Bacteriophage levels and associated characteristics in selected temperate water systems

L Bothma

22207694

Dissertation submitted in fulfilment of the requirements for the degree *Magister Scientiae* in Environmental Sciences at the Potchefstroom Campus of the North-West University

Supervisor: Prof. CC Bezuidenhout

Assistant supervisor: Dr. R Adeleke

May 2017

AKNOWLEDGEMENTS

I would like to express my sincere appreciation and gratitude to the following people and institutions for their contribution and support towards the completion of this study:

Prof. C.C. Bezuidenhout for his patience, guidance, encouragement, time, support and valuable input into making this possible.

Dr. R. Adeleke for his patience, encouragement and support in making this study possible.

The Water Research Commission (WRC) of South Africa (K5/2347//3), the Agricultural Research Council – Professional Development Programme (ARC-PDP) and the North-West University post graduate (PUK-bursary) for financing this study.

Dr. Jaco Bezuidenhout for his assistance with the statistical analysis of this study.

Dr. Anine Jordaan for her assistance with the Transmission Electron Microscopy analysis of this study.

Dr. Charlotte Mienie for her assistance with any molecular method queries.

Dr. Juan Jofre for providing positive control viruses for the molecular analysis of this study.

Dr. Lesego Molale for her assistance in content editing and always having answers to all my many questions.

For the WRC research group of (2015) for the shared physico-chemical analyses (Alewyn, Mzy, Adele, Janita, Rohan, Vivienne, Carla, Maxi, Roelof, Tamryn, Magnus, and Karoline)

My parents Chris and Trudi Bothma for their prayers, love, emotional and financial support as well as believing in me always.

My friends Lesego and Janita, for their motivation and assistance with this dissertation.

My friends Jeanette, Jurgens, Lineke, Konstanze and Landi for their continued emotional support throughout my study.

ABSTRACT

Bacteriophages are studied in an effort to establish the viral safety of water as well as gather information as to what source of faecal pollution is dominant in a water system. The aim of this study was to determine bacteriophage levels and associated characteristics in selected temperate water systems. The methods used in this study were the double-agar–layer plaque assay, for enumeration of somatic coliphage and F-RNA phage levels. Transmission Electron Microscopy was used for characterisation and identification of somatic coliphages. RT-PCR was employed for identification of F-RNA phages. Physical parameter levels of the water systems were measured on site by multi-meter probes. Chemical parameter levels of the water systems were measured in the laboratory using a spectrophotometer. Six water systems in the North West Province of South Africa was studied. These water systems were: Mooi River, Harts River, Barberspan, Crocodile River, Marico River, and Schoonspruit River. Somatic coliphages were found at 34 of the 37 sites that were sampled in this study. The two sites that had no somatic coliphage detected in their water were both in the Crocodile River. The Delarey site in the Harts River was not analysed for phage levels. The highest somatic coliphage level detected in this study was 23 000.00 ± 989.95 pfp/100 mL in the Schoonspruit River. The highest F-RNA phage level recorded during the period of this study was 4 270.00 ± 11.84 pfp/100 mL in the Barberspan water system. The Marico River was the system least affected by environmental and species variables. Barberspan was the water system most severely impacted by F-RNA phage pollution sources. TEM images of plaques showed 3 different morphologies which could indicate a possible link between virion size of somatic coliphages and plaque morphology. However, definite statements regarding this is premature and requires further investigation. Human faecal pollution is entering Barberspan near the hotel sampling site as well as near the outflow of Barberspan into Leeupan sampling site. The physico-chemical parameter levels of the six water systems studied were all indicative of temperate water systems. Considering the bacteriophage and physicochemical parameter levels it is evident that all six water systems studied were being impacted by pollution from domestic and/or agricultural sources.

Keywords: Somatic coliphages, F-RNA bacteriophages, faecal pollution, TEM, plaque morphology, RT-PCR.

DECLARATION

I, Leani Bothma, declare that this dissertation is my own work in design and execution. It is being submitted for the degree Master of Science in Environmental Science at the North West University, Potchefstroom Campus. It has not been submitted before for any degree or examination at this or any other university. All material contained herein has been duly acknowledged.

Leani Bothma Date

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION AND PROBLEM STATEMENT

Viruses have been found wherever there is cellular life and are regarded as important players in various ecological processes, such as nutrient cycling, gene transfer, and biodiversity (Sandaa, 2009). Viruses are the most numerous and probably the most diverse biological entities in the environment. There are an estimated 10^{31} viruses on earth, and the greater majority of them infect bacteria (Sandaa, 2009). This fact is a clear indication of their biological and environmental importance and versatility. Viruses are found in different and sometimes strange environments. For instance, viruses are capable of infecting other microorganisms such as bacteria. Viruses that infect bacteria are called bacteriophages, or phagest (Douglas, 1975; DWAF, 1996a). The term bacteriophage was coined by F. d'Herelle. F.W. Twort discovered phages in 1915, and independent from Twort's research, F. d'Herelle discovered them in 1917 (Douglas, 1975). Phages of *E. coli* are known as coliphages (Douglas, 1975).

Phages provide a model of behaviour for researchers whereby viruses with important influence on human activity can be better understood (Douglas, 1975). Faecal polluted water harbours a great variety of viruses originating primarily from the gastro-intestinal tract. This includes phages, and more specifically coliphages (Bosch, 1998; AWPRC, 1991). The target water quality range (TWQR) for coliphages in recreational water is 0 – 20 counts / 100 mL (DWAF, 1996a). No TWQR guidelines for any bacteriophages exist for agricultural or aquatic health environments. The risk of being infected by human enteric pathogens correlates with the level of contamination of the water and the amount of contaminated water consumed. Viruses have a considerably lower minimum infectious dose than bacteria, i.e. 1 -10 viral particles, as compared to 10 – 1000 bacteria cells, respectively (DWAF, 1996a). This means that even at low levels of viral pollution, a high risk of infection exists. Hence, contamination of water resources intended for use by general population with enteric viruses could pose a public health challenge (Ganesh *et al*., 2013).

Bacteriophages are generally accepted as indicator organisms for testing water safety, especially regarding human enteric viruses. However, routine examination of water samples for the presence of enteric viruses is not largely performed (Ganesh *et al*., 2013). Humans are exposed to enteric viruses through various routes: food crops grown on land irrigated with wastewater, sewage-polluted recreational water and contaminated drinking water, etc. (Bosch, 1998; Lucena *et al*, 2003). Enteric viruses may also be potentially transmitted by recreational activities in polluted waters (Bosch, 1998). Enteric viruses are common casual agents for

diarrhoeal disease and their resistant characteristics allow them to survive in changing environmental conditions indefinitely. Routine environmental surveillance of human enteric viruses in water sources can enhance understanding of the actual burden (community well-being and socio-economic repercussions) of disease on those who might be using the water directly without treatment (Ganesh *et al*., 2013).

Bacteriophages can be used as easy, cost effective, and fast faecal pollution source tracking organisms (Bosch, 1998). Researchers identified specific RNA coliphages (i.e., bacteriophages that infect *E. coli*) from human and non-human faecal material, suggesting that these phages can also be used to distinguish between human and non-human faecal sources of pollution (Havelaar & Hogeboom, 1984; Cole *et al*., 2003; Luther & Fujioka, 2004; Dryden *et al*., 2006). However, natural processes in the environment such as sedimentation, absorption, various physico-chemical and biological factors affect the survival rate of phages in the environment (Durán *et al*., 2002) and thus extreme caution must be taken when interpreting the data provided by phage assays.

The surveillance of the microbiological quality of environmental water is a major public health and economic issue (Servais & Billing, 1990; Skraber *et al*., 2002). According to DACE (2002) the main aim of the North West State of Environmental Water Report (SOER) is to provide valuable environmental information to support sustainable development in the North West Province. According to SOER (October 2011 to September 2012), water systems in the North West Province are considered to be, generally, in good health (DWA, 2013). Is this assumption of the state of water in the North West Province correct or is the assumption based largely on a lack of information? Information gathered in his study aims to help the government, by providing information not yet gathered and published in reports such as this previously. This is an important aspect to help improve the knowledge, health, and sustainability of rivers in the North West Province as healthy rivers provide goods and services such as water supply, breakdown of pollutants, conservation, flood attenuation, natural products, recreation and spiritual rituals (DWAF, 2009). This in turn contributes to human welfare and economic growth (DWAF, 2007).

The purpose of this study was to increase the current information available on an aspect of faecal pollution in water systems in the North West Province. Based on importance and size, there are eight main rivers in the North West Province of South Africa. They are the Crocodile, Groot Marico, Hex, Elands, Vaal, Mooi, Harts and Molopo Rivers (DACE, 2002). This study included four of these rivers: Mooi River, Upper Harts River, Crocodile River (west) and Marico Rivers. Two other water systems were also screened: Barberspan and Schoonspruit River. Priority was placed on the information relating to levels of bacteriophage in the water system.

1.2 RESEARCH AIM AND OBJECTIVES

The aim of this study was to determine bacteriophage levels and associated characteristics in selected temperate water systems.

Specific objectives of this study were to:

- Enumerate F-RNA bacteriophage and somatic coliphage in selected water systems.
- Determine the levels of selected physico-chemical parameters in selected water systems.
- Characterize the F-RNA bacteriophages and somatic coliphages.
- Determine possible pollution sources based on phage genogroups.

CHAPTER 2

LITERATURE REVIEW

2.1 VIRUSES

Only with the realization of the nature of bacteria in the middle of the nineteenth century did the principal difference between bacteria and the various poisons and toxins became clear. Beijerinck in 1898 proclaimed a new concept from which the understanding of the nature of viruses slowly grew (Fraenkel-Conrat, 1969). From this time, the definition of a virus has evolved into an infectious agent consisting of a genome of one or several molecules of DNA or RNA (Fraenkel-Conrat *et al*., 1988). It is usually surrounded by a coat known as a capsid of several or one protein and in many instances it is coated by more complex envelopes (AWPRC, 1991). These agents are able to transmit their nucleic acid between host cells by superimposing their genetic information on that of the host cell (Fraenkel-Conrat *et al*., 1988).

Viruses have been found in every environment in which there is cellular life, from polar ice caps (López-Bueno *et al*., 2009) to hot springs (Breitbart *et al*., 2004; Bolduc *et al*., 2012). As a scientific discipline, water virology was born in New Delhi between December 1955 and January 1956 (Sandaa, 2009). Environmental virology began with efforts to detect poliovirus in water around half a century ago (Bosch, 1998). Presently, viral ecology is the study of interactions of viruses with other organisms and the environment (Sandaa, 2009). The concept of viruses as a separate natural phenomenon is just over 120 years old while the actual understanding of their nature is just over 70 years old. The realization that many diseases of plants, animals, people, insects and even amoebae can be attributed to this type of agent is even more recent. So also is the recognition that similar agents kill bacteria (bacteriophages) (Fraenkel-Conrat *et al*., 1988).

Human enteric viruses and bacteriophages enter the water environment in high numbers through the discharge of sewage contaminated water (Havelaar *et al*., 1993; DWAF, 1996a). Current water treatment practices fail to ensure the complete removal of viral pathogens. Consequently, viruses become environmental pollutants (Bosch, 1998). Faecally polluted water harbour a great variety (over a hundred species) of viruses originating primarily from the gastro-intestinal tract (Bosch, 1998; AWPRC, 1991). These viruses are often conveniently described as enteric viruses (AWPRC, 1991). Enteric viruses can cause a large range of human illnesses such as paralysis, meningitis, gastroenteritis, fever, rash, respiratory diseases, myocarditis, congenital abnormalities, conjunctivitis, epidemic vomiting, diarrhoea and hepatitis (DWAF, 1996a; Bosch, 1998; Ganesh *et al*., 2013). These viruses are relatively resistant to inactivation by natural and treatment processes (Havelaar *et al*., 1993). Viral diseases are difficult to identify

by current diagnostic techniques such as ELISA, PCR, NGS (Ganesh *et al*., 2013). These methods are also relatively laborious and require well-trained, specialised personnel with sophisticated facilities, and are therefore not well suited for monitoring purposes (Havelaar *et al*,. 1993; Ganesh *et al*., 2013). It is also impractical (considering technical and economic reasons) for monitoring the presence of all viral pathogens. These facts propelled the search for an appropriate enteric virus indicator organism.

2.2 BACTERIOPHAGES

An indicator organism is an organism that is related to the occurrence of the surrogate micro-organism and is a model that has behavioural characteristics similar to those of the original micro-organism. It also has the same or greater resistance to environmental stresses than the original organism (Durán *et al*., 2002). Because of the concern associated with viruses transmitted through the faecal oral route, microorganisms present in the faecal micro-biota were proposed (Havelaar *et al.*, 1993). For routine monitoring purposes of water pathogens, model organisms that behave like waterborne viruses but are readily detectible by simple, rapid, and inexpensive methods were selected (Havelaar *et al.*, 1993; DWAF, 1996a). A good indicator organism should fulfil the following requirements according to Gerba *et al*. (1975); Wyer *et al*. (1995); DWAF (1996a); Durán *et al*. (2002); Skraber *et al*. (2004); Harwood *et al*. (2005):

1. Should be associated with the source of the pathogen and should be absent in unpolluted areas.
2. Should occur in greater numbers than the pathogen.
3. Should not multiply out of the host.
4. Should be at least equally resistant to natural and artificial inactivation as the viral pathogen.
5. Should be detectable by means of easy, rapid and inexpensive procedures.
6. Should not be pathogenic.

Bacteriophages share many properties with human enteric viruses, particularly – composition, morphology, habitat, and structure (Ganesh *et al*., 2013). Also, the survival and incidence of bacteriophages in water environments resembles that of human enteric viruses more closely than most other indicators commonly used (DWAF, 1996a). Bacteriophages have long been considered as attractive candidates for indicators of enteric viral behaviour and of faecal pollution in environmental waters (Ogorzaly & Gantzer, 2006). Three bacteriophage groups appeared promising candidates: Somatic coliphages, F-RNA bacteriophages and *Bacteroides fragilis* bacteriophages (Morinigo *et al.,* 1992; Bosch, 1998; Leclerc *et al*., 2000; Grabow, 2001; Ogorzaly & Gantzer, 2006; Ganesh *et al*., 2013).

Results obtained during a study by Baggi *et al*. (2001) showed correlations between enteric viruses and bacteriophage levels in river systems receiving sewage effluent. Somatic coliphages and F-RNA coliphages, especially 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 by faeces, than any other indicator organism (Havelaar *et al.*, 1993; Bosch, 1998; Durán *et al*., 2002; Contreras-Coll *et al*., 2002; Ganesh *et al*., 2013). These results confirmed the role of bacteriophages as indicators for viral contamination.

2.3 SOMATIC COLIPHAGES

Somatic coliphages are a diverse group of phages which infect *E. coli* and other certain closely-related bacteria (DWAF, 1996a). The different degrees of homogeneity of somatic coliphages are a heterogenous group that comprises *Myoviridae, Siphoviridae, Podoviridae* and *Microviridae* (Durán *et al*., 2002; Ganesh *et al*., 2013). Bacteriophages are classified into families with respect to their morphology and size (Jończyk *et al*., 2011). *Myoviridae, Siphoviridae, Podoviridae* belong to the Caudovirales order while *Microviridae* is an unassigned family (ICTV, 2011). The *Myoviridae, Siphoviridae*, and *Podoviridae* families consist of phages with linear double stranded DNA, while the *Microviridae* family consists of circular single stranded DNA phages (Elbreki *et al*., 2014). Tail lengths give information about phage stability and resistance in the environment. Short and not-tailed phages are generally more resistant while long tails tend to be prone to breakage, resulting in loss of infectious activity (Aprea *et al*., 2015). The 9th International Committee on Taxonomy of Viruses (ICTV) (2011) report explains how Caudovirales phages transfer their DNA through the tail tube by injecting it into a cell (ICTV, 2011).

Myoviridae include four genera of Enterobacter phages: Mu-, P1-, P2-, and T4-like viruses (Lavigne *et al*., 2009; ICTV, 2011). They are non-enveloped and have contractile tails (Elbreki *et al*., 2014). They have icosahedral capsids, large genomes, as well as a base plate with terminal fibers (Jończyk *et al*., 2011). *Siphoviridae* include the four genera of Enterobacter phages: Lambda- (λ), T1-, T5-, and N15-like viruses (ICTV, 2011). They are non-enveloped and have long non-contractile tails (Elbreki *et al*., 2014). They have a genome size of approximately 100 kb (Jończyk *et al*., 2011). *Podoviridae* include the four genera of Enterobacterphages: P22-, Phieco (Φ)32- , SP6-, and T7- like viruses, as well as the coliphage N4-like viruses (ICTV, 2011). They are non-enveloped and have short non-contractile tails, as well as long hexagonal capsids (Jończyk *et al*., 2011; Elbreki *et al*., 2014). *Microviridae* includes only one genus (*Microvirus*) that infects Enterobacter bacterial species: phi (Φ) X174 (ICTV, 2011). They are non-enveloped, don't have any tails and are isometric in form (Elbreki *et al*., 2014)

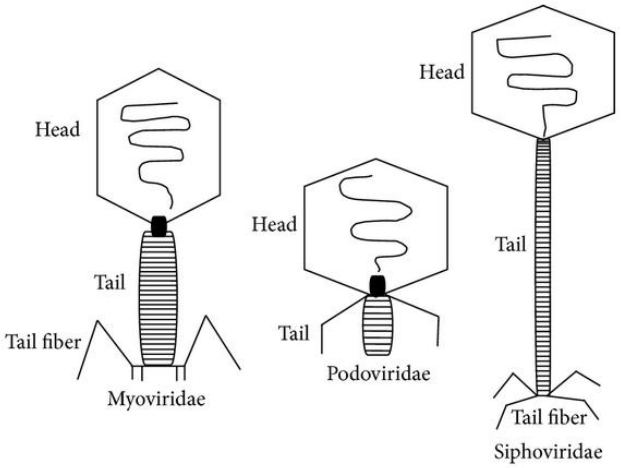


Figure 2-1 Three tailed bacteriophage families (Myoviridae, Siphoviridae, and Podoviridae). (Image courtesy of Elbreki *et al*., 2014)

Figure 2-2 Non-tailed DNA bacteriophage. (Image courtesy of Elbreki *et al*., 2014)

The term somatic coliphage is used to describe the counts obtained by using female *E. coli* host strain, which is infected by phages absorbing to receptors situated in the cell wall (somatic receptors) (Rodríguez *et al*., 2012). Theoretically, the use of a male (F+ or Hfr) host strain would result in the detection of both somatic and F-RNA phages. In practice, results using male *E. coli* strains can be interpreted as somatic coliphages counts. This is due to several studies showing that only a minute portion of plaques are produced by F-RNA phages upon subsequent identification (Grabow, 2001; Rodríguez, *et al*., 2012).

Somatic coliphages have the advantage of being used as indicator organism because they are very abundant, even in water with low levels of faecal indicators (Durán *et al*., 2002; Contreras-Coll *et al*., 2002). One reason for this abundance is that they possess the ability to multiply in unpolluted waters (AWPRC, 1991). Even though replication of somatic coliphages in aquatic environments has been established, most studies concerned with viral safety of water still make use of them (DWAF, 1996a; Grabow, 2001; Rodríguez, *et al*., 2012).

2.4 F-RNA BACTERIOPHAGES

All F-RNA bacteriophages belong to the family *Levivirdae* (Franki *et al*., 1991; Durán *et al*., 2002; Ogorzaly, & Gantzer, 2006; Skraber *et al*., 2009) and can be divided into two genera: *Levivirus* and *Allolevivirus (*Bollback & Huelsenbeck, 2001; Ogorzaly, & Gantzer, 2006). Although they may differ in certain features (Furuse, 1987), they constitute a homogeneous group (Durán *et al*., 2002). These virus genomes consists of positive-sense linear single-stranded RNA (Ogorzaly & Gantzer, 2006; Elbreki *et al*., 2014).

Figure 2-3 F-RNA bacteriophage (Image courtesy of Elbreki *et al*., 2014).

F-RNA coliphages are a restricted group of coliphages which only infect *E. coli* and related hosts which produce fertility fimbriae during the logarithmic growth phase at temperatures greater than 30 °C (DWAF, 1996a; Grabow, 2001; Rodríguez, *et al*., 2012). These phages can therefore not replicate in natural environments. This implies that they are highly specific indicators of faecal pollution (Ogorzaly & Gantzer, 2006). Their numbers in sewage are generally lower than those of somatic coliphages and their behaviour and indices in water environments seem to resemble that of human viruses even closer than somatic coliphages (Gironés *et al*., 1989; Havelaar *et al*., 1993; Bosch, 1998; Muniesa *et al*., 1999; Contreras-Coll *et al*., 2002; Ganesh *et al*., 2013). It was shown that for monitoring purposes, F-RNA phages can indicate the possible presence of human pathogenic enteric viruses where somatic coliphages only indicate human enteric viruses (Ganesh *et al*., 2013).

F-RNA phages primarily infect Gram-negative bacteria which possess a plasmid coding for an F or sex pilus (Ogorzaly & Gantzer, 2006). F-RNA phages specifically absorb to the sex pili coded for by the classical K-12 F-plasmid of *E. coli* and related plasmids of the $IncF^-$ incompatibility group (AWPRC, 1991; Grabow, 2001; Rodríguez, *et al*., 2012). Havelaar & Hogeboom (1984) developed a single reliable method for F-RNA phage enumeration and isolation. A *Salmonella typhimurium* strain was used, because this strain would consistently yield low plaque counts of some phages even when examining raw sewage. After obtaining a naladixic acid resistant mutant, an F-plasmid (F'42 *lac*::Tn5) was introduced (Havelaar & Hogeboom, 1984). The sex pili are only produced under specific conditions and elevated temperature, similar to that found

in the gastro intestinal tract of humans and other warm blooded animals (Havelaar *et al.*, 1993; Bosch, 1998). This makes them highly specific indicators for faecal pollution by warm-blooded animals, including humans (Durán *et al*., 2002; Contreras-Coll *et al*., 2002). F-RNA phages generally also have a greater resistance to disinfection processes than somatic coliphages (Ganesh *et al*., 2013).

For the sake of simplicity, F-RNA coliphages can be grouped into four main serotypes, which, with few exceptions, show overall comparability with genotypes (Beekwilder *et al*, 1996; Hsu *et al*., 1995; Muniesa *et al*., 2009). These phages are grouped on the basis of their serological cross-reactivity (Vinjé *et al*., 2004), replicase template activity (Miyake *et al*., 1971) and phylogenetic analysis (Bollback & Huelsenbeck, 2001; Ogorzaly & Gantzer, 2006). Using genotyping, several researchers have demonstrated that subgroups of F-RNA phages can be used to distinguish human inputs from those of warm-blooded animals (Hsu *et al*., 1995; Blanch *et al*., 2006).

The genus *Levivirus* contains the genogroup I (MS2-like phage) and the genogroup II (GA-like phage), whereas *Allolevivirus* genus contains the genogroup III (Qβ-like phage) and the genogroup IV (SP-like phage) (Osawa *et al*., 1981; Ogorzaly & Gantzer, 2006). Groups II and III are associated with human sources while groups I and IV are associated with animal sources (Osawa *et al*., 1981; Brion *et al*., 2002; Ogorzaly, & Gantzer, 2006). This can help selecting effective remediation strategies to bring chronically polluted waters into compliance with regulatory policies (Skraber *et al*., 2004). GII (GA-like) phages are routinely found in pig faeces, but never in any other animal faeces. This exception is explained by the fact that pigs have a gastrointestinal physiology and flora similar to humans, partly because of their close living conditions (Hsu *et al*., 1995; Ogorzaly & Gantzer, 2006). GIII (Qβ-like) phages are found in chicken and pig wastewater (Sundram *et al*., 2006). On the other hand, F-RNA phages GI and GIV are specific for animal wastewater (Ogorzaly, & Gantzer, 2006).

2.5 BACTERIOPHAGES IN ENVIRONMENTAL WATER

Somatic coliphages are consistently and significantly isolated in higher numbers than F-RNA coliphages (Sundram *et al*., 2002; Ganesh *et al*., 2013). Changes in the relative numbers may result from phage replication, inputs of faecal contamination other than urban sewage and from a greater proportion of faecal bacteria affected by environmental stresses. Somatic coliphages may replicate in the environment, outside the human and animal gut. However, there are uncertainties on the conditions necessary for replication, such as the host and phage concentrations required and the physiological state of the host bacteria (Contrerass-Coll *et al*., 2002). Furthermore, the fact that somatic coliphage levels are only higher than *E. coli* levels in water with high levels of faecal pollution seems to minimise the importance of coliphage

replication in the environment (Contrerass-Coll *et al*., 2002). Multiple studies (Gironés *et al*., 1989; Chung & Sobsey, 1993; Hill & Sobsey, 1998; Muniesa *et al*., 1999; Sinton *et al*., 1999; Contrerass-Coll *et al*., 2002) explain the difference in the proportions between different bacteriophages and that between bacteriophages and the bacterial indicator studied. In studies conducted by Havelaar *et al*. (1993) and Baggi *et al*. (2001) regression equations for F-RNA phages compared to other enteric viruses in river water and lake water were statistically equivalent. These relationships support the possibility that enteric virus concentrations can be predicted from F-RNA phage data (Havelaar *et al*., 1993).

Baggi *et al*. (2001) investigated the different effects of sewage treatment on viral contamination in rivers, which receive water from treatment plants without a final sand filtration step. The study showed that these waters were highly contaminated with human enteric viruses and bacteriophages. The persistent discharge of treated sewage is one of the most obvious sources of degradation of urban freshwater ecosystems (Luger & Brown, 1999). However, these relatively constant impacts are intensified by emergency events like intermittent spillages of raw sewage due to power failures, pump -, or pipe failures of blockages, and inadequate hydraulic capacity during high rainfall events (DWAF, 2009). The impact of non-compliant wastewater discharges from a wastewater treatment plant is considered to be a major contributor to salinity, eutrophication and microbiological problems currently observed (DWAF, 2009). It is estimated that 2-10% of phytoplankton primary production is channelled through “the viral shunt” in the microbial food web. Cell lysis implies that organic material (nitrogen and phosphate) is lost from the grazing food chain and becomes available to bacteria, which thrive on dissolved organic and material and nutrients. This means that phage activity has a direct effect on the carbon budget of aquatic systems (Sandaa, 2009).

Phages may also be released at a time when the physico-chemical conditions of the water have changed (temperature, pH, UV light) so the fate of viruses may depend on whether they have formerly interacted with biofilms or not (Skraber *et al*., 2009). Several studies have shown that infectious viruses persist longer when associated with solids (specifically organic solids) instead of free in the water (Smith *et al*., 1978;, Gersberg *et al*., 1987; Chung & Sobsey, 1993; Sakoda *et al*., 1997; Karim *et al*., 2004). Not considering the potential role of biofilms in the fate of enteric pathogens may lead to false assumptions in risk assessment, modeling research or epidemiological investigations (Skraber *et al*., 2009). In a study by Skraber *et al*. (2009) it was demonstrated that F-RNA phage levels were still stable two months after any new faecal pollution input into the water system.

2.6 MORPHOLOGIES OF PLAQUES

Plaques are formed by disbanding (lysing) of the bacteria cells. A single viral particle infects a single host cell. There it multiplies within it and causes it to lyse (Nishihara, 2002). This liberates many progeny phages which diffuse away from the original site infecting and lysing more cells. This process is repeated until a plaque grows to be visible, eventually, to the naked eye (Douglas, 1975). Some bacteriophages lyse the cell in which they have multiplied by producing lysozyme. Lysozyme attacks the murein of the cell wall, weakening it so that it bursts and liberates the phage within (Douglas, 1975). Other phages uses a low-molecular-mass hydrophobic protein to trigger lysis of the F-RNA coliphages host in genogoup I and II phages (Kastelein *et al*., 1982; Coleman *et al*., 1983; Nishihara, 2002). In genogroup III phages, it has been shown that the maturation (A2) protein of Qβ page induces *E. coli* cell lysis by itself (Karnik & Billeter, 1983; Winter & Gold, 1983; Nishihara, 2002). This A2 (maturation/lysis) protein of RNA phage Qβ blocks cell-wall biosynthesis (Nishihara, 2002). These different methods of lysing and different degree of osmotic pressure in the cells may attribute to distinctive plaque morphologies. MS2 and GA phages show harsh rupture of host cells (Nishihara, 2002). The different proteins responsible for lysing of cells have been studied in depth (van Duin, 1998; Kastelein *et al*., 1982; Coleman *et al*., 1983; Karnik & Billeter, 1983; Winter & Gold, 1983; Young, 1992; Young *et al*., 2000; Bernhardt *et al*., 2000, 2001). Yet, no study could be found linking these means of lysing to different morphologies of lysis zones.

Plaque characteristics can be quite useful in distinguishing different phages. The size may vary in limits for any particular phage, but whereas some phages typically produce plaques of 5 mm diameter or more, others rarely exceed 1 mm. The shape of most plaques is circular. The plaque margin may be sharp or diffuse and there may be a zone of turbidity surrounding it (Douglas, 1975; Nur ilida *et al*., 2013). In each case, the size and appearance of a plaque can give important information about the virus responsible for it. Thus the local lesion response is useful for purposes of diagnosis and quantitation (Fraenkel-Conrat, 1969). As yet, there has been no methodical experimental investigation on how different phage characteristics influence the formation of plaques (Gallet *et al*., 2011).

In 2013, a review article was published by Nur ilida *et al*. that reviewed all the different proteins used by phages for lysis. It was clear from the article that as soon as molecular techniques were standardised to identify phages, there has been little research focus on identification of phages using plaque morphology. In the same year Gallet *et al*., also observed this and tried to fill this research gap. It was establishing that absorption rate, lysis rate, and virion size (this includes the presence of appendages) has an effect on plaque size and formation.

2.7 ENUMERATION OF BACTERIOPHAGES

Each phage has a limited range of host bacteria that it can infect and lyse. Viable count techniques are based on the lysis of a cultivable host. The method available for performing viable counts is the plaque-forming unit (PFU). The PFU method is used to determine the number of viruses that cause lysis of bacteria cells that grow on solid medium (Sandaa, 2009). This is an infectious quantitative assay, seeing as it only quantifies the number of virus particles present in an inoculum that gives rise to plaques. Plaques are a localised area of virus-induced cell pathology (Burleson *et al*., 1992). Each plaque represents a single virus in the original sample that infected a bacteria cell and created an ever widening circle as progeny phages infect new host cells and lysed them (Levine, 1992).

The PFU double-layer-agar method is useful for quantification and isolation of coliphages (ISO, 1995; ISO, 2000). In this method, solutions containing bacteriophage are mixed with an excess of susceptible bacteria and appropriate semi-solid agar, plating the mixtures on solid agar in petri dishes, and incubating them (Douglas, 1975; Fraenkel-Conrat *et al*., 1988; ISO, 1995; ISO, 2000; Sandaa, 2009). In these assays, each infectious particle induces an area of focus of cell killing or transformation. Direct proportionality between virus particle count and number of foci of infection is generally accepted for bacteriophages. Nevertheless, biological variability, chance and other factors affect this quantification method. To compensate for this effect, the quantitative data is referred to as plaque-forming particles (pfp). This refers to the amount of viruses that were able to infect host cells under the specific conditions of the experiment and not necessarily to the statistical significant amount of viruses present in the sample (Douglas, 1975; Fraenkel-Conrat *et al*., 1988).

International standardisation of methods for bacteriophage detection and enumeration was necessary to promote further development of this field (AWPRC, 1991). The double-layer-agar phage plaque assay has the advantage of technical simplicity and low cost (AWPRC, 1991). The choice of a suitable bacterial host is of paramount importance (AWPRC, 1991; Ganesh *et al*., 2013). Wild *E. coli* strains are normally poor hosts for phage enumeration in water, and best results are obtained with rough/semi-rough mutants. Several studies have compared host strains and *E. coli* C was generally found to yield the highest plaque counts (Havelaar & Hogeboom, 1984). The *E. coli* K12 Hfr and Salmonella typhimurium (lac+, nal+) WG 49 is generally used for quantification of F-RNA phages (Havelaar & Hogeboom, 1984; ISO 1995; Baggi *et al*., 2001; Ganesh *et al*., 2013). For reproducible counts it is essential to use host cultures in the early exponential growth phase (Havelaar & Hogeboom, 1984).

2.8 TRANSMISSION ELECTRON MICROSCOPY

In 1939, the first virus was visualised using electro-microscopy (Douglas, 1975; Fraenkel-Conrat *et al*., 1988). To visualise a virus directly by electron microscopy requires at least a million particles (Fraenkel-Conrat *et al*., 1988). Thus, sufficient replication and survival of the phage must occur. Many viruses, however, replicate by low titers and may decompose during attempts to isolate or transmit them (Fraenkel-Conrat *et al*., 1988).

The preparation of the specimen is of great importance (Fraenkel-Conrat *et al*., 1988). The electron microscope operates in high vacuum; hence the specimen must be free from water and other volatiles (Douglas, 1975). Negative staining yields quite clear and informative images (Fraenkel-Conrat, 1969; Fraenkel-Conrat *et al*., 1988). The process consists of adding a heavy-atom electron-opaque salt stain (e.g. uranyl acetate or phospho tuncstic acid) to the neutral aqueous virus solution and allowing the mixture to dry on the specimen film (Douglas 1975; Fraenkel-Conrat *et al*., 1988; Sandaa, 2009). Under these conditions the stain does not combine with the viral components but stains the background, including all holes and crevices, so that the particles are revealed in great detail (Douglas 1975; Fraenkel-Conrat, 1969; Fraenkel-Conrat *et al*., 1988). For Transmission Electron Microscopy (TEM), phages are harvested directly from a plaque onto electron microscopy copper grids or phage suspensions are centrifuged and concentrated by ultra-filtration and then transferred to copper grids (Sandaa, 2009).

2.9 PREPARING FOR PCR OF PHAGES

Different viruses contain either RNA or DNA, but not (substantial amounts of) both; each can be double- or single stranded nucleic acid chains (Douglas , 1975; Fraenkel-Conrat *et al*., 1988). In this study, with regards to the molecular aspects, focus was placed on the detection of single-stranded RNA bacteriophages. This was done based on the fact that entroviruses are small single-stranded RNA viruses (Bourlet *et al.,* 2003).

To separate viruses from cellular proteins, the particulate nature of viruses was exploited, as well as their higher buoyant density which is attributed to their nucleic acid component (Fraenkel-Conrat, 1969). Because of their small size, phages are kept in suspension by diffusion and will not sediment of their own accord, no matter how long a preparation is allowed to stand (Douglas, 1975). Normal bench centrifuges are used to separate viruses from other debris in the sample. This is followed by ultracentrifugation with which phages are sedimented into compact pellets using centrifugal forces higher than 5000 rpm (Douglas, 1975, Sandaa, 2009).

2.10 SELECTED TEMPERATE WATER SYSTEMS IN THE NORTH WEST PROVINCE, SOUTH AFRICA

The majority of the North West Province falls within the Savannah Biome, while the remainder falls within the Grassland Biome (DACE, 2002). The climate of the Province is characterised by well-defined seasons with hot summers and cool sunny winters. The climate and rainfall vary from the more mountainous and wetter eastern region to the drier, semi-desert plains of the Kalahari in the west. The rainy season usually occurs from October to March (DACE, 2002). Most of the rainfall occurs as thunderstorms during the summer period of October to April (DEAT, 2005). Maximum temperatures (± 31°C) in this region occur in January, while minimum temperatures (± 3°C) occur in July (DEAT, 2005). Water temperatures in this region range between (18 and 12°C) (DWAF, 2009). In the North West Province of South Africa the Mooi River, Upper Harts River and Schoonspruit River have waste water treatment plants integrated to those water systems. Thus these catchments must ensure the development of water quality management strategies to manage the impacts originating from them, thereby alleviating the stress currently being placed on the Rivers. However, routine viral monitoring is not required for 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 (Ganesh *et al*., 2013).

A map of the North West Province including all its water systems and major towns can be viewed in section 3.1 (Figure 3.1). The main challenges on the environment of the North West Province are from land-uses such as formal and informal urbanization contributing faecal contamination, agriculture (nutrient as well as faecal contamination), mining, industry and other economic activities (DACE, 2002; DWAF, 2009). Mining activities, especially platinum and gold mining form the back-bone of the provincial economy, agriculture is the second-most important sector, while maize and sunflowers are the most important crops grown. In addition, cattle and game farming are also well-established (DACE, 2002). Agriculture in the eastern, wetter parts of the province largely comprises livestock and crop farming, the central and southern regions are dominated by wheat and maize farming, while livestock and wildlife farming occurs in the semi-arid western region of the Province. The surface waters in the Province are in the form of rivers, dams, pans, wetlands and dolomitic eyes fed by aquifers. The North West Province relies heavily on ground water resources to meet its needs (DACE, 2002). Main groundwater water quality issues in the province include high levels of dissolved mineral levels, nitrates and fluoride concentrations in certain areas, due to both natural and human-induced factors (DACE, 2002).

2.10.1 MOOI RIVER

Gold mining operations on the West Rand have led to significant contamination of the Mooi River (DWAF, 2009). The Mooi River catchment area includes the mining areas of Westonaria, Carletonville and Potchefstroom (DWA, 2013). The major utilisation of the Mooi River water is irrigation (DWAF, 2009). Land use in this Water Management Area (WMA) is characterised by expansive urban, mining and industrial areas in the northern and western parts. Other development in the WMA is dry land agriculture and livestock farming (DWAF, 2009). Urbanization and agriculture in this region are contributing faecal contamination to the water system (DWAF, 2009). There are many informal urbanisations within the Mooi River region, giving rise to diffuse sources of pollution, especially faecal pollution, and possible consumption of unsafe surface and groundwater (DWAF, 2006; DWA, 2013). Some of this water is also subtracted by farmers along the lower reaches of the river for livestock watering and domestic supplies. The Mooi River is further used for angling and general recreational purposes (DWAF, 2006). At one point the Mooi River tributuary brings with it large return flows from mine discharges and seepage, sewage effluent, and irrigation return flows.

Historically (DWAF, 2009; DWA, 2013) The Mooi River had the following parameter values: Nitrogen concentrations in this river is historically relatively low (mean of 0.854 mg/l) yet fluctuate significantly. Dissolved salts and total dissolved solids (TDS) concentrations are historically high (448 – 560 mg/L). While the sulphate concentration in the river historically averages 104 mg/L. pH ranges from 8 – 8.7. Phosphate has a mean average of 0.89 mg/L. Typical of a temperate water system DWA (2009) notes that the water quality of the Mooi River worsens during dry weather flows. DWAF (2009) notes the Mooi River as a key area requiring attention in the Vaal WMA.

2.10.2 UPPER HARTS RIVER

Most of the negative impacts on quality of water in Upper Harts River are associated with dry land agriculture, livestock farming and abstraction due to limited centre pivot irrigation. Golder Associates (2010) noted that two sewage treatment plants illegally release untreated sewage into the Upper Harts River. This may have a degrading effect on water quality of Barberspan, downstream. Barberspan, a Ramsar site, is important for recreational activities and is fed by the Upper Harts River. The population density is low. The area hosts mining, manufacturing and irrigation agriculture sectors (DWA, 2013). Historically (DWAF, 2009), the Harts River has had the following mean average parameter levels: pH 8.2 – 8.4, phosphate 0.03 mg/L, TDS of 1118 mg/L, sulphate of 230 mg/L and nitrite of 0.36 mg/L.

2.10.3 BARBERSPAN

The Barberspan is a natural, shallow, alkaline lake which became perennial after it was artificially connected in 1918 with the Harts River. It is one of the few permanent natural water bodies on the western Highveld of South Africa (Golder Associates, 2010). This site was listed during 1975 as a wetland of international importance according to the Ramsar Convention (DWA, 2013). Barberspan has not dried up since this modification (Golder Associates, 2010). Migrating aquatic bird species use the pan as an important stop-over. Barberspan is registered as an Important Bird Area of South Africa (SA026) because it regularly supports a significant number of globally threatened or near-threatened species (Barnes, 1998; Golder Associates, 2010). In 1954 it was proclaimed that the whole of the pan is protected as a Provincial Nature Reserve and therefore State controlled (Golder Associates, 2010). This imposes on the government a level of additional responsibility for ensuring that it is adequately protected (Cumming, 2009). The area is used for research on birds, and an angling resort and recreational area (Allan *et al*., 1996; Golder Associates, 2010).

Barberspan receive both surface and groundwater flows, which accumulate in the depression owing to a generally impervious underlying layer, which prevents the water draining away (Goudie & Thomas, 1985; Marshall & Harmse, 1992). The most important manner in which water leaves the pans is by evaporation off the pan surface and by transpiration from plants in the vicinity (Golder Associates, 2010). One of the reasons this water system was included in this study is due to the concern that excessive nutrients and organic pollution is entering Barberspan. This may be a huge threat to the long term maintenance of Barberspan and possibly Leeupan. Legislation and its effective enforcement should be considered in order to stop the environmental degradation of Barberspan (Golder Associates, 2010). Historically, (Golder Associates, 2010), Barberspan had TDS levels ranging from 445 ppm at its inflow to 1200 ppm at its outflow, DO levels ranging from 2 mg/L to 14 mg/L and COD levels ranging from 10 mg/L to 124 mg/L.

2.10.4 CROCODILE (WEST) RIVER

The main sources of pollution in the Crocodile (west) River area is sewage effluent, urbanised and informal settlements, agriculture and industries (DEAT, 2005). Small open-cast stone and sand quarries, and a number of large platinum and chrome mines are common in this area. (DEAT, 2005). Cumulative impacts arising from the upper and middle Crocodile River catchments have seriously affected the flow regime of the lower Crocodile River. In most years, the river stops flowing and the diversity of aquatic fauna is declining (DEAT, 2005). For most of its length, the river flows through game and cattle country (DEAT, 2005). DEAT (2005) concluded that water quality of the Crocodile (west) River was poor, have low to intermediate

levels of nutrients, and is heavily contaminated by organic pollution attributed to high agricultural return flow. Historically the Crocodile (west) River has parameter level mean averages of: TDS 455 ppm - 1950 ppm, pH 6.5 – 7.5 and nitrite levels greater than 10 mg/L are sometimes found (DWAF, 2004).

2.10.5 MARICO RIVER

The Marico River has two sources – the first (Groot Marico) being a complex of three dolomitic eyes. These eyes pour crystal clear, pristine waters into the start of the river. The second source (Klein Marico) is the catchment area immediately next to the Marico eyes and several other springs (Anon, 2010). The main sources of pollution in the Marico River area are sewage effluent, urbanised and informal settlements, as well as agriculture and industry runoff (DEAT, 2005).

Groot Marico's water quality is good. The nitrogen and phosphate levels are low and intermediate and free from significant organic pollution (DEAT, 2005). Klein Marico's water quality in general is fair - flows have intermediate levels of nutrients and there is some evidence of organic pollution (DEAT, 2005). Middle Marico's water quality is averagely acceptable - flows have low to intermediate levels of nutrients and are free from significant organic pollution (DEAT, 2005). Lower Marico's water quality is reduced because of irrigation return flows (DEAT, 2005). More than half of the total water use in the WMA comprises urban, industrial and mining use, approximately a third is used by irrigation and the remainder of the water requirements are for rural water supplies and power generation (DEAT, 2005). Historically the Crocodile (west) River has parameter level mean averages of: TDS 455 ppm - 1950 ppm, pH 6.5 – 7.5 and nitrite levels greater than 10 mg/L are sometimes found (DWAF, 2004).

2.10.6 SCHOONSPRUIT RIVER

Land use around Schoonspruit River is predominately gold mining, dryland and limited irrigated agriculture and urbanization (DACE, 2002; DWA, 2013). The Schoonspruit River dolomitic eye acts as source for the upper part of the catchment, providing water for irrigation agriculture and the Ventersdorp settlement, as well as base flow in the river. There is also substantial irrigation abstractions through boreholes from the dolomitic compartments feeding the eye (DWA, 2013). Major impacts include mining and agricultural runoff (containing high levels on nitrogen sulphate, phosphate and faecal contamination), flow regulation for irrigation use, and water quality related problems due to urbanization, mining and agriculture such as sewage and agricultural runoff pollutants (DWA, 2013). In 2005, there was a significant increase in faecal indicators, which indicated sewage pollution and improper disinfection of treated sewage effluent (DWAF, 2009). Historically (DWAF, 2009), Schoonspruit River has mean average

parameter levels of: 168 mg/L sulphate, 4.11 mg/L nitrate, 0.95 – 1.08 mg/L phosphate, 676 ppm TDS, pH range of 7.7 – 9.3, and COD of 7.05 mg/L.

2.11 PHYSICO-CHEMICAL PARAMETERS.

The term water quality describes the physical, chemical, biological and aesthetic properties of water that determine its fitness for a variety of uses and for the protection of aquatic ecosystems (DWAF, 1996a). Many of these properties are controlled or influenced by constituents who are either dissolved or suspended in water (DWAF, 1996a; b). Few specific recommendations on the general chemical characteristics of recreational and irrigational waters are available and where they are, the full range of possible irritants and toxicants cannot practically be addressed (DWAF, 1996a; b; c). Various external physical and chemical elements, such as temperature, pH, salinity, and ions, determine the occurrence and viability of bacteriophages. These factors can also inactivate a phage through damage of its structural elements (head, tail, and/or envelope), lipid loss, and/or nucleic acid structural changes (Ackermann *et al*. 2004). The most significant factors that affect inactivation of phages are temperature, suspended solids, biological activity and sunlight (DWAF, 1996a).

2.11.1 TOTAL DISSOLVED SOLIDS (TDS) & SALINITY

One of the most important parameters to describe water quality is the total amount of material dissolved in it. This property is measured as Total Dissolved Solids (TDS) in mg/l (Golder Associates, 2010). TDS consists mainly of inorganic salts such as potassium, magnesium, sodium, calcium, bicarbonates, sulphates and chlorides, but contains small amounts of organic matter as well (WHO, 2011; Heydari & Bidgoli, 2012). Salinity is thus also a measurement of the amount of TDS present in the water (CSIR, 2010). Natural sources, sewage, urban runoff and industrial wastewater impact TDS in water (WHO, 2011). There is no reliable data on possible health effects associated with the ingestion of reasonable levels of TDS in drinking water (WHO, 2011). Domestic and industrial effluent discharges and surface runoff from urban, industrial and cultivated areas are examples of the types of return flows that may contribute to increased TDS concentrations. High TDS concentrations in surface waters are also caused by evaporation in water bodies which are isolated from natural drainage systems (DWAF, 1996a). Osmotic shock (radical change in salinity/TDS levels) inactivates bacteriophages by causing the phage nucleic acid to eject from the tail or their heads to break (Jończyk *et al*., 2011).

2.11.2 PH

The pH of most unpolluted water lies between 6.5–8.5. pH is an important operational water quality parameter (WHO, 2011). This is also the TWQR of recreational water (DWAF, 1996a) and crop irrigation water (DWAF, 1996c). The direct health effects of low and high pH levels

include acid and alkali burns, respectively (DWAF, 1996a). pH of greater or less than 7, has the ability to cause irritation of eyes, skin, ears and mucous membranes of the nose, mouth and throat of humans and animals. Ideally, water used for direct contact recreational activities should be as close to pH 7.4 as possible (DWAF, 1996a). The TWQR of pH for recreational water is between 6.5 and 8.5 (DWAF, 1996a).

When assessing the potential effect of a change in pH, it is important to note that some streams are naturally more acidic than others and their biotas are often adapted to these conditions. A change in pH from the normally encountered pH values in un-impacted streams may have severe effects upon the biota (DWAF, 1996d). Indirect pH changes include changes in the availability of toxic substances such as ammonia and aluminium. Most tailed viruses are stable at pH 5–9; a few are stable at pH 2 or pH 11 (ICTV, 2011, Taj *et al*., 2014). pH affects virus survival indirectly by influencing the extent of viral attachment and absorption to other particles and surfaces (Gerba, 1984; Taj *et al*., 2014). Feng *et al*. (2003) investigated the survivability of coliphages (MS2 and Qβ) in water and wastewater with regard to the effects of different pH on the phages. It was found that MS2 survives better in an acidic than in an alkaline environments, while the opposite was true for Qβ.

2.11.3 SULPHATE

Sulphur is essential for life, mainly as a component of amino acids, saliva, bile and the hormone insulin (DWAF, 1996c). Since most sulphates are soluble in water, it tends to accumulate to progressively increasing concentrations. Typically the concentration of sulphate in un-polluted water is 5 mg/L (DWAF, 1996c). A dietary deficiency of sulphur can depress microbial numbers and reduce microbial digestion and protein synthesis in animals. Adverse effects of deficiency are mainly due to a reduced amount of sulphur-containing amino acids necessary for protein synthesis. Symptoms associated with insufficient dietary sulphur are retarded growth and reduced wool growth (DWAF, 1996c). No adverse effects are found when livestock ingest sulphate at the concentration between 0 and 1000 mg/L (DWAF, 1996b). Sulphate is a key component in heparan sulphate (HS). This is very commonly expressed on the surfaces of virtually all cell types (including bacterial cells), making it an ideal receptor for viral infection (Zhu *et al*., 2011). The availability of sulphate in aquatic environments can thus influence the expression of this protein receptor and consequently influence the rate at which phages can infect host cells as well as replicate and survive in an environment.

2.11.4 NITRATE & NITRITE

Nitrate- and nitrite-ions are part of the nitrogen cycle and therefore occur naturally in the environment (WHO, 2007). Wastewaters and agricultural as well as urban runoff are natural

sources contributing nitrate to water. The largest contributor to anthropogenic nitrogen, in environmental water, is - nitrogen fertilizer and faecal pollution. These are also the main sources of nitrate in water in rural areas (Chang *et al*., 2010). Nitrate itself is not toxic, but the microbial reduction of nitrate to nitrite in the intestine is toxic (Adam, 1980; WHO, 2007). Under oxidising conditions nitrite is converted to nitrate, which is the most stable positive oxidation state of nitrogen and far more common in the aquatic environment than nitrite. Nitrate in water used by livestock is of concern, in that it can be readily converted in the gastrointestinal tract to nitrite, as a result of bacterial reduction (DWAF, 1996c). Nitrate does not cause direct toxic effects, but its reduced form, nitrite, does and is 10 - 15 times more toxic than nitrate. Nitrite is formed through the biological reduction of nitrate in the rumen or caecum, thus ruminants and horses are therefore susceptible to nitrite poisoning (DWAF, 1996c). Nitrate has no adverse effects on livestock, when concentrations between 0 – 100 mg/L are consumed (DWAF, 1996b). Nitrate and nitrite concentrations, in aquatic environments, also have no significant impact on the survival of phages (Yates *et al*., 1985).

In view of their co-occurrence and rapid inter-conversion, nitrite and nitrate are usually measured and considered together. Inorganic nitrogen is seldom present in high concentrations in un-impacted natural surface waters. This is because inorganic nitrogen is rapidly taken up by plants and converted to protein and other organic forms of nitrogen in plant cells. Inorganic nitrogen concentrations in un-impacted aerobic surface waters are usually less than 0.5 mg/L, while highly enriched waters concentrations may be as high as 5 - 10 mg/L (DWAF, 1996b). Since nitrogen is one of the major plant nutrients, plants actively absorb nitrate and ammonium ions from the soil solution, as do soil micro-organisms. Nitrate ions that remain in the soil solution can leach into irrigation water, and thus may pollute ground water (DWAF, 1996b). Crop yield starts decreasing when the TWQR of 5 mg/L nitrogen is exceeded (DWAF, 1996c).

2.11.5 CHEMICAL OXYGEN DEMAND (COD)

Chemical Oxygen Demand (COD) is an indirect indicator of organic matter in the water body (Hur *et al*., 2010). High levels of COD are an indication of serious water pollution (Kawabe & Kawabe, 1997; Yin *et al*., 2011). Industrial, agricultural and domestic wastes are the sources of organic matter in aquatic environments. Organic matter, present in dissolved form, causes undesirable tastes and odours of the water (DWAF, 1996a). COD levels of below 75 mg/L are acceptable for environmental water (DPW, 2012). Organic matter can promote phage survival as it promotes biofilm formation and in affect niches for phage replication and survival (Baggi *et al*., 2001). However in some instances it can interfere with phage binding sites and thus deter phage infections and replication (Pieper *et al*. 1997).

2.11.6 PHOSPHOROUS

Phosphorus in environmental water can be contributed by nonpoint pollution such as agricultural runoff due to excess fertilizer (Capece *et al*., 2007). A slight increase of phosphorous in the water can increase microbial growth (this includes phage replication) significantly (Lehtola *et al*., 2002), as phosphorus is a limiting growth factor. Phosphorus itself is harmless, but the toxic algal blooms that grows due to excessive available phosphorus, are toxic (Carpenter *et al*., 1998). Phosphorous in water systems promotes biofilm formation (Fang *et al*., 2009), and thus promotes phage survival. Concentrations between 0.1 and 0.5 mg/L are commonly found in environmental surface water (DWAF, 1996d). Elevated levels of phosphorus may result from point-source discharges such as domestic and industrial effluent (waste water treatment plants (WWTP's)), and from surface and subsurface non-point drainage. Non-point sources include atmospheric precipitation, urban runoff, and drainage from agricultural land. During rainfall events, phosphorus levels may be elevated by runoff from the land, and by re-suspension and flushing of deposited material from the river bed to the water column (DWAF, 1996d).

2.11.7 TEMPERATURE

Temperature affects almost all physico-chemical equilibriums and biological reactions (Delpla *et al*., 2009). Water temperature can have a direct or indirect effect on physical and biological parameters (LeChevallier *et al*., 1996; Park *et al*., 2010). Water temperature can affect the rates by which chemical reactions occur and therefore also the metabolic rates and distribution of aquatic organisms (DWAF, 1996d). Natural variations in water temperature occur in response to seasonal and day light cycles and organisms use these changes as cues for activities such as migration, emergence and spawning. Temperatures of inland waters in South Africa generally range from 5 - 30 °C throughout the year's seasons (DWAF, 1996d). Anthropogenic sources which result in changes in water temperature include discharge of heated: industrial effluents, effluents below power stations, return-flows of irrigation water; and removal of riparian vegetation cover (DWAF, 1996d). Higher temperatures reduce the solubility of dissolved oxygen in water, decreasing its concentration and thus its availability to aquatic organisms (Dallas & Day, 2004). The toxicity of most substances and the vulnerability of organisms are intensified as water temperature increases (DWAF, 1996d). Most organisms associated with freshwater, excluding birds and mammals, are unable to control their body temperatures and are therefore highly dependent on ambient water temperature and very susceptible to changes in water temperature. (DWAF, 1996d)

Heat sensitivity is variable, but the following general temperature effects has been established regarding phage activity (Taj *et al*., 2014): 1) Temperature lower than 4°C inhibit lysis. 2) lysis

takes place at temperatures between 15 and 41°C. 3) Ideal lytic activity occurs at 37°C. 4) At temperatures between 45 and 70°C phages become completely inactive. A study by Prigment *et al*., (2005) showed that under large temperature fluctuations, phages belonging to the *Myoviridae* family can protect themselves through intercellular location in pseudo-lysogens or confinement in a bacterial biofilm. Temperature plays a fundamental role in attachment, penetration, multiplication, and the length of the latent period of bacteriophages (Jończyk *et al*., 2011). An increase in temperature decreases the survival and activity of phages (Basdew & Laing, 2014). At lower than optimal temperatures, fewer phage genetic material penetrate into bacterial host cells, while higher temperatures can prolong the length of the latent stage (Tey *et al.,* 2009). F-RNA phages are less resistant to temperature changes than somatic coliphages (Mocé-Llivina *et al.,* 2003).

2.11.8 DISSOLVED OXYGEN

Gaseous oxygen (O_2) from the atmosphere dissolves in water and is also generated during photosynthesis by aquatic plants and phytoplankton. Oxygen is moderately soluble in water. Saturation solubility varies non-linearly with temperature, salinity and atmospheric pressure. The maintenance of adequate dissolved oxygen (DO) concentrations is critical for the survival and functioning of the aquatic biota because it is required for the respiration of all aerobic organisms. Therefore, the DO concentration provides a useful measure of the health of an aquatic ecosystem (DWAF, 1996d). As phages are not living organisms, DO has no direct impact on their survival. Indirect effects however, of DO concentrations in aquatic environments, include the survival of bacterial hosts needed for replication. In effect, if DO is not contributing to host survival, it is not contributing to phage survival (Vega *et al*., 2003). Aerobic organisms are dependent for respiration on the presence of dissolved oxygen in water. Anoxic or hypoxic conditions may be lethal within short time scales (minutes to hours) (DWAF, 1996d).

In unpolluted surface waters, dissolved oxygen concentrations are usually close to saturation (9.03 mg/L). Reduction in the concentration of dissolved oxygen can be caused by several factors (e.g. Re-suspension of anoxic sediments, turnover or release of anoxic bottom water from a deep lake or reservoir, presence of oxidizable organic matter). COD is used as a routine measurement for effluents, and is a measure of the amount of oxygen likely to be used in the degradation of organic waste. The amount of suspended material in the water affects the saturation concentration of dissolved oxygen, either chemically, through the oxygen-scavenging attributes of the suspended particles, or physically through reduction of the volume of water available for solution. Dissolved oxygen concentrations can be increased by natural diffusion of gaseous oxygen from the atmosphere into water (DWAF 1996d).

2.11.9 SUMMARY OF THE LITERATURE

The literature provided an overview that demonstrated that bacteriophages are well established as good indicator organisms. The information is related to viral safety of water systems as well as faecal pollution of aquatic ecosystems. In addition, presence of somatic coliphages and F-RNA bacteriophages can indicate faecal pollution of water systems. Genogroups of F-RNA phage can provide information regarding sources of faecal pollution, while phage plaques may provide more information about phage quantity. By establishing links between plaque morphology and phage virion morphology, plaque morphology may aid in preliminary identification of somatic coliphages. Characteristics of the rivers that were part of the study (Mooi River, Harts River, Baberspan, Crocodile River, Marico River, and Schoonspruit River) were also provided from the infrequent data sets that are available.

CHAPTER 3

MATERIALS & METHODS

3.1 STUDY SITE

Based on importance to the surrounding community and size, there are eight main rivers in the North West Province of South-Africa. They are the Crocodile, Groot Marico, Hex, Elands, Vaal, Mooi, Harts and Molopo Rivers (DACE, 2002). This study included four of these rivers: Mooi River, Upper Harts River, Crocodile River (west) and Marico River. Two other water systems were also screened: Barberspan and Schoonspruit River. These six water systems were chosen due to proximity as well as speculated specific possible pollution impacts and activities in and around these water systems. The pollution impacts and activities include: waste water treatment plant (WWTP) pollution, agricultural runoff, mine activities, industrial and settlement discharge, as well as runoff. A total number of 37 sampling points were identified and utilised in this study. Their co-ordinates are listed in Appendix A. Figure 3-1 is a map, of the six respective water systems, illustrating the locations of the 37 sampling points.

3.2 SAMPLE COLLECTION

Samples for each site were collected once in dry and once in wet season in order to assess the effects of seasonal-variations in source loading and water system dynamics. The experimental period was from April 2014 to October 2015. Sampling was conducted over three seasons. Two seasons during 2014 (April and June & October and November) as well as once during 2015 (April to May & October). During each sampling event, environmental samples were directly collected into sterile 50 ml glass (Schott Duran, Germany) bottles. These bottles were then transported to the NWU (North-West University) Potchefstroom campus laboratories in polystyrene boxes in order to prevent ambient air changing the temperature of the collected water. Sampling was done as prescribed by the ISO sampling guide (ISO, 2006). Water samples were always analysed within 24 hours of sampling.

The physico-chemical and general microbiological data form part of a WRC funded research project (K5/2347//3). The candidate was one of the members of the research team that collected some of the data. It was agreed that all participants would use data from the set and it is thus unavoidable that overlaps of the actual data in this dissertation, some M.Sc dissertations and the WRC final report will exist.

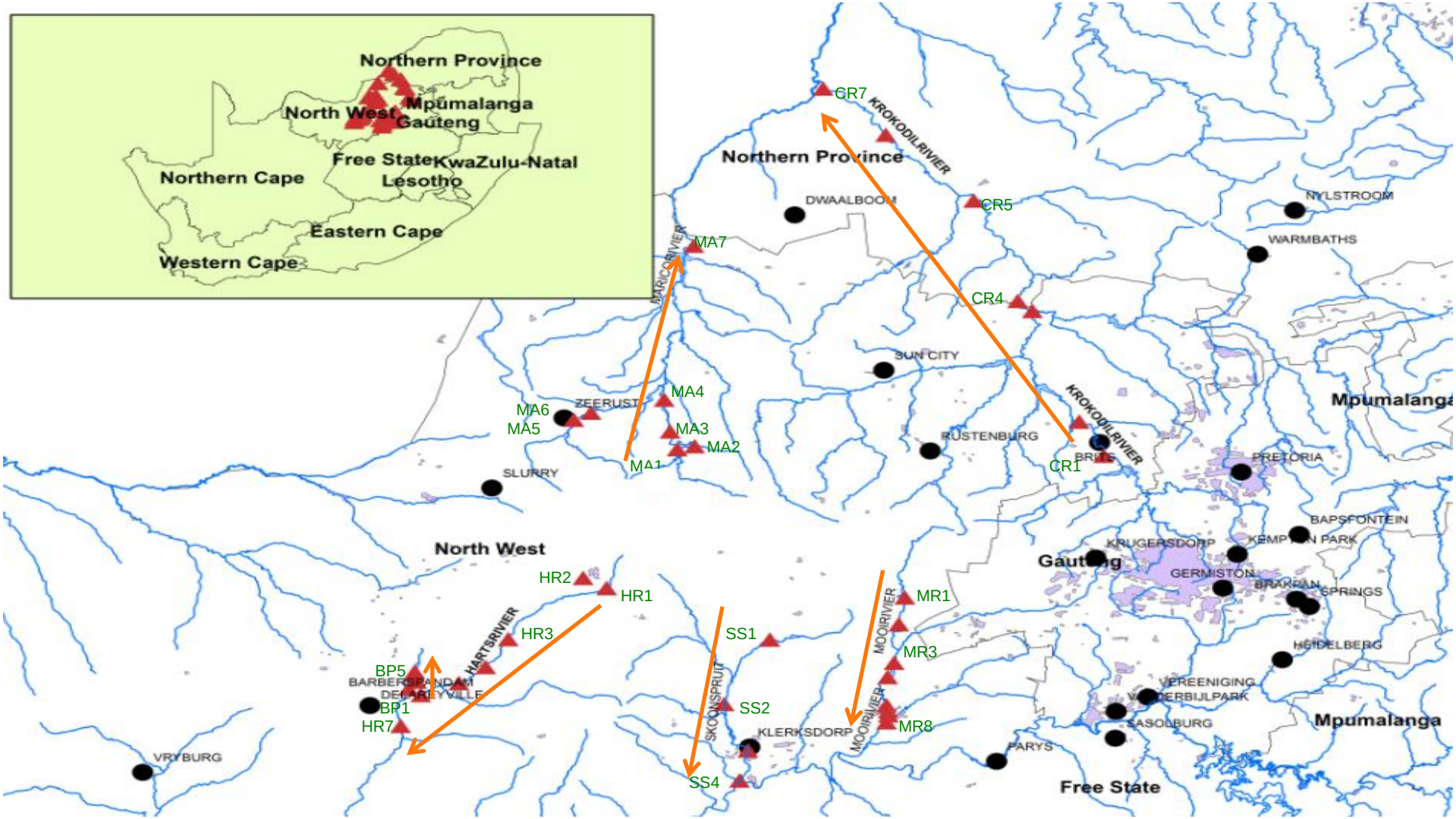


Figure 3-1 **A map of all the sites sampled during this study. The red triangles indicate the sampling sites. The green labels give some indication of specific sample site names (see Appendix A). The black circles represent major towns. The orange arrows indicate the direction of river flow.**

3.3 BACTERIAL STRAINS AND BACTERIOPHAGES

For somatic coliphages enumeration and characterization (TEM) purposes, *E. coli* strain WG 5 (ATCC 700078) was used as a host strain and grown in a Tryptone soy broth (TSB) (Merck, Germany) for 3 hours to optimal density. A nalidixic acid-resistant mutant, WG5, ATCC 13706 was used because it is more susceptible to a wide range of phages (Green *et al*., 1999; ISO, 2000; Ogorzaly *et al.,* 2009). This is due to its ability to allow the deletion of the genes that code for nuclease enzymes which serve as a defence mechanism for the host. This is due to the destruction of the host by nucleic acid of phages when injected into it (Grabow, 2001).

For F-RNA phage enumeration purposes *Salmonella enterica* serovar Typhimurium strain WG49 (ATCC 700730) was used as a host strain and grown in a Tryptone soy broth (TSB) (Merck, Germany) for 16 hours to optimal density. This strain possesses a plasmid from *Escherichia coli* K-12 which enables it to produce F-pili to which the F-RNA coliphages can adhere. Another advantage for using this strain is that it is no longer pathogenic as the genes that code for its pathogenicity have been removed, rendering this bacterium safe to work with in the laboratory (Grabow, 2001; Mooijman *et al*., 2002). The host culture must be in a logarithmic, or log phase of growth to ensure the formation of the fertility-pili.

Bacteriophages GA and Qβ were used as positive control references in all Reverse Transcription – Polymerace Chain Reaction (RT-PCR) experiments for groups II and III, respectively. Bacteriophages GA and Qβ were provided by J. Jofre (University of Barcelona, Spain). These stock cultures were kept at 4°C.

3.4 ENUMERATION OF PHAGES

For the somatic coliphage and F-RNA bacteriophage enumeration assays, the double-agar layer plaque assay was conducted and any plaques that formed were counted. The method was a modified version of the respective ISO 10705-1 (1995) & -2 (2000). This method was adopted from a method used by Midvaal Water Company – In-house Method (MWC-IM) (MWC-IM, 2010) and the respective ISO 10705-1 (1995) & -2 (2000). Midvaal Water Company is an accredited scientific services provider. The method makes use of a semi-solid top layer of agar mixed with bacterial host and sample containing phage. Semi-solid (ss) top agar layer is used to increase the diffusion potential of the phages as it provides less resistance than solid-agar. Increasing the size of a plaque as well as decreasing the margin of error in quantification on principle of one plaque one virus in original sample. The bottom layer of solid agar is the main source of nutrients for the bacteria in the semi-solid top agar by way of diffusion (Douglas, 1975).

Initially the host was grown in a liquid medium (TSB), and then mixed with a sample that contains the virus. The mixture of virus and host was then combined with molten ss-agar and poured onto a plate in which the agar content was high and contained a medium that the host can utilise. After incubation, the plaques (clear circular zones), which were areas in which cells have been killed, were counted and the number of infective virus particles in the original suspension estimated as plaque forming particles (pfp) (Sandaa, 2009).

3.4.1 SOMATIC COLIPHAGES ANALYSES

For the bottom agar plates, Tryptone Yeast Glucose-extract Agar (TYGA) was autoclaved for 15 min at 121ºC and then poured into 90 mm petri dishes and stored at 4ºC. For the top agar, semi-solid Tryptone Yeast Glucose-extract Agar (ssTYGA) was made and autoclaved for 15 min at 121ºC and kept, for later use, in a water bath at 60ºC to keep from solidifying.

Three pure colonies of WG5, which was the host bacterium for the somatic coliphage analysis, was inoculated into 50 mL of Tryptone Soy Broth (TSB) (Merck, Germany) in an 250 mL Erlenmeyer flask. The TSB was then incubated at 37°C on a shaker (100 rpm) for 3 hours at which time it had reached its optimal density to form a solid bacterial mat, when plated in a 90 mm petri dish. Into a sterile test tube 1.5 mL of the appropriate bacterial host and 2.5 mL of a test sample (or an appropriate dilution) was pipetted. The test tube containing the phage-host mixture was lightly rolled between the palms to mix thoroughly. After mixing the phage-host mixture, 6 ml ssTYGA was slowly pipetted into the test tube. Again the test tube was rolled between the palms to mix thoroughly and then rapidly poured over the bottom agar. This mixture was plated with minimum delay as plaque counts obtained start decreasing within 10 minutes from the time substances were mixed (Mooijman *et al*., 2002; Ogorzaly *et al*., 2009). The plates were quickly swirled to spread the molten top agar evenly over the bottom agar before solidifying. After the ssTYGA solidified, the plates were then inverted and incubated at 37ºC for 18 plus minus 2 hours. This was done in triplicate for each sample of each site. After the incubation period, the plates were viewed under ambient indirect light and any plaques that had formed were counted. The average number of plaques observed from each sample site were multiplied by a factor of 4, so the phage enumeration results could be given as plaque forming particles (pfp) per 10 mL of water sample (Sundram *et al*., 2002).

3.4.2 F-RNA COLIPHAGES ANALYSES

A modified protocol was followed as for the somatic coliphages assay (as described in section 3.4.1). The difference was using *Salmonella enterica* serovar Typhimurium WG49 (ATCC 700730) as bacterial host. Three pure colonies of WG49 was inoculated into 50 mL of Tryptone Soy Broth (TSB) (Merck, Germany) in an 250 mL Erlenmeyer flask. The broth was then

incubated at 37°C on a shaker (100 rpm) for 16 hours at which time it has reached its optimal density to form a solid bacterial mat, when plated in a 90 mm petri dish. Once optimal density and log phase was achieved after 16 hours of incubation the Erlenmeyer flask with log phase culture was incubated on melting ice or in a 4ºC fridge and used within 2 hours to ensure the presence of fertility-pili.

Similar to the somatic coliphages enumeration assay, 1.5 mL of the appropriate bacterial host (WG49) and 2.5 mL of a test sample (or an appropriate dilution) was pipetted into a sterile test tube. The test tube with the phage-host mixture was lightly rolled between the palms to mix thoroughly. After mixing the phage-host mixture, 6 ml ssTYGA was slowly pipetted into the test tube. Again the test tube was rolled between the palms to mix thoroughly and then rapidly poured over the bottom agar. This mixture was plated with minimum delay as plaque counts obtained start decreasing within 10 minutes from the time substances were mixed (Mooijman *et al*., 2002; Ogorzaly *et al*., 2009). Plates were quickly swirled to spread the molten top agar evenly over the bottom agar before solidifying. After the ssTYGA solidified, the plates were then inverted and incubated at 37ºC for 18 plus minus 2 hours. This was done in triplicate for each sample. After the incubation period the plates were viewed under ambient indirect light and any plaques that had formed were counted. The average number of plaques observed from each sample site was multiplied by a factor of 4, so the phage enumeration results could be given as plaque forming particles (pfp) per 10 mL of water sample (Sundram *et al*., 2002).

3.5 RT-PCR BACTERIOPHAGE IDENTIFICATION

3.5.1 REVERSE TRANSCRIPTION – POLYMERASE CHAIN REACTION (PCR) PROCEDURE

Water samples of Barberspan at the Hotel, North of Goosepan, and Outflow into Leeupan was sampled and molecular characterization was done of F-RNA phages found at the respective sites. Due to a drought in the North West Province at the time of sampling (October 2015) (SAWS, 2016) the three afore mentioned sites were the only sites at Barberspan that still had any water left in them. In this study, primers specific to a subgroup of F-RNA-phage for a RT-PCR technique was used to differentiate between faecal pollution sources without the need for viral isolation and membrane hybridization (Dryden *et al*., 2006). The reverse transcription – PCR procedure was as described by Ogorzaly & Gantzer (2006). For reverse transcription (RT), a litre of sample water was taken from 3 sites at Barberspan. The sites sampled were (in order of water flow): 1) BP3 (Hotel), 2) BP4 (North of Goosepan) and, 3) BP5 (Outflow into Goosepan). The respective 1 L samples were in turn filtered through 0.2 mm membrane filters and then through 0.45 µm membrane filters. The membrane filters were then cut up and washed with 2 mL of respective sample water. The 2 mL of sample for each site was then treated with chloroform and centrifuged for 20 min at 5000 rpm to remove any debris still left.

The top 0.5 mL of the sample was then placed in a new sterile Eppendorf tube and centrifuged at 12000 rpm (g-force: 8870.4) at 4 °C for one hour, or until a pellet was formed in the bottom of the Eppendorf tube.

Following this, 7.5 µl of extracted viral RNA was mixed with 1 µl of 10 mM dNTPs (Bio-Rad, USA), 1 µl of both reverse and forward primer (See Table 3-1) at 10 µM (Bio-Rad, USA) and 2.5 µl of DNase-RNase free water (Bio-Rad, USA). The first RT step was carried out at 65°C for 5 minutes and immediately put on ice for at least 1 minute. A reaction mixture consisting of 4 µl of 5x First-Strand Buffer (BioRad, USA), 1µl of 0.1 M DTT (BioTech), 1 µl (40 U/µl) of RNase Inhibitor (BioRad, USA), and 2 µl (100 U/µl) of iScript Reverse Transcriptase (BioRad, USA) was added in the reaction tube to give a total volume of 18 µl. This RT mixture was heated at 50°C for 1 hour, followed by 15 minutes at 70°C in order to inactivate the reaction. The RT products (cDNA) were immediately cooled to 4°C (Ogorzaly & Gantzer, 2006). PCR was performed on the cDNA obtained through RT as described by Ogorzaly & Gantzer (2006). The PCR protocol was as follows: 50°C for 2 minutes, 10 minutes at 95°C, followed by 50 cycles of 15 seconds at 95°C and 60 seconds at 60°C. This was done in an Techne Prime Elite thermocycler (Cambridge, UK), in 0.2 mL reaction tubes.

Primers designed by Ogorzaly & Gantzer (2006) and tested in urban raw wastewater were used (see Table 3-1). These primers were designed to target all sequenced bacteriophages belonging to one genogroups, without cross-reactivity with other genogroups, for each specific RT-PCR (Ogorzaly & Gantzer, 2006). The primers, without specifically targeting any given region of the genome, enabled identification of a specific regions preserved in each genogroup (Ogorzaly & Gantzer, 2006).

Table 3-1 Primer sequences for RT-PCR for detection of three F-specific RNA phage genogroups

Source	Strain	Genogroup		Sequence (5'-3')	Nucleotides	Location
Human	GA	GII	Forward	TGCAAACCTAACTCGGAATGG	21-bp	3273-3293[a]
			Reverse	AGGAGAGAACGCAGGCCTCTA	21-bp	3325-3345[a]
Human	Qβ	GIII	Forward	CCGCGTGGGGTAAATCC	17-bp	1412-1428[b]
			Reverse	TTCTTACGATTGCGAGAAGGCT	22-bp	1506-1527[b]

[a] Nucleotide positions based on Genbank accession number NC_001426: GA phage.
[b] Nucleotide positions based on Genbank accession number AF059242: MX1 phage.

3.5.2 AGAROSE GEL ELECTROPHORESIS OF PCR AMPLIFICATION PRODUCTS

The presence or absence of F-RNA cDNA amplifications was confirmed by electrophoresis of the PCR products on a 2% (w/v) agarose gel (SeaKem, US) in 1 x TAE buffer [20 mM Acetic acid, 40 mM Tris and 1 mM EDTA at pH 8.0). A mixture of 5 µl PCR amplification product and 5 µl 6 x orange loading dye (with added Gel Red) (Fermentas Life Science, US) was loaded onto each well of the gel. The fragment sizes of the PCR amplification products were confirmed by

loading a 100 bp molecular marker (O'GeneRuler, Fermentas Life Science, US) into the gel. Electrophoresis conditions were set at 70 V for 45 minutes. The gel images were captured under a UV light using a Bio-Rad ChemiDoc imaging system (Hercules, CA, USA) and analysed using Image Lab software (version 4.0.1).

3.6 TRANSMISSION ELECTRON MICROSCOPY (TEM)

A drop of distilled water was placed directly onto a somatic coliphage plaque. A carbon-coated copper formvar grid was then placed on the water droplet and left there for 5 minutes. This was done to allow phages to adsorb to the copper grid. The copper grid was then removed and excessive water was drawn off with filter paper. After this, the grid was negatively stained by placing a drop of Phospho Tungsten Acid (PTA) on the copper grid for 1 minute (Barreto-Vieira & Barth, 2015). The excessive PTA was drawn off by filter paper. The copper grid was then left for a further 10 minutes to ensure it was completely died. Phages were visualized and images captured by A. Jordaan of the Electron Microscope Laboratory at the North-West University, South-Africa. A FEI Technai G^{12} High Resolution Transmission Electroscope was used at 120 kV, supported by Digital Micrograph software and a GATAN bottom mount camera to visualize the somatic coliphages.

3.7 PHYSICO-CHEMICAL ANALYSES

The temperature, pH, salinity, and TDS, were taken onsite with a multi-meter (PCSTestr 35, Eutech Instruments Pte Ltd, Singapore). DO was also taken on site by a multi-meter (Orbeco Hellige Series 150 Multi-Parameter E-Chem Instrument). Before each measurement was taken a measurement beaker was cleaned with distilled water and rinsed with the specific water sample. The probe was also cleaned with distilled water before and after each measurement.

The concentrations of nitrate (method 8039), nitrite (method 8153), sulphate (method 8051), phosphorous (method 8178), and COD (method 8000) were measured at the NWU laboratories using the Hach Lange DR 2800 spectrophotometer (Güler & Alpaslan, 2009). Manufacturers' prescription for the various methods were followed (Hach Company, 2007).

CHAPTER 4

RESULTS

The following chapter contains the different results obtained from six different water systems in the North West Province, South Africa (Mooi River, Harts River, Barberspan, Crocodile River, Marico River, and Schoonspruit River). In section 4.1 the average bacteriophage levels for 2014 and 2015 in the six water systems are recorded. In section 4.2 different plaque morphologies obtained in this study are shown. Section 4.3 contain results obtained by RT-PCR of F-RNA phages found in Barberspan and Somatic coliphages by visualized TEM are illustrated. In section 4.4 the average results of the physical parameter (Temperature, TDS, Salinity, DO, and pH) for 2014 and 2015 are recorded, followed by the average chemical parameters (NO_2^-, NO_3^-, SO^{4-}, PO_4^{3-}, COD) results of 2014 and 2015. Finally, section 4.5 contains the correlation results between the physico-chemical parameters and selected microbiological organisms.

4.1 BACTERIOPHAGE LEVELS

Table 4-1 is a summary of average phage levels of six water systems in the North West Province. The data is for sampling periods in 2014 and 2015. Any values measured on the specific sampling dates are provided in Appendix B. Any value in Table 4-1 that exceeds 0 pfp/100 mL in the F-RNA phage column or the value of 20 pfp/100 mL in the somatic coliphage column, did not comply with TWQR for recreational and agricultural water systems (DWAF, 1996a, b, c). No universal ranges for coliphages in environmental water has been determined yet. The water systems in this study were thus considered as low, moderate, high, or very high in reference to individual sites in each system itself, as well as in reference to the other systems in this study.

In Table 4-1 it is depicted that the F-RNA phage levels (32.20 pfp/100 mL) from the Mooi River at Klerkskraal were higher than the somatic coliphages levels (22.90 pfp/100 mL). The somatic phage levels were higher at the Muiskraal (266.70 pfp/100mL) and Boskop dam inflow (Boskop A; 663.80 pfp/100mL). The phage levels were lower at the Boskop outflow (Boskop B; 84.40 pfp/100 mL). The somatic phage levels of between 134 and 154 pfp/100 mL at two of the urban sampling points (Thabo Mbeki bridge and Trimpark bridge) were 6 to 8 times lower than at the sampling points in the southern part of the catchment (Pedestrian Bridge and Viljoen Bridge). Overall the F-RNA levels were relatively constant and moderate at the respective sites (See Appendix B Table 6-2). Somatic coliphages levels in the Mooi River were considerably higher in 2014 wet season than in 2015 dry season (Appendix B Table 6-2).

Table 4-1 Average phage levels of the six water systems monitored in this study for the time period of 2014 and 2015.

Sample name	Water system	F-RNA phages (pfp/100 ml)	Somatic coliphage (pfp/100ml)
Klerkskraal	Mooi River	32.2	22.90
Muiskraal	Mooi River	0.00	266.70
Boskop A	Mooi River	4.40	663.80
Boskop B	Mooi River	7.08	84.40
Thabo Mbeki Bridge	Mooi River	1.10	134.30
Trim Park Bridge	Mooi River	1.10	154.30
Pedestrian Bridge	Mooi River	4.40	680.90
Viljoen Bridge	Mooi River	15.30	840.70
Lichtenburg Bridge	Harts River	10.00	2990.00
Village	Harts River	213.50	483.50
Biesiesvlei	Harts River	40.00	866.00
Vermaas	Harts River	160.00	2840.00
Sannieshof Bridge	Harts River	10.00	1690.00
Harts before Barberspan	Barberspan	945.00	1500.00
Train Bridge	Barberspan	370.00	330.00
Hotel	Barberspan	55.00	613.00
North of Goosepan	Barberspan	266.50	533.00
Outflow into Leeupan	Barberspan	2135.00	230.00
Before Brits	Crocodile River	3.30	0.00
After Brits	Crocodile River	8.30	66.50
Pienaars River	Crocodile River	11.70	53.00
Koedoeskop Bridge	Crocodile River	0.00	0.00
Croc Thaba	Crocodile River	0.00	3.30
Croc after Thabazimbi	Crocodile River	5.00	6.70
Croc turn	Crocodile River	1.70	35.30
Marico Eye	Marico River	0.00	356.80
Before Sterkstroom	Marico River	11.70	433.00
Sterkstroom	Marico River	0.00	1453.30
Before Bosveld Dam	Marico River	0.00	871.50
Below Klein-Maricopoort (Bospoort) Dam.	Marico River	1.70	750.00
After Marico Bosveld Dam.	Marico River	1.70	166.70
Derdepoort.	Marico River	1.70	309.80
Bodenstein	Schoonspruit River	0.00	454.50
Confluence with Brakspruit	Schoonspruit River	0.00	2806.50
Voortrekker	Schoonspruit River	0.00	529.00
Orkney	Schoonspruit River	238.70	20000.00

In the Harts River at the Lichtenburg Bridge site, somatic coliphage levels were very high (2990 pfp/100 mL somatic coliphage). There were lower somatic coliphage levels at the Village site (483.5 pfp/100 mL). However, the F-RNA phage level at this site (Village site) was extremely high (213.5 pfp/100 mL). This was possibly due to the impacts of the wastewater treatment plant that is upstream from the sampling site. High levels of somatic coliphage and the presence of F-RNA phage, were found at all the down-stream sites. There was an increase of F-RNA phage levels (from 40 pfp/100 mL to 160 pfp/100 mL) at Vermaas (see Table 4-1). No seasonal trend could be established for the Harts River in this study, due to severe drought in this area causing the river to run dry in 2014.

At Barberspan very high levels of F-RNA and high levels of somatic coliphage was detected (Table 4-1). Somatic coliphage levels were very high at the Harts before Barberspan site (1500 pfp/100 mL). Higher F-RNA phage levels (370 pfp/100 mL) compared to the somatic coliphage levels (330 pfp/100 mL) were found at Train Bridge. Very high levels of F-RNA phage (2135 pfp/100 mL) was detected at the Outflow into Leeupan site. No trend regarding phage levels could be established for Barberspan between the year of 2014 and 2015 due to severe drought in the area leading to severe decrease in water levels. However, in 2014 phage levels were considerably higher in the dry season than in the wet season (See Appendix B; Table 6-3).

In the Crocodile River, all phage levels were relatively low (Table 4-1 and Appendix B) in comparison to the other water systems screened and not the TWQR standards (DWAF, 1996a). The most significant results in this system was the average phage levels found at the site after Brits (66.5 pfp/100 mL somatic coliphage and 8.3 pfp/100 mL F-RNA phage) and Pienaars River (53 pfp/100 mL somatic coliphage and 11.7 pfp/100 mL F-RNA phage) sites. Phages were not detected at the Koedoeskop Bridge. After this site, phage levels started steadily increasing again, as the river flows north (Table 4-1 and Figure 3-1). Phage levels were consistently higher after the wet season, compared to phage levels after the dry season in 2015 in the Crocodile River (Tabel 4.1; Appendix B).

All the sites in the Marico River had very high levels of somatic phages (166.70 to 1453.3 pfp/100 mL). One of the sources of the Marico River is the Marico eye, which produces crystal clear spring water. It was thus surprising to find high somatic coliphage levels (356.8 pfp/100 mL) at this site. The last sampling points of the Groot- and Klein- Marico Rivers, before they converge to form the Middle Marico, have high somatic coliphage levels (Sterkstroom (1453.3 pfp/100 mL) and Below Klein-Maricopoort (Bospoort) Dam (750 pfp/100 mL)). However, after their convergence the average somatic coliphage levels were remarkably lower (After Marico Bosveld Dam (166.7 pfp/100 mL). Once again as is in the case of the Crocodile River, phage levels were consistently higher after the wet season, compared to phage levels after the dry season in 2015 in the Marico river (Table 4-1; Appendix B).

Alarmingly high somatic coliphage (20 000 pfp/100 mL) and F-RNA phage (238.7 pfp/100 mL) levels were detected at the Orkney site, in the Schoonspruit River. High somatic coliphage levels (450 pfp/100 mL, 2806.5 pfp/100 mL, and 529 pfp/100 mL. The rest of this water system also showed, when considering these consistently high somatic coliphage results, it is unusual that no F-RNA phage were ever found at any site in the Schoonspruit River except Orkney. Phage levels were consistently higher after the wet season, compared to phage levels after the dry season in 2015 in the Schoonspruit River (Appendix B).

4.2 PLAQUE MORPHOLOGIES

4.2.1 CLEAR, CLEAN EDGE PLAQUE

In Figure 4-1 is a photo illustrating a Petri dish with a solid mat growth of WG5 can be seen, with several phage lysis zones of different diameters. These results were obtained using the double-layer-agar plaque technique.

In Figure 4-1, plaques with clear, clean edges to the lysis zone were observed. The lysis zone is clearly visible with or without an ambient light source. Sizes of plaques that displayed this morphology varied greatly from very small (1 mm in diameter) to large (18 mm in diameter). No trend could be established about the frequency, size, sample sites, or seasonal variation regarding the formation of this plaque morphology. This plaque morphology was most commonly found when somatic coliphage were assayed.

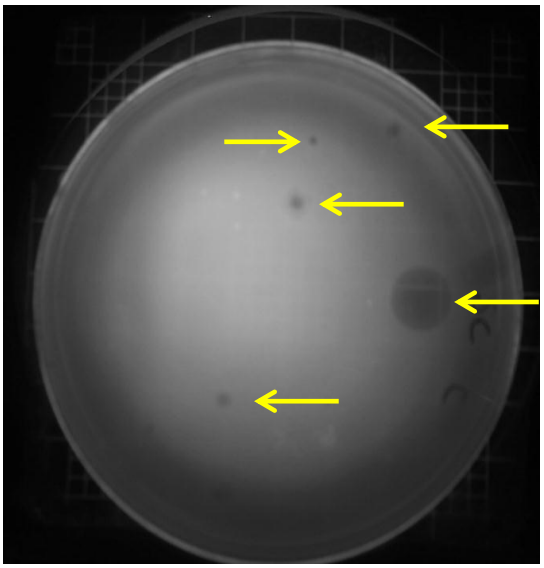


Figure 4-1: Photo of a Petri-dish containing one big clear, clean edge somatic phage plaque and four smaller clear, clean edge plaques obtained during this study.

4.2.2 FRIED EGG PLAQUE

Figure 4-2 is a photo illustrating a Petri dish with a solid mat growth of WG5. It also has several phage lysis zones of different diameters. These results were obtained using the double-layer-agar plaque technique. The fried egg plaques were composed of an inner circle which had clear edges. This inner circle was surrounded by a less clear outer circle, which presented sharp, round, definable edges. This gave it the appearance of a fried egg. As presented in Figure 4-2 this plaque morphology is clearly visible with or without an ambient light source. The size of the inner circle (1 mm – 5 mm in diameter), outer circle (4mm – 13 mm) and plaque as a whole varied. Once again no trend could be established about the frequency, size, sample sites, or seasonal variation regarding the formation of this plaque morphology. This plaque morphology was most commonly found when somatic coliphage were assayed, yet not quite as common as the clear, clean edges plaque formation.

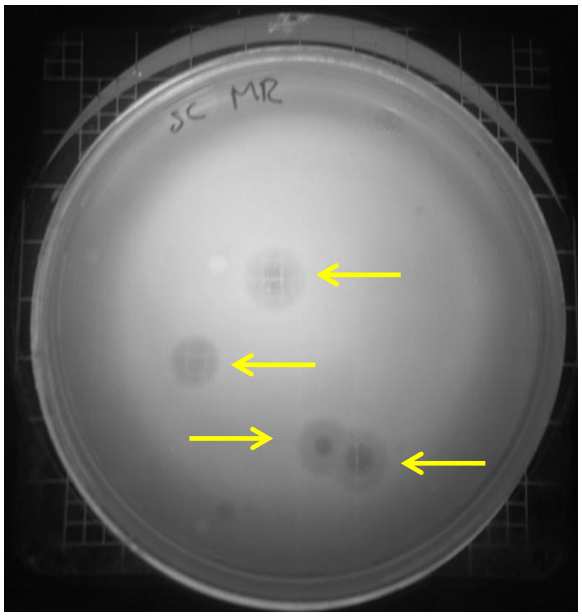


Figure 4-2 Photo showing clearly four somatic coliphage plaques of the fried egg morphology in the Petri-dish.

4.2.3 JAGGED EDGE, VAGUE PLAQUE

Figure 4-3 is a photo illustrating a Petri dish with a solid mat growth of WG5. It also has several phage lysis zones of different diameters. These results were obtained using the double-layer-agar plaque technique. Literature suggests that these plaques are most likely due to lysogenic life cycle than a lytic one (Grabow, 2001; Mullan, 2002).

Figure 4-3 shows examples of this jagged edge, vague plaque. These jagged edges and different areas of protrusions of the lysis zone can be seen. Lysis zones are also not as clear as the plaques described in Section 4.2.1 and 4.2.2. These jagged edge, vague plaques were not

visible by an ambient light source. Only by holding it against indirect white light could the plaques be seen. Sizes of plaques that showed this morphology varied from small (2 mm in diameter) to quite large (32 mm in diameter). No trend could be established about the frequency, size, sample sites, or seasonal variation regarding the formation of this plaque morphology. This plaque morphology was most commonly found when F-RNA coliphage were assayed. These plaques were more commonly larger than 10 mm in diameter.

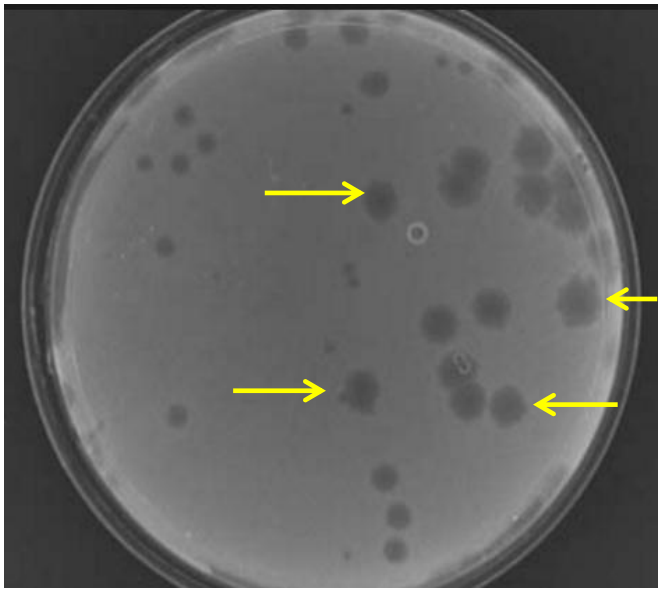


Figure 4-3 Photo showing multiple jagged edge, vague somatic coliform plaques.

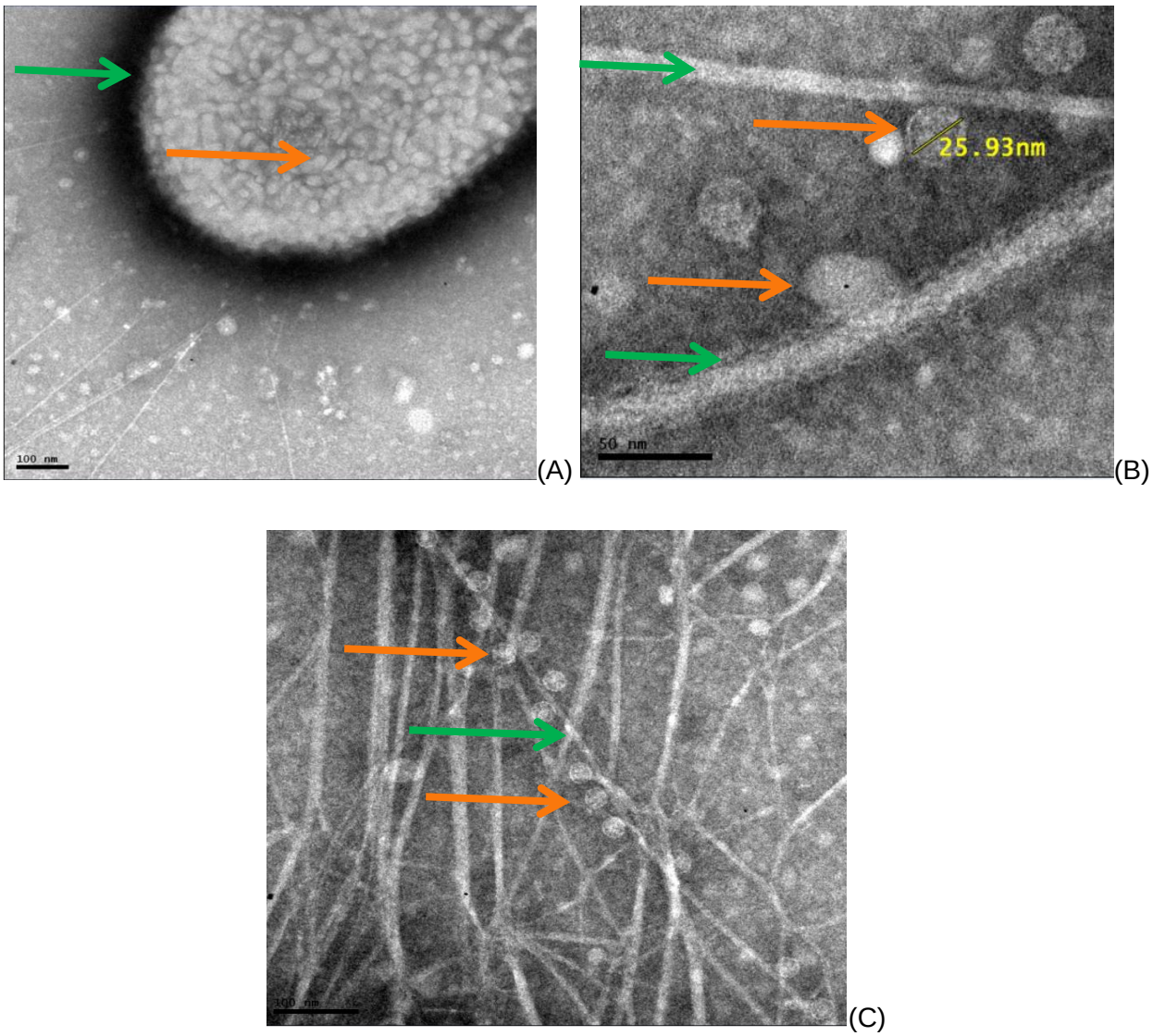
4.3 PHAGE CHARACTERIZATION

4.3.1 CHARACTERIZATION OF SOMATIC COLIPHAGE BY TEM

TEM was performed on phages found in each of the three respective plaque morphologies. This was done to determine if there was a link between specific plaque morphologies and specific phage types.

4.3.1.1 Clear, clean edge plaque

Figure 4-4 depicts three TEM images showing the attachment of somatic coliphages to WG5, as well as the morphology of the phages. These phages are from clear, clean edge plaques.



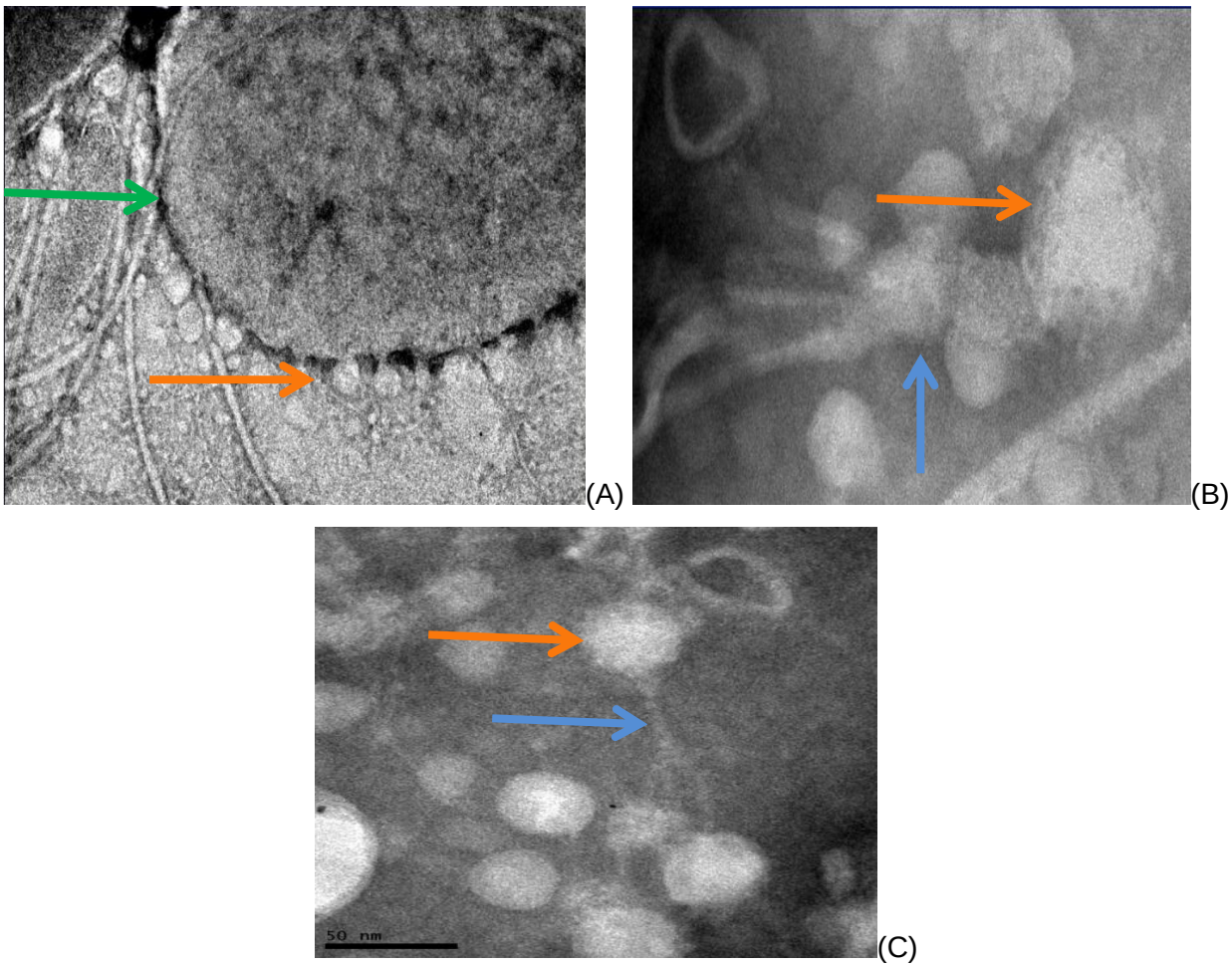
(A) (B) (C)

Figure 4-4 TEM images of phages found in a clear, clean edge plaque. Green arrows indicate bacteria cell structures. Orange arrows indicate phage heads.

Figure 4-4 (A) shows how multiple somatic coliphages adhere to the cell wall of an *E. coli* cell. In Figure 4-4 (B) the size of the head of the average phage found in the clear, clean plaque is indicated as 25.93 nm. These phages had the largest heads compared to the phages visualized in the other plaque morphologies (Figure 4-5 and 4-6). This characteristic influences the overall size of the virion. This may be important to consider when, possible reasons for plaque morphology is addressed. The phages found in this plaque had no tails as seen in Figure 4-4 (C), this again had an effect on virion size and thus diffusion in the agar, directly influencing plaque morphology. The greater majority of the phages in this plaque were adhered to the cell wall of the *E. coli* host cells.

4.3.1.2 Fried egg plaque

Figure 4.5 depicts TEM images showing the attachment of somatic coliphages to WG5, as well as the morphology of the phages themselves responsible for producing fried egg plaque.



(A) (B) (C)

Figure 4-5 TEM images of phages found in a fried egg plaque. The green arrow indicates an *E. coli* cell. The orange arrows indicate phage heads. Blue arrows indicate phage tails with appendages.

Figure 4-5 (A) shows how multiple somatic coliphages adhere to the cell wall of an *E. coli* cell. There was a marked difference in the number of phages that were adhered to cell walls in this plaque morphology compared to the plaque morphology in Section 4.3.1.1 The majority of phages in this plaque morphology were unattached. Figure 4-5 (B) and (C) show unattached, short tailed phages found in the fried egg plaque morphology. Important factors to notice in these photographs are the presence of short tails as well as tail fibers. These structural characteristics influences the overall size of the virion, and may affect the diffusion in the agar and may thus also explain the plaque morphology.

4.3.1.3 Jagged edge, vague plaque

Four TEM micrographs are depicted in Figure 4-6 are TEM photo's showing the attachment of somatic coliphages to WG5. The morphology of the phages responsible for producing jagged edge, vague plaque are also depicted.

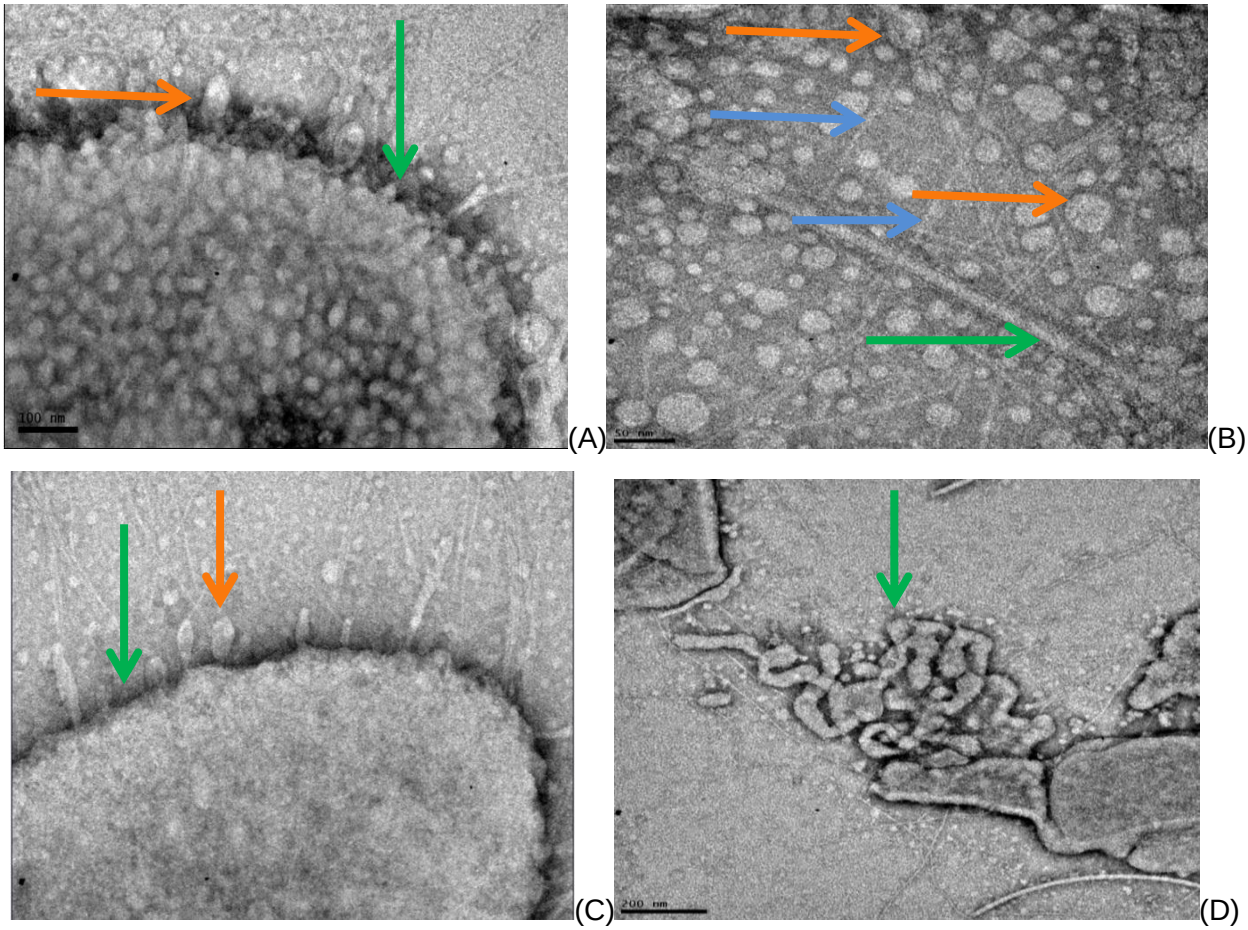


Figure 4-6 TEM images of phages found in a fried egg plaque. The green arrows indicate *E. coli* cell structures. The orange arrows indicate phage heads. Blue arrows indicate phage tails.

Figure 4-6 (A) shows how multiple somatic coliphages adhere to the cell wall of an *E. coli* cell. These phages were different from the phages in the other plaque morphologies in two regards. Firstly, their heads appeared to not have the rigid hexagonal structure after attachment to a cell wall. This can be seen in Figure 4-6 (C). Secondly, these phages had long tails. Several of these long tails are illustrated in Figure 4-6 (B). In Figure 4-6 (D) the dramatic evidence of phage infection, and destruction, by lysing its bacterial host, can be seen.

4.3.2 CHARACTERIZATION OF F-RNA PHAGE FOUND IN BARBERSPAN

Figure 4-7 is a 2% (w/v) agarose gel negative image showing cDNA fragments loaded into several lanes. These cDNA fragments were generated and amplified from F-RNA phage samples collected from the Barberspan water system. The cDNA shows that GA and Qß of F-RNA phages were detected at the Hotel, North of Goosepan, and Outflow into Leeupan sites. These two F-RNA phages are indicators of human faecal pollution.

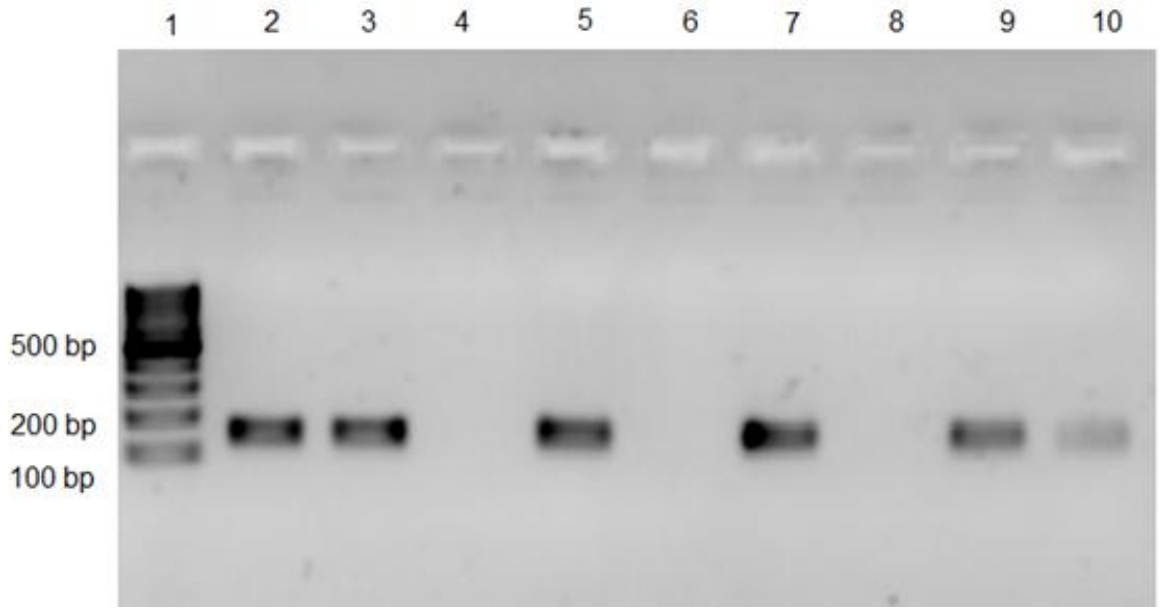


Figure 4-7 A 2 % (w/v) agarose gel showing the amplified Qß (Lanes 2 to 5) and GA (7 to 10) cDNA F-RNA phage results of samples from the Hotel (Lanes 3 & 9), North of Goosepan (Lanes 4 & 8) (which contained no amplified genes), and Outflow into Leeupan (Lane 5 & 7) sites, of Barberspan, after reverse transcription-PCR was performed. The non-template control was in Lane 6. The positive controls were in Lanes 2 and 10. Lane 1 contains a 100 bp molecular ladder. cDNA fragments were approximately 150 bp in size.

Detection of Qß and GA phage at the Hotel and Outflow into Leeupan samples demonstrate that these sites have potential human faecal inputs. The North of Goosepan site, which is situated between the other two sites no GA or Qß cDNA were detected. This indicates possible point pollution of human faecal matter at the Hotel and Outflow into Leeupan sites only and this may not be due to general or diffused pollution.

4.4 PHYSICO-CHEMICAL RESULTS

4.4.1 PHYSICAL PARAMETERS

Table 4-2 shows the lowest, highest and average levels of the selected physical parameters screened for in this study. These measurements demonstrate the temperate nature of the water systems monitored in this study.

From the data in Table 4-2, it is evident that the mean water temperatures in the river systems varied between 14.64±1.37 and 21.79 ± 2.36°C. The average salinity of Marico River (145.45 ± 67.54 ppm) was notably lower than the other systems monitored (range: 287.00±61.12 and 463.00 ± 84.95 mg/L). An extreme alkaline (11.20) condition was detected at the Vermaas site in Harts River during the dry season in 2014 (Appendix C; Table 6-8). Seasonal impacts on the Harts River is evident by the fact that all the maximum values (27.50°C, 527.00 ppm, 11.20, 732.00 ppm, 43.00 mg/L) for this river were recorded in the warm dry season, and the minimum values (19.00°C, 302.00 mg/mL, 7.44, 203.00 mg/mL, 17.16 mg/L) in the wet season sampling run (refer to Appendix C Table 6-8). All the water systems had average pH in the low alkaline range. The Orkney site in the Schoonspruit River consistently had the highest TDS values in the study (959 ± 12.77 mg/mL, 977.67 ± 3.06 mg/mL) (Appendix C; Table 6-12). The average TDS of Marico River (269.56 ± 126.48 ppm) was markedly lower than the other water systems investigated in the study. The total ranges of DO for Mooi River (4.30 ± 0.30 mg/L to 41.10 ± 0.00 mg/L) and Harts River (17.16 ± 0.00 mg/L to 43.00 ± 0.00 mg/L) varied more compared to the other water systems in the study.

4.4.2 CHEMICAL PARAMETERS

Table 4-3 shows the lowest, highest and average levels of the selected chemical parameters screened for in this study. These measurements were selected in order to show the temperate nature of the water systems monitored in this study.

Table 4-3 shows extreme maximum levels of nitrite (136.00 ± 7.07 mg/L) and nitrate (51.25 ± 18.17 mg/L) at Muiskraal (refer to Appendix D Table 6-15) in the Mooi River water system. The average nitrite level (15.97 ± 33.89 mg/L) measured in the Mooi River was high in comparison to the other water systems screened in the wet season. Sulphate levels in the Crocodile River were elevated yet similar across sampling sites and during the sampling periods. Levels ranged between 41.50 ± 6.36 mg/L and 78.50 ±3.54 mg/L (Table 4-3). Phosphate levels in the Marico River were low (average: 0.27 ± 0.22 mg/L) compared to the other water systems monitored in this study. The COD levels of Harts River (57.27 ± 26.16 mg/L) and Barberspan (67.00 ± 18.77) were relatively high compared to the other systems (averages from 11.09 ±11.85 to 31.86 ±38.53 mg/L; Table 4-3).

Table 4-2 Minimum, maximum, and average levels of selected physical parameters of the six temperate water systems throughout the study (2014-2015).

Water System	Temperature (°C) ± SD			Salinity (ppm) ± SD			pH ± SD			TDS (ppm) ± SD			DO (mg/L) ± SD		
	Min	Max	Avg	Min	Max	Avg	Min	Max	Avg	Min	Max	Avg	Min	Max	Avg
Mooi River	16.80	27.77	20.90	162.00	422.00	287.00	5.90	9.20	8.17	43.07	590.00	431.53	4.30	41.10	19.42
	±0.30	±0.06	±2.67	± 0.00	± 0.00	±61.12	±3.44	±0.00	±0.66	±1.16	±0.00	±133.74	±0.30	±0.00	±14.50
Harts River	19.00	27.50	21.79	302.00	527.00	434.40	7.44	11.20	8.85	203.00	732.00	544.00	17.16	43.00	30.49
	±0.00	±0.00	±2.36	±0.00	±0.00	±92.49	±0.00	±0.00	±1.11	±0.00	±0.00	±173.67	±0.00	±0.00	±8.38
Barberspan	12.80	25.60	19.42	304.00	585.00	463.00	7.87	9.97	9.08	443.00	824.00	648.56	38.40	47.30	42.73
	±0.00	±0.00	±5.23	±0.00	±0.00	±84.95	±0.00	±0.00	±0.65	±0.00	±0.00	±115.05	±0.00	±0.00	±3.65
Crocodile	11.93	25.07	18.49	251.00	377.00	314.95	7.96	9.09	8.56	456.33	745.67	580.69	1.50	8.03	4.59
River	±0.25	±0.67	±4.50	±5.29	±2.65	±46.14	±0.05	±0.01	±0.27	±0.58	±7.57	±91.92	±0.10	±0.25	±2.87
Marico River	13.93	21.10	17.17	45.87	267.00	145.45	7.52	8.56	7.99	81.50	513.33	269.56	1.97	12.27	7.35
	±1.23	±0.92	±2.66	±0.64	±4.36	±67.54	±0.33	±0.07	±0.31	±0.36	±3.21	±126.48	±0.23	±0.31	±2.38
Schoonspruit	12.63	16.60	14.64	278.67	541.67	379.84	7.50	8.53	7.93	510.67	977.67	694.63	2.63	12.37	6.85
River	±0.12	±0.44	±1.37	±0.58	±2.08	±96.18	±0.03	±0.01	±0.40	±0.58	±3.06	±176.34	±0.40	±0.96	±3.58

Abbreviations in the table above have the following meaning: Min: Minimum, Max: Maximum, Avg: Average

Table 4-3 Minimum, maximum, and average levels of selected chemical parameters of the six temperate water systems throughout the study (2014-2015).

Water System	NO_2^- (mg/L) ± SD			NO_3^-(mg/L) ± SD			SO_4^{2-}(mg/L) ± SD			PO_4^{3-}(mg/L) ± SD			COD (mg/L) ± SD		
	Min	Max	Avg	Min	Max	Avg	Min	Max	Avg	Min	Max	Avg	Min	Max	Avg
Mooi River	0.35 ±0.07	136.00 ±7.07	15.97 ±33.89	0.30 ±0.28	51.25 ±18.17	7.35 ±10.88	0.00 ±0.00	89.00 ±1.41	49.18 ±37.68	0.21 ±0.02	6.23 ±0.00	1.81 ±2.03	0.00 ±0.00	45.50 ±4.95	11.09 ±11.85
Harts River	0.00 ±0.00	11.00 ±0.00	1.67 ±3.30	0.00 ±0.00	6.00 ±0.00	1.91 ±2.02	9.00 ±0.00	90.00 ±0.00	42.80 ±31.40	0.86 ±0.00	4.43 ±0.00	2.14 ±1.55	31.00 ±0.00	98.00 ±0.00	57.27 ±26.16
Barberspan	0.00 ±0.00	7.00 ±0.00	1.22 ±2.21	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	1.00 ±0.00	79.00 ±0.00	35.33 ±23.52	1.32 ±0.00	10.4 ±0.00	3.82 ±2.67	35.00 ±0.00	87.00 ±0.00	67.00 ±18.77
Crocodile River	0.00 ±0.00	15.50 ±0.71	7.93 ±4.80	0.00 ±0.00	3.37 ± 1.21	1.22 ±1.11	41.50 ±6.36	78.50 ±3.54	66.04 ±10.93	0.22 ±0.06	3.09 ±0.21	1.66 ±0.83	3.00 ±1.41	58.50 ±7.78	23.50 ±14.13
Marico River	0.00 ±0.00	13.50 ±0.71	2.46 ±3.77	0.00 ±0.00	12.00 ±1.41	3.24 ±4.16	0.00 ±0.00	80.00 ±0.00	19.00 ±27.82	0.05 ±0.00	0.77 ±0.89	0.27 ±0.22	0.00 ±0.00	143.50 ±10.61	31.86 ±38.53
Schoonspruit River	0.05 ±0.07	21.50 ±0.71	8.11 ±8.60	0.20 ±0.00	9.00 ±4.24	3.49 ±3.31	1.00 ±0.00	118.50 ±2.12	54.94 ±48.82	0.04 ±0.00	4.05 ±0.07	1.11 ±1.47	0.00 ±0.00	101.50 ±2.12	24.94 ±36.12

Abbreviations in the table above have the following meaning: Min: Minimum, Max: Maximum, Avg: Average

Seasonal changes appeared to impact on the nitrogen cycle in the Harts River as nitrite was consistently more abundant than nitrate after the wet season and less abundant after the dry season (Appendix D; Table 6-15). Average nitrite levels were generally higher than nitrate levels (Table 4-3) The highest nitrite value in Barberspan was at the Outflow into Leeupan (7.00 ± 0.00 mg/L). At several of the sites over the sampling period, the measured nitrate and nitrite levels were very low thus causing the average levels to be low. However, the general trend was that the average nitrite levels were generally higher than the nitrate levels. This is probably due to bacterial activity (Jordaan & Bezuidenhout, 2016).

Seasonal impacts were evident in the Crocodile River as low levels for all (0.00 mg/L nitrite, 0.00 mg/L nitrate, 41.50 mg/L sulphate, 0.22 mg/L phosphate, 3.00 mg/L COD) were detected after the wet season and higher levels (15.50 mg/L nitrite, 3.37 mg/L nitrate, 78.50 mg/L sulphate, 3.09 mg/L phosphate) (except COD) after the dry season (refer to Appendix D Table 6-17). This phenomenon could potentially be due to dilution-concentration effects during the wet and dry seasons respectively. Overall COD, nitrates, phosphates, sulphates were detected at all the sites suggesting the maintenance of heterotrophic microbial population (Jordaan & Bezuidenhout, 2016). This may include faecal indicator bacteria that are the host of phages. In the present study, the focus was on the levels and diversity of phages at the various sites. It was thus necessary to also investigate the relationship of phage levels and physico-chemical parameters.

4.5 CORRELATION OF PHAGE AND PHYSICO-CHEMICAL PARAMETER

The results in this section show positive and negative correlations between environmental variables, phage species and sampling sites in redundancy analysis (RDA) graphs (Figure 4-8).

Figure 4-8 shows that the sampling sites from each of water systems monitored associated with one another. Yet most the systems generally associated different from one another. These associations are highlighted in the RDAs. Furthermore, it shows that somatic coliphage levels have a positive correlation to both *E. coli* and F-RNA phage. However, F-RNA phage and *E. coli* do not have a positive correlation to one another, and thus provide different information regarding the water systems.

Figure 4-8 also shows a strong correlation between somatic coliphage and the following physico-chemical parameters: TDS, Salinity, and pH. A weaker but still positive correlation exists between somatic coliphage and COD, and sulphate. Furthermore, it also demonstrates that nitrite levels had a negative correlation to somatic coliphage counts in the systems monitored. With regard to F-RNA phage levels Figure 4-8 depicts that there was a strong correlation between F-RNA phage and physico-chemical parameters such as DO, phosphate, and COD. It also shows that temperature and nitrate, had negative correlation to F-RNA phage levels, and that sulphate and F-RNA levels are not

correlated to each other. Figure 4-8 also illustrates that Marico River was least impacted by pollution inputs.

Figure 4-8 (A) shows that Barberspan is highly impacted by COD, DO and phosphate, and F-RNA pollution. More specifically Outflow into Leeupan (B_OL) and Hotel (B_HB) seem to be mostly affected by F-RNA pollution. Lichtenburg bridge site (H_LB) seems to be highly impacted by *E. coli* and phosphate pollution. This site has a large swallow population, particularly in the warm wet season.

Village site (H_V) in Harts river seems to be the site most affected by F-RNA pollution. This site is downstream from the Lichtenburg WWTP. Vermaas (H_VM) and Biesiesvlei (H_BV) show a close positive correlation with somatic coliphage pollution. These sites are relatively close to each other. Figure 4-8 (B) illustrates that of the Crocodile River is highly impacted by nitrogen pollution. It is also illustrated that Crocodile River and Marico River had a negative correlation with DO, COD, phosphate, TDS, salinity and pH. Below Klein-Maricopoort (Bospoort) Dam (MA_BK) and Croc Thaba (CCT) sites show strong positive correlation to *E. coli* and sulphate pollution.

In Figure 4-8 (C) it is demonstrated that Trim Park Bridge (Mooi river-_TPB) and Voortrekker (Schoonspruit river_VT) are highly impacted by *E.coli* and sulphate pollution. The Orkney site (S_OR) in the Schoonspruit River showed a strong positive correlation to salinity and TDS, with signs of somatic coliphage pollution as well. Klerkskraal (M_KK) in the Mooi River is not correlated to any specific pollution indices. Pedestrian Bridge site (M_PB) showed strong correlation to nitrite impacts, while Boskop B (M_BB) and Thabo Mbeki Bridge (M_TMB) showed strong correlation to nitrate levels. Boskop A (M_BA) and Viljoen Bridge (M_VB) showed the closest correlation to COD and DO levels in the Mooi River In the Mooi River system, water at the Viljoen Bridge (M_VB) showed the strongest correlation to F-RNA pollution.

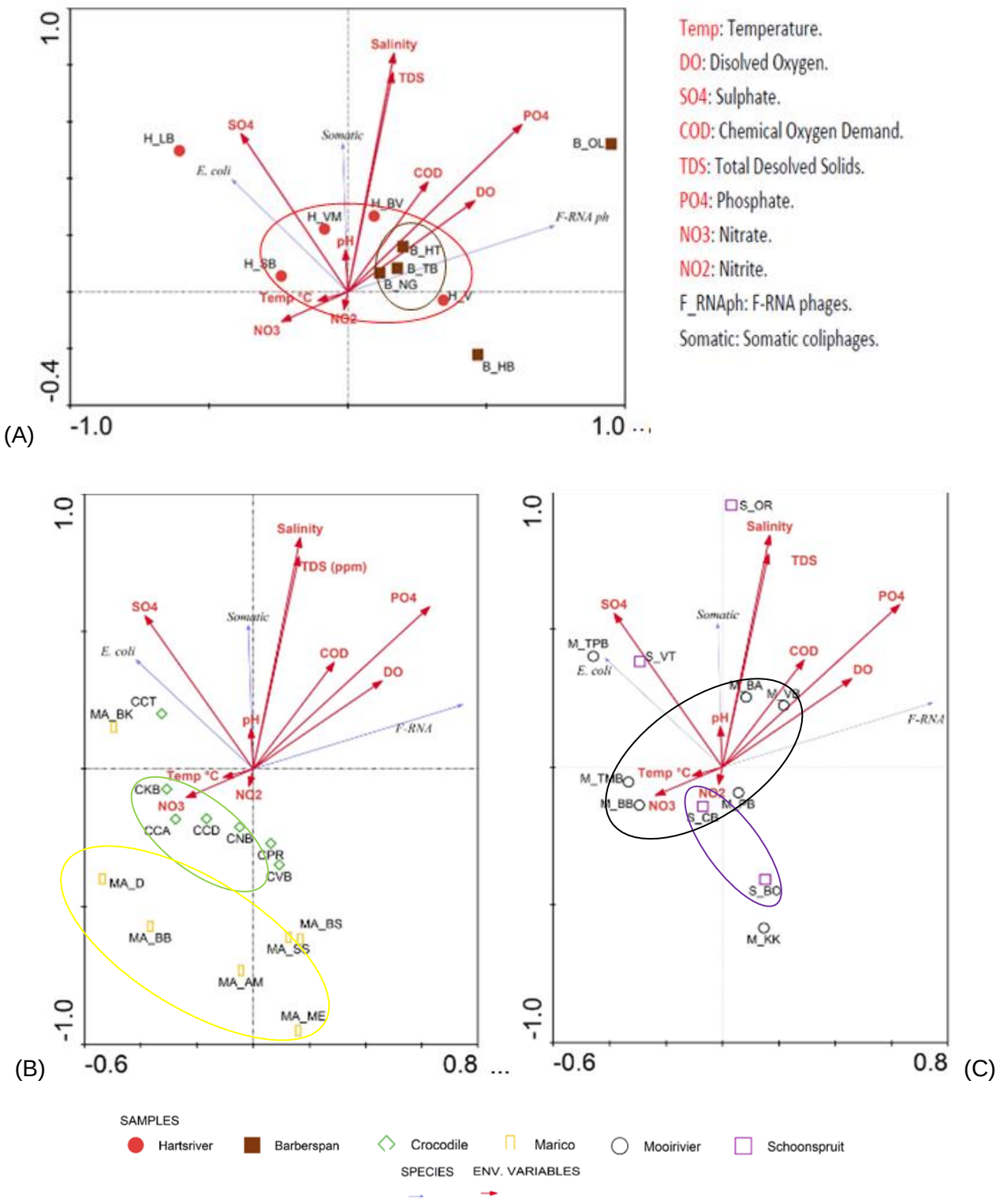


Figure 4-8 **RDA triplots indicating the correlation between the physico-chemical (red vectors), microbiological variables (blue vectors) and sample sites.**

4.6 SUMMARY OF RESULTS

Somatic coliphages were found at 34 of the 36 sites that were analysed for their presence. The two sites that had no detectable somatic coliphage were located just Before Brits and Koedoeskop Bridge sites (Table 4-1). Both of these sites are in the Crocodile River. The highest somatic coliphage level ever detected in this study was 23 000.00 ± 989.95 pfp/100 ml (Table 6-7 in Appendix B). This level was detected in the dry season in the Schoonspruit River system at the Orkney site. The highest F-RNA phage level recorded during the period of this study was 4 270.00 ± 11.84 pfp/100 ml (Table 6-4 in Appendix B). This level was also detected in the dry season and, it was found in the Barberspan water system at the Outflow into Leeupan site. Most commonly, higher phage levels could be seen after or during the dry season, with a few exceptions in the rain season. F-RNA phage levels were much more stable and had a smaller average spread than that of somatic coliphage in this study.

In comparing faecal pollution impacts in the water systems in Figure 4-8. The Harts River and Barberspan could clearly be seen as the water systems most severely impacted by faecal pollution (Figure 4-8). Barberspan could be seen as the water system with the most severe impacted water due to F-RNA phage pollution (Figure 4-8). Marico River was the water system least impacted by any of the environmental- or species variables.

Physico-chemical parameter results showed that the six water systems were typical temperate water systems. The systems were influenced by seasonal variations as well as rainfall events. Of all the parameters monitored, only sulphate and nitrite levels seemed to have no impact on F-RNA phage survival. Temperature and nitrate levels seemed to have no major impact on somatic coliphage survival. The Marico River seemed to be the least impacted by any pollution sources.

CHAPTER 5

DISCUSSION

5.1 ENUMERATION OF F-RNA BACTERIOPHAGE AND SOMATIC COLIPHAGE IN THE SELECTED WATER SYSTEMS.

Douglas (1975) describes double-layer-agar plaque method as simple, rapid and accurate, but also indicated that it is not 100% efficient, as not every viral particle added to the lawn of bacteria forms a plaque. This is because some phages infect dead bacteria in which case they are unable to develop further, others encounter random hazards during sample processing that may damage them. With this in mind, one can assume that the phage levels might very well be higher than what can be detected with this enumeration method. For specific values of the dry and wet seasons respectively, as well as standard deviations, and accompanying *E.coli* counts of which levels in Table 4-1 are the averages of, see Appendix B.

The seasonal impacts of evaporation and heavy rainfall had similar effects on concentration or disturbance of sedimented phage levels in this study. This is in agreement to the study conducted by Riou *et al*. (2007) who studied microbial impacts of small tributaries. Their results showed that F-RNA phage concentrations were relatively stable between dry and wet conditions and somatic coliphage levels increased significantly after rainfall periods. Clear faecal sources of water pollution could be identified by assessing the phage level results with those of other studies concerning different faecal pollution sources into river systems (Donnison & Ross, 1995; Ogoraly *et al*., 2009).

In the sections that follow certain results and trends obtained from the different water systems in this study was discussed. Focus was placed on bacteriophage levels and selected physico-chemical parameters in established temperate water systems.

5.1.1 MOOI RIVER

Literature shows that F-RNA phage are an indication that pathogenic human enteric viruses may also be present in the same aquatic environment, while somatic coliphage are an indication of enteric viruses (*Ganesh et al*., 2013). The fact that F-RNA phage levels are higher than somatic coliphage levels at the Klerkskraal site, in the Mooi River system may have indicated a disruption of a biofilm (Wingender & Flemming, 2011; Harper *et al*., 2014) on the dam wall at this site. This deduction is made because this site does not have major urban (or peri-urban) developments close to it. There is a major dam upstream from the site and no major agricultural impacts that could contribute direct faecal pollution. Harper *et al*. (2014) describe the role of phages in a biofilm, with the main focus on using phages to control biofilms. This is achieved through phage replication in the biofilm. However, the conditions (temperature) noted at this site was not conducive for replication of

F-RNA phages. Novotny & Lavin (1971) established the minimum temperature for the production of the F-pilli to be 25°C. As can be seen in Table 6-8 (Appendix C) this temperature was never reached. In conclusion normal lytic activity of F-RNA phages in a biofilm could not be the cause of this anomaly, at this site.

Furthermore, according to previous studies (Donnison & Ross, 1995; Ogorzaly *et al*., 2009), F-RNA phage levels only outnumber somatic coliphage levels in areas that are dominantly impacted by human faecal matter. As there is no basis for this being the cause at this site, this possibility was also ruled out and a more plausible explanation would be disruption of a biofilm prior to sampling. This deduction is supported by the results obtained by Fujioka *et al*. (1999). Their study evaluated the role of sand filtration in removing viruses (including bacteriophages) from water after a WWTP by comparing WWTP effluent with and without a sand filtration step as tertiary treatment step. The location of the sampling site in the present study was just before the point of release of the water into environmental surface water. It was discovered that absorption to organic material is the most probable reason responsible for the removal of viruses from the environmental water, and that the presence of other biological entities in this filtering system promotes the absorption (Yanko *et al*., 1999). This is very effective when the filtration system (now including biofilms) remains undisturbed. However, when there is disturbance, some of these biological entities would have been released and the probable cause of the levels that was observed at this site for that wet season sampling run (Appendix B, Table 6-2). This occurence was also addressed by Skraber *et al* (2009), which made them to conclude that biofilms may contribute to the persistence and dispersal of pathogenic viruses outside the usual epidemic periods.

The difference in seasonal somatic coliphage levels indicate spatial and temporal variation in phage pollution patterns in the Mooi River (Riou *et al*., 2007). The high somatic coliphage levels in the Mooi River at Pedestrian Bridge and Viljoen Bridge could be due to the fact that there is a lot of birds' activities (especially duck) at the Pedestrian Bridge and some agricultural activities (livestock farming) between the Pedestrian Bridge and Viljoen Bridge sites (DWAF, 2009, Ogorzaly *et al*., 2009). These impacts were observed during the sampling periods. Historically the Mooi River has not complied with management criteria concerning physico-chemical parameters (DWA, 2013) and it seems that this is also the case concerning viral contamination of this system. There are no historical data available for phage levels in the Mooi River. The strong correlation between Viljoen Bridge site and F-RNA levels could be because of human faecal pollution from sources upstream from the sampling site. This is an aspect that needs to be further investigated. Potentially this could be due to leakage from the system that transports sewage to the Tlokwe municipal WWTP. Viral particles might have seeped through the groundwater and into the river near the Viljoen Bridge site (Bosch, 1998, Baggi *et al*., 2001, Ganesh *et al*., 2013). Kivaisi (2001), in a review article, made the substantiated claim that wetlands have proven to be efficient technologies for wastewater treatment.

In a previous study Bothma *et al*. (2013) has demonstrated that, at this specific site in the Mooi River, the artificial wetland (OPM Prozesky Bird Sanctuary) reduced somatic coliphages levels were observed when compared to the levels that enter the wetland. However, as Kivaisi (2001) pointed out, lack of adequate planning and training in wetland reclamation systems in South Africa, may be the cause of certain wetlands not functioning as expected. One specific aspect of Prosesky that might be controversial for leakage of phage particles can be deducted from studying the findings of Yanko *et al*. (1999). In their study the movement of coliphage particles through soil was determined. Yanko *et al*. (1999) mentioned that F-RNA phage absorb poorly to soil particles. Their study examined the occurrence of coliphages in groundwater samples from wells at different distances from reclaimed water discharge sites. Somatic coliphages were found in wells as far as 1704 meters from the discharge site. From these findings, it is not impossible that the short distances between the effluent discharge point WWTP and the Viljoen Bridge site, in the Mooi River could be responsible for phage migration through the soil with regard to the somatic coliphages levels at the Viljoen Bridge site.

5.1.2 HARTS RIVER

Results of the Harts River presented in this study could not be analysed as a single flowing system, but rather as closed water sources (large ponds). This is due to the fact that the area was suffering a severe drought from 2013 to 2015 (SAWS, 2016). In 2014, low annual rainfall was insufficient and this resulted in extremely low water level in this system. The drought was so severe that no research could be done on this water system in 2015, because even the isolated pockets of water present in 2014 had completely dried up and no water could be found in the Upper Harts River.

The results obtained demonstrated that prior or at the Lichtenburg Bridge site very high average somatic coliphage levels were detected indicating that faecal pollution, most likely from animal sources, is entering the system (Bosch, 1998; Yahya *et al*., 2015). The conclusion that animal sources are the faecal contributors, is supported by the study done by Ogorzaly *et al*. (2009). The latter study showed that in water systems where humans are the main source of faecal pollution contributors – F-RNA phages outnumber somatic coliphage levels. Whereas, in water systems where birds and cattle are the main sources of contributing faecal matter to the system, somatic coliphages outnumber F-RNA phage levels. The Lichtenburg Bridge site is located near a bridge where birds have built habitations underneath the overpass and cattle farms are upstream from the sites. During sampling periods numerous cow tracks were observed on the river bank.

The lower average somatic coliphage levels, at the Village site, could be an indication that the WWTP is working well or that the semi-marsh area that receives the wastewater is removing phages. These results are supported by the findings of Fujioka *et al*. (1999), who evaluated the effects of adding a sand filtration step as tertiary process at WWTP's in the reclamation process of

water. They found that sand filtration has the potential to reduce the viral load of the treated wastewater by up to 99%. However, elevated levels of F-RNA phage observed at this site could have been introduced through faecal pollution from the informal settlements found at this site (Village site) as well as the Vermaas site (Donnison & Ross 1995; Ogorzaly *et al*., 2009, Ganesh *et al*., 2013). Poor sanitation facilities may thus be responsible because human faecal pollution was probably entering the system. The residents from the informal settlement, on the banks of the river use this water for religious purposes, livestock farming, washing of clothes, etc. This is of concern when considering the water is virally unsafe according to TWQR standards (DWAF, 1996a).

The somatic coliphage prevalence at the Vermaas and Biesiesvlei sites were also strong indications of livestock faecal matter entering the system (Ogorzaly *et al*., 2009; Ganesh *et al.,* 2013). Results of Ogorzaly *et al*. (2009) as well as Donnison & Ross (1995) supported the findings of the present study. In the present study of the Harts River, predominant human pollution sources by means of inefficiently working WWTP or faecal pollution from informal settlements could have contributed to bacteriophage levels along this river. This was however, not the only pollution source. Animals (wild and agricultural) could also be contributors to the pollution. In the mentioned studies (Ogorzaly *et al*., 2009; Donnison & Ross, 1995), phage levels and ratios in river systems affected by either human or animal sources were compared. These authors specifically studied rivers with no associated WWTPs and those that have WWTPs decanting treated effluent into it. They found that F-RNA phage levels were higher than somatic coliphage levels in the rivers that receive treated WWTP effluent. The Harts River contains input from both human and animal sources, yet the sites were isolated due to the drought. Point specific pollution sources could thus be identified by comparing these findings at each site in the Harts River with the findings of Donnison & Ross (1995) and Ogorzaly *et al*. (2009).

5.1.3 BARBERSPAN

A previous study (Golder Associates, 2010) suggests that Barberspan may be threatened by organic pollution via the Harts River. However, for the duration of this current study, there was no water flowing from the Harts River into Barberspan, making any claims of such nature invalid. In Barberspan high levels of both F-RNA and somatic coliphage were detected throughout the system. It was noted, when sampling the Harts before Barberspan site, that there were birds' (swallow and sparrow) activities around and under the bridge. This observation could be the reason for the high somatic coliphage levels at this particular site (Ogorzaly *et al*., 2009). The stretch of river between the first two sites, Harts before Barberspan and Train Bridge of this system was completely dried up (SAWS, 2016). Thus Barberspan should be seen as a closed water system, from the Train Bridge site onwards, when interpreting any results of this study. Any indications of pollution at Barberspan must then only be attributed to the direct surrounding environment of Barberspan itself.

The presence of a disturbed biofilm in the aquatic vegetation growing at the Train Bridge site could be the cause of higher F-RNA than somatic coliphage level. The reasoning for this would be exactly the same as previously stated in section 5.1.1 (Klerkskraal site in the Mooi River system). There was no basis to state that the pollution was due to urban or peri-urban influences,yet there was dense aquatic vegetation in this area (Donnison & Ross, 1995; Novotny & Lavin, 1971; Yanko *et al*., 1999; Fujioka *et al*., 1999; Ogoraly *et al*., 2009; Skraber *et al*., 2009; Wingender & Flemming, 2011; Harper *et al*., 2014).

Very high levels of F-RNA phage were found at the Outflow into Leeupan site. There are two possible reasons for this. The first being an active biofilm in the aquatic vegetation surrounding this site (Harper *et al*., 2014). The second might be a defective septic tank sewage system situated near the site, that was contributing human faecal matter into Barberspan at this site. The latter explanation seems to be the more probable. However, both suggested reasons could probably be true. Statistical analysis showed that there was a strong correlation between the Hotel and Outflow into Leeupan sites with F-RNA levels (Ganesh *et al*., 2013). A study by Donnison & Ross (1995) showed that where F-RNA levels outnumber somatic coliphage levels in water systems similar to lagoons (as is the case with Barberspan) domestic sewage pollution is the main source of pollution. At both the sites (Hotel and the Outflow into Leeupan), it is known that septic tanks are situated near this site and are the most likely source of the problem. Appropriate distances for septic tanks from water sources were evaluated in a study by Pang *et al*. (2003), in New Zealand. It was found that in areas where surface water is prevalent, the minimal recommended setback distance is 16 meters. However, a minimum setback distance of 48 meters is recommended for the sake of extra precaution.

A study by Wingender & Flemming (2011) about the potential of biofilms that could act as environmental reservoirs for pathogenic microorganisms, makes our findings in Barberspan very important. Especially considering that Barberspan is used for various recreational activities and human contact with the water is prevalent. There is thus potential that biofilms in the system could act as reservoirs for potential pathogenic viruses and could become dislodged and end up in the bulk water, presenting users with a risk of contamination.

5.1.4 CROCODILE RIVER

In the Crocodile River, all phage levels were relatively low when compared to the other systems and not the TWQR standards for recreational-, and agricultural water (DWAF, 1996a, DWAF, 1996b, DWAF, 1996c). The problems in this system, which indicated potential viral faecal pollution could be attributed to the following: Firstly, the After Brits site (CNB), which is just downstream from the town of Brits the phages could originate from the WWTP of this town (DWAF, 1996a; Baggi *et al*, 2001); Secondly, the Pienaars River site (CPR) could be contributing to phage pollution; Thirdly low levels

of phage could be introduced by grazing livestock in the area after Koedoespoort area. Another reason could be that of people living and defecating under the bridges of the Crocodile River (DWAF, 1996a). These findings can all be supported by the studies conducted by Donnison & Ross (1995) as well as Ogorzaly *et al*. (2009). However, the Sundays River confluence with the Crocodile river is providing a near permanent supply of relatively clean water to the lower Crocodile river, possibly diluting polluted water and thus soften the impacts in this section of the river. This flow is viewed as an important factor in maintaining the status of the Crocodile River in the Thabazimbi area and for maintaining the river in the Ben Alberts Reserve (DEAT, 2005).

5.1.5 MARICO RIVER

Faecal point pollution affects the Marico River in the Marico eye area. This is evident because "crystal clear" water is flowing through dolomite rock to become the source of Marico river (Anon, 2010). It was thus anticipated that if any phages were present in ground water, it would have been filtered out before reaching the spring, as was demonstrated in the study of Fujioka *et al*. (1999). Yet, high levels of somatic coliphage were detected at the Marico eye and this suggest faecal pollution at this site.

There were lower somatic coliphage levels at the After Marico Bosveld Dam site. These phage levels can be ascribed to very high levels of plant growth in this dam. Thus the biofilm on the vegetation in the dam may be absorbing the phages and acting as a filter, similar to what happens in some artificial wetlands after WWTPs (Fujioka *et al*., 1999). The findings, in this regard, agreed with the findings of several researchers. Sinton *et al*. (2010) demonstrated that the application of filtration through wetlands promote the settling of phages. Ghermandi *et al*. (2007) came to the conclusion that wetlands (natural or artificial) are a key element in the reclamation and reuse practices of water. Finally in the review article by Kivaisi (2001), it was concluded that with good planning, management and maintenance practices, wetlands can be a great way to reclaim water in developing countries.

5.1.6 SCHOONSPRUIT RIVER

Untreated sewage flow freely into the Schoonspuit River between the Klerksdorp and Orkney sites. This is clearly shown by the very high levels of both somatic coliphage and F-RNA phages. The Orkney site is immediately downstream from the Orkney WWTP. A lot of floating organic plant material was also observed at the Voortrekker site, which could contribute to phage survival at that site (Yang & Griffiths, 2013). The somatic coliphage levels at the Confluence of Brakspruit and Bodenstein sites could be due to agricultural runoff as well as some other anthropogenic activities (DWAF, 1996b; c).

5.2 DETERMINING THE LEVELS OF SELECTED PHYSICO-CHEMICAL PARAMETERS IN THE SELECTED WATER SYSTEMS.

5.2.1 PHYSICAL PARAMETERS

Average temperatures of the six monitored systems are in the optimal range for phage survival (10 – 25°C; Silva *et al*., 2014). Barberspan had the highest salinity and average pH of all the systems, this was probably due to the nature of a perennial pan that had high rates of evaporation and no new, substantial inflow of water during the study period (DWAF, 1996b; Golder Associates, 2010). Leeupan is known for its high salinity levels (Golder Associates, 2010) and thus it was no surprise that the Outflow into Leeupan, in the Barberspan system, had the highest average salinity of all the sites in this study. Moreover, Barberspan also had the highest average of DO compared to the other five water systems monitored. The most likely reason for the DO levels in Barberspan are the larger ratio of water to atmosphere contact as well as the presence of small waves in the system, demonstrating potential mixing of air and the surface water (DWAF, 1996d).

The high salinity, TDS and low DO at Orkney in the Schoonspruit River was a clear indication of some sort of severe pollution entering the system at or prior to Orkney (DWAF 1996b,c). As indicated, this site is immediately downstream from a WWTP and operational problems had been experienced at this WWTP. The Marico River showed the lowest average salinity and most neutral pH average of the six water systems monitored in this study. This corresponds with the historical information of only low levels of nutrient pollution entering the Marico river (DEAT, 2005) The physical parameter levels of the trends in the Crocodile River does not point to severe, above average pollution levels when compared to the other systems in this study. This observation support the historical data for the Crocodile River (DEAT, 2005). However, the low levels of DO does raise concerns about the health of the aquatic ecosystem in this system (DWAF, 1996d).

The high salinity and TDS values at Trim Park Bridge in the Mooi River is most probably as a result of a confluence with Wasgoedspruit. Upstream, this latter tributary of the Mooi River flows through the informal settlements as well as an urban section that had been affected by sewage pollution (DWAF, 1996a; b; c). There are also vagrants living in the area of the Trim Park Bridge site. There was a decline in every physical parameter at the Village site in Barberspan, from the previous site (Lichtenburg Bridge) except DO. This could be an indication of a working WWTP situated between these two sites (Baggi *et al*., 2001). A decline in every single physical parameter from one site (Before Sterkstroom) to the next (Sterkstroom) in the Marico River is an indication of dilution (Dallas & Day, 2004).

5.2.2 CHEMICAL PARAMETERS

The low levels of nitrite and nitrate in the Barberspan system are a clear indication that agricultural runoff is not entering the system (Chang *et al*., 2010). High levels of nitrite and nitrate in the Mooi River, Crocodile River and Schoonspruit River, pose an economic risk for some sensitive cultivars that are irrigated via these systems (DWAF, 1996c). Elevated sulphate levels of the Mooi River after Klerkskraal is possibly due to mine drainage entering the Mooi River before Boskop A. Agricultural runoff can most probably be blamed for the sulphate levels in Harts- and Crocodile River. In the Schoonspruit River high sulphate levels is possibly due to agricultural runoff (Confluence with Brakspruit), anthropogenic activities (Voortrekker) and direct sewage pollution (Orkney). Phosphates were detected at elevated levels (DWAF, 1996d) in all the systems except the Marico River. This was expected (Capece *et al*., 2007) as most of the land use around all these systems are used for agricultural activities (DEAT, 2005). Industrial, agricultural and domestic wastes are the sources of organic matter in aquatic environments (DWAF, 1996a). Organic matter present in dissolved form causes undesirable odours of the water (DWAF, 1996a). COD levels were high in all the systems except the Mooi River. This means that organic pollution is entering all these systems continuously (Hur *et al*., 2010). The average COD levels at Barberspan were the highest, with all the sites in the closed system. COD levels were, in some cases, unacceptable for environmental water use (DWP, 2012). The Marico Bosveld Dam was potentially filtering out the organic pollution (Baggi *et al*., 2001) and thus improving the quality of water and decreasing the COD levels at sites below this sampling point.

5.3 CHARACTERIZATION OF THE F-RNA BACTERIOPHAGES AND SOMATIC COLIPHAGES.

5.3.1 CHARACTERIZATION OF SOMATIC COLIPHAGES

The morphologies of plaques isolated from various surface water systems were investigated and three different morphologies were consistently noted throughout the study. Contrastingly, studies in literature have widely documented only two morphologies: (i) clear, clean edge plaque and (ii) fried egg plaque) (Hadley, 1924; Donnison & Ross, 1995; ISO, 2000; Nur ilida *et al.,* 2013). The third plaque morphology identified in this study (jagged edge, vague plaque) has not been widely documented in literature (Hayes, 1968, Grabow, 2001).

Transmission electron microscopy was performed on the three different plaque morphologies. This was done to determine if there was a link between specific plaque morphologies and specific phage types. Such an approach may render the double-layer-agar method more valuable than just serving as an enumeration assay for phages. It may thus also be used as a basic phage classification assay. Abedon and Yin (2009) argued that plaque studies, particularly morphology, could be

valuable, but is under-appreciated by scientists working on phages because of the complex naure of interpretation. Molecular techniques, as opposed to visualisation techniques, are thus more commonly employed to identify phages (Nur ilida *et al*., 2013). Combining these two methods could thus provide a powerful approach to study enviromental phages. Results of the present study demonstrated that somatic coliphages that were found to have clear, clean edge plaque morphologies had reasonably larger heads than the phages found in the other plaque morphologies. Also, the phages with the clear, clean edge plaques morphologies had no apparent tails. Combining these observations with literature (Douglas, 1975; ICTV, 2011, Singh *et al*., 2016), it could be speculated that these were possible *Podoviridae* or *Microviridae* phages. Somatic coliphages observed in the fried egg plaque assay area showed the presence of short contractile tails. The presence of appendages was also noted. Furthermore, they had heads a bit smaller than those found among the clear, clean edge plaque morphology phages. Combining these facts with literature (Durán *et al*., 2002; Fattouh *et al*., 2002; Sandaa, 2009; Colomer-Lluch *et al*., 2011, ICTV, 2011), it could be speculated that somatic coliphages were of the *Caudovirales* order (isohedrical head), *Myoviridae* family (presence of collar) and of morphotype 2 (presence of appendages) form fried egg plaques. Somatic coliphages observed in the jaqqed edge, vague plaque had remarkable long straight tails. If this observation is compared to what is in the literature (Durán *et al*., 2002; Fattouh *et al*., 2002; Sandaa, 2009; Colomer-Lluch *et al*., 2011; ICTV, 2011), it could be speculated that phages were of the *Caudovirales* order (isohedrical head), *Siphoviridae* family (straight tail) form jagged edge, vague plaques.

Combining the results of the two assays (double-layer-agar plaque assay and TEM) in this study, a possible trend was observed. The longer the tail and smaller the head the more unclear (vague) zone were present in the plaque. Possible reasons for this could be: If a phage had a small head and no appendages, less drag was created when diffusing through the agar (Gallet *et al*, 2011). So, phages of that morphology could spread out faster, wider, and non-uniform thus creating vague jagged edge plaques. However, phages with very short or no tails and big heads were dependent on lysogenic forces, osmotic pressures and diffusion portability of the top agar for their spread in the bacterial mat. This caused a higher concentrated area of phages and thus a clearer plaque (Gallet *et al*, 2011). Finally, if a phage then has a relatively big head, and appendages it will experience more resistance in spreading through the top layer of agar than a phage without these would experience (Gallet *et al*, 2011). Also, if a relatively long tail is present, migration may be limited, but the tails will still be able to infect phage a relatively long distance away from the head of the virion. In these cases a mixture of a clear, clean plaque close to the original infected bacterial host might form, yet a jagged edge, vague area will form as the plaque further protrudes away from the original infected host cell site. This will form the fried egg plaque morphology. Although these are speculations, such approach could provide the basis for further study. Viron size is only one of a long list of phage characteristics that influence plaque morphology (Mullan, 2002; Gallet *et al*, 2011).

5.3.2 CHARACTERIZATION OF F-RNA PHAGES AND IDENTIFYING POLLUTION SOURCES

The results of the RT-PCR of water samples taken from these sites showed the presence of GA- and Qβ phage at both the Hotel and Outflow into Leeupan site. This is an indication of human faecal contamination of the Barberspan water system. These results are further supported by the facts that these F-RNA genes excreted only in human faecal matter (Dryden *et al*., 2006; Yahya *et al*., 2015) were not detected at the North of Goosepan site, which is situated between the other two sites (Golder Associates, 2010) of the Barberspan water system. The sites in Barberspan which were highly impacted by human activity (Hotel and Outflow into Leeupan), were the two sites that showed the closest correlation to F-RNA levels in this study (Ganesh *et al*., 2013). These findings should be seriously considered by the management of Barberspan as well as the national government, as it proves that human activity around Barberspan is aiding in the declining of water quality and aquatic ecosystem health at this RAMSAR site.

F-RNA phages have been proposed to identify the origin of faecal pollutions (Hsu *et al*., 1995; Beekwilder *et al*., 1996; Dryden *et al*., 2006). GA and Qβ phage belong respectively to genogroups II and III (Miyake *et al*, 1969; Ogorzaly & Gantzer, 2006; Yahya *et al*., 2015). Literature (Osawa *et al*., 1981; Havelaar *et al*., 1990; Scott *et al*., 2002; Yahya *et al*., 2015) states that these genogroups (II and III) are specific to human faecal matter. The presence of these phage genogroups in water is thus clearly an indication of human faecal pollution of the system (Dryden *et al*., 2006). For years the degradation of Barberspan's water quality has been blamed on the WWTP's of Lichtenburg and Sannieshof (Golder Associates, 2010). During the period of the present study (2014 - 2015), the North West Province of South Africa was experiencing extreme droughts (SAWS, 2016) and no water of the Upper Harts River (and thus these WWTP's) ever flowed into Barberspan. With this in mind, source(s) of pollution of Barberspan water system is/are likely coming from the immediate area of Barberspan.

CHAPTER 6

CONCLUSIONS & RECOMMENDATIONS

Health and sustainability of rivers in the North West Province is important economically and socially particularly as healthy rivers help provide essential goods and services (DWAF, 2007). This is why this study and the results gathered are of importance especially when considering SOER (DWA, 2013) and the possible impact it can have on remediation and sustainability programmes in the North West Province.

6.1 CONCLUSION

The aim of this study was to determine bacteriophage levels and associated characteristics in selected temperate water systems. This was done successfully and some conclusions could be made concerning the water systems as explained in the following paragraphs.

6.1.1 ENUMERATION OF F-RNA BACTERIOPHAGES AND SOMATIC COLIPHAGES

Somatic coliphage and F-RNA phage levels for each water system was established. F-RNA and Somatic coliphage were found in all water systems studied. F-RNA levels in the Mooi River was not of great concern, but due to the somatic coliphage levels of the Mooi River, it was concluded that the water may not be suitable for livestock or irrigation. The Harts River showed clear indications of agricultural- and anthropogenic faecal pollution being introduced into the system at various points. It was concluded that the water in the Upper Harts River may not be suitable for livestock farming. Molecular and culture based assays indicated human faecal pollution was entering the system through areas where people are living on the Barberspan premises. It was concluded that the water in Barberspan may be unsafe for recreational purposes. In comparison to the Crocodile River, the Marico River had better water quality. This was also the case when compared to the other river systems studied. In spite of this quality being better than other water systems studied, the Marico River was still not in excellent condition. Clear indications of agricultural runoff was indicated by the somatic coliphage levels. It was concluded that the water in the Marico River may not be safe for livestock farming or irrigation if strict water safety guidelines are used. The first three sites of the Schoonspruit River could be classified as a system of moderate health, with some indications of agricultural and anthropogenic pollution. However, the Orkney site had extremely high phage levels which is an indications of sewage entering the system. As the Schoonspruit River runs southward, the water quality is immensely degraded in just a short stretch. Considering all these facts, it was concluded that the Schoonspruit River was not safe for irrigation purposes of either livestock or cultivars.

6.1.2 LEVELS OF SELECTED PHYSICO-CHEMICAL

All six water systems in this study showed typical physico-chemical characteristics of temperate water systems. Seasonal changes (including ambient temperature and rainfall) have affected the physico-chemical parameter levels measured. Many of the physical parameter levels of Harts River were high, most probably due the concentrating effect of the drought. At Barberspan low levels of nitrite and nitrate were measured. This showed that no agricultural runoff was entering the system during the study period. The Crocodile River was greatly polluted by agricultural and domestic runoff. This had a clear effect on the biota of the river, as the river was not able to oxidize nitrite or perhaps levels of nitrate were bacteriologically converted to nitrites. It was concluded that the water of the Crocodile River may not be safe for livestock farming or crop irrigation. In the Marico River, indications of agricultural runoff was indicated by nitrite and nitrate levels. Once again with the exception of the Orkney site in the Schoonspruit River, the sites upstream from Klerksdorp had physico-chemical levels that suggest that the river is in relatively good health.

6.1.3 CHARACTERIZATION OF F-RNA BACTERIOPHAGES AND SOMATIC COLIPHAGES

The characterization of F-RNA phages by RT-PCR was a suitable method in identifying the faecal source pollution at Barberspan. The characterization of somatic coliphage was done by TEM and possible links between diversity in plaque morphologies and phage virion characteristics could be proposed.

6.1.4 DETERMINATION OF POSSIBLE POLLUTION SOURCES BASED ON PHAGE GENOGROUPS

Human feacal pollution could be identified at the Hotel and Outflow into Leeupan sites in Barberspan, by positive identification of GA and Qβ phage genogroup cDNA fragments.

6.2 RECOMMENDATIONS

- The effect of wetlands and/or vegetation in a water system could be seen in this study in the Marico River before and after the Marico Bosveld Dam. This was also evident at other sites. If more artificial wetlands could be introduced in these water systems it should help better the quality of water downstream.
- Action should be taken to stop the contamination of Barberspan water system caused by anthropogenic activities, especially human faecal matter contamination. Barberspan is a RAMSAR site and should be treated with the utmost care. One of the shortcomings of this study was the number of sampling times within a year and season. The frequency of sampling in monitoring these systems should be increased from what was applied in this study. This will enable the robustness of the data as well as better interpretation of the results. One would also get a better idea of how each system and site reacts to specific

environmental impacts. The intensive monitoring of these systems should be set forth and done on a continual basis to ensure remediation activities of these systems are working and to catch pollution sources as soon as possible. The South African Government and its provinces have numerous environmental reporting obligations. The National Environmental Management Act (NEMA; Act No. 107 of 1998) states that “every person is entitled to have access to information held by the State relating to the state of the environment, and actual and future threats to the environment.” Access to environmental information at a national, provincial and local level is essential to upholding these rights. NEMA also stipulates that the Minister must compile an Annual Performance Report, whose purpose is to provide an audit of the government’s performance in respect of implementing Agenda 21 to the Commission for Sustainable Development (CSD). South Africa is obliged to report to the UN on implementation of Agenda 21 programmes. (DEAT, 2002). Hopefully, some of the results found in this study will help in aiding these government structures with a better understanding of the health of water systems in the North West Province

- Real-time amplification techniques are currently used to determine the viral contamination of many kind of samples including fresh water, wastewater and sewage (Ogorzaly & Gantzer, 2006). It would be good if these methods could be applied to the water systems in this study to assess the effectiveness of the culture dependent assays in enumerating phage levels. Phages may play a vital role in horizontal gene transfer of antibiotic resistance genes in these waters systems (Sandaa, 2009). To elucidate this hypothesis, sequencing of and identification of phages found in these water systems are recommended.
- Bethnic viral abundance is typically 10 – 100 times higher than in the water column (Sandaa, 2009). In addition, the total diversity of the viral particles is higher in sediments than in the overlaying water column (Sandaa, 2009). In 2009 Sandaa made the statement that to date; virtually all information on the interaction between environmental viruses and their hosts has been gathered from aquatic systems, while little information is available on the importance of viruses in soil. This is of great importance and a good motivation to do soil studies of these water systems. This is necessary because of some activities, such as cattle grazing in some of the water sites, thus disturbing and releasing sedimented viruses back into the flow system. Correlations between viral abundances and suspended solids have been shown, suggesting that viruses are absorbed to suspended material in the water column that may settle out and contribute to the bethnic viral population (Sandaa, 2009). Inclusion of more parameters such as total suspended solids, magnesium and calcium concentrations, to the routine monitoring of these sites would be very beneficial in elucidating the survival of phages in these water systems.
- To determine public health risks associated with pollution, there is a need to identify sources of faecal contamination, develop measures to remediate polluted waterways, and establish

legal responsibility for remediation. Source-tracking provides a means to identify the origin of faecal pollution in a particular water body (Malakoff, 2002; Muniesa *et al.,* 2009). As shown with the Barberspan F-RNA molecular results in this study, phages will be very helpful organisms in endeavours tracking source pollution in temperate water systems. Many phages have a very definite requirement for divalent cations particularly calcium, which promotes adsorption and without which infection may fail to occur at all. This explains why they are inhibited by, for example citrates and oxalates which form insoluble compounds with calcium in the medium and render it unavailable. Magnesium may be used as a replacement for calcium by some, but not all phages with this requirement (Douglas, 1975). This shows the importance calcium and magnesium concentrations in a water system can have on phage survival. It is thus recommended that these two chemical parameters be added to the list for regular monitoring purposes of these water systems. Is there a definite relation to phage plaques and the morphology of plaques? If this question could be answered the double-layer-agar plaque method could be used not only for enumeration and isolation of phages, but also for preliminary identification of phages.

REFERENCE LIST

Abedon, S.T. & Yin, J. 2009. Bacteriophage plaques: theory and analysis. *Methods in Molecular Biology*, 501: 161–174.

Ackermann, H.W., Tremblay, D. & Moineau, S. 2004. Long-term bacteriophage preservation. *WFCC Newsletter*, 38:35–40.

Adam, J.W.H. 1980. Health aspects of nitrate in drinking-water and possible means of denitrification. *Water SA*, 6(2): 79–84.

Allan, G.D., Seaman, M.T. & Kaletja, B. 1996. The endorheic pans of South Africa.(*In* Cowan, G.I., *ed.* Wetlands of South Africa, South African wetlands conservation programme series. Pretoria: Department of Environmental Affairs and Tourism. p. 75-101).

Anon. 2010. The Groot Marico is under treat. http://www.marico.co.za/marico-petition.htm Date of access: May 2015.

Aprea, G., D'Angelo, D.R., Prencipe, V.A. & Migliorati, G. 2015. Bacteriophage morphological characterization by using transmission electron microscopy. *Journal of Life Science*, 9: 214–220.

AWPRC: Study Group on Health Related Water Microbiology. 1991. Bacteriophages as model viruses in water quality controlag. *Water Research*, 25(5): 529–545.

Baggi, F., Demarta, A. & Peduzzi, R. 2001. Persistence of viral pathogens and bacteriophages during sewage treatment: lack of correlation with indicator bacteria. *Research in Microbiology*, 152: 743–751.

Barreto-Vieira, D.F. & Barth, O.M. 2015. Negative and positive staining in transmission electron microscopy for virus diagnosis. http://www.intechopen.com/books/ microbiology-in-agriculture-and-human-health/negative-and-positive-staining-in-transmission-electron-microscopy-for-virus-diagnosis Date of access: October 2015.

Barnes, K.N. 1998. The important bird areas of southern Africa. Johannesburg: BirdLife South Africa.

Basdew, I.H. & Laing, M.D. 2014. Stress sensitivity assays of bacteriophages associated with *Staphylococcus aureus*, causal organism of bovine mastitis. *African Journal of Microbiology Research*, 8(2): 200-210.

Beekwilder, J., Niewenhuizen, R., Havelaar, A., van Duin, J. 1996. An oligonucleotide hybridization assay for the identification and enumeration of F-specific RNA phages in surface waters. *Journal of Applied Bacteriology*, 80: 179–186.

Bernhardt, T.G., Roof, W.D & Young, R. 2000. Genetic evidence that the bacteriophage phi X174 lysis protein inhibits cell wall synthesis. *Proceedings of the National Academy of Sciences of the United States of America,* 97(8): 4297-4302.

Bernhardt, T.G., Struck, D.K., & Young, R. 2001. The lysis protein E of phi X174 is a specific inhibitor of the MraY-catalyzed step in peptidoglycan synthesis. *Journal of Biological Chemistry*, 276(9): 6093–6097.

Blanch, A.R., Belanche – Muñoz, L., Bonjoch, X., Ebdon, J., Gantzer, C., Lucena, F., Ottoson, J., Kourtis, C., Iversen, A., Kühn, I., Moce, L., Muniesa, M., Schwartzbrod, J., Skraber, S., Papageorgiou, G., Taylor, H.D., Wallis, J. & Jofre, J. 2006. Integrated analysis of established and novel microbial and chemical methods for microbial source tracking. *Applied Environmental Microbiology*, 27: 5915–5926.

Bolduc, B., Shaughnessy, D.P., Wolf, Y.I., Koonin, E.V., Roberto, F.F. & Young, M. 2012. Identification of novel positive-strand RNA viruses by metagenomic analysis of archaea-dominated yellowstone hot springs. *Journal of Virology*, 86(10): 5562–5573.

Bollback, J.P. & Huelsenbeck, J.P. 2001. Phylogeny, genome evolution, and hast specificity of single-stranded RNA bacteriophage (Family *Leviviridae*). *Journal of Molecular Evolution*, 52(2): 117–128.

Bosch, A 1998. Human enteric viruses in the water environment: a minireview. *International Microbiology*, 1:191–196.

Bothma, L., Bezuidenhout, C.C. & Adeleke, R. 2013. Determining levels of bacteriophages present in treated sewage effluent and receiving water bodies. Potchefstroom: NWU. (*Mini-dissertation-BSc.Hons)*.

Bourlet, T., Caro, V., Minjolle, S., Jusselin, I., Pozetto, B., Crainic, R. & Colimon, R. 2003. New PCR test that recognizes all human prototypes of enterovirus: application for clinical diagnosis. *Journal of Clinical Microbiology*, 41(4): 1750-1752.

Breitbart, M., Wegley, L., Leeds, S., Schoenfeld, T. & Rohwer, F. 2004. Phage community dynamics in hot springs. *Applied and Environmental Microbiology*, 70(3): 1633–1640.

Brion, G.M., Meschke, J.S. & Sobsey, M.D. 2002. F-specific RNA coliphages: occurrence, types, and survival in natural waters. *Water Research*, 36: 2419–2425.

Burleson, F.G., Chambers, T.M. & Wiedbrauk, D.L. 1992. Virology: A laboratory manual. California: Academic Press Inc.

Carpenter, S.R., Caraco, N.F., Correl, D.L., Howarth, R.W., Sharpley, A.N. & Smith, V.H. 1998. Nonpoint pollution of surface waters with phosphorus and nitrogen. *Ecological Applications*, 8(3): 559-568.

Capece, J.C., Campbell, K.L., Bohlen, P.J., Graetz, D.A. & Portier, K.M. 2007. Soil phosphorus, cattle stocking rates, and water quality in subtropical pastures in Florida, USA. *Rangeland Ecology and Management*, 60(1): 19-30.

Chang, C., Chen, C., Wu, D. & Yang, C. 2010. Nitrates in drinking water and the risk of death from rectal cancer: does hardness in drinking water matter? *Journal of Toxicology and Environmental Health Part A*, 73: 1337-1347.

Chung, H. & Sobsey, M.D. 1993. Comparitive survival of indicator viruses and enteric viruses in seawater and sediment. *Water Science and Technology*, 27(3–4): 425–429.

Cole, D., Long, S.C. & Sobsey, M.D. 2003. Evaluation of F-RNA and DNA coliphages as source-specific indicators of faecal contamination in surface waters. *Applied Environmental Microbiology*, 69(11): 6507–6514.

Coleman, J., Inouye, M. & Atkins, J. 1983. Bacteriophage MS2 lysis protein does not require coat protein to mediate cell lysis. *Journal of Bacteriology*, 153: 1098–1100.

Colomer-Lluch, M., Jofre, J. & Muniesa, M. 2011. Antibiotic resistance genes in the bacteriophage DNA fraction of environmental samples. *PLoS ONE,* 6(3): e17549.

Contreras-Coll, N., Lucena, F., Mooijman, K., Havelaar, A., Pierzo, V., Boque, M., Gawler, A., Höller, C., Lambiri, M., Mirolo, G., Moreno, B., Niemi, M., Sommer, R., Valentin, B., Wiedenmann, A., Young, V & Jofre, J. 2002. Occurrence and levels of indicator bacteriophages in bathing waters throughout Europe. *Water Research*, 36:4963–4974.

CSIR: Council for Scientific and Industrial Research. 2010. The current status of water quality in South Africa: A review. Pretoria.

Cumming, G.S. 2009. SA-Gains: Final report. Cape Town: UCT.

DACE: Department of Agriculture, Conservation and Environment. 2002. Overview. State of the Environment Report, North West Province. Pretoria.

Dallas, H.F. & Day, J.A. 2004. The effect of water quality variables on aquatic ecosystems: A review. Pretoria: Water Research Commission.

DEAT: Department: Environmental Affairs and Tourism. 2005. State-of-Rivers Report: Monitoring and managing the ecological state of rivers in the Crocodile (West) Marico Water Management Area. Pretoria.

Delpla, I., Jung, A.V., Baures, E., Clement, M. & Thomas, O. 2009. Impacts of climate change on surface water quality in relation to drinking water production. *Environmental International*, 35:1225-1233.

Donnison, A.M. & Ross, C.M. 1995. Somatic and F-specific coliphages in New Zealand waste treatment lagoons. *Water Research*, 29(4): 1105-1110

Douglas, J. 1975. Bacteriophages. London: *Chapman and Hall Ltd*.

DPW: Department of Public Works. 2012. Small waste water treatment works - DPW design guidelines. Pretoria.

Dryden, S.K., Ramaswami, B.,Yuan, Z., Giammar, D.E. & Angenent, L.T. 2006. A rapid reverse transcription-PCR assay for F^+ RNA coliphages to trace fecal pollution in Table Rock Lake on the Arkansas-Missouri border. *Water Research*, 40: 3719– 3724.

Durán, A.E., Muniesa, M., Méndez, X., Valero, F., Lucena, F. & Jofre, J. 2002. Removal and inactivation of indicator bacteriophages in fresh water. *Journal of Applied Microbiology*, 92: 338–347.

DWA: Department of Water Affairs. 2009. Adopt-a-River program phase II: Development of an implementation plan. Pretoria.

DWA: Department of Water Affairs. 2013. The annual national state of water resources report. Pretoria.

DWAF: Department of Water Affairs and Forestry. 1996a. South African water quality guidelines: Recreational use. 2nd edition. Pretoria.

DWAF: Department of Water Affairs and Forestry. 1996b. South African water quality guidelines: Agricultural water use: Livestock. 2nd edition. Pretoria.

DWAF: Department of Water Affairs and Forestry. 1996c. South African water quality guidelines: Agricultural water use: Irrigation. 2nd edition. Pretoria.

DWAF: Department of Water Affairs and Forestry. 1996d. South African water quality guidelines: Aquatic Ecosystems. 2nd edition. Pretoria.

DWAF: Department of Water Affairs and Forestry. 2004. Crocodile (West) Marico Water Management Area: Internal strategic perspective for Marico, Upper Molopo and Upper Ngotwane catchments. Pretoria.

DWAF: Department of Water Affairs and Forestry. 2006. River Health Programme: Annual champions symposium. Pretoria.

DWAF: Department of Water Affairs and Forestry. 2007. Habitat Integrity. Pretoria.

DWAF: Department of Water Affairs and Forestry. 2009. Task 2: Water quality status assessment. Pretoria.

Elbreki, M., Ross, R.P., Hill, C., O'Mahony, J., McAuliffe, O. & Coffey, A. 2014. Bacteriophages and their derivatives as biotherapeutic agents in disease prevention and treatment. *Journal of Viruses,* 2014: 382539.

Fang, W., Hu, J.Y. & Ong, S.L. 2009. Influence of phosphorus on biofilm formation in model drinking water distribution systems. *Journal of Applied Microbiology*, 106:1328-1335.

Fattouh, F.A., Saeed, H. & Wifi, S.M. 2002. PCR-based DNA fingerprinting analysis of coliphages isolated from sewage polluted seawater in Alexandria. *Pakistan Journal of Biological Sciences*, 5(9): 938–942.

Feng, Y.Y., Ong, S.L., Hu, J.Y., Tan, X.L. & Ng, W.J. 2003. Effects of pH and temperature on the survival of coliphages MS2 and Qbeta. *Journal of Industrial Microbiology & Biotechnology*, 30:549–552.

Fraenkel-Conrat, H. 1969. The chemistry and biology of viruses. New York: Academic Press.

Fraenkel-Conrat, H., Kimball. P.C. & Levy, J.A. 1988. Virology. 2nd edition. New Jersey: Prentice Hall.

Franki, R.I.B., Fauquet, C.M., Knudson, D.L. & Brown, F. 1991. Classification and nomenclature of viruses. Vienna: Springer-Verlag.

Fujioka, R.S. Bonilla, A.J. & Rijal, G.K. 1999. The microbial quality of a wetland reclamation facility used to produce an effluent for unrestricted non-potable reuse. *Water Science and Technology*, 40(4-5): 369-374.

Furuse, K. 1987. Phage Ecology. New York: John Wiley.

Gallet, R., Kannoly, S. & Wang, I. 2011. Effects of bacteriophage traits on plaque formation. *BioMed Central Microbiology*, 11(181): 1-16.

Ganesh, A., Lin, J. & Singh, M. 2013. Detecting virus-like particles from the Umgeni River, South Africa. *Clean – Soil, Air, Water*, 41: 1–15.

Gerba, C.P. 1984. Applied and theoretical aspects of virus absorption to surfaces. *Advances in Applied Microbiology:* 30: 133–168.

Gerba, C.P., Wallis, C. & Melnick, J.L. 1975. Fate of wastewater bacteria and viruses in soil. *Journal of the Irrigation and Drainage Division*, 101: 157–174.

Gersberg, R.M., Lyon, S.R., Brenner, R. & Elkins, B.V. 1987. Fate of viruses in artificial wetlands. *Applied and Environmental Microbiology*, 53(4): 731–736.

Ghermandi, A., Bixio, D. & Thoeye, C. 2007. The role of free water surface constructed wetlands as polishing step in municipal wastewater reclamation and reuse. *Science of the Total Environment*, 380: 247-258.

Gironés, R., Jofre, J. & Bosch, A. 1989. Natural inactivation of enteric viruses in seawater. *Journal of Environmental Quality*, 18: 34–39.

Golder Associates. 2010. Risk assessment of the Barberspan bird sanctuary. Roodepoort.

Goudie, A.S. & Thomas, D.S.G. 1985. Pans in southern Africa with particular reference to South Africa and Zimbabwe. *Zeitschrift fur Geomorphologie* NF, 29: 1–9.

Grabow, W.O.K. 2001. Bacteriophages: Update in application as models for viruses in water. *Water SA*, 27(2): 251–268.

Green, J., Bruce, K., Conner, C. & Mutesi, R. 1999. The APHA standard for enumeration of somatic coliphages in water has low efficiency of plating. *Water Research*, 34(3): 759–762.

Güler, C. & Alpaslan, M. 2009. Mineral content of 70 bottled water brands sold on the Turkish market: Assessment of their compliance with current regulations. *Journal of Food Composition and Analysis*, 22: 728-737.

Hach Company. 2007. DR 2800 spectrophotometer: Procedures manual. 2nd edition. Germany.

Hadley, P. 1924. The variation in size of lytic areas and its significance. *Journal of Bacteriology*, 9(4): 397-403.

Harper, D.R., Parracho, H.M.R.T., Walker, J., Sharp, R., Hughes, G., Werthén, M, Lehman, S. & Morales, S. 2014. Bacteriophages and biofilms. *Antibiotics*, 3: 270-284.

Harwood, V.J., Levine, A.D., Scott, T.M., Chivukula, V., Lukasik, J., Farrah, S.R. & Rose, J.B. 2005. Validity of the indicator organism paradigm for pathogen reduction in reclaimed water and public health protection. *Applied and Environmental Microbiology*, 71(6): 3163-3170.

Havelaar, A.H. & Hogeboom, W.M. 1984. A method for the enumeration of male-specific bacteriophages in sewage. *Journal of Applied Bacteriology*, 56: 439–447.

Havelaar, A.H., Pot – Hogeboom, W.M., Furuse, K., Pot, R. & Hormann, M.P. 1990. F-specific RNA bacteriophages and sensitive host strains in faeces and wastewater of human and animal origin. *Journal of Applied Microbiology*, 69(1): 30–37.

Havelaar, A.H., Van Olphen, M. & Drost, Y.C. 1993. F-specific RNA bacteriophages are adequate model organisms for enteric viruses in fresh water. *Applied and Environmental Microbiology*, 59(9): 2956–2962.

Hayes, W. 1968. The genetics of bacteria and their viruses. 2nd edition. Oxford: Blackwell Scientific Publications.

Heydari, M.M. & Bidgoli, H.N. 2012. Chemical analysis of drinking water of Kashan district, central Iran. *World Applied Science Journal,* 16(6): 799–805.

Hill, V.R. & Sobsey, M.D. 1998. Microbial indicator reductions in alternative treatment systems for swine wastewater. *Water Science and Technology*, 38(12): 119–122.

Hsu, F.C., Shieh, Y.S.C., van Duin, J., Beekwilder, M.J. & Sobsey, M.D., 1995. Genotyping male-spesific coliphages by hybridization with oligonucleotide probes. *Applied and Environmental Microbiology*, 61: 3960–3966.

Hur, J., Lee, B., Lee, T. & Park, D. 2010. Estimation of biological oxygen demand and chemical oxygen demand for combined sewer systems using synchronous fluorescence spectra. *Sensors*, 10: 2460-2471.

ICTV: International Committee on Taxonomy of Viruses. 2011. Virus taxonomy: Classification and nomenclature of viruses. 9th edition. Oxford: Elsevier.

ISO: International Organization for Standardization. 1995. Enumeration of F-specific RNA bacteriophages. Pretoria: SABS, Standards division (SANS 10705).

ISO: International Organization for Standardization. 2000. Enumeration of somatic coliphages. Pretoria: SABS, Standards division (SANS 10705).

ISO: International Organization for Standardization. 2006. Sampling. Pretoria: SABS, Standards division (SANS 5667).

Jończyk, E., Kłak, M., Międzybrodzki, R. & Górski, A. 2011. The influence of external factors on bacteriophages—review. *Folia Microbiologica*, 56: 191–200.

Jordaan, K. & Bezuidenhout, C.C. 2016. Bacterial community composition of an urban river in North West Province, South Africa, in relation to physico-chemical water quality. *Environmental Science and Pollution Research*, 23: 5868-5880.

Karim, M. R., Manshadi, F. D. Karpiscak, M. M. & Gerba, C. P. 2004. The persistence and removal of enteric pathogens in constructed wetlands. *Water Research*, 38 (7): 1831–1837.

Karnik, S. & Billeter, M. 1983. The lysis function of RNA bacteriophage Qβ is mediated by the maturation (A2) protein. *EMBO journal*, 2: 1521–1526.

Kastelein, R.A., Remaut, E., Fiers, W. & van Duin, J. 1982. Lysis gene expression of RNA phage MS2 depends on a frameshift during translation. *Nature*, 276: 35–41.

Kawabe, M. & Kawabe, M. 1997. Temporal and spatial characteristics of chemical oxygen demand in Tokyo Bay. *Journal of Oceanography*, 53:19–26.

Kivaisi, A.K. 2001. The potential for constructed wetlands for wastewater treatment and reuse in developing countries: a review. *Ecological Engineering*, 16: 545-560.

Lavigne, R., Darius, P., Summer, E.J., Seto, D., Mahadevan, P., Nilsson, A.S., Ackermann, H.W. & Kropinski A.M. 2009. Classification of *Myoviridae* bacteriophages using protein sequence similarity. *BMC Microbiology*, 9: 224.

LeChevallier, M.W., Welch, N.J. & Smith, D.B. 1996. Full-scale studies of factors related to coliform regrowth in drinking water. *Applied and Environmental Microbiology*, 62(7):2201-2211.

Leclerc, H., Schwartzbrod, L. & Dei-Cas, E. 2002. Microbial agents associated with waterborne diseases. *Critical Reviews in Microbiology*, 28(4):371-409.

Lehtola, M., Miettinen, I.K., Vartiainen, T. & Martikainen, P.J. 2002. Changes in content of microbially available phosphorus, assimilable organic carbon and microbial growth potential during drinking water treatment processes. *Water Research*, 36:3681-3690.

Levine, A.J. 1992. Viruses. New York: Scientific American Library.

López-Bueno, A., Tamames, J., Velázquez, D., Moya, A., Quesada, A. & Alcamí, A. 2009. High diversity of the viral community from an Antarctic lake. *Science*, 326 (5954): 858–861.

Lucena, F., Méndez, X., Morón, A., Calderón, E., Campos, C., Guerrereo, A., Cárdenas, M., Gantzer, C., Shwartzbrood, L., Skraber, S. & Jofre, J. 2003. Occurence and densities of bacteriophages proposed as indicators and bacterial indicators in river waters from Europe and South America. *Journal of Applied Microbiology*, 94: 808–815.

Luger, M. & Brown, C. 1999. The impact of treated sewage effluent on urban rivers. http://www.southernwaters.co.za Date of access: March 2014.

Luther, K. & Fujioka, R. 2004. Usefulness of monitoring tropical streams for male-specific RNA coliphages. *Journal of Water and Health*, 2(3): 171-81.

Malakoff, D. 2002. Microbiologists on the trail of polluting bacteria. *Science*, 295: 2352-2353.

Marshall, T.R. & Harmse, J.T. 1992. A review of the origin and propagation of pans. *SA Geographer,* 19:9-21.

Miyake, T., Haruna, I., Shiba, T., Itoh, Y.H., Yamane, K. & Watanabe, I. 1971. Genotyping of RNA phages based n the template specificity of their RNA replicases. *Proceedings of the National Acadamy of Science of the United States of America*, 68(9): 2022–2024.

Miyake, T., Shiba, T., Sakurai, T. & Watanabe, I. 1969. Isolation and properties of two new RNA phages SP and FI. *Japanese Journal of Microbiology*, 13: 375–382.

Mocé-Llivina, L., Muniesa, M., Pimenta-Vale, H., Lucena, F. & Jofre, J. 2003. Survival of bacterial indicator species and bacteriophages after thermal treatment of sludge and sewage. *Applied and Environmental Microbiology*, 69:1452–1456.

Mooijman, K.A. Bahar, M., Muniesa, M. & Havelaar, A.H. 2002. Optimisation of ISO 10705-1 on enumeration of F specific bacteriophages. *Journal of Virological Methods*, 103: 129–136.

Morinigo, M.A., Wheeler, D., Berry, C., Jones, C., Munoz, M.A., Cornax, R. & Borrego, J.J. 1992. Evaluation of different bacteriophage groups as faecal indicators in contaminated natural waters in Southern England. *Water Research*, 26: 267–271.

Mullan, W.M.A. 2002. Factors Affecting Plaque Formation. https://www.dairyscience.info/index.php /enumeration-of-lactococcal-bacteriophages/factors-affecting-plaque-formation.html Date of access: October 2016.

Muniesa, M., Lucena, F. & Jofre, J. 1999. Comparative survival of free Shiga toxin 2-encoded phages and *E. coli* strains outside the gut. *Applied Environmental Virology*, 65(12): 5615–5618.

Muniesa, M., Payan, A., Moce-Llivina, L., Blanch, A.R. & Jofre, J. 2009. Differential persistence of F-specific RNA phage subgroups hinders their use as single tracers for faecal source tracking in surface waters. *Water Research*, 43(6): 1559–1564.

MWC-IM: Midvaal Water Company – Inhouse Method. 2010. Somatic coliphage analysis. Klerksdorp.

Nishihara, T. 2002. Various morphological aspects of *Escherichia coli* lysis by two distinct RNA bacteriophages. *Journal of General Virology*. 83: 2601–2606.

Novotny, C.P. & Lavin, K. 1971. Some effects of temperature on the growth of F pili. *Journal of Bacteriology*, 107(3): 671-682.

Nur ilida, M., Tan, G.H., Nur Baizura, S. & Mohd Effendi, M.N. 2013. Isolation and virion morphology classification of coliphages from local foods. *Journal of Tropical Agriculture and Food Science*, 41(2): 349–357.

Ogorzaly, L. & Gantzer, C. 2006. Development of real-time RT-PCR methods for specific detection of F-specific RNA bacteriophage genogroups: Application to urban raw wastewater. *Journal of Virological Methods*, 138: 131–139.

Ogorzaly, L., Tisser, A., Bertand, I., Maul, A. & Gantzer, C. 2009. Relationship between F-specific RNA phage genogroups, faecal pollution indicators and human adenoviruses in river water. *Water Research*, 43: 1257–1264.

Osawa, S., Furuse, K. & Watanabe, I. 1981. Distribution of ribonucleic acid coliphages in animals. *Applied and Environmental Microbiology*, 41(1): 164–168.

Pang, L., Close, M., Goltz, M., Sinton, L., Davies, H., Hall, C. & Stanton, G. 2003. Estimation of septic tank setback distances based on transport of *E.coli* and F-RNA phages. *Environment International*, 29:907-921.

Park, J., Duan, L., Kim, B., Mitchell, M.J. & Shibata, H. 2010. Potential effects of climate change and variability on watershed biogeochemical processes and water quality in Northeast Asia. *Environmental International*, 36:212-225.

Pieper, A.P., Ryan, J.N., Harvey, R.W., Amy, G.L., Illangasekare, T.H. & Metge, D.W. 1997. Transport and recovery of bacteriophage PRD1 in a sand and gravel aquifer: Effect of sewage-derived organic matter. *Environmental Science and Technology*, 31: 1163-1170.

Prigent, M., Leroy, M., Confalonieri, F., Dutertre, M. & DuBow, M.S. 2005. A diversity of bacteriophages forms and genomes can be isolated from the surface sands of Sahara Desert. *Extremophiles*, 9:289–296.

Riou, P., Le Saux, J.C., Dumas, F., Caprais, M.P., Le Guyader, S.F. & Pommepuy, M. 2007. Microbial impact of small tributaries on water and shellfish quality in shallow coastal areas. *Water Research*, 41: 2774–2786.

Rodríguez, R.A., Love, D.C., Steward, J.R., Tajuba, J., Dickerson Jr, J.W., Webster, L.F. & Sobsey, M.D. 2012. Comparison of methods for the detection of coliphages in recreational water at two California, United States beaches. *Journal of Virological Methods*, 181:1–7.

Sakoda, A., Sakai, Y., Hayakawa, K. & Motoyuki, S. 1997. Adsorption of viruses in water environment onto solid surfaces. *Water Science and Technology*, 35(7): 107–114.

Sandaa, R. A. 2009. Viruses, Environmental. (*In* Encyclopedia of Microbiology, Volume 1. 3rd edition. p. 553-567).

SAWS: South African Weather Service. 2016. Historical rain maps. http://www.weathersa.co.za/climate/historical-rain-maps Date of access: January 2016.

Scott, T.M., Rose, J.B., Jenkins, T.M., Farrah, S.R. & Lukasik, J. 2002. Microbial source tracking: current methodology and future directions. *Applied and Environmental Microbiology*, 68(12): 5796-5803.

Servais, P. & Billing, G. 1990. Control of the bacteriological quality of baig waters. *Water Tribune* 543: 23–28.

Silva, Y.J., Costa, L., Pereìra, C., Cunha, Ā., Calado, R., Gomes, N.C.M. & Almeida A. 2014. Influence of environmental variables in the efficiency of phage therapy aquaculture. *Microbial Biotechnology*; 7(5): 401–413.

Singh, V., Jain, P. & Dahiya, S. 2016. Isolation and characterization of bacteriophage from waste water against *E. coli*, a food borne pathogen. *Asian Journal of Microbiology Biotechnology and Environmental Sciences*, 18(1): 163–170.

Sinton, L.W., Finlay, R.K. & Lynch, P. 1999. Sunlight inactivation af faecal bacteriophages and bacteria in sewage-polluted waters. *Applied Environmental Microbiology*, 65 (12): 3605–3613.

Sinton, L.W., Mackenzie, M.L., Karki, N., Braithwaite, R.R., Hall, C.H. & Flintoft, M.J. 2010. Transport of *Escherichia coli* and F-RNA bacteriophages in a 5 m column of saturated pea gravel. *Journal of Contaminant Hydrology*, 117: 71-81.

Skraber, S., Gantzer, C., Maul, A. & Schwartzbrod, L. 2002. Fates of bacteriophages and bacterial indicators in the Moselle river (France). *Water Research*, 36: 3629–3637.

Skraber, S., Gassilloud, B. & Gantzer, C. 2004. Comparison of coliforms and coliphages as tools for assessment of viral contamination in river water. *Applied and Environmental Microbiology*, 70 (6): 3644–3649.

Skraber, S., Ogorzaly, L., Helmi, K., Maul, A., Hoffmann, L., Cauchie, H. & Gantzer, C. 2009. Occurrence and persistence of enteroviruses, noroviruses and F-specific RNA phages in natural wastewater biofilms. *Water Research*, 43(19): 4780–4789.

Smith, E.M., Gerba, C.P. & Melnick, J.L. 1978. Role of sediment in the persistence of enteroviruses in the estuarine environment. *Applied and Environmental Microbiology*, 35(4): 685–689.

Sundram, A., Donnelly, L., Ehlers, M.M., Very, A., Grabow, W.O.K. & Bailey, I.W. 2002. Evaluation of F-RNA coliphages as indicators of viruses and the sources of faecal pollution. *Water SA Special Edition: WISA Proceedings,* 2002: 86–91.

Sundram, A., Jumanlal, N. & Ehlers, M.M. 2006. Genotyping of F-RNA coliphages isolated from wastewater and river water samples. *Water SA*, 32(1): 65–70.

Taj, M. K., Ling, J.X., Bing, L.L., Qi, Z., Taj, I., Hassani, T.M., Samreen, Z. & Yunlin, W. 2014. Effects of dilution, temperature and pH on the lysis activity of T4 phage against *E.coli* BL21. *Journal of Animal and Plant Sciences*, 24(4): 1252–1255.

Tey, B.T., Ooi, S.T., Yong, K.C., Tan Ng, M.Y., Ling, T.C.& Tan, W.S. 2009. Production of fusion m13 phage bearing the disulphide constrained peptide sequence (C-WSFFSNI-C) that interacts with hepatitis B core antigen. *African Journal of Biotechnology*, 8: 268–273.

Van Duin, J. 1998. Bacteriophages. New York: Plenum Press.

Vega, E., Lesikar, B. & Pillai, S.D. 2003. Transport and survival of bacterial and viral tracers through submerged-flow constructed wetland and sand-filter system. *Bioresource Technology*, 89: 49–56.

WHO: World Health Organization. 2007. Nitrate and nitrite in drinking-water. Geneva.

WHO: World Health Organisation. 2011. Guidelines for drinking-water. 4th edition. Geneva.

Winter, R.B. & Gold, L. 1983. Overproduction of bacteriophage Qβ maturation (A2) protein leads to cell lysis. *Cell*, 33: 877–885.

Wingender, J. & Flemming, H.C. 2011. Biofilms in drinking water and their role as reservoir for pathogens. *International Journal of Hygiene and Environmental Health*, 214(6): 417-423.

Wyer, M.D., Kay, D., Jackson, G.F., Dawson, H.M., Yeo, J. & Tanguy, L. 1995. Indicator organism sources and coastal water quality: A catchment study on the island of Jersey. *The Journal of Applied Bacteriology*, 78(3): 290–296.

Yahya, M., Hmaied, F., Jebri, S., Jofre, J. & Hamdi, M. 2015. Bacteriophages as indicators of human and animal faecal contamination in raw and treated wastewaters from Tunisia. *Journal of Applied Microbiology*, 118: 1217–1225.

Yang, Y. & Griffiths, M.W. 2013. Comparative persistence of subgroups of F+RNA phages in river water. *Applied Environmental Microbiology*, 79(15): 4564-4567.

Yanko, W.A., Jackson, J.L., Williams, F.P., Walker, A.S. & Castillo, M.S. 1999. An unexpected temporal pattern of coliphage isolation in groundwaters sampled from wells at varied distances from reclaimed water recharge sites. *Water Research*, 33(1): 53-64.

Yates, M.V., Gerba, C.P. & Kelley, L.M. 1985. Virus persistence in groundwater. *Applied and Environmental Microbiology*, 49: 778–781.

Yin, Y., Zhang, Y., Liu, X., Zhu, G., Qin, B., Shi, Z. & Feng, L. 2011. Temporal and spatial variations of chemical oxygen demand in Lake Taihu, China, from 2005 to 2009. *Hydrobiologia*, 665:129-141.

Young, I, Wang, I. & Roof, W.D. 2000. Phages will out: strategies of host cell lysis. *Trends in Microbiology*, 8(3): 8120–8128.

Young, R. Y. 1992. Bacteriophage lysis: mechanism and regulation. *Microbiological Reviews*, 56(3): 56430–56481.

Zhu, W., Li, J. & Liang, G. 2011. How does cellular heparan sulfate function in viral pathogenicity? *Biomedical and Environmental Sciences*, 24(1): 81–87.

APPENDIX

APPENDIX A – GPS CO-ORDINATES OF SAMPLING SITES

Table 6-1: Table containing site number, site name, water system designation and Global Positioning System (GPS) co-ordinates for sites monitored in this study.

Water system	Site name	Sampling map reference	RDA graph reference	GPS-Coord. S	GPS-Coord. E
Mooi River	Klerkskraal	MR1	M_KK	26°15'11.4"	27°09'31.4"
	Muiskraal	MR2		26°21'36.4"	27°08'20.7"
	Boskop A	MR3	M_BA	26°30'52.3"	27°07'28.5"
	Boskop B	MR4	M_BB	26°34'18.5"	27°06'13.0"
	Thabo Mbeki Bridge	MR5	M_TMB	26°41'05.2"	27°06'00.8"
	Trim Park Bridge	MR6	M_TPB	26°42'29.3"	27°06'20.6"
	Pedestrian Bridge	MR7	M_PB	26°43'29.2"	27°06'21.2"
	Viljoenskroon Bridge	MR8	M_VB	26°45'09.2"	27°06'00.9"
Harts River	Lichtenburg Bridge	HR1	H_LB	26°12'54.7"	26°12'30.3"
	Village	HR2	H_V	26°10'29.0"	26°08'01.6"
	Biesiesvlei	HR3	H_BV	26°25'11.6"	25°53'42.4"
	Vermaas	HR4	H_VM	26°31'51.0"	25°49'21.9"
	Sannieshof	HR5	H-SB	26°35'43.8"	25°44'16.3"
	Delareyville	HR6		26°46'01.7"	25°33'07.9"

Water system	Site name	Sampling map reference	RDA graph reference	GPS-Coord. S	GPS-Coord. E
Barberspan	Harts before Barberspan	BP1	B_HB	26°38'36.9''	25°36'58.4''
	Train Bridge	BP2	B_TB	26°37'05.0''	25°34'37.9''
	Hotel	BP3	B_HT	26°35'24.1''	25°36'09.7''
	North of Goosepan	BP4	B_NG	26°33'37.5''	25°35'46.6''
	Outflow into Leeupan	BP5	B_OL	26°33'00.0''	25°35'48.5''
Crocodile River	Before Brits	CR1	CVB	25°41'09.7''	27°47'33.6''
	After Brits	CR2	CNB	25°32'58.0''	27°42'50.8''
	Pienaars River	CR3	CPR	25°06'24.7''	27°33'55.2''
	Koedoeskop Bridge	CR4	CKB	25°03'59.6''	27°31'06.2''
	Croc Thaba	CR5	CCD	24°39'53.6''	27°22'40.4''
	Croc after Thabazimbi	CR6	CCA	24°24'05.3''	27°05'51.7''
	Croc turn	CR7	CCT	24°12'57.3''	26°53'54.3''
Marico River	Marico Eye	MA1	MA_ME	25°47'22.1''	26°21'59.8''
	Before Sterkstroom	MA2	MA_BS	25°39'34.6''	26°26'01.1''
	Sterkstroom	MA3	MA_SS	25°38'50.8''	26°29'20.5''
	Before Bosveld Dam	MA4	MA_BB	25°35'19.3''	26°24'38.9''
	Below Klein-Maricopoort (Bospoort) Dam.	MA5	MA_BK	25°30'49.3''	26°09'30.8''
	After Marico Bosveld Dam.	MA6	MA_AM	25°27'42.5''	26°23'30.9''
	Derdepoort.	MA7	MA_D	24°50'42.6''	26°29'11.1''

Water system	Site name	Sampling map reference	RDA graph reference	GPS-Coord. S	GPS-Coord. E
Schoonspruit River	Bodenstein	SS1	S_BO	26°25'16.4"	26°43'42.8"
	Confluence with Brakspruit	SS2	S_CB	26°40'46.0"	26°34'58.7"
	Voortrekker	SS3	S_VT	26°51'54.4"	26°39'30.3"
	Orkney	SS4	S_OR	26°59'09.1"	26°37'54.7"

APPENDIX B – SEASONAL RESULTS OF ENUMERATION OF PHAGES

Table 6-2 Results of *E. coli*, F-RNA phage and somatic coliphage enumeration in the Mooi River during this study.

Sampling date	Sample name	Water system	*E. coli* (cfu/100 ml)			F-RNA phages (pfp/100 ml) ± SD			Somatic coliphages (pfp/100ml) ± SD		
2014/11/25	**Klerkskraal**	Mooi River	-		-	0.00	±	0.00	12.0	±	0.00
2014/11/25	**Muiskraal**	Mooi River	-		-	-		-	-		-
2014/11/25	**Boskop A**	Mooi River	-		-	0.00	±	0.00	1868.00	±	0.00
2014/11/25	**Boskop B**	Mooi River	-		-	0.00	±	0.00	160.00	±	0.00
2014/11/25	**Thabo Mbeki Bridge**	Mooi River	-		-	0.00	±	0.00	293.00	±	0.00
2014/11/25	**Trim Park Bridge**	Mooi River	-		-	0.00	±	0.00	253.00	±	0.00
2014/11/25	**Pedestrian bridge**	Mooi River	-		-	0.00	±	0.00	1826.00	±	0.00
2014/11/25	**Viljoen Bridge**	Mooi River	-		-	26.00	±	0.00	2386.00	±	0.00
2015/03/09	**Klerkskraal**	Mooi River	-		-	76.70	±	5.03	3.30	±	0.58
2015/03/09	**Muiskraal**	Mooi River	-		-	0.00	±	0.00	266.70	±	5.51
2015/03/09	**Boskop A**	Mooi River	-		-	13.30	±	2.31	40.00	±	2.65
2015/03/09	**Boskop B**	Mooi River	-		-	3.30	±	0.58	13.30	±	0.58
2015/03/09	**Thabo Mbeki Bridge**	Mooi River	-		-	3.30	±	0.58	76.70	±	4.93
2015/03/09	**Trim Park Bridge**	Mooi River	-		-	0.33	±	0.58	206.70	±	4.93
2015/03/09	**Pedestrian bridge**	Mooi River	-		-	13.30	±	0.58	176.70	±	4.93
2015/03/09	**Viljoen Bridge**	Mooi River	-		-	6.70	±	0.58	36.00	±	1.15
2015/05/12	**Klerkskraal**	Mooi River	40.00	±	1.73	20.00	±	2.65	53.30	±	1.53
2015/05/12	**Muiskraal**	Mooi River	-		-	-		-	-		-
2015/05/12	**Boskop A**	Mooi River	1613.30	±	18.04	0.00	±	0.00	83.30	±	1.15
2015/05/12	**Boskop B**	Mooi River	2053.30	±	20.53	20.00	±	1.73	80.00	±	6.56
2015/05/12	**Thabo Mbeki Bridge**	Mooi River	>300	±	-	0.00	±	0.00	33.30	±	1.53
2015/05/12	**Trim Park Bridge**	Mooi River	>300	±	-	0.00	±	0.00	3.30	±	0.58
2015/05/12	**Pedestrian bridge**	Mooi River	2120.00	±	10.58	0.00	±	0.00	40.00	±	1.73
2015/05/12	**Viljoen Bridge**	Mooi River	3733.30	±	53.27	13.30	±	1.53	100.00	±	1.73

Table 6-3 Results of *E. coli*, F-RNA phage and somatic coliphage enumeration in the Harts River during this study.

Sampling date	Sample name	Water system	*E. coli* (cfu/100 ml)	F-RNA phages (pfp/100 ml) ± SD	Somatic coliphages (pfp/100ml) ± SD
2014/10/22	**Lichtenburg bridge**	Harts River	28400 ± 0.00	10 ± 0.00	2990 ± 0.00
2014/10/28	**Village**	Harts River	4000 ± 0.00	227 ± 0.00	267 ± 0.00
2014/10/28	**Biesiesvlei**	Harts River	2030 ± 0.00	40 ± 0.00	866 ± 0.00
2014/10/28	**Vermaas**	Harts River	6530 ± 0.00	160 ± 0.00	2840 ± 0.00
2014/10/22	**Sannieshof bridge**	Harts River	4940 ± 0.00	10 ± 0.00	1690 ± 0.00

Table 6-4 Results of *E. coli*, F-RNA phage and somatic coliphage enumeration in Barberspan during this study.

Sampling date	Sample name	Water system	E. coli (cfu/100 ml)	F-RNA phages (pfp/100 ml) ± SD	Somatic coliphages (pfp/100ml) ± SD
2014/06/18	**Harts before Barberspan**	Barberspan	- -	1860 ± 17.99	1790 ± 16.46
2014/06/18	**Train bridge**	Barberspan	- -	650 ± 5.12	470 ± 9.64
2014/06/18	**Hotel**	Barberspan	- -	110 ± 0.96	920 ± 7.16
2014/06/18	**North of Goosepan**	Barberspan	- -	373 ± 8.50	960 ± 8.83
2014/06/18	**Outflow into Leeupan**	Barberspan	- -	4270 ± 11.84	460 ± 5.45
2014/10/22	**Harts before Barberspan**	Barberspan	- -	-	- -
2014/10/22	**Train bridge**	Barberspan	1730 ± 0.00	30 ± 0.00	1210 ± 0.00
2014/10/22	**Hotel**	Barberspan	80 ± 0.00	90 ± 0.00	190 ± 0.00
2014/10/22	**North of Goosepan**	Barberspan	80 ± 0.00	0 ± 0.00	306 ± 0.00
2014/10/22	**Outflow into Leeupan**	Barberspan	180 ± 0.00	160 ± 0.00	106 ± 0.00

Table 6-5 Results of *E. coli*, F-RNA phage and somatic coliphage enumeration in the Crocodile River during this study.

Sampling date	Sample name	Water system	*E. coli* (cfu/100 ml)	F-RNA phages (pfp/100 ml) ± SD	Somatic coliphages (pfp/100ml) ± SD
2015/04/21	**Voor Brits**	Crocodile River	1256.7 ± 21.55	6.7 ± 1.15	0 ± 0.00
2015/04/21	**Na Brits**	Crocodile River	11120 ± 90.07	16.7 ± 1.15	70 ± 2.65
2015/04/21	**Pienaars rivier**	Crocodile River	3700 ± 22.63	23.3 ± 1.15	60 ± 2.00
2015/04/21	**Koedoeskop brug**	Crocodile River	2713.3 ± 13.32	0 ± 0.00	0 ± 0.00
2015/04/21	**Croc Thaba**	Crocodile River	1020 ± 11.14	0 ± 0.00	6.7 ± 1.15
2015/04/21	**Croc na Thabazimbi**	Crocodile River	453.3 ± 4.93	10 ± 1.00	13.3 ± 1.53
2015/04/21	**Croc draai**	Crocodile River	5046.7 ± 20.53	3.3 ± 0.58	46.7 ± 0.58
2015/08/03	**Voor Brits**	Crocodile River	266.7 ± 9.87	0 ± 0.00	0 ± 0.00
2015/08/03	**Na Brits**	Crocodile River	1353.3 ± 20.23	0 ±	63 ± 1.60
2015/08/03	**Pienaars rivier**	Crocodile River	880 ± 5.29	0 ± 0.00	46 ± 0.50
2015/08/03	**Koedoeskop brug**	Crocodile River	1206.7 ± 6.43	0 ± 0.00	0 ± 0.00
2015/08/03	**Croc Thaba**	Crocodile River	1640 ± 87.43	0 ± 0.00	0 ± 0.00
2015/08/03	**Croc na Thabazimbi**	Crocodile River	100 ± 4.00	0 ± 0.00	0 ± 0.00
2015/08/03	**Croc draai**	Crocodile River	133.3 ± 3.06	0 ± 0.00	24 ± 0.53

Table 6-6 Results of *E. coli*, F-RNA phage and somatic coliphage enumeration in the Marico River during this study.

Sampling date	Sample name	Water system	*E. coli* (cfu/100 ml)	F-RNA phages (pfp/100 ml) ± SD	Somatic coliphages (pfp/100 ml) ± SD
2015/05/04	**Marico Eye**	Marico River	10 ± 1.73	0 ± 0.00	0 ± 0.00
2015/05/04	**Before Sterkstroom**	Marico River	420 ± 4.36	23.3 ± 2.52	24.2 ± 0.13
2015/05/04	**Sterkstroom**	Marico River	1233.3 ± 20.50	0 ± 0.00	0 ± 0.00
2015/05/04	**Before Bosveld Dam**	Marico River	290 ± 5.66	0 ± 0.00	0 ± 0.00
2015/05/04	**Below Klein-Maricopoort (Bospoort) Dam.**	Marico River	>3000 ±	3.3 ± 0.58	4.6 ± 0.17
2015/05/04	**After Marico Bosveld Dam.**	Marico River	903.3 ± 51.43	3.3 ± 0.58	4.6 ± 0.17
2015/05/04	**Derdepoort.**	Marico River	665 ± 6.36	3.3 ± 0.58	4.6 ± 0.17
2015/08/10	**Marico Eye**	Marico River	33.3 ± 0.58	0 ± 0.00	0 ± 0.00
2015/08/10	**Before Sterkstroom**	Marico River	50 ± 3.00	0 ± 0.00	13 ± 0.40
2015/08/10	**Sterkstroom**	Marico River	113.3 ± 6.11	0 ± 0.00	0 ± 0.00
2015/08/10	**Before Bosveld Dam**	Marico River	153.3 ± 1.15	0 ± 0.00	0 ± 0.00
2015/08/10	**Below Klein-Maricopoort (Bospoort) Dam.**	Marico River	30 ± 1.73	0 ± 0.00	0 ± 0.00
2015/08/10	**After Marico Bosveld Dam.**	Marico River	0 ± 0.00	0 ± 0.00	0 ± 0.00
2015/08/10	**Derdepoort.**	Marico River	256.7 ± 6.11	0 ± 0.00	0 ± 0.00

Table 6-7 Results of *E. coli*, F-RNA phage and somatic coliphage enumeration in the Schoonspruit River during this study.

Sampling date	Sample name	Water system	*E. coli* (cfu/100 ml)	F-RNA phages (pfp/100 ml) ± SD	Somatic coliphages (pfp/100ml) ± SD
2015/05/19	**Orkney**	Schoonspruit River	1233.3 ± 7.23	273.3 ± 6.43	23000 ± 989.95
2015/05/19	**Voortrekker**	Schoonspruit River	650 ± 10.54	0 ± 0.00	575 ± 17.68
2015/05/19	**Confluence with Brakspruit**	Schoonspruit River	716.7 ± 3.51	0 ± 0.00	5200 ± 0.00
2015/05/19	**Bodenstein**	Schoonspruit River	226.7 ± 7.23	0 ± 0.00	533.3 ± 25.17
2015/08/17	**Orkney**	Schoonspruit River	3000 ± 0.00	204 ± 3.62	17000 ± 375.45
2015/08/17	**Voortrekker**	Schoonspruit River	726.7 ± 6.11	0 ± 0.00	483 ± 16.43
2015/08/17	**Confluence with Brakspruit**	Schoonspruit River	36.7 ± 0.58	0 ± 0.00	413 ± 12.34
2015/08/17	**Bodenstein**	Schoonspruit River	120 ± 3.46	0 ± 0.00	375.7 ± 16.37

APPENDIX C – RESULTS OF PHYSICAL PARAMETERS

Table 6-8 Results for selected physical properties monitored in the Mooi River during this study.

Sampling date	Sample name	Water system	Temp (°C) ± SD			Salinity (ppm) ± SD			pH ± SD			TDS (ppm) ± SD			DO (mg/L) ± SD		
2014/11/25	Klerkskraal	Mooi River	20.60	±	0.00	162.00	±	0.00	9.20	±	0.00	234.00	±	0.00	39.70	±	0.00
2014/11/25	Muiskraal	Mooi River	-		-	-		-	-		-	-		-	-		-
2014/11/25	Boskop A	Mooi River	21.00	±	0.00	271.00	±	0.00	8.38	±	0.00	392.00	±	0.00	41.10	±	0.00
2014/11/25	Boskop B	Mooi River	23.10	±	0.00	268.00	±	0.00	8.93	±	0.00	380.00	±	0.00	38.70	±	0.00
2014/11/25	Thabo Mbeki Bridge	Mooi River	22.50	±	.	270.00	±	0.00	8.96	±	0.000	383.00	±	0.00	39.50	±	0.00
2014/11/25	Trim Park Bridge	Mooi River	21.90	±	0.00	422.00	±	0.00	8.68	±	0.00	294.00	±	0.00	38.80	±	0.00
2014/11/25	Pedestrian bridge	Mooi River	23.30	±	0.00	313.00	±	0.00	8.65	±	0.00	444.00	±	0.00	39.10	±	0.00
2014/11/25	Viljoen Bridge	Mooi River	21.80	±	0.00	300.00	±	0.00	8.28	±	0.00	430.00	±	0.00	40.60	±	0.00
2015/03/09	Klerkskraal	Mooi River	22.20	±	0.10	-		-	8.18	±	0.02	274.00	±	0.00	7.23	±	1.17
2015/03/09	Muiskraal	Mooi River	27.77	±	0.06	-		-	7.50	±	0.08	43.07	±	1.16	6.77	±	0.15
2015/03/09	Boskop A	Mooi River	20.50	±	0.10	-		-	7.78	±	0.02	504.33	±	3.79	6.87	±	0.64
2015/03/09	Boskop B	Mooi River	21.67	±	0.31	-		-	8.13	±	0.01	426.33	±	11.50	6.30	±	0.20
2015/03/09	Thabo Mbeki Bridge	Mooi River	21.73	±	0.12	-		-	8.05	±	0.01	442.33	±	9.45	8.37	±	0.49
2015/03/09	Trim Park Bridge	Mooi River	21.33	±	0.06	-		-	7.68	±	0.06	542.67	±	10.21	4.30	±	0.30
2015/03/09	Pedestrian bridge	Mooi River	22.83	±	0.12	-		-	7.77	±	0.02	531.67	±	4.04	5.13	±	0.15
2015/03/09	Viljoen Bridge	Mooi River	22.70	±	0.10	-		-	8.01	±	0.01	506.67	±	9.71	7.87	±	0.60
2015/05/12	Klerkskraal	Mooi River	17.20	±	0.53	191.00	±	2.00	8.28	±	0.02	350.00	±	4.58	13.63	±	1.44
2015/05/12	Muiskraal	Mooi River	-		-	-		-	-		-	-		-	-		-
2015/05/12	Boskop A	Mooi River	17.77	±	0.55	312.33	±	3.06	5.90	±	3.44	565.67	±	7.09	13.87	±	0.93
2015/05/12	Boskop B	Mooi River	20.83	±	0.51	301.67	±	14.22	8.32	±	0.04	548.33	±	23.09	13.20	±	0.36
2015/05/12	Thabo Mbeki Bridge	Mooi River	17.93	±	0.51	303.33	±	3.21	8.42	±	0.02	549.67	±	2.08	14.07	±	0.85
2015/05/12	Trim Park Bridge	Mooi River	17.43	±	0.51	317.33	±	3.06	8.18	±	0.02	579.00	±	7.21	13.60	±	0.61
2015/05/12	Pedestrian bridge	Mooi River	16.80	±	0.30	264.67	±	102.77	8.23	±	0.04	483.00	±	187.07	14.50	±	5.84
2015/05/12	Viljoen Bridge	Mooi River	16.93	±	0.49	321.67	±	3.51	8.20	±	0.02	590.00	±	0.00	14.10	±	1.48

Table 6-9 Results for selected physical properties monitored in the Harts River during this study.

Sampling date	Sample name	Water system	Temp (°C) ± SD	Salinity (ppm) ± SD	pH ± SD	TDS (ppm) ± SD	DO (mg/L) ± SD
2014/04/15	Lichtenburg bridge	Harts River	19.40 ± 0.00	- -	8.26 ± 0.00	494.00 ± 0.00	20.35 ± 0.00
2014/04/15	Village	Harts River	19.00 ± 0.00	- -	7.44 ± 0.00	678.00 ± 0.00	17.16 ± 0.00
2014/04/15	Biesiesvlei	Harts River	21.90 ± 0.00	- -	8.08 ± 0.00	687.00 ± 0.00	27.66 ± 0.00
2014/04/15	Vermaas	Harts River	21.70 ± 0.00	- -	8.23 ± 0.00	375.00 ± 0.00	28.86 ± 0.00
2014/04/15	Sannieshof bridge	Harts River	23.50 ± 0.00	- -	8.45 ± 0.00	401.00 ± 0.00	- -
2014/04/15	Delareyville	Harts River	21.20 ± 0.00	- -	8.01 ± 0.00	203.00 ± 0.00	- -
2014/10/22	Lichtenburg bridge	Harts River	23.00 ± 0.00	476.00 ± 0.00	9.94 ± 0.00	732.00 ± 0.00	28.10 ± 0.00
2014/10/28	Village	Harts River	19.80 ± 0.00	302.00 ± 0.00	8.39 ± 0.00	466.00 ± 0.00	43.00 ± 0.00
2014/10/28	Biesiesvlei	Harts River	21.80 ± 0.00	490.00 ± 0.00	9.72 ± 0.00	683.00 ± 0.00	35.40 ± 0.00
2014/10/28	Vermaas	Harts River	20.90 ± 0.00	527.00 ± 0.00	11.20 ± 0.00	730.00 ± 0.00	36.40 ± 0.00
2014/10/22	Sannieshof bridge	Harts River	27.50 ± 0.00	377.00 ± 0.00	9.60 ± 0.00	535.00 ± 0.00	37.50 ± 0.00

Table 6-10 Results for selected physical properties monitored in Barberspan during this study.

Sampling date	Sample name	Water system	Temp (°C) ± SD	Salinity (ppm) ± SD	pH ± SD	TDS (ppm) ± SD	DO (mg/L) ± SD
2014/06/18	Harts before Barberspan	Barberspan	18.40 ± 0.00	304.00 ± 0.00	8.92 ± 0.00	443.00 ± 0.00	45.10 ± 0.00
2014/06/18	Train bridge	Barberspan	14.50 ± 0.00	460.00 ± 0.00	8.55 ± 0.00	649.00 ± 0.00	46.10 ± 0.00
2014/06/18	Hotel	Barberspan	14.90 ± 0.00	470.00 ± 0.00	8.92 ± 0.00	667.00 ± 0.00	46.60 ± 0.00
2014/06/18	North of Goosepan	Barberspan	15.50 ± 0.00	474.00 ± 0.00	9.12 ± 0.00	680.00 ± 0.00	43.00 ± 0.00
2014/06/18	Outflow into Leeupan	Barberspan	12.80 ± 0.00	585.00 ± 0.00	7.87 ± 0.00	824.00 ± 0.00	47.30 ± 0.00
2014/10/22	Harts before Barberspan	Barberspan	- -	- -	- -	- -	- -
2014/10/22	Train bridge	Barberspan	25.60 ± 0.00	408.00 ± 0.00	9.53 ± 0.00	592.00 ± 0.00	38.40 ± 0.00
2014/10/22	Hotel	Barberspan	23.40 ± 0.00	470.00 ± 0.00	9.97 ± 0.00	595.00 ± 0.00	40.00 ± 0.00
2014/10/22	North of Goosepan	Barberspan	24.30 ± 0.00	421.00 ± 0.00	9.86 ± 0.00	590.00 ± 0.00	39.50 ± 0.00
2014/10/22	Outflow into Leeupan	Barberspan	25.40 ± 0.00	575.00 ± 0.00	9.02 ± 0.00	797.00 ± 0.00	38.60 ± 0.00

Table 6-11 Results for selected physical properties monitored in the Crocodile River during this study.

Sampling date	Sample name	Water system	Temp (°C) ± SD	Salinity (ppm) ± SD	pH ± SD	TDS (ppm) ± SD	DO (mg/L) ± SD
2015/04/21	Voor Brits	Crocodile River	21.77 ± 0.47	251.33 ± 0.58	7.96 ± 0.05	456.33 ± 0.58	6.57 ± 0.55
2015/04/21	Na Brits	Crocodile River	21.50 ± 0.26	301.00 ± 1.00	8.22 ± 0.02	543.67 ± 0.58	7.60 ± 0.10
2015/04/21	Pienaars rivier	Crocodile River	20.77 ± 0.29	262.33 ± 0.58	8.84 ± 0.06	476.33 ± 0.58	6.53 ± 0.40
2015/04/21	Koedoeskop brug	Crocodile River	22.83 ± 1.10	301.00 ± 0.00	8.56 ± 0.08	545.33 ± 2.08	7.29 ± 1.75
2015/04/21	Croc Thaba	Crocodile River	25.07 ± 0.67	356.00 ± 1.00	8.44 ± 0.02	639.67 ± 2.08	8.03 ± 0.25
2015/04/21	Croc na Thabazimbi	Crocodile River	22.57 ± 0.46	304.67 ± 1.53	8.71 ± 0.07	549.67 ± 2.89	7.40 ± 0.30
2015/04/21	Croc draai	Crocodile River	23.17 ± 0.06	366.00 ± 33.29	8.48 ± 0.13	689.33 ± 8.39	7.87 ± 1.16
2015/08/03	Voor Brits	Crocodile River	14.23 ± 0.23	251.00 ± 5.29	8.62 ± 0.09	468.67 ± 0.58	2.03 ± 0.06
2015/08/03	Na Brits	Crocodile River	14.90 ± 1.21	285.33 ± 3.21	8.52 ± 0.12	530.33 ± 4.16	2.17 ± 0.06
2015/08/03	Pienaars rivier	Crocodile River	11.93 ± 0.25	290.67 ± 1.15	9.09 ± 0.01	541.67 ± 0.58	2.03 ± 0.06
2015/08/03	Koedoeskop brug	Crocodile River	12.53 ± 0.35	318.00 ± 3.61	8.73 ± 0.04	578.00 ± 5.29	1.87 ± 0.06
2015/08/03	Croc Thaba	Crocodile River	17.93 ± 0.23	376.33 ± 36.12	8.53 ± 0.07	745.67 ± 7.57	1.90 ± 0.10
2015/08/03	Croc na Thabazimbi	Crocodile River	14.40 ± 0.52	377.00 ± 2.65	8.63 ± 0.01	688.00 ± 5.29	1.53 ± 0.06
2015/08/03	Croc draai	Crocodile River	15.20 ± 0.30	368.67 ± 5.13	8.50 ± 0.16	677.00 ± 7.94	1.50 ± 0.10

Table 6-12 Results for selected physical properties monitored in the Marico River during this study.

Sampling date	Sample name	Water system	Temp (°C) ± SD	Salinity (ppm) ± SD	pH ± SD	TDS (ppm) ± SD	DO (mg/L) ± SD
2015/05/04	Marico Eye	Marico River	20.83 ± 0.23	122.33 ± 0.58	7.66 ± 0.03	222.67 ± 1.53	12.27 ± 0.31
2015/05/04	Before Sterkstroom	Marico River	16.87 ± 0.47	127.33 ± 0.58	7.93 ± 0.05	234.33 ± 1.53	7.03 ± 0.61
2015/05/04	Sterkstroom	Marico River	14.67 ± 0.40	45.87 ± 0.64	7.52 ± 0.33	81.50 ± 0.36	6.57 ± 0.68
2015/05/04	Before Bosveld Dam	Marico River	20.63 ± 0.47	133.33 ± 0.58	7.72 ± 0.07	243.67 ± 1.15	6.00 ± 0.26
2015/05/04	Below Klein-Maricopoort (Bospoort) Dam.	Marico River	14.23 ± 0.25	279.00 ± 2.65	7.90 ± 0.03	513.33 ± 3.21	6.77 ± 3.75
2015/05/04	After Marico Bosveld Dam.	Marico River	19.53 ± 0.68	119.67 ± 1.53	7.67 ± 0.21	219.00 ± 2.00	6.23 ± 0.74
2015/05/04	Derdepoort.	Marico River	16.73 ± 0.51	189.33 ± 2.52	8.24 ± 0.27	349.33 ± 4.04	6.07 ± 0.35
2015/08/10	Marico Eye	Marico River	21.10 ± 0.92	123.33 ± 1.53	7.77 ± 0.32	227.00 ± 1.73	1.97 ± 0.23
2015/08/10	Before Sterkstroom	Marico River	14.17 ± 0.78	128.33 ± 1.53	8.01 ± 0.09	240.67 ± 0.58	10.17 ± 0.12
2015/08/10	Sterkstroom	Marico River	13.93 ± 1.23	56.73 ± 1.00	8.14 ± 0.20	104.00 ± 1.73	9.40 ± 0.20
2015/08/10	Before Bosveld Dam	Marico River	51.37 ± 67.23	124.00 ± 1.00	8.22 ± 0.18	241.33 ± 3.79	7.43 ± 0.15
2015/08/10	Below Klein-Maricopoort (Bospoort) Dam.	Marico River	7.93 ± 0.21	267.00 ± 4.36	8.46 ± 0.03	505.33 ± 8.14	8.87 ± 0.40
2015/08/10	After Marico Bosveld Dam.	Marico River	19.03 ± 1.21	117.33 ± 2.52	8.08 ± 0.14	217.33 ± 3.79	6.80 ± 0.20
2015/08/10	Derdepoort.	Marico River	15.30 ± 0.10	202.67 ± 0.58	8.56 ± 0.07	374.33 ± 2.52	7.27 ± 0.25

Table 6-13 Results for selected physical properties monitored in the Schoonspruit River during this study.

Sampling date	Sample name	Water system	Temp (°C) ± SD	Salinity (ppm) ± SD	pH ± SD	TDS (ppm) ± SD	DO (mg/L) ± SD
2015/05/19	Orkney	Schoonspruit River	16.60 ± 0.44	516.00 ± 17.58	7.50 ± 0.03	959.00 ± 12.77	2.63 ± 0.40
2015/05/19	Voortrekker	Schoonspruit River	15.73 ± 0.81	326.00 ± 3.61	7.59 ± 0.03	585.67 ± 17.90	4.93 ± 0.65
2015/05/19	Confluence with Brakspruit	Schoonspruit River	15.53 ± 0.78	356.67 ± 7.51	8.14 ± 0.08	648.67 ± 13.65	6.63 ± 1.27
2015/05/19	Bodenstein	Schoonspruit River	14.23 ± 0.06	340.33 ± 2.08	8.29 ± 0.07	624.00 ± 4.36	8.93 ± 0.35
2015/08/17	Orkney	Schoonspruit River	14.13 ± 0.06	541.67 ± 2.08	7.61 ± 0.05	977.67 ± 3.06	2.83 ± 0.12
2015/08/17	Voortrekker	Schoonspruit River	12.63 ± 0.12	367.67 ± 3.21	7.61 ± 0.03	677.33 ± 0.58	5.63 ± 0.45
2015/08/17	Confluence with Brakspruit	Schoonspruit River	15.23 ± 0.40	278.67 ± 0.58	8.53 ± 0.01	510.67 ± 0.58	12.37 ± 0.96
2015/08/17	Bodenstein	Schoonspruit River	13.07 ± 0.21	311.67 ± 0.58	8.19 ± 0.01	574.00 ± 1.00	10.83 ± 0.45

Table 6-14 Average physical parameter levels of the six water systems monitored in this study for the time period of 2014 and 2015.

Sample name	Water system	Temp (°C)	Salinity (ppm)	pH	TDS (ppm)	DO (mg/L)
Klerkskraal	Mooi River	19.70	176.50	8.23	312.00	10.43
Muiskraal	Mooi River	27.77	-	7.50	43.07	6.77
Boskop A	Mooi River	19.13	291.67	6.84	535.00	10.37
Boskop B	Mooi River	21.25	284.83	8.23	487.33	9.75
Thabo Mbeki Bridge	Mooi River	19.83	286.67	8.24	496.00	11.22
Trim Park Bridge	Mooi River	19.38	369.67	7.93	560.83	8.95
Pedestrian Bridge	Mooi River	19.82	288.83	8.00	507.33	9.82
Viljoen Bridge	Mooi River	19.82	310.83	8.11	548.33	10.98
Lichtenburg Bridge	Harts River	21.20	413.00	9.10	613.00	24.23
Village	Harts River	19.40	302.00	7.92	572.00	30.08
Biesiesvlei	Harts River	21.85	490.00	8.90	685.00	31.53
Vermaas	Harts River	21.30	527.00	9.72	552.50	32.63
Sannieshof Bridge	Harts River	25.50	377.00	9.03	468.00	37.50
Harts before Barberspan	Barberspan	18.40	304.00	8.92	443.00	45.10
Train Bridge	Barberspan	20.05	434.00	9.04	620.50	42.25
Hotel	Barberspan	19.15	470.00	9.45	631.00	43.30
North of Goosepan	Barberspan	19.90	447.50	9.49	635.00	41.25
Outflow into Leeuwpan	Barberspan	19.10	580.00	8.45	810.50	42.95
Before Brits	Crocodile River	18.00	251.17	8.29	462.50	4.30
After Brits	Crocodile River	18.20	293.17	8.37	537.00	4.89
Pienaars River	Crocodile River	16.35	276.50	8.96	509.00	4.28
Koedoeskop Bridge	Crocodile River	17.68	309.50	8.65	561.67	4.58
Croc Thaba	Crocodile River	21.50	366.17	8.49	692.67	4.97
Croc after Thabazimbi	Crocodile River	18.48	340.83	8.67	618.83	4.47
Croc turn	Crocodile River	19.18	367.34	8.49	683.17	4.68
Marico Eye	Marico River	20.97	122.83	7.71	224.83	7.12
Before Sterkstroom	Marico River	15.52	127.83	7.97	237.50	8.60
Sterkstroom	Marico River	14.30	51.30	7.83	92.75	7.98
Before Bosveld Dam	Marico River	18.00	128.67	7.97	242.50	6.72
Below Klein-Maricopoort (Bospoort) Dam.	Marico River	11.08	273.00	8.18	509.33	7.82
After Marico Bosveld Dam.	Marico River	19.28	118.50	7.88	218.17	6.52
Derdepoort.	Marico River	16.02	196.00	8.40	361.83	6.65

Sample name	Water system	Temp (°C)	Salinity (ppm)	pH	TDS (ppm)	DO (mg/L)
Bodenstein	Schoonspruit River	13.65	326.00	8.24	599.00	9.88
Confluence with Brakspruit	Schoonspruit River	15.38	317.67	8.33	579.67	9.50
Voortrekker	Schoonspruit River	14.18	346.84	7.60	631.50	5.28
Orkney	Schoonspruit River	15.37	528.84	7.56	968.34	2.73

APPENDIX D – SEASONAL RESULTS OF CHEMICAL PARAMETERS

Table 6-15 Results of selected chemical properties monitored in the Mooi River during the study.

Sampling date	Sample name	Water system	NO_2^- (mg/L) ± SD	NO_3^-(mg/L) ± SD	SO_4^{2-}(mg/L) ± SD	PO_4^{3-}(mg/L) ± SD	COD (mg/L) ± SD
2014/11/25	Klerkskraal	Mooi River	- -	6.00 ± 0.00	9.00 ± 0.00	2.11 ± 0.00	21.00 ± 0.00
2014/11/25	Muiskraal	Mooi River	- -	- -	- -	- -	- -
2014/11/25	Boskop A	Mooi River	- -	12.00 ± 0.00	34.00 ± 0.00	3.62 ± 0.00	5.00 ± 0.00
2014/11/25	Boskop B	Mooi River	- -	6.00 ± 0.00	11.00 ± 0.00	2.48 0.00	13.00 0.00
2014/11/25	Thabo Mbeki Bridge	Mooi River	- -	5.00 ± 0.00	8.00 ± 0.00	0.76 ± 0.00	15.00 ± 0.00
2014/11/25	Trim Park Bridge	Mooi River	- -	14.00 ± 0.00	20.00 ± 0.00	3.53 ± 0.00	26.00 ± 0.00
2014/11/25	Pedestrian bridge	Mooi River	- -	9.00 ± 0.00	<5 ± 0.00	4.79 ± 0.00	17.00 ± 0.00
2014/11/25	Viljoen Bridge	Mooi River	- -	5.00 ± 0.00	<5 ± 0.00	6.23 ± 0.00	11.00 ± 0.00
2015/03/09	Klerkskraal	Mooi River	12.00 ± 5.66	0.50 ± 0.28	1.50 ± 0.71	- -	22.50 ± 6.36
2015/03/09	Muiskraal	Mooi River	136.00 ± 7.07	51.25 ± 18.17	0.00 ± 0.00	- -	45.50 ± 4.95
2015/03/09	Boskop A	Mooi River	11.50 ± 4.95	1.05 ± 0.07	89.00 ± 1.41	- -	8.00 ± 2.83
2015/03/09	Boskop B	Mooi River	7.00 ± 1.41	0.45 ± 0.07	85.00 ± 2.83	- -	7.00 ± 1.41
2015/03/09	Thabo Mbeki Bridge	Mooi River	8.00 ± 1.41	0.30 ± 0.28	68.00 ± 1.41	- -	29.50 ± 26.16
2015/03/09	Trim Park Bridge	Mooi River	11.00 ± 2.83	1.50 ± 0.28	87.00 ± 4.24	- -	2.00 ± 0.00
2015/03/09	Pedestrian bridge	Mooi River	13.00 ± 1.41	1.00 ± 0.28	88.00 ± 1.41	- -	7.50 ± 0.71
2015/03/09	Viljoen Bridge	Mooi River	23.00 ± 9.90	0.45 ± 0.21	87.50 ± 0.71	- -	4.00 ± 2.83
2015/05/12	Klerkskraal	Mooi River	0.85 ± 0.07	4.50 ± 0.71	4.00 ± 4.24	0.07 ± 0.02	0.00 ± 0.00
2015/05/12	Muiskraal	Mooi River	- -	- -	- -	- -	- -
2015/05/12	Boskop A	Mooi River	1.11 ± 0.15	8.50 ± 0.71	80.00 ± 0.00	0.21 ± 0.02	0.00 ± 0.00
2015/05/12	Boskop B	Mooi River	0.45 ± 0.07	17.00 ± 0.00	80.00 ± 0.00	0.42 ± 0.01	0.00 ± 0.00
2015/05/12	Thabo Mbeki Bridge	Mooi River	0.35 ± 0.07	5.00 ± 0.00	80.00 ± 0.00	0.22 ± 0.04	8.50 ± 6.36
2015/05/12	Trim Park Bridge	Mooi River	0.70 ± 0.28	4.50 ± 0.71	80.00 ± 0.00	0.21 ± 0.04	0.00 ± 0.00
2015/05/12	Pedestrian bridge	Mooi River	0.55 ± 0.07	8.50 ± 0.71	80.00 ± 0.00	0.30 ± 0.05	0.00 ± 0.00
2015/05/12	Viljoen Bridge	Mooi River	14.00 ± 0.00	0.25 ± 0.07	80.00 ± 0.00	0.35 ± 0.01	1.50 ± 2.12

Table 6-16 Results of selected chemical properties monitored in the Harts River during the study.

Sampling date	Sample name	Water system	NO_2^- (mg/L) ± SD	NO_3^-(mg/L) ± SD	SO_4^{2-}(mg/L) ± SD	PO_4^{3-}(mg/L) ± SD	COD (mg/L) ± SD
2014/04/15	Lichtenburg bridge	Harts River	0.00 ± 0.00	1.00 ± 0.00	- -	- -	42.00 ± 0.00
2014/04/15	Village	Harts River	0.80 ± 0.00	3.50 ± 0.00	- -	- -	35.00 ± 0.00
2014/04/15	Biesiesvlei	Harts River	0.20 ± 0.00	6.00 ± 0.00	- -	- -	32.00 ± 0.00
2014/04/15	Vermaas	Harts River	0.20 ± 0.00	3.00 ± 0.00	- -	- -	31.00 ± 0.00
2014/04/15	Sannieshof bridge	Harts River	0.10 ± 0.00	2.50 ± 0.00	- -	- -	32.00 ± 0.00
2014/04/15	Delareyville	Harts River	0.10 ± 0.00	4.00 ± 0.00	- -	- -	43.00 ± 0.00
2014/10/22	Lichtenburg bridge	Harts River	0.00 ± 0.00	0.00 ± 0.00	90.00 ± 0.00	1.13 ± 0.00	81.00 ± 0.00
2014/10/28	Village	Harts River	3.00 ± 0.00	0.20 ± 0.00	22.00 ± 0.00	1.21 ± 0.00	67.00 ± 0.00
2014/10/28	Biesiesvlei	Harts River	3.00 ± 0.00	0.10 ± 0.00	9.00 ± 0.00	3.05 ± 0.00	93.00 ± 0.00
2014/10/28	Vermaas	Harts River	11.00 ± 0.00	0.60 ± 0.00	54.00 ± 0.00	4.43 ± 0.00	98.00 ± 0.00
2014/10/22	Sannieshof bridge	Harts River	0.00 ± 0.00	0.10 ± 0.00	39.00 ± 0.00	0.86 ± 0.00	76.00 ± 0.00
2014/10/28	Delareyville	Harts River	- -	- -	- -	- -	- -

Table 6-17 Results of selected chemical properties monitored in Barberspan during the study.

Sampling date	Sample name	Water system	NO_2^- (mg/L) ± SD	NO_3^-(mg/L) ± SD	SO_4^{2-}(mg/L) ± SD	PO_4^{3-}(mg/L) ± SD	COD (mg/L) ± SD
2014/06/18	Harts before Barberspan	Barberspan	0.40 ± 0.00	- -	39.00 ± 0.00	2.87 ± 0.00	38.00 ± 0.00
2014/06/18	Train bridge	Barberspan	1.00 ± 0.00	- -	44.00 ± 0.00	3.11 ± 0.00	35.00 ± 0.00
2014/06/18	Hotel	Barberspan	1.10 ± 0.00	- -	79.00 ± 0.00	5.32 ± 0.00	67.00 ± 0.00
2014/06/18	North of Goosepan	Barberspan	0.80 ± 0.00	- -	59.00 ± 0.00	2.42 ± 0.00	63.00 ± 0.00
2014/06/18	Outflow into Leeupan	Barberspan	0.70 ± 0.00	- -	22.00 ± 0.00	4.16 ± 0.00	87.00 ± 0.00
2014/10/22	Harts before Barberspan	Barberspan	- -	- -	- -	- -	- -
2014/10/22	Train bridge	Barberspan	0(-4) ± 0.00	0.00 ± 0.00	17.00 ± 0.00	1.32 ± 0.00	75.00 ± 0.00
2014/10/22	Hotel	Barberspan	0.00 ± 0.00	0.00 ± 0.00	35.00 ± 0.00	2.06 ± 0.00	76.00 ± 0.00
2014/10/22	North of Goosepan	Barberspan	0.00 ± 0.00	0.00 ± 0.00	22.00 ± 0.00	2.85 ± 0.00	80.00 ± 0.00
2014/10/22	Outflow into Leeupan	Barberspan	7.00 ± 0.00	0.00 ± 0.00	1.00 ± 0.00	10.24 ± 0.00	82.00 ± 0.00

Table 6-18 Results of selected chemical properties monitored in the Crocodile River during the study.

Sampling date	Sample name	Water system	NO_2^- (mg/L) ± SD	NO_3^-(mg/L) ± SD	SO_4^{2-}(mg/L) ± SD	PO_4^{3-}(mg/L) ± SD	COD (mg/L) ± SD
2015/04/21	Voor Brits	Crocodile River	0.00 ± 0.00	1.95 ± 0.21	71.00 ± 12.73	1.22 ± 0.02	5.00 ± 0.00
2015/04/21	Na Brits	Crocodile River	3.50 ± 0.71	1.80 ± 0.14	74.50 ± 7.78	1.39 ± 0.52	3.00 ± 1.41
2015/04/21	Pienaars rivier	Crocodile River	0.50 ± 0.71	0.00 ± 0.00	41.50 ± 6.36	2.03 ± 0.06	17.00 ± 1.41
2015/04/21	Koedoeskop brug	Crocodile River	4.00 ± 4.24	0.00 ± 0.00	52.50 ± 4.95	1.55 ± 0.22	27.00 ± 7.07
2015/04/21	Croc Thaba	Crocodile River	8.50 ± 2.12	0.95 ± 0.35	59.00 ± 2.83	1.13 ± 0.07	58.50 ± 7.78
2015/04/21	Croc na Thabazimbi	Crocodile River	9.00 ± 4.24	0.00 ± 0.00	68.50 ± 2.12	0.57 ± 0.06	30.00 ± 1.41
2015/04/21	Croc draai	Crocodile River	10.00 ± 2.83	0.00 ± 0.00	74.50 ± 3.54	0.22 ± 0.06	24.00 ± 7.07
2015/08/03	Voor Brits	Crocodile River	15.00 ± 4.24	3.37 ± 1.21	65.50 ± 3.54	3.04 ± 0.23	19.50 ± 9.19
2015/08/03	Na Brits	Crocodile River	12.50 ± 6.36	3.20 ± 0.92	66.50 ± 0.71	2.32 ± 0.05	35.50 ± 4.95
2015/08/03	Pienaars rivier	Crocodile River	7.00 ± 2.83	1.37 ± 1.02	53.00 ± 1.41	3.09 ± 0.21	29.00 ± 4.24
2015/08/03	Koedoeskop brug	Crocodile River	8.00 ± 4.24	1.57 ± 1.23	67.00 ± 1.41	1.15 ± 0.08	20.00 ± 2.83
2015/08/03	Croc Thaba	Crocodile River	6.50 ± 2.12	1.07 ± 0.76	78.50 ± 2.12	1.51 ± 0.02	32.00 ± 5.66
2015/08/03	Croc na Thabazimbi	Crocodile River	11.00 ± 2.83	1.10 ± 0.40	74.00 ± 2.83	1.97 ± 0.13	20.00 ± 4.24
2015/08/03	Croc draai	Crocodile River	15.50 ± 0.71	0.67 ± 0.76	78.50 ± 3.54	2.10 ± 0.07	8.50 ± 4.95

Table 6-19 Results of selected chemical properties monitored in the Marico River during the study.

Sampling date	Sample name	Water system	NO_2^- (mg/L) ± SD	NO_3^-(mg/L) ± SD	SO_4^{2-}(mg/L) ± SD	PO_4^{3-}(mg/L) ± SD	COD (mg/L) ± SD
2015/05/04	Marico Eye	Marico River	0.80 ± 0.14	12.00 ± 1.41	0.00 ± 0.00	0.17 ± 0.06	143.50 ± 10.61
2015/05/04	Before Sterkstroom	Marico River	1.60 ± 0.14	5.00 ± 1.41	0.00 ± 0.00	0.22 ± 0.01	53.00 ± 8.49
2015/05/04	Sterkstroom	Marico River	0.55 ± 0.07	0.00 ± 0.00	0.00 ± 0.00	0.19 ± 0.08	60.50 ± 0.71
2015/05/04	Before Bosveld Dam	Marico River	0.30 ± 0.14	4.50 ± 2.12	0.50 ± 0.71	0.14 ± 0.01	47.00 ± 5.66
2015/05/04	Below Klein-Maricopoort (Bospoort) Dam.	Marico River	0.00 ± 0.00	3.00 ± 1.41	80.00 ± 0.00	0.13 ± 0.02	38.00 ± 15.56
2015/05/04	After Marico Bosveld Dam.	Marico River	0.45 ± 0.07	8.50 ± 0.71	1.50 ± 0.71	0.25 ± 0.10	10.00 ± 14.14
2015/05/04	Derdepoort.	Marico River	0.20 ± 0.00	10.00 ± 2.83	21.00 ± 1.41	0.10 ± 0.06	43.00 ± 9.90
2015/08/10	Marico Eye	Marico River	13.50 ± 0.71	0.75 ± 0.07	3.00 ± 1.41	0.15 ± 0.07	8.50 ± 6.36
2015/08/10	Before Sterkstroom	Marico River	1.50 ± 0.71	0.60 ± 0.14	1.00 ± 0.00	0.77 ± 0.89	0.00 ± 0.00
2015/08/10	Sterkstroom	Marico River	0.00 ± 0.00	0.25 ± 0.07	39.50 ± 2.12	0.36 ± 0.40	0.00 ± 0.00
2015/08/10	Before Bosveld Dam	Marico River	5.00 ± 0.00	0.25 ± 0.07	7.50 ± 0.71	0.65 ± 0.60	0.00 ± 0.00
2015/08/10	Below Klein-Maricopoort (Bospoort) Dam.	Marico River	6.00 ± 0.00	0.00 ± 0.00	76.00 ± 0.00	0.15 ± 0.08	20.50 ± 2.12
2015/08/10	After Marico Bosveld Dam.	Marico River	4.50 ± 0.71	0.50 ± 0.00	7.50 ± 2.12	0.49 ± 0.04	3.50 ± 4.95
2015/08/10	Derdepoort.	Marico River	0.00 ± 0.00	0.00 ± 0.00	28.50 ± 0.71	0.05 ± 0.00	18.50 ± 4.95

Table 6-20 Results of selected chemical properties monitored in the Schoonspruit River during the study.

Sampling date	Sample name	Water system	NO_2^- (mg/L) ± SD	NO_3^-(mg/L) ± SD	SO_4^{2-}(mg/L) ± SD	PO_4^{3-}(mg/L) ± SD	COD (mg/L) ± SD
2015/05/19	Orkney	Schoonspruit River	0.90 ± 0.28	9.00 ± 4.24	85.50 ± 0.71	2.75 ± 0.00	58.50 ± 26.16
2015/05/19	Voortrekker	Schoonspruit River	0.45 ± 0.07	4.50 ± 0.71	88.00 ± 1.41	0.41 ± 0.00	101.50 ± 2.12
2015/05/19	Confluence with Brakspruit	Schoonspruit River	0.05 ± 0.07	4.50 ± 2.12	28.50 ± 0.71	0.61 ± 0.06	9.00 ± 4.24
2015/05/19	Bodenstein	Schoonspruit River	0.45 ± 0.07	7.00 ± 1.41	1.00 ± 0.00	0.50 ± 0.16	0.00 ± 0.00
2015/08/17	Orkney	Schoonspruit River	14.00 ± 2.83	1.80 ± 0.99	118.50 ± 2.12	4.05 ± 0.07	5.50 ± 4.95
2015/08/17	Voortrekker	Schoonspruit River	15.50 ± 4.95	0.55 ± 0.07	104.00 ± 2.83	0.27 ± 0.01	3.50 ± 0.71
2015/08/17	Confluence with Brakspruit	Schoonspruit River	12.00 ± 7.07	0.40 ± 0.14	9.00 ± 0.00	0.24 ± 0.01	7.00 ± 5.66
2015/08/17	Bodenstein	Schoonspruit River	21.50 ± 0.71	0.20 ± 0.00	5.00 ± 1.41	0.04 ± 0.00	14.50 ± 10.61

Table 6-21 Average chemical parameter levels of the six water systems monitored in this study for the time period of 2014 and 2015.

Sample name	Water system	NO_2^- (mg/L)	NO_3^- (mg/L)	SO_4^{2-} (mg/L)	PO_4^{3-} (mg/L)	COD (mg/L)
Klerkskraal	Mooi River	6.43	2.50	2.75	1.09	11.25
Muiskraal	Mooi River	-	-	-	-	-
Boskop A	Mooi River	6.30	4.78	84.50	1.91	4.00
Boskop B	Mooi River	3.73	8.73	82.50	1.45	3.50
Thabo Mbeki Bridge	Mooi River	4.18	2.65	74.00	0.49	19.00
Trim Park Bridge	Mooi River	5.85	3.00	83.50	1.87	1.00
Pedestrian Bridge	Mooi River	6.78	4.75	84.00	2.54	3.75
Viljoen Bridge	Mooi River	18.50	0.35	83.75	3.29	2.75
Lichtenburg Bridge	Harts River	0.00	0.50	90.00	1.13	61.50
Village	Harts River	1.90	1.85	22.00	1.21	51.00
Biesiesvlei	Harts River	1.60	3.05	9.00	3.05	62.50
Vermaas	Harts River	5.60	1.80	54.00	4.43	64.50
Sannieshof Bridge	Harts River	0.05	1.30	39.00	0.86	54.00
Harts before Barberspan	Barberspan	0.40	0.00	39.00	2.87	38.00
Train Bridge	Barberspan	0.50	0.00	30.50	2.22	55.00
Hotel	Barberspan	0.55	0.00	57.00	3.69	71.50
North of Goosepan	Barberspan	0.40	0.00	40.50	2.64	71.50
Outflow into Leeuwpan	Barberspan	3.85	0.00	11.50	7.20	84.50
Before Brits	Crocodile River	7.50	2.66	68.25	2.13	12.25
After Brits	Crocodile River	8.00	2.50	70.50	1.86	19.25
Pienaars River	Crocodile River	3.75	0.69	47.25	2.56	23.00
Koedoeskop Bridge	Crocodile River	6.00	0.79	59.75	1.35	23.50
Croc Thaba	Crocodile River	7.50	1.01	68.75	1.32	45.25
Croc after Thabazimbi	Crocodile River	10.00	0.55	71.25	1.27	25.00
Croc turn	Crocodile River	12.75	0.34	76.50	1.16	16.25
Marico Eye	Marico River	7.15	6.38	1.50	0.16	76.00
Before Sterkstroom	Marico River	1.55	2.80	0.50	0.50	26.50
Sterkstroom	Marico River	0.28	0.13	19.75	0.27	30.25
Before Bosveld Dam	Marico River	2.65	2.38	4.00	0.40	23.50
Below Klein-Maricopoort (Bospoort) Dam.	Marico River	3.00	1.50	78.00	0.14	29.25
After Marico Bosveld Dam.	Marico River	2.48	4.50	4.50	0.37	6.75
Derdepoort.	Marico River	0.10	5.00	24.75	0.08	30.75

Sample name	Water system	NO_2^- (mg/L)	NO_3^- (mg/L)	SO_4^{2-} (mg/L)	PO_4^{3-} (mg/L)	COD (mg/L)
Bodenstein	Schoonspruit River	10.98	3.60	3.00	0.27	7.25
Confluence with Brakspruit	Schoonspruit River	6.03	2.45	18.75	0.42	8.00
Voortrekker	Schoonspruit River	7.98	2.53	96.00	0.34	52.50
Orkney	Schoonspruit River	7.45	5.40	102.00	3.40	32.00