 2.9 | 2 | 3.0 | 0.3 |
| **$10^{-3}$** | 4 | 8.0 | 8.0 | 6 | 12.0 | 12.0 | 2 | 4.2 | 4.2 | 2 | 3.6 | 3.6 | 1 | 2.0 | 2.0 |
| | 2 | 4.7 | 4.7 | 4 | 7.6 | 7.6 | 2 | 3.2 | 3.2 | 2 | 3.0 | 3.0 | 0 | 0.0 | 0.0 |
| **Average (x $10^{-3}$)** | | | 3.6 | | | 4.6 | | | 3.2 | | | 3.1 | | | 0.5 |
| **SD** | | | 0.9751 | | | 2.6417 | | | 0.6785 | | | 0.3818 | | | 0.1595 |
| **Faecal Coliforms Count** | | | | | | | | | | | | | | | |
| | **U1** | | | **U2** | | | **U3** | | | **U4** | | | **U5** | | |
| | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** |
| **$10^{-1}$** | 132 | 264.0 | 2.6 | 137 | 274.0 | 2.7 | 76 | 152.0 | 1.5 | 89 | 178.0 | 1.8 | 54 | 108.0 | 1.1 |
| | 101 | 202.0 | 2.0 | 120 | 240.0 | 2.4 | 98 | 196.0 | 2.0 | 74 | 148.0 | 1.5 | 41 | 82.0 | 0.8 |
| **$10^{-2}$** | 21 | 42.0 | 4.2 | 29 | 58.0 | 5.8 | 5 | 10.0 | 1.0 | 17 | 34.0 | 3.4 | 2 | 4.0 | 0.4 |
| | 15 | 30.0 | 3.0 | 20 | 40.0 | 4.0 | 3 | 6.0 | 0.6 | 11 | 22.8 | 2.3 | 1 | 2.0 | 0.2 |
| **$10^{-3}$** | 2 | 4.0 | 4.0 | 3 | 6.0 | 6.0 | 1 | 2.0 | 2.0 | 4 | 8.0 | 8.0 | 1 | 2.0 | 2.0 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2.0 | 2.0 | 1 | 2.0 | 2.0 | 1 | 1.4 | 1.4 | 1 | 2.0 | 2.0 | 0 | 0.0 | 0.0 |
| **Average (x $10^{-3}$)** | | | 3.3 | | | 3.9 | | | 1.1 | | | 2.2 | | | 0.5 |
| **SD** | | | 1.0132 | | | 1.9009 | | | 0.5311 | | | 0.8439 | | | 0.3164 |
| **Faecal Streptococci Count** | | | | | | | | | | | | | | | |
| | **U1** | | | **U2** | | | **U3** | | | **U4** | | | **U5** | | |
| | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** |
| **$10^{-1}$** | 15 | 30.0 | 0.3 | 30 | 60.0 | 0.6 | 18 | 36.0 | 0.4 | 14 | 28.0 | 0.3 | 6 | 12.0 | 0.1 |
| | 11 | 22.2 | 0.2 | 21 | 42.0 | 0.4 | 17 | 34.0 | 0.3 | 8 | 16.0 | 0.2 | 3 | 6.0 | 0.1 |
| **$10^{-2}$** | 8 | 16.0 | 1.6 | 3 | 5.2 | 0.5 | 2 | 4.0 | 0.4 | 1 | 2.6 | 0.3 | 1 | 2.0 | 0.2 |
| | 5 | 10.0 | 1.0 | 7 | 14.0 | 1.4 | 2 | 3.8 | 0.4 | 1 | 2.0 | 0.2 | 0 | 0.0 | 0.0 |
| **$10^{-3}$** | 2 | 4.0 | 4.0 | 1 | 2.0 | 2.0 | 1 | 2.0 | 2.0 | 0 | 0.0 | 0.0 | 0 | 0.0 | 0.0 |
| | 0 | 0.6 | 0.6 | 1 | 2.0 | 2.0 | 0 | 0.0 | 0.0 | 0 | 0.0 | 0.0 | 0 | 0.0 | 0.0 |
| **Average (x $10^{-3}$)** | | | 0.4 | | | 0.5 | | | 0.4 | | | 0.2 | | | 0.1 |
| **SD** | | | 0.1996 | | | 0.0902 | | | 0.0258 | | | 0.1361 | | | 0.1155 |
| **Enterococci Count** | | | | | | | | | | | | | | | |
| | **U1** | | | **U2** | | | **U3** | | | **U4** | | | **U5** | | |
| | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** |
| **$10^{-2}$** | 79 | 158.0 | 1.6 | 65 | 130.0 | 1.3 | 67 | 134.0 | 1.3 | 72 | 144.0 | 1.4 | 65 | 130.0 | 1.3 |
| | 75 | 150.0 | 1.5 | 52 | 104.0 | 1.0 | 62 | 124.0 | 1.2 | 58 | 116.0 | 1.2 | 17 | 34.0 | 0.3 |
| **$10^{-3}$** | 13 | 25.0 | 2.5 | 7 | 14.0 | 1.4 | 12 | 24.0 | 2.4 | 11 | 21.6 | 2.2 | 2 | 4.6 | 0.5 |
| | 9 | 18.0 | 1.8 | 1 | 2.0 | 0.2 | 5 | 9.0 | 0.9 | 6 | 12.0 | 1.2 | 2 | 4.0 | 0.4 |
| **Average (x $10^{-3}$)** | | | 1.9 | | | 1.2 | | | 1.2 | | | 1.5 | | | 0.4 |
| **SD** | | | 0.5557 | | | 0.1858 | | | 0.2307 | | | 0.4635 | | | 0.5378 |
| ***Vibrio cholerae* Count** | | | | | | | | | | | | | | | |
| | **U1** | | | **U2** | | | **U3** | | | **U4** | | | **U5** | | |
| | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** |
| **$10^{-1}$** | 136 | 272.0 | 2.7 | 150 | 299.2 | 3.0 | 112 | 224.0 | 2.2 | 95 | 190.0 | 1.9 | 82 | 55.0 | 0.6 |
| | 158 | 316.0 | 3.2 | 167 | 334.6 | 3.3 | 120 | 240.0 | 2.4 | 79 | 158.0 | 1.6 | 67 | 42.0 | 0.4 |
| **$10^{-2}$** | 15 | 30.0 | 3.0 | 23 | 46.0 | 4.6 | 12 | 23.9 | 2.4 | 14 | 28.0 | 2.8 | 17 | 3.1 | 0.3 |
| | 18 | 36.0 | 3.6 | 19 | 38.0 | 3.8 | 19 | 37.8 | 3.8 | 10 | 19.8 | 2.0 | 28 | 1.0 | 0.1 |
| **$10^{-3}$** | 2 | 4.0 | 4.0 | 2 | 4.0 | 4.0 | 1 | 2.0 | 2.0 | 1 | 2.0 | 2.0 | 1 | 0.0 | 0.0 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2.0 | 2.0 | 1 | 2.0 | 2.0 | 4 | 8.0 | 8.0 | 1 | 2.0 | 2.0 | 3 | 0.0 | 0.0 |
| **Average ($x 10^{-3}$)** | | | 3.1 | | | 3.7 | | | 2.6 | | | 2.2 | | | 0.4 |
| **SD** | | | 0.3681 | | | 0.6942 | | | 2.7467 | | | 0.4034 | | | 0.1226 |
| ***Salmonella spp.*Count** | | | | | | | | | | | | | | | |
| | **U1** | | | **U2** | | | **U3** | | | **U4** | | | **U5** | | |
| | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** |
| **$10^{-1}$** | 55 | 110.0 | 1.1 | 47 | 23.0 | 0.2 | 15 | 30.0 | 0.3 | 49 | 98.0 | 1.0 | 0 | 0.0 | 0.0 |
| | 64 | 128.0 | 1.3 | 72 | 39.0 | 0.4 | 11 | 22.0 | 0.2 | 57 | 114.0 | 1.1 | 0 | 0.0 | 0.0 |
| **$10^{-2}$** | 8 | 15.2 | 1.5 | 15 | 3.0 | 0.3 | 1 | 2.0 | 0.2 | 2 | 4.6 | 0.5 | 0 | 0.0 | 0.0 |
| | 6 | 12.0 | 1.2 | 11 | 4.0 | 0.4 | 1 | 1.6 | 0.2 | 2 | 4.0 | 0.4 | 0 | 0.0 | 0.0 |
| **$10^{-3}$** | 1 | 1.2 | 1.2 | 2 | 2.0 | 2.0 | 0 | 0.0 | 0.0 | 1 | 2.0 | 2.0 | 0 | 0.0 | 0.0 |
| | 1 | 1.6 | 1.6 | 2 | 1.0 | 1.0 | 0 | 0.0 | 0.0 | 0 | 0.4 | 0.4 | 0 | 0.0 | 0.0 |
| **Average ($x 10^{-3}$)** | | | 1.4 | | | 0.3 | | | 0.2 | | | 0.4 | | | 0.0 |
| **SD** | | | 0.2104 | | | 0.0804 | | | 0.0589 | | | 0.0346 | | | 0.0000 |
| ***Shigella spp.*** Count | | | | | | | | | | | | | | | |
| | **U1** | | | **U2** | | | **U3** | | | **U4** | | | **U5** | | |
| | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** |
| **$10^{-1}$** | 76 | 152.0 | 1.5 | 63 | 126.0 | 1.3 | 51 | 102.0 | 1.0 | 37 | 74.0 | 0.7 | 16 | 32.0 | 0.3 |
| | 66 | 132.0 | 1.3 | 46 | 92.0 | 0.9 | 38 | 76.0 | 0.8 | 28 | 56.0 | 0.6 | 9 | 18.0 | 0.2 |
| **$10^{-2}$** | 17 | 34.0 | 3.4 | 8 | 16.0 | 1.6 | 7 | 14.0 | 1.4 | 11 | 22.0 | 2.2 | 3 | 6.2 | 0.6 |
| | 13 | 26.0 | 2.6 | 5 | 10.0 | 1.0 | 5 | 10.0 | 1.0 | 7 | 14.0 | 1.4 | 1 | 2.0 | 0.2 |
| **$10^{-3}$** | 1 | 2.0 | 2.0 | 1 | 1.2 | 1.2 | 1 | 1.0 | 1.0 | 2 | 3.2 | 3.2 | 0 | 0.0 | 0.0 |
| | 1 | 2.1 | 2.1 | 0 | 0.0 | 0.0 | 0 | 0.0 | 0.0 | 1 | 2.0 | 2.0 | 0 | 0.0 | 0.0 |
| **Average ($x 10^{-3}$)** | | | 1.735 | | | 1.180 | | | 1.040 | | | 2.200 | | | 0.330 |
| **SD** | | | 0.3750 | | | 0.3038 | | | 0.2653 | | | 0.7483 | | | 0.2030 |

SD – standard deviation

cfu/ml – colony forming units per millilitre

Shaded areas highlight the values used to calculate average

**Table 2: Presumptive bacterial indicator counts for water samples collected along the Umgeni River during June-July 2011 (Winter)**

| **Total Heterotrophic Bacterial Count** | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **U1** | | | **U2** | | | **U3** | | | **U4** | | | **U5** | | |
| | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-6}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-6}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-6}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-6}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-6}$** |
| **$10^{-4}$** | 143 | 286.0 | 2.9 | 104 | 208.0 | 2.1 | 128 | 256.0 | 2.6 | 78 | 156.0 | 1.6 | 54 | 108.0 | 1.1 |
| | 123 | 246.0 | 2.5 | 119 | 238.0 | 2.4 | 154 | 308.0 | 3.1 | 109 | 218.0 | 2.2 | 76 | 152.0 | 1.5 |
| **$10^{-5}$** | 18 | 36.0 | 3.6 | 17 | 34.0 | 3.4 | 24 | 48.0 | 4.8 | 13 | 26.0 | 2.6 | 3 | 6.0 | 0.6 |
| | 16 | 32.0 | 3.2 | 15 | 30.0 | 3.0 | 19 | 38.0 | 3.8 | 16 | 32.0 | 3.2 | 2 | 4.0 | 0.4 |
| **$10^{-6}$** | 2 | 4.0 | 4.0 | 2 | 4.0 | 0.4 | 2 | 4.0 | 4.0 | 1 | 2.0 | 2.0 | 0 | 0.0 | 0.0 |
| | 3 | 6.0 | 6.0 | 0 | 0.0 | 0.0 | 1 | 2.0 | 2.0 | 0 | 0.0 | 0.0 | 0 | 0.0 | 0.0 |
| **Average (x $10^{-6}$)** | | | 3.1 | | | 2.7 | | | 3.9 | | | 2.1 | | | 0.9 |
| **SD** | | | 0.6541 | | | 1.1114 | | | 0.7073 | | | 0.4309 | | | 0.5023 |
| **Total Coliforms Count** | | | | | | | | | | | | | | | |
| | **U1** | | | **U2** | | | **U3** | | | **U4** | | | **U5** | | |
| | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** |
| **$10^{-1}$** | 87 | 174.0 | 1.7 | 123 | 246.0 | 2.5 | 67 | 134.0 | 1.3 | 100 | 200.0 | 2.0 | 11 | 22.0 | 0.2 |
| | 98 | 196.0 | 2.0 | 132 | 264.0 | 2.6 | 55 | 110.0 | 1.1 | 78 | 156.0 | 1.6 | 13 | 26.0 | 0.3 |
| **$10^{-2}$** | 15 | 30.0 | 3.0 | 19 | 37.6 | 3.8 | 10 | 20.0 | 2.0 | 16 | 32.0 | 3.2 | 1 | 1.0 | 0.1 |
| | 11 | 22.0 | 2.2 | 17 | 33.4 | 3.3 | 7 | 14.0 | 1.4 | 13 | 25.8 | 2.6 | 1 | 1.0 | 0.1 |
| **$10^{-3}$** | 2 | 4.0 | 4.0 | 3 | 6.0 | 6.0 | 2 | 4.0 | 4.0 | 2 | 4.0 | 4.0 | 1 | 1.0 | 1.0 |
| | 2 | 4.0 | 4.0 | 2 | 4.6 | 4.6 | 1 | 2.0 | 2.0 | 1 | 2.0 | 2.0 | 0 | 0.0 | 0.0 |
| **Average (x $10^{-3}$)** | | | 3.3 | | | 3.9 | | | 2.4 | | | 2.9 | | | 0.4 |
| **SD** | | | 0.8718 | | | 0.6416 | | | 1.1358 | | | 0.8572 | | | 0.0833 |
| **Faecal Coliforms Count** | | | | | | | | | | | | | | | |
| | **U1** | | | **U2** | | | **U3** | | | **U4** | | | **U5** | | |
| | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** |
| **$10^{-1}$** | 45 | 90.0 | 0.9 | 65 | 130.0 | 1.3 | 43 | 86.0 | 0.9 | 48 | 96.0 | 1.0 | 4 | 8.0 | 0.1 |
| | 33 | 66.0 | 0.7 | 78 | 156.0 | 1.6 | 76 | 152.0 | 1.5 | 34 | 68.0 | 0.7 | 6 | 12.0 | 0.1 |
| **$10^{-2}$** | 16 | 32.0 | 3.2 | 21 | 42.0 | 4.2 | 3 | 12.0 | 1.2 | 13 | 26.0 | 2.6 | 1 | 2.0 | 0.2 |
| | 11 | 22.0 | 2.2 | 15 | 30.0 | 3.0 | 3 | 6.0 | 0.6 | 12 | 23.0 | 2.3 | 1 | 2.0 | 0.2 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **$10^{-3}$** | 2 | 4.0 | 4.0 | 2 | 4.0 | 4.0 | 1 | 2.0 | 2.0 | 3 | 6.0 | 6.0 | 0 | 0.0 | 0.0 |
| | 1 | 2.0 | 2.0 | 2 | 4.0 | 4.0 | 0 | 0.0 | 0.0 | 1 | 2.0 | 2.0 | 0 | 0.0 | 0.0 |
| **Average ($x\ 10^{-3}$)** | | | 2.9 | | | 3.8 | | | 0.9 | | | 2.3 | | | 0.1 |
| **SD** | | | 0.9292 | | | 0.5416 | | | 0.3009 | | | 0.3000 | | | 0.0611 |
| **Faecal Streptococci Count** | | | | | | | | | | | | | | | |
| | **U1** | | | **U2** | | | **U3** | | | **U4** | | | **U5** | | |
| | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** |
| **$10^{-1}$** | 49 | 98.0 | 1.0 | 66 | 132.0 | 1.3 | 93 | 186.0 | 1.9 | 23 | 46.0 | 0.5 | 1 | 1.0 | 0.0 |
| | 54 | 108.0 | 1.1 | 51 | 102.0 | 1.0 | 79 | 158.0 | 1.6 | 18 | 36.0 | 0.4 | 1 | 1.0 | 0.0 |
| **$10^{-2}$** | 18 | 36.0 | 3.6 | 22 | 44.0 | 4.4 | 32 | 64.0 | 6.4 | 3 | 6.0 | 0.6 | 0 | 0.0 | 0.0 |
| | 9 | 18.0 | 1.8 | 11 | 22.0 | 2.2 | 49 | 98.0 | 9.8 | 2 | 3.0 | 0.3 | 0 | 0.6 | 0.1 |
| **$10^{-3}$** | 3 | 6.0 | 6.0 | 3 | 6.0 | 6.0 | 2 | 4.0 | 4.0 | 1 | 2.0 | 2.0 | 0 | 0.0 | 0.0 |
| | 2 | 4.0 | 4.0 | 1 | 1.0 | 1.0 | 1 | 2.0 | 2.0 | 0 | 0.0 | 0.0 | 0 | 0.0 | 0.0 |
| **Average ($x\ 10^{-3}$)** | | | 1.3 | | | 1.4 | | | 2.4 | | | 0.4 | 0 | 0.0 | 0.02 |
| **SD** | | | 0.4474 | | | 0.5627 | | | 1.1072 | | | 0.0808 | | | 0.0271 |
| **Enterococci Count** | | | | | | | | | | | | | | | |
| | **U1** | | | **U2** | | | **U3** | | | **U4** | | | **U5** | | |
| | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** |
| **$10^{-2}$** | 4 | 8.0 | 0.8 | 6 | 12.0 | 1.2 | 2 | 4.0 | 0.4 | 1 | 2.0 | 0.2 | 1 | 2.0 | 0.2 |
| | 3 | 6.0 | 0.6 | 1 | 2.0 | 0.2 | 1 | 2.0 | 0.2 | 1 | 2.0 | 0.2 | 1 | 2.0 | 0.2 |
| **$10^{-3}$** | 0 | 0.4 | 0.4 | 0 | 1.0 | 1.0 | 0 | 0.2 | 0.2 | 0 | 0.6 | 0.6 | 0 | 0.4 | 0.4 |
| | 0 | 0.0 | 0.0 | 0 | 0.1 | 0.1 | 0 | 0.0 | 0.0 | 0 | 0.2 | 0.2 | 0 | 0.0 | 0.0 |
| **Average ($x\ 10^{-3}$)** | | | 0.4 | | | 0 | | | 0.2 | | | 0.3 | | | 0.2 |
| **SD** | | | 0.4000 | | | 0.4933 | | | 0.1633 | | | 0.2000 | | | 0.1633 |
| ***Vibrio cholerae*** Count | | | | | | | | | | | | | | | |
| | **U1** | | | **U2** | | | **U3** | | | **U4** | | | **U5** | | |
| | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** |
| **$10^{-1}$** | 115 | 230.0 | 2.3 | 158 | 316.0 | 3.2 | 103 | 206.0 | 2.1 | 96 | 192.0 | 1.9 | 9 | 18.0 | 0.2 |
| | 105 | 210.0 | 2.1 | 162 | 324.0 | 3.2 | 88 | 176.0 | 1.8 | 84 | 168.0 | 1.7 | 6 | 12.0 | 0.1 |
| **$10^{-2}$** | 36 | 72.0 | 7.2 | 98 | 196.0 | 19.6 | 21 | 42.0 | 4.2 | 45 | 90.0 | 9.0 | 3 | 6.0 | 0.6 |
| | 23 | 46.0 | 4.6 | 78 | 156.0 | 15.6 | 32 | 64.0 | 6.4 | 41 | 82.0 | 8.2 | 2 | 3.2 | 0.3 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **$10^{-3}$** | 3 | 6.0 | 6.0 | 7 | 14.0 | 14.0 | 3 | 6.0 | 6.0 | 2 | 4.0 | 4.0 | 0 | 0.0 | 0.0 |
| | 1 | 2.0 | 2.0 | 2 | 4.0 | 4.0 | 1 | 1.4 | 1.4 | 1 | 2.0 | 2.0 | 0 | 0.0 | 0.0 |
| **Average ($x\ 10^{-3}$)** | | | 2.1 | | | 3.5 | | | 1.7 | | | 1.9 | | | 0.2 |
| **SD** | | | 0.1528 | | | 0.5374 | | | 0.3305 | | | 0.1665 | | | 0.1330 |
| ***Salmonella spp.*** Count | | | | | | | | | | | | | | | |
| | **U1** | | | **U2** | | | **U3** | | | **U4** | | | **U5** | | |
| | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** |
| **$10^{-1}$** | 37 | 74.0 | 0.7 | 31 | 62.0 | 0.6 | 12 | 24.0 | 0.2 | 15 | 30.0 | 0.3 | 1 | 2.0 | 0.0 |
| | 21 | 42.0 | 0.4 | 29 | 58.0 | 0.6 | 8 | 16.0 | 0.2 | 12 | 24.0 | 0.2 | 0 | 0.0 | 0.0 |
| **$10^{-2}$** | 9 | 18.0 | 1.8 | 8 | 16.0 | 1.6 | 2 | 4.0 | 0.4 | 11 | 22.0 | 2.2 | 0 | 0.2 | 0.0 |
| | 4 | 7.2 | 0.7 | 6 | 11.4 | 1.1 | 1 | 2.4 | 0.2 | 8 | 16.0 | 1.6 | 0 | 0.0 | 0.0 |
| **$10^{-3}$** | 1 | 2.0 | 2.0 | 1 | 2.0 | 2.0 | 1 | 2.0 | 2.0 | 1 | 1.2 | 1.2 | 0 | 0.0 | 0.0 |
| | 0 | 0.0 | 0.0 | 0 | 0.4 | 0.4 | 0 | 0.0 | 0.0 | 0 | 0.0 | 0.0 | 0 | 0.0 | 0.0 |
| **Average ($x\ 10^{-3}$)** | | | 0.6 | | | 0.7 | | | 0.2 | | | 0.6 | | | 0.013 |
| **SD** | | | 0.1793 | | | 0.3181 | | | 0.0462 | | | 0.5262 | | | 0.0115 |
| ***Shigella spp.*** Count | | | | | | | | | | | | | | | |
| | **U1** | | | **U2** | | | **U3** | | | **U4** | | | **U5** | | |
| | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** |
| **$10^{-1}$** | 54 | 108.0 | 1.1 | 66 | 132.0 | 1.3 | 16 | 32.8 | 0.3 | 54 | 108.0 | 1.1 | 3 | 5.0 | 0.1 |
| | 36 | 72.0 | 0.7 | 59 | 118.0 | 1.2 | 12 | 23.0 | 0.2 | 48 | 96.0 | 1.0 | 2 | 3.2 | 0.0 |
| **$10^{-2}$** | 14 | 28.0 | 2.8 | 22 | 44.0 | 4.4 | 3 | 6.0 | 0.6 | 14 | 28.0 | 2.8 | 1 | 1.0 | 0.1 |
| | 9 | 18.0 | 1.8 | 17 | 34.0 | 3.4 | 1 | 2.0 | 0.2 | 9 | 18.6 | 1.9 | 0 | 0.0 | 0.0 |
| **$10^{-3}$** | 2 | 3.0 | 3.0 | 1 | 2.0 | 2.0 | 0 | 0.0 | 0.0 | 3 | 6.0 | 6.0 | 0 | 0.0 | 0.0 |
| | 4 | 8.0 | 8.0 | 2 | 3.0 | 3.0 | 0 | 0.0 | 0.0 | 1 | 2.0 | 2.0 | 0 | 0.0 | 0.0 |
| **Average ($x\ 10^{-3}$)** | | | 1.7 | | | 1.9 | | | 0.3 | | | 1.3 | | | 0.061 |
| **SD** | | | 0.5499 | | | 0.8311 | | | 0.2828 | | | 0.4887 | | | 0.0352 |

SD – standard deviation
cfu/ml – colony forming units per milliliter
Shaded areas highlight the values used to calculate average

**Table 3: Presumptive bacterial indicator counts for water samples collected along the Umgeni River during September -October 2011 (Spring)**

| **Total Heterotrophic Bacterial Count** | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **U1** | | | **U2** | | | **U3** | | | **U4** | | | **U5** | | |
| | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-6}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-6}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-6}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-6}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-6}$** |
| **$10^{-4}$** | 178 | 356.0 | 3.6 | 147 | 294.0 | 2.9 | 134 | 268.0 | 2.7 | 156 | 312.0 | 3.1 | 125 | 250.0 | 2.5 |
| | 197 | 394.0 | 3.9 | 187 | 374.0 | 3.7 | 146 | 292.0 | 2.9 | 148 | 296.0 | 3.0 | 109 | 218.0 | 2.2 |
| **$10^{-5}$** | 55 | 110.0 | 11.0 | 49 | 98.0 | 9.8 | 29 | 58.0 | 5.8 | 21 | 42.0 | 4.2 | 17 | 34.0 | 3.4 |
| | 38 | 76.0 | 7.6 | 42 | 84.0 | 8.4 | 33 | 66.0 | 6.6 | 19 | 38.0 | 3.8 | 15 | 30.0 | 3.0 |
| **$10^{-6}$** | 5 | 10.0 | 10.0 | 3 | 6.0 | 6.0 | 3 | 6.0 | 6.0 | 1 | 2.0 | 2.0 | 1 | 2.0 | 2.0 |
| | 4 | 8.0 | 8.0 | 4 | 8.0 | 8.0 | 1 | 2.0 | 2.0 | 1 | 2.0 | 2.0 | 2 | 4.0 | 4.0 |
| **Average (x $10^{-6}$)** | | | 8.9 | | | 8.7 | | | 6.1 | | | 3.3 | | | 2.8 |
| **SD** | | | 1.9 | | | 0.9 | | | 0.4 | | | 1.2 | | | 0.7 |
| **Total Coliforms Count** | | | | | | | | | | | | | | | |
| | **U1** | | | **U2** | | | **U3** | | | **U4** | | | **U5** | | |
| | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** |
| **$10^{-1}$** | 201 | 402.0 | 4.0 | 209 | 418.0 | 4.2 | 122 | 244.0 | 2.4 | 113 | 226.0 | 2.3 | 44 | 88.0 | 0.9 |
| | 196 | 392.0 | 3.9 | 187 | 374.0 | 3.7 | 145 | 290.0 | 2.9 | 102 | 204.0 | 2.0 | 45 | 90.0 | 0.9 |
| **$10^{-2}$** | 31 | 62.0 | 6.2 | 59 | 118.0 | 11.8 | 15 | 30.0 | 3.0 | 46 | 92.0 | 9.2 | 51 | 102.0 | 10.2 |
| | 27 | 53.2 | 5.3 | 36 | 71.2 | 7.1 | 14 | 28.6 | 2.9 | 43 | 86.0 | 8.6 | 48 | 96.0 | 9.6 |
| **$10^{-3}$** | 4 | 8.0 | 8.0 | 11 | 22.0 | 22.0 | 6 | 12.0 | 12.0 | 3 | 6.0 | 6.0 | 4 | 8.0 | 8.0 |
| | 3 | 6.8 | 6.8 | 9 | 18.0 | 18.0 | 3 | 6.0 | 6.0 | 1 | 1.0 | 1.0 | 1 | 1.0 | 1.0 |
| **Average (x $10^{-3}$)** | | | 4.9 | | | 5.0 | | | 2.9 | | | 1.8 | | | 0.9 |
| **SD** | | | 1.1 | | | 1.8 | | | 0.1 | | | 0.7 | | | 0.1 |
| **Faecal Coliforms Count** | | | | | | | | | | | | | | | |
| | **U1** | | | **U2** | | | **U3** | | | **U4** | | | **U5** | | |
| | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** |
| **$10^{-1}$** | 189 | 378.0 | 3.8 | 201 | 402.0 | 4.0 | 39 | 78.0 | 0.8 | 111 | 222.0 | 2.2 | 25 | 50.0 | 0.5 |
| | 178 | 356.0 | 3.6 | 195 | 390.0 | 3.9 | 33 | 66.0 | 0.7 | 134 | 268.0 | 2.7 | 23 | 46.0 | 0.5 |
| **$10^{-2}$** | 98 | 196.0 | 19.6 | 76 | 152.0 | 15.2 | 11 | 22.0 | 2.2 | 21 | 42.0 | 4.2 | 6 | 12.0 | 1.2 |
| | 101 | 202.0 | 20.2 | 83 | 166.0 | 16.6 | 7 | 14.0 | 1.4 | 20 | 39.6 | 4.0 | 3 | 6.8 | 0.7 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **$10^{-3}$** | 5 | 10.0 | 10.0 | 3 | 6.0 | 6.0 | 3 | 6.0 | 6.0 | 11 | 22.0 | 22.0 | 1 | 2.0 | 2.0 |
| | 3 | 5.7 | 5.7 | 3 | 5.5 | 5.5 | 1 | 1.3 | 1.3 | 4 | 8.0 | 8.0 | 0 | 0.0 | 0.0 |
| **Average ($x\ 10^{-3}$)** | | | 4.3 | | | 4.8 | | | 0.9 | | | 3.3 | | | 0.7 |
| **SD** | | | 1.2 | | | 1.0 | | | 0.4 | | | 1.0 | | | 0.3 |
| **Faecal Streptococci Count** | | | | | | | | | | | | | | | |
| | **U1** | | | **U2** | | | **U3** | | | **U4** | | | **U5** | | |
| | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** |
| **$10^{-1}$** | 55 | 110.0 | 1.1 | 78 | 156.0 | 1.6 | 15 | 30.0 | 0.3 | 6 | 12.0 | 0.1 | 1 | 2.0 | 0.0 |
| | 67 | 134.0 | 1.3 | 53 | 106.0 | 1.1 | 16 | 32.0 | 0.3 | 2 | 4.0 | 0.0 | 1 | 1.0 | 0.0 |
| **$10^{-2}$** | 4 | 8.0 | 0.8 | 6 | 12.0 | 1.2 | 2 | 3.4 | 0.3 | 0 | 0.4 | 0.0 | 0 | 0.2 | 0.0 |
| | 3 | 6.0 | 0.6 | 4 | 8.0 | 0.8 | 2 | 4.2 | 0.4 | 0 | 0.2 | 0.0 | 0 | 0.4 | 0.0 |
| **$10^{-3}$** | 1 | 1.8 | 1.8 | 1 | 1.6 | 1.6 | 0 | 0.2 | 0.2 | 0 | 0.0 | 0.0 | 0 | 0.0 | 0.0 |
| | 1 | 1.2 | 1.2 | 1 | 1.3 | 1.3 | 0 | 0.6 | 0.6 | 0 | 0.0 | 0.0 | 0 | 0.0 | 0.0 |
| **Average ($x\ 10^{-3}$)** | | | 0.9 | | | 1.2 | | | 0.4 | | | 0.0 | 0 | 0.0 | 0.0 |
| **SD** | | | 0.3055 | | | 0.3304 | | | 0.1669 | | | 0.0115 | | | 0.0126 |
| **Enterococci Count** | | | | | | | | | | | | | | | |
| | **U1** | | | **U2** | | | **U3** | | | **U4** | | | **U5** | | |
| | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** |
| **$10^{-2}$** | 6 | 12.0 | 1.2 | 10 | 20.0 | 2.0 | 7 | 14.6 | 1.5 | 7 | 14.0 | 1.4 | 1 | 2.8 | 0.3 |
| | 4 | 8.6 | 0.9 | 6 | 12.0 | 1.2 | 5 | 10.0 | 1.0 | 5 | 10.0 | 1.0 | 2 | 4.0 | 0.4 |
| **$10^{-3}$** | 1 | 1.8 | 1.8 | 1 | 1.2 | 1.2 | 1 | 2.0 | 2.0 | 1 | 1.6 | 1.6 | 0 | 0.2 | 0.2 |
| | 1 | 1.0 | 1.0 | 1 | 1.8 | 1.8 | 1 | 1.0 | 1.0 | 0 | 0.0 | 0.0 | 0 | 0.0 | 0.0 |
| **Average ($x\ 10^{-3}$)** | | | 1.2 | | | 1.4 | | | 1.2 | | | 1.3 | | | 0.3 |
| **SD** | | | 0.5657 | | | 0.3464 | | | 0.3253 | | | 0.2884 | | | 0.1007 |
| ***Vibrio cholerae*** Count | | | | | | | | | | | | | | | |
| | **U1** | | | **U2** | | | **U3** | | | **U4** | | | **U5** | | |
| | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** |
| **$10^{-1}$** | 128 | 256.0 | 2.6 | 134 | 268.0 | 2.7 | 123 | 246.0 | 2.5 | 127 | 254.0 | 2.5 | 14 | 28.0 | 0.3 |
| | 134 | 268.0 | 2.7 | 165 | 330.0 | 3.3 | 124 | 248.0 | 2.5 | 120 | 240.0 | 2.4 | 12 | 24.0 | 0.2 |
| **$10^{-2}$** | 17 | 33.4 | 3.3 | 20 | 40.2 | 4.0 | 12 | 24.6 | 2.5 | 12 | 24.0 | 2.4 | 1 | 2.8 | 0.3 |
| | 15 | 30.8 | 3.1 | 19 | 38.6 | 3.9 | 11 | 22.2 | 2.2 | 10 | 20.6 | 2.1 | 1 | 2.8 | 0.3 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **$10^{-3}$** | 3 | 5.2 | 5.2 | 3 | 6.2 | 6.2 | 2 | 3.2 | 3.2 | 1 | 2.0 | 2.0 | 0 | 0.2 | 0.2 |
| | 2 | 4.4 | 4.4 | 3 | 5.8 | 5.8 | 1 | 2.4 | 2.4 | 1 | 2.0 | 2.0 | 0 | 0.0 | 0.0 |
| **Average ($x\ 10^{-3}$)** | | | 4.0 | | | 5.0 | | | 2.6 | | | 2.1 | | | 0.3 |
| **SD** | | | 0.9801 | | | 1.2023 | | | 0.1237 | | | 0.1921 | | | 0.0197 |
| ***Salmonella spp.*** Count | | | | | | | | | | | | | | | |
| | **U1** | | | **U2** | | | **U3** | | | **U4** | | | **U5** | | |
| | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** |
| **$10^{-1}$** | 63 | 126.0 | 1.3 | 21 | 42.0 | 0.4 | 11 | 22.0 | 0.2 | 31 | 62.0 | 0.6 | 2 | 4.0 | 0.0 |
| | 54 | 108.0 | 1.1 | 18 | 36.0 | 0.4 | 7 | 13.0 | 0.1 | 24 | 48.0 | 0.5 | 1 | 2.0 | 0.0 |
| **$10^{-2}$** | 9 | 18.0 | 1.8 | 4 | 8.0 | 0.8 | 1 | 2.0 | 0.2 | 3 | 6.0 | 0.6 | 0 | 0.2 | 0.0 |
| | 8 | 15.6 | 1.6 | 3 | 6.0 | 0.6 | 1 | 2.0 | 0.2 | 7 | 14.0 | 1.4 | 0 | 0.0 | 0.0 |
| **$10^{-3}$** | 1 | 1.0 | 1.0 | 0 | 0.9 | 0.9 | 0 | 0.0 | 0.0 | 1 | 2.0 | 2.0 | 0 | 0.0 | 0.0 |
| | 0 | 0.0 | 0.0 | 0 | 0.0 | 0.0 | 0 | 0.0 | 0.0 | 0 | 0.0 | 0.0 | 0 | 0.0 | 0.0 |
| **Average ($x\ 10^{-3}$)** | | | 1.4 | | | 0.5 | | | 0.2 | | | 0.6 | | | 0.0 |
| **SD** | | | 0.3189 | | | 0.2684 | | | 0.0473 | | | 0.0757 | | | 0.0115 |
| ***Shigella spp.*** Count | | | | | | | | | | | | | | | |
| | **U1** | | | **U2** | | | **U3** | | | **U4** | | | **U5** | | |
| | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** |
| **$10^{-1}$** | 31 | 62.0 | 0.6 | 27 | 54.0 | 0.5 | 15 | 30.0 | 0.3 | 15 | 30.0 | 0.3 | 4 | 7.0 | 0.1 |
| | 24 | 48.0 | 0.5 | 24 | 48.0 | 0.5 | 12 | 24.0 | 0.2 | 13 | 26.0 | 0.3 | 4 | 8.0 | 0.1 |
| **$10^{-2}$** | 10 | 20.2 | 2.0 | 1 | 2.0 | 0.2 | 1 | 2.0 | 0.2 | 2 | 4.0 | 0.4 | 0 | 0.0 | 0.0 |
| | 9 | 17.6 | 1.8 | 1 | 2.0 | 0.2 | 2 | 4.0 | 0.4 | 1 | 2.0 | 0.2 | 0 | 0.0 | 0.0 |
| **$10^{-3}$** | 1 | 2.2 | 2.2 | 0 | 0.4 | 0.4 | 0 | 0.0 | 0.0 | 0 | 0.0 | 0.0 | 0 | 0.0 | 0.0 |
| | 0 | 0.2 | 0.2 | 0 | 0.0 | 0.0 | 0 | 0.0 | 0.0 | 0 | 0.0 | 0.0 | 0 | 0.0 | 0.0 |
| **Average ($x\ 10^{-3}$)** | | | 0.4 | | | 0.3 | | | 0.2 | | | 0.3 | | | 0.1 |
| **SD** | | | 0.2117 | | | 0.1039 | | | 0.0503 | | | 0.0721 | | | 0.0435 |

SD – standard deviation

cfu/ml – colony forming units per milliliter

Shaded areas highlight the values used to calculate average

**Table 4: Presumptive bacterial indicator counts for water samples collected along the Umgeni River during December 2011-January 2012 (Summer)**

| **Total Heterotrophic Bacterial Count** | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **U1** | | | **U2** | | | **U3** | | | **U4** | | | **U5** | | |
| | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-6}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-6}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-6}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-6}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-6}$** |
| **$10^{-4}$** | 156 | 312.0 | 3.1 | 189 | 378.0 | 3.8 | 125 | 250.0 | 2.5 | 127 | 254.0 | 2.5 | 56 | 112.0 | 1.1 |
| | 172 | 344.0 | 3.4 | 198 | 396.0 | 4.0 | 153 | 306.0 | 3.1 | 132 | 264.0 | 2.6 | 74 | 148.0 | 1.5 |
| **$10^{-5}$** | 84 | 168.0 | 16.8 | 63 | 126.0 | 12.6 | 41 | 82.0 | 8.2 | 46 | 92.0 | 9.2 | 16 | 32.0 | 3.2 |
| | 71 | 142.0 | 14.2 | 59 | 118.0 | 11.8 | 28 | 56.0 | 5.6 | 37 | 74.0 | 7.4 | 11 | 22.0 | 2.2 |
| **$10^{-6}$** | 5 | 10.0 | 10.0 | 12 | 24.0 | 24.0 | 2 | 4.0 | 4.0 | 3 | 6.0 | 6.0 | 1 | 2.0 | 2.0 |
| | 2 | 4.0 | 4.0 | 6 | 12.0 | 12.0 | 0 | 0.0 | 0.0 | 1 | 2.0 | 2.0 | 0 | 0.0 | 0.0 |
| **Average (x $10^{-6}$)** | | | 13.7 | | | 12.1 | | | 5.9 | 0 | | 7.5 | | | 2.5 |
| **SD** | | | 3.4312 | | | 0.4163 | | | 2.1197 | | | 1.6042 | | | 0.6429 |
| **Total Coliforms Count** | | | | | | | | | | | | | | | |
| | **U1** | | | **U2** | | | **U3** | | | **U4** | | | **U5** | | |
| | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** |
| **$10^{-1}$** | 135 | 270.0 | 2.7 | 198 | 396.0 | 4.0 | 154 | 308.0 | 3.1 | 146 | 292.0 | 2.9 | 165 | 330.0 | 3.3 |
| | 140 | 280.0 | 2.8 | 185 | 370.0 | 3.7 | 167 | 334.0 | 3.3 | 155 | 310.0 | 3.1 | 138 | 276.0 | 2.8 |
| **$10^{-2}$** | 27 | 54.2 | 5.4 | 22 | 44.6 | 4.5 | 14 | 28.4 | 2.8 | 22 | 44.2 | 4.4 | 10 | 19.6 | 2.0 |
| | 27 | 54.8 | 5.5 | 21 | 42.2 | 4.2 | 12 | 24.8 | 2.5 | 21 | 41.8 | 4.2 | 9 | 17.2 | 1.7 |
| **$10^{-3}$** | 6 | 11.4 | 11.4 | 6 | 11.2 | 11.2 | 2 | 3.0 | 3.0 | 2 | 3.2 | 3.2 | 1 | 1.6 | 1.6 |
| | 4 | 7.2 | 7.2 | 3 | 6.8 | 6.8 | 2 | 4.0 | 4.0 | 3 | 6.0 | 6.0 | 2 | 4.0 | 4.0 |
| **Average (x $10^{-3}$)** | | | 6.0 | | | 5.2 | | | 2.8 | | | 3.9 | | | 1.8 |
| **SD** | | | 1.0108 | | | 1.4253 | | | 0.2663 | | | 0.6463 | | | 0.6742 |
| **Faecal Coliforms Count** | | | | | | | | | | | | | | | |
| | **U1** | | | **U2** | | | **U3** | | | **U4** | | | **U5** | | |
| | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** |
| **$10^{-1}$** | 111 | 222.0 | 2.2 | 125 | 250.0 | 2.5 | 157 | 314.0 | 3.1 | 123 | 246.0 | 2.5 | 86 | 172.0 | 1.7 |
| | 123 | 246.0 | 2.5 | 143 | 286.0 | 2.9 | 143 | 286.0 | 2.9 | 154 | 308.0 | 3.1 | 102 | 204.0 | 2.0 |
| **$10^{-2}$** | 18 | 35.6 | 3.6 | 14 | 28.0 | 2.8 | 8 | 15.8 | 1.6 | 10 | 19.8 | 2.0 | 8 | 15.2 | 1.5 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 12 | 24.8 | 2.5 | 16 | 32.0 | 3.2 | 7 | 13.0 | 1.3 | 8 | 15.2 | 1.5 | 5 | 9.8 | 1.0 |
| **$10^{-3}$** | 2 | 3.6 | 3.6 | 1 | 2.0 | 2.0 | 1 | 1.6 | 1.6 | 2 | 3.0 | 3.0 | 1 | 1.6 | 1.6 |
| | 2 | 4.0 | 4.0 | 7 | 14.0 | 14.0 | 2 | 4.0 | 4.0 | 3 | 6.0 | 6.0 | 0 | 0.6 | 0.6 |
| **Average ($x\ 10^{-3}$)** | | | 3.2 | | | 2.7 | | | 1.5 | | | 2.2 | | | 1.2 |
| **SD** | | | 0.6354 | | | 0.6110 | | | 0.1677 | | | 0.7575 | | | 0.4720 |
| **Faecal Streptococci Count** | | | | | | | | | | | | | | | |
| | **U1** | | | **U2** | | | **U3** | | | **U4** | | | **U5** | | |
| | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** |
| **$10^{-1}$** | 21 | 42.0 | 0.4 | 37 | 74.0 | 0.7 | 21 | 42.0 | 0.4 | 8 | 16.0 | 0.2 | 2 | 4.4 | 0.0 |
| | 19 | 38.0 | 0.4 | 33 | 66.0 | 0.7 | 16 | 32.0 | 0.3 | 5 | 10.0 | 0.1 | 2 | 3.6 | 0.0 |
| **$10^{-2}$** | 4 | 8.8 | 0.9 | 5 | 10.0 | 1.0 | 2 | 4.0 | 0.4 | 1 | 1.4 | 0.1 | 0 | 0.8 | 0.1 |
| | 6 | 11.6 | 1.2 | 6 | 11.2 | 1.1 | 2 | 4.0 | 0.4 | 1 | 1.4 | 0.1 | 1 | 1.0 | 0.1 |
| **$10^{-3}$** | 3 | 6.0 | 6.0 | 1 | 1.0 | 1.0 | 0 | 0.8 | 0.8 | 0 | 0.0 | 0.0 | 0 | 0.0 | 0.0 |
| | 1 | 1.4 | 1.4 | 3 | 6.0 | 6.0 | 0 | 0.0 | 0.0 | 0 | 0.0 | 0.0 | 0 | 0.0 | 0.0 |
| **Average ($x\ 10^{-3}$)** | | | 1.1 | | | 1.0 | | | 0.5 | | | 0.1 | | | 0.1 |
| **SD** | | | 0.2603 | | | 0.0693 | | | 0.2309 | | | 0.0306 | | | 0.0302 |
| **Enterococci Count** | | | | | | | | | | | | | | | |
| | **U1** | | | **U2** | | | **U3** | | | **U4** | | | **U5** | | |
| | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** |
| **$10^{-2}$** | 9 | 18.0 | 1.8 | 9 | 18.0 | 1.8 | 13 | 26.0 | 2.6 | 12 | 24.0 | 2.4 | 3 | 6.0 | 0.6 |
| | 11 | 22.0 | 2.2 | 4 | 8.0 | 0.8 | 11 | 22.0 | 2.2 | 7 | 14.0 | 1.4 | 7 | 14.0 | 1.4 |
| **$10^{-3}$** | 0 | 0.8 | 0.8 | 1 | 1.5 | 1.5 | 1 | 1.0 | 1.0 | 1 | 1.0 | 1.0 | 0 | 0.1 | 0.1 |
| | 0 | 0.4 | 0.4 | 1 | 1.3 | 1.3 | 0 | 0.8 | 0.8 | 0 | 0.4 | 0.4 | 0 | 0.2 | 0.2 |
| **Average ($x\ 10^{-3}$)** | | | 1.0 | | | 1.2 | | | 0.9 | | | 0.9 | | | 0.3 |
| **SD** | | | 0.7211 | | | 0.3747 | | | 0.1414 | | | 0.5033 | | | 0.2646 |
| ***Vibrio cholerae* Count** | | | | | | | | | | | | | | | |
| | **U1** | | | **U2** | | | **U3** | | | **U4** | | | **U5** | | |
| | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** |
| **$10^{-1}$** | 135 | 270.0 | 2.7 | 145 | 290.0 | 2.9 | 92 | 184.0 | 1.8 | 114 | 228.0 | 2.3 | 33 | 66.0 | 0.7 |
| | 142 | 284.0 | 2.8 | 157 | 314.0 | 3.1 | 98 | 196.0 | 2.0 | 123 | 246.0 | 2.5 | 24 | 48.0 | 0.5 |
| **$10^{-2}$** | 22 | 44.2 | 4.4 | 21 | 42.2 | 4.2 | 9 | 18.0 | 1.8 | 14 | 28.0 | 2.8 | 1 | 2.0 | 0.2 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 17 | 33.4 | 3.3 | 19 | 38.8 | 3.9 | 7 | 14.0 | 1.4 | 10 | 20.0 | 2.0 | 1 | 2.0 | 0.2 |
| **$10^{-3}$** | 3 | 6.4 | 6.4 | 3 | 5.6 | 5.6 | 2 | 4.0 | 4.0 | 2 | 4.0 | 4.0 | 0 | 0.4 | 0.4 |
| | 2 | 4.2 | 4.2 | 2 | 3.8 | 3.8 | 1 | 2.0 | 2.0 | 1 | 2.0 | 2.0 | 0 | 0.0 | 0.0 |
| **Average ($x\ 10^{-3}$)** | | | 4.6 | | | 4.4 | | | 1.8 | | | 2.7 | | | 0.3 |
| **SD** | | | 1.2935 | | | 0.8367 | | | 0.2430 | | | 0.9452 | | | 0.1424 |
| ***Salmonella spp.*Count** | | | | | | | | | | | | | | | |
| | **U1** | | | **U2** | | | **U3** | | | **U4** | | | **U5** | | |
| | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** |
| **$10^{-1}$** | 76 | 152.0 | 1.5 | 65 | 130.0 | 1.3 | 18 | 36.0 | 0.4 | 54 | 108.0 | 1.1 | 4 | 8.0 | 0.1 |
| | 75 | 150.0 | 1.5 | 67 | 134.0 | 1.3 | 13 | 26.0 | 0.3 | 51 | 102.0 | 1.0 | 3 | 6.0 | 0.1 |
| **$10^{-2}$** | 6 | 12.0 | 1.2 | 4 | 8.0 | 0.8 | 1 | 2.2 | 0.2 | 5 | 10.0 | 1.0 | 0 | 0.8 | 0.1 |
| | 4 | 8.0 | 0.8 | 3 | 6.0 | 0.6 | 2 | 4.0 | 0.4 | 2 | 4.0 | 0.4 | 0 | 0.0 | 0.0 |
| **$10^{-3}$** | 1 | 2.0 | 2.0 | 0 | 0.9 | 0.9 | 0 | 0.2 | 0.2 | 1 | 1.0 | 1.0 | 0 | 0.0 | 0.0 |
| | 1 | 2.0 | 2.0 | 0 | 0.6 | 0.6 | 0 | 0.2 | 0.2 | 1 | 1.0 | 1.0 | 0 | 0.0 | 0.0 |
| **Average ($x\ 10^{-3}$)** | | | 1.5 | | | 0.7 | | | 0.2 | | | 0.8 | | | 0.1 |
| **SD** | | | 0.6000 | | | 0.1500 | | | 0.0115 | | | 0.3464 | | | 0.0115 |
| ***Shigella spp.* Count** | | | | | | | | | | | | | | | |
| | **U1** | | | **U2** | | | **U3** | | | **U4** | | | **U5** | | |
| | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** | **COUNT** | **cfu/100ml** | **(cfu/100ml) x $10^{-3}$** |
| **$10^{-1}$** | 45 | 90.0 | 0.9 | 41 | 82.0 | 0.8 | 11 | 22.0 | 0.2 | 19 | 38.0 | 0.4 | 11 | 22.0 | 0.2 |
| | 39 | 78.0 | 0.8 | 38 | 76.0 | 0.8 | 17 | 34.0 | 0.3 | 18 | 36.0 | 0.4 | 9 | 18.0 | 0.2 |
| **$10^{-2}$** | 7 | 14.4 | 1.4 | 11 | 22.2 | 2.2 | 1 | 2.0 | 0.2 | 7 | 14.0 | 1.4 | 1 | 2.0 | 0.2 |
| | 5 | 10.8 | 1.1 | 9 | 17.0 | 1.7 | 1 | 2.0 | 0.2 | 4 | 8.0 | 0.8 | 1 | 2.0 | 0.2 |
| **$10^{-3}$** | 1 | 2.4 | 2.4 | 1 | 2.2 | 2.2 | 0 | 0.0 | 0.0 | 1 | 2.0 | 2.0 | 0 | 0.2 | 0.2 |
| | 0 | 0.0 | 0.0 | 1 | 1.2 | 1.2 | 0 | 0.0 | 0.0 | 0 | 0.0 | 0.0 | 1 | 2.0 | 2.0 |
| **Average ($x\ 10^{-3}$)** | | | 1.2 | | | 1.8 | | | 0.2 | | | 1.4 | | | 0.2 |
| **SD** | | | 0.9914 | | | 0.4840 | | | 0.0673 | | | 0.6000 | | | 0.0000 |

SD – standard deviation

cfu/ml – colony forming units per millilitre

Shaded areas highlight the values used to calculate average

**Table 5: Somatic phage plaque count for water samples collected along the Umgeni River**

| **Plaque Forming Units Count - Autumn** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | U1 | | U2 | | U3 | | U4 | | U5 | |
| | COUNT | pfu/ml | COUNT | pfu/ml | COUNT | pfu/ml | COUNT | pfu/ml | COUNT | pfu/ml |
| **0** | 233 | 2.3 | 145.0 | 1.5 | 115.0 | 1.2 | 53.0 | 0.5 | 19.0 | 0.2 |
| | 251 | 2.5 | 121.0 | 1.2 | 138.0 | 1.4 | 46.0 | 0.5 | 10.0 | 0.1 |
| **10** | 64 | 6.4 | 48.0 | 4.8 | 59.0 | 5.9 | 6.0 | 0.6 | 1.0 | 0.1 |
| | 55 | 5.5 | 36.0 | 3.6 | 35.0 | 3.5 | 2.0 | 0.2 | 1.0 | 0.1 |
| **100** | 4 | 4.0 | 2.0 | 2.0 | 3.0 | 3.0 | 1.0 | 1.0 | 0.0 | 0.0 |
| | 1 | 1.0 | 0.0 | 0.0 | 1.0 | 1.0 | 0.0 | 0.0 | | 0.0 |
| Average ($x\ 10^{-2}$) | | 5.3 | | 3.5 | | 4.1 | | 0.6 | | 0.1 |
| SD | | 1.21244 | | 1.40475 | | 1.55027 | | 0.40000 | | 0.00000 |
| **Plaque Forming Units Count - Winter** | | | | | | | | | | |
| | U1 | | U2 | | U3 | | U4 | | U5 | |
| | COUNT | pfu/ml | COUNT | pfu/ml | COUNT | pfu/ml | COUNT | pfu/ml | COUNT | pfu/ml |
| **0** | 281 | 2.8 | 156.0 | 1.6 | 208.0 | 2.1 | 143.0 | 1.4 | 27.0 | 0.3 |
| | 236 | 2.4 | 176.0 | 1.8 | 132.0 | 1.3 | 120.0 | 1.2 | 17.0 | 0.2 |
| **10** | 42 | 4.2 | 34.0 | 3.4 | 37.0 | 3.7 | 54.0 | 5.4 | 7.0 | 0.7 |
| | 33 | 3.3 | 26.0 | 2.6 | 29.0 | 2.9 | 32.0 | 3.2 | 4.0 | 0.4 |
| **100** | 1 | 1.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 0.0 | 0.0 |
| | 0 | 0.0 | 1.0 | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Average ($x\ 10^{-2}$) | | 3.4 | | 2.7 | | 2.9 | | 1.5 | | 0.3 |
| SD | | 0.70501 | | 0.70238 | | 0.81002 | | 0.41187 | | 0.11533 |
| **Plaque Forming Units Count - Spring** | | | | | | | | | | |
| | U1 | | U2 | | U3 | | U4 | | U5 | |
| | COUNT | pfu/ml | COUNT | pfu/ml | COUNT | pfu/ml | COUNT | pfu/ml | COUNT | pfu/ml |
| **0** | 376 | 3.8 | 398.0 | 4.0 | 212.0 | 2.1 | 198.0 | 2.0 | 111.0 | 1.1 |
| | 331 | 3.3 | 365.0 | 3.7 | 235.0 | 2.4 | 184.0 | 1.8 | 85.0 | 0.9 |
| **1** | 124 | 12.4 | 132.0 | 13.2 | 64.0 | 6.4 | 36.0 | 3.6 | 22.0 | 2.2 |
| | 57 | 5.7 | 77.0 | 7.7 | 48.0 | 4.8 | 24.0 | 2.4 | 14.0 | 1.4 |
| **2** | 6 | 6.0 | 6.0 | 6.0 | 4.0 | 4.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| | 1 | 1.0 | 2.0 | 2.0 | 1.0 | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Average ($x\ 10^{-2}$) | | 5.2 | | 5.9 | | 5.1 | | 2.7 | | 1.1 |
| SD | | 1.21595 | | 1.86229 | | 1.22202 | | 0.84071 | | 0.28431 |
| **Plaque Forming Units Count - Summer** | | | | | | | | | | |
| | U1 | | U2 | | U3 | | U4 | | U5 | |
| | COUNT | pfu/ml | COUNT | pfu/ml | COUNT | pfu/ml | COUNT | pfu/ml | COUNT | pfu/ml |
| **0** | 387 | 3.9 | 332.0 | 3.3 | 298.0 | 3.0 | 288.0 | 2.9 | 167.0 | 1.7 |
| | 338 | 3.4 | 276.0 | 2.8 | 276.0 | 2.8 | 264.0 | 2.6 | 132.0 | 1.3 |
| **10** | 103 | 10.3 | 91.0 | 9.1 | 52.0 | 5.2 | 55.0 | 5.5 | 26.0 | 2.6 |
| | 79 | 7.9 | 65.0 | 6.5 | 49.0 | 4.9 | 39.0 | 3.9 | 19.0 | 1.9 |
| **100** | 14 | 14.0 | 4.0 | 4.0 | 11.0 | 11.0 | 9.0 | 9.0 | 2.0 | 2.0 |
| | 8 | 8.0 | 1.0 | 1.0 | 6.0 | 6.0 | 4.0 | 4.0 | 0.0 | 0.0 |
| Average ($x\ 10^{-2}$) | | 6.6 | | 6.5 | | 5.4 | | 4.5 | | 2.2 |
| SD | | 2.35612 | | 2.55016 | | 0.56862 | | 0.89629 | | 0.37859 |

SD – standard deviation

pfu/ml – plaque forming units per millilitre

Shaded areas highlight the values used to calculate average

**Table 6: F-RNA phage plaque count for water samples collected along the Umgeni River**

| **Plaque Forming Units Count - Autumn** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | U1 | | U2 | | U3 | | U4 | | U5 | |
| | COUNT | pfu/ml | COUNT | pfu/ml | COUNT | pfu/ml | COUNT | pfu/ml | COUNT | pfu/ml |
| **0** | 221 | 2.2 | 126.0 | 1.3 | 192.0 | 1.9 | 15.0 | 0.2 | 0.0 | 0.0 |
| | 194 | 1.9 | 97.0 | 1.0 | 95.0 | 1.0 | 3.0 | 0.0 | 0.0 | 0.0 |
| **10** | 64 | 6.4 | 32.0 | 3.2 | 48.0 | 4.8 | 2.0 | 0.2 | 0.0 | 0.0 |
| | 50 | 5.0 | 19.0 | 1.9 | 27.0 | 2.7 | 1.0 | 0.1 | 0.0 | 0.0 |
| **100** | 4 | 4.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 0.0 | 0.0 |
| | 2 | 2.0 | 0.0 | 0.0 | 0.0 | 0.0 | | 0.0 | | 0.0 |
| Average ($x\ 10^{-2}$) | | 3.7 | | 2.1 | | 3.1 | | 0.1 | | 0.0 |
| SD | | 1.52753 | | 0.98853 | | 1.48956 | | 0.08544 | | 0.00000 |
| **Plaque Forming Units Count - Winter** | | | | | | | | | | |
| | U1 | | U2 | | U3 | | U4 | | U5 | |
| | COUNT | pfu/ml | COUNT | pfu/ml | COUNT | pfu/ml | COUNT | pfu/ml | COUNT | pfu/ml |
| **0** | 106 | 1.1 | 85.0 | 0.9 | 65.0 | 0.7 | 52.0 | 0.5 | 15.0 | 0.2 |
| | 83 | 0.8 | 67.0 | 0.7 | 87.0 | 0.9 | 27.0 | 0.3 | 3.0 | 0.0 |
| **10** | 13 | 1.3 | 10.0 | 1.0 | 9.0 | 0.9 | 7.0 | 0.7 | 2.0 | 0.2 |
| | 9 | 0.9 | 8.0 | 0.8 | 6.0 | 0.6 | 4.0 | 0.4 | 1.0 | 0.1 |
| **100** | 2 | 2.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.0 | 1.0 |
| | 0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | | 0.0 |
| Average ($x\ 10^{-2}$) | | 1.1 | | 0.9 | | 0.8 | | 0.5 | | 0.1 |
| SD | | 0.20133 | | 0.10408 | | 0.16523 | | 0.15100 | | 0.08544 |
| **Plaque Forming Units Count - Spring** | | | | | | | | | | |
| | U1 | | U2 | | U3 | | U4 | | U5 | |
| | COUNT | pfu/ml | COUNT | pfu/ml | COUNT | pfu/ml | COUNT | pfu/ml | COUNT | pfu/ml |
| **0** | 278 | 2.8 | 254.0 | 2.5 | 221.0 | 2.2 | 76.0 | 0.8 | 17.0 | 0.2 |
| | 249 | 2.5 | 117.0 | 1.2 | 113.0 | 1.1 | 60.0 | 0.6 | 10.0 | 0.1 |
| **10** | 53 | 5.3 | 42.0 | 4.2 | 57.0 | 5.7 | 10.0 | 1.0 | 4.0 | 0.4 |
| | 21 | 2.1 | 29.0 | 2.9 | 31.0 | 3.1 | 8.0 | 0.8 | 1.0 | 0.1 |
| **100** | 5 | 5.0 | 5.0 | 5.0 | 4.0 | 4.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| | 3 | 3.0 | 2.0 | 2.0 | 1.0 | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Average ($x\ 10^{-2}$) | | 4.4 | | 3.2 | | 4.3 | | 0.8 | | 0.2 |
| SD | | 1.37652 | | 0.87323 | | 1.32035 | | 0.20000 | | 0.17321 |
| **Plaque Forming Units Count - Summer** | | | | | | | | | | |
| | U1 | | U2 | | U3 | | U4 | | U5 | |
| | COUNT | pfu/ml | COUNT | pfu/ml | COUNT | pfu/ml | COUNT | pfu/ml | COUNT | pfu/ml |
| **0** | 301 | 3.0 | 275.0 | 2.8 | 221.0 | 2.2 | 221.0 | 2.2 | 98.0 | 1.0 |
| | 279 | 2.8 | 265.0 | 2.7 | 187.0 | 1.9 | 187.0 | 1.9 | 73.0 | 0.7 |
| **10** | 59 | 5.9 | 74.0 | 7.4 | 65.0 | 6.5 | 45.0 | 4.5 | 23.0 | 2.3 |
| | 43 | 4.3 | 51.0 | 5.1 | 42.0 | 4.2 | 28.0 | 2.8 | 18.0 | 1.8 |
| **100** | 5 | 5.0 | 4.0 | 4.0 | 5.0 | 5.0 | 3.0 | 3.0 | 3.0 | 3.0 |
| | 1 | 1.0 | 2.0 | 2.0 | 1.0 | 1.0 | 1.0 | 1.0 | 0.0 | 0.0 |
| Average ($x\ 10^{-2}$) | | 5.1 | | 5.5 | | 4.3 | | 3.4 | | 1.6 |
| SD | | 0.80208 | | 1.73494 | | 2.14687 | | 0.92916 | | 0.80206 |

SD – standard deviation
pfu/ml – plaque forming units per millilitre
Shaded areas highlight the values used to calculate average

**Table 7: Virus like particle count for water samples collected along the Umgeni River during autumn**

| REPLICATE COUNTS | U1 | U2 | U3 | U4 | U5 |
|---|---|---|---|---|---|
| | VLPs/ml | | | | |
| **a** | 177 | 132 | 306 | 805 | 166 |
| **b** | 145 | 158 | 226 | 557 | 171 |
| **c** | 221 | 117 | 492 | 452 | 257 |
| **d** | 266 | 138 | 358 | 513 | 161 |
| **e** | 259 | 198 | 380 | 506 | 186 |
| **f** | 135 | 137 | 401 | 538 | 170 |
| **g** | 280 | 158 | 397 | 463 | 138 |
| **h** | 177 | 193 | 417 | 476 | 136 |
| **i** | 217 | 163 | 344 | 462 | 125 |
| **j** | 199 | 131 | 300 | 385 | 234 |
| Average (x $10^0$) | 207.6 | 152.5 | 362.1 | 515.7 | 174.4 |
| SD | 48.8800 | 26.1061 | 71.6798 | 109.725 | 41.0781 |

**Table 8: Virus like particle count for water samples collected along the Umgeni River during winter**

| REPLICATE COUNTS | U1 | U2 | U3 | U4 | U5 |
|---|---|---|---|---|---|
| | VLPs/ml | | | | |
| **a** | 192 | 118 | 267 | 256 | 243 |
| **b** | 238 | 284 | 292 | 153 | 232 |
| **c** | 189 | 193 | 224 | 254 | 250 |
| **d** | 150 | 336 | 328 | 221 | 222 |
| **e** | 146 | 236 | 357 | 216 | 257 |
| **f** | 195 | 245 | 332 | 250 | 151 |
| **g** | 259 | 224 | 316 | 219 | 168 |
| **h** | 196 | 278 | 347 | 231 | 301 |
| **i** | 206 | 241 | 326 | 201 | 268 |
| **j** | 185 | 198 | 331 | 214 | 166 |
| Average (x $10^0$) | 195.6 | 235.3 | 312.0 | 221.5 | 225.8 |
| SD | 33.4576 | 57.6104 | 39.267 | 29.655 | 47.9645 |

SD – standard deviation

VLP/ml – virus like particles per millilitre

**Table 9: Virus like particle count for water samples collected along the Umgeni River during spring**

| REPLICATE COUNTS | U1 | U2 | U3 | U4 | U5 |
|---|---|---|---|---|---|
| | VLPs/ml | | | | |
| **a** | 793 | 1918 | 817 | 335 | 326 |
| **b** | 966 | 1568 | 814 | 412 | 486 |
| **c** | 968 | 1489 | 814 | 426 | 385 |
| **d** | 696 | 1737 | 937 | 363 | 496 |
| **e** | 789 | 1996 | 851 | 562 | 496 |
| **f** | 813 | 2118 | 942 | 449 | 342 |
| **g** | 778 | 2001 | 856 | 429 | 480 |
| **h** | 882 | 1445 | 875 | 421 | 396 |
| **i** | 825 | 1659 | 925 | 398 | 432 |
| **j** | 846 | 1842 | 794 | 456 | 462 |
| Average (x $10^0$) | 835.6 | 1777.3 | 862.5 | 425.1 | 430.1 |
| SD | 82.2131 | 227.3175 | 53.8755 | 59.0992 | 62.5063 |

**Table 10: Virus like particle count for water samples collected along the Umgeni River during summer**

| REPLICATE COUNTS | U1 | U2 | U3 | U4 | U5 |
|---|---|---|---|---|---|
| | VLPs/ml | | | | |
| **a** | 2278 | 1557 | 1008 | 445 | 334 |
| **b** | 1737 | 1784 | 688 | 546 | 387 |
| **c** | 1875 | 1453 | 656 | 558 | 394 |
| **d** | 2004 | 1743 | 1132 | 334 | 437 |
| **e** | 2115 | 1651 | 866 | 534 | 406 |
| **f** | 1998 | 1834 | 857 | 487 | 443 |
| **g** | 2567 | 1639 | 987 | 443 | 398 |
| **h** | 2114 | 1754 | 886 | 442 | 411 |
| **i** | 2146 | 1659 | 901 | 415 | 386 |
| **j** | 2028 | 1585 | 869 | 412 | 417 |
| Average (x $10^0$) | 2086.2 | 1665.9 | 885.0 | 461.6 | 401.3 |
| SD | 219.474 | 112.687 | 137.439 | 68.2275 | 29.75 |

SD – standard deviation

VLP/ml – virus like particles per millilitre

**Table 11: Percentage cell survival of VLP infected HEP G2 cell lines during all seasons tested along the Umgeni River**

| | U1 | | | | | U2 | | | | | U3 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **R1** | **R2** | **AV** | **SD** | **%** | **R1** | **R2** | **AV** | **SD** | **%** | **R1** | **R2** | **AV** | **SD** | **%** |
| **AUTUMN** | 1.523 | 1.663 | 1.593 | 0.099 | 79.551 | 1.245 | 1.265 | 1.255 | 0.014 | 62.672 | 1.645 | 1.547 | 1.596 | 0.069 | 79.700 |
| **WINTER** | 1.425 | 1.527 | 1.476 | 0.072 | 73.708 | 1.362 | 1.456 | 1.409 | 0.066 | 70.362 | 1.360 | 1.387 | 1.374 | 0.019 | 68.589 |
| **SPRING** | 1.326 | 1.369 | 1.348 | 0.030 | 67.291 | 1.106 | 1.169 | 1.138 | 0.045 | 56.804 | 1.152 | 1.124 | 1.138 | 0.020 | 56.829 |
| **SUMMER** | 1.325 | 1.251 | 1.288 | 0.052 | 64.320 | 1.003 | 1.104 | 1.054 | 0.071 | 52.609 | 1.265 | 1.365 | 1.315 | 0.071 | 65.668 |
| | **U4** | | | | | **U5** | | | | | | | | | |
| | **R1** | **R2** | **AV** | **SD** | **%** | **R1** | **R2** | **AV** | **SD** | **%** | | | | | |
| **AUTUMN** | 1.919 | 1.866 | 1.893 | 0.037 | 94.507 | 1.889 | 1.795 | 1.842 | 0.066 | 91.985 | | | | | |
| **WINTER** | 1.869 | 1.745 | 1.807 | 0.088 | 90.237 | 1.758 | 1.884 | 1.821 | 0.089 | 90.936 | | | | | |
| **SPRING** | 1.235 | 1.139 | 1.187 | 0.068 | 59.276 | 1.858 | 1.752 | 1.805 | 0.075 | 90.137 | | | | | |
| **SUMMER** | 1.263 | 1.285 | 1.274 | 0.016 | 63.620 | 1.381 | 1.675 | 1.528 | 0.208 | 76.305 | | | | | |
| | **CONTROLS** | | | | | | | | | | | | | | |
| **COXSACKIEVIRUS** | 1.828 | 1.787 | 1.808 | 0.029 | 90.262 | | | | | | | | | | |
| **ADENOVIRUS** | 1.363 | 1.254 | 1.309 | 0.077 | 65.343 | | | | | | | | | | |
| **ROTAVIRUS** | 1.451 | 1.472 | 1.462 | 0.029 | 72.984 | | | | | | | | | | |
| **CONTROL** | **2.012** | **1.993** | **2.003** | | | | | | | | | | | | |

R1- Absorbance Reading 1

R2- Absorbance Reading 2

AV-Average

SD-Standard Deviation

%- Percentage of cell survival

**Table 12: Percentage cell survival of VLP infected HEK 293 cell lines during all seasons tested along the Umgeni River**

| | U1 | | | | | U2 | | | | | U3 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **R1** | **R2** | **AV** | **SD** | **%** | **R1** | **R2** | **AV** | **SD** | **%** | **R1** | **R2** | **AV** | **SD** | **%** |
| **AUTUMN** | 1.234 | 1.054 | 1.144 | 0.127 | 66.223 | 0.729 | 1.084 | 0.907 | 0.251 | 52.475 | 1.319 | 1.012 | 1.166 | 0.217 | 67.467 |
| **WINTER** | 1.219 | 1.366 | 1.293 | 0.104 | 74.819 | 1.074 | 1.156 | 1.115 | 0.058 | 64.544 | 1.036 | 1.058 | 1.047 | 0.016 | 60.608 |
| **SPRING** | 0.959 | 0.966 | 0.963 | 0.005 | 55.716 | 0.956 | 0.773 | 0.865 | 0.129 | 50.043 | 0.945 | 1.056 | 1.001 | 0.078 | 57.916 |
| **SUMMER** | 1.234 | 1.054 | 1.144 | 0.127 | 66.223 | 0.729 | 1.084 | 0.907 | 0.251 | 52.475 | 1.319 | 1.012 | 1.166 | 0.217 | 67.467 |
| | **U4** | | | | | **U5** | | | | | | | | | |
| | **R1** | **R2** | **AV** | **SD** | **%** | **R1** | **R2** | **AV** | **SD** | **%** | | | | | |
| **AUTUMN** | 1.623 | 1.604 | 1.614 | 0.013 | 93.401 | 1.629 | 1.654 | 1.642 | 0.018 | 95.022 | | | | | |
| **WINTER** | 0.985 | 0.971 | 0.978 | 0.010 | 56.614 | 1.674 | 1.656 | 1.665 | 0.013 | 96.382 | | | | | |
| **SPRING** | 0.966 | 1.053 | 1.010 | 0.062 | 58.437 | 1.656 | 1.573 | 1.615 | 0.059 | 93.459 | | | | | |
| **SUMMER** | 0.973 | 0.883 | 0.928 | 0.064 | 53.719 | 0.693 | 0.689 | 0.691 | 0.003 | 40.000 | | | | | |
| | **CONTROLS** | | | | | | | | | | | | | | |
| **COXSACKIEVIRUS** | 0.537 | 0.636 | 0.587 | 0.070 | 33.951 | | | | | | | | | | |
| **ADENOVIRUS** | 0.963 | 1.084 | 1.024 | 0.086 | 59.247 | | | | | | | | | | |
| **ROTAVIRUS** | 1.728 | 1.687 | 1.708 | 0.029 | 98.842 | | | | | | | | | | |
| **CONTROL** | **1.752** | **1.703** | **1.728** | | | | | | | | | | | | |

R1- Absorbance Reading 1

R2- Absorbance Reading 2

AV-Average

SD-Standard Deviation

%- Percentage of cell survival

**Table 13: Percentage cell survival of VLP infected VERO cell lines during all seasons tested along the Umgeni River**

| | U1 | | | | | U2 | | | | | U3 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **R1** | **R2** | **AV** | **SD** | **%** | **R1** | **R2** | **AV** | **SD** | **%** | **R1** | **R2** | **AV** | **SD** | **%** |
| **AUTUMN** | 1.139 | 1.112 | 1.126 | 0.019 | 58.180 | 1.123 | 1.167 | 1.145 | 0.031 | 59.188 | 1.136 | 1.165 | 1.151 | 0.021 | 59.473 |
| **WINTER** | 1.136 | 1.108 | 1.122 | 0.020 | 57.999 | 1.021 | 1.098 | 1.060 | 0.054 | 54.769 | 1.059 | 1.121 | 1.090 | 0.044 | 56.345 |
| **SPRING** | 0.845 | 0.968 | 0.907 | 0.087 | 46.860 | 0.658 | 0.789 | 0.724 | 0.093 | 37.400 | 0.911 | 0.821 | 0.866 | 0.064 | 44.766 |
| **SUMMER** | 0.623 | 0.678 | 0.651 | 0.039 | 33.626 | 0.424 | 0.369 | 0.397 | 0.039 | 20.496 | 0.569 | 0.238 | 0.404 | 0.234 | 20.858 |
| | **U4** | | | | | **U5** | | | | | | | | | |
| | **R1** | **R2** | **AV** | **SD** | **%** | **R1** | **R2** | **AV** | **SD** | **%** | | | | | |
| **AUTUMN** | 1.126 | 1.109 | 1.118 | 0.012 | 57.767 | 1.121 | 1.211 | 1.166 | 0.064 | 60.274 | | | | | |
| **WINTER** | 1.106 | 1.197 | 1.152 | 0.064 | 59.524 | 1.201 | 1.214 | 1.208 | 0.009 | 62.419 | | | | | |
| **SPRING** | 1.003 | 1.098 | 1.051 | 0.067 | 54.303 | 1.109 | 1.036 | 1.073 | 0.052 | 55.441 | | | | | |
| **SUMMER** | 1.045 | 0.966 | 1.006 | 0.056 | 51.977 | 1.002 | 1.121 | 1.062 | 0.084 | 54.872 | | | | | |
| | **CONTROLS** | | | | | | | | | | | | | | |
| **COXSACKIEVIRUS** | 0.237 | 0.236 | 0.237 | 0.001 | 12.225 | | | | | | | | | | |
| **ADENOVIRUS** | 1.828 | 1.787 | 1.808 | 0.029 | 93.435 | | | | | | | | | | |
| **ROTAVIRUS** | 0.653 | 0.757 | 0.705 | 0.074 | 36.444 | | | | | | | | | | |
| **CONTROL** | **1.882** | **1.987** | **1.935** | | | | | | | | | | | | |

R1- Absorbance Reading 1

R2- Absorbance Reading 2

AV-Average

SD-Standard Deviation

%- Percentage of cell survival

**Table 14: Percentage cell survival of VLP infected HELA cell lines during all seasons tested along the Umgeni River**

| | U1 | | | | | U2 | | | | | U3 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **R1** | **R2** | **AV** | **SD** | **%** | **R1** | **R2** | **AV** | **SD** | **%** | **R1** | **R2** | **AV** | **SD** | **%** |
| **AUTUMN** | 0.141 | 0.138 | 0.140 | 0.002 | 36.047 | 0.201 | 0.158 | 0.180 | 0.030 | 46.382 | 0.248 | 0.185 | 0.217 | 0.045 | 55.943 |
| **WINTER** | 0.048 | 0.035 | 0.042 | 0.009 | 10.724 | 0.193 | 0.332 | 0.263 | 0.098 | 67.829 | 0.105 | 0.123 | 0.114 | 0.013 | 29.457 |
| **SPRING** | 0.251 | 0.241 | 0.246 | 0.007 | 63.566 | 0.172 | 0.151 | 0.162 | 0.015 | 41.731 | 0.180 | 0.258 | 0.219 | 0.055 | 56.589 |
| **SUMMER** | 0.153 | 0.152 | 0.153 | 0.001 | 39.406 | 0.147 | 0.145 | 0.146 | 0.001 | 37.726 | 0.106 | 0.117 | 0.112 | 0.008 | 28.811 |
| | **U4** | | | | | **U5** | | | | | | | | | |
| | **R1** | **R2** | **AV** | **SD** | **%** | **R1** | **R2** | **AV** | **SD** | **%** | | | | | |
| **AUTUMN** | 0.005 | 0.047 | 0.026 | 0.030 | 6.718 | 0.101 | 0.152 | 0.127 | 0.036 | 32.687 | | | | | |
| **WINTER** | 0.301 | 0.411 | 0.356 | 0.078 | 91.990 | 0.284 | 0.275 | 0.280 | 0.006 | 72.222 | | | | | |
| **SPRING** | 0.244 | 0.256 | 0.250 | 0.008 | 64.599 | 0.307 | 0.267 | 0.287 | 0.028 | 74.160 | | | | | |
| **SUMMER** | 0.197 | 0.147 | 0.172 | 0.035 | 44.444 | 0.307 | 0.363 | 0.335 | 0.040 | 86.563 | | | | | |
| | **CONTROLS** | | | | | | | | | | | | | | |
| **COXSACKIEVIRUS** | 0.152 | 0.166 | 0.159 | 0.010 | 41.085 | | | | | | | | | | |
| **ADENOVIRUS** | 0.034 | 0.033 | 0.034 | 0.001 | 8.656 | | | | | | | | | | |
| **ROTAVIRUS** | 0.114 | 0.052 | 0.083 | 0.044 | 21.447 | | | | | | | | | | |
| **CONTROL** | 0.382 | 0.392 | 0.387 | | | | | | | | | | | | |

R1- Absorbance Reading 1

R2- Absorbance Reading 2

AV-Average

SD-Standard Deviation

%- Percentage of cell survival

**Table 15: Percentage cell survival of VLP infected PLC/PRF/5 cell lines during all seasons tested along the Umgeni River**

| | U1 | | | | | U2 | | | | | U3 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **R1** | **R2** | **AV** | **SD** | **%** | **R1** | **R2** | **AV** | **SD** | **%** | **R1** | **R2** | **AV** | **SD** | **%** |
| **AUTUMN** | 0.200 | 0.233 | 0.217 | 0.023 | 63.959 | 0.294 | 0.279 | 0.287 | 0.011 | 84.638 | 0.287 | 0.301 | 0.294 | 0.010 | 86.854 |
| **WINTER** | 0.314 | 0.297 | 0.306 | 0.012 | 90.251 | 0.205 | 0.219 | 0.212 | 0.010 | 62.629 | 0.227 | 0.276 | 0.252 | 0.035 | 74.298 |
| **SPRING** | 0.154 | 0.135 | 0.145 | 0.013 | 42.688 | 0.163 | 0.174 | 0.169 | 0.008 | 49.778 | 0.214 | 0.222 | 0.218 | 0.006 | 64.402 |
| **SUMMER** | 0.114 | 0.132 | 0.123 | 0.013 | 36.337 | 0.140 | 0.164 | 0.152 | 0.017 | 44.904 | 0.114 | 0.110 | 0.112 | 0.003 | 33.087 |
| | **U4** | | | | | **U5** | | | | | | | | | |
| | **R1** | **R2** | **AV** | **SD** | **%** | **R1** | **R2** | **AV** | **SD** | **%** | | | | | |
| **AUTUMN** | 0.258 | 0.232 | 0.245 | 0.018 | 72.378 | 0.311 | 0.325 | 0.318 | 0.010 | 93.944 | | | | | |
| **WINTER** | 0.270 | 0.256 | 0.263 | 0.010 | 77.696 | 0.184 | 0.180 | 0.182 | 0.003 | 53.767 | | | | | |
| **SPRING** | 0.235 | 0.254 | 0.245 | 0.013 | 72.230 | 0.277 | 0.260 | 0.269 | 0.012 | 79.321 | | | | | |
| **SUMMER** | 0.118 | 0.128 | 0.123 | 0.007 | 36.337 | 0.263 | 0.234 | 0.249 | 0.021 | 73.412 | | | | | |
| | **CONTROLS** | | | | | | | | | | | | | | |
| **COXSACKIEVIRUS** | 0.154 | 0.160 | 0.157 | 0.004 | 46.381 | | | | | | | | | | |
| **ADENOVIRUS** | 0.020 | 0.025 | 0.023 | 0.004 | 6.647 | | | | | | | | | | |
| **ROTAVIRUS** | 0.084 | 0.094 | 0.089 | 0.007 | 26.292 | | | | | | | | | | |
| **CONTROL** | **0.348** | **0.329** | **0.339** | | | | | | | | | | | | |

R1- Absorbance Reading 1

R2- Absorbance Reading 2

AV-Average

SD-Standard Deviation

%- Percentage of cell survival

**Table 16: Percentage cell survival of VLP infected A549 cell lines during all seasons tested along the Umgeni River**

| | U1 | | | | | U2 | | | | | U3 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **R1** | **R2** | **AV** | **SD** | **%** | **R1** | **R2** | **AV** | **SD** | **%** | **R1** | **R2** | **AV** | **SD** | **%** |
| **AUTUMN** | 0.357 | 0.364 | 0.361 | 0.005 | 64.203 | 0.371 | 0.363 | 0.367 | 0.006 | 65.361 | 0.424 | 0.459 | 0.442 | 0.025 | 78.629 |
| **WINTER** | 0.175 | 0.091 | 0.133 | 0.059 | 23.687 | 0.318 | 0.345 | 0.332 | 0.019 | 59.038 | 0.176 | 0.161 | 0.169 | 0.011 | 30.009 |
| **SPRING** | 0.239 | 0.248 | 0.244 | 0.006 | 43.366 | 0.196 | 0.203 | 0.200 | 0.005 | 35.530 | 0.311 | 0.308 | 0.310 | 0.002 | 55.120 |
| **SUMMER** | 0.088 | 0.092 | 0.090 | 0.003 | 16.028 | 0.061 | 0.048 | 0.055 | 0.009 | 9.706 | 0.005 | 0.002 | 0.004 | 0.002 | 0.623 |
| | **U4** | | | | | **U5** | | | | | | | | | |
| | **R1** | **R2** | **AV** | **SD** | **%** | **R1** | **R2** | **AV** | **SD** | **%** | | | | | |
| **AUTUMN** | 0.116 | 0.135 | 0.126 | 0.013 | 22.351 | 0.440 | 0.357 | 0.399 | 0.059 | 70.971 | | | | | |
| **WINTER** | 0.125 | 0.140 | 0.133 | 0.011 | 23.598 | 0.210 | 0.196 | 0.203 | 0.010 | 36.153 | | | | | |
| **SPRING** | 0.095 | 0.110 | 0.103 | 0.011 | 18.255 | 0.366 | 0.345 | 0.356 | 0.015 | 63.313 | | | | | |
| **SUMMER** | 0.245 | 0.287 | 0.266 | 0.030 | 47.373 | 0.402 | 0.379 | 0.391 | 0.016 | 69.546 | | | | | |
| | **CONTROLS** | | | | | | | | | | | | | | |
| **COXSACKIEVIRUS** | 0.311 | 0.288 | 0.300 | 0.016 | 53.339 | | | | | | | | | | |
| **ADENOVIRUS** | 0.236 | 0.194 | 0.215 | 0.030 | 38.290 | | | | | | | | | | |
| **ROTAVIRUS** | 0.188 | 0.182 | 0.185 | 0.004 | 32.947 | | | | | | | | | | |
| **CONTROL** | **0.580** | **0.543** | **0.562** | | | | | | | | | | | | |

R1- Absorbance Reading 1

R2- Absorbance Reading 2

AV-Average

SD-Standard Deviation

%- Percentage of cell survival

**Table 17: Percentage cell death and cell survival of VLPs infected on all cell lines during all seasons**

| CELL LINE | SAMPLING SITE and SEASON | % OF CELL DEATH | | | | | | % OF CELL SURVIVAL | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | U1 | U2 | U3 | U4 | U5 | | U1 | U2 | U3 | U4 | U5 |
| HEK 293 | AUTUMN | 33.78 | 47.53 | 32.53 | 6.60 | 4.98 | | 66.22 | 52.47 | 67.47 | 93.40 | 95.02 |
| | WINTER | 25.18 | 35.46 | 39.39 | 43.39 | 3.62 | | 74.82 | 64.54 | 60.61 | 56.61 | 96.38 |
| | SPRING | 44.28 | 49.96 | 42.08 | 41.56 | 6.54 | | 55.72 | 50.04 | 57.92 | 58.44 | 93.46 |
| | SUMMER | 67.12 | 54.21 | 47.87 | 46.28 | 60.00 | | 32.88 | 45.79 | 52.13 | 53.72 | 40.00 |
| HEP G2 | AUTUMN | 5.49 | 37.33 | 12.81 | 5.49 | 8.01 | | 94.51 | 62.67 | 87.19 | 94.51 | 91.99 |
| | WINTER | 9.76 | 29.64 | 31.41 | 9.76 | 9.06 | | 90.24 | 70.36 | 68.59 | 90.24 | 90.94 |
| | SPRING | 40.72 | 43.20 | 38.18 | 33.23 | 9.86 | | 59.28 | 56.80 | 61.82 | 66.77 | 90.14 |
| | SUMMER | 36.38 | 47.39 | 43.17 | 28.89 | 23.70 | | 63.62 | 52.61 | 56.83 | 71.11 | 76.30 |
| VERO | AUTUMN | 41.82 | 40.81 | 40.53 | 42.23 | 39.73 | | 58.18 | 59.19 | 59.47 | 57.77 | 60.27 |
| | WINTER | 42.00 | 45.23 | 43.65 | 40.48 | 37.58 | | 58.00 | 54.77 | 56.35 | 59.52 | 62.42 |
| | SPRING | 53.14 | 62.60 | 55.23 | 45.70 | 44.56 | | 46.86 | 37.40 | 44.77 | 54.30 | 55.44 |
| | SUMMER | 66.37 | 79.50 | 79.14 | 48.02 | 45.13 | | 33.63 | 20.50 | 20.86 | 51.98 | 54.87 |
| HELA | AUTUMN | 63.95 | 53.62 | 44.06 | 93.28 | 67.31 | | 36.05 | 46.38 | 55.94 | 6.72 | 32.69 |
| | WINTER | 89.28 | 32.17 | 70.54 | 8.01 | 27.78 | | 10.72 | 67.83 | 29.46 | 91.99 | 72.22 |
| | SPRING | 36.43 | 58.27 | 43.41 | 35.40 | 25.84 | | 63.57 | 41.73 | 56.59 | 64.60 | 74.16 |
| | SUMMER | 60.59 | 62.27 | 71.19 | 55.56 | 13.44 | | 39.41 | 37.73 | 28.81 | 44.44 | 86.56 |
| PLC | AUTUMN | 36.04 | 15.36 | 13.15 | 27.62 | 6.06 | | 63.96 | 84.64 | 86.85 | 72.38 | 93.94 |
| | WINTER | 9.75 | 37.37 | 25.70 | 22.30 | 46.23 | | 90.25 | 62.63 | 74.30 | 77.70 | 53.77 |
| | SPRING | 57.31 | 50.22 | 35.60 | 27.77 | 20.68 | | 42.69 | 49.78 | 64.40 | 72.23 | 79.32 |
| | SUMMER | 63.66 | 55.10 | 66.91 | 63.66 | 26.59 | | 36.34 | 44.90 | 33.09 | 36.34 | 73.41 |
| A549 | AUTUMN | 35.80 | 34.64 | 21.37 | 77.65 | 29.03 | | 64.20 | 65.36 | 78.63 | 22.35 | 70.97 |
| | WINTER | 76.31 | 40.96 | 69.99 | 76.40 | 63.85 | | 23.69 | 59.04 | 30.01 | 23.60 | 36.15 |
| | SPRING | 56.63 | 64.47 | 44.88 | 81.75 | 36.69 | | 43.37 | 35.53 | 55.12 | 18.25 | 63.31 |
| | SUMMER | 83.97 | 90.29 | 99.38 | 52.63 | 30.45 | | 16.03 | 9.71 | 0.62 | 47.37 | 69.55 |

**Table 18: Standard deviations of VLPs infected on all cell lines during all seasons**

| CELL LINE | SAMPLING SITE and SEASON | AVERAGE STANDARD DEVIATIONS | | | | | | STANDARD DEVIATIONS | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | U1 | U2 | U3 | U4 | U5 | | U1 | U2 | U3 | U4 | U5 |
| | AUTUMN | 7.36783 | 14.53099 | 12.56624 | 0.77772 | 1.02331 | | 0.12728 | 0.25102 | 0.21708 | 0.01344 | 0.01768 |
| | WINTER | 6.01706 | 3.35645 | 0.90051 | 0.57305 | 0.73678 | | 0.10394 | 0.05798 | 0.01556 | 0.00990 | 0.01273 |
| | SPRING | 0.28653 | 7.49062 | 4.54349 | 3.56112 | 3.39739 | | 0.00495 | 0.12940 | 0.07849 | 0.06152 | 0.05869 |
| HEK 293 | SUMMER | 3.35645 | 8.02275 | 1.84196 | 3.68391 | 0.16373 | | 0.05798 | 0.13859 | 0.03182 | 0.06364 | 0.00283 |
| | AUTUMN | 4.94233 | 0.70605 | 3.45963 | 1.87103 | 3.31842 | | 0.09899 | 0.01414 | 0.06930 | 0.03748 | 0.06647 |
| | WINTER | 3.60084 | 3.31842 | 0.95316 | 4.37750 | 4.44810 | | 0.07212 | 0.06647 | 0.01909 | 0.08768 | 0.08910 |
| | SPRING | 1.51800 | 2.22405 | 0.98847 | 3.38903 | 3.74205 | | 0.03041 | 0.04455 | 0.01980 | 0.06788 | 0.07495 |
| HEP G2 | SUMMER | 2.61238 | 3.56554 | 3.53024 | 0.77665 | 10.37890 | | 0.05233 | 0.07142 | 0.07071 | 0.01556 | 0.20789 |
| | AUTUMN | 0.98666 | 1.60789 | 1.05975 | 0.69432 | 3.28887 | | 0.01909 | 0.03111 | 0.02051 | 0.01344 | 0.06364 |
| | WINTER | 1.02320 | 2.81381 | 2.26567 | 0.51160 | 0.47506 | | 0.01980 | 0.05445 | 0.04384 | 0.00990 | 0.00919 |
| | SPRING | 4.49479 | 4.78713 | 3.28887 | 3.17924 | 2.66764 | | 0.08697 | 0.09263 | 0.06364 | 0.06152 | 0.05162 |
| VERO | SUMMER | 2.00986 | 2.00986 | 12.09573 | 3.28887 | 4.34862 | | 0.03889 | 0.03889 | 0.23405 | 0.06364 | 0.08415 |
| | AUTUMN | 0.10963 | 1.57135 | 2.30221 | 1.53481 | 1.86369 | | 0.00212 | 0.03041 | 0.04455 | 0.02970 | 0.03606 |
| | WINTER | 0.47506 | 5.07948 | 0.65777 | 4.01973 | 0.32889 | | 0.00919 | 0.09829 | 0.01273 | 0.07778 | 0.00636 |
| | SPRING | 0.36543 | 0.76740 | 2.85035 | 0.43852 | 1.46172 | | 0.00707 | 0.01485 | 0.05515 | 0.00849 | 0.02828 |
| HELA | SUMMER | 0.03654 | 0.07309 | 0.40197 | 1.82715 | 2.04641 | | 0.00071 | 0.00141 | 0.00778 | 0.03536 | 0.03960 |
| | AUTUMN | 1.20592 | 0.54814 | 0.51160 | 0.95012 | 0.51160 | | 0.02333 | 0.01061 | 0.00990 | 0.01838 | 0.00990 |
| | WINTER | 0.62123 | 0.51160 | 1.79061 | 0.51160 | 0.14617 | | 0.01202 | 0.00990 | 0.03465 | 0.00990 | 0.00283 |
| | SPRING | 0.69432 | 0.40197 | 0.29234 | 0.69432 | 0.62123 | | 0.01344 | 0.00778 | 0.00566 | 0.01344 | 0.01202 |
| PLC | SUMMER | 0.65777 | 0.87703 | 0.14617 | 0.36543 | 1.05975 | | 0.01273 | 0.01697 | 0.00283 | 0.00707 | 0.02051 |
| | AUTUMN | 0.25580 | 0.29234 | 1.27900 | 0.69432 | 3.03307 | | 0.00495 | 0.00566 | 0.02475 | 0.01344 | 0.05869 |
| | WINTER | 3.06961 | 0.98666 | 0.54814 | 0.54814 | 0.51160 | | 0.05940 | 0.01909 | 0.01061 | 0.01061 | 0.00990 |
| | SPRING | 0.32889 | 0.25580 | 0.10963 | 0.54814 | 0.76740 | | 0.00636 | 0.00495 | 0.00212 | 0.01061 | 0.01485 |
| A549 | SUMMER | 0.14617 | 0.47506 | 0.10963 | 1.53481 | 0.84049 | | 0.00283 | 0.00919 | 0.00212 | 0.02970 | 0.01626 |

**Table 19: Percentage cell death and cell survival of control viruses infected on all cell lines during all seasons**

| CELL LINE | CONTROL VIRUSES | CONTROL | | | |
|---|---|---|---|---|---|
| | | % OF CELL DEATH | % OF CELL SURVIVAL | AVERAGE STANDARD DEVIATIONS | STANDARD DEVIATIONS |
| HEK 293 | COXSACKIEVIRUS | 66.049 | 33.951 | 4.0523051 | 0.0700036 |
| | ADENOVIRUS | 40.753 | 59.247 | 4.9528174 | 0.0855599 |
| | ROTAVIRUS | 1.158 | 98.842 | 1.6782274 | 0.0289914 |
| HEP G2 | COXSACKIEVIRUS | 9.738 | 90.262 | 1.4473978 | 0.0289914 |
| | ADENOVIRUS | 34.657 | 65.343 | 3.84796 | 0.0770746 |
| | ROTAVIRUS | 27.016 | 72.984 | 1.4473978 | 0.0289914 |
| VERO | COXSACKIEVIRUS | 87.775 | 12.225 | 0.036543 | 0.0007071 |
| | ADENOVIRUS | 6.565 | 93.435 | 1.4982624 | 0.0289914 |
| | ROTAVIRUS | 63.556 | 36.444 | 3.8004706 | 0.0735391 |
| HELA | COXSACKIEVIRUS | 58.915 | 41.085 | 0.5116018 | 0.0098995 |
| | ADENOVIRUS | 66.796 | 33.204 | 0.3288869 | 0.006364 |
| | ROTAVIRUS | 78.553 | 21.447 | 2.2656651 | 0.0438406 |
| PLC | COXSACKIEVIRUS | 53.619 | 46.381 | 0.2192579 | 0.0042426 |
| | ADENOVIRUS | 68.390 | 31.610 | 0.5846878 | 0.0113137 |
| | ROTAVIRUS | 73.708 | 26.292 | 0.3654299 | 0.0070711 |
| A549 | COXSACKIEVIRUS | 46.661 | 53.339 | 0.8404887 | 0.0162635 |
| | ADENOVIRUS | 61.710 | 38.290 | 1.5348054 | 0.0296985 |
| | ROTAVIRUS | 67.053 | 32.947 | 0.2192579 | 0.0042426 |

**Table 19a:Detection of Adenovirus, Rotavirus, Enterovirus and Hepatits B virus genomes in the various infected cell line**

| | ADENOVIRUS | ENTEROVIRUS | ROTAVIRUS | HEPATITIS B VIRUS |
|---|---|---|---|---|
| **HEK 293** | 80 | 100 | 0 | 40 |
| **A549** | [a] | 85 | 100 | [a] |
| **VERO** | [a] | 100 | 100 | [a] |
| **HELA** | [a] | 90 | 100 | [a] |
| **PLC** | 85 | 100 | 0 | 55 |
| **HEP G2** | 100 | 95 | [b] | 20 |
| | | | | |

[a]-Not Determined

[b]- Inconclusive

**Table 20: Size range of tailed phage's for water samples collected along the Umgeni River during autumn**

| SAMPLE SEASON | NO. OF PHAGE OBSERVED BY TEM | HEAD DIAMETER (nm) | TAIL LENGTH (nm) | TOTAL LENGTH (nm) |
|---|---|---|---|---|
| | | **RANGE** | RANGE | **RANGE** |
| **AUTUMN** | 7 | 48.71 | 97.95 | 150.16 |
| | | 49.76 | 186 | 239.78 |
| | | 46.69 | 95.84 | 139.05 |
| | | 66.72 | 89.2 | 169.93 |
| | | 48.7 | 67.8 | 84.57 |
| | | 69.20 | 109.67 | 132.9 |
| | | 68.72 | 115.43 | 156.7 |
| | **MEAN** | **49.76** | 97.95 | **150.16** |

**Table 21: Size range of tailed phage's for water samples collected along the Umgeni River during winter**

| SAMPLE SEASON | NO. OF PHAGE OBSERVED BY TEM | HEAD DIAMETER (nm) | TAIL LENGTH (nm) | TOTAL LENGTH (nm) |
|---|---|---|---|---|
| | | **RANGE** | **RANGE** | **RANGE** |
| **WINTER** | 3 | 47.63 | 93.38 | 134.7 |
| | | 56.59 | 180.57 | 249.72 |
| | | 58.73 | 112.76 | 180.97 |
| | **MEAN** | **56.59** | **112.76** | **180.97** |

**Table 22: Size range of tailed phage's observed by electron microscopy for water samples collected along the Umgeni River during spring**

| SAMPLE SEASON | NO. OF PHAGE OBSERVED BY TEM | HEAD DIAMETER (nm) | TAIL LENGTH (nm) | TOTAL LENGTH (nm) |
|---|---|---|---|---|
| | | **RANGE** | **RANGE** | **RANGE** |
| **SPRING** | 19 | 48.65 | 262.83 | 301.68 |
| | | 64.45 | 218.39 | 289.76 |
| | | 60.07 | 240.91 | 302.28 |
| | | 52.16 | 209.87 | 315.87 |
| | | 67.60 | 198.80 | 279.10 |
| | | 41.97 | 121.22 | 185.90 |
| | | 63.79 | 123.24 | 186.23 |
| | | 67.15 | 120.29 | 269.61 |
| | | 48.18 | 115.04 | 169.93 |
| | | 58.93 | 137.66 | 184.57 |
| | | 47.30 | 167.44 | 193.38 |
| | | 63.79 | 203.42 | 260.57 |
| | | 60.44 | 261.39 | 429.28 |
| | | 56.59 | 227.39 | 479.91 |
| | | 48.73 | 158.10 | 316.80 |
| | | 54.48 | 121.25 | 334.68 |
| | | 66.46 | 161.39 | 256.87 |
| | | 56.73 | 227.39 | 306.25 |
| | | 43.65 | 210.91 | 311.76 |
| | **MEAN** | **56.73** | **198.80** | **289.76** |

**Table 23: Size range of tailed phage's observed by electron microscopy for water samples collected along the Umgeni River during summer**

| SAMPLE SEASON | NO. OF PHAGE OBSERVED BY TEM | HEAD DIAMETER (nm) | TAIL LENGTH (nm) | TOTAL LENGTH (nm) |
|---|---|---|---|---|
| | | RANGE | RANGE | RANGE |
| **SUMMER** | 31 | 48.65 | 120.87 | 149.72 |
| | | 49.20 | 121.25 | 180.97 |
| | | 55.73 | 130.94 | 223.51 |
| | | 59.98 | 180.57 | 293.53 |
| | | 60.07 | 212.76 | 272.17 |
| | | 61.08 | 123.24 | 158.10 |
| | | 51.79 | 97.95 | 137.24 |
| | | 50.46 | 149.67 | 208.29 |
| | | 50.97 | 114.43 | 209.64 |
| | | 65.96 | 167.44 | 218.39 |
| | | 66.08 | 203.42 | 349.56 |
| | | 69.75 | 213.43 | 376.59 |
| | | 52.16 | 135.66 | 227.39 |
| | | 71.10 | 350.86 | 514.36 |
| | | 56.84 | 139.94 | 234.56 |
| | | 56.85 | 171.12 | 214.69 |
| | | 46.92 | 92.76 | 143.18 |
| | | 57.43 | 189.50 | 262.89 |
| | | 42.25 | 119.86 | 223.51 |
| | | 58.34 | 178.50 | 272.17 |
| | | 78.80 | 256.20 | 421.87 |
| | | 62.07 | 184.06 | 280.25 |
| | | 51.43 | 107.86 | 209.64 |
| | | 59.49 | 154.67 | 194.28 |
| | | 49.20 | 120.55 | 194.60 |
| | | 49.41 | 131.24 | 199.01 |
| | | 61.08 | 243.42 | 300.24 |
| | | 61.14 | 215.43 | 274.48 |
| | | 42.88 | 97.66 | 153.14 |
| | | 48.22 | 158.10 | 186.76 |
| | | 48.65 | 161.39 | 178.97 |
| | **MEAN** | **56.84** | **154.67** | **218.39** |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| THB | Between Groups | 98.595 | 3 | 32.865 | 4.105 | .024 |
| | Within Groups | 128.084 | 16 | 8.005 | | |
| | Total | 226.679 | 19 | | | |
| TC | Between Groups | 4.941 | 3 | 1.647 | .631 | .606 |
| | Within Groups | 41.796 | 16 | 2.612 | | |
| | Total | 46.737 | 19 | | | |
| FC | Between Groups | 1.998 | 3 | .666 | .310 | .818 |
| | Within Groups | 34.347 | 16 | 2.147 | | |
| | Total | 36.345 | 19 | | | |
| VC | Between Groups | 2.305 | 3 | .768 | .324 | .808 |
| | Within Groups | 37.970 | 16 | 2.373 | | |
| | Total | 40.275 | 19 | | | |
| SHIG | Between Groups | .302 | 3 | .101 | .179 | .909 |
| | Within Groups | 8.980 | 16 | .561 | | |
| | Total | 9.282 | 19 | | | |
| SAL | Between Groups | .152 | 3 | .051 | .206 | .891 |
| | Within Groups | 3.939 | 16 | .246 | | |
| | Total | 4.091 | 19 | | | |
| FS | Between Groups | 1.628 | 3 | .543 | 1.541 | .242 |
| | Within Groups | 5.634 | 16 | .352 | | |
| | Total | 7.262 | 19 | | | |
| EC | Between Groups | 2.415 | 3 | .805 | 5.180 | .011 |
| | Within Groups | 2.486 | 16 | .155 | | |
| | Total | 4.901 | 19 | | | |
| TEMP | Between Groups | 182.514 | 3 | 60.838 | 13.892 | .000 |
| | Within Groups | 70.072 | 16 | 4.379 | | |
| | Total | 252.586 | 19 | | | |
| pH | Between Groups | 1.124 | 3 | .375 | 1.382 | .284 |
| | Within Groups | 4.337 | 16 | .271 | | |
| | Total | 5.460 | 19 | | | |
| Turbidity | Between Groups | 157.644 | 3 | 52.548 | 3.075 | .058 |
| | Within Groups | 273.390 | 16 | 17.087 | | |
| | Total | 431.035 | 19 | | | |
| BOD | Between Groups | 4.225 | 3 | 1.408 | .247 | .862 |
| | Within Groups | 91.261 | 16 | 5.704 | | |
| | Total | 95.486 | 19 | | | |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| COD | Between Groups | 72733.371 | 3 | 24244.457 | .485 | .698 |
| | Within Groups | 800311.105 | 16 | 50019.444 | | |
| | Total | 873044.476 | 19 | | | |
| Conductivity | Between Groups | 2043005.174 | 3 | 681001.725 | .309 | .819 |
| | Within Groups | 35311741.772 | 16 | 2206983.861 | | |
| | Total | 37354746.946 | 19 | | | |
| Phosphate | Between Groups | .199 | 3 | .066 | .194 | .899 |
| | Within Groups | 5.474 | 16 | .342 | | |
| | Total | 5.673 | 19 | | | |
| Nitrate | Between Groups | 2.403 | 3 | .801 | .870 | .477 |
| | Within Groups | 14.720 | 16 | .920 | | |
| | Total | 17.122 | 19 | | | |
| Ammonia | Between Groups | 184.313 | 3 | 61.438 | .755 | .535 |
| | Within Groups | 1301.533 | 16 | 81.346 | | |
| | Total | 1485.846 | 19 | | | |
| Chloride | Between Groups | 28671926.857 | 3 | 9557308.952 | .325 | .807 |
| | Within Groups | 4.706E8 | 16 | 29410471.227 | | |
| | Total | 4.992E8 | 19 | | | |
| Sulphate | Between Groups | 166884.414 | 3 | 55628.138 | .184 | .906 |
| | Within Groups | 4836113.092 | 16 | 302257.068 | | |
| | Total | 5002997.506 | 19 | | | |

**Correlations**

| | | THB | TC | FC | VC | SHIG | SAL | FS | EC |
|---|---|---|---|---|---|---|---|---|---|
| THB | Pearson Correlation | 1 | .955 | .325 | .862 | -.554 | .999 | -.401 | .319 |
| | Sig. (2-tailed) | | .045 | .675 | .138 | .446 | .001 | .599 | .681 |
| | N | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| TC | Pearson Correlation | .955* | 1 | .094 | .775 | -.373 | .966* | -.461 | .343 |
| | Sig. (2-tailed) | .045 | | .906 | .225 | .627 | .034 | .539 | .657 |
| | N | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| FC | Pearson Correlation | .325 | .094 | 1 | .688 | -.176 | .289 | -.468 | .578 |
| | Sig. (2-tailed) | .675 | .906 | | .312 | .824 | .711 | .532 | .422 |
| | N | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| VC | Pearson Correlation | .862 | .775 | .688 | 1 | -.254 | .850 | -.725 | .706 |
| | Sig. (2-tailed) | .138 | .225 | .312 | | .746 | .150 | .275 | .294 |
| | N | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| SHIG | Pearson Correlation | -.554 | -.373 | -.176 | -.254 | 1 | -.538 | -.482 | .495 |
| | Sig. (2-tailed) | .446 | .627 | .824 | .746 | | .462 | .518 | .505 |
| | N | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| SAL | Pearson Correlation | .999** | .966* | .289 | .850 | -.538 | 1 | -.403 | .315 |
| | Sig. (2-tailed) | .001 | .034 | .711 | .150 | .462 | | .597 | .685 |
| | N | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| FS | Pearson Correlation | -.401 | -.461 | -.468 | -.725 | -.482 | -.403 | 1 | -.986* |
| | Sig. (2-tailed) | .599 | .539 | .532 | .275 | .518 | .597 | | .014 |
| | N | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| EC | Pearson Correlation | .319 | .343 | .578 | .706 | .495 | .315 | -.986* | 1 |
| | Sig. (2-tailed) | .681 | .657 | .422 | .294 | .505 | .685 | .014 | |
| | N | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |

*. Correlation is significant at the 0.05 level (2-tailed).
**. Correlation is significant at the 0.01 level (2-tailed).

**Correlations**

| | | TEMP | pH | Turbidity | BOD | COD | Conductivity |
|---|---|---|---|---|---|---|---|
| TEMP | Pearson Correlation | 1 | .572 | .377 | .000 | .282 | .702 |
| | Sig. (2-tailed) | | .428 | .623 | 1.000 | .718 | .298 |
| | N | 4 | 4 | 4 | 4 | 4 | 4 |
| pH | Pearson Correlation | .572 | 1 | .961* | .813 | -.369 | .310 |
| | Sig. (2-tailed) | .428 | | .039 | .187 | .631 | .690 |
| | N | 4 | 4 | 4 | 4 | 4 | 4 |
| Turbidity | Pearson Correlation | .377 | .961* | 1 | .925 | -.356 | .039 |
| | Sig. (2-tailed) | .623 | .039 | | .075 | .644 | .961 |
| | N | 4 | 4 | 4 | 4 | 4 | 4 |
| BOD | Pearson Correlation | .000 | .813 | .925 | 1 | -.546 | -.205 |
| | Sig. (2-tailed) | 1.000 | .187 | .075 | | .454 | .795 |
| | N | 4 | 4 | 4 | 4 | 4 | 4 |
| COD | Pearson Correlation | .282 | -.369 | -.356 | -.546 | 1 | -.228 |
| | Sig. (2-tailed) | .718 | .631 | .644 | .454 | | .772 |
| | N | 4 | 4 | 4 | 4 | 4 | 4 |
| Conductivity | Pearson Correlation | .702 | .310 | .039 | -.205 | -.228 | 1 |
| | Sig. (2-tailed) | .298 | .690 | .961 | .795 | .772 | |
| | N | 4 | 4 | 4 | 4 | 4 | 4 |

*. Correlation is significant at the 0.05 level (2-tailed).

**Correlations**

| | | Phosphate | Nitrate | Ammonia | Chloride | Sulphate |
|---|---|---|---|---|---|---|
| Phosphate | Pearson Correlation | 1 | -.913 | -.079 | -.075 | -.005 |
| | Sig. (2-tailed) | | .087 | .921 | .925 | .995 |
| | N | 4 | 4 | 4 | 4 | 4 |
| Nitrate | Pearson Correlation | -.913 | 1 | -.234 | .436 | .235 |
| | Sig. (2-tailed) | .087 | | .766 | .564 | .765 |
| | N | 4 | 4 | 4 | 4 | 4 |
| Ammonia | Pearson Correlation | -.079 | -.234 | 1 | -.951* | -.964* |
| | Sig. (2-tailed) | .921 | .766 | | .049 | .036 |
| | N | 4 | 4 | 4 | 4 | 4 |
| Chloride | Pearson Correlation | -.075 | .436 | -.951* | 1 | .862 |
| | Sig. (2-tailed) | .925 | .564 | .049 | | .138 |
| | N | 4 | 4 | 4 | 4 | 4 |
| Sulphate | Pearson Correlation | -.005 | .235 | -.964* | .862 | 1 |
| | Sig. (2-tailed) | .995 | .765 | .036 | .138 | |
| | N | 4 | 4 | 4 | 4 | 4 |

*. Correlation is significant at the 0.05 level (2-tailed).

**Correlations**

| | | SomaticPhage | FRNAPhage | VLP |
|---|---|---|---|---|
| SomaticPhage | Pearson Correlation | 1 | .991** | .884* |
| | Sig. (2-tailed) | | .001 | .046 |
| | N | 5 | 5 | 5 |
| FRNAPhage | Pearson Correlation | .991** | 1 | .838 |
| | Sig. (2-tailed) | .001 | | .076 |
| | N | 5 | 5 | 5 |
| VLP | Pearson Correlation | .884* | .838 | 1 |
| | Sig. (2-tailed) | .046 | .076 | |
| | N | 5 | 5 | 5 |

**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

**Correlations**

| | | | SomaticPhage | FRNAPhage | VLP |
|---|---|---|---|---|---|
| Spearman's rho | SomaticPhage | Correlation Coefficient | 1.000 | .900* | .900* |
| | | Sig. (2-tailed) | . | .037 | .037 |
| | | N | 5 | 5 | 5 |
| | FRNAPhage | Correlation Coefficient | .900* | 1.000 | .700 |
| | | Sig. (2-tailed) | .037 | . | .188 |
| | | N | 5 | 5 | 5 |
| | VLP | Correlation Coefficient | .900* | .700 | 1.000 |
| | | Sig. (2-tailed) | .037 | .188 | . |
| | | N | 5 | 5 | 5 |

*. Correlation is significant at the 0.05 level (2-tailed).

**Correlations**

| | | SomaticPhage | FRNAPhage | VLP | THB |
|---|---|---|---|---|---|
| SomaticPhage | Pearson Correlation | 1 | .991** | .884* | .878 |
| | Sig. (2-tailed) | | .001 | .046 | .050 |
| | N | 5 | 5 | 5 | 5 |
| FRNAPhage | Pearson Correlation | .991** | 1 | .838 | .902* |
| | Sig. (2-tailed) | .001 | | .076 | .036 |
| | N | 5 | 5 | 5 | 5 |
| VLP | Pearson Correlation | .884* | .838 | 1 | .584 |
| | Sig. (2-tailed) | .046 | .076 | | .301 |
| | N | 5 | 5 | 5 | 5 |
| THB | Pearson Correlation | .878 | .902* | .584 | 1 |
| | Sig. (2-tailed) | .050 | .036 | .301 | |
| | N | 5 | 5 | 5 | 5 |

**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

**Correlations**

| | | | SomaticPhage | FRNAPhage | VLP |
|---|---|---|---|---|---|
| Spearman's rho | SomaticPhage | Correlation Coefficient | 1.000 | .900 | .900 |
| | | Sig. (2-tailed) | . | .037 | .037 |
| | | N | 5 | 5 | 5 |
| | FRNAPhage | Correlation Coefficient | .900* | 1.000 | .700 |
| | | Sig. (2-tailed) | .037 | . | .188 |
| | | N | 5 | 5 | 5 |
| | VLP | Correlation Coefficient | .900* | .700 | 1.000 |
| | | Sig. (2-tailed) | .037 | .188 | . |
| | | N | 5 | 5 | 5 |
| | THB | Correlation Coefficient | .700 | .900* | .600 |
| | | Sig. (2-tailed) | .188 | .037 | .285 |
| | | N | 5 | 5 | 5 |

| | | | THB |
|---|---|---|---|
| Spearman's rho | SomaticPhage | Correlation Coefficient | .700 |
| | | Sig. (2-tailed) | .188 |
| | | N | 5 |
| | FRNAPhage | Correlation Coefficient | .900* |
| | | Sig. (2-tailed) | .037 |
| | | N | 5 |
| | VLP | Correlation Coefficient | .600 |
| | | Sig. (2-tailed) | .285 |
| | | N | 5 |
| | THB | Correlation Coefficient | 1.000 |
| | | Sig. (2-tailed) | . |
| | | N | 5 |

*. Correlation is significant at the 0.05 level (2-tailed).

**Correlations**

| | | SomaticPhage | FRNAPhage | VLP | THB | Turbidity |
|---|---|---|---|---|---|---|
| SomaticPhage | Pearson Correlation | 1 | .991** | .884* | .878 | .683 |
| | Sig. (2-tailed) | | .001 | .046 | .050 | .204 |
| | N | 5 | 5 | 5 | 5 | 5 |
| FRNAPhage | Pearson Correlation | .991** | 1 | .838 | .902* | .682 |
| | Sig. (2-tailed) | .001 | | .076 | .036 | .204 |
| | N | 5 | 5 | 5 | 5 | 5 |
| VLP | Pearson Correlation | .884* | .838 | 1 | .584 | .561 |
| | Sig. (2-tailed) | .046 | .076 | | .301 | .326 |
| | N | 5 | 5 | 5 | 5 | 5 |
| THB | Pearson Correlation | .878 | .902* | .584 | 1 | .464 |
| | Sig. (2-tailed) | .050 | .036 | .301 | | .431 |
| | N | 5 | 5 | 5 | 5 | 5 |
| Turbidity | Pearson Correlation | .683 | .682 | .561 | .464 | 1 |
| | Sig. (2-tailed) | .204 | .204 | .326 | .431 | |
| | N | 5 | 5 | 5 | 5 | 5 |
| BOD | Pearson Correlation | .795 | .806 | .489 | .809 | .840 |
| | Sig. (2-tailed) | .108 | .100 | .403 | .097 | .075 |
| | N | 5 | 5 | 5 | 5 | 5 |
| Conductivity | Pearson Correlation | .526 | .550 | .421 | .299 | .959** |
| | Sig. (2-tailed) | .362 | .337 | .480 | .625 | .010 |
| | N | 5 | 5 | 5 | 5 | 5 |
| TEMP | Pearson Correlation | .948* | .956* | .697 | .968** | .654 |
| | Sig. (2-tailed) | .014 | .011 | .191 | .007 | .231 |
| | N | 5 | 5 | 5 | 5 | 5 |
| pH | Pearson Correlation | -.223 | -.270 | -.077 | -.078 | -.824 |
| | Sig. (2-tailed) | .718 | .660 | .902 | .900 | .087 |
| | N | 5 | 5 | 5 | 5 | 5 |
| Nitrate | Pearson Correlation | .294 | .240 | .147 | .524 | -.207 |
| | Sig. (2-tailed) | .631 | .697 | .814 | .365 | .738 |
| | N | 5 | 5 | 5 | 5 | 5 |
| Ammonia | Pearson Correlation | .338 | .424 | -.031 | .714 | -.106 |
| | Sig. (2-tailed) | .578 | .477 | .961 | .176 | .865 |
| | N | 5 | 5 | 5 | 5 | 5 |
| Chloride | Pearson Correlation | .520 | .543 | .418 | .290 | .959* |
| | Sig. (2-tailed) | .369 | .344 | .484 | .636 | .010 |
| | N | 5 | 5 | 5 | 5 | 5 |

**Correlations**

| | | BOD | Conductivity | TEMP | pH | Nitrate |
|---|---|---|---|---|---|---|
| SomaticPhage | Pearson Correlation | .795 | .526 | .948 | -.223 | .294 |
| | Sig. (2-tailed) | .108 | .362 | .014 | .718 | .631 |
| | N | 5 | 5 | 5 | 5 | 5 |
| FRNAPhage | Pearson Correlation | .806 | .550 | .956* | -.270 | .240 |
| | Sig. (2-tailed) | .100 | .337 | .011 | .660 | .697 |
| | N | 5 | 5 | 5 | 5 | 5 |
| VLP | Pearson Correlation | .489 | .421 | .697 | -.077 | .147 |
| | Sig. (2-tailed) | .403 | .480 | .191 | .902 | .814 |
| | N | 5 | 5 | 5 | 5 | 5 |
| THB | Pearson Correlation | .809 | .299 | .968** | -.078 | .524 |
| | Sig. (2-tailed) | .097 | .625 | .007 | .900 | .365 |
| | N | 5 | 5 | 5 | 5 | 5 |
| Turbidity | Pearson Correlation | .840 | .959** | .654 | -.824 | -.207 |
| | Sig. (2-tailed) | .075 | .010 | .231 | .087 | .738 |
| | N | 5 | 5 | 5 | 5 | 5 |
| BOD | Pearson Correlation | 1 | .707 | .900* | -.554 | .268 |
| | Sig. (2-tailed) | | .182 | .038 | .333 | .663 |
| | N | 5 | 5 | 5 | 5 | 5 |
| Conductivity | Pearson Correlation | .707 | 1 | .484 | -.935* | -.463 |
| | Sig. (2-tailed) | .182 | | .408 | .020 | .432 |
| | N | 5 | 5 | 5 | 5 | 5 |
| TEMP | Pearson Correlation | .900* | .484 | 1 | -.237 | .433 |
| | Sig. (2-tailed) | .038 | .408 | | .701 | .466 |
| | N | 5 | 5 | 5 | 5 | 5 |
| pH | Pearson Correlation | -.554 | -.935* | -.237 | 1 | .611 |
| | Sig. (2-tailed) | .333 | .020 | .701 | | .274 |
| | N | 5 | 5 | 5 | 5 | 5 |
| Nitrate | Pearson Correlation | .268 | -.463 | .433 | .611 | 1 |
| | Sig. (2-tailed) | .663 | .432 | .466 | .274 | |
| | N | 5 | 5 | 5 | 5 | 5 |
| Ammonia | Pearson Correlation | .347 | -.147 | .527 | .158 | .406 |
| | Sig. (2-tailed) | .568 | .814 | .361 | .800 | .498 |
| | N | 5 | 5 | 5 | 5 | 5 |
| Chloride | Pearson Correlation | .703 | 1.000** | .477 | -.936* | -.467 |
| | Sig. (2-tailed) | .186 | .000 | .416 | .019 | .428 |
| | N | 5 | 5 | 5 | 5 | 5 |

**Correlations**

| | | Ammonia | Chloride | COD |
|---|---|---|---|---|
| SomaticPhage | Pearson Correlation | .338 | .520 | .581 |
| | Sig. (2-tailed) | .578 | .369 | .304 |
| | N | 5 | 5 | 5 |
| FRNAPhage | Pearson Correlation | .424 | .543 | .610 |
| | Sig. (2-tailed) | .477 | .344 | .275 |
| | N | 5 | 5 | 5 |
| VLP | Pearson Correlation | -.031 | .418 | .452 |
| | Sig. (2-tailed) | .961 | .484 | .445 |
| | N | 5 | 5 | 5 |
| THB | Pearson Correlation | .714 | .290 | .373 |
| | Sig. (2-tailed) | .176 | .636 | .537 |
| | N | 5 | 5 | 5 |
| Turbidity | Pearson Correlation | -.106 | .959* | .963** |
| | Sig. (2-tailed) | .865 | .010 | .008 |
| | N | 5 | 5 | 5 |
| BOD | Pearson Correlation | .347 | .703 | .745 |
| | Sig. (2-tailed) | .568 | .186 | .149 |
| | N | 5 | 5 | 5 |
| Conductivity | Pearson Correlation | -.147 | 1.000** | .996** |
| | Sig. (2-tailed) | .814 | .000 | .000 |
| | N | 5 | 5 | 5 |
| TEMP | Pearson Correlation | .527 | .477 | .546 |
| | Sig. (2-tailed) | .361 | .416 | .341 |
| | N | 5 | 5 | 5 |
| pH | Pearson Correlation | .158 | -.936* | -.919* |
| | Sig. (2-tailed) | .800 | .019 | .027 |
| | N | 5 | 5 | 5 |
| Nitrate | Pearson Correlation | .406 | -.467 | -.426 |
| | Sig. (2-tailed) | .498 | .428 | .475 |
| | N | 5 | 5 | 5 |
| Ammonia | Pearson Correlation | 1 | -.157 | -.068 |
| | Sig. (2-tailed) | | .801 | .914 |
| | N | 5 | 5 | 5 |
| Chloride | Pearson Correlation | -.157 | 1 | .995** |
| | Sig. (2-tailed) | .801 | | .000 |
| | N | 5 | 5 | 5 |

**Correlations**

| | | SomaticPhage | FRNAPhage | VLP | THB | Turbidity |
|---|---|---|---|---|---|---|
| COD | Pearson Correlation | .581 | .610 | .452 | .373 | .963** |
| | Sig. (2-tailed) | .304 | .275 | .445 | .537 | .008 |
| | N | 5 | 5 | 5 | 5 | 5 |

**Correlations**

| | | BOD | Conductivity | TEMP | pH | Nitrate |
|---|---|---|---|---|---|---|
| COD | Pearson Correlation | .745 | .996** | .546 | -.919* | -.426 |
| | Sig. (2-tailed) | .149 | .000 | .341 | .027 | .475 |
| | N | 5 | 5 | 5 | 5 | 5 |

**Correlations**

| | | Ammonia | Chloride | COD |
|---|---|---|---|---|
| COD | Pearson Correlation | -.068 | .995 | 1 |
| | Sig. (2-tailed) | .914 | .000 | |
| | N | 5 | 5 | 5 |

**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

**Correlations**

| | | | SomaticPhage | FRNAPhage | VLP |
|---|---|---|---|---|---|
| Spearman's rho | SomaticPhage | Correlation Coefficient | 1.000 | .900* | .900* |
| | | Sig. (2-tailed) | . | .037 | .037 |
| | | N | 5 | 5 | 5 |
| | FRNAPhage | Correlation Coefficient | .900* | 1.000 | .700 |
| | | Sig. (2-tailed) | .037 | . | .188 |
| | | N | 5 | 5 | 5 |
| | VLP | Correlation Coefficient | .900* | .700 | 1.000 |
| | | Sig. (2-tailed) | .037 | .188 | . |
| | | N | 5 | 5 | 5 |
| | THB | Correlation Coefficient | .700 | .900* | .600 |
| | | Sig. (2-tailed) | .188 | .037 | .285 |
| | | N | 5 | 5 | 5 |
| | Turbidity | Correlation Coefficient | .900* | .700 | .800 |
| | | Sig. (2-tailed) | .037 | .188 | .104 |
| | | N | 5 | 5 | 5 |
| | BOD | Correlation Coefficient | .700 | .900* | .400 |
| | | Sig. (2-tailed) | .188 | .037 | .505 |
| | | N | 5 | 5 | 5 |
| | Conductivity | Correlation Coefficient | .900* | 1.000** | .700 |
| | | Sig. (2-tailed) | .037 | . | .188 |
| | | N | 5 | 5 | 5 |
| | TEMP | Correlation Coefficient | .821 | .975** | .667 |
| | | Sig. (2-tailed) | .089 | .005 | .219 |
| | | N | 5 | 5 | 5 |
| | pH | Correlation Coefficient | .000 | -.100 | .400 |
| | | Sig. (2-tailed) | 1.000 | .873 | .505 |
| | | N | 5 | 5 | 5 |
| | Nitrate | Correlation Coefficient | .000 | .100 | .100 |
| | | Sig. (2-tailed) | 1.000 | .873 | .873 |
| | | N | 5 | 5 | 5 |
| | Ammonia | Correlation Coefficient | .600 | .800 | .500 |
| | | Sig. (2-tailed) | .285 | .104 | .391 |
| | | N | 5 | 5 | 5 |
| | Chloride | Correlation Coefficient | .900* | 1.000** | .700 |
| | | Sig. (2-tailed) | .037 | . | .188 |
| | | N | 5 | 5 | 5 |

**Correlations**

| | | | THB | Turbidity | BOD |
|---|---|---|---|---|---|
| Spearman's rho | SomaticPhage | Correlation Coefficient | .700 | .900 | .700 |
| | | Sig. (2-tailed) | .188 | .037 | .188 |
| | | N | 5 | 5 | 5 |
| | FRNAPhage | Correlation Coefficient | .900* | .700 | .900* |
| | | Sig. (2-tailed) | .037 | .188 | .037 |
| | | N | 5 | 5 | 5 |
| | VLP | Correlation Coefficient | .600 | .800 | .400 |
| | | Sig. (2-tailed) | .285 | .104 | .505 |
| | | N | 5 | 5 | 5 |
| | THB | Correlation Coefficient | 1.000 | .400 | .800 |
| | | Sig. (2-tailed) | . | .505 | .104 |
| | | N | 5 | 5 | 5 |
| | Turbidity | Correlation Coefficient | .400 | 1.000 | .600 |
| | | Sig. (2-tailed) | .505 | . | .285 |
| | | N | 5 | 5 | 5 |
| | BOD | Correlation Coefficient | .800 | .600 | 1.000 |
| | | Sig. (2-tailed) | .104 | .285 | . |
| | | N | 5 | 5 | 5 |
| | Conductivity | Correlation Coefficient | .900* | .700 | .900* |
| | | Sig. (2-tailed) | .037 | .188 | .037 |
| | | N | 5 | 5 | 5 |
| | TEMP | Correlation Coefficient | .975** | .564 | .872 |
| | | Sig. (2-tailed) | .005 | .322 | .054 |
| | | N | 5 | 5 | 5 |
| | pH | Correlation Coefficient | .200 | -.100 | -.300 |
| | | Sig. (2-tailed) | .747 | .873 | .624 |
| | | N | 5 | 5 | 5 |
| | Nitrate | Correlation Coefficient | .300 | .100 | .300 |
| | | Sig. (2-tailed) | .624 | .873 | .624 |
| | | N | 5 | 5 | 5 |
| | Ammonia | Correlation Coefficient | .900* | .200 | .600 |
| | | Sig. (2-tailed) | .037 | .747 | .285 |
| | | N | 5 | 5 | 5 |
| | Chloride | Correlation Coefficient | .900* | .700 | .900* |
| | | Sig. (2-tailed) | .037 | .188 | .037 |
| | | N | 5 | 5 | 5 |

**Correlations**

| | | | Conductivity | TEMP | pH |
|---|---|---|---|---|---|
| Spearman's rho | SomaticPhage | Correlation Coefficient | .900 | .821 | .000 |
| | | Sig. (2-tailed) | .037 | .089 | 1.000 |
| | | N | 5 | 5 | 5 |
| | FRNAPhage | Correlation Coefficient | 1.000** | .975** | -.100 |
| | | Sig. (2-tailed) | . | .005 | .873 |
| | | N | 5 | 5 | 5 |
| | VLP | Correlation Coefficient | .700 | .667 | .400 |
| | | Sig. (2-tailed) | .188 | .219 | .505 |
| | | N | 5 | 5 | 5 |
| | THB | Correlation Coefficient | .900* | .975** | .200 |
| | | Sig. (2-tailed) | .037 | .005 | .747 |
| | | N | 5 | 5 | 5 |
| | Turbidity | Correlation Coefficient | .700 | .564 | -.100 |
| | | Sig. (2-tailed) | .188 | .322 | .873 |
| | | N | 5 | 5 | 5 |
| | BOD | Correlation Coefficient | .900* | .872 | -.300 |
| | | Sig. (2-tailed) | .037 | .054 | .624 |
| | | N | 5 | 5 | 5 |
| | Conductivity | Correlation Coefficient | 1.000 | .975** | -.100 |
| | | Sig. (2-tailed) | . | .005 | .873 |
| | | N | 5 | 5 | 5 |
| | TEMP | Correlation Coefficient | .975** | 1.000 | .051 |
| | | Sig. (2-tailed) | .005 | . | .935 |
| | | N | 5 | 5 | 5 |
| | pH | Correlation Coefficient | -.100 | .051 | 1.000 |
| | | Sig. (2-tailed) | .873 | .935 | . |
| | | N | 5 | 5 | 5 |
| | Nitrate | Correlation Coefficient | .100 | .205 | .500 |
| | | Sig. (2-tailed) | .873 | .741 | .391 |
| | | N | 5 | 5 | 5 |
| | Ammonia | Correlation Coefficient | .800 | .872 | .100 |
| | | Sig. (2-tailed) | .104 | .054 | .873 |
| | | N | 5 | 5 | 5 |
| | Chloride | Correlation Coefficient | 1.000** | .975** | -.100 |
| | | Sig. (2-tailed) | . | .005 | .873 |
| | | N | 5 | 5 | 5 |

**Correlations**

| | | | Nitrate | Ammonia | Chloride | COD |
|---|---|---|---|---|---|---|
| Spearman's rho | SomaticPhage | Correlation Coefficient | .000 | .600 | .900 | .900 |
| | | Sig. (2-tailed) | 1.000 | .285 | .037 | .037 |
| | | N | 5 | 5 | 5 | 5 |
| | FRNAPhage | Correlation Coefficient | .100 | .800 | 1.000** | 1.000** |
| | | Sig. (2-tailed) | .873 | .104 | . | . |
| | | N | 5 | 5 | 5 | 5 |
| | VLP | Correlation Coefficient | .100 | .500 | .700 | .700 |
| | | Sig. (2-tailed) | .873 | .391 | .188 | .188 |
| | | N | 5 | 5 | 5 | 5 |
| | THB | Correlation Coefficient | .300 | .900* | .900* | .900* |
| | | Sig. (2-tailed) | .624 | .037 | .037 | .037 |
| | | N | 5 | 5 | 5 | 5 |
| | Turbidity | Correlation Coefficient | .100 | .200 | .700 | .700 |
| | | Sig. (2-tailed) | .873 | .747 | .188 | .188 |
| | | N | 5 | 5 | 5 | 5 |
| | BOD | Correlation Coefficient | .300 | .600 | .900* | .900* |
| | | Sig. (2-tailed) | .624 | .285 | .037 | .037 |
| | | N | 5 | 5 | 5 | 5 |
| | Conductivity | Correlation Coefficient | .100 | .800 | 1.000** | 1.000** |
| | | Sig. (2-tailed) | .873 | .104 | . | . |
| | | N | 5 | 5 | 5 | 5 |
| | TEMP | Correlation Coefficient | .205 | .872 | .975** | .975** |
| | | Sig. (2-tailed) | .741 | .054 | .005 | .005 |
| | | N | 5 | 5 | 5 | 5 |
| | pH | Correlation Coefficient | .500 | .100 | -.100 | -.100 |
| | | Sig. (2-tailed) | .391 | .873 | .873 | .873 |
| | | N | 5 | 5 | 5 | 5 |
| | Nitrate | Correlation Coefficient | 1.000 | -.100 | .100 | .100 |
| | | Sig. (2-tailed) | . | .873 | .873 | .873 |
| | | N | 5 | 5 | 5 | 5 |
| | Ammonia | Correlation Coefficient | -.100 | 1.000 | .800 | .800 |
| | | Sig. (2-tailed) | .873 | . | .104 | .104 |
| | | N | 5 | 5 | 5 | 5 |
| | Chloride | Correlation Coefficient | .100 | .800 | 1.000 | 1.000** |
| | | Sig. (2-tailed) | .873 | .104 | . | . |
| | | N | 5 | 5 | 5 | 5 |

**Correlations**

| | | | SomaticPhage | FRNAPhage | VLP |
|---|---|---|---|---|---|
| Spearman's rho | COD | Correlation Coefficient | .900* | 1.000** | .700 |
| | | Sig. (2-tailed) | .037 | . | .188 |
| | | N | 5 | 5 | 5 |

**Correlations**

| | | | THB | Turbidity | BOD |
|---|---|---|---|---|---|
| Spearman's rho | COD | Correlation Coefficient | .900* | .700 | .900* |
| | | Sig. (2-tailed) | .037 | .188 | .037 |
| | | N | 5 | 5 | 5 |

**Correlations**

| | | | Conductivity | TEMP | pH |
|---|---|---|---|---|---|
| Spearman's rho | COD | Correlation Coefficient | 1.000 | .975 | -.100 |
| | | Sig. (2-tailed) | . | .005 | .873 |
| | | N | 5 | 5 | 5 |

**Correlations**

| | | | Nitrate | Ammonia | Chloride | COD |
|---|---|---|---|---|---|---|
| Spearman's rho | COD | Correlation Coefficient | .100 | .800 | 1.000** | 1.000 |
| | | Sig. (2-tailed) | .873 | .104 | . | . |
| | | N | 5 | 5 | 5 | 5 |

*. Correlation is significant at the 0.05 level (2-tailed).
**. Correlation is significant at the 0.01 level (2-tailed).

# APPENDIX iii

## Tissue Culture Nested PCR/RT-PCR and Blast Analysis

**Tissue culture PCR/RT-PCR**

## A) Adenovirus

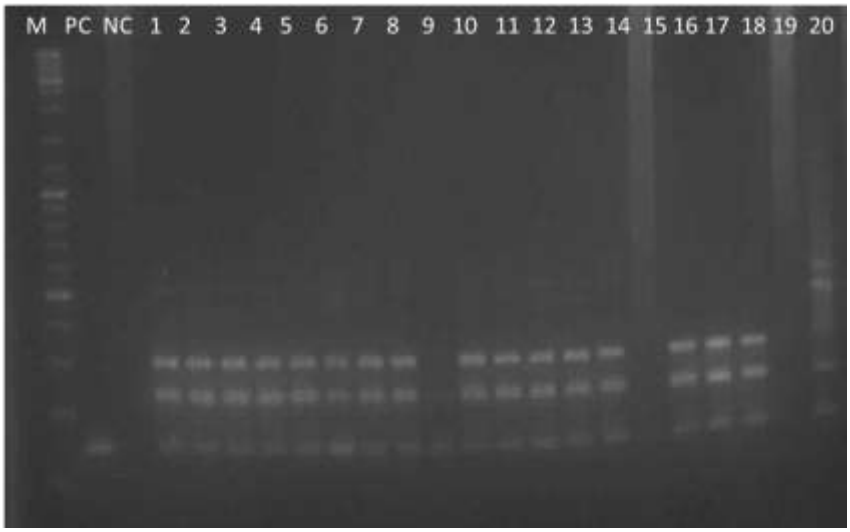



Figure 1: Tissue cell HEK 293 nested PCR amplification of the hexon gene region of 47 different Adenovirus serotypes. M: Molecular weight marker, NC: No template control, PC: Adenovirus Control, 1-5: Points for autumn season, 6-10: Points for winter season, 11-15: Points for spring season, 16-20: Points for summer season.

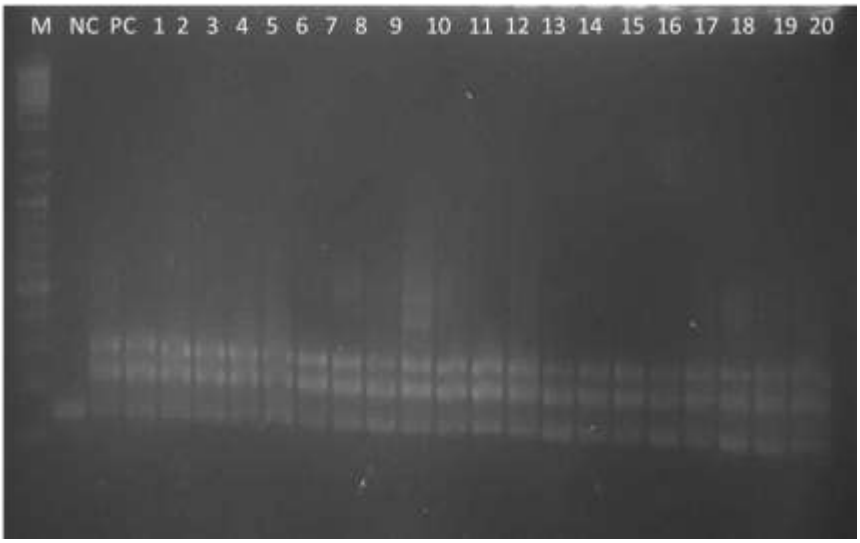



Figure 2: Tissue cell HEP G2 nested PCR amplification of the hexon gene region of 47 different Adenovirus serotypes. M: Molecular weight marker, NC: No template control, PC: Adenovirus Control, 1-5: Points for autumn season, 6-10: Points for winter season, 11-15: Points for spring season, 16-20: Points for summer season.

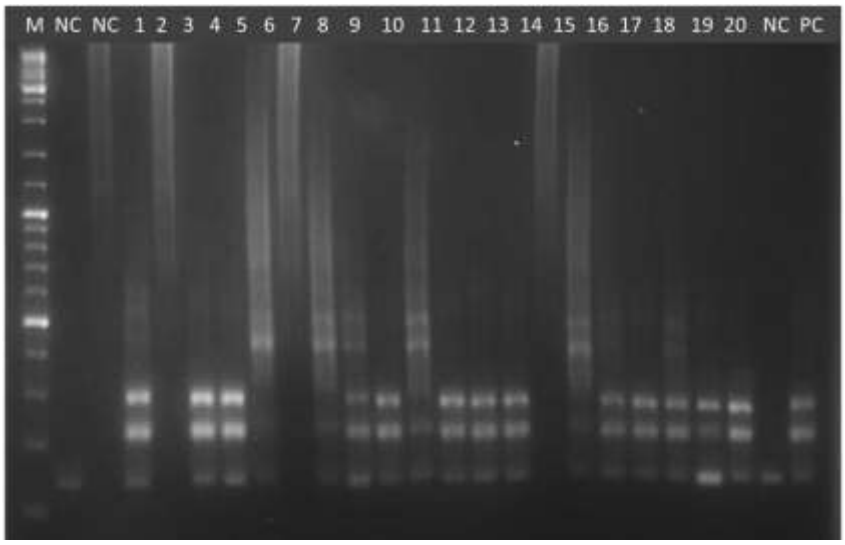



Figure 3: Tissue cell PLC/PRF/5 nested PCR amplification of the hexon gene region of 47 different Adenovirus serotypes. M: Molecular weight marker, NC: No template control, PC: Adenovirus Control, 1-5: Points for autumn season, 6-10: Points for winter season, 11-15: Points for spring season, 16-20: Points for summer season.

**B) Hepatitis B virus**

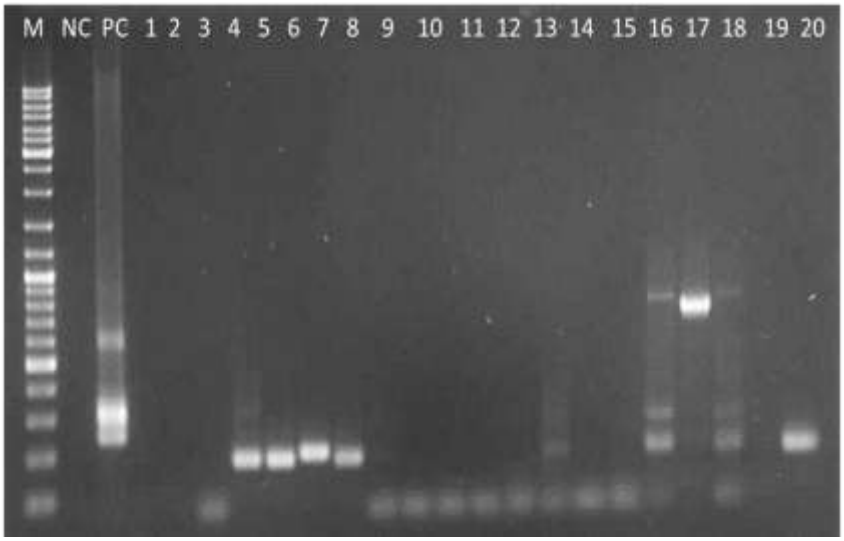



Figure 4: Tissue cell HEK 293 nested PCR amplification of the S gene region of Hepatitis B viruses. M: Molecular weight marker, NC: No template control, PC: Hepatitis B virus Control, 1-5: Points for autumn season, 6-10: Points for winter season, 11-15: Points for spring season, 16-20: Points for summer season.


Figure 5: Tissue cell HEP G2 nested PCR amplification of the S gene region of Hepatitis B viruses. M: Molecular weight marker, NC: No template control, PC: Hepatitis B virus Control, 1-5: Points for autumn season, 6-10: Points for winter season, 11-15: Points for spring season, 16-20: Points for summer season.


Figure 6: Tissue cell PLC/PRF/5 nested PCR amplification of the S gene region of Hepatitis B viruses. M: Molecular weight marker, NC: No template control, PC: Hepatitis B virus Control, 1-5: Points for autumn season, 6-10: Points for winter season, 11-15: Points for spring season, 16-20: Points for summer season.

**C) Enterovirus**

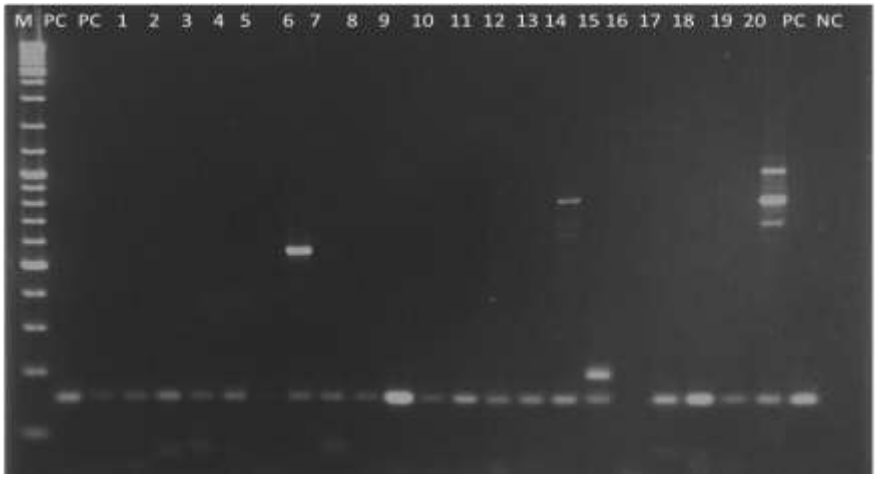



Figure 7: Tissue cell A549 nested RT-PCR amplification of the 5'-untranslated region of the Enterovirus genome detecting at least 25 different Enteroviruses. M: Molecular weight marker, NC: No template control, PC: Coxsackie B virus Control, 1-5: Points for autumn season, 6-10: Points for winter season, 11-15: Points for spring season, 16-20: Points for summer season.

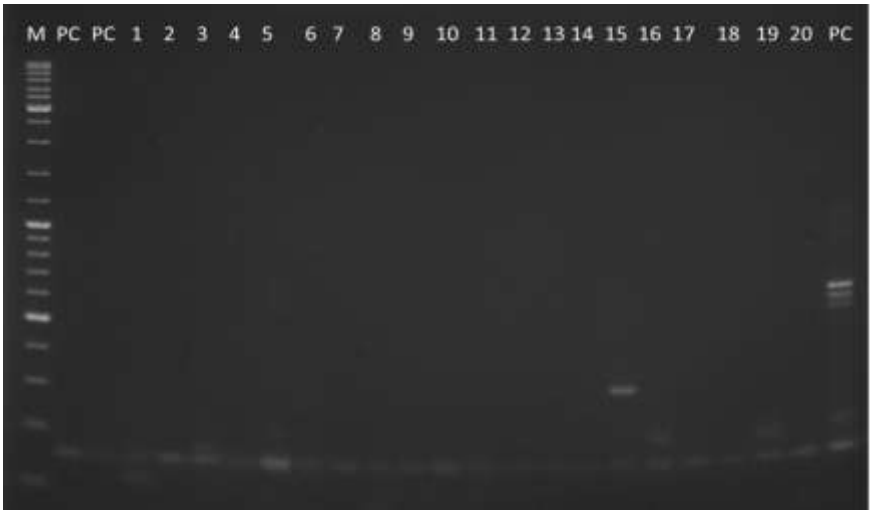



Figure 8: Tissue cell VERO nested RT-PCR amplification of the 5'-untranslated region of the Enterovirus genome detecting at least 25 different Enteroviruses. M: Molecular weight marker, NC: No template control, PC: Coxsackie B virus Control, 1-5: Points for autumn season, 6-10: Points for winter season, 11-15: Points for spring season, 16-20: Points for summer season.

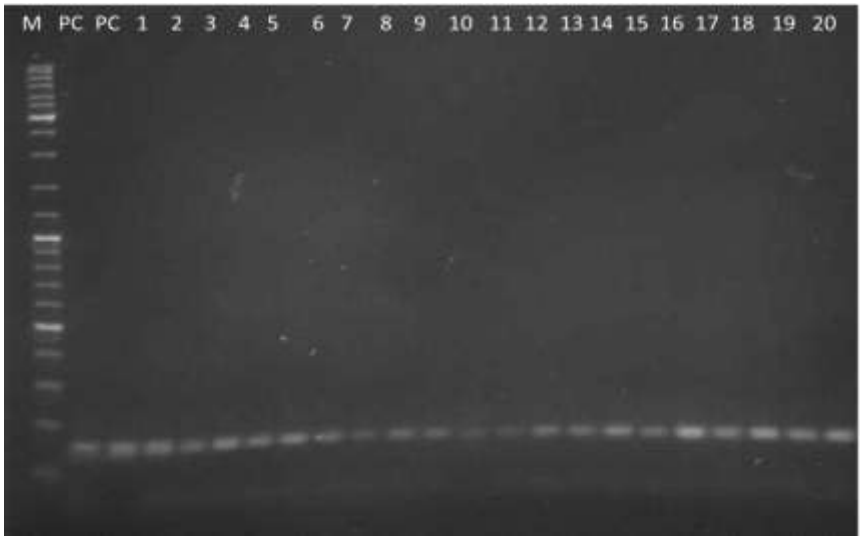



Figure 9: Tissue cell HEK 293 nested RT-PCR amplification of the 5'-untranslated region of the Enterovirus genome detecting at least 25 different Enteroviruses. M: Molecular weight marker, NC: No template control, PC: Coxsackie B virus Control, 1-5: Points for autumn season, 6-10: Points for winter season, 11-15: Points for spring season, 16-20: Points for summer season.

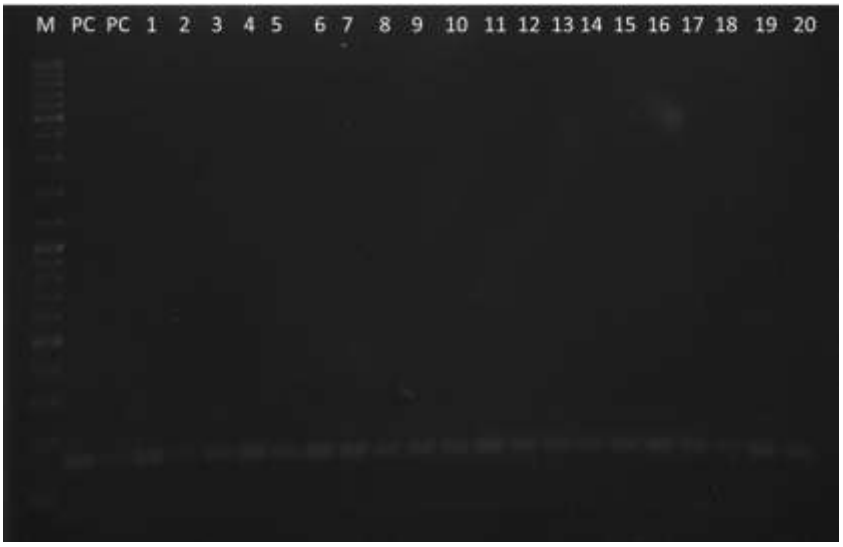



Figure 10: Tissue cell HEP G2 nested RT-PCR amplification of the 5'-untranslated region of the Enterovirus genome detecting at least 25 different Enteroviruses. M: Molecular weight marker, NC: No template control, PC: Coxsackie B virus Control, 1-5: Points for autumn season, 6-10: Points for winter season, 11-15: Points for spring season, 16-20: Points for summer season.

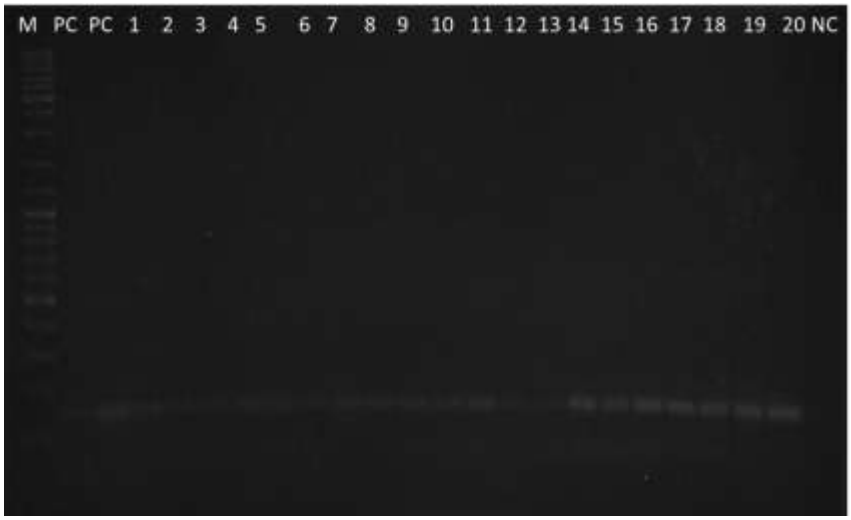



Figure 11: Tissue cell PLC/PRF/5 nested RT-PCR amplification of the 5'-untranslated region of the Enterovirus genome detecting at least 25 different Enteroviruses. M: Molecular weight marker, NC: No template control, PC: Coxsackie B virus Control, 1-5: Points for autumn season, 6-10: Points for winter season, 11-15: Points for spring season, 16-20: Points for summer season.

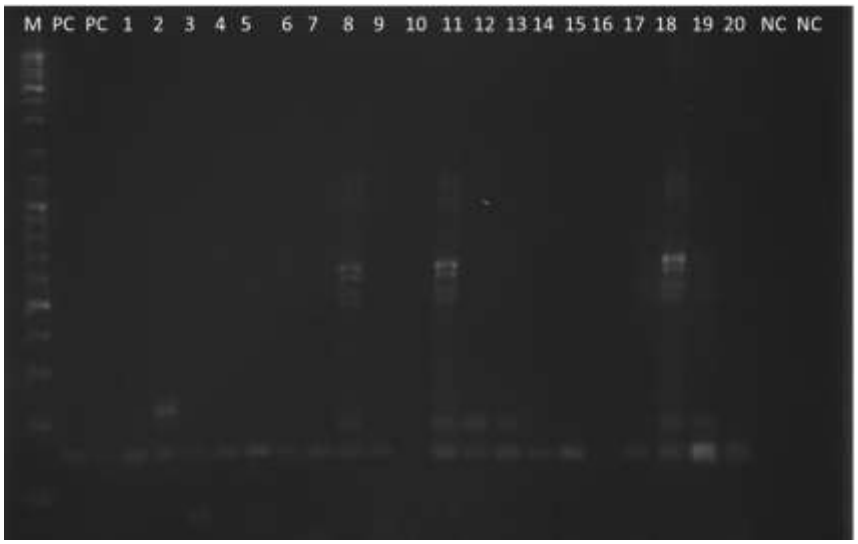



Figure 12: Tissue cell HELA nested RT-PCR amplification of the 5'-untranslated region of the Enterovirus genome detecting at least 25 different Enteroviruses. M: Molecular weight marker, NC: No template control, PC: Coxsackie B virus Control, 1-5: Points for autumn season, 6-10: Points for winter season, 11-15: Points for spring season, 16-20: Points for summer season.

**D) Rotavirus**

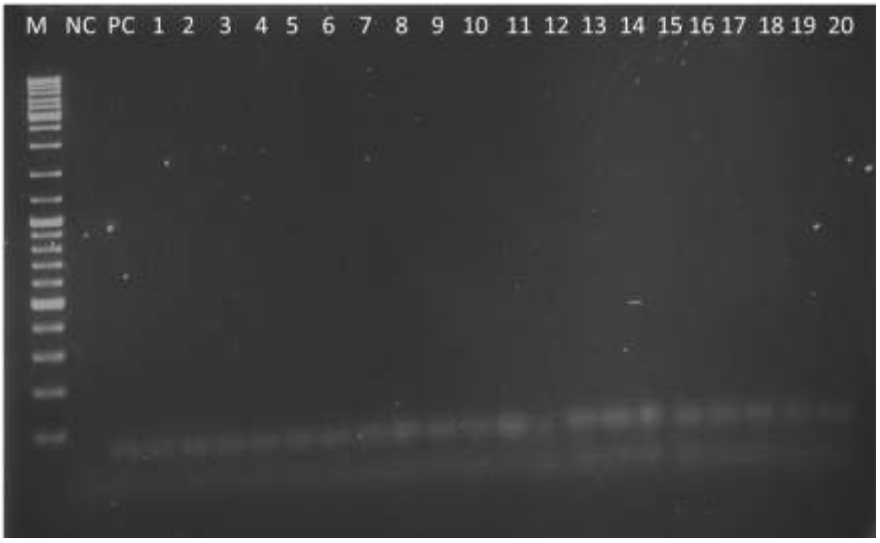



Figure 13: Tissue cell VERO nested RT-PCR amplification of the VP7 gene of group A Rotaviruses. M: Molecular weight marker, NC: No template control, PC: Coxsackie B virus Control, 1-5: Points for autumn season, 6-10: Points for winter season, 11-15: Points for spring season, 16-20: Points for summer season.

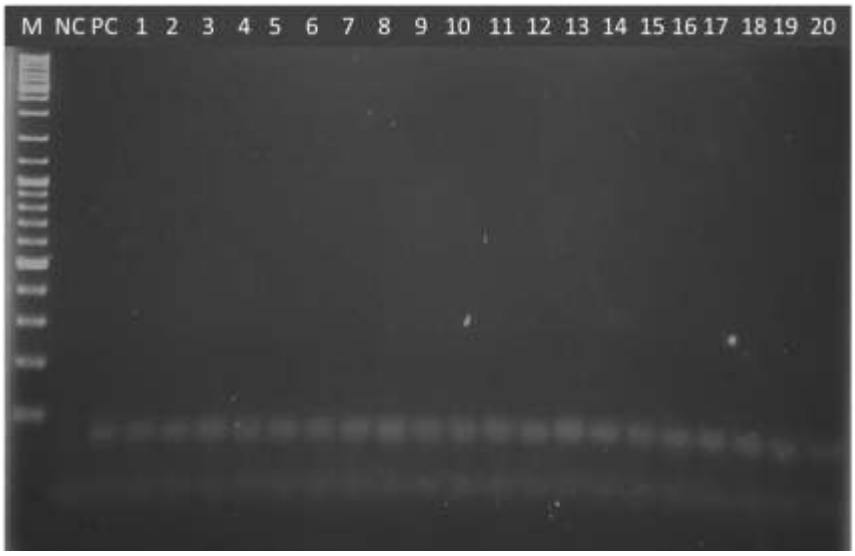



Figure 14: Tissue cell A549 nested RT-PCR amplification of the VP7 gene of group A Rotaviruses. M: Molecular weight marker, NC: No template control, PC: Coxsackie B virus Control, 1-5: Points for autumn season, 6-10: Points for winter season, 11-15: Points for spring season, 16-20: Points for summer season.


Figure 15: Tissue cell PLC/PRF/5 nested RT-PCR amplification of the VP7 gene of group A Rotaviruses. M: Molecular weight marker, NC: No template control, PC: Coxsackie B virus Control, 1-5: Points for autumn season, 6-10: Points for winter season, 11-15: Points for spring season, 16-20: Points for summer season.


Figure 16: Tissue cell HEP G2 nested RT-PCR amplification of the VP7 gene of group A Rotaviruses. M: Molecular weight marker, NC: No template control, PC: Coxsackie B virus Control, 1-5: Points for autumn season, 6-10: Points for winter season, 11-15: Points for spring season, 16-20: Points for summer season.


Figure 17: Tissue cell HEK 293 nested RT-PCR amplification of the VP7 gene of group A Rotaviruses. M: Molecular weight marker, NC: No template control, PC: Coxsackie B virus Control, 1-5: Points for autumn season, 6-10: Points for winter season, 11-15: Points for spring season, 16-20: Points for summer season.

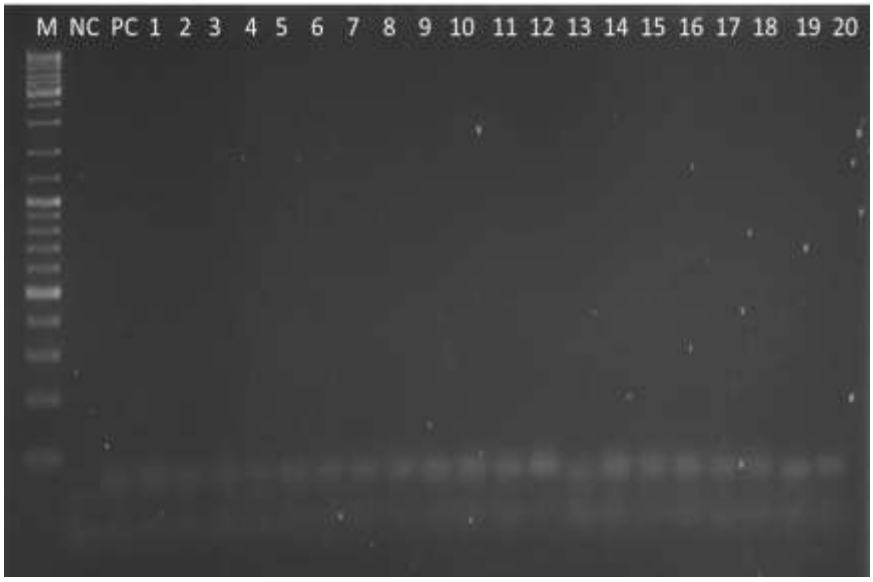



Figure 17: Tissue cell HELA nested RT-PCR amplification of the VP7 gene of group A Rotaviruses. M: Molecular weight marker, NC: No template control, PC: Coxsackie B virus Control, 1-5: Points for autumn season, 6-10: Points for winter season, 11-15: Points for spring season, 16-20: Points for summer season

**BLAST ANALYSIS SHOWING FIRST 3 HITS OF EACH RAW SEQUENCE DATA**

**Adenovirus Nested PCR Sequences from Different Sampling Points and Seasons Along The Umgeni River**

**U1-AUTUMN**

TAGAACCACGGTGGCACCTACGCACGACGTAACCACAGACCGGTCCCAGCGTTTGACGCTGCGGTTCAT
CCCTGTGGACCGCGAGGATACCGCGTACTCGTACAAAACGCAGCGTCAACGCTGGGACCGGTCTGTGGT
TACGTCGTGCGTAGGTGCCACCGTGGGGTTTCTAAACTTGTTATTCAGGCTGAAGTACKTCTCGGTGGCC
CCYASGCMCGACGTAACCMCRGACSGGTCCCRGCGTTTGACGCTGCGSTTCTTGWRSGCSGGGRAAAAC
CMMYTACTCAACCAAA

| Accession | Description | E value | Max ident |
|---|---|---|---|
| EF205290.1 | Human adenovirus 2 isolate TWT003 hexon gene, partial cds | 1e-44 | 99% |

**U2-AUTUMN**

TTAGTACCCCACGTGTGAMTCYRMSCACGAYGTAACCACAGACMGGTCMCAGCGWYTGACGCTGCGRT
TCRTSCCYGTSGACCGCGAGGATACCGCGTACTCGTACAAAGCAGCGTCAAYSCTKGYGACCKGTCTGT
GGTTACRTCGTGSGTRGGWGCCACMGTGGGGTTTCTAGAACTTGTTAYCYCAGGCTRAAGTACGTCYCG
GTGRCCMCTACGCACGACGTAACCACAGACSGGTCCCRGSGTTTGACGCTGSGGTTCATCCCKGTGGAC
CGMRASGATAYCGMRWAAAAA

| Accession | Description | E value | Max ident |
|---|---|---|---|
| FJ943610.1 | Human adenovirus 2 strain RKI-1366/07 hexon gene, partial cds | 9e-06 | 97% |

**U3-AUTUMN**

AACCCACGTGTGGCMTCYAMSCACGAYGTAACCACAGACMGGTCMCAGCGWYTGACGCTGCGSTTCRT
SCCYGTSGACCGCGAGGATACCGCGTACTCGTACAAAGCAGCGTCAAYSCCTKGYGACCKGTCTGTGGT
TACATCGTGSGTRGGAGCCACMGTGGGGTTTCTAAAACTTGTTAYCCCAGGCTRAAGTACGTCYCGGTG
MCCMCTAMGCACGACGTAACCMCAGACSGGTCCRGSGTTTGASGCTGSGGTTCATCCCKGTGGACCGM
GAGGATACCGMRWAMWCGTACAA

| Accession | Description | E value | Max ident |
|---|---|---|---|
| FJ943585.1 | Human adenovirus 2 strain RKI-0414/02 hexon gene, partial cds | 8e-06 | 97% |

**U4-AUTUMN**

TTRGTAACCCACGTGTGCMTCYAMSCACGAYGTAACCACAGACMGGTCMCAGCGWYTGACGCTGCGRT
TCRTSCCYGTSGACCGCGAGGATACCGCGTACTCGTACAAAACGCAGCGTCAAYSCYKGYGACCKGTCT
GTGGTTACRTCGTGSGTMGGAGCCACMGTGGGGTTTCTAAACTTGTTAYCYCAGGCTRAAGTACGTCYC
GGTGRCCMCTAMGCACGACGTAACCMCAGACSGGTCCCRGSGTTTGACGCTGSGGTTCATCCCKGTGGA
CCGMRAGGATACCGMGWAMWCGTACAA

| Accession | Description | E value | Max ident |
|---|---|---|---|
| EU867467.1 | Human adenovirus 2 strain RKI-0135/05 hexon gene, partial cds | 4e-24 | 88% |

**U5-AUTUMN**

ATAGTACCCACGYGTGGCTCTAMSCACGAYGTAACCACAGACMGGTCMCAGCGWYTGACGCTGCGRTT
CRTSCCYGTSGACCGCGAGGATACCGCGTACTCGTACAAAGCAGCGTCAAYSSYTGGGACCKGTCTGTG
GTTACRTCGTGSGTRGGAGCCACMGTGGGGTTTCTAGACTTGTTAYCYCAGGCTGAAGTACGTCTCGGT

| Accession | Description | E value | Max ident |
|---|---|---|---|
| FJ943610.1 | Human adenovirus 2 strain RKI-1366/07 hexon gene, partial cds | 6e-06 | 97% |

**U1-WINTER**

TTARGACCCACGYGTGGMTCTAMSCACGAYGTAACCACAGACMGGTCMCAGCGWYTGACGCTGCGSTT
CRTSCCYGTSGACCGCGAGGATACCGCGTACTCGTACAAATCAGCGTCAAYSCTGGGACCKGTCTGTGGT
TACRTCGTGSGTRGGAGCCACCGTGGGGTTTCTARACTTGTTAYYCAGGCTGAAGTACGTCTCGGTGGM

| Accession | Description | E value | Max ident |
|---|---|---|---|

| Accession | Description | E value | Max ident |
|---|---|---|---|
| AM920719.1 | Human adenovirus 41 partial L4 gene for hexon protein | 7e-25 | 90% |

**U2-WINTER**

TTAGGAACCACSGTGGCTCTAMSCACGAYGTAACCACAGACMGGTCCCAGCGWYTGACGCTGCGSTTC
WTSCCYGTSGACCGCGAGGATACCGCGTACTCGTACAAAGCAGCGTCAAYSCTKGGACCKGTCTGTGGT
TACRTCGTGSGTRGGWGCCACCGTGGGGTTTCTARACTTGTTMTYCAGGCTGAAGTACGTCTCGGTGGM

| Accession | Description | E value | Max ident |
|---|---|---|---|
| AY288441.1 | Human adenovirus AdV/Berlin004/02/DE hexon protein gene, partial cds | 2e-25 | 90% |

**U3-WINTER**

TWRTACCCACGYGTGGCYCYAMSCACGAYGTAACCACAGACMGGTCCCAGCGWYTGACGCTGCGGTT
CRTSCCYGTSGACCGCGAGGATACCGCGTACTCGTACAAAGCAGCGTCAAYSCYKGYGACCKGTCTGTG
GTTACRTCGTGSGTRGGWGCCACCGTGGGGTTTCTARACTTGTTAYCYCAGGCTRAAGTACKTCYCGGT
GGACCCYASGCACGACGTAACCMCAGACSGGTCCCRGSGTTTGACGCTGCGGTTCATCCCKGTGGACCG
YWMSGATWWTWAAAAAAAA

| Accession | Description | E value | Max ident |
|---|---|---|---|
| JX173077.1 | Human adenovirus C strain human/ARG/A8649/2005/2[P2H2F2], complete genome | 9e-06 | 97% |

**U4-WINTER**

GACCCACGTGTGGMTCYAMSCACGAYGTAACCACAGACMGGTCMCAGCGWYTGACGCTGCGRTTCRTS
CCYGTSGACCGCGAGGATACCGCGTACTCGTACAAAACGCAGCGTCAAYSCYTGGGACCKGTCTGTGGT
TACRTCGTGSGTRGGTAGCCACCGTGGGGTTTCTAGACTTGTTAYCYCAGGCTGAAGTACGTCTCGGTGG

| Accession | Description | E value | Max ident |
|---|---|---|---|
| JX173084.1 | Human adenovirus C strain human/USA/VT5544/2003/2[P2H2F2], complete genome | 2e-28 | 87% |

**U5-WINTER**

AACCCCACGTGTGGCATCTAMSCACGAYGTAACCACAGACMGGTCCCAGCGWYTGACGCTGCGGTTCR
TSCCYGTSGACCGCGAGGATACCGCGTACTCGTACAAAGCAGCGTCAAYSCTGGGACCKGTCTGTGGTT
ACRTCGTGSGTRGGTAGCCACCGTGGGGTTTCTARACTTGTTAYCYCAGGCTGAAGTACGTCTCGGTGG

| Accession | Description | E value | Max ident |
|---|---|---|---|
| FJ943610.1 | Human adenovirus 2 strain RKI-1366/07 hexon gene, partial cds | 6e-06 | 97% |

**U1-SPRING**

AACCCCACGTGTGGCRTCTAMSCACGAYGTAACCACAGACMGGTCMCAGCGWYTGACGCTGCGRTTCR
TSCCYGTSGACCGCGAGGATACCGCGTACTCGTACAAAACGCAGCGTCAAYSCYTGGGACCKGTCTGTG
GTTACRTCGTGSGTRGGAGCCACCGTGGGGTTTCTARACTTGTTAYCYCAGGCTGAAGTACGTCTCGGTG
GM

| Accession | Description | E value | Max ident |
|---|---|---|---|
| JX173084.1 | Human adenovirus C strain human/USA/VT5544/2003/2[P2H2F2], complete genome | 3e-32 | 87% |

**U2-SPRING**

TTARGTACCCACGGTGGCAYCTAMGCACGAYGTAACCACAGACCGGTCCCAGCGWYTGACGCTGCGGT
TCATSCCTGTGGACCGCGAGGATACCGCGTACTCGTACAAAACGCAGCGTCAAYSCTKGGACCKGTCTG
TGGTTACRTCGTGCGTAGGWGCCACCGTGGGGTTTCTAAACTTGTTATYCAGGCTGAAGTACGTCTCGG
TGGACMCCWACCCAGAAGTTACCACAGACGGGTCCCRGSGTTTGACGCTGCGGTTCYTGCSGGTGGAA
WGCGAGGATWCCGCAAAAAAAGT

| Accession | Description | E value | Max ident |
|---|---|---|---|
| JX173084.1 | Human adenovirus C strain human/USA/VT5544/2003/2[P2H2F2], complete | 4e-39 | 93% |

| Accession | Description | E value | Max ident |
|---|---|---|---|
| | genome | | |

**U3-SPRING**

TTAGAACCCACGGTGGCAYCTAMSCACGAYGTAACCACAGACCGGTCCCAGCCCAGCGTCAACSCTGGG
ACCGGTCTGTGGTTACRTCGTGSGTAGGTGCCACCGTGGGGTTTCTAAACTTGTTATTCAGGCTGAAGTA
CGTCTCGGTGGCCGWYTGACGCTGCGGTTCATSCCTGTGGACCGCGAGGATACCG CGTACTCGTACAAA

| Accession | Description | E value | Max ident |
|---|---|---|---|
| JX173084.1 | Human adenovirus C strain human/USA/VT5544/2003/2[P2H2F2], complete genome | 4e-37 | 96% |

**U4-SPRING**

TWRGTACCCCACGGTGGCATCTAMGCACGAYGTAACCACAGACMGGTCMCAGCGWYTGACGCTGCGS
TTCWTSCCYGTSGACCGCGAGGATACCGCGTACTCGTACAAATCAGCGTCAAYSCYTGGGACCKGTCTG
TGGTTACRTCGTGSGTRGGAGCCACCGTGGGGTTTCTARACTTGTTAYCYCAGGCTGAAGTACGTCTCGG
TGGM

| Accession | Description | E value | Max ident |
|---|---|---|---|
| FJ943608.1 | Human adenovirus 2 strain RKI-1114/07 hexon gene, partial cds | 8e-05 | 100% |

**U5-SPRING**

TTTRAAACCAMGGTGGCATCTAMSCACGAYGTAACCACAGACMGGTCMCAGCGWYTGACGCTGCGRTT
CRTSCCYGTSGACCGCGAGGATACCGCGTACTCGTACAAATCAGCGTCAAYSCYKGGACCKGTCTGTGG
TTACRTCGTGSGTRGGTAGCCACCGTGGGGTTTCTARACTTGTTAYCYCAGGCTGAAGTACGTCTCGGTG
GM

| Accession | Description | E value | Max ident |
|---|---|---|---|
| FJ943592.1 | Human adenovirus 2 strain RKI-1157/02 hexon gene, partial cds | 7e-05 | 100% |

**U1-SUMMER**

ATAGACCCACGTGTGGCATCTAMSCACGAYGTAACCACAGACMGGTCMCAGCGWYTGACGCTGCGGTT
CWTSCCYGTSGACCGCGAGGATACCGCGTACTCGTACAAAGCAGCGTCAAYSCTKGGACCKGTCTGTGG
TTACRTCGTGSGTRGGTAGCCACCGTGGGGTTTCTARACTTGTTAYCYCAGGCTGAAGTACGTCTCGGTG
GM

| Accession | Description | E value | Max ident |
|---|---|---|---|
| FJ943592.1 | Human adenovirus 2 strain RKI-1157/02 hexon gene, partial cds | 6e-06 | 97% |

**U2-SUMMER**

AYCCCAASCCYGACKGTAMCCACGAYGTAACCACAGACMGGTCRCAGCGWYTGACGCTGCGGTTRCCC
KGTGGACCGCGAGGATACCGCGTACTCGTACKGSACCTACGCASGCSKWAACAGCCGGKCCCGTTTAGA
TGCGKTTTCTCTTGAGCCGGAGAAKCTCTTATAAAAAAAACGCAGCGTCAAYSCTKGGACCKGTCTGTG
GTTACRTCGTGSGTMGGAGCCACCGTGGGGTTTYTARACTTGTTAYTCAGGCTGAAGTACGTCTCGGTG
GM

| Accession | Description | E value | Max ident |
|---|---|---|---|
| FJ943610.1 | Human adenovirus 2 strain RKI-1366/07 hexon gene, partial cds | 1e-04 | 100% |

**U3-SUMMER**

TACCCACGCGCTGGGYGCYACGCACGAYGTAACCACAGACCGGTCRCAGCGWYTGACGCTGCGGTTCR
CCCKGKGGACCGCGAGGATACCGCGTACTCATACAACCCCTACGCAGASKWACCSAGGGGCCGTTTTAR
RGGGTTTTCTGTGGACGCCGGATACTCTTTTCCCAAAAAAACGCAGCGTCAAYSCTKGGACCKGTCTGTG
GTTACRTCGTGSGTMGGWGCCACCGTGGGGTTTCTARACTTGTTATYCAGGCTGAAGTACGTCTCGGTG
GM

| Accession | Description | E value | Max ident |
|---|---|---|---|
| EU867464.1 | Human adenovirus 2 strain RKI-1947/03 hexon gene, partial cds | 1e-04 | 100% |

**U4-SUMMER**

GACCCAACSGGTTGGGTTCKWACGCACGAKGTAACCACAGACCGGTCACAGCGTTTGACGCTGCGGTTC
ACCCKGTGGACCGCGAGGATACCGCGTAYTCGTACAGACCTACGCAGAGTTACGAGGGGGTCGGTTTM
MTGGGTGCTCCTTGGGGGCGAGTGCTGGGTTGTSAAAAAACGCAGCGTCAAYGCTKGACCGGTCTGTGG
TTACRTCKKGCAGTAGGWGCCACCGTGGGGTTTCTAAACTTGTTATTCAGGCTGAAGTACGTCTCGGTG
GC

| Accession | Description | E value | Max ident |
|---|---|---|---|
| JX173084.1 | Human adenovirus C strain human/USA/VT5544/2003/2[P2H2F2], complete genome | 6e-32 | 91% |

**U5 –SUMMER**

TACCCCAACGSGKTGGGKTCTACSCACGATGTAACCACAGACCGGTCCCAGCGKTTGACGCTGCGGTTC
RCCCWGTGGACCGCGAGGATACCGCGTACTCGTACAAAACCTACGCACGAGTTAWGMGGGGRWACGT
GTTCTTYTTTCTTTCWCYGCTTTMATCCCGACCATGAAAWGCCGCGTGGTCTTATTTCACWTKTGGGGA
GCGATGCCTCGAAGTGTTGAGCCCCGGGGTGGGGGGGTMTGCCCGTGCCAACGAGTCTCCTTWCCCYG
AGAAACAAGGTAAGAAACCCCCCGGGTGGACGTTGCACGTGTTAGTGTTAACGGGGGCGAGGGTGCAC
CGTGGGGTTTCTTGMTGTGGACGCGGGGAGACGTSTYTTCGAGAACGCAGCGTCAAYGCTKGGACCGGT
CTGTGGTTACRTCGTGCGTAGGAGCCACCGTGGGGTTTCTAAACTTGTTAYYCAGGCTGAAGTACGTCTC
GGTGGC

| Accession | Description | E value | Max ident |
|---|---|---|---|
| FJ943592.1 | Human adenovirus 2 strain RKI-1157/02 hexon gene, partial cds | 3e-07 | 97% |

**ADENOVIRUS CONTROL**

AACCWCCAARCGTGGCWKCTACGCACGAYGTAACCACAGACCGGTCCCAGCGTTTGACGCTGCGGTTC
ATCCSKGKGGACCGCGAGGATACCGCGTACTCATACAAAAACCTACGCACGACTAACCCCGRGGGTCGT
GTTWGCTCTGYTTTTCTTYTGTGGACGGATACCCKCTTTTAAAAAAAGCAKACCGCAGCGTCAACSCTG
GGACCGGTCTGTGGTTACRTCGTGCGTAGGWGCCACCGTGGGGTTTCTAAACTTGTTATTCAGGCTGAA
GTACGTCTCGGTGGC

**Enterovirus Nested RT-PCR Sequences from Different Sampling Points and Seasons Along The Umgeni River**

**U1-AUTUMN**

GGRMGTGCCTCCATTCCAGGRGGTGGCGTGTCGTAACGGGCAACTCTGCAGCGGAACCGACTACTTTGG
KTGTCCGTGTTTCTTTTAATTTTATACTGGTCGCTTATGGTGACAATKRAAAGATTGYTGCCATATAGCT
ATTGGATTGGCCATCCGGTKATGACCGGACACCCAAAGTAGTCGGTTCCGCTGCAGAGTTGCCCGTTAC
GACACGCCACCYCCTGGAATGGAGGCACGTGCTCCGCAGTTAGGATTAGCCGCATTCAGGGGCGGAGG
A

| Accession | Description | E value | Max ident |
|---|---|---|---|
| AJ295197.1 | Human coxsackievirus B5 genomic RNA for partial polyprotein gene, isolate VR-1036, ATCC | 9e-65 | 98% |

**U2-AUTUMN**

TGRAMGTGCCTCCATTCCAGGGGGTGGCGTGTCGTAACGGGCAACTCTGCAGCGGAACCGACTACTTTG
GGTGTCCGTGTTTCTTTTAATTTTATACTGGTCGCTTATGGTGACAATGACCGGACACCCAAAGTAGTCG
GTTCCGCTGCAGAGTTGCCCGTTACGACACGCCACCCCCTGGAATGGAGGCACGTGCTCCGCAGTTAGG
ATTAGCCGCATTCAGGGGCGGAGGA

| Accession | Description | E value | Max ident |
|---|---|---|---|
| AY843300.1 | Human enterovirus 82 strain USA/CA64-10390, complete genome | 7e-46 | 97% |

**U3-AUTUMN**

GGRAMAGTGCCTCCATTCCAGGGGGTGGCGTGTCGTAACGGGCAACTCTGCAGCGGAACCGACTACTTT
GGKTGTCCGTGTTTCTTTTAATTTTATACTGGTCGCTTATGGTGACAATGACCGGGCACACCCAAAGTAG
TCGGTTCCGCTGCAGAGTTGCCCGTTACGACACGCCACCCCCTGGAATGGAGGCACGTGCTCCGCAGTT
AGGATTAGCCGCATTCAGGGGCGGAGGA

| Accession | Description | E value | Max ident |
|---|---|---|---|
| AJ295197.1 | Human coxsackievirus B5 genomic RNA for partial polyprotein gene, isolate VR-1036, ATCC | 1e-47 | 99% |

**U4-AUTUMN**

CCGAMGCTGCCTCCATTCCAGGGGGTGGCGTGTCGTAACGGGCAACTCTGCAGCGGAACCGACTACTTT
GGGTGTCCGTGTTTCTTTTAATTTTATACTGGTCGCTTATGGTGACAATTACCGGCACACCCAAAGTAGT
CGGTTCCGCTGCAGAGTTGCCCGTTACGACACGCCACCCCCTGGAATGGAGGCACGTGCTCCGCAGTTA
GGATTAGCCGCATTCAGGGGCGGAGGA

| Accession | Description | E value | Max ident |
|---|---|---|---|
| AJ295197.1 | Human coxsackievirus B5 genomic RNA for partial polyprotein gene, isolate VR-1036, ATCC | 4e-48 | 98% |

**U5-AUTUMN**

GGGACMGTGCCTCCATTCCAGGGGGTGGCGTGTCGTAACGGGCAACTCTGCAGCGGAACCGACTACTTT
GGGTGTCCGKGTTTCTTTTAATTTTATACTGGYCGCTTATGGTGACAAAYRAAAGATTGTTGCCATATAG
CTATTGGATTGGCCATCCGGTAAGAACCGGACACCCAAAGTAGTCGGTTCCGCTGCAGAGTTGCCCGTT
ACGACACGCCACCCCCTGGAATGGAGGCACGTGCTCCGCAGTTAGGATTAGCCGCATTCAGGGGCGGA
GGA

| Accession | Description | E value | Max ident |
|---|---|---|---|
| AF114383.1 | Coxsackievirus B5 strain Faulkner, complete genome | 2e-66 | 98% |

**U1-WINTER**

GGAMSGTGCCTCCMTTCCAGGGGGTGGCGTGTCGTAACGGGCAACTCTGCAGCGGAACCGACTACTTY
GKGTGTCCGTGTTTCTTTTAATTTTATACWGGTCGCTTATGGTGACAATAGACCGGACACCCAAAGTAGT
CGGTTCCGCTGCAGAGTTGCCCGTTACGACACGCCACCCCCTGGAATGGAGGCACGTGCTCCGCAGTTA
GGATTAGCCGCATTCAGGGGCGGAGGA

| Accession | Description | E value | Max ident |
|---|---|---|---|
| AJ295197.1 | Human coxsackievirus B5 genomic RNA for partial polyprotein gene, isolate VR-1036, ATCC | 3e-49 | 99% |

**U2-WINTER**

GKCCTCCATTCCAGGGGGTGGCGTGTCGTAACGGGCAACTCTGCAGCGGAACCGACTACTTTGGTGTCC
GTGTTTCTTTTTATTTTATTCTGGTCKCTCATGGTGACCATAKRAAAGATTGTTGCCATATAGCTATKGGA
TKGGCCATCCGGWAAGACCCGGACACCCAAAGTAGTCGGTTCCGCTGCARAGTTGCCCGTTACRACACG
CCACCCCCTGGAATGGAGGCACGTGCTCCGCAGTTAGGATTAGCCGCATTCAGGGGCGGAGG

| Accession | Description | E value | Max ident |
|---|---|---|---|
| AJ295197.1 | Human coxsackievirus B5 genomic RNA for partial polyprotein gene, isolate VR-1036, ATCC | 5e-52 | 97% |

**U3-WINTER**

TGRAMGTGCCTCATTCCAGGGGGTGGCGTGTCGTAACGGGCAACTCTGCAGCGGAACCGACTACTTTGG
GTGTCCGTGTTTCTTTTAATTTTATACTGGTCGCTTATGGTGACAAAYRAAAGATTGTTGCCATATAGCT
ATTGGATTGGCCMTCCGGTAAAACCGCACACCCAAAGTAGTCGGTTCCGCTGCAGAGTTGCCCGTTACG
ACACGCCACCCCCTGGAATGGAGGCACGTGCTCCGCAGTTAGGATTAGCCGCATTCAGGGGCGGAGGA
CACKMMYCCRTTCCATAGACTCKTCTGTCCWAGTCCAAATCTAAGCSTACCATGGATTGCCCCTTGGGT
ATTTCSCTTACTTTTCCACCGCTCCTKATAGCCGCGTGAAATGCGGTGACTCATCSACCTGATCTACACTG
GGAATGTGCTGARTGAAACACTCCGCAATTTCCATGGTGTTACTGGGTTTCTCGAAGTAGTTAGTCGGGT
AACGGACGTTTTCTCCTTCSACCGCGCAAGCAGTTTATTGATACTCAGTCCGGGGAAACAGAAGTGCTTG
ATCAAGACATGGCTGGCGTGTCATGCCTACTATTGATCGAGGTGTATTGCTTCTAAGTTTCAAATTGGGG
AGAGGGTTTTAAAACAGGCGCACAAAGGTACCGTGATACCAGARTGCTGGCGCCCAGTGGGCCCTGCG
GGTGGGAACAACCCACAGGCTGTTTTAAAACCCTCTCCCCAATTTGAAACTTAGAAGCAATACACCTCG
ATCAATAGTAGGCATGACACGCCAGCCATGTCTTGATCAAGCACTTCTGTTTCCCCGGACTGAGTATCAA
TAAACTGCTTGCGCGGTCGAAGGAGAAAACGTCCGTTACCCGACTAACTACTTCGAGAAACCCAGTAAC
ACCATGGAAATTGCGGAGTGTTTCACTCAGCACATTCCCAGTGTAGATCAGGTCGATGAGTCACCGCAT
TCCCCACGGGTGACCGTGGCGGTGGCTGCGCTGGCGGCCTGCCCATGGGGCAACCCATGGGACGCTTCA
ATATGGACATGGTGTGAAGAGTCTATTGAGCTAGTTAGTAGTCCTCCGCCCCTGAATGCGGCTAATCMT
AACTGCGAAGCACGTGCCTWCAATTWCAAGGGGGGTGGMCGTGTCGATRMCGGGCAAMTMTGCAGCA
GAACMCGAMCTACATAC

| Accession | Description | E value | Max ident |
|---|---|---|---|
| AJ295197.1 | Human coxsackievirus B5 genomic RNA for partial polyprotein gene, isolate VR-1036, ATCC | 0.0 | 96% |

**U4-WINTER**

TKCCTCCATTCCAGGGGGTGGCGTGTCGTAACGGGCAACTCTGCAGCGGAACCGACTACTTTGGGTGTC
CGTGTTTCTTTTAATTTTATACTGGTCGCTTATGGTGACAATAACCGGACACCCAAAGTAGTCGGTTCCG
CTGCAGAGTTGCCCGTTACGACACGCCACCCCCTGGAATGGAGGCACGTGCTCCGCAGTTAGGATTAGC
CGCATTCAGGGGCGGAGGA

| Accession | Description | E value | Max ident |
|---|---|---|---|
| AJ295197.1 | Human coxsackievirus B5 genomic RNA for partial polyprotein gene, isolate VR-1036, ATCC | 8e-50 | 98% |

**U5-WINTER**

AGGRCMGTGCTCCATTCCAGGGGGTGGCGTGTCGTAACGGGCAACTCTGCAGCGGAACCGACTACTTTG

GKTGTCCGTGTTTCTTTTAATTTTATACTGGTCGCTTATGGTGACAATAAACCCGGACACCCAAAGTAGT

CGGTTCCGCTGCAGAGTTGCCCGTTACGACACGCCACCCCCTGGAATGGAGGCACGTGCTCCGCAGTTA

GGATTAGCCGCATTCAGGGGCGGAGGA

| Accession | Description | E value | Max ident |
|---|---|---|---|
| AJ295197.1 | Human coxsackievirus B5 genomic RNA for partial polyprotein gene, isolate VR-1036, ATCC | 2e-50 | 98% |

**U1-SPRING**

AGRMMGTGCCTCCATTCCAGGGGGTGGCGTGTCGTAACGGGCAACTCTGCAGCGGAACCGACTACTTY
GGKTGTCCGTGTTTCTTTTAATTTTATACTGGTCGCTTATGGTGACAATATACMCGGACACCCAAAGTAG
TCGGTTCCGCTGCAGAGTTGCCCGTTACGACACGCCACCCCCTGGAATGGAKGCACGTGCTCCGCAGTT
AGGATTAGCCGCATTCAGGGGCGGAGGA

| Accession | Description | E value | Max ident |
|---|---|---|---|
| AF114383.1 | Coxsackievirus B5 strain Faulkner, complete genome | 4e-48 | 98% |

**U2-SPRING**

AGRAMGTGCCTCCATTCCAGGGGGTGGCGTGTCGTAACGGGCAACTCTGCAGCGGAACCGACTACTTTG
GKTGTCCGTGTTTCTTTTAATTTTATACTGGTCGCTTATGGTGACAATAACCGGACACCCAAAGTAGTCG

GTTCCGCTGCAGAGTTGCCCGTTACGACACGCCACCCCCTGGAATGGAGGCACGTGCTCCGCAGTTAGG
ATTAGCCGCATTCAGGGGCGGAGGA

| Accession | Description | E value | Max ident |
|---|---|---|---|
| AJ295197.1 | Human coxsackievirus B5 genomic RNA for partial polyprotein gene, isolate VR-1036, ATCC | 8e-50 | 98% |

**U3-SPRING**

TGAMMGTGCCTCCATTCCAGGGGGTGGCGTGTCGTAACGGGCAACTCTGCAGCGGAACCGACTACTTTG
GKTGTCCGTGTTTCTTTTAATTTTATACTGGTCGCTTATGGTGACAATAACCGGGACACCCAAAGTAGTC
GGTTCCGCTGCAGAGTTGCCCGTTACGACACGCCACCCCCTGGAATGGAGGCACGTGCTCCGCAGTTAG
GATTAGCCGCATTCAGGGGCGGAGGA

| Accession | Description | E value | Max ident |
|---|---|---|---|
| AY843300.1 | Human enterovirus 82 strain USA/CA64-10390, complete genome | 2e-45 | 97% |

**U4-SPRING**

TGAMMGGTGCCTCATTCCAGGGGGTGGCGTGTCGTAACGGGCAACTCTGCAGCGGAACCGACTACTTT
GGGTGTCCGTGTTTCTTTTAATTTTATACTGGTCGCTTATGGTGACAATAACCGGACACCCAAAGTAGTC
GGTTCCGCTGCAGAGTTGCCCGTTACGACACGCCACCCCCTGGAATGGAGGCACGTGCTCCGCAGTTAG
GATTAGCCGCATTCAGGGGCGGAGGA

| Accession | Description | E value | Max ident |
|---|---|---|---|
| AJ295197.1 | Human coxsackievirus B5 genomic RNA for partial polyprotein gene, isolate VR-1036, ATCC | 8e-50 | 98% |

**U5-SPRING**

CCGAMGAGCCTCCATTCCAGGGGGTGGCGTGTCGTAACGGGCAACTCTGCAGCGGAACCGACTACTTTG
GGTGTCCGTGTTTCTTTTAATTTTATACTGGTCGCTTATGGTGACAATGACCGGACACCCAAAGTAGTCG
GTTCCGCTGCAGAGTTGCCCGTTACGACACGCCACCCCCTGGAATGGAGGCACGTGCTCCGCAGTTAGG
ATTAGCCGCATTCAGGGGCGGAGGA

| Accession | Description | E value | Max ident |
|---|---|---|---|
| AJ295197.1 | Human coxsackievirus B5 genomic RNA for partial polyprotein gene, isolate VR-1036, ATCC | 3e-49 | 99% |

**U1-SUMMER**

AGGAMGTGCCTCCATTCCAGGGGGTGGCGTGTCGTAACGGGCAACTCTGCAGCGGAACCGACTACTTY
GKKTGTCCGTGTTTCTTTTAATTTTATACTGGTCGCTTATGGTGACAATAACCGGACACCCAAAGTAGTC
GGTTCCGCTGCAGAGTTGCCCGTTACGACACGCCACCCCCTGGAATGGAGGCACGTGCTCCGCAGTTAG
GATTAGCCGCATTCAGGGGCGGAGGA

| Accession | Description | E value | Max ident |
|---|---|---|---|
| AJ295197.1 | Human coxsackievirus B5 genomic RNA for partial polyprotein gene, isolate VR-1036, ATCC | 8e-50 | 98% |

**U2-SUMMER**

CGACMGTGCCTCCATTCCAGGGGGTGGCGTGTCGTAACGGGCAACTCTGCAGCGGAACCGACTACTTYG
GKTGTCCGTGTTTCTTTTAATTTTATACTGGTCGCTTATGGTGACAATAACCGGACACCCAAAGTAGTCG
GTTCCGCTGCAGAGTTGCCCGTTACGACACGCCACCCCCTGGAATGGAGGCACGTGCTCCGCAGTTAGG
ATTAGCCGCATTCAGGGGCGGAGGA

| Accession | Description | E value | Max ident |
|---|---|---|---|
| AJ295197.1 | Human coxsackievirus B5 genomic RNA for partial polyprotein gene, isolate VR-1036, ATCC | 8e-50 | 98% |

**U3-SUMMER**

TGACMGTGCCTCCATTCCAGGGGGTGGCGTGTCGTAACGGGCAACTCTGCAGCGGAACCGGACCGGAC
ACCCAAAGTRGTCGGTTCCGCTGCAGAGTTGCCCGTTACGACACGCCACCCCCTGGAATGGAGGCACGT
GCTCCGCAGTTAGGATTAGCCGCATTCAGGGGCGGAGGAACTACTTTGGGTGTCCGTGTTTCTTTTAATT
TTATACTGGTCGCTTATGGTGACAAT

| Accession | Description | E value | Max ident |
|---|---|---|---|
| AJ295197.1 | Human coxsackievirus B5 genomic RNA for partial polyprotein gene, isolate VR-1036, ATCC | 8e-50 | 100% |

**U4-SUMMER**

TKCCATCCATTCCAGGGGGTGGCGTGTCGTAACGGGCAACTCTGCAGCGGAACCGACTACTTTGGGTGT
CCGTGTTTCTTTTAATTTTATACTGGTCGCTTATGGTGACAATAACCGGTACACCCAAAGTAGTCGGTTC
CGCTGCAGAGTTGCCCGTTACGACACGCCACCCCCTGGAATGGAGGCACGTGCTCCGCAGTTAGGATTA
GCCGCATTCAGGGGCGGAGGA

| Accession | Description | E value | Max ident |
|---|---|---|---|
| GU198758.1 | Human coxsackievirus A6 isolate NUH0026/SIN/08 5' UTR | 4e-38 | 99% |

**U5 –SUMMER**

TKCCCTCCATTCCAGGGGGTGGCGTGTCGTAACGGGCAACTCTGCAGCGGAACCGACTACTTTGGGTGT
CCGTGTTTCTTTTAATTTTATACTGGTCGCTTATGGTGACAATCACCGGACACCCAAAGTAGTCGGTTCC
GCTGCAGAGTTGCCCGTTACGACACGCCACCCCCTGGAATGGAGGCACGTGCTCCGCAGTTAGGATTAG
CCGCATTCAGGGGCGGAGGA

| Accession | Description | E value | Max ident |
|---|---|---|---|
| AJ295197.1 | Human coxsackievirus B5 genomic RNA for partial polyprotein gene, isolate VR-1036, ATCC | 3e-49 | 99% |

**COXSACKIEVIRUS Control**

TTCCTCCATTCCAGGGGGTGGCGTGTCGTAACGGGCAACTCTGCAGCGGAACCGACTACTTTGGGTGTC
CGTGTTTCTTTTAATTTTATACTGGTCGCTTATGGTGACAATGACCGGTACACCCAAAGTAGTCGGTTCC
GCTGCAGAGTTGCCCGTTACGACACGCCACCCCCTGGAATGGAGGCACGTGCTCCGCAGTTAGGATTAG
CCGCATTCAGGGGCGGAG

**Hepatitis B virus Nested PCR Sequences from Different Sampling Points and Seasons Along The Umgeni River**

**U1-AUTUMN**

CCACAGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGATCACCCGTGTGTCTTGGCCAAAAT
TCGCAGCCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGT
GTCTGCGGCGTTTTATCATATTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTCGCATAGAG
GATATGATAAACGCCGCAGACACATCCAGCGATAACCAGGACAAATTGGAGGACAGGAGGTTGGTGAG
TGATTGGAGGTTGGGGGCTGCGAATTTTGGCCAAGACACACGGGTGATCCCCCTAGAAAATTGAGAGAA
GTCCACCACGAGTCTAGACTCTGCGGTATTGTGAGGATTCTTGTCAACAAGAAAAACCCCGCCG

| Accession | Description | E value | Max ident |
|---|---|---|---|
| JX144294.1 | Hepatitis B virus isolate SHH043A large S protein (S) gene, complete cds | 2e-98 | 99% |

**U2-AUTUMN**

CACACAGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGATCACCCGTGTGTCTTGGCCAAAA
TTCGCAGCCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGT
GTCTGCGGCGTTTTATCATATTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTCAGATAGAG
ATATGATAAACGCCGCAGACACATCCAGCGATAACCAGGACAAATTGGAGGACAGGAGGTTGGTGAGT
GATTGGAGGTTGGGGGCTGCGAATTTTGGCCAAGACACACGGGTGATCCCCCTAGAAAATTGAGAGAA
GTCCACCACGAGTCTAGACTCTGCGGTATTGTGAGGATTCTTGTCAACAAGAAAAACCCCGCCG

| Accession | Description | E value | Max ident |
|---|---|---|---|
| FN545837.1 | Hepatitis B virus complete genome, strain 42145 | 6e-98 | 99% |

**U3-AUTUMN**

CCACAGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGATCACCCGTGTGTCTTGGCCAAAAT
TCGCAGCCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGT

GTCTGCGGCGTTTTATCATATTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTCAGGRTAGA
GATATGATAAACGCCGCAGACACATCCAGCGATAACCAGGACAAATTGGAGGACAGGAGGTTGGTGAG
TGATTGGAGGTTGGGGGCTGCGAATTTTGGCCAAGACACACGGGTGATCCCCCTAGAAAATTGAGAGAA
GTCCACCACGAGTCTAGACTCTGCGGTATTGTGAGGATTCTTGTCAACAAGAAAAACCCCGCSGAG

| Accession | Description | E value | Max ident |
|---|---|---|---|
| JX144294.1 | Hepatitis B virus isolate SHH043A large S protein (S) gene, complete cds | 8e-97 | 99% |

**U4-AUTUMN**

CCACAGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGATCACCCGTGTGTCTTGGCCAAAAT
TCGCAGCCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGT
GTCTGCGGCGTTTTATCATATTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTCAGATAGAG
GATATGATAAACGCCGCAGACACATCCAGCGATAACCAGGACAAATTGGAGGACAGGAGGTTGGTGAG
TGATTGGAGGTTGGGGGCTGCGAATTTTGGCCAAGACACACGGGTGATCCCCCTAGAAAATTGAGAGAA
GTCCACCACGAGTCTAGACTCTGCGGTATTGTGAGGATTCTTGTCAACAAGAAAAACCCCGCCGGT

| Accession | Description | E value | Max ident |
|---|---|---|---|
| JX144297.1 | Hepatitis B virus isolate SHH270A large S protein (S) gene, complete cds | 8e-97 | 99% |

**U5-AUTUMN**

CRTAGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGATCACCCGTGTGTCTTGGCCMAAWTT
CGCAGCCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGTG
TCTGCGGCGTTTTATCATATTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTCAGGRTAGAG
GATATGATAAACGCCGCAGACACATCCAGCGATAACCAGGACAAATTGGAGGACAGGAGGTTGGTGAG
TGATTGGAGGTTGGGGGCTGCGAATTTTGGCCAAGACACACGGGTGATCCCCCTAGAAAATTGAGAGAA
GTCCACCACGAGTCTAGACTCTGCGGTATTGTGAGGATTCTTGTCAACAAGAAAAACCCCGCCG

| Accession | Description | E value | Max ident |
|---|---|---|---|
| JX144294.1 | Hepatitis B virus isolate SHH043A large S protein (S) gene, complete cds | 2e-98 | 99% |

**U1-WINTER**

TRCAGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGATCACCCGTGTGTCTTGGCCAAAATT
CGCAGCCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGTG
TCTGCGGCGTTTTATCATATTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTCAGRTAGAGA
TATGATAAAACGCCGCAGACACATCCAGCGATAACCAGGACAAATTGGAGGACAGGAGGTTGGTGAGT
GATTGGAGGTTGGGGGCTGCGAATTTTGGCCAAGACACACGGGTGATCCCCCTAGAAAATTGAGAGAA
GTCCACCACGAGTCTAGACTCTGCGGTATTGTGAGGATTCTTGTCAACAAGAAAAACCCCGCCG

| Accession | Description | E value | Max ident |
|---|---|---|---|
| EF690525.1 | Hepatitis B virus isolate 161-CW S protein (S) gene, complete cds | 6e-98 | 99% |

**U2-WINTER**

CRCAGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGATCACCCGTGTGTCTTGGCCAAAATT
CGCAGCCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGTG
TCTGCGGCGTTTTATCATATTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTCAGAKAGAGG
ATATGATAAACGCCGCAGACACATCCAGCGATAACCAGGACAAATTGGAGGACAGGAGGTTGGTGAGT
GATTGGAGGTTGGGGGCTGCGAATTTTGGCCAAGACACACGGGTGATCCCCCTAGAAAATTGAGAGAA
GTCCACCACGAGTCTAGACTCTGCGGTATTGTGAGGATTCTTGTCAACAAGAAAAACCCCGCCRAGTCT
AGACTCGTGGTGGACTTYTCTCWATTTTYTAGGGGGATMWCMCGTGTGTCTTGGCCAAAATTCGCAGC
CCCCAACCTCCAATCACTCACCMACCTCCTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGTGTCTGCG
GCGTTTTATCATATTCCTCYTCMTCCTGCTGCYATGCCTCATCTTMTTGTTGGTTCA

| Accession | Description | E value | Max ident |
|---|---|---|---|
| JX144294.1 | Hepatitis B virus isolate SHH043A large S protein (S) gene, complete cds | 3e-98 | 99% |

**U3-WINTER**

CRCAGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGATCACCCGTGTGTCTTGGCCAAAATT
CGCAGCCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGTG
TCTGCGGCGTTTTATCATATTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTCAGGAKCASA
GSATATGATAAACGCCGCAGACACATCCAGCGATAACCAGGACAAATTGGAGGACAGGAGGTTGGTGA
GTGATTGGAGGTTGGGGGCTGCGAATTTTGGCCAAGACACACGGGTGATCCCCCTAGAAAATTGAGAGA

AGTCCACCACGAGTCTAGACTCTGCGGTATTGTGAGGATTCTTGTCAACAAGAAAAACCCCGCCRAGTC
TAGACTCGTGGTGGACTTYTCTCWMTTTTYTMGGGGGATMWCMCGTGTGTCTTGGCCAAAATTCGCAG
CCCCCAACCTCCAATCACTCMCCAACCTCCTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGTGTCTGC
GGCGTTTTATCAYATTCCTCTTCATCCTGCTGCYATGCCTCATCTTCWTGTTGGTTCA

| Accession | Description | E value | Max ident |
|---|---|---|---|
| HQ589429.1 | Hepatitis B virus isolate M3T3-12 from Argentina polymerase gene, partial cds | 1e-96 | 99% |

**U4-WINTER**

CCACAGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGATCACCCGTGTGTCTTGGCCAAAAT
TCGCAGCCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGT
GTCTGCGGCGTTTTATCATATTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTCAGGRTAGA
GGATATGATAAACGCCGCAGACACATCCAGCGATAACCAGGACAAATTGGAGGACAGGAGGTTGGTGA
GTGATTGGAGGTTGGGGGCTGCGAATTTTGGCCAAGACACACGGGTGATCCCCCTAGAAAATTGAGAGA
AGTCCACCACGAGTCTAGACTCTGCGGTATTGTGAGGATTCTTGTCAACAAGAAAAACCCCGCSG

| Accession | Description | E value | Max ident |
|---|---|---|---|
| JX144294.1 | Hepatitis B virus isolate SHH043A large S protein (S) gene, complete cds | 6e-98 | 99% |

**U5-WINTER**

GCATAGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGATCACCCGTGTGTCTTGGCCAAAAT
TCGCAGCCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGT
GTCTGCGGCGTTTTATCATATTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTCGATAGAGG
ATATGATAAACGCCGCAGACACATCCAGCGATAACCAGGACAAATTGGAGGACAGGAGGTTGGTGAGT
GATTGGAGGTTGGGGGCTGCGAATTTTGGCCAAGACACACGGGTGATCCCCCTAGAAAATTGAGAGAA
GTCCACCACGAGTCTAGACTCTGCGGTATTGTGAGGATTCTTGTCAACAAGAAAAACCCCGCCRAGTCT
AGACTCGTGGTGGACWTYTCTCWMTTTTYTMGGGGGATMWCMCGTGTGTCTTGGCCAMAATTCGCAG
CCCCCAACCTCCAATCACTCMCCMACCTCCTGTCCTCCAMTTTGTCCTGGTTATCGCTGGATGTGTCTGC
GGCGTTTTATCAYWTYCCTCTTCATCCTGCTGCYATGCCTCATCTTCATGTTGGTTCA

| Accession | Description | E value | Max ident |
|---|---|---|---|
| JX144294.1 | Hepatitis B virus isolate SHH043A large S protein (S) gene, complete cds | 3e-98 | 99% |

**U1-SPRING**

CGCAGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGATCACCCGTGTGTCTTGGCCAAAATT
CGCAGCCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGTG
TCTGCGGRTAGAGATATGATAAAACGCCGCAGACACATCCAGCGATAACCAGGACAAATTGGAGGACA
GGAGGTTGGTGAGTGATTGGAGGTTGGGGGCTGCGAATTTTGGCCAAGACACACGGGTGATCCCCCTAG
AAAATTGAGAGAAGTCCACCACGAGTCTAGACTCTGCGGTATTGTGAGGATTCTTGTCAACAAGAAAAA
CCCCGCCRGCGTTTTATCATATTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTCA

| Accession | Description | E value | Max ident |
|---|---|---|---|
| JX144294.1 | Hepatitis B virus isolate SHH043A large S protein (S) gene, complete cds | 5e-99 | 100% |

**U2-SPRING**

CRCAGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGATCACCCGTGTGTCTTGGCCAAAATT
CGCAGCCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGTG
TCTGCGGCGTTTTATCATATTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTCGGAKAGAGA
TATGATAAACGCCGCAGACACATCCAGCGATAACCAGGACAAATTGGAGGACAGGAGGTTGGTGAGTG
ATTGGAGGTTGGGGGCTGCGAATTTTGGCCAAGACACACGGGTGATCCCCCTAGAAAATTGAGAGAAGT
CCACCACGAGTCTAGACTCTGCGGTATTGTGAGGATTCTTGTCAACAAGAAAAACCCCGCSRAGTCTAG
ACTCGTGGTGGACWTCTCTCAATTTTCTAGGGGGATMACMCGTGTGTCTTGGCCAAAATTCGCAGCCCC
CAACCTCCAATCACTCACCAACCTCCTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGTGTCTGCGGCG
TTTTATCATATTCCTCTTCATCCTGCTGCTATGCCTCATCTTMWWGTTGGTTC

| Accession | Description | E value | Max ident |
|---|---|---|---|
| JX144294.1 | Hepatitis B virus isolate SHH043A large S protein (S) gene, complete cds | 1e-96 | 99% |

**U3-SPRING**

CRTGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGATCACCCGTGTGTCTTGGCCAAAATTC
GCAGCCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGTGT

CTGCGGCGTTTTATCATATTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTCGAKAGAGGAT
ATGATAAACGCCGCAGACACATCCAGCGATAACCAGGACAAATTGGAGGACAGGAGGTTGGTGAGTGA
TTGGAGGTTGGGGGCTGCGAATTTTGGCCAAGACACACGGGTGATCCCCCTAGAAAATTGAGAGAAGTC
CACCACGAGTCTAGACTCTGCGGTATTGTGAGGATTCTTGTCAACAAGAAAAACCCCGCYRAGTCTAGA
CTCGTGGTGGACWTYTCTCWMTTTYTAGGGGGATMWCMCGTGTGTCTTGGCCAMAATTCGCAGCCCCC
AACCTCCAATCACTCACCAACCTCCTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGTGTCTGCGGCGTT
TWATCMTATTCCTCTTYATCCTGCTGCTMTGCCTCATCTTCYTGTTGGTTCA

| Accession | Description | E value | Max ident |
|---|---|---|---|
| JX096953.1 | Hepatitis B virus isolate 12-8957, complete genome | 4e-96 | 99% |

**U4-SPRING**

CGCCRCAGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGATCACCCGTGTGTCTTGGCCAAA
ATTCGCAGCCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCAATTTGTCCTGGTTATCGCTGGAT
GTGTCTGCGGCGTTTTATCATATTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTCAGRTAG
AGGATATGATAAACGCCGCAGACACATCCAGCGATAACCAGGACAAATTGGAGGACAGGAGGTTGGTG
AGTGATTGGAGGTTGGGGGCTGCGAATTTTGGCCAAGACACACGGGTGATCCCCCTAGAAAATTGAGAG
AAGTCCACCACGAGTCTAGACTCTGCGGTATTGTGAGGATTCTTGTCAACAAGAAAAACCCCGCCGAGT
CTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGATCACCCGTGTGTCTTGGCCAAAATTCGCAGC
CCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGTGTCTGCG
GCGTTTTATCATATTCCTCTTCATCCTGCTGCTATGCCTCATCWWMTTGTTGGTTCA

| Accession | Description | E value | Max ident |
|---|---|---|---|
| AB713894.1 | Hepatitis B virus PreS1/S2/S, p gene for envelope protein, polymerase, partial cds, isolate: YOGHhbv121 | 1e-96 | 99% |

**U5-SPRING**

CRTAGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGATCACCCGTGTGTCTTGGCCAAAATT
CGCAGCCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGTG
TCTGCGGCGTTTTATCATATTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTCGGRTAGAGG
ATATGATAAAACGCCGCAGACACATCCAGCGATAACCAGGACAAATTGGAGGACAGGAGGTTGGTGAG
TGATTGGAGGTTGGGGGCTGCGAATTTTGGCCAAGACACACGGGTGATCCCCCTAGAAAATTGAGAGAA
GTCCACCACGAGTCTAGACTCTGCGGTATTGTGAGGATTCTTGTCAACAAGAAAAACCCCGCCRAGTCT
MGACTCGTGGTGGRCWTYTCTCWMTTTTYTAGGGGGATWCMCGTGTGTCTTGGCCAAAATTCGCAGCC
CCCAACCTCCAATCACTCACYMACCTCCTGTCYTCCAMTTTGTCCTGGTTATCGCTGGATGTGTCTGCGG
CGTTWTATCWTATTYCTCYTSMTCYKGCTGCYWTGCCTCWTCTTCTTGTTGGTTCA

| Accession | Description | E value | Max ident |
|---|---|---|---|
| JX144294.1 | Hepatitis B virus isolate SHH043A large S protein (S) gene, complete cds | 6e-100 | 99% |

**U1-SUMMER**

CCRTGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGATCACCCGTGTGTCTTGGCCAAAATT
CGCAGCCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGTG
TCTGCGGCGTTTTATCATATTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTCAGGRKAGAG
GATATGATAAAACGCCGCAGACACATCCAGCGATAACCAGGACAAATTGGAGGACAGGAGGTTGGTGA
GTGATTGGAGGTTGGGGGCTGCGAATTTTGGCCAAGACACACGGGTGATCCCCCTAGAAAATTGAGAGA
AGTCCACCACGAGTCTAGACTCTGCGGTATTGTGAGGATTCTTGTCAACAAGAAAAACCCCGCCRAGTC
TAGACTCGTGGTGGACWTCTCTCAATTTTCTAGGGGGATMACCCGTGTGTCTTGGCCAAAATTCGCAGC
CCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGTGTCTGCG
GCGTTTTATCATATTCCTCTTCATCCTGCTGCTATGCCTCATCWWCTTGTTGGTTC

| Accession | Description | E value | Max ident |
|---|---|---|---|
| JX144294.1 | Hepatitis B virus isolate SHH043A large S protein (S) gene, complete cds | 6e-100 | 99% |

**U2-SUMMER**

CAGTGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGATCACCCGTGTGTCTTGGCCAAAATT
CGCAGCCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGTG
TCTGCGGCGTTTTATCATATTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTCAGCRKAGAG
GATATGATAAAACGCCGCAGACACATCCAGCGATAACCAGGACAAATTGGAGGACAGGAGGTTGGTGA
GTGATTGGAGGTTGGGGGCTGCGAATTTTGGCCAAGACACACGGGTGATCCCCCTAGAAAATTGAGAGA
AGTCCACCACGAGTCTAGACTCTGCGGTATTGTGAGGATTCTTGTCAACAAGAAAAACCCCGCCRAGTC
TAGACTCGTGGTGGACWTYTCTCAATTTTCTAGGGGGATMACMCGTGTGTCTTGGCCAAAATTCGCAGC
CCCCAACCTCCAATCACWCACCMACCTCCTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGTGTCTGCG
GCGTTTTATCATATTCCTCTTCMTCYTGCTGCYATGCCTCATCTTCATGTTGGTTC

| Accession | Description | E value | Max ident |
|---|---|---|---|
| JX144294.1 | Hepatitis B virus isolate SHH043A large S protein (S) gene, complete cds | 6e-100 | 99% |

**U3-SUMMER**

CRCAGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGATCACCCGTGTGTCTTGGCCAAAATT
CGCAGCCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGTG
TCTGCGGCGTTTTATCATATTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTCGGRTAGAGA
TATGATAAAACGCCGCAGACACATCCAGCGATAACCAGGACAAATTGGAGGACAGGAGGTTGGTGAGT
GATTGGAGGTTGGGGGCTGCGAATTTTGGCCAAGACACACGGGTGATCCCCCTAGAAAATTGAGAGAA
GTCCACCACGAGTCTAGACTCTGCGGTATTGTGAGGATTCTTGTCAACAAGAAAAACCCCGCSRAGTCT
MGACTCGTGGTGGRCWTYTCTCWMTTTTYTMGGGGGATMWCMCGTGTGTCTTGGCCAMAATTCGCAG
CCCCCAACCTCCAATCACTCACYAMCCTCCTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGTGTCTGC
GGCGTTTTATCWYWTTCCTCYTCMTCYTGCTGCYWTGCCTCWTCTTCTTGTTGGTTCA

| Accession | Description | E value | Max ident |
|---|---|---|---|
| EF690525.1 | Hepatitis B virus isolate 161-CW S protein (S) gene, complete cds | 3e-97 | 99% |

**U4-SUMMER**

CCACAGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGATCACCCGTGTGTCTTGGCCAAAAT
TCGCAGCCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGT
GTCTGCGGCGTTTTATCATATTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTCGGATAGAG
GATATGATAAACGCCGCAGACACATCCAGCGATAACCAGGACAAATTGGAGGACAGGAGGTTGGTGAG
TGATTGGAGGTTGGGGGCTGCGAATTTTGGCCAAGACACACGGGTGATCCCCCTAGAAAATTGAGAGAA
GTCCACCACGAGTCTAGACTCTGCGGTATTGTGAGGATTCTTGTCAACAAGAAAAACCCCGCSG

| Accession | Description | E value | Max ident |
|---|---|---|---|
| JX144294.1 | Hepatitis B virus isolate SHH043A large S protein (S) gene, complete cds | 6e-98 | 99% |

**U5 –SUMMER**

CRCAGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGATCACCCGTGTGTCTTGGCCAAAATT
CGCAGCCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGTG
TCTGCGGCGTTTTATCATATTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTCGGRTAGAGG
ATATGATAAACGCCGCAGACACATCCAGCGATAACCAGGACAAATTGGAGACAGGAGGTTGGTGAGTG
ATTGGAGGTTGGGGGCTGCGAATTTTGGCCAAGACACACGGGTGATCCCCCTAGAAAATTGAGAGAAGT
CCACCACGAGTCTAGACTCTGCGGTATTGTGAGGATTCTTGTCAACAAGAAAAACCCCGCCRAGTCTAG
ACTCGTGGTGGACWTCTCTCAATTTTCTAGGGGGATMACCCGTGTGTCTTGGCCAAAATTCGCAGCCCC
CAACCTCCAATCACTCACCAACCTCCTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGTGTCTGCGGCG
TTTTATCATATTCCTCTTCATCCTGCTGCTATGCCTCATCWWCTTGTTGGTTC

| Accession | Description | E value | Max ident |
|---|---|---|---|
| JX144294.1 | Hepatitis B virus isolate SHH043A large S protein (S) gene, complete cds | 1e-96 | 99% |

**HEPATITIS B VIRUS Control**

CRCAGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGATCACCCGTGTGTCTTGGCCAAAATT
CGCAGCCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGTG
TCTGCGGCGTTTTATCATATTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTCGGRTAGAGG
ATATGATAAAACGCCGCAGACACATCCAGCGATAACCAGGACAAATTGGAGGACAGGAGGTTGGTGAG
TGATTGGAGGTTGGGGGCTGCGAATTTTGGCCAAGACACACGGGTGATCCCCCTAGAAAATTGAGAGAA
GTCCACCACGAGTCTAGACTCTGCGGTATTGTGAGGATTCTTGTCAACAAGAAAAACCCCGCC

# APPENDIX iv

## Real-Time PCR Raw Data

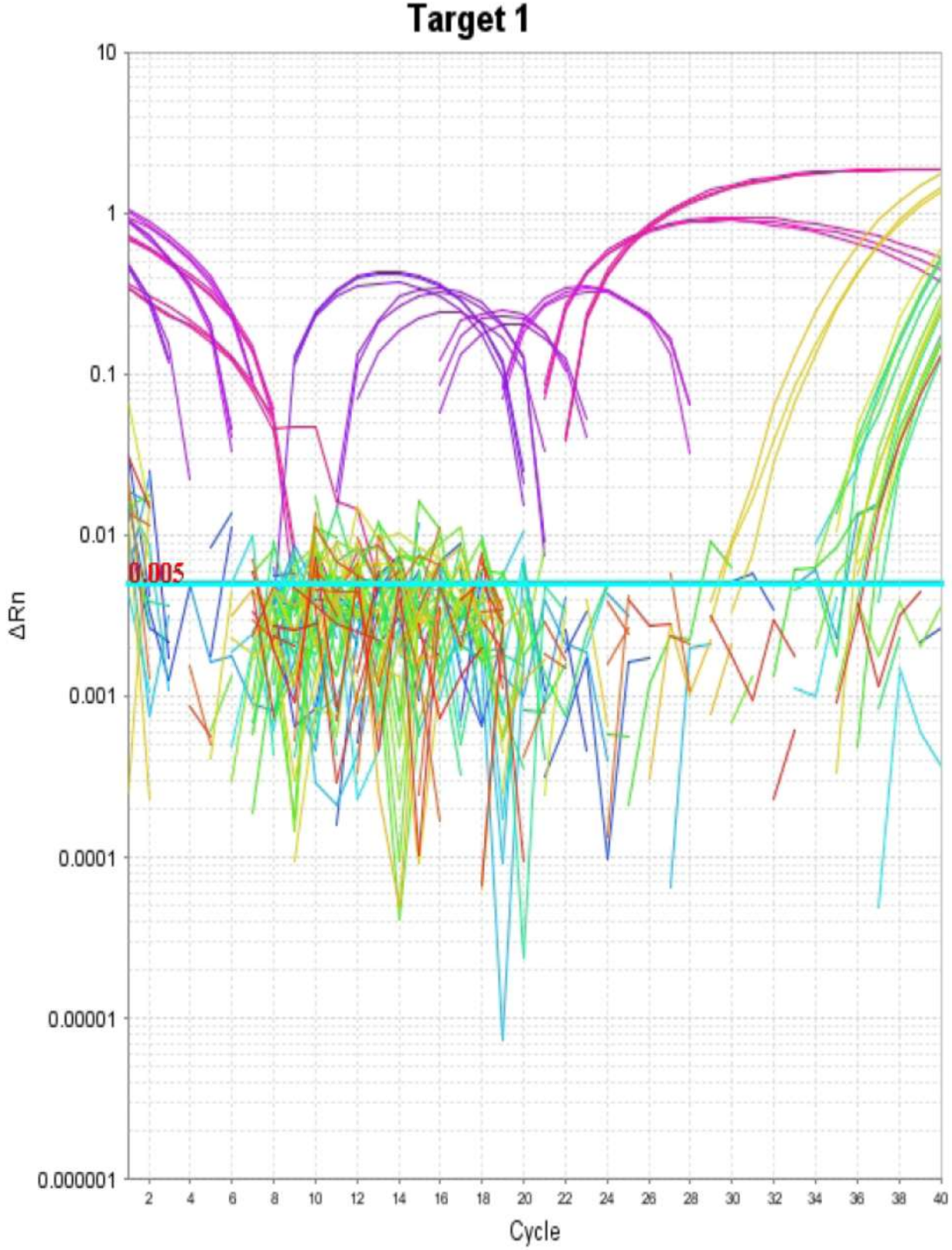
**Table 24: Real- time quantitative raw data for Hepatitis B virus**

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Well | Sample Name | Target Name | Task | Reporter | Quencher | CT | CT Mean | CT SD | Quantity | Quantity Mean | Quantity SD |
| A1 | NEG | Target 1 | NTC | FAM | NFQ-MGB | 9.646913 | 19.5605 | 14.53847 | | | |
| A2 | NEG | Target 1 | NTC | FAM | NFQ-MGB | 12.78452 | 19.5605 | 14.53847 | | | |
| A3 | NEG | Target 1 | NTC | FAM | NFQ-MGB | 36.25005 | 19.5605 | 14.53847 | | | |
| A4 | U1 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | Undetermined | 17.5229 | | 9.01E+13 | 4.51E+13 | 6.36E+13 |
| A5 | U1 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 17.5229 | 17.5229 | | 1.77E+11 | 4.51E+13 | 6.36E+13 |
| A6 | U1 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | Undetermined | 17.5229 | | | | |
| A7 | U2 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 13.24059 | 14.37111 | 1.598799 | 1.32E+11 | 7.63E+10 | 7.9E+10 |
| A8 | U2 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 15.50163 | 14.37111 | 1.598799 | 2.04E+10 | 7.63E+10 | 7.9E+10 |
| A9 | U2 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | Undetermined | 14.37111 | 1.598799 | | | |
| A10 | U3 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 15.88466 | 12.1581 | 3.364733 | 1.49E+10 | 1.33E+12 | 1.74E+12 |
| A11 | U3 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 11.24663 | 12.1581 | 3.364733 | 6.85E+11 | 1.33E+12 | 1.74E+12 |
| A12 | U3 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 9.342996 | 12.1581 | 3.364733 | 3.3E+12 | 1.33E+12 | 1.74E+12 |
| B1 | U4 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 9.470512 | 11.46359 | 3.213807 | 2.97E+12 | 1.79E+12 | 1.55E+12 |
| B2 | U4 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 15.17109 | 11.46359 | 3.213807 | 2.69E+10 | 1.79E+12 | 1.55E+12 |
| B3 | U4 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 9.749174 | 11.46359 | 3.213807 | 2.36E+12 | 1.79E+12 | 1.55E+12 |
| B4 | U5 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 29.7396 | 30.0392 | 0.567006 | 160785.2 | 134061.8 | 52855.61 |
| B5 | U5 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 30.69316 | 30.0392 | 0.567006 | 73180.52 | 134061.8 | 52855.61 |
| B6 | U5 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 29.68484 | 30.0392 | 0.567006 | 168219.7 | 134061.8 | 52855.61 |
| B7 | U1 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 35.74088 | 21.08501 | 13.33942 | 1134.453 | 8.53E+11 | 1.47E+12 |
| B8 | U1 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 17.86127 | 21.08501 | 13.33942 | 2.91E+09 | 8.53E+11 | 1.47E+12 |
| B9 | U1 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 9.652899 | 21.08501 | 13.33942 | 2.55E+12 | 8.53E+11 | 1.47E+12 |
| B10 | U2 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 36.27124 | 35.7269 | 1.002922 | 732.2349 | 1469.187 | 1311.563 |
| B11 | U2 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 34.56951 | 35.7269 | 1.002922 | 2983.47 | 1469.187 | 1311.563 |
| B12 | U2 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 36.33996 | 35.7269 | 1.002922 | 691.855 | 1469.187 | 1311.563 |
| C1 | U3 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 35.73211 | 26.08971 | 15.16161 | 1142.695 | 2.01E+12 | 3.48E+12 |
| C2 | U3 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 33.92326 | 26.08971 | 15.16161 | 5086.311 | 2.01E+12 | 3.48E+12 |
| C3 | U3 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 8.613763 | 26.08971 | 15.16161 | 6.02E+12 | 2.01E+12 | 3.48E+12 |
| C4 | U4 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 35.32763 | 20.69168 | 13.01753 | 1595.651 | 4.6E+11 | 7.88E+11 |
| C5 | U4 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 10.40761 | 20.69168 | 13.01753 | 1.37E+12 | 4.6E+11 | 7.88E+11 |
| C6 | U4 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 16.3398 | 20.69168 | 13.01753 | 1.02E+10 | 4.6E+11 | 7.88E+11 |
| C7 | U5 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | Undetermined | 13.09214 | 1.675643 | | | |

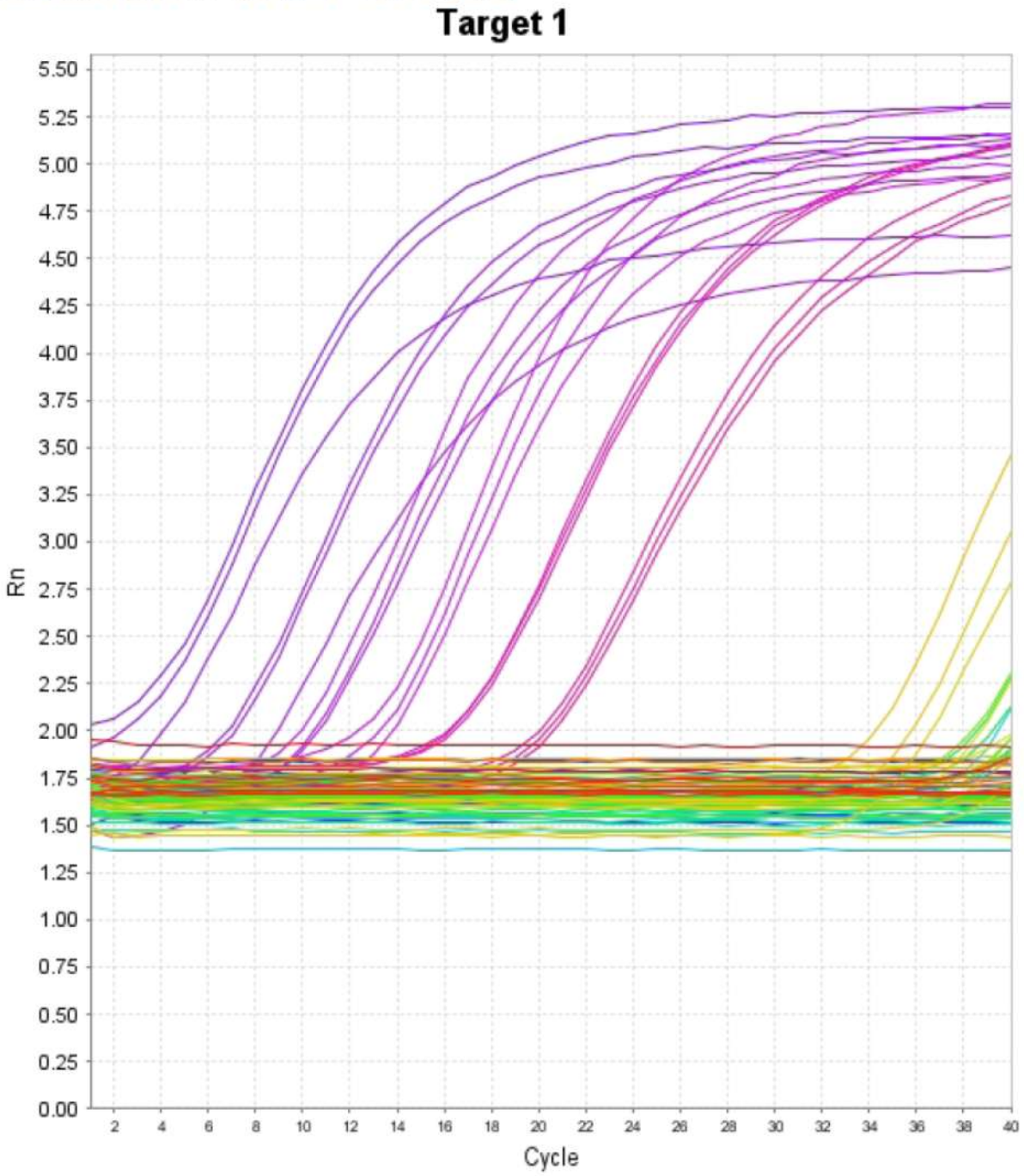
| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| C8 | U5 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 11.90729 | 13.09214 | 1.675643 | 3.97E+11 | 2.27E+11 | 2.41E+11 |
| C9 | U5 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 14.277 | 13.09214 | 1.675643 | 5.62E+10 | 2.27E+11 | 2.41E+11 |
| C10 | U1 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 9.438799 | 26.51267 | 14.89402 | 3.05E+12 | 1.02E+12 | 1.76E+12 |
| C11 | U1 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 36.8368 | 26.51267 | 14.89402 | 459.0924 | 1.02E+12 | 1.76E+12 |
| C12 | U1 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 33.26242 | 26.51267 | 14.89402 | 8776.374 | 1.02E+12 | 1.76E+12 |
| D1 | U2 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 34.44961 | 26.12386 | 14.44817 | 3293.865 | 1.01E+12 | 1.76E+12 |
| D2 | U2 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 34.4814 | 26.12386 | 14.44817 | 3208.547 | 1.01E+12 | 1.76E+12 |
| D3 | U2 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 9.440557 | 26.12386 | 14.44817 | 3.04E+12 | 1.01E+12 | 1.76E+12 |
| D4 | U3 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 7.485636 | 10.67407 | 3.73572 | 1.53E+13 | 5.89E+12 | 8.22E+12 |
| D5 | U3 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 14.78444 | 10.67407 | 3.73572 | 3.7E+10 | 5.89E+12 | 8.22E+12 |
| D6 | U3 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 9.752144 | 10.67407 | 3.73572 | 2.35E+12 | 5.89E+12 | 8.22E+12 |
| D7 | U4 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 36.99039 | 28.95684 | 13.30797 | 404.4234 | 3.29E+10 | 5.69E+10 |
| D8 | U4 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 13.59553 | 28.95684 | 13.30797 | 9.86E+10 | 3.29E+10 | 5.69E+10 |
| D9 | U4 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 36.2846 | 28.95684 | 13.30797 | 724.2068 | 3.29E+10 | 5.69E+10 |
| D10 | U5 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 11.76389 | 10.50328 | 1.782777 | 4.47E+11 | 2.02E+12 | 2.22E+12 |
| D11 | U5 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 9.242662 | 10.50328 | 1.782777 | 3.58E+12 | 2.02E+12 | 2.22E+12 |
| D12 | U5 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | Undetermined | 10.50328 | 1.782777 | | | |
| E1 | U1 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 8.689716 | 12.35232 | 6.270679 | 5.66E+12 | 3.64E+12 | 3.16E+12 |
| E2 | U1 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 8.774336 | 12.35232 | 6.270679 | 5.28E+12 | 3.64E+12 | 3.16E+12 |
| E3 | U1 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 19.59291 | 12.35232 | 6.270679 | 6.98E+08 | 3.64E+12 | 3.16E+12 |
| E4 | U2 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 33.76657 | 19.55718 | 12.39546 | 5788.648 | 3.13E+11 | 4.8E+11 |
| E5 | U2 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 13.9416 | 19.55718 | 12.39546 | 7.41E+10 | 3.13E+11 | 4.8E+11 |
| E6 | U2 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 10.96337 | 19.55718 | 12.39546 | 8.66E+11 | 3.13E+11 | 4.8E+11 |
| E7 | U3 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 19.76775 | 14.07811 | 8.046365 | 6.04E+08 | 3.63E+12 | 5.13E+12 |
| E8 | U3 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | Undetermined | 14.07811 | 8.046365 | | | |
| E9 | U3 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 8.388468 | 14.07811 | 8.046365 | 7.25E+12 | 3.63E+12 | 5.13E+12 |
| E10 | U4 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 10.71548 | 9.489644 | 1.733599 | 1.06E+12 | 4.55E+12 | 4.93E+12 |
| E11 | U4 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 8.263804 | 9.489644 | 1.733599 | 8.04E+12 | 4.55E+12 | 4.93E+12 |
| E12 | U4 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | Undetermined | 9.489644 | 1.733599 | | | |
| F1 | U5 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 13.18381 | 13.86703 | 0.96623 | 1.39E+11 | 9.17E+10 | 6.62E+10 |
| F2 | U5 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | Undetermined | 13.86703 | 0.96623 | | | |
| F3 | U5 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 14.55026 | 13.86703 | 0.96623 | 4.48E+10 | 9.17E+10 | 6.62E+10 |
| F4 | 21 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 5.336665 | 9.110584 | 5.337128 | | | |
| F5 | 21 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 12.8845 | 9.110584 | 5.337128 | | 3.85E+09 | 3.85E+09 |
| F6 | 21 | Target 1 | UNKNOWN | FAM | NFQ-MGB | Undetermined | 9.110584 | 5.337128 | | | |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| G1 | HEP B | Target 1 | STANDARD | FAM | NFQ-MGB | Undetermined | 8.011415 | | 6.4E+12 | | |
| G2 | HEP B | Target 1 | STANDARD | FAM | NFQ-MGB | Undetermined | 8.011415 | | 6.4E+12 | | |
| G3 | HEP B | Target 1 | STANDARD | FAM | NFQ-MGB | 8.011415 | 8.011415 | | 6.4E+12 | | |
| G4 | 10^1 | Target 1 | STANDARD | FAM | NFQ-MGB | Undetermined | | | 6.4E+11 | | |
| G5 | 10^1 | Target 1 | STANDARD | FAM | NFQ-MGB | Undetermined | | | 6.4E+11 | | |
| G6 | 10^1 | Target 1 | STANDARD | FAM | NFQ-MGB | Undetermined | | | 6.4E+11 | | |
| G7 | 10^2 | Target 1 | STANDARD | FAM | NFQ-MGB | Undetermined | | | 6.4E+10 | | |
| G8 | 10^2 | Target 1 | STANDARD | FAM | NFQ-MGB | Undetermined | | | 6.4E+10 | | |
| G9 | 10^2 | Target 1 | STANDARD | FAM | NFQ-MGB | Undetermined | | | 6.4E+10 | | |
| G10 | 10^3 | Target 1 | STANDARD | FAM | NFQ-MGB | Undetermined | | | 6.4E+09 | | |
| G11 | 10^3 | Target 1 | STANDARD | FAM | NFQ-MGB | Undetermined | | | 6.4E+09 | | |
| G12 | 10^3 | Target 1 | STANDARD | FAM | NFQ-MGB | Undetermined | | | 6.4E+09 | | |
| H1 | 10^4 | Target 1 | STANDARD | FAM | NFQ-MGB | 20.57124 | 20.5785 | 0.040551 | 6.4E+08 | | |
| H2 | 10^4 | Target 1 | STANDARD | FAM | NFQ-MGB | 20.54207 | 20.5785 | 0.040551 | 6.4E+08 | | |
| H3 | 10^4 | Target 1 | STANDARD | FAM | NFQ-MGB | 20.62219 | 20.5785 | 0.040551 | 6.4E+08 | | |
| H4 | 10^5 | Target 1 | STANDARD | FAM | NFQ-MGB | 21.77596 | 21.78241 | 0.005585 | 64000000 | | |
| H5 | 10^5 | Target 1 | STANDARD | FAM | NFQ-MGB | 21.78554 | 21.78241 | 0.005585 | 64000000 | | |
| H6 | 10^5 | Target 1 | STANDARD | FAM | NFQ-MGB | 21.78573 | 21.78241 | 0.005585 | 64000000 | | |
| H10 | | Target 1 | NTC | FAM | NFQ-MGB | Undetermined | 9.259275 | | | | |
| H11 | | Target 1 | NTC | FAM | NFQ-MGB | 9.259275 | 9.259275 | | | | |

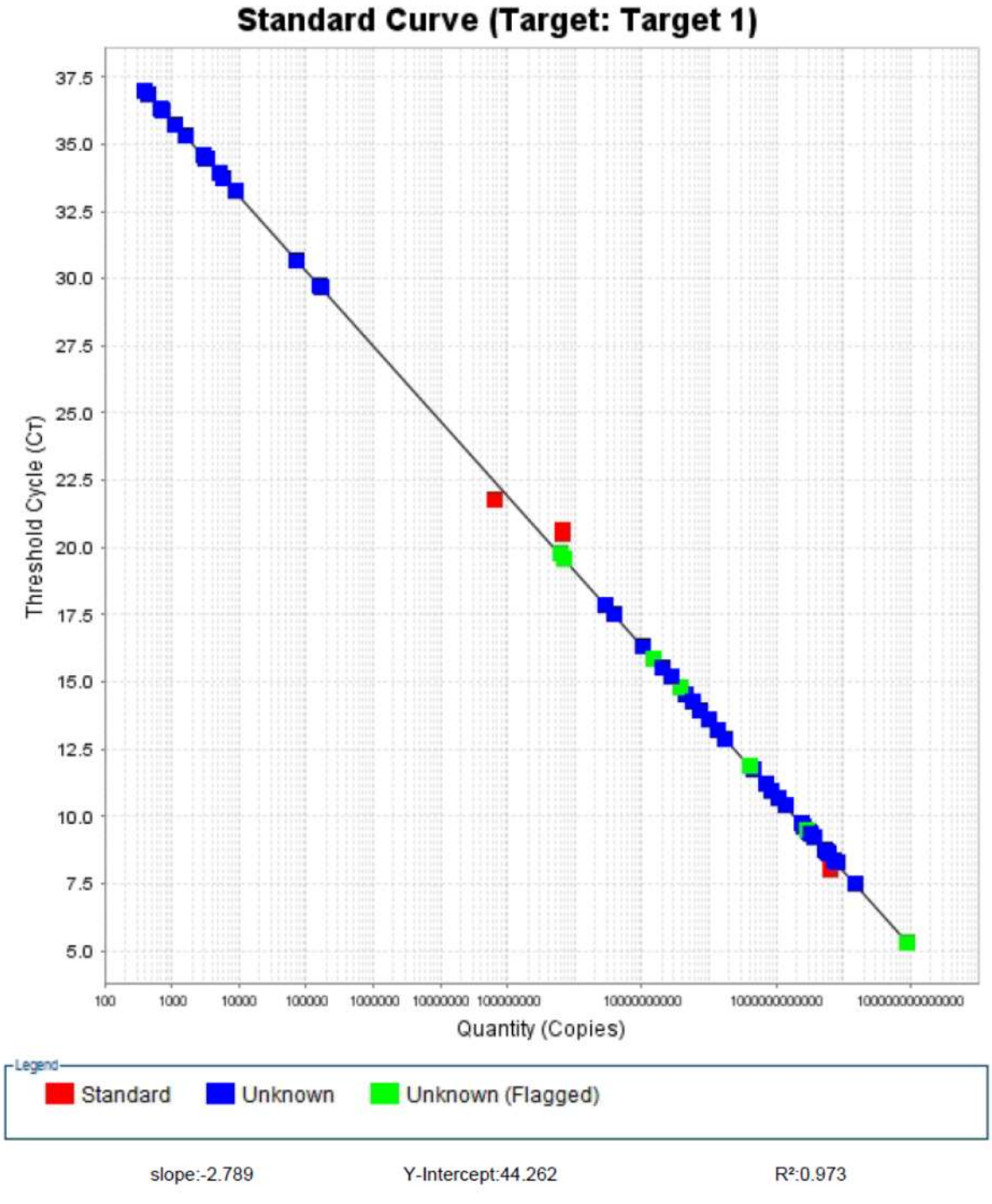
# Amplification Plot (Rn vs. Cycle)



## Amplification Plot (Rn vs. Cycle)



# Standard Curves




slope:-2.789 Y-Intercept:44.262 $R^2$:0.973

**Table 25: Real- time quantitative raw data for Adenovirus**

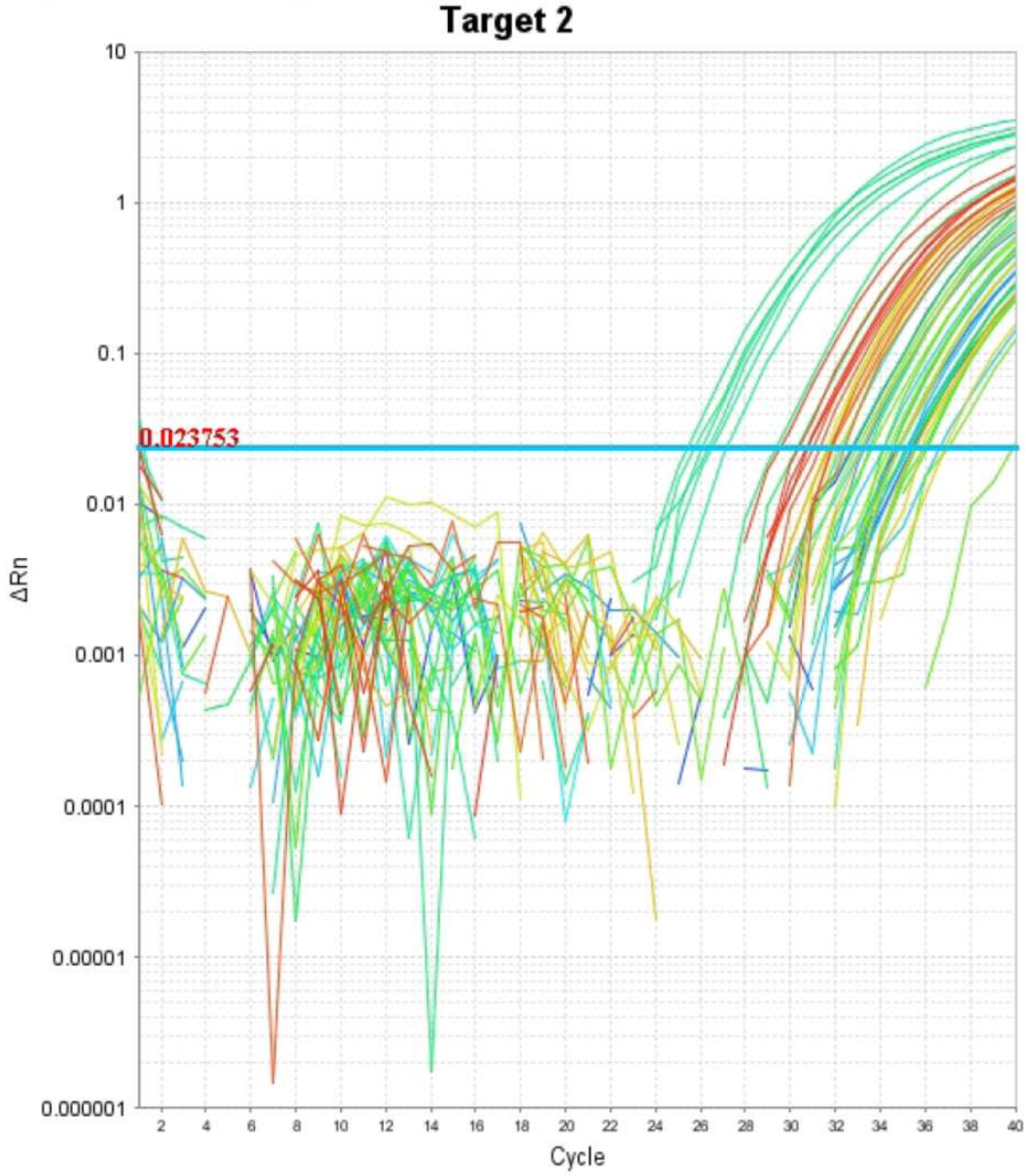
| Well | Sample Name | Target Name | Task | Reporter | Quencher | Cт | Cт Mean | Cт SD | Quantity | Quantity Mean | Quantity SD |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A1 | NEG 1 | Target 1 | NTC | FAM | NFQ-MGB | Undetermined | | | | | |
| A2 | NEG 1 | Target 1 | NTC | FAM | NFQ-MGB | Undetermined | | | | | |
| A3 | NEG 1 | Target 1 | NTC | FAM | NFQ-MGB | Undetermined | | | | | |
| A4 | U1 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 30.81612 | 30.42069 | 0.663727 | 599272.6 | 841194.9 | 410346.8 |
| A5 | U1 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 30.79153 | 30.42069 | 0.663727 | 609325.1 | 841194.9 | 410346.8 |
| A6 | U1 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 29.65442 | 30.42069 | 0.663727 | 1314987 | 841194.9 | 410346.8 |
| A7 | U2 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 30.47865 | 30.94426 | 0.439814 | 752955.3 | 566159.8 | 171157.4 |
| A8 | U2 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 31.00143 | 30.94426 | 0.439814 | 528667.7 | 566159.8 | 171157.4 |
| A9 | U2 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 31.35269 | 30.94426 | 0.439814 | 416856.5 | 566159.8 | 171157.4 |
| A10 | U3 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 31.95011 | 31.95011 | | 278272.6 | 278272.6 | 46435.88 |
| A11 | U3 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 31.80785 | 31.80785 | | 306382.7 | 306382.7 | 93826.65 |
| A12 | U3 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 32.32684 | 32.32684 | | 215671 | 215671 | 55949.58 |
| B1 | U4 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 31.8216 | 33.98447 | 1.795362 | 303546.5 | 118763.5 | 130828.7 |
| B2 | U4 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 35.59332 | 33.98447 | 1.795362 | 23666.56 | 118763.5 | 130828.7 |
| B3 | U4 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 33.20078 | 33.98447 | 1.795362 | 119409.5 | 118763.5 | 130828.7 |
| B4 | U5 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | | | | | | |
| B5 | U5 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | | | | | | |
| B6 | U5 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 35.84652 | 35.84652 | | 19941.03 | 19941.03 | 104012 |
| B7 | U1 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 36.87417 | 35.53592 | 1.159762 | 9950.278 | 29120.03 | 16639.81 |
| B8 | U1 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 34.90991 | 35.53592 | 1.159762 | 37576.29 | 29120.03 | 16639.81 |
| B9 | U1 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 34.82367 | 35.53592 | 1.159762 | 39833.53 | 29120.03 | 16639.81 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| B10 | U2 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 31.7248 | 31.90676 | 0.366811 | 324088.3 | 292191.7 | 66857.14 |
| B11 | U2 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 32.32898 | 31.90676 | 0.366811 | 215359.6 | 292191.7 | 66857.14 |
| B12 | U2 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 31.6665 | 31.90676 | 0.366811 | 337127 | 292191.7 | 66857.14 |
| C1 | U3 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 34.40226 | 33.68553 | 0.698476 | 52973.25 | 92462.63 | 41745.49 |
| C2 | U3 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 33.00686 | 33.68553 | 0.698476 | 136147.4 | 92462.63 | 41745.49 |
| C3 | U3 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 33.64749 | 33.68553 | 0.698476 | 88267.23 | 92462.63 | 41745.49 |
| C4 | U4 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 37.01447 | 37.138 | 2.621982 | 9049.329 | 19126.4 | 24420.74 |
| C5 | U4 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 39.81956 | 37.138 | 2.621982 | 1356.787 | 19126.4 | 24420.74 |
| C6 | U4 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 34.57996 | 37.138 | 2.621982 | 46973.09 | 19126.4 | 24420.74 |
| C7 | U5 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | | | | | | |
| C8 | U5 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | | | | | | |
| C9 | U5 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 34.12017 | 34.12017 | | 64110.67 | 64110.67 | 42516 |
| C10 | U1 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 35.88993 | 35.82374 | 0.241988 | 19363.99 | 20435.7 | 3435.238 |
| C11 | U1 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 36.02575 | 35.82374 | 0.241988 | 17664.06 | 20435.7 | 3435.238 |
| C12 | U1 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 35.55555 | 35.82374 | 0.241988 | 24279.03 | 20435.7 | 3435.238 |
| D1 | U2 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 32.74383 | 33.85843 | 1.304556 | 162661.2 | 95565.72 | 66836.05 |
| D2 | U2 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 35.29329 | 33.85843 | 1.304556 | 28992.16 | 95565.72 | 66836.05 |
| D3 | U2 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 33.53814 | 33.85843 | 1.304556 | 95043.79 | 95565.72 | 66836.05 |
| D4 | U3 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 30.42104 | 29.97263 | 0.634157 | 782879.4 | 1109474 | 461873.8 |
| D5 | U3 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 29.52421 | 29.97263 | 0.634157 | 1436068 | 1109474 | 461873.8 |
| D6 | U3 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | | | | | | |
| D7 | U4 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 25.4783 | 25.81607 | 0.327065 | 22172740 | 17934866 | 3988005 |
| D8 | U4 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 26.13126 | 25.81607 | 0.327065 | 14255641 | 17934866 | 3988005 |
| D9 | U4 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 25.83864 | 25.81607 | 0.327065 | 17376218 | 17934866 | 3988005 |

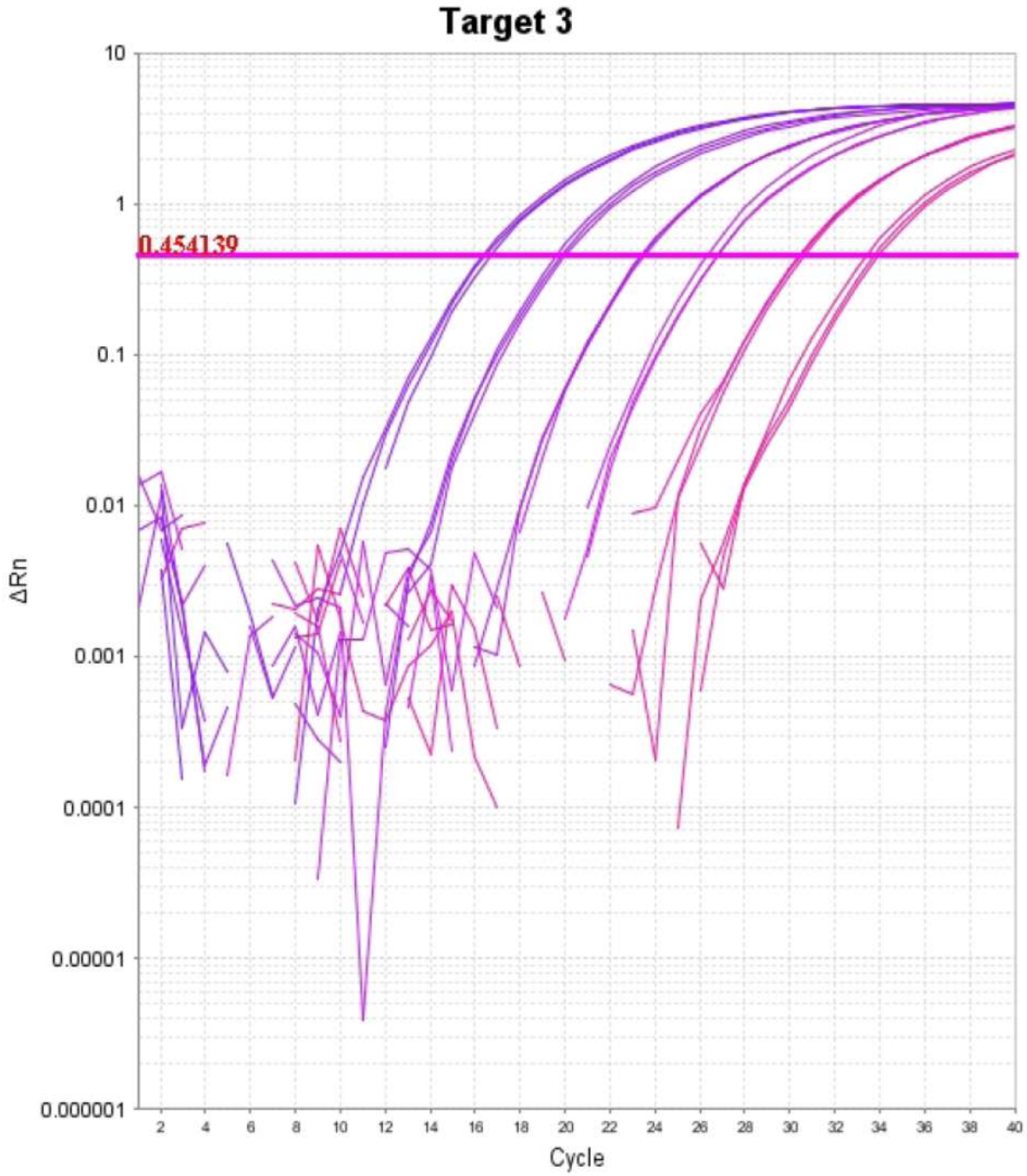
| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| D10 | U5 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 26.59744 | 26.72033 | 0.438527 | 10399854 | 9842221 | 2721835 |
| D11 | U5 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 26.35636 | 26.72033 | 0.438527 | 12242055 | 9842221 | 2721835 |
| D12 | U5 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 27.2072 | 26.72033 | 0.438527 | 6884755 | 9842221 | 2721835 |
| E1 | U1 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 34.80896 | 33.45615 | 1.254596 | 40231.98 | 124164.8 | 87630.23 |
| E2 | U1 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 32.33093 | 33.45615 | 1.254596 | 215075.2 | 124164.8 | 87630.23 |
| E3 | U1 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 33.22855 | 33.45615 | 1.254596 | 117187.2 | 124164.8 | 87630.23 |
| E4 | U2 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | | | | | | |
| E5 | U2 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 34.11317 | 35.38132 | 1.793427 | 64414.81 | 37999.3 | 37357.16 |
| E6 | U2 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 36.64946 | 35.38132 | 1.793427 | 11583.8 | 37999.3 | 37357.16 |
| E7 | U3 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 35.19484 | 35.23681 | 0.109505 | 30988.75 | 30175.74 | 2192.921 |
| E8 | U3 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 35.36109 | 35.23681 | 0.109505 | 27692.43 | 30175.74 | 2192.921 |
| E9 | U3 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 35.1545 | 35.23681 | 0.109505 | 31846.06 | 30175.74 | 2192.921 |
| E10 | U4 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 34.88142 | 34.26216 | 0.802043 | 38307.46 | 64590.93 | 37468.9 |
| E11 | U4 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 34.54891 | 34.26216 | 0.802043 | 47970.23 | 64590.93 | 37468.9 |
| E12 | U4 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 33.35616 | 34.26216 | 0.802043 | 107495.1 | 64590.93 | 37468.9 |
| F1 | U5 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 34.73524 | 34.31375 | 1.364379 | 42289.29 | 75592.39 | 71651.88 |
| F2 | U5 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 35.41764 | 34.31375 | 1.364379 | 26653.02 | 75592.39 | 71651.88 |
| F3 | U5 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 32.78836 | 34.31375 | 1.364379 | 157834.9 | 75592.39 | 71651.88 |
| G1 | ADENO | Target 1 | STANDARD | FAM | NFQ-MGB | 12.36774 | 11.90823 | 0.40759 | 2.1E+11 | | |
| G2 | ADENO | Target 1 | STANDARD | FAM | NFQ-MGB | 11.76663 | 11.90823 | 0.40759 | 2.1E+11 | | |
| G3 | ADENO | Target 1 | STANDARD | FAM | NFQ-MGB | 11.59033 | 11.90823 | 0.40759 | 2.1E+11 | | |
| G4 | 10^1 | Target 1 | STANDARD | FAM | NFQ-MGB | 15.06919 | 15.20705 | 0.182622 | 2.1E+10 | | |
| G5 | 10^1 | Target 1 | STANDARD | FAM | NFQ-MGB | 15.13779 | 15.20705 | 0.182622 | 2.1E+10 | | |
| G6 | 10^1 | Target 1 | STANDARD | FAM | NFQ-MGB | 15.41418 | 15.20705 | 0.182622 | 2.1E+10 | | |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| G7 | 10^2 | Target 1 | STANDARD | FAM | NFQ-MGB | 18.88432 | 18.96081 | 0.119655 | 2.1E+09 | | |
| G8 | 10^2 | Target 1 | STANDARD | FAM | NFQ-MGB | 18.89941 | 18.96081 | 0.119655 | 2.1E+09 | | |
| G9 | 10^2 | Target 1 | STANDARD | FAM | NFQ-MGB | 19.0987 | 18.96081 | 0.119655 | 2.1E+09 | | |
| G10 | 10^3 | Target 1 | STANDARD | FAM | NFQ-MGB | 21.9893 | 22.17828 | 0.164091 | 2.1E+08 | | |
| G11 | 10^3 | Target 1 | STANDARD | FAM | NFQ-MGB | 22.28464 | 22.17828 | 0.164091 | 2.1E+08 | | |
| G12 | 10^3 | Target 1 | STANDARD | FAM | NFQ-MGB | 22.26089 | 22.17828 | 0.164091 | 2.1E+08 | | |
| H1 | 10^4 | Target 1 | STANDARD | FAM | NFQ-MGB | 25.2515 | 25.61484 | 0.328319 | 21000000 | | |
| H2 | 10^4 | Target 1 | STANDARD | FAM | NFQ-MGB | 25.89022 | 25.61484 | 0.328319 | 21000000 | | |
| H3 | 10^4 | Target 1 | STANDARD | FAM | NFQ-MGB | 25.70279 | 25.61484 | 0.328319 | 21000000 | | |
| H4 | 10^5 | Target 1 | STANDARD | FAM | NFQ-MGB | 28.99348 | 28.84691 | 0.17017 | 2100000 | | |
| H5 | 10^5 | Target 1 | STANDARD | FAM | NFQ-MGB | 28.88695 | 28.84691 | 0.17017 | 2100000 | | |
| H6 | 10^5 | Target 1 | STANDARD | FAM | NFQ-MGB | 28.66029 | 28.84691 | 0.17017 | 2100000 | | |
| H11 | NEG 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 35.32215 | | | | | |
| H12 | | | | | | | | | | | |

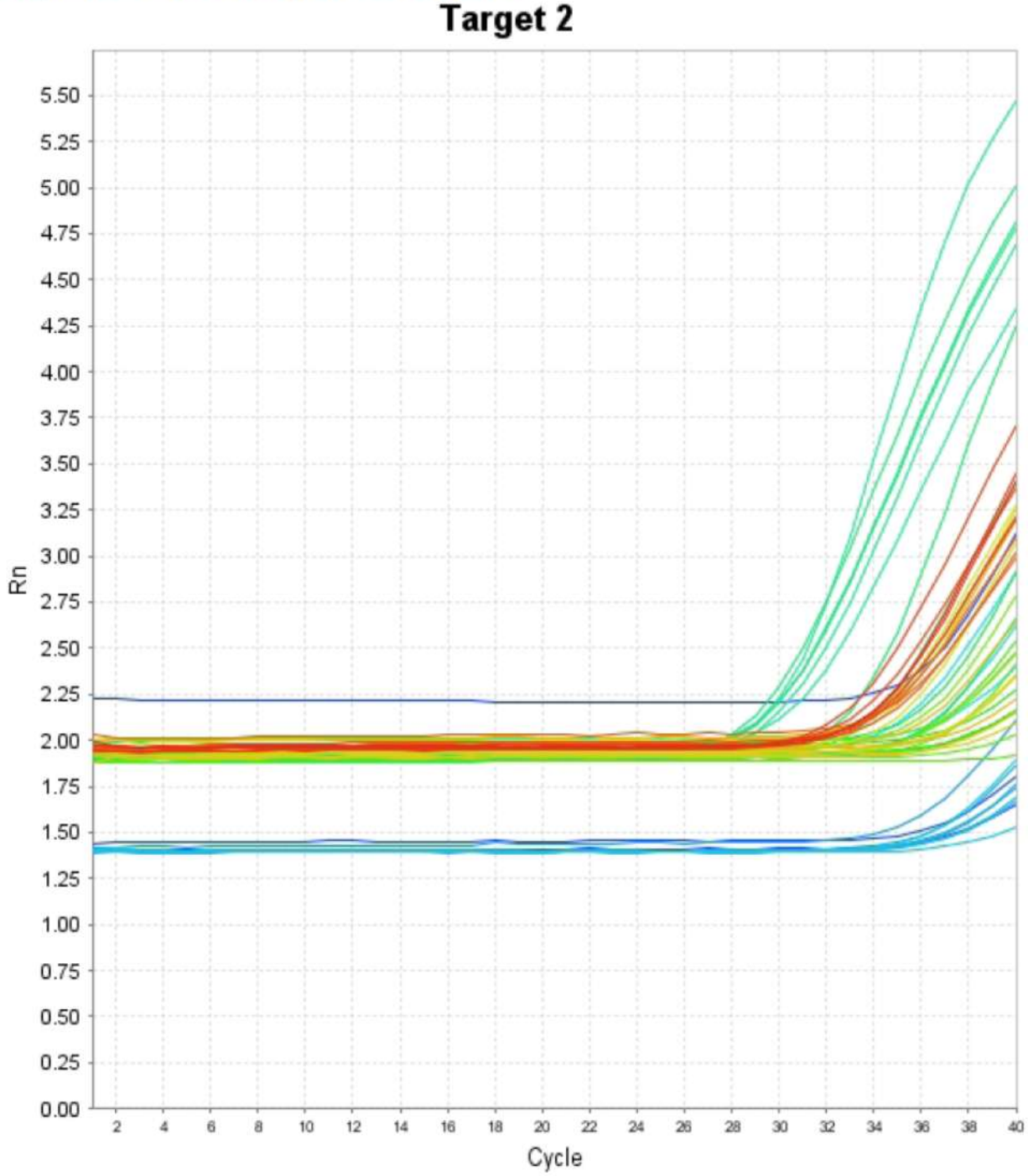
## Amplification Plot (Rn vs. Cycle)



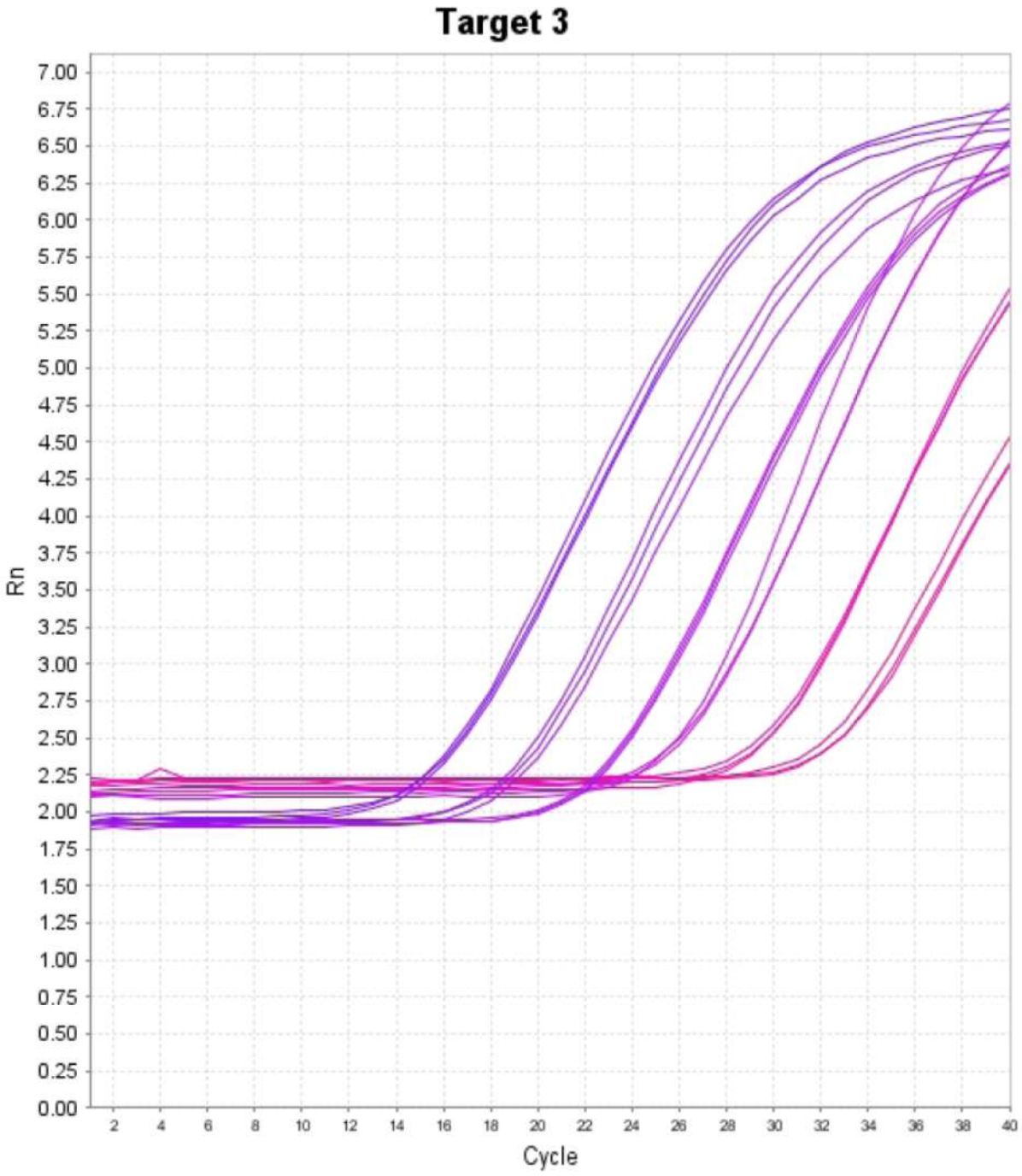
## Amplification Plot (Rn vs. Cycle)

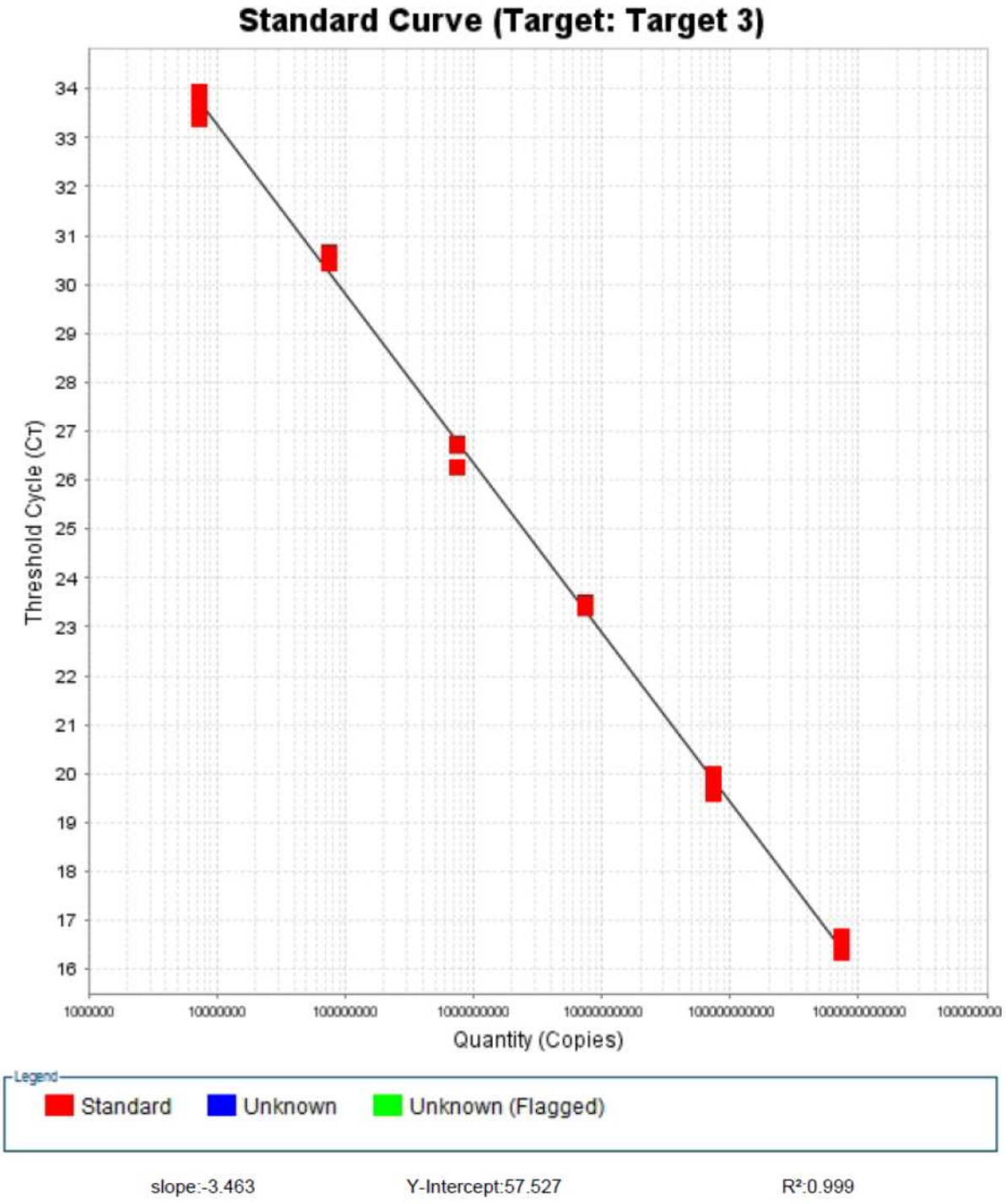


## Amplification Plot (Rn vs. Cycle)



## Amplification Plot (Rn vs. Cycle)






slope:-3.463 Y-Intercept:57.527 $R^2$:0.999

**Table 26: Real- time quantitative raw data for Enterovirus**

| Well | Sample Name | Target Name | Task | Reporter | Quencher | Cт | Cт Mean | Cт SD | Quantity | Quantity Mean | Quantity SD |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A1 | NEG 1 | Target 1 | NTC | FAM | NFQ-MGB | 35.34897 | 35.34897 | | | | |
| A2 | NEG 2 | Target 1 | NTC | FAM | NFQ-MGB | 36.0848 | 36.0848 | | | | |
| A3 | NEG 3 | Target 1 | NTC | FAM | NFQ-MGB | 34.62985 | 34.62985 | | | | |
| A4 | U1 AUT 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 31.60907 | 31.57488 | 0.055095 | 4.2E+08 | 447697280 | 44180952 |
| A5 | U1 AUT 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 31.60424 | 31.57488 | 0.055095 | 4.24E+08 | 447697280 | 44180952 |
| A6 | U1 AUT 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 31.51132 | 31.57488 | 0.055095 | 4.99E+08 | 447697280 | 44180952 |
| A7 | U2 AUT 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 30.46377 | 30.1704 | 0.267706 | 3.11E+09 | 5547676672 | 2335136512 |
| A8 | U2 AUT 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 29.93935 | 30.1704 | 0.267706 | 7.76E+09 | 5547676672 | 2335136512 |
| A9 | U2 AUT 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 30.10808 | 30.1704 | 0.267706 | 5.78E+09 | 5547676672 | 2335136512 |
| A10 | U3 AUT 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 32.38565 | 31.86034 | 0.455641 | 1.08E+08 | 322314624 | 186276464 |
| A11 | U3 AUT 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 31.5722 | 31.86034 | 0.455641 | 4.48E+08 | 322314624 | 186276464 |
| A12 | U3 AUT 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 31.62318 | 31.86034 | 0.455641 | 4.1E+08 | 322314624 | 186276464 |
| B1 | U4 AUT 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 30.82852 | 30.86135 | 0.456666 | 1.64E+09 | 1887825792 | 1347046016 |
| B2 | U4 AUT 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 30.42198 | 30.86135 | 0.456666 | 3.34E+09 | 1887825792 | 1347046016 |
| B3 | U4 AUT 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 31.33354 | 30.86135 | 0.456666 | 6.8E+08 | 1887825792 | 1347046016 |
| B4 | U5 AUT 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 28.62948 | 28.63618 | 0.071913 | 7.64E+10 | 75892580352 | 9427492864 |
| B5 | U5 AUT 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 28.71121 | 28.63618 | 0.071913 | 6.62E+10 | 75892580352 | 9427492864 |
| B6 | U5 AUT 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 28.56785 | 28.63618 | 0.071913 | 8.51E+10 | 75892580352 | 9427492864 |
| B7 | U1 WIN 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 31.51415 | 32.42484 | 0.790594 | 4.96E+08 | 196006272 | 260022560 |
| B8 | U1 WIN 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 32.82523 | 32.42484 | 0.790594 | 50295284 | 196006272 | 260022560 |

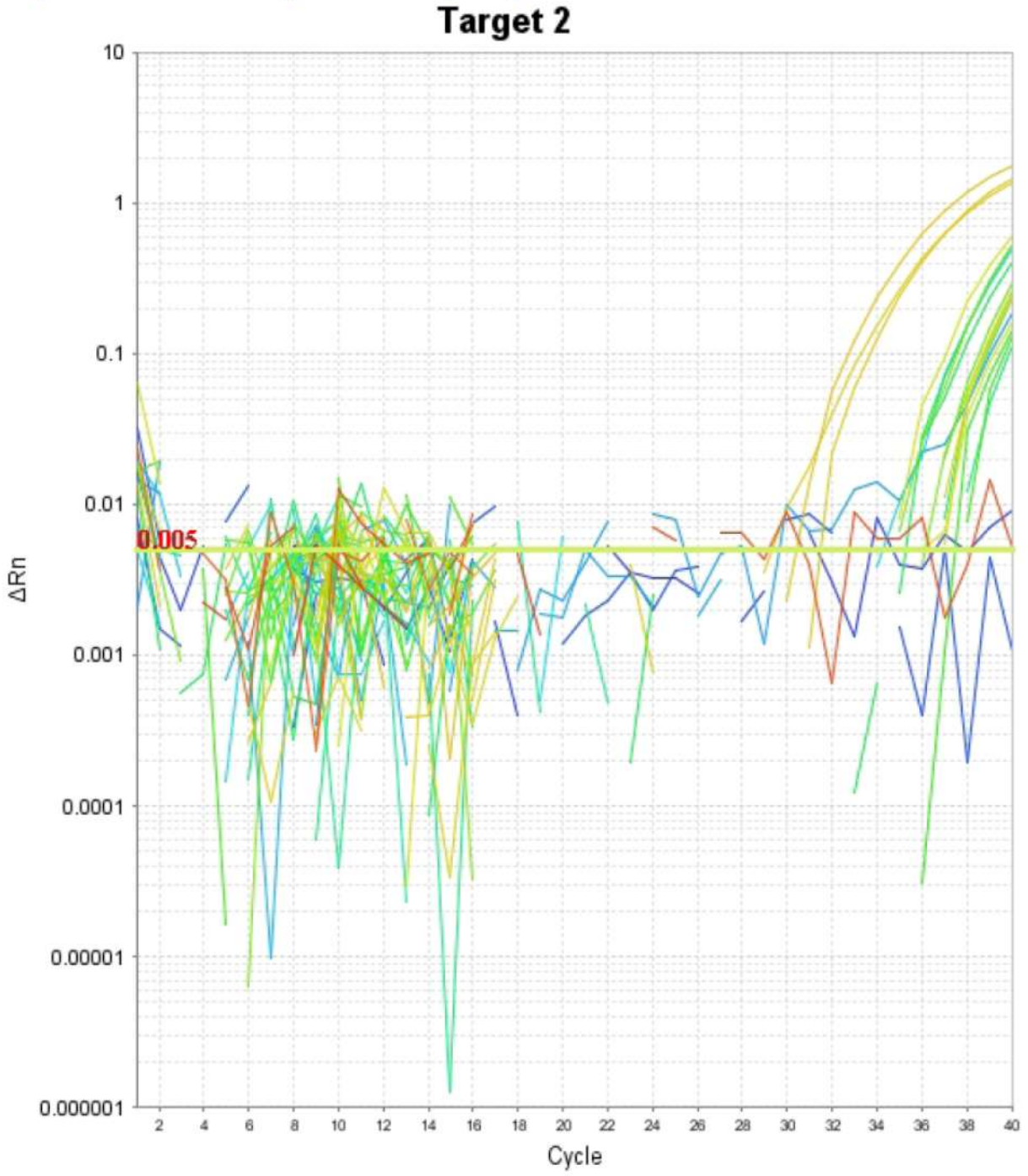
| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| B9 | U1 WIN 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 32.93515 | 32.42484 | 0.790594 | 41511900 | 196006272 | 260022560 |
| B10 | U2 WIN 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 31.84782 | 30.83657 | 2.124334 | 2.77E+08 | 38444699648 | 66232659968 |
| B11 | U2 WIN 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 32.26636 | 30.83657 | 2.124334 | 1.33E+08 | 38444699648 | 66232659968 |
| B12 | U2 WIN 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 28.39553 | 30.83657 | 2.124334 | 1.15E+11 | 38444699648 | 66232659968 |
| C1 | U3 WIN 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 35.94728 | 35.18367 | 1.013657 | 215864.8 | 2243840.25 | 3339839.5 |
| C2 | U3 WIN 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 35.5701 | 35.18367 | 1.013657 | 417050.6 | 2243840.25 | 3339839.5 |
| C3 | U3 WIN 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 34.03364 | 35.18367 | 1.013657 | 6098605 | 2243840.25 | 3339839.5 |
| C4 | U4 WIN 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 31.61007 | 31.44116 | 0.149511 | 4.2E+08 | 576009088 | 139892000 |
| C5 | U4 WIN 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 31.32578 | 31.44116 | 0.149511 | 6.89E+08 | 576009088 | 139892000 |
| C6 | U4 WIN 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 31.38764 | 31.44116 | 0.149511 | 6.19E+08 | 576009088 | 139892000 |
| C7 | U5 WIN 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 31.33319 | 32.06499 | 1.091435 | 6.81E+08 | 391406112 | 337065888 |
| C8 | U5 WIN 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 31.54231 | 32.06499 | 1.091435 | 4.72E+08 | 391406112 | 337065888 |
| C9 | U5 WIN 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 33.31948 | 32.06499 | 1.091435 | 21220412 | 391406112 | 337065888 |
| C10 | U1 SPR 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 28.40607 | 31.52917 | 2.746334 | 1.13E+11 | 37638156288 | 65116319744 |
| C11 | U1 SPR 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 32.6142 | 31.52917 | 2.746334 | 72700512 | 37638156288 | 65116319744 |
| C12 | U1 SPR 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 33.56722 | 31.52917 | 2.746334 | 13768830 | 37638156288 | 65116319744 |
| D1 | U2 SPR 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 31.35712 | 31.22527 | 1.376212 | 6.53E+08 | 3614743296 | 5629984768 |
| D2 | U2 SPR 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 32.53082 | 31.22527 | 1.376212 | 84094704 | 3614743296 | 5629984768 |
| D3 | U2 SPR 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 29.78788 | 31.22527 | 1.376212 | 1.01E+10 | 3614743296 | 5629984768 |
| D4 | U3 SPR 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 29.81845 | 29.89091 | 0.254344 | 9.58E+09 | 8974814208 | 3556237056 |
| D5 | U3 SPR 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 29.68066 | 29.89091 | 0.254344 | 1.22E+10 | 8974814208 | 3556237056 |
| D6 | U3 SPR 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 30.17362 | 29.89091 | 0.254344 | 5.15E+09 | 8974814208 | 3556237056 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| D7 | U4 SPR 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 31.82084 | 31.00027 | 1.872546 | 2.9E+08 | 17236561920 | 29498370048 |
| D8 | U4 SPR 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 28.85752 | 31.00027 | 1.872546 | 5.13E+10 | 17236561920 | 29498370048 |
| D9 | U4 SPR 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 32.32244 | 31.00027 | 1.872546 | 1.21E+08 | 17236561920 | 29498370048 |
| D10 | U5 SPR 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 35.87987 | 35.54799 | 0.410492 | 242826.6 | 518691.5 | 390939.1875 |
| D11 | U5 SPR 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 35.08897 | 35.54799 | 0.410492 | 966070.8 | 518691.5 | 390939.1875 |
| D12 | U5 SPR 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 35.67512 | 35.54799 | 0.410492 | 347177.1 | 518691.5 | 390939.1875 |
| E1 | U1 SUM 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 31.00066 | 31.85751 | 2.13714 | 1.22E+09 | 1829176320 | 2196820224 |
| E2 | U1 SUM 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 34.2901 | 31.85751 | 2.13714 | 3897278 | 1829176320 | 2196820224 |
| E3 | U1 SUM 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 30.28175 | 31.85751 | 2.13714 | 4.27E+09 | 1829176320 | 2196820224 |
| E4 | U2 SUM 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 31.76148 | 32.73804 | 0.856676 | 3.22E+08 | 124521720 | 171303424 |
| E5 | U2 SUM 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 33.36282 | 32.73804 | 0.856676 | 19673818 | 124521720 | 171303424 |
| E6 | U2 SUM 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 33.08984 | 32.73804 | 0.856676 | 31687102 | 124521720 | 171303424 |
| E7 | U3 SUM 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 27.52448 | 28.49202 | 0.900391 | 5.26E+11 | 279544960 | 180761648 |
| E8 | U3 SUM 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 28.64624 | 28.49202 | 0.900391 | 7.42E+10 | 279544960 | 180761648 |
| E9 | U3 SUM 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 29.30534 | 28.49202 | 0.900391 | 2.35E+10 | 279544960 | 180761648 |
| E10 | U4 SUM 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 32.29549 | 31.80281 | 0.445602 | 1.27E+08 | 357288320 | 225174272 |
| E11 | U4 SUM 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 31.68494 | 31.80281 | 0.445602 | 3.68E+08 | 357288320 | 225174272 |
| E12 | U4 SUM 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 31.42799 | 31.80281 | 0.445602 | 5.77E+08 | 357288320 | 225174272 |
| F1 | U5 SUM 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 32.30028 | 32.13804 | 0.190413 | 1.26E+08 | 173401616 | 59966908 |
| F2 | U5 SUM 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 32.18544 | 32.13804 | 0.190413 | 1.54E+08 | 173401616 | 59966908 |
| F3 | U5 SUM 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 31.92841 | 32.13804 | 0.190413 | 2.41E+08 | 173401616 | 59966908 |
| F4 | 21 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 32.3908 | 32.35559 | 0.097846 | 1.07E+08 | 115334232 | 20396796 |

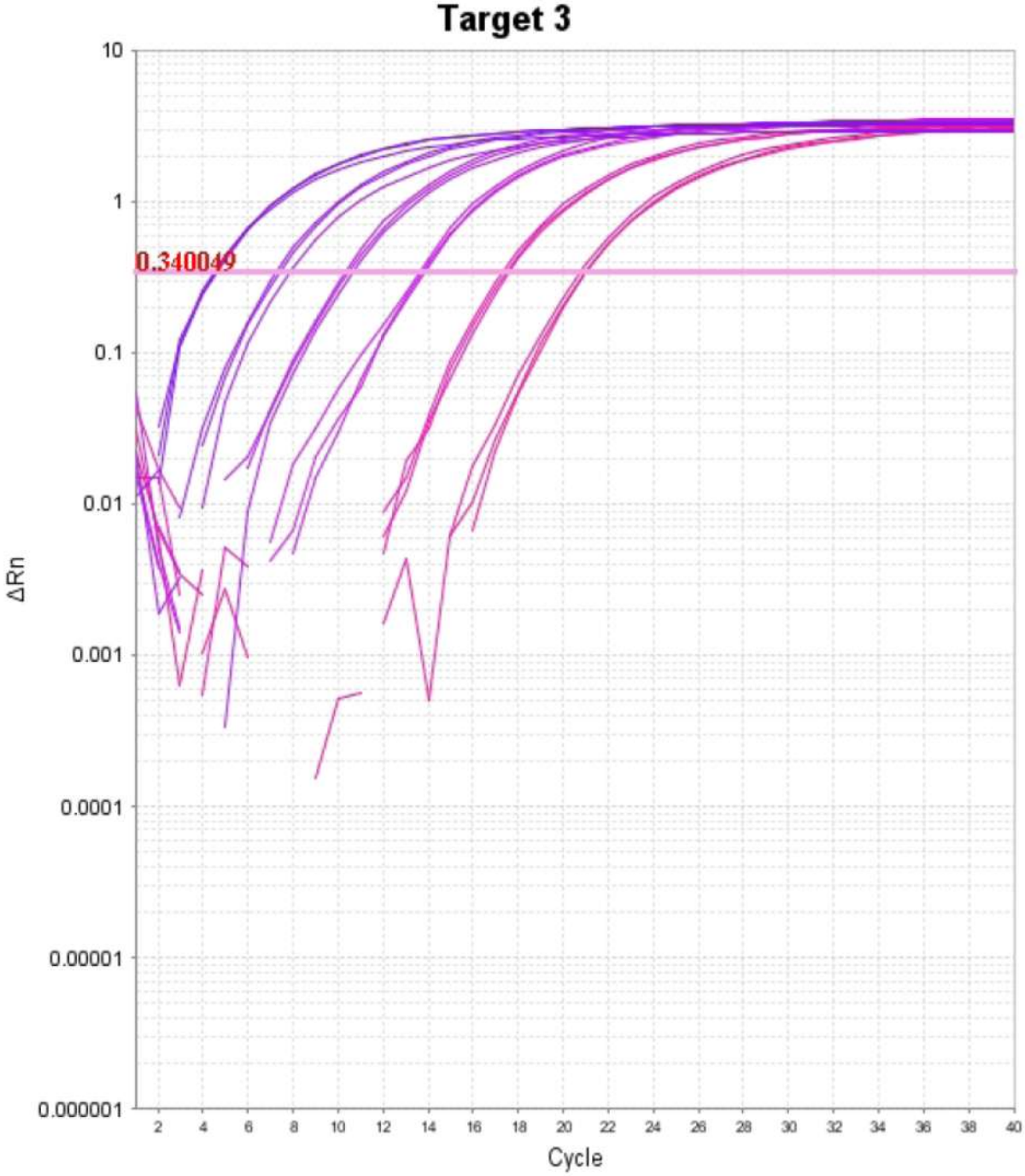
| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| F5 | 21 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 32.43097 | 32.35559 | 0.097846 | 1E+08 | 115334232 | 20396796 |
| F6 | 21 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 32.24502 | 32.35559 | 0.097846 | 1.39E+08 | 115334232 | 20396796 |
| F7 | 22 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 31.3075 | 32.51584 | 1.128429 | 7.12E+08 | 263008080 | 389429536 |
| F8 | 22 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 33.54225 | 32.51584 | 1.128429 | 14382410 | 263008080 | 389429536 |
| F9 | 22 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 32.69778 | 32.51584 | 1.128429 | 62830052 | 263008080 | 389429536 |
| F10 | 23 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 31.58673 | 31.96571 | 0.508542 | 4.37E+08 | 27954470 | 180761648 |
| F11 | 23 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 32.54366 | 31.96571 | 0.508542 | 82230384 | 27954470 | 180761648 |
| F12 | 23 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 31.76675 | 31.96571 | 0.508542 | 3.19E+08 | 27954470 | 180761648 |
| G1 | cox 1 | Target 1 | STANDARD | FAM | NFQ-MGB | 23.2895 | 23.39782 | 0.101342 | 1.13E+12 | | |
| G2 | cox 1 | Target 1 | STANDARD | FAM | NFQ-MGB | 23.41365 | 23.39782 | 0.101342 | 1.13E+12 | | |
| G3 | cox 1 | Target 1 | STANDARD | FAM | NFQ-MGB | 23.49032 | 23.39782 | 0.101342 | 1.13E+12 | | |
| G4 | 10^1 1 | Target 1 | STANDARD | FAM | NFQ-MGB | 27.74774 | 27.84608 | 0.288513 | 1.13E+11 | | |
| G5 | 10^1 1 | Target 1 | STANDARD | FAM | NFQ-MGB | 27.6196 | 27.84608 | 0.288513 | 1.13E+11 | | |
| G6 | 10^1 1 | Target 1 | STANDARD | FAM | NFQ-MGB | 28.17091 | 27.84608 | 0.288513 | 1.13E+11 | | |
| G7 | 10^2 1 | Target 1 | STANDARD | FAM | NFQ-MGB | 31.83701 | 31.70143 | 0.439998 | 1.13E+10 | | |
| G8 | 10^2 1 | Target 1 | STANDARD | FAM | NFQ-MGB | 31.2096 | 31.70143 | 0.439998 | 1.13E+10 | | |
| G9 | 10^2 1 | Target 1 | STANDARD | FAM | NFQ-MGB | 32.05769 | 31.70143 | 0.439998 | 1.13E+10 | | |
| G10 | 10^3 1 | Target 1 | STANDARD | FAM | NFQ-MGB | 35.60445 | 36.47247 | 0.779047 | 1.13E+09 | | |
| G11 | 10^3 1 | Target 1 | STANDARD | FAM | NFQ-MGB | 37.11097 | 36.47247 | 0.779047 | 1.13E+09 | | |
| G12 | 10^3 1 | Target 1 | STANDARD | FAM | NFQ-MGB | 36.70201 | 36.47247 | 0.779047 | 1.13E+09 | | |
| H1 | 10^4 1 | Target 1 | STANDARD | FAM | NFQ-MGB | 39.06717 | 36.25026 | 2.49319 | 1.13E+08 | | |
| H2 | 10^4 1 | Target 1 | STANDARD | FAM | NFQ-MGB | 34.32727 | 36.25026 | 2.49319 | 1.13E+08 | | |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| H3 | 10^4 1 | Target 1 | STANDARD | FAM | NFQ-MGB | 35.35633 | 36.25026 | 2.49319 | 1.13E+08 | | |
| H4 | 10^5 1 | Target 1 | STANDARD | FAM | NFQ-MGB | 35.69355 | 26.63273 | 16.48768 | 11300000 | | |
| H5 | 10^5 1 | Target 1 | STANDARD | FAM | NFQ-MGB | 7.601634 | 26.63273 | 16.48768 | 11300000 | | |
| H6 | 10^5 1 | Target 1 | STANDARD | FAM | NFQ-MGB | 36.603 | 26.63273 | 16.48768 | 11300000 | | |
| H7 | 24 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 32.27652 | 32.4458 | 0.25644 | 1.31E+08 | 103625888 | 39564084 |
| H8 | 24 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 32.74085 | 32.4458 | 0.25644 | 58278400 | 103625888 | 39564084 |
| H9 | 24 1 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 32.32005 | 32.4458 | 0.25644 | 1.22E+08 | 103625888 | 39564084 |
| H10 | | | | | | | | | | | |
| H11 | NEG 4 | Target 1 | NTC | FAM | NFQ-MGB | 7.0949 | | | | | |
| H12 | NEG 5 | Target 1 | NTC | FAM | NFQ-MGB | Undetermined | | | | | |

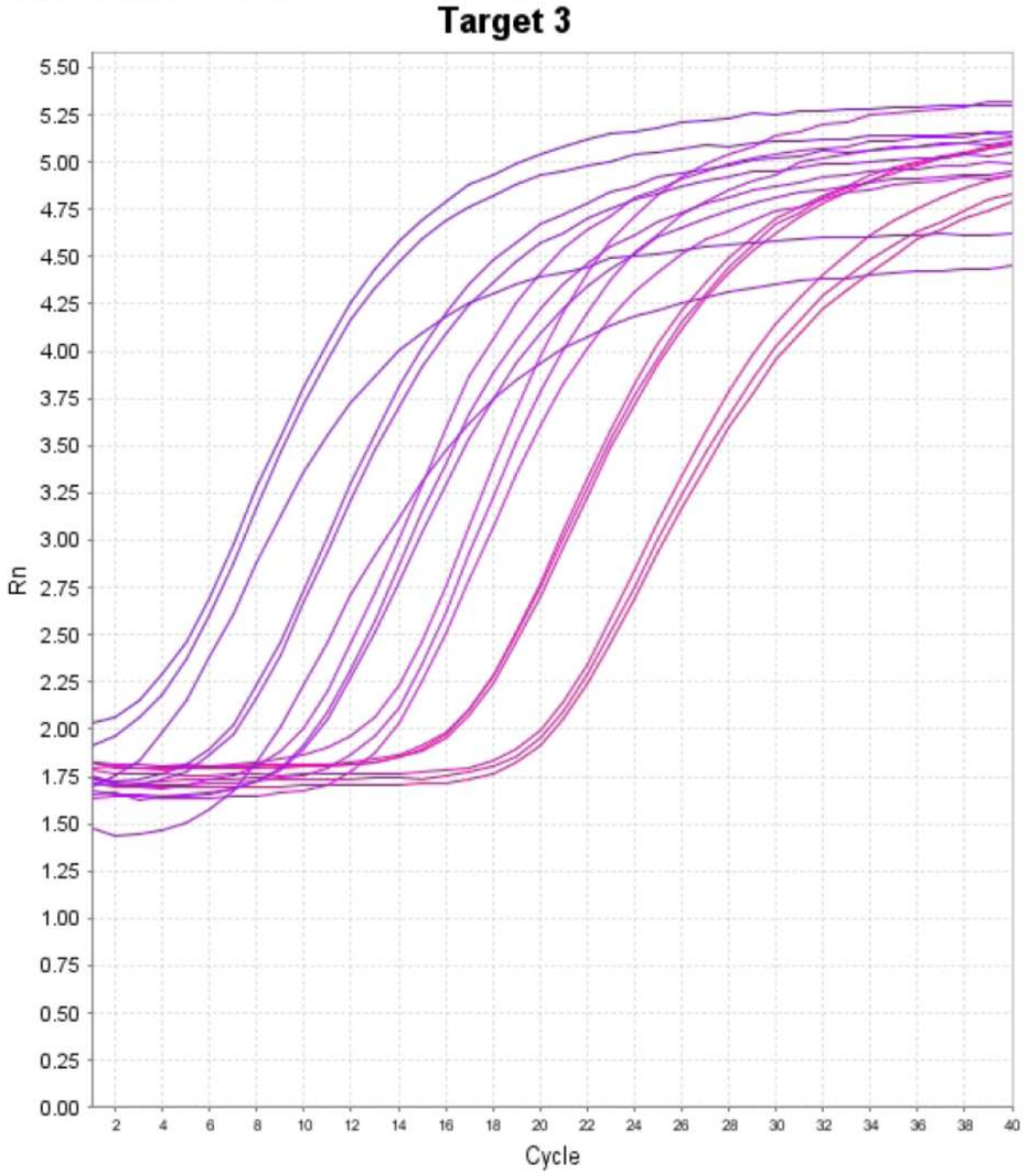
## Amplification Plot (Rn vs. Cycle)

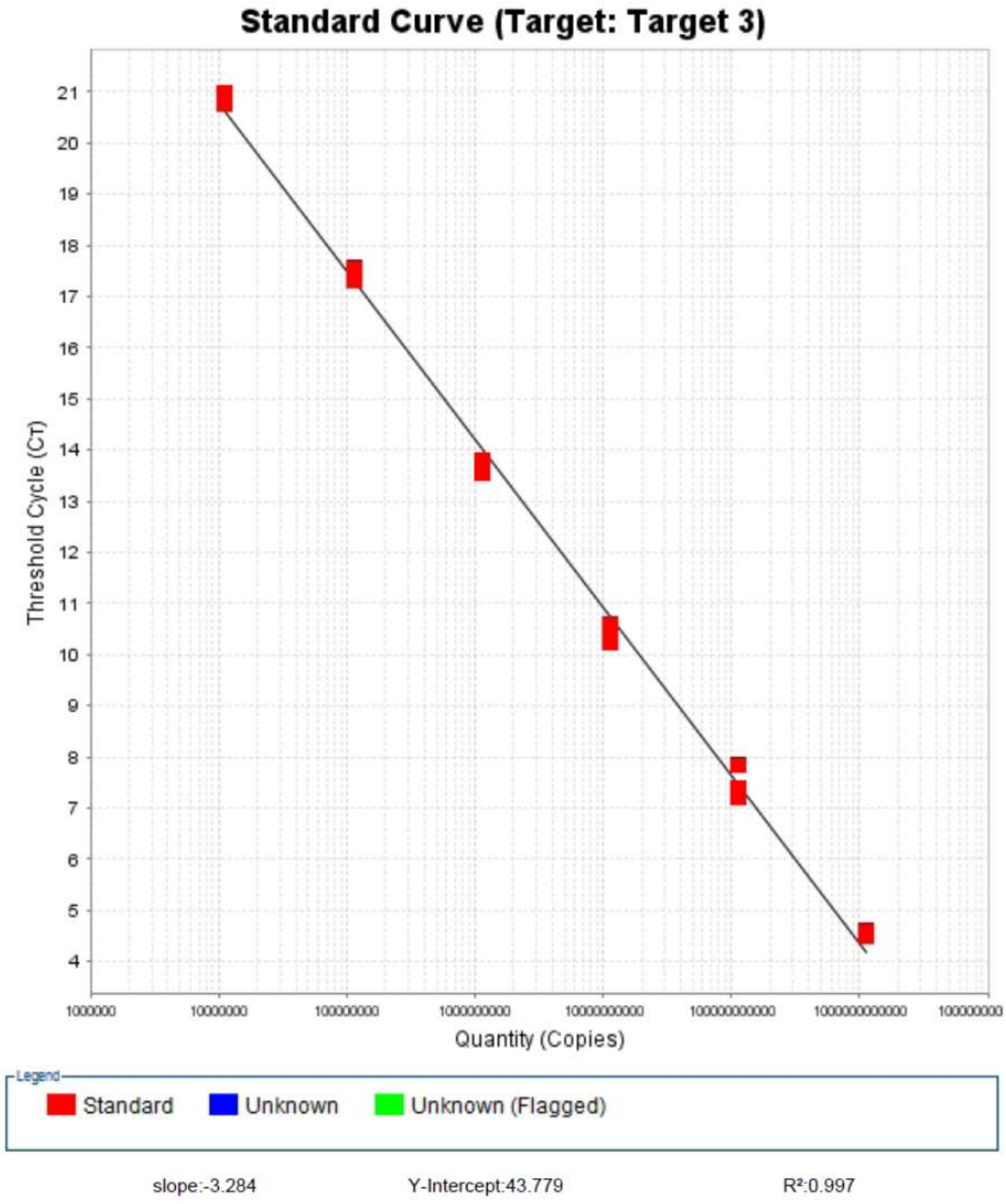


## Amplification Plot (Rn vs. Cycle)



## Amplification Plot (Rn vs. Cycle)






slope:-3.284 Y-Intercept:43.779 $R^2$:0.997

**Table 27: Real- time quantitative raw data for Rotavirus**

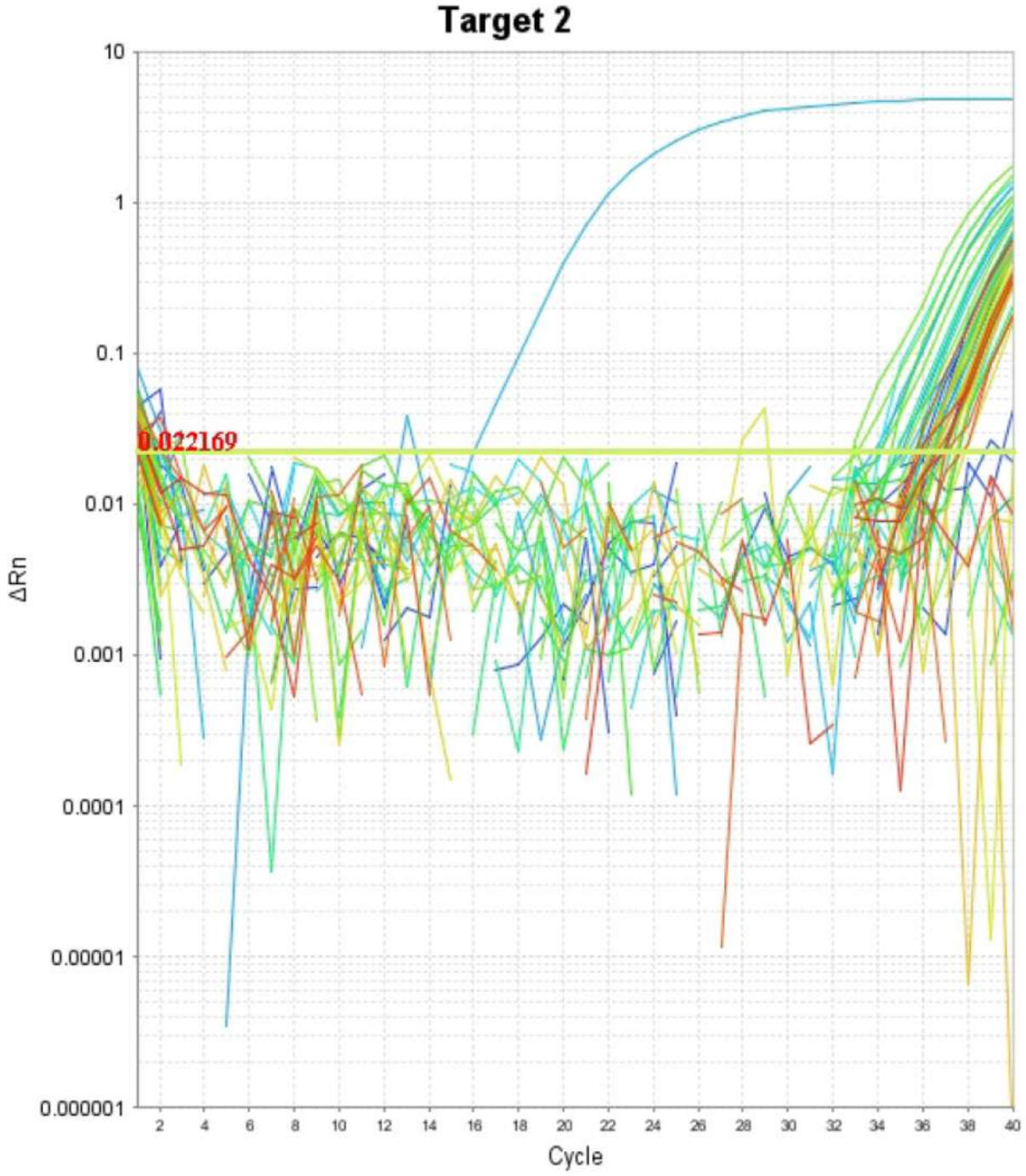
| Well | Sample Name | Target Name | Task | Reporter | Quencher | Cт | Cт Mean | Cт SD | Quantity | Quantity Mean | Quantity SD |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A1 | NEG | Target 1 | UNKNOWN | FAM | NFQ-MGB | Undetermined | 35.46468 | 0.146296 | | | |
| A2 | NEG | Target 1 | UNKNOWN | FAM | NFQ-MGB | 35.36123 | 35.46468 | 0.146296 | 173.5052 | 161.7471 | 16.62843 |
| A3 | NEG | Target 1 | UNKNOWN | FAM | NFQ-MGB | 35.56813 | 35.46468 | 0.146296 | 149.9891 | 161.7471 | 16.62843 |
| A4 | U1 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 36.96601 | 36.4606 | 0.714759 | 56.06472 | 85.14005 | 41.11872 |
| A5 | U1 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | Undetermined | 36.4606 | 0.714759 | | | |
| A6 | U1 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 35.95519 | 36.4606 | 0.714759 | 114.2154 | 85.14005 | 41.11872 |
| A7 | U2 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 35.62294 | 36.74555 | 1.040897 | 144.3121 | 78.5204 | 58.16169 |
| A8 | U2 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 36.935 | 36.74555 | 1.040897 | 57.3022 | 78.5204 | 58.16169 |
| A9 | U2 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 37.67871 | 36.74555 | 1.040897 | 33.94687 | 78.5204 | 58.16169 |
| A10 | U3 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 37.51649 | 37.51649 | | 38.0533 | 38.0533 | 10225 |
| A11 | U3 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | Undetermined | 37.51649 | | | | |
| A12 | U3 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | Undetermined | 37.51649 | | | | |
| B1 | U4 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 36.4048 | 36.57104 | 0.235097 | 83.2272 | 74.54333 | 12.28085 |
| B2 | U4 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | Undetermined | 36.57104 | 0.235097 | | | |
| B3 | U4 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 36.73728 | 36.57104 | 0.235097 | 65.85946 | 74.54333 | 12.28085 |
| B4 | U5 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | Undetermined | 37.49845 | | | | |
| B5 | U5 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | 37.49845 | 37.49845 | | 38.53984 | 38.53984 | |
| B6 | U5 AUT | Target 1 | UNKNOWN | FAM | NFQ-MGB | Undetermined | 37.49845 | | | | |
| B7 | U1 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 36.37704 | 37.15104 | 0.820705 | 84.86959 | 54.67841 | 29.07961 |
| B8 | U1 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 37.06447 | 37.15104 | 0.820705 | 52.31047 | 54.67841 | 29.07961 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| B9 | U1 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 38.01159 | 37.15104 | 0.820705 | 26.85517 | 54.67841 | 29.07961 |
| B10 | U2 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 36.93188 | 36.47256 | 0.64959 | 57.4279 | 83.53548 | 36.9217 |
| B11 | U2 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 36.01323 | 36.47256 | 0.64959 | 109.6431 | 83.53548 | 36.9217 |
| B12 | U2 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | Undetermined | 36.47256 | 0.64959 | | | |
| C1 | U3 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 36.77206 | 36.02757 | 1.052865 | 64.26679 | 123.7925 | 84.18204 |
| C2 | U3 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 35.28308 | 36.02757 | 1.052865 | 183.3182 | 123.7925 | 84.18204 |
| C3 | U3 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | Undetermined | 36.02757 | 1.052865 | | | |
| C4 | U4 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 33.24081 | 33.50415 | 0.816621 | 771.936 | 707.9021 | 343.8065 |
| C5 | U4 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 32.85168 | 33.50415 | 0.816621 | 1015.19 | 707.9021 | 343.8065 |
| C6 | U4 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 34.41994 | 33.50415 | 0.816621 | 336.5805 | 707.9021 | 343.8065 |
| C7 | U5 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | Undetermined | 37.50657 | | | | |
| C8 | U5 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | 37.50657 | 37.50657 | | 38.32013 | 38.32013 | |
| C9 | U5 WIN | Target 1 | UNKNOWN | FAM | NFQ-MGB | Undetermined | 37.50657 | | | | |
| C10 | U1 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 34.33947 | 35.95699 | 1.436631 | 356.1996 | 162.8567 | 168.0759 |
| C11 | U1 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 36.44695 | 35.95699 | 1.436631 | 80.79404 | 162.8567 | 168.0759 |
| C12 | U1 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 37.08454 | 35.95699 | 1.436631 | 51.5765 | 162.8567 | 168.0759 |
| D1 | U2 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 37.2268 | 36.56396 | 0.738569 | 46.66175 | 81.57366 | 43.51059 |
| D2 | U2 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 36.69729 | 36.56396 | 0.738569 | 67.74 | 81.57366 | 43.51059 |
| D3 | U2 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 35.76782 | 36.56396 | 0.738569 | 130.3192 | 81.57366 | 43.51059 |
| D4 | U3 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 37.58411 | 37.30225 | 0.3986 | 36.28454 | 45.1216 | 12.49749 |
| D5 | U3 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | Undetermined | 37.30225 | 0.3986 | | | |
| D6 | U3 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 37.0204 | 37.30225 | 0.3986 | 53.95867 | 45.1216 | 12.49749 |

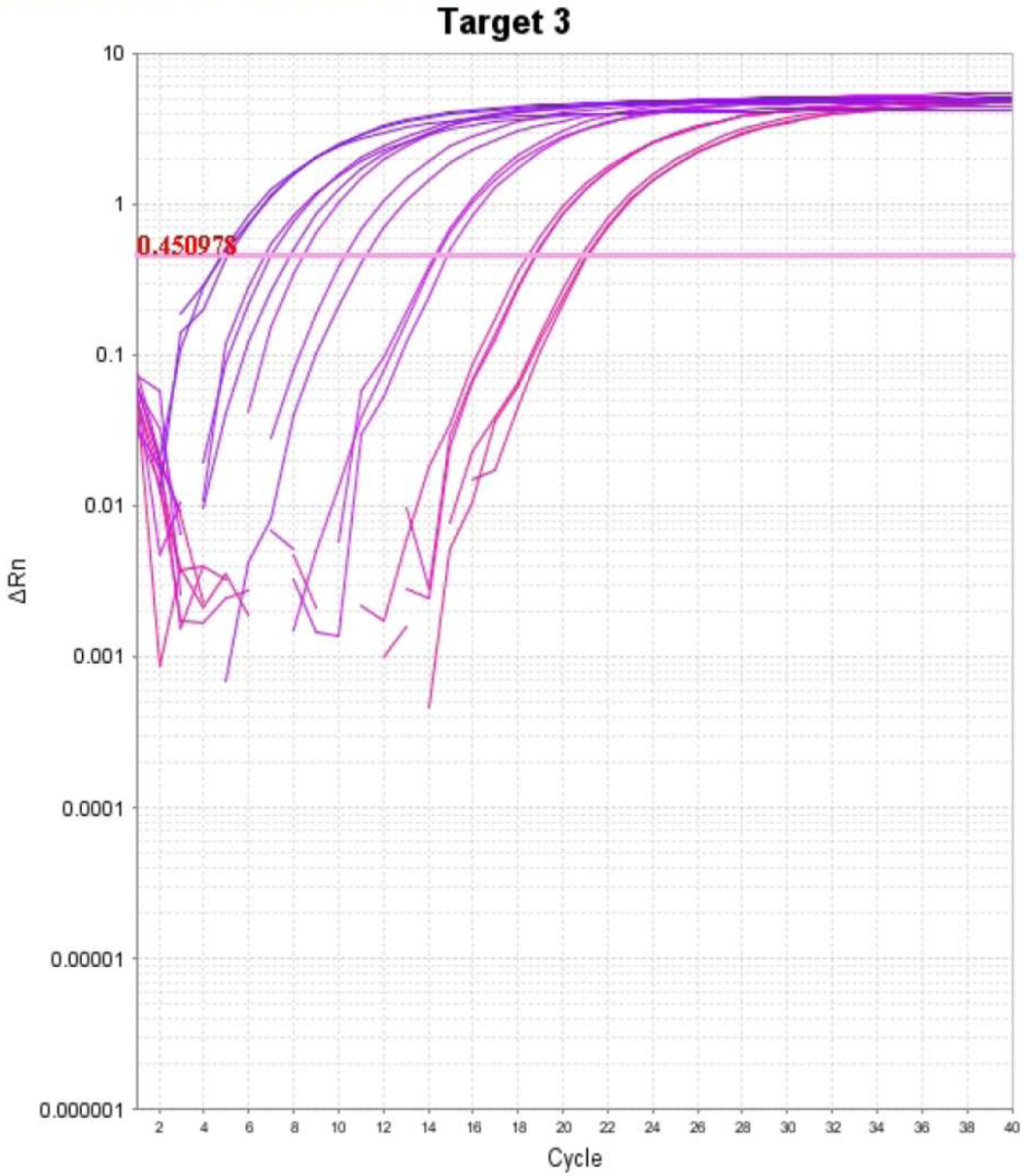
| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| D7 | U4 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | Undetermined | 35.03394 | 0.362983 | 74.80189 | 98.99002 | 24.97497 |
| D8 | U4 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | Undetermined | 35.03394 | 0.362983 | 97.48447 | 98.99002 | 24.97497 |
| D9 | U4 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 35.03394 | 35.03394 | 0.362983 | 124.6837 | 98.99002 | 24.97497 |
| D10 | U5 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 34.89225 | 35.41529 | 0.739691 | 241.3753 | 178.4771 | 88.95155 |
| D11 | U5 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | 35.93833 | 35.41529 | 0.739691 | 115.5788 | 178.4771 | 88.95155 |
| D12 | U5 SPR | Target 1 | UNKNOWN | FAM | NFQ-MGB | Undetermined | 35.41529 | 0.739691 | | | |
| E1 | U1 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 33.93731 | 35.95532 | 1.886054 | 472.7641 | 199.7698 | 238.2165 |
| E2 | U1 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 37.67349 | 35.95532 | 1.886054 | 34.07172 | 199.7698 | 238.2165 |
| E3 | U1 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 36.25515 | 35.95532 | 1.886054 | 92.47353 | 199.7698 | 238.2165 |
| E4 | U2 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 36.40289 | 36.118 | 0.421192 | 83.33948 | 105.0175 | 33.14645 |
| E5 | U2 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 36.31691 | 36.118 | 0.421192 | 88.53924 | 105.0175 | 33.14645 |
| E6 | U2 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 35.63419 | 36.118 | 0.421192 | 143.1738 | 105.0175 | 33.14645 |
| E7 | U3 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 33.89303 | 28.09763 | 10.26656 | 487.7306 | 40447828 | 70056912 |
| E8 | U3 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 34.15606 | 28.09763 | 10.26656 | 405.2898 | 40447828 | 70056912 |
| E9 | U3 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 16.2438 | 28.09763 | 10.26656 | 1.21E+08 | 40447828 | 70056912 |
| E10 | U4 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | Undetermined | 35.38677 | 0.275663 | | | |
| E11 | U4 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 35.5817 | 35.38677 | 0.275663 | 148.5632 | 172.0205 | 33.17369 |
| E12 | U4 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 35.19185 | 35.38677 | 0.275663 | 195.4779 | 172.0205 | 33.17369 |
| F1 | U5 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 38.70914 | 37.74277 | 1.36665 | 16.43502 | 40.25277 | 33.68338 |
| F2 | U5 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | 36.7764 | 37.74277 | 1.36665 | 64.07052 | 40.25277 | 33.68338 |
| F3 | U5 SUM | Target 1 | UNKNOWN | FAM | NFQ-MGB | Undetermined | 37.74277 | 1.36665 | | | |
| F4 | 21 | Target 1 | UNKNOWN | FAM | NFQ-MGB | Undetermined | 36.18908 | | | | |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| F5 | 21 | Target 1 | UNKNOWN | FAM | NFQ-MGB | Undetermined | 36.18908 | | | | |
| F6 | 21 | Target 1 | UNKNOWN | FAM | NFQ-MGB | 35.83062 | 36.18908 | | 124.6837 | 98.99002 | 24.97497 |
| G1 | ROT | Target 1 | STANDARD | FAM | NFQ-MGB | Undetermined | | | 6.4E+12 | | |
| G2 | ROT | Target 1 | STANDARD | FAM | NFQ-MGB | Undetermined | | | 6.4E+12 | | |
| G3 | ROT | Target 1 | STANDARD | FAM | NFQ-MGB | Undetermined | | | 6.4E+12 | | |
| G4 | 10^1 | Target 1 | STANDARD | FAM | NFQ-MGB | 4.614129 | 4.292876 | 0.28239 | 6.4E+11 | | |
| G5 | 10^1 | Target 1 | STANDARD | FAM | NFQ-MGB | 4.180641 | 4.292876 | 0.28239 | 6.4E+11 | | |
| G6 | 10^1 | Target 1 | STANDARD | FAM | NFQ-MGB | 4.083858 | 4.292876 | 0.28239 | 6.4E+11 | | |
| G7 | 10^2 | Target 1 | STANDARD | FAM | NFQ-MGB | 7.492037 | 6.736282 | 0.877904 | 6.4E+10 | | |
| G8 | 10^2 | Target 1 | STANDARD | FAM | NFQ-MGB | 6.943505 | 6.736282 | 0.877904 | 6.4E+10 | | |
| G9 | 10^2 | Target 1 | STANDARD | FAM | NFQ-MGB | 5.773306 | 6.736282 | 0.877904 | 6.4E+10 | | |
| G10 | 10^3 | Target 1 | STANDARD | FAM | NFQ-MGB | 10.99808 | 10.64236 | 0.317967 | 6.4E+09 | | |
| G11 | 10^3 | Target 1 | STANDARD | FAM | NFQ-MGB | 10.38576 | 10.64236 | 0.317967 | 6.4E+09 | | |
| G12 | 10^3 | Target 1 | STANDARD | FAM | NFQ-MGB | 10.54324 | 10.64236 | 0.317967 | 6.4E+09 | | |
| H1 | 10^4 | Target 1 | STANDARD | FAM | NFQ-MGB | 14.81107 | 14.72498 | 0.223241 | 6.4E+08 | | |
| H2 | 10^4 | Target 1 | STANDARD | FAM | NFQ-MGB | 14.89236 | 14.72498 | 0.223241 | 6.4E+08 | | |
| H3 | 10^4 | Target 1 | STANDARD | FAM | NFQ-MGB | 14.47151 | 14.72498 | 0.223241 | 6.4E+08 | | |
| H4 | 10^5 | Target 1 | STANDARD | FAM | NFQ-MGB | 16.08947 | 16.65344 | 0.593893 | 64000000 | | |
| H5 | 10^5 | Target 1 | STANDARD | FAM | NFQ-MGB | 17.27331 | 16.65344 | 0.593893 | 64000000 | | |
| H6 | 10^5 | Target 1 | STANDARD | FAM | NFQ-MGB | 16.59755 | 16.65344 | 0.593893 | 64000000 | | |
| H10 | NEG | Target 1 | NTC | FAM | NFQ-MGB | 35.65716 | | | | | |
| H11 | NEG | Target 1 | NTC | FAM | NFQ-MGB | 36.8895 | | | | | |

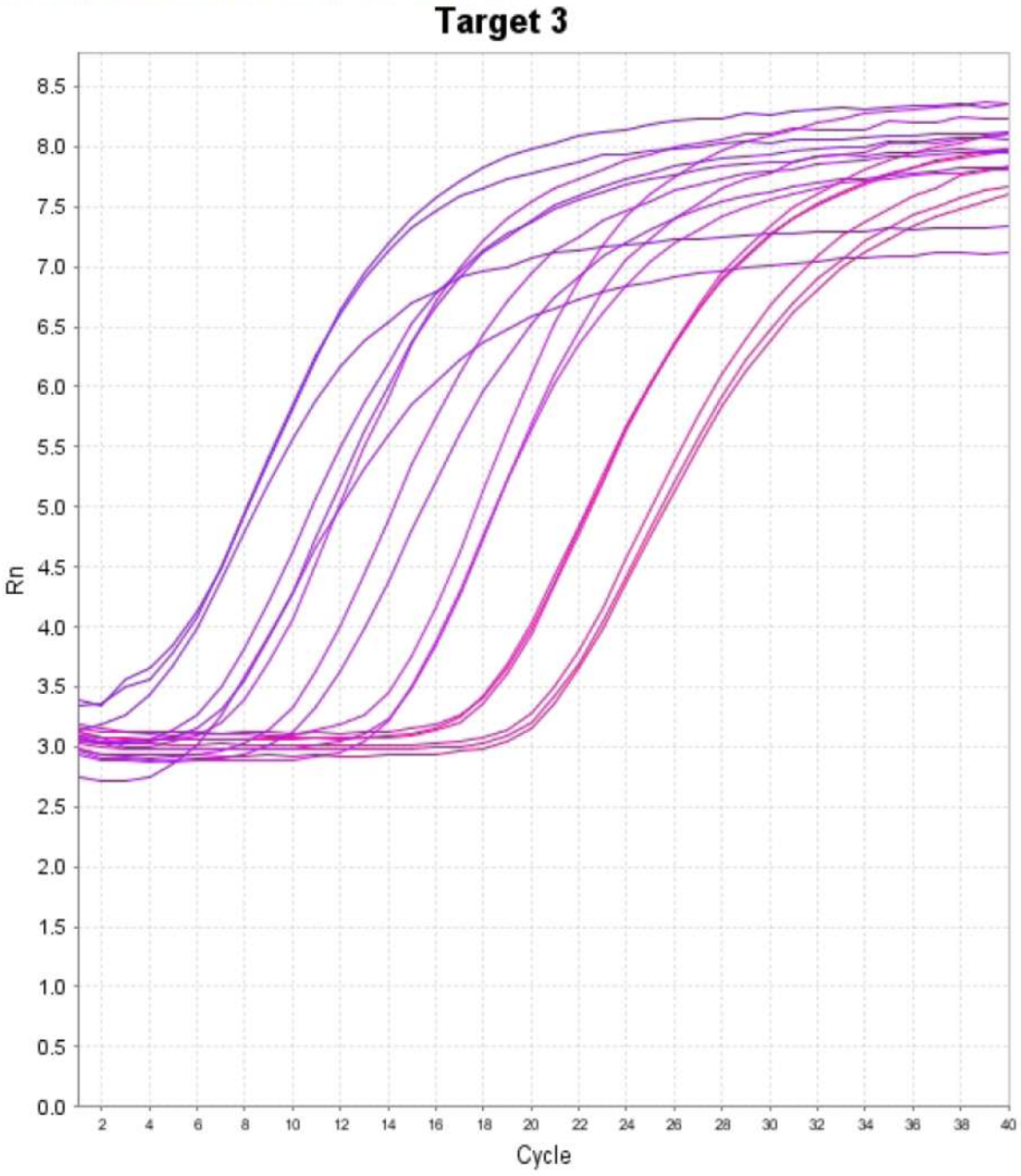
## Amplification Plot (Rn vs. Cycle)

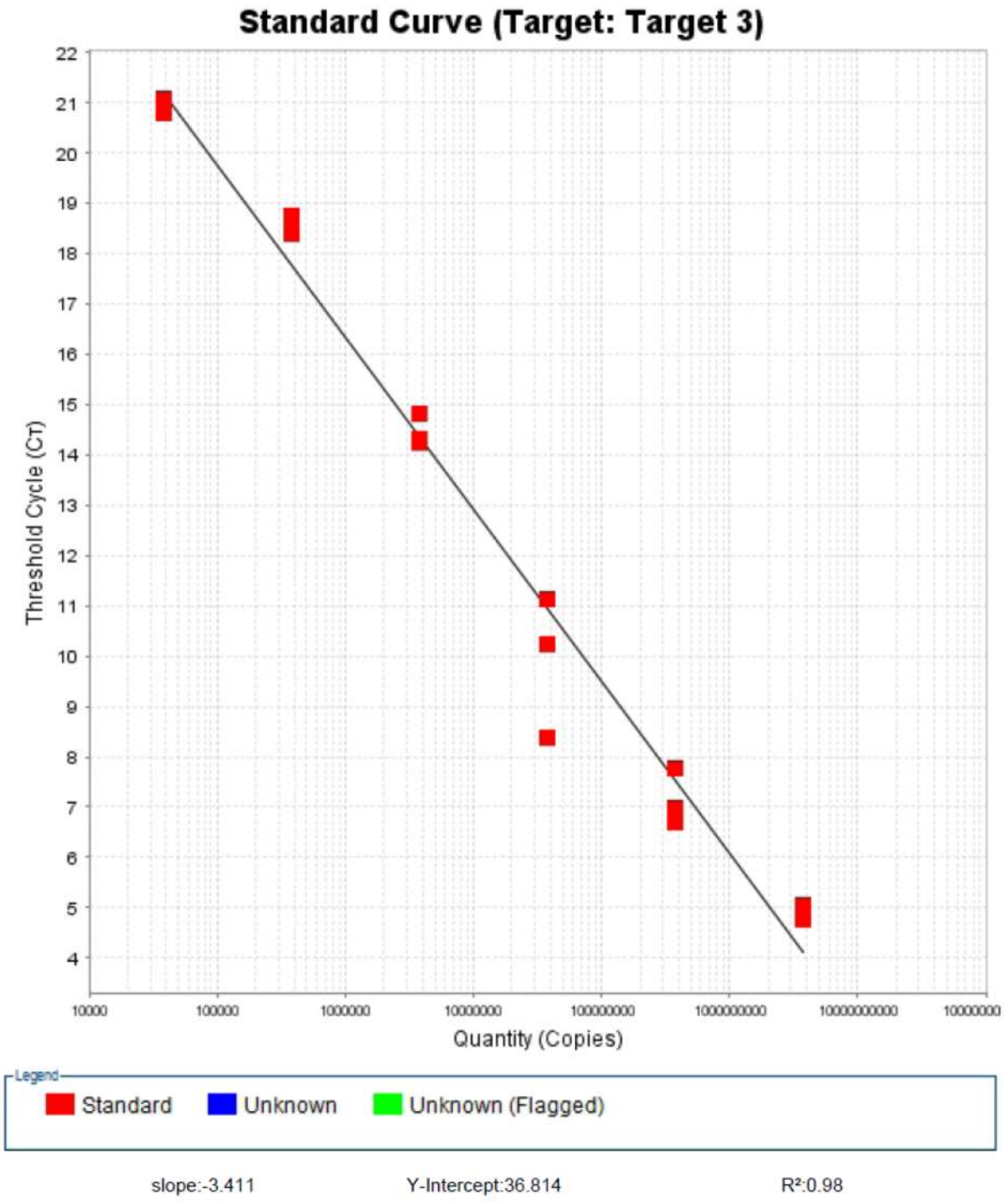


## Amplification Plot (Rn vs. Cycle)



## Amplification Plot (Rn vs. Cycle)






slope:-3.411 Y-Intercept:36.814 $R^2$:0.98