DETECTION AND INFECTIVITY OF HUMAN ADENOVIRUS IN WASTEWATER EFFLUENT, BIOSOLIDS, AND SHELLFISH, AND ITS PERSISTENCE IN ESTUARINE WATER

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by
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Project Abstract

The United States Environmental Protection Agency (USEPA) has established the Total Coliform Rule as an indication of health risks associated with microbial contamination of drinking and ground water. In addition, the fecal coliform test is used as an indicator to reflect the suitability of use by consumers of class A biosolids. However, numerous studies have shown that bacterial indicators are not predictive of enteric viruses, such as human adenovirus (HAdV), which are much more resistant to treatment methods than bacteria. Enteric viral contamination of estuarine waters and locally-harvested shellfish as a result of receiving effluent from wastewater treatment plants, as well as run-off from agricultural land treated with biosolids, can have serious implications for human health. Preliminary results suggest that HAdV is present in biosolids, wastewater effluent, estuarine waters receiving effluent and shellfish harvested from these receiving waters. The density, persistence and infectivity of human adenovirus in these environmental matrices are not known. The focus of this research was to address the presence, persistence and viability of HAdV in all four matrices.

Presence and density of the virus was established through the use of a nested polymerase chain reaction (PCR) and quantitative PCR (qPCR). HAdV DNA was detected in 21 of the 26 biosolid samples and 21 of the 24 effluent samples assayed. The treatment method employed in the processing of the samples appeared to have an effect on the detection and concentration of HAdV DNA. Persistence of HAdV DNA in estuarine water was addressed in an in situ study using seeded microcosms containing either sterile river water or unfiltered river water under various environmental conditions during the spring, summer and fall. Unfiltered river water collected during the summer had the greatest deleterious effect on HAdV DNA persistence. HAdV DNA was most persistent, under all environmental treatments, during the fall. An in vitro study of sterile river water confirmed that temperature, not salinity, had a greater effect on HAdV DNA degradation. Laboratory tank studies revealed that oysters are capable of filtering and retaining HAdV from contaminated water. In each of the three tank studies conducted, HAdV DNA was detected in tissue samples from oysters exposed to seeded river water for 18 hours. It was also established that the oysters could depurate the virus, in an open system, in as little as three days.

Integrated cell culture (ICC) - qPCR was used to determine the viability of detected viral particles. No direct correlation between the detection of HAdV DNA and the presence of viable viruses was found. Frequently, samples that contained HAdV DNA failed to produce viable virions. Current research corroborates these results, suggesting the detection and persistence of viral DNA is not sufficient evidence to support the assumption of viability.

DETECTION AND INFECTIVITY OF HUMAN ADENOVIRUS IN WASTEWATER EFFLUENT, BIOSOLIDS, AND SHELLFISH, AND ITS PERSISTENCE IN ESTUARINE WATER

Introduction

Contamination of coastal and estuarine waters by fecal pollution can lead to serious human health risks. According to the World Heath Organization, waterborne or water related diseases comprise over 88% of the 1.8 million annual diarrheal related fatalities worldwide (http://www.who.int/water_sanitiation_health/publications/facts2004/en/index.html). A proportion of these deaths can be attributed to enteric viruses that are shed in human feces and contaminate environmental waters through the release of insufficiently treated effluent from wastewater treatment plants and, to a lesser extent, terrestrial runoff from agricultural lands treated with biosolids. Exposure to these viruses may occur through use of, direct ingestion, or recreational exposure to contaminated water or through the consumption of contaminated shellfish (Beller et al., 1997; Jiang et al., 2001; Simmons et al., 2001; Fromiga-Cruz et al., 2002; CDC, 2005; Haramoto et al., 2007). Infection by human enteric viruses can result in respiratory illnesses, conjunctivitis, hemorrhagic cystitis, and/or gastroenteritis (Parshionikar et al., 2003; Haramoto et al., 2007). One enteric virus of growing interest and concern with regard to environmental transmission is human adenovirus (HAdV) (Nwachuku and Gerba, 2004). While human adenoviruses, specifically species F, cause gastroenteritis, infections are usually asymptomatic among healthy adults due to conferred immunity (Jiang, 2006). It is much more common for symptoms to occur in children and immune compromised individuals. Adenovirus is

second only to rotavirus as the primary cause of pediatric gastroenteritis (Chapron et al., 2000; Jiang, 2006).

Human adenovirus is a non-enveloped, icosahedral virus belonging to the family Adenoviridae and genus *Mastadenovirus*. It is a double stranded DNA virus with a genome size of 30-36 kilobases, depending on the species, and encodes 30-40 genes (Russell, 2009). The virus is comprised of capsid proteins, minor proteins, and core proteins. The capsid contains 252 capsomers (240 hexons and 12 pentons) and 12 fiber proteins (Russell, 2009). The hexon monomers (including minor proteins) function as structural and stabilizing proteins while the fiber proteins allow for host cell recognition and binding and the penton bases enable host penetration. The core proteins are involved in DNA packaging and replication (Berk, 2007; Russell, 2009). Initially, classification of human adenoviruses was based on hemagglutination properties and the various strains were referred to as serotypes. However, due to a number of cases of cross-reactivity, perhaps due to high incidence of intraspecific recombination, and to a lesser extent, interspecific recombination, this method of classification has largely been abandoned in favor of molecular methods that exploit variability in the hexon gene, with as many as nine hypervariable regions having been found by comparative sequence analysis (Ebner et al., 2005; Madisch et al., 2005; Jones et al., 2007 Russell, 2009). These variable regions of the hexon gene are currently used for the identification and classification of human adenovirus. Over the last six years, due to the increase in genotyping as a means of identification, the number of identified adenovirus species has increased from six to seven and the

number of identified genotypes has increased from 51 to 57 (Table 1). (Madisch et al, 2005; Robinson et al., 2011; Walsh et al, 2011; Jones et al 2007). However, it is highly debated whether a new human adenovirus species, called species G, is truly a valid species. (Jones, et al., 2007, deJong and Osterhaus, 2008).

While it is widely accepted that transmission of HAdV can occur through inadequately chlorinated swimming pools and drinking water, less in known about the direct transmission and fate of HAdV in the marine environment. Studies have reported that human pathogenic viruses can be found in marine sediments with a concentration 10 - 10,000 times greater than in the overlaying marine water (Bosch et al., 2005). The adsorption of the virus to the marine sediment serves to protect it from environmental inactivation and may result in unanticipated resuspension due to physical or chemical disruptions of the sediment (Van Donsel & Geldreich, 1971; Bosch et al., 2005). Consequently, viruses that desorb from sediment in areas known to be polluted with fecal contamination may be transported through the water to unpolluted areas (Griffin et al., 2003). Conflicting data regarding the viability of HAdV in marine waters may be due to different environmental conditions present at various study sites. Variables known to impact virus viability include temperature, pH, salinity, light, presence of solids, and indigenous microbiota (Girones et al., 1989; Sobsey et al., 1998; Bosch et al., 1998). However, to our knowledge, no studies have been conducted, under controlled conditions, to address the exact impact that each these conditions have, either alone or in combination, on the viability of HAdV in marine waters. Given that HAdV is a dsDNA virus, while many other

enteric viruses such as norovirus, coxsackievirus and poliovirus are RNA viruses, and that DNA is generally more stable that RNA, HAdV may be better able to persist in the environment. Determining the impact that various environmental parameters have on HAdV persistence and its viability in marine waters is vital to appropriately addressing human health risks associated with HAdV contamination. Due in large part to this genomic structure, adenovirus has been recognized as more resistant to UV exposure than many other waterborne pathogens (Linden et al., 2007). Additionally, adenoviruses can survive for extended periods outside of their animal hosts (Horowitz and Mold, 2007). This has led to adenoviruses inclusion on the EPA's Contaminant Candidate List, which identifies particular pathogens as priorities for drinking water research and fecal contamination monitoring (USEPA 1998, 2005). Another important attribute, unlike other enteric viruses such as norovirus, adenovirus can be cultured as a means of assessing viral infectivity (Piña et al., 1998; Puig et al., 1994; Formiga-Cruz et al., 2002; Muscillo et al., 2008; Gerba et al., 2002; Nwachuku, et al., 2005; Straub et al., 2007; Griffin et al., 2008; Dong et al., 2009). Given these and other favorable characteristics, adenovirus is currently considered as a candidate viral indicator over other viruses that may be present in fecally-contaminated waters.

Assessing the health risk in both treated and ground water is challenging when viruses are involved. The Environmental Protection Agency (EPA) has established two methods for determining drinking water health as it relates to microbial contamination, the Total Coliform Rule, a measure of fecal coliform counts, and the Information Collection Rule (ICR) (USEPA 1989, 1995, 2003). The widely accepted

Total Coliform Rule is not effective in determining viral load since much research indicates that bacterial indicators are not a good proxy for viruses (Fong et al, 2005). The ICR stipulates that enteric viruses must be detected and quantified via the total culturable virus assay, most-probable- number (TCVA-MPN) method (USEPA, 1995). The TCVA-MPN method as generally implemented employs detection of total viral cytopathic effects in Buffalo green monkey kidney cells, which can reduce its effectiveness in detecting enteric viral contamination. For example, many adenovirus strains do not produce cytopathic effects during replication, and other enteric viruses, such as astrovirus cannot grow in Buffalo green monkey kidney cells (Chapron et al., 2000). Consequently, determining the "true" level of viral contamination in any water sample by employing either of the two above-mentioned methods can yield inaccurate results due to false positives or false negatives. This can in turn lead to water safety concerns with potentially serious health consequences. When one considers that adenoviruses are less susceptible to UV radiation, extreme shifts in pH, ionic strength, and are more persistent than bacteria in estuarine water and sediment, the limitations of the TCVA-MPN assay become especially troublesome (Symonds et al., 2009).

The specific mode of transmission, as well as clinical manifestation, varies between species of adenovirus (Heim et al., 2003). Although many species exhibit waterborne transmission, species F, genotypes 40 and 41, are of primary concern when addressing the effectiveness of wastewater treatment facilities in producing safe effluent and biosolids. These strains cause gastroenteritis in humans and are

transmitted via oral/fecal contact, which can occur as the result of ingestion of contaminated water during recreational activities or by consumption of shellfish harvested from contaminated waters (Jiang et al., 2001; Fromiga-Cruz et al., 2002; CDC, 2005; Haramoto et al., 2007). After infection, some adenovirus strains may be shed in feces for months or even years (Jiang, 2006). Current studies indicate that 100% of raw sewage samples contained adenovirus, while the frequency of adenovirus- positive wastewater effluent samples ranged from 25% - 82%, with little seasonal variation (Piña et al., 1998; Haramoto et al., 2007; Symonds et al., 2009).

Wastewater that is received by a treatment plant is referred to as influent. The influent is treated by a number of chemical, physical and biological processes and then returned as effluent to local waterways, such as an estuary or river. Typically, wastewater treatment plants employ the same basic steps to processing influent, but may differ in the tertiary phase. Initially influent undergoes a preliminary screening treatment that removes large debris. The second phase, or primary treatment, removes solids and organic matter by allowing solid particles to sink and low-density organic matter such as oil to float. The organics are skimmed off the top and the solids are removed. The influent then passes on to secondary aerobic treatment in which microorganisms assimilate macromolecules such as sugars, proteins and detergents. Tertiary or inorganic nutrient removal, following secondary treatment, may be used by some plants to remove additional phosphorous and nitrogen. Finally, a disinfecting step typically involves chlorinating the effluent to kill microbial pathogens and dechlorinating before the treated water is released into the

environment as final effluent. One variation that is being seen more frequently is a shift from chlorine disinfection to ultraviolet (UV) disinfection. UV disinfection is appealing because it is typically effective in destroying bacteria, protozoa and viruses found in wastewater, is a physical process as opposed to a chemical process so there are no toxic or hazardous chemicals to be handled, and it does not produce chemical by-products that could be harmful to aquatic life (Fahey, 1990; Blatchley, 1996). Unfortunately, a number of studies have found that UV treatment is ineffective in destroying human adenoviruses, as well as some bacteria such as *Pseudomonas aeruginosa* (Thompson et al., 2003; Thurston-Enriquez et al., 2003; Nwachuku and Gerba, 2004).

Locally in Virginia, the Hampton Roads Sanitation District (HRSD) still relies on chlorine disinfection, although each of its treatment plants may use different secondary and tertiary treatments. The research described in this dissertation focused on three Virginia plants that employ different secondary and tertiary treatment strategies. The Virginia Initiative Plant in Norfolk, VA uses Biological Nutrient Removal (BNR) technology to remove nitrogen and phosphorous, while the Williamsburg Treatment Plant in Williamsburg, VA uses oxidation towers, a type of biological filter, to treat influent which is dominated by brewery waste and the James River Plant in Newport News, VA, does not currently use any additional treatment for nutrient removal, but is in the process of testing Integrated Fixed Film in Activated Sludge (IFAS) for future use (http://www.hrsd.com/treatmentplants.htm). The effluent from each of these plants was assessed for HAdV presence and viability.

International studies have confirmed that HAdV contamination has been greatly underestimated (Jiang, 2006). The advent of molecular techniques has led to a significant increase in the number of reports of human adenovirus contamination in river, coastal and surface waters worldwide (Chapron et al., 2000; Jiang et al., 2001; Greening et al., 2002; Lee et al., 2005, Haramoto et al., 2005). Therefore, it is important to determine the adenoviral load in wastewater treatment plant (WWTP) effluents and specifically, to measure the infectivity and persistence of viruses in waters receiving these effluents. WWTP effluents released directly into the marine environment can potentially contaminate receiving waters used for recreation purposes, as well as adjacent shellfish beds, thereby compromising the public-health safety of locally harvested shellfish and affecting their commercial utilization.

In order to ensure that shellfish harvested for direct marketing to the public are safe for human consumption, shellfish growing waters must be classified as "enteric-pathogen free." The National Shellfish Sanitation Program (NSSP) uses a microbiological standard outlined by the United States Food and Drug Administration (FDA) to ensure that shellfish are safe to consume. The standard is based on total or fecal coliform counts for classifying waters for shellfish harvesting. The current fecal coliform standard for shellfish growing water is an MPN equal to or less than 14 fecal coliforms/100mL, with no more than 10% of samples exceeding 43 MPN fecal coliforms/100 mL (U.S. FDA, 2009).

There are no national or regional requirements, however, to test directly for the presence of human viral contamination. A study conducted by the FDA found that shellfish accumulated large quantities of enteric viruses and that consumption of contaminated raw shellfish posed a public health risk (Lees et al., 2010). Once viruses are released into the marine environment, they may persist for months in the water column and accumulate in the sediment (Bosch, 1998; Formiga-Cruz et al., 2002). Shellfish take in the viruses while filter feeding and concentrate them in their edible tissues (Bosch, 1998). Outbreaks of enteric viral gastroenteritis have been linked to the consumption of contaminated shellfish worldwide (Ng et al., 2004; David et al., 2007; Sala et al., 2009). While norovirus- and rotavirus-contaminated shellfish are often linked to outbreaks of gastroenteritis, adenovirus is seldom implicated in outbreaks, even though it is present (Kukkula, et al. 1997) in oysters or infected individuals. This could be because adenovirus infections are typically asymptomatic among healthy adults due to conferred immunity (Jiang, 2006). In a study of Korean oysters, Choo and Kim (2006) employed integrated cell culture-quantitative polymerase chain reaction (ICC-qPCR) to detect adenoviral and enteroviral contamination. They found 50.9% of oysters collected from the Noryangjin fishery wholesale market were contaminated with infective adenovirus while 30.9% were contaminated with infective enteroviruses. In addition, Kukkula et al. (1997) and Formiga-Cruz et al. (2002) report that adenovirus is often found in concert with other enteric viruses, such as norovirus. Therefore, although adenovirus infection is typically asymptomatic in healthy adults, its occurrence in bivalve

shellfish may prove to be a useful indicator of enteric viral contamination, and by extension, the waters from which they are harvested (Formiga-Cruz et al., 2002).

Commercial depuration, the process of attempting to clear viruses by placing shellfish in uncontaminated water, does not appear to effectively reduce the quantity of infective viral particles in shellfish. In fact, depurated shellfish typically retain higher levels of adenoviruses than coliform bacteria (Gerba et al., 1979; DeLeon and Jaykus, 1997; Pina et al., 1998; Nasser and Omar, 1999; Formiga-Cruz et al., 2002). Formiga-Cruz et al., (2002) compared the effectiveness of commercial depuration of *Crassostrea gigas* for *E. coli* and human adenovirus contamination and reported that depuration, although effective for removal of *E. coli*, was ineffective for removal of human adenovirus. Twenty eight percent of the non-depurated oysters analyzed were positive for adenovirus while 25% of the depurated oysters were positive. Depuration was much more effective for clearing *E. coli* contamination; 61% positive in the non-depurated sample compared to 6% positive for the depurated group. Additionally, an *in situ* study of adenovirus persistence in bivalve shellfish (Hernroth and Allard, 2006) demonstrated that not only do oysters concentrate adenovirus in their gills and digestive gland, but it is detectable for 6-10 weeks after contamination and appears to remain infective for 2-4 weeks at 18°C and up to 6 weeks at 4°C in these tissues. Furthermore, Dancho and Kinsley (2010) recently reported that enteric viruses have been found to persist in the hemocytes of the Eastern oyster (*C. virginica*), perhaps the basis of ineffective depuration.

Adenovirus containing WWTP effluent is not the only potential source of environmental viral contamination. Wastewater treatment plants also produce biosolids, which have been shown to contain infective adenoviruses (Wei et al., 2009; Wong et al., 2010). Biosolids is the term used to designate sewage sludge that has been processed to reduce or eliminate pathogens and organic content, so that it can be used in land application, which is defined by EPA as "...the spreading, spraying, injection or incorporation of sewage sludge, including a material derived from sewage sludge (e.g., compost and pelletized sewage sludge), onto or below the surface of the land to take advantage of the soil enhancing qualities of the sewage sludge" (http://www.epa.gov/owm/mtb/biosolids/sludge.pdf). Biosolids are classified by EPA as either class A or class B. Class A biosolids are deemed pathogen-free following treatment as determined using the fecal coliform indicator, and can be sold directly to the public, while Class B biosolids may contain detectable levels of pathogens, but they have been treated, and along with site access restrictions implemented following application, are presumed to be a low public health risk. Class B biosolids are used by commercial applicators on forest and agricultural lands.

Several different methods are used for generating biosolids from sewage sludge including separation, aerobic and anaerobic digestion, alkaline stabilization, composting, ultraviolet radiation, and heating (Jenkins et al., 2007). Each wastewater treatment facility determines, independently, the method or combination of methods that it will use (Evanylo, 2003). As mentioned above, the USEPA has established the Total Coliform Rule for determining health risks associated with drinking and ground

water (USEPA 1989, 2003a) and fecal coliform density is also used as an indicator for biosolids to determine treatment effectiveness although as with the water treatments, enteric viruses are more resistant than bacteria to biosolids treatment methods (Symonds et al., 2009).

Although several studies have addressed the risks associated with aerosolization of pathogens from biosolids (Brooks et al., 2005; Baertsch et al., 2007; Jenkins et al., 2007; Low et al., 2007), little work has been done to assess the risk of pathogen contamination, particularly by enteric viruses, to ground water, rivers, lakes and estuaries by runoff from biosolids applied to agricultural lands and forests (Jenkins et al., 2007). Persistent viruses could pose a health risk if they remain intact after application and are capable of transport through runoff to ground or surface waters. "The longer a pathogen survives in the environment, the greater the chance of its transmission to a susceptible host" (Committee on Toxicants and Pathogens in Biosolids Applied to Land, National Research Council, 2002). Transport of fecal coliforms and other microorganisms, including adenovirus, from source biosolids to marine receiving waters through runoff could have potential human health consequences.

Human adenoviruses have been shown to be resistant to many currently employed wastewater treatment methods. They have been detected in effluent and biosolids released from treatment plants and in oysters and other bivalves grown in effluent receiving waters. Limited data also suggest that these viruses are persisting in an

infectious state. However, further development and validation of molecular screening techniques to assess persistence of human adenovirus in local marine waters and shellfish is needed. The main goals of this dissertation were to: 1) detect and quantify human adenovirus (HAdV) in pre- and post-chlorinated effluent produced by three Virginia wastewater treatment plants (WWTP) during all four seasons; 2) detect and quantify HAdV in sentinel oysters exposed to James River WWTP effluent outfall during all four seasons; 3) detect and quantify HAdV in biosolids produced by WWTPs across the United States that employ various treatment methods; 4) assess infectivity of HAdV present in effluent, sentinel oysters and biosolids; and 5) determine the seasonal persistence of HAdV using microcosms exposed *in situ* to an estuarine environment and under *in vitro* conditions.

While the overarching goal of this research was to assess the presence, viability and fate of human adenovirus throughout the wastewater treatment process, each of these topics will be addressed in separate chapters. A final concluding chapter will provide a concise review of the effectiveness of three Virginian WWTPs in the treatment and removal of HAdV through each step of the process, as well as an assessment of the persistence of HAdV under various environmental conditions.

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Chapter 1: Occurrence and infectivity of human adenovirus in secondary clarifier and final effluents from three different local wastewater treatment facilities in Virginia

Abstract

Water contaminated by human enteric viruses, such as human adenovirus (HAdV), can result in respiratory illnesses, conjunctivitis, hemorrhagic cystitis, and gastroenteritis. One means by which adenoviruses can enter coastal and estuarine waters is through ineffective treatment of influent by wastewater treatment plants resulting in the release of contaminated effluent into the environment. Secondary clarifier (SCE) and final (FNE) effluents from the James River, Virginia Initiative, and Williamsburg wastewater treatment plants (WWTPs) in Hampton Roads, VA were collected in January, April, July and October for two years and assayed for the presence and infectivity of human adenovirus. Nested PCR and qPCR were used to detect HAdV DNA. Both assays allowed for the detection of HAdV DNA, but qPCR also allowed for the quantification of viral density. Integrated cell culture-qPCR (ICC-qPCR) was used to assess infectivity of HAdV in final effluents from the James River wastewater treatment plant. Nested PCR detected HAdV DNA in 13/24 (54%) of the SCE and 8/24 (36%) of the FNE, while qPCR detected HAdV DNA in 9/24 (37.5%) of the SCE and 12/24 (50%) of the FNE. Of the three WWTPs, the James River facility produced significantly more positive effluent samples. No significant seasonal variation was detected. Viable virus was detected by ICC-qPCR in 1/8 FNE samples (12.5%) from the James River WWTP.

Introduction

Contamination of estuarine water with enteric viruses has tremendous implications for human health. Enteric viruses such as adenovirus, norovirus, rotavirus, and enteroviruses can be transmitted through exposure to water contaminated with human feces resulting in outbreaks of respiratory illnesses, conjunctivitis, hemorrhagic cystitis, and gastroenteritis (Parshionikar et al., 2003; Haramoto et al., 2007). Estuarine waters can become contaminated with these viruses through terrestrial runoff and effluents released by wastewater treatment plants (Jiang et al., 2001; Fong et al., 2010; Viau et al., 2011). While terrestrial runoff may be difficult to control and predict, assessing the microbiological quality of effluents produced by wastewater treatment facilities can be achieved using current molecular techniques (Puig et al., 1994; Fong et al., 2005).

Of the many enteric viruses that may be present in contaminated water, adenoviruses are extremely stable in the environment, resistant to UV radiation, do not appear to have substantial seasonal variation, and can persist, based on genome detection, for extended periods outside of their animal hosts (Piña et al., 1998; Puig et al., 1994; Formiga-Cruz et al., 2002; Muscillo et al., 2008; Gerba et al., 2002; Nwachuku et al., 2005; Horowitz and Mold, 2007; Straub et al., 2007; Griffin et al., 2008; Dong et al., 2009). Consequently, adenoviruses are on the Environmental Protection Agency's (EPA) Contaminant Candidate List, which identifies particular pathogens as priorities for drinking water research and fecal contamination monitoring (USEPA 1998, 2005). In addition, human adenoviruses have been identified as emerging waterborne

pathogens that can be life threatening in children and immunocompromised individuals (Nwachuku and Gerba, 2004; Jiang, 2006).

Recent studies have indicated that adenoviruses, specifically human adenoviruses (HAdV), can be found in 100% of raw sewage samples, while 25% - 82% of wastewater effluent samples have been shown to contain HAdV (Piña et al., 1998; Haramoto et al., 2007; Symonds et al., 2009). In addition, HAdV is more prevalent in sewage than any of the other enteric viruses (Piña et al, 1998). Despite the prevalence of HAdV in wastewater effluents, little research has been conducted in the United States to characterize adenovirus presence and infectivity in estuaries receiving effluents (Jiang et al 2001; Fong et al, 2010, Tong and Lu, 2011).

The goal of this study was to detect and quantify HAdV occurrence in both secondary clarifier effluent (SCE) and final effluent (FNE) produced by three wastewater treatment plants (WWTP) located in Hampton Roads Virginia: James River, Virginia Initiative and Williamsburg. The Williamsburg and James River WWTPs release their final effluents directly into the James River and the Virginia Initiative WWTP releases its final effluent into the Elizabeth River. Both of these tributaries empty into the Chesapeake Bay. While numerous previous studies have reported high concentration of HAdV DNA in effluents, few have addressed the viability of the viruses being detected. Therefore, for this study the viability of HAdV detected in the James River WWTP FNE was assessed using an integrated cell culture-quantitative

polymerase chain reaction (ICC-qPCR) technique (Schlindwein et al., 2010; Fongaro et al., 2013).

While all three WWTPs whose effluents were examined use chlorination/dechlorination for disinfection of the effluent, each of these facilities employs different treatment regimes before discharging effluent directly into estuarine receiving waters. The Virginia Initiative Plant (Norfolk, VA) has an average daily input of 34 million gallons and uses biological nutrient removal (BNR) technology to remove nitrogen and phosphorous. The Williamsburg Treatment Plant (Williamsburg, VA) has an average daily input of 10.5 million gallons and uses oxidation towers, a type of biological filter, to treat a large volume of brewery waste received by the plant. The James River Plant (Menchville, VA) has an average daily input of 13.7 million gallons and does not currently use tertiary treatment for nutrient removal; however, the plant is testing Integrated Fixed Film in Activated Sludge (IFAS) for future use (http://www.hrsd.com/treatmentplants.htm). Given this information, it was hypothesized that the Williamsburg and Virginia Initiative plants, with their additional tertiary processing, would exhibit lower densities and frequencies of HAdV DNA detection in SCE and FNE samples compared to those from the James River plant.

Materials and Methods

Sample collection and concentration. SCE and FNE samples were supplied from three Virginia Hampton Roads Sanitation District (HRSD) WWTPs in January, April, July and October from July 2010-April 2012; the James River WWTP located at

Menchville, the Virginia Initiative Plant located in Norfolk, and the Williamsburg WWTP located in Williamsburg (Figure 1.1). In addition, FNE samples were collected from the James River WWTP for eight consecutive days (February 5, 2012 – February 12, 2012). All samples were immediately frozen (-80°C) and processed within 24 hours of receipt. One hundred and fifty ml volumes of effluent were concentrated following the protocol described by Katayama et al. (2002). Briefly, 2.5 mol l^{-1} $MgCl_2$ was added to each sample to achieve a 25 mmol l^{-1} concentration. The solution was vacuum filtered through electronegative nitrocellulose membrane filters (HAWG047SO- Millipore, USA), the filter was washed to remove salts with 0.5 mmol l^{-1} H_2SO_4 (pH 3.0), and the adsorbed virus particles were eluted from the filter with 1.0 mmol l^{-1} NaOH (pH 10.8). The eluate was neutralized with the addition of 1.0 mmol l^{-1} H_2SO_4 (pH 1.0) and 100X Tris-EDTA buffer (pH 8.0). The eluate was concentrated using Vivaspin (Sartorius Stedim, Goettingen, Germany) 6 ml concentrator columns by centrifugation for 10 minutes at 1500 x g. Total genomic DNA was extracted from the concentrate using the QIAGEN DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA) per manufacturer's protocol. Negative controls were run for the concentration step and the DNA extraction step using sterile deionized water as the blank. Positive controls to verify viral recovery were generated by spiking a 150 ml effluent sample with five µl of HAdV F-41 (ATCC, Manassas, VA). Five µl aliquots of the resulting DNA were used for both nested and real-time quantitative PCR (qPCR) assays as described below. In addition, a 50µl aliquot of viral concentrate from the James River WWTP FNE was used for integrated cell culture (ICC).

Nested polymerase chain reaction. Nested PCR was used for all samples, in duplicate (analytical), using primers designed to amplify a hypervariable region in an open reading frame of the hexon gene encoding this protein (or capsid) coat component (Puig et al, 1994). Amplifications were performed in 25µl reactions on an MJ Research PTC 200 Peltier Thermal Cycler (MJ Research Inc., Reno, NV). Invitrogen PCR reagents were used for all nested PCR reactions. Final concentrations for PCR reagents were as follows: 1X PCR buffer, 0.2 mg/ml BSA, 1.5 mM $MgCl_2$, each dNTP at 1.2mM, 0.08 pmol/µl each primer, hexAA 1885 and hexAA 1913 (Table 1.1) (Allard et al., 1990; Puig et al., 1994), 0.02 U ml^{-1} Taq polymerase. Thermocycling parameters were as follows: 94°C for 4 m followed by 30 cycles of 94°C for 90 s, 55°C for 90 s, 72°C for 2 m, with a final elongation step of 72°C for 10 m. The first round of amplification resulted in a 300bp product. The second round of amplification used primers designed to bind within the primary product. The 300bp product was used as the template and the resulting PCR fragment was 143bp. Amplification parameters remained the same as the first round, however, the primer concentration (nehexAA 1893 and nehexAA 1905, Table 1.1) was increased to 0.16 pmol/µl (Puig et al., 1994; Piña et al., 1998), and 0.5 µl of the primary PCR product was used as the template. The final PCR product was run on a 1.5% agarose gel and visualized under UV light using ethidium bromide stain.

Quantitative polymerase chain reaction (qPCR). qPCR was conducted on all samples in (analytical) duplicate. Primers and a FAM/TAMRA labeled TaqMan

probe designed to amplify the same region of the capsid gene as the nested PCR primers were used in this assay (Heim et al., 2003). Ten µl reactions were run on an Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA). Final concentrations of PCR reagents for a 10µl reaction were as follows: 1X TaqMan Universal Master Mix (Invitrogen, Grand Island, New York), 0.2 mg/ml BSA, each primer at 0.5 µM, 0.4 µM TaqMan FAM/TAMRA labeled probe (Table 1.1). Thermocycling parameters were as follows: 95°C for 20 s, followed by 45 cycles of 95°C for 15 s, 55°C for 10s, 60°C for 1min. A standard curve was generated in the following manner: HAdV F41 DNA was amplified using the above outlined nested PCR protocol. The PCR product was run on a 1.5% agarose gel, the band was excised and cleaned using the QIAquick PCR purification kit (Valencia, CA) per manufacturer's protocol. The resulting DNA was quantified using the NanoDrop 2000 UV-Vis Spectrophotometer (ThermoScientific,Wilmington, DE). Based on the fragment size of 143bp, the mass of one copy of the fragment, a genomic equivalent (ge), was calculated to be 1.56×10^{-10} ng/ge. By dividing the concentration of the DNA by the mass of one ge, the ge/µl of purified fragment was obtained. Tenfold serial dilutions were performed and were used to create a standard curve ranging from 3.81 ge/µl to 3.81×10^{7}ge/µl. For both nested PCR and qPCR, negative controls were run using deionized water as the blank. Positive controls were run using 3 µl (1.95×10^{6} genome equivalents) HAdV 41 Tak strain DNA (ATCC, Manassas, Virginia).

ICC-qPCR. The infectivity of HAdV in FNE from the James River WWTP was determined using an integrated cell culture-quantitative polymerase chain reaction (ICC-qPCR) technique following a combination of protocols from Choo and Kim (2006) and Gallagher and Margolin (2007). Caco-2 cells were grown to 75-90% confluence in t25 tissue culture flasks using Minimum Essential Medium Eagle (MEM) with 10% fetal bovine serum (FBS). Once the cells reached the appropriate confluence, the spent medium was removed and the cells were washed with fresh medium. Four flasks were inoculated for each WWTP sample by adding 50 µl of effluent viral concentrate to each flask containing washed Caco-2 cells. After inoculation, the cells were rocked every 15 minutes for 60 minutes. Following this, 5 ml of MEM was added to each culture flask. Two of the four flask cultures for each sample were immediately frozen (-80°C) (T_0), the remaining two were incubated at 37°C for 4 days (T_4). Following the 4 day incubation period the culture flasks were stored at -80°C until DNA extraction. Prior to DNA extraction, each culture flask was subjected to three freeze/thaw cycles. Two hundred µl of the supernatant containing thawed cells was used to extract total genomic DNA using the QIAGEN Blood and Tissue Kit (QIAGEN Valencia, CA) following the manufacturer's protocol. Five µl out of 60 µl total extraction volume of DNA was used for qPCR as outlined previously. T_0 and T_4 duplicates for each sample were compared to determine if the number of viral particles increased over the 4 day incubation period. An increase in HAdV DNA was interpreted as meaning adenoviruses in a given sample proliferated and therefore, were infective. Negative controls were run for each sample by inoculating control flasks with 50 µl of sterile MEM medium. Positive controls

were run for each sample by inoculating control flasks with 50 µl (3.25×10^7 genomic equivalents) HAdV F41 strain Tak from ATCC (Manassas, Virginia). .

Quantification calculations. To determine the genomic equivalents per sample, the initial volume of effluent, the final volume of concentrated effluent, the volume of concentrated effluent for DNA extraction, the final volume of DNA eluate, and the volume of DNA added to the reaction are all taken into consideration. When calculating the genomic equivalents present in ICC-qPCR samples, the addition of the medium was also accounted for. Below are examples for each:

qPCR: 150 ml of effluent ➔ 400 µl concentrated effluent ➔ 200 µl concentrate sampled for DNA extraction ➔ elution of the DNA into 60 µl ➔ 3 µl of eluate added to each qPCR reaction. From this the following equation would be used: genomic equivalents as reported by qPCR assay based on standard curve × 2 × 20 ÷ 150 = genomic equivalents/ml.

ICC-qPCR: 150 ml of effluent ➔ 400 µl concentrated effluent ➔ 100 µl inoculum ➔ 5 mls culture medium added ➔ 200 µl lysate for extraction ➔ elution of the DNA into 60 µl ➔ 3 µl of eluate added to each qPCR reaction. From this the following equation would be used: genomic equivalents as reported by qPCR assay based on standard curve × 4 ×25 ×20 ÷ 150 = genomic equivalents /ml. It is important to note that calculations must be adjusted for each sample. Specifically, because the volume obtained during the concentration step was variable, these calculations must be adjusted accordingly.

Data analysis. When comparing presence/absence data obtained for the SCE and FNE samples, and comparing the standard nested PCR assay to the quantitative PCR assay, unpaired t-tests were used to determine significance at the 95% level. Paired t-tests were used to assess seasonal variability. When assessing the concentration of virus, the mean number of virus particles per samples were $\log_{10}$ transformed and plotted using 95% confidence intervals. Since it was unknown whether a value of 0 genomic equivalents/L (ge/L) was due to the absence of HAdV DNA, inhibition, or a sample below the detection limit of an assay, all zeros were removed prior to $\log_{10}$ transformation and then put back in as zeros. When confidence intervals overlapped, no significant variation between samples was assumed. However, when they did not overlap, one-way analysis of variance testing (ANOVA) was used to confirm significant differences. Statistical analyses were performed using Excel version 14.2.3 (Microsoft Corporation, 2010) and an online ANOVA calculator (http://www.physics.csbsju.edu/stats/anova.html).

Results

Comparison of nested and qPCR assays. Between July 2010 and April 2012, sixteen 150 ml samples of effluent (eight SCE and eight FNE) were collected from each of the three WWTPs. Viral concentrates from these samples were amplified with both standard nested PCR and qPCR to compare methods to detect the presence of adenoviral capsid DNA. Due to the variability in final volume obtained after concentration for each sample, two to eight reactions were run for each of the assays (Table 1.2). Initially, all nested data were pooled, as were the qPCR data, to look for

an overall difference in detection between the two assays. No significant differences in detection of HAdV capsid DNA compared across all samples, including both the FNE and SCE data were found (unpaired t-test $p = 0.4337$, $t = 0.7863$). Again, when individual months were compared for both SCE and FNE samples, there were no significant differences between nested and qPCR (Table 1.3). However, when SCE and FNE samples were separated and compared by month, nested PCR was shown to be significantly more sensitive for detection of HAdV DNA ($p = 0.04$) during April 2011 with respect to SCE samples (Table 1.3). No significant differences between the two assay methods were found with respect to the FNE data (Table 1.3). However, in a side-by-side comparison seven samples where nested PCR detected HAdV DNA and qPCR did not, and seven samples when qPCR detected HAdV DNA when nested did not, were observed (Table 1.4).

Differences between WWTPs in HAdV DNA detected via nested PCR and qPCR.

Presence/absence data for each of the collection dates (July 2010 – April 2012) were pooled for each of the WWTPs and analyzed by ANOVA. No significant differences in the detection of HAdV capsid DNA in SCE samples between the three WWTPs were found with respect to either the nested PCR assay or the qPCR assay ($p= 0.717$ and $p= 0.919$, respectively, $n=8$). However, a significant difference between the three WWTPs was detected in the FNE samples in both the nested and qPCR assays ($p = 0.005$ and 0.017, respectively, $n=8$). Taking into consideration the restrictions of the analytical methodology, the James River WWTP appears to have significantly more

positive samples. In the nested assay, the James River WWTP tested positive in 16 out of 30 amplifications, compared to six out of 30 and five out of 30 for VIP WWTP and Williamsburg WWTP, respectively. With respect to the qPCR assay, the James River WWTP had 17 positive amplifications out of 28, while Virginia Initiative and Williamsburg WWTP had six out of 28 and two out of 28 positive samples, respectively. Statistical analysis comparing the genomic equivalents per liter of FNE between each of the WWTPs over the study period showed no significant differences based on 95% confidence intervals (Figure 1.2). Despite an apparent lack of overlap between the confidence intervals in both April 2011 and April 2012 with the FNE data, comparing the mean concentrations of genomic equivalents indicated no significant differences between plants ($p = 0.1811$ and $p = 0.055$, respectively).

HAdV DNA detection and quantification in FNE versus SCE for each WWTP. Concentrations of viral capsid DNA in both FNE and SCE for each of the eight collection months were determined via qPCR. Mean virus concentrations in monthly samples were calculated, $\log_{10}$ transformed and plotted with 95% confidence interval error bars to assess whether differences existed between the FNE and SCE samples (Figure 1.3). For the Virginia Initiative WWTP, HAdV capsid DNA was detected in the SCE and the FNE in three of the eight collection months. In the SCE it was detected in July 2010 (3.16 ge/L), July 2011 (0.95 ge/L) and April 2012 (1.48 ge/L). In the FNE it was detected in January 2011 (1.21 ge/L), April 2011(1.03 ge/L), and April 2012 (1.99 ge/L). Based on the overlap of the error bars, differences in concentration values for the April 2012 samples were not significant. This

conclusion was also supported with an unpaired t-test ($p = 0.6553$). HAdV DNA was detected in October 2010 (1.16 ge/L and 1.02 ge/L), January 2011 (0.92 ge/L and 0.96 ge/L) and April 2012 (2.45 ge/L and 0.72 ge/L) in both the SCE and FNE collected from the Williamsburg WWTP. There were no significant differences in concentration between the SCE and FNE for any of the samples (unpaired t-test, $p = 0.9364$, $p = 0.9787$, and $p = 0.166$, respectively). The James River WWTP had the highest monthly incidence of HAdV DNA detection. SCE samples were positive in four out the eight months; July 2010 (0.80 ge/L), April 2011 (3.37 ge/L), July 2011 (1.00 ge/L), and April 2012 (3.32 ge/L). FNE samples were positive in six out of the eight months; July 2010 (1.43 ge/L), October 2010 (2.56 ge/L), January 2011 (1.04 ge/L), April 2011 (3.13 ge/L), July 2011 (0.81 ge/L), and April 2012 (1.99 ge/L). Interestingly, in both April 2011 and April 2012, significantly higher HAdV DNA densities were detected in the SCE than in the FNE ($p = 0.021$, and $p = .044$, respectively), whereas there were no significant differences found in either July 2010 or July 2011 ($p = 0.8958$ and $p = 0.5792$, respectively).

Inter-annual and seasonal variation in HAdV DNA detected in FNE samples via qPCR.

To test whether there was significant inter-annual variation in HAdV DNA detection (Table 1.5) the $\log_{10}$ transformed concentrations of viral HAdV DNA (ge/l) in FNE samples collected at each WWTP in January 2010 and January 2011, April 2011 and April 2012, July 2010 and July 2011, and October 2010 and October 2011 were compared using a paired t-test. Results indicated a significant difference between

October 2010 and October 2011 with respect to the James River WWTP only. There were no other significant differences detected. These data imply no significant inter-annual variation in two of the three WWTP and in only one sampling month in the third WWTP. In addition, data for each season were pooled by WWTP and a pairwise analysis (paired t-test) was conducted to determine if there were significant seasonal differences in HAdV DNA detection (Table 1.6). For both the JR and VIP WWTPs, differences between spring and summer values were significant. This was due to high concentrations of HAdV detected in April 2012 in the effluent from both plants. There was no significant difference in HAdV detection between seasons in effluents from the Williamsburg WWTP.

Viability of HAdV in the James River WWTP FNE

ICC-qPCR of FNE from the James River WWTP obtained during each of the sampling dates indicated that infectious virions were present in effluent from January 2011. The initial (T_0) concentrations were 0 ge/L for both replicates, whereas the average four day post inoculation (T_4) for both replicates was 54.5 ge/L. Infectious virions were not detected on any of the other sampling dates.

One-week time series

FNE samples were collected from the JR WWTP for eight consecutive days, from February 5, 1012 to February 12, 2012 to determine if there was a daily difference in HAdV DNA concentration within this sampling month. The concentration of HAdV DNA in each sample was determined by qPCR, $\log_{10}$ transformed and plotted to

determine consistency of HAdV presence over the course of a week (Figure 1.4). HAdV DNA was detected in three of the eight days at concentrations ranging from 110-210 ge/L.

Discussion

Based on the presence/absence data, there was no significant difference in the detection of the HAdV viral DNA with respect to the nested PCR and qPCR assays over the course of the two-year study when all data for each assay was pooled. Even when the data were separated by season, there was no significant difference in the detection of HAdV DNA using these two methods. Heim et al (2003) demonstrated that both standard PCR and qPCR have identical detection capabilities in clinical samples. During this study, when viewed in terms of detection versus no detection, there were seven samples where nested PCR detected HAdV DNA and qPCR did not, and seven samples when qPCR detected HAdV DNA when nested did not (Table 1.6). This demonstrated that, in terms of over all effectiveness in detecting the target viral DNA, neither assay is inherently superior, a position defended by Bastien et al. (2008). However, there was one instance when the difference in detection with respect to method was statistically significant. When the data was further separated by FNE and SCE samples and then by month, there was a significant difference in SCE samples for one of the eight sampling months, April 2011. In this case, nested PCR detected HAdV in 83% of the samples (n=12), whereas the qPCR detected HAdV in 0% of the samples (n=12). None of the other sampling months showed significant differences (Table 1.2). Because this appeared to be an isolated event,

perhaps due to inhibitory compounds, it does not convincingly indicate that nested PCR is better at detecting HAdV in SCE samples. Furthermore, there could be a number of other reasons for this result. Nested PCR has much higher risk of carry over contamination since the amplicon from the first amplification must be used as the template for the second amplification (Heim et al., 2003). The reaction tubes must be opened after the first amplification and only a single copy of target DNA may be required for detection. While the negative controls for this reaction were consistently negative, the possibility that carryover occurred cannot be ruled out. Secondly, Dong et al., (2009) have demonstrated that qPCR can have up to a 1 log lower detection sensitivity when compared to nested PCR in the detection of HAdV in partially treated wastewater. This reduced sensitivity in environmental samples could be due to the presence of enzymatic inhibitors present in the effluent concentrate (Bofill-Mas et al., 2006). Therefore, the possibility exists that HAdV DNA presence in these specific SCE samples was overestimated by the nested assay due to carryover contamination or that it was underestimated in the qPCR assay due to inhibition. Given that each assay has its strength and weaknesses, one is not inherently better than the other, therefore, it would be recommended, in light of these data, that both assays be run to avoid the risk of false negatives.

In several cases, adenovirus was detected in the final effluent, but not the secondary clarifier effluent. This occurred three times in samples from the James River facility and the Virginia Initiative facility and once in samples from the Williamsburg facility. These results are consistent with reports by other researchers where final

effluents had higher concentration of adenovirus than upstream effluents (He and Jiang, 2005; Fong et al., 2010). PCR inhibition, caused by trace amounts of inhibitors in the secondary effluent, is believed to be a possible explanation. However, this cannot be confirmed due to the costs associated with running internal spiked controls for each sample. Importantly, HAdV DNA concentrations between the FNE and SCE were not significantly different for any of the samples.

There were significant differences in detection of HAdV DNA when the wastewater treatment plants were compared. Nested and qPCR HAdV DNA detection in the final effluent from the James River facility was significantly higher (53% nested, 61% qPCR) than from the Williamsburg or Virginia Initiative facilities (20%, 21% and 17%, 7%, respectively). This could be related to the absence of an additional secondary treatment process at the James River facility. While all three WWTP plants use chlorination (sodium hypochlorite) for disinfection, the Williamsburg and Virginia Initiative plants use additional tertiary treatments (HRSD, personal communication). Williamsburg had the lowest number of positive detections by both nested and qPCR. This plant employs oxidation towers to reduce the biochemical oxygen demand from the input of brewery waste from the Williamsburg Anheuser-Busch Brewery. Current research has shown that adenoviruses are sensitive to oxidation (Nwachcuku and Gerba, 2004; Bounty et al., 2012). The viral load in the Williamsburg final effluent was the lowest of the three plants studied and could be related the oxidizing activity of both chlorine disinfection and oxygenation to remove biological oxygen demand (BOD). The Virginia Initiative facility uses an advanced

biological nutrient removal (BNR) system as an additional tertiary treatment process. The aim of this treatment is to remove additional nitrogen and phosphorous from the effluent before it is released into the receiving water. The first step in this process is the nitrification of ammonia to nitrate by autotrophic bacteria under aerobic conditions (Jeyanayagam, 2005). This requires additional oxidation of the effluent. Perhaps, as with the Williamsburg facility, additional exposure to an oxidative process leads to a reduction in the HAdV viral load of the secondary effluent.

Interestingly, although the presence/absence detection of HAdV shows a significantly higher detection rate in the final effluent from the James River plant, a quantitative assessment does not. When the concentrations of HAdV in genomic equivalents per liter (ge/L) in the final effluents produced by the three plants were compared based on qPCR results, there was no significant difference based on 95% confidence intervals or by ANOVA (Table 1.2). Although the incidence of detection was greater in the James River effluent, HAdV DNA concentrations were not significantly higher. This variation may be due to a number of factors. First, for each sample, only 150 ml of effluent was collected for concentration. It is quite possible that concentrations observed were biased due to insufficient sample volume. Additionally, based on the daily sampling series, the concentration of HAdV in the effluent varies day to day. It may also stand to reason, the concentration may vary hour to hour. Finally, levels of inhibitors may also vary from sample to sample. So while presence/absence detection may be consistent, concentration values may not be. Other researchers have reported final effluent concentrations as high as$10^2 – 10^4$ ge/L (Bofill-Mas et al., 2006; Fong et

al., 2010), whereas concentrations observed in this study ranged from 10^1–10^2 ge/L. This may simply be reflective of the timing of our sample draws, the volume of sample supplied, viral recovery efficiency, inhibitor variability, or inherent differences in the treatment processes at various WWTPs. While additional studies need to be conducted to elucidate the nature of the low concentration values, this study demonstrated detectable concentrations of HAdV DNA were present in final effluents discharged by the three WWTPs in southeastern Virginia coastal waters.

Over the course of the two year study, samples were obtained twice during each of the following months; January, April, July and October. This allowed for both inter-annual and seasonal comparisons for the presence and quantitative detection of adenovirus DNA. Results did not show an overall significant inter-annual or seasonal variation. This was consistent with the literature and one of the hallmark characteristics of adenovirus occurrence (Piña et al., 1998; Haramoto et al., 2007). It is worth noting that, due to high concentrations of adenovirus DNA in April 2012 samples from both the James River and Virginia Initiative plants, there was a significant variation in HAdV DNA detection comparing the spring and summer only, while no seasonal difference was detected for the Williamsburg plant. The cause for the April spike is not known. A review of weather records does not indicate unusually high rainfall in the areas surrounding the James River or Virginia Initiative WWTPs during April 2012. While the Hampton Roads Sanitation District does have separate sanitary and storm sewer systems, extreme rainfall can lead to infiltration of

storm water into the sanitary sewer system (http://www.hrsd.com/EPAWWCD.shtml).

In discussing the lack of inter-annual and seasonal variation, it is important to point out a limitation of this study. For each of the months, only one sample was processed for a single monthly time point from each of the WWTPs. This information was then used to draw conclusions about patterns seen over entire seasons. Unfortunately, it was analytically cost prohibitive to sample every day during the entire course of the month. To illustrate how this could affect the data, a one week time series study conducted illustrated the variability in detection within the week, let alone the month or season. When samples pulled each day for eight consecutive days in February 2012 from the James River facility were tested, HAdV DNA was only detected on three days. Therefore, a randomly selected sample one day that week to determine if HAdV was present that month, would exhibit a 37.5% chance of obtaining a positive result and a 62.5% of obtaining a negative result.

The final concern, and perhaps the most important, is the question of viral viability. The final effluent from the James River facility was used in ICC-qPCR analysis to determine if HAdV viruses in the discharged effluent remained viable. Because adenovirus is culturable, ICC-qPCR provided a direct means of assessing infectivity. In addition, coupling qPCR with cell culture also provided a quantitative measure of viral viability. Human adenovirus, specifically the enteric genotypes HAdV F40 and 41, do not produce easily discernable cyptopathic effects on host assay cells (Chapron

et al., 2000). Therefore, relying on visual changes in cell morphology for viability assessments would be inaccurate. Integrating cell culture with qPCR allowed for detection of HAdV proliferation over the four-day incubation period. In all but one of the sample months (n=8), ICC-qPCR indicated that the viruses detected were either not infective or below detection limits. In these seven instances, initial viral concentrations did not change discernably after four days of inoculation. A detectable increase in viral concentration occurred in the January 2011 sample indicating viability. Although these results were equivalent to a relatively low positive infective frequency of 12.5%, the fact that one sample was infective suggests that a larger scale study on the FNE produced by the James River WWTP should be undertaken. Such a study should include larger volumes of effluent collected more frequently.

In conclusion, human adenovirus DNA was detected by both qPCR and nested PCR in effluents from each of the wastewater treatment plants studied, with no significant seasonal variation as reported in the previous literature. Moreover, as hypothesized, HAdV DNA was detected with greater frequency in one plant, in particular, the James River WWTP. Finally, use of ICC-PCR revealed viable HAdV virions were detected in one sample of final effluent from the James River WWTP. Given the small sample size and limited sampling frequency, from a risk perspective these results likely underestimate both the occurrence and viability of HAdV in final effluents.

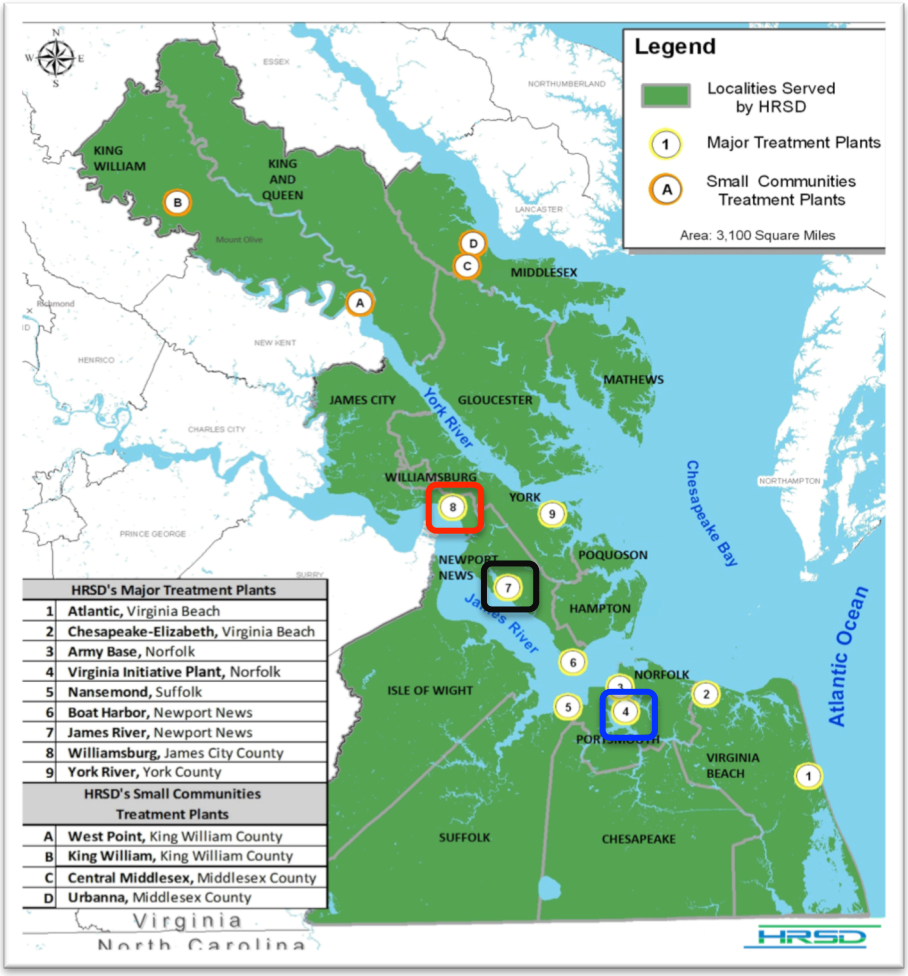
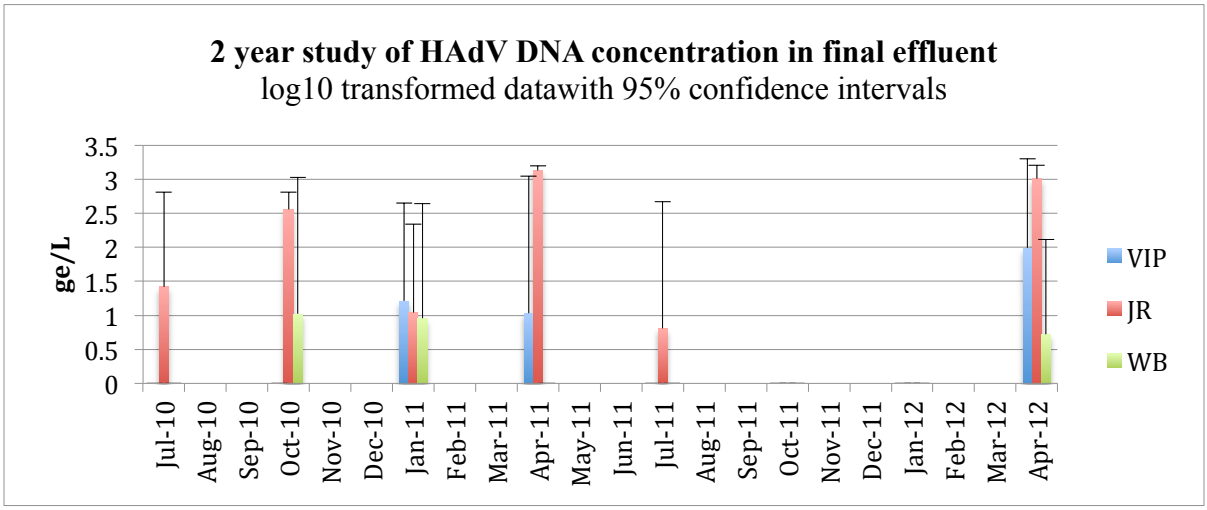


Figure 1.1. Locations of Hampton Roads Sanitation District wastewater treatment plants. Red box = Williamsburg WWTP, Black box = James River WWTP, Blue box = Virginia Initiative Treatment WWTP.
http://www.hrsd.com/images/FastFactsServiceAreaMap2.jpg



VIP – Virginia Initiative wastewater treatment plant; JR – James River wastewater treatment plant; WB – Williamsburg wastewater treatment plant

Figure 1.2. HAdV DNA concentrations detected by qPCR in the final effluent (FNE) produced by three wastewater treatment plants (WWTP) over the course of two years. Samples were collected in January, April, July and October.

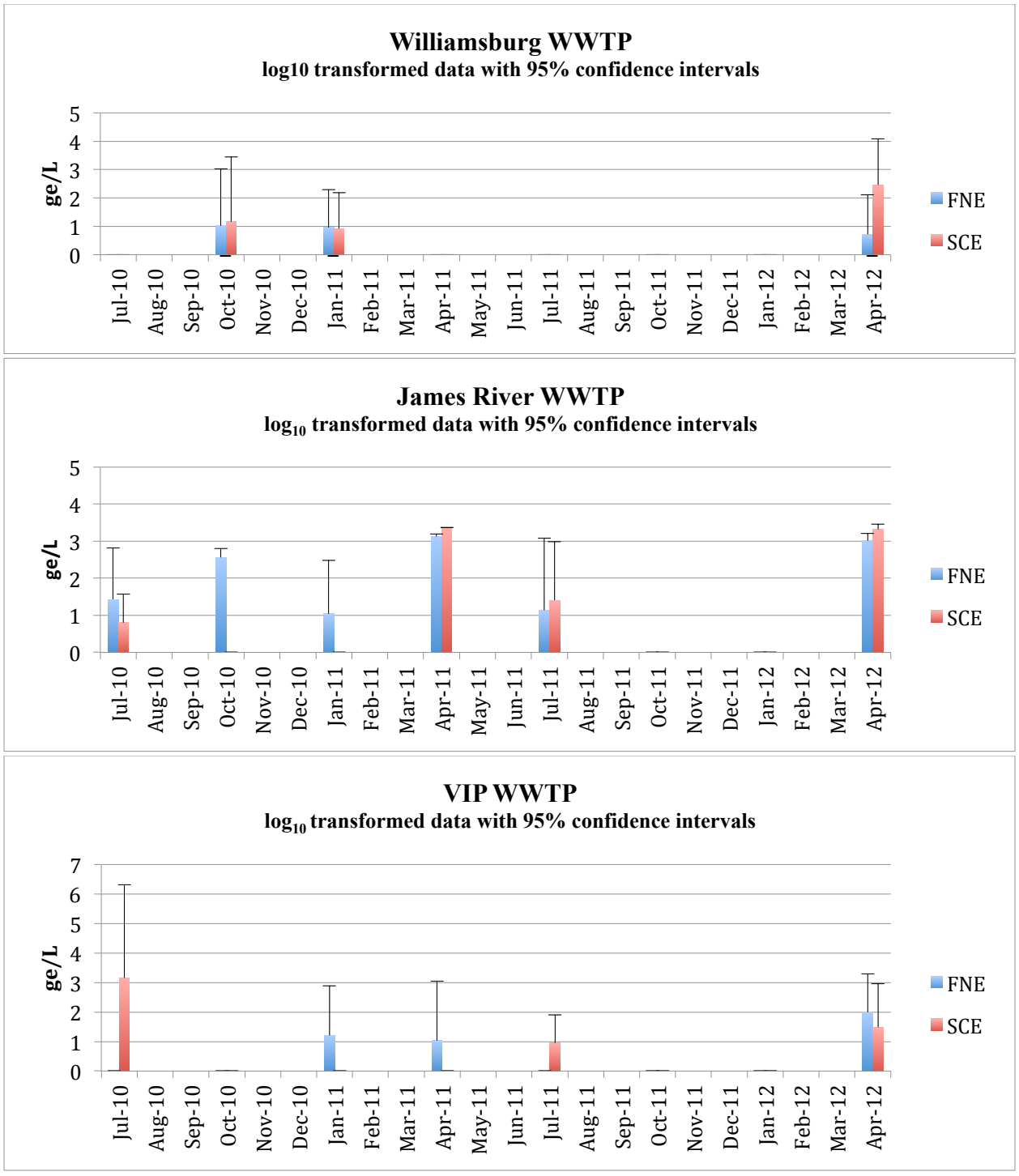


Figure 1.3. Comparison of HAdV DNA concentration by qPCR in the secondary clarifier (SCE) and final (FNE) effluents produced by the three wastewater treatment plants.

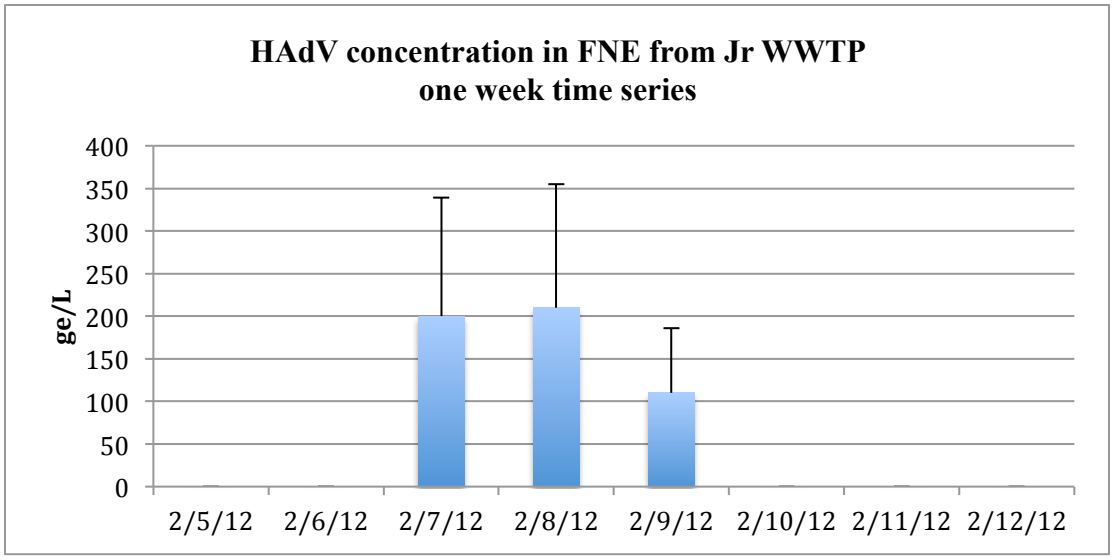


Figure 1.4. The average concentration (genomic equivalents per liter) of HAdV DNA detected via qPCR for final effluent (FNE) samples collected from the James River WWTP over the course of eight consecutive days. Error bars represent 95% confidence intervals.

Table 1.1 Primer sequences and amplicon lengths for nested PCR and qPCR assays.

	Sequence	Amplicon length	reference
nested PCR			
hexAA1885	5'-GCCGCAGTGGTCTTACATGCACATC-3'	300bp	Allard et al., 1990
hexAA1913	5'-CAGCACGCCGCGGATGTCAAAGT-3'		
nehexAA1893	5'-GCCACCGAGACGTACTTCAGCCTG-3'	142bp	
nehexAA1905	5'-TTGTACGAGTACGCGGTATCCTCGCGGTC-3'		
qPCR			
Adenoquant (AQ1)	5'-GCCACGGTGGGGTTTCTAAACTT-3'	131bp	Heim et al., 2003
Adenoquant (AQ2)	5'-GCCCCAGTGGTCTTACATGCACATC-3'		
Adenoprobe (AP)	5'-TGCACCAGACCCGGGCTCAGGTACTCCGA-3'		

Table 1.2 Nested PCR detection compared to qPCR detection for total DNA extracted from concentrated pre- and post-chlorinated effluent samples from three WWTPs in southeastern Virginia.

sample	secondary treatment method	number of positives by nested PCR								number of positives by qPCR							
		07/10	10/10	01/11	04/11	07/11	10/11	01/12	04/12	07/10	10/10	01/11	04/11	07/11	10/11	01/12	04/12
JR SCE	phos removal	0/4	2/4	0/8	4/4	1/4	0/2	0/2	2/2	2/4	0/4	0/4	0/4	2/4	0/2	0/2	4/4
VIP SCE	BNR	4/4	0/4	8/8	2/4	1/4	0/2	0/2	2/2	4/4	0/4	0/4	0/4	2/4	0/2	0/2	2/4
WB SCE	oxidation	2/4	0/4	5/8	4/4	1/4	0/2	0/2	0/2	0/4	2/4	1/4	0/4	0/4	0/2	0/2	3/4
JR FNE	phos removal	3/4	4/4	3/8	4/4	2/4	0/2	0/2	2/2	2/4	4/4	1/4	4/4	2/4	0/2	0/2	4/4
VIP FNE	BNR	0/4	0/4	6/8	0/4	0/4	0/2	0/2	0/2	0/4	0/4	1/4	2/4	0/4	0/2	0/2	3/4
WB FNE	oxidation	0/4	0/4	3/8	0/4	0/4	0/2	0/2	0/2	0/4	1/4	1/4	0/4	0/4	0/2	0/2	1/4

JR – James River WWTP, Newport News, VA; VIP – Virginia Initiative WWTP, Norfolk, Va; WB – Williamsburg1/4 WWTP, Williamsburg, VA
SCE – Secondary clarifier effluent (pre-chlorination)
FNE – Final Effluent (post-chlorination)

Table 1.3 Comparison of paired t-test results (α = 0.05) by month for nested PCR versus qPCR in the detection of HAdV capsid DNA in secondary clarifier and final effluents. Red text indicates samples that are statistically significant.

	SCE and FNE* pooled			SCE			FNE		
Sample Date*	Nested PCR	qPCR	P value	Nested PCR	qPCR	**P value**	Nested PCR	qPCR	**P value**
July 2010	9/24	8/24	**0.77**	6/12	6/12	**1.0**	3/12	2/12	**0.42**
October 2010	2/24	7/24	**0.36**	2/12	2/12	**1.0**	4/12	5/12	**1.0**
January 2011	25/48	4/24	**0.06**	13/24	1/12	**0.26**	12/24	3/12	**0.18**
April 2011	14/24	6/24	**0.24**	10/12	0/12	**0.04**	4/12	6/12	**0.42**
July 2011	5/24	6/24	**0.61**	3/12	4/12	**0.67**	2/12	2/12	**1.0**
October 2011	0/12	0/12	**N/A**	0/6	0/6	**N/A**	0/6	0/6	**N/A**
January 2012	0/12	0/12	**N/A**	0/6	0/6	**N/A**	0/6	0/6	**N/A**
April 2012	2/12	17/24	**0.34**	4/6	9/12	**0.84**	2/6	8/12	**0.27**

*SCE – secondary clarifier effluent FNE – Final effluent

Table 1.4. Comparison of nested PCR and qPCR positives and negatives.

		SCE			FNE			
Date	Assay	JR	VIP	WB	JR	VIP	WB	
7/10	nested	-	+	+	+	-	-	+1
	qPCR	+	+	-	+	-	-	+1
10/10	nested	+	-	-	+	-	-	+1
	qPCR	-	-	+	+	-	+	+2
1/11	nested	-	+	+	+	+	+	+1
	qPCR	-	-	+	+	+	+	0
4/11	nested	+	+	+	+	-	-	+3
	qPCR	-	-	-	+	+	-	+1
7/11	nested	+	+	+	+	-	-	+1
	qPCR	+	+	-	+	-	-	0
10/11	nested	-	-	-	-	-	-	0
	qPCR	-	-	-	-	-	-	0
1/12	nested	-	-	-	-	-	-	0
	qPCR	-	-	-	-	-	-	0
4/12	nested	+	+	-	+	-	-	0
	qPCR	+	+	+	+	+	+	+3
				Total nested positive when qPCR negative				**7**
				Total nested positive when qPCR negative				**7**

Table 1.5. P values for month-to-month comparison (paired t-test at a $\alpha = 0.05$) of HAdV DNA detected by qPCR in the final effluent produced by each wastewater treatment plant to assess significant inter-annual variation. Red text indicates samples that are statistically significant.

	James River	Virginia Initiative	Williamsburg
July 2010 to July 2011	0.62	no detection	no detection
October 2010 to October 2011	0.03	no detection	0.50
January 2011 to January 2012	0.50	0.50	0.50
April 2011 to April 2012	0.45	0.50	0.50

Table 1.6. Paired t-test (α = 0.05) comparison of HAdV DNA detection by season for the final effluent produced by each of the wastewater treatment plants. Red text indicates samples that are statistically significant.

James River WWTP				
	Spring	Summer (P)	Fall (P)	Winter (P)
Spring		0.01	0.22	0.05
Summer			0.62	0.27
Fall				0.32
Winter				

Virginia Initiative WWTP				
	Spring	Summer (P)	Fall (P)	Winter (P)
Spring		0.02	0.07	0.09
Summer			not detected	0.30
Fall				0.41
Winter				

Williamsburg WWTP				
	Spring	Summer (P)	Fall (P)	Winter (P)
Spring		0.39	0.96	0.79
Summer			0.17	0.30
Fall				0.75
Winter				

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Chapter 2: Concentration and depuration of human adenovirus in oysters

Abstract

Oysters grown in water contaminated with human enteric viruses can concentrate the viruses in their tissues, leading to a public health risk when consumed raw. Human adenovirus (HAdV), though often not directly associated with outbreaks of gastrointestinal diseases, is proposed as an indicator of viral contamination in estuarine waters due to its persistence characteristics and because HAdV can be cultured, the viability of the concentrated viruses can be assayed. In a small pilot study, three *in vitro* tank contamination studies were conducted to determine if oysters (*Crassostrea virginica*) were able to concentrate HAdV when allowed to feed for 18 hours in water spiked with viable HAdV. HAdV DNA concentrations were determined with qPCR and infectivity of detected HAdV assayed using ICC-qPCR. Finally, the ability of oysters to depurate the viruses in an open system was assessed through relay of tank contaminated oysters in the spring and fall to the York River, Gloucester Point, VA. Results showed that oysters were able to accumulate viable HAdV in their tissues after 18 hour exposures to York River water that was spiked with viable HAdV F41. In addition, it appears that the oysters were able to depurate the virus after as little as three days in the York River. This pilot study sets the groundwork for additional studies.

Introduction

The consumption of raw shellfish contaminated with human pathogens has been associated with several gastrointestinal illnesses (Myrmel et al, 2004; Sala et al., 2009). Oysters, being filter feeders, are capable of pumping large volumes of water across their gills, making them very effective at concentrating water borne contaminants. Among these contaminants are pathogenic enteric viruses, such as norovirus, hepatitis A and adenovirus (Bosch, 1998). This is of concern since recent studies have demonstrated that wastewater effluent being released into estuarine receiving waters can contain these viruses (Fong et al., 2010). In an attempt to ensure the safety of oysters harvested for direct marketing to the public, the National Shellfish Sanitation Program has established a standard based on fecal coliform detection by which shellfish growing waters are classified as "enteric pathogen free". (http://www.vdh.state.va.us/EnvironmentalHealth/Shellfish/classification/index.htm). However, this standard is based on bacterial contamination and does not address viral contamination. In a study conducted by Christensen et al. (1998), it was demonstrated that even when oysters are grown in waters that meet the European fecal coliform standards, oysters have still been implicated in outbreaks of viral gastroenteritis. In a separate study conducted by the World Health Organization, it was concluded that shellfish are capable of concentrating large quantities of enteric viruses and that, if consumed raw, these shellfish posed a public health risk (Lees et al., 2010). Due to the fact that viruses are more resistant to disinfection and inactivation, it is now widely accepted that bacteria are not good indicators of viral contamination. Of the viruses mentioned above, human adenovirus (HAdV) is of particular interest due to

its demonstrated presence in WWTP effluents at reasonably elevated levels, resistance to UV inactivation and environmental stability. These very characteristics suggest HAdV might be a good candidate indicator of enteric viral contamination in oysters. And, since it is culturable, assays such as integrated cell culture-quantitative PCR (ICC-qPCR), allow its viability to be determined. This is especially important when assessing the health risk of depurated oysters.

Traditionally, depuration in a closed system has been used as a means to clear human pathogens from shellfish intended for market. Depuration involves placing the oysters in a closed system comprised of tanks of clean seawater, under various conditions, and allowing them to purge the concentrated contaminants as they pump the clean water through their systems (Lee et al., 2008). The addition of chlorine, or the use of UV light, for example, in conjunction with clean seawater is used to inactivate pathogenic contaminants. However, viruses are more difficult to depurate than bacteria (Schwab et al., 1998; Formiga-Cruz et al., 2002). Furthermore, there has been a push to move away from chlorine as a disinfecting agent for a number of reasons. In terms of oysters, chlorine can have a toxic affect on the oyster, impairing pumping and thus hampering depuration (Rodrick and Schneider, 2003). Hence, the current trend is to employ UV radiation as a means of disinfection (De Abreu Corrêa et al., 2012) despite the recognition that viruses such as HAdV are highly resistant to UV radiation (Mena and Gerba, 2009). Ultimately, when depurated in a closed system, this could lead to recontamination of shellfish with viable HAdV that was released during the depuration process.

The purpose of this pilot study was to determine if oysters grown in seawater contaminated with HAdV concentrated and retained the virus in their tissues, and if so, did the virus remain infective. Finally, rather than depurating the oysters in a closed system, after exposure to the virus oysters were placed in flexible mesh bags and deployed into the York River in order to determine if HAdV could be depurated in an open system and how long it would take. Given the potential of reuptake of released viable virus in a closed system, it was anticipated that the open system should provide shorter, more effective, elimination. In addition, effective elimination in an open system avoids the need for chlorination or UV radiation treatments.

Materials and Methods

Tank assembly and oyster exposure. Three separate tank studies were conducted over the course of 6 months. For each study, twenty market-sized oysters (*Crassostrea virginica*) approximately 7.8 cm long, were obtained from the Aquaculture Genetics and Breeding Technology Center at the Virginia Institute of Marine Science, Gloucester Point, VA. The oysters were divided into four 2 L beakers (five oysters to a beaker) and covered with York River water containing a 1% algal solution made with Reed Mariculture algal paste (Campbell, CA). The oysters were allowed to feed over night to ensure that they were actively pumping. After 18 hours, the cleared water was removed and one oyster from each beaker (N=4) was removed and kept at 4°C for the duration of the tank exposure. These oysters represented the "no exposure" negative controls. The oysters in beakers #1-3 (N=12)

were covered with a solution comprised of 300 mls raw wastewater influent from the James River Wastewater Treatment Plant in Newport News, VA, 1700 mls York River water, 20 mls 1% algal solution and 500µl HAdV F41 ($3.25x10^8$ genomic equivalents), strain Tak from ATCC (Manassas, Virginia). The oysters in beaker #4 (N=4) were covered with a negative control solution comprised of 1980 mls York River water and 20 mls 1% algal solution. Air stones were put into each beaker and the beakers were covered with aluminum foil and placed under an environmental hood and allowed to feed for 18 hours. Oysters from beakers #1 and 4, as well as the "no exposure" negative control oysters, were processed immediately upon completion of this exposure phase. They were scrubbed with a 1% bleach solution, then shucked and digestive and gill tissue were dissected from each oyster under aseptic conditions. Tissues from all four of the oysters from each treatment condition was pooled and processed for viral elution and concentration as outlined below. Oysters from beakers #2 and 3 were placed in mesh bags for relay in the York River.

James River deployed oysters. Twenty market-sized oysters (roughly 74 – 77 mm in length) were divided into two bags (10 oysters/bag) and placed in a small off-bottom wire oyster cage. The cage was deployed directly adjacent to the effluent outfall from the James River Wastewater Treatment plant on April 25, 2011. One bag of oysters was collected seven days after deployment and the second bag of oysters was collected 14 days after deployment. Immediately upon returning to the lab on each collection day, the oysters were divided into three groups of three oysters each. The oysters were scrubbed and shucked under sterile conditions. Tissue from the three

oysters in each group were pooled and processed for viral elution and concentration as outlined below.

Viral elution and concentration. The weight of each pooled oyster sample from the laboratory and field deployment studies was determined, 0.25N sterile glycine buffer (pH10) added, (1:5 wt/vol) and each sample was homogenized for 60 seconds in a sterile blender. The homogenate from the "no exposure" laboratory treatment was divided in half and half was spiked with 3 µl (1.95×10^6 genome equivalents) HAdV F41 strain Tak from ATCC (Manassas, Virginia) to provide a positive control for the procedure. Homogenates were then mixed by magnetic stirring for 15 minutes, the pH adjusted to 7.0 ± 0.2, and clarified by centrifugation at 2,170 x *g* for 15 minutes at 4°C. Supernatants were transferred to sterile centrifuge bottles and spun at 3,800 x *g* for 45 min at 4°C. Supernatants from this last low-speed centrifugation were transferred to sterile ultracentrifuge tubes and spun at 81,584 x *g* for 1 hour at 4°C. The resulting pellets were washed with sterile 1X phosphate buffered saline (PBS), resuspended in 3 mls of sterile 1X PBS, and stored at -20°C for no more than 48 hours prior to DNA extraction.

Depuration of oysters. Oysters for relay were suspended in the York River from the research pier at the Virginia Institute of Marine Science. Oysters from the first two tank studies were relayed for 7 and 14 days, while oysters from the final tank study were relayed for 3, 6, and 10 days. At each time point retrieved oysters were scrubbed with a 1% bleach solution and processed as described above.

Nucleic acid extraction. Total genomic DNA was extracted from the viral concentrates using the QIAGEN DNeasy Blood and Tissue Kit (Valencia, CA) per manufacturer's protocol. The final volume of DNA obtained was 60 µl. Negative controls were run for the concentration step and the DNA extraction step using sterile deionized water as the blank. Five µl aliquots of the resulting DNA were used for real-time quantitative PCR (qPCR) assays as described below. In addition, 100µl aliquots of viral concentrate from each of the four treatment conditions (York River water only, York River water with HAdV, no exposure negative control, and spiked positive control) were used for integrated cell culture (ICC).

Quantitative polymerase chain reaction (qPCR). qPCR was conducted on all samples. Primers and a FAM/TAMRA labeled TaqMan probe developed by Heim et al. (2003) were used in this assay. Ten µl reactions were run on an Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA). TaqMan Fast Universal Master Mix was used for all qPCR reactions. Final concentration of PCR reagents was as follows: 1X TaqMan Universal Master Mix, 0.2 mg/ml BSA, each primer at 0.5 µM, 0.4 µM TaqMan FAM/TAMRA labeled probe (Table 1.1). Thermocycling parameters were as follows: 95°C for 20 s, followed by 45 cycles of 95°C for 15 s, 55°C for 10s, 60°C for 1m. A standard curve was generated in the following manner: HAdV F41 DNA was amplified using the above outlined nested PCR protocol. The PCR product was run on a 1.5% agarose gel, the band excised and cleaned using the QIAquick PCR purification kit (Valencia,

CA) per manufacturer's protocol. The resulting DNA was quantified using the NanoDrop 2000 UV-Vis Spectrophotometer (ThermoScientific,Wilmington, DE). Based on the fragment size of 143bp, the mass of one copy of the fragment, a genomic equivalent (ge), was calculated to be 1.56×10^{-10} ng/ge. By dividing the concentration of the DNA by the mass of one ge, we were able to obtain the ge/µl of our purified fragment. Tenfold serial dilutions were performed and were used to create a standard curve.

Negative controls were run using deionized water as the blank. Positive controls were run using 3 µl (1.95×10^6 genome equivalents) HAdV 41 DNA, strain Tak from ATCC (Manassas, Virginia).

ICC-qPCR. The infectivity of HAdV in all samples was determined using an integrated cell culture-quantitative polymerase chain reaction (ICC-qPCR) technique following a combination of protocols from Choo and Kim (2006) and Gallagher and Margolin (2007). Caco-2 cells were grown to 75-90% confluence in t25 tissue culture flasks using Minimum Essential Medium Eagle (MEM) with 10% fetal bovine serum (FBS). Once the cells reached the appropriate confluence, the medium was removed and the cells were washed with fresh medium. Each sample was cultured in quadruplicate by adding 50 µl viral concentrate to a flask containing washed Caco-2 cells. After inoculation, the cells were rocked every 15 minutes for 60 minutes. Following this, 5 ml of MEM was added to each culture flask. Two of the four flask cultures for each sample were frozen (-80°C) immediately (T_0), the remaining two were incubated at 37°C for 4 days (T_4). Following the 4 day incubation period the

culture flasks were stored at -80°C until DNA extraction. Prior to DNA extraction, each culture flask was subjected to three freeze/thaw cycles. Two hundred µl of the lysate containing thawed cells was used to extract total genomic DNA using the QIAGEN Blood and Tissue Kit (Valencia, CA). Five µl of DNA was used for qPCR as outlined previously. The T_0 and T_4 duplicates for each sample were compared to determine if the number of viral particles increased over the 4 day incubation period. An increase in viral particles was interpreted as meaning the adenoviruses contained in a given sample were viable and therefore remained infective. Negative controls were run for each sample by inoculating negative control flasks with 50 µl of MEM medium. Positive controls were run for each sample by inoculating control flasks with with 50 µl (3.25×10^7 genomic equivalents) HAdV F41 strain Tak from ATCC (Manassas, Virginia).

Quantification calculations. To determine the genomic equivalents per sample, the total mass of tissue, the volume of tissue viral concentrate, the volume of viral concentrate used for DNA extraction, the final volume of DNA eluate, and the volume of DNA added to the reaction were all taken into consideration. When calculating the genomic equivalents present in ICC-qPCR samples, the addition of the medium was also accounted for. Below are examples for each:

<u>qPCR:</u> X grams of tissue (mass varied by sample) ➔ concentration of the virus into a 5 ml viral concentrate ➔ 200 µl concentrate sampled for DNA extraction ➔ elution of the DNA into 60 µl ➔ 3 µl of eluate added to each qPCR reaction. From this the following equation would be used to determine the genomic

equivalent/gram of tissue: genomic equivalents as reported by qPCR assay based on standard curve × 25 × 20 ÷ X (X = the number of grams of tissue) = genomic equivalents/gram.

ICC-qPCR: X grams of tissue (mass varied by sample) ➔ concentration of the virus into a 5 ml viral concentrate ➔ 100 µl inoculum ➔ 5 mls culture medium added ➔ 200 µl aliquot sampled for DNA extraction ➔ elution of the DNA into 60 µl ➔ 3 µl of eluate added to each qPCR reaction. From this the following equation was used: genomic equivalents as reported by qPCR assay based on standard curve × 50 × 25 ×20 ÷ X (X = the number of grams of tissue) = genomic equivalents/gram. It is important to note that calculations must be adjusted on a per sample basis as the weight of oyster tissue obtained per sample was variable.

Results

Tank studies. qPCR results from three separate pilot tank studies confirmed that oysters were able to filter and retain HAdV spiked in algal enriched York River water. During the study conducted on May 10, 2012, the average HAdV DNA concentration in oyster tissue taken from animals allowed to feed on HAdV F41 spiked York River water for 18 hours was $5.76x10^2$ genomic equivalents per gram of tissue (ge/gram). In addition, ICC-qPCR conducted on viral concentrates from tissues of these animals confirmed that the animals filtered and retained viable virus. Over the course of the four day incubation period, the concentration of HAdV DNA detected by qPCR increased by an order of magnitude, from $1.05x10^3$ ge/g to

$1.06x10^4$ ge/g (Table 2.1). Tissue from animals that were exposed to York River water only during the same 18 hour period were negative for HAdV DNA.

During the May 17, 2012 tank study, an average of 89 ge/gram was recovered from tissue of animals exposed to algal enriched York River water spiked with HAdV. However, the ICC-qPCR of the viral concentrates from the tissues of these animals did not result in any detectable infectious HAdV. These results were repeated in the November 15, 2012 study. In November study, the concentration of HAdV DNA from the animals exposed to the enriched, spiked York River water was $4.17x10^2$ ge/gram, but the ICC-qPCR assay did not detect any infectious HAdV (Table 2.1).

For all three studies, oyster homogenates were spiked with 3 µl (1.95×10^6 genome equivalents) viable HAdV F41 prior to the viral elution and concentration procedure to serve as positive controls. Additionally, negative controls for each study were obtained by selecting four oysters from the oysters received from the Aquaculture Genetics and Breeding Technology Center (Gloucester Point, VA) and processing them immediately. Results for the positive and negative controls, as well as the experimental treatments are shown in Figure 2.1.

Elimination studies. Oysters from the May experiments that were exposed to York River water spiked with virus were relayed to the York River for seven and 14 days. qPCR did not detect HAdV DNA in oyster tissues recovered from each of these relays. Also, ICC-qPCR of the seven-day relay samples was negative for viable

viruses. However, in both the May 10 and May 17 experiments, ICC-qPCR of day 14-relay samples revealed the presence of infective HAdV. The HAdV DNA concentration at T_0 in both experiments was 0.00 ge/gram, but the T_4 concentrations were $3.91x10^3$ and $1.23x10^3$, respectively (Table 2.1). Oysters exposed to spiked York River water during the November tank studies were relayed for three, six and ten days in the York River. qPCR results for each of the relay days, as well as ICC-qPCR for each were negative for HAdV DNA and viable HAdV (Table 2.1).

Field deployed oysters. qPCR analysis of tissue from oysters deployed directly adjacent to the outfall of the effluent from the James River Wastewater Treatment Plant was negative for HAdV DNA, regardless of whether the oysters were examined at seven days or 14 days post deployment.

Discussion

The objective of this small pilot study was two fold. First, to ascertain if oysters exposed to human adenovirus would filter the virus from the water and retain it in a viable state in their tissues. And second, if the virus was retained, could it be eliminated by relay in an open system. In order to accomplish this laboratory treatment tanks were used to control exposure of the oysters to the virus and to ensure that the oysters were adequately pumping water. York River water was used in tank studies and depuration trials were conducted in the York River during the spring and fall to account for possible seasonal differences in depuration efficiency.

All three of the tank studies confirmed that oysters were able to filter the virus from the York River water. For the May 10th experiment, the concentration of viral DNA recovered from the oyster tissue was 576 ge/gram, while the viral DNA concentration recovered during the May 17th and November 17th experiments were 89 ge/gram and 417 ge/gram, respectively. All the animals used in the experiments were of the same approximate size. Furthermore, conditions under which the animals were treated experimentally were the same in each experiment, including water temperature. Water from the York River collected on the day of the study was allowed to equilibrate to room temperature (24°C) before animals were placed into the tanks. The principle difference between the experiments would have been the composition of the York River water and its constituents. Tank water was spiked with the same concentration of HAdV F41 and algal paste, although, conditions such as salinity, pH, and dissolved oxygen, were different comparing May and November study dates. (http://www3.vims.edu/vecos/StationDetail.aspx?param=YRK005.40&program=CMON). During May 2012, the salinity of the York River remained approximately the same, 18.5 psu, whereas, in November of 2012, the salinity was approximately 22.3 psu. May dissolved oxygen concentrations, 7.5 mg/l and 6.5 mg/l (May 10, 2012 and May 17, 2012, respectively) were fairly comparable, but were slightly higher in November (9.5 mg/l). Finally, the pH remained around 7.9 during May but increased to 8.35 during November. However, even though there were seasonal differences in the chemistry of the water, all of these values fell well within tolerance ranges for the oyster. Since the tank water was not assayed to determine the concentration of HAdV DNA that remained unfiltered or released by the animals, it was not possible to

determine if the lower viral concentration in the May 17th experiment was the result of reduced uptake efficiency, or if the elution and concentration procedure was less efficient in recovering HAdV DNA from the tissue.

While water temperature was not a factor in the initial contamination, it may have been an important factor in the elimination process. The spring studies were conducted during the month of May. The average water temperature during this period was 20°C for the May 10, 2012 experiment and 23°C for the May 17, 2012 (http://www3.vims.edu/vecos/StationDetail.aspx?param=YRK005.40&program=CMON). While these values are slightly lower than the optimal temperature for maximum pumping, they are well within the tolerance temperature range for the oyster species used (Loosanoff, 1958). The average water temperature during the fall experiment was 11°C, considerably lower than the May studies and the oysters would have been pumping at a slower rate (Loosanhoff, 1958). (http://www3.vims.edu/vecos/StationDetail.aspx?param=YRK005.40&program=CMON). Based on this, a slower rate of elimination would be anticipated in the fall than in spring. This was not the case. In the spring experiments, there was no evidence of HAdV in the tissues after seven days of relaying the oysters to the York River. Interestingly, we do see evidence of viable HAdV in the ICC-qPCR assay of the 14 day relayed oysters. This was unexpected since both the seven and 14 day relayed oysters were qPCR negative and the seven day relayed oysters were negative by ICC-qPCR. However, both May relays were negative in the ICC-qPCR at T_0 for the 14 day relayed animals, but were positive (i.e., 3910 ge/gram and 1230 ge/gram HAdV

DNA) at T_4. This suggests that there was at least one virion that remained in the tissue and was infective. Although the limit of detection (LOD) of pure HAdV DNA by qPCR was measured to be 1 ge/µl, environmental samples do not behave as pure viral extracts when amplifying the DNA. It is not possible to account for any number of inhibitors that may be in any given sample. Therefore, it is plausible that viable virions that were present in the seven day relayed oysters could not be detected. Also complicating this is the fact that the negative control for the May 10th 14 day ICC-qPCR assay was apparently contaminated and produced a positive signal. However, the May 17th negative control was not contaminated. As for the November depuration study, given the lower water temperature, we expected to be able to detect HAdV during the relay period. Consequently, we increased the number of sample days to include a three, six and ten day sampling point. All of the qPCR and ICC-qPCR assays on oysters from each of the samples at all time points were negative. This begs the question of whether complete elimination occurred, or if the amount of HAdV DNA was simply below the limit of detection of the assays.

Ideally, we would have been able to test if oysters were able to concentrate viable HAdV directly from estuarine water receiving contaminated effluent from a wastewater treatment plant (WWTP). Initially, our experimental plan did involve the deployment of oysters to the James River, directly adjacent to the James River WWTP effluent outfall. Unfortunately, several of our deployed oyster samples were lost. This resulted in very incomplete data. The only deployment experiment from which we were able to get a fairly complete data set for was the one conducted in

April 2011. For this deployment we were able to retrieve animals after seven and 14 days of exposure to the effluent, however, qPCR assays of all of the animals were negative for HAdV DNA, hence, no depuration relay studies were conducted.

In conclusion, this small pilot study confirmed that oysters are capable of filtering HAdV from river water while feeding and that the virus is retained, in an infective state, in the oyster tissues. In addition, depuration of the virus in an open system, such as a river, appears to be achievable in less than three days under optimal conditions. It is important to note that a higher viral load would most likely take longer to depurate (Pommepuy et al., 2003). Also, the water temperature would have a significant impact on the depuration process. Oysters do not actively pump below 6-7 °C (Loosanoff, 1958). While these temperatures are not common in the York River, they do occur (http://www3.vims.edu/vecos/StationDetail.aspx?param=YRK005.40&program=CMON). However, due to contamination in our negative control of one of the depuration experiments, it is not clear whether the positive signal obtained in the ICC-qPCR assay of the May 14 day relay T4 was due to viable virus or contamination. Therefore, additional elimination studies will need to be conducted. This pilot study established a foundation to design a full-scale study on the efficiency of HAdV filtration and concentration by oysters feeding in contaminated waters and the effectiveness of viral elimination of contaminated oysters in an open river system.

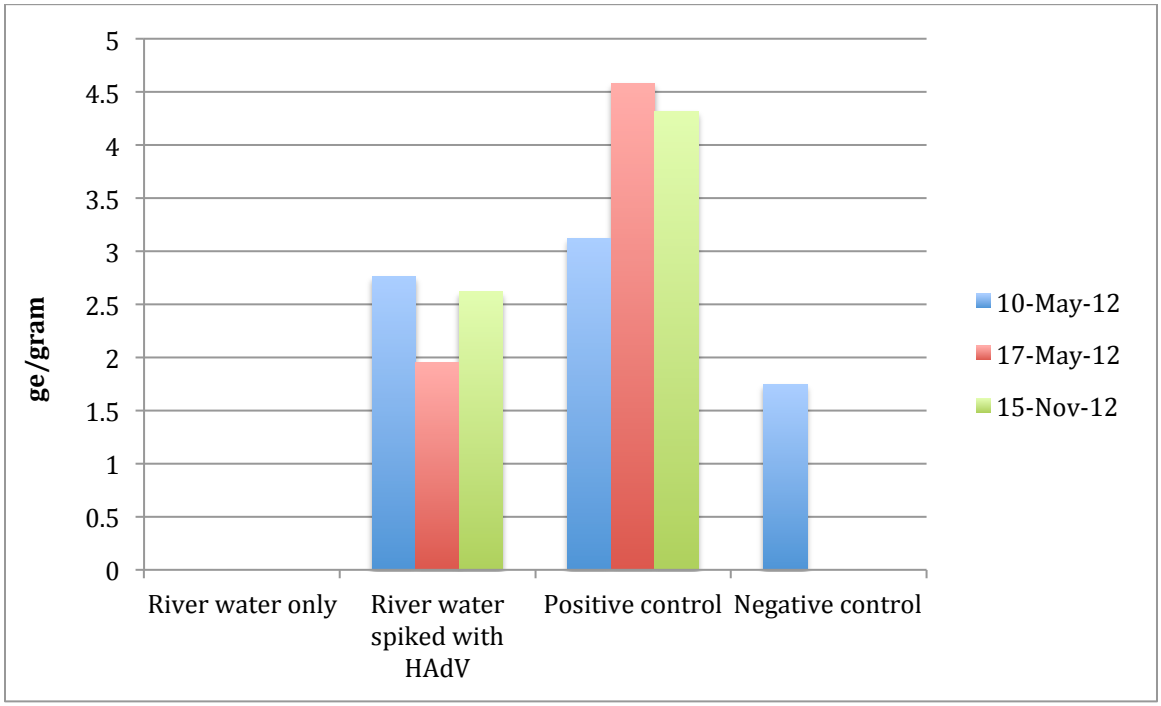

Figure 2.1. Log_{10} transformed concentration of HAdV DNA obtained by qPCR for oysters subjected to each treatment condition. River water only oysters were exposed to York River water for 18 hours in the absence of added HAdV. Negative control samples were not exposed to any water treatment. Spiked samples were exposed to 2 L river water containing 500µl HAdV F41 ($3.25x10^8$ genomic equivalents), strain Tak from ATCC (Manassas, Virginia). Positive control samples contained 3 µl HAdV F41 (1.95×10^6 genome equivalents), strain Tak from ATCC (Manassas, Virginia), which was added to the homogenized tissue prior to viral elution and concentration.

Table 2.1. Tank exposure studies with York River water spiked with 500 ul of HAdV / 2 l. Concentration of detected viral DNA is recorded in genomic equivalents per gram of tissue. *Negative control was contaminated.

Tank Study	River water spiked with HAdV			Day 7 Relay			DAY 14 Relay		
		ICC-qPCR		qPCR	ICC-qPCR		qPCR	ICC-qPCR	
	18 hour post feeding	T_0	T_4		T_0	T_4		T_0	T_4
May 10, 2012	576.0 ge/gram	1050 ge/gram	1060 ge/gram	0.00	0.00	0.00	0.00	0.00	3920 ge/gram*
May 17, 2012	89.0 ge/gram	0.00 ge/gram	0.00 ge/gram	0.00	0.00	0.00	0.00	0.00	1230 ge/gram

Tank Study	River water spiked with HAdV			Day 3 Relay			Day 6 Relay			Day 10 Relay		
		ICC-qPCR		qPCR	ICC-qPCR		qPCR	ICC-qPCR		qPCR	ICC-qPCR	
	18 hour post feeding	T_0	T_4		T_0	T_4		T_0	T_4		T_0	T_4
Nov. 15, 2012	4170 ge/gram	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

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Chapter 3: Detection and viability of human adenovirus in biosolids produced by wastewater treatment plants employing various treatment and stabilization processes

Abstract

Biosolids are used as fertilizer for agricultural fields with increasing frequency worldwide. As this use continues to rise, so does the risk of environmental contamination with human pathogens, including enteric viruses such as human adenovirus (HAdV). Enteric HAdV (F40 and F41) are considered to be more resistant to disinfection than many other enteric viruses. In addition, HAdV are quite stable in the environment and thus of potential health concern when biosolids are applied for land use where there is the risk of runoff into environmental waters or aerosolization. Various treatment and stabilization methods are employed by WWTPs to produce biosolids throughout the United States. Twenty-six blinded biosolids samples were obtained from six different states employing five different treatment methods: plate and frame process, belt process, alkaline stabilized, centrifugation and "unknown". Using qPCR analysis 22/26 samples (84.6%) had detectible HAdV DNA. There was a significant difference among samples in the concentration of detectable HAdV DNA based on treatment method ($p = 0.000$). Alkaline stabilized (lime treated) samples had the lowest concentration (1.73 ge/g), while plate-framed thickened samples had the highest (3.58 ge/g). Although the plate-frame thickened samples had the highest concentrations of detectable HAdV DNA, viable HAdV particles were not detected by an integrated cell culture-quantitative PCR (ICC-qPCR) assay applied to test viability. Two samples generated

by “unknown” treatment methods were the only samples in which viable viruses were detected.

Introduction

The term biosolids was first used in the early 1990s to refer to sewage sludge that had undergone various chemical and physical treatments to ensure its safety as an agricultural soil fertilizer (Lu et al., 2012). Biosolids are classified by EPA as either class A or class B. Class A biosolids can be sold commercially to homeowners, and thus, must meet stringent standards with regard to bacterial, viral and protozoan pathogen contamination. Class B biosolids are not held to these standards and may still contain detectable concentrations of pathogens (U.S. E.P.A, 2003). Since adsorption of viruses to solid surfaces decreases their susceptibility to degradation or inactivation, it is inherently more difficult to disinfect biosolids when compared to effluent. Disinfection methods to produce each class of biosolids can vary, but the standards must be met. The primary treatment methods are divided into two categories: The processes to significantly reduce pathogens (PSRPs) and the processes to further reduce pathogens (PFRPs). The PSRPs include anaerobic digestion, aerobic digestion and alkaline stabilization (liming). The PFRPs include heat drying, composting and thermophilic aerobic digestion (Smith and Reimers, 2006). In addition, dewatering, using the plate and frame process, belt process or centrifugation of raw sludge, can be used as a treatment, or in conjunction with other treatment methods. It is estimated that the United States produces 6.5 million dry

metric tons of Class B biosolids annually. In 2004, approximately 4 million dry metric tons were applied to US agricultural lands (NEBRA, 2007; Lu et al., 2012).

Of the human pathogens detected in Class B biosolids, human adenovirus (HAdV) is among the most abundant (Jenkins and et al., 2007; Viau and Peccia, 2009). Human adenoviruses belong to a group of DNA viruses responsible for acute respiratory tract infections, conjunctivitis, hemorrhagic cystitis, and gastroenteritis (Haramoto et al., 2007; CDC, 2005). The modes of transmission vary for each clinical presentation. Adenovirus strains that cause respiratory infections are typically transmitted via contact with fomites contaminated with respiratory secretions, while conjunctivitis is associated with contaminated swimming pools (Jiang, 2006; Artieda et al, 2009). The strains that cause gastroenteritis are transmitted via waterborne or fecal-oral contact, which could occur as the result of inadequate wastewater treatment plant (WWTP) disinfection of wastewater and sewage sludge, leading to contaminated estuarine and recreational waters (Jiang et al., 2001; CDC, 2005; Haramoto et al., 2007). These adenovirus strains, HAdV F40 and F41, are considered emerging pathogens and are second only to rotavirus as the causative agent of viral pediatric diarrhea (Jiang, 2006). In addition, they can have up to a 50% case fatality rate among immunosuppressed and immunocompromised patients (Jenkins et al., 2007).

Despite the evidence that HAdV DNA has been detected in Class B biosolids, few studies have addressed its viability in biosolids, and even fewer have addressed how different treatment methods affect its viability (Hansen et al., 2007; Viau and Peccia,

2009; Wei et al., 2009; Wong et al.; 2010) and to date, none have employed integrated cell culture-quantitative PCR (ICC-qPCR) as a technique for determining viability. Wong et al (2010) assessed infectivity of human adenoviruses in Class B biosolids using ICC-PCR, while Wei et al. (2009) relied on cell culture coupled with reverse transcription-PCR. The main difference between theses two methods and ICC-qPCR is that they do not use the quantitative capability of qPCR to determine viability. Instead, they rely on the most-probable-number (MPN) method, which is based on the observation of cytopathic effects in the cell culture. Since, human adenoviruses F40 and F41 have been shown to not cause significant cytopathic effects in cell culture regardless of the cell line used, the use of MPN methods to provide accurate quantitative evidence of viability is limited (Chapron et al., 2000).

The objective of this study was to detect, quantify and assess the infectivity of human adenoviruses found in Class B biosolids processed under various stabilization methods; plate and frame, belt processing, centrifugation and liming. To accomplish this, viral particles were isolated from biosolids samples and used in ICC – qPCR assays. Due to the ability of adenovirus to withstand drastic shifts in pH and persist under various environmental conditions, detection of HAdV DNA and the presence of viable virus were expected regardless of treatment method.

Materials and Methods

Sample collection. Freshly collected biosolids samples were kindly provided by Dr. Aaron Margolin of the University of New Hampshire. These samples were collected

from various wastewater treatment plants in the United States and their sources were blinded. The only information provided was the state of origin and the treatment method employed. Biosolids samples were shipped in insulated containers and kept chilled using blue ice. Upon receipt, biosolids samples were kept under refrigeration (4°C) at VIMS and processed within 24 hours. Unused biosolids were archived at -80°C.

Viral concentration and DNA extraction. Two methods of direct DNA extraction from biosolids samples were compared to determine which would provide the best viral recovery. The MO BIO PowerSoil DNA kit (Mo Bio Laboratories, Carlsbad, CA) was used for DNA isolation directly from the sample and results were compared to a those obtained using a modification of an elution/concentration protocol developed by Balkin and Margolin (2010) followed by DNA extraction of the concentrated sample using the Qiagen DNeasy Blood and Tissue kit (Valencia, CA). The Mo Bio PowerSoil protocol was done following manufacture's directions. For each sample, 0.5 grams of biosolids was used and recovered DNA was eluted into 100 µl elution buffer and stored at -20°C.

The revised elution/concentration protocol for obtaining infectious virions was kindly provided by A. Margolin (personal communication). Thirty grams of biosolids sample was added to 60 ml of 10% beef extract and stirred for 30 minutes, followed by centrifugation at 2500 x g for 30 minutes at 4°C. The eluate was collected and the beef extract concentration was diluted to 3% with sterile reagent grade water. The

eluate was stirred while the pH was adjusted to 3.5 ± 0.1 causing a flocculate to form. The flocculated suspension was centrifuged at 2500 x g for 15 min at 4°C and the resulting pellet was resuspended with Na_2HPO_4 to a final volume of 1/20 of the volume of the original diluted beef extract suspension. The pH was adjusted to 7.2 ± 0.2 and the antibiotics antimycotic and gentamicin were added to the final concentrate at a concentration of 10^{-1} and 10^{-2} (v/v), respectively. The concentrate was incubated for 2 hours at 37°C and stored at -80°C until further analysis. At this time, the concentrate was used to inoculate cells in ICC-qPCR and to generate genomic DNA for nested and qPCR assays. Total genomic DNA was extracted from 200 µl of the concentrate using the QIAGEN DNeasy Blood and Tissue Kit per manufacturer's protocol.

Although the MO BIO PowerSoil kit has been used in previous studies, a comparative analysis was conducted to determine whether the MO BIO PowerSoil was more effective than the Qiagen DNeasy kit in recovering HAdV DNA from viral concentrates. This was done by spiking 30 g of each biosolids sample with 100 µl HAdV F41, strain Tak from ATCC (Manassas, Virginia). The spiked samples were concentrated using the above described beef flocculation protocol. Two hundred µl of the concentrate was then extracted using either the MO BIO PowerSoil kit or the Qiagen DNeasy kit per the respective manufacturer's directions. DNA obtained from each protocol was then subjected to qPCR (as described below) to assess viral DNA recovery.

Detection of HAdV by nested PCR. Nested PCR was conducted on all samples, in duplicate, using primers designed to amplify a hypervariable region in an open reading frame of the hexon gene encoding this protein (or capsid) coat component (Puig *et al*, 1994) as described in chapter 1.

Detection of HAdV by qPCR. The qPCR assay was run for all samples in duplicate. Primers and a FAM/TAMRA labeled TaqMan probe developed by Heim et al. (2003) were used in this assay. Ten µl reactions were run on an Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA). TaqMan Fast Universal Master Mix was used for all qPCR reactions. Final concentration of PCR reagents was as follows: 1X TaqMan Universal Master Mix, 0.2 mg/ml BSA, each primer at 0.5 µM, 0.4 µM TaqMan FAM/TAMRA labeled probe (Table 1.1). Thermocycling parameters were as follows: 95°C for 20 s, followed by 45 cycles of 95°C for 15 s, 55°C for 10s, 60°C for 1min. A standard curve was generated in the following manner: HAdV F41 DNA was amplified using the above outlined nested PCR protocol. The PCR product was run on a 1.5% agarose gel, the band excised and cleaned using the QIAquick PCR purification kit (Valencia, CA) per manufacturer's protocol. The resulting DNA was quantified using the NanoDrop 2000 UV-Vis Spectrophotometer (ThermoScientific,Wilmington, DE). Based on the fragment size of 143bp, the mass of one copy of the fragment, a genomic equivalent (ge), was calculated to be 1.56×10^{-10} ng/ge. By dividing the concentration of the DNA by the mass of one ge, the ge/µl of our purified fragment was calculated. Tenfold serial dilutions were performed and used to create a standard curve.

Negative controls were run using deionized water as the blank. Positive controls were run using 3 µl (1.95×10^6 ge) HAdV F41 DNA, strain Tak from ATCC (Manassas, Virginia).

ICC-qPCR. The infectivity of HAdV in biosolids samples was determined using an integrated cell culture-quantitative polymerase chain reaction (ICC-qPCR) technique following a combination of protocols from Choo and Kim (2006) and Gallagher and Margolin (2007). Initially, two cell lines, Buffalo Green monkey kidney (BGMK) cells and Caco-2 cells, were tested for their efficiency in HAdV propagation. While both cell lines were permissive to HAdV infection, Caco-2 cells were chosen for ICC-qPCR based on ease of maintenance and a 10^2 increase in viral concentration relative to the BGMK cells over a four-day incubation period. Caco-2 cells were grown to 75-90% confluence in t25 tissue culture flasks using Minimum Essential Medium Eagle (MEM) with 10% fetal bovine serum (FBS). Once the cells reached the appropriate confluence, the medium was removed and the cells were washed with fresh medium. Each sample was cultured in quadruplicate by adding 50 µl of a biosolids viral concentrate prepared as above to a flask containing washed Caco-2 cells. Inoculations for each biosolids sample were carried out with undiluted and serial dilutions of concentrate (10^{-1}, 10^{-2}, 10^{-3}) to evaluate the possibility that inhibition of Caco-2 cell growth by substances contained within the concentrate was affecting the assay. After inoculation, the cells were rocked every 15 minutes for 60 minutes. Following this 5 ml of MEM was added to each culture flask. Two of the four flask cultures for each sample were frozen (-80°C) immediately (T_0) the

remaining two were incubated at 37°C for 4 days (T_4). Following the 4 day incubation period the culture flasks were stored at -80°C until DNA extraction. Prior to DNA extraction, each culture flask was subjected to three freeze/thaw cycles. Total genomic DNA was extracted from 200 µl of the supernatant containing thawed cells using the QIAGEN Blood and Tissue Kit (Valencia, CA) following the manufacturer's protocol and eluted in 60ul buffer. Five µl of DNA was used for qPCR as outlined previously. The T_0 and T_4 duplicates for each sample were compared to determine if the number of viral particles increased over the 4-day incubation period. An increase in viral particles was interpreted as meaning the adenoviruses contained in a given sample were viable and therefore remained infective. Negative controls were run for each sample by inoculating control flasks with 50 µl of MEM medium. Positive controls were run for each sample by inoculating control flasks with 50 µl (3.25×10^7 ge) HAdV F41, strain Tak from ATCC (Manassas, Virginia). In addition to inoculating with serial dilutions of viral concentrate, a subsample of 10^{-1} concentrate dilutions were filtered through a 0.2 µm cellulose acetate membrane sterile syringe filter to remove potential particulate inhibitors. The filtered, diluted concentrates were then used to inoculate cells as outlined above.

Quantification calculations. To determine the genomic equivalents per sample, the initial mass of biosolids sampled, the final volume of biosolids concentrate, the volume of concentrated biosolids for DNA extraction, the final volume of DNA eluate, and the volume of DNA added to the reaction are all taken into consideration.

When calculating the genomic equivalents present in ICC-qPCR samples, the addition of the medium is also accounted for. Below are examples of these calculations:

qPCR: 30 g biosolids ⇒ 8 (variable) ml viral concentrate ⇒ 200 µl concentrate sampled for DNA extraction ⇒ elution of the DNA into 60 µl ⇒ 3 µl of eluate added to each qPCR reaction. From this the following equation would be used: genomic equivalents as reported by qPCR assay based on standard curve × 40 (variable) × 20 ÷ 30 = genomic equivalents/g.

ICC-qPCR: 30 g biosolids ⇒ 8 (variable) ml viral concentrate ⇒ 100 µl inoculum ⇒ 5 mls medium added ⇒ 200 µl lysate for extraction ⇒ elution of the DNA into 60 µl ⇒ 3 µl of eluate added to each qPCR reaction. From this the following equation would be used: genomic equivalents as reported by qPCR assay based on standard curve × 80 ×25 ×20 ÷ 30 = genomic equivalents /ml. It is important to note that calculations must be adjusted for each sample. Specifically, the volume obtained during the concentration step is variable, thus the calculations must be adjusted accordingly.

Data analysis. All quantitative genomic equivalents data were $\log_{10}$ transformed prior to analysis. Analysis of Variance (ANOVA) was used to test for differences in HAdV DNA concentration between the various stabilization methods. A paired T-test was used to detect differences in presence/absence of HAdV DNA comparing results of assays using the DNA from the direct MO BIO extraction to those with the

DNA from the beef flocculation/Qiagen DNA extraction methods for both nested and qPCR assays. P values ≤0.05 indicated significant differences.

Results

Method comparison for viral concentration and DNA extraction. Each sample of biosolids received from the Margolin lab was processed using the MO BIO PowerSoil DNA kit to extract DNA directly and by a modification of a beef flocculation elution and concentration protocol (Balkin and Margolin, 2009) using the Qiagen DNeasy Blood and Tissue kit for DNA extraction. Nested and qPCR were used to detect presence or absence of HAdV DNA for each sample (Table 3.1). Six percent (6%) of the samples that were processed with the MO BIO kit were positive for HAdV DNA by qPCR assay compared to 20.2% using the beef flocculation/Qiagen DNA extraction method. Paired t-test analysis indicated that this difference was statistically significant ($p = 0.001$). However, when the same samples were assayed with the nested PCR assay, there wasn't a significant difference in the detection of HAdV ($p = 0.63$). The MO BIO method produced positive results in 8.3% of the samples, while the beef flocculation/Qiagen method produced positive results in 10.6% of the samples. Given that qPCR is an integral component to ICC-qPCR, the beef flocculation/Qiagen method was chosen for subsequent processing of samples.

A side-by-side comparison of viral DNA recovery efficiency and inhibitor removal capacity of the Qiagen DNeasy Blood and Tissue and MO BIO Powersoil kits was conducted by extracting 200 µl of HAdV spiked biosolids concentrate with each kit.

The DNA from each kit was then analyzed by qPCR to obtain viral genomic equivalents/µl. The Qiagen kit performed significantly better than the MO BIO kit ($p = 0.0062$) based on a paired t-test, with average recovered concentrations of $1.65x10^4$ ge/µl and $1.25x10^3$ ge/µl of HAdV DNA, respectively (Table 3.2).

Comparison of stabilization methods in processing biosolids. Twenty-six biosolids samples were collected from six different states employing a total of five different stabilization methods to disinfect the biosolids (Table 3.3). Viral elution and concentration followed by DNA extraction was accomplished with the beef flocculation/Qiagen method based on results presented above. HAdV DNA present in each sample was quantified with qPCR. All biosolids processed by centrifugation were negative for the presence of HAdV DNA. Whereas, 100% of the samples processed by all other methods were positive for HAdV DNA. Limed samples had an average HAdV DNA concentration of 1.63 ge/g whereas samples processed by plate and frame and belt processing had average concentrations of 3.58 ge/g and 3.32 ge/g, respectively. Five of the 26 samples provided to us did not contain information describing the stabilization process used. These were labeled as "stabilization unknown" and had an average of 1.73ge/g. Analysis of variance of the $\log_{10}$ transformed concentration data indicated a statistically significant difference in recovered HAdV concentration between all stabilization methods ($p = 0.000$). However, a pairwise comparison of plate and frame processing and belt processing indicated no significant difference between the two dewatering methods (unpaired *t*-test, $p = 0.369$).

ICC-qPCR with biosolids concentrates. Caco-2 cells were used to culture HAdV from each of 25 biosolids samples (sample 26 was not cultured). Eighteen of 25 had detectable HAdV DNA at time zero (T_0). Of these, nine were from samples processed with the plate/frame thickening method (100% positive), six were from belt processed dewatered samples (100% positive), and three were from samples of unknown processing method (60% positive). None of the centrifugally dewatered or lime stabilized samples had any detectable HAdV DNA at T_0 (Table 3.4). After four days of incubation (T_4), three samples had detectable levels of HAdV DNA via qPCR assay. Two of these, one belt processed and one of "unknown" method, had lower concentrations of HAdV DNA at T_4 than at T_0. One sample from an "unknown" method had a slightly higher concentration of HAdV DNA at T_4 implying that this sample contained viable virus. Paired t-tests indicated that there was no significant difference in the concentration of HAdV detected in the T_0 and the T_4 assays for each of these three samples.

ICC-qPCR with diluted and filtered biosolids concentrates. Caco-2 cells were inoculated with serial dilutions of each biosolids concentrate (10^{-1}, 10^{-2}, 10^{-3}) to identify if inhibitors/toxics present in an undiluted concentrate were detrimental to HAdV proliferation (Table 3.5). At time zero (T_0), ten of the 25 samples inoculated with the 10^{-1} dilution had detectable HAdV DNA, five inoculated with 10^{-2} dilutions had detectable HAdV DNA and five inoculated with the 10^{-3} dilution had detectable HAdV DNA. Fifteen unique samples accounted for these 20 positive detections and

included samples that underwent all but one of the stabilization processes, dewatering by centrifugation. Eleven were positive at only one dilution, three were positive at two dilutions and one was positive at all three dilutions. Of these 15 samples, only three were positive at one or more dilutions but negative in the undiluted assay. One of these three positives (limed) was not detectable at any dilution in the T_4 assays, suggesting that it was not infective. The remaining two (liming and "unknown" stabilization) were only detectable in T_4 assays, suggesting they contained viable virions. One sample (belt processing) that was positive in the undiluted T_0 assay as well as in the 10^{-3} T_0 assay, showed an increase in virion concentration in the 10^{-1} and 10^{-3} T_4 assays, again implying viability. Finally, of the initial 25 samples assayed, three remained negative regardless of dilution (liming, centrifugation and unknown stabilization) and 12 were positive in both the diluted and undiluted assays.

Filtration to remove particulates did not improve detection of viable virions. A subsample of five biosolids concentrates was chosen to be diluted and filtered prior to inoculation on Caco-2 cells. This subsample included all four of the samples that were positive in at least one of the T_0 and T_4 dilution assays and the one sample that was negative in all T_0 dilution assays, but positive in the T_4 assay. Only two of these samples contained detectable HAdV DNA in the T_0 assay and none of them contained detectable HAdV DNA in the T4 assay (Table 3.6).

Discussion

The use of Class B biosolids as a source of nutrient amendment for agricultural lands has been steadily increasing (Wong et al., 2010). With increased use comes the increased potential risk of environmental contamination with human pathogens. Enteric viruses, pathogenic bacteria and protozoa are all found in biosolids (Sidhu and Toze, 2009). However, due to a lack of consistency in detection and quantification, it is difficult to compare relative abundance and persistence of human pathogens in biosolids samples, regardless of treatment method (Sidhu and Toze, 2009). Human adenovirus is a pathogen of special concern since it has been demonstrated to be particularly stable and resistant to many forms of disinfection (Thompson et al., 2003; Symonds et al., 2009). This study addressed the incidence of human adenovirus in biosolids samples from various wastewater treatment facilities within the United States employing four different treatment methods; liming (alkaline stabilization), plate and frame processing (dewatering), belt processing (dewatering) and centrifugation (dewatering). A fifth grouping of samples included those for which the treatment method was unknown. While liming is considered a PSRP, dewatering is not. In many instances, biosolids may be dewatered to reduce volume prior to further treatment (U.S. EPA, 2000). Based on this information, it was hypothesized that the limed samples would have comparatively lower concentration of detectable adenovirus compared to dewatered material.

Of the 26 blinded biosolids samples that were received, nine were dewatered (two by centrifugation and seven by belt process), three underwent alkaline stabilization by

liming, eight were thickened by the plate and frame process and the stabilization methods of the remaining five were unknown. Of the treatment processes examined, centrifugation was the only method yielding no detection of HAdV DNA. This was surprising since centrifugation is a form of dewatering. Dewatering of biosolids has been shown to be effective in lowering nitrogen and potassium concentrations and improving the ease with which the biosolids are handled and transported, however, it is not considered particularly effective at removing pathogen loads (U.S. EPA, 1984). The other dewatering method addressed in this study, belt processing, was far less effective in reducing detectable adenovirus DNA based on the samples provided. It should be noted that only two samples that were identified as having been centrifuged were received, compared to eight that were belt processed. If the centrifuged samples were included with the belt processed samples, an average HAdV DNA concentration value of 3.19×10^{2} ge/g would result, well below that of the plate/frame thickened samples (7.10×10^{3} ge/g) but higher than alkaline stabilized samples (85 ge/g).

Interestingly, while there is evidence that liming is viewed as an effective means by which to eliminate pathogen load in biosolids, few studies have been conducted to address the effects of liming on the detectability and viability of human adenovirus 40 and 41 (Farrell et al., 1974, Jimenez et al., 2000). Two laboratory-scale studies have looked at the effects of liming in various spiked biosolids matrices on the inactivation of HAdV 5, a proxy for type 40 and 41due to its mode of transmission. These studies suggest that human adenovirus is susceptible to the affects of liming and that, regardless of the composition of the matrix, HAdV 5 was inactivated within 24 hours.

We found that HAdV DNA was detected in two out of the three lime stabilized samples that we received, but at a considerably lower average HAdV DNA concentration, 85 ge/g (SD = ±90.8), compared to dewatered, 3.19×10^3 ge/g (SD = $\pm2.4\times10^3$), and thickened 7.10×10^3 ge/g (SD = $\pm7.05\times10^3$) samples. However, detection of the HAdV DNA does not address the question of viability.

ICC-qPCR of 25 of the 26 samples revealed that only three, two from unknown treatment processes and one from plate/frame thickening yielded detectable concentrations of DNA in both the T_0 (immediately after inoculation) and the T_4 (four days after inoculation). Of these, only one showed an increase in concentration of HAdV, indicating that the sample contained viable virus. This happened to be one of the samples lacking information on the treatment process. The remaining two samples demonstrated a decrease in virus concentration at T_4. This likely indicated that there was detectable HAdV DNA present in the concentrate, but the DNA degraded during incubation. None of the lime treated samples had detectable HAdV DNA in either the T_0 or T_4 assays. As discussed above, the concentration of HAdV DNA detected by qPCR was considerably lower in ICC samples than in any other sample (Table 3.2). Therefore, it is likely that adding the viral concentrate from these samples to the cell culture in order to perform ICC-qPCR also diluted the DNA concentration to below the detection limit. This was the only stabilization method in which this occurred. In all of the dewatered and thickened samples, if HAdV DNA was detected by qPCR on DNA extracted directly from the biosolids concentrate, it was also detected in the time zero ICC-qPCR assay. This further substantiates the

claims made by Hansen et al. (2007) and Bean et al. (2007) that alkaline stabilization is effective in the inactivation and removal of human adenovirus from biosolids.

It was unexpected that only one sample appeared to contain viable virions. All ICC-qPCR assays were run with positive controls, as indicated above, lending confidence to the assay integrity. Consequently, questions of inhibition and toxicity were addressed to consider the possibility of false negatives. Environmental samples may contain high levels of such compounds (Greening et al., 2002). Biosolids concentrates likely contain many inhibitors and toxics that could interfere with direct PCR and cell culture, including polysaccharides, humic acids, bile salts, collagen, heavy metals and others (Reynolds et al., 1996; Abbaszadegan et al., 2006; Rock et al., 2010). However, the combined technique of cell culture and qPCR has been shown to reduce effects of inhibitors and toxins (Reynolds et al., 1996). Dilution of the viral concentrate, and thereby dilution of any inhibitor or toxicant, occurred by virtue of adding it to the much larger volume of cell culture medium. However, Greening et al. (2002) did demonstrate that while overt toxicity usually results in cell death, reduced toxicity due to the dilution of the concentrate with the medium can still result in the inability of viable viruses to attach to the host cell in culture, leading to false negatives. Therefore, to address this possibility we conducted ICC-qPCR with serial dilutions of all the concentrates (10^{-1},10^{-2}, 10^{-3}) to address potential toxicity and inhibition.

An obvious draw back to this approach is the possibility that viruses present in the sample could be diluted below the assay detection limit or reduced so low that detectable proliferation would not occur over the incubation period. After inoculation with serially diluted concentrates, four of the original seven samples that did not contain detectable HAdV DNA at T_0, still did not contain detectable HAdV DNA at any dilution at T_0 or T_4. One of the samples had detectable HAdV DNA in the 10^{-3} T_0 culture only and not T_4, and two samples had detectable levels in one or more of the dilutions in the T_4 cultures, indicating viable virus. Furthermore, three samples that did not yield detectable levels of HAdV DNA in the undiluted T_4 assay produced detectable levels in one or more of the diluted T_4 assays, again indicating viability. By diluting the concentrates prior to inoculation, the number of samples shown to contain viable HAdV increased from one to six, including sample #6, an alkaline stabilized (limed) sample. Of the three lime stabilized samples, sample #6 had the highest initial HAdV DNA concentration; one order of magnitude higher than the other two samples. However, it was negative when assayed by ICC-qPCR using undiluted concentrate. Of the remaining five positive samples, two were from dewatering facilities and three were from "unknown" treatments.

To possibly reduce or remove inhibitors or toxins, a subset of 10^{-1} diluted concentrates were also membrane filtered prior to inoculation. Filtration did not enhance detectability of HAdV DNA. Potential limitations with filtration include loss of adsorbed HAdV retained on particulates and ineffective removal of soluble inhibitors that were able to pass through the filter.

One potential problem with ICC-qPCR when inoculating with viral concentrates from environmental samples, and especially biosolids, that has not been addressed in this study, is the presence of other viruses. Biosolids have been demonstrated to contain many other enteric viruses including enteroviruses. If the sample concentrates contained other viable viruses that could compete with viable adenoviruses for host cells, the latter's ability to infect host cells and propagate would be reduced, resulting in false negatives. Lee and Kim (2002) concluded that enteroviruses proliferated so much more rapidly than adenoviruses, that when in culture together, adenoviruses are infrequently detected. One way to test for this would be run a number of viral qPCR or RT-qPCR assays on the nucleic acid extracted from the cell lysate to look for other enteric viral, and specifically enteroviral, signatures.

Viral recovery from an environmental sample is critical, not only to merely assess presence/absence, but especially in achieving accurate estimations of viral concentrations. Thus, two commonly used methods for total DNA isolation and extraction from biosolids samples were tested to determine which method provided the greatest HAdV DNA recovery. The Mo Bio PowerSoil, a commercially available kit for isolation and purification of DNA was compared with the "traditional" beef flocculation/elution and concentration protocol followed by DNA extraction with the DNeasy Blood and Tissue kit (Qiagen). The Mo Bio PowerSoil kit is frequently used to extract total DNA from biosolids samples without concentrating the sample first (Abbaszadegan et al., 2006; Viau and Peccia, 2009; Iker et al., 2013). In a side-by-

side comparison of kits used to recover viral nucleic acids from environmental samples, Abbaszadegan et al, (2006) found that the Mo Bio PowerSoil kit and the Qiagen QIAamp DNA Mini Kit performed equally well in isolating human adenovirus DNA from unconcentrated biosolids samples. Iker et al. (2013) found that the Mo Bio PowerVirus Environmental DNA/RNA Extraction kit out performed other commercially available kits. While this kit is not the same as the PowerSoil kit, the basic chemistry appears the same. However, in a side-by-side comparison with a beef flocculation concentration/DNA extraction protocol, the Mo Bio kit did not perform as well. Only 15.0% of the samples extracted with the Mo Bio kit resulted in successful HAdV DNA detection, whereas, 76.0% of the same samples that were first eluted and concentrated via beef flocculation and then subjected to DNA extraction with the Qiagen DNeasy Blood and Tissue kit resulted in HAdV DNA amplification by qPCR. These results were likely due to the amount of biosolids sample that was used for each method. The Mo Bio kit employs a combination of bead beading and filtration using microcentrifuge tubes, which greatly limits the amount of sample that can be used for extraction. For our purposes, only 0.5 g of raw, unconcentrated biosolids was extracted. The beef flocculation protocol is a combination of viral elution and concentration followed by DNA extraction. Since the initial elution and concentration steps were not limited to microcentrifuge tube-methods, the amount of starting material was much larger, i.e., 30 g/sample, equivalent to a 3000% increase compared with the Mo Bio procedure. The difference in starting material could have greatly increased the detection frequency.

Comparing DNA extracted from HAdV virus-spiked concentrates using each of the kits the Qiagen DNeasy kit out performed the Mo Bio PowerSoil kit. As stated above, the Mo Bio PowerSoil kit has been frequently used to extract viral DNA from biosolids samples and numerous side-by-side tests have supported its use. However, this study is to the best of our knowledge the first side-by-side comparison of the Mo Bio PowerSoil kit with the Qiagen DNeasy Blood and Tissue kit for HAdV recovery. Furthermore, unlike other comparisons, this is the first time that a comparison has been based on starting with a biosolids concentrate, as opposed to raw biosolids.

The beef flocculation method is much more time consuming than the MO BIO method and requires a number of pH adjustments and centrifugation steps to elute and isolate the viruses. This of course makes this method less "fool proof." However, the larger volume of starting material increases the probability of isolating virus from the sample and reduces the chance of a false negative result. The final DNA extraction was accomplished using the Qiagen DNeasy Blood and Tissue kit, which employs a silica filter, like the MO BIO kit, to aid in the removal of inhibitors. Thus, the combined method provided a more reliable means of DNA isolation and better detection and quantification via qPCR.

In conclusion, the treatment process used by aWWTP affected detection of HAdV DNA and viable virus. Based on our study, there appears to be a significant difference in HAdV DNA concentration based on the treatment method used to produce the biosolids. Alkaline stabilized biosolids had significantly less detectable

HAdV DNA when compared to dewatered and thickened biosolids. However, of the methods we tested, plate/frame thickened biosolids were the only ones in which HAdV DNA was detected but viable virions were not. ICC-qPCR performed with undiluted viral concentrate indicated only one of the 25 samples contained viable adenovirus, however, serial dilution of the concentrate increased that number to six. This appeared to indicate that while ICC-qPCR may reduce the risk of inhibition or toxicity, it does not eliminate it, and serial dilutions are necessary to avoid possible false negatives. Filtration of the diluted concentrate did not improve detection. In fact, of the two filtered samples that had detectable HAdV in the T_0 assay, concentrations were lower than when assayed without filtration. This would appear to suggest that perhaps filtering removed virions that may have been adsorbed to particulates. Another concern is due to the fastidious nature of enteric adenovirus, other enteric viruses may infect host cells before adenovirus. The only way to ensure that this is not happening is to assay the cell lysate for other viral pathogens.

Table 3.1. Presence-absence comparison of HAdV DNA detection with nested PCR and qPCR using the Mo Bio PowerSoil Kit and viral concentration via beef flocculation coupled with DNA extraction via the Qiagen DNeasy kit.

Sample	Mo Bio PowerSoil qPCR	Concentration/ Qiagen DNeasy qPCR	Mo Bio PowerSoil nested PCR	Concentration/ Qiagen DNeasy nested PCR
Biosolid #1	0/4	4/4	0/4	0/4
Biosolid #2	4/4	4/4	2/2	0/4
Biosolid #3	0/4	2/4	0/2	0/4
Biosolid #4	0/4	0/4	0/2	2/4
Biosolid #5	0/4	4/4	0/2	0/4
Biosolid #6	0/4	3/4	0/2	0/4
Biosolid #7	0/4	4/4	2/2	0/4
Biosolid #8	1/2	4/4	2/2	0/4
Biosolid #9	2/2	4/4	2/2	0/4
Biosolid #10	0/4	2/4	2/2	0/4
Biosolid #11	0/4	0/4	0/4	0/4
Biosolid #12	2/4	4/4	4/4	0/4
Biosolid #13	0/4	4/4	4/4	2/4
Biosolid #14	4/4	4/4	4/4	0/4
Biosolid #15	0/4	0/4	0/4	0/4
Biosolid #16	2/4	4/4	2/4	2/4
Biosolid #17	0/4	4/4	0/4	1/4
Biosolid #18	0/4	0/4	0/4	1/4
Biosolid #19	0/4	0/4	1/4	0/4
Biosolid #20	0/4	4/4	0/4	1/4
Biosolid #21	0/4	4/4	0/4	0/4
Biosolid #22	0/4	4/4	0/4	4/4 (weak)
Biosolid #23	0/4	4/4	0/4	4/4(weak)
Biosolid #24	0/4	4/4	0/4	4/4 (weak)
Biosolid #25	0/4	4/4	0/4	4/4 (weak)
Biosolid #26	0/4	4/4	0/4	4/4(weak)
percent positives	15.0%	76.0%	25.6%	28.0%
P value (α>0.05)		**0.001**		0.6829

Table 3.2. qPCR analysis of HAdV DNA concentration obtained by two different DNA extraction kits performed on beef flocculated biosolids concentrate.

	replicate 1 (ge/µl)	replicate 2 (ge/µl)	replicate 3 (ge/µl)	Average (ge/µl)	Standard deviation
Qiagen DNeasy Blood and Tissue	1.54×10^{4}	1.46×10^{4}	1.93×10^{4}	$1.65\text{x}10^{4}$	2.05×10^{3}
Mo Bio Power Soil kit	9.7×10^{2}	1.0×10^{3}	1.75×10^{3}	1.25×10^{3}	3.60×10^{2}
p = 0.0062					

Table 3.3. Concentration of (ge/g) of HAdV DNA detected by qPCR for each of the samples received from the Margolin lab.

Location	Stabilization method	HAdV DNA concentration (ge/g)	Standard deviation
Tennessee	centrifuge	0.00	
Tennessee	centrifuge	0.00	0.00
Texas	unknown	1700	
Pennslyvania	unknown	65.6	
Texas	unknown	4160	
Arkansas	unknown	0.00	
Arkansas	unknown	0.00	1623.4
Oklahoma	lime	30.3	
Oklahoma	lime	11.7	
Iowa	lime	213	90.8
Texas	belt process	7910	
Texas	belt process	2410	
Texas	belt process	2180	
Texas	belt process	2430	
Texas	belt process	1400	
Texas	belt process	6210	
Texas	belt process	155	
Texas	belt process	2860	2401.2
Texas	plate/frame	8550	
Texas	plate/frame	6450	
Texas	plate/frame	420	
Texas	plate/frame	1300	
Texas	plate/frame	1330	
Texas	plate/frame	4180	
Texas	plate/frame	11800	
Texas	plate/frame	22800	6992.7

Table 3.4. HAdV DNA concentration (ge/g) determined by ICC-qPCR using Caco-2 cells inoculated with undiluted biosolids concentrates. Bold values represent detected viability

Stabilization method	T0	T4
centrifuge	0	0
centrifuge	0	0
unknown	0	0
unknown	0	0
unknown	2800	0
unknown	10800	395
unknown	**2750**	**4290**
lime	0	0
lime	0	0
lime	0	0
belt process	10900	0
belt process	1320	0
belt process	3630	0
belt process	3420	388
belt process	3870	0
belt process	11900	0
belt process	1080	0
plate/frame	1170	0
plate/frame	65900	0
plate/frame	9250	0
plate/frame	6110	0
plate/frame	36700	0
plate/frame	61500	0
plate/frame	3060	0
plate/frame	2050	0

Table 3.5. ICC-qPCR using serial dilutions of the biosolids concentrate. Concentration values measured as genomic equivalents/g (ge/g)

		T0			T4		
Sample	stabilization method	10^{-1} dilution	10^{-2} dilution	10^{-3} dilution	10^{-1} dilution	10^{-2} dilution	10^{-3} dilution
1	plate/frame	1930	-	-	-	-	-
2	belt process	1050	307	280	-	-	-
3	lime	-	-	-	-	-	-
4	lime	-	-	360	-	-	-
5	plate/frame	299	411	-	-	-	-
6	**lime**	**-**	**-**	**-**	**-**	**707**	**358**
7	belt process	-	-	587	1300	456	1110
8	belt process	1120	-	-	614	612	-
9	unknown	-	779	-	327	-	569
10	unknown	10000	-	-	457	601	-
11	centrifuge	-	-	-	-	-	-
12	plate/frame	-	-	-	-	-	-
13	belt process	-	-	-	-	-	-
14	unknown	-	-	-	-	-	-
15	centrifuge	-	-	-	-	-	-
16	plate/frame	473	-	-	-	-	-
17	belt process	-	-	-	-	-	-
18	unknown	-	-	-	-	-	-
19	**unknown**	**-**	**-**	**-**	**363**	**-**	**-**
20	belt process	786	-	-	-	-	-
21	plate/frame	-	-	5900	-	-	-
22	belt process	-	-	576	-	-	-
23	plate/frame	923	-	-	-	-	-
24	plate/frame	3530	848	-	-	-	-
25	plate/frame	3790	675	-	-	-	-

Table 3.6. HAdV DNA concentration obtained by ICC-qPCR using 10^{-1} diluted and membrane-filtered biosolids concentrate.

Sample	stabilization method	HAdV DNA concentration T_0	HAdV DNA concentration T_4
7	belt process	497	0.00
8	belt process	0.00	0.00
9	unknown	0.00	0.00
10	unknown	32.5	0.00
19	unknown	0.00	0.00

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Chapter 4: In situ and in vitro analysis of the persistence of enteric human adenovirus in estuarine water under various environmental conditions.

Abstract

The persistence of human enteric viruses in environmental waters is a global health concern. Waterborne or water related diseases comprise over 88% of the 1.8 million annual diarrheal related fatalities worldwide. Human adenovirus (HAdV) is of special concern since, due to its structure, it has been shown to be far more stable than other enteric viruses. To assess the stability of HAdV in estuarine water under various environmental conditions, both *in situ* and *in vitro* exposure studies were conducted to assess the persistence and infectivity of HAdV in response to seasonal temperature, light, salinity and indigenous microbiota over time. Results indicated that the *in situ* degradation rate for HAdV was significantly higher during the summer in microcosms with the indigenous microbiota compared with the other exposure seasons ($p = 0.000$, rANOVA). Integrated cell culture – quantitative PCR analysis (ICC-qPCR) confirmed four out of 24 (17%) of the *in situ* treatment samples contained viable viruses after 28 days. Under *in vitro* conditions, ANCOVA analysis indicated that salinity did not have a significant effect on the persistence of HAdV ($p = 0.255$), while, temperature did ($p = 0.000$).

Introduction

Despite the evidence that adenovirus may be a useful indicator of viral contamination in wastewater effluents and shellfish harvested from wastewater effluent contaminated waters, very little research has been conducted in the United States to characterize adenovirus persistence and retention of infectivity in aquatic environments (Jiang, 2006; Tong and Lu, 2011). Over the last twenty years, a great deal of effort has gone into developing molecular assays for the detection of adenovirus in environmental samples. This has lead to a greater ability to rapidly identify and quantify human adenovirus contamination in coastal, ground and recreational waters (Chapron et al., 2000; Jiang et al., 2001; Greening et al., 2002; Lee et al., 2005, Haramoto et al., 2005; Tong et al., 2011). Surprisingly little data on the infectivity of HAdV in environmental waters have been reported (Enriquez et al., 1995; Rodriguez et al., 2008). Genome detection via various PCR methods confirms HAdV contamination of environmental waters, however, it doesn't address infectivity of the virus, and as such, has limited value as a tool for assessing public health risks (Jiang, 2006). To add to the confusion, the term persistence is often used when referring to detection of HAdV DNA in an environmental sample, as in this study, but may be misinterpreted as viability (Fong and Lipp, 2005). In recent years, studies conducted to correlate HAdV DNA occurrence (persistence) with infectivity have had mixed results (Enriquez et al., 1995, Charles et al., 2009; Ogorzaly et al., 2010). Many factors may contribute to this ambiguity. HAdV is a double stranded DNA virus, so its nucleic acid (or fragments thereof) may remain detectable in the environment after the virus is no longer viable. HAdV can be cultured as a means to

assess viability. However, if researchers use different cell lines as the host and various HAdV strains, different results may be observed (Choo and Kim, 2006; Jiang et al., 2009). Enteric adenoviruses (F40 and F41) are fastidious and if present can be easily out-competed by other pathogenic viruses that may be present in environmental samples. Therefore, if there are not enough assay cells available to establish infection, it could lead to false negatives (Choo and Kim, 2006). Additionally, a study by Ko and colleagues (2003) indicates that carry over of DNA fragments in the inoculate may lead to false positives when assessing infectivity via cell culture.

Therefore, the development and validation of molecular screening techniques to assess both DNA persistence and infectivity of adenovirus in estuarine waters under various environmental conditions can enable research designed to correlate detection and viability, thereby providing a means to assess the safety of wastewater treatment plant (WWTP) receiving waters. By determining how long adenovirus persists and remains infective in estuarine waters under various seasonal temperature and light conditions, potential health risks and possible steps to decrease health concerns could be better addressed.

In this study, the persistence of human adenovirus F41 (HAdV F41) in environmental waters under various conditions was assessed using a quantitative polymerase chain reaction (qPCR) assay. *In situ* exposure experiments were deployed during the months of January, July and November and addressed the role of temperature, light exposure and indigenous microbiota in the persistence of HAdV F41 over the course

of 28 days. In addition, integrated cell culture-quantitative polymerase chain reaction (ICC-qPCR) was used to assess the viability of the HAdV after 28 days following exposure treatments. *In vitro* studies, in the laboratory exclusively, addressed the role of temperature and salinity on DNA persistence and viability under controlled exposure conditions over the course of 55 days. These experiments provided information on the inherent responses and fate of enteric HAdV introduced into estuarine receiving waters through the discharge of contaminated wastewater effluent.

Materials and Methods:

***In situ* Experiments**

Two exposure arrays were constructed using 16 mm mesh rigid TENAX plastic mesh (TENAX Corporation, Baltimore, MD) to enable the deployment of twelve experimental microcosms in each array. One array housed microcosms containing unfiltered river water (URW) spiked with HAdV and the other array housed microcosms containing sterile river water (SRW) spiked with HAdV. The arrays were designed so as to allow for the assessment of persistence and viability under various environmental conditions. Both the URW and SRW arrays were deployed at the same time, approximately 4 feet apart. They were hung from a movable line that ran through a pulley between a piling and the pier (latitude: 37.259484°, longitude:-76.467604°) and allowed the arrays to be moved out into open water so as to not be shaded by the pier or piling at any point during the day (Figure 4.1).

Unfiltered river water (URW) array preparation: Approximately 2 liters of York River water containing the ambient microbiota and other consituents was collected prior to each exposure experiment by wading 10 feet into the river directly adjacent to the research pier at the Virginia Institute of Marine Science, Gloucester Point, VA (latitude: 37.247586°, longitude:-76.499618°). Four hundred milliliters was measured by graduated cylinder and placed in a sterile flask. Forty microliters of human adenovirus F41, strain Tak (ATCC Manassas, Virginia) was added to the unfiltered river water and mixed well. The microcosms were made using twelve sterile, transparent Permalife (OriGen) culture bags with sterile ports that were filled with 30 ml of the spiked river water/virus mixture. These bags are transparent to both visible and UV light. Six bags were wrapped in heavy aluminum foil as dark controls, the remaining six bags remained unwrapped and exposed to light. Microcosms were deployed horizontally in plastic mesh racks in floating arrays (Figure 4.1) as follow: 3 light and 3 dark suspended just below the surface of the York River and 3 light and 3 dark suspended one meter below the surface.

Sterile river water (SRW) array preparation: Sterile river water was prepared by measuring four hundred milliliters of York River water into a graduate cylinder and successively filtering it through 0.45 μm and 0.22 μm membrane (Whatman) filters into a sterile collection flask. The flask was covered and autoclaved for 15 minutes on liquid cycle setting and allowed to cool completely. The SRW microcosms were constructed as outlined above for the URW arrays.

Sample collection: Bags were retrieved at days 0, 3, 7, 10, 14, 17, 20, 24, and 28 of exposure and 1 ml samples were aseptically removed from each bag, placed in 1.5 ml sterile microcentrifuge tubes, labeled, and stored at 4°C. Samples were processed within 24 hours. Any unused sample volume was placed in -20°C. Deployed "light" bags were gently scrubbed and rinsed in river water after each sampling event to remove any fouling. Care was taken during sampling and deployment to ensure the integrity of the foil covers on the dark bags and the foil was replaced as necessary. Continuous water temperature data was collected using two HOBO® (Onset Computer Corp.) pendant waterproof temperature loggers. One logger was attached to the surface rack of the array and one logger was attached to the depth rack (one meter below the surface). Seasonal light penetration (as extinction, k_d) was measured as photosynthetically active radiation (PAR) and recorded each sampling day using a LiCor® underwater PAR sensor and the Quantum deck PAR sensor. Readings were taken at 10 cm increments from the surface to a meter depth. Surface and bottom readings were also recorded. These data were then used to calculate the relative light attenuation coefficient (k_d) for each collection day.

In vitro Experiment

In order to assess the effect of temperature and salinity on HAdV persistence and viability in the absence of light and the ambient microbiota, four salinities of sterile river water were spiked with HAdV and incubated at three different controlled temperatures for 55 days in the dark.

*Sample preparation***.** Five liters of high salinity (35 psu) Chesapeake Bay water were collected from Wachapreague VA. Using reagent grade sterile water, three one liter volumes of this water were adjusted to the following salinities: 30 psu, 20 psu, and 10 psu. Each of the samples was put through a series of filters: Whatman paper (No. 41) and 0.45 μm and 0.02 μm Whatman membrane filters. In addition, one liter of sterile reagent grade water (0 psu) was similarly filtered. Two hundred microliters of human adenovirus F41, strain Tak from ATCC (Manassas, Virginia) was added to each of four 200 ml water samples at salinities of 0 psu, 10 psu, 20 psu and 30 psu, and mixed well. To create the microcosms, twelve sterile UV transparent Permalife (OriGen) culture bags with sterile ports were filled with 30 ml of virus spiked water as follows: three with 30 psu, three with 20 psu, three with 10 psu, three with 0 psu. The bags were divided into 3 complete sets containing a sample at each of the four salinities. Each set was placed in a Nalgene® tub and covered with foil to prevent light from reaching the bags. One set was incubated at 10°C, one set was incubated at 20C° and the final set was incubated at 30°C.

*Sample collection***.** On days 0, 3, 6, 13, 26, and 55 three separate one ml samples were aseptically collected from each of the four bags at each of the three temperatures (n=12). All samples were shielded from direct light during the collection process, placed in sterile 1.5 ml microcentifuge tubes and stored at 4°C until processing (within <24 hours).

Methods common to both *in situ* and *in vitro* studies

DNA extraction. DNA from 200 µl of each water sample was extracted using the Qiagen DNeasy Blood and Tissue kit (Valencia, CA) per manufacturer's instruction. DNA was eluted in 60 µl elution buffer and stored at 4°C. Negative controls for DNA extractions were performed using sterile deionized water.

Nested polymerase chain reaction (PCR). Nested PCR was conducted on all *in situ* day 28 samples to assess if HAdV DNA was still detectable. This assay was performed, in duplicate, as described in Chapter 1.

Quantitative polymerase chain reaction (qPCR). qPCR was conducted on all *in situ* and *in vitro* samples at each sampling day to measure the quantity of detectable HAdV DNA present, as described in Chapter 1.

ICC-qPCR of in situ day 28 samples. The infectivity of HAdV under each *in situ* treatment condition was determined for all day 28 samples using an integrated cell culture-quantitative polymerase chain reaction (ICC-qPCR) technique. Water samples for each like treatment (Table 4.2) were pooled and 100 µl was withdrawn for inoculation. ICC-qPCR was conducted following a combination of protocols from Choo and Kim (2006) and Gallagher and Margolin (2007). Caco-2 cells were grown to 75-90% confluence in t25 tissue culture flasks using Minimum Essential Medium Eagle (MEM) with 10% fetal bovine serum (FBS). Once the cells reached the appropriate confluence, the medium was removed and the cells were washed with

fresh medium. Each treatment sample was cultured in quadruplicate by adding 100 µl of water sample to a flask containing washed Caco-2 cells. After inoculation, the cells were rocked every 15 minutes for 60 minutes. Following this, 5 ml of MEM was added to each culture flask. Two of the four flask cultures for each sample were frozen (-80°C) immediately (T_0), the remaining two were incubated at 37°C for 4 days (T_4). Following the 4 day incubation period the culture flasks were stored at -80°C until DNA extraction. Prior to DNA extraction, each culture flask was subjected to three freeze/thaw cycles. Two hundred µl of the supernatant containing thawed cells was used to extract total genomic DNA using the QIAGEN Blood and Tissue Kit (Valencia, CA). Five µl of DNA was used for qPCR as outlined previously. The (T_0) and (T_4) duplicates for each treatment sample were compared to determine if HAdV DNA increased over the 4 day incubation period. An increase in HAdV DNA was interpreted as meaning the adenoviruses contained in a given sample had proliferated and therefore remained infective. Negative controls were run for each sample by inoculating negative control flasks with 50 µl of MEM medium. Positive controls were run for each sample by inoculating control flasks with 50 µl (3.25×10^7 genomic equivalents) HAdV F41, strain Tak from ATCC (Manassas, Virginia).

Quantification calculations. To determine the genomic equivalents per sample, the volume of water in the microcosm (bag), the volume of water withdrawn when sampling, the volume of water used for DNA extraction, the final volume of DNA eluate, and the volume of DNA added to the reaction are all taken into consideration.

When calculating the genomic equivalents present in ICC-qPCR samples, the addition of the medium is also accounted for. Below are examples for each:

qPCR: 30 ml (initial volume, decreased by 1ml after each sampling event) ⇒ 1 ml water removed per sampling event ⇒ 200 µl concentrate sampled for DNAextraction ⇒ elution of the DNA into 60 µl ⇒ 3 µl of eluate added to each qPCR reaction. From this the following equation would be used: genomic equivalents as reported by qPCR assay based on standard curve × 30 (decreases by 1 after each sampling) × 5 × 20 = genomic equivalents/microcosm.

ICC-qPCR: 30 ml (initial volume, decreased by 1ml after each sampling event) ⇒ 1 ml water removed per sampling event ⇒ 100 µl inoculum ⇒ 5 mls medium added ⇒ 200 µl lysate sampled for DNA extraction ⇒ elution of the DNA into 60 µl ⇒ 3 µl of eluate added to each qPCR reaction. From this the following equation would be used: genomic equivalents as reported by qPCR assay based on standard curve × 30 (decreases by 1 after each sampling) × 10 × 25 ×20 = genomic equivalents /microcosm. It is important to note that calculations must be adjusted per sample. Specifically, the volume in each microcosm decreases by 1 ml after each sampling event.

Data analysis. Repeated measures analysis of variance (rANOVA) and analysis of covariance (ANCOVA) were used to evaluate persistence of HAdV DNA both between and within sampling seasons under the various treatment conditions, with $P \leq 0.05$ being considered significant. For both the *in vitro* and *in situ* persistence experiments, degradation rates were calculated using both linear and non-linear

regression models. Log_{10} transformed data of the viral concentration (as ge/l) for each treatment condition and time were used to generate these regression curves with 95% confidence intervals calculated for the upper and lower limits. Degradation rates for different treatments were considered to be not significant if the confidence intervals overlapped. All analyses were run using Systat Software (Chicago, IL)

Results

In situ experiments

The average attenuation coefficients (k_d) and the high, low and average water temperatures for each exposure season are listed in Table 4.1. The persistence of HAdV measured as HAdV DNA via qPCR varied by season. A significant difference was detected in the persistence of HAdV comparing January, July and November by repeated measures analysis of variance (rANOVA) ($p= 0.000$), with July having the lowest rate of persistence. Also, persistence was significantly different as a function of the type of water (SRW or URW) spiked with HAdV ($p=0.000$), although whether the virus persisted in SRW or URW depended on season (Figure 4.2 - 4.4). While water temperatures were reported for each season, the effect of the water temperature alone could not be separated from the other variables. The effect of temperature on the persistence of HAdV is addressed in the controlled *in vitro* experiments.

Over the course of the November exposure period (11/11/2011 – 12/06/2011), HAdV DNA was detected in each of the eight treatment conditions through day 21. By day 24, it was undetectable in two of the three SRW replicates exposed to light at the

surface and by day 28, all SRW replicates were below the detection limit. Also, by day 28, HAdV DNA was not detected in one of the three SRW replicates exposed to light at 1 m below the surface. For all other treatment conditions during November, HAdV DNA was detectable through day 28. Figure 4.2 shows the average $\log_{10}$ transformed concentrations of HAdV over the 28-day sampling period.

During the February sampling period (2/17/2012 – 3/15/2012), HAdV DNA was detected in each of the eight treatments conditions through day 7. By day 9, HAdV DNA in one of the three SRW replicates exposed to light at the surface was undetectable: by day 21 HAdV was undetectable in all three SRW surface replicates. In addition, by day 23, HAdV was undetectable in two of the three URW replicates exposed to light at the surface. HAdV persisted in all other treatment conditions through day 28 (Figure 4.3).

In contrast, over the course of the July sampling period (7/16/2012 – 8/09/2012), HAdV DNA was detectable in all treatment conditions only through day 4. By day 7, HAdV was not detectable in any of the URW surface samples exposed to light. By day 14, HAdV was not detectable in any of the URW samples, regardless of light exposure or depth. In comparison, HAdV was detectable at day 7 in two of the three SRW surface samples exposed to light but by day 14, HAdV was undetectable in all SRW treatments regardless of depth or light exposure. (Figure 4.4).

Analysis of light exposure and depth showed no significant relationship with HAdV persistence when analyzed independently for all seasons combined ($p=0.079$ and $p=0.301$, respectively). However, when a multivariate rANOVA was applied to analyze the relationship of multiple variables together (season, water, light exposure, and depth) on HAdV persistence, a significant relationship ($p=0.017$) was identified.

The degradation of HAdV, the decrease in viral DNA concentration over exposure time , was expressed as -k, where -k is the rate coefficient of HAdV degradation with units of day^{-1}. HAdV degradation data were analyzed using three approaches; linear regression, non-linear exponential regression and non-linear quadratic regression (Tables 4.3 – 4.5). Of these, the non-linear quadratic regression provided the best model for degradation. Therefore, non-linear exponential regression and linear regression were omitted from further analyses. When the degradation coefficients for each treatment condition were analyzed within each season, differences related to water treatment and season were evident. Significance was determined using 95% confidence intervals where overlapping confidence intervals were interpreted as indicating no significant difference in rate coefficients. Using this approach, no significant treatment differences were observed during the fall (November). Degradation coefficients fell between -0.210 and -0.334 and confidence intervals were all overlapping (Figure 4.5). During the winter (February) there were no significant differences in the degradation coefficients for HAdV within each water treatment (SRW and URW). However, there were significant differences in values of -k between SRW and URW exposed to light at the surface, and SRW and URW

exposed to light at 1 m, with HAdV in the SRW showing a higher value of -k (Figure 4.5). During the summer (July), there was a significant difference in -k values between the SRW and URW in the dark at 1 m, with HAdV in the latter showing a higher degradation coefficient (Figure 4.5). In addition, -k in SRW exposed to light at the surface was significantly higher compared to SRW in the dark at 1 m.

When all three seasons were compared, HAdV rate coefficients in URW during July were significantly higher in three out of the four treatments (light/surface, light/deep, and dark/surface). The HAdV degradation rate coefficient in the URW dark/surface treatment in November, while significantly lower than in July, was significantly higher than in February. The degradation rate coefficient in the SRW light/surface, while not significantly different between February and July, was significantly lower in November (Figure 4.5). These observations explain the rANOVA results that indicated HAdV degradation was significantly different as a function of season (month) and water treatment (URW vs. SRW).

Viability of HAdV for each treatment was assessed at day 28 using ICC-qPCR. For the month of November, seven of the eight treatment conditions were negative for HAdV DNA at both T_0 and T_4, indicating a lack of infectivity of viral particles contained in these bags. However, one treatment condition, SRW exposed to light at 1 m had detectable HAdV DNA at T_4, and none at T_0, supporting the presence of viable virions. In addition, the quantity of virions per microcosm was nearly 1.5 orders of magnitude greater at T_4 of ICC-qPCR when compared to the standard qPCR

result on day 28 (Table 4.6). During the month of February, two of the eight treatment conditions evidenced detectable HAdV DNA in both T_0 and T_4 ICC-qPCR assays. However, in both cases, the concentrations were lower in T_4 than in T_0, suggesting that the viruses did not proliferate and were not viable (Table 4.6). In July, all but two treatments, SRW exposed to light at 1 m and URW exposed to light at the surface, had detectable HAdV DNA in T_0, T_4, or both. URW exposed to light at depth, and both surface SRW treatments (light and dark) had detectable HAdV DNA at T_0, but not at T_4, again indicating no proliferation. Three treatments had detectable HAdV DNA at T_4, but not at T_0; URW exposed to light at the surface, URW dark at depth, and SRW dark at depth. The SRW dark at 1 m was the only treatment during this month to show an increase in the quantity of genomic equivalent/bag (ge/bag) in the T_4 ICC-qPCR compared to the standard day 28 qPCR (Table 4.6).

In vitro experiments comparing the persistence of HAdV as a combined function of temperature and salinity over a 55 day time period were conducted under dark conditions to remove the effect of light. Figures 4.6 and 4.7 show the $\log_{10}$ transformed concentrations of HAdV as a function of temperature (Figure 4.6) and salinity (Figure 4.7) over time. Ninety five percent confidence intervals showed significant variation in concentrations at each time point under each condition. An initial analysis of covariance (ANCOVA) comparing degradation rate coefficients for each of the salinity treatments at each temperature suggested that salinity, temperature and time were significantly related to HAdV persistence ($p = 0.00$, $p = 0.026$, $p =$

0.00, respectively). However, the sterile reagent grade water (0 psu) treatment was significantly different from the other treatments and once it was removed from the analysis, salinity was no longer a significant variable ($p = 0.255$), whereas, temperature and time remained significantly related to the persistence of HAdV ($p = 0.004$ and $p = 0.000$, respectively). Degradation coefficients for each salinity treatment as a function of temperature are shown in Figure 4.8. The 95% confidence intervals overlap for all salinity treatments except 0 psu, suggesting that temperature did not have a significant effect on degradation. However, when linear regression lines were used to evaluate if temperature was predictive of degradation, it was predictive in the 10 psu and 20 psu treatment, but not in the 30 psu treatment (Figure 4.9). The degradation coefficient in the 30 psu treatment was lower at 30°C, with an R^2 value of 0.172. Additionally, -k values for each temperature as a function of salinity are shown in Figure 4.10. Based on the 95% confidence intervals, only the -k for 10 psu at 10 °C was significantly different. Linear regression analysis revealed that salinity was predictive of the rate coefficient at 20°C only when the 0 psu treatment is removed. The values of k actually were smaller at 30 psu under 10°C and 30°C conditions, although not significantly (Figure 4.11). However, when the 0 psu treatment is included in the analysis, salinity was slightly more predictive of degradation, as reported by the ANCOVA (Figure 4.12)

Discussion

Water borne transmission of human pathogenic enteric viruses, such as human adenovirus, is a significant concern worldwide. Human adenovirus has been

identified as an emerging waterborne pathogen with potentially fatal outcomes for immunocompromised individuals (Jiang, 2006). While a number of studies have addressed the presence of human adenovirus in various aquatic environments, including recreational, surface and coastal waters, few have addressed its persistence and factors that affect its autecology. When assessing public health concerns, being able to differentiate between the detection of non-viable "naked" viral DNA and infectious virions is critical. A few studies have addressed this question by examining the correlation between genome detection via PCR and detection of infectious virus via cell culture or RT-PCR, with conflicting results (Enriquez et al., 1995, Charles et al., 2009; Ogorzaly et al., 2010). Ogorzaly et al. (2010) and Enriquez et al. (1995) both reported that there was little variation in HAdV-2 and HAdV-41 genome detection and viability and that virus persisted in an infectious state for greater than 300 days in ground water and tap water. Furthermore, Enriquez et al. (1995) demonstrated that HAdV-41 detection and infectivity persisted for 85 days in seawater. However, Charles et al. (2009) reported that HAdV-2, while detectable via qPCR, had significant reduction in infectivity by 21 days in spiked ground water. The apparent differences may be due to a number of factors including the strain of adenovirus used, the composition of the water and the exact treatment conditions. To our knowledge, the work described herein is the first study to assess the correlation of genome detection via qPCR and infectivity detection via ICC-qPCR under various controlled *in situ* and *in vitro* conditions in order to evaluate the effects of season, temperature, sunlight, salinity and the indigenous microbiota on the detection, persistence and rate of degradation of human adenovirus in estuarine water.

Persistence in unfiltered river water

Although studies have shown that HAdV can be detected in environmental samples with little seasonal variation (Symonds et al., 2009; Fong et al., 2010), we found there to be significant seasonal differences in the persistence of HAdV, based on DNA detection, under varying *in situ* conditions. The HADv degradation coefficients during the month of July were significantly higher in unfiltered river water (URW) in three out of four treatment conditions when compared to other seasons. Both of the treatment microcosms exposed to light (surface and depth), as well as the treatment shielded from light at the surface exhibited significantly higher -k values than the same treatment conditions in February and November. This was not surprising for several reasons. The average light attenuation coefficient recorded in July ($k_d = 1.874$) was higher than in November and February ($k_d = 1.25$ and $k_d = 1.02$, respectively), as the water was more turbid during July. This turbidity is due, in part, to the presence of indigenous microbiota and natural organic matter (NOM). Since the treatment bags were filled with unfiltered river water collected during the same time period, they also contained the indigenous microbiota, including bacteria, algae and protists, as well as NOM. Though the mechanisms are not fully understood, a number of studies have shown that many marine bacteria have antiviral properties. Among these bacteria are *Vibrio* sp, *Aeromonas* sp., *Pseudomonas* sp. and *Moraxella* (Kamei et al., 1998; Girones et al., 1989; Balcazar et al., 2006). Furthermore, in a study by Deng and Cliver (1995), extracellular proteases were implicated in antiviral activity. In addition, it has been reported that protists graze on prokaryotes (Pernthaler, 2005;

Jürgens and Massana, 2008; Montagnes et al., 2008) and viruses (González and Suttle, 1993). Together, the virucidal activity of the bacterial community and possibly grazing by protists, could affect HAdV numbers and persistence over time, independently of direct insolation effects.

The role of NOM in aquatic viral degradation is complex. Organic matter (particulate and dissolved) can be protective for viruses by attenuating light, absorbing shorter wavelengths, or providing a surface for adsorption (LaBelle and Gerba, 1979; Kelble et al., 2005). However, it can also have a negative effect by acting as a photosensitizer and can contribute to the production of reactive oxygen species (ROS) (Silverman et al., 2013). In a study addressing photodegradation of human virus in coastal waters, Silverman et al. (2013) demonstrated that human adenovirus are susceptible to exogenous UV degradation although due to its double stranded DNA structure, HAdV is particularly resistant to UV damage (Nwachuku et al., 2005). It is also capable of persisting with DNA damage and upon infection can use a host cell's molecular machinery to repair pyrimidine dimers that have formed in its DNA as a result of UV exposure. However, ROS produced from NOM through photodegradation or microbial processes can damage HAdV's capsid protein leaving the virus unable to repair itself (Silverman et al., 2013).

The effects of naturally occurring microbiota, both virucidal and as a source for the production of ROS, may account for the higher rates of degradation in most URW treatments. The only July URW treatment that did not have a significantly higher rate

of degradation when compared to the other seasons was the dark 1 m treatment. However, the degradation coefficient in this treatment, was in fact, not significantly different from those of the other three July URW treatments. The only reason that it was not considered significant compared to the other seasons is due to an exceedingly large confidence interval in the November treatment. Furthermore, looking at the degradation coefficients for the URW in February and November, there was no significant seasonal variation, except for one case. The rate for the February URW treatment exposed to light at 1 m depth was significantly lower compared with that in November. Comparing the February URW exposed to light at 1 m to the February SRW treatment under the same condition, the SRW treatment exhibited a significantly higher value of -k. This observation also held true comparing the February URW exposed to light at the surface and the SRW under the same condition. Perhaps the URW in February, while not harboring the seasonally-active microbiota typical of spring and summer, could have contained natural organic matter providing a protective effect, as opposed to a deleterious effect, as hypothesized happening in July. HAdV virions could have adsorbed to the organic matter, thereby being protected from light degradation (Zuang and Jin, 2003).

Persistence in sterile river water

In contrast, when the July SRW treatments were compared to SRW treatments in November and February, three of the four treatments (surface dark, light, and dark at 1 m) showed no seasonal variation. However, the SRW treatments exposed to surface light, showed seasonal variation in values of -k, with July and February

values significantly higher than in November. It would not be unexpected perhaps to expect more photodegradation in July compared to February and November if the intensity of light at the surface was significantly stronger. An ANOVA of surface PAR (measured in μmol s^{-1} m^{-2}) for the study dates of all three seasons, reveals that there were no significant differences ($p = 0.749$). However, this data is limited. PAR readings were taken during each sampling day and there was not a continuous record of PAR over the course of the study period. Thus, factors such as cloud cover during the sampling, moisture content of the air, and time of day could have influenced the PAR measurements. While there was seasonal variation with respect to URW treatments, the surface, light exposed treatment was the only instance of significant difference in SRW degradation rates between the three seasons.

Viability based on ICC-qPCR

To evaluate whether HAdV virions were still viable in the *in situ* exposure studies, ICC-qPCR was conducted on day 28 samples only. Since the arrays for each study contained three replicates for each treatment (Fig 4.1), all like experimental replicates were pooled prior to inoculating the tissue culture cells. Interestingly, over the course of the three seasons, only four samples had higher concentrations of HAdV DNA following tissue culture in the T_4 compared to the T_0, indicating the presence of viable HAdV (Table 4.6). Three of these samples were shielded from the light, and the fourth, while exposed to light, was at 1 m depth during July when the light attenuation was the greatest, suggesting that light insolation was an important factor in the inactivation of viable viruses. In five samples, HAdV was detected in T_0 and

T_4, but the concentration of HAdV DNA was greater in the T_0 assay. This would suggest that while the HAdV DNA was detected, the virus was not viable and that the DNA was degrading over the four-day incubation period, resulting in lower concentrations.

This is surprising, as previous researchers have reported viable HAdV to be quite stable in some environments (Enriquez et al., 1995; Ogorzaly et al., 2010). It was anticipated that the viruses would have retained infectivity over the course of 28 days in more than four microcosms. However, due to monetary constraints, water from like-treatment microcosms was pooled and only the end point (day 28) was analyzed by ICC-qPCR. Additionally, the viruses were not concentrated in the pooled water samples prior to inoculation, resulting in a limited pilot study. It is plausible that the inoculum was too dilute in most samples to generate any detectable results in the four-day incubation. This supposition is supported by the results for July's SRW surface light sample. In this case, HAdV DNA was detected by qPCR at day 28 but was not detected at T_0. The day 28 qPCR and the T_0 ICC-qPCR assayed an aliquot from the same water sample. However, the ICC-qPCR sample was mixed with 5 ml medium prior to DNA extraction. In order to determine if the viruses were truly non-viable at day 28 or if the assay, as conducted with unconcentrated sample, lacks the sensitivity to provide accurate assessment, additional ICC-qPCR assays will need to be conducted on archived water samples for earlier sampling days. This could reveal the time points when infectivity, or detection, was lost.

Correlation of persistence and viability

Based on a comparison of the persistence values from the qPCR assays and ICC-qPCR values from the day 28 samples of the in situ experiments, simple detection of HAdV DNA was not always a valid indicator of infectivity. In July only 7/24 microcosms (29%) had detectable DNA at day 28. However, ICC-qPCR revealed that 3/8 (38%) of pooled microcosms had viable virions, with only one microcosm sample (SRW, dark at 1 m depth) having both detectable DNA and viable virions at day 28. In both November and February, 10/12 (83%) of the microcosms had persistent HAdV DNA. Whereas 1/8 (12.5%) and 0/8 (0%) of the ICC-qPCR assays for November and February, respectively, were positive for infective virus. This supports the notion of persistence beyond viability due to the double stranded DNA nature of adenovirus.

In vitro persistence

The HAdV *in vitro* persistence studies were designed to remove the light and indigenous microbiota effects so that the role of salinity and temperature on the degradation of HAdV could be conservatively addressed, and to facilitate interpretation of the *in situ* studies. The initial ANCOVA in which the variables salinity, temperature and time, were taken into consideration demonstrated that all variables grouped together had a significant effect on HAdV degradation coefficient values in each of the treatments. However, once the 0 psu treatments were removed and the analysis was rerun, ANCOVA analysis indicated that salinity, (at least at 10 psu and above), was not, while temperature and time were, significantly related to

viral degradation. To an extent, this result was also confirmed by linear regression analysis. Once the 0 psu treatment was removed from the analysis, the only temperature at which salinity was predictive of degradation was 20°C (Figure 4.11). However, even though the R^2 was ≥ 0.8, the line is still rather flat, suggesting that the effect of salinity was minimal because the absolute differences in degradation rate coefficients, although significant, were rather small. This is supported by previous studies that found that salinity has little impact on viral persistence in environmental waters (Gantzer et al., 1998; Wetz et al., 2004). Furthermore, the regression analysis as a function of temperature showed that at both 10 and 20 psu temperature was predictive of degradation (Figure 4.9). The fact that the 0 psu treatments did not show any degradation at any temperature over the course of the study suggests that HAdV was thermally stable between 10 – 30° C and for 55 days in the absence of other treatment variables. One possibility is that there is an interaction of temperature and salinity leading to an increased rate of degradation.

In conclusion, HAdV persistence in estuarine water was significantly influenced by components in the water, which included the indigenous microbiota and ambient NOM. The *in situ* experiments indicated that during the summer season when the activity of microbiota and NOM concentrations would be considered high (Rhodes and Kator, 1988), HAdV degradation rate coefficients were significantly larger than in the fall or winter. Temperature, salinity and light, taken individually, in the absence of NOM or microbiota, did not have significant effects on HAdV degradation in either sterile or unfiltered river water. However, results from the *in vitro*

experiments suggested that the interaction of temperature and salinity did have an effect on degradation. As salinity increased, temperature became predictive of degradation. Similar results have been reported for another human enteric virus, poliovirus. Wetz and colleagues (2004) reported that survival rates for poliovirus in unfiltered seawater were much shorter than in filtered or artificial seawater, regardless of temperature, while Skraber et al (2004) demonstrated a greater seasonal effect on the persistence of poliovirus RNA and infectious poliovirus. Viable poliovirus survived better than fecal coliform bacteria when seeded into river water obtained during the winter, regardless of incubation temperature, while the opposite was true when seeded into river water obtained during the summer. Furthermore, Suttle and Chen (1992) analyzed decay rates for three marine bacteriophages in natural seawater, artificial seawater, filtered seawater and cyanide treated seawater. While they concluded that solar radiation was responsible for the majority of viral decay, removal of the phages from the water column due to adsorption to particulates (both living and nonliving) and protozoan grazing contributed to loss of viable virus. Importantly, Suttle and Chen (1992) stress that solar inactivation of the virus does not remove the virus components from the water, whereas grazing does. Therefore, the viral genome may still be detectable long after the loss of viability. The July URW samples in the current study showed rapid loss of genome signal, as well as lack of infectivity, whereas in the July SRW samples the genome signal persisted in all but one microcosm (surface light) through day 28, suggesting more was at play than just photodegradation. The data provided by this study are evidence that HAdV is stable outside of its host and genome detection may persist for extended periods of time

under various environmental conditions, even after loss of infectivity. That said, HAdV is most vulnerable to degradation and removal in the presence of the indigenous microbiota/organic matter in estuarine waters during the summer months. Further ICC-qPCR analysis should be conducted for each sampling day to determine the exact point at which loss of viability occurs. While other studies have reported genome persistence of HAdV well beyond 28 days, none have employed ICC-qPCR to address genome detection and infectivity and most have examined less fastidious, non-enteric strains of HAdV (Charles et al., 2009; Ogorzaly et al., 2010).

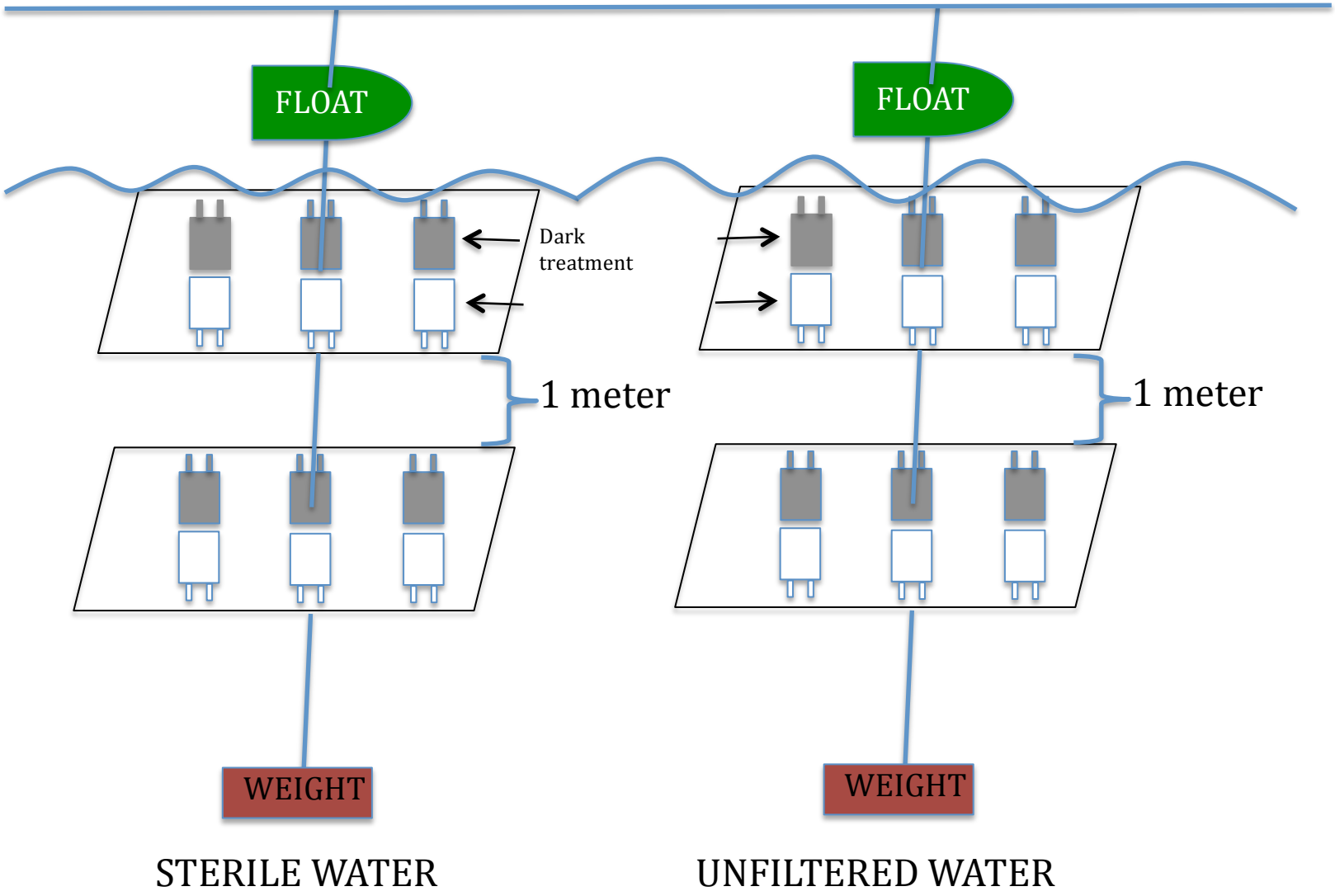

Figure 4.1. Diagram of *in situ* experimental array. Grey bags represent foil covered dark treatments. White bags represent light exposed treatments. The top rack was suspended below the surface of the York River. The bottom rack was suspended 1 meter below surface of the York River.

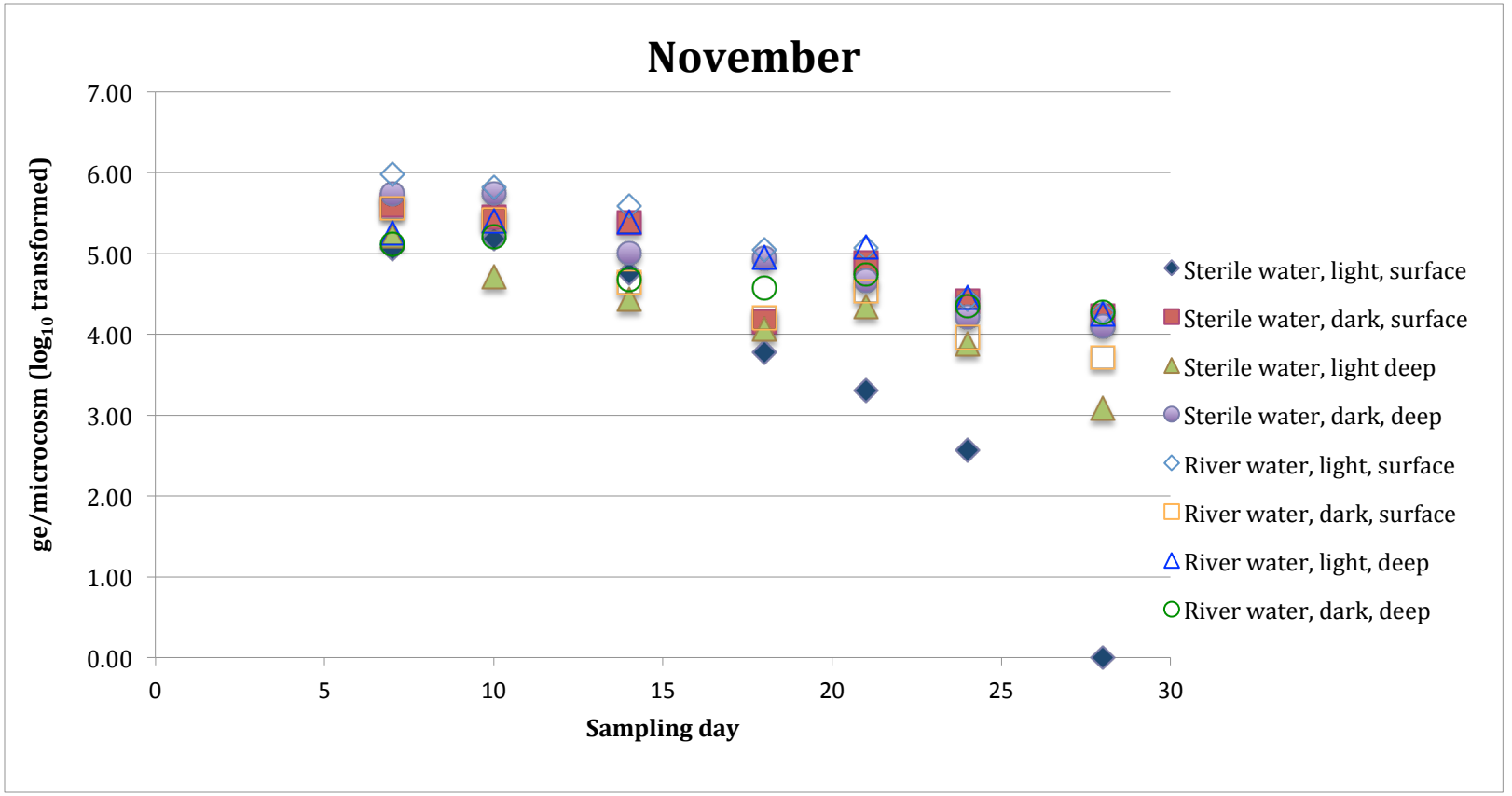

Figure 4.2. *In situ* $\log_{10}$ transformed viral concentrations (genomic equivalents per microcosm) over the course of 28 days in November.

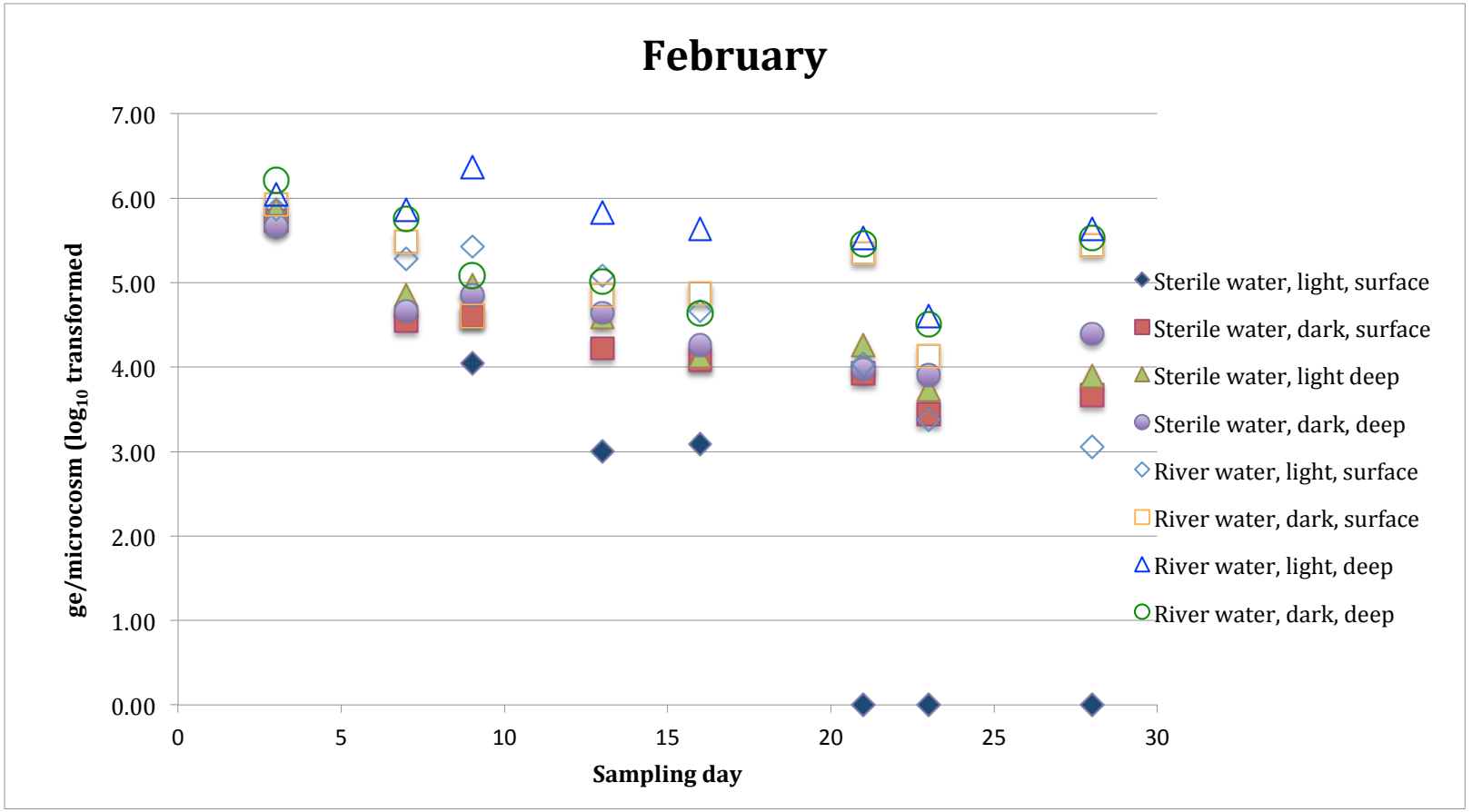

Figure 4.3. *In situ* $\log_{10}$ transformed viral concentrations (genomic equivalents per microcosm) over the course of 28 days in February.

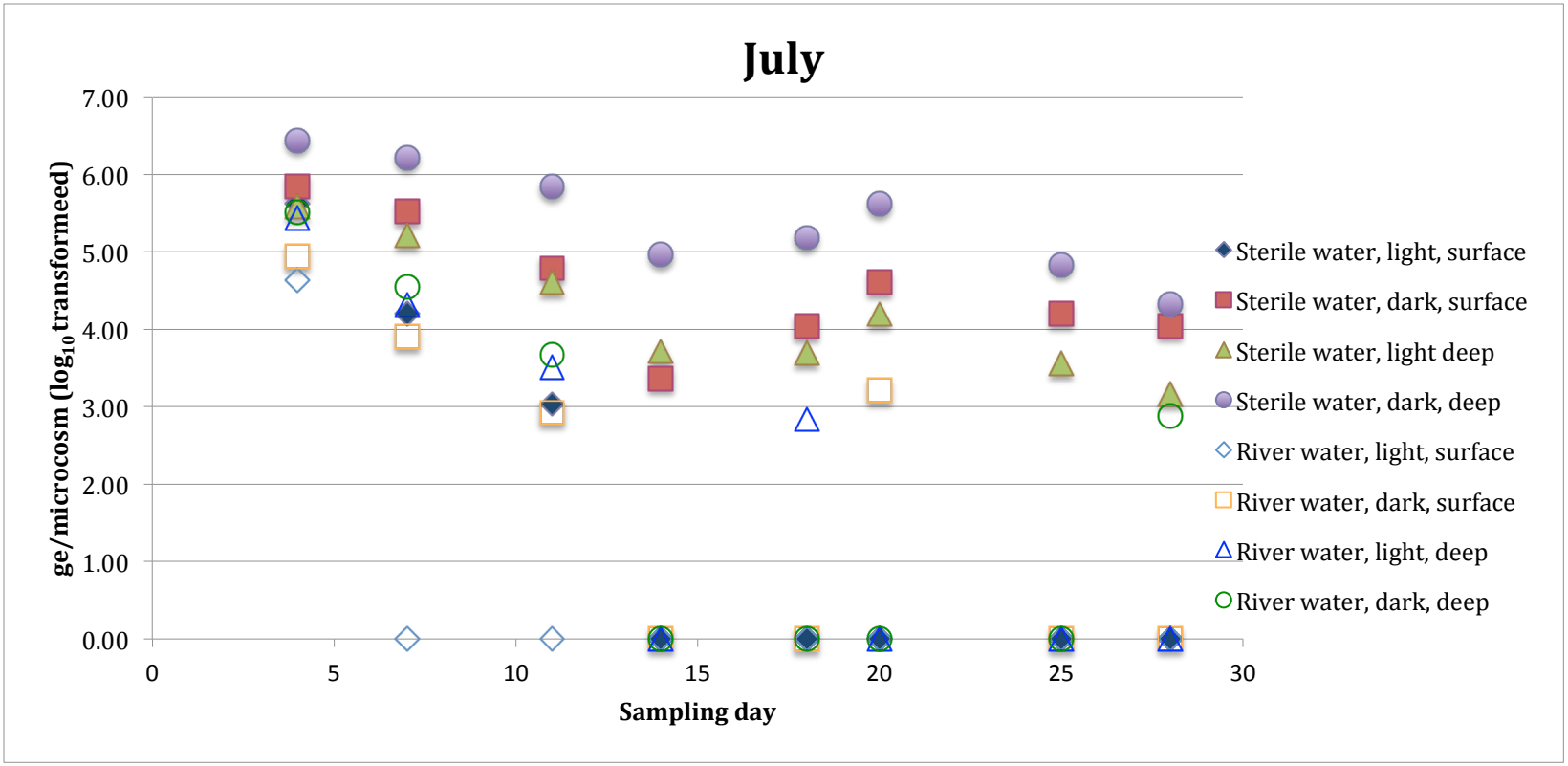

Figure 4.4. *In situ* $\log_{10}$ transformed viral concentrations (genomic equivalents per microcosm) over the course of 28 days in July.

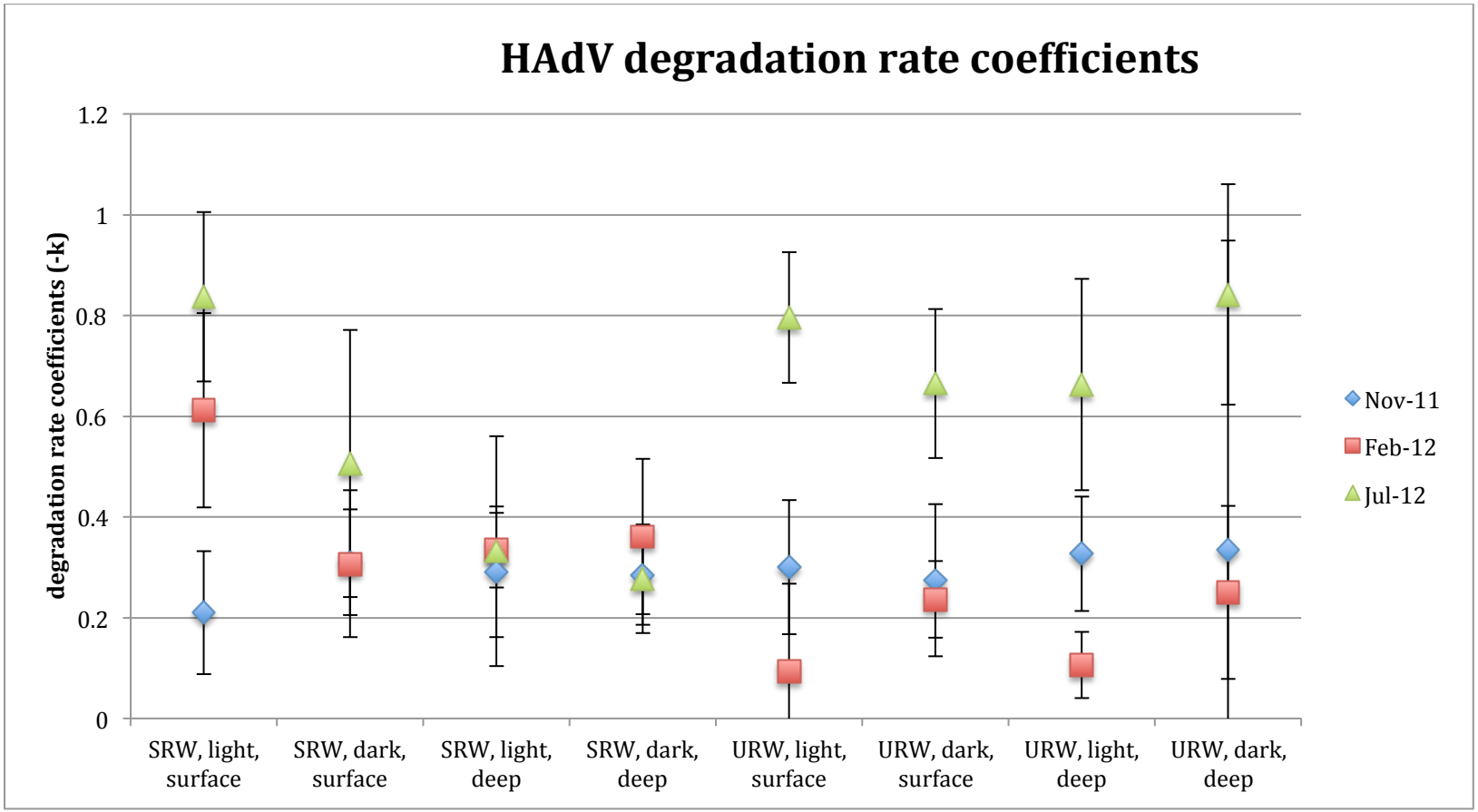

Figure 4.5. HAdV degradation rate coefficients in units per day (d^{-1}) for all three season. Degradation rate coefficients were calculated using the non-linear quadratic regression with 95% confidence intervals. Lack of overlap between confidence intervals represents significant differences in rate coefficients.

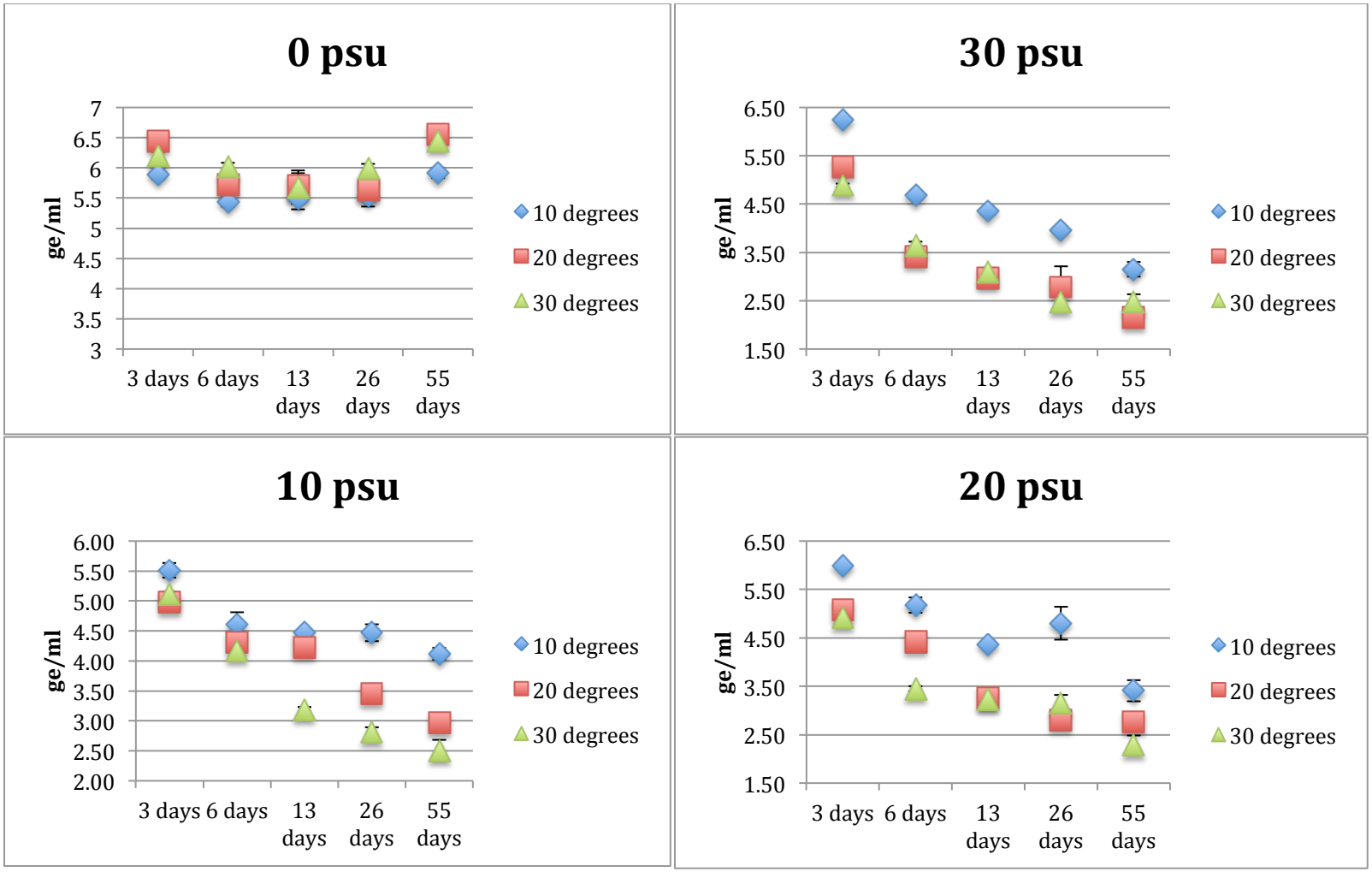

Figure 4.6. *In vitro* $\log_{10}$ transformed viral concentrations (genomic equivalents per milliliter) over the course of 55 days as a function of temperature at a given salinity.

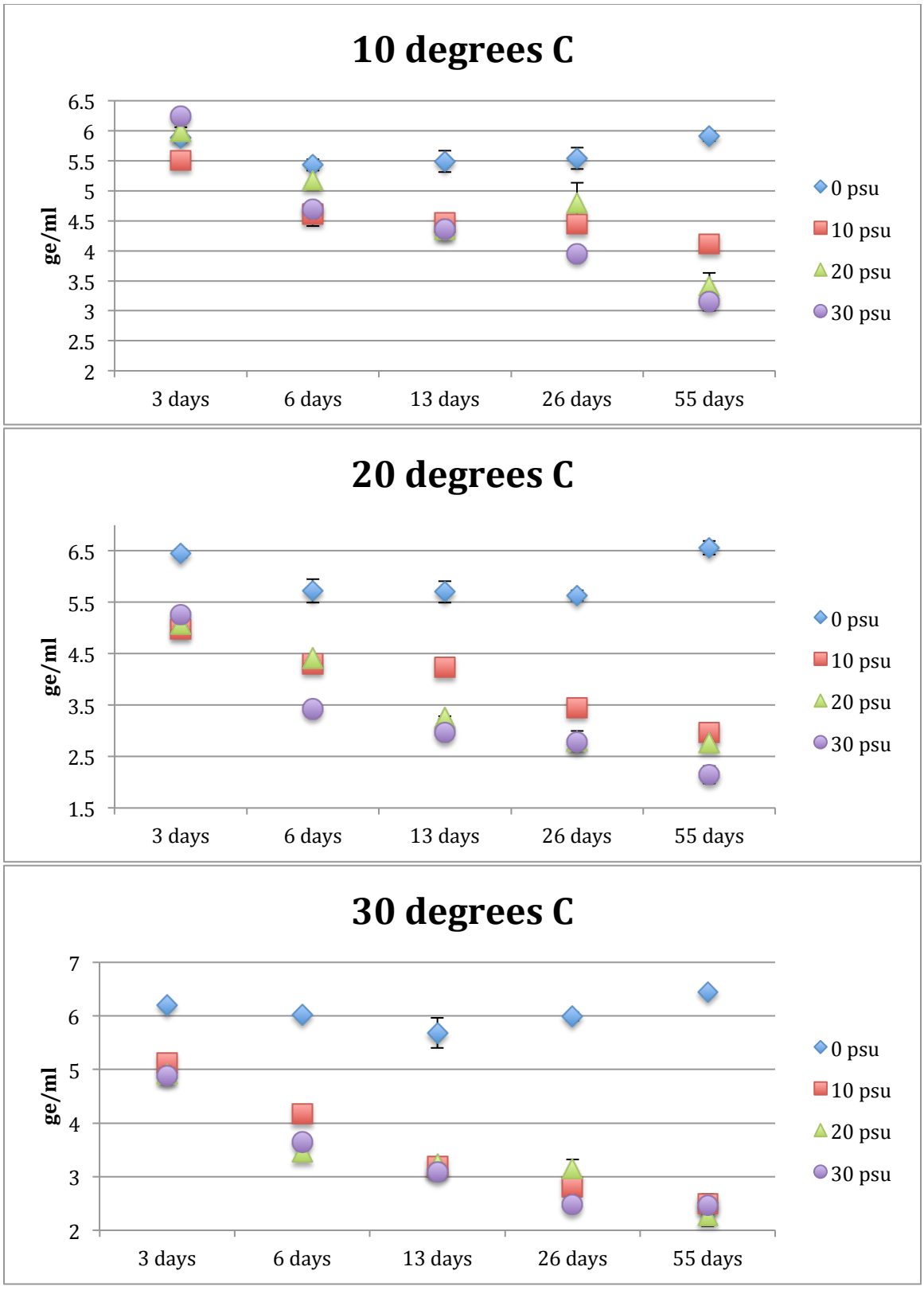

Figure 4.7. *In vitro* $\log_{10}$ transformed viral concentrations (genomic equivalents per milliliter) over the course of 55 days as a function of salinity at a given temperature.

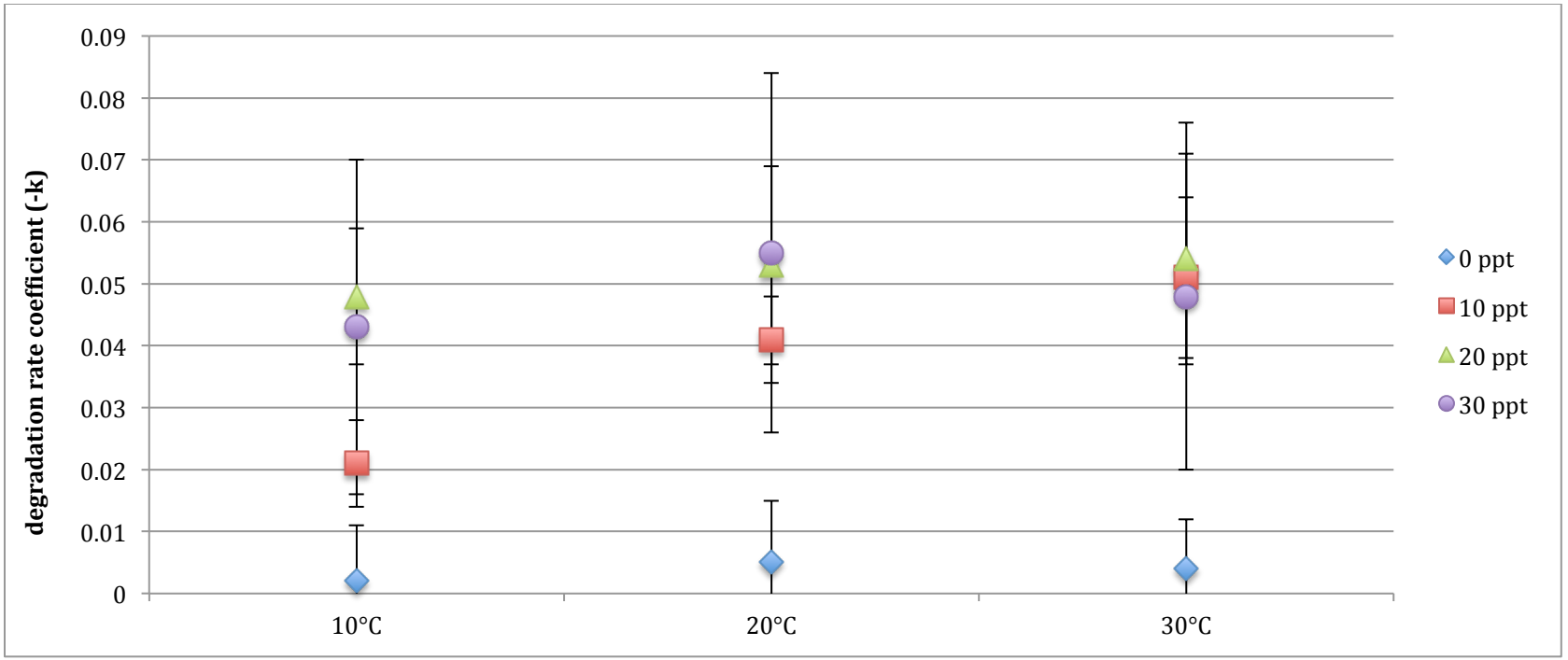

Figure 4.8. Degradation rate constant in units per day (d^{-1}) for each salinity treatment as a function of temperature including 95% confidence intervals. Overlapping confidence intervals signifies lack of significant difference in degradation rate coefficients.

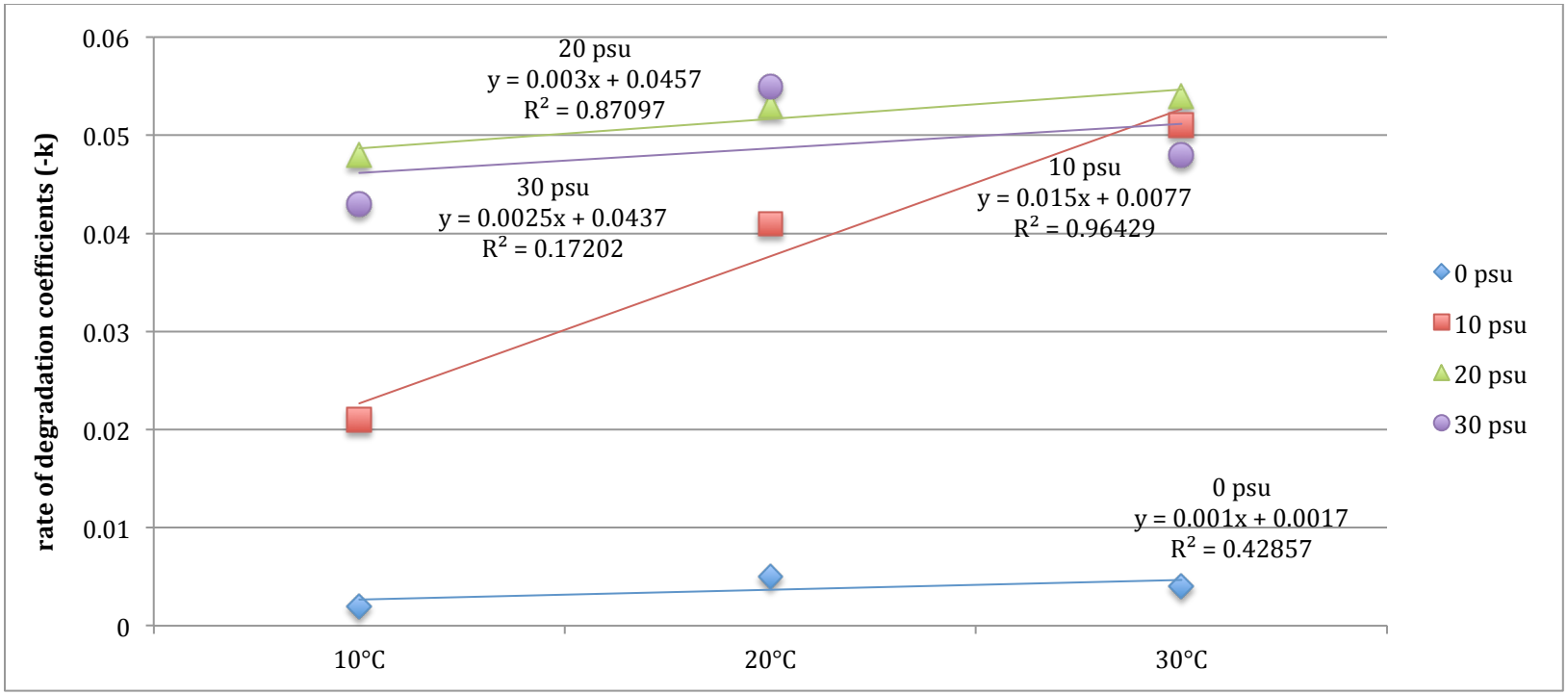

Figure 4.9. Linear regression of the degradation rate coefficients (in units d^{-1}) for each salinity treatment as a function of temperature. $R^2 \geq 0.8$ indicates that temperature is a predicative of the degradation rate.

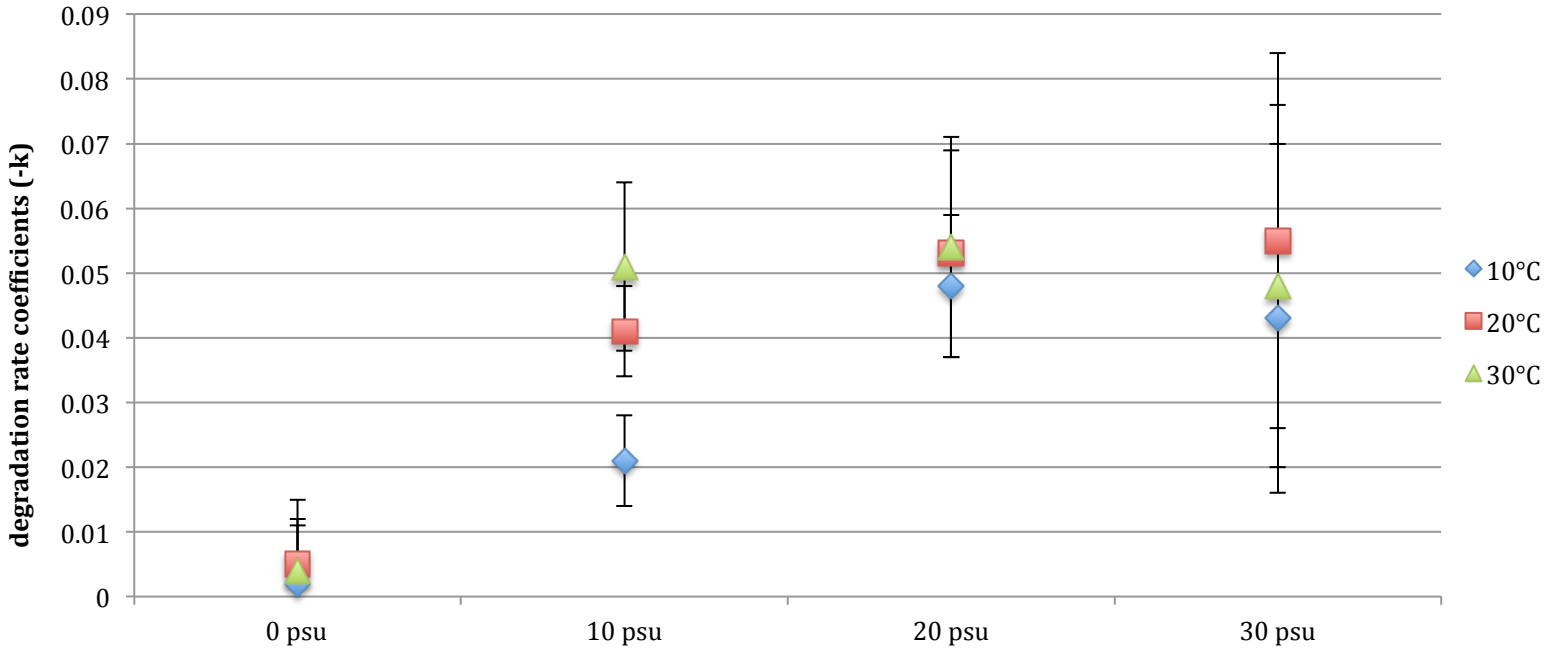

Figure 4.10. Degradation rate constant in units per day (d^{-1}) for each temperature treatment as a function of salinity including 95% confidence intervals. Overlapping confidence intervals signifies lack of significant difference in degradation rate coefficients.

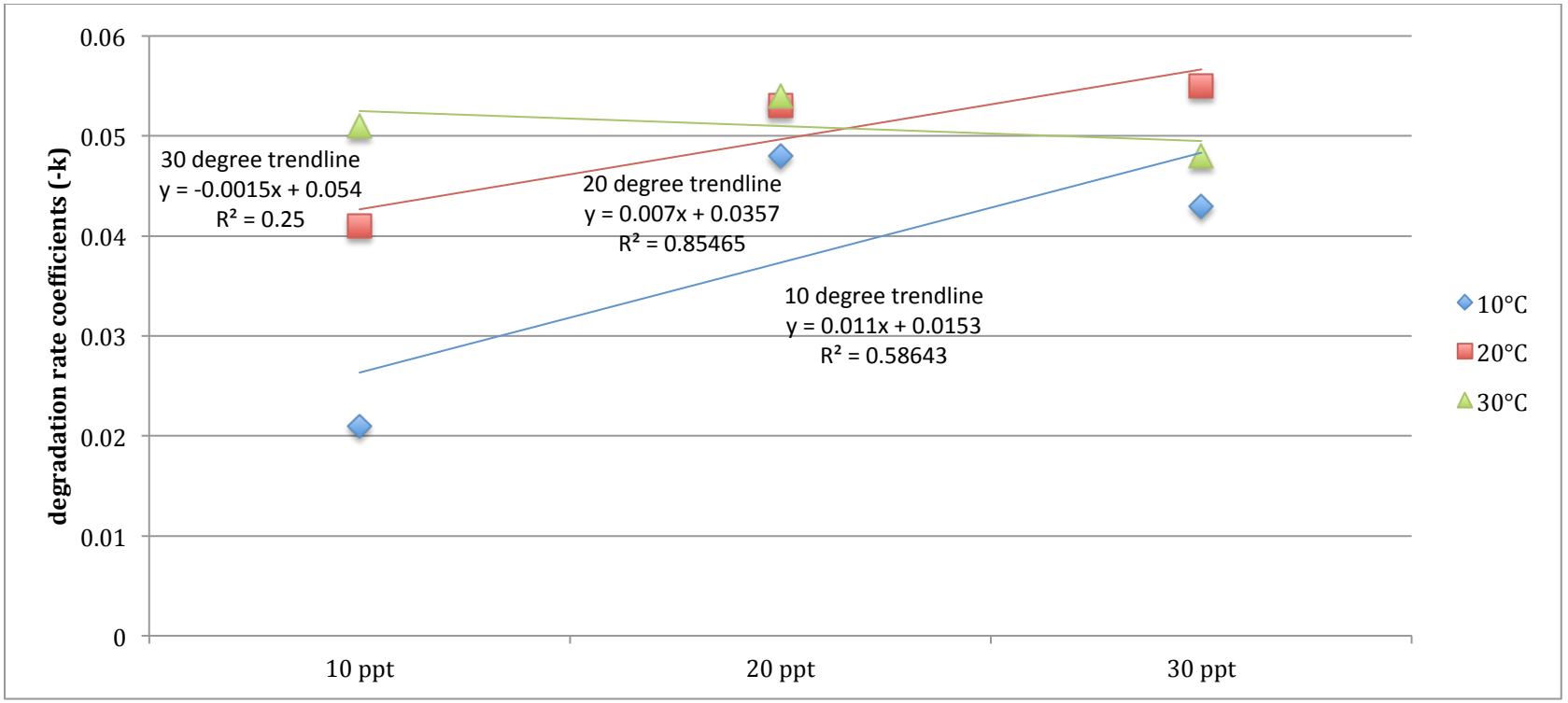

Figure 4.11. Linear regression of the degradation rate constant (in units d^{-1}) for each temperature treatment as a function of salinity. $R^2 \geq 0.8$ indicates that salinity is a predicative of the degradation rate. 0 psu is excluded.

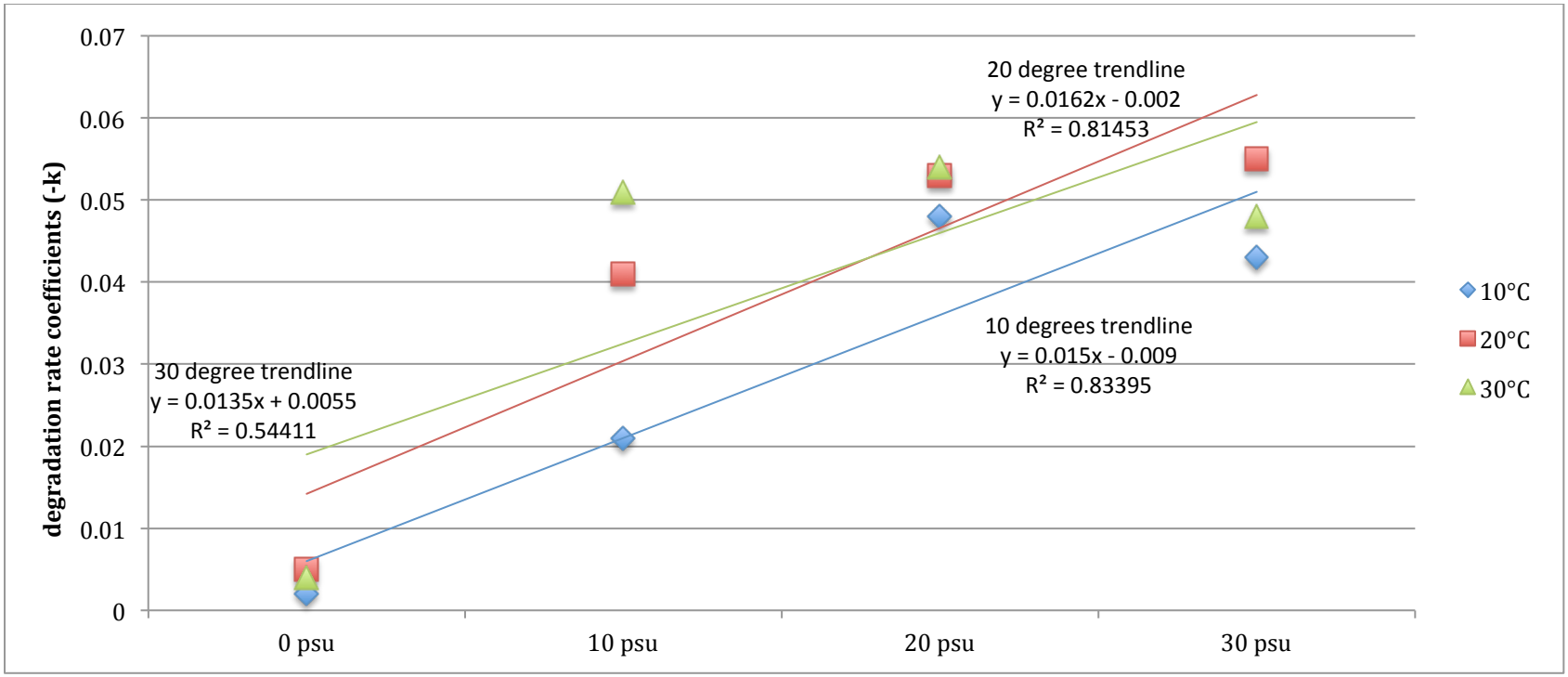

Figure 4.12. Linear regression of the degradation rate constant (in units d^{-1}) for each temperature treatment as a function of salinity. $R^2 \geq 0.8$ indicates that salinity is a predicative of the degradation rate. 0 psu is included.

Table 4.1. Light attenuation coefficient ($k_d = \ln(I_2-I_1)/d_2-d_1$) and average temperature recorded by HOBO® pendant waterproof temperature loggers attached to the top of the array and the bottom of the array. * HOBO® was lost during the course of the experiment. Photosynthetically active radiation (PAR) readings used to calculate light attenuation coefficients were recorded at the same time each day.

		average temperature (°C)	
	light attenuation coefficient (k_d)	surface array	bottom array
November 2011	1.25	13.06	13.42
February 2012	1.02	10.9	data lost*
July 2012	1.87	30.64	30.04

Table 4.2. Treatment conditions for each *in situ* sample bag.

Sample	Treatment	
Bag 1	Sterile river water, light, surface	Array 1
Bag 2	Sterile river water, light, surface	
Bag 3	Sterile river water, light, surface	
Bag 4	Sterile river water, dark, surface	
Bag 5	Sterile river water, dark, surface	
Bag 6	Sterile river water, dark, surface	
Bag 7	Sterile river water, light deep	
Bag 8	Sterile river water, light deep	
Bag 9	Sterile river water, light deep	
Bag 10	Sterile river water, dark, deep	
Bag 11	Sterile river water, dark, deep	
Bag 12	Sterile river water, dark, deep	
Bag 13	Unfiltered river water, light, surface	Array 2
Bag 14	Unfiltered river water, light, surface	
Bag 15	Unfiltered river water, light, surface	
Bag 16	Unfiltered river water, dark, surface	
Bag 17	Unfiltered river water, dark, surface	
Bag 18	Unfiltered river water, dark, surface	
Bag 19	Unfiltered river water, light, deep	
Bag 20	Unfiltered river water, light, deep	
Bag 21	Unfiltered river water, light, deep	
Bag 22	Unfiltered river water, dark, deep	
Bag 23	Unfiltered river water, dark, deep	
Bag 24	Unfiltered river water, dark, deep	

Table 4.3. Viral inactivation in relation to time, where -k is the decrease in viral density (log_{10} transformed) per day (d). Inactivation rate was calculated as the slope (b) of the linear regression for log_{10} (virus density) = constant + b*time.

Treatment	inactivation rate -k/d ± se	95% confidence interval
November 2011		
SRW, light, surface	0.255 ± 0.016	0.034
SRW, dark, surface	0.123 ± 0.081	0.036
SRW, light, deep	0.161 ± 0.019	0.039
SRW, dark, deep	0.126 ± 0.016	0.032
URW, light, surface	0.103 ± 0.022	0.045
URW, dark, surface	0.174 ± 0.022	0.045
URW, light, deep	0.090 ± 0.030	0.062
URW, dark, deep	0.041 ± 0.024	0.051
February 2012		
SRW, light, surface	0.277 ± 0.032	0.066
SRW, dark, surface	0.142 ± 0.021	0.045
SRW, light, deep	0.119 ± 0.016	0.032
SRW, dark, deep	0.121 ± 0.025	0.05
URW, light, surface	0.194 ± 0.024	0.048
URW, dark, surface	0.051 ± 0.015	0.03
URW, light, deep	0.052± 0.009	0.019
URW, dark, deep	0.079 ± 0.025	0.051
July 2012		
SRW, light, surface	0.260 ± 0.40	0.083
SRW, dark, surface	0.169 ± 0.40	0.082
SRW, light, deep	0.183 ± 0.31	0.065
SRW, dark, deep	0.112 ± 0.017	0.035
URW, light, surface	0.231 ± 0.037	0.076
URW, dark, surface	0.251 ± 0.031	0.065
URW, light, deep	0.277 ± 0.031	0.014
URW, dark, deep	0.205 ± 0.039	0.081

Table 4.4. HAdV inactivation rates based on non-linear exponential regression where -k is the decrease in viral density (log_{10} transformed) per day (d). Inactivation rate was calculated as the slope (b) of the exponential equation log_{10} (virus density) = $c_1 + c_2 * e^{-(btime)}$.

Treatment	inactivation rate -k/d
November 2011	
SRW, light, surface	0.001
SRW, dark, surface	0.000
SRW, light, deep	0.000
SRW, dark, deep	0.000
URW, light, surface	0.000
URW, dark, surface	0.000
URW, light, deep	0.000
URW, dark, deep	0.000
February 2012	
SRW, light, surface	0.088
SRW, dark, surface	0.170
SRW, light, deep	0.204
SRW, dark, deep	0.220
URW, light, surface	nd
URW, dark, surface	0.265
URW, light, deep	0.074
URW, dark, deep	0.123
July 2012	
SRW, light, surface	nd
SRW, dark, surface	nd
SRW, light, deep	nd
SRW, dark, deep	0.138
URW, light, surface	0.256
URW, dark, surface	0.135
URW, light, deep	0.105
URW, dark, deep	0.135

Table 4.5. Viral degradation in relation to time using a non-linear quadratic regression, where -k is the decrease in viral density (log_{10} transformed) over time (d, day). Degradation rate was calculated as the slope (b_1) of the multiple regression for: $log_{10}(\text{virus density}) = \text{constant} + b_1*\text{time} + b_2*\text{time}^2$. In most cases b_2 was not significantly different from zero.

Treatment	degradation rate -k/d ± se	95% confidence interval
November 2011		
SRW, light, surface	0.210 ± 0.059	0.122
SRW, dark, surface	0.310 ± 0.051	0.105
SRW, light, deep	0.291 ± 0.063	0.130
SRW, dark, deep	0.284 ± 0.047	0.098
URW, light, surface	0.300 ± 0.065	0.133
URW, dark, surface	0.274 ± 0.074	0.515
URW, light, deep	0.327 ± 0.369	0.114
URW, dark, deep	0.334 ± 0.296	0.615
February 2012		
SRW, light, surface	0.612 ± 0.093	0.193
SRW, dark, surface	0.307 ± 0.071	0.146
SRW, light, deep	0.334 ± 0.036	0.074
SRW, dark, deep	0.361 ± 0.074	0.154
URW, light, surface	0.094 ± 0.084	0.174
URW, dark, surface	0.236 ± 0.037	0.076
URW, light, deep	0.106 ± 0.032	0.066
URW, dark, deep	0.250 ± 0.083	0.172
July 2012		
SRW, light, surface	0.837 ± 0.081	0.168
SRW, dark, surface	0.506 ± .0128	0.265
SRW, light, deep	0.332 ± 0.111	0.228
SRW, dark, deep	0.277 ± 0.052	0.108
URW, light, surface	0.796 ± 0.063	0.130
URW, dark, surface	0.665 ± 0.072	0.148
URW, light, deep	0.663 ± 0.101	0.210
URW, dark, deep	0.842 ± 0.105	0.219

Table 4.6. HAdV DNA concentration (ge/microcosm) obtained by ICC-qPCR using Caco-2cells inoculated with undiluted day 28 water samples. Bold concentration values signify significance.

Treatment	T0	T4	day 28 qPCR
November 2011			
SRW, light, surface	0	0	0
SRW, dark, surface	0	0	1.68E+04
SRW, light, deep	0	0	1.23E+03
SRW, dark, deep	**0**	**1.93E+05**	**1.26E+4**
URW, light, surface	0	0	1.85E+04
URW, dark, surface	0	0	5.17E+03
URW, light, deep	0	0	1.79E+04
URW, dark, deep	0	0	1.89E+04
February 2012			
SRW, light, surface	0	0	4.60E+03
SRW, dark, surface	0	0	7.88E+03
SRW, light, deep	5.06E+04	1.94E+04	7.89E+03
SRW, dark, deep	3.45E+04	2.31E+04	2.47E+04
URW, light, surface	0	0	1.15E+03
URW, dark, surface	0	0	2.81E+05
URW, light, deep	0	0	4.35E+05
URW, dark, deep	0	0	3.40E+05
July 2012			
SRW, light, surface	3.91E+04	0	0
SRW, dark, surface	1.81E+05	0	1.11E+04
SRW, light, deep	0	0	1.46E+03
SRW, dark, deep	**0**	**4.30E+04**	**2.9E+04**
URW, light, surface	0	0	0
URW, dark, surface	**0**	**3.77E+04**	**0**
URW, light, deep	**0**	**4.65E+04**	**0**
URW, dark, deep	4.37E+05	0	7.59E+02

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Conclusions

Environmental transmission of human adenovirus through contaminated recreational and estuarine waters has been linked to insufficient treatment of sewage waste by wastewater treatment facilities (Fong et al., 2010). Previous studies have demonstrated that HAdV DNA is able to persist for long periods of time in various environmental matrices, including effluent, biosolids and water (Bosch 1998, Ogorzaly et al., 2010). However, detection and persistence of viral DNA does not give comprehensive indication of risk because DNA may be detected when virions are not viable or infective. Therefore, the question of viral viability and infectivity must be addressed before the threat of HAdV contamination to human health can be fully assessed. Thus, the focus of this study was to determine whether HAdV DNA could be detected in various environmental matrices and to assess the persistence, viability and infectivity of HAdV in products of the wastewater treatment process including biosolids and effluent, and in estuarine receiving waters and shellfish exposed to HAdV-contaminated waters.

Results from this current study indicated that for WWTP biosolids and effluent samples, the treatment method employed during their production had an effect on whether HAdV DNA was detected and whether the virus was found to be viable and infective. Biosolids that were produced using centrifugation as their treatment method did not contain any detectable HAdV DNA, nor were viable virions detected. However, plate and frame thickened samples contain detectable HAdV DNA 100% of the time, but viability and infectivity of virions could not be confirmed. Viable virions could not be detected in effluents produced by plants that employed a tertiary treatment method (Biological

Nutrient Removal or oxidation towers), although, detection of viral DNA was possible during at least one sampling season. Therefore, based on the results from both biosolids and effluents, we were able to conclude that the detection of HAdV DNA does not necessarily correlate with viability of the virus. That is to say, just because the DNA is found in the sample, does not confirm that the sample represents a risk for HAdV infection. During this study detection of the viral DNA occurred with far greater frequency than the confirmation of viable and infective virions. Therefore, when determining the optimal treatment method, whether for biosolids production or effluent processing, the final product should be assayed with an integrated cell culture – quantitative PCR approach. Detection of DNA alone is an insufficient measure of health risk.

The question of how long the HAdV DNA can persist in various environmental matrices, whether viable or not, was also addressed in this study. However, because each of the biosolids and effluent samples represented a single time point, it was not possible to determine persistence of the HAdV DNA over time in either of them. This was accomplished through the *in situ* and *in vitro* estuarine water persistence study. The most striking reduction in persistence occurred during July in unfiltered river water (URW) samples. While the February and November sampling months had similar levels of persistence, viral DNA was completely undetectable in all URW samples from July after two weeks. We concluded that this complete degradation of the virus was the enhanced by indigenous microbiota and/or natural organic matter. In addition, regardless of the sampling month, HAdV DNA was undetectable in sterile river water (SRW) samples

exposed to surface light, whereas it could still be detected in URW samples exposed to surface light during February and November. This supports the idea that photodegradation of viruses that are not adsorbed to a substrate, as would be found in URW, was an important removal mechanism in these treatments. In terms of the correlation of viral genome detection and persistence with viability, as with the biosolids and effluent samples, there was none. It is important to note, however, that the assay for viability was only conducted on the day 28 samples for each treatment microcosm during each sampling month. While we are able to conclude that persistence of the DNA does not reflect viability of the virion, we were not able to ascertain when the virus lost its viability for each of the treatment microcosms. This should be addressed in future studies.

Conclusions as to the detection, persistence and viability of HAdV in oysters grown in receiving waters could not be drawn from this study. Unfortunately, our experimental oysters were removed from our study site, without our permission, resulting in incomplete data. A small scale pilot study involving laboratory exposure of oysters to spiked river water did confirm that oysters do filter viable HAdV from contaminated water and retain it in their tissue. Relay studies also confirmed that the virus could be depurated from the oyster in as little as three days, in an open system. A larger scale study involving the deployment of oysters in a well safeguarded natural setting needs to be done before any conclusions can be drawn as to the ability of oysters to filter HAdV and retain infective HAdV from estuarine waters.

While there are several avenues for future work stemming from this study, particular attention should be paid to persistence of HAdV in estuarine water and its correlation to viability. This study has demonstrated that viable HAdV is released from WWTPs into the environment. The fate of this virus, once it reaches the estuarine receiving waters, should be thoroughly elucidated in order to further substantiate its role as a marker for viral contamination, and potential human health risk, in estuarine and recreational waters.

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