CHAPTER 1

INTRODUCTION

1.1 General introduction

In the last decade, increased attention has been given to the physical and biological integrity of our nation's water bodies because of the indispensable value and role in people's lives. The 2000 National Water Quality Inventory reported that 39% of the rivers and streams evaluated were polluted, and pathogens are among the top three causes of impairment (Cole *et al*., 2003; USEPA, 2000). Factors contributing to water impairment include increased land use and rural development which place receiving waterbodies at high risk of contamination (Rose *et al*., 2001) and alterations in the hydrologic cycle, associated with global climate change, that are increasing the magnitude and frequency of runoff events (Lipp *et al*., 2001; Rose *et al*., 2001).

Water borne pathogens originate from human and animal feces and these include protozoa, bacteria and viruses (Ostioch *et al*., 2010). Microbiological indicators have been used for decades to monitor fecal pollution of water (Grabow, 1996) as well as the possibility of the presence of other microbiological pathogens (Medema *et al*., 2003). Despite the shortcomings of fecal coliforms for indicating the presence of resistant organisms, they are still reliable indicators for the possible presence of mainly bacterial pathogens such as pathogenic *E. coli* and Salmonella that may be present in water (Grabow, 1996; Steyn and Jagals, 2000).

The reuse of wastewater is one of the main options being considered as a new source of water in regions where water is scarce. The standards required for the safe use of wastewater and the amount and type of wastewater treatment needed are contentious (WHO, 1999). Enteric

bacteria have traditionally been used as indicators of fecal contamination in source, drinking, and recreational waters (Cole *et al*., 2003), however, research has demonstrated that these bacteria may not be appropriate indicators of pathogenic viruses and protozoa (Nwachuku *et al*., 2002; Payment *et al*., 1985; Rose *et al*., 1986), and the need for reliable index or indicator organisms for these pathogens has been widely recognized. Hence the need to monitor final effluents, receiving waterbodies, source waters, recreational waters and drinking water for the presence of pathogenic enteric viruses is of utmost importance to public health. U.S. Environmental Protection Agency (USEPA) has proposed the use of coliphages as a viral indicator of fecal contamination of groundwater (USEPA, 2000), and male-specific (F+) coliphages may provide the additional benefit of distinguishing human and animal fecal sources of pollution. Most importantly, wastewater treatment plants have been found to be a veritable source of enteric viruses in environmental water through the leakage of sewage and septic systems as well as the discharge of inadequately treated wastewater (Fong and Lipp, 2005). Conventional wastewater and drinking water treatment methods have proved to be inadequate in eliminating viral pathogens (Gutiérrez *et al*., 2007).

The presence of human enteric viruses in water used for drinking, recreation, or growing shellfish may pose a risk to human health (Havelaar *et al*., 1993). Water is in fact a vehicle for the transfer of a wide range of diseases of microbial origin including Hepatitis A virus and polio. It has been reported that more than 120 different types of virus are known to be excreted in feces by infected persons (Monceyron and Grinde, 1994; Li *et al*., 1998). Several of these viruses have been associated with disease transmitted by water (Li *et al*., 1998). Viruses exist in natural water or tap water with a much lower concentration because viruses cannot reproduce in water, in

addition, water treatment, dilution and natural inactivation all can reduce virus numbers. Even in low concentrations, they are still capable of causing diseases when ingested (Li *et al*., 1998).

Viruses reach a much lower concentration in water samples and thus, to reveal the presence of viruses in this type of samples a concentration step must be incorporated prior to the use of the detection techniques (Gosalvez *et al*., 2003). Commonly studied groups of enteric viruses belong to the families Picornaviridae (polioviruses, enteroviruses, coxsakieviruses, hepatitis A virus, and echoviruses), Adenoviridae (adenoviruses), Caliciviridae (noroviruses, caliciviruses, astroviruses, and small round-structured viruses), and Reoviridae (reoviruses and rotaviruses) (Fong and Lipp, 2005).

Although enteric virus infections are associated primarily with diarrhea and self-limiting gastroenteritis in humans, they may also cause respiratory infections, conjunctivitis, hepatitis, and diseases that have high mortality rates, such as aseptic meningitis, encephalitis, and paralysis in immunocompromised individuals (Fong and Lipp, 2005; Kocwa-Haluch, 2001a). In addition, some enteric viruses have been linked to chronic diseases such as myocarditis and insulindependent diabetes (Griffin *et al*., 2003; Kocwa-Haluch, 2001a).

1.2 Justification of this research

Enteric viruses including polioviruses, hepatitis A virus (HAV) and rotaviruses are commonly found in fecally-contaminated water and food and pose an important public health concern (Kittigul *et al*., 2000). In this regard, disposal of inadequately treated wastewater to surface water recipients is one of the main sources of pathogens in the environment, more so in a water scarce

country like South Africa with a very high population of the immunocompromised especially in the impoverished Eastern Cape Province. In the Eastern Cape Province (which is mostly non-urban, poor and without adequate infrastructure) a significant proportion of the rural communities lack pipe-born water, and as such depend on streams, rivers, groundwater and other available waterbodies for drinking and domestic purposes. Many of these waterbodies are often impacted by inadequately treated effluents from municipal wastewater treatment plants as receiving waterbodies (Fatoki *et al*., 2003). Our recent studies (Igbinosa *et al*., 2009; Odjadjare and Okoh, 2009; Okoh *et al*., 2007) have shown that the wastewater treatment facilities in the Eastern Cape Province of South Africa are a veritable source of pathogens in aquatic environments of this study area and negatively impact physicochemical quality of receiving watershed (Igbinosa and Okoh, 2009) therefore it is highly probable that they might also be a source of enteric viruses in the aquatic environment. The attendant consequences of the impact of such negative practices are the compromising of the primary health of people especially with death threatening diarrheal diseases, caused by invasive viral pathogens. Hence, the need to ensure protection of waterbodies in the province against contamination by invasive pathogens from wastewater treatment plants effluents becomes imperative. Much of the work done in relation to compliance with stipulated standards have been based on using conventional pollution indicator organisms like culturable total and feacal coliforms and coliphages, which are grossly inadequate for monitoring the presence of pathogenic viruses that have been known to be more resistant to wastewater treatment than vegetative bacteria. This study is therefore designed to obtain information on the prevalence of enterovirus and hepatitis A virus, as examples of viral pathogens, in relation to the fecal indicator bacteria (FIBs) and physicochemical qualities of the final effluents of wastewater treatment plants in the Eastern Cape Province.

1.3 Aims and objectives for this study

The broad aim of this research is to carry out a comparative assessment of the occurrence of viral and feacal indicator bacteria in the final effluents of peri-urban wastewater treatment facility in the Eastern Cape Province, South Africa. The working hypothesis would be that wastewater treatment plants in the Eastern Cape Province are sources of microbial pathogens in the aquatic environment. The specific objectives include:

1. To investigate the prevalence of enteric viruses in the municipal wastewater final effluents.
2. To investigate the prevalence of feacal indicator bacteria (total coliform, feacal coliform and Enterococci) in the municipal wastewater final effluents.
3. To assess the physicochemical qualities of the final effluents of the municipal wastewater treatment facility.
4. To assess the correlation between microbial and the physiochemical qualities of the effluents.
5. To determine the antibiogram characteristics of *E. coli* isolates obtained from the final effluents.

CHAPTER TWO

LITERATURE REVIEW

2.1 Wastewater

Municipal wastewater is a mixture of human excreta (sewage), suspended solids, debris and a variety of chemicals that originate from residential, commercial and industrial activities (Argaw, 2004; Tchobanoglous *et al*., 2003). Raw sewage is a major carrier of disease causing agents, particularly enteric pathogens (Bosch, 1998). Wastewater is characterized by both physicochemical and biological/ or microbiological characters. The physicochemical characteristics of wastewater include: temperature, color, odor, suspended solids, turbidity, total dissolved solids, pH, dissolved oxygen (DO), biological oxygen demand (BOD), nutrients and toxic substances, organics, alkalinity, metals and chlorides (Integrated Publishing, 2008; Drinan and Whiting, 2001). Biologically, wastewater contains various microorganisms, but the ones of concern to human health are bacteria, protozoa, helminthes and viruses (Tchobanoglous *et al*., 2003), plants and animals (Spellman and Drinan, 2008). The biological characteristics are of fundamental importance, because they include disease causing pathogenic bacteria and viruses of human origin (Nagulapallay, 2007).

Municipal wastewater is mainly comprised of water (99.9%) together with relatively small concentrations of suspended and dissolved organic and inorganic solids (Sperling, 2006), that ends up in wastewater treatment plants (WWTPs). Different techniques that can be applied in wastewater treatment include: (a) source control, (b) biological techniques (activated sludge, biofilms, and anaerobic systems), (c) as well as physical or physical-chemical techniques

(sedimentation, separation, filtration, precipitation, ion exchange techniques, treatment with ozone, use of activated carbon, etc.) (Pons *et al*., 2004).

2.1.1 Wastewater treatment

Wastewater treatment is of particular concern to all governments worldwide. The processes and technologies used to remove contaminants from wastewater and to improve and protect water quality are similar all around the world (CRC, 2007). In order to achieve different levels of contaminant removal, individual wastewater treatment procedures are combined into a variety of systems, classified as primary, secondary, and tertiary wastewater treatment. More rigorous treatment of wastewater includes the removal of specific contaminants as well as the removal and control of nutrients (UN, 2003 (b)). The safe treatment of sewage is thus crucial to the health of any community. In subjecting municipal wastewater to treatment before discharge to the environment, the goal is to remove pollutants, both chemical and biological, from the water in order to decrease the possibility of detrimental impacts on humans and the rest of the ecosystem (DeBusk, 1999).

2.1.1.1 Primary treatment

Primary wastewater treatment typically involves screening, grit chamber and sedimentation tank. Screening removes large objects such as stones, sticks, and plastics etc. that could plug lines or block tank inlets. Grit chamber slows down the flow to allow grit to fall out.

Sedimentation tank (settling tank or clarifier) allows settleable solids to settle out and are pumped away, while oils floats to the top and are skimmed off (ADS, 2004).

2.1.1.2 Secondary treatment

Secondary or biological treatment is used to convert the finely divided and dissolved organic matter in wastewater into flocculent settleable organic and inorganic solids. In these processes, micro-organisms, particularly bacteria, convert the colloidal and dissolved carbonaceous organic matter into various gases and into cell tissue which is then removed in sedimentation tanks. Biological processes are usually used in conjunction with physical and chemical processes, with the main objective of reducing the organic content (measured as BOD, TOC or COD) and nutrient content (notably nitrogen and phosphorus) of wastewater (UN, 2003(b)). This process includes the following options: activated sludge process, Aerated lagoon, and trickling filters (UN, 2003(b)).

The activated sludge process is the biological method of wastewater treatment that exploits the mixed community of microorganisms in an aerobic aquatic environment. The success of this process depends on the ability of these microbes to remove and consume organic material present in the sludge, in a process known as bioflocculation. This process consists of two liquid stream unit processes: a biological conversion of pollutants in a biological reactor and solids separation (gravity clarifier. It is the one that is commonly used today and the wastewater treatment plants under study utilize it (Jenkins *et al*., 2004).

2.1.1.3 Tertiary treatment

Tertiary treatment may include processes to remove nutrients such as nitrogen and phosphorus, and carbon adsorption to remove chemicals (ADS, 2004). Disinfection process in tertiary treatment is of importance in wastewater treatment owing to the nature of wastewater, which harbours a number of human enteric organisms that are associated with various waterborne diseases (UN, 2003(b)). Commonly used means of disinfection include the following: physical agents such as heat and light; mechanical means such as screening, sedimentation, filtration, and so on; radiation, mainly gamma rays; chemical agents including chlorine and its compounds, bromine, iodine, ozone, phenol and phenolic compounds, alcohols, heavy metals, dyes, soaps and synthetic detergents, quaternary 13 ammonium compounds, hydrogen peroxide, and various alkalis and acids. The most common chemical disinfectants are the oxidizing chemicals, and of these, chlorine is the most widely used (UN, 2003(b)). As useful the above mentioned wastewater treatment processes can be, they nonetheless fail to adequately eliminate more resistant pathogens such as protozoa, Giardia cysts, helminthes eggs, clostridia and more importantly enteric viruses which can be eliminated only up to 50% (Cloette *et al.*, 1998).

2.2 Feacal Indicator Bacteria (FIB)

Fecal indicator bacteria (FIBs) are used to assess the microbiological quality of water because, although not typically disease causing, they are correlated with the presence of several waterborne disease-causing organisms (Myers *et al.*, 2007). FIBs reside in the gastrointestinal tract of humans and animals (Anderson *et al.*, 2005), and are comprised of Total Coliforms (TC),

Fecal Coliforms (FC) and members of the genus Enterococci. Indicator bacteria are frequently used as the source identifier in microbial source tracking (MST) methods, which are designed to determine the source(s) of fecal pollution and/ or indicator organisms in water (Anderson *et al*., 2005). The presence of fecal indicator bacteria (FIB) in surface waters is a prevalent concern for many municipalities, health departments, and regulatory agencies (Tiefenthaler *et al*., 2009), and an ideal indicator should have the following characteristics: (1) quick, easy, affordable detection; (2) not naturally present in the environment; (3) concentrations in the environment correlated to the amount of fecal pollution present; and (4) decay rate comparable to pathogen(s) of interest (USEPA, 2006).

2.2.1 Total Coliforms

Total Coliforms include bacteria that are found in the soil, in water that has been influenced by surface water, and in human or animal waste. The extent to which total coliforms are present in the source water can indicate the general quality of that water and the likelihood that the water is fecally contaminated (USEPA, 2009). Total coliforms are currently controlled in drinking water regulations because their presence above the standard indicates problems in treatment or in the distribution system. EPA requires all public water systems to monitor for total coliforms in distribution systems (USEPA, 2009).

2.2.2 Faecal Coliforms

Faecal coliform bacteria are primarily used as indicators of faecal pollution and are more specific than total coliforms. They are mainly used for the assessment of faecal pollution of wastewater, raw water supplies and natural water environments used for recreational purposes (DWAF, 1996). These indicators are consistently released by all humans and in some instances by animals, because they are members of the normal microbial flora and are always present in sewage polluted water with numbers relatively close related to the levels of faecal pollution and the time since pollution has taken place (DWAF, 1996).

Higher concentrations of faecal coliforms in water will indicate a higher risk of contracting waterborne disease, even if small amounts of water are consumed. They are used to indicate the presence of bacterial pathogens such as *Salmonella spp*., *Shigella spp*., *Vibrio cholera*, *Campylobacter coli*, *Yersinia enterocolitica* and pathogenic *E.coli*, that can be transmitted via the faecal/oral route by contaminated or poorly treated drinking water (DWAF, 1996). These pathogens can cause diseases such as gastroenteritis, salmonellosis, dysentery, cholera and typhoid fever.

2.2.3 Enterococci

Enterococci are gram positive facultative anaerobes and originated as a separate species in the mid 1980's. They have emerged as important nosocomial and community-acquired pathogens in recent years and it has also been demonstrated that they have the capacity to exchange genetic information by conjugation, a process known to take place in the gastrointestinal tract, and thus acquiring virulence and antimicrobial resistance factors (Mundy *et al*., 2000; Grammenou *et al*., 2006). They are members of the natural intestinal flora of animals

and humans and are being released into the environment directly or via sewage outlets (Grammenou *et al*., 2006) thus they are being recognized as good indicators of faecal contamination in water quality studies (Godfree *et al*., 1997). Enterococci can persist and survive for longer periods in the environment due to their resistance to environmental stress and chlorination; however they are unable to multiply in water.

Enterococci are well known as antibiotic resistant opportunistic pathogens and are well established as etiological agents of endocarditis and urinary tract infection (Murray, 2000) as well as enterococcal bacteremia, neonatal infections, central nervous system infections, intraabdominal and pelvic infections and nosocomial infections and superinfections (Murray, 1990). Among enterococci strains *Enterococcus feacalis* (*E. faecalis*) and *Enterococcus faecium* (*E. faecium*) are the well known ones with the former species being the common cause of nosocomial infections and the latter for its resistance to vancomycin (Murray, 2000). The isolation of nosocomial enterococcus resistant to multiple antibiotics, especially vancomycin, has become worrisome worldwide (Talebi *et al*., 2008).

2.3 *Escherichia coli* (*E. coli*)

Escherichia coli was first described by Theodor Escherich in 1885 as *Bacterium coli commune*, which he isolated from the feaces of newborns (Todar, 2008). In 1935 a strain of *E. coli* was shown to be the causative agent in an outbreak of diarrhea in infants, although for many years it was considered a simple commensal bacterium of the large intestines (Todar, 2008). *E. coli* is now used as an indicator of faecal pollution that originates from human and warm-blooded animals (Omar and Barnard, 2010). *E. coli* survives in drinking water for between 4 and 12

weeks, depending on environmental conditions (temperature, microflora, etc.). *E. coli* is found in mammal faeces at concentrations of 10^9 cfu/100 ml, but it does not multiply appreciably in the environment (Edberg *et al*., 2000). Although usually harmless, various *E. coli* strains have acquired genetic determinants (virulence genes) rendering them pathogenic for both humans and animals. These pathogens are responsible for three main types of clinical infections: (i) enteric and diarrheal diseases, (ii) urinary tract infections, and (iii) sepsis and meningitis (Bekal et al., 2003). Virulence factors involved in pathogenic mechanisms of E. coli include adhesins, host cell surface-modifying factors, invasins, toxins, and secretion systems (Bekal *et al*., 2003). *E. coli* is the most common bacterial pathogen associated with endemic forms of childhood diarrhea in developing countries (Okeke *et al*., 2000) including South Africa, especially in regions with poor sanitation.

Based on the presence of different chromosomal or plasmid- encoded virulence genes, their pattern of interaction with epithelial cells and tissue culture monolayers, *E. coli* enteropathogens are classified into six categories: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) (Okeke *et al*., 2000; Nataro and Kaper, 1998).

2.3.1 Enterotoxigenic *E. coli* (ETEC)

Enterotoxigenic *E. coli* is the most common pathotype, particularly in developing countries and is recognized as an emerging enteric pathogen (Osode and Okoh, 2008; WHO,

2000). ETEC strains produce heat-labile toxin (LT), heat-stable toxin or both toxins (Benenson, 1995) and colonization factors allowing the organism to colonize the small intestine with subsequent development of diarrhea (Osode and Okoh, 2008; Wolf, 1997). A number of different O serogroups and H serotypes are represented among ETEC strains (Mahdy *et al*., 2010). ETEC is the common cause of traveler's diarrhea, weanling diarrhea in children in the developing world (Nataro and Kaper, 1998) and adults (4.5%) presenting to emergency departments and inpatient units in the United States (Osode and Okoh, 2008; Cohen *et al*., 2005; Nataro *et al*., 2006).

Laboratory techniques that can be applied to identify ETEC include: gene probes designed to detect the toxins or the toxin genes, tissue culture and immunochemical test (Zuber, 1999). The incubation period for ETEC is between 14 to 50 hrs (Nataro and Kaper, 1998) causing watery diarrhea similar to that of *Vibrio cholera*, usually without blood or mucus (Benenson, 1995), fever may not be present and the disease is self-limiting (Benenson, 1995; Beutin *et al*., 1998). The most common vehicles for ETEC infection are food and water contaminated with feaces (Zuber, 1999). There are about 400 million episodes of diarrhea associated with ETEC that occur annually worldwide, with an estimated 700 000 deaths (Mahdy *et al*., 2010; Putnam *et al*., 2004).

2.3.2 Enterohemorrhagic *E. coli* (EHEC)

Enterohemorrhagic *E. coli* also known as *E. coli* 0157:H7 surfaced in the last decade as an important food-borne pathogen with 73 000 cases of annual infection in the United States (Rangel *et al*., 2005; Zhao *et al*., 2006). Several studies have shown cattle to be the major

reservoir of *E. coli* 0157:H7, thus the cattle drinking water troughs are an important source of this pathogen on farms (Bach *et al*., 2004; Bach *et al*., 2005; Al-Saigh *et al*., 2004; Bosilevac *et al*., 2004; Low *et al*., 2005; Sheng *et al*., 2004).

Most studies indicate that *E. coli* 0157:H7 can survive in cattle drinking water up to 12 months and that the pathogen can be easily disseminated to other cattle (Faith *et al*., 1996; LeJeune *et al*., 2005; Shere *et al*., 2002) and subsequently to the environmental source waters used for human consumption.

2.3.3 Enteropathogenic *E. coli* (EPEC)

Enteropathogenic *E. coli* (EPEC) strains are the oldest recognized category of diarrheogenic *E. coli* (Zuber, 1999) and are well known prominent cause of diarrhea, particularly in children in less developed countries (Tennant *et al*., 2009; Trabulsi *et al*., 2002). EPEC are characterized in part by their ability to induce attaching-effacing (A/E) lesions which are comprised of bacteria attached to the intestinal mucosa at sites of cytoskeletal rearrangements leading to characteristic morphological changes (Tennant *et al*., 2009). Furthermore EPEC have inhibitory effect on macrophage phagocytosis and thereby escapes the mucosal immune system (Yosuke *et al*., 2007; Goosney *et al*., 1999), and these processes are mediated by the locus of enterocyte effacement (LEE) pathogenicity island (Yosuke *et al*., 2007). LEE encodes transcriptional regulators, components of a type III secretion system (TTSS), effectors that are injected by TTSS into infected cells, chaperones for these effectors, and the intimin outer membrane protein (Yosuke *et al*., 2007) .

EPEC is divided into typical and atypical subtypes, with typical EPEC (tEPEC) strains carrying a ca. 90-kb adherence factor plasmid (pEAF) that encodes type IV-like bundle-forming pili (BFP) (Yosuke *et al*., 2007; Tobe *et al*., 1999). The latter (atypical EPEC (aEPEC)) serves as a facilitator in the adherence of bacteria to the intestinal mucosa and to each other, thus allowing them to form micro-colonies on epithelial cells in vitro and in vivo (Yosuke *et al*., 2007; Cleary *et al*., 2004; Tobe and Sasakawa, 2002). Trabulsi *et al*., 2002 made an interesting observation that there is evidence that a subset of EPEC strains known as atypical EPEC (aEPEC), which lack pEAF and BFP are also pathogenic. This evidence came after studies carried out with adult volunteers have demonstrated that intimin, pEAF and BFP are essential virulence determinants of EPEC (Bieber *et al*., 1998; Donnenberg *et al*., 1993; Levine *et al*., 1985).

2.3.4 Enteroinvasive *E. coli* (EIEC)

Enteroinvasive *E. coli* (EIEC) was first shown in 1971 to be one of the etiological agents of diarrheal diseases in otherwise healthy volunteers in a study carried out by DunPont et al.(1971). EIEC causes shigellosis-like symptoms in both adults and children, dysentery, however is the less widely reported then other etiological agents in studies of diarrhea worldwide (Vieira *et al*., 2007). Vieira *et al*., 2007 have observed that despite the acknowledgement status of EIEC as a human pathogen, very little research has been conducted to identify individual risk factors for infection, possible reservoirs, and infectious rates.

EIEC are highly invasive and utilize adhesin proteins to bind to and enter intestinal cells, hence the name enteroinvasives. EIEC resembles a genetic hybrid between *E. coli* and *Shigella*,

carry plasmids with extensive homology to the virulence plasmid of *Shigella* and cause a diarrheal disease that is clinically similar to dysentery caused by *Shigella* (Maurelli *et al*., 1998).

2.3.5 Enteroaggregative *E. coli* (EAEC)

Enteroaggregative *E. coli* (EAEC) surfaced in 1985 and is recognized by its distinctive adherence to HEp-2 cells in an aggregative, stacked brick-like pattern (Harrington *et al*., 2006). It causes both acute and persistent diarrhea among children, adults and HIV positive persons, in both developing and developed countries (Huang *et al*., 2006). EAEC is the second most common cause of traveler's diarrhea, and is a common cause of acute diarrheal illness in children and adults (4.5%). The basic strategy of EAEC comprise colonization of the intestinal mucosa, probably predominantly that of the colon, followed by secretion of enterotoxins and cytotoxins (Weintraub, 2007). The USA National Institutes of Health has categorized EAEC as a category B potential bioterrorism agent (Kaur *et al*., 2010). A study from Brazil identified EAEC infection as the most common cause of diarrhea in small children, and was found to be more frequently associated with diarrhea in children less than 2 years of age (Araujo *et al*., 2007; Kaur *et al*., 2010).

EAEC pathogenesis involves three stages: (1) adherence to the intestinal mucosa by aggregative adherence fimbriae (AAF) and adherence factors, (2) increased production of mucus that encrusts EAEC on the surface of enterocytes; and (3) release of toxins and elicitation of an inflammatory response, mucosal toxicity, and intestinal secretion (Kaur *et al*., 2010; Nataro, 2005). The most commonly reported symptoms of EAEC infections are watery diarrhea with or without blood and mucus, abdominal pain, nausea, vomiting, and low-grade fever (Kaur *et al*.,

2010; Adachi *et al*., 2002), and these clinical manifestation vary from individual to individual, depending upon genetic composition of the host (Jiang *et al*., 2003).

2.3.6 Diffusely adherent *E. coli* (DAEC)

Diffusely adherent *Escherichia coli* strains are identified by their diffuse adherence (DA) pattern, in which the bacteria uniformly cover the entire cell surface (Scaletsky *et al*., 2002), on cultured epithelial cells in vitro and have been recognized as the sixth class of diarrheagenic *E. coli* (Lopes *et al*., 2005). DAEC is most commonly associated with age-dependent diarrhea and in children less than 12 months of age (Scaletsky *et al*., 2002), however most case-control studies have demonstrated that the association of DAEC as a diarrhea causing agent remains controversial. DAEC is believed to comprise a heterogeneous group of organisms of variable enetropathogenicity (Arikawa *et al*., 2005).

DAEC strains can be classified into two large groups of Afa/Dr and non-Afa/Dr DAEC before considering their etiological role (Meraz *et al*., 2008). The DAEC strains possess afimbrial adhesions called afimbrial adhesive sheaths (Afas) that are encoded by *afa* gene clusters which consists of a*faA, afaB, afaC, afaD* and afaE genes (Hussain *et al*., 2010). It has been found that DAEC strains possessing *afa / dr* adhesins may not only be adherent but invasive to epithelial cells and capable of inducing an inflammatory response (Meraz *et al*., 2008; Arikawa *et al*., 2005; Betis *et al*., 2003; Meraz *et al*., 2006).

There are a limited number of studies regarding the virulence and pathogenic mechanisms of DAEC. It is presumed that the virulence factor of the organism is diffuse

attachment to the intestinal lining of the infected host (Sarkar, 2008). In a study carried out by Meraz et al., 2008, the major clinical symptoms of the patients carrying DAEC strains were found to include watery diarrhea in some instances with mucus and blood, vomiting, with vomiting being more prominent as compared to diarrhea among children.

2.4 Enteric Viruses (EVs)

The enteric viral pathogens commonly found in human feces include noroviruses, enteroviruses, adenoviruses, hepatitis A virus (HAV), hepatitis E virus (HEV), rotaviruses and astroviruses (Greening, 2007). There are three main types of diseases caused by enteric viruses; gastroenteritis, enterically transmitted hepatitis and illnesses that can affect other parts of the body such as the eye, the respiratory system, and the central nervous system. Enteric viruses are obligate pathogens and require living cells to multiply, thus they are inert particles in the environment, they do not replicate or metabolize. They are thought to be related to endemic disease, with sporadic, undetected outbreaks of infection (Black *et al*., 2007).

While most enteric virus infections may be non-symptomatic or result in mild illness, some of the illnesses caused can be more serious and include myocarditis, infectious hepatitis, paralysis, meningitis, and severe gastroenteritis (Black *et al*., 2007). Relatively little is known about many of the viruses in this group because many enteric viruses are difficult to culture or are not cultureable and produce diseases that are not readily identifiable. Enteric viruses are stable in the environment and are resistant to chlorine and ultraviolet (UV) disinfection (Payment *et al*., 1985; Batigelli *et al*., 1993; Vivier *et al*., 2004). In the light of recent epidemiological evidence it has been indicated that enteric viruses, in particular noroviruses (NV), which cause

acute gastroenteritis, but also hepatitis A virus (HAV) and rotavirus (RV), are the leading cause of food-borne illness in developed countries (Butot *et al*., 2007; Fleet *et al*., 2000; Koopmans and Duizer, 2004; Widdowson *et al*., 2005).

2.4.1 Enteroviruses

Human enteroviruses are members of the family *Picornaviridae*, which consist of nonenveloped virus particles containing a 7,500-nucleotide single-stranded positive sense RNA genome protected by an icosahedral capsid (Okoh *et al*., 2010; Nasri *et al*., 2007). Enteroviruses consist of poliovirus, coxsackieviruses, echoviruses, and the numbered enteroviruses. As of 2003, 89 serotypes of enteroviruses have been identified and ratified by the Executive Committee of the International Committee on Taxonomy of Viruses (Fong and Lipp, 2005). Enteroviruses are positive sense single-stranded RNA viruses, meaning that they can act as messenger RNA (mRNA), with an icosahedral capsid ranging from 20 to 30 nm in diameter and can cause a wide spectrum of diseases in humans and animals (Fong and Lipp, 2005). About 70% (62 serotypes) of nonpoliovirus enteroviruses have been associated with human infections, and 30% have been associated with animal infections. Enteroviral infections in humans are reported to peak in summer and early fall, which also coincides with increased water recreational activities and water contact.

Enteroviruses are characterized by their stability, both in the gastrointestinal tract and in the environment, and thus are excreted in feces in large amounts and persist in the environment for long times (Jiménez-Clavero et al., 2005). They are one of the best-studied groups of enteric viruses belonging to the family *Picornaviridae* and have been recognized as indicators of fecal

contamination (Jiménez-Clavero et al., 2005). EV bearers are sewage, sewage sediments, rivers receiving sewage (Kocwa-Haluch, 2001a; Berg, 1967; Carducci, 1995; Green and Lewis, 1999; Kocwa-Haluch, 2001b; Melnick *et al*., 1958), as well as treated sewage (Grinstein *et al*., 1970; Irving and Smith, 1981; Lund *et al*., 1969).

Enteroviruses can be transmitted by the fecal–oral route from any surface or ground water such as estuarine water, seawater, rivers, springs and drinking water that directly or indirectly receives treated or untreated wastewater (Chen *et al*., 2007; Yates *et al*., 1985; Sobsey *et al*., 1986; Rose *et al*., 1987; Pallanch and Ross, 2001; Vivier *et al*., 2004; Fong and Lipp 2005). Enteroviruses have been implicated in recent waterborne gastroenteritis outbreaks in many countries (Pianetti *et al*., 2000; Griffin *et al*., 2003; Lee *et al*., 2005a,b), and human enteroviruses have been included in the European Union regulations governing water quality, as a parameter for evaluating the degree of viral pollution of a water body (Kocwa-Haluch 2001b).

Polioviruses are not associated with waterborne transmission; however they are typically transmitted via faecal-oral route. Thus raising 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). On the other hand, echoviruses are comprised of 33 serotypes with serotypes 22 and 23 being considered to be reclassified as a distinct picornaviral genus because of the substantial genetic divergence from the other viruses in the group (Lutwick and Hussain, 2006).

Water virology is therefore an important aspect of water quality standards and affects public health in many ways. The presence of enteric viruses in water, apart from causing diseases, negatively and indirectly affects a country's socio-economic status. Thus the detection,

identification and monitoring of pathogenic viruses from wastewater final effluents, raw source water and drinking water become imperative.

2.4.2 Noroviruses (NVs)

Noroviruses are members of the family Caliciviridae that contain a single-stranded positive sense RNA genome of approximately 7.7 kb which is organized into three open reading frames (ORFs) (Carter, 2005). They are a major cause of acute viral gastroenteritis that impact on people of all ages worldwide (Okoh *et al*., 2010; Hutson *et al*., 2004) with either seasonal or sporadic outbreaks that occur throughout the year (Maguire *et al*., 1999) in semi closed settings such as schools, families, elderly people's homes, hospitals, hotels and cruise ships (Ike *et al*., 2006). They are estimated to cause up to 66% of all food-borne illnesses attributable to known causes in the United States (Koopmans *et al*., 2000; Koopmans *et al*., 2001; Allwood *et al*., 2003). NV outbreaks are commonly associated with the consumption of fresh produce and other ready-to-eat foods contaminated by infected food workers or shellfish harvested from waters contaminated by sewage (Allwood *et al*., 2003; Frankhauser *et al*., 2002; Glass *et al*., 2001).

2.4.3 Adenoviruses

Adenoviruse belongs to the family *Adenoviridae*, with a double-stranded DNA genome and is portrayed as a possible candidate as an index virus for human viral pollution (Choi and Jiang, 2005). They display a great deal of resistance to UV treatments and this resistance is attributable to the double stranded DNA genome that allows for repair of damaged DNA by

using the host cell enzymes (Choi and Jiang, 2005; Kallenbach *et al*., 1989; Harm, 1980). Adenovirus is frequently found in urban rivers and polluted coastal waters (Choi and Jiang, 2005; Jiang *et al*., 2005), however the diversity of human adenovirus serotypes in the aquatic environment is not yet known (Choi and Jiang, 2005). He and Jiang 2005, demonstrated that approximately 0.1% of 10^5 adenovirus genomes per liter in primary treated sewage were infectious by plaque assay of HEK-293A tissue culture.

There are about 8 serotypes of adenoviruses and may cause respiratory diseases, conjunctivitis and gastroenteritis. Adenoviruses are divided into subgroups A-F, with members of the A-E subgroups causing respiratory infections and members of the F sub-group causing enteric infections. Infections are common and affect primarily children with symptoms and epidemiological patterns varying between subgroups and even for different species and are endemic throughout the world (Fong *et al*., 2010). It has been observed that human adenoviruses (HAdVs) are present at a higher frequency in sewage than any other enteric virus (54) and that are excreted in high concentrations from infected patients (up to 10^{11} viral particles per gram of feces (Fong *et al*., 2010).

2.4.4 Rotaviruses

Rotaviruses are small (75-nm in diameter), round, unenveloped viruses with a three layer protein capsid. The genome is composed of 11 double stranded RNA segments and surrounded by the inner capsid proteins VP1, VP2, and VP3 (Gutièrrez-Aguirre *et al*., 2008). On the basis of antigenic characteristics of the VP6 protein, a protein that surround the inner capsid, six serogroups (A to G) of rotaviruses are distinguished (Gutièrrez-Aguirre *et al*., 2008). Group A

rotaviruses are the most important causes of acute diarrhea in young children worldwide. They are estimated to cause about 500,000 to 600,000 yearly deaths worldwide among children under the age of 5 years (Gutièrrez-Aguirre *et al*., 2008). They are also important pathogens in some animals (Estes, 2001) and this brought about a postulation that animals are a potential source of newly emerging genotypes in humans (Cook *et al*., 2004). Severe rotavirus disease is preventable by vaccination (Cunliffe *et al*., 2001); however this can be negatively impacted by the possibility of inter-species transmission (Cooney *et al*., 2001).

2.5 Hepatitis A and E Viruses

Hepatitis is a collective term for several diseases of the liver caused by viruses, five of which have been reasonably characterized to come from a wide range of families (Hunt and Saab 2009). Although hepatitis A and E are non enteric viruses, they are nonetheless of important public health concern and are thus worth monitoring in environmental water samples. Hepatitis A virus (HAV) is a 7.5 kilo base (kb) positive sense single-stranded RNA virus, surrounded by a naked icosahedral capsid that is 28 nm in diameter, belonging to the family *Picornaviridae* (Ching *et al*., 2002; Melnick, 1982; Gust *et al*., 1983) and has been designated a member of the genus *Hepatovirus* (Ching *et al*., 2002; Minor, 1991). Current studies report that there is only one serotype of HAV (Hunt and Saab, 2009). Hepatitis A virus is a significant cause of morbidity, life-threatening and attendant socio-economic losses in many parts of the world especially in developing countries (Kittigul *et al*., 2000).

Hepatitis E virus is round, icosahedral, non enveloped positive strand RNA virus which is approximately 34 nm in diameter, causes non-A, non-B hepatitis with a genomic sequence that

is more similar to rubella which is a Togavirus (Hunt and Saab, 2009). Its genome consists of three overlapping open reading frames (ORF), each in a different coding frame, with ORF1 involved in enzymatic activity and ORF2 serving structural functions (Hunt and Saab, 2009). Particularly, these two viruses have been implicated in outbreaks of waterborne diseases (Gilgen *et al*., 1997).

2.5.1 Epidemiological Background

In Sierra Leone, Africa, during the spring and summer seasons of 1988, two young female missionaries associated with a rural hospital, reportedly developed a fulminant hepatitis A disease (Ching *et al*., 2002). In an investigative study carried out by Ching et al. (2002), they have deduced that one of the characteristics of HAV that facilitates in its transmission is its extreme resistance to environmental conditions and that it remains infectious in the dried state for 1-2 months (Abad *et al*., 1994). The HAV disease is hyper-endemic in regions such as the Amazon basin in South America, most of Africa, the Middle East and Central Asia, and the Indian subcontinent, with the majority of infections occurring during childhood (Ching *et al*., 2002). In the United States, hepatitis A accounts for approximately 20% of cases of viral hepatitis reported annually to the Centers for Disease Control and Prevention. In young children HAV infection is usually asymptomatic whereas symptomatic disease occurs more commonly among adults (WHO, 1999). Humans are the only host, and hence the only source, of HAV (Kumar *et al*., 2010).

2.5.2 Pathogenesis

Viral hepatitis is caused mainly by infection with one of the five hepatitis viruses (designated as A to E), which use the liver as their primary site of replication (Kumar *et al*., 2010). Hepatitis A infects the small intestines where it presumably replicates and reaches the liver through portal circulation. While in the liver, the virus uses hepatocytes as their major replication site. After entering the cell, the entire open reading frame (ORF) on the genomic RNA is translated into protein, which is subsequently processed into 11 proteins through the actions of 3C protease and unidentified cellular proteases (Kumar *et al*., 2010). When the entire replication process is complete the newly formed virions are secreted across the apical surface of hepatocytes into the liver sinusoids and bile canaliculi from where they enter small intestine and are excreted in faeces (Kumar *et al*., 2010).

2.5.3 Clinical symptoms and manifestations

Hepatitis is a collective term for several diseases of the liver caused by viruses and can be categorized into two types, namely acute and chronic hepatitis. Hepatitis viruses cause similar clinical manifestations during the acute phase of infection but vary in their ability to cause chronic infection (Kumar *et al*., 2010). Hepatitis A and E are the main culprits in acute hepatitis, which is a common viral infection, found throughout the world and is spread principally via the fecal/oral route. It is characterized by atypical manifestations such as relapse, cholestasis, rash, and arthralgia also have been described in patients with hepatitis A, but the pathophysiology of these phenomena has not been elucidated (Tong *et al*., 1995). Tong *et al*., 1995 reported that 59 subjects under their study showed signs of hepatitis A symptoms including dark urine (81%),

fatigue (80%), gastrointestinal problems including anorexia, nausea and abdominal pain (75% to 41%), and fever (58%). They also reported less common complaints such as myalgia (32%), pruritus (29%), chills (27%), diarrhea (24%), headache (22%), arthralgia (19%), sore throat (7%), and rash (7%). The average incubation period is 28 to 30 days but can range from 15 to 50 days. The symptoms last for several weeks on average and usually not longer than 2 months

Chronic viral hepatitis is frequently hidden due to the asymptomatic nature of liver disease in a large proportion of people and the slowness of progression to advanced liver disease. Most cases of chronic viral hepatitis are caused by hepatitis B, C or D virus (Ryder and Beckingham, 2001). It is the principal cause of chronic liver disease, cirrhosis, and hepatocellular carcinoma in the world and now ranks as the chief reason for liver transplantation in adults (Hoofnagle and Bisceglie, 1997). The clinical presentation of hepatitis E is comparable to hepatitis A.

2.5.4 Clinical diagnosis

A number of very sensitive and specific serologic tests for viral hepatitis are commercially available, but the relative inconvenience of obtaining blood samples and the risk of disease transmission associated with needle stick injuries makes serologic testing somewhat unattractive (Thieme *et al*., 1992). To compensate for the disadvantages of serological tests, several studies have used saliva which has proved to be a veritable source of antibodies for the diagnostic testing of HAV and HVB (Bull *et al*., 1989; Parry *et al*., 1989). Also, a study by Thieme *et al*., 1992 has demonstrated the presence of anti-HAV IgM in oral samples from 51 subjects with recent HAV infection.

2.5.5 Treatment, prevention and control

Persons infected with hepatitis A do not need any specific treatment because the disease is self-limiting and it confers strong life-long protection (Kumar *et al*., 2010). Management and control is mainly supportive and hospitalization is recommended only to patients with another serious medical problem (Kumar *et al*., 2010). Central to hepatitis A prevention are improved hygiene safe water supply and adequate disposal of human excreta. There are two different vaccines currently available against hepatitis A - Havrix^{TM} (Glaxo Smith-Kline) and VAQTA^{TM} (Merck) that have been proven to be highly immunogenic and safe.

2.6 Coxsackieviruses

Coxsackie viruses are divided into two main groups, coxsackievirus A (CVA) and B (CVB). They are composed of four structural proteins (VP1-VP4) with VP1-3 having no sequence homology but same topology (Maghsoudi *et al*., 2007). With group A having 23 serotypes and group B, with 6 serotypes based on their pathogenicity in mice. Group A Coxsackieviruses cause a flaccid paralysis, while group B causes a spastic paralysis (Palacios and Oberst, 2005). Coxsakievirus B (CVB) consists of six serotypes with coxsackievirus B3 (CVB3) being considered as the most important infectious agent involved in viral heart muscle disease and may induce acute and chronic forms of human myocarditis, which can be a life-threatening disease with adverse acute and long-term clinical outcomes (Maghsoudi *et al*., 2007). Coxsakievirus A is the common cause of hand, foot and mouth (HFM) disease in infants and children which is characterized by fever and vesicles in the mouth and on distal extremities (Frydenberg and Starr, 2003).

Coxsakieviruses pathogenicity is the same as poliovirus and targets the maninges and occasionally the cerebrum, causes about 60% subclinical aseptic meningitis and encephalitis with coxsakie A and B viruses mostly associated with muscle weakness and paralysis and coxsakievirus B3, in particular, has been linked to chronic dilated cardiomyopathy, a common cause of progressive heart failure and sudden death (Liu *et al*., 2000).

2.6.1 Epidemiology

Enterovirus infections generally go unnoticed, or symptoms are 'flu'-like and therefore are not recognized (Mulders *et al*., 2000). Coxsackieviruses B are in particular, been associated with juvenile type 1 diabetes (Mulders *et al*., 2000). It is also known that coxsackievirus B4 and B5 infections are wide spread in humans and are readily detected from the environment (Mulders *et al*., 2000). They have been encountered throughout the world, especially in infants under 15 days old in Netherlands, and are frequently recovered during summer and early fall (Verboon-Maciolek *et al*., 2002).

2.6.2 Pathogenesis

Like all enteroviruses, the pathogenicity of coxsackievirus infections, through oral ingestion of the virus (Daley *et al*., 2005), is mediated by an arginine-glycine-aspartic acid (RGD) motif found on the viral capsid proteins of the *picornavirus* family. Among the seven distinct receptors for different enteroviruses identified from human cells, the one specific for

coxsackievirus is coxsackievirus-adenovirus receptor (CAR) (Okoh *et al.*, 2010; Palacios and Oberst, 2005).

The primary site of infection is the epithelial cells of the respiratory or gastrointestinal tract, from there, the viruses may spread to secondary sites particularly following viremia. As viremia spreads, the infection of dendritic cells and macrophages can aid the transport of the viruses across the blood-brain barrier or transport along neural pathways to infect brain cells (Daley *et al.*, 2005). Secondary infection of the central nervous system results in aseptic meningitis or, rarely, encephalitis or paralysis (Okoh *et al.*, 2010). In general, group A coxsackieviruses tend to infect the skin and mucous membranes, causing herpangina, acute hemorrhagic conjunctivitis (AHC), and hand-foot-and-mouth (HFM) disease. Group B coxsackieviruses tend to infect the heart, pleura, pancreas, and liver, causing pleurodynia, myocarditis, pericarditis, and hepatitis.

2.6.3 Clinical symptoms and manifestations

Most enterovirus infections are asymptomatic or result in only mild illnesses, such as non-specific febrile illness or mild upper respiratory tract infections. However, enteroviruses can also cause a wide variety of clinical illnesses including acute haemorrhagic conjunctivitis, aseptic meningitis, undifferentiated rash, acute flaccid paralysis, myocarditis and neonatal sepsis-like disease (Okoh *et al.*, 2010; Palacios and Oberst, 2005). Most infections result in tissue specific cell destruction, although some disease manifestations can be a result of host immune response. In an investigative study carried out by Verboon-Maciolek et al. (2002), they reported

that about 87% of infants developed fever and 21% had diarrhea after coxsackievirus B infection.

2.6.4 Clinical diagnosis

Diagnosis is made by viral isolation from stool, nasopharyngeal swab, cerebrospinal fluid, and blood. Verboon-Maciolek et al. (2002), performed clinical diagnosis of sepsis in 35% infants and diagnosed meningitis in 24% of the total investigated cases.

2.6.5 Treatment, prevention and control

Coxsackievirus infections are usually self-limiting and do not require hospitalization, as a result do not require any kind of treatment. Treatment is mainly supportive and that there is no existing Federation for Food and Drug Administration (FDA) approved therapy for the treatment of enteroviral infections (Rotbart and Webster, 2001). Control of coxsackievirus infection may be achieved by patient and general public education on good hygiene practices to avoid transmission. Padalko et al. (2004) explored the role of peroxynitrite in the host immune response to coxsackievirus infection and have found that it inhibits viral replication in vitro, in part by inhibiting viral RNA entry into the host cell and particularly by inhibiting S-nitrosylation of viral cysteine proteases (Zaragoza *et al*., 1997). This agent is known to be harmful to the host at high concentrations, however in low concentrations may be beneficial to the host during pathogen infection (Padalko *et al.*, 2004).

2.7 Detection Methods of Viruses in Environmental Water Samples

The ability to measure the quantity of enteric viruses in a water sample is an important factor in determining the degree of pollution, the effectiveness of virus removal or disinfection procedures, and the safety of food products which may become intrinsically contaminated during growth or contaminated during handling or processing, as is seen with shellfish or salad crops (Laverick *et al*., 2004). Due to low concentration of viral particles in water samples, the concentration of viruses is crucial prior to any detection method. The adsorption-elution from porous filters is the most promising technique and has been recommended as a standard method for virus concentration from water and wastewater samples (Kittigul *et al*., 2006; American Public Health Association (APHA), 1998).

Methods that can be used to detect enteric viruses in environmental samples generally fall into two categories: infectivity in cell culture, and molecular detection methods such as polymerase chain reaction (PCR) amplification followed by nucleic acid hybridization (Abbaszadegan *et al*., 1993; Griffin *et al*., 2003; Fuhrman *et al*., 2005; Chen *et al*., 2007). Cell culture method, the PCR method, immunological assay, oligonucleotide microarray have long since been employed to detect viruses from environmental samples.

2.7.1 Cell culture method

Cell culture method is theoretically capable of detecting a single viable virus in relatively large volumes of sample, but the major disadvantage of this method is that, the time required for

confirmed results with conventional cell culture makes it an impractical method for routine monitoring of environmental samples. Furthermore, cell culture does not detect noncytopathogenic viruses (viruses that are viable, infecting cells, and continually spreading to neighboring cells but that do not cause a visible cytopathogenic effect [CPE] on the cell monolayer). Rotavirus and most wild-type hepatitis A viruses (HAV) are infectious to cell cultures but do not produce a clear CPE (Reynolds, 2004). Cell culture of environmental samples is further complicated by the presence of organic and inorganic materials that are toxic to the cell (Reynolds *et al*., 1996).

2.7.2 Polymerase Chain Reaction (PCR) methods

PCR is a nucleic acid amplification technique that gives high sensitivity in the detection of viruses in water samples (Kittigul *et al*., 2006; Tsai *et al*., 1993). There are quite a number of PCR versions that have been developed to detect viruses from environmental samples and these include conventional reverse transcriptase (RT)-PCR, antigen-capture PCR, immune-magnetic capture PCR, integrated cell culture/PCR (ICC/qRT-PCR), semi-nested RT-PCR, nested RT-PCR, and real-time(q) RT-PCR.

The ICC/qRT-PCR is a proven method for the rapid detection of infective enteroviruses in environmental waters and it allows low virus concentration to be propagated to increase target nucleic acid (Balkin and Margolin, 2010). The advantage of this method is that it does not only detect viruses but also demonstrate its infectivity, which is vital for public health concern and risk management of viral infections. The disadvantage may be that it cannot be used for viruses that do not grow in monolayer cell cultures.

The real-time qRT-PCR is the PCR version that is mostly used for the detection and quantification of virus densities in environmental waters. It is highly sensitive and rapid thus it is useful in assessing human health risk associated with recreational use of environmental waters (Brooks *et al*., 2005).

CHAPTER 3

METHODOLOGY

3.1 Study Area

Eastern Cape Province is one of the poorest and second largest provinces in South Africa and mainly comprised of rural settlements with little or no adequate sanitary facilities. It is divided into six district municipalities, namely, Alfred Nzo, Amatole, Chris Hani, Ukhahlamba, O.R. Tambo and Cacadu, including the Nelson Mandela Metropolitan Municipality. The district municipalities are further sub-divided into several magisterial districts, with O.R. Tambo and Amatole being the most populated municipalities. The province has a very low Human Development Index (HDI) of 0.52 and high percentage of people living in poverty (67.4%) (Eastern Cape Department of Social Development, 2008). A map of the Eastern Cape Province is as shown in Figure 3.1.

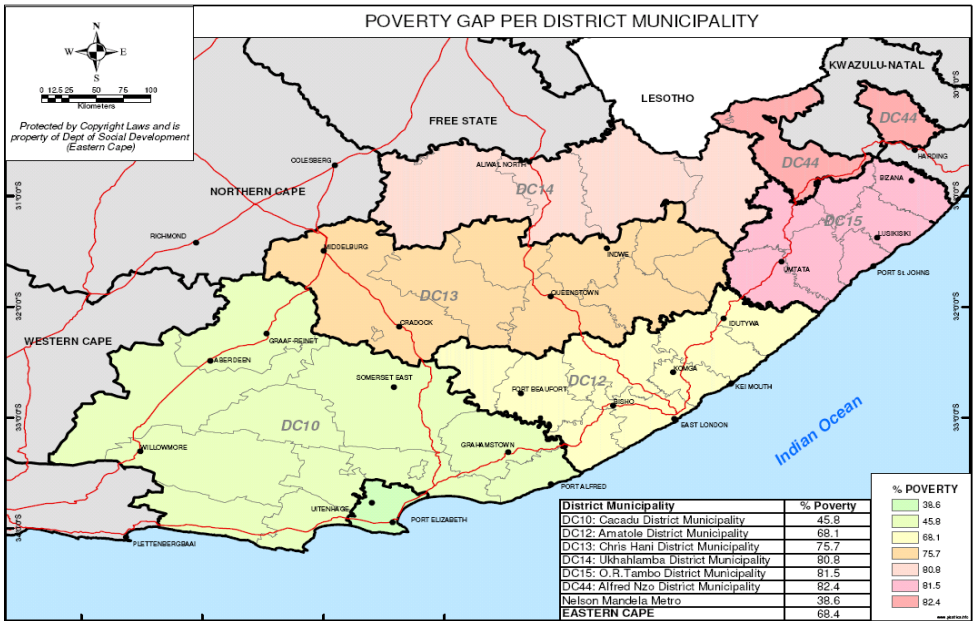

Figure 3.1: The Eastern Cape Province (Eastern Cape Department of Social Development, 2008).

The Amatole district is situated along the coastline of the south-eastern part of the Eastern Cape and incorporates the former magisterial districts of Willowvale, Idutywa and Elliotdale (Mbashe); Butterworth, Nqamakwe and Centani (Mnquma); Komga (Great Kei); Keiskammahoek, Catcart and Stutterheim (Amahlati); East London, King William's Town, Zwelitsha and Mdantsane (Buffalo City); Peddie and Hamburg (Ngqushwa); Fort Beaufort, Alice, Middledrift and Seymour (Nkonkobe) and Bedford and Adelaide (Nxuba).

Buffalo City municipality has a population of approximately 1 million people and is highly industrialized with a rather adequate wastewater and water treatment infrastructure.

3.2 Sampling sites description

Two wastewater treatment plants were selected to represent typical peri-urban (Dimbaza Sewage Treatment Works, Dimbaza, King William's Town) and rural (Alice Sewage Treatment Works, Alice) settlements of the province and these discharge their final effluents into the Tembisa River, and the Tyume River, respectively. From each of the two sampling locations, two liter wastewater samples were collected from the final effluent (FE).

The Alice Sewage Treatment Works is situated at geographical coordinates of 32^{o}50' 36'' S, 26^{o}55' 00'' E and approximately 1 km East of Alice town, has a design capacity of 2000 m3/day receives domestic sewage, some light industrial wastewater, as well as run-off water (Igbinosa and Okoh, 2009). Its treatment is based on the activated sludge system and the treated

final effluent is discharged into the Tyume River. Some communities downstream the river use the water for irrigation and other domestic purposes.

The Dimbaza Sewage Treatment Works is situated within the geographic coordinates 32°51'274"S and 27°14'167"E, receives municipal domestic sewage and wastewater containing a heavy industrial contribution (Osode and Okoh, 2009). The plant has two aeration tanks, each equipped with three vertically mounted mechanical aerators two anaerobic tanks and two sedimentation tanks. The plant has a waste mixed liquor pump station which pumps the waste mixed liquor from the aeration tank to two sludge lagoons (Osode and Okoh, 2009). The plant is designed to treat an average flow of 7 000 m^3/day and an average wet weather flow of 21 000 m^3/day.

Basically both of these wastewater treatment plants apply the activated sludge treatment system with, of course, the Alice plant being smaller in carrying capacity compared to the Dimbaza Plant.

3.3 Sample Collection

Wastewater samples were collected once monthly from August to December 2010 from the final effluent (FE) of each of the two wastewater treatment plants. Two liter samples were collected from each point by grab sample method, from which 500 ml was used for viral concentration and detection and the remaining 1500 ml for physicochemical analysis. For the final effluent samples, 1ml of 0.1 M sodium thiosulphate was added in each 2 L sterile strew cap plastic bottle prior to sampling and transported to the laboratory on ice for processing.

3.4 Physicochemical analysis

The pH, temperature, conductivity, total dissolved solids (TDS), salinity, dissolved oxygen were determined *on site* using a multi-parameter ion specific meter (Hanna-BDH laboratory supplies). The chlorine residual and turbidity were measured on site using a free and total ion specific meter (Hanna-BDH laboratory supplies), and a microprocessor turbidity meter (HACH company, model 2100P), respectively. Conductivity will be determined using a conductivity meter (CRISON CM35, Crison instrument). On arrival at the laboratory, the concentration of orthophosphate as phosphorus (P) and total nitrogen (nitrate and nitrite as N) will be determined by the standard photometric method (DWAF, 1992) using a Spectroquant NOVA 60 photometer (Merck Pty Ltd). Dissolved oxygen (DO) will be measured with Merck DO meter, Model Ox 330 (Merck Pty Ltd). Biological oxygen demand (BOD) will be determined using the Oxitop WTW BOD meter (Merck Pty Ltd) and a 5 day incubation period.

3.4.1 Biological oxygen demand analysis

Biological oxygen demand was measured using HACH (model HQ40d) following the manufacture's protocol. Briefly, about 300 ml of sample is measured into the BOD bottle, filled up to the top to prevent any air bubbles that might be trapped inside the cup. Then the BOD was measured for all the samples followed by a five day incubation period in the dark at room temperature. After five days BOD was measured and this measurement was subtracted from the reading before incubation for each sample. The blank was treated the same way, except that deionized water was used in place of sample.

3.4.2 Chemical oxygen demand analysis

Samples for chemical oxygen demand (COD) were digested with a Thermo reactor Model TR 300 (Merck Pty Ltd). Method 014 (14) with concentration range of 10 to 150 mg/L was used for the determination of COD. Briefly, 300 µl of solution A and 2850 µl of solution B were added to a clean empty COD reaction tube and mixed thoroughly. Then 3000 µl of sample was added to the tube and the contents were mixed. The reaction tube was then placed in a thermo reactor and digested at 148 ^{o}C for two hours. The blank was prepared in the same way but 3000 µl of deionized water was added instead of the sample. The blank was read first followed by the samples, using a Spectroquant NOVA 60 photometer (Merck Pty Ltd). The blank measurement was recorded, and subsequently subtracted from the measurements of the samples in order to get the actual COD measurement of each sample.

3.4.3 Determination of nitrates

Method number 14773 (NO_3^-)' measuring range of 0.5 to 20.00 NO_3-N mg/L of the NOVA 60 photometer was used to measure nitrate concentration in all the samples. Briefly, one level spoonful of reagent NO_3^{-1} was placed into a dry test tube followed by addition of 5 ml reagent NO_3^{-2} and shaken vigorously until reagent NO_3^{-1} was completely dissolved. Then 1.5 ml of sample was slowly and carefully added onto the reagent mixture with vigorous shaking, and allowed to stand for ten minutes. The sample was filled into a cuvette and measured using a Spectroquant NOVA 60 photometer (Merck Pty Ltd) in accordance with the manufacturer's protocol.

3.4.4 Determination of nitrites

Method number 14776 (NO_2^-), measuring range of 0.02 to 1.00 NO_2-N mg/L of the NOVA 60 photometer was used to measure nitrite concentration in all the samples. This method is analogous to EPA 354.1, US Standard Methods 4500-NO_2-B, and EN 26 777. Briefly, 5 ml sample was measured into a test tube followed by the addition of 1 level spoonful of reagent NO_2^{-1} and mixed vigorously until reagent was completely dissolved. The sample was allowed reaction time of 10 minutes, then filled into a cuvette and measured using a Spectroquant NOVA 60 photometer (Merck Pty Ltd) in accordance with the manufacturer's protocol.

3.4.5 Determination of phosphates

Method number 14848 P, measuring range of 0.05 to 5.00 mg/L was used to measure phosphate concentration in all the samples. This method is analogous to EPA 365.2+3, US Standard Methods 4500-PE, ISO 6878/1, and EN 1189. Briefly, five milliliters of sample was pipetted into a clean test tube followed by the addition of five drops of reagent PO_4^{-1} and thorough mixing. Thereafter, one level spoonful (provided with the bottle) of reagent PO_4^{-2} was added followed by vigorous shaking until the reagent were completely dissolved. The reaction mixture was left to stand for five minutes. The sample was then filled into a cuvette and the concentration of phosphate measured using a Spectroquant NOVA 60 photometer (Merck Pty Ltd) in accordance with the manufacturer's protocol.

3.5 Microbiological analysis

The microbiological wastewater quality was assessed in terms measuring indicator organisms such as total coliforms, feacal coliforms and enterococci. Each sample was diluted tenfold and 100 µl of the 10^{-1} and 10^{-2} (and in some exceptional cases 10^{0}) dilution were inoculated onto mFC agar (biolab, Merck) for feacal coliforms; m-Endo Agar Les (m-Endo) (biolab, Merck) for total coliforms; and on Enterococcus Selective Agar (ESA) (biolab, Merck) for enterococci. Each dilution was inoculated in duplicates. The inoculums were then spread on the surfaces of the agars to dryness using sterile glass spreaders. Thereafter, the plates for enterococci and total coliforms were incubated in an inverted position at 37 ± 2^{o}C for 24 hours, and the plates for feacal coliforms were incubated at 44 ± 2 ^{o}C for 24 hours. The plates were taken out of the incubator and colonies that shown metallic sheen on m-Endo were counted as presumptive total coliforms on the other hand those that appeared as white colonies with a blackening circumference on ESA agar were counted as presumptive enterococci; and those that shown blue color on mFC were counted as feacal coliforms. Counts were recorded as colony forming units (cfu)/ml per 100 ml of wastewater sample.

3.5.1 Isolation and presumptive identification of *E.coli*

The presumptive distinct blue colonies that grew on mFC agar were aseptically transferred to Chromocult Coliform Agar (CCA) (Merck) for presumptive identification of *E. coli* isolates. About ten distinct blue colonies were taken from each mFC agar plate and this was done only for the final effluent samples. When cultured on Chromocult agar, *E. coli* exhibits a dark-blue to violet color. To ensure purity the isolates were subcultured three consecutive times on CCA and

once on nutrient agar (NA) (biolab, Merck). A loopful of the isolate from NA was then aseptically inoculated into nutrient broth (NB) (biolab, Merck), incubated with vigorous shaking at 37 ^{o}C for 6 hours and preserved in 20% sterile glycerol at -80^{o}C. Nutrient agar slants of the isolates were also prepared and refrigerated for routine use.

3.5.2 Confirmative identification of E. coli isolates

The Analytical Profile Index (API) 20E (BioMerieux-API products) system was used in this study for the confirmation of *E. coli* isolates. API 20E system is used for the identification of anterobacteriaceae and its incubation period is 24 hrs. Young (12-24 hrs) cultures were transferred into test tubes containing 5 ml of sterile normal saline (8.5%) and adjusted to 1.0 McFarland standard solution. The API 20E strips were inoculated with the standardized normal saline suspension of each *E. coli* isolate and incubated at 37^{o}C for 24 hrs. Oxidase supplementary test, as suggested by the manufacturer was performed on commercially available oxidase strips using 12-24 hr cultures. The results were analyzed by APIweb^{TM} standalone version 1.2.1(BioMerieux, 2006/04).

3.5.3 Antimicrobial susceptibility test

Antimicrobial susceptibility testing was done on Mueller-Hinton agar (MH) (Merck biolab, Gauteng) by the standard disc diffusion method recommended by the Clinical and Laboratory Standards Institute (CLSI, 2006). Fresh cultures (about 22 hrs old) were transferred into test tubes containing 5 ml sterile normal saline. The turbidity of the suspension was adjusted to be to

0.5 McFarland standards. Sterile swabs were then deepen into the bacterial suspensions and used to inoculate the MH agar plates by spreading uniformly on the surface of the agar, after which the antibiotic discs were impregnated on the bacterial lawn and the plates were incubated at 35 ± 2^{o}C for 18 to 24 hrs. The antibiotics used in this study are shown in Table 3.1 below. A total of 24 isolates, was used for antimicrobial susceptibility testing against the 15 test antibiotics used. After incubation the plates were examined for zones of inhibition which were then measured and interpreted using the minimal inhibitory concentration (MIC) breakpoints for Enterobacteriaceae (CLSI, 2006) Table 3.1.

Table 3.1: Zone Diameter Interpretative Standards and Equivalent Minimal Inhibitory Concentration (MIC) Breakpoints for Enterobacteriaceae. Source: (CLSI/NCCLS, 2006)

Report/Test Group	Antimicrobial Agent	Disc content	Zone Diameter nearest whole (mm)			Equivalent MIC Breakpoints (µg/ml)	
			R	I	S	R	S
O	Streptomycin (S)	10 µg	≤ 11	12-14	≥ 15	-	-
B	Ciprofloxacin (CIP)	5 µg	≤ 15	16-20	≥ 21	≥ 4	≤ 1
A	Ampicillin (AP)	25 µg	≤ 13	14-16	≥ 17	≥ 32	≤ 8
	Erythromycin (E)	15 µg	≤ 13	14-22	≥ 23	≤ 8	≤ 0.5
C	Chloramphenicol (C)	30 µg	≤ 12	13-17	≥ 18	≥ 32	≤ 8

B	Meropenem (MEM)	10 µg	≤ 13	14-15	≥ 16	≥ 16	≤ 4
B	Cefuroxime (CXM)	30 µg	≤ 14	15-17	≥ 18	≥ 32	≤ 8
U	Norfloxacin (NOR)	10 µg	≤ 12	13-16	≥ 17	≥ 16	≤ 4
C	Kanamycin (K)	30 µg	≤ 13	14-17	≥ 18	≥ 25	≤ 6
A	Gentamycin (GM)	10 µg	≤ 12	13-14	≥ 15	≥ 8	≤ 4
O	Doxycycline (DXT)	30 µg	≤ 12	13-15	≥ 16	≥ 16	≤ 4
B	Imipenem (IMI)	10 µg	≤ 13	14-15	≥ 16	≥ 16	≤ 4
B	Amikacin (AK)	30 µg	≤ 14	15-16	≥ 17	≥ 32	≤ 16
C	Tetracycline (T)	10 µg	≤ 14	15-18	≥ 19	≥ 16	≤ 4
B	Cefotaxime (CTX)	30 µg	≤ 14	15-17	≥ 23	≥ 64	≤ 8

3. 6 Detection of Hepatitis A virus and Coxsackie A virus

The presence of the enteric viruses - human Coxsackievirus and Hepatitis virus in the wastewater samples were investigated. Human Coxsackievirus A 2; strain Fleetwood, and the Hepatovirus, Hepatitis A Virus, Strain PA21, were used as positive controls in viral concentration, RNA extraction and RT-PCR steps. These positive controls were prepared in serial 10-fold dilutions from a stock solutions of each viral strain and each dilution was added to autoclaved deionized

water (same volume as the test sample volume) following the protocol described below for virus recovery, and 10 µl of the ZR Viral RNA Kit™ (Zymo Research) extracts from concentrated samples was used for RT-PCR assay using the optimized condition of seminested RT-PCR. Negative controls were included in nucleic acid extraction, reverse transcription, seminested PCR and agarose gels and this was done by adding nuclease free water in place of sample.

3.6.1 The adsorption-elution method

The concentration of viruses was done following the method of Haramoto et al. (2005), with some modifications. All samples were pre-filtered through glass filter prior to the concentration step as schematically represented in Figure 2. Five milliliters of 250 mM aluminum chloride ($AlCl_3$) were passed through an HA filter (0.45-µm pore size and 90-mm diameter, Millipore) to form a cation (Al^{3+})-coated filter, and then 500 ml of water sample was passed through the filter. The filter was rinsed with 200 ml of 0.5 mM sulphuric acid (H_2SO_4) (pH 3.0) to remove aluminum ions, followed by elution of viruses with 10 ml of 0.1 mM sodium hydroxide (NaOH) (pH 10.8). The filtrate was recovered in a tube containing 50 µl of 100 mM H_2SO_4 (pH1.0) and 100 µl of 100X Tris-EDTA buffer (pH 8.0) for neutralization, followed by centrifugation using a Centriprep YM-50 (Millipore). The Centriprep YM-50 is a centrifugation unit equipped with an ultrafiltration membrane, which can achieve the high concentration efficiency needed for viruses. The filtrate was added to the Centriprep YM-50 and centrifuged according to the manufacturer's protocol; centrifugation at 2,500 rpm for 10 min, followed by removal of the sample that passed through the ultrafiltration membrane (8 ml) and further centrifugation at 2,500 rpm for 5 min to

obtain a final volume of 700 µl. The final concentrated sample was stored at -80 ^{o}C until needed for further analysis.

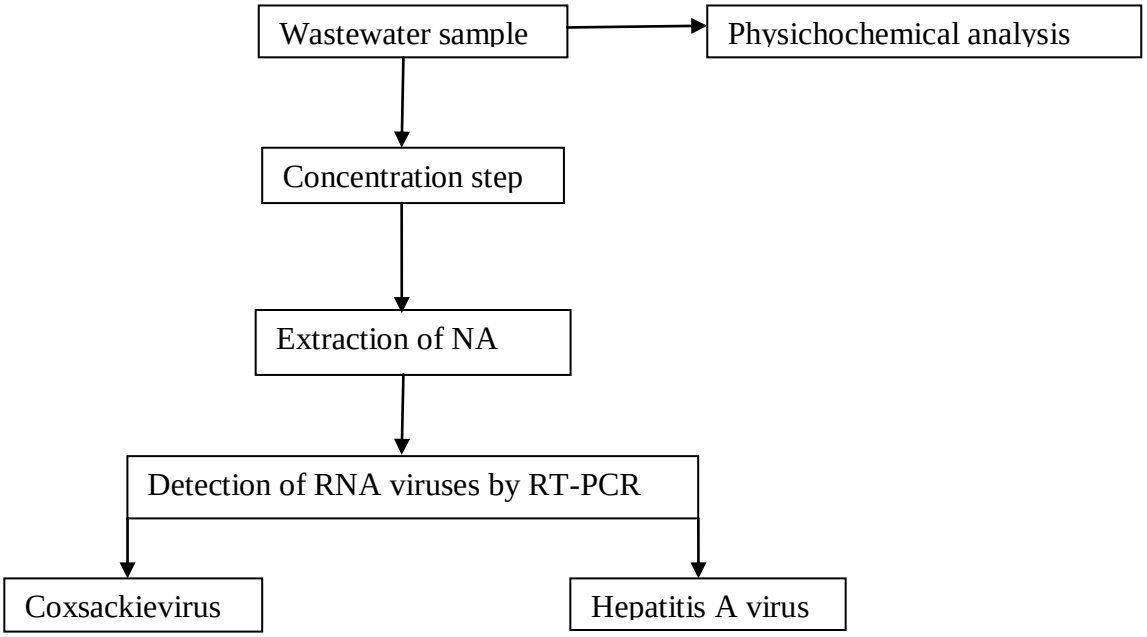

Figure 3.2: Schematic representation of analysis of water samples for the detection of enteric viruses.

3.6.2 RNA extraction

For the detection of Hepatitis A virus and Coxsackievirus A, RNA was extracted from 100 µl of the final concentrated sample using ZR Viral RNA Kit^{TM} (Zymo Research) to obtain a final volume of 10 µl following the manufacturer's instructions. Briefly, 300 µl of ZR Viral RNA Buffer was added to 100 µl of concentrated water sample contained in a 1.5 ml microcentrifuge tube. The mixture was transferred to a Zymo-Spin^{TM} IC Column in a collection and centrifuged at 12 000 ×g for 2 minutes. The flow-through was discarded from the collection tube. Three

hundred microliters of RNA Wash Buffer was added to the column and centrifuged at 12 000 ×g for 30 seconds. The flow-through was discarded and the Zymo-Spin™ IC Column was placed back into the collection tube. The washing step was repeated 2 times. The Zymo-Spin™ IC Column was centrifuged at 12 000 ×g for 2 minutes in an empty collection tube to ensure complete removal of the wash buffer. The Zymo-Spin™ IC Column was placed into a provided DNase/RNase free tube and then 10 µl of RNase/DNase free water was added into the column and was left to stand at room temperature for 1 minute. The Zymo-Spin™ IC Column was centrifuged at 12 000 ×g for 1 minute to elute the RNA which was then used immediately for reverse transcription.

3.6.3 Reverse transcription

Reverse transcription was carried out following the protocol for Maxima Reverse Transcriptase (Fermentas, life sciences) (Fermentas International Inc.). The ingredients for reverse transcription were added following the order depicted in Table 3.2 below.

Table 3.2: Recommended reaction components for reverse transcription of viral RNA.

Ingredient/ reagent	Concentration/quantity
Template RNA	10 µl
Random Hexamer (#S0142)	1 µl
dNTP Mix, 10 mM each (#R0191)	1 µl
Water, nuclease free	2.5 µl

5X RT buffer	4 µl
RiboLock™ RNase Inhibitor (#E00381)	0.5 µl
Maxima Reverse Transcriptase	1 µl
Total volume	20 µl

The mixture was mixed thoroughly by centrifugation and was incubated for 10 minutes at 25°C followed by 5 minutes at 85°C to terminate the reaction. The RT product was used immediately to perform the PCRs.

3.6.4 PCR Detection of Hepatitis A virus and Coxsackie A virus

The PCR assay used 20 µl of the concentrated copy DNA (cDNA) in a total reaction volume of 50 µl, containing 25 µl of PCR master mix, 0.5 µl of primer I, 0.5 µl of primer II, and 4 µl of nuclease free water. The thermal cycling conditions for coxsackievirus are given in Table 3.3 and those for hepatitis A virus are in Table 3.4. The primer sets for both coxsackievirus and hepatitis A virus seminested PCR are given in Table 6 together with their oligonucleotide sequence and the expected fragment length.

Table 3.3 The recommended thermal cycling conditions for coxsackie A virus detection.

Segment	Temperature	Duration	Number of cycles
Initial denaturation	94°C	3 min	1

Denaturation	94°C	1 min	40
Annealing	56°C	1 min	
Primer extention	72°C	40 s	
Final extention	72°C	10 min	1

Table 3.4 The recommended thermal cycling conditions for Hepatitis A virus detection.

Segment	Temperature	Duration	Number of cycles
Initial denaturation	94°C	3 min	1
Denaturation	94°C	1 min	40
Annealing	57°C	1 min	
Primer extention	72°C	40 s	
Final extention	**72°C**	**10 min**	**1**

Table 3.5 Oligonucleotide sequences used for detection of hepatitis A virus and coxsackie A virus

Virus	**Sequence**	**Size**
HAVC-L	5′-CAGCACATCAGAAAGGTGAG-3′	20 bp
HAVC-R	5′-CTCCAGAATCATCTCCAAC-3′	19 bp
CAV6vp1-R	5′-ACTCGCTGTGTGATGAATCG-3′	20 bp
CAV6vp1_l	5′-CGTCAAAGCGCATGTATGTT-3′	20 bp

3.7 Agarose gel electrophoresis

After amplification, PCR products were analyzed by gel electrophoresis. Seventeen microliters of amplified products and 3 µl of loading dye was electrophoresed at 10 V/cm for 90 minutes through 2.5% agarose gels (Progma) in 0.5x TBE buffer (1x TBE; 89 mM Tris borate, 2 mM EDTA, pH 8.2). Gels were stained with 5 µl (per 50 ml of gel) of GRGreen DNA stain (Fermentas) and visualized by 2UV transilluminator Model LM-26E (UVP, BioDoc-It^{TM} System, Upland, USA). Positive, negative, and reagent controls were always added in every gel. The presence of band of the expected size was assessed by comparison with a molecular size marker (100 bp DNA Ladder, New England Biolabs, Inc, USA).

CHAPTER 4

RESULTS

4.1 Physicochemical analyses

Table 4.1 shows the results of the physicochemical analyses of the two wastewater treatment plants during the study period. The temperature of the final effluents of both wastewater treatment plants ranged between 14.2 and 25.7^{o}C and fell within the DWAF (1996), recommended limits of ≤ 25^{o}C for most of the sampling period except in one case in the rural community (RC) based plant (25.7^{o}C during the December sampling period). Also, the conductivities of the final effluents of both treatment plants ranged from 229 to 373.5 µS/cm, while turbidity varied from 1.49 to 6.98 NTU. The pH of the final effluents of the two wastewater treatment plants fall within the acceptable limits and ranged between 6.0 and 9.0. The residual chlorine concentration of the final effluent of the two wastewater treatment plants was strikingly unacceptable, and showed a high degree of variability ranging from no residual chlorine to 2.97 mg/L.

With regards to chemical parameters, the nitrate and nitrite concentration of the final effluents of the peri-urban community (PUC) based treatment plant ranged between 1.55 and 4.95 mg/L, while those of the rural community (RC) based plant varied between 0.02 and 0.7 mg/L. Salinity on the other hand ranged between 0.12 and 0.2 psu. The dissolved oxygen (DO) concentration throughout the sampling period generally fell short of the acceptable limit which is ≥ 5 mg/L for sustaining aquatic life (Fatoki *et al*., 2003) and ranged from 0.1 to 18.33 mg/L.

Also, the BOD of the effluent from the RC based plant ranged between 3.47 and 4.48 mg/L and that of the PUC based plant varied from 3.68 to 7.81 mg/l. Total dissolved solids on the other hand ranged from 114.5 to 187 mg/L, and the Chemical oxygen demand (COD) concentrations ranged from 0.45 to 45 mg/L.

4.2 Microbiological analyses

4.2.1 Indicator microorganisms

The results of the bacteriological counts of the final effluents are given in Table 4.4. During the August sampling period, the PUC based plant had zero counts for total coliform, feacal coliform and enterococcus. On the other hand, the RC based plant had high counts of these FIBs during the same sampling period viz. total coliform (4.5×10^2 cfu/100 ml), feacal coliform (0.5×10^3 cfu/100 ml), and enterococcus (0.5×10^3 cfu/100 ml). Opposite contrary trend was observed during the month of September wherein RC recorded zero bacterial count while the PUC based plant had counts of 3.95×10^4 cfu/100 ml, 1.25×10^4 cfu/100 ml and 5.0×10^3 cfu/100 ml for total coliform, feacal coliform and enterococcus respectively. This trend was observed through to December with the two plants alternating in months for their counts and these observations suggests that both plants are inefficient in reducing the FIBs in their treatment processes. The lowest bacterial counts were observed during the months of November and December.

Table 4.1: Physicochemical qualities of the final effluents of the rural community (RC) and the peri-urban community (PUC) based wastewater treatment plants.

Parameter	August		September		October		November		December		Range	
	RC	PUC	RC	PUC	RC	PUC	RC	PUC	RC	PUC	RC	PUC
Turbidity (NTU)	3.81±0.08	2.96±0.01	1.49±0.05	6.98±1.91	4.88±0.13	3.47±0.72	3.28±0.75	5.78±0.71	3.35±0.49	2.38±0.31	1.49-4.88	2.38-6.98
Salinity (psu)	0.16±0.0	0.13±0.01	0.16±0.0	0.13±0.0	0.17±0.0	0.13±0.01	0.20±0.0	0.13±0.0	0.18±0.01	0.12±0.0	0.16-0.2	0.12-0.18
pH	6.7±0.2	5.9±0.1	6.5±0.1	6.4±0.1	7.8±0.3	7.5±0.2	7.2±0.0	7.2±0.2	6.5±0.0	7.2±0.0	6.5-7.8	5.9-7.5
Temp (^{o}C)	18.1±0.1	14.2±0.2	18.4±0.6	17.2±0.1	19.3±0.1	17.7±0.0	21.2±0.0	18.9±0.1	25.7±0.0	20.4±0.0	18.1-25.7	14.2-20.4
EC (µS/cm)	292.5±0.71	235.5±14,85	305.5±2.12	250±1.41	318±2.83	233.50±7.78	373.5±0.71	239±0.0	338±2.83	231±0.0	292.5-373.5	233.50-250.0
DO (mg/L)	0.10±0.0	0.12±0.13	0.50±0.04	0.12±0.0	1.07±0.14	13.48±0.13	0.64±0.0	0.78±0.01	0.53±0.0	0.69±0.0	0.10-1.07	0.12-13.48
TDS (ppm)	146.5±0.7	117.5±7.78	153±1.41	125.5±0.7	161.5±2.12	117±4.24	187±0.0	119.5±0.71	170±0.0	115±0.0	146.5-187.0	114.5-125.5
COD (mg/L)	ND	ND	-5.95±5.59	4.05±0.92	10.55±4.45	0.45±7.42	45±32.53	19±14.14	3±0.0	100±0.0	-5.95-45	0.45-19
BOD (mg/L)	ND	ND	ND	ND	3.52±0.50	5.37±3.34	7.27±0.27	7.69±0.42	7.48±0.08	7.81±0.18	3.52-7.48	5.37-7.81
Phosphates (mg/L)	3.2±0.49	3.77±0.30	3.71±0.06	3.61±0.03	3.08±0.19	0.51±0.04	4.85±0.05	1.92±0.04	3.32±0.06	0.92±0.02	3.08-4.85	0.51-3.77
Nitrates (mg/L)	4.62±0.92	4.16±0.41	4.61±0.13	1.55±1.17	3.5±0.28	5.3±0.14	5.45±0.07	3.75±0.07	6.65±0.49	4.95±0.07	3.50-6.7	1.55-4.95
Nitrites (mg/L)	0.03±0.0	0.02±0.02	0.03±0.0	0.04±0.0	0.83±0.01	0.04±0.01	0.40±0.0	0.08±0.01	0.15±0.03	0.05±0.0	0.03-1.15	0.02-0.07
Free Chlorine	0.34±0.01	2.97±0.01	0.37±0.09	0.00±0.0	0.18±0.01	1.2±0.12	0.41±0.04	0.38±0.01	0.07±0.01	0.29±0.06	0.07-0.75	0.0-2.97

Legend: DO – Dissolved Oxygen; TDS – Total dissolved solute; COD-Chemical oxygen demand; BOD-Biological oxygen demand, Temp-Temperature; EC-electrical conductivity. The standard limit for TDS: 0 – 450 mg/L; EC: 0 – 70 mg/L; DO: $\geq$ 5 mg/L; pH: 6 – 9; nitrate and nitrite: 0 – 6; and COD: 30 mg/L.

Table 4.2: Correlation of the physicochemical and biochemical parameters for the PUC based wastewater treatment plant.

Parameters	Turbidity (NTU)	Salinity (psu)	pH	Temp. (^oC)	EC (µS/cm)	DO (mg/L	TDS (mg/L)	COD (mg/L)	BOD (mg/L)	Phosphates (mg/L)	Nitrates (mg/L)	Nitrites (mg/L)	Free chlorine (mg/L)	TC	FC	EC
Turbidity (NTU)	1															
Salinity (psu)	.548	1														
pH	-.049	-.310	1													
Temp. (^oC)	.026	-.674	.805	1												
EC (µS/cm)	.930*	.593	-.375	-.247	1											
DO (mg/L)	-.249	.222	.485	-.035	-.303	1										
TDS (mg/L)	.924*	.513	-.395	-.198	.993**	-.411	1									
COD (mg/L)	.573	.271	.391	.352	.280	-.280	.300	1								
BOD (mg/L)	-.530	-.727	.829	.787	-.747	.371	-.735	-.023	1							
Phosphates (mg/L)	.433	.457	-.904*	-.666	.666	-.648	.696	-.007	-.937*	1						
Nitrates (mg/L)	-.866	-.383	.460	.164	-.957*	.541	-.984**	-.248	.720	-.743	1					
Nitrites (mg/L)	.393	-.164	.696	.761	.046	-.202	.084	.875	.404	-.363	-.065	1				
Free chlorine (mg/L)	-.530	.315	-.604	-.828	-.319	.082	-.350	-.404	-.378	.334	.352	-.696	1 5			
TC	.756	.250	-.360	-.087	.890*	-.280	.898*	-.059	-.553	.545	-.908*	-.109	-.449	1		

FC	.756	.250	-.360	-.087	.890*	-.280	.898*	-.059	-.553	.545	-.908*	-.109	-.449	1.000**	1	
EC	.756	.250	-.360	-.087	.890*	-.280	.898*	-.059	-.553	.545	-.908*	-.109	-.449	1.000**	1.000**	1

*. Correlation is significant at the 0.05 level (2-tailed).
**. Correlation is significant at the 0.01 level (2-tailed).
Legend: DO – Dissolved Oxygen; TDS – Total dissolved solute; COD-Chemical oxygen demand; BOD-Biological oxygen demand, Temp-Temperature; EC- electrical conductivity; TC- Total coliform; FC- Feacal coliform; EC- Enterococcus counts.

Table 4.3: Correlation of the physicochemical and biochemical parameters for the RC based wastewater treatment plant.

Parameters	Turbidity (NTU)	Salinity (psu)	pH	Temp. (°C)	EC (µS/cm)	DO (mg/L	TDS (mg/L)	COD (mg/L)	BOD (mg/L)	Phosphates (mg/L)	Nitrates (mg/L)	Nitrites (mg/L)	Free chlorine (mg/L)	TC	FC	EC
Turbidity (NTU)	1															
Salinity (psu)	.145	1														
pH	.651	.476	1													
Temp. (°C)	.031	.442	-.147	1												
EC (µS/cm)	.083	.960**	.407	.640	1											
DO (mg/L)	.685	-.085	.813	-.249	-.078	1										
TDS (mg/L)	.081	.978**	.451	.560	.995**	-.061	1									
COD (mg/L)	.237	.955*	.591	.174	.840	.014	.884*	1								

BOD (mg/L)	.253	.829	.336	.837	.930*	-.009	.893*	.655	1							
Phosphates (mg/L)	-.360	.813	.165	.099	.721	-.412	.768	.818	.435	1						
Nitrates (mg/L)	-.309	.390	-.509	.871	.529	-.692	.462	.143	.639	.295	1					
Nitrites (mg/L)	.706	.352	.974**	-.068	.333	.902*	.360	.434	.334	-.027	-.500	1				
Free chlorine (mg/L)	-.428	.153	-.049	-.654	-.066	-.367	.020	.340	-.406	.628	-.293	-.253	1			
TC	.718	-.132	.788	-.283	-.137	.996**	-.120	-.021	-.056	-.461	-.714	.879*	-.365	15		
FC	.204	-.442	-.400	-.418	-.610	-.274	-.598	-.292	-.552	-.335	-.165	-.434	.257	-.191	1	
EC	.767	-.200	.743	-.372	-.239	.967**	-.218	-.056	-.154	-.512	-.764	.825	-.309	.985**	-.019	1

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

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

Table 4.4: Feacal indicator bacteria counts of the wastewater final effluents from both plants.

Sampling month	FIB counts (CFU/100ml)					
	Enterococci		Total Coliform		Feacal Coliform	
	RC	PUC	RC	PUC	RC	PUC
August	0.5×10^3	0	4.5×10^2	0	0.5×10^3	0
September	0	5.0×10^3	0	3.95×10^4	0	1.25×10^4
October	2.13×10^3	0	6.88×10^3	0	0	0
November	0.3×10^2	0	0.3×10^1	0	0	0
December	0	0	8.7×10^1	0.2×10^1	0	0

Key: RC (Rural community); PUC (Peri-urban community).

4.2.2 Correlation data for all tested parameters

The correlation coefficients of all tested parameters for both plants are presented in Tables 4.2 and 4.3. In the RC based plant, there were significant ($P < 0.01$) positive correlation between EC and salinity, TDS and enterococci, enterococci and TC, enterococci and DO. The correlation between BOD, EC, TDS, DO, Nitrites, and TC was significant ($P < 0.05$). There was no significant correlation observed between salinity and turbidity, pH and turbidity, salinity and pH, EC, pH and temperature, DO, pH and turbidity, temperature and TDS. Also, there was no significant correlation between BOD, turbidity, salinity, pH, and temperature, as well as between

COD, salinity, temperature, EC, and DO. It also should be noted that there was a negative correlation between residual chlorine and most of other parameters, except for TDS, COD, and phosphates which had no significant correlation. There was also a negative correlation between feacal coliforms and total coliforms, feacal coliforms and enterococci.

In the PUC based plant, there were strong positive correlations ($P < 0.01$) between TDS and EC, feacal coliforms and total coliforms, total coliforms and enterococci, enterococci and feacal coliforms; and a strong negative correlation ($P < 0.01$) between nitrates and COD. The negative correlation was significant ($P < 0.05$) between nitrites, total coliforms, feacal coliforms and enterococci. Correlation between feacal coliform, total coliform, enterococci, DO and COD was significant ($P < 0.05$), while that between turbidity, salinity, temperature, BOD, phosphates, nitrates, and free chlorine were insignificant.

4.2.3 Identification and antibiogram of *E. coli* isolates

A total of 24 presumptive *E.coli* isolates obtained from the PUC and RC wastewater final effluents were identified using the commercial API 20E kit. *E. coli* ATCC 25922 was used as a positive control. About 55.4% (13 out of 24) of the presumptive *E. coli* isolates were confirmed to be *E. coli*. The antibiotic susceptibility patterns of the isolates are presented in Table 4.5. All the isolates were susceptible to Meropenem (MEM), Cefuroxime (CXM), Norfloxacin (NOR), Gentamycin (GM), Imipenem (IMI), Amikacin (AK), and Cefotaxime (CTX), and exhibited varying degrees of susceptibilities to the other antibiotics. The highest resistance (72%) observed in this study was to erythromycin, while the highest indeterminate response (57%) was observed against tetracycline.

Table 4.5: Antibiogram of *E. coli* isolates from wastewater final effluents.

Percentage reaction	Antibiotic Susceptibility Pattern														
	S10	CIP5	AP25	E15	C30	MEM10	CXM30	NOR10	K30	GM10	DXT30	IMI10	AK30	T10	CTX30
%R	**28**	**0**	**43**	**72**	**7**	**0**	**0**	**0**	**0**	**0**	**28**	**0**	**0**	**21**	**0**
%I	**0**	**14**	**0**	**28**	**0**	**0**	**0**	**0**	**7**	**0**	**43**	**0**	**0**	**57**	**0**
%S	**72**	**86**	**57**	**0**	**93**	**100**	**100**	**100**	**93**	**100**	**29**	**100**	**100**	**21**	**100**

Legend: R-resistance; S-susceptible; I-intermediate

4.3 Detection of Hepatitis A virus and Coxsackie A virus

The results of the virological analyses are shown in Table 4.6. All the four months samples from the RC based plant were positive for Hepatitis A virus (HAV), while four of the five samples collected from the PUC based plant were found to be positive for Hepatitis A virus. The prevelance of Coxsackie A virus (CAV) in the RC based plant was found to be 75 % (3/4) and that of the PUC based plant was found to be 80 % (4/5) of the collected samples.

Table 4.6 Prevelance of Hepatitis A virus and Coxsackie A virus in wastewater final effluents from both plants.

Sampling month	**Hepatitis A**		**Coxsackie A virus**	
	RC	**PUC**	**RC**	**PUC**
August	+	-	+	-
September	+	+	-	+
October	+	+	+	+
November	+	+	+	+
December	ND	+	ND	+
% Occurence	100	80	75	80

Key: + (present); - (absent); ND- not determined

CHAPTER 5

DISCUSSION, CONCLUSION AND FUTURE DIRECTIONS

5.1 Discussion

Acheiving adequately treated wastewater final effluents remains a challenge in developing countries including South Africa. Several studies (Fatoki *et al*., 2003, Osode and Okoh, 2009, Igbinosa and Okoh, 2009) on wastewater final effluents in the Eastern Cape Province of South Africa has proved wastewater treatment plants to be inefficient in reducing nutrient load and pathogenic microorganisms. Routine monitoring of these final effluents becomes imperative as most communities, especially in rural areas rely on the receiving water bodies.

The physicochemical qualities of the RC and PUC based wastewater final effluents were assessed in this studies and some were within the WHO and South African set guidelines with DO falling short of the acceptable limit of ≥ 5 mg/L for sustaining aquatic life (Fatoki *el al*., 2003). Though there is no health guideline for DO content in water, low concentration of DO in water supplies may encourage the microbial reduction of nitrate to nitrite and sulfate to sulfite and can also cause an increased ferrous iron concentration in solution with subsequent discoloration at the tap point (WHO, 2006).

The BOD range for the RC based plant was 3.47 to 4.48 mg/L and that of the PUC based plant as 3.68 to 7.81 mg/l. The BOD concentrations obtained in this study are similar to those reported by Momba *et al*. (2006). Though there are no South African guidelines for BOD in final effluents, the BOD concentrations of these two plants' final effluents far exceeded the EU stipulated target limits of 3.0 to 6.0 mg/L for water intended for fisheries and aquatic life

(Chapman, 1996). As such, the BOD quality of these effluents disqualifies their discharge into receiving water bodies that support aquatic ecosystems.

Salinity concentration showed some degree of variation throughout the sampling period for both the RC and PUC based plants. Excess salinity has been reported to contribute to reduction in crop yield, increased formation of scale and corrosion in domestic water use and increased pretreatment of water for selected industrial use (DEAT, 2000; Igbinosa and Okoh, 2009). The recorded salinity values for both facilities in this study do not pose any problem with regards to the environment and public health (SANCOR, 1984; Whitfield and Bate, 2007).

Turbidities for the RC based plant were within the South African recommendations for water quality guidelines for domestic use (DWAF, 1996), and that of the PUC based plant was above this recommended limits. Previous works (EPA, 2009; Momba *et al*., 2002) strongly associates higher levels of turbidity with higher levels of pathogenic microorganisms such as viruses, parasites and some bacteria. Thus, the occurrence of higher indicator bacterial numbers during the month of September (Table 4.4) could be related to the higher turbidity levels, although, the November month samples revealed absence of these microorganisms. Also, high turbidity is counter-productive to water disinfection (DWAF, 1998) and may lead to formation of trihalomethane (TMH) precursors when chlorine is applied (Igbinosa and Okoh, 2009).

The conductivities of the final effluents from the PUC based treatment plant was within the South African recommended guidelines (250 µS/cm) for effluent meant to be discharged into receiving water bodies, with reference to the 1984 South African Government Gazette (Gazette No. 9225,1984; Igbinosa and Okoh, 2009). Similarly, the South African Government set an acceptable limit of conductivity for water intended for domestic use to be 70 µS/cm (DWAF,

1996), however, the conductivity profiles for the RC based plant's effluents were higher than the set value thus, rendering this plant's effluent quality noncompliant.

The nutrient load of the wastewater final effluents of both the RC and PUC based plants was measured by assessing the concentration of nitrates, nitrites and phosphate. The high nitrate concentrations in the RC based plant may contribute to eutrophication, due to accelerated growth of algae and the occurrence of algal blooms, because nitrate together with phosphate stimulate plant growth (DWAF, 1996). Higher nitrate and nitrite levels were recorded in the RC based treatment plant during the November month. The high levels of nitrite impacted upon receiving water bodies by the discharge of wastewater final effluents is considered to pose a problem to communities that use the water bodies for domestic purposes (Igbinosa and Okoh, 2009).

The high concentrations and the high degree of variation in the residual chlorine concentrations of both RC and PUC based plants are unacceptable. In South Africa, there is no limit set for free chlorine residual in wastewater final effluents to be discharged to receiving water bodies. In September, the PUC facility had no residual chlorine residue in its final effluent, whereas high concentration of residual chlorine residue was detected in the PUC based plant during the month of August. The concentration of residual chlorine from the RC based plant are similar to those reported by Odjadjare *et al*. (2010) on a study carried out in the final effluent of a wastewater treatment works in the same locality. Very low concentration of residual chlorine, like the one observed in the PUC facility, poses a very high environmental and health risk due to the fact that chlorination is the only means employed by these facilities to reduce the levels of pathogenic microorganisms. Also, there is a risk of the water becoming unacceptably salty and

could result in nausea and electrolyte problems associated with high chlorine levels in the final effluents discharged into the receiving waterbodies intended for domestic use (DWAF, 1996).

The COD values obtained in this study are relatively within the South African guideline for COD (30 mg/L) in effluents to be discharged into the receiving waterbodies (Government Gazette, 1984; Igbinosa and Okoh, 2009). COD together with BOD are the most important biochemical parameters in that they reflect the organic load in wastewater (Uz *et al*., 2004; Huertasa *et al*., 2008; Al-Turk, 2010).

Wastewater is one of the major contributors of pathogens in the environment as it carries lots of feacal matter especially that of human and animal origin. One of the primary purposes of wastewater treatment plants is therefore the removal of parasites, bacterial and viral pathogens and thus protects the receiving waterbodies from such pathogens (Osode and Okoh, 2009). The high levels of FIBs, and possibly high levels of pathogenic microorganisms, obtained from these two wastewater treatment plants during the course of the study period suggests that these wastewater treatment plants are inefficient with regards to the removal of pathogens. This consequently results in both environmental and public health risk.

Feacal coliforms have since been used as indicators of feacal pollution (Davies-Colley et al., 1994). High numbers (1.25×10^4 cfu/100 ml) of feacal coliform in the PUC based plant during September sampling and 5×10^2 cfu/100 ml in the RC based plant during August sampling period implies that these plants were highly polluted during these periods. At this point it may be difficult to tell whether the pollution is of animal or human origin unless microbial source tracking (MST) studies are undertaken. Total coliform and enterococcus were the most abundant of the three studied FIBs in the RC based treatment plant.

E.coli, one of the most common feacal coliform and cause of waterborne diarrhea suggests a high risk of illness associated with consumption of feacal contaminated waters. Other enteric pathogens apart from *E. coli* that were identified from this study include *Enterobacter spp*., *Klebsiella spp*., *Citrobacter spp*., which are of public health concern. There is a strong correlation between the risk of being infected by these microbial pathogens and the level of contamination and the amount of contaminated water consumed (DWAF, 1996). Enterococcus is relatively an indicator of feacal pollution and tends to survive longer in the water environment than coliform bacteria (DWAF, 1996).

Many studies have proved that the assessment of FIBs that fall within the recommended limits do not necessarily implies that the tested wastewater final effluents are free of pathogens. Indeed, though they did not assess for the presence of FIBs in their study, Igbinosa and Okoh (2009), and Odjadjare and Okoh (2009) reported the presence of Vibrio and Listerial species from wastewater final effluents.

In the RC based plant, there was strong positive correlation (P<0.01) between total coliforms and DO. On the other hand the correlation between TC and nitrites was significant at P<0.05. However the correlation between total coliforms and the rest of the parameters was considered insignificant. There was a strong positive correlation (P < 0.01) between enterococci and DO. Previous works agreed with this observation that under low DO condition nitrate may be reduced to ammonia, and this reduction may occur under high FIB counts as the DO positively correlated with enterococci and TC (He *et al*., 2007). While the correlation between enterococci and the rest of the parameters were considered insignificant, on the other hand the

correlation between FC and all the tested parameters were not. For the RC based plant, DO seems to be the only parameter that had a positive correlation with both TC and enterococci.

In the PUC based plant, the correlation between TC, EC, and TDS was positively significant ($P < 0.05$). This observation is in agreement with report elsewhere (Tiefenthaler *et al*., 2009) which states that EC is closely correlated to TDS, which in turn are a potential source of BOD, thus promoting bacterial growth and decomposition of organic matter. BOD is used to measure the strength of the wastewater and is the direct measure of the amount of oxygen required by microorganisms to breakdown the wastewater within 5 days (Al-Rekabi *et al*., 2007). This positive correlation between EC and TDS is evident of high microbial activity that takes place during the decomposition of the organic content of the wastewater as it correlates to TC counts. It has been reported that increased turbidity and TDS enhance feacal coliform abundance, and in particular *E. coli* attachment, thus making it more resistant to chlorine disinfection (Maier *et al.*, 2000).

E. coli was isolated from the final effluents and assessed for their antibiotic susceptibilities, and 72 % of the *E. coli* isolates were resistant to erythromycin, while 28 % were intermediate. Forty-six percent of the *E. coli* isolates were multi-resistant to at least five of the antimicrobial agents with 2 isolates being resistant to Streptomycin, Ampicillin, Erythromycin, Doxycycline and Cefotaxime. This observation is in agreement with the study by Meng *et al*. (1998) which showed *E. coli* isolates from cattle being resistant to six antibiotics. Also, Okeke *et al*. (2000) reported the prevalence of feacal *E. coli* strains resistant to tetracycline, Ampicillin, Chloramphenicol, and Streptomycin, which further supports the findings of this present study. Okeke *et al*. (2000) also revealed that resistance against these antibiotics has shown a rocketing

increase from 9 % in 1986 to 100 % in 1998. Also, about 57 % of the isolates had intermediate response to tetracycline. In a related report, Wilkerson *et al*. (2004) showed that 98 % of bovine *E. coli* isolates and 59 % of human *E. coli* isolates were resistant to tetracycline. A study by Carrasco *et al*. (1997) reported multiple antibiotic resistant (MAR) *E. coli* isolated from a site receiving sewage effluent. This present study revealed about 46 % resistant of *E. coli* isolates to MAR.

Previous works have reported that correlation between the occurrence of FIBs, coliphages and viral pathogen is not well understood. Wastewater effluents and combined sewer overflows have been found in several studies to be sources of viruses and could negatively impact on the quality of receiving waters and thus threaten public health (Aslan *et al*., 2011; Veldhuis *et al*., 2010; Kay *et al*.,2004). Some studies reported polluted drinking and recreactional waters to have transmitted viruses (Bosch *et al*., 2011; Maynula *et al*,. 2005; Cannon *et al*,. 1991). In this present study, hepatitis A virus was detected in all the samples from the RC based plant and in 80 % of the samples from the PUC based plant, while coxsackie A virus was detected in 75 % of the samples from the RC based plant and in 80 % of the samples from the PUC based plant. The presence of these viruses in the final effluents suggest the inefficiency of the plants in the removal of viruses during the treatment process and posses a potential health burden on the receiving water body.

There are reported cases of viral hepatitis caused by hepatitis A virus and these are associated with feacal contaminated waters (Hutin *et al*,. 1999; Niu *et al*,. 1992). Though, the infectious dose for hepatitis A virus is estimated to be about 10 to 100 infectious particles, however, irrespective of this, estimated low concentrations often associated with contaminated

water and foods can result in infection and illness (Bosch *et al*,. 2011). A study by Halliday *et al*. (1991) reported an outbreak of 300,000 cases of hepatitis A virus and 25,000 cases of viral gastroenteritis in Shanghai, China, and this has, according to Catley-Carlson (1993), contributed to an estimate of about 50,000 people who die each day due to waterborne diseases world wide. There was also another outbreak of 79,000 cases of hepatitis E virus in Kanpur, India which was ascribed to polluted drinking water (Grabow *et al*., 1994). Bosch et al. (2011) reported that between 1980 and 1994 there were about 28 reported outbreaks and 11,195 confirmed cases of waterborne viral diseases in the United States, 9038 out of these cases were attributed to HAV.

Coxsackievirus A (CVA), more specifically serotype A16, is the major etiological agent of the hand, foot and mouth (HFMD) diseases worldwide and may lead to normal benign and fatal central nevervous system complications (Bendig *et al*., 2001). Early identification of the cause of HFMD may be of prognostic and epidemiological value since VCA-16 is closely related to enterovirus 71 (EV71), another causative agent of normal and central nervous system complication (Bendig *et al*., 2001; Ho *et al*., 1999; Shimizu *et al*., 1999). However, infections caused by CVA are usually mild (Gjøen and Bruu, 1997). In Vietnam, during 2005, about 764 children diagnosed with HFMD were hospitalised and 52.1 % of the isolates from specimens collected from stools, vesicle fluid and throat swabs of these children were identified as CVA16 (van Tu *et al*., 2007). Most of the samples in this study were positive for CVA. Consodering that these effluents are discharged into the receiving waterbodies which children use for swimming during hot days, the risk of infection through body contact and/or ingestion becomes evidently high. Our result clearly indicate the need for regular routine monitoring of enteric viruses in wastewater final efflunts as this might have major health risk implications.

5.2 Conclusion and Future directions

Water scarcity has, in the past decades, become one of the challenges faced by all the countries in the world, with the Southern regions of Africa being one of the most impacted and affected. This has been made more severe by the impact of poorly treated wastewater effluents on receiving waterbodies that serve in most parts South Africa as a source of drinking water supply. These water sources are often negatively impacted by effluents from such point-sources as wastewater treatment plants as shown in this study.

The two wastewater treatment plants under study showed high degree of inconsistency in their effluents qualities. Of particular importance is the chlorine under-dosing or over-dosing which has serious negative effects in the environment and public health. Also, the high densities of FIBs in the effluents further corroborate the inefficiencies of the treatment plants. The presence of hepatitis A virus and coxsackie A virus suggest that these two wastewater treatment plants are sources of enteric viral pathogens in the aquatic environment of the Eastern Cape Province. Assessment of enteric viral pathogens in raw source water and wastewater final effluents has gained worldwide attention as they are etiological of viral gastroenteritis and other viral infections.

It is therefore recommended that enteric viruses be included as one of the parameters used in routine analysis of water and wastewater final effluent quality. Future studies could consider extended viral targets as well as determine their infectivity and risk assessments. Also, there is urgent need of intervention by appropriate regulatory agencies to ensure regular

monitoring of the qualities of final effluents of wastewater treatment facilities in the Eastern Cape Province and ensure compliance to established guidelines.

REFERENCES

Abad FX, Pintó RM, Bosch A (1994). Survival of enteric viruses on environmental fomites. *J. Appl. Environ. Microbiol. 60: 3704-3710.*

Abbaszadegan M, Huber MS, Gerba CP, Pepper IL (1993). Detection of enteroviruses in ground water by PCR. *Appl. Environ. Microbiol. 59: 1318-1324.*

Adachi JA, Ericsson CD, Jiang ZD, DuPont MW, Pallegar SR, DuPont HL (2002). "Natural history of enteroaggregative and enterotoxigenic Escherichia coli infection among US travelers to Guadalaralar, Mexico". *J. Infect. Dis. 185: 1681-1683.*

Allwood PB, Malik YS, Hedberg CW, Goyal SM (2003). Survival of F-specific RNA coliphages, feline calicivirus, and Escherichia coli in water: a comparative study. *Appl. Environ. Microbiol. 69: 5707-5710.*

Al-Rekabi WS, Qiang H, Qiang WW (2007). Review on sequencing batch reactors. *Pakistan J. Nutr. 6: 11-19.*

Al-Saigh H, Zweifel C, Blanco J, Blanco JE, Usera MA, Stephan R (2004). Fecal shedding of Escherichia coli 0157:H7, Salmonella, and Campylobacter in Swiss cattle at slaughter. *J. Food Prot. 67: 679-684.*

Al-Turki AI (2010). Assessment of effluent quality of tertiary wastewater treatment plant at Buraidah city and its reuse in irrigation. *J. Appl. Sci. 10: 1723-1731.*

American Public Health Association (APHA), American Water Works Association and Water Environment Federation (1998). *Standard Methods for the Examination of Water and Wastewater,* 20th edn. Washington, DC: American Public Health Association.

Anderson K L, Whitlock JE, Harwood VJ (2005). Persistence and differential survival of fecal indicator bacteria in subtropical waters and sediments. *Appl. Environ. Microbiol. 71: 3041-3048.*

Application Data Sheet (ADS) (2004). Primary wastewater treatment: Influent monitoring. Water and Wastewater Industry. *ADS 4950-12/rev. A.*

Araujo JM, Tabarelli GF, Aranda KRS, et al. (2007). "Typical enteroaggregative and atypical enteropathogenic types of Escherichia coli are the most prevalent diarrhea-associated pathotypes among children". *J. Clin. Microbiol. 45: 3396-3399.*

Argaw N (2004). Chapter 6: Wastewater Sources and Treatment. In Renewable Energy in Water and Wastewater Treatment Applications; National Renewable Energy Laboratory, U.S. *Department of Energy Laboratory: Golden, CO, USA; 38-46.*

Arikawa K, Meraz IM, Nishikawa Y, et al. (2005). Interleukin-8 secretion by epithelial cells infected with diffusely adherent Escherichia coli possessing Afa adhesin-coding genes. *Microbiol. Immun. 49: 493-503.*

Aslan A, Xagoraraki I, Simmons FJ, Rose JB, Dorevitch S (2011). Occurance of adenoviruses and other enteric viruses in limited-contact freshwater recreactional areas and bathing waters. *J. Appl. Microbiol. 111: 1250-1261.*

Bach SJ, McAllister TA, Mears GJ, Schwartzkopf-Genswein KS (2004). Long-haul transport and lack of preconditioning increases fecal shedding of Escherichia coli 0157:H7 by calves. *J. Food Prot. 67: 672-678.*

Bach SJ, Selinger LJ, Stanford K, McAllister TA (2005). Effect of supplementing corn- o barley-based feed lot diets with canola oil on faecal shedding of Escherichia coli 0157:H7 by steers. *J. Appl. Microbiol. 98: 464-475.*

Balkin HB, Margolin AB (2010). Detection of poliovirus by ICC/qPCR in concentrated water samples has greater sensitivity and less costly using BGM cells in suspension as compared to monolayers. *Virol. J . 7: 282.*

Batigelli DA, Sobsey MD, Libe DC (1993). The inactivation of hepatitis A virus and other model viruses by UV irradiation. *Water Sci. Technol. 27: 339-342.*

Bekal S, Brousseau R, Masson L, Préfontaine G, Fairbrother JM, Harel J (2003). Rapid identification of Escherichia coli pathotypes by virulence gene detection with DNA microarray. *J. Clin. Microbiol. 41: 2113-2125.*

Bendig JWA, O'Brien PS, Muir P (2001). Serotype-specific detection of Coxsackievirus A16 in clinical specimens by reverse transcription-nested PCR. *Am. J. Clin. Microbiol. 39: 3690-3692.*

Benenson AS (ed.) (1995). Control of communicable diseases Manual, sixteenth Edition. *United Book press, Baltimore. 140-150.*

Berg G (1967). Transmission of viruses by the water route. *Willey, New York.*

Betis F, Brest P, Hofman V, et al. (2003). Afa/Dr adhesins of diffusely adherent Escherichia coli stimulate interleukin-8 secretion, activate mitogen-activated protein kinases, and promote polymorphonuclear transepithilial migration in T84 polarized epithelial cells. *Infect. Immun. 71: 1068-1074.*

Beutin L, Zimmermann S, Gleier K (1998). Human infections with Shiga toxin-producing Escherichia coli other than serogroup 0157 in Germany. *Emerg. Infect. Dis. 4940: 635-639.*

Bieber D, Ramer SW, Wu CY, Tobe T, Fernandez R, Schoolnik GK (1998). Type IV pili, transient bacterial aggregates, and virulence of enteropathogenic Escherichia coli. *Sci. 280: 2114-2118.*

Black LE, Brion GM, Freitas SJ (2007). Multivariate logistic regression for predicting total culturable virus presence at the lake of a potable-water treatment plant: novel application of the etypical coliform/total coliform ratio. *Appl. Environ. Microbiol. 73: 3965- 3974.*

Bosch A (1998). Human enteric viruses in the water environment: A minireview. *Int. Microbiol. 1: 191-196.*

Bosch A, Sánchez G, Abbaszadegan M, Carducci A, Guix S, Le Guyader FS, et al. (2011). Analytical methods for virus detection in water and food. *Food Analyt. Methods. 4: 4-12.*

Bosilevac JM, Arthur TM, Wheeler TL, Shackelford SD, Rossman JO, Koohmaraie M (2004). Prevalence of Escherichia coli 0157 and levels of aerobic bacteria and enterobacteriaceae are reduced when hides are washed and treated with cetylpyridinium chloride at a commercial beef processing plant. *J. Food Prot. 67: 646-650.*

Brooks HA, Gersberg RM, Dhar AK (2005). Detection and quantification of hepatitis A virus in sea water via real-time RT-PCR. *J. Virol. Methods, 127: 109-118.*

Bull AR, Kimmance KJ, Parry JV, Perry KR (1989). Investigation of an outbreak of hepatitis A simplified by salivary antibody testing. *Epidemiol. Infect. 103: 371-376.*

Butot S, Putallaz T, Sánchez G (2007). Procedure for rapid concentration and detection of enteric viruses from berries and vegetables. *Appl. Environ. Microbiol. 73: 186-192.*

Cannon RO, Poliner JR, Hirschhorn RB, Rodeheaver DC, Silverman PR, et al. (1991). A multistate outbreak of Norwalk virus gastroenteritis associated with consumption of commercial ice. *J. Infect. Dis. 164: 860-863.*

Carducci A, Arrighi S, Ruschi A (1995). Detection of coliphages and enteroviruses in sewage and aerosols from an activated sludge wastewater treatment plant. *Lett. in Appl. Microbiol. 21: 207-209.*

Carrasco CE, Alvarez HJ, Ortiz N, Bisbal M, Arias W, Santo Domingo JW, Hazen TC (1997). Multiple antibiotic resistant Escherichia coli from a tropical rain forest stream in Puerto Rico. *Carrib. J. Sci. 33:191-197.*

Carter MJ (2005). Enterically infecting viruses: Pathogenicity, transmission, and significance for food and waterborne infection. *J. Appl. Microbiol. 98: 1354-1380.*

Catley-Carlson M (1993). Foreword. In: *Water Supply and Sanitation Sector Monitoring Report 1993.*Water supply and sanitation collaborative council. *World Health Organization, Geneva, 57.*

Chapman D (1996). Water Quality Assessments- A guide to Use of Biota, Sediments and Water in Environmental Monitoring- *Second edition.*

Chen CH, Hsu BM, Wan MT (2007). Molecular detection and prevalence of enterovirus within environmental water in Taiwan. *J. Appl. Microbiol. 104: 817-823.*

Ching KZ, Nakano T, Chapman LE, Demby A, Robertson BH (2002). Genetic characterization of wild-type genotype VII hepatitis A virus. *J. Gen. Virol. 83: 53-60.*

Choi S, Jiang SC (2005). Real-time PCR quantification of human adenoviruses in urban rivers indicates genome prevalence but low infectivity. *Appl. Environ. Microbiol. 71: 7426-7433.*

Cleary J, Lai LC, Shaw RK, Straatman-Iwanowska A, Donnenberg MS, Frankel G, Knutton S (2004). Enteropathogenic *Escherichia coli* (EPEC) adhesion to epithelial cells: role of bundle-forming pili (BFP), EspA filaments and intimin. *Microbiol. 150: 527-538.*

Clinical Laboratory Standards Institute (CLSI) (2006). Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Approved Standard-Third Edition. *M31-A3, 28.*

Cloette TE, Da Silva E, Nel LH (1998). Removal of waterborne human enteric viruses and coliphages with oxidized coal. *Curr. Microbiol. 37: 23-27.*

Cohen MB, Nataro JP, Bernstein DI, Hawkins J, Roberts N, Staat MA (2005). Prevalence of diarrheagenic Escherichia coli in acute childhood enteritis: a perspective controlled study. *J. Pediatr. 146: 54-61.*

Cole D, Long SC, Sobsey MD (2003). Evaluation of F+ RNA and DNA Coliphages as Source Specific Indicators of Fecal Contamination in Surface Waters. *Appl. Environ. Microbiol. 69: 6507-6514.*

Cook NJ, Kendall BK, Iturriza-Gómara M, El-Attar L, Gray J (2004). The zoonotic potential of rotavirus. *J. Infect. 48: 289-302.*

Cooney MA, Gorrell RJ, Palambo EA (2001). Characterisation and phylogenetic analysis of the VP7 proteins of serotype G6 and G8 human rotaviruses. *J. Med. Microbiol. 50: 462-467.*

Cooperative Research Centre (CRC) (2007). Water Quality and Treatment, Drinking Water Quality. www.waterquality.crc.org.au/ - 12k.

Cunliffe NA, Dove W, Bunn JEG, Ramadam MB, Nyangao JWO, Riveron RL, Cuevas LE, Hart CA (2001). Expanding global distribution of rotavirus serotype G9: Detection in Libya, Kenya, and Cuba. *Emerg. Infect. Dis. 7: 890-892.*

Daley JK, Gechman LA, Skipworth J, Rall GF (2005). Poliovirus replication and spread in primary neuron cultures. *Virol. 340: 10-20.*

Davies-Colley RJ, Bell RG, Donnison AM (1994). Sunlight inactivation of enterococci and feacal coliforms in sewage effluent diluted in seawater. *Appl. Environ. Microbiol. 6: 2049-2058.*

DeBusk WF (1999). Wastewater treatment wetlands: contaminant removal processes. *University of Florida, IFAS Extension: Gainesville, FL, USA: 1-5.*

DEAT (2000). White paper on integrated pollution and waste management for South Africa: A policy on pollution prevention, waste minimization, impact management and remediation, Department Of Environmental Affairs and Tourism, *80*.

Department of Water Affairs and Forestry (DWAF) (1996). *South African Water Quality Guidelines-Domestic Water Use. 1-8.*

Department of Water Affairs & Forestry (DWAF) (1992). *Analytical Methods Manual*, DWAF, Pretoria.

Donnenberg MS, Tacket CO, James SP, Losonsky G, Nataro JP, Wasserman SS, Kaper JB, Levine MM (1993). Role of the eaeA gene in experimental enteropathogenic Escherichia coli infection. *J. Clin. Invest. 92: 1412-1417.*

Drinan J, Whiting NE (2001). Wastewater Regulations, Parameters, and Characteristics. *CRC Press LLC: 117-126.*

DuPont HL, Formal SB, Hornick RB, Snyder MJ, Libonati JP, Sheahan DG, LaBrec EH, Kolas JP (1971). Pathogenesis of Escherichia coli diarrhea. *N. Engl. J. Med. 285: 1-9.*

Edberg SC, Rice EW, Karlin RJ, Allen MJ (2000). Escherichia coli: the best biological drinking water indicator for public health protection. *J. Appl. Microbiol. 88: 106-116.*

Estes MK (2001). Rotavirus and their replication. In Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Strais SE (ed.), *Fields virology*, 4th ed. Lippincot William and Wilkins, Philadelphia, PA. *1747-1785.*

Faith NG, Shere JA, Brosch R, Arnold KW, Ansay SE, Lee MS, Luchansky JB, Kaspar CW (1996). Prevalence and clonal nature of Escherichia coli 0157:H7 on dairy farms in Winsconsin. *Appl. Environ. Microbiol. 62: 1519-1525.*

Fatoki OS, Gongwana P, Ogunfowakan AO (2003). Pollution assessment in the Keiskamma river and in the impoundment downstream. *Water SA. 29: 183-188.*

Fleet GH, Heiskanen P, Reid I, Buckle KA (2000). Foodborne viral illness-status in Australia. *Int. J. Food Microbiol. 59: 127-136.*

Fong TT, Lipp EK (2005). Enteric viruses of human and animals in aquatic environments: health risk, detection, and potential water quality assessment tools. *Microbiol. Mol. Biol. Rev. 69: 357-371.*

Fong TT, Phanikumar MS, Xagoraraki I, Rose JB (2010). Quantitative detection of human adenoviruses in wastewater and combined sewer overflows influencing a Michigan river. *Appl. Environ. Microbiol. 76: 715-723.*

Frankhauser RL, Monroe SS, Noel JS, Humphrey CD, Bresee JS, Parashar UD, Ando T, Glass RI (2002). Epidemiologic and molecular trends of "Norwalk-like viruses" associated with outbreaks of gastroenteritis in the United States. *J. Infect. Dis. 186: 1-7.*

Frydenberg A, Starr M (2003). Hand, foot and mouth disease. *Aust. Fam. Physician, 32: 594-595.*

Fuhrman JA, Liang X, Noble RT (2005). Rapid detection of enteroviruses in small volumes of natural waters by real-time quantitative reverse transcriptase PCR. *Appl. Environ. Microbiol. 71: 4523-4530.*

Gilgen M, Germann D, Lüthy J, Hübner Ph. (1997). Three-step isolation method for sensitive detection of enterovirus, rotavirus, hepatitis A virus, and small round structured viruses in water samples. *Int. J. Food Microbiol. 37: 189-199.*

Gjøen KV, Bruu AL (1997). Specific detection of coxsackie virus A by the polymerase chain reaction. *Clin Diagn. Virol. 8: 183-188.*

Glass RI, Bresee J, Jiang B, Gentsch J, Ando T, Fankhauser R, Noel J, Parashar U, Rosen B, Monroe SS (2001). Gastroenteritis viruses: an overview. *Novartis Found. Symp. 238: 525.*

Godfree AF, Kay D, Wyer MD (1997). Feacal streptococci as indicators of feacal contamination in water. *J. Appl. Microbiol. 83: 110-119.*

Goosney DL, Celli J Kenny B, Finlay BB (1999). Enteropathogenic Escherichia coli inhibits phagocytosis. *Infect. Immun. 67: 490-495.*

Gosalvez B, Navarro JA, Lorca A, Botella F, Sánchez-Pina MA, Pallas V (2003). Detection of Melon necrotic spot virus in water samples and melon plants by molecular methods. *J. Virol. Methods, 113:87-93.*

Grabow WOK, Favorov MO, Khudyakova NS, Taylor MB, Fields HA (1994). Hepatitis E seroprevalence in selected individuals in South Africa. *J. Med. Virol. 44: 384-388.*

Grabow WOK (1996). Waterborne diseases: Update on water quality assessment and control. *Water SA, 22: 193-202.*

Grammenou P, Spiliopoulou I, Sazakli E, Papapetropoulou M (2006). PFGE analysis of enterococcus isolates from recreational and drinking water in Greece. *J. Water and Health, 4: 263-269.*

Green DH, Lewis GD (1999). Comparative detection of enteric viruses in wastewater, sediment and oysters by reverse transcription - PCR and cell culture. *Water Res. 33: 1195-1200.*

Greening GE, Lewis GD (2007). Objective 2: Virus prevalence in shellfish. *ESR Report FW0659.*

Griffin DW, Donaldson KA, Paul JH, Joan BR (2003). Pathogenic human virus in coastal waters. *Clin. Microbiol. Rev. 16:129–143.*

Grinstein S, Melnick JL, Wallis C (1970). Virus isolation from sewage and from a stream receiving effluents of sewage treatment plants. *Bull. World Health Organization, 42: 291-296.*

Gust ID, Coulepis AG, Feinstone SM, Lorcanini SA, Moritsugu Y, Najera R, Siegl G (1983). Taxonomic classification of hepatitis A virus. *Intervirol. 20: 1-7.*

Gutiérrez MF, Alvarado MV, Martınez E, Ajami NJ (2007). Presence of viral proteins in drinkable water-Sufficient condition to consider water a vector of viral transmission? *Water Res. 41: 373-378.*

Gutièrrez-Aguirre I, Steyer A, Boben J, Gruden K, Poljšak-Prijatelj, Ravnikar M (2008). Sensitive detection of multiple rotavirus genotypes with a single reverse transcription real-time quantitative PCR assay. *J. Clin. Microbiol. 46: 2547-2554.*

Halliday ML, Kang LY, Zhou TK, Pan QC, Fu TY, Huang YS, Hu SL (1991). An epidemic of hepatitis A attributable to the ingestion of raw clams in Shangai, China. *J. Infect. Dis. 164: 852-859.*

Harm W (1980). Biological effects of ultraviolet radiation. *Cambridge University Press,* Cambridge, United Kingdom.

Harrington SM, Dudley EG, Nataro JP (2006). Pathogenesis of enteroaggregative Escherichia coli infection. *FEMS Microbiol. Lett. 254: 12-18.*

Harrington SM, Strauman MC, Abe MC, Nataro PJ (2005). "Aggregative adherence fimbriae contribute to inflammatory response of epithelial cells infected with enteroaggregative Escherichia coli." *Cell. Microbiol. 7: 1565-1578.*

Havelaar AH, van Olphen M, Drost YC (1993). F-Specific RNA Bacteriophages Are Adequate Model Organisms for Enteric Viruses in Fresh Water. *Appl. Environ. Microbiol. 59: 2956-2962.*

He JW, Jiang S (2005). Quantification of enterococci and human adenoviruses in environmental samples by real-time PCR. *Appl. Environ. Microbiol. 71: 2250-2255.*

Ho M, Chen ER, Hsu KH, Twu SJ, Chen KT, Tsai SF, Wang JR, Shih SR (1999). An epidemic of enterovirus 71 infection in Taiwan. *N. Engl. J. Med. 341: 929-935.*

Hoofnagle JH, Biscegile AM (1997). The treatment of chronic viral hepatitis. *N Engl. J. Med. 336: 347-356.*

Huang DB, Mohanty A, DuPont HL, Okhuysen PC, Chiang T (2006). A review of an emerging enteric pathogen: enteroaggregative Escherichia coli*. J. Med. Microbiol. 55: 1303-1311.*

Huertasa E, Salgota M, Hollenderb J, Weberb S, Dottb W et al. (2008). Key objectives for water reuse concepts. *Desalination, 218: 120-131.*

Hunt DR, Saab S (2009). Viral hepatitis in incarcerated adults: a medical and public health concern. *Am. J. Gastroenterol. 104: 1024-1031.*

Hussain MI, Wani SA, Nagamani K, Fayaz I, Mir MA, Nabi A, Qureshi K, Khan MA, Nishikawa Y (2010). Isolation and characterization of enteroaggregative, enterotoxigenic, diffusely adherent *Escherichia coli* and *Salmonella* Warthington from human diarrhoeic feacal samples in Kashmir and Secunderabad, India. *World J. Microbiol. Biotechnol. 26: 1883-1889.*

Hutin Y, Pool V, Cramer E, Nainan O, Weth J, Williams I et al. (1999). A multistate foodborne outbreak of hepatitis A. National hepatitis A investigation team*. N. Engl. J. Med. 340: 595-602.*

Hutson AM, Atmar RL, Estes MK (2004). Norovirus disease: changing epidemiology and host susceptibility factors. *Trends Microbiol. 12: 279-287.*

Igbinosa EO, Obi LC, Okoh AI (2009). Occurrence of potentially pathogenic vibrios in the final effluents of a wastewater treatment facility in a rural community of the Eastern Cape Province of South Africa. *Res. Microbiol. 160: 531-537.*

Igbinosa EO, Okoh AI (2009). Impact of discharge wastewater effluents on the physic-chemical qualities of a receiving watershed in a typical rural community. *Int. J. Environ. Sci. Tech. 6: 175-182.*

Ike AC, Brockmann SO, Hartelt K, Marschang RE, Contzen M, Oehme RM (2006). Molecular epidemiology of norovirus in outbreaks of gastroenteritis in southwestern Germay from 2001to 2004. *J. Clin. Microbiol. 44: 1262-1267.*

Integrated Publishing (2008). Physical characteristics of wastewater. Available from: *http//www.tpub.com/utilities/71.htm.*

Irving LG, Smith FA (1981). One-year survey of enteroviruses, adenoviruses and reoviruses isolated from effluent at an activated-sludge purification plant. *Appl. Environ. Microbiol. 41: 51-59.*

Jenkins D, Richard MG, Daigger GT (2004). Manual on the Causes and Control of Activated Sludge Bulking, Foaming, and Other Solids Separation Problems. *IWA Publishing, CRC Press,* London, UK. ISBN 1-84339-046-9.

Jiang S, Noble R, Chu W (2005). Real time quantitative PCR for enteric adenovirus serotype 40 in environmental waters. *Can. J. Microbiol. 51: 393-398.*

Jiang ZD, Okhuysen PC, Guo DC, et al. (2003). "Genetic susceptibility to enteroaggregative Escherichia coli diarrhea: polymorphism in the interleukin-8 promotor region". J. *Infect. Dis. 188: 506-511.*

Jiménez-Clavero MA, Escribano-Romero E, Mansilla C, Gómez N, Córdoba L, Roblas N, Ponz F, Ley V, Sáiz JC (2005). Survey of Bovine Enterovirus in Biological and Environmental Samples by a Highly Sensitive Real-Time Reverse Transcription-PCR. *Appl. Environ. Microbiol. 71: 3536-3543.*

Kallenbach NR, Cornelius PA, Negus D, Montgomerie D, Englander S (1989). Inactivation of viruses by ultraviolet light. *Cur. Stud. Hematol. Blood Transfus. 56: 70-82.*

Kaur P, Chakraborti A, Asea A (2010). Enteroaggregative Escherichia coli: An emerging enteric foodborne pathogen. *Int. Persp. Infect. Dis. ID 254159, doi:10.1155/2010/254159.*

Kay D, Bartram J, Pruss A, Ashbolt N, Wyer MD, Fleisher JM, Fewtrell L, Rogers A et al. (2004). Derivation of numerical values for the World Health Organization guidelines for recreational waters. *Water Res. 38: 1296-1304.*

Kittigul L, Raengsakulrach B, Siritantikorn S, et al. (2000). Detection of poliovirus, Hepatitis a Virus, and Rotavirus from Sewage and Water Samples. *Southeast Asian J. Trop. Med Public Health, 31: 41-46.*

Kittigul L, Uthaisin A, Ekchaloemkiet S, Utrarachkij F, Luksamijarulkul P (2006). Detection and characterization of hepatitis A virus in water samples in Thialand. *J. Appl. Microbiol. 100: 1318-1323.*

Kocwa-Haluch R (2001a). Waterborne enteroviruses as a hazard for human health. *Pol. J. Environ. Stud. 10: 485–487.*

Kocwa-Haluch R (2001b). Viruses and their occurrence in waters and wastewater. *In Polish*. Tech. University of Kielce.

Koopmans M, Duizer E (2004). Foodborne viruses: an emerging problem. *Int. Food Microbiol. 90: 23-41.*

Koopmans M, Vinje J, de Wit M, Leenen I, van der Poel W, van Duynhoven Y (2000). Molecular epidemiology of human caliciviruses in Netherlands. *J. Infect. Dis. 181: 262-269.*

Koopmans M, Vinje J, Duizer E, de Wit M, van Duijnhoven Y (2001). Molecular epidemiology of human enteric caliciviruses in The Netherlands. *Novartis Found. Symp. 238: 197-218.*

Kumar V, Das S, Jameel S (2010). The biology and pathogenesis of hepatitis viruses. *Curr. Sci. 98: 312-325.*

Laverick MA, Wyn-Jones AP, Carter MJ (2004). Quantitative RT_PCR for the enumeration of noroviruses (Norwalk-like viruses) in water and sewage. *Lett. Appl. Microbiol. 39: 127-136.*

Lee SG, Lin TL, Ko YF, Yang WC, Wu SL, Wang SF, Yang SY, Yang CY, et al. (2005a). Analysis of epidemic serotypes of enterovirus in Taiwan during 1998- 2004. *Epidemiol. Bull. 21:96-117.*

Lee SH, Lee C, Lee KW, Cho HB, Kim SJ, (2005b). The simultaneous detection of both enteroviruses and adenoviruses in environmental water samples including tap water with an integrated cell culture-multiplex-nested PCR procedure. *J. Appl. Microbiol. 98: 1020-1029.*

LeJeune JT, Besser TE, Hancock DD (2005). Effect of sand and sawdust bedding materials on the fecal prevalence of Escherichia coli 0157:H7 in dairy cows. *Appl. Environ. Microbiol. 71: 326-330.*

Levine MM, Nataro JP, Karch H, Baldini MM, Kaper JB, Black RE, Clements ML, O'Brien AD (1985). The diarrheal response of humans to some classic serotypes of enteropathogenic Escherichia coli is dependent on a plasmid encoding an anteroadhesiveness factor. *J. Infect. Dis. 152: 550-559.*

Li JW, Wang XW, Rui QY, Song N, Zhang FG, Ou YC, Chao FH (1998). A new and simple method for concentration of enteric viruses from water. *J. Virol. Methods, 74: 99-108.*

Lietzau S, Raum E, von Baum H, Marre R, Brenner H (2006). Clustering of antibiotic resistance of E. coli in couples: suggestion for a major role of conjugal transmission. *BMC Infect. Dis. 6:119-124.*

Lipp EK, Schmidt N, Luther M, Rose JB (2001). Determining the effects of El Nin˜o-southern oscillation events on coastal water quality. *Estuaries, 24: 491-497.*

Liu P, Aitken K, Kong YY et al. (2000). The tyrosine kinase p561ck is essential in coxsackievirus B3-mediated heart disease. *Nat. Med. 6: 429-434.*

Lopes LM, Fabbricotti SH, Ferreira AJP, Kato MAMF, Michalski J, Scaletsky CA (2005). Heterogeneity among strains of diffusely adherent Escherichia coli isolated in Brazil. *J. Clin. Microbiol. 43: 1968-1972.*

Low JC, Mckendrick IJ, Fenlon D, Naylor SW, Currie C, Smith DGE, Allison L, Gally DL (2005). Rectal carriage of enterohemorrhagic Escherichia coli 0157 in slaughtered cattle. *Appl. Environ. Microbiol. 71: 93-97.*

Lund E, Hedstrom C, Jantzen W (1969). Occurrence of enteric viruses in waste water after activated sludge treatment. *J. Water Pollut. Contr. Fed. 41: 169.*

Lutwick LI, Hussain S (2006). Echoviruses. eMedicine Infectious Diseases, *An update.*

Maghsoudi N, Khalipour A Kamali M, Zeinoddini M (2007). Cloning and expression of coxsackievirus B3 viral protein-1 in *E. coli*. *Iranian Biomed. J. 11: 147-152.*

Maguire AJ, Green J, Brown DWG, Desselberger U, Gray JJ (1999). Molecular epidemiology of outbreaks of gastroenteritis associated with small round structured viruses in East Anglia, United Kingdom, during the 1996-1997 season. *J. Clin. Microbiol. 37: 81-89.*

Mahdy HM, Fareid MA, Negm ME (2010). Toxin of Enterotoxigenic E. coli Causing Gastroenteritis in Children. J. *Appl. Sci. Res. 6: 756-776.*

Maier RM, Pepper IL (2000). Wastewater engineering: treatment and reuse. *Environ. Microbiol. Second edition.*

Maunula L, Miettinen IT, von Bonsdorff CH (2005). Norovirus outbreaks from drinking water. *Emerg. Infect. Dis. 11: 1716-1721*.

Maurelli AT, Fernández RE, Bloch CA, Rode CK, Fasano A (1998). "Black hole" and bacterial pathogenicity: A large genomic deletion that enhances the virulence of Shigella spp. and enteroinvasive Escherichia coli. *Proc. Natl. Acad. Sci. 95: 3943-3948.*

Medema GJ, Payment P, Dufour A, Robertson W, Waite M, Hunter P, Kirby R, Andersson Y (2003). Safe drinking water: an ongoing challenge. In: Assessing microbial safety of drinking water: Improving approaches and methods. DUFOUR A. et al. (Editors), WHO Drinking Water Quality Series, OECD – WHO, Paris, France *(IWA Publishing, London),* Chapter 1.

Melnick JL (1982). Classification of hepatitis A virus as enterovirus type 72 and of hepatitis B virus as hepadnavirus type 1. *Intervirol. 18: 105-106.*

Melnick JL, Emmons I, Opton E, Coffey JH (1958). Coxsackieviruses from sewage. *Am. J. Hyg. 59: 185.*

Meng J, Zhao S, Doyle MP, Joseph SW, (1998). Antibiotic resistance of Escherichia coli 0157:H7 and 0157: NM isolated from animals, food, and humans. *J. Food Prot. 61: 1511-1514.*

Meraz IM, Arikawa K, Ogasawara J et al. (2006). Epithelial cells secrete interlekin-8 in response to adhesion and invasion of diffusely adhering Escherichia coli lacking Afa/Dr genes. *Microbiol. Immun. 50: 159-169.*

Meraz IM, Jiang ZD, Ericsson CD, Bourgeois AL, Steffen R, Taylor DN, Hernandez N, DuPont HL (2008). Enterotoxigenic Escherichia coli and diffusely adherent E. coli as likely

causes of a proportion of pathogen-negative traveler's diarrhea- A PCR based study. *J. Travel Med. 15: 412-418.*

Minor PD (1991). Picornaviridae. In Classification and Nomenclature of Viruses. Fifth Report of the International Committee on Taxonomy of Viruses. Edited by Francki RIB, Fauquet CM, Kundson DL, Brawn F. Vienna & New York: *Springer Verlag, 320–326.*

Momba MNB, Malakate VK, Theron J (2006). Abundance of pathogenic *Escherichia coli, Salmonella typhimurium* and *Vibrio cholerae* in Nkonkobe drinking water sources. *J. Water Health, 4: 289-296.*

Monceyron C, Grinde B (1994). Detection of hepatitis A virus in clinical and environmental samples by immunomagnetic separation and PCR. *J. Virol. Methods, 46: 157-166.*

Mulders MN, Salminen M, Kalkkinen N, Hovi T (2000). Molecular epidemiology of coxsackievirus B4 and disclosure of the correct VP1/2A^{pro} cleavage site: evidence for high genomic diversity and long term endemicity of distinct genotypes. *J. Gen. Virol. 81: 803-812.*

Mundy LM, Sahm DF, Gilmore M (2000). Relationships between enterococcal virulence and antimicrobial resistance. *Clin. Microbiol. 13: 513-522.*

Murray BE (1990). The Life and Times of the Enterococcus. *Clin. Microbiol. Rev. 3: 46-65.*

Murray BE (2000). Vancomycin-resistant enterococcal infections. *N. Engl. J. Med. 342: 710-721.*

Myers DN, Stoeckel DM, Bushon RN, Francy DS, Brady AMG (2007). Fecal indicator bacteria: *U.S. Geological Survey Techniques of Water-Resources Investigations*, book 9, chap. A7, section 7.1 (version 2.0), available from http://pubs.water.usgs.gov/twri9A/.

Nagulapallay SR (2007). Antibiotic resistance patterns in municipal wastewater bacteria-A thesis. *Kansas State University: 1-89.*

Nasri D, Bouslama L, Omar S, Saoudin H, Bourlet T, Aouni M, Pozzetto B, Pillet S (2007). Typing of human enterovirus by partial sequencing of VP2. *J. Clin. Microbiol. 45: 2370 -2379.*

Nataro JP (2005). "Enteroaggregative Escherichia coli pathogenesis." *Curr. Opin. Gastroentero. 21: 4-8.*

Nataro JP, Kaper JB (1998). Diarrheagenic Escherichia coli. *Clin. Microbiol. Rev. 11: 142-*201.

Nataro JP, Mai V, Johnson J, Blackwelder WC, Heimer R, Tirrell S, et al. (2006). Diarrheagenic Escherichia coli infection in Baltimore, Maryland, and New Haven, Connecticut. *Clin. Infect. Dis. 43: 402-407.*

Niu M, Polish L, Robertson B, Khanna B, Woodruff B, Shapiro C et al. (1992). Multistate outbreak of hepatitis A associated with frozen strawberries. *J. Infect. Dis. 166: 518-524.*

Nwachuku N, Craun FC, Calderon RL (2002). How effective is the TCR in assessing outbreak vulnerability. *J. Am. Water Works Assoc. 94: 88-96.*

Odjadjare EEO, Okoh AI (2009). Prevalence and distribution of Listeria pathogens in the final effluents of a rural wastewater treatment facility in the Eastern Cape Province of South Africa. *World J. Microbiol. Biotech. 26: 297-307.*

Odjadjare EEO, Igbinosa EO, Okoh AI (2010). Microbial and physichochemical quality of an urban reclaimed wastewater used for irrigation and aquaculture in South Africa. *African J. Microbiol. Res. 5: 2179-2186.*

Okeke IN, Fayinka ST, Lamikanra A, (2000). Antibiotic resistance trends in *E. coli* from apparently healthy Nigerian students (1986-1998). *Emerg. Infect. Dis. 6: 393-396.*

Okeke IN, Lamikanra A, Steinrück H, Kaper JB (2000). Characterization of Escherichia coli Strains from Cases of Childhood Diarrhea in Provincial Southwestern Nigeria. *J. Clin. Microbiol. 38: 7-12.*

Okoh AI, Odjadjare EEO, Igbinosa EO, Osode AN (2007). Wastewater treatment plants as a source of microbial pathogens in the receiving watershed. *Afr. J. Biotech. 6: 2932-2944.*

Okoh AI, Sibanda T, Gusha SS (2010). Inadequately treated wastewater as a source of human enteric viruses in the environment. *Int. J. Environ. Res. Public Health, 7: 2620-2637.*

Omar KB, Barnard TG (2010). The occurrence of Pathogenic Escherichia coli in South African wastewater treatment plants as detected by multiplex PCR. *Water SA (Online)*; Young Water Professionals Special Edition 2010, *36: 172-176.*

Osode AN, Okoh AI (2008). Enterotoxigenic Escherichia coli (ETEC): A Recurring Decimal in Infants and traveler's diarrhea. *Rev. Environ. Health, 23: 135-148.*

Ostioch M, Aimo E, Fassina D, Barbaro J, Vazzoler M, Soccorso C, Rossi C (2010). Biological impact on the coastal belt of the province of Venice (Italy, Northern Adriatic Sea): preliminary analysis for the characterization of the bathing water profile. *Environ. Sci. Pollut. Res. DOI 10.1007/s11356-010-0368-1.*

Padalko E, Ohnishi T, Matsushita K, Sun H, Fox-Talbot K, Bao C, Baldwin III WM, Lowenstein CJ (2004). Peroxynitrite inhibition of Coxsackievirus infection by prevention of viral RNA entry. *PNSA, 101: 11731-11736.*

Palacios G, Oberst MS (2005). Enteroviruses as agents of emerging infectious diseases. *J. Neur. Virol. 11: 424-233.*

Pallanch MA, Ross RP (2001). Enteroviruses: polioviruses, coxsackievirus, echoviruses and newer enteroviruses. *In Fields Virology, 4th edn ed. Knipe D, Howley:723–775*. Baltimore, MD: Williams and Wilkins Publishers.

Parry JV, Perry KR, Panday S, Mortimer PP (1989). Diagnosis of hepatitis A and B by testing saliva. *J. Med. Virol. 28: 255-260.*

Pavlov DN, Van Zyl WB, Van Heerden J, Grabow WOK, Ehlers MM (2005). "Prevalence of vaccine-derived polioviruses in sewage and river water in South Africa". *Water Res. 39: 3309-3319.*

Payment P, Tremblay M, Trudel M (1985). Relatively resistance to chlorine of poliovirus and coxsackievirus isolated from environmental sources and drinking water. *Appl. Environ. Microbiol. 49: 981-983.*

Payment P, Trudel M, Plante R (1985). Elimination of virus and indicator bacteria at each step of treatment during preparation of drinking water at seven water treatment plants. *Appl. Environ. Microbiol. 49:1418-1428.*

Pianetti A, Baffone W, Citterio B, Casaroli A, Bruscolini F, Salvaggio L (2000). Presence of enteroviruses and reoviruses in the waters of the Italian coast of the Adriatic Sea. *Epidemiol. Infect. 125: 455-462.*

Pons MN, Spanjers H, Baetens D, Nowak O, Gillot S, Nouwen J, Schuttinga N (2004). Wastewater characteristics in Europe- A survey. *Euro. Water Assoc. 4: 1-10.*

Putnam S, Riddle M, Wierzba T, Pittner B, Elyazeed R, El-Gendy A, Ruo M, Clemens J, Frenck R (2004). Antimicrobial susceptibility trends among Escherichia coli and Shigella spp. Isolated from rural Egyptian pediatric populations with diarrhea between 1995 and 2000. *Clin Microbiol. Infect. 10: 804-814.*

Rangel JM, Sparling PH, Crowe C, Griffin PM, Swerdlow DL (2005). Epidemiology of Escherichia coli 0157:H7 outbreaks, United States, 1982-2002. *Emerg. Infect. Dis. 11: 603 609.*

Government Gazette No. 9225 (1984). Requirement for the purification of wastewater or effluent, *Regulation No. 991.*

Reynolds KA, Gerba CP, Pepper IL (1996). Detection of infectious enteroviruses by an integrated cell culture-PCR procedure. *Appl. Environ. Microbiol. 62: 1424-1427.*

Reynolds KA (2004). Integrated cell culture/PCR detection of enteric viruses in environmental samples. *Methods Mol. Biol. 268: 69-78.*

Rose JB, Epstein PR, Lipp EK, Sherman BH (2001). Climate variability and change in the United States: potential impacts on water- and foodborne diseases caused by microbiologic agents. *Environ. Health Perspect. 109: 211-221.*

Rose JB, Gerba CP, Singh SN, Toranzos GA, Keswick B (1986). Isolation of entero- and rotaviruses from a drinking water facility. *J. Am. Water Works Assoc. 78: 56-61.*

Rose JB, Mullinax RL, Singh SN, Yates MV, Gerba CP (1987). Occurrence of rotaviruses and enteroviruses in recreational waters of Oak Creek, Arizona. *Water Res. 21: 1375-1381.*

Rotbart HA, Webster AD (2001). Treatment of potentially life-threatening enterovirus infections with pleconaril. *Clin. Infect. Dis. 32: 228-235.*

Ryder S, Beckingham I (2001). "Acute hepatitis". *BMJ, 322: 151-153.*

Sarkar S (2008). "Diffusely adherent Escherichia coli (DAEC) as a cause of acute diarrhea". Texas Medical Center dissertations (via ProQuest). *Paper AAI 1459796.*

SANCOR (1984). Report of the hocworking committee. Lusher JA (Ed). Established by the Marine Pollution Committee of South African National Committee for Oceanographic Research, South African Network for Coastal and Oceanic Research, *25.*

Scaletsky ICA, Fabbricotti SH, Aranda KR, Morais MB, Fagundes-Neto U (2002). Comparison of DNA hybridization and PCR assays for detection of putative pathogenic enteroadherent Escherichia coli. *J. Clin. Microbiol. 40: 1254-1258.*

Sheng H, Davis MA, Knecht HJ, Hovde CJ (2004). Rectal administration of Escherichia coli 0157:H7: novel model for colonization of ruminants. *Appl. Environ. Microbiol. 70: 4588-4595.*

Shere JA, Kaspar CW, Bartlett KJ, Linden SE, Norell B, Francey S, Schaefer DM (2002). Shedding of Escherichia coli 0157:H7 in dairy cattle housed in a confined environment following waterborne inoculation. *Appl. Environ. Microbiol. 68: 1947-1954.*

Shimizu H, Utama A, Yoshii K, Yoneyama T et al. (1999). Enterovirus 71 from fatal and nonfatal cases of hand, foot and mouth disease epidemics in Malaysia, Japan and Taiwan in 1997-1998. *Japan J. Infect. Dis. 52: 12-15.*

Sobsey MD, Shields PA, Hauchman FS, Hazard RL, Caton LW (1986). Survival and transport of hepatitis A virus in soils, groundwater and wastewater. *Water Sci. Tech. 18: 97-106.*

Spellman FP, Drinan J (2008). Wastewater Treatment Plant Operations Made Easy. *Deshtech Publishers: 98.*

Sperling V (2006). Wastewater characteristics, Treatment, and Disposal. *IWA Publishing: 28.*

Steyn M, Jagals P (2000). Guidelines for the Re-Use of Treated Wastewater: Public Health Protection or Denial of Essential Resources? *Presented at WISA 2000 Biennial Conference,* Sun City, South Africa.

Talebi M, Rahimi F, Katouli M, Möllby R, Pourshafie MR (2008). Epidemiological Link Between Wastewater and Human Vancomycin-Resistant *Enterococcus faecium* Isolates. *Curr. Microbiol. 56: 468-473.*

Tchobanoglous G, Burton FL, Stensel HD (2003). Metcalf & Eddy, Inc.'s Wastewater Engineering: Treatment, Disposal, and Reuse, 4th Edition. *McGraw-Hill, Inc.*, New York: 1819.

Tennant SM, Tauschek M, Azzopardi K, Bigham A, Bennett-Wood V, Hartland EL, Qi W, Whittam TS, Robins-Browne RM (2009). Characterization of atypical enteropathogenic *E. coli* strains of clinical origin. *BioMed Central (BMC) Microbiol. 9: 117.*

Thieme T, Yoshihara P, Piacentini S, Beller M (1992). Clinical evaluation of oral fluid samples for diagnosis of viral hepatitis. *J. Clin. Microbiol. 30: 1076.*

Tiefenthaler LL, Stein ED, Lyon GS (2009). Fecal indicator bacteria (FIB) levels during dry weather from Southern California reference streams. *Environ. Monit. Assess. 155: 477-492.*

Tobe T, Hayashi T, Han CG, Schoolnik GK, Ohtsubo E, Sasakawa C (1999). Complete DNA sequence and structural analysis of the enteropathogenic *Escherichia coli* adherence factor plasmid. *Infect. Immun. 67: 5455-5462.*

Tobe T, Sasakawa C (2002). Species-specific cell adhesion of enteropathogenic *Escherichia coli* is mediated by type IV bundle-formong pili. *Cell. Microbiol. 4: 29-42.*

Todar K (2008). Pathogenic *Escherichia coli*. *In: Todar's Online Textbook on bacteriology*. University of Wisconsin, Madison, Dept. of Bacteriology.

Tong MJ, el-Farra NS, Reikes AR Co RL (1995). Clinical outcomes after transfusion-associated hepatitis C. *N. Engl. J. Med. 332: 1463-1466.*

Trabulsi LR, Keller R, Gomes TAT (2002). Typical and atypical enteropathogenic *Escherichia coli*. *Emerg. Infect. Dis. 8: 508-513.*

Tsai YL, Sobsey M, Sangermano LR, Palmer CJ (1993). Simple method of concentrating enteroviruses and hepatitis A virus from sewage and ocean water for rapid detection by reverse transcriptase-polymerase chain reaction. *Appl. Environ. Microbiol. 59: 3488-3491.*

U.S. Environmental Protection Agency (USEPA) (2000). Proposed ground water rule EPA 815 F-00–003. Office of Ground Water. *Environmental Protection Agency*, Washington, D.C. http: //www.epa.gov/305b/2000report/).

U.S. Environmental Protection Agency (USEPA) (2006). Implementing the BEACH Act of 2000: Report to Congress. *EPA-823-R-06-001, 111 pp.*

U.S. Environmental Protection Agency (USEPA) (2009). *Analytical methods approved for drinking water compliance monitoring under the total coliform rule*. National Primary Drinking Water Regulations. *1-18.*

United Nations (UN) (2003a). Committee on Economic Social and Cultural Rights. General Comment No. 15 (2002). *The Right to Water. E/C.12/2002/11*. United Nations Social and EconomicCouncil. Available from website :www.unhchr.ch/tbs/doc.nsf/0/a5458d1d1bbd713fc1256cc400389e94?

United Nations (UN) (2003b). Wastewater treatment technologies- *A general review.* New York, 03-0695.

Uz G, Turak F, Afsar H (2004). Research of BOD and COD values of wastewaters that contain certain organic materials. Adnan Menderes University, *4th AACD congress*, Sept. 29-Oct. 3, Kupadasy-AYDIN, Turkey Proceedings book 177.

Van Tu P, Thao NTT, Perera D, Truong KH, Tien NTK et al. (2007). Epidemic and virologic investigation of hand, foot and mouth disease, Southern Vietnam, 2005. *Emerg. Infect. Dis.* (serial on internet). Available from: http://wwwnc.cdc.gov/eid/article/13/11/07-0632.htm.

Veldhuis JAE, Clemens FHLR, Sterk G, Berends BR (2010). Microbial risk associated with exposure to pathogens in contaminated urban flood water. *Water Res. 44: 2910-2918.*

Verboon-Maciolek MA, Krediet TG, van Loon AM, Kaan J, Galama JMD, Gerards LJ, Fleer A (2002). Epidemiological survey of neonatal non-polio enterovirus infection in the Netherlands. *J. Med. Virol. 66: 241-245.*

Vieira N, Bates SJ, Solberg OD, Ponce K, Howsmon R, Cevallos W, Trueba G, Riley L, Eisenberg JNS (2007). High prevalence of enteroinvasive *Escherichia coli* isolated in a remote region of northern coastal Ecuador. *Am. J. Med. Hyg. 76: 528-533.*

Vivier JC, Ehlers MM, Grabow WOK (2004). Detection of enteroviruses in treated drinking water. *Water Res. 38l: 2699-2705.*

Weintraub A (2007). Enteroaggregative Escherichia coli: epidemiology, virulence and detection. *J. Med. Microbiol. 56: 4-8.*

Widdowson MA, Sulka A, Bulens SN, et al. (2005). Norovirus and foodborne disease, United States, 1991-2000. *Emerg. Infect. Dis. 11: 95-102.*

Whitefield A, Bate G (1997). A review on information on temporarily open/closed estuaries in the warm and cool temperate biogeographic regions of South Africa, with particular emphasis on the influence of river flow on these systems. *WRC Report No. 1581/1/07.*

Wilkerson C, Samadpour M, van Kirk N, Roberts MC (2004). Antibiotic resistance and distribution of tetracycline resistance genes in *Escherichia coli* 0157:H7 isolates from humans and bovines. *Antimicrob. Agents and Chemothe. 48:1066-1067.*

Wolf MK (1997). Occurrence, distribution and associations of O and H serogroups, colonization factor antigens, and toxins of enterotoxigenic *Escherichia coli*. *Clin. Microbiol. Rev. 10: 569-584.*

World Health Organization (WHO) (2006). *Guidelines for Drinking-Water Quality. 1*

World Health Organization (WHO) (1999). New frontiers in the development of vaccines against enterotoxigenic (ETEC) and enteroheamorrhagic (EHEC) *E. coli* infections. *Weekly Epidemiol. Rec. 13: 98-100.*

Yates MV, Gerba CP, Kelley LM (1985). Virus persistence in groundwater. *Appl. Environ. Microbiol. 49: 778–781.*

Yosuke I, Sagara H, Yasuaki K, Azuma M, Kume K, Ogawa M, Nagai T, Gillespie PG, Sasakawa C, Handa H (2002). The Enteropathogenic *E. coli* Effector EspB Facilitates Microvillus Effacing and Antiphagocytosis by Inhibiting Myosin Function. *Cell Host & Microbe, 2: 383-392.*

Zaragoza C, Ocampo CJ, Saura M, McMillan A, Lowenstein CJ (1997). Nitric oxide inhibition of Coxsackie replication in vivo. *J. Clin. Invest. 100: 1760-1767.*

Zhanel GG, Weibe R, Dilay L, Thomson K, Rubinstein E, Hoban DJ, Noreddin AM, Karlowsky JA (2007). Comparative review of the carbapenems. *Drugs, 67: 1027-1052.*

Zhao T, Zhao P, West JW, Bernard JK, Cross HG, Doyle MP (2006). Inactivation of Enterohemorrhagic Escherichia coli in Rumen Content- or feces-Contaminated Drinking Water of Cattle. *Appl. Environ. Microbiol. 72: 3268-3273.*

Zuber M (1999). *E. coli*: Serotypes other than 0157:H7. MSPH DOH, Regional epidemiologist.

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