Cape Peninsula
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THE ESTABLISHMENT OF A ROUTINE MONITORING TECHNIQUE FOR DETECTING THE MOST PREVALENT PATHOGENIC VIRUSES IN RIVER WATER, WESTERN CAPE, SOUTH AFRICA

by

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DECLARATION

I, Michael John Saayman, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

30/11/2012

Signed **Date**

ABSTRACT

In many developed countries worldwide the provision of safe, clean water is an expected commodity. In South Africa however, as in most developing countries, the access and supply of water safe for human consumption is challenged or complicated by pollution and more recently water availability. Point-source pollutants in surface- and groundwater are normally the most concentrated closest to the pollutant source (such as the end of a pipe or an underground injection system). Examples of point-source pollution are commercial and industrial businesses, that often discharge waste such as solvents and heavy metals from their operations. In contrast, non-point-source pollution occurs due to runoff moving across or through the ground and absorbing and accumulating pollutants which eventually end up in streams, rivers and dams. The lack of waste removal and adequate sanitation facilities results in the disposal of faecal matter and sewage into storm water drains which flow directly into the river systems contributing to the incidence of diseases such as gastroenteritis, diarrhoea and chronic lung ailments, caused by waterborne pathogenic bacteria, viruses and fungi. Routine water quality analysis however, does not include monitoring for viral contaminants, as this process is hampered by the lack of simple, reliable, time- and cost-effective testing methods to concentrate and detect viral pathogens. The primary aim of this study was thus to establish and optimise routine monitoring techniques for the detection of rota-, adeno- and enteroviruses in the Berg- and Plankenburg Rivers, Western Cape. Initially, various concentration and extraction methods were compared for the optimum recovery of viruses from spiked water samples. One hundred milliliter water samples were spiked with one milliliter rotavirus and two milliliters adenovirus control virions (Coris Bioconcept, Gembloux, Belgium). Optimisation testing of enterovirus was however, not completed due to the unavailability of a positive control. Four viral concentration techniques, namely the Silicon dioxide (SiO_2) method, positively charged, negatively charged and the mixed-ester filters, were compared. Various nucleic acid extraction methods were also employed to establish which method would provide optimum yields for both DNA and RNA nucleic acids. The extraction techniques included the TRIzol method (Invitrogen, California, USA) for RNA extraction, the Roche High Pure PCR Template Preparation kit (Roche, Mannheim, Germany) for DNA extraction, and the QIAamp Ultrasens Virus kit (Qiagen GmbH, Hilden, Germany) for simultaneous RNA and DNA extraction. The use of virus specific primers within the PCR technique was also optimised. In addition, gene specific primers and oligo$(dT)_{15}$ primers were tested and compared to establish which primers would yield the best results since gene specific primers are said to be more sensitive than oligo$(dT)_{15}$ primers (van Pelt-Verkuil *et al.,* 2008) when synthesising cDNA (rotavirus). The SiO_2 concentration method yielded variable results when it was used with the various nucleic acid extraction techniques in this study, since positive PCR results were obtained when used in combination

with some techniques, while negative results were obtained with others. Similarly, variable results were also obtained when negatively charged filters were used to concentrate virus particles, and when this method was used in conjunction with various virus nucleic acid extraction techniques to identify different viruses by RT-PCR and PCR. Results for the non-charged mixed-ester filter were comparable to the positively charged filters when used in conjunction with the various nucleic acid extraction techniques in this study. Both these techniques yielded the highest viral particle concentration from the spiked water samples. Pilot study results indicated the presence of rotavirus and adenovirus detected by RT-PCR and PCR respectively, when filtering through the positively charged filter. The positively charged filter/QIAamp UltraSens virus kit combination was found to be the optimum combination when analysing the spiked water results and was then employed for the concentration of virus particles in the river water samples collected from the Plankenburg- and Berg River systems throughout the study period. The expected PCR product of 346 bp for rotavirus was absent in all 72 river water samples analysed for both river systems. In contrast to the PCR results obtained for rotavirus, the expected product of 261 bp for adenovirus was detected in 22 (30.5%) samples collected throughout the study period. Fifteen of the 22 adenovirus positive samples were found in the Plankenburg River (distributed over all sites), while seven of the 22 adenovirus positive samples were found in the Berg River (all sites). A nested PCR was used to detect enterovirus in the river water samples collected from both river systems throughout the study period. In the first round of the enterovirus PCR 15 river water samples (at various sites for both river systems) yielded a faint 513 bp product. Further amplification by nested PCR then yielded 13 (18.1%) positive nested PCR products of 297 bp. The incidence of adenovirus and enterovirus in river waters reported in the current study and the Van Heerden *et al*. (2003) investigation motivates for similar studies to be conducted in drinking water, dam water used for recreational purposes as well as rainwater, which is gaining popularity as a sustainable water source.

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DEDICATION

This thesis is dedicated to my late mother Eileen Elizabeth Saayman and sister Denise Roseline Saayman

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ABBREVIATIONS

ANZECC	Australian and New Zealand Environment and Conservation Council
CCME	Canadian Council of Ministers of the Environment
DWA	Department of Water Affairs
DWAF	Department of Water Affairs and Forestry (DWAF name changed to DWA in Government Gazette, 2009)
EPA	Environmental Protection Agency
ICTV	International Committee on Taxonomy of Viruses
MAP	Mean Annual Precipitation
NCBI	National Centre for Biotechnology Information
PCCL	Pathogen Contaminant Candidate List
SABS	South African Bureau of Standards
WBDO	Waterborne Disease Outbreak
WHO	World Health Organisation

CHAPTER ONE
LITERATURE REVIEW

LITERATURE REVIEW

1.1 INTRODUCTION

In many developed countries worldwide the provision of safe, clean water is an expected commodity. In South Africa however, as in most developing countries, the access and supply of water safe for human consumption is challenged or complicated by pollution and more recently water availability (Turton, 2008; Department of Water Affairs, 2010).

South Africa has variable rainfall patterns with potentially high evaporation rates of 2000 to 3000 mm per annum experienced in parts of the country. With an average annual precipitation of 437 mm (**Figure 1.1**) South Africa can be designated a relatively dry country (Earle *et al.*, 2005; Turton, 2008). The Western Cape region of South Africa in particular has a Mediterranean climate, with cool, wet winters and warm, dry summers (Le Maitre, 1996). In 2009 the Eden District Municipality in the Western Cape experienced the lowest annual rainfall (477 mm) since 1921, which accounted for 63% of its annual rainfall (DWA, 2010). As a consequence, severe water shortages were experienced and water restrictions were implemented. In 1994 approximately 12 to 14 million people in South Africa did not have access to a municipal water supply and 21 million inhabitants had no formal sanitation infrastructure (Turton, 2008). Also, in 2006 the Department of Water Affairs and Forestry estimated that 15.7 million South Africans did not have access to basic water resources and very little infrastructure was in place to supply water to especially people falling into the lower socio-economic income bracket (Dungumaro, 2007).

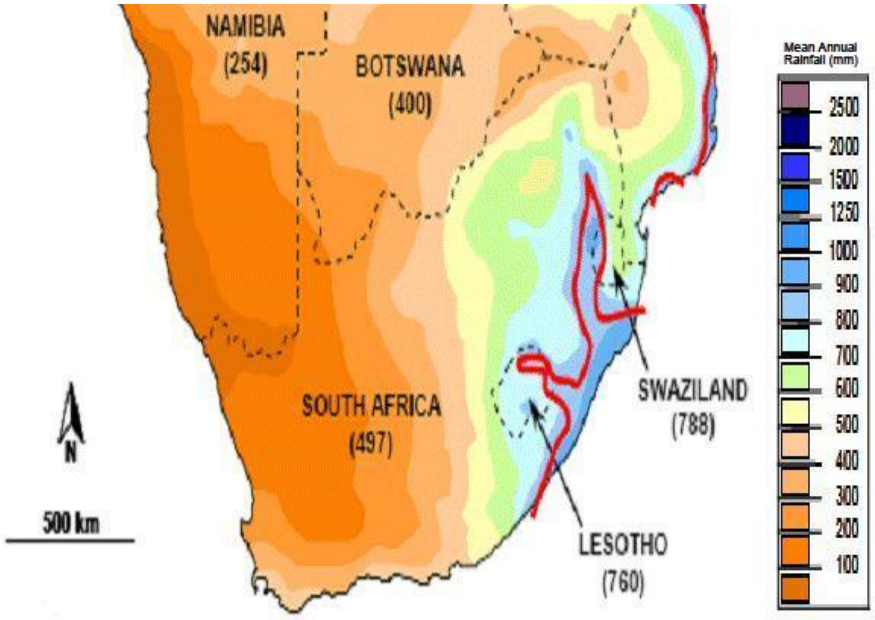

Figure 1.1: Mean annual precipitation (MAP) in the Southern African region (Adapted from Turton, 2008).

The lack of adequate water resources could further be ascribed to industrial development and urbanisation, which leads to the direct competition for limited water sources. Urbanisation in South Africa has led to the formation of informal housing schemes, constructed close to untreated surface water sources, such as rivers and dams. In many cases the lack of waste removal and adequate sanitation facilities results in the disposal of faecal matter and sewage into storm water drains which flow directly into the river systems (Gerba, 1996). Diseases such as gastroenteritis, diarrhoea and chronic lung ailments, caused by waterborne pathogenic bacteria, viruses and fungi, are thus rife in the informal settlements especially among infants and the immuno-compromised (Bachmann *et al.,* 1996). Diarrhoea however, remains a common cause of illness worldwide, despite the provision of safer food and potable water supplies, improved sanitation and the promotion of non-invasive interventions such as oral rehydration (Hendrickx *et al.*, 2008; Lindesmith *et al.*, 2008). As a direct result, approximately 1.8 million children worldwide die annually due to diarrhoea related disease (**Figure 1.2**). This relates to about 15% of the major causes of death in children, with 1% of neonatal deaths resulting from diarrhoea associated disease (Bryce *et al.,* 2005; Black *et al*., 2010).

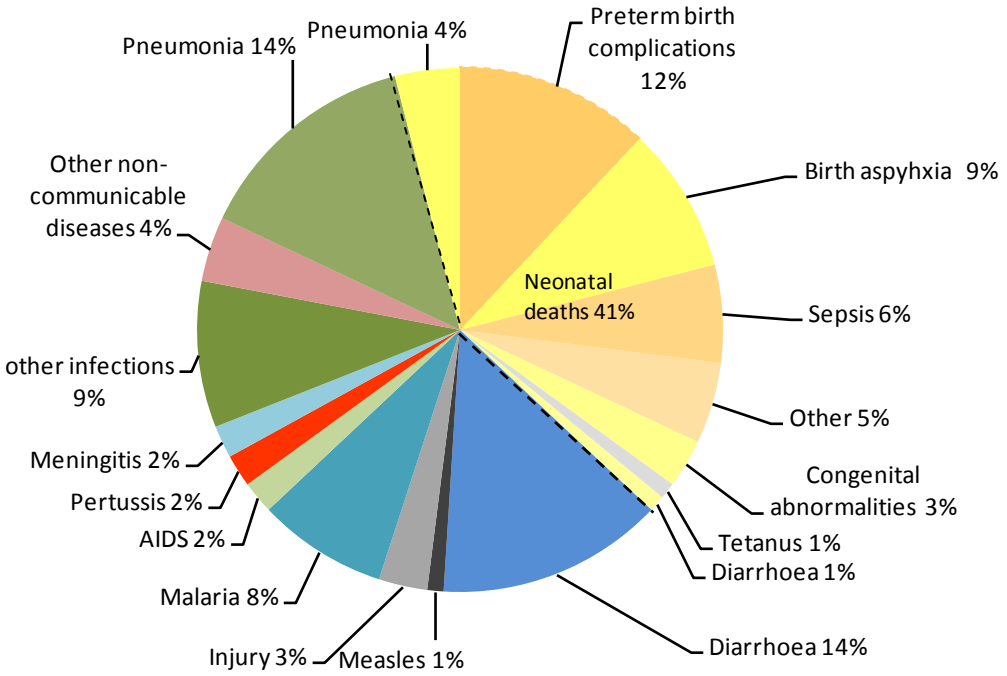

Figure 1.2: Global causes of child deaths. Data are separated by dotted line into deaths of neonates aged 0-27 days to the right, and children aged 1-59 months (Adapted from Black *et al*., 2010).

1.2 SOURCES OF WATER POLLUTION

Water resources in South Africa are mainly collected in dams and water abstraction schemes for use in agriculture, industry and for domestic purposes (Langwaldt and Puhakka, 2000). Moreover, surface water, such as water in streams, rivers, lakes, swamps and the ocean, is lost through evaporation and seepage into groundwater and reclaimed by precipitation. Additionally, groundwater is found in crevices and sand, gravel, silt or clay under the ground, saturating the empty spaces (Hoyle, 2005). In the Green Drop Report released by the Department of Water Affairs (2009), an audit of the waste water treatment plants in South Africa, it was found that about 50% of the waste water treatment plants were operating below standard. These results imply that large amounts of untreated or inadequately treated sewage (mainly those of small towns) flow into South Africa's rivers and streams (DWA, 2009; Tladi, 2010). Furthermore, in informal settlements, toilet facilities are usually shared by two or more families and in many instances the "bucket system" is still employed. Accordingly, inadequate sanitation and faecal waste removal facilities then leads to contamination of surface- and groundwater sources (Barnes, 2003). Various factors influencing the potential health risk to humans due to polluted waters are illustrated in **Figure 1.3**. The diagram depicts the impact that the lack of sanitation, in and around dense settlements of communities, may have on land use which leads to contaminated water sources from run-off. The overloading of water treatment plants, together with the lack of maintenance, leads to ineffective water treatment resulting in contaminated discharge into drinking water. Moreover, when contaminated water is ingested this leads to an increase in health risks, which in turn leads to corrective action costs which places a burden on the economy (Murray, 1999).

The Department of Water Affairs and Forestry (DWAF, 1996; DWAF name changed to DWA in Government Gazette, 2009) reported that municipal and industrial waste, such as poultry processing operations, slaughter houses, agricultural run-off and food processing factories, are the main sources of river water pollution. In addition, informal settlements may serve as point sources of pollution to rivers, resulting in an increased microbial, organic and inorganic substance load in the water source (Gerba, 1996). Groundwater reservoirs, such as wells or boreholes are generally assumed to be safe and free from pollutants. However, wells may be vulnerable to contamination with viruses in particular, and with other tenacious microorganisms (West Midlands Environment Agency, 2000).

Point-source pollutants in surface- and groundwater are normally the most concentrated closest to the pollutant source (such as the end of a pipe or an underground injection system). Examples of point-source pollution are commercial and industrial businesses, that often discharge waste such as solvents and heavy metals from their operations (Gumbo *et al*., 2003;

Manders et al., 2009). In contrast, non-point-source pollution occurs due to runoff moving across or through the ground and absorbing and accumulating pollutants which eventually end up in streams, rivers and dams. An example of non-point-source pollution is the use of pesticides and fertilisers in agriculture; municipal sources include wastewater treatment plants, and landfills. Furthermore, in residential areas waste such as oils, grease and toxic materials can be picked up by storm water runoff, while bacteria and other microorganisms are common in agricultural and residential wastes, all of which contributes to non-point-source pollution (Chowdary, 2005).

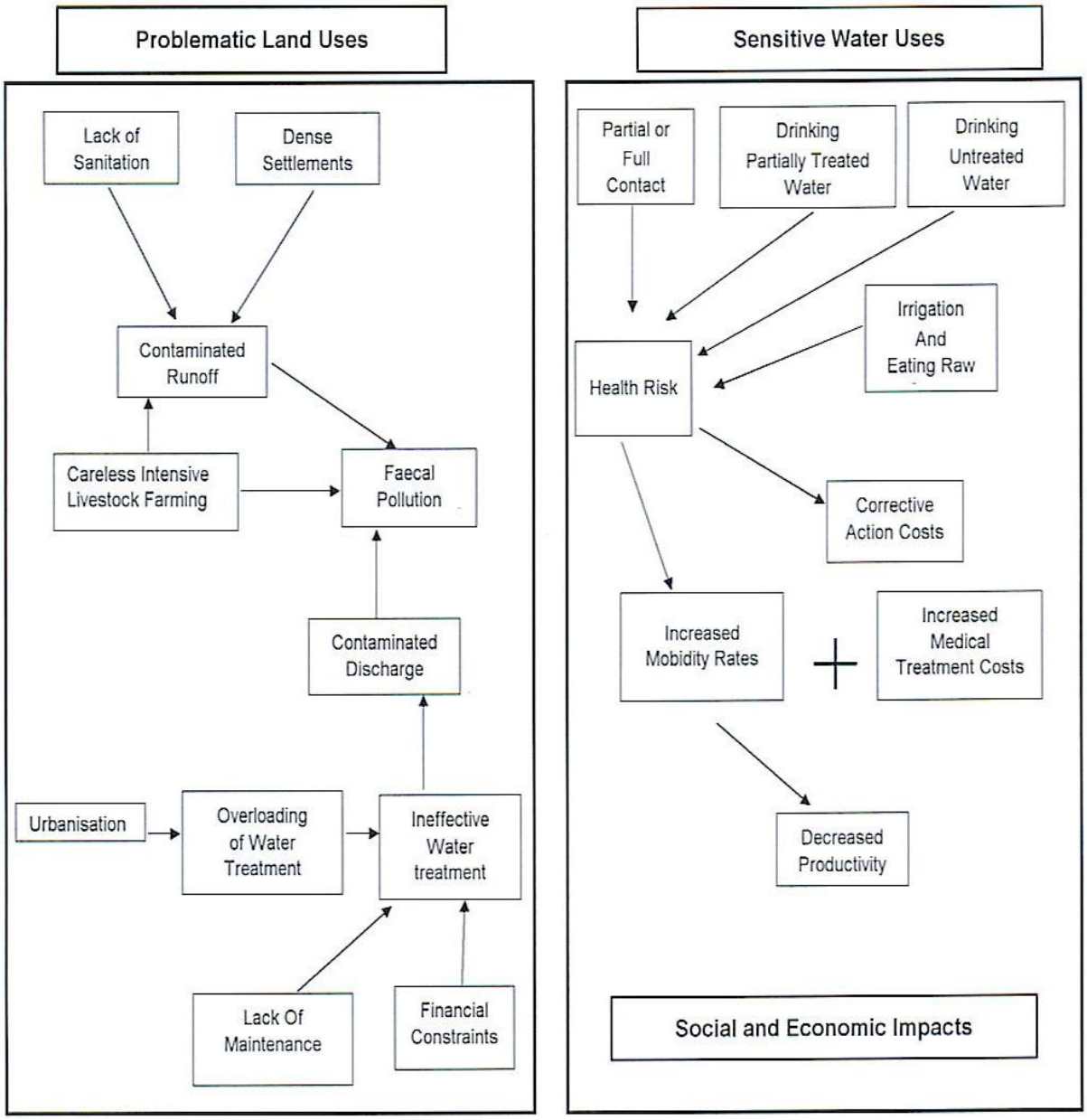

Figure 1.3: Diagrammatic illustration of problematic land uses, sensitive water uses and impacts of health risk (Adapted from Murray, 1999)

1.3 RIVER WATER QUALITY

The use of water in the domestic environment is common to all consumers and includes water for drinking, food preparation, bathing and personal hygiene, washing (for example dishes and laundry) and gardening (irrigation). Domestic water consumers can therefore experience a range of consequences as a result of changes in water quality, which include; health impacts; economic impacts - such as increased costs of water treatment; and aesthetic impacts - such as changes in water taste, odour or colour. The microbial levels in potable and river water sources in South Africa are however, routinely tested by local municipalities and water monitoring bodies. This allows for the monitoring of the quality of the water sources which should meet the criteria as stipulated by the South African Bureau of Standards (SABS), Act No 24 of 1945 and the Department of Water Affairs and Forestry (1996). Various indicator organisms that are used to determine the quality of domestic -, recreational - and irrigational water are listed in **Table 1.1**. These guidelines stipulate that when surface water is used for domestic purposes, the total coliform counts should not exceed five total coliforms per 100 ml, no faecal coliforms or *E.coli* must also be present in the water source (DWAF, 1996).

Table 1.1 Lists the established water quality guidelines for indicator bacteria in domestic water use (Adapted from DWAF, 1996).

Microorganism	**DWAF (1996) (CFU/100ml)**
Total coliforms	~5 (Domestic purposes)
Faecal coliforms	0 (Domestic purposes) ~2000 (Recreational purposes) ~10000 (Irrigational purposes)
Enterococci	0 (Domestic purposes) ~230 (Intermediate contact recreational purposes) ~30 (full contact recreational purposes)
Escherichia coli	0 (Domestic purposes) ~130 (recreational purposes) ~1 (Irrigational purposes)

Moreover, the total water resources available in South Africa are utilised in different sectors such as; for agricultural activities, where 52% of the total water resources are used; in industry, mining and power generation where 12.5% of the total water resources are used; and for domestic and municipal usage, where 12% of the total water resources are used (Holtzhausen,

2002; Mack *et al.*, 2004). The DWAF (1996) indicated that the reference used as a guideline for enteric viruses in domestic water should be the impact on human health since ingesting drinking water should not induce illness in humans. Guidelines indicating the effects of enteric viruses on human health are listed in **Table 1.2**.

Table 1.2 The effects of enteric viruses on human health (Adapted from DWAF, 1996)

Enteric virus range ($TCID_{50}$/10ml)*	**Effects**
0	Negligible risk of infection expected
1	Slight risk of enteric virus infection for continuous exposure; minimal risk for short or occasional exposures
1-10	Medium risk of enteric virus infection for continuous exposure; probable low risk for occasional exposure
>10	Risk of enteric virus infection is significant and increases as virus levels increase

* $TCID_{50}$/10ml = Tissue culture infectious dose required to cause 50 % infection/10 ml

Routine water quality analysis does not include monitoring for viral contaminants, as this process is hampered by the lack of simple, reliable, time- and cost-effective testing methods to concentrate and detect viral pathogens. Consequently the quality of the water sources relating to virus contamination is not known (Bosch *et al*., 2008). However, pathogenic viruses can be indirectly detected by the analyses and presence of viruses which infect and replicate in *Escherichia coli* (*E. coli*) and related coliform bacteria. Hence, coliphages have been proposed as indicators of faecal pollution due to their constant presence in water sources, and because they do not multiply outside a host. *Escherichia coli* phages and *Bacteroides fragilis* phages are somatic coliphages which yield better resistance and persistence than the common indicators, making them a preferential tool for the monitoring of the presence of viruses in polluted water (Grabow, 2001; Baldini and Brezina, 2008). In addition, coliforms serve as indicators of faecal contamination, as they are present in large numbers in the faeces of warm-blooded animals and humans. **Table 1.3** illustrates the effects of coliphages on human health as described by the Department of Water Affairs and Forestry, (1996). When the coliphage range is 0-1 coliphages per 100 ml then no sewage pollution is indicated and negligible risk to viral infection is declared. However, when more than 100 coliphages per 100 ml is detected then significant sewage pollution and an increased risk of viral infection is implied. Nevertheless, phages and enteric viruses can multiply only in host cells, where they utilise the host ribosomes, protein-producing

factors, amino acids and energy generating systems to reproduce. Phages can also only multiply in metabolising host bacteria (Grabow, 2001).

Table 1.3 The effects of coliphages on human health (Adapted from DWAF, 1996)

Coliphage Range (Counts/100ml)	Effects
Target Water Quality Range 0 – 1	Sewage pollution is not indicated. Negligible risk of viral infection is indicated.
1-10	Slight probability of sewage pollution. A very slight risk of viral infection is indicated for continuous exposure, but negligible risk is expected for short, occasional exposure.
10-100	Probable sewage pollution. A low risk of viral infection is indicated with continuous exposure; minimal effects expected for occasional exposure.
>100	Significant sewage pollution and increasing risk of viral infection as coliphage levels increased.

The use of *E. coli* as a viral indicator is however hampered by the fact that numerous studies have shown that viruses persist longer than *E. coli* in natural water sources (Nasser *et al.*, 1993; Bosch, 1998; Jiang *et al.*, 2000). The development of direct methods to monitor for human viral pathogens is thus crucial as studies have also shown that rotaviruses are the most common cause of gastroenteritis worldwide and is responsible for the most severe form of viral gastroenteritis in humans (Gerba *et al.,* 1996; Ahn *et al.,* 2006; Mazari-Hiriart *et al.,* 2009). Viral groups which also cause waterborne diseases include the enterovirus, adenovirus and caliciviruses.

1.4 WATERBORNE PATHOGENS

Surface water may act as a reservoir of various types of pathogens ranging from viruses such as norovirus, adenovirus, hepatitis A virus, to bacteria such as *Salmonella spp., Shigella spp., Vibrio cholera*, and parasites such as *Cryptosporidium, Giardia, and Entamoeba* (Bouzid *et al*., 2008; Canepari and Pruzzo, 2008). Presently, bacteria have been identified as the causative agent in the majority of the waterborne outbreaks in the developed world, where the contribution of pathogenic bacteria to waterborne outbreaks is increasing because of changes in life style and the emergence of several new bacterial pathogens (WHO, 2003; Liang *et al*., 2006). In addition, the re-emergence of bacterial pathogens in water sources can be attributed to several

reasons; firstly, the organism may become resistant to treatment; secondly, the environmental complex including the transmission route and global warming could impact source water quantity and quality; thirdly, the treatment procedure and the drinking water supply system may allow organisms through; and finally the combined effect of all three complexes (Nwachcuku and Gerba 2004). In addition to the well-known pathogens, there are groups of bacteria that are regarded as emerging risk groups for drinking water (**Table 1.4**). The most prominent ones are epsilon-*proteobacteria,* which include *Campylobacter jejuni (C.* Jejuni*), Campylobater coli (C. Coli), Helicobacter pylori* and *Arcobacter butzleri* (Miller *et al*., 2007; Nakagawa *et al*., 2007). Moreover, some organisms which were not perceived as waterborne pathogens a decade ago, such as *H. pylori, C. jejuni*, and *C. coli*, and several species of *Arcobacter,* are now classified as waterborne pathogens as they have been detected not only in private wells but also in bulk water and in biofilms present in public drinking water system supplies (Moreno *et al*., 2004; Vandenberg *et al*., 2004). Their susceptibility to oxidative disinfection is still under debate and whether the epsilon-*proteobacteria*, as detected by molecular means, are viable and infective or dead is unclear (Moreno *et al*., 2004; Vandenberg *et al*., 2004; Watson *et al*., 2004).

Table 1.4 Major target taxa of waterborne bacterial pathogens to be considered in drinking water sources and supplies (Adapted from Brettar and Hofle, 2008)

Pathogenic species	Health significance	Relative infective dose
Pathogenic *Escherichia coli*	High	Low
Shigella flexneri	High	Low
Salmonella enterica	High	High
Vibrio cholerae	High	High
Yersinia enterocolitica	High	Variable
Campylobacter jejuni, C. coli	High	Low
Helicobacter pylori	Unknown	Unknown
Arcobacter butzleri	High	Moderate
Legionella pneumophila	High	Variable
Mycobacterium avium complex	Unknown	Unknown
Francisella tularensis	High	Low

In addition to bacteria which can cause serious illness in people, the parasitic protozoa may have similar debilitating effects. The three major waterborne protozoan diseases are cryptosporidiosis, giardiasis and amoebiasis (**Figure 1.4**). Seven *Cryptosporidium* species (*C. hominis, C. parvum, C. meleagridis, C. felis, C. canis, C. suis and C. muris*) can cause the diarrhoeal disease cryptosporidiosis in humans, but *C. parvum* and *C. hominis* are responsible for the vast majority of cases and outbreaks (Caccio *et al*., 2005).

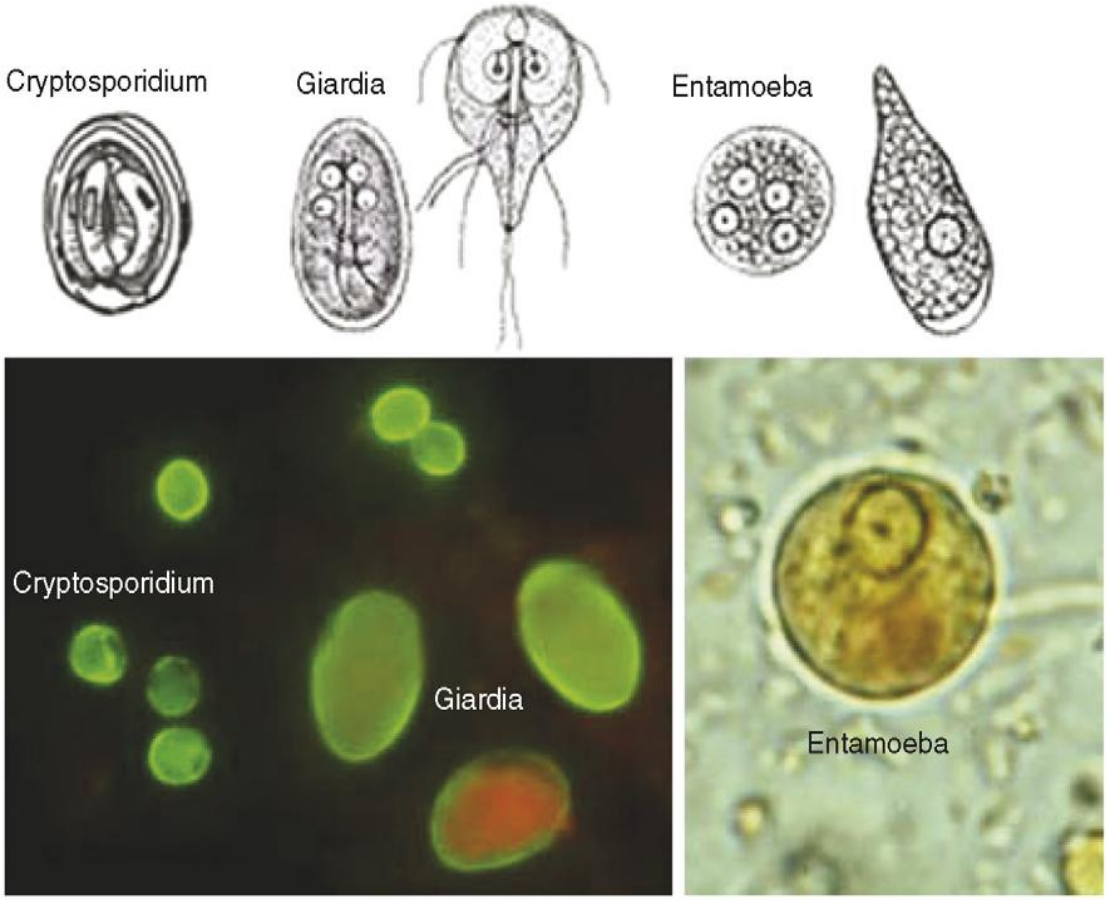

Figure 1.4: Waterborne enteric protozoa, upper panel shows representations of *Cryptosporidium oocyst, Giardia cyst* and trophozoite and *Entamoeba cyst* and trophozoite (left to right). The lower panel shows immunofluorescence images of *Cryptosporidium oocysts* and *Giardia cysts* (left) and a concentrated wet mount image stained with iodine of an *Entamoeba cyst* (Adapted from Bouzid *et al*., 2008).

Giardia duodenalis (syn. *G.lamblia, G. intestinalis*) is arguably the most widespread protozoan causing diarrhoea, with more than 200 million symptomatic individuals worldwide (WHO, 2006). However, most *G. duodenalis* infections are asymptomatic, and prevalence is 2–5% in industrialised countries and 20–30% in developing countries. *Giardia duodenalis* is currently categorised into seven genotypes: A, B, C, D, E, F and G (Thompson and Monis, 2004). Only genotypes A and B have been detected in humans but both also infect other mammals.

Entamoeba histolytica is responsible for amoebic dysentery, but other free-living amoebae can cause fatal amoebic encephalitides. Amoebic encephalitides includes symptoms which cause inflammatory necrosis of brain tissue due to amoebic infiltrates. It appears rarely and infections occur worldwide with some 500 cases reported between 1960 and 2000. Amoebic dysentery furthermore occurs worldwide with higher incidence in tropical and subtropical regions, with over 500 million people infected and around 100 000 deaths each year (Schuster and Visvesvara, 2004).

1.5 WATERBORNE VIRUSES

Equally important to pathogenic bacteria and parasites is the presence of human viruses in water, which poses an additional threat to human health. Furthermore, several virus genera such as enterovirus, norovirus, rotavirus, astrovirus, and hepatitis A virus can survive and persist for long periods of time in water (Canepari and Pruzzo, 2008). It is also widely recognised that waterborne viruses pose a potential health risk to humans as illustrated by the extensive list compiled by the Environment Protection Agency (EPA, 2008) in the USA called the Pathogen Contaminant Candidate List (PCCL) (**Table1.5**).

Table 1.5 Pathogens on the PCCL (Adapted from EPA, 2008)

Pathogen	***WBDO**	**Pathogen**	***WBDO**
Naegleria fowleri	4	*Mycobacterium avium*	4
Legionella pneumophila	5	Rotavirus	4
Escherichia coli (0157)	5	*Yersinia enterocolitica*	5
Hepatitis A virus	5	*Arcobacter butzleri*	4
Shigella sonnei	5	*Fusarium solani*	1
Helicobacter pylori	1	*Plesiomonas shigelloides*	4
Campylobacter jejuni	5	Hepatitis E virus	2
Salmonella enterica	5	*Toxoplasma gondii*	2
Caliciviruses	5	*Aspergillus fumigatus* group	1
Entamoeba histolytica	5	*Exophiala jeanselmei*	1
Vibrio cholera	5	*Aeromonas hydrophila*	1
Adenovirus	2	Astrovirus	2
Enterovirus	2	Microsporidia	1
Cyclospora cayetanensis	4	*Iospora belli*	2

***Waterborne disease outbreak

Contaminants considered as high priority for the development of detection technology and inclusion in water-quality specifications are listed in the PCCL. Rotavirus, adenovirus, enteroviruses, caliciviruses (includes norovirus), and hepatitis A are five of only seven of the viral pathogens on the PCCL (EPA, 2008). As indicated in **Table 1.5** the EPA (2008) also allocated a score based on the number of documented outbreaks or occurrences in the United States of America. In general a score of 1 (on a scale of 1-5) indicates that the organism has never caused waterborne disease outbreaks in any state, while a score of 5 indicates that multiple documented cases have been reported.

Human sewage may also contain a variety of viruses which may find its way into water treatment plants (Bosch *et al*., 2008). A list of viruses known to be found in water, their popular name and the disease they cause is listed in **Table 1.6.**

Table 1.6 Human viruses documented to be found in the water environment (Adapted from Bosch *et al*., 2008)

Genus (genome)	**Popular name**	**Disease caused**
Enterovirus (ssRNA)	Poliovirus	Paralysis, meningitis, fever
	Coxsackie A and B virus	Meningitis, fever, respiratory disease, hand-foot-and-mouth disease, myocarditis, pleurodynia, diabetes
	Echovirus	Meningitis, fever, respiratory disease, gastroenteritis
Hepatovirus (ssRNA)	Hepatitis A virus	Hepatitis
Reovirus (dsRNA)	Human reovirus	Unknown
Rotavirus (dsRNA)	Human rotavirus	Gastroenteritis
Norovirus (ssRNA)	Norovirus	Gastroenteritis
Sapovirus (ssRNA)	Sapporo-like virus	Gastroenteritis
Hepevirus (ssRNA)	Hepatitis E virus	Hepatitis
Mamastrovirus (ssRNA)	Human astrovirus	Gastroenteritis
Coronavirus (ssRNA)	Human coronavirus	Gastroenteritis, respiratory disease, SARS
Orthomyxovirus (ssRNA)	Influenza virus	Influenza, respiratory disease
Parvovirus (ssDNA)	Human parvovirus	Gastroenteritis
Mastadenovirus (dsDNA)	Human adenovirus	Gastroenteritis, respiratory disease, conjunctivitis
Polyomavirus (dsDNA)	Polyomavirus	Progressive multifocal leucoencephalopathy, diseases of urinary tract
Circovirus (ssDNA)	TT(Torque Teno) virus	Hepatitis, unknown

Some of the viruses in **Table 1.7** may be shed in high numbers from people suffering from diarrhoea who may excrete up to 10^{13} virus particles per gram of stool (Caballero *et al*., 2003; Costafreda *et al*., 2006; Ozawa *et al*., 2007).

In a separate study conducted by Maunula *et al*., (2009) it was found that PCR analysis indicated that norovirus, astrovirus, rotavirus, adenovirus and enterovirus were present in treated drinking water (**Table 1.7**). Hence, depending on the amount of virus particles present and the host's immune status, people's health may have been compromised if the virus contaminated water was ingested.

Table 1.7 Types of enteric viruses found in contaminated water and treated wastewater (Adapted from Maunula *et al*., 2009)

Enteric virus	**Drinking water**	**Treated wastewater**
Norovirus		
Genogroup I	Negative	Positive
Genogroup II	Positive	Positive
Astrovirus	Positive	Positive
Rotavirus	Positive	Positive
Adenovirus	Positive	Positive
Enterovirus	Positive	Negative
Hepatitis A virus	Negative	Negative

Since current water treatments do not ensure complete removal of virus particles, they become contaminants of the water environment if their particle numbers are high enough to represent a public health threat. The viral particle level may however, be below the detection limit (Bosch *et al*., 2008). Some of the RNA viruses causing water-related gastroenteritis are rotavirus in children and norovirus in adults (Bosch *et al*., 2008). Norovirus is well documented in causing waterborne gastroenteritis (Kukkula *et al*., 1999; Hewitt *et al*., 2007). Other enteric viruses such as astrovirus, sapovirus, hepatitis A virus and hepatitis E virus have also been implicated in gastroenteritis outbreaks (Bosch, *et al*., 2008; Maunula *et al*., 2009). Furthermore, statistics indicate that hepatitis A is responsible worldwide for approximately 50% of the total hepatitis cases. Despite hepatitis A infections being self-limiting and seldom causing death it may incapacitate patients for months (Pinto and Saiz, 2007). Another waterborne virus causing serious illness is hepatitis E which is less prevalent than hepatitis A, but has a higher mortality

rate than hepatitis A. It is also one of the leading causes of acute hepatitis in adults throughout Asia, the Middle East and Africa in contrast to industrialised countries where hepatitis E infections are sporadic (Purcell and Emerson, 2008).

The reasons why waterborne viruses continue to emerge include; globalisation of commerce and travel; an increase in the sensitivity to infections: molecular methods for detection and source tracking methods are continually being developed; changes in drinking water treatment technology; changes in food supply production; and the genetic re-assortment (evolution) of viruses (Nwachcuku and Gerba, 2004). Despite advances in technology, adenovirus still appears to be the most difficult to control by conventional drinking water treatment methods (Nwachcuku and Gerba, 2004). Adenovirus, although sensitive to inactivation by oxidising disinfectants, is known to be the most resistant waterborne pathogen to inactivation by UV light (Gerba *et al*., 2002; Gerba *et al*., 2003). This is because of the double-stranded DNA genome, which allows adenoviruses to use the host-cell repair enzymes during replication to repair damage in the DNA caused by the UV light (Gerba *et al*., 2002). The results of this study suggest that double-stranded DNA viruses are likely the most resistant viruses to UV light disinfection. Consequently, DNA viruses have been responsible for both drinking water and recreational waterborne disease outbreaks (Hurley and Roscoe, 1983; Kukkula *et al*., 1997; Maunula *et al*., 2009).

1.5.1 RNA viruses

Rotavirus is one of the leading causes of severe, acute gastroenteritis worldwide and may infect infants, young children and adults, with infants and children experiencing the worst symptoms. If left untreated, rotavirus infection may lead to death (Bosch *et al*., 2008; Cunliffe *et al*., 2009). Norovirus, enterovirus and hepatitis A virus were similarly implicated in severe, acute gastroenteritis mostly in developing countries (Bosch *et al*., 2008). Detecting rotavirus before it can cause disease may aid in improving patient care and state health resources could be better utilised.

1.5.1.1 Rotavirus

Rotaviruses are classified under the genus rotavirus and the family *Reoviridae.* They have an icosahedral symmetry and have a characteristic wheel-like appearance when viewed by electron microscopy (rota means “wheel” in Latin). Furthermore, rotaviruses are approximately 75 nm in diameter. The triple layered capsid is composed of 11 segments of double-stranded, linear, non-enveloped RNA, where each segment codes for one protein with the exception of segment

11, which codes for two proteins (Morris and Estes, 2001). Of these 12 proteins **(Figure 1.5)**, six are structural (VPs) and six are non-structural proteins (NSPs).

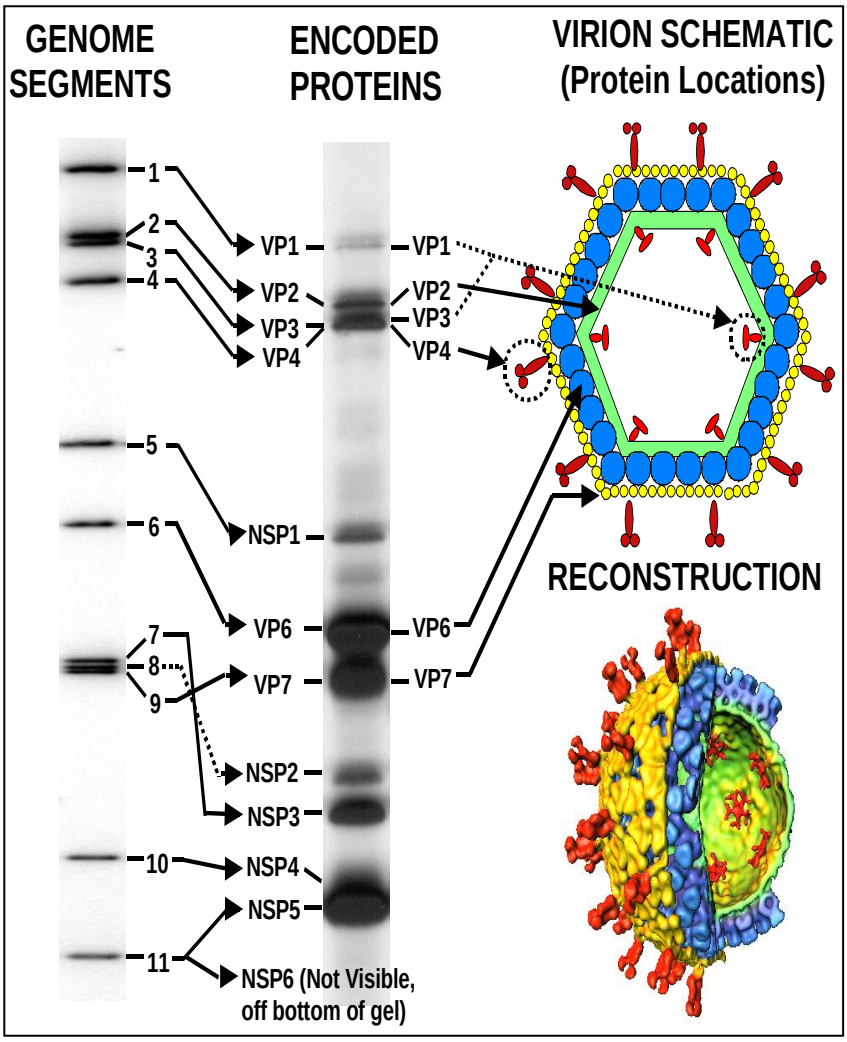

Figure 1.5 Coding assignments and virion locations of rotavirus and 3D structure of the rotavirus particle (Adapted from Pesavento *et al.*, 2006).

The rotavirus capsid is also composed of three concentric protein layers that enclose the genome (Prasad and Estes, 2000). Rotaviruses infect the cells of the intestinal epithelium. The outermost layer of the capsid, which is required for cell attachment, membrane penetration and cell entry, is composed of two structural proteins (VP4 and VP7). The VP4 protein is implicated in cell attachment, cell penetration, haemagglutination, neutralisation and virulence, while VP7 may also perform a similar role (Estes, 2001; Morris and Estes, 2001). Viral proteins three (VP3) and six (VP6) encodes for proteins required for RNA transcription and correct viral structure. Meanwhile, non-structural proteins may facilitate viral replication and thereby increase

the efficiency of viral formation. However, NSP4 has been shown to promote calcium-mediated enterotoxigenic effects linked to diarrhoea (Estes, 2001; Suguna and Rao, 2010).

1.5.1.1.1 Mode of Transmission

Rotaviruses are shed in high concentrations (>10^{12} particles/gram) and persist for many days in the stools and vomit of infected individuals (Staat *et al.*, 2005). Symptoms appear approximately two to three days after infection, which is the time period when children between the ages of three months and two years are most likely to display the symptoms. The virus spreads rapidly, presumably through person-to-person contact, airborne droplets, or possibly contact with contaminated toys. In animal studies it was shown that rotavirus infects the mature enterocytes of the villi in the small intestine (Morris and Estes, 2001; Widdowson *et al*., 2005). It can also infect a wide range of cell types in tissue culture such as bone, breast, stomach and lung cell lines (Ciarlet and Estes, 2001). The absorptive capacity of the gut then decreases due to virus-induced cell death, which leads to sloughing of the villus epithelium and an increase in secretory crypt cells (Widdowson *et al*., 2005). This results in fluid and electrolyte loss into the lumen and a resultant decrease of digestive enzymes such as sucrase and isomaltase, which in turn leads to the accumulation of sugars in the gut. Consequently, fluid secretion into the lumen is favoured by the osmotic gradient (Ramig, 2004). Symptoms include vomiting and watery diarrhoea, often with fever and abdominal pain. Oral rehydration therapy is recommended since no specific drug treatment exists. According to the WHO (2007) two rotavirus vaccines were licensed in 2006 which exhibited good safety and efficacy profiles in large clinical trials. The full potential of the current vaccines have however, not been confirmed in all the regions of the world, particularly Asia and Africa and the WHO therefore does not recommend that these vaccines be included into national immunisation programmes (WHO, 2007).

1.5.1.1.2 Disease Distribution

A three year longitudinal study conducted by Bishop *et al.* (1983) showed that neonatal rotavirus infection did not restrict re-infection but it did confer significant resistance to disease severity during re-infection. Bishop *et al*. (1983) also observed that rotavirus infection can occur at a very early age (even in one day old babies). The infection in neonates is usually asymptomatic, and neonatal infection may confer protection against severe rotavirus gastroenteritis. Parashar *et al.* (2003) reviewed studies on global illness and deaths caused by rotavirus disease in children from 1986 to 2000 in developing and developed countries. It was found that each year, rotavirus causes in excess of 100 million cases of gastroenteritis where home care is required, 25 million will visit their local clinic, 2 million will be hospitalised, and about 440 000 children

younger than five years old will die. It is also estimated that nearly every child would have experienced rotavirus gastroenteritis by age 5, 20% would have visited a clinic, one in 65 will be admitted to hospital, and about one in 293 will die. The study also indicates that 82% of rotavirus deaths are from children in poverty stricken countries, with the WHO also estimating an annual average death rate as high as 527 500 due to rotavirus infections (Parasha *et al.*, 2003).

In Africa alone, nearly 150 000 children younger than 5 years die annually of rotavirus disease (Page, 2006). Moreover, in Sub-Saharan Africa, rotavirus causes approximately 25% of diarrhoeal deaths and on average rotavirus causes 25% of hospitalisations due to diarrhoeal disease (Cunliffe *et al*., 1998). In a study conducted by Steele *et al*., (2003), with regard to epidemiology and surveillance of rotavirus in South Africa, it was found that rotavirus accounts for approximately 25% of diarrhoeal hospitalisations. The most commonly identified strain was VP7 serotype G1, followed by the G2 strains. In addition, the G1 strain was found to be the most prevalent internationally to infect children <5 years old (Steele *et al*., 2003). However, in a study conducted by Jere *et al*., (2011), on the characterisation of rotavirus strains in Sierra Leone, it was found that approximately 50% were G2 serotypes. It was reported by Reddy (2006) that the mortality rate from diarrhoea is 14% in Durban, moreover, 22% of the mortality cases in a local study of diarrhoea incidence at King Edward Hospital were ascribed to rotavirus infection.

1.5.1.2 Norovirus

During an epidemic of gastroenteritis in Norwalk, Ohio it was discovered that a small round structured calicivirus was the causative agent. It was subsequently called the Norwalk virus which was renamed norovirus by the International Committee on Taxonomy of Viruses (ICTV) in 2002 (Wang *et al.*, 1994; Kapikian, 2000). Norovirus is the prototype human calicivirus (Smit *et al.*, 1997) and is classified under the genus noroviruses and the family *Caliciviridae*. It has a non-enveloped capsid that is round and icosahedral in shape and has a diameter that is 35-39 nm (**Figure 1.6**). Noroviruses comprise of five genogroups, with two major groups, i.e. genogroup I and II consisting of 15 and 18 genotypes, respectively (Lopman *et al.*, 2008; Okabayashi *et al.*, 2008). The genome is a linear positive-sense, single-stranded RNA that is not segmented. It is about 7.5 kb long and has a guanine/cytosine content of 48% to 56%. A genome-linked protein (VPg) is situated at the 5'-end and the 3'-terminus has a poly (A) tract. The norovirus genomic nucleic acid on its own could also be infectious (Haramoto *et al.*, 2004; ICTVdb, 2006).

1.5.1.2.1 Mode of Transmission

Transmission of norovirus occurs via the faecal/oral route. The viral particles cause damage to the microvilli in the small intestine, thereby causing malabsorption. In addition, delayed gastric motility and gastric emptying due to the virus-mediated change causes vomiting. Infected persons leave no histopathologic lesions in the gastric mucosa. Faecal leucocytes are absent and bloody stools are rare because the virus does not invade the colon (Khan *et al.*, 2009).

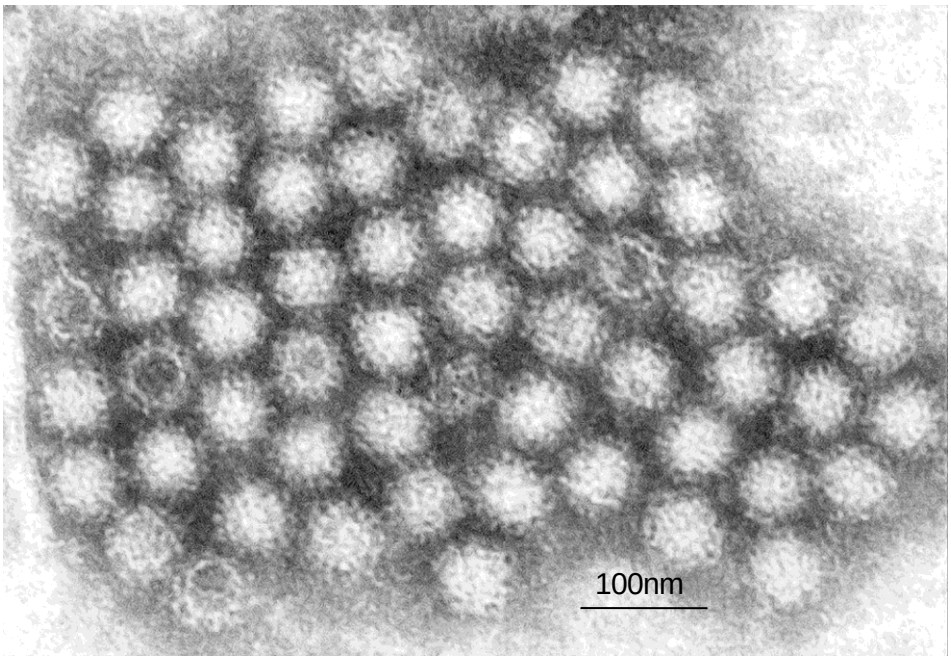

Figure 1.6 Transmission electron micrograph of norovirus (Adopted from CDC, 2012)

Noroviruses (NVs) cause gastroenteritis in patients of all age groups worldwide. It is estimated that over 90% of acute viral gastroenteritis cases throughout the world are associated with NV infections (Donaldson *et al.*, 2008; Patel *et al.*, 2008). In a study conducted by Patel *et al.* (2008), in estimating the prevalence of norovirus disease in children, it was found that annually, in developing countries, more than one million hospitalisations and ~ 210 000 deaths, of children less than five years old, can be attributed to norovirus infections.

Norovirus has also been detected in diverse environmental water samples such as sewage, river water, well water, seawater, and mineral waters (Haramoto *et al.*, 2004). It is normally associated with foodborne and waterborne outbreaks with minute amounts of virus associated with a high risk of infection (close to 49% for a single infectious NV particle), hence the highly infectious nature of NV according to Teunis *et al.* (2008), who also cautiously estimates the infectivity of norovirus as similar to that of rotavirus. Rohayem *et al.* (2004) also reports that after rotavirus infections, calicivirus infections are the second most common cause

of viral infections. Treatment is symptomatic and includes replacing fluid and electrolyte loss. Noroviruses can survive in the environment under difficult conditions and infections can be prevented by practicing good hygiene or infection control such as frequent washing of hands (Donaldson *et al.*, 2008).

1.5.1.2.2 Disease Distribution

Advances in molecular techniques have shown that norovirus is the leading cause of epidemic gastroenteritis in all age groups with >90% of non-bacterial epidemic gastroenteritis worldwide attributed to the virus as demonstrated in **Table 1.8**. A high percentage (>95%) of deaths due to diarrhoeal diseases also occured mainly in developing countries (Patel, 2008). A lapse in infection control procedures, which resulted in the deaths of six newborn babies, was ascribed to norovirus infection at the Charlotte Maxeke Johannesburg Academic Hospital in early 2010 (Maphumulo and Van Schie, 2010).

Table 1.8 Estimates of annual number of episodes of norovirus-associated diarrhoea among children <5 years of age in industrialised and developing countries (Adapted from Patel, 2008).

Environment	Annual no. diarrhoea-associated events	Pooled proportion of episodes attributable to noroviruses, %	Total no. norovirus episodes	Annual incidence per 100,000 children
Industrialised countries	7,743,000	11.7	906,000	1,685
Outpatient	531,000	12.1	64,200	118
Inpatient	9,015,000	12.1	1,091,000	197
Developing countries §				
Inpatient Deaths	1,800,000	12.1	218,000	39
§ Data from developing countries were sparse on fraction of norovirus-associated diarrhoea episodes in the outpatient.				

1.5.1.3 Enterovirus

Enteroviruses (EVs) were initially classified into four groups on the basis of their pathogenesis in humans and laboratory animals, i.e. polioviruses, coxsackie A viruses, coxsackie B viruses and echoviruses. However, significant similarities in the biological properties narrowed it down to three groups (Oberste *et al.*, 1999), and polio-, echo-, and coxsackie viruses are thus classified

under the genus enteroviruses, and the family *Picornaviridae*. Their genome consists of a linear, positive-sense single stranded RNA of about 7.5 kb. There is a long (600-1200 base pair) untranslated region (UTR) at the 5' end and a shorter 3' untranslated region (50-100 bases). The 5' UTR contains a "clover-leaf" secondary structure known as the Internal Ribosome Entry Site (IRES). The rest of the genome encodes a single "polyprotein" of between 2100-2400 amino acids. The 5' end has a genome-linked protein (VPg) and the 3' end has a poly (A) tract and these viruses have five structural proteins which are not glycosylated. The virion is infectious and serves as both the genome and the viral messenger RNA (**Figure 1.7**). Enteroviruses are well characterised and have been known to cause meningitis, paralysis, rash, fever, myocarditis, respiratory disease, and diarrhoea in humans (van Regenmortel *et al.*, 2000; Kubo *et al.*, 2002).

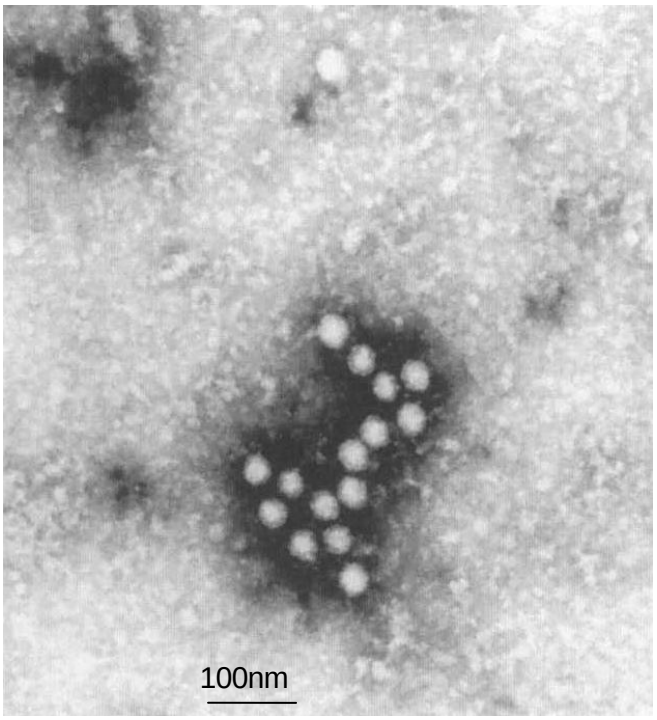

Figure 1.7 Immune-complexed electron micrograph of enterovirus (Adopted from Lee *et al*., 1996)

1.5.1.3.1 Mode of Transmission

Enteroviruses are spread predominantly via the faecal/oral route, entering the oropharynx where they multiply in the submucosal tissues of the distal pharynx and alimentary tract. The infected individual may be asymptomatic for days as the viral particles are shed in faeces and in upper respiratory secretions. Three to ten days is the average incubation period, during which time the virus migrates to the regional lymphoid tissue and replicates. The onset of symptoms appears

and the virus spreads to the spleen, liver and bone marrow. Dissemination to target organs, i.e. skin, heart and central nervous system causes major viremia (Bennett *et al.*, 2009). Enteroviruses replicate in the gastrointestinal tract and are excreted into the environment via sewage. Normal sewage treatment processes are not able to remove these viruses; hence they are transmitted via sewage-polluted water, sewage, groundwater or sludge-amended soils (Hubner *et al.*, 1997; Borchardt *et al.*, 2002). Enteroviruses are also distributed throughout the world but some serotypes may be endemic.

1.5.1.3.2 Disease Distribution

Enteroviruses are responsible for about 30 million cases of gastroenteritis per year in the United States, with children under ten years most affected (Ehlers *et al.*, 2005). They are well characterised, since they cause meningitis, paralysis, rash, fever, myocarditis, respiratory disease, and diarrhoea in humans. They can spread from person to person, usually on unwashed hands and surfaces contaminated by faeces, where they can persist for several days. In tropical parts of the world, they infect people year-round, but in cooler climates, outbreaks of coxsackie virus most often occur in the summer and autumn (van Regenmortel *et al.*, 2000; Sawyer, 2002). Poliomyelitis, a disease caused by enterovirus has significantly decreased due to improved global economic conditions and the availability of vaccines. According to a report by the WHO in February 2006, there remain only four countries which are polio endemic, namely Nigeria, India, Pakistan and Afghanistan. Namibia and Angola were polio-free since 1996 and 2001 respectively, but a polio outbreak in Namibia in 2006 was traced to a strain from Angola which originated in India, indicating the infectious nature of enterovirus (CDC, 2006).

1.5.1.4 Hepatitis A virus

Hepatitis A (HAV) viruses are also classified under the genus enteroviruses and the family *Picornaviridae*. The hepatitis A virus is non-enveloped and icosahedral in shape measuring about 28 nm in diameter (**Figure 1.8**). Moreover, the genome consists of linear, single stranded positive-sense RNA of about 7.5 kb. It has a large polyprotein which is expressed from a large open reading frame which extends through most of the genomic RNA. This polyprotein is subsequently cleaved by a viral protease (3Cpro) to form three (possibly four) capsid proteins and several non-structural proteins (Lemon, 1994; Stapleton and Lemon, 1994; Hollinger and Ticehurst, 1996; Koff, 1998). The virus can survive denaturation by ether, acid (pH 3.0), drying, 56ºC, and freezing temperatures (-20ºC). Inactivation of HAV requires one minute contact time

with household bleach, and exposing it to heat higher than 85ºC for at least one minute also results in inactivation (Melnick, 1992; Nainan *et al.*, 2006).

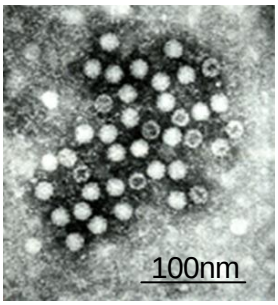

Figure 1.8 Hepatitis A virus as viewed through an electron microscope (Adopted from Gilroy *et al*., 2009)

1.5.1.4.1 Mode of Transmission

Hepatitis A is transmitted via the faecal-oral route from person to person, and through contaminated water, food supplies and occasionally via blood transfusions. This virus spreads easily in areas where sanitation is poor and living conditions are crowded. The HAV can survive in the environment for long periods (Biziagos *et al.,* 1988). The virus is excreted in high concentration one to three weeks before the onset of illness, and may be excreted for a number of weeks at lower concentrations after jaundice occurs as illustrated in **Figure 1.9**.

In acute hepatitis A the anti-HAV IgM is detectable about three weeks after exposure, while its concentration peaks after about two months and declines to undetectable levels normally within six months of infection. Hepatitis A IgA and IgG antibodies are detected within a few days after the onset of symptoms and IgA antibodies decrease to undetectable levels in a few months while IgG antibodies remain in the patient's circulation for years and imparts lifelong immunity to the patient (WHO, 2000a). Treatment is symptomatic as the HAV infection is self limiting (Previsani and Lavanchi, 2000).

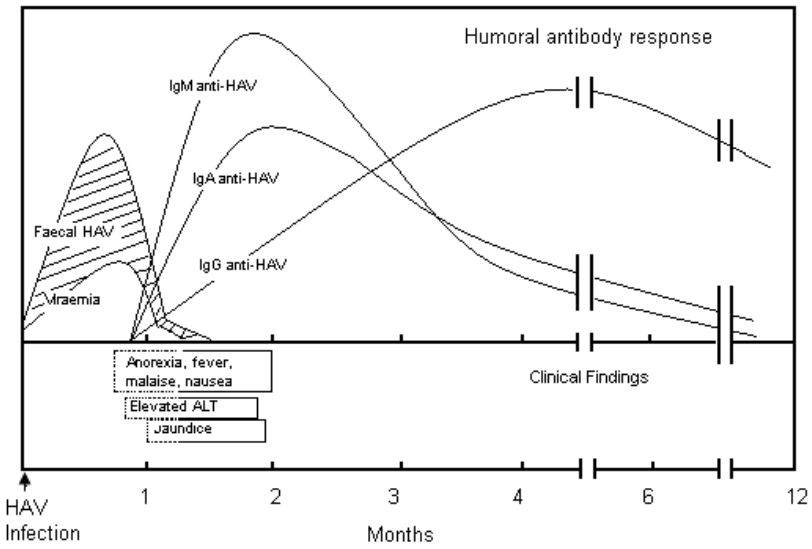

Figure 1.9 Summary of clinical, virologic, and serologic findings in uncomplicated acute hepatitis A (Adapted from WHO, 2000a)

1.5.1.4.2 Disease Distribution

The hepatitis A virus is one of the major causes of acute hepatitis and is spread throughout the world (Villar *et al.*, 2006). A report published in 1994 indicated that the majority of black adults in South Africa below twenty years of age, tested positive for antibodies to HAV (Melnick, 1995). In contrast, only thirty to forty percent of white adults had antibodies to HAV by age twenty years, increasing to about sixty percent between forty and forty nine years of age (Taylor, 1997; Poovorawan *et al.*, 2002). Hepatitis A virus infection often appears asymptomatic among children, while symptoms are clearly visible with infected adults (Lemon, 1997; Hendrickx *et al.*, 2008). Overcrowding, poor sanitation, and lack of a reliable clean water supply, predispose people to acquiring hepatitis A infection (Nainan *et al.*, 2006). The liver cells of humans are the only site where hepatitis A replicates. After entry into the hepatocyte, the host ribosomes bind to the released viral RNA to form polysomes. Copies are made of the viral genome and shed into the biliary tree to be excreted into faeces. Transmission of the virus is highest 14 – 21 days after infection, corresponding to the period of highest shedding of the virus and after the development of jaundice (Lemon, 1997; Gilroy *et al.*, 2008). A notable case of acute hepatitis A infection was recorded at a Pennsylvania restaurant where the source of the virus was traced to green onions that were used to make a mild salsa. Tracing the contamination to the onions before it arrived in the United States of America, illustrated the resilience of the virus to survive and spread (Gilroy *et al.*, 2008).

Dual infection can also occur with hepatitis E virus since both viruses have a similar clinical presentation, and are transmitted via the same route. Inflammation of the liver (hepatitis)

can be caused by a variety of viruses such as hepatitis A, B, C, D and E with a characteristic feature being jaundice. Testing the patient's serum for the presence of specific anti-viral antibodies will indicate the causative agent. A single serotype of hepatitis A virus exists in humans despite its genetic "heterogeneity" at the nucleotide level. This single serotype virus imparts immunity in its host (Melnick, 1992; Jacobsen and Koopman, 2004; Nainan *et al.*, 2006). Vaccines are available which may reduce disease incidence and potentially eliminate infection transmission (WHO, 2000b; Pickering *et al.,* 2006a; Nainan *et al.*, 2006). The lack of reporting of hepatitis A incidence in South Africa results in severe underestimation of statistics (DOH, 2005). The South African Department of Health reported 1612 hepatitis A cases between January 2001 and December 2005. A decrease in the mortality rate from 1.1% in 2001 to zero in 2005 was also reported. The decrease in the fatality rate could, however, be ascribed to the vaccination program launched by the Department of Health (DOH, 2005).

1.5.2 Adenovirus

Studies have suggested that adenovirus may be the most common enteric virus in sewage and may survive longer than any other enteric virus, along with hepatitis A virus (Enriquez *et al*., 1995; Pina *et al*., 1998). Other DNA viruses also found in the water environment and causing gastroenteritis and other disease are parvovirus, polyomavirus and circovirus (Bosch et al., 2008).

Adenoviruses are classified under the family *Adenoviridae.* They are divided into four genera, namely *Mastadenovirus, Aviadenovirus, Atadenovirus and Siadenovirus*. The majority of adenoviruses isolated from mammals are grouped under the family *Mastadenovirus*, while the family *Aviadenovirus* includes adenoviruses isolated from avian species. Furthermore, the family *Atadenovirus* contains viruses isolated from reptiles, birds, marsupials and mammals. In addition, adenoviruses isolated from turkeys and frogs are grouped under the family *Siadenovirus.* Human adenovirus serotypes 8, -19 and -37 belong to species D and may be the causative agents for nosocomial infections and cause sporadic cases and outbreaks of severe epidemic keratoconjunctivitis (Reddy *et al*., 2006). They are non-enveloped icosahedral viruses (**Figure 1.10**) and their genome consists of double stranded, linear DNA. It has a virus-coded terminal protein which is covalently linked to the 5'-end of each strand (Van Heerden *et al.*, 2005).

The genome of human adenovirus has about 35 000 bp and contains an inverted terminal repetition (ITR) of 103 bp. Its guanine and cytosine (G+C) content also varies between 34% and 60%. To date 51 human adenovirus serotypes have been identified and are classified into six species, A-F. Adenovirus classification is based on their hemagglutination properties and

biophysical and biochemical criteria are also used (Shenk, 1996; De Jongh *et al*., 1999; Van Heerden *et al.*, 2003).

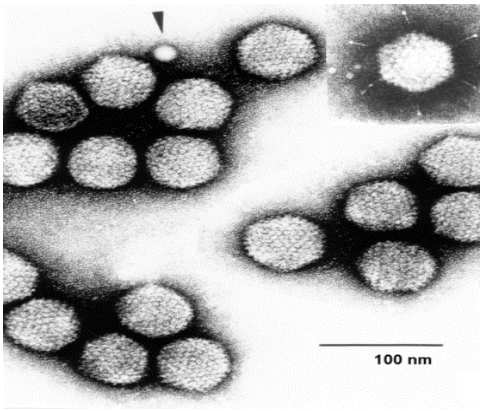

Figure 1.10 Electron micrograph of a human adenovirus, courtesy of C. Büchen-Osmond (Adopted from ICTV, 2002)

1.5.2.1 Mode of Transmission

Adenoviruses are transmitted by direct contact, the faecal-oral route, and through waterborne transmission. It causes diarrhoea, eye infections, and respiratory disease in humans (Kapikian & Chanock, 1995; Horwitz, 1995; Kapikian, 1997; Foy, 1997; Van Regenmortel, *et al.*, 2000). Adenovirus infections vary by serotype and some serotypes causes persistent asymptomatic infections in the tonsils, adenoids and the gut of infected persons (Russell, 2009). It has also been shown that insufficiently chlorinated swimming pools and small lakes are linked to epidemics of febrile disease and conjunctivitis. This was in turn ascribed to waterborne transmission of some adenovirus serotypes. The clinical manifestation of disease associated with the infection of certain adenovirus serotypes depend on the site of infection as well as the mode of entry into the body. Severe lower respiratory tract disease, for example, is associated with serotype 7 if acquired by inhalation, while oral transmission of the virus causes no or only mild disease. Adenovirus infections can occur throughout the year despite adenovirus-associated respiratory illness being more prevalent in late winter (Pickering *et al.,* 2006b). Enteric serotypes 40 and 41 of adenovirus cause gastroenteritis, mostly in children. Adenovirus can be excreted for prolonged periods and may cause infections throughout the year, however respiratory illness caused by adenovirus generally occur in winter, spring, and early summer. Outbreaks of adenovirus-associated diseases can also be prevented from spreading by good infection-control practices such as the regular washing of hands (Horwitz, 1995; Foy, 1997; Pickering *et al.,* 2006b).

1.5.2.2 Disease Distribution

The prevalence of adenovirus were shown to be the same as statistics reported throughout the world, with serotypes 40 and 41 constituting between 38% and 100% of adenovirus serotypes (Enriquez, 1995; Moore, *et al.*, 2000). Adenovirus is regarded as a robust virus, found almost everywhere in human and animal populations. It is endemic throughout the year and can survive for long periods outside a host. This virus was first isolated in the 1950's in adenoid tissue-derived cell cultures. Adenovirus causes gastroenteritis, respiratory illness, conjunctivitis, cystitis, and various other illnesses depending on the infecting serotype. Respiratory infection caused by adenovirus may display as the common cold syndrome, pneumonia, croup or bronchitis. In addition, adenovirus infections may cause severe complications in patients whose immune system is compromised (Pickering *et al.,* 2006b; Jones *et al.*, 2007).

1.6 Aims of Study

The aim of this study was to improve the efficiency of the current concentration and extraction techniques used to recover enteric viruses from surface water sources. The efficiency of the technique was optimised under various laboratory conditions and subsequently surface water was collected at various points along the Plankenburg- (Stellenbosch) and Berg Rivers (Paarl) every month for a period of one year.

The specific aims were:

1. To isolate DNA/RNA virus particles (adenovirus and rotavirus) from spiked water samples using various concentration methods, that is, the Silicon dioxide technique, positively charged-, negatively charged- and mixed-ester non-charged filtration techniques;
2. To optimise various DNA/RNA extraction methods i.e. the TRIzol method, Roche High Pure PCR Template Preparation kit and the QIAamp Ultrasens Virus kit;
3. To optimise the polymerase chain reaction (PCR) to detect adenovirus and reverse transcriptase PCR to detect rotavirus from spiked water.
4. To determine whether rotavirus, enterovirus and adenovirus are present in the river water samples by applying the optimised concentration and nucleic acid extraction methods.

CHAPTER TWO
MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 Overview of Methodology

2.1.1 Isolation and Concentration of Viruses

Viruses can adhere to many charged surfaces due to their polarity. Surfaces such as filter membranes or glass powder thus allow for the isolation of the virus by adsorption onto such matrices. The sedimentation of viruses by ultracentrifugation and concentration by ultrafiltration is also achieved due to their relatively high molecular mass ($M_r > 10^6$). **Table 2.1** outlines various methods generally used for the concentration of viruses from water, based on their general properties, such as their polarity (Wyn-Jones and Sellwood, 2001). This table also details the various concentration principles employed, such as adsorption/elution or entrapment/ultrafiltration, etc. The filtration methods predominantly used such as electronegative- and electropositive membranes, glass wool, etc. are also listed (Cashdollar and Dahling, 2006). In addition, information on the water quality, i.e. whether the specific method performs optimally with low turbidity or all types of water quality samples is included. **Table 2.1** also indicates the effectiveness and relative costs of the methods. Block and Schwartzbrod (1989) also established criteria for an ideal concentration method as follows:

- it must be technically easy and quick,
- must recover quantitatively high concentrations of virus,
- must be able to concentrate all types of viruses,
- must yield a small volume of concentrate,
- must be cheap,
- be able to process large volumes of water,
- be repeatable (within a laboratory) and reproducible (between laboratories).

Ideally, the presence of viruses should be accurately predicted from a microbial or chemical indicator, thereby avoiding the expense, time and technical expertise involved with virus testing. The concentration methods (**Table 2.1**) should also attempt to yield a simplified procedure for the advancement of efficient viral recovery which simultaneously decreases the cost of the method (Cashdollar and Dahling, 2006).

Villar *et al.* (2006) used negatively charged filters to concentrate hepatitis A virus from four different water sources, i.e. coastal water, river water, tap water and mineral water. Their results indicated that this concentration method worked well when performing quantitative

Table 2.1. Summary of concentration techniques for viruses in water and water–related materials (adapted from Wyn-Jones and Sellwood, 2001)

Technique	Method	Water Quality	Initial volume	Relative virus content	Recovery	Capital Cost	Revenue cost	Secondary Concentration required	Comments
Adsorption/ Elution	Gauze Pads	Sewage or effluent	Large	High	Low to medium	Nil	Very low	No	Not quantitative
	Electronegative Membranes	All waters	1-1000 liters	Low to medium	50-60% with practice	Medium	Medium	Yes	High volumes require dosing pumps
	Electropositive Membranes	All waters	1-1000 liters	Low to medium	50-60% with practice	Medium	High	Yes	No preconditioning required
	Electronegative Cartridges	Any low turbidity	1-50 liters	Low to medium	Variable: higher with clean waters	Low	Low	Yes	Clogs more quickly than membranes
	Electropositive Cartridges	All waters	1-1000 liters	Low to medium	Variable	Medium	High	Yes	Wide range of viruses
	Glass Wool	All waters	1-1000 liters	Low to medium	Variable	Low	Very low	Yes	No preconditioning required
	Glass Powder	All waters	<100 liters	Any	20-60%	Medium	Low	If vol > 100 liters	Special apparatus
Entrapment/ Ultrafiltration	Alginate Membranes	Clean only	Low	High	Good	Low	Low	No	Very slow, clogs rapidly if turbid
	Single Membranes	Clean	Low	Any	Variable	Medium	Low	No	Slow
	Tangential (=cross) flow and hollow fibres	Treated effluents or better	High	Low	Variable	High	Medium	Sometimes	Prefilter turbid waters
	Vortex flow	Treated effluents or better	High	Low	Unknown	High	Medium	Unknown	Undeveloped
Hydroextraction	PEG or Sucrose	Any	Low	High	Variable (toxicity)	Negligible	Very low	No	High virus loss in waste waters
Ultracentrifugation		Clean	Low	High	Medium	High	Medium	No	Wide range but use impractical
Other techniques	Fe oxide Flocculation	All	Low	Any	Variable	Low	Low	No	
	Biphasic Partition	All	<7 liters	Any	Variable	Low	Low	No	Toxic to cells
	Immunoaffinity and Magetic Beads	Unknown	Low	Low	High	High	Low	No	New method

PCR (detecting the desired segment and the quantity present), however poor recovery was obtained with qualitative PCR (detecting the desired segment only). Unlike quantitative PCR, qualitative PCR might be more sensitive to inhibitors which may lead to poor recovery of a virus (Villar *et al.,* 2006). Liu *et al.* (2007) developed a flocculation-dissolution concentration procedure using an uncharged filter, which did not require beef extract for the detection of norovirus in drinking water and compared this method to one which required a positively charged filter and elution using beef extract buffer. They then concluded that more viruses were detected (as evaluated with dilution experiments) with the uncharged filter method than the positively charged filter method. Another concentration method used adsorption-elution on sodocalcic glass wool as described by Hot *et al.* (2003) and the West Midlands Environment Agency (2000). This method was described as user friendly and economical since it could process relatively large volumes of water. Kittigul *et al.* (2001) employed a negatively charged filter to concentrate viruses from water samples. The eluate from the negatively charged filter was further concentrated using a SpeedVac concentrator for 4–5 hours. The authors concluded that using negatively charged filters and further concentrating using a SpeedVac concentrator method was more efficient to detect rotavirus in environmental water samples. In a subsequent study by Kittigul *et al.* (2005), a negatively charged filter method was also used to detect rotavirus in environmental water samples.

There are many studies that prefer utilising either positive- or negatively-charged filters, however Rose *et al.* (1984) showed no significant disparity in recovery between negatively- or positively charged filters. A disadvantage of negatively charged filters is the addition of cations and the conditioning of the water by adjusting the pH which makes the procedure burdensome (Ma *et al.*, 1994). Positively charged filters are easier to work with since pre-conditioning of water is not required, unless there is chlorine present. However, the cost of positively charged filters makes it prohibitive for routine monitoring. Cashdollar and Dahling (2006) compared new and used filters and found that the positively charged filter can be re–used up to three times with sterilisation and rinsing in-between usage. Zurbriggen *et al*. (2008) performed an efficiency of virus recovery on a silicon dioxide concentration method and found a 60% virus recovery with 30% recovery variability. The authors applied this concentration method to isolate poliovirus from wastewater and found 46 strains (11.5%) positive for poliovirus. Furthermore, when the isolates that were negative for poliovirus were cell cultured, a further 16 poliovirus strains were detected, indicating the inefficiency of the concentration technique. For the purposes of this study, a silicon dioxide method, positively-, negatively- and mixed-ester non-charged filters were evaluated for optimal DNA and RNA concentration.

2.1.2 Nucleic Acid Extraction Techniques

Most DNA viruses develop in the nucleus while most RNA viruses develop in the cytoplasm of the host cell (Strauss and Strauss, 2008). In addition, RNA viruses are more vulnerable to genetic variation than DNA viruses leading to increased pathogenecity by RNA viruses (Dimmock *et al*., 2007). The genetic variation could be due to mutation and errors made by RNA polymerases, which are more error prone than DNA polymerases. In addition, recombination of genetic material could occur due to dual infections of similar viruses of different strains. Gene rearrangement is another cause of genetic variation in viruses caused by the chronic re-infection of a virus, resulting in a different strain emerging months later (Strauss and Strauss, 2008; Dimmock *et al*., 2007). Currently there are 21 families of viruses described infecting humans, of which 70% are RNA viruses (Gelderblom, 1996).

An extraction technique should yield highly purified DNA or RNA samples without any inhibiting substances to effect any downstream applications such as PCR (Kok *et al.*, 2000). A variety of methods may be employed to extract DNA/RNA from such samples. Sambrook *et al.* (1989) describe a method using phenol–chloroform followed by ethanol precipitation to extract DNA/RNA. Chomczynski and Sacchi (1987) described a method employing guanidinium isothiocyanate followed by ethanol precipitation to extract RNA from cells and microorganisms. Both methods minimised inhibiting substances when performing nucleic acid amplification by PCR (Kok *et al.*, 2000). In a study conducted by Barnes and Taylor (2004), two extraction techniques, namely a Qiagen QIAamp Viral extraction kit and TRIZOL reagent, were evaluated for best results to extract RNA from samples concentrated from fruit washings, water and sewage. It was found that the method employing TRIZOL reagent was most suitable for the type of samples used. However, when the Qiagen QIAamp Ultrasens Virus kit was subsequently evaluated, it was found to be a better method than the TRIZOL reagent method since RNA extraction efficiency was improved and larger sample volumes could be used (Barnes and Taylor, 2004). Various extraction kits made by different manufacturers each have specific applications available for extracting DNA and RNA, respectively. Ehlers *et al.* (2005) used the RNeasy Mini Kit (Qiagen, Hilden, Germany) to successfully extract enterovirus from treated and untreated drinking water in South Africa. Van Heerden *et al.* (2005) used the High Pure Nucleic Acid Kit (Roche) to extract DNA viruses from river water and drinking water and reported that their results were in agreement with other extraction techniques. Additionally, Fong *et al*., (2010) used the DNeasy tissue kit (Qiagen) to extract DNA viruses from wastewater and reported that their objectives were met. Other researchers reported satisfaction with the performance and efficiency of the QIAamp viral RNA extraction kit (Qiagen, Courtaboeuf, France) (Burgener *et al.*, 2003; Hot *et al.*, 2003). Burgener *et al.* (2003) compared four kits namely the QIAamp UltraSens Virus kit (Qiagen, Hilden, Germany), NucliSens Isolation kit (Organon Technica, Boxtel, Netherlands),

NucleoSpin RNA virus F (Machery–Nagel, Dueren, Germany) and the QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany), for the extraction of RNA from environmental water samples. Their results showed that the QIAamp Viral RNA Mini kit and the NucliSens isolation kit were the methods of choice for application in water monitoring (Burgener *et al.*, 2003). For the purposes of this study, a TRIzol extraction method, the Roche High Pure PCR Template Preparation kit and the QIAamp Ultrasens virus kit were compared for optimal DNA and RNA extraction.

2.1.3 Virus Detection Methods

Detection of viruses in groundwater relies on various factors, such as the recovery method employed, recovery efficiency, virus type, sample volume, storage, transport conditions and the type of viral assay used in the laboratory (West Midland Environment Agency, 2000). Techniques to detect these viruses include cell culture, PCR, reverse-transcription PCR, multiplex reverse-transcription PCR, nested PCR and molecular beacons. While cell culture and PCR is commonly used, both have restrictions for detecting viruses in water environments (USEPA, 1996; Cho *et al.,* 2000; Hot *et al.,* 2003). False negative results may be obtained with cell culture methods due to different viral strains present in water (Lee *et al.*, 2005). Some viruses may also not grow in any cell line leading to more false-negative results (Metcalf *et al.,* 1995) and often, cell toxicity may lead to false-positive results with cell culture methods (Murrin and Slade, 1997).

Viruses have genetic material that consists of double stranded DNA (such as adenovirus), single stranded DNA (such as Parvovirus B19), double stranded RNA (such as rotavirus), and single stranded RNA (such as enterovirus). Since the PCR technique utilises DNA and not RNA as the starting template (van Pelt-Verkuil *et al., 2008)*, it follows that the RNA should then be converted to DNA prior to PCR. An initial conversion step utilises an enzyme called reverse transcriptase to transcribe RNA to complementary DNA (cDNA). The primers in reverse transcription reactions can be gene specific, oligo(dT) or random primers. The RT-PCR can also be performed in two-step or one-step procedures. In the two-step RT-PCR, the reverse transcription reaction and the PCR reaction are completed separately under optimal conditions. Complementary DNA synthesis would be performed first, and a small sample of the reaction would subsequently be removed and used in PCR. In one-step RT-PCR, the reverse transcription and PCR occurs in one tube under optimal conditions (Viljoen *et al., 2005;* van Pelt-Verkuil *et al., 2008*).

The overall sensitivity and specificity of the PCR technique relies largely on the first few rounds of thermal cycling (Ruano *et al.,* 1991) and the success of specific amplification would depend on the rate at which primers anneal to their target. Factors hindering optimal

annealing include poorly designed primers, the annealing temperature, and sub-optimal buffer composition (Elnifro *et al.,* 2000). The extension of the specific primer-target compound would also depend on the activity of the enzyme. In addition, the availability of dNTPs and the nature of the target DNA are vital.

The PCR is used in many applications such as detecting infectious agents, genetic disease diagnosis, genomic DNA or cDNA cloning, RNA amplification by reverse transcription PCR (RT-PCR), forensic sample studies, analysis and allele sequence variation(Viljoen *et al., 2005*). Soil samples and treated drinking water may contain substances such as humic acids and metals which may interfere with the chemical components of the PCR reaction (Abbaszadegan *et al.,* 1999; Donaldson *et al*., 2002). Humic acid is a mixture of acids containing carboxyl and phenolate groups which can form complexes with ions in various environments (Baigorri, 2009). Polymerase chain reaction inhibition is however, considered negligible with certain concentration and extraction techniques which utilise filters (Van Heerden *et al.,* 2005).

In some diagnostic settings, the use of PCR is limited by cost and occasionally inadequate test sample volume. A variant of PCR, termed multiplex PCR, attempts to overcome the lack of sample and improve the diagnostic capacity of PCR. More than one primer included in the multiplex PCR would be required to amplify more than one target sequence. This however increases the likelihood of false amplification products leading to primer dimer formation (Brownie *et al.*, 1997). Poor sensitivity or specificity of certain specific targets in multiplex PCR may also create numerous problems when optimising the reaction (Polz & Cavanaugh, 1998). However, in a multiplex PCR study done by Rohayem *et al.* (2004), in which the detection of norovirus, astrovirus and adenovirus in clinical stool samples was described, it was shown that multiplex PCR was equally sensitive and specific when compared to simplex PCR. The detection limits of the multiplex PCR and the simplex PCR were determined using 10-fold serial dilutions of norovirus, astrovirus and adenovirus controls and were found to be similar, i.e. about 10^2 copies of virus particles could be detected. Multiplex PCR has also been applied productively in many areas of nucleic acid diagnostics. Examples of such applications would be RNA detection, mutation and polymorphism analysis and gene deletion analysis (Elnifro *et al.*, 2000). The multiplex PCR technique was also shown to be a valuable method to identify viruses, bacteria, fungi and parasites.

Nested PCR refers to two pairs of primers being used to amplify a single fragment. The first pair amplifies the fragment in a normal PCR, while the second pair binds within the first PCR product. Incorrect initial amplification would yield little or no PCR product by a second primer

pair and thus the nested PCR technique is more specific than a normal PCR. The sensitivity of the nPCR could be enhanced by using 'hot' nPCR whereby one of the nested primers can be labelled with radio- or non-radioactive markers. Applications include identity confirmation and epidemiological studies (Viljoen *et al., 2005).*

Molecular beacons can be described as oligonucleotide probes which fluoresce upon hybridisation, thereby aiding with real-time monitoring of the target amplicon during the PCR reaction (Yates and Chen, 2003). Studies have shown that the technique is very sensitive, but not very specific, since it could detect low copies of hepatitis A but could not detect other enteric viruses. The principle of molecular beacons rests on intercalating fluorescence dyes which may partially prevent non-specific DNA synthesis by using labelled primers with a stem-loop structure (**Figure 2.1**). The native structure of the molecular beacon oligonucleotide folds into a hairpin loop. The beacon loses its hairpin loop structure upon PCR thermocycling where hybridisation to the PCR product occurs. The reporter and quencher molecules are spatially separated upon hybridisation, resulting in the quencher molecule being unable to absorb fluorescence from the reporter molecule. The reporter molecule could therefore be detected using fluorescence measurement procedures (van Pelt-Verkuil *et al., 2008).* Molecular beacons are affected by various factors which may lead to false-positive or false-negative results. The most significant factor would be the distance between fluorescence– and quenching groups (Li *et al.*, 2008). Other factors include the purity of the molecular beacons, and the temperature and pH at which molecular beacons are stored (Li *et al.*, 2008; van Pelt-Verkuil *et al., 2008*).

Many variations/adaptations of the standard qualitative PCR have been developed. The hot-start PCR involves heating the PCR components to a certain temperature (e.g. 95ºC) before adding the polymerase enzyme, which reduces non-specific binding. Other variations to the PCR protocol include booster PCR and time release PCR (van Pelt-Verkuil *et al.,* 2008*).*

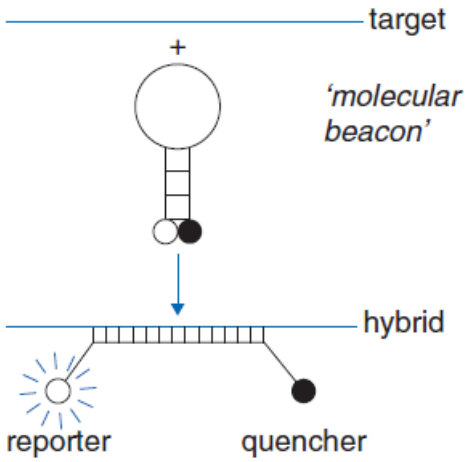

Figure 2.1. General structure of a molecular beacon (van Pelt-Verkuil *et al., 2008)*

2.2 Pilot Study: Optimisation of Methods

2.2.1 Water Samples for Pilot Study

Twelve sterile Schott bottles containing 100 ml sterile distilled water were spiked with a 1 ml rotavirus control (Coris Bioconcept, Gembloux, Belgium) and a 2 ml adenovirus control (Coris Bioconcept, Gembloux, Belgium). The rotavirus concentration was higher than adenovirus concentration, requiring higher concentrations of the adenovirus control for the spiked water samples. The 12 bottles of 100 ml water containing rotavirus and adenovirus controls were concentrated using the SiO_2 method, positively charged-, negatively charged, and non–charged mixed ester filters, respectively (three for each respective method). The optimisation of enterovirus was not completed due to the unavailability of a positive control.

2.2.2 Concentration Techniques

2.2.2.1 Concentration of Viruses by Silicon Dioxide (SiO_2) Method

Silicon dioxide particles (Appendix A) were prepared according to the protocol described by Boom *et al.* (1990). The method as outlined by Baggi and Peduzzi (2000) and Baggi *et al.* (2001) was followed to concentrate the viruses from water. The spiked water sample was firstly analysed for the presence of rotavirus and adenovirus by means of a Combi-Strip (Coris Bioconcept, Gembloux, Belgium). Briefly, 3 x 100 ml of spiked water (Section 2.2.1) was then acidified to pH 3.5 with acetic acid. Sterile SiO_2 (20 µl) and 100 µl aluminium chloride solution (0.5 M) was added and mixed on a magnetic stirrer for 30 minutes at room temperature. The samples were placed at 4ºC for 24 hours to allow the SiO_2 to settle undisturbed. Approximately 90 ml of the supernatant was discarded and the remaining 10 ml was centrifuged at 7500 x g for 10 minutes to pellet the virus using an Avanti JE centrifuge (Beckman Coulter, USA). The supernatant was discarded by decanting and the viruses were recovered from the pellet by homogenising it with beef extract glycine buffer (0.25 N glycine and 3% beef extract at pH 9.5). The samples were incubated in a 64ºC oven for 10 minutes. The suspension was transferred to a sterile labelled micro-centrifuge tube and centrifuged at 1600 x g for 2 minutes. The supernatant containing virus was removed and centrifuged at 12500 x g for one hour at 4ºC. The supernatant was again discarded and the pellet was resuspended in 150 µl nuclease–free water. Fifty microliters of each sample was used for transmission electron microscopy (TEM). RNA extraction was performed using the TRIzol extraction method (Section 2.2.3.1), DNA extraction was performed using the High Pure PCR Template Preparation kit (Section 2.2.3.2), while the QIAamp Ultrasens Virus kit (Section 2.2.3.3) was utilised for the simultaneous extraction of DNA and RNA.

2.2.2.2 Concentration of viruses using Positively Charged Filters

For the concentration of virus particles using positively charged filters triplicate samples of the 100 ml of spiked water (Section 2.2.1) were centrifuged at 3000 rpm for 30 minutes to

pellet the debris. The supernatant was analysed for the presence of rotavirus and adenovirus by means of a Combi-Strip (Coris Bioconcept, Gembloux, Belgium) according to the manufacturer's instructions. The supernatant (about 90 ml) was then passed through a 47 mm, 0.45 µm pore size Zetapore positively charged filter (Cuno Inc., Meriden, Conn. USA) to which viral particles adhere. The Zetapore membrane was placed in a 50 ml centrifuge tube containing 4 ml of 50 mM glycine (pH 9.5) and 1% beef extract. The virus particles were eluted from the filter by shaking at 500 rpm for 20 minutes at room temperature. The virus-containing buffer was adjusted to pH 8 with HCl, and micro-concentrated with the Amicon Ultra centrifugal filter device (Millipore). The volume was adjusted to 200 µl with phosphate buffered saline (PBS) and a 50 µl aliquot was removed for TEM. The concentrate was again checked for the presence of rotavirus and adenovirus using the Combi-Strip. RNA extraction was performed using the TRIzol extraction method (Section 2.2.3.1), DNA extraction was performed using the High Pure PCR Template Preparation kit (Section 2.2.3.2), while the QIAamp Ultrasens Virus kit (Section 2.2.3.3) was utilised for the simultaneous extraction of DNA and RNA.

2.2.2.3 Concentration of viruses using Negatively Charged Filters

For the concentration of virus particles using negatively charged filters triplicate samples of the 100 ml of spiked water (Section 2.2.1) were also centrifuged at 3000 rpm for 30 minutes to pellet the debris. The supernatant was analysed for the presence of rotavirus and adenovirus antigens by means of a Combi-Strip. To prepare the filters, 2 ml of 250 mM $AlCl_3$ was passed through the GF/F negatively charged filter (Whatman International Ltd, England). The supernatant (about 90 ml) was removed and then passed through the $AlCl_3$ treated 47 mm, 0.45 µm pore size GF/F negatively charged filter. The filter was then rinsed with 200 ml of 0.5 mM H_2SO_4 (pH 3.0) to remove aluminium ions. The filter was rinsed with 4 ml of a 1 mM NaOH (pH 10.8) solution to elute the viral particles and the filtrate was placed in a 25 ml sterile conical tube containing 25 µl of 100 mM H_2SO_4 (pH 1.0) and 50 µl 100 X Tris-EDTA (TE) buffer (pH 8.0). The virus-containing buffer was micro-concentrated with the Amicon Ultra centrifugal filter device (Millipore). The volume was adjusted to 150 µl with 1x PBS and a 50 µl aliquot was removed for TEM. This filtrate was once again tested for the presence of rotavirus and adenovirus antigens using the Combi-Strip test. RNA extraction was performed using the TRIzol extraction method (Section 2.2.3.1), DNA extraction was performed using the High Pure PCR Template Preparation kit (Section 2.2.3.2), while the QIAamp Ultrasens Virus kit (Section 2.2.3.3) was utilised for the simultaneous extraction of DNA and RNA.

2.2.2.4 Concentration using Non-charged Mixed-ester Filters

Triplicate samples of the 100 ml of spiked water (Section 2.2.1) were centrifuged at 3000 rpm for 30 minutes to pellet the debris, for the concentration of virus particles using non-charged mixed-ester filters. The supernatant was analysed for the presence of rotavirus and adenovirus antigens by means of a Combi-Strip. The supernatant (about 90 ml) was transferred to a sterile 100 ml Schott bottle and the sediment was discarded. Subsequently 1 ml of 1 M $CaCl_2$ and 1 ml of 1 M Na_2HPO_4 was added. The mixture was stirred for 5 minutes to allow flocculation and filtered through a 47 mm, 0.45 µm pore size non-charged mixed-ester filter membrane (Whatman GmbH, Germany). The membrane was transferred to a 9 cm diameter petri dish and soaked in 4 ml of a 0.3 M citrate buffer (pH 3.5) for 3 minutes after which the membrane was discarded. The virus-containing buffer was micro-concentrated with the Amicon Ultra centrifugal filter device (Millipore). The volume was adjusted to 200 µl with phosphate buffered saline and a 50 µl aliquot was removed for TEM. This solution was again checked for rotavirus and adenovirus antigens using the Combi-Strip test. RNA extraction was performed using the TRIzol extraction method (Section 2.2.3.1), DNA extraction was performed using the High Pure PCR Template Preparation kit (Section 2.2.3.2), while the QIAamp Ultrasens Virus kit (Section 2.2.3.3) was utilised for the simultaneous extraction of DNA and RNA.

2.2.3 Viral Extraction Techniques

2.2.3.1 TRIzol method

Viral RNA was extracted using the protocol described by Viviers *et al.* (2004). One hundred microliters of the concentrated virus water sample (Sections 2.2.2.1, 2.2.2.2, 2.2.2.3 and 2.2.2.4, respectively) was mixed with 500 µl TRIzol reagent (Invitrogen, California, USA). The TRIzol-sample mixture was incubated at room temperature for 5 minutes. Thereafter, 100 µl chloroform was added and the mixture was allowed to incubate at room temperature for 3 minutes. The sample mixture was centrifuged at 1200 x g at 4ºC for 15 minutes. The aqueous phase (about 300 µl) of each sample was transferred to a 2 ml tube. One tenth volume of the aqueous phase of 3 M sodium acetate (pH 5.2) and twice the volume of the aqueous phase of absolute ethanol were added. The samples were incubated at -20°C overnight. Thereafter, the samples were centrifuged at 12000 x g at 4°C for 5 minutes. The supernatant was discarded and the pellet was washed with 300 µl of 70% cold ethanol and centrifuged at 12000 x g at 4°C for 5 min and allowed to air dry. The pellet was re-suspended in 30 µl of nuclease-free water. A 5 µl aliquot was visualised on a 2% Tris acetic acid EDTA (TAE) agarose gel containing 2 µl of a 10 mg/ml ethidium bromide solution for 1 hr at 90 V to confirm whether any RNA was present. Aliquoted samples were stored at 4ºC for subsequent RNA analysis.

2.2.3.2 High Pure PCR Template Preparation Kit (Roche)

Deoxyribonucleic acid was extracted from 100 µl samples (Sections 2.2.2.1-2.2.2.4 respectively) as described in the Roche High Pure PCR Template Preparation kit (Roche, Mannheim, Germany). The final volume was approximately 200 µl which was stored at 4ºC. The presence of genomic DNA was confirmed by gel electrophoresis and 5 µl was run on a 2% TAE agarose gel containing 2 µl of a 10 mg/ml ethidium bromide solution for 1 hr at 90 V.

2.2.3.3 QIAamp UltraSens Virus Kit (Qiagin)

Deoxyribonucleic acid and RNA were extracted from 100 µl samples (Sections 2.2.2.1-2.2.2.4 respectively) using the QIAamp Ultrasens virus kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The final volume was approximately 56 µl, of which 20 µl was stored at 4ºC for adenovirus identification. Aliquots (6 µl) were stored in the -80ºC freezer for rotavirus identification. The presence of genomic DNA/RNA was confirmed by gel electrophoresis with 5 µl run on a 2% TAE agarose gel containing 2 µl of a 10 mg/ml ethidium bromide solution for 1 hr at 90 V.

2.2.4 Reverse Transcription/cDNA Synthesis

2.2.4.1 Rotavirus cDNA synthesis

Complementary DNA from the double stranded RNA (Section 2.2.3.1 and 2.2.3.3) of rotavirus was synthesised using the Improm-II reverse transcription system (Promega Corp, USA) according to the manufacturer's instructions. To account for the dsRNA nature of rotavirus, an additional denaturation step was included as suggested by Gouvea *et al.* (1990) and Gilgen *et al.* (1997). A variety of parameters were optimised for the cDNA synthesis. Both oligo$(dT)_{15}$ primers and a gene specific primer (RV3, **Table 2.2**) were employed for first strand cDNA synthesis. Magnesium chloride concentrations (2 mM – 6 mM) and initial RNA concentrations (1 µl – 10 µl) were also compared. For the optimised protocol, a 20 µl final volume was used and 4 µl RNA was denatured at 97ºC for five minutes in the presence of 1 µl RV3 primer (10.0 µM). The mixture was immediately placed on ice for five minutes. In a separate tube, a mixture containing 5 mM $MgCl_2$, 0.5 µM dNTP mix, 1x Improm-II reaction buffer, 20 units RNasin ribonuclease inhibitor and 1 µl Improm-II reverse transcriptase (according manufacturer's recommendations) was used. Reverse transcription was completed at 50ºC for 60 minutes with the reverse transcriptase enzyme heat inactivated at 70ºC for 15 minutes. Complementary DNA was used immediately or stored at 4ºC for later use. **Table 2.2** also outlines all the primer oligonucleotide sequences used in the optimisation study as well as in the working methodology employed for virus investigation in river water.

Table 2.2. Primer oligonucleotide sequence used throughout the study

Virus and oligonucleotide	Region	Sequence 5' ⟶ 3'[a]	Localisation on respective gene	Product size
Rotavirus group A[b] RV3 RV4	VP7 gene	 TGTATGGTATTGAATATACCAC ACTGATCCTGTTGGCCAWCC	 50 - 71 395 - 376	346bp
Adenovirus[c] Adhex1 Adhex2	Hexon gene	GCCACCGATACGTACTTCAGCCTG GGCAGTGCCGGAGTAGGGTT TAAA	99 - 123 360 – 336	261bp
Enterovirus[d] EP1 EP2 EP3 EP4	5' untranslated	 ATTGTCCACCATAAGCAGCCA ACCTTTGTACGCCTGTT AAGCACTTCTGTTTCCC ATTCAGGGGCCGGAGGA	 577 – 596 67 - 83 166 - 182 447 - 463	 513bp 297bp

a Mixed bases in degenerated primers: W = A/T
b Described in Gilgen *et al.* (1997).
c Descibed in Rohayem *et al.(* 2004).
d EP1 is a universal primer from the 5' untranslated region of enteroviruses, EP2, EP3 and EP4 are obtained from a fragment of homologous sequence of *Picomaviridae* (Kuan, 1997).

2.2.5 Polymerase Chain Reaction (PCR)

2.2.5.1 Templates for PCR

Table 2.3 indicates the templates used in the PCR procedure throughout the study, which were obtained from different concentration and extraction methods applied to spiked water containing rotavirus and adenovirus controls.

Table 2.3. The PCR templates used throughout the study, obtained from different concentration and extraction methods employed

Virus	Templates used in PCR	Extraction methods	SiO_2 method	Positively charged filter	Negatively charged filter	Mixed-ester membrane
Rotavirus	cDNA	TRIzol	√	√	√	√
Adenovirus	DNA	High Pure PCR Template Preparation Kit	√	√	√	√
Rotavirus	cDNA	QIAamp Ultrasens Virus Kit	√	√	√	√
Adenovirus	DNA	QIAamp Ultrasens Virus Kit	√	√	√	√

2.2.5.2 Amplification of rotavirus cDNA

The PCR mixture for rotavirus contained the following reagents; 5 µl cDNA as template (prepared from Section 2.2.4.1), a final concentration of 0.2 mM dNTP mix, 1.5 mM $MgCl_2$, 0.5 µM RV3 and RV4 primers (see **Table 2.2**) respectively. All PCR's were performed with 1.25 units of Go*Taq* Flexi (Promega Corp, USA) DNA polymerase in a final volume of 50 µl. The amplification was performed for 35 cycles at 95ºC for 1 minute, 45ºC for 1 minute, and 72ºC for 1 minute. A rotavirus positive control (346 bp) and a negative control were also included. All the PCR thermal cycling reactions were done in a MyCycler (Biorad). Both the annealing temperatures (30ºC - 55 ºC) and the $MgCl_2$ (0.5 mM – 6.0 mM) concentrations of all PCR reactions were compared to determine optimal PCR parameters.

2.2.5.3 Amplification of adenovirus DNA

The adenovirus PCR mixture contained 6 µl DNA as template (prepared from Sections 2.2.3.1, 2.2.3.2, 2.2.3.3), 0.2 mM dNTP mix, 2.5 mM $MgCl_2$, 1.25 units of Go*Taq* Flexi polymerase, 0.3 µM adhex 1 and adhex 2 primers (**Table 2.2**) in a final volume of 50 µl. The PCR mixture was amplified for 35 cycles of 94ºC for 30 seconds, 55ºC for 1 minute, and 72ºC for 1 minute. In addition an adenovirus positive control (261 bp) and a negative control were included.

2.2.6 Agarose Gel Electrophoresis

For all PCR analyses individual samples (20 µl) were mixed with 5 µl loading buffer and transferred to a 2% 1x TAE agarose gel containing 2 µl of a 10 mg/ml ethidium bromide solution. The gel was submerged in 1x TAE pH 7.0 buffer and electrophoresed for 90 minutes at 100 V. The separated products in the gel were visualised by ultraviolet transillumination for the presence of the virus amplicon.

2.2.7 Sequencing

The PCR bands corresponding to the respective base pairs (**Table 2.2**) were purified using the Minelute Gel Extraction Kit and sent for sequencing at the Stellenbosch University DNA Sequencing Facility where the BigDye Terminator V3.1 Sequencing Kit (Applied Biosystems) was used. Sequences were identified using the Basic Local Alignment Tool (BLASTn) (Altschul *et al*., 1997) obtained from the National Centre for Biotechnology Information (NCBI) website.

2.3 Working Methodology

2.3.1 Sampling Sites

Sampling sites as outlined in Paulse *et al*. (2007) and (2009) were utilised in the current study. Three sampling sites were identified along the Plankenburg River (Stellenbosch) location as indicated in **Figure 2.2**: Site A (agricultural farming and residential areas) – Before site; Site B (Informal settlement of Kayamandi) – At site and Site C (Industrial area substation) – After site. Sampling sites on the Berg River are indicated in **Figure 2.3**: Site A (agricultural farming area); Site B (Mbekweni informal settlement); Site C (Newton pumping station).

2.3.2 Sample Collection

Sampling started in August 2005 and continued for one year until July 2006. Water samples (one litre at each site) were collected on a monthly basis in sterile polypropylene bottles (Cole-Palmer Instrument Company). The bottles were submerged deep enough into the river system to obtain a representative surface water sample. A total of 72 samples was collected over the study time period. The temperature and pH of the river water at the various sampling locations were measured using a YSI 100 portable pH/temperature meter (YSI Environmental Inc.).

2.3.3 Concentration Technique

2.3.3.1 Concentration using Positively Charged Filters

Based on results obtained in the pilot study (Section 2.2), positively charged filters were used for the concentration of all samples collected along the Berg- and Plankenburg Rivers. One litre water was centrifuged at 3000 rpm for 30 minutes to pellet the debris. The supernatant was checked for the presence of rotavirus and adenovirus by means of a Combi-Strip. The supernatant (about 900ml) was removed and passed through a 47 mm, 0.45 µm pore size Zetapore positively charged filter (Cuno Inc., Meriden, Conn. USA). The Zetapore membrane was placed in a 50 ml centrifuge tube containing 4 ml of a 50 mM Glycine (pH adjusted to 9.5 using 10 M NaOH) and 1% beef extract. The virus particles were eluted from the filter by shaking at 500 rpm for 20 minutes at room temperature. The virus-containing buffer was adjusted to pH 8 with HCl, and micro-concentrated with the Amicon Ultra centrifugal filter device (Millipore, Ireland). The volume was adjusted to 200 µl with phosphate buffered saline (PBS) and an aliquot (30 µl) was removed for transmission electron microscopy (TEM). The concentrate was again checked for the presence of rotavirus and adenovirus using the Combi-Strip.

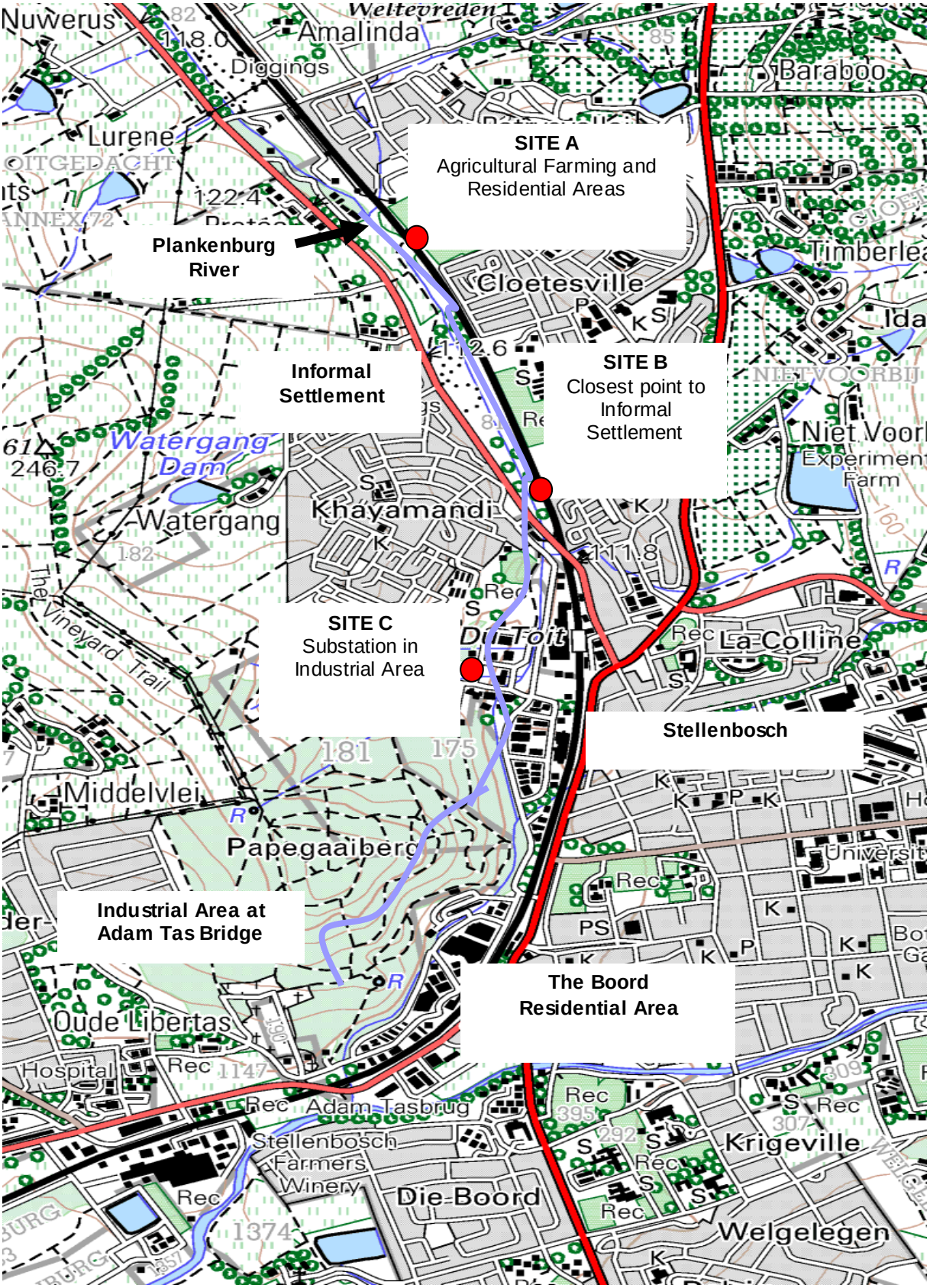

Figure 2.2. Map of the Plankenburg River indicating different sampling points. Site A - agricultural farming areas (Before site); Site B- closest point to the informal settlement of Kayamandi (At site) and Site C- substation in industrial area (After site) (Paulse *et al*., 2009).

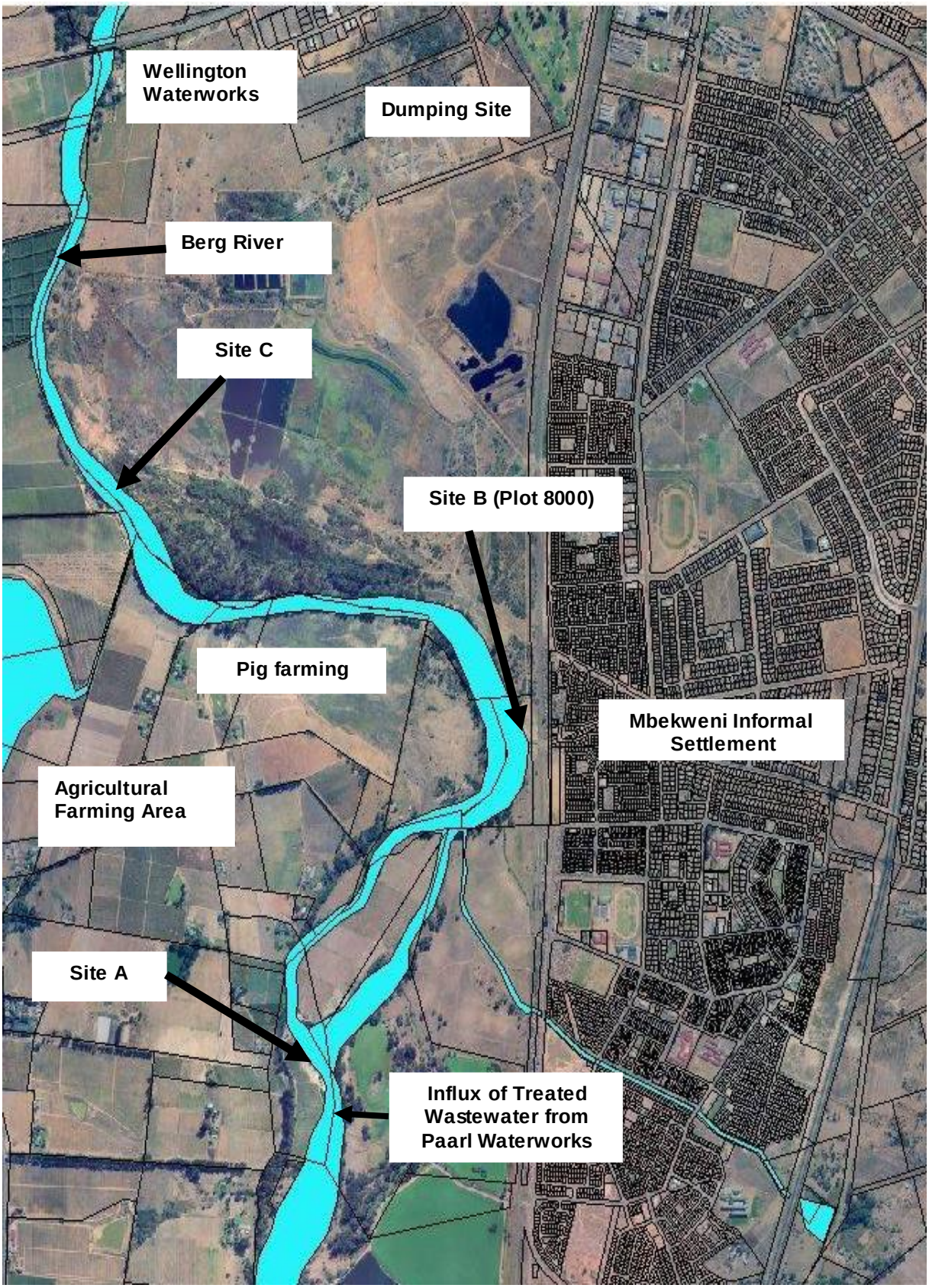

Figure 2.3. Map of the Berg River indicating the different sampling points. Site A –agricultural farming areas (Before site); Site B (plot 8000) - close to the informal settlement of Mbekweni (At site) and Site C – Newton pumping station (After site) (Paulse *et al*,, 2007).

2.3.4 Viral Extraction Techniques

2.3.4.1 QIAamp UltraSens Virus Kit (Qiagen)

Based on results obtained in the pilot study (Section 2.2), the QIAamp Ultrasens Virus kit was used for the extraction of DNA and RNA from all samples collected along the Berg- and Plankenburg Rivers. The final volume was approximately 56 µl, of which 20 µl was stored at 4ºC for adenovirus identification. Aliquots (6 µl) were stored in the -80ºC freezer for rotavirus and enterovirus identification. The presence of genomic DNA/RNA was confirmed by gel electrophoresis (Section 2.3.7).

2.3.5 Reverse Transcription/cDNA synthesis

2.3.5.1 Enterovirus cDNA synthesis

Enterovirus is a known contaminant which can be found in diverse water environments (Bosch *et al*., 2008; Maunula *et al*., 2009), and it was thus decided to analyse the collected environmental samples for the presence of this virus. Complementary DNA from the single stranded RNA of enterovirus was generated using the Improm-II reverse transcription system (Promega Corp, USA) according to the manufacturer's instructions. Complementary DNA was made from RNA extracted using the QIAamp UltraSens Virus kit, using an oligo$(dT)_{15}$ primer and a gene specific primer to ascertain which primer would yield the best PCR product. A variety of reverse transcription extension temperatures were also assessed. Enterovirus cDNA was synthesised using a gene specific primer EP1 (see **Table 2.2**). Complementary DNA synthesis was performed in a 20 µl final volume, 4 µl RNA was denatured at 70ºC for five minutes in the presence of 1 µl of EP1 primer (10.0 µM) and the mixture immediately placed on ice for five minutes. In a separate tube, a mixture containing 3.75 mM $MgCl_2$, 0.5 µM dNTP mix, 1x Improm-II reaction buffer, 20 units RNasin ribonuclease inhibitor and 1 µl Improm-II reverse transcriptase was prepared. Reverse transcription was completed at 42ºC for 60 minutes and the enzyme inactivated at 70ºC for 15 minutes. Samples were used immediately in a PCR reaction (Section 2.3.6.1) or stored at 4ºC for later use.

2.3.5.2 Rotavirus cDNA synthesis

Complementary DNA was synthesised using gene specific primers using the Improm-II reverse transcription system according to manufacturer's instructions. Complementary DNA synthesis was performed in a 20 µl final volume, 4 µl RNA was denatured at 97ºC for five minutes in the presence of 1 µl RV3 primer (10 µM) (see **Table 2.2**) and the mixture immediately placed on ice for five minutes. In a separate tube, a mixture containing 5.0 mM $MgCl_2$, 0.5 µM dNTP mix, 1x Improm-II reaction buffer, 20 units RNasin ribonuclease inhibitor and 1 µl Improm-II reverse transcriptase was used. Reverse transcription was completed at

50ºC for 60 minutes with the reverse transcriptase enzyme heat inactivated at 70ºC for 15 minutes. Complementary DNA was used immediately in the PCR reaction (2.3.6.2) or stored at 4ºC for later use.

2.3.6 Polymerase Chain Reaction (PCR)

2.3.6.1 Amplification of enterovirus cDNA

Enterovirus was amplified in a final volume of 50 µl, with 2 µl cDNA, 0.2 mM dNTP mix, 3.6 mM $MgCl_2$, 1 unit of Go*Taq* Flexi polymerase, 0.5 µM EP1 and EP2 (**Table 2.2**), respectively. The PCR mixture was amplified for 35 cycles of 94ºC for 1 minute, 42ºC for 40 seconds, and 72ºC for 2 minutes. For the nested PCR, 2 µl of the enterovirus PCR product was used with primers EP3 and EP4 (**Table 2.2**). The PCR components are described above (Section 2.3.6.1) except for the different primer pair, and the template DNA (Section 2.3.6.1). A negative control was included; however, no enterovirus positive control was included as no enterovirus control could be sourced.

2.3.6.2 Amplification of rotavirus cDNA

The PCR mixture for rotavirus contained the following reagents: 2.5 µl cDNA, a final concentration of 0.2 mM dNTP mix, 1.5 mM $MgCl_2$, 0.5 µM RV3 and RV4 primers (**Table 2.2**), respectively. PCR was performed with 1.25 units of Go*Taq* Flexi (Promega) DNA polymerase in a final volume of 50 µl. The amplification was performed for 35 cycles at 95ºC for 1 minute, 45ºC for 1 minute, and 72ºC for 1 minute. A rotavirus positive control (346 bp) and a negative control were included. All the PCR thermal cycling reactions were completed in a MyCycler (Biorad).

2.3.6.3 Amplification of adenovirus DNA

The adenovirus PCR mixture in a final volume of 50 µl contained 6 µl DNA, 0.2 mM dNTP mix, 2.5 mM $MgCl_2$, 1.25 units of Go*Taq* Flexi polymerase, 0.3 µM adhex 1 and adhex 2 (**Table 2.2**). The PCR mixture was amplified with 35 cycles of 94ºC for 30 seconds, 55ºC for 1 minute, and 72ºC for 1 minute. An adenovirus positive control (262 bp) and a negative control were included.

2.3.7 Agarose Gel Electrophoresis

Individual PCR samples (20 µl) for the respective viruses were mixed with 5 µl loading buffer and transferred to a 2% 1x TAE agarose gel containing 2 µl of a 10 mg/ml ethidium bromide solution. The gel was covered in 1x TAE pH 7.0 buffer and ran for 90 minutes at 100 V. The separated products on the gel were visualised by ultraviolet transillumination for the presence of the virus amplicon.

2.3.8 Sequencing

The PCR bands corresponding to the respective base pairs (**Table 2.2**) were purified using the Minelute Gel Extraction Kit and sent for sequencing at the Stellenbosch University DNA Sequencing Facility where the BigDye Terminator V3.1 Sequencing Kit (Applied Biosystems) was used. Sequences were identified using the Basic Local Alignment Tool (BLASTn) (Altschul *et al*., 1997) obtained from the National Centre for Biotechnology Information (NCBI) website.

CHAPTER THREE
RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

3.1 Introduction

Virus detection has traditionally been done in cell culture where the sensitivity is low, the method is tedious, expensive and labour intensive, and where some enteric viruses, such as hepatitis A virus, are difficult to cultivate (Gilgen *et al.*, 1997). While, PCR assays are faster and more reliable for virus detection, investigating for their presence in water samples requires a reliable method to extract and concentrate these virus particles. This will then enable sufficient virus nucleic acid yield which will allow for optimum detection by PCR assays.

Similar to the traditional cell culture techniques, some of the concentration and extraction methods utilised in virus detection, such as the silicon dioxide/TRIzol method, may be time consuming and labour intensive (Rasool *et al*., 2002). Furthermore, the numerous steps required when completing this technique may result in virus samples being lost due to procedures that are not properly followed. The goal of the present study was to compare various concentration and extraction methods for the recovery of viruses such as rotavirus and adenovirus from river water. The use of virus specific primers within a PCR was also optimised. In addition, gene specific primers and oligo(dT)$_{15}$ primers were tested and compared to establish which primers would yield the best results since gene specific primers are said to be more sensitive than oligo(dT)$_{15}$ primers (van Pelt-Verkuil *et al.,* 2008) when synthesising cDNA (rotavirus). This is because a specific gene sequence of the template is targeted in the sample when gene specific primers are used for cDNA preparation, while only the poly(A) in the template is targeted when oligo(dT)$_{15}$ primers are used to prepare cDNA for PCR assays. In the pilot study (Section A) various methodologies were tested to determine optimal recovery of viruses from spiked water samples and in Section B this information was used to investigate viruses present in two rivers from the Western Cape, South Africa.

SECTION A

3.2. Pilot Study

3.2.1 Viral extraction and concentration techniques

As described in the Materials and Methods section 2.2.1, distilled water (100 ml) samples were spiked with one milliliter rotavirus and two milliliters adenovirus control virions (Coris Bioconcept, Gembloux, Belgium). Optimisation testing of enterovirus was not completed due to the unavailability of a positive control. Various sedimentation and concentration methods were

employed to establish which method would yield optimum results when analysing the spiked water samples.

3.2.2.1 Concentration of Viruses by Silicon Dioxide (SiO_2) Method

Table 3.1 shows the results of the antigen test (Combi Strip Test) for rotavirus and adenovirus on a concentrated sample after the initial centrifugation but before the addition of silicon dioxide and $AlCl_3$. Transmission electron microscope (TEM) micrographs were used to determine whether any intact viruses could be observed.

Table 3.1 Concentration of viruses by SiO_2 method

Optimisation of Methods (spiked)	Results
Combi Strip Test after supernatant removal	
rotavirus	Positive
adenovirus	Positive
Transmission electron microscopy	
SiO_2 method	No viruses observed

The positive antigen tests for rota- and adenovirus indicated that the viruses were present before concentration with the silicon dioxide method. The final concentrated sample was however not tested for rota- and adenovirus antigens (using the Combi Strip test) since there was a small sample size and the small volume had to be used for virus nucleic acid extraction. When the concentrated sample was viewed using the TEM, no viral particles could be seen, possibly due to low amounts of viral particles present in the final concentrated sample (**Figure 3.1**).

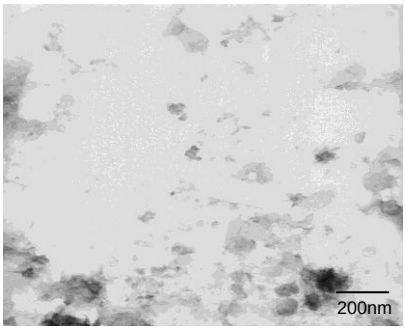

Figure 3.1 Transmission electron micrograph after concentration of viruses from spiked water using the SiO_2 method (X30 000 magnification)

The TEM generally has a detection limit of $10^5 – 10^6$ particles/ml (Roingeard, 2008). The limit of rotavirus and adenovirus antigen detection for the Combi Strip Test is not indicated. However, it is reported that the adenovirus antigen test is 100% sensitive and specific to the groups A and F hexon-antigens of adenovirus in stools (Depierreux *et al*., 2000). Adenoviruses are grouped into species A through F with the hexon antigen being the most prominent (Ferreyra *et al*., 2010). For rotavirus, the antigen test is 92.4% specific and 100% sensitive to group A antigens of rotavirus in stools (Depierreux *et al*., 2000).

3.2.2.2 Concentration of Viruses by Filtration

3.2.2.2.1 Concentration using Positively Charged Filters

The presence of rotavirus and adenovirus antigens was confirmed with the Combi Strip Test at specific points in the protocol. Both rotavirus and adenovirus were detected in the water samples after centrifugation and before filtering through the positively charged filter. The viruses were also present in the concentrate that passed through the positively charged filter (**Table 3.2)**. Transmission electron analysis confirmed the presence of icosahedral and round particles suggesting the presence of both adenovirus and rotavirus particles respectively, which can be distinguished based on their size and morphology (**Figure 3.2)**.

Table 3.2 Concentration of viruses using positively charged filter

Optimisation of Methods (spiked)	Results
Combi Strip Test after centrifugation	
rotavirus	Positive
adenovirus	Positive
Combi Strip Test after filtration	
rotavirus	Positive
adenovirus	Positive
Transmission electron microscopy	
Positively charged filter	Viruses observed

The concentration step is based on adsorption of viral particles onto a positively charged nylon membrane. The capsid of viruses is generally considered negatively charged, hence it would be attracted to a positively charged membrane and viruses would therefore attach by adsorption to the positively charged membrane (Sobsey and Meschke, 2003). Beef extract was also added to enrich the virus. The elution step with a high pH buffer containing beef extract was optimised by Gilgen *et al*. (1997). Results showed that beef extract improved the recovery rate at least ten times with no inhibitory effects on RT-PCR (rotavirus) (Gilgen *et al.*, 1997). A limiting factor

when using filter membranes is the 0.45 µm pore size, since the isolation of the viruses depends on their adsorption onto the membrane. It may be difficult to isolate all the viruses present in a sample since virus particles are small (20-200 nm in diameter) (Langlet *et al*., 2009). In addition, the virion of the majority of enteric viruses consists of a nucleocapsid consisting of weak acids and base groups that are ionisable (Brown and Sobsey 2009). Factors controlling the adhesion of viruses include the type of viruses and their associated properties such as pH, ionic strength and the presence or absence of organic matter which may either absorb the viruses or compete with it for adsorption sites (Gerba, 1984; Straub and Chandler, 2003; Chu *et al*., 2003; John and Rose, 2005). Also, the Environmental Protection Agency (EPA) of the USA also advised optimising the pH based on the specific virus types and water tested, since waterborne viruses have different isoelectric points and adsorption-desorption behaviours (Lambertini *et al*., 2008; Gerba, 1984).

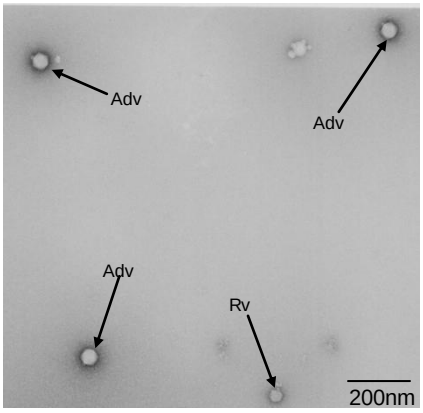

Figure 3.2 Transmission electron micrograph showing rotavirus (Rv) and adenovirus (Adv) after filtration through a positively charged filter (X30 000 magnification)

3.2.2.2.2 Concentration using Negatively Charged Filters

The negatively charged filter method was also tested using the Combi Strip Test at specific points in the procedure. Rotavirus and adenovirus antigens were present in the water after initial centrifugation and before filtering through the negatively charged filter. However, after filtration and micro–concentration, no rotavirus and adenovirus antigens were found in the concentrate that had passed through the negatively charged filter (**Table 3.3**). Furthermore, no viral particles could be seen when viewed using the TEM (**Figure 3.3)**. This could indicate that the virus particles did not pass through the filter, thus insufficient virus particles were present after filtration to be detected by either the Combi Strip antigen test or to be viewed by the TEM. While Haramoto *et al.* (2004) successfully used negatively charged filters to concentrate enteric viruses from tap water, the negatively charged filters must be rinsed with cations in order to

allow viruses to adsorb to these filters. During this procedure the negatively charged filters were rinsed with H_2SO_4 (pH3.0) to remove magnesium ions and other PCR inhibitory substances. Despite these additions, no viruses were detected post filtration (**Table 3.3**).

Table 3.3 Concentration of viruses using negatively charged filter

Optimisation of Methods (spiked)	Results
Combi Strip Test after centrifugation	
rotavirus	Positive
adenovirus	Positive
Combi Strip Test after filtration	
rotavirus	Negative
adenovirus	Negative
Transmission electron microscopy	
Negatively charged filter	No viruses observed

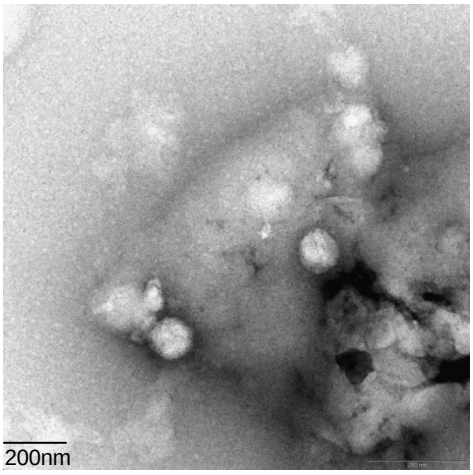

Figure 3.3 Transmission electron micrograph after filtration through a negatively charged filter (X30 000 magnification)

3.2.2.2.3 Concentration using Non–Charged Mixed–Ester Filters

The concentration of rotavirus and adenovirus particles was again tested using the Combi Strip Test at specific points in the method and compared to the above mentioned filter methods. It was found that rota- and adenovirus were present in the water after initial centrifugation and before filtering through the mixed-ester filter (**Table 3.4**). The viruses were also present in the concentrate that passed through the mixed–ester filter. Transmission electron microscopy further confirmed the presence of viral particles in the concentrate (**Figure 3.4**). The results thus indicated a successful concentration of viruses using the non-charged mixed-ester filter.

Similarly, Liu *et al.* (2007), also successfully employed non-charged mixed-ester filters to concentrate viruses from tap water.

Table 3.4 Concentration of viruses using non-charged mixed-ester filter

Optimisation of Methods (spiked)	Results
Combi Strip Test after centrifugation	
rotavirus	Positive
adenovirus	Positive
Combi Strip Test after filtration	
rotavirus	Positive
adenovirus	Positive
Transmission electron microscopy	
Mixed–ester filter	Viruses present

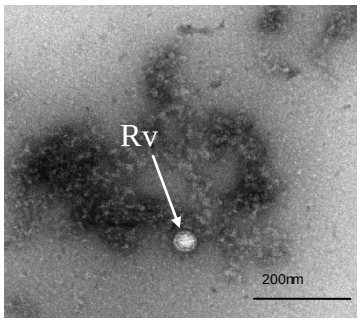

Figure 3.4 Transmission electron micrograph after filtration through a non-charged mixed-ester filter (X30 000 magnification)

For the pilot study, the best concentration methods for RNA/DNA viruses were the positively charged filter and the mixed-ester filter which yielded a positive antigen test result together with TEM micrographs. In contrast, the flocculation concentration method (SiO_2) and the negatively charged filter showed poor retrieval of the viruses. Numerous studies also preferred to use electropositive filters for virus concentration from water (Haramoto *et al*., 2005; Lambertini *et al*., 2008; Locas *et al*., 2008; Verheyen *et al*., 2009; etc.), with one study mentioning the preference of mixed-ester filters (Liu *et al*., 2007).

3.2.3 Viral Nucleic Acid Extraction

Various nucleic acid extraction methods were also employed to establish which method would provide optimum yields for both DNA and RNA nucleic acids. The extraction techniques included the TRIzol method (Invitrogen, California, USA) for RNA extraction, the Roche High Pure PCR Template Preparation kit (Roche, Mannheim, Germany) for DNA extraction, and the

QIAamp Ultrasens Virus kit (Qiagen GmbH, Hilden, Germany) for simultaneous RNA and DNA extraction.

3.2.3.1 TRIzol Method for Nucleic acid Extraction

Viral nucleic acid (rotavirus) were visualised on a 2% agarose gel after nucleic acid extraction using the TRIzol method for RNA. **Figure 3.5** shows the agarose gel of viral nucleic acids (rotavirus) extracted using TRIzol after concentration using the SiO_2 method, positively charged, negatively charged -, and mixed-ester filter methods. No viral RNA could be visualised when the TRIzol extraction method was employed for any of the concentration techniques. This might indicate that no viral RNA was extracted or the RNA concentration too low to be visualised on an agarose gel. In a study done by Triant and Whitehead (2009), different extraction methods were compared, namely the TRIzol method, Qiagen AllPrep DNA/RNA Micro kit, and the MagMax-96 Total RNA Isolation kit (Applied Biosystems/Ambion, Austin, Tx) for RNA and DNA extraction from tissue samples. This study found that high quality RNA was consistently produced with all the extraction methods, however, the TRIzol method employed by Triant and Whitehead (2009) had an additional step whereby they employed Qiagen RNeasy spin columns for higher nucleic acid yield. In addition, their TRIzol method was modified to extract DNA by adding several steps and by utilising a phenol phase and incubating overnight at -80ºC. The homogenate was then divided for use in a RNA extraction and DNA extraction protocol respectively, to prevent the spin columns from clogging due to the high viscosity of the buffer employed in their protocol. While Triant and Whitehead (2009) concluded that their modified TRIzol method delivered a high RNA and DNA yield, incorporation of more steps and long incubation periods may lead to errors.

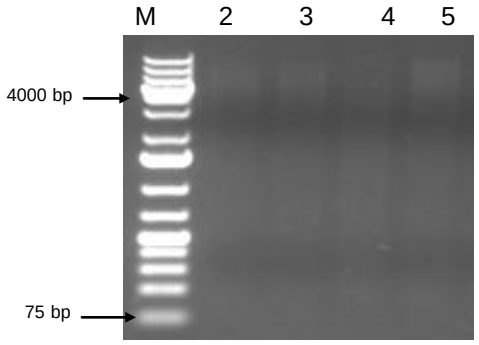

Figure 3.5 Total RNA extracted from spiked water samples using the TRIzol method. A 1kb Plus ladder was used as a molecular size marker (M) with the sizes indicated in base pairs. Total RNA was concentrated using the: SiO_2 method in lane 2, positively charged filter method in lane 3, negatively charged method in lane 4, and mixed-ester filter method in lane 5

3.2.3.2 Roche High Pure PCR Template Preparation Kit for nucleic acid extraction

Viral nucleic acid (adenovirus) was visualised on a 2% agarose gel after nucleic acid extraction using the Roche High Pure PCR Template Preparation kit for DNA extraction. **Figure 3.6** indicates the genomic nucleic acids (adenovirus) concentrated using the SiO_2 method, positively charged-, negatively charged-, and mixed-ester filter methods.

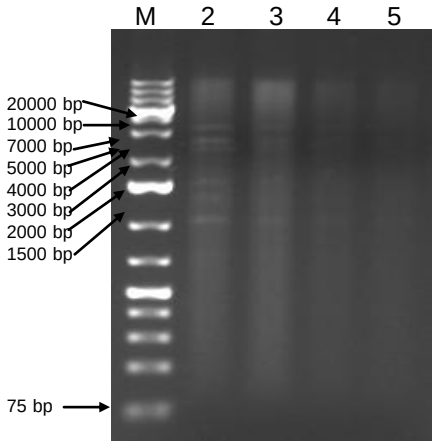

Figure 3.6 Genomic DNA extracted from spiked water samples using the Roche High Pure PCR Template Preparation kit. A 1kb Plus ladder was used as a molecular size marker (M) with the sizes indicated in base pairs. Water samples were concentrated using the: SiO_2 method in lane 2, positively charged filter method in lane 3, negatively charged method in lane 4, and mixed-ester filter method in lane 5

In **Figure 3.6** successful extraction of genomic DNA was observed when the SiO_2 – and positively charged filter concentration methods was used, while the negatively charged and mixed-ester filters showed faint genomic smears. In a study by Kok *et al*. (2000) different extraction methods for viral RNA and DNA were compared. Among them a High Pure PCR Template Preparation kit (Roche) and a QIAamp DNA mini kit (Qiagen) were used. The results as reported by Kok *et al*. (2000) showed similar yield and sensitivity when viral DNA was extracted with the High Pure PCR Template Preparation kit and the QIAamp DNA mini kit, respectively.

3.2.3.3 QIAamp Ultrasens Virus Kit for nucleic acid extraction

Viral nucleic acid (rota- and adenovirus) was visualised on a 2% agarose gel after using the QIAamp Ultrasens Virus Kit for DNA and RNA. **Figure 3.7** indicates the viral nucleic acids (A–RNA and B-DNA) concentrated using the SiO_2 method, positively charged-, negatively charged-, and mixed-ester filter methods, respectively.

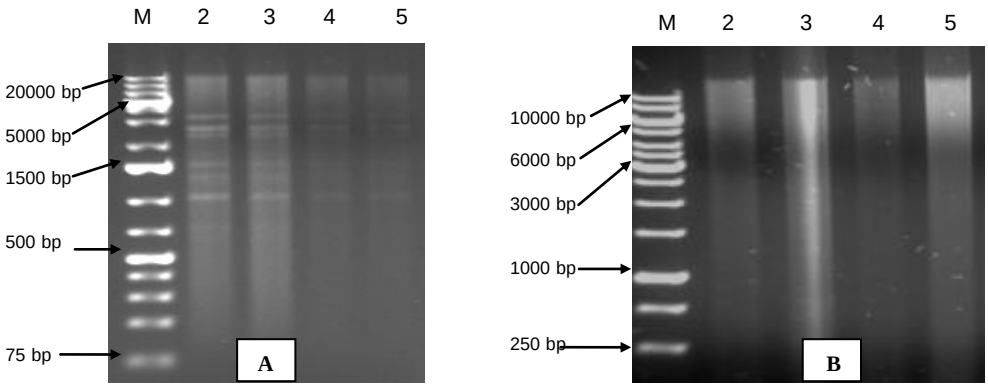

Figure 3.7 Rotavirus (A) and adenovirus (B) genomes extracted from spiked water samples using the QIAamp Ultrasens Virus kit. A 1kb Plus ladder (A) and a 1kb DNA ladder (B) was used as a molecular size marker (M) with the sizes indicated in base pairs. Total RNA and DNA was concentrated using the: SiO_2 method in lane 2, positively charged filter method in lane 3, negatively charged method in lane 4, and mixed-ester filter method in lane 5

Figure 3.7 indicates successful extraction of genomic RNA (A: rotavirus) and DNA (B: adenovirus) when the SiO_2 and positively-, negatively- and non-charged mixed-ester filter concentration methods were used. In **Figure 3.7 (A)** the total RNA extracted when the SiO_2/QIAamp method (lane 2), and the positively charged filter/QIAamp method (lane 3) yielded higher resolution than the RNA extracted with the negatively charged filter/QIAamp method (lane 4) and the mixed-ester filter/QIAamp method (lane 5). In contrast, in **Figure 3.7 (B)** the total DNA extracted showed improved resolution when the positively charged filter/QIAamp method (lane 3) and the mixed-ester filter/QIAamp method (lane 5) were used, while low intensity smears were visible when the SiO_2/QIAamp method (lane 2), and the negatively charged filter/QIAamp method (lane 4) were employed.

Four concentration methods and three extraction methods were assessed to determine the most suitable concentration and nucleic acid extraction combination. In a study by Burgener *et al*. (2003) different viral RNA extraction kits, namely the QIAamp Viral RNA Mini Kit (Qiagen), QIAamp UltraSens Virus Kit (Qiagen), NucliSens Isolation Kit (Organon Technica) and NucleoSpin RNA Virus F Kit (Machery-Nagel) were compared for the isolation of viral RNA from water samples. It was found that the detection limit was the lowest with the QIAamp Viral RNA Mini Kit, while the QIAamp UltraSens Virus Kit only had a better detection limit than the NucleoSpin RNA Virus F kit. According to literature, it was found that the QIAamp Ultrasens Virus kit also yielded superior results than the TRIzol method for nucleic acids (Barnes and Taylor, 2004). Overall, the QIAamp Viral RNA Mini Kit showed the best performance relating to sensitivity and low inhibitors, however this kit is specific for viral RNA while the QIAamp

UltraSens Virus Kit has the advantage that it can extract both viral DNA and RNA, simultaneously.

3.2.4 Polymerase Chain Reaction – Optimisation of Concentration and Extraction Methods

The concentrated and extracted RNA and DNA samples were tested for rotavirus and adenovirus by using RT-PCR and PCR, respectively. The SiO_2 method, positively charged, negatively charged, and mixed-ester filter methods were applied to obtain a concentrated virus sample. Each concentrated virus sample obtained was used to extract rotavirus RNA using the TRIzol method, adenovirus DNA using the Roche High Pure PCR Template Preparation kit and the QIAamp Ultrasens Virus kit, for simultaneous extraction of DNA and RNA. Complementary DNA was prepared from the rotavirus RNA (TRIzol method and QIAamp Ultrasens Virus kit) using both an oligo$(dT)_{15}$ primer and a gene-specific primer (reverse primer for rotavirus **Table 2.2**). The PCR for rotavirus was performed on each cDNA sample. The PCR for adenovirus was also performed from samples extracted using the Roche High Pure PCR Template Preparation kit as well as samples extracted from the QIAamp UltraSens Virus kit. All PCR products were sequenced and results confirmed by BLAST analysis.

3.2.4.1 PCR Results of Concentration and Extraction Methods

3.2.4.1.1 PCR from the Concentration using SiO_2 Method

For the rotavirus samples extracted using the TRIzol method **Figure 3.8,** no rotavirus was detected from cDNA when the oligo$(dT)_{15}$ primer was used (lane 2), however a positive 346 bp result was obtained when cDNA was made with a gene-specific primer (lane 3). For RNA samples extracted using the QIAamp Ultrasens Virus kit the PCR results for rotavirus illustrated in **Figure 3.8** indicated that rotavirus was detected when cDNA was prepared using both the oligo$(dT)_{15}$ (lane 4) primer and a gene specific primer (lane 5), using the SiO_2 concentration method.

A low intensity PCR product (261 bp) was detected for adenovirus when DNA samples were extracted using the Roche High Pure PCR Template Preparation kit (lane 2 of **Figure 3.9**), and when samples were extracted using the QIAamp Ultrasens Virus kit (lane 3 of **Figure 3.9**), using the SiO_2 concentration.

Silicon dioxide was used as a flocculation concentration method and the silicon dioxide/TRIzol method was used in the pilot study to concentrate and extract rotavirus from spiked water

samples, after which the samples were tested by PCR assay for virus presence. The RT-PCR results for rotavirus was negative when Oligo(dT)$_{15}$ primers were used but positive when gene-specific primers were used (**Figure 3.8**). This is most likely due to the higher sensitivity of the gene specific primer. As previously mentioned, while the Combi Strip test for rotavirus was positive before the addition of SiO_2 and $AlCl_3$ to the spiked water, no virus particles could be seen when the concentrate was viewed using the TEM. Hence, results obtained from this concentration/extraction method combination yielded less reliable results than filtration methods, possibly due to the multiple steps necessary to perform this technique, even though care was taken to follow the technique diligently.

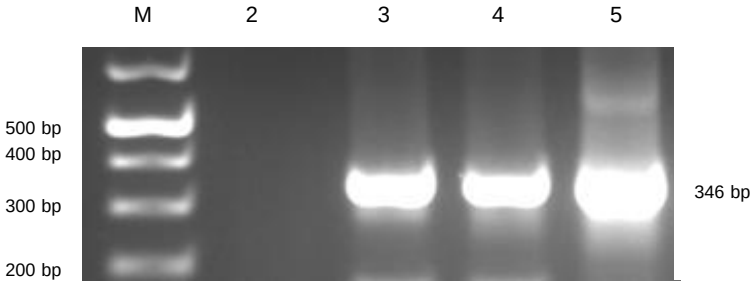

Figure 3.8 Gel electrophoresis (2%) of rotavirus PCR product after concentration of viruses using the SiO_2 method and extracted using the TRIzol method (lanes 2 and 3) and the QIAamp Ultrasens Virus kit (lanes 4 and 5). The cDNA sample of lane 2 and 4 was prepared with oligo(dT)$_{15}$, while the cDNA of lanes 3 and 5 were prepared with a gene-specific primer. A 1kb Plus ladder was used as a molecular size marker (M) with the sizes indicated in base pairs

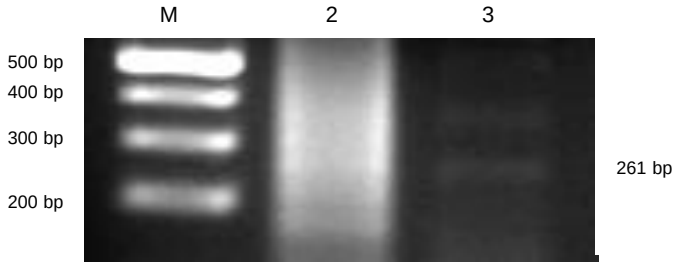

Figure 3.9 Gel electrophoresis (2%) of adenovirus PCR product after concentration of viruses using the SiO_2 method and extracted using the Roche High Pure PCR Template Preparation kit (lane 2) and the QIAamp Ultrasens Virus kit (lane 3). A 1kb Plus ladder was used as a molecular size marker (M) with the sizes indicated in base pairs

The QIAamp UltraSens Virus kit together with the silicon dioxide method yielded a 346 bp PCR product for rotavirus when cDNA was prepared with either the oligo(dT)$_{15}$ primer and the gene specific primer (**Figure 3.8**). These results showed that the QIAamp Ultrasens Virus kit had the ability to deliver sufficient quantities of RNA for detection by PCR when used with the SiO_2

method for detection of rotavirus. When the Roche High Pure PCR Template Preparation Kit was used together with the SiO_2 concentration method the PCR for adenovirus yielded a low intensity product (**Figure 3.9**). Similarly, while this method yielded the expected 261 bp PCR fragment for adenovirus (**Figure 3.9,** lane 3) when DNA was extracted using the QIAamp Ultrasens Virus kit, a faint band was observed. This indicates that either inadequate virus particles were concentrated with the SiO_2 method, or low concentrations of DNA were extracted from the concentrated sample.

3.2.4.1.2 PCR for Concentration using Positively Charged Filters

When spiked water samples were concentrated using positively charged filters, the rotavirus PCR of RNA samples extracted using the TRIzol method (**Figure 3.10**) was positive when cDNA was synthesised with both an oligo$(dT)_{15}$ primer (lane 2) and the gene-specific primer (lane 3) was used. Furthermore, samples extracted using the QIAamp Ultrasens Virus kit **(Figure 3.10)** indicated that rotavirus was detected when cDNA was prepared using oligo$(dT)_{15}$ (lane 4) and with a gene-specific primer (lane 5).

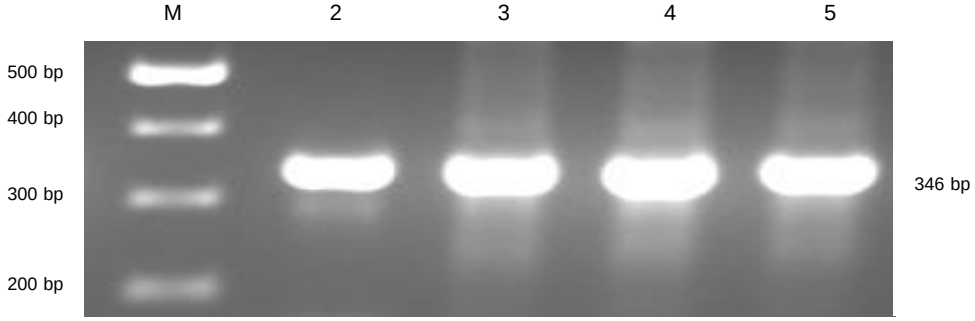

Figure 3.10 Gel electrophoresis (2%) of rotavirus PCR product after concentration of viruses using the positively charged method and extracted using the TRIzol method (lanes 2 and 3) and the QIAamp Ultrasens Virus kit (lanes 4 and 5). The cDNA sample of lane 2 and 4 was prepared with oligo$(dT)_{15}$, while the cDNA of lanes 3 and 5 were prepared with a gene-specific primer. A 1kb Plus ladder was used as a molecular size marker (M) with the sizes indicated in base pairs

After concentration using positively charged filters the polymerase chain reaction was also positive when DNA samples were extracted from adenovirus using the Roche High Pure PCR Template Preparation kit (**Figure 3.11**, lane 2) and the QIAamp Ultrasens Virus kit (**Figure 3.11**, lane 3).

The positively charged filter concentration method yielded consistent results. In the pilot study, the PCR results for rotavirus were positive when oligo$(dT)_{15}$ primers as well as gene-specific primers were used (**Figure 3.10**), as the expected 346 bp product for rotavirus was obtained. The expected 261 bp PCR product for adenovirus (**Figure 3.11**) was also obtained. Positive

results were also obtained for viral presence in both the pre- and post-filtration samples as tested with the Combi Strip test (**Table 3.2**) and confirmed by TEM (**Figure 3.2**). This indicated that the positive filter was better able to retain the viruses present in the water samples tested. Similarly, Haramoto *et al*. (2005) reported recovery of adenovirus from cation-coated filters in addition to the conventional positively charged filters. The results obtained in the current study thus show that the positively charged filter method was superior to the SiO_2 concentration technique since it yielded a positive PCR result for both viruses.

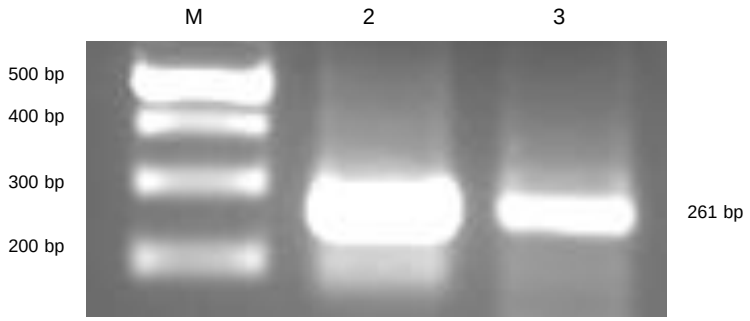

Figure 3.11 Gel electrophoresis (2%) of adenovirus PCR product after concentration of viruses using the positively charged filter method and extracted using the Roche High Pure PCR Template Preparation kit (lane 2) and the QIAamp Ultrasens Virus kit (lane 3). A 1kb Plus ladder was used as a molecular size marker (M) with the sizes indicated in base pairs

3.2.4.1.3 PCR for Concentration using negatively charged filters

The PCR of the rotavirus RNA samples extracted using the TRIzol method (**Figure 3.12)** indicated that this virus was not detected from cDNA when an oligo$(dT)_{15}$ primer (lane 2) or when a gene-specific primer was used (lane 3). In addition, for the RNA samples extracted using the QIAamp Ultrasens Virus kit (**Figure 3.12**), no rotavirus was detected when cDNA was prepared using oligo$(dT)_{15}$ (lane 4) primer. The PCR results were however positive, when a gene-specific primer (**Figure 3.12**, lane 5) was used to prepare the cDNA.

Figure 3.12 Gel electrophoresis (2%) of rotavirus PCR product after concentration of viruses using the negatively charged method and extracted using the TRIzol method (lanes 2 and 3) and the QIAamp Ultrasens Virus kit (lanes 4 and 5). The cDNA sample of lanes 2 and 4 were prepared with oligo$(dT)_{15}$,

while the cDNA of lanes 3 and 5 were prepared with a gene-specific primer. A 1kb Plus ladder was used as a molecular size marker (M) with the sizes indicated in base pairs

After concentration using negatively charged filters, positive adenovirus PCR results were obtained when DNA was extracted from spiked water samples using the Roche High Pure PCR Template Preparation kit (**Figure 3.13**, lane 2) while a low intensity adenovirus PCR product was formed when the QIAamp UltraSens Virus kit (**Figure 3.13**, lane 3) was used, in conjunction with the negatively charged filter.

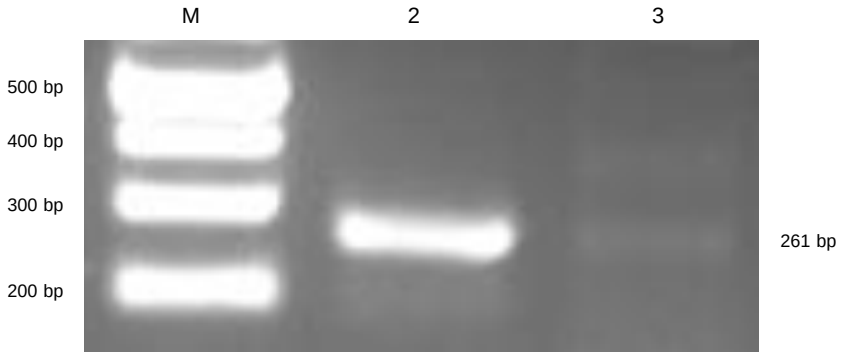

Figure 3.13 Gel electrophoresis (2%) of adenovirus PCR product after concentration of viruses using the negatively charged filter method and extracted using the Roche High Pure PCR Template Preparation kit (lane 2) and the QIAamp Ultrasens Virus kit (lane 3). A 1kb Plus ladder was used as a molecular size marker (M) with the sizes indicated in base pairs

In the pilot study, rotavirus antigens was shown to be present in the pre-filtration samples when using the negatively charged filter/TRIzol method but not in the post-filtration samples (**Table 3.3**), which indicated a loss of virus particles upon filtration. The absence of post-filtration rotavirus was confirmed by TEM (**Figure 3.3)**. These findings showed that insufficient virus particles passed through the filter. The PCR results for rotavirus testing was however positive when gene specific primers were used on RNA extracted using the QIAamp UltraSens Virus kit but negative for when a gene-specific primer was used for TRIzol extracted RNA and when oligo$(dT)_{15}$ primers (**Figure 3.12**) were used on all RNA sample. Research has shown that to improve the recovery yield of viruses from negatively charged filters, adjustment of the pH (Ma *et al.,* 1994; Haramoto *et al.,* 2004) and/or the addition of cations such as $MgCl_2$ or $AlCl_3$ (Ma *et al.,* 1994) might optimise virus adsorption. However, these techniques may limit the application of these electronegative filters as acidifying the water may cause the filter to clog due to the presence of humic acid. In waters with high turbidities and organic matter, high adsorption and recovery efficiencies may be lost due to the viruses being associated with solids, which may then compete for adsorption sites (Gerba, 1984; Sobsey and Meschke, 2003).

Positively charged filters offer an advantage over negatively charged filters, as preconditioning of the water sample is not required. Positively charged filters have also shown great versatility for concentrating bacteria and endotoxins as well as viruses (Hou *et al*., 1980). Due to the inherent difficulties associated with negatively charged filters, Rose *et al.* (1984) proposed that electropositive filters should be used to detect viruses in water environments.

3.2.4.1.4 PCR for Concentration using Non–Charged Mixed–Ester Filters

When concentration of virus particles using non-charged mixed-ester filters were used the rotavirus PCR of RNA extracted using the TRIzol method identified rotavirus with the oligo$(dT)_{15}$ primer prepared cDNA (**Figure 3.14**, lane 2). Positive results were also observed when cDNA prepared with a gene-specific primer was used (lane 3). **Figure 3.14** also indicates the presence of rotavirus with the QIAamp Ultrasens Virus kit extracted RNA with oligo$(dT)_{15}$ primer prepared cDNA (lane 4) and a gene-specific primer (lane 5), respectively.

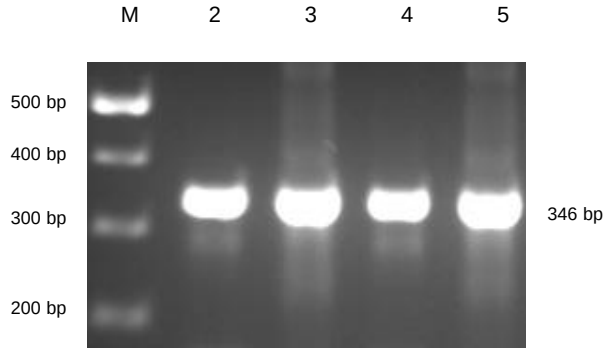

Figure 3.14 Gel electrophoresis (2%) of rotavirus PCR product after concentration of viruses using the mixed-ester filter method and extracted using the TRIzol method (lanes 2 and 3) and the QIAamp Ultrasens Virus kit (lanes 4 and 5). The cDNA sample of lane 2 and 4 was prepared with oligo$(dT)_{15}$, while the cDNA of lanes 3 and 5 were prepared with a gene-specific primer. A 1kb Plus ladder was used as a molecular size marker (M) with the sizes indicated in base pairs

The PCR procedure also yielded positive results when DNA samples were extracted from adenovirus using the Roche High Pure PCR Template Preparation kit (**Figure 3.15** lane 2) and the QIAamp Ultrasens Virus kit (**Figure 3.15**, lane 3), after concentration of virus particles using non-charged mixed-ester filters.

The PCR assay for rotavirus was positive when both Oligo$(dT)_{15}$ and gene-specific primers were used (**Figure 3.14**), showing that fragments with sufficient quantity and integrity was concentrated and amplified with the mixed-ester filter/TRIzol method. In addition, the presence of virus particles was confirmed with TEM (**Figure 3.4**) as well as with an antigen test (Coris

Combi strip test) which was used to detect the two different virus particles (**Table 3.4**). Therefore, similar to findings with the positively charged filter, the mixed-ester filter together with either the TRIzol extraction method or the QIAamp Ultrasens Virus kit yielded positive results for rotavirus in all samples tested by RT-PCR (**Figure 3.14**).

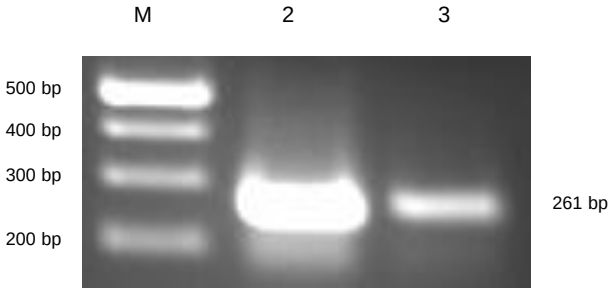

Figure 3.15 Gel electrophoresis (2%) of adenovirus PCR product after concentration of viruses using the mixed-ester filter method and extracted using the Roche High Pure PCR Template Preparation kit (lane 2) and the QIAamp Ultrasens Virus kit (lane 3). A 1kb Plus ladder was used as a molecular size marker (M) with the sizes indicated in base pairs

The method to concentrate viruses from water using the mixed-ester filter used in this study was developed by Liu *et al.* (2007) who concluded that this concentration method has a high ability to recover viruses from water samples and provides certain advantages over the use of the positively charged filter. In this method, the viruses are adsorbed and enveloped by an inorganic flocculate in contrast to the adsorption onto a positively charged filter. Liu *et al.* (2007) reported, while the release from the positively charged filter by elution with beef extract may be incomplete, the dissolution with a citrate buffer from the mixed-ester filter is sufficient and rapid. The detection limit of the mixed-ester filter method of one litre drinking water seeded with the virus is one RT-PCR unit which equalled to a 10^{-6} dilution, which gave this method a five-fold higher sensitivity than the positively charged filter method (Liu *et al.,* 2007). However, while Liu *et al*. (2007) concluded that non-charged mixed-ester filters were optimum for high yield viral concentration, numerous other studies utilised positively charged filters for the concentration of viral particles from diverse sample sources (Haramoto *et al*., 2005; Lambertini *et al*., 2008; Locas *et al*., 2008; Verheyen *et al*., 2009).

3.2.5 Summary: Pilot Study

The pilot-study showed that the best concentration methods were the positively charged filter as well the mixed-ester filter which yielded more consistent resolution of RNA/DNA genomic fragments and PCR results than both the flocculation concentration method (SiO_2) and the negatively charged filter which showed poor retrieval of the virus (**Table 3.4.1** and **Table 3.4.2**). The more efficient nucleic acid extraction from these concentrated viral samples was obtained

with the QIAamp UltraSens virus kit which could be utilised to extract DNA and RNA nucleic acids simultaneously, while both the TRIzol extraction method and the Roche High Pure PCR Template Preparation Kit could only extract either RNA or DNA respectively. These findings showed that using either the positively charged filter or the mixed-ester filter together with the QIAamp UltraSens virus kit would be the best option to use when investigating virus presence in river water. Numerous studies preferred to use electropositive filters for virus concentration from water (Haramoto *et al*., 2005; Lambertini *et al*., 2008; Locas *et al*., 2008; Verheyen *et al*., 2009; etc). For this reason the positively charged filter/QIAamp UltraSens Virus kit combination was applied when analysing river water samples (Section B).

Table 3.4.1 Comparison of results for concentration and extraction methods for RNA RT-PCR

	Oligo(dT)$_{15}$ primers	**Gene specific primer**
TRIzol extraction		
SiO_2	-ve	+ve
+ve Filter	+ve	+ve
-ve Filter	-ve	-ve
Mixed-ester Filter	+ve	+ve
QIAamp UltraSens Virus kit		
SiO_2	+ve	+ve
+ve Filter	+v e	+ve
-ve Filter	-ve	+ve
Mixed-ester Filter	+ve	+ve

Table 3.4.2 Comparison of results for concentration and extraction methods for DNA PCR

	Roche High Pure PCR Template Preparation Kit	**QIAamp UltraSens Virus kit**
SiO_2	faint	faint
+ve Filter	+ve	+ve
-ve Filter	-ve	faint
Mixed-ester Filter	+ve	faint

SECTION B

3.3 Environmental Water Samples Analysis

The presence of rotavirus, adenovirus and enterovirus was tested at strategic points along the Plankenburg River (Stellenbosch) as indicated in **Figure 2.2**; Site A (agricultural farming and residential areas) represented the site before the possible point source of pollution, while Site B (informal settlement of Kayamandi) represented the site closest to the pollution source and Site C (industrial area substation) was situated downstream from the pollution source. Sampling sites along the Berg River (Paarl) are indicated in **Figure 2.3** and included**;** Site A – before point source of pollution (agricultural farming area), Site B – at point source of pollution (Mbekweni informal settlement) and Site C – after point source of pollution (Newton pumping station). The Newton pumping station services the residential area of Newton as well as certain sections of Mbewkeni informal settlement, which borders the Paarl/Wellington, Boland region. Samples collected from the Plankenburg River were labelled 1-36 (three samples per month for 12 months), while surface water samples collected from the Berg River were labelled 37-72 (three samples per month for 12 months).

The results from the pilot study indicated that the positively charged filter method and the mixed-ester filter method were most suitable and comparable for the concentration of virus particles. However, many authors reported using positively charged filters for routine virus concentration (Haramoto *et al*., 2005; Lambertini *et al*., 2008; Locas *et al*., 2008; Verheyen *et al*., 2009; etc.) and this method was incorporated in the present study. The most suitable nucleic acid extraction procedure was the QIAamp Ultrasens Virus kit due to its ease of use and it being able to extract both DNA and RNA from environmental water samples. Furthermore, the optimum procedure to obtain complementary DNA from the RNA samples was the utilisation of gene specific primers instead of an Oligo$(dT)_{15}$ primer. The QIAamp UltraSens Virus kit and gene specific primers were then used in the analyses of the water samples collected from the above mentioned river systems.

3.3.1 Detection of Viral Antigens and Particles using the Positively Charged Filters

The results for the Combi-Strip analyses, which tested for the presence of antigens, as well as the TEM analyses for all water samples collected from the Plankenburg- and Berg River systems during the sampling period are represented in **Table 3.5.** The river water collected at the respective study sites were tested for rotavirus and adenovirus antigens after centrifugation, but before filtration, and again after filtration using the positively charged filters. Negative rotavirus and adenovirus antigen results were obtained for all 72 samples collected from both river

systems, before and after filtration, using the Combi-Strip technique (**Table 3.5**). However, when viewed under the TEM the filtered water sample concentrate revealed the presence of viruses in all 72 samples (collected from both river systems) tested (**Table 3.5**) as indicated by the representative TEM micrograph for the water sample collected from the At site, Berg River, in October 2005 (**Figure 3.16**).

Table 3.5 Presence of viruses in environmental water samples collected from August 2005 to July 2006 from the Plankenburg- and Berg River systems.

Application of Methods	Results
Combi Strip Test after centrifugation	
rotavirus	Negative on all 72 river water samples
adenovirus	Negative on all 72 river water samples
Combi Strip Test after filtration	
rotavirus	Negative on all 72 river water samples
adenovirus	Negative on all 72 river water samples
Transmission electron microscopy	
Positively charged filter	Viral particles present in all 72 river water samples

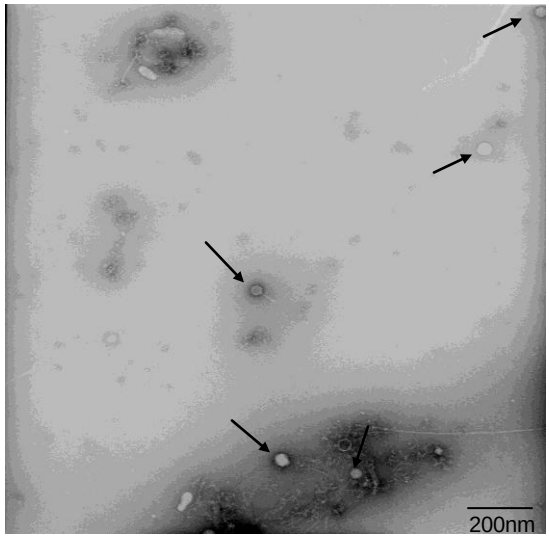

Figure 3.16 Transmission electron micrograph for the At site, Berg River (October 2005) with arrows indicating virus particles micro–concentrated from water after filtration through a positively charged filter (X30 000 magnification)

3.3.2 QIAamp Ultrasens Virus Kit for Nucleic Acid Extraction

Lanes 1-20 (**Figure 3.17**) are representative samples of nucleic acids extracted from water collected from the Plankenberg River during the study period using the QIAmp Ultrasens Virus kit. As indicated this kit can be utilised for the simultaneous extraction of DNA and RNA from diverse samples.

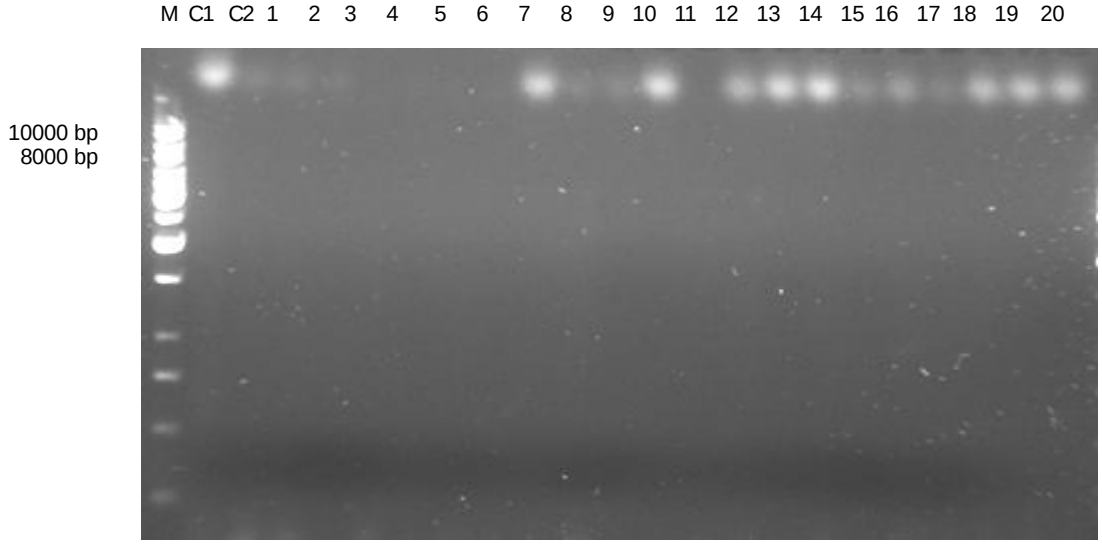

Figure 3.17 Example of genomic nucleic acids (DNA and RNA) extracted from environmental water samples with the QIAmp Ultrasens Virus Kit. A 1kb ladder was used as a molecular size marker (M) with the sizes indicated in base pairs. Lane C1, rotavirus positive control; lane C2, adenovirus positive control, lanes 1-20, environmental water samples extracted with QIAamp Ultrasens Virus Kit.

The nucleic acids extracted from the river water did not show distinguishable bands (**Figure 3.17**). This may be due to low concentrations of viruses that appear in environmental waters (Bosch *et al.,* 2008), as viruses are able to survive within the water but not replicate. Thus the concentration of nucleic acid in the river water samples may have been too low to be detected on an agarose gel.

3.3.3 Testing for Virus Presence using PCR

3.3.3.1 Rotavirus

The PCR products were loaded onto the gel according to their sample numbers, namely, lanes 1 to 36 refers to water samples collected from the Plankenburg River over the 12-month sampling period, while the water samples collected from the Berg River over the sampling period were loaded in lanes 37-72 (**Figures 3.18–3.21**). The expected PCR product of 346 bp for rotavirus was absent in all 72 river water samples analysed for both river systems. Prominent PCR products seen on the gel electrophoresis (510 to 750 bp), were extracted from the gel using the Minelute Gel Extraction kit (Qiagen, Hilden, Germany). This PCR band range of 510 to 750 bp

was sent for sequencing at the Stellenbosch University, DNA Sequencing Facility. The results were analysed using the BLAST program (Altschul *et al*., 1997) where the sequences were compared to similar sequences within the NCBI database. No similarity to any rotavirus genome was found, however diverse hits, ranging from HIV to human virus, were recorded. The primers were then compared to the database on NCBI and they were confirmed as the correct primer set for rotavirus.

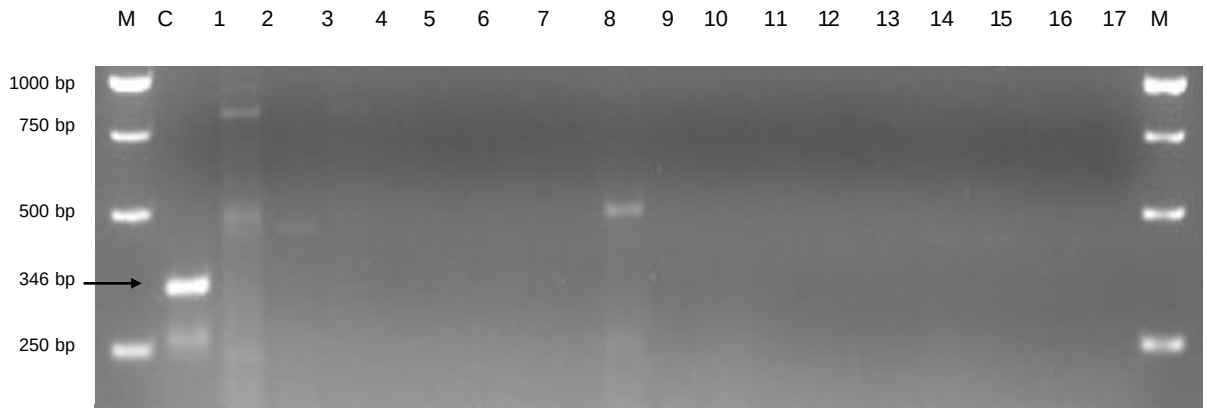

Figure 3.18 Gel electrophoresis (2%) of rotavirus PCR. A 1kb ladder was used as a molecular size marker (M) with the sizes indicated in base pairs. Lane C is the rotavirus control while lanes 1–17 corresponds to water samples collected from the Plankenburg River over the 12-month sampling period.

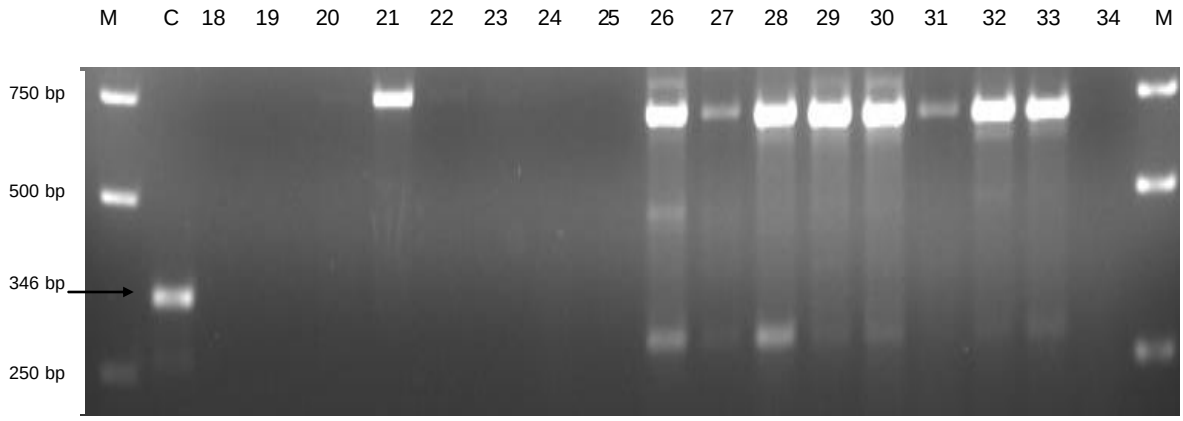

Figure 3.19 Gel electrophoresis (2%) of rotavirus PCR. A 1kb ladder was used as a molecular size marker (M) with the sizes indicated in base pairs. Lane C is the rotavirus control while lanes 18–34 corresponds to water samples collected from the Plankenburg River over the 12-month sampling period.

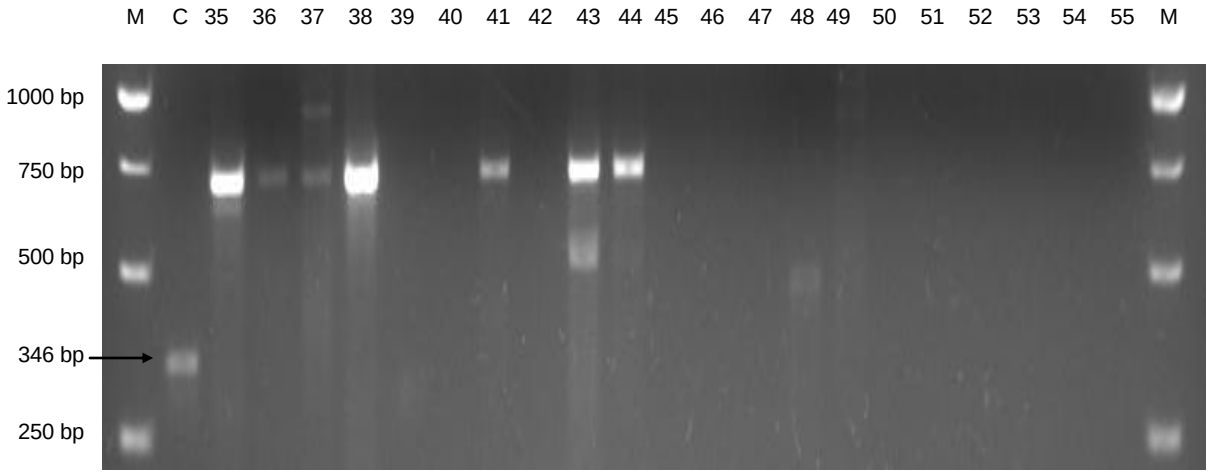

Figure 3.20 Gel electrophoresis (2%) of rotavirus PCR. A 1kb ladder was used as a molecular size marker (M) with the sizes indicated in base pairs. Lane C is the rotavirus control while lanes 35-36 corresponds to water samples collected from the Plankenburg River (12-month period) and lanes 37–55 correspond to water samples collected from the Berg River over the 12-month sampling period.

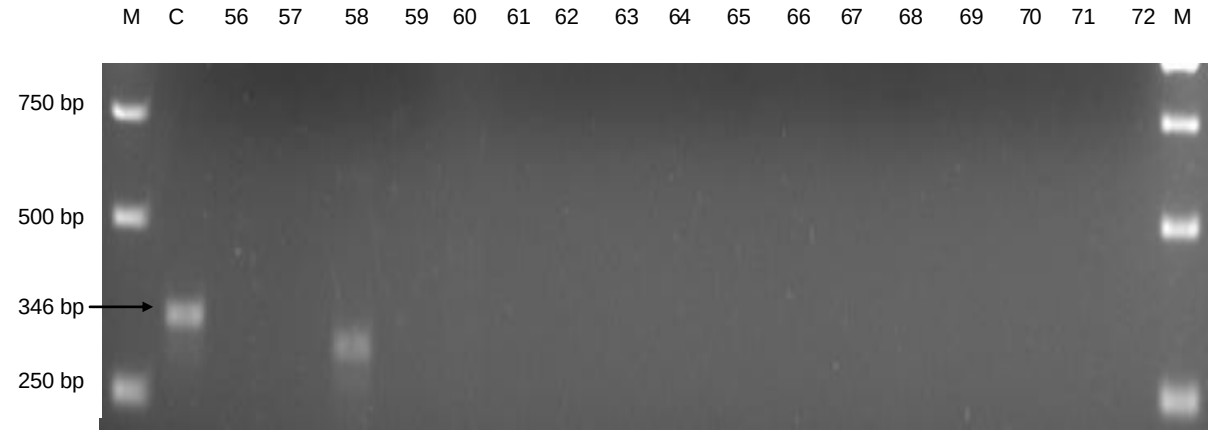

Figure 3.21 Gel electrophoresis (2%)of rotavirus PCR. A 1kb ladder was used as a molecular size marker (M) with the sizes indicated in base pairs. Lane C is the rotavirus control while lanes 56–72 corresponds to water samples collected from the Berg River over the 12-month sampling period.

The absence of rotaviruses in surface water samples was in contrast to previous results obtained by other groups who were able to detect rotaviruses (Abbaszadegan *et al.*, 1999; Kittigul *et al.*, 2005; Miagostovich *et al.,* 2008) in water samples. This difference might be explained by virus characteristics such as, resistance to environmental influences and/or fewer circulating viruses in the study area. Borchardt *et al.* (2003) conducted a study in the USA where private household wells were tested for the presence of enteric viruses. Eight percent of the 50 wells tested were positive for hepatitis A virus, rotavirus and enterovirus. In a separate study Abbaszadegan *et al*. (1993) analysed 150 samples from municipal wells in 35 USA states and found that approximately 30%, 14%, and 9% were positive for enteroviruses, rotavirus, and hepatitis A virus, respectively. In the current study, the possibility exists that the virus was present at too low copy numbers to have been effectively detected within the water supplies.

3.3.3.2 Adenovirus

The PCR products for adenovirus detection were also loaded onto the gel according to their sample numbers, namely, lanes 1 to 36 refers to water samples collected from the Plankenburg River over the 12-month sampling period, while the water samples collected from the Berg River over the sampling period were loaded in lanes 37-72 (**Figures 3.22 – 3.25)**. In contrast to the PCR results obtained for rotavirus, the expected product of 261 bp for adenovirus was detected in 22 (30.5%) samples collected throughout the study period. Fifteen of the 22 adenovirus positive samples were found in the Plankenburg River (distributed over all sites), while seven of the 22 adenovirus positive samples were found in the Berg River (all sites). The PCR products viewed by gel electrophoresis were extracted from the gel using the Minelute Gel Extraction kit (Qiagen, Hilden, Germany). The cleaned PCR product was sent for sequencing at the Stellenbosch University, DNA Sequencing Facility. The results were analysed using the BLAST program where the sequences were compared to similar sequences within the NCBI database (Altschul *et al*., 1997). For all 22 positive PCR samples, the BLAST analyses revealed a 94-100% sequence similarity to human adenovirus type 40.

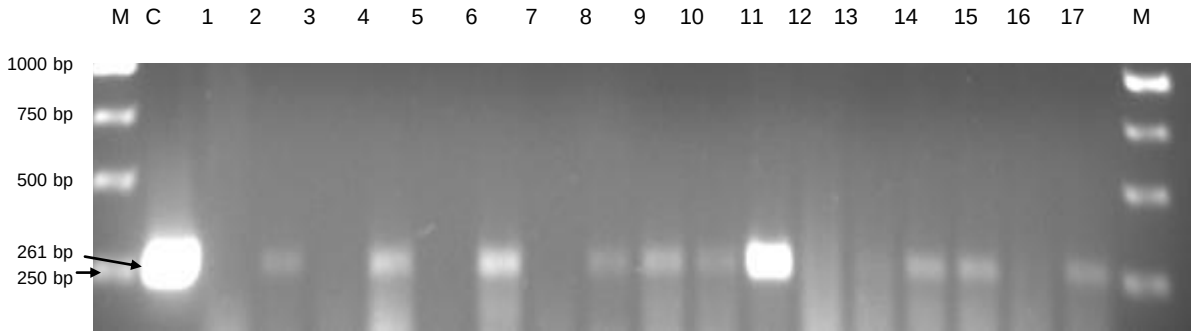

Figure 3.22 Gel electrophoresis (2%) of adenovirus PCR. A 1kb ladder was used as a molecular size marker (M) with the sizes indicated in base pairs. Lane C is the adenovirus control while lanes 1-17 corresponds to water samples collected from the Plankenburg River over the 12-month sampling period.

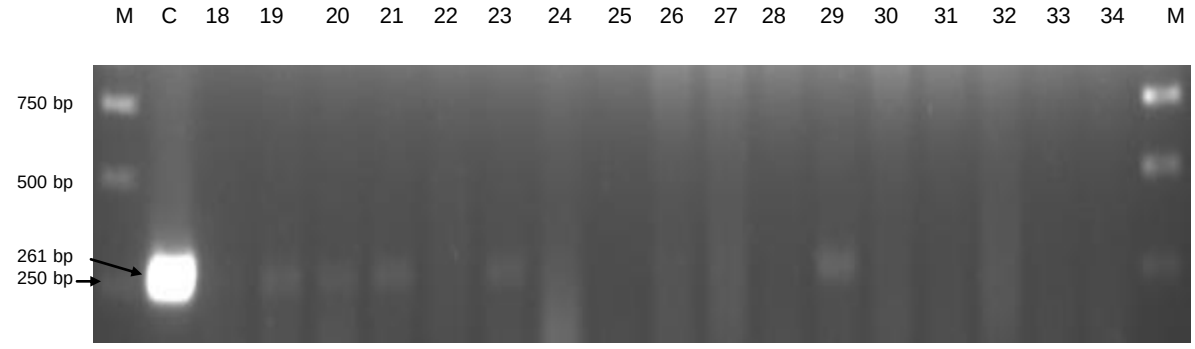

Figure 3.23 Gel electrophoresis (2%) of adenovirus PCR. A 1kb ladder was used as a molecular size marker (M) with the sizes indicated in base pairs. Lane C is the adenovirus control while lanes 18-34 corresponds to water samples collected from the Plankenburg River over the 12-month sampling period.

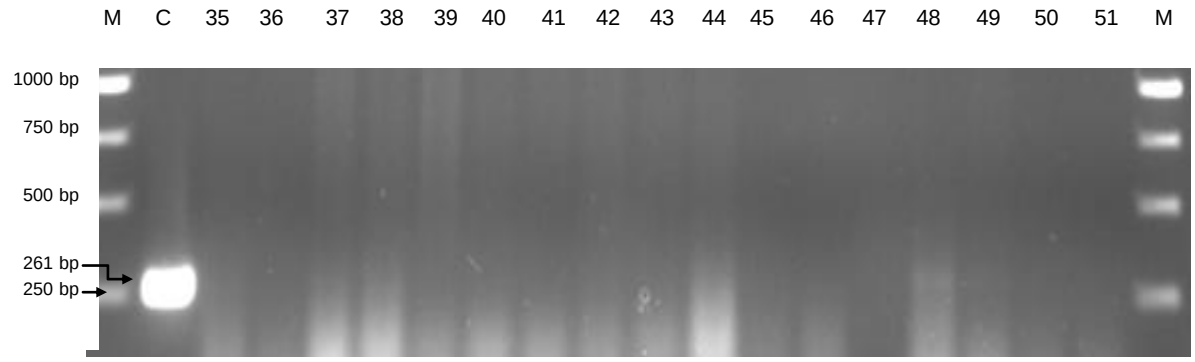

Figure 3.24 Gel electrophoresis (2%) of adenovirus PCR. A 1kb ladder was used as a molecular size marker (M) with the sizes indicated in base pairs. Lane C is the adenovirus control while lanes 35-36 corresponds to water samples collected from the Plankenburg River (12-month period) and lanes 37–51 correspond to water samples collected from the Berg River over the 12-month sampling period.

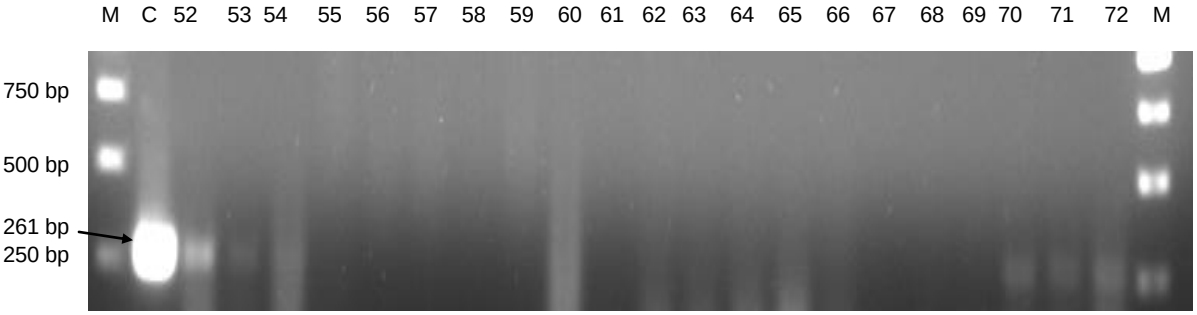

Figure 3.25 Gel electrophoresis (2%) of adenovirus PCR. A 1kb ladder was used as a molecular size marker (M) with the sizes indicated in base pairs. Lane C is the adenovirus control while lanes 52–72 corresponds to water samples collected from the Berg River over the 12-month sampling period.

These findings are in agreement with other studies which showed that adenovirus is present in environmental waters (van Heerden *et al.,* 2005; Jothikumar, 2005; Pickering *et al*., 2006) as well as in surface water sources (Jiang *et al.,* 2001; Jiang *et al*., 2000; Verheyen *et al.,* 2009). A 30.5% detection frequency of adenovirus in the river water samples as obtained in the present study correlated to a study conducted by Van Heerden *et al*. (2003) who detected on average 43% adenovirus frequency in a different river system within South Africa. In Van Heerden *et al*. (2003) the authors used a primary glass wool concentration method and a secondary polyethylene glycol concentration step. In addition, after concentration the authors isolated adenovirus using cell monolayers and later extracted the DNA with a Roche High Pure Nucleic Acid Kit. It is important therefore to note that the incidence of adenovirus in river waters reported in the current study and the Van Heerden *et al*. (2003) investigation motivates for similar studies to be conducted in drinking water, dam water used for recreational purposes as well as rainwater, which is gaining popularity as a sustainable water source.

Verheyen *et al.* (2009) indicated that the presence of latrines within a 50 m radius of testing sites was a significant risk factor for viral contamination of pumps and wells. This distance was corroborated by findings of an outbreak of hepatitis A virus, which occurred due to a leaking sewage tank located 60 m from public water wells. It has also been hypothesised that under optimised conditions, viruses can travel in surface water and crevices at distances greater than 1000 m (Robertson and Edberg, 1997). Although the risk of microbiological contamination through latrines in the vicinity of drinking water sources is recognised in developed first-world countries, it is not broadly recognised in most developing third-world countries (Robertson and Edberg, 1997; De Serres *et al.*, 1999). In the current investigation however, the sampling sites for both the Plankenburg- and Berg Rivers were located before, at and after an identifiable point source of pollution, where storm water (containing sewage effluent etc.) from a bordering informal settlement flowed into the river systems (**Figures 2.2 and 2.3**). The other sampling sites also border agricultural, residential and industrial areas. In this particular case, the storm water and other effluent sources, containing faecal matter, among other contaminants, could possibly have served as a source of viral contamination.

3.3.3.3 Enterovirus

A nested PCR was used to detect enterovirus in the river water samples collected from both river systems throughout the study period. The PCR products for intitial enterovirus detection were loaded onto the gel according to their sample numbers, namely, lanes 1 to 36 refers to water samples collected from the Plankenburg River over the 12-month sampling period, while the water samples collected from the Berg River over the sampling period were loaded in lanes 37-72 (**Figures 3.26–3.29**). In the first round of the enterovirus PCR 15 river water samples (at various sites for both river systems) yielded a faint 513 bp product. Another prominent PCR product was also seen on the gel electrophoresis (360 bp) and was extracted from the gel using the Minelute Gel Extraction kit (Qiagen, Hilden, Germany). This 360 bp PCR product was sent for sequencing at the Stellenbosch University, DNA Sequencing Facility. The results were analysed using the BLAST program where the sequences were compared to similar sequences within the NCBI database (Altschul *et al*., 1997). No similarity to any enterovirus strain in the database was obtained. Further amplification by nested PCR then yielded 13 (18.1%) positive nested PCR products of 297 bp (**Figures 3.30)**. The prominent nPCR products seen on the agarose gel were extracted from the gel using the Minelute Gel Extraction kit (Qiagen, Hilden, Germany). The cleaned PCR product was sent for sequencing at the Stellenbosch University, DNA Sequencing Facility. The results were then confirmed using the BLAST program where the

sequences were compared to similar sequences within the NCBI database (Altschul *et al*., 1997).

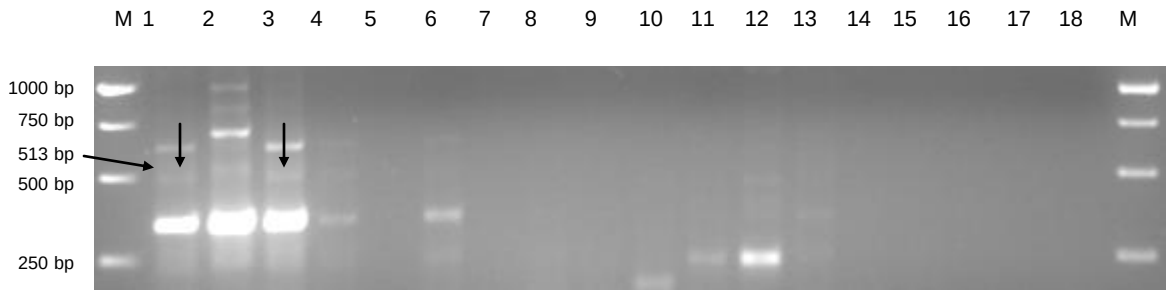

Figure 3.26 Gel electrophoresis (2%) of enterovirus PCR. A 1kb ladder was used as a molecular size marker (M) with the sizes indicated in base pairs. Lanes 1-18 corresponds to water samples collected from the Plankenburg River over the 12-month sampling period.

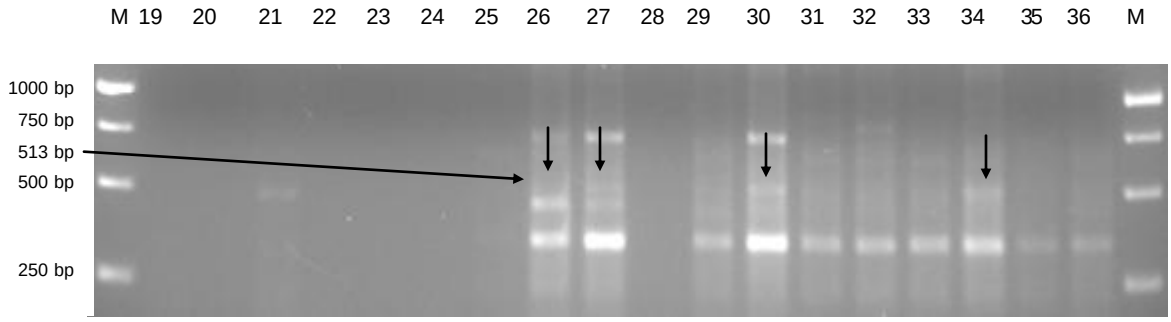

Figure 3.27 Gel electrophoresis (2%) of enterovirus PCR. A 1kb ladder was used as a molecular size marker (M) with the sizes indicated in base pairs. Lanes 19-36 corresponds to water samples collected from the Plankenburg River over the 12-month sampling period.

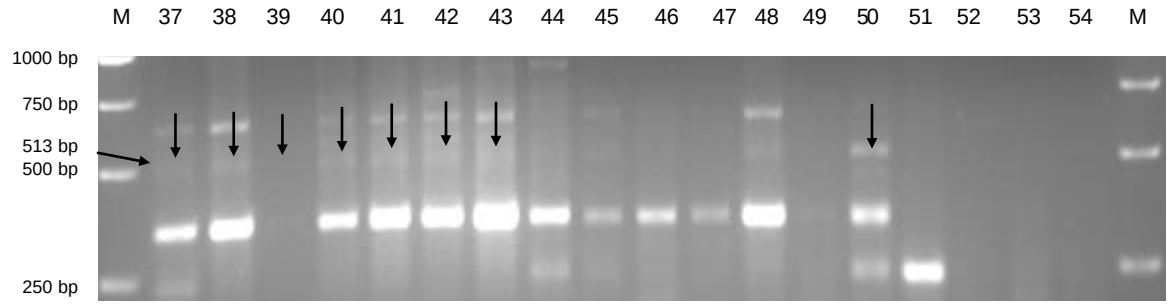

Figure 3.28 Gel electrophoresis (2%) of enterovirus PCR. A 1kb ladder was used as a molecular size marker (M) with the sizes indicated in base pairs. Lanes 37-54 corresponds to water samples collected from the Berg River over the 12-month sampling period.

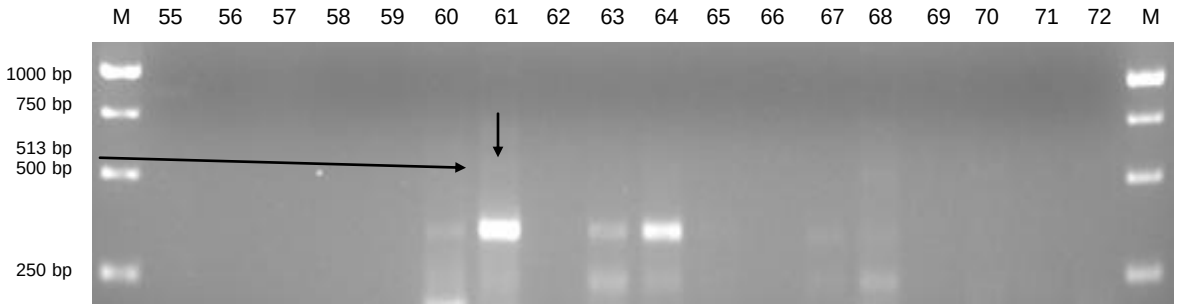

Figure 3.29 Gel electrophoresis (2%) of enterovirus PCR products. A 1kb ladder was used as a molecular size marker (M) with the sizes indicated inbase pairs. Lanes 55-72 corresponds to water samples collected from the Berg River over the 12-month sampling period.

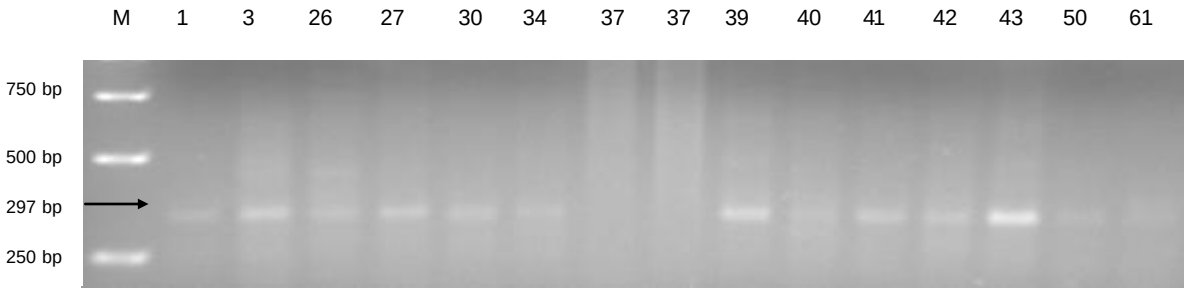

Figure 3.30 Gel electrophoresis (2%) of enterovirus nPCR products. A 1kb ladder was used as a molecular size marker (M) with the sizes indicated in base pairs. Enterovirus strains obtained by nested PCR for both river systems are loaded in the respective lanes.

Ehlers *et al*. (2005) conducted a study in South Africa from July 2000 to June 2002 in sewage, river-, borehole-, spring- and dam water as well as in drinking water supplies. The purpose of the study was to detect enterovirus in both treated and untreated water. The authors found that enterovirus was present in 42.5% of sewage, 28.5% of river-, 26.7% of dam-/spring-, 25.3% of borehole-water and 18.7% of drinking water. Grabow *et al*. (2000) and Payment *et al*. (1997) reported similar results in their respective studies. Enteroviruses detected by Ehlers *et al.* (2005) in treated and untreated surface water were also culturable (tissue culture techniques) and were thus classified as potentially infectious, indicating a potential health risk. Results from this study therefore showed that enterovirus presence was slightly lower (21%) than was reported by Ehlers *et al*. (2005) (28.5%) for river water in South Africa. Therefore, results showed that while adenovirus presence was increased, that of enteroviruses were slightly lower than previously reported. However, it is known that enteroviruses may be outnumbered by other viruses such as adenoviruses, reoviruses and caliciviruses and that while these viruses may not be readily detectable (Grabow *et al*., 2000), they may well compete for occupancy in the rivers tested.

3.4 Additional factors that may have influenced detection of viral presence

Tables 3.6 and 3.7 summarises the main results for the detection of rota-, adeno- and enteroviruses in the Plankenburg- and Berg River systems, respectively. In addition, the pH and temperature obtained for both river systems throughout the study period is also listed. The pH and temperature in the Plankenburg River (**Table 3.6**) ranged from 6 to 8.4 and 11°C to 28°C, respectively, while the pH and temperature in the Berg River system (**Table 3.7**) ranged from 5.9 to 8.5 and 10.5°C to 25°C, respectively. Research has shown that, depending on temperature, pH and salinity of the experimental conditions, viruses such as rotaviruses, can persist for several weeks. In addition, increased water temperatures lead to decrease virus persistence (Brown *et al.*, 2009; Lebarbenchon *et al.*, 2011). Lebarbenchon *et al.* (2011) also showed that viruses could still be detected in water one year after inoculation at temperatures as low as 4°C. In a study done by Nazir *et al*. (2010), where the pathogenecity of viruses at temperatures ranging from -10°C to 30°C was tested, it was found that viruses lose their infectivity at higher temperatures. In a separate study Guan *et al.* (2003) also showed that the electrostatic adsorption was sensitive to water pH. In addition, factors that were determined to have a statistically significant effect on waterborne virus survival included pH from 6.0 to 7.8, temperatures from 4 to 37°C, chloride from 0.5 to 16.3 mg/l, total organic carbon from 1 to 17 mg/l, water hardness from 29 to 339 mg/l, calcium carbonate and turbidity from 2.5 to 36 nephelometric turbidity units (NTU) (John and Rose 2005). Another factor influencing the detection of viruses is their isoelectric point, which refers to the point when there is no net surface charge on the virus (Sobsey and Meschke, 2003). The degree to which viruses will adsorb to soil particles or filter membranes will vary since the isoelectric points also vary to a large degree even between strains of a particular virus type (Sobsey and Meschke, 2003).

As indicated in **Tables 3.6 and 3.7**, rotavirus was not detected in either of the river systems, while adeno-and enterovirus were detected sporadically at various sampling sites throughout the study period in the Plankenburg- and Berg River systems. Adenovirus was however, more prevalent in the Plankenburg River where 15 of the 22 positive samples were found. It was detected in every month in the Plankenburg River, except the 9th, 11th and 12th month of sampling (April, June and July), where no adenovirus was detected. In the Berg River, adenovirus was only detected in months four, six and twelve (November, January and July). Enterovirus was detected uniformly in both river systems however, was detected in the 1st, 9th and 10th months (August, April and May) of sampling in the Plankenburg River, while it was detected in the 1st, 2nd and 3rd months (August, September and October) of sampling in the Berg

River. The larger incidence of adenovirus could be as a result of the runoff into the Plankenberg River from the farms, factories, and the informal settlement bordering the river system. A higher than usual water table during the months April, June and July could also have contributed to the fact that adenovirus was not detected during those sampling months. The Berg River generally has a higher water table than the Plankenburg River, which could have contributed to the low incidence of adenovirus in this river system.

Table 3.6 PCR, pH and temperature results for samples collected from the Plankenburg system.

Sample no	Month and Location of Sample Collection	Rotavirus RT-PCR	Adenovirus PCR	Enterovirus nPCR	pH	Temperature °C
1	K1A	Negative	Negative	+	8.4	11.0
2	K1B	Negative	+	Negative	8.3	11.0
3	K1C	Negative	Negative	+	8.1	11.0
4	K2A	Negative	+	Negative	6.9	16.8
5	K2B	Negative	Negative	Negative	6.9	16.0
6	K2C	Negative	+	Negative	6.8	16.8
7	K3A	Negative	Negative	Negative	7.0	18.0
8	K3B	Negative	+	Negative	7.0	19.0
9	K3C	Negative	+	Negative	6.5	18.0
10	K4A	Negative	+	Negative	6.0	21.0
11	K4B	Negative	+	Negative	6.5	21.0
12	K4C	Negative	Negative	Negative	7.0	21.0
13	K5A	Negative	Negative	Negative	7.0	18.0
14	K5B	Negative	+	Negative	7.0	20.0
15	K5C	Negative	+	Negative	7.0	21.0
16	K6A	Negative	Negative	Negative	7.0	25.0
17	K6B	Negative	+	Negative	7.0	24.0
18	K6C	Negative	Negative	Negative	7.0	28.0
19	K7A	Negative	+	Negative	6.0	23.0
20	K7B	Negative	+	Negative	7.0	24.0
21	K7C	Negative	+	Negative	6.0	23.0
22	K8A	Negative	Negative	Negative	6.8	20.0
23	K8B	Negative	+	Negative	6.8	23.0
24	K8C	Negative	Negative	Negative	7.1	20.9
25	K9A	Negative	Negative	Negative	6.9	17.7
26	K9B	Negative	Negative	+	7.1	18.0
27	K9C	Negative	Negative	+	7.1	25.0
28	K10A	Negative	Negative	Negative	6.7	13.0
29	K10B	Negative	+	Negative	7.3	13.3
30	K10C	Negative	Negative	+	7.1	13.1
31	K11A	Negative	Negative	Negative	6.9	14.1
32	K11B	Negative	Negative	Negative	7.5	13.9
33	K11C	Negative	Negative	Negative	7.5	13.8
34	K12A	Negative	Negative	+	7.5	11.3
35	K12B	Negative	Negative	Negative	7.6	12.4
36	K12C	Negative	Negative	Negative	7.5	12.1

Key: K = Kayamandi informal settlement, 1-12 = month, A = Before informal settlement, B = At informal settlement, C = After informal settlement

Table 3.7 PCR, pH and temperature results results for samples collected Berg River system

Sample no	Month and Location of Sample Collection	Rotavirus RT-PCR	Adenovirus PCR	Enterovirus nPCR	pH	Temperature
37	M1A	Negative	Negative	Negative	7.1	12.0
38	M1B	Negative	Negative	Negative	8.5	11.0
39	M1C	Negative	Negative	+	8.1	10.5
40	M2A	Negative	Negative	+	6.3	18.0
41	M2B	Negative	Negative	+	6.4	18.0
42	M2C	Negative	Negative	+	6.4	18.0
43	M3A	Negative	Negative	+	6.0	19.0
44	M3B	Negative	Negative	Negative	6.0	20.0
45	M3C	Negative	Negative	Negative	6.0	19.0
46	M4A	Negative	Negative	Negative	6.0	25.0
47	M4B	Negative	Negative	Negative	6.0	23.0
48	M4C	Negative	+	Negative	6.0	23.0
49	M5A	Negative	Negative	Negative	6.0	24.0
50	M5B	Negative	Negative	+	6.0	24.0
51	M5C	Negative	Negative	Negative	6.0	22.0
52	M6A	Negative	+	Negative	6.0	25.0
53	M6B	Negative	+	Negative	6.0	25.0
54	M6C	Negative	+	Negative	6.0	24.0
55	M7A	Negative	Negative	Negative	6.0	23.0
56	M7B	Negative	Negative	Negative	6.0	23.0
57	M7C	Negative	Negative	Negative	6.0	22.0
58	M8A	Negative	Negative	Negative	6.3	25
59	M8B	Negative	Negative	Negative	5.9	23.0
60	M8C	Negative	Negative	Negative	6.3	20.4
61	M9A	Negative	Negative	+	6.3	25.0
62	M9B	Negative	Negative	Negative	6.3	22.0
63	M9C	Negative	Negative	Negative	6.4	25.0
64	M10A	Negative	Negative	Negative	6.4	14.5
65	M10B	Negative	Negative	Negative	6.3	12.3
66	M10C	Negative	Negative	Negative	6.5	12.8
67	M11A	Negative	Negative	Negative	7.5	13.5
68	M11B	Negative	Negative	Negative	7.5	13.5
69	M11C	Negative	Negative	Negative	7.5	14.1
70	M12A	Negative	+	Negative	6.9	11.8
71	M12B	Negative	+	Negative	7.3	11.9
72	M12C	Negative	+	Negative	7.4	12.4

Key: M = Mbekweni informal settlement, 1-12 = month, A = Before informal settlement, B = At informal settlement, C = After informal settlement

CHAPTER FOUR
GENERAL CONCLUSIONS

GENERAL CONCLUSIONS

Despite the provision of safer food and potable water supplies, improved sanitation and the promotion of non-invasive interventions such as oral rehydration, diarrhoeal disease remains a common cause of illness worldwide (Hendrickx *et al.*, 2008; Lindesmith *et al.*, 2008). This remains a challenge for devoloping countries where industrial development and urbanisation, has resulted in direct competition for limited water sources. The supply of potable water is further complicated by pollution and more recently water availability (Turton, 2008; DWA, 2010).

Massive urbanisation in South Africa has led to the formation of informal housing schemes, constructed close to untreated surface water sources, such as rivers and dams. The lack of waste removal and adequate sanitation facilities results in the disposal of faecal matter and sewage into storm water drains which flow directly into the river systems (Paulse *et al.,* 2009) contributing to the incidence of diseases such as gastroenteritis, diarrhoea and chronic lung ailments, caused by waterborne pathogenic bacteria, viruses and fungi. The Department of Water Affairs and Forestry (DWAF, 1996) reported that municipal and industrial waste, such as poultry processing operations, slaughter houses, agricultural run-off and food processing factories, are the main sources of river water pollution. In addition, informal settlements may serve as point sources of pollution to rivers, resulting in an increased microbial, organic and inorganic substance load in the water source (Paulse *et al.,* 2009).

Routine water quality analysis does not include monitoring for viral contaminants, as this process is hampered by the lack of simple, reliable, time- and cost-effective testing methods to concentrate and detect viral pathogens. Consequently the quality of the water sources relating to virus contamination is not known (Bosch *et al*., 2008). No data on viral contamination of river water in the Western Cape region is also available despite the high level of bacterial contamination observed in some studies.

In order to determine the viral contamination of the Berg- and Plackenburg Rivers, various concentration and extraction methods for the recovery of viruses from water were compared. As described in the Materials and Methods section 2.2.1, water (100 ml) samples were spiked with one milliliter rotavirus and two milliliters adenovirus control virions (Coris Bioconcept, Gembloux, Belgium). Optimisation testing of enterovirus was not completed due to the unavailability of a positive control. Four viral concentration techniques were compared which included the Silicon

dioxide (SiO_2) method, positively charged, negatively charged and the mixed-ester filter techniques. Various nucleic acid extraction methods were also employed to establish which method would provide optimum yields for both DNA and RNA nucleic acids. The extraction techniques included the TRIzol method (Invitrogen, California, USA) for RNA extraction, the Roche High Pure PCR Template Preparation kit (Roche, Mannheim, Germany) for DNA extraction, and the QIAamp Ultrasens Virus kit (Qiagen GmbH, Hilden, Germany) for simultaneous RNA and DNA extraction. The use of virus specific primers within a PCR was also optimised. In addition, gene specific primers and oligo$(dT)_{15}$ primers were tested and compared to establish which primers would yield the best results since gene specific primers are said to be more sensitive than oligo$(dT)_{15}$ primers (van Pelt-Verkuil *et al.,* 2008) when synthesising cDNA (rotavirus). This is because a specific gene sequence of the template is targeted in the sample when gene specific primers are used for cDNA preparation, while only the poly(A) in the template is targeted when oligo$(dT)_{15}$ primers are used to prepare cDNA for PCR assays.

The SiO_2 concentration method yielded variable results when it was used with the various nucleic acid extraction techniques in this study, since positive PCR results were obtained when used in combination with some techniques, while negative results were obtained with others. The Combi Strip test for rota- and adenovirus indicated that the viruses were present before concentration with the silicon dioxide method commenced. However, when viewed using the TEM, no viral particles could be seen, possibly due to low amounts of viral particles present. The concentrated samples were not tested for rota- and adenovirus antigens after the concentration method was completed to preserve sample volume for virus extraction. The rotavirus PCR samples extracted using the SiO_2/TRIzol method indicated no rotavirus detected from cDNA when oligo$(dT)_{15}$ was used, but positive when cDNA was made with a gene-specific primer (**Table 3.4.1**). When the Roche High Pure PCR Template Preparation Kit was used together with the silicon dioxide concentration method the PCR for adenovirus yielded a low intensity product (**Table 3.4.2**). The QIAamp Ultrasens Virus Kit together with the SiO_2 method yielded a desired PCR product for rotavirus when cDNA was prepared with either Oligo$(dT)_{15}$ primer and gene specific primer (**Table 3.4.1**). Similarly, this method yielded the expected PCR product for adenovirus (**Table 3.4.2**). These results showed that the QIAamp Ultrasens virus kit had the ability to deliver sufficient quantities of RNA for detection by RT-PCR when used with the SiO_2 method for detection of both rotavirus and adenovirus by PCR.

Similarly variable results were obtained when a negatively charged filter was used to concentrate virus particles, used in conjunction with various virus nucleic acid extraction

techniques to identify different viruses by RT-PCR and PCR. Rotavirus and adenovirus antigens were present in the water after initial centrifugation and before filtering through the negatively charged filter. After filtration and micro–concentration, no rotavirus and adenovirus antigens were found in the concentrate that had passed through the negatively charged filter. Furthermore, while viral particles could be seen when viewed using the TEM, mostly unidentifiable material was observed. The rotavirus PCR of RNA samples extracted using the negative filter/TRIzol method indicated that the rotavirus was not detected from cDNA when an oligo$(dT)_{15}$ primer, or when a gene-specific primer was used (**Table 3.4.1**). The PCR result for adenovirus also showed a prominent PCR product when the Roche High Pure PCR Template Preparation kit was used together with the negatively charged filter in contrast to a low intensity PCR product obtained when the QIAamp Ultrasens virus kit was used (**Table 3.4.2**). The higher intensity PCR product for adenovirus suggested better retrieval of the required virus fragments with the negative filter/Roche High Pure PCR Template Preparation Kit than that obtained with the negative filter/QIAamp Ultrasens virus kit. The negatively charged filter/QIAamp Ultrasens virus kit yielded a PCR product for rotavirus when cDNA was prepared with gene specific primers, but not when oligo$(dT)_{15}$ primers were used (**Table 3.4.1**). Also, the PCR for adenovirus showed a low intensity PCR product with this method (**Table 3.4.2**).

A good virus concentration technique is the non-charged mixed-ester filter which yielded similar results to the positively charged filter when used in conjunction with the various nucleic acid extraction techniques in this study. The Combi Strip test indicated that rota- and adenovirus were present in the water after initial centrifugation and before filtering through the mixed-ester filter. After filtration and micro–concentration the presence of rotavirus and adenovirus antigens were found in the concentrate that passed through the mixed–ester filter. Furthermore, when viewed under the TEM the concentrate showed the presence of viral particles. The results thus indicated a successful concentration of viruses by using the non-charged mixed-ester filter. The PCR assay for rotavirus was positive when both oligo$(dT)_{15}$ and gene-specific primers were used, showing that fragments with sufficient quantity and integrity was concentrated and extracted with the mixed-ester filter/TRIzol method (**Table 3.4.1**). The Roche High Pure PCR Template Preparation kit yielded a PCR product for adenovirus when the mixed-ester filter was used (**Table 3.4.2**). This indicated the ability of the mixed-ester filter to concentrate sufficient quantities of either DNA or RNA nucleic acids. Similarly, the mixed-ester filter when used together with the QIAamp UltraSens virus kit yielded a PCR product for rotavirus when cDNA was prepared with either oligo$(dT)_{15}$ primers or gene specific primers (**Table 3.4.1**). In addition, the PCR for adenovirus yielded a desired PCR product (**Table 3.4.2**).

Another effective concentration method for viruses from river water investigated in this study was found to be positively charged filters for concentration of viruses from water. Results indicated the presence of rotavirus and adenovirus antigens in the water samples after centrifugation and before filtering through the positively charged filter. After filtration and micro–concentration the presence of rotavirus and adenovirus antigens were found in the concentrate that passed through the positively charged filter. When viewed under the TEM, the concentrate clearly showed the presence of viral particles. When used together with the positively charged filter the RT-PCR for rotavirus was positive (**Table 3.4.1**) when both primers were used to yield cDNA with the TRIzol extraction method. The polymerase chain reaction was also positive when DNA samples were extracted from adenovirus using the Roche High Pure PCR Template Preparation kit (**Table 3.4.2**). Together with the positively charged filter the QIAamp Ultrasens virus kit yielded the expected PCR product for rotavirus when cDNA was prepared with either Oligo(dT)$_{15}$ primer or gene specific primer (**Table 3.4.1**) as well as the expected product for the adenovirus PCR (**Table 3.4.2**).

These findings showed that using either the positively charged filter or the mixed-ester filter would be the best option to use when investigating virus presence in water samples. Numerous studies also preferred to use electropositive filters for routine virus concentration from water (Haramoto *et al*., 2005; Lambertini *et al*., 2008; Locas *et al*., 2008; Verheyen *et al*., 2009; etc). The positively charged filter was thus applied when analysing river water samples.

An extraction technique should yield highly purified DNA or RNA samples without any inhibiting substances to effect any downstream applications such as PCR (Kok *et al.*, 2000). Various extraction kits made by different manufacturers each have specific applications available for extracting DNA and RNA, respectively. Furthermore, none of the various virus extraction techniques reviewed, yielded superior results for the extraction of both DNA and RNA viruses separately and simultaneously. The QIAamp Ultrasens Virus kit was the most suitable for extraction of DNA and RNA simultaneously when compared to the TRIzol method and the Roche High Pure PCR Template Preparation kit. For this reason the positively charged filter/QIAamp UltraSens Virus kit combination was applied when analysing river water samples.

The presence of rotavirus, adenovirus and enterovirus was tested at strategic points along the Plankenburg River (Stellenbosch) as indicated in **Figure 2.2**; Site A (agricultural farming and residential areas) represented the site before the possible point source of pollution, while Site B (informal settlement of Kayamandi) represented the site closest to the pollution source and Site

C (industrial area substation) was situated downstream from the pollution source. Sampling sites along the Berg River (Paarl) are indicated in **Figure 2.3** and included**;** Site A – before point source of pollution (agricultural farming area), Site B – at point source of pollution (Mbekweni informal settlement) and Site C – after point source of pollution (Newton pumping station). The Newton pumping station services the residential area of Newton as well as certain sections of Mbewkeni informal settlement, which borders the Paarl/Wellington, Boland region. Samples collected from the Plankenburg River were labelled 1-36 (three samples per month for 12 months), while surface water samples collected from the Berg River were labelled 37-72 (three samples per month for 12 months). The positively charged filter together with the QIAamp Ultrasens Virus kit was used to investigate for the virus presence in river water.

The river water collected at the respective study sites were tested for rotavirus and adenovirus antigens using the Combi-Strip technique after centrifugation, but before filtration, and again after filtration using the positively charged filters. Negative rotavirus and adenovirus antigen results were obtained for all 72 samples collected from both river systems, before and after filtration (**Table 3.5**). However, when viewed under the TEM the filtered water sample concentrate revealed the presence of viruses in all 72 samples (collected from both river systems) tested (**Table 3.5**) as indicated by the representative TEM micrograph for the water sample collected from the At site, Berg River, in October 2005 (**Figure 3.16**). When the QIAmp Ultrasens Virus kit was used to extract nucleic acids from viruses within the river water no distinguishable bands were observed (**Figure 3.17**).

The expected PCR product of 346 bp for rotavirus was absent in all 72 river water samples analysed for both river systems. Prominent PCR products seen on the gel electrophoresis (510 to 750 bp), were extracted from the gel using the Minelute Gel Extraction kit (Qiagen, Hilden, Germany). This PCR band range of 510 to 750 bp was sent for sequencing at the Stellenbosch University, DNA Sequencing Facility. The results were analysed using the BLAST program (Altschul *et al*., 1997) where the sequences were compared to similar sequences within the NCBI database. No similarity to any rotavirus genome was found, however diverse hits, ranging from HIV to human virus, were recorded.

In contrast to the PCR results obtained for rotavirus, the expected product of 261 bp for adenovirus was detected in 22 (30.5%) samples collected throughout the study period. Fifteen of the 22 adenovirus positive samples were found in the Plankenburg River (distributed over all sites), while seven of the 22 adenovirus positive samples were found in the Berg River (all sites).

The PCR products viewed by gel electrophoresis were extracted from the gel using the Minelute Gel Extraction kit (Qiagen, Hilden, Germany). The cleaned PCR product was sent for sequencing at the Stellenbosch University, DNA Sequencing Facility. The results were analysed using the BLAST program where the sequences were compared to similar sequences within the NCBI database (Altschul *et al*., 1997). For all 22 positive PCR samples, the BLAST analyses revealed a 94-100% sequence similarity to human adenovirus type 40.

A nested PCR was used to detect enterovirus in the river water samples collected from both river systems throughout the study period. In the first round of the enterovirus PCR 15 river water samples (at various sites for both river systems) yielded a faint 513 bp product. Another prominent PCR product was also seen on the gel electrophoresis (360 bp) and was extracted from the gel using the Minelute Gel Extraction kit (Qiagen, Hilden, Germany). This 360 bp PCR product was sent for sequencing at the Stellenbosch University, DNA Sequencing Facility. The results were analysed using the BLAST program where the sequences were compared to similar sequences within the NCBI database (Altschul *et al*., 1997). No similarity to any enterovirus strain in the database was obtained. Further amplification by nested PCR then yielded 13 (18.1%) positive nested PCR products of 297 bp (**Figures 3.30)**. The prominent nPCR products seen on the agarose gel were extracted from the gel using the Minelute Gel Extraction kit (Qiagen, Hilden, Germany). The cleaned PCR product was sent for sequencing at the Stellenbosch University, DNA Sequencing Facility. The results were then confirmed using the BLAST program where the sequences were compared to similar sequences within the NCBI database (Altschul *et al*., 1997).

In the current study 30.5% of adenovirus and 18.1% of enterovirus in the two rivers investigated compared well with a study conducted by Van Heerden *et al*. (2003) who detected on average 43% of adenovirus in a different river. It has also been hypothesised that under optimised conditions, viruses can travel in surface water and crevices at distances greater than 1000 m (Robertson and Edberg, 1997). Although the risk of microbiological contamination through latrines in the vicinity of drinking water sources is recognised in developed first-world countries, it is not broadly recognised in most developing third-world countries (Beller *et al*., 1997; Robertson and Edberg, 1997; De Serres *et al*., 1999). In the current investigation however, the sampling sites for both the Plankenburg- and Berg Rivers were located before, at and after an identifiable point source of pollution, where storm water (containing sewage effluent etc.) from a bordering informal settlement flowed into the river systems (**Figures 2.2 and 2.3**). The other sampling sites also border agricultural, residential and industrial areas. In this particular case, the storm

water and other effluent sources, containing faecal matter, among other contaminants, could possibly have served as a source of viral contamination.

Rotavirus was not detected in any of the river water samples. The absence of rotaviruses in our study was in contrast to data reported by other groups (Kittigul *et al*., 2005; Miagostovich *et al*., 2008) who found about 20% and 44% in river water, respectively. This discrepancy might be explained by virus characteristics like poor resistance to environmental influences and/or fewer circulating viruses in this area. During this study, we could not determine any seasonal pattern for the distribution of the enteroviruses found. Similarly, Ehlers *et al*. (2005) did not observe any seasonal pattern, probably due to the mild temperatures of autumn and winter months in South Africa.

4.1 LIMITATIONS

Epidemiological data has been reported where enteric infections were associated with drinking water (Payment *et al*., 1997). A limitation of this study was that we did not compare viral presence in river water investigated with disease outcome in the areas surrounding these rivers. Another limitation of this study was that virus detection was only performed on one sample per sampling site per month for 12 months.

4.2 RECOMMENDATIONS

The incidence of adenovirus and enterovirus in river waters reported in the current study and the Van Heerden *et al*. (2003) investigation motivates for similar studies to be conducted in drinking water, dam water used for recreational purposes as well as rainwater, which is gaining popularity as a sustainable water source. Furthermore, these methods may be used in follow-up studies to monitor the presence of different viruses in more of the rivers surrounding Cape Town, as well as in drinking water supplies and in sewage waste in the Western Province South Africa.

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APPENDIX

Appendix A: Preparation of silicon dioxide particles

- Sixty gram silicon dioxide (particle size 0.5-10 μm; approx. 80% between 1-5 μm; Sigma Chemical Co.) were suspended into 500 ml demineralized water in a glass cylinder.
- The suspension was allowed to sediment at unit gravity for 24 hrs at room temperature.
- A 430 ml of the supernatant was removed by careful suction and discarded.
- A 430 ml demineralised water was added to make top up to 500 ml.
- The silica pellet was resuspended by shaking forcefully.
- The silica particles were allowed to sediment at room temperature for 5 hrs.
- A 440 ml of the supernatant was removed by careful suction.
- The pH was adjusted to 2 by adding 600 μl HCl (32%, wt/vol).
- The remaining 60 ml silica coarse should be sufficient for about 1500 nucleic acid purifications (Boom *et al.*, 1990).
- Small portions (4 ml) was aliquoted into glass bottles, autoclaved for 20 minutes at 121°C to destroy any contaminating nucleic acid.
- Silica coarse is stable for at least 6 months when stored at room temperature in the dark (Boom *et al.*, 1990).