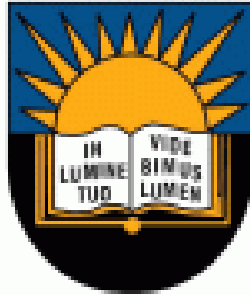


**QUALITY INDICES OF THE FINAL EFFLUENTS OF TWO SUB-URBAN-BASED
WASTEWATER TREATMENT PLANTS IN AMATHOLE DISTRICT MUNICIPALITY
IN THE EASTERN CAPE PROVINCE OF SOUTH AFRICA.**



University of Fort Hare
Together in Excellence

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SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE IN MICROBIOLOGY

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2014

DECLARATION

I, the undersigned, hereby declare that this dissertation submitted to the University of Fort Hare for the degree of Master of Science in Microbiology in the Faculty of Science and Agriculture, is my original work with the exception of the citations. I also declare that this work has not been submitted to any other University in partial or entirety for the award of any degree. I certify that this dissertation is devoid of any element of plagiarism and in the event that element(s) of plagiarism is/are detected in this dissertation I and I alone will be held responsible for the offence

ONELE GCILITSHANA

SIGNATURE

DATE

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DEDICATION

This is dedicated to my beloved mother; Mrs N. Gcilitshana and my late father; Mr A. Z. Gcilitshana, all that I am and will be, I owe it to them.

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LIST OF ABBREVIATIONS

ADRV	- Adult diarrhoea rotavirus
AEMREG	- Applied and Environmental Microbiology Research Group
ATCC	- American Type Culture Collection
BOD	- Biochemical oxygen demand
CCME	- Canadian Council of Ministers of the Environment
cDNA	- Complementary Deoxyribonucleic acid
COD	- Chemical oxygen demand
DO	- Dissolved oxygen
DNA	- Deoxyribo-nucleic Acid
DWAF	- Department of water affairs
EC	-Electrical conductivity
EtBr	- Ethidium bromide
GSCN	- Guadium thiocyanate
HAV	- Hepatitis A virus
ICC-RTPCR	- Intergrated cell-culture quantitative reverse transcription PCR
PCR	- Polymerase chain reaction
RNA	- Ribonucleic Acid
TDS	- Total dissolved solids
UN	- United Nations
UNECA	- United Nations Economic Commission for Africa
UNEP	- United Nations Environmental Programme
WHO	- World health organization
WRC	- Water research commission
WWTP	- Wastewater treatment plant

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ABSTRACT

Worldwide, water reuse is promoted as an alternative for water scarcity, however, wastewater effluents have been reported as possible contaminants to surface water. The failure of some wastewater treatment processes to completely remove organic matter and some pathogenic microorganisms allows them to initiate infections. This manifests more in communities where surface water is used directly for drinking. To assess water quality, bacteria alone cannot be used as it may be absent in virus-contaminated water. This study was carried out to assess the quality of two wastewater treatment plant effluents from the Eastern Cape Province of South Africa. Physicochemical parameters and microbiological parameters like faecal coliforms, adenovirus, rotavirus, hepatitis A virus, norovirus and enterovirus were evaluated over a projected period of one year. Physicochemical parameters were measured on site using multiparameters, faecal coliforms enumerated using culture-based methods and viruses are detected using both conventional and real-time PCR.

Physicochemical parameters like electrical conductivity, turbidity, free chlorine and phosphates were incompliant with the standards set by the Department of Water affairs for effluents to be discharged. Faecal coliform counts were nil for one plant (WWTP-R) where they correlated inversely ($P < 0.01$) with the high free chlorine. For WWTP-K, faecal coliforms were detected in 27% of samples in the range of 9.9×10^1 to 6.4×10^4 CFU/100ml. From the five viruses assessed, three viruses were detected with Rotavirus being the most abundant (0-2034176 genome copies/L) followed by Adenovirus (0-275 genome copies/L) then Hepatitis A virus (0-71 genome copies/L) in the WWTP-K while none of the viruses was detected in WWTP-R. Species

B, species C and Adv41 serotypes were detected from the May 2013 and June 2013 samples where almost all parameters were incompliant in the plant.

The detection of these viruses in supposedly treated effluents is suggestive of these being the sources of contamination to surface water and therefore renders surface waters unsafe for direct use and to aquatic life. Although real-time PCR is more sensitive and reliable in detection of viruses, use of cell-culture techniques in this study would have been more efficient in confirming the infectivity of the viruses detected, hence the recommendation of these techniques in future projects of this nature.

CHAPTER ONE

BACKGROUND STUDY

Water forms the backbone of the world's economy and is essential for living systems, industrial processes, agricultural production, and domestic uses (Hu, 2009). According to a report by DWA (1996a), water has been predicted to be one of the scarcest resources worldwide due to the increasing world population. Years later, Brulliard (2009) reported that "*South Africa's available freshwater resources are already almost fully-utilised and under stress and the rapidly increasing population growth rate and economic development rate, make it highly unlikely for the projected demand on water resources in South Africa to be sustainable*", thus suggesting the need for reuse of wastewater after proper purification. Therefore, worldwide, reuse of wastewater has become a solution to water scarcity because access to water, especially adequately treated water, is a basic right and need to all humans (WHO, 2003).

Due to the increasing demand for water as a result of economic expansion and population growth, wastewater and sewage treatment plants increasingly operate under stress exerting pressure on water and sanitation authorities to find ways to sustain the quality of water resources (Mema, 2009). This situation itself raises concern regarding the quality of South Africa's water resources (Turton, 2008) and the contribution of untreated effluent or poorly treated waste water discharges to the deteriorating water quality.

Assessment of water and wastewater is very crucial to safeguard public health and the environment (Okoh et al., 2005; 2007). Momba et al., (2006) reported the poor operational state

and inadequate maintenance of most municipalities' sewage treatment works as leading to the pollution of various water bodies thereby posing very serious health and socio-economic threats to the dependants of such water bodies. When wastewater treatment systems are simple and inefficient, sewage discharges contribute to oxygen demand and nutrient loading of the water bodies, promoting toxic algal blooms and leading to a destabilised aquatic ecosystem (DWAF and WRC, 1995; WRC, 2000).

Water quality is a term used to describe the physical, chemical, biological and aesthetic properties of water which determine its fitness for a variety of uses controlled and influenced by constituents dissolved or suspended in water (Walmsley, 1998). The measurement and determination of these parameters is important to assess the impact of the wastewater effluents on the receiving water bodies before discharge to eliminate any health threatening substances (Harty, 2001).

1.1. PHYSICOCHEMICAL PARAMETERS

Physicochemical parameters include both physical and chemical parameters of water that affect the water quality aesthetically and as chemical pollutants respectively. These parameters have been reported in Hacıoglu and Dulger (2009) to influence the biochemical reactions within water systems and in certain concentrations these indicate changes in water quality thus compromising it for beneficial use.

The effect of such parameters is manifested in cases like excessive loading of nitrogen (N) and phosphorus (P) into water bodies resulting in eutrophication (Stow et al., 2001), and impairing both the physical and biologic integrity of the fluvial systems. Eutrophication causes undesirable

algal blooming, reduced water transparency, anaerobic hypolimnions, taste and odor problems, and increasing cost of water treatment (Drenner et al., 1997). The physical properties of water (turbidity, temperature, pH etc) largely determine its aesthetic properties (DWAF, 1996b).

1.2. MICROBIOLOGICAL PARAMETERS

Faecal coliforms are the most commonly used bacterial indicators to indicate faecal pollution (DWAF, 1996a) when one assesses the microbiological quality of water. Faecally contaminated water has high chances of containing a large number of viruses, which could be released into the environment through human faeces and urine (Puig et al., 1994). These viruses, known as enteric viruses, infect and replicate in the human gastrointestinal tract and are then released with the faeces.

According to Haramoto *et al.* (2005), enteric viruses are excreted in the faeces of infected patients in high concentrations and transmitted mainly by the faecal-oral route via contaminated food and water. Enteric viruses are mostly RNA viruses that belong to the following families: *Picornaviridae* (which include enteroviruses e.g. poliovirus, coxsackievirus, echovirus, hepatitis A virus), *Caliciviridae* (noroviruses, caliciviruses and small round structured viruses), *Astroviridae* (astroviruses) , *Reoviridae* (rotaviruses) and DNA viruses belonging to the family *Adenoviridae* (adenoviruses) (Fong and Lipp, 2005; Fuhrman et al., 2005; Hamza et al., 2009; Karim *et al.*, 2009). Waterborne human enteric viruses are a major public health concern since they often lead to fatal epidemics of, among other infections, non-bacterial gastroenteritis, conjunctivitis and hepatitis A infections in children, pregnant mothers and immunocompromised

individuals (Debartolomeis and Cabelli, 1991). They are estimated to cause about 30% to 90% of gastroenteritis cases worldwide (Haramoto et al., 2005).

1.3. PROBLEM STATEMENT

According to Mackintosh and Colvin (2002), despite the improvement in the treatment process of sanitation systems, a large number of water works in South Africa have difficulties in providing adequately treated water for their communities. They subsequently discharge significant amounts of pathogenic microorganisms into the receiving water bodies, deteriorating the quality of those water sources thus putting the consumers at risk of waterborne diseases (Dungeni et al., 2010). The inadequate treatment of water becomes a serious concern because most communities use this water for multiple purposes like recreational use, agricultural use and in some cases, drinking it straight from the receiving waterbodies (Okoh et al., 2007).

A cholera outbreak was reported in September 2007, in the Eastern Cape where people were seriously ill. Such cases prove how inadequately treated water can be health threatening.

1.4. JUSTIFICATION OF RESEARCH

Wastewater in rural areas and small towns of South Africa often receives partial or minimal treatment (DWAF, 1996c). However, even in the sub-urban and urban areas with expectedly a high degree of treatment, pathogens and some chemicals may still be released into the environment (LeChevallier and Kwok-Keung, 2004). The inadequately treated effluents from municipal wastewater treatment plants often impact so many receiving water bodies (Fatoki et al., 2003). The discharge of inadequately treated sewage water has a direct impact on the

microbiological quality of surface waters and consequently the potable water derived from it (Okoh, 2010). This makes the inadequately treated wastewater a source of pathogens in the environment thus a threat to human health. Diarrhoea has been observed as the primary manifestation of such health hazards and it has been recognised that the deadly diarrhoeal diseases are associated with viral infections (Griffin et al., 2003).

It is therefore needful that both the microbiological and physicochemical qualities of such wastewater be investigated periodically to make sure that they comply with the standards set by Department of Water Affairs and thus prevent or reduce incidences of waterborne disease outbreaks in this province.

1.5. HYPOTHESIS

The working hypothesis of this study was that wastewater treatment facilities in the Eastern Cape Province are sources of pathogenic microorganisms in the environment.

1.6. AIM AND OBJECTIVES

The aim of the study was to evaluate the final effluents of the two wastewater treatment plants in the Eastern Cape Province as sources of physicochemical and microbiological pollutants in the environment. The specific objectives were:

- To evaluate the physicochemical qualities of the final effluents of the two wastewater treatment plants.
- To assess the incidence of human viral pathogens and their serotypes in the two selected wastewater treatment plant effluents.
- To assess the occurrence of faecal indicator bacteria
- To correlate viral and bacterial pathogens occurrence with physicochemical qualities of the selected wastewater treatment plants effluents.

CHAPTER TWO

LITERATURE REVIEW

2.1 WASTEWATER IN A WATER-SCARCE COUNTRY

Water scarcity is a global challenge so much that it is estimated that in the next 11 years (2025) more than half of the world's population may suffer water shortages with most countries suffering from the water stress or water scarcity being from Africa (UNEP, 2008). All this negatively affects the economic development of these countries. The figure below shows the estimate of water availability between 1990 and 2025 in African Countries.

Africa is in the second driest continent after Australia and has been identified as a water-scarce country. It only receives average rainfall of 450 mm/annum, which is less than half of the world's average (DWA, 1996a). This water scarce country is a developing country with urbanization and increasing population putting a strain on the available water resource.

Hence, water reuse has come as an alternative (WHO, 2003) in trying to meet the country's increasing water demand, hence the need for wastewater treatment. However, domestic sewage and agricultural runoff are major sources of nutrients that when in excess causes of eutrophication. According to Chislock (2013), eutrophication has been identified, globally as the most prevalent water quality problem. A graph showing the water availability trend of African countries is shown in Figure 2.1.

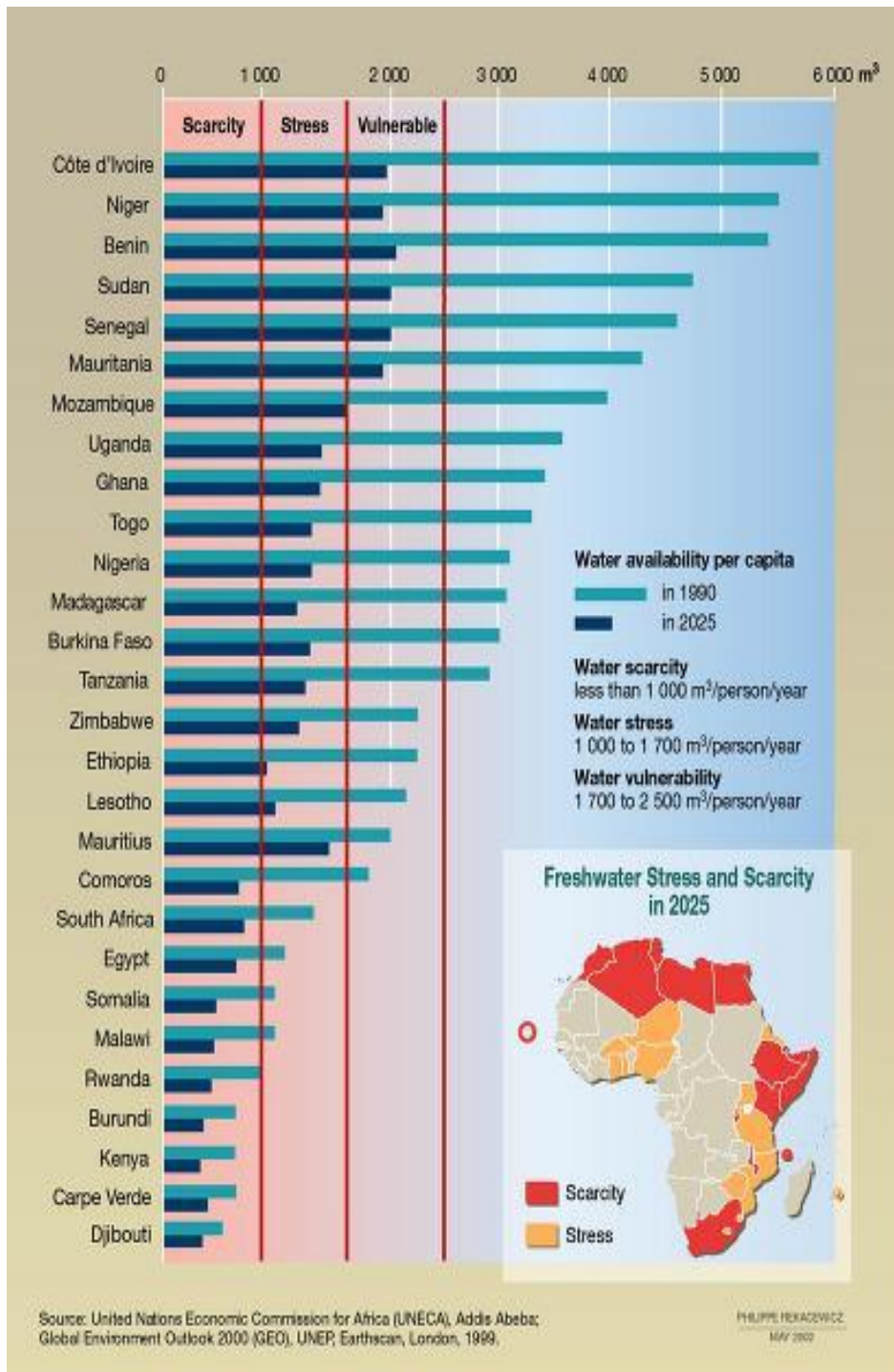


Figure 2.1: A graph showing the water availability trend of African countries (UNECA, 1999).

2.2 WASTEWATER TREATMENT

Wastewater treatment is a process of removing solids, organic matter and pathogenic organisms so as to release an effluent of good quality into the environment (Tansel, 2008). This treatment process differs with countries but follows a series of steps, ie, preliminary stage, primary stage, secondary stage, tertiary stage and the disinfection stage (Doorn et al., 2006). The wastewater treatment can be summarized as shown in the figure below;

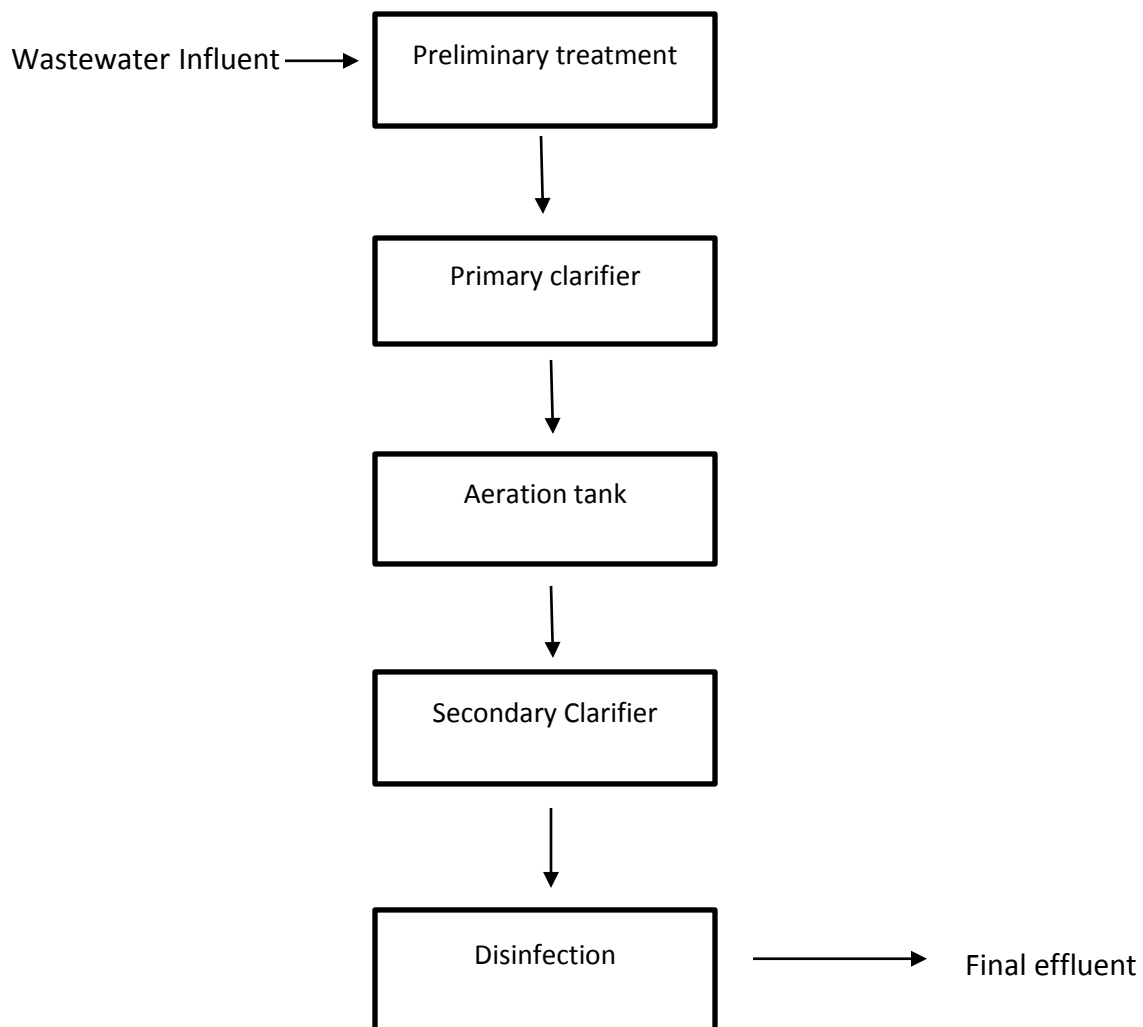


Figure 2.2: Flow diagram showing a typical wastewater treatment process using activated sludge process

2.2.1. PRELIMINARY TREATMENT

Preliminary treatment is the removal of floating materials and solids such as rags, plastic, sticks and tree branches that may interfere with the treatment process by damaging or clogging the equipment involved (Topare et al., 2011). This process is achieved by use of screens or communicators that have grinders to permit only the water while retain the solids before they can grind them to suspended solids that pass through to the primary stage (Okoh et al., 2007). The screening and removal of these solids also helps in reduction of Biochemical Oxygen Demand by about 15 to 30% and removal of oils and grease from water (Topare et al., 2011).

2.2.2 PRIMARY TREATMENT

The wastewater from the preliminary stage often carries the suspended solids and a high Biochemical oxygen demand of about 60%. Primary stage sometimes merged as on with the preliminary, is the removal of most of the suspended solids and removal of about 50 to 70% of organic matter by physical operations such as sedimentation in the settling basins (Sonune and Ghante et al., 2004; Topare et al., 2011). The primary stage acts as a precursor for the secondary step wherein more advanced systems are used in chemicals and filtration to enhance sedimentation, removal of dissolved solids and lighter suspended solids (Metcalf and Eddy, 2003).

2.2.3. SECONDARY TREATMENT

This is often referred to as the biological treatment process where organic matter is biodegraded by use of biological processes either under aerobic or anaerobic conditions (Tapero et al., 2011).

These biological processes usually work with physical and chemical processes to reduce both organic matter and nutrient content usually measured as phosphorus and nitrogen (UN, 2003). In an activated sludge system, as that of the plants under study, this process consists of two processes being the biological conversion of pollutants in a bioreactor and solid separation that uses gravity clarifier (Jenkins et al., 2004).

In secondary treatment, ninety percent is achieved by use of microorganisms that are usually cultivated and added. The microorganisms absorb the organic matter as their food source or convert them into various gases and cell tissues that are removed in the sedimentation tanks (UN, 2003; Okoh et al., 2007). The common secondary treatment methods are attached growth processes, suspended growth processes and lagoon systems (Okoh et al., 2007).

2.2.4. TERTIARY TREATMENT

This is also known as the advanced wastewater treatment because although the suspended solids, organic matter and oxygen demand may be removed in the earlier treatment stages, this is required to kill all pathogenic bacteria and remove any remaining toxic compounds and suspended solids (Prabu et al., 2011; Tapero et al., 2011). The advanced wastewater treatment stage employs a variety of chemicals, physical and biological processes such as chemical coagulation, flocculation, sedimentation and filtration (Okoh et al., 2007; Tapero et al., 2011).

2.2.5. DISINFECTION

Due to the nature of wastewater, disinfection is an important stage of the wastewater treatment process to ensure that an effluent of good quality is produced. A wide variety of disinfection

methods are available such as use of physical agents like heat and light; mechanical processes like filtration; radiation and use of chemical agents such as chlorine, ozone, ammonium compounds, alcohols, etc. The most common means of disinfection is the use of chemicals with chlorine being the most widely used, but ozone and ultraviolet radiation are also used (Hijnen et al., 2006; UN, 2003). Chlorine can be added as either solid or gas killing microorganisms by destroying their cellular materials.

Although chlorine is the most widely used its effect may be limited in cases of high turbidity and removal of protozoans, and the remains of the chemical may be toxic to aquatic life and may produce hazardous products that are harmful to both humans and animals, hence the emphasis on having extremely low levels of free chlorine in water to be discharged (Hijnen et al., 2006; Okoh et al, 2007). According to Snyman (2006), 67% of South African wastewater treatment plants that use chlorination method of disinfection in their final effluents still experience challenges such as inadequately designed disinfection systems, inappropriate technology employed and improper operation of chlorine stock.

In spite of its negative effects, chlorine is still used because as much as ozone can destroy viruses and bacteria without producing hazardous byproducts; ozone is high in energy costs. The Ultraviolet radiation cannot be used in large scale basis because it is not only expensive but unreliable and has a lot of maintenance problems (Hoyer, 2004). Despite the other alternatives, chlorination is still the ideal disinfectant used in water treatment because it has a lasting residual disinfection effect that is used to measure the effectiveness of disinfection and protect the water from downstream microbial contaminants (Betancourt and Rose, 2004).

2.3 PHYSICOCHEMICAL PARAMETERS

In surface water and wastewater quality assessment, physicochemical parameters are used. These indices include the nutrient load measured as nitrite, nitrate and phosphates, oxygen demand measured as dissolved oxygen (DO), biochemical oxygen demand (BOD) and chemical oxygen demand (COD), and dissolved solids measured as turbidity and total dissolved solids. In addition, pH, temperature and chlorine added during the treatment process also affect the quality of water.

pH is a measurement of the acidity or alkalinity of a specific solution with acidic water containing extra hydrogen ions (H⁺) while basic water contains extra hydroxyl (OH⁻) ions. A change in pH may impair the water use and affect its biota (Morrison et al., 2001). For many aquatic organisms, the optimal pH ranges between 6.5 and 9 (Mesner et al., 2010). High pH values of water may also trigger toxicity of other pollutants available in the water (DWAF, 1996b; Morrison et al., 2001).

Turbidity of water limits the amount of light penetrating through the water by absorbing it (DWAF, 1996b). When the light is scattered and absorbed in by the turbidity which measures the suspended solids, it affects the photosynthesis process by aquatic plants and thus depleting the food source of aquatic animals (Palmer et al., 2004). High turbidity can be associated with microbial contamination as it makes the disinfection process almost impossible by shielding the microorganisms (Fatoki et al., 2001). Chlorination of highly turbid water does not only become ineffective but also increases the formation of trihalomethane precursors which are carcinogenic compounds that have serious health impacts to both aquatic life and humans exposed to them (Odjadjare and Okoh, 2009). In addition highly turbid water is aesthetically unappealing.

Aquatic life requires certain temperature values to survive in the water. This temperature governs by regulating chemical and biological reactions in the water and regulating the maximum dissolved oxygen concentration of the water. All these being indices of water quality are therefore greatly influenced by any changes in the temperature such as high temperatures increasing the rate of chemical and metabolic reactions (DWA, 1996b). Together with the metabolic reactions affected, high temperatures may promote the growth of pollutants in the water thus rendering the water unsafe for use.

In water, oxygen balance is maintained by the dissolved oxygen. This dissolved oxygen is an important index of water because the degree of oxygenation of water affects the concentration of solutes in the water (Slack, 2012). This dissolved oxygen undergoes fluctuation and therefore requires monitoring procedures so that it does not affect the organisms that need it for respiration (DWA, 1996b). Low dissolved oxygen levels can be the result of elevated temperature and thus the inability of the water to hold the available oxygen thus adverse effects to the aquatic ecosystem (Fatoki et al., 2003; Hacioglu and Dulger, 2009). Low dissolved oxygen levels can also indicate an excessive demand on the oxygen in the system. All of this might then disturb the survival of the aquatic animals through the increase of their susceptibility to diseases; distort their swimming ability; altering their feeding, migration and reproductive behavior, which may ultimately lead to death (Environment Canada, 2001; Odjadjare and Okoh, 2009).

To measure the potential of organisms to deplete oxygen in the water, biochemical oxygen demand (BOD) and chemical oxygen demand (COD) are used. BOD is oxygen is needed by the water to completely oxidize its organic pollution load and is therefore also defined as a measure of organic matter present in the water resource (Fatoki et al., 2003). COD measures the amount of oxygen needed to fully degrade and oxidise the organic matter in the water using a strong

chemical oxidant (Morrison et al., 2001). Increased levels of the BOD and/or COD may deplete the oxygen dissolved and therefore affecting the aquatic life (Fatoki et al., 2003).

Nitrite, nitrate and phosphate are the most commonly used to measure nutrient loading in water. Nitrate and nitrite occur naturally as part of the nitrogen cycle with nitrate ion being the stable form of combined nitrogen while nitrite ion contains nitrogen in an unstable oxidation state (WHO, 2004). At appropriate concentrations, the inorganic sources of nitrogen, nitrates, support the growth and development of living organisms, mainly found in plant cells (Odjadjare and Okoh, 2009; Palmer et al., 2004). In wastewater, usually there are high concentrations of nitrate because of the presence of ammonium nitrogen in untreated wastewater which later becomes oxidized to nitrates by the microbes in the water (Morrison et al., 2001).

High nitrate and nitrite levels may result in excessive nutrient enrichment in water systems (eutrophication) that leads to ecosystem degradation as a result of excessive growth of plants mainly algal blooms, oxygen depletion and the reduction of sunlight penetration (CCME, 2006; Odjadjare and Okoh, 2009). In human, high nitrate concentrations may result in methanoglobinemia in infants and pregnant women and formation of carcinogenic nitrosamines (Akan et al., 2007; Fatoki et al., 2001). Methanoglobinemia, however, is said to be very rare and may result if the concentration of nitrate is more than $30\text{mgNO}_3\text{-N/l}$ (Morrison et al., 2001).

The effects of nitrites in water resources are similar to those described for nitrates because they are both nitrogen compounds that cause adverse effects on the aquatic ecosystem at high concentrations. However, assessment of nitrite should be undertaken immediately due to its instability in water (WHO, 2004).

Phosphorous occurs inorganically as orthophosphate. Orthophosphates, along with nitrates and nitrites, are important growth limiting factors in eutrophication, which result in undesirable ecological effects within the receiving water resource (DWAF, 1996b; Morrison et al., 2001).

2.4. INDICATORS OF WATER QUALITY

Worldwide, indicator bacteria have been used to assess the microbiological quality and risk of exposure to diseases of water used for drinking, recreational use and industrial use (Anderson et al., 2005; Noble et al., 2003). Within the indicator bacteria, faecal coliforms have been identified as more specific indicators of faecal pollution especially from warm-blooded animals due to their hemophilic property (Leclerc et al., 2001; Ashbolt et al., 2001).

2.4.1 FAECAL COLIFORMS

Faecal coliforms reside in the gastrointestinal tracts of living organisms where from they are replicate to cause intestinal infections. These organisms have been used as the standard indicators of bacterial contamination and quality measures of water (Griffin et al., 2001; Jiang et al., 2007). These have been used worldwide because of their ability to survive even high temperatures (44.5 °C) producing gas from lactose (Jamieson et al., 2002) where only coliforms of faecal origin can grow hence the name faecal coliforms (Tallon et al., 2005). Comprising these faecal coliforms are *Escherichia coli* and *Klebsiella pneumoniae* being the less dominant than *Escherichia coli* (Bitton, 2005). The most dominant coliform (*E.coli*) is unlike other coliforms

that are harmless to human health, an indicator of the presence of more harmful pathogens in water due to its thermotolerant property (DWAF, 1996b).

Although these have been suggested as indicators of water pollution, their ability to grow in naturally tropical environments may produce erroneous results (Boehm, 2003). In addition, these alone cannot be used to assess microbiological water quality because they do not correlate with the presence of other pathogens such as the protozoan parasites and enteric viruses which are very health threatening (Harwood et al., 2005).

2.4.2 VIRUSES

Some wastewater treatment processes fail to completely remove organic matter and some resistant viruses allowing them to initiate infections when used after the inadequate treatment. The ability for these viruses to survive under a wide range of pH values also contributes to these being associated with a number of diseases like meningitis, gastroenteritis, hepatitis and paralysis thus increasing the mortality rate (Fong and Lipp, 2005). Therefore, although the use of enteric viruses as good indicators for water quality is time consuming and laborious, it is more efficient than the bacterial indicators (Connell et al., 2012). According to literature (Dubois et al., 1997; Vantarakis, A., and M. Papapetropoulou. 1999; Van Heerden et al., 2003; Lodder et al., 2005; Van den Berg et al., 2005), adenoviruses, noroviruses, reoviruses, rotaviruses, and other enteroviruses (e.g., polioviruses, coxsackie viruses, and echoviruses) have been identified as indicators of faecal pollution in raw sewage in Australia, Europe, and South Africa.

2.4.2.1. ENTEROVIRUSES

These viruses are transmitted through faecal-oral routes from faecally contaminated water and may cause further infections (Rajtar et al., 2008). The enteroviruses are a group from the *Picornaviridae* family, non-enveloped and small sized viruses of 20-30 nm in diameter. These survive in human faeces for longer periods and therefore, humans have become the only reservoirs of these viruses (Kocwa-Haluchi, 2001). Enteroviruses consist of polioviruses, echoviruses, coxsackieviruses and numbered enteroviruses, out of these, 89 serotypes have been identified by the International Committee of Taxonomy of viruses (Fong and Lipp, 2005).

Both humans and animals may be infected by these viruses that cause both polio and non-polio related diseases but the spread of polio has decreased since the introduction of polio vaccines in the 1950s (Rajtar et al., 2008). These polioviruses may not be associated with waterborne transmission; however they are typically transmitted via faecal-oral route, all this raises some concerns not to underestimate the infection risk by exposure to the viruses in water more so in rural communities which use sewage-polluted river water for domestic purposes (Pavlov et al., 2005).

All the above statements leave the non-polio enteroviruses as the main causative agents of enteroviral diseases. The resistance of these viruses to sewage treatment plant conditions, chlorination and UV disinfection (Vivier et al., 2004) makes these a threat to human health after consumption of contaminated water. Even in low levels, enteroviruses can initiate infections such as myocarditis and meningitis (Muir et al., 1997), contribute to chronic diseases like diabetes and also result in paralysis like poliomyelitis because they infect the central nervous system of the human (Kocwa-Haluchi, 2001). Because of the large number of reported outbreaks

by this virus, it has now been used as the parameter for evaluation of the degree of viral pollution of water by the European Union (Kocwa-Haluchi, 2001).

2.4.2.2. ADENOVIRUS

Adenoviruses were identified, after first isolation from humans in the 1950's as the causative agents of respiratory diseases (Rowe et al., 1953; Hilleman et al., 1954; Fong et al., 2010). A Human adenovirus is a non-enveloped, double stranded DNA genome-containing virus of 90 nm sizes in diameter (Vellinga et al., 2005). Adenovirus is reported as the most prevalent of enteric viruses in water environments worldwide (Pina et al., 1998, Sibanda and Okoh, 2012). Its DNA genome makes it very much more thermostable than all other enteric viruses outlasting them in environmental waters (Jiang et al., 2004).

Human adenoviruses (HAdV) have been identified as a major cause of clinical infections such as gastroenteritis and respiratory diseases being the second most important viral pathogens of gastroenteritis in infants and children after rotavirus (Fong et al., 2010). So far, 51 adenovirus serotypes have been identified and further sub-divided into six species of A to F based on hemagglutination properties, oncogenicity in rodents, DNA homology, and genomic organization (Gray et al., 2007). Out of these, 30% are pathogenic in humans with serotypes 40 and 41 being agents of viral gastroenteritis in children and endemic in developing countries (Griffin et al., 2003). Adenovirus species 3 and 7 do not only cause outbreaks in children but also in adults worldwide as manifest in Japan, Korea, the United States, South America and China (Metzgar et al., 2005) while Adenovirus 1, 2, 5 and 6 are reported as causative agents of 5 – 10% childhood respiratory diseases (Jiang, 2006). Persistent outbreaks of acute respiratory disease caused by

Ad4 have been reported mainly in the United States (Fong and Lipp, 2005), this serotype together with serotype 21 have been reported as responsible for epidemics febrile acute respiratory diseases in military training facilities worldwide (Metzgar et al., 2007).

Because of their recognition as critical emerging viruses in waterborne transmission as observed by researchers (Swenson et al., 2003; Xagoaraki et al., 2007), human adenoviruses have been included in the list of contaminant candidates of the United States Environmental Protection Agency.

2.4.2.3. ROTAVIRUS

Rotaviruses have been identified as the most common diarrheal pathogens in infants and children worldwide causing about 600 000 deaths of children per year (Grimwood and Bines, 2007, Parashar et al., 2003) especially in low-income countries where they are transmitted by faecal-oral route. These double-stranded RNA viruses have been identified as the most resistant to UV radiation among all the enteric viruses (Kocwa-Haluch and Zalewska, 2002) but they are more sensitive to inactivation by the chlorination process. According to Griffin (2003), up to 150 deaths caused by rotavirus occur in 1 to 4 year old children in the United States while in South Africa, rotavirus is responsible for the deaths of a quarter of children who die aged 1 - 5 years (de Villiers François P. R. and Driessen M., 2012). A large number of these cases of rotaviral infections are reported to occur in winter and early spring temperatures (Gerba et al., 1996; Kocwa-Haluch and Zalewska, 2002). According to Hoshino and Kapikian (2000), rotaviruses are divided into seven groups from A to G with their composition and distribution varying in certain socioeconomic and geographic regions of the world.

Most Human rotavirus (HRV)-associated infections are predominantly caused by group A, and less commonly by group B or C, hence the emphasis of vaccine development has been targeted at group A rotavirus associated disease. Following Group A, Group B rotavirus (adult diarrhea rotavirus [ADRV]) which caused large outbreaks in China in the 1980s was later (1997 and 1998) detected in patients with diarrhea in India (Hoshino and Kapikian, 2000).

The physicochemical characteristics of the G serotypes, composing of major strains, make them resistant to harsh environmental conditions, hence their abundance in wastewater (Dubois et al., 1997). There have been some efforts to limit the transmission of this virus but the rotaviral outbreaks may be difficult to control because rotaviruses are not only infectious in low doses but can survive in the environmental surfaces for days (Ruiz-Palacios et al., 2006).

2.4.2.4. HEPATITIS A VIRUS

Hepatitis a virus, transmitted by faecal-oral route and ingestion of contaminated food and water has been reported as among the waterborne disease causing agents worldwide especially in developing countries where water supply and sanitation are still poor and there is no routine vaccination of this virus due to its high costs (Cuthberth, 2001; Kittigul et al., 2005). Hepatitis A virus is single-stranded RNA virus of 7.5 kb in length that belongs to the genus Hepatovirus (Feinstone, 1996; Murray et al., 2005). Among the other viruses causing gastrointesteritis, this virus causes acute inflammation in the liver where it replicates and multiplies, destructs the liver cells and thus the inflammation of the liver, affecting the blood stream and thus causing jaundice and darkening of urine (Cuthberth, 2001; WHO, 2002).

Grabow (1997) once reported that this virus is rarely fatal; however, Cuthberth (2001) reports that this virus is endemic in developing countries and it becomes an economic burden especially where vaccination has not been practised. Worldwide, Hepatitis virus outbreak reports have been estimated to be around 1.5 million each year (WHO; 2003) with specific countries like Thailand and China where 300 000 clinical cases and 30 deaths were reported due to ingestion of faecally contaminated shellfish (Grabow et al., 2002; Nenonen et al., 2006). A study by Cristina and Costa-Mattioli (2007) showed that the incidences of Hepatitis A virus in travellers are 500 times higher than those of cholera.

2.4.2.5. NOROVIRUS

These are non-enveloped, single-stranded RNA viruses of sizes of 27-32nm in diameter, belonging to the family of *Caliciviridae* (Zheng et al., 2006; La Rosa et al., 2010). The norovirus is a diverse genus comprising of five genogroups (GI to GV), divided by their sequence similarity of the capsid protein which are further subdivided into their genotypes (Flannery et al., 2012). According to Bon et al. (2005) and Atmar et al. (2006), Norovirus is the leading cause of gastroenteritis in adults worldwide with its GII and GI accounting for the majority of those gastroenteritis cases. The human norovirus (GII and GI) have a significant health impact because they do not only cause gastroenteritis in adults but also cause non-bacterial gastroenteritis in children. A large number of norovirus cases are reported to occur mostly in winter (da Silva et al., 2007). Because it is shed in large numbers (up to 10^8 viruses) in the faeces of humans, Norovirus is abundant in wastewater. Its reported resistance to wastewater treatment processes further explains its common detection in municipal water and shell fish (da Silva et al., 2007;

Flannery et al., 2012). Norovirus outbreaks have been reported worldwide including a recent detection of the human norovirus from wastewater effluents in Kenya (Kiulia et al., 2010). In South Africa, the first outbreak was reported in 1993 (Taylor et al., 1993), however, recently there have been no reports of Norovirus-related outbreaks in this country.

2.5 DETECTION OF VIRUSES IN WATER

Viruses exist in small quantities in water such that further concentration by various methods such as ultrafiltration, adsorption-elution, glass-wool, glass powder, flocculation and chromatography. Among these techniques, glass wool adsorption has been preferred because of its low costs while adsorption-elution by membrane filters has high recovery efficiency (Taylor et al., 2001; Haramoto et al., 2005). However, amongst all these techniques, none could be regarded as superior than the other because of their variations in efficiency, constancy of performance, robustness, cost and complexity (Bosch et al., 2010).

A number of methods for concentration of viruses (Rodriguez et al., 2009; Nordgern et al., 2009; Lambertini et al., 2008; Brassard et al., 2005) have been employed but have difficulties in recovering viruses from wastewater due to high costs and time such that there is a need for rapid and economical methods for recovering of these viruses from low quality waters. Currently, adsorption-elution method is the most commonly used with the recovery efficiency of $56 \pm 32\%$ (Katayama et al., 2008, Haramoto et al., 2005).

Cell-cultures were used as the detection techniques until the invention of polymerase chain reaction and PCR proved to be more efficient than the cell culture-based because of its low costs, specificity and easy processing of results (Abbaszadegan et al., 1999).

Besides the costs and easy performance of PCR, it can detect very low titres of viruses in water and can be used to identify specific viruses but despite the advances in molecular techniques, these molecular-based methods still fail to distinguish between infectious and non-infectious virions, but the detection of viral nucleic acids in polluted water suggests the presence of infective virus (Miagostovich et al., 2008) as survival of naked nucleic acid in aquatic environment is limited (Lambertini et al., 2008). PCR is not only qualitative but quantitative, enabling quantitative hazard risk assessment by use of Real-time PCR quantification or most probable number by conventional PCR. After detection, viruses can be further characterized into their respective species and serotypes (Bosch et al., 2011).

The limitations of PCR for virus detection such as presence of inhibitory factors and inability to determine the infectious state of a virus make the cell culture a gold standard to examine the viability of viruses after exposure to chlorine, UV light and extreme temperatures (Richards, 1999; Greening et al., 2002). Therefore, susceptible cell-lines that allow viruses to propagate and produce cytopathic effects observed under a light microscope are needed. But even this method has a shortfall in infectivity detection because not all viruses grow and cell culture and viruses have varying capabilities to propagate in a given cell line (Nitsche, 2007). For reliable detection of infectious viruses, factors such as duration of exposure to host cell, volume of inocula and age of the cells need to be considered in a cell culture (Rodríguez et al., 2009). The cost implications and time and inability to culture certain viruses became a major drawback for the culture-based method suggesting the PCR as an ideal detection method for contamination of water by viruses.

2.6. REVIEW OF CURRENT INDICATORS

Literature (Fewtrell et al., 2005; Clasen et al., 2007; Levy et al., 2012) suggests that review of the current measures of water contamination may improve the water quality and thus reducing the rate of reported cases on waterborne pathogens. Due to increasing reports on cases of waterborne disease outbreaks, one may see the need for review of the currently used indicators of water contamination. This may be supported by recent findings that these currently used indicators occur naturally in some areas where diarrhea is manifest (Levy et al., 2012), which raises concern as to whether their detection in water is of faecal contamination or natural existence. In addition, the absence of indicator organisms is not the direct implication of the absence of microbial pathogens (Wu et al., 2011).

According to WHO (2003), indicators may differ in countries but they all imply that the faecal contamination assessed is associated with human origin leaving the effect of non-human contamination unaddressed. Although these faecal indicators are widely used, they cannot be highly correlated with the presence of the pathogens depending on the source of pollution (Savhichtcheva and Okabe, 2006). Because of cost implications, time and other challenges, it is impractical to monitor all organisms in water quality. However, Dufour (2012) suggests that application of both the conventional and alternative indicators may help increase the sensitivity and specificity of water quality assessment. These alternative indicators that comprise of bacterial anaerobes, viral indicators, chemical compounds and other anthropogenic markers of human activity, when combined with conventional indicators enhance the ability to predict health risks associated with waterborne pathogens (Savhichtcheva and Okabe, 2006).

CHAPTER THREE

MATERIALS AND METHODS

3.1. DESCRIPTION OF STUDY SITE

Two wastewater treatment plants were used and are located in the Amathole district municipality and for confidentiality reasons are designated as WWTP-K and WWTP-R and both have the geographical S32°34'37.34" E27°53'31.20" and 32°97'S & 27°87' E respectively. WWTP-K has a design capacity of 0.63ML/d while WWTP-R has a design capacity of 2.5ML/d. Both plants use activated sludge treatment system and discharge their final effluents into the Kei and Buffalo Rivers, respectively.

3.2. SAMPLE COLLECTION

Water samples were collected monthly from the final effluents of the selected wastewater treatment plants for a period of twelve months between September 2012 and August 2013. Sterile 1.7 ml bottles containing 1% sodium thiosulphate were used in sampling. All samples were transported to the Applied and Environmental Microbiology Research Group (AEMREG) laboratory, at the University of Fort Hare, Alice in South Africa, in a cooler box for analyses.

3.3. PHYSICOCHEMICAL ANALYSIS

Total dissolved solids, pH, temperature and electrical conductivity were measured *on site* using a multi-parameter ion specific meter (Hanna_BDH laboratory supplies), while turbidity was measured using a microprocessor turbidity meter (HACH company, model 2100P). The free chlorine residual was also measured *on site* using HACH free chlorine meter (Crison MM40). Samples for COD analysis were digested for two hours with a Thermo Reactor Model TR 300 (Merck Pty Ltd) and then analyzed using Spectroquant NOVA 60 photometer (Merck Pty Ltd). Dissolved oxygen and Biochemical oxygen demand (BOD₅) were determined using the Oxitop WTW BOD meter (Merck Pty Ltd) after five days incubation in accordance with the description of the Standard Methods for the Examination of Water and Wastewater (1998).

3.4. FAECAL COLIFORM COUNTS

Faecal coliform counts were done using the membrane filtration method as described by Clesceri et al. (1998). Commercially available m-FC Agar was prepared according to the manufacturer's procedure and used for the cultivation of faecal coliforms. Samples were serially diluted and 100 ml filtered through Millipore (0.45µm pore size 47 mm diameter) sterile membrane filter, placed on the cultivation medium and incubated for 24 hours and incubated at 44.5°C. Colonies with characteristic blue coloration on m-FC agar were enumerated as faecal coliforms.

3.5. VIROLOGICAL ANALYSIS

3.5.1 CONCENTRATION OF VIRUSES

The viral nucleic acids were concentrated by the absorption-elution method of Fong et al. (2005) using a negatively charged Millipore filter membrane. Briefly, 5 ml of 250 mM AlCl_3 solution was passed through the filter membrane placed over sterile filters. This was followed by filtration of five hundred millilitres of water sample. Al^+ ions were then washed off using 200 ml of 0.5 mM H_2SO_4 . All of the steps through adsorption were aided with the use of the suction pump to speed up the whole process while elution of the adsorbed viral particles was done using 10 ml of 1 mM NaOH solution. The eluate was recovered in a Centriprep YM-50 (Millipore) tube containing 50 μl of 100 mM H_2SO_4 (pH 1.0) and 100 μl of 100X Tris-EDTA buffer (pH 8.0) for neutralisation, followed by centrifugation. The Centriprep YM-50 is a centrifugation unit equipped with an ultrafiltration membrane, which can achieve the high concentration efficiency needed for the viral particles. The final concentrated samples were stored at -80°C until further analyses.

3.5.2 EXTRACTION OF VIRAL NUCLEIC ACIDS

Extraction of viral nucleic acid was done in accordance with the method of Boom et al. (1990) using commercially available extraction kits. For adenoviruses, DNA was extracted using Quick-gDNATM Mini Prep (Zymo Research, USA). For the RNA-containing viruses (Enteroviruses, Rotaviruses, Noroviruses and Hepatitis A viruses) extraction was carried out using Quick-RNATM MiniPrep (Zymo Research, USA). The concentrated samples were first lysed in a

reaction vessel containing a solid nucleic acid carrier (silica gel based membrane) in the presence of the chaotropic agent Guadium thiocyanate (GSCN) that inactivates RNase and ensures the isolation of intact viral RNA. The mixture was then centrifuged briefly to aid the selective adsorption of viral RNA to the silica gel membrane. The bound viral RNA was washed free of contaminants in two steps before elution in RNase-free water containing sodium-azide to prevent microbial growth and subsequent recontamination with RNase. The eluates were transcribed into cDNA.

As suggested by Jothikumar et al. (2009), for rotavirus, prior to reverse transcription, sample RNA was subjected to denaturation at 95°C for five minutes and flash chilled in ice for two minutes to ensure complete separation of double stranded RNA.

All the RNA viruses were transcribed by mixing 10µl of the template, 1µl of Random Hexamer primer, 1 µl dNTP mix, 2.5 µl DEPC-treated water, 4 µl 5X RT buffer, 0.5 µl Ribolock RNase inhibitor and 1 µl RevertAid Premium Reverse Transcriptase (Fermentas Life Sciences) were added in the indicated order into a 0.5 ml PCR tube on ice. The mixture was briefly vortexed and centrifuge before incubation at 25°C for 10 minutes, followed by 30 minutes at 60°C and terminated by heating at 85°C for 5 minutes.

3.5.3 DETECTION OF VIRAL GENOMES BY REAL-TIME PCR

The concentration of Adenoviruses, Hepatitis A viruses, and Rotaviruses was estimated by using quantitative PCR with TaqMan probes. For the RNA viruses (Rotavirus and Hepatitis A virus), reverse transcription QPCR (RT-PCR) was performed using a StepOnePlus PCR System

(OPTIPLEX 755, Applied Biosystems) while Adenovirus quantification was done following a one-step reaction in a 96-well. The PCR assay used 5 µl of the concentrated copy DNA (cDNA) in a total reaction volume of 25 µl, containing 12.5 µl of PCR master mix, 0.5 µl of primer I, 0.5 µl of primer II, 0.5 µl of Probe and 6 µl of nuclease free water. The reaction mixture was loaded into 96-well plates, loaded into the thermocycler and data collected at the end of two hours. Primer pairs and probes used for each virus are shown in Table 3.1.

Table 3.1: Primers and probes for Real-Time RT-PCR and qPCR

Enteric Virus	Primers and Labelled TaqMan Probe	Reference
Hepatitis A Virus	HAV68 (F): 5'-TCA CCG CCG TTT GCC TAG-3' HAV240 (R): 5'-GGA GAG CCC TGG AAG AAA G-3' HAV150 (P): 5'-FAM-CCT GAA CCT GCA GGA ATT AA-MGBNFQ-3'	(Costafreda et al., 2006) (Pintó et al., 2009)
Rotavirus	JVK (F): 5'-CAGTGGTTGATGCTCAAGATGGA-3' JVK (R): 5'-TCATTGTAATCATATTGAATACCCA-3' JVK (P): 5'-FAM-ACA ACTGCAGCTTCAA AAGAAGWGT-BHQ-3'	(Jothikumara et al., 2009)
Adenovirus	HEX(F): 5'-ACCCACGATGTAACCACAGAC-3' HEX(R1): 5'-ACTTTGTAAGAGTAGGCGGTTTC-3' HEX(R2): CACTTTGTAAGAATAAGCGGTGTC HEX(P): 5'-CGACKGGCACGAAKCGCAGCGT-3'	(Fredrick & Irene, 2011)

Abbreviations: F, forward/sense; R, reverse/antisense; P, probe

The thermal cycling protocols used were as follows:

HAV: 10 min at 95°C for *Taq* activation, 45 cycles of denaturation at 94°C for 15 sec, annealing at 60°C for 1 min, and extension at 70°C for 1 min.

Rotavirus: 15 min at 95°C for *Taq* activation, 45 cycles of denaturation at 95°C for 15 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec.

Adenovirus: 15 min at 95°C for *Taq* activation, 45 cycles of denaturation at 95°C for 10 sec, annealing at 55°C for 30 sec, and extension at 72°C for 20 sec.

For standard curve formulation, nucleic acid extracts from ATCC control strains were quantified using Qubit Fluorometer. To get a range of known concentration, Ten-fold serial dilutions were prepared. The serial dilutions, treated as standard together with the samples were subjected to real-time PCR and analyzed using SDS software (Applied Biosystems) for quantitative data of all viruses.

3.5.4 DETECTION OF NOROVIRUS AND ENTEROVIRUS BY CONVENTIONAL PCR

Because of the absence of their positive controls, Norovirus and Enterovirus were detected by conventional PCR. The PCR assays used 5 µl of the concentrated copy DNA (cDNA) in a total reaction volume of 25 µl, containing 12.5 µl of PCR master mix, 0.5 µl of primer I, 0.5 µl of primer II, and 6.5 µl of nuclease free water. The primer sets used are shown in Table 3.2

Enterovirus cycling conditions were as follows: denaturation for 4 min at 94°C, 30 cycles of amplification; 90 sec at 92°C, annealing for 90 sec at 55°C and extension for 2 min at 72°C.

Norovirus : 3 min at 94°C to activate the *Taq* polymerase followed by 40 cycles of 1 min at 94°C, 1.5 min at 37°C, 1 min at 72°C, and a final extension of 72°C for 7 min.

Table 3.2: Primers for conventional PCR detection of Norovirus and Enterovirus.

Enteric Virus	Primers and Labelled TaqMan Probe	Reference
Norovirus	JV13I (F): 5'-TCATCATCACCATAGAAIGAG -3' JV12Y (R): 5'-ATACCACTATGATGCAGAYTA -3'	La Rosa et al., 2009
Enterovirus	Ent1 (F): 5'-CGGTACCTTTGTACGCCTGT -3' Ent2 (R): 5'-ATTGTCACCATAAGCAGCCA -3' neEnt1 (F): 5'-TCCGGCCCCTGAATGCGGCTA-3' neEnt2 (R): 5'-GAAACACGGACACCCAAAGTA-3'	Puig et al., 1994

Abbreviations: F, forward/sense; R, reverse

3.5.5 DETECTION OF VIRAL SPECIES AND SEROTYPES

Rotavirus species, A, B, and C were detected by conventional PCR following a description of Lai et al., (2005). The primers sequences used for these detections are described in Table 3.3 below;

Table 3.3: Primers for Detection of Rotavirus Groups (Gouvea et al., 1991)

Species	Primer	Sequence (5' – 3')	Amplicon size (bp)
A	Beg9	GGCTTTAAAAGAGAGAATTTCCGTCTGG	1062
	End9	GGTCACATCATACAATTCTAATCTAAG	
B	GRB-1F	CTATTCAGTGTGTCG TGAGAGG	498
	GRB-1R	CGTGGCTTTGGAAAATTCTTG	
C	G8S	GGCATTTAAAAAAGAAGAAGCTGT	1063
	G8A	AGCCACATGATCTTGTTTACGC	

The PCR assay was run as follows, for group A and B species: 3 min at 95°C followed by 30 cycles of 1 min at 95°C, 2min at 42°C and 1 min at 72°C and a final extension step of 7 min at 72°C. The cycling conditions for group C were as follows: 3 min at 95°C followed by 30 cycles of 1 min at 95°C, 2 min at 48°C and 2 min at 72°C and then final extension step of 7 min at 72°C. The PCR products were visualized on an ethidium bromide stained 1% agarose gel.

The primers described by Xu et al. (2000) targeting the fibre region of the viral genome were used for the detection of adenovirus species A to F as shown in Table 3.4. The PCR assays for the species detection was carried out as described by Metzgar et al. (2005) and Tiemessen et al., (1991) but now as single-plex PCR.

Table 3.4: Primers for detection of Adenovirus serotypes

Species	Serotype	Primer	Sequence (5' to 3')	Source
B	Ad3	Ad3F	GGTAGAGATGCTGTTGCAGGA	Metzgar et al., (2005).
		Ad3R	CCCATCCATTAGTGTCATCGGT	
	Ad7	Ad7F	GGAAAGACATTACTGCAGACA	
		Ad7R	AATTCAGGCGAAAAAGCGTCA	
	Ad21	Ad21F	GAAATTACAGACGGCGAAGCC	
		Ad21R	AACCTGCTGGTTTTGCGGTTG	
C		AdCF	TGCTTGCGCTHAAAATGGGCA	Metzgar et al., (2005).
	Ad1	Ad1R	CGAGTATAAGACGCCTATTTACA	
	Ad2	Ad2R	CGCTAAGAGCGCCGCTAGTA	
	Ad5	Ad5R	ATGCAAAGGAGCCCCGTAC	
	Ad6	Ad6R	CTTGCAGTCTTTATCTGAAGCA	
	E	Ad4	Adeno4.U3	
Adeno4.L1			TTAGCATAGAGCATGTTCTGGC	
F		AdF1	ACTTAATGCTGACACGGGCAC	Tiemessen et al., (1996).
	Ad40	K402	CACTTAATGCTGACACG	
	Ad41	K403	ACTGGATAGAGCTAGCG	

Abbreviations: F, forward/sense; R, reverse

The cycling conditions for the Adenovirus serotype B, C, and E were as listed in Table 3.5 using conventional PCR while for the F serotype, the conditions were as follows: denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 45 sec and extension at 72°C for 2 min.

Table 3.5: Cycling conditions for species B, C and E Adenovirus as suggested by Metzgar et al., (2005).

PCR step	No. of cycles	B serotypes	C serotypes	E Serotype
Denaturation	1	5:00 (95)	5:00 (95)	5:00 (95)
Denaturation	35	1:00 (94)	1:00 (94)	0:40 (94)
Annealing	35	1:00 (56)	1:00 (47)	0:40 (53)
Extension	35	1:00 (72)	2:00 (72)	1:30 (72)

3.6 STATISTICAL ANALYSIS

Obtained data was analyzed using Statistical Package for Social Sciences (IBM SPSS Statistics release 19; IBM, USA). Calculation of means and standard deviations and Correlations among the various physicochemical parameters was done using this application.

CHAPTER FOUR

RESULTS

4.1. PHYSICOCHEMICAL ANALYSIS

Most physicochemical parameters fluctuated appreciably throughout the sampling period. Temperature varied from 13°C to 28°C with higher values in WWTP-K than at WWTP-R. Turbidity measured in NTU was significantly higher in WWTP-K with extreme values in the month of June and August while WWTP-R had alarming high values of free chlorine with a maximum value of 8.8 mg/L. pH was stable in both plants and within the recommended limits of 6 to 9.5 except for one month in the WWTP-R. Nutrients (Nitrites, Nitrates and Orthophosphates) were mostly compliant to standards set by DWA (1996c) for recreational water. Electrical conductivity in all the months sampled, was above the set limits ≤ 250 (Government Gazette, 1984). A summary of these results is presented in the following graphs.

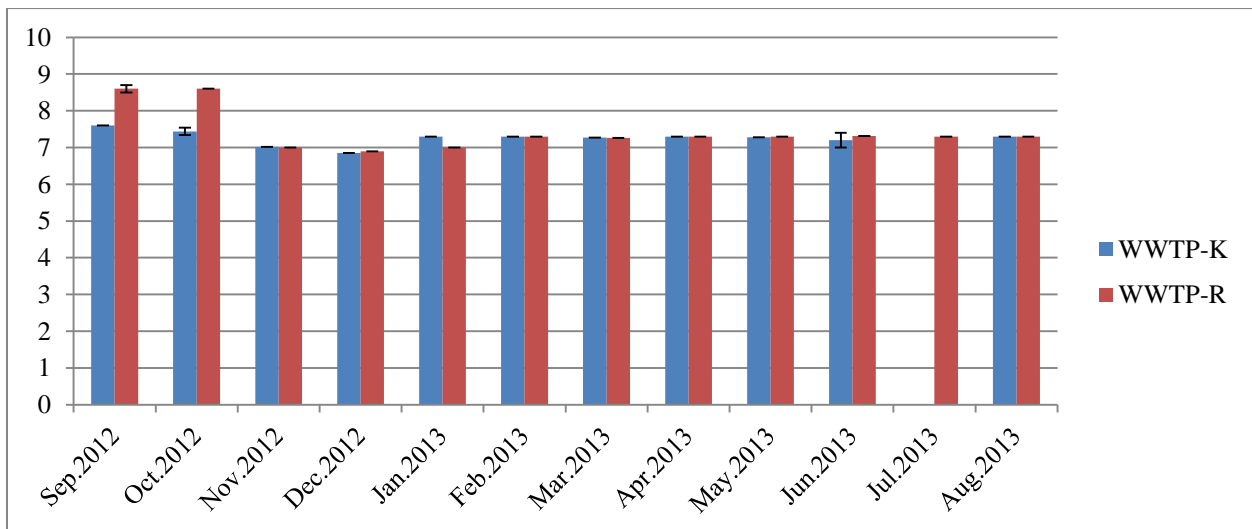


Figure 4.1: pH values of the two wastewater treatment plants (error bars = standard deviation).

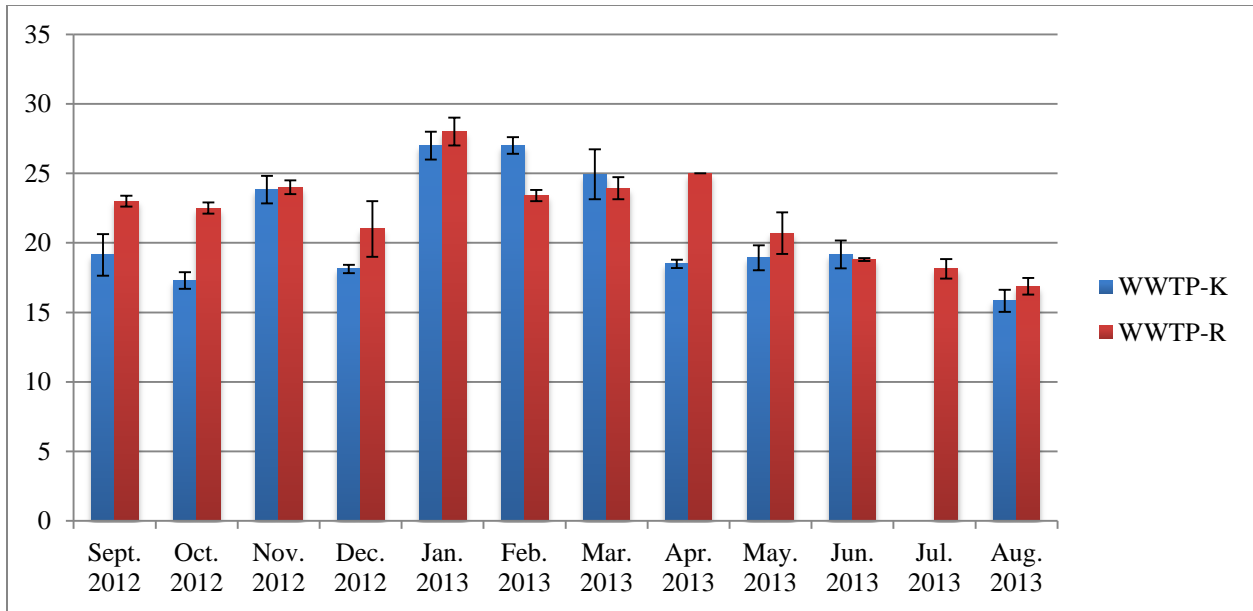


Figure 4.2: Temperature (°C) of the two wastewater treatment plants measured *on site* (error bars = standard deviation)

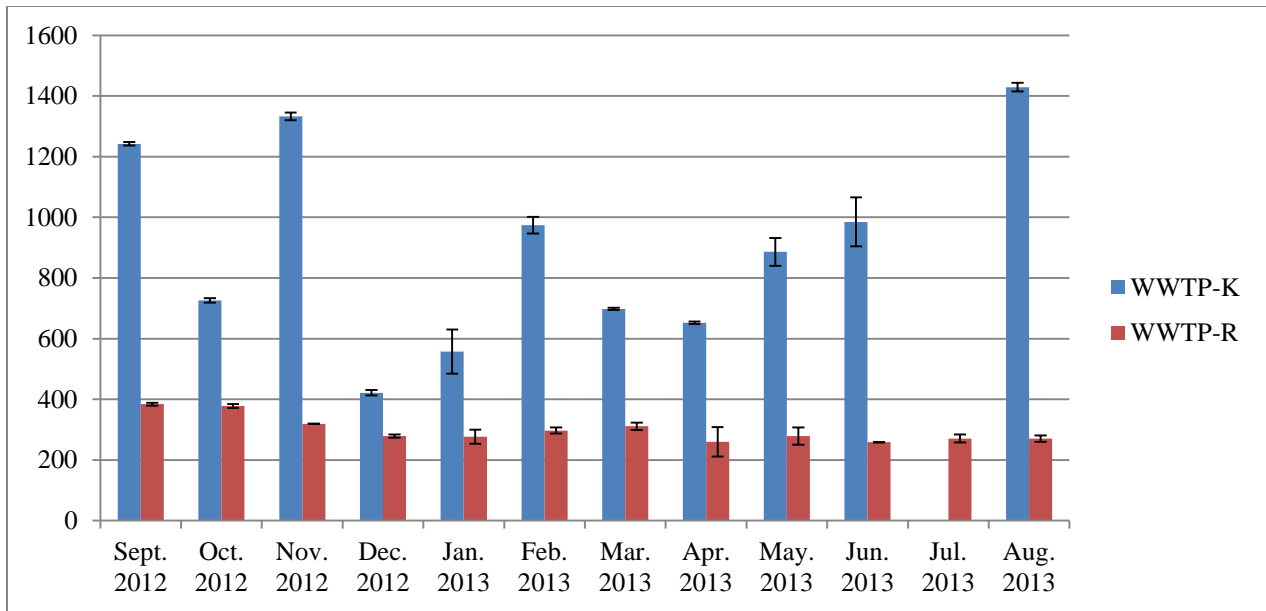


Figure 4.3: Electrical conductivity (µS/cm) values of the two wastewater treatment plants measured *on site* (error bars = standard deviation)

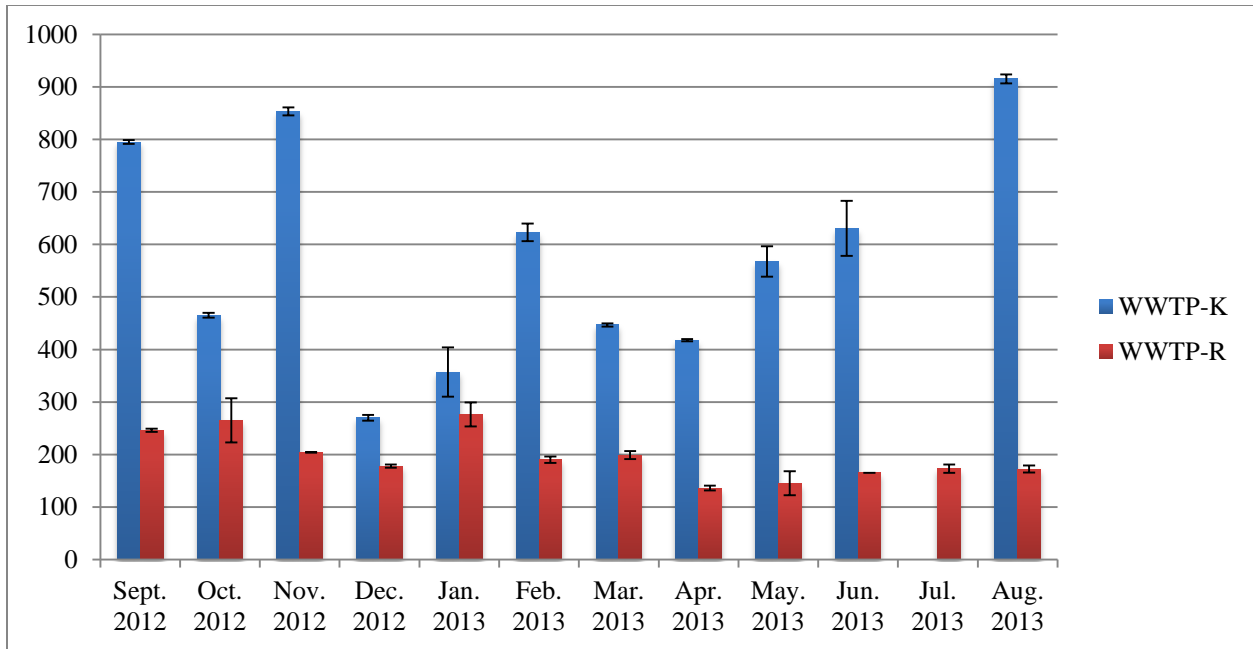


Figure 4.4: Measurement of the Total dissolved solids (mg/L) for both plants sampled, measured *on site* (error bars = standard deviation)

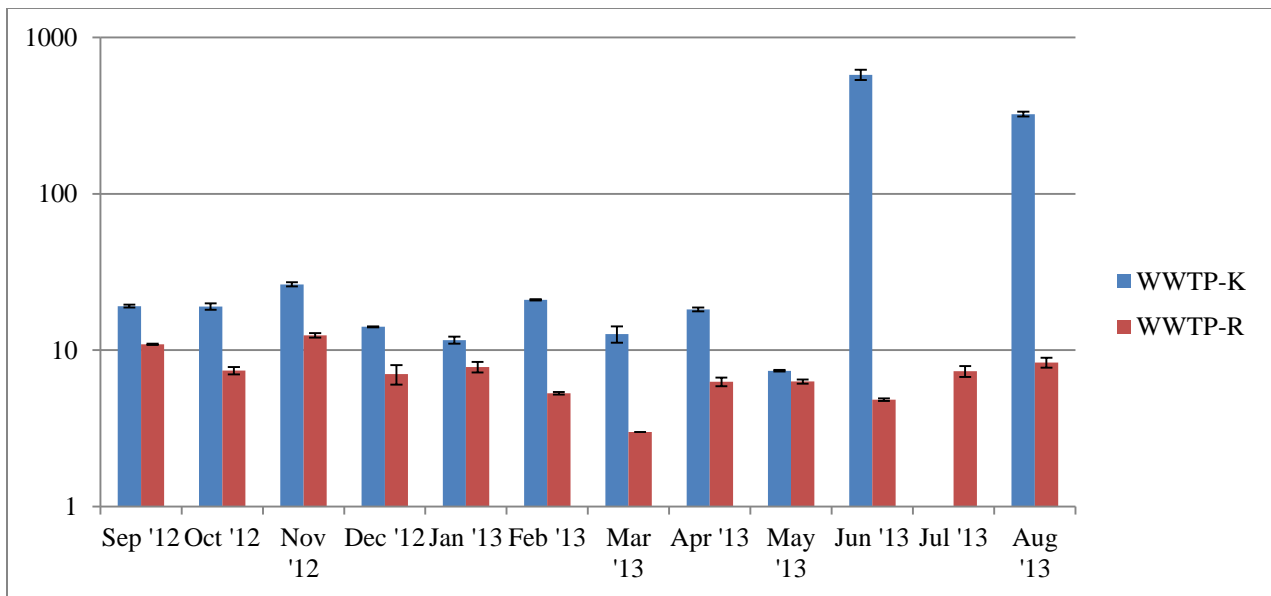


Figure 4.5: Turbidity (NTU) of the plants measured *on site* (error bars = standard deviation)

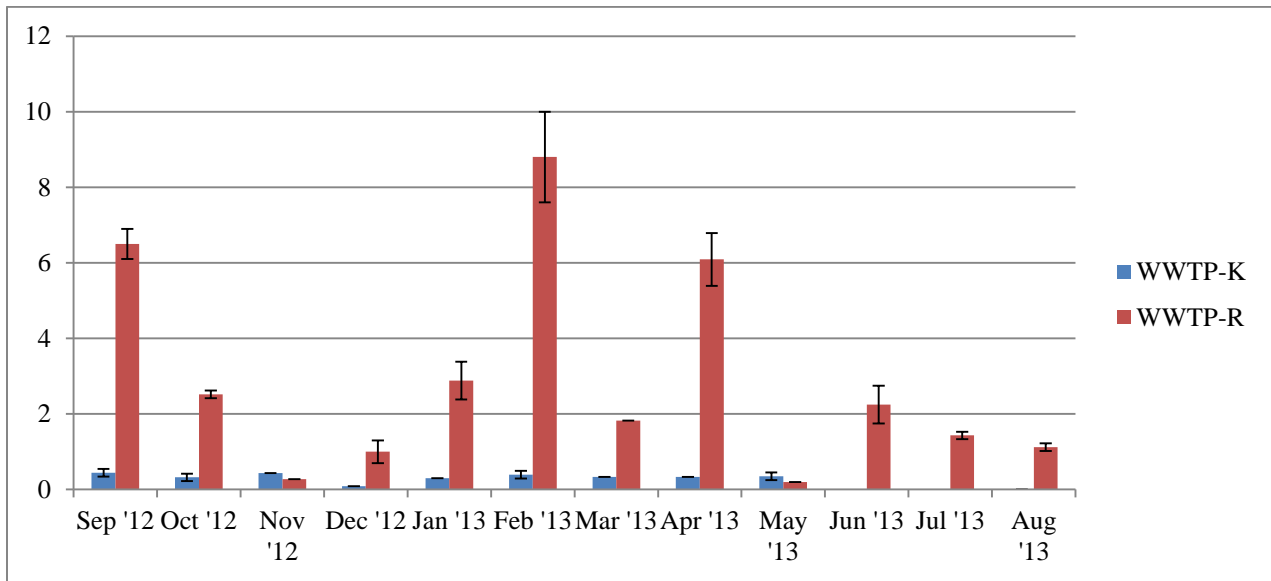


Figure 4.6: Concentrations of free chlorine (mg/L) in both plants (error bars = standard deviation)

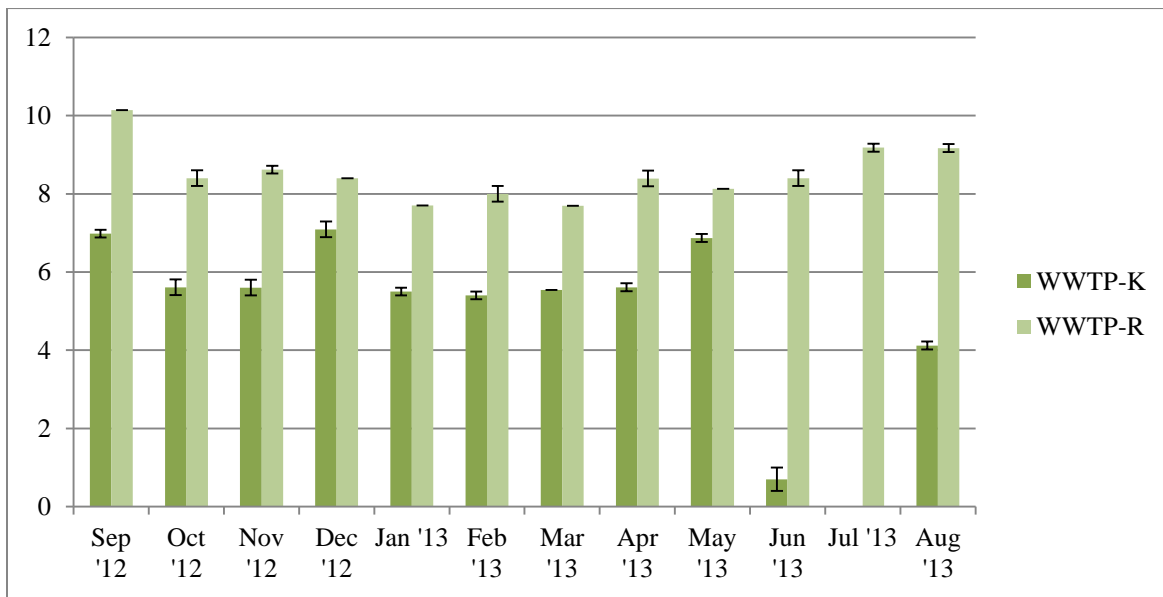


Figure 4.7: Concentrations of Dissolved Oxygen (mg/l) in both plants (error bars = standard deviation)

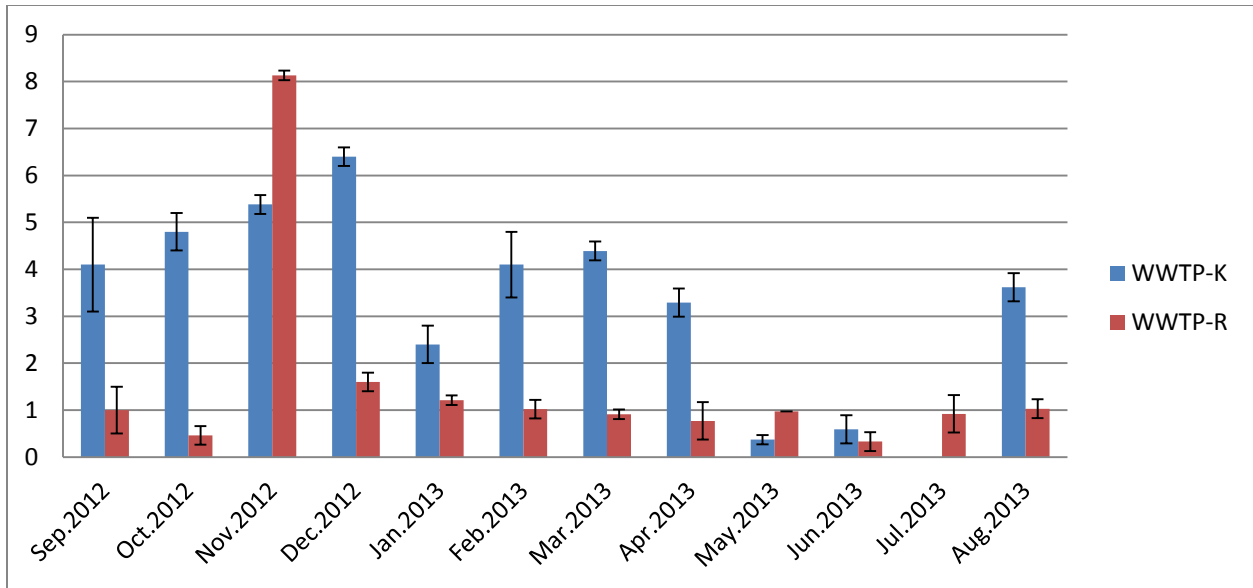


Figure 4.8: Concentrations of Biochemical Oxygen Demand (mg/L) in both plants (error bars = standard deviation)

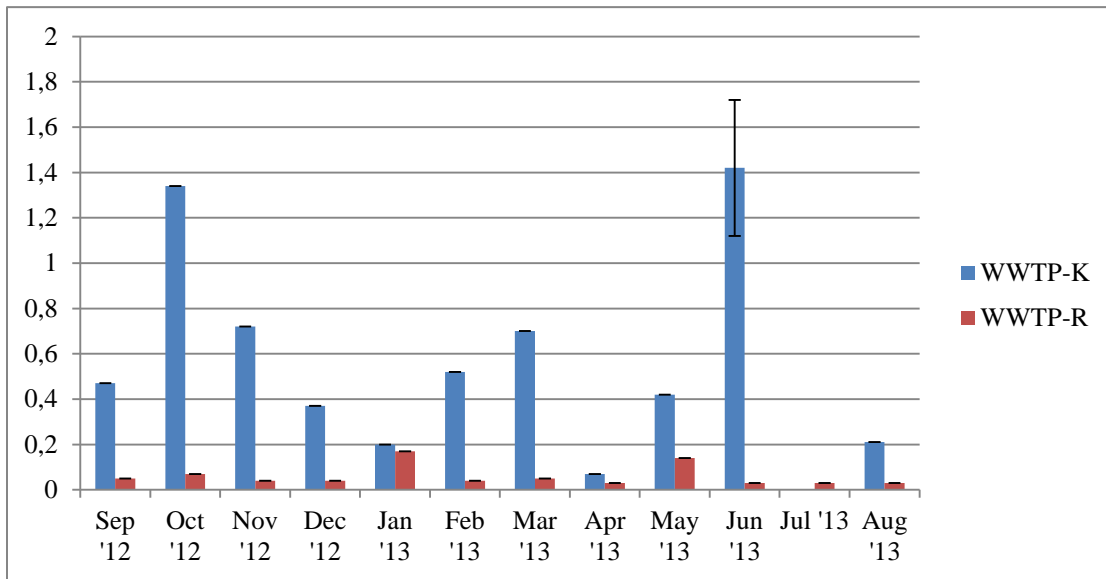


Figure 4.9: Measurement of the Nitrites (mg/L) for both plants sampled, (error bars = standard deviation)

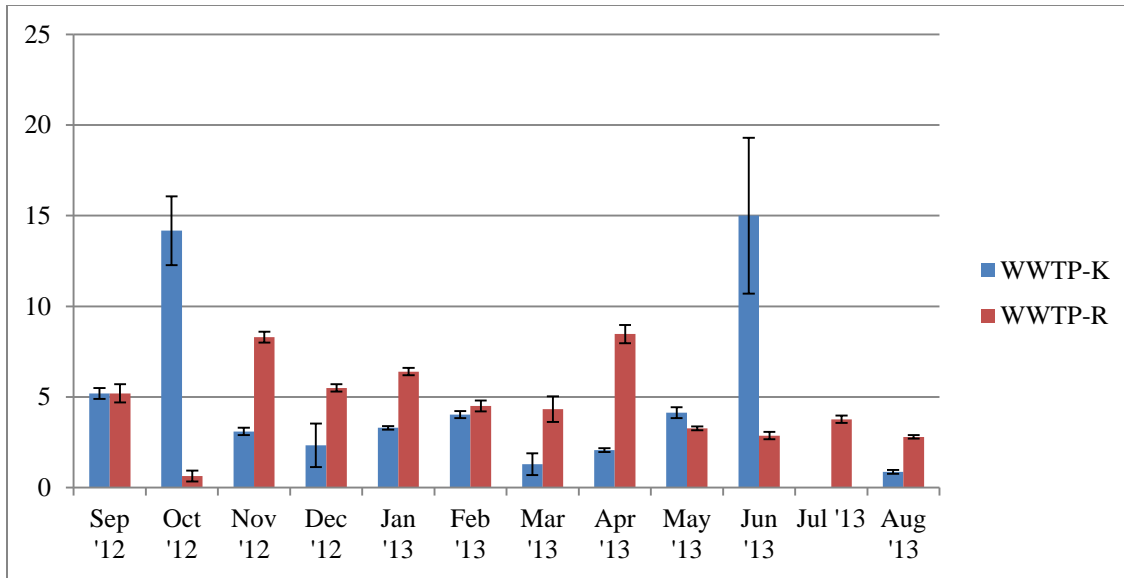


Figure 4.10: Measurement of the Nitrates (mg/L) for both plants sampled, (error bars = standard deviation)

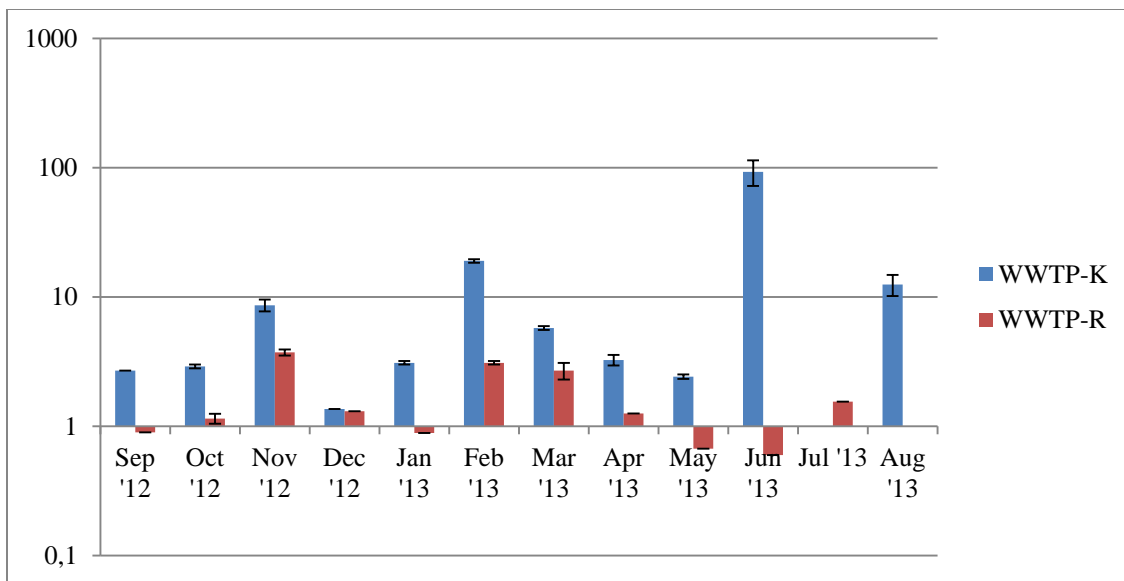


Figure 4.11: Phosphate concentrations (mg/L) for both plants sampled, (error bars = standard deviation)

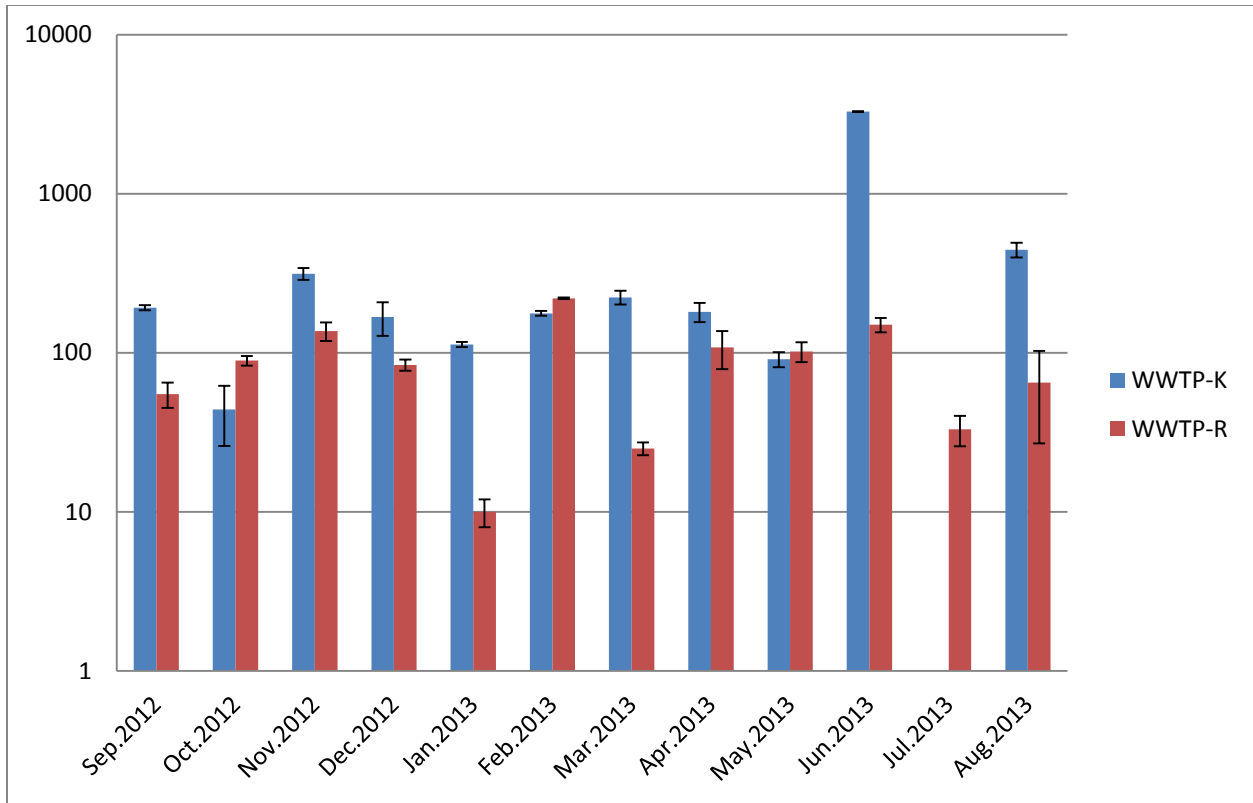


Figure 4.12: Measurement of the Chemical Oxygen Demand (mg/L) for both plants sampled, (error bars = standard deviation).

4.2. BACTERIOLOGICAL ANALYSIS

The results of the faecal indicator bacteria are shown as the mean of the triplicates in Table 4.1. For the WWTP-R, zero counts of faecal indicator bacteria were observed for the entire sampling period. In WWTP-K, out of 27% of samples where faecal indicator bacteria was detected, the month of June 2013 accounted for maximum counts of 6.4×10^4 CFU/100ml.

Table 4.1: Faecal indicator bacterial counts (CFU/100ml) of the wastewater final effluents from both plants.

Month	WWTP-K	WWTP-R
September 2012	0	0
October 2012	0	0
November 2012	0	0
December 2012	0	0
January 2013	0	0
February 2013	0	0
March 2013	9.9×10^4	0
April 2013	0	0
May 2013	0	0
June 2013	6.4×10^4	0
July 2013	No sampling	0
August 2013	1.4×10^4	0

4.3. DETECTION OF VIRUSES FROM THE FINAL EFFLUENTS OF THE WASTEWATER TREATMENT PLANTS

Five viruses were assessed and out of these, Enterovirus and Norovirus were never detected in both plant effluents, while Adenovirus, Rotavirus and Hepatitis were only detected in WWTP-K. Figure 4.13 shows quantitative detection of the above mentioned viruses. Rotavirus was detected in 55% (6/11) and concentrations of 10678 to 2034176 genome copies/L while Adenovirus and Hepatitis A Virus were detected in 18% (2/11) of the samples at concentrations of 45 – 276 and 23 – 71 genome copies/L respectively.

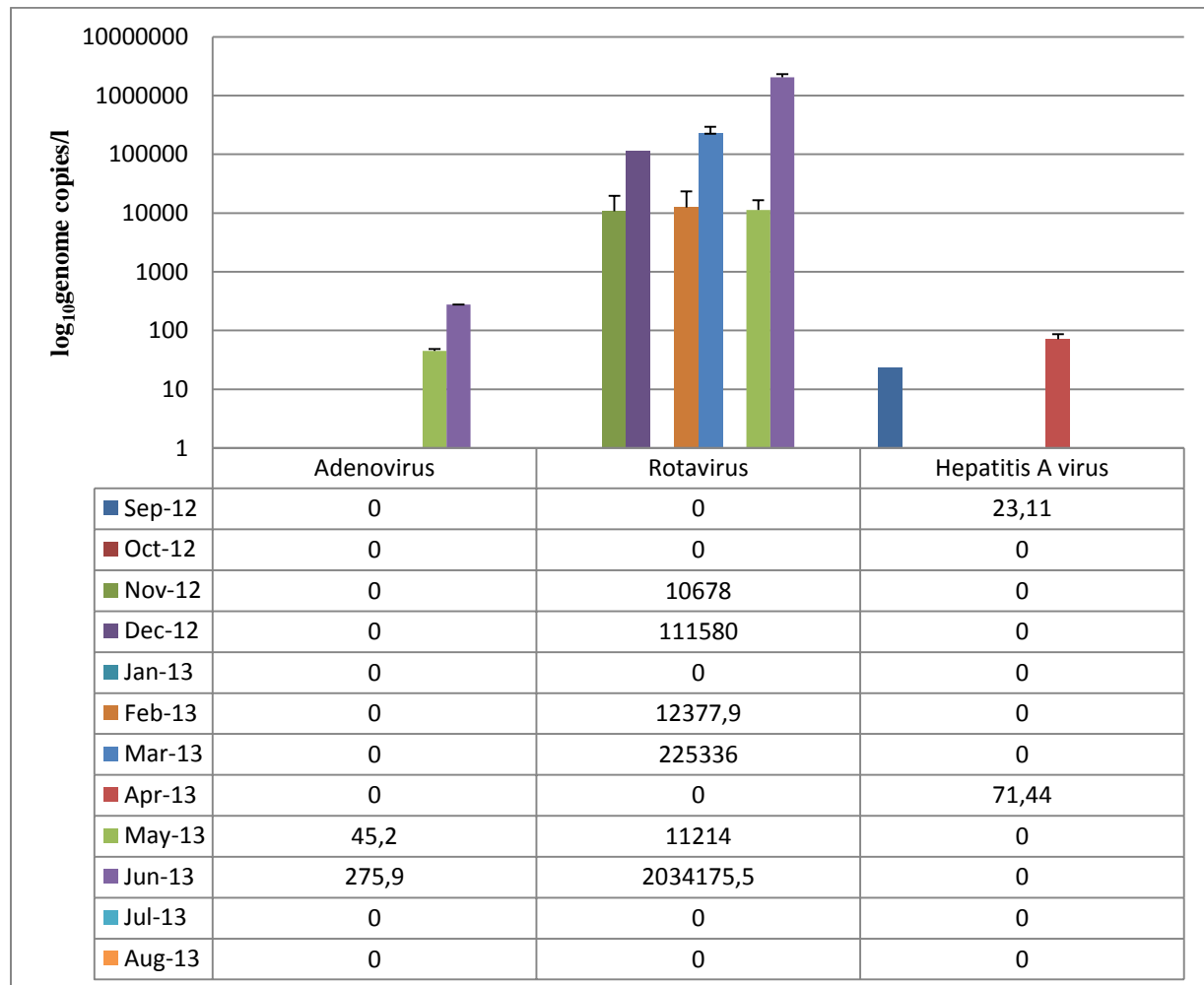


Figure 4.13: Quantitative detection of viruses by Real-time PCR

4.4. CHARACTERIZATION OF VIRUSES BY CONVENTIONAL PCR

In this study, 3 rotavirus serogroups (A, B, and C) were assessed; none of them was detected from the rotavirus positive samples. All the adenovirus positive samples were observed to contain for species F, serotype 41 while only 50% of samples was positive for species C (Adv 6 and Adv 2). Also species B Adv 7 was detected in 50% of the samples positive for Adenovirus from real-time PCR. The following figures show gel pictures of only the positive samples in the characterization experiments.

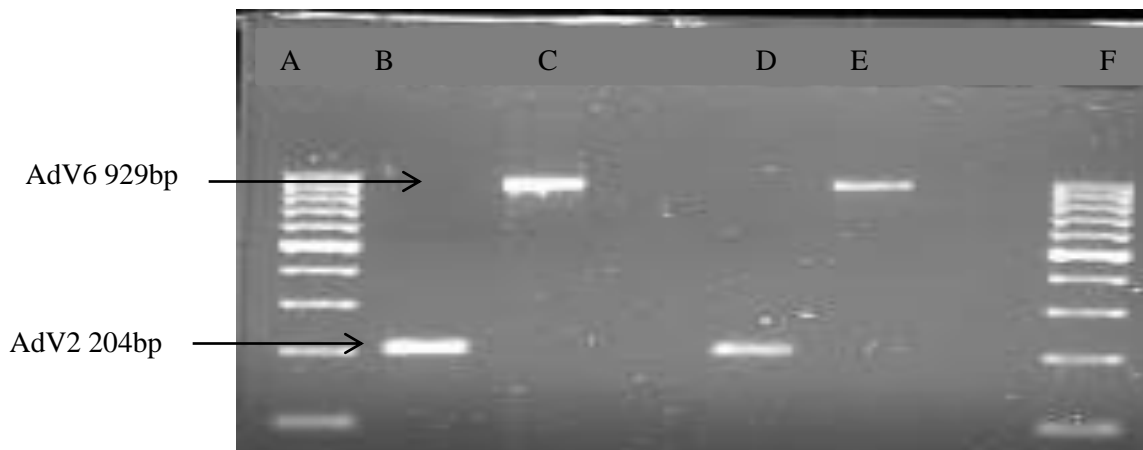


Figure 4.14: EtBr stained gel picture showing AdV species C serotype 2 and 6 detected from WWTP-K. A and F = DNA Ladder; B=positive control (Ad2); C = positive control (Ad6); D= June 2013; E= June 2013.

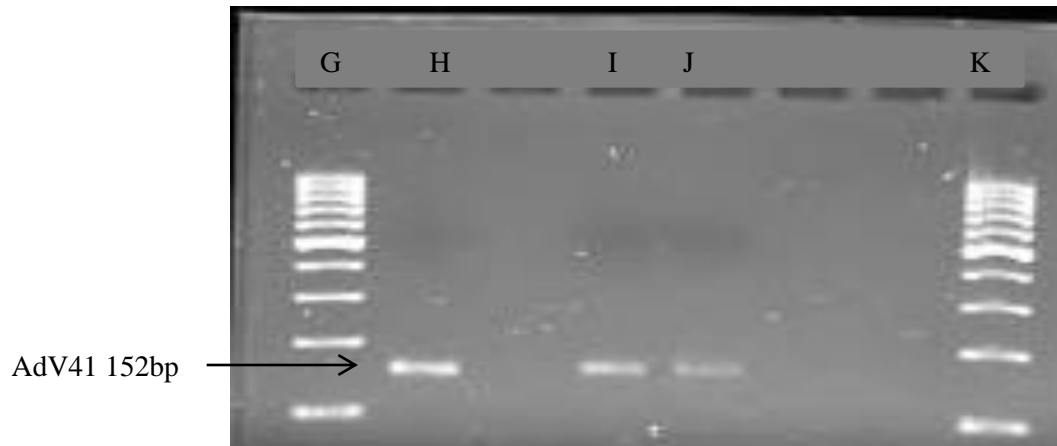


Figure 4.15: EtBr stained gel picture showing AdV species F serotype 41 detected from WWTP-K. G and K = DNA Ladder; H=positive control (Ad 41); I= May 2013; J= June 2013.

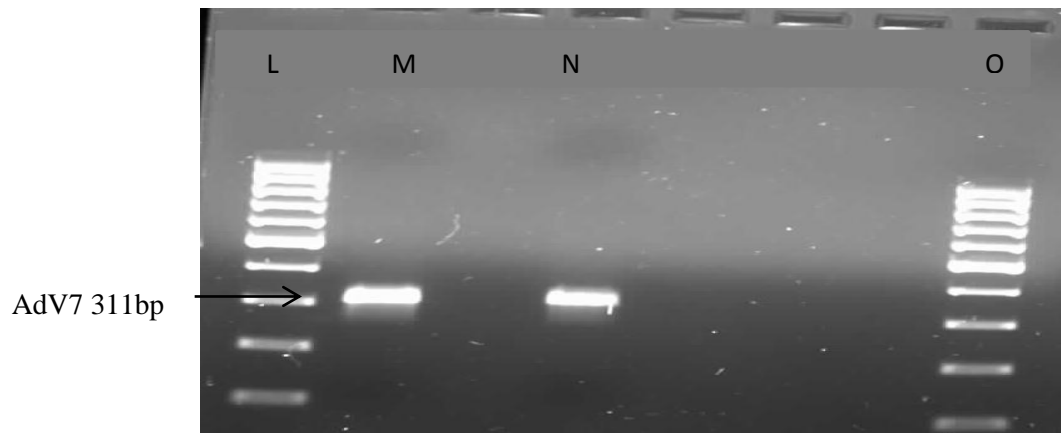


Figure 4.16: EtBr stained gel picture showing AdV species B serotype 7 detected from WWTP-K. L and O = DNA Ladder; M=positive control (Ad 7); N= May 2013.

4.5. CORRELATION OF PHYSICOCHEMICAL PARAMETERS AND MICROBIOLOGICAL INDICATORS.

Correlation coefficients of the assessed parameters of this study are shown in table 4.2 and 4.3 for WWTP-R and WWTP-K respectively. In table 4.2, only the physicochemical parameters are correlated as there are microbiological indicators detected from the plant. In WWTP-R, there was significant positive correlation ($P < 0.01$) between TDS and EC, DO and pH, BOD and COD, Turbidity and DO. Significant ($P < 0.01$) inverse correlation existed between pH and BOD, pH and phosphate, DO and temperature, phosphate and COD, phosphate and free chlorine.

Table 4.3 shows significant positive correlation ($P < 0.01$) between EC, TDS, COD and Turbidity. DO, COD and Nitrite correlated positively ($P < 0.01$) with faecal coliforms while these coliforms had no positive correlation with the viruses except for the significant inverse correlation ($P < 0.01$) with Hepatitis A virus. The viruses did not positively correlate with each other or with the water temperature, but hepatitis A virus had an inverse correlation significant at $P < 0.01$ with the water temperature. Adenovirus showed significant positive correlation ($P < 0.01$) with phosphate but inversely correlated ($P < 0.01$) with BOD. Rotavirus did not correlate with any of the parameters assessed while hepatitis A virus showed positive correlation with pH and DO, however, its correlation with temperature, free chlorine and phosphate was negative.

Table 4.2: Correlation coefficient half-matrix of physicochemical indicators of WWTP-R

Parameters	pH	TDS	EC	DO	COD	BOD	Temp.	Turbidity	FreeChl.	Nitrite	Nitrate	Phosphate
pH	1											
TDS	-0.106	1										
EC	0	.850**	1									
DO	.482**	0.148	0.063	1								
COD	0.022	-0.252	-0.178	0.137	1							
BOD	-0.508**	-0.095	-0.187	-0.076	.144**	1						
Temp	-0.171	0.084	0.227	-.471**	-0.125	0.057	1					
Turbidity	0.141	.338*	0.276	.625**	-0.09	.153*	-0.058	1				
FreeChl	0.182	0.174	0.118	-0.191	-0.088	-0.383	0.405	-0.293	1			
Nitrite	-0.099	0.253	.405*	-.420*	-0.146	0.032	.339*	.209*	-0.058	1		
Nitrate	-.342*	0.036	0.132	-0.092	-.036*	0.382	0.74	0.224	.171*	0.083	1	
Phosphate	-.429**	0.217	0.107	0.16	-.111**	0.172	-0.028	0.199	-.150**	-0.324	0.147	1

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

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

Abbreviations: TDS, Total dissolved solids; EC, electrical conductivity; DO, dissolved oxygen; COD, chemical oxygen demand; BOD, biochemical oxygen demand.

Table 4.3: Correlation coefficient half-matrix of physicochemical and microbiological indicators WWTP-K

Parameters	pH	TDS	EC	DO	COD	BOD	Temp.	Turbidity	FreeChl.	Nitrite	Nitrate	Phosphate	FC	RV	ADV	HAV
pH	1															
TDS	0.266	1														
EC	0.266	1.000**	1													
DO	0.013	-0.332	-0.332	1												
COD	-0.061	.607**	.607**	-.514**	1											
BOD	-0.303	-0.063	-0.063	0.242	-0.116	1										
Temperature	-.392*	-0.098	-0.098	-0.117	-0.021	-0.056	1									
Turbidity	0.128	.687**	.687**	-.589**	.689**	0.105	-0.213	1								
Free Chlorine	0.089	0.194	0.194	.398*	-0.22	0.214	0.456	-0.158	1							
Nitrite	-0.017	0.295	0.296	-0.184	0.14	0.198	0.102	0.359	0.177	1						
Nitrate	.380*	0.113	0.113	0.032	-0.1	-0.281	.018*	0.161	0.203	0.557	1					
Phosphate	-0.064	.503**	.504**	-.851**	.655**	-0.145	0.266	.715**	-.181**	.313**	-.034**	1				
FC	-0.017	0.259	0.259	-.611**	.600**	-0.264	-0.153	0.414	-0.583	.267**	-.169**	0.605	1			
RV	-0.161	-0.077	-0.077	0.173	0.318	0.002	0.14	0.081	0.003	0.296	0.141	0.051	0.349	1		
ADV	-0.016	0.104	0.104	-0.176	0.234	-.658**	-0.066	0.066	-0.293	0.284	0.477	.133**	0.385	0.356	1	
HAV	.483**	-0.041	-0.041	.354*	0.026	-0.11	-.141**	-0.028	0.304	-.404*	0.005	-0.239	-.282**	0.212	-0.219	1

* Correlation is significant at the 0.05 level (2-tailed). ** Correlation is significant at the 0.01 level (2-tailed). Abbreviations: TDS, Total dissolved solids; EC, electrical conductivity; DO, dissolved oxygen; COD, chemical oxygen demand; BOD, biochemical oxygen demand; Temp., temperature; Freechl., free chlorine FC, faecal coliforms; RV, rotavirus; ADV, adenovirus; HAV, Hepatitis A virus.

CHAPTER FIVE

DISCUSSION

5.1. GENERAL DISCUSSION

Wastewater final effluents are discharged to surface waters which are often abstracted for domestic and other purposes in many communities, especially poor communities of South Africa. However, these sewage discharges are a major component of water pollution, contributing to oxygen demand and nutrient loading of the water bodies (WRC, 2000; Fatoki et al., 2001). Henceforth, poor quality wastewater final effluents remain a major public health concern. The final effluents from this study were discharged into the rivers which are used for various purposes such as irrigation, direct domestic uses and also a habitat for a wide diversity of aquatic life. Therefore, the quality of these final effluents needs to adhere to the set limits so as to reduce health risks associated with inadequately treated wastewater.

The results of these measured wastewater quality parameters showed significant variations. However, in July 2013, at WWTP-K, samples could not be taken due to construction works taking place therein that led to unavailability of water samples while in the month of June and August 2013 they were discharged rather untreated due to their inability to control the influent levels. The influence of this infrastructure upgrade works became notable in the results shown in Chapter 4.

Electrical conductivity (EC) is a measure the presence of ions in water that can carry an electric charge and also an indicator of the salinity of water or total salt content (DWAF, 1996a; Morrison et al., 2001). In this study, the ECs was above the South African target limit of 250

$\mu\text{S}/\text{cm}$ for effluents meant to be discharged into a water source (Government Gazette, 1984) and also above the revised maximum limit of $150 \mu\text{S}/\text{cm}$ (DWAF, 2004). In the case of irrigation, water's suitability and purity is determined by two major factors measured as electrical conductivity (EC) and concentration of total dissolved solids (TDS). Hence, EC influences crop yield, as crops are very sensitive to high salinity. In this study, these two parameters are non-compliant for irrigation purpose especially at the rural community located WWTP-K with a range of $297 - 1429 \mu\text{S}/\text{cm}$. When the EC concentration is above $540 \mu\text{S}/\text{cm}$, only low percentage yield of the salt-tolerant crops is expected (DWAF, 1996c).

The total dissolved solids (TDS) are a measure of the quantity of various inorganic salts dissolved in water with its concentration directly proportional to the electrical conductivity (EC) of water. The TDS concentrations of this study fell below the recommended limit of $2000 \text{ mg}/\text{L}$ for discharged effluents as indicated by WHO standards (Akan et al., 2008). However, with regards to the DWAF (1996c) standards of 0 to $450 \text{ mg}/\text{L}$, the WWTP-K had its TDS concentration non-compliant (above $450 \text{ mg}/\text{L}$).

pH which is a measure of the acid-base equilibrium of dissolved compounds is affected by temperature (DWAF, 1996b). The pH of water does not have direct consequences except at extremes and in this study; it was within the recommended limits of $5.5-9.5$ (DWAF, 2004). When the pH is extremely high, it solubilizes toxic heavy metals and protonate or deprotonate certain ions which in turn affects aquatic life and humans that rely on that water body (DWAF, 1996b; Odjadjare and Okoh, 2009).

In this study, the turbidity of most of the samples was above the WHO set limit of $\leq 5 \text{ NTU}$. Only 8% (March 2013 and June 2013) of samples which are from Reeston WWTP had their

turbidity levels within the set limit. The occurrence of higher indicator bacterial numbers in the samples of June 2013 and August 2013 from WWTP-K (Table 4.1.) could be related to the higher turbidity levels. High turbidity hinders water disinfection (DWAF, 1996a) and may lead to formation of the toxic trihalomethane (TMH) precursors when chlorine is applied (Igbinsosa and Okoh, 2009). In addition, these high turbidities (3–567 NTU) also impact negatively on the aesthetic characteristic of recreational waters.

As well known, the effluents discharged into rivers may in some cases be used directly for drinking, and therefore, in this regard the DO was above the acceptable target limits for drinking water purposes (6 mg/l) in the WWTP-R while for WWTP-K, 50% of the samples complied with the standard. The standard set for sustaining of aquatic life (4 mg/l) which in this study none of the plants complied with but mostly complied with that of unpolluted water (8-10 mg/l) (DWAF, 1996a, Igbinsosa and Okoh, 2009). The extremely low DO levels (0.7 mg/L) recorded from the June 2013 sample is suggestive of high levels of pollution and nutrient loading which negatively affects the aquatic life of the water body. This is further corroborated by the high nitrate, nitrite and orthophosphate concentrations in the same sample, possibly due to the failure of the primary wastewater treatment stage to remove these nutrients.

Although there are no South African guidelines for BOD in final effluents, the EU standard of 3 to 6 mg/L for water intended for fisheries and aquatic life is adopted for wastewater final effluents (Chapman, 1996). The BOD range for the WWTP-K was 0.37 to 5.4 mg/L and that of the WWTP-R was 0.46 to 8.13 mg/l. According to Momba et al., (2006), these BOD levels disqualify the final effluents from being discharged into an aquatic environment. The results of this study depict the inefficiency of these treatment plants in removing the chemical oxygen-demanding substances during the treatment process. The COD of this study was extremely above

30 mg/l which is the South African guideline for COD in effluents to be discharged into the river (Government Gazette, 1984).

Temperature of wastewater effluents recommended to be used for domestic purposes has to be $\leq 25^{\circ}\text{C}$ (Government Gazette, 1984; DWAF, 1996a). The temperatures of the effluents in this study were compliant to the target limit but varied and increased with seasons, with the highest temperatures observed in the summer season, January to be exact (28°C) in both treatment plants. When temperatures do not comply with the standards, the effluents have a potential to upset the homeostatic balance of the receiving watershed (Odjadjare and Okoh, 2009), and in this study, the effluents do not constitute any risk.

Free chlorine is used as a biocide for disinfecting and sterilizing water (DWAF, 1996c). In this study, the free chlorine was above the limit set for treated water (0.25 mg/L). For the WWTP-K, free chlorine was not detected in some months and as such pose a very high health. Although free chlorine is important, when in excess, it is detrimental to aquatic life such as fish. Aesthetically, highly chlorinated water is unappealing as detected in WWTP-R which ranged between 0.2 and 7 mg/L.

The concentrations of nutrients; nitrate, nitrite and orthophosphates were measured as the best parameters in this study. According to Government Gazette (1984), the limit for treated effluent for nitrate was 1.5 mg/L of which only 13% of the samples complied. Also, DWAF (1996a) recommended 6 mg/L for water intended for domestic use and 6-10 mg/L for recreational use. The nitrate concentration range in effluents from WWTP-K ranged between 0.9 and 15 mg/L and for WWTP-R it varied from 0.64 to 8.5 mg/L. These high nitrate loads could result in the

excessive growth of aquatic plants and algal blooms that may be toxic to the aquatic environment and even surrounding animals (DWAF, 1996b). In addition, high concentrations of nitrate (>10 mg/L) are indicative of pollution by industrial or agricultural wastes containing toxic substances (DWAF, 1996c). Contact with water with phosphates may cause methanoglobinemia at concentrations higher than the set limits (Morrison et al., 2001; Odjadjare and Okoh, 2009).

Nitrite is mostly supplied to surface water by sewage wastes (DWAF, 1996b). When it is in excess, it causes eutrophication and thus impacting negatively on growth of aquatic life. Most samples in this study fell within the recommended limit (< 0.5 mg/L) except for the WWTP-K samples in October 2012, November 2012, March 2013 and June 2013.

Orthophosphates stimulate growth of both algae and aquatic macrophytes, therefore, excess phosphate leads to eutrophication and increased oxygen demand. There may be no safety limits for phosphates in water for recreational use and domestic use (Momba et al., 2006), exposure to water intoxicated by eutrophication is a health risk. In this study, 78% samples fell below the recommended limit (<5 mg/L) for survival of aquatic life.

Although water reuse is promoted, it has been generally acknowledged that the greatest hazard associated with it is the potential presence of microbial pathogens that constitutes a risk for the transfer of infections to humans or animals if they are exposed to pathogens in the wastewater effluents (Morrison et al., 2001). Wastewater has been regarded as a potential source of pathogens for the receiving watershed when inadequately treated. When wastewater effluents have high nutrient loads, as detected in this study, the presence of high concentrations of pathogens becomes likely, thus increasing the risk of infections either from direct water use or from food irrigated by the contaminated water.

Faecal coliforms being the ideal indicators of pollution in sewage water were assessed in this study using culture methods. In our data, the faecal coliforms correlated with chlorine dosage, especially in WWTP-R where chlorine dose was high and no faecal coliforms were detected. For WWTP-K samples, faecal coliforms were detected in 27.3% (3/11) where chlorine dosage was at its minimum throughout the sampling period at the plant. The faecal coliform counts ($9.9 \times 10 - 6.4 \times 10^4$ CFU/100 ml) suggest that the plant was highly contaminated during the three months of March 2013, June 2013 and August 2013. The presence of these faecal coliforms suggests the presence of other pathogenic microorganisms known to be major causes of waterborne diseases worldwide (Griffin et al., 2003).

In this study, PCR (both Real-time and conventional) were used to determine the presence of five viruses and their serotypes. In WWTP-R, none of the five viruses were detected throughout the study while in WWTP-K samples; three out of the five virus types were detected by real-time PCR and includes Adenovirus, Rotavirus and Hepatitis A Virus.

Rotavirus was detected in 55% of the samples assessed. Previous studies have reported that rotavirus is more prevalent in autumn and winter (Gerba et al., 1996; Brassard et al., 2005; Li et al., 2011) but in our study it was detected atleast once in all seasons although in higher concentrations in the months of March (autumn) and June (Winter). This could be because the rotaviruses are more stable in lower temperature. The presence of rotavirus in the effluents poses a threat to human health as they can persist for up to 32 days in water. Also, the presence of this virus in wastewater effluent in all seasons is suggestive of possible circulation of rotaviruses in the human environment throughout the year.

Adenovirus was detected in 18% of the samples collected and during the cold season of the year in line with the optimal temperature for virus survival ($< 23^{\circ}\text{C}$). Adenovirus serotypes, Adv2, Adv6 and Adv7 were detected at least once while Adv41 was detected in all of the adenovirus positive samples. Adv41 has replaced Adv40 as the predominant serotype isolated from gastroenteritis patients worldwide (Shinozaki et al., 1991; Yamashita et al., 1995; Shimizua et al., 2007; Fong et al., 2010). This study confirms these reports as Adv40 was not detected.

Hepatitis A virus was also detected in 18% of the samples. When Hepatitis A virus infects a person, it replicates and multiplies, destroys the liver cells causing inflammation of the liver, affecting the blood stream and causing jaundice and darkening of urine.

The viruses detected are a public health hazard because of their high infectious nature that even in low dosages they are harmful. Detection of these viruses does not only question the efficiency of the plant but also triggers concern on the resistance of these viruses to harsh conditions such as the treatment process. Most of the treatment plants in the Eastern Cape, including the one under study, use activated sludge technology for treatment of their wastewater (Katamaya et al., 2002) and helps in removal and inactivation of viruses. This might explain why in this study, some viruses were not detected.

Rotavirus was detected in significantly higher quantities compared to Adenovirus and Hepatitis A virus over the same period. Detection of Rotavirus was also independent (not season dependent) since it was detected at least once every season. However, the correlation between rotavirus and other parameters was considered insignificant while adenovirus only correlated positively with phosphates ($P < 0.01$) which is one of the nutrients known as indicators of organic contamination. Hepatitis A virus inverse correlation with temperature ($P < 0.01$) confirms the

survival strategies of viruses which are favoured by low temperatures. In addition, HAV inversely correlated with free chlorine which serves as a disinfection but this HAV and other viruses could not correlate with the faecal coliforms suggesting that faecal coliforms alone are not adequate indicators of faecal pollution of water.

5.2. CONCLUSION AND RECOMMENDATIONS

The results obtained from this study showed that wastewater treatment still favours the urban-based treatment plants (WWTP-R) than the rural-based treatment plant. These two wastewater treatment plants showed fluctuation in their effluent qualities especially the WWTP-K. The lack of skilled man-power in one of these wastewater treatment plants and infrastructure inadequacy are factors contributing to such inefficiency.

According to this study, the chlorine under-dosing or over-dosing is particularly a serious concern. Although high chlorine doses are effective in significant virus inactivation in treated wastewater, the production of chlorinated byproducts and the cost of additional dechlorination might become a limiting factor. Therefore, when designing wastewater disinfection processes, reduction of pathogens should certainly not be the only concern.

The real-time PCR assay used in the current study was not able to evaluate the viability of viruses detected after disinfection. In addition, when viruses are concentrated for real-time PCR, the compounds matrices in sewage effluents are also concentrated thus inhibiting the extraction, reverse transcription and PCR resulting in less positive samples (Ijzerman et al. 1997; Ko et al. 2003; Li et al., 2011). For this reason, an alternative method, Intergrated cell-culture quantitative reverse transcription PCR (ICC-RT-qPCR) may be used. This method involves pre-infection to increase sensitivity and reduce the PCR inhibitors in concentrated samples. ICC-RT-qPCR method also assists in evaluating the infectivity of viruses in treated wastewater effluents.

The detection of faecal coliforms and viruses in this study suggests that wastewater treatment plants are potential sources of pathogens in surface waters and the insignificant correlation of faecal coliforms and virus detection necessitates that enteric viruses be assessed routinely as one

of the parameters used in analysis of water quality. In addition, this study provided new information on the fate and susceptibility of viruses to different levels of wastewater treatment processes and demonstrated the impact of wastewater discharge on the receiving surface water quality in terms of potential spreading of infectious viruses. Therefore, the fate of viruses in wastewater treatment processes needs further evaluation, with the goals to improve virus removal.

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