ABSTRACT

Field Evaluation of Improved Methods to Detect Infectious Group F Adenoviruses in source water

By

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Adenoviruses (Ads) are non-enveloped respiratory and enteric viruses containing a dsDNA genome. Group F ads 40 and 41 are of particular interest due to their persistence and abundance in ambient waters. Therefore, it is important to develop, verify, and apply a sensitive, specific, and efficient method to detect and quantify infectious adenoviruses in environmental samples of water and sewage. In this study the proposed method for infectivity assay is a combination of cell culture and mRNA reverse transcription (RT)-PCR for the viral hexon gene. In addition, the use of a housekeeping gene, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), as an internal control for mRNA expression was used to evaluate the performance of this method. Source water samples of 20 L were collected three times from six water treatment plants (WTP) and concentrated to small volumes suitable for molecular analysis by hollow fiber ultrafiltration (HFUF) for primary concentration and polyethylene glycol (PEG) precipitation, chloroform extraction, and ultracentrifugation for secondary concentration. Because ads 40 and 41 are difficult to culture, newly developed transactivated 293 cell lines (293 CMV and 293 RAS) were compared to the STD G293 cell line to examine the levels of detectable viral mRNA expression and the incubation time required. The overall results indicated that infectious ads can be successfully detected from environmental source water and sewage samples using the new

transactivated and standard cell lines. The housekeeping gene, GAPDH, as a positive control for the performance of CC/mRNA RT-PCR was found to be effective.

Keywords: *Adenoviruses, Concentration, Cell culture Infectivity, Viral mRNA, Reverse Transcription (RT)-PCR, Housekeeping Gene*

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ad – Adenovirus

CCL – 3 – Candidate Contaminant List 3

CC/mRNA RT-PCR – Cell Culture and mRNA Reverse Transcription Polymerase Chain Reaction

CPE – Cytopathic effect

Ct – Cycle Threshold (when referring to PCR results)

FC – Fecal coliforms

FIOs – Fecal Indicator Organisms

GAPDH – Glyceraldehyde-3-phosphate-dehydrogenase

GC – Genome copies

HAdvs – Human adenoviruses

HFUF – Hollow Fiber Ultracentrifugation

HK – Housekeeping gene

IU – Infectious units

PBS - Phosphate-Buffered Saline

PEG – Polyethylene glycol precipitation

US EPA – United States Environmental Protection Agency

TC – Total Coliforms

VP – Viral particles

WHO – World Health Organization

WTP – Water Treatment Plant

WWTP – Wastewater Treatment Plant

BACKGROUND AND INTRODUCTION

Contaminated water is the cause of high morbidity and mortality; especially in children under five. More than 1.4 million children die each year due to diarrheal related illnesses and 3.575 million people die each year from water-related disease. For this reason two of the targets in the United Nation's Millennium Development Goals include the improvement of access to safe water and the reduction in child mortality. Human pathogens such as bacteria, viruses, and parasites can be easily transmitted via water and collectively impact the health of large human populations. Water treatment is an important barrier to reduce the spread of pathogens through water used for drinking and other beneficial purposes where human exposure occurs. Standards have been developed using primarily bacterial indicators of fecal contamination to determine the microbiological safety of water, including source water quality, water treatment processes efficacy, and treated water quality. Many standards have been implemented to attempt to monitor water sources, the treatment of drinking water, and finished water quality in developed countries. Over the years, these standards have been mainly based on required testing for bacteria, like *E. coli* and coliforms with the hypothesis or assumption that these bacteria are good indicators for other microorganisms, like viruses. Most recently and certainly since the development of the Safe Drinking Water Act by US EPA, viruses, especially enteric viruses and their presence in drinking water sources and supplies, have been a concern. Researchers have been investigating the possibility of testing water for certain human pathogenic viruses that are suspected to be present in drinking water supplies; including human adenoviruses. Therefore, the focus of this research is on human adenoviruses and their public health importance as potential waterborne pathogens.

Adenoviruses have been included in the United States Environmental Protection Agency's drinking water contaminant candidate list (USEPA-CCL), they are second only to rotaviruses as the cause of death in children under five, and ad types 40 and 41 account for two-thirds of the diarrhea caused by adenoviruses (Ko, Cromeans, & Sobsey, 2003). In addition to direct drinking water concerns about adenovirus exposure and health risk, there is a broader concern that documents their presence in wastewater and the ease of becoming exposed to and acquiring disease via the fecal-oral route of transmission associated with waterborne exposures. Interest in adenovirus as a waterborne pathogen is also driven by its resistance to UV disinfection and its widespread occurrence in surface waters. If wastewater is not properly treated, adenoviruses can spread via waste water discharge and result in waterborne exposure that can cause illnesses. The occurrence of adenoviruses in waste waters is important to public health because waste water is often indirectly recycled and reused as a drinking water source. Therefore, if it is not properly treated, wastewater can become a source that releases adenoviruses into the water environment where human waterborne exposures can contribute to illness.

Research is needed that investigates the specific role viruses, such as adenoviruses play in water-borne illnesses. Enteric viruses show different resistance in comparison to indicator microorganisms such as *E. coli* bacteria to water treatment processes due to their different chemical and physical characteristics. Despite their potential importance as waterborne viral pathogens, information on waterborne viruses is limited because they are found in such low concentrations, requiring large volumes to detect them and the higher numbered ones, types 40 and 41, have been difficult to culture; especially from water. Therefore, while considered a high priority for regulation, adenoviruses have not been high on the radar for testing due to the difficulties in detecting them in water and no drinking water standards have been developed for

them. It is necessary that we continue to investigate the impact that waterborne viruses, especially enteric viruses, have on people and our environmental resources. It is the goal of this project to improve and develop methods for the detection and quantification of infectious human adenoviruses found in environmental samples such as sewage and water. It is important to develop the analytical tools needed and then apply them to provide knowledge about the risk associated with adenovirus' presence in water sources. Information on adenovirus presence and concentrations in drinking water sources and supplies makes it important to consider the need for and possible development and implementation of regulatory standards that ensure the safety of drinking water with respect to these viruses.

GENERAL PROJECT OBJECTIVE

To develop, verify, and apply a sensitive, specific, and efficient method to detect and quantify infectious adenoviruses in environmental samples of water and sewage.

Specific Objectives

1. Compare the detection of adenovirus in a new transactivated G293 cell line with adenovirus in the standard G293 cell line based on infectivity.
2. Demonstrate by using adenoviruses isolated from sewage the capability of the combined use of cell culture infectivity and mRNA reverse transcription real-time PCR for detecting infectious adenovirus in the aquatic environment as well as concurrent mRNA reverse transcription real-time PCR of a cell-HK gene as an internal control on the infectivity assay procedure.
3. Quantify the effectiveness of HFUF for primary concentration, PEG precipitation and ultracentrifugation for secondary concentration and the combined use of cell culture and mRNA RT-PCR for the detection of infectious adenovirus from source water.
4. Determine if suspended solids affect the recovery of adenoviruses in source waters. If adenoviruses tend to attach to suspended solids in source water, then describe a method for the elution of adenovirus from solids.
5. Determine if there are relationships between bio-indicators and the presence of infectious adenovirus in source waters.

EXPERIMENTAL DESIGN

In order to achieve the overall project goal of developing and applying methods to better detect and quantify enteric human adenoviruses (group F) in water, several tasks needed to be accomplished to verify and test the methods for recovery. Six water treatment plants were identified and their source water samples were collected and processed three different times as described in the respective methods sections. Having the ability to efficiently filter and concentrate large volumes of water while retaining the target microbes in the retentate is essential for an effective adenovirus recovery method in this project. Hence, prior to this study research had been done in our lab at UNC on the development and improvement of the hollow fiber ultrafiltration (HFUF) method. This method has been proven in previous studies to yield good virus recovery and was used for this present study. In addition, to address the difficulties that have been experienced in detecting human enteric ads using infectivity assays, improved cell culture and mRNA RT-PCR methods will be used. These methods will detect infectious adenoviruses, unlike direct PCR that only detects the presence/absence or concentrations of virus nucleic acid and providing no information regarding virus infectivity. The concentration steps involving HFUF, PEG precipitation, chloroform extraction, and ultrafiltration reduce the sample volume, while retaining the virus, to a volume that is suitable for molecular analysis by direct PCR or combined cell culture and mRNA RT-PCR. The source water samples were further analyzed to determine the capacity of fecal indicator microorganisms to predict the presence of infectious adenovirus in raw source water. The fecal indicator bacteria analyzed were *Escherichia coli,* enterococci, *Clostridium perfringens,* and *Bacteroides spp.* and the fecal virus indicators were somatic and male-specific F+ coliphages.

To accomplish objective two, a method was needed that could detect infectious adenoviruses found in environmental samples. Because laboratory strains can grow more efficiently than clinical strains or isolates from the environment, it is important to demonstrate that the cell culture-mRNA approach can be used for the detection of adenovirus found in environmental samples. Sewage samples are an appropriate source to evaluate the effectiveness of these methods for infectivity detection and quantification because adenoviruses are found in abundant amounts in human feces of infected persons and they are typically present in municipal sewage. Sewage samples were collected from Chapel Hill, Raleigh, and Durham, North Carolina and they were quantified for adenoviruses by direct PCR and CC/mRNA RT-PCR. In addition, the development of an internal control for the infectivity assays was needed to assist with validating the effectiveness of the methods used. The house keeping (HK) gene GAPDH was a shown to be a good candidate to achieve this goal and was incorporated into this study.

Source water samples that are high in suspended solids pose additional challenges for adenovirus recovery because the viruses may adsorb to suspended solids and either be lost during processing or difficult to detect by either cell culture infectivity or direct PCR. It has been well documented that turbidity or particles in water can interfere with the detection of viruses by cell culture infectivity and molecular amplification assays (field report). To better detect solids-associated adenoviruses in source water samples, a method had to be developed, applied and evaluated to elute the adenoviruses from the solids to improve adenovirus recovery. This method had to be evaluated for performance before applying it to the source water field samples. Based on the relatively high turbidity and the amount of suspended solids in certain raw source waters, it is anticipated that if viruses are present they would be present on or would adsorb to these solids. Such solids-association would present problem detecting these viruses in water sources

with high turbidity. To address the concern about recovery of solid-associated viruses by a new method, additional samples were collected from two WTP locations that had high levels of turbidity, New Orleans and St. Louis, to determine the partitioning of viruses. These samples were processed by a modification of the sample processing method in which the solids in the HFUF concentrate were removed by centrifugation and then eluted from the separated solids using a 10% beef extract solution in sodium phosphate citric acid buffer (pH 7.2). Both the aliquots of the source water HFUF retentate supernatant and the beef extract eluate of the separated suspended solids will be analyzed by real-time PCR for human adenoviruses.

Because enteric adenoviruses are usually difficult to culture due to the amount of time that is required to see CPE, additional research was done that compared the use of different G293 cell lines, the standard cell line and a genetically modified version intended to improve cell culture susceptibility to virus infection and thereby improve virus detection. UNC collaborated with Dr. GwangPyo Ko at Seoul National University in South Korea where he developed a new and improved transactivated G293 cell lines. Based in prior research by Dr. Ko's lab, it is suggested that this transactivated G293 cell line is better for supporting enteric adenovirus infectivity, resulting in a higher virus recovery in a shorter incubation time. In this study three cell lines, standard and two transactivated ones, are compared in our lab parallel with Dr. Ko's lab for the ability to detect enteric adenoviruses in environmental samples. Based on the results, recommendations will be made as to which cell line is better for the detection of infectious adenoviruses in environmental field samples.

Note: Details of all sampling, detection, and analysis methods mentioned are found in the respective methods section.

Figure 1. Water Treatment Plant (WTP) Water Sample Analysis

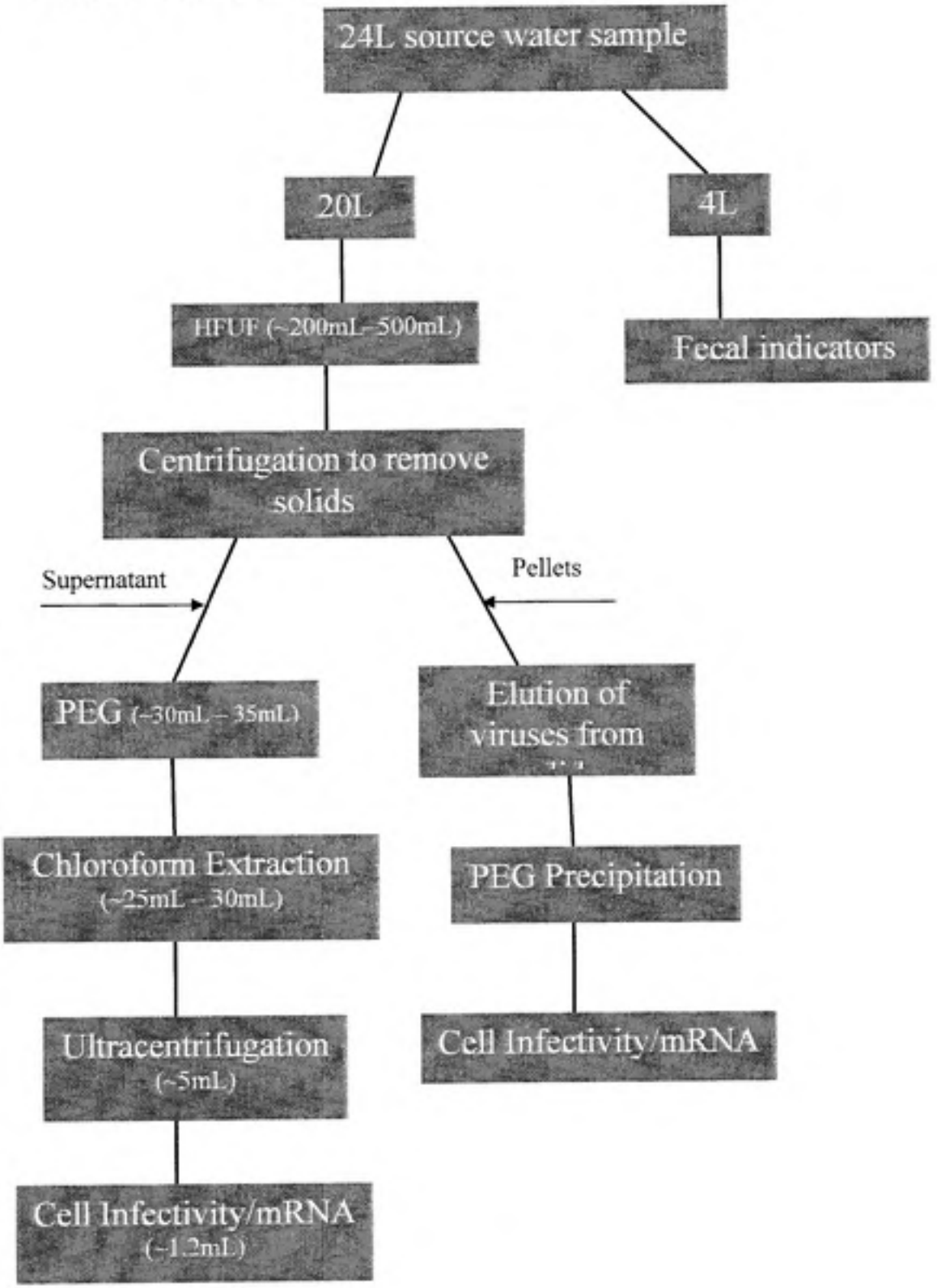


Figure 2. Virus Concentration from Sewage

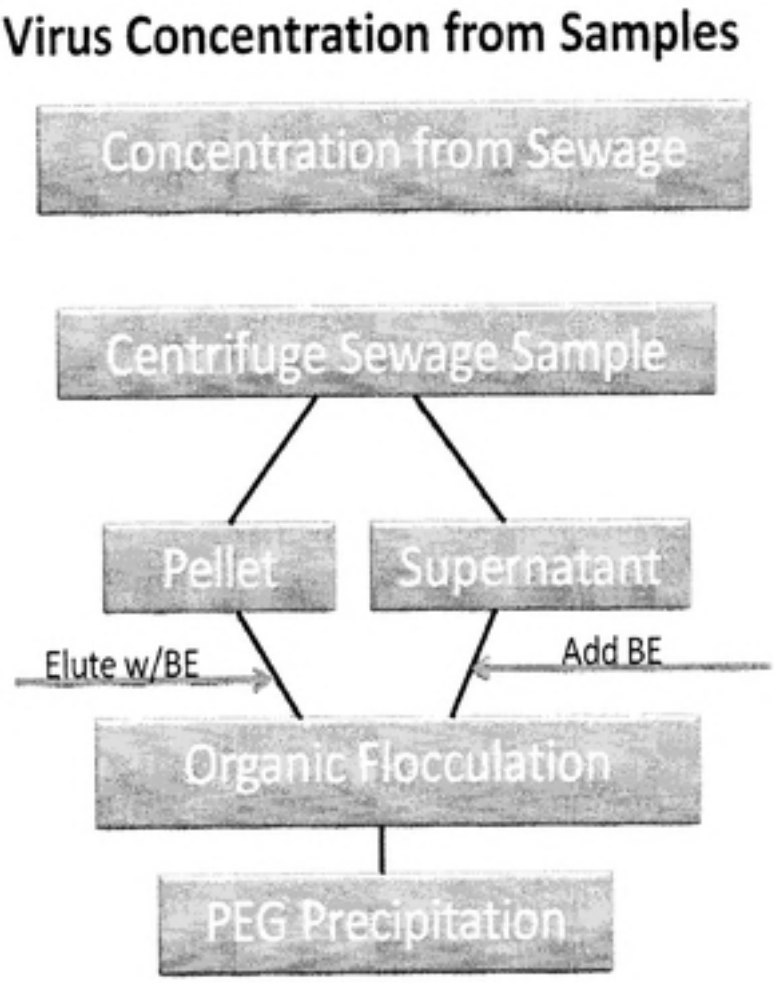
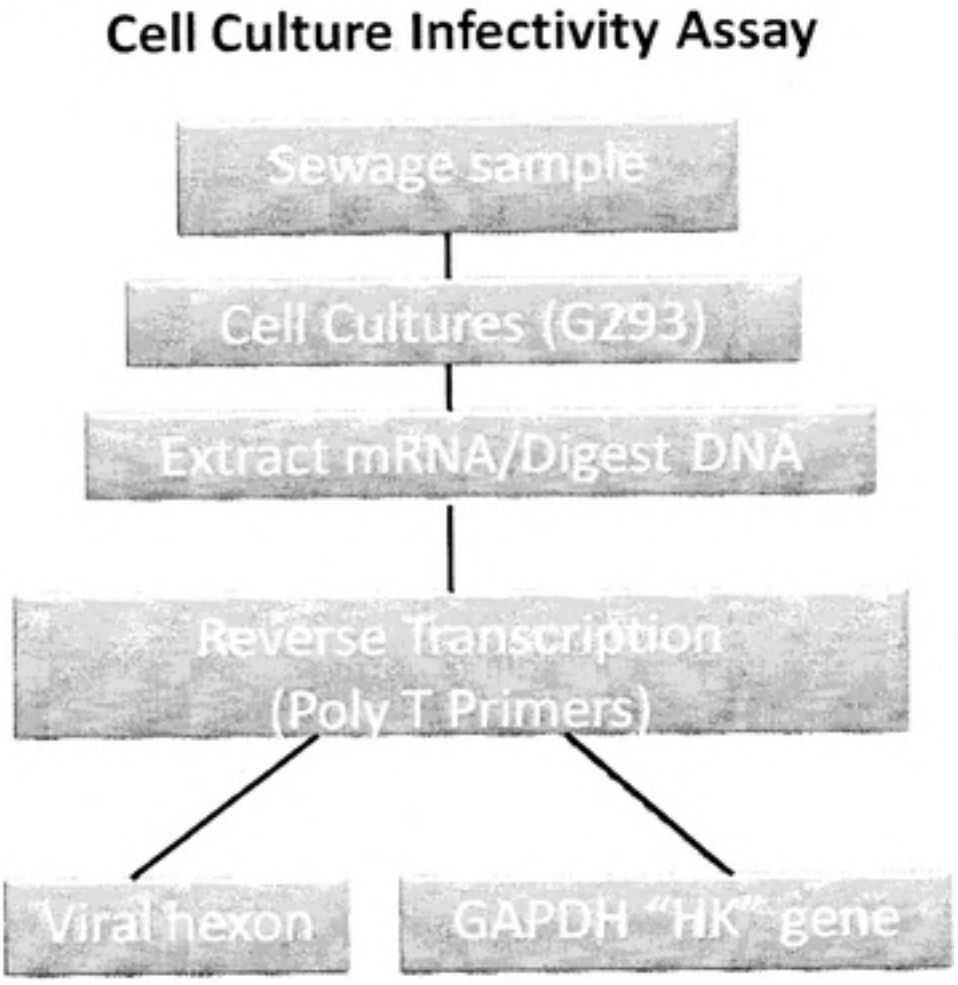


Figure 3. Cell Culture Infectivity Assay*



*(demonstrated using sewage but same method for water samples)

LITERATURE REVIEW

Waterborne Enteric Viruses

Waterborne enteric viruses are spread via fecal-oral transmission and due to the large quantities that are shed over long periods of time they are constantly found in surface water, ground water, and drinking water (Bosch, 2010; Cho, Lee, Cho, & Kim, 2000; Gerba & Rose, 1990). As a result, water sources are compromised; thus, increasing the chance for humans to acquire gastroenteritis, respiratory illness, diarrhea, and other adverse effects that are associated with these viruses. Enteric viruses contribute to the global burden of disease due to the number of deaths per year from diarrheal disease (1.7-2.5 million), with rotavirus being the leading cause of death (527,000 children under five, mostly in developing countries) (World Health Organization (WHO), 2010). Detecting and quantifying human enteric viruses in the water environment (wastewater, surface water, ground water, etc) is essential to understanding and quantifying their health impact on humans.

Some enteric viruses are thought to be more resistant than other viruses to the standard treatment processes for water and wastewater which contributes to their survival in the environment (Gerba & Rose, 1990; Payment, Armon, & Gerba, 1989). This resistance could be due to their physical and chemical properties. They are non-enveloped and they tend to have the ability to withstand various temperatures and environmental stresses. The viral particle is composed of two components, (the capsid and the genome), where the capsid is responsible for protecting the degradation of the genome (Rodriguez, Pepper, & Gerba, 2009). The genome includes positive-stranded RNA viruses, double-stranded RNA viruses, and double-stranded DNA viruses and each viral group has its own mechanism of translation and replication (Rodriguez et al., 2009). With the exception of two, adenovirus and parvovirus all contain RNA

genome. Enteric viruses, as do most viruses, require a host cell to replicate. They have the ability to enter a living host cell and essentially take over; replicating, producing more viral particles that will eventually be shed in the environment. In the case of enteric viruses, infected persons can shed as high as 109 viral particles per gram of stool during the acute phase and in some cases, like adenoviruses, viruses can be continuously released for months after the symptoms have subsided. This presents a public health problem because at low concentrations these viruses can cause illness if ingested (Ward, Akin, & D□Alessio, 1984). However, water bodies are not routinely monitored for the presence of human enteric viruses due to some of the challenges associated with performing these test. There are technical difficulties associated with the detection of human pathogenic viruses in environmental samples such as water and sewage.

Adenoviruses

Adenoviruses (Ads) are members in the Adenoviridae family and the genius is the Mastadenovirus. Adenoviruses are commonly divided into 6 serogroups (A-F); within these groups, there are 51 serotypes that infect humans. They are non-enveloped with a medium capsid in size (90-100 nm) and icosahedral in shape. Adenoviruses' nucleic acids are linear dsDNA. The capsid that encloses the genome is comprised of capsomers (240 hexons and 12 pentons) with fibers that are projected that are specific for each serotype (Mena & Gerba, 2009). The following characteristics may assist with adenoviruses' ability to survive outside of the host cells: stability in the presence of many physical and chemical agents as well as adverse pH conditions, resistance to lipid solvents due to lack of lipid structures, resistance to temperature conditions (Mena & Gerba, 2009), and UV inactivation (Ko, Cromeans, & Sobsey, 2005; Thurston-Enriquez, Haas, Jacangelo, Riley, & Gerba, 2003); presenting a challenge for disinfection by WTPs in which adenovirus are continuously detected from disinfected sewage

(Rodriguez, Gundy, & Gerba, 2008). Group F contains the enteric serotypes 40 and 41 that are important waterborne viruses due to their great resistance to sewage treatment (Ko et al., 2003), their presence in drinking water, surface water, ground water, and recreational waters (Gerba & Rose, 1990; Heerden, Ehlers, Vivier, & Grabow, 2005; S. C. Jiang, 2006; Keswick, Gerba, DuPont, & Rose, 1984), and to their inclusion in the United States Environmental Protection Agency's drinking water contaminant candidate list (CCL) (US Environmental Protection Agency, 2010). The EPA developed this CCL 3 list of 104 chemical contaminants and 12 microbiological contaminants that are not currently regulated but are known or anticipated to be found in public water systems; therefore, future regulation might be needed to meet the Safe Drinking Water Act (SDWA). The impact that human adenoviruses (HAdvs) (types 40 and 41) have on public health is significant; they are second to rotaviruses as a leading cause of acute gastroenteritis (Allard, Girones, Juto, & Wadell, 1990; Cruz et al., 1990; Enriquez, Hurst, & Gerba, 1995; Jothikumar et al., 2005; Logan, O'Leary, & O'Sullivan, 2006; Mena & Gerba, 2009; Topkaya, Aksungar, Özakkafl, & Çapan, 2006; Uhnoo, Wadell, Svensson, & Johansson, 1984) and the number of deaths in children under five (Ko et al., 2003). In addition, Ad 40 and 41 account for two-thirds of the diarrhea caused by adenoviruses (Cromeans, Lu, Erdman, Humphrey, & Hill, 2008). The human exposure to adenoviruses can be from consumption of contaminated water sources or from inhalation of aerosolized droplets during recreational activities (S. C. Jiang, 2006). Due to this public health impact, it is important to develop methods for the detection and quantification of infectious HAdvs found in environmental samples such as sewage and water that could potentially pose a threat to humans.

Adenovirus Life Cycle

The infectious cycle of adenovirus can be divided into two phases, with DNA replication occurring between the two phases. During adsorption, the fibers on the adenovirus virus attach itself to specific cell surface receptors on the host and through endocytosis the viron is engulfed into the host cell. Inside the cell, the capsid proteins disassemble, releasing the viral genome into the cytoplasm where they cross the nucleus to begin transcription. A single strand of the DNA is transcribed to mRNAs in the nucleus. The viral proteins are produced outside the nucleus and carried back into the nucleus where they are assembled before release into the cytoplasm. The virons mature and lyse the cell, releasing new adenoviruses. It is important to note that viral infectivity is dependent on an undamaged viral capsid which provides protection for the viral genome against degradation (by nucleases, pH, UV radiation, and temperature) and the steps of replication and translation of the viral genome to viral proteins and enzymes is critical components for detection of infectious ads (Rodriguez et al., 2009). The early gene, EA1 and the late hexon gene play an important role in the infectivity process during cell culturing (Kim, Lim, & Ko, 2010; Rodriguez et al., 2009).

Use of Fecal Indicator Organisms

Fecal indicators consist of a group of organisms that are used as a measure to determine the presence or absence of fecal contaminants that threat public water systems. In 1974, according to the Safe Drinking Water Act, the EPA had to develop a standard of measure that all water treatment facilities followed identifying indicators and maximum contaminant levels as a means of protecting public drinking water (US Environmental Protection Agency, 2011b). If these levels exceeded the maximum, it could indicate that the public water system was compromised and public health was potentially at risk. The methods used to test water for these indicators are fairly inexpensive, quick, and easy to perform. Currently, the indicators are turbidity which test

the cloudiness of water that could interfere in the disinfection process; total coliforms (TC) which are naturally found in the environment; fecal coliforms (FC) or *Escherichia coli* which are found in animal and human intestines and feces; and fecal indicators that include coliphages, which are viruses that infect the bacterium *E. coli* and Enterococci, which indicates fecal contamination (US Environmental Protection Agency, 2011a). These indicators have been used as measures to monitor water systems, however, the ability of these indicators being used to predict the occurrence and concentration of human enteric viruses is questionable simply based on the differences in physical and chemical traits of the two groups of microorganisms (S. C. Jiang, 2006).

Testing water sources for viruses requires more resources, including adequate labs and personnel, money, and time which presents a problem for testing water sources for viral contaminants; hence, the idea to use microorganisms that are transmitted fecal-orally as bacterial indicators for fecal-oral transmitted viruses (Bosch, 2010). Despite the need, there is reason to believe that they might not be good indicators of enteric viruses due to viruses being more resistant to water treatment processes (S. C. Jiang, 2006; M. D. Sobsey, 1989), their ability to survive longer in natural environments than bacteria (Berg & Metcalf, 1978), and the seasonal distribution of most viruses is different than bacterial indicators (APHA 1995). Therefore, relying on these indicators to predict the presence or absence of enteric viruses in water is not guaranteed and a more reliable choice should be found (Bosch, 2010). There are certain requirements for an ideal indicator: (i) should be associated with the source of the pathogen and should be absent in unpolluted areas, (ii) should occur in greater numbers than the pathogen, (iii) should not multiply out of the host, (iv) should be at least equally resistant to natural and

artificial inactivation as the viral pathogen, (v) should be detectable by means of easy, rapid, and inexpensive procedures, and (vi) should not be pathogenic (Bosch, 2010).

There is a need for methods of monitoring the viral quality of water (Cho et al., 2000). This will help with regulating public water systems that, if contaminated by viral pollutants could pose a threat to public health. The regulatory method should be cost effective, easy to administer, and accurate in its ability to predict viral contamination. According to research, a promising indicator is within the three bacteriophage groups: somatic coliphages, F-specific bacteriophages, and *Bacteroides fragilis* bacteriophages (Bosch, 2010; Havelaar, 1993). Adenoviruses are known for being more stable than fecal indicator bacteria during UV treatment processes (S. C. Jiang, 2006) and it has been suggested that they might fit the criteria and be a good indicator of viral pathogens due to their high prevalence in water (Fong, Phanikumar, Xagoraraki, & Rose, 2010; S. Jiang, Dezfulian, & Chu, 2005); hence, the importance of this research for the development of an efficient method to detect and quantify adenoviruses in environmental samples.

Occurrence of adenovirus in the Environment

Research indicates that adenoviruses are ubiquitous in the environment. Having the unique quality of being a DNA virus makes it a thermostable virus and it is suspected that this contributes to their ability to survive for long periods of time in the environment (Enriquez et al., 1995; He & Jiang, 2005). Adenovirus infections occur year-round and there is very little seasonal variation in shedding (He & Jiang, 2005). According to several reports, adenoviruses have been detected in environmental waters (recreational, surface, ground, wastewater) and drinking water (Cho et al., 2000; Gerba & Rose, 1990; Heerden et al., 2005; S. C. Jiang, 2006; Muscillo, 2008). Human ads occurrence studies have been limited due to the lack of data that demonstrates proper recovery from environmental samples (Cromeans et al., 2008). When sampling environmental

samples there are a number of inhibitors that could influence detection; therefore, developing and improving methods is essential in accurately documenting recovery of adenoviruses. For this experiment the use of environmental samples (WTPs and sewage samples) will contribute to science by providing additional data on the occurrence of adenoviruses in environmental samples using the HFUF and cell culture mRNA-RT PCR as the method of choice.

Concentration of Adenoviruses

The process of concentrating the virus is an essential component in the recovery and detection of infectious adenoviruses. These viruses are found in water and sewage sources at low concentrations. Therefore, large volumes (10L to >100L) have to be concentrated to reduce the volume to a suitable quantity for molecular (100µL) and cell culture analysis (10-20mL); while retaining the microbes and viruses for successful recovery. Methods that have been developed and improved at UNC that combine the use of hollow fiber ultrafiltration (HFUF) and polyethylene glycol precipitation (PEG) to concentrate the adenoviruses in water. This unpublished laboratory experiment demonstrates high virus recovery efficiency in water samples spiked with adenovirus. Additional concentration and purification steps were used to remove any inhibiting factors that could interfere with detection and quantification processes.

Methods of detection

Due to the "fastidious" characteristics of serotypes 40 and 41 it has made detection, culturing, and quantification difficult. Ad 40 and Ad41 are difficult to isolate, in contrast to other ads due to how they grow in cell culture (Jothikumar et al., 2005). Cell culturing is the standard for detecting infectious enteric viruses due to some viruses having the ability to propagate and produce cytopathic effects (CPE) (Dahling, 1991; Rodriguez et al., 2009; Tiemessen & Kidd, 1995). CPE are when you notice changes in a cell monolayer, in some cases it is a visible plague

that indicates viral infectivity. Detection of viruses in cell culture is dependent on assay conditions (i.e. duration of exposure to host cells, volume if inocula, age of the cells, and the presence of inhibitory or toxic substances) (Rodriguez et al., 2009). In addition, every cell line does not propagate viruses similarly (Rodriguez et al., 2009) and some infectious viruses simply cannot be detected using cell culture assays because these viruses are not cultivable (Cho et al., 2000). Understanding the infectivity of slow-growing or non-cultivable viruses and how they survive in environmental samples is necessary to properly address the public health risk (Rodriguez et al., 2009).

Many researchers use the Graham 293 (G293) cell line for propagating ads (Cromeans et al., 2008), but they have also been cultured in many other cell lines including HeLa, HEK, A549, PLC/PRF/5, and Caco-2 cells and because these viruses can infect cells without producing any cytopathic effects (CPE) or grow slow, requiring up to 10-20 days of incubation to see CPE has made detection and quantification difficult (S. C. Jiang, 2006; Jothikumar et al., 2005; Ko et al., 2003; Ko, Jothikumar, Hill, & Sobsey, 2005; Rodriguez et al., 2008). Having to wait until CPE are visible before infectivity can be determined becomes problematic when fast, accurate measures need to be performed to determine the human health risks associated with adenoviruses in environmental sources. The discovery of PCR as a method for detection advanced our ability to detect, especially those viruses that are difficult to culture (Cho et al., 2000; Rodriguez et al., 2009). Polymerase chain reaction (PCR), a molecular detection method amplifies a single copy of target DNA to millions of copies of DNA that can easily be quantified. To develop real-time PCR methods for adenoviruses, degenerate primers and a TaqMan® probe targeting a 163-bp region of the hexon gene were designed to amplify enteric serotype 40 and 41 (He & Jiang, 2005). Although direct PCR provides no information about viral infectivity it can be used for

presence absence of ads (S. Jiang, Noble, & Chu, 2001; Ko, Jothikumar et al., 2005; M. Sobsey, Battigelli, Shin, & Newland, 1998). A combination of cell culture and PCR methods has been used for the detection of low grown viruses such as some enterovirus, hepatitis A virus, and adenovirus (Ko et al., 2003). In the case of Adenovirus the combination of cell culture with the specific detection of viral mRNA with RT PCR can be used for the rapid detection of enteric adenovirus (Ko et al., 2003). With this method the mRNA that was produced during replication will allow for the detection of low numbers of infectious ads to be detected within 3 to 5 days (Ko, Jothikumar et al., 2005). The disadvantage of the nested PCR was the risk of contamination because the procedure required the tubes to be opened post PCR (Ko, Jothikumar et al., 2005). The study that Ko et al 2005 later conducted, eliminated the need for post PCR required for nested PCR and integrated cell culture-TaqMan® RT-PCR method that rapidly and sensitively detected infectious ads in clinical and environmental samples.

Control for infectivity assays

Despite improvements in quantification and detection time, mRNA-RT PCR and cell culture infectivity assay requires robust positive and negative controls that currently do not exist. It is essential to be able to control for error, which can be caused from input samples to reverse transcription when you are detecting RNA expression (Dheda et al., 2004). Therefore, it is proposed that the use of the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), which has previously been used to standardize mRNA expression assays, has the ability to serve this purpose (Dheda et al., 2004). In doing so, the expression levels for the housekeeping gene should remain constant between the cells of different tissues and under different conditions (Thellin et al., 1999). Research indicates that an internal control (i.e. housekeeping gene) can be used to ensure the results are not false negative for infectivity

(Rodriguez et al., 2009). It is important to note that there is some speculation that certain housekeeping genes might be inappropriate and should be chosen based on specific experiments due to the variability seen in certain samples (Dheda et al., 2004).

Development of transactivated cell line

For this research, the need for group F ads to effectively infect and replicate in cell culture is crucial for developing efficient methods for detection and quantification. A team of researchers at Seoul National University has developed stable cell lines (CMV and RAS) that they believe consistently express high levels of viral transactivating proteins that promote the transcription and growth of group F ads during cell culture propagation and assay (Kim et al., 2010). Viral transactivated proteins can activate and stimulate viral genes and transcription factors which aid in the increase of levels of target mRNA and to promote multiplication of fastidious human adenoviruses in cell culture (Kim et al., 2010). This experiment analyzed the effect that the viral transactivator proteins IE1 from cytomegalovirus (CMV) and hepatitis B virus (HBV) X had on the multiplication of human ads. The transactivated 293 CMV significantly increased levels of adenovirus DNA and mRNA during cell culture when compared with the G293 (Kim et al., 2010).

MATERIALS AND METHODS

Source water sample collection. We selected six geographically distinct water treatment plants within the United States that agreed to participate in our project. There were three different sample times per plant in September 2009, November 2009, and February 2010 for a total of eighteen sample sets. Each facility was provided six 4L containers that totaled 24L, in which they collected source water samples, stored them overnight at 4˚C, and next day air expressed them to UNC. Upon arrival at the virology lab at the University of North Carolina Chapel Hill, five – 4L water sample containers were transferred to a 20L polyethylene cubic container and the viruses were concentrated using the Hollow Fiber Ultrafiltration (HFUF), polyethylene glycol precipitation (PEG), and ultracentrifugation method to a final volume of approximately 5mL and stored in -80˚C for future cell culture analysis. The remaining 4L container was used to conduct microbial indicator analysis for total coliforms, fecal coliforms, coliphages, *Clostridium perfringens* spores, and *Bacteroides* spp.

The six water treatment plants (WTPs) were selected based on their potential to contain adenovirus in their raw water. These plants are representative of different U.S. geographical locations. The water treatment plants identified were in Cary, North Carolina; Raleigh, North Carolina; St. Louis, Missouri; New Orleans, Louisiana; Ann Arbor, Michigan; and Las Vegas, Nevada.

- Cary, North Carolina: The source water for the town of Cary is Jordan Lake. This lake is fed by the Haw River, which is receiving water for wastewater effluent from the town of Graham and the City of Burlington. In addition, it receives water from Northeast Creek (a

wastewater effluent discharge point for the City of Durham) and Morgan Creek (a wastewater discharge point for the town of Chapel Hill). It also receives surface and storm water runoff from heavily urbanized areas of the Jordan Watershed. Jordan Lake has been classified as impaired by the state of North Carolina.

- Raleigh, North Carolina: The E.M. Johnson Water Treatment plant receives its source water from the Falls Lake. They treat and pump on average 50-60 million gallons a day. This plant is not under the direct influence of a major NPDES-permitted wastewater treatment plant effluent and it does not have the magnitude of urban development of Jordan Lake. However, it does present similar concerns with potential sewage impacts and potentially contaminated surface water runoff as does Jordan Lake. Little Lick Creek, a tributary contributing to Falls Lake is classified as impaired by the State of North Carolina from excess storm water, lack of riparian vegetation, malfunctioning septic systems, and pollutants from urban development from a population of more than 17,000 people. Ellerbe Creek, a predominately urban watershed located in Durham, NC also is a tributary to Falls Lake, has been classified by the State of North Carolina as an "impaired stream" because it does not sufficiently support aquatic life. Possible causes impairment include: (1) excess storm water from urban development, (2) lack of riparian vegetation in many areas, (3) storm drain discharges that carry pollutants from urbanized areas, (4) "illicit discharges," including chemical or sewage spills and (5) impacts from past channel straightening. The watershed has a population of >47,500 people (as of 2000) and permitted wastewater discharges (in MGD, million gallons per day) includes North Durham Water Reclamation Facility (20 MGD); Days Inn Wastewater Treatment Plant (0.018 MGD) and Lake Ridge Aero Park (0.016 MGD).

- St. Louis, Missouri: The City of St. Louis actually has two water treatment plants that collect water from two rivers. For the purpose of this research we selected the Chain of Rocks Plant, where the influent is the Mississippi River. This particular location is 11 miles north of the center of the city and 5 miles downstream from the Missouri confluence. This particular plant has the capacity to produce 240 million gallons day but averages 120MGD daily. It is all surface water runoff. The effluent of the Lower Meramec River Wastewater Treatment Plant flows into the Mississippi River at the confluence of the Meramec River through an underground pipe.

- New Orleans, Louisiana: The City of New Orleans receives raw water from the Mississippi River and it is pumped to the Carrollton Water Purification Plant. The source water is under the influence of wastewater, industrial, and storm water discharges that are upstream from their location. The plant treats approximately 135 million gallons of water per day.

- Ann Arbor, Michigan: The source water for the town of Ann Arbor's drinking water treatment plant is the Huron River. The river receives direct surface runoff from the town of Ann Arbor and other urbanized areas nearby, and sections have been classified as impaired by the state, with Total Maximum Daily Load (TMDL) limits established for *E. coli*.

- Las Vegas, Nevada: There are two water treatment facilities that service Nevada, but for the purpose of this experiment, the samples were collected from the River Mountains Water Treatment Facility. They currently service approximately 110 MGD. The plants' raw water source is received from Lake Mead and the Colorado River. The intake is at

the bottom of the lake in that Colorado River channel. At this specific location, the intake pipe is submerged; therefore, it is not under the influence of contaminated effluent from a waste water treatment facility. It is important to note that the Colorado River is primarily snow melt from the Rocky Mountains and is considered to be high quality source water.

Identifying water treatment plant-specific factors of vulnerability. Each plant was only able to provide the following specific characteristics about their source water during each sample cycle: alkalinity, hardness, turbidity, TOC, pH, and temperature (Table 15) that was used as a tool for predicting Group F ads presence and concentrations. This data was used as a possible predictor of vulnerability based the level of turbidity reported. From this information, water treatment plants with high levels of turbidity received additional analysis using the pellets recovered after centrifugation of the ultrafilter retentate because it was suspected that there is a solids-association when detecting viruses which could impact the ability to recover and detect the viruses. Microbial data was not collected from these plants; however, microbial testing was performed upon arrival at UNC.

Microbial indicator analysis for fecal contamination:

IDEXX Quanti-Tray Colilert and Enterolert. Using the Colilert and Enterolert US EPA approved methods that are included in Standard Methods for Examination of Water and Wastewater; we tested the concentrations of *Escherichia coli*, fecal coliforms, and enterococcus in the source water samples. Per 100mL volume of source water, the growth medium provided in each kit, was mixed thoroughly with each 100 ml sample, poured into a Quanti-Tray, that detected up to 200 MPN/100mL, and sealed using the Quanti-Tray Sealer prior to being placed in the appropriate temperature based incubator depending on the bacteria tested (see below) for 24 hours. The control bacteria used for

the Colilert testing was *Klebsiella* (coliform) and *E. coli B* and for Enterolert testing, *Enterococcus faecalis*. Using a sterile wooden applicator stick, an aliquot (this only required a small portion due to it being highly concentrated) of each bacterium from a frozen broth stock was scrapped and placed in 100mL of DI for the controls and 100mL of source water for each WTP and processed as described. For Colilert, the trays were placed in the incubator for 4 hours at 37˚C and then moved to 44˚C (testing for fecal coliforms) for the remainder of the 24 hour incubation period. The Enterolert trays were placed in the incubator for the entire 24 hour period at 44˚C as described by the manufacturer. These methods were based on Defined Substrate Technology (DST); therefore, during this incubation period, the defined substrate nutrients served as visible indicators by the observable change of color and/or fluorescence when metabolized by the bacteria if they are present in the sample and grow. This allowed for the trays to be read by examining the color and fluorescence of each well. For Colilert, yellow wells indicated the presence of coliforms, and with the aid of a long wavelength UV lamp, the wells were observed for blue fluorescence which indicated the presence of *E. coli*. The UV lamp was also used to observe the Enterolert trays; fluorescent wells indicated the presence of enterococci. The positive big and small wells were counted for each tray and the most probable number (MPN) was calculated (Table 4) using an MPN calculator.

Coliphage Testing. Method 1602 was used to detect male-specific and somatic coliphage in water by the Single Agar Layer (SAL) procedure prepared by the US EPA. A 100mL source water sample was assayed by adding $MgCl_2$ (magnesium chloride), log-phase host bacteria (*E. coli* Famp for F^+ coliphage and *E. coli* CN-13 for somatic coliphage), and 100mL of double-strength molten tryptic soy agar (TSA) to the sample. The sample was

thoroughly mixed and the total volume was poured into 4 plates each for male-specific and somatic coliphages testing per source water sample. After overnight incubation, phage induced lysis zones (plaques) were counted and totaled for all plates from a single sample. The quantity of coliphage in a sample was expressed as plaque forming units (PFU)/100mL (US Environmental Protection Agency, 2001).

Clostridium perfringens spores. The most probable number assay using iron milk medium was used to test for *Clostridium perfringens* spores. The medium was prepared by aseptically adding 175mL of evaporated milk to a 250mL graduated cylinder and the volume was brought to 250mL by adding sterile DI water. A 0.2% ferrous sulfate septahydrate solution was prepared in a 500mL bottle by adding 1g of ferrous sulfate to 250mL of sterile DI water and allowed to mix. After the milk was mixed with the ferrous sulfate solution, volumes of 10mL were dispensed into 15 sterile tubes (per sample). Each row of 5 tubes corresponded to the undiluted sample and 11-fold sample dilutions in series through 1:121. The serial 11-fold dilutions were made in 15mL tubes by adding 5mL of sterile DI water and then 0.5mL of sample. For each sterile tube set, 1mL from the corresponding dilution series was added to each 10mL sterile culture tube. Water samples were prepared by heating 10mL of the sample to 70˚C for 10mins. This was done to kill any vegetative bacteria while leaving only the spores. The tubes were incubated at 42˚C for 18-24 hrs and then observed for stormy fermentation; indicative of *Clostridium perfringens* spores.

Bacteroides sp. For the analysis of *Bacteroides sp.* in the source water samples, 100mL of each collected source water was passed through a 0.22 micron fiberglass filter. The filters were stored at -80˚C until DNA extraction and real-time PCR analysis was

performed. The DNA was extracted from the filter using the MO BIO Ultraclean® Soil DNA Isolation Kit (MO BIO Laboratories, Inc Carlsbad, CA USA). Briefly, the filter was put into the bead solution tube provided in the kit and the procedure was performed as described in the kit instructions. The real-time PCR for the detection of *Bacteroides* sp used primers and probe targeting the 16s DNA, as previously described by Layton et al., 2006. The PCR mixture consisted of 12.5pmoles of each primer and 5pmoles of the probe, 12.5µL of 2X Quantitech probe PCR mix (Qiagen) and water to a volume of 25µL. The real-time PCR reaction was run in a Smartcycler system (Chepeid) and the amplification conditions were as follows: initial activation step of 95°C for 15minutes, and 50 cycles of 95°C for 10 seconds, and 60°C for 30 seconds. A positive DNA control was kindly provided by Professor Jill Stewart of UNC. This was a quantified DNA sample of *Bacteroides* sp. and it was used to make dilutions and assayed by real-time PCR for quantification. The Ct values were used to make a calibration curve that was used to calibrate the Ct values retrieved from the source water samples.

Concentration of adenovirus in environmental samples:

Adenovirus Stock. The adenovirus stock used was previously prepared in 2007. The viruses are commonly propagated in 150 cm^2 flask when the flask is at least 80% confluent, corresponding to ~10^7 cells/flask, and using multiplicity of infection, two infectious viruses per cell are infected into the G293 cell line and harvested for 10 days post infection or until at least 50% CPE was observed. The monolayer was incubated with 2% fetal bovine serum complete MEM at 37°C. The viruses were recovered by disrupting the infected cell monolayer with two freeze/taw cycles and the cells were

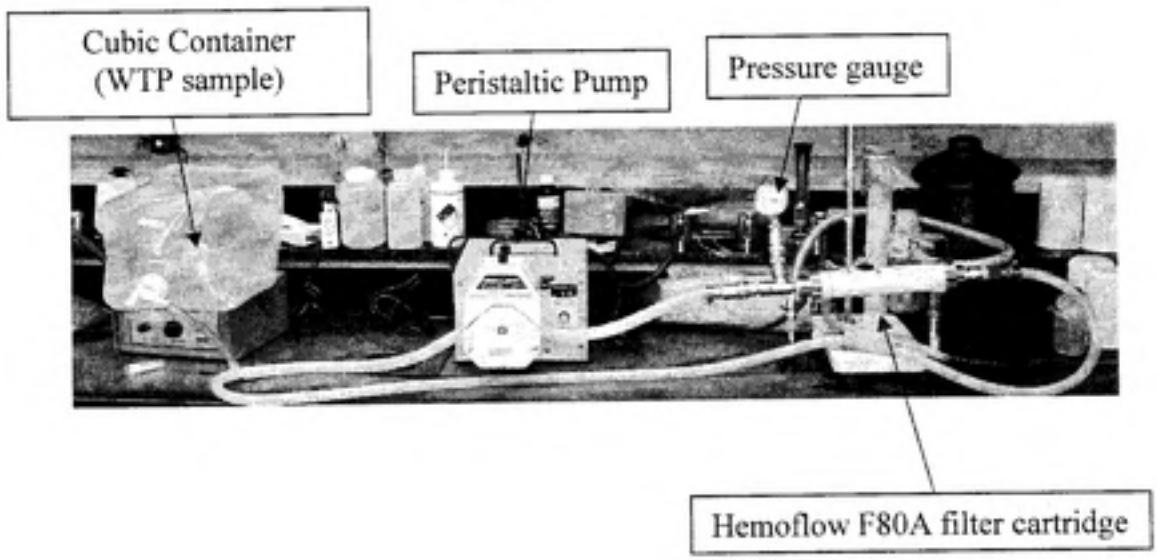
removed by centrifugation (3500 x g, 30 minutes, 4°C). The supernatant was retained as virus stock and stored at -80°C.

*Primary Concentration using Hollow Fiber Ultrafiltration (HFUF).**

- Source water. Five 4L containers (totaling 20L) were transferred to a polyethylene cubic container and allowed to mix using a stir bar as the sample passed through a Hemoflow F80A sterile, disposable, medical grade HFUF cartridge that retains any viruses present in the water. This Hemoflow F80A polysulfone dialysis filter has a parallel flow through with a MWCO (molecular weight cut off) of 15,000 to 20,000. The surface area of the filter is 1.8 m^2 and the fiber inner diameter is 200 µm. A peristaltic pump was used to recirculate the water sample initially at low pressures (5-10 psi) for approximately 5 minutes to ensure no leakage from the tubing. The pressure was increased between 15-25 psi until the sample volume was reduced, by continuous recirculation, indicated by the water being emptied in the cubic container. To recover the microbes on the filter, the viruses were eluted by adding 250mL HFUF Eluting Solution (1XPBS/1% Laureth-12) that was prepared by adding 10g/L of Laureth-12 and 50µL antifoam-A in 1 L of PBS (pH 7.4). The elution solution recirculated slowly through the filter at 5-10 psi until the sample volume was again reduced to the hold-up volume of the filter and the hoses. The resulting volume of retentate was approximately 200-300mL. This filtration process took approximately 30 minutes per water sample. To remove and recover the sample suspended solids before PEG precipitation, the retentate was divided into two 250mL Corning centrifuge bottles and the samples were then centrifuged at 3,000 X g for 20 minutes at 4°C. The resulting supernatant was recovered and further concentrated using PEG

precipitation as described in secondary concentration steps below. After removing the supernatant, the centrifuge bottles containing the resulting pellets of solids sediment were stored at -20°C until further processing and analysis was completed..

Figure 4. Hollow Fiber Ultrafiltration (HFUF)



- Sewage Samples. Sewage samples were collected from three waste water treatment plants in Chapel Hill, Raleigh, and Durham, North Carolina. One liter was collected and concentrated using organic flocculation by adding 200mL of 10% beef extract solution while adjusting the pH of the sample to 3.5 and agitating it for 20 minutes. The flocculated viruses were centrifuged at 3,000 X g for 30 minutes resulting in a pellet. The pellets were resuspended into 50mL of 0.45 M Na_2HPO_4 followed by readjustment of the pH to 7.2. The sample was further concentrated by PEG precipitation (6% PEG, 0.3 N NaCl, pH 7.0) with overnight orbital shaking at 4°C and 200rpm followed by chloroform extraction of the resulting supernatant (the supernatant from the BE used to elute the viruses from the pellets and the supernatant that was originally formed after separation of pellets before BE was added) of PEG precipitation. This resulted in a final sample volume of 5mL that was later assayed for viruses.

*Secondary concentration using PEG Precipitation, Chloroform Extraction, and Ultracentrifugation.**

Additional concentration steps were needed to remove bacterial contamination and to reduce sample toxicity in the HFUF retentate. The HFUF buffer contains 1% W/V Laureth-12 which causes cytotoxicity and the sample itself may have accumulated other cytotoxic constituents in the water samples. Therefore, these additional sample processing steps were needed for further virus concentration and purification.

Recipe for 1X phosphate-buffered saline (PBS)/L (100mM, pH 7.5)
8 g NaCl
0.2 g KCl
0.91 g Na_2HPO_4 (anhydrous)
0.12 KH_2PO_4, and bring volume up to 1 L with reagent; autoclaved

Polyethylene Glycol Precipitation (PEG). Based on the sample volume, PEG and NaCl were added to final concentrations of 9% PEG and 0.3 M NaCl. The PEG/NaCl was added in a sterile 250mL centrifuge bottle to the primary concentrate, not to exceed 225mL. The bottles were placed on an orbital shaker and allowed to shake overnight at 4˚C and 200 rpm. The next day, the bottles were centrifuged at 4200 rpm for 1 hour at 4˚C. The supernatant was decanted and the pellets were resuspended using 15mL of PBS (pH 7.5). The resulting concentrates per treatment plant sample were combined and stored in a 50mL centrifuge tube.

Chloroform Extraction. To kill any bacteria and to remove certain soluble impurities, 5mL of chloroform were added to the concentrated sample from step 2 (PEG precipitation) and mixed to emulsify by inverting the tubes in vertical movement for one minute. The samples were centrifuged for 20mins at 3000 x g/4˚C. The supernatant was recovered from the top without disturbing the thin film layer above the chloroform and the volumes were recorded. The supernatant was stored at -40˚C or ultracentrifugation directly followed this step.

Ultracentrifugation. The volume of the supernatant that was recovered after chloroform extraction (in the previous step) was adjusted to 50mL by adding PBS (pH 7.5). The sample was transferred to a 75mL ultracentrifuge tube and

centrifuge at 100,000 x g at 4°C for 2 hours. Ultracentrifugation was used to separate the viruses from the buffer and further reduce the sample volume to 5mL. The supernatant was immediately decanted and the pellet of sedimented viruses was resuspended in 5mL of PBS (pH 7.5). The concentrated samples were aliquoted in 4-1.2mL tubes and stored at -80°C for virus infectivity assay.

Recovery, detection, and quantification of adenoviruses from environmental samples:

A combined CC/mRNA RT-PCR method was used because specific detection of adenovirus mRNA in inoculated cell culture indicates active viral replication in cells. This method of analysis allowed for potential detection of infectious virus before cytopathic effect (CPE) became visible; therefore, providing a faster approach to detection by shortening the incubation time necessary before being able to detect the presence of infectious viruses in cell culture. The mRNA was detected using a two step approach. In the first step, mRNA from RNA extraction of inoculated cells was subjected to reverse transcription using a poly T primer. Next, the reverse transcribed nucleic acid was subjected to real-time PCR with specific primers targeting the hexon gene for adenoviruses A-F and another that specifically targets the hexon gene for the group F ads 40 and 41. This approach is a specific and sensitive method that yields high recovery for viral infectivity assays. The advantage of combining the cell culture and detection of mRNA by RT-PCR allowed for archiving the amplified cDNA for further analysis if needed and for measuring the mRNA activity of the cellular housekeeping gene as a reference gene during infectivity analysis for adenoviruses in inoculated cell cultures.

An experiment was conducted that verified the performance of recovery for viruses that adsorb to suspended solids. The first method proceeded with the initial process that

was used to concentrate the spiked water samples using HFUF and several other concentration steps; including PEG, beef extract followed by organic flocculation, and ultracentrifugation on the pellets collected. This was done to determine if the recovery of viruses attached to suspended solids would be efficient and this method be accepted for use on the field samples. This yielded a large loss in virus recovery (5%) at each of the secondary concentration steps (HFUF, PEG, and ultracentrifugation). It was suspected that this was due to the lack of removal of viruses from the suspended solids at the centrifugation steps; confirming that adsorption is responsible for low viral recovery. On the other hand, when beef extract was used as the tool for elution, 93% of the viruses adsorbed were recovered.

From this preliminary experiment we determined that viruses do adsorb to suspended solids and the use of beef extract to elute them yielded the best recovery. It was determined that this approach would be taken using the field samples collected from the WTPs.

It is important to note that the resulting pellets were frozen and processed in a later experiment that determined the effect of suspended solids on the recovery of adenoviruses and verified a sufficient method to elute the viruses.

Elution of viruses from suspended solids. Viruses were eluted from the previously collected pellets from centrifuged HFUF retentate by adding 100mL of 19% beef extract solution in 0.05 M sodium-citrate buffer (pH 7.2). The pellet suspension was agitated for 30 minutes at room temperature. The solids were removed by centrifugation at 3,000 X g for 20 minutes at 4°C. The supernatant was collected and further concentrated by PEG precipitation. The PEG

precipitation conditions were 6% PEG and 0.3 M NaCl, with incubation overnight at 4˚C at 200rpm and centrifugation at 4200 X g for 1 hour at 4˚C. The pellet of the same sample were combined and resuspended into 10mL of PBS (pH 7.5) and stored at -80˚C until further analysis.

Cell culture infections. For infectivity assays, G293 cell monolayers were used after reaching at least 80% confluency which usually happens by 4 to 5 days after planting. Before infecting the cells, the growth medium was removed and the cell monolayer was washed with PBS (pH 7.5). The inoculum was produced by diluting 350 µL of concentrated sample with 1050 µL complete MEM medium without serum and containing 10 µg of kanamicin, 50µg of gentamicin, and 20 µg of nystatin per mL of medium for a 1.5 mL inoculums volume and 2 mL of the inoculua was applied in a 25cm² flask to the cell monolayer and allowed to incubate for 1 hour at 35˚C for virus adsorption. The inoculum was not redistributed over the cells during this 1 hour incubation period because the cells had the tendency to detach if they were disturbed; therefore, no additional rotations were performed. After inoculation and adsorption, the inoculua was removed, 6 mL of complete MEM media with 2% fetal bovine serum was added to each flask, and the cell cultures were incubated at 35˚C and 4% CO_2 in a water-jacked incubator for 3 days when using the CMV G293 cell line and for 5 days when using the STD G293 cell line.

Extraction of nucleic acids. The QIAGEN® RNeasy® Mini Kit (50) was used for extracting the nucleic acids from the cell monolayers. Before extraction, the cell medium was removed and the cell monolayer was disrupted with 1mL of PBS

(pH 7.5) by pipetting up and down. The cells were collected in a 1.5 eppendorf microcentrifuged tube and centrifuged in a microcentrifuge at full speed for 2 minutes at room temperature, forming a pellet. The supernatant was discarded and the cells were resuspended using the cell lysis buffer [RLT buffer provided in the QIAGEN® RNeasy® kit (cat. No. 74104), Qiagen, Valencia, CA plus mercaptoethanol (500 µL per 50 mL of RLT buffer)]. The mercaptoethanol was used to prevent the activity of RNAses during purification. The cell suspension was homogenized using the procedure and tubes provided in the QIAshredder™ (50) kit (cat. No. 79654, Qiagen, Valencia CA). Then, the nucleic acid extraction was performed as described in the RNeasy® Kit and the purified nucleic acids were obtained in 50µL of nuclease free water. For DNAse treatment, an aliquot of 15µL of the purified nucleic acids were treated with 2µL of RQ-1 RNAse-free DNAse (Promega, Madison WI), 2µL of 10X PCR reaction buffer containing 15mM of $MgCl_2$ (Qiagen, Valencia CA) and 20 units of RNAse inhibitor (Promega, Madison WI). The reaction was incubated for 30 minutes at 37°C, after which the DNAse was inactivated by adding 2µL of DNAse Stop treatment and incubating at 65°C for 10 minutes and immediately chilled on ice, followed by reverse transcription.

Reverse transcription of mRNA. For reverse transcribing mRNA to cDNA, the reverse transcription was performed using poly T primers (dT_{15}) and the M-MLV reverse transcriptase (Invitrogen, Carlsbad, California). Per reaction the following was added: 1.25µL of 50µM oligo dT15 solution, 2.5µL of 10µM each dNTPs solution and 13.5µL of RNA extract. The sample was heated at 65°C for 3

minutes and chilled on ice for 10 minutes. Then 2μL of 0.1 M DTT solution (Invitrogen RT enzyme only, provided by the manufacturer), 5μL of 5X RT buffer, 0.25μL of RNAse inhibitor (40units/μL), and 0.3μL of RT enzyme (200 units/μL) were added to the sample. The sample was incubated for 50 minutes at 37˚C and 15 minutes at 65˚C followed by PCR detection.

Real-Time PCR for the quantification of adenovirus hexon gene. The real-time PCR mixture for the qualification of adenovirus A-F hexon gene consisted of 1X QuantiTect® probe PCR mix (Qiagen, Valencia CA), 1μM of each primer (forward and reverse), 0.1μM of dual-labeled probe, and 2μL of nucleic acid target for a reaction volume of 25μL. The real-time RT-PCR conditions were as follows: 50˚C for 30 minutes, 94˚C for 15 minutes, and then 45 cycles of 94˚C for 15 seconds, 58˚C for 30 seconds, and 72˚C for 15 seconds (Jothikumar et al., 2005).

Real-time PCR for quantification of expression of cellular HK gene. The real-time PCR mixture consisted of 1X QuantiTect® probe, 0.5μM of each primer (forward and reverse), 0.2μM of Dual-labeled probe, 2μL of target for a reaction volume of 25μL. The conditions were 15 minutes at 95˚C, 50 cycles of 30 seconds at 95˚C, and 30 seconds at 60˚C. The primers used were: GAP-L 5' GGAAGGACTCATGACCACCACAG 3', GAP-R 5' CAGTAGAGGCAGGGATGATG 3' and the dual-labeled probe was GAP-P 5' CATCCACAGTCTTCTGGGTG 3'. The expected size of the amplified final product was 120bp. For creating a DNA control for quantification of the cDNA, the PCR product was cloned into a TOPO vector (Invitrogen, Carlsbad, CA) as

described by the manufacturer and the clones were purified using the QIAGEN® plasmid miniprep kit (Qiagen, Valencia CA). The concentration of the plasmid was determined by spectrophotometry using a Smart Spec Plus (Bio-Rad) instrument.

Comparison of transactivated and standard G293 cell lines for the detection of adenovirus from sewage samples. The concentration of adenovirus in sewage samples was determined using infectivity assays with cell culture/reverse transcription real-time PCR in the standard G293 cell line and the transactivated cell lines293 CMV and 293 RAS. These experiments were performed in 6-well trays and the cells were infected in parallel using adenovirus concentrations of 10 infectious units (IU), 2 IU, and 0.8 IU per well. The sewage concentration was measured using a calibration curve created for ad41 using the CC/mRNA real time PCR. The propagation of adenoviruses was determined by measuring viral mRNA and viral DNA at 2 days post infection (DPI), 3DPI, and 5DPI. Those measurements were performed as described before.

Assay of concentrated source water samples in the transactivated 293 CMV' cell line. CC/mRNA RT-PCR assays were performed using the same procedures as described in the methods section for recovering and quantifying infectious ads from source water with the exception of the using the new cell line, CMV. Three 25cm² flasks were used. Per each flask, 350µL of inoculums were diluted with 1050µL complete MEM medium without serum for a 1.5mL volume of inoculum. The use of media in the inoculua reduced the detachment of the cells after infection, which was a problem observed when the concentrated samples were

diluted with PBS. Three flasks were used for each sample for a total of 1.2mL of concentrate per sample for 3 days post infection. No additional medium was added nor was the medium changed during this infectivity assay.

Statistical analysis for fecal indicators. Statistical analysis was conducted using the fecal indicators to attempt to determine a connection between these indicators and the water treatment plants that were positive for adenoviruses (infectious or noninfectious). An analysis of variance (ANOVA) was performed for all of the indicators checking for differences in the levels of each group variable (all ads, yes or no; infectious ads, yes or no; etc). A logistic regression was done taking all ads, infectious ads, and noninfectious ads checking them against the indicator variables. This model was done to assess whether the increase in the amount of a particular indicator could be a predictor of a positive all ads, infectious ads, or noninfectious ads.

Quality assurance and control. The water samples received from WTPs were processed and analyzed in a lab room dedicated only for field samples. This room is used exclusively for processing of field samples, and no work with positive control or reference strains of microbes or their nucleic acids takes place in it. Nucleic acid extractions and assays of samples in cell culture were also done in the field samples room. Positive controls were processed in a separate lab room which is dedicated to working with known pathogens, and involving the use of positive control and reference strains. The different stages in the analysis of water samples were done on a schedule such that all samples being processed were at the same stage of analysis and no work at different stages of analysis was performed simultaneously. The analysis of PCR products

was the last step and it was conducted in a separate lab room designated for PCR replications only.

Samples of source water were cooled at 4°C and shipped overnight in coolers and a chain of custody form was kept for each sample. All the sample sheets, forms, supporting information and results were archived. All equipment and reusable lab ware used was autoclaved or decontaminated with sodium hypochlorite, depending on the nature of the equipment and lab ware.

RESULTS

Validation of cell culture assays and the use of HK gene, GAPDH, as a positive control.

The initial steps for this project included the validation of the cell culture assays for the target nucleic acids including the use of the housekeeping (HK) gene, GAPDH, as a reference gene for mRNA assays and adenovirus detection. To validate the performance of cell culture assays to detect and quantify the target nucleic acids, the G293 cell line was infected with adenovirus and a calibration curve was calculated. This was done to establish a baseline for the detection of infectious adenovirus using the G293 cell line. In addition, the performance of the mRNA reverse transcription-PCR was validated by the use of the GAPDH housekeeping gene as a reference gene for mRNA detection. The efficiency of the real-time PCR for the quantification of the adenovirus hexon gene and the host HK gene was determined using DNA standards (**Figure 5** and **Figure 6**). Based on the results, the conditions of the real-time PCR were sufficient for the quantification of the target gene copies in the assay.

In order to demonstrate that the quantification of the mRNA from the GAPDH gene is related to the number of cells assayed, we performed mRNA reverse transcription PCR using different concentrations of host cells as starting material. The results demonstrated that real-time PCR can quantify mRNA at different amounts of cells; as the number of host cells increased, the concentrations of mRNA HK gene detectable increased (**Figure 7**). There was a linear relationship between the number of cells assayed and the cDNA quantified for GAPDH and it correlates to having a lower Ct value due to the increase in the number of detectable cDNA.

Another validation test demonstrated the number of days post infection that was required to predict better infectivity. **Figure 8** illustrates the amount of mRNA detectable in 10-fold ad stock dilutions by mRNA/RT-PCR as described by a quantitative dose-response relationship. At each dilution of the stock, beginning at the most dilute (-7) and ending at the most concentrated (-1), the amount of mRNA detected using mRNA/RT-PCR was recorded. The more concentrated the sample, the more copies of viral DNA detected. From these results, we concluded that at 5 day, viral mRNA expression was generally higher, less variable, and more consistent than at day 3.

Next, we determined if adenovirus infection at different inoculums concentrations would affect the quantification of the reference gene GAPDH (**Figure 9**). This experiment was conducted using the standard G293 cell line. With increasing concentrations of virus inocula, the viral hexon gene mRNA detected by RT-PCR increased (Ct values decreased). Therefore, the efficacy of the mRNA/RT-PCR assay for viral mRNA is log-linear, with lower Ct values at higher IU. In contrast, the concentration of GAPDH mRNA detectable by mRNA RT-PCR remains essentially the same. This illustrated that GAPDH is a suitable control because it remained detectable at the same levels despite changing mRNA RT-PCR detection with changing adenovirus 41 IU levels in the cell culture inocula.

In addition to lab based experiments, the performance of this assay in detection and quantification of infectious human adenoviruses using environmental samples is a crucial validation step for this research (**Figure 10**). The graph demonstrated the effect of a known environmental sample, sewage, had on the detection of viral mRNA and the efficacy of using HK gene, GAPDH, as a good positive control during adenovirus

infectivity assays. When the dilution of sewage increased, the Ct value increased due to lower adenovirus IUs. It is important to note that detectable levels of the HK gene remained nearly constant (which is good evidence that GAPDH mRNA is a suitable positive control), while the detection of HAdv hexon gene mRNA varied depending on the volume of sewage assayed (with greater detection at higher sewage volumes, indicated by lower Ct values).

The experiment identified in **Table 1**, showed the effectiveness and sensitivity of the assay to detect infectious units from environmental samples. In addition to the detection of enteric group F adenovirus that is typically present in sewage, the detection of group A and group E was observed in one of the samples. The results for sewage sample 1A, which yielded a ratio of 381:1 genomes per IU, indicated the number of genomes that were detected; which could have included detectable viral DNA.

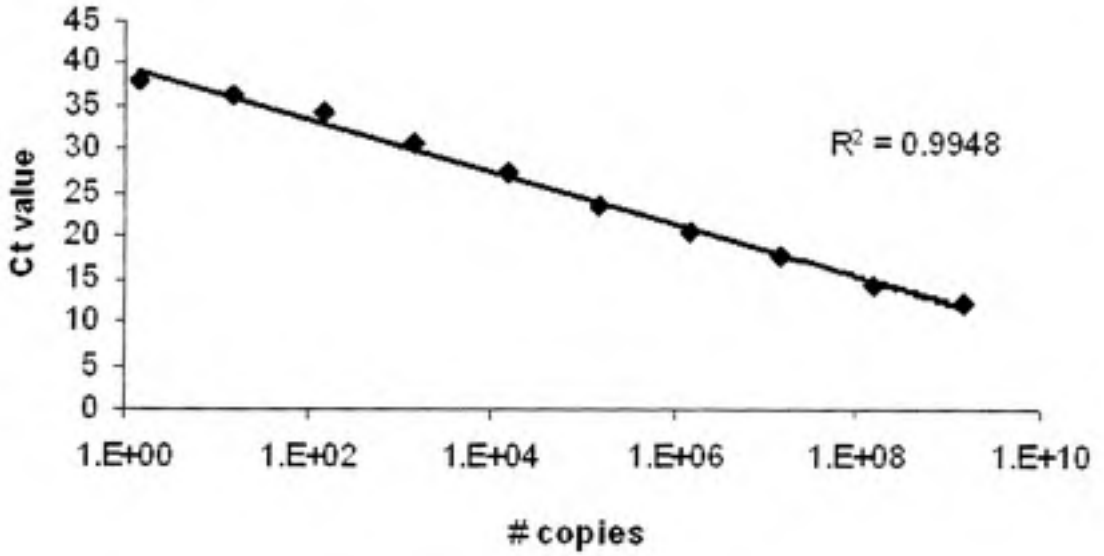


Figure 5. Calibration of the real-time PCR for the quantification of adenovirus hexon gene using a DNA standard.

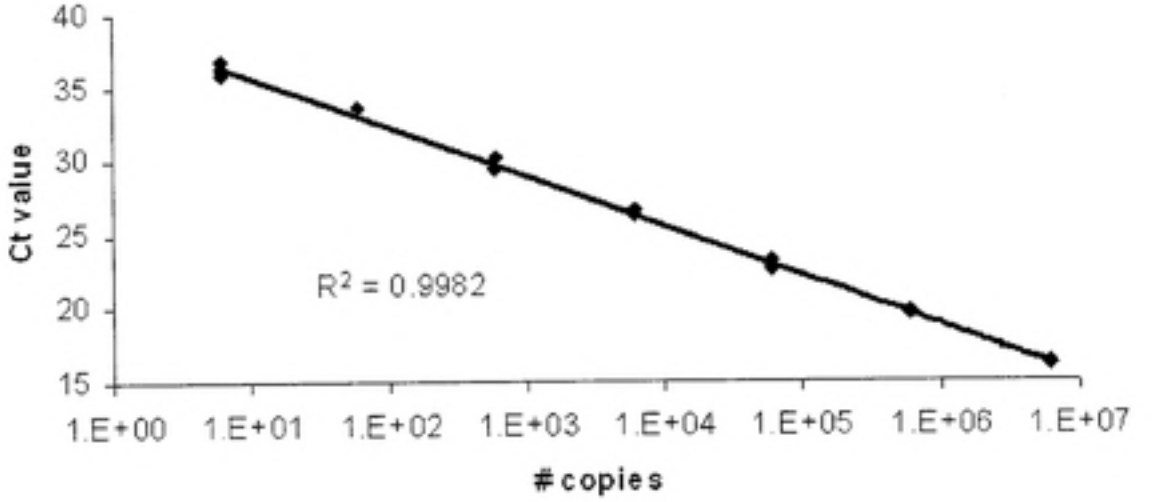


Figure 6. Calibration of the real-time PCR for the quantification of the GAPDH gene using a DNA standard.

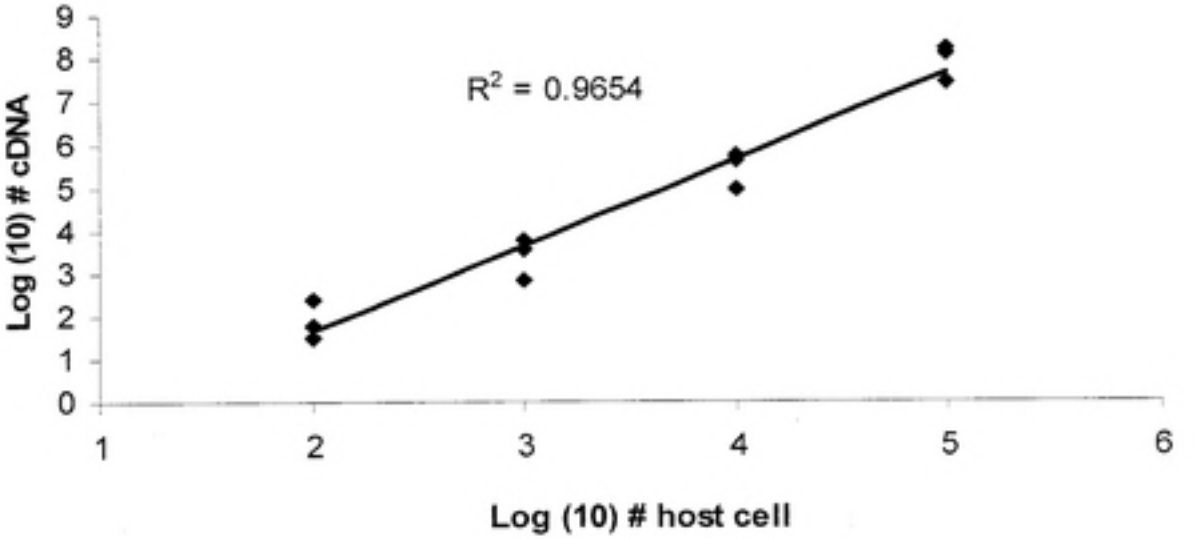


Figure 7. Quantification of the mRNA expression levels of the GAPDH gene at different concentrations of host cells.

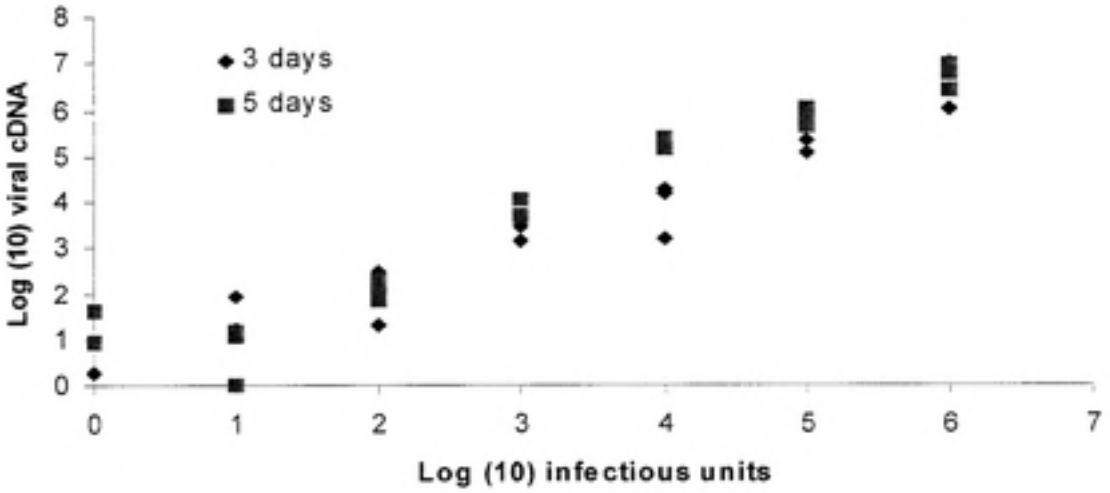


Figure 8. Quantification of the hexon gene mRNA at a range of infectious adenovirus 41 concentrations (n=3).

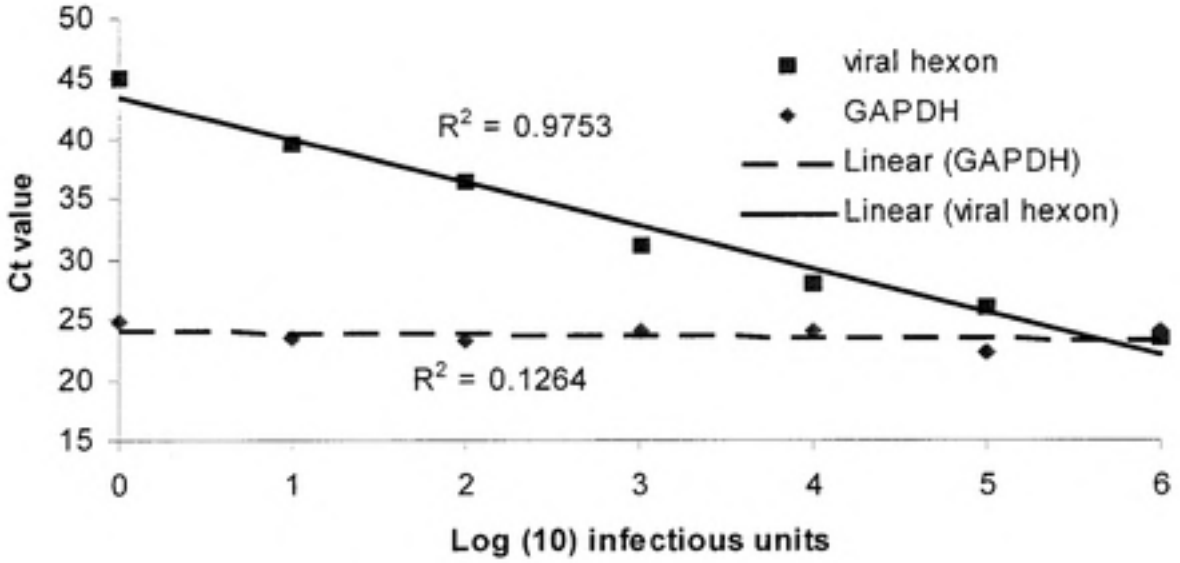


Figure 9. Quantification of viral mRNA versus host HK mRNA at a range of adenovirus 41 concentrations.

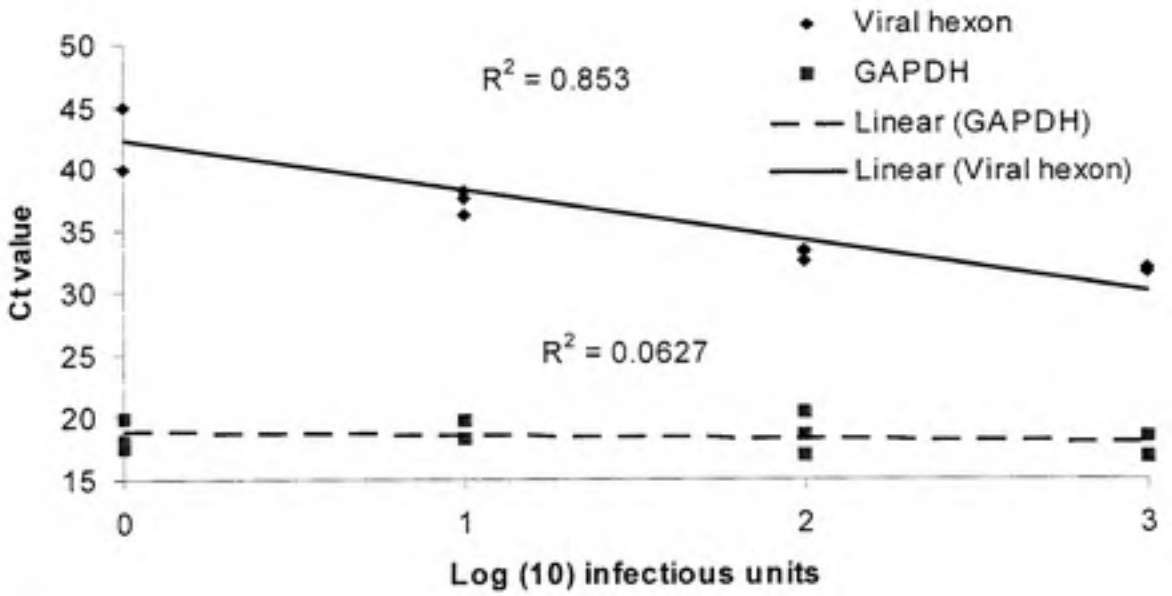


Figure 10. Quantification of adenovirus mRNA and host cell HK gene at different concentrations of infectious adenovirus isolated from sewage.

Inoculums		Infectious Units (IU)	Viral genomes	Ratio (gen/IU)	Ad Type(s)**	HK control sing (p)***
Adenovirus 41 positive control stock		4.7×10^7 IU/mL	1.0×10^9 gen/mL	21/1	F	>0.1
Sewage	1* A	3.8×10^5 IU/L	1.5×10^6 gen/L	381/1	A, E, F	>0.1
	1 B	1.0×10^3 IU/L	2.2×10^4 gen/L	22/1	F	>0.1
	3	1.7×10^2 IU/L	4.0×10^3 gen/L	26/1	F	>0.1
	4	7.2×10^1 IU/L	7.6×10^2 gen/L	11/1	F	>0.1

*waste water treatment plant
**Adenovirus was genotyped from DNA extracted from positive cell culture flask using the primers described by Xu et al. 2000
***Significance difference between the expression of the HK host gene in infected flask vs. non-infected flask, as p value

Table 1. Quantification of adenovirus by direct real-time PCR and cell culture/mRNA RT-PCR from concentrated and purified sewage samples.

Comparison of the performance of the new transactivated cell lines CMV and RAS to STD 293 for the propagation of adenovirus 41

The objective of this comparison was to demonstrate that the performance of the transactivated cell lines have not been affected after cryopreservation, shipment, and resuscitation in the UNC laboratory. Two different viral inocula were used for these demonstration experiments and they were confirmed using real-time RT-PCR targeting the adenovirus hexon gene. The two viral concentrations used were 1000 viral particles (as genome copies GC, **Figure 11**) and 100 viral particles (**Figure 12**). The viral mRNA was measured after 2 days post infection (DPI) and 3 DPI. The reported concentrations are the average of three experiments. In **Figure 11,** the expression of the viral hexon gene at the 1000 viral particles inoculum after 2 DPI was 1.3 $\log_{10}$ higher in 293 CMV than in the G293 cell line and at 3 DPI the viral mRNA expression was 0.4 $\log_{10}$ higher in 293 CMV than in the G293 cell line. The same trends were observed for the 100 viral particles inoculums, with the viral mRNA gene expression higher in 293 CMV than G293 for both 2 DPI and 3 DPI (**Figure 12**). These results demonstrate that the transactivated cell line 293 CMV has higher levels of expression of the adenovirus hexon gene than the standard G293 cell line as determined by reverse transcription real-time PCR of target hexon gene mRNA. These results also indicate that the new transactivated 293 CMV cell line is capable of producing more rapid, higher levels of detectable viral hexon gene mRNA at 2DPI than at 3DPI. The 293 CMV detection of viral hexon gene mRNA will peak and remain consistent after day 3, yielding more efficient expression earlier than the

required 5DPI for STD G293. The STD G293 needs a longer incubation time to yield hexon gene mRNA results similar to 293 CMV results.

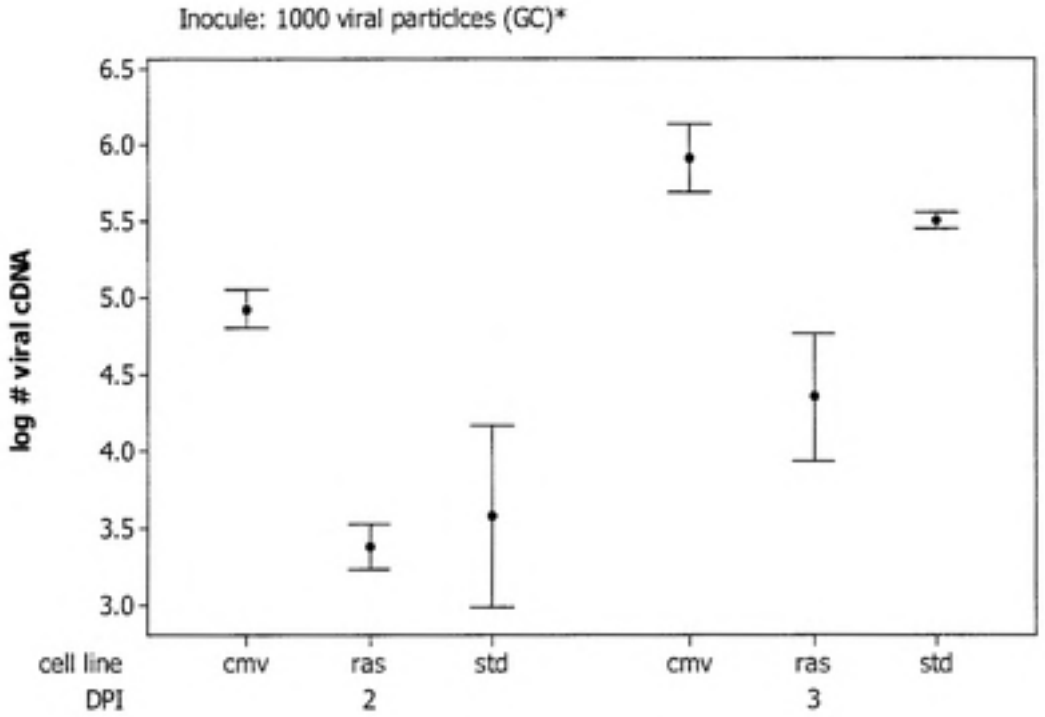


Figure 11. Comparison of the transactivated 293 CMV and 293 RAS cell lines with the standard G293 cell line (STD) for the propagation of adenovirus 41 determined by measuring the production of viral hexon gene mRNA in the cell monolayer at an initial concentration of 1000 genome copies. Reported concentrations are the average of three experiments.

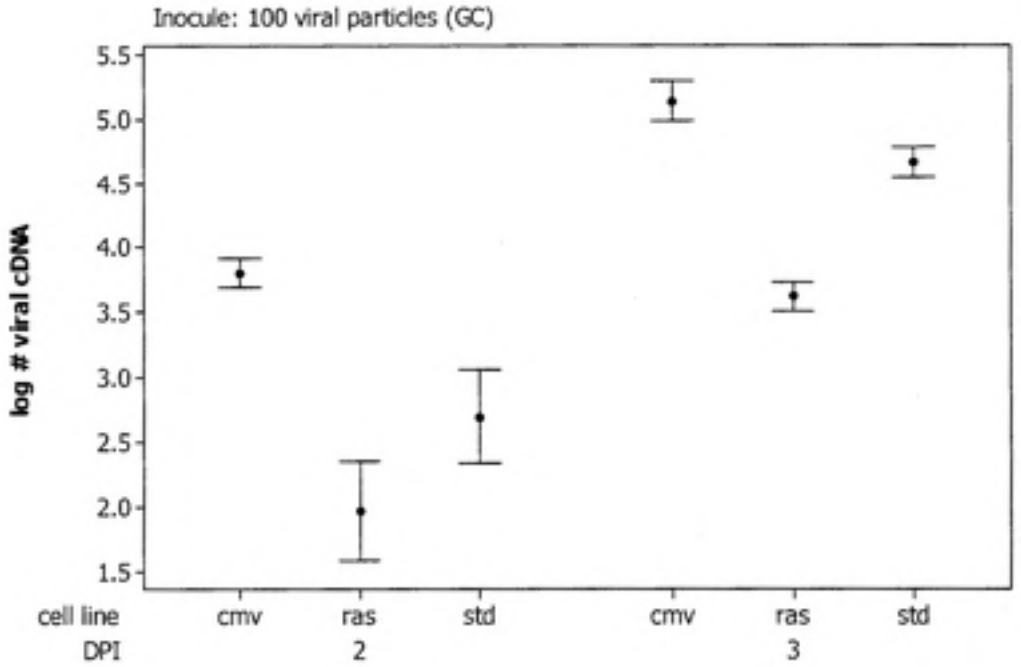


Figure 12. Comparison of the transactivated 293 CMV and 293 RAS cell lines with the standard G293 cell line (STD) for the propagation of adenovirus 41 determined by measuring the viral hexon gene mRNA in the cell monolayer at an initial concentration of 100 genome copies. Reported concentrations are the average of all three experiments.

A Normality test was performed on the $\log_{10}$ conversions of the data and analysis of variance determined that the difference observed in $\log_{10}$ mRNA per cell line was significant ($P < 0.05$, **Table 2**).

Table 2. Analysis of variance results for the comparison of different cell lines for the propagation of adenovirus 41 determined by measuring the viral mRNA by RT-PCR.

Source	DF[1]	Seq SS[2]	Adj SS[3]	Adj MS[4]	F[5]	P[6]
Cell line	2	15.71	15.71	7.855	33.74	<0.001
Dilution	1	8.299	8.299	8.299	35.65	<0.001
DPI	1	19.546	19.546	19.546	83.97	<0.0010
Error	31	7.216	7.216	0.233		
Total	35	50.772				

[1] Degrees of freedom
[2] Sequential sum of squares
[3] Adjusted sum of squares
[4] Adjusted mean squares
[5] F statistics
[6] P value

Comparison of the transactivated cell lines (293 CMV and 293 RAS) and the standard G293 cell line for the detection of human adenovirus in sewage

The purpose of these experiments was to evaluate the efficiency of the transactivated 293 cell lines compared to the standard G293 cell line in propagating adenoviruses from environmental samples. Sewage samples were collected from 3 wastewater treatment plants in Chapel Hill, Durham and Raleigh, North Carolina. These samples were collected, concentrated, and purified for infectious adenoviruses. The concentration of adenovirus naturally present in sewage was determined using direct PCR and by combining cell culture with viral mRNA detection by reverse transcription PCR in the G293 standard cell line for different dilutions. Using this approach, the concentration of infectious adenovirus and adenovirus genome used for infectivity assays ranged from 11 to 0.8 IU and from 240 genome copies (gc) to 11 gc per well, respectively. The propagation of the viruses was determined by measuring the expression of adenovirus

hexon gene targeting the viral mRNA by reverse transcription PCR and the replication of viral genome as determined by real-time PCR. The peak of expression of the viral hexon gene was observed for samples containing estimated virus concentrations ranging from 11 IU to 2 IU at 3DPI for the 293 CMV cell line. In **Figure 13,** the comparison of all three cell lines (CMV, RAS, and STD) using the samples obtained from OWASA at 2 DPI, 3 DPI, and 5 DPI are shown. The peak for the expression of viral hexon gene mRNA was 3 days post infection (DPI) in the transactivated 293 CMV cell line. The levels of expression were higher than those observed in the standard G293 cell line or the RAS cell line. Compared to the 3 DPI levels, the viral hexon gene mRNA levels were lower at day 5 for all of the cell lines.

In addition to the expression of viral hexon gene, the replication of the viral genome was monitored throughout the period of infection by PCR of adenovirus hexon gene DNA in infected cells (**Figure 14**). These results showed that compared to the standard cell line, the transactivated cell line consistently produced higher concentrations of viral mRNA that targeted the hexon region within the genome in the infected cell monolayers at each time point measured. Analysis of variance was conducted and it demonstrated that this higher replication of adenovirus was significant ($p < 0.01$) (**Table 3**). The highest difference observed between the G293 CMV cells and the STD G293 cells was at 2 DPI in which the concentration of adenovirus genome was 0.9 $\log_{10}$ higher in the 293 CMV cell monolayer than in the G293 cell monolayer. The lowest difference was observed at 5 DPI in which the concentration of adenovirus genome was 0.2 $\log_{10}$ higher in the 293 CMV monolayer than in the G293 cell monolayer.

The results presented in **Table 4** for the collected sewage samples demonstrate the efficiency of the new 293 CMV transactivated cell line in propagating adenovirus from environmental samples, even when the viruses are wild types present at very low concentrations. OWASA samples demonstrated better levels based on the number of positive samples detected when targeting the viral hexon mRNA and the viral hexon DNA genes for all cell lines; therefore, normal distribution and additional graphs were performed for these samples only. Upon observation of the data, Raleigh samples only had 2 IU detected from the concentrated samples measuring the mRNA versus 11 IU detected in the OWASA samples. The infectivity assays conducted for Raleigh across cell lines yielded detectable values for both viral mRNA and viral DNA for the new transactivated CMV cell line at each of the days post infection. When comparing the 293 CMV with the STD G293 for Raleigh, the viral mRNA was detected at all DPI for 293 CMV but only one was positive for STD G293, which was at 5 DPI. The detectable copy number was the same number that was detected for CMV at the same day post infection. It is also important to observe that the level of viral DNA detected for STD G293 5DPI, which was the only day with detectable results, falls within the range of detectable values for 293 CMV. It appears that the STD G293 cell line eventually reaches similar detectable levels of adenovirus hexon gene mRNA as for the 293 CMV cell line if the incubation time is longer versus the faster detectable time required for the 293 CMV cell line. The results for the Durham samples yielded mostly non-detectable values; therefore, this sample was not used to do a quantitative comparison. An observable difference in the sample collected from Durham versus the other samples is the level of infectious units

detected (0.8 IU) which was very low in comparison; yielded low concentrations and low detectable levels.

Table 3. Analysis of variance of the cell comparison for adenovirus detection in OWASA sewage samples by measuring viral DNA by real-time PCR.

Source	DF[1]	Seq SS[2]	Adj SS[3]	Adj MS[4]	F[5]	P[6]
Cell line	2	6.0044	6.1375	3.0687	9.08	0.007
DPI	2	7.0536	7.0536	3.5268	10.43	0.005
Error	9	3.0432	3.0432	0.3381		
Total	13	16.1012				

[1] Degrees of freedom
[2] Sequential sum of squares
[3] Adjusted sum of squares
[4] Adjusted mean squares
[5] F statistics
[6] P value

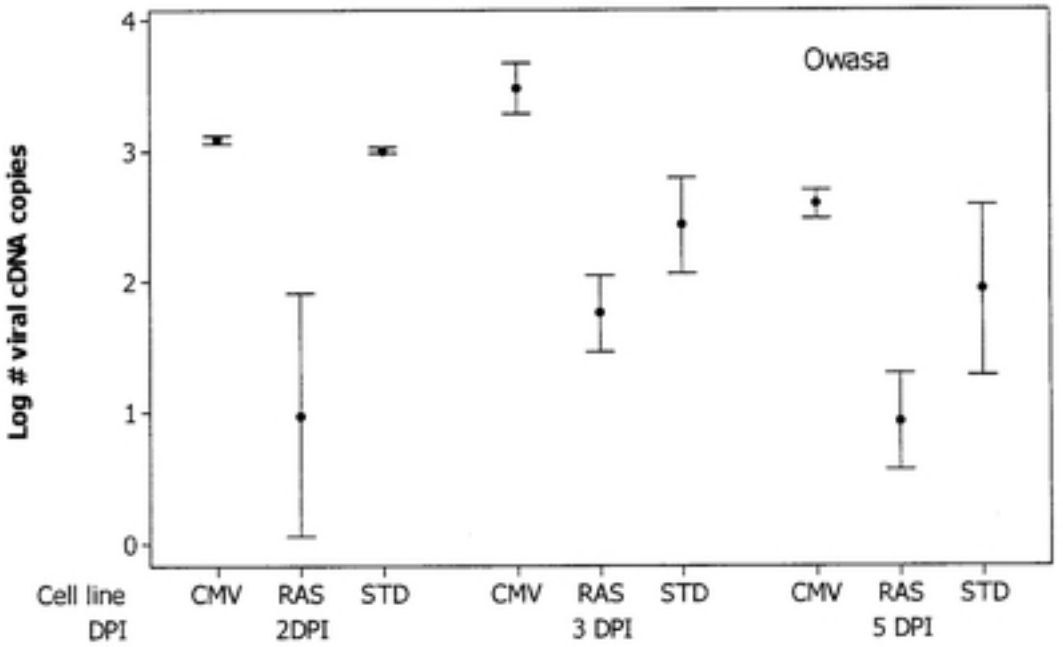


Figure 13. Comparison of the different G293 cell lines for the propagation of adenovirus from sewage sample collected from the OWASA WWTP as determined by measuring the viral hexon gene mRNA.

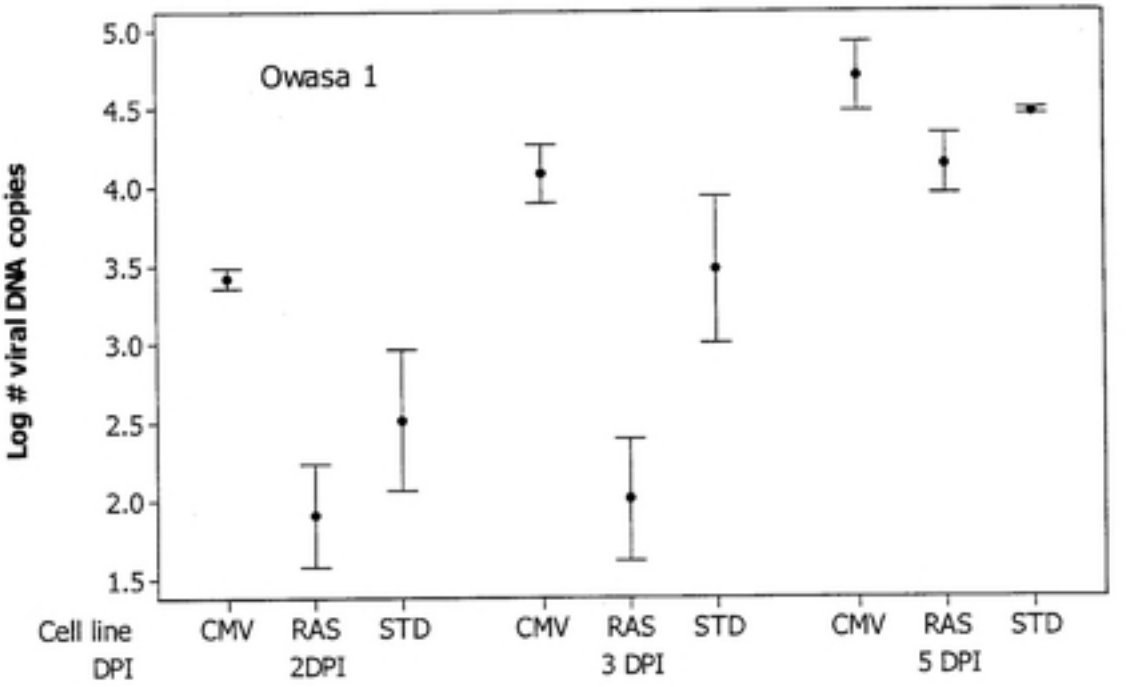


Figure 14. Comparison of the different G293 cell lines for the propagation of adenoviruses in sewage samples from OWASA WWTP as determined by measuring viral genomic DNA from the hexon region that was detected by real-time PCR.

Table 4. Comparison of standard (STD) G293 cell line and the transactivated cell lines 293 CMV and 293 RAS for the detection of group F human adenovirus in sewage.

Sewage sample	Initial adenovirus concentration		Cell line	DPI	Adenovirus replication outcome			
					viral mRNA (total 3 wells)		viral DNA (total 3 wells)	
	Infectious Units (IU)[a]	Genome copies (GC)[b]			Pos	Copies (±STD)	Pos	Copies (±STD)
OWASA	11	240	CMV	2	3	3.1 ±0.1	3	3.4 ±0.1
				3	3	3.5 ±0.3	3	4.1 ±0.3
				5	3	2.6 ±0.2	3	4.7 ±0.4
			RAS	2	2	1.0	2	1.9
				3	2	1.7	3	2.0 ±0.7
				5	2	0.9	3	4.1 ±0.3
			STD	2	3	3.0 ±0.0	3	2.5 ±0.8
				3	3	2.4 ±0.6	3	3.5 ±0.8
				5	3	1.9 ±0.9	3	4.5 ±0.0
Raleigh	2	44	CMV	2	2	1.7	2	1.9
				3	3	2.5 ±0.8	3	3.2 ±0.7
				5	2	1.6	3	3.6 ±0.7
			RAS	2	2	2.3	2	1.9
				3	0	None detected	1	1.5
				5	0	None detected	3	2.8 ±0.1
			STD	2	0	None detected	0	None detected
				3	0	None detected	0	None detected
				5	2	1.6	3	3.5 ±0.6
Durham	0.8	11	CMV	3	0	None detected	0	None detected
				5	1	0.7	2	2.2
			RAS	3	0	None detected	1	3.6
				5	1	0.5	1	1.2
			STD	3	0	None detected	1	1.3
				5	0	None detected	2	1.6

[a] Infectious Units were determined using ICC/mRNA Reverse Transcription PCR in G293 cell lines as described previously

[b] Genome copies were determined using real-time PCR as described previously

Comparison of the transactivated cell line 293 CMV with the standard G293 cell line for the detection of infectious adenovirus from source waters.

Three 20-liter volumes of source water samples from 6 water utilities were concentrated for viruses to a final 5 ml volume using hollow fiber ultrafiltration, PEG precipitation, chloroform extraction, and ultracentrifugation. These concentrated samples were used for comparing the performance of the transactivated and standard G293 cell lines in detecting adenovirus from source water samples. The adenovirus infection was determined by measuring the viral DNA by real-time PCR and viral mRNA by RT-PCR of inoculated cell cultures as described in the methods section. Initial testing for sample compatibility with cell culture infectivity assay using the standard G293 cell line was conducted on the WTP samples in order to determine possible interferences from sample cytotoxicity. Concentrated sample #2 from St. Louis showed high toxicity that manifested as cell death; therefore, this sample was diluted prior to performing the infectivity assays. All other concentrated samples did not show toxicity when infected with 350 µL sample volumes per cell culture. **Table 5,** summarizes all of the results from this initial analysis, and two samples were positive for infectious adenovirus using the standard G293 cell line; one from Ann Arbor and one from St. Louis.

Based on comparing the new transactivated cell lines (CMV and RAS) to standard G293 cells for infectivity detection of adenovirus type 41 and wild-type adenoviruses in sewage samples, it was determined that 293 CMV yielded better results vs. 293 RAS. Therefore, all WTPs source waters were analyzed using the 293 CMV cell line (**Table 6**). The inoculated cells were incubated for 3 days as previous results showed the highest expression of viral hexon gene was observed at this incubation time for CMV G293 cells. The equivalent water volume

analyzed for each sample was 5 L. The only exception was when inoculating the 2nd sample collected from St. Louis, for which the sample volume was reduced to 1.6 L in order to reduce cell toxicity. Two samples from St. Louis were positive for viral mRNA and three for viral DNA using this new transactivated 293 CMV cell line.

A side-by-side comparison of the standard G293 cell line and the transactivated CMV G293 cell line was conducted using all source water samples (**Table 7**). All incubation times were equivalent, at 5 DPI due to the standard cell line requirements to achieve maximum adenovirus detection. St. Louis source water showed two samples positive for viral mRNA using CMV and one positive using the STD and Ann Arbor showed negative for viral mRNA using CMV G293 but one positive for STD G293. When comparing the viral DNA detected between cell lines, St. Louis yielded three positive samples using the 293 CMV cell line and two positive samples when using the STD G293 cell line. The other positive results were observed using the STD G293 cell line only, where one sample from Nevada was positive and one sample from Ann Arbor was positive. As indicated by **Table 7**, six samples were positive for either viral mRNA or viral DNA when using the STD G293 cell line and five samples were positive for either viral mRNA or viral DNA when using the 293 CMV cell line. Based on the results, there is not a clear indication that one cell line is better than the other in the detection of infectious adenovirus genome. It is important to note that all cell lines and samples were verified for performance of infection assays and reverse transcription steps by using the housekeeping gene, GAPDH, which proved to be a suitable control for detection of infectious adenoviruses (refer to data presented in previous section – *Validation of cell culture assays and the use of HK gene, GAPDH, as a positive control*).

Table 5. Infectivity assays of the concentrated source water samples for the detection of adenovirus using ICC/ mRNA real time RT-PCR in the standard G293 cell line.

WTP	Sample	Conc. sample vol. Assayed per flask	Total conc. Vol. Assayed	Eq. Sample Vol. Assayed	Detection of Ads mRNA[a] Pos flask/ total flask	ct value	mRNA "HK" CT value (±SD)
	Negative Control[b]				0/6	Nd[c]	24 ±5
Cary	1	300uL/flask	1.2mL	5 L	0/4	Nd	22 ±2
	2	300uL/flask	1.2mL	5 L	0/4	Nd	22 ±2
	3	350uL/flask	1.05mL	4.2 L	0/3	Nd	
Raleigh	1	300uL/flask	1.2mL	5 L	0/4	Nd	21 ±2
	2	300uL/flask	1.2mL	5 L	0/4	Nd	24 ±2
	3	350uL/flask	1.05mL	4.2 L	0/3	Nd	
Nevada	1	300uL/flask	1.2mL	5 L	0/4	Nd	19 ±2
	2	300uL/flask	1.2mL	5 L	0/4	Nd	25 ±3
	3	350uL/flask	1.05mL	4.2 L	0/4	Nd	
Ann Arbor	1	300uL/flask	1.2mL	5 L	0/3	Nd	20 ±2
	2	300uL/flask	1.2mL	5 L	1/4	40.43	23 ±3
	3	350uL/flask	1.05mL	4.2 L	0/3	Nd	
New Orleans	1	300uL/flask	1.2mL	5 L	0/4	Nd	19 ±1
	2	300uL/flask	1.2mL	5 L	0/4	Nd	25 ±1
	3	350uL/flask	1.05mL	4.2 L	0/3	Nd	
St Louis	1	300uL/flask	1.2mL	5 L	0/4	Nd	20 ±2
	2	120uL/flask	0.480mL	1.9 L	0/4	Nd	30 ±2
	3	350uL/flask	1.05mL	4.2 L	1/3	41.95	

[a] detection of viral mRNA in tissue culture. [b] cell culture flask with no sample, PBS was used instead. [c] Not Detected

Table 6. Detection of infectious adenovirus from source water samples using combined cell culture and viral mRNA detection by reverse transcription real-time PCR with the 293 CMV cell line.

WTP	Sample	Eq. Vol. Assayed	mRNA assays			Detection of viral DNA	
			Adenovirus mRNA				
			Pos flask /total flask	ct value	Cell "HK" mRNA	Pos flask /total flask	ct value
	PBS		0/6		17.4 ±1.1	0/6	
Cary	1	5 L	0/3		17.0 ±0.5	0/3	
	2	5 L	0/3		17.1 ± 0.3	0/3	
	3	5 L	0/3		16.7 ±0.4	0/3	
Raleigh	1	5 L	0/3		17.8 ±0.8	0/3	
	2	5 L	0/3		17.1 ±0.2	0/3	
	3	5 L	0/3		17.6 ±0.8	0/3	
Nevada	1	5 L	0/3		18.5 ±0.4	0/3	
	2	5 L	0/3		18.4 ±1.3	0/3	
	3	5 L	0/3		17.4 ±0.6	0/3	
Ann Arbor	1	5 L	0/3		18.8 ±1.5	0/3	
	2	5 L	0/3		18.4 ±1.0	0/3	
	3	5L	0/3		16.9 ±0.3	0/3	
New Orleans	1	5 L	0/3		19.0 ±0.9	0/3	
	2	5 L	0/3		18.0 ±0.4	0/3	
	3	5 L	0/3		16.9 ±0.4	0/3	
St Louis	1	5 L	0/3		17.9 ±0.2	0/3	
	2	1.6 L	1/3	42.7	23.4 ±0.9	1/3	39.4
	3	5 L	1/3	37.4	17.1 ±0.4	2/3	41.2/39.7

Table 7. Comparison of Standard G293 and transactivated 293 CMV cell lines for the detection of infectious adenovirus in source water from WTPs as determined by measuring two different genomic targets, viral mRNA and viral DNA, in inoculated cell monolayers.

		viral mRNA				viral DNA			
		CMV		STD G293		CMV		STD G293	
WTP	Sample #	pos/total	Ct value	pos/total	Ct value	pos/total	CT Value	pos/total	CT value
Cary	1	0/3		0/4		0/3		0/4	
	2	0/3		0/4		0/3		0/4	
	3	0/3		0/3		0/3		0/3	
Raleigh	1	0/3		0/4		0/3		0/4	
	2	0/3		0/4		0/3		0/4	
	3	0/3		0/3		0/3		0/3	
Nevada	1	0/3		0/4		0/3		0/4	
	2	0/3		0/4		0/3		0/4	
	3	0/3		0/3		0/3		**1/3**	41.48
Ann Arbor	1	0/3		0/4		0/3		0/4	
	2	0/3		**1/4**	40.4	0/3		0/4	
	3	0/3		0/3		0/3		**1/3**	40.69
St. Louis	1	0/3		0/4		0/3		0/4	
	2	**1/3**	42.7	0/4		**1/3**	39.4	0/3	
	3	**1/3**	37.4	**1/3**	42.0	**2/3**	41.2, 39.7	**2/3**	37.7, 36.6
New Orleans	1	0/3		0/4		0/3		0/4	
	2	0/3		0/4		0/3		0/4	
	3	0/3		0/3		0/3		0/3	

The effect of suspended solids on the recovery of adenovirus from source water samples.

The purpose of these experiments was to determine the proportion of adenovirus remaining attached to source water suspended solids and the effects of suspended solids on recovery, detection, and quantification of infectious adenoviruses. Only field source water samples with a high probability of finding adenoviruses, based on turbidity levels, and were assayed. Those included samples from Ann Arbor, New Orleans and St. Louis. There were no samples of suspended solids from other WTPs that were separated and analyzed because they showed only low amounts of turbidity. These results are presented separately for each cell line (**Table 8 and 9**) and **Table 10** includes both cell lines in addition to direct detection by real-time PCR. Using direct real-time PCR, adenovirus DNA was detected in the eluted suspended solid pellets from 4 samples out of a total of 18, specifically in 2 samples from New Orleans and 2 samples from St. Louis (**Table 10**). For the cell culture assays, none of the suspended solids samples were positive for adenoviruses by infectivity assays using the 293 CMV cell line either for detection the viral mRNA or viral DNA (**Table 9**). However, one suspended solids sample was positive for infectious adenovirus when analyzed using the STD G293 cell line (**Table 8 and 9**), and this sample was also positive for adenoviruses by direct real-time PCR.

These results suggest a proportion of adenoviruses and/or their nucleic acids actually adsorb to the suspended solids and their DNA can be detected by direct PCR. It is important to note that the viruses that were detected by direct PCR may be non infectious as measured by negative cell culture assays. Again, only one sample from New Orleans resulted in a positive by cell culture infectivity using the standard G293 cell line despite two samples showing positive for direct PCR DNA detection. The reasons for the discrepancies between detection by cell culture infectivity and direct PCR are uncertain. It could be that the concentrations of the viruses and their nucleic

acids in the samples were low, such that some aliquots contained adenoviruses or adenovirus DNA and others did not. It is also possible that some of the adenovirus signal detected by direct PCR was from freed adenovirus DNA or inactivated adenoviruses. We are unable to determine if some of the adenoviruses became non-infectious due to the performance of the elution methods; therefore, not detected using infectivity assays. Additional research should be done to determine the effect of the elution methods on recovering infectious adenoviruses. It is important to note that the housekeeping gene, GAPDH, was used as an internal control for the methods used to detect infectious adenoviruses as indicated previously. The results yielded the expected activity for this housekeeping gene; the typical expression of mRNA for all samples tested. This indicates that the method used to assay these samples was effectively performed.

Table 8. Detection of infectious adenovirus from source water suspended solids elutes using combined cell culture and viral mRNA detection by reverse transcription Real-Time PCR with the standard G293 cell line.

WTP	Sample	mRNA assays			Detection of viral DNA	
		Adenovirus mRNA		Cell "HK" mRNA		
		Pos flask /total flask	Ct value	Ct value	Pos flask /total flask	Ct value
	PBS	0/3			0/3	
Ann Arbor	1	0/2		18.8	0/2	
	2	0/2		19.3	0/2	
	3	0/2		18.8	0/2	
New Orleans	1	0/2			0/2	
	2	0/2		18.0	0/2	
	3	1/2	40.24	19.8	0/2	
St Louis	1	0/2		17.9	0/2	
	2	0/2		24.9	0/2	
	3	0/2		17.5	0/2	

Table 9. Detection of infectious adenovirus from source water suspended solids elutes using combined cell culture and viral mRNA detection by reverse transcription Real-Time PCR with the new transactivated 293 CMV cell line.

WTP	Sample	Adenovirus mRNA			Cell "HK" mRNA Ct value	
	PBS	Pos flask /total flask				
		0/3		Ct value	Pos flask/ total flask	Ct value
Ann Arbor	1	0/2			0/3	
	2	0/2		0	0/2	19.4
	3	0/2		0	0/2	18.7
New Orleans	1	0/2		0	0/2	17.9
	2	0/2		0	0/2	
	3	0/2		0	0/2	18.7
St Louis	1	0/2		0	0/2	16.6
	2	0/2		0	0/2	18.1
	3	0/2		0	0/2	18.9

Table 10. Comparative detection of adenoviruses in source water suspended solids elutes by direct real-time PCR and by infectivity assays in standard and CMV G293 cells.

WTP	Sample	Real-time PCR results	Infectivity results	
			STD G293	CMV G293
Cary	1	nd[a]	NA[b]	NA
	2	nd	NA	NA
	3	nd	NA	NA
Raleigh	1	nd	NA	NA
	2	nd	NA	NA
	3	nd	NA	NA
Nevada	1	nd	NA	NA
	2	NA	NA	NA
	3	NA	NA	NA
Ann Arbor	1	nd	nd	nd
	2	nd	nd	nd
	3	nd	nd	nd
New Orleans	1	41.2	nd	nd
	2	nd	nd	nd
	3	40.0	positive	nd
St. Louis	1	39.5	nd	nd
	2	nd	nd	nd
	3	40.9	nd	nd

[a] Not detected means that no viral genome was detected by Real-time PCR and no viral genome or viral mRNA was detected on successful infectivity assays.
[b] Not analyzed because of the low turbidity of the sample

Fecal indicators in source waters and their relationships to the presence of adenovirus.

The eighteen water treatment source water samples were analyzed for the following fecal indicators: enterococcus, coliforms, *E. coli*, F+ coliphages, somatic coliphages, *Bacteroides* sp., and *Clostridium perfringens* spores. All results are listed in **Table 13** along with physical and chemical water quality data in **Table 14**; both are in the Appendix section of this report. A key question posed in this study was whether or not fecal indicator microorganisms could be sufficient predictors of infectious adenovirus contamination based on their co-occurrence in source water samples. Statistical analysis was done to compare indicator occurrence and concentrations with infectious and total adenoviruses detected using PCR methods. An ANOVA was considered to be the best approach to achieving this goal due to the few data points. Source water samples collected from two water treatment plants located in St. Louis and New Orleans consistently showed higher concentrations of fecal indicator organisms than those from the other plants. In addition, based on the water quality data, these plants had consistently higher turbidity levels.

In **Table 11** are the mean values for the fecal indicators tested at each site. As indicated, the fecal indicators at New Orleans and St. Louis were significantly higher than those at the other sites; with St. Louis having the highest levels detected. **Table 12** displays the calculated p values that compared any observable differences based on the sites sampled, the time of sampling, and the detection of adenoviruses (infectious or non-infectious). These variables were used to do an ANOVA output for all fecal indicators tested. The term "site" refers to the six different WTPs as water sources. The "sample" refers the three different samples taken per site, and the variable "all ads" corresponds to a positive detection of adenoviruses; either infectious or non-infectious. If the p value is lower than 0.05 then there is a significant difference for that particular variable.

The results of this test indicate a significant difference when testing for coliforms (p=.0298), somatic coliphages (p=.0184), and *E. coli* (p=.0401) when sampling across the different WTPs. In addition, when looking at the all ads variable that is categorized as 1 vs. 0, where 1 is positive for adenoviruses (infectious or non-infectious) and 0 is negative for adenoviruses, it is observed that there is a significant difference in the detectable adenoviruses as well as it correlates to the levels of coliforms tested (p=.0500). There is no significant difference in samples per site, but this is to be expected due to consistency in analysis techniques.

It is important to note that with assistance of a biostatistician, a multiple regression analysis was conducted, but the results not reported due to the few data points (i.e. eighteen sample sites). The multiple regression results conflicted with the ANOVA outputs in that nothing was significant. It was advised that more variables are needed to better proceed with this type of an analysis; therefore, the only results reported here are those using ANOVA.

WTP	Enterococcus MPN/100mL Mean (SD)	Coliforms MPN/100mL Mean (SD)	*E. coli* MPN/100mL Mean (SD)	F+ coliphages MPN/100mL Mean (SD)	Somatic coliphages MPN/100mL Mean (SD)
Cary	2.0(1.8)	29.2(33.9)	3.8(3.3)	1.3(0.58)	40.0(34.1)
Raleigh	2.7(1.6)	11.9(16.2)	2.0(1.8)	3.3(4.0)	27.3(37.3)
Nevada	1.0(0)	1.0(0)	1.0(0)	3.3(3.2)	5.0(6.1)
St. Louis	438.6(509.7)	461.0(394.0)	357.6(326.4)	10.0(7.0)	448.0(346.5)
Ann Arbor	1.7(1.2)	17.6(22.5)	5.0(5.3)	1.3(0.58)	10.0(6.1)
New Orleans	34.8(12.7)	186.2(44.8)	70.5(44.8)	3.7(3.8)	65.7(49.6)

Table 11. Means for fecal indicators analyzed at each WTP

	Group Variables [P-value]				
	ALLADS*	**INFADS****	**NONINFADS*****	**SITE******	**SAMPLE*******
Enterococcus	0.1358	0.1612	0.8762	0.1287	0.5149
Coliforms	**0.0500**	0.8018	**0.0020**	**0.0298**	0.5201
E. coli	0.0616	0.9763	0.0159	**0.0401**	0.5821
F+ coliphages	0.0549	0.0839	0.4245	0.1354	0.4456
Somatic coliphages	0.1300	0.1345	0.6184	**0.0184**	0.5708
Bacteroides sp.	0.5921	0.6384	0.7861	0.4646	0.3235
Clostridium perfringens	0.6231	0.3594	0.2101	0.1465	0.9970

Variables used to conduct ANOVA analysis include:
*ALLADS – indicates a positive sample (both infectious and noninfectious)
**INFADS – infectious adenoviruses
***NONINFADS – noninfectioius adenoviruses
****SITE – the WTPs
*****SAMPLE – the source water sampled and analyzed (n=3)

Table 12. ANOVA for all fecal indicator organisms analyzed using source water samples from the six WTPs including all p values (p value < 0.05 is significant).

DISCUSSION

Project Overview

To achieve the overall objective of the project which was to develop and apply methods to better detect and quantify enteric human adenoviruses in environmental source water samples collected from six water treatment plants, several sub objectives were needed to satisfy the demands of the project. The achievement of this goal was important for the advancement of knowledge in that it provided a sensitive and specific detection method to concentrate; purify; detect; and quantify ads from environmental samples, it demonstrated the successful propagation of human ads in a new cell line (293 RAS and 293 CMV) while comparing it to the standard G293 cell line, and it provided a necessary suitable internal control for the performance of CC/mRNA RT-PCR methods. In addition, it raised questions relating to viruses and solids-association; including improving elution methods and the ability to detect infectious adenoviruses from these suspended pellet solids.

To begin with, several methods have been developed and proven to be time consuming, difficult to execute, expensive, and not as effective in recovering certain microbes. Some of these methods included continuous flow centrifugation (Borchardt & Spencer, 2002; Swales & Wright, 2000), immune-affinity concentration, charged filtration, and charged modified filtration for enteric viruses (M. D. Sobsey & Glass, 1980; M. D. Sobsey & Glass, 1984). Research has been conducted at UNC's virology lab that developed and improved an alternative method that is capable of effectively recovering and concentrating certain microbes, including adenovirus 41, found in large volumes of water. The method identified used the hollow fiber ultrafiltration (HFUF) as the primary concentration step with polyethylene glycol (PEG), chloroform

extraction, and ultracentrifugation as secondary concentration steps needed to reduce the sample volume to suitable volumes required for molecular analysis. Based on research conducted, the combined use of cell culture and mRNA RT-PCR was performed based on research that concluded this method to be effective in detecting and quantifying viruses that are difficult to culture (Ko et al., 2003).

Adenoviruses are difficult to culture and researchers have been investigating different methods and techniques that will enhance the ability of these viruses to propagate in cell culture (Jothikumar et al., 2005). A lot has to do with the line of cells used. Of all the cell lines attempted, the standard G293 cell line has been accepted by researchers as the one that yields the best performance for propagating ads. Recently, Dr. GwangPyo Ko and his research team at Seoul National University in South Korea have developed a new transactivated cell line that expresses viral transactivating proteins. These proteins are capable of stimulating a variety of genes (including the viral genes) and other transcription factors which enhance the propagation and detection of infectious ads in cell cultures (Kim et al., 2010). This is suspected to be true due to there being an increase in the level of viral mRNA that replicates during the infection process; therefore, improving the detection methods of the infectious ads in water. A stable 293 CMV cell line was demonstrated to enhance the replication of enteric adenovirus when compared with the G293 (Kim et al., 2010; Ko, Jothikumar et al., 2005). The UNC lab team received frozen samples of the new cell lines (CMV and RAS) and begin the passaging process required to reach confluence. We encountered problems in the beginning of this process due to the G293 CMV cells not reaching complete confluence. After consulting Dr. Ko, our cell culturist was able to get the cell monolayer to grow better and adenoviruses propagated efficiently in the cell line; therefore, the new cell lines were used to begin the comparison.

This research is different from some studies in that it used environmental samples as the source for detection of infectious adenoviruses and in doing so, certain natural inhibitors have to be considered that are not required when conducting lab based experiments. The natural constituents found in environmental waters could interfere in recovering, detecting, and quantifying infectious adenoviruses. Turbidity, one of the water quality parameters has an effect on the levels of detection because in this experiment, high turbidity levels resulted in viruses adsorbing to the suspended solids. To overcome this, additional methods were added to separate the suspended solids and to elute the viruses from these pellets; resulting in solid concentrates that had to be analyzed. In addition, when using environmental sources, it is important to have a robust positive and negative internal control used to verify the performance of the infectivity assay. To our knowledge, there is not a control that has demonstrated success at achieving this when analyzing environmental samples. The separate experiments conducted connect to the overall objective in that it provided the foundation necessary that verified and applied the proposed methods to additional sample sources with the intentions of addressing possible interferences that have previously been researched when analyzing environmental water samples.

Validation of cell culture assays and the use of HK gene, GAPDH, as a positive control.

Research was conducted that combined cell culture with real-time RT-PCR for the detection of adenovirus mRNA (Ko, Jothikumar et al., 2005), however, this combined use of cell culture and viral mRNA RT-PCR detection lacked a positive control that could be used for monitoring the performance of this assay. More specific, it would be used to monitor the status of inoculated cells during infection and the RNA upon extraction and reverse transcription. This project demonstrated the usefulness of measuring a house keeping gene of the cell as a positive control for adenovirus infectivity assays. The gene that encodes for the house keeping gene, GAPDH

was used due to HK genes being constantly expressed at the same level in the cell; therefore, they can be used as a reference for determining the expression of other genes that are not constantly expressed, such as the expression of the viral genes during infection and replication. The expression of viral genes will only occur during infection and their detection is a definitive confirmation of viral infectivity. However, in cases where no viral genes are detected during the infectivity assay, there is no way to know if the lack of detection was due to no infection or due to there being unexpected problems during RNA production, processing, and recovery. Therefore, the incorporation of a reference gene as a positive control during infectivity assays allows for the determination of true negative samples. Our results demonstrated that the expression of the GAPDH gene remained relatively constant during the adenovirus infectivity assay of the host cell at different concentrations of adenovirus inocula. Therefore, it is reasonable to conclude that the expression of the host cell housekeeping gene is not affected by adenovirus infectivity because it needs an active cell for viral propagation, despite it using other cell resources for virus replication.

Comparison of the performance of the new transactivated cell lines 293 CMV and 293 RAS to STD G293 for the propagation of adenovirus 41

The STD G293 cell line was developed from HEK 293 cell line. A new stable transactivated G293 cell line that expressed viral transactivating proteins of cytomegalovirus was created and applied to this study. A comparison study was conducted for sewage samples, concentrated source water samples, and suspended solid pellets from source water samples. Upon investigation of the two new cell lines, it was determined that the 293 CMV demonstrated better performance when compared to the STD G293 cell line for detecting infectious adenovirus in sewage samples; in which virus concentrations are relatively low. The sewage samples were a

good environmental source that could be used to verify the performance of the methods proposed for processing the water field samples collected from the WTPs. There was a difference in the growth kinetics of adenovirus between the cell lines. When observing the virus using the STD G293 cell line, the virus reached optimum performance at 5 DPI versus the 293 CMV cell line that was able to detect maximum ad hexon gene mRNA at 3 DPI. When the cells were collected for both cell lines at 5 DPI, detection of ad hexon gene mRNA was very similar, yielding maximum detection. With the 293 CMV, the incubation period required for detection was earlier than the STD G293, but both cell lines appeared to plateau at the same point, when there are no longer cells to propagate the virus. This difference in growth kinetics that was observed could be a result of the transactivating proteins that were added to the new 293 CMV cell line to enhance propagation and replication of the adenovirus; yielding shorter incubation time required for detection. Despite the similarity in the ability to detect the gene at 5 DPI it was important to develop a method, including a new cell line that was capable of propagating adenovirus from environmental samples at lower concentrations and shorter incubation times. This is useful information for the water treatment plants, enabling them to obtain pertinent information that could be used as a tool for early detection; protecting public health. In addition, government agencies like the EPA could use the new and improved method as a means for detection and quantification of viruses that are potential threats to the public. There is not a current method that is required to regulate drinking water for viral contamination; therefore, if a method is developed that is efficient in performance and cost it could be a useful tool for management.

Sewage samples comparison across cell lines

Before WTP water samples were processed using the different cell lines, an experiment was conducted with the sewage samples collected from three wastewater treatment plants (Cary, Raleigh, and Durham, North Carolina). This experiment demonstrated the performance of all three cell lines on detecting infectious adenoviruses in environmental samples. This resulted in better detection when using the CMV line vs. the RAS line; therefore, we decided to use the CMV cell line when doing the comparison of the WTP samples against the STD 293 line. This experiment yielded positive recovery using the CC/mRNA RT-PCR assay. However, when using the new transactivated cell line for infectivity assays of sewage samples, it showed the ability to detect wild-type adenoviruses at low concentrations (0.8 IU by infectivity assays and 11 genome copies by direct PCR). Adenoviruses isolated from the sewage were typed, indicating the presence of additional groups A and E. These findings confirmed the importance of having reliable methods for detecting and quantifying adenoviruses found in the environment. Due to adenoviruses having the ability to survive in the environment, even after certain treatment processes, it is important that water treatment plants be made aware and be cautious of adenoviruses' fate as a measure of protecting water systems that could potentially place the public at risk. In addition, this demonstrated the days post infection that yielded the best adenovirus hexon gene mRNA detection results for 293 CMV cells, which 3 DPI for maximum detection. This time of maximum infectious adenovirus hexon gene mRNA detection is different from the 5 days for maximum that were required for standard G293 cell line. This difference could be attributed to the transactivating proteins produced by the 293 CMV cell line that enhance the replication mechanisms of the virus during the virus replication cycle. This improved ability to replicate adenoviruses from sewage is significant because environmental

strains of adenovirus may differ from laboratory strains. Therefore, developing and documenting the performance of a method and cell line by the ability to propagate these ads at lower concentrations and shorter incubation times is important for the advancement of science and the security of public health.

Source water samples comparison across cell lines

The source water samples collected from the WTPs were concentrated and adenovirus was detected and quantified using both cell lines (CMV and STD). The propagation of adenovirus was determined by measuring viral mRNA and viral DNA. It is important to note, based on the sewage experiment described above that the CMV cell line was better able to propagate infectious adenoviruses based on higher expressions of mRNA at a shorter incubation time. The sufficient detectable level observed for CMV was at 3 DPI but due to the STD cell line requiring 5 DPI to achieve sufficient detection levels we decided to incubate both lines at 5 DPI when source water samples were analyzed. This was considered necessary when trying to do a true comparison between the two cell lines as all parameters should be equivalent due to possible changes in mRNA levels of specific adenovirus genes in infected cells over time.

Concentrated water samples

The comparison was made and it was determined that the CMV cell line was able to detect the virus faster (3 DPI) vs. STD (5 DPI), but we could not definitively comment on the performance ability of one line to detect the infectious viruses better than the other. This is especially true when looking at the results that show two samples positive for viral mRNA using CMV and two positive using STD. The difference is in the samples. The samples from St. Louis yielded two

positive results using CMV and one sample from St. Louis and one sample from Ann Arbor was positive using STD. In addition, infectious adenoviruses viral mRNA was detected in two samples collected from St. Louis and viral DNA was detected in three samples collected from St. Louis using the 293 CMV cell line.

Concentrated pellet samples

For the comparison of cell lines when detecting infectious adenoviruses adsorbed to suspended solids, we could not conclude that either cell line was efficient in its ability to propagate the virus by the detection of viral mRNA. There was only one positive result for infectivity out of thirty-six samples tested across both cell lines; 293 CMV and standard G293.

The use of "HK" gene, GAPDH as an internal control for CC/mRNA RT-PCR assays

The suitable use of the housekeeping gene, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control for adenovirus infectivity assays to verify the methods used for expressing mRNA (extraction and reverse transcription steps) was documented in this study. This enzyme is constantly expressed in the cell and the gene is commonly used as a positive control for gene expression assays. The mRNA extracted from the cell monolayer was transcribed to cDNA using a poly T primer and the cellular gene GAPDH and the hexon gene were quantified using specific PCR assays. From these experiments, we were able to demonstrate that with increasing number of cells, the concentration of mRNA increases, but the CT value decreases indicating that the more cells present, the easier it is to detect that specific gene. This is what we expect when evaluating the HK gene and its relationship to host cell numbers available for detection. What was most important, was the effect the HK gene had when coupled with the changes in concentrations of sewage. The sewage was diluted, changing the concentration levels of

adenoviruses able to be detected using the hexon gene but not the number of cells. If the sample is less concentrated then there is a decrease in the level of adenovirus hexon gene mRNA detected, but the HK gene should remain constant because it is dependent on the number of cells available for expression. These relationships held true, because the results indicated a constant expression of the GAPDH gene at different concentrations of sewage and adenoviruses; therefore, providing good evidence that this is a suitable internal control for the performance of CC/mRNA RT-PCR on environmental samples.

HFUF and the combined use of CC/mRNA RT-PCR for the detection of infectious ads in source water.

The first phase of this project, conducted at UNC virology lab, demonstrated that the methods incorporating hollow fiber ultrafiltration (HFUF) and polyethylene glycol precipitation (PEG) for concentrating ads in laboratory experiments with spiked water samples were effective for viral recovery. It was essential to verify the performance of these methods using lab based water samples before applying the method to field samples that were proposed for this second, filed sample phase of the project. This phase of the project applied the use of HFUF, PEG precipitation, ultracentrifugation, and improved cell cultures integrated with mRNA reverse-transcriptase PCR, as an efficient method for the detection of group F ads in source waters collected from six water treatment plants. This method is suitable to concentrate large volumes of water (10L - 100L) down to volumes suitable for molecular and cell culture analysis (10mL - 20mL), without effecting virus recovery.

Concentration and recovery using HFUF, PEG precipitation, and UC

The water passes through a cartridge with recirculation, retaining the viruses, and an elution buffer is used to detach any viruses adsorbed to the filter medium and place them back in solution (retentate). For this project, we concentrated 20L of source water. Based on previous experiments with lab spiked water samples, the HFUF elution buffer in the PEG precipitated secondary concentrate was found to be toxic to cell cultures unless largely diluted prior to cell culture inoculation. To eliminate the negative effects cytotoxicity would have on the field samples, precautionary measures were taken and the additional processing steps of chloroform extraction and ultracentrifugation were added to the concentration procedure. Ultracentrifugation separates the viruses from the buffer and further reduces the sample volume to approximately 10mL. After the samples were concentrated to ~5mL, four equal aliquots were frozen for virus infectivity; one was assayed using the standard G293 cell line at UNC, one was assayed using the new transactivated 293 CMV cell line sent from Dr. Ko's lab, one was sent to Dr. Ko's lab for independent but parallel viral infectivity analysis, and one was archived for any future analysis that might be needed.

It is important to address any conditions that could potentially interfere in the recovery efficacy of the virus, especially when using field sampled due to the natural inhibitors that are present in the samples, as well as those added during sample processing, such as ingredients in the reagents in which the viruses are suspended. It is also essential to verify the analyst's performance of techniques in order to eliminate any chances that the analyst lack of proficiency in performing the method is interfering in the virus recovery process rather than the adverse

effects on virus recovery and detection of natural inhibitors present in the sample or inhibitors added as reagents during sample processing.

Cell culture and mRNA RT-PCR method

The only other problem we encountered while cell culturing was with the toxicity of one of that samples (St. Louis) that lead to cell death. To address this, we diluted the sample and then preceded with cell culture assays.

The effect of suspended solids on the recovery of adenoviruses

It is well documented that turbidity or suspended solids found in water can interfere with detection methods for viruses (Moore, Sagik, & JF Jr, 1975; M. D. Sobsey, Dean, Knuckles, & Wagner, 1980). Based on the levels of some of the initial water quality parameters (**Table 15**) obtained as data from the water treatment plants at the time of sampling, we suspected that sample matrix interference could inhibit recovery and detection. To determine the effect turbidity has on viral infectivity, a method to elute the viruses from the suspended solids was performed and verified using additional samples collected from New Orleans and St. Louis by spiking the water samples with adenovirus. These particular WTPs showed higher levels of source water turbidity; New Orleans (>50 NTU) and St. Louis (>30 NTU) than did the source waters of the other WTPs. Understanding the interfering role of turbidity and suspended solids on virus recovery and detection and addressing this interference by adjustments in the sample processing and assay methods is essential to the overall success of this project. We needed to understand what effects the association of adenoviruses with suspended solids in the concentrated samples had on recovering the viruses and performing cell culture infectivity assays and molecular detection. In addition, to ensure we were not underestimating infectious group F

adenoviruses in source water samples due to the presence of suspended solids, understanding the possible interfering effects of high turbidity needed to be understood prior to proceeding with further processing and analysis of the WTP pellets collected during the HFUF, PEG precipitation, and ultracentrifugation steps.

From the results in **Table 8**, adenoviruses are attaching to the suspended solids and elution from the solids enable the detection of DNA by direct PCR. Four of the samples were detected for the presence/absence of adenoviruses (two samples from St. Louis and two samples from New Orleans; both having high turbidity levels). However, it is unknown if the eluted viruses were inactivated, becoming non-infectious due to them not being detected in cell culture; with the exception of one sample, New Orleans. We do not have sufficient evidence to state that using CC/mRNA RT-PCR as the method for detecting infectious adenoviruses in suspended solids is an effective approach.

Fecal Indicators and their relationship to the presence of adenovirus

It was proposed to sample drinking water treatment plant source waters likely to contain group F ads based on their geographical locations which are intended to be representative of the United States. The sites that were chosen were: Raleigh and Cary, North Carolina; St. Louis, Missouri; Ann Arbor, Michigan; New Orleans, Louisiana; and Las Vegas, Nevada. It was agreed upon by UNC and WateRF to sample each plant three separate times over a one year period (September 2009, November 2009, and February 2010), to account for any seasonal variations that could occur with adenoviruses.

In addition, it is important to note that some of the WTPs selected were under the influence of wastewater treatment plant discharges. If WTPs received effluent from WWTPs and/or if there were other environmental conditions that influenced the source water used by the WTPs, then it

could impact the detection of adenoviruses and the use of fecal indicators as a true indicator for viral contamination. Adenoviruses are able to survive in waters at low temperatures and through water treatment processes.

The results of this study did not demonstrate any positive relationships between the presence and concentrations of fecal indicator organisms (FIOs) and the detection of infectious adenovirus. The only water quality parameters that showed significant associations were turbidity and somatic coliphages. When examining the mean values for the FIOs **(Table 11)**, the samples from St. Louis and New Orleans had higher FIO values than the other samples, but between these two sites, samples from St. Louis resulted in positive ads detection. In contrast, Ann Arbor and Nevada had relatively good FIOs but a sample from these WTPs was positive for adenovirus. Due to the discrepancy between fecal indicators and positive detection of ads, it is very difficult to establish a clear relationship between the FIOs and the presence of infection adenovirus. Therefore, there was no way to produce a general model that could predict the presence of adenovirus in water using FIO data. What could be suggested is that adenoviruses may be more persistent in those waters sampled with relatively low FIOs (but positive ads detection) than the actual bacteria analyzed.

In addition to FIOs, it was also observed that water with high turbidity is a potentially greater risk because there is a potential for viruses to adsorb to suspended solids and interfere in detection and recovery. Nevertheless, high turbidity source waters also were more likely to contain adenoviruses and it is recommended that additional studies be conducted that examine these relationships on a larger scale, proving more data enabling more appropriate and effective statistical analysis to be done.

After statistical consultation, it was determined that the suggested multiple regression analysis would not be meaningful due to the small data set collected. Unfortunately, due to limited resources, additional water sampling and analysis was not within the scope of this project. However, additional research could be built on the data we have obtained. The results for this study objective are inconclusive in that it is not possible to provide a reliable answer that will verify the use of FIOs as a predictive tool for determining the risk for contamination from adenoviruses in drinking water. Such a predictive ability would be helpful due to the methods used to test for FIOs being relatively inexpensive, easy and widely in practice. It would be beneficial if levels of FIOs could signal water facilities when further analysis of their source water is needed and more aggressive action is needed due to increased virus risks; either prior to treatment or prior to it reaching the consumer. It would be beneficial for current regulatory practices used to monitor water quality by checking levels of FIOs, pH, turbidity, and temperature to determine the degree of virus risk of source waters.

Limitations and Recommendations

There were some limiting factors and recommendations that should be considered when discussing this project. The sample size was small which presented problems in performing statistical analysis. It would be beneficial to conduct a larger experiment, including additional locations; providing a wider spread of geographical variations which might impact results. It would be recommended to perform more replications of each individual water treatment plant spaced throughout the calendar year to see if there is some seasonal variation in detecting infectious adenoviruses. For example, it would be important to sample during the summer months when people tend to use rivers and lakes for recreational purposes to compare levels of recovery and detection. Natural environmental inhibitors present in the samples could have

impeded virus assays. I would also recommend that finished water be collected and analyzed from the water treatment plants that yielded positive findings for infectious adenoviruses. In addition, more analysis needs to be performed evaluating the use of fecal indicators as predictors of viral contamination.

In addition to the environmental sampling, more spiked water samples need to be conducted to examine the solids-association that was observed when processing the field water source samples. A better understanding of the methods to recover, elute and detect viruses that have adsorbed to solids would be recommended with particular focus on the affect elution solutions have on the viruses' infectivity.

Additional analysis of the source water sample concentrates are currently being done at Dr. GwangPyo Ko's lab to further evaluate the use of the new transactivated cell lines. There should be some comparison of the data set between the two labs; UNC and Seoul University. I would also recommend additional samples to be analyzed using the new cell lines with other environmental sources to see if the cell lines can be improved. To my knowledge we were one of the first labs to use these transactivated cell lines on environmental samples so it would be recommended that a larger study be conducted that can more effectively evaluate the performance of these cell lines in comparison to the standard cell line.

CONCLUSIONS

- Source water samples positive for infectious adenoviruses were found using the source water concentrates from St. Louis and Ann Arbor; using both the new transactivated G293 CMV cell line and the standard G293 cell line. Two out of the eighteen samples tested were positive for infectivity.
- Source water samples positive for infectious adenoviruses were found using source water suspended solid pellets concentrates from New Orleans only. Of the nine samples tested, due to higher levels of turbidity, only one was positive using the standard G293 cell line. We suspect that the viruses could have become inactivated during the process of eluting the viruses from the suspended solids. Further tests need to be conducted to evaluate the effect elution solutions have on viruses.
- There is a solids-association for virus adsorption that was observed. Despite a substantial amount of negative samples for infectivity using these suspended solid pellets, we can confidently conclude that viruses adsorb and that this affects recovery and detection of infectious adenoviruses
- The viruses recovered from suspended solid pellets that detected the presence/absence of the hexon gene for viral DNA using real time PCR yielded four positive out of sixteen samples analyzed. These samples were from New Orleans and St. Louis.
- The use of HFUF as a primary concentration method and PEG precipitation, chloroform extraction, and ultracentrifugation as a secondary concentration method was effective in recovering and concentrating the viruses in the samples tested.

- The method of CC/mRNA RT-PCR used to conduct infectivity assays demonstrated effective in detecting and quantifying infectious adenoviruses from all sample sources tested (environmental sewage and water and spiked lab based samples). This method was sensitive and specific for the detection of infectious adenoviruses.
- The evaluation of the housekeeping gene, GAPDH as a control for the performance of the infectivity assays using environmental samples is a suitable control.
- The new transactivated cell line, G293 CMV is a promising new cell line for the combination of cell culture and mRNA RT-PCR based on the decreases time required for incubation (3 DPI). This reduction in detection time is important when determining the impact a virus has on human health. Reducing the time required to detect a virus without compromising the level of mRNA expression is critical, and this cell line was effective in achieving this.
- We cannot conclude that there is a significant difference, other than DPI, between the cell lines in terms of the detection of viral mRNA. Both the STD G293 and the CMV G293 effectively detected viral mRNA.
- We can conclude that we were able to develop, verify, and apply a method that was sensitive and specific in the recovery, concentration, purification, detection, and quantification of infectious adenoviruses; using environmental samples (water and sewage).

APPENDIX

Table 13. Fecal indicator microorganisms' concentrations in source waters collected from six water treatment plants (WTP).

WTP	Sampling	Enterococcus MPN/100mL	Coliforms MPN/100mL	*E. coli* MPN/100mL	F+ coliphages MPN/100mL	Som. Coliphages MPN/100mL	*Bacteroides* sp. (GC/100mL)	Clostridium spores (MPN/mL)
Cary	1st	1	15.8	<1.0	<1	<1	2.2×10^{2}	<0.18
	2nd	4.1	67.7	7.4	2	64	4.9×10^{2}	<0.18
	3rd	1	4.1	3	<1	55	1.5×10^{3}	<0.18
Raleigh	1st	<1.0	4.1	<1.0	<1	<1	5.3×10^{1}	<0.18
	2nd	3.1	30.5	4.1	8	11	7.1×10^{2}	<0.18
	3rd	4.1	1	<1.0	1	70	4.1×10^{1}	0.45
Nevada	1st	<1.0	<1.0	<1.0	2	12	7.4×10^{1}	<0.18
	2nd	<1.0	<1.0	<1.0	<1	2	$< 2.5 \times 10^{1}$	<0.18
	3rd	<1.0	<1.0	<1.0	7	<1	7.4×10^{1}	<0.18
St. Louis	1st	270	913.9	721.5	5	284	2.5×10^{3}	0.45
	2nd	1011.2	272.3	260.3	18	846	3.1×10^{3}	2.3
	3rd	34.5	196.8	90.9	7	214	5.7×10^{2}	<0.18
Ann Arbor	1st	<1.0	43.2	11	2	13	9.4 x 10 2	<0.18
	2nd	3.1	8.6	3.1	1	3	1.1 x 10 2	<0.18
	3rd	1	1	<1	<1	14	4.1 x 10 1	<0.18
New Orleans	1st	42	248.9	81.7	1	14	2.2×10^{2}	1.8
	2nd	20.1	116.2	21.1	2	113	1.3×10^{4}	0.45
	3rd	42.2	193.5	108.6	8	70	5.7×10^{2}	0.2

Table 14. Physical and Chemical Water Quality Data for WTPs

WTP	Sampling	Alkalinity (mg/L)	Hardness (mg/L)	Turbidity (NTU)	pH	Temperature (°C)	TOC (mg/L)
Cary	1st	42.7	43.35	5.57	7	27	6.8
	2nd	39.25	32.61	18.3	7	16.1	5.8
	3rd	27		9.2	6.8	7	7.2
Raleigh	1st	31.47	31.8	1.46	6.56	25.1	6.19
	2nd	26.7	36.86	5.12	6.39	15.5	104
	3rd	17		14.6	6.62	5.4	8.58
Nevada	1st	140		0.29	8	19	2.5
	2nd	133	160	0.4	7.9	17.2	2.9
	3rd			0.4	8.15	13.2	2.2
St. Louis	1st	166	220	33	8.26	23.9	4.23
	2nd	100	125	330	7.83	14	6.37
	3rd	175	224	39	8.08	3	3.68
Ann Arbor	1st	209	286	1.88	8.3	18.2	5.9
	2nd	224	284	0.88	8.3	8.4	5.43
	3rd	256	308	1.23	8.3	5.8	5.36
New Orleans	1st	116	159	53	7.74	26.5	3.3
	2nd	108	132	100	7.72	15.8	4.4
	3rd	120	167	117	7.91	4.9	3.2

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