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Research Article

Detecting Virus-Like Particles from the Umgeni River, South Africa

It is important to consider viruses in water quality because of their incidence as causal agents for diarrhoeal disease, and due to their characteristics, which allow them to survive in changing environmental conditions indefinitely. This study assessed the viral quality of the Umgeni River in South Africa seasonally. A two-step tangential flow filtration process was setup to remove the bacteria and to concentrate the virus populations from large volume water samples. The concentrated water samples contained up to 659 and 550 pfu/mL of somatic and F-RNA coliphages, respectively. Several virus families including *Adenoviridae*, *Herpesviridae*, *Orthomyxoviridae*, *Picornaviridae*, *Poxviridae* and *Reoviridae* were found in the river based on the morphologies examined under transmission electron microscopy. All concentrated water samples produced substantial cytopathic effects on the Vero, HEK 293, Hela and A549 cell lines. These results indicate the potential of viruses in the water samples especially from the lower catchment areas of the Umgeni River to infect human hosts throughout the year. The present study highlights the importance of routine environmental surveillance of human enteric viruses in water sources. This can contribute to a better understanding of the actual burden of disease on those who might be using the water directly without treatment.

Keywords: Coliphages; Cytopathic effects; Diarrhoeal disease; Tangential flow filtration; Viral infection

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1 Introduction

Despite the advancement in water and wastewater management proficiency, faecal pollution of surface waters is still a cause of concern for the general public and can lead to waterborne illnesses which have community well being and socio-economic repercussions in both the industrialised and developing world [1, 2]. Waterborne diseases are frequently caused by enteric pathogens, which belong to the group of organisms transmitted by the faecal–oral route [3]. The World Health Organisation (WHO), states that there are ~2.2 million deaths related with hazardous water conditions, sanitation and hygiene, and millions more suffer numerous incidents of non-fatal diarrhoea every year [4]. Water quality concerns have increased in recent years, in part due to recurrent contamination of surface water resources by waterborne bacterial, viral and protozoan pathogens [5].

Human enteric viruses are imperative causative agents of waterborne illness, however, viral diseases are difficult to identify by current diagnostic techniques [6, 7]. The Centres for Disease Control and Prevention [8] projected that viral infection may be responsible for almost half of all acute gastrointestinal diseases, and therefore, viral contamination of recreational coastal water is of paramount importance and is a rising public health concern [9]. Enteric viruses have a relatively low infectious dose and can be responsible for a large range of human illnesses such as paralysis, meningitis, respiratory diseases, myocarditis, congenital abnormalities, epidemic vomiting, diarrhoea and hepatitis [10, 11]. Nevertheless, it is now accepted that viral contamination should be definitely taken into account in the microbiological analysis of water and food [12].

Currently microbiological quality standards for water include mostly bacterial faecal indicators, which are not correlated with the presence of waterborne viruses [13]. Routine viral monitoring is not required for drinking or recreational waters and neither is it required for wastewater that is discharged into the environment. This lack of a monitoring effort is due largely to the lack of methods that can rapidly and sensitively detect infectious viruses in environmental samples [14]. Phages share many properties with human viruses, particularly composition, morphology and structure [15, 16]. Phages, especially somatic coliphages and F-RNA coliphages, have been recommended as alternate microbial indicators as they behave more like the human enteric viruses which pose a health risk to water consumers if water has been contaminated with human faeces [17, 18].

Somatic coliphages have been described as being a heterogeneous group of viruses, which could originate from faecal sources [17, 19]. The presence of these viruses in faecally contaminated water means that they can serve as indicators of faecal pollution and may indicate the coexisting presence of pathogenic viruses. Due to their resistance against environmental factors, somatic coliphages

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Abbreviations: **CCA**, canonical correspondence analysis; **CPE**, cytopathic effect; **EM**, epifluorescence microscopy; **TEM**, transmission electron microscopy; **TFF**, tangential flow filtration; **VLP**, virus-like particle

are more applicable than faecal bacteria for indicating faecal contamination of water [15, 16, 20].

The occurrence of F-RNA phages in surface waters generally indicates pollution by human or animal faeces [20–23]. It was shown that for monitoring purposes, F-specific RNA bacteriophages can indicate the possible presence of human pathogenic enteric viruses as they behave like waterborne viruses and their morphology and survival characteristics closely resemble that of some of the important human enteric viruses [17, 24, 25]. The phage plaque assay has the advantages of technical simplicity and low cost but the choice of a suitable bacterial host is of paramount importance [26].

One of the requirements for the virological analysis of water is an efficient and simple virus concentration method. It is well recognised that monitoring the presence of enteric viruses could be challenging due to the relatively low level of infectious viral particles towards the respective host species and small viral particle size existing in environmental waters, thus making it essential to start with a large water sample volume and concentrate it to several orders of magnitude [27–31]. However, this limitation can be overcome by the use of improved methods for aquatic sample concentration, viral infectivity and more sensitive viral detection techniques [32]. Tangential-flow and hollow-fibre ultrafiltration (UF) have been used to investigate microbial contamination of drinking water [33, 34]. The virus concentration method should be applicable to a broad spectrum of enteric viruses to facilitate concurrent downstream study [29].

The present study was conducted to optimise procedures to extract and enumerate indigenous virus-like particles (VLPs) and to determine the community structures and infectivity of these viruses from river water. A setup for the concentration of viruses from large (20 L) volume water samples using a two-step tangential flow filtration (TFF) process was established. The presence of VLPs was determined using epifluorescence microscopy (EM), transmission electron microscopy (TEM) and their infectivity was determined on cell culture.

2 Materials and methods

2.1 Sample collection

Focus in this study was on the Umgeni River (five points along the river as shown in Fig. 1) due to it being the largest catchment (5000 km^2) in the KwaZulu-Natal region of South Africa. The sampling seasons and sites included five vast areas of the river starting from a Dam water source, passing through: a nature reserve, waste treatment works, informal human settlement and finally ending of at the river mouth (were the river and ocean meet). Sampling points were designated as follows: U1 (Umgeni River Mouth-estuarine/brackish water), U2 (Reservoir Hills-informal settlement/domestic waste), U3 (New Germany Wastewater Works-treated water after chlorination that enters the adjoining Umgeni River water), U4 (Krantzkloof Nature Reserve-vegetation and conservation area) and U5 (Inanda Dam-restricted water containment).

Water samples from the River were collected during autumn (March–April 2011), winter (June–July 2011), spring (September–October 2011) and summer (December 2011–January 2012) months, to cover the four seasons of the year. Water samples were collected in 25 L plastic drums with caps. Prior to the sample collections, the alcohol-sterilised drums were rinsed with the water from the river source. The water samples were collected knee deep (±0.5 m) below the water surface facing away from the water current. All samples were protected from direct sunlight and transported to the laboratory within one hour of collection, and stored at 4°C until further analysis.

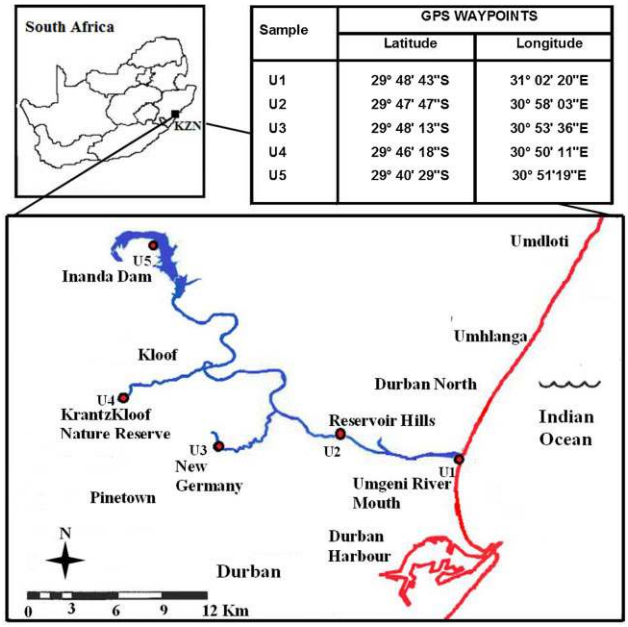

Figure 1. Map of the study region within Durban and surrounding areas. Shown are the locations approximate sampling points of the Umgeni River investigated in this study [35].

2.2 Bacteriophage determinations

2.2.1 Preparation of bacterial hosts for bacteriophage detection

Escherichia coli ATCC 13786 (American type culture collection) was used as the host for the somatic coliphage. The bacterial host was grown overnight at 37°C on a shaking incubator at 100 rpm, in nutrient broth. *Salmonella typhimurium* WG49 (provided by Dr. Maite Muniesa, University of Barcelona, Spain) was used as the host for F-specific coliphage. The *S. typhi* host was grown in Tryptone Yeast Extract broth and incubated at 37°C with shaking at 100 rpm until the F-pili developed (ISO 1995). This growth suspension was monitored at 30 min intervals from time 0 against a blank reference at wavelength of 560 nm until an absorbance of 0.75 was obtained indicating that the sex pili were produced [18].

2.2.2 Double agar overlay plate assay

Bacteriophages were enumerated by the double agar layer technique following the ISO 10705-2 standard (ISO 2000) for enumeration of somatic coliphages and ISO 10705-1 (ISO 1995) for enumeration of F-specific RNA bacteriophages (F-RNA). One hundred microliters of the concentrated samples were mixed with 1 mL of overnight bacterial host and 8 mL of soft agar and the mixture was poured over agar bottom plates [36]. Plaques were enumerated after 12–24 h of

incubation at 37°C and plaque forming units (pfu) were recorded per millilitre.

2.3 TFF for viral recovery

Viral community samples (virioplankton) were concentrated using a two-step TFF process as given in Fig. 2, according to the method of Wommack et al. [37], with some modifications. Concentration of virus particles from large volume water samples occurred in four steps: (i) twenty litres of river water were first pumped through a 25 μm string-wound polypropylene sediment cartridge filter to remove large particles and cells at a flow rate of 230 mL/min; (ii) a peristaltic pump (Masterflex) was used at a flow rate of 530 mL/min with pressure (P_{in} = 7 psi and P_{out} = 6 psi) to pump the pre-treated water samples through a 0.5 m^2, 0.22 μm Pellicon TFF cartridge filter (microporous filtration membrane) (Millipore) to remove all bacterial cells; (iii) viruses in the cell-free permeate was then further concentrated to a final volume of 500 mL using 10 m^2 100-kDa spiral-wound TFF filter (Helicon, Millipore); (iv) the retentate was allowed to re-circulate through the two step system until only 500 mL to 1 L of sample remained in the original vessel. Retentates were then reconcentrated by ultracentrifugation. For ultracentrifugation, 3 runs of 3 h 30 min each at 130 000 × *g* (i.e. 29 000 rpm), 4°C with a SW-32 Ti rotor (Optima L-100 XP, Beckman Coulter ultracentrifuge) were necessary to ultracentrifuge 500 mL (6 tubes of 30 mL filled with 28 mL of samples per run) of the ultrafiltered retentate [38]. Viral pellets were then resuspended in 5 mL of 1 × phosphate buffered saline (pH 7).

Normal water permeability (NWP) testing was performed regularly on the TFF filter membranes before and after running the water samples through the system to ensure filter quality. Proper maintenance and preservation of filter quality was carried out according to manufactures instruction with some modifications. NWP was calculated using the following equation:

$$\mathrm{NWP} = \frac{RF}{A\left\{\frac{P_{in} + P_{out}}{2}\right\}P_p} \quad (1)$$

where R is the permeate flow rate (L/h), P_{in} the feed inlet pressure (psi), P_{out} the retentate discharge pressure (psi), P_p the permeate discharge pressure (psi), A the total filter area (m^2) and F is the temperature correction factor (based on water fluidity relative to 25°C).

2.4 Enumeration and visualisation of VLPs

2.4.1 Epifluorescent microscopy

SYBR gold staining coupled with epifluorescent microscopy [39–41] was used to enumerate VLPs from the Umgeni River virus filtrate. After UF one millilitre (100 kDa cut-off) of the concentrated water samples were fixed with 40 μL of a 2% paraformaldehyde solution and then filtered through 0.02 μm Anodisc filters (Whatman, Maidstone, Kent, UK) with vacuum pressure ≤20 kPa. The Anodisc filters were allowed to air dry and were then stained with 2 × SYBR Gold (Invitrogen, Carlsbad, CA, USA) for 15 min in the dark. After staining the filters were wicked to remove any remaining solution and air dried. The filters were mounted onto glass slides, and counted digitally at 1000× magnification under blue-green light excitation (Fitch filter, excitation at 480–495 nm) with a NIKON Eclipse (80*i*)

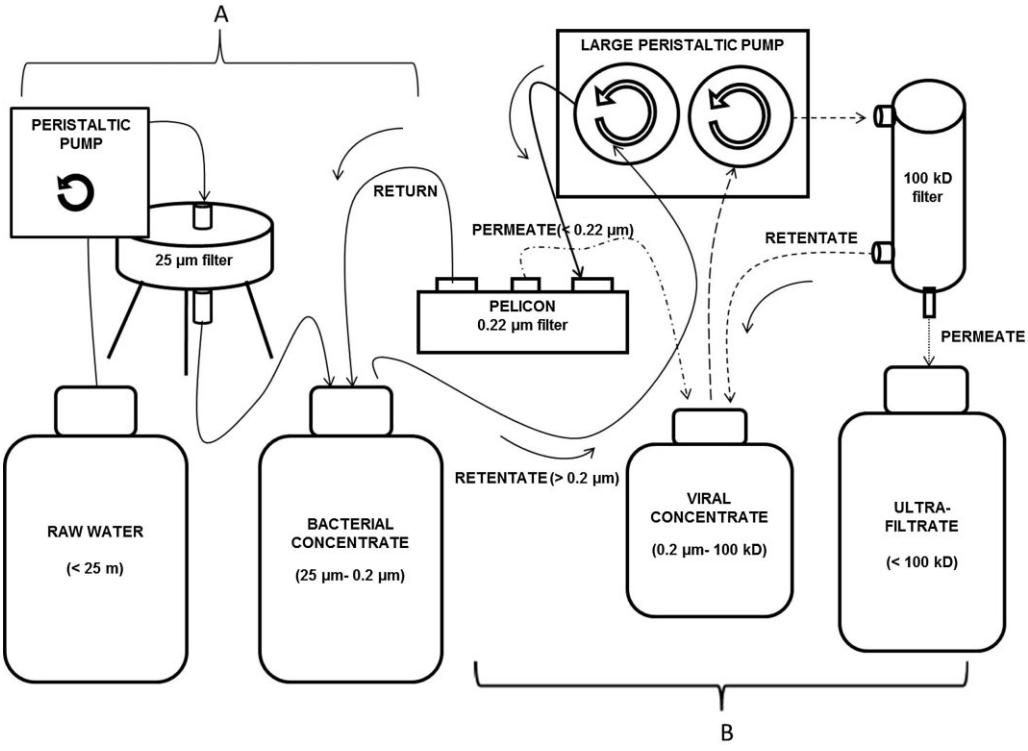

Figure 2. Experimental set-ups for TFF concentration of viruses from large volume water samples. (A) Pre-filtration of initial water sample and (B) the two-step TFF procedures for virus concentration.

epifluorescent microscope in at least eight fields of view for each sample [39]. Images obtained were then analysed using the iTEM software and NIS-D Elements software (D 3.2) to digitally count the fluorescent green VLP spots and compute the results.

2.4.2 TEM

TEM was used to examine the structures and morphology of VLPs in the river water. These TEM images were then compared to known viral images of human origin where possible [42]. Briefly one drop of freshly prepared VLPs was spotted onto a Formvar–carbon-coated 200-mesh TEM grid (Electron Microscopy Sciences, Fort Washington, PA). The edge of the grid was gently blotted with a piece of Whatman filter paper to drain away the excess fluid, and the grid was then stained with a 1% phospho-tungstic acid (PTA) solution or a 2% uranyl acetate solution for 30 s, washed with 1 drop of deionised water for 10 s, and air-dried before examination with a TEM (JEOL). Photomicrographs of viruses were taken at magnifications of 150 000× to 600 000×. Morphological characteristics of VLPs were compiled from multiple photomicrographs of phage particles in order to minimise size or shape anomalies.

2.5 Viral infectivity using cell-culture

Cell-culture (where cell lines were available) was used to determine infectivity of VLPs. The total cultivable virus method as described in US EPA [43] was used as the infectivity protocol. The concentrated VLPs from various water sources were fed into the various cell lines. Three known viruses: human adenovirus, group A rotavirus and coxsackie B virus were used as positive controls. Amongst the cell lines used for human virus investigations in water were, the A549 (adenocarcinomic human alveolar basal epithelial cell), HEK293 (human embryonic kidney cell), Hela (human – cervical cancer cell), HepG2 (human hepatocellular carcinoma cell), PLC/PRF/5 (human primary liver carcinoma cell) and Vero (African green monkey kidney cell). Cells were grown in 10% Dulbecco's modified essential medium (GIBCO) supplemented with 10% foetal calf serum (GIBCO) containing a penicillin/streptomycin/fungizone mix (1:1:1) by volume, to confluent monolayers in 24 well tissue culture plates (Corning, USA). Approximately 200 μL of viral concentrate was overlaid onto the monolayers of appropriate cell lines and incubated at 37°C for 5–7 days. The development of cytopathic effect (CPE) that is indicative of a viral infection in the cell cultures was monitored for up to 7 days. Presence or absence of CPE (loss of cell to cell contact and detachment) was confirmed as described in US EPA [43]. After three freeze–thaw cycles, CPE positive and negative samples were filtered through 0.22 μm syringe filters and were inoculated in new A549, HEK293, Hela, HepG2 and PLC/PRF/5 cells for another seven days. The CPE of cell culture was inspected under an Olympus microscope at 400× magnification. Samples that showed CPE at the end of the confirmation step were reported as positive for infectivity. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was performed using the cell lines mentioned above [44]. When performing the MTT assay, the cells were plated into 24-well plates and infected by the viral samples (each sample in triplicate) approximately 24 h later. After 6–7 days, the medium was removed and MTT solution was added to the wells, and further incubated at 30°C for 4 h. Thereafter the MTT solution was removed and DMSO was added to the wells. The cell viability (%) was determined by the optical density of cells at 570 nm using a Mindray 96A microplate reader (Vacutec, South Africa) according to the method of Heldt et al. [44]. The CPE was calculated as the percentage of cell death due to the viral infection comparing to the control.

2.6 Statistical analysis

Pearson's correlation test was used to evaluate the correlations between the bacteriophages, VLPs and environmental variables. Data comparisons were performed using analysis of variance (post hoc tests) and the Student's *t*-test. Probability (significant level) was set at 0.05. The SPSS program version 19 (SPSS, Illinois) was used for the statistical analyses [45]. Canonical correspondence analysis (CCA) was used to reveal the association amongst the bacteriophages, VLPs and the physical and chemical water quality variables, which were measured from the same sites and seasons in concurrent studies performed in this laboratory [46], with a view to defining the significant variables accountable for the observed spatial and temporal distribution of the communities. CCA ordination allows assessment of the relationships between biological and environmental data, specifically the extent to which variation in biological data can be accounted for by the measured environmental variables. On CCA ordinations, environmental variables are represented by arrows whose lengths reflect their relative importance in structuring the biological sample data [47]. A Monte Carlo permutation test with 499 random permutations was used to establish the environmental axis that significantly correlated with the biological variables. CCA analysis was performed using the computer programme Canoco for Windows version 4.5 [48].

3 Results

3.1 Enumeration of bacteriophages and VLPs

Somatic coliphage counts ranged from 10 pfu/mL (U5–autumn) to 659 pfu/mL (U1–summer) and F-RNA coliphage counts from 0 pfu/mL (U5–autumn) to 550 pfu/mL (U2–summer), respectively (Fig. 3). VLPs were detected using EM (electrostatic force microscopy). Point U1 during summer had the highest population of 2086 VLP/mL and point U4 and U5 had the lowest VLP counts of 221.5 VLP/mL (U4–winter) and 190.1 VLP/mL (U5–autumn), respectively. Mean concentrations of somatic coliphages and F-RNA coliphages were comparable between the autumn and spring seasons and correlation analysis resulted in a strong Pearson's correlation coefficient of 0.977 ($p < 0.01$). These coliphages had inverse correlations ($r = -0.536$) with the VLPs detected during autumn but correlated ($r = 0.795$) with the VLPs found during spring along the Umgeni River.

3.2 CCA

In this study, CCA was used to investigate the relationships between the water quality variables and the VLPs, as well as the relationships between the bacteriophages and the physico-chemical parameters at all sites and seasons. CCA ordination plot (Fig. 4) revealed strong relationships between the overall VLP and phage populations at different sites and seasons measured as well as with the physico-chemical water quality variables. CCA plot (blue circle), temperature, biochemical oxygen demand in five days, turbidity, pH, conductivity, orthophosphate and sulphate were the most important variables

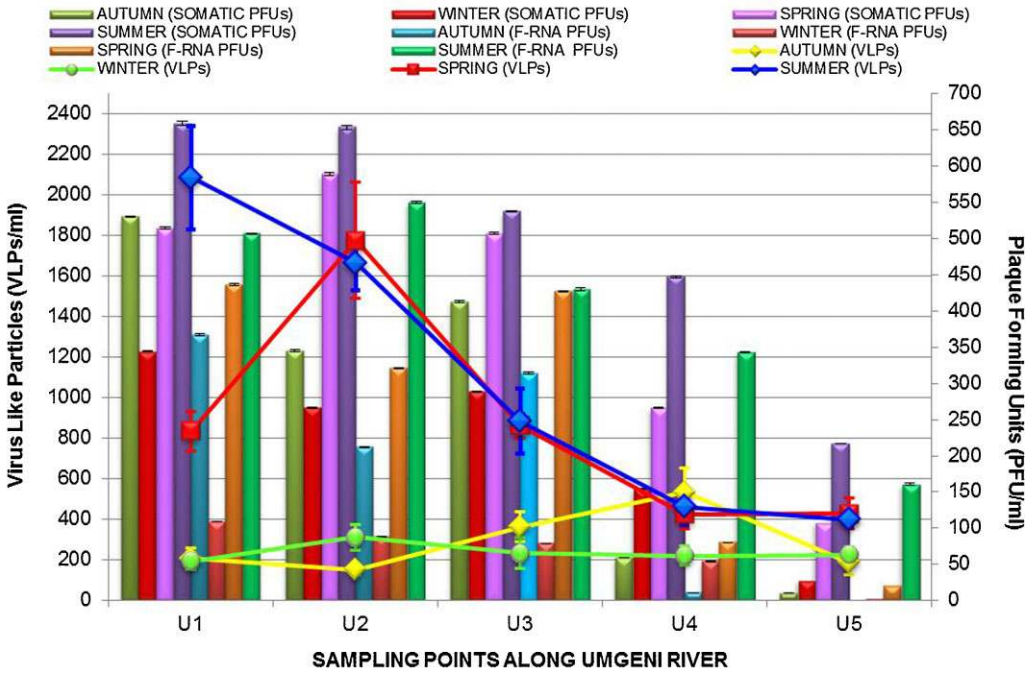

Figure 3. Presumptive counts of VLPs and plaque forming unit's counts for the Umgeni River at the different sampling sites during autumn, winter, spring and summer. Line plot indicates the average of replicate samples ($n = 8$ or 10) for VLPs and bars indicates the average of replicate samples for PFUs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

(Fig. 4, long arrows) that impacted the community structures significantly at sites 1, 2 and 3 during the autumn, summer and spring seasons. Site 2, 4, 5 during autumn, winter and spring seasons correlated with the nitrate/nitrite profiles (green circle). Site 1 and 3 in autumn and winter showed no correlations with the rest of the sites and variables measured.

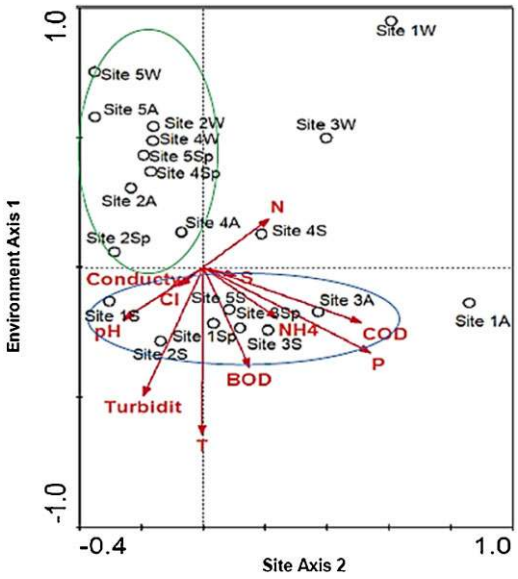

Figure 4. CCA ordination plot for all the water quality variables and the total viral and bacteriophage growth at the five study sites and during autumn, winter, spring and summer seasons. (1) Umgeni River mouth, (2) informal settlement at reservoir hills, (3) new Germany waste treatment works, (4) Krantzkloof Nature Reserve, (5) Inanda Dam. Seasons: A = autumn, W = winter, Sp = spring, S = summer. BOD, biochemical oxygen demand; COD, chemical oxygen demand. The arrows indicate the direction of maximum change of that variable across the diagram.

CCA axis 1 (Fig. 4) accounted for 77.6% of total variance of the species data set and in total the species – environment relation accounted for 94.1% of the cumulative variance suggesting that there may be a correlation between the VLP and phage populations at the various sites and water quality variables during all seasons. Positive significant ($p < 0.05$) Pearson correlation of species and environmental scores for axis one and two were $r = 0.955$ and 0.962, respectively. The ordination plot revealed that the Somatic phage, F-RNA phage and VLPs had little relationships with each other and existed independently (Fig. 5). The sample scores were scattered in the ordination with a number of denser clusters (physico-chemical) spread out around the origin. CCA axis 1 (Fig. 5) accounted for 60.5% of total variance of the species data set and in total, the species – environment relation ordination accounted for 83.7% of the cumulative variance. Eigen values for axis point one and two were 0.043 and 0.021, respectively. Positive significant ($p < 0.05$) Pearson correlation of species and environmental scores for axis one and two were $r = 0.759$ and 0.980, respectively.

3.3 Visualisation of VLPs by TEM

Table 1 classifies the different type of bacteriophage that could be detected in the Umgeni River by TEM. Most of the detected phages (33%) had isometric heads and long non-contractile tails, belonging to morphotype B1 (*Siphoviridae*) (Tab. 1). Members of morphotypes A1 (*Myoviridae*) were detected in 25% of the samples, and C1 (*Podoviridae*) was present in substantial (20%) numbers. The phages tagged as A1/B1 were not conclusive in morphological appearance and could not be classified. Table 2 illustrates the size range of the bacteriophages that could be observed during all four seasons. Phage head diameters

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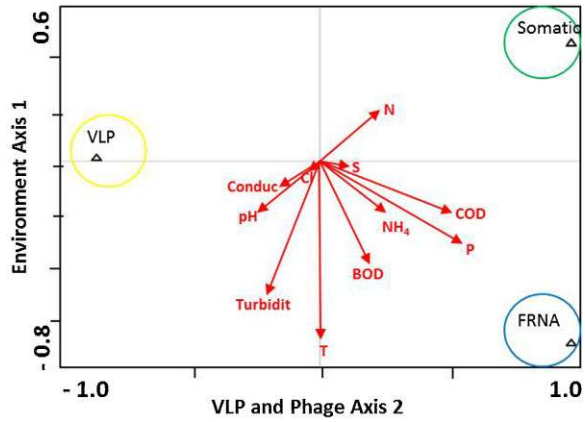

Figure 5. CCA ordination plot for bacteriophage and virus populations and water quality variables at the five study sites during autumn, winter, spring and summer. BOD, biochemical oxygen demand; COD, chemical oxygen demand.

ranged from 48–59 nm (winter) to 42–79 nm (summer), with the general mean value of phage heads being 57 nm. The tail length of the phage populations varied throughout all seasons from 69 nm in autumn to 352 nm in summer. Interesting to note was that the total length of phages varied extensively, ranging from 150 nm (autumn), 181 nm (winter), 290 nm (spring) to 218 nm (summer).

Figures 6 and 7 resemble bacteriophages detected in the Umgeni River water samples. Phage heads examined, appeared to be hexagonal in outline, with six sided profiles which were regular, with three symmetrical axes (e.g. Fig. 6a, b, c, e and f) while others were irregular, with only one symmetrical axis (e.g. Fig. 6i and k). However, these data were not adequate to differentiate between icosahedra, octahedra and dodecahedra classification. Figure 6c and h closely resemble members of the family *Myoviridae* and *Podoviridae*, respectively. Figure 6l and p resemble members of the family B2 *Siphoviridae* and B1 *Siphoviridae*, respectively. Figure 7 represents phage particles detected in the Umgeni River at sites U1, U2 and U3 during spring and summer seasons that resemble known bacteriophages found in literature. These include known phages of: 71A-6 of *Vibrio vulnificus* phage, phage T4 and T4-like *Vibrio parahaemolyticus* phage as shown by Ackermann and Heldal [50].

Figures 8–12 illustrate several presumptive virus types that were found in the Umgeni River during all seasons. These presumptive viruses were compared to their known structures illustrated in literature. Presumptive naked *Adenoviridae*-like particles ranging in size from 66.97 to 74.40 nm are shown in Fig. 8 and these images are compared to known adenoviruses (70–90 nm) (Fig. 8g). Figure 9 represents TEM images of presumptive naked *Picornaviridae* (enterovirus)-like particles ranging in size from 26 to 30 nm, compared to a known coxsackievirus (Fig. 9f). *Poxviridae*-like particles (Fig. 10a–c) and *Herpesviridae*-like particles (Fig. 10e–g) were also detected in the Umgeni River water samples and these were found to be similar in literature to known poxvirus (Fig. 10d) and known herpesvirus (Fig. 10h). Figure 11 illustrates presumptive *Reoviridae*-like particles ranging in size from 18 to 20 nm (Fig. 11a–c) compared to a known rotavirus (20–30 nm) (Fig. 11d). Presumptive *Caliciviridae*-like particles are illustrated in Fig. 11e–g and these had a size of 35 nm, similar to that of a known norovirus (Fig. 11h) of 30–40 nm. Presumptive

Table 1. Frequency of phage morphotypes found in the Umgeni River water, classified according to the scheme of Ackermann and Eisenstark [49] – [International Committee on Taxonomy of Viruses (ICTV)]

Sample season	No. of phages belonging to morphotypes									
	A1		B1		A1/B1[a)]	B2		C1		Total
Autumn	2		1		1	0		3		7
Winter	1		1		0	0		1		3
Spring	7		4		3	2		3		19
Summer	5		14		7	0		5		31
Total	15		20		11	2		12		60

[a)] Classification uncertain, A1 – Myoviridae – short capsid contractile tail, B1 – Siphoviridae – short capsid, non-contractile long tail, B2 – Siphoviridae – long capsid, non-contractile long tail, C1 – Podoviridae – short tail.

Table 2. Size range of tailed phage's observed by electron microscopy

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

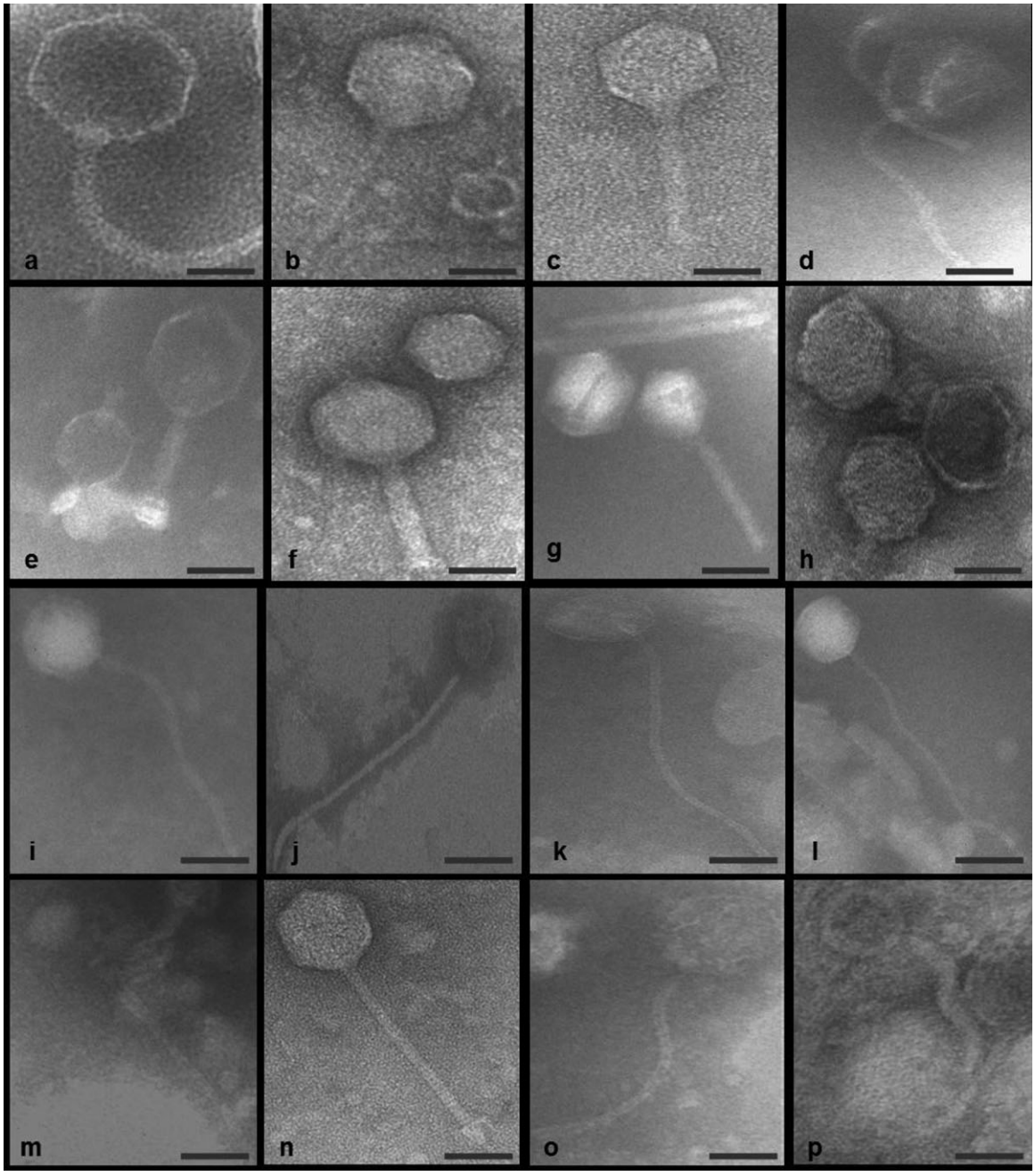

Figure 6. TEM images of various phage morphotypes particles present in the Umgeni River at all the sampling sites and seasons tested. Images captured at 300 000–400 000× magnification. Scale bar; 100 nm.

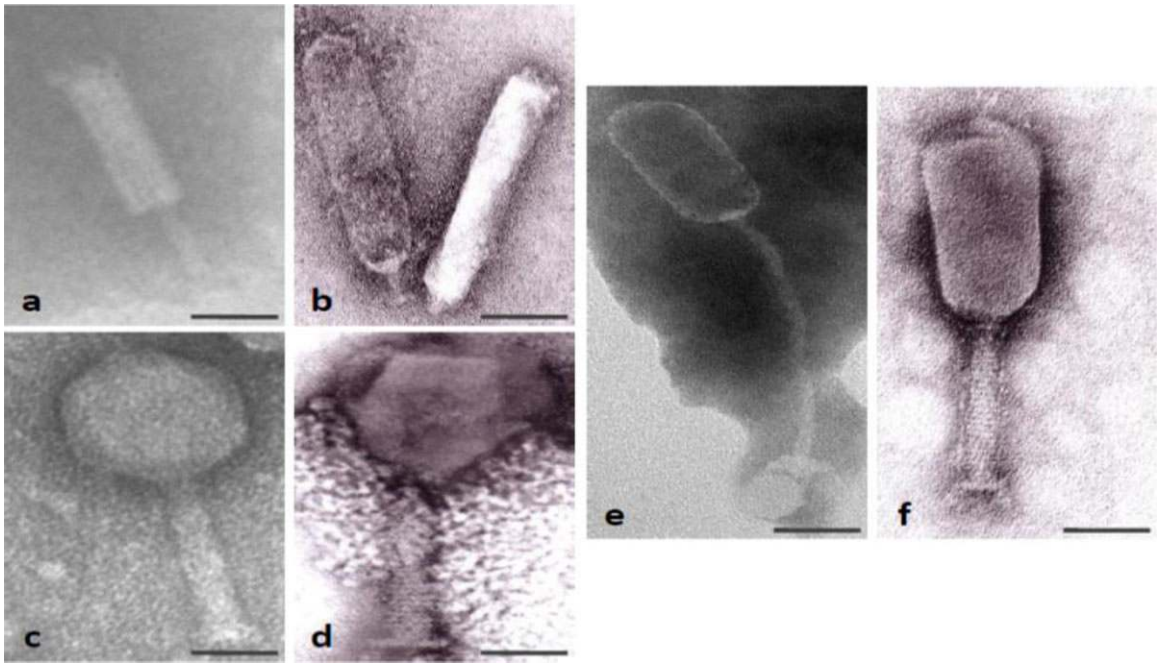

Figure 7. TEM images of phage morphotypes (a, c, e) present downstream of the Umgeni River at the sampling sites U1, U2 and U3, respectively, during spring and summer, (b) known 71A-6 of *Vibrio vulnificus* phage, (d) known phage T4, (f) known T4-like *Vibrio parahaemolyticus* phage [50]. Images captured at 300 000–400 000× magnification. Scale bar: 100 nm

enveloped *Coronaviridae*-like particles (Fig. 12a–c) were found to be similar to a known coronavirus (Fig. 12d), with presumptive *Orthomyxoviridae*-like particles being similar to a known influenza virus (Fig. 12h).

3.4 Viral infectivity using cell-culture

The CPE is an observable morphological (shape) change in tissue cells due to viral infection. Figure 13 illustrates the results obtained for the

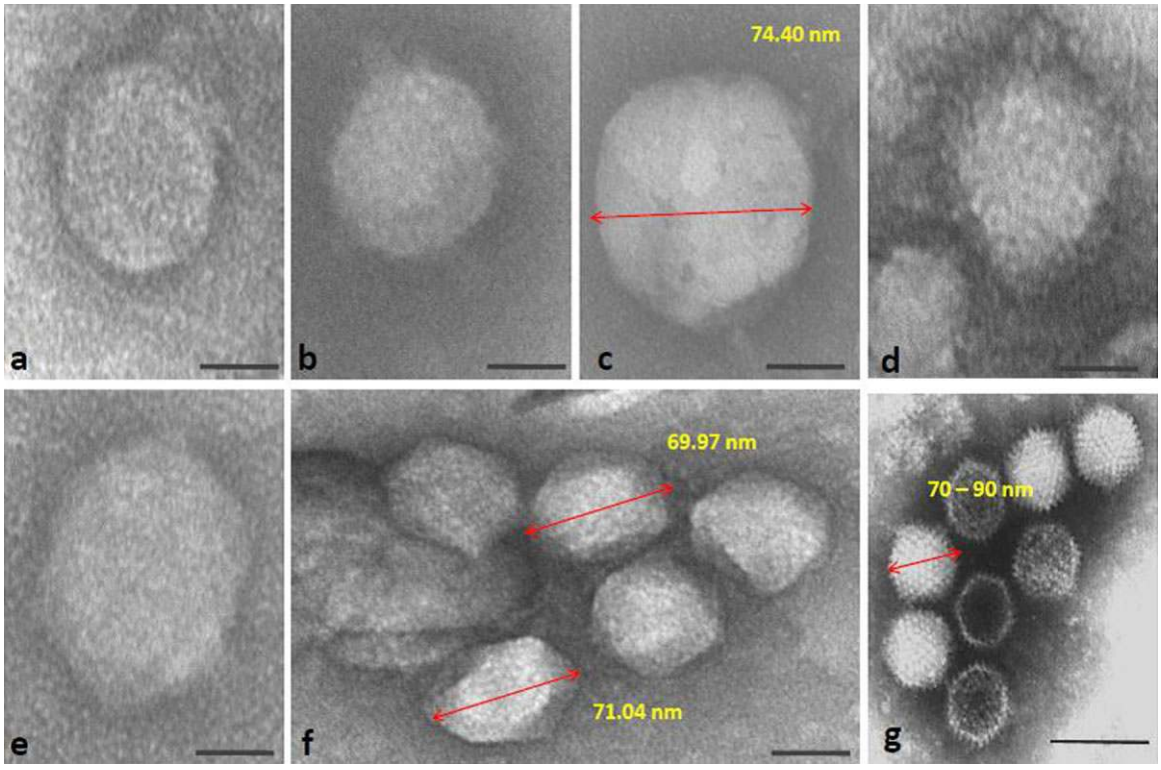

Figure 8. TEM images of presumptive naked *Adenoviridae*-like particles and (g) a known adenovirus [51], present in the Umgeni River at the sampling sites U1, U2, U3, U4 and U5 during autumn, spring and summer tested. Images captured at 400 000–600 000× magnification. Scale bar; 100 nm.

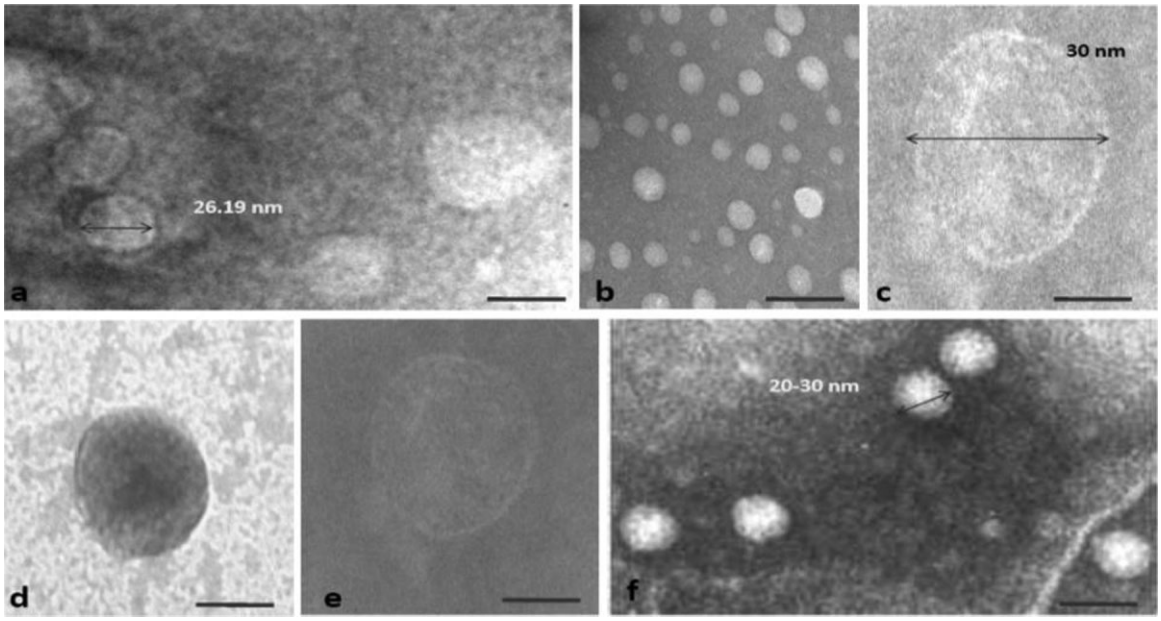

Figure 9. TEM images of presumptive naked *Picornaviridae* (enterovirus)-like particles and (f) a known coxsackievirus [52], present in the Umgeni River at sampling sites U1, U2, and U3 during spring and summer tested. Images captured at 500 000–600 000× magnification. Scale bar: 50 nm.

CPE of the VLPs isolated from the Umgeni River using six tissue cell lines. The identification of CPE in the cell cultures included the occurrence of elongated, granulated cells, loss of cell to cell contact and the appearance of vacuoles.

All VLPs isolated from all water samples during all four seasons were capable of inducing some CPE (cell death) on all tissue cell lines tested. The VLP samples from sites U3, U4 and U5 produced no CPE on the Hep-G2 cell line during autumn. VLPs from site U5 produced CPE

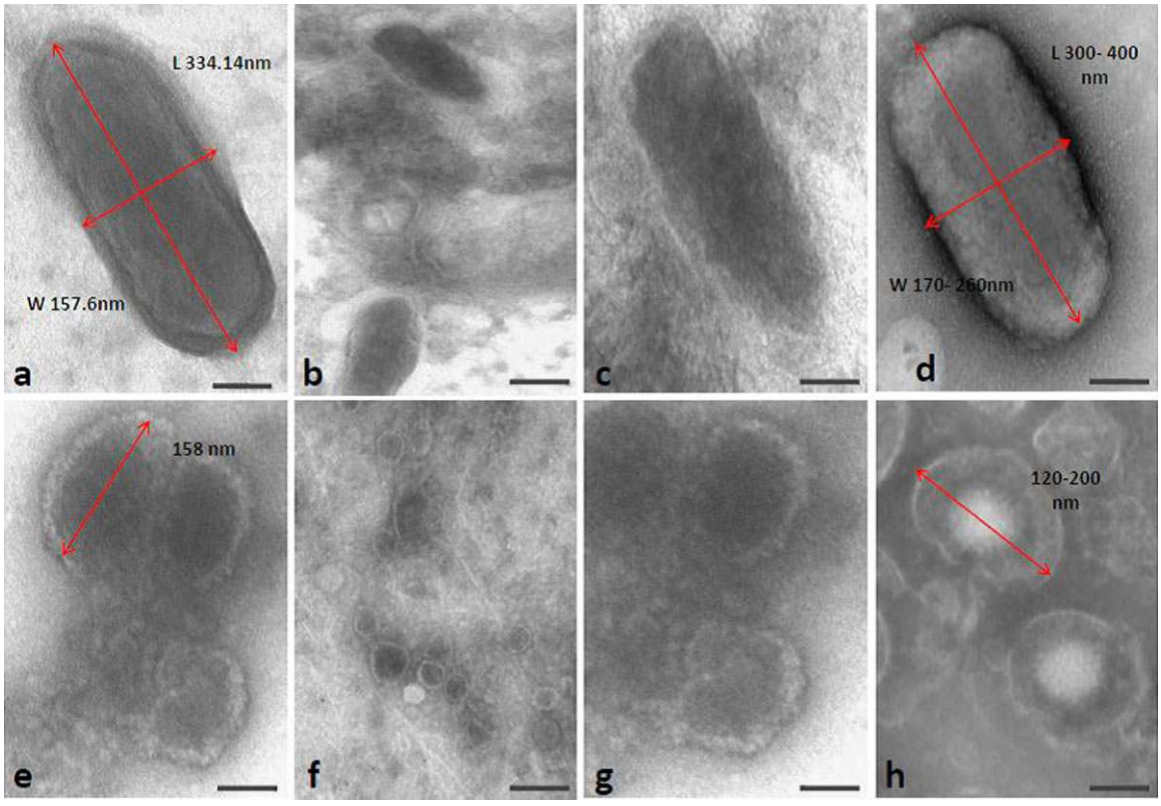

Figure 10. TEM images of enveloped presumptive (a, b, c) *Poxviridae*-like particles, (d) known poxvirus [53] and (e, f, g) *Herpesviridae*-like particles, (h) known herpesvirus [53], present in the Umgeni River at site U3 during summer. Images captured at 300 000–500 000× magnification. Scale bar: 200 nm.

CLEAN Soil Air Water

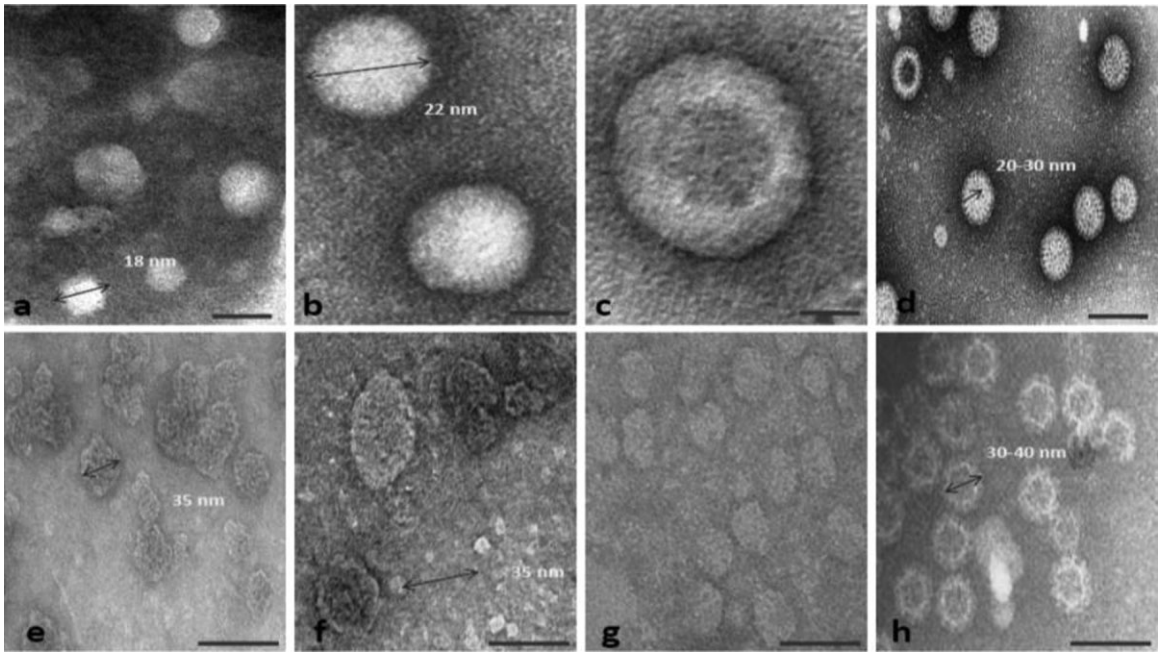

Figure 11. TEM images of presumptive (a, b, c) *Reoviridae* VLPs, (d) known rotavirus [54], (e, f, g) presumptive *Caliciviridae* VLPs, (h) known norovirus [55], present in the Umgeni River at the different sampling sites during all seasons tested. Images captured at 500 000–600 000× magnification. Scale bar: 50 nm.

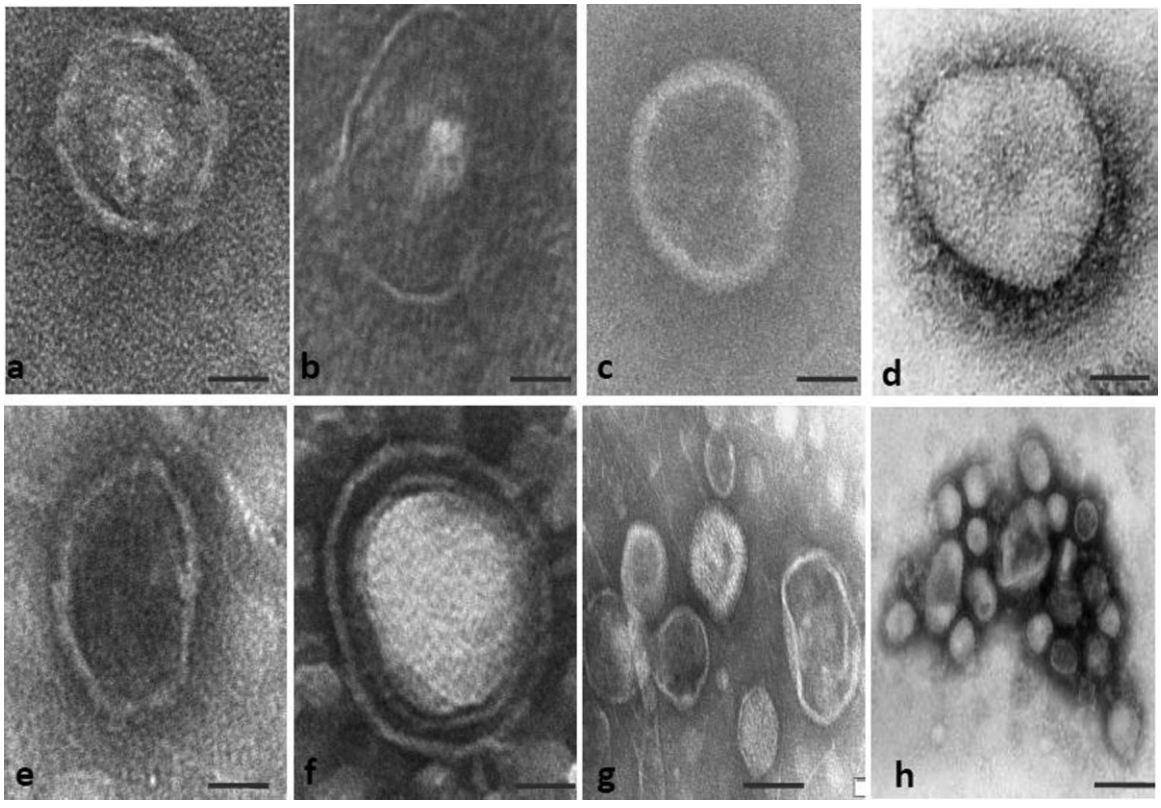

Figure 12. TEM images of presumptive enveloped VLPs (a, b,c) *Coronaviridae* VLPs, (d) known coronavirus [52], (e, f, g) presumptive *Orthomyxoviridae* VLPs, (h) known influenzavirus [52], present in the Umgeni River at the different sampling sites during all seasons tested. Images captured at 400 000–600 000× magnification. Scale bar: 100 nm.

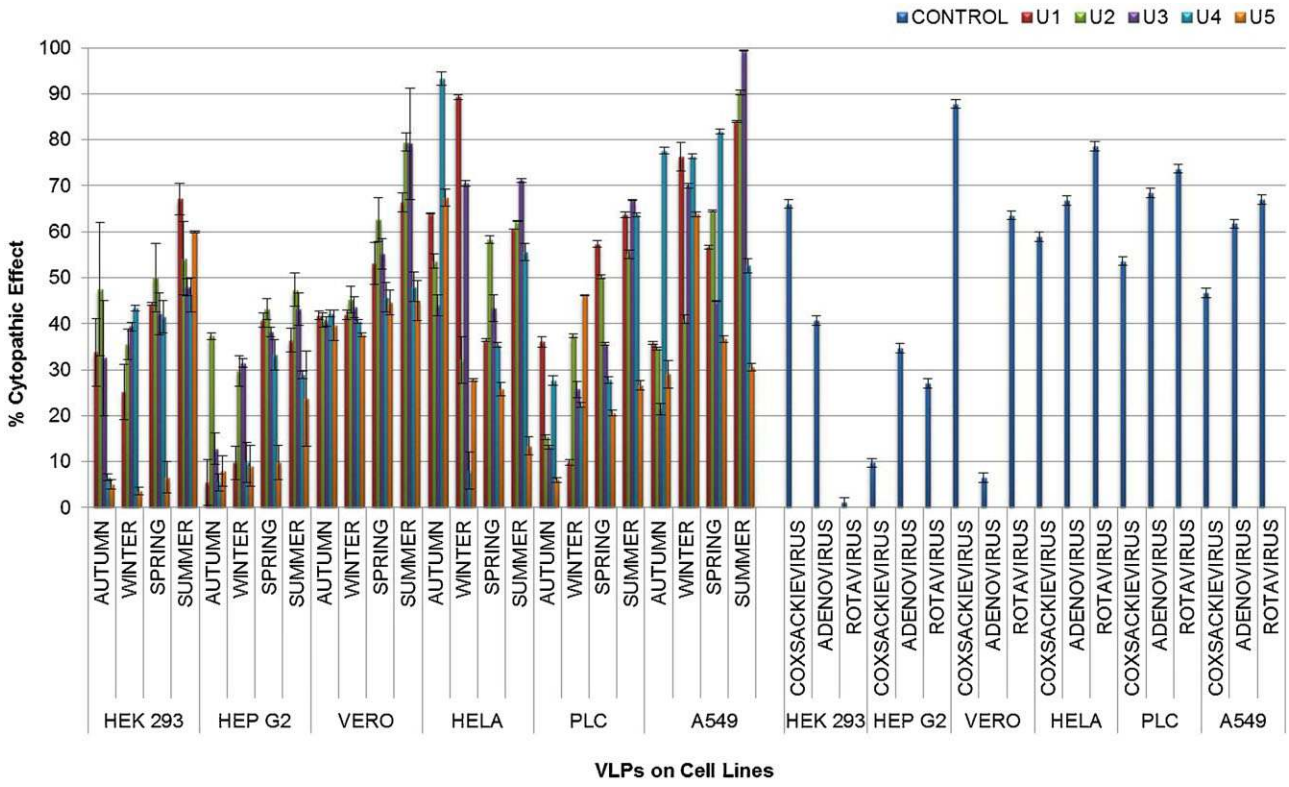

Figure 13. CPE of the concentrated VLPs on six cell lines for the Umgeni River at the different sampling sites during all seasons tested. Bars indicate the average of replicate samples ($n = 2$ or 3) while the error bars show the standard deviation.

on the Hep-G2 during summer only and failed to cause CPE on the PLC cell line during autumn. The VLPs caused substantial CPEs on the Vero, Hela and A549 cell lines during all seasons and sites tested. CPE values for Vero cells ranged from 66.37, 79.50 and 79.14% at sites U1, U2 and U3, respectively, during summer. Site U4 (autumn), U1 (winter) and U3 (summer) seasons had 93.28, 89. 28 and 71.19% CPE, respectively, on the Hela cell line (Fig. 13). Sites U1, U2, U3, U4 and U5 had relatively high CPEs for the A549 cell line during winter as compared to the other cell lines during that season. Sites U1, U2 and U3 had CPEs of 83.97, 90.29 and 99.38%, respectively, on the A549 cell line during summer. HEK293 and HepG2 cell lines showed minimal cytopathic activity from the VLPs. Propagation of coxsackievirus, rotavirus A and adenovirus controls were favoured on the Vero, Hela, PLC and A549 cell lines (Fig. 13).

4 Discussion

Microbiological contamination of water has long been a concern to the public and contamination of water resources, intended for use by general population, with enteric viruses is a great public health problem. Although the occurrence of human enteric viruses has been demonstrated in surface water bodies as well as in drinking water supplies throughout the world and several outbreaks of enteric viral diseases (attributed to either contamination of drinking water or vegetables and seafood grown in such waters) have been recorded [56, 57]. Routine examination of water samples for the presence of enteric viruses is not largely performed in both developed and developing countries [57]. In this study, the populations of somatic and F-RNA coliphages as well as various enteric viruses were monitored using traditional techniques such as electron microscopy and tissue culture.

Phages have been proposed as microbial indicators of water quality, as they share many fundamental properties with human enteric viruses which pose a health risk, if present in water contaminated with human faeces [18]. All sampling points throughout all seasons tested positive for the presence of somatic coliphages which ranged from 10 pfu/mL (U5–autumn) to 659 pfu/mL (U1–summer) especially in the lower reaches of the river and during summer. F-RNA coliphage counts were significantly lower compared to those of somatic coliphage counts. F-RNA coliphages, from 0 pfu/mL (U5–autumn) to 550 pfu/mL (U2–summer), were also detected more frequently in the lower reaches of the river. Mean concentrations of somatic coliphages and F-RNA coliphages were comparable between autumn and spring and correlation analysis resulted in a strong Pearson's correlation coefficient of 0.977 ($p < 0.01$). Bacteriophages are not a stable part of water micro-flora and their presence is usually associated with wastewater inflow rich in animal and human excrement [58]. Their survivability depends upon physico-chemical environmental conditions [59]. Somatic coliphages have been found to outnumber F-RNA phages in waste water and raw water sources by a factor of about 5, and cytopathogenic human viruses by about 500 [60, 61], thus making them valuable indicators for assessing the behaviour of and the possible presence of enteric viruses in water environments. Male-specific (F-RNA) coliphages are highly specific for sewage pollution and cannot be replicated in water environments, but detection methods are more complicated [62].

VLPs were detected using EM at all sampling sites throughout all seasons, with point U1 during summer having the highest population

of 2086 VLP/mL. Umgeni River points U1, U2 and U3 were more contaminated with phage and virus-like populations than points U4 and U5. Increasing in the phage and VLP populations from upstream to the river mouth were observed in all seasons. This was probably because the river flows through the more urbanised areas of Durban and is subject to higher surface runoff [63]. It can be speculated that increased human activity as well as informal settlements and trading activities in the storm water drain catchment areas contributed to the high levels of phage populations downstream the river at points U1, U2 and U3. The thicket and bushland that enclosed both the Krantzkloof Nature Reserve (U4) and Inanda Dam (U5) can serve as vegetated pervious buffers as passive treatment systems allowing agricultural and storm water runoff to reach the river and dam in a less impaired state [35]. Mallin et al. [64] found that human development along the land–seawater interface is considered to have significant environmental consequences resulting in a difference in abundance and distribution of the enteric pathogen indicator microbes and can often pose an increased human health risk.

All variables tested had positive correlations with each other during all seasons. Significant positive correlations were observed between the somatic phage and F-RNA phage ($r = 0.991$, $p = 0.001$) and somatic phage and VLP ($r = 0.884$, $p = 0.46$). A correlation between phage quantity and degree of faecal pollution has been found elsewhere [65]. Similar results were also obtained by Armon and Knott [60] who found positive correlations of phage number and bacterial pollution indicator factors.

In this study, TFF with the Pellicon Cassette System (Millipore) for concentrating VLPs from 20 L river water samples was used. The two-step filtration procedure used in our laboratory involved fluid flowing parallel to the 0.22 μm TFF filter surface. Particles with molecular weights (M_w) smaller than the exclusion size (0.22 μm) of the filter passed through and collected (filtrate) in a reservoir which was further concentrated through a 100 kDa spiral wound TFF unit. Particles with larger molecular weights were retained and recycled to the original reservoir (retentate). Thus, as the filtration continued, the retentate volume decreased until the desired volume was attained. The TEM study demonstrated that the two-step TFF procedure coupled with ultracentrifugation produced a viral concentrate devoid of bacteria, with most viruses being intact. The phage/VLPs concentrations in every sample were high enough to view on formvar coated electron microscopic grids without enrichment. All water samples in this study contained a mixture of morphologically different tailed phage viruses, which were regarded as bacteriophages. It was possible to observe viral particles with long tails (B1-*Siphoviridae*), short tails (A1-*Myoviridae* and C1-*Podoviridae*), and without tails. The phages particles observed without tails may belong to a wide range of hosts, including eukaryotes [66]. Most of the phages from the Umgeni River were intact, with discrete structures such as tail fibres, base plates, and other appendages which are pertinent for the recognition of and interaction with the host cell. These accessories may be an indication that a significant proportion of the phages are suspended in the water environment as potentially infective particles. The main purposes of phage classification are generalisation and simplification, which facilitates comparisons and understanding of viruses [67]. The high morphological diversity of phage communities corresponds well with the great variability and dynamics of bacterial populations obtained in this current study. Electron microscopy provides a direct insight into the morphological variability of phage present in the water environment, without being dependent on the isolation of suitable host strains. This is important, as bacteria which are not cultivable under laboratory conditions usually dominate aquatic environments and may be important phage hosts [68].

Great morphological variability was observed in the virus assemblage's from the Umgeni River, which suggests that these viruses may also be diverse in terms of the hosts that they infect. Most of the viruses were detected downstream of the river towards the river mouth area during the spring and summer seasons. The viral contamination of these widely used areas could be attributed to storm water discharge, surface runoff, sewage discharge and overflows, recreational exposure and other anthropogenic activities [63]. The detection of viruses by TEM in the sampling points upstream of the Umgeni River was relatively low or non-existent, probably due to the stagnant waters and limited recreational exposure. Several presumptive virus types including *Adenoviridae*, *Caliciviridae* (norovirus), *Coronaviridae*, *Herpesviridae*, *Orthomyxoviridae* (influenza virus), *Picornaviridae* (enterovirus), *Poxviridae* and *Reoviridae* (rotavirus) were found in the Umgeni River during all seasons based on the morphologies under TEM. Human viruses seen in negative stains fall into one of two major morphological categories: enveloped or naked [64]. Enveloped viruses have a nucleocapsid (the nucleic acid held together by some structural proteins) inside, whilst naked viruses are icosahedral, their protein coat or capsid is more rigid and withstands the drying process well to maintain their spherical structure in negative stains [50]. Naked human viruses are of three size ranges: (i) 22–35 nm (e.g. parvoviruses, enteroviruses and caliciviruses), (ii) 40–55 nm (polyomaviruses and papillomaviruses) and (iii) 70 × 90 nm (reoviruses, rotaviruses and adenoviruses) [69]. As a general pattern, the virus population was dominated by small forms (<40 nm), and tails were rarely seen, which is in agreement with the results reported by several authors [66, 70–72]. The negative staining technique for TEM allowed for the examination of particulate material including determination of structure and size of particles and has proved important in virological studies [50]. All water samples analysed were environmental in nature, thus natural degradation may have altered the morphological features of the viruses substantially [50].

The application of PCR-based molecular technology and TEM has advanced our knowledge of the occurrence and prevalence of human viruses in water. However, it has provided no information on viral viability and infectivity in a specific environment. The tissue culture assay is the only US EPA approved method for virus infectivity monitoring in aquatic samples [73]. Virology laboratories have traditionally used tissue culture cell lines to amplify the amount of virus present in a water sample, express the viral antigens and in many cases monitor the cell death as a consequence of the viral infection producing characteristic CPEs in the cell monolayer. The tissue culture technique has been used extensively for virus replication and infectivity studies and plaque assays [74]. It has been established that for the recovery of a maximum number of infectious viruses, it is often necessary to inoculate each sample type into several different cell culture lines because no single cell culture is available with susceptibility to all enteric virus types [74]. In the present study six cell lines (the A549, HEK293, Hela, HepG2, PLC/PRF/5 and Vero) were used to determine CPE of the VLPs from the Umgeni River during four seasons. All VLP samples from all sites and seasons produced substantial CPEs on the Vero, HEK293, Hela and A549 cell lines. This effect was tested against positive controls of three known viruses (voxsackievirus, rotaviruses and adenovirus) in the same cell lines. Vero cells are sensitive to infection with many

different viruses [74]. Human embryonic kidney cell line 293A and human lung carcinoma cell line A549 were the most penetrating to viral infection, especially to enteric adenovirus 40 and 41 [75]. Grabow et al. [76] reported the propagation of both enteric adenoviruses 40 and 41 in the primary liver carcinoma (PLC/PRF/5) cell line. Hep-G2 cell lines are often used to study Hepatitis B, C and other viruses [74]. CPE of the VLPs was examined by microscopy every day for the first week of growth to maximise the detection of viral progression. Certain viruses take one day to three weeks to yield a CPE dependent on the initial concentration and virus type. CPE can be swelling, shrinking, rounding of cells to grouping or whole destruction of the monolayer [77]. All the VLPs isolated from water samples were capable of inducing the CPEs on six tissue cell lines. The ability of these viruses to infect susceptible host cells and to replicate their DNA/RNA, confirms that they are viable and infectious and therefore constitute a health risk which is due to be investigated [18, 78].

5 Concluding remarks

In this study, different approaches to determine viral pollution during four seasons and at five sampling points in the Umgeni River were comparatively evaluated. A setup for the concentration of viruses from large (20 L) volume water samples using a two-step TFF process was successfully established. Electron microscopy illustrated the presence of VLPs in concentrated water samples at all sites sampled, and their infectivity was established based on their CPE on various cell lines. The results produced suggest the infectious potentials of the VLPs in the Umgeni River water samples, especially river water from the lower catchment areas, to infect the human hosts throughout the year. These observations have serious health care implications if raw untreated water is used for human consumption. However, it should also be considered that not all viral particles detected in this study correspond to infectious viral particles and a high percentage of non-infectious viral particles may be present in the environment [76]. The present study thus highlights the importance of routine environmental surveillance of human enteric viruses for a better understanding of the actual burden of these viral infections on those who might be using the water directly without treatment.

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